Single molecule microscopy to profile the effect of zinc status on transcription factor dynamics

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The regulation of transcription is a complex process that involves binding of transcription factors (TFs) to specific sequences, recruitment of cofactors and chromatin remodelers, assembly of the pre-initiation complex and recruitment of RNA polymerase II. Increasing evidence suggests that TFs are highly dynamic and interact only transiently with DNA. Single molecule microscopy techniques are powerful approaches for tracking individual TF molecules as they diffuse in the nucleus and interact with DNA. Here we employ multifocus microscopy and highly inclined laminated optical sheet microscopy to track TF dynamics in response to perturbations in labile zinc inside cells. We sought to define whether zinc-dependent TFs sense changes in the labile zinc pool by determining whether their dynamics and DNA binding can be modulated by zinc. We used fluorescently tagged versions of the glucocorticoid receptor (GR), with two C4 zinc finger domains, and CCCTC-binding factor (CTCF), with eleven C2H2 zinc finger domains. We found that GR was largely insensitive to perturbations of zinc, whereas CTCF was significantly affected by zinc depletion and its dwell time was affected by zinc elevation. These results indicate that at least some transcription factors are sensitive to zinc dynamics, revealing a potential new layer of transcriptional regulation.
fold of the zinc finger domain and thereby enables interaction with DNA. It is well established that zinc finger TFs require their Zn²⁺ cofactor to bind DNA, but whether these TFs are sensitive to physiological changes in the labile Zn²⁺ pool in cells has not been examined. Multiple studies have shown that the labile Zn²⁺ pool in the cytosol and nucleus of mammalian cells is on the order of hundreds of picomolar, but that the level of Zn²⁺ changes with an ever-expanding number of cellular processes. For example, it has been shown that cells experience changes in the labile Zn²⁺ pool during immune cell activation, development, neuronal stimulation, and the cell cycle, and that these fluctuations are important for cell physiology. Evaluating whether zinc-binding proteins, such as transcription factors, sense these fluctuations in Zn²⁺ is an important step in dissecting the mechanism of how Zn²⁺ fluctuations alter cell physiology.

The only known TF to exhibit sensitivity to the cellular Zn²⁺ pool is the metal-responsive transcription factor (MTF1). MTF1 senses high Zn²⁺ through its array of six zinc fingers and in the presence of high Zn²⁺, translocates to the nucleus to regulate metal buffering proteins (metallothionines) and metal transporters. The apparent dissociation constant (Kₐ₅ₐ₅) for Zn²⁺ in the full length MTF1 protein was found to be 31 pM. However, biochemical studies have shown that Zn²⁺ fingers five and six are the most reactive and have the weakest affinity for cobalt (which is often used to measure affinities of Zn²⁺ binding proteins), suggesting that these may sense much higher levels of Zn²⁺. Most TFs that bind Zn²⁺ have Kₐ₅ₐ₅ in the hundreds of picomolar range. For example, it's been shown that the nuclear hormone receptors glucocorticoid receptor (GR) and estrogen receptor (ER) contain two C4 Zn²⁺ fingers bind Zn²⁺ with Kₐ₅ₐ₅ of 316 and 501 pM, respectively. Despite the fact that these Kₐ₅ₐ₅ are similar to the concentration of the labile Zn²⁺ pool, these TFs are generally not thought to be metal responsive. However, this has not been tested in live cells.

In this work, we sought to determine whether zinc finger TFs are susceptible to changes in the labile Zn²⁺ pool. We used fluorescently tagged versions of GR, with two C4 Zn²⁺ finger domains, and CCCTC-binding factor (CTCF), with eleven C2H2 Zn²⁺ finger domains, and single molecule fluorescence microscopy to monitor their mobility within live cells. We found that CTCF, but not GR, shows increases in the mean squared displacement and apparent diffusion coefficient when cellular Zn²⁺ is chelated with Tris(2-pyridylmethyl)amine (TPA), suggesting that CTCF is more dynamic when cellular Zn²⁺ is low. Both Zn²⁺ and TPA decreased the dwell times for CTCF. On the other hand, GR was largely unaffected by perturbation of Zn²⁺. These results suggest that some TFs are sensitive to changes in the labile Zn²⁺ pool while others are not.

