A genetic strategy to identify targets for the development of drugs that prevent bacterial persistence

Jee-Hyun Kim, Kathryn M. O’Brien, Ritu Sharma, Helena I. M. Boshoff, German Rehren, Sumit Chakraborty, Joshua B. Wallach, Mercedes Monteleone, Daniel J. Wilson, Courtney C. Aldrich, Clifton E. Barry III, Kyu Y. Rhee, Sabine Ehr, and Dirk Schnapp

Antibacterial drug development suffers from a paucity of targets whose inhibition kills replicating and nonreplicating bacteria. The latter include phenotypically dormant cells, known as persisters, which are tolerant to many antibiotics and often contribute to failure in the treatment of chronic infections. This is nowhere more apparent than in tuberculosis caused by Mycobacterium tuberculosis, a pathogen that tolerates many antibiotics once it ceases to replicate. We developed a strategy to identify proteins that Mycobacterium tuberculosis requires to both grow and persist and whose inhibition has the potential to prevent drug tolerance and persister formation. This strategy is based on a tunable dual-control genetic switch that provides a regulatory range spanning three orders of magnitude, quickly depletes proteins in both replicating and nonreplicating mycobacteria, and exhibits increased robustness to phenotypic reversion. Using this switch, we demonstrated that depletion of the nicotinamide adenine dinucleotide synthetase (NadE) rapidly killed Mycobacterium tuberculosis under conditions of standard growth and nonreplicative persistence induced by oxygen and nutrient limitation as well as during the acute and chronic phases of infection in mice. These findings establish the dual-control switch as a robust tool with which to probe the essentiality of Mycobacterium tuberculosis proteins under different conditions, including those that induce antibiotic tolerance, and NadE as a target with the potential to shorten current tuberculosis chemotherapies.

Target-based approaches have emerged as a major paradigm of modern drug development, but they largely failed to discover new antibacterials (1, 2). The reasons for this are multifactorial. Nonetheless, a fundamental requirement of target-based approaches is the need to identify targets whose inhibition can selectively and quickly improve the pathophysiologic phenotype of interest. For the development of antibacterials, this need was initially thought to be addressable with recombinant genetic technologies that enabled both untargeted genome-wide mutations and targeted gene deletions (2). Subsequent advances achieved conditional transcriptional silencing of genes, which allowed for the identification of targets whose functions are essential both to normal physiology of the cell and to metabolism in the context of the pathophysiology of the disease of interest (3–6). Notwithstanding, such advances have failed to address that drugs act on a time scale much faster than those associated with changes in de novo transcription and that many genetic approaches only achieve narrow ranges of regulation (6, 7). Furthermore, existing genetic approaches are also limited by their inability to identify proteins required not only for growth but also for the survival and persistence of nongrowing bacteria. Bacteria that are not actively dividing often include persisters, phenotypic variants that withstand bactericidal antibiotics and complicate the treatment of many chronic infections including cystic fibrosis-associated lung infections, salmonellosis, and tuberculosis (TB) (8–11). We sought to address these issues by improving the ability of current genetic technologies to more closely mimic the kinetics and magnitude of chemical inhibition and thus facilitate the more accurate identification of new potential drug targets. To this end, we engineered a dual-control (DUC) switch in which a single inducer—anhidrotetracycline (atc) or doxycycline (doxy)—simultaneously triggers transcriptional repression of a target gene and degradation of the encoded protein and demonstrated that this switch can identify proteins required by both replicating and nonreplicating Mycobacterium tuberculosis (Mtb).

TB remains the leading cause of death from a single bacterial infection worldwide, and drug-resistant Mtb strains continue to evolve and spread (12). Drug-sensitive TB can be cured by uninterrupted treatment with multiple drugs for at least 6 mo. The requirement for such a long therapy is, in part, due to bacilli that persist in a slow-growing or nongrowing state and are recalcitrant to killing by most TB drugs. However, the specific activities Mtb requires to persist in a drug-tolerant state remain largely unexplored. A genetic strategy to identify targets for the development of drugs that prevent bacterial persistence

Significance

Chronic bacterial infections, such as those caused by Mycobacterium tuberculosis (Mtb), continue to claim the lives of millions of people. New antibiotics are needed to treat these infections, but their development is hindered by a lack of targets whose inhibition quickly eradicates bacterial pathogens and prevents the survival of drug-tolerant persisters. We describe a unique dual-control (DUC) switch that combines repression of transcription and controlled proteolysis to silence gene activities in Mtb. By conditionally inactivating Mtb’s nicotinamide adenine dinucleotide synthetase, we demonstrate that the DUC switch can identify proteins that this pathogen requires for growth and nonreplicating persistence in vitro and during infections. Targeting such proteins holds the promise of yielding drugs that shorten the duration of antibacterial chemotherapies.


The authors declare no conflict of interest. This article is a PNAS Direct Submission.

1J.-H.K. and K.M.O. contributed equally to this work.
2To whom correspondence should be addressed. E-mail: dia2003@med.cornell.edu.

