

Control of Mycobacterium tuberculosis protein secretion by ESX-4 and the outer membrane EsxUT-EsxEF complex

Corresponding Author: Professor Michael Niederweis

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The manuscript of Nair et al. addresses the significant question of how proteins are transported/secreted across the unique mycobacterial cell wall, which could help identify novel drug targets. The authors present compelling data for outer membrane localization and secretion of the EsxUT proteins by the ESX-4 system and EsxEF. They also demonstrate that EsxEF and EsxUT proteins form a complex that is proposed to function as a channel for protein secretion. Most interestingly, the authors demonstrate that Esx proteins of the other ESX systems (Esx1-3, 5) require EsxUT and EsxEF for surface location and secretion into the host cell cytoplasm in Mtb infected macrophages. This dependence of other Esx proteins on the Esx-4 system is an important finding. Lastly, the authors demonstrate that EsxC and EsxU join EsxA in playing roles in promoting phagosome permeabilization. The experiments are well performed with appropriate controls and the data is compelling due to the use of multiple methods, flow cytometry and fluorescence microscopy, to assess surface localization and protein secretion.

The authors conclude and state in the title that the EsxUT EsxEF proteins are master controllers of protein secretion (line 380, outer membrane protein complex for general protein secretion). While the data is compelling for Esx proteins depending on EsxUT and EsxEF, it is unclear if this one mechanism is responsible (i.e. controls) all protein secretion by Mtb. If the authors want to draw that conclusion, they should assess secretion of additional proteins such as Esp, PPE and non-Esx secreted proteins such as Sec secreted proteins. This concern, however, is relatively minor given the significance of discovering a role for Esx proteins of the Esx-4 system in secretion of other Esx proteins. Additionally, if EsxUT and EsxEF are needed for secretion of all Esx proteins of all five Esx systems then the EsxUT and cpnT operon mutants should exhibit phenotypes reported for each of the esx mutants (i.e. attenuation in mice and macrophages, defective in iron and zinc limited media etc). This is not addressed in the manuscript.

Specific comments

There are typos/awkward sentence in line 27-28 and there are some problems with references to figures (ex. Line 83 and 85, should this be Fig S1d?)

In supplemental figure 1, why is EsxU running high on gels from WT Mtb but not the complemented strain? Also, there is a faint signal corresponding to that aberrantly high species in controls. Similarly, the authors should discuss the expected size versus observed size of fusion proteins in Fig 2a.

Fig 1B should have microscopy images to go along with the flow assay to provide more compelling data as the mean fluorescence shifts are relatively minor.

Have the authors confirmed that in cpnT operon mutants EsxU protein is made at normal levels?

Supplemental Fig 2 should be moved to the main body of the manuscript.

Data shown in supplemental figure 10 that Esx1 is not required for EsxU localization should also be moved to the main body of the manuscript.

The authors need to review the statement in Line 363. The cited references do not support their assertion that PknG and

SapM reach the cytoplasm of macrophages as a consequence of ESX mediated permeabilization.

It is not clear if the authors propose that all Esx proteins localized to the outer membrane/surface similarly act as channel proteins for their respective systems. Lines 275-284. Line 358. The authors should refine this part of the discussion.

Reviewer #2

(Remarks to the Author)

The five type VII secretion systems (ESX) are essential for virulence and nutrient acquisition in *M. tuberculosis*. These systems are well known to secrete distinctive families of substrates, in particular the Esx proteins, characterized by a YXXXD/E and WXG motif. While the structures and assembly of some of these ESX inner membrane machineries have received considerable interest recently, little is known regarding the outer membrane components, allowing the secretion of the ESX protein substrates. In this study, Nair et al. show that the ESX-4 substrates, EsxU-EsxT, can form a non-canonical complex with the orphan EsxE-EsxF proteins encoded in the *cpnT* operon, that encodes also the major TNT toxin in *M. tuberculosis*.

The authors first demonstrate that EsxU, along with EsxE-EsxF, is associated with the mycobacterial membranes and is required to the secretion of TNT. They next established that, EsxU plays a role as an outer membrane component of ESX-4 and that this surface localization is dependent of EsxE-EsxF. Through the use of biochemical studies, they show that EsxU-EsxT physically interacts with EsxE-EsxF, presumably by forming non-canonical complexes in the *M. tuberculosis* outer membrane. The formation of these complexes is required to the function of ESX-4 and is also dependent on the EsxE-EsxF pore-forming activity. They next provided evidence that the surface accessibility and secretion into the macrophage of all small Esx proteins associated with the five ESX systems largely rely on the formation of the EsxEF-EsxUT supercomplexes as an essential outer membrane component of ESX-4.

The major finding of this study is to show that ESX-4 (the ESX ancestor) is essential for the secretion of the Esx substrates of all ESX systems, highlighting its critical vulnerability, opening the door to future studies devoted to drug/vaccine developments. While the manuscript is well written, not all the conclusions raised are supported by their findings and additional control experiments are necessary to consolidate these conclusions.

