LipidII interaction with specific residues of *Mycobacterium tuberculosis* PknB extracytoplasmic domain governs its optimal activation

Prabhjot Kaur\(^1\), Marvin Rausch\(^{2,3}\), Basanti Malakar\(^1\), Uchenna Watson\(^4\), Nikhil P. Damle\(^1,6\), Yogesh Chawla\(^1,7\), Sandhya Srinivasan\(^5\), Kanika Sharma\(^5\), Tanja Schneider\(^2,3\), Gagan Deep Jhingan\(^5\), Deepak Saini\(^4\), Debasisa Mohanty\(^1\), Fabian Grein\(^{2,3}\) & Vinay Kumar Nandicoori\(^1\)

The *Mycobacterium tuberculosis* kinase PknB is essential for growth and survival of the pathogen in vitro and in vivo. Here we report the results of our efforts to elucidate the mechanism of regulation of PknB activity. The specific residues in the PknB extracytoplasmic domain that are essential for ligand interaction and survival of the bacterium are identified. The extracytoplasmic domain interacts with mDAP-containing LipidII, and this is abolished upon mutation of the ligand-interacting residues. Abrogation of ligand-binding or sequestration of the ligand leads to aberrant localization of PknB. Contrary to the prevailing hypothesis, abrogation of ligand-binding is linked to activation loop hyperphosphorylation, and indiscriminate hyperphosphorylation of PknB substrates as well as other proteins, ultimately causing loss of homeostasis and cell death. We propose that the ligand-kinase interaction directs the appropriate localization of the kinase, coupled to stringently controlled activation of PknB, and consequently the downstream processes thereof.
Protein phosphorylation has come forth as a preeminent circuitry regulating a vast number of physiological processes in the bacterial kingdom. A particular class of receptor-type serine-threonine kinase called PASTA (Penicillin binding proteins And Serine Threonine Associated) kinase is widespread across gram-positive firmicutes and actinomycetes and is known for its functions associated with bacterial cell growth. These protein kinases have an intracellular kinase domain, which shows sequence and structural homology to the eukaryotic serine/threonine kinases, and an extracytoplasmic (Ec) domain made up of varying number of PASTA domains. PASTA kinases are usually required by bacteria under stress conditions like nutrient starvation, antibiotic stress, biofilm formation etc., and are non-essential for their vegetative growth. However, in the pathogenic bacterium *Mycobacterium tuberculosis* (*Mtib*) the PASTA kinase PknB (*PknBMtb*) is an essential gene and is proposed to be one of the master regulators of serine/threonine phosphorylation-mediated signaling.

The essential nature of PknB in mycobacteria stems from its ability to influence the activity of a large repertoire of substrates involved in cell wall synthesis, cell growth, cellular metabolism, transcription, and translation. Over-expression or deletion of PknB impacts cellular morphology and survival of *Mtib* which suggests that the expression and activity of this kinase must be critically fine-tuned inside the bacterium. PknB levels are modulated under different conditions of mycobacterial growth: for instance its levels are down regulated during dormancy and nutrient starvation and are up regulated during exponential growth and resuscitation. The dynamicity of PknB regulation implies that the receptor kinase actively monitors its environment and responds accordingly, in an effort to provide survival advantage. PknB is believed to respond to environmental signals through PASTA domain interactions with the specific ligand, identified to be non-crosslinked peptidoglycan (PG) fragments called muropeptides. In line with this, purified PASTA domains of PknB interact and bind with a synthetic muropeptide containing isoglutamine (iGln) and meso-diaminopimelic acid (mDAP) residues at the second and third position of the stem peptide in vitro. The prevailing hypothesis suggests that the interaction of the extracytoplasmic domain with the ligand results in the dimerization of intracellular kinase domain, which is required for the activation of the kinase through activation loop phosphorylations. The hypothesis is based on the front-to-front and back-to-back dimeric crystal structures of cytosolic kinase domain and surface plasmon resonance-based in vitro binding experiments of PASTA domain with the muropeptides. In consonance with this we have previously reported that the extracytoplasmic PASTA domains are indispensable for the function of PknB and survival of *Mtib*. Deletion of the terminal PASTA domain (PASTA4) alone results in compromised survival, suggesting that it plays a leading role in kinase-ligand interactions. To date, the hypothesis with respect to PknB activation has not been tested in vivo. Here we set out to answer the following questions: (i) Is PASTA4 sufficient for PknB activation? (ii) What are the ligand binding residues in the extracytoplasmic domain? (iii) What is the impact of ablating ligand binding on the localization and activation of PknB? (iv) What are the physiologial ligands that interact with PASTA domains? and (v) What is the impact of ablating ligand binding on the phosphorylation of target substrates of PknB?

Here we identify ligand interacting residues and show that mutating these residues caused abolition of ligand binding. Abrogation of ligand binding triggers aberrant localization and hyperactivation of PknB, which in turn results in hyperphosphorylation of both canonical and non-canonical downstream target substrates, eventually leading to cell death. Results suggest that interaction with the ligand is critical for appropriate localization and regulation of the kinase activity.

**Results**

**PASTA4 and domain length are essential for PknB function.**

We have previously shown that the deletion of the PASTA4 domain (Fig. 1c; *PknB-123*) compromises the in vivo functionality of PknB. The terminal PASTA domain of StkP, a *Streptococcus pneumoniae* ortholog of the PknBMtb, was demonstrated to be necessary and sufficient for its signaling. Thus we sought to determine the role of the terminal PASTA4 in the context of shorter total domain length. We employed previously described *Mtib* conditional mutant of pknB (*RvΔAB*), wherein the native locus has been modified to bring its expression under pristinamycin inducible promoter, which allows the bacterium to grow efficiently in the presence of pristinamycin but not in its absence (Supplementary Fig. 1a). To assess the effect of PknB mutations, we generated wild type or mutant constructs in pNit-3F (Supplementary Fig. 1b), which could be induced with isovaleronitrile and owing to the presence of 3X-FLAG tag, the ectopically expressed PknB migrates slower compared with the endogenous protein (Supplementary Fig. 1c).

To investigate the role of PASTA4 as well as the domain length, we have generated PknB-234, lacking PASTA1 wherein the terminal PASTA4 is retained, and PASTA-1212, a chimera of appropriate domain length where PASTA1-2 were repeated (Fig. 1c). Western blot analysis confirmed the efficient depletion of PknB in the absence of pristinamycin as well as efficient expression of 3X-FLAG tagged wild-type and mutant proteins (Fig. 1d). On examining the ability of wild-type and mutant PknB proteins to complement the in vivo functionality of PknB (Fig. 1e), it was observed that vector-transformed *RvΔAB* showed significantly compromised survival in the absence of pristinamycin while the ectopic expression of 3F-PknB rescued the growth defects (Fig. 1e). Even though PknB-234 was marginally better compared to either PknB-123 or PknB-1212 (~1.5 vs 2 log fold) in rescuing the growth defects, growth was significantly compromised in all three strains when compared with the wild type (Fig. 1e). To assess the impact in an infection scenario, we evaluated the survival in differentiated THP1 cells (Fig. 1f). The data resembled the in vitro growth results, wherein PknB-234 showed slightly better survival as compared with PknB-1212 or PknB-123. These results suggest that while the PASTA4 domain is quite distinct and important, yet the appropriate length of the entire domain is also vital for efficient PknB function.

**mDAP and iGln interacting residues influence the survival.**

The structure of the PASTA domains has been derived with the help of NMR as well as X-ray crystallography. Nonetheless, the residues that are responsible for the interaction with the proposed muropeptide ligand have not yet been identified. As PASTA4 is critical for PknB functionality, we examined a possible role for it in ligand binding. As PknB is hypothesized to dimerize upon ligand binding, we assumed that the PASTA domains interact with the muropeptide as a dimer. With the help of in silico molecular simulations, we identified a potential dimerization interface between the PASTA4 domains (Fig. 2a), which predominantly comprises of residues that are conserved across the PASTA4 domains of prokaryotic kinases (Supplementary Fig. 2). The initial simulation data suggested that ligand binding might be extending into the PASTA3 domain; hence, the dimeric PASTA3-4 domain was employed for further analysis. The ligand-binding domain majorly comprised of residues in the linker regions between PASTA3 and 4 domains (Fig. 2a & **
Supplementary Fig. 2). Data suggested that Ser556 and Lys557 (SK) interact with the carboxy terminal region of the iGln and Asn559 and Gln560 (NQ) interact with the mDAP residues in the muropeptide ligand (Fig. 2a & Supplementary Fig. 2a). We set out to assess the impact of mutating these putative iGln and mDAP binding residues in modulating PknB functionality. We generated a PknB tetra mutant (PknB-GM), wherein all four putative ligand interacting residues have been mutated simultaneously (Fig. 2b). The RvΔB strain was transformed with pNit-3F-PknB and pNit-3F-PknB-GM and the expression of both proteins was confirmed (Fig. 2c). While the wild type could successfully rescue the phenotype both in vitro and ex vivo, the tetra mutant failed to do so (Fig. 2d, e). The growth phenotype did not vary even when the expression of PknB from pNit-3F constructs was not induced.
with isovaleronitrile, suggesting that the results are not due to overexpression artifacts (Supplementary Fig. 2b, c). Thus, putative iGln and mDAP interacting residues in the PASTA domain of PknB seem to be necessary for its functionality.

iGln/mDAP interacting residues are independently essential.

