Bacteria respond dynamically to the changes in zinc availability. Repression by the *Bacillus subtilis* transcription factor Zur requires Zn(II), which binds with negative cooperativity to two regulatory sites per dimer to form, sequentially, Zur$_2$:Zn$_3$ and Zur$_2$:Zn$_4$ forms of the repressor. Here we show that, as cells transition from zinc sufficiency to deficiency, operons regulated by Zur are derepressed in three distinct waves. The first includes the alternative RpmEB(L31*) and RpmGC(L33*) ribosomal proteins, which mobilize zinc from the ribosome, whereas the second includes the ZnuACB uptake system and the YciC metallochaperone. Finally, as zinc levels decrease further, the Zur$_2$:Zn$_3$ form loses Zn(II) leading to derepression of RpsNB(S14*) and FolE2, which allow continued ribosome assembly and folate synthesis, respectively. We infer that zinc mobilization from intracellular zinc stores takes priority over energy-dependent import, and our results link the biochemistry of zinc sensing by Zur to the molecular logic of the zinc deprivation response.
A hallmark of life is the ability to adapt to changing environmental conditions, often by altering the expression of DNA through the action of transcription factors. In many systems, we understand in atomic detail how activators and repressors are converted from their inactive to their active forms, and genomics-level approaches, such as transcriptomics and chromatin immunoprecipitation (ChIP), provide an overview of the complete suite of genes (regulon) that is affected by individual transcription factors. A quantitative understanding of transcriptional control mechanisms also requires an appreciation for the ways in which transcription factors are regulated (often involving integration of multiple inputs), the combinatorial nature of their interactions at specific regulatory regions and the graded responses resulting from variations in operator occupancy, as a function of transcription factor activity. Here we focus on a relatively simple system, the regulation of the zinc deficiency response mediated by the Bacillus subtilis zinc uptake regulator (Zur)1–3.

B. subtilis Zur is representative of a large class of bacterial metal-sensing transcription factors (metalloregulatory proteins) whose DNA-binding activity is regulated by the reversible binding of metal ions4. These proteins therefore function as ‘one-component’ regulators that serve to directly couple changes in concentration of a simple ligand (an inorganic ion) to DNA occupancy5. B. subtilis Zur, like other members of the Fur family of metalloregulatory proteins, is a dimer in solution and requires a structural Zn(II) ion for protein folding and dimerization5. When Zn(II) levels are sufficient, Zur(II) is additionally bound to a regulatory site within each monomer and the resulting, fully metallated protein (designated Zur2:Zn4) is an active repressor. Zur is expressed at relatively uniform level across a range of growth conditions6, and its activity is regulated primarily, if not exclusively, by the reversible binding of Zn(II) ions to this regulatory binding site. As cells transition from Zur(II) to Zur(II) deficiency, Zur transitions first to a partially metallated dimer (ZurZn2:Zn4) and finally to the inactive resting form with Zur(II) bound only at the structural sites, Zur2:Zn2. The presence of the Zur2:Zn4 intermediate results from the ~20-fold negative cooperativity between the dimer of the first and second Zur(II) ions to the regulatory sites in the Zur dimer3.

Zur is known to regulate at least seven operons encoding proteins that facilitate adaptation to Zn(II) limitation7–9. As might be anticipated for a stress response activated when an essential metal becomes limiting for growth, a key part of this adaptive response is the expression of a high-affinity ABC transporter system encoded by the zuuABC operon, which likely functions together with a Zn(II) scavenging lipoprotein, Znt10–12, and a highly abundant member of the COG0523 family of metallochaperones (YciC) conserved in Bacteria, Eukarya and Archaea13. Adaptation to Zur(II) deficiency also commonly involves the expression of alternative ribosomal protein paralogues that functionally replace ribosomal proteins that require Zur(II) for function14,15. In B. subtilis, two Zur-regulated ribosomal proteins are paralogues of L31 (designated L31* and encoded by rpmEB) and L33 (designated L33*, encoded by rpmGC). Induction of L31* and L33* serves to displace the cognate Zur(II) containing proteins from the surface of the assembled ribosome thereby mobilizing Zur(II) into the cytoplasm for redistribution16,17. The expression of a third ribosomal protein parologue (S14*, encoded by rpsNB) allows for de novo ribosome synthesis, which might otherwise become limited by the cell’s ability to synthesize the Zn-requiring S14 protein18. Another process that becomes limiting when cells are zinc deficient is folate biosynthesis due to the Zn dependence of the FolE1 GTP cyclohydrolase. Zur also regulates an alternate, Zn-independent FolE2 enzyme19. In general, the replacement of enzymes dependent on one metal ion by an alternative enzyme dependent on a different metal or a non-metal cofactor is a widely conserved mechanism to facilitate adaptation to metal-limiting growth conditions20.

We previously speculated that the presence of negative cooperativity in Zur(II) binding might provide a mechanism for a graded response to changing Zn(II) levels3. Here we have tested this hypothesis and monitored the relative sensitivity of each of the Zur-regulated operons to zinc deprivation. Our results indicate that as cells transition from zinc sufficiency to zinc limitation operons regulated by Zur are derepressed in three distinct waves. First, induction of RpmEB(L31*) and RpmGC(L33*) mobilizes Zur(II) from the ribosome and Znt1 primes the cell for Zur(II) import. Next, the high-affinity ZnuABC transporter for Zur(II) import is expressed together with the YciC metallochaperone. Finally, RpsNB(S14*) and FolE2 are expressed. Studies with strains expressing a mutant Zur protein (C84S) that can form the partially active ZurZn2:Zn3 form, but which is impaired in its ability to transition to the fully active ZurZn2:Zn4 form, indicate that this partially active form is still sufficient to repress the rpsNB and folEB genes in vivo and still binds with high affinities to the corresponding regulatory regions in vitro. We conclude that the negative cooperativity in Zur(II) binding to Zur is responsible for the transition between the middle and late genes derepressed as part of the zinc limitation adaptive response.

