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Mycobacterium tuberculosis WhiB1 is an essential DNA-binding protein with a nitric oxide sensitive iron-sulphur cluster

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Running title: Characterization of *M. tuberculosis* H37Rv WhiB1

Abbreviations used: BSA, bovine serum albumin; CRP^{Mt}, cyclic-AMP receptor protein Rv3676; DNIC, dinitrosyl-iron thiol complex; DTNB, 5,5'-dithiobis (2-nitrobenzoic acid); DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EMSA, electrophoretic mobility shift assay; EPR, electron paramagnetic resonance; Gent^R, gentamycin resistant; Hyg^R, hygromycin resistant; IRP1, iron-regulatory protein-1; Kan^R, kanamycin resistant; PBS, phosphate buffered saline; *PrpfA*, the *rpfA* promoter region; *PwhiB1*, the *whiB1* promoter region; RNAP, RNA polymerase; rpm, revolutions per min; TBE, Tris-borate EDTA; SDS, sodium dodecyl sulphate; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; TraSH, transposon site hybridization; X-Gal bromo-chloro-indoyl-galactopyranoside

1 *Mycobacterium tuberculosis* is a major pathogen that has the ability to establish, and emerge from, a
2 persistent state. Wbl family proteins are associated with developmental processes in actinomycetes, and *M.*
3 *tuberculosis* has seven such proteins. Here it is shown that the *M. tuberculosis* H37Rv *whiB1* gene is
4 essential. The WhiB1 protein possesses a [4Fe-4S]²⁺ cluster that is stable in air but reacts rapidly with eight
5 equivalents of nitric oxide to yield two dinuclear dinitrosyl-iron thiol complexes. The [4Fe-4S] form of
6 WhiB1 did not bind *whiB1* promoter DNA, but the reduced and oxidized apo-WhiB1, and nitric oxide-
7 treated holo-WhiB1 did bind to DNA. *Mycobacterium smegmatis* RNA polymerase induced transcription of
8 *whiB1 in vitro*; however in the presence of apo-WhiB1 transcription was severely inhibited, irrespective of
9 the presence or absence of the CRP protein Rv3676, which is known to activate *whiB1* expression.
10 Footprinting suggested that autorepression of *whiB1* is achieved by apo-WhiB1 binding at a region that
11 overlaps the core promoter elements. A model incorporating regulation of *whiB1* expression in response to
12 nitric oxide and cAMP is discussed with implications for sensing two important signals in establishing *M.*
13 *tuberculosis* infections.

14
15 Key words: actinomycetes, iron-sulphur cluster, nitric oxide, TB, Wbl protein

16 INTRODUCTION

17
18 Approximately one third of the world's population is infected by *Mycobacterium tuberculosis* [1]. Most
19 infected individuals are essentially asymptomatic, a state characterized by the presence of *M. tuberculosis* in
20 a persistent non-replicating state. However, 1 in 10 infected individuals will become ill with active
21 tuberculosis during their lifetimes, contributing to ~1.8 million deaths in 2008 [1]. Thus, prevention of
22 reactivation tuberculosis is a major healthcare challenge and understanding the reprogramming of gene
23 expression that facilitates entry into, and emergence from, the persistent state is an important research goal
24 [2,3].

25 The *M. tuberculosis* cyclic AMP receptor protein Rv3676 (CRP^{Mt}) is a global regulator that is
26 required for virulence [4]. Amongst the genes regulated by Rv3676 (CRP^{Mt}) are *rpfA* and *whiB1* [4,5]. The
27 *rpfA* gene encodes a protein that is involved in reviving dormant bacteria [6], and *whiB1* encodes a member
28 of a family of proteins (Wbl) that are involved in developmental processes in actinomycetes [7]. Wbl
29 proteins possess four conserved cysteine residues that bind a redox-sensitive iron-sulphur cluster [7-9]. It is
30 assumed that the iron-sulphur cluster will prove essential in controlling Wbl protein function. This
31 assumption is supported by experiments showing that all four conserved cysteine residues are essential for
32 WhiD function in *S. coelicolor* [8]. Recombinant expression and aerobic isolation of all seven Wbl proteins
33 of *M. tuberculosis* resulted in proteins partially occupied by [2Fe-2S] clusters, but after anaerobic
34 reconstitution these proteins contained [4Fe-4S] clusters [9]. The presence of a predicted helix-turn-helix,
35 combined with phenotypic studies of mutants, led to the suggestion that Wbl proteins are DNA-binding
36 transcription factors [7, and references therein]. Consistent with this suggestion, *M. tuberculosis* WhiB3
37 interacts with promoter DNA of genes known to be differentially regulated in a *whiB3* mutant, and with the
38 essential sigma factor SigA [10-12]. It has also been suggested that Wbl proteins, including *M. tuberculosis*
39 WhiB1, function as protein disulphide reductases [13], although WhiD from *Streptomyces coelicolor* was
40 shown to lack such activity [14].

41 The *M. tuberculosis* WhiB3 protein contributes to virulence [10] and *whiB3* expression is induced in
42 mouse lungs and macrophages [15]. The WhiB3 [4Fe-4S] cluster is slowly degraded in the presence of O₂
43 resulting in total cluster loss, in contrast to the rapid reaction with O₂ of the *Escherichia coli* O₂-sensing
44 transcription factor FNR which proceeds by cluster conversion from the [4Fe-4S] to the [2Fe-2S] form [16-
45 18]. The WhiB3 and FNR [4Fe-4S] clusters also reacted with nitric oxide (NO) to form dinitrosyl-iron thiol
46 complexes (DNIC) [16, 19]. Thus, it was suggested that WhiB3 acts as a sensor of the physiologically
47 significant gases O₂ and NO to control expression of genes involved in intermediary metabolism.

48 Here it is shown that the *M. tuberculosis* H37Rv *whiB1* gene is essential, and that the WhiB1 protein
49 possesses a [4Fe-4S]²⁺ cluster that reacts rapidly with 8NO molecules per cluster. Furthermore, apo-WhiB1
50 and NO-treated holo-WhiB1 (but not holo-WhiB1) bind at the *whiB1* promoter (P*whiB1*) to repress
51 transcription. Therefore, it is concluded that the WhiB1 [4Fe-4S] cluster is a NO sensor, and that exposure
52 to NO converts holo-WhiB1 from a non-DNA-binding form to a DNA-binding form capable of regulating
53 transcription.

54

55 MATERIALS AND METHODS

56 Bacterial strains, plasmids and growth conditions

57 Bacterial strains and plasmids are listed in Table 1. *Escherichia coli* cultures were grown in Luria-Bertani
58 (LB) medium [20] in a 1:5 volume/flask ratio at 37°C with shaking at 250 rpm. For isolation of WhiB1, the
59 expression plasmid (pGS2164) was selected for by addition of kanamycin (50 µg ml⁻¹). DNA was
60 manipulated using standard methodologies [20]. The *whiB1* open reading frame was amplified by PCR
61 using primers MS40 (5'-TTTTTTGAATTCGATTGGCGCCACAAGGCGGT-3') and MS41 (5'-
62 TTTTTTCTCGAGTCAGACCCCGGTACGGGCTT-3') containing engineered EcoRI and XhoI sites
63 respectively. The amplified DNA was ligated into the corresponding sites of pET28a. The authenticity of
64 the resulting plasmid (pGS2164) was confirmed by DNA sequencing and encoded a His₆-WhiB1 fusion
65 protein.

66 *Mycobacterium tuberculosis* cultures (100 ml) were grown in 1 litre polycarbonate culture bottles
67 (Techmate) in a Bellco roll-in incubator (2 rpm) at 37°C in Dubos broth containing 0.05% (v/v) Tween 80
68 supplemented with 0.2% (v/v) glycerol and 4% Dubos medium albumin. Middlebrook 7H11 agar was used
69 for growth on plates. Where required, kanamycin was added at a final concentration of 25 µg ml⁻¹,
70 hygromycin at 50 µg ml⁻¹, and gentamycin at 15 µg ml⁻¹.

