DISCOVERY REPORT

The *Mycobacterium tuberculosis* PE15/PPE20 complex transports calcium across the outer membrane

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Abstract

The mechanisms by which nutrients traverse the *Mycobacterium tuberculosis* (*Mtb*) outer membrane remain mostly unknown and, in the absence of classical porins, likely involve specialized transport systems. Calcium ions (Ca²⁺) are an important nutrient and serve as a second messenger in eukaryotes, but whether bacteria have similar Ca²⁺ signaling systems is not well understood. To understand the basis for Ca²⁺ transport and signaling in *Mtb*, we determined *Mtb*’s transcriptional response to Ca²⁺. Overall, only few genes changed expression, suggesting a limited role of Ca²⁺ as a transcriptional regulator. However, 2 of the most strongly down-regulated genes were the pe15 and ppe20 genes that code for members of a large family of proteins that localize to the outer membrane and comprise many intrinsically disordered proteins. PE15 and PPE20 formed a complex and PPE20 directly bound Ca²⁺. Ca²⁺-associated phenotypes such as increased ATP consumption and biofilm formation were reversed in a pe15/ppe20 knockout (KO) strain, suggesting a direct role in Ca²⁺ homeostasis. To test whether the PE15/PPE20 complex has a role in Ca²⁺ transport across the outer membrane, we created a fluorescence resonance energy transfer (FRET)-based Ca²⁺ reporter strain. A pe15/ppe20 KO in the FRET background showed a specific and selective loss of Ca²⁺ influx that was dependent on the presence of an intact outer cell wall. These data show that PE15/PPE20 form a Ca²⁺-binding protein complex that selectively imports Ca²⁺, show a distinct transport function for an intrinsically disordered protein, and support the emerging idea of a general family-wide role of PE/PPE proteins as idiosyncratic transporters across the outer membrane.

Introduction

Second messengers are a class of signaling molecules that permit a fast response and swift amplification of signals intracellularly. Among second messengers, calcium ions (Ca²⁺) are a particularly versatile signal in eukaryotes [1]. The functions of hundreds of human proteins are directly regulated by Ca²⁺, and almost every cellular process is affected by Ca²⁺ [1,2]. Ca²⁺
signaling is initially facilitated by Ca\textsuperscript{2+} flux along membrane Ca\textsuperscript{2+} gradients that are carefully maintained by pumps and transporters.

Several bacteria also maintain a similar Ca\textsuperscript{2+} gradient between the inside and outside of the cell [3]. What’s more, Ca\textsuperscript{2+} has anecdotally been linked to bacterial processes such as motility, spore formation, gene expression [4,5], and, in the case of Yersinia, also to virulence [6], suggesting a common bacterial Ca\textsuperscript{2+} sense-and-response system. However, while some components of Ca\textsuperscript{2+} signaling have been identified in bacteria, their number remains small. Ca\textsuperscript{2+} transporters have been annotated but few experimentally tested. Similarly, Ca\textsuperscript{2+}-binding proteins have been predicted, but few experimentally confirmed. As a result, Ca\textsuperscript{2+} remains a poorly understood signaling mechanism in bacteria, and many fundamental questions about Ca\textsuperscript{2+} homeostasis remain unanswered, from the triggers of Ca\textsuperscript{2+} influx, the Ca\textsuperscript{2+}-binding proteins, to the eventual cellular outcomes.

*Mycobacterium tuberculosis* (*Mt*) is surrounded by a highly impermeable outer cell wall that is composed primarily of the complex phthiocerol dimycocerosates (PDIMs) that form an outer membrane [7], creating a structure not unlike that of the outer membrane of gram-negative bacteria. While transport through the outer membrane in gram-negative bacteria is facilitated by characteristic beta barrel porins [8], no equivalent porins have been identified in *Mt*. The impermeable outer membrane and lack of porins raise the question how *Mt* transports small molecules such as nutrients, metabolites, but also Ca\textsuperscript{2+} across the outer membrane. In this way, the very first step in *Mt* Ca\textsuperscript{2+} signaling remains unknown.

The mycobacterial PE/PPE proteins have long been a mystery. They are predominantly found in pathogenic mycobacteria, where they take up a substantial share of the coding capacity [9], and many are substrates of a type VII secretion system [10,11]. The PE/PPE proteins are associated with the outer membrane of the mycobacterial cell wall [12,13]. Many PE/PPE proteins have been implicated in aspects of tuberculosis pathogenesis, but the molecular mechanisms have not been conclusively identified [14]. A recent milestone study showed that several PE/PPE protein pairs function as channels for nutrient transport across the outer mycobacterial membrane [15], defining an idiosyncratic transport system and suggesting a new and perhaps shared family-wide function for the PE/PPE proteins as small molecule transporters.

Here, we sought to further explore Ca\textsuperscript{2+}-mediated processes in *Mycobacterium tuberculosis*. We identified regulation of ATP levels and biofilm formation by Ca\textsuperscript{2+}. The transcriptional response to Ca\textsuperscript{2+} was narrow, and the most highly regulated genes were *pe15* and *ppe20*. We show that PE15/PPE20 form a complex, directly bind Ca\textsuperscript{2+}, and facilitate the influx of Ca\textsuperscript{2+} into *Mt* across the outer membrane. These data point to a functional Ca\textsuperscript{2+} sense-and-response system, identify physiologic processes regulated by Ca\textsuperscript{2+} and identify a new, specific PE/PPE Ca\textsuperscript{2+} import system across the outer membrane.

