Research Paper

*Mycobacterium tuberculosis* SufR responds to nitric oxide via its 4Fe–4S cluster and regulates Fe–S cluster biogenesis for persistence in mice

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**A R T I C L E   I N F O**

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**A B S T R A C T**

The persistence of *Mycobacterium tuberculosis* (*Mtb*) is a major problem in managing tuberculosis (TB). Host-generated nitric oxide (NO) is perceived as one of the signals by *Mtb* to reprogram metabolism and respiration for persistence. However, the mechanisms involved in NO sensing and reorganizing *Mtb*’s physiology are not fully understood. Since NO damages iron-sulfur (Fe–S) clusters of essential enzymes, the mechanism(s) involved in regulating Fe–S cluster biogenesis could help *Mtb* persist in host tissues. Here, we show that a transcription factor SufR (*Rv1460*) senses NO via its 4Fe–4S cluster and promotes persistence of *Mtb* by mobilizing the Fe–S cluster biogenesis system; *suf* operon (*Rv1460-Rv1466*). Analysis of anaerobically purified SufR by UV–visible spectroscopy, circular dichroism, and iron-sulfide estimation confirms the presence of a 4Fe–4S cluster. Atmospheric O<sub>2</sub> and H<sub>2</sub>O<sub>2</sub> gradually degrade the 4Fe–4S cluster of SufR. Furthermore, electron paramagnetic resonance (EPR) analysis demonstrates that NO directly targets SufR 4Fe–4S cluster by forming a protein-bound dinitrosyl-iron-dithiol complex. DNase I footprinting, gel-shift, and *in vitro* transcription assays confirm that SufR directly regulates the expression of the *suf* operon in response to NO. Consistent with this, RNA-sequencing of *MtbΔsufR* demonstrates deregulation of the *suf* operon under NO stress. Strikingly, NO inflicted irreversible damage upon Fe–S clusters to exhaust respiratory and redox buffering capacity of *MtbΔsufR*. Lastly, *MtbΔsufR* failed to recover from a NO-induced non-growing state and displayed persistence defect inside immune-activated macrophages and murine lungs in a NO-dependent manner. Data suggest that SufR is a sensor of NO that supports persistence by reprogramming Fe–S cluster metabolism and bioenergetics.

1. Introduction

About 90% people infected with *Mycobacterium tuberculosis* (*Mtb*) remain asymptomatic for tuberculosis (TB). This indicates that the host immunity effectively suppresses the bacterial replication without eradicating the pathogen. The ability of the host to produce nitric oxide (NO) by an inducible nitric oxide synthase (iNOS) is known to modulate immunity [1] and microbial physiology [2], thereby controlling diverse infections [3,4] including TB [5]. Upon infection with *Mtb*, lesional macrophages in humans and macaques express functional iNOS [6,7], and exhaled breath of TB patient contains NO [8]. Importantly, iNOS activity seems to control TB in humans [9,10]. Mechanistically, NO inhibits respiration and arrests growth of *Mtb* [2]. The bacterial mechanisms responsible for sensing NO and mobilizing adaptation programs are poorly understood.

Previous models suggested that *Mtb* exploits a three-component system, DosR/S/T, to induce transcriptional changes, growth arrest, and a switch from aerobic to anaerobic respiration under NO stress [11, [9,10]].
K. Anand et al.

suf shown to coordinate a 2Fe standing the molecular underpinning of operon represents a potential adaptive defense against NO. Despite these degradation and calibrated regeneration of Fe

In this context, a recent study demonstrated active degradation of several iron-sulfur (Fe–S) cluster proteins coordinating respiration, central metabolism, and amino acid biosynthesis in NO-treated Mtb [13]. Importantly, a seven-gene operon, the Suf system (Rv1460-Rv1466) that is likely involved in Fe–S cluster biogenesis/repair [16], showed prolonged, elevated expression in response ([13]. Importantly, a seven-gene operon, the Suf system

was quantified and assessed for purity by a 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany). RNA samples with an RIN (RNA Integrity Number) value > 8 were processed further for sequencing. Ribosomal RNA (16s and 23s rRNA) was removed by hybridization with magnetic beads-coupled oligonucleotide (MICROBExpress Kit, Life Technologies, USA) and concentration of enriched mRNA was quantified by Qubit RNA HS Assay Kit (Life Technologies, USA). RNA-seq was performed as described [23]. In brief, libraries were prepared using NEB Next Ultra Directional RNA Library Prep Kit for Illumina (New England Biolabs, USA), according to manufacturer’s instructions. The library size distribution and quality were assessed using a high sensitivity DNA Chip (Agilent Technologies, USA) and sequenced in HiSeq 2500 platform (Illumina, USA) sequencer using 1X50 bp single-end reads with 1% PhiX spike-in control.

2.4. Differential gene expression and statistical analysis for RNA-Seq

Raw reads were obtained for Mtb H37Rv strain as fastq files. The reference genome sequence (.fna) and annotation (.gff) files for the same strain (accession number: NC_000962.3) were downloaded from the ncbi ftp website (“ftp.ncbi.nlm.nih.gov”). The annotation file was customized with the addition of annotations for non-coding RNAs [24]. The format of the annotation file (.gff) was changed to .bed format using an in-house python script. The raw read quality was checked using the Fast QC software (version v0.11.5; http://www.bioinformatics.babraham.ac.uk/projects/fastqc). BWA (version 0.7.12-r1039) [25] was used to index the reference genome. Reads with raw read quality >= 20 were aligned using BWA aln -q option. SAMTOOLS (version 0.1.19-96b5f2294a) [26] was used to filter out the multiply mapped reads. BEDTOOLS (version 2.25.0) [27] was used to calculate the Reads count per gene using the annotation file (.bed). The normalization and differential gene expression analysis for the conditions were carried out using edgeR as mentioned previously [28]. Genes with at least 10 reads were selected for each comparative analysis. DGE analysis was done in RStudio (1.1.447) with R version 3.4.4 (http://www.rstudio.com/).

2.5. Aconitase assay

The activity of aconitase (Acn) was measured by monitoring the disappearance of cis-aconitate at 240 nm in a UV spectrophotometer (Thermo Scientific Biomat 35, USA) as described [29]. One unit (U) of aconitase activity is defined as 1 μmol cis-aconitate formed or converted per minute. Reaction mixtures (1 mL) for Acn contained 25 mM Tris-HCl (pH 8.0), 100 mM NaCl, and 50 μg Mtb cell lysates. Reactions were initiated by adding 0.15 mM cis-aconitate and monitored by following the disappearance of cis-aconitate at 240 nm after every 15 s for total 30 min. Absorbance at 240 nm was plotted against time. Acn activity was calculated from linear portion of the curve in initial 5 min when reaction follows 0th order of reaction. An extinction coefficient of 3, 500 M⁻¹cm⁻¹ was used to calculate the rates.

