CRISPR interference identifies vulnerable cellular pathways with bactericidal phenotypes in *Mycobacterium tuberculosis*.

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Abstract

Mycobacterium tuberculosis remains a leading cause of death for which new drugs are needed. The identification of drug targets has been advanced by high-throughput and targeted genetic deletion strategies. Each though has limitations including the inability to distinguish between levels of vulnerability, lethality and scalability as a molecular tool. Using mycobacterial CRISPR interference in combination with phenotypic screening we have overcome these individual issues to investigate essentiality, vulnerability and lethality for 96 target genes from a diverse array of cellular pathways, many of which are potential antibiotic targets. Essential genes involved in cell wall synthesis and central cellular functions were equally vulnerable and often had bactericidal consequences. Conversely, essential genes involved in metabolism, oxidative phosphorylation or amino acid synthesis were less vulnerable to inhibition and frequently bacteriostatic. In conclusion, this study provides novel insights into mycobacterial genetics and biology that will help to prioritise potential drug targets.

30 **Introduction**

31 Infections from *Mycobacterium tuberculosis* remain a leading cause of death[1]. *M.*
32 *tuberculosis* is currently treated with a combination of four drugs for six months.
33 However, the evolution and spread of drug resistance highlights a need for new drugs.
34 Novel drugs with unique molecular targets that are (i) essential for growth, (ii) are
35 highly vulnerable to inhibition (i.e. require low levels of inhibition to produce a bacterial
36 phenotype)[2-4] and (iii) are lethal when inhibited are likely to have the greatest impact
37 on reducing treatment times[5].

38 Transposon-saturation mutagenesis coupled to next-generation sequencing (i.e. Tn-
39 seq) provides a high-throughput assessment of fitness costs associated with gene
40 disruption. Tn-seq in *M. tuberculosis* has made significant contributions to the
41 identification of essential genes [6-8]. Despite this, Tn-seq does not distinguish between
42 bactericidal and bacteriostatic outcomes. Although improved statistical analysis has
43 allowed for the identification of differences in genetic requirements between clinical
44 isolates[9], there is also no standardized Tn-seq methodology to determine which genes
45 are more vulnerable to inhibition[10]. Dual-control (DUC) switches that combine
46 transcriptional repression and proteolysis of target genes overcomes issues
47 associated with Tn-seq[3]. DUC switches can also partially knockdown target genes to
48 generate hypomorphic strains for compound mechanism of action studies and for
49 investigations into pathway vulnerability[2-4]. Despite this, the construction of DUC
50 switches is resource intensive, prohibiting the parallel construction of DUC switches
51 for multiple genes of interest. Notable exceptions include the large collaborative efforts
52 of the PROSPECT screen[11].

53 Mycobacterial CRISPR interference (CRISPRi) is an alternative genetic platform that
54 transcriptionally represses target gene expression[12-15]. CRISPRi plasmids are easy to

55 construct as (i) they only require the cloning of only a 20 bp segment of
56 complementarity to target genes (i.e. sgRNA sequences) and (ii) require only a single
57 transformation as CRISPRi plasmids also express the necessary catalytically dead
58 Cas9 (i.e. dCas9) [13,15]. We and others have shown that CRISPRi can (i) probe gene
59 essentiality, (ii) provide information on the lethality and (iii) can generate partial
60 knockdown strains[12-16]. Using mycobacterial CRISPRi, this current study has
61 phenotypically screened target genes from a diverse spectrum of cellular pathways in
62 *M. tuberculosis* to investigate variations in gene essentiality, vulnerability and lethality.
63 These results demonstrate that the majority of screened genes involved in cell wall
64 synthesis and core cellular functions (e.g. transcription and translation) are essential,
65 equally vulnerable to inhibition and bactericidal when inhibited. Conversely, the
66 majority of essential genes involved in metabolism or amino acid synthesis were
67 subject to buffering effects, requiring higher levels of repression to inhibit growth.
68 Furthermore, the inhibition of several genes previously defined as non-essential
69 inhibited bacterial growth suggesting a delay between target inhibition and the
70 necessary metabolic remodelling to facilitate growth. In conclusion, this study will help
71 to prioritise potential drug targets as well as reiterate the utility of CRISPRi in
72 investigating mycobacterial genetics and physiology.
73

Results

Mycobacterial CRISPRi to assess genetic essentially: Using CRISPRi we investigated the phenotypes (i.e. growth and viability) of 96 genes (encoding 94 proteins and 2 ribosomal RNAs) from a diverse array of biological processes, many of which are considered potential drug targets. Mycobacterial CRISPRi uses a type I dCas9 from *Strepoccous thermophilus* that recognizes 15 permissible non-canonical PAM sequences to repress target gene expression[15]. Both sgRNA and dCas9 are expressed in response to the addition of anhydrotetracycline (ATc). There is a linear relationship between the level of ATc added and the level of transcriptional repression [13,16]. Two sgRNA sequences per target gene were selected and cloned (Table S1). Some genes have only a single sgRNA due to cloning difficulties. Bacterial phenotypes following target gene repression were screened in 96 well plates using an ATc gradient (Figure 1). OD_{600} was determined 7-days post the addition of ATc and genes were defined as either essential (i.e. <25% growth relative to no ATc control), growth impairing (i.e. <50% growth relative to no ATc control) or non-essential (i.e. >50% growth relative to no ATc control). Essentiality calls were compared with published Tn-seq experiments as available in the mycobrowser database[6]. CFUs were determined 5-days post the addition of ATc as previous work demonstrated that this allows for the detection of bacterial killing prior to the emergence of non-responsive CRISPRi mutants[13,16]. Essential or growth impairing sgRNAs that produced ≥1 $\log_{10}$ reduction in CFU/ml were defined as bactericidal, whilst <1 $\log_{10}$ reduction was bacteriostatic[13,16].

