Organisms have a myriad of strategies for sensing, responding to, and combating reactive oxygen species, which are unavoidable consequences of aerobic life. In the heterocystous cyanobacterium *Nostoc* sp. PCC 7120, one such strategy is the use of an ArsR-SmtB transcriptional regulator RexT that senses H$_2$O$_2$ and upregulates expression of thioredoxin to maintain cellular redox homeostasis. Different from many other members of the ArsR-SmtB family which bind metal ions, RexT has been proposed to use disulfide bond formation as a trigger to bind and release DNA. Here, we present high-resolution crystal structures of RexT in the reduced and H$_2$O$_2$-treated states. These structures reveal that RexT showcases the ArsR-SmtB winged-helix-turn-helix fold and forms a vicinal disulfide bond to orchestrate a response to H$_2$O$_2$. The importance of the disulfide-forming Cys residues was corroborated using site-directed mutagenesis, mass spectrometry, and H$_2$O$_2$-consumption assays. Furthermore, an entrance channel for H$_2$O$_2$ was identified and key residues implicated in H$_2$O$_2$ activation were pinpointed. Finally, bioinformatics analysis of the ArsR-SmtB family indicates that the vicinal disulfide “redox switch” is a unique feature of cyanobacteria in the *Nostocales* order, presenting an interesting case where an ArsR-SmtB protein scaffold has been evolved to showcase peroxidatic activity and facilitate redox-based regulation.
The diversity of environments that photosynthetic microorganisms are equipped to survive in is unparalleled. These organisms inhabit environments that contain different amounts of molecular oxygen (O₂) and experience different wavelengths and intensities of light. To thrive in these environments, photosynthetic organisms must have strategies to perceive and defend against the production of reactive oxygen species (ROS), a class of O₂-derived species that includes superoxide (O₂⁻), peroxide (O₂²⁻), hydroxyl radicals (OH⁻), singlet oxygen (¹O₂), and hydrogen peroxide (H₂O₂)1,2. ROS are generally proposed to respond to H₂O₂ stress through formation of a disulfide bond that initiates transcription of the thioredoxin-encoding gene 23,24.

For Nostoc sp. PCC 7120, it is known that thioredoxin plays a critical role in the oxidative stress response15-17. In particular, transcription of the gene that encodes thioredoxin A2 (TrxA2) is induced upon exposure to H₂O₂ by the redox-sensing transcriptional regulator RexT17, an annotated member of the ArsR-SmtB family of transcriptional regulators. This protein family was named after the founding members, E. coli arsenic and antimony regulatory protein (EaArsR)18 and Synechococcus elongatus PCC 7942 Zn²⁺-dependent regulatory protein (SeSmtB)19, and many of its members are recognized as metal-responsive transcriptional regulators (Fig. 1a). However, there are also members in this protein family that deviate from the metal-sensing role and instead have been shown to sense ROS or reactive sulfur species20,21. For example, as observed with the OxyR transcriptional repressor homolog9, RexT binds DNA under reducing conditions and dissociates from DNA following treatment with H₂O₂17 (Fig. 1b). The latter dissociation step permits transcription of trxA2 and is suggested to be caused by intramolecular disulfide bond formation between two of the three RexT Cys.

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**Fig. 1** The ArsR-SmtB family of transcriptional regulators act as repressors of their target genes. a The metal-binding members of the ArsR-SmtB class act as repressors of the target gene in the apo-state. In contrast, when bound to a metal ion, a conformational change ensues, and transcription of the target gene is initiated23,24. b RexT is a non-canonical member of the ArsR-SmtB family of transcriptional regulators. Rather than binding metal ions, RexT is proposed to respond to H₂O₂ stress through formation of a disulfide bond that initiates transcription of the thioredoxin-encoding gene trxA227.
residues (Cys40, Cys41, and Cys105)\textsuperscript{17}. Once produced, TrxA2 can reduce disulfide bonds in target proteins and restore the ability of RexT to bind DNA via disulfide bond reduction, meaning that RexT is a reversible sensor of oxidative stress\textsuperscript{17}. This ability of RexT to sense and respond to ROS and contribute to the maintenance of cellular redox homeostasis has led to its identification as a potential target for promoter development in applications aimed at metabolically engineering cyanobacteria to fix nitrogen or produce hydrogen\textsuperscript{22}. However, there are still many missing details regarding how it functions. For example, it is unknown how RexT binds DNA, which residues RexT uses to sense and react to H$_2$O$_2$, and how the formation of a disulfide bond results in de-repression of transcription (Fig. 1).

To investigate the metal-independent regulatory mechanism by which RexT senses and propagates a response to H$_2$O$_2$, we used X-ray crystallography, site-directed mutagenesis, mass spectrometry, fluorescence anisotropy, and electrophoretic mobility shift assays (EMSA) to establish the molecular details of the RexT-based mode of DNA regulation. We identified key residues involved in DNA binding and pinpointed residues involved in the binding and activation of H$_2$O$_2$ and the formation of the regulatory disulfide bond. In addition, we established that the RexT-based mode of regulation is ubiquitous among organisms in the Nostocales order. Importantly, this mechanism expands the known regulatory mechanisms for sensing H$_2$O$_2$ and adds an extra layer of complexity to the ArsR-SmtB family as it shows that a common ancestral protein scaffold can evolve to showcase peroxidatic activity and respond to oxidative stress or sense metal ions.

### Results

**RexT has a dimeric ArsR-SmtB-like helix-turn-helix fold but lacks metal-binding motifs.** The crystal structure of RexT was determined in two stages. First, a lower-resolution 2.50-Å resolution structure of SeMet labeled RexT was solved using single-wavelength anomalous dispersion (SAD) phasing. Second, this SeMet structure was used as a model for the molecular replacement to determine a higher resolution structure of native RexT to 1.95-Å resolution (Table 1). The asymmetric unit of the RexT crystal structure contains a homodimer of RexT, which is consistent with the oligomeric state observed in solution using size-exclusion chromatography (Fig. 2a, Supplementary Fig. 1a). Each monomeric unit of RexT contains five α-helices and a pair of antiparallel β-strands, which is comparable to the α1-α2-α3-α4-β1-β2-α5 topology that is typical of the ArsR-SmtB superfamily of transcription factors\textsuperscript{23,24} (Fig. 2a, b). The dimer interface of RexT is formed by a coiled-coil interaction between the α1 helices (residues 13–22) and between the α5β5 helices (residues 91–96) of each protomer (Fig. 2a, b and Supplementary Fig. 1b). The buried interface area is 707.6 Å$^2$, ~11.5-percent of the total surface area of each protomer (Supplementary Fig. 1b). The α3 helix (residues 41–43), which houses two of the three aforementioned Cys residues (Cys40 and Cys41), the α4 helix (also known as the recognition helix, αR, residues 50–62), and the loop connecting them form the helix-turn-helix motif, whereas the antiparallel β-strands (residues 66-71 and 74-79) form the “wing” of the winged helix-turn-helix (wHTH) architecture\textsuperscript{25} (Fig. 2a, b). The third Cys residue, Cys105, is located near the flexible C-terminus of the protein. This region is disordered in both monomeric units. As such, Cys105 is missing from chain A and is the last modeled residue in chain B.