Next we sought to assess the impact of individually mutating the amino acid pairs, which we believed to interact with iGln or mDAP residues. Hence we generated PknB-G and PknB-M, wherein SK or NQ residues were mutated to AA or DE residues, respectively (Fig. 3a). Western blot showed efficient expression of 3F-PknB, 3F-PknB-G, and 3F-PknB-M in the complemented strains (Fig. 3b). Compared with the tetra mutant (Fig. 2d), strains complemented with either PknB-G or PknB-M showed partial growth defects, albeit to different extents (Fig. 3c, e). PknB-G (Rv\(\Delta B\)-B-G) showed one and half fold poorer survival compared with PknB (Rv\(\Delta B\)-B), while it showed ~3 log folds better survival compared with the control (Rv\(\Delta B\)-V) (Fig. 3c). On the other hand, PknB-M (Rv\(\Delta B\)-B-M) showed 3 log fold lower survival compared with Rv\(\Delta B\)-B and one and half log fold better survival compared with the control (Fig. 3e). These results suggested that mDAP interacting residues play a more critical role compared with iGln interacting residues. However in a THP1 infection model (Fig. 3d, f), both the mutants were equally abrogated and showed defects similar to the tetra mutant (Fig. 2e). THP1 infection experiment performed at lower MOI (1:4) showed similar defects as those observed at higher MOI (1:10) (Figs 2e and 3d, f). Data suggests that both iGln and mDAP interacting residues are individually critical for PknB functionality, especially during the ex vivo infection scenario where even marginal perturbations in ligand binding are not endured.

**Fig. 1** PASTA4 and domain length are essential for PknB function. a Chemical structure of suggested muropeptide ligand for PknB. b Schematic outline of prevailing hypothesis vis-a-vis PknB activation, wherein the ligand binding is proposed to result in dimerization and phosphorylation of the activation loop residues in the intracellular kinase domain. c Schematic representation of full-length PknB, PknB-PASTA domain deletion mutants, and the chimera. d Rv\(\Delta B\) strain was electroporated with pNit-3F, pNit-3F-PknB, pNit-3F-PknB-123, pNit-3F-PknB-234, and pNit-3F-PknB-1212 to generate Rv\(\Delta B\)-V, Rv\(\Delta B\)-B, Rv\(\Delta B\)-123, Rv\(\Delta B\)-234, and Rv\(\Delta B\)-212. Whole-cell lysates (WCLs) were prepared from cultures initiated at A\(_{600}\) of ~0.6 and grown for 5 days in the presence or absence of pristinamycin. Ivn was added in the absence of pristinamycin to the cultures to induce the expression of episomal copy. Ten microgram each of WCLs were resolved on 8% SDS-PAGE, transferred to nitrocellulose membrane, and western blotted with antibodies. e Rv and Rv\(\Delta B\) transformants were inoculated at initial A\(_{600}\) of ~0.05 and grown in the presence or absence of pristinamycin. Ivn (0.2 \(\mu\)M) was added in the absence of pristinamycin to the cultures to induce the expression of episomal copy. After 6 days in vitro growth, Colony forming Units (CFUs) were enumerated by plating appropriate dilutions on 7H11 agar plates containing pristinamycin. Data is representative of one of the two biologically independent experiments and each experiment was performed in triplicates.

**Mutations in the PknB-Ec abrogate its binding to LipidII.** PG synthesis at the poles and septum region involves two stages. In the first stage, which happens in the cytoplasm, LipidII is synthesized from UDP-GlcNAc precursor by sequential action of multiple Mur family enzymes. LipidII is composed of N-acetylmuramic acid (NAM)-pentapeptide (stem peptide) connected to the membrane anchored decaprenyl phosphate through a pyrophosphate link (Supplementary Fig. 3a). LipidII is then translocated/filipped into the periplasmic region, which provides the NAG-NAM-pentapeptide moieties to the growing PG layer. The nature of the lipid moiety, the amino acids in the stem peptides and modification in the NAG and NAM sugars, vary from species to species. The *Staphylococcus aureus* PknB ortholog (Pkn\(B_{sa}\)) has been shown to interact strongly with LipidII through PASTA domains (1:2 molar ratio of LipidII:PknB\(B_{sa}\)). Thus we sought to explore the possibility of an interaction between PknB\(B_{Ec}\) and LipidII. Unlike *S. aureus*, wherein the Lipid II stem peptide contains a lysine residue at the third position, the Lipid II in *Mtb* carries mDAP at the anomalous position in the stem peptide. Statistical analysis was performed with the unpaired t-test using Graphpad software. Data shown represents mean + standard deviation (SD). *p < 0.005, ****p < 0.0005. Source data are provided as a Source Data file.

Abrogation of ligand binding perturbs localization of PknB.

Nascent PG biosynthesis takes place at the poles and mid cell (septum) regions and hence the precursors such as LipidII and muropeptides are anticipated to be concentrated at these niches. Interestingly, PknB also localizes to both polar and mid-cell regions and the extracytoplasmic PASTA domains govern the localization. Thus the rational supposition would be that the localization of PknB is probably dictated by the interaction between PASTA domain and PG precursors. However, the hypothesis has not been tested till date, perhaps due to the non-availability of PknB mutants that fail to interact with the ligand. We examined the localization of GFP-PknB and GFP-PknB-GM mutants in *M. smegmatis* conditional mutant (m\(c^2\Delta B\)), following the reasoning that in the absence of endogenous PknB, GFP-PknB or GFP-PknB-GM would be the sole PknB in the cell.
Fig. 2 mDAP and iGln interacting residues influence the survival. 

a Model of Mtb PknB PASTA3-4 dimer in complex with the muropeptide ligand. Inset panel shows a magnified view of the interactions between the mDAP and iGln residues of the muropeptide with polar amino acids between the PASTA3 and 4 domains of PknB. 

b Schematic representation of extra-cytoplasmic PASTA domain of PknB and the residues in the PASTA3-4 linker region suggested to be involved in interaction with iGln (SK) and mDAP (NQ) residues in the muropeptide. The mutations introduced in the linker region to generate PknB-GM are indicated.

c Western blot analysis of WCLs prepared from Rv and RvΔB transformants. The transformants were seeded at A600 of ~0.05 in the presence of pristinamycin or IVN as indicated for 5 days and WCLs were resolved on 8% SDS-PAGE and probed with α-PknB and α-GroEL1 antibodies. 

d Cultures of Rv and RvΔB transformants were initiated at A600 of ~0.05 and grown in the presence of 1.5 μg/ml pristinamycin or 0.2 μM IVN as indicated for 6 days. CFUs were evaluated by plating appropriate dilution on 7H11 agar plates containing pristinamycin. Data are representative of the three biologically independent experiments and each experiment was performed in triplicates. 

e Human monocytic cell line THP-1 was differentiated to macrophages and was infected at 1:10 M.O.I. with Rv and RvΔB transformants grown to A600 of ~0.6–1.0 in the presence of pristinamycin. IVN was added in the media to induce the expression of episomal copy and CFUs were enumerated 72 h p.i. Data are representative of one of the two biological replicates and each experiment was performed in triplicates. Data represents mean ± SD. Statistical analysis was performed with the unpaired t-test using Graphpad software. *** p < 0.0005. Source data are provided as a Source Data file.
and hence phenotypic impact in terms of localization can be clearly visualized. Although GFP-PknB shows a strong punctate distribution at the polar and mid cell regions, GFP-PknB-GM mutant shows aberrant localization throughout the cell (Fig. 5a), suggesting that abrogation of ligand binding results in aberrant localization. Quantification of pole/septa vs. aberrant localization drives home the message (Fig. 5b). If this was indeed accurate, the converse experiment wherein the ligand is sequestered should also result in aberrant localization. Thus, we asked what would happen to PknB localization when LipidII is sequestered into a complex with antibiotic “nisin”, which binds through the pyrophosphate group resulting in membrane pore formation and

---

**ARTICLE NATURE COMMUNICATIONS | https://doi.org/10.1038/s41467-019-09223-9**

---

[Diagram and graphs not rendered here]
eventual cell death. The antibiotic isoniazid (INH) that does not interact with LipidII was used as the control (Fig. 5c).

