Novel Role of Phosphorylation-Dependent Interaction between FtsZ and FipA in Mycobacterial Cell Division

Kamakshi Sureka, Tofajjen Hossain, Partha Mukherjee, Paramita Chatterjee, Pratik Datta, Manikuntala Kundu, Joyoti Basu

Department of Chemistry, Bose Institute, Kolkata, India

Abstract

The bacterial divisome is a multiprotein complex. Specific protein-protein interactions specify whether cell division occurs optimally, or whether division is arrested. Little is known about these protein-protein interactions and their regulation in mycobacteria. We have investigated the interrelationship between the products of the Mycobacterium tuberculosis gene cluster Rv0014c-Rv0019c, namely PknA (encoded by Rv0014c) and FtsZ-interacting protein A, FipA (encoded by Rv0019c) and the products of the division cell wall (dcw) cluster, namely FtsZ and FtsQ. M. smegmatis strains depleted in components of the two gene clusters have been complemented with orthologs of the respective genes of M. tuberculosis. Here we identify FipA as an interacting partner of FtsZ and FtsQ and establish that PknA-dependent phosphorylation of FipA on T77 and FtsZ on T343 is required for cell division under oxidative stress. A fipA knockout strain of M. smegmatis is less capable of withstanding oxidative stress than the wild type and showed elongation of cells due to a defect in septum formation. Localization of FtsQ, FtsZ and FipA at mid-cell was also compromised. Growth and survival defects under oxidative stress could be functionally complemented by fipA of M. tuberculosis but not its T77A mutant. Merodiploid strains of M. smegmatis expressing the FtsZ(T343A) showed inhibition of FtsZ-FipA interaction and Z ring formation under oxidative stress. Knockdown of FipA led to elongation of M. tuberculosis cells grown in macrophages and reduced intramacrophage growth. These data reveal a novel role of phosphorylation-dependent protein-protein interactions involving FipA, in the sustenance of mycobacterial cell division under oxidative stress.

Introduction

Cell growth and division involves exquisite temporal and spatial regulation of a succession of events which include choice of the site of division, positioning of the Z-ring at mid-cell, and formation of the septum [1–3]. The fidelity of cell division occurring at mid-cell depends on the assembly of a macromolecular complex at the site of division involving recruitment of proteins in a hierarchical manner [4–6]. In most Gram-negative and Gram-positive bacteria, genes whose products are involved in cell division, cluster in a chromosomal region termed the dcw (division and cell wall) cluster [7]. The principal player in driving cytokinesis is FtsZ, a GTP-binding protein considered to be the bacterial counterpart of eukaryotic tubulin [8,9]. It is encoded by a gene within the dcw cluster. It forms the Z-ring [10] which serves as a cytoskeletal scaffold for the sequential recruitment and assembly of the multiprotein complex that comprises the divisome [11,12]. Mycobacteria share many components of the cell division machinery identified in other bacteria. However, it is likely that there are several unique features in the mechanism of cell division in mycobacteria, considering that assisters of Z ring formation such as FtsA and ZipA have not been identified, nor have counterparts of the MinCD system which ensures that division occurs at midcell. For example, it is possible that direct interaction between FisZ and FisW stabilizes FisZ at the membrane and links cell division to septal peptidoglycan biosynthesis [13,14].

A family of 11 serine/threonine protein kinases (STPKs) are likely sensors of environmental signals in mycobacteria [15,16]. In spite of an interest in this family of kinases in recent years, there is limited understanding of the signals that they sense and the consequences of such sensing. The STPKs of mycobacteria with the exception of PknG and PknK, have extracellular C-terminal sensory domains and intracellular N-terminal kinase domains. Cell wall biogenesis, cell division, central metabolic processes such as the TCA cycle and gene expression, are among the growing body of processes that are regulated by STPK-mediated phosphorylation in mycobacteria [16–18].

Both PknA and PknB are involved in regulation of cell shape [19,20]. These two kinases reside in a genomic region (encompassing the ORFs Rv0014c-Rv0019c in Mycobacterium tuberculosis H37Rv) that is conserved across all species of mycobacteria for which genome sequences are available. This region also encodes PstP, the cognate phosphatase of PknA and PknB; PBPA, a penicillin-binding transpeptidase [19], and RodA, a putative
FtsZ protein A (FipA). The FipA-FtsZ complex. FtsZ fails to localize at mid-cell in knock down of amino acid residue T77 and FtsZ on T343. We demonstrate that interaction is dependent on PknA which phosphorylates FipA on encoded by regulation of cell division in mycobacteria. This report focuses on the forkhead-associated domain (FHA)-containing protein encoded by Rv0019c. A role for this gene product in cell division was tested by knocking out its counterpart in M. smegmatis. The increased susceptibility of the mutant to oxidative stress and the altered cell morphology, further supported our contention that this particular FHA-domain-containing protein could regulate cell division. Using a variety of biochemical and genetic approaches, we demonstrate that in mycobacteria, FtsZ, FtsQ, FtsU and the product of the ORF Rv0019c, form a ternary complex. In view of its ability to interact with FtsZ, we name this gene product, FtsZ-interacting protein A (FipA). The FipA-FtsZ interaction is dependent on PknA which phosphorylates FipA on amino acid residue T77 and FtsZ on T343. We demonstrate that knock down of pknA impairs the formation of the FtsZ-FtsQ-FipA complex. FtsZ fails to localize at mid-cell in fipA knock out M. smegmatis cells under oxidative stress and localization is rescued by complementation with FipAMSMEG. An important role of FipA is further suggested by the fact that depletion of FipA leads to reduced growth of M. tuberculosis in macrophages.

