Concentration- and chromosome-organization-dependent regulator unbinding from DNA for transcription regulation in living cells

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Binding and unbinding of transcription regulators at operator sites constitute a primary mechanism for gene regulation. While many cellular factors are known to regulate their binding, little is known on how cells can modulate their unbinding for regulation. Using nanometer-precision single-molecule tracking, we study the unbinding kinetics from DNA of two metal-sensing transcription regulators in living Escherichia coli cells. We find that they show unusual concentration-dependent unbinding kinetics from chromosomal recognition sites in both their apo and holo forms. Unexpectedly, their unbinding kinetics further varies with the extent of chromosome condensation, and more surprisingly, varies in opposite ways for their apo-repressor versus holo-activator forms. These findings suggest likely broadly relevant mechanisms for facile switching between transcription activation and deactivation in vivo and in coordinating transcription regulation of resistance genes with the cell cycle.
binding and unbinding of transcription regulators at operator sites constitutes a primary mechanism for regulating gene expression, and they are often rate-determining for regulatory responses. For binding to an operator site, its rate is readily modulated by many cellular factors such as regulators’ cellular concentration and the chromosome organization. For unbinding from an operator site, it is unclear whether these factors can modulate its rate for regulation. At least, regulator unbinding from an operator site on DNA is presumed to be a unimolecular reaction and thus independent of regulators’ cellular concentration. Surprisingly, recent in vitro studies revealed that CueR, a Cu\(^{+}\)-sensing efflux regulator, can undergo assisted dissociation and direct substitution processes on its cognate DNA; both lead to its concentration-dependent unbinding rate from a recognition site on DNA and may facilitate CueR in deactivating transcription. This unusual concentration-dependent unbinding was also reported recently for nonspecific chromosomal organization proteins, DNA polymerase, and a single-strand-DNA-binding protein, all functionally unrelated to CueR. Moreover, using force to apply tension, which changes DNA conformation, can modulate protein unbinding kinetics from DNA.

Despite the above discoveries in vitro, it remains unknown whether concentration- and DNA-conformation-dependent protein unbinding from DNA is relevant in vivo. Here using stroboscopic single-molecule tracking (SMT) to image protein–DNA interactions in real time and at nanometer spatial resolution, we study the quantitative DNA interaction kinetics of CueR and its Zn\(^{2+}\)-sensing homologue ZntR in living Escherichia coli (E. coli) cells. CueR and ZntR, both belonging to the MerR-family regulators, bind tightly to their respective dyad-symmetry recognition sequences within \(\sigma^{70}\)-dependent suboptimal promoters, either repressing or, with Cu\(^{+}\) or Zn\(^{2+}\) bound (\(10^{-21} \) M affinity and \(10^{-15} \) M affinity, respectively), activating the transcription of efflux genes to defend against metal stress. We find that in living E. coli cells, CueR and ZntR show striking concentration-dependent unbinding kinetics from chromosomal recognition sites in both their apo and holohormones. Unexpectedly, their unbinding kinetics further varies with the extent of chromosome condensation, and more surprisingly, varies in opposite ways for apo-repressor versus holomediator forms. These findings suggest novel mechanisms for facilitating transcription deactivation and activation in vivo and in coordinating transcription regulation of resistance genes with the cell cycle, which are likely broadly relevant for gene regulation.

Results

Concentration-dependent regulator residence time. To visualize CueR or ZntR in living cells, we made its functional fusion with the photoconvertible fluorescent protein mEos3.2 (refs 22,23; that is, CueR\(^{\text{mE}}\) or ZntR\(^{\text{mE}}\)) at its chromosomal locus as well as a plasmid for varying its cellular concentration (Methods and Supplementary Note 2). We used time-lapse stroboscopic imaging to track the positions over time of single photoconverted mEos3.2-tagged proteins in a cell at tens of nanometer precision until their mEos3.2 tags photobleached (Fig. 1a; Methods and Supplementary Note 5.2). For each tracked protein molecule, we obtained its time trajectory of displacement \(r\) per time-lapse (that is, the distance the protein molecule travelled between two consecutive images) (Fig. 1a,b). This SMT approach, along with single-cell total fluorescence counting, also enabled us to quantify the copy number of CueR or ZntR in each cell (Methods and Supplementary Note 5.3). By sorting the cells into groups of similar cellular protein concentrations, we could analyse protein-concentration-dependent processes without being limited by the large cell-to-cell heterogeneity in protein expression levels (Supplementary Note 5.3).

