Cyclic di-GMP (c-di-GMP) is a second messenger that modulates multiple responses to environmental and cellular signals in bacteria. Here we identify CdbA, a DNA-binding protein of the ribbon-helix-helix family that binds c-di-GMP in *Myxococcus xanthus*. CdbA is essential for viability, and its depletion causes defects in chromosome organization and segregation leading to a block in cell division. The protein binds to the *M. xanthus* genome at multiple sites, with moderate sequence specificity; however, its depletion causes only modest changes in transcription. The interactions of CdbA with c-di-GMP and DNA appear to be mutually exclusive and residue substitutions in CdbA regions important for c-di-GMP binding abolish binding to both c-di-GMP and DNA, rendering these protein variants non-functional in vivo. We propose that CdbA acts as a nucleoid-associated protein that contributes to chromosome organization and is modulated by c-di-GMP, thus revealing a link between c-di-GMP signaling and chromosome biology.
n bacteria, nucleotide-based second messengers fulfill key functions in the generation of output responses to changing environmental and cellular cues. Among them, cyclic dinucleotide bis-(3′-5′)-cyclic dimeric GMP (c-di-GMP) stands out as widespread and highly versatile regulating a multitude of diverse processes including biofilm formation, motility, adhesion, exopolysaccharide (EPS) synthesis, cell cycle progression, development, and virulence. Signal transduction by c-di-GMP involves its regulated synthesis and degradation by diguanylate cyclases (DGCs) and phosphodiesterases (PDEs), respectively while output responses are generated by c-di-GMP binding to and allosteric regulation of downstream receptors. Receptors include riboswitches and proteins that regulate processes at the transcriptional, translational or post-translational level. Proteinaceous receptors comprise a variety of proteins with no or little sequence homology including catalytically inactive DGCs and PDEs, PilZ-domain proteins, MshEN domain proteins, different transcription factor families, and ATPases of flagella and type III as well as type VI secretion systems. Typically, bacterial genomes encode multiple DGCs and PDEs. For some DGCs and PDEs distinct functions have been defined; however, for many, no function has been assigned. Similarly, the number of DGCs and PDEs often exceeds that of known c-di-GMP receptors impeding a complete understanding of the biological functions of c-di-GMP signaling and how effects of c-di-GMP are implemented. These observations also support that additional functions of c-di-GMP regulation remain to be uncovered.

The Gram-negative deltaproteobacterium Myxococcus xanthus is a model organism for studying social behavior in bacteria. In the presence of nutrients, M. xanthus cells generate coordinately spreading colonies. In response to nutrient depletion, development is initiated resulting in the formation of multicellular, spore-filled fruiting bodies. c-di-GMP accumulates during growth and development and the level increases ~10-fold during development. During growth, c-di-GMP regulates T4P-dependent motility and the composition of the extracellular matrix. During development, the increase in c-di-GMP level is essential for EPS accumulation, fruiting body formation, and sporulation. Little is known about c-di-GMP receptors in M. xanthus and only the NtrC-like transcriptional regulator Epsl/Nla24, which is important for motility and EPS accumulation, and the histidine protein kinase SgmT, which possesses an enzymatically inactive DGC domain and regulates extracellular matrix composition, have been identified as c-di-GMP receptors.

During growth, M. xanthus cells divide at midcell and each daughter cell contains a single, fully replicated chromosome with the origin and terminus regions in the subpolar regions close to the old and new cell pole, respectively. Replication and chromosome segregation is initiated soon after cell division. Segregation depends on the ParABS system in which ParB binds to parS sites close to the origin while the ParA ATPase and ParB mediate segregation. Duplication of the origin region, one ParB/parS complex remains in the subpolar region of the old pole while the second copy translocates to the subpolar region of the new pole. In parallel, the terminus region translocates to midcell where it is replicated. In the subpolar regions, the ParB/parS complexes are anchored to a scaffold composed of the three BacNOP bacterofilins and the PadC adaptor protein that together form a complex that extends ~1 μm away from the cell pole. The ParABS system is essential for chromosome segregation. By contrast, BacNOP and PadC anchor the origin region and in their absence the nucleoid is more compact but BacNOP and PadC are not essential.

Here, we identify two small, paralogous proteins, CdbA and CdbB, as c-di-GMP receptors. We show that CdbA is a tetrameric ribbon-helix-helix DNA binding protein and changes conformation upon c-di-GMP binding. The regions in CdbA important for c-di-GMP binding are also important for DNA binding. Consistently, c-di-GMP and DNA binding are mutually exclusive. In vivo CdbA is essential, while CdbB is not, and depletion of CdbA causes defects in chromosome organization and segregation resulting in defects in cell division. CdbA is abundant and binds globally to the M. xanthus chromosome. These observations are in agreement with a model whereby CdbA is a ligand-regulated nucleoid-associated protein (NAP) important for chromosome organization and segregation and in which CdbA activity is modulated by c-di-GMP.

**Results**

**CdbA and CdbB bind c-di-GMP in vitro.** We identified c-di-GMP binding proteins using the unbiased c-di-GMP capture compound technology and cell extracts of growing wild-type (WT) M. xanthus cells (Methods). Here, we focused on MXAN_4361 and MXAN_4362 (renamed CdbA and CdbB, respectively for c-di-GMP binding protein A and B), which were enriched in the experimental samples compared to the controls, due to their lack of homology to known c-di-GMP receptors and because in vivo analyses demonstrated that CdbA is essential (see below).

CdbA and CdbB are small paralogs (67 and 86 amino acids, respectively; 50.6/57.3% identity/similarity) and encoded in an operon (Fig. 1a, Supplementary Fig. 1a–c). All fully sequenced Myxococcales genomes encode at least one ortholog, and when only one is present, then based on sequence identity/similarity, it is CdbA-like (Supplementary Fig. 1b). CdbA/CdbB homologs were not identified outside the Myxococcales. We confirmed the interaction with c-di-GMP employing purified CdbA and CdbB proteins in a differential radial capillary action of ligand assay (DRAcALA), where both C-terminally His-tagged CdbA and CdbB specifically bound [32P]-labeled c-di-GMP (Fig. 1b), i.e., binding was outcompeted by excess unlabeled c-di-GMP, but not by unlabeled GTP (Fig. 1b). Based on isothermal titration calorimetry (ITC), CdbA binds c-di-GMP with high affinity (K_d of ~83 nM) and a stoichiometry of 0.5 molecules of c-di-GMP per 1 molecule of CdbA (Fig. 1c). By analytical size-exclusion chromatography (SEC) in the absence of c-di-GMP, CdbA had an apparent molecular mass of ~38 kDa suggesting that CdbA is a tetramer (Fig. 1d). In the presence of c-di-GMP, CdbA had an apparent molecular mass of ~52 kDa (Fig. 1d) suggesting that c-di-GMP binding does not alter CdbA oligomerization but modulates the conformation of tetrameric CdbA resulting in a more open conformation than in the unbound form.

