Delineating FtsQ mediated regulation of cell division in *Mycobacterium tuberculosis*

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Key words: FtsQ, cell division, growth regulation, tuberculosis, phosphorylation, mycobacteria, dcw operon, divisome, septation, cytokinesis

Identifying and characterizing the individual contributors to bacterial cellular elongation and division will improve our understanding of their impact on cell growth and division. Here, we delineated the role of ftsQ, a terminal gene of the highly conserved division cell wall (dcw) operon, in growth, survival, and cell length maintenance in the human pathogen *Mycobacterium tuberculosis* (Mt). We found that FtsQ overexpression significantly increases the cell length and number of multiseptate cells. FtsQ depletion in Mt resulted in cells that were shorter than WT cells during the initial growth stages (4 days after FtsQ depletion), but were longer than WT cells at later stages (10 days after FtsQ depletion), and compromised the survival in vitro and in differentiated THP1 macrophages. Overexpression of N- and C-terminal FtsQ regions altered the cell length, and the C-terminal domain alone complemented the FtsQ depletion phenotype. MS analyses suggested robust FtsQ phosphorylation on Thr-24, and although phospho-ablative and -mimetic mutants rescued the FtsQ depletion-associated cell viability defects, they failed to complement the cell length defects. MS and co-immunoprecipitation experiments identified 63 FtsQ-interacting partners, and we show that the interaction of FtsQ with the recently identified cell division protein SepIVA is independent of FtsQ phosphorylation and suggests a role of FtsQ in modulating cell division. FtsQ exhibited predominantly septal localization in both the presence and absence of SepIVA. Our results suggest a role for FtsQ in modulating the length, division, and survival of Mt cells both in vitro and in the host.

Cell division is fundamental to all living cells. Most of the proteins either involved or thought to be involved in this process are essential for in vitro growth of *Mycobacterium tuberculosis* (Mt) (1). In *E. coli*, the divisome, a macromolecular assembly arranged in a multilayered toroid shape for septation and cytokinesis, is governed by a set of ~30 proteins that function in coordinating partition of chromosome with septa initiation, divisome stabilization followed by the segregation of mother cell into two daughter progenies (2-6). Appropriate positioning of divisome assembly requires existence of specialized systems such as MinCDE or nucleoid occlusion system (SlmA in *E. coli* and Noc in *B. subtilis*) (7,8). Sequential recruitment of proteins in divisome occurs at two distinct stages with a short delay in between (9). Proto-ring comprising FtsZ, FtsA, ZipA are the early recruits to the divisome that ensure the constriction initiation and its stabilization (10). This is followed by recruitment of FtsK, FtsQ in complex with FtsB and FtsL (FtsQBL), peptidoglycan remodelers such as FtsW (transglycosylase), FtsI (transpeptidase), FtsN, carboxypeptidases, endopeptidases and number of other accessary protein, which are necessary for chromosome segregation and final cytokinesis (11-17). Recently, FtsN driven switch of FtsA and FtsQBL between on and off states has been proposed, implicating their functions in divisome activation (18,19). Additional recent emergence of FtsEX as a regulator of FtsA and amidases, indicates multistep regulation of divisome assembly (20). However, the precise functions of individual components and mechanism of signal transfer between components remains elusive.

Tight regulation of cell division is necessary for Mt to sustain bouts of active infection, dormancy and reactivation in the host. Heterogeneous cell population of Mt during growth is thought to be one among the primary reasons for prolonged treatment (21). Even though mycobacterium lacks specialized systems to ensure the accurate positioning of divisome, the combination of directional chromosome...
translocation along with unequal bipolar growth have been suggested as a compensatory mechanism (22). While homologs of FtsZ, FtsK, FtsB, FtsL, FtsQ, FtsI, and FtsW are annotated in mycobacteria, it lacks homologs for FtsA, ZipA and FtsN proteins found in *E. coli* (23,24). Presence of these homologous proteins and similar sequence of recruitment at mid cell suggests the partial preservation of elementary complexes and their functions in mycobacteria (24). In mycobacteria, FtsZ is the first protein to assemble at the mid-cell, which polymerizes and serves as an initiating site for recruitment of peptidoglycan (PG) remodelling proteins (25). Ternary complex comprising of FtsZ, FtsW (probable lipidII flippase), and FtsI (transpeptidase) is thought to stabilize the divisome assembly and regulate septal PG biosynthesis (26).

Homolog of FtsK in *M. smegmatis* (*Msmeg*) plays a role in translocation of chromosome prior to cytokinesis (22). In addition to the conserved proteins, other non-conserved proteins such as CrgA, Ssd, SepF and SepIVA have been identified to be involved in division (24,27,28). CrgA has been shown to interact with various other divisome members like FtsZ, FtsI, PbpA, FtsQ and CwsA, and is co-expressed along with mur genes, which suggests its function as a sensor of completion of DNA replication period in *M. smegmatis* (34). Structural investigation of the *ftsQ* gene has suggested further subdomain organization of the periplasmic region into alpha (α), beta (β) and gamma (γ) domains (35). Sequence alignment of FtsQ from *G. stearothermophilus* with its homologs showed preservation of this subdomain organization structure across bacterial kingdoms (35). These periplasmic subdomains play a role in appropriate localization and interactions with other cell division members (36,37). Even though interaction of FtsQ*<sub>Stb</sub>* with FtsZ*<sub>Stb</sub>* through Fip*<sub>Stb</sub>* has been demonstrated in mycobacterium (38), the characterization of FtsQ for its role in cell division, shape maintenance and viability has not been investigated. In this report, we investigated the functionality of FtsQ in mycobacteria by overexpressing and conditionally depleting FtsQ.

**Results**

**Overexpression of FtsQ increases the average cell length.**

The *fitQ* gene in mycobacteria lies in a conserved division or cell wall cluster (dcw) operon and is co-expressed along with *mur* genes, which allows coordinated cell wall synthesis and division (Fig 1a). FtsQ contains N-terminal cytosolic domain connected through a transmembrane domain to the extracytoplasmic domain (Fig 1b). Based on the sequence alignment of FtsQ<sub>Mtb</sub> with its homologs from *E. coli*, *B. subtilis* and *Streptococcus pneumoniae* (data not shown), the periplasmic domain of FtsQ could be further divided into alpha (α), beta (β) and gamma (γ) domains (Fig 1b). To delineate the function of FtsQ<sub>Mtb</sub> first we sought to investigate the impact of FtsQ overexpression in mycobacterial growth and survival. *Msmeg* was electroporated with either pNit1 (vector) or pNit-FtsQ construct, wherein *fitQ* is cloned under the isovaleronitrile (IVN) inducible promoter. Western blot analysis of lysates prepared from *Msmeg::pNit* and *Msmeg::pNit-fitQ* cells grown in the presence or absence of 5 µM IVN showed significant expression of FtsQ in the presence of IVN (Fig 1c). Colony forming units (CFU) enumerated at 0, 14 and 28 h showed ~100 fold lower survival of the strain overexpressing FtsQ as compared to the wild type at 14 h, which reduced to ~10 fold at 28 h (Fig 1d). These results suggest an initial delay in the growth upon overexpression, which eventually seems to be converging with the wild type strain. Such growth patterns are typically observed upon overexpression of proteins critical for cell division (39). Interesstingly, analysis of cell length with the help of scanning electron microscopy (SEM) showed significant increase in the average mean cell length upon overexpression of FtsQ from 3.2 µm to 4.9 µm (Fig 1e & f).
Next, we wanted to determine the minimum levels of overexpression of FtsQ that is necessary and sufficient to exhibit increase in the average mean cell length. Towards this, the expression of FtsQ in Msmeg harbouring episomal copy of the gene was induced with different concentrations of IVN, which led to differential level of FtsQ expression, as evaluated by real time PCR and western blot (Fig 2a & b). With increasing concentration of IVN from 0.2, 1 and 5 µM, we observed ~10, 27 and 37 fold induction of the expression of ftsQ, respectively, as comparison with no inducer (Fig 2a). In agreement with this, we could detect expression of FtsQ only in lysates from 1 and 5 µM IVN induced cultures (Fig 2b). SEM analysis showed statistically significant increase in the cell length at all the concentrations including in the absence of inducer (3.8 vs 3.0 µm), suggesting that the leaky over-expression from episomal construct was sufficient to alter the average mean cell length (Fig 2c & d). To evaluate the impact of overexpression on the septum formation, we performed transmission electron microscopy (TEM) analysis. Interestingly, we observed multi-septate phenotype in cells overexpressing FtsQ. In order to quantify the percentage of such cells, we evaluated the septation pattern for ~60-65 cells / sample. We observed a significant increase from 4 to 14% in cells containing bi or multi-septa phenotype upon overexpression of FtsQ (Fig 2e & f). Taken together, our data indicate that overexpression of FtsQ results in elongated and multi-septate cells, implicating a crucial role of FtsQ in regulation of cell division.

