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Chemical-genetic interaction mapping links carbon metabolism and cell wall structure to tuberculosis drug efficacy [preprint]

Item Type	Preprint
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Citation	<p>bioRxiv 2021.04.08.439092; doi: https://doi.org/10.1101/2021.04.08.439092 . Link to preprint on bioRxiv.</p>
DOI	10.1101/2021.04.08.439092
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Link to Item	https://hdl.handle.net/20.500.14038/29818

1 **Chemical-genetic interaction mapping links carbon metabolism and cell wall**
2 **structure to tuberculosis drug efficacy**

3
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38

39 **Abstract**

40 Current chemotherapy against *Mycobacterium tuberculosis* (*Mtb*), an important human
41 pathogen, requires a multidrug regimen lasting several months. While efforts have been
42 made to optimize therapy by exploiting drug-drug synergies, testing new drug
43 combinations in relevant host environments remains arduous. In particular, host
44 environments profoundly affect the bacterial metabolic state and drug efficacy, limiting
45 the accuracy of predictions based on *in vitro* assays alone. In this study, we utilize
46 conditional *Mtb* knockdown mutants of essential genes as an experimentally-tractable
47 surrogate for drug treatment, and probe the relationship between *Mtb* carbon
48 metabolism and chemical-genetic interactions (CGI). We examined the anti-tubercular
49 drugs isoniazid, rifampicin and moxifloxacin, and found that CGI are differentially
50 responsive to the metabolic state, defining both environment-independent and –
51 dependent synergies. Specifically, growth on the *in vivo*-relevant carbon source,
52 cholesterol, reduced rifampicin efficacy by altering mycobacterial cell surface lipid
53 composition. We report that a variety of perturbations in cell wall synthesis pathways
54 restore rifampicin efficacy during growth on cholesterol, and that both environment-
55 independent and cholesterol-dependent *in vitro* CGI could be leveraged to enhance
56 bacterial clearance in the mouse infection model. Our findings present an atlas of novel
57 chemical-genetic-environmental synergies that can be used to optimize drug-drug
58 interactions as well as provide a framework for understanding *in vitro* correlates of *in*
59 *vivo* efficacy.

60

61

62 **Significance**

63 Efforts to improve tuberculosis therapy include optimizing multi-drug regimens to take
64 advantage of drug-drug synergies. However, the complex host environment has a
65 profound effect on bacterial metabolic state and drug activity, making predictions of
66 optimal drug combinations difficult. In this study, we leverage a newly developed library
67 of conditional knockdown *Mycobacterium tuberculosis* mutants in which genetic
68 depletion of essential genes mimics the effect of drug therapy. This tractable system
69 allowed us to assess the effect of growth condition on predicted drug-drug interactions.
70 We found that these interactions can be differentially sensitive to the metabolic state
71 and select *in vitro*-defined synergies can be leveraged to accelerate bacterial killing
72 during infection. These findings suggest new strategies for optimizing tuberculosis
73 therapy.

74

75

76 **Introduction**

77 The current chemotherapeutic regimen for tuberculosis (TB) is the product of many
78 decades of basic and clinical research. Since the first trials of streptomycin
79 monotherapy in 1948 were rapidly followed by the emergence of antibiotic resistant
80 clones (1), multidrug regimens to both suppress resistance and accelerate bacterial
81 killing have become standard. The current regimen used against drug-sensitive strains
82 of *Mycobacterium tuberculosis* (*Mtb*) consists of four antibiotics, isoniazid (INH),
83 rifampicin (RIF), pyrazinamide (PZA), and ethambutol (EMB), and was optimized in a
84 series of clinical trials in the 1970s (2, 3). While this “short-course regimen” has been
85 credited with curing over 50 million patients, its delivery is complicated by the need for
86 6-9 months of drug administration (4). Furthermore, even in clinical trial settings where
87 the delivery of this extended regimen is assured, 5-10% of patients fail therapy (5). The
88 frequent transmission of antibiotic resistant *Mtb* strains has further complicated TB
89 treatment options, requiring the use of less optimized drug combinations that are
90 administered for even longer periods (6).

91
92 The factors that necessitate prolonged therapy are complex and specific to the infection
93 environment. While antibiotics such as INH and RIF cause rapid cell death *in vitro*, their
94 antimicrobial activities are much slower during infection (7). Limited drug penetration
95 into *Mtb*-containing tissue lesions may reduce the efficacy of some drugs. For example,
96 the intralesional RIF concentrations are significantly lower than what is achieved in the
97 plasma (8, 9), and clinical studies suggest that increased RIF dosing improves bacterial
98 clearance (10, 11). However, drug penetration is unlikely to fully account for the reduced

99 antibiotic efficacy in host tissue, as this complex and stressful environment has also
100 been shown to alter the physiology of the pathogen to induce a drug-tolerant state (12–
101 14). While a number of host immune-related stresses may be involved in this process,
102 simple changes in macronutrient availability can have important consequences. *Mtb* has
103 access to a mixture of glycolytic carbon sources, fatty acids and cholesterol in host
104 tissue (15–17), and altering the availability of these carbon sources *in vitro* can change
105 the efficacy of anti-tubercular compounds (18–23). A role for differential carbon
106 catabolism in determining drug efficacy is also supported by the identification of natural
107 genetic variants in clinical *Mtb* isolates that enhance drug tolerance by altering either
108 glycerol, lipid, or sterol catabolism (24, 25). The profound effect of the host environment
109 on bacterial metabolic state and drug activity makes it difficult to predict the ultimate
110 efficacy of an antibiotic regimen based on *in vitro* assays alone.

111
112 The most advanced efforts to accelerate TB therapy involve optimizing multi-drug
113 regimens to take advantage of drug-drug synergies. These pharmacological
114 interactions can improve therapy by increasing drug exposure, decreasing MIC, or
115 enhancing the maximal effect of the treatment (26). However, despite the demonstrated
116 benefits of synergistic regimens in many therapeutic realms, the combinatorial burden of
117 testing all potential multi-drug combinations remains cumbersome. While recent
118 advances to more efficiently predict synergies *in vitro* have proven valuable (27, 28), it
119 remains unclear whether the environmental influences that so profoundly alter the
120 efficacy of individual drugs will also influence drug-drug interactions (29, 30).

121

122 In this study, we leveraged a newly developed genetic resource to understand the effect
123 of growth conditions on potential drug-drug synergies. This “hypomorph library” consists
124 of individually DNA barcoded strains in which individual essential genes are tagged with
125 the DAS+4 sequence that targets proteins for degradation upon inducible induction of
126 the SspB adapter (31). Graded *sspB* expression produces different degrees of depletion
127 to model the effect of partial chemical inhibition (32). This approach allowed for highly
128 parallel assessment of chemical-genetic interactions (CGI) between individual
129 hypomorphic alleles and TB drugs under different growth conditions. We report that
130 drugs of distinct classes produce synergies that are differentially sensitive to the
131 environment, and that both condition-independent and –dependent CGIs can be
132 identified for all drugs tested. In particular, RIF efficacy was impaired as a result of cell
133 surface lipid alterations that occur during growth in cholesterol, and the cell could be
134 resensitized through a condition-specific interaction with the cell wall synthetic
135 machinery both *in vitro* and in the mouse lung. These observations provide a
136 compendium of chemical-genetic synergies that can be exploited to enhance therapy
137 and begin to define *in vitro* correlates of *in vivo* efficacy.

138

139 **Results**

140 **Genetic strategy to define essential bacterial functions that alter drug efficacy *in*** 141 ***vitro*.**

142 Host environmental factors alter antibiotic efficacy against TB (12, 24, 33, 34). To
143 determine if carbon source availability specifically affects antibiotic efficacy, we used the
144 *Mtb* strain H37Rv in minimal medium with glycerol, acetate or cholesterol as sole

145 carbon sources in a growth inhibition assay with the TB drugs isoniazid (INH), rifampicin
146 (RIF) and moxifloxacin (MOX). As these carbon sources support different growth rates,
147 endpoint-based antibiotic activity measurements such as MIC assays can be difficult to
148 interpret. Therefore we quantified growth rates in each carbon source over multiple
149 concentrations of antibiotics to determine the concentration that decreased the growth
150 rate by 50% (GR_{50}) (24, 35). We found different carbon sources to alter the GR_{50} of
151 these drugs in distinct ways. For INH, glycerol-dependent growth increased efficacy,
152 relative to the non-glycolytic carbon sources. In contrast, growth in cholesterol was
153 found to decrease RIF efficacy, relative to the other conditions. Media composition had
154 a more modest effect on MOX, altering GR_{50} by only approximately 2-fold (**Fig1A**).
155 These findings show that carbon metabolism plays an important role in antibiotic
156 efficacy and has distinct effects on different drugs.

