Programmable transcriptional repression in mycobacteria using an orthogonal CRISPR interference platform

Jeremy M. Rock¹, Forrest F. Hopkins¹, Alejandro Chavez^{2,3,4}, Marieme Diallo¹, Michael R. Chase¹, Elias R. Gerrick¹, Justin R. Pritchard¹, George M. Church^{2,4}, Eric J. Rubin¹, Christopher M. Sassetti⁵, Dirk Schnappinger⁶ and Sarah M. Fortune^{1,7*}

The development of new drug regimens that allow rapid, sterilizing treatment of tuberculosis has been limited by the complexity and time required for genetic manipulations in *Mycobacterium tuberculosis*. CRISPR interference (CRISPRi) promises to be a robust, easily engineered and scalable platform for regulated gene silencing. However, in *M. tuberculosis*, the existing *Streptococcus pyogenes* Cas9-based CRISPRi system is of limited utility because of relatively poor knockdown efficiency and proteotoxicity. To address these limitations, we screened eleven diverse Cas9 orthologues and identified four that are broadly functional for targeted gene knockdown in mycobacteria. The most efficacious of these proteins, the CRISPR1 Cas9 from *Streptococcus thermophilus* (dCas9_{Sth1}), typically achieves 20- to 100-fold knockdown of endogenous gene expression with minimal proteotoxicity. In contrast to other CRISPRi systems, dCas9_{Sth1}-mediated gene knockdown is robust when targeted far from the transcriptional start site, thereby allowing high-resolution dissection of gene function in the context of bacterial operons. We demonstrate the utility of this system by addressing persistent controversies regarding drug synergies in the mycobacterial folate biosynthesis pathway. We anticipate that the dCas9_{Sth1} CRISPRi system will have broad utility for functional genomics, genetic interaction mapping and drug-target profiling in *M. tuberculosis*.

he treatment of drug-susceptible Mycobacterium tuberculosis requires a regimen of four drugs taken for six months. One of the overarching goals of the M. tuberculosis research community is to develop a regimen of novel agents that can treat both drug-susceptible and drug-resistant M. tuberculosis in less than two weeks. This will necessitate the development of new genetic tools to identify drug target synergies that accelerate pathogen clearance while avoiding drug target antagonisms. Current genetic approaches for drug target characterization include promoter replacement and inducible protein degradation systems that allow the regulation of target protein levels over two orders of magnitude¹⁻³, but can take months to target a single gene. Transposon-sequencing (Tn-seq) allows the simultaneous assessment of hundreds of thousands of loss-of-function mutants, but as currently implemented in M. tuberculosis is not conditional and thus does not allow the manipulation of essential genes⁴. Furthermore, neither method provides a simple mechanism to modulate multiple genes simultaneously and thereby elucidate genetic interactions.

The re-purposing of the bacterial CRISPR-Cas (clustered regularly interspersed short palindromic repeats–CRISPR associated proteins) system as a site-specific transcriptional repressor could fill this methodological gap^{5–7}. CRISPR systems use small RNAs to target and cleave foreign nucleic acid in a sequence-specific manner^{8–11}. The best-characterized system is the type IIA system from *Streptococcus pyogenes*. Here, a single nuclease Cas9 and two small RNAs, a CRISPR RNA (crRNA) and a partially complementary *trans*-activating crRNA (tracrRNA), are necessary and sufficient for RNA-guided silencing of foreign DNA^{9,11,12}. This dual RNA system can be further simplified by the fusion of the crRNA and tracrRNA to generate a chimaeric single guide RNA (sgRNA)¹¹. Targeting specificity is determined both by Watson–Crick base pairing of the sgRNA and target DNA as well as a short DNA motif (protospacer adjacent motif, PAM) within the target DNA sequence^{8,13,14}. To repurpose Cas9 for transcriptional repression (CRISPR interference or CRISPRi), Cas9 nuclease activity is inactivated by point mutations within its HNH and RuvC nuclease domains^{5,7}. Co-expression of the nuclease-dead S. *pyogenes* Cas9 protein (dCas9_{Spy}) with an sgRNA designed with 20 nucleotides of complementarity to the target gene juxtaposed to an appropriate PAM yields specific silencing of the gene of interest (Fig. 1a)^{5,7}.

CRISPRi has been extensively characterized in the model bacteria *Escherichia coli* and *Bacillus subtilis*^{5,7,15}. In these organisms, the dCas9_{Spy}-sgRNA complex represses target gene transcription by either occluding RNA polymerase from the target promoter or by causing a steric block to transcription elongation (Fig. 1a). When targeting the promoter, sgRNAs specific for either the template or non-template strand can be used, whereas an elongation block is most effective when targeting the non-template strand, producing up to 300-fold repression^{5,7}. However, despite successes in *E. coli* and *B. subtilis*, the first attempts to introduce a similar

NATURE MICROBIOLOGY 2, 16274 (2017) | DOI: 10.1038/nmicrobiol.2016.274 | www.nature.com/naturemicrobiology

¹Department of Immunology and Infectious Diseases, Harvard T.H. Chan School of Public Health, Boston, Massachusetts 02115, USA. ²Wyss Institute for Biologically Inspired Engineering, Harvard University, Cambridge, Massachusetts 02138, USA. ³Department of Pathology, Massachusetts General Hospital, Boston, Massachusetts 02114, USA. ⁴Department of Genetics, Harvard Medical School, Boston, Massachusetts 02115, USA. ⁵Department of Microbiology and Physiological Systems, University of Massachusetts Medical School, Worcester, Massachusetts 01655, USA. ⁶Department of Microbiology and Immunology, Weill Cornell Medical College, New York, New York 10065, USA. ⁷The Ragon Institute of MGH, Harvard and MIT, Cambridge, Massachusetts 02139, USA. *e-mail: sfortune@hsph.harvard.edu

