Published in final edited form as:

Nat Med. 2007 December ; 13(12): 1515–1520. doi:10.1038/nm1683.

***In vivo* gene silencing identifies the *Mycobacterium tuberculosis* proteasome as essential for persistence in mice**

Sheetal Gandotra^{1,2}, Dirk Schnappinger^{1,3}, Mercedes Monteleone¹, Wolfgang Hillen⁴, and Sabine Ehrh^{1,2,*}

¹Department of Microbiology and Immunology, Weill Cornell Medical College, New York, NY 10021, USA

²Program in Immunology and Microbial Pathogenesis, Weill Graduate School of Medical Sciences of Cornell University, New York, NY 10021, USA

³Program in Molecular Biology, Weill Graduate School of Medical Sciences of Cornell University, New York, NY 10021, USA

⁴Lehrstuhl für Mikrobiologie, Friedrich-Alexander-Universität Erlangen-Nürnberg, 91058 Erlangen, Germany

Abstract

The success of *Mycobacterium tuberculosis* (Mtb) as a human pathogen relies on its ability to resist eradication by the immune system of healthy individuals. Identification of mechanisms that enable Mtb to persist is key to limiting latent tuberculosis, which affects one third of the world's population. Here we demonstrate that conditional gene silencing makes it possible to determine if an Mtb gene that is required for optimal growth *in vitro* is also important for virulence, and during which phase of an infection it is required. Application of this approach to *prcBA*, which encode the core of the mycobacterial proteasome, reveals an unpredicted essentiality of the core proteasome for persistence of Mtb during the chronic phase of infection in mice. Proteasome depletion also attenuated Mtb in interferon- γ -deficient mice, pointing to a function of the proteasome beyond defence against the adaptive immune response. Genes that are essential for growth *in vitro* and/or *in vivo* account for approximately 20% of Mtb's genome. Conditional gene silencing could therefore facilitate the validation of up to 800 potential Mtb drug targets and improve our understanding of host-pathogen dynamics.

Many life-threatening infections are due to pathogens that commonly establish long-lasting latent infections before causing clinical disease. Approximately 1.8 billion people are estimated to be latently infected with one such pathogen, *Mycobacterium tuberculosis* (Mtb), and at least 5% of these infected individuals will eventually develop tuberculosis and require chemotherapy¹. Mtb survives in an infected host despite the development of strong cell-mediated immunity. In mice, persistence of Mtb is observed as prolonged survival in lungs at nearly constant bacterial titres resulting in a chronic infection². Mtb mutants that are attenuated during chronic infections in experimental animals can identify pathways that may be targeted for the eradication of persistent bacteria. Unfortunately, relatively little is known

*Correspondence and request for materials should be addressed to S.E. (sae2004@med.cornell.edu).

Supplementary Information is linked to the online version of the paper at www.nature.com/nature

Author Contributions S.G. performed experiments. D.S. guided experimental design and analyses. M.M. helped with initial experiments. W.H. provided constructs and advice. S.E. helped with experiments and guided the study. S.G., D.S., S.E. wrote the paper. All authors discussed the results and commented on the manuscript.

The authors declare no competing financial interest.

about the genetic repertoire that Mtb requires to avoid clearance by the immune system and to persist in its host. So far, persistence mutants - including those affected in intermediary metabolism³, cell envelope modification⁴, or heat shock control⁵ - have primarily been identified using deletion or transposon mutagenesis. However, this approach can neither determine which of the many genes that are essential for growth *in vitro*⁶ are required *in vivo*, nor identify which of the genes that are essential for growth *in vivo*⁷ are also required for persistence.

To facilitate the analysis of genes that are essential for growth we previously generated regulated mycobacterial expression systems that are controlled by tetracycline repressors (TetRs)⁸⁻¹⁰. These expression systems allowed the construction of conditional knockdown mutants of *M. smegmatis* and *M. bovis* BCG, in which expression of a single target gene can be silenced either by the addition (Tet-OFF system) or removal of tetracyclines (Tet-ON system)^{8, 10, 11}. For this study we aimed to determine if Tet-ON and Tet-OFF systems also allow generating conditional knockdown mutants for virulent Mtb and if silencing of Mtb genes can be achieved during mouse infections. As the target for gene silencing we selected the mycobacterial 20S proteasome.