Both N and C-terminal domains of FtsQ are critical for cell length maintenance.

N-terminus region of FtsQ including the transmembrane domain is 123 aa in length. The carboxy terminal domain of FtsQ in E. coli, B. subtilis and other bacterial kingdoms contains crucial alpha (α), beta (β) and gamma (γ) domains thought to be important for its function (35,40,41). In order to evaluate the impact of overexpressing N or C-terminal regions, we have cloned the respective fragment along with the membrane anchoring sequences into pNit1 vector. While the full length and N-terminal domain showed significant overexpression, the C-terminal fragment displayed relatively minimal overexpression (Fig 3a). The expression of full length, N- and C-terminal fragments led to compromised growth (Fig 3b), albeit to different extents. These observations were also reflected in the cellular morphology, wherein their expression led to increased cell length (Fig 3c & d). We think that the differences in the extent of growth defect or cell lengths upon N- or C-terminal fragment overexpression are most likely due to variations in the expressions of these fragments (Fig 3a). Thus, results suggest a definitive role for both N and C-terminal regions of FtsQ in regulating the cell division.

FtsQ is essential for bacterial viability in Mtb.

We sought to investigate the consequence of absence of FtsQ on the bacterial growth, morphology, and survival in the host. High throughput transposon based mutagenesis experiments suggested ftsQ to be an essential gene for the in vitro growth of the bacteria (42). Thus, we set out to alter the expression of ftsQ at its native locus into pristinamycin inducible expression. Towards this, we cloned -20 to 680 bp of ftsQ gene under pristinamycin inducible pptr promoter in a pAZ vector (43) that lacks Mtb origin of replication (suicide delivery vector) (Fig 4a). Single homologous recombination of the construct at the native locus replaces the native expression of ftsQ into a pristinamycin inducible gene (Fig 4a), which in absence of any inducer, would act as FtsQ knockdown mutant, RvΔfQ. Recombination at the native locus was confirmed by PCR analysis using different primers sets (Fig 4b). Streaking of Rv and RvΔfQ cultures on plates in presence or absence of inducer clearly demonstrated the inability of RvΔfQ strain to sustain growth in the absence of inducer (Fig 4c). CFU analysis (Fig 4d) showed compromised survival of the mutant from day 6, eventually resulting in ~1.5 log fold decrease in the survival on day 10 (Fig 4d). Next, we investigated the role of FtsQ in modulating the survival of pathogen in the host. Towards this the Rv or RvΔfQ grown in the presence or absence of pristinamycin were used for infecting differentiated THP1 cells. We enumerated CFUs at 0, 96 and 120 h post infection and found that 120 h post infection, intracellular bacillary survival was compromised upto 10 fold in FtsQ depleted sample (Fig 4e).

FtsQ depletion alters the mean cell length.

Overexpression of FtsQ in Msmeg resulted in elongated cells, indicating crucial role of FtsQ in regulating cell wall morphology (Fig 1). This prompted us to investigate the effect of FtsQ depletion in Mtb on the maintenance of average mean cell length. We performed SEM and TEM analysis of samples on day 4 and 10 (early and late log phase cultures; Fig 5a-c). While the cells looked quite healthy on day 4, they looked more ruffled with indentations in day 10, which was lot more palpable in FtsQ depleted cells (Fig 5a). Intriguingly, we observed two distinct phenotypes in cell lengths with respect to time. After 4 days post depletion, the cells appeared smaller when compared with the wild type
in FtsQ depleted samples, with the average cell length decreasing from 2.3 in wild type cells to 1.8 \( \mu m \) in FtsQ depleted samples (Fig 5a & b). On the other hand, upon 10 days of depletion, the average cell length increased from 2.2 \( \mu m \) in \( H37Rv \) cells to 2.7 \( \mu m \) in \( \Delta \text{fQ} \) in the presence of pristinamycin (inducer). Importantly, the cell length increased to 3.1 \( \mu m \) in \( \Delta \text{fQ} \) in the absence of inducer wherein FtsQ was depleted (Fig 5a & b). We think that the cell length differences observed could be because of differences in the levels of FtsQ protein in the cell. In the subsequent experiments, we used 4 days post depletion SEM analysis of samples to assess the functionality of FtsQ. TEM analysis showed presence of septum even in the smaller cells observed on 4th day after depletion, suggesting that division may be happening before cells reached appropriate length. To confirm that the defects are indeed due to depletion of FtsQ, we generated complementation strain in which the protein was expressed from the episomal pNit-FtsQ construct. While expression of vector alone could not rescue the survival defect of FtsQ mutant, complementation with vector, expressing FtsQ could restore the growth on plates in the absence of inducer (Fig 5d). Furthermore, CFU analysis of wild type, mutant, and complemented strains, showed restoration of viability defects upon complementation (Fig 5e). This ability of episomal FtsQ to functionally complement the phenotypic defects of FtsQ mutant was also reflected in the SEM analysis (performed 4 days after depletion), wherein the shorter cell length phenotype observed was restored to wild type lengths (Fig 5f).

Based on the above data, we suggest that the presence of shorter cells in the initial and longer at the later stages is indicative of regulatory role played by FtsQ in elongation and division.

**Carboxy terminal domains are critical for the functionality of FtsQ.**

Overexpression of either N- or C-terminal fragments of FtsQ increased the cell length of \( M.\text{smeg} \) (Fig 3). Thus we wanted to decipher, whether the phenotypes observed upon depletion of FtsQ in \( \Delta \text{fQ} \) strain could be complemented by the expression of either N- or C-terminal fragments. \( \Delta \text{fQ} \) strain was electroporated with pNit or pNit-FtsQ or pNit-FtsQ-N or pNit-FtsQ-C constructs to generate different complementation strains. We assessed the survival of these complementation strains on 7H11 plates with or without pristinamycin (Fig 6a). While the complementation with full length and C-terminal fragments resulted in growth in the absence of pristinamycin, neither the vector nor the N-terminal fragment could rescue the growth defects (Fig 6a). Next, we determined the CFU’s 10 days post depletion in these samples. In concurrence with the above data, while the C-terminal fragment successfully rescued the ~2.5 log fold decrease in the survival upon FtsQ depletion, N-terminal fragment failed to do so (Fig 6b). SEM analysis of 4 day depleted sample also suggested that episcopal expression of both full length FtsQ and the C-terminal fragment could restore the cell length to wild type level (Fig 6c). It is apparent from the data that C-terminal fragment is necessary and sufficient to rescue observed FtsQ depletion phenotypes. Since, C-terminal region contains alpha (\( \alpha \)), beta (\( \beta \)) and gamma (\( \gamma \)) domains; we further evaluated the impact of deleting either \( \gamma \) or both \( \beta-\gamma \) domains. Analysis of growth on 7H11 plates with or without pristinamycin suggested that the expression of either deletion mutants could not rescue the growth in the absence of inducer. The results obtained were in concurrence with the above conclusion, when we analyzed CFU’s 10 days post depletion, or the cell lengths 4 days post depletion (SEM) (Fig 6e & f). Together these results suggest that C-terminal domain and all the subdomains within are essential for functionality of FtsQ.

**FtsQ is phosphorylated at T24 residue and phosphorylation influences cell division**

We have performed high-throughput phosphoproteomics to identify novel targets of protein kinases in \( Mtb \) (data not shown). FtsQ is among the substrates that we identified consistently in every biological replicate. Moreover, FtsQ has also been identified as a target in other phosphoproteomic studies (44-46). We obtained one phospho-peptide corresponding to precursor mass of 2140.92, a doubly charged peptide from residues 13-32. MS/MS analysis of the precursor clearly identified T24 to be the phosphorylation site on FtsQ (Fig 7a). In order to determine the stoichiometry of phosphorylation, \( Mtb \) was electroporated with pNit-FtsQ, which contains N-terminal FLAG tag, and FLAG-FtsQ was immunoprecipitated and processed for LC-MS analysis. The sum of all isotopic peaks at MS1 level for any peptide is indicative of its quantity. We calculated the area of peaks for phospho and the corresponding unphosphorylated peptide using Precursor Ions Area Detector Node. The ratio of the area for the total phosphorylated peptide with respect to the corresponding area for the total unphosphorylated peptide provides the stoichiometry. Based on these calculations from two biologically independent experiments, the stoichiometry of T24 phosphorylation was observed to be ~28-30% (Fig 7b). To understand the role of phosphorylation, if any, in modulating the function...
of FtsQ, we generated phosphoablative (T24A) and phosphomimetic (T24E) mutants and electroporated \textit{Rv::pN}\textsuperscript{FtsQ} strain with pNit constructs expressing the above mutants. In order to evaluate the ability of mutants to complement the growth, we analyzed three different aspects; the growth on 7H11 plates in the absence of pristinamycin (Fig 7e), growth in liquid media upon depletion (Fig 7d) and cell length 4 days post depletion (Fig 7e). Both phosphoablative and phosphomimetic mutants complemented the growth on 7H11 (Fig 7c) as well as in the liquid media (Fig 7c). While the phosphomimetic mutant could partially complement cell length defects observed 4 days post depletion, phosphoablative mutant completely failed to do so (Fig 7e). Based on the above data, we speculate that the cell viability and cell length defects associated with FtsQ depletion are independent traits, and are regulated at different levels. Phosphorylation seems to be playing a role in modulating the cell length defects but has no apparent role in cell viability.

