DNA recognition and transcriptional regulation by the WhiA sporulation factor

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Sporulation in the filamentous bacteria *Streptomyces coelicolor* is a tightly regulated process involving aerial hyphae growth, chromosome segregation, septation and spore maturation. Genetic studies have identified numerous genes that regulate sporulation, including WhiA and the sigma factor WhiG. WhiA, which has been postulated to be a transcriptional regulator, contains two regions typically associated with DNA binding: an N-terminal domain similar to LAGLIDADG homing endonucleases, and a C-terminal helix-turn-helix domain. We characterized several in vitro activities displayed by WhiA. It binds at least two sporulation-specific promoters: its own and that of parABp2. DNA binding is primarily driven by its HTH domain, but requires full-length protein for maximum affinity. WhiA transcription is stimulated by WhiG, while the WhiA protein binds directly to WhiG (leading to inhibition of WhiG-dependent transcription). These separate activities, which resemble a possible feedback loop, may help coordinate the closely timed cessation of aerial growth and subsequent spore formation.

*S. coelicolor* is a filamentous soil-dwelling bacteria that initiates a sporulation program in response to certain environmental cues1. This process of differentiation begins with the formation of aerial hyphae that sprout from the vegetative mycelium, which then undergo multiple rounds of DNA replication in the absence of cell division to generate multigenomic syncitia (recently reviewed in2). Subsequently a coordinated process that includes the cessation of hyphal growth, chromosome segregation and septum formation results in the formation of pre-spore compartments containing individual bacterial chromosomes. A key point in this pathway appears to be the tight coupling of growth cessation with subsequent segregation and compartmentalization of individual bacterial chromosomes into early spore precursors. Later maturation events, including thickening of the cell wall, rounding of the spore’s shape, and the accumulation of the WhiE polyketide pigment, culminates in long chains of robust, desiccation-resistant spores.

A variety of genetic screens have identified genes that are required for this tightly choreographed differentiation process (Figure 1). Those required for the initial formation of aerial hyphae are termed ‘Bld’ (corresponding to *bld* mutants that display a ‘bald’ phenotype under sporule-inducing environmental conditions)3.4. Genes required for the subsequent generation of spores are termed ‘Whi’ (corresponding to *whi* mutants that remain white, rather than accumulating the normal grey color associated with production of polyketide pigment)5.6. *whi* mutants can be further classified into early (WhiA, -B, -G, -H and –I) and late (WhiD, -E) genes, depending on whether mutations result in the complete inability to septate or only an inability to form mature spores1,7. Several additional genes that function during vegetative growth also play important roles in spore formation, including ParA and ParB (an ATPase and a DNA binding protein required for proper chromosome segregation)8,9, FtsZ (a bacterial tubulin homologue required for septation)10,11 and several spore-specific surface proteins12.

WhiG is a bacterial sigma factor (also termed σ\(^{WhiC}\)) that directs the transition to a sporulation-specific mode in aerial hyphae5,4. It is expressed throughout all stages of vegetative growth and differentiation13, but undergoes a burst of transcription during the onset of aerial hyphal formation16. WhiG activates transcription of at least two genes—the autoregulatory ‘early’ Whi-genes Whil and Whih16,17. However, there is a significant lag between the burst of WhiG transcription and subsequent expression of Whih16, suggesting that WhiG is regulated post-transcriptionally. Several groups have speculated that an anti-sigma factor may be involved in inhibiting WhiG activity until the proper window during sporulation18. Consistent with this possibility, WhiG is a member of the family of sigma factors that regulate flagellar formation in Salmonella, including FliA (which shares 41% sequence identity to WhiG); FliA is negatively regulated by an anti-sigma factor
encoded by the FlgM gene. Constraining WhiG activity to the former domain is associated predominantly in eukaryotic and archael homing endonucleases (invasive genes that are typically invasive to have a three-dimensional structure, a biochemical examination of the molecular activities of this protein seemed appropriate. Here we describe several in vitro DNA- and protein-binding and transcriptional activities of purified WhiA from *Streptomyces coelicolor* that are consistent with WhiA coordinating cessation of aerial hyphal growth with later sporulation events, both by confining WhiG activity to a short window at the onset of sporulation and presumably acting as a general transcription factor in regulating genes required for subsequent steps.