**Results**

CTCF, but not GR, shows significantly greater mean squared displacements and diffusion coefficient in low Zn²⁺. While it is well established that zinc finger transcription factors require Zn²⁺ to bind DNA in vitro, an open question is whether the Zn²⁺ occupancy and hence DNA binding capacity in cells is dependent on levels of cellular Zn²⁺. We applied 3D single molecule microscopy to investigate whether zinc finger transcription factors have altered mobility when Zn²⁺ is perturbed, where mobility is routinely used as a proxy for DNA binding. Specifically, we used multifocus microscopy (MFM) that allows for simultaneous acquisition of particles in 9 Z-planes separated by approximately 430 nm (~3.9 µm total axial depth). This enabled us to track labeled transcription factors at rapid acquisition rates (25 Hz) and did not result in truncated trajectories as they diffused along the Z-axis in the nucleus, a common limitation of 2D single molecule tracking.

To test the ability of MFM to detect differences in TF dynamics, we examined the mean squared displacement and dwell times for the glucocorticoid receptor (GR) treated with the known activator dexamethasone or hydrocortisone. Previously it has been shown that dexamethasone is a more potent activator of GR than hydrocortisone and this leads to longer dwell times, presumably because GR is more strongly associated with target sites on DNA. U2OS cells expressing HaloTag-GR (stable overexpression) were stained with JF549-HaloTag ligand and treated with 100 nM of either dexamethasone or hydrocortisone for 30 min. Figure 1a reports the mean squared displacements (MSDs) as a function of time for HaloTag-GR treated with each activator and the computed diffusion characteristics for each. GR activated by hydrocortisone diffuses more quickly than dexamethasone (diffusion coefficients of 0.335 ± 0.011 µm²/s and 0.289 ± 0.019 µm²/s for hydrocortisone and dexamethasone, respectively). These values are the diffusion coefficients for the entire population and most previous studies divide the populations into “fast” diffusing and “slow” diffusing populations. Therefore, we cannot directly compare these values to previous literature values. However, our observation that hydrocortisone results in more diffusive GR compared to dexamethasone is consistent with previous literature reports and confirms that our analysis is valid for analyzing TF dynamics. Additionally, we calculated the dwell (residence) times for GR treated with the above hormones. Our rationale was that particles with longer dwell times may correspond to those that are bound to DNA rather than freely diffusing or experiencing short, transient interactions. We used the calculated MSDs from each trajectory to identify displacements of less than 200 nm that corresponded to bound particles. This 200 nm threshold has previously been used as a conservative estimate of general chromatin movement, as measured using fluorescently labeled histone H2B. Additionally, binding events had to last at least 8 frames (320 ms) to be considered bound; this eliminated transient, non-specific interactions that were sometimes observed. We found that hydrocortisone had slightly shorter dwell times than dexamethasone (Fig. 1b), consistent with our diffusion analysis and the model that GR activated by hydrocortisone would be bound to DNA for a shorter period of time than GR treated with the more potent activator dexamethasone. Taken together, these findings support that MFM is a valid technique for measuring differences in TF dynamics upon a chemical perturbation.

To evaluate whether perturbation of Zn²⁺ alters dynamics of candidate TFs, we continued to interrogate GR as it contains two C4 Zn²⁺ finger domains. Additionally, we selected the CCCTC-binding factor (CTCF), a chromatin binding protein that contains eleven C2H2 Zn²⁺ finger domains. Both transcription factors have been previously characterized by 2D single molecule microscopy and have DNA dwell times that span an approximate
order of magnitude (3–8 s for GR34, 60 s for CTCF32). U2OS cells stably expressing HaloTag-GR or HaloTag-CTCF (CRISPR-edited endogenous expression) were treated with either 50 µM of the Zn²⁺ chelator TPA to deplete free Zn²⁺, 30 µM ZnCl₂ to increase free Zn²⁺, or a media-only control for 30 min. Previously, we have shown that these perturbations decrease labile Zn²⁺ to < 1 pM or increase intracellular labile Zn²⁺ to 30 nM, respectively19,20,40. Under our imaging conditions, we acquired between 493–1080 total tracks for HaloTag-GR and between 5065–12,770 tracks for HaloTag-CTCF (Table 1), with most tracks lasting for less than 40 frames (1.6 s) (Supplemental Figure S1).

