Bulk-level maps of pioneer factor binding dynamics during Drosophila maternal-to-zygotic transition

Sadia Siddika Dima[a], Gregory T. Reeves[a,b*]

[a] Department of Chemical Engineering, Texas A&M University, College Station, TX 77843

[b] Faculty in Genetics and Genomics, Texas A&M University, College Station, TX 77843

[*] To whom correspondence should be addressed:

100 Spence Street

Texas A&M University, Chemical Engineering Dept

College Station, Texas 77843 MS 3122

979-862-4021

gtreeves@tamu.edu

Abstract

Gene regulation by transcription factors (TFs) binding cognate sequences is of paramount importance. For example, the TFs Zelda (Zld) and GAGA factor (GAF) are widely acknowledged for pioneering gene activation during zygotic genome activation (ZGA) in *Drosophila*. However, quantitative dose/response relationships between bulk TF concentration and the DNA binding, an event tied to transcriptional activity, remain elusive. Here, we map these relationships during ZGA: a crucial step in metazoan development. To map the dose/response relationship between nuclear concentration and DNA binding, we performed raster image correlation spectroscopy, a method that can measure concentration and binding of fluorescent molecules. We found that, although Zld concentration increases over time, its DNA binding in the transcriptionally active regions decreases, consistent with its function as an activator for early genes. In contrast, GAF DNA binding is nearly linear with its concentration, which sharply increases during the major wave, implicating it in the major wave. This study provides key insights into the properties of the two factors and puts forward a quantitative approach that can be used for other TFs to study transcriptional regulation.

Introduction

In *Drosophila*, ZGA begins with the transcription of a handful of genes during the minor wave of ZGA, followed by the major wave when thousands of genes are transcribed[1–4]. The TF Zld has the ability to bind nucleosomal DNA[5,6] and subsequently to facilitate the binding of other TFs[7–13]: the two defining features of a special class of TFs known as pioneer factors[14–16]. The maternally encoded TF GAF also possesses pioneer-like properties[10,11,17–20].

The role of TFs binding DNA for gene regulation is widely accepted in the context of development and homeostasis[21–23]. However, the relationship between the concentration of TFs such as these pioneer factors and gene expression remains an open question[24,25]. Fluorescent imaging of live and fixed tissues can give relative concentrations of TFs, which are often used to predict TF activity by assuming some nonlinear relationship between concentration and activity[26–32]. However, validation of these relationships using dynamic, quantitative data in live cells is currently lacking. Furthermore, the binding of the TFs to DNA –required for transcriptional regulation – is dependent

not only on their nuclear concentration, but also on factors such as chromatin accessibility and saturation kinetics. On the other hand, recent studies have identified hubs of TF binding at DNA sites, which may be more direct measures of TF activity[33–36]. However, the relationship between these hubs and the bulk TF concentration (experimentally accessible from fluorescence measurements) is unknown. Thus, knowing only total concentration is inadequate to predict TF binding, and direct measurements of TF hubs lack generalizability; an input/output map between the two, based on quantitative measurements of concentration and binding, is required.

To bridge this gap, we used raster image correlation spectroscopy (RICS) to quantify the concentration and binding of the pioneer-like factors Zld and GAF in live embryos over the course of nc 10-14[37–42]. These measurements allowed us to construct dose/response relationships which suggest that the binding of Zld to transcriptionally active sites is dependent on factors other than its concentrations alone, whereas the GAF concentration is the primary driver of its binding. Furthermore, we found that GAF must bind and saturate its sites in the inactive regions of the DNA before it can bind to active DNA sites, resulting in a delay in its role in ZGA. Similar approaches can be used to obtain comprehensive quantitative picture of dynamics of other TFs for gene regulation studies.

Results and discussion

Raster-scanned confocal images have a fast-scanning direction (pixel-to-pixel) and a slow-scanning direction (line retracing; Figure 1A). RICS analysis uses intensity fluctuations of GFP-tagged molecules, correlated in time and space, to build an autocorrelation function (ACF) in two dimensions, corresponding to the fast (Δx) and slow (Δy) scanning directions, respectively (Figure 1B; see Methods). The amplitude of the ACF, A, is inversely proportional to the GFP concentration (Figure 1C), while the shape of the ACF, especially in the slow direction, is determined by the fraction of GFP that is freely diffusible vs. that which is immobile (Figure 1D). Furthermore, cross-correlations in the fluctuations between GFP and RFP (tagged to His2Av) allows us to calculate the fraction of GFP-tagged molecules that are bound to the same structure as the His2Av-RFP (likely to be DNA[43,44]; Figure 1E). Therefore, RICS allows us to quantify not only the absolute concentration of GFP molecules, but also the fraction freely diffusible, the fraction immobile, and the fraction correlated to His2Av (Figure 1F).

Zld levels bound to DNA decrease while nuclear concentration increases

We performed RICS analysis on the nuclei of blastoderm-stage embryos expressing sfGFP-Zld [45] (Figure 2A and Video S1) to measure the dynamics of the nuclear concentration of Zld over time. We found the total nuclear concentration of Zld increased from one nuclear cycle to the next, suggesting an increase in the Zld levels with time (Figure 2B). Increase in Zld levels have been reported previously using immunoblotting[46,47]. Furthermore, the nuclear concentrations of Zld vary significantly within each nc, as more sfGFP-Zld enters nuclei after mitosis and fills it during the interphase. The longer duration of nc 14 allows Zld levels to reach a steady state, unlike the other, shorter ncs. In contrast, we observed that the nuclear Zld concentration drastically decreases from the end of one interphase to the beginning of the next (during mitosis), in agreement with previous observations that, unlike most pioneer factors, Zld is not mitotically retained on the chromosomes[36].

RICS analysis also allowed us to determine ϕ, the fraction of Zld which is immobile (or nearly so) due to either binding to immobile structures (such as DNA) or forming large aggregates having very low diffusivity (Equation 4). The immobile fraction of Zld remains nearly the same from nc 10-13, after which it reaches a steady state at a lower value of ~0.5 at nc 14 (Figure 2C). In each nc, at the beginning of interphase, the immobile concentration of Zld, calculated as the product of total nuclear concentration and the immobile fraction, increases perhaps because the chromatin decondenses and Zld binds the replicated DNA[5,18,48,49] and it starts decreasing after reaching a maximum as chromatin starts condensing before mitosis (Figure S1A).

We also used cross-correlation RICS (ccRICS) to determine ψ, the fraction of the pioneer factors that correlate with His2Av-RFP (Equation 9 in Methods). In *Drosophila*, the enrichment of the histone variant His2Av has been found to correlate with transcriptional potential[50,51], and therefore, the pool correlated with His2Av-RFP (hereafter referred to as the active pool) is likely responsible for pioneering gene activation. As the immobile fraction always measured as larger than the active fraction (Figures 2C, D), we inferred that the immobile fraction is composed of two pools: one that correlates with His2Av-RFP and one that does not. Hereafter, the pool that does not correlate with His2Av but is immobile, either due to binding to inactive chromatin regions or due to low

diffusivity from the formation of large clusters, is referred to as the inactive pool. We found the active fraction decreases from nuclear cycle to nuclear cycle (Figure 2D). Consequently, the active concentration of Zld, which is the product of the total nuclear concentration and the active fraction of Zld, also decreases, albeit weakly due to the increasing total nuclear concentration (Figure S1B). Roughly 5 min into nc 14, the concentration of active Zld reaches a steady value around 20-25 nM (Figure S1B).

Our measurements lend themselves to a general analysis in which the relationship between Zld total nuclear concentration and its binding could be constructed. According to standard thermodynamic equilibrium binding models (see Methods), an increase in the total Zld concentration should lead to an increase in the bound Zld concentration. However, our data suggest that the active concentration of Zld is a weakly *decreasing* function of total Zld (Figure 2E) in which nc 10-12 has a high active concentration of Zld despite the low total Zld concentration, while in nc 14, there is a high total Zld concentration and a slightly lower active concentration. As such, our results suggest that Zld binding is not influenced by Zld concentration alone, and additional factors, such as chromatin structure, must be taken into account. Using our data and ChIP-seq results reported by Harrison et al.[52], the dissociation constant (Kd) for Zld can be estimated to be ~17 nM (see Methods). Using this value, fitting a Hill function to our data suggests that the Zld sites accessible for binding reduce over time (Figure 2F), which might result from the chromatin becoming more densely packed during the later cycles[8,52,53]. On the other hand, the inactive concentration of Zld increases with time (Figure S1C), and thereby is correlated with the increase in total Zld concentration as well, and is functionally consistent with standard thermodynamic equilibrium binding models, suggesting the inactive fraction may result from binding that is saturating in nature. Fitting a Hill function with a Hill coefficient of 2 yielded the Kd of ~24 nM and a max concentration of 34 nM (Figure 2E). These values of Kd suggest that the binding of Zld to DNA or to immobile structures/aggregates is moderately strong. Overall, the dose/response relationship between total and bound Zld concentration is consistent with the previously-observed hubs of Zld bound to DNA. Zld molecules within these hubs have short residence times on DNA (on the order of seconds[34]), which is expected for our Kd measurements if either the local concentration of Zld within the hub[34], or the local Zld diffusivity[54], is high (see Methods).