Major points:

-Fig S1a: line 71, "... only background levels are observed in strains lacking *esxUT* genes or the *eccC4* ATPase...". This is not true when looking carefully at the figure as one can easily distinguish bands corresponding to EsxU in the membrane fraction in the *esxUT* and *eccC4* deletion mutants. How is it possible to detect the EsxU band in the mutant in which *esxUT* has been removed? This raises the question of the specificity of the EsxU peptide antibodies. An easy explanation is that this Ab cross-reacts with other Esx proteins (or other proteins). The authors should provide a Western blott analysis of the different antibodies raised in this study and probe them with all other Esx proteins to define their specificity. Second, since ESX4-dependent secretion is abolished in the *eccC4* mutant, how to explain the presence of a EsxU reactive band in the membrane of the mutant? Again, this could be linked to cross-reactivity of the antibody with other proteins. Alternatively, secretion of EsxU does not rely only on ESX-4 but can be channeled through other ESX systems in the *eccC4* mutant (as already demonstrated in ESX mutants in *M. marinum*). Explain in the legend what "M" and "S" mean in Fig. S1a. In addition, the MW of EsxU which is a small secreted protein should not appear at 26 kDa, although smaller bands can be seen in the least last lanes. Does the band at 26 kDa correspond to the EsxU-EsxT heterodimer? but then the complex should be separated into monomers in an SDS-PAGE. Clearly, these different bands/complexes should be better identified and subcellular localization addressed using additional antibodies to strengthen the claims. Once update, this figure could be added as a main figure in the manuscript.

-In many places in the text/figure, the authors are stating that EsxU has a dual role as an outer membrane component of ESX-4 and a secreted protein. However, they never investigated the role EsxT in their experiments. Since EsxT is known to form a heterocomplex with EsxU prior its secretion through ESX-4, the contribution of EsxT should also be investigated. For instance, is EsxT also important in *CpnT* export? This could be addressed by complementing the *EsxUT* mutant with a functional *esxT* gene. To complete the story, it is important to analyze and report the contribution of EsxU AND EsxT in the different phenotypes addressed in this work.

-Fig S2: lane 181, "... while no protein was detected in strain producing only one of these operons...". This contradicts with the microscopy analyses showing that EsxU can be observed in more than 25% of the *esxUT* mutant, corresponding to almost half of the % found in the WT strain (Fig. S2b) (same remark for the *eccC4* mutant). This cannot be considered as "background" since the *esxUT* mutant is unable to produce EsxU. Again, these results are questioning the quality and specificity of the EsxU antibodies used. This needs to be fully addressed with additional antibodies or control experiments.

-Fig. 2a: The pull-down assays require more explanations. Numerous bands corresponding to proteins with different sizes react with the different antibodies (anti-His, HA and Myc), with no clear designation of the reactive bands. The authors should highlight and identify each band corresponding to EsxF, E, U and T. Why are there two bands to EsxU and EsxT? Are these heterocomplexes? How to explain they are found around 43 kDa, which is much higher than their expected sizes.

-Fig. 2b: I cannot see the EsxU labeling in blue, therefore the co-localization between EsxF and EsxU is not convincing and uncertain.

-Fig 3. While most experiments performed analyzed the surface accessibility of the various Esx substrates, they did not investigate whether these substrates are secreted in the culture filtrate. Are EsxA, C, H and N secreted in the various

mutants studied (cpbT, esxUT and complemented strains). This needs to be addressed by doing fractionation studies and Western blott analysis (presence or not of the proteins in the cell culture filtrates). It would nicely complete the study and making it more robust. This is also important to show secretion using in vitro cultures before showing secretion in infected macrophages (Fig. 4), which again is only based on microscopic studies.

Minor points:

-the title is misleading. The authors have not shown that ESX-4 is a master regulator in the secretion of all proteins as they only focused on the Esx substrates. Therefore, the title should be more precise, such as "Master control of Esx protein secretion by *M. tuberculosis*".

-lane 28: 'tuberculosis' is missing in the sentence.

-legend Fig. S7. These high resolution images correspond to Fig. 3A and not 5A.

-Material and Method (page 26). "... the best peptide sequences for EsxC and EsxT were determined (Table S4). This table does not show the different peptide sequences used to generate the antibodies... same for EsxF and EsxU. It is important to provide the sequences of the peptides used. As already mentioned, a quality-control Western blott is needed to highlight the specificity of the different antibodies used with the different Esx proteins.

-Homogenize the nomenclature of the genes/proteins used in the text and figures. Sometimes EsxEF is used, sometimes EsxFE etc...

Version 1:

Reviewer comments:

Reviewer #2

(Remarks to the Author)

The authors have considerably revised the manuscript. Additional controls, figures and supplemental information have been added to the manuscript to strengthen the message of the study.

I just found a few minor points that need to be rectified.