Results

Effect of Inactivation of FipAMSMEG and Knock Down of FipA in M. tuberculosis

The genomic region encompassing the ORFs Rv0014c to Rv0019c (Figure S1A) of M. tuberculosis, is conserved across mycobacterial species. Its role in cell division and the function of some of the genes encoded by this region has been conjectured over the years. The genes Rv0014c-Rv0016c are organized in an operon. The ORF Rv0016c encodes PBPA, a transpeptidase that regulates cell morphology in a manner dependent on phosphorylation by the product of the ORF Rv0014c, PknB [19]. The product of the ORF Rv0015c, PknA phosphorylates Wag31 thereby regulating cell morphology [20]. Rv0015c encodes PstP, a phosphatase which dephosphorylates substrates such as phospho-PBPA. The function of FipA (the product of the adjacent transcriptional unit Rv0019c), a putative FHA protein, remains obscure. Computational analysis of genome-wide functional linkages, suggests that FipA has a role in cell envelope biosynthesis in M. tuberculosis [24]. Considering that FipA is conserved across pathogenic and non-pathogenic mycobacterial species, we chose M. smegmatis, the fast-growing non-pathogenic strain in order to understand the role of FipA in mycobacterial physiology. The deduced amino acid sequences of FipAMSMEG and FipAMTB (Figure S1B) show that the proteins are 80% identical in amino acid sequence. To test the role of FipA in cell growth and division, fipA was inactivated at its native locus in M. smegmatis. The absence of FipA was confirmed by Western blotting (Figure S2A). We also confirmed by Western blotting that the expression of PknB, PknA, PBPA and PsP encoded by neighboring genes, was not affected (Figure S2A). The mutated strain will be referred to as FipA-KO.

The morphology and the growth rates of wild type M. smegmatis mc^155 and FipA-KO were compared. FipA-KO exhibited reduced growth compared to the wild type in Middle Brook 7H9 broth supplemented with Tween 80. The wild type reached mid-log phase at 20 h, whereas the mutant reached mid-log phase at 24 h (Fig. 1A). At stationary phase, the growth of the mutant was lower than that of the wild type. Considering the lowered growth of the mutant particularly at stationary phase, we speculated that it was possibly less capable of handling stressful conditions than the wild type. We tested its susceptibility to conditions of stress. FipA-KO was more susceptible to H2O2 stress than the wild type (Fig. 1B). In order to test whether FipA is required for cell division under H2O2-induced oxidative stress, we determined cell lengths in the wild type and FipA-KO. In the absence of exogenous stress, cells of the wild type and FipA-KO were of similar length. However, approximately 40% of the cells were >6 μm in length in the knockout strain subjected to oxidative stress (Fig. 1C). In comparison, <5% of the wild type cells were >6 μm in length under similar oxidative stress. These results suggest that FipA is required to sustain cell division in bacteria subjected to oxidative stress. In order to assess the ability of FipA of M. tuberculosis to complement FipA-KO, a single copy of fipA of M. tuberculosis was integrated into the chromosome of FipA-KO. Complementation of FipA was confirmed by Western blotting (Figure S2A). FipA-KO complemented with fipAMTB was visualized by microscopy before and after subjecting to oxidative stress. Complementation with fipAMTB restored the cell length of FipA-KO to resemble that of the wild type (Fig. 1C). In addition, growth defect was rescued (Fig. 1A), and the complemented strain could withstand oxidative stress (Fig. 1B) to an extent similar to the wild type. This also confirmed that the difference in phenotypic behavior between the wild type and FipA-KO was attributable specifically to the absence of FipA.

The division defect occurring after H2O2 treatment in FipA-KO could possibly be due to induction of the SOS response. RecA and LexA are two principal proteins involved in the SOS response [25,26]. We checked the transcription levels of recA and lexA after treatment with H2O2. No increase in lexA or recA was observed in either the wild type or FipA-KO or FipA-KO complemented with fipAMTB (Figure S2B, C). O’ Sullivan et al. [27] have reported that recA and lexA transcription increases after exposure of M. tuberculosis to mitomycin C, a compound that alkylates and crosslinks DNA and induces the SOS response. Mitomycin C exposure was therefore used as a positive control for induction of the SOS response. In this case, recA and lexA transcription increased in the wild type, FipA-KO and FipA-KO complemented with fipAMTB (Figure S2B, C). Taken together, this suggests that the division defect in FipA-KO cells after H2O2 treatment is not due to induction of the SOS response.

In order to assess the role of FipA in M. tuberculosis growing in macrophages, we knocked down fipA of M. tuberculosis (Fig. 1D). This knock down compromised the ability of M. tuberculosis to multiply within macrophages after two days (Fig. 1E). This argued in favor of a role of FipA in mycobacterial multiplication in macrophages. A defect in cell division in the fipA-knocked down
cells in macrophages was confirmed by the observation that the percentage of macrophage-grown cells over 6 μm in size was consistently higher in fipA-knocked down cells compared to the wild type over a period of 6 days (Fig. 1F, G). This suggests that FipA is required to sustain mycobacterial cell division in macrophages.

FipA Interacts with FtsZ In Vivo

In view of the fact that FtsZ provides the driving force of cytokinesis by forming the Z-ring at the septum [1], and that knock out of fipA is associated with a septation defect, we tested the possibility that FtsZ and FipA are interacting partners in vivo. In order to analyze the interaction of FipA with FtsZ, FtsZ was immunoprecipitated from cell lysates of M. tuberculosis or M. smegmatis, and the presence of FipA in the immunoprecipitates was tested using anti-FipA antibody. FtsZ immunoprecipitates pulled down endogenous FipA (Fig. 2A). In order to understand the role of FHA domain in the interaction of FipA with FtsZ, FipA-KO was complemented with wild type fipAMTB or fipAMTB harboring mutations in conserved amino acid residues of the FHA domain encompassing amino acid residues P82 to V132. FHA domain mutants S101A, H104A and N123A of FipA were impaired in their ability to interact with FtsZ in vivo (Fig. 2B). On the other hand, the FipA (D118A) mutant retained the ability to interact with FtsZ. These results suggest that the FHA domain of FipA plays a role in its interaction with FtsZ in vivo.

