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# Proximity-dependent biotin identification links cholesterol catabolism with branched-chain amino acid degradation in *Mycobacterium smegmatis*

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1           **Proximity-dependent biotin identification links cholesterol**  
2           **catabolism with branched-chain amino acid degradation in**  
3                           *Mycobacterium smegmatis*

4  
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16  
17 **Abbreviations**

18 BCAA, branched-chain amino acid; BioID, proximity-dependent biotin identification; BirA, *E. coli*  
19 biotin ligase; HRP, horseradish peroxidase; LB, lysogeny broth; MCC, methylcitrate cycle; MMP,  
20 methylmalonyl pathway; *Mtb*, *Mycobacterium tuberculosis*; OADC, oleic  
21 acid/albumin/dextrose/catalase; PPI, protein-protein interactions; TB, tuberculosis.

## 23 **Abstract**

24 Cholesterol is a crucial component in *Mycobacterium tuberculosis* virulence as it is required for  
25 phagocytosis of mycobacteria by macrophages. In addition, the tubercle bacilli can grow using  
26 cholesterol as the sole carbon source. Thus, cholesterol catabolism represents a valuable target for the  
27 development of new antitubercular drugs. However, the molecular partners of cholesterol catabolism  
28 remain elusive in mycobacteria. Here, we focused on HsaC and HsaD, enzymes involved in two  
29 consecutive steps of cholesterol ring degradation and identified putative partners, using a BirA-based  
30 proximity-dependent biotin identification (BioID) approach in *Mycobacterium smegmatis*. In rich  
31 medium, the fusion protein BirA-HsaD was able to fish the endogenous cognate HsaC, thus validating  
32 this approach to study protein-protein interactions and to infer metabolic channelling of cholesterol  
33 ring degradation. In chemically defined medium, both HsaC and HsaD interacted with four proteins,  
34 BkdA, BkdB, BkdC and MSMEG\_1634. BkdA, BkdB and BkdC are enzyme that participate in the  
35 degradation of branched-chain amino acids. As cholesterol and branched-chain amino acid catabolism  
36 both generate propionyl-CoA, which is a toxic metabolite for mycobacteria, this interconnection  
37 suggests a compartmentalization to avoid dissemination of propionyl-CoA into the mycobacterial  
38 cytosol. Moreover, the BioID approach allowed us to decipher the interactome of MSMEG\_1634 and  
39 MSMEG\_6518, two proteins of unknown function, which are proximal to the enzymes involved in  
40 cholesterol and branched-chain amino acid catabolism. In conclusion, BioID is a powerful tool to  
41 characterize protein-protein interactions and to decipher the interconnections between different  
42 metabolic pathways, thereby facilitating the identification of new mycobacterial targets.

43

## 44 **Keywords**

45 *Mycobacterium*, tuberculosis, protein-protein interactions, cholesterol, catabolism, BioID

46

## 47 **1. Introduction**

48 *Mycobacterium tuberculosis* (*Mtb*) is the etiological agent of tuberculosis (TB), which causes 1.6  
49 million deaths per year. There is an alarming increase of TB cases caused by *Mtb* strains, which are  
50 (multi)resistant to available antibiotics. Therefore, there is an urgent need to identify new targets and  
51 to develop new anti-TB agents. *Mtb* is capable of infecting, surviving and multiplying within the  
52 alveolar macrophages of the infected host, and cholesterol is required for the phagocytosis of  
53 mycobacteria by macrophages (1), as mycobacteria enter phagocytes through cholesterol-enriched  
54 membrane microdomains (2). Moreover, during the intramacrophagic stage, access to nutrients is very  
55 limited for mycobacteria. Host cholesterol is an essential carbon and energy source for mycobacterial  
56 growth (3), and cholesterol metabolism is required to maintain optimal *Mtb* infection in animal models  
57 (4-7). Thus, cholesterol catabolism plays a major role in both the establishment and the development  
58 of *Mtb* infection.

59 Cholesterol is composed of a ring and a side chain, whose degradation is ensured by a distinct set of  
60 enzymes (for review, (8-11)). Furthermore, cholesterol degradation generates potentially toxic  
61 metabolic intermediates for mycobacteria, such as propionyl-CoA, which requires a very tight  
62 regulation of this degradation pathway (12). Recently, several studies have shown that chemical  
63 compounds, able to block or perturb cholesterol utilization, inhibit *Mtb* growth (13-15). Thus,  
64 cholesterol catabolism may represent a valuable target for the development of new antitubercular  
65 drugs.

66 We have recently shown that proximity-dependent biotin identification (BioID) is a powerful tool to  
67 detect and characterize protein-protein interactions (PPI) in mycobacteria by defining the heparin-  
68 binding hemagglutinin HbhA interactome (16). Briefly, after generating a fusion protein between the  
69 protein of interest and the *Escherichia coli* biotin ligase (BirA), proximity labelling allows the  
70 identification of proteins that strongly, weakly or transiently interact or are closely associated (within  
71 10 to 20 nm) with the fusion protein (17), allowing to define its proxisome (or proximal interactome)  
72 (18). In addition, this technology can help to identify multi-partner complexes directly in the organism  
73 of interest, preserving the intact subcellular structures, the presence of enzyme cofactors and post-  
74 translational modifications (for review, (19-22)).

75 In this study, we used the BioID approach in saprophytic *Mycobacterium smegmatis* mc<sup>2</sup>155 in order  
76 to characterize the proxisome of two consecutive enzymes, HsaC (MSMEG\_6036) and HsaD  
77 (MSMEG\_6037). In *Mtb*, HsaC (Rv3568c) and HsaD (Rv3569c) are required for growth on  
78 cholesterol (23), as they are both involved in cholesterol ring catabolism (8, 10), and HsaD was shown  
79 to be required for mycobacterial survival within macrophages (24). In addition, it was shown that in  
80 the absence of HsaC mycobacterial survival is impaired in mice and guinea pigs (7). We show here  
81 that in rich medium, such as Lysogeny Broth (LB), the fusion protein BirA-HsaD is able to interact  
82 with endogenous HsaC in *M. smegmatis*, thus validating the BioID approach. In a chemically defined  
83 medium, such as 7H9 supplemented with glycerol, both HsaC and HsaD were closely associated with  
84 BkdA, BkdB and BkdC, which are involved in branched-chain amino acid (BCAA) degradation, such  
85 as leucine, isoleucine and valine. In addition, MSMEG\_1634 interacted with both HsaC and HsaD. By  
86 defining the MSMEG\_1634 proxisome, encompassing proteins involved in BCAA degradation and  
87 MSMEG\_6518, the BioID technology allowed us to characterize mycobacterial proteins of unknown  
88 function. Thus, BioID proves to be useful for the characterization of metabolic pathways, leading to  
89 the identification of an interconnection between cholesterol ring catabolism and BCAA degradation in  
90 *M. smegmatis* grown in 7H9.