**Results**

**Ca\textsuperscript{2+} affects ATP levels and biofilm formation**

The role of Ca\textsuperscript{2+} in *Mt* physiology is almost entirely unknown. We initially tested for parallels with Ca\textsuperscript{2+} effects on other bacteria. Ca\textsuperscript{2+} transport in *Escherichia coli* depends on ATP, and Ca\textsuperscript{2+} in turn increases intracellular ATP levels [16]. To test if Ca\textsuperscript{2+} has a similar effect on ATP levels in *Mt*, we exposed *Mt* to increasing concentrations of extracellular Ca\textsuperscript{2+} and measured the intracellular ATP levels. ATP levels increased by >2.5-fold in the presence of 1 mM Ca\textsuperscript{2+} and by >4-fold in the presence of 10 mM Ca\textsuperscript{2+} (Fig 1A). These changes are larger than those observed in *E. coli*, where 10 mM Ca\textsuperscript{2+} resulted in 30% elevation in ATP [16]. To test the reverse effect, we cultured *Mt* in the presence of EGTA, a Ca\textsuperscript{2+}-specific chelator and observed a reduction in ATP levels by approximately 50% (Fig 1A).
In some bacteria, a link between biofilm formation and Ca$^{2+}$ has been proposed [17,18]. To test this idea in Mtb, we measured the generation of biofilm in response to increasing concentrations of Ca$^{2+}$ in vitro. Indeed, 1 mM Ca$^{2+}$ led to a 4-fold increase in biofilm when compared

Fig 1. Ca$^{2+}$ affects Mtb cellular processes and down-regulates pe15 and ppe20 transcripts. (A) Effect of Ca$^{2+}$ on cellular ATP levels. Mtb was grown in detergent-free CTSM containing increasing concentration of Ca$^{2+}$ (10 μM–10 mM) or 1 mM EGTA at 37°C, treated with 0.1% Tween-80 and ATP was quantified using BacTiter-Glo reagent. Fold change was calculated by comparing to the Ca$^{2+}$-free condition. Data are from biological triplicates, error bars show standard deviation. (B) Ca$^{2+}$ promotes biofilm formation. Biofilms were grown in the presence of increasing concentrations of Ca$^{2+}$ (10 μM–10 mM) and 1 mM EGTA and were quantified using crystal violet. The experiment was repeated 3 times and a picture of a representative experiment is shown. Error bars show standard error. RNA-seq analysis of Mtb transcripts after exposure to 1 mM Ca$^{2+}$ for (C) 1 h, (D) 6 h, (E) 1 day, (F) 3 days. Significant down-regulation of pe15 and ppe20 transcripts occurred at 1 and 3 days. Few other genes are significantly changed. The data underlying all the plots in this figure are included in S1 Data. Ca$^{2+}$, calcium ion; CTSM, Chelex-treated Sauton’s medium.

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In some bacteria, a link between biofilm formation and Ca$^{2+}$ has been proposed [17,18]. To test this idea in Mtb, we measured the generation of biofilm in response to increasing concentrations of Ca$^{2+}$ in vitro. Indeed, 1 mM Ca$^{2+}$ led to a 4-fold increase in biofilm when compared
to low Ca\(^{2+}\) conditions (Fig 1B). To rule out that this increase is simply a result of increased growth and thus biomass in high Ca\(^{2+}\) conditions, we tested for differences in growth of *Mtb* in the same Ca\(^{2+}\) concentrations as above. We detected no differences in growth (S1A and S1B Fig), showing that the biofilm effects are specifically due to biofilm generation, not to differences in biomass.

**pe15/ppe20 transcripts are down-regulated in response to Ca\(^{2+}\)**

In eukaryotes and in some bacteria, Ca\(^{2+}\) affects the transcription of a large number of genes [19]. To test for transcriptional effects of Ca\(^{2+}\) in *Mtb* and to identify genes potentially involved in Ca\(^{2+}\) homeostasis, we grew *Mtb* with and without 1 mM Ca\(^{2+}\) and determined transcriptional effects by RNA-seq. At early time points, few transcripts changed abundance in response to Ca\(^{2+}\), although we detected a larger response after 3 days of Ca\(^{2+}\) exposure. All changes in transcript abundance are given in Table A in S1 Table. Interestingly, 2 genes with reduced transcript abundance were apparent as early as day 1 and were the most down-regulated genes by day 3: *pe15* (Rv1386) and *ppe20* (Rv1387) (Fig 1C–1F). This behavior in response to Ca\(^{2+}\) was reminiscent of metal transporter genes that are often regulated in response to changing metal concentrations, with importers typically down-regulated and exporters typically up-regulated in high metal concentrations.

**PE15/PPE20 form a complex, bind Ca\(^{2+}\), and localize to the cell wall**

Several PE/PPE proteins have been shown to form protein complexes, typically those coding in the same operon [20]. The *pe15* and *ppe20* genes are also co-operonic, suggesting that they could be a functional protein pair. According to previously published data, recombinant expression of PE15 and PPE20 individually in *E. coli* failed to produce soluble protein [20]. However, we could readily obtain soluble, recombinant protein when the 2 were expressed together, also indicating a potential interaction. To conclusively show a PE15 and PPE20 interaction, we tested for an association by reciprocal pulldowns with tagged proteins. For all biochemical experiments, we co-expressed His-tagged PE15 and Strep II-tagged PPE20 from a dual expression plasmid and precipitated separately with beads binding to each tag. Both proteins were efficiently pulled down by both beads, indicating binding between PE15 and PPE20 (Fig 2A). To further test the interaction, we co-expressed both proteins and visualized them by native PAGE. Both proteins migrated together, as shown by imaging for the respective tags, further confirming that they form a complex (Fig 2B).