FHA domains are known to interact with ser/thr kinases [21]. Therefore, we tested whether FipA of M. smegmatis (FipAMSmeg, encoded by MSMEG_0034), is phosphorylated in vivo. FipA was immunoprecipitated from cell lysates of M. tuberculosis or M. smegmatis followed by Western analysis using anti-phosphothesine antibody. The results confirmed that FipAMTB and FipAMMSMEG are phosphorylated in vivo on threonine (Fig. 3A). Incubation of immunoprecipitates with purified PstP resulted in dephosphorylation of FipA.
aggarose-bound FipA<sub>MTB</sub> and FipA<sub>MSMEG</sub> (Fig. 3A). In a reverse experiment, capture of proteins from lysates of <i>M. smegmatis</i> on phosphothreonine agarose (B), left untreated (-) or treated (+) with S-tagged PstP<sub>post</sub>, followed by immunoblotting with anti-phosphothreonine (A) or anti-FipA (B) antibody as indicated; and reprobing with anti-FipA antibody for panel A. C. Cell lysates were fractionated and membrane and cytosolic fractions were immunoblotted with anti-FipA antibody followed by reprobing with anti-FtsZ (marker for the cytosol) or with anti-FtsW (membrane marker). Blots shown are representative of three separate experiments. doi:10.1371/journal.pone.0008590.g003

Subcellular Localization of FipA

In order to understand the function of FipA and in view of the fact that it interacts with FtsZ, we felt it imperative to localize FipA in cells. Its topology predicted using the program HMMTOP [28] suggest that it is a transmembrane protein. Fractionation of lysates into membranes and cytosolic components, followed by Western blotting confirmed that FipA localizes in mycobacterial membranes (Fig. 3C). Crosscontamination of membrane and cytosolic fractions was ruled out by the fact that membranes were positive for the membrane protein FtsW but not for the cytosolic protein FtsZ, whereas the cytosol fraction was positive for FtsZ but not for FtsW.

FipA Is Phosphorylated by PknA

In order to test whether FipA is phosphorylated by the neighbouring ser/thr kinase PknA, we expressed FipA in <i>E. coli</i> with an N-terminal hexahistidine or S-tag. Purified His-tagged FipA migrated as a protein of approximately 20 kDa (Fig. 4A). We expressed and purified the kinase domain of PknA encompassing Y13 to A273 (PknA<sub>13–273</sub>) as a GST-tagged protein (Fig. 4B). Recombinant FipA could be phosphorylated by PknA<sub>13–273</sub> in the presence of Mg<sup>2+</sup> and [γ-<sup>32</sup>P] ATP (Fig. 4C). The kinase-dead K42A mutant of PknA<sub>13–273</sub> [20] was unable to phosphorylate FipA (Fig. 4C). In order to evaluate the role of FHA domain amino acid residues S101, H104, D118 and N123 on interaction of FipA with PknA and its phosphorylation, these residues were individually mutated to alanine. None of the mutants other than the D118A mutant, could interact with PknA<sub>13–273</sub> (Fig. 4D), nor were these mutants (with the exception of D118A) phosphorylated by PknA (Fig. 4E). These results confirmed the importance of the FHA domain residues S101, H104, and N123 in interaction with PknA and in PknA-mediated phosphorylation of FipA.

The NET Phos program [29] predicted threonine residues at positions 77 and 142 as putative phosphorylatable residues. Mutation of T77 abrogated PknA<sub>13–273</sub>-dependent phosphorylation of FipA (Fig. 4F) but not its interaction with PknA (Fig. 4D). This identified T77 as a possible site of PknA-dependent phosphorylation. Mutation of T142 had no effect in PknA<sub>13–273</sub>-mediated phosphorylation (data not shown). In order to confirm that FipA is phosphorylated on a single threonine residue by PknA<sub>13–273</sub>, mass analysis of His-FipA and the protein phosphorylated by PknA<sub>13–273</sub>, was undertaken. The increase in the molecular mass of the intact protein (Figure S3), confirmed incorporation of a single phosphate residue by PknA<sub>13–273</sub>.

In order to investigate the role of phosphorylation of FipA on its interaction with FtsZ, FipA<sub>KO</sub> was complemented with <i>fipA</i><sub>MTB</sub> or <i>fipA</i><sub>MSMEG</sub> (Fig. 3A). Wild type FipA<sub>MTB</sub> could immunoprecipitate FtsZ (Fig. 2B) while the T77A mutant could not, suggesting the requirement of FipA phosphorylation for its interaction with FtsZ. In addition, phosphorylation-defective FipA<sub>T77A</sub> could not restore the growth defect of the FipA<sub>KO</sub> (Fig. 1A) or its ability to withstand H<sub>2</sub>O<sub>2</sub> stress (Fig. 1B). This suggests that phosphorylation of FipA on T77 is required to support survival under oxidative stress.

FipA Is Phosphorylated In Vivo by PknA in Mycobacteria

In order to demonstrate PknA-dependent phosphorylation of FipA in <i>vivo</i>, we performed gradual depletion of the protein in <i>M. smegmatis</i> by acetamide-inducible expression of an antisense construct of <i>pknA</i>. Depletion of PknA was confirmed by immunoblotting of lysates with anti-PknA antibody (Fig. 5A). Gradual depletion of PknA was concomitant with gradual decrease in threonine phosphorylation of FipA (Fig. 5B), suggesting that PknA mediates phosphorylation of FipA in <i>vivo</i>.

Interaction of FtsZ with FipA Is Required for Septal Localization of FtsZ under Oxidative Stress

Considering that FipA interacts with FtsZ, <i>vivo</i> (Fig. 2), we attempted to understand whether this interaction is necessary for the localization of FtsZ at mid-cell in dividing cells. Immunolocalization of FtsZ in FipA<sub>KO</sub> showed that FtsZ was compromised in its ability to localize at mid-cell in FtsZ-KO cells subjected to oxidative stress (Fig. 6A, B), but could localize to mid-cell in the absence of oxidative stress (data not shown). Complementation of FtsZ<sub>KO</sub> with <i>fipA</i><sub>MTB</sub> restored the localization of FtsZ at mid-cell even under oxidative stress (Fig. 6C, D). The frequency of Z ring formation in the absence or presence of oxidative stress, was measured by microscopy. The data presented in Table 1 clearly indicate that Z ring formation under oxidative stress requires FipA. No Z ring formation could be observed in FipA<sub>KO</sub> under oxidative stress. However, complementation with <i>fipA</i><sub>MTB</sub> restored Z ring formation in 63 out of 81 FtsZ-positive cells (Table 1). We
hypothesize that FipA facilitates localization of FtsZ at mid-cell under H2O2 stress.