72 Creation of a *M. tuberculosis* H37Rv conditional *whiB1* mutant

73 Approximately 1.5 kb of DNA sequence from each side of the *whiB1* gene (Rv3219) was amplified from *M.*
74 *tuberculosis* genomic DNA using *PfuUltra* (Stratagene) with the following primer pairs: for the 5' side
75 Myc642 (5'-GCGGATCCCGAACAGGCACAGCATCA-3') and Myc643 (5'-
76 GCGGATCCCGCAATCCATTAGTCGT-3') (bp 3594224–3595722), and for the 3' side Myc644 (5'-
77 CGGCGGCCGCGGTCTGACGACTCAGTTCT-3') and Myc645 (5'-
78 CGGCGGCCGCGACGGTGTCTGGTGTGC-3') (bp 3595961–3597391). The 5' fragments were cloned
79 into the BamHI site and 3' fragments into the NotI site of the suicide gene delivery vector p2NIL, which is
80 capable of replication within *E. coli* but lacks a mycobacterial origin of replication [21]. A hygromycin
81 cassette was cloned into the KpnI site of p2NIL to replace the *whiB1* gene. A PacI fragment from pGOAL17
82 containing the *lacZ* and *sacB* genes was inserted to give the completed suicide delivery vector p*whiB1*-KO
83 (Table 1). This vector contains the *sacB* gene from *Bacillus subtilis* that causes lethality when expressed in
84 *M. tuberculosis* making it an effective counter-selectable marker, and a *lacZ* gene. The virulent *M.*
85 *tuberculosis* strain H37Rv was transformed with p*whiB1*-KO and selection was made for potential single
86 crossovers as kanamycin- and hygromycin-resistant colonies expressing the *lacZ* gene. The veracity of the
87 single crossover event was demonstrated by PCR with the following primers internal to the hygromycin
88 cassette and external to the regions of homology: Myc152 (in Hyg cassette, 5'-CGTTAGAACGCGGCTAC-
89 3') and Myc1786 (5'-GCCATTGACGATGTGCTG-3'); Myc153 (in Hyg cassette, 5'-
90 GGTCAGCGAACCAATCA-3') and Myc1789 (5'-GATTACCTGATGTGGGTTCGC-3'); Myc1786 and
91 Myc1787 (5'-GCACCGATTACAGACCAGT-3'); and Myc1789 and Myc1788 (5'-
92 GCAACGCCCGCACGAAAGCC-3'). These colonies were streaked onto plates containing hygromycin to
93 allow for double crossovers to occur and then serially diluted onto plates containing hygromycin, sucrose
94 and X-Gal. No colonies with a double crossover phenotype (Hyg^R, sucrose^S and white on X-Gal) were
95 obtained at this stage suggesting that the *whiB1* deletion was lethal to the bacteria. Accordingly, a single-
96 cross strain was transformed with a complementing plasmid (pDMH1) carrying the *whiB1* gene plus 293 bp
97 5'-flanking and 14 bp 3'-flanking DNA cloned into the EcoRI site of pKP203 (gentamycin-resistant), an
98 integrase-negative derivative of the integrating vector pMV306. This was prepared by PCR of genomic DNA
99 using the primers Myc1777 (5'-GCGAATTC-GCAAGAAAGCGGATCTGAGC-3') and Myc1778 (5'-
100 GCGAATTC-AGAAGTCTGAGTCGTCAGACC-3'). Plasmid pDMH1 was co-transformed into the *whiB1*
101 single crossover strain along with plasmid pBS-int carrying the integrase gene necessary to achieve
102 integration of the plasmid into the chromosome. The pBS-int plasmid lacks a mycobacterial origin of
103 replication and is therefore lost from the bacterium. Using this complemented strain for selection of double
104 crossover events resulted in colonies with the required null phenotype (Gent^R, Hyg^R, sucrose^S and white on
105 X-Gal) of which after testing by PCR as above, seven out of eight colonies were genuine double crossovers.
106 This deletion removes all but the initial 11 5' DNA bases and 3 3' bases of the *whiB1* gene. Subsequently the
107 complementing plasmid in the *whiB1* null mutant was switched [22] using pKP186 (kanamycin-resistant)
108 plasmids with 1192 bp DNA 5' to *whiB1* (pDMH2, using primers Myc1779 (5'-GCGAATTC-

109 GCCGCGACCTGCTGGCGCAC-3') and Myc1778) and 1192 bp DNA 5' and 535 bp DNA 3' to the *whiB1*
110 gene (pDMH3, using primers Myc1779 and Myc1785 (5'-GCGAATTC-CACGATGCGTTGTTCGATGTC-
111 3') to give faster growing colonies (see *Results*).

112

113 **Quantification of *whiB1* expression by qRT-PCR**

114 RNA isolated as previously described [4] was used to prepare cDNA using a QuantiTect reverse
115 transcription kit (Qiagen). Real-time quantitative PCR on this cDNA was carried out on the ABI Prism 7700
116 Sequence Detection system using the Fast SYBR Green Master Mix (Applied Biosystems). The primers
117 were designed using the Primer Express software (Applied Biosystems). The forward *whiB1* primer was
118 Myc947 (5'-TTCTTCCCGGTAGGAAACAGTG-3'), the reverse primer was Myc948 (5'-
119 ATTACAGACCAGTTTCGCGTCA-3'). The forward primer from *sigA*, the normalizer gene, was Myc1790
120 (5'-TCGGTTCGCGCCTACCT-3'), the reverse primer Myc1791 (5'-GGCTAGCTCGACCTCTTCT-3').

121

122 **Overproduction and purification of WhiB1**

123 For isolation of recombinant WhiB1, cultures of the *E. coli* expression strain (JRG6050) were grown at 37°C
124 in Lennox broth [20] to an OD of ~ 0.6, when IPTG (120 µg ml⁻¹) was added and the cultures were incubated
125 at 25°C for a further 2 h. Cells were lysed after resuspension in 20 mM sodium phosphate buffer (pH 7.4)
126 containing 0.5 M NaCl by two passages through a French pressure cell at 37 MPa. The lysate was cleared by
127 centrifugation and the resulting cell-free extract was applied to a nickel-charged Hi-Trap chelating column
128 (GE Healthcare). The recombinant His₆-WhiB1 protein was eluted using a linear imidazole gradient (0-500
129 mM in 20 ml). WhiB1 containing fractions were either used immediately or stored at -20°C after addition of
130 1 mM DTT.

131

132 **Reconstitution of WhiB1**

133 WhiB1 was reconstituted under anaerobic conditions, in a Mark3 anaerobic workstation (DW Scientific).
134 Protein samples were typically incubated with 25 mM Tris buffer (pH 7.4) L-cysteine (1 mM), DTT (2.5
135 mM) and a 10-fold molar excess of ammonium ferrous sulphate and an aliquot of NifS (225 nM final
136 concentration) as described by Crack *et al.* [23]. Reactions were incubated at room temperature for up to 16
137 h before purification of the reconstituted protein by chromatography on a 1 ml HiTrap heparin column [24].

138

139 **Total amino acid analysis, iron and reactive thiol assays**

140 Amino acid analysis was carried out by Alta Bioscience (University of Birmingham, UK) by complete acid
141 hydrolysis for 24 h at 110°C of aliquots of WhiB1 that had been previously assayed for protein concentration
142 using the BioRad protein reagent with bovine serum albumin as the standard. The results of the analyses
143 were used to determine that the BioRad assay overestimated the amount of protein present in the samples and
144 a correction factor of 0.86 was required to obtain an accurate measurement of protein concentration.

145

146 Aliquots of reconstituted WhiB1 were used to determine their iron content. Samples were boiled in
147 1% (w/v) trichloroacetic acid for 5 min. The clarified supernatants were retained and mixed with saturated
148 sodium acetate, bathophenanthroline sulphonic acid (0.3%) and sodium ascorbate (1.8%). After
149 centrifugation the absorbance at 595 nm was measured. The amount of iron present was estimated by
150 comparison to a standard curve prepared using an iron standard solution (BDH).

151

152 Reactive thiols were measured according to Thelander [25] using ~4 µM apo-WhiB1 protein in each
153 reaction. Where indicated the apo-WhiB1 protein was denatured by pre-treatment with SDS to a final
154 concentration of 1% (w/v).

155

156 **Electron paramagnetic resonance (EPR) analysis**

157 WhiB1 samples were transferred to EPR tubes and, where indicated, a 27-fold molar excess of proline
158 NONOate was added for the indicated times before the samples were frozen in liquid nitrogen. EPR spectra
159 were recorded with a Bruker EMX spectrometer equipped with a TE-102 microwave cavity and an ESR-900
160 helium flow cryostat (Oxford Instruments). Spectra were recorded at both 20 K and 77 K. No [3Fe-4S]¹⁺
161 signals were detected in any of the nitrosylated samples (not shown). The microwave power and frequency
162 were 2 mW and 9.68 GHz, respectively, and the field modulation amplitude was 1 mT. Spectra were
163 normalized to the same receiver gain. Spin intensities of paramagnetic samples were estimated by double
164 integration of EPR spectra using 1 mM Cu(II), 10 mM EDTA as the standard.

165

164 **Optical spectroscopy**

165 Scanning spectrometry was carried out using a Cary 50 Bio UV-Vis spectrophotometer using Hellma 10 mm
166 cuvettes with a screw-top lid to maintain anaerobic conditions. Oxygen or NO was injected into the cuvette
167 as air saturated buffer or anaerobic solution of proline NONOate through the Hellma silicone seal in the lid
168 using a Hamilton syringe. The concentration of NO released by aliquots of proline NONOate was measured
169 using a NO electrode (ISO-NOTM Mark II; World Precision Instruments, Stevenage, UK) calibrated using
170 dilutions of a saturated solution of NO gas in H₂O (1.91 mM at 20°C).
171

172 **Protein disulphide reductase activity**

173 The protein disulphide reductase activity of WhiB1 was measured as described by Holmgren [26] with
174 modifications described by Crack *et al.* [14], except that WhiB1 was used in place of WhiD.
175

176 **Electrophoretic mobility shift assays (EMSA), DNase I footprints and *in vitro* transcription** 177 **reactions**

178 The EMSAs were done using the indicated promoter DNA as described by Stapleton *et al.* [5] except that
179 His₆-WhiB1 was used in place of CRP^{Mt}. Apo-WhiB1, reconstituted holo-WhiB1 or holo-WhiB1 treated
180 with a 20-fold molar excess of proline NONOate was used as indicated. Radiolabelled DNA (~2 nM) was
181 incubated with 0-50 μM His₆-WhiB1 in the presence of 40 mM Tris pH 8.0, 1 mM EDTA, 114 mM NaCl, 1
182 mM DTT, 10 mM MgCl₂, 0.25 mg ml⁻¹ BSA and 1 μg calf thymus DNA, for 5 min on ice. Where indicated
183 apo-WhiB1 was oxidized by treatment with 5 mM diamide before analysis. The resulting complexes were
184 then separated on 6% polyacrylamide gels buffered with 0.5X TBE (45 mM Tris-borate, 1 mM EDTA). The
185 sizes of the DNA fragments used were: *whiB1*, -289 to -4, obtained from pGS2060; and *rpfa*, -531 to -349;
186 *3616c*, -1119 to -948, and -217 to -16; and *ahpC*, -127 to +1 (all obtained by PCR with genomic DNA as the
187 template). Numbering is relative to the ATG start codons. For the competition assays 100-fold excess
188 unlabelled *whiB1* or *rpfa* DNA was included in the EMSA reactions with radiolabelled *whiB1* as the target.
189