157
158 To understand the mechanisms linking drug efficacy and metabolism, we utilized a
159 barcoded-hypomorph library consisting of 465 essential genes to identify CGI with these
160 drugs (32). The library was grown in minimal media with glycerol, acetate or cholesterol,
161 and antibiotics were added at concentrations ranging from 0.05X to 1X GR_{50} for each.
162 Bacteria were subjected to these conditions in 96-well plates for 14 days, at which point
163 the relative abundance of individual mutants was assessed through multiplex PCR and
164 Next-Generation Sequencing (**Fig1B, S.Table 1**). Relative abundances of individual
165 mutants were calculated by normalizing the mutant's barcode count to the total barcode
166 count and comparing the relative abundance of each mutant in different drug
167 concentrations with the untreated control samples.

168

169 We initially investigated the validity of this dataset by determining if genetic inhibition of
170 each drug's target produced the expected CGI with that compound in standard glycerol-
171 dependent growth conditions. RIF and MOX interact with the RNA polymerase subunit,
172 RpoB or the DNA gyrase subunit, GyrA, respectively. As expected, *rpoB* and *gyrA*
173 hypomorphic mutants were hypersensitive to RIF or MOX, and the CGI increased with
174 drug concentration (**Fig1C**). The major target of INH, *InhA*, was not present in our
175 mutant pool. However, the library did contain a *ndh* mutant. Deficiency in this NADH-
176 dehydrogenase is known to decrease INH efficacy, likely due to the inefficient formation
177 of the active INH-NAD adduct (36). Consistent with these observations, we found that
178 the *ndh* hypomorphic mutant showed an INH dose-dependent increase in abundance in
179 our dataset (**Fig1C**).

180

181 The hypomorph library was constructed to contain up to five different versions of each
182 DAS+4 tagged mutant, which express different levels of *sspB* and therefore produce
183 graded levels of protein depletion (31, 37). To investigate the relationship between
184 target protein abundance and phenotype, we correlated the strength of *sspB* expression
185 with drug sensitivity for a number of genes that were expected to alter antibiotic efficacy
186 (**Fig1D**). For RIF, we concentrated on *rpoB*. For INH, we examined *ndh* as well as
187 genes associated with mycolate biosynthesis, and found *kasA* and *hadB* mutants to be
188 significantly hypersensitive to increasing INH concentrations. For MOX, in addition to
189 *gyrA*, depletion of proteins associated with DNA integrity or deoxynucleotide production,
190 *dnaN*, *lexA* and *ndrF2*, showed significantly decreased fitness over increasing MOX

191 concentrations. For some mutants, particularly those involved in DNA metabolism, we
192 noted a dose-dependent effect of *sspB* expression, with larger phenotypes
193 corresponding to greater degrees of depletion. However, this was not a universal
194 phenomenon, and we often found that independent mutants expressing different *sspB*
195 levels produced consistent phenotypic effects. The observed differences in sensitivity to
196 protein depletion likely reflect the unique biochemistry of each pathway (38). As a result,
197 we considered each mutant corresponding to a target protein independently in the
198 following analyses.

199

200 **CGI profiles are determined by the combination of carbon source and drug**

201 To understand the relative importance of carbon metabolism and drug on bacterial
202 physiology, we compared the “fitness profiles” of each condition, which represented the
203 relative fitness of each mutant strain. We first selected the fold change in abundance
204 (\log_2FC) values of every mutant at 0.65X GR_{50} drug concentration that showed a
205 statistically significant fitness difference when at least one treated sample was
206 compared to the untreated control (918 mutants, $Q < 0.05$). 0.65X GR_{50} drug
207 concentration displayed the largest number of mutants with a statistically significant
208 fitness difference across most conditions. Upon hierarchical clustering of both genes
209 and conditions, we found three major condition clusters that corresponded to each
210 antibiotic (**Fig2A**). These condition clusters were differentiated by two distinct clusters of
211 mutants; one large gene set differentiated MOX from the other drugs (Fig 2A bottom
212 cluster), and differential fitness effects on a second smaller gene set differentiated INH

213 and RIF (Fig2A top cluster). Within each condition cluster, fitness effects due to carbon
214 source were apparent.

215
216 To catalog the genes that differentiate these conditions, we compared each drug-
217 treated sample (0.65X GR₅₀) with the corresponding untreated control and chose genes
218 with log₂FC < -1 and $p < 0.05$ for each carbon source. For all antibiotics tested, there
219 were genes that showed synergy across all carbon sources as well as those associated
220 with a single carbon source (**Fig2B**). Notably, MOX produced the largest number of
221 synergies in a condition-independent manner, while INH and RIF produced the largest
222 number of synergies during acetate and cholesterol-dependent growth respectively. To
223 more quantitatively assess the relative contribution of carbon source and drug, we used
224 Principal Component Analysis (PCA) to examine the amount of variance contributed by
225 each, concentrating only on the INH and RIF conditions, which produced the most
226 condition-specific effects. PC1 aligned to distinguish the carbon sources, while PC2
227 differentiated among drug treatments (**Fig2C**). The first and second principal
228 components accounted for 37.6% and 23.7% respectively, of the overall variance
229 across the 9 data sets, indicating that drug and carbon source played a relatively equal
230 role in shaping bacterial physiology.

231

232 **Condition-independent CGI can be reproduced with individual mutants.**

233 Genes found to alter antibiotic efficacy from the high-throughput hypomorph analysis
234 were validated using individual mutant strains. For initial validation studies, we
235 concentrated on mutants predicted to show drug-hypersensitivity under all media

236 conditions. The repression of a drug's target is thought to increase sensitivity to
237 inhibition by reducing the fraction of the target that needs to be inhibited to reduce
238 growth (39). As such, we anticipated that drug target inhibition would result in condition-
239 independent effects. This prediction was verified using a *rpoB* hypomorphic mutant,
240 which our screening data predicted to increase RIF efficacy in all carbon sources
241 **(Fig3A)**. When the MIC of RIF was determined using a standard microplate growth
242 assay, we verified that this degree of RpoB depletion decreased the RIF MIC by 4 to 16-
243 fold in each of the three media conditions **(Fig3A)**. In addition to known drug targets, a
244 number of additional genes were predicted to enhance RIF efficacy in a carbon source-
245 independent manner, including the transcriptional regulator, Rv0472c. This gene has
246 been named, mycolic acid desaturase regulator (*madR*), based on its specific effect on
247 the mycolate-modifying *desA1* and *desA2* genes (40). As we found for RpoB, MadR
248 depletion sensitized the bacterium to RIF in all tested carbon sources in the single strain
249 assay **(Fig3B)**. Finally, to ensure the specificity of these CGI, we investigated whether
250 they could be reversed by specific metabolite complementation. As predicted by the
251 screen, depletion of the lysine biosynthetic enzyme, LysA, produced RIF
252 hypersensitivity in both glycerol and cholesterol media, and we found this phenotype
253 was largely reversed by supplementation with 3mM lysine **(Fig3C)**. These data support
254 the predictions of the primary screen, and demonstrate that certain CGI are relatively
255 unaffected by carbon source.

256

257 **Depletion of cell-wall biosynthetic genes produces condition-specific RIF synergy**

258 As cholesterol represents a primary carbon source for *Mtb* during infection (16, 41), and
259 RIF efficacy is thought to be limited by drug exposure (42–44), we probed the
260 hypomorph fitness profiles for clues to the mechanism underlying the cholesterol-
261 dependent increase in RIF MIC. We found that inhibition of genes associated with
262 different components of the mycobacterial cell wall, including arabinogalactan (*embC*
263 and *aftB*), and mycolic acid (*kasA*) (45, 46) specifically sensitized the bacterium to RIF
264 during cholesterol growth (**Fig4A**). These mutants were significantly underrepresented
265 by up to 8-fold upon RIF treatment specifically in cholesterol growth conditions across
266 multiple *sspB* doses and drug concentrations. To further explore the effect of cell wall
267 inhibition, we tested individual mutants with defects in peptidoglycan (*dapE*),
268 arabinogalactan (*aftB*), and mycolic acid (*hadB*) synthesis (45, 47–49). In this format,
269 we found that perturbation in each of these cell wall layers produced a similar effect, as
270 they all preferentially sensitized the bacterium to RIF in cholesterol growth conditions
271 (**Fig4BCD**). These observations suggest that alterations in cell wall structure might
272 underlie the relationship between cholesterol catabolism and reduced RIF efficacy.

