

# 1 Minimal synthetic enhancers reveal 2 control of the probability of 3 transcriptional engagement and its 4 timing by a morphogen gradient

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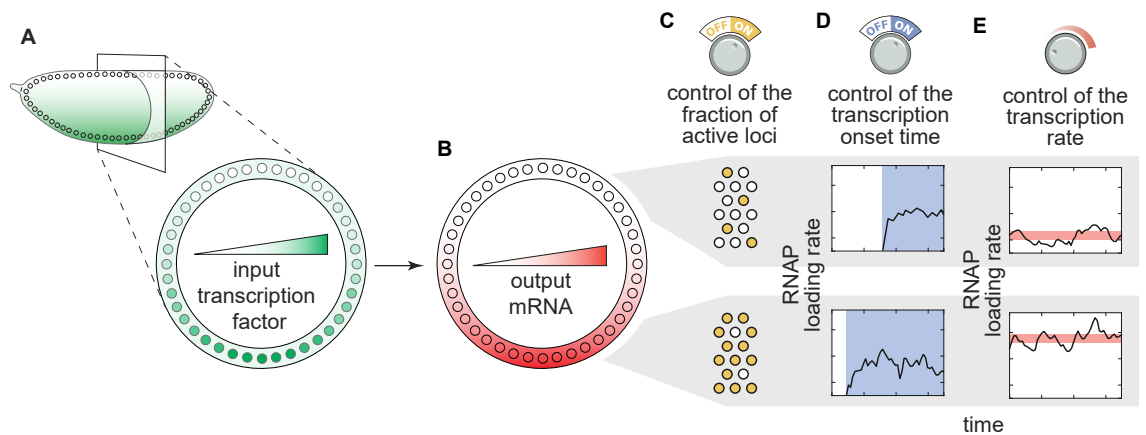
14 **Abstract** How enhancers interpret morphogen gradients to generate spatial patterns of gene  
15 expression is a central question in developmental biology. Although recent studies have begun to  
16 elucidate that enhancers can dictate whether, when, and at what rate a promoter will engage in  
17 transcription, the complexity of endogenous enhancers calls for theoretical models with too many  
18 free parameters to quantitatively dissect these regulatory strategies. To overcome this limitation,  
19 we established a minimal synthetic enhancer system in embryos of the fruit fly *Drosophila*  
20 *melanogaster*. Here, a gradient of the Dorsal activator is read by a single Dorsal binding site. By  
21 quantifying transcriptional activity using live imaging, our experiments revealed that this single  
22 Dorsal binding site is capable of regulating whether promoters engage in transcription in a Dorsal  
23 concentration-specific manner. By modulating binding-site affinity, we determined that a gene's  
24 decision to engage in transcription and its transcriptional onset time can be explained by a simple  
25 theoretical model where the promoter has to traverse multiple kinetic barriers before transcription  
26 can ensue. The experimental platform developed here pushes the boundaries of live-imaging in  
27 studying gene regulation in the early embryo by enabling the quantification of the transcriptional  
28 activity driven by a single transcription factor binding site, and making it possible to build more  
29 complex enhancers from the ground up in the context of a dialogue between theory and  
30 experiment.

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## 32 1 Introduction

33 The adoption of distinct cellular identities in multicellular organisms relies on the formation of  
34 spatial gene expression domains driven, in large part, by transcriptional regulatory programs. The  
35 positional information giving rise to these mRNA patterns is typically provided by transcription  
36 factor gradients (Fig. 1A) whose concentrations are interpreted by enhancer DNA sequences that,  
37 in turn, regulate transcription of developmental genes (*Wolpert, 1969; Briscoe and Small, 2015*). A  
38 long-standing goal in quantitative developmental biology is to precisely predict gene expression  
39 from knowledge of the DNA regulatory sequence and morphogen concentration (*Garcia et al.,*

40 **2020; Vincent et al., 2016**). Achieving this predictive understanding requires theoretical models  
 41 that calculate how DNA sequence dictates the functional relation between input morphogen con-  
 42 centration and output transcriptional activity, and calls for testing these predictions by measuring  
 43 input-output functions (**Garcia et al., 2020**). Precise genetic manipulations (**Venken and Bellen,**  
 44 **2005; Bier et al., 2018**) and powerful imaging technologies (**Gregor et al., 2005; Garcia et al., 2013;**  
 45 **Mir et al., 2017**) have rendered the early embryo of the fruit fly *Drosophila melanogaster* (*Drosophila*)  
 46 a prime model system for quantitatively dissecting these input-output functions in development.  
 47 In recent years, several studies have reported that *Drosophila* enhancers can control various,  
 48 potentially independent aspects of transcriptional dynamics in early embryonic development (Fig. 1;  
 49 **Lucas et al. (2013); Garcia et al. (2013); Fukaya et al. (2016a); Lammers et al. (2020); Fuqua et al.**  
 50 **(2020); Eck et al. (2020); Berrocal et al. (2020); Fukaya (2021); Harden et al. (2021)**). First, for a  
 51 given gene, a fraction of loci remain transcriptionally inactive throughout entire mitotic cycles in  
 52 development, even when exposed to the same activator concentration as active loci (Fig. 1B)—a  
 53 behavior usually quantified through the fraction of active nuclei or loci. This stochastic decision for  
 54 a locus to become active is a ubiquitous and potentially important regulatory feature for shaping  
 55 gene-expression patterns in the embryo (**Garcia et al., 2013; Dufourt et al., 2018; Lammers et al.,**  
 56 **2020; Harden et al., 2021**). However, it remains unclear whether this feature constitutes a regulatory  
 57 ‘knob’ or whether inactive loci are artifacts of experimental detection thresholds. Second, the timing  
 58 of transcription onset (and cessation, which is not addressed in the present investigation) can  
 59 also be controlled by input transcription-factor dynamics (Fig. 1C; **Desponds et al. (2016); Tran**  
 60 **et al. (2018); Dufourt et al. (2018); Eck et al. (2020); Lammers et al. (2020); Desponds et al. (2020);**  
 61 **Harden et al. (2021)**). Finally, the rate of transcriptional initiation in active loci is under regulatory  
 62 control (Fig. 1D) and has been the focus of most studies to date (e.g., **Garcia et al. (2013); Fukaya**  
 63 **et al. (2016b); Park et al. (2019); Lammers et al. (2020); Berrocal et al. (2020); Fukaya (2021)**). Thus,  
 64 multiple regulatory strategies together realize gene-expression patterns in space and time (Fig. 1E).



**Figure 1. Transcriptional regulatory strategies of enhancers in response to transcription factor concentration gradients. (A)** A *Drosophila* embryo with a transcription factor gradient along its dorsoventral axis. **(B)** This input transcription factor dictates the emergence of output gene-expression patterns by controlling a combination of three enhancer regulatory ‘knobs’: **(C)** the probability of loci becoming transcriptionally active, **(D)** the transcriptional onset time, and **(E)** the mean transcription rate of active loci. (RNAP, RNA polymerase II).

65 Intense theoretical scrutiny (**Desponds et al., 2016; Fakhouri et al., 2010; Sayal et al., 2016;**  
 66 **Estrada et al., 2016; Scholes et al., 2017; Dufourt et al., 2018; Park et al., 2019; Eck et al., 2020;**  
 67 **Cheng et al., 2021**) has generated a compelling hypothesis: that the regulation of transcriptional  
 68 dynamics can be separated into two stages. First, a promoter must pass through a series of kinetic  
 69 barriers consisting of reactions catalyzed by transcription factors in order for for loci to engage in  
 70 transcription. Previous analyses of the mean and distribution in transcriptional onset times have

71 suggested that the number of inactive promoter states can range from one to three (*Dufourt et al.*,  
72 *2018*; *Eck et al.*, *2020*; *Harden et al.*, *2021*). These reactions could be associated with, for example,  
73 the stepwise unwrapping of DNA from nucleosomes (*Desponds et al.*, *2016*; *Dufourt et al.*, *2018*;  
74 *Eck et al.*, *2020*) and/or the sequential recruitment of general transcriptional cofactors (*Zhou et al.*,  
75 *1998*). Second, after initial promoter activation, the rate of mRNA production is proportional to  
76 the probability of finding RNA polymerase II (RNAP) bound to the promoter. Statistical mechanical  
77 (also called thermodynamic) models have been used to calculate this probability of finding RNAP  
78 bound to the promoter, and have successfully use to predict mRNA production rates in bacteria  
79 (*Razo-Mejia et al.*, *2018*). However, whether they can be applied to the more complex context of  
80 eukaryotic transcriptional regulation—let alone to the dynamical processes of cellular decision-  
81 making in development—is still an open question (*Polach and Widom*, *1995*; *Schulze and Wallrath*,  
82 *2006*; *Lam et al.*, *2008*; *Li et al.*, *2008*; *Kim and O’Shea*, *2008*; *Levine*, *2010*; *Fussner et al.*, *2011*; *Bai*  
83 *et al.*, *2011*; *Li et al.*, *2014*; *Hansen and O’Shea*, *2015*; *Estrada et al.*, *2016*; *Li and Eisen*, *2018*; *Park*  
84 *et al.*, *2019*; *Eck et al.*, *2020*).