We first examined CueR\(^{\text{apo}}\) and ZntR\(^{\text{apo}}\), whose metal-binding cysteines were mutated to make them permanently locked in the apo-repressor forms (that is, C112S for CueR\(^{\text{apo}}\) and C115S for ZntR\(^{\text{apo}}\)) from ~450 and 250 cells containing a total of ~12,000 and 10,000 molecules, respectively. The solid lines are empirical fits with \(r = (c0 \times P_{\text{apo}} + b) \times 10^7\) (Supplementary Note 8), except for mEos3,2, for which the line is a horizontal eye guide. x, y error bars are s.d. and s.e.m., respectively. Relevant data for ZntR\(^{\text{apoE}}\) and ZntR\(^{\text{apoZn}}\) are in Supplementary Figs 16 and 19.

Figure 1 | Protein-concentration-dependent residence time of CueR and ZntR on chromosome from stroboscopic single-molecule tracking in living E. coli cells. (a) Position trajectory of a CueR\(^{\text{apo}}\) molecule in a living cell. Dash line is the cell boundary. Zoom-in insets: locations (that is, residence sites) associated with the two residence times in b. Displacement \(r\) per time-lapse is the distance the molecule travelled between two consecutive images as shown in the \(t_2\) inset. Scale bars in a and in the insets are 500 nm and 80 nm, respectively. (b) Dispersion \(r\) per time-lapse \((T_{\text{avg}} = 60 \mu\text{s})\) versus time trajectory for the molecule in a. \(t_1\) and \(t_2\), whose lengths are denoted by grey shades and double-headed arrows, are two microscopic residence times thresholded by \(r_0 = 220 \mu\text{m}\) (horizontal red dashed line). (c) Correlation of average residence time \(\tau\) and total protein concentration in each cell for CueR\(^{\text{apo}}\) and CueR\(^{\text{apoZn}}\) from ~450 and 250 cells containing a total of ~12,000 and 10,000 molecules, respectively. (d) Dependence of \(\tau\) on cellular protein concentration for CueR\(^{\text{apoE}}\), CueR\(^{\text{apoZn}}\) or free mEos3.2 as a control. \(\tau\) of individual cells from c are grouped every ~150 nM along the x-axis by their cellular protein concentrations and averaged within each group. Note 1 nM corresponds approximately to one protein molecule per cell volume (about 1.5 fL). The solid lines are empirical fits with \(\tau = (c0 \times P_{\text{apo}} + b) \times 10^7\) (Supplementary Note 8), except for mEos3,2, for which the line is a horizontal eye guide. x, y error bars are s.d. and s.e.m., respectively. Relevant data for ZntR\(^{\text{apoE}}\) and ZntR\(^{\text{apoZn}}\) are in Supplementary Figs 16 and 19.
site. Each microscopic residence time $\tau$ starts when $r$ drops below $r_0$ and ends when $r$ jumps above $r_0$ (for example, $\tau_1$ in Fig. 1b) or when the mEos3.2-tag photobleaches/blinks (for example, $\tau_2$ in Fig. 1b). By measuring many individual $r$‘s from a large number of single-molecule displacement trajectories, we obtained the average residence time $\langle \tau \rangle$ for each cell, whose cellular concentration of CueR or ZntR (or ZntRmE) was also determined (Fig. 1c).

Strikingly, the average residence time $\langle \tau \rangle$ decreases with increasing cellular concentration of CueRmE or ZntRmE (Fig. 1c,d). This trend is independent of the value of the thresholding $r_0$ and persists after correcting for the contribution of mEos3.2 photobleaching/blinking kinetics (Supplementary Note 8). Free mEos3.2, which does not bind to DNA and exhibits much shorter apparent $\langle \tau \rangle$, does not show this trend in the cell (Fig. 1d).

We next examined un-mutated CueRmE and ZntRmE in cells grown with $100 \mu M$ Cu$^2+$ or Zn$^2+$ in the medium (that is, CueRmCu and ZntRmZn). These metal concentrations cause maximal induction of the cueR or zntR regulons in the cell$^{30,31}$, and the regulators are largely metallated. Their $\langle \tau \rangle$‘s show differences from those of CueRmE apo and ZntRmE apo, indicating that we indeed observe the behaviour of holo-regulators (Fig. 1c,d). Importantly, their average residence times on chromosome still exhibit a decreasing trend with increasing cellular protein concentrations (Fig. 1c,d). Therefore, for both apo and holo forms of CueR or ZntR, higher cellular protein concentrations shorten their average residence times on chromosome, suggesting concentration-enhanced protein unbinding kinetics from DNA.

