Supporting Information

Molecular connectivity between extra-cytoplasmic sigma factors and PhoP accounts for coupled mycobacterial stress response

Harsh Goar[1], Partha Paul[1], Hina Khan[1], and Dibyendu Sarkar[1,*]

[1]CSIR-Institute of Microbial Technology, Sector 39 A, Chandigarh 160036, India

Running title: Coupling mycobacterial pH stress and redox stress response

Key Words: Mycobacterium; PhoP; Redox stress; SigH; pH stress; thiol homeostasis

*Address correspondence to: Dibyendu Sarkar, CSIR-Institute of Microbial Technology,

Tel.: 091-172-2880258; Fax: 091-172-2690585; E-mail: dibyendu@imtech.res.in

Table S4

Oligonucleotide primer sequences and plasmids used for amplifications and cloning in this study

Primers	Sequence (5'-3') or relevant genotype	Reference
GST-phoPstart	CCTGGATCCATGCGGAAAGGGGTT	(1)
RPphoPN	AATAATCTCGAGGCGTCGCAGGATGA	This study
FPphoPC	AATAATGGATCCAAGGGCAACAAGGAACCA	This study
GST-phoPstop	GGTCTCGAGTCGAGGCTCCCGCAG	(1)
FPsigH19K	AATAATGGATCCATGGCCGACATC	This study
RPsigH19K	AATAAGCTTTCACTTGTCGTCATCGTCTTTGTA GTCTGACGACACCCCCT	This study
FPrshA	AATAATCAATTGGTGAGCGAAAAT	This study
RPrshA	AATAATAAGCTTCTAGGGCCCTCC	This study
Plasmids		
pGEX-4T-1[a]	*E. coli* cloning vector	GE-Healthcare
pGEX-*phoP*	PhoP residues 1-247 cloned in pGEX-4T-1	(1)
pGEX-*phoPN*	PhoP residues 1-141 cloned in pGEX-4T-1	This study
pGEX-*phoPC*	PhoP residues 141-247 cloned in pGEX-4T-1	This study
pET-28c[c]	*E. coli* cloning vector	Novagen
pET-SigE	His_6-tagged SigE expression plasmid	(2)
pET-SigH	His_6-tagged SigH expression plasmid	(2)
p19Kpro[b]	Mycobacterial expression vector	(3)
p19Kpro-*phoP*-His	PhoP residues 1–247 cloned in p19Kpro with His_6-tag	(4)
p19Kpro-*phoP*-FLAG	PhoP residues 1–247 cloned in p19Kpro with FLAG tag	(5)
p19Kpro-*sigH*-FLAG	SigH residues 1-216 cloned in p19Kpro with FLAG tag	This study
Mrx1-roGFP2[b]	Mycoredoxin gene cloned upstream of redox sensitive GFP	(6)
pSM128[d]	Integrative promoter probe vector for mycobacteria	(7)
pSM-trxB1up	trxB1up-*lacZ* fusion in pSM128	This work
pUAB300[b]	Episomal mycobacteria-*E. coli* shuttle plasmid	(8)
pUAB300-*sigE*	SigE residues 1-257 cloned in pUAB300	(2)
pUAB300-*sigH*	SigH residues 1-216 cloned in pUAB300	(2)
pUAB400[c]	Integrative mycobacteria-*E. coli* shuttle plasmid	(8)
pUAB400-*phoP*	PhoP residues 1–247 cloned in pUAB400	(9)
pUAB400-*phoPN*	PhoP residues 1–141 cloned in pUAB400	(5)
pUAB400-*phoPC*	PhoP residues 141–247 cloned in pUAB400	(5)
pUAB400-*cfp10*	CFP10 residues 1-100 cloned in pUAB400	(2)
pUAB400-*rshA*	RshA residues 1-101 cloned in pUAB400	This study

[a] ampicillin resistance; [b] hygromycin resistance; [c] kanamycin resistance; [d] streptomycin resistance