273

274 **RIF efficacy correlates with propionate catabolism and branched chain lipid**
275 **abundance.**

276 *Mtb* catabolizes cholesterol to pyruvate, propionyl-CoA and acetyl-CoA (17, 50).
277 Propionyl-CoA is a precursor of branched-chain lipid synthesis, and catabolism of either
278 cholesterol or propionate increases cellular propionyl-CoA levels causing an increase in
279 both the abundance and chain-length of cell wall lipids, such as diacyltrehalose (DAT),
280 polyacyltrehalose (PAT), and sulfolipid-1 (SL-1) (50–53). To understand whether these

281 alterations in cell wall lipids could be responsible for altering RIF efficacy, we compared
282 RIF GR₅₀ values in cells grown in cholesterol to those grown in butyrate media
283 supplemented with increasing concentrations of propionate. We observed that GR₅₀
284 values increased up to 8-fold with increasing propionate levels, and the addition of 0.1X
285 propionate, reflecting a 1:10 ratio of propionate to butyrate (weight:weight), mimicked
286 the effect of cholesterol (**Fig5A**). To more quantitatively associate branched-chain lipid
287 abundance with drug efficacy, we performed relative quantification of the abundance of
288 SL-1 during growth in butyrate, butyrate and propionate, and cholesterol using mass
289 spectrometry. As expected, we found that growth in either propionate or cholesterol
290 increased both the *m/z* range of SL-1 and its total abundance, and the effect of
291 propionate was dose-dependent in the tested range (**Fig5B**). This dose-dependency
292 allowed us to correlate changes in sulfolipid abundance with changes in rifampicin
293 efficacy. At multiple propionate concentrations, we calculated the GR₅₀ for RIF and the
294 total abundance of SL-1. We found a correlation between these values, as 0.003X
295 propionate supplementation had no effect on either SL-1 abundance or RIF GR₅₀, and
296 0.1X increased both values (**Fig5C**). These findings implied that the propionyl-CoA
297 derived from cholesterol alters rifampicin efficacy through modification of mycobacterial
298 surface lipids.

299
300 The different branched chain lipid species of *Mtb* are produced by distinct biosynthetic
301 pathways, and inhibiting the synthesis of one lipid can produce a compensatory
302 increase in others (52). Therefore, to assess whether increased lipid abundance was
303 causally related to RIF efficacy, we employed an *Mtb* mutant lacking the PhoPR

304 regulatory system, which is required for the synthesis of multiple branched chain lipid
305 species (54–56). In contrast to wild type *Mtb*, a Δ *phoPR* deletion mutant showed little
306 change in rifampicin efficacy with propionate supplementation or cholesterol compared
307 to butyrate growth conditions, and possessed significantly decreased sulfolipid levels
308 compared to WT (**Fig6AB**). Similar to the effects seen with the Δ *phoPR* mutant, cell wall
309 defective hypomorphs (*aftB*, *hadB*, *dapE*) also maintained a relatively consistent RIF
310 GR50 in all media conditions, compared to wild type (**Fig6CD**). These findings suggest
311 that cholesterol catabolism reduces RIF efficacy via a propionyl-CoA driven increase in
312 synthesis of cell surface lipids, and that this effect can be reversed by perturbing the
313 structure of the cell envelope.

314

315 ***In vitro* CGI predict drug efficacy *in vivo***

316 To determine whether synergies identified *in vitro* predict strategies to accelerate
317 bacterial killing in the lung, we infected C57BL/6J mice via the aerosol route with a
318 pooled culture consisting of 3 barcoded WT strains and a number of select hypomorph
319 mutants. We concentrated on genes that either displayed carbon source-independent
320 synergy with RIF (*rpoB*, *madR*), or mutants that disrupt the peptidoglycan (*dapE*, *murA*)
321 or arabinogalactan (*aftB*) layer of the cells wall and would be expected to reverse the
322 effect of host-derived cholesterol catabolism (**Fig3AB, Fig4BC, S.Fig1**). To ensure that
323 the animals were treated with a relevant dose of antibiotic, we measured the plasma
324 concentration of RIF in mice and adjusted the dosing to match the 24-hour exposure
325 observed during clinical TB therapy (**Fig7A**) (57). The mice were fed doxycycline chow
326 starting 3 days before infection to repress *sspB* expression in the mutants upon

327 infection, and allow them to grow normally. At 3 weeks post-infection, doxycycline was
328 withdrawn to initiate protein degradation, and two weeks of RIF treatment was
329 administered (**Fig7B**) (58). The antibiotic regimen effectively decreased the total
330 number of lung CFU (**Fig7C**). Lung homogenates were washed free of RIF and plated
331 on 7H10 plates with supplementation to recover viable clones. The relative abundance
332 of individual mutants was normalized to the average abundance of three barcoded WT
333 strains for each mouse and fitness changes were calculated by comparing to those from
334 untreated mice. Each of the mutants showed significant decrease in abundance in RIF
335 treated animals, relative to wild type. AftB and MurA depletion increased bacterial
336 clearance to the same degree as RpoB depletion. Depleting MadR had an even greater
337 effect, and the abundance of this mutant was reduced below our limit of detection in four
338 out of five animals (**Fig7D**). DapE depletion mutant was below our limit of detection in
339 both untreated and treated animals. These findings show that CGI identified *in vitro* can
340 accelerate bacterial clearance during infection, and highlight the importance of the
341 environment for defining relevant interactions.

342

343 **Discussion**

344 Optimizing combination therapy is critical to improving TB treatment. While efficient
345 strategies for predicting and quantifying drug-drug interactions under *in vitro* conditions
346 have been developed, the impact of bacterial environment on these interactions has not
347 been determined (27, 28). As a result, it remains unclear how well these *in vitro* data
348 predict the effects of combination therapy in the infection environment. In this study, we
349 modeled drug-drug interactions using a newly developed genetic hypomorph library to

350 assess the impact of bacterial carbon metabolism on CGI. This work revealed that
351 synergies with distinct drugs are differentially sensitive to the environment, suggesting
352 tailored approaches to optimization.

353

354 While global analysis of our chemical-genetic-environmental dataset suggested that
355 drug mechanism and environment both play critical roles in shaping chemical-genetic
356 interactions, all drugs were not equally affected by condition. In particular, the individual
357 effect of the fluoroquinolone, MOX, was unaffected by carbon source, and this relative
358 indifference to the environment was also observed for CGI with MOX. These
359 observations contrast with RIF and INH, where carbon source plays an important role in
360 determining the MIC of the drug alone, as well as shaping chemical-genetic interaction
361 profiles. While we can only speculate on the mechanistic basis for these differences
362 between drugs, we found that the cholesterol-dependent decrease in RIF sensitivity is
363 due to the profound effect of carbon source on cell envelope structure that is likely to
364 alter RIF penetration. Similarly, the activation of the prodrug, INH, is affected by the
365 redox state of the cell (59), another process that is impacted by carbon catabolism. In
366 contrast, MOX is not a prodrug and we speculate that it may not be limited by cellular
367 penetration as strongly as RIF. More practically, these observations suggest that drug-
368 drug interactions with MOX may be particularly robust to changes in condition, and
369 therefore may translate well between *in vitro* and *in vivo* conditions.

370

371 While RIF synergies were complex and condition-dependent compared to MOX, we
372 found that the cholesterol-specific effects translate well to the infection environment and

373 may be particularly relevant to treatment. As reduced RIF exposure limits the efficacy of
374 this critical component of our standard regimen, even small increases in MIC during
375 infection could have a significant effect on bacterial killing. A number of observations
376 indicate that RIF efficacy is limited by similar mechanisms *in vivo* and in cholesterol-
377 containing media. Firstly, the increase in branched-chain lipid abundance that is
378 associated with reducing RIF efficacy in cholesterol- or propionate-containing media has
379 also been observed during infection, where similar carbon sources are utilized (16, 52).
380 Furthermore, genetic perturbation of the same cell wall synthetic enzymes sensitized
381 *Mtb* to RIF in both conditions. This effect may underlie the increased cell-associated RIF
382 upon simultaneous INH and ethambutol treatment *in vitro* (60). While the importance of
383 cell wall architecture and cell envelope lipid production suggests that cellular
384 permeability to RIF may be altered during growth on cholesterol, we note that lipid
385 synthesis also has important effects on central carbon metabolic pathways that could
386 influence antibiotic susceptibility (15). Additional enzymes identified in this study may
387 also be attractive targets for further drug development, such as AftB, a
388 arabinofuranosyltransferase distinct from EmbA and EmbB that are targets of
389 ethambutol (46). Similarly, enzymes in the DAP (diaminopimelic acid) biosynthesis
390 pathway produce precursors for the synthesis of peptidoglycan and lysine (61), and the
391 loss of either sensitizes the cell to RIF.

392

393 Using a chemical-genetic system, we determined that cellular environments can have a
394 profound effect on drug-drug interactions, and that even the use of relatively simple *in*
395 *vitro* culture conditions can identify synergies that are relevant during infection. These

396 studies provide a tractable system that can incorporate more complex culture systems,
397 or even cellular models, to identify additional *in vivo*-relevant synergies. Expanding this
398 CGI atlas promises to elucidate the processes limiting drug efficacy during infection and
399 guide drug development and regiment optimization efforts.

400

401 **Material and methods**

402 **Bacterial cultures**

403 *M. tuberculosis* (H37Rv) cells were cultured in Middlebrook 7H9 medium supplemented
404 with 10% oleic acid-albumin-dextrose-catalase (OADC), 0.5% glycerol, and 0.05%
405 Tween-80, or in minimal medium supplemented with 0.1% Tyloxapol and variable levels
406 of glycerol, acetate, butyrate, propionate, or cholesterol up to 0.1%. Minimal medium
407 was made as previously described but with ferric chloride (100uM) replacing ferric
408 ammonium citrate (50).

409 Cells were also cultured on 7H10 agar medium supplemented with 10% OADC and
410 0.5% glycerol. Streptomycin (20ug/mL) was supplemented when necessary.