Results

The Zur regulon is derepressed in a stepwise manner. We used an S1 nuclease protection assay to monitor the induction of six different Zur-regulated operons in cells exposed to the zinc chelator TPEN for various periods of time (Fig. 1a). The results reveal that Zur’s target genes are induced in three waves which we assign as early (znuT and rpmEB), middle (ycic and znuA) and late genes (folEB and rpsNB) with respect to their time of induction after chelator addition. While these genes differ in their basal level of expression under zinc sufficient conditions (no added TPEN), they are all highly induced by TPEN but with different kinetics. On the basis of these findings, we extended this analysis to include an additional Zur-regulated gene, rpmGC, which is a pseudogene in B. subtilis 168 due to a frameshift mutation. Although rpmGC does not encode a functional protein in this genetic background, its expression is still regulated by Zur3. Since the rpmEB gene (encoding the L31* ribosomal protein) is induced early, we hypothesized that rpmGC (encoding L33*) would also be induced early. Indeed, rpmGC is induced at a time intermediate between the two early genes and the middle genes noted above (Fig. 1a). These results are supported by studies in which cells were treated for a fixed time (5 min) with increasing amounts of chelator (Fig. 1b). Average values from three independent experiments are presented in Supplementary Figs 1 and 2 and representative full gel images are shown in Supplementary Figs 7 and 8, which revealed a similar stepwise derepression.

Stepwise derepression correlates with operator occupancy. The simplest model to account for the stepwise induction of the Zur regulon is that binding of Zur to its various operator sites is differentially affected as a function of zinc depletion. To monitor the occupancy of Zur protein on its operator sites in vivo, we used ChIP. As predicted, the operator occupancy at the six tested sites declined in parallel with the observed increase of mRNA expression level (Fig. 2). Indeed, the same three sets of early, middle and late genes can be discerned. These results are supportive of a model in which mRNA levels of Zur-regulated
Role of negative cooperativity of Zn(II) binding to Zur.

Previously, we demonstrated that Zur forms a dimer and that efficient dimerization requires a structural Zn(II) site. The Zur$_2$:Zn$_2$ protein binds operator DNA with low affinity and mutations that prevent binding of Zn(II) to the regulatory site are non-functional in repression in vivo. The affinity of Zur for a cognate operator site (folEB) was shown to increase by 1000-fold in the presence of Zn(II) to support the active Zur$_2$:Zn$_4$ form of the repressor. Biochemical measurements revealed that the binding of Zn(II) to the regulatory sites (one in each monomer) occurs with negative cooperativity with a ~20-fold difference in the measured affinities. Further, we found that a C84S mutation to one of the Zn(II) ligands of the regulatory metal binding site did not significantly affect binding of the first Zn(II), but greatly impaired the second binding event (a decrease in affinity of ~10$^{4}$-fold). Since the C84S mutant protein was still able to repress a Zur-regulated target (yciC) in vivo, we concluded that this protein likely retained some repressor activity despite being impaired in the transition from the Zur$_2$:Zn$_3$ to the Zur$_2$:Zn$_4$ form of the repressor.

One possible mechanism to explain why repression of Zur-regulated genes would be differential activity of the Zur$_2$:Zn$_3$ and Zur$_2$:Zn$_4$ forms of the repressor. To explore this possibility, we compared the ability of FLAG-tagged variants of the wild-type (WT) Zur and the C84S mutant to repress each of the six Zur-regulated operons using S1 nuclease protection (Fig. 3; full gel images in Supplementary Fig. 9). As controls, we also included Zur mutants shown previously to be defective for binding the structural Zn(II) ion (C98S) or compromised for protein dimerization (H124A). To monitor the basal level of repressor activity in standard growth conditions, cells were grown to mid-logarithmic phase (OD$_{600}$ = 0.5) in Luria-Bertani (LB) medium. Zinc limitation was imposed by the treatment with 2 mM EDTA for 1 h, and washed cells were then resuspended in LB medium amended with 25 µM Zn(II) for 1 h to restore repression (Fig. 3a). Unlike TPEN, EDTA does not enter cells and imposes zinc limitation by chelating extracellular zinc. Under these conditions, WT Zur was able to repress all six operons with the ambient zinc levels in our LB medium (~18 µM), consistent with the previous work. All six tested operons were induced by EDTA, leading to mRNA accumulation, and this was reversed by resuspension in zinc-amended medium (Fig. 3b, lanes 1–3). In contrast, in cells expressing the C84S Zur mutant both the early and middle genes were partially derepressed in LB medium, whereas the late genes (folEB and rpsNB) were fully repressed. Again, all six operons were derepressed by EDTA and repression was complete in the zinc-amended medium (lanes 4–6). These results suggest that the C84S mutant, which is likely to be largely restricted to the Zur$_2$:Zn$_3$ form in unamended medium, is still active in repression of the late genes, but not the early and middle genes. In contrast to the C84S mutant, a Zur protein defective for dimerization (H124A), is reduced in activity at all six genes (lanes 7–9), and a mutant defective for coordinating the structural zinc ion (C98S) is grossly defective in function (lanes 10–12), despite similar levels of expression of all Zur proteins under all tested conditions (Supplementary Fig. 3e).
Stepwise Zn(II) binding and operator discrimination. The above results are consistent with the hypothesis that decreasing zinc availability leads to a decrease in fully metallated Zur (Zur2:Zn4) and that this leads to a selective induction of early and middle genes, whereas the remaining Zur2:Zn3 population may suffice for repression of late genes. To further explore the basis for this graded response, we sought to determine the biochemical affinity of Zur proteins for each operator. All four Zur proteins (WT, C84S, H124A and C98S) were purified after overproduction in Escherichia coli and biochemically characterized using assays to monitor purity, dimerization and Zn content as described previously for Zur and other Fur family proteins3,21,22 (Supplementary Fig. 4). Next, the DNA-binding affinity was monitored for each active protein (the C98S protein was inactive in binding DNA) at the six operator sites using an electrophoretic mobility shift assay (EMSA) optimized with WT Zur (Supplementary Figs 5 and 6). The results, summarized in Table 1, indicate that WT Zur bound to all six operator sites with similar affinities (Kd values of ~12–25 nM) that differed by no more than approximately twofold. The H124A Zur protein, which was very poorly active as a repressor in vivo (Fig. 2), bound with much reduced affinity (Kd values ~100 nM). Of note, the C84S mutant protein was essentially unaffected in its ability to bind to the operators of the two late genes, folEB and rpsNB, but had reduced affinity for the other four operator sites. These findings support the inference that declining cellular zinc levels lead to an initial transition of Zur2:Zn4 to Zur2:Zn3, and that this leads to a selective induction of early genes. However, the operator affinities in this assay do not provide an explanation for the difference in sensitivity between early and middle genes. We note that early and middle genes clearly differ in their operator occupancy as a function of zinc depletion in vivo as a repressor in vitro that can account for these differences, at least under fully zinc sufficient conditions (Table 1).

