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Review

In Vivo Methods to Study Protein-Protein Interactions as Key Players in *Mycobacterium tuberculosis* Virulence

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Abstract: Studies on Protein-Protein interactions (PPI) can be helpful for the annotation of unknown protein functions and for the understanding of cellular processes, such as specific virulence mechanisms developed by bacterial pathogens. In that context, several methods have been extensively used in recent years for the characterization of *Mycobacterium tuberculosis* PPI to further decipher TB pathogenesis. This review aims at compiling the most striking results based on *in vivo* methods (yeast and bacterial two-hybrid systems, protein complementation assays) for the specific study of PPI in mycobacteria. Moreover, newly developed methods, such as in-cell native mass resonance and proximity-dependent biotinylation identification, will have a deep impact on future mycobacterial research, as they are able to perform dynamic (transient interactions) and integrative (multiprotein complexes) analyses.

Keywords: tuberculosis; *Mycobacterium*; protein-protein interactions; virulence

1. Introduction

Mycobacterium tuberculosis (*Mtb*) is the main causative agent of human tuberculosis (TB), which is the first leading global cause of death due to a single infectious agent. In 2017, TB killed an estimated 1.6 million people, according to the World Health Organization. In addition, there is an alarming increase in multi-resistant drug TB cases (0.6 million cases in 2017). Therefore, actions to fight TB have to be urgently taken and understanding the mechanisms underpinning mycobacterial virulence may be useful to tackle TB.

Proteins perform various key roles in bacteria (enzymatic reactions, transport, DNA replication…), either alone or in association with other partners as a part of stable or dynamic complexes. Thus, elucidating the role of individual proteins is essential to understand the physiology of bacteria, including *Mtb*. Moreover, deciphering Protein-Protein Interactions (PPI) is a crucial step not only to understand the physiology of the bacteria but also to elucidate host-pathogen interactions [1]. In addition, studying PPI may facilitate the discovery of unknown protein functions by the 'guilty by association' principle, implying that the partner(s) of a protein with an unknown function may provide valuable information about its function [2]. This may potentially lead to the identification of new antibacterial drug targets.

The aim of this review is to provide an overview of the *in vivo* methods used for the characterization of PPI in mycobacteria and to highlight the pros and cons for each method. Several examples will illustrate how these studies contributed to decipher the mycobacterial interactome, providing worthy insights into *Mtb* virulence mechanisms. This review will focus only on *in vivo*

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methods, and *in vitro* methods, such as co-precipitation, surface plasma resonance or isothermal titration calorimetry will not be discussed here.

2. Yeast two-hybrid (Y2H) system

2.1. Principle

The Y2H system is based on the reconstitution of an active transcriptional activator (TA) in yeast (e.g. GAL4 or LexA) [3]. The proteins of interest (POIs) are produced in fusion with the DNA-binding domain (BD) or the activating domain (AD) of the TA. If the two proteins under investigation interact, the BD and AD are close enough to each other to allow the transcription of reporter genes, usually auxotrophic markers (*HIS3*, *ADE2* and *MEL1*) or *lacZ*, which in turn allows yeast colonies to grow on selective media or to change color on colorimetric media.

2.2. The Y2H system to study mycobacterial PPI

2.2.1. Signaling pathways

Sigma factors are subunits of the RNA polymerase complex required for transcriptional initiation of specific sets of genes. As rapid adaptation is key to the success for bacterial pathogens, sigma factors play a critical role in *Mtb* physiology and virulence [4]. Among the dozen of sigma factors in *Mtb*, SigA, also called RpoV, is essential for growth and is involved in the transcription of housekeeping genes [5]. To study mycobacterial PPI involving SigA, the Y2H system was used to screen a *Mtb* H37Rv library and identified the transcription regulator WhiB3 as an interactor of SigA/RpoV [6]. In addition, a single amino acid change in SigA/RpoV (R515H) was sufficient to abolish its interaction with WhiB3 in the Y2H system [6]. Another transcriptional regulator, WhiB1, was shown to interact with the alpha-glucan branching enzyme GlgB [7]. SigF is the general stress response sigma factor of *Mtb* and is responsible for the regulation of genes involved in cell wall protein synthesis and survival of the bacilli in the host [8,9]. The Y2H system was also used to study the interactions between anti-anti-sigma factor, anti-sigma factor RsbW and sigma factor SigF [10].