411 Anhydrotetracycline (ATC, 500ng/mL) was supplemented periodically until cultures
412 reach exponential growth. ATC was removed from cultures prior to antibiotic growth
413 inhibition assays by washing in PBS supplemented with 0.1% Tyloxapol. *ΔphoPR*
414 mutant was generated as described previously (62).

415

416 **Multiplexed library screening**

417 Library pools of hypomorph mutants were prepared as described previously (32). Pools
418 were grown in minimal medium with 0.1% glycerol, 0.1% acetate, or 0.1% cholesterol

419 on 96-well plates for 2 weeks. Isoniazid and moxifloxacin were used at 1ug/mL and
420 rifampicin was used at 0.062 ug/mL. Antibiotics were subsequently serially diluted in a
421 2-fold manner. An untreated growth condition was included for each study. Samples
422 were inoculated at OD600 0.05 and growth was monitored using OD600. Upon
423 completion, 96-well plates were heat-inactivated at 85 degrees for 2 hours. Barcodes
424 were PCR amplified as described previously (32). Individual libraries were mixed with
425 1:1 20% DMSO and heated for 10 minutes at 98 degrees prior to PCR reaction.
426 Amplified barcodes were purified using SPRI-based purification methods and
427 sequenced using Next-generation sequencing methods. Sequence alignment and
428 analysis were conducted using Bowtie software package with index mismatch set to 2
429 bases and barcode mismatch set to 1 base. Relative abundance of every mutant was
430 calculated as mean ratio of barcode abundance of mutant relative to total barcode
431 abundance of library. Log₂FC of a mutant was calculated as the log₂ of the ratio of the
432 mean relative abundances in a given antibiotic condition relative to its untreated control.
433 Hierarchical clustering and principal component analysis were conducted using ClustVis
434 (63). Hierarchical clustering was applied to vectors of Log₂FC of each gene across all
435 conditions. PCA was performed on relative abundances across all conditions. Statistical
436 significance was determined by unpaired *t*-test with Benjamini-Hochberg multiple testing
437 correction.

438

439 **Antibiotic growth inhibition assay**

440 *Mtb* cells were grown in minimal medium with 0.1% glycerol, 0.01% acetate, 0.01%
441 butyrate, 0.01% propionate, or 0.01% cholesterol on 96-well plates. Isoniazid and

442 moxifloxacin were used at 1ug/ml and rifampicin was used at 0.062 ug/mL. Antibiotics
443 were subsequently serially diluted in a 2-fold manner. Untreated condition was included
444 for each study. Cells were inoculated at OD600 0.05 and growth was monitored using
445 OD600. Antibiotic efficacy was determined using growth rate inhibition as done
446 previously (24). The exponential growth constant (k) value was determined for all
447 conditions. The k -value of each antibiotic concentration was normalized to the k -value of
448 the no-drug control. The GR₅₀ value was defined as the concentration of antibiotic that
449 resulted in 50% decrease in growth rate. L-lysine (Sigma) was added to growth
450 conditions to final concentration of 3mM.

451

452 **Lipid extraction and mass spectrometry**

453 *Mtb* cells were grown in minimal medium with 0.01% butyrate, propionate or cholesterol.
454 Cells were inoculated at OD600 0.1 and grown to final OD600 of 0.7. Cells were
455 pelleted and heat-inactivated at 85 degrees for 45 minutes. Cells were washed in 10%
456 glycerol to remove residual detergents from growth media. Lipid extraction was
457 conducted with methyl tert-butyl ether (MTBE) as described previously (64). 1.5mL
458 methanol and 5mL MTBE were mixed with the cell pellet and placed into a glass tube
459 with a Teflon-lined cap. The mixture was incubated at room temperature for 5 hours
460 while shaking. Phase-separation was achieved by adding 0.75mL water to the tubes,
461 incubating for 10 minutes at room temperature, then centrifuging at 1000g for 10
462 minutes. The 2.5mL of the top phase was collected and transferred to a fresh glass tube
463 with Teflon-lined cap. Samples were dried using a nitrogen evaporator and stored in -20
464 degrees prior to injection to mass spectrometer. After evaporation of MTBE, 300 μ L of

465 2:1 methanol:chloroform was added to each tube followed by vigorous vortex.
466 Solubilized lipids were then transferred to a new, pre-weighed tube, dried using a
467 nitrogen evaporator, and then tubes were re-weighed to determine the lipid mass in
468 each sample. Then 1.5 μL of 100 ng/ μL N-omega-CD₃-octadecanoyl
469 monosialoganglioside GM₂ (Matreya, State College, PA) was added to each sample for
470 normalization and samples were reconstituted in 300 μL of 2:1 methanol: chloroform for
471 mass spectrometry analysis.
472 Samples were analyzed by direct infusion on a syringe pump to an Orbitrap Velos Pro
473 (Thermo Fisher Scientific, Waltham, MA) mass spectrometer operating in the negative
474 electrospray ionization mode. Mass spectra were acquired at a flow rate of 10 $\mu\text{L}/\text{min}$
475 for 2 minutes in two different mass ranges, m/z 200-2000 and m/z 300-3000, acquiring
476 two replicates for each range using a resolution of 30,000 (m/z 200), an AGC target
477 population of $5e^5$ and a maximum ion injection time of 100 ms. Data were analyzed in
478 Xcalibur 2.2 (Thermo Scientific). Briefly, mass spectra were averaged over the entire
479 acquisition range (0-2 minutes), producing one averaged spectrum for each analysis.
480 Peak lists were exported in Excel and intensity data was normalized to the intensity of
481 the GM2 ganglioside spike.

482

483 **Plasma pharmacokinetic analysis**

484 C57BL/6J mice were purchased from Jackson Laboratories. Housing and
485 experimentation were done in accordance with the guidelines set by the Department of
486 Animal Medicine of University of Massachusetts Medical School and Institutional Animal
487 Care and Use Committee and adhered to the laws of the United States and regulations

488 of the Department of Agriculture. Eight- to 12-week mice were administered 0.1g/L RIF
489 through drinking water for three days. Blood was collected during a 12-hour period 24
490 hours post RIF administration, kept on ice, centrifuged at approximately 2500 x g for 5
491 minutes. After centrifugation, plasma was collected and stored at -80°C until analysis.

492 Neat 1 mg/mL DMSO stocks for RIF were serial diluted in 50/50 acetonitrile/water to
493 create neat spiking stocks. Standards and quality control (QC) samples were created by
494 adding 10 µL of spiking stock to 90 µL of drug free plasma. Ten microliters of control,
495 standard, QC, or study sample were added to 100 µL of acetonitrile/methanol 50/50
496 protein precipitation solvent containing 20 ng/mL RIF-d8. Extracts were vortexed for 5
497 minutes and centrifuged at 4000 RPM for 5 minutes. 100 µL of supernatant was
498 combined with 5 µL of 75 mg/mL ascorbic acid to stabilize RIF. 100 µL of mixture was
499 combined with 100 µL of Milli-Q water prior to HPLC-MS/MS analysis. Mouse control
500 plasma (K2EDTA) was sourced from Bioreclamation. Mouse control lungs were
501 collected in house. RIF was sourced from Sigma Aldrich and RIF-d8 was purchased
502 from Toronto Research Chemicals.

503 LC-MS/MS analysis was performed on a Sciex Applied Biosystems Qtrap 6500+ triple-
504 quadrupole mass spectrometer coupled to a Shimadzu Nexera X2 UHPLC system to
505 quantify each drug in plasma. Chromatography was performed on a Agilent SB-C8
506 (2.1x30 mm; particle size, 3.5 µm) using a reverse phase gradient. Milli-Q deionized
507 water with 0.1% formic acid was used for the aqueous mobile phase and 0.1% formic
508 acid in acetonitrile for the organic mobile phase. Multiple-reaction monitoring of
509 precursor/product transitions in electrospray positive-ionization mode was used to
510 quantify the analytes. Sample analysis was accepted if the concentrations of the quality