CdbA has a ribbon-helix-helix fold. CdbA and CdbB do not share sequence homology with known c-di-GMP binding proteins; however, sequence analysis suggested that they belong to the ubiquitous ribbon-helix-helix (RHH) superfamily of transcription factors, named so after the order of secondary structure elements (β-strand/α-helix1/α-helix2) in each subunit of the dimeric RHH DNA binding domain. In this domain, two intertwined chains form a β-sheet consisting of the two anti-parallel β-strands that insert into the DNA major groove to make sequence-specific contacts. Additionally, the loop between α-helix1 and α-helix2 together with the N-terminus of α-helix2 make contacts to the DNA phosphate backbone. RHH proteins typically bind DNA as tetramers recognizing direct or inverted repeat sequences.
To gain insights into the mechanism of CdbA and CdbB, we determined the CdbA crystal structure (Supplementary Table 1). We obtained two crystal forms of full-length CdbA-His6. The first structure (traced residues 5–52) verified that CdbA is a RHH superfamily member, with crystallographic symmetry generating a classical RHH dimer (Fig. 2a). This observation was confirmed by DALI analysis38, which suggested the streptococcal RHH repressor CopG as the closest structural homolog (PDB: 2CPG, r.m.s.d. of 1.9 Å over 45 residues39). In CdbA, the exposed face of the β-strand projects residues K8, S10 and Y12, while L11 and F13 are facing the hydrophobic core of the dimer. The β-strand is terminated by P14, leading into α-helix1 (residues 16–28), followed by a loop and then α-helix2 (residues 32–48), with a strongly kinked region at A43/R44, and a C-terminal tail traceable to A52 (Fig. 2a). The two α2 helices run antiparallel, with kink residues I42 and A43 packing against their counterparts in the opposing chain (Fig. 2a).

**Fig. 1** CdbA and CdbB bind c-di-GMP in vitro. a cdbA-B locus. Start and stop codons are indicated. +1 indicates first nucleotide in the cdbA start codon. Arrows indicate direction of transcription. MXAN locus tags are indicated for the genes flanking cdbA-B. See Supplementary Fig. 1 for annotation of these genes. b CdbA and CdbB binding to 32P-c-di-GMP in DRaCALA in the absence and presence of unlabeled c-di-GMP and GTP. Similar results were obtained in two independent experiments. Source data are provided as a Source Data file. c ITC of c-di-GMP-binding to CdbA variants. In each experiment, the original titration traces (top panel) and integrated data (bottom panel) are shown. Solid line in the bottom panel represents the fit of the integrated titration peaks to a one-site binding model. Dissociation constants (K_D) and thermodynamic parameters are listed for CdbAWT for which binding was detected. d Analytical SEC of CdbA variants in the presence and absence of c-di-GMP. Arrows indicate elution volume and mass of molecular weight standards. n = 5 for CdbAWT, n = 2 for CdbA^K8A/S10A and CdbA^R27A/R30A. Table indicates calculated and apparent molecular mass of CdbA variants. Source data are provided as a Source Data file.
Successful model building of the first form allowed us to solve the second form via molecular replacement. This form has three copies of the protein in the asymmetric unit, one of which forms a tetramer via crystallographic symmetry (four copies of same chain) and two of which form a dimer within the asymmetric unit, which then tetramerizes over the final crystallographic axis. These two tetramers are independent yet essentially identical and we interpret these to represent the physiological tetramer identified by SEC. The tetramer centers on residue F50 of the two innermost chains, and the C-terminal tails can be traced five residues further than in form one, and with the C-terminal tail of the innermost chains in a dimer contacting an opposing dimer (Fig. 2b). Protein–protein contacts mediated by the tail region include a hydrophobic component from F50 and V53 in one chain projecting towards a pocket in the opposing dimer formed by W34/I42/I47, a second hydrophobic interaction between V56 and P14/M17, and a more polar interface between the free carbonyl groups of A52/V53/D55 and the side chains of Q38 and K41 (Fig. 2b). Upon solving this crystal form, we realized that a similar, but more loosely-packed, tetramer can be observed when considering the crystal symmetry of form one. The arrangement of the two dimers in the tetramer places their β-sheets ~35 Å apart (measured between Cα atoms of equivalent Y12 residues).

Using the RHH protein Arc, which binds DNA as a tetramer and with the two β-sheets separated by 26 Å, as a template, we created a model of how CdbA could bind DNA (Fig. 2c, d). Consistent with the idea that the two β-sheets in the tetramer make DNA contacts, a surface electrostatic map of CdbA revealed that this region is positively charged (Fig. 2c). Because the β-sheets are ~35 Å apart in CdbA, this model suggests that CdbA bends DNA upon binding (Fig. 2d).

c-di-GMP binding to CdbA induces conformational changes. Because we were unsuccessful in obtaining the crystal form of c-di-GMP-bound CdbA, we performed hydrogen-deuterium exchange (HDX) mass-spectrometry (MS) in the presence and absence of c-di-GMP to identify regions in CdbA involved in c-di-GMP binding or undergoing conformational changes upon c-di-GMP binding. We obtained a total of 68 peptides in both states that cover the entire CdbA sequence with ~11-fold redundancy per amino acid (Supplementary Data 1, Supplementary Fig. 2a). The HDX profile in the absence of c-di-GMP is in agreement with the crystal structure, i.e., the RHH fold (amino acids 1–48) exchanges hydrogen to deuterium more slowly than the disordered C-terminal region (Supplementary Fig. 2a). The difference in HDX between c-di-GMP-bound and apo-CdbA revealed changes that were restricted to the RHH fold (Fig. 3a, Supplementary Fig. 2b). Specifically, the N-terminus, the β-strand, a portion of α-helix1, the loop between α-helices 1 and 2, and the N-terminal tip of α-helix2 had reduced HDX in the presence of c-di-GMP (Fig. 3a–c). When illustrating the location of three...
representative peptides that exhibit substantial differences in deuterium incorporation (Fig. 3b) onto the crystal structure of a CdbA dimer or tetramer (Fig. 3c), two of the three peptides (3–13 and 29–34) converge providing a polar pocket formed by residues Y12 and N54 from the two innermost chains and K6, K8, D29, R30, and S31 from the two outermost chains (Fig. 3d). Because HDX-MS cannot discriminate between peptides originating from the inward- and outward-facing chains, we cannot dissect whether the copies of the two peptides that do not engage in formation of this pocket have altered HDX profiles upon c-di-GMP binding. Nevertheless, given the proximity of the β-strand (peptide 3–13) and the helix α1–α2 loop including the N-terminal tip of α-helix2 (peptide 29–34), we hypothesized that they might represent the c-di-GMP binding site. From hereon, we refer to these two regions as interface −1 and −2, respectively (Fig. 3e).