The Mtb 20S proteasome core consists of 14 α subunits encoded by *prcA* and 14 β subunits encoded by *prcB*, which assemble in an $\alpha_7\beta_7\beta_7\alpha_7$ complex^{12,13}. Bacterial proteasomes, which are unique to actinomycetes^{14, 15}, are not essential for *in vitro* growth of *Streptomyces coelicolor*, *S. lividans*, or *M. smegmatis*¹⁶⁻¹⁸. In contrast, *prcA* and *prcB* were predicted to be essential for optimal *in vitro* growth of Mtb⁶. Inactivation of two proteasome-associated genes, *mpa* and *paflA*, reduced Mtb virulence in mice, suggesting that the proteasome is also important for pathogenicity^{19, 20}. *Mpa* encodes a proteasomal ATPase, which is homologous to ATPases found in the regulatory cap of the eukaryotic 26S proteasome and likely involved in substrate recognition, unfolding and translocation into the proteasome core^{15,21,22}. A biochemical activity for PafA remains to be discovered, but it appears to play a role similar to that of Mpa in protein degradation²³. Here we report the construction of mutants that allow conditional silencing of the proteasome α and β subunits and analyzed the role of the Mtb proteasome for *in vitro* growth, for survival under protein damaging conditions and during *in vivo* growth and persistence in mice.

Results

Construction of conditional *prcBA* knockdown mutants

To generate mutants that allow conditional silencing of the Mtb proteasome, we integrated P_{myc1}tetO, a tetracycline responsive mycobacterial promoter⁸, 5' of *prcBA*, generating strain H37Rv P_{myc1}tetO:*prcBA* (Fig. 1a, b). Placement of P_{myc1}tetO 5' of *prcBA* did not alter proteasome expression or growth of Mtb (Supplementary Fig. 1). Next, we transformed H37Rv P_{myc1}tetO:*prcBA* with plasmids expressing wild type Tet repressor (wtTetR) or reverse TetR (revTetR)¹⁰. wtTetR represses P_{myc1}tetO in the absence of tetracyclines, whereas revTetR requires tetracyclines as cofactors for binding to and silencing of P_{myc1}tetO²⁴. Addition of anhydrotetracycline (atc) thus induces expression of *prcBA* in H37Rv P_{myc1}tetO:*prcBA*/wtTetR (hereafter, PrcBA_{Tet-ON}) and silences expression of *prcBA* in H37Rv P_{myc1}tetO:*prcBA*/revTetR (PrcBA_{Tet-OFF}).

We analyzed efficiencies and kinetics of *prcBA* silencing in PrcBA_{Tet-ON} and PrcBA_{Tet-OFF} using immunoblot, mRNA quantification and proteasome activity assays (Fig. 1c-f). By immunoblot, the β subunit was reduced to levels below the limit of detection following removal of atc from PrcBA_{Tet-ON} cultures or addition of atc to PrcBA_{Tet-OFF} cultures (Fig. 1c). *PrcBA* transcripts were reduced 15- to 20-fold after silencing in both mutants (Fig. 1d). Atc did not affect PrcB protein or *prcBA* transcript levels in the absence of wtTetR or

revTetR (Fig. 1c, d), demonstrating that silencing of *prcBA* was TetR-dependent. To analyze proteasome activity we measured the peptidolytic activity of Mtb lysates against succinyl-Leu-Leu-Val-Tyr-aminomethyl coumarin (Suc-LLVY-Amc), a fluorescent peptide substrate of the Mtb proteasome¹³. Four days after silencing was initiated, proteasome activity was reduced ~100 and 10-fold in the PrcBA_{Tet-OFF} and PrcBA_{Tet-ON} mutants, respectively (Fig. 1e). By 7 days proteasome activity was below the level of detection in both mutants. A similar difference in the kinetics of silencing proteasome expression between the Tet-ON and Tet-OFF strains was found in immunoblot analyses (Fig. 1f). Together, these experiments demonstrate that PrcBA_{Tet-ON} and PrcBA_{Tet-OFF} allowed rapid and efficient silencing of the mycobacterial proteasome by removing (wtTetR, PrcBA_{Tet-ON}) or adding (revTetR, PrcBA_{Tet-OFF}) atc, respectively. Silencing was slightly more efficient in the Tet-ON mutant but somewhat faster in the Tet-OFF mutant. These differences are likely due to the lower activity of revTetR compared to wtTetR in mycobacteria¹⁰ and residual atc bound to Mtb after transfer of a Tet-ON strain into medium not containing atc.

Impact of *prcBA* silencing on *in vitro* growth and resistance to nitrosative and oxidative stress

We next sought to determine the impact of *prcBA* silencing on *in vitro* growth and survival under nitrosative stress because previous studies predicted that the Mtb proteasome is essential for optimal growth on agar plates and required for defence against reactive nitrogen intermediates (RNI)^{6,19}. Proteasome depletion impaired growth of Mtb on agar plates and, to a small degree, in liquid culture (Fig. 2a), and increased susceptibility to RNI (Fig. 2b). After exposure to acidified nitrite for 3 days, silencing in the PrcBA_{Tet-ON} mutant led to a 10-fold decrease in survival ($p=0.005$, Fig. 2b, left panel). *PrcBA* silencing in the Tet-OFF mutant did not significantly reduce the number of bacteria recovered after RNI stress ($p=0.116$, Fig. 2b, middle panel) suggesting that silencing in this mutant was not sufficient to cause decreased survival in this assay.

