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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* mc²155 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* mc²155 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* mc²155 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* mc²155 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* mc²155 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* mc²155 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_{600nm}=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* mc²155
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 ≥ 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* mc²155 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* mc²155. 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 μ 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 μ 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 mc²155. 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 μ 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 μ L 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 μ 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 ≥ 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* mc²155, 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 α -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* mc²155 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* mc²155 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* mc²155 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* mc²155 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* mc²155 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_{600nm} values at 16 and 40 hours from cultures of
 653 *M. smegmatis* mc²155 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* mc²155
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* mc²155 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* mc²155 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_{600nm} values at 16 and 40 hours from cultures of *M.*
671 *smegmatis* mc²155 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* mc²155 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* mc²155 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.

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

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

MSMEG_4711	Rv2496c	Probable branched-chain keto acid dehydrogenase E1 component, BkdB	24
MSMEG_4710	Rv2495c	Probable branched-chain keto acid dehydrogenase E2 component, BkdC	15
MSMEG_6518		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.

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

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

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

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