PknA-Mediated Phosphorylation of FtsZ

The present study shows that FipA is phosphorylated by PknA on T77 (Fig. 4E) and phosphorylation is required for its interaction with FtsZ (Fig. 2B). Thakur and Chakraborti (2006) had earlier demonstrated that FtsZ is phosphorylated by PknA without identifying the site of PknA-dependent FtsZ phosphorylation. In order to further understand the role of PknA-dependent phosphorylations, we attempted to identify the site of phosphorylation of FtsZ. Computational analysis using the Net Phos program [29], suggested that T343 is a putative site of phosphorylation of FtsZ. Recombinant PknA13–273 could phosphorylate recombinant wild type His-FtsZ MTB but not the T343A mutant in vitro (Fig. 7A). In addition, mass analysis of FtsZMTB after phosphorylation by PknA13-273 showed an increase in mass corresponding to phosphorylation on a single residue by (Figure S4). The T343A mutant, on the other hand, showed no increase in mass after treatment with PknA13-273, supporting our contention that T343 represents the only site of PknA-mediated phosphorylation of FtsZ. In order to confirm PknA-mediated threonine phosphorylation of FtsZ in vivo, pknA was partially knocked down by inducing the expression of an antisense RNA to pknA driven by the acetamidase promoter. Knock down of pknA inhibited phosphorylation of FtsZ on threonine (Fig. 7B), just as in the case of FipA.

Figure 4. FipA interacts with, and is phosphorylated by PknA in vitro. A, B. Coomassie blue-stained gels of uninduced (1), or induced (2) cells of E. coli expressing FipA (A), GST-PknA13-273 (B), purified FipA (A, lane 3) and GST-PknA13-273 (B, lane 3). C. Purified FipA was phosphorylated by GST-PknA13-273 or its K42A mutant in vitro in the presence of [γ-32P] ATP followed by autoradiography. D. Recombinant S-tagged FipA or its mutants were incubated with GST-PknA13-273 bound to glutathione-Sepharose beads. Proteins bound to the beads were detected by immunoblotting with anti-FipA antibody. The blot was reprobed with anti-GST antibody. E. Purified FipA or its mutants were phosphorylated by GST-PknA13-273 in vitro in the presence of [γ-32P] ATP followed by autoradiography. The first lane represents autophosphorylated GST-PknA13-273. Blots shown are representative of three separate experiments. Lower blots of panels C and E are Coomassie blue-stained gels showing the levels of FipA.

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Figure 5. A, B. FipA is phosphorylated in vivo in a PknA-dependent manner. A, B. M. smegmatis was transformed with a construct carrying pknA in antisense orientation under an acetamide-inducible promoter. Transformed cells were induced with 0.2% acetamide for the indicated periods of time in order to downregulate pknA expression. Antisensing was followed by immunoblotting of cell lysates with anti-PknA antibody, followed by reprobing with anti-FtsW antibody (A); or cell lysates at different periods of time were immunoprecipitated with anti-FipA antibody followed by immunoblotting with anti-phosphothreonine antibody and reprobing with anti-FipA antibody (B). Blots shown are representative of three separate experiments. IB: immunoblotting; IP: immunoprecipitation.

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Formation of a Complex between FtsZ, FipA and FtsQ and the Role of PknA

Considering that FipA appeared likely to play a role in cell division, we tested whether FipA influences interaction of FtsZ with any divisomal protein which follows it to the site of division. Among the proteins which have been recognized as following FtsZ and preceding FtsW to the site of division is FtsQ of E. coli (or DivIB of B. subtilis). The ORFs Rv2151c and MSMEG_4229 (Figure S5) encode M. tuberculosis and M. smegmatis FtsQ, respectively [30]. Like FtsZ, both FipA and FtsQ localize to mid-cell in dividing M. smegmatis (Figure S6) as observed by
immunofluorescence microscopy. Numerical data show that FipA localizes to the septum to a similar extent in untreated and H_2O_2-treated cells (Table 2).

We tested the possibility that FipA is part of a larger divisomal complex including FtsQ and FtsZ. FtsQ could be pulled down in a complex with FtsZ in wild type M. smegmatis as well as in FipA-KO (Fig. 8A) in the absence of stress. However, under H_2O_2 stress, FtsQ immunoprecipitated with FtsZ only in the wild type, but not in FipA-KO (Fig. 8A), suggesting that FipA is required for FtsZ to interact with FtsQ in oxidatively stressed cells, whereas FipA likely plays a redundant role in the absence of oxidative stress. Support of this contention, FtsQ-FipA-KO complemented with fipA unordered with S-tagged FtsQMTB, beads were washed, and FtsZMTB was subsequently added, neither FipA unordered nor FtsZMTB could be pulled down with FtsQMTB (Fig. 9C, lane 2). These observations suggest that prior interaction between FtsZ and FipA is necessary for formation of the FtsZ-FipA-FtsQ ternary complex. In support of this, FtsZ immunoprecipitates which contained FipA were able to pull down FtsZ (Fig. 9C, lane 3). When FipA was incubated with immobilized FtsQMTB, beads were washed, and FtsZMTB was subsequently added, neither FipA unordered nor FtsZMTB could be pulled down with FtsQMTB (Fig. 9C, lane 2). These observations suggest that prior interaction between FtsZ and FipA is necessary for formation of the FtsZ-FipA-FtsQ ternary complex. In support of this, FtsZ immunoprecipitates which contained FipA were able to pull down FtsZ (Fig. 9C, lane 3), whereas FtsZ immunoprecipitates alone could not pull down FtsZ (Fig. 9C, lane 5). These results suggest that FtsZ interacts with FipA influences the ability of FtsZ to interact with partner divisomal proteins, and that initial interaction between FtsZ and FipA is necessary for ternary FtsZ-FipA-FtsQ complex formation.