To test whether PE15 and/or PPE20 directly bind Ca\(^{2+}\), we incubated recombinantly expressed PE15/PPE20 with Ca\(^{2+}\) and tested for protein stability using a thermal shift assay with a gel readout (Fig 2C). After heating and precipitation, PPE20 showed clear differential stability when incubated with Ca\(^{2+}\), indicating Ca\(^{2+}\) binding. By using different concentrations of Ca\(^{2+}\) in the same assay, we determined a Ca\(^{2+}\) denaturation curve and estimated a \(K_D\) of 383.7 ± 34.08 μM (Fig 2D and 2E). Interestingly, in a departure from typical ligand-induced stability changes, Ca\(^{2+}\) binding decreased thermal stability of PPE20. PE15 did not produce interpretable results in this assay. Ligand binding in the context of transport suggests that the PE15/PPE20 complex may be a specific channel, as channels typically bind to their ligands [21]. To confirm Ca\(^{2+}\) binding of PE15/PPE20 in an orthogonal assay, we used equilibrium dialysis of Ca\(^{2+}\) in the presence and absence of the PE15/PPE20 complex (Fig 2F). After complete equilibration, the fluid chamber containing PE15/PPE20 retained 30% more Ca\(^{2+}\) than the fluid chamber without protein as determined by inductively coupled plasma optical emission spectrometry (ICP-OES) (Fig 2G). Controls showed a similar effect of a known Ca\(^{2+}\)-binding protein and no effect for a protein that does not bind Ca\(^{2+}\) (Figs 2G and S2).
Fig 2. PE15 and PPE20 form a complex and PPE20 binds Ca\(^{2+}\).

(A) Western blot of PE15 with an N-terminal His tag and PPE20 with a C-terminal Strep tag shows the 2 co-purify. W: wash, E: elution. (B) PE15 and PPE20 co-migrate on a native PAGE gel, also indicating complex formation. (C) Thermal shift experiment with western blot readout. (D) PPE20 remains pelleted at 65°C. (E) Thermal shift experiment with western blot readout. (F) Dialysis membrane experiment with calcium concentration difference. (G) Graph showing %AC values.
To test if PE15/PPE20 localize to the outer membrane as do other PE/PPE pairs [15, 22], we tested for PPE20 expression in different Mtb cell fractions. PPE20 was only detected in the cell wall fraction (Fig 3A).

**PE15/PPE20 KO reverses Ca\(^{2+}\)-dependent phenotypes**

To test for a phenotypic link of the PE15/PPE20 protein complex in Ca\(^{2+}\)-dependent processes, we generated an Mtb pe15/ppe20 knockout (KO) strain by recombineering [23]. We next show that PE15/PPE20 affects Ca\(^{2+}\) associated processes and facilitates Ca\(^{2+}\) uptake.

(A) Western blot showing localization of PPE20 to the cell wall. Subcellular fractions from the complemented pe15/ppe20 KO strain expressing PPE20-FLAG were probed with α-FLAG antibody. Cyt: cytosolic fraction, CM: cell membrane fraction, CW: cell wall fraction. LAM is a cell envelope marker and FtsZ is a cytosolic marker. (B) PE15/PPE20 affect cellular ATP levels. Mtb was treated with 1 mM Ca\(^{2+}\) at 37°C and ATP quantified using BacTiter-Glo reagent. Fold change was calculated by comparing to the Ca\(^{2+}\)-free condition. Data shown are from 4 experiments, error bars show standard deviation (*p > 0.05, **p < 0.05, ***p < 0.001). (C) PE15/PPE20 affect biofilm formation. Cultures were grown with increasing concentrations of Ca\(^{2+}\) and biofilm was quantified by crystal violet (D). The experiment was repeated 3 times, error bars show standard error (*p > 0.05, *p < 0.05, **p < 0.01). The data underlying all the plots and uncropped images in this figure are included in S1 Data and S1 Raw images. Ca\(^{2+}\), calcium ion; Comp, complement; KO, knockout; WT, wild type.

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tested the pe15/ppe20 KO strain for altered ATP levels. The KO showed reduced Ca\(^{2+}\)-dependent increase in ATP production that was reverted to wild-type (WT) levels by complementation with pe15/ppe20 (Comp) (Fig 3B). We next tested whether the PE15/PPE20 complex also affects Ca\(^{2+}\)-dependent biofilm formation. The KO blocked the effect of Ca\(^{2+}\) on biofilm formation, and complementation with pe15/ppe20 restored the phenotype (Fig 3C and 3D). These data show that the PE15/PPE20 complex is not only transcriptionally responsive to, but regulates functions related to Ca\(^{2+}\).

**The PE15/PPE20 complex is a Ca\(^{2+}\) importer**

A recent study showed selective channel function of PE/PPE proteins [15], and the decrease of transcript levels of pe15/ppe20 in the presence of Ca\(^{2+}\) was consistent with the behavior of a Ca\(^{2+}\) channel. To directly test the idea that PE15/PPE20 has a role in Ca\(^{2+}\) transport, we created an *Mtb* reporter strain using a fluorescence resonance energy transfer (FRET) system that detects intracellular Ca\(^{2+}\), Twitch [24]. Twitch is based on a minimal Ca\(^{2+}\)-binding domain from troponin C that is optimized for maximal Ca\(^{2+}\) selectivity and ratiometric signal and that has a *K*D for Ca\(^{2+}\) of 200 nM [24] (Fig 4A). We ectopically expressed Twitch in the H37Rv background (*Mtb-twitch*) and tested for Twitch expression in different cell fractions. Twitch was expressed only in the cytoplasm, indicating that it only reports on cytoplasmic Ca\(^{2+}\) (S3A Fig). We could only obtain a robust FRET signal in Ca\(^{2+}\)-free Sauton’s medium, not 7H9 medium which already contains Ca\(^{2+}\) (Figs 4B, S1C and S1D). We thus used Sauton’s medium for all Ca\(^{2+}\) measurements. To further test the Twitch reporter in *Mtb*, we measured the Ca\(^{2+}\) signal at different Ca\(^{2+}\) concentrations. The FRET signal was robust, dose dependent, and comparable to that of Twitch in previously described nonbacterial systems [24] (Fig 4C). We next tested the selectivity of the probe over the closest Earth alkali metal neighbor, Mg\(^{2+}\). Mg\(^{2+}\) did not generate a FRET signal (S3B Fig). These data show that Twitch is a sensitive probe to continuously measure intracellular Ca\(^{2+}\) in *Mtb*, establish conditions for detecting intracellular Ca\(^{2+}\) changes, show that *Mtb* readily takes up extracellular Ca\(^{2+}\), and reveal a similar *Mtb* response to extracellular Ca\(^{2+}\) to that previously observed in *E. coli* [25].