Figure 1. Dynamic properties of HaloTag-GR as measured by MFM. (a) Mean squared displacement (MSD) curves for HaloTag-GR treated with either 100 nM dexamethasone (Dex) or 100 nM hydrocortisone (HyD) for 30 min. Below, the calculated diffusion coefficients and α factors for each curve. (b) Dwell time analysis for HaloTag-GR treated with either 100 nM dexamethasone or 100 nM hydrocortisone. For both (a) and (b), a total of 236 and 151 trajectories were analyzed for dexamethasone and hydrocortisone treatment, respectively.

| Cell line      | Treatment | Microscope | Exposure time (ms) | Acquisition interval (ms) | Number of cells | Total number of tracks | Total number of tracks – diffusion | Total number of Dwell times |
|----------------|-----------|------------|--------------------|---------------------------|----------------|-----------------------|-----------------------------------|-----------------------------|
| U2OS HaloTag-GR| 50 µM TPA | MFM        | 40                 | 40                        | 5              | 493                   | 262                               |                             |
| U2OS HaloTag-GR| Ctrl      | MFM        | 40                 | 40                        | 5              | 948                   | 419                               |                             |
| U2OS HaloTag-GR| 30 µM ZnCl₂ | MFM  | 40                 | 40                        | 5              | 1080                  | 601                               |                             |
| U2OS HaloTag-CTCF| 50 µM TPA | MFM        | 40                 | 40                        | 11             | 11,704                | 5046                              |                             |
| U2OS HaloTag-CTCF| Ctrl     | MFM        | 40                 | 40                        | 10             | 12,770                | 4985                              |                             |
| U2OS HaloTag-CTCF| 30 µM ZnCl₂ | MFM  | 40                 | 40                        | 7              | 5065                  | 1975                              |                             |
| U2OS HaloTag-GR| 50 µM TPA | N-STORM    | 100                | 100                       | 5              | 17,511                | N/A                               | 7702                        |
| U2OS HaloTag-GR| Ctrl      | N-STORM    | 100                | 100                       | 11             | 45,748                | N/A                               | 10,994                      |
| U2OS HaloTag-GR| 30 µM ZnCl₂ | N-STORM  | 100                | 100                       | 9              | 24,544                | N/A                               | 20,121                      |
| U2OS HaloTag-CTCF| 50 µM TPA | N-STORM    | 100                | 500                       | 5              | 12,452                | N/A                               | 2721                        |
| U2OS HaloTag-CTCF| Ctrl     | N-STORM    | 100                | 500                       | 6              | 44,628                | N/A                               | 12,430                      |
| U2OS HaloTag-CTCF| 30 µM ZnCl₂ | N-STORM  | 100                | 500                       | 7              | 21,725                | N/A                               | 6179                        |

Table 1. Summary statistics for all cell lines and imaging conditions used in this study. Here, MFM denotes experiments conducted to generate 3D particle tracking data and N-STORM denotes experiments conducted to generate 2D particle tracking data.
coefficients than the control cells (12.4% larger in high Zn2+, 5.8% larger in low Zn2+). A similar analysis for HaloTag-CTCF revealed that the MSDs upon Zn2+ depletion were significantly greater than either the control or Zn2+ treatment (Fig. 2b), resulting in a diffusion coefficient that was 211% higher than the control. Elevated Zn2+ resulted in a very small increase in the diffusion coefficient (9.1% compared to control). These results suggest that the dynamics of GR are minimally sensitive to changes in the labile Zn2+ pool, while CTCF is sensitive to Zn2+ depletion.

The above analysis gives the diffusion coefficient for the entire population of molecules. However, proteins in cells are more apt to exhibit anomalous diffusion rather than purely Brownian motion, and sub-populations with different diffusion properties have been observed. The sub-populations of molecules with different diffusion behavior can be inferred by using the MSD curves to extract the $\alpha$ parameter. A value of $\alpha = 1$ indicates purely Brownian motion, while $\alpha < 1$ indicates subdiffusive behavior and $\alpha > 1$ indicates superdiffusive behavior. Within our populations of HaloTag-GR and HaloTag-CTCF trajectories, we found that the aggregate $\alpha$ for the population hovered around 1 (Fig. 2). We separated these populations into those exhibiting subdiffusive or superdiffusive behaviors by fitting the individual MSD curve for each trajectory to the above diffusion model and extracted $\alpha$ values for each trajectory. Trajectories with $\alpha < 1$ were labeled as subdiffusive and trajectories with $\alpha > 1$ were labelled superdiffusive, and the diffusion coefficients were calculated for the two groups of molecules. For HaloTag-GR, there was a small increase in diffusion coefficient (D) for both subdiffusive (D was 7.1% larger in low Zn2+, 6.1% larger in high Zn2+) and superdiffusive particles (D was 8.3% larger in low Zn2+, 22.5% larger in high Zn2+) compared to control conditions (Fig. 3a). For HaloTag-CTCF, in low Zn2+ conditions there was a significant increase in D for both subdiffusive (215% larger compared to control) and superdiffusive particles (202% larger compared to control) (Fig. 3b). In high Zn2+ conditions, there was a small increase in D for subdiffusive particles (23.7% larger compared to control) and no change for superdiffusive particles.