2.6. Western blot

Whole cells lysate (50 μg) was separated on 12% SDS-PAGE and then transferred onto a PVDF membrane (GE Healthcare, Piscataway, NJ, USA). Membrane were blocked in 5% (w/v) nonfat dry milk and incubated for 3 h at room temperature with primary antibody (Acn and Cbs
using ECL Advance Western blotting detection kit (BioRad, USA). (1:10000 dilution) for 1 h. The autoradiography signals were visualized using ECL advance Western blotting detection kit (BioRad, USA).

2.7. OCR and ECAR measurements

The Mtb strains adhered to the bottom of a XF cell culture microplate (Agilent technologies, USA), at 2X10\(^6\) bacilli per well by using Cell-Tak (a cell adhesive). Oxygen Consumption Rate (OCR) and Extracellular Acidification Rate (ECAR) were measured using Agilent XF Extracellular Flux Analyser. Assays were carried out in unbuffered 7H9 media (pH 7.35) with glucose 2 mg/mL as carbon source. Basal OCR and ECAR were measured for initial 21 min before the automatic addition of freshly prepared DETA-NO (0 mM, 0.25 mM, 0.5 mM and 1 mM) in 7H9 unbuffered media, through port A of cartridge plate. Three measurements were taken after 1 h of incubation. Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) (Sigma-Aldrich, India) was added at 10 μM concentration to achieve maximum rate of respiration. Raw data of OCR and ECAR was CFU normalized for 2X10\(^6\) CFU/well. Spare respiratory capacity, was calculated from % OCR value, by subtracting third basal reading (normalized as 100%) from first point after CCCP addition.

2.8. CellROXDeep Red staining and flow cytometry

Logarithmically growing Mtb strains were treated with DETA-NO (0 mM, 0.25 mM, 0.5 mM and 1 mM) and then incubated at 37 °C with shaking for 2 h. 200 μL cells were treated with CellROX® Deep Red reagent (Thermo Fisher, USA) at a final concentration of 5 μM for 30 min at 37 °C and analyzed on BD FACSVerse flow cytometer with 640/665 nm excitation and emission respectively. We collected 5000-10,000 events for each sample wherever possible.

2.9. Animal experiments

For the chronic model of infection, 5- to 6-week-old female BALB/c, C57BL/6 and INOS\(^{-}\) mice (n = 6 per group) were infected by aerosol with approximately 100 bacilli per mouse with the Mtb strains using a Madison chamber aerosol generation. At indicated times post infection, mice were euthanized, and the lungs were harvested for bacillary load, tissue histopathology analysis, and pathological scoring as described [23]. The remaining tissue samples from each mouse were homogenized and bacillary load was quantified by plating serial dilutions of tissue homogenates onto Middlebrook 7H11-OADC agar plates supplemented with lyophilized BBL MGIT PANTA antibiotic mixture (polymyxin B, amphotericin B, nalidixic acid, trimethoprim, and azlocillin, as supplied as lyophilized BBL MGIT PANTA antibiotic mixture (polymyxin B, amphotericin B, nalidixic acid, trimethoprim, and azlocillin, as supplied by BD; USA). Colonies were observed and counted after 4 weeks of incubation at 37 °C.

2.10. Construction of Mtb SufT knockdown strain

For construction of Mtb SufT knockdown strain (SufT-KD), we followed CRISPR interference (CRISPRi) technology as described previously [30]. Anhydrotetracycline (ATc, Cayman, USA) 200 μg/mL was added for the induction of sufT specific-guide RNA (sgRNA) and dCas9 every 48 h. SufT-KD culture was divided equally when ATc concentration reached 0.1–0.2 and cultured in the presence or absence of ATc. The dCas9 was expressed by pRH2502 from a TetR-regulated uTRetO promoter and sgRNA in pRH2521 under the control of a TetR-regulated smyc promoter (Pmyc1tetO). To deplete sufT, gene specific sgRNAs were designed for two regions between 98-127 bp and 171–191 bp of sufT and cloned into pRH2521. Depletion of sufT was verified by qRT-PCR and based on the significant repression of sufT, we chose sgRNA targeting sufT region between 98 and 127 bp for further study. For qRT-PCR analysis, total RNA was extracted from SufT-KD and control strains after 24 h of ATc treatment. After DNase treatment, total 600 ng of RNA was used for cDNA synthesis by using Random hexamer oligonucleotide primer (iScript Select cDNA Synthesis Kit, BioRad, USA). Gene specific primers (Table S2) and iQ SYBER Green Supermix (BioRad, USA) were used for RT-PCR (StepOne Plus, Thermo, USA). Gene expression was normalized to Mtb 16S rRNA expression level.

2.11. NO exposure and recovery

Logarithmically grown Mtb strains (OD\(_{600}\) of 0.8) were diluted to an OD\(_{600}\) of 0.1 and exposed to six doses of 100 μM DETA-NO, once every 6 h. Recovery from exposure to DETA-NO was monitored by recording the OD\(_{600}\) after each addition of DETA-NO, as well as every 24 h after addition. Two biological replicates were used for each strain. For single dose experiments, different doses of DETA-NO (0.5 mM, 1.25 mM, and 2.5 mM) were added. After and 24 h cells were harvested, washed with 1X PBS and plated on OADC-7H11 plates. Colonies were counted after 3–4 weeks of incubation at 37 °C.

2.12. Cell line experiments

RAW264.7 murine macrophage cell line was activated by treatment with IFN\(_\gamma\) (100U/μL, Invitrogen, USA) for 12 h before infection and LPS (100 ng/mL, Sigma-Aldrich, India) for 2 h before infection. Activated RAW264.7 macrophages were infected with wt Mtb, Mtb\(_{sufR}\), and sufR-Comp strains at multiplicity of infection (MOI) 2 for 2 h, followed by washing thoroughly to remove extracellular bacteria with warm DMEM medium and suspended in the same containing 10% FBS. For CFU determination, macrophages were lysed using 0.06% SDS-7H9 medium diluted in PBS/Tween and plated on OADC-7H11 at indicated time points. Colonies were counted after 3–4 weeks of incubation at 37 °C.

2.13. Purification of SufR under anaerobic conditions

The entire ORF of Mtb sufR (Rv1460) was PCR-amplified using gene-specific oligonucleotides (pET28asauRFF and pET28asauRR; Table S2), digested with Ndel-HindIII, and ligated into similarly digested His-tag-based expression vector, pET28a (TAKARA BIO, Clontech Laboratories, CA, USA) to generate pET28a:SufR. A N-terminal histidine-tagged SufR was overexpressed in E. coli BL21 I DE3 by 0.6 mM IPTG [Isopropyl β-D-1-thiogalactopyranoside; MP Biomedicals, USA (60 min, 30 °C)]. To facilitate Fe–S cluster formation, cultures were incubated on ice for 18 min prior to induction and were supplemented with 300 μM ferric ammonium citrate and 75 μM 1-methionine (Amresco, USA) and purified as described [31]. Purification was performed under strict anaerobic conditions inside an anaerobic glove box (Plas-Labs, Lansing, MI, USA) maintaining ≥2.0 ppm O\(_2\) by volume, and buffers and solutions were appropriately deoxygenated. The sufR gene on pET28a:SufR was mutated using oligonucleotide-based site-directed mutagenesis approach to create individual cysteine to alanine substitutions. After the PCR, DpnI was added into the reaction mixture to digest the wild-type plasmid that was used as the template. The reaction mixture containing the mutated sufR gene was used to transform E. coli BL21 I DE3. Sequences of primers used to create mutations are shown in (Table S2). Resulting clones were verified by sequencing, and the mutant Cys variants of the wt SufR were purified as described earlier. In order to generate apo-SufR, the holo-SufR was incubated with EDTA (Ethylendiaminetetraacetic acid, Sigma-Aldrich, India) and potassium ferricyanide in a molar ratio of protein: EDTA: ferricyanide in 1:50:20 at 25 °C and incubated for 20–30 min till the extensive loss of color. The solution was passed through PD10 desalting column and stored at −80 °C.