85 One of the main challenges to systematically testing these models is the complexity of en-  
86 dogenous regulatory regions (*Fakhouri et al.*, *2010*; *Foo et al.*, *2014*; *Sayal et al.*, *2016*; *Dufourt*  
87 *et al.*, *2018*; *Park et al.*, *2019*; *Eck et al.*, *2020*). Because endogenous enhancers contain multiple  
88 binding sites for different transcription factors, accounting for these sites and their interactions  
89 leads to a combinatorial explosion of model parameters (*Garcia et al.*, *2016*, *2020*); determin-  
90 ing the values of these parameters from simple experiments constitutes a computational—and  
91 conceptual—challenge (*Vincent et al.*, *2016*; *Garcia et al.*, *2016*, *2020*). To render complex transcrip-  
92 tional regulatory systems tractable to theory, minimal synthetic enhancers have been engineered  
93 in bacteria (*Garcia and Phillips*, *2011*; *Brewster et al.*, *2014*; *Razo-Mejia et al.*, *2018*; *Phillips et al.*,  
94 *2019*), eukaryotic cells (*Popp et al.*, *2020*), and developing organisms (*Fakhouri et al.*, *2010*; *Sayal*  
95 *et al.*, *2016*). In such experiments, a short, synthetic DNA sequence with only one to a few binding  
96 sites for a single transcription factor drives the expression of a reporter gene. Measuring the  
97 concentration of the transcription-factor input and reporter mRNA output makes it possible to test  
98 models of transcriptional regulation and to infer molecular parameters that can be used to predict  
99 the behavior of more complex regulatory architectures (*Phillips et al.*, *2019*).

100 Here we sought to use synthetic minimal enhancers to challenge our integrated model of  
101 transcriptional control using the dorsoventral patterning system in *Drosophila* embryos, in which  
102 a concentration gradient of the Dorsal transcription factor specifies spatial domains of transcrip-  
103 tion, as a case study. To test the integrated model of transcriptional dynamics (Fig. 2A,B), we  
104 performed simultaneous quantitative live-cell measurements of Dorsal concentration (input) and  
105 transcription (output) driven by minimal synthetic Dorsal-dependent enhancers in single nuclei.  
106 By repurposing the *parS*-ParB DNA labeling technology (*Germier et al.*, *2017*; *Chen et al.*, *2018*) to  
107 quantify transcriptional activity independent of RNA detection, we determined that the inactive  
108 loci described by our model constitute a distinct transcriptional state under regulatory control and  
109 are not the result of detection artifacts. Further, our theoretical model predicted how, through  
110 the Dorsal-mediated catalysis of reactions prior to transcriptional onset, regulatory architecture  
111 dictates both the transcriptional onset time and the fraction of active loci. Finally, once promoters  
112 turn on, we found that our measurements are compatible with an equilibrium model. Thus, the  
113 present investigation provides quantitative evidence supporting a unified model of transcriptional  
114 regulation in eukaryotes that accounts for whether loci become transcriptionally active, when this  
115 activity ensues, and, once transcription ensues, at what rate nascent RNA molecules are produced.  
116 More generally, our work demonstrates the feasibility of using minimal synthetic enhancers to  
117 engage in a dialogue between theory and experiment in the context of transcriptional control in  
118 development.

## 119 2 Results

### 120 2.1 An integrated model of transcriptional dynamics driven by a single activator 121 binding site

122 To probe the transcriptional regulatory strategies (Fig. 1) of a minimal synthetic enhancer, we posit  
123 a theoretical model that predicts the fraction of loci that will become active, their transcriptional  
124 onset time, and RNAP loading dynamics once transcription ensues. Specifically, we consider a  
125 simplified case in which only one activator is present and can only bind to one site only a few base  
126 pairs away from the promoter (Fig. 2).

127 In order to explain the transcriptional onset dynamics of a locus and the probability of loci  
128 becoming active, we invoke recent experiments leading to a ‘kinetic barrier’ model (*Desponds et al.*,  
129 *2016*; *Dufourt et al.*, *2018*; *Eck et al.*, *2020*) proposing that, after exiting mitosis, all promoters are in  
130 an inactive state. In this state, labeled as ‘OFF<sub>1</sub>’ in Figure 2A, transcription is not possible. Promoters  
131 must then traverse a series of distinct inactive states (labeled ‘OFF<sub>2</sub>’ to ‘OFF<sub>n</sub>’ in Fig. 2A) before  
132 reaching an active state in which transcription proceeds (labeled ON in Fig. 2A).

133 The temporal evolution of the enhancer-promoter system as it traverses the states shown in  
134 Figure 2A can be simulated by computing the probability that the promoter occupies each state.  
135 Here, the transition rates between states,  $k$ , determines how the states probability spreads from  
136 the initial condition where the promoter is in state OFF<sub>1</sub> to the active state as time passes (see  
137 Section S1.1 for details).

138 We propose that a transcriptional activator such as Dorsal can catalyze the transition between  
139 states in an affinity-dependent manner via binding to its cognate site in the enhancer. Because we  
140 assume that Dorsal binding and unbinding is faster than the transition rate  $k$ , we posit that  $k$  is a  
141 linear function of the equilibrium Dorsal occupancy at the enhancer such that

$$k(t) = c \cdot \frac{\frac{[Dl](t)}{K_D}}{1 + \frac{[Dl](t)}{K_D}}, \quad (1)$$

142 where  $c$  is a rate constant,  $[Dl](t)$  is the Dorsal concentration at time  $t$ , and  $K_D$  is the Dorsal-DNA  
143 dissociation constant.

144 Because Dorsal is time-varying, the model cannot be solved analytically. As a result, we numeri-  
145 cally calculated the probability of the promoter being in each state as a function of time using a  
146 particular set of model parameters (see details in Section S1.1). As seen in Figure 2C, because  
147 individual loci must traverse a sequence of intermediate states before reaching the ON state, this  
148 model introduces a delay in activation.

149 This kinetic barrier model accounts for loci that never transcribe during the nuclear cycle.  
150 Specifically, if nuclear cycles lasted indefinitely, all promoters would eventually reach the ON state  
151 as shown in Figure 2C. However, due to the rapid mitotic cycles that characterize early embryonic  
152 development in *Drosophila*, this duration is limited: transcription cannot initiate during mitosis  
153 and thus is only permissible during a time window within interphase (Fig. 2C, vertical dashed line;  
154 *Shermoen and O’Farrell (1991)*; *Garcia et al. (2013)*; *Eck et al. (2020)*). Consequently, if the time  
155 it takes a promoter to reach the ON state is longer than the duration of this window, then this  
156 hypothetical promoter will not initiate transcription at all during the nuclear cycle (Fig. 2C, horizontal  
157 dashed line).

158 The kinetic barrier model can be used to predict two of the three regulatory strategies, fraction  
159 of active loci and transcription onset times, that we aim to dissect quantitatively (Fig. 1). First, the  
160 model predicts how the fraction of active loci is determined by Dorsal nuclear concentration and  
161 binding affinity (Fig. 2D, left y-axis). Second, this same model calculates the mean transcriptional  
162 onset time of those loci that turn on as a function of these same Dorsal parameters (Fig. 2D, right  
163 y-axis).

164 To model a locus once it is active, we follow *Eck et al. (2020)* and propose a simple thermody-  
165 namic model (*Bintu et al., 2005b,a*) that assumes that the RNAP loading rate,  $R$ , is proportional to



166 the probability of finding RNAP bound to the promoter  $p_{bound}$ , such that

$$R = R_{max} \cdot p_{bound}, \quad (2)$$

167 where  $R_{max}$  is a constant coefficient that dictates the maximum possible polymerase loading rate.

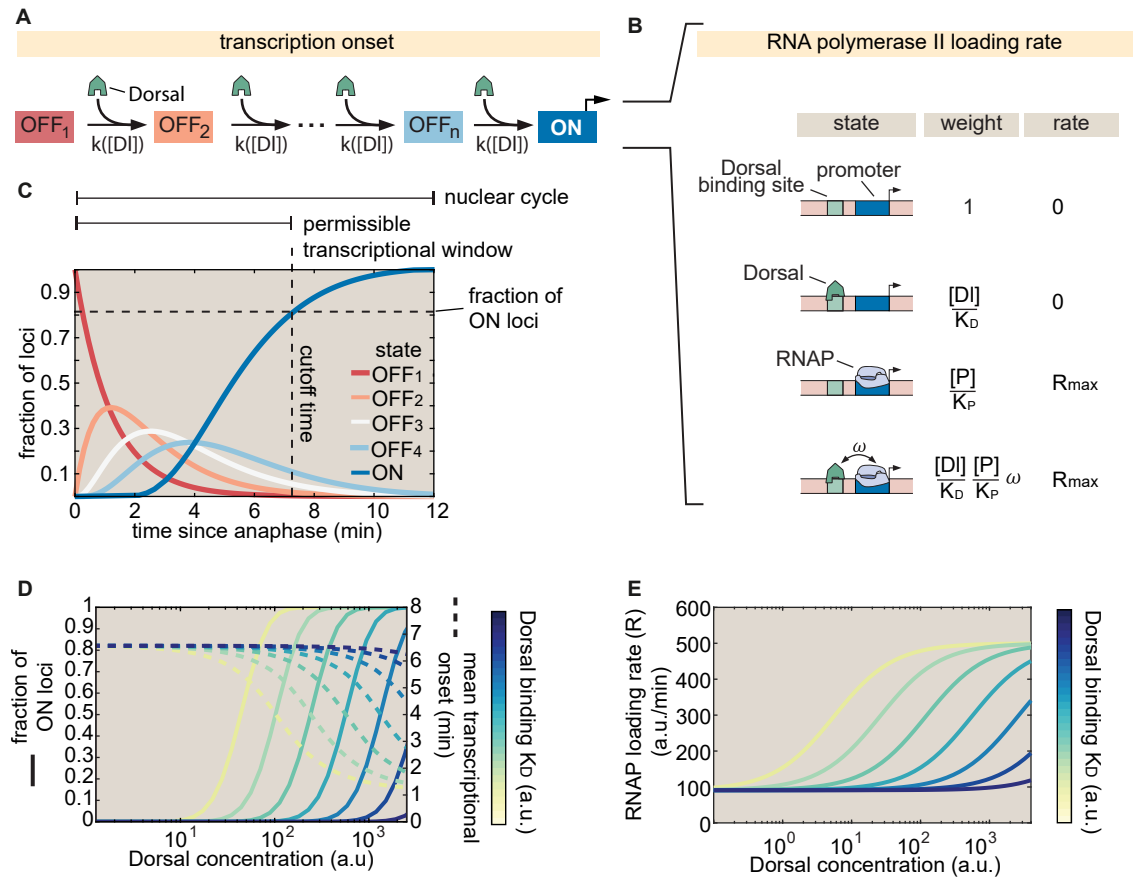
168 Thermodynamic models enable the calculation of  $p_{bound}$  by assigning a statistical weight to each  
169 possible state in which the regulatory system can be found. In the case of a minimal enhancer  
170 with one activator binding site, the enhancer-promoter DNA can be empty, occupied by Dorsal,  
171 occupied by RNAP, or simultaneously bound by Dorsal and RNAP (Fig. 2B). The statistical weight  
172 associated with each of these terms is shown in Figure 2B. Here,  $[DI]/[K_D]$  is the statistical weight  
173 associated with finding Dorsal (with concentration  $[DI]$  and binding dissociation constant  $K_D$ ) bound  
174 to the promoter alone, while  $[P]/[K_P]$  is the weight of finding RNAP (with concentration  $[P]$  and  
175 binding dissociation constant  $K_P$ ) bound to the promoter alone. Note that the weight of having  
176 both Dorsal and RNAP bound simultaneously includes an extra glue-like cooperativity coefficient,  $\omega$ ,  
177 that determines how strongly Dorsal recruits RNAP to the promoter. The value of  $\omega$  is constrained  
178 to be  $> 1$  so that higher Dorsal occupancy leads to higher RNAP occupancy.