**Table 1.** Presence of Z ring in cells.

|                  | Total | septum | poles | others |
|------------------|-------|--------|-------|--------|
| WT without stress| 122   | 102    | 18    | 2      |
| WT after H_2O_2  | 105   | 79     | 17    | 9      |
| FipA-KO without stress | 101   | 81     | 10    | 10     |
| FipA-KO after H_2O_2 stress | -     | -      | -     | -      |
| FipA-KO complemented with fipA unordered with stress | 102   | 75     | 21    | 6      |
| FipA-KO complemented with fipA unordered after H_2O_2 stress | 81    | 63     | 14    | 4      |

FtsZ positive cells were counted.  
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Discussion

The gene cluster encompassing the products of the ORFs *Rv0014c-Rv0019c* is conserved throughout the genus *Mycobacterium*. It encompasses two transmembrane sensor kinases PknA and PknB, a phosphatase PstP, a transpeptidase PBPA and the FHA domain protein encoded by *Rv0019c* (FipA). There has been speculation that this gene cluster plays a role in cell division [31]. However, knowledge of the roles of the individual gene products of this cluster in the genus *Mycobacterium*, is scanty. The *dsc* cluster is conserved across the genus *Mycobacterium* and includes genes such as *ftsZ*, *ftsQ* and *ftsW* whose products are central to the process of cell division. Here we establish two important findings. In the first instance we demonstrate a synergy of action between two products of the first gene cluster, (PknA and the FHA domain protein FipA), and two products of the *dsc* cluster (*ftsZ* and *ftsQ*) in maintaining cells in a division-competent state under oxidative stress. We uncover a novel role of phosphorylated FipA in the process of cell division. Here we establish two important findings. In the first instance we demonstrate a synergy of action between two products of the first gene cluster, (PknA and the FHA domain protein FipA), and two products of the *dsc* cluster (*ftsZ* and *ftsQ*) in maintaining cells in a division-competent state under oxidative stress. We uncover a novel role of phosphorylated FipA in maintaining cells in a division-competent state under oxidative stress. In the second instance, we demonstrate that the network of interactions between FtsZ, FipA and FtsQ, is critically dependent on the serine/threonine kinase PknA. These findings are discussed below.

*M. tuberculosis* resides within host macrophages following inhalation into the alveolar spaces of the lung. The bacterium is exposed to adverse environmental conditions and toxic agents such as reactive oxygen intermediates (ROI). It is logical that the bacterium must be endowed with mechanisms of withstanding the stressful conditions of its intracellular niche to an extent that allows bacteria to survive within macrophages. Control of cell division as well as maintenance of cell shape are critical factors for the survival of mycobacteria within the host. After replication, the multiprotein complex that comprises the divisome, assembles at mid-cell in order to facilitate cell division [32]. In mycobacteria, the repertoire of proteins required for cell division of mycobacteria within macrophages, remains incompletely understood. In order to test whether the FHA domain protein FipA plays a role in supporting intramacrophage growth of *M. tuberculosis*, *fipA* was knocked down. Knockdown of *fipA* was associated with a cell division defect of *M. tuberculosis* in macrophages after 5 days of infection, providing evidence that FipA is required to sustain growth of *M. tuberculosis* within macrophages. The role of FipA was investigated using *M. smegmatis* as the model. Knockout of *fipA* resulted in a cell division defect in *M. smegmatis* exposed to H$_2$O$_2$. In *E. coli*, cell division block is frequently associated with induction of recA-dependent SOS response [33]. In *M. tuberculosis*, ROI produced by macrophages can damage DNA and induce the SOS response. In the classical pathway, DNA damage induces recA and lexA-dependent SOS response [34]. However, in this instance we could rule out the possibility of induction of a classical SOS response at the concentrations and times of H$_2$O$_2$ exposure used. Consequently, we investigated other causes of FipA-linked cell division block under oxidative stress, focusing on the interaction of FipA with well-characterized proteins of the divisome.

Our results indicate that FipA interacts with FtsZ in a phosphorylation-dependent manner. Earlier studies have focused on biochemical characterization of PknA [35], and identified substrates such as MurD [36]. Kang et al. [20] reported that in mycobacteria, partial knock down of pknA or pknB leads to the formation of narrow and elongated cells, suggesting a role of these protein kinases in cell division. However, the significance of PknA-dependent phosphorylation of divisomal proteins is still not clear. Unlike Thakur and Chakrabarti [35] we did not observe phosphorylation-dependent inhibition of the GTPase activity of FtsZ. It is pertinent to point out that Thakur and Chakrabarti [35] used GST-FtsZ, whereas we have worked with His-FtsZ. It may be mentioned that Schulz et al. [37] failed to observe PknA-dependent inhibition of polymerization of *Corynebacterium glutamicum* FtsZ. During infection, membrane-bound kinases are thought to transmit environmental signals inside bacteria. In fact, *pknB* and *pknA* are upregulated by more than 10-fold when THP-1 cells are infected with *M. tuberculosis* [38]. It therefore appears likely that the serine/threonine kinases of mycobacteria play important roles during

### Table 2. Localization of FipA in *M. smegmatis*.

| Growth condition     | Septum | Poles | ND* |
|----------------------|--------|-------|-----|
| Untreated            | 52     | 9     | 39  |
| Subjected to H$_2$O$_2$ stress | 48     | 10    | 42  |

In each set, FipA localization was counted among 100 cells.

*Not determined.

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Figure 7. FtsZ is phosphorylated in a PknA-dependent manner. A. Purified FtsZ or its T343A mutant was phosphorylated by GST-PknA$_{13-273}$ in the presence of [$\gamma$-$\text{32P}$] ATP followed by autoradiography. The lower blot is a Coomassie blue-stained gel showing the levels of FtsZ. B. Conditional knockdown of *pknA* was induced by growing *M. smegmatis* expressing an antisense construct of *pknA* (under the control of an acetamide-inducible promoter) in the absence (-) or in the presence (+) of acetamide for 12 h. Cells were lysed and lysates were immunoprecipitated with preimmune sera (first lane) or anti-FtsZ antibody, followed by immunoblotting with anti-phosphothreonine and reprobing with anti-FtsZ antibody. Blots shown are representative of three separate experiments.