190 For DNase I footprinting, radiolabelled P*whiB1* DNA (~60 ng) was incubated with 20 μM apo-
191 WhiB1 and 2.5-20 μM CRP^{Mt} in the presence of 40 mM Tris pH 8.0, 75 mM KCl, 10 mM MgCl₂, 0.5 mM
192 EDTA, 5 % (v/v) glycerol, 1 mM DTT and 250 mg ml⁻¹ BSA. The complexes were then digested with 1 unit
193 of DNase I for 15-60 sec at 25°C. Reactions were stopped by the addition of 50 mM EDTA, followed by
194 phenol/chloroform extraction. The DNA was ethanol-precipitated and resuspended in loading buffer (80%
195 v/v formamide, 0.1% w/v SDS, 10% v/v glycerol, 8 mM EDTA, 0.1% w/v bromophenol blue, 0.1% w/v
196 xylene cyanol) for electrophoretic fractionation on 6% polyacrylamide-urea gels and autoradiographic
197 analysis. Maxam and Gilbert G tracks of the DNA fragments were used to provide a calibration [27].
198

199 *In vitro* transcription reactions were as described by Stapleton *et al.* [5] except that apo-WhiB1 was
200 included as indicated. Apo-WhiB1 protein preparations lacked significant deoxyribonuclease and
201 ribonuclease activities.

201 **RESULTS**

202 **The *M. tuberculosis whiB1* gene is essential**

203 Attempts to create a *M. tuberculosis whiB1* deletion mutant were unsuccessful. Although single crossovers
204 were obtained it was not possible to select for double crossover events. Therefore, a conditional mutant was
205 created by including a wild-type copy of the *whiB1* gene on a plasmid (pDMH1) together with 293 bp DNA
206 upstream of the *whiB1* open reading frame. When pDMH1 was present, double crossovers that disrupted the
207 chromosomal *whiB1* gene were isolated. When the vector (pKP203) was present, double crossover events
208 were not be selected. Therefore, it was concluded that under the conditions used here the *whiB1* gene is
209 essential for *M. tuberculosis* H37Rv. However, even in the presence of plasmid-encoded WhiB1 (pDMH1),
210 the conditional *whiB1* mutant grew slowly, forming small colonies on 7H11 medium. This growth defect
211 was accounted for by the low level of expression of *whiB1* from pDMH1 in the *whiB1* mutant, which was
212 only ~20% of that obtained from the chromosomal copy of the wild-type strain, as indicated by qRT-PCR.
213 Complementation by a plasmid containing an extra 899 bp upstream of *whiB1* (pDMH2) resulted in an
214 improved growth rate, the formation of normal sized colonies, and an increase in expression of *whiB1*
215 (equivalent to ~65% of the wild type). A plasmid that in addition also contained an extra 521 bp downstream
216 of *whiB1* (pDMH3) resulted in a further increase in *whiB1* expression (equivalent to ~130% of wild type).
217 These observations suggest the presence of positive regulatory elements located upstream of -293 bp in the

218 *whiB1* promoter, and also up to 521 bp downstream of the *whiB1* gene, or that the local structure of the
219 chromatin for the chromosome and plasmid are sufficiently different to facilitate enhanced expression of
220 chromosomal *whiB1* compared to plasmid-borne *whiB1*. The *whiB1* gene has its own promoter [5,29], and
221 the gene downstream of *whiB1* (Rv3220c) has the opposite orientation. Hence, it is unlikely that polar
222 effects could account for either, the apparent essentiality of *whiB1* or the observation that DNA downstream
223 of *whiB1* is needed for full complementation of *whiB1* expression. It should be noted that *whiB1* has
224 previously been reported as a non-essential gene based on transposon site hybridization (TraSH) screening
225 [30], but there are several possible reasons for false negative results, including transposon insertion in non-
226 essential regions either in or adjacent to essential genes resulting in TraSH probes that are still able to
227 hybridize. This could be a particular problem for small genes such as *whiB1*.

228 229 **WhiB1 contains an O₂-insensitive [4Fe-4S]²⁺ cluster**

230 The essential nature of *M. tuberculosis whiB1* causes difficulty in assigning function. Several members of the
231 Wbl family of proteins, including WhiB1, have been shown to possess iron-sulphur clusters [9]. Although
232 the properties of these iron-sulphur clusters are relatively poorly characterized, it is assumed that they are
233 important for Wbl protein function. Therefore, the *M. tuberculosis* H37Rv WhiB1 protein was overproduced
234 as a soluble His₆-tagged protein in *E. coli* BL21(λDE3) transformed with pGS2164. On the affinity column
235 a dark brown band was formed, which eluted as pale yellow-brown fractions, with UV-visible spectra typical
236 of a [4Fe-4S] cluster (not shown). Upon longer term storage under aerobic conditions the preparations lost
237 their colour, and in the absence of reducing agents the WhiB1 protein was prone to aggregation. Analysis by
238 non-reducing SDS-PAGE showed the presence of different species with molecular masses and N-terminal
239 amino acid sequences consistent with the presence of monomeric, dimeric and trimeric WhiB1 containing
240 inter- and intra-molecular disulphide bonds (not shown). The presence of disulphide bonds in apo-WhiB1
241 was confirmed by measuring the amount of reactive thiol using 5,5'-dithio-bis-nitrobenzoic acid (DTNB) in
242 the absence of reducing agents under native conditions and under denaturing conditions (after treatment with
243 1% SDS). This revealed the presence of two (1.88±0.27, n=9) reactive thiols in the native soluble apo-
244 protein, implying the presence, on average, of one disulphide per apo-WhiB1. The number of reactive thiols
245 per apo-WhiB1 decreased to 1.15±0.15 (n=9) after treatment with SDS, implying that unfolding the protein
246 promoted additional disulphide bond formation (on average 1.5 per monomer). This propensity to form
247 disulphide bonds accounts for the pattern of WhiB1 migration on non-reducing SDS-PAGE.

248 Reconstitution of the WhiB1 iron-sulphur cluster under anaerobic conditions was accompanied by an
249 increase in absorbance around 420 nm (Figure 1A). Total amino acid analysis confirmed the authenticity of
250 the isolated protein and allowed accurate estimation of protein content such that after removing excess
251 reagents from reconstitution mixtures by chromatography on heparin Sepharose, the iron content of the
252 reconstituted protein was measured as 3.57±0.06 Fe atoms per WhiB1 protein (n=3). In this form the protein
253 was stable for several days if kept under anaerobic conditions and exhibited no tendency to aggregate, unlike
254 apo-WhiB1 (see above). The reconstituted WhiB1 absorbance band at 420 nm was bleached by the addition
255 of the reductant dithionite (Figure 1B). Exposure of the reconstituted protein to O₂, by addition of air-
256 saturated buffer equivalent to a final concentration of 110 μM O₂ for up to 2 h, only slightly decreased
257 absorbance at 420 nm (Figure 1C). Thus, it appears that the WhiB1 [4Fe-4S] cluster is more stable in the
258 presence of O₂ than those of other Wbl proteins (WhiD and WhiB3), which degrade significantly when
259 exposed to air [14,16]. The anaerobic CD spectrum of WhiB1 had positive features at 427 nm and 509 nm
260 (Figure 1D) that were similar to those of WhiD [14], but different from those of the O₂ sensor FNR [23],
261 indicating that the environment of the O₂-insensitive WhiB1 iron-sulphur cluster is different from that of the
262 O₂-sensitive FNR iron-sulphur cluster.

263 264 **The WhiB1 iron-sulphur cluster is highly sensitive to NO**

265 Nitric oxide is a key component of the host response to mycobacterial infection, and iron-sulphur clusters
266 serve as sensors of NO in some bacterial regulatory proteins (e.g. NsrR and FNR) [31]. Therefore,
267 reconstituted WhiB1 was titrated with the NO releasing compound proline NONOate under anaerobic
268 conditions and the response of the iron-sulphur cluster was monitored by obtaining UV-visible spectra
269 (Figure 2A). Before addition of NO, the extinction coefficient of the WhiB1 iron-sulphur cluster was
270 estimated to be ~18,000 M⁻¹ cm⁻¹, which is similar to reported values for other [4Fe-4S] containing proteins.
271 Nitric oxide addition caused decreased absorbance at 420 nm and increased absorbance at ~355 nm, with

272 apparent isobestic points at 395 nm and 480 nm, indicative of formation of DNICs [19]. Under these
273 conditions 8NO (7.5-8.2) molecules reacted per WhiB1 [4Fe-4S] cluster (Figure 2B), suggesting the
274 formation of an unusual octa-nitrosylated iron-sulphur cluster. The spectral properties of the product and the
275 observed stoichiometry of the reaction are consistent with the formation of two dinuclear DNICs.
276 Accordingly, using the extinction coefficient ($\sim 8,500 \text{ M}^{-1} \text{ cm}^{-1}$) determined by Cruz-Ramos *et al.* [19], the
277 absorbance increase at 360 nm upon completion of the NO titration was equivalent to the formation of ~ 2.5
278 dinuclear DNICs per iron-sulphur cluster. Remarkably, unlike the reaction of WhiB1 with O_2 , the spectral
279 changes upon exposure to NO were extremely rapid. Using stopped flow to monitor absorbance changes at
280 364 nm upon mixing of a 92-fold molar excess of NO with holo-WhiB1 (7 μM iron-sulphur cluster) the
281 reaction was complete within 30 sec of mixing; the kinetic details of the reaction of WhiB1 with NO will be
282 reported elsewhere.