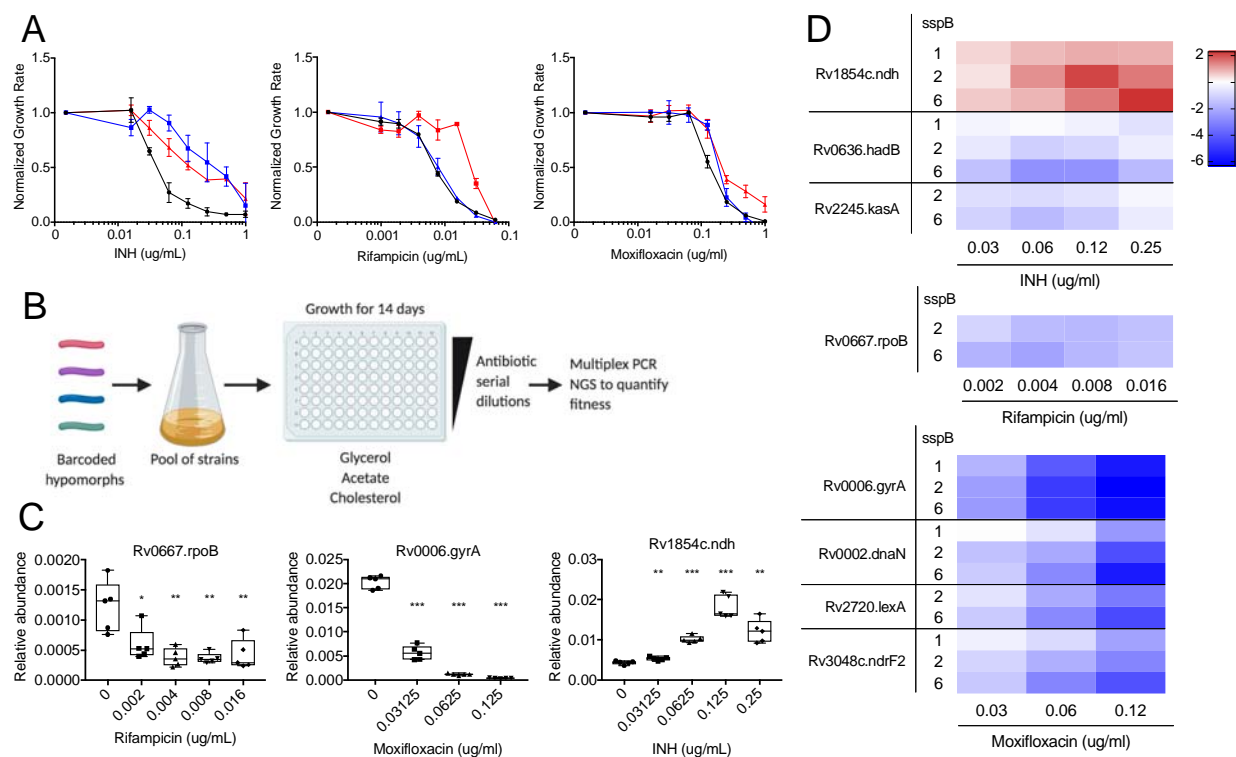
511 control samples were within 20% of the nominal concentration. The compounds were
512 ionized using ESI positive mode ionization and monitored using masses RIF
513 (823.50/791.60) and RIF-d8 (831.50/799.60). Data processing was performed using
514 Analyst software (version 1.6.2; Applied Biosystems Sciex).

515

516 ***In vivo* antibiotic susceptibility**

517 C57BL/6J mice were purchased from Jackson Laboratories. Housing and
518 experimentation were done in accordance with the guidelines set by the Department of
519 Animal Medicine of University of Massachusetts Medical School and Institutional Animal
520 Care and Use Committee and adhered to the laws of the United States and regulations
521 of the Department of Agriculture. Eight- to 12-week mice were infected with pools of
522 strains at equal ratios through the aerosol route (500 to 1000 CFU/mouse). Mice were
523 fed doxycycline-containing chow (Purina 5001 with 2000 ppm doxycycline, Research
524 Diets C11300-2000i) starting 3 days pre-infection to 21 days post-infection. At 21 days
525 post-infection, 0.1g/L rifampicin was administered through drinking water for 14 days. At
526 35 days post-infection, mice were sacrificed, spleen and lungs were isolated and
527 homogenized, and CFU was determined by plating dilutions on 7H10 agar with 50ug/mL
528 streptomycin and 500ng/mL ATC. For library recovery, approximately 1 million CFU per
529 mouse were plated on 7H10 agar with 50ug/mL streptomycin and 500ng/ml ATC.
530 Genomic DNA was extracted and normalized as done previously (34) and sequenced
531 using multiplex PCR methods. Sequence alignment and analysis were conducted using
532 Bowtie software package as described above. Relative abundance of every mutant was
533 calculated as mean ratio of barcode abundance of mutant relative to the average

534 barcode abundance of three barcoded H37Rv strains. Log₂FC of a mutant was
535 calculated as the log₂ of the ratio of the mean relative abundances of rifampicin treated
536 mice relative to the untreated mice. Statistical significance was determined by unpaired
537 *t*-test.
538
539



540

541 **Figure 1. Genetic strategy to define essential bacterial functions that alter drug**

542 **efficacy *in vitro*.** (A) Normalized growth inhibition of WT *Mtb* across increasing

543 concentrations of INH, RIF and MOX in minimal media with glycerol (black), acetate

544 (blue) and cholesterol (red) as sole carbon sources. Results shown as means from 3

545 biological replicates with standard deviations. (B) Barcoded hypomorph mutants were

546 pooled and grown in 96-well plates containing minimal media with glycerol, acetate or

547 cholesterol as the sole carbon source for 14 days. Antibiotics were added to individual

548 wells as well as untreated controls. Chromosomal barcodes were PCR amplified and

549 pooled for Illumina Next Generation Sequencing (NGS). Barcodes were analyzed to

550 quantify changes in fitness of individual strains from different conditions. (C) Boxplot

551 representing changes in relative abundances of *rpoB*, *gyrA* and *ndh* mutants during RIF,

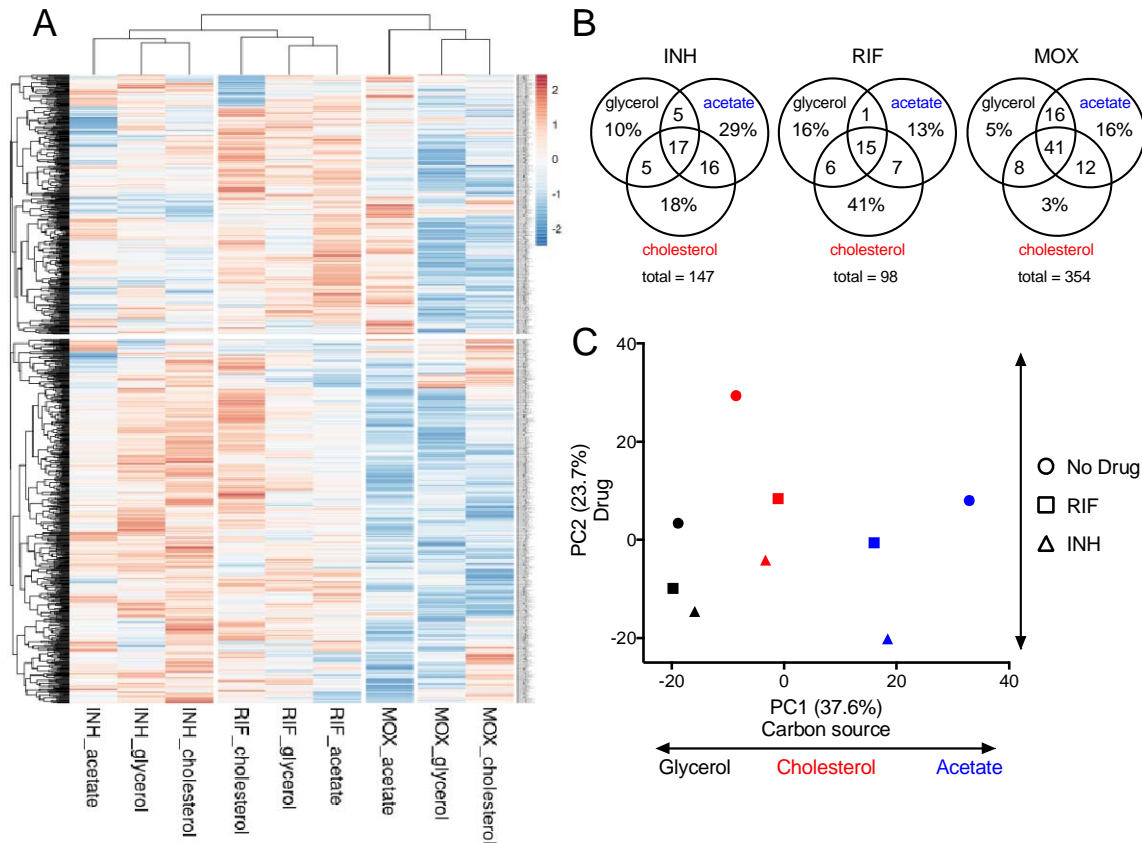
552 MOX and INH treatment respectively. Data represents 5 biological replicates.

553 Significance was calculated using unpaired t-test and compared to untreated conditions,

554 * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. **(D)** Heat map representing changes in fitness of
555 individual mutants, shown as Log2 fold change, during INH, RIF and MOX treatment in
556 glycerol growth conditions. Mutants were chosen from previous association with
557 respective antibiotic. *sspB* numbers denote *sspB* expression level of mutant. Results
558 shown as means from 5 biological replicates.