Cell wall synthesizing genes are essential and bactericidal when inhibited: The majority of sgRNAs targeting genes involved in cell wall synthesis in our screen were essential (Figure 2A). Essentiality and non-essentiality (e.g. *rv3032* and *treS*) calls are

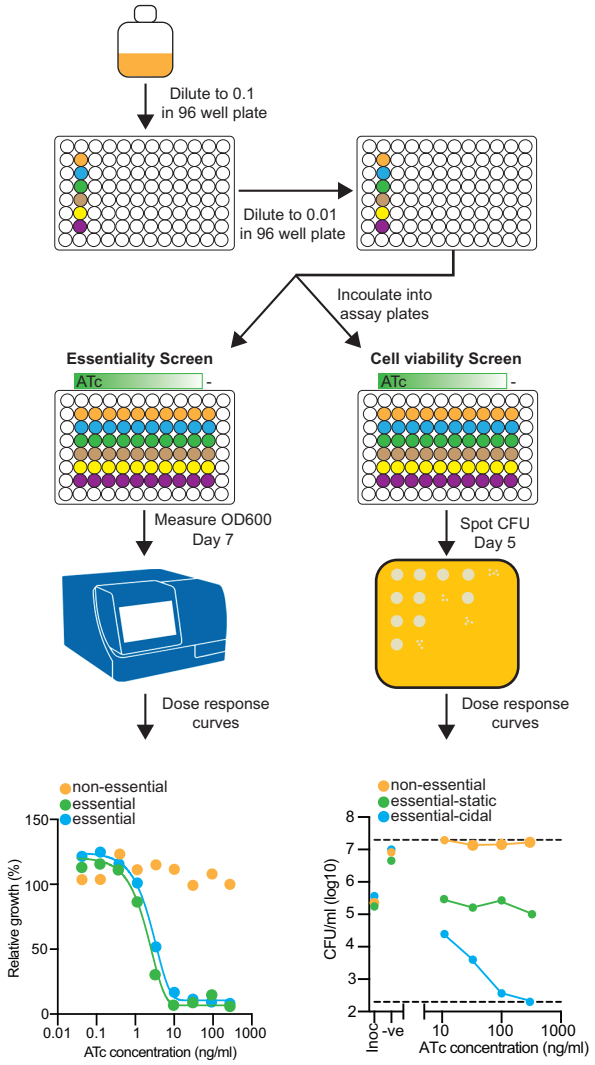

Figure 1: Experimental workflow for CRISPRi assessment of bacterial phenotypes. Cultures of *M. tuberculosis* mc^{2}6230 expressing CRISPRi plasmids that express a single sgRNA were maintained in 10 ml cultures. Cultures were diluted to an OD_{600} of 0.01 in a deep well 96 well plate and then inoculated into 96 well assay plates that contain a dilution gradient of ATc to induce expression of both the dCas9 and sgRNA at a starting OD_{600} of 0.005. Assays plates used to determine if sgRNAs inhibited bacterial growth were assessed after 7 days by determining the OD_{600} relative to a no ATc control. Plates used to determine bacterial viability were assessed after 5 days by spotting for CFUs. Previous work has demonstrated that day 5 allows for the detection of bacterial killing prior to the emergence of non-responsive CRISPRi mutants[13,16]. Further details for experimental workflow are described in the materials and methods

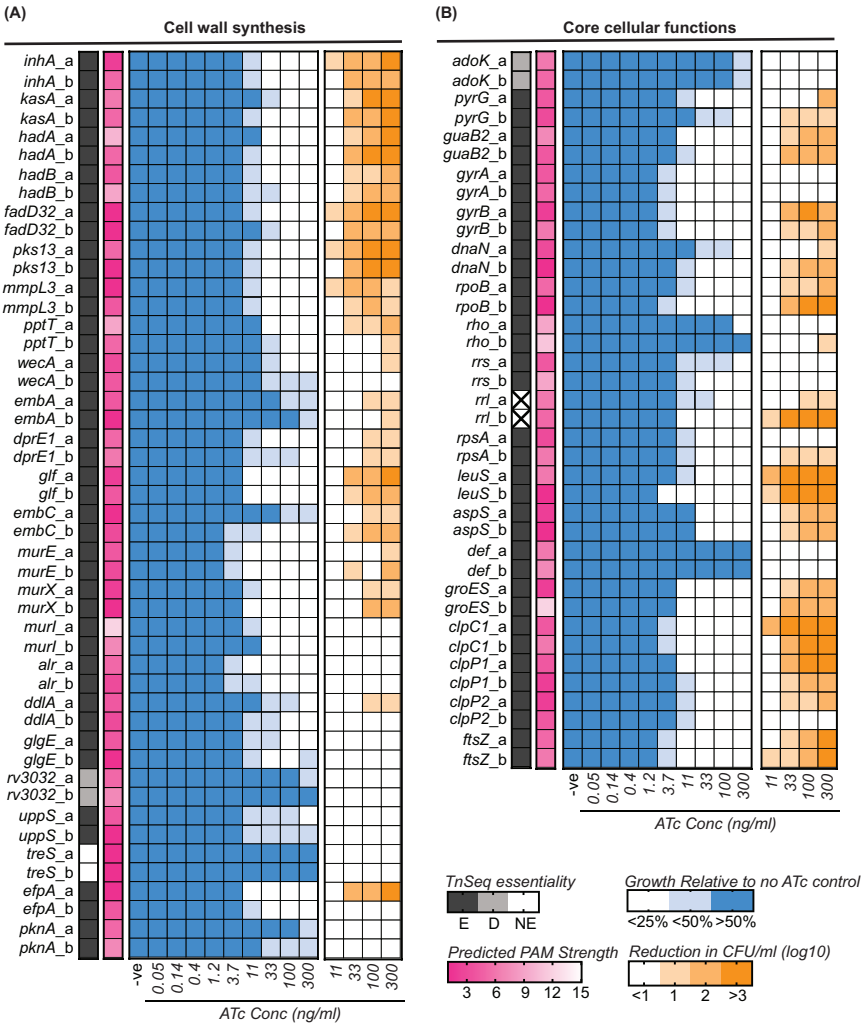

Figure 2: Bacterial phenotypes following the transcriptional inhibition of genes involved in cell wall synthesis or core cellular processes. (A and B) Each sgRNA is designated by the target gene name and whether it is the first or second designed sequence (i.e. a or b). Gray shaded heatmap describes the results from previous TnSeq experiments[6] as presented in the mycobrowser database (https://mycobrowser.epfl.ch/) and whether it is essential (E), non-essential (NE) or whether it has a growth defect (D). X denotes genes for which there is no essentiality call. Pink shaded heatmap describes the predicted strength of PAM scores ranked between 1-15 based on previous studies by Rock et al[15]. Blue shaded heatmap describes the level of growth inhibition at increasing ATc concentrations relative to a no ATc control well. The level of inhibition is the mean of at least four replicate experiments. Orange shaded heatmap describes the $\log_{10}$ reduction in CFU/ml for each sgRNA between day 0 and day 5 at increasing concentrations of ATc. The $\log_{10}$ reduction in CFU/ml of inhibition is the mean of at least four replicate experiments.