283 Reconstituted WhiB1 under anaerobic conditions was EPR silent, consistent with the presence of a
284 diamagnetic $[4\text{Fe-4S}]^{2+}$ cluster (Figure 3). However, monomeric DNICs have characteristic EPR spectra
285 [32]. After treatment with a 27-fold molar excess of proline NONOate under anaerobic conditions for 10
286 min an axial EPR spectrum ($g = 2.048, 2.03, \text{ and } 2.022$) was detected, indicative of the presence of 6 μM
287 monomeric DNIC arising from 17 μM iron-sulphur cluster (i.e. $\sim 10\%$ of total Fe was present as an EPR
288 detectable monomeric DNIC). Because DTT was present in the reaction buffer, some of this intensity may
289 have arisen from a DTT-coordinated DNIC species. The intensity of the 2.03 signal decreased to that
290 equivalent to 2.8 μM monomeric DNIC (equivalent to 4% total Fe as monomeric DNIC) after further
291 incubation. Taken together with the UV-visible spectra, to which both mononuclear and dinuclear DNICs
292 contribute, the EPR data suggest that exposure of holo-WhiB1 to NO results in the formation of a mixture of
293 EPR active monomeric and EPR silent DNICs, the latter presumed to be the dinuclear form, with the EPR-
294 silent form as the major species. Similar observations were made with the *E. coli* FNR protein after
295 exposure to NO [19].

296 Under anaerobic conditions the DNIC form of WhiB1 was stable for at least 2 h at 25°C in the
297 presence of 1 mM DTT (Figure 4, spectrum 2). Introduction of air caused the absorbance at 360 nm to
298 decrease by $\sim 20\%$ after 2 h (Figure 4, spectrum 3), and only after >24 h was the DNIC form degraded to
299 apo-WhiB1 (Figure 4, spectrum 4).

301 WhiB1 is an NO-responsive DNA-binding protein

302 It has been suggested that Wbl proteins are transcription factors and/or general protein disulphide reductases
303 (see above). Therefore, the protein disulphide reductase activity of apo-WhiB1, prepared by prolonged
304 exposure to air, was tested in an insulin reduction assay [26]. Upon reduction of disulphide bonds the
305 insulin-B chain precipitates, which can be monitored at 600 nm. For comparison *E. coli* thioredoxin was
306 used as a *bone fide* general protein disulphide reductase. Although increased insulin reduction was observed
307 as the concentration of apo-WhiB1 increased, the rates of reaction were slow compared to those obtained
308 with a much lower concentration of thioredoxin and were similar to the rate obtained when both thioredoxin
309 and WhiB1 were omitted from the reactions (Table 2). Garg *et al.* reported significant WhiB1 protein
310 disulphide reductase activity using a similar assay, and later showed that WhiB1 specifically interacted with
311 and was able to reduce disulphide bonds in the $\alpha(1,4)$ -glucan branching enzyme GlgB [13,33]. However, the
312 data reported here indicate that *M. tuberculosis* WhiB1 is not a general protein disulphide reductase, but do
313 not exclude the possibility that apo-WhiB1 could act as a specific protein disulphide reductase.

314 The DNA-binding ability of WhiB1 was tested using electrophoretic mobility shift assays (EMSA)
315 with the *whiB1* promoter (*PwhiB1*) as the target. Apo-WhiB1 (0.08 ± 0.04 Fe atoms per monomer) in the
316 presence of the reducing agent DTT bound *PwhiB1* (Figure 5A, lanes 2-5). The smeared appearance of the
317 EMSAs at lower protein concentrations suggests that the complex is relatively unstable and dissociates
318 during electrophoresis (Figure 5A, lane 3). DNA-binding by WhiB1 was inhibited by the presence of the
319 $[4\text{Fe-4S}]$ cluster (Figure 5A, lanes 6-9), but was restored after treatment of holo-WhiB1 with NO (Figure
320 5B). Furthermore, DNA-binding was abolished following reconstitution of an iron-sulphur cluster into NO-
321 treated WhiB1 (not shown). In the absence of additional reducing agent (DTT) NO-treated WhiB1 formed a
322 second retarded species of lower mobility (Figure 5B, lane 5), which was absent following the addition of
323 DTT (Figure 5B, lane 10). A similar species was also present when apo-WhiB1 was analyzed (Figure 5A,
324 lane 5). Oxidation of apo-WhiB1 by treatment with 5 mM diamide increased the amount of the slow
325 migrating DTT-sensitive complex (Figure 5C, compare lanes 2 and 5). These data, and the relative stability

326 of the DNIC-WhiB1 (Figures 3 and 4), suggests that the aerobic EMSAs shown in Figure 5B contained
327 mostly dinuclear DNIC-WhiB1 mixed with some reduced and oxidized apo-WhiB1. These results are
328 consistent with the assignments of the slower migrating complex as that formed between *PwhiB1* and
329 oxidized (disulphide) WhiB1, and the faster migrating complex as that formed between *PwhiB1* and reduced
330 (dithiol) apo-WhiB1 and/or DNIC-WhiB1. Specificity of the DNA-binding was demonstrated by the
331 inability of apo-WhiB1 to bind *rpfA*, *ahpC* and *Rv3616c* promoter DNA (not shown). Specificity of DNA-
332 binding was further demonstrated by competition experiments in which a 100-fold excess of unlabelled
333 *PwhiB1* DNA inhibited binding of both oxidized (Figure 5C, compare lanes 2 and 3) and reduced (Figure
334 5D, compare lanes 2 and 3) apo-WhiB1 at labelled *PwhiB1*, whereas 100-fold excess unlabelled *rpfA*
335 promoter DNA did not (Figure 5C, compare lanes 2 and 4; Figure 5D, compare lanes 2 and 4). Thus, it was
336 concluded that DNA-binding by WhiB1 is complex and is affected by the oxidation state of the apo-protein
337 and the chemical form ([4Fe-4S] or DNIC) of its cofactor.
338

339 DNase I footprinting of apo-WhiB1 at *PwhiB1*

340 Footprinting of apo-WhiB1 at *PwhiB1* revealed a distinctive DNase I cleavage pattern characterized by a
341 protected region (W1) located at -42 to -3 relative to the transcript start (Figure 6). The footprint also
342 exhibited several hypersensitive sites, suggesting that apo-WhiB1 causes significant distortion of *PwhiB1*
343 (Figure 6). W1 was located downstream of the activating Rv3676 binding site (CRP1, centred at -58.5, [5])
344 and overlapped the repressing Rv3676 binding site (CRP2, centred at -37.5) and the -10 element of *PwhiB1*.
345

346 Apo-WhiB1 represses *whiB1* transcription *in vitro*

347 Previous work has shown that *whiB1* expression is activated by intermediate concentrations (2.5 μ M) of the
348 *M. tuberculosis* cAMP receptor protein (Rv3676), but that higher concentrations (20 μ M) result in repression
349 of *whiB1* expression [5]. *In vitro* transcription reactions confirmed this pattern of regulation (Figure 7,
350 compare lanes 1, 5 and 9). In the absence of Rv3676, apo-WhiB1 significantly inhibited *whiB1* transcription
351 at concentrations as low as 2 μ M (Figure 7, lanes 1-4). In the presence of activating concentrations of
352 Rv3676 (Figure 7, lanes 5-8), or in the presence of repressing concentrations of Rv3676 (Figure 7, lanes 9-
353 12), apo-WhiB1 still repressed *whiB1* transcription. It was therefore concluded that apo-WhiB1 acts as an
354 inhibitor of *whiB1* expression, and that this inhibition is more severe than that mediated by high
355 concentrations of Rv3676 and silences any Rv3676-dependent activation of *whiB1* expression.
356

357 Discussion

358 Nitric oxide reacts with proteins that contain iron-sulphur clusters, non-haem iron, haem and thiols [34], and
359 the consequent toxic effects are exploited by host immune systems in the response to pathogenic bacteria
360 [35], providing the selection pressure for such bacteria to evolve mechanisms for NO detoxification [36]. To
361 mount an effective defence to counter the effects of NO, bacteria must reprogram gene expression. Several
362 NO-sensing regulators are known, including: the non-haem iron protein NorR; the haem-containing DosS-R
363 and DosT-R two-component systems; and the iron-sulphur proteins FNR, IRP1, NsrR, SoxR, and WhiB3
364 [19,31,37-44]. Reaction of NO with the [4Fe-4S] cluster of the O₂ sensor FNR yields a DNIC form that is
365 incapable of DNA binding [19]. Similarly, NO nitrosylates the [2Fe-2S] cluster of NsrR to inactivate the
366 protein [39]. In contrast, reaction of the SoxR [2Fe-2S] cluster with NO to form a protein bound DNIC
367 activates the protein [40]. The [4Fe-4S] cluster of WhiB3 reacts with NO to form DNIC species, but it is the
368 cluster-free oxidized form that binds DNA with high affinity [12]. IRP1 is a post-transcriptional regulator of
369 iron homeostasis in higher organisms [41]. Like WhiB3, the apo-form of IRP1 binds nucleic acid (in this
370 case mRNA), and its [4Fe-4S] cluster reacts with NO to yield protein bound DNIC [41,42]. Exposure of
371 IRP1 to NO activates mRNA binding, probably by promoting cluster disassembly [43,44]. Here it is shown
372 that the WhiB1 iron-sulphur cluster reacts specifically and very rapidly with NO to convert the protein from
373 a non-DNA-binding form to a DNA-binding form capable of transcription regulation *in vitro*. Expression of
374 *whiB1* in response to NO *in vivo* has not been specifically tested, but the evidence obtained thus far suggests
375 that the essential protein WhiB1 is likely to play a role in establishing *M. tuberculosis* infections by
376 controlling gene expression in response to macrophage-generated or endogenous NO.
377