FP, forward primer; RP, reverse primer

Table S5

Oligonucleotide primers used in RT-qPCR and ChIP experiments of this study

Primers	Sequence or description (5'-3')	Reference
FPtrxB1RT	GAAACCATCCAAAGCAGCGA	This study
RPtrxB1RT	GGGGATGGATCGGATCTGAG	This study
FPtrxB2RT	GTGGAGAACTACCCGGGATT	This study
RPtrxB2RT	CCCGTGAAGTGATACCGACT	This study
FPtrxCRT	GGTTGACTTTTGGGCGACAT	This study
RPtrxCRT	TCGAGACGACCTGGAAGTTG	This study
FPphoPRT	GCCTCAAGTTCCAGGGCTTT	This study
RPphoPRT	CCGGGCCCGATCCA	This study
FPpks2RT	GTTGTGGAAGGCGTTGTTAC	(10)
RPpks2RT	GTCGTAGAACTCGTCGCAAT	(10)
FPgapdhRT	AGTAGGCATCAACGGGTTTG	(9)
RPgapdhRT	GTGCTGTTGTCGGTGATGTC	(9)
FPtrxB1up	TGGCCTCGATCGCCGGGGTC	This study
RPtrxB1up	GTGTTTTTCCGACGACTCGGCAA	This study
FPtrxB2up	ACGCTCGGCCCGCCGTACTGCTGG	This study
RPtrxB2up	GGCACGGGCGGCGTAGAGCG	This study
FPtrxC ORF	GTACCACCAGCACCTCACT	This study
RPtrxC ORF	CCATCTTGCAAGGTCCACAC	This study
FPespAup	CGTGATCTTGATACGGCTCG	(4)
RPespAup	GTTGTTGGTACCCTCGGCAAGATCGGC	(4)
FP16SrDNAup	CTGAGATACGGCCCAGACTC	(9)
RP16SrDNAup	CGTCGATGGTGAAAGAGGTT	(9)