To directly test for a role of the PE15/PPE20 complex in Ca\(^{2+}\) transport, we measured Ca\(^{2+}\) uptake in the *Mtb-twitch* strain and a strain expressing *Mtb-twitch* in the pe15/ppe20 KO background. The WT produced a robust FRET signal as before upon addition of 5 mM Ca\(^{2+}\). The KO, however, showed reduced influx, consistent with the loss of a selective import channel (Fig 4D). Complementation of the KO with pe15/ppe20 expressed from an extrachromosomal plasmid restored Ca\(^{2+}\) levels to those in WT. PDIM is one of the most abundant cell wall lipids and is often lost in strains grown in vitro [26]. To rule out PDIM differences between WT and mutant strains that might affect Ca\(^{2+}\) transport, we tested all strains for PDIM. All strains produced PDIM at comparable levels (S3C Fig). To further test if PE15/PPE20’s transport role is associated with the cell wall/outer membrane, we permeabilized the *Mtb* cell wall by treatment with lysozyme and Triton-X100 [27]. The difference in Ca\(^{2+}\) influx between WT and KO was reduced by permeabilization, indicating that PE15/PPE20 facilitate transport across the outer membrane (S3D Fig).

**Discussion**

Ca\(^{2+}\) signaling is ubiquitous in eukaryotes [1] but in bacteria, only few components and functions of Ca\(^{2+}\) signaling have been described. In *Mtb*, the outer membrane generally prevents passage of charged solutes, presenting a hurdle for Ca\(^{2+}\) uptake not encountered in most bacteria. In the absence of typical porins, the transport processes that allow for transfer through the outer membrane have long been unknown. Here, we identify a specific Ca\(^{2+}\) channel that
consists of PE15 and PPE20. In addition, we identify several phenotypes associated with elevated \( \text{Ca}^{2+} \) in \( \text{Mtb} \): \( \text{Ca}^{2+} \) leads to an increase in cellular ATP concentrations, an effect that has also been reported in \( \text{E. coli} \) and was suggested to be a mechanism to sustain the increased activity of \( \text{Ca}^{2+} \) ATPase efflux pumps required to reset \( \text{Ca}^{2+} \) levels [16]. We also show a clear contribution of \( \text{Ca}^{2+} \) to biofilm formation. The role of biofilms for \( \text{Mtb} \) pathogenesis and tuberculosis treatment has long been unclear, but recent evidence supports the presence of biofilms in infected lungs of nonhuman primates and human patients and a role in \( \text{Mtb} \) pathogenesis and drug susceptibility [28].

The PE/PPE proteins have long been the subject of much speculation. They are specific not only to mycobacteria but are predominantly found in pathogenic or slow-growing mycobacteria. Although they make up approximately 10% of \( \text{Mtb} \)’s genetic coding potential, their function has long remained unclear [14]. Their large number and sequence variation is reminiscent of variable surface proteins that serve as antigenic decoys in other pathogens. Consistent with this idea, the PE/PPE proteins have generally been linked to the cell wall, although mass spectrometry-based proteomic studies not always identify PE/PPE proteins in cell wall fractions. The difficulties to detect PE/PPE proteins by MS are likely due to a

Fig 4. PE15/PPE20 facilitate \( \text{Ca}^{2+} \) uptake. (A) Schematic of the \( \text{Ca}^{2+} \) FRET probe and its ratiometric signal. (B) \( \text{Ca}^{2+} \) FRET signals in different media show that \( \text{Ca}^{2+} \) presents in standard 7H9 compromise \( \text{Ca}^{2+} \) detection. FRET ratio was calculated by calculating the green:blue fluorescence ratio and was plotted against time. (C) The FRET probe generates a robust, dose dependent FRET signal. Emission scan of \( \text{Mtb-twitch} \) incubated with increasing concentrations of \( \text{Ca}^{2+} \) for 30 min at 37°C. (D) FRET trace over time shows reduced \( \text{Ca}^{2+} \) influx in the \( \text{pe15/ppe20} \) KO strain. The data underlying all the plots in this figure are included in S1 Data. \( \text{Ca}^{2+} \), calcium ion; FRET, fluorescence resonance energy transfer; KO, knockout; WT, wild type.

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Ca^{2+} transport by the Mycobacterium tuberculosis PE15/PPE20 proteins

combination of the relative lack of trypsin cleavage sites in the repetitive sequences and the challenges to assign repeat-derived peptides to individual PE/PPE proteins [29–31]. However, phylogenetic analyses showed that sequence variation does not arise through immunogenic pressure and does not support a role in providing antigenic variation [14]. While mycobacterial ESX secretion systems have been associated with nutrient import before [32,33], the responsible proteins and mechanisms were not known. A recent hallmark study showed channel-like function of PE/PPE complexes specifically transporting several carbon sources, Mg^{2+}, and phosphate across the outer mycobacterial cell membrane [15]. We now show a PE/PPE complex with such a channel-like function for Ca^{2+}. What’s more, we show direct binding of PPE20 to the cargo, which is also more consistent with a channel than for example, a porin. The selectivity of PE15/PPE20 for Ca^{2+} over even the closely related Mg^{2+} further argues for a specific channel rather than a porin, which often show more indiscriminate transport [34]. In fact, another PE/PPE pair, PE20/PPE31 specifically transports Mg^{2+} [15]. Interestingly, several other PE-PGRS proteins bind Ca^{2+} [35,36], could also contribute to Ca^{2+} import, and could explain the residual Ca^{2+} import seen in the pe15/ppe20 KO strain. A link of PE15/PPE20 to pathogenicity is highly plausible given the association of PE/PPE proteins with the outer mycobacterial cell membrane [37], the presence of PE/PPE proteins primarily in pathogenic mycobacteria, their association with the type VII secretion system, and previous studies directly implicating other PE/PPE proteins in virulence [14]. What’s more, the source of Ca^{2+} for the PE/PPE channel is likely host Ca^{2+}. In this way, Mtb may be able to eavesdrop on the many host cell signaling events that involve Ca^{2+}, for example phagocytosis, which is Ca^{2+}-dependent [38] and marks the beginning of Mtb’s intracellular stage.