179 To calculate  $p_{bound}$ , we divide the sum of the weights featuring a bound RNAP molecule by the  
180 sum of all possible weights. Substituting this calculation into Equation 2 yields

$$R = R_{max} \cdot p_{bound} = R_{max} \cdot \frac{\frac{[P]}{K_P} + \frac{[DI]}{K_D} \frac{[P]}{K_P} \omega}{1 + \frac{[DI]}{K_D} + \frac{[P]}{K_P} + \frac{[DI]}{K_D} \frac{[P]}{K_P} \omega}, \quad (3)$$

181 which is plotted in Figure 2E. As shown in the figure, increasing  $K_D$  shifts the concentration at which  
182 the RNAP loading rate reaches half its maximum value toward higher Dorsal concentrations, but  
183 does not change the overall shape of the curve. We also note the presence of a non-zero baseline  
184 of RNAP loading rate due to the Dorsal-independent  $[P]/[K_P]$  term in the numerator of Equation 3.  
185 This baseline suggests that it could be possible for a promoter in the 'ON' state to produce low,  
186 basal-level transcription in the absence of bound Dorsal.

187 Together, the kinetic barrier model outlined in Figure 2A and the thermodynamic model's  
188 Equation 3 define a comprehensive quantitative framework that predicts how the fraction of active  
189 loci, the transcriptional onset time, and the RNAP loading rate as a function of Dorsal concentration  
190 vary as model parameters such as the Dorsal dissociation constant  $K_D$  are modulated (Fig. 2D,E).  
191 These predictions constitute hypotheses that we experimentally tested throughout the remainder  
192 of this work.



**Figure 2. Integrated kinetic and thermodynamic model of simple activation by Dorsal.** (A) The promoter undergoes kinetic transitions from transcriptionally inactive states (OFF<sub>1</sub> to OFF<sub>n</sub>) to an active state (ON) with Dorsal accelerating the transition rate,  $k$ , by a factor proportional to the Dorsal occupancy at the promoter. (B) Thermodynamic states and weights for the simple activator model. The probability of finding RNAP bound to the promoter can be calculated from the statistical weights associated with all possible occupancy states of the enhancer-promoter system. (C) Visualization of a particular solution of the kinetic scheme from (A) showing the probability of finding a given locus in each of the states for an illustrative, representative set of parameters ( $[D] = 1000$  a.u.,  $K_D = 1000$  a.u.,  $c = 10/\text{min}$ ,  $n = 4$  states, and 7 min nuclear cycle duration). The predicted fraction of active loci (dashed horizontal line) is calculated as the probability of being in the ON state by the end of the permissible time window (dashed vertical line) that is determined by mitotic repression. (D) Predictions for the fraction of active loci (solid lines plotted against the left y-axis) and mean transcriptional onset times (dashed lines plotted against the right y-axis) as a function of Dorsal concentration for different, illustrative values of the Dorsal dissociation constant  $K_D$ . (E) Rate of mRNA production across active loci as a function of Dorsal concentration for different values of  $K_D$  based on the model in (B) ( $R_{max} = 1000$  a.u., Dorsal  $K_D$  ranging from 10 a.u. to  $10^5$  a.u.,  $\omega = 10$ ,  $[P]/[K_P] = 0.1$ ).

## 193 2.2 Establishing a minimal synthetic enhancer system to test theoretical predic- 194 tions

195 To test our model's predictions, we constructed single binding site enhancers driven by the Dorsal  
196 activator. Dorsal is one of the best characterized transcription factors in *Drosophila* and a classic  
197 example of a morphogen (Roth et al., 1989; Reeves et al., 2012). Dorsal is provided maternally and  
198 forms a dorsoventral gradient of nuclear localization (Fig. 3A) (Gilbert, 2010), acting as an activator  
199 by default (Thisse et al., 1991; Jiang et al., 1991) and as a repressor in the presence of nearby  
200 binding sites for corepressors (Kirov et al., 1993; Papagianni et al., 2018). Prior to activation of  
201 the zygotic genome (up to the 12th mitotic cycle), Dorsal is the only transcription factor with a  
202 nuclear protein gradient across the dorsoventral axis (Sandler and Stathopoulos, 2016; Dufourt

203 *et al., 2020*). Thus, the Dorsal nuclear concentration is the sole source of dorsoventral positional  
204 information for developmental enhancers at this stage in development. These features, combined,  
205 make Dorsal an ideal input transcription factor for activating a minimal synthetic reporter system.

206 In order to relate output transcriptional activity to the time-variant input Dorsal concentration  
207 throughout development, we measured the instantaneous Dorsal concentration in live embryos  
208 by creating a CRISPR knock-in Dorsal-mVenus fusion allele based on a previous Dorsal fusion  
209 (*Reeves et al., 2012*) that rescues embryonic development (*Kremers et al. (2006); Gratz et al. (2015);*  
210 *Materials and methods*). Further, in order to increase the dynamic range of Dorsal concentration in  
211 our experiments, we further combined this CRISPR allele with a Dorsal-mVenus transgene (*Reeves*  
212 *et al., 2012*), resulting in a line that will hereafter be referred to as 2x Dorsal flies. This fusion  
213 made it possible to quantify the concentration dynamics of the Dorsal protein input (Fig. 3A,B) in  
214 individual nuclei (Video S4, left; *Materials and methods*). Dorsal-mVenus nuclear fluorescence  
215 time traces quantified over nuclear cycle 12 confirmed the dynamic nature of Dorsal concentration  
216 and were quantitatively similar to previous measurements (Fig. 3B; *Reeves et al. (2012)*; details of  
217 Dorsal-mVenus quantification in Fig. S5A,B). Nuclear cycle 12 nuclei in 2x Dorsal flies experience a  
218 Dorsal concentration gradient spanning multiple orders of magnitude, from less than 1 nM to  $\approx$   
219 400 nM (Fig. 3B; details of Dorsal-mVenus calibration in Fig. S6).

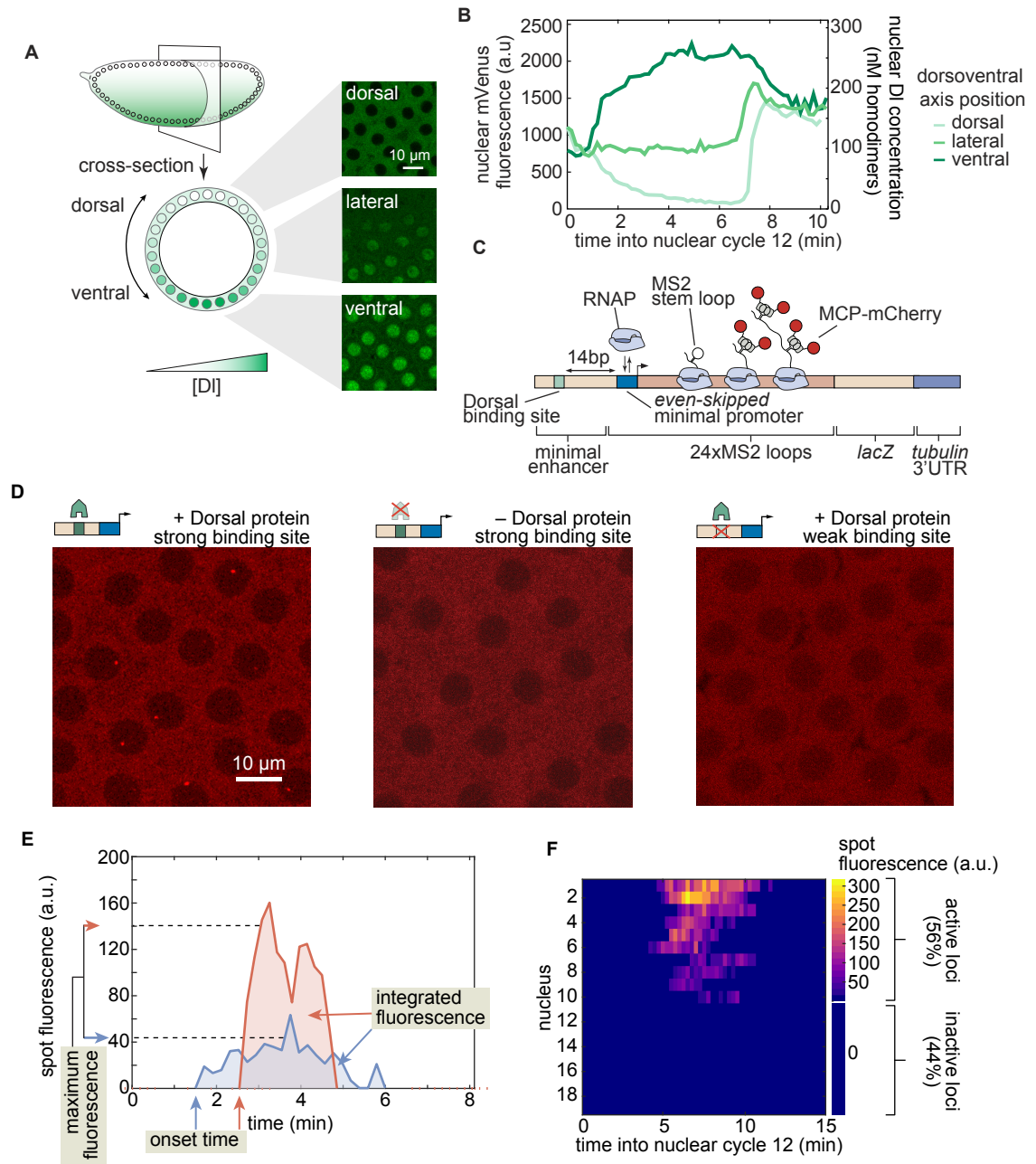
220 To visualize the dynamics of Dorsal-dependent transcription, we developed a reporter transgene  
221 containing a minimal synthetic enhancer consisting of a single high affinity, consensus binding site  
222 for the Dorsal transcription factor (*Ip et al., 1992; Jiang and Levine, 1993; Szymanski and Levine,*  
223 *1995*) (Fig. 3C). Hereafter we refer to this strong site enhancer as as DBS\_6.23 for Dorsal Binding  
224 Site, followed by its binding affinity score according to the Patser algorithm (*Stormo and Hartzell*  
225 *(1989)*; *Materials and methods*). To quantify the transcriptional activity of this enhancer, we used  
226 the MS2-MCP system to fluorescently label nascent RNA molecules in our reporter constructs, which  
227 appear as nuclear fluorescent puncta (hereafter “transcription spots”) in laser-scanning confocal  
228 microscopy movies (Video S4, right; *Bertrand et al. (1998); Garcia et al. (2013); Lucas et al. (2013)*).  
229 We performed image analysis of the MS2 movies using a custom data analysis pipeline in Matlab  
230 and Fiji (*Materials and methods*; (*Schindelin et al., 2012; Lammers et al., 2020*)).