Although we observed distinct puncta with mc2ΔAB::GFP-B in the absence of any antibiotic or in the presence of INH, aberrant number/mislocalized puncta were observed (>3) upon nisin treatment (Fig. 5c). Quantification showed a significant increase in the number of cells with more than 3 puncta or localization all through the cell (Fig. 5d). These results suggest that LipidII is likely to be the major intracellular ligand of PknB, and importantly, ligand binding is essential for the appropriate localization. Since the PknB-GM fails to interact with the ligand and does not localize to pole and septum, we reasoned that it might impact the diffusion dynamics. We investigated the rate of recovery of GFP-PknB and GFP-PknB-GM upon photobleaching by performing FRAP (Fluorescence Recovery After Photobleaching) experiments (Fig. 5e). Although GFP-PknB showed relatively slower recovery (t1/2 = 111 s), GFP-PknB-GM mutant showed substantially higher dynamics with t1/2 = 90.56 s (Fig. 5f), suggesting that ligand binding also plays a role in modulating molecular dynamics.

**PknB-GM is hyper-phosphorylated in the activation loop.** If the prevailing hypothesis with respect to the activation of PknB shown in Fig. 1b is accurate, abrogation of ligand binding should result in decreased phosphorylation of the activation loop residues. PknB is auto-phosphorylated at the T171 and T173 residues within the activation loop in vitro, and mutating these residues significantly diminishes its activity. PknB is also phosphorylated in vitro in the juxtamembrane domain; however, there are no reports about the influence of these phosphorylations its function. To scrutinize the roles of T171 and T173 residues, we generated PknB-TATA (T171A, T173A) mutant (Supplementary Figs. 4a and 6a), and the activity was analyzed using GarA as the substrate (Supplementary Fig. 4b-d). In agreement with the previous report, the PknB-TATA mutant significantly compromised activity (Supplementary Fig. 4c, d). To examine their in vivo role, PknB or PknB-TATA clones made using integrative vector as backbone were electroporated into RvΔB cells. The expression of PknB and PknB-TATA from the integrated construct was comparable to that of inducible PknB at the native locus (Fig. 6b). Compared with RvΔB::V, RvΔB::B-TATA showed better survival; nonetheless, the mutant was significantly compromised when compared with RvΔB::B, indicating the essentiality of activation loop phosphorylations (Fig. 6c). Importantly, in the ex vivo model of infection, both RvΔB::V and RvΔB::B-TATA were found to be equally compromised (Fig. 6d).

To determine the extent of activation loop and juxtamembrane phosphorylations, we resorted to isobaric TMT labeling (Fig. 6e). Western blot and quantitative mass spectrometry analysis of total proteome demonstrated depletion of PknB and similar expression of 3F-PknB and 3F-PknB-GM (Fig. 6f, g). However, the levels of PknB protein in RvΔB::B & RvΔB::B-GM were ~1.2–1.5 (log2) fold higher compared with PknB in RvΔB + pristinamycin; Fig. 6g; Supplementary Table 1a). Intriguingly, contrary to the current belief, we observed hyperphosphorylation of both activation loop and juxtamembrane domain in the 3F-PknB-GM mutant compared with 3F-PknB (Fig. 6h & Supplementary Table 1b). Although the role of activation loop phosphorylation is known, the role of juxtamembrane phosphorylation needs further investigation. Thus the data suggests that abrogation of ligand binding results in hyper phosphorylation of PknB, strongly suggesting that the ligand binding plays a regulatory role.

**PknB-GM hyperphosphorylates specific & non-specific targets.** We identified a total of 632 (Supplementary Fig. 6a & Supplementary Data 1) and 258 (Supplementary Fig. 6b) proteins in the proteomic and phosphoproteomic samples, respectively. Of the 258 phosphoproteins, 242 had common phosphopeptides (total = 390 phosphopeptides) in all three replicates. Among them, 147 proteins encompassing 257 phosphopeptides were present in both, proteomic and phosphoproteomic samples (Supplementary Fig. 6c & Supplementary Data 2). The intensity ratios of these 257 phosphopeptides were normalized with respect to the whole-protein intensity ratios (Supplementary Data 1) and the mean normalized ratios (Supplementary Data 3) were converted to log2 values (Supplementary Table 2) and plotted as a heatmap (Fig. 7a). The 147 phosphoproteins were distributed across all the functional categories (Fig. 7b) with a majority of the phosphorylations on threonine (68%), followed by serine (~23%) and tyrosine (~9%), residues, a trend universal to all mycobacterial phosphoproteomic studies. Among the 257 phosphopeptides, a total of 111 phosphopeptides mapping to 73 different proteins (which included multiple well-characterized substrates such as Ef-Tu, Wag31, and HupB) were potentially the products of PknB-mediated phosphorylation (Fig. 7b, c; Supplementary Table 3a). Recently, Carette et al. have identified 46 potential targets of PknA and PknB with the help of a specific inhibitor. Nine of these identified targets were found to be among the 73 potential targets of PknB identified in this study (Supplementary Table 3b). Interestingly, we also identified 8 putative PknB-dependent tyrosine phosphorylated peptides (Fig. 7d; Supplementary Table 4a, b), in agreement with a previous study where PknB was suggested to be a dual specificity kinase.

The analysis of the activation loop phosphorylations in PknB suggested that PknB-GM is hyperphosphorylated (Fig. 6h), which led us to infer it to be a hyperactive kinase. In line with this prediction, complementation with the PknB-GM mutant seems to have resulted in the remarkable hyperphosphorylation of cellular proteins (Fig. 7a; Supplementary Table 2). The data in Fig. 7a can be further divided into three clusters (Supplementary Table 4c): cluster 1 where the phosphorylation is unperturbed by depletion of PknB as well as complementation with ectopic expression of PknB or PknB-GM; cluster 2 representing direct...
targets of PknB; and cluster 3 represents the proteins that are not the direct targets of PknB, wherein phosphorylation does not alter significantly upon depletion or complementation with the wild-type PknB. (Fig. 7e; Supplementary Table 4c). Interestingly, we observed that both cluster 2 and cluster 3 proteins were hyperphosphorylated by the mutant PknB-GM, exemplifying both specific as well as promiscuous hyperphosphorylation (Fig. 7e; Supplementary Table 4c).

Hyperphosphorylation is a result of increased activity. To confirm that mutant is indeed hyper-phosphorylated in the activation loop (Fig. 6), we raised phosphospecific antibodies that are capable of recognizing phosphorylated T171 and T173 residues (Fig. 8a). The specificity and sensitivity of the antibodies were characterized (Supplementary Fig. 5). Consistent with mass spectrometry data, we observed ~1.5-fold increase in the normalized activation loop phosphorylation in the

Fig. 4 Abrogation of ligand binding perturbs localization of PknB. a Schematic depicting hexa-His tagged extracytoplasmic region of PknB (PknB-Ec) and PknB-Ec mutants. b His-PknB wild type and mutants were purified as described in methods and 2 μg of purified PknB-Ecwt/mutant proteins were resolved on SDS-PAGE and stained with commassie. The purified proteins were used for the CD experiment in c. c CD spectrum of PknB-ECwt/mutant proteins in far-UV range (200-250 nm) CD data is depicted as MRE values (deg cm² dmol⁻¹) in the Y-axis plotted against wavelength (nm) in X-axis. d 1 μg each of His-PknB-ECwt/mutant purified for the LipidII binding experiment were resolved on SDS-PAGE and stained with coommassie as a loading control for experiments shown in e, f. e 2 nmol of mDAP or Lys containing LipidII were incubated with increasing mole:mole ratio of His-PknB-ECwt with respect to LipidII. Addition of Trypsin to degrade His-PknB-ECwt in the reaction mixture is indicated. The samples were extracted with BuOH/PyrAc and resolved on TLC to analyze the presence of extractable LipidII. LipidII trapped in a stable complex with PknB Ecwt resides in the water phase and free LipidII is extracted and migrates to a defined position on the chromatogram. The intensity of the LipidII bands relative to the control is shown. f 2 nmol of mDAP containing LipidII was incubated with increasing mole:mole ratio of His-PknB-ECwt/mutant. The samples were extracted with organic solvent and resolved on TLC to analyze the presence of extractable LipidII. Source data are provided as a Source Data file.
ligand binding mutant PknB-GM (Fig. 8b). GarA has previously been demonstrated to be a robust in vitro substrate for PknA, PknB, and PknG, hence we performed in vitro kinase assays with immunoprecipitated 3F-PknB and 3F-PknB-GM using GarA as the substrate. It is apparent from the data that PknB-GM showed higher activity compared with the PknB (Fig. 8c, d), which could be a combinatorial effect of higher phosphorylation of loop as well as the juxtamembrane residues. Even though GarA is a robust in vitro substrate for PknB, in vivo it is majorly phosphorylated by PknG on T21 residue. In agreement with this we observed that phosphorylation of GarA on T21 is unperturbed by depletion of PknB as well as complementation (Fig. 7e). On the other hand, phosphorylation of TatA on T60 was found to be PknB dependent, which showed hyperphosphorylation upon complementation with PknB-GM (Fig. 7e). In an independent