2.14. UV–Vis, CD analysis, and gel filtration of SufR

The UV–visible absorption spectroscopy was carried out in a Thermo scientific spectrophotometer (Thermo scientific, USA) at 25 °C.
Absorption spectra of SuFR WT (native/holo) and mutants were recorded immediately on the elution fractions were collected during the purification. In order to study the effect of air oxidation, on Fe–S cluster stability, freshly purified holo-SuFR was transferred to an anaerobic quartz cuvette, exposed to air by opening the cap and mixing by pipetting for 2 min. The cuvette was then sealed and monitored by UV-visible spectroscopy over time (Thermo scientific, USA). To study the effect of NO, DTH and H₂O₂ on [4Fe–4S] cluster of SuFR, the absorption spectra of freshly purified protein were recorded at indicated concentration and different time intervals in anaerobic quartz cuvette. CD measurements were conducted in a Jasco J-715 spectropolarimeter (Jasco, USA). Far-UV spectra were measured from 190 to 250 nm range and near-UV spectra from 300 nm to 650 nm range. Protein concentration used for the far-UV CD measurements was 10–20 μM and for the near-UV measurements was 140–150 μM. Cells of 1.0 cm path length were used for the measurements of the far- and near-UV spectra, respectively. Three repeat scans were obtained for each sample. The averaged baseline spectrum was subtracted from the averaged sample spectrum. The protein was dissolved in 5 mM phosphate buffer pH 7.4 and 150 mM NaCl. Results are expressed as molar ellipticity [θ] (deg cm² dmol⁻¹), calculated from the following formula [θ]λ = 0/([c]*l)*10*n, where θ is the measured ellipticity in degrees at wavelength λ, c is the protein concentration in mg/mL, l is the light path length in centimeters and n is the number of amino acids. CD Pro software was used to analyze the data.

To determine the molecular mass of apo- and holo-SuFR protein, analytical size-exclusion chromatography experiments were performed with Superdex 200 1000, 10/300 GL analytical columns (GE Healthcare Life Sciences, USA). The column was pre-equilibrated and eluted with the running buffer (10 mM phosphate buffer, 10% glycerol and 0.1 mM EDTA). The reaction mixture was heated to 95 °C for 5 min and then cooled in a thermal cycler. Annealed mixtures were resolved by 10% native PAGE in 1X Tris-borate EDTA (TBE). Analyzed to ensure the reproducibility and three dilution of each sample were considered.

2.17. Electrophoretic mobility shift assay (EMSA)

For EMSA, promoter fragment of *sufR (~170 bp) and *blaC (~100 bp) were PCR amplified from the *Mtb* genome. The 5’ end was labeled by [γ-32P]-ATP using T4 polynucleotide kinase (MBI Fermentas, USA) as per the manufacturer’s instructions. The labeled oligonucleotides were passed through a 1 ml Tris-EDTA, pH 7.5 equilibrated Sephadex G-50 standard solution in the range of 52–260 μM of S²⁻. Protein sample/standard (200 μL) was mixed with 0.6 mL of zinc acetate (1% w/v) followed by addition of 50 μL of NaOH (12% w/v). For iron and sulfide estimation, three independent preparations of holo-SuFR were used to analyze the reproducibility and three dilution of each sample was considered.

2.18. In vitro transcription assays

The DNA templates (170 bp) including the *sufR* promoter regions were PCR amplified using primers *PsuR* F1/PsSuR R1 (Table S2). The ampiclons (50 nM) were pre-incubated with different concentration of holo- and apo-SuFR in the transcription buffer (50 mM Tris HCl, (pH 8.0), 10 mM magnesium acetate, 100 mM EDTA, 100 mM DTT, 50 mM KCl, 50 mg/mL BSA, and 5% glycerol) for 30 min at room temperature. Single-round transcription reactions were initiated with the addition of 100 nM *Mtb* RNAp-α holo enzyme, 100 μM NTPs, 1 μCi [γ-32P]-UTP, 50 μg mL⁻¹ heparin and incubated at 37 °C for 20 min. The reactions were terminated by addition of 2X formamide dye (95% formamide, 0.025% (w/v) bromophenol blue, 0.025% (w/v) xylene cyanol, 5 mM EDTA and 0.025% SDS and 8 M urea) and heated at 95 °C for 5 min.
followed by snap chilling in ice for 2 min. The transcripts were resolved on an 8% TBE-urea-PAGE gel. To study the effect of NO, transcription assays were performed as mention above after treating the holo-SuFR with proline NONOate for 5 min followed by purification. All the treatments and the reactions were performed inside anaerobic glove box under anaerobic condition.

2.19. DNase I footprinting

5 nM of $^{32}$P-labeled DNA substrate was incubated with increasing concentration of purified holo-SuFR in binding buffer (25 mM Tris- HCl (pH 7.5), 1 mM DTT, 100 µg/ml BSA, 5 mM MgCl2, 5 mM CaCl2), and samples were incubated for 30 min at 4 °C inside glove box under anaerobic condition. Reactions were initiated by the addition of DNase I to a final concentration of 0.05 units and incubated for 2 min at room temperature. The reactions were terminated by the addition of 150 µl of TE (pH 7.5) followed by incubation at 75 °C for 15 min to deactivate DNase I enzyme. The sample was further subjected to vacuum evaporation and the pellet thus obtained was re-suspended in loading dye (80% (v/v) formamide, 0.1% (v/v) BBP, and 0.1% (v/v) xylene cyanol) and analyzed on a denaturing 15% polyacrylamide gel containing 7 M urea. The gel was dried, and the bands were visualized with a Typhoon FLA-9000 phosphor imager (GE Healthcare Life Sciences, USA). Maxam and Gilbert A + G ladder was prepared as described previously [34].

2.20. Ethics

Animal experimentation: This study was carried out in strict accordance with the guidelines provided by the Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA), Government of India. The protocol of animal experiment was approved by the Institute’s Animal Ethical Committee (IAEC), Indian Institute of Science (IISc), Bangalore, India (Approval number: CAF/Ethics/544/2017). All efforts were made to minimize the suffering.