To probe the participation of interface −1 and −2 in c-di-GMP binding, we substituted residues in both interfaces that show 100% conservation in CdbA homologs (Supplementary Fig. 1c, Fig. 3e), to generate CdbAK8A/S10A and CdbAR27A/R30A (Fig. 3e). In SEC, both variants, eluted with an apparent molecular mass of ~35 kDa (Fig. 1d) indicating correct tetramer formation; however, they did not detectably bind c-di-GMP as estimated by ITC (Fig. 1c) and SEC (Fig. 1d) suggesting involvement of both interface-1 and -2 in c-di-GMP binding. Of note, the two interfaces important for c-di-GMP binding overlap with the regions of CdbA predicted to be involved in DNA binding (Fig. 2d).
CdbA is essential for viability while CdbB is dispensable. We attempted to generate in-frame deletions in cdbA and cdbB. We readily obtained the in-frame deletion of cdbB and the mutant was indistinguishable from WT with respect to growth, cell morphology, motility and development (Fig. 4a–c, Supplementary Fig. 3a). By contrast, we were unable to generate an in-frame deletion of cdbA (∆cdbA). We, therefore, constructed two merodiploid derivatives of WT in which mCherry (mCh)-tagged CdbA was expressed. In these strains, CdbA expression was induced with the indicated concentrations of vanillate for 24 h before cells were harvested. Protein from the same number of cells was loaded per lane. Molecular mass marker is indicated on the left. Similar results were obtained in two independent experiments. Source data are provided as a Source Data file.
CdbA was expressed ectopically. In one strain, cdbA-mCh was expressed from the native cdbA promoter in a single copy from the Mx8 attB site (WT/Pnat_cdbA-mCh) and in the second, cdbA-mCh was expressed from the vanillate-inducible promoter in a single copy from the MXAN_18/19 locus (WT/Pvan_cdbA-mCh). In immunoblots, CdbA-mCh accumulated in both strains; however, even at the highest concentration of vanillate (500 µM), the level of CdbA-mCh was ~9-fold lower when expressed from the vanillate-inducible promoter than when expressed from the native promoter (Fig. 4d). In the absence of vanillate, CdbA-mCh was undetectable by immunoblotting in the WT/Pvan_cdbA-mCh strain verifying that Pvan tightly regulates cdbA-mCh expression (Fig. 4d). Using the WT/Pvan_cdbA-mCh strain, we successfully generated an in-frame deletion of cdbA at the native locus in the presence of 500 µM vanillate (∆cdbA/Pvan_cdbA-mCh). This strain grew in the presence of 500 µM vanillate but at a reduced rate compared to WT (Fig. 4a) while a derivative of this strain in which the Pnat_cdbA-mCh construct was also integrated at the Mx8 attB site grew like WT in the absence of vanillate (Supplementary Fig. 3b, c). We conclude that the CdbA-mCh protein is fully active and that the reduced growth rate of the ∆cdbA/Pvan_cdbA-mCh strain is likely caused by the lower accumulation of CdbA-mCh.

Removal of vanillate caused growth arrest of ∆cdbA/Pvan_cdbA-mCh cells after 24 h correlating with the earliest time-point at which CdbA-mCh was no longer detectable by immunoblotting and also caused a four-log defect in plating efficiency (Fig. 4a, e). Cells of the CdbA-mCh depletion strain were slightly longer than WT cells in the presence of vanillate, while cells grown under CdbA-mCh depletion conditions for 24 h were highly filamentous (Fig. 4b, c). These cells still formed constrictions at midcell but failed to complete division and eventually lysed (Fig. 4b). We conclude that CdbA is essential for viability and that lack of CdbA causes a cell division defect.

To investigate the relationship between CdbA and CdbB, we generated a ∆cdbA∆B double deletion in the strain containing the Pvan_cdbA-mCh construct (∆cdbA∆B/Pvan_cdbA-mCh). In the presence as well as in the absence of vanillate, cells of this strain had an even more significant growth and cell division defect than the ∆cdbA/Pvan_cdbA-mCh cells (Fig. 4a–c) suggesting that CdbB can partially substitute CdbA. Interestingly, CdbA and CdbB interact in a bacterial two-hybrid assay suggesting that they can form heterooligomers (Supplementary Fig. 3d). Altogether, these observations indicate that CdbA and CdbB have similar functions. However, CdbA alone is required and sufficient for viability while CdbB is neither required nor sufficient for viability.

CdbA binds the nucleoid globally and sequence-specifically. Because CdbA is a member of the RHH family of DNA binding proteins, we asked whether CdbA binds DNA. In fluorescence microscopy analyses of WT/Pnat_cdbA-mCh cells, CdbA-mCh colocalized with the DAPI-stained nucleoid in M. xanthus (Fig. 5a). Similarly, CdbA-mCh colocalized with the nucleoid in E. coli (Fig. 5b) supporting that CdbA binds DNA in vivo.

We used ChIP-seq to identify CdbA binding sites at a genome-wide scale in M. xanthus using a strain in which cdbA at the native locus was replaced with an allele encoding a C-terminally 3xFLAG-tagged CdbA variant. This strain grew like WT, indicating that the protein was fully functional (Supplementary Fig. 4a). As a positive control, we used a strain expressing 3xFLAG-tagged ParB from the native locus and as a negative control, WT without a FLAG-tagged protein. In the two replicates, the WT control only showed a few peaks enriched ≥4-fold over input (Fig. 5c), while the ParB-3xFLAG control showed a broad peak in the region containing the parS sites (Fig. 5c). The ChIP-seq peaks for CdbA-3xFLAG revealed broad occupancy over the M. xanthus chromosome (Fig. 5c). For both 3xFLAG-tagged proteins, the ChIP-seq peaks were strongly correlated between the two replicates while no correlation was observed for the WT control (Fig. 5c).

We detected 569 peaks with ≥4-fold enrichment over input for CdbA in intergenic regions and within structural genes (Fig. 5c, Supplementary Data 2). To experimentally verify the ChIP-seq results, we performed electrophoretic mobility shift assays (EMSA) with purified CdbA-His6 and 250 bp fragments corresponding to peak rank #1, #8, #161, and a fragment with no peak as a control. CdbA bound to the peak-fragments with an affinity that decreased with decreasing peak rank (Kd from ~0.4 to ~0.9 µM) and the fragment with no peak had significantly lower affinity (Kd of ~4 µM) (Fig. 6a) suggesting that CdbA binds with some sequence specificity and non-specifically at high concentration. Consistently, at the highest concentration of CdbA-His6 (4 µM) the shifted complexes tended to run higher in the gels than at lower concentrations supporting that more than one CdbA molecule would bind to a DNA fragment (Fig. 6a).

To identify the CdbA consensus DNA binding sequence, we searched the 100-bp peak sequences centered around each peak summit using the MEME-ChIP web tool. Using the top 100 peak sequences, we identified a direct repeat motif separated by four bp, close to the peaks summits (Fig. 6b), while only half of this motif was detected when we used the top 500 peak sequences (Fig. 6b).

To analyze the importance of the direct repeat, we performed EMSAs with a DNA fragment from peak rank #1 containing the WT sequence or fragments in which the two most conserved bp had been mutated in one or both repeats (Fig. 6c). While the WT sequence had a Kd of ~0.4 µM, the fragment with one repeat mutated had a Kd of ~1.1 µM and the fragment with two repeats mutated had a Kd of ~2.5 µM, close to that for unspecific binding by CdbA (Kd ~ 4 µM; Fig. 6a, c). These observations support that the identified motif is important for sequence-specific DNA binding by CdbA and that its conservation dictates CdbA affinity for DNA. The repeat fits well to the model of CdbA DNA binding in which the two DNA binding regions are separated by one helical turn. Using a hidden Markov model, we searched the M. xanthus genome for the presence of the direct repeat motif and detected 8917 sequences with a p-value < 0.00077. Out of the 569 ChIP-seq peaks, 296 (52%) have at least one identified direct repeat motif present in a distance ±50bp from the peak summit (Supplementary Fig. 4c, Supplementary Data 2).

The model of DNA bound tetrameric CdbA, predicts that CdbA binds DNA when bound to the direct repeat (Fig. 2d). To test this prediction, we performed a circular permutation analysis using 250 bp fragments from peak rank #1 in which the direct repeat was systematically placed at different locations along the 250 bp. All DNA fragments showed the same migration with no CdbA bound (Supplementary Fig. 4d) while CdbA binding induced a more pronounced shift in the DNA fragment containing the motif towards the center of the fragment in comparison to fragments in which the binding site was positioned towards the end, demonstrating that CdbA binds DNA upon binding (Supplementary Fig. 4d).