FP, forward primer; RP, reverse primer

Figure S1

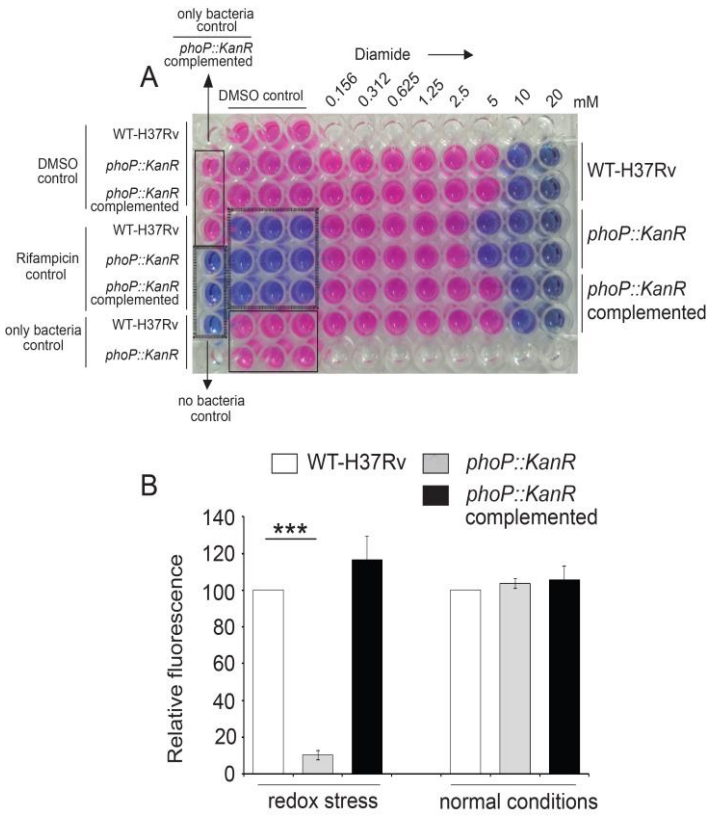

Fig. S1: PhoP contributes a major role in mycobacterial survival under oxidative stress. (A) In this experiment, we compared metabolic activity of WT, and *phoP::KanR* mutant grown in presence of increasing concentrations of diamide by using Alamar Blue assay. Because reduction of Alamar Blue correlates with the change of a non-fluorescent blue to a fluorescent pink appearance, mycobacterial metabolic activity could be quantitatively assessed by monitoring fluorescence. (B) Bacterial metabolic activity in presence of carry-over concentrations of DMSO and in presence of 5 mM diamide were assessed relative to corresponding WT-H37Rv cultures (considered as 100%). The following controls were included in the Alamar Blue assays: (i) DMSO control of WT-H37Rv, *phoP::KanR* mutant and the complemented mutant, grown in presence of carryover DMSO, (ii) Rifampicin control (indicated by a dotted square), showing mycobacterial growth inhibition to confirm validity of the assay, (iii) only bacteria as a control indicated by a square at the left bottom of the plate and another rectangle at the left top corner, and (iv) no bacteria control shown by a dotted rectangle and indicated at the left bottom corner of the plate.