2.21. Statistical analysis

All data were graphed and analyzed with Prism v8.0 (GraphPad) unless otherwise stated. Statistical analyses were performed using Student’s t-test (two-tailed). Where comparison of multiple groups was made either one-way or two-way ANOVA with Bonferroni multiple comparison was performed. Differences with a p value of <0.05 were considered significant. Statistical significance for RNA-seq was calculated using the QL F-test followed by Benjamini-hochberg method of multiple testing correction.

2.22. Miscellaneous

The molar extinction coefficient per [4Fe–4S]$^{2+}$ cluster ($\epsilon_{413} = 16200 \text{ M}^{-1} \text{ cm}^{-1}$) was determined from in vitro reconstituted protein. Protein concentration of SuFR throughout the study was calculated using A$\text{280}$ reading. Anti-SuFR polyclonal antibody was used to monitor the expression and purification of wt SuFR and mutant proteins by Western blot. Samples (5 mg of protein per slot) were resolved by 12% reducing SDS-PAGE and transferred to nitrocellulose membrane. Blots were probed with primary and secondary (horseradish peroxidase-conjugated anti-rabbit IgG) antibodies and processed.

2.23. Data availability

The RNA-sequencing data presented in the manuscript are deposited into the NCBI Gene Expression Omnibus (GEO) (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=equals;GSE154169 with the accession number GSE154169.

3. Results and discussion

3.1. Mtb SuFR contains a 4Fe–4S cluster

To understand the role of Mtb SuFR in sensing NO and regulating Fe–S cluster homeostasis, we first examined the biochemical and biophysical characteristics of the SuFR Fe–S cluster. We anaerobically purified histidine-tagged SuFR from E. coli cultured in growth conditions optimized for the maximum incorporation of Fe–S clusters in vivo [31]. As isolated, native SuFR (holo-SuFR) displayed a characteristic straw brown color, with an absorption maximum indicative of a 4Fe–4S cluster at 413 nm (molar absorption coefficient $\epsilon$ at 413 = 16200 M$^{-1}$ cm$^{-1}$) (Fig. 1A and Fig. S1 A-C) [32]. Treatment of holo-SuFR with a one-electron donor, sodium dithionite (DTH), caused partial bleaching of brown color and loss of absorbance at 413 nm, consistent with the presence of a redox-responsive 4Fe–4S cluster [32] (Fig. 1A). Gel filtration performed under anaerobic conditions suggested a molecular mass of ~54 kDa for native SuFR, consistent with a dimer. Clusterless SuFR (apo-SuFR) also eluted as a dimer, indicating that the Fe–S cluster does not bridge SuFR monomers (Fig. 1B). Moreover, the identity of cysteine residues that coordinates the 4Fe–4S cluster was experimentally validated. The alanine mutant of two putative cysteine ligands (C$_{37}$-A$_{38}$-G$_{40}$) was sufficient to disrupt the 4Fe–4S cluster as evident from the loss of absorbance at 413 nm (Fig. 1C).

A recent study using aerobically purified SuFR, demonstrated that SuFR is a monomer and coordinates a 2Fe–2S cluster upon Fe–S cluster reconstitution in vitro [19]. In general, Fe–S clusters are inherently unstable under aerobic conditions [35] and stabilize only under anaerobic conditions [31]. Fe–S clusters can be assembled in vitro, but this process has poor yield [31]. Therefore, the technique of assembling the Fe–S cluster in vivo followed by anaerobic purification is more likely to provide native configuration of SuFR Fe–S cluster. We clarified this by analyzing native SuFR using circular dichroism (CD). The far-UV CD-spectrum showed two minima at 208 and 224 nm and indicated 70% α-helical content in the secondary structure (Fig. 1D). The near-UV CD-spectrum displayed two characteristic maxima at 330 and 420 nm and two minima near 350 nm and 550 nm, as described earlier for 4Fe–4S cluster (Fig. 1E) [31,36]. As expected, the near-UV CD-spectrum of apo-SuFR did not show a 4Fe–4S cluster (Fig. 1E). Lastly, the estimation of iron and sulfide ions in the native SuFR revealed the association of 3.23–3.75 iron atoms per SuFR monomer and similar amount of sulfide ions per SuFR monomer (Fig. S1D). In sum, we demonstrate that Mtb SuFR contains a 4Fe–4S cluster.

3.2. SuFR responds to NO through its 4Fe–4S cluster

We exposed native SuFR to the fast-releasing NO donor; proline NONOate (T$_{1/2}$=1.8 s, pH 7.4) and subjected it to UV-visible spectroscopy. Treatment with proline NONOate gradually reduced absorbance at 413 nm and formed a new chromophoric feature at ~350 nm over time with a clear isosbestic point (Fig. 1F). These spectral features were consistent with a dinitrosyl-iron dithiol complex (DNIC), wherein the nitroso group in NO donates a one-electron acceptor to the Fe$^{3+}$ ion, resulting in the rapid loss of absorption at 413 nm (Fig. 1F). A plot of $\Delta$A$_{413}$nm against time revealed the loss of ~75% of the 4Fe–4S cluster only at 20 h post-NO exposure. However, exposure of native SuFR to 1 mM H$_2$O$_2$ resulted in the rapid loss of absorption at 413 nm (Fig. 1I). A plot of $\Delta$A$_{413}$nm against time revealed the loss of ~75% of the 4Fe–4S...
cluster in 20 min. Higher concentrations of H$_2$O$_2$ (10 mM and 100 mM) resulted in the loss of the cluster within 1–2 min (Figs. S2A–C). Taken together, these data demonstrate that the 4Fe–4S cluster of SufR is sensitive to NO and H$_2$O$_2$.

### 3.3. SufR binds upstream of suf operon promoter and represses expression

To investigate if SufR mediates Fe–S cluster biogenesis by regulating the suf operon expression, we first assessed the DNA-binding properties of SufR. We carried out electrophoretic mobility shift assays (EMSA) using a radioactively ($^32$P)-labeled 170 bp DNA fragment encompassing the promoter region of suf operon [19]. We used four different forms of
SufR; holo-SufR, apo-SufR, NO-treated SufR, and H$_2$O$_2$-treated SufR. Holo-SufR bound to the suf promoter region, whereas apo-SufR, NO-treated SufR, and H$_2$O$_2$-treated SufR showed no DNA binding (Fig. 2A–C). DNA binding was outcompeted by 50-100-fold excess of unlabeled suf promoter DNA, but not by an unrelated promoter fragment (blaC) (Fig. 2D). These findings suggest that Mtb SufR is an NO- and H$_2$O$_2$-responsive, sequence-specific DNA-binding protein.