378 Iron-sulphur clusters are intrinsically sensitive to O₂ and thus the stability of the WhiB1 iron-sulphur
379 cluster in air is worthy of comment. The highly conserved CXXCXXXXXC motif in Wbl proteins, which
contains three of the four Cys ligands of the iron-sulphur cluster, is also present in the extremely sensitive O₂

380 sensor FNR (Figure 8) [17]. In the latter, the O₂-sensitivity of the iron-sulphur cluster is determined by the
381 protein fold [17,18,45,46]. A systematic study of FNR in which the amino acid (Ser) located downstream of
382 Cys 2 (labelled 2 in Figure 8) was replaced by all possible alternatives showed that Pro in this position
383 enhanced the stability of the FNR iron-sulphur cluster in air [18]. The presence of Pro immediately adjacent
384 to Cys 2 may go some way to account for the relative O₂-insensitivity of the iron-sulphur cluster of WhiB1
385 and other Wbl proteins. A second stabilizing substitution is replacement of the Leu residue located upstream
386 of Cys 3 by His [45]. In WhiB1 and WhiD this position is occupied Glu; however a systematic study of the
387 effects of amino acid replacements at this position has not been reported and so the effects of the presence of
388 Glu rather than Leu at this position are unknown.

389 In spite of its insensitivity to O₂, the WhiB1 [4Fe-4S] cluster is remarkably sensitive to NO, reacting
390 extremely rapidly to generate DNIC species. The CD spectrum of WhiB1 is different from that of the O₂
391 sensor FNR [23], indicating that the environment of the O₂-insensitive WhiB1 iron-sulphur cluster is
392 different from that of the O₂-sensitive FNR iron-sulphur cluster. Further detailed biochemical analysis will
393 be necessary to determine the molecular basis underpinning the different responses of these two gas sensors.

394 In contrast to previous reports [13], the data presented here indicate that WhiB1 is not a general
395 protein disulphide reductase (Table 2). Nevertheless, the propensity of the apo-protein to form intra- and
396 inter-molecular disulphide bonds suggests that such redox chemistry is possible, but might require an as yet
397 unidentified reductase and specific target proteins for reduction [33]. WhiB1 interacts with the $\alpha(1,4)$ -
398 glucan branching enzyme GlgB, which is essential for optimal growth of *M. tuberculosis* [30], and thus
399 WhiB1 might be bifunctional, acting as both a gene regulator and a specific GlgB reductase.

400 The apo-WhiB1 footprint at *PwhiB1* showed a 37 bp protected region (-40 to -3, relative to the
401 transcript start) that contained related DNA sequences (⁻³²TGACA⁻²⁸ and ⁻¹⁴TGACA⁻¹⁰), but it would be
402 premature to propose an apo-WhiB1 binding site sequence; further footprints and/or SELEX experiments are
403 necessary. The footprint was characterized by hypersensitive sites spanning a region that was too large to be
404 accounted for by simple binding of an apo-WhiB1 monomer, suggesting multiple apo-WhiB1-DNA
405 interactions (Figure 6). Based on the location of the apo-WhiB1 footprint the simplest explanation for
406 repression of *whiB1* transcription is that apo-WhiB1 blocks RNA polymerase (RNAP) binding.
407 Alternatively, by analogy with WhiB3 [10], apo-WhiB1 could interact with the sigma factor, SigA, to repress
408 *whiB1* transcription by inhibiting RNAP activity or by preventing RNAP from escaping the promoter.

409 It has recently been shown that upon infection of macrophages a mycobacterial-derived cAMP burst
410 promotes bacterial survival by interfering with host signalling pathways [47]. Moreover, cAMP is important
411 in *M. tuberculosis* gene regulation [4,5,48]. Dual regulation of *whiB1* expression by the cAMP-responsive
412 regulator CRP^{Mt} (Rv3676) and the NO-responsive WhiB1 protein might provide a mechanism to integrate
413 the transcriptional response to two important signals associated with infection (Figure 9). Whatever the
414 detailed mechanism proves to be, the evidence thus far suggests that in the macrophage environment (high
415 NO, high cAMP) transcription of the essential gene *whiB1* will be inhibited. This is consistent with entry
416 into the persistent state. At low NO concentrations (immunocompromised state) holo-WhiB1 is formed, de-
417 repressing *whiB1* transcription, consistent with emergence from the dormant state. The reason for the
418 essentiality of *whiB1 in vitro* is not established, but the data suggest that either sufficient NO is generated
419 during *in vitro* culture of *M. tuberculosis* to activate WhiB1, or that the [4Fe-4S] form regulates as yet
420 unidentified essential gene(s), or that the interaction between WhiB1 and GlgB [30,33] is essential for
421 normal growth. Recall that WhiB2 is also essential and binds DNA in the apo-form [49,50], and that the
422 transcription factor IscR regulates distinct groups of genes in its [2Fe-2S] and apo-forms [51].

423 In conclusion, it is shown that *M. tuberculosis* WhiB1 is an essential [4Fe-4S] protein that acts as a
424 specific NO-sensing DNA-binding protein. The essential nature of the *whiB1* gene and switching of WhiB1
425 DNA-binding activity in response to NO has implications for the control of *M. tuberculosis* gene expression
426 in the host environment. Although high NO concentrations kill *M. tuberculosis* [52], lower concentrations
427 promote entry into the latent state [53], and thus NO sensing and gene expression reprogramming by WhiB1
428 could contribute to developmental adaptations in response to host generated NO.

430 AUTHOR CONTRIBUTION

431 Laura Smith, Melanie Stapleton, Gavin Fullstone, Jason Crack, Salvatore Adinolfi, Debbie Hunt
432 and Evelyn Harvey contributed to the experimental design, carried out the experiments, analyzed
433 the data, and helped with the drafting of the paper. Andrew Thomson, Nick Le Brun, Roger Buxton

434 and Jeffrey Green initiated the project, contributed to the experimental design, data analysis and
435 preparation of the paper.

436

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Figure legends

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Figure 1 The *M. tuberculosis* WhiB1 protein iron-sulphur cluster can be reconstituted under anaerobic conditions and is O₂ stable

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Figure 2 The WhiB1 iron-sulphur cluster reacts with NO

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Figure 3 EPR spectra of WhiB1 and the response to NO

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Figure 4 Stability of the DNIC form of WhiB1

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Figure 5 WhiB1 is a DNA-binding protein

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(A) Binding of WhiB1 at the *whiB1* promoter (*PwhiB1*) region. Radiolabelled *PwhiB1* was incubated with the indicated amounts of apo- or holo-WhiB1 before separation of protein-DNA complexes (*PwhiB1*-WhiB1) by electrophoresis. (B) Binding of NO-treated holo-WhiB1 to *PwhiB1*. A 20-fold molar excess of NO (as proline NONOate) was added to holo-WhiB1 and after incubation for 1 min at 20°C an aliquot was removed and DTT was added to a final concentration of 30 mM. After further incubation at 20°C for 3 min EMSAs were done using the DTT-treated and untreated samples as described in *Materials and Methods*. The final concentrations of WhiB1 protein are

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646 indicated. (C) Oxidation of WhiB1 results in the formation of a second retarded species. Binding of
 647 oxidized apo-WhiB1 (20 μ M, pre-treated with 5 mM diamide) to radiolabelled *PwhiB1* (lane 2) was
 648 challenged by the presence of 100-fold excess unlabelled *PwhiB1* DNA (lane 3) or 100-fold excess
 649 unlabelled *PrpfA* DNA (lane 4). Lane 5 shows the EMSA with reduced (1 mM DTT) apo-WhiB1 and
 650 *PwhiB1*. (D) Apo-WhiB1 binding at *PwhiB1* is specific. Binding of apo-WhiB1 (20 μ M) in the
 651 presence of DTT (1 mM) to radiolabelled *PwhiB1* (lane 2) was challenged by the presence of 100-
 652 fold excess unlabelled *PwhiB1* DNA (lane 3) or 100-fold excess unlabelled *PrpfA* DNA (lane 4).
 653

654 **Figure 6 DNase I footprint of apo-WhiB1 at *PwhiB1***

655 (A) Increasing concentrations of apo-WhiB1, as indicated above each lane, were incubated with
 656 *PwhiB1* before DNase I digestion as described in *Materials and Methods*. Hypersensitive sites
 657 associated with interaction with apo-WhiB1 are arrowed; numbering is relative to the transcriptional
 658 start site. The locations of apo-WhiB1 interaction (W1), the previously identified Rv3676 (CRP^{Mt})
 659 binding sites (CRP1 and CRP2), and the -10 element are indicated. Lane 5 shows a Maxam and
 660 Gilbert G track. (B) Nucleotide sequence of *PwhiB1* showing: the transcript start (+1, block arrow),
 661 the -10 and -35 elements (underlined), the tandem Rv3676 (CRP^{Mt}) binding sites (boxed, CRP1
 662 and CRP2), the hypersensitive sites that characterize interaction with apo-WhiB1 (bold upper
 663 case), the location of the protected region (W1; overlined), and two related DNA sequences within
 664 the protected region (bold lower case) are indicated.
 665