1. labeling of the different panels in Figure S8a are missing

2. In the Discussion, the authors mention that "Since the ESX systems are the only know protein secretion systems in mycobacteria....". This is not true. Other proteins secretion systems, such as the SecA and TAT secretions systems, have been described previously in mycobacteria. This needs to be changed in the text.

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Rebuttal

“Master control of protein secretion by *Mycobacterium tuberculosis*”

Manuscript number: NCOMMS-24-85015

Reviewer 1

The manuscript of Nair et al. addresses the significant question of how proteins are transported/secreted across the unique mycobacterial cell wall, which could help identify novel drug targets. The authors present compelling data for outer membrane localization and secretion of the EsxUT proteins by the ESX-4 system and EsxEF. They also demonstrate that EsxEF and EsxUT proteins form a complex that is proposed to function as a channel for protein secretion. Most interestingly, the authors demonstrate that Esx proteins of the other ESX systems (Esx1-3, 5) require EsxUT and EsxEF for surface location and secretion into the host cell cytoplasm in Mtb infected macrophages. This dependence of other Esx proteins on the Esx-4 system is an important finding. Lastly, the authors demonstrate that EsxC and EsxU join EsxA in playing roles in promoting phagosome permeabilization. The experiments are well performed with appropriate controls and the data is compelling due to the use of multiple methods, flow cytometry and fluorescence microscopy, to assess surface localization and protein secretion.

1. The authors conclude and state in the title that the EsxUT EsxEF proteins are master controllers of protein secretion (line 380, outer membrane protein complex for general protein secretion). While the data is compelling for Esx proteins depending on EsxUT and EsxEF, it is unclear if this one mechanism is responsible (i.e. controls) all protein secretion by Mtb. If the authors want to draw that conclusion, they should assess secretion of additional proteins such as Esp, PPE and non-Esx secreted proteins such as Sec secreted proteins. This concern, however, is relatively minor given the significance of discovering a role for Esx proteins of the Esx-4 system in secretion of other Esx proteins. Additionally, if EsxUT and EsxEF are needed for secretion of all Esx proteins of all five Esx systems then the EsxUT and *cpnT* operon mutants should exhibit phenotypes reported for each of the *esx* mutants (i.e. attenuation in mice and macrophages, defective in iron and zinc limited media etc). This is not addressed in the manuscript.

It is gratifying to read that the reviewer finds our data revealing the master control of Esx proteins by the EsxEF-EsxUT supercomplex compelling. We agree with his/her suggestion to extend our study to other protein substrates of ESX systems as additional control experiments. To this end, we performed a substantial number of new experiments. Specifically, we show now that:

1. EspB is a secreted ESX-1 substrate protein required for the interaction with host phospholipids and for killing macrophages (Mol Microbiol 89, 1154; 2013; Embo J. 42, e113490; 2023). We now show that secretion of EspB also requires EsxEF-EsxUT (new Fig. 6a, b), demonstrating that ESX-1 substrates other than EsxAB depend on EsxEF-EsxUT. This is now described in the manuscript (lines:304-308).
2. Since no protein secretion substrates other than EsxGH are known for the ESX-3 system we examined whether ESX-3 dependent iron acquisition depends on EsxEF-EsxUT. Indeed, the *M. tuberculosis* strains lacking EsxEF or EsxUT show growth defects in iron-limiting conditions, while their growth rates in medium with replete iron are identical (new Fig. 6c, d), demonstrating that ESX-3 dependent proteins required for iron uptake depend on EsxEF-EsxUT. This is now described in the manuscript (lines:308-316).
3. Previously, we showed that the outer membrane protein PPE62 is required for heme uptake by *M. tuberculosis* (MBio 8: e01720; 2017). However, we had no direct evidence whether PPE62 is dependent on the ESX-5 system. Hence, we first show that PPE62 is indeed dependent on ESX-5 using a conditional *eccD5* mutant (new Fig. 6e). We then show that no PPE62 is detectable in *M. tuberculosis* strains lacking

EsxEF or EsxUT in contrast to the parent strain (see new Fig. 6), demonstrating that also ESX-5 substrates other than the associated EsxMN proteins depend on EsxEF-EsxUT. This is now described in the manuscript (lines: 316-325). These results highlight the essential role of EsxEF-EsxUT in controlling secretion of not only small Esx proteins, but also other proteins secreted by the ESX systems of *M. tuberculosis*. The abstract, introduction (lines 56-58), the discussion (lines: 499-502) and Fig. 9 were changed accordingly.

Notes:

- No protein secretion substrates other than EsxC and EsxD or functions for the ESX-2 system are known. Thus, we could not examine other ESX-2-dependent secretion substrates (Figs. 4 and S8).
- We did not perform infection experiments because we show that EsxAB secretion is dependent on EsxEF-EsxUT and is required for virulence of *M. tuberculosis*. Thus, any expected virulence defect could not be interpreted easily.

Specific comments

1. There are typos/awkward sentence in line 27-28 and there are some problems with references to figures (ex. Line 83 and 85, should this be Fig S1d?)