We next performed in vitro transcription using Mtb RNA polymerase as described [39]. A single round of transcription of the suf promoter fragment (170 bp [-63 to +107 bp]) produced a single transcript of 107 nucleotides, consistent with the leaderless transcription of the suf operon [40]. Addition of holo-SufR repressed transcription from the suf promoter in a dose-dependent manner, whereas apo-SufR did not (Fig. 2E–F). Importantly, the treatment of holo-SufR with proline NONOate (NO) reversed the repression of the suf promoter (Fig. 2G). These results indicate that holo-SufR binds to the suf promoter and represses expression, whereas NO-damaged or clusterless apo-SufR lacks DNA-binding and transcription repression. The data suggest that occupancy of Fe–S cluster on SufR serves as an indicator of Fe–S cluster-sufficient or -deficient conditions. During low Fe–S cluster demand, holo-SufR represses the Suf system to limit excessive assembly of Fe–S clusters. In contrast, loss or damage of the Fe–S cluster in SufR (apo-SufR) signals heightened demand for Fe–S clusters that results in the de-repression of the suf operon and mobilization of Fe–S cluster assembly. In support of this, we depleted an essential gene of the suf operon (Rv1466; sufT) involved in Fe–S cluster maturation [41] using CRISPR interference (CRISPRi-sufT). The depletion of SufT is expected to perturb Fe–S cluster biogenesis and increase the pool of apo-SufR, which could result in the de-repression of the suf operon in Mtb. Consistent with this, expression of the suf operon was induced in CRISPRi-sufT as compared to wild-type Mtb, indicating that Mtb de-represses the suf operon in response to abnormal Fe–S cluster biogenesis caused by SufT depletion (Fig. S3).

Lastly, we performed DNase I footprinting to identify the binding site of holo-SufR on the promoter region of the suf operon (Fig. 3A). A clear region of protection from DNase I digestion was evident with increasing molar ratios of SufR:DNA (Fig. 3A–B). The protected region contains a perfect inverted repeat (ACACT-NTTTTGTCACACT-NNN) separated by 5 bp (Fig. 3A–B). This inverted repeat forms a part of a larger inverted repeat (ACACT-NTTTTGTCACACT-NNN). Consistent with the footprinting data, mutations in the inverted repeat completely abolished binding, thus confirming that the SufR binds to the palindromic ACACAGTTT-TGTGAAAAT. Altogether, using multiple techniques, we confirmed that SufR functions as a NO-sensitive DNA-binding transcription factor in Mtb.

### 3.4. NO regulates expression of the suf operon and Fe–S cluster pathways in Mtb

Having shown that SufR responds to NO via its 4Fe–4S cluster and directly regulates the transcription of suf operon promoter, we next asked if SufR coordinates Mtb’s adaptation under NO stress. To examine
this possibility, we first investigated the role of SufR in regulating transcriptome of \textit{Mtb} in response to NO. All genes of the \textit{suf} operon are essential except \textit{sufR} \cite{16}. Therefore, we utilized \textit{sufR}-deficient (\textit{Mtb} \textit{ΔsufR}) strain of \textit{Mtb} \cite{23}. We generated the \textit{sufR}-complemented strain (\textit{sufR-Comp}) by integrating \textit{sufR} (\textit{Rv1460}) along with its native promoter (~500 bp upstream of \textit{sufR}) in the genome of \textit{Mtb} \textit{ΔsufR}. Using qRT-PCR, we confirmed the restoration of \textit{sufR} expression to wild type (wt) \textit{Mtb} levels in \textit{sufR-Comp} (Fig. S4.). Since exposure to 0.5 mM of NO donor diethylenetriamine-nitric oxide (DETA-NO) arrested the growth of wt \textit{Mtb}, \textit{MtbΔsufR}, and \textit{sufR-Comp} without affecting viability (Fig. S5), we performed RNA-sequencing (RNA-seq) on \textit{Mtb} strains treated with 0.5 mM of DETA-NO for 4 h.

Our RNA-seq data recapitulated the previously published NO-responsive transcriptome of \textit{Mtb}, with the induction of the DosR, IdeR, Zur, and CsoR regulons, and reduced expression of RNA and protein biosynthesis pathways (fold change $\geq 2$; FDR $\leq 0.05$; Fig. S6 and Table S1A) \cite{13}. These pathways were similarly affected in \textit{MtbΔsufR} and \textit{sufR-Comp} in response to NO (Fig. S6 and Tables S1B–C). Since Fe–S
cluster proteins are the most susceptible targets of NO [42], we found that the expression of ~50% of genes encoding Fe–S cluster proteins was altered in wt Mtb, MtbΔsufR, and sufR-Comp under NO stress (Fig. 4 and Table S1D). The strong reactivity of NO towards heme iron of primary cytochrome oxidase is thought to arrest respiration [2]. However, we found that NO does not affect the expression of genes encoding primary terminal oxidase (cytochrome bc1–aa3 complex; qcrABC) in Mtb (Fig. 4). Nonetheless, downregulation of Fe–S cluster-containing NADH dehydrogenase complex 1 (nuo operon) and other genes encoding Fe–S cluster proteins involved in central metabolism (acn, pyrA, and ugdA) are consistent with the inhibition of primary respiration by NO. Moreover, the expression of genes associated with alternate form of respiration (e.g., NADH dehydrogenase type II [nadH], cytochrome BD oxidase [cydABCD], nitrate reductase [narH], sulfite reductase [sirA], hydrogenase [hycP]) [43], carbon catabolism (korAB, frdB, icl1, and pckA), and branched chain amino acid (BCAA) biosynthesis [44] were induced by NO (Fig. 4).

The association between NO, respiration, and metabolism is further indicated by the upregulation of Fe–S cluster enzymes associated with the biosynthesis of respiratory cofactors such as isoprenoid (lytB2 and gcpE), molybdopterin (moaA2Z), thiamin (thiC), quinolinate (nadaA-B), and sulfur metabolites (cysH) [45–48] (Fig. 4; Table S1D). Also, NO induced the expression of transcription factors containing Fe cluster biogenesis. In agreement, expression of the suf operon (sufBDCSUT; Rv1461-Rv1466) was stimulated 15- to 25-fold by NO in Mtb (Fig. 4). Also, NO induces the cysteine biosynthetic machinery that supplies sulfur for Fe–S cluster biogenesis (Fig. 4). However, genes coordinating biosynthesis of Fe-binding heme (hemZ) and encoding Fe-storage proteins (bacterioferretin; bfrA-A) were downregulated (Fig. 4). These observations suggest that Mtb prioritizes the assembly of Fe–S clusters under NO stress. In contrast to wt Mtb, the suf operon remains basally expressed in NO-treated MtbΔsufR (Fig. 4). A direct comparison of the expression data confirmed a uniformly reduced expression of the suf operon in MtbΔsufR as compared to wt Mtb under standard growing conditions and NO stress (Fig. 5A–D). Since SuFR is a putative repressor of the suf operon [19], the operon’s diminished expression in MtbΔsufR was unexpected. One possibility is that by replacing 345 bp fragment internal to the suf operon (±308 to +653 bp) with the hygromycin resistance cassette (lox-hyg-gfp-lox) [23], we might have interrupted the NO-inducibility of the downstream suf genes (sufBDC-SUT) in MtbΔsufR (Fig. 5A). Consistent with this, the partial transcript of sufR originated from the undelated region (+1 to +307 bp) that is present upstream to the deleted fragment (+308 to +653 bp) retained NO-inducibility in MtbΔsufR (Fig. 5C–D). The basal expression of the sufBDCSUT genes in MtbΔsufR is likely due to alternative transcription start site (TSS2) present upstream of sufB (Fig. 5A). As expected, sufR-Comp expressing a native copy of sufR restored the induction of sufR in response to NO, whereas rest of the operon remainedbasally expressed (Fig. 5B–D and Fig. S7). While the suf operon was not induced, the expression of pathways requiring Fe–S clusters remained induced in NO-treated MtbΔsufR and sufR-Comp. Thus, we anticipate that the heightened demand for Fe–S clusters under NO stress is unlikely to be satisfied in MtbΔsufR or in sufR-Comp. The restoration of NO-inducibility of sufR but not of the suf operon in sufR-Comp provides an opportunity to assess the contribution of SuFR other than regulating Fe–S cluster homeostasis under NO stress. Altogether, the transcriptomic data indicate that SuFR is mainly required to adjust the NO-responsive expression of the suf operon in Mtb.
the undelated (UD) region of sufR. (C, D) qRT-PCR data showing the expression of the suf genes where MtbΔsufR and sufR-Comp were compared with untreated and DETA-NO-treated wt Mtb. Results are expressed as mean ± standard deviation (Mean ± SD).