NATURE MICROBIOLOGY



Figure 1 | *In vivo* screen for functional Cas9 orthologues in mycobacteria. **a**, Schematic of CRISPRi-mediated transcriptional repression (adapted from ref. 15, Cell Press). Anhydrotetracycline (ATc)-inducible (P_{Tet}) *dcas*9 is directed to specific DNA targets by ATc-inducible or constitutively (P_{con}) expressed sgRNA, which prevents transcription initiation or elongation. RNAP, RNA polymerase. **b**, Phylogenetic tree of selected Cas9 orthologues. Listed are the Cas9 name abbreviations, size (amino acids) and sub-type classification. **c**, dCas9 orthologues show variable efficacy in mycobacteria. The *Renilla* reporter 5'UTR was engineered such that the optimal PAM for each dCas9 protein could be tested with an identical sgRNA targeting sequence (UTR1) for all dCas9 proteins. The *x* axis denotes each dCas9 protein tested and the *y* axis shows the ratio of the luminescence repression observed with the UTR1 targeting sgRNA normalized to the luminescence repression observed with a non-targeting control (ctrl) sgRNA. The fold inhibition is shown in white type for each column. **d**, sgRNAs targeting NGG PAMs were co-expressed with dCas9_{spy} (+ATc) and the effects on the *Renilla* target were measured by luciferase assay. Shown above the graph is a schematic depicting the *Renilla* gene (yellow arrow) and transcriptional start site (black arrow) and the relative targeting position of each sgRNA (P, promoter; UTR, 5' untranslated region; NT, *Renilla* non-template strand; RLU, relative light units). The Δ RL promoter sample depicts basal levels of luminescence e, sgRNAs targeting NNAGAAW PAMs were co-expressed with dCas9_{sth1} because of the less frequent PAM occurrence in the *Renilla* target. **f**, sgRNAs targeting NGGNG PAMs were co-expressed with dCas9_{sth3} (the *cas9* allele derived from the CRISPR3 locus; +ATc) and luminescence repression was measured as in **d**. Fewer sgRNAs were tested for dCas9_{sth3} (the cas9 allele derived from the CRISPR3 locus; +ATc) and luminescence repression was targeting



Figure 2 | dCas9_{Spy} sensitizes *M. smegmatis* to sublethal drug treatment. **a**, dCas9_{Spy} can mediate high-level knockdown of endogenous targets in *M. smegmatis.* sgRNAs targeting *dnaE1* (Ms3178) were co-expressed with dCas9_{Spy} or dCas9_{Sth1} (+ATc). Gene knockdown was quantified by qRT-PCR; the consequences of *dnaE1* knockdown were monitored by spotting dilutions of each culture on the indicated media. Error bars are 95% confidence intervals of three technical replicates. **b**, sgRNAs targeting *pptT* (Ms2648) in *M. smegmatis* were co-expressed with the indicated dCas9 protein (+ATc) and plated as in **a. c**, The indicated dCas9 proteins were haemagglutinin (HA)-tagged, co-expressed with a non-targeting control sgRNA (+ATc) and monitored by western blot. RpoB protein levels are shown as loading controls. *Background anti-HA cross-reactive band. **d**, dCas9_{Spy} sensitizes *M. smegmatis* to sub-minimum inhibitory concentration (sub-MIC) drug treatment. Non-targeting control sgRNAs were co-expressed with dCas9_{Spy} or dCas9_{Sth1} (+ATc); the consequences of dCas9 expression in the presence of sub-MIC concentrations of the indicated drugs were monitored by spotting serial dilutions of each culture as in **a**. Approximately 5,000 cells are deposited in the first spot and each subsequent spot is a tenfold serial dilution. AMK, amikacin; NAT, nourseothricin; STR, streptomycin; RIF, rifampicin. Data in **b-d** are representative of three independent experiments.

dCas9_{Spy}-based CRISPRi system in mycobacteria were less impressive, typically resulting in an approximately fourfold knockdown of gene expression^{16,17}.

Results

Limited utility of first-generation dCas9_{Spy} CRISPRi in mycobacteria. We sought to develop a CRISPRi system for targeted gene knockdown in mycobacteria. Given the efficacy of dCas9_{Spy} CRISPRi in model bacteria^{5,7,15}, we first attempted to build a similar dCas9_{Spy}-based system for mycobacteria. Although some degree of dCas9_{Spy} toxicity has been reported, dCas9_{Spy} is in general well tolerated in *E. coli*¹⁸. In contrast, high-level expression of dCas9_{Spy} with a non-genome targeting control sgRNA was lethal in *M. smegmatis*, and this lethality was not relieved by site-specific mutations that abrogate PAM interaction (Supplementary Fig. 1a)¹⁹. While lowering dCas9_{Spy} expression levels largely eliminated the growth defect, this first-generation dCas9_{Spy} CRISPRi system resulted in poor target gene knockdown (approximately three- to fourfold repression; Supplementary Fig. 1b,c), consistent with published data^{16,17}.

Identification of efficacious Cas9 alleles. While fourfold gene knockdown is sufficient to generate loss-of-function phenotypes for some mycobacterial genes, many genes appear to require higher-level knockdown to reveal a phenotype^{3,20–23}. Thus, we sought to develop a more efficacious CRISPRi system for mycobacteria. Given the extensive sequence diversity across the Cas9 family²⁴, we hypothesized that orthologous Cas9 proteins might function more robustly than dCas9_{Spy} in mycobacteria. We selected 11 diverse Cas9 proteins from the Type IIA and Type IIC

subfamilies based on their relatively small size (range 984–1,388 amino acids) and availability of data to predict crRNA, tracrRNA and PAM sequences (Fig. 1b and Supplementary Fig. 2)^{24–26}. We built single-plasmid platforms that expressed a mycobacterial codon-optimized nuclease-dead *dcas9* allele under the control of an anhydrotetracycline (ATc)-inducible promoter and the cognate sgRNA under the control of a strong, constitutive promoter. To evaluate the performance of each dCas9 protein, we developed a *Renilla* luciferase-based reporter assay. The *Renilla* 5′ untranslated region (UTR) was engineered such that the optimal PAM for each dCas9 protein could be tested with an identical sgRNA targeting sequence (UTR1) for all dCas9 proteins. Each dCas9 orthologue was then co-expressed with its cognate sgRNA (UTR1), and gene silencing was measured by luciferase assay.

We observed a wide range of gene silencing mediated by the dCas9 proteins (Fig. 1c). Of the 11 tested dCas9 proteins, only five produced greater than fivefold gene knockdown. The effective dCas9 proteins included all five Type IIA but none of the Type IIC proteins, which may reflect the recently described limited double-stranded DNA helicase activity of Type IIC Cas9 proteins²⁷. We next tested multiple Renilla-targeting sgRNA sequences to control for variation in sgRNA efficacy and, where relevant, variations in the PAM sequence. Although there was variability in the fold knockdown observed across different sgRNA sequences for each dCas9 protein, this more extensive evaluation broadly confirmed the results obtained with the UTR1 sgRNA (Fig. 1d-g and Supplementary Fig. 3). The dCas9 protein derived from the CRISPR1 locus of Streptococcus thermophilus²⁸ (dCas9_{Stb1}) produced by far the most robust and consistent gene silencing in our Renilla reporter system (Fig. 1c,e), although, interestingly, the expression



Figure 3 | *In vivo* identification of permissive PAM position variants for dCas9_{sth1}. The *Renilla* 5'UTR from Fig. 1c was modified to contain the indicated PAM sequences listed below the *x* axis. Each PAM contains a single-base mutation within the context of the consensus NNAGAAT dCas9_{sth1} PAM. dCas9_{sth1} was co-expressed with the UTR1 sgRNA and luminescence repression was measured as in Fig. 1c. Red and grey colouring depicts robust and weak *Renilla* target repression, respectively. Error bars are standard deviations of three technical replicates.