**Concentration-enhanced regulator unbinding.** To quantify the variable motions of individual CueR or ZntR molecules in a cell, we determined their cumulative distribution functions (CDFs) of displacement $r$ (Fig. 2a)$^{32}$. Regardless of the metallation state of CueR or ZntR, global analysis of CDFs across all cellular protein concentrations resolves minimally three diffusion states with effective diffusion constants of $\sim 3.7 \mu m^2 s^{-1}$, $0.7 \mu m^2 s^{-1}$ and $0.04 \mu m^2 s^{-1}$ (referred to as $D_{FD}$, $D_{NB}$ and $D_{SB}$, respectively; Methods and Supplementary Note 11). No subcellular localization or protein aggregation was observed, and hence these two are not the reasons for the presence of the resolved three diffusion states (Supplementary Note 13). We assigned these three diffusion states as proteins that are: freely diffusing (FD) in the cytoplasm; nonspecifically bound (NB) to and moving on chromosome (the contributions from nonspecific interactions with the plasmids in the cell is $<7\%$ and thus negligible; Supplementary Note 20.5); and specifically bound (SB) to chromosomal recognition sites, whose slow motions reflect the chromosome dynamics (and measurement uncertainties). Both experimental evidences and simulations (details in Supplementary Notes 12 and 15) support these assignments: (1) CueR or ZntR can bind to DNA specifically and nonspecifically$^{12,21,33,34}$. (2) The three effective D’s are

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**Figure 2** | **Protein-concentration-dependent unbinding of CueR and ZntR from recognition sites.** (a) Cumulative-distribution-function (CDF) of displacement $r$ (plotted against $r^2/4D_t$) of CueRmE apo at [CueRmE apo $_{\text{cell}}$] = 180 ± 34 nM; fitting (black line) with three diffusion states gives effective diffusion constants (and fractional populations) (equation (1)): $D_{FD} = 3.7 ± 0.2 \mu m^2 s^{-1}$ (23.2 ± 0.3%), $D_{NB} = 0.70 ± 0.03 \mu m^2 s^{-1}$ (49.2 ± 0.4%) and $D_{SB} = 0.036 ± 0.009 \mu m^2 s^{-1}$ (26.7 ± 0.5%), which are also plotted individually. (b) Histogram of displacement $r$ and the corresponding resolved FD, NB and SB states as in a. The solid lines are the overall probability density function (PDF) (black; equation (2)) and the resolved three components of $r$ (red, green and blue as in a), all multiplied by a scaling factor to account for the actual number of measured displacements. The vertical red dash line denotes $r_0 = 220 \mu m$, as in Fig. 1b. (c) Protein-concentration-dependent fractional populations ($A_k$, in percentage) of FD, NB and SB states for CueRmE apo. For example, $A_{SB}$ is equivalent to (number of proteins bound at recognition sites)/(total number of proteins in the cell). Data for ZntR are summarized in Supplementary Tables 4 and 5. (d) Fractional populations of the SB state ($A_{SB}$) for CueRmE and ZntRmE in wild-type (WT) or promoter knockout strains at cellular protein concentrations of $\sim 100 \mu M$. (e) Kinetic model for regulator – DNA interactions in a cell. The direct interconversions between NB and SB states are assumed to be negligible, but indicated (grey arrows). Parameters are defined in detail in Methods and Supplementary Fig. 29. (f) Histogram in log scale of residence time $\tau$ for CueRmE apo at [CueRmE apo $_{\text{cell}}$] = 180 nM; fitting with a three-state kinetic model (black line, equation (6)) gives the apparent unbinding constant ($k_{d}^{\text{app}}$) from recognition sites. Contribution from each state is also plotted. (g) Dependences of $k_{d}^{\text{app}}$ on free protein concentration ($C = A_{FD}[P]_{\text{cell}}$) in cells for CueRmE apo and ZntRmE apo, and for CueRmE apo and ZntRmE apo. Lines are fits with $k_{d}^{\text{app}} = k_{d}[P]_{FD} + k_{-1}$ (equation (8)). All error bars are s.d.
consistent with reported values for proteins freely diffusing in the cytoplasm, nonspecifically bound or specifically bound to chromosome$^{2,3,25,28,32,35}$ when the cell confinement effect$^{24}$ and the time-lapse effect of imaging$^{32}$ are both taken into account. Note that the magnitude of the effective diffusion constant $D_{FD}$ here is smaller than that measured for free diffusing protein in vitro$^{36}$; this apparent difference results from the cell confinement effect (Supplementary Note 15.2 and Supplementary Figs 35 and 36). (3) Control experiments on tracking free mEos3.2 in cells with variable time lapses show similar effective diffusion constants as the FD state (Supplementary Fig. 23). (4) Simulations of molecular diffusion in confined cell geometries. (5) Short-time movement analysis for the SB state agrees with the literature reported chromosome diffusion constant (Supplementary Fig. 26).  