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FipA in Cell Division

In each set, FipA localization was counted among 100 cells.

*Not determined.

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infection. Understanding their function could in the long run provide newer directions for development of antituberculosis drugs. With this in view, we pursued investigation on the possible role of PknA in cell division. We now establish that FipA is phosphorylated on T77 in a PknA-dependent manner, and elucidate a functional role for such a phosphorylation event. Coimmunoprecipitation assays confirm that the FHA domain of FipA is necessary for its interaction with PknA. FipA<sub>MTB</sub> could complement FipA-KO with restoration of growth and survival under oxidative stress. The phosphorylation-defective T77A mutant failed to support cell division in FipA-KO under oxidative stress, underscoring the importance of PknA-dependent phosphorylation of FipA in sustaining cell division under oxidative stress.

**Table 3.** Localization of FtsQ in FipA-KO and complemented strains.

| Cell type and treatment | punctate * | septum | poles | ND** |
|-------------------------|------------|--------|-------|------|
| WT (−) stress            | 13         | 64     | 21    | 2    |
| WT (+) stress            | 30         | 52     | 13    | 5    |
| KO (−) stress            | 19         | 58     | 19    | 4    |
| KO (+) stress            | 67         | 0      | 3     | 30   |
| KO with <i>fipA<sub>MTB</sub></i> (−) stress | 15 | 61 | 15 | 9 |
| KO with <i>fipA<sub>MTB</sub></i> (+) stress | 35 | 45 | 12 | 8 |

*pCells showing punctate distribution of FtsQ in the membrane.
**Not determined.

In *E. coli*, FtsQ interacts with a large number of divisomal proteins [39] and is required for divisome assembly [40]. Recruitment of FtsZ, FtsQ, and FtsA to mid-cell is required for the septal localization of FtsW [41]. In mycobacteria, FtsQ is required for localization of FtsW to the septum in dividing cells (unpublished observations) suggesting that it plays an important role in mycobacterial cell division. We provide evidence that FipA interacts with FtsZ and FtsQ in a PknA-dependent manner. We hypothesize that FipA is required for divisomal protein assembly under oxidative stress, in a manner that is dependent on phosphorylation of FipA mediated by PknA. We show that in the absence of oxidative stress, FtsQ interacts with FtsZ in vitro both in the wild type as well as in FipA-KO, although the two proteins do not interact directly with each other (Fig. 9C). This suggests that FipA, as a member of the divisomal complex, plays a

**Table 4.** Localization of FtsQ in *M. smegmatis*-F1 and *M. smegmatis*-F2.

| Cell type and treatment | punctate * | septum | poles | ND** |
|-------------------------|------------|--------|-------|------|
| *M. smegmatis*-F1 (−) stress | 19 | 67 | 7 | 7 |
| *M. smegmatis*-F1 (+) stress | 29 | 55 | 10 | 6 |
| *M. smegmatis*-F2 (−) stress | 26 | 53 | 13 | 8 |
| *M. smegmatis*-F2 (+) stress | 64 | 0 | 8 | 28 |

*pCells showing punctate distribution of FtsQ in the membrane.
**Not determined.

In each set, FtsQ localization was scored in 100 cells.

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by washing and further incubation with FtsZMTB (lane 2); or incubated dependent phosphorylation events fine tune cell division and play gene cluster Rv0014c-Rv0019c and the first time that in mycobacteria (i) there is synergy between the cellular milieu of the host. Our observations provide knowledge for specifically required for survival of mycobacteria in the intracellular environment. FipA proteins are present across numerous genera. It is likely that FipA functions under oxidative stress. FipA assumes a critical role in sustaining septation of growing cells even in the absence of FipA. Additional, unidentified proteins are probably unique to mycobacteria, even though FtsZ and FtsQ are present across numerous genera. It is likely that FipA supports cell division in these conditions in the absence of FipA. It therefore seems reasonable to contend that FipA supports cell division in conditions in the absence of any in vivo experiments. It remains to be investigated whether PknA-mediated phosphorylation of FipA on Thr77 and PknB-mediated phosphorylation on Thr36 are interdependent, whether PknB-dependent phosphorylation is constitutive or inducible, and whether it too plays a role in cell division. It is possible that site-specific phosphorylations of FipA by two different kinases regulate interaction with different substrates. Another FHA domain containing protein, GarA [43] has been found to be phosphorylated by two kinases, PknG and PknB which differentially regulate its interaction with other proteins. Detailed investigations to resolve these unanswered question are in progress. In summary, our observations offer new insight into how targeting FtsZ-FipA interaction could block cell division to an extent that would compromise mycobacterial multiplication in macrophages.

Figure 9. FipA is a bridging partner for interactions between FtsZ and FtsQ. A. B. M. smegmatis before or after conditional knockdown of pknA, was subjected to oxidative stress, lysed and immunoprecipitated with preimmune sera (lane 1) or anti-FipA antibody (lanes 2 and 3), followed by immunoblotting with anti-FtsQ (A) or –FtsZ (B) antibody and reprobing with anti-FipA antibody. C. Recombinant S-tagged FtsQMTB immobilized on S-agarose was incubated with buffer only (lane 1) or with recombinant FipAMTB followed by washing and further incubation with FtsZMTB (lane 2); or incubated with FipAMTB and FtsZMTB simultaneously (lane 3). Resin-bound proteins were separated by SDS-PAGE and immunoblotted with different antibodies as indicated. For lanes 4 and 5, Ni2+ bound His-tagged FtsZMTB was incubated with (lane 4) or without (lane 5) FipAMTB followed by washing and further incubation with FtsQMTB. Bound proteins were identified by immunoblotting with the indicated antibodies. The blot was reprobed with anti-FtsZ antibody. Blots shown are representative of three separate experiments.