**Fig. 5 Abrogation of ligand binding perturbs localization of PknB.**

a. M. smegmatis pknB conditional mutant (mcΔB) was electroporated with pNit-GFP-PknB or pNit-GFP-PknB-GM constructs to generate mcΔB::GFP-B or mcΔB::GFP-B-GM strains. The strains were cultured in the presence of 50 ng per ml ATc and 0.2 μM IVN till A600 of ~0.8. The cultures were washed thrice with PBS to remove ATc and the cultures were grown for 6 hours in 7H9 media containing 1 μM IVN. Florescence images were captured using ×100 oil-immersion Zeiss Imager. M1 microscope. Scale bar- 5 µm.

b. 200 mcΔB::gB or mcΔB::gB-GM cell from a were analyzed for the localization of GFP-PknBwt/mutant. The aberrant puncta collectively represents cells without any distinct localization or showing puncta at regions other than poles and septa.

c. mcΔB::gB strain cultured till A600 of ~0.8 were washed and grown for 3 hours in 7H9 media containing 1 μM IVN in the presence or absence of 25 μg per ml nisin or 250 ng per ml INH. Scale bar- 5 µm.

d. Between 120 and 174 cells (as indicated) of mcΔB::gB cell from c were analyzed for the localization of GFP-PknBwt. The no puncta phenotype has been mentioned as a distinct feature from aberrant puncta in this case for better analysis of phenotype. Scale bar- 5 µm.

e. M. smegmatis mc155 strain was electroporated with GFP-PknB or GFP-PknB-GM to generate mc::gB or mc::gB-GM. Cultures of mc::gB or mc::gB-GM grown in the presence of 0.2 μM IVN were used for the FRAP analysis. The graph represents the mean fluorescence intensity plotted as a function of time by normalizing the intensity at each time point (Iₜ) to the first time point (I₀, t = 0 s) i.e., Iₜ/I₀. The values obtained were subtracted from the intensity at the time point of bleaching (Iₜ₋). The time frames from 50 to 600 s are depicted, which highlight the difference in recovery times of GFP-PknB and GFP-PknB-GM. The t₁/₂ values of recovery obtained are an indication of the time taken for half the maximal recovery after bleaching in three biologically different experiments. Statistical analysis was performed with the unpaired t-test using Graphpad software. Source data are provided as a Source Data file.
study, phosphorylation of TatA on T60 was shown to be dependent on PknA and PknB\(^{34}\).

We sought to validate the data by quantitating the peak area in an independent mass spec experiment for phosphopeptides corresponding to GarA(T21) and TatA(T60). Depletion of PknB in the absence of inducer and expression of PknB and PknB-GM was confirmed by western blots (Fig. 8e). In concurrence with the TMT data (Fig. 7), phosphopeptide corresponding to GarA-T21 showed similar peak area in Rv\(\Delta\)B & Rv\(\Delta\)B::B samples, with slight decrease in Rv\(\Delta\)B::B sample (Fig. 8f). On the other hand, phosphopeptide corresponding to TatA showed distinct hyperphosphorylation in Rv\(\Delta\)B::B-GM compared with Rv\(\Delta\)B & Rv\(\Delta\)B::B (Fig. 8f). To further substantiate the data, we performed parallel reaction monitoring (PRM) to quantitate the TatA-T60 phosphopeptide (Supplementary Figs. 7 and 8g). Quantitation of TatA-T60 phosphopeptide with respect to the corresponding heavy peptide using PRM evidently demonstrated ~2-fold (31.2 fmoles) increase in its levels in Rv\(\Delta\)B::B-GM sample compared with Rv\(\Delta\)B & Rv\(\Delta\)B::B (18.6 and 16.4 fmoles) samples. Collectively, these data demonstrate that the abrogation of ligand binding perturbs the normal regulatory circuits of PknB, resulting in aberrant localization, hyperactivation of the kinase, and indiscriminate target-specific and promiscuous phosphorylation events, leading to eventual cell death (Fig. 8h)

**Discussion**

Since the domain structure of bacterial STPKs are similar to their eukaryotic counterparts\(^{28,38,39}\), the hypotheses with respect to...
their activation and regulation are influenced by the findings in eukaryotic kinases. There are two major mechanisms by which the activity of a protein kinase is regulated: (a) by modulating protein expression levels and (b) by limiting the levels of activity through phosphorylation and dephosphorylation of the activation loop residues. In case of PknB, the following findings have cumulatively led to the formulation of the activation mechanism hypothesis presented in Fig. 1b: (i) PknB<sub>Mtb</sub> is autophosphorylated in the activation loop and this phosphorylation is necessary and sufficient for its activity in vitro<sup>37</sup>. (Supplementary Fig. 4). (ii) PknB<sub>Mtb</sub> forms both back-to-back<sup>15</sup> and front-to-front PknB<sup>14</sup> dimers, and dimerization is a pre-requisite for activation loop phosphorylations<sup>13</sup> (iii). The PASTA domain interacts with mDAP-containing muropeptides, and this domain is adequate for appropriate localization of the protein<sup>11</sup>. Interestingly, PknB protein expression levels are downregulated during dormancy<sup>9</sup> and nutrient starvation<sup>10</sup> and are upregulated during exponential growth<sup>8</sup> and resuscitation<sup>9</sup>, suggesting that PknB activity may also be regulated through the modulation of its expression pattern.

The PASTA domains across the bacterial kingdom share a highly conserved globular structure, although their sequences are diverse<sup>40</sup>. Various PASTA kinases have been demonstrated to harbor specific unique features. For example, a conserved arginine in PASTA3 has been shown to be a determining factor for ligand-binding in PrkC, the Bacillus subtilis PknB ortholog<sup>41</sup>. Similarly, three putative muropeptide binding sites in the hinge regions have been suggested to be the ligand binding pockets in StkP<sup>42</sup>. Recently, a unique citrate-binding site<sup>19</sup> and a hydrodase (LytB)-binding region<sup>16</sup> have been defined in the terminal PASTA domains of PknB and StkP, respectively, thus implicating other roles for the domain in addition to muropeptide binding. In S. pneumoniae, the terminal PASTA domain of StkP is both necessary and sufficient for its function<sup>16</sup>. In Mtb, we have previously reported that the terminal PASTA4 is absolutely essential for the function of PknB<sup>19</sup>. However, unlike in S. pneumoniae, we observe that the terminal PASTA4 is not sufficient; rather, appropriate length of the total PASTA domain is also vital (Fig. 1). The fact that PASTA4 plays an indispensible role is reinforced by the finding that SK and NQ residues in the PASTA3-4 linker region, serve as the putative ligand interacting residues (Fig. 2). In contrast to the tetra mutant that shows a drastic phenotype (Fig. 2), we observed varying levels of compromise in case of double mutants, with the data suggesting a greater role for mDAP-interacting residues compared with iGln-interacting residues (Fig. 3). Interestingly, both the double mutants as well the tetra mutant are similarly compromised in their ability to functionally complement PknB deletion ex-vivo, suggesting that PknB plays a very stringent role at the time of infection and under these circumstances even minor perturbations are not tolerated (Figs. 2 and 3).

Muropeptides are widely acknowledged as the ligands for the PASTA domains of PknB<sub>Mtb</sub><sup>11,12</sup>. However, some experimental observations suggest that they may not be the primary ligand: for example, in vitro binding affinity of muropeptides to purified PknB-Ec is relatively weak, with micromolar concentrations of muropeptide being required for the binding<sup>21,12</sup>, and the growth inhibition due to overexpression of PknB-Ec could not be ameliorated upon the addition of muropeptides mixtures<sup>43</sup>. Optimal binding occurs only when the MurNAc sugar and the stem-pentapeptide (muramyl pentapeptide) are present in the ligand. The other possible source of PknB ligand is peptidoglycan precursors such as LipidIII, also present in the periplasmic space. LipidIII possesses all the signatures of the muropeptide ligand and is also spatio-temporally localized to the same niche as PknB. In line with this, PknB<sub>Mtb</sub> has been shown to bind very efficiently with LipidIII molecules<sup>22</sup>. Our data also demonstrates efficient binding of PknB-Ec with mDAP-LipidII (Fig. 4). The weaker binding affinity compared to PknB<sub>Ec</sub> could be either due to the fact that we tested only the PknB-Ec, or could be due to the lack of additional modifications (such as amidation), known to be present in mDAP as well as iGlu residues in Mtb<sup>44</sup>. Importantly, all three PknB-Ec mutants failed to interact with the mDAP-LipidII, clearly showing abrogation of ligand binding (Fig. 4).