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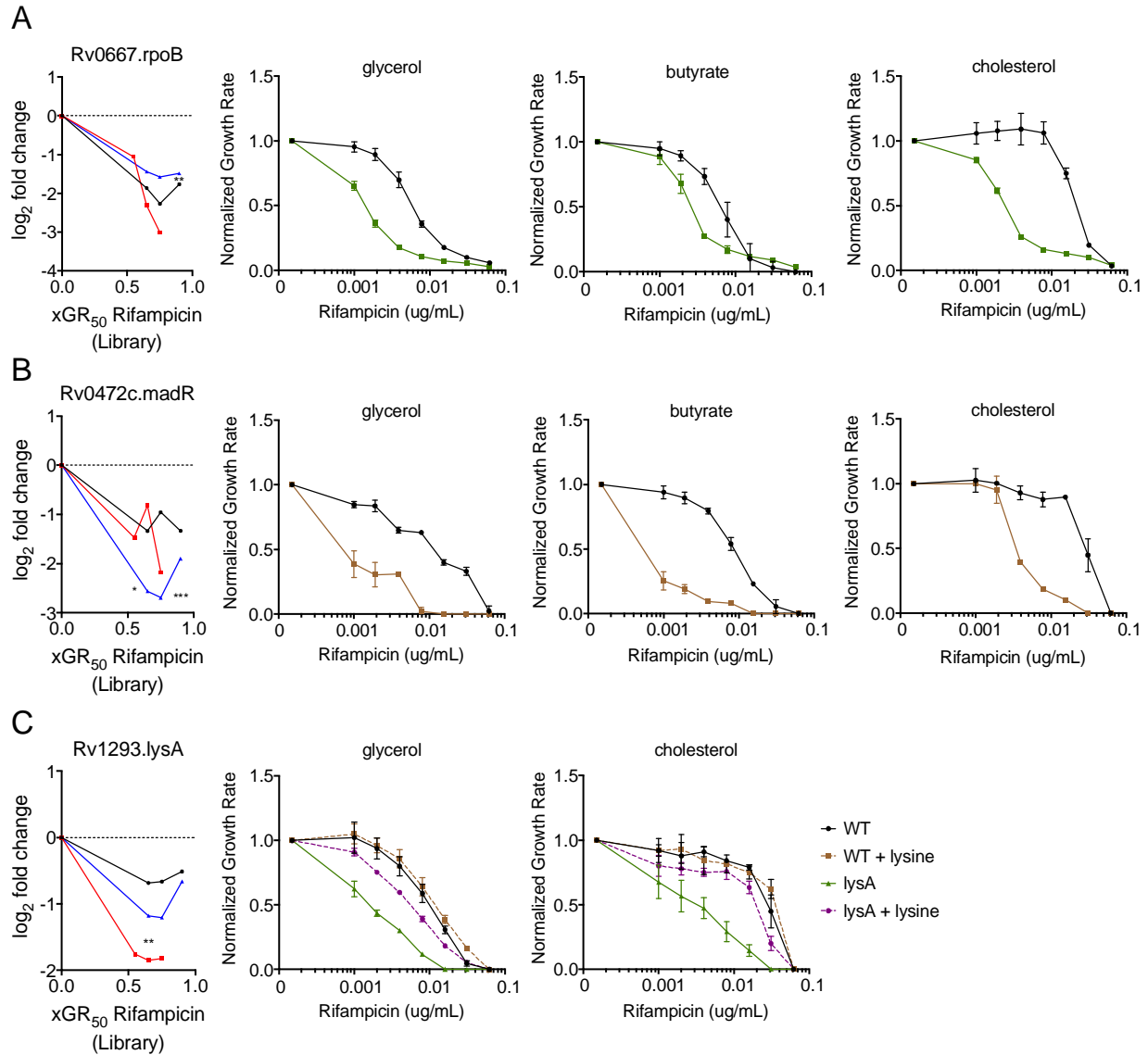


561
 562 **Figure 2. Carbon sources mediate antibiotic-genetic interactions of essential**
 563 **bacterial functions. (A)** Heat map of hypomorph mutants shown as Log₂ fold change
 564 of 0.65X GR₅₀ of INH, RIF and MOX against untreated controls. Results shown as
 565 means from 5 biological replicates. Each antibiotic (INH, RIF and MOX) in single carbon
 566 source (glycerol, acetate and cholesterol) conditions were compared using hierarchical
 567 clustering methods. **(B)** Venn diagrams showing the total number of genes with log₂FC
 568 > -1 and *p*<0.05 at 0.65X GR₅₀ of INH, RIF and MOX, and the % of genes associated
 569 with specific conditions. **(C)** Relative abundance datasets of the hypomorph libraries
 570 from glycerol (black), acetate (blue) and cholesterol (red) growth conditions with 0.65X
 571 GR₅₀ INH (triangle) and RIF (square) treatment as well as untreated controls (circle)

572 were examined using principal component analysis. Units shown on axes are arbitrary

573 values in principal component space.

574



575

576 **Figure 3. Condition-independent chemical-genetic synergies can be reproduced**

577 **with individual mutants. (A)** RpoB depletion shows decreased fitness compared to the

578 library over increasing concentrations of RIF for glycerol (black), acetate (blue) and

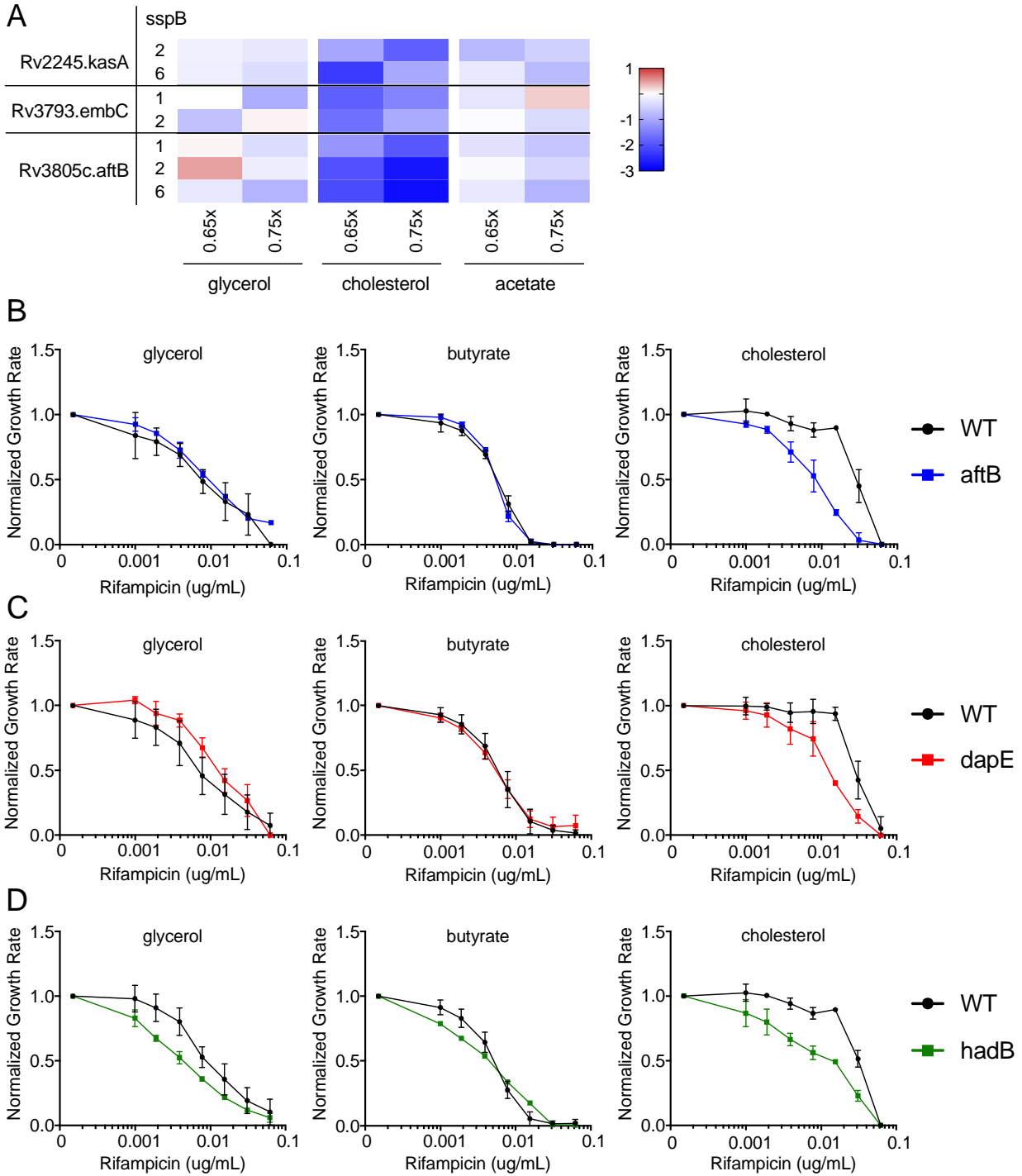
579 cholesterol (red). Normalized growth inhibition assay shows *rpoB* hypomorph mutant

580 (green) has decreased RIF MIC compared to WT (black) across multiple carbon

581 conditions. **(B)** MadR depletion shows decreased fitness compared to the library over

582 increasing concentrations of rifampicin for glycerol (black), acetate (blue) and