Dwell time analysis reveals that CTCF is more mobile in low Zn2+ conditions. As noted in Supplemental Figure S1, many of the MFM trajectories we detected lasted for less than 40 frames (1.6 s) due to photobleaching of the JF dye under the illumination conditions of the MFM. Literature reports for the dwell time of GR and CTCF are 7 seconds36 and 70 seconds32 respectively. Therefore, to determine whether the dwell times for GR and CTCF are altered by labile Zn2+, we performed a similar experiment using a Nikon N-STORM microscope in highly-inclined laminated optical sheet (HILO)41 mode with longer acquisition times and substantially less laser intensity. Using HILO we were able to image for 5 min at 10 Hz (for HaloTag-GR) and 20–30 min at 2 Hz (for HaloTag-CTCF) rather than the 1–2 min acquisition periods on the MFM. Additionally, these conditions allowed us to bias all detected particles towards bound particles, rather than freely diffusing particles. As
such, we calculated the particle dwell time to be equal to \( \text{tracklength} \times \text{framerate} \) (s), with a minimum track length of 5 to be considered in the analysis.

For HaloTag-GR, treatment with TPA and \( \text{ZnCl}_2 \) resulted in very small changes in dwell times relative to the control (Fig. 4a). This correlates with the diffusion data we measured using the MFM. We first fit the survival curves to a biexponential decay (Fig. 4a, right) to extract the kinetic parameters \( k_1 \) and \( k_2 \), which correlate to the off rates for non-specific and specific interactions for the TF of interest. The inverse of \( k_2 \) \((1/k_2)\) is widely used in the literature as the dwell time, \( \tau \), of the TF. Through this method, we measured photobleaching corrected GR dwell times of 7.65 ± 0.35, 5.44 ± 0.38, and 3.94 ± 0.26 s for untreated, TPA-treated, and \( \text{ZnCl}_2 \)-treated cells (Table 2). These values are consistent with previously reported GR dwell times which range from 3–8 s\(^34,36\). Similarly, we find that perturbing \( \text{Zn}^{2+} \) with either TPA or \( \text{ZnCl}_2 \) results in reduced dwell times for CTCF (Fig. 4b). This resulted in dwell times of 97.5 ± 11.03, 38.1 ± 4.43, and 30.1 ± 1.89 s for untreated, TPA-treated, and \( \text{ZnCl}_2 \)-treated cells, respectively (Table 2). Our measured dwell time for CTCF is slightly longer than the literature values (63–66 s)\(^32\). However, both TPA-treated and \( \text{ZnCl}_2 \)-treated cells exhibit dwell times substantially less than our untreated cells, indicating greater mobility of CTCF upon perturbation of \( \text{Zn}^{2+} \).

An alternative approach for comparing TF dwell times has recently emerged where the photobleaching-corrected dwell time survival curve (see methods) is fit to a power law (\( f(t) = At^{-\beta} \)), rather than a biexponential decay\(^42\). The biexponential decay model assumes that a given TF occupies one of three states: diffusive; fast, or non-specific interactions; and slow, or specific interactions. This does not account for the transition from the fast state to the slow state, which a TF is likely to experience as it searches for its target site. When using a power law to fit dwell time survival curves, the physical model proposed is that of a broad distribution of affinities where there are microenvironments that contain energy wells of different depths. This model stems from the recognition that the chromatin landscape is heterogeneous due to physical constraints and motif degeneracy, and this heterogeneity affects TF binding. The power law exponent, \( \beta \), is a measure of the skewness of the distribution where a smaller value of \( \beta \) correlates with a longer dwell time. A previous study found that that \( \beta \) values for GR and CTCF were 0.828 ± 0.004 and 0.55 ± 0.02, respectively\(^42\). When fitting our GR data to a power law, we found that the \( \beta \) values were approximately 1.2 for all treatment conditions (Fig. 5a, Table 3). While these values of \( \beta \) are larger than previously reported, they reveal no change in GR upon perturbation of \( \text{Zn}^{2+} \). When fitting our CTCF data to a power law, we found that the \( \beta \) for untreated cells was 0.62 ± 0.03 (Fig. 5b, Table 3), which aligns closely with the previously reported \( \beta \) of 0.55. We found that the \( \beta \) for TPA treated cells was 0.95 ± 0.03 and the \( \beta \) for \( \text{ZnCl}_2 \)-treated cells was 0.88 ± 0.02 (Fig. 5b, Table 3), indicating a decrease in DNA binding under these conditions.