systems used in this second-generation platform improved even dCas9_{Spy} gene silencing relative to our first-generation system (Fig. 1d and Supplementary Fig. 1b,c).

dcas9_{sth1} enables robust gene knockdown in mycobacteria. We next extended our analysis of this second-generation CRISPRi platform by targeting endogenous *M. smegmatis* genes with dCas9_{Sth1} and, as a point of comparison, dCas9_{Spy}. We initially targeted two essential genes, *dnaE1* and *pptT* (refs 21,29). In both cases, dCas9_{Sth1} allowed robust target gene knockdown with resulting inhibition of bacterial growth (Fig. 2a,b). In the case of *dnaE1*, our second-generation dCas9_{Spy} platform was capable of achieving high-level knockdown of endogenous gene expression that was roughly comparable to levels seen with dCas9_{Sth1} (Fig. 2a). However, targeting *pptT*, which requires ~95% knockdown in *M. tuberculosis* in order to reveal a growth defect²¹, was successful with seven out of eight sgRNAs with dCas9_{Sth1} but only one out of eight sgRNAs with dCas9_{Spy} (Fig. 2b).

These results were surprising in light of two published reports in E. coli and human cells where Cas9_{Spy} is more efficacious than Cas9_{Sth1} (refs 26,30). Thus, we next sought to explore the basis for the relative underperformance of dCas9_{Spy} relative to dCas9_{Sth1} in mycobacteria. To determine if this difference in efficacy could be the result of differences in dCas9 expression, we performed western blots to monitor the expression of both dCas9 proteins. Despite induction from identical promoters and 5'UTRs, we consistently saw higher levels of full-length dCas9_{Sth1} than dCas9_{Spy} (Fig. 2c), although both proteins showed some degree of degradation. However, this relatively poor expression of dCas9_{Spv} was not phenotypically silent, because expression of dCas9_{Spy} sensitized cells to sub-minimum inhibitory concentrations (sub-MIC) of aminoglycosides and rifampicin, which cause proteotoxic stress by inducing mis-translation and inhibiting transcription, respectively (Fig. 2d). Thus, it appears that under ideal conditions (for example, defined medium at 37 °C), the second-generation dCas9_{Spy} platform can produce high-level gene knockdown, but it also sensitizes mycobacteria to stress, which is likely to affect both bacterial viability and gene knockdown levels under desired screening conditions. Such target-independent dCas9_{Spv} phenotypes could confound the identification of target-dependent phenotypes.

| , Suit | | |
|------------|-----------------|--------------------|
| PAM | Fold repression | Standard deviation |
| NNAGAAG | 216.7 | 10.0 |
| NNAGAAT | 216.2 | 10.4 |
| NNAGAAA | 158.1 | 22.8 |
| NNGGAAG | 145.2 | 5.3 |
| NNAGAAC | 120.5 | 7.9 |
| NNGGAAA | 110.5 | 26.4 |
| NNAGCAT | 84.6 | 5.2 |
| NNAGGAG | 82.2 | 9.2 |
| NNAGGAT | 64.7 | 8.7 |
| NNAGCAA | 53.4 | 9.9 |
| NNGGAAC | 51.5 | 6.2 |
| NNGGAAT | 47.3 | 3.3 |
| NNAGCAG | 42.2 | 7.0 |
| NNAGGAA | 38.5 | 5.2 |
| NNAGGAC | 25.5 | 0.8 |
| NNGGGAG | 24.7 | 1.9 |
| NNGGGAT | 24.2 | 3.4 |
| NNGGGAA | 12.3 | 0.8 |
| NNAGCAC | 11.9 | 1.2 |
| NNGGGAC | 7.9 | 1.0 |
| NNGGCAT | 6.7 | 0.9 |
| NNGGCAG | 4.0 | 0.3 |
| NNGGCAA | 3.3 | 0.3 |
| NNGGCAC | 2.7 | 0.3 |
| ctrl sgRNA | 1.3 | 0.1 |

Table 1 | Permissive PAM sequences for dCas9

The *Renilla* 5'UTR from Fig. 1c was modified to test all PAM sequence combinations from the permissive PAM position variants identified in Fig. 3. dCas9_{sth1} was then co-expressed with the UTR1 sgRNA and luminescence repression was measured as in Fig. 1c.

Identification of functional PAM variants for dCas9_{Sth1}. Given its robust performance, we chose to move forward with a dCas9_{Sth1}-based CRISPRi system. The published Cas9_{Sth1} PAM consensus sequence (NNAGAAW) allows limited targeting opportunities in GC-rich mycobacterial genomes^{13,14,24}. However, recent work has highlighted the ability of Cas9_{Sth1} to tolerate deviations from this consensus sequence^{26,31-33}. Thus, we defined the repertoire of PAMs available to dCas9_{Sth1} in vivo. Leveraging our Renilla reporter system, we created single-base mutations in Renilla 5'UTR within the context of the consensus the NNAGAAT PAM while maintaining the same UTR1 sgRNA sequence. We found that dCas9_{Sth1} functions with 'non-canonical' PAM sequences (Fig. 3), tolerating a G at position 3, G or C at position 4, and any base at position 7. Given that each possible permissive PAM position variant may not be functional with all other variants, we next tested all 24 possible PAM combinations (Table 1). These PAM variants produced a range of target knockdowns, spanning 2.7- to 216.7-fold. The more divergent the PAM sequence was from the consensus, the less well the PAM functioned in gene silencing (Table 1). By defining a cutoff score of >25-fold knockdown, this analysis increased the number of possible dCas9_{Sth1} PAMs from 2 to 15, thereby increasing the number of possible target sites in the M. tuberculosis genome from 4,624 to 83,091, or approximately one targetable site every 53 base pairs.

Parameters for silencing endogenous genes. We next sought to assess our $dCas9_{Sth1}$ system by silencing a larger panel of endogenous genes. To confirm the functionality of the alternative $dCas9_{Sth1}$ PAMs, we chose to benchmark our system by targeting the same genes investigated by Choudhary *et al.* which produced, on average, three- to fourfold knockdown with a first generation $dCas9_{Spy}$ CRISPRi system¹⁶. In our first attempts to target these essential genes with $dCas9_{Sth1}$, we found that for some sgRNAs, leaky expression of $dCas9_{Sth1}$ from the ATc-regulated promoter resulted in growth inhibition, even in the absence of CRISPRi induction. We therefore built and optimized a new set of ATc-regulated promoters that

minimize leaky expression without compromising high-level induction and used these to control expression of dCas9_{Sth1} and the sgRNA (Supplementary Fig. 4).