We have rephrased this sentence (lines 28-29) and fixed the figure numbers to Fig. S1d (lines 86, 89).

2. In supplemental figure 1, why is EsxU running high on gels from WT Mtb but not the complemented strain? Also, there is a faint signal corresponding to that aberrantly high species in controls. Similarly, the authors should discuss the expected size versus observed size of fusion proteins in Fig 2a.

We agree that the appearance of multiple bands in the Western blots with the EsxU antibody is confusing. However, we previously observed that Esx proteins have a strong tendency to form SDS-stable oligomers. This is described extensively for the EsxEF proteins, which not only form pentameric structures of heterodimers, but also form smaller oligomers and long filaments. Thus, only minor quantities of monomers or heterodimers are observed (Tak et al., 2021; *Nat Commun* **12**: 394). Similar results have been obtained in our lab for the EsxAB proteins (unpublished).

It is also correct that the Western blot of endogenous EsxU in wt Mtb shows a band at ~26 kDa unlike the EsxUT complemented strain which shows a band at ~10 kDa. This is most likely an overexpression artefact, since the *esxUT* operon is expressed from strong promoter from an integrated plasmid in contrast to the *esxUT* genes which are expressed together with the genes of the ESX4 system, which are essential for export and secretion of EsxUT. Additional evidence for the aberrant production of EsxU and EsxT proteins in the overexpressing strain is obtained in the new subcellular fractionation experiments (new Fig. S2), revealing large quantities of water-soluble EsxU in contrast to the wt strain, in which most EsxU is membrane-associated (Fig. S1a).

It is an astute observation by the reviewer that there is a faint signal in the EsxU Western blots in the *esxUT* deletion mutant (Fig. S1a). This is likely due to a weak cross-reactivity of the EsxU antiserum with similar epitopes of EsxU homologs in *M. tuberculosis*. This issue has been addressed in the surface accessibility experiments by labeling EsxU using an HA tag (new Fig. S2c, d)

We want to clarify that the EsxEF and EsxUT proteins are co-expressed in *E. coli* and not fused. To increase clarity the observed sizes of the Esx proteins in the eluted fraction from the pull-down experiment are now labelled according to their expected sizes in the revised Figs. 3 and S6a. In addition, we used several

approaches to denature the high molecular weight Esx proteins oligomers in the eluted fractions, which have been shown to denature other SDS-resistant proteins. For example, we boiled the eluted proteins in 80% DMSO or precipitated the eluted proteins in 12% trichloroacetic acid and dissolved them in 6 M urea. However, the Esx oligomers were mostly resistant to these treatments. However, we did observe small quantities of monomeric EsxT_{Myc} in the Myc-specific Western blot. Thus, we now present evidence of monomeric proteins for all four Esx proteins in the pulldown experiments demonstrating that they interact with each other in a tetra-heteromeric complex as shown in revised Fig. 3a. This is now described in the revised manuscript (lines: 206-224).

3. Fig 1B should have microscopy images to go along with the flow assay to provide more compelling data as the mean fluorescence shifts are relatively minor.

We agree that the fluorescence shifts in flow cytometry experiments with bacteria are small. This is due to their small size in contrast to eukaryotic cells, for which flow cytometers were developed. However, these small shifts are reproducible and hence a valid indicator of differences. Nevertheless, we did additional experiments and analyzed the same strains by fluorescence microscopy. These new images are consistent with the flow cytometry analysis and have been added to the revised manuscript (Fig. 2b).

4. Have the authors confirmed that in *cpnT* operon mutants EsxU protein is made at normal levels?

Considering the cross-reactivity of EsxU antiserum with the other Esx homologs, we now constructed Mtb WT, $\Delta eccC4$, $\Delta cpnT_{op}$ and $\Delta esxUT$ strains producing HA-EsxU/EsxT_{FLAG}. The membrane localization of both tagged EsxU and EsxT were considerably reduced in the *cpnT_{op}* deletion mutant but severely reduced in the *eccC4* deletion mutant. This showed that EsxUT are indeed the substrates of the ESX-4 system but required EsxEF for the membrane localization.

5. Supplemental Fig 2 should be moved to the main body of the manuscript.

This figure is now Fig. 1a in the revised Fig. 1 as requested.

6. Data shown in supplemental figure 10 that Esx1 is not required for EsxU localization should also be moved to the main body of the manuscript.

This figure is now Fig. 5d in the revised Fig. 5 as requested.

7. The authors need to review the statement in Line 363. The cited references do not support their assertion that PknG and SapM reach the cytoplasm of macrophages as a consequence of ESX mediated permeabilization.