Previous studies showed that deletion of the 11 \( \text{Zn}^{2+} \) fingers in CTCF resulted in long displacements consistent with free diffusion of CTCF\(^32\). This suggests that without \( \text{Zn}^{2+} \) fingers, CTCF is unable to effectively bind DNA. We set out to determine whether depletion of \( \text{Zn}^{2+} \) can alter the bound versus unbound trajectories for both HaloTag-GR and HaloTag-CTCF. The fraction bound was determined by dividing the number of trajectories...
Figure 4. The effect of Zn$^{2+}$ on dwell times, as measured by HILO. (a) Left: Raw 2D dwell time survival curves (1-CDF) for HaloTag-GR cells treated with 50 µM TPA (red), 30 µM ZnCl$_2$ (blue), or a media-only control (black). Right: photobleaching-corrected survival curves for each treatment & their respective biexponential decay fits. (b) same as in (a), but with HaloTag-CTCF cells.

Table 2. Photobleaching-corrected 2D dwell time fits for HaloTag-GR and HaloTag-CTCF. $k_1$ and $k_2$ are the rate constants for the fast and slow components, respectively. $\tau$ is the dwell time calculated using $k_2$, and $\tau_{\text{corrected}}$ is the photobleaching corrected dwell time calculated using H2B dwell times calculated at the same frame rate.
longer than 5 frames by the total number of trajectories. While our imaging conditions did bias detections towards bound tracks, we only computed dwell times for trajectories lasting longer than 5 frames. For HaloTag-GR, we found that the fraction bound did not vary significantly across treatments (Fig. 6a). For HaloTag-CTCF, we found that the fraction bound for TPA treated cells was less than the control and ZnCl₂ treated cells, with a p value = 0.0572 (Fig. 6b). We didn’t observe any difference between the cells treated with ZnCl₂ and the untreated control.

Discussion
Proper transcriptional regulation is essential for the cell’s ability to respond to the demands of its environment. Over the past several decades, various genomic techniques have been developed to monitor TF activity and the downstream consequences on gene expression, but most of these techniques monitor a heterogenous population of cells at fixed time points. SM microscopy allows for assessment of individual TFs within single cells, where different experimental approaches can be used to access different temporal regimes. For example, MFM is capable of rapid (> 25 Hz) acquisition in 3D due to simultaneous acquisition of multiple Z-planes. Thus, MFM is ideally suited for measuring fast TF characteristics such as diffusion coefficients. On the other hand, measurement of dwell times requires much longer measurement windows to observe the dynamics and heterogeneity of binding.

Table 3. Power law fits for HaloTag-GR and HaloTag-CTCF. β is a measure of the skewness of the distribution and is therefore proportional to dwell time.

| Factor       | Treatment | A     | β       |
|--------------|-----------|-------|---------|
| HaloTag-GR   | 50 µM TPA | 0.36 ± 0.006 | 1.27 ± 0.03 |
| HaloTag-GR   | Ctrl      | 0.37 ± 0.005 | 1.20 ± 0.02 |
| HaloTag-GR   | 30 µM ZnCl₂ | 0.36 ± 0.006 | 1.29 ± 0.03 |
| HaloTag-CTCF | 50 µM TPA | 2.08 ± 0.11  | 0.95 ± 0.03 |
| HaloTag-CTCF | Ctrl      | 1.36 ± 0.03  | 0.62 ± 0.03 |
| HaloTag-CTCF | 30 µM ZnCl₂ | 2.01 ± 0.09  | 0.88 ± 0.02 |