Figure S2

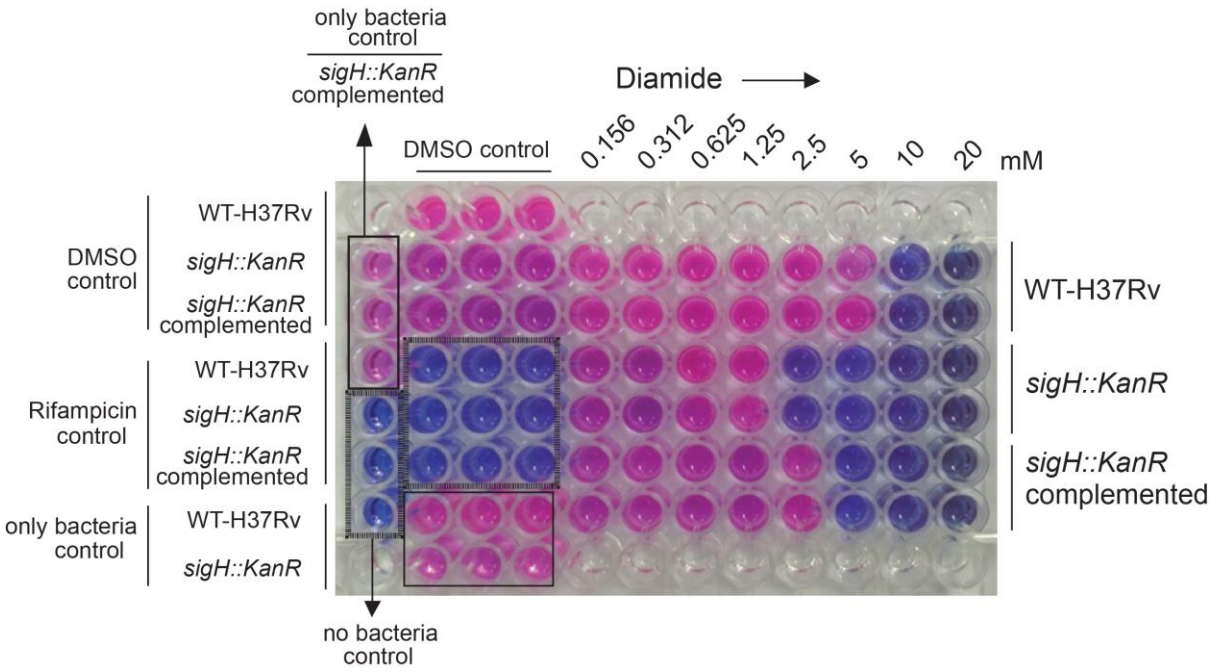

Fig. S2: SigH plays a major role in mycobacterial survival under oxidative stress. To examine sensitivity of *sigH::KanR* to oxidative stress, we compared metabolic activity of WT-H37Rv, and the mutant bacilli to increasing concentrations of diamide by using Alamar Blue assay, as described in the Experimental procedures. Because reduction of Alamar Blue correlates with the change of a non-fluorescent blue to a fluorescent pink appearance, mycobacterial metabolic activity could be quantitatively assessed by monitoring fluorescence. The following controls were included in the Alamar Blue assays: (i) DMSO control of WT-H37Rv, *sigH::KanR* mutant and the complemented mutant, grown in presence of carryover DMSO, (ii) Rifampicin control (indicated by a dotted square), showing mycobacterial growth inhibition to confirm validity of the assay, (iii) only bacteria as a control indicated by a square at the left bottom of the plate and another rectangle at the left top corner, and (iv) no bacteria control shown by a dotted rectangle and indicated at the left bottom corner of the plate.