This article contains supporting information online at www.pnas.orglookup/suppl/doi:10.1073/pnas.1315860110/-/DCSupplemental.

www.pnas.org/cgi/doi/10.1073/pnas.1315860110

PNAS | November 19, 2013 | vol. 110 | no. 47 | 19095–19100
unknown. Using the DUC switch, we demonstrated that *Mtb* depends on nicotinamide adenine dinucleotide (NAD) synthesis for growth and survival during nonreplicating persistence and identified the NAD synthetase (NadE) as an essential persistence target whose inactivation is bactericidal. Moreover, depletion of NadE during both acute and chronic infections rapidly eliminated *Mtb* from mice. These findings thus validate the potential of this genetic strategy to identify targets whose inhibition is capable of killing the *Mtb* persistor population and potentially shortening the duration of TB chemotherapies.

**Results**

**Construction of a Genetic Switch That Combines Transcriptional and Proteolytic Silencing.** To develop a switch in which atc simultaneously represses transcription and induces proteolysis, we first designed two tetracycline repressors (TetRs), T38 and TSC10, to recognize different tet operators (tetO and tetO-4C5G), not form heterodimers, and respectively be activated and inactivated by atc (Fig. S1A). T38 was derived from TetR28 (13), which requires atc to repress transcription, by introducing two mutations—E37A and P39K—that prevent binding to tetO and allow binding to tetO-4C5G (14). TSC10 was constructed by converting TetR10, which is inactivated by atc (13), into a single-chain repressor to prevent heterodimerization with T38 (15). We confirmed that TSC10 and T38 only repress promoters that contain tetO (PettO) and tetO-4C5G (PettO-4C5G), respectively, using a *Mycobacterium smegmatis* (Msm) dual-reporter strain (Fig. S1 B and C).

SspB is an *Escherichia coli*-derived protein that can deliver proteins containing the DAS+4-tag to the degradation machinery in mycobacteria (16, 17). In an *Msm* strain in which transcription of sspB and the luciferase encoding luxAB-DAS operon were controlled by TSC10 and T38, respectively (Fig. 1A), we expected that atc (i) represses transcription of luxAB-DAS (via inactivation of PettO-4C5G by the T38–atc complex), (ii) causes proteolysis of the DAS+4-tagged luciferase subunit LuxB-DAS (via the atc-induced release of TSC10 from and activation of PettO-sspB), and (iii) thus reduces luciferase activity more efficiently than when luciferase activity is controlled by either repression of transcription or controlled proteolysis alone. We confirmed these expectations first using an episomal PettO-4C5G-luxAB-DAS construct (Fig. 1B). When luciferase was inactivated by either inducible proteolysis or transcriptional repression alone, atc reduced luminescence by up to 270-fold and 420-fold, respectively, whereas their combination reduced luminescence up to 5,800-fold. All three regulatory systems showed similar atc dose–response. Similar results were obtained with *Msm* expressing chromosomally integrated luxAB-DAS and *Msm* and *Mtb* expressing a red fluorescent protein (Fig. S2).

Next, we analyzed the kinetics of luciferase inactivation and reactivation in replicating *Msm*. Half-maximal repression of luciferase activity was reached in less than 1 h by controlled proteolysis on its own and when proteolysis was combined with transcriptional repression (Fig. S3A). Transcriptional silencing, in comparison, was slow and did not reach the half-maximum until 10 h after the addition of atc. When *Msm* was first grown with atc and then transferred to medium without atc, luciferase activity reappeared with similar kinetics in all strains (Fig. S3B). To analyze the kinetics of gene silencing in nonreplicating *Msm*, we adopted the PBS starvation model developed for *Mtb* (18). Luciferase activity decreased slowly when inactivation solely depended on transcriptional repression (Fig. S3C). In contrast, half-maximal repression was reached after approximately 3 h when controlled proteolysis was used alone or in combination with transcriptional repression. Only the DUC switch achieved a reduction in luciferase activity to background levels. In summary, these experiments established that the DUC switch mediates efficient, reversible, tunable, and rapid inactivation of different reporter genes in replicating and, more importantly, nonreplicating mycobacteria.