Mutants in the proteasome-associated genes *mpa* and *paflA* were more resistant to H₂O₂ than wild type Mtb¹⁹ and similarly, depletion of the proteasome resulted in increased resistance to oxidative stress ($p=0.002$ for PrcBA_{Tet-ON} and $p=0.007$ for PrcBA_{Tet-OFF}, Fig. 2c). The proteasome did not confer resistance or susceptibility to another physiological stress, acidic pH (data not shown). Atc did not affect survival of the control strain (H37Rv P_{myc1}tetO:*prcBA*/no TetR) in the presence of acidified nitrite or H₂O₂ (Fig. 2b, right panel). In summary, these data support the proposed essentiality of *prcBA* for optimal growth on agar plates and the proposed functional association of Mpa and PrcBA.

Impact of *prcBA* silencing on growth and persistence of Mtb in mice

We next used PrcBA_{Tet-ON} and PrcBA_{Tet-OFF} to determine the role of the proteasome during growth and persistence of Mtb in mice. Tet-ON systems have been applied to infection models for pathogens such as *Staphylococcus aureus* and *Yersinia pestis*^{25,26} but have not heretofore allowed analysis of Mtb mutants during mouse infections. Moreover, application of revTetR-dependent Tet- OFF systems to the *in vivo* analysis of a bacterial pathogen has not been reported. We first constructed an Mtb reporter strain containing *gfp* (encoding green fluorescent protein) controlled by P_{myc1}tetO and wtTetR (GFP_{Tet-ON}). Feeding GFP_{Tet-ON} infected mice doxycycline, a tetracycline derivative, induced green fluorescence in pulmonary Mtb, while no fluorescent bacteria were detected in mice that had not received doxycycline (Fig. 3). This demonstrated that the mycobacterial Tet-ON system allows control of Mtb gene expression during mouse infections. Next, we infected mice with the *prcBA* mutants and initiated proteasome silencing at day 1 (PrcBA_{Tet-ON}), day 7 (PrcBA_{Tet-OFF}) or day 21 (PrcBA_{Tet-OFF}) in different mice (Fig. 4). In C57BL/6 mice, the onset of the adaptive immune response at around 3 weeks post infection results in control of

Mtb replication, such that titres remain stable for many months²⁷ (Fig. 4d). In contrast, bacterial counts in lungs measured 112 days post infection were reduced by 140-fold (PrcBA_{Tet-ON}) and 500-fold (PrcBA_{Tet-OFF}) as a consequence of *prcBA* silencing ($p=0.028$ and $p=0.0038$) (Fig. 4a, b). Even when *prcBA* silencing was only initiated at the onset of the chronic phase of the infection (day 21, PrcBA_{Tet-OFF}), bacterial loads in lungs and spleens were 36-fold and 22-fold lower than those of the control groups at day 112 (Fig. 4b, c). Thus, the core proteasome is essential for persistence of Mtb in mice.

A less dramatic effect of *prcBA* silencing was observed for growth of Mtb during the acute phase. Colony forming units obtained at day 21 were 2-fold reduced after silencing of *prcBA* in the PrcBA_{Tet-ON} mutant. This difference was larger (7-fold) for the PrcBA_{Tet-OFF} mutant, presumably due to the faster kinetics of proteasome silencing in this strain (Fig. 4a, b). The reduced growth of proteasome-depleted strains during the acute phase suggested that depletion of the Mtb proteasome during an infection of immune-compromised mice may extend their lifespan. IFN γ ^{-/-} mice cannot control growth of Mtb²⁸. We infected these mice with the PrcBA_{Tet-OFF} mutant, allowed bacterial replication for 10 days and then induced *prcBA* silencing. This extended the mean survival time of the mice from 54 days to 81 days ($p<0.0001$) (Fig. 4f). The Mtb proteasome is thus also essential for optimal *in vivo* growth and its function goes beyond providing defence against the IFN γ -dependent elements of the host's immune response.