Known secreted virulence factors of *M. tuberculosis* are TNT, PknG, SapM and PtpA. These proteins interact with host factors in the cytosol of infected macrophages demonstrating that these proteins are secreted (*Nat Struct Mol Biol* **22**, 672-678, (2015); *EMBO Rep* **22**, e52175, (2021); *Infect Immun* **92**, e0021724, (2024); *PNAS* **108**, 19371-19376, (2011). We do not claim that the access of these proteins to the cytosol is solely dependent on Esx-mediated permeabilization of the phagosomal membrane. It is also possible that proteins are secreted immediately after phagosomal permeabilization and/or after the bacteria enter the host cytosol. This is now described in the revised manuscript (lines: 502-505).

8. It is not clear if the authors propose that all Esx proteins localized to the outer membrane/surface similarly act as channel proteins for their respective systems. Lines 275-284. Line 358. The authors should refine this part of the discussion.

We have demonstrated that all Esx proteins encoded in the *esx* loci of *M. tuberculosis* are surface localized and associated with membranes, demonstrating that they are localized in the outer membrane (lines: 251-255; 261-263). The dual function of Esx proteins as secreted effector proteins and as structural components of their respective ESX systems is clearly mentioned in several parts of the manuscript (lines: 385-389), extensively discussed (lines: 392-421) and shown in the model based on our data (Fig. 9).

Reviewer #2

The five type VII secretion systems (ESX) are essential for virulence and nutrient acquisition in *M. tuberculosis*. These systems are well known to secrete distinctive families of substrates, in particular the Esx proteins, characterized by a YXXXD/E and WXG motif. While the structures and assembly of some of these ESX inner membrane machineries have received considerable interest recently, little is known regarding the outer membrane components, allowing the secretion of the ESX protein substrates. In this study, Nair et al. show that the ESX-4 substrates, EsxU-EsxT, can form a non-canonical complex with the orphan EsxE-EsxF proteins encoded in the *cpnT* operon, that encodes also the major TNT toxin in *M. tuberculosis*.

The authors first demonstrate that EsxU, along with EsxE-EsxF, is associated with the mycobacterial membranes and is required to the secretion of TNT. They next established that, EsxU plays a role as an outer membrane component of ESX-4 and that this surface localization is dependent of EsxE-EsxF. Through the use of biochemical studies, they show that EsxU-EsxT physically interacts with EsxE-EsxF, presumably by forming non-canonical complexes in the *M. tuberculosis* outer membrane. The formation of these complexes is required to the function of ESX-4 and is also dependent on the EsxE-EsxF pore-forming activity. They next provided evidence that the surface accessibility and secretion into the macrophage of all small Esx proteins associated with the five ESX systems largely rely on the formation of the EsxEF-EsxUT supercomplexes as an essential outer membrane component of ESX-4.

The major finding of this study is to show that ESX-4 (the ESX ancestor) is essential for the secretion of the Esx substrates of all ESX systems, highlighting its critical vulnerability, opening the door to future studies devoted to drug/vaccine developments. While the manuscript is well written, not all the conclusions raised are supported by their findings and additional control experiments are necessary to consolidate these conclusions.

Major points:

1. Fig S1a: line 71, "... only background levels are observed in strains lacking *esxUT* genes or the *eccC4* ATPase...". This is not true when looking carefully at the figure as one can easily distinguish bands corresponding to EsxU in the membrane fraction in the *esxUT* and *eccC4* deletion mutants. How is it possible to detect the EsxU band in the mutant in which *esxUT* has been removed? This raises the question of the specificity of the EsxU peptide antibodies. An easy explanation is that this Ab cross-reacts with other Esx proteins (or other proteins). The authors should provide a Western blott analysis of the different antibodies raised in this study and probe them with all other Esx proteins to define their specificity.

We agree with the reviewer that the EsxU antibody used in this study has a weak cross-reactivity, probably with EsxU homologs, despite the fact that the antibodies were affinity purified. This is what we called

“background” activity in our manuscript. To improve the detection specificity, we have constructed a new Mtb strain which produces tagged $_{HA}EsxU/EsxT_{FLAG}$ proteins. Subcellular localization experiments with wt Mtb and the $\Delta eccC4$, $\Delta cpnT_{op}$ and $\Delta esxUT$ strains using the tag-specific antibodies **demonstrate that the tagged EsxU and EsxT proteins are detected very specifically with no visible background bands in Western blots (new Fig. S2a)**. For this reason it is not necessary to test the reactivity of the EsxU antibody against all other Esx proteins of Mtb. This is now described in lines 136-141. However, a caveat of using antibodies to detect proteins is that sometimes the recognized epitope is not accessible in complexes. This applies to both protein-specific and tag-specific antibodies. For instance, the EsxT antibody failed to detect EsxT in the purified EsxUT complex (data not shown). See co-expression experiments in *E. coli* (Response to 6).

2. Second, since ESX4-dependent secretion is abolished in the $\Delta eccC4$ mutant, how to explain the presence of a EsxU reactive band in the membrane of the mutant ? Again, this could be linked to cross-reactivity of the antibody with other proteins. Alternatively, secretion of EsxU does not rely only on ESX-4 but can be channeled through other ESX systems in the $\Delta eccC4$ mutant (as already demonstrated in ESX mutants in *M. marinum*). Explain in the legend what “M” and “S” mean in Fig. S1a.