91

## 92 **2. Materials and Methods**

### 93 *2.1. Bacterial strains and culture conditions*

94 *Escherichia coli* TOP10 (Invitrogen, Carlsbad, CA, USA) used for the cloning steps was cultured in  
95 LB medium (MP Biomedicals, Illkirch, France) at 37 °C supplemented with ampicillin (100 µg/ml) or  
96 kanamycin (25 µg/ml), when required. *M. smegmatis* mc<sup>2</sup>155 and the recombinant strains constructed  
97 in this study (Table S1) were grown in Middlebrook 7H9 broth containing glycerol (without OADC)  
98 or on 7H11 agar plates supplemented with glycerol and OADC enrichment (Becton Dickinson, Le  
99 Pont-de-Claix, France), containing kanamycin (25 µg/mL), when required.

100

101 2.2. Genetic constructions

102 The *hsaC* (MSMEG\_6036), *hsaD* (MSMEG\_6037) and *MSMEG\_1634* genes were PCR amplified  
103 from *M. smegmatis* mc<sup>2</sup>155 genomic DNA using primers pMV361\_*hsaC*\_dir and pMV361\_*hsaC*\_rev,  
104 or pMV361\_*hsaD*\_dir and pMV361\_*hsaD*\_rev, or pMV361\_MS1634\_dir and pMV361\_MS1634\_rev,  
105 respectively (Table S2). The corresponding amplicons were digested by MunI and HindIII, and  
106 inserted into pMV361 digested by the same enzymes, to yield pMV361\_*hsaC*, pMV361\_*hsaD* and  
107 pMV361\_MSMEG\_1634.

108 To generate the vectors expressing the fusion between *hsaC* or *hsaD* and *birA*, the genes were PCR  
109 amplified from *M. smegmatis* mc<sup>2</sup>155 genomic DNA using primers pcDNA\_*birA*\_*hsaC*\_dir and  
110 pcDNA\_*birA*\_*hsaC*\_rev, or pcDNA\_*birA*\_*hsaD*\_dir and pcDNA\_*birA*\_*hsaD*\_rev, or  
111 pcDNA\_*hsaC*\_*birA*\_dir and pcDNA\_*hsaC*\_*birA*\_rev or pcDNA\_*hsaD*\_*birA*\_dir and  
112 pcDNA\_*hsaD*\_*birA*\_rev (Table S2). For N-terminal fusions with BirA, the *hsaC* and *hsaD* amplicons  
113 were digested by HindIII and NotI and inserted into pcDNA3.1 mycBioID (Addgene, (25)) digested  
114 with the same enzymes to yield pcDNA3.1\_*birA*\_*hsaC* and pcDNA3.1\_*birA*\_*hsaD*, respectively. For  
115 C-terminal fusions with BirA, the *hsaC* and *hsaD* amplicons were digested by BamHI and Kpn2I and  
116 inserted into pcDNA3.1 MCS-BirA(R118G)-HA (Addgene, (25)) digested with the same enzymes to  
117 yield pcDNA3.1\_*hsaC*\_*birA* and pcDNA3.1\_*hsaD*\_*birA*, respectively. The *birA*\_*hsaC* and *birA*\_*hsaD*  
118 fragments were amplified from pcDNA3.1\_*birA*\_*hsaC* and pcDNA3.1\_*birA*\_*hsaD* using primers  
119 pMV361\_*birA*\_XXX\_dir and pcDNA\_*birA*\_*hsaC*\_rev, or pMV361\_*birA*\_XXX\_dir and  
120 pcDNA\_*birA*\_*hsaD*\_rev (Table S2), digested by HindIII and inserted into pMV361, previously  
121 digested by HindIII and PvuII, to yield pMV361\_*birA*\_*hsaC* and pMV361\_*birA*\_*hsaD*. The  
122 *hsaC*\_*birA* fragment was obtained after digestion of pcDNA3.1\_*hsaC*\_*birA* by BstBI and PmeI and  
123 inserted into pMV361, digested by BstBI and KspAI, to yield pMV361\_*hsaC*\_*birA*. The *hsaD*\_*birA*  
124 fragment was obtained after digestion of pcDNA3.1\_*hsaD*\_*birA* by MunI and PmeI and inserted into  
125 pMV361, digested by MunI and KspAI, to yield pMV361\_*hsaD*\_*birA*.

126 To construct the vectors for production of HsaC with a N-terminal 6His-tag, the gene was PCR  
127 amplified from *M. smegmatis* mc<sup>2</sup>155 genomic DNA using primers pMV361\_6His\_*hsaC*\_dir and

128 pMV361\_6His\_hsaC\_rev (Table S2). The corresponding amplicon was digested by HindIII and  
129 inserted into pMV361 digested by PvuII and HindIII to yield pMV361\_6His\_hsaC.

130 To generate the vectors expressing the fusion between *MSMEG\_1634* and *birA*, the gene was PCR  
131 amplified from *M. smegmatis* mc<sup>2</sup>155 genomic DNA using primers pcDNA\_birA\_MS1634\_dir and  
132 pcDNA\_birA\_MS1634\_rev, or pcDNA\_MS1634\_birA\_dir and pcDNA\_MS1634\_birA\_rev (Table S2).  
133 For N-terminal fusions with BirA, the *MSMEG\_1634* amplicon was digested by HindIII and EcoRV  
134 and inserted into pcDNA3.1 mycBioID (Addgene, (25)) digested with the same enzymes to yield  
135 pcDNA3.1\_birA\_MSMEG\_1634. For C-terminal fusions with BirA, the *MSMEG\_1634* amplicon was  
136 digested by EcoRI and KspAI and inserted into pcDNA3.1 MCS-BirA(R118G)-HA (Addgene, (25))  
137 digested with the same enzymes to yield pcDNA3.1\_MSMEG\_1634\_birA. The *birA\_MSMEG\_1634*  
138 fragment was amplified from pcDNA3.1\_birA\_MSMEG\_1634 using primers pMV361\_birA\_XXX\_dir  
139 and pcDNA\_birA\_MS1634\_rev (Table S2), digested by HindIII and inserted into pMV361, previously  
140 digested by HindIII and PvuII, to yield pMV361\_birA\_MSMEG\_1634. The *MSMEG\_1634\_birA*  
141 fragment was obtained after digestion of pcDNA3.1\_MSMEG\_1634\_birA by BstBI and PmeI and  
142 inserted into pMV361, digested by BstBI and KspAI, to yield pMV361\_MSMEG\_1634\_birA. All the  
143 constructions were checked by DNA sequencing.

144

### 145 2.3. BioID protocol with *M. smegmatis* cultures

146 Fifty-ml of medium were inoculated at OD<sub>600nm</sub>=0.025 from 3-days saturated cultures of *M. smegmatis*  
147 and grown overnight (16 hours) at 37°C under agitation. To avoid any depletion during exponential  
148 phase of growth, biotin was added at a final concentration of 200 μM and cultures were grown for 24  
149 additional hours. After 40 hours of growth, cultures were centrifuged at 4,000 x g for 10 min and then  
150 rinsed twice with Phosphate-Buffered Saline (PBS). Each pellet was resuspended in 1 ml of PBS per  
151 300 mg of bacteria. One ml of each culture was transferred into Lysing Matrix B tubes (MP  
152 Biomedicals) and the tubes were subjected to FastPrep (MP Biomedicals) treatment (6 cycles at  
153 maximum speed for 1 min, 1 min of incubation on ice). After quick spin, 700 μl of lysates was

154 transferred to new Eppendorf tubes and centrifuged at 16,500 x g for 1 hr at 4°C. Dynabeads MyOne  
155 Streptavidin C1 (ThermoFisher Scientific, Illkirch, France) were rinsed three times with PBS, and 100  
156 µL of beads was incubated at 4°C with each cleared lysate using a rotating wheel. After overnight  
157 incubation, beads were washed three times with PBS containing 0.1% bovine serum albumin and two  
158 times with PBS (to decrease the bovine serum albumin signal during mass spectrometry analysis).  
159 Finally, enriched biotinylated proteins were eluted using Laemmli buffer.