Consistent with our *Renilla* assay results, the optimized $dCas9_{Sth1}$ system produced robust knockdown (11.1- to 226-fold) of all five *M. smegmatis* genes tested (Fig. 4a–e). This high-level knockdown is quantitatively similar to that produced with CRISPR-based gene knockdown platforms in both *E. coli* and *B. subtilis*^{5–7,15}. The robust knockdown achieved with our system was sufficient to silence genes that had previously been difficult to target. For example, we found that *yidC* is indeed essential in *M. smegmatis*, as it is in *M. tuberculosis*, in contrast to published results achieved with a dCas9_{Spy} system (Fig. 4b)¹⁶. In addition, target mRNA knockdown kinetics were rapid, with near complete knockdown occurring in 1–2 h or less than one cell cycle after ATc addition (Supplementary Fig. 5a,b).

Given that the dCas9_{Sth1} system effectively silenced endogenous targets, we next sought to test its specificity. To do this, we tested the ability of the homologous M. tuberculosis dnaE1 gene²⁹ to complement the dCas9_{Sth1}-mediated silencing of the endogenous M. smegmatis dnaE1 gene (Fig. 4f). All three M. smegmatis dnaE1 targeting sgRNAs (sgRNA1-3) have perfect PAM matches to M. tuberculosis dnaE1 and either one (sgRNA1), two (sgRNA2) or four (sgRNA3) mismatches between the sgRNA targeting sequence and M. tuberculosis dnaE1. Our results indicate that as few as two mismatches between the sgRNA and the target are sufficient to abrogate phenotypic silencing (Fig. 4f)⁵. Thus, off-target silencing with the dCas9_{Stb1} system would require near-perfect sgRNA sequence complementarity, an appropriately spaced PAM, as well as requiring targeting of the promoter or non-template strand (Supplementary Fig. 5c), the combination of which is likely to be exceedingly rare in the context of compact bacterial genomes.

Gene silencing with CRISPRi in *M. tuberculosis.* Having developed this system in the model mycobacterium *M. smegmatis*, we then confirmed that our $dCas9_{Sth1}$ system was effective in *M. tuberculosis.* As in *M. smegmatis*, the use of optimized ATc-regulated promoters was essential to prevent leaky expression of CRISPRi from inhibiting bacterial growth (Supplementary Fig. 4). We designed sgRNAs to target eight essential genes in H37Rv (ref. 4), including genes known or predicted to require high-level gene knockdown in order to reveal a growth defect^{3,21}. Consistent with our results in *M. smegmatis*, dCas9_{Sth1}-mediated silencing prevented growth and produced robust gene knockdown for all genes and PAM variants tested (Fig. 4g and Supplementary Fig. 5d). Moreover, our dCas9_{Sth1} system reached maximal mRNA knockdown within one to two cell divisions after the addition of ATc (Supplementary Fig. 5e).

Interestingly, in both M. smegmatis and M. tuberculosis, dCas9_{Stb1} achieved high-level gene knockdown when targeted far from the predicted transcriptional start site (TSS); for example, yidC is predicted to be the fourth gene in an operon in M. tuberculosis and shows a syntenic operon structure in M. smegmatis³⁴. This high-level silencing was somewhat unexpected, given published observations describing an inverse correlation between repression efficacy and sgRNA targeting site distance from the TSS (refs 5,35). To explore this relationship, we quantified knockdown for 26 sgRNAs tiled along the pptT gene operon in M. smegmatis and 60 sgRNAs tiled along the groES-groEL1 operon or dnaE1 gene in *M. tuberculosis*³⁴. First, these results further confirmed that the permissive PAM variants identified in our Renilla assay (Table 1) were also functional in the context of endogenous gene targeting in both M. smegmatis and M. tuberculosis (Fig. 4h,i, Supplementary Fig. 5f and Supplementary Table 2). Second, in contrast to published work with dCas9_{Spy}, we observed no correlation between repression efficacy and the sgRNA targeting site distance from the TSS (Fig. 4h,i and Supplementary Fig. 5f)⁵. However, consistent with published work, CRISPRi is polar: any operonic gene downstream of the dCas9 binding site will be silenced in addition to the targeted gene (Fig. 4h,i)⁵. In addition to a 'downstream' polar effect, an 'upstream' polar effect of CRISPRi was also recently reported in *B. subtilis*, in which the targeting of a downstream gene in an operon represses the transcription of the upstream genes¹⁵. To query for this effect, we quantified the expression level of the upstream gene in the *pptT* and *groES-groEL1* operon targeting sgRNAs (Fig. 4h,i). Knockdown of the upstream gene occurred only when the sgRNAs were targeted within or very close to the 3' end of the upstream gene coding sequence (Supplementary Fig. 5g,h).

Identification of synergistic interactions in the folate biosynthesis pathway. Finally, we tested the ability of our optimized CRISPRi system to interrogate genetic and chemical interactions in an essential metabolic pathway (Fig. 5a). In both prokaryotes and eukaryotes, reduced folate species serve as essential cofactors in the transfer of one-carbon groups in pathways for the synthesis of DNA, RNA and protein. Although mammals lack the de novo folate synthesis pathway, many microbes are unable to acquire folates from their environment and rely entirely on de novo folate biosynthesis³⁶. The dichotomy in essentiality of this pathway makes it an ideal target for the development of antibiotics. Two such antifolate antibiotics, trimethoprim (TMP) and sulfamethoxazole (SMX), have been used clinically for 40 years. TMP and SMX inhibit successive steps of the folate synthesis pathway (Fig. 5a). SMX inhibits dihydropteroate synthase FolP1, which catalyses the addition of dihydropterin diphosphate to p-aminobenzoic acid. The product of FolP1, 7,8-dihydropteroate, reacts with glutamate in a FolC-catalysed reaction to form dihydrofolate. Dihydrofolate is then reduced to tetrahydrofolate by the dihydrofolate reductase FolA, the target of TMP. The combination of SMX and TMP has been demonstrated to be synergistic in many bacteria³⁷. However, the efficacy of TMP, SMX and whether or not these drugs act synergistically is controversial in mycobacteria. Initially, M. tuberculosis was reported to be resistant to the combination of TMP+SMX (ref. 37). Consistent with this, TMP is a relatively weak in vitro inhibitor of M. tuberculosis FolA (ref. 38). However, subsequent studies demonstrated that the combination of TMP and SMX is effective against *M. tuberculosis*³⁹. One study evaluated the synergy between these two drugs and found that sub-MIC concentrations of SMX sensitized cells to TMP (ref. 40); in contrast, a parallel study found that SMX alone was responsible for the observed M. tuberculosis inhibition, with no improvement by the addition of TMP (ref. 41).