Stepwise repression is independent of local chromosomal context. Although our working model is that the temporal induction of genes in the Zur regulon is due exclusively to binding of Zur to the promoter proximal operator site(s), we cannot exclude the possibility that other factors might influence their regulation and, specifically, their sensitivity of zinc depletion. For example, it is increasingly appreciated that chromosome position can affect gene function in bacteria23, and regulators and nucleoid-associated proteins can function over extended distances to impact regulation. To determine whether the local sequence context of each operon is sufficient to determine its sensitivity to zinc-mediated repression, we generated transcriptional fusions of representative early, middle and late promoters to fluorescent protein reporters and integrated these at three distinct sites around the chromosome. In both strains tested, the reporter fusions retained their relative sensitivity to zinc depletion (Fig. 4). This observation indicates that chromosome context is not

To confirm that the effects observed at the level of mRNA are reflective of Zur operator occupancy, we conducted ChIP experiments using the four FLAG-tagged Zur variants (Fig. 3c). The fractional enrichment of each operator site (relative to that observed for WT Zur in LB medium) is consistent with the observed mRNA levels: EDTA treatment leads to a substantial (but not full) decrease in operator occupancy (approximately twofold) for both WT and C84S, consistent with the observed derepression. Further, the difference in repression efficiency in LB medium noted for C84S Zur (Fig. 3b) was also reflected in the promoter occupancy levels (Fig. 3c, lane 4). As expected, the H124A Zur (dimerization defective) had decreased operator occupancy under most conditions (but could be rescued by excess zinc), and the C98S Zur (disrupted in the structural Zn-binding site essential for protein folding and dimerization) was essentially inactive in this operator binding in this assay.

Figure 3 | Comparative repressor activity of mutant Zur proteins. (a,b) We compared the ability of FLAG-tagged variants of the wild-type (WT) Zur and the C84S mutant to repress each of the six Zur-regulated operons using S1 nuclease protection. (a) In WT, the general expression pattern of rpmEB in LB liquid medium (repressed), after treatment with 2 mM EDTA (derepressed), and then subsequently resuspended in LB medium amended with 25 μM ZnSO4 (repressed). (b) Expression profiles of six Zur target genes in complemented strains with FLAG-tagged WT or mutant Zur proteins under the same conditions as (a). Average values from three independent experiments are presented in Supplementary Fig. 3a–c. Transcripts of the zur gene and ribosomal RNA were used as constitutive expression controls. (c) To monitor the in vivo DNA-binding activity of FLAG-tagged Zur variants, cells were grown under the same condition as a and b, and were analysed by in vivo crosslinking and immunoprecipitation as described in Fig. 2. The normalized values from three independent experiments were determined relative to input (1%) and are presented as per cent (%) of DNA binding relative to the untreated control cells.

To confirm that the effects observed at the level of mRNA are reflective of Zur operator occupancy, we conducted ChIP experiments using the four FLAG-tagged Zur variants (Fig. 3c). The fractional enrichment of each operator site (relative to that observed for WT Zur in LB medium) is consistent with the observed mRNA levels: EDTA treatment leads to a substantial (but not full) decrease in operator occupancy (approximately twofold) for both WT and C84S, consistent with the observed derepression. Further, the difference in repression efficiency in LB medium noted for C84S Zur (Fig. 3b) was also reflected in the promoter occupancy levels (Fig. 3c, lane 4). As expected, the H124A Zur (dimerization defective) had decreased operator occupancy under most conditions (but could be rescued by excess zinc), and the C98S Zur (disrupted in the structural Zn-binding site essential for protein folding and dimerization) was essentially inactive in this operator binding in this assay.