\textbf{FtsQ interacts with SepIVA protein in a phosphorylation independent manner}

In \textit{E. coli}, FtsQ is known to be part of a macromolecular divisome assembly. We were interested in identifying interacting partners of \textit{Mtb} FtsQ in order to gain possible insights into its involvement in cell division. We electroporated \textit{Rv} with either pNit1, pNit-FtsQ, pNit-FtsQ-T24A or pNit-FtsQ-T24E constructs to generate \textit{Rv::pN}, \textit{Rv::pN-FtsQ}, \textit{Rv::pN-FtsQ-T24A} and \textit{Rv::pN-FtsQ-T24E} strains, wherein the expression of FLAG-FtsQ could be induced with IVN. The whole cell lysates prepared from three biological replicates were independently immunoprecipitated (IP) with FLAG-M2 beads. Western blot analysis of the samples clearly established efficient IP (Fig 8a). The IPed samples were loaded on SDS-PAGE, and a short while after the samples entered resolving gel, the run was terminated and the gel pieces were sliced out. The gel slices were trypsinized and the peptides were identified with the help of LC-MS/MS. Each sample was run twice through the mass spec and peptides that were found in the vector transformed \textit{Mtb} were subtracted from those found in the corresponding FLAG-FtsQ IP. We identified 117, 122 and 111 interacting partners in three independent replicates of FLAG-FtsQ IP, wherein 63 proteins were common to all three experiments (Fig 8b; Sup Table1). Similarly, three replicates of FLAG-FtsQ-T24A and FLAG-FtsQ-T24E IP samples were analyzed to investigate phosphorylation dependent interactions (Fig. 8c; Sup Table 1). We have found 6 and 24 interacting proteins common in all three biological replicates of FLAG-FtsQ-T24A and FLAG-FtsQ-T24E samples, respectively. After applying these stringent criteria, we identified four interacting partners in all nine samples from 18 mass spectrometry runs (Fig 8d; Sup Table 1). Presence of these four proteins in both phosphoablative and phosphomimetic IPed samples indicates that interactions of these proteins with FtsQ is phosphorylation independent. We have also identified one protein that specifically associated with FtsQ-T24E but not with FtsQ or FtsQ-T24A. We have identified 38 interacting partners that are not found either with T24A or with T24E. We do not know the implications of these findings yet. One of the four interacting partner that was consistently found is a recently characterized cell division protein, SepIVA (24). Similar to Wag31, SepIVA has a characteristic DivIVA domain, found in cell division proteins across bacterial kingdom (47). We sought to validate the interactome data by probing FtsQ and FtsQ-T24A and FtsQ-T24E IP samples with anti-SepIVA antibodies (Fig 8e). It is apparent from the data that SepIVA interacted with both wild type and phosphomutants of FtsQ, validating the interactome data. Taken together, we have identified cell division protein SepIVA to be novel phosphorylation independent interacting partner of FtsQ.

\textbf{FtsQ shows predominantly septal localization in both the presence and absence of SepIVA protein.}

As a constituent of divisome assembly, localization of FtsQ protein at the septal region is demonstrated in rod shaped bacteria (48). However, in polar growing bacteria like mycobacteria where divisome and elongosome components overlap at times, it is important to investigate the localization of proteins to get an insight about their involvement. Localization of Wag31 and FtsZ proteins at subpolar and septum, is thought to be suggestive of their roles in elongosome and divisome complexes, respectively (30). To investigate the localization of FtsQ, we have electroporated pN-GFP-FtsQ into \textit{mc2}\textsuperscript{c}\textsuperscript{FGQ} strain. We observed that FtsQ localizes at septal, subpolar and polar regions of cells (Fig. 9a). However, when we quantitated the localization of FtsQ in 150 independent cells, we observed that in \textsim\text{40}\% of the population it is localized to septal region, which is indicative of its probable role in divisome assembly (Fig. 9b).

We have consistently identified interaction between FtsQ and SepIVA, a recently identified cell division protein that contains a DivIVA domain (24). DivIVA domain containing proteins are membrane curvature sensitive proteins (49). We sought to
investigate whether the interaction between SepIVA and FtsQ is important for septal localization of FtsQ. Based on the high throughput transposon mutagenesis studies, SepIVA is identified to be essential for the in vitro growth of pathogen (42). Thus, we first integrated anhydrotetracycline (ATc) regulatable copy (tet off) of sepIVA<sub>Δlum</sub> into the L5 site to generate a merodiploid mc2::sep<sub>Δlum</sub> strain (Fig 9c). Next we replaced the native copy of sepIVA<sub>Δlum</sub> with hyg<sup>+</sup> and the replacement of sepIVA<sub>Δlum</sub> at the native locus was confirmed by performing multiple PCR reactions (Fig 9e). Western blot analysis of lysates prepared from mc2::sep<sub>Δlum</sub> strain at different time points post ATc addition clearly showed efficient depletion of FLAG-SepIVA<sub>Δlum</sub> by 12 h (Fig 9f). 18 h post ATc addition, we observed significantly compromised growth (Fig 9g), confirming essentiality of SepIVA for the in vitro growth. So as to assess the localization of FtsQ in the presence and absence of SepIVA, we electroporated pN-GFP-FtsQ into mc2::sep<sub>Δlum</sub> strain to generate mc2::sep<sub>Δlum</sub>;gfpQ. Localization of GFP-FtsQ was determined in the presence and absence of ATc. We have quantitated ~100 independent cells in the presence and absence of SepIVA (Fig 9h). We have not observed any statistically significant changes in the localization of GFP-FtsQ upon depletion of SepIVA (Fig 9h & i). These results suggest that recruitment of FtsQ during cell division precedes recruitment of SepIVA. Taken together, our data suggests a definitive role for FtsQ in modulating cell length, cell division, and eventual survival during in vitro growth.

**Discussion**

The ability of an organism to maintain its cell length necessitates stringent regulation of cellular elongation and division, which in turn is reliant on coordinated involvement of macromolecular elongosome and divisome assemblies, respectively. Identification and characterization of individual contributors to these complexes is necessary to understand the sequence of recruitment and their impact on the cell growth and division. Divisome assembly driven septation and cytokinesis mark the decisive last step of cell cycle and are well regulated for precise cell division. The divisomal components are known to maintain a constant stoichiometry by exhibiting intrinsic regulation. Elevated levels of divisomal components like FtsA, ZipA, ZapC (stabilizer of FtsZ bundling), FtsQ in *E. coli* (FtsQ<sub>Ec</sub>) and FtsZ, CrgA, Ssd (Rv3660c) in mycobacterium have been shown to cause filamentation, suggesting concentration to be a major determinant for optimal cell division (25,27,28,50-52). Interestingly, it was observed that overexpression of FtsZ alleviates the cell division blockage induced by overexpression of FtsA, suggesting that appropriate ratios of cell division proteins is necessary in determining the rate and timing of cell division (53-55). In accordance with these observations, results showed that overexpression as well as the depletion of FtsQ led to changes in the average mean cell length (Fig 1 & 4). Interestingly, the perturbation in the levels of FtsQ need not to be very drastic; as we observed subtle changes in the level of FtsQ resulted in altered mean cell length (Fig 2 & 4).

The observed elongated phenotype upon FtsQ overexpression (Fig 1) could either be due to its role in peripheral PG biosynthesis (elongation) or due to inadequacy in undergoing septation (division). Inadequacy in undergoing septation could either be due to incompetent initiation or completion of septation process. Overexpression of ZipA in *E. coli* causes formation of smooth filaments, which is characterized by the absence of any visible invagination, suggesting that block is at early stage of cell division (51). On the other hand, overexpression of FtsQ in *E. coli* displays multiseptate pattern (52), suggesting block at completion of septation process. In our studies, we have observed increased proportion of bi-septate filamentous cells upon FtsQ overexpression, indicating that cell division was likely blocked at later stages of cell division and cells are able to initiate formation of second septa before completion of first, implying altered coordination among cell division members (Fig 2).