160

#### 161 2.4. In vivo pulldown in *M. smegmatis*

162 *M. smegmatis* containing empty pMV361 or producing HsaC with a N-terminal 6His-tagged was  
163 grown in 100 ml of 7H9 until OD600 reaches ~1. Cultures were split in two, centrifuged at 3,000 x g  
164 and rinsed twice with PBS containing 0.05% Tween80. The pellets were resuspended in 1 ml of PBS,  
165 transferred into Lysing Matrix B tubes (MP Biomedicals) and lysed using a FastPrep (MP  
166 Biomedicals) treatment (6 cycles at maximum speed for 1 min, 1 min of incubation on ice). Lysates  
167 were harvested and SDS was added to a final concentration of 1%. The samples were further incubated  
168 for 30 min at room temperature under gentle rotation. Cell debris were eliminated by centrifugation at  
169 5,000 x g for 15 min at 4°C. The supernatants were then incubated with 16 µl of PureCube INDIGO  
170 Ni-MagBeads (Cube Biotech, Monheim, France, dilution 1:4) for 1 h at room temperature under  
171 gentle rotation. After incubation, the beads were rinsed two times with PBS, two times with PBS  
172 containing 50 mM imidazole, and the proteins were finally eluted with 100 µl PBS containing 300  
173 mM imidazole.

174

#### 175 2.5. Western-blot analysis

176 Ten µL of total protein extract was resolved by SDS-PAGE using 12% acrylamide gels and then  
177 electro-transferred onto a nitrocellulose membrane. The membrane was saturated with Tris-Buffered  
178 Saline (TBS), pH 7.5, containing 0.05% Tween 80 and 5% milk and probed overnight at 4 °C with  
179 anti-HA monoclonal antibody (Sigma-Aldrich, Saint-Quentin-Fallavier, France, dilution 1:1,000) or



180 with anti-Myc monoclonal antibody (Thermo Fisher Scientific, dilution 1:1,000) in TBS-Tween-3%  
181 milk. Finally, the membrane was incubated for 1 hr at room temperature with goat anti-mouse  
182 horseradish peroxidase (HRP)-conjugated secondary antibodies (Abcam, Paris, France, diluted  
183 1:5,000) in TBS-Tween-3% milk. Alternatively, the biotinylated proteins were directly probed  
184 overnight with HRP-conjugated streptavidin (BD Pharmingen, San Diego, CA, USA, dilution  
185 1:1,000). The blots were developed using the Amersham ECL Prime Western-Blotting Detection  
186 Reagent (GE Healthcare, Velizy-Villacoublay, France) and chemiluminescence was detected using the  
187 Amersham Imager 600 (GE Healthcare).

188

## 189 2.6. Mass spectrometry proteomic analysis

190 Raw data collected during nanoLC-MS/MS analyses were processed and converted into a \*.mgf peak  
191 list format with Proteome Discoverer 1.4 (Thermo Fisher Scientific). MS/MS data were analysed  
192 using search engine Mascot (version 2.4.0, Matrix Science, London, UK) installed on a local server.  
193 Searches were performed with a tolerance on mass measurement of 10 ppm for precursor and 0.02 Da  
194 for fragment ions, against a composite target-decoy database built with a *M. smegmatis* mc<sup>2</sup>155  
195 UniProt database (strain ATCC 700084/mc(2)155, taxo 246196, July 20172021, 12666 entries) fused  
196 with recombinant trypsin and a list of classical contaminants (118 entries). Cysteine  
197 carbamidomethylation, methionine oxidation, protein N-terminal acetylation and cysteine  
198 propionamidation were searched as variable modifications. Up to one missed trypsin cleavage was  
199 allowed. The identification results were imported into Proline 2.0 software installed on a local server  
200 (<http://proline.profi-proteomics.fr>) for validation (26). Peptide spectrum matches taller than nine  
201 residues and ion scores >10 were retained. The false discovery rate was then optimized to be below  
202 2% at the protein level using the Mascot Modified Mudpit score. Spectral counting analyses were  
203 performed with Proline.

204 For BioID analyses, we introduced a corrective factor in order to compare the results, since the total  
205 number of spectra may vary between independent biological replicates. For each condition, the  
206 number of spectra for one protein was normalized against Replicate 1 and was calculated as follows:

207 Normalized number of spectra (Protein X) = Number of spectra (Protein X) in Replicate 1 + [Number  
208 of spectra (Protein X) in Replicate 2 \* Total number of spectra in Replicate 1 / Total number of spectra  
209 in Replicate 2] + [Number of spectra (Protein X) in Replicate 3 \* Total number of spectra in Replicate  
210 1 / Total number of spectra in Replicate 3]. Furthermore, as the expression of *birA* alone may result in  
211 nonspecific biotinylation of mycobacterial proteins (16), the ratio was calculated as follows: Ratio  
212 (Protein X) = Normalized number of spectra with HsaC, HsaD or MSMEG\_1634 in fusion with BirA /  
213 Normalized number of spectra with BirA alone. For calculation purpose, when the Normalized  
214 number of spectra with BirA alone for Protein X was zero, it was arbitrarily set to 0.5 (between 0 and  
215 1 spectrum) in order to calculate a ratio. Only proteins with a normalized number of spectra  $\geq 25$  and a  
216 ratio  $> 10$  were considered as meaningful, implying that Protein X is enriched in that specific  
217 condition in comparison to the control (BirA alone). As the heterologous expression of *hsaC* and *hsaD*  
218 alone could also potentially lead to non-specific biotinylation of proteins, the ratio of HsaC or HsaD  
219 fused to BirA/BirA should be at least two times superior to the ratio HsaC or HsaD alone/BirA.

220 For pulldown experiments, the ratio was determined as the number of spectra for Protein X in the  
221 strain producing recombinant 6His-HsaC divided by the number of spectra for Protein X in the strain  
222 containing empty pMV361. When the number of spectra for Protein X was zero in case of *M.*  
223 *smegmatis* containing empty pMV361, it was arbitrarily set to 0.5 (between 0 and 1 spectrum) in order  
224 to calculate a ratio.