Our data suggest that Zld may not retain its ability to pioneer chromatin accessibility throughout ZGA. Consistent with this, it was recently shown that, in larval type II neuroblasts, Zld binding is influenced by chromatin accessibility[55]. Thus, our observed decrease in the active concentration may imply that Zld primarily regulates transcription during the minor wave. Indeed, the removal of Zld activity affects early gene expression patterns, resulting in a delay in, but not complete loss of the transcription of patterning genes[47,56]. For example, in the Dorsal (Dl) network, the target genes *sna*, *twi*, *rho*, *brk*, *sog* are delayed in embryos lacking maternal Zld[47,56], and the removal of Zld binding sites from the *sog* neurogenic ectoderm enhancer results in delayed and sporadic *sog* expression[9,57]. Furthermore, mathematical models that account for decreasing Zld levels concurrent with increasing Dl levels with time[58–60], and can explain Dl target gene expression[27,61]. Therefore, our results suggest that, during the minor wave of ZGA (nc 10-13), a high concentration of bound Zld might be needed to facilitate the binding of low Dl levels. On the other hand, during the major wave at nc 14, either the high level of Dl, or the presence of other pioneer-like factors, may be sufficient to drive Dl binding and target gene expression when the bound Zld levels have decreased. This raises the question of whether other factors are present in during the major wave that may continue to facilitate the binding of other developmental TFs.

GAF levels increase suddenly in nc 14

GAF, encoded by the *Trithorax-like* (*Trl*) gene, plays an essential role in ZGA along with Zld[17,62]. GAF motifs along with Zld motifs are enriched in the highly occupied target (HOT) regions characterized by open chromatin and bound by many TFs[63–65]. GAF binds to clustered motifs[66–68] and possesses the properties of a pioneer factor, such as the ability to bind nucleosomal DNA and to create regions of chromatin accessibility by functioning with chromatin remodelers to facilitate the binding of other TFs[17,20,69–72].

To measure the dynamics of the nuclear concentration of GAF over time, we performed RICS analysis on the nuclei of blastoderm-stage embryos expressing GAF-sfGFP[17] (Figure 3A and Video S2). The results suggest that the total nuclear concentration of GAF remained nearly constant and very low (~5-10 nM) from nc 10-13, then showed a sudden increase during nc 14 to ~30 nM (Figure 3B). We saw that the nuclear concentration at the beginning of an interphase was

similar to that at the end of the previous interphase (Figure 3B). This observation is consistent with previous work showing GAF is mitotically retained on the chromosomes[17], and is in contrast to Zld (Figure 2B), which is not mitotically retained[36].

The immobile fraction of GAF remained nearly the same during nc 10-13, then decreased slightly during nc 14 (Figure 3C). The sudden increase in GAF total nuclear concentration, together with only a slight decrease in its immobile fraction, resulted in an increase in the immobile concentration of GAF during nc 14 (Figure S2A). Although the active fraction of GAF, the pool expected to be bound near the actively transcribed genes, remained approximately the same on average from nc 10-14, it varied significantly within each nc (Figure 3D).

As with Zld, we sought to use our data to map the relationship between GAF nuclear concentration and binding. We noted that, unlike Zld, both active and inactive concentrations of GAF increase with an increase in the total concentration of GAF (Figure 3E). Both pools appeared to be saturating functions of total GAF concentration, with the inactive pool saturating quickly at a low overall concentration (Figure 3E and Figure S2B-C). In contrast, the active pool required higher levels of total GAF to saturate and had a higher capacity. Fitting Hill functions to the data bore out these observations: the inactive pool had a Kd of 0.4 nM and a max concentration of 9 nM, while the active pool had a Kd of 5 nM and a max concentration of 35 nM. The Kd values represent a strong affinity of GAF for the binding sites, roughly 4-fold and 60-fold greater than for Zld, consistent with previous reports of stable GAF/DNA binding[70,73,74]. Interestingly, the higher affinity/lower capacity inactive pool may in part explain the sudden increase in the lower affinity/higher capacity active pool in nc 14. It appears that, as GAF concentration slowly increases, the majority is apportioned to the inactive pool due to its high affinity. However, because of the low capacity of the inactive pool, upon entering nc 14, the active pool is suddenly able to increase. If the active pool corresponds to transcriptionally active regions of the DNA (*i.e.*, euchromatin), the relative affinities and capacities ensure that GAF acts as a pioneer factor solely during the later, major wave of ZGA. Furthermore, it has been shown that GAF associates to the GA/CT-rich repeats in the heterochromatin regions throughout cell cycles[68,75], potentially driving transcriptional silencing and the euchromatin association drives activation during ZGA[68]. As such, the inactive pool may represent GAF bound to heterochromatin.

Our measurements of the biophysical parameters of the pioneer factors and their dynamics allowed the construction of input/output maps between total concentration of the factors and their DNA binding. While a subset of the pioneer factor bound regions has been found to stay inaccessible[76,77], binding of pioneer factors to DNA is an indispensable step for establishing and maintaining chromatin accessibility. Using the input/output maps we can infer that Zld acts as a global activator of early genes enabled by the high active concentration of Zld during the minor wave of ZGA, and GAF comes into play during the major wave, as indicated by the high active concentration of GAF during this time (Figure 4). This is supported by the fact that the regions that gain accessibility early during ZGA are enriched for Zld binding, whereas the ones that gain accessibility late are enriched for GAF binding[18]. A similar quantitative approach, in which accurate measurements of biophysical parameters of TFs and their dynamics enable the construction of input/output map between TF concentration and transcriptional activity, is expected to aid other gene expression studies.

Acknowledgements

We thank Melissa M. Harrison for kindly providing fly stocks and for helpful discussion of the manuscript. This work was supported in part by NSF grant MCB-2105619.

Author contributions

S.S.D and G.T.R designed the experiments and wrote the manuscript. S.S.D conducted the experiments, made Zld and GAF mathematical models. G.T.R developed the RICS analysis pipeline.

Declaration of interests

The authors declare no competing interests.

Methods

***Drosophila* strains**

The fly strain used for the Zld imaging is *sfGFP-zld; His2Av-RFP (II).* The *sfGFP-zld* mutant allele was generated using Cas9-mediated genome engineering by Hamm et al.[45] The fly strain used for the GAF imaging is *His2Av-RFP (II); GAF-sfGFP(C) (III)*. The *GAF-sfGFP(C) (III)* mutant allele was generated using Cas9-mediated genome engineering by Gaskill, Gibson et al.[17]

Sample preparation

Flies were raised on standard cornmeal-molasses-yeast medium at 25°C. Fly cages were prepared with desired fly strains and kept at room temperature for 2 days before imaging. Grape juice agar plates streaked with yeast paste were placed onto the bottoms of the cages for oviposition. For imaging, the flies were allowed to lay eggs for 1 hour after which the plates were removed for embryo collection. The embryos were transferred from the plates to mesh baskets, dechorionated using bleach for 30 s, and washed with deionized water to remove residual bleach[78].

Live imaging

After dechorionation, the embryos were mounted in 1% low melting point agarose (IBI Scientific, IB70051) in deionized water on a glass bottom Petri dish (MatTek, P35G-1.5-20-C) and deionized water was poured into the Petri dish over the solidified low melting point agarose to cover the samples. The low melting point agarose rendered mechanical stability to the embryos while remaining transparent submerged in water[79,80]. All images were collected on an LSM 900 (Carl Zeiss, Germany) confocal laser scanning microscope. For the image acquisitions, C-Apochromat 40x/1.2 water immersion Korr objective, 488 nm laser for sfGFP, 561 nm laser for RFP, and GaAsP-PMT detector were used. The detector was operated at 650 V with 1x gain and 0% offset. Emission was detected in the range of 410-546 nm for sfGFP and 595-700 nm for RFP. All the images had 1024 × 1024 pixels resolution. The images were collected at 5x zoom, resulting in pixel size of 31.95 nm, and a pixel dwell time of 2.06 µs, which corresponds to a frame time of 5.06 s. Image acquisition was started when the embryos were at nc 10 and continued till gastrulation as indicated by the nuclear morphology.