FtsN, late cell recruiter protein of *E. coli*, contains a cytoplasmic amino terminal region, and a carboxy terminal region containing sporulation related repeat domain (SPOR). The N-terminal region of FtsN plays a role in interaction with FtsA (early divisome protein) and the carboxy terminal SPOR domain is responsible for sensing PG and mediating mid cell localization (56-58). FtsQ, bitopic protein, encompasses N-terminal cytoplasmic region connected through a transmembrane domain to the C-terminal periplasmic region (Fig 1). FtsQ<sub>Ec</sub> localizes to the mid cell and is involved in recruitment of interacting proteins necessary for the division (37). While the periplasmic region of FtsQ was found to be essential for its interaction with FtsB/FtsL in *E. coli* and PBP2B in *B. subtilis*, the functionality of N-terminal cytosolic region has not...
yet been elucidated (41,59). We observed that overexpression of both N- and C-terminal domains of mycobacterial FtsQ led to elongation of cells (Fig 3), suggesting that both the domains are independently important for its functionality. However, it remains elusive whether both N and C terminus domains interact with similar or differential set of proteins. We noticed that the number of bands detected in the western blot for FtsQ varied from one to three (Fig 1, 2, 3 and 8). The appearance of these additional bands is dependent on amount of protein loaded, resolution of the gel and the ECL exposure times. FtsQ is a single transmembrane containing protein and we speculate that it may be undergoing spontaneous cleavage during the storage at susceptible sites, which is likely to be responsible for multiple bands.

Deletion of many Fts proteins in E. coli is known to cause filamentation and hence they are categorized as divisome proteins (60,61). In contrast, a point mutation in FtsL (E88K) of E. coli shows smaller cells and enhanced cell division rate, subsequently leading to cell lysis. We observed that depletion of FtsQ in Mtb for 4 and 10 days, respectively gave two distinct phenotypes. While we observed shorter cells with distinct septa on day 4, the cells were longer at 10 days post depletion, eventually leading to cell death (Fig 4 & 5). The elongation phenotype observed at day 10 is consistent with a recent report, wherein depletion of FtsQ in M. megg was shown to result in elongated and branched cells (24).  We speculate that distinct morphologies could be due to differences in the protein levels of FtsQ.

Depletion of FipA and PonA1/PonA2 is known to reduce bacterial replication in macrophages and murine infection models, respectively (38,62,63). Recent identification of C terminal and full length Wag31 induced production of T cell cytokines such as IL-10 and IL-17, suggest crucial role of cell division proteins in maintenance of bacilli survival during infection (64). The observed reduction in bacilli growth during macrophage infection with FtsQ depletion strain indicates that FtsQ plays an important role in maintenance of cell division and hence, persistent survival of pathogen during host infection (Fig 4). However, we observed marginal changes, if any, in the cell length upon infection in the presence or absence of FtsQ (data not shown).

We observed that overexpression of both N and C-terminal regions impacted the cell morphology (Fig 3). On the contrary, while both full length and C-terminal domain could complement the FtsQ functionality, N-terminal domain failed to do so (Fig 5 & 6). Similar observations were noted in both E. coli and B. subtilis, wherein cytoplasmic domain was found to be dispensable for the function of FtsQ and DivIB, respectively (65,66). Interestingly, a point mutation in the C-terminal α domain (V92D) of FtsQEc, made the cytoplasmic N-terminal domain essential for its functionality, suggesting possible crosstalk between domains (67). Thus, even though the N-terminal region of FtsQ fails to complement (Fig 6), its possible role in the optimal functionality of FtsQ cannot be overruled. In E. coli sub domains of FtsQ, 1-135 and 136-276 aa both complemented the mutant, suggesting that many of the interactions may have been mediated by both the domains (68). However, in Mtb the loss of viability as well as cell length defects upon FtsQ depletion, could not be complemented even in the absence of C-terminal γ domain, indicating that all the C-terminal sub domains are independently essential for its functionality (Fig 6).

Phosphorylation of cell division proteins such as FtsZ and Wag31 are shown to be important for modulating their functionality (69,70). Essential mycobacterial serine/threonine protein kinases PknA, PknB and the sole phosphatase PstP play an important role in regulating cell division and cell wall synthesis processes (71-74). Phosphorylation of FtsZ by PknA was reported to regulate its GTPase activity (69). Furthermore, PknA mediated phosphorylation of FtsZ on T343 and FipA on T77 are necessary for cell division under oxidative stress conditions (38). Phosphorylation of Wag31, a regulator of cell shape and cell wall synthesis, alters the growth rate as well as its interaction with its kinase PknA (70,75). In addition to FtsZ, FipA and Wag31, which have been biochemically shown to be the targets of PknA, high throughput phosphoproteomic studies have identified phosphorylation sites on other cell division proteins such as FtsI, FtsK and FtsQ (44-46). However, the functional implications of phosphorylation have not been elucidated. Our studies revealed that FtsQ is phosphorylated on T24 residue in the N-terminal domain with ~30% stoichiometry (Fig 7). Even though there were no significant changes in the viability upon complementation with phosphomimetic and ablative mutants, we observed that phosphoablative (T24A) mutant failed to complement the cell length defects observed (Fig 7).  We hypothesize that the phosphorylation at T24 in FtsQ may be important in the context of recruitment of one or more proteins necessary for regulating cell length.
Previous studies have demonstrated a pairwise interaction between a numbers of cell division members such as LamA-PonA1, FtsZ-FtsW, FtsW-FtsI, FtsI-Wag31, Wag31-CwsA, CwsA-CrgA, CrgA-PBPA, CrgA-FtsQ, SepF-FtsZ and FtsZ-FipA-FtsQ, suggesting a complex and multiple interactions among the members of divisome in mycobacteria (26,27,30,38,76-78). In this report with the help of mass spectrometry, we have identified novel interacting partners for FtsQ (Fig. 8). Since mass spectrometry based identification is very sensitive, we have applied highly stringent criteria for the analysis. We have only considered those proteins, which were identified in all three biological replicates but not in the corresponding control IPs. We have identified 63 interacting proteins in all three biological replicates with wild type FtsQ. Significant numbers of these interacting partners were ribosomal proteins (Sup Table 1), which may be present because of their relative abundance. Interestingly, we did not find Wag31 or FtsZ in even one of these runs. However, one cannot rule out the possibility of transient interaction between FtsQ and the other cell division proteins at the pole or septum at specific stages of cell division.

While this manuscript was under consideration, Wu et al., reported characterization of novel septal factors in Msmeg (24). They have performed pull down experiments with cross-linked FtsQ-Strep and identified 48 Msmeg proteins, among which 41 proteins had homologs in Mtb. When we compared our list with that of Wu et al., we found only two proteins, namely SepIVA and FhaA to be common (Sup Table 1 & (24)). The limited overlap may be due to differences in the pull down protocol (vis a vis cross linking) and/or the species used for the experiment (Mtb vs Msmeg). We have identified four interacting proteins, which were found with wild type as well as phosphosite mutants. In addition to newly identified cell division protein SepIVA (24), we have identified transketolase, a metabolic enzyme; single stranded binding protein, involved in DNA replication; and a conserved hypothetical protein, whose function is unknown. Further, we have identified Rv3140 only in FtsQ-T24E sample. Rv3140 is annotated as a probable acyl-CoA dehydrogenase, which has not yet been characterized. With the exception of SepIVA, we have neither validated or explored the implications of these interactions any further.

Localization of FtsQ at septal region in E. coli indicates its predominant function during division (48). We have also detected predominant septal localization of FtsQ in mycobacteria indicating its possible function during division (Fig 9). These results are in agreement with a recent report, in which FtsL, FtsQ, FtsL and FtsB proteins were found to be at the septum (24). With the exception of SepIVA, we have not identified any other protein involved in cell division process as an interacting partner (Sup Table 1). However, we cannot exclude the possibility of transient spatio temporal interactions between FtsQ and other cell division proteins. SepIVA protein is known to possess a conserved DivIVA domain and is recently reported to be localized to the septum and its depletion results in elongated cells, thus indicating its involvement in cell division process (24). Since both FtsQ and SepIVA are localized to the septum and they interact with each other, we investigated the localization of FtsQ in the presence or absence of SepIVA. We did not find significant differences in the localization of FtsQ in the absence of SepIVA (Fig 9). These results suggest that SepIVA is recruited to the septum after FtsQ. The domains of FtsQ and SepIVA involved in their interaction and the biological impact of abrogating their interaction on cell division would be investigated in future.