**Biological Utility of the DUC Switch for Conditional Inactivation of *Mtb* Genes.** The 7,8-diaminopelargonic acid synthase (BioA) is required by *Mtb* to synthesize biotin, grow in biotin-free media, and multiply and persist in mice, but *Mtb* is also remarkably resistant to partial inactivation of this enzyme (7). To determine if the DUC switch could efficiently inactivate BioA, we generated *Mtb* BioA-T38, in which atc turns off bioA via transcriptional repression; *Mtb* BioA-SspB, in which atc inactivates BioA via controlled proteolysis; and *Mtb* BioA-DUC, in which BioA is regulated by the DUC switch. For all mutants we used a strong translation initiation site that causes approximately 10-fold overexpression of BioA (7). Addition of atc to *Mtb* BioA-SspB and *Mtb* BioA-DUC depleted BioA protein below the level of detection within 48 h and stopped growth thereafter (Fig. 2). In *Mtb* BioA-T38, BioA protein was undetectable 72 h after the addition of atc, but growth remained unaffected. When growth was monitored for a longer period, it became apparent that atc only reduced growth of *Mtb* BioA-T38, whereas it abolished growth of *Mtb* BioA-SspB and *Mtb* BioA-DUC for 15 d (Fig. S4 A and B). During this period the colony-forming units (cfu) of *Mtb* BioA-SspB and *Mtb* BioA-DUC decreased by >90% (Fig. S4C). After 15 d, the OD of *Mtb* BioA-SspB cultures increased (Fig. S4B) due to atc-resistant revertants. Such revertants also occurred in *Mtb* BioA-DUC but at a lower frequency. When we analyzed the appearance of revertants in 96 cultures of both mutants, we found revertants in 8% of the BioA-SspB cultures but none of the BioA-DUC cultures after 27 d (Fig. S4D). After
knockdown mutants, the infections also included WT in multistrain mouse infections (19). In addition to the three DNA tags, which we used to analyze their growth and persistence PrcB (loading control).

Left

tracted from the cultures shown in medium with atc added on day 5. (after exposure to different atc concentrations. (Fig. 3).

Fig. 2. Kinetics of BioA inactivation. (Left) Growth in biotin-free Sauton’s medium with atc added on day 5. (Right) Immunoblot of proteins extracted from the cultures shown in Left using antisera against BioA and PrcB (loading control).

35 d, 60% of the BioA-SspB and 40% of the BioA-DUC cultures contained revertants.

The bioA mutants constructed for this study contain unique DNA tags, which we used to analyze their growth and persistence in multistrain mouse infections (19). In addition to the three knockdown mutants, the infections also included WT Mtb, to which the abundance of all other strains was normalized; Mtb ΔbioA; and Mtb DUC-control. In Mtb DUC-control, which contains WT bioA in addition to bioA-DAS, atc depleted DAS-tagged BioA protein but did not interfere with biotin synthesis due to continued expression of WT BioA protein. The relative abundance of Mtb BioA DUC-control was similar to that of WT Mtb in mice with or without doxy (Fig. S5A). Inactivation of BioA-DAS by the DUC switch thus did not exert phenotypic off-target or dominant-negative effects. ΔbioA was strongly attenuated in all mice, whereas the abundance of Mtb BioA-T38 was similar to that of WT (Fig. S5 B and C). Mtb BioA-SspB and Mtb BioA-DUC were both strongly attenuated when doxy was administered throughout the infection, but when doxy was given later, Mtb BioA-DUC showed a stronger phenotype than Mtb BioA-SspB (Fig. S5 D and E). For example, in mice that started receiving doxy 10 d postinfection, the relative abundance of Mtb BioA-SspB decreased to 0.2% on day 135, whereas that of Mtb BioA-DUC decreased to 0.02% (P < 0.01). Taken together, these experiments demonstrated that the DUC switch can achieve near-complete inactivation of an Mtb protein in vitro and during infections and increases the resistance of conditional knockdown mutants to phenotypic reversion.

Fig. 3. Impact of depleting NadE on NAD synthesis. (A) NAD biosynthesis pathways and quantification of NaMN, NaAD, and NAD in extracts of NadE-DUC after exposure to different atc concentrations. (B) Growth with different atc concentrations. OD, optical density; data are means ± SEM of three replicates.

Consequences of Inactivating NadE for Growth and Survival of Mtb in Vitro and During Infections. NadE is an essential cofactor for numerous biochemical reactions (20) and a substrate for DNA ligases (21), ADP ribosylases (22), and protein deacetylases (23). Mtb can synthesize NAD via a de novo pathway and recycle it via a salvage pathway (Fig. 3A). Both pathways share the reactions catalyzed by NadE and nicotinic acid mononucleotide adenyltransferase (encoded by nadD) (24, 25). Transposon mutagenesis identified nadD and nadE as required for optimal in vitro growth (26, 27). This essentiality for replication so far precluded an analysis of their requirement for viability in nonreplicating or slowly replicating Mtb such as those associated with the chronic phase of infection. The importance of NAD synthesis for survival during nonreplicating persistence was questioned by experiments which showed that Mtb mutants incapable of synthesizing NAD de novo (but still capable of NAD recycling) survived unimpaired in the absence of exogenous nicotinamide when their replication had been arrested for up to several weeks by incubation under hypoxic conditions or nutrient starvation (24). Mtb also remained virulent in mice after either the salvage or the de novo pathway had been inactivated (24, 25). To determine the consequences of inactivating both the de novo and salvage pathways for survival of nonreplicating Mtb in vitro and for growth and persistence in mice, we constructed Mtb NadE-DUC, in which the DUC switch allowed inducible depletion of NadE. In nutrient-rich media, atc prevented growth of Mtb NadE-DUC. rapidly depleted NadE, and caused cfu to decrease to less than 0.1% within 24 h (Fig. S6). Inhibition of growth was atc dose-dependent and was also accompanied by a dose-dependent decrease in NAD and an increase in the NadE substrate NaAD (Fig. 3). Depletion of NadE furthermore caused NaMN to accumulate, possibly due to product inhibition of NadD or deadenylation of NaAD (28, 29). Next, reliance of nonreplicating Mtb on NadE for survival was analyzed during starvation and hypoxia. During starvation in PBS, inactivation of NadE decreased cfu to
~2% within the first 24 h (Fig. 4A). Inactivation of NadE during hypoxia sterilized a culture of $10^8$ cfu/mL (Fig. 4B).