Figure 5. (a) Power law fits for HaloTag-GR for cells treated with 50 µM TPA (top), a media-only control (middle), or 30 µM ZnCl₂ (bottom). Raw survival distributions were corrected for photobleaching using the survival curves from untreated U-2 OS cells stably expressing H2B-HaloTag. (b) Same as in (a), but for HaloTag-CTCF.
we cannot rule out the possibility that perturbation of $\text{Zn}^{2+}$ leads to changes in chromatin architecture. Besides needed to deconvolve the complexity of transcriptional regulation. With respect to global changes in chromatin, chromatin architecture proteins, may therefore be more strongly affected by perturbations in $\text{Zn}^{2+}$ than GR, due to changes in chromatin accessibility or compaction that alter the dynamics of TFs. CTCF, as one of the key targets. It is important to note that the dwell times we observe do not necessarily indicate functional binding events. As noted, CTCF can potentially bind 80,000 $\pm$ sites in the genome, and GR ChIP-seq experiments have shown 10,000 $\pm$ potential binding sites across multiple cell types. However, of the ~7000 sites found in A549 (lung adenocarcinoma) cells, only 928 (13.5%) of these sites were shown to truly be glucocorticoid responsive. It is therefore plausible that some of the binding events we observed via single molecule microscopy may be dominated by transient, non-specific interactions that occur when a TF is searching for its target rather than specific, productive binding events. This is where novel quantitative methods and kinetic models for assessing TF dwell time, such as fitting distributions to a power law to account for a continuum of binding affinities, are needed to deconvolve the complexity of transcriptional regulation. With respect to global changes in chromatin, we cannot rule out the possibility that perturbation of $\text{Zn}^{2+}$ leads to changes in chromatin architecture. Besides TFs, $\text{Zn}^{2+}$ is a required cofactor for histone acetyltransferases, histone deacetylases, histone demethylases, and DNA methyltransferases. It may be that the sheer number of $\text{Zn}^{2+}$ binding proteins in the nucleus all contribute to changes in chromatin accessibility or compaction that alter the dynamics of TFs. CTCF, as one of the key chromatin architecture proteins, may therefore be more strongly affected by perturbations in $\text{Zn}^{2+}$ than GR, perhaps because of the number of genomic binding sites. Whether the effect of $\text{Zn}^{2+}$ is direct (modulating the $\text{Zn}^{2+}$ occupancy of the TF of interest) or indirect (changing chromatin architecture in a way that affects some TFs

**Figure 6.** The effect of $\text{Zn}^{2+}$ on fraction bound. (a) Fraction bound analysis for HaloTag-GR cells. Fraction bound was calculated as the number of tracks that were present for at least 5 frames (500 ms) divided by the total number of detected tracks. (b) Same as in (a), except with HaloTag-CTCF cells and with tracks that were present for at least 5 frames (2.5 s). Numbers indicate the $p$-value calculated using a one-way ANOVA.
more than others), the implication of our results is that some TFs are affected by physiological changes in the labile Zn\(^{2+}\) pool and hence their function could be altered during Zn\(^{2+}\) fluctuations.

Our work suggests that a subset of TFs may be sensitive to changes in cellular Zn\(^{2+}\). While further studies are necessary to determine whether changes in TF dynamics and mobility are correlated with altered function, our results show for the first time that canonical zinc-finger TFs (outside of MTF1) can be sensitive to changes in the labile Zn\(^{2+}\) pool. Given the noted advantages of SM microscopy, it would be valuable to pair these techniques with other fluorescent microscopy techniques to assess the downstream consequences of this sensing. GR, as a canonical TF, could be paired with the incorporation of promoter arrays to more readily assess specific binding\(^4\), or it could be coupled with nascent RNA imaging\(^1\) to examine the consequences on its target gene products. CTCF, as a regulator of chromatin architecture, could be paired with microscopy techniques that measure chromatin compaction\(^5\) to see if this is perturbed with changes in Zn\(^{2+}\). These tools will better allow us to understand precisely how changes in TF dynamics translate into changes in TF function.

Materials and methods

Plasmid generation. To generate PiggyBac-GR-HaloTag, PB-CMV-MCS-EF1α-Puro (System Bioscience #PB510B-1) was linearized using EcoRI and BamHI. The GR insert was amplified from pK7-GR-GFP (Addgene #15534) to generate overhangs with both PB-CMV-MCS-EF1α-Puro and the HaloTag. The HaloTag insert was amplified from pcDNA3.1-3xFLAG-HaloTag-2xNLS (Daniel Youmans, Cech lab, CU Boulder) using the primers listed in the Key Resources table to generate overhangs with both PB-CMV-MCS-EF1α-Puro and the HaloTag. Stable clones were selected by growing in DMEM containing 0.5 µg/µL puromycin (Sigma-Aldrich #P8833-25MG) for 7 days, after which they were transferred to normal DMEM.