231 To validate this minimal synthetic system, we determined that DBS\_6.23-MS2 drives detectable  
232 and quantifiable levels of transcription, and that this transcriptional activity is mainly governed by  
233 Dorsal. We compared the transcriptional activity of DBS\_6.23-MS2 in embryos laid by 2x Dorsal  
234 females with the activity in embryos laid by females homozygous for a *dorsal* null allele. While  
235 transcription spots were clearly present in the 2x Dorsal background (Fig. 3D, left), they were  
236 extremely rare in *dorsal* null embryos (Fig. 3D, middle): not a single transcription spot was detected  
237 during nuclear cycle 12 in any of 4 replicates containing > 60 nuclei in total. Dorsal is therefore  
238 necessary for transcriptional activity in our reporter constructs.

239 We next sought to determine whether the detected transcriptional activation is solely due to  
240 Dorsal interacting with the binding site explicitly engineered into the construct or whether there  
241 are cryptic Dorsal binding sites contributing to gene expression. We generated a second reporter,  
242 DBS\_4.29-MS2 in which the Dorsal binding site was strongly perturbed using known point mutations  
243 (*Ip et al., 1992*). Transcription was rarely detectable in DBS\_4.29-MS2 embryos (Fig. 3D, right), with  
244 the average transcriptional activity (mean instantaneous fluorescence) per detected spot being less  
245 than 10% of the optimal DBS\_6.23 enhancer at any Dorsal concentration (Fig. S9). Thus, the Dorsal  
246 site placed within the synthetic enhancer is necessary for robust activation and is the main driver  
247 of this transcriptional activity.

248 Next, we identified which observable features in the MS2 signal could be used as metrics for  
249 quantifying Dorsal-dependent transcriptional activity. We collected DBS\_6.23-MS2 time traces of  
250 MCP-mCherry fluorescence from transcription spots during nuclear cycle 12 along with four metrics  
251 of transcriptional activity (Fig. 3E,F). First, the maximum spot fluorescence corresponds to the 95th  
252 percentile of intensity over time, which is proportional to the transcription rate (Section S1 .2).  
253 Second, the transcriptional onset time is defined as the time since the previous mitosis at which a

254 transcription spot is first detected (Fig. S3). Third, the integrated spot fluorescence corresponds  
255 to the time integral of the spot fluorescence and is directly proportional to the amount of mRNA  
256 produced by the locus (*Garcia et al., 2013*) (Materials and methods). Finally, as previously observed  
257 in other genes in flies (*Garcia et al., 2013; Dufourt et al., 2018; Lammers et al., 2020; Harden et al.,*  
258 **2021**), not all nuclei exposed to the same average nuclear Dorsal concentration exhibited detectable  
259 transcription (Fig. 3F). As a result, we quantified the fraction of active loci—regardless of their level  
260 of activity or temporal dynamics—by measuring the number of nuclei with observable transcription  
261 signal in at least one movie frame throughout nuclear cycle 12, divided by the total number of  
262 nuclei in the field of view. Thus, we have established quantitative metrics that enable us to engage  
263 in a dialogue between experiment and a theory of Dorsal-driven transcriptional dynamics.



**Figure 3. Simultaneously measuring transcription factor protein input and transcriptional output. (A)** Schematic of the Dorsal protein gradient in early *Drosophila* embryos. Dorsal protein accumulates in ventral nuclei and is progressively excluded from more dorsal nuclei. Example snapshots show Dorsal-mVenus in various positions along the dorsoventral axis. **(B)** Representative time traces of nuclear Dorsal-mVenus fluorescence in various positions along the dorsoventral axis. The right y-axis shows the nuclear Dorsal concentration according to the calibration described in Figure S6. **(C)** Schematic of minimal synthetic enhancer system containing a single binding site for Dorsal that drives transcription of a reporter tagged with MS2 loops, which are visualized through the binding of MCP-mCherry. The Dorsal binding site is placed 14 bp upstream of the *even-skipped* minimal promoter. **(D)** Snapshots from embryos containing an optimal binding-site reporter in the presence (left) or absence (middle) of Dorsal, or containing a strongly mutated Dorsal binding site (right). **(E)** Example fluorescence time traces and quantitative metrics of transcriptional activity. **(F)** Fluorescence of all transcription spots in individual nuclei in the field of view of one embryo as a function of time. If a transcription spot was detected within a nucleus at any point during the interphase of nuclear cycle 12, then the locus was considered active; otherwise, the locus was classified as inactive.



### 264 **2.3 Transcriptionally active and inactive loci correspond to functionally distinct** 265 **populations**

266 Before attempting to predict Dorsal-driven transcriptional dynamics, it is important to ensure  
267 that the fact that only some loci engage in transcription is the result of Dorsal action and not of  
268 limitations of our experimental setup. Transcriptionally silent loci that remain inactive throughout  
269 interphase, such as those revealed by our experiment (Fig. 3F), have been observed using MS2 (and  
270 its sister mRNA labeling tool, PP7) in live-imaging experiments in flies (*Garcia et al., 2013; Lammers*  
271 *et al., 2020; Berrocal et al., 2020*), plants (*Alamos et al., 2020*), and mammalian cells (*Hafner et al.,*  
272 *2020*). However, it has not been possible to determine whether these inactive loci correspond to a  
273 separate transcriptional state from active loci, or whether they are an artifact of the fluorescence  
274 detection thresholds associated with various microscopy techniques.

275 To answer this question, it is necessary to quantify MS2 fluorescence at these inactive loci  
276 and determine whether they differ from loci not exposed to activators, which do not transcribe  
277 (Fig. 3F). However, to date this approach has not been feasible because most MS2 measurements  
278 have relied on the presence of an MS2 signal itself to segment and quantify the fluorescence of  
279 transcription spots. We hypothesized that, if undetected loci correspond to a distinct and weaker,  
280 Dorsal-independent state, then detected and undetected spots in embryos carrying wild-type  
281 Dorsal would appear as two distinct populations. In this scenario, the mCherry fluorescence of  
282 undetected spots corresponding to inactive loci in wild-type Dorsal embryos would be similar to  
283 that observed in Dorsal null embryos, and clearly distinct from the mCherry fluorescence of active  
284 loci in the presence of Dorsal.