Raster image correlation spectroscopy (RICS) image analysis

RICS analysis, a derivative of fluctuation correlation spectroscopy, entails constructing autocorrelation functions (ACFs) from imaging data and fitting ACF models to these data-derived ACFs. We performed these analyses according to previous protocol
s[40,42]. In brief, live imaging time courses were divided into groups of 7-12 frames. The frames within a group were averaged together to create an averaged frame for that group, which was used for two purposes. First, the nuclei in the averaged frame were segmented according to a watershed algorithm. This segmented nuclear mask was used for each frame in the group. Second, the immobile variation within each frame was then removed by subtracting by the averaged frame on a pixel-by-pixel basis, then the scalar average intensity value of the averaged frame was added back [37,38]. Two dimensional (2D) RICS autocorrelation functions of the nuclear fraction of each frame were built using a fast Fourier transform protocol in Matlab, and these ACFs were averaged together for all frames in a given group. The result was a time series of 2D ACFs, each of which corresponded to a given grouping of 7-12 frames.

Fitting RICS autocorrelation functions (ACFs) to estimate concentration and mobility

RICS autocorrelation functions generally contain two orthogonal pieces of information: the amplitude determines the concentration of the species and the shape determines the mobility. For a purely diffusing species, the theoretical, normalized ACF, $\hat{G}(\Delta x, \Delta y)$, is the following:

$$\hat{G}(\Delta x, \Delta y) = \left(\frac{1}{1 + \frac{4D(\tau_p \Delta x + \tau_\ell \Delta y)}{w_0^2}} \right) \sqrt{\frac{1}{1 + \frac{4D(\tau_p \Delta x + \tau_\ell \Delta y)}{w_z^2}}} \exp\left(-\frac{(\Delta x^2 + \Delta y^2)\Delta r^2}{w_0^2 + 4D(\tau_p \Delta x + \tau_\ell \Delta y)} \right). \qquad (1)$$

where D is the diffusivity. This equation has five microscope parameters: w_0 and w_z are the radii of the point spread function (PSF) in the xy plane and the axial (z) direction, respectively; Δr is the xy size of a pixel; and τ_p and τ_ℓ are the pixel dwell time (determined by the scan speed) and line time (determined by a combination of the scan speed and number of pixels in the width of the image), respectively. The two independent variables, Δx and Δy, are the pixel shifts in the fast and slow directions, respectively.

The non-normalized ACF includes the amplitude A, which is equal to the reciprocal of $\bar{n}$ (the average number of molecules in the confocal volume):

$$G(\Delta x, \Delta y) = A\hat{G}(\Delta x, \Delta y). \qquad (2)$$

In practice, $A = \gamma/\bar{n}$, where $\gamma = \sqrt{2}/4$ is a factor that accounts for the uneven illumination airy unit[41]. Note that $\bar{n}$ can be converted to $\bar{c}$, the average concentration in the confocal volume, V_{PSF}, by $\bar{n} = V_{PSF}\bar{c}$, where $V_{PSF} = \pi^{\frac{3}{2}} w_0^2 w_z$.

The 2D ACFs for sfGFP-Zld or GAF-sfGFP for each time point were then used to fit two different models. First, the fast direction cut of the 2D ACF, $G_s(\Delta x, 0)$, was used to fit a Gaussian equation that approximates the microscope's point spread function (PSF). Because $\Delta y = 0$ along this cut, and $4D\tau_p\, \Delta x/w_z^2 \ll 1$, Equations (1-2) simplify to:

$$G(\Delta x, 0) \approx (A - B) \exp\left(-\frac{\Delta x^2 \Delta r^2}{w_0^2}\right) + B, \qquad (3)$$

where, for robustness of fit, a small, adjustable background constant, B, was added and w_0 was allowed to vary slightly. This first fitting step resulted in accurate estimates of the ACF amplitude, A.

Second, holding A fixed, the entire 2D ACF was then used to fit a two component model, $G_{2c}(\Delta x, \Delta y)$, which is a linear combination of a freely diffusing fraction and an immobile fraction[42]:

$$\hat{G}_{2c}(\Delta x, \Delta y) = \phi \hat{G}_{bind}(\Delta x, \Delta y) + (1 - \phi)\hat{G}_{diff}(\Delta x, \Delta y), \qquad (4)$$

where $\hat{G}_i(\Delta x, \Delta y)$ is given by Equation 1 (with $\hat{G}_{bind}(\Delta x, \Delta y)$ having $D = 0$ and $\hat{G}_{diff}(\Delta x, \Delta y)$ having D non-zero; see Figure 1D), the linear combination weight, ϕ, is the fraction immobile, and $\hat{G}_{2c}$ is the normalized two-component ACF, such that:

$$G_{2c}(\Delta x, \Delta y) = (A - B)\hat{G}_{2c}(\Delta x, \Delta y) + B. \qquad (5)$$

Note that the parameter B in Equation 5 may have a different value from the one found in Equation 3. The 2D ACF for His2Av-RFP for each time point was used to fit only the 2D PSF to get a measure of the ACF amplitude.

Fitting ccRICS cross-correlation functions (CCFs) to estimate correlated binding

Like the 2D ACF, the 2D cross correlation function (CCF) between either sfGFP-Zld or GAF-sfGFP and His2Av-RFP was computed through a fast Fourier transform protocol in Matlab. This 2D CCF was then used to fit a 2D model of cross correlation[42]:

$$G_{cc}(\Delta x, \Delta y) = (A_{cc} - B)\exp\left(-\frac{(\Delta x \Delta r - d_x)^2 + \left(\Delta y \Delta r - d_y\right)^2}{\widehat{w}_0^2}\right) + B. \qquad (6)$$

Because cross correlation uses two lasers, the two PSFs are different in size and their centers are generally not concurrent. In Equation 6, the parameters d_x, d_y represent the displacements between the centers of the two PSFs in the *xy* plane. The axial displacement is predicted to be small and was ignored. Fitting Equation 6 to the CCF data resulted in an estimate of the CCF amplitude, which is defined as:

$$A_{cc} = \frac{\bar{c}_2}{\pi^{3/2}\widehat{w}_0^2\widehat{w}_z(\bar{c}_1 + \bar{c}_2)(\bar{c}_2 + \bar{c}_3)}, \qquad (7)$$

where the average PSF sizes are defined as $\widehat{w}_0^2 = 0.5\left(w_{0,g}^2 + w_{0,r}^2\right)$ and $\widehat{w}_z^2 = 0.5\left(w_{z,g}^2 + w_{z,r}^2\right)$, and the subscripts g and r denote the green and red channels, respectively, and where $\bar{c}_1$ is the average concentration of the sfGFP-containing species that does not cross-correlate with the RFP-containing species (in the rest of the paper, this is the free pool plus the inactive pool); $\bar{c}_2$ is the average concentration of the active pool, and $\bar{c}_3$ is the average concentration of the RFP-containing species that does not cross-correlate with the sfGFP-containing species.

To obtain ψ, the fraction of sfGFP-Zld or GAF-sfGFP correlated to His2Av-RFP, we calculate the ACF amplitude of the red channel, $G_{red}(\Delta x, \Delta y)$, then use it to fit Equation 3, yielding the amplitude, $G_{red}(0,0) = A_{red} = 1/\left(\pi^{3/2}w_{0,r}^2 w_{z,r}(\bar{c}_2 + \bar{c}_3)\right)$. Then the ratio of A_{cc} to A_{red} gives:

$$\frac{A_{cc}}{A_{red}} = \frac{w_{0,r}^2 w_{z,r}}{\widehat{w}_0^2\widehat{w}_z}\frac{\bar{c}_2}{\bar{c}_1 + \bar{c}_2} = \frac{w_{0,r}^2 w_{z,r}}{\widehat{w}_0^2\widehat{w}_z}\psi. \qquad (8)$$

Therefore, we can calculate ψ as:

$$\psi = \frac{\widehat{w}_0^2\widehat{w}_z}{w_{0,r}^2 w_{z,r}}\frac{A_{cc}}{A_{red}}. \qquad (9)$$

Thermodynamic equilibrium model

When the binding of a TF to DNA is in equilibrium, the probability of the TF binding can be modeled as[81]:

$$P\{TF\ bound\} = \frac{C_{free}}{C_{free} + K_D}. \qquad (10)$$

where C_{free} is the nuclear concentration of the free TF, K_D is the dissociation constant, $P\{TF\ bound\} = C_{bound}/C_B$, C_{bound} is the concentration of the bound TF, and C_B is the concentration of binding sites.