A search of the protein data bank using the DALI server\textsuperscript{26} identified the Cd$^{2+}$-binding regulator from *Staphylococcus aureus* (SaCadC)\textsuperscript{27}, the ArsR homolog from *Acidithiobacillus ferrooxidans* (AfArsR)\textsuperscript{28}, and two HlyU regulators proposed to be involved in redox-based regulation of virulence genes\textsuperscript{29,30}, as the closest related structural homologs to RexT (Supplementary Fig. 2a–d). Pairwise superposition of these structures and that of SmtB\textsuperscript{31} (Supplementary Fig. 2e) with RexT shows that the core wHTH architecture is well conserved but some structural deviations are apparent in the flexible N- and C-terminal domains (Supplementary Fig. 2). Differences are also seen in the α5 helix of RexT, which is split into two segments by a three-amino-acid turn (Phe-Pro-Gly), and in the α3 helix of RexT, which consists of only three amino-acids (Fig. 2b). Most noticeably, RexT lacks the metal-binding motifs typical of this superfamily, including the N-terminal Cys residue and the conserved ELCVCD motif on the α3 helix for binding Cd$^{2+}$ observed in SaCadC\textsuperscript{27} (Supplementary Fig. 2a, c). Likewise, RexT does not contain the three Cys binding motif on the C-terminus found in AfArsR\textsuperscript{28}, or the CXCXXC motif on the α3 helix found in other ArsR homologs\textsuperscript{24} (Supplementary Fig. 2a, d). RexT also lacks the Asp/Glu and His-rich motif that resides on the α5 helix for binding Zn$^{2+}$ found in SmtB\textsuperscript{19,31} (DXXH$_x$HX$X$, Supplementary Fig. 2a, e). Consistent with these observations, an EMSA was used to probe the effect of As (III), Cd$^{2+}$, and Zn$^{2+}$ on the ability of RexT to bind DNA (Supplementary Fig. 3a, Supplementary Table 1–2). As expected, based on the structure of RexT and the lack of the typical residues involved in metal ion binding, none of these ions, even at a five-fold excess, caused changes in DNA binding. A similar trend was also observed in a fluorescence anisotropy experiment, which again revealed no significant changes in DNA binding affinity in the presence of Cd$^{2+}$ (Supplementary Fig. 3b). Last, despite a similar proposed role of sensing oxidative stress, RexT also lacks the functional Cys residues found on the α2 helix of HlyU (Supplementary Fig. 2a, b)\textsuperscript{29}.

### Identification of RexT structural features involved in DNA binding.

In the dimeric structure of RexT, the wHTH region

| Table 1 Data collection and refinement statistics. |
|----------------------------------|------------------|------------------|------------------|
|                                   | SeMet-RexT$^3$   | RexT             | RexT$^\text{ox}$ |
| **Data collection**               |                  |                  |                  |
| Space group                       | P6$_3$           | P6$_3$           | P6$_3$           |
| Cell dimensions a, b, c (Å)       | 100.46, 100.46, 36.05 | 99.89, 99.89, 36.47 | 100.46, 100.46, 36.05 |
| α, β, γ (°)                       | 90, 90, 120      | 90, 90, 120      | 90, 90, 120      |
| Resolution (Å)                   | 3.3–2.50         | 3.3–2.50         | 3.3–2.50         |
| $R_{	ext{merge}}$                | 0.157 (1.93)     | 0.150 (1.045)    | 0.104 (1.431)    |
| No. of atoms                     | 1679             | 1679             | 1679             |
| Protein                          | 1616             | 1679             | 1697             |
| μ (Å)                            | 1.66             | 1.69             | 1.62             |
| Chloride                         | 4                | 4                | 4                |
| Chloride                         | 4                | 4                | 4                |
| Water                            | 59               | 173              | 102              |
| **Reﬁnement**                   |                  |                  |                  |
| Resolution (Å)                   | 2.50             | 1.95             | 2.16             |
| Unique reflections               | 7402             | 15063            | 11401            |
| $R_{	ext{merge}}/R_{	ext{free}}$| 0.229, 0.258     | 0.179, 0.222     | 0.200, 0.256     |
| No. of atoms                     | 1679             | 1679             | 1679             |
| Protein                          | 1616             | 1679             | 1697             |
| μ (Å)                            | 1.66             | 1.69             | 1.62             |
| Chloride                         | 4                | 4                | 4                |
| Chloride                         | 4                | 4                | 4                |
| Water                            | 59               | 173              | 102              |
| **B-factors**                    |                  |                  |                  |
| Overall                          | 47.72            | 27.33            | 50.13            |
| σ (Å)                            | 47.38            | 25.95            | 50.02            |
| Chloride                         | 47.16            | 37.17            | /                |
| Chloride                         | 57.31            | 35.17            | /                |
| Water                            | 47.77            | 35.85            | 51.77            |
| H$_2$O$_2$                       | /                | /                | 62.52            |
| R.m.s. deviations                | 0.012            | 0.010            | 0.009            |
| Bond lengths (Å)                 | 1.71             | 1.19             | 1.15             |

Values in parentheses are for the highest-resolution shell. Bijvoet pairs were not merged during data processing.
Fig. 2 RexT has an ArsR-SmtB-type winged helix-turn-helix fold. a The crystal structure of RexT reveals that it is a homodimer. Each protomer of RexT has an α1-α2-α3-α4-β1-β2-α5/α6 architecture. Chains A and B are colored in light blue and light gray, respectively. Two of the three potential redox sensing Cys residues are located on the α3 helix (Cys40 and Cys41). Due to the dynamic nature of the residues following Cys105 in chain B, residues 106–112, like residues 102–112 in chain A were unable to be included in the final model of RexT. b A topology diagram of RexT shows the position of the three Cys residues (yellow circles), the dimeric interface (blue circle), and the wHTH motif (light blue secondary structure). c The calculated electrostatic potential of RexT reveals positively charged patches. The two-Cys residues and two positively charged residues that are proposed to be involved in DNA-binding are highlighted. d RexT is shown overlaid with a DNA-bound NolR structure (PDB: 4ON0, RexT is shown with DNA from the NolR structure following an alignment performed in PyMol). This overlay reveals potential residues involved in interacting with DNA and showcases that a complementary interaction can be formed between the positively charged region of RexT and the negative backbone of DNA. In each monomer, αα is colored in dark purple. e A labeled DNA probe shows changes in fluorescence anisotropy following the addition of RexT. These differences allowed for the calculation of the DNA binding affinity of wild-type RexT and the different variants used in this work (Supplementary Table 2). Compared to wild-type RexT (gray, Kd = 1.08 ± 0.07 μM), the K50A (orange, Kd = 5.29 ± 1.86 μM) and R26A (red, Kd = 5.18 ± 1.22 μM) RexT variants show decreased DNA binding affinity whereas the C40S (blue) and C41S (green) variants don’t have any significant change. f A sequence alignment of RexT with NolR shows a conserved DNA-binding architecture and key residues. The sequences are aligned by Clustal W,89 and colored by Jalview90 based on the percent identity with dark blue indicating sequence identity. DNA binding residues in NolR are indicated by orange (interaction with DNA backbone phosphate) and blue (base-specific interaction) dots. The alignment is annotated based on the secondary structure and residue numbers of RexT. In e, data was measured using n = 3 independent experiments and is presented with the individual measurements (open shapes) and as the mean value of these measurements ± SD (closed shapes). Source data are provided as a Source Data file.