Methods
Reagents, bacterial strains, and growth conditions.
Restriction/modification enzymes were procured from New England Biolabs (NEB) and MBI Fermentas (Thermo Scientific). Oligonucleotide primers were procured from Sigma Aldrich. Analytical grade chemicals were purchased from Sigma Aldrich, Amresco, Merck or Biobasic Canada. Pristinamycin 1A was purchased from Molcon Corporation, Canada. pENTR/Directional TOPO Cloning Kit was purchased from Invitrogen. pNit1 vector (79) was a kind gift from Christopher M. Sassetti. Anti-FLAG monoclonal antibody was purchased from Sigma. Electron microscopy chemicals were obtained from Electron Microscopy Sciences and growth media components were acquired from BD. E. coli DH5a strain (Invitrogen) was used for cloning, which was grown in LB broth at 200 revolutions per minute (rpm) or LB-agar in the presence of either Kanamycin (50 µg/ml) or Hygromycin (150 µg/ml). Msmeg mc2155 or Mtb H37Rv strains were grown at 100 rpm/ 37°C in Middlebrook 7H9 broth (BD) supplemented with 10% ADC (5% bovine serum albumin fraction V, 2% Dextrose, 0.85% NaCl, catalase 0.004%) and 0.05% Tween-80 in the absence or presence of Kanamycin (25 µg/ml) or Hygromycin (100 µg/ml). Msmeg mc2155 or Mtb strains were plated on Middlebrook 7H10 plates supplemented with OADC (ADC + 0.06% oleic acid).
Generation of ftsQ expression constructs and 
Rv ΔfQ gene replacement mutant.

Full length Mtb H37Rv ftsQ was amplified from 
H37Rv genomic DNA using gene specific primers and 
Phusion DNA polymerase (NEB), and the 
amplicons were cloned into the NdeI-HindIII sites in 
pNit1 vector. pNit-FtsQ-N (1-369 bp), pNit-FtsQ-C 
(300-945 bp), pNit-FtsQ-Cα (1-579 bp), pNit-FtsQ-
Cβ (1-783bp) were generated by amplifying the 
respective regions using specific forward and reverse 
primers and cloning the amplicons into the NdeI-
HindIII sites on pNit1 vector. All point mutations 
were generated by overlapping PCR using 
appropriate mutagenic primers and the mutations 
were confirmed by DNA sequencing. To generate 
the gene replacement mutant in Mtb, -20 to 680 bp 
region of ftsQ was PCR amplified and the amplicons 
were cloned into the NeoI-SphI sites under the 
pristinamycin inducible promoter in the vector 
pAZI9479 (43) to generate pAZ -ftsQ. The suicide 
delivery vector pAZ-ftsQ was electroporated into 
H37Rv, and colonies were selected on 7H10 agar 
containing hygromycin and pristinamycin (2 
µg/ml). Gene replacement at the native locus was confirmed 
with the help of specific PCRs.

Growth kinetics, SEM and TEM experiments.

To analyze the growth pattern of wild type and 
recombinant strains of Msmeg, cultures grown till 
A600 ~0.8 were washed once with PBS containing 0.05% 
Tween80 and were seeded at an initial A600 of 0.02 or 
0.05 in the absence or presence of varied 
concentrations of IVN (0.2 to 5 µM). The growth 
was monitored every 3 h for 30 h. Rv or RvΔfQ 
mutant or RvΔfQ complemented strains were grown 
till A600~0.8 in presence of inducer pristinamycin 1A 
and then seeded at A600 of 0.05 in presence or 
absence of pristinamycin 1A or 0.2 µM IVN. The 
growth was monitored every 24 h for 10 days. To 
evaluate the bacteriostatic or bactericidal effects of 
FtsQ, Rv or RvΔfQ mutant or RvΔfQ complemented 
strains were withdrawn on 0 & 10 days and different 
dilutions were spotted on plates containing 
pristinamycin. SEM and TEM experiments were 
performed as described previously (80,81). For SEM, 
cells were visualized under the microscope with 10000X 
or 15000X magnifications after coating with gold 
particles (Carl Zeiss, Evo LS SEM). For TEM, cells 
were sliced and stained, followed by its examination 
under Tecnai G2 20 twin (FEI) transmission electron 
microscope.

THP1 macrophage infection experiments.

Bacterial cultures Rv and RvΔfQ grown in the 
presence of pristinamycin were seeded in presence or 
absence of pristinamycin in 7H9 media at A600~0.1. 
THP1 cells were cultured, maintained in RPMI-1640 + 
10% FBS (heat inactivated) + 1% Pen-Strep 
medium. For CFU enumeration, 2 x 10^5 Cells / 24 
well plates were seeded in triplicates for each sample. 
The cells were differentiated with phorbol 12-
myristate 13- acetate (PMA) and were infected as 
described previously (80) at MOI of 1:5 with Rv and 
RvΔfQ in presence or absence of pristinamycin. Cells 
were lysed after 96 and 120 h, and CFUs were 
enumerated.

Identification of interacting partners and 
phosphorylation sites.

In order to identify interacting partners, the 
cultures of Rv::pN-FtsQ, Rv::pN-FtsQ-T24A 
and Rv::pN-FtsQ-T24E were seeded at A600 ~0.1 in 
the presence of 5 µM IVN. Western blot was 
performed to evaluate the expression and 1 mg of 
lysate was immunoprecipitated using FLAG-M2 
beads (Sigma). The bound FtsQ and interacting 
partners were eluted by adding 2X SDS sample dye 
to the beads. Samples were resolved on 10% 
polyacrylamide gel till the dye front is ~1.5 cm into 
the resolving gel. Gel pieces were sliced and 
processed for trypsinization as described earlier (82). 
MS and MS/MS analysis was performed as described 
previously to determine the stoichiometry of 
phosphorylation and interacting partners (83).

Validation of SepIVA-FtsQ interaction.

The coding sequence of sepIVA was PCR 
amplified using H37Rv genomic DNA as the 
template. The amplicon was digested with NdeI-
HindIII enzymes and cloned into the corresponding 
sites in pQE2 vector (Qiagen). The construct was 
transformed into E. coli BL21-DE3 codon plus strain 
and expression was induced with 0.1 mM IPTG at 
22°C for 12-16 h. His tagged protein was purified as 
described earlier (84). To prepare antiserum against 
SepIVA, 4 Balb/c mice were immunized 
subcutaneously with a mixture containing 50 µg 
protein and equal volume of Freund's incomplete 
adjuvant (Sigma). The immunization procedure was 
repeated twice (2 weeks apart). Two weeks after the 
last immunization, mice were euthanized and the sera 
were collected. In order to validate the interaction of 
FtsQ with SepIVA, 1 mg whole cell lysates (WCLs) 
from strains expressing FLAG-FtsQ wild type and 
mutants, were IPed. 1/10th IP was probed with a-
FLAG antibody and 9/10th IP was probed with a-
SepIVA antibody (1:2000 d).
Generation of mc2::sep mutant.

To generate the integrating construct sepIVA was amplified from mc2 genomic DNA using gene specific primers and the amplicon was cloned into the Ndel-HindIII sites in pST-KirT vector (80). The construct was electroporated in mc2 to generate a mc2::sep merodiploid strain. Two independent mc2::sep merodiploid colonies were assessed for depletion of FLAG-SepIVA upon ATc addition (200 ng/ml) by probing WCLs with anti-FLAG antibody. 5’ and 3’ genomic flank sequences of sepIVA (~800 bps each) were amplified and allelic exchange substrate (AES) was generated as described earlier (85,86). Linearization of AES, electroelution, mutant generation and confirmation was performed as previously described (87).

Localization of GFP-FtsQ in Msmeq and mc2::sep deletion strains.

The genes encoding gfp and ftsQ were amplified from plasmids pMN437 (88) and pNit-FtsQ, respectively. The gfp and ftsQ amplicons were digested with Ndel-SapI and SapI-HindIII, respectively and cloned into the Ndel-HindIII sites in pNit1 to generate pN-GFP-FtsQ construct. The construct was electroporated into Msmeq and mc2::sep strains to generate mc2::gfp and mc2::sep::gfp strains. Culture of mc2::gfp were seeded at A600~0.1 at 30°C for 14 h to express GFP fused FtsQ protein. mc2::sep::gfp strain was seeded at A600~0.25 in the presence and absence of 200 ng/ml ATc for overnight at 37°C. These cultures were subcultured at A600~0.25 at 30°C for 10 h in the presence or absence of ATc (25 ng/ml) and GFP-FtsQ expression was induced with 0.2 µM IVN. Cells were harvested, fixed with 4% paraformaldehyde and imaged using LSM 510 Meta Zeiss confocal microscope, as described in previous reports (72).

Acknowledgements

This work was supported by the funding provided by Department of Science and Technology (DST), Government of India (EMR/2014/000877) to VKN. We thank Prof. Christopher M. Sassetti and Dr. Francesca Forti for their kind gift of pNit1 and pAZI9479 vectors. We thank the Scanning Electron Microscopy, confocal microscopy and Mass spectrometry facilities at National Institute of Immunology (NII) and Mrs. Rekha Rani and Mrs. Shanta Sen for their support in managing these facilities. We thank the Transmission Electron Microscopy Facility at Institute of Genomics and Integrative Biology (IGIB).

Author Contributions

P.J. designed and performed the experiments. V.K.N. supervised the design and execution of the experiments. P.J and V.K.N wrote the manuscript. B.M and P.J. prepared, analyzed the samples and results of mass spectrometry M.Z.K. generated and characterized the sepIVA mutant and antibody. P.J. and S.L. performed microscopy. A.S. performed TEM.