The equation was solved and adjustable parameters (dissociation constant and binding site concentrations) were determined using the least-squares fitting algorithm, lsqcurvefit, and the global optimization solver, MultiStart in Matlab.

To express the concentration of active or inactive bound TF as a function of total nuclear concentration, we used the conservation relation between different pools of the TF:

$$C_{tot} = C_{free} + C_{active} + C_{inactive}, \qquad (11)$$

where C_{tot} is the total nuclear concentration, C_{active} is the concentration of the active pool, and $C_{inactive}$ is the concentration of the inactive pool.

Using Equation (10) this can be written as:

$$C_{tot} = C_{free} + \frac{C_{free}C_{B_a}}{C_{free} + K_{D_a}} + \frac{C_{free}C_{B_i}}{C_{free} + K_{D_i}}, \qquad (12)$$

where C_{B_a} and C_{B_i} are the concentrations of binding sites for the active and inactive pool respectively. K_{D_a} and K_{D_i} are the dissociation constants for the active and inactive pool respectively.

In the case of Zld, C_{B_a} can be expressed as a function of C_{free} (see Figure S1D). Empirically, we modeled this relationship as a power law:

$$C_{B_a} = aC_{free}^{-k}, \qquad (13)$$

where $a = 420$ and $k = 0.7$ were the best-fit parameters (see Figure S1D). The value of C_{B_a} from Equation (13) can be substituted into Equation (12). Equation (12) can then be used to determine the concentrations of free TF corresponding to a given total concentration to construct the dose/response maps in Figs. 2E, 3E. For these calculations, nc 10 is ignored due to its short

duration and because data points near the start of each nc are excluded due to equilibrium not being established during these time points.

Zld accessible sites

Pooled data from nc 8, 13, and 14 identified 12,135 Zld ChIP-Seq peaks[52]. We assumed that, during the early ncs, the chromatin is fairly open and all the ChIP-Seq identified sites are accessible for binding, such that the number of Zld sites accessible for binding during nc 11 is also assumed to be 12,135. Given that the nuclear volume during nc 11 is roughly $435\ \mu m^3$;[82], the concentration of Zld sites accessible for binding, averaged over the whole nucleus, is roughly, L = $\frac{12135\ peaks}{435\ \mu m^3} = 46.34\ nM$. However, given that *Drosophila* is diploid, we set L = 2×46.34 = 92.68 nM. Using these estimates, Equation (10), and our data for nc 11, we arrive at K_D = 17 nM. Using this K_D and our data in Equation (10), the change in Zld sites accessible for binding over time was estimated.

Residence time of Zld

Dissociation constants are typically defined as $K_D = \frac{k_{off}}{k_{on}}$; where k_{on} and k_{off} are the on and off rates for the TF-immobile structure interaction. For protein binding events in which the on-rate is limited by three dimensional diffusion, k_{on} is roughly 10^{-4} $nM^{-1}s^{-1}$ (ref [83]). Using this rule of thumb for the k_{on} value, and $K_D \sim 20\ nM$ (valid for both the active and inactive pools of Zld), the residence time, $\tau = \frac{1}{k_{off}} = \frac{1}{k_{on}K_D}$ can be estimated to be: $\tau \sim 8\ min$. However, if the diffusivity is higher, as would be the case of one-dimensional sliding along the DNA[54], k_{on} is closer to 10^{-1} $nM^{-1}s^{-1}$, resulting in $\tau \sim 0.5\ s$. Furthermore, if the local concentration of Zld is increased, as would be the case inside the Zld hubs localized to DNA, a transient interaction ($\tau \sim 5\ s$)[34] could appear more stable with respect to the bulk concentration.

Supplemental information

Document S1. Figures S1–S2

Figure S1. Concentration of different pools of Zld, related to Figure 2

Figure S2. Concentration of different pools of GAF, related to Figure 3

Video S1. Live imaging of sfGFP-Zld embryo from nc 10 to nc 14 used for RICS analysis, related to Figure 2. Acquisitions for the interphases of different ncs have been concatenated to generate the video, as mitoses were not used for the analysis.

Video S2. Live imaging of GAF-sfGFP embryo from nc 10 to nc 14 used for RICS analysis, related to Figure 3. Acquisitions for the interphases of different ncs have been concatenated to generate the video, as mitoses were not used for the analysis.

References

1. Pritchard, D.K., and Schubiger, G. (1996). Activation of transcription in Drosophila embryos is a gradual process mediated by the nucleocytoplasmic ratio. Genes Dev *10*, 1131–1142. https://doi.org/10.1101/gad.10.9.1131.
2. Schulz, K.N., and Harrison, M.M. (2019). Mechanisms regulating zygotic genome activation. Nat Rev Genet *20*, 221–234. https://doi.org/10.1038/s41576-018-0087-x.
3. Harrison, M.M., Marsh, A.J., and Rushlow, C.A. (2023). Setting the stage for development: the maternal-to-zygotic transition in Drosophila. Genetics *225*, iyad142.
4. Vastenhouw, N.L., Cao, W.X., and Lipshitz, H.D. (2019). The maternal-to-zygotic transition revisited. Development *146*, dev161471.
5. McDaniel, S.L., Gibson, T.J., Schulz, K.N., Fernandez Garcia, M., Nevil, M., Jain, S.U., Lewis, P.W., Zaret, K.S., and Harrison, M.M. (2019). Continued Activity of the Pioneer Factor Zelda Is Required to Drive Zygotic Genome Activation. Mol Cell *74*, 185-195.e4. https://doi.org/10.1016/j.molcel.2019.01.014.
6. Garcia, M.F., Moore, C.D., Schulz, K.N., Alberto, O., Donague, G., Harrison, M.M., Zhu, H., and Zaret, K.S. (2019). Structural features of transcription factors associating with nucleosome binding. Mol Cell *75*, 921–932.
7. Satija, R., and Bradley, R.K. (2012). The TAGteam motif facilitates binding of 21 sequence-specific transcription factors in the Drosophila embryo. Genome Res *22*, 656–665. https://doi.org/10.1101/gr.130682.111.
8. Li, X.Y., Harrison, M.M., Villalta, J.E., Kaplan, T., and Eisen, M.B. (2014). Establishment of regions of genomic activity during the Drosophila maternal to zygotic transition. Elife *3*, 1–20. https://doi.org/10.7554/eLife.03737.
9. Foo, S.M., Sun, Y., Lim, B., Ziukaite, R., O'Brien, K., Nien, C.Y., Kirov, N., Shvartsman, S.Y., and Rushlow, C.A. (2014). Zelda potentiates morphogen activity by increasing chromatin accessibility. Current Biology *24*, 1341–1346. https://doi.org/10.1016/j.cub.2014.04.032.
10. Sun, Y., Nien, C.Y., Chen, K., Liu, H.Y., Johnston, J., Zeitlinger, J., and Rushlow, C. (2015). Zelda overcomes the high intrinsic nucleosome barrier at enhancers during Drosophila zygotic genome activation. Genome Res *25*, 1703–1714. https://doi.org/10.1101/gr.192542.115.
11. Schulz, K.N., Bondra, E.R., Moshe, A., Villalta, J.E., Lieb, J.D., Kaplan, T., McKay, D.J., and Harrison, M.M. (2015). Zelda is differentially required for chromatin accessibility, transcription factor binding, and gene expression in the early Drosophila embryo. Genome Res *25*, 1715–1726. https://doi.org/10.1101/gr.192682.115.
12. Yáñez-Cuna, J.O., Dinh, H.Q., Kvon, E.Z., Shlyueva, D., and Stark, A. (2012). Uncovering cis-regulatory sequence requirements for context-specific transcription factor binding. Genome Res *22*, 2018–2030. https://doi.org/10.1101/gr.132811.111.
13. Xu, Z., Chen, H., Ling, J., Yu, D., Struffi, P., and Small, S. (2014). Impacts of the ubiquitous factor Zelda on Bicoid-dependent DNA binding and transcription in Drosophila. Genes Dev *28*, 608–621. https://doi.org/10.1101/gad.234534.113.
14. Iwafuchi-Doi, M., and Zaret, K.S. (2014). Pioneer transcription factors in cell reprogramming. Genes Dev *28*, 2679–2692. https://doi.org/10.1101/gad.253443.114.
15. Zaret, K.S., and Carroll, J.S. (2011). Pioneer transcription factors: Establishing competence for gene expression. Genes Dev *25*, 2227–2241. https://doi.org/10.1101/gad.176826.111.