shows an overall positive electrostatic potential and appears well-poised to interact with DNA (Fig. 2c). An overlay with a related DNA-bound ArsR-SmtB member NolR,32 which exhibits a DNA binding competent conformation that is unusual for the protein family, reveals that a complementary interaction can be formed between the overall positive electrostatic potential region of RexT and the overall negative region of the DNA backbone (Fig. 2c, d). Specifically, both α4 helices (α4) of the dimer are comparably spaced relative to those in NolR and are positioned to interact with the major groove of DNA, and both antiparallel “wings” are situated to interact with the DNA minor groove (Fig. 2d and Supplementary Fig. 4).32,33 In the structure of NolR, binding interactions with the DNA phosphate backbone are largely contributed by residues from the α2 and α3 helices as well as the β wing, whereas base-specific interactions mostly come from the α4 helix (Fig. 2d, f).32 For example, Arg31 on the α2 helix of NolR interacts with the phosphate group on the DNA backbone.32 Changing this residue into an alanine residue was demonstrated to abolish the ability of NolR to interact with DNA using isothermal titration calorimetry.32 The corresponding residue in RexT is Arg26 (Fig. 2f). To investigate whether this residue participates in an analogous protein-DNA interaction, an R26A RexT variant was made using site-directed mutagenesis (Supplementary Table 3). This R26A variant was then shown by EMSA and fluorescence anisotropy to have a significantly weaker affinity for DNA than wild-type RexT (Fig. 2e, Supplementary Fig. 5a, Supplementary Table 1–3). Gln56 from the N-terminal end of the α4 helix in NolR is involved in base-specific interactions with DNA and interacts with either an adenine or thymine base, facilitating the recognition of the NolR consensus motif (A/T)TAG-N5-A(T/A).32 Replacement of this residue with alanine in NolR causes a less than two-fold change in its affinity for DNA.32 At the corresponding position in RexT is Lys50 (Fig. 2e, f). Change of this residue in RexT into alanine (K50A) also weakens the affinity of RexT for DNA (Fig. 2e, Supplementary Fig. 5a, and Supplementary Tables 1–3). Lys and Arg residues are known to predominantly interact with guanine bases, forming the most favorable interaction among the possible amino
acid–nucleotide interactions. Correspondingly, RexT has been shown to bind a palindromic sequence ATTCG-N$_{15}$-CGAAT and we hypothesize that Lys50 is important for DNA binding through an interaction with guanine. Finally, in NoR the main-chain amide of Gly46 on the α3 helix forms a hydrogen bond with the backbone phosphate of DNA. Similarly, in both monomeric units of the reduced RexT structure, the main-chain amide of the corresponding Cys41 interacts with a molecule, which is best modeled as a chloride anion from the crystallization buffer (Supplementary Fig. 6). Here, the sidechain changes in the C40S and C41S RexT variants have only minor impacts on the ability of RexT to interact with DNA (Fig. 2e, Supplementary Fig. 5b, and Supplementary Tables 1–3).

**Identification of cysteine residues involved in sensing oxidative stress.** To identify the Cys residues that are involved in disulfide bond formation, the reactivity of three RexT Cys-to-Ser variants (C40S, C41S and C105S) and wild-type RexT towards H$_2$O$_2$ was probed using an assay to monitor consumption of H$_2$O$_2$ via the ferrous oxidation of xylene orange (FOX assay) over time$^9$. Using similar conditions to those described for OxyR$^9$, it was determined that when 200 μM of H$_2$O$_2$ was mixed with 100 μM of either wild-type RexT or C105S RexT, approximately 50 percent of the added H$_2$O$_2$ was consumed in 200 s (Fig. 3b, c). In contrast, the C40S and C41S variants showed an impaired ability to consume H$_2$O$_2$ relative to wild-type RexT (Fig. 3d, e). These results along with our structural results, which show that Cys40 and Cys41 are located adjacent to each other on the α3 helix, suggest that oxidative stress results in disulfide bond formation between Cys40 and Cys41 (Fig. 3a). The identification of the disulfide bond forming Cys residues was further explored using mass spectrometry (Supplementary Fig. 7). These experiments showed the formation of a disulfide bond in wild-type RexT and the C105S variant, but not in the C40S or C41S variants (Supplementary Fig. 7a, c–f).

Furthermore, the larger impairment of the C41S variant to consume H$_2$O$_2$, suggests it functions as the peroxidatic Cys residue, or the residue that is converted to a sulfenic acid moiety in the process of disulfide bond formation. This finding also suggests that Cys40 is the resolving Cys, or the residue that reacts with the formed sulfenic acid moiety to make a disulfide bond. This hypothesis was probed using mass spectrometry to look for incorporation of the small molecule dimedone into RexT during treatment with H$_2$O$_2$. Dimedone modifies sulfenic acid moieties and was shown to not be incorporated into the C105S variant (Supplementary Fig. 7b, f). In contrast, these experiments revealed that one equivalent of dimedone reacted with both wild-type RexT and the C41S variant (Supplementary Fig. 7c, e) and two equivalents of dimedone reacted with the C40S variant (Supplementary Fig. 7d). Collectively, the mass spectrometry experiments show that a sulfenic acid moiety can be formed at Cys41 and Cys105 in response to H$_2$O$_2$. The latter modification is likely formed due to the reactive nature of the non-disulfide bonded Cys residue in RexT. On the other hand, the C41 modification is observed only in the case where the proposed resolving Cys residue (Cys40) is absent (Supplementary Fig. 7d). This result is consistent with disulfide bond formation being faster than dimedone incorporation. The location of the Cys105 residue, which is only modeled in chain B of the determined structure is located ~20 Å away from both Cys40 and Cys41 (Fig. 3a). Based on this observation, the wild-type levels of H$_2$O$_2$ consumed by the C105S RexT variant, and a circular dichroism experiment that shows no large structural rearrangements following the addition of H$_2$O$_2$ to wild-type RexT, we conclude that Cys105 is not involved in the RexT-mediated oxidative stress response (Fig. 3c and Supplementary Fig. 8). Intriguingly, despite the widespread use of Cys residues to coordinate metal ions in the ArsR-SmtB class of proteins, adjacent Cys residues are rarely used as metal-binding ligands$^{35}$. Likewise, although there are cases in this protein family where adjacent Cys residues are used to coordinate methyl-As(III) or As(III), all of the involved cysteine residues are found at the end of α5 and the flexible region of the C-terminus$^{28,36}$. These observations suggest that RexT has evolved as a redox sensor through formation of a specific sensory site for responding to H$_2$O$_2$.