Moreover, the resolved CDF also gives the fractional populations (that is, percentages) of the CueR (or ZntR) in the FD, NB and SB states among all CueR (or ZntR) protein molecules in the cell. With increasing cellular protein concentrations, the fractional populations of $D_{FD}$ and $D_{NB}$ increase, while that of $D_{SB}$ decreases (Fig. 2c); these trends are consistent with that at higher protein concentrations, each protein molecule will spend more time freely diffusing or nonspecifically bound to the chromosome than specifically bound at recognition sites, as more protein molecules compete for the limited number of recognition sites. Deleting the cueO promoter ($\Delta P_{cueO}$), one of the many CueR operator sites, expectedly did not cause much perturbation, including CueR$^{\text{apo}}$'s fractional population at the SB state (Fig. 2d and Supplementary Note 18.3). In contrast, deleting the zntA promoter ($\Delta P_{zntA}$), the only known ZntR operator site in E. coli, did decrease, but surprisingly did not abolish, ZntR$^{\text{apo}}$'s SB state (Fig. 2d and Supplementary Note 18.3). This observation motivated us to identify many additional possible ZntR recognition sites in the E. coli genome (Supplementary Note 22.4).  

The resolved CDF of $r$ concurrently gives the corresponding resolved probability density function (PDF) of $r$ (Fig. 2b), in which the three resolved peaks correspond to the FD, NB and SB states. The resolution of these three states in this PDF of $r$ immediately justifies the $r_0$ value that thresholds the displacement-versus-time trajectories—for $r$ smaller than $r_0 \approx 220 \text{ nm}$, >99% of SB states of CueR (or ZntR) are included, and the thresholded residence times $\tau$ are thus dominated by contributions from proteins specifically bound at chromosomal recognition sites (and those nonspecifically bound to chromosome at higher cellular protein concentrations), with very little contribution from freely diffusing proteins in the cytoplasm. To quantitatively deconvolute all possible contributions to $\tau$, we formulated a minimal mechanistic model comprising the FD, NB and SB states (Fig. 2e and Supplementary Note 14). This model enabled us to analyse the distribution of $\tau$ to obtain $k_{\text{on,app}}$, the apparent unbinding rate constant from recognition sites (Fig. 2f), as well as the fractional populations of different states to obtain other kinetic constants for protein binding and unbinding at recognition sites and nonspecific DNA sites (Methods, Table 1, and Supplementary Tables 7 and 8).  

Strikingly, $k_{\text{on,app}}$, the apparent unbinding rate constant from recognition sites, increases linearly with increasing cellular concentrations of the free (or total) CueR$^{\text{apo}}$ or ZntR$^{\text{apo}}$ (Fig. 2g), in contrast to unimolecular reaction kinetics of protein unbinding from DNA where the unbinding rate constant is independent of free protein concentration. Linear fitting of $k_{\text{on,app}}$ versus free protein concentration gives the slope $k_0$ and intercept $k_{-1}$, the facilitated (second order) and spontaneous (first order) unbinding rate constants from recognition sites, respectively. For CueR$^{\text{apo}}$ or ZntR$^{\text{apo}}$, the slopes are even steeper (Fig. 2g). Therefore, both apo and holo forms of CueR and ZntR have concentration-dependent unbinding kinetics from chromosomal recognition sites, where the dependence likely results from the assisted dissociation or direct substitution mechanism that we discovered for CueR $-$ DNA interactions in vitro$^{12}$ (see Discussion and Supplementary Fig. 41b and Supplementary Note 18.2)$^{15,37-40}$.  

### Different extents of chromosome condensation among cells.

We next examined the spatial distribution in a cell of the residence sites associated with the residence times of CueR and ZntR, which are dominated by their binding to chromosomal recognition sites and nonspecific sites (Fig. 1a insets). This distribution can reflect the chromosome organization$^{41}$ because: (1) The recognition sites of CueR and ZntR spread across the chromosome randomly (Supplementary Note 22.3), (2) their nonspecific binding sites are expected to scatter across the chromosome randomly as well (Supplementary Note 20.6) and (3) contributions from nonspecific binding to the plasmids in the cell are negligible (<7%; Supplementary Note 20.5).  