As mentioned above our EsxU antibody has a weak cross-reactivity probably with EsxU homologs, although it was affinity-purified. To specifically detect EsxU and EsxT we constructed wt, $\Delta eccC4$, $\Delta cpnT_{op}$ and $\Delta esxUT$ Mtb strains producing tagged $_{HA}EsxU/EsxT_{FLAG}$ proteins. The tagged EsxU and EsxT proteins were both membrane localized in wt Mtb (new Fig. S2). Importantly, deletion of $\Delta eccC4$ severely impaired the membrane localization of these tagged proteins, demonstrating that EsxU and EsxT are indeed substrates of the ESX-4 system. These results are consistent with those obtained with the untagged proteins and the EsxU-antibody. The faint signals detected in the $\Delta eccC4$ strain likely result from an overexpression artefact. This assumption is supported by the detection of the tagged EsxU/EsxT in the water-soluble fraction after overexpression (new Fig. S2).

3. In addition, the MW of EsxU which is a small secreted protein should not appear at 26 kDa, although smaller bands can be seen in the least last lanes. Does the band at 26 kDa correspond to the EsxU-EsxT heterodimer? but then the complex should be separated into monomers in an SDS-PAGE. Clearly, these different bands/complexes should be better identified and subcellular localization addressed using additional antibodies to strengthen the claims. Once update, this figure could be added as a main figure in the manuscript.

EsxU in the WT membrane fraction (Fig. S1a) is detected at ~26kDa. This is probably due to the interaction of EsxU (10 kDa), EsxT (6kDa) and EsxU or an EsxU homolog generating a heterotrimeric complex at ~26 kDa. The expected molecular weight of the heterotrimer is now included in the figure legend. As discussed in the response to Reviewer 1, these high molecular weight complexes of EsxU correlate with the SDS-stable oligomers formed by EsxEF proteins, as previously reported (Tak et al., 2021; *Nat Commun* **12**: 394). In addition, we employed various denaturation strategies to denature the Esx protein complexes obtained by the pull-down experiment (Figs. 3 and S6A). However, these Esx protein complexes are almost completely resistant to denaturation with boiling in 80% DMSO or TCA precipitation and 6 M urea (lines: 216-221). These experiments underlined the stability of the interactions observed in the Esx protein complexes, making it difficult to observe the monomers on an SDS-PAGE. To further validate the subcellular localization using EsxU antiserum, we used antibodies targeting tagged Esx proteins (new Fig. S2a).

4. In many places in the text/figure, the authors are stating that EsxU has a dual role as an outer membrane component of ESX-4 and a secreted protein. However, they never investigated the role EsxT in their experiments. Since EsxT is known to form a heterocomplex with EsxU prior its secretion through ESX-4, the contribution of EsxT should also be investigated. For instance, is EsxT also important in CpnT export ? This could be addressed by complementing the EsxUT mutant with a functional *esxT* gene. To complete the story, it is important to analyze and report the contribution of EsxU AND EsxT in the different phenotypes addressed in this work.

Heterodimer formation and co-dependent secretion has been established for many Esx protein pairs in mycobacteria (*Science* **313**, 1632; 2006; *Proc Natl Acad Sci USA* **102**, 10676; 2005). Importantly, it has been shown that co-expression is required to obtain stable Esx proteins. In the absence of their cognate partner protein no proteins were detected for EsxA/EsxB (*Science* **313**, 1632; 2006; *Embo J* **42**, e113490; 2023), EsxG/H (*PNAS* **113**, E348; 2016), EsxF/E (*Nat Commun* **12**, 394; 2021) and EsxN (*Mol Microbiol* **83**, 1195; 2012) indicating protein degradation when export/secretion is impaired. Therefore, expression of *esxT* in the absence of *esxU* would almost certainly result in impaired EsxT export and protein degradation and, subsequently, failure to export CpnT. Instead we examined the presence of EsxT in Mtb producing tagged EsxT_{FLAG}, which, unfortunately, was not detectable on the cell surface in an Mtb strain producing HA_{EsxU}/EsxT_{FLAG}, probably due to the inaccessible FLAG tag in complex with EsxU, EsxE and EsxF. This interpretation is supported by our finding that some oligomers are detected only by antibodies specific for tagged EsxU, while other oligomers are only detected by antibodies specific for tagged EsxT after coexpression in *E. coli* (Fig. 3a; see also response to critique 6). Importantly, we detected EsxT as a membrane-associated protein in Mtb (Fig. S2a), strongly indicating a functional role of EsxT in transport of ESX-4 substrates as EsxU. For these reasons we believe that both EsxU and EsxT are essential for the CpnT export and TNT secretion and for the other functions of the ESX-4 system. For these reasons, we did not repeat all experiments with an Mtb strain expressing only *esxT*.