**Results**

**Promoter Binding.** Because WhiA is a putative transcriptional regulator and contains two protein domains that are widely utilized for DNA recognition (an N-terminal domain present in the LAGLIDADG class of homing endonucleases and a C-terminal HTH domain), we tested the ability of purified WhiA protein to bind several candidate promoter sequences in vitro using an electrophoretic mobility shift assay (EMSA, see materials and methods). We expressed full-length WhiA as a soluble protein in *E. coli* containing an N-terminal, 6XHis affinity tag that was removed during purification (Supplementary Figure S1 and Materials and Methods). On the final step of purification--size exclusion chromatography--the protein eluted as a single monodisperse peak with an elution time consistent with a monomer (data not shown). Further analyses using dynamic light scattering and small angle X-ray scattering (SAXS) also demonstrated that full-length WhiA is a monomer in solution (data not shown). For the gel-shift assays we selected candidate promoters from genes whose sporulation-specific expression had been shown to be significantly decreased in ΔWhiA strains, including WhiA itself and the Parp2 promoter, which directs the sporulation-specific expression of the ParAB genes.

We also tested two promoter sequences in which there was no prior published evidence of WhiA dependence: the Whi promoter (characterized in[5]); and the Parp1 promoter, which lies downstream of the Parp2 promoter but is not up-regulated in a sporulation-specific manner. The Parp2 promoter is identical to the Parp1 promoter but contains an additional 185 basepairs (see Supplemental Figure S2 for a list and summary of promoter regions).

![Figure 1](http://www.nature.com/scientificreports)  
**Figure 1 | Sporulation regulatory network in *Streptomyces coelicolor*.** Previous studies described in the introduction have indicated that the sigma factor whiG (σ†spor), which orchestrates the transition from aerial hyphal growth to a sporulation-specific program, is expressed in all stages of growth but becomes active only during a short window early in the sporulation process. "σ†spor" indicates active WhiG; inactive WhiG is in brackets. The transcriptional regulator BldD is required to limit WhiG activity prior to sporulation, although the exact mechanism is not understood. Once active, WhiG directs the transcription of at least two sporulation factors, Whil and WhiH, which themselves do not become active until later in sporulation. WhiA and the Fe-S cluster protein WhiB mutually regulate each other’s expression, and WhiA is also required in vivo to activate its own sporulation-specific transcription. Two other factors—the ParAB and FtsZ genes—are required for hyphal chromosome segregation and septation, respectively. WhiA has been shown in vivo to be required for the sporulation-specific expression of both ParAB and FtsZ. Other sporulation factors required for the subsequent spore maturation include WhiD, WhiE and σ†. As described in this paper and indicated with grey lines in the figure, WhiA physically binds to its own promoter and to the Parp2 promoter (i.e. sporulation-specific), consistent with a role in gene activation, and also binds to WhiG. WhiG activates expression of WhiA. A model consistent with those findings is that when WhiA accumulates, it binds to and inhibits WhiG activity, thereby forming a feedback loop that contributes to the inactivation of WhiG and itself. Other factors may also be required to fully limit WhiG activity.
WhiA bound its own promoter and the ParP₂, but not the ParP₁ or WhiI promoters (Figure 2a; Supplementary Figure S3). Based on the averaging of numerous independent experiments, we estimate the $K_d$ of WhiA for its own promoter to be approximately 300 nM (Supplementary Figure S3). The affinity for the WhiA promoter was at least 2 to 3 fold tighter than for ParP₂. In binding experiments with the WhiA promoter, several discrete bands of lower mobility appeared with increasing concentrations of WhiA protein, perhaps indicative of cooperative binding (Figure 2A, lanes 1–5). A single shifted band was consistently observed in binding reactions with the ParP₂ promoter, but not the multiple bands as seen with the WhiA promoter. The observed binding of the WhiA protein to the ParP₂, but not the ParP₁ promoter, is consistent with in vitro expression analyses, where the sporulation-specific expression of the ParAB genes is driven by the ParP₂ promoter in a WhiA-dependent manner. These in vitro results of WhiA binding provide a biochemical correlation to previous in vivo expression studies showing that WhiA was required for sporulation-specific expression. We note that the affinity of WhiA for these promoters is low, perhaps indicating that WhiA collaborates with other factor(s) in regulating transcription.