Beside sigma factors, the *Mtb* genome encodes a dozen two-component systems (TCS), allowing gene expression to adapt in response to a wide variety of signals. Some of these TCS were shown to be involved in the regulation of virulence [11]. The Kdp signal transduction pathway appears to be the primary response mechanism to osmotic stress, which is mediated by differences in the potassium concentrations in the bacteria. The N-terminal sensing module of the histidine kinase KdpD interacts with a portion thought to be cytosolic of two membrane lipoproteins, LprF and LprJ, to modulate *kpd* expression [12]. Another study assessed pairwise interactions in the Y2H system between histidine kinases and response regulators of all the mycobacterial TCS in order to analyze crosstalks between the different TCS [13].

The *Mtb* genome also encodes eleven Serine/Threonine Protein Kinases (STPK), from PknA to PknL. As the *Mtb* phosphoproteome includes hundreds of Ser- and Thr-phosphorylated proteins that participate in many aspects of *Mtb* biology (signal transduction, cell wall synthesis, pathogenesis…), STPK are critical in regulating *Mtb* physiology [14]. In an extensive study, 492 STPK interactants were identified by a *Mtb* proteome microarray [15]. To confirm the *in vitro* screening, the Y2H system was further used to assess the interactions between 75 randomly-selected interactants with PknB, PknD, PknG and PknH. However, only 52% (39 out 75) of the STPK interactants could be confirmed by the Y2H system, which may be due to the fact that PknB, PknD and PknH were tested without their membrane domain, as this could have been detrimental in the Y2H system [15].

2.2.2. *Mtb* cell division

As for other living organisms, cell growth and division needs to be tightly organized and regulated in mycobacteria [16]. In particular, divisome assembly depends on the proper localization

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of FtsZ in order to form the Z ring structure [16]. Thus, a Y2H screening was performed using *Mtb* FtsZ as a bait, which led to the identification of SepF (Rv2147c), an essential protein of the division machinery in mycobacteria [17].

Reactivation of dormant *Mtb* requires the resuscitation-promoting factors (Rpf), which are peptidoglycan–hydrolyzing enzymes [18]. The Y2H system was used to identify a RpfB and RpfE interactant, named RipA for Rpf-interacting protein A [19]. Additional work performed with RipA as a bait in the Y2H system further identified the protein PBP1/PonA1 as a new partner, potentially modulating the RipA-RpfB cell wall degradation activity [20].

2.2.3. *Mtb* cell wall composition

Mycolic acids are essential lipid components of the mycolic acid-arabinogalactan-peptidoglycan complex (MAPc) in the *Mtb* cell wall and they contribute directly to the pathogenicity of *Mtb* [21]. The Y2H system was extensively used to demonstrate that the discrete enzymes of the Fatty Acid Synthase-II (FAS-II) system interact with each other during the mycolic acid biosynthesis, suggesting the existence of specialized and interconnected protein complexes [22-24].

Another study using the Y2H system showed that Rv2623, a universal stress-response protein, and Rv1747, a putative ABC transporter, interact with each other to regulate mycobacterial growth by potentially impeding Rv1747 function as a phosphatidylinositol mannoside (PIM) transporter [25]. PIM are immunologically active lipids that can modulate the host immune response [26,27].

2.2.4. Secretion of *Mtb* virulence factors

ESAT-6 and CFP-10 are both secreted antigens, which play a key role in *Mtb* virulence [28,29]. The Y2H system helped to demonstrate that EccCa1, EccCb1 and EccD1, which all are components of Type VII Secretion System ESX-1 [30,31], are required for ESAT-6/CFP-10 secretion [32]. In addition, a single amino acid change in the C-terminal region of CFP-10 was enough to abolish the CFP-10/EccCb1 interaction in the Y2H system, and to prevent the secretion of the ESAT-6/CFP-10 complex [33]. Similarly to CFP-10, the C-terminal region of EspC, another ESX-1 substrate, was shown to interact with Rv3868, a cytosolic ATPase, by a Y2H approach [34].