The extracytoplasmic PASTA domain by itself has previously been shown to be sufficient for appropriate localization of PknB, which suggested that the interaction of this domain with the ligand may dictate the localization<sup>11</sup>. The data presented in Figs. 4 and 5 of this study suggest that LipidIII may be the primary intracellular ligand, as incubating the cells with sub-lethal doses of the drug “nisin” results in an aberrant distribution pattern of PknB in the cell. Previous studies have shown that the addition of purified intracellular PASTA domain to the culture also inhibits the growth of Mtb<sup>43</sup>. Considering this fact with the data in Fig. 5, it is evident that incubating the cells with purified extracytoplasmic PASTA domain or nisin results in sequestration of available LipidIII, thus compromising the functionality of PknB. The NMR structure of PASTA domain suggests that it is a linear domain. It is not clear as to how the muropeptide moiety in the LipidIII interacts with the linear PASTA domain. It is possible that the interaction occur once the muropeptide is cleaved from the decaprenyl moiety. Alternatively, in the cellular context the PASTA domains may not be linear in structure. The regulation
Fig. 7 Ligand binding mutation causes global hyperphosphorylation of specific and non-specific targets. a TMT intensities of phosphopeptides in RvΔB-pristinamycin (PknB depleted sample, labeled with TMT 127) or in RvΔB-B (complemented with 3F-PknB, labeled with TMT 128) or RvΔB-B-GM (complemented with 3F-PknB-GM, labeled with TMT131), with respect to RvΔB+pristinamycin as the reference comparator (control strain, labeled with TMT 126). The intensities of phosphopeptides in each case were normalized with respect to the corresponding absolute protein intensities and the values were converted to log2 values. Data were sorted with respect to RvΔB-B sample and heatmap of the data were generated using online tool Morpheus. b The 257 phosphopeptides detected in TMT experiment belonged to 147 unique proteins, which were classified according to their functional category with reference to mycobrowser database. The phosphopeptides were categorized as probable PknB substrates if the TMT log2 phosphointensity upon depletion was < −0.32 and upon complementation was >1. 111 phosphopeptides were classified as probable PknB substrates, which belonged to 73 unique proteins. The 73 PknB targets were also functionally characterized according to mycobrowser database. c Normalized TMT intensities of all 111 phosphopeptides which are probable PknB targets were converted to log2 values and data were sorted with respect to RvΔB-B. Heatmap was generated using online tool Morpheus. d Normalized TMT intensities of 5 phosphopeptides each belonging to cluster 1, cluster 2, and cluster 3. e Normalized TMT intensities of PknB-dependent tyrosine phosphorylations are represented. Source data are provided as a Source Data file.
of the synthesis and continuous remodeling of PG is a fundamental process of the bacterial cell involving multiple proteins. PknBMtb is a well-known modulator of multiple substrates involved in cell division and cell wall synthesis, phosphorylating MviN and FhaA proteins among others, a molecular event that is linked to reduced PG biosynthesis45,46. Thus, a feedback loop regulation mechanism intertwining PknB activity and peptidoglycan synthesis may exist.
If the ligand binding is required for the activation of PknB, abrogation of binding should result in compromised loop phosphorylation. Contrary to this supposition, we observed significant hyperphosphorylation of activation loop and juxtamembrane regions in the PknB-GM mutant (Fig. 6). PrkCβ, localizes to the division site and interacts and phosphorylates GspB, which in turn regulates its activity by inhibiting its auto/transphosphorylations.

We hypothesize that upon binding of ligand, the kinase would be localized to the appropriate niche, whereupon a combination of other regulatory proteins/partners (including PstP) would ensure tight regulation of autophosphorylation levels, and by extension, kinase activity. In consonance, results (Fig. 8) showed that 3F-PknB-GM to be a more active kinase compared with 3F-PknB. The mis-regulated kinase results in hyperphosphorylation events targeting proteins, which are both canonical and non-canonical substrates that may be leading to aberrant functionality, leading to eventual death (Fig. 7). PRM analysis showed ~2-fold increase in the phosphorylation of the substrate of TatA on T60 residue upon complementation with PknB-GM, thus validating the data (Fig. 8).

There are few examples in literature wherein phosphomimetic mutant of PknB substrates such as InhA, KasB, PcaA, and CwMl have been shown to have significant impact on the catalytic function and/or survival defects. Complementation with phosphomimetic mutant of KasB (T334D/T336D) results in loss of acid fastness character and also causes loss of virulence. Phosphomimetic mutant of InhA (T266E) fails to rescue Msneg/Mtb inhA conditional mutant upon deletion and Pca-A (T168D)/T183D phosphomimetic mutant shows reduced bacterial survival and defective mycolic acid profile. Recently, a double phosphomimetic mutant of a major substrate of PknB, CwMl (T382D + T386D) was shown to be defective in complementing the mutant strain.

The hyperphosphorylation of the juxtamembrane domain could be linked to the hyperphosphorylation of the activation loop. Although the specific role of PknB juxtamembrane phosphorylation is unknown, it might be critical for transducing the ligand-mediated signal to the intracellular kinase domain, or for recruitment of regulatory interacting partners. Based on in vitro phosphorylation assays, Sassetti’s group suggested that PknB and PknH are master regulators that are capable of phosphorylating multiple other kinases. It is possible that promiscuous hyperphosphorylation could be due to aberrant activation of other STPKs by mislocalized PknB. Even though, we did not find any other STPKs in our final phosphoproteome, we cannot negate this prospect. These aspects need further investigation. Future PknB interactome studies may provide possible insights into how PknB activation loop and juxtamembrane phosphorylations are regulated.

**Methods**

**Generation of plasmid constructs.** pknB was amplified from the genomic DNA of Mtb and the amplicon was cloned into NdeI-HindIII sites of pNit-151 and pNit-3F vectors to generate pNit-B and pNit-3F-B, respectively. PknB up to transmembrane region (NdeI-SapI) and PASTA-234 (SapI-HindIII) were PCR amplified and the digested amplicons were ligated with Ndel-HindIII or 0.2 µM pNit-3F to generate PknB-233. Similarly, pknB-12 and PASTA-212 were ligated with pNit-3F to generate PknB-1212. PknB activation loop mutants and PASTA point mutants were generated by overlapping PCR. The codon optimized nucleotide sequence of superfolder gfp was commercially synthesized from Genechem. pknB or PknB-GM were amplified using specific primers containing SapI and HindIII sites, and gfp was amplified from pUC57-GFP using specific primers containing Ndel and SapI sites. The amplicons were digested and ligated with pNit-3F vector digested with Ndel-HindIII to generate pNit-GFP-PknB and pNit-GFP-PknB-GM. The extracto"xml:namespace prefix = "m1" ns = "http://www.mathmlcentral.org/OnTheWeb/dl/mathml" m1:lens = "Math" offset = "0" occurrence = "0" position = "0" orientation = "0" stroke = "black" stroke-width = "1" stroke-linecap = "round" stroke-linejoin = "round" style = "" title = ""/m1"" /ns/m1"" offset = "0" occurrence = "0" position = "0" orientation = "0" stroke = "black" stroke-width = "1" stroke-linecap = "round" stroke-linejoin = "round" style = "" title = ""/m1"" m1:lens = "Math" offset = "0" occurrence = "0" position = "0" orientation = "0" vspace = "0" vspace = "0" hspace = "0" hspace = "0" lang = "en-US" xml:lang = "en-US" xml:id = "m1:tag0" title = """>

**Analysis of growth, isolation of lysates, and western blot.** pknB conditional mutants, mcΔΔ and rvΔΔ (Rv-ppt-ΔB)14,15 were electroporated with pNit, pNit-3F or pST-CTG16-derived constructs (Supplementary Material). Transforms were grown in 7H9 media containing ADC (10%) and pristinamycin 1A (1.5 µg per ml; Molcon Corp) till A600 reached ~1.0. To determine the ability of mutants to rescue growth, the cultures were washed thrice with equal volumes of PBS (1X PBS with 0.05% tween 80), diluted to A600 of ~0.05, and grown for 6 days in the presence or absence of 1.5 µg per ml pristinamycin or 0.2 µM isovaleronitrile (IVN) (Sigma-Aldrich). CFUs were enumerated after 6 days of growth. To evaluate the expression of 3X-FLAG tagged wild type and mutant PknB proteins, the transforms were grown in the absence of pristinamycin and presence of IBN for 5 days and probed with anti-PknB (α-PknB), anti-phospho-PknB (α-PknB), or anti-GroEL1 (α-GroEL1) antibodies. α-PknB and α-GroEL1 antibodies were raised in rabbits and were used at 1:10,000 dilutions. The rabbit polyclonal phospho-specific antibodies were custom generated by PhosphoSolutions (Aurora, CO) using the antigenic peptide “[C]GNSVT(P)QT(P)AAV” a sequence derived from the PknB activation loop. For the α-PknB blot the membrane was blocked with 5% BSA followed by overnight incubation with α-PknB (1:250 dilution in 5% BSA) at 4 °C. Monoclonal α-FLAG M2 (Sigma-F1804) antibody was used at 1:2500 dilution.