Zinc perturbations. To manipulate labile Zn\(^{2+}\), cells were treated with either 30 µM ZnCl\(_2\) (Sigma-Aldrich #39059-100ML-F) or 50 µM Tris(2-pyridylmethyl)amine (TPA, Sigma-Aldrich #723134-250MG) for 30 min prior to imaging. For experiments involving HaloTag-GR, Zn\(^{2+}\) perturbations were followed by hormone activation with either 100 nM dexamethasone (Sigma-Aldrich #D4902-100MG) or 100 nM hydrocortisone (Sigma-Aldrich #H4001-1G).

3D single particle tracking. Three dimensional single particle tracking was performed on the Multifocus Microscope (MFM) at the Janelia Advanced Imaging Center (HHMI)\(^3\). Briefly, an epi-fluorescent microscope equipped with a multi-focal diffraction grating (MFG) allows for the collection of 9 aberration-corrected focal planes. Chromatic aberrations are corrected using a separate chromatic correction grating and prism. The MFG allows for a total axial detection depth of approximately 4 µm. For each experimental day, a calibration with TetraSpeck 0.2 um fluorescent beads (ThermoFisher #T7279) was used to determine the precise Z-spacing between each focal plane, with an average AZ of 430 nm. Additionally, the calibration allowed for measurement of the point spread function of the microscope, which allowed for image deconvolution (see below). All images were acquired using a 100 × 1.45 NA TIRF objective (Nikon), a 561 nm laser (Cobolt Jive 300, ), a Di01-R405/488/561/635 dichroic (Semrock), a FF01-593/40 (Semrock) emission filter, and an iXon3-DU897E EMCCD (Andor Technologies).

Image acquisition: Cells were labeled with 1 µM of JaneliaFluor (JF) 549 HaloTag ligand (Janelia Research Campus) for 5 min at 37 °C, rinsed three times with Dulbecco's phosphate-buffered saline (D-PBS), and then incubated for 30 min in FluoroBrite DMEM. Image acquisition was performed using a 561 nm laser at typical irradiance of 3-4 kW/cm², with 40 ms exposure times for an effective frame rate of 25 Hz. Movies, on average, were acquired for 2 min (3000 frames).

Post processing: Following acquisition, movies were cropped and laterally registered using a pre-determined affine transformation determined via TetraSpeck bead data (described above) to correct for the 3 × 3 image into a 9 Z-plane stack, spanning approximately 3.8 µm in the Z-dimension. To improve signal-to-noise ratio and particle localization, local background was subtracted using a rolling ball correction (radius = 7 px), followed by 5 iterations of the Richardson-Lucy deconvolution algorithm within MATLAB R2019a (Mathworks). Following deconvolution, images were smoothed using a Gaussian filter (radius = 0.7 px) to improve particle detection. Additionally, the first 500 frames of each movie were removed, as these tended to have dense labeling that did not allow for robust tracking.

3D particle tracking: Particle trajectories were generated using the MosaicSuite ImageJ plugin\(^5\) with the following parameters: radius = 3 px; cutoff = 0.001; threshold = 750; max link range = 1 frame; max displacement = 500 nm; dynamics = Brownian. Additionally, any tracks that did not exist for at least 10 frames (400 ms) were discarded.

Diffusion analysis: Diffusion coefficients for HaloTag-GR and HaloTag-CTCF were calculated by first computing the mean squared displacement (MSD) for each trajectory across the entire length of the track. The first 9 displacements (corresponding to the first 10 frames of each trajectory) of all tracks in each condition were then assembled into the final plasmid using a homemade Gibson assembly master mix.\(^2\)
subsequently averaged to generate an aggregate MSD curve across the population. This curve was then fit to the equation \( MSD = \gamma D \Delta t^\alpha \), where MSD is the mean squared displacement, \( \gamma \) is the number of dimensions (3) multiplied by 2, \( D \) is the apparent diffusion coefficient, \( \Delta t \) is the time delay between frames (here, 0.040 s), and \( \alpha \) defines whether the population exhibits superdiffusive (\( \alpha > 1 \)) or subdiffusive (\( \alpha < 1 \)) behavior. In addition to calculating the diffusion coefficients for each population, we further divided the population into trajectories exhibiting superdiffusive and subdiffusive behaviors by fitting the MSD curves of individual trajectories to the above equation and filtering according to the \( \alpha \) measured for each.

**2D single particle tracking.** Equipment: 2D single particle images were acquired on a Nikon N-STORM imaging system equipped with a Nikon Ti-E microscope, a Nikon CFI Apo TIRF 100X oil immersion objective (1.49 NA), a N-STORM 647 nm laser (Agilent), an iXon 897 Ultra EMCCD (Andor Technologies), and a cage incubator (Okolab).