A vicinal disulfide-bond mediated conformational change in RexT. To visualize the structural consequence of Cys residue oxidation, a 2.16 Å resolution structure of H$_2$O$_2$-treated RexT was solved (RexT$_\text{Ox}$, Table 1). These crystals were prepared by removing crystals of reduced RexT from the anaerobic chamber and immersing them in a solution of cryoprotectant that contained H$_2$O$_2$. The oxidized structure shares a similar overall architecture with that of reduced RexT (rmsd of 0.380 Å for 1258 atoms). In chain A of the RexT dimer, a slight orientation difference is observed for both the Cys40 and Cys41 sidechains relative to the reduced structure. In contrast, in the α3 helix region of chain B, in agreement with the above-described biochemical results, the electron density is consistent with the presence of a disulfide bond between Cys40 and Cys41 (Fig. 4a, Supplementary Fig. 9, Supplementary Fig. 10a–d). To form this disulfide bond, Cys41 undergoes a large conformational change: the sidechain flips −167.8° and moves 8.8 Å away from its position in the reduced structure (Fig. 4a). The observed disulfide bond belongs to a rare group of disulfide bonds known as vicinal disulfide bonds. Based on the succession of sidechain dihedral angles and the handedness of the sulfur–sulfur bond, this vicinal disulfide bond has a Trans-Z (Tz) geometry$^{35}$ (Fig. 4b). Formation of this vicinal disulfide bond results in the creation of an eight-membered ring with the peptide backbone and distortion of the peptide bond geometry. To bring the two sulfur atoms close together, the ω peptide bond in the Tz conformation twists −35° from the ideal 180° geometry. This distortion is larger than the average value of −17°$^{35}$ and similar to that observed (−29°) in 1,5-alpha-L-arabinanase$^{37}$ (PDB ID: 3CU9), the first protein for which the Tz conformation was documented (Fig. 4b). In addition to this observed peptide bond distortion, the movement of Cys41 disrupts the interactions seen in the reduced structure between the backbone and sidechain of Cys41 and residues His75 and Asn77 from the wing of the wHTH motif (Fig. 4c). The lack of a disulfide bond in chain A is proposed to be due to crystal packing, which would prohibit a comparable movement of these residues (Supplementary Fig. 9). Finally, both the oxidized and reduced structures of RexT have the highest B factors in the DNA binding features (Fig. 4d). This result may suggest that consistent with that observed for other wHTH motifs, this region is flexible in solution (Fig. 4d, Supplementary Fig. 10e, and Table 1).

**Identification of key residues involved in H$_2$O$_2$ binding and activation.** As described above, the formation of a disulfide bond by H$_2$O$_2$ typically starts with the oxidation of the peroxidatic Cys residue to a sulfenic acid. A subsequent step using a resolving Cys completes the process$^{9,38}$. To visualize how H$_2$O$_2$ could access Cys40 and Cys41 in RexT to facilitate disulfide bond formation, the cavities present in the structures of RexT were calculated using PyMOL (Fig. 5a). Through this analysis, it was found that a cavity leads from the surface of the protein to a cluster of amino acids that includes Cys41 (Fig. 5a). This cavity contains one...
molecule of H$_2$O$_2$ in chain A of the H$_2$O$_2$-treated structure and one molecule of glycerol in chain B of the reduced structure, suggesting the possibility that H$_2$O$_2$ gains access to Cys41 using this route (Fig. 5a, b and Supplementary Fig. 11). Importantly, along this calculated cavity, a few polar and charged residues are arranged in a way that is reminiscent of the catalytic residues found in Corynebacterium glutamicum OxyR$^9$ (CgOxyR, Fig. 5c).

For example, in RexT, the positioning of Arg61 and Thr68 resembles the conserved catalytic residues Arg278 and Thr107 in CgOxyR that contribute key hydrogen bonds for the binding of H$_2$O$_2$ (Fig. 5b, c). The sidechain of Asn77 is close to Cys41, suggesting that like Gln210 in CgOxyR, it could be involved in stabilizing a negatively charged Cys41 thiolate. Additionally, there is potential for His75 to act as a proton acceptor, either for the

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**Fig. 3** Cys40 and Cys41 form an intramolecular disulfide bond in response to H$_2$O$_2$ stress. **a** The positions of the Cys residues in the reduced structure of RexT. The two sulfur atoms of Cys40 and Cys41 are located ~7 Å apart in both monomeric units of RexT. The sulfur atoms of Cys40 and Cys41 are located 21.9 Å and 23.0 Å away from the sulfur atom of Cys105, the last modeled residue in the crystal structure, respectively (shown for chain B of the structure). There are two measurements shown in chain A since the Cys41 residue sidechain shows two orientations of the sidechain. **b** Using the FOX assay, wild-type RexT was shown to consume H$_2$O$_2$ over time. Two control reactions are also included in this panel that shows H$_2$O$_2$ is not consumed in the absence of RexT or in the presence of the protein bovine serum albumin (BSA). **c** The C105S RexT variant consumes H$_2$O$_2$ similarly to wild-type RexT, suggesting it is not involved in mediating the oxidative stress response. **d** In contrast, the C40S variant of RexT shows a decreased ability to consume H$_2$O$_2$ relative to wild-type RexT. **e** As observed for the C40S variant, the C41S RexT variant is also impaired in its ability to consume H$_2$O$_2$, albeit to a greater extent. In b–e, data was measured using $n = 3$ independent experiments and is presented with the individual measurements (open shapes) and as the mean value of these measurements ± SD (closed shapes). Source data are provided as a Source Data file.
Cys41 thiol group or for H$_2$O$_2$ (Fig. 5b, c). Likewise, Gln74, although located outside of the cavity, is found in the vicinity of Cys40 and may also be involved in disulfide bond formation (Fig. 5b and Supplementary Fig. 11). To test the involvement of these residues in H$_2$O$_2$-mediated disulfide bond formation, five variants of RexT (R61A, T68A, N77A, Q74A, and H75A) were created and subjected to the above-described H$_2$O$_2$ consumption assay (Fig. 5d–h and Supplementary Table 3). The results of this experiment show that Arg61, Thr68, and Asn77 are important players in permitting RexT to consume H$_2$O$_2$ (Fig. 5d–f). Gln74 and His75, on the other hand, have little effect on the peroxidatic reactivity of RexT (Fig. 5g, h). Thus, remarkably, unlike other members of the ArsR-SmtB family of regulators, RexT has evolved to contain a Cys-Arg-Thr-Asn tetrad of residues to activate and reduce H$_2$O$_2$ and facilitate the formation of a sulfenic acid moiety.