**THP-1 infections.** Rv or RvΔB transforms grown up to A600 of ~0.8 in the presence of 1.5 µg per ml pristinamycin were washed once with PBS (1X PBS with 0.2% Triton X100) and centrifuged at 1000 rpm for 10 min. The cells were resuspended with 10% FBS (Invitrogen) supplemented with 10% FBS (Invitrogen). THP1 infections were performed with 5 × 10^7 cells seeded in 24-well plates that were differentiated with 10 nM PMA for 24 h. Differentiated cells were allowed to recover for 12 h prior infection with Mtb at 1:10 or 1:4 MOI. The extracellular bacteria were removed 4 h post infection by washing the cells thrice with sterile PBS, and this was considered as the zero time point. 0.2 µM IVN was added in the media wherever RvΔB-ΔBwt/mutant transforms were used for the infection. Cells were lysed in 1 ml of 0.1% triton-X100 at 0 and 72 h post infection and CFUs were enumerated on 7H11 plates containing 1.5 µg per ml pristinamycin.
Identification of the muropeptide binding site. The coordinates of the PASTA domains of mycobacterial PknB were obtained from the NMR structure (2KUI)18. The PASTA-3-4 domains were docked using HADDOCK web server55,56. Surface exposed hydrophobic residues – Met356, Val593, Val604, and Ile619 – were treated as active residues. To investigate possible binding modes of muropeptide to the dimeric model of PASTA-3-4 domains, muropeptide (N-acetylglucosaminyl-N-acetylmuramyl-L-alanyl-D-isoglutamic acid-Ala-alanine) was docked using AutoDock47. Coordinates of the muropeptide were generated using the Babel program of Open Babel package58 and PyMOL software was used for visualization (The PyMOL Molecular Graphics System, Version 1.5.0.4 Schrödinger). The ligand was allowed maximum possible flexibility with 27 rotatable bonds and Gasteiger atomic charges assigned to it. Coordinates of dimeric model of PASTA-3-4 domains were kept rigid, and the ligand was allowed to explore the entire surface through the construction of a grid box, within the docking grid of 60 × 45 × 29 Å3 with a grid spacing of 0.475. The docking simulation involved 27,000 generations, and population size of 150 in each genetic algorithm (GA) run. At each evaluation step of the rescoring function, the best individual was chosen from each iteration of the 250 Lamarckian search GA runs. The rate of genetic mutations and cross-overs were set to 0.02 and 0.8, respectively. The most stable binding mode of the ligand had a free energy of −4.29 kcal/mol. The ligand–receptor interface on the PASTA dimer was defined as residues from each of the PASTA-3-4 monomers, which had at least one atom within 5 Å of any atom of the ligand.

Purification and circular dichroism. E. coli BL21(DE3) (Strategene) cells transformed with the appropriate recombinant plasmid (pET28a- pknB-Ec, pET28a-pknB-Ec-M, and pET28a-pknB-Ec-GM) were grown in 1 L LB medium (50 µg per ml kanamycin) at 37 °C. At an A600 of 0.6, IPTG was added at a final concentration of 1 mM to induce expression of the recombinant protein at 30 °C. The purification of His6-tagged protein was described as described earlier59. Jasco J-815 spectropolarimeter was used for analyzing the ellipticity changes for each protein in far-UV (195–250 nm) wavelength range at 20 °C. The ellipticity changes were converted into MRE (Molar residual ellipticity) values and plotted against wavelength using Sigmaplot version 10.0.

In vitro LipidII interaction studies. In vitro LipidII binding assay was performed using purified His6-pknB-Ec or PknB-Ecmutatant, His6-pknB-Ec or PknB-Ecmutatant were incubated with 2 nmol LipidII at molar ratios ranging from 0.5 to 8:1 (PknB:LipidII) in 50 mM Tris/HCi pH 7.0, 5 mM MgCl2, 60 min at 30°C. The reaction mixture was extracted with an equal volume of butanol/pyridine acetate (95% Water, 5% Acetonitrile, 0.1% Formic acid). All experiments were performed with pRS score >50 and PEP <0.05 were only considered for further analysis. The p-sites were focused and one end of each bacteria (covering 30 °21, 22) to the fth frame (50 s) and the fluorescence recovery was monitored up to 600 s from at least three biologically independent experiments. The mean relative intensity obtained was plotted as a function of time (in seconds) to calculate the rate of recovery (τ1/2, the time taken to attain half of the maximum intensity) in the bleached areas of the bacteria and plotted using the Non-linear fit equation in Prism 6.0.

Immunoprecipitation and in vitro kinase assays. RvΔB strains electroporated with pNiT3E-PknBWT/GM were incubated at A600 of 0.05 and were grown in the absence of pristinamycin and presence of 0.2 µM lVAN for 72 h. Cultures were centrifuged at 4000 rpm for 5 min and the pellets were resuspended in PBS (pH 7.4) and washed at 13,000 rpm in g PBS. Cells were lysed using 0.1 mm zincium beads (Biospec) for 10 min with 1 min intervals on ice using Biospec minibeadbeater. The lysates were clarified at 13,000 rpm at 4 °C and the concentration of supernatant whole-cell lysates (WCLs) were estimated. 0.1 mg of WCL was used for FLG immunoprecipitation (IP) using FlG-GFP antibodies and the 3F-PknB or 3F-PknB-GM were eluted with 0.1 M glycine (pH 2.2) and the eluate was neutralized by addition of 1/10th vol 1 M Tris-HCl (pH 8.0). The IPed proteins were resolved on SDS-PAGE and subjected to western blotting. The western blots were probed with 1:10,000 & 1:25 dilution of α-PknB and α-p-PknB antibodies. The ratio of phospho-PknB band to that of PknB was measured as described above (Figure S8).

TMT labeling and MS/MS analysis. Cultures pellets were resuspended in SDS lysis buffer (2% SDS, 50 mM Tris(hydroxymethyl)aminomethane bicarbonate buffer (TBAE), Sigma), PhosSTOP tablets (Roche) in PBS (pH 7.4) and resuspended in 5% glycerol (vglbe) (vglbe weight weight in gbuffer) ratio. Samples were heated at 95°C for 20 min followed by 10 cycles of beadbeating. The lysates were clarified and the concentration of supernatant WCLs were estimated with the help of BCA reagent (Pierce). WCL (250 µg) from each samples shown in Fig. 6c were used. TMT labeling was performed using 10 mM tris (2-carboxyethyl) phosphine at 55 °C for 1 h and alkylating using 10 mM iodoacetamide for 30 min at 25 °C. Samples were acetic acid precipitated and the pellet was resuspended in 100 µM TEAB and digested with 6 µg Trypsin (Promega) for 16 h at 37 °C. TMT labeling (Thermo Fisher Scientific) was performed as per manufacturer’s instructions. Peptides from RvΔB−V::pristinamycin; RvΔB−V::pristinamycin; RvΔB−V::pristinamycin; and RvΔB−V::pristinamycin were resolved on 12% SDS-PAGE, transferred to nitrocellulose membrane and autoradiographed. The bands corresponding to pGARa were excised, incubated overnight in the scintillation cocktail (spectrochem) and counts per minute (CPM) were determined using Perkin Elmer microbeta TriLux 1450 LSC & Luminescence counter.