Figure S3

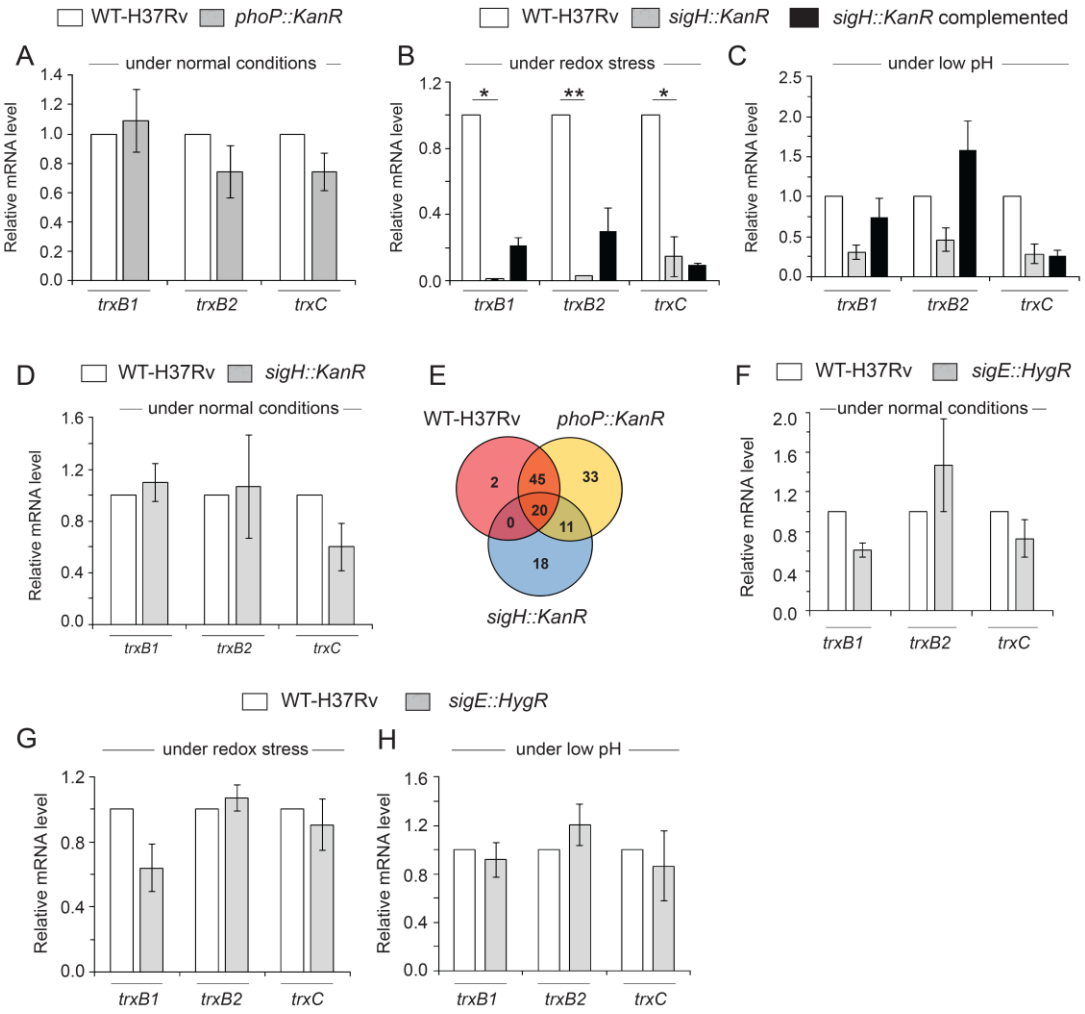

Fig. S3: Regulation of mycobacterial thioredoxin gene expression. (A-D and F-H), expression of redox-active thioredoxin genes in WT-H37Rv, and indicated *M. tuberculosis* H37Rv mutants, grown under normal conditions, or under specific stress conditions (as indicated on the figure) were examined by RT-qPCR, and the results were analysed as described in the legend to Fig. 2B. The average fold difference in expression levels were determined from three independent RNA preparations (**P*<0.05; ***P*<0.01); nonsignificant difference is not indicated. (E) Venn diagram of genes upregulated (>2-fold; p< 0.05) in diamide treated WT-H37Rv and *phoP::KanR* against their corresponding normal controls, as determined by RNA sequencing analysis [Table S5 (Excel spreadsheet)], significantly overlap with the genes which belong to *sigH* regulon (11,12). The analyses of results involve comparison of genes annotated in the H37Rv genome.