Discussion

The genes encoding the Mtb core proteasome, *prcB* and *prcA*, were among the approximately 600 genes of the Mtb genome predicted to be essential or required for optimal growth *in vitro*⁶. This suggested that *prcBA* deletion mutants cannot be obtained and we therefore used conditional gene silencing to determine if *prcBA* are important for virulence of Mtb. Both Tet-ON and Tet-OFF systems allowed efficient *prcBA* silencing and resulted in significant decreases in proteasome transcript and protein levels as well as proteasome activity. The silencing kinetics of the Tet-OFF system were slightly faster than those achieved with the Tet-ON system even though the final proteasome level was lower after silencing with the Tet-ON system. Silencing of *prcBA* significantly delayed growth of Mtb on agar plates, which confirmed that the proteasome is required for optimal growth on this medium. However, *prcBA* silencing hardly affected growth in liquid culture even though *prcBA* transcript levels were reduced more than 10-fold and proteasome activity was reduced approximately 100-fold. This suggests that *prcBA* may not be essential for growth in liquid medium.

We further validated the efficiency of *prcBA* silencing by demonstrating that depletion of the proteasome rendered Mtb more susceptible to RNI and increased its resistance to H₂O₂. These results are consistent with the phenotypes of mutants of the proteasome associated genes *mpa* and *pafA*¹⁹. Increased resistance to organic peroxide has also been reported for *S. coelicolor* proteasome mutants and was accompanied by increased levels of haloperoxidase²⁹. The mechanisms by which the core proteasome protects Mtb against nitrosative damage may include removal of nitrosylated proteins, as demonstrated in mammalian cells³⁰. However, our and previous data suggest that the role of the Mtb proteasome extends beyond defence against RNI. Infection of IFN γ -deficient mice with proteasome-depleted Mtb extended their life span compared to mice infected with proteasome-expressing Mtb. Similarly, an Mtb *mpa* mutant was not fully virulent in iNOS-deficient mice¹⁹.

Silencing of *prcBA* transcription during mouse infections demonstrated that the Mtb proteasome is not only required for optimal *in vivo* growth but is also essential during the

chronic phase of the infection, when the pathogen replicates slowly or not at all^{31,32}. The eukaryotic proteasome participates in protein turnover, transcription and DNA repair³³. It is unclear by which of these processes Mtb's proteasome allows the pathogen to persist in its host. Mutants of the proteasomal ATPase Mpa suggested that the Mtb core proteasome is required for growth of Mtb in mice^{19,20} but did not predict a role for the proteasome during the chronic phase of infection.

Analyses of *prcBA* provided proof-of-principle that transcriptional silencing permits inactivation of Mtb genes during different stages of an infection in mice. Reporter gene studies and experiments with other mutants demonstrated that TetR-mediated gene silencing is atc-dose dependent^{8,10}. This suggests that gene silencing could determine to which extent a gene has to be inactivated before mycobacterial growth or survival are impaired. Both of these features, conditionality and dose-responsiveness, distinguish gene silencing from deletion and transposon mutagenesis and are not only important for functional analyses but also relevant to the development of new drugs against tuberculosis. Chemotherapy must be administered during different phases of an infection, including chronic disease, and is likely most effective if it inhibits processes that are essential during all stages of an infection³⁴. Conditional gene silencing facilitates the genetic identification of such processes. In addition, the development of drugs that completely inactivate a target is difficult. Experiments that use gene silencing to partially inactivate a gene might therefore help to focus drug development on targets whose inactivation is effective even if it is incomplete.

In addition to the opportunities that gene silencing offers, the approach also has its challenges. The true impact of target inactivation might be masked by compensatory mutations, epigenetic changes or the residual transcription that occurs despite silencing. Careful analysis of bacterial populations after gene silencing, for example with respect to the frequency of suppressor mutations and the residual expression of the target protein, are required to interpret experiments in which gene silencing does not lead to a phenotypic effect. In addition, for some genes it might be necessary to tailor the activity of the regulated promoter to the target gene's native expression level to achieve promoter replacement. This could be accomplished with a library of well-regulated promoters that differ in their induced activities. The continued improvement of gene silencing tools for mycobacteria should therefore further facilitate the validation of targets for the development of new drugs against tuberculosis.

METHODS

Strains, media and culture conditions

Wild type Mtb (H37Rv) was obtained from Dr. Robert North, Trudeau Institute. Mycobacteria were grown in Middlebrook 7H9 medium (Difco) with 0.2% glycerol and 0.05% Tween 80, supplemented with 0.5% BSA, 0.2% dextrose and 0.085% sodium chloride (ADN). Recombinant mycobacteria were selected on 50 µg/ml hygromycinB (hyg) and 15 µg/ml kanamycin (kan) as required. Anhydrotetracycline (atc) was used at 100 ng/ml and replenished every 4 days in liquid culture.