225

## 226 **3. Results**

### 227 *3.1. Production of HsaD fusion proteins in M. smegmatis in LB medium*

228 HsaD (MSMEG\_6037) from *M. smegmatis* mc<sup>2</sup>155 was fused to the N-terminal or the C-terminal end  
229 of BirA(R118G) in order to catalyse protein biotinylation in a proximity-dependent fashion (25). The  
230 corresponding coding sequences were inserted into the mycobacterial vector pMV361 under the  
231 control of the strong *hsp60* promoter. All constructions, as well as pMV361\_*birA* and pMV361\_*hsaD*,  
232 were introduced into electro-competent cells of *M. smegmatis* mc<sup>2</sup>155. Western-blot analysis using

233 anti-Myc (when BirA was fused to the N-terminal end of HsaD) or anti-HA (for unfused BirA or when  
234 BirA was fused to the C-terminal end of HsaD) antibodies confirmed that the fusion proteins were  
235 produced in *M. smegmatis* when grown in LB medium (Figure 1A).

236 For BioID analyses, each *M. smegmatis* culture was grown overnight (16 hours) in LB medium, and  
237 200  $\mu$ M of biotin was added to the culture medium to avoid any biotin depletion during exponential  
238 growth. The cultures were then grown for 24 additional hours. At 16 and 40 hours, the growth of *M.*  
239 *smegmatis* was not affected by the production of recombinant HsaD or the fusion proteins with BirA,  
240 as the OD was similar for all strains of *M. smegmatis* (Figure 1B).

241 The cultures were then subjected to bacterial lysis and the biotinylated proteins were purified using  
242 magnetic beads coated with streptavidine. After releasing from the beads, 10  $\mu$ L of each sample was  
243 analysed by Western-blotting, and biotinylated proteins were detected by using HRP-conjugated  
244 streptavidin (Figure 1C). Monomers and dimers of bovine serum albumin, which was used to saturate  
245 streptavidine free sites on beads, were detected as 66- and 132-kDa proteins, respectively, in all the  
246 samples. Additional biotinylated proteins (shown by the asterisks) were detected in *M. smegmatis*  
247 producing the HsaD-fused proteins, but absent with BirA alone, suggesting specific biotinylation of  
248 proteins closely associated with HsaD.

249

### 250 3.2. Identification of HsaD interactants by BioID in LB medium

251 To identify proteins that interact with HsaD, each sample of biotinylated proteins from three  
252 independent biological replicates was analysed by mass spectrometry (Table S3). From our previous  
253 study (16) and as confirmed by Western-blot analyses (Figure 1C), overexpression of *birA* alone may  
254 result in non-specific biotinylation of mycobacterial proteins. Therefore, candidate proteins, as  
255 quantified using spectral counting, were selected by comparing their enrichment in *M. smegmatis*  
256 producing the bait of interest, BirA-HsaD or HsaD-BirA, over *M. smegmatis* producing BirA alone.  
257 Only a normalized number of spectra equal or above 25 was considered as representative, and only  
258 proteins that displayed a ratio superior to 10 of enrichment in the presence of BirA-HsaD or HsaD-

259 BirA compared to BirA alone were selected. Moreover, as recombinant expression of *hsaD* alone  
260 could lead to a non-specific increase in the amounts of biotinylated proteins, we only considered  
261 proteins for which the BirA-HsaD/BirA or HsaD-BirA/BirA ratios were at least two times superior to  
262 the HsaD alone/BirA ratio.

263 These stringent criteria led to the identification of proteins closely associated with HsaD (Table 1).  
264 When BirA was fused to the C-terminal end of HsaD, very few interactants were isolated, inferring  
265 that a free C-terminal end is important to maintain PPI. However, the HsaD-BirA fusion was still able  
266 to interact with HsaD itself (99% identical to BphD), inferring *in vivo* multimerization of the protein  
267 or auto-biotinylation of the HsaD-BirA fusion. When BirA was fused to the N-terminal end of HsaD  
268 (BirA-HsaD), two interactants were identified, HsaD itself and HsaC, confirming that HsaC and HsaD  
269 interact with each other *in vivo* and demonstrating that the BioID approach is a robust tool to study  
270 PPI.

271

### 272 3.3. Production of HsaC and HsaD fusion proteins in *M. smegmatis* in 7H9 medium

273 In order to compare different growth conditions, we also searched for HsaD interactants when *M.*  
274 *smegmatis* was grown in the chemically-defined 7H9 medium, supplemented with glycerol (and  
275 without OADC). As HsaC was detected as a HsaD interactant, it was added to the study in order to  
276 compare its proxisome with that of HsaD. Similar to HsaD, HsaC (MSMEG\_6036) was fused to the  
277 N-terminal or the C-terminal end of BirA(R118G) and the corresponding coding sequences were  
278 inserted into pMV361. All constructs were introduced into electro-competent cells of *M. smegmatis*  
279 mc<sup>2</sup>155. Western-blot analysis using anti-Myc (when BirA is fused to the N-terminal end of HsaC or  
280 HsaD) and anti-HA (when BirA is fused to the C-terminal end of HsaC or HsaD) antibodies confirmed  
281 that the fusion proteins were produced in *M. smegmatis* when grown in 7H9 medium supplemented  
282 with glycerol (Figure 2A).

283 Each *M. smegmatis* culture was grown overnight (16 hours) to reach the beginning of exponential  
284 phase, and 200  $\mu$ M of biotin was added to the culture medium to avoid any biotin depletion during

285 exponential growth. The cultures were then grown for an additional 24 additional hours, until the  
286 beginning of stationary phase. The OD values were similar at 16 and 40 hours for the *M. smegmatis*  
287 strains that produced the recombinant HsaC, HsaD or the fusion proteins (Figure 2B), indicating that  
288 the over-expression of the recombinant proteins did not affect mycobacterial growth.

289 The cultures were then subjected to bacterial lysis and the biotinylated proteins were purified using  
290 magnetic beads coated with streptavidine. After releasing from the beads, 10 µL of each sample was  
291 analysed by Western-blotting, and biotinylated proteins were detected by using HRP-conjugated  
292 streptavidin (Figure 2C). The profiles of recombinant *M. smegmatis* containing pMV361\_ *hsaC* and  
293 pMV361\_ *hsaD* were similar to each other and few biotinylated proteins were detected. However, like  
294 shown above for LB, further biotinylated proteins (shown by the asterisks) were detected in *M.*  
295 *smegmatis* producing the HsaC- or HsaD-fusion proteins, but were absent with BirA alone, suggesting  
296 specific biotinylation of proteins closely associated with HsaC or HsaD.

297

#### 298 3.4. Identification of HsaC and HsaD partners by BioID in 7H9 medium

299 To identify the proteins that interact with HsaC and HsaD, each sample of biotinylated proteins from  
300 three independent biological replicates was analysed by mass spectrometry (Tables S4 and S5).  
301 Protein abundance was evaluated using spectral counting and candidate proteins were selected by  
302 comparing their enrichment in *M. smegmatis* producing the proteins of interest (BirA-HsaC, BirA-  
303 HsaD, HsaC-BirA or HsaD-BirA) over *M. smegmatis* overexpressing *birA* alone. When BirA was  
304 fused to the C-terminal end of HsaC or HsaD, very few interactants were identified (Tables S4 and  
305 S5), suggesting that a free C-terminal end is required to maintain PPI for both proteins. The HsaC-  
306 BirA and HsaD-BirA fusions were able to interact with themselves, reflecting their probable ability to  
307 multimerize *in vivo* or to auto-biotinylate.