583 cholesterol (red). Normalized growth inhibition assay shows *madR* hypomorph mutant
584 (brown) has decreased RIF MIC compared to WT (black) across multiple carbon
585 conditions. **(C)** *LysA* depletion shows decreased fitness compared to the library over
586 increasing concentrations of rifampicin for glycerol (black), acetate (blue) and
587 cholesterol (red). Normalized growth inhibition assay shows *lysA* hypomorph mutant
588 (green) has decreased RIF MIC compared to WT (black), which can be reversed by
589 3mM lysine supplementation (purple). Library results are shown as means from 5
590 biological replicates, and significance was calculated using unpaired t-test with
591 Benjamini-Hochberg multiple testing correction, * $q < 0.05$, ** $q < 0.01$, *** $q < 0.001$.
592 Normalized growth inhibition results are shown as means from 3 biological replicates
593 with standard deviations.
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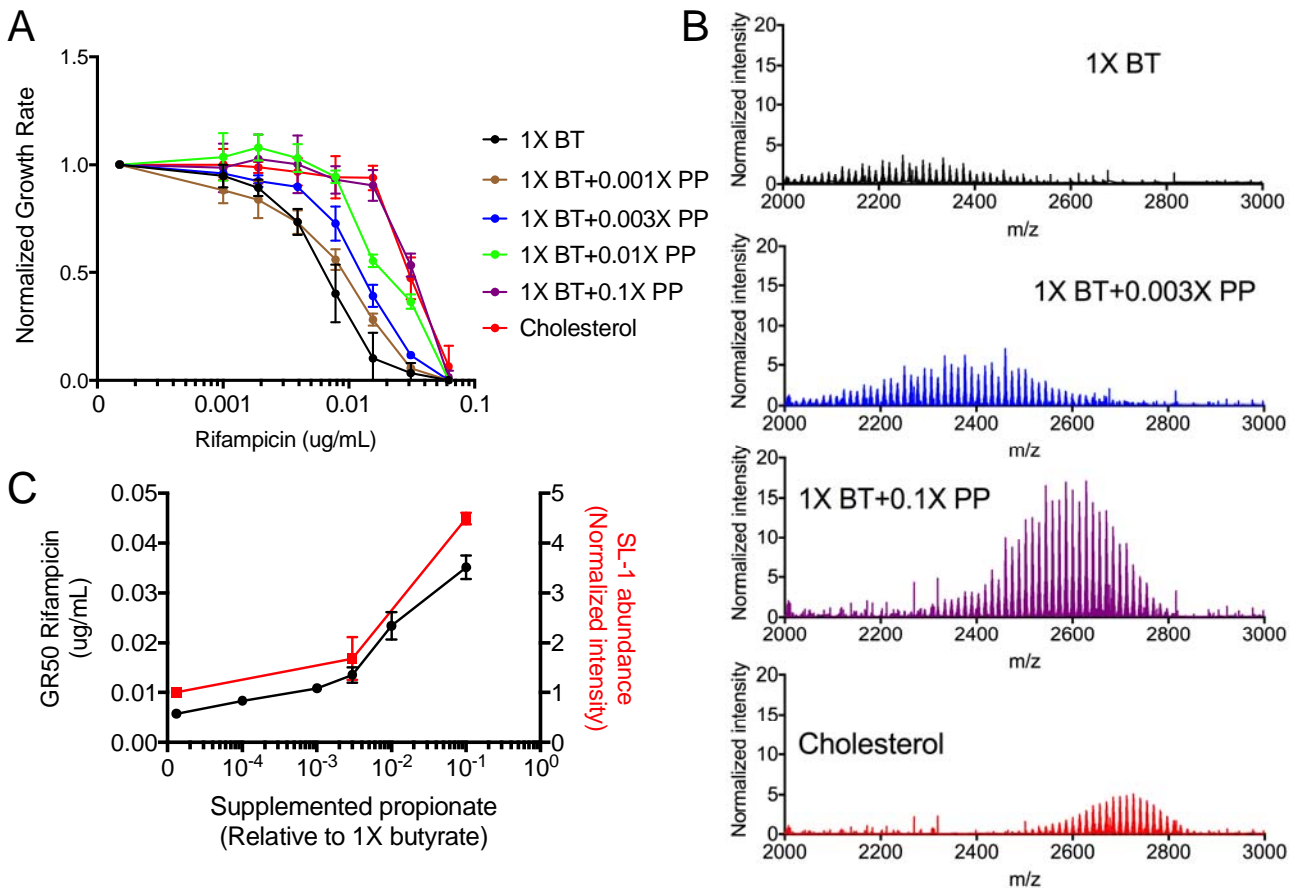
596

597 **Figure 4. Cell-wall biosynthesis hypomorph mutants show altered RIF efficacy**

598 **during cholesterol growth conditions. (A)** Heat map showing hypomorph mutants of

599 select mycobacterial cell-wall biosynthesis genes and pathways with significant changes

600 in fitness during cholesterol growth, shown as Log₂ fold change at 0.65X and 0.75X
601 GR₅₀ of RIF against untreated controls. Results shown as means from 5 biological
602 replicates. Classification of mutants was done using Mycobrowser. **(B-D)** Normalized
603 growth inhibition of WT and select mutants across increasing concentrations of RIF in
604 minimal media with sole carbon sources. Depletion of AftB (blue), DapE (red) and HadB
605 (green) show decreased RIF MIC compared to WT (black) only during cholesterol
606 growth conditions. Results shown as means from 3 biological replicates with standard
607 deviations.
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612 **Figure 5. Propionate and cholesterol catabolism decreases RIF efficacy in**

613 **correlation with increased branched chain lipid length and abundance. (A)**

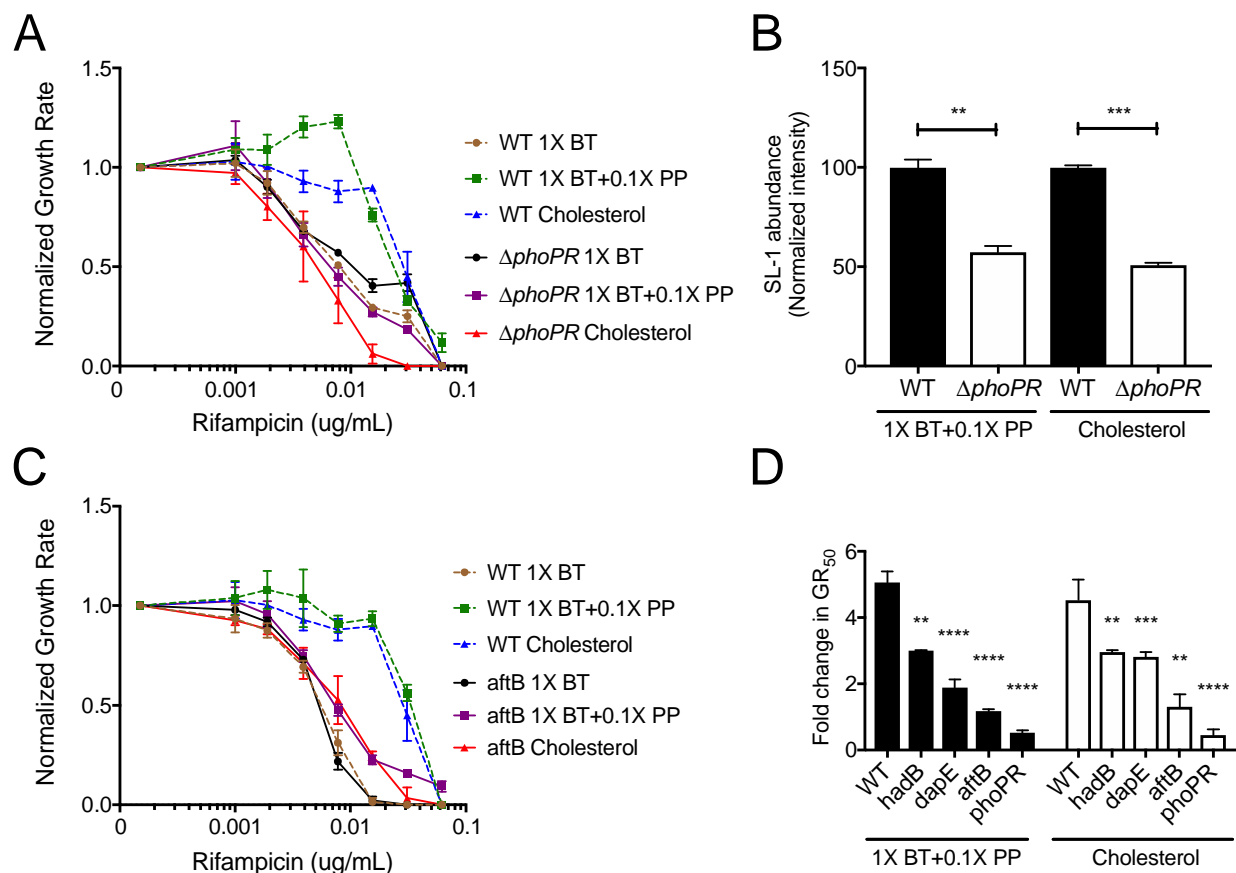
614 Normalized growth inhibition of WT across increasing concentrations of RIF in minimal
615 media with 1X butyrate (BT) and corresponding propionate (PP) supplementations as
616 well as cholesterol. Results shown as means from 3 biological replicates with standard
617 deviations. **(B)** Normalized mass spectra of extracted sulfolipids from WT grown in
618 minimal media with 1X butyrate (BT) and corresponding propionate (PP)
619 supplementations as well as cholesterol. MS spectra intensities were normalized using
620 GM2 ganglioside internal standard and cell density. Results shown as representative
621 from 2 biological and 2 technical replicates. **(C)** Plotted GR₅₀ values (black) and relative

622 sulfolipid abundances (red) across increasing propionate levels. Relative sulfolipid
623 abundances were determined by the combined normalized mass spectra intensities
624 from m/z 2000-2800 for each growth condition. Results shown as means with standard
625 deviations.