16. Zaret, K.S., and Mango, S.E. (2016). Pioneer transcription factors, chromatin dynamics, and cell fate control. Curr Opin Genet Dev *37*, 76–81. https://doi.org/10.1016/j.gde.2015.12.003.
17. Gaskill, M.M., Gibson, T.J., Larson, E.D., and Harrison, M.M. (2021). GAF is essential for zygotic genome activation and chromatin accessibility in the early Drosophila embryo. Elife *10*, 1–30. https://doi.org/10.7554/eLife.66668.
18. Blythe, S.A., and Wieschaus, E.F. (2016). Establishment and maintenance of heritable chromatin structure during early Drosophila embryogenesis. Elife *5*, e20148. https://doi.org/10.7554/eLife.20148.
19. Blythe, S.A., and Wieschaus, E.F. (2015). Zygotic genome activation triggers the DNA replication checkpoint at the midblastula transition. Cell *160*, 1169–1181. https://doi.org/10.1016/j.cell.2015.01.050.
20. Judd, J., Duarte, F.M., and Lis, J.T. (2021). Pioneer-like factor GAF cooperates with PBAP (SWI/SNF) and NURF (ISWI) to regulate transcription. Genes Dev *35*, 147–156. https://doi.org/10.1101/GAD.341768.120.
21. Spitz, F., and Furlong, E.E.M. (2012). Transcription factors: from enhancer binding to developmental control. Nat Rev Genet *13*, 613–626.
22. Weidemüller, P., Kholmatov, M., Petsalaki, E., and Zaugg, J.B. (2021). Transcription factors: Bridge between cell signaling and gene regulation. Proteomics *21*, 2000034.
23. Upadhyaya, A. (2024). Transcription factor dynamics in gene expression—the long and short of it. Biophys J *123*, 8a.
24. Kim, H.D., and O'shea, E.K. (2008). A quantitative model of transcription factor–activated gene expression. Nat Struct Mol Biol *15*, 1192–1198.
25. Ay, A., and Arnosti, D.N. (2011). Mathematical modeling of gene expression: a guide for the perplexed biologist. Crit Rev Biochem Mol Biol *46*, 137–151.
26. Manu, Surkova, S., Spirov, A. V, Gursky, V. V, Janssens, H., Kim, A.-R., Radulescu, O., Vanario-Alonso, C.E., Sharp, D.H., and Samsonova, M. (2009). Canalization of gene expression in the Drosophila blastoderm by gap gene cross regulation. PLoS Biol *7*, e1000049.
27. Kanodia, J.S., Liang, H.L., Kim, Y., Lim, B., Zhan, M., Lu, H., Rushlow, C.A., and Shvartsman, S.Y. (2012). Pattern formation by graded and uniform signals in the early Drosophila embryo. Biophys J *102*, 427–433. https://doi.org/10.1016/j.bpj.2011.12.042.
28. O'Connell, M.D., and Reeves, G.T. (2015). The presence of nuclear cactus in the early Drosophila embryo may extend the dynamic range of the dorsal gradient. PLoS Comput Biol *11*, e1004159.
29. Jaeger, J., Surkova, S., Blagov, M., Janssens, H., Kosman, D., Kozlov, K.N., Manu, n, Myasnikova, E., Vanario-Alonso, C.E., and Samsonova, M. (2004). Dynamic control of positional information in the early Drosophila embryo. Nature *430*, 368–371.
30. Papatsenko, D., and Levine, M. (2005). Gene Regulatory Networks Special Feature: Quantitative analysis of binding motifs mediating diverse spatial readouts of the Dorsal gradient in the Drosophila embryo. Proc Natl Acad Sci U S A *102*, 4966.
31. Zinzen, R.P., Senger, K., Levine, M., and Papatsenko, D. (2006). Computational models for neurogenic gene expression in the Drosophila embryo. Current Biology *16*, 1358–1365.
32. Papatsenko, D., and Levine, M. (2011). The Drosophila gap gene network is composed of two parallel toggle switches. PLoS One *6*, e21145.

33. Mir, M., Reimer, A., Haines, J.E., Li, X.-Y., Stadler, M., Garcia, H., Eisen, M.B., and Darzacq, X. (2017). Dense Bicoid hubs accentuate binding along the morphogen gradient. Genes Dev *31*, 1784–1794.
34. Mir, M., Stadler, M.R., Ortiz, S.A., Hannon, C.E., Harrison, M.M., Darzacq, X., and Eisen, M.B. (2018). Dynamic multifactor hubs interact transiently with sites of active transcription in Drosophila embryos. Elife *7*, e40497.
35. Yamada, S., Whitney, P.H., Huang, S.K., Eck, E.C., Garcia, H.G., and Rushlow, C.A. (2019). The Drosophila Pioneer Factor Zelda Modulates the Nuclear Microenvironment of a Dorsal Target Enhancer to Potentiate Transcriptional Output. Current Biology *29*, 1387-1393.e5. https://doi.org/10.1016/j.cub.2019.03.019.
36. Dufourt, J., Trullo, A., Hunter, J., Fernandez, C., Lazaro, J., Dejean, M., Morales, L., Nait-Amer, S., Schulz, K.N., Harrison, M.M., et al. (2018). Temporal control of gene expression by the pioneer factor Zelda through transient interactions in hubs. Nat Commun *9*, 1–13. https://doi.org/10.1038/s41467-018-07613-z.
37. Digman, M.A., Brown, C.M., Sengupta, P., Wiseman, P.W., Horwitz, A.R., and Gratton, E. (2005). Measuring fast dynamics in solutions and cells with a laser scanning microscope. Biophys J *89*, 1317–1327. https://doi.org/10.1529/biophysj.105.062836.
38. Digman, M.A., Sengupta, P., Wiseman, P.W., Brown, C.M., Horwitz, A.R., and Gratton, E. (2005). Fluctuation correlation spectroscopy with a laser-scanning microscope: Exploiting the hidden time structure. Biophys J *88*, L33–L36. https://doi.org/10.1529/biophysj.105.061788.
39. Digman, M.A., and Gratton, E. (2009). Analysis of diffusion and binding in cells using the RICS approach. Microsc Res Tech *72*, 323–332.
40. Schloop, A.E., Hiremath, S.V., Shaikh, R., Williams, C.M., and Reeves, G.T. (2024). Spatiotemporal dynamics of NF-κB/Dorsal inhibitor IκBα/Cactus in Drosophila blastoderm embryos. bioRxiv, 2022–2024.
41. Brown, C.M., Dalal, R.B., Hebert, B., Digman, M.A., Horwitz, A.R., and Gratton, E. (2008). Raster image correlation spectroscopy (RICS) for measuring fast protein dynamics and concentrations with a commercial laser scanning confocal microscope. J Microsc *229*, 78–91. https://doi.org/10.1111/j.1365-2818.2007.01871.x.
42. Al Asafen, H., Clark, N.M., Goyal, E., Jacobsen, T., Siddika Dima, S., Chen, H.-Y., Sozzani, R., and Reeves, G.T. Dorsal/NF-κB exhibits a dorsal-to-ventral mobility gradient in the Drosophila embryo. https://doi.org/10.1101/320754.
43. Bacia, K., and Schwille, P. (2007). Practical guidelines for dual-color fluorescence cross-correlation spectroscopy. Nat Protoc *2*, 2842–2856.
44. Weidemann, T., Wachsmuth, M., Tewes, M., Rippe, K., and Langowski, J. (2002). Analysis of ligand binding by two-colour fluorescence cross-correlation spectroscopy. Single Molecules *3*, 49–61. https://doi.org/10.1002/1438-5171(200204)3:1<49::AID-SIMO49>3.0.CO;2-T.
45. Hamm, D.C., Larson, E.D., Nevil, M., Marshall, K.E., Bondra, E.R., and Harrison, M.M. (2017). A conserved maternal-specific repressive domain in Zelda revealed by Cas9-mediated mutagenesis in Drosophila melanogaster. PLoS Genet *13*, 1–22. https://doi.org/10.1371/journal.pgen.1007120.
46. Harrison, M.M., Botchan, M.R., and Cline, T.W. (2010). Grainyhead and Zelda compete for binding to the promoters of the earliest-expressed Drosophila genes. Dev Biol *345*, 248–255.