Using quantitative immunoblot analysis, we determined the number of CdbA monomers per cell to ~7000 corresponding to a concentration of 2.2 µM for the tetramer supporting that CdbA is able to broadly occupy the M. xanthus chromosome (Supplementary Fig. 4e). We conclude that CdbA binds to >500 sites along the M. xanthus chromosome, that CdbA likely binds DNA as a tetramer with a consensus sequence consisting of a direct repeat and causes DNA bending upon binding.
CdbA depletion causes only minor changes in transcription. 198 of the CdbA ChIP-seq peaks (~35%) mapped to intergenic regions, and among the top 100 peaks, 47% were intergenic (Supplementary Data 2) while less than 10% of the M. xanthus genome is intergenic.42 Genes downstream from the 198 intergenic peaks encode proteins annotated as hypothetical or involved in various cellular functions and no specific functional category was enriched (Supplementary Data 2). Cell division defects resulting in cell filamentation can be caused by defects in divisome assembly29,30,43,44, peptidoglycan synthesis29, DNA replication as well as DNA damage45–49, chromosome segregation or chromosome organization50,51. Because CdbA-depletion caused cell filamentation with incomplete cell division, we searched all 569 peaks for genes known to be involved in such functions and with a peak in the predicted promoter region but did not identify any (Supplementary Data 2).

To determine whether CdbA functions as a transcriptional regulator, we performed RT-qPCR analysis on total RNA isolated from WT and CdbA-mCh-depleted cells. Among the nine genes tested, which all had a ChIP-seq peak in the predicted promoter

**Fig. 5 CdbA binds multiple sites across the M. xanthus genome.** a, b CdbA-mCh localization and DAPI staining of the nucleoid in WT M. xanthus and E. coli. In the demographs, cells were sorted according to cell length and fluorescence profiles of individual cells stacked with the shortest cell at the top and the longest at the bottom. n is indicated for each strain. Below, images of representative cells. Scale bars, 5 μm. c Genome-wide ChIP-seq profiles of CdbA-3xFLAG-bound and ParB-3xFLAG-bound regions on the M. xanthus chromosome. WT was used as a negative control. The log2 enrichment ratio was calculated from IP DNA and input DNA and plotted against location on the 9.28 Mb M. xanthus chromosome for one replicate. Grey line indicates no difference between sample and input. Red line indicates 4-fold difference (log2 = 2) set as a significance threshold for peaks. Right panels, scatter plots show correlation between two replicates for each strain. The log2 enrichment over input for replicate 1 is plotted against log2 enrichment over input for replicate 2 for each genomic position. The correlation coefficient, R^2, is indicated for each strain.
region, three showed minor but significant decreases in transcription upon CdbA-mCh depletion, but this effect did not correlate with peak rank; four were unaffected by CdbA depletion and two were not detectably expressed under the conditions tested (which were identical to those under which the ChIP-seq experiment was performed) (Fig. 6d). Thus, even though CdbA binds in the promoter regions of genes, it does not function as a classical transcriptional regulator consistent with the observation that most ChIP-seq peaks are within structural genes.

c-di-GMP inhibits DNA binding by CdbA. As noted, the regions in CdbA involved in c-di-GMP binding overlap with the regions predicted to be important for DNA binding (Figs. 2d and 3e). We analyzed whether c-di-GMP affects DNA binding by CdbA using a DNA fragment that covers 250 bp upstream of the cdbA translational start codon. CdbA-His6 binds this fragment with a K_d of 0.9 µM (Fig. 6a). Increasing c-di-GMP concentrations reduced CdbA DNA binding and at 16 µM, DNA binding was completely abolished (Fig. 7a). Because CdbA binds c-di-GMP with a K_d of 83 nM while c-di-GMP only reduced CdbA DNA binding in the µM range, we performed an order of addition experiments in which we first added c-di-GMP to CdbA followed by DNA or vice versa. Independently of the order of addition, c-di-GMP had the same inhibitory effect on CdbA DNA binding (Supplementary Fig. 5). This effect was specific to c-di-GMP as none of five other tested nucleotides had an effect on
CdbA DNA binding (Fig. 7b). Similarly, c-di-GMP abolished binding to the other DNA fragments tested in EMSAs (Fig. 6a).

CdbA^K8A/S10A-His6 and CdbA^R27A/R30A-His6, which do not detectably bind c-di-GMP (Fig. 1c, d), also failed to bind the cdBA promoter in EMSAs (Fig. 7c). These observations are in agreement with a model in which the same regions in CdbA are important for DNA binding and c-di-GMP binding and that binding of these two ligands is mutually exclusive.

To determine whether DNA binding and/or c-di-GMP binding by CdbA is important in vivo, we generated three strains in which (A) the native cdBA gene was deleted (ΔcdBA), (B) CdbA^WT-mCh was expressed from the vanillate-inducible promoter, and (C) CdbA^WT-mCh, CdbA^K8A/S10A-mCh or CdbA^R27A/R30A-mCh were expressed from the native promoter from the Mx8 attB site. Upon removal of vanillate, these three strains accumulate CdbA^WT-mCh, CdbA^K8A/S10A-mCh or CdbA^R27A/R30A-mCh, respectively (Fig. 7d). While the strain accumulating CdbA^WT-mCh grew as WT (Fig. 7e), the two strains accumulating CdbA^K8A/S10A-mCh or CdbA^R27A/R30A-mCh had a growth defect (Fig. 7e) and became filamentous (Fig. 7f) demonstrating that the variants are inactive in vivo and suggesting that DNA and/or c-di-GMP binding is essential for CdbA function in vivo. The two variants also failed to localize over the nucleoid in M. xanthus (Fig. 7f), confirming the in vitro result that they have a DNA binding defect (Fig. 7c).

CdbA depletion affects chromosome organization and segregation. Having ruled out that CdbA functions as a classical transcription factor and inspired by its abundance and global occupancy over the M. xanthus chromosome and essentiality, we hypothesized that CdbA could be a nucleoid-associated protein (NAP). We, therefore, analyzed nucleoid organization and segregation in CdbA-mCh-depleted cells using DAPI staining to assess nucleoid organization and a ParB-YFP fluorescent fusion as a marker for the origin and to assess chromosome organization and segregation.

In the presence of vanillate, WT and the CdbA-mCh depletion strain showed similar DAPI staining patterns with a single nucleoid in short cells and two fully replicated and segregated nucleoids in longer cells (Fig. 8a). As previously observed, most WT cells had two ParB-YFP signals at 25 and 75% of the cell length while slightly more cells of the CdbA-mCh depletion strain had more than two ParB signals (Fig. 8a, b). Also, the distance from the ParB-YFP clusters to the nearest cell pole was significantly longer in cephalxin treated cells compared to WT (2.2 ± 0.8 vs 1.8 ± 0.6 μm) (Fig. 8c). As a control for the CdbA-mCh depletion strain in the absence of vanillate, WT cells were artificially elongated with 8 h of cephalxin treatment, which inhibits cell division without affecting chromosome replication, organization and segregation.

As reported, cephalxin treated WT cells had a regular distribution of nucleoids and ParB-YFP signals along the cell length. By contrast, in the CdbA-mCh depleted cells, nucleoids appeared more condensed, less well separated and mostly localizing in the center of the long cells (Fig. 8a, b, Supplementary Fig. 6a). Also, ParB-YFP foci were not regularly distributed along the cell length but clustered in the center of cells paralleling the localization of the nucleoid (Fig. 8a, b, Supplementary Fig. 6a). Consistently, in these cells, the distance from the ParB-YFP clusters to the nearest cell pole was significantly larger than in cephalxin treated cells (5.3 ± 2.4 vs 3.7 ± 2.5 μm) (Fig. 8c, Supplementary Fig. 6b). This phenotype is different from ΔbacNOP and ΔapadC strains where localization of ParB-YFP foci was also affected but no cellular filamentation was observed.