We next tested if either isolated structural domain of WhiA was able to bind the WhiA promoter by itself. For this purpose we purified N-terminal His-tagged versions of the LAGLIDADG domain of WhiA (amino acids 1–216) and the HTH domain (amino acids 221–328; Supplementary Figure S1). Both isolated domains were easily overexpressed and purified, and both were well-behaved in solution at high concentrations. The HTH domain, but not the LAGLIDADG domain, bound the WhiA promoter (Figure 2b). The affinity of the HTH domain was significantly lower than full-length WhiA—a gel-shift was observed at 2 μM but not 500 nM WhiA protein—suggesting that while the HTH domain may play a dominant role in DNA binding, the full-length protein is required for maximum affinity (although we cannot formally exclude the possibility that the isolated HTH domain binds more weakly simply due to poor folding behavior). As well, in the binding assay with the HTH domain only a single discrete shifted band is observed, as opposed to the multiple shifted bands can clearly be visualized with full-length WhiA.

We then employed several independent experimental strategies to more precisely define the binding region of WhiA to its own promoter. First, we performed gel-shift competition assays in which the labeled WhiA promoter used in Figure 2 was incubated with a 10-fold excess of a series of 40 basepair, double-stranded unlabeled oligonucleotides corresponding to individual regions of the WhiA promoter (Figure 3a). The competing oligonucleotides were designed to overlap their neighbor by 10 basepairs so that the entire promoter region could be tested for competition. We reasoned that 40 basepairs would span a sufficient length to encompass the WhiA binding site if both domains of WhiA were involved in binding to DNA. Using this approach, an oligonucleotide corresponding to –4 to –44 basepairs relative to the WhiA transcription start site significantly competed for binding with the full-length probe. The neighboring 5’ oligonucleotide (basepairs –34 to +6) also competed, albeit to a lesser extent.

Next, we examined the specificity of WhiA binding to its promoter by DNase I footprint analyses. We used a double-stranded oligonucleotide probe corresponding to basepairs –70 to +20 relative to the transcriptional start site (defined by; Figure 3b, materials and methods). With increasing concentration of WhiA several regions were protected from DNase I digestion, including –33 to –39 and –22 to –27 on the plus strand; and –19 to –25 on the minus strand (Figure 3B, 3D). Additionally, several sites were hypersensitive to DNase I digestion, including –52 and –17 on the plus strand; and –15, –16, –26, –27 and –28 on the minus strand (Figure 3B and 3D).
Figure 3 | WhiA binding specificity across the WhiA promoter. (a) WhiA(FL) binds to its own promoter in a region between -4 and -44 relative to the transcriptional start. 40mer double-stranded oligonucleotides corresponding to the WhiA promoter (overlapping each neighbor by 10 basepairs) were used in competition gel-shift assays at a 10-fold higher concentration than the 32P-labeled WhiA promoter in Figure 2. The "*" indicates the shifted band seen with no competitor (left lane). Numbers above lanes indicate the region of the competing oligonucleotide relative to the transcription start. (b) DNA footprinting assay with the WhiA promoter. A duplex oligo corresponding to the WhiA promoter (-70/+20 relative to the transcriptional start) was labeled on the plus strand (left) or the minus strand (right) and incubated with increasing concentrations of WhiA. For the plus-stranded reaction three
Finally, we assayed the specificity of the DNA binding interaction exhibited by WhiA against its own promoter. Specificity was tested at each individual basepair position from basepair -44 to -1, using a high-throughput, parallel assay previously developed in our laboratory to describe the binding specificity of homing endonucleases (see Materials and Methods and references 28,29). Using this assay, we determined that the identity of five basepairs in particular (positions -22, -21, and -20, and to a lesser extent -19 and -18) are most important for WhiA binding affinity (Figure 3c and 3d). The deleterious effect of any basepair substitution on binding affinity is relatively uniform at four out of five of these positions (-18 through -21). At the fifth position (-22) the protein tolerates either the wild-type G:C basepair or a substitution to A:T. The length of the DNA sequence region that is specifically recognized by WhiA appears to correspond to the typical profile of sequence-specific recognition displayed by a helix-turn-helix DNA binding domain. The sequence of the most strongly recognized DNA region bound by WhiA was non-palindromic, consistent with the apparent monomeric structure of the WhiA protein.