In some cells, these residence sites localize to a small region (Fig. 3a), reflecting that in these cells the chromosomes are highly condensed. In contrast, in some other cells, the residence sites spread over the cell (Fig. 3b), reflecting that the cells’ chromosomes are less condensed. We further directly imaged the Hoechst-dye-stained chromosomes in the cells. Consistently, some cells have compact chromosomes (Fig. 3d), while others have their chromosomes spread over the entire cell volume (Fig. 3e), reflecting again the different extents of chromosome condensation among the individual cells.  

To quantify the extent of chromosome condensation for each cell, we computed the average pairwise distance ($d_{ij}$) between the

| Table 1 | Kinetic and thermodynamic parameters for CueR$^\text{apo}$ – DNA interactions in E. coli cells$^\text{a}$. |
|-----------------|-----------------|-----------------|
| Parameters      | CueR$^\text{apo}$ | CueR$^\text{apo}$ | CueR$^\text{apo}$ (dividing cells) |
| $k_0$ ($\mu M^{-1}s^{-1}$) | 214 ± 6 | 54 ± 96 | 219 ± 77 |
| $k_1$ ($s^{-1}$) | 8.2 ± 0.9 | 4.6 ± 0.8 | 6.4 ± 1.1 |
| $K_D$ (mM) | 31.9 ± 6.9 | 55.0 ± 8.5 | 20.1 ± 8.7 |
| $f_+$ | 0.037 ± 0.028 | 0.038 ± 0.058 | 0.029 ± 0.011 |
| $f_-$ | 3.6 ± 1.9 | 4.9 ± 3.8 | 1.6 ± 6.1 |
| $f_{-2}$ ($s^{-1}$) | 2.5 ± 0.1 | 4.1 ± 0.1 | 2.6 ± 0.2 |
| $K_{D2}$ (mM) | 0.69 ± 0.38 | 0.83 ± 0.65 | 1.6 ± 6.3 |
| $K_{D3}$ (mM) | 0.06 ± 0.03 | 0.04 ± 0.05 | 0.02 ± 0.08 |
| $N_{NB}$ | 2,605 ± 1,381 | 2,588 ± 1,976 | 8,941 ± 34,406 |
| $N_{SB}$ | 130 ± 17 | 121 ± 89 | 153 ± 36 |

$^a$See Fig. 2e for definition of kinetic parameters. $N_{NB}$ and $N_{SB}$ are the effective numbers of specific recognition sites and nonspecific binding sites on the chromosome. All error bars are s.d. Relevant data for ZntR variants and other control strains are in Supplementary Tables 7 and 8.
residence sites as a measure. We validated this measure by comparing with results from direct imaging of dye-stained chromosomes (Supplementary Note 20.2). The distributions of $\langle d_{ij}\rangle$ among cells are broad (Fig. 3g, h), reflecting that the extent of chromosome condensation differs significantly from cell to cell. For cells expressing CueR^{apo} and ZntR^{apo}, for example, two subpopulations can be resolved, corresponding to those with more or less condensed chromosomes (that is, smaller or larger $\langle d_{ij}\rangle$), respectively; Fig. 3g).

**Chromosome-organization-dependent regulator unbinding.** We sorted the cells into three groups based on their $\langle d_{ij}\rangle$ to examine how the unbinding of CueR and ZntR from recognition sites may relate to chromosome organization. Within each group, we determined the dependence of $k_{app}$ on the apparent unbinding rate constant from recognition sites, on the cellular protein concentration. Remarkably, for CueR^{apo} and ZntR^{apo}, while their $k_{app}$ preserve the protein-concentration dependence in each group, their $k_{app}$ in cells with more condensed chromosome (that is, smaller $\langle d_{ij}\rangle$) are up to three times smaller than those in cells with less condensed chromosome (that is, larger $\langle d_{ij}\rangle$); Fig. 4a and Supplementary Fig. 50c. As a control, we treated the cells expressing CueR^{apo} and ZntR^{apo} with the drug chloramphenicol, which is known to cause chromosome compaction. Consistently, the chloramphenicol treatment leads to a decrease of $k_{app}$ across all accessible cellular protein concentrations (Supplementary Note 21.5).