RexT represents a distinct class of regulatory redox sensors in the ArsR-SmtB family. To investigate the prevalence of the RexT-based mode of gene regulation in the ArsR-SmtB family, we constructed a sequence similarity network (SSN) based on the conserved HTH ArsR-type DNA-binding domain (IPR001845). This network mainly comprises two Pfam groups to which most of the previously characterized ArsR-SmtB regulators belong (Fig. 6, Supplementary Fig. 12, and Supplementary Table 4). At a cut-off value of $e^{-20}$, a main cluster of nodes can be visualized that contains most of the canonical metal- and arsenite-binding regulators. As described above for SeSmtB, regulators that contain Asp/Glu/His-rich metal-binding motifs on the $\alpha_5$ helix are found in two connected subgroups (Fig. 6, circle Ia-Ib, Supplementary Fig. 13). The first subgroup (circle Ia) includes canonical metal-binding regulators SmtB19,31, CzrA31, and NmtR40 that contain a DXHX$_{10}$HXXE/H metal-binding motif on the $\alpha_5$ site. Regulators that contain the $\alpha_5$ site and an additional Cys-rich metal-binding motif that bridges the N-terminus of one subunit with the $\alpha_3$ helix of a second subunit ($\alpha_3N$ site), including CadC27, ZiaR41, AztR42, and BxmR43, are also found in this subgroup. The second subgroup (circle Ib) is less well characterized, and contains three regulators from Mycobacterium tuberculosis, one of which (MtKmtR44), is known to use an HX$_6$DHX$_5$EX$_6$HH metal-binding motif on a5. Aside from these
regulators, the main cluster of nodes contains a second region rich in regulators that use two or three Cys residues to bind an arsenite or a methyl-arsenite anion (Fig. 6, circle II). These Cys residues are found in different locations, including the α3 helices of EcArsR and the arsenite-binding regulator AseR from Bacillus subtilis (BsAseR). They are also found on the N-terminus and the loop connecting the α2 and α3 helices of the ArsR homolog from Corynebacterium glutamicum (CgArsR), or on the C-terminal extension of AfArsR and the ArsR homolog from Shewanella putrefaciens (SpArsR) (Supplementary Fig. 13). Each of these regulators highlights the flexibility and versatility of the wHTH-ArsR architecture (Supplementary Fig. 13, Supplementary Table 4). Finally, the main cluster of nodes also contains an emerging class of regulators that do not bind a metal or arsenite ion at all, further emphasizing the adaptability of the protein fold (Fig. 6, circle III). In this group, BigR and SqrR are implicated in responding to the presence of hydrogen sulfide (H2S) and reactive sulfur species (RSS) using two-Cys residues from the α2 helix and the α5/C-terminal extension. This pair of Cys residues are also conserved in HlyU and YgaV, which are found in a similar region of the main cluster and have been suggested to be involved in sensing ROS using an unknown regulatory mechanism (Supplementary Fig. 13). Interestingly, NolR, which is highlighted in Supplementary Fig. 4, is a global regulator for symbiotic nodulation and is also found close to these nonmetal-binding regulators. However, NolR does not contain any Cys residues and the environmental signal that triggers gene regulation is unknown (Supplementary Fig. 13, Supplementary Table 4).
and HlyU, RexT is separated from the main cluster of nodes, representing a distinct class of its own. The RexT cluster in the SSN contains 7085 non-redundant protein sequences in the UniProtKB database (Fig.6). To date, RexT is the only member of this cluster that has been biochemically and structurally investigated. However, as it is known that the formation of a disulfide bond under oxidative stress in RexT induces the expression of a thioredoxin-encoding gene, it was hypothesized that the co-occurrence of rexT and trx genes is a genomic signature of the disulfide bond-based redox sensor (Supplementary Fig. 14, Supplementary Table 4). Analysis of the genomic neighborhood diagram of the 7085 sequences that belong to the RexT cluster revealed that 105 sequences are likely to behave similarly to RexT and act as a redox regulator that responds to ROS (Supplementary Fig. 14, 15a, Supplementary Table 5).

Remarkably, all 105 sequences contain at least two-Cys residues: Cys41 is found in all 105 putative RexT proteins whereas Cys40 is found in only 85 of the sequences (based on RexT numbering, Supplementary Fig. 15a, b, Supplementary Table 6). Of the 105 sequences that contain Cys41, two are from unclassified bacteria, one is from Abditibacteria, and 102 are from Cyanobacteria, suggesting the disulfide bond-based redox switch is unique to Cyanobacteria (Supplementary Fig. 14, Supplementary Table 5). Further analysis shows that the 102 sequences mainly fall into three orders of Cyanobacteria: 84 sequences come from Nostocales, nine sequences come from Synechoccocales, three sequences come from Oscillatoriales, and the remaining six sequences are from unclassified Cyanobacteria species. The dual Cys40-Cys41 sequence signature on the α3 helix is conserved in 83 of the 84 Nostocales sequences (Supplementary Fig. 15b). In contrast, the dual-Cys motif is not observed in Synechoccocales or Oscillatoriales (Supplementary Fig. 15c). In the latter two orders, conserved Cys residues are found either at positions 41 and 68 (8 out of 11), positions 9 and 41.
(5 out of 11), or at positions 41, 44, and 105 (Supplementary Fig. 15c, Supplementary Table 5).

Consistent with these sequence-specific observations, a rooted phylogenetic tree of RexT homologs shows that sequences of *Nostocales* form different clades than the *Synechococcales* or *Oscillatoriales* order sequences (Supplementary Fig. 16). Interestingly, the one *Nostocales* sequence that does not contain the Cys40-Cys41 motif (but instead contains C40 and C105) lies at the branching point between the *Nostocales* and the *Synechococcales/Oscillatoriales* sequences, suggesting an evolutionary diverging point between these orders (Supplementary Fig. 16).

**Discussion**

Here, we used biochemical, structural, and bioinformatics studies to elucidate the molecular details of how RexT senses H2O2 and initiates an oxidative stress response. It was found that RexT senses H2O2 through the formation of a vicinal disulfide bond and the molecular details of the disulfide bond-based redox regulation process were revealed. Importantly, RexT differs from members of the ArsR-SmtB family of regulators that have been thus far characterized and represents the only sensor in this family that has been shown to employ catalytic residues to facilitate a response to ROS. Thus, this work adds to the regulatory repertoire of the ArsR-SmtB metalloregulator superfamily.

Mechanistically speaking, most H2O2-mediated disulfide bond-forming reactions proceed through an attack of the deprotonated-negatively charged Cys sidechain thiolate on H2O2 to form an unstable sulfenic acid moiety (Fig. 7).

This transient species can subsequently react with a nearby Cys residue to form an intramolecular disulfide bond (Fig. 7). For RexT, based on the mutagenesis studies, mass spectrometry experiments, and the X-ray crystal structures determined in this work, we posit that Cys41 is the peroxidatic Cys and that Asn77, due to it being within hydrogen-bonding distance of Cys41, is involved in forming or stabilizing the negatively charged Cys41 thiolate (Fig. 5b and Fig. 7). Arg61 and Thr68, on the other hand, are suggested to form hydrogen bonds with H2O2 and favor its reduction by polarizing the O=O bond and facilitating the requisite S=O2 reaction (Fig. 5b and Fig. 7). Consistent with the importance of these residues in disulfide bond formation, Asn77 and Arg61 are completely conserved in all identified RexT homologs that are adjacent to a thioredoxin gene (Supplementary Fig. 15a). Thr68, although not completely conserved, is replaced by a Ser residue in some sequences from the *Nostocales* order or intriguingly substituted by a Cys residue in some RexT homologs from *Synechococcales* and *Oscillatoriales* that lack Cys40 (Supplementary Fig. 15b, c).

As described above, our mechanistic proposal for RexT is reminiscent of that put forward for OxyR,