A comparison of the sequence in the WhiA promoter region that is bound by WhiA with the corresponding region of Parp2, that was also sufficient for WhiA binding (Materials and Methods and references 28,29). However, it is possible that those transcripts could have arisen from transcription from WhiA's constitutive, whiG-independent promoter, rather than its sporulation-specific promoter. Regardless, the in vitro biochemical results in this current study (Figure 4b) clearly demonstrate WhiG-dependent transcription from WhiA's sporulation-specific promoter.

Given that WhiA binds to its own promoter, and is required in vivo for transcription from its promoter22, we next tested whether WhiA could itself function as a sigma factor and direct its own transcription in vitro. This possibility has previously been postulated22. However, WhiA clearly did not direct transcription from its own promoter (Figure 4b, lanes 6–9). We also tested WhiA for sigma factor activity using the Parp2 promoter and again did not observe transcription activity (data not shown). It should be noted that although the E. coli RNA polymerase worked well for H6-WhiG-directed transcription in our assays, and has been used successfully with many other cross-species sigma factors in the literature, these results do not rule out the possibility that WhiA indeed functions as a sigma factor but has a strict requirement for the S. coelicolor RNA polymerase.

We next tested whether WhiA affected WhiG-directed transcription from the WhiA promoter. WhiA, H6-WhiG and RNA polymerase were pre-incubated with each other, followed by an incubation with the PCR-amplified promoter template prior to addition of ribonucleotides to initiate the reaction. Under this reaction scheme, WhiA inhibited transcription in a dose-dependent manner (Figure 4B, lanes 10–13). The inhibition of transcription by WhiA was not complete in this assay—in three different experiments where H6-WhiG was used at a concentration of 63 nM and WhiA at 200 nM, the average inhibition was 45% (with a standard deviation of 14%).

We then tested whether order of addition of WhiG and WhiA in the experiment above influenced inhibition of transcription by first preincubating H6-WhiG and RNA polymerase, followed by addition of the DNA template, then WhiA, then ribonucleotides to initiate the reaction (Figure 4c). Under this reaction scheme we observed no inhibitory effect by WhiA. Therefore, WhiA appears to inhibit transcription by blocking the association between WhiG and RNA polymerase.

WhiA may inhibit WhiG-directed transcription by binding directly to either WhiG or to RNA Polymerase. To test this we utilized a pulldown assay in which untagged WhiA was incubated with His-tagged WhiG, pulled down by Ni-NTA resin, resolved by SDS PAGE and visualized by silver staining (materials and methods). Under these conditions WhiA and WhiG clearly interacted physically (Figure 5). WhiA and with E. coli RNA polymerase did not react in a parallel assay in which His-tagged-WhiA was pulled down with Ni-NTA beads (data not shown). As discussed above, it remains a possibility that WhiA interacts with the S. coelicolor RNA Polymerase but not the E. coli RNA polymerase used in these assays; or that WhiA and the E. coli polymerase interact with a low affinity not detectable in this assay. In summary, the binding results are consistent with WhiA-mediated inhibition of transcription directed by σWhiG.

dilutions of DNAsel I were used (1: 100, 1: 300 and 1: 1000); for the minus-stranded reaction two concentrations were used (1: 300 and 1: 1000). For the plus-strand, reactions with no WhiA or 1000 nM WhiA were rerun for clarity with the highest concentration of DNAsel I. For the plus-strand reactions, a 25 basepair standard ladder is indicated on the left; on the minus-stranded reactions a 10 basepair ladder is on the right. Bars indicate regions of protection; hypersensitive sites are indicated by **.*. (c) The specificity of WhiA DNA recognition was tested across basepair positions -44 to -1 using a fluorescent competition binding assay. WhiA displays sequence specificity at positions -22, -21 and -20, and to a lesser extent -19 and -18 (arrows). The relative affinity (rKa) for each competitor was calculated as described in Methods; a reduced value indicates a lower binding affinity and preference for the wild-type basepair. Each position was run in triplicate (error bars are standard deviation). Individual colors correspond to the base at each position: A=blue, T=red, G=green, C=purple. (d) Summary of WhiA specificity for the WhiA promoter. Ovals indicate regions of protection identified by DNAsel I footprinting; asterisks indicate hypersensitive regions in the DNasel assay; arrows indicate basepair positions identified by fluorescence competition as being essential for recognition by WhiA.
Discussion