Image acquisition: Cells were stained with JaneliaFluor (JF) 646 HaloTag ligand at either 100 pM (U-2 OS HaloTag-CTCF) for 1 min or 1 nM (U-2 OS HaloTag-GR) for 5 min, rinsed three times with D-PBS, and then incubated for 30 min in FluoroBrite DMEM prior to Zn\(^{2+} \) perturbations. Image acquisition occurred with low laser intensities (5–10%) and 100 ms exposure times. Frame rates were chosen to bias towards only detecting bound particles: for HaloTag-CTCF, effective frame rate = 2 Hz; for HaloTag-GR, effective frame rate = 10 Hz. Because CTCF is known to have long residence times on DNA, these movies were collected on average for 20 min (2400 frames), while movies for GR were typically acquired for 2–5 min.

Post processing: Movies were post-processed within Nikon Elements to subtract background using the rolling ball method (radius = 50), and then subjected to 5 iterations of the Richardson-Lucy deconvolution algorithm within Nikon Elements. Following deconvolution, images were smoothed using a Gaussian filter (radius = 0.7 px) to improve particle detection. Additionally, the first 500 frames of each movie were excluded, as these tended to have dense labeling that did not allow for robust tracking.

**2D particle tracking.** Particle trajectories were generated using the MosaicSuite ImageJ plugin with the following parameters: radius = 3 px; cutoff = 0; absolute threshold = 500–1000, depending on the experiment; max link range = 1 frame; max displacement = 300 nm; dynamics = Brownian. Additionally, any tracks that did not exist for at least 5 frames (2.5 s for CTCF and H2B; 0.5 s for HaloTag-GR) were excluded from further analysis.

**Dwell time analysis:** Because the effective frame rate of each movie and tracking parameters were biased towards only detecting bound molecules, we inferred that the only particles detected were bound. Therefore, we calculated dwell time as the length of the track divided by the frame rate, and the aggregate dwell times were used to generate dwell time survival curves. These were then fit to a biexponential decay \( f(t) = a_1 e^{-(k_1 t)} + a_2 e^{-(k_2 t)} \), where \( a_1 \) and \( a_2 \) are the fraction sizes of the two components and \( k_1 \) and \( k_2 \) are the off rates for non-specific and specific binding. For the traditional photobleaching correction of these data, we assumed that the calculated value \( k_2 \) is skewed due to photobleaching as previously noted. Therefore, we used H2B dwell time data acquired at the same frame rate (10 Hz for GR, 2 Hz for CTCF; Supplemental Figure S2) to estimate \( k_{bias} \), where \( k_{bias} = k_{2,GR}/k_{2,H2B} \). This then enabled us to calculate \( k_{2,true} = k_2 - k_{bias} \). Dwell times for each component were subsequently computed as \( t_{corrected} = \frac{t}{k_{bias}} \).

For fitting the dwell times to a power law, photobleaching was first corrected using H2B-HaloTag as previously described. Briefly, the raw H2B-HaloTag dwell time survival curves fit to a triple exponential of the form:

\[
P(\tau_{his} \geq t) = f_1 e^{-\gamma_1 t} + f_2 e^{-\gamma_2 t} + f_3 e^{-\gamma_3 t}
\]

Here, \( \gamma_1 \) and \( \gamma_2 \) are related to the dynamics of histones, but \( \gamma_3 \) corresponds to the rate of photobleaching. This subsequently allowed us to correct the survival distribution for a TF of interest, \( P(\tau_{TF} > t) \), by calculating:

\[
P(\tau_{TF,real} \geq t) = \frac{P(\tau_{TF} \geq t)}{e^{-\gamma_3 t}}
\]

The survival distribution was then fit to a Power law, \( f(t) = At^{-\beta} \), where \( \beta \) is proportional to the dwell time of the TF.

**Data availability**

The datasets generated and analyzed during the current study are not publicly available due the large size of the time-lapse imaging data but are available from the corresponding author on request.

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**Author contributions**
L.J.D. and A.E.P. conceived of the study. L.J.D. carried out experiments. J.A. assisted with multifocus microscopy data collection, processing and analysis. L.J.D. and A.E.P. analyzed data and wrote the paper. L.J.D., A.E.P. and J.A. edited the paper.

**Competing interests**
The authors declare no competing interests.

**Additional information**

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