5. Fig S2: lane 181, “.. while no protein was detected in strain producing only one of these operons...”. This contradicts with the microscopy analyses showing that EsxU can be observed in more than 25% of the *esxUT* mutant, corresponding to almost half of the % found in the WT strain (Fig. S2b) (same remark for the *eccC4* mutant). This cannot be considered as “background” since the *esxUT* mutant is unable to produce EsxU. Again, these results are questioning the quality and specificity of the EsxU antibodies used. This needs to be fully addressed with additional antibodies or control experiments.

It is important to realize that the background activity in Western blots and in the microscopy experiments is dominated by different mechanisms. In Western blots the background activity is mainly due to cross-reactivity of antibodies. This is nicely demonstrated by the complete absence of any bands using the HA antibody in wt Mtb (Fig. S2a) versus a faint band using the EsxU-specific antiserum in the Δ *esxUT* mutant (Fig. S1a). Not surprisingly the background activity in the fluorescence microscopy experiments is higher since antibodies, in addition to potential cross-reactivities can bind unspecifically to cell surfaces or can get trapped in cell clumps which are typical in experiments with Mtb as evident in our images. We also did flow cytometry experiments which not only provide a more accurate quantification but are also less affected by cell clumps which are not included in the analysis. Importantly, these three different experimental approaches consistently and clearly demonstrate that EsxUT are dependent on the ESX-4 system and have two essential functions as outer membrane proteins of the ESX-4 system and as secreted effector proteins. In addition, all experiments have been done in biological triplicates (except for flow cytometry which were done twice). Furthermore, we have repeated these experiments with tagged HA-

EsxU–EsxT-FLAG to address the concerns of the weak cross-reactivity of our protein –specific EsxU antibody. Again, these new experiments confirmed our previous results. Thus, we have fully addressed the specificity issue and have provided plenty of controls.

6. Fig. 2a: The pull-down assays require more explanations. Numerous bands corresponding to proteins with different sizes react with the different antibodies (anti-His, HA and Myc), with no clear designation of the reactive bands. The authors should highlight and identify each band corresponding to EsxF, E, U and T. Why are there two bands to EsxU and EsxT? Are these heterocomplexes? How to explain they are found around 43 kDa, which is much higher than their expected sizes.

We agree with the reviewer that the pull-down assay requires more information. We have now labeled each of these bands according to their expected molecular masses in the blots in the revised manuscript (Fig. 3a). Based on the predicted molecular masses the following protein bands were assigned: M, monomer; HD, heterodimer; HT, heterotrimer; HO, hetero-oligomer. A heterodimer (~18 kDa) consists of tagged EsxF homolog (11 kDa) and a tagged EsxE homolog (7 kDa). Heterotrimers consist of either two EsxF homologs and an EsxE homolog with a molecular mass of ~29 kDa or of one EsxF homolog and two EsxE homologs with a molecular mass of ~25 kDa. These assignments correspond nicely with the electrophoretic mobilities in the gel electrophoretic analysis and were added in the legend of Fig. 3a. Based on our previous findings demonstrating oligomer formation by Esx protein pairs (Tak et al., 2021), any bands with molecular masses larger than 30 kDa were labeled as hetero-oligomers (HO). Interestingly, different oligomeric forms of the EsxUT-EF supercomplex are detected only by one or two tag-specific antibodies but are not detectable by other antibodies (Fig. 3a). For example, the hetero-trimeric band is recognized only by EsxF- and EsxE-tag specific antibodies, while the larger hetero-oligomers are only recognized by the EsxT-tag specific antibody. The exclusive recognition of different oligomeric Esx complexes by specific antibodies indicates that the epitope tags are not accessible in certain oligomers, but are accessible in others. These results clearly show a fascinating capacity of the small Esx proteins to form a wide variety of large non-canonical complexes with each other with different stoichiometries. Furthermore, in additional experiments we tried to denature these complexes to detect the EsxT monomer by boiling with 80% DMSO or treatment with 6 M urea subsequent to 12% trichloroacetic acid precipitation. Interestingly these Esx complexes were mostly resistant to denaturation, explaining while they are detectable as oligomers in SDS gels. However, we were able to detect monomeric EsxT (new subpanel added to Fig. 3a) after a partial denaturation of oligomeric complexes detected by the Myc antibody specific for EsxT. This analysis was added to the results section (lines: 216-222).

7. Fig. 2b: I cannot see the EsxU labeling in blue, therefore the co-localization between EsxF and EsxU is not convincing and uncertain.

The blue color for tagged EsxU was not visible in the merged images because it completely overlaps with the red color of tagged EsxF yielding a purple color in the merged images in Fig. 3. However, the blue color is clearly visible in the single channel fluorescent microscopy images shown in Fig. S6. To avoid this confusion we added the single channel fluorescent microscopy images for the Mtb strain co-expressing His8EsxFE and MycEsxUT, which clearly show co-localization of EsxF and EsxU on the Mtb cell surface. However, surface exposure of EsxF was strongly reduced in the absence of EsxU, and vice versa (Fig. 3d, e; S6b, c). These experiments demonstrate that both EsxFE and EsxUT are mutually dependent for surface exposure in Mtb.