Sporulation in *S. coelicolor* is tightly regulated, both temporally and spatially, and clearly involves one or more discrete points in the organism’s lifecycle where the progression through the differentiation program is carefully coordinated. One particularly important transition is the cessation of growth and of DNA replication in the
be quite relevant to subsequent events in sporulation. We found that in addition to binding its own promoter, WhiA bound the ParABp2 promoter, which directs the sporulation-specific (and WhiA-dependent) expression of ParA and ParB genes through a short burst of LAGLIDADG domain.

A notable feature of the early Whi genes (−A, −B, −H, −I, −G) is that they encode regulatory factors, and it is critical that they are active only during the window in which they are required for the sporulation program. All of these factors except WhiG are expressed in a sporulation-specific manner, but other regulatory mechanisms are likely to be in place to also ensure their proper timing of activation. This appears to be the case for WhiH and WhiI—with both genes there is a delay between when they are transcribed and when they become active. WhiG transcript levels are relatively constant throughout differentiation, with the exception of a short burst of WhiG transcription around the time of aerial hyphal formation, and WhiG transcripts are not restricted to the aerial hyphae, as is found for many other sporulation factors. These features of WhiG have led others to postulate that WhiG activity is likely to be regulated post-transcriptionally, perhaps by an anti-sigma factor. Two features of WhiA and WhiG that we have demonstrated in vitro—the ability of WhiG to direct transcription of WhiA, and the ability of the WhiA protein to inhibit WhiG-directed transcription—are sufficient to create a feedback loop to confine WhiG activation to a short window during the transition from hyphal growth to a sporulation program (Figure 1). It is also possible that WhiA plays a role in constraining WhiG activity during vegetative growth since it is constitutively expressed (albeit at a much lower level than during sporulation). While we consistently observed WhiA-mediated inhibition of WhiG in the in vitro transcription assays, the inhibition was not complete. Given the central role that WhiG plays in directing the sporulation program, regulation of its activity is likely to be complex and require multiple regulatory factors. One such factor is the transcriptional regulator BldD, which binds to the WhiG promoter and appears to repress its expression prior to sporulation onset.

While WhiA accumulation might suppress the whiG-dependent "early" steps in sporulation, an additional activity that WhiA displays—binding to several sporulation specific promoters—could

Figure 5 | WhiA binds directly to h6-WhiG. 2 μg of untagged WhiA was incubated with 2 μg h6-WhiG, purified with Ni-NTA resin, washed 5 times and separated on SDS PAGE followed by silver staining. Lanes 4 and 5 contain 1/20 the amount of protein used in the pull-down reactions. An "*" indicates a breakdown product of WhiA that corresponds to the LAGLIDADG domain.

Methods

Constructs for protein expression and in vitro transcription assays. The sequence of S. coelicolor WhiA (WhiA++) was codon-optimized for expression in E. coli (Blue Heron) and subcloned into the pET15 vector that was modified to contain a thrombin-cleavable, N-terminal 6Xhis tag (pET15(HE)-WhiA). WhiA truncation constructs (amino acids 221–328 and 1–216) were subcloned by PCR into a pET22b+ vector-modified to also contain a cleavable N-terminal 6Xhis tag. WhiG was amplified from genomic S. violaceoruber DNA (ATCC) (having the same sequence as annotated for S. coelicolor A3(2)) and cloned into pET22b+ with an N-terminal His-tag.

Promoter sequences used for EMSA and in vitro transcription assays were PCR amplified from genomic DNA (Supplemental Figure 2) and cloned into the BamHI site of pGEM3T.