### 3.5. NO irreversibly damages Fe–S clusters of aconitase in MtbΔsufR

Altered expression of the Fe–S pathways involved in metabolism and respiration by NO indicates that NO might modulate these processes in Mtb. To investigate this idea, we evaluated a 4Fe–4S-containing enzyme aconitase (Acn) activity, which functions as a critical gatekeeper of the TCA cycle, and shows sensitivity to NO due to a solvent-exposed Fe atom [51]. A gradual decrease in Acn activity over time was observed in Mtb exposed to 0.5 mM of DETA-NO, indicating Fe–S cluster damage. At 12 h post-exposure, DETA-NO triggered a 40% reduction in Acn activity without decreasing its abundance (Fig. 6A). Importantly, re-culturing DETA-NO-treated Mtb in a DETA-NO-free medium significantly restored Acn activity, indicating efficient mobilization of Fe–S cluster regeneration machinery (Fig. 6A).

We next examined if defective induction of the suf operon impairs regeneration of NO-damaged Fe–S clusters in MtbΔsufR. The Acn activity in MtbΔsufR was similar to wt Mtb under aerobic growing conditions (Fig. 6B). Moreover, like wt Mtb, Acn activity decreased in MtbΔsufR under NO stress over time (Fig. 6B). However, in contrast to wt Mtb, reactivation of Acn upon removal of NO stress was absent in MtbΔsufR (Fig. 6B). Importantly, sufR-Comp that maintains sufR expression but lacks NO-inducibility of the suf operon also failed to reinitiate Acn activity (Fig. S8A). Data suggest that NO-mediated induction of the suf operon rather than SufR alone is critical for the repair of NO-damaged Fe–S clusters in MtbΔsufR. A previous study indicated that Acn activity is dependent upon a stand-alone cysteine desulfurase (IscS) in Mtb under standard culture conditions [52]. These findings, along with our data, suggest that Mtb prefers IscS under aerobic conditions and Suf system under NO stress for biogenesis of Fe–S clusters. Similar roles were assigned for Isc and Suf systems in E. coli [53].

### 3.6. NO depletes spare respiratory capacity and perturbs redox homeostasis of MtbΔsufR

Fe–S cluster-containing enzymes are crucial for maintaining carbon catabolism, oxidative phosphorylation (OXPHOS), and redox balance [45,54]. Therefore, we exploited Seahorse XF Flux technology to analyze the influence of NO on oxygen consumption rate (OCR) and extracellular acidification rate (ECAR), which are measurable readouts of OXPHOS and glycolysis, respectively [55]. To quantify the basal and maximum rates of OCR and ECAR, we cultured Mtb and MtbΔsufR in 7H9-glucose in an XF microchamber, then exposed it to DETA-NO, and finally to the uncoupler carbonyl cyanide m-chlorophenyl hydrazine (CCCP). Addition of CCCP stimulates respiration to the maximal capacity manageable by Mtb. The difference between basal and CCCP-induced OCR provides an estimate of the spare respiratory capacity (SRC) available for sustaining stress-mediated bioenergetics (e.g., nitrosative and oxidative conditions) [56]. Under normal growing conditions, Mtb displayed a basal OCR of 20 ± 0.14 pmol/min, which increased to 85 ± 11.7 pmol/min in response to uncoupling stress by CCCP (Fig. 6C). This indicates that Mtb normally functions at a sub-maximal OXPHOS (~25%) capacity. Under similar conditions, MtbΔsufR utilizes ~ 35% of its maximal respiratory capacity, which is more than wt Mtb (Fig. 6D). Similar to the uncoupler CCCP, NO also depolarizes the cytoplasmic membrane to arrest respiration and growth [57,58]. Consistent with this, and as seen with CCCP, pretreatment with DETA-NO also increased basal OCR of wt Mtb and MtbΔsufR (Fig. 6C, D). However, while 1 mM of DETA-NO was required to increase basal OCR of wt Mtb significantly, 0.25 mM was sufficient for MtbΔsufR (Fig. 6C, D). Data suggest that NO stimulated basal OCR, possibly by collapsing proton motive force (PMF), and that MtbΔsufR is more sensitive to membrane depolarization by NO.

We also found that DETA-NO pretreatment progressively reduced the ability of bacteria to increase OCR in response to CCCP (Fig. 6C, D). As a result, DETA-NO significantly decreased SRC in a dose-dependent manner in both Mtb and MtbΔsufR (Fig. 6E). However, SRC of MtbΔsufR was significantly lower than wt Mtb under normal culture conditions and upon exposure to 0.25 mM DETA-NO (Fig. 6E). These results indicate that Mtb mobilizes its reserved respiratory capacity to sustain bioenergetics in response to NO. Data also suggest that the inherently reduced SRC of MtbΔsufR due to diminished Fe–S cluster biogenesis increased its vulnerability towards bioenergetic exhaustion by NO.

Measurement of basal ECAR with and without CCCP treatment indicated that wt Mtb and MtbΔsufR operate at a suboptimal glycolytic capacity of 20% and 15%, respectively (Figs. S8B-C). Similar to OCR, DETA-NO pretreatment progressively reduced the ability of Mtb to increase ECAR in response to CCCP (Fig. S8B). However, unlike wt Mtb, MtbΔsufR significantly increased basal ECAR and retains CCCP-induced ECAR in response to 0.25 mM and 0.5 mM of DETA-NO (Fig. S8C). This suggests an increased reliance of MtbΔsufR on glycolysis to handle the bioenergetic needs under NO stress. Similar to MtbΔsufR, the NO-
induced changes in OCR, SRC, and ECAR were recapitulated in sufR-Comp (Figs. S8D–F), indicating that the increased expression of the suf operon rather than sufR alone is critical for Mtb’s response to NO.