Conflict of Interest

The authors declare that they have no conflicts of interest with the contents of this article.

Statistical analysis

Two way Anova was used to analyze the significance of results, unless otherwise specified. GraphPad Prism version 5.0 was used for the plotting the results and modified using adobe illustrator CS5.1.
Role of FtsQ in cell division

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Role of FtsQ in cell division

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Figure Legends-

Figure-1. Overexpression of FtsQ increases the average cell length. a. Schematic representation of the Rv2160c-ftsQ operon. b. Schematic depiction of various domains of FtsQ. N-terminal (N), transmembrane (TM), carboxy terminal regions (C), the subdomains α, β and γ in the C-terminal regions are indicated. c. Fresh cultures of mc2::pNit and mc2::pN-FtsQ were seeded at A600~0.02 and induced with 5 µM IVN for 14 h. 30 µg and 10 µg of the WCLs prepared from these samples were resolved, transferred to nitrocellulose membrane and probed with α-FLAG and α-PknB antibodies, respectively. d. Cultures of mc2, mc2::pNit and mc2::pN-FtsQ were seeded at A600~0.02 and induced with 5 µM IVN. CFUs were enumerated at 0, 14 and 28 h, grown in the presence of 5 µM IVN. e. Cultures of mc2, mc2::pNit and mc2::pN-FtsQ strains were seeded at A600~0.02 and induced with 5 µM IVN for 14 h. The samples were processed for SEM and morphologies were observed at 15000X. f. The cell lengths for ~200 cells / samples from (e) were measured using Smart Tiff software and plotted as scattered dot plot. Mean and standard deviation (SD) were calculated using GraphPad Prism6. Mean cell lengths obtained are

Figure-2. Overexpression of FtsQ results in multi-septate phenotype. a. Quantification of differential level of FtsQ expression in mc2::pN-FtsQ overexpression strain, induced with differential concentration of IVN. Fold change in mRNA levels of ftsQ at 0.2, 1 and 5 µM IVN, calculated with respect to the transcript levels in the absence of inducer by qRT-PCR. Mean with standard deviation is from three replicates. b-d. Cultures of mc2, mc2::pNit and mc2::pN-FtsQ strains were seeded at A600~0.02 and grown in the presence of 5, 1, 0.2 µM or no IVN for 14 h. b. 30 µg and 10 µg of the WCLs prepared from these samples were resolved, transferred to nitrocellulose membrane and probed with α-FLAG and α-PknB antibodies, respectively. * indicates band due to probable cleavage of full length FtsQ. c. Samples were processed for SEM analysis and representative images at 15000X are shown. Scale bar, 1 µm. d. Cell lengths of ~200 cells / sample from (c) were measured independently using Smart Tiff software and plotted scattered dot plot with mean and SD values. Mean cell lengths obtained are me 2 - 3.1 µm; mc2::pNit - 3.3 µm; mc2::pN-FtsQ - 3.9 µm; mc2::pN-FtsQ + 0.2 µM - 3.9 µm; mc2::pN-FtsQ + 1.0 µM - 3.7 µm; mc2::pN-FtsQ + 5 µM - 4.1 µm. e. Samples were processed for TEM analysis and the representative TEM images are shown. Scale bar, 0.5 µm. f. 65 to 75 cells/ sample from (e) were analyzed to calculate the percentage of non-septate, uni-septate and bi/multiseptate cells. 1- mc2 + 5 µM; 2- mc2::pNit + 5 µM; 3- mc2::pN-FtsQ + 5 µM; 4- mc2::pN-FtsQ + 1.0 µM; 5- mc2::pN-FtsQ + 0.2 µM and 6- mc2::pN-FtsQ. Statistical analysis was performed with the help of two way ANOVA. *** P<0.0001; ns, non-significant.

Figure 3- Both N and C-terminal domains of FtsQ are critical for cell length maintenance. a. Fresh cultures of mc2, mc2::pNit, mc2::pN-FtsQ, mc2::pN-FtsQ-N and mc2::pN-FtsQ-C were seeded at A600~0.05 and induced with 5 µM IVN for 14 h. 30 µg and 10 µg of WCLs prepared from these samples were resolved, transferred and probed with α-FLAG and α-PknB antibodies, respectively. Bands indicated by white arrows arose probably due to cleavage of full length FtsQ. b. Cultures of me2, mc2::pNit, mc2::pN-FtsQ, mc2::pN-FtsQ-N and mc2::pN-FtsQ-C strains were seeded at A600~0.05 and grown in the presence of 5 µM IVN. CFUs were enumerated at 0, 14 and 28 h. Error bars represent standard error. c. The samples were processed for SEM after 14 h and morphologies were observed at 15000 X. Scale bar - 1 µm. d. The cell lengths for ~200 cells / samples from (c) were measured using Smart Tiff software and plotted as scattered dot plot. Mean and SD were calculated using GraphPad Prism6. Mean cell lengths obtained were mc2 - 3.1 µm; mc2::pNit - 3.2 µm; mc2::pN-FtsQ - 5.7 µm; mc2::pN-FtsQ-N - 5.9 µm and mc2::pN-FtsQ-C - 4.4 µm. Data is representative of two biologically independent experiments. Statistical analysis was performed with the help of two way ANOVA. *** P<0.001, ****P<0.0001.

Figure-4. FtsQ is essential for bacterial viability in Mtb a. Schematic depiction of the methodology used to generate gene replacement mutant (RdsQ). Primer pairs used for PCR confirmation are indicated. b. 1% agarose gel showing PCR amplification using genomic DNA from Rv and RdsQ as templates. Mr. denotes 1 kb ladder. Left panel shows ftsQ (0.94 kb) gene amplicon in both Rv and RdsQ. Middle panel shows differential PCR amplicon of (1.1kb) obtained only in RdsQ. Right panel shows specific amplification of 0.8 kb fragment in RdsQ. c-d. Rv and RdsQ grown in the presence of 1 µg/ml pristinamycin till A600~0.8 were washed twice with PBS containing 0.05% tween-80 and fresh cultures were seeded at A600~0.05 in triplicates. c. 10 µl of freshly inoculated cultures was streaked on 7H11 agar plates in the presence or absence of 1 µg/ml
pristinamycin. d. CFUs were enumerated at 0, 2, 4, 6, 8 and 10 days. f. Intra-macrophage survival of RvΔftsQ (+/- p) was compared with Rv (+/- p) strain after infection in PMA differentiated THP1. Data is representative of one of the two biologically independent experiments. Statistical analysis was performed with the help of two way ANOVA. ** P<0.01; *** P<0.001; **** P<0.0001; ns- non significant.

Figure-5. FtsQ depletion alters the mean cell length a-c. Fresh cultures of Rv, RvΔftsQ were seeded at A600~0.05 and grown in the presence (+p) or absence (-p) of inducer. Samples were harvested after day 4 and 10. a. The samples were processed for SEM and morphologies were observed at 15000 X. Representative images are shown. Scale bar - 1 µm. b. The cell lengths for ~200 cells / samples from (a) were measured and plotted. Mean cell lengths obtained at day 4 are Rv +p - 2.3 µm; RvΔftsQ +p - 2.2 µm and RvΔftsQ -p - 1.8 µm and at day 10 are Rv +p - 2.1 µm; RvΔftsQ +p - 2.7 µm and RvΔftsQ -p - 3.2 µm. c. Representative TEM images for Rv +p, RvΔftsQ +p and RvΔftsQ -p at day 4 and day 10. Scale bar, 0.5 µm. d. RvΔftsQ strain was transformed with pNit and pNit-FtsQ constructs to generate RvΔftsQ:pN and RvΔftsQ:ftsQ, respectively. Cultures were seeded at A600~0.05 and 10 µl were streaked on 7H11 agar in absence or presence of 1 µg/ml pristinamycin. e. Cultures were seeded at A600~0.05 in the presence or absence of 1 µg/ml pristinamycin or 0.2 µg/ml IVN as indicated. CFUs were enumerated at 0, 10 days. Error bars represent standard error. f. Experiment was performed as described above except that cells were harvested at day 4 and processed for SEM. The cell lengths for ~200 cells / samples were measured and plotted. Mean cell length values were; Rv +p -2.2 µm; RvΔftsQ:pN +p 2.3 µm; RvΔftsQ:pN -p -1.8 µm; and RvΔftsQ:ftsQ -p - 2.2 µm. Data is representative of two biologically independent experiments. Statistical analysis was performed with the help of two-way ANOVA. ** P<0.01; **** P<0.0001; ns- non significant.