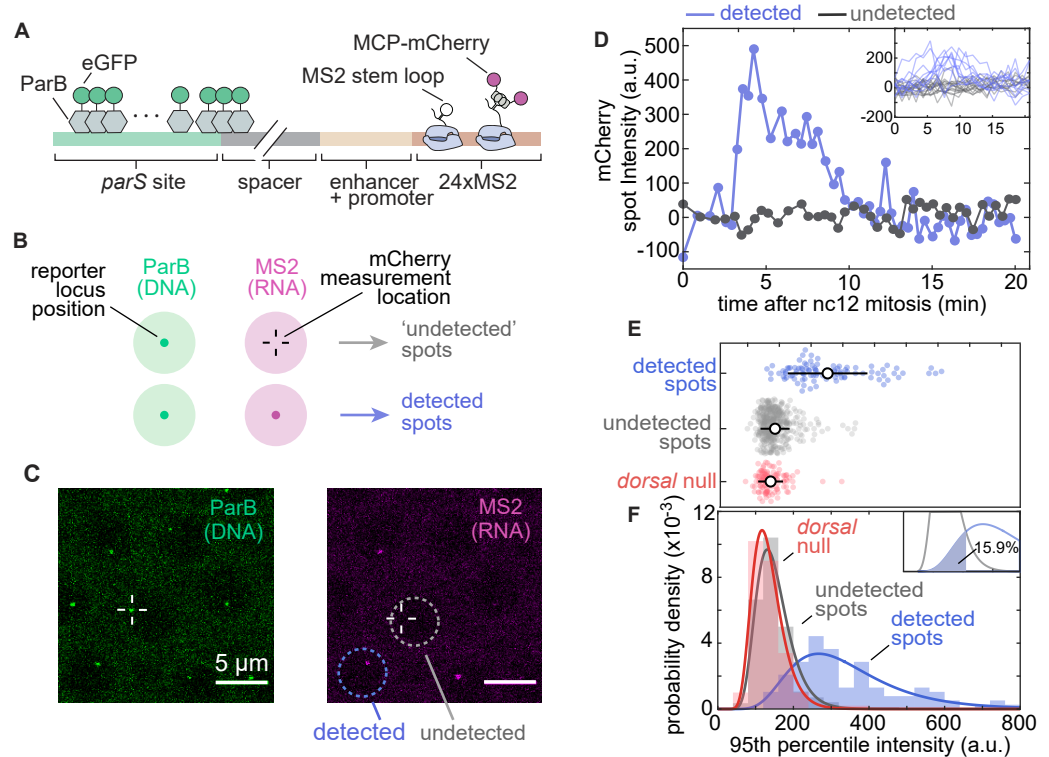
285 To quantify MS2 fluorescence independently of whether a MS2 spot was detected, we im-  
286 plemented the *parS*-ParB DNA labeling system (*Germier et al., 2017; Chen et al., 2018*). Here,  
287 fluorescently labeled ParB proteins bind the *parS* DNA sequence resulting in a fluorescence spot  
288 appearing at the locus independently of the transcriptional state of the locus (Fig. 4A). We created  
289 flies with and without functional Dorsal expressing ParB2-eGFP (subsequently referred to as ParB-  
290 eGFP) and MCP-mCherry to label our locus DNA and nascent RNA, respectively. We crossed flies  
291 containing *parS*-DBS\_6.23-MS2 to flies carrying ParB-eGFP and MCP-mCherry to generate embryos  
292 that have our locus of interest labeled with ParB-eGFP colocalized with the transcriptional signal in  
293 the MCP-mCherry channel (Fig. 4A,B; Video S4).

294 Guided by the spatial positions reported by ParB-eGFP, we measured the MCP-mCherry signal  
295 at all DBS\_6.23 reporter loci in embryos carrying wild-type Dorsal (Fig. 4C) or laid by mothers  
296 homozygous for the *d<sup>l</sup>* null allele (Dorsal null embryos). We then classified loci from wild-type  
297 Dorsal embryos into two categories, detected and undetected, depending on whether they were  
298 identified as spots in the MCP-mCherry channel by our analysis pipeline (Fig. 4B,C; Section 4.5). As  
299 shown in the the examples presented in Figure 4D, there are clear qualitative differences between  
300 MCP-mCherry fluorescence time traces corresponding to detected or undetected transcriptional  
301 spots from wild-type embryos. Thus, our analysis made it possible to quantify MS2 fluorescence in  
302 three populations: all loci in Dorsal null embryos, undetected loci in wild-type Dorsal embryos, and  
303 detected loci in wild-type Dorsal embryos.

304 To compare these populations, we computed the 95th percentile value over each locus' MCP-  
305 mCherry fluorescence time trace (Fig. 4E). The distribution of mCherry fluorescence from undetected  
306 spots in wild-type Dorsal embryos largely overlapped with that of all spots in Dorsal-null embryos  
307 (Fig. 4F), consistent with these two populations corresponding to loci expressing Dorsal-independent  
308 levels of activity. Moreover, both distributions were clearly distinct from the distribution of detected  
309 spots in wild-type Dorsal embryos (Fig. 4E,F). Thus, our results provide strong evidence that inactive  
310 loci are not artifacts of the detection limit of our imaging techniques. Rather, loci can belong to  
311 one of two distinct populations: those that transcribe at a high, Dorsal-dependent level and those  
312 that are transcriptionally inactive (or active at a low, undetectable level that is comparable to that of  
313 embryos lacking Dorsal). We therefore conclude that the decision to transcribe made by each locus

314 is an additional regulatory strategy controlled by Dorsal.

315 From the observations in Figure 4E and F, we estimated our error in classifying loci as inactive.  
 316 This false-negative detection rate, corresponding to the area under the curve shaded in the inset  
 317 of Figure 4F, is estimated as 15.9%. However, this false-negative rate is likely an underestimation.  
 318 For example, this rate may depend on Dorsal concentration, which cannot be controlled for in  
 319 this experiment. Additionally, the presence of ParB in the locus may itself affect transcriptional  
 320 dynamics, impacting the false-negative rate. For these reasons, we do not attempt to correct our  
 321 measurements of the fraction of active loci using this estimated false-negative rate.



**Figure 4. Transcriptionally independent ParB labeling confirms that transcriptionally inactive loci are functionally distinct from active loci.** (A) Schematic of ParB-eGFP construct. ParB-eGFP molecules bind and polymerize out from *parS* sequences, which are placed ~400 bp upstream of the enhancer. The enhancer and promoter together drive transcription of MS2 loops that subsequently bind MCP-mCherry. (B) Schematic of the experiment. Loci are located by detecting a signal in the ParB-eGFP channel; these locations were used to fit a 2D Gaussian to the same area in the MS2-mCherry channel to estimate fluorescence intensity regardless of whether an MS2-mCherry signal was detected (Materials and methods Sec. 4.4). (C) Example images of ParB-eGFP (left) and MCP-mCherry (right) channels. Detected and undetected transcriptionally active loci solely based on the MCP-mCherry signal alone are shown. (D) Example time traces of MCP-mCherry fluorescence over time at the ParB-eGFP loci in nuclei with (blue) and without (grey) detected MS2-mCherry spots of the DBS\_6.23 enhancer showing clear qualitative differences between the two populations. Inset, all detected and undetected fluorescence traces obtained in the same embryo. Negative intensity values are due to spot intensities very close to the background fluorescence. (E) Swarm plots of 95th percentile MCP-mCherry fluorescence at loci with detected (blue; N = 125) and undetected MS2-mCherry transcription (grey; N = 425) driven by the DBS\_6.23 enhancer in wild-type Dorsal embryos. Red (N = 96), maximum fluorescence of all loci in Dorsal null embryos, defined as the 95th percentile of intensity over time (black circles, mean; bars, standard deviation). Detected spots are significantly different from both null (ANOVA,  $p < 0.01$ ) and undetected spots (ANOVA,  $p < 0.01$ ) (F) Histograms of the data shown in (E). Solid lines correspond to log-normal fits performed for ease of visualization. Inset, undetected and detected distribution fits and the area used to estimate the false-negative detection rate of 15.9%.

## 322 **2.4 Dorsal-dependent kinetic barriers explain transcription onset dynamics and** 323 **modulation of the fraction of active loci**

324 Having established that transcriptionally inactive promoters mostly constitute a separate population  
325 from transcriptionally active promoters (Fig. 4), we sought to test whether our theoretical model  
326 (Fig. 2A) can quantitatively recapitulate the fraction of active loci and their transcription onset  
327 times. Tuning transcription factor-DNA binding affinity has been a powerful tool to test models  
328 of transcriptional regulation in the past (*Meijsing et al., 2009; Phillips et al., 2019*). Inspired by  
329 these previous works, we probed our model by adjusting the Dorsal-DNA interaction energy in our  
330 minimal synthetic enhancer.

331 We constructed a series of enhancers containing a single binding site with varying affinities for  
332 Dorsal. Building on the optimal DBS\_6.23 and the mutated DBS\_4.29 sites (Fig. 3D, left vs. right), we  
333 created five additional enhancers of varying intermediate strengths by introducing point mutations  
334 into the consensus Dorsal binding motif to obtain a range of predicted affinities (Fig. 5A,B; Materials  
335 and methods Section 4.1). As described above, we refer to these enhancers as DBS, followed by  
336 their corresponding Patser score.

337 For the purpose of quantifying output transcriptional activity as a function of Dorsal concentra-  
338 tion, we assigned a single Dorsal concentration value to each nucleus corresponding to the mVenus  
339 fluorescence in the center of that nucleus at a fiducial time point halfway through each nucleus's  
340 lifetime, approximately in the middle of nuclear cycle 12 when Dorsal levels are relatively stable  
341 (Fig. S5A,B). We next grouped nuclei into 17 linearly spaced bins that span the dorsoventral axis  
342 based on their fiducial fluorescence (Fig. S5B).

343 We assessed whether these point mutations were sufficient to generate a graded response to  
344 Dorsal and to determine the dynamic range of gene expression afforded by these enhancers. To  
345 make this possible, we integrated the total mRNA output over nuclear cycle 12 of each enhancer as  
346 a function of Dorsal concentration across all nuclei exposed to a given Dorsal concentration. The  
347 integrated mRNA output of the four weakest enhancers changed little across the dorsoventral axis  
348 (Fig. 5C). However, an appreciable trend in integrated mRNA was observed for the three strongest  
349 affinities (Fig. 5C). Further, plotting the total mRNA integrated across the entire dorsoventral axis of  
350 the embryo as a function of Patser score revealed that binding-site affinity (as reported by Patser  
351 score) is strongly correlated with transcriptional output in our single binding site enhancers (Fig. 5C,  
352 inset). In the case of this measure, there was also a threshold affinity: enhancers containing binding  
353 sites with affinities below that of DBS\_5.13 showed no substantial differences in transcriptional  
354 activity (inset, Fig. 5C).