2.2.5. Regulation of mycobacterial protease activity

Mycobacterial proteases play critical roles in pathogenesis [35]. For instance, the site-2 protease Rip1 (Rv2869c) is a major virulence determinant in *Mtb* [36,37]. A Y2H screening performed using the Rip1 PDZ domain against a *Mtb* library led to identification of PDZ-interacting protease regulators 1 and 2 (Ppr1 and Ppr2, corresponding to Rv3333c and Rv3439c, respectively) and these interactions are thought to prevent nonspecific activation of the Rip1 pathway [38].

2.3. Pros and cons

The Y2H system allows direct assessment of pairwise interactions between partners in an *in vivo* context. However, as the PPI occurs in the nucleus of the yeast cell, the Y2H system requires nuclear translocation of the proteins under study. Thus, membrane-associated proteins are difficult if not impossible to study in this system (Table 1). Furthermore, only two (or three in the case of a Y3H system) partners can be studied at a time. In addition, the Y2H system is not suitable for the study of PPI in their natural cellular context, and specific mycobacterial Post-Translational Modifications (PTM) or cofactors may be lacking in yeast (Table 1).

Table 1. Comparison of the different techniques used to study mycobacterial PPI.

Methods	Contact	Membrane proteins	Nature of the interaction	Cellular context	PTM and cofactors	HTS [1]

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Y2H	direct	no	binary	no	no	yes
BACTH	direct	yes	binary	yes/no	yes/no	yes
M-PFC	direct	yes	binary	yes	yes	yes
Split-Trp	direct	yes	binary	yes	yes	yes
Crosslinking	direct	yes	complex	yes	yes	no
In-cell NMR	proximity	yes	complex	yes/no	yes/no	no
Biotinylation	proximity	yes	complex	yes	yes	yes

[1] HTS, High-Throughput Screening

3. Bacterial Adenylate Cyclase-based Two-Hybrid (BACTH) system

3.1. Principle

The BACTH system is based on the interaction-mediated reconstitution of an active *Bordetella pertussis* adenylate cyclase (CyaA) in *Escherichia coli* [39-41]. POIs are genetically fused to the N-terminal or C-terminal ends of the subunits T18 or T25 of CyaA. The enzyme is inactive when T18 and T25 are physically separated. If the POIs interact, the proximity of T18 and T25 allows the generation of cyclic AMP (cAMP), which then binds to the Catabolite Activator Protein (CAP). This cAMP/CAP complex then activates the transcription of reporter genes (*lac* and *mal* operons). As *lac* and *mal* operons are involved in lactose and maltose catabolism, respectively, this confers the ability to *E. coli* to grow on media on which lactose or maltose is the unique carbon source.

3.2. The BACTH system to study mycobacterial PPI

3.2.1. Signaling pathways

Mtb SigE plays an important role in the intracellular life of the mycobacteria and regulates several genes that are important for maintaining the integrity of the cell envelope during stress, particularly during macrophage infection, since SigE is required to arrest phagosome maturation [42,43]. SigE interacts with the anti-sigma factor RseA in the BACTH system, and using this system residues C70 and C73 of RseA have been shown to be required for full interaction, which prevents the transcription of genes that are controlled by SigE [44].

The BACTH system was also used to study the interactions between components of TCS, such as the C-terminal domain of the response regulator MtrA and the histidine kinase MtrB [45]. Although, the environmental signals of MtrA/B are unknown, this TCS is essential for mycobacterial growth, but overexpression of *mtrA* level was shown to impede *in vivo* proliferation of *Mtb* [46,47]. In another study, the sensor kinase KdpD was found to interact in the BACTH system with the membrane peptide KpdF, potentially altering KdpABC transporter function [48]. In the same study, a screening was performed using KdpF as a bait against a *Mtb* H37Rv DNA library and identified as interactors the proteins MmpL7 and MmpL10 [48], which are members of the MmpL protein family involved in lipid and iron transport in mycobacteria [49,50]. It was further shown that KdpF also interacts with the nitrosative stress detoxification proteins NarI and NarK2, as well as with a protein highly induced upon nitrosative stress, Rv2617c [51]. This network of PPI suggests that the KdpF peptide could promote the degradation of these partners involved in nitrosative stress, leading to decreased intracellular multiplication of the mycobacteria [51].