308 When BirA was fused to the N-terminal end of HsaC or HsaD, the stringent criteria led to the  
309 identification of five proteins for BirA-HsaC and six proteins for BirA-HsaD (Tables 2 and 3). For  
310 BirA-HsaC, the interactants were MSMEG\_4712, MSMEG\_1634, MSMEG\_4711, MSMEG\_4710

311 and MSMEG\_6518. For the BirA-HsaD construct, HsaD (or BphD) itself, MSMEG\_4711,  
312 MSMEG\_4712, GlgE/MSMEG\_4916, MSMEG\_1634 and MSMEG\_4710 were detected.

313 Interestingly, four proteins (MSMEG\_4712, MSMEG\_4711, MSMEG\_4710 and MSMEG\_1684)  
314 were common to both the HsaC and HsaD proxisomes, highlighting a potential interconnection  
315 between these proteins. MSMEG\_4712, MSMEG\_4711 and MSMEG\_4710 are encoded by *bkdA*,  
316 *bkdB* and *bkdC*, respectively, which form an operon that is conserved in most mycobacteria, including  
317 *M. smegmatis* and *Mtb* (27). The corresponding enzymes, BkdA, BkdB and Bkdc, belong to the  
318 Branched-Chain Keto Acid DeHydrogenase (BCKADH) complex, required for *Mtb* virulence and  
319 involved in the catabolism of BCAA (28). The additional protein, MSMEG\_1684, is annotated as  
320 probable forkhead-associated protein of unknown function.

321

### 322 3.5. BioID approach to characterize proteins of unknown function such as MSMEG\_1634

323 The use of BioID may be helpful to investigate proteins with unknown function by characterizing their  
324 close protein environment. For this purpose, BirA-MSMEG\_1634 and MSMEG\_1634-BirA were  
325 produced in *M. smegmatis* (Figure 3A). Their production only slightly affected *M. smegmatis* growth  
326 at 16 and 40 hours (Figure 3B). After purification using streptavidin beads, 10  $\mu$ L of biotinylated  
327 protein solution was analysed by Western-blotting and detected using HRP-conjugated streptavidin  
328 (Figure 3C). Several biotinylated proteins (shown by the asterisks) were detected in *M. smegmatis*  
329 producing the MSMEG\_1634-fusion proteins, and were absent with BirA alone, suggesting specific  
330 biotinylation of proteins closely associated with MSMEG\_1634.

331 Protein abundance was then evaluated using spectral counting, and candidate proteins were selected by  
332 comparing their enrichment in *M. smegmatis* producing the fusion proteins of interest (BirA-  
333 MSMEG\_1634 and MSMEG\_1634-BirA) over *M. smegmatis* overexpressing *birA* alone (Table S6).  
334 Only two interactants, MSMEG\_1634 and MSMEG\_6518, were identified when MSMEG\_1634 was  
335 fused to the N-terminal part of BirA (Table 4), highlighting again the importance of a free C-terminal  
336 end to maintain PPI. Interestingly, MSMEG\_6518 displays 56% of identity with MSMEG\_1634 and

337 both proteins display a very similar structure as predicted by Alphafold (29, 30) (Figure S1). Thus,  
338 BioID is able to detect homologous proteins, which may reflect the ability of MSMEG\_1634 to form  
339 *in vivo* heteromultimers with MSMEG\_6518. Moreover, MSMEG\_6518 was also identified as an  
340 interactant of BirA-HsaC (Table 2).

341 For BirA-MSMEG\_1634, eight interactants were identified, MSMEG\_1634 itself,  
342 BkdA/MSMEG\_4712, BkdB/MSMEG\_4711, MSMEG\_6518, MSMEI\_1212 (corresponding to  
343 MSMEG\_1247), HrpA/MSMEG\_6587, MSMEG\_3726 and BkdC/MSMEG\_4710 (Table 4).  
344 Interestingly, MSMEG\_3726, a putative alcohol dehydrogenase, was previously described as being  
345 upregulated in the presence of cholesterol (11). As shown above, five out of the eight BirA-  
346 MSMEG\_1634 interactants (BkdA, BkdB, BkdC, MSMEG\_1634 and MSMEG\_6518) were common  
347 to the proxisome of either HsaC or HsaD.

348 Surprisingly, all the interactants identified with MSMEG\_1634-BirA or BirA-MSMEG\_1634 were  
349 also enriched (ratio  $\geq 7.8$ ) when MSMEG\_1634 was produced alone (Table S6). These proteins were  
350 not over-represented when HsaC or HsaD were produced alone (Table S4 and S5), nor in our previous  
351 study with HbhA (16). Moreover, BkdA, BkdB, BkdC, HrpA and MSMEG\_3726 were not over-  
352 represented in the *M. smegmatis* strain overproducing MSMEG\_1634-BirA (Table S6). These results  
353 discard any non-specific binding to the streptavidin beads or any natural biotinylation of these  
354 proteins. Hence, the over-representation of these interactants appears to be driven by *MSMEG\_1634*  
355 overexpression alone.

356

### 357 3.6. *In vivo* pulldown using a strain of *M. smegmatis* producing 6His-HsaC

358 The BioID approach allows the detection of PPI in 10- to 20-nm range (17), without necessarily a  
359 direct interaction. Nevertheless, we developed an *in vivo* pulldown assay, which requires a direct  
360 interaction between protein partners, in order to confirm the PPI isolated with the BioID approach. For  
361 this purpose, we generated a *M. smegmatis* strain containing empty pMV361 or producing HsaC with  
362 a N-terminal 6His-tag, as only HsaC with a N-terminal BirA was prone to interact with other proteins

363 (Table 2). After purification on nickel beads, we analysed the protein content by mass spectrometry,  
364 and their abundance was compared to that present in *M. smegmatis* carrying the empty vector (Table  
365 S7). Endogenous HsaC or MSMEG\_1634 were not able to bind nickel beads (with 1 and 0 spectrum,  
366 respectively) in *M. smegmatis* containing the empty vector. As expected, using two independent  
367 biological replicates, the recombinant 6His-HsaC was the most enriched protein with a ratio of 360 in  
368 *M. smegmatis* producing 6His-HsaC over *M. smegmatis* containing the empty vector. In the same  
369 strain, endogenous MSMEG\_1634 was isolated among the top 15 most enriched proteins with a ratio  
370 of 52, inferring that HsaC and MSMEG\_1634 may be in direct contact with each other in *M.*  
371 *smegmatis* grown in 7H9 and confirming our results obtained with the BioID approach (Table S6).