Elucidation of the mycobacterial folate synthesis pathway has been hampered by the inability to generate conditional loss-of-function alleles of folate biosynthesis genes. Previous attempts to target FolA with an inducible protein degradation system achieved ~97% knockdown, but no significant reduction in growth rate³. We therefore first sought to determine whether our optimized CRISPRi system would be robust enough to block growth when targeting folate biosynthesis genes. We designed a panel of sgRNAs against *folP1, folC* and *folA*. Due to the operonic nature of these genes, the targeting of *folP1* is also expected to knockdown the expression of *folB* and *folK* (Supplementary Fig. 6). Strikingly, we identified sgRNAs for all three genes that produced knockdown of sufficient magnitude to inhibit growth, consistent with these genes being essential in *M. smegmatis* (Fig. 5b).

To investigate genetic synergy within this pathway, we tuned gene knockdown by using 'weaker' $dCas9_{Sth1}$ PAM variants to generate 'hypomorphic' sgRNAs, which could then be combined to assess pairwise knockdown phenotypes. These hypomorphic sgRNAs cause partial knockdown of their targeted genes but not

NATURE MICROBIOLOGY



Figure 4 | dCas9_{Sth1} **CRISPRi is highly active against endogenous genes in mycobacteria. a-e**, dCas9_{Sth1} achieves high-level knockdown of endogenous targets in *M. smegmatis.* sgRNAs targeting the indicated genes were co-expressed with dCas9_{Sth1} (+ATc). Gene knockdown was quantified and visualized as in Fig. 2a. Error bars are 95% confidence intervals of three technical replicates. **f**, dCas9_{Sth1}-mediated target knockdown is specific. Three sgRNAs targeting *M. smegmatis dnaE1* (Ms3178) were co-expressed with dCas9_{Sth1} (+ATc) in wild-type *M. smegmatis* strains or strains complemented with *M. tuberculosis dnaE1* (Rv1547). All three *M. smegmatis dnaE1* targeting sgRNAs have perfect PAM matches to *M. tuberculosis dnaE1* and either one, two or four mismatches between the sgRNA targeting sequence and *M. tuberculosis dnaE1*, as indicated to the right of each strain. Positions of the sgRNA-*M. tuberculosis dnaE1* mismatches (position 1 is closest to the target PAM): sgRNA1 (5), sgRNA2 (6,21), sgRNA3 (7,9,15,19). Strains were spotted as in Fig. 2d. n/a, not applicable. **g**, dCas9_{Sth1} achieves high-level knockdown of endogenous targets in *M. tuberculosis*. sgRNAs targeting the indicated genes were co-expressed with dCas9_{Sth1} (+ATc). Gene knockdown was quantified as in Fig. 2a (fold knockdown ± 95% confidence interval is shown beneath the +ATc plates). The consequences of gene knockdown were monitored by plating dilutions of each culture on the indicated media. Data shown in **a-g** are representative of three independent experiments. **h**, dCas9_{Sth1}. Gene knockdown far from the transcriptional start site (TSS) in *M. smegmatis*. sgRNAs targeting the *pptT* operon were co-expressed with dCas9_{Sth1}. Gene knockdown of *pptT* was quantified as in Fig. 2a. The first two genes of the *pptT* gene operon are depicted at scale below the graph; the black bar beneath the *pptT* gene marks the site of the qPCR amplicon. **i**, dCas9_{Sth1} achieves robust target knockdown far from the transcriptional start site o



Figure 5 | **Functional profiling of the mycobacterial folate synthesis pathway. a**, Schematic of mycobacterial folate metabolism. The targeted genes (*folP1* Ms6103; *folC*, incorrectly annotated as pseudogene in *M. smegmatis*; and *folA* Ms2671) and the inhibitors of those genes (SMX, sulfamethoxazole; TMP, trimethoprim) are shown by coloured text. GTP, guanosine-5'-triphosphate; p.tase, phosphatase; DHPPP, 7,8-dihydropterin pyrophosphate; pABA, *para*-aminobenzoic acid; H₂Pte, dihydropteroate; DHF, dihydrofolate; THF, tetrahydrofolate. **b**, dCas9_{Sth1}-induced growth inhibition due to knockdown of folate pathway targets. sgRNAs targeting the indicated genes were co-expressed with dCas9_{Sth1} (+ATc) and spotted as in Fig. 2d. **c**, Multiplexed targeting reveals synthetic lethal interactions in the folate pathway. The consequences of partial knockdown of folate pathway targets, individually or in combination, were monitored by co-expressing 'hypomorphic' sgRNAs targeting the indicated genes and dCas9_{Sth1} (+ATc). Strains were spotted as in Fig. 2d. **d**, Partial knockdown of folate pathway targets sensitizes cells to TMP and SMX. sgRNAs from Fig. 5c were co-expressed with dCas9_{Sth1} (+ATc) and spotted on plates containing sub-MIC concentrations of TMP or SMX. Data shown in **b-d** are representative of three independent experiments.

to levels high enough to completely inhibit growth (Fig. 5c). Whereas *folA*, *folP1* and *folC* hypomorphic sgRNAs induced moderate growth defects individually, they were synthetic lethal when co-expressed (Fig. 5c). These genetic data suggest that knockdown of these targets should sensitize cells to TMP and SMX. Consistent with this hypothesis, partial knockdown of all three targets sensitized *M. smegmatis* to TMP and SMX (Fig. 5d). Thus, CRISPRi-mediated silencing demonstrates that drugs targeting successive steps within the mycobacterial folate biosynthesis pathway (*folP1, folC* and *folA*) can interact to produce a synergistic antimicrobial effect.

Discussion

We report here an optimized CRISPRi platform for mycobacteria and believe that this system presents a number of advantages relative to more conventional methods of gene perturbation. First, our system represents the simplest and fastest method for programmable gene regulation in mycobacteria. With our single-plasmid platform, all that is required for gene knockdown is the cloning of a unique ~20 bp targeting region into the sgRNA scaffold. Second, CRISPRi is scalable. With advances in array-based oligo synthesis, generating large pools of oligos that contain unique ~20 bp targeting regions is fast and inexpensive. Third, unlike complementary methods like Tn-seq, CRISPRi is inducible, thereby allowing the facile manipulation of essential genes. Fourth, the magnitude of CRISPRi knockdown is tunable, either by varying targeted PAM 'strength' in the case of dCas9_{Sth1} (Table 1 and Fig. 5c,d), varying the concentration of the small-molecule CRISPRi inducer¹⁵, modulating the degree of sgRNA-target complementarity⁵, or truncating the sgRNA targeting region⁵. Finally, CRISPRi can be multiplexed, which is of particular importance in slow-growing pathogens like *M. tuberculosis* in which serial genetic manipulation is prohibitively time consuming.