In time-lapse recordings of non-motile cells, we observed that segregation of ParB clusters was significantly slower and more erratic after CdbA-mCh depletion compared to that in WT (Supplementary Fig. 6c). Despite the abnormal localization of ParB-YFP clusters and the abnormal morphology and localization of the nucleoid, the number of ParB-YFP clusters per cell length increased similarly for cephalxin treated WT and the CdbA-mCh depletion strain (Fig. 8a) suggesting that DNA replication is unaffected by depletion of CdbA-mCh. These observations demonstrate that CdbA is important for chromosome organization and segregation in agreement with the idea that CdbA is a NAP.

Because DNA and c-di-GMP binding by CdbA is mutually exclusive in vitro, we speculated that if the essential function of CdbA lies in its NAP activity, then changing the cellular concentration of c-di-GMP would affect CdbA DNA binding and, therefore, chromosome organization and segregation. To test this hypothesis, we analyzed cell length distribution and nucleoid morphology in a strain that accumulates the heterologous DGC DgcA of Caulobacter crescentus, and which accumulates c-di-GMP at a 7-fold higher level than WT and in a strain that accumulates the heterologous PDE PA5295 of Pseudomonas aeruginosa and which accumulates c-di-GMP at a 2-fold lower level than WT.

Discussion

Here, we report the identification of the DNA binding RHH protein CdbA in M. xanthus, a previously undescribed type of c-di-GMP receptor, that globally binds the M. xanthus chromosome and is essential for viability. Cells depleted for CdbA have severe defects in chromosome organization and segregation and are impaired in cell division and highly filamentous. Taken together our data support a model whereby CdbA is an essential protein affecting chromosome replication, organization and segregation.
Fig. 7 C-di-GMP and DNA binding is mutually exclusive and essential for CdbA function in vivo. a EMSA experiment with CdbA in the presence of c-di-GMP. The fragment used covers the cdbAB promoter (see also Fig. 5a). 4 µM CdbA was incubated with indicated concentrations of c-di-GMP for 10 min. prior to addition of DNA fragment. Similar results were obtained in two independent experiments. Source data are provided as a Source Data file. b EMSA experiment with CdbA in the presence of different nucleotides. Experiment was done as in a and a concentration of 16 µM for all nucleotides was used. The same results were obtained in two independent experiments. Source data are provided as a Source Data file. c EMSA experiment with CdbAK8A/S10A and CdbAR27A/R30A. Experiments were done as in a; CdbA concentrations are indicated. The same results were obtained in two independent experiments. Source data are provided as a Source Data file. d Immunoblot analysis of accumulation of CdbA-mCh variants in indicated strains after 24 h of vanillate depletion. PilC was used as a loading control. Protein from the same number of cells was loaded per lane. For both proteins, samples are from the same blot and lanes were removed for presentation purposes. Molecular mass marker is indicated on the left. Similar results were obtained in two independent experiments. Source data are provided as a Source Data file. e Growth of indicated strains on solid surface in the presence and absence of vanillate driving synthesis of CdbAWT-mCh. Plates were incubated for 4 days. All the strains were spotted on the same plates. Similar results were obtained in two independent experiments. Source data are provided as a Source Data file. f CdbA-mCh localization and DAPI staining of the nucleoid in representative cells of indicated genotypes grown in the presence and absence of vanillate. In the absence of vanillate, cells were imaged 24 h after vanillate removal. Scale bars, 10 µm. Similar results were obtained in two independent experiments.
and ligand-regulated NAP, the activity of which is modulated by c-di-GMP binding.

Generally, NAPs are small, abundant and dissimilar proteins that bind DNA with moderate sequence specificity causing bending, wrapping or bridging of DNA and, typically, only regulate transcription on a small scale. The moderate effects on gene expression distinguishes NAPs from classical transcriptional regulators whose target genes are normally more dramatically regulated. For example, Fis, a NAP of Escherichia coli, recognizes ~900 regions across the genome, bends DNA, and generally only causes minor changes in transcription. While Fis is non-essential for viability, in its absence, cells are elongated, have chromosome segregation defects, and less condensed nucleoids compared to WT. H-NS, a NAP and global repressor of
transcription in enterobacteria, shows sequence-specific DNA binding to ~500 AT-rich regions and affects chromosome organization and transcription by forming DNA loops. Garp in C. crescentus is an essential NAP that recognizes AT-rich sequences, binds to ~600 regions across the genome preferentially associating with intergenic regions. Garp depletion causes formation of filaments—and minicells but does not cause major changes in transcription. By comparison CdbA binds DNA with some sequence specificity and a Kd of 0.4–4.0 µM, which is significantly lower than for other RHH proteins that have Kd in the range of 10–200 nM. Forty-seven percent of the top 100 ChIP-seq peaks are in intergenic regions; however, RT-qPCR experiments comparing transcription in WT and CdbA-depleted cells either revealed modest effect, no effect or no expression of genes with a CdbA binding site in the promoter region suggesting that CdbA does not function as a classical transcriptional regulator. Also, the putative functions of proteins encoded by genes with a ChIP-seq peak in the promoter region did not show enrichment of functional categories as would be expected for a classical transcriptional regulator. Moreover, these genes did not include those encoding proteins involved in cell growth, cell division, chromosome organization and/or segregation that could explain the chromosome and cell division defects in cells depleted of CdbA.

Additionally, CdbA binds the 9.28 Mb M. xanthus genome at >500 sites, bends DNA upon binding, is highly abundant with ~1700 tetramers per cell, and is essential. Altogether, these observations are consistent with the notion that CdbA is a NAP with an essential function in chromosome organization and segregation. According to this model, the primary function of CdbA is that of a NAP to assist in organizing the chromosome and support chromosome segregation while the effects on transcription and cell division are likely secondary to the defect in chromosome organization. In contrast to other NAPs, the CdbA consensus binding site is GC-rich. We speculate that this might be explained not only by the GC-rich nature of M. xanthus genome (69% GC content) but also by the mutually exclusive binding of DNA and c-di-GMP potentially allowing the guanine bases of c-di-GMP and GC-rich DNA fragment to compete for the same binding site on CdbA (see below). Other NAPs are typically regulated by abundance, whether CdbA is also regulated by abundance is not known; however, our data support the notion that CdbA’s DNA binding activity is regulated by c-di-GMP, i.e., CdbA is a ligand-regulated NAP.