372

## 373 **4. Discussion**

374 The BioID technology is a new and powerful tool helpful for the characterization of the proxisome of  
375 proteins of interest. In this study, we focused on the two enzymes, HsaC and HsaD, which are  
376 involved in two consecutive steps of ring cholesterol catabolism in *M. smegmatis* (11) and *Mtb* (8-10).  
377 Cholesterol catabolism in mycobacteria is a subject of growing interest, as it is a key process for  
378 mycobacterial pathogenesis (3), thereby representing a promising therapeutic target. Using the  
379 mycobacterial surrogate strain *M. smegmatis* mc<sup>2</sup>155, we show here that HsaC and HsaD are able to  
380 interact *in vivo* with each other when the bacilli are grown in LB medium, demonstrating the validity  
381 of the BioID approach. This interaction between HsaC and HsaD probably reflects the metabolic  
382 channelling of the cholesterol ring degradation. However, no interaction between HsaC and HsaD was  
383 detected when a medium specific for mycobacterial growth, such as 7H9 supplemented with glycerol  
384 (but no OADC), was used. These results highlight the importance of the medium when carrying out  
385 experiments to characterize the proxisome of mycobacterial proteins and suggest that the use of the  
386 chemically defined 7H9 medium with glycerol as the sole source of carbon may favour the activation  
387 of specific metabolic pathways. This is consistent with other findings showing that the growth of wild-  
388 type *M. smegmatis* and a *MSMEG\_3811* deletion mutant were reversed when grown in a complete



389 (LB) compared to a defined medium with a single carbon source (31). Here, the use of complete  
390 medium containing multiple carbon sources, such as LB, is likely to activate many catabolic pathways,  
391 as shown by the differential proxisomes detected in the two growth conditions. It would have been of  
392 interest to use a cholesterol-containing medium as sole carbon source for further characterization of  
393 the HsaC and HsaD proxisomes. Thus, the lack of a direct comparison of BioID analyses performed  
394 in 7H9 supplemented with glycerol with those performed in 7H9 supplemented with cholesterol, due  
395 to the severe growth defect in the latter medium (data not shown), constitutes a limitation of our study.  
396 However, this subject may be part of future work.

397 When *M. smegmatis* was grown in 7H9 supplemented with glycerol, HsaC and HsaD were both  
398 strongly interconnected with BkdA, BkdB and BkdC, which are involved in the catabolism of BCAA.  
399 This interconnection was observed only in 7H9 medium and not in LB medium. Since 7H9 contains  
400 large amounts of L-glutamate (0.5 g/L), which is used by IlvE to catalyse the final step of leucine,  
401 isoleucine and valine biosynthesis (32), degradation of excessive BCAA may be a way for the bacilli  
402 to feed its central carbon metabolism by increasing the presence of BkdA, BkdB and BkdC, when  
403 grown in 7H9. As cholesterol and BCAA degradation both lead to the generation of propionyl-CoA,  
404 which is highly toxic for mycobacteria, this interconnection might help to sequester propionyl-CoA  
405 into specialized compartments. Moreover, the interplay between pathways, such as the methylcitrate  
406 cycle (MCC), the methylmalonyl pathway (MMP) and the incorporation into methyl-branched cell  
407 wall lipids, involved in propionyl-CoA detoxification has been suggested previously (12). Hence, such  
408 a compartmentalization may avoid dissemination of toxic metabolites within the cytosol of  
409 mycobacteria and would allow the metabolic channelling of propionyl-CoA directly from upstream  
410 pathways, such as cholesterol and BCAA degradation, to downstream pathways, such as MCC, MMP  
411 and the incorporation into the methyl-branched cell wall lipids (Figure 4).

412 There is additional evidence indicating that mycobacteria display compartmentalization of their  
413 metabolic pathways. For example, the lipid biosynthetic enzymes involved in phosphatidylinositol  
414 mannoside biosynthesis were suggested as being compartmentalized at the mycobacterial plasma  
415 membrane in *M. smegmatis* (33). More recently, the compartmentalization of peptidoglycan precursors

416 was also demonstrated in *M. smegmatis*, and this organization may be applicable to rod-shaped  
417 bacteria that are phylogenetically distant (34). This concept has now been extended to the biosynthesis  
418 of several cell wall components of *Mtb* (35). Metabolic compartmentalization is not restricted to lipid  
419 biosynthesis, as it was also suggested for the co-catabolism of carbon substrates, and the formation of  
420 multi-protein complexes in mycobacteria may occur in response to the nutritional environment and the  
421 physiological needs (36). Hence, in order to identify these multi-protein complexes, the use of BioID  
422 may be a particularly interesting approach.

423 In our previous BioID study, we identified an interaction between HsaC and HbhA, when HbhA-BirA  
424 was overproduced in *M. smegmatis* (16). However, we were not able to detect the reverse interaction  
425 using HsaC fused to BirA. This may be due to the low abundance of endogenous HbhA in *M.*  
426 *smegmatis* (37, 38). In agreement with that hypothesis, the orthologous HbhA from *M. smegmatis*  
427 (MSMEG\_0919) was not detected by mass spectrometry in any of the samples. Moreover, the growth  
428 conditions (7H9 or LB) may not be optimal for production of endogenous HbhA and for the detection  
429 of the HsaC-HbhA interaction. Alternatively, as BirA covalently binds biotin to lysine residues of the  
430 proximal protein (39), and as HbhA from *M. smegmatis* is methylated on most lysine residues (37),  
431 these post-translational modifications may have decreased the efficacy of biotinylation by BirA.

432 Finally, the BioID approach may help into characterize bacterial proteins of unknown function. We  
433 show here that MSMEG\_1634 is interconnected with cholesterol and BCAA degradation, and is  
434 closely associated with the homologous MSMEG\_6518 protein. The function of MSMEG\_1634 is not  
435 assigned and blast analyses do not provide any further information. However, the protein contains a  
436 probable forkhead-associated domain, which is a signalling motif that mediates PPI and modulates a  
437 wide variety of biological processes in many organisms, including mycobacteria (40). Thus, we  
438 hypothesize that MSMEG\_1634 and MSMEG\_6518 act as regulators of cholesterol and/or BCAA  
439 catabolism, possibly by playing a structural role at the interface of the complexes formed by HsaC,  
440 HsaD, BkdA, BkdB and BkdC (Figure 4). A similar situation has been described for the mycobacterial  
441 forkhead-associated domain-containing protein GarA (Rv1827), which was shown to interact and to  
442 regulate several enzymes of the tricarboxylic acid cycle (41). Since propionyl-CoA and its metabolites

443 are highly toxic for mycobacteria, destabilizing the formation of Hsa/Bkd complexes would constitute  
444 a potential anti-mycobacterial target, as it would lead to their dissociation and drive diffusion of  
445 propionyl-CoA into the cytosol, thereby causing self-poisoning of the mycobacteria. Moreover,  
446 identifying interconnected pathways may help to design synergizing drugs.