3.2.2. Cell division

The BACTH system was also used to characterize the mycobacterial cell division, identifying interactions between FtsW, FtsZ and PbpB [52]. Another study demonstrated that FtsZ is able to interact with ClpX, the substrate recognition domain of the ClpXP protease, potentially modulating Z-ring structure formation and negatively regulating FtsZ polymerization activity [53]. FtsZ also

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interacts with CrgA (Rv0011c), a protein that possibly facilitates septum formation [54]. Another study showed that the membrane protein CwsA (Rv0008c) interacts with CrgA and Wag31, both involved in mycobacterial peptidoglycan biosynthesis [55]. Together these studies highlight the value of the BACTH system to study and further characterize the mycobacterial divisome [56].

3.2.3. *Mtb* cell wall composition

A BACTH screening using as a bait KasA, a component of FAS-II system, revealed that KasA interacts with PpsB and PpsD, which are two enzymes involved in the biosynthesis of lipid phthiocerol dimycoserosate (PDIM). This suggests a possible transfer of lipids between the FAS-II system and the PDIM biosynthetic pathways [57], highlighting the importance of PPI in the course of mycobacterial cell wall biosynthesis. Similar to mycolic acid, PDIM are involved in mycobacterial virulence [58,59].

EccA1 is an ATPase and belongs to ESX-I, the mycobacterial type VII secretion system [30]. It was shown that the *Mycobacterium marinum* EccA1 activity is required for optimal mycolic acid biosynthesis, probably through its interaction with FAS-II components (KasA and KasB), the mycolic acid condensase Pks13 and slightly with the mycolic acid methyltransferase MmaA4 [60]. In addition, EchA6, a possible enoyl-CoA hydratase, also interacts with several members of FAS-II (KasA and InhA), suggesting a possible role in feeding FAS-II with long-chain fatty acids [61].

The BACTH system was also used to detect interactions between the transporter-like Rv3789 and the galactosyltransferase Glft1, involved in arabinogalactan biosynthesis, another component of the mycobacterial MAPc [62].

Recently, a *Mtb* genome-wide screening using MmpL3 as a bait in BACTH system identified several interactants related to mycolic acid biosynthesis (MmpL11 and Rv0228=TmaT), PG biosynthesis (Rv3909, Rv3910 and Rv1337), glycolipid biosynthesis (Rv0227c, Rv0236c=AftD and Rv1457c) or cell division (CrgA) [63].

3.2.4. *Mtb* virulence factors

The BACTH system was used to search for partners of the virulence-associated factor Erp, which is required for optimal multiplication of *Mtb* in macrophages and in mice [64]. This led to the identification of two putative membrane proteins, Rv1417 and Rv2617c [65], the functions of which remain yet to be established.

MgtC is a virulence factor that participates to the adaptation of the mycobacteria to magnesium deprivation [66]. The BACTH system was used to assess the interactions between MgtC from *Mtb* and a MgtR peptide from *Salmonella typhimurium* [67], known to promote MgtC degradation in *Salmonella* [68]. Thus, the BACTH system is also adequate to evaluate the antivirulence activity of peptides (or proteins) towards mycobacterial proteins.

HbhA is a surface-exposed adhesin that is involved in the binding of mycobacteria to non-phagocytic cells, a necessary process for *Mtb* dissemination [69], and in the formation of intracellular lipid inclusions [70]. The BACTH system was used to demonstrate that HbhA interacts with Rv0613c and MmpL14 [71]. In addition, deletion of the orthologous gene of *rv0613c* in *Mycobacterium smegmatis* prevents cell-surface exposure of HbhA [71].

A three-hybrid system was developed in *E. coli* and helped to confirm the interaction between ESAT-6, CFP-10 and EccCb1 [72], as previously described with individual binary interactions identified in the Y2H system [32,33].

3.2.5. High-Throughput screening applied to BACTH

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The BACTH system has mostly been used to study pairwise interactions between a limited number of proteins. However, a global *Mtb* PPI network was also studied using the BACTH system. By using the nearly complete *Mtb* gene sets, it led to the identification of more than 8,000 interactions involving 2,907 mycobacterial proteins [73]. All these potential interactions now require further validation and characterization using complementary approaches.