626

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630 **Figure 6. Disruption of the cell envelope reverses propionate and cholesterol**

631 **catabolism dependent decrease in RIF efficacy. (A)** Normalized growth inhibition of

632 $\Delta phoPR$ mutant across increasing concentrations of RIF in minimal media compared to

633 WT. Results shown as means from 3 biological replicates. **(B)** Relative sulfolipid

634 abundances of WT and $\Delta phoPR$ mutant grown in minimal media with 1X butyrate and

635 0.1X propionate (1X BT+0.1X PP), and cholesterol. Relative sulfolipid abundances were

636 determined by the combined normalized mass spectra intensities from *m/z* 2000-2800

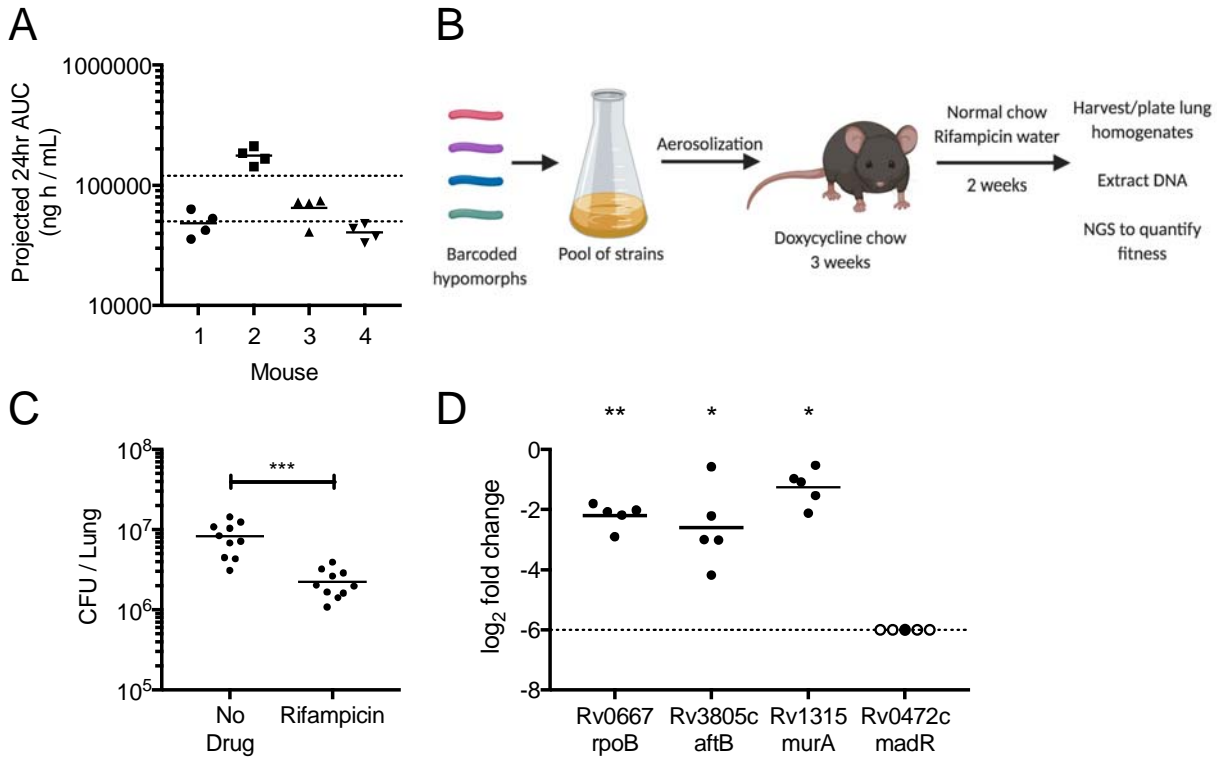
637 for each growth condition. MS spectra intensities were normalized using GM2

638 ganglioside internal standard and cell density. Results shown as means with standard

639 deviations from 2 biological and 2 technical replicates. Significance was calculated

640 using unpaired t-test, * $p < 0.05$, ** $p < 0.01$. **(C)** Normalized growth inhibition of *aftB* mutant

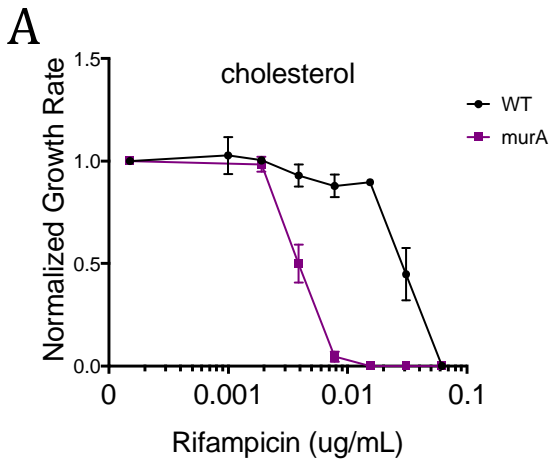
641 across increasing concentrations of RIF in minimal media compared to WT. Results
642 shown as means from 3 biological replicates. **(D)** Calculated fold change in GR₅₀ values
643 of 1X butyrate and 0.1X propionate (1X BT+0.1X PP) and cholesterol conditions against
644 1X butyrate alone for WT and mutant strains. Significance was calculated using
645 unpaired t-test, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.
646



647

648 **Figure 7. Essential bacterial functions that alter drug efficacy *in vivo*.** (A) RIF
649 plasma concentrations were measured in mice over 12-hour period 24 hours post RIF
650 administration (0.1g/L). Results shown as Area Under the Concentration (AUC) time
651 profile. Dotted lines indicate RIF plasma range observed during clinical TB therapy (57).
652 (B) C57BL/6J mice were infected through the aerosol route with a pooled culture of
653 individual hypomorph mutants and barcoded WT strains. Mice were fed doxycycline
654 chow starting 3 days before infection to 3 weeks post infection. Mice were subsequently
655 switched to normal chow and water with or without RIF (0.1g/L) for 2 additional weeks.
656 Lungs were harvested, homogenized and plated for *Mtb* outgrowth. Upon DNA
657 extraction of grown colonies, chromosomal barcodes were PCR amplified and pooled
658 for Illumina Next Generation Sequencing (NGS). Barcode abundances of individual
659 mutants were normalized to WT strains and analyzed to quantify changes in fitness

660 during RIF treatment. **(C)** RIF treated mice show significant decrease in *Mtb* CFU in
661 lungs compared to untreated controls. Each dot represents a single mouse. Significance
662 was calculated using unpaired t-test, *** $p < 0.001$. **(D)** Depletion of RpoB, AftB and MurA
663 show significant decrease in fitness, shown as Log₂ fold change, during rifampicin
664 treatment compared to untreated controls. Each dot represents a single mouse and
665 empty dot indicates that the relative abundance was below the limit of detection (Dotted
666 line). Significance was calculated using unpaired t-test, * $p < 0.05$, ** $p < 0.01$
667
668



669

670 **Supplementary Figure 1. MurA hypomorph mutant shows altered RIF efficacy**

671 **during cholesterol growth conditions. (A)** Normalized growth inhibition of WT and

672 MurA hypomorph mutant across increasing concentrations of RIF in minimal media with

673 cholesterol as the sole carbon source. Depletion of MurA (purple) show decreased RIF

674 MIC compared to WT (black) during cholesterol growth conditions. Results shown as

675 means from 3 biological replicates with standard deviations.

676

677 **Acknowledgements**

678 We are thankful to the members of the Sasseti lab for both technical assistance and
679 helpful discussion. We thank Sovie Lavalette-Levy and Curtis Engelhart for technical
680 help. This work was supported by the Office of the Assistant Secretary of Defense for
681 Health Affairs through the Peer Reviewed Medical Research Program, Focused
682 Program Award, under award no. W81XWH-17-1-0692. Opinions, interpretations,
683 conclusions, and recommendations are those of the author and are not necessarily
684 endorsed by the Department of Defense. The work was additionally supported by the
685 NIH (AI095208). Model figures were created with BioRender.com

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