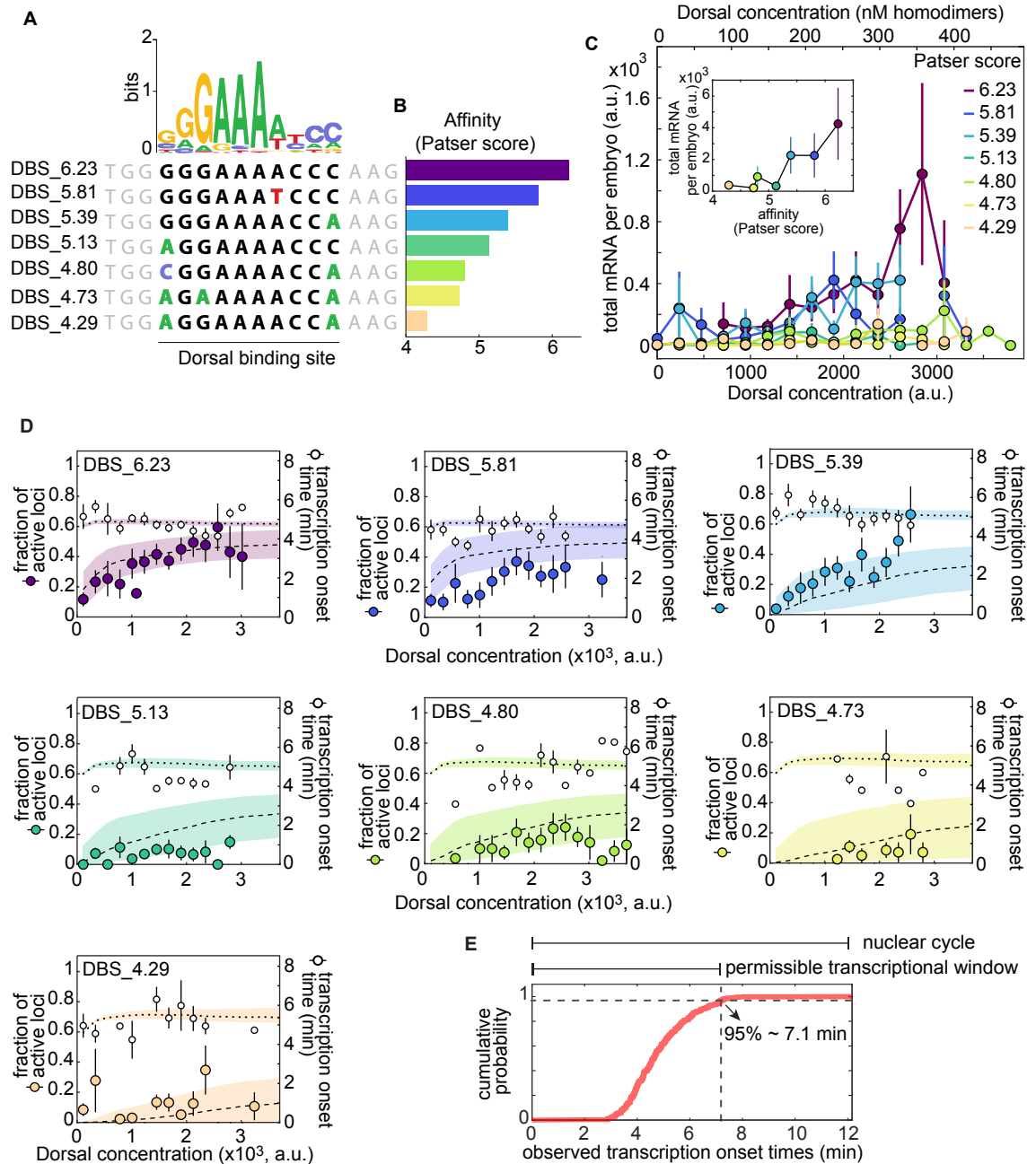
355 We used these constructs to measure mean transcriptional onset time as a function of Dorsal  
356 concentration and binding affinity, one of the key magnitudes predicted by our model (Fig. 2D). The  
357 measured mean onset time was relatively constant at  $\approx 5$  minutes across all Dorsal concentrations  
358 and enhancer constructs (Fig. 5D, dotted lines). This value is consistent with the measured onset  
359 times of other early embryonic genes such as the minimal *hunchback* promoter P2P (*Garcia et al.,*  
360 *2013; Lucas et al., 2013; Eck et al., 2020*).

361 We also determined that the fraction of active loci is highly sensitive to Dorsal concentrations  
362 and Dorsal binding-site affinity (Fig. 5D, dashed lines). The strongest Dorsal binding sites showed  
363 a large modulation of the fraction of active loci across Dorsal concentrations, while the weakest  
364 drove a relatively constant and low fraction of active loci across all Dorsal concentrations (Fig. 5D).

365 Our kinetic barrier model assumes that loci which fail to become active during the permissible  
366 transcription time window will remain inactive during the rest of the nuclear cycle (Fig. 2C). As a  
367 result, to determine whether the kinetic barrier model recapitulates the observations in Figure 5D, it  
368 was necessary to assign a value to this time window. We reasoned that the end of this time window  
369 determines the time point at which new transcription spots can no longer appear, possibly due  
370 to the onset of the next round of mitosis. To estimate the time point when nearly all spots have  
371 turned on, we calculated the 95th percentile of the observed spot onset times across all affinities:

372  $\approx 7.1$  min after the previous anaphase (Fig. 5E).

373 Using the measured time window of permissible transcription, we performed a simultaneous  
374 fit to the fraction of active loci and mean transcription onset times across all enhancers using  
375 the kinetic barrier model from Section 2.1 (Fig. 5D). Consistent with our model, we forced all  
376 enhancers to share the same value for  $c$ , and only letting the Dorsal dissociation constant,  $K_D$ ,  
377 vary for each enhancer separately. By systematically exploring models with different numbers  
378 of OFF states  $n$  (Fig. S10, Fig. S11), we determined that a biochemical cascade with at least 3 to  
379 4 rate-limiting OFF states is capable of capturing the qualitative behavior of our observations: a  
380 Dorsal concentration- and binding affinity-dependent fraction of active loci (dashed lines in Fig. 5D)  
381 and a mean transcription onset time that is mostly constant across Dorsal concentrations and  
382 affinities (dotted lines in Fig. 5D). Interestingly, alternative functional forms for  $k$ , such as modeling  
383 this transition rate as depending linearly on Dorsal concentration, instead of depending on Dorsal  
384 DNA occupancy, resulted in worse fits to the fraction of active loci at saturating concentrations of  
385 Dorsal (Section S1.5; Fig. S4). Thus, our observations can be explained by a model in which Dorsal,  
386 through DNA binding, accelerates the promoter's transition through a sequence of kinetic barriers  
387 to a state of active transcription.



**Figure 5. A multi-step kinetic barrier model predicts the Dorsal-dependent fraction of active loci with constant mean transcriptional onset times. (A)** Top: Dorsal positional weight matrix logo from *Ivan et al. (2008)*. Bottom: Sequence of the Dorsal binding sites engineered into minimal synthetic enhancers. Bold letters, 10 bp Dorsal motif. Black letters, consensus bases; colored letters, mutated bases; gray letters, sequence context. **(B)** Relative affinities of Dorsal binding sites estimated from the Patser algorithm using the Dorsal position weight matrix. **(C)** Overall transcriptional activity driven by the enhancers containing the binding sites in (A) measured as the total produced mRNA (fluorescence integrated over nuclear cycle 12) as a function of Dorsal concentration. Error bars, SEM over  $N > 3$  embryos containing 3 or more nuclei belonging to that fluorescence bin. The top x-axis shows the estimated nuclear Dorsal concentration according to the calibration described in Figure S6. Caption continues on next page.



**Figure 5. Continued from previous page: A multi-step kinetic barrier model explains the Dorsal-dependent fraction of active loci with constant mean transcriptional onset times.** (D) Data and model fits for the fraction of active loci (left y-axis) and mean transcription onset time (right y-axis) for each enhancer. Empty black circles, experimentally observed mean transcription onset time; filled circles, experimentally observed mean fraction of active loci. Fitted curves are represented as dashed lines (fraction of active loci) and dotted lines (mean onset times), corresponding to predictions using median parameter values from the joint posterior distribution. Shaded areas, 95% credible interval (see Table S1 for inferred parameter values). Error bars, SEM over  $N > 3$  embryos containing 3 or more nuclei belonging to that fluorescence bin. (E) Cumulative distribution of mean spot detection times per Dorsal fluorescence bin across all embryos and enhancers ( $N = 344$  spots). Vertical dashed line, time at which 95% of spots have turned on ( $\approx 7.1$  min) and end of the permissible transcription time window.