3.3. Pros and cons

The BACTH system also permits to test direct interaction of pairwise partners in an *in vivo* environment. Furthermore, unlike the Y2H system, membrane-associated proteins can be studied in the BACTH system, as long as T18 and T25 reside on the cytoplasmic side of the bacteria. However, the BACTH system can only detect binary interactions (or potentially ternary interactions in the case of the bacterial three-hybrid system). The bacterial cellular context is partially maintained but it lacks the specificity of the mycobacterial cell wall organization (Table 1). Finally, some PTM and cofactors may be present in *E. coli*, however, all specific mycobacterial PTM and cofactors are absent (Table 1).

4. Methods developed for use with live mycobacteria

4.1. The mycobacterial protein fragment complementation (M-PFC)

The Y2H and BACTH systems have their limitations, as the identified interactions do not necessarily take place in their natural environment. In addition, neither system can take care of the specific mycobacterial cell wall organization, and some of the specific PTM and cofactors (Table 1). Hence, systems to directly assess PPI in a mycobacterial environment have been developed. The mycobacterial protein fragment complementation (M-PFC) technology relies on the functional reconstitution of a murine dihydrofolate reductase (mDHFR) in *Mycobacterium M. smegmatis* [74]. The POIs are fused to complementary fragments of mDHFR. If the POIs interact, the reconstitution of an active mDHFR confers resistance to the antibiotic trimethoprim. This system was validated by confirming the interactions between ESAT-6 and CFP-10, membrane-associated DosS and cytosolic DosR, and membrane-associated KdpD and cytosolic KdpE [74]. The authors performed a screen using a *Mtb* library and CFP-10 as a bait, confirming interactions of CFP-10 with ESAT-6 and identifying new interactions of CFP-10 with Rv0686, FtsQ, ClpC1, Pks13 and Rv2240c [74]. Interestingly, the interaction between CFP-10 and mycolic acid condensase Pks13 could not be reproduced in Y2H system, inferring that this interaction requires a specific mycobacterial environment to be detected [74].

4.1.1. Signaling pathways

M-PFC was also used to demonstrate interactions between PknH and the response regulator DosR, demonstrating a convergence between STPK and TCS signaling in *Mtb* [75]. In combination with *Mtb* proteome microarrays and Y2H approaches, M-PFC was used to further validate interactions between STPK protein interactants and two selected STPK, PknB and PknD [15].

4.1.2. Cell division

In agreement with the BACTH system, M-PFC confirmed interactions between ClpX and FtsZ [53]. M-PFC also confirmed interactions between FtsZ and SepF [76], independently of the screening performed in the Y2H system using FtsZ as a bait, as mentioned above [17].

4.1.3. Peptidoglycan biosynthesis

Mur synthases (MurC-F), which are essential and involved in peptidoglycan biosynthesis in mycobacteria [77], interact with regulatory proteins and proteins involved in cell division, such as PknA and PknB [78].

4.2. The split-protein sensor (Split-Trp)

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Split-Trp (or protein fragment complementation assay) requires a tryptophan biosynthetic pathway, which is present in mycobacteria. It relies on the reconstitution of an active Trp1p enzyme, only if the POIs interact with each other. This will then allow a tryptophan auxotrophic strain of *M. smegmatis* Δ*hisA* to grow on media without tryptophan [79]. The validity of Split-Trp was assessed by confirming interaction between ESAT-6 and CFP-10, and the homodimerization of GlfT1 and RegX3 [79].

In parallel with M-PFC, Split-Trp was used to evaluate interactions between PknH and DosR. However, only the phosphorylation-defective form of DosR (T198A/T205A) was able to interact with PknH in this system, indicating that Split-Trp seems to be a less sensitive than M-PFC [75].

4.3. In vivo crosslinking in live mycobacteria

In vivo crosslinking was developed to directly address PPI in a natural environment in order to limit false positive interactions or miss transient interactions. It relies on the use of crosslinking agents, such as formaldehyde or (sulfo-)disuccinimidyl suberate, generating covalent adduct of two spatially-close proteins. Using formaldehyde as a crosslinking agent, *Mtb* subunit E1 of the pyruvate dehydrogenase complex was shown to interact with nine *M. smegmatis* proteins [80]. Nonetheless, this approach could generate false positives, as naturally biotinylated mycobacterial proteins may interfere with the purification protocol [80].