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Materials and Methods

Molecular Biological Procedures

Standard procedures for cloning and analysis of DNA, PCR, electroporation and transformation were used. Enzymes used to manipulate DNA were from Roche Applied Science, Mannheim, Germany. All constructs made by PCR were sequenced to verify their integrity. E. coli strains were routinely grown in Luria broth (LB). Details of strains and plasmids are provided in Table S1.

Construction of Suicidal Delivery Vector for Inactivation of fipA in M. smegmatis

The two-step homologous recombination strategy described by Parish and Stoker [44] was used to disrupt MSMEG_0034, the counterpart of Rv0019c at its native locus in M. smegmatis. Briefly, a suicide plasmid was constructed in three steps. First, an 860 bp DNA fragment containing the 5’ end of MSMEG_0034 along with upstream sequence was PCR amplified using the genomic DNA of wild type M. smegmatis mc²155 as template and the primer pair FipA 1F sense and FipA 1F antisense (Table S2). The amplicon was cloned between the asymmetric HindIII and BamHI sites of p2NIL to give pJB110. In the next step, a 900 bp fragment bearing the 3' end of MSMEG_0034 and its downstream region was amplified using the primer pair FipA 2F sense and FipA 2F antisense (Table S2) and cloned between the BamHI and PacI sites of pJB110 to give pJB111 carrying a truncated fipA gene. The hygromycin cassette was excised from pUC-HY−INT [Mehenthiralingam et al., 1998] and introduced into pJB111 to give pJB112. Finally, a 6.1 kb PacI cassette carrying the lacZ, aph and sacB genes, was isolated from pGOAL17 and inserted into pJB112 to generate the suicide plasmid pJB113.

Isolation of the FipA Knockout Mutant

Denatured pJB113 was electroporated into electrocompetent cells of M. smegmatis mc²155. Cells were plated on Lemo agar supplemented with hygromycin B, kanamycin and 50 µg/ml x-gal. Blue colonies were streaked onto Lemo agar without any selection to enhance the recombination process. Cells were then plated onto Lemo agar supplemented with 2% sucrose and 50 µg/ml x-gal. The white colonies were restreaked onto replica plates containing hygromycin, x-gal and sucrose, either without or
with kanamycin. White, kan^S, hyg^R, suc^R colonies generated by double crossover represented the *fipA*–inactivated strain (*FipA*-KO). Expression of FipA was checked using antibody raised against FipA.

**Complementation of FipA*<sub>MTB</sub> in FipA-KO Using an Integrating Vector**

An integrating vector carrying His-FipA (or its mutants) of *M. tuberculosis* under the control of the hsp60 promoter was constructed as described earlier [19]. Complementation with *fipA*<sub>MTB</sub> was achieved by electroporation of the integrating vector into FipA–KO. The presence of His-FipA was confirmed by Western blotting using anti-His antibodies.

**Cloning and Expression of M. tuberculosis H37Rv Genes**

*FipA* was amplified from genomic DNA of *M. tuberculosis* H37Rv using the sense primer “a” and antisense primers “d” (for pET28a+) and “e” (for pET29a+) depicted in Table S2. The amplicons were cloned between asymmetric BamHI and HindIII sites in pET28a or the BamHI and EcoRI sites in pET29a+ to generate pBJ120 and pBJ121 respectively. *E. coli* C41 (DE3) harbouring the respective constructs was grown to an Abs<sub>600</sub> of 0.6 at 37°C, IPTG was added at a concentration of 0.25 mM, and growth was continued for 2–3 h. His- or S-tagged proteins were purified from cell lysates by chromatography on Ni<sup>2+</sup>-NTA- or S-agarose respectively. Mutants of *FipA*<sub>MTB</sub> were generated by overlap extension PCR. Initial rounds of PCR were performed using primers “a” and “b” or primers “c” and “d” (or “e”) (Table S2). Using the products of the initial round as template, the second round of PCR was performed using primers “a” and “d” (or “e”). Products were cloned in pET28a+ or pET29a+. The T343A mutant of FtsZ was generated using pBJ101 (Datta et al., 2002) as template and primers “a” and “d” (Table S2).

The kinase domain of *pknA* (*pknA*(13–273)) was amplified using the sense and antisense primers shown in Table S2, and cloned either in pET28a+ or in pET29a+ between the BamHI and HindIII sites of pET29a+ as described earlier [19]. The *fipA* gene was cloned into pUC19 using KpnI and HindIII sites. Expression was carried out in *E. coli* C41 (DE3) using 0.1 mM IPTG for 4 h at 37°C.

The kinase domain of *pknA* (*pknA*(13–273)) was amplified using the sense and antisense primers shown in Table S2, and cloned between the BamHI and EcoRI sites of pGEX-2T to generate the plasmid pBJ122. Expression was carried out in *E. coli* BL21 by induction with 0.1 mM IPTG at 37°C for 4 h, and the expressed protein was purified by chromatography on glutathione Sepharose. The K42A mutant was generated by overlap extension PCR using primers “b” and “c” depicted in Table S2. His-*PknB*-<sub>276</sub> was purified as described earlier [19]. The *psIP* (Rv0013c) gene was amplified using the sense and antisense primer pair shown in Table S2, and cloned between the BamHI and HindIII sites of pET29a+. N-terminal S-tagged PsrP was obtained by induction for 3 h at 37°C with 50 μM IPTG and purified by chromatography on S-agarose.