Stepwise repression is independent of local chromosomal context. Although our working model is that the temporal induction of genes in the Zur regulon is due exclusively to binding of Zur to the promoter proximal operator site(s), we cannot exclude the possibility that other factors might influence their regulation and, specifically, their sensitivity of zinc depletion. For example, it is increasingly appreciated that chromosome position can affect gene function in bacteria23, and regulators and nucleoid-associated proteins can function over extended distances to impact regulation. To determine whether the local sequence context of each operon is sufficient to determine its sensitivity to zinc-mediated repression, we generated transcriptional fusions of representative early, middle and late promoters to fluorescent protein reporters and integrated these at three distinct sites around the chromosome. In both strains tested, the reporter fusions retained their relative sensitivity to zinc depletion (Fig. 4). This observation indicates that chromosome context is not
determinative, and further reveals that all information needed for the stepwise induction of the Zur regulon is contained within the ~500 bp regions used for fusion construction.

### Discussion

The observation that Zur-regulated genes are derepressed in a distinct temporal order as cells transition from sufficiency to deficiency provides new insights into the molecular logic of the zinc deprivation response (Fig. 5). Our results indicate that as intracellular zinc availability declines the first response is the mobilization of zinc from surface-associated, zinc-associated ribosomal proteins. Both L31 and L33 have been previously implicated as part of a widely distributed but underappreciated mechanism of zinc storage2,14,16,17,24, just as cells may store iron in times of sufficiency within ferritin and ferritin-like proteins, many bacteria appear to use small zinc-binding proteins that associate with the surface of the ribosome as a mobilizable Zn(II) store15. These storage proteins are notable since they are small peptides (<50 amino acids in length) that coordinate Zn(II) through two Cys–X–X–Cys motifs. In many organisms, there are paralogues of nearly identical sequence that lack many or all of these cysteine ligands and these non-Zn(II) binding paralogues are nearly always regulated by Zur or analogous sensors of zinc deprivation15. Biochemical studies reveal that these paralogous ribosomal proteins (for example, L31*) can displace the endogenous ribosomal protein from the surface of the ribosome to mobilize the associated Zn(I)16. Genetic and physiological studies support a model in which this mobilized Zn(II) is important for growth under conditions of zinc limitation7. It is presently unknown whether Zn(II) exchange from the displaced L31 is spontaneous or whether it is facilitated by specific low-molecular-weight ligands or protein chaperones or by proteolysis. Regardless of mechanism, due to the high abundance of ribosomes in bacterial cells (~2–6 × 10^4 per cell depending on growth rate), it can be calculated that ribosomally associated Zn(II) represents a substantial fraction of total cell-associated Zn(II). The other protein induced as part of the early response to Zn(II) deprivation is Znt. Znt is a surface-associated lipoprotein hypothesized to function as an accessory factor for Zn(II) import10–12. It is somewhat surprising that Znt would be induced before the ZnuACB uptake system with which it presumably functions. It may be that Znt primes the cell for Zn(II) import by scavenging Zn(II), or Znt may function with other transporters.

As cellular zinc levels decline further, and Zn(II) mobilized from the ribosomal pool is consumed by continued protein synthesis, the middle genes are induced. These include the high-affinity Zn(II) ABC transporter, ZnuACB, and the putative metallochaperone YciC. The function of YciC is presently unclear, but this protein is representative of a highly conserved family of GTPases implicated as cofactors for metal insertion into target metalloproteins13. YciC may be involved in the intracellular trafficking of Zn(II), which likely becomes more critical when zinc is limiting for growth. One possible role of YciC is to prime the cell for Zn(II) uptake (ZinT), utilization by making sure that Zn(II) is delivered to those enzymes essential for growth. An analogous problem arises with respect to iron deprivation. In this case, B. subtilis activates an iron-sparing response involving the small RNA FsrA and accessory proteins PbpABC25. This system translationally represses numerous abundant iron-containing enzymes and complexes including succinate dehydrogenase, glutamate synthase and lactate dehydrogenase with the presumed function of enabling the limiting amounts of iron to be available for incorporation to more essential enzymes. An analogous iron-sparing response is present in E. coli mediated by the RyhB sRNA26,27.

The ability of the ZnuACB system to overcome Zn(II) limitation relies, of course, on the availability of extracellular zinc. As zinc levels fall further, the final two genes are induced, rpsNB and folEB. Repression of these genes is maintained at intermediate levels of intracellular Zn(II) by their unique ability to be efficiently repressed in vivo by the Zur–Zn_\text{II} form of Zur. The rpsNB gene encodes an S14 parologue (S14*). Unlike the L31 and L33 proteins, which are dispensable, S14 is essential and is required at an early stage of ribosome biogenesis. Thus, if cellular Zn(II) levels decline to levels that no longer support Zn(II) acquisition by S14 cells will be unable to assemble new ribosomes. The induction of rpsNB allows the synthesis of a replacement, Zn-independent S14* protein that enables new ribosome synthesis (a ‘failsafe’ pathway)18. Mutant strains lacking rpsNB can still grow under severe zinc limitation, but their inability to synthesize new ribosomes results in linear rather than exponential growth7, consistent with the prediction that each new daughter cell inherits only half the ribosome complement of its parent. Like rpsNB, folEB encodes a replacement function for a protein that fails when zinc levels are low. The FolE1 enzyme (product of folEA) encodes a Zn(II)-dependent GTP cyclohydrolase required for folate biosynthesis. The folEB product is a non-orthologous replacement that allows continued folate synthesis even under conditions of severe zinc depletion19.