Lastly, we asked whether NO perturbed redox homeostasis in Mtb. We used a genetic biosensor (Mrx1-roGFP2) to measure the redox potential of a physiologically relevant antioxidant, mycothiol (MSH), as a proxy for the cytoplasmic redox potential ($E_{MSH}$) of Mtb [59].

Ratiometric measurements of emission at 510 nm after excitation at 405 and 488 nm can easily quantify any changes in redox physiology [59]. In response to an oxidant or a reductant, the biosensor ratio showed a rapid increase or decrease, respectively [59]. Msb and MtbΔsufR expressing Mrx1-roGFP2 were treated with 0.25 mM, 0.5 mM, 1 mM DETA-NO, and the biosensor ratio was measured. Exposure of Mtb to NO did not increase the Mrx1-roGFP2 ratio, indicating that wt Mtb robustly maintains
cytoplasmic E\textsubscript{SSi} in response to NO (Fig. 7A). In contrast, NO induces a slightly higher oxidative shift in E\textsubscript{SSi} of \textit{Mtb}\textsubscript{ΔsufR} than wt \textit{Mtb} in a dose-dependent manner (Fig. 7A). Using a ROS-sensitive fluorescent dye Cell ROX, we confirmed that NO induces oxidative stress in \textit{Mtb}\textsubscript{ΔsufR} but not in wt \textit{Mtb} (Fig. 7B). Altogether, our data indicate that the \textit{suf} operon’s NO-mediated induction is required to regenerate Fe–S clusters, maintain respiratory reserves, and buffer redox imbalance.

3.7. \textit{SufR} is required to recover from NO-induced growth arrest and persistence in vivo

Next, we investigated the biological consequence of compromised Fe–S homeostasis and bioenergetics by assessing the phenotype of \textit{Mtb}\textsubscript{ΔsufR} under NO stress. First, we investigated the survival phenotype of \textit{Mtb}\textsubscript{ΔsufR} under NO stress in \textit{vitro}. A single dose of various concentrations of DETA-NO (0.5 mM, 1.25 mM, and 2.5 mM) did not influence the survival of \textit{Mtb}\textsubscript{ΔsufR} (Fig. 8A and Fig. S9). Repeated exposure to low doses of NO is known to arrest \textit{Mtb}’s growth for an extended duration [60]. Administration of 0.1 mM of DETA-NO every 6 h for 36 h induces an extended period of growth arrest followed by recovery of wt \textit{Mtb} at day 7 post-treatment (Fig. 8B). In contrast, \textit{Mtb}\textsubscript{ΔsufR} resumed growth only at day 16 post-treatment with NO (Fig. 8B). The \textit{sufR-Comp} strain showed a recovery defect largely similar to \textit{Mtb}\textsubscript{ΔsufR}, reinforcing that the NO inducibility of the entire \textit{suf} operon rather than only \textit{sufR} is necessary for the timely resumption of growth. We confirmed this using CRISPRi-\textit{sufT} strain, which expresses reduced levels of another Fe–S cluster assembly factor- SuT. Similar to \textit{Mtb}\textsubscript{ΔsufR}, diminished levels of \textit{sufT} delayed recovery of \textit{Mtb} from NO-induced growth inhibition (Fig. 8C).

Immunologically activated macrophages are known to induce nitrosative stress in \textit{Mtb} [61]. In line with this, \textit{Mtb}\textsubscript{ΔsufR} showed 10- and 15-fold reduced survival in immune-activated RAW 264.7 macrophages as compared to wt \textit{Mtb} at day 2 and 4 post-infection, respectively (Fig. 8D). These observations prompted us to investigate the NO-dependent phenotype of \textit{Mtb}\textsubscript{ΔsufR} in vivo. Previous studies have reported the requirement of \textit{SufR} for persistence of \textit{Mtb} in mice [22,62]. However, it remains to be addressed if \textit{SufR} coordinates pathogen’s persistence in response to NO. wt\textit{Mtb} and \textit{Mtb}\textsubscript{ΔsufR} showed comparable growth in the lungs of BALB/c mice during the acute phase of infection (0–2 weeks) (Fig. 8E). However, \textit{Mtb}\textsubscript{ΔsufR} was cleared progressively from the lungs, with a more than 1.5-log decline in bacterial burden by 8 weeks (Fig. 8E). The histopathological changes observed in animals’ lungs at 8 weeks post-infection were proportionate to the bacterial burden (Fig. S10). The magnitude of pulmonary damage was highest in case of wt \textit{Mtb} (2.75 ± 0.5), intermediate in \textit{sufR-Comp} (2.0 ± 0.8), and lowest in \textit{Mtb}\textsubscript{ΔsufR} (0.25 ± 0.50) (Fig. S10B). Lastly, to clarify \textit{NO}’s role in the persistence defect of \textit{Mtb}\textsubscript{ΔsufR}, we infected a highly susceptible mouse strain lacking inducible nitric oxide (iNOS\textsuperscript{−/−}) [5]. We found that the persistence defect of \textit{Mtb}\textsubscript{ΔsufR} was abolished in iNOS\textsuperscript{−/−} mouse, indicating the requirement of \textit{SufR} for the persistence of \textit{Mtb} in response to NO (Fig. 8F–G).

Surprisingly, the persistence defect of \textit{Mtb}\textsubscript{ΔsufR} was somewhat rescued in animals and macrophages infected with \textit{sufR-Comp} (Fig. 8D–E). Since \textit{suf} operon’s expression is also responsive to Fe-limitation encountered in vivo (41), it is possible that \textit{SufR} induces the expression of \textit{suf} operon under Fe-limitation from a promoter that is distinct from NO-responsive promoter in \textit{sufR-Comp}. Consistent with this, overexpression of \textit{SufR} led to its binding inside the ORF of \textit{Rv1461} (\textit{sufB}) in \textit{Mtb} [63]. Moreover, several other transcription factors (e.g., \textit{Rv0081}, \textit{Rv0023}, \textit{Rv1189}, \textit{Rv3765c}, \textit{Rv3849}, \textit{Rv0260c}, and \textit{glnR}) bind and alter the expression of \textit{suf} operon [63,64]. Some of these transcription factors, along with \textit{SufR}, could regulate persistence in \textit{Mtb} (e.g., \textit{iscS}) (42) partially counterbalance the repressed Suf system’s effect in \textit{sufR-Comp} in vivo. Consistent with this, our unpublished data suggest that \textit{iscS} and \textit{Suf} systems compensate for the loss of each other in mediating \textit{Mtb}’s survival inside macrophages. Altogether, data show that \textit{SufR} enables NO-dependent persistence of \textit{Mtb} during infection.

4. Conclusions

Fe–S cluster production is tightly regulated to promote Fe–S formation when the necessity for the clusters is heightened (e.g., ROI/RNI/iron-limitation) and to limit unnecessary production when the demand is low (e.g., hypoxia) (43). Deregulation of Fe–S cluster biogenesis can lead to toxic accumulation of iron and polysulfides inside cells (43). Therefore, the calibrated expression of Fe–S cluster biogenesis is important. Here we show that \textit{Mtb} \textit{SufR} is required to regulate Fe–S cluster biogenesis in \textit{Mtb} under NO stress. Our findings provide mechanistic insights into how \textit{Mtb} exploits Fe–S cluster regulation and biogenesis under NO stress to favor the pathogen’s persistence.