TMX labeling and MS/MS analysis. Cultures pellets were resuspended in SDS lysis buffer (2% SDS, 50 mM Tris(hydroxymethyl)aminomethane bicarbonate buffer (TEAB), Sigma), PhosSTOP tablets (Roche) in PBS (pH 7.4) and resuspended in 5% glycerol (vglbe) (vglbe weight weight in gbuffer) ratio. The desalted samples for the total proteome analysis as well as the enriched peptide samples for phosphoproteomics analysis were reconstituted in Buffer A (95% Water, 5% Acetonitrile, 0.1% Formic acid). All experiments were performed using EASY-nLC system (Thermo Fisher Scientific) coupled to LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific) equipped with nanoelectrospray ion source. A 10-cm PicoFrit Self-Pack microcapillary column (New Objective) was used to resolve the peptide mixture and the peptides were eluted at a flow rate of 300 nl per min for 120 min. The acetonitrile containing (0.1% formic acid) gradient used for the run was 0–40% for 70 min, 40–80% for 10 min, 80% for 5 min, and 0% for last 25 min. LCQ orbitrap was used for the full MS scan. The peptides were dissociated with both HCD and CID for better MS/MS spectra. The collision energy induced dissociation of X ion precursors was performed at 35 for CID and 40 for HCD. Both MS and MS/MS data were acquired using scan range of 20–2000 M/Z ratios. The dynamic exclusion was performed with TMT 6 plex method. TMT labeling and MS/MS analysis was performed on a MALDI-TOF/TOF (After TMT labeling and MS/MS analysis was performed on a MALDI-TOF/TOF) instrument. TMT labeling and MS/MS analysis was performed on a MALDI-TOF/TOF instrument.
common proteins represented in the total proteome and phosphoproteome were analyzed using venn diagram generating tool venny5. The phosphophotensity ratio of each phosphopeptide was normalized against the whole-protein intensity ratio of the corresponding protein. The normalized phosphophotensity from three replicates was averaged and the average phosphophotensity ratios were converted into log2 values. Heatmap was generated using online tool Morpheus (https://software.broadinstitute.org/morpheus/). The functional characterization of individual phosphopeptides identified in the study was done using mycobrowser database. The TMT-phosphoproteomics data has been submitted to the ProteomeXchange Consortium (http://proteomcentral.proteomeexchange.org) and can be accessed using data set identifier PXD012180 via the PRIDE partner repository.

Targeted proteomics. Synthetic isotopically labeled (SIL) peptides (Maxi Spiked-Tides QL, AAA-peptides) with C-terminus 15N and 13C-labeled arginine were purchased from JPT Peptide Technologies GmbH (Berlin, Germany). The synthetic peptides were resuspended in 0.1% formic acid at a final concentration of 1.7 µg per µl and working concentrations of 0.6, 6, 60, and 600 fmol per µl were prepared. All the samples were analyzed using EASY-nLC 1000 system (Thermo Fisher Scientific, San Jose, CA). Raw data were processed using data-dependent acquisition method with a non-scheduled parallel reaction monitoring (PRM) event targeting the doubly charged precursor ion of the SIL peptides. The PRM event was performed with an orbitrap resolution of 17500 at (m/z 200), a target AGC value of 1e6, and maximum fill times of 100 ms. Fragmentation was acquired with a normalized collision energy of 27 eV and MS/MS scan range of 180-2000. A target AGC value of 1e4 was used in data analysis (Fig. 8) are submitted as Supplementary Data 4-6. Images of unprocessed western blots, coomassie stained gels, TLC images, and autoradiograms used in the study are provided in Supplementary Fig. 8. The source data underlying Figs. 1a, f, 2d, 3c, 4g, 5b–d, 6c, 7d, e, and 8d are provided as a Source Data file. The source data underlying Fig. 6 are provided in Supplementary Table 1.