In summary, our results indicate the transition from zinc sufficiency to zinc deprivation derepresses (i) early proteins for the mobilization Zn(II) from the surface of the ribosome (L31* and L33*) and to prime the cell for zinc uptake (ZinT), (ii) middle proteins for high-affinity zinc import (ZnuACB) and an accompanying metallochaperone (YciC), and (iii) late proteins to replace critical functions that fail as zinc levels decline further, RpsNB and FolE2. Numerous other sites in B. subtilis are thought to be associated with Zur in vivo, as judged by ChIP, but the significance of this extended regulon is not clear, and many sites did not appear to significantly affect gene expression18. A graded response to zinc deprivation is also likely to occur in other bacteria. Indeed, a graded response of Zur has been previously documented in Streptomyces coelicolor29, although the molecular basis for this response, and its physiological implications, were not clear. Similarly, the E. coli Zur regulon contains operator sites

---

**Table 1 | DNA-binding affinity of wild-type and mutant Zur proteins on target promoters.**

| Promoters | WT | C84S (site II) | H124A (site III) |
|-----------|----|---------------|-----------------|
| zinT      | 15.6 ± 3.5 | 75.5 ± 5.3  | 96.0 ± 1.3    |
| rpmEB     | 14.3 ± 2.6 | 77.9 ± 4.0  | 101.0 ± 2.3   |
| yciC      | 25.4 ± 1.1 | 80.6 ± 3.2  | 129.0 ± 3.8   |
| znuA      | 12.5 ± 4.0 | 73.0 ± 2.0  | 110.2 ± 3.0   |
| folEB     | 21.9 ± 3.9 | 28.6 ± 2.5  | 100 ± 4.0     |
| rpsNB     | 14.3 ± 2.5 | 16.5 ± 2.5  | 99.0 ± 1.6    |

*Average K_D values (± s.d., n = 3) against B.zur monomer. Shaded cells correspond to high-affinity interactions that correlate to in vivo repression. Each K_D value was calculated by fitting data (representative data are shown in Supplementary Fig. 6) to a Hill equation, using SigmaPlot 2001 software (SPSS Inc.).

1[Average K_D values (± s.d., n = 3) against B.zur monomer. Shaded cells correspond to high-affinity interactions that correlate to in vivo repression. Each K_D value was calculated by fitting data (representative data are shown in Supplementary Fig. 6) to a Hill equation, using SigmaPlot 2001 software (SPSS Inc.).

1[Average K_D values (± s.d., n = 3) against B.zur monomer. Shaded cells correspond to high-affinity interactions that correlate to in vivo repression. Each K_D value was calculated by fitting data (representative data are shown in Supplementary Fig. 6) to a Hill equation, using SigmaPlot 2001 software (SPSS Inc.).

1[Average K_D values (± s.d., n = 3) against B.zur monomer. Shaded cells correspond to high-affinity interactions that correlate to in vivo repression. Each K_D value was calculated by fitting data (representative data are shown in Supplementary Fig. 6) to a Hill equation, using SigmaPlot 2001 software (SPSS Inc.).

1[Average K_D values (± s.d., n = 3) against B.zur monomer. Shaded cells correspond to high-affinity interactions that correlate to in vivo repression. Each K_D value was calculated by fitting data (representative data are shown in Supplementary Fig. 6) to a Hill equation, using SigmaPlot 2001 software (SPSS Inc.).

1[Average K_D values (± s.d., n = 3) against B.zur monomer. Shaded cells correspond to high-affinity interactions that correlate to in vivo repression. Each K_D value was calculated by fitting data (representative data are shown in Supplementary Fig. 6) to a Hill equation, using SigmaPlot 2001 software (SPSS Inc.).
of widely varying affinities and this has been correlated with the overall magnitude of the derepression response but not yet with a defined temporal order of induction.

It is likely that graded responses are controlled by a combination of factors that ensure that genes are derepressed in an optimal order. Here we have documented the role of negative cooperativity in zinc binding to the two regulatory sites in each dimer in helping distinguish early from middle genes from late genes. However, our results have not yet provided a mechanism to explain the transition from early to middle genes, although this transition is also correlated with Zur operator occupancy (Fig. 2).

In the case of some other Fur homologues (including B. subtilis Fur, S. coelicolor Zur and Magnetospirillum gryphiswaldense Fur) a second regulatory site within each monomer (site 3) may serve to fine-tune operator binding, with some sites requiring occupancy of this additional site and others not. An added level of complexity results from cooperativity in binding of multiple dimers to a single regulatory region. Although Fur proteins were originally proposed to bind to a 19 bp Fur box recognition sequence, subsequent studies demonstrated that each a minimal Fur-binding site is a 7–1–7 inverted repeat and the classic 19 bp Fur box represents two overlapping repeats that can bind a dimer of dimers.

In the case of some other Fur homologues (including B. subtilis Fur, S. coelicolor Zur and Magnetospirillum gryphiswaldense Fur) a second regulatory site within each monomer (site 3) may serve to fine-tune operator binding, with some sites requiring occupancy of this additional site and others not. An added level of complexity results from cooperativity in binding of multiple dimers to a single regulatory region. Although Fur proteins were originally proposed to bind to a 19 bp Fur box recognition sequence, subsequent studies demonstrated that each a minimal Fur-binding site is a 7–1–7 inverted repeat and the classic 19 bp Fur box represents two overlapping repeats that can bind a dimer of dimers. Other Fur family members likely share a similar architecture, at least at some operators. Recent structural studies have highlighted the role of electrostatic interactions between the two dimers in cooperative binding by E. coli Zur and M. gryphiswaldense Fur. Thus, another possible mechanism to impart a preferred order for derepression is a variable number of dimers or a variable level of cooperativity between dimers at a particular operator region. Indeed, in the B. subtilis Fur regulon, some operons are regulated by a single 7–1–7 repeat and the classic 19 bp Fur box represents two overlapping repeats that can bind a single Fur dimer, whereas other operons, such as dhh, are tightly repressed by a dimer of dimers.