Construction of *prcBA*_{Tet-ON} and *prcBA*_{Tet-OFF} mutants

P_{myc1}tetO was integrated 5' of *prcBA* on the Mtb chromosome via homologous recombination following transduction with temperature sensitive mycobacteriophage³⁵. To achieve this, 768 bp 5' of the start codon of *prcB* and the first 675 bp of *prcB* were amplified by PCR from H37Rv genomic DNA. The DNA fragments were cloned to flank the hygromycin resistance gene in pJSC284, a derivative of pYUB854 containing a lambda cos site, and a unique PacI site³⁵ (gift of J.S. Cox). P_{myc1}tetO was cloned 5' of the start codon of

prcB. A transcriptional terminator was cloned 3' of the hygromycin resistance gene. The plasmid was ligated with temperature-sensitive phage Φ 87^{4,35} and the resulting phage was used to infect Mtb as previously described^{4,36}. Hyg-resistant transductants selected on 7H11 agar plates with 50 μ g/ml hyg for 3 weeks were analyzed by Southern blot. Recombinants were transformed by electroporation with plasmids encoding wtTetR, revTetR, or no TetR and transformants selected on hyg and kan, in the presence (wtTetR) and absence (revTetR, no TetR) of atc.

Analysis of *prcBA* mRNA by quantitative real time PCR (qRTPCR) and PrcB protein by immunoblot

PrcBA_{Tet-ON} and PrcBA_{Tet-OFF} mutants were grown in the presence or absence of atc to OD_{580nm} of 0.8–1.0. RNA was prepared from 10 ml cultures and cDNA was synthesized using random hexamers. A reaction lacking reverse transcriptase was performed for every RNA sample. Real-time PCR was performed as described³⁶ using specific TaqMan probes for *prcA*, *sigA*, *16S rRNA*, and *rpoB*. For immunoblots, cell lysates were prepared by bead-beating in PBS containing protease inhibitor cocktail (Complete Mini, Roche). 15 μ g cell lysates were subjected to SDS237 PAGE, followed by transfer to a nitrocellulose membrane. Blots were probed with anti-PrcB and anti-DlaT rabbit sera and developed using Immobilon Western Chemiluminescent HRP substrate (Millipore).

Proteasome activity assay

Bacteria were grown to OD_{580nm} 1.0 in the presence or absence of atc and cell pellets from 10 ml cultures were lysed in PBS containing protease inhibitor cocktail (Complete Mini, Roche) using a bead beater. Clarified lysates were sterilized by passage through a 0.2 μ m filter. Proteasome activity was determined as described elsewhere¹³. Briefly, 50 μ g of lysate was incubated with 100 μ M Succinyl-Leu-Leu-Val-Tyr-aminomethyl coumarin (Suc-LLVY-AMC) in 20 mM HEPES, 0.5 mM EDTA buffer and fluorescence was monitored at excitation of 370 nm and emission of 430 nm at 37°C over 60 minutes.

In vitro stress susceptibility assays

Mtb cultures were grown for 7 days to log phase (OD_{580nm} 0.6) in the presence and absence of 100 ng/ml atc. Single cell suspensions of cultures were prepared by centrifugation at 800 rpm for 10 minutes and subsequently diluted to OD_{580nm} 0.01. To test susceptibility to RNI, cultures were diluted and incubated at pH 5.5 with or without 3mM NaNO₂ for 3 days at 37°C. To test susceptibility to H₂O₂, cultures were incubated at pH 6.8 with or without 5mM H₂O₂ for 4h at 37°C. Cultures containing atc were maintained in medium with atc during the course of the experiment. To determine viability, serial dilutions of cultures were plated on 7H11 plates with atc for H37Rv PrcBA_{Tet-ON} and on plates without atc for all other strains.

Animal infections

The PrcBA_{Tet-ON} mutant was grown in the presence of atc, the PrcBA_{Tet-OFF} mutant and H37Rv/revTetR were grown in the absence of atc to early log phase and washed single cell suspensions were used to infect female wild type and IFN γ -deficient (Jackson Laboratory) C57/BL6 mice via aerosol, as described previously³⁶. Groups of infected mice received 1 mg/ml doxycycline (doxy) in the drinking water containing 5% sucrose. Doxy-containing water was kept in light-protected bottles and changed twice a week. Bacterial burden in lungs and spleen was determined by plating for colony forming units (cfu) at indicated times. The log-rank test was used to determine statistical significance of survival differences observed in PrcBA_{Tet-OFF} mutant infected IFN γ ^{-/-} mice (GraphPad Prism 4.0).