47. Nien, C.Y., Liang, H.L., Butcher, S., Sun, Y., Fu, S., Gocha, T., Kirov, N., Manak, J.R., and Rushlow, C. (2011). Temporal coordination of gene networks by Zelda in the early Drosophila embryo. PLoS Genet *7*. https://doi.org/10.1371/journal.pgen.1002339.
48. Yuan, K., Shermoen, A.W., and O'Farrell, P.H. (2014). Illuminating DNA replication during Drosophila development using TALE-lights. Preprint, https://doi.org/10.1016/j.cub.2014.01.023 https://doi.org/10.1016/j.cub.2014.01.023.
49. Antonin, W., and Neumann, H. (2016). Chromosome condensation and decondensation during mitosis. Curr Opin Cell Biol *40*, 15–22.
50. Mavrich, T.N., Jiang, C., Ioshikhes, I.P., Li, X., Venters, B.J., Zanton, S.J., Tomsho, L.P., Qi, J., Glaser, R.L., Schuster, S.C., et al. (2008). Nucleosome organization in the Drosophila genome. Nature *453*, 358–362. https://doi.org/10.1038/nature06929.
51. Leach, T.J., Mazzeo, M., Chotkowski, H.L., Madigan, J.P., Wotring, M.G., and Glaser, R.L. (2000). Histone H2A.Z is widely but nonrandomly distributed in chromosomes of Drosophila melanogaster. Journal of Biological Chemistry *275*, 23267–23272. https://doi.org/10.1074/jbc.M910206199.
52. Harrison, M.M., Li, X.Y., Kaplan, T., Botchan, M.R., and Eisen, M.B. (2011). Zelda binding in the early Drosophila melanogaster embryo marks regions subsequently activated at the maternal-to-zygotic transition. PLoS Genet *7*. https://doi.org/10.1371/journal.pgen.1002266.
53. Lowenhaupt, K., Cartwright, I.L., Keene, M.A., Zimmerman, J.L., and Elgin, S.C.R. (1983). Chromatin structure in pre-and postblastula embryos of Drosophila. Dev Biol *99*, 194–201.
54. Mirny, L., Slutsky, M., Wunderlich, Z., Tafvizi, A., Leith, J., and Kosmrlj, A. (2009). How a protein searches for its site on DNA: the mechanism of facilitated diffusion. J Phys A Math Theor *42*, 434013.
55. Larson, E.D., Komori, H., Gibson, T.J., Ostgaard, C.M., Hamm, D.C., Schnell, J.M., Lee, C.Y., and Harrison, M.M. (2021). Cell-type-specific chromatin occupancy by the pioneer factor Zelda drives key developmental transitions in Drosophila. Nat Commun *12*. https://doi.org/10.1038/s41467-021-27506-y.
56. Liang, H.-L., Nien, C.-Y., Liu, H.-Y., Metzstein, M.M., Kirov, N., and Rushlow, C. (2008). The zinc-finger protein Zelda is a key activator of the early zygotic genome in Drosophila. Nature *456*, 400–403. https://doi.org/10.1038/nature07388.
57. Liberman, L.M., and Stathopoulos, A. (2009). Design flexibility in cis-regulatory control of gene expression: synthetic and comparative evidence. Dev Biol *327*, 578–589.
58. DeLotto, R., DeLotto, Y., Steward, R., and Lippincott-Schwartz, J. (2007). Nucleocytoplasmic shuttling mediates the dynamic maintenance of nuclear Dorsal levels during Drosophila embryogenesis.
59. Reeves, G.T., Trisnadi, N., Truong, T. V., Nahmad, M., Katz, S., and Stathopoulos, A. (2012). Dorsal-Ventral Gene Expression in the Drosophila Embryo Reflects the Dynamics and Precision of the Dorsal Nuclear Gradient. Dev Cell *22*, 544–557. https://doi.org/10.1016/j.devcel.2011.12.007.
60. Liberman, L.M., Reeves, G.T., and Stathopoulos, A. (2009). Quantitative imaging of the Dorsal nuclear gradient reveals limitations to threshold-dependent patterning in Drosophila. Proceedings of the National Academy of Sciences *106*, 22317–22322.
61. Bandodkar, P.U., Al Asafen, H., and Reeves, G.T. (2020). Spatiotemporal control of gene expression boundaries using a feedforward loop. Developmental Dynamics *249*, 369–382.

62. Farkas, G., Gausz, J., Galloni, M., Reuter, G., Gyurkovics, H., and Karch, F. (1994). The Trithorax-like gene encodes the Drosophila GAGA factor. Nature *371*, 806–808.
63. Kvon, E.Z., Stampfel, G., Yáñez-Cuna, J.O., Dickson, B.J., and Stark, A. (2012). HOT regions function as patterned developmental enhancers and have a distinct cis-regulatory signature. Genes Dev *26*, 908–913.
64. Slattery, M., Ma, L., Spokony, R.F., Arthur, R.K., Kheradpour, P., Kundaje, A., Negre, N., Crofts, A., Ptashkin, R., and Zieba, J. (2014). Diverse patterns of genomic targeting by transcriptional regulators in Drosophila melanogaster. Genome Res *24*, 1224–1235.
65. Consortium, T., Roy, S., Ernst, J., Kharchenko, P. V, Kheradpour, P., Negre, N., Eaton, M.L., Landolin, J.M., Bristow, C.A., Ma, L., et al. (2011). Identification of Functional Elements and Regulatory Circuits by Drosophila modENCODE. Science (1979) *330*, 1787–1797. https://doi.org/10.1126/science.1198374.Identification.
66. Katsani, K.R., Hajibagheri, M.A.N., and Verrijzer, C.P. (1999). Co-operative DNA binding by GAGA transcription factor requires the conserved BTB/POZ domain and reorganizes promoter topology. EMBO J *18*, 698–708.
67. Van Steensel, B., Delrow, J., and Bussemaker, H.J. (2003). Genomewide analysis of Drosophila GAGA factor target genes reveals context-dependent DNA binding. Proc Natl Acad Sci U S A *100*, 2580–2585. https://doi.org/10.1073/pnas.0438000100.
68. Gaskill, M.M., Soluri, I. V., Branks, A.E., Boka, A.P., Stadler, M.R., Vietor, K., Huang, H.Y.S., Gibson, T.J., Mukherjee, A., Mir, M., et al. (2023). Localization of the Drosophila pioneer factor GAF to subnuclear foci is driven by DNA binding and required to silence satellite repeat expression. Dev Cell *58*, 1610-1624.e8. https://doi.org/10.1016/j.devcel.2023.06.010.
69. Tsukiyama, T., Becker, P.B., and Wu, C. (1994). ATP-dependent nucleosome disruption at a heat-shock promoter mediated by binding of GAGA transcription factor. Nature *367*, 525–532. https://doi.org/10.1038/367525a0.
70. Tang, X., Li, T., Liu, S., Wisniewski, J., Zheng, Q., Rong, Y., Lavis, L.D., and Wu, C. (2022). Kinetic principles underlying pioneer function of GAGA transcription factor in live cells. Nat Struct Mol Biol *29*, 665–676. https://doi.org/10.1038/s41594-022-00800-z.
71. Moshe, A., and Kaplan, T. (2017). Genome-wide search for Zelda-like chromatin signatures identifies GAF as a pioneer factor in early fly development. Epigenetics Chromatin *10*, 1–14. https://doi.org/10.1186/s13072-017-0141-5.
72. Fuda, N.J., Guertin, M.J., Sharma, S., Danko, C.G., Martins, A.L., Siepel, A., and Lis, J.T. (2015). GAGA Factor Maintains Nucleosome-Free Regions and Has a Role in RNA Polymerase II Recruitment to Promoters. PLoS Genet *11*, 1–22. https://doi.org/10.1371/journal.pgen.1005108.
73. Espinás, M.L., Jiménez-García, E., Vaquero, A., Canudas, S., Bernués, J., and Azorín, F. (1999). The N-terminal POZ domain of GAGA mediates the formation of oligomers that bind DNA with high affinity and specificity. Journal of Biological Chemistry *274*, 16461–16469. https://doi.org/10.1074/jbc.274.23.16461.
74. Bellec, M., Dufourt, J., Hunt, G., Lenden-Hasse, H., Trullo, A., Zine El Aabidine, A., Lamarque, M., Gaskill, M.M., Faure-Gautron, H., and Mannervik, M. (2022). The control of transcriptional memory by stable mitotic bookmarking. Nat Commun *13*, 1176.
75. Raff, J.W., Kellum, R., and Alberts, B. (1994). The Drosophila GAGA transcription factor is associated with specific regions of heterochromatin throughout the cell cycle. EMBO Journal *13*, 5977–5983. https://doi.org/10.1002/j.1460-2075.1994.tb06943.x.