We found that NO more severely inhibits spare respiratory capacity of \textit{Mtb}\textsubscript{ΔsufR} as compared to wt \textit{Mtb}. Since spare respiratory capacity depends mainly on the recruitment of previously inactive respiratory complexes (55), the SUF system’s sustained activation can provide a reserve of Fe–S cluster-containing respiratory complexes to maintain electron transfer in response to NO. Induction of the \textit{suf} operon in response to conditions that damage Fe–S clusters (e.g., H\textsubscript{2}O\textsubscript{2}, NO, iron-starvation, antibiotics, phagosomal pH, and sputum) [22,23,38,39, 405/488 nm) was measured using flow cytometry. Data (Fig. 7A) were the result of three independent experiments performed in triplicate (mean ± SD). One-way analysis of variance (ANOVA) with Bonferroni’s post hoc test was employed to determine statistical significance. (B) wt \textit{Mtb} and \textit{Mtb}\textsubscript{ΔsufR} treated with the indicated concentrations of DETA-NO for 2 h and stained with CellROX Deep Red reagent to measure endogenous ROS. Data shown are the result of three independent experiments performed in triplicate (mean ± SD). Two-way analysis of variance (ANOVA) with Bonferroni’s post hoc test was employed to determine statistical significance between different doses of DETA-NO. Symbols: (*) comparison to \textit{Mtb}, (#) comparison to untreated \textit{Mtb}\textsubscript{ΔsufR}. *** p<0.01 *** p<0.001 ***
Redox Biology 46 (2021) 102062

K. Anand et al.

nation to maintain persistence. The capacity of SufR to sense and respond to a range of cues, such as NO, H₂O₂, and iron limitation, possibly empowers Mtb to transduce different redox signals into transcriptional responses crucial for persistence in vivo.

One limitation of our study is the lack of complementation in various in vitro assays. Since the polar effects of sufR disruption interfered with NO-inducibility of the downstream suf genes, the phenotypic changes exhibited by MtbΔsufR were mainly due to basal expression of the suf operon. Consistent with this, the restoration of expression of sufR alone did not rescue the phenotype of MtbΔsufR in vitro. Surprisingly, while both MtbΔsufR and sufR-Comp showed defective recovery from NO-mediated growth arrest, the mutant strain recovered earlier than the complemented strain. In this context, we noticed that NO exposure upregulated the DOS dormancy regulon more in MtbΔsufR as compared to wt Mtb but below 2-fold (FDR < 0.05) cutoff (Fig. S11). Notably, the expression of the DOS regulon was restored to wt Mtb levels in sufR-Comp under NO stress (Fig. S11). Therefore, marginally better recovery of MtbΔsufR than sufR-comp could be a consequence of elevated DOS regulon in the mutant. Agreeing to this, a Mtb strain lacking DOS dormancy regulator (MtbΔdosR) showed defective recovery from NO-mediated growth cessation [60]. Interestingly, a previous study reported overexpression of the suf operon in MtbΔdosR under hypoxia [15], signifying a regulatory loop between SufR and DosR in Mtb. In addition to SufR and DosR/S/T system, the Fe–S cluster containing regulators such as WhiB3 and WhiB1 also respond to NO [32,50,69]. Further, using bacterial-one-hybrid system, another study reported binding of WhiB3 to the promoter region of suf [64], suggesting that further experiments are needed to fully understand the mechanism underlying the regulation of the suf operon in Mtb.

Previous studies on the sufR mutant did not clarify the polar effects on the downstream suf genes. Pandey et al., reported a marginal induction of sufD, sufC, and sufT and a basal expression of sufB, sufS, and sufU in the sufR-deleted strain (ΔsufR < [22]. ΔsufR Δflew similar to wt Mtb under standard growth conditions but showed survival defect under redox stress and inside macrophages [22]. The mutant also displayed persistence defect in mice [22]. In contrast to our findings, Pandey et al., reported a significantly better survival of the sufR-complemented strain (ΔsufR <Δf<) than wt Mtb under diverse in vitro stress conditions and macrophages, and full rescue of the persistence defect in mice [22]. Another study generated three identical truncated mutants of sufR (ΔRv1460stop_1.19, ΔRv1460stop_5.19, and ΔRv1460stop_5.20) by introducing a premature stop codon at position 122 [19]. Surprisingly, ΔRv1460stop_1.19 and ΔRv1460stop_5.19 grew slowly than wt Mtb under standard growing conditions, whereas growth of ΔRv1460stop_5.20 was comparable to wt Mtb. Intriguingly, the activity of Fe–S cluster enzymes was not affected in ΔRv1460stop_5.20 but diminished in the reported sufR-complemented strain [19]. None of these studies examined the expression of full suf operon both in the sufR mutant and the complemented strain under normal and/or NO stress conditions to rule out polar effects. We believe that the reported discrepancies in the sufR complementation could be due to the use of non-native promoters (e.g., mycobacterium optimum promoter [MOP] [22] and hsp60 [19]) to restore SufR expression in previous studies rather than the NO-responsive native sufR promoter used in this study. Altogether, future work is required to explore the breadth of SufR-mediated gene regulation and the role of additional regulators in coordinating the expression of the suf operon.

Lastly, the suf operon was uniformly induced in clinical isolates of Mtb belonging to five globally circulating lineages during survival inside macrophages [70]. These results indicate that regulation of Fe–S cluster biogenesis is a part of core processes that remain conserved in diverse Mtb lineages evolved under selection pressure inside the human host. Altogether, we propose a new model of mycobacterial persistence in which SufR senses NO through its Fe–S cluster to coordinate Fe–S cluster biogenesis and regulate metabolism, respiration, and redox balance.
Author contributions

KA, AT, and AS participated in the design of the study. KA, AT, KS, NM, AJ, RKJ, RSR, and SNC, carried out the experiments. AR, VN, BG, GN, and ASN contributed to reagents and analyzed the data. KA and AS conceived the study, supervised the project, analyzed the data and drafted the manuscript. All authors read and approved the final manuscript.

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Notes

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Abbreviations

| Abbreviation | Definition                                      |
|--------------|-------------------------------------------------|
| Mtb          | Mycobacterium tuberculosis                      |
| NO           | nitric oxide                                    |
| iNOS         | inducible nitric oxide synthase                  |
| DNIC         | dinitrosoyl-iron dithiol complex                 |
| DTH          | sodium dithionite                               |
| CD           | circular dichroism                              |
| EMSA         | electrophoretic mobility shift assay            |
| DETA-NO      | diethylenetriamine-nitric oxide                 |
| OCR          | Oxygen Consumption Rate                         |
| SRC          | spare respiratory capacity                      |
| CCCP         | carbonyl cyanide m-chlorophenyl hydrzone        |
| ECAR         | extracellular acidification rate                |

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.redox.2021.102062.

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