447 Two mycobacterial enzymes have been shown to be lipoylated in *Mtb*, DlaT and  
448 BkdC/MSMEG\_4710 (28). Lipoic acid and biotin display very similar structures and properties (for  
449 review, (42)). Therefore, lipoylation may increase non-specific binding of the *M. smegmatis* DlaT  
450 analogue (also known as SucB/MSMEG\_4283) and BkdC/MSMEG\_4710 to streptavidin beads. When  
451 MSMEG\_1634 alone was overproduced, it interacted with BkdA, BkdB and BkdC, leading to  
452 enhanced binding to the streptavidin beads and potentially creating a snowball effect in which  
453 multimers of MSMEG\_1634 play a bridging role between the different BCKADH complexes.  
454 Moreover, the mycobacterial enzyme Lpd interacts with both DlaT and the BCKADH complex in *Mtb*  
455 (28), which could amplify this phenomenon in *M. smegmatis*. Consistent with this hypothesis, all the  
456 components of the  $\alpha$ -ketoglutarate dehydrogenase complex (DlaT/SucB/MSMEG\_4283,  
457 Kgd/SucA/MSMEG\_5049 and LpdA/MSMEG\_0903) were slightly over-represented (ratios between  
458 2.8 and 4.4) in *M. smegmatis* overexpressing *MSMEG\_1634* or *birA\_MSMEG\_1634* (Table S6), but  
459 not in the strain overexpressing *MSMEG\_1634\_birA* (ratios between 1.0 and 1.1). As a free C-terminal  
460 end of MSMEG\_1634 appears to be required to maintain PPI, the MSMEG\_1634-BirA construct  
461 would no longer be able to play a bridging role, thereby precluding the snowball effect.

462 The bioinformatics tool STRING (<https://string-db.org/>) allows to deciphering the functional  
463 characterization of protein-protein networks (43). When applied to MSMEG\_1634, the strongest  
464 predicted functional partner is Kgd/SucA/MSMEG\_5049 with a score of 0.987. Interestingly, when  
465 applied to BkdA/MSMEG\_4712 and BkdB/MSMEG\_4711, the strongest predicted functional partner  
466 (outside the BCKADH complex) is also Kgd/SucA/MSMEG\_5049 with a score of 0.993 and 0.998,  
467 respectively. Thus, using a totally unrelated approach, these predictions also suggests that BkdA,  
468 BkdB and MSMEG\_1634 may be closely associated, around SucA, in *M. smegmatis*, which is in  
469 agreement with the MSMEG\_1634 proxisome defined here by the BioID technology. In addition, as

470 mentioned above, Kgd/SucA/MSMEG\_5049 was slightly over-represented in the strains producing  
471 either MSMEG\_1634 or BirA-MSMEG\_1634 (Table S6).

472

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477

## 478 **Conflict of Interest Statement**

479 The authors declare no conflicts of interest.

480

## 481 **Author Contributions**

482 R. Veyron-Churlet conceived, designed and analysed the research; S. Lecher, A.S. Lacoste, J.M.  
483 Saliou and R. Veyron-Churlet performed the research and acquired the data; C. Locht and R. Veyron-  
484 Churlet interpreted the data and wrote the manuscript. All authors were involved in drafting and  
485 revising the manuscript.

486

## 487 **Data Availability Statement**

488 The data that support the findings of this study are available on request from the corresponding author.

489

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630 **Figure Legends**

631 **Figure 1. HsaD, fused with BirA, mediates biotinylation of *M. smegmatis* proteins grown in LB**  
 632 **medium. (A)** Western-blot analysis using anti-Myc (left panel) or anti-HA (right panel) antibodies of  
 633 total lysates of *M. smegmatis* mc<sup>2</sup>155 transformed with pMV361\_birA, pMV361\_birA\_hsaD,  
 634 pMV361\_hsaD or pMV361\_hsaD\_birA, as indicated. The molecular size markers (M) with their  
 635 respective sizes expressed in kDa are shown in the left margins. The positions of Myc-BirA-HsaD  
 636 (69.8 kDa), HsaD-BirA-HA (68.9 kDa) and BirA-HA (37.4 kDa) are indicated by the short horizontal  
 637 bars. **(B)** Growth curves in LB medium of *M. smegmatis* mc<sup>2</sup>155 transformed with pMV361\_birA (in  
 638 black), pMV361\_birA\_hsaD (in red), pMV361\_hsaD (in green) and pMV361\_hsaD\_birA (in blue).  
 639 **(C)** Western-blot analysis using HRP-conjugated streptavidin of biotinylated proteins from total  
 640 lysates of *M. smegmatis* mc<sup>2</sup>155 transformed with pMV361\_birA, pMV361\_birA\_hsaD,  
 641 pMV361\_hsaD or pMV361\_hsaD\_birA, as indicated after enrichment on streptavidin beads. The  
 642 molecular size markers (M) with their respective sizes expressed in kDa are shown in the left margin.  
 643 The asterisks indicate proteins present in the lysates of *M. smegmatis* mc<sup>2</sup>155 producing BirA-HsaD or  
 644 HsaD-BirA, but not in the others. The pictures are representative of three independent experiments.

645 **Figure 2. HsaC and HsaD, fused to BirA, mediate biotinylation of *M. smegmatis* proteins grown**  
 646 **in 7H9 medium. (A)** Western-blot analysis using anti-Myc (upper panels) or anti-HA (lower panels)  
 647 antibodies of total lysates for two independent clones of *M. smegmatis* mc<sup>2</sup>155 transformed with  
 648 pMV361\_hsaC, pMV361\_birA\_hsaC or pMV361\_hsaC\_birA (left panels), and with pMV361\_hsaD,  
 649 pMV361\_birA\_hsaD or pMV361\_hsaD\_birA (right panels). The molecular size markers (M) with  
 650 their respective sizes expressed in kDa are shown in the left margins. The positions of Myc-BirA-  
 651 HsaC (71.1 kDa), Myc-BirA-HsaD (69.8 kDa), HsaC-BirA-HA (71.0 kDa) and HsaD-BirA-HA (68.9  
 652 kDa) are indicated by the short horizontal bars. **(B)** OD<sub>600nm</sub> values at 16 and 40 hours from cultures of  
 653 *M. smegmatis* mc<sup>2</sup>155 transformed with pMV361\_birA (in black), pMV361\_birA\_hsaC (in red),  
 654 pMV361\_hsaC (in green) or pMV361\_hsaC\_birA (in blue) (left panel), or with pMV361\_birA (in

655 black), pMV361\_birA\_hsaD (in red), pMV361\_hsaD (in green) or pMV361\_hsaD\_birA (in blue)  
656 (right panel), when grown in 7H9 supplemented with glycerol. (C) Western-blot analysis using HRP-  
657 conjugated streptavidin of biotinylated proteins from total lysates of *M. smegmatis* mc<sup>2</sup>155  
658 transformed with pMV361\_birA, pMV361\_birA\_hsaC, pMV361\_hsaC or pMV361\_hsaC\_birA (left  
659 panel), or with pMV361\_birA, pMV361\_birA\_hsaD, pMV361\_hsaD or pMV361\_hsaD\_birA (right  
660 panel) after enrichment on streptavidin beads. The molecular size markers (M) with their respective  
661 sizes expressed in kDa are shown in the left margins. The asterisks indicate proteins present in the  
662 lysates of *M. smegmatis* mc<sup>2</sup>155 producing HsaC or HsaD fused to BirA, but not in the others. The  
663 pictures are representative of three independent experiments.