132 consistent with previous experiments (Figure 2A)[6,17,18]. All genes involved in mycolic
133 acid synthesis (i.e. *kasA*, *inhA*, *hadA/B, pkS13, fadD32*, *mmpL3 and pptT*) were
134 essential and bactericidal when inhibited (Figure 2A). The majority of mycolic acid
135 synthesis genes caused a >3 log reduction in CFU/ml at the maximum ATc
136 concentration (i.e. 300 ng/ml) (Figure 2A). Genes involved in arabinogalactan
137 synthesis (i.e. *wecA*, *embA*, *dprE1* and *glf*) had either an essential or growth impairing
138 phenotype (Figure 2A). Inhibition of *wecA*, *embA* and *dprE1* had a weak bactericidal
139 phenotype (i.e. 1 log reduction in CFU/ml) at ATc concentrations >100 ng/ml (Figure
140 2A). Inhibition of *glf* had a stronger bactericidal phenotype (i.e. >2 log reduction in
141 CFU/ml at lower ATc concentrations) (Figure 2A). All genes in peptidoglycan synthesis
142 (i.e. *murE*, *murX*, *murI*, *alr*, *ddlA*) were essential for growth (Figure 2A). The inhibition
143 of *murE*, *murX* and *ddlA* had a bactericidal phenotype, whilst *murI* and *alr* were
144 bacteriostatic (Figure 2A). With the exception of *glgE* that had an essential-
145 bacteriostatic phenotype, all genes involved in glucan biosynthesis (i.e. *rv3032* and
146 *treS*) were individually non-essential (Figure 2A). The predicted efflux pump, *efpA*, had
147 an essential-bactericidal phenotype, whilst inhibition of the serine/threonine protein
148 kinase *pknA* had a growth impairing phenotype (Figure 2A). In conclusion, genes
149 involved in cell wall synthesis are predominately essential for growth, although there
150 is variation in whether their inhibition is bactericidal.

151 **Core cellular processes are essential and have lethal phenotypes when**
152 **inhibited**: The majority of sgRNAs targeting genes involved in core cellular processes
153 (i.e. transcription, translation, nucleotide metabolism, protein homeostasis and cell
154 division) were essential and consistent with previous Tn-seq experiments[6] (Figure 2B).
155 With the exception of *adoK*, genes involved in nucleotide biosynthesis (i.e. *pyrG* and
156 *guaB2*) were essential-bactericidal (Figure 2B). Both *gyrA* and *gyrB* were individually

157 essential, yet only the inhibition of *gyrB* had a bactericidal phenotype (Figure 2B).
158 Inhibition of DNA polymerase (i.e. *dnaN*) and RNA polymerase (i.e. *rpoB*) were
159 essential and bactericidal, with the phenotypically strongest sgRNAs having a >2 log
160 reduction in CFU/ml. Inhibition of the transcriptional terminator, *rho*, inhibited bacterial
161 growth but only at the highest ATc concentration (Figure 2B). Ribosomal RNAs (i.e.
162 16S-*rrs* and 23S-*rrl*) were essential for bacterial growth, but only the inhibition of *rrl*
163 resulted in reduced bacterial viability. The ribosomal protein *rpsA* was also essential
164 for growth, with the phenotypically strongest sgRNA reducing bacterial viability.
165 Similarly the transcriptional inhibition of genes encoding tRNA synthetase (i.e. *aspS*
166 and *leuS*), genes involved in protein homeostasis (i.e. *groES, clpC1, clpP1* and *clpP2*)
167 and cell division (i.e. *ftsZ*) were essential and had strong bactericidal phenotypes
168 (Figure 2B). The peptide deformylase encoded by *def* that was previously defined as
169 essential failed to inhibit bacterial growth following transcriptional repression (Figure
170 2B). In conclusion, genes involved in central cellular processes are essential for the
171 growth of *M. tuberculosis*, and frequently have a bactericidal phenotype.

172 **Genes involved in metabolic process and oxidative phosphorylation are**
173 **generally bacteriostatic:** Transcriptional inhibition of genes involved metabolic
174 processes or oxidative phosphorylation that had previously been defined as essential
175 resulted in growth inhibition (i.e. *fba*, *glcB*, *folP1*, *folC*, *atpE* and *atpB*) (Figure 3A and
176 B). However, only the inhibition of *glcB* and *atpE/B* significantly reduced bacterial
177 viability (Figure 3A and B). Some sgRNAs targeting genes previously defined as
178 essential failed to inhibit bacterial growth (e.g. *fum*, *menA*, *menD* and *birA*), whilst only
179 the strongest sgRNA targeting *trxB2* was able impair bacterial growth (Figure 3A and
180 B). Furthermore, some sgRNA targeting genes previously defined as non-essential
181 inhibited bacterial growth in our study (i.e. *eno*, *dlaT*, *mqo*, *mdh*, *icl1*, *pckA*, *ndh*, *qcrB*,