In vitro c-di-GMP binding by the CdbA tetramer induces conformational changes. C-di-GMP and DNA binding are mutually exclusive and the two ligands appear to compete for the same binding interfaces on CdbA. In other RHH proteins the β-sheet provides specificity in DNA binding, while the loop region between α-helix1 and α-helix2 together with the N-terminus of α-helix2 make DNA backbone contacts and increases the affinity for DNA. Precisely these two regions show a modified response upon addition of c-di-GMP in the HDX experiment. The 1:2 ratio of c-di-GMP:CdbA, HDX data of binding interfaces, and our structure of a tetramer formed from two RHH dimers allow the postulation of two models for c-di-GMP binding. The first model would place one c-di-GMP molecule directly over each β-sheet in the tetramer; this singular dinucleotide would adopt either the extended conformation observed in EAL PDEs or the clamp-like conformations adopted for example in the HD-GYP domain protein PmGH. In this model, the alterations in HDX of the α-helix 1–2 loop could be explained by a change in the relative orientation of the two CdbA dimers in the tetramer upon c-di-GMP binding. A second, intriguing possibility is that the unique dimer-dimer pocket formed at the center of the tetramer acts to bind the commonly encountered intercalated dimer form of c-di-GMP, and the two β-sheets flex inward to explain their implication in the HDX data. Flexation would also allow the helical turn (residues 29–34) in the center of the tetramer to recognize the c-di-GMP phosphate group in the same manner that RHH proteins use to bind the DNA backbone. The tetramer interface displays different hydrophobic packing between the two crystal forms we observed here, suggesting that it may have inherent plasticity that allows it to undergo this change. In total, the first model would rely on steric blockage to enable c-di-GMP to license CdbA release from DNA, whereas the second model could modulate DNA binding via c-di-GMP alteration of the β-sheet-β-sheet distance. RHH domains are found as stand-alone RHH domains or together with other domains. Ligand binding and modulation of DNA binding by other RHH proteins has only been demonstrated for RHH proteins with additional domains and the ligands bind to these extra domains. Thus, CdbA is the first RHH protein that binds a ligand (other than DNA) via the RHH domain. In P. aeruginosa AmrZ, one of the best studied RHH proteins, is involved in regulating the c-di-GMP level. However, we did not observe c-di-GMP binding to AmrZ (Supplementary Fig. 8) documenting that c-di-GMP binding is not a general feature of RHH proteins.

In contrast to CdbA, other c-di-GMP binding transcriptional regulators use distinct domain for c-di-GMP and DNA binding. C-di-GMP may stimulate DNA binding by stimulating oligomer formation as is the case for VpsT of Vibrio cholerae, BldD of Streptomyces coelicolor and CuxR of Sinorhizobium meliloti. Alternatively, upon c-di-GMP binding, FleQ of Pseudomonas aeruginosa undergoes a conformational change causing reduced DNA binding.

In the absence of DNA, CdbA binds c-di-GMP with a Kd ~ 83 nM; and CdbA binds DNA fragments with Kd ~ 0.4–4.0 µM (depending on the fragment used). Nevertheless, in vitro c-di-GMP only reduced CdbA DNA binding when added in the µM range indicating the complexity of this interplay; there is a possibility that DNA-binding changes CdbA structure/dynamics such that c-di-GMP binding is less favorable than when DNA is absent (i.e., this is not represented by a simple competition model, and can be enacted by an extensive interface for DNA binding...
that is more than just the two β-sheets. The estimated c-di-GMP concentration in *M. xanthus* cells in rich medium is ~1.4 ± 0.5 μM, suggesting that c-di-GMP binding by CdbA should be relevant in vivo. However, strains in which the c-di-GMP level was artificially increased or decreased 7- or 2-fold, respectively, have no defects in growth, chromosome organization/segregation or cell division. Based on these observations and the observation that inactivation of CdbA has a dramatic effect on viability, we speculate that alteration in c-di-GMP may not elicit an all-or-none response with respect to CdbA DNA binding but rather a graded response in which c-di-GMP binding to CdbA modulates the response with respect to CdbA DNA binding but rather a graded response in which c-di-GMP binding to CdbA modulates the

Operon mapping. Total RNA was isolated using a Trizol (Sigma) extraction method. RNA was treated with DNase I (Inviogene) and purified with the RNeasy kit (Qiagen). PCR analysis was used to confirm that the RNA was DNA free. One microgram of RNA was used to synthesize cDNA with the High capacity cDNA Archive Kit (Applied Biosystems) using random hexamer primers. For the operon mapping, following primer pairs were used: “4361 qPCR forw”/ “4361 qPCR rev” (fragment 1), “4362 qPCR forw”/ “4362 qPCR rev” (fragment 2) and “4361 qPCR forw”/ “4362 qPCR rev” (fragment 3). Genomic DNA and RNA were used as positive and negative controls, respectively.

Real-time PCR. Total RNA was isolated and cDNA was synthesized as described for operon mapping. qRT-PCR was performed in 25 μl reaction volume using SYBR green PCR master mix (Applied Biosystems) and 0.1 μM primers specific to the target gene in a 7500 Real Time PCR System (Applied Biosystems). Relative gene expression levels were calculated using the comparative Ct method. All experiments were done with three biological replicates each with three technical replicates.

Immunoblot analysis. Immunoblots were carried out as described. Briefly, rabbit polyclonal α-FLAG (Rockland, dilution 1:2000), α-mCherry (Biovision, dilution 1:5000) antibodies were used together with horseradish-conjugated goat anti-rabbit immunoglobulin G (Sigma-Aldrich, dilution 1:10000) as secondary antibody. Blots were developed using Luminata Crescendo Western HRP Substrate (Millipore) and visualized using a LAS-4000 luminance image analyzer (Fujiﬁlm).

Determination of protein copy number per cell. Number of CdbA molecules per cell was determined using a quantitative immunoblot analysis. Exponentially growing WT culture was used to prepare cell lysates for immunoblot analysis. Different amounts of cell lysates and purified protein were separated on a 15% SDS-gel, transferred to a nitrocellulose membrane and probed with α-FLAG antibodies. Signal intensities of the lysate bands were quantified using Fiji and compared against a standard curve generated from known amounts of His6_CdbA_3×FLAG from the same Immunoblot. Experiment was performed in two independent biological replicates.

Protein purification. For DrA_CALA, EMSA and determination of copy number proteins were expressed in *E. coli* Rosetta 2 (DE3) growing in 2×TY medium at 37 °C using 0.5 mM IPTG induction for 3 h. His6-tagged proteins were purified using Ni-NTA affinity purification. Briefly, cells were harvested by centrifugation (3500 × g, 20 min, 4 °C), resuspended in buffer A (50 mM Tris-HCl, 150 mM NaCl, 10 mM imidazole, 1 mM DTT, 10% glycerol, pH 8.0) and lysed using a French pressure cell. After centrifugation (1 h, 48,000 × g, 4 °C) lysates were loaded on a Ni-NTA agarose column (Qiagen) and washed with 20× column volume using buffer B (50 mM Tris-HCl, 150 mM NaCl, 20 mM imidazole, 1 mM DTT, 10% glycerol, pH 8.0). Proteins were eluted with buffer C (50 mM Tris-HCl, 150 mM NaCl, 50-500 mM imidazole, 1 mM DTT, 10% glycerol, pH 8.0). For ITC, analytical SEC and HDX proteins were essentially purified as described previously. Briefly, *Esherichia coli* BL21 (DE3) Express carrying the expression plasmids were grown in lysogeny-broth medium supplemented with 50 μg ml⁻¹ kanamycin and 12.5 μg ml⁻¹ (D+)-lactose-monohydrate for 20 h at 30 °C under rigorous shaking. Cells were harvested by centrifugation (3500 × g, 20 min, 4 °C), resuspended in lysis buffer (20 mM of HEPEs-Na pH 8.0, 250 mM NaCl, 20 mM MgCl₂, 20 mM KCl, 40 mM imidazole) and broken by one passage through the M-110L Microfluidizer (Microfluidics). After centrifugation (47,850 × g, 20 min, 4 °C), the supernatant was loaded on a 5 ml HisTrap FF column (GE Healthcare) equilibrated with 5-column volumes (CV) lysis buffer. After washing with 5 CV of lysis buffer, the proteins were eluted with 5 CV elution buffer (lysis buffer containing 500 mM imidazole). Proteins were concentrated (Amicon Ultra-0.5 ml-1, Millipore) and applied to size-exclusion chromatography (SEC) on a HiLoad 16/60 Superdex 200 column (GE Healthcare). Protein-containing fractions were pooled, again concentrated (Amicon Ultra-0.5 ml-1, Millipore), deep-frozen in liquid nitrogen and stored at −80 °C. For structure determination a single transformant was grown overnight in LB at 37 °C and used to inoculate 1 liter of auto-induction medium which was then incubated at 37 °C for three hours before the temperature was decreased to 18 °C for 18 h before cells were harvested by centrifugation. The resulting cell pellets were then homogenized in Buffer A (20 mM imidazole-HCl, pH 7.5, 400 mM NaCl) supplemented with 0.05% Tween20 and 100 μg ml⁻¹ lysozyme. Resuspended cells were lysed by sonication and the lysate was clarified by centrifugation at 48,000 × g for 60 min. Soluble proteins were loaded onto a 5 ml HiTrap FF column (GE Healthcare) pre-equilibrated in Buffer A, washed extensively with Buffer A, and
then eluted in buffer B (400 mM imidazole-HCl, pH 7.5, 400 mM NaCl). Fractions containing pure recombinant CdbA were pooled and dialysed overnight in dialysis buffer (20 mM HEPES-NaOH, pH 7.0, 300 mM NaCl) at 4 °C.