To assess the effect of silencing during acute and chronic infection in mice, NadE was inactivated by administering doxy beginning at day 1, 10, 28, or 56. In mice that received doxy throughout the infection, *Mtb* NadE-DUC could only be recovered from the lungs immediately after the infection, and no pathological signs of infection were observed (Fig. 5 and Fig. S7). When doxy administration was started later, cfu declined rapidly in lungs and spleens of both acutely and chronically infected mice. Decreases in cfu were accompanied by decreases in lung pathology (Fig. 5 and Fig. S7). Inactivation of NadE-DUC by doxy had no impact on growth or survival of *Mtb* in vitro or during infections in the presence of an additional WT copy of *nadE* that is not controlled by the DUC switch (Fig. S8). These results thus revealed that inactivation of both NAD recycling and de novo synthesis not only is bactericidal for *Mtb* during growth and nonreplicating persistence in vitro but also rapidly eradicates cultivable *Mtb* from mouse lungs and spleens during acute and chronic infections.

**Discussion**

Effective and rapid genetic inactivation has the potential to identify targets for the development of drugs that prevent bacterial persistence and can better treat chronic bacterial infections. However, this is often prevented by transcriptional leakiness, slow or incomplete depletion of transcriptionally regulated gene products, mutations that interfere with regulation, or a combination thereof (6, 7, 30). Here we demonstrated that these problems can be drastically reduced or overcome entirely with a switch that combines transcriptional repression with regulated proteolysis. The DUC switch reduced reporter gene activities by up to 5,800-fold and accelerated gene inactivation in growing and nonreplicating bacteria, yet did not compromise reactivation of gene expression or atc dose-responsiveness (Fig. 1 and Figs. S2 and S3). We first demonstrated that these features facilitate analyses of essential gene functions in clinically relevant pathogens by targeting BioA in *Mtb*. We previously demonstrated that *Mtb* is phenotypically resistant to partial depletion of BioA, which greatly complicated the isolation of knockdown mutants capable of revealing the consequences of efficient BioA depletion in vitro and during infections (7). In contrast, BioA-DUC allowed rapid and bactericidal depletion even though efficient translation caused an ∼10-fold increase in BioA, which indicated that the DUC switch regulated BioA by ∼1,000-fold, from ∼10-fold above the WT expression level to ∼99-fold below the WT expression level required to stop growth (7). The DUC switch furthermore generated a BioA knockdown mutant that is less susceptible to phenotypic reversion (Fig. S4D). This is significant because the accumulation of revertants often complicates the construction, maintenance, and analysis of knockdown mutants for in vitro essential genes.

Degradation of proteins by ClpXP, the protease complex that mediates controlled proteolysis via the DUC switch, consumes ATP (31). This ATP consumption itself did not impair growth or survival of *Mtb* as is evident from two control strains. In the first, *Mtb* DUC-control, atc caused degradation of DAS-tagged BioA but did not interfere with biotin synthesis due to the continued expression of WT BioA. Even with doxy, *Mtb* DUC-control grew and persisted like WT in mice (Fig. S5A). In the second strain, WT *nadE* was used to complement *Mtb* NadE-DUC, which led to normal growth in vitro and during infections (Figs. S6 and S8). These controls also demonstrated that depletion of BioA or NadE by the DUC switch caused no other dominant-negative effects.