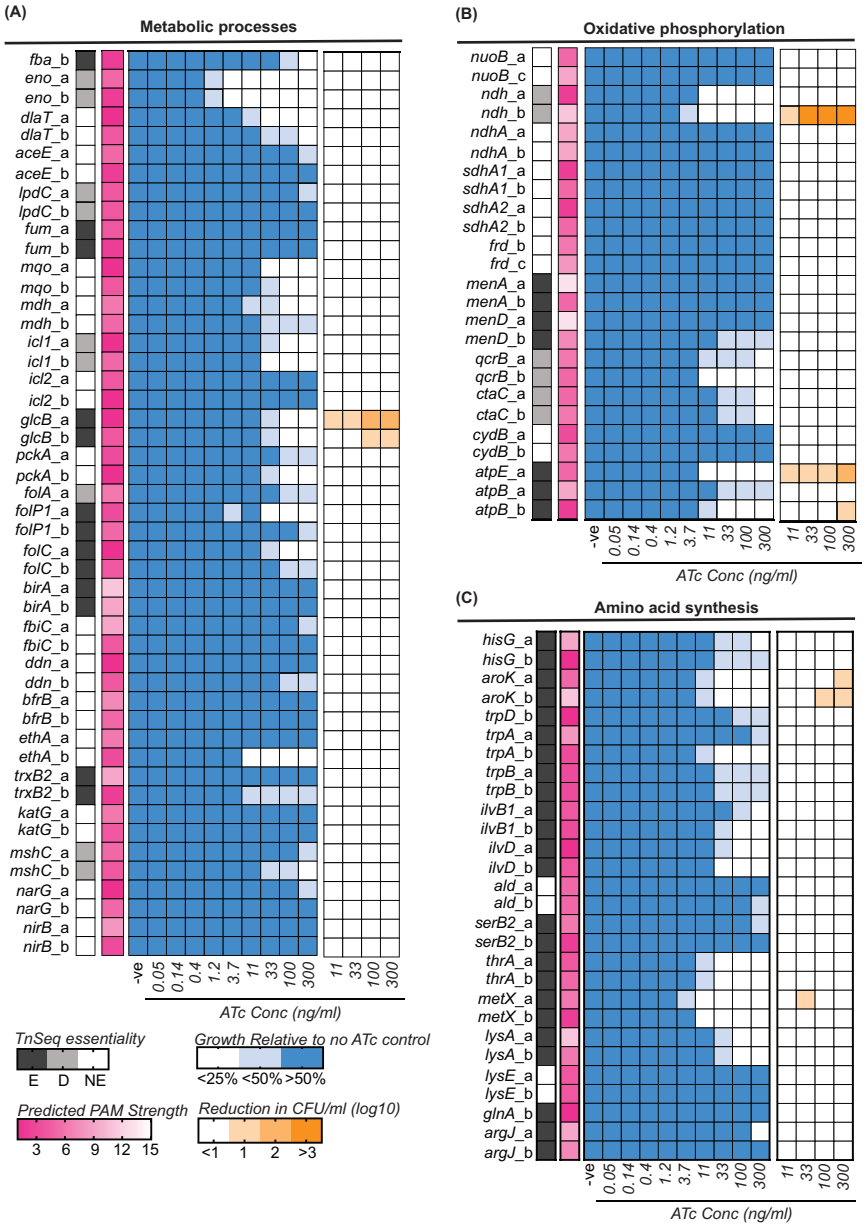

Figure 3: Bacterial phenotypes following the transcriptional inhibition of genes involved in metabolic processes, oxidative phosphorylation or amino acid synthesis. (A and B) Each sgRNA is designated by the target gene name and whether it is the first or second designed sequence (i.e. a or b). Gray shaded heatmap describes the results from previous TnSeq experiments[6] as presented in the mycobrowser database (https://mycobrowser.epfl.ch/) and whether it is essential (E), non-essential (NE) or whether it has a growth defect (D). Pink shaded heatmap describes the predicted strength of PAM scores ranked between 1-15 based on previous studies by Rock et al[15]. Blue shaded heatmap describes the level of growth inhibition at increasing ATc concentrations relative to a no ATc control well. The level of inhibition is the mean of at least four replicate experiments. Orange shaded heatmap describes the $\log_{10}$ reduction in CFU/ml for each sgRNA between day 0 and day 5 at increasing concentrations of ATc. The $\log_{10}$ reduction in CFU/ml of inhibition is the mean of at least four replicate experiments.

199 *ctaC* and *ethA*). Whilst the majority were bacteriostatic the strongest *ndh* sgRNA was
200 bactericidal (Figure 3A and B). In conclusion, the transcriptional inhibition of many
201 genes involved in metabolic processes or oxidative phosphorylation inhibit or impair
202 bacterial growth, but have a largely bacteriostatic phenotype.

203 **Genes involved in amino acid synthesis are essential but generally**
204 **bacteriostatic:** Transcriptional inhibition of genes involved in amino acid biosynthesis
205 generally inhibited bacterial growth (i.e. *hisG*, *aroK*, *trpD/A/B*, *ilvB1/D*, *thrA*, *metX*,
206 *lysA*). These essential phenotypes are consistent with previous Tn-seq experiments
207 (Figure 3C)[6]. The majority were bacteriostatic with only *aroK* being bactericidal.
208 Although the sgRNA *metX*_a reduced bacterial viability at 33 ng/ml ATc (i.e. 1.02 ±
209 0.92 $\log_{10}$ reduction), this phenotype was not observed across other concentrations or
210 sgRNAs (Figure 3C). Only a single *argJ* sgRNA was able to inhibit bacterial growth at
211 the highest ATc concentration, whilst sgRNAs targeting *serB2* and *glnA* failed to inhibit
212 bacterial growth (Figure 3C)[6]. Non-essentiality calls for *ald* and *lysE* are consistent
213 with previous experiments (Figure 3C)[6]. In conclusion, the transcriptional inhibition of
214 genes involved in amino acid synthesis generally inhibit bacterial growth, but have a
215 bacteriostatic phenotype.

216 **Comparison of sgRNA activity identifies pathways of altered vulnerability:** To
217 identify cellular pathways more vulnerable to inhibition (i.e. requiring less ATc to inhibit
218 bacterial growth) all sgRNAs were grouped according to biological function and the
219 ATc MIC of each active sgRNA (i.e. that inhibited bacterial growth) was compared. To
220 account for potential variation between experiments, the ATc MIC of sgRNAs was
221 compared to the ATc MIC of the *mmpL3*_a sgRNA that was used as a positive
222 knockdown control in all experiments (Figure 4B). The majority of sgRNAs targeting
223 cell wall synthesis or core cellular process were as active as *mmpL3*_a (i.e. within a

224 log2 change) (Figure 4A). Several sgRNAs targeting cell wall synthesis, core process
225 and metabolic genes were more active (i.e. >-1 log2 change), representing pathways
226 of potentially increased vulnerability (Figure 4A-B, red symbols). Interestingly, *eno*,
227 which converts 2-phosphoglycerate to phosphoenolpyruvate as part of glycolysis, was
228 the most vulnerable of all assessed sgRNAs (Figure 4A-B). A significant proportion of
229 sgRNAs targeting metabolism, oxidative phosphorylation and amino acid synthesis
230 genes were less active than *mmpL3*_a (i.e. >1 log2 change), representing biological
231 pathways of potentially reduced vulnerability (Figure 4A-B, blue symbols). Dose
232 response curves for five sgRNAs of either increased or reduced vulnerability highlight
233 this shift in ATc MIC (Figure 4B).
234

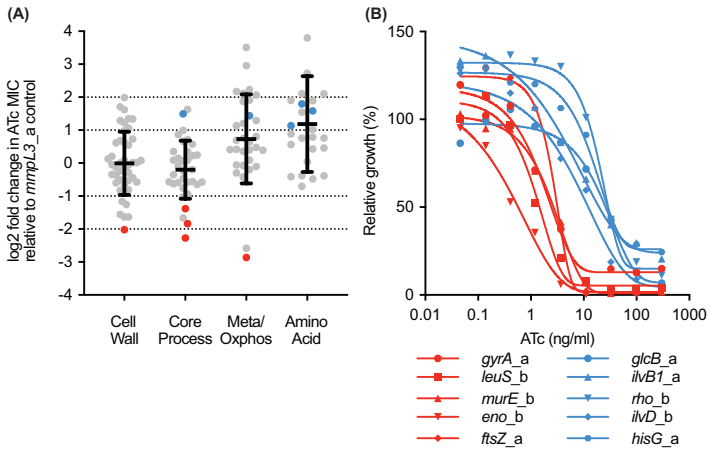

Figure 4: Comparison of target vulnerability across cellular pathways. (A) Plotted data is the average log2 fold change in ATc MIC compared to the *mmpL3*_a sgRNA for each sgRNA that results in at least a growth impairment (i.e. <50% relative to no ATc control) at the maximum ATc concentration. Data is the average fold change from at least two experiments that individually included biological duplicates (i.e. at least 4 replicates). sgRNAs are grouped according to their predicted or validated cellular function. Average sgRNA fold change values are presented in table S1. The average log2 fold change for a cellular pathway is represented by a solid black line. (B) Dose response curves for selected sgRNAs. Red and blue dots in A correspond to red and blue lines in B. Dose response curves are the average of at least 4 replicate experiments.