The primary disadvantage of CRISPRi is the polar effect—any operonic gene downstream of the dCas9 binding site will be silenced in addition to the targeted gene (Fig. 4h,i)⁵. The fact that genes in an operon typically function in the same biological pathway partially mitigates this problem. Moreover, the fact that dCas9_{Sth1} can mediate robust gene knockdown irrespective of distance from the TSS (Fig. 4h,i and Supplementary Fig. 5f) with a minimal upstream polar effect (Supplementary Fig. 5g,h) means that dCas9_{Sth1} CRISPRi can be used to target deep into bacterial operons and thereby increase the resolution of phenotyping.

In addition to facilitating the study of *M. tuberculosis* biology, our $dCas9_{Sth1}$ CRISPRi system will have broad utility in *M. tuberculosis* drug discovery. High-level gene knockdown can be used to define essential drug targets and here CRISPRi will be a complementary and confirmatory approach to Tn-seq. The ability to tune the level of gene knockdown can then be used to define drug target vulnerability (that is, the degree of knockdown required to inhibit growth) as well as serve as a platform for target-based whole-cell screening to identify novel small molecules active against the desired drug target. Furthermore, the ability to generate hypomorphic alleles of essential genes will enable chemical and genetic interaction studies to define the interactions among essential drug targets, including the identification of drug target synergies. Using large-scale chip

synthesis of unique sgRNA targeting regions, it will be possible to perform these studies at genome scale. The identification of such drug target synergies will be a significant step towards the goal of developing novel drug regimens to shorten the duration of tuberculosis chemotherapy.

Methods

Bacterial strains. All *M. tuberculosis* strains are derivatives of H37Rv, and all *M. smegmatis* strains are derivatives of mc²155.

Media. *M. tuberculosis* and *M. smegmatis* were grown at 37 °C in Middlebrook 7H9 broth or 7H10 plates supplemented with 0.5% glycerol, 0.05% Tween80, 1× albumin dextrose catalase (ADC) (*M. smegmatis*) or oleic acid ADC (*M. tuberculosis*) and the appropriate antibiotics. Where indicated, antibiotics or small molecules were used at the following concentrations: ATc (100 ng ml⁻¹ anhydrotetracycline); AMK (25 ng ml⁻¹ amikacin); NAT (800 ng ml⁻¹ nourseothricin); STR (100 ng ml⁻¹ streptomycin); RIF (1.25 µg ml⁻¹ rifampicin); TMP (5 µg ml⁻¹ trimethoprim); SMX (625 ng ml⁻¹ sulfamethoxazole).

Plasmid construction and cloning. Cas9 sequences were identified by Blast⁴² or obtained from published studies²⁴⁻²⁶. Cas9 nuclease activity was inactivated by making the homologous D10A and H840A (S. pyogenes numbering) mutations in the RuvC and HNH nuclease domains for each Cas9 protein. dcas9 alleles were then codon optimized for mycobacterial expression with Jcat (ref. 43) and synthesized by Genscript. dCas9 protein sequences used in this study are listed in Supplementary Table 1. Single-plasmid platforms were generated for all dcas9 alleles that contained (1) the dcas9 allele under the control of a Tet repressor (TetR)-regulated UV15-Tet promoter¹ or an optimized, synthetic TetR-regulated promoter (Supplementary Fig. 4 and Supplementary Table 1); (2) the cognate sgRNA under the control of a minimal synthetic constitutive promoter or an optimized, synthetic TetR-regulated promoter (Supplementary Fig. 4 and Supplementary Table 1); (3) a Tet repressor; (4) a single-copy L5-integrating backbone⁴⁴; (5) a pBR322-derived E. coli replication origin; and (6) a kanamycin-selectable marker. sgRNA scaffold sequences were obtained from published studies^{25,26} or engineered by fusing the crRNA direct repeat to the tracrRNA in an analogous manner to the S. pyogenes sgRNA (ref. 11). We attempted to enrich for highly active dCas9_{sp}, sgRNAs by selecting top-scoring sgRNAs based on the algorithm described by Doench and co-authors⁴⁵. All sgRNA scaffold sequences used in this study are listed in Supplementary Table 1.

To clone sgRNA targeting sequences, the sgRNA scaffolds were designed with two unique BsmBI restriction sites immediately 5' to the sgRNA scaffold sequence. Complementary sgRNA targeting oligos (N20-25) were then annealed and ligated (T4 DNA ligase, NEB) into the BsmBI-digested CRISPRi vector backbone. To clone multiple sgRNAs into the same vector, the CRISPRi vector backbones were designed with a SapI-based Golden Gate cloning site 3' to the first sgRNA scaffold. sgRNA targeting sequences are listed in Supplementary Table 2.

To construct the *Renilla* luciferase reporter plasmid, the *Renilla* gene was cloned downstream of the G13 promoter from *Mycobacterium marinum*⁴⁶ with a synthetic 5'UTR in a single-copy Giles integrating vector⁴⁷. The *Renilla* 5'UTR was engineered such that the optimal PAM for each dCas9 protein could be tested with an identical sgRNA targeting sequence (UTR1) for all dCas9 proteins. The *Renilla* reporter sequences used for each dCas9 protein are shown in Supplementary Table 1.

Phenotying. To visually monitor the effects of CRISPRi-mediated gene silencing, cultures were grown to log phase and plated on solid media with or without ATc. Plates were incubated at 37 °C for 3–4 days (*M. smegmatis*) or 21 days (*M. tuberculosis*). For the complementation experiment shown in Fig. 4f, *M. smegmatis* was complemented with a single-copy Giles integrating vector expressing *M. tuberculosis dnaE1* (Rv1547) under the control of the endogenous Rv1547 promoter.

Western blots. HA-dCas9_{Sth1} and HA-dCas9_{Spy} were detected using an anti-HA antibody (Cell Signaling, C29F4) at a 1:1,000 dilution, and RpoB was detected using an anti-RpoB antibody (Neoclone, WP023) at a 1:1,000 dilution. IRDye-800 antimouse and IRDye-680 anti-rabbit were used at 1:15,000 dilution (Licor). Immunoblots were imaged on a Licor Odyssey scanner. The full scans for the western blot shown in Fig. 2c are provided as Supplementary Fig. 7.

Cas9 phylogeny. Representative Cas9 sequences were pooled from published sources^{48,49} and aligned with FastTree⁵⁰ with the default parameters. Tree visualization was performed with FigTree.