NAD biosynthesis enzymes have been proposed as targets for the development of drugs against several pathogens (24, 25, 32). However, *Mtb* mutants unable to either recycle or synthesize NAD de novo remained virulent in mice, and *Mtb* lacking the de novo synthesis pathway remained viable during nonreplicating persistence even without external NAD (24, 25). These results left the essentiality of NAD biosynthesis in nonreplicating or slowly replicating *Mtb* populations in vivo unresolved. Our studies established that depletion of NadE in *Mtb* NadE-DUC occurred rapidly, prevented growth, and caused atc dose-dependent reduction of NAD and atc dose-dependent increases of the substrates of NadE (NAD and NAD (NaMN) (Fig. 3). Strong depletion of NadE was bactericidal for *Mtb* during growth in broth (Fig. S6B), nonreplicating persistence (Fig. 4), and infections (Fig. 5). For its validation as a potential drug target, it is significant how efficiently depletion of NadE killed *Mtb* during hypoxia-induced nonreplicating persistence because tuberculous granulomas are often hypoxic (33), and current TB drugs are not very effective against hypoxia-adapted *Mtb* (34). This and the impact of NadE depletion during starvation suggested that inhibiting this enzyme could kill *Mtb* in several if not all of its metabolic states during infections. In accordance with this expectation, depletion of NadE strongly attenuated *Mtb* in vivo during both acute and chronic infections. The rates with which cfu declined in mouse lungs and spleens were fast and very similar irrespective of when
depletion was initiated (Fig. 5). This is remarkable because the existing TB drugs are generally either more effective in the acute phase (e.g., isoniazid) or chronic phase of the infection (e.g., rifampicin and pyrazinamide) but not in both (35, 36). Targeting NadE thus provides one rational approach for the development of shorter anti-TB chemotherapies.

The development of new classes of antibacterial drugs represents an urgent need and a major challenge, which cannot be met with the validation of new targets alone. The identification of novel small molecules that penetrate the bacterial cell envelope and efficiently and selectively inactivate a bacterial target remains a major bottleneck. Target-based whole-cell screens, which use bacteria underexpressing a selected protein to bias hit compounds toward inhibitors of that protein (37–41), promise to widen this bottleneck and are best applied to proteins that represent well-validated targets. The strategy we used to identify NadE as such a validated target is scalable and can be applied, in principle, to any gene in the *Mtb* genome. We expect that the promising regulatory targets will increase the frequency with which phenotypically well-regulated conditional knockdown mutants can be constructed. Such mutants will help to define more broadly the genes and activities *Mtb* requires not only to grow in vitro and during acute infections but also to persist despite the chemical insults it faces from an activated immune system or chemotherapy. The regulatory components of the DUC switch can control gene expression in several bacteria and eukaryotes (42), which broadens its utility to studying other organisms and diseases.

**Methods**

**Plasmids, Bacterial Strains, and General Procedures.** Bacteria and plasmids are listed in [Table S1](#). Mycobacteria were generally grown at 37 °C on Middlebrook 7H11 agar with 0.5% glycerol or in Middlebrook 7H9 medium with 0.5% glycerol and 0.02% tylosapox (Sigma). For *Mtb*, 7H11 was supplemented with 10% (vol/vol) Middlebrook oleic acid-albumin-dextrose-catalase (Becton Dickinson); 7H9 was supplemented with 0.2% glycerol, 0.05% tylosapox, 0.5% BSA, 0.2% dextrose, and 0.085% NaCl. Preparation of cell lysates; immunoblot analyses; and measurements of fluorescence, luminescence, and β-galactosidase activity were performed as described previously (7, 13, 16, 43). To measure the kinetics of luciferase inactivation during starvation, *Msm* was transferred into P85 with 0.05% tylosapox (P85). After incubating at 37 °C for 32 h, atc (Sigma) was added to half of the cultures, and luciferase activity was measured periodically. Single-strain and multistrain infections were performed as described (19). Procedures involving mice were reviewed and approved by the Institutional Animal Care and Use Committee of Weill Cornell Medical College.

**Survival of Mtb During Nonreplicating Persistence.** To obtain nonreplicating *Mtb* by starvation, bacteria were grown in 7H9 medium, washed twice with PBST, and suspended in PBST to an OD of 0.6. After 10 d, atc was added to half of the cultures, and survival was determined by periodically plating serial dilutions on agar plates. For hypoxia experiments, bacteria were grown in glycerol-free Dubos medium to an OD of 0.5. Cells were filtered through a 5-μm filter to remove clumps and diluted to an OD of ∼0.005 in glycerol-free Dubos medium. Cells were transferred to septum sealed tubes with a headspace ratio of 0.5. A control tube for each strain contained methylene blue to ensure that cells became hypoxic at the expected time. Tubes were placed on a multitube stir plate at 37 °C and covered in foil. The methylene blue tubes decolorized at day 9. When indicated, tubes were transferred to an anaerobic chamber, and atc or solvent without atc was injected through the rubber septum. For each time point, three tubes from each strain were opened, and serial dilutions prepared and plated on agar plates.

**Analysis of atc Resistance in Conditional BioA Knockdown Mutants.** BioA mutant cultures in 7H9 medium were washed and used to inoculate 100 μL biotin-free Sauton’s medium containing atc with ∼5,000 cfu. OD was measured by a SpectraMax M5 plate reader. Aliquots of BioA mutants that had grown to OD > 0.1 were streaked out on biotin-free 7H9 agar plates with or without atc to confirm loss of regulation.