**Construction of ftsZ Replacement Vectors in M. smegmatis**

A construct for replacement of the chromosomal *ftsZ* of *M. smegmatis* was generated using the method of described above [44]. A suicide plasmid with a 488 bp internal deletion in the *ftsZ* gene was constructed in two steps. In the first step, a 1244 bp DNA fragment encompassing the 5’ end of *ftsZ* and its downstream region was PCR amplified using the primer pair *FtsZ* 1F sense and *FtsZ* 1F antisense (Table S2) and cloned in pBluescript SK between the asymmetric BamHI and EcoRI sites to give rise to pBJ114. In the next step 1153 bp of DNA bearing the 3’ end of *ftsZ* and its downstream flanking region was amplified using the primer pair FtsZ 2F sense and FtsZ 2F antisense (Table S2) and cloned between the EcoRI and HindIII sites of pBJ114 to generate pBJ115. The truncated *ftsZ* gene was excised from pBJ115 by digesting with BamHI and HindIII and cloned into p2NIL between the same sites to generate pBJ116. Finally, the 6.1 kb PacI fragment was isolated from pGOAL19 and inserted into pBJ116 to generate the suicide plasmid pBJ117.

**Isolation of M. smegmatis Strains Carrying Wild Type or Mutated ftsZ<sub>MTB</sub>**

*M. smegmatis* was electroporated with denatured pBJ117. Single cross over (SCO)s were selected on agar plates containing kanamycin and x-gal. Kanamycin-resistant blue SCOs were isolated. In the next step, His-tagged *ftsZ<sub>MTB</sub>* (or its mutant) was cloned under the control of the hsp60 promoter in pUC19 using KpnI and HindIII sites as described above. The 3.75kb Hyg-integrate cassette was excised from pUC-HY-INT [45] and inserted into the above construct to generate an integrating vector. Before inactivation of *ftsZ* at its native location, *ftsZ<sub>MTB</sub>* in the integrating vector was electroporated into the SCOs. The resultant merodiploid strains carrying His-tagged *ftsZ<sub>MTB</sub>* (*M. smegmatis-FtsZ<sub>1</sub>*) or *ftsZ<sub>MTB</sub>-F1343A* (*M. smegmatis-F2*) were then screened for double cross over (DCO) as described earlier [46]. White kan^R* Hyg^R* and sucrose-resistant DCOs were analyzed by PCR.

**In Vitro Kinase Assay**

In vitro kinase assays were performed with the purified kinase domain of *PknA*<sub>13–273</sub> (1 μg) in 25 mM Tris/HCl, pH 7.5, 5 mM β-glycerophosphate, 2 mM DTT, 2 mM MnCl<sub>2</sub>, 0.1 mM sodium orthovanadate as described by Dasgupta et al. (2006). Dephosphorylation assays were carried out in 50 mM HEPES buffer pH 7.5 containing 0.1 mM EDTA, 1 mM DTT and 5 mM MnCl<sub>2</sub> as described earlier [19]. In some experiments, proteins separated by SDS-PAGE were electrotransferred and Western blotting was performed using monoclonal anti-phospho-threonine (Sigma Chemical Co.) or anti-phosphotyrosine (Cell Signaling Technology) antibody.

**Conditional Inactivation of pknA in Mycobacteria**

The *pknA* gene was amplified using the primer pair pknA sense and pknA antisense (Table S1) followed by cloning of the PCR product in reverse orientation in the vector pSD29 [47] between the BamHI and EcoRV sites to generate pBJ118. In order to conditionally inactivate *pknA*, *M. smegmatis* was electroporated with pBJ118. Transformants were grown up to mid log phase and induced with 0.2% acetylamide for different periods of time. Western blotting was performed to confirm the antisensing of *PknA*.

**Knock Down of FipA in M. tuberculosis**

For knock down of *fipA<sub>ATB</sub>* an antisense construct (pBJ119) of *fipA* was generated by PCR amplification of the *fipA* gene using the primer pair *FipA*<sub>MTB</sub> sense and *FipA*<sub>MTB</sub> antisense (Table S2) followed by cloning of the PCR product in reverse orientation under the control of the hsp60 promoter using the strategy described by Surek et al. [40]. Western blotting using anti-FipA antibody was performed to confirm the antisensing of *fipA*.

**Induction of Oxidative Stress and Treatment with Mitomycin C**

Cells were subjected to H<sub>2</sub>O<sub>2</sub>-mediated oxidative stress as described earlier [48]. Briefly, cells were grown to an OD<sub>600</sub> of 0.3 to 0.4, diluted into fresh medium to an OD<sub>600</sub> of 0.2 and treated...
with 50 μM hydrogen peroxide for 1 h, followed by addition of hydrogen peroxide to 5 mM and incubation for the desired period of time. Mitomycin C treatment was carried out similarly by exposing cells (suspended at an OD_{600} of 0.2) to mitomycin C (0.2 μg/ml) for 4 h.

Preparation of Cell Lysate, Antibodies and Immunoprecipitations

Cell lysates were prepared by disrupting cells in a Mini Bead Beater (BioSpec Products) as described earlier [48]. Immunoprecipitations were carried out by overnight incubation of lysates with antibodies at a dilution of 1:1,000 at 4°C, followed by precipitation with Protein A/G agarose for 3 h. Antibodies against the kinase domain of PknA, PknB, PBPA, PstP, PtsQ, FipA and FtsZ were raised by Imgenex, Bhubaneswar.

Fluorescence Microscopy

Immunostaining was performed as described earlier [14]. Briefly, cells were fixed by incubation for 15 minutes at room temperature followed by 45 minutes on ice in 2.5% (v/v) paraformaldehyde, 0.4% (v/v) glutaraldehyde, 30 mM sodium phosphate (pH 7.5). After washing in PBS, the cells were permeabilized by exposing to 2% toluene for 2 min, and immediately transferred to slides. The slides were washed with PBS, air-dried, dipped in methanol (−20°C) for 5 min and then in acetone (−20°C) for 30 s and allowed to dry. After rehydration with PBS, the slides were blocked for 2 h at room temperature with 2% (w/v) BSA-PBS and incubated for 1 h with appropriate dilutions of primary antibody in BSA-PBS. The slides were washed extensively with PBS and then incubated with a 1:1,000 dilution of anti-FipA or anti-FtsQ antibody followed by staining with Alexa 488-conjugated rabbit IgG and visualization by fluorescence microscopy.

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Author Contributions

Conceived and designed the experiments: MK JB. Performed the experiments: KS TH PM PC PD. Analyzed the data: KS MK JB. Wrote the paper: MK JB.

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