Fluorescent microscopy of Mtb in mouse lungs

Mice were infected by aerosol with 100 cfu of Mtb transformed with $P_{myc1}tetO-gfp$ or $P_{myc1}tetO-gfp/wtTetR$. Two weeks post infection one group of mice received 1mg/ml doxy in the drinking water containing 5% sucrose while the control groups were kept without doxy. Six days later mice were sacrificed, lung tissues were fixed for 24–36 hrs in 10% formalin and frozen 10 mm thick sections of lung tissues were analyzed microscopically. Images were acquired with a Leica DMIRB widefield microscope using a 40× 1.25 and a 100× 1.25 numerical aperture objective and a 1.5× magnification changer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank G. Lin and C. Nathan for anti-PrcB antiserum; R. Bryk and C. Nathan for anti-DlaT antiserum, L. M. Pierini for help with fluorescent microscopy; E. Hwang for technical support; C. Nathan and G. Lin for helpful discussions; and K.H. Darwin and C. Nathan for review. Supported by NIH AI63446 (S.E.), the I.T. Hirsch Trust (S.E.), the Ellison Medical Foundation (D.S.), Deutsche Forschungsgemeinschaft through SFB 473 (W.H.), Fonds der Chemischen Industrie (W.H.), a grant from the Bill and Melinda Gates Foundation and the Wellcome Trust through the Grand Challenges in Global Health Initiative (S.E., D.S.). The Dept. of Microbiology and Immunology acknowledges the support of the William Randolph Hearst Foundation.

References

1. Corbett EL, et al. The growing burden of tuberculosis: global trends and interactions with the HIV epidemic. *Arch Intern Med*. 2003; 163:1009–21. [PubMed: 12742798]
2. Gomez JE, McKinney JDM. tuberculosis persistence, latency, and drug tolerance. *Tuberculosis (Edinb)*. 2004; 84:29–44. [PubMed: 14670344]
3. McKinney JD, et al. Persistence of Mycobacterium tuberculosis in macrophages and mice requires the glyoxylate shunt enzyme isocitrate lyase. *Nature*. 2000; 406:735–8. [PubMed: 10963599]
4. Glickman MS, Cox JS, Jacobs WR Jr. A novel mycolic acid cyclopropane synthetase is required for cording, persistence, and virulence of Mycobacterium tuberculosis. *Mol Cell*. 2000; 5:717–27. [PubMed: 10882107]
5. Stewart GR, et al. Overexpression of heat-shock proteins reduces survival of Mycobacterium tuberculosis in the chronic phase of infection. *Nat Med*. 2001; 7:732–7. [PubMed: 11385512]
6. Sassetti CM, Boyd DH, Rubin EJ. Genes required for mycobacterial growth defined by high density mutagenesis. *Mol Microbiol*. 2003; 48:77–84. [PubMed: 12657046]
7. Sassetti CM, Rubin EJ. Genetic requirements for mycobacterial survival during infection. *Proc Natl Acad Sci U S A*. 2003; 100:12989–94. [PubMed: 14569030]
8. Ehrt S, et al. Controlling gene expression in mycobacteria with anhydrotetracycline and Tet repressor. *Nucleic Acids Res*. 2005; 33:e21. [PubMed: 15687379]
9. Ehrt S, Schnappinger D. Controlling gene expression in mycobacteria. *Future Microbiol*. 2006; 1:177–84. [PubMed: 17661663]
10. Guo XV, et al. Silencing essential protein secretion in Mycobacterium smegmatis using tetracycline repressors. *J Bacteriol*. 2007; 189:4614–4623. [PubMed: 17483222]
11. Chalut C, Botella L, de Sousa-D'Auria C, Houssin C, Guilhot C. The nonredundant roles of two 4'-phosphopantetheinyl transferases in vital processes of Mycobacteria. *Proc Natl Acad Sci U S A*. 2006; 103:8511–6. [PubMed: 16709676]
12. Hu G, et al. Structure of the Mycobacterium tuberculosis proteasome and mechanism of inhibition by a peptidyl boronate. *Mol Microbiol*. 2006; 59:1417–28. [PubMed: 16468986]
13. Lin G, et al. Mycobacterium tuberculosis prcBA genes encode a gated proteasome with broad oligopeptide specificity. *Mol Microbiol*. 2006; 59:1405–16. [PubMed: 16468985]