which has been suggested to also rely on Thr and Arg residues to activate H2O2 for reduction (Fig. 5b, c). However, unlike OxyR, the rate of H2O2 consumption by RexT appears to be slower. Specifically, it was observed that it took wild-type RexT about 200 s to consume half of the added H2O2 in our assays (Fig. 3 and Fig. 5). For OxyR, using the same assay, an equimolar amount of H2O2 is consumed within 10 sec. The magnitude of difference between the rates of H2O2 consumption in these proteins is proposed to correlate with the different levels of cellular H2O2 in the organisms that use these regulators. For example, OxyR is mainly found in Gram-negative bacteria where the H2O2 levels are maintained at a low level by the efficient antioxidant activity of catalase. In *E. coli*, this statement means that OxyR is activated when exogenous H2O2 levels accumulate to ~3 μM or intracellular levels near a concentration of 0.2 μM. In contrast, it has been shown that the RexT-mediated expression of thioredoxin occurs at H2O2 concentrations above 1 mM. This higher concentration can presumably be attributed to studies that show *Anabaena* (from the order *Nostocales*) predominantly uses two-Cys peroxiredoxin proteins to cope with detoxification of H2O2 rather than catalase. These peroxiredoxin proteins showcase modest catalytic rates and are sensitive to overoxidation, which allows higher concentrations of H2O2 to accumulate. Similarly, as it is currently proposed that the RexT-TrxA2 system could function in both vegetative cells and heterocysts, it is worth noting that in heterocysts, detoxification of H2O2 involves a rubrerythrin that showcases peroxidase activity. Recent work on this rubrerythrin revealed a measured *K₉* of 2 mM H2O2. Thus, regardless of whether RexT functions in vegetative cells or in heterocysts, it appears optimally tuned to start the signaling process at higher H2O2 levels than OxyR. At the molecular level, it is also possible that the lower observed rate of H2O2 consumption in RexT is due to the different positioning of H2O2 in the active site relative to that seen in OxyR. In OxyR, the oxygen atom of H2O2 is attacked by the nucleophilic Cys residue engages in multiple hydrogen-bonding interactions with active site residues (Fig. 5c). These interactions presumably increase the electrophilicity of the oxygen atom and thereby facilitate the S=O₂ displacement reaction. The equivalent oxygen atom of H2O2 observed in RexT does not engage in any such interactions (Fig. 5b).

Last, it was determined that Cys41 is the peroxidatic Cys residue, whereas Cys40 is the resolving Cys residue (Figs. 3, 5, and 7). Consistently, we find that Cys41 is absolutely conserved among all “true” RexT homologs or those that are adjacent to a thioredoxin-encoding gene (Supplementary Fig. 15). In contrast, despite conservation of most of the identified residues involved in dimer formation, DNA binding (Arg26 and Lys50), and the required residues for H2O2 reduction (Asn77 and Arg61), Cys40 is not
conserved in all these identified homologs (Supplementary Fig. 15). Intriguingly, CyeR\textsuperscript{35}, which is also found in the RexT cluster of the SSN, upregulates the expression of the old-yellow enzyme-family protein-encoding gene \textit{cye1} in response to oxidative stress (Fig. 6 and Supplementary Fig. 17). This CyeR-based mode of regulation is native to \textit{Mycobacterium} and \textit{Streptomyces} and its inactivation is known to rely on one Cys residue that is found in an equivalent position to Cys\textsubscript{41} in RexT (Supplementary Fig. 17). This protein has been suggested to function as a monomer and form an intramolecular disulfide bond with a second Cys that is found seven residues away, indicating the possibility that some RexT homologs could function through the formation of an alternate disulfide linkage\textsuperscript{35} (Supplementary Fig. 15 and 17). The conservation of just one Cys residue among RexT homologs also parallels what is known about OxyR, for which there exist both two-Cys and one-Cys containing homologs\textsuperscript{36,37}. As with the CyeR-mediated response, the molecular details of how these one-Cys OxyR homologs sense and respond to \textit{H}\textsubscript{2}O\textsubscript{2} remain enigmatic.

In this work, we also found that the dimeric architecture and DNA binding domains of RexT regions of RexT are relatively unchanged between the reduced and oxidized states (Fig. 2c and Supplementary Fig. 18a). This result is unlike the conformational changes observed in OxyR, where formation of a disulfide bond causes a global conformational change in the protein architecture\textsuperscript{39} (Supplementary Fig. 18e, f). Instead, in RexT, the formation of an eight-membered vicinal disulfide bond results in only a local reorganization of the \textalpha{} helix and disruption of its interactions with the “wing” of the wHTH motif (Fig. 4). The lost interactions between the \textalpha{} helix and \beta{} strand in the wHTH motif in the oxidized structure, suggest that Cys\textsubscript{41} is a redox switch: its movement propagates as a series of small movements in the \textalpha{} helix that disrupt interactions with the other structural elements required for DNA binding (Fig. 4 and Supplementary Fig. 10c). In line with this statement, a vicinal disulfide bond has also been observed in redox-cycling proteins as well as in proteins where the rearrangement to form the strained eight-membered ring constitutes the required conformational change for protein inactivation\textsuperscript{35}. Collectively, these changes should disfavor DNA binding, and initiate the transcription of \textit{trxA2}. We acknowledge that the magnitude of the observed conformational change may be constrained by the crystal lattice, but the absence of a large conformational change upon signal recognition is not uncommon for ArsR-SmtB family\textsuperscript{58} (Supplementary Fig. 8 and Supplementary Fig. 18b–d). For example, in the Zn-binding \textit{CzrA} and SmtB regulators, small changes in the internal motions have been linked to the formation of a protein conformation that has a lower affinity for DNA\textsuperscript{33,59} (Supplementary Fig. 18b, d). Likewise, in SqrR, it has been proposed that receptor of the environmental signal results in small structural perturbations that energetically disfavor DNA binding\textsuperscript{47} (Supplementary Fig. 18c). Finally, it is also important to point out that formation of a disulfide bond in RexT has other implications. It has been previously shown that TrxA2 is the native reducing agent for oxidized RexT\textsuperscript{17}. In the oxidized structure, the newly formed disulfide bond is oriented towards the protein surface and perhaps coupled with the architectural changes in RexT, more favorably interacts with reduced TrxA2 and permits subsequent resolving of the disulfide linkage (Fig. 4a).

With regards to the third Cys residue found in RexT (Cys\textsubscript{105}), our bioinformatics analysis revealed that only 38 out of 85 \textit{Nostocales} sequences have this residue, and its occurrence seems to be randomly distributed among different families under the \textit{Nostocales} order (Supplementary Fig. 16). Interestingly, a multiple sequence alignment between different types of ArsR-SmtB regulators shows that this C-terminal Cys residue is conserved in a few cases. For example, the equivalent of Cys\textsubscript{105} is found as part of the arsenite-binding motif of \textit{AfaArsR} (Supplementary Fig. 13). Likewise, although not perfectly aligned, a C-terminal Cys residue of CmiR is known to be involved metal ion binding (Supplementary Fig. 13). A C-terminal Cys residue has also been shown in BigR and SqrR to be involved in binding a reactive sulfur species in partnership with another Cys residue on the \textalpha{} helix (Supplementary Fig. 13). There are also several examples (EcArsR\textsuperscript{36} and BsAseR\textsuperscript{35}), in line with what we hypothesized for RexT, which don’t seem to have a function for this C-terminal Cys residue (Supplementary Fig. 13). Assuming multiple ArsR-type paralogs within an organism arise from gene duplication during evolution (like \textit{M. tuberculosis}, which contains 12 putative Ars-R-SmtB regulators\textsuperscript{46,61}), it seems likely that the Cys\textsubscript{105} residue is present in an ancestral ArsR sequence and is an evolutionary remnant in RexT homologs. On the other hand, the phylogenetic tree suggests that the Cys\textsubscript{105} residue may be a gain of function for the \textit{Nostocales} organisms, which unlike \textit{Synechococcales} and \textit{Oscillatoriales}, use heterocysts for nitrogen fixation and require thioredoxin to maintain an anaerobic environment (Supplementary Fig. 16).