249 **Discussion**

250 There remains an urgent global need for novel therapeutic agents to treat both drug
251 susceptible and resistant strains of *M. tuberculosis*. Biological pathways that are
252 essential, highly vulnerable to inhibition and have a bactericidal outcome are likely to
253 have the greatest clinical impact. High-throughput and targeted genetic deletion
254 strategies have been useful in predicting gene essentiality. However, each strategy is
255 subject to individual limitations that include an inability to determine target
256 vulnerability, differentiation between lethal and static outcomes or issues with
257 scalability. By coupling CRISPRi to assessments of growth and viability this study
258 overcomes these individual limitations to identify variations in target vulnerability and
259 consequences on cellular viability for a diverse array of biological pathways.

260 There was a strong correlation between genetic essentiality and CRISPRi mediated
261 growth inhibition when targeting genes involved in cell wall synthesis or core cellular
262 processes. The majority of cell wall synthesis or core cellular encoding genes were
263 equally vulnerable to transcriptional inhibition and bactericidal. Genes involved in
264 mycolic acid synthesis, DNA metabolism, transcription, translation, tRNA synthesis,
265 protein homeostasis or cell division had the strongest bactericidal phenotype (i.e. both
266 level of reduction and levels of ATc needed to induce killing). Inhibition of
267 peptidoglycan and arabinogalactan biosynthesis had differing effects on cell viability
268 depending on the gene being targeted, with bactericidal phenotypes generally being
269 weaker. Due to the polar effects of CRISPRi[15,16] the repression of *murE* and *murX* is
270 likely to repress multiple downstream genes within the *dcw* operon (including, *murD*,
271 *murF*, *murG*, *ftsW* and *ftsQ*)[19]. Consequently, bactericidal phenotypes for *murE* and
272 *murX* may not be reflective of single target gene repression. Similarly, the polar effects
273 of CRISPRi suggest that transcriptional inhibition of both gyrase subunits, as would

274 be facilitated by the *gyrB* sgRNA on the *gyrBA* operon but not *gyrA* sgRNA, is needed
275 for lethality. Furthermore, both sgRNAs targeting *rho* have low PAM scores (i.e. 9 and
276 11), which may explain the bacteriostatic phenotype compared to previously published
277 bactericidal *rho*-DUC mutants[20]. Combined, the results of this study support the
278 continued development of novel drugs that target proteins involved in cell wall
279 synthesis and core cellular processes.

280 Genes involved in metabolic processes, oxidative phosphorylation or amino acid
281 synthesis, were on average less vulnerable to transcriptional inhibition. Genes that
282 inhibited bacterial growth were generally bacteriostatic, with the bactericidal
283 exceptions of *glcB* (i.e. malate synthase)[21], *atpE/B* (i.e. ATP synthase operon)[16], *aroK*
284 (i.e. shikimate biosynthesis) and *ndh* (i.e. NDH2 dehydrogenase). Recent studies have
285 demonstrated that the consequences of inhibiting individual metabolic genes when
286 using CRISPRi can be mitigated by metabolic buffering, to limit global effects on
287 bacterial metabolism[22]. Consequently, several cell divisions may be needed to deplete
288 enzyme products below critical levels. Previous studies in *E. coli* demonstrated that
289 metabolic buffering resulted in time dependent delays in inhibition of bacterial growth,
290 with only a small proportion of metabolic genes having rapid inhibitory phenotypes[22].
291 We hypothesize that metabolic buffering in *M. tuberculosis* may explain why many
292 predicted essential metabolic, oxidative phosphorylation or amino acid synthesis
293 genes fail to inhibit bacterial growth or have a significantly reduced vulnerability when
294 targeted by CRISPRi. Metabolic buffering may also explain why *metX*, *thrA* and *ilvB1*
295 were bacteriostatic, yet in previous studies deletion mutants had bactericidal
296 phenotypes [2,23,24]. Consistent with this, previous observations of *metX* and *thrA*
297 lethality are observed post 5-days, whilst our assay assessed viability at 5-days [2,23].

298 Media choice also plays an important role in the observation of phenotypes associated
299 with metabolic mutants [21,25,26]. Consequently, the use of OADC (i.e. glucose and oleic
300 acid as carbon sources) and exogenous pantothenic acid in our experiments to
301 supplement *M. tuberculosis* mc^{2}6230 (Δ*panCD*) may alter or mitigate the phenotypes
302 of some metabolic, oxidative phosphorylation or amino acid transcriptional
303 knockdowns. Furthermore, the lack of phenotype for sgRNAs targeting *birA* is likely
304 due to the presence of biotin in 7H9 media.

305 In our study, numerous previously defined non-essential genes had significant impacts
306 on bacterial growth. In contrast to the metabolic buffering that may mitigated the
307 phenotypes of essential genes, we hypothesize that a lack of metabolic buffering in
308 some cases may delay the necessary metabolic remodelling that is required to
309 facilitate growth in the absence of these non-essential genes. Consequently,
310 previously constructed knockouts or Tn-seq mutants of of *icl1*[27], *qcrB*[25], *ctaC*[25], *dlaT*[28]
311 and *ndh*[25] may reflect an adapted metabolic state that in our study are defined as
312 being essential for bacterial growth.

313 We acknowledge that variations in genetic vulnerability may be a result of variation in
314 CRISPRi transcriptional repression. It is also possible that differences in bacterial
315 phenotypes between sgRNAs (e.g. one static and one cidal) may be due to differences
316 in sgRNA efficacy. Previous studies have highlighted the influence of PAM sequence,
317 location of sgRNA within the genetic target and sgRNA sequence on dCas9
318 *Streptococcus pyogenes* activity[29-31]. Whilst we selected the strongest predicted PAM
319 sequence and sgRNA location does not influence activity of the dCas9$_{Sth1}$
320 orthologue[15], more work is required to determine the influence of sgRNA sequence on
321 dCas9$_{Sth1}$ activity. Off-target effects are also unlikely to be a confounding factor, as
322 previous work as suggested limited off-targeting for the dCas9$_{Sth1}$ orthologue[14].

323 Despite this, we speculate that variable levels of transcriptional repression are not a
324 confounding factor on our overall conclusions as CRISPRi studies in *E. coli* and *B.*
325 *subtilis* have demonstrated that genes involved in peptidoglycan synthesis and
326 translation are more vulnerable to inhibition than metabolic genes[31].