More surprisingly, an opposite trend applies to the metallated holo-proteins with regard to the dependences of their unbinding kinetics on the extent of chromosome condensation. For CueR^{apo} and ZntR^{apo}, their $k_{app}$ are larger in cells with more condensed chromosomes (that is, smaller $\langle d_{ij}\rangle$) at any cellular protein concentration (Fig. 4b and Supplementary Fig. 50f).

We further obtained $k_i$ and $k_{-i}$, the second-order facilitated and first-order spontaneous unbinding rate constants from recognition sites, for cells with different extents of chromosome condensation (Fig. 4a, b). Correspondingly, $k_i$ and $k_{-i}$ both show opposite dependences on $\langle d_{ij}\rangle$ between the apo and holo forms of CueR or ZntR; and they can change by up to nine times with $\langle d_{ij}\rangle$ ranging from ~0.5 to 1.2 μm (Fig. 4d, e, and Supplementary Note 21.3). Altogether, these results suggest that chromosome organization modulates both the facilitated and spontaneous unbinding of CueR and ZntR from recognition sites, but in opposite directions depending on the regulators’ metallation state.

**Discussion**

The concentration- and chromosome-organization-dependent unbinding of CueR and ZntR from their recognition sites open up new possibilities for regulating transcription of their associated metal resistance genes. The concentration-dependent unbinding of CueR and ZntR, together with their spontaneous unbinding from DNA, may facilitate the switching between the transcriptionally activated state (bound holo-protein) and the repressed state (bound apo-protein), which both require protein binding to the same operator site (Supplementary Note 18.2). Transcription deactivation by these regulators will likely involve the unbinding of a promoter-bound holo-activator from DNA followed by binding of an apo-repressor, rather than the dissociation of the tightly bound metal from the promoter-bound holo-protein, which is expected to be slow. For CueR^{apo}, just the basal concentration range of ~17–240 nM (Supplementary Fig. 17) can already change its unbinding rate from a recognition site from ~4.7 to 7.9 s^{-1}, a ~70% increase. On the other hand, transcription activation will likely involve the unbinding of a promoter-bound apo-repressor followed by binding of a holo-activator, rather than the direct metallation of the promoter-bound apo-repressor, which faces competition for the metal by many other apo-repressors in the cell. For CueR^{apo}, the basal concentration range of ~14–185 nM can change its unbinding from a recognition site from ~8.3 to 9.8 s^{-1}.

Mechanistically, concentration-dependent unbinding of CueR or ZntR from chromosomal recognition sites likely results from their assisted dissociation or direct substitution process, which we discovered previously in studying CueR interactions with a specific DNA in vitro (Supplementary Note 18.2). In the assisted dissociation process, a protein from the surrounding...
solution (for example, the cytoplasm) helps carry away the incumbent protein bound at the recognition site (Fig. 5d); in the direct substitution process, the incoming protein directly replaces the incumbent one (Fig. 5e); both processes depend on the protein concentration in the surrounding. Both of these processes likely involve a ternary complex as a common intermediate, in which two protein molecules each partially bind to the recognition site (Fig. 5c), as we proposed previously\(^\text{37}\). This ternary complex is possible because of the homodimeric nature of CueR and ZntR: they can form bivalent contacts with DNA in which their two DNA-binding domains bind to the two halves of their dyad-symmetric recognition sequences (Fig. 5a). Under thermal fluctuation, one of the DNA-binding domains of a protein molecule could transiently detach, allowing another protein from solution to bind to the vacant half dyad sequence, leading to a ternary complex (Fig. 5c). This ternary complex could then proceed in two possible pathways: both proteins fall off, resulting in an assisted dissociation (Fig. 5d), or one of the two proteins falls off, where 50% of the chance would result in a direct substitution (Fig. 5e). Although no evidence exists for higher-order oligomeric CueR or ZntR complexes on regular DNA, our previous in vitro study has revealed a related ternary complex in CueR interactions with an engineered DNA Holliday junction\(^\text{37,45}\). Relatedly, ternary complexes involving multivalent apo and CueR\(^\text{mE}\) in nondividing cells with different average \(\langle d_{ij}\rangle\) (\(d_{ij}\)). Lines are linear fits with \(k_{\text{app}} = k_{\text{P}}/C_0 + k_{-1}\), as in Fig. 2g. (c) Same as a but for dividing cells, in comparison with \(k_{\text{app}}\) for nondividing cells from Fig. 2g. (d,e) \(\langle d_{ij}\rangle\)-dependent facilitated unbinding and spontaneous unbinding rate constants \(k_1\) and \(k_{-1}\) of CueR\(^\text{apo}\) and CueR\(^\text{apo}\) in nondividing cells. Lines are linear fits (Supplementary Note 21.3). Data for ZntR are in Supplementary Note 21. All error bars are s.d.