666 **Figure 7 Apo-WhiB1 inhibits transcription of *whiB1* in vitro**

667 Reactions contained: 0.1 pmoles *PwhiB1*, 1 pmole *M. smegmatis* RNAP, 40 mM Tris-Cl pH 8.0, 10
 668 mM MgCl₂, 70 mM NaCl, 1 mM EDTA, 1 mM DTT, 250 μ g ml⁻¹ BSA, 5% glycerol. Proteins
 669 (Rv3676 and apo-WhiB1) were pre-incubated with *PwhiB1* for 10 min at 37°C before addition of
 670 nucleotide triphosphates and further incubation for 10 min at 37°C. The location of the *whiB1*
 671 transcript, the loading control, and the concentrations of the Rv3676 and apo-WhiB1 proteins, are
 672 indicated.
 673

674 **Figure 8 Alignment of the conserved cysteine-rich motif in Wbl proteins and the O₂-sensor FNR**

675 The protein sequences aligned are the Wbl-family proteins WhiB1 and WhiB3 from *M. tuberculosis*,
 676 WhiD from *S. coelicolor*, and the O₂-sensing transcription factor FNR from *E. coli*. The sequences
 677 are shown in single letter amino acid code and the Cys residues are labelled 1-3 for ease of
 678 identification in the text.
 679

680 **Figure 9 Nitric oxide- and cAMP-mediated regulation of *whiB1* expression**

681 The forms of WhiB1 are represented by labelled circles, where no specific form is indicated this
 682 represents a mixture of oxidized and reduced apo-WhiB1 and DNIC-WhiB1. The *whiB1* promoter
 683 DNA (*PwhiB1*), Rv3676 (labelled circles), NO and iron-sulphur cluster biosynthesis machinery are
 684 also indicated. From bottom left, when cAMP levels are low and NO is absent, transcription of
 685 *whiB1* occurs at basal levels. At intermediate concentrations of cAMP, expression of *whiB1* is
 686 activated by the *M. tuberculosis* CRP protein (Rv3676) binding at class I site to allow increased
 687 synthesis of apo-WhiB1 [5]. At high concentrations of cAMP, Rv3676 occupies tandem sites at
 688 *PwhiB1* and represses *whiB1* expression [5]. Apo-WhiB1 protein acquires a [4Fe-4S]²⁺ cluster by
 689 the action of the iron-sulphur cluster biosynthesis system (Figure 1), resulting in the non-DNA-
 690 binding form. Each WhiB1 [4Fe-4S]²⁺ cluster reacts extremely rapidly with 8NO (Figure 2) to
 691 produce a mixture of mononuclear and dinuclear dinitrosyl-iron complexes (DNIC; Figures 2 and
 692 3). In the presence of O₂, the DNIC form of WhiB1 slowly degrades to yield apo-WhiB1 (Figure 4).
 693 DNA-binding activity has been demonstrated for reduced and oxidized apo-WhiB1 and NO-treated
 694 WhiB1 (Figures 5 and 6), but it is not yet known whether the minority mononuclear and majority
 695 dinuclear DNIC forms possess DNA-binding activity, or whether they are intermediate species that
 696 yield the proven DNA-binding form, apo-WhiB1; however, the relative stability of the DNIC form
 697 (Figure 4) and the EPR spectra (Figure 3), suggest that the EMSAs with NO-treated WhiB1 contain
 698 mostly dinuclear DNIC WhiB1. The DNA-binding apo-WhiB1 severely represses *whiB1* expression
 699

700 *in vitro* in the presence and absence of the *M. tuberculosis* CRP, Rv3676, by interactions at site(s)
701 overlapping the -10 and -35 elements of the promoter (Figures 6 and 7). Apo-WhiB1 can be
702 switched off by the action of the iron-sulphur cluster assembly machinery to regenerate the non-
703 DNA-binding holo-WhiB1 resulting in derepression of *whiB1* expression and synthesis of apo-
704 WhiB1 protein.

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705 **Table 1 Bacterial strains and plasmids**

Strain or plasmid	Relevant characteristics	Source or reference
<i>E. coli</i> strains		
BL21 (λ DE3)	Lysogen of λ DE3 carrying a copy of the T7 RNAP under the control of the IPTG-inducible <i>lacUV5</i> promoter	Novagen
JRG6050	BL21 (λ DE3) pGS2164	This work
<i>E. coli</i> plasmids		
pET28a	His ₆ -tag overexpression vector; Kan ^R	Novagen
pGS2060	pCR4Blunt-TOPO containing 285 bp upstream of the <i>whiB1</i> coding region	[5]
pGS2164	pET28a derivative encoding His ₆ -WhiB1 fusion protein; Kan ^R	This work
<i>M. tuberculosis</i> strains		
H37Rv	Wild-type virulent strain	[28]
Δ <i>whiB1</i> /pDMH1	Complemented <i>whiB1</i> null mutant	This work
Δ <i>whiB1</i> /pDM2	Complemented <i>whiB1</i> null mutant (more 5')	This work
Δ <i>whiB1</i> /pDM3	Complemented <i>whiB1</i> null mutant (more 5' and 3')	This work
<i>M. tuberculosis</i> shuttle plasmids		
p2NIL	Suicide gene delivery vector, <i>oriE</i> , <i>kan</i>	[21]
p <i>whiB1</i> -KO	Final <i>whiB1</i> knock out p2NIL derivative (see <i>Materials and Methods</i>)	This work
pKP203	Integrase-negative derivative of the integrating vector pMV306, Gent ^R	K.G. Papavinasasundaram
pKP186	Integrase-negative derivative of the integrating vector pMV306, Kan ^R	K.G. Papavinasasundaram
pBS-int	Integrase-positive plasmid lacking a <i>M. tuberculosis</i> origin of replication	B. Springer
pDMH1	<i>whiB1</i> complementing derivative of pKP203 (293 bp 5'-14 bp 3')	This work
pDMH2	<i>whiB1</i> complementing derivative of pKP186 (1192 bp 5'-14 bp 3')	This work
pDMH3	<i>whiB1</i> complementing derivative of pKP186 (1192 bp 5'-535 bp 3')	This work

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Table 2 General protein disulphide reductase activity of WhiB1

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The data shown are the mean values from three or four experiments with mean standard errors of less than ± 0.04 .

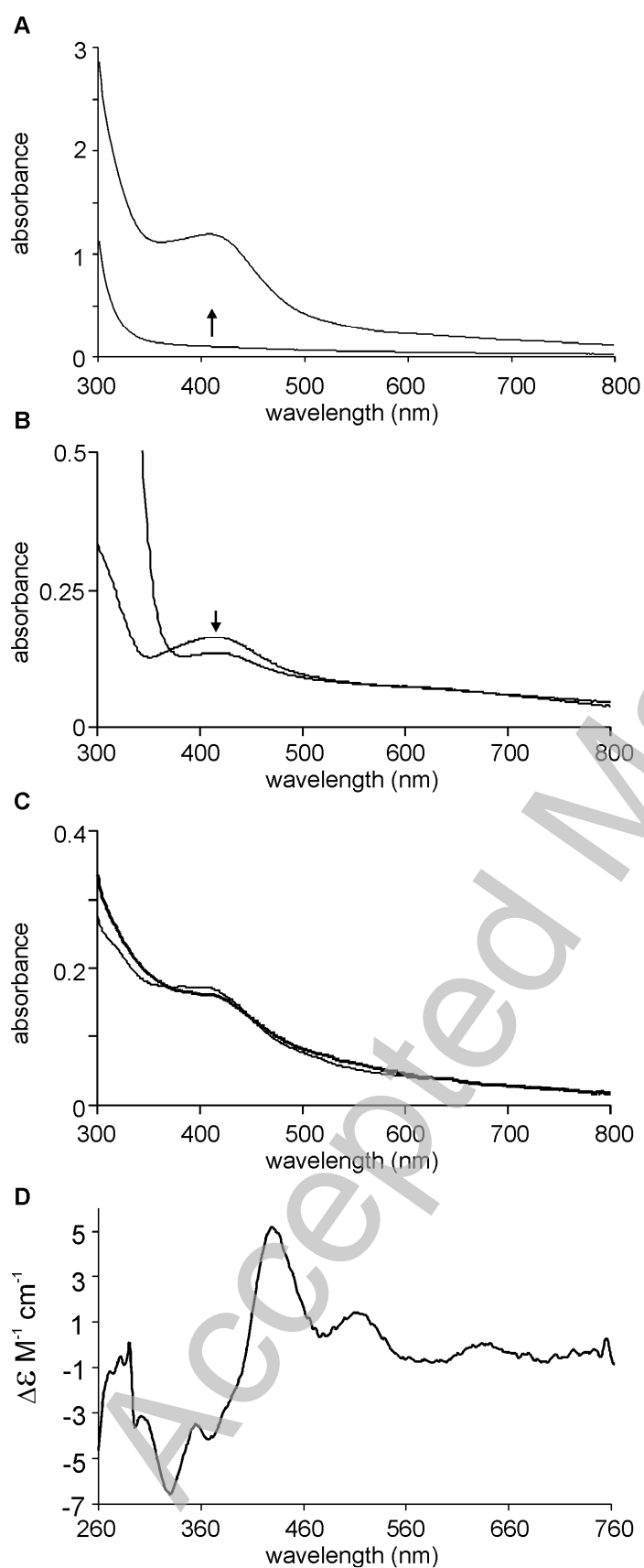
Protein	Concentration (μM)	Rate ($\Delta A_{600} \text{ min}^{-2}$) ($\times 10^{-3}$)
none	-	0.07
thioredoxin	2.5	1.30
apo-WhiB1	5.0	0.10
apo-WhiB1	10	0.43
apo-WhiB1	20	0.45
holo-WhiB1	5.0	0.07
apo-WhiB1 after treatment with NONOate (100 μM)	5.0	0.07
holo-WhiB1 after treatment with NONOate (100 μM)	5.0	0.02