The continuous recycling, repurposing, and adaptation of a common protein fold to a new function has been suggested to account for how a proposed group of <1000 protein folds can give rise to the vast number of proteins found in living organisms\textsuperscript{62,63}. This concept is embodied by the ArsR-SmtB family of regulators, which allow organisms to adapt to environmental conditions and respond to environmental stressors. To date, members of the ArsR-SmtB family of regulators family, have been shown to exploit a common protein scaffold to sense and respond to environmental stimuli, including the availability of metal ions, arsenite ions, and RSS (Fig. 6). In this work, we describe how Nature has yet again exploited the ArsR-SmtB regulator architecture to sense, activate, and reduce \textit{H}\textsubscript{2}O\textsubscript{2} to begin the oxidative stress response in cyanobacteria (Fig. 5). In this case, it was found that placement of two-Cys residues adjacent to one another, an arrangement that is typically not conducive to metal-binding\textsuperscript{35}, allows for formation of a vicinal disulfide bond in response to increased levels of \textit{H}\textsubscript{2}O\textsubscript{2}. This result, the identification of RexT homologs that may rely on a different set of Cys residues, and the vast uncharted territory in the ArsR-SmtB SSN (Fig. 6), makes it clear that we will continue to be surprised by examples of how the ArsR-SmtB fold can be reused, repurposed, and reinvented.

**Methods**

**Protein expression and purification for RexT and selenium-methionine-labeled RexT (SeMet-RexT).** The 	extit{Nostoc (Anabaena)} sp. PCC 7120 rexT gene (Locus: ali18679, GenBank: BA0000192) was codon optimized for \textit{E. coli} expression, commercially synthesized, and sub-cloned into pET21d(+)-vector with Ncol and XhoI restriction sites by GenScript. The resulting construct of RexT contains an N-terminal 6x-His-tag and a TEV protease cleavage site (ENLYFQG). \textit{E. coli} BL21(DE3) cells were transformed with the plasmid carrying the rexT gene and selected on LB agar plates containing 50 \textmu{}g/ml ampicillin. A single colony was inoculated into a 5 mL LB starter culture and grown at 37 °C for 16 h. The 5 mL starter culture was subsequently diluted into 1 L of LB medium that contained 50 \textmu{}g/ml ampicillin and grown at 37 °C with a shaking rate of 200 rpm until the OD\textsubscript{600} reached 0.6. At this point, the 1 L culture was cooled to 16 °C and isopropyl \textbeta{}-D-1-thiogalactopyranoside (IPTG) was added to the culture at a final concentration of 100 \mu{}M to induce protein expression. After 16 h of incubation at 16 °C with a shaking rate of 200 rpm, the cells were harvested by centrifugation (3000 x g, 20 min) at 4 °C. 2.5 g of wet cell pellet was typically obtained from a 1 L growth.

To purify His-tagged RexT (His-RexT), the cell pellets (~5 g) were resuspended in 50 mL of Buffer A (20 mM tris(hydroxymethyl)aminomethane (Tris, pH 8.0), 1 M NaCl, 25 mM imidazole, 10 mM \textbeta{}-mercaptoethanol (BME), and 5% v/v glycerol which was supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF). The resuspended cells were sonicated on ice with a 3 s pulse followed by 15 s rest for a total pulse time of 6 min at 30% output (Fischerbrand Model 120 Sonic Dismembrator). The cell debris was removed by centrifugation (12,000 x g, 20 min) twice. The supernatant was loaded on a gravity Nickel column (Ni
Sepharose 6 Fast Flow resin, (Cytsa, I) with a bed volume of 10 mL, which was pre-equilibrated with Buffer A. After the supernatant passed through the column, the column was washed three times with 10 mL of Buffer A. His-RexT was eluted with 30 mL of Buffer B (20 mM Tris (pH 8.0), 1 M NaCl, 200 mM imidazole, 10 mM BME, and 5% v/v glycerol). Ethylenediaminetetraacetic acid (EDTA, pH 8.0) and dithiothreitol (DTT) were added to the eluate to a final concentration of 0.5 mM and 2 mM, respectively. To remove imidazole, the eluate was concentrated by centrifugal filtration using the CentriSpin-200 column (Millipore) and loaded into a Superdex 75/260 10/100 GL column (HiLoad 16/600 Superdex 200 pg column, Cygma) pre-equilibrated with Buffer F (50 mM HEPES (pH 7.5), 100 mM NaCl, 5% v/v glycerol, and 2 mM DTT). The flow-through fraction was collected, and the column was washed with Buffer D and eluted with a 0–100% gradient of Buffer E (20 mM HEPES (pH 8.0), 1 M NaCl 5% v/v glycerol, and 2 mM DTT). TEV was eluted at a concentration above 40% Buffer F, whereas tag-free RexT (RexTelt) existed mainly in the flow-through fraction and a lesser extent in the lower Buffer F gradient fractions. Fractions of RexTelt were pooled, concentrated and loaded onto a size-exclusion column (HiLoad 16/600 Superdex 200 pg column, Cygma) pre-equilibrated with Buffer F (50 mM HEPES (pH 7.5), 100 mM NaCl, 5% v/v glycerol, and 2 mM DTT).

The dimeric fraction of RexTelt eluted from the size-exclusion column was concentrated, flash-frozen in liquid N2, and stored in a ~80 °C freezer. RexTelt used for crystallization and X-ray structure determination was further buffer-exchanged into Buffer G (20 mM HEPES (pH 7.5), 0.2 M NH4Cl, 20% (v/v) PEG3350, and 24% glycerol and quickly looped and frozen into a nitrogen stream. The native RexTelt crystal was harvested similarly. To obtain SeMet-RexT, the SeMet-RexT structure was used to solve a higher-resolution structure of native RexTelt by molecular replacement. The native RexTelt structure was used as a search model in the Phenix software suite (version 2021-10-26) with the PDB ID 6JTM. All plasmid sequences were checked by the DNASP sequence alignment tools (Genewiz). Conditions for expression and purification of each RexTelt variant were identical to that described above for wild-type RexTelt.

Crystallization and X-ray structure determination of SeMet-RexTelt, RexTelt, and oxidized RexTelt (RexTelt-ox).

A mosquito pipetting robot (TTP LabTech) was used to screen crystallization conditions anaborexic for RexTelt. First RexTelt crystals were observed following anarobic incubation in a 20 °C incubator located in a Coy chamber (O2 < 20 ppm, Coy Lab) at 20 °C for a week. This condition consisted of 0.3 uL/min of gaseous mixture and 0.3 uL of culture solution (0.2 mM of isomorphous replacement in Phenix). The OD500 reached 0.6, the growth medium was removed by centrifugation and replaced with the same medium supplemented with 40 μL/L-selenomethionine (TCI). The purification procedure of SeMet-RexTelt is identical to that of RexTelt.

The conditions for overexpression of selenomethionine-labeled RexTelt (SeMet-RexTelt) are similar to those of RexTelt, except that a methionine auxotrophic E. coli B834 (DE3) cell line was used. Consequently, a growth medium which consisted of SelenoMet medium base and nutrient mix (Molecular Dimensions) was used in place of LB, with 40 μg/mL L-methionine added before IPTG induction. The OD500 was established at 0.6, and the growth medium was removed by centrifugation and replaced with the same medium supplemented with 40 μL/L-selenomethionine (TCI). The purification procedure of SeMet-RexTelt is identical to that of RexTelt.