76. Freund, M.M., Harrison, M.M., and Torres-Zelada, E.F. (2024). Exploring the reciprocity between pioneer factors and development. Development *151*, dev201921.
77. Gibson, T.J., Larson, E.D., and Harrison, M.M. (2024). Protein-intrinsic properties and context-dependent effects regulate pioneer factor binding and function. Nat Struct Mol Biol *31*, 548–558. https://doi.org/10.1038/s41594-024-01231-8.
78. Carrell, S.N., and Reeves, G.T. (2015). Imaging the dorsal-ventral axis of live and fixed Drosophila melanogaster embryos. Methods Mol Biol *1189*, 63–78. https://doi.org/10.1007/978-1-4939-1164-6_5.
79. Huisken, J., and Stainier, D.Y.R. (2009). Selective plane illumination microscopy techniques in developmental biology. Development *136*, 1963–1975. https://doi.org/10.1242/dev.022426.
80. Kaufmann, A., Mickoleit, M., Weber, M., and Huisken, J. (2012). Multilayer mounting enables long-term imaging of zebrafish development in a light sheet microscope. Development (Cambridge) *139*, 3242–3247. https://doi.org/10.1242/dev.082586.
81. Bintu, L., Buchler, N.E., Garcia, H.G., Gerland, U., Hwa, T., Kondev, J., and Phillips, R. (2005). Transcriptional regulation by the numbers: Models. Preprint at Elsevier Ltd, https://doi.org/10.1016/j.gde.2005.02.007 https://doi.org/10.1016/j.gde.2005.02.007.
82. Foe, V.E., and Alberts, B.M. (1985). Reversible chromosome condensation induced in Drosophila embryos by anoxia: visualization of interphase nuclear organization. J Cell Biol *100*, 1623–1636.
83. Wittrup, K.D., Tidor, B., Hackel, B.J., and Sarkar, C.A. (2019). Quantitative Fundamentals of Molecular and Cellular Bioengineering (The MIT Press).

Figures

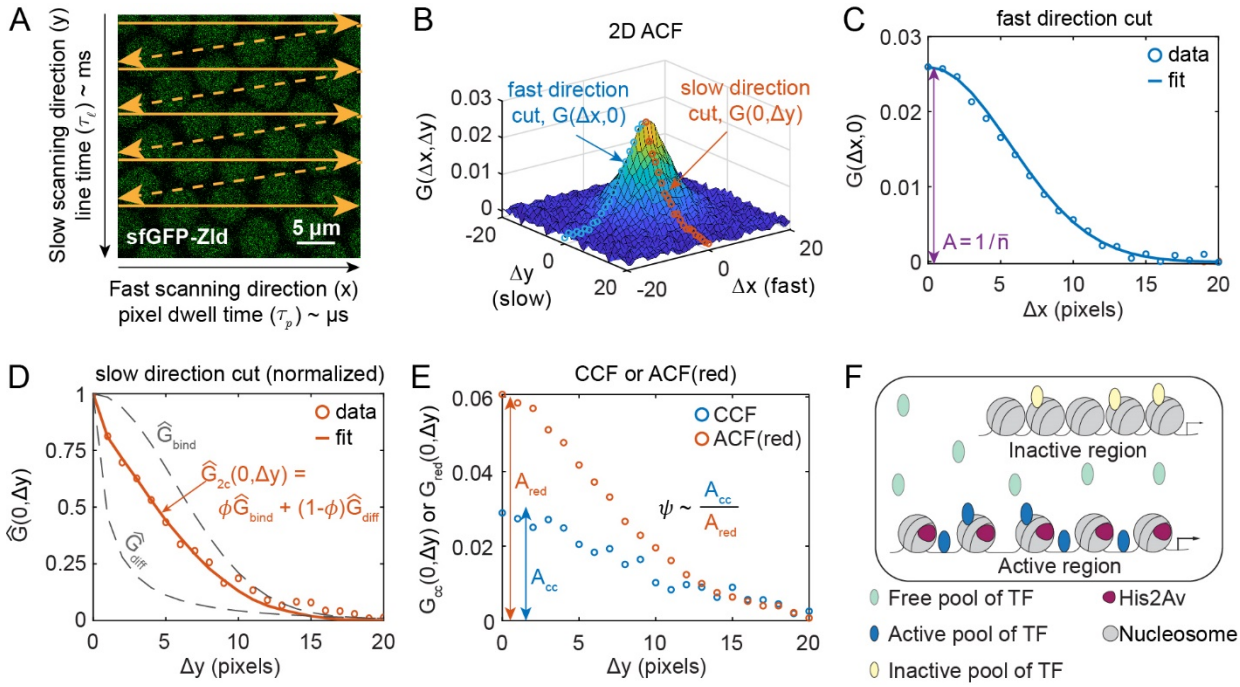

Figure 1. Raster Image Correlation Spectroscopy (RICS).

(A) Laser scanning confocal microscopes build images by raster scan, with a fast scanning direction (x direction, solid arrows), and a slow scanning direction (y direction) due to line retracing (dotted arrows). The time between horizontally-neighboring pixels is ~microseconds, while that between vertically-neighboring pixels is ~milliseconds. Image: mid-nc14 embryo expressing sfGFP-Zld.

(B) Two-dimensional ACF from embryo depicted in (A). Cuts along the fast (blue circles) and slow (orange circles) directions are depicted.

(C) Cut of ACF along the fast direction. Solid curve: fit of Gaussian-shaped PSF, used to estimate the ACF amplitude, A.

(D) Plot of the slow direction data (circles) and the fit to the slow direction (solid curve), composed of a linear combination between two ACFs (gray dotted curves): an immobile ACF ($\hat{G}_{bind}$) and a diffusible ACF ($\hat{G}_{diff}$). The linear combination weight is ϕ, the fraction immobile.

(E) Plots of cuts of the cross correlation function (blue circles) and of the ACF in the red channel (orange circles). The ratio of the amplitudes (A_{cc}/A_{red}) is proportional to ψ, the fraction bound in active regions of the DNA.

(F) Illustration of the different pools of TF: freely diffusible (light blue), bound to active regions (dark blue), and bound to inactive regions (yellow). His2Av (red) is associated with the active regions of DNA.