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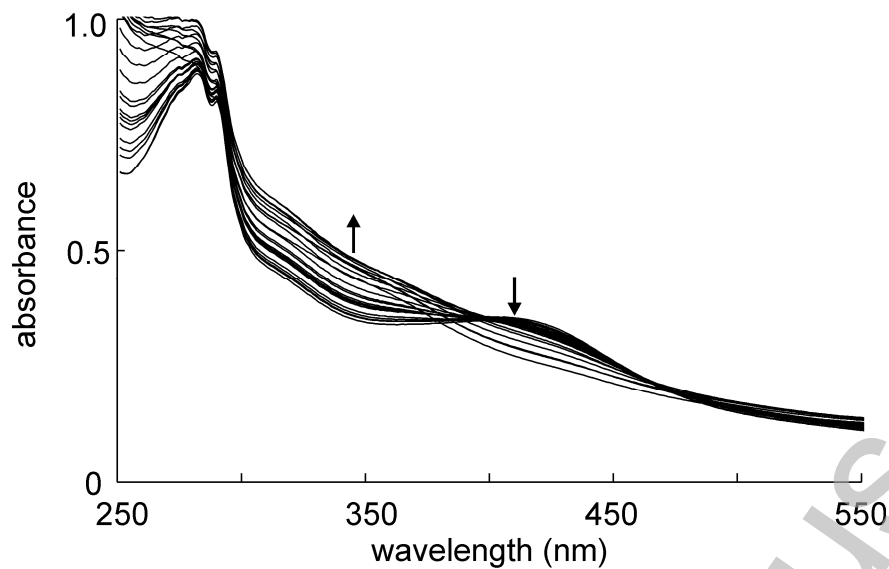
712 Figure 1
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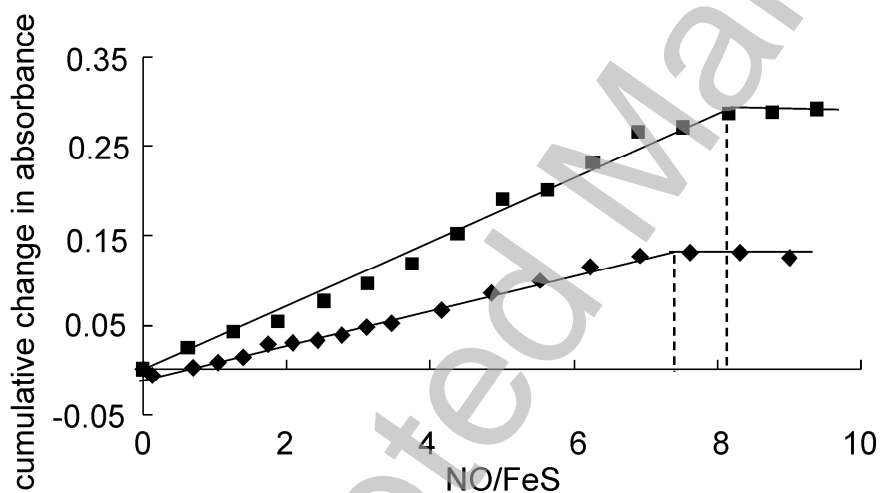
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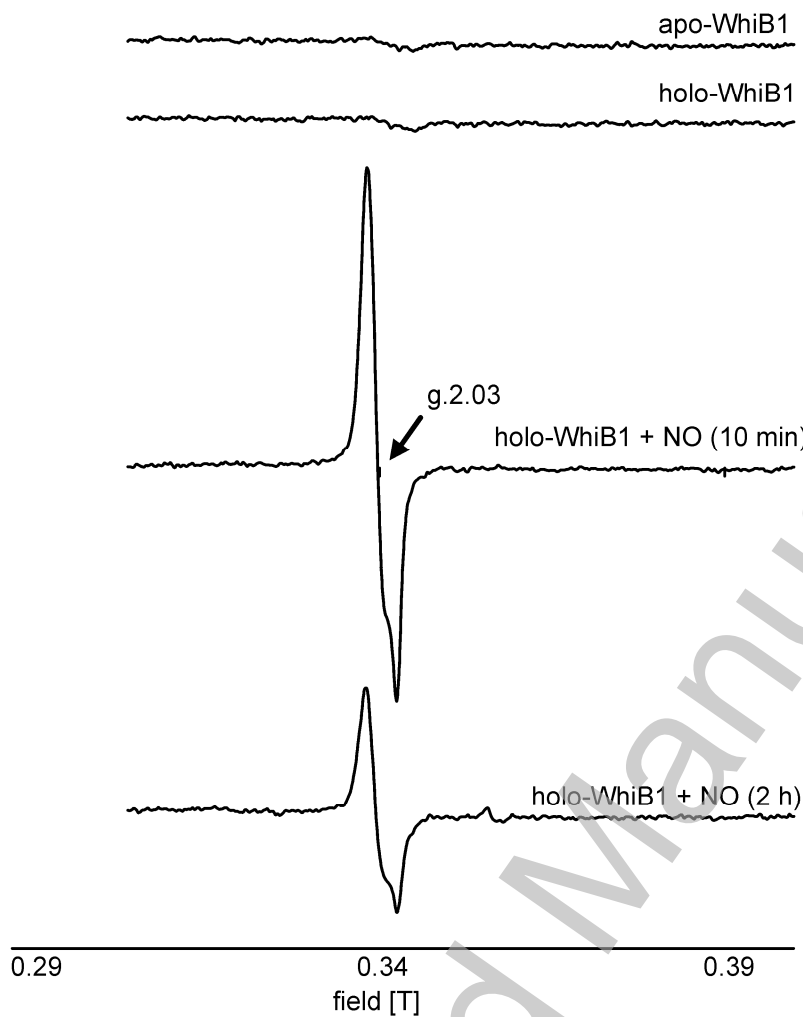
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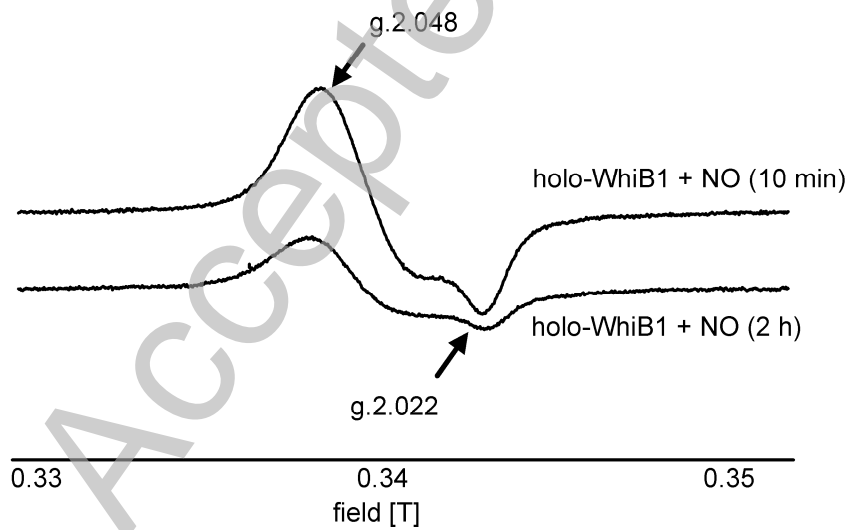
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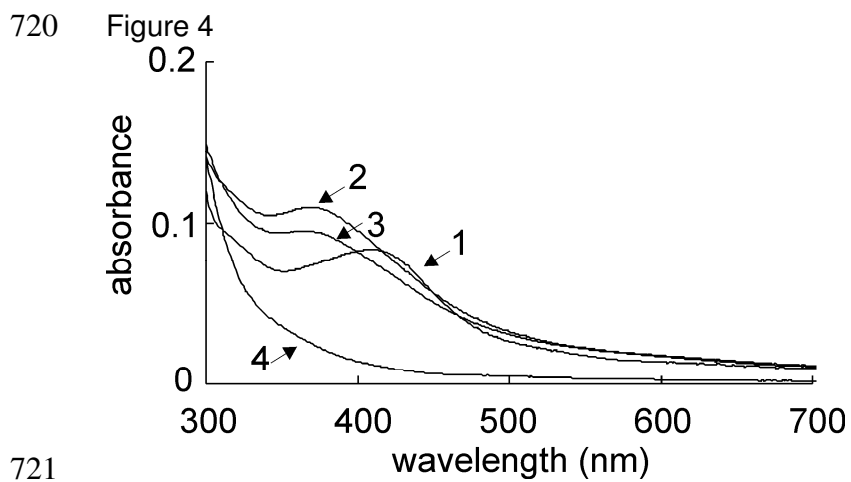
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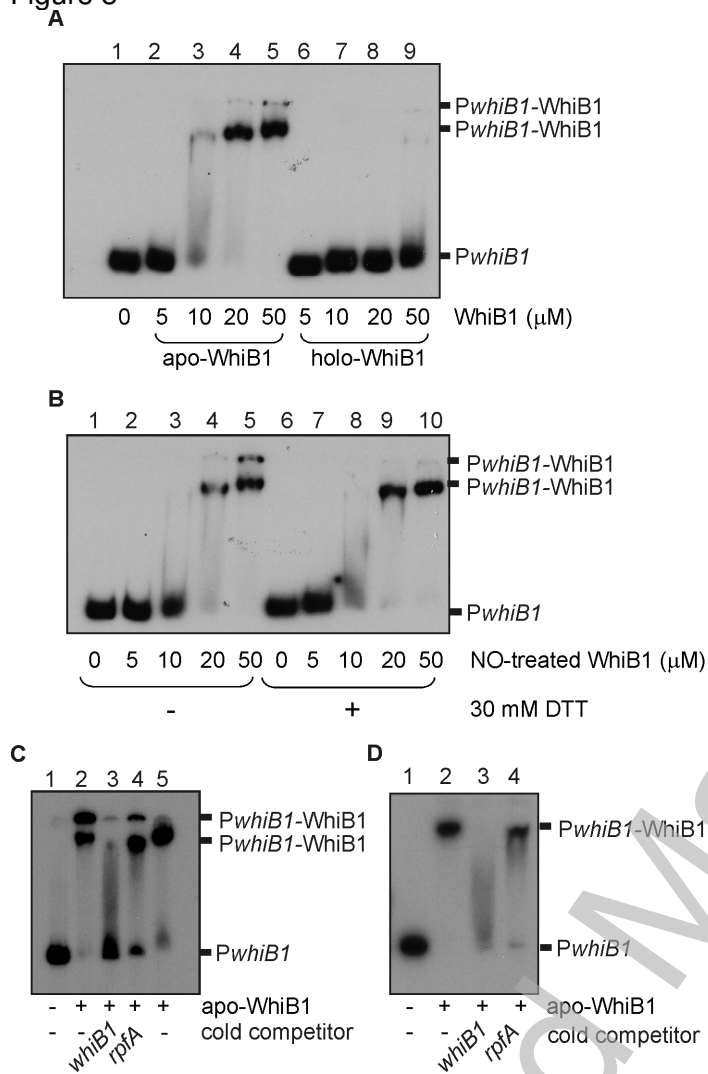
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722 Figure 5