Figure-6. Carboxy terminal domains are critical for the functionality of FtsQ. a-c. RvΔftsQ was electroporated with pNit, pNit-FtsQ, pNit-FtsQ-N and pNit-FtsQ-C to generate RvΔftsQ:pN, RvΔftsQ-FtsQ, RvΔftsQ-fqtsQ-N and RvΔftsQ-fqtsQ-C. Cultures were seeded at A600~0.05 in the presence of 0.2 µM IVN and in the presence (+p) or absence (-p) of pristinamycin. a. 10 µl of freshly inoculated cultures were streaked on 7H11 agar in absence or presence of 1 µg/ml pristinamycin. b. CFUs were enumerated at 0, 10 days. Error bars represent standard error. c. Cells were harvested at day 4 and processed for SEM. The cell lengths for ~200 cells / samples were measured and plotted. Mean cell lengths obtained at day 4; Rv +p - 2.5 µm; RvΔftsQ:pN +p - 2.6 µm; RvΔftsQ:pN -p - 1.8 µm; RvΔftsQ:ftsQ -p - 2.5 µm; RvΔftsQ:ftsQ-N -p - 1.6 µm and RvΔftsQ:ftsQ-C -p - 2.5 µm. d. RvΔftsQ was electroporated with pNit-FtsQs, pNit-FtsQ-C, pNit-FtsQ-Cα and pNit-FtsQ-Cβ to generate RvΔftsQ::ftsQ, RvΔftsQ::ftsQ-C, RvΔftsQ::ftsQ-Cα and RvΔftsQ::ftsQ-Cβ. 10 µl of freshly inoculated cultures were streaked on 7H11 agar in absence or presence of 1 µg/ml pristinamycin. e. CFUs of Rv +p, RvΔftsQ:pN +p, RvΔftsQ:pN -p, RvΔftsQ:ftsQ -p, RvΔftsQ::ftsQ-Cα -p, RvΔftsQ::ftsQ-Cβ -p were enumerated at 0, 10 days. Error bars represent standard error. f. Cells were harvested at day 4 and processed for SEM. The cell lengths for ~200 cells / samples were measured and plotted. Mean cell lengths obtained at day 4 are RvΔftsQ::ftsQ -p - 2.1 µm, RvΔftsQ::ftsQ-Cα -p - 1.9 µm, RvΔftsQ::ftsQ-Cβ -p - 1.8 µm. Data is representative of two biologically independent experiments. Statistical analysis was performed with the help of two way ANOVA. ** P<0.01; *** P<0.001; **** P<0.0001; ns- non significant.

Figure-7. FtsQ is phosphorylated at T24 residue and phosphorylation influences cell division. a-b. RvΔftsQ:T24 strain was inoculated at A600~0.1, induced with 5 µM IVN and grown till A600~0.8. FLAG tagged FtsQ was immunoprecipitated, fragmented using trypsin and subjected to LC-MS as described previously (83). a. MS/MS spectrum of precursor m/z 1070.45949 (+2) and MH + 2140.91899 Da, of the semi-tryptic MS/MS spectrum of precursor m/z 1070.45949 (+2) and MH + 2140.91899 Da, of the semi-tryptic phosphopeptide VADDAAEEAV(εT)ELPLATESK. The unambiguous location of the intact phosphate group on T24 was determined by the presence of the b and y ion series containing b6,11, b13, b15:7 and y4, y8, Y1:7. b. Tabular representation of identified stoichiometry of phosphorylation for T24 site. c-e. RvΔftsQ was electroporated with pNit, pNit-FtsQ, pNit-FtsQ-T24E and pNit-FtsQ-T24A to generate RvΔftsQ::pN, RvΔftsQ::ftsQ, RvΔftsQ::ftsQ-T24E and RvΔftsQ::ftsQ-T24A. Cultures were seeded at A600~0.05 in the presence of 0.2 µM IVN and in the presence (+p) or absence (-p) of pristinamycin. c. 10 µl of freshly inoculated cultures were streaked on 7H11 agar in absence or presence of 1 µg/ml pristinamycin. d. CFUs were enumerated at 0, 10 days. Error bars represent standard error. e. Cells were harvested at day 4 and processed for SEM. The cell lengths for ~200 cells / samples were measured and plotted. Mean cell lengths obtained at day 4; Rv +p -2.3 µm, RvΔftsQ::pN +p - 2.2 µm, RvΔftsQ::pN -p -1.7 µm, RvΔftsQ::ftsQ -p - 2.2 µm, RvΔftsQ::ftsQ-T24E -p - 1.9 µm.
and RsAfQ::ftsQ-T24A -p - 1.7 µm. Data is representative of two biologically independent experiments. Statistical analysis was performed with the help of two-way ANOVA. * P<0.1, ** P<0.01; *** P<0.001; **** P<0.0001; ns- non significant.

Figure-8. *FtsQ* interacts with *SepIVA* protein in a phosphorylation independent manner. a. 30 and 10 µg of WCLs prepared from Rs:pN, Rs::pN-ftsQ, Rs::pN-ftsQ-T24A and Rs::pN-ftsQ-T24E strains, in the presence of 5 µM IVN, were resolved, transferred on SDS-PAGE and probed with α-FLAG and α-PknB antibodies, respectively. 1 mg of WCL was immunoprecipitated (IP) and 1/10th of IP was probed with α-FLAG. b. Venn diagram showing 63 interacting partners, found common in three biological replicates of FLAG-ftsQ, each containing 117, 122 and 111 unique proteins. Unique proteins were obtained after subtracting the proteins identified in the corresponding control IP. c. 63, 24 and 24 interacting proteins were identified in all three biological sets of FLAG-ftsQ, FLAG-ftsQ-T24A and FLAG-ftsQ-T24E, after subtracting the proteins identified in the corresponding control IP. Venn diagram showing 4 interacting partners, found common between FLAG-ftsQ, FLAG-ftsQ-T24A and FLAG-ftsQ-T24E. d. Table showing the list of four interacting partners. e. 30 µg of WCLs prepared from Rs:pN, Rs::pN-ftsQ, Rs::pN-ftsQ-T24A and Rs::pN-ftsQ-T24E strains in the presence of 5 µM IVN, were resolved, transferred on SDS-PAGE and probed with α-FLAG and α-SepIVA antibodies, respectively. 1 mg WCL from each sample was IPed and 1/10th of IP was probed with α-FLAG antibodies and 9/10th of the IP was probed with α-SepIVA antibodies. * indicates band due to probable cleavage of full length FtsQ.

Figure-9. *FtsQ* shows predominantly septal localization both in the presence and absence of *SepIVA* protein. a-b. Fresh cultures of mc2::gfQ were seeded at A600~0.1 and grown for 14 h at 30°C. Cells were harvested, fixed with 4% paraformaldehyde and washed with 1X PBS. Cells were imaged with the help of Zeiss Imager.M1 microscope. b. Bar graph represents percentage distribution of GFP-FtsQ in different localization patterns. ~150 cells were counted to calculate the percentage distribution. c-g. Generation and characterization of mc2::sep deletion strain in mc2. a. Schematic depiction of the methodology used to generate gene replacement mutant (mc2::sep). Primer pairs used for PCR confirmation are indicated. d. mc2 cultures were electroporated with pST-KirT-sep to generate merodiploid strain mc2::sep. Cultures of merodiploid strain was seeded at A600~0.1 in presence and absence of ATc. 30 and 10 µg of lysates were prepared, resolved, transferred and probed with α-FLAG and α-PknB antibodies, respectively. e. Genomic DNA was prepared from mc2 and mc2::sep mutant and PCR amplifications were performed with the primers as indicated. Left panel shows the amplification with primers specific for rodAMsm gene, which was used as control. The PCR products were resolved on 1% agarose gel; M-denotes 1 kb ladder. Second and third panel show the PCR amplification with F1-R1 and F2-R2 primers, respectively. 1.1 and 1.2 kb PCR product is expected only in the mc2::sep mutant, but not in the mc2. Right panel shows the PCR amplification using sepIVAMsm gene forward and reverse primers. Both mc2 and mc2::sep amplify ~0.7 kb sepIVAMsm gene due to the presence of native and integrated copy of the gene, respectively. However in case of mc2::sep we expect a 1.7 kb additional band due to presence of hygromycin at the native locus. f. mc2::sep mutant strain was seeded at A600~0.1 in presence or absence of ATc (200 ng/ml). 30 and 10 µg of WCLs from cultures grown in the absence or in the presence of ATc for 6 and 12 h were resolved, transferred and probed with α-FLAG and α-GroEL antibodies, respectively. g. Photographs of mc2::sep cultures grown in the absence or presence of ATc for 18 h. h. Fresh culture of mc2::sep::gfQ were seeded at A600~0.25 in the presence or absence of ATc (200 ng/ml) at 37°C for 12 h. These cultures were subcultured at A600~0.25 at 30°C for 10 h in the presence or absence of ATc (25 ng/ml) and GFP-FtsQ expression was induced with 0.2 µM IVN. Cells were harvested, fixed and imaged using LSM 510 Meta Zeiss confocal microscope. i. Bar graph represents percentage distribution of GFP-FtsQ in different localization patterns. ~100 cells were counted to calculate the percentage distribution.
Table 1. Strains used in the study.