Analytical size-exclusion chromatography (SEC). Prior analytical SEC, 200 μM CdbA wild-type or its variants were incubated with or without 200 μM c-di-GMP for 1 minute at 25 °C. Analytical SEC was carried out using a Superdex 200 Increase 10/300 GL column (GE Healthcare) at 0.5 ml/min flow rate with SEC buffer. A vanadium-blue dye curve for molecular mass determination was obtained using a mixture of thymoglobin (669 kDa), ferritin (440 kDa), carbonhydrate (158 kDa), aldolase (75 kDa), ovalbumin (44 kDa), conalbumin (29 kDa), and RNase A (13.7 kDa).

Electrophoretic mobility shift assay (EMSA). The HEX-labelled DNA fragments were generated by PCR. Reactions were carried out in 10× EMSA buffer (380 mM HEPES-NaOH, pH 7.0, 500 mM KAc, 200 mM NaCl, 0.1% Nonidet P-40, 10 mM DTT, 1 mM EDTA, 1× protease inhibitor cocktail). The reaction was centrifuged (10 min, 15,000 × g) and the supernatant was used for the c-di-GMP binding assay.

In vitro c-di-GMP binding assays. In the DrsA CALA assay36,44, c-di-GMP was incubated with 20 μM of the relevant protein and incubated for 10 min at 30 °C in binding buffer (10 mM Tris-HCl pH 8.0, 100 mM NaCl, 5 mM MgCl2). Ten microliters of this mixture was transferred to a nitrocellulose filter (GE Healthcare), allowed to dry and imaged using a STORM 840 Scanner (Amersham Biosciences). For competition experiments, 0.4 mM unlabeled c-di-GMP (BioLog) or GTP (Sigma-Aldrich) was added. ITC was performed with the ITC200 (MicroCal) at 25 °C. CdbA (200 μM, 25 mM) was titrated with 2 μl per injection of 150 μM c-di-GMP. The baseline of 19 injections was then subtracted. The injection was started by mixing 1 μl of 3x and individual injections were separated by breaks of 180 s. To subtract dilution heat of c-di-GMP, a measurement with the same protocol was performed using only SEC buffer in the sample cell. Data analysis was done with NTITPIC 1.2.26 and the experimental data fitted using SEDPHAT 1.12B89. The final image was generated with GUSSI 1.3.2.7.

Hydrogen-deuterium exchange mass-spectrometry (HDX-MS). Sample preparation for HDX-MS was carried out by a two-arm robotic autosampler (LEAP Technologies). CdbA (7.5 µl, 50 µM) with or without 500 µM c-di-GMP (BioLog) was mixed with 67.5 µl D2O-containing SEC buffer (20 mM of HEPES-Na, pH 7.5, 200 mM NaCl 20 mM MgCl2, 20 mM KCl). After incubation for 10/30/95/1000/10000 s at 25 °C, 55% of the HDX reaction was added to 55 μl quench solution (400 mM KH2PO4/K2HPO4, 20 mM guanidine-HCl, pH 2.2) kept at 1 °C and 95% of the mixture injected into an ACQUITY UPLC M-class system with HDX technology (Waters).88 Peptides were generated online using immobilized peptase at 12 °C and 100 μl/min flow rate of water < 0.1% (v/v) formic acid and the resulting peptides trapped on a C18 column (Waters) kept at 0.5 °C. After 3 min, the trap column was placed in line with an ACQUITY UPLC BEH C18 1.7 μm 100 × 100 nm column (Waters) and the peptides eluted at 0.5 °C using a gradient of water + 0.1% (v/v) formic acid (eluent A) and acetonitrile + 0.1% (v/v) formic acid (eluent B) at 30 μl/min flow rate: 0-95% A: 7-8 min, 65-15% A, 8-10 min/15% A, 10-11 min/5% A, 11-16 min/95% A. Undeuterated samples were obtained by injecting extra buffer through the H2O-containing SEC buffer. HDX spectra were recorded in positive ion mode using a Synapt G2-Si HDMS mass spectrometer equipped with an ESI source (Waters) in HDMS (High Definition-MS) or HDMS2 (Enhanced High Definition MS)90,91 mode for deuterated and undeuterated samples, respectively. [Glu1]–Fibrinopeptide B standard (Waters) was used for lock mass correction. The peptide column was washed three times with 80 μl of 4% (v/v) acetonitrile and 0.5 M guanidine-HCl during each run and additionally blank runs were performed between each sample to avoid peptide carry-over. All measurements were performed in triplicates. Peptide identification and assignment of deuterium incorporation was carried out as described previously91,92 aided by PLGS and DYNAX 3.0 softwares (Waters). A total of 68 peptides were obtained that covered the entire sequence of CdbA with 11-fold redundancy per amino acid. HDX-MS data can be found in the Supplementary Data 194,95.
550 µl ChIP buffer with 0.01% sodium dodecyl sulfate (SDS) and pre-cleaned with 30 µl of protein A magnetic beads (Thermo Scientific) previously washed with PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 15 mM KH2PO4) plus 1 mg/ml bovine serum albumin (BSA). 10 µl of monoclonal α-FLAG (Rockland) antibodies was added, incubated at 4 °C overnight with rotation and immunoprecipitated (2 h, 4 °C) with rotation with 30 µl of protein A magnetic Dynabeads previously washed with PBS plus 1 mg/ml BSA. The beads were washed with low salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris pH 8.1, 150 mM NaCl), with high salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris pH 8.1, 0.5 M NaCl), with LiCl buffer (0.5 M LiCl, 1% NP-40, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris pH 8.1), and twice with TE buffer (10 mM Tris pH 8.0, 1 mM EDTA, 0.1% SDS, 1% Triton X-100). The protein–DNA complex eluted from the beads in two 100 µl fractions of elution buffer (1% SDS, 0.1 M NaHCO3) and incubated at 65 °C overnight in the presence of 20 µl 3 M NaCl to disrupt the crosslinks, treated with 0.5 µl proteinase K (50 mg/ml) at 42 °C for 2 h and with 2 µl RNase A (10 mg/ml) at 37 °C for 20 min, followed by DNA isolation using PCR product purification kit (Macherey-Nagel). The input sample was also subjected to this cross-link reversal and DNA extraction procedure. FS primers were prepared and sequenced (paired-end, 2×150 bp) on an Illumina HiSeq3000 instrument at the Max Planck-Genome-centre Cologne.