**Metabolite Analyses.** Bacteria were grown in 7H9 medium and transferred onto 0.22-μm nitrocellulose filters under vacuum filtration. After growing on 7H11 plates for 6 d, the *Mtb*-laden filters were transferred to 7H11 plates containing varying concentrations of atc and incubated for an additional 2 d before they were plunged into buffer containing 10% (vol/vol) acetonitrile, 0.15 mM ammonium acetate, and 0.5 mM ascorbic acid (pH 6.0), precooled to −40 °C. The metabolite extraction was achieved by mechanically lysing the bacteria containing solution with 0.1-μm Zirconia beads in a Precellys tissue homogenizer for 8 min under continuous cooling. Lyssates were clarified by centrifugation, filtered across a 0.22-μm filter, and passed through a centrifugal column (Nanosep 10K Omega). Residual protein content of metabolite extracts was measured to normalize samples to cell biomass. Metabolites were separated in an Agilent Accurate Mass 6520 quadrupole time-of-flight coupled to an Agilent 1200 liquid chromatography system on a Cogent Diamond Hydride Type C column using the following mobile phase: solvent A [50% (vol/vol) methanol, 0.05% formic acid] and solvent B [90% (vol/vol) acetonitrile, 1.5 mM ammonium acetate, pH 6.8]. Dynamic mass axis calibration was achieved by continuous infusion of a reference mass solution using an isocratic pump with 0.05% formic acid and solvent B [90% (vol/vol) acetonitrile, 1.5 mM ammonium acetate, pH 6.8]. Metabolite identities were assigned using a mass tolerance of 0.005 Da. Metabolites were quantified using a calibration curve generated with chemical standard spiked into

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**Fig. 5.** Impact of depleting NadE on *Mtb* during acute and chronic infections. (A) Growth and survival in mouse lungs (Left) and spleens (Right). Mice received doxy-containing food from day 1 (black), day 10 (orange), day 28 (purple), day 56 (green), or not at all (gray). Dotted lines end in data points for which the titer was below the limit of detection of 1 cfu/organ. Data are means ± SEM of three or four mice. (B) Gross pathology of lungs 168 d postinfection.
homologous mycobacterial extract to correct for matrix-associated ion suppression effects. This approach allowed quantification of nicotinamide, NaMN, NaAD, and NAD. Nicotinamide levels did not change in response to inactivation of NadE, and the changes that occurred for NaMN, NaAD, and NAD are shown in Fig. 3. Concentrations of the other metabolites of the NAD biosynthesis pathway were below the level of detection.

Fig. S1. Regulation of gene expression by T38, TSC10, tetO, and tetO-4C5G. (A) The nucleotide sequences of tetO and tetO-4C5G are shown on the left, and the amino acid sequences of T38 and TSC10 are on the right. Nucleotides that distinguish tetO and tetO-4C5G are in red. TSC10 is a single-chain TetR that recognizes tetO but not tetO-4C5G. The two monomers are connected by a peptide linker (underlined and italicized) to prevent heterodimerization when TSC10 is coexpressed with T38. T38 is a reverse TetR that recognizes tetO-4C5G but not tetO. The amino acids that cause the reverse phenotype (E15A, L17G, L25V, Q152T, and V153A) are in red; those that determine DNA binding specificity (E37A and P39K) are in blue. (B) Regulation of transcription by T38 and TSC10. tsc10, lacZ, tetR38, luxA, and luxB-DAS were integrated into the chromosome of Mycobacterium smegmatis (Msm). LuxAB and lacZ encode luciferase and β-galactosidase, respectively. tsc10 and tetR38 were transcribed constitutively; transcription of lacZ and luxAB-DAS was repressed by TSC10 and T38, respectively. (C) Relative fluorescence units (RFUs) (Left) and relative luminescence units (RLU) (Right) indicate β-galactosidase and luciferase activities, respectively. Data are means ± SEM of six replicates from two independent experiments.
The dual-control (DUC) switch efficiently inactivates chromosomally expressed luciferase and red fluorescent protein (RFP) in an anhydrotetracycline (atc) dose-dependent manner. (A) RLU of strains in which chromosomally integrated luciferase was repressed by SspB (red), T38 (blue), or SspB and T38 (purple) grown with different atc concentrations. Black squares represent Msm strains expressing luxAB-DAS constitutively without any regulatory components. (B) tsc10 and sspB were integrated into the chromosome of Msm; tetR38 and rfp-DAS were expressed by an episomally replicating plasmid. RFUs were measured for strains in which RFP was repressed either by SspB (red), T38 (blue), or SspB and TSC10 (purple). Black squares represent Msm strains expressing RFP constitutively without any regulatory components. (C) Same as B but in Mycobacterium tuberculosis (Mt) Data are means ± SEM of six replicates from two independent experiments. The gray areas represent autoluminescence and autofluorescence.