327 There is good correlation between the phenotypes of transcriptional inhibition and
328 many known antibiotics. For example, the bactericidal phenotype of isoniazid, a
329 cornerstone of current treatment regimens, correlates well with transcriptional
330 inhibition of its target *inhA*[32]. Interestingly, transcriptional inhibition of *rpoB* produced
331 a strong bactericidal phenotype, whilst rifampicin, a known inhibitor of RpoB, only
332 achieves bacterial killing when in significant excess of the MIC[32]. Whilst it is possible
333 that there are differences between the consequences of transcriptional and chemical
334 inhibition, these results suggest that rifampicin may be a poor inhibitor of RNA
335 polymerase *in vitro*. Combined, the results of our current study have provided novel
336 insights into the vulnerability and lethality of inhibiting different biological processes in
337 *M. tuberculosis*. These results will be a valuable resource for the mycobacterial
338 research community that will help to advance both basic biology and the advancement
339 of novel drug targets.
340

341 **Methods**

342 **Bacterial strains and growth conditions:** *Escherichia coli* MC1061 was grown in or
343 on luria broth (LB) media or agar (1.5%) at 37°C and shaking at 200 rpm when
344 required. *M. tuberculosis* strain mc^{2}6230 (Δ*panCD*, ΔRD1) [33-36], was grown and
345 maintained in 7H9 liquid media or on 7H11 solid media supplemented with OADC
346 (0.005% oleic acid, 0.5% bovine serum albumin, 0.2% dextrose, 0.085% catalase) and
347 pantothenic acid (25 µg/ml) and incubated at 37°C. Liquid cultures were supplemented
348 with 0.05% tyloxapol (Sigma) and grown with shaking at 140 rpm. *M. tuberculosis*
349 strain mc^{2}6230 is a BSL2 avirulent auxotroph that has been approved for use under
350 BSL2 containment at the University of Otago. When necessary media was
351 supplemented with Kanamycin at 50 µg/ml for *E. coil* and 25 µg/ml for *M. tuberculosis.*
352 Anhydrotetracycline (ATc, Sigma) was solubilized in 70% ethanol and added to
353 experiments at the stated concentrations.

354 **Construction and transformation of CRISPRi plasmids:** A 20-25 bp sequence
355 downstream of permissible PAM sequences targeting the non-template strand of
356 target genes of interest were identified[15]. For each target gene, the two sgRNAs based
357 on PAM score were manually selected. Target sequences were ordered as oligos with
358 GGGA and AAAC overhangs respectively (Table S1), and cloned into pJLR965 using
359 BsmB1 and golden gate cloning as previously described[13]. Plasmids were cloned into
360 *E. coli* and validated with sanger sequencing. Confirmed CRISPRi plasmids were
361 electroporated into *M. tuberculosis* strain mc^{2}6230 following previously established
362 protocols[13,16].

363 **CRISPRi phenotypic assessment of essentiality and viability:** To determine the
364 consequences of targeted gene repression on bacterial growth, phenotypic assays
365 were performed as follows (Figure 1). *M. tuberculosis* mc^{2}6230 strains containing

366 CRISPRi plasmids were grown and maintained in 7H9-supplemented media with KAN.
367 Cultures were diluted initially to an OD_{600} of 0.1 in 7H9-supplemented media (+KAN)
368 in a deep well 96 well plate. Cultures were diluted again to a final OD_{600} of 0.01 in a
369 deep well 96 well plate. 96 well assay plates were prepared with a 3-fold dilution of
370 ATc along the X-axis starting at 300 ng/ml of ATc in column 2 with a starting inoculum
371 of OD_{600} 0.005 (Figure 1). This was achieved by adding 50 µl of 7H9-supplemented
372 media (+KAN) to all wells of columns 3-11 except row A-H. 75 µl of 7H9-supplemented
373 media (+KAN) containing the starting concentration of ATc (i.e. 600 ng/ml ATc) was
374 added to column 2 except row A-H. ATc was diluted along the horizontal axis of the
375 96 well plate, transferring 25 µl between columns, down to column 10. Column 11 was
376 used as a no ATc control. Columns 1 and 12 and rows A-H contained 100 µl of 7H9-
377 supplemented media (+KAN) as contamination and background controls. Fifty µl of
378 culture adjusted to an OD_{600} 0.01 was added to wells 2-11 of individual rows of a 96
379 well plate to achieve a starting OD_{600} of 0.005. Each row represents the ATc dilution
380 gradient for a single *M. tuberculosis* $mc^2$6230 strain expressing a unique CRISPRi
381 plasmids. All experiments included a non-targeting sgRNA (i.e. pJR965) and a
382 *mmpL3*_a targeting sgRNA as a negative and positive essential-bactericidal control[13].

383 To assess gene essentially, duplicate plates were grown at 37°C without shaking for
384 7-days. OD_{600} was measured using a Varioskan-LUX microplate reader. The minimal
385 inhibitory concentration (MIC) of ATc was determined using OD_{600} reads from
386 duplicate plates relative to the growth of the no-ATc control, using a non-linear fitting
387 of data to the Gompertz equation[37]. Targeted genes were defined as either essential
388 (i.e. <25% growth relative to no ATc control), growth impairing (i.e. <50% growth
389 relative to no ATc control) or non-essential (i.e. >50% growth relative to no ATc
390 control). In this study, the vulnerability of genes to transcriptional inhibition is defined

391 by the ATc MIC, i.e. the lower the MIC the more vulnerable that gene is to CRISPRi
392 mediated transcriptional inhibition.

393 Duplicate 96 well assay plates were also set up to determine the consequences of
394 targeted gene repression on bacterial viability by taking colony forming units on day 0
395 and day 5. Previous work has demonstrated that day 5 allows for the detection of
396 bacterial killing prior to the emergence of non-responsive CRISPRi mutants[13,16].
397 Briefly, at day 0 hrs a 4-point ten-fold dilution of the 0.01 diluted culture was performed
398 in 7H9 base media (i.e. not supplemented), with 5 µl of each dilution spotted onto to
399 7H11-supplemented (+KAN) agar plates. At Day 5, approximately 100 µl of culture
400 was removed from columns 2-5 of each row and transferred to a new 96 well plate to
401 be diluted. A 4-point ten-fold dilution gradient was performed in 7H9 base media (i.e.
402 not supplemented), and 5 µl of each dilution was spotted onto to 7H11-supplemented
403 (+KAN) agar plates. Plates were incubated at 37°C for 4-5 weeks, at which point
404 colonies were counted. Essential genes with bacteriostatic consequences resulted in
405 no change in CFU/ml relative to 0 hrs, whilst bactericidal consequences produced at
406 least a 1 log reduction in CFU/ml relative to the day 0 inoculum.
407

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References

1 WHO. Global Tuberculosis Report 2018. (2018).

2 Hasenoehrl, E. J., Rae Sajorda, D., Berney-Meyer, L., Johnson, S., Tufariello, J. M., Fuhrer, T., Cook, G. M., Jacobs, W. R., Jr. & Berney, M. Derailing the aspartate pathway of *Mycobacterium tuberculosis* to eradicate persistent infection. *Nat Commun* 10, 4215, (2019).