Figure 4 | Chromosome-organization-dependent unbinding of CueR and ZntR from recognition sites. (a,b) Protein-concentration-dependent \(k_{\text{app}}\) of CueR\(^\text{apo}\) and CueR\(^\text{apo}\) in nondividing cells with different average \(\langle d_{ij}\rangle\). Lines are linear fits with \(k_{\text{app}} = k_{\text{P}}/C_0 + k_{-1}\), as in Fig. 2g. (c) Same as a but for dividing cells, in comparison with \(k_{\text{app}}\) for nondividing cells from Fig. 2g. (d,e) \(\langle d_{ij}\rangle\)-dependent facilitated unbinding and spontaneous unbinding rate constants \(k_1\) and \(k_{-1}\) of CueR\(^\text{apo}\) and CueR\(^\text{apo}\) in nondividing cells. Lines are linear fits (Supplementary Note 21.3). Data for ZntR are in Supplementary Note 21. All error bars are s.d.

Regarding the correlation between the unbinding kinetics of CueR (or ZntR) and chromosome organization, as well as the opposite trends between their apo-repressor versus holo-activator forms, we postulate that they may help the cell modulate transcription of metal resistance genes during the growth cycle. The unbinding of apo- and holo-CueR (or ZntR) from recognition sites is important for activation and deactivation of transcription, respectively. Under optimal growth conditions without metal stress, cells divide frequently. The dividing cells for cell division. On the other hand, under metal stress conditions, cells barely divide and their chromosomes are less condensed (Fig. 3h versus Fig. 3i). Here apo-CueR or apo-ZntR unbinds faster from promoters, leading to more facile transcription activation to defend against metal stress. Moreover, holo-CueR or holo-ZntR unbinds slower here, which would keep activating transcription longer. Supporting this postulate, we found that the apparent unbinding rate constants \((k_{\text{app}})\) of CueR\(^\text{apo}\) and ZntR\(^\text{apo}\) in dividing cells are indeed significantly slower than those in nondividing cells across all cellular protein concentrations (Fig. 4c and Supplementary Fig. 52c).
Mechanistically, the chromosome-organization-dependent unbinding of CueR and ZntR from recognition sites likely stems from their ability to impose DNA structural distortions. The holo forms of these regulators bend and unwind DNA at the recognition site in the chromosome, making the complex susceptible to chromosome organization and associated tension in DNA. Green colour on chromosome denotes the two halves of the dyad-symmetric recognition sequence. The bound regulator (blue) can unbind spontaneously (b), Alternatively, another regulator (red) from the cytoplasm can bind to one half of the recognition sequence, which is vacated by the incipient one, forming a ternary complex as an intermediate (c), which can then proceed to result in assisted dissociation (d) or direct substitution (e), giving rise to concentration-dependent unbinding.

Figure 5 | Schematic mechanism for concentration- and chromosome-organization-dependent unbinding from recognition sites. On binding to a recognition site in the chromosome, the homodimeric protein CueR or ZntR distorts the DNA structure (a), making the complex susceptible to chromosome organization and associated tension in DNA. Green colour on chromosome denotes the two halves of the dyad-symmetric recognition sequence. The bound regulator (blue) can unbind spontaneously (b), Alternatively, another regulator (red) from the cytoplasm can bind to one half of the recognition sequence, which is vacated by the incipient one, forming a ternary complex as an intermediate (c), which can then proceed to result in assisted dissociation (d) or direct substitution (e), giving rise to concentration-dependent unbinding.
(2) Dividing the total fluorescence intensity by the average fluorescence intensity of a single mEos3.2 (which was derived from the earlier SMT for the same single cell). Note that in step (1), depending on the amount of remaining mEos3.2, the EM gain of the camera was adjusted accordingly to ensure the fluorescence signal was within the linear regime of camera response. The fluorescence signal was then corrected by the linear regime of camera response. The fluorescence signal was then corrected by the EM gain to obtain the total fluorescence intensity for quantification. An important feature here was to use the single mEos3.2 fluorescence intensity determined in the same cell to eliminate the need to correct for power density differences of the imaging laser across the illumination area. The photobleaching efficiency of mEos3.2 and the protein oligomeric state (CueR and ZntR are stable homodimers) were also included in determining the total copy number of CueR or ZntR in each cell. The protein copy number was converted to protein concentration by using the cell volume determined from its transmission image; the cell boundary was fitted with the model of a cylinder with two hemispherical caps as reported (Supplementary Fig. 13a). The quantification of each cell’s CueR or ZntR in Supplementary Note 15.4)