Inactivation and reactivation kinetics. (A) Msm was grown in liquid media without atc to early log phase and diluted in fresh medium with atc. Luminescence was normalized to the strain expressing luxAB-DAS constitutively without any regulatory components at time 0. (B) Msm was grown with atc, harvested, washed, and diluted into medium without atc. Msm was grown with atc, harvested, washed, and diluted into medium without atc. Luminescence was normalized to the strain expressing luxAB-DAS constitutively without any regulatory components at the end of the experiment. (C) Similar to A but the bacteria were nonreplicating when atc was added. Filled symbols represent measurements of Msm grown with atc, and open symbols represent measurement from Msm without atc. Data are means ± SEM of 7–11 replicates from two to three independent experiments.
Fig. S4. Growth and survival of BioA cKD mutants. (A) Growth without biotin and without atc. (B) Growth without biotin and with atc. (C) Growth and survival in biotin-free atc-containing medium. atc was present from the beginning of the experiment. Data points were excluded when the cfu recovered on biotin-free, atc-containing plates were more than 10% of those recovered on atc-free plates (identified by *). Data are means ± SEM of four replicates. (D) Emergence of atc resistance in Mtb BioA knockdown mutants. Cultures without revertants are shown as black circles; cultures with revertants are shown in red and purple. The dashed line indicates the limit of detection. OD, optical density; cfu, colony-forming units.

Fig. S5. Relative growth and survival of Mtb BioA mutants in mouse lungs. Mice received doxycycline (doxy)-containing food from day 1 (black), day 10 (orange), day 28 (blue), or not at all (gray). Data are averages of means ± SEM of three or four mice. Dotted lines end in data points for which the relative abundance of a mutant was below the limit of detection in at least two mice. The relative abundances of (A) DUC-control (squares), (B) ΔbioA (triangles), and (C–E) the three bioA mutants (circles) are shown.
Fig. S6. Impact of depleting nicotinamide adenine dinucleotide synthetase (NadE) on replicating Mtb. (A) Growth in 7H9 medium without atc (white symbols) or with atc (black symbols). (B) Survival in atc-containing 7H9 medium. (C) Immunoblot of NadE (Upper) and PrcB (loading control) (Lower) in extracts isolated from the cultures shown in B.
Fig. S7. Histopathology of lungs 224 d postinfection. Each panel shows a section from one lobe at 4× (left column) and 20× magnification (right column).
Fig. S8. Complementation of Mtb NadE-DUC. (A) Survival of Mtb NadE-DUC complemented with a WT copy of nadE during starvation in PBS with and without atc. (B) Survival of WT Mtb, Mtb NadE-DUC, and complemented Mtb NadE-DUC during hypoxia without atc and after adding atc after adaptation to hypoxic conditions for 20 d. (C) Growth of WT Mtb and complemented Mtb NadE-DUC mutant in lungs of doxy-fed mice. Data are means ± SEM of three or four mice.
Table S1. Bacteria and plasmids

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<tr>
<th>Strains and Plasmids</th>
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<tr>
<td>M. tuberculosis H37Rv</td>
<td>Mycobacterium tuberculosis H37Rv</td>
<td>(2)</td>
</tr>
<tr>
<td>Mtb ΔbioA</td>
<td>H37Rv in which bioA was deleted</td>
<td>(3)</td>
</tr>
<tr>
<td>Mtb ΔbioA complemented</td>
<td>Kan’ Nou’ derivative of ΔbioA in which the native copy of bioA is deleted but bioA-DAS+4 was integrated into the attachment site of the phage L5</td>
<td>This work</td>
</tr>
<tr>
<td>Mtb BioA-SspB</td>
<td>Kan’ Nou’ derivative of ΔbioA in which atc induces expression of SspB and causes degradation of BioA</td>
<td>This work</td>
</tr>
<tr>
<td>Mtb BioA-T38</td>
<td>Kan’ Nou’ derivative of ΔbioA in which atc induces repression of bioA by T38</td>
<td>This work</td>
</tr>
<tr>
<td>Mtb BioA-DUC</td>
<td>Kan’ Nou’ derivative of ΔbioA in which atc induces repression of bioA by T38 and degradation of BioA by SspB (dual control)</td>
<td>This work</td>
</tr>
<tr>
<td>Mtb DUC-control</td>
<td>Kan’ Nou’ derivative of H37Rv in which atc induces repression of the nonnative bioA by T38 and degradation of nonnative BioA by SspB but does not affect a second, constitutively expressed copy of bioA</td>
<td>This work</td>
</tr>
<tr>
<td>Mtb ΔnadE attL5</td>
<td>H37Rv in which nadE was deleted from a merodiploid strain</td>
<td>This work</td>
</tr>
<tr>
<td>Mtb NadE-SspB</td>
<td>Zeo’ Nou’ derivative of ΔnadE attL5 in which atc induces expression of SspB and causes degradation of NadE</td>
<td>This work</td>
</tr>
<tr>
<td>Mtb NadE-T38</td>
<td>Zeo’ Nou’ derivative of ΔnadE attL5 in which atc induces repression of nadE by T38</td>
<td>This work</td>
</tr>
<tr>
<td>Mtb NadE-DUC</td>
<td>Zeo’ Nou’ derivative of ΔnadE attL5 in which atc induces repression of nadE by T38 and degradation of NadE by SspB (dual control)</td>
<td>This work</td>
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<tr>
<td>Mtb NadE-DUC complemented</td>
<td>Zeo’ Nou’ Kan’ derivative of NadE-DUC containing a second, constitutively expressed copy of nadE</td>
<td>This work</td>
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</tbody>
</table>