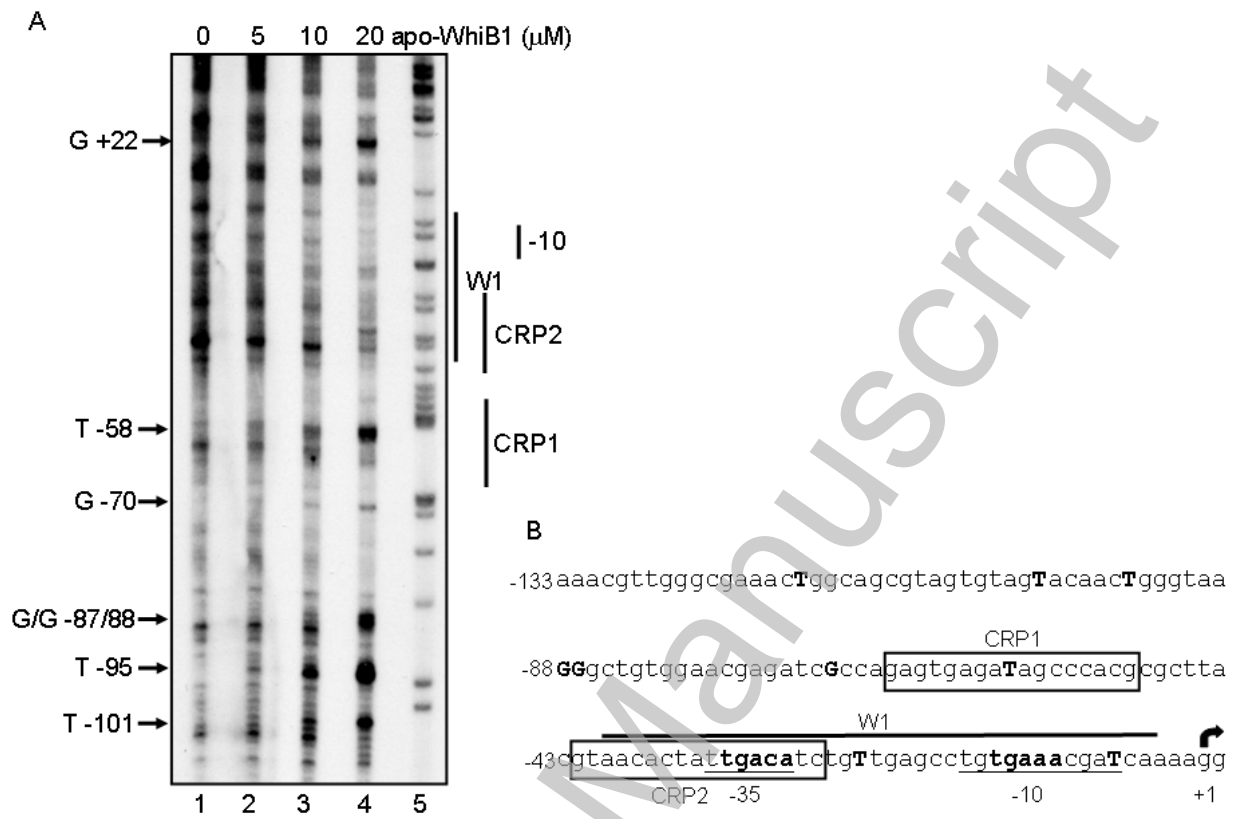


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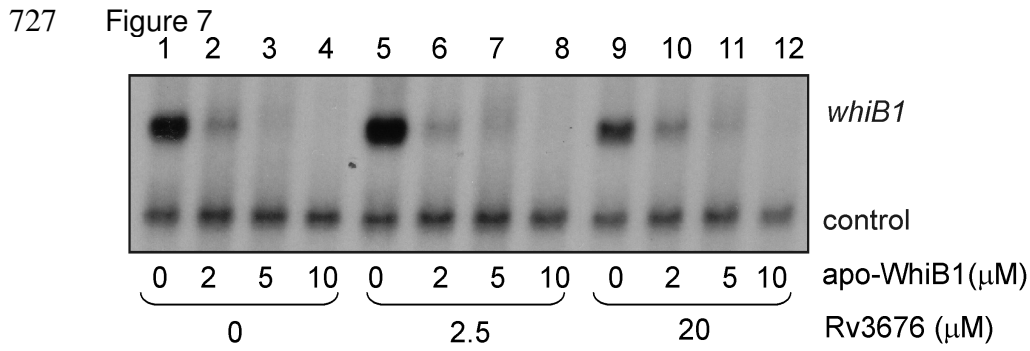
725 Figure 6



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730 **Figure 8**
WhiB1: LVCNRCPVTTECL

WhiB3: EMCRRCPVIEACR

WhiD: EVCMRCPVRAECA

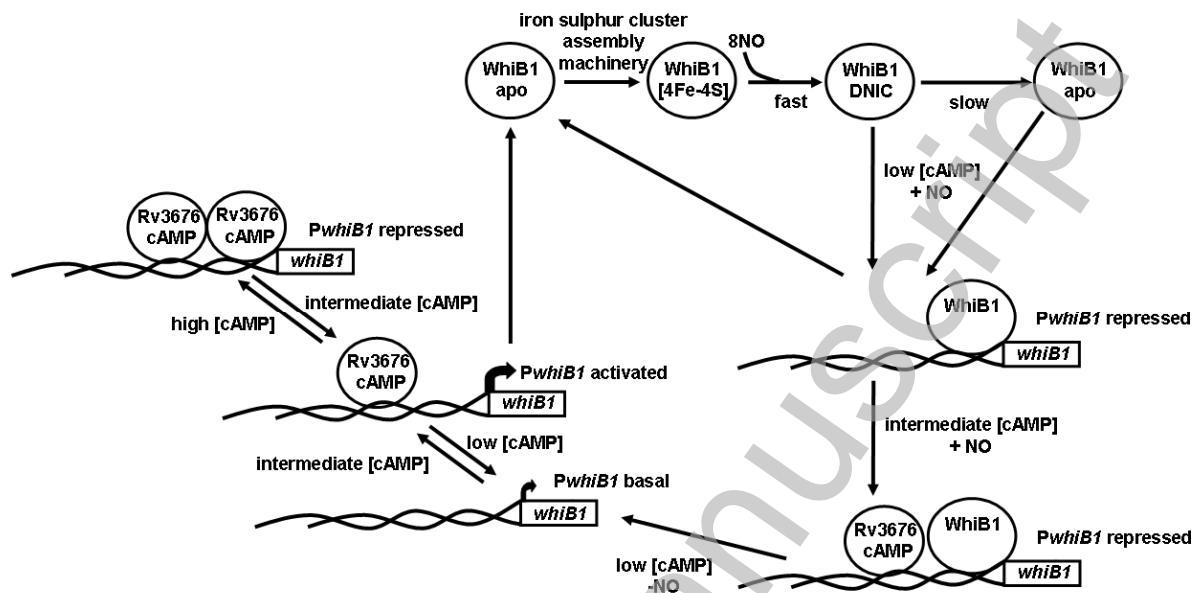
FNR: IHCQDCSISQLCI

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733 Figure 9



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