3 Kim, J.-H., O'Brien, K. M., Sharma, R., Boshoff, H. I. M., Rehren, G., Chakraborty, S., Wallach, J. B., Monteleone, M., Wilson, D. J., Aldrich, C. C., Barry, C. E., Rhee, K. Y., Ehrt, S. & Schnappinger, D. A genetic strategy to identify targets for the development of drugs that prevent bacterial persistence. *Proceedings of the National Academy of Sciences of the United States of America* 110, 19095-19100, (2013).

4 Li, W., Obregon-Henao, A., Wallach, J. B., North, E. J., Lee, R. E., Gonzalez-Juarrero, M., Schnappinger, D. & Jackson, M. Therapeutic potential of the *Mycobacterium tuberculosis* mycolic acid transporter, MmpL3. *Antimicrobial agents and chemotherapy* 60, 5198-5207, (2016).

5 Wei, J. R., Krishnamoorthy, V., Murphy, K., Kim, J. H., Schnappinger, D., Alber, T., Sassetti, C. M., Rhee, K. Y. & Rubin, E. J. Depletion of antibiotic targets has widely varying effects on growth. *Proceedings of the National Academy of Sciences* 108, 4176-4181, (2011).

6 DeJesus, M. A., Gerrick, E. R., Xu, W., Park, S. W., Long, J. E., Boutte, C. C., Rubin, E. J., Schnappinger, D., Ehrt, S., Fortune, S. M., Sassetti, C. M. & Ioerger, R. Comprehensive Essentiality Analysis of the Mycobacterium tuberculosis Genome via Saturating Transposon Mutagenesis. *mBio* 8, 1-17, (2017).

7 Sassetti, C. M., Boyd, D. H. & Rubin, E. J. Genes required for mycobacterial growth defined by high density mutagenesis. *Molecular Microbiology* 48, 77-84, (2003).

8 Sassetti, C. M., Boyd, D. H. & Rubin, E. J. Comprehensive identification of conditionally essential genes in mycobacteria. *Proceedings of the National Academy of Sciences of the United States of America* 98, 12712-12717, (2001).

9 Carey, A. F., Rock, J. M., Krieger, I. V., Chase, M. R., Fernandez-Suarez, M., Gagneux, S., Sacchettini, J. C., Ioerger, T. R. & Fortune, S. M. TnSeq of *Mycobacterium tuberculosis* clinical isolates reveals strain-specific antibiotic liabilities. *PLOS Pathogens* 14, e1006939, (2018).

10 Rock, J. Tuberculosis drug discovery in the CRISPR era. *PLOS Pathogens* 15, (2019).

11 Johnson, E. O., LaVerriere, E., Office, E., Stanley, M., Meyer, E., Kawate, T., Gomez, J. E., Audette, R. E., Bandyopadhyay, N., Betancourt, N., Delano, K., Da Silva, I., Davis, J., Gallo, C., Gardner, M., Golas, A. J., Guinn, K. M., Kennedy, S., Korn, R., McConnell, J. A., Moss, C. E., Murphy, K. C., Nietupski, R. M., Papavinasasundaram, K. G., Pinkham, J. T., Pino, P. A., Proulx, M. K., Ruecker, N., Song, N., Thompson, M., Trujillo, C., Wakabayashi, S., Wallach, J. B., Watson, C., Ioerger, T. R., Lander, E. S., Hubbard, B. K., Serrano-Wu, M. H., Ehrt, S., Fitzgerald, M., Rubin, E. J., Sassetti, C. M., Schnappinger, D. & Hung, D. T. Large-scale chemical-genetics yields new *M. tuberculosis* inhibitor classes. *Nature* 571, 72-78, (2019).

12 de Wet, T. J., Winkler, K. R., Mhlanga, M., Mizrahi, V. & Warner, D. F. Arrayed CRISPRi and quantitative imaging describe the morphotypic landscape of essential mycobacterial genes. *Elife* 9, (2020).
13 McNeil, M. & Cook, G. Utilization of CRISPR interference to validate MmpL3 as a drug target in *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother* 63, (2019).
14 de Wet, T. J., Gobe, I., Mhlanga, M. M. & Warner, D. F. CRISPRi-Seq for the Identification and Characterisation of Essential Mycobacterial Genes and Transcriptional Units. *bioRvix*, (2018).
15 Rock, J. M., Hopkins, F. F., Chavez, A., Diallo, M., Chase, M. R., Gerrick, E. R., Pritchard, J. R., Church, G. M., Rubin, E. J., Sassetti, C. M., Schnappinger, D. & Fortune, S. M. Programmable transcriptional repression in mycobacteria using an orthogonal CRISPR interference platform. *Nature Microbiology* 2, 16274, (2017).
16 McNeil, M., Ryburn, H., Harold, L. K., Tirados, J. & Cook, G. Transcriptional Inhibition of the F1F0-Type ATP Synthase Has Bactericidal Consequences on the Viability of Mycobacteria. *Antimicrobial Agents and Chemotherapy* In press, (2020).
17 Stadthagen, G., Sambou, T., Guerin, M., Barilone, N., Boudou, F., Kordulakova, J., Charles, P., Alzari, P. M., Lemassu, A., Daffe, M., Puzo, G., Gicquel, B., Riviere, M. & Jackson, M. Genetic basis for the biosynthesis of methylglucose lipopolysaccharides in *Mycobacterium tuberculosis*. *J Biol Chem* 282, 27270-27276, (2007).
18 Lee, J. J., Lee, S. K., Song, N., Nathan, T. O., Swarts, B. M., Eum, S. Y., Ehrt, S., Cho, S. N. & Eoh, H. Transient drug-tolerance and permanent drug-resistance rely on the trehalose-catalytic shift in Mycobacterium tuberculosis. *Nat Commun* 10, 2928, (2019).
19 Munshi, T., Gupta, A., Evangelopoulos, D., Guzman, J. D., Gibbons, S., Keep, N. H. & Bhakta, S. Characterisation of ATP-dependent Mur ligases involved in the biogenesis of cell wall peptidoglycan in *Mycobacterium tuberculosis*. *PLoS One* 8, e60143, (2013).
20 Botella, L., Vaubourgeix, J., Livny, J. & Schnappinger, D. Depleting *Mycobacterium tuberculosis* of the transcription termination factor Rho causes pervasive transcription and rapid death. *Nature Communications* 8, (2017).
21 Puckett, S., Trujillo, C., Wang, Z., Eoh, H., Ioerger, T. R., Krieger, I., Sacchettini, J., Schnappinger, D., Rhee, K. Y. & Ehrt, S. Glyoxylate detoxification is an essential function of malate synthase required for carbon assimilation inMycobacterium tuberculosis. *Proceedings of the National Academy of Sciences* 114, E2225-E2232, (2017).
22 Donati, S., Kuntz, M., Pahl, V., Farke, N., Beuter, D., Glatter, T., Gomes-Filho, J. V., Randau, L., Wang, C. Y. & Link, H. Multi-omics Analysis of CRISPRi-Knockdowns Identifies Mechanisms that Buffer Decreases of Enzymes in E. coli Metabolism. *Cell Syst*, (2020).
23 Berney, M., Berney-Meyer, L., Wong, K.-W., Chen, B., Chen, M., Kim, J., Wang, J., Harris, D., Parkhill, J., Chan, J., Wang, F. & Jacobs, W. R. Essential roles of methionine and S-adenosylmethionine in the autarkic lifestyle of *Mycobacterium tuberculosis*. *Proceedings of the National Academy of Sciences*, 201513033, (2015).
24 Awasthy, D., Gaonkar, S., Shandil, R. K., Yadav, R., Bharath, S., Marcel, N., Subbulakshmi, V. & Sharma, U. Inactivation of the *ilvB1* gene in