The idea of a graded response occurring as a function of increasing levels of a functional transcription factor is well preceded, but in only a minority of cases is the underlying logic apparent. One notable example is the regulation of gene expression in response to declining nutrient availability by the Spo0A transcription factor in B. subtilis. This is a complex system, involving multiple kinases, phosphotransfer proteins and
phosphatases that integrates a variety of stress signals. As the level of the active Spo0A → P transcription factor increases, cells are proposed to sequentially activate a motility response (sliding), biofilm formation and ultimately they enter into sporulation28. In this and related systems, a remaining challenge is to understand the molecular basis of this sequential gene regulation that may involve different forms of the transcription factor and variable affinities of activated factor for its operator sites, the molecular basis of this sequential gene regulation that may involve different forms of the transcription factor and variable affinities of activated factor for its operator sites.

Methods

**Bacterial strains and culture conditions.** All *B. subtilis* strains used in this study were isogenic with common laboratory strains listed in Supplementary Table 1. *B. subtilis CUI065* was grown on LB medium and modified glucose minimal medium (20 g l\(^{-1}\) (NH\(_4\))\(_2\)SO\(_4\), 183 g l\(^{-1}\) K\(_2\)HPO\(_4\), 3H\(_2\)O, 60 g l\(^{-1}\) KH\(_2\)PO\(_4\), 2 g l\(^{-1}\) MgSO\(_4\)*\(_7\)H\(_2\)O, 10 g l\(^{-1}\) sodium citrate, 0.5% glucose, 0.5 mM CaCl\(_2\) and 5 mM MnCl\(_2\)) was used for PY79 at 37 °C. When appropriate, antibiotics were included at: 100 µg ml\(^{-1}\) of lincomycin, 5 µg ml\(^{-1}\) of chloramphenicol, 10 µg ml\(^{-1}\) of kanamycin, 5 µg ml\(^{-1}\) of tetracyclin and 1 µg ml\(^{-1}\) of erythromycin plus 25 µg ml\(^{-1}\) lincomycin for the selection of various *B. subtilis* strains. DNA was transformed into *B. subtilis* using a modified version of a previously published protocol27. *E. coli* DH5x was used for routine DNA cloning28. Unless indicated otherwise, liquid media were inoculated from an overnight pre-culture and incubated at 37 °C with shaking at 200 r.p.m.

**Preparation of total RNA.** Total RNA was isolated from *B. subtilis* strains that were cultured to mid-logarithmic phase (at an OD\(_{600}\) of 0.4–0.5) in LB medium. For Zn(II)-depleted condition, various amounts of TPEN were treated for 5 or 40 min. Total RNA was extracted by the ‘hot phenol method’ as described29. The total amount of RNA in its quality were measured by absorbance spectroscopy and confirmed by resolving RNA samples on 1.3% formaldehyde agarose gels.

**S1 nuclease mapping analysis.** Gene-specific DNA oligonucleotide probes for zur, zinT, znaA, yciC, folEB, rpsNB and rpmE transcripts were used for PCR amplification using the wild-type genome as template. Together, appropriate primer pairs are listed in Supplementary Table 2. An amount of 100 µg of total RNA was pelleted and liophylized. Each specific DNA probe was radiolabelled with (γ-32P) ATP and T4 polynucleotide kinase, and 30,000–40,000 c.p.m. of labelled probe was used in each reaction. The total RNA pellet was carefully resuspended in 400 µl of 7 M urea. The dried gels were exposed to a phosphor imaging screen (Typhoon FLA 7000; GE) and bands were quantified using Multi Gauge software. gels containing 7 M urea. The dried gels were exposed to a phosphor imaging screen (Typhoon FLA 7000; GE) and bands were quantified using Multi Gauge software.

**Quantitative in vivo crosslinking and immunoprecipitation.** In vivo crosslinking of DNA to Zur proteins and subsequent immunoprecipitation with specific antibody to FLAG were carried out. Cells were grown in LB medium to mid-logarithmic phase (at an OD\(_{600}\) of 0.5–0.6) and each 30 ml aliquots were spun down and then treated at 80 °C for 10 min. For experimental control, reduced and fully denatured protein samples were prepared by sonication and ultra-centrifugation at 20,000 g for 30 min. Cell extracts were loaded onto a nickel-charged NTA column and then washed with six volumes of binding buffer followed by six volumes of washing buffer (20 mM Tris–HCl (pH 7.9), 0.5 M NaCl and 10 mM imidazole). Zur was eluted with 10 volumes of elution buffer (20 mM Tris–HCl (pH 7.9) and 0.5 M NaCl) containing linear imidazole gradients from 20 to 300 mM. Fractions containing Zur proteins were pooled and dialysed against buffer A (20 mM Tris–HCl (pH 7.8), 250 mM NaCl, 5% (vol/vol) glycerol and 4 mM EDTA) to remove imidazole and nickel. For holoto Zur proteins, dialysed proteins in buffer A were further dialysed against buffer B (20 mM Tris–HCl (pH 7.8), 100 mM NaCl, 10% glycerol and 0.2 mM dithiothreitol (DTT)) and buffer C (20 mM Tris–HCl (pH 7.8), 50 mM NaCl, 1 M MgCl\(_2\) and 25 mM ZnSO\(_4\)). The purified proteins were concentrated by centrifugal filter devices (Millipore, 3,000 MW CO) before injection onto High load TM (16/60) pg Superdex G75 column in FPLC system (Pharmacia). The column was equilibrated with buffer G (20 mM Tris–HCl (pH 7.8), 50 mM NaCl, 2 mM DTT and 0.1 mM ZnSO\(_4\)) so that the final buffer consisted of EMGA. Eluted fractions were monitored through ultraviolet detector. Concentrations of purified wild-type Zur and variant proteins were estimated in triplicates by Bradford assay (Bio-Rad) using bovine serum albumin (Sigma-Aldrich) as the calibration standard at A595. Measurement of ultraviolet absorbance at 280 nm, combined with calculated molar extinction coefficient (ε\(_{280}\) = 765 M\(^{-1}\) cm\(^{-1}\); http://expasy.org/cgi-bin/protparam), gave nearly identical values. The purity of the purified proteins was confirmed through three-dimensional blue staining of loaded protein samples in the SDS–polyacrylamide gel electrophoresis (SDS–PAGE) gel. The protein was stored in final storage buffer S (20 mM Tris–HCl (pH 7.8), 50 mM NaCl, 2 mM DTT, 30% glycerol and 0.1 mM ZnSO\(_4\)) at −80 °C. To avoid metal contamination, all buffers were prepared through chexel-100 column (Bio-Rad).