Fluorescence anisotropy experiments. The DNA duplex fluorescence probe (Supplementary Table 1) was synthesized with one 5'-end labeled with 6-carboxyfluorescein (5'-FAM, IIDT). Different concentrations of RexTelt and its variants were prepared by two-fold serial dilution to achieve a final concentration of 16.525 nM to 32 μM (up to 64 μM for R26A and K50A) and were incubated with 100 nM DNA fluorescence probe in binding buffer (20 mM HEPES (pH 8.0), 100 mM NaCl, 10 mM MgCl₂, 10% v/v glycerol, and 1 mM DTT) for 30 min. To evaluate the effect of possible metal or arsenite binding on DNA binding, 200 nM, 500 nM, and 1000 nM of ZnCl₂, CdCl₂, and sodium arsenite were added to the 100 nM dimeric RexTelt sample. Samples were analyzed by electrophoresis on 7% non-SDS polyacrylamide gels (Bio-Rad) at 45 V for 120 min using a Tris-Borate-EDTA (TBE) buffer. DNA fragments were visualized by staining with GelRed® nucleic acid gel stain (Biotium).

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model, shown in equation (3).

\[
V = \frac{A_{max} \times X}{K_M + X}
\]  

(3)

H₂O₂ consumption assay. To compare how H₂O₂ was consumed by RexT and its molecular variants, a modified version of a previously published ferrous oxidation of xylenol orange (FOX) assay was used87. RexT or its variants were first buffer-exchanged into the reaction buffer (100 mM sodium phosphate, pH 7.4) with a desalting column (Bio-Spin 6 Gel Columns, Bio-Rad) to remove the TNT present in the storage buffer right before being added into the reaction mixture at a final concentration of 100 µM. To initiate the reaction, H₂O₂ was added to the mixture to a final concentration of 200 µM. 20 µl aliquots of the mixture were taken after 10, 20, 30, 40, 60, 90, 150, 300, and 500 s, mixed with 980 µl of the FOX agent (100 µl xylenol orange, 250 µM ammonium ferrous sulfate, 100 µM sodium citrate, and 25 mM H₂SO₄), and incubated for 30 min at room temperature in the dark. At the end of the reaction, the absorbance at 560 nm was measured on a 96-well plate reader (Epoch 2 microplate spectrophotometer, BioTek). Negative controls were included by using the reaction buffer, or 1.3 µg/ml bovine serum albumin (BSA) in the place of RexT. The concentration of H₂O₂ was determined by an H₂O₂ standard curve.

Statistics and reproducibility. All biochemical experiments were typically performed in independent triplicates, with two or three different batches of protein or DNA samples. For H₂O₂ consumption assays and fluorescence anisotropy experiments, the results are presented as the mean ± SD with individual data points overlaid in the same graph. For EMSA, mass spectrometry, and CD experiments, representative images and graphs are presented. Data for protein X-ray crystallography were collected on different crystals produced from different batches of protein samples with similar results.

Mass spectrometry experiments. Liquid chromatography-mass spectrometry (LC-MS) analysis was performed for RexT and its variants in their as-purified, H₂O₂-treated, and H₂O₂/dimedone-treated forms. As-purified samples were prepared by diluting protein stock solutions with water to a final concentration of 5 µM. H₂O₂-treated samples were prepared by mixing 10 µM RexT or its variants with 400 µM H₂O₂ at a 1:1 ratio and incubated at 30 °C for 2 h. For dimedone-modified protein samples with 10 mM dimedone added (Tokyo Chemical Industry) were mixed with 400 µM H₂O₂ at a 1:1 ratio and incubated at 30 °C for 14 h. The samples were then injected into LC-MS for further analysis.

LC-MS experiments were performed on an Agilent G6545A liquid chromatography/quadrupole-time of flight (LC/Q-TOF) equipped with a dual AJS ESI source and an Agilent 1290 Infinity series diode array detector, autosampler, and binary pump. An Aeris WIDEPOR C4 column (2.1 x 50 mm, 3.5 µm, 200 Å) (Phenomenex) was used for sample separations, with Solvent A = water with 0.1% formic acid and Solvent B = 95% acetonitrile, 5% water, and 0.1% formic acid. The employed chromatographic method used (i) 5% Solvent B 0–2 min, (ii) a linear gradient to 90% Solvent B over 5 min, and (iii) a final step that used 90% Solvent B for 1 min. 50.5 µl injections were made for each sample and the column was run at 0.3 mL/min. MS data were analyzed and tabulated in Agilent MassHunter BioConfirm.

Circular dichroism (CD) experiments. Stock solutions of wild-type RexT were diluted to 10 µM using a buffer containing 50 mM HEPES (pH 8.0) and 150 mM NaCl. H₂O₂ was added into the protein solution with a final concentration of 1:1 or 2:1 to protein concentration. This mixture was incubated at room temperature for 5, 15, and 30 min and the CD spectrum of each sample was taken subsequently. For each CD measurement, 400 µl of the diluted RexT–H₂O₂ solution was transferred into a 10 mm quartz cuvette (Hellma). The CD spectra were recorded using a Jasco J-1500 CD spectrometer with 0.1 nm data pitch and 20 nm/min scan speeds. The baseline was measured with the same buffer used to do dilution (50 mM HEPES (pH = 8.0) and 150 mM NaCl). Each sample spectrum is an average of five cumulative spectra.

Bioinformatics analysis. The protein family classification for RexT was analyzed through the InterPro database88 by searching the full amino acid sequence of RexT. The 6-99 amino acid region was identified as the HTH ArsR-type DNA-binding domain (IPR001845), which was shared with typical members of the ArsR-SmtB family. A sequence similarity network (SSN) for representative sequences of the ArsR-SmtB family was generated by EF-Enzyme Similarity Tool89,90. The resultant SSN contained 20616 UniRef5091 sequences, or proteins that share 50% sequence identity in the UniProt database. These sequences were clustered by Cytoscape83 at a cut-off value of e = 20. Biochemically and/or structurally characterized proteins were manually highlighted in the network. Key amino acid sequences from the network were aligned by Clustal W84.

To explore the cluster that contains RexT in more detail, 7085 of the original amino acid sequences were analyzed using the EFI Genome Neighborhood Tool81,85,86. The genome neighborhood diagrams show that only 105 out of the 7085 clustered proteins are adjacent to a thioredoxin gene. Sequence logos of these 105 proteins were generated by WebLogo 387. A phylogenetic tree of proteins from three orders of cyanobacteria was built by MEGA X90.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. Protein coordinates and structure factors have been submitted to the Protein Data Bank under accession codes 7TXO (SmtR-RexT), 7TXM (Reduced RexT), and 7TXN (Oxidized RexT). The source data underlying Figs 2, 3, and 5 and Supplementary Fig. 3 are provided as a source data file. Other data are available in the Supplementary Information and from the corresponding authors upon reasonable request.

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Author contributions

B.L., M.I., J.L., J.T., and J.B.R. contributed to the design of the experiments and wrote the manuscript. B.L. and M.I. expressed and purified all native and SeMet proteins used in this work. B.L. performed all H2O2 consumption, fluorescence anisotropy, and EMSA experiments. B.L. performed all crystal optimization, crystallographic data collection, and solved the three X-ray crystallographic structures determined in this work. B.L. conducted sequence similarity network and phylogenetic analysis. J.L. performed mass spectrometry experiments. J.T. performed the CD experiments. R.C. performed initial crystallographic screening and optimization experiments.

Competing interests

The authors declare no competing interests.

Additional information

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