Mycobacterium tuberculosis leads to branched-chain amino acid auxotrophy and attenuation of virulence in mice. *Microbiology (Reading)* 155, 2978-2987, (2009).
25 Beites, T., O'Brien, K., Tiwari, D., Engelhart, C. A., Walters, S., Andrews, J., Yang, H. J., Sutphen, M. L., Weiner, D. M., Dayao, E. K., Zimmerman, M., Prideaux, B., Desai, P. V., Masquelin, T., Via, L. E., Dartois, V., Boshoff, H. I., Barry, C. E., 3rd, Ehrt, S. & Schnappinger, D. Plasticity of the *Mycobacterium tuberculosis* respiratory chain and its impact on tuberculosis drug development. *Nat Commun* 10, 4970, (2019).
26 Ruecker, N., Jansen, R., Trujillo, C., Puckett, S., Jayachandran, P., Piroli, G. G., Frizzell, N., Molina, H., Rhee, K. Y. & Ehrt, S. Fumarase Deficiency Causes Protein and Metabolite Succination and Intoxicates Mycobacterium tuberculosis. *Cell Chem Biol* 24, 306-315, (2017).
27 Munoz-Elias, E. J. & McKinney, J. D. *Mycobacterium tuberculosis* isocitrate lyases 1 and 2 are jointly required for in vivo growth and virulence. *Nat Med* 11, 638-644, (2005).
28 Shi, S. & Ehrt, S. Dihydrolipoamide acyltransferase is critical for *Mycobacterium tuberculosis* pathogenesis. *Infect Immun* 74, 56-63, (2006).
29 Cui, L., Vigouroux, A., Rousset, F., Varet, H., Khanna, V. & Bikard, D. A CRISPRi screen in E. coli reveals sequence-specific toxicity of dCas9. *Nat Commun* 9, 1912, (2018).
30 Gasiunas, G., Young, J. K., Karvelis, T., Kazlauskas, D., Urbaitis, T., Jasnauskaite, M., Grusyte, M. M., Paulraj, S., Wang, P. H., Hou, Z., Dooley, S. K., Cigan, M., Alarcon, C., Chilcoat, N. D., Bigelyte, G., Curcuru, J. L., Mabuchi, M., Sun, Z., Fuchs, R. T., Schildkraut, E., Weigele, P. R., Jack, W. E., Robb, G. B., Venclovas, C. & Siksnys, V. A catalogue of biochemically diverse CRISPR-Cas9 orthologs. *Nat Commun* 11, 5512, (2020).
31 Hawkins, J. S., Silvis, M. R., Koo, B.-M., Peters, J. M., Osadnik, H., Jost, M., Hearne, C. C., Weissman, J. S., Todor, H. & Gross, C. A. Mismatch-CRISPRi Reveals the Co-varying Expression-Fitness Relationships of Essential Genes in Escherichia coli and Bacillus subtilis. *Cell Systems* 11, 523-535, (2020).
32 de Steenwinkel, J. E. M., de Knegt, G. J., ten Kate, M. T., van Belkum, A., Verbrugh, H. A., Kremer, K., van Soolingen, D. & Bakker-Woudenberg, I. A. J. M. Time-kill kinetics of anti-tuberculosis drugs, and emergence of resistance, in relation to metabolic activity of *Mycobacterium tuberculosis*. *Journal of Antimicrobial Chemotherapy* 65, 2582-2589, (2010).
33 Sambandamurthy, V. K., Wang, X., Chen, B., Russell, R. G., Derrick, S., Collins, F. M., Morris, S. L. & Jacobs, W. R., Jr. A pantothenate auxotroph of *Mycobacterium tuberculosis* is highly attenuated and protects mice against tuberculosis. *Nature Medicine* 8, 1171-1174, (2002).
34 Mayer, O., Jain, P., Weisbrod, T. R., Biro, D., Ho, L., Jacobs-Sera, D., Hatfull, G. F. & Jacobs, W. R., Jr. Fluorescent Reporter DS6A Mycobacteriophages Reveal Unique Variations in Infectibility and Phage Production in Mycobacteria. *J Bacteriol* 198, 3220-3232, (2016).
35 Dalecki, A. G., Haeili, M., Shah, S., Speer, A., Niederweis, M., Kutsch, O. & Wolschendorf, F. Disulfiram and Copper Ions Kill *Mycobacterium tuberculosis* in a Synergistic Manner. *Antimicrob Agents Chemother* 59, 4835-4844, (2015).
36 Jain, P., Hartman, T. E., Eisenberg, N., O'Donnell, M. R., Kriakov, J., Govender, K., Makume, M., Thaler, D. S., Hatfull, G. F., Sturm, A. W., Larsen,

M. H., Moodley, P. & Jacobs, W. R., Jr. phi(2)GFP10, a high-intensity fluorophage, enables detection and rapid drug susceptibility testing of *Mycobacterium tuberculosis* directly from sputum samples. *J Clin Microbiol* 50, 1362-1369, (2012).

37 Lamert, R. & Pearson, J. Susceptibility testing- accurate and reproducible minimum inhibitory concentration (MIC) and non-inhibitory concentration (NIC) values. *Journal of Applied Microbiology* 88, 784-790.