Figure S4

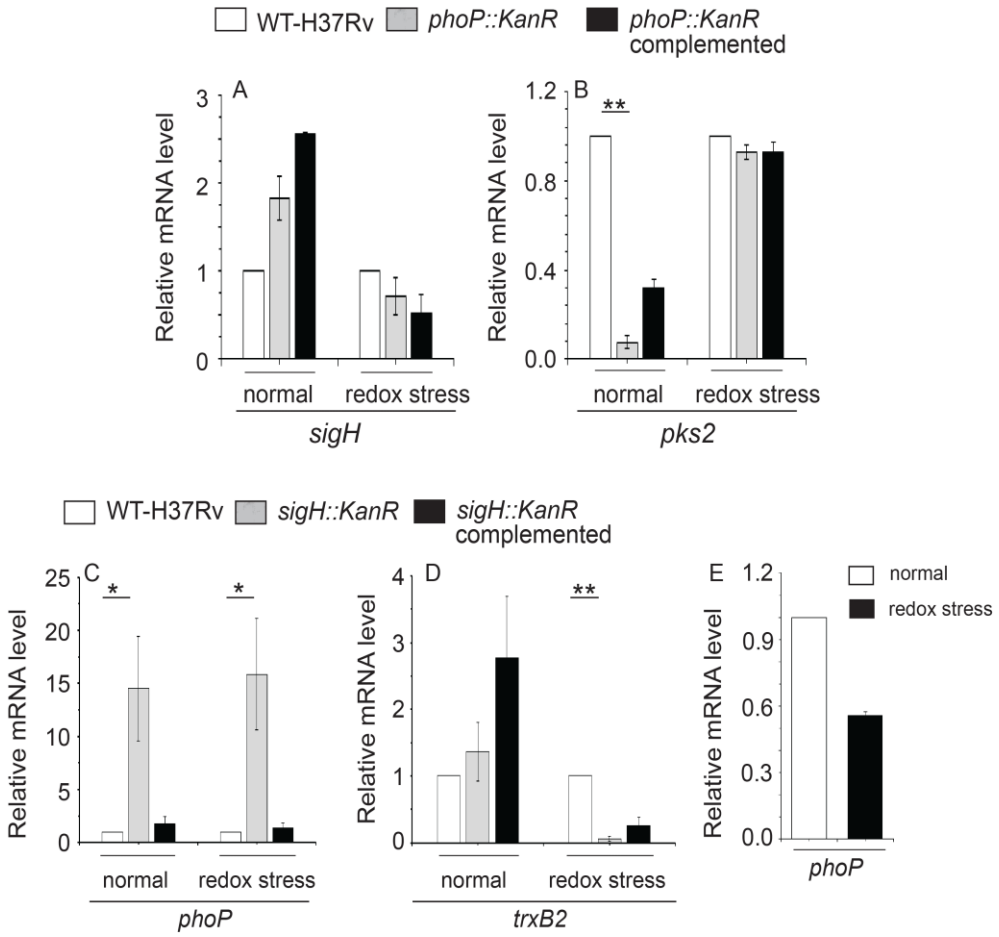

Fig. S4: PhoP expression appears to be under the control of mycobacterial *sigH* locus. mRNA levels of (A) *sigH*, and (C) *phoP* was compared by RT-qPCR using indicated mycobacterial strains, grown under normal conditions or in presence of 5 mM diamide. As controls, panels B and D show expression levels of mycobacterial *pks2*, a member of the PhoP regulon, and *trxB2*, a representative gene of the SigH regulon, respectively. Panel E measured expression of *phoP* in WT *M. tuberculosis* H37Rv, grown under normal and redox conditions. In contrast to *sigH*, expression level of *phoP* was lower under redox stress relative to normal conditions of mycobacterial growth. Relative fold difference in gene expression levels were determined as described in the legend to Fig. 2B. Nonsignificant difference is not indicated.

Figure S5

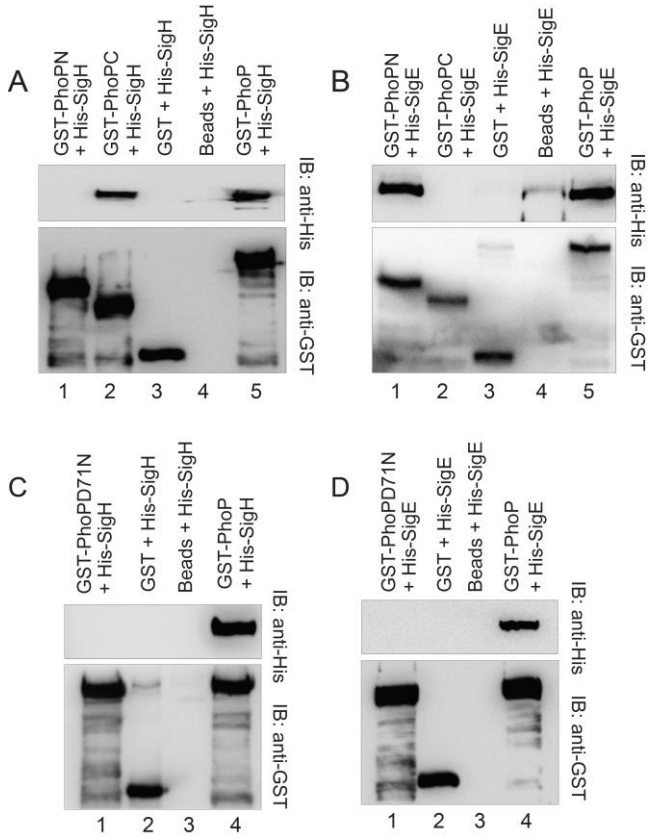

Fig. S5: Probing SigH/SigE interacting domains of PhoP. To examine protein-protein interaction by *in vitro* pull-down assays, recombinant His_6-tagged SigH (A) or SigE (B) was incubated with glutathione-Sepharose, previously immobilized with GST-tagged PhoPN (lane 1), GST-tagged PhoPC (lane 2), GST alone (lane 3), resin alone (lane 4), or GST-tagged PhoP (lanes 5). Likewise, to examine the effect of phosphorylation of PhoP on PhoP-SigH/SigE interactions, recombinant His_6-tagged SigH (C) or SigE (D) was incubated with glutathione-Sepharose, previously immobilized with GST-tagged PhoPD71N, a mutant PhoP unable to be phosphorylated *in vitro* (lane 1), GST alone (lane 2), resin alone (lane 3), or GST-tagged PhoP (lanes 4). In all cases, fractions of bound proteins were analysed by Western blot using anti-His (upper panel) or anti-GST antibody (lower panel), as described in the Experimental procedures.