14. De Mot R, Nagy I, Walz J, Baumeister W. Proteasomes and other self307 compartmentalizing proteases in prokaryotes. *Trends Microbiol.* 1999; 7:88–92. [PubMed: 10081087]
15. Butler SM, Festa RA, Pearce MJ, Darwin KH. Self-compartmentalized bacterial proteases and pathogenesis. *Mol Microbiol.* 2006; 60:553–62. [PubMed: 16629660]
16. Knipfer N, Shrader TE. Inactivation of the 20S proteasome in *Mycobacterium smegmatis*. *Mol Microbiol.* 1997; 25:375–83. [PubMed: 9282749]
17. Nagy I, et al. Characterization of a novel intracellular endopeptidase of the alpha/beta hydrolase family from *Streptomyces coelicolor* A3(2). *J Bacteriol.* 2003; 185:496–503. [PubMed: 12511496]
18. Hong B, et al. Inactivation of the 20S proteasome in *Streptomyces lividans* and its influence on the production of heterologous proteins. *Microbiology.* 2005; 151:3137–45. [PubMed: 16151224]
19. Darwin KH, Ehrt S, Gutierrez-Ramos JC, Weich N, Nathan CF. The proteasome of *Mycobacterium tuberculosis* is required for resistance to nitric oxide. *Science.* 2003; 302:1963–6. [PubMed: 14671303]
20. Lamichhane G, et al. Deletion of a *Mycobacterium tuberculosis* proteasomal ATPase homologue gene produces a slow-growing strain that persists in host tissues. *J Infect Dis.* 2006; 194:1233–40. [PubMed: 17041849]
21. Darwin KH, Lin G, Chen Z, Li H, Nathan CF. Characterization of a *Mycobacterium tuberculosis* proteasomal ATPase homologue. *Mol Microbiol.* 2005; 55:561–71. [PubMed: 15659170]
22. Pickart CM, Cohen RE. Proteasomes and their kin: proteases in the machine age. *Nat Rev Mol Cell Biol.* 2004; 5:177–87. [PubMed: 14990998]
23. Festa RA, Pearce MJ, Darwin KH. Characterization of the proteasome accessory factor (paf) operon in *Mycobacterium tuberculosis*. *J Bacteriol.* 2007; 189:3044–50. [PubMed: 17277063]
24. Berens C, Hillen W. Gene regulation by tetracyclines. Constraints of resistance regulation in bacteria shape TetR for application in eukaryotes. *Eur J Biochem.* 2003; 270:3109–21. [PubMed: 12869186]
25. Ji Y, et al. Identification of critical staphylococcal genes using conditional phenotypes generated by antisense RNA. *Science.* 2001; 293:2266–9. [PubMed: 11567142]
26. Lathem WW, Price PA, Miller VL, Goldman WE. A plasminogen-activating protease specifically controls the development of primary pneumonic plague. *Science.* 2007; 315:509–13. [PubMed: 17255510]
27. North RJ, Jung YJ. Immunity to tuberculosis. *Annu Rev Immunol.* 2004; 22:599–623. [PubMed: 15032590]
28. Flynn JL, et al. An essential role for interferon gamma in resistance to *Mycobacterium tuberculosis* infection. *J Exp Med.* 1993; 178:2249–54. [PubMed: 7504064]
29. De Mot R, Schoofs G, Nagy I. Proteome analysis of *Streptomyces coelicolor* mutants affected in the proteasome system reveals changes in stress-responsive proteins. *Arch Microbiol.* 2007
30. Grune T, et al. Peroxynitrite increases the degradation of aconitase and other cellular proteins by proteasome. *J Biol Chem.* 1998; 273:10857–62. [PubMed: 9556559]
31. Rees RJ, Hart PD. Analysis of the host-parasite equilibrium in chronic murine tuberculosis by total and viable bacillary counts. *Br J Exp Pathol.* 1961; 42:83–8. [PubMed: 13740304]
32. Munoz-Elias EJ, et al. Replication dynamics of *Mycobacterium tuberculosis* in chronically infected mice. *Infect Immun.* 2005; 73:546–51. [PubMed: 15618194]
33. Demartino GN, Gillette TG. Proteasomes: machines for all reasons. *Cell.* 2007; 129:659–62. [PubMed: 17512401]
34. Duncan K, Barry CE 3rd. Prospects for new antitubercular drugs. *Curr Opin Microbiol.* 2004; 7:460–5. [PubMed: 15451500]
35. Bardarov S, et al. Specialized transduction: an efficient method for generating marked and unmarked targeted gene disruptions in *Mycobacterium tuberculosis*, *M. bovis* BCG and *M. smegmatis*. *Microbiology.* 2002; 148:3007–17. [PubMed: 12368434]
36. Shi S, Ehrt S. Dihydrolipoamide acyltransferase is critical for *Mycobacterium tuberculosis* pathogenesis. *Infect Immun.* 2006; 74:56–63. [PubMed: 16368957]

Figure 1. Tet-ON and Tet-OFF systems allow efficient and rapid silencing of proteasome expression