How do PE/PPE proteins facilitate transport? Crystal structures show that these proteins, unlike classical porins, fold into long alpha helices, with no structural information available for the long repetitive C-terminal sequences of the PPE proteins that are intrinsically disordered. A potential model for how the alpha helical sections of PE/PPE proteins may form pores was recently suggested by structures of the ESX type VII secretion system component EspB [39], which is a naturally occurring fusion of a PE and a PPE protein that forms a donut-shaped heptamer with a large central pore. Although the helices that potentially traverse the cell membrane did not have the requisite outward-facing hydrophobic residues, other models in which the PPE C-termini provide a hydrophobic sheath or association with other membrane-spanning proteins of the ESX complex are possibilities. In our studies, Ca^{2+} binding led to an atypical decrease in thermal stability of PPE20. This change could indicate relaxation of the protein concomitant with channel opening. The precise nature of this unfolding event and how it impacts transport remains to be identified, as do the larger transport mechanics of the PE/PPE proteins and how the intrinsically disordered regions of the PPE proteins contribute to transport. Although our data show that PE15/PPE20 are necessary for efficient Ca^{2+} uptake, they may not be sufficient. The genetic and functional association of the PE/PPE proteins with the ESX secretion system suggests that other ESX components such as an ATPase could be required for PE/PPE transport functions.

The PE and PPE proteins can be further stratified into subfamilies by C-terminal sequence motifs [14] that perhaps also indicate distinct functions. While all currently known PE/PPE channels including PE15 contain a minimal PE-only protein, the PPE proteins identified as components of transporters prior to our study belong to the PPE-SVP subfamily. However, PPE20 belongs to the PPE-PPW subgroup and is the first example of a PE/PPE channel outside of the PPE-SVP group. Similarly, the previous PE/PPE pairs with channel function are associated with the ESX-5 secretion system [40], while PE15/PPE20 is associated with ESX-3 [37]. The PE/PPE channels appear to be highly specific, which could explain their large number, as Mtb requires access to many different nutrients without compromising the outer membrane’s
barrier function. The full set of PE/PPE transporters and cargos remains to be identified. However, our data support the emerging theme that the PE/PPE protein family is a mycobacterial transporter family that solves the conundrum of an outer cell membrane so impermeable as to exclude vital nutrients.

**Methods**

**Media and growth conditions**

*Mtb H37Rv* was used as a parental strain for generation of all mutants and was cultured at 37°C in either Middlebrook 7H9 medium (Difco) with 10% (vol/vol) oleic acid-albunin-dextrose-catalase (OADC) enrichment (BBL; Becton Dickinson), 0.5% glycerol (referred to as “7H9+GO”), and 0.05% Tween 80 or 0.05% Tyloxapol (referred to as “7H9+GOT or 7H9+-GOTy”) or 7H10 agar supplemented with 10% OADC and 0.5% glycerol or Chelex-treated Sauton’s medium (CTSM) with additional supplements as indicated. CTSM consisted of 0.5 g KH$_2$PO$_4$, 4 g L-asparagine, 2 g citric acid, 6% glycerol, adjusted to pH 7, and was treated overnight with Chelex-100 resin (10 g/L) (Sigma) to remove trace metal ion contaminants including Ca$^{2+}$. After filtration, 0.5 g/L of MgSO$_4$, 0.05 g/L ferric ammonium citrate, 0.1 ml of 1% zinc sulfate, and 0.05% Tween 80 or 0.05% Tyloxapol was added. The pH was adjusted to 6.9 and the medium was sterilized by filtration. Strains bearing antibiotic cassettes were cultured with 50 μg/mL hygromycin or 30 μg/mL kanamycin or 25 μg/mL zeocin as appropriate. Ca$^{2+}$ concentrations in media were determined using ICP-OES (Perkin Elmer Optima 8300). Media were treated with nitric acid (trace metal grade) for 1 h at 65°C and diluted with Chelex-treated water. Ca$^{2+}$ intensities in each of the digested sample were measured 3 times by ICP-OES at 317.933 nm. Ca$^{2+}$ standards were measured to generate a standard curve of absorbance versus concentration (S1C Fig). Intensities were converted to Ca$^{2+}$ concentration using the standard curve.

**Biofilm formation**

*Mtb* biofilms were generated and quantified according to a published protocol [41] in 48-well polystyrene plates. Briefly, cells were grown to an OD$_{600}$ of 0.8–1 in 7H9+GOT medium, washed twice with CTSM, and diluted to an OD$_{600}$ of 0.01 in CTSM without detergent and added to each well supplemented with varying concentrations of Ca$^{2+}$. Outer wells were filled with water and the plate was incubated at 37°C for 4 weeks without shaking. The plates were photographed and the processed for crystal violet staining. The medium was removed, biofilms were dried and incubated with 500 μl of 1% crystal violet for 10 min. Wells were washed 3 times with water and dried again. Absolute ethanol (1 ml) was added to each well and incubated for 10 min. Then, 3-fold serial dilutions were read at A$_{600}$ on a spectrophotometer in a 96-well plate. The represented bar graph is the average readings of 4 biological replicates and Welch’s *t* test was applied to determine the significance (*p*-value).