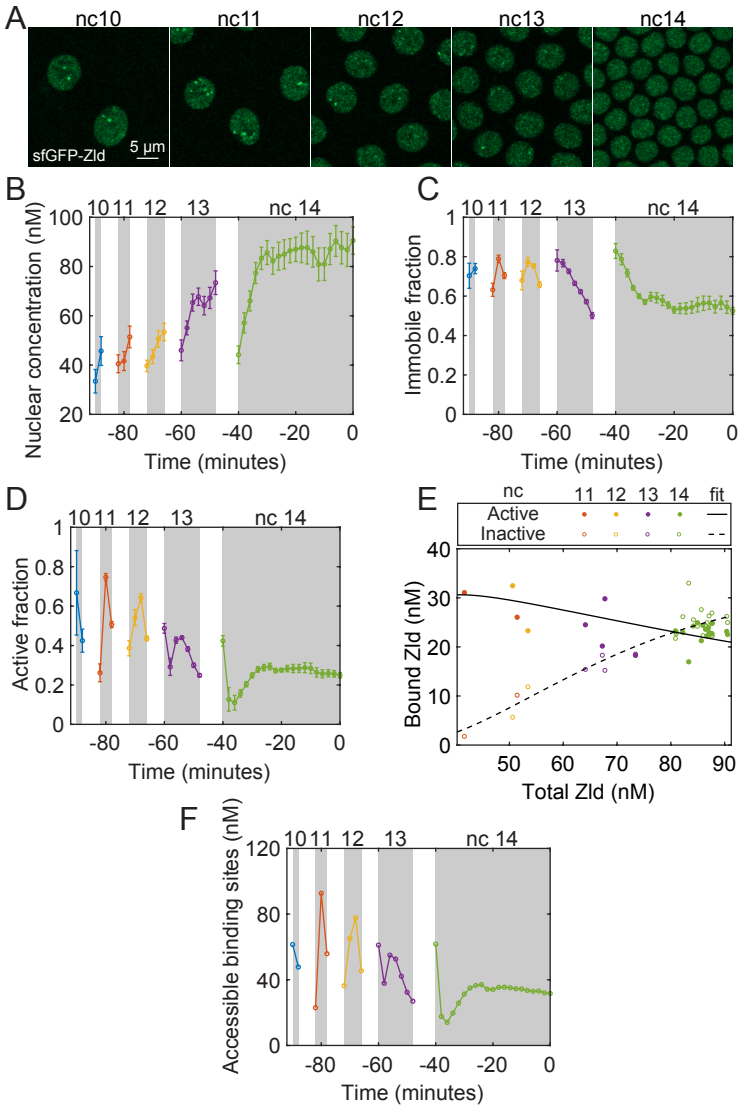

Figure 2. Quantification of the biophysical parameters and dynamics of Zld.

(A) Representative images of sfGFP-Zld embryo used for RICS analysis from nc 10 to nc 14 as marked.

(B-D) Dynamics of different pools of sfGFP-Zld from nc 10 until gastrulation including total nuclear concentration (B), immobile fraction (C), and active fraction (D). Curves: mean values (n = 20 embryos). Error bars: S.E.M.

(E) Dose/response map between total nuclear concentration of Zld and the immobile (both active and inactive) concentration of Zld.

(F) Change of Zld sites accessible for binding over time. See also Figure S1 and Video S1.

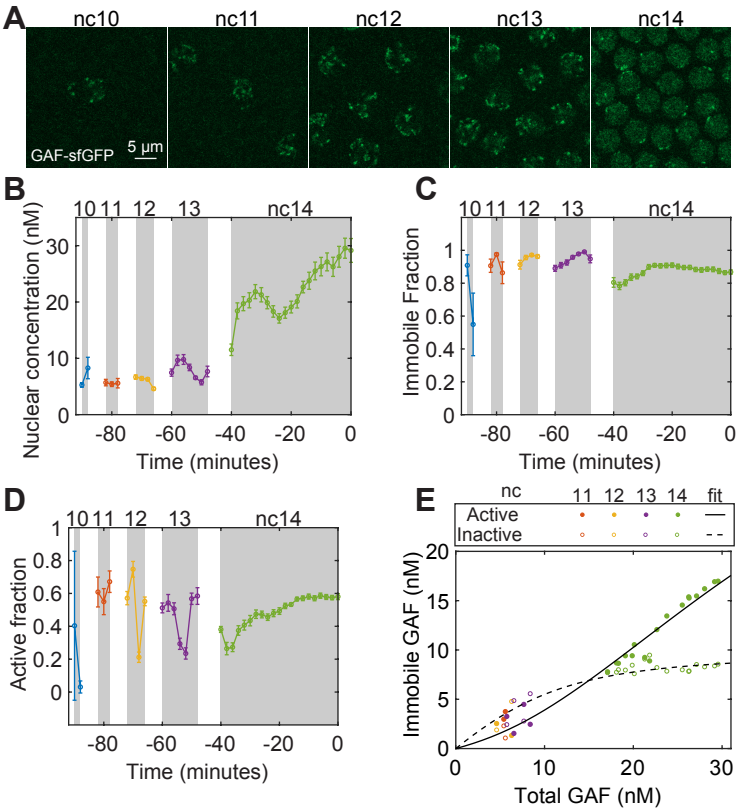

Figure 3. Quantification of the biophysical parameters and dynamics of GAF.

(A) Representative images of GAF-sfGFP(C) embryo used for RICS analysis from nc 10 to nc 14 as marked.

(B-D) Dynamics of the parameters from nc 10 until gastrulation including total nuclear concentration (B), immobile fraction (C), and active fraction (D). Curves: mean values (n = 19 embryos). Error bars: S.E.M.

(E) Dose/response map between total nuclear concentration of GAF and the immobile (both active and inactive) concentration of GAF. See also Figure S2 and Video S2.

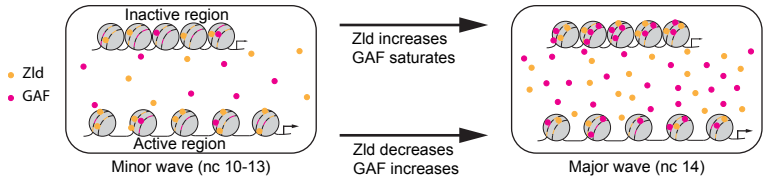

Figure 4. Role of pioneer factors Zld and GAF during *Drosophila* ZGA.

During the minor wave of ZGA high concentration of Zld (yellow circles) bound to its sites (yellow lines) in the active regions allows it to act as the main activator of early genes. This active bound concentration reduces during the major wave when the concentration of GAF (magenta circles) bound to the sites (magenta lines) in the active regions increases, after saturating the sites in the inactive regions, allowing GAF to act as the main activator of the genes that begin expressing during the major wave.

Supplemental Figures

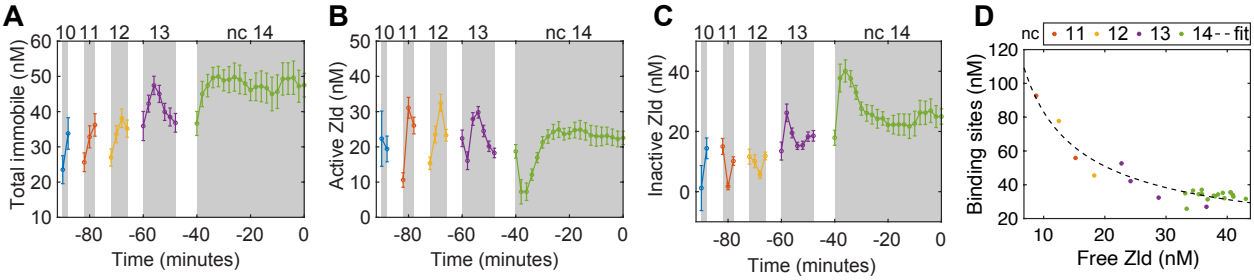

Figure S1. Dynamics of concentration of different pools of Zld from nc 10 until gastrulation.

(A) Immobile concentration.
(B) Active concentration.
(C) Inactive concentration. Line plots indicate the mean values for the measurements from 20 embryos. Error bars represent the standard errors of the mean.
(D) Power Law relation between concentration of binding sites and free Zld concentration.

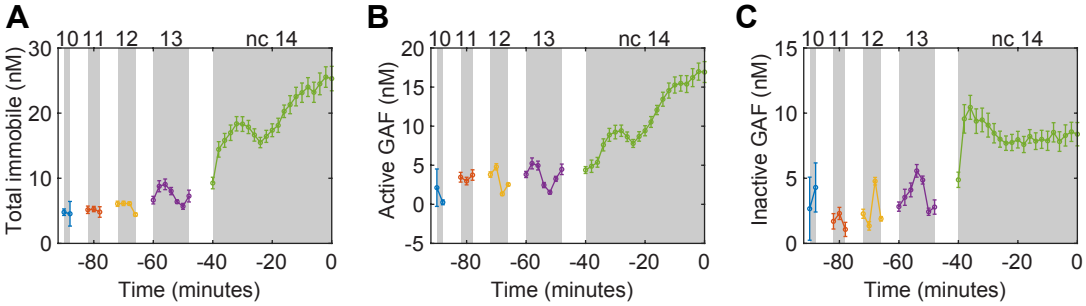

Figure S2. Dynamics of concentration of different pools of GAF from nc 10 until gastrulation.

(A) Immobile concentration.
(B) Active concentration.
(C) Inactive concentration. Line plots indicate the mean values for the measurements from 19 embryos. Error bars represent the standard errors of the mean.