**Gel-based dimerization assay.** To confirm oligomeric forms of purified wild-type and mutant Zur proteins in solution1, total 7 µg of purified Zur proteins were loaded on the native non-reducing gel and visualized by the coomassie blue staining method. To keep multicellular forms of protein, non-boiled protein samples were loaded after mixed with non-reducing loading buffer (no DTT or β-mer). For fully reduced and denatured proteins, reducing buffer containing 140 mM β-mer and 2% SDS was added and then boiled at 95 °C for 10 min. For experimental control, the non-reducing protein samples were loaded on to 13% SDS–PAGE after being mixed with the same amount of non-boiled proteins40.

**Purification of BzZur wild-type and mutant proteins.** Wild-type, C84S, C95S and H124A mutant Zur proteins were purified from *E. coli* BL21 (DE3) cells containing pET3a-based recombinant plasmid. The coding sequence for WT BzZur was cloned into pET3a (Novagen) between the NdeI and BamHI restriction sites. For BzZur mutants, plasmids were constructed using quick change site-directed mutagenesis and transformed into *E. coli* strain BL21 (DE3/pLYS). For the purification of Zur, an overnight culture from a single colony was used to inoculate 1 liter of LB medium. Cells were grown with vigorous shaking at 37 °C to an optical density at 600 nm (OD\(_{600}\)) of 0.5 and were induced with 1 mM isopropylβ-D-thiogalactopyranoside with 25 mM ZnSO\(_4\) for 6 h at 30 °C. Collected cells were resuspended with binding buffer (20 mM Tris–HCl (pH 7.9), 0.5 M NaCl, 1 mM TCEP (tris(2-carboxyethyl)-phosphine) and 5 mM imidazole) and cell extracts were prepared by sonication and ultra-centrifugation at 20,000 g for 30 min. Cell extracts were loaded onto a nickel-charged NTA column and then washed with six volumes of binding buffer followed by six volumes of washing buffer (20 mM Tris–HCl (pH 7.9), 0.5 M NaCl and 10 mM imidazole). Zur was eluted with 10 volumes of elution buffer (20 mM Tris–HCl (pH 7.9) and 0.5 M NaCl) containing linear imidazole gradients from 20 to 300 mM. Fractions containing Zur proteins were pooled and dialysed against buffer A (20 mM Tris–HCl (pH 7.8), 250 mM NaCl, 5% (vol/vol) glycerol and 4 mM EDTA) to remove imidazole and nickel. For holoto Zur proteins, dialysed proteins in buffer A were further dialysed against buffer B (20 mM Tris–HCl (pH 7.8), 100 mM NaCl, 10% glycerol and 0.2 mM dithiothreitol (DTT)) and buffer C (20 mM Tris–HCl (pH 7.8), 50 mM NaCl, 1 M MgCl\(_2\) and 25 mM ZnSO\(_4\)). The purified protein was concentrated by centrifugal filter devices (Millipore, 3,000 MW CO) before injection onto High load TM (16/60) pg Superdex G75 column in FPLC system (Pharmacia). The column was equilibrated with buffer G (20 mM Tris–HCl (pH 7.8), 50 mM NaCl, 2 mM DTT and 0.1 mM ZnSO\(_4\)) so that the final buffer consisted of EMGA. Eluted fractions were monitored through ultraviolet detector. Concentrations of purified wild-type Zur and variant proteins were estimated in triplicates by Bradford assay (Bio-Rad) using bovine serum albumin (Sigma-Aldrich) as the calibration standard at A595. Measurement of ultraviolet absorbance at 280 nm, combined with calculated molar extinction coefficient (ε\(_{280}\) = 765 M\(^{-1}\) cm\(^{-1}\); http://expasy.org/cgi-bin/protparam), gave nearly identical values. The purity of the purified proteins was confirmed through three-dimensional blue staining of loaded protein samples in the SDS–polyacrylamide gel electrophoresis (SDS–PAGE) gel. The protein was stored in final storage buffer S (20 mM Tris–HCl (pH 7.8), 50 mM NaCl, 2 mM DTT, 30% glycerol and 0.1 mM ZnSO\(_4\)) at −80 °C. To avoid metal contamination, all buffers were prepared through chexel-100 column (Bio-Rad).
Detection of Zn(II) by PAR staining assay. To check Zn(II) occupancy in multimeric forms of Zur protein, resolved non-reducing SDS–PAGE gel was soaked in buffer ZZ (20 mM Tris–HCl (pH 7.8), 50 mM NaCl and 5% glycerol) containing final 10 mM 4-(2-pyridylazo) resorcinol (PAR) for 20 min after washing with Milli-Q water two times. To release of Zn(II) ions from coordinating ligands, final 50 mM H$_2$O$_2$ was added and incubated for 20 min. PAR-stained images were taken on the white light every minute since the addition of H$_2$O$_2$.