**ATP measurement**

*Mtb* cells from a 7H9+GOT medium culture (OD$_{600}$~1) were washed twice and diluted to an OD$_{600}$ of 0.01 in CTSM without detergent supplemented with different concentrations of CaCl$_2$ or 1 mM EGTA. Cultures were grown in a 48-well plate at 37°C for 14 days. Cells were treated with 0.1% Tween-80 overnight to create a homogenous suspension. ATP was quantified by incubating 50 μl of the bacteria in triplicate with 50 μl of the BacTiter-Glo reagent for 15 min followed by a luminescence reading. ATP production in the Ca$^{2+}$-treated cultures were calculated as fold change compared to the calcium-free condition. The represented bar graph
is the average fold change of 4 biological replicates and Welch’s t test was applied to determine the significance (p-value).

**RNA sequencing**

*Mtb* H37Rv was grown to an OD$_{600}$ of approximately 1 in 7H9+GOT medium. Cells were washed twice with CTSM, subcultured in CTSM starting at an OD$_{600}$ of 0.05, and grown to an OD$_{600}$ of approximately 1. The culture was again subcultured in CTSM starting at an OD$_{600}$ of 0.05 and incubated at 37˚C to an OD$_{600}$ of 0.2. The cultures were supplemented with or without 1 mM CaCl$_2$ in triplicate. At the indicated times, cells were pelleted at 4,000 g for 5 min at 4˚C, resuspended in Trizol and lysed by bead beating for 30 s at 6 m/s for 3 cycles with intermittent cooling on ice. Cell debris were pelleted at 20,000 g for 1 min, and the supernatant was transferred to a heavy phase lock gel tube containing 300 µl chloroform. The tubes were inverted for 2 min and centrifuged at 20,000 g for 5 min. RNA in the aqueous phase was precipitated using 300 µl isopropanol and 300 µl high salt solution (0.8 M Na citrate, 1.2 M NaCl). RNA was purified using QIAGEN RNeasy kit and ribosomal RNA was depleted using the Ribo-Zero rRNA removal magnetic kit (Illumina). The cDNA library was generated using the NEBNext Ultra II RNA Library Prep Kit, and each replicate was barcoded in the DNA library using the NEBNext Multiplex Oligos for Illumina. Libraries were quantified using the KAPA qPCR quantification kit, pooled, and sequenced at the University of Washington Northwest Genomics Center with the Illumina NextSeq 500 High Output v2 Kit. Read alignment was performed using the Bowtie 2 custom processing pipeline (https://github.com/robertdouglasmorrison/DuffyNGS, https://github.com/robertdouglasmorrison/DuffyTools). Gene expression changes were identified using a combination of 5 differential expression (DE) tools within DuffyTools. The 5 DE tools included round robin, RankProduct, significance of microarrays (SAMs), EdgeR, and DeSeq2. Each DE tool measurement was combined using the weighted average of fold change and significance (p-value). Genes with averaged absolute fold change more than 1.5-fold and p-value <0.01 were considered differentially expressed. RNA-seq data is available at NCBI-GEO (accession no. GSE214266).

**Cloning, co-expression, and purification of recombinant PE15 and PPE20 proteins**

pETDuet dual expression plasmid was used to co-express PE15 and PPE20. PE15 was cloned in the MCS-1 region with N-terminal His tag and PPE20 was cloned in the MCS-2 region with a C-terminal Strep II tag. *Mtb* Rv1386 (PE15) and Rv1387 (PPE20) genes were amplified from *Mtb* H37Rv genomic DNA using the primers Duet 1–5 that included Gibson overlap sequence (Table B in S1 Table). The linearized pETDuet plasmid and the region between MCS-1 and MCS-2 were prepared by PCR amplification using the primers Duet 6,7 and Duet 8,9, respectively (Table B in S1 Table). Finally, all 4 purified PCR products, i.e., PE15, PPE20, region between MCS-1/MCS-2, and linearized pETDuet plasmid were ligated using the Gibson Assembly (NEB) to generate the pETDuet pe15/ppe20 plasmid. Cloning was confirmed by sequencing. The vector was transformed into *E. coli* BL21(DE3) and a single colony was picked and was grown at 37˚C in Terrific Broth medium containing ampicillin. Protein expression was induced by addition of 0.5 mM isopropyl-β-d-thiogalactoside (IPTG) at OD$_{600}$ of approximately 0.4; culture was then maintained at 16˚C for 16 h. Cells were harvested, and pellets were processed for purification. For complex purification through His-PE15, pellets were resuspended in buffer A (50 mM Tris (pH 8.0), 150 NaCl, and 10% glycerol) containing 20 mM imidazole and 1 mM AEBSF and lysed by sonication. Lysate was centrifuged at 35,000 g for 30 min at 4˚C and the supernatant was loaded on Ni-NTA column. The column was
thoroughly washed with buffer A containing 20 mM imidazole followed by elution with buffer A containing 250 mM imidazole. For PPE20-strep II tag, pellets were resuspended in buffer A containing 1 mM AEBSF and lysed by sonication. Lysate was centrifuged at 35,000 g for 30 min at 4°C and the supernatant was loaded on Strep-tactin column. The column was thoroughly washed with buffer A followed by elution with buffer A containing 2.5 mM desthiobiotin. Both the purified proteins were immediately dialyzed against buffer A and stored at −80°C. Interaction of PE15/PPE20 was confirmed by western blot. The wash and elution fraction from the Ni-NTA and strep tag purification were loaded on either SDS-PAGE or Native-PAGE, and the proteins were transferred onto a nitrocellulose membrane. Blots were probed with mouse α-His antibody followed by IRDye 680RD Goat α-Mouse IgG Secondary Antibody (LI-COR) to detect the His tagged PE15 and were also probed with IRDye 800CW Streptavidin to detect the PPE20-strep tag. The blots were scanned on LI-COR Odyssey platform using the 700 nm and 800 nm channel.