A more recent approach consists in the incorporation of the UV-crosslinking unnatural amino acid *p*-benzoylphenylalanine, added to the culture medium, via nonsense suppression in the sequence of the protein under study [81]. Upon UV irradiation of live cells, this allows the formation of a covalent adduct between the studied protein and any interactant, thus capturing physiological interactions in a native environment. This method was applied to the lipoprotein LprG [82], which is involved in cell surface exposure of lipoarabinomannan, the regulation of triacylglycerol levels, phagolysosomal fusion and *Mtb* virulence [83-85]. Among 23 identified interactants, the authors focused on the site-specific interactions of LprG with LppI and LppK, as well as the physical and functional interactions between LprG and the mycoloyltransferase Ag85A conditioning cell growth and mycolic acid composition [82].

4.4. Pros and cons

Methods developed for use with live mycobacteria are devoted to test direct interactions between potential partners within the mycobacterial environment, in the presence of an adequate cellular organization and the potentially required cofactors. M-PFC and Split-Trp can be used to characterize pairwise interactions, whereas *in vivo* crosslinking may be useful to demonstrate the existence of protein complexes (Table 1). However, this latter technique is hardly amenable for the development of a high-throughput screening system (Table 1).

5. Conclusion and perspectives

All the methods listed above greatly contributed to the understanding of *Mtb* virulent mechanisms by focusing on PPI. However, despite the tremendous amount of data generated by these different technologies, deciphering mycobacterial PPI in terms of multiprotein and dynamic complexes requires more specific and more appropriate systems. In that regard, novel methods, such as in-cell Native Mass Resonance (NMR) spectroscopy or proximity-dependent biotinylation assay, appear to be very promising (Table 1).

In-cell NMR allows studying the conformation and the dynamics of biological macromolecules (such as protein complexes) under physiological conditions (i.e. in living cells) [86]. For instance, in-cell NRM was used to studying the intrinsically disordered mycobacterial protein Pup, a functional analog of ubiquitin [87]. Pup targets mycobacterial proteins for proteasome-mediated degradation, a process that is directly involved in *Mtb* virulence [88]. Pup was studied for its interaction in *E. coli*

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with the mycobacterial proteasomal ATPase Mpa and with the intact mycobacterial proteasome (Mpa plus *Mtb* proteasome core particle), showing that the proteasome complex had a higher affinity for Pup than Mpa alone [89]. However, the application of in-cell NMR directly in living mycobacteria remains to be tested and further developed.

Proximity-dependent biotinylation assays [90] consist in generating a hybrid protein between the POI and a biotin ligase (e.g. a variant of *E. coli* BirA [91] or *A. aeolicus* biotin ligase [92]) or an engineered ascorbic acid peroxidase (e.g. APEX [93,94]). APEX catalyzes the conversion of its substrate, biotin-phenol, into short-lived and highly reactive radicals, leading to the covalent attachment of biotin to electron-rich amino acids (such as tyrosine) of proximal proteins [90]. As the technique is directly performed in the organism of interest, whose subcellular structures are kept intact, it greatly minimizes false-positive identification. The hybrid protein can properly localize, perform its function and add a biotin residue to all potential partners in spatial proximity (in a 10- to 20-nm radius). Once the biotin is covalently bound to the proximal proteins, classical lysis methods are not expected to interfere in the process, in contrast to other approaches, such as co-precipitation or tandem affinity purification. The bacterial lysate can then be subjected to purification using streptavidin-based beads or columns. After stringent washes, elution and tryptic digestion, the samples can be subjected to mass spectrometry analysis to detect which biotinylated proteins are enriched in the samples. This method may be particularly suitable for the study of PPI in a natural context, for particular subcellular structures or for proteins involved in specific mycobacterial processes (such as cell wall biosynthesis or virulence mechanisms). Although *Mtb* possesses a biotin synthesis pathway [95] that could interfere with this technique, the use of relevant controls (e. g. a similar production of the POI not in fusion) would allow the identification of a specific subset of enriched biotinylated proteins, representing either direct interactants or spatially-close partners. This technology has not yet been applied to mycobacteria, but may be worthwhile to be tested for the study of *Mtb* PPI.

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