**Plasmids**

<table>
<thead>
<tr>
<th>Plasmids</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pTE-mcs</td>
<td>Hyg⁺; replicates episomally</td>
<td>(4)</td>
</tr>
<tr>
<td>pTC-mcs</td>
<td>Km⁺; integrates into the attachment site of the phage L5 (att-L5)</td>
<td>(4)</td>
</tr>
<tr>
<td>pGMCTnq1-mcs</td>
<td>Nou’; replicates episomally</td>
<td>This work</td>
</tr>
<tr>
<td>pGMCTn-0X1-lacZ</td>
<td>Nou’; contains PtetO-lacZ; integrates into att-Tweety</td>
<td>This work</td>
</tr>
<tr>
<td>pGMCTn-T5C10M1-lacZ</td>
<td>Nou’; contains PtetO-lacZ and tsc10; integrates into att-Tweety</td>
<td>This work</td>
</tr>
<tr>
<td>pGMCK-T0X750-luxAB-DAS+4</td>
<td>Km⁺; contains PtetO-4CSG-luxAB-DAS+4; integrates into att-L5</td>
<td>This work</td>
</tr>
<tr>
<td>pGMCK-T38538-750-luxAB-DAS+4</td>
<td>Km⁺; contains PtetO-4CSG-luxAB-DAS+4 and tetR38; integrates into att-L5</td>
<td>This work</td>
</tr>
<tr>
<td>pGMEH-T0X750-luxAB-DAS+4</td>
<td>Hyg⁺; contains PtetO-4CSG-luxAB-DAS+4 and tetR38; replicates episomally</td>
<td>This work</td>
</tr>
<tr>
<td>pGMEH-T38538-750-luxAB-DAS+4</td>
<td>Hyg⁺; contains PtetO-4CSG-luxAB-DAS+4 and tetR38; replicates episomally</td>
<td>This work</td>
</tr>
<tr>
<td>pGMEH-T0X750-RFP-DAS+4</td>
<td>Hyg⁺; contains PtetO-4CSG-rfp-DAS+4; replicates episomally</td>
<td>This work</td>
</tr>
<tr>
<td>pGMEH-T38538-750-RFP-DAS+4</td>
<td>Hyg⁺; contains PtetO-4CSG-rfp-DAS+4 and tetR38; replicates episomally</td>
<td>This work</td>
</tr>
<tr>
<td>pGMCTn-T5C10M1-sspB</td>
<td>Nou’; contains PtetO-sspB and tsc10; integrates into att-Tweety</td>
<td>This work</td>
</tr>
<tr>
<td>pGMCKq17-0X0X</td>
<td>Kan’; contains qtag20; integrates into att-L5.</td>
<td>This work</td>
</tr>
<tr>
<td>pGMCKq20-T38538-0X</td>
<td>Kan’; contains qtag20 and tetR38; integrates into att-L5.</td>
<td>This work</td>
</tr>
<tr>
<td>pGMCKq22-0X750-bioA-DAS+4</td>
<td>Kan’; contains qtag22, PtetO-4CSG-bioA-DAS+4; integrates into att-L5.</td>
<td>This work</td>
</tr>
<tr>
<td>pGMCKq23-0X750-bioA-DAS+4</td>
<td>Kan’; contains qtag23, PtetO-4CSG-bioA-DAS+4; integrates into att-L5.</td>
<td>This work</td>
</tr>
<tr>
<td>pGMCKq24-T38538-750-bioA-DAS+4</td>
<td>Kan’; contains qtag24, PtetO-4CSG-bioA-DAS+4 and tetR38; integrates into att-L5.</td>
<td>This work</td>
</tr>
<tr>
<td>pGMCKq25-T38538-750-bioA-DAS+4</td>
<td>Kan’; contains qtag25, PtetO-4CSG-bioA-DAS+4 and tetR38; integrates into att-L5.</td>
<td>This work</td>
</tr>
<tr>
<td>pGMCKq26-T38538-750-bioA-DAS+4</td>
<td>Kan’; contains qtag26, PtetO-4CSG-bioA-DAS+4 and tetR38; integrates into att-L5.</td>
<td>This work</td>
</tr>
<tr>
<td>pGMCM-T38538-750-nadE-DAS+4 Zeo’</td>
<td>Zeo’; contains PtetO-4CSG-nadE-DAS+4 and tetR38; integrates into att-L5.</td>
<td>This work</td>
</tr>
<tr>
<td>pGMCKK-PnadE</td>
<td>Kan’; contains constitutively expressed nadE; integrates into the attachment site of the mycobacteriophage Gaul</td>
<td>This work</td>
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</tbody>
</table>