The approach of using a linear combination of diffusion terms in the CDF (or the corresponding PDF, Fig. 2b) of τ was also applied previously by others to analyse SMT results and resolve multiple diffusion states of a protein in E. coli or mammalian cells (24,27,32,35,59-61). This linear combination approach assumes an approximation of a quasi-static system, that is, interconversion between the diffusion states is slower than the experimental time resolution (see justification of this approximation for CueR or ZntR in Supplementary Note 18.4). In the global fit, the effective diffusion constants (that is, DSB, D0SB, and DNB) were expected to be concentration independent and thus were shared across the cellular protein concentrations, while their fractional populations (that is, ASB, ASB and A0SB = 1-ASB-ASB) were allowed to differ (Supplementary Note 11.2). This global fit was critical for the reliability of determining the minimal number of diffusion states, and their fitted diffusion constants and fractional populations, as compared with fitting the CDFs individually. Exemplary fitted fractional populations versus cellular protein concentrations are shown in Fig. 2c. The extraction of the number of diffusion states and their effective diffusion constants was validated through multistate diffusion simulations within the confined cell geometry, followed by the quantitative analysis of the simulated results in parallel to analysis of the experimental results (Supplementary Note 15).

Determination of k^{BP} from chromosomal recognition sites. A three-state kinetic model (Fig. 2e) was used to analyse the distributions of the microscopic residence time τ, thresholded from the displacement-versus-time trajectory to extract the apparent unbinding rate constant k^{BP} of mEos3.2-tagged regulators from chromosomal recognition sites (Supplementary Note 14). The three states are the freely diffusing CueR or ZntR proteins in the cytoplasm, nonspecifically bound to DNA and specifically bound to chromosomal recognition sites. This kinetic model includes the reversible binding/unbinding of a regulator to chromosomal recognition sites and nonspecific sites, as well as the photobleaching/blinking of the mEos3.2 tag, but the direct interconversion between the SB and the NB states is assumed to be sufficiently slow to be kinetically negligible (justification and validation of this approximation in Supplementary Note 14.5).

With a given displacement threshold τo, the effective diffusion constants (D5), the unbinding rate constant (k-5) from the NB state, the apparent unbinding rate constant (k5) in the concentration range and the photobleaching/blinking rate constant k5o, we derived the respective probability distribution functions φ(τ) of the thresholded residence time τ for the FD, NB and SB states (that is, φFD(τ), φNB(τ) and φSB(τ), respectively; Supplementary Note 14.1):
\[ \frac{A_{SP}}{A_{TD}} \text{ versus } [P]_{FD}, \frac{A_{SB}}{A_{TD}} \text{ versus } [P]_{FD}, \frac{A_{SP}}{A_{NB}} \text{ versus } [P]_{FD}, \frac{A_{SB}}{A_{NB}} \text{ versus } [P]_{NB} \]

with equations (9–11) as in Supplementary Fig. 30 to obtain the dissociation constants of SB (Kd) and NB (Kd) and their states together with k_c, k_{dud}, k_{dsd}, and k_{ds} (Table 1 and Supplementary Tables 7 and 8). The determined dissociation constants (K_i’s) at the recognition sites and nonspecific sites (tens and hundreds of nM, respectively) are consistent with literature [8,12], further supporting the validity of our analyses. An interesting note here: for both CueR and Znth, their affinity differences between recognition and nonspecific sites mainly come from kinetic differences in binding rather than unbinding (Supplementary Note 18.2).

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Acknowledgements
We acknowledge the National Institutes of Health (GM109993, GM106420 and AI117295) for funding, J. Xiao and P. Xu for gifts of mEos2 and mEos3.2 genes, X. Su, H. Lin, J. Peters, Y. Aye and C. Kinsland for helping with molecular biology and/or E. coli genetics.

Author contributions
T.-Y.C., A.G.S. and P.C. designed the experiments; T.-Y.C., A.G.S. and W.J. performed the experiments; I.K., F.Y. and D.J.M. contributed to the experiments; J.D.H. contributed to discussions; T.-Y.C., A.G.S., W.J. and P.C. analysed the data and wrote the manuscript.

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: Chen, T.-Y. et al. Concentration- and chromosome-organization-dependent regulator unbinding from DNA for transcription regulation in living cells. Nat. Commun. 6:7445 doi: 10.1038/ncomms8445 (2015).

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