**Thermal shift assay and equilibrium dialysis to determine Ca²⁺ binding**

Purified PE15/PPE20 protein was incubated with or without 1 mM CaCl₂ in a thermocycler for 5 min at 25°C followed by incubation at varying temperature (55 to 70°C) for 3 min. The reaction was cooled to 4°C and centrifuged at 20,000 g for 30 min at 4°C to separate the native (supernatant) and the denatured (pellet) fractions. Pellet fraction was solubilized by boiling with 2xSDS loading buffer. PPE20 in each of the supernatant and pellet fraction was detected by western blot using rabbit α-strep II tag antibody followed by IRDye 800CW Goat α-Rabbit IgG Secondary Antibody. To determine the K_D value, PPE20 was incubated with varying concentration of CaCl₂ (12.5 μM to 3.2 mM) for 5 min at 25°C, 3 min at 65°C followed by incubation at 4°C. PPE20 in the pellet fraction was detected by western blot as described above. The band intensities were calculated using the Image studio software and converted to fraction denatured relative to the total protein. The graph of fraction denatured v/s log Ca²⁺ concentration was plotted using GraphPad Prism 9.3, and the K_D value was determined by fitting a dose-response curve using the least square regression method. For equilibrium dialysis, Ca²⁺-free PE15/PPE20 was prepared by EGTA treatment and dialysis against Chelex-treated buffer A. Protein (10 μM) was placed in the upper chamber and dialyzed against 50 mM Tris (pH 7.4), 150 mM NaCl buffer containing 200 μM of Ca²⁺ in the lower chamber for 24 h and the Ca²⁺ concentration in both chambers was determined by ICP-OES. Samples (100 μl) were digested with 100 μl of nitric acid (trace metal grade) for 1 h at 65°C and diluted with 2.8 ml of Chelex-treated water. Ca²⁺ intensities were measured 3 times by ICP-OES and converted to Ca²⁺ concentration using the standard curve described above. Percentage ΔC was calculated as per the following formula:

\[
\%\Delta C = \frac{Ca^{2+}(upper \ chamber) - Ca^{2+}(lower \ chamber)}{Ca^{2+}(lower \ chamber)} \times 100.
\]

The thermal shift and equilibrium dialysis experiments were repeated with buffer control (no protein), calcium-binding protein (CBP) from *Encephalitozoon cuniculi* as a positive control (obtained from SSGCID # EncuA.01276.a.A1.PS00484), and Rv0831c as a negative control. The bar graph in Fig 2G is the average %ΔC of 3 technical replicates. Student’s t test was applied to determine the p-value.

**Creation of pe15/ppe20 knock out and complemented strain in Mtb H37Rv**

The KO strain was created by recombineering as described previously [23]. Initially 500 bp upstream of pe15 (primers KO1 and KO2), 500 bp downstream of ppe20 (primers KO3 and
KO4) and hygromycin cassette (primers KO5 and KO6) were amplified separately (Table B in S1 Table). Both PCR fragments were Gibson ligated to the 5' and 3' of a hygromycin-resistance cassette, respectively, to generate the recombineering cassette. This linear recombineering cassette was PCR amplified, purified, and electroporated into Mtb H37Rv strain carrying recombineering plasmid pNIT [42]. Hygromycin positive colonies were screened, and the positive clone was confirmed by DNA sequencing. For the complemented strain, both pe15 (His-tag) and ppe20 (FLAG-tag) genes were separately PCR-amplified using primers Comp 1–3 and Comp 4, 5, respectively (Table B in S1 Table), and Gibson cloned in pDTCF plasmid (Zeocin) separately under an anhydrotetracycline (ATc)-inducible promoter. The ATc promoter along with the ppe20 gene was amplified using primers Comp 6, 7 (Table B in S1 Table) from pDTCF-PPE20 vector and Gibson cloned in pDTCF-PE15 vector downstream of pe15 gene. The resulting plasmid pDTCF-pe15/ppe20 carries both genes with individual ATc promoters. The plasmid was electroporated into the KO strain and positive clones were selected by growth in hygromycin and zeocin. This strain expressed His-PE15 and PPE20-FLAG proteins when induced with 100 ng/ml ATc.

Creation of Mtb Ca\(^{2+}\) FRET reporter strain

The Ca\(^{2+}\) FRET reporter Twitch-2B was PCR amplified from the plasmid Twitch-2B pRSETB obtained from Addgene [24] using the primers T2B1 and T2B2 (Table B in S1 Table) that included Gibson overlap sequence. The PCR product was Gibson cloned in the E. coli-myco-bacterial episomal shuttle vector pDTCF (kanamycin) under the anhydrotetracycline (ATc)-inducible promoter. The plasmid was electroporated into wild type Mtb H37Rv (WT), pe15/ppe20 KO and pe15/ppe20 complemented strain (Comp), and the expression of Twitch was induced using 100 nM ATc. The expression of Twitch was confirmed by measuring the fluorescence of uninduced and induced cells using a plate reader at 3 different wavelengths (432/475 nm, 432/532 nm, and 488/532 nm). The localization of Twitch and PPE20 was confirmed by western blot. Briefly, 100 ml culture of uninduced and induced Mtb-twitch complemented with the pe15/ppe20 genes was grown in 7H9+GO medium. Cells were washed twice in PBS and resuspended in lysis buffer (50 mM Tris (pH 7.4), 150 mM NaCl) containing protease inhibitor cocktail. Cells were lysed by bead beating and the following fractions were prepared according to published protocol [43]: cytosolic (CYT), cell membrane (CM), and cell wall (CW) fraction. Briefly, the lysate was sequentially centrifuged at 3,000 g to separate the beads and cell debris, followed by spin at 20,000 g to pellet the CW fraction and finally at 100,000 g to separate the pellet, i.e., CM fraction and supernatant CYT fraction. CM and CW fractions were washed once and resuspended in lysis buffer. The expression of Twitch protein (His-tagged) was detected by western blot using a mouse α-His antibody, PPE20 was detected by mouse α-Flag antibody and the purity of cellular fractions was confirmed by using α-LAM (CM and CW fraction) and α-FtsZ (CYT) antibodies (obtained from BEI resources, NIAID, NIH).