8. Fig 3. While most experiments performed analyzed the surface accessibility of the various Esx substrates, they did not investigate whether these substrates are secreted in the culture filtrate. Are EsxA, C, H and N secreted in the various mutants studied (cpbT, esxUT and complemented strains). This needs to be addressed by doing fractionation studies and Western blott analysis (presence or not of the proteins in the cell culture filtrates). It would nicely complete the study and making it more robust. This is also important to show secretion using in vitro cultures before showing secretion in infected macrophages (Fig. 4), which again is only based on microscopic studies.

We have now included the secretion analyses of the tagged Esx proteins in the culture filtrates of the different Mtb strains (Fig. 5 a-c). Both the strains lacking EsxEF ($\Delta cpnT_{op}$) and EsxUT showed impaired secretion of the tagged Esx proteins into the culture filtrate, consistent with their reduced surface accessibilities (Fig. 4). Strikingly, the $\Delta esxUT$ strain exhibited a more pronounced defect in secretion, indicating a dominant role of EsxUT in the secretion of small Esx proteins.

Minor points:

- The title is misleading. The authors have not shown that ESX-4 is a master regulator in the secretion of all proteins as they only focused on the Esx substrates. Therefore, the title should be more precise, such as “Master control of Esx protein secretion by *M. tuberculosis*”.

We agree with the reviewer and tested whether known protein substrates of the ESX-1, ESX-3 and ESX-5 systems other than Esx proteins would also depend on the EsxUT-EsxEF supercomplex. Importantly, we show now that secretion of EspB (ESX-1-dependent), PPE62 (ESX-5 dependent) and unknown proteins of the ESX-3 system involved in iron uptake also depend on the EsxUT-EsxEF, confirming our model (new Fig. 6).

We also agree that a more specific title would be a better fit and decided to change the title to “**Control of *Mycobacterium tuberculosis* protein secretion by ESX-4 and the outer membrane EsxUT-EsxEF complex**”.

-lane 28: ‘tuberculosis’ is missing in the sentence.

This sentence was fixed in the revised manuscript.

-legend Fig. S7. These high resolution images correspond to Fig. 3A and not 5A.

The legend of Fig. S7 was corrected.

-Material and Method (page 26). “... the best peptide sequences for EsxC and EsxT were determined (Table S4). This table does not show the different peptide sequences used to generate the antibodies... same for EsxF and EsxU. It is important to provide the sequences of the peptides used. As already mentioned, a quality-control Western blott is needed to highlight the specificity of the different antibodies used with the different Esx proteins.

Table S1 in the supplement of the revised manuscript shows the peptide sequences used to generate the respective antisera.

-Homogenize the nomenclature of the genes/proteins used in the text and figures. Sometimes EsxEF is used, sometimes EsxFE etc...

The nomenclature of the Esx genes/proteins is now consistent in the revised manuscript.

Summary of significant changes:

To address the critiques of the reviewers we did a substantial number of new experiments:

1. To improve the detection specificity of EsxU we have constructed new Mtb strains which produce tagged $_{HA}EsxU/EsxT_{FLAG}$ proteins. Subcellular localization experiments with wt Mtb and the $\Delta eccC4$, $\Delta cpnT_{op}$ and $\Delta esxUT$ strains using the tag-specific antibodies **demonstrate that the tagged EsxU and EsxT proteins are membrane associated and are detected very specifically with no visible background bands in Western blots (new Fig. S2a).**
2. We constructed new Mtb strains and showed that **secretion of HA-tagged EsxA, EsxN and EsxH proteins is impaired in Mtb grown *in vitro* in the absence of EsxEF and EsxUT (new Fig. 5, new Fig. S11).**
3. We showed that HA-tagged EspB (ESX-1) is not secreted by Mtb in the absence of EsxUT or EsxEF in macrophage infection experiments. We identified that PPE62 is an ESX-5 dependent protein and showed that it is not exported to the outer membrane without EsxUT or EsxEF. We revealed that iron utilization by Mtb depends largely on EsxUT, and to a lesser extent on EsxEF. **These results (new Fig. 6) demonstrate that secreted/exported proteins other than Esx proteins are also dependent on EsxUT-EsxEF confirming its essential role in controlling protein secretion in *M. tuberculosis*.**
4. We did extensive denaturation experiments to show that EsxT monomers are also present in Esx complexes formed in *E. coli* after co-expression of esxUT and EsxEF (extension of Fig. 3a).
5. We showed that tagged HA-EsxU enabled export of CpnT to the cell surface to the same extent as untagged EsxUT in Mtb demonstrating that the HA tag does not impair the function of EsxU (new Fig. S10).
6. The entire manuscript was carefully edited to improve clarity. The title was changed to be more specific.

Altogether, we added **two new main figures and three new figures to the Supplementary Information** to support our study.