Electrophoretic mobility shift assays. Each Zur promoter DNA probes of ~85 bp containing Zur-binding sites were isolated using crude and soaking method from the polycrylamide gel after annealing with each primer pairs in Supplementary Table 2. The purified DNAs were labelled at 5'-ends with (γ-32P) ATP using T4 polynucleotide kinase. Binding reactions were performed with ~1 fmol of labelled DNA fragments and 0.075–0.16 nM of purified Zur proteins in 20 μl of the reaction buffer (20 mM Tris–HCl (pH 6.4), 50 mM KCl, 1 mM DTT, 0.1 mg of bovine serum albumin per ml, 5% glycerol and 0.1 μg of poly(dI-dC), with 0.1 μM ZnSO$_4$). Following incubation at room temperature for 20 min, the binding mixture was subjected to electrophoresis at 4°C on a 5% polyacrylamide gel in TA (pH 6.4) buffer. After electrophoresis, the dried gels were exposed and quantified by a phosphor image analyser (Typhoon FLA 7000). A band intensity of unbound DNA probes was measured against Zur concentration using Multi Gauge V3.0 software. Digitalized data were fitted to binding curves through SigmaPlot 2001 program (SPSS Inc.). Apparent $K_d$ values, corresponding to the concentration of variables (Zur) at half-maximal upshift of DNA probes, were determined from at least three independent sets of experiments.

Construction of fluorescence fusion strains. For construction of reporter fusions between the representative Zur target promoters corresponding to groups with differential Zn(II) sensitivity and the three different fluorescent protein encoding genes (ecfp, egfp and mCherry), each promoter region was amplified by PCR using DNA oligonucleotides shown in Supplementary Table 2. To integrate at different loci, the PCR products were digested with Xmal restriction enzyme and ligated into three different integration vectors digested by same restriction enzyme. Constructs were confirmed by Sanger sequencing and transformed into B. subtilis PT79. For selection of correctly integrated strains, appropriate antibiotics were used and candidates were verified using diagnostic genomic DNA PCR and Sanger sequencing of PCR products.

Fluorescence spectroscopy. For measurement of fluorescence intensity from reporter fusion strains, fresh cultured cells were used to inoculate 5 ml of modified glucose minimal media. That was cultured overnight while shaking at 37°C on a shaker. Next day, 1 ml of the reaction buffer (20 mM Tris–HCl (pH 6.4), 50 mM KCl, 1 mM DTT, 0.1 mg of bovine serum albumin per ml, 5% glycerol and 0.1 μg of poly(dI-dC), with 0.1 μM ZnSO$_4$) containing final 10 mM 4-(2-pyridylazo)resorcinol (PAR) for 20 min after releasing Zn(II) ions from coordinating ligands, final 50 mM H$_2$O$_2$ was added and incubated for 20 min. PAR-stained images were taken on the white light every minute since the addition of H$_2$O$_2$. That was cultured overnight while shaking at 37°C on a shaker. Cells were prepared under the same condition, as described in Fig. 2 for Supplementary Fig. 3d or Fig. 3b for Supplementary Fig. 3e. The cell pellet was resuspended in 0.5 ml of PBS buffer (pH 7.4). Fluorescence intensity of each 1 ml sample was taken with a Perkin-Elmer LS55 luminescence spectrometer.

Western blot analysis of BsZur-FLAG. Cells were prepared under the same condition, as described in Fig. 2 for Supplementary Fig. 3d or Fig. 3b for Supplementary Fig. 3e. The cell pellet was resuspended in 0.5 ml of PBS buffer and lysed by sonication. The cell lysates were removed by centrifugation and the resulting supernatant was mixed with SDS–PAGE loading buffer, and boiled at 95°C for 10 min before being resolved by 13% SDS–PAGE. The proteins were transferred to a membrane at 60 mA for 40 min. The membrane was then blocked with blocking solution (Dhy Milk dissolved in 20 mM Tris–HCl (pH 7.8), 130 mM NaCl and 0.1% Triton X-100) for overnight. The membrane was incubated with a 1:10,000 dilution of polyclonal anti-FLAG Antibody (Sigma Chemical Co., SAB4301135) or 1h, washed with TBST (20 mM Tris, 150 mM NaCl and 0.1% Triton X-100), and incubated with 1:5,000 dilution of alkaline phosphatase antibody conjugated with alkaline phosphatase (Santa Cruz Biotech, SC-2004) for 1h. The membrane was then washed with 5 ml AP buffer (100 mM Tris-TCl (pH 9.5), 100 mM NaCl and 5 mM MgCl$_2$), 1:100 dilution of NBT, and BCIP. The Zur-FLAG protein has a molecular weight of ~17kDa, in agreement with the observed mobility.

Data availability. The authors declare that the data supporting the findings of this study are available within the article and its Supplementary Information files, or from the corresponding author on request.
Acknowledgements

We thank Dr Peng Chen (Cornell Univ.) for helpful insights and Dr Xiaojuan Huang (Cornell Univ.) for information regarding the level of Zn(II) in our LB medium. Research reported in this publication was supported by the National Institute of General Medical Sciences under award numbers R01GM059323 (to J.D.H.) and R01GM109993 (to Peng Chen and J.D.H.).

Author contributions

J.H.S. performed all the experiments and data analysis, and wrote the paper. J.D.H. contributed to the data analysis and wrote the paper. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Additional information

Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Shin, J.-H. & Helmann, J. D. Molecular logic of the Zur-regulated zinc deprivation response in Bacillus subtilis. Nat. Commun. 7:12612 doi: 10.1038/ncomms12612 (2016).

© The Author(s) 2016

This work is licensed under a Creative Commons Attribution 4.0 International License. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder to reproduce the material. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/