## 388 **2.5 The experimentally measured RNAP loading rate are compatible with a ther-** 389 **modynamic binding model**

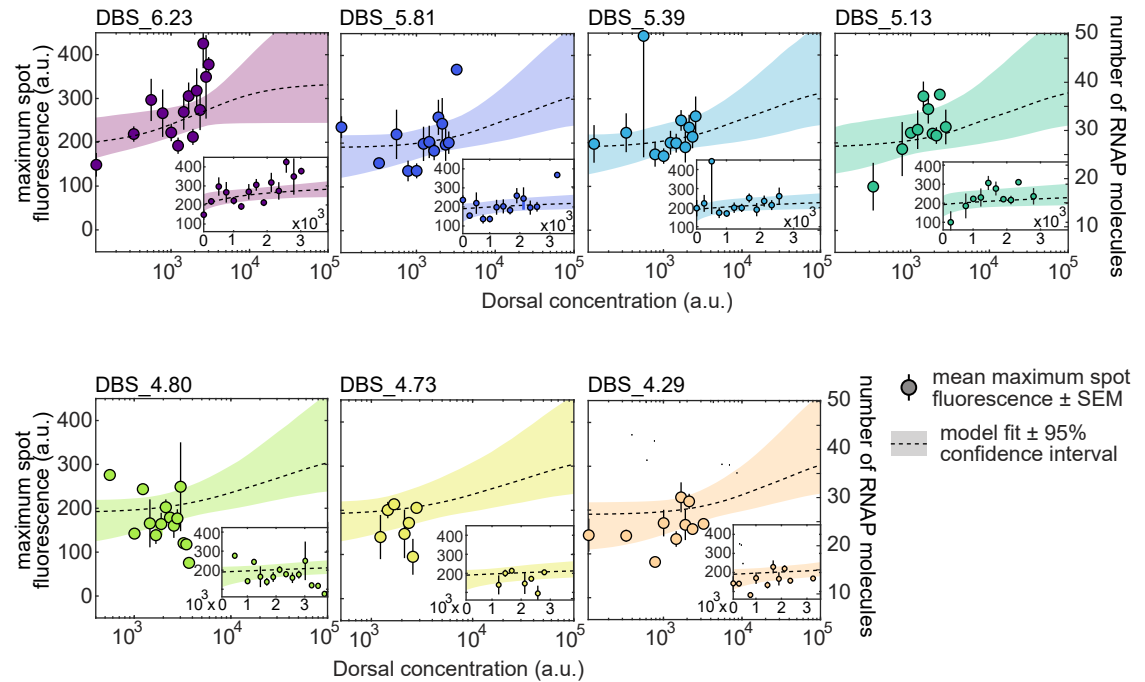
390 As a next step in our theoretical dissection, we tested the performance of our theoretical model  
391 in explaining the rate of transcription after loci become active. Typically, in MS2 experiments, the  
392 loading rate is measured from the initial slope of spot fluorescence traces (*Garcia et al., 2013; Eck*  
393 *et al., 2020; Liu et al., 2021*). However, due to the weak expression driven by our enhancers, it  
394 was not possible to perform this analysis with confidence (Fig. S8). In lieu of directly measuring  
395 the transcription rate, we evaluated a related, more robust and readily observable quantity: the  
396 maximum trace fluorescence (Fig. 3E). We approximately relate the RNAP loading rate predicted  
397 by the simple activator model (Equation 3) to the maximum fluorescence by a constant factor  
398 (Appendix S1 .2), enabling direct comparison between theoretical predictions and experimental  
399 data.

400 Measurements of the maximum spot fluorescence over time as a function of Dorsal concentra-  
401 tion for each of our seven minimal synthetic enhancers revealed that the maximum fluorescence is  
402 relatively constant across Dorsal concentration for most binding sites—particularly for the weakest  
403 of them, DBS\_5.13, DBS\_4.73, and DBS\_4.23 (Fig. 6). However, the sparse and noisy nature of our  
404 data makes it challenging to draw confident conclusions from the fits, even for the stronger binding  
405 sites (i.e. DBS\_6.23, DBS\_5.81, and DBS\_5.39). In the case of the lower affinity binding sites, the  
406 constant maximum fluorescence suggests that the Dorsal concentration level in our embryos is far  
407 below the Dorsal dissociation constant  $K_D$ , even after increasing the Dorsal dosage by a factor of  
408 two as in our 2x Dorsal line. The effect of very low Dorsal concentrations relative to their respective  
409  $K_D$  values can be clearly seen in Equation 3 and in Figure 2, where, for  $[D]/K_D \ll 1$ , the RNAP  
410 loading rate,  $R$ , adopts a basal level given by

$$R = R_{max} \frac{\frac{P}{K_p}}{1 + \frac{P}{K_p}} \quad (4)$$

411 that is independent of Dorsal concentration and binding affinity.

412 As shown on the right y-axes in Figure 6, this basal level corresponds to  $\approx 20$  RNAP molecules  
413 actively transcribing the gene ( $\approx 15\%$  of the maximum number of RNAPs that can fit on the gene, as  
414 described in Section S1 .3). For ease of visual comparison to the thermodynamic model predictions,  
415 we also plotted best-fit theoretical curves on top of the data using dashed curves (the insets in Fig. 6  
416 show the same plots but zoomed into the measured data and plotted on a linear scale). These fits  
417 further underscore that our data do not explore a wide dynamic range with the precision necessary  
418 to determine the magnitude of  $K_D$  for each construct and to thoroughly test the thermodynamic  
419 model.



**Figure 6. Testing RNAP loading rate predictions of the thermodynamic model.** Mean maximum spot fluorescence as a function of Dorsal concentration for minimal synthetic enhancers with different affinities for Dorsal (filled circles). The right y-axis denotes the calibrated number of actively transcribing RNAP molecules (for details of calibration, see Section S1.3 and Fig. S2). Dashed curves correspond to a simultaneous Markov Chain Monte Carlo curve fit to all data using Equation 3. Fits share all parameters except  $K_D$ . Shaded areas, 95% prediction intervals. Insets, same data and fits plotted on a linear scale with axis ranges zoomed in on the data. See Table S2 for inferred parameter values. Error bars, SEM across  $N > 3$  embryos containing 3 or more nuclei in a given fluorescence bin.

### 3 Discussion

A major obstacle to uncovering the mechanistic and quantitative underpinnings of enhancer action is the inherent complexity of endogenous regulatory sequences. Synthetic minimal enhancers are powerful alternatives to the complex experimental reality faced by modeling efforts in endogenous enhancers (Garcia et al., 2016, 2020). Synthetic minimal enhancers contain binding sites for one or a handful of transcription factors, making them more amenable to theoretical dissection (Fakhouri et al., 2010; Sayal et al., 2016; Crocker and Ilsley, 2017) and revealing the complex interplay among activators, repressors, and pioneer factors, as well as their contribution to mRNA transcript accumulation (Fakhouri et al., 2010; Sayal et al., 2016; Crocker and Ilsley, 2017). However, previous synthetic-based efforts to dissect enhancer function always involved fixed-embryo measurements, which cannot reveal the three inherently dynamical roles dictated by enhancer sequences (Fig. 1).

Here we augmented previous synthetic approaches by quantifying the real-time action of minimal enhancers with one binding site for the Dorsal activator in single cells of living, developing *Drosophila* embryos using the MS2 system. Contrary to theoretical speculations that single binding sites within eukaryotic genomes lack enough information to be recognized by transcription factors in the absence of other nearby binding sites (Wunderlich and Mirny, 2009), we demonstrated that Dorsal can drive expression when bound to single binding sites (Fig. 3D). Additionally, we demonstrated that the fraction of active loci is a feature under regulatory control in our synthetic system (Fig. 3F; Fig. 4F), confirming the important role of this regulatory strategy in shaping the expression dynamics of endogenous enhancers (Garcia et al., 2013; Dufourt et al., 2018; Lammers et al., 2020; Harden et al., 2021). Thus, while the signal driven by our minimal synthetic constructs

441 is weak (Fig. 6), it can be quantified and recapitulates biologically relevant dynamic features of  
442 transcription that are also at play in endogenous enhancers.

443 It is important to note that the uncovering of a fraction of inactive loci in many reporter systems  
444 by us and others (*Garcia et al., 2013; Dufourt et al., 2018; Lammers et al., 2020; Harden et al.,*  
445 *2021*) did not necessarily imply that this modulation of transcriptional engagement constitutes  
446 a biological control variable. Indeed, because live cell imaging techniques typically lack single-  
447 molecule resolution, it was unclear whether undetected loci in our study—and all previous studies—  
448 corresponded to a distinct population or were a detection artifact. By simultaneously labeling  
449 the locus with the transcription-independent reporter ParB-eGFP and nascent mRNA with MCP-  
450 mCherry (Fig. 4A), we demonstrated that a significant number of loci categorized as inactive do not  
451 constitute an experimental artifact and instead correspond to a distinct transcriptional state that is  
452 comparable to that measured in the absence of Dorsal protein (Fig. 4). In the future, conducting all  
453 live transcription measurements with DNA loci labeled by ParB could make it possible to confidently  
454 quantify the activity of all loci regardless of their activity.

455 Our minimal synthetic constructs and our validation of a distinct population of inactive loci  
456 enabled us to test an emerging theoretical model of enhancer action in development: a kinetic  
457 barrier model of transcriptional engagement (Fig. 2A; *Fritzsch et al. (2018); Dufourt et al. (2018);*  
458 *Eck et al. (2020)*). Importantly, our model deviated from previous theoretical efforts that assumed  
459 that the transition rates between states preceding transcriptional engagement were either constant  
460 (*Dufourt et al., 2018*) or depended linearly on activator concentration (*Eck et al., 2020*). Instead,  
461 in order to account for the effects of Dorsal binding affinity on transcriptional dynamics, we  
462 assumed that this rate was proportional to Dorsal occupancy at its target DNA site. Thus, while the  
463 mechanisms underlying several aspects of this model, such as the molecular identity of the various  
464 OFF states, remain unknown, this model can generate predictions for how the fraction of active  
465 loci and the transcriptional onset time are modulated by the Dorsal concentration and its binding  
466 affinity (Fig. 2C-E).

467 We systematically challenged this model by generating a small collection of minimal synthetic  
468 enhancers spanning a large range of affinities for Dorsal (Fig. 5A). Comparing the fraction of  
469 active loci and the transcription onset times of these enhancers revealed that the kinetic barrier  
470 model recapitulated our measurements (Fig. 5D). In past studies probing transcription dynamics  
471 in the *Drosophila* embryo (*Dufourt et al., 2018; Eck et al., 2020*), the pioneer factor Zelda was  
472 found to be largely responsible for ensuring constant transcription factor onset times and for  
473 determining the fraction of active loci. We cannot rule out the potential existence of distant or  
474 low-affinity Zelda binding sites (*Rushlow and Shvartsman, 2012*) in our constructs. Alternatively,  
475 as it was recently demonstrated for the Bicoid activator *Hannon et al. (2017)*, Dorsal could also  
476 have a pioneering activity. Indeed, the Dorsal homolog NF- $\kappa$ B has been recently shown to displace  
477 nucleosomes (*Cheng et al., 2021*). To further test the kinetic barrier model, it would be informative  
478 to directly perturb the temporal dynamics of nuclear Dorsal concentration to affect transcriptional  
479 engagement. For example, several optogenetics systems have been successfully deployed in the  
480 early fly embryo to inactivate transcription factors during discrete time windows (*Huang et al., 2017;*  
481 *McDaniel et al., 2019; Irizarry et al., 2020*). In the future, a version of one of these systems may  
482 dissect how the temporal dynamics of Dorsal concentration affect transcriptional activation.