PDIM analysis

Cultures were grown in 50 ml 7H9+GO for 7 days and washed 3x with PBS. Cell pellets were resuspended in 9 ml chloroform-methanol (2:1) and incubated overnight at room temperature with shaking. The extract was filtered through 0.2 μm PTFE filters and dried under a stream of nitrogen. The total lipid was weighed and resuspend at a concentration of 20 mg/ml in chloroform-methanol (2:1). Lipids (500 μg) were spotted onto aluminum-backed TLC plate and run in petroleum ether-ethyl acetate (98:2) as the solvent system. Control PDIM was obtained from BEI resources, solubilized in chloroform-methanol (2:1), and 100 μg was run on TLC.
The TLC plate was dried, and the lipids were stained by spraying 5% sulfuric acid in methanol followed by heating.

**Ca\(^{2+}\) uptake assay**

*Mtb-twitch* was grown in either 7H9+GOT or CTSM to an OD\(_{600}\) of 1. Cells (50 μl) were aliquoted in duplicate wells and spiked with Ca\(^{2+}\) (5 mM) and EGTA (5 mM) at indicated time points and fluorescence was continuously measured at wavelengths 432/475 nm (blue) and 432/532 nm (green) at 37°C. Fluorescence readings were corrected by subtracting the readings from wells containing uninduced culture. The FRET ratio was calculated as the green:blue fluorescence ratio and plotted against time. An increase in the FRET ratio indicates an increased intracellular Ca\(^{2+}\) concentration. To determine the dose-dependent effect of Ca\(^{2+}\) on the FRET signal, *Mtb-twitch* was incubated with increasing concentration of Ca\(^{2+}\) for 30 min at 37°C and an emission scan (from Em450-550 nm) was recorded using excitation at 432 nm.

*Mtb* H37Rv (WT), pe15/ppe20 KO and complemented strains (Comp) expressing *twitch* were grown to OD\(_{600}\) of approximately 0.5 in CTSM/tyloxapol with or without 100 nM ATc. Cells were washed twice and resuspended in CTSM/tyloxapol at a dilution of 1 OD/ml. Cells were spiked with Ca\(^{2+}\) (5 mM) at indicated time point and blue and green fluorescence were measured continuously at 37°C. FRET ratios were calculated and plotted against time. The assay was repeated by treating cells with permeabilizing reagent [27] (0.1% Triton-X100 and 2 μg/μl lysozyme) at time 0 min. Experiments were performed using 3 biological replicates and 2 technical replicates.

**Supporting information**

**S1 Fig. Growth curves of Mtb H37Rv and Ca\(^{2+}\) quantification in different media.** (A) CTSM and (B) 7H9 medium containing glycerol, OADC, and Tween-80 (7H9+GOT) supplemented with different concentrations of CaCl\(_2\) shows no effect of Ca\(^{2+}\) on growth. (C) ICP-OES standard curve for the determination of Ca\(^{2+}\) concentrations. (D) Ca\(^{2+}\) concentrations in standard 7H9 and Sauton’s media. The data underlying all the plots in this figure are included in S1 Data. Ca\(^{2+}\), calcium ion; CTSM, Chelex-treated Sauton’s medium; ICP-OES, inductively coupled plasma optical emission spectrometry. (TIF)

**S2 Fig. Thermal shift-based analysis of Ca\(^{2+}\) binding to control proteins.** (A) A known CBP from *Encephalitozoon cuniculi* shows a Ca\(^{2+}\)-dependent increase in the melting temperature and concomitant precipitation. (B) A protein not known to bind Ca\(^{2+}\) shows no difference in melting temperature and resulting precipitation. Uncropped images in this figure are included in S1 Raw images. Ca\(^{2+}\), calcium ion; CBP, calcium-binding protein. (TIF)

**S3 Fig. Transport by PE15/PPE20 is associated with the cell wall and is specific for Ca\(^{2+}\).** (A) Western blot showing localization of Twitch-2B (His tagged) in the cytosolic fraction. The same cell fractions as in Fig 3A were used, see controls therein. Cyt: cytosolic fraction, CM: cell membrane fraction, CW: cell wall fraction. (B) The FRET signal is highly specific for Ca\(^{2+}\) over the congener Mg\(^{2+}\). The His-tagged Twitch protein was purified from the cytosolic fraction using Ni-NTA column, treated with EDTA and desalted. An emission scan of the purified protein was recorded upon incubation with either Ca\(^{2+}\) or Mg\(^{2+}\). (C) Thin-layer chromatography of the cell wall component PDIM shows no difference in PDIM content between WT and pe15/ppe20 KO strains. (D) Permeabilization of the cell wall diminishes PE15/PPE20’s effect on Ca\(^{2+}\) import. WT, pe15/ppe20 KO, and complemented strain were transformed with
Twitch, permeabilized (P) with lysozyme and Triton-X100, and the Ca\(^{2+}\)-FRET signal measured. The data underlying all the plots and uncropped images in this figure are included in S1 Data and S1 Raw images. Ca\(^{2+}\), calcium ion; FRET, fluorescence resonance energy transfer; KO, knockout; PDIM, phthiocerol dimycocerosate; WT, wild type.

S1 Table. List of differentially expressed genes and primers. (A) Differentially expressed genes in response to Ca\(^{2+}\) in Mtb and (B) primers used in this study.

S1 Data. Numerical data points underlying presented graphs.

S1 Raw images. Uncropped western blot images.

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References