Protein Production. Codon-optimized WhiA constructs were expressed in BL21(DE3) cells, and WhiG was expressed in BL21 (DE3) RIL cells. For induction, a 1 mL starter culture was grown at 37°C overnight in LB media supplemented with 100 μg/mL ampicillin, 0.5% glucose and 1 mM MgCl2. The next morning this culture was diluted into ~200 mL of prewarmed media with ampicillin, grown 5–6 hours, then 25 mL of this culture was diluted into six flasks containing 1.5 L of prewarmed media with ampicillin. When the OD600 reached 0.6 the cultures were chilled on ice for 20 minutes, followed by induction with 0.5 mM IPTG (final concentration). The cultures were grown overnight at 16°C, pelleted by centrifugation and stored at −20°C. Pellets were thawed and lysed by sonication on ice in 300 mM NaCl, 50 mM Tris pH 7.5, 20 mM imidazole, 0.1% Triton X-100 and 1 mM PMSF. The lysate was centrifuged 45 min in an SS34 rotor at 19,000 rpm and the cleared lysate was loaded onto a 9 L prep (2 mL of resin was used) using a peristaltic pump (Biorad) in the cold room. The column was then hooked up to an FPLC (Aka, Amersham) and washed with at least 30 column volumes of 50 mM Tris (pH 7.5), 300 mM NaCl, 20 mM imidazole. When the A280 trace
flat-lined the protein was eluted in 50 mM Tris pH 7.5, 300 mM NaCl, 200 mM imidazole. The peak fractions were immediately diluted 1:1 with 50 mM Tris pH 7.5, loaded onto a 1 mL Heparín HiTrap column (GE Healthcare) in 200 mM NaCl/50 mM Tris (pH 7.5), and eluted using 200 mM to 1 M NaCl gradient (in 50 mM Tris, 7.5) over 20 column volumes. h6-WhiA elutes at ~500 mM NaCl. The peak fraction was then concentrated to ~4 mL in a centrifugal filter with 10 K MWCO (Millipore) and this tag was cleaved adding biotinylated thrombin (Novagen, 1 U per ~0.5 mg of h6-WhiA) and incubating at 15°C overnight. The next morning 15 μl of packed streptavidin agarose (Novagen) was added per U of thrombin and incubated at room temperature for 15 minutes, then filter purified through 0.22 μm centrifugal filter (Millipore) and loaded onto a prep grade HiLoad 16/10 Superdex200 gel filtration column (GE Healthcare) equilibrated in 150 mM (NH4)2SO4/50 mM Tris pH 7.5. The peak fractions were pooled, concentrated to ~2–3 mg/mL supplemented with 10% glycerol, and stored at ~80°C. A typical final yield of untagged WhiA under these conditions was ~0.5 mg L of culture.

The purification of h6-tagged WhiA variants (1–216 and 221–328) was as described above, except that thrombin-cleavage and Heparín HiTrap steps were omitted. h6-WhiG was expressed as a soluble protein under the same conditions described above, except the thrombin-cleavage and Heparin HiTrap column steps had been prerun for at least 30 minutes at 7 W (constant wattage). The samples were electrophoresed for 2 hrs at 8 W (constant wattage), with temperature monitoring that 1 unit of polymerase in this reaction volume is roughly equivalent to 100 nM WhiA protein (or WhiA truncations) were incubated with 30 nM (a 30-fold excess) of unlabeled double-stranded oligos specifically labeled on one strand. The WhiA protein (at final concentrations indicated in Figure 3) was incubated with 2.5 ng of labeled oligonucleotide, 25 ng of cold ds-oligonucleotide and 500 ng of S/HindIII fragments in EMSA binding buffer (20 μM final concentration) for 15°C at room temperature. 1 μl of DNAsel was then added at various dilutions (optimized in prior reactions), incubated for 5 minutes, and the reaction was stopped by adding 100 μl of 20 mM EDTA followed by 100 μl of phenol/chloroform. Samples were centrifuged 5 minutes at top speed in an Eppendorf centrifuge, and the aqueous layer was extracted with 100 μl of chloroform/isobutanol precipitation and washed with 70% ethanol. The dried pellet was resuspended in 5 μl of water followed by four volumes of 95% formamide/10 mM EDTA containing bromphenol blue and xylene cyanol. The reactions were then separated on an 8% acrylamide sequencing gel (Sequagel) at 20W (constant wattage, ~30 minutes), dried and exposed to film.

Fluorescence competition DNA binding specificity assay. Determination of the DNA binding specificity by WhiA across the ~44 to 4 region of its promoter was carried out as previously described. Details of the exact method and calculations used for the assay can be found in that reference and its supplementary information. Bacteria that had been grown to mid-log phase were infected with mCherry::WhiA and were grown in individual wells of a 96-well, Talon-coated plate and incubated with a fluorescently labeled oligonucleotide containing the wild-type ~44~4 region of the WhiA promoter. The measurements of the retained fluorescent signal for each mismatch sequence variant were then converted to relative binding affinities as compared to the wild-type target site as previously described.