References

1. Gupta, S., Pathak, A., Sinha, A., and Sarkar, D. (2009) Mycobacterium tuberculosis PhoP recognizes two adjacent direct-repeat sequences to form head-to-head dimers. *J Bacteriol* **191**, 7466-7476
2. Bansal, R., Anil Kumar, V., Sevalkar, R. R., Singh, P. R., and Sarkar, D. (2017) Mycobacterium tuberculosis virulence-regulator PhoP interacts with alternative sigma factor SigE during acid-stress response. *Mol Microbiol* **104**, 400-411
3. De Smet, K. A., Kempsell, K. E., Gallagher, A., Duncan, K., and Young, D. B. (1999) Alteration of a single amino acid residue reverses fosfomycin resistance of recombinant MurA from Mycobacterium tuberculosis. *Microbiology* **145 (Pt 11)**, 3177-3184
4. Anil Kumar, V., Goyal, R., Bansal, R., Singh, N., Sevalkar, R. R., Kumar, A., and Sarkar, D. (2016) EspR-dependent ESAT-6 Protein Secretion of Mycobacterium tuberculosis Requires the Presence of Virulence Regulator PhoP. *J Biol Chem* **291**, 19018-19030
5. Singh, P. R., Vijjamarri, A. K., and Sarkar, D. (2020) Metabolic Switching of Mycobacterium tuberculosis during Hypoxia Is Controlled by the Virulence Regulator PhoP. *J Bacteriol* **202**
6. Bhaskar, A., Chawla, M., Mehta, M., Parikh, P., Chandra, P., Bhave, D., Kumar, D., Carroll, K. S., and Singh, A. (2014) Reengineering redox sensitive GFP to measure mycothiol redox potential of Mycobacterium tuberculosis during infection. *PLoS Pathog* **10**, e1003902
7. Dussurget, O., Timm, J., Gomez, M., Gold, B., Yu, S., Sabol, S. Z., Holmes, R. K., Jacobs, W. R., Jr., and Smith, I. (1999) Transcriptional control of the iron-responsive fxbA gene by the mycobacterial regulator IdeR. *J Bacteriol* **181**, 3402-3408
8. Singh, A., Mai, D., Kumar, A., and Steyn, A. J. (2006) Dissecting virulence pathways of Mycobacterium tuberculosis through protein-protein association. *Proc Natl Acad Sci U S A* **103**, 11346-11351
9. Singh, R., Anil Kumar, V., Das, A. K., Bansal, R., and Sarkar, D. (2014) A transcriptional co-repressor regulatory circuit controlling the heat-shock response of Mycobacterium tuberculosis. *Mol Microbiol* **94**, 450-465
10. Goyal, R., Das, A. K., Singh, R., Singh, P. K., Korpole, S., and Sarkar, D. (2011) Phosphorylation of PhoP protein plays direct regulatory role in lipid biosynthesis of Mycobacterium tuberculosis. *J Biol Chem* **286**, 45197-45208
11. Manganelli, R., Voskuil, M. I., Schoolnik, G. K., Dubnau, E., Gomez, M., and Smith, I. (2002) Role of the extracytoplasmic-function sigma factor sigma(H) in Mycobacterium tuberculosis global gene expression. *Mol Microbiol* **45**, 365-374
12. Voskuil, M. I., Bartek, I. L., Visconti, K., and Schoolnik, G. K. (2011) The response of mycobacterium tuberculosis to reactive oxygen and nitrogen species. *Front Microbiol* **2**, 105