Received: 5 June 2018 Accepted: 28 February 2019
Published online: 15 March 2019

References

1. Jones, G. & Dyson, P. Evolution of transmembrane protein kinases implicated in coordinating remodeling of gram-positive peptidoglycan: inside versus outside. J. Bacteriol. 188, 7470–7476 (2006).
2. Manuse, S., Fleurié, A., Zucchiini, L., Lesterlin, C. & Grangeasse, C. Role of eukaryotic-like serine/threonine kinases in bacterial cell division and morphogenesis. FEBS Microbiol. Rev. 40, 41–56 (2016).
3. Sassetti, C. M., Boyd, D. H. & Rubin, E. J. Comprehensive identification of conditionally essential genes in mycobacteria. Proc. Natl Acad. Sci. USA 98, 12712–12717 (2001).
4. Fernández, P. et al. The Ser/Thr protein kinase PknB is essential for sustaining mycobacterial growth. J. Bacteriol. 188, 7778–7784 (2006).
5. Chawla, Y. et al. Protein kinase B (PknB) of Mycobacterium tuberculosis is essential for growth of the pathogen in vitro as well as for survival within the host. J. Biol. Chem. 289, 13858–13875 (2014).
6. Baer, C. E., Lavarone, A. T., Alber, T. & Sassetti, C. M. Biochemical and spatial coincidence in the provision Ser/Thr protein kinase interaction network of Mycobacterium tuberculosis. J. Biol. Chem. 289, 20422–20433 (2014).
7. Richard-Greenblatt, M. & Av-Gay, Y. Epigenetic phosphorylation control of Mycobacterium tuberculosis infection and persistence. Microbiol. Spectr. 5, 10.1128/microbiolspec.TBYB2-0005-2015 (2017).
8. Kang, C. M. et al. The Mycobacterium tuberculosis serine/threonine kinases PknA and PknB: substrate identification and regulation of cell shape. Genes Dev. 19, 1692–1704 (2005).
9. Ortega, C. et al. Mycobacterium tuberculosis Ser/Thr protein kinase B mediates an oxygen-dependent replicative switch. PLoS Biol. 12, e1001746 (2014).
10. Betts, J. C., Lukey, P. T., Robb, L. C., McAdam, R. A. & Duncan, K. Evaluation of a nutrient starvation model of Mycobacterium tuberculosis persistence by gene and protein expression profiling. Mol. Microbiol. 43, 717–731 (2002).
11. Mir, M. et al. The extracytoplasmic domain of the Mycobacterium tuberculosis Ser/Thr kinase PknB binds specific muropeptides and is required for PknB localization. PLoS Pathog. 7, e1002182 (2011).
12. Wang, Q. et al. A comprehensive study of the interaction between peptidoglycan fragments and the extracellular domain of Mycobacterium tuberculosis Ser/Thr kinase PknB. ChemBiochem 18, 2094–2098 (2017).
13. Lombana, T. N. et al. Allosteric activation mechanism of the Mycobacterium tuberculosis receptor Ser/Thr protein kinase, PknB. Structure 18, 1667–1677 (2010).
14. Miezckowski, C., Lavarone, A. T. & Alber, T. Auto-activation mechanism of the Mycobacterium tuberculosis PknB receptor Ser/Thr kinase. EMBO J. 27, 3186–3197 (2008).
15. Wehenkel, A. et al. The structure of PknB in complex with mitoxantrone, an ATP-competitive inhibitor, suggests a mode of protein kinase regulation in mycobacteria. FEBS Lett. 580, 3018–3022 (2006).
16. Zucchiní, L. et al. PASTA repeats of the protein kinase StkP interconnect cell constriction and separation of Streptococcus pneumoniae. Nat. Microbiol. 3, 197–209 (2018).
17. Forti, F., Crosta, A. & Ghisotti, D. P. Rstaminycin-inducible gene regulation in mycobacteria. J. Bacteriol. 140, 270–277 (2009).
18. Bärth, P., Mukamolova, G. V., Roumestand, C. & Cohen-Gonsaud, M. The structure of PknB extracellular PASTA domain from mycobacterium tuberculosis suggests a ligand-dependent kinase activation. Structure 18, 606–615 (2010).
19. Prigozhin, D. M. et al. Structural and genetic analyses of the Mycobacterium tuberculosis Protein Kinase B sensor domain identify a potential ligand-binding site. J. Biol. Chem. 291, 22961–22969 (2016).
20. Crick, D. C., Mahapatra, S. & Brennan, P. J. Biosynthesis of the arabinogalactan-peptidoglycan complex of Mycobacterium tuberculosis. Glycobiology 14, 1078–1148 (2001).
21. Mahapatra, S. et al. Mycobacterial lipid II is composed of a complex mixture of modified muramyl and peptido moieties linked to decaprenyl phosphate. J. Bacteriol. 187, 2747–2757 (2005).
22. Hardt, P. et al. The cell wall precursor lipid II acts as a molecular signal for the sensing of Mycobacterium tuberculosis PknB of Staphylococcus aureus. Int. J. Med. Microbiol. 307, 1–10 (2017).
23. Jani, C. et al. Regulation of peptidoglycan biosynthesis by Wag31 phosphorylation in Mycobacteria. BMC Microbiol. 10, 327 (2010).
24. Breukink, E. et al. Use of the cell wall precursor lipid II by a pore-forming peptide antibiotic. Science 286, 2361–2364 (1999).
25. Wiedmann, I. et al. Specific binding of nisin to the peptidoglycan precursor lipid II combines pore formation and inhibition of cell wall biosynthesis for potent antibiotic activity. J. Biol. Chem. 276, 1772–1779 (2001).
26. Hsu, S. T. et al. The nisin-lipid II complex reveals a pyrophosphate cage that provides a blueprint for novel antibiotics. Nat. Struct. Mol. Biol. 11, 963–967 (2004).
27. Duran, R. et al. Conserved autophosphorylation pattern in activation loops and juxtamembrane regions of Mycobacterium tuberculosis Ser/Thr protein kinases. Biochem. Biophys. Res. Commun. 333, 858–867 (2005).
28. Young, T. A., Delagoutte, B., Endrizzi, J. A., Falick, A. M. & Alber, T. Structure of Mycobacterium tuberculosis PknB supports a universal activation mechanism for Ser/Thr protein kinases. Nat. Struct. Biol. 10, 168–174 (2003).
29. Prisci, S. et al. Extensive phosphorylation with overlapping specificity by Mycobacterium tuberculosis serine/threonine protein kinases. Proc. Natl Acad. Sci. USA 107, 7521–7526 (2010).
30. Fortuin, S. et al. Phosphoproteomics analysis of a clinical Mycobacterium tuberculosis H37Ra and H37Rv strains of Mycobacterium tuberculosis. Proc. Natl Acad. Sci. USA 107, 1632–1645 (2010).
31. Sajid, A. et al. Interaction of Mycobacterium tuberculosis elongation factor Tu with GTP is regulated by phosphorylation. J. Bacteriol. 193, 5347–5358 (2011).
32. Gupta, M. et al. HepII, a nucleoid-associated protein of Mycobacterium tuberculosis, is modified by serine/threonine protein kinases in vivo. J. Bacteriol. 196, 2646–2657 (2014).
33. Carette, X. et al. Multisystem Analysis of Mycobacterium tuberculosis Reveals Kinase-Dependent Remodeling of the Pathogen-Environment Interface. MBio 7(6), e02333-17 (2018).
34. Kusebauch, U. et al. Mycobacterium tuberculosis supports protein tyrosine phosphorylation. Proc. Natl Acad. Sci. USA 111, 9265–9270 (2014).
36. O’Hare, H. M. et al. Regulation of glutamate metabolism by protein kinases in mycobacteria. *Mol. Microbiol.* **70**, 1408–1423 (2008).
37. Khan, M. Z. et al. Protein kinase G confers survival advantage to *Mycobacterium tuberculosis* during latency-like conditions. *J. Biol. Chem.* **292**, 16093–16108 (2017).
38. Zhang, C. C. Bacterial signalling involving eukaryotic-type protein kinases. *Mol. Microbiol.* **20**, 9–15 (1996).
39. Orioli, D. E., M. P., R., B., & Alzari, P. M. Crystal structure of the catalytic domain of the PknB serine/threonine kinase from *Mycobacterium tuberculosis*. *J. Biol. Chem.* **278**, 13094–13100 (2003).
40. Yeats, C., Finn, R. D. & Bateman, A. The PASTA domain: a beta-lactam-binding domain. *Trends Biochem. Sci.* **27**, 438 (2002).
41. Shah, I. M., Laaberki, M. H., Popharn, D. L. & Dworkin, J. A. eukaryotic-like Ser/Thr kinase signals bacteria to exit dormancy in response to peptidoglycan fragments. *Cell 135*, 486–496 (2008).
42. Righino, B. et al. Structural model of the full-length Ser/Thr protein kinase Skp from *S. pneumoniae* and its recognition of peptidoglycan fragments. *J. Biomol. Struct. Dyn. 7*, 1–14 (2017).
43. Turapov, O. et al. The external PASTA domain of the essential serine/threonine protein kinase PknB regulates mycobacterial growth. *Open Biol.* **5**, 150025 (2015).
44. Mahapatra, S., Crick, D. C., McNeil, M. R. & Brennan, P. J. Unique structural features of the peptidoglycan of *Mycobacterium leprae*. *J. Bacteriol.* **190**, 655–661 (2008).
45. Ge, C. L. et al. A phosphorylated pseudokinase complex controls cell wall synthesis in mycobacteria. *Sci. Signal*. **5**, r27 (2012).
46. Turapov, O. et al. Two faces of CwpM, an essential PknB substrate, in mycobacterial cell wall. *Cell Rep.* **25**, 57–67 e55 (2018).
47. Pompeo, F., Fouquier, E., Serrano, B., Grangeasse, C. & Galinier, A. Phosphorylation of the cell division protein GpsB regulates PknB kinase activity through a negative feedback loop in *Bacillus subtilis*. *Mol. Microbiol.* **97**, 139–150 (2015).
48. Khan, S. et al. Phosphorylation of enoyl-acyl carrier protein reductase InhA impacts mycobacterial growth and survival. *J. Biol. Chem.* **285**, 37860–37871 (2010).
49. Molle, V. et al. Phosphorylation of InhA inhibits mycolic acid biosynthesis and growth of *Mycobacterium tuberculosis*. *Mol. Microbiol.* **78**, 1591–1605 (2010).
50. Vlček, C. et al. Phosphorylation of KasB regulates virulence and acid-fastness in *Mycobacterium tuberculosis*. *PLoS Pathog.* **10**, e1004115 (2014).
51. Corrales, R. M. et al. Phosphorylation of mycobacterial PcaA inhibits mycolic acid cyclopropanation: consequences for intracellular survival and for phagosomal maturation block. *J. Biol. Chem.* **287**, 26187–26199 (2012).
52. Pandey, A. K. et al. Nitrile-inducible gene expression in mycobacteria. *Tuberculosis (Edinb.*) **89**, 12–16 (2009).
53. Nagarajan, S. N. et al. Protein kinase A (PknA) of *Mycobacterium tuberculosis* is independently activated and is critical for growth in vitro and survival of the pathogen in the host. *J. Biol. Chem.* **290**, 9626–9645 (2015).
54. Puri, R. V., Reddy, P. V. & Tyagi, A. K. Secreted acid phosphatase (SapM) of *Mycobacterium tuberculosis* is indispensable for arresting phagosomal maturation and growth of the pathogen in guinea pig tissues. *PLoS ONE* **8**, e70514 (2013).
55. Domínguez, C., Boelens, R. & Bonvin, A. M. HADDOCK: a protein-protein docking approach based on biochemical or biophysical information. *J. Am. Chem. Soc.* **125**, 1731–1737 (2003).
56. de Vries, S. J. et al. HADDOCK versus HADDOCK: new features and performance of HADDOCK2.0 on the CAPRI targets. *Proteins* **69**, 726–733 (2007).
57. Morris, G. M. et al. Automated docking using a Lamarckian genetic algorithm and an empirical binding free energy function. *J. Comput. Chem.* **19**, 1639–1662 (1998).
58. O’Boyle, N. M. et al. Open Babel: an open chemical toolbox. *J. Cheminform.* **3**, 33 (2011).
59. Jain, R., Watson, U., Vasudevan, L. & Saini, D. K. ERK activation pathways downstream of GPCRs. *Int. Rev. Cell Mol. Biol.* **338**, 79–109 (2018).
60. Schneider, C. A., Rasband, W. S. & Eliceiri, K. W. NIH Image to Image: 25 years of image analysis. *Nat. Methods* **9**, 671–675 (2012).
61. Dephoure, N. & Gygi, S. P. A solid phase extraction-based platform for rapid phosphoproteomic analysis. *Methods* **54**, 379–386 (2011).
62. Shaik, A. A. et al. Phosphoprotein network analysis of white adipose tissues unveils deregulated pathways in response to high-fat diet. *Sci. Rep.* **6**, 25844 (2016).
63. Oliveros, J. C. VENNY. An interactive tool for comparing lists with Venn’s diagrams (2007–2015).
64. Vizcaino, J. A. et al. 2016 update of the PRIDE database and its related tools. *Nucleic Acids Res.* **44**, D447–D456 (2016).

Acknowledgements

This work was supported by the funding provided by Department of Biotechnology, Government of India (DST/INT/TUNISIA/P-17/2017 & BT/PR13522/COE/34/27/2015) to V.K.N.; P.K. is a Senior Research Fellow. We thank Dr. Francesca Forti for kindly gifting RvB14 conditional mutant. We thank the Central Mass Spec facility of NII and Mrs. Shanta Sen for her support in managing the facility. We thank Dr. Swati Saha for critical reading of the manuscript. We are grateful to Dr. Sudeepa Rajan for her help with CD experiments and Dr. Savita Lochab, Dr. Mansoor Hussain for their assistance in microscopy.

Author contributions

P.K., M.R., B.M., U.W., N.P.D., Y.C., S.S., and K.S. were involved in execution of experiments, data acquisition, analysis, and providing raw data for figures. T.S., G.D.J., D.S.M., F.G., and V.K.N. provided the scientific overview. P.K. and V.K.N. were involved in overall experimental design, manuscript writing, and making figures. V.K.N. guided the study.

Additional information

Supplementary Information accompanies this paper at https://doi.org/10.1038/s41467-019-09223-9.

Competing interests: The authors declare no competing interests.

Reprints and permission information is available online at http://npg.nature.com/reprintsandpermissions/

Journal peer review information: *Nature Communications* thanks Nelson da Cruz Soares and Robert Husson for their contribution to the peer review of this work. Peer reviewer reports are available.

Publisher’s note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.