483 Although the kinetic barrier model predicted the fraction of active loci and onset times (Fig. 5D)  
484 relatively well, we were unable to use our data to conclusively test the thermodynamic model's  
485 predictions of the rate of mRNA production (Fig. 6). Such limitation stemmed from the fact that only  
486 a fraction of loci display detectable transcription that can be used to quantify the mRNA production  
487 rate. Further, among these loci, the rate of transcription was found to be highly variable. As a  
488 result, our statistics were limited such that it was not possible to perform an unequivocal test of the  
489 thermodynamic model.

490 The apparent lack of substantial Dorsal concentration dependence observed in our measure-  
491 ments of RNAP loading rate could be explained in two possible ways. First, it is possible that there

492 is a modulation of this rate in our measurements, but that this modulation is obscured by our  
493 experimental noise. Second, the Dorsal concentrations accessed by our experiment could be below  
494 the  $K_D$  of our binding sites. In this scenario, a modulation in the mRNA production rate would  
495 become apparent only at Dorsal concentrations higher than those attainable by our experimental  
496 system. While our embryos contained double the genetic dosage of Dorsal compared to wild type,  
497 perhaps 5-10 times the wild-type Dorsal concentration could be needed to exceed the  $K_D$  and  
498 modulate the rate of mRNA production. To express this high Dorsal concentration, which is certain  
499 to affect normal embryonic development, genetic approaches to increase Dorsal dosage in the  
500 embryos similar to those recently applied to flatten the Bicoid gradient might be necessary (*Hannon*  
501 *et al., 2017*).

502 It is important to note that, despite not seeing a modulation in the rate of mRNA production, we  
503 do see a significant change in the fraction of active loci as Dorsal concentration is varied (Fig. 5).  
504 This seeming contradiction could be explained through the presence of two dissociation constants  
505 in our model (Fig. 2): one dissociation constant for the first part of the model governing the onset  
506 of transcription, and a different dissociation constant for the second part of the model dictating  
507 the rate of RNAP loading once transcription has ensued. Interestingly, previous works quantifying  
508 transcriptional dynamics of a minimal Bicoid-activated *hunchback* P2 enhancers also hint at the  
509 existence of these two distinct dissociation constants (*Garcia et al., 2013*).

510 Further, this model is consistent with our surprising observation of a basal level of transcription  
511 in the presence of even extremely weak binding sites (Fig. 6) despite the lack of detected transcrip-  
512 tion in the absence of Dorsal protein (Fig. 3D, middle). This observation could be explained if Dorsal  
513 acted as both as a pioneer-like transcription factor triggering the onset of transcription, even at low  
514 concentrations relative to its  $K_D$ , and as an activator of the transcription rate at high concentrations.

515 Going forward, synthetic minimal enhancers could constitute the foundation for exploring the  
516 behavior of more complex regulatory regions. Independently inferring biophysical parameters  
517 such as Dorsal-DNA binding and dissociation constants could help constrain models of Dorsal  
518 participating in the activation of promoters with additional activators and repressors (*Fakhouri et al.,*  
519 *2010; Sayal et al., 2016*). Indeed, while Dorsal is the sole maternal nuclear-localized input specifying  
520 dorsoventral position in *Drosophila*, it rarely acts alone in endogenous enhancers (*Hong et al., 2008*).  
521 For example, the interaction of Dorsal with Twist is a classic example of positive cooperativity in  
522 development (*Szymanski and Levine, 1995*). Dorsal can also act as a repressor depending on the  
523 presence of nearby Capicua binding sites (*Shin and Hong, 2014*). The minimal synthetic enhancers  
524 presented here could be used as scaffolds for more complex minimal enhancers incorporating a  
525 second binding site for Twist or Capicua, for example.

526 In conclusion, we have developed a minimal synthetic enhancer system that has shed light  
527 on the fundamental assumptions about transcription in development. By engaging in a dialogue  
528 between theory and experiment, we have advanced our understanding of how kinetic processes  
529 give rise to important features of transcriptional dynamics in the embryo and made progress toward  
530 predictive understanding of how regulatory DNA sequence dictates the functional relation between  
531 input transcription factor dynamics and output transcriptional activity in development.

## 532 **4 Methods and materials**

### 533 **4.1 Plasmids and reporter design**

534 To design our minimal construct (Fig. 3), we placed the 10 bp consensus Dorsal binding site  
535 (*Markstein et al., 2002*) upstream of the *even-skipped* core promoter. This enhancer-promoter  
536 construct drives the expression of the MS2v5 sequence containing 24 nonrepetitive MS2 loops  
537 (*Tutucci et al., 2018*) followed by the *lacZ* coding sequence and the *tubulin* 3'UTR. (*Garcia et al.,*  
538 *2013*).

539 In addition to the consensus Dorsal binding site (DBS\_6.23), we created six enhancers of varying  
540 strength by introducing point mutations to the consensus Dorsal binding motif. Some of these

541 binding sites were taken from known validated Dorsal motifs (*Markstein et al., 2002*), while others  
542 were generated based on mutations known to decrease Dorsal binding (*Ip et al., 1992; Jiang et al.,*  
543 *1991*). To guide the design of these binding sites, we used an already existing position weight matrix  
544 computed with the MEME algorithm (*Ivan et al., 2008; Bailey et al., 2006*) using motifs generated  
545 by DNase I footprinting assays (*Bergman et al., 2005*) and quantified the information content of  
546 each base pair using Patser (*Hertz and Stormo, 1999*).

547 All plasmid sequences used in this study are shown in Table 1 and can be accessed from a public  
548 [Benchling](#) folder. Injections were carried out by Rainbow inc. or Bestgene inc.

## 549 **4.2 Flies**

550 Reporter plasmids were injected into BDSC fly line 27388 containing a landing site in position  
551 38F1. Transgene orientation was confirmed by PCR using primers 18.8 (ggaacgaaggcagtagttgt) and  
552 Ori-Seq-F1 (tagttccagtgaatccaagcattttc) binding outside of the 5' 38F1 *attP* site and the *even-skipped*  
553 promoter, respectively. All reporter lines were confirmed to be in the same orientation. All flies  
554 used in this study can be found in Table 2.

555 To generate the embryos used in the experiments shown in all figures except for Figure 4, we  
556 crossed 2x Dorsal or 1x Dorsal virgins to males carrying synthetic enhancers. The genotype of  
557 2x Dorsal flies is *yw;DI-mVenus (CRISPR), MCP-mCherry; Dorsal-mVenus, MCP-mCherry, His2Av-iRFP*.  
558 The genotype of 1x Dorsal flies is *yw;dl[1], MCP-mCherry; Dorsal-mVenus, MCP-mCherry, His2Av-iRFP*.  
559 Because there does not seem to be a difference in transcriptional activity between the CRISPR  
560 knock-in and the transgene Dorsal-mVenus alleles (Fig. S7), we combined the 1x Dorsal and 2x  
561 Dorsal data for some enhancers.

562 MCP-mCherry and His-iRFP were described before by (*Liu et al., 2021*). The Dorsal-mVenus  
563 transgene was developed by *Reeves et al. (2012)*.

564 To generate the Dorsal-Venus knock-in allele we used the CRISPR/Cas9 protocol described by  
565 (*Gratz et al., 2015*). We generated a donor plasmid containing the mVenus sequence followed  
566 by a stop codon and a 3xP3-dsRed marker flanked by PiggyBac recombinase sites. This insert  
567 was flanked by two  $\approx 1$  kbp homology arms matching  $\approx 2$  kbp surrounding the Dorsal stop codon  
568 (plasmid DI-mVenus-dsRed in Table 1). The Cas9 expressing BDSC line 51324 was injected with  
569 the donor plasmid in combination with a plasmid carrying a sgRNA targeting the sequence GTTGT-  
570 GAAAAAGGTATTACG in the C-terminus of Dorsal (plasmid pU6-DlgRNA1 in Table 1). Survivors  
571 were crossed to *yw* and the progeny was screened for dsRed eye fluorescence. Several independent  
572 lines were established and tested for rescue. The insertion was confirmed by PCR using primers  
573 flanking the homology arms OutLHA (ccattaaaacggaaccaagaggtgag) and OutDIRHA (tctaacaatggctc-  
574 gatttttgcca). The dsRed eye marker cassette was flipped out of rescuing lines via crossing with a  
575 piggyBac recombinase line. The resulting Dorsal-mVenus locus was then resequenced using the  
576 same primers.

577 The data shown in Figure 4 were obtained from embryos laid by *yw;ParB2-eGFP, eNosx2-MCP-*  
578 *mCherry*;+ (wild-type Dorsal mothers) or *yw;ParB2-eGFP, eNosx2-MCP-mCherry, dl[1]*;+ (Dorsal null  
579 mothers).

## 580 **4.3 Microscopy**

581 Fly cages were allowed to lay for 90 to 120 minutes prior to embryo collection. Embryos were then  
582 mounted on microscopy slides in Halocarbon 27 oil (Sigma-Aldrich, H8773) in between a coverslip  
583 and breathable membrane as described in (*Garcia et al., 2013; Bothma et al., 2014; Garcia and*  
584 *Gregor, 2018*).

585 Confocal microscopy was performed on a Leica SP8 with HyD detectors and a White Light  
586 Laser. We used a 63x oil objective, and scanned bidirectionally with a scan rate of 420 Hz and a  
587 magnification of 3.4x zoom. We did not use line or frame accumulation. Time-lapse z-stacks were  
588 collected with  $\sim 10$  s frame rate and 106 nm x-y pixel dimensions and 0.5  $\mu\text{m}$  separation between  
589 z-slices (7  $\mu\text{M}$  range, 16 slices). x-y resolution was 512x512 pixels. Pinhole was set to 1.0 Airy units at



590 600 nm. mVenus was excited by a 510 nm laser line calibrated to 5  $\mu$ W using the 10x objective and  
591 detected in a 520-567 nm spectral window. mCherry was excited by a 585 nm laser line calibrated  
592