| Strains         | Description                                                                 | Source                     |
|-----------------|-----------------------------------------------------------------------------|----------------------------|
| mc2155          | Wild type *M. smegmatis* mc2155 strain                                       | ATCC, 700084               |
| mc^2::pNit      | *mc^2* strain electroporated with ectopic, IVN inducible pNit vector; Kan^r  | This study                 |
| mc^2::pN-FtsQ   | *mc^2* strain electroporated with pNit-FtsQ (aa 1-315) construct             | This study                 |
| mc^2::pN-FtsQ-N | *mc^2* strain electroporated with pNit-FtsQ-N (aa 1-123) construct           | This study                 |
| mc^2::pN-FtsQ-C | *mc^2* strain electroporated with pNit-FtsQ-C (aa 101-315) construct         | This study                 |
| mc^2::gfQ       | *mc^2* strain electroporated with pN-GFP-FtsQ construct                      | This study                 |
| mc^2::sep       | *mc^2* strain electroporated with pST-KirT-SepIVA construct                 | This study                 |
| mc^2::sep::gfQ  | *mc^2*::sep electroporated with linearized AES                               | This study                 |
| mc^2::sep::gfQ  | *mc^2*::sep strain electroporated with pN-GFP-FtsQ                          | This study                 |
| Rv              | Wild type *M. tuberculosis* H37Rv strain                                     | ATCC                       |
| Rv::pN          | Rv electroporated with pNit vector                                           | This study                 |
| Rv::pN-ftsQ     | Rv electroporated with pNit-FtsQ construct                                   | This study                 |
| Rv::pN-ftsQ-T24A| Rv electroporated with pNit-FtsQ-T24A construct                             | This study                 |
| Rv::pN-ftsQ-T24E| Rv electroporated with pNit-FtsQ-T24E construct                             | This study                 |
| RvΔfQ           | *Rv* ftsQ conditional mutant. *ftsQ* gene expression is under the regulation of pristinamycin inducible *pptr* promoter. | This study                 |
| RvΔfQ::pN       | RvΔfQ electroporated with pNit vector                                        | This study                 |
| RvΔfQ::ftsQ     | RvΔfQ electroporated with pNit-FtsQ construct                               | This study                 |
| RvΔfQ::ftsQ-N   | RvΔfQ electroporated with pNit-FtsQ-N construct                             | This study                 |
| RvΔfQ::ftsQ-C   | RvΔfQ electroporated with pNit-FtsQ-C construct                             | This study                 |
| RvΔfQ::ftsQ-Cα  | RvΔfQ electroporated with pNit-FtsQ-Cα construct                            | This study                 |
| RvΔfQ::ftsQ-β   | RvΔfQ electroporated with pNit-FtsQ-β construct                             | This study                 |
| RvΔfQ::ftsQ-T24A| RvΔfQ electroporated with pNit-FtsQ-T24A construct                          | This study                 |
| RvΔfQ::ftsQ-T24E| RvΔfQ electroporated with pNit-FtsQ-T24E construct                          | This study                 |

Role of FtsQ in cell division
Figure 1

a. Arrangement of Rv2160c-ftsQ operon

b. FtsQ

c. mc::pN-FtsQ

mc::pNit

mc^2

+ +

kDa

27

35

51

91

72

19

Cell length (μm)

****

ns

42

d. CFU Log10 / ml

0 h 14 h 28 h

Time

mc^2

mc::pNit

mc::pN-FtsQ
Figure 2

a. Graph showing fold change.

b. Bar graph showing cell length (μm) with various concentrations of IVN.

c. Images of cells with different treatments.

d. Graph showing cell length (μm) with various treatments.

e. Images of cells with different treatments.

f. Bar graph showing percentage of cells with different treatments.
Figure 4

a. Diagram of homologous recombination between murG, murC, and ftsQ in RvΔfQ and murG, murC, and oriC in RvΔfQ::pN.

b. Gel electrophoresis showing bands for F1-R1, F2-R1, and F2-R2.

c. Petri dishes showing + pristinamycin and - pristinamycin conditions.

d. Bar graph showing CFU Log10/ml over days 0 to 10 for Rv, RvΔfQ + p, and RvΔfQ - p.

e. Bar graph showing CFU Log10/ml over 0 h, 96 h, and 120 h for pristinamycin treatments.
Figure 6

(a) Comparison of RvΔfQ::ftsQ and RvΔfQ::ftsQ-C, with and without Pristinamycin.

(b) Bacterial counts (CFU) for three different strains: RvΔfQ::ftsQ, RvΔfQ::ftsQ-N, and RvΔfQ::ftsQ-C.

(c) Cell length measurements for each strain.

(d) Cloning experiment showing the growth of RvΔfQ::ftsQ and RvΔfQ::ftsQ-C.

(e) Further comparison of bacterial counts for strains RvΔfQ::ftsQ, RvΔfQ::ftsQ-N, and RvΔfQ::ftsQ-C.

(f) Additional data on cell length for each strain.
Figure 7

a. Schematic representation of the mass spectrometric analysis with a diagram showing m/z values and intensity on the x and y axes, respectively. The spectrum includes peaks at m/z 464.22, 1055.52, 1225.73, 1298.61, 1396.57, 1508.72, 1598.88, 1677.71, 1778.93, 2027.15, and 2817.86.

b. Table showing replicate peptide sequence, PSM (protein sequence matching) area, and stoichiometry:

| Replicate | Peptide Sequence | PSM | Area       | Stoichiometry |
|-----------|------------------|-----|------------|---------------|
| 1         | VADDADEAVTEPATESK | 167 | 9.394 x 10^4 | 28.46%        |
|           | VADDADEAVpTEPATESK | 25  | 3.737 x 10^4 |               |
| 2         | VADDADEAVTEPATESK | 172 | 1.575 x 10^5 | 30.80%        |
|           | VADDADEAVpTEPATESK | 101 | 7.007 x 10^4 |               |

c. Photographs showing bacterial colonies with and without pristinamycin treatment.

d. Graphs showing CFU (colony-forming units) log10/ml for different treatments:

- Rv + p
- RvΔfQ::pN - p
- RvΔfQ::ftsQ  - p
- RvΔfQ::ftsQ-T24E  - p
- RvΔfQ::ftsQ-T24A - p

Comparison between Day 0 and Day 10 treatments across all conditions.

e. Graphs showing cell length (μm) for different treatments:

- Rv + p
- RvΔfQ::pN - p
- RvΔfQ::ftsQ  - p
- RvΔfQ::ftsQ-T24E  - p
- RvΔfQ::ftsQ-T24A - p

Comparison between Day 0 and Day 10 treatments across all conditions.
**Figure 8**

| Rv number | Protein Name                                      | Cellular Process                                      |
|-----------|---------------------------------------------------|-------------------------------------------------------|
| Rv2927    | SepIVA                                            | DivIVA domain containing cell division protein       |
| Rv1449    | Transketolase                                     | Intermediary Metabolism                               |
| Rv2744    | 35kDa alanine rich antigen                        | Conserved hypothetical                                |
| Rv0054    | Single stranded binding protein                   | Information pathway                                   |

**Diagram a.**

- $Rv::pN$-ftsQ
- $Rv::pN$-ftsQ-T24A
- $Rv::pN$-ftsQ-T24E

**Diagram b.**

- FtsQ-Replicate 1
- FtsQ-Replicate 2
- FtsQ-Replicate 3

**Diagram c.**

- FtsQ
- FtsQ-T24A

**Diagram d.**

- FtsQ-T24E

**Diagram e.**

- $Rv::pN$-ftsQ-T24E
- $Rv::pN$-ftsQ-T24A
- $Rv::pN$-ftsQ

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Figure 9

a. GFP-FtsQ

b. 

| Percentage of cells | polar | subpolar | septal | polar + septal | septal |
|---------------------|-------|----------|--------|----------------|--------|
|                     | 0     | 10       | 20     | 30             | 40     |

c. 

d. 

e. mc\text{Δ}sep M M' pc-ATc pc + 12 h pc + 18 h

f. 

g. 

h. GFP-FtsQ

i. 

| Percentage of cells | polar | subpolar | septal | polar + septal | subpolar + septal |
|---------------------|-------|----------|--------|----------------|-------------------|
|                     | 0     | 5         | 30     | 40             | 10               |
Delineating FtsQ mediated regulation of cell division in Mycobacterium tuberculosis
Preeti Jain, Basanti Malakar, Mehak Zahoor Khan, Savita Lochab, Archana Singh and Vinay K Nandicoori

J. Biol. Chem. published online June 14, 2018

Access the most updated version of this article at doi: 10.1074/jbc.RA118.003628

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