664 **Figure 3. MSMEG\_1634, fused to BirA, mediates biotinylation of *M. smegmatis* proteins grown**  
665 **in 7H9 medium.** (A) Western-blot analysis using anti-Myc (upper panel) or anti-HA (lower panel)  
666 antibodies of total lysates for two independent clones of *M. smegmatis* mc<sup>2</sup>155 transformed with  
667 pMV361\_MSMEG\_1634, pMV361\_birA\_MSMEG\_1634 or pMV361\_MSMEG\_1634\_birA. The  
668 molecular size markers (M) with their respective sizes expressed in kDa are shown in the left margins.  
669 The positions of Myc-BirA-MSMEG\_1634 (58.8 kDa) and MSMEG\_1634-BirA-HA (58.4 kDa) are  
670 indicated by the short horizontal bars. (B) OD<sub>600nm</sub> values at 16 and 40 hours from cultures of *M.*  
671 *smegmatis* mc<sup>2</sup>155 transformed with pMV361\_birA (in black), pMV361\_birA\_MSMEG\_1634 (in red),  
672 pMV361\_MSMEG\_1634 (in green) or pMV361\_MSMEG\_1634\_birA (in blue), when grown in 7H9  
673 supplemented with glycerol. (C) Western-blot analysis using HRP-conjugated streptavidin of  
674 biotinylated proteins from total lysates of *M. smegmatis* mc<sup>2</sup>155 transformed with  
675 pMV361\_MSMEG\_1634, pMV361\_birA\_MSMEG\_1634, pMV361\_MSMEG\_1634\_birA or  
676 pMV361\_birA after enrichment on streptavidin beads. The molecular size markers (M) with their  
677 respective sizes expressed in kDa are shown in the left margins. The asterisks indicate proteins present  
678 in the lysates of *M. smegmatis* mc<sup>2</sup>155 producing BirA-MSMEG\_1634 or MSMEG\_1634-BirA, but  
679 not in the others. The pictures are representative of three independent experiments.

680 **Figure 4. Proposed global scheme of the complex formed by HsaC, HsaD, BkdA, BkdB, BkdC,**  
681 **MSMEG\_1634 and MSMEG\_6518 in *M. smegmatis*.** HsaC and HsaD, involved in the cholesterol

682 ring catabolism, are able to multimerize and interact with each other *in vivo*. They are closely  
683 associated with BkdA, BkdB and BkdC, which are involved in the catabolism of BCAA (leucine,  
684 isoleucine and valine). We propose that MSMEG\_1634 and MSMEG\_6518 acts as regulators or  
685 pivotal links between cholesterol and BCAA degradation complexes. The compartmentalization of  
686 these pathways would avoid diffusion of toxic propionyl-CoA into the mycobacterial cytosol and may  
687 allow metabolic channelling of propionyl-CoA directly from upstream pathways (cholesterol and  
688 BCAA catabolism) to downstream pathways (MCC, MMP and incorporation into the methyl-branched  
689 cell wall lipids).

690

## 691 **Tables**

692 **Table 1.** List of *M. smegmatis* proteins interacting or closely associated with HsaD-BirA or BirA-  
693 HsaD and displaying a ratio > 10, when bacteria were grown in LB medium.

<b>Construct</b>	<b>Interactant</b>	<b><i>Mtb</i></b>	<b>Product</b>	<b>Ratio</b>
<b>HsaD-BirA</b>	<b>MSMEG_6037</b>	Rv3569c	HsaD (BphD)	36
<b>BirA-HsaD</b>	<b>MSMEG_6037</b>	Rv3569c	HsaD (BphD)	33
	<b>MSMEG_6036</b>	Rv3568c	HsaC	13

694

695 **Table 2.** List of *M. smegmatis* proteins interacting or closely associated with HsaC-BirA or BirA-  
696 HsaC and displaying a ratio > 10, when bacteria were grown in 7H9 medium supplemented with  
697 glycerol.

<b>Construct</b>	<b>Interactant</b>	<b><i>Mtb</i></b>	<b>Product</b>	<b>Ratio</b>
<b>HsaC-BirA</b>	<b>MSMEG_6036</b>	Rv3568c	HsaC	25
<b>BirA - HsaC</b>	<b>MSMEG_4712</b>	Rv2497c	Probable branched-chain keto acid dehydrogenase E1 component, BkdA	76
	<b>MSMEG_1634</b>		Probable forkhead-associated protein	31

<b>MSMEG_4711</b>	Rv2496c	Probable branched-chain keto acid dehydrogenase E1 component, BkdB	24
<b>MSMEG_4710</b>	Rv2495c	Probable branched-chain keto acid dehydrogenase E2 component, BkdC	15
<b>MSMEG_6518</b>		Conserved hypothetical protein	14

698

699 **Table 3.** List of *M. smegmatis* proteins interacting or closely associated with HsaD-BirA or BirA-  
700 HsaD and displaying a ratio > 10, when bacteria were grown in 7H9 medium supplemented with  
701 glycerol.

<b>Construct</b>	<b>Interactant</b>	<b><i>Mtb</i></b>	<b>Product</b>	<b>Ratio</b>
<b>HsaD-BirA</b>	<b>MSMEG_6037</b>	Rv3569c	HsaD (BphD)	240
	<b>MSMEG_6037</b>	Rv3569c	HsaD (BphD)	183
	<b>MSMEG_4711</b>	Rv2496c	Probable branched-chain keto acid dehydrogenase E1 component, BkdB	107
<b>BirA-HsaD</b>	<b>MSMEG_4712</b>	Rv2497c	Probable branched-chain keto acid dehydrogenase E1 component, BkdA	79
	<b>MSMEG_4916</b>	Rv1327c	M1P-dependent maltosyltransferase, GlgE	40
	<b>MSMEG_1634</b>		Probable forkhead-associated protein	16
	<b>MSMEG_4710</b>	Rv2495c	Probable branched-chain keto acid dehydrogenase E2 component, BkdC	16

702

703 **Table 4.** List of *M. smegmatis* proteins interacting or closely associated with MSMEG\_1634-BirA or  
704 BirA-MSMEG\_1634 and displaying a ratio > 10, when bacteria were grown in 7H9 medium  
705 supplemented with glycerol.

<b>Construct</b>	<b>Interactant</b>	<b><i>Mtb</i></b>	<b>Product</b>	<b>Ratio</b>
<b>MSMEG_1634-</b>	<b>MSMEG_1634</b>		Probable forkhead-associated protein	43

<b>BirA</b>	<b>MSMEG_6518</b>		Conserved hypothetical protein	19
	<b>MSMEG_1634</b>		Probable forkhead-associated protein	59
	<b>MSMEG_4712</b>	Rv2497c	Probable branched-chain keto acid dehydrogenase E1 component, BkdA	27
	<b>MSMEG_4711</b>	Rv2496c	Probable branched-chain keto acid dehydrogenase E1 component, BkdB	21
<b>BirA-</b>	<b>MSMEG_6518</b>		Conserved hypothetical protein	15
<b>MSMEG_1634</b>	<b>MSMEI_1212</b> ( <b>MSMEG_1247</b> )		HEPN_Swt1 domain-containing protein	14
	<b>MSMEG_6587</b>		ATP-dependent helicase, HrpA	14
	<b>MSMEG_3726</b>		Alcohol dehydrogenase	12
	<b>MSMEG_4710</b>	Rv2495c	Probable branched-chain keto acid dehydrogenase E2 component, BkdC	11

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707