**ChIP-seq data analysis.** CLC workbench 12.0 (Qiagen, Hilden, Germany) was used for computational processing of sequencing data. The functions “Trim Reads”, “Map Reads to Reference”, “Duplicate Mapped Reads Removal”, “Transcription Factor ChIP-Seq” & “Annotation with Nearby Gene Information” were applied with default settings. ChIP-seq peak calling in CLC workbench is based on a shape learning algorithm [36]. Here every two samples were compared individually to their respective controls where the genome assembly of *Mycobacterium xanthus* DK1622 (Accession: NC_008095) obtained from NCBI was used as reference. Sequence coverage of peaks was inferred via samtools [37]. Peaks were considered significant if the enrichment in the sample over input was ≥ 2-fold. The DNA binding consensus of CdbA was identified using sequences from the top 100 or top 500 peaks (peak summit ±50 bp) and the MEME-Chip web tool [38]. The probability matrix from the MEME-Chip analysis using the top 100 peaks served as an input to create the Hidden Markov Model used for the genome-wide search for putative CdbA binding sites with a given score. P-value of identified sequences was determined based on siimilc simulation. In this simulation 1,000,000 of 18 bp random sequences with the same GC content as *M. xanthus* genome were screened for the presence of the same probability matrix with a given score. A motif was assigned to the peak if it was found within a range of a peak summit ±50 bp.

**Bioinformatics.** Homology based structure prediction for CdbA and CdbB was performed using HHPref [39]. 16 s rRNA and protein sequences were aligned with ClustalW using MEGA7 [40] and the 16 s rRNA phylogenetic tree was generated using the Maximum Likelihood method. CdbA/B homologs were identified using BLASTp analysis and the sequence identity/similarity was calculated using EMBOSS Needle software [41] (pairwise sequence alignment).

**Statistics.** All statistics were performed using a two-tailed Student’s t-test for samples with unequal variances using Snapmaplot 12.5 and Microsoft Excel 2013.

**Plasmid construction.** Primers used in this study are listed in Supplementary Table 4. pDJS 83 (for overexpression and purification of CdbB), cdbB was amplified from DK1622 genomic DNA with the primer pair “4362 F+/4362 -stop R”, digested with Ndel and HindIII, cloned into pET24b+ digested with the same enzymes and sequenced.

pDJS 85 (for generation of in-frame deletion of cdbB), up- and downstream fragments were amplified from DK1622 genomic DNA using the primer pairs “4361-2_A/4361-2_B (5aa)” and “4361-2_C (5aa)/4361-2_D”, as described [42]. The AB and CD fragments were used for overlapping PCR with the primer pair “4361-2_A/4361-2_D (20µl)”, the AD fragment was digested with KpnI and HindIII, cloned into pKT25 digested with the same enzymes and sequenced.

pDJS 86 (for overexpression and purification of CdbA), cdbA was amplified from DK1622 genomic DNA with the primer pair “4361 F+/4361 -stop Rev”, digested with Ndel and HindIII, cloned into pET24b+ digested with the same enzymes and sequenced.

pDJS 92 (for generation of in-frame deletion of cdbA), up- and downstream fragments were amplified from DK1622 genomic DNA using the primer pairs “4362_A/4362_B (5aa)” and “4362_C (5aa)/4362_D”, as described [42]. The AB and CD fragments were used for overlapping PCR with the primer pair “4362_A/4362_D (20µl)”, the AD fragment was digested with KpnI and XbaI, cloned into pBluescript digested with the same enzymes and sequenced.

pDJS 99 (for generation of in-frame deletion of cdbA), up- and downstream fragments were amplified from DK1622 genomic DNA using the primer pairs “4361-2_A/4361-2_B (5aa)” and “3831_C (5aa)/4361_D (5aa)”, as described [42]. The AB and CD fragments were used for overlapping PCR with the primer pair “4361-2_A/4361-2_B (5aa)” to generate the AD fragment. The AD fragment was digested with KpnI and XbaI, cloned into pBluescript digested with the same enzymes and sequenced.
KpnI “4361 - str R BamHI”, digested with KpnI and BamHI, cloned into pNG62 digested with the same enzymes and sequenced.

pDJS 155 (for expression of Pnat_cdbA_R27/A30A 3xFLAG) was digested with the same enzymes and sequenced.

digested with pDJS 170 with the primer pair “Pnat - str R BamHI”, digested with KpnI and BamHI, cloned into pNG62 digested with the same enzymes and sequenced.

pDJS 170 (replacement for cdBA with cdA-3xFLAG at native site), up- and downstream fragments were amplified from DK1622 genomic DNA using the primer pairs “4361-2_A”/4361 3xFLAG rev and “4361 3xFLAG fl”/4361-2_D”. Resulting fragments were used as a template for overlapping PCR with the primer pair “4361-2_A”/4361-2_D”. Resulting fragment was digested with KpnI and XbaI, cloned into pBRI114 digested with the same enzymes and sequenced.

pDJS 177 (for overexpression and purification of His6_cdbA_3xFLAG), the cdBA_3xFLAG fragment was amplified from pDJS 170 with the primer pair “4361 no start NdeI”/4361 3xFLAG stop BamHI”, digested with NdeI and BamHI, cloned into pET28a – digested with the same enzymes and sequenced.

pDJS 179 (for replacement of parB with parB_3xFLAG at native site), up- and downstream fragments were amplified from DK1622 genomic DNA using the primer pairs “ParB 3xFLAG A”/ParB 3xFLAG B” and “ParB 3xFLAG C”/ParB 3xFLAG D”. Resulting fragments were used as a template for overlapping PCR with the primer pair “ParB 3xFLAG A”/ParB 3xFLAG D”. Resulting fragment was digested with EcoRI and XbaI, cloned into pBRI114 digested with the same enzymes and sequenced.

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

Data availability
Co-ordinates and structure factors have been deposited with the RCSB accession codes of 6SBW (form one) and 6SRX (form two). The ArrayExpress accession number for the ChIP-seq experiment is E-MTAB-8535. The HDX-MS data have been deposited to the ProteinChange Consortium via the PRIDE partner repository with the dataset identifier PXD018028. The source data underlying Figs. 1b, d, e, c, a, d, e, c, d, c and Supplementary Figs. 3b–d, 4a, d, e, 5, 6b, 7a, 8a, b are provided as a Source Data file.

Code availability
Custom MATLAB scripts used for ChIP-seq data analysis and for microscopy analysis are available from the corresponding author upon request.

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Author contributions

D.S. and L.S.-A. conceptualized the study. D.S., W.S., and I.T.C. conducted the investigation. D.S. and D.Sz. developed the methodology, analyzed microscopy images, and ChIP-seq data. D.S. and L.S.-A. wrote the original draft of the manuscript. G.B., A.L.L., W.S., D.Sz., and I.T.C. reviewed and edited the manuscript. G.B. and L.S.-A. acquired funding. G.B., A.L.L., and L.S.-A. provided supervision.

Competing interests

The authors declare no competing interests.

Additional information

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Competing interests

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