In vitro transcription reactions. Promoters for in vitro run-off transcription reactions were generated from PCR using GoldenGate vectors (see Supplemental Figure S2). Reaction conditions. 10 μl of in vitro transcription reactions contained 40 mM Tris-HCl (pH 7.5), 150 mM KCl, 10 mM MgCl2, 0.01% TX-100, 2 mM DTT; 300 mM ATP, GTP and CTP, 50 mM UTP, and 0.1 μl of α-32P-UTP, 1 unit of E. coli core RNA polymerase (Epitrite Biotechnologies); we estimate based on silver-stained gel visualization that 1 unit of polymerase in this reaction volume is roughly equivalent to 1 ng of polymerase (100 32P-UTP). A cocktail containing the transcription buffer and NTP’s (including radioactively-labeled UTP) was then added, and the reaction was incubated for 30°C at 37°C. The reaction was stopped by the addition of an equal volume of 8 M Urea, 50 mM EDTA, 90 mM Tris-borate buffer, pH 8.3, 0.02% bromphenol blue and 0.02% xylene cyanol. The samples were heated to 90°C for 3 min, and 10 μl was then electrophoresed on an 8% polyacrylalamide (29:1 acrylamide:bisacrylamide)/8 M urea gel in 1X TBE Buffer. The gel was prerun for at least 20 minutes at 25°C (constant), then ran for 1 hr at 40 W constant with temperature monitoring not to exceed 25°C. The gels were dried and exposed to a phosphorimager plate.

Pull-down protein binding assay. 2 μg of h6-WhiG was incubated with 2 μg of untagged WhiA protein in 150 mM NaCl/50 mM Tris pH 7.5/20 mM Imidazole to a 10 μl volume for 15°C at room temperature. A slurry containing 5 μl of Ni-NTA resin (Qiagen) was then added in a volume of 100 μl of the same buffer, and the sample was incubated for 5 min at 4°C. The sample was collected by centrifugation, the supernatant was carefully aspirated with a 25 gauge needle, and then 15 μl of 2X SDS PAGE sample buffer was added to the beads. The samples were heated at 95°C for 5 minutes, and all of the sample was loaded onto a 12% NuPAGE Bis-Tris Gel (Invitrogen) and electrophoresed in MES buffer (Invitrogen). The gel was stored in 50% ethanol for at least one hour prior to silver staining using standard protocols.

Each individual competitive binding assay consists of one of three unlabeled oligonucleotides versus the same labeled wild-type target: (1) the unlabeled wild type promoter competing against the labeled version of the same sequence; (2) a completely randomized unlabeled sequence used as a negative control to account for competition by a nonspecific DNA sequence; and (3) each of the target sequences in the substrate matrix, containing a single basepair mismatch, competing against the labeled wild-type promoter. All competition experiments were performed in triplicate.

H6-tagged WhiA was immobilized onto nickel-coated 96 well plates (Ni-NTA HisSorb™ plates) by incubating 100 μl of 300 nM WhiA in TBS/BSA buffer (50 mM Tris pH 7.5, 150 mM NaCl, 0.2% BSA) in wells for 2 hours at room temperature. The plates were washed four times with TBS/Tween-20 (50 mM Tris pH 7.5, 150 mM NaCl, 0.02% poly dI-dC, 10 mM CaCl2). The plates were washed four times with TBS (50 mM Tris pH 7.5, 150 mM NaCl, 0.02 mg/mL poly dl-dC, 10 mM CaCl2). The fluorescent signal retained from each test well was counted using a SpectraMax® M5/M5s micro-plate reader (Molecular Devices; excitation: 510 nm, emission: 565 nm, cutoff: 550 nm). All measurements were performed in triplicate. Additional negative control experiments performed in the absence of protein indicated that no significant detectable fluorescent signal was retained after the protocol described above was completed.

The measurements of the retained fluorescent signal for each mismatch sequence variant (F(i,j)) were then converted to relative binding affinities as compared to the wild-type target site as previously described.

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