Luciferase assays. For each sample, cultures were grown to log phase and then diluted back with or without 100 ng ml⁻¹ ATc to maintain the cultures in the log phase and induce CRISPRi. *Renilla* knockdown was allowed to proceed for 24 h. One OD₆₀₀ (optical density at 600 nm) equivalent of cells was harvested by centrifugation and processed for *Renilla* luciferase assay according to the manufacturer's instructions (Promega Renilla Luciferase Assay system). Luciferase activity was quantified in 96-well white plates (Costar; 3362) using a VarioSkan Flash plate reader (Thermo Scientific). Error bars represent standard deviations of three technical replicates.

mRNA quantification. For each sample, cultures were grown to log phase and then diluted back in the presence of 100 $\rm ng~ml^{-1}$ ATc to maintain the cultures in the log phase and induce CRISPRi. Target knockdown was allowed to proceed for 14 h (M. smegmatis) or 4 days (M. tuberculosis). One-to-two OD₆₀₀ equivalents of cells from each culture were harvested by centrifugation, resuspended in TRIzol (Thermo Fisher), and disrupted by bead beating (Lysing Matrix B, MP Biomedicals). Total RNA was isolated by RNA miniprep (Zymo Research), residual contaminating genomic DNA was digested with TURBO DNase (Ambion), and samples were cleaned with RNA clean-up columns (Zymo Research). cDNA was prepared with random hexamers as per manufacturer instructions (Life Technologies Superscript IV). RNA was then removed by alkaline hydrolysis and cDNA was purified by a spin column (Qiagen). cDNA levels were then quantified by quantitative real-time PCR (qRT-PCR) on a Viia7 light cycler (Applied Biosystems) using iTaq Universal SYBR Green Supermix (BioRad). All qPCR primer pairs were verified to be >95% efficient and cDNA masses tested were experimentally validated to be within the linear dynamic range of the assay. Signals were normalized to the housekeeping sigA transcript for both M. tuberculosis (Rv2703) and M. smegmatis (Ms2758) and quantified by the $\Delta\Delta$ Ct method. Error bars are 95% confidence intervals of three technical replicates.

M. smegmatis operon predictions. The homologues of *M. tuberculosis* folate biosynthesis genes were identified in *M. smegmatis* by Blast⁴²; note that *folC* is incorrectly annotated as a pseudogene in $mc^{2}155$. Operon predictions from the TB Database⁵¹ or primary data⁵² were used to predict syntenic operons in *M. smegmatis*. Gene essentiality predictions are based on essentiality predictions of *M. tuberculosis* homologues⁴.

Data availability. All data that support the findings of this study are available from the corresponding author upon request.

Received 14 June 2016; accepted 21 December 2016; published 6 February 2017

References

- Ehrt, S. et al. Controlling gene expression in mycobacteria with anhydrotetracycline and Tet repressor. Nucleic Acids Res. 33, e21 (2005).
- Kim, J. H. *et al.* Protein inactivation in mycobacteria by controlled proteolysis and its application to deplete the beta subunit of RNA polymerase. *Nucleic Acids Res.* 39, 2210–2220 (2011).
- 3. Wei, J.-R. *et al.* Depletion of antibiotic targets has widely varying effects on growth. *Proc. Natl Acad. Sci. USA* **108**, 4176–4181 (2011).
- Zhang, Y. J. et al. Global assessment of genomic regions required for growth in Mycobacterium tuberculosis. PLoS Pathogens 8, e1002946 (2012).
- Qi, L. S. et al. Repurposing CRISPR as an RNA-guided platform for sequencespecific control of gene expression. Cell 152, 1173–1183 (2013).
- Rath, D., Amlinger, L., Hoekzema, M., Devulapally, P. R. & Lundgren, M. Efficient programmable gene silencing by Cascade. *Nucleic Acids Res.* 43, 237–246 (2015).
- Bikard, D. et al. Programmable repression and activation of bacterial gene expression using an engineered CRISPR-Cas system. Nucleic Acids Res. 41, 7429–7437 (2013).
- Wright, A. V., Nuñez, J. K. & Doudna, J. A. Biology and applications of CRISPR systems: harnessing nature's toolbox for genome engineering. *Cell* 164, 29–44 (2016).
- Gasiunas, G., Barrangou, R., Horvath, P. & Siksnys, V. Cas9-crRNA ribonucleoprotein complex mediates specific DNA cleavage for adaptive immunity in bacteria. *Proc. Natl Acad. Sci.* 109, E2579–86 (2012).
- 10. Garneau, J. E. *et al.* The CRISPR/Cas bacterial immunesystem cleaves bacteriophage andplasmid DNA. *Nature* **468**, 67–71 (2010).
- 11. Jinek, M. et al. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* **337**, 816–821 (2012).
- 12. Deltcheva, E. et al. Crispr RNA maturation by trans-encoded small RNA and host factor RNase III. Nature 471, 602–607 (2011).
- 13. Horvath, P. et al. Diversity, activity, and evolution of CRISPR loci in *Streptococcus thermophilus. J. Bacteriol.* **190**, 1401–1412 (2008).
- Deveau, H. et al. Phage response to CRISPR-encoded resistance in Streptococcus thermophilus. J. Bacteriol. 190, 1390–1400 (2008).
- Peters, J. M. et al. A comprehensive, CRISPR-based functional analysis of essential genes in bacteria. Cell 165, 1493–1506 (2016).
- Choudhary, E., Thakur, P., Pareek, M. & Agarwal, N. Gene silencing by CRISPR interference in mycobacteria. *Nat. Commun.* 6, 6267 (2015).
- Singh, A. K. et al. Investigating essential gene function in Mycobacterium tuberculosis using an efficient cRISPR interference system. Nucleic Acids Res. 44, e143 (2016).
- Nielsen, A. A. & Voigt, C. A. Multi-input CRISPR/Cas genetic circuits that interface host regulatory networks. *Mol. Syst. Biol.* 10, 763–763 (2014).
- Anders, C., Niewoehner, O., Duerst, A. & Jinek, M. Structural basis of PAMdependent target DNA recognition by the Cas9 endonuclease. *Nature* 513, 569–573 (2014).
- Park, S. W. et al. Evaluating the sensitivity of Mycobacterium tuberculosis to biotin deprivation using regulated gene expression. PLoS Pathogens 7, e1002264 (2011).