(a) Map of the *prcBA* genomic region in H37Rv wild type (top) and in H37Rv *P_{myc1tetO}:prcBA* (bottom). Probe location and EcoRV restriction sites are indicated. (b) Southern blot of EcoRV digested genomic DNA from wild type H37Rv and from three transductants of H37Rv *P_{myc1tetO}:prcBA* (t1, t2, t3) probed with the DNA fragment indicated in (a). (c) PrcB levels analyzed by immunoblot in H37Rv *P_{myc1tetO}:prcBA* without TetR and transformed with wtTetR (*PrcBA_{Tet-ON}*, left panel) and with revTetR (*PrcBA_{Tet-OFF}*, right panel) after growth for 7 days in the presence and absence of anhydrotetracycline (atc). Dihydrolipoamide acyltransferase (DlaT) was used as loading control. (d) *PrcBA* transcript levels after transformation of H37Rv *P_{myc1tetO}:prcBA* with wtTetR (*PrcBA_{Tet-ON}*, left panel) and with revTetR (*PrcBA_{Tet-OFF}*, right panel) and growth

for 7 days in the presence of atc (white bars) and in the absence of atc (black bars). H37Rv $P_{\text{myc1tetO}}:prcBA$ not transformed with TetR (no TetR) was included as control; normalization of *prcBA* transcript to *rpoB* and *16S rRNA* instead of *sigA* gave similar results (data not shown); data are means $\pm$ s.e. of triplicate samples. **(e)** Proteasome activities in $\text{PrcBA}_{\text{Tet-ON}}$ (circles, left panel) and $\text{PrcBA}_{\text{Tet-OFF}}$ (diamonds, right panel) mutants in presence (open symbols) and absence (filled symbols) of atc; nd = not detected. **(f)** PrcB levels in $\text{PrcBA}_{\text{Tet-ON}}$ (left panel) and $\text{PrcBA}_{\text{Tet-OFF}}$ (right panel) mutants in the presence and absence of atc at indicated time points. DlaT was used as loading control.

Figure 2. *In vitro* phenotypes caused by *prcBA* silencing

(a) Growth in liquid culture determined by optical density and on agar plates (insets) of PrcBA_{Tet-ON} (left panel) and PrcBA_{Tet-OFF} (middle panel) mutants and wild type Mtb (H37Rv, no TetR, right panel) in the presence (open symbols, +) and absence (filled symbols, -) of anhydrotetracycline (atc). (b) Susceptibility of PrcBA_{Tet-ON} and PrcBA_{Tet-OFF} mutants to RNI and H₂O₂. The indicated strains were grown for 7 days in the presence or absence of atc and then exposed to 3 mM NaNO₂ at pH 5.5 for 3 days and 5 mM H₂O₂ at pH 6.8 for 4 hours at 37°C in the presence (white bars) and absence (black bars) of atc and enumerated by plating for colony-forming-units (cfu); data are means ± s.e. of 2 independent experiments each with triplicate cultures (exposure to NaNO₂) or 1 experiment representative of 2 with triplicate cultures (exposure to H₂O₂).

Figure 3. Induction of GFP expression in Mtb in mouse lungs

(a) Lung sections from mice infected with Mtb transformed with *P_{myc1}tetO-gfp* (upper panels) and *P_{myc1}tetO-gfp/wtTetR* (*GFP_{Tet-ON}*, middle and lower panels). Two weeks post infection a group of mice (lower panels) received doxycycline (doxy) for 6 days in the drinking water while the control groups were kept without doxy (middle panels). The left panels depict lung tissues after rhodamine-auramine staining to detect Mtb (magnification: 60×). The middle and right panels show unstained fluorescent images obtained with a GFP filter at two magnifications (60× and 150×). (b) Bacterial titres in lungs of mice infected with *P_{myc1}tetO-GFP* (green bars) and *P_{myc1}tetO/wtTetR* (*GFP_{Tet-ON}*). CfU from mice treated with doxy starting 2 days and 14 days post infection are depicted as yellow and orange bars, respectively. Control groups were kept without doxy (green and black bars). Data are means $\pm$ s.d. from 4 mice per group and time point. CfU counts from mice treated with doxy at day 12 were not significantly different from the other two data points according to a Student's t-test.

Figure 4. The proteasome is essential for optimal growth and persistence of *Mtb* in mice
(a) Mice were infected by aerosol with the PrcBA_{Tet-ON} mutant and administered doxycycline (doxy, green squares) in the drinking water or given regular water (blue diamonds). Bacterial loads in lungs were enumerated by plating for cfu at indicated time points. **(b, c, d, e)** Mice were infected by aerosol with **(b, c)** the PrcBA_{Tet-OFF} mutant and **(d, e)** wild type H37Rv transformed with revTetR. Mice were given doxy starting on day 7 (green squares, bars) and day 21 (orange triangles, bars) or given regular water (blue diamonds, bars). Bacterial loads in lungs **(b, d)** and spleens **(c, e)** were enumerated by plating for cfu at indicated time points. Data are means $\pm$ s.d. of 4 mice per group and time point and are representative of 2 independent experiments. **(f)** Survival of IFN γ ^{-/-} mice infected by aerosol with the PrcBA_{Tet-OFF} mutant. At day 10 post infection half (n=10) of the infected mice received doxy in their drinking water (green squares) the other half received regular water (blue diamonds).