NATURE MICROBIOLOGY

- Leblanc, C. et al. 4'-Phosphopantetheinyl transferase PptT, a new drug target required for mycobacterium tuberculosis growth and persistence in vivo. PLoS Pathogens 8, e1003097 (2012).
- Lin, K. et al. Mycobacterium tuberculosis thioredoxin reductase is essential for thiol redox homeostasis but plays a minor role in antioxidant defense. PLoS Pathogens 12, e1005675 (2016).
- Reddy, B. K. K. et al. Assessment of Mycobacterium tuberculosis pantothenate kinase vulnerability through target knockdown and mechanistically diverse inhibitors. Antimicrob. Agents Chemother. 58, 3312–3326 (2014).
- Fonfara, I. *et al.* Phylogeny of Cas9 determines functional exchangeability of dual-RNA and Cas9 among orthologous type II CRISPR-Cas systems. *Nucleic Acids Res.* 42, 2577–2590 (2014).
- Ran, F. A. et al. In vivo genome editing using Staphylococcus aureus Cas9. Nature 520, 186–191 (2015).
- Esvelt, K. M. et al. Orthogonal Cas9 proteins for RNA-guided gene regulation and editing. Nat. Methods 10, 1116–1121 (2013).
- Ma, E., Harrington, L. B., O'Connell, M. R., Zhou, K. & Doudna, J. A. Singlestranded DNA cleavage by divergent CRISPR-Cas9 enzymes. *Mol. Cell* 60, 398–407 (2015).
- 28. Barrangou, R. *et al.* CRISPR provides acquired resistance against viruses in prokaryotes. *Science* **315**, 1709–1712 (2007).
- Rock, J. M. et al. DNA replication fidelity in *Mycobacterium tuberculosis* is mediated by an ancestral prokaryotic proofreader. *Nat. Genet.* 47, 677–681 (2015).
- Chari, R., Mali, P., Moosburner, M. & Church, G. M. Unraveling CRISPR-Cas9 genome engineering parameters via a library-on-library approach. *Nat. Methods* 12, 823–826 (2015).
- Kleinstiver, B. P. et al. Engineered CRISPR-Cas9 nucleases with altered PAM specificities. Nature 523, 481–485 (2015).
- Leenay, R. T. et al. Identifying and visualizing functional PAM diversity across CRISPR-Cas systems. Mol. Cell 62, 137–147 (2016).
- Karvelis, T. et al. Rapid characterization of CRISPR-Cas9 protospacer adjacent motif sequence elements. Genome Biol. 16, 253 (2015).
- Caspi, R. et al. The MetaCyc database of metabolic pathways and enzymes and the BioCyc collection of pathway/genome databases. Nucleic Acids Res. 36, D623–D631 (2007).
- Larson, M. H. et al. CRISPR interference (CRISPRi) for sequence-specific control of gene expression. Nat. Protoc. 8, 2180–2196 (2013).
- Minato, Y. et al. Mycobacterium tuberculosis folate metabolism and the mechanistic basis for para-aminosalicylic acid susceptibility and resistance. Antimicrob. Agents Chemother. 59, 5097–5106 (2015).
- Bushby, S. Trimethoprim-sulfamethoxazole: *in vitro* microbiological aspects. J. Infect. Dis. 128(Suppl), 442–462 (1973).
- White, E. L., Ross, L. J., Cunningham, A. & Escuyer, V. Cloning, expression, and characterization of *Mycobacterium tuberculosis* dihydrofolate reductase. *FEMS Microbiol. Lett.* 232, 101–105 (2004).
- Forgacs, P. et al. Tuberculosis and trimethoprim-sulfamethoxazole. Antimicrob. Agents Chemother. 53, 4798–4793 (2009).
- Vilcheze, C. & Jacobs, W. R. The combination of sulfamethoxazole, trimethoprim, and isoniazid or rifampin is bactericidal and prevents the emergence of drug resistance in *Mycobacterium tuberculosis*. *Antimicrob. Agents Chemother.* 56, 5142–5148 (2012).
- 41. Ong, W. et al. Mycobacterium tuberculosis and sulfamethoxazole susceptibility. Antimicrob. Agents Chemother. 54, 2748–2749 (2010).
- Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. Basic local alignment search tool. J. Mol. Biol. 215, 403–410 (1990).

- Grote, A. et al. JCat: a novel tool to adapt codon usage of a target gene to its potential expression host. Nucleic Acids Res. 33, W526–W531 (2005).
- 44. Lee, M. H., Pascopella, L., Jacobs, W. R. & Hatfull, G. F. Site-specific integration of mycobacteriophage L5: integration-proficient vectors for mycobacterium smegmatis, mycobacterium tuberculosis, and *Bacille Calmette-Guérin. Proc. Natl Acad. Sci. USA* 88, 3111–3115 (1991).
- Doench, J. G. et al. Rational design of highly active sgRNAs for CRISPR-Cas9mediated gene inactivation. Nat. Biotechnol. 32, 1262–1267 (2014).
- 46. Barker, L. P., Porcella, S. F., Wyatt, R. G. & Small, P. L. The *Mycobacterium marinum* G13 promoter is a strong sigma 70-like promoter that is expressed in *Escherichia coli* and mycobacteria species. *FEMS Microbiol. Lett.* 175, 79–85 (1999).
- Morris, P., Marinelli, L. J., Jacobs-Sera, D., Hendrix, R. W. & Hatfull, G. F. Genomic characterization of mycobacteriophage Giles: evidence for phage acquisition of host DNA by illegitimate recombination. *J. Bacteriol.* **190**, 2172–2182 (2008).
- Makarova, K. S. et al. An updated evolutionary classification of CRISPR-Cas systems. Nat. Rev. Microbiol. 13, 722–736 (2015).
- Chylinski, K., Makarova, K. S., Charpentier, E. & Koonin, E. V. Classification and evolution of type II CRISPR-Cas systems. *Nucleic Acids Res.* 42, 6091–6105 (2014).
- Price, M. N., Dehal, P. S. & Arkin, A. P. Fasttree 2—approximately maximumlikelihood trees for large alignments. *PLoS ONE* 5, e9490 (2010).
- Reddy, T. B. K. et al. TB database: an integrated platform for tuberculosis research. Nucleic Acids Res. 37, D499–D508 (2009).
- Fivian-Hughes, A. S., Houghton, J. & Davis, E. O. *Mycobacterium tuberculosis* thymidylate synthase gene *thyX* is essential and potentially bifunctional, while *thyA* deletion confers resistance to *p*-aminosalicylic acid. *Microbiology* **158**, 308–318 (2012).

Acknowledgements

The authors thank L. Gilbert for advice. This work was supported by a Helen Hay Whitney fellowship to J.M.R., an NIH Director's New Innovator Award 1DP20D001378, subcontracts from NIAID U19 AI107774 and a Doris Duke Charitable Foundation Grant 2010054 to S.M.F. A.C. was funded by the National Cancer Institute grant no. 5T32CA009216-34. G.M.C. acknowledges support from the US National Institutes of Health National Human Genome Research Institute grant no. P50 HG005550 and the Wyss Institute for Biologically Inspired Engineering.

Author contributions

S.M.F. supervised the project. J.M.R. and J.R.P. conceived the study. J.M.R. designed and executed the study. F.F.H. and M.D. cloned CRISPR constructs and assisted with luciferase assays. A.C., M.R.C., E.R.G., G.M.C., E.J.R., C.M.S. and D.S. contributed reagents and expertise. J.M.R. and S.M.F. wrote the manuscript with input from all co-authors.

Additional information

Supplementary information is available for this paper.

Reprints and permissions information is available at www.nature.com/reprints.

Correspondence and requests for materials should be addressed to S.M.F.

How to cite this article: Rock, J. M. *et al.* Programmable transcriptional repression in mycobacteria using an orthogonal CRISPR interference platform. *Nat. Microbiol.* **2**, 16274 (2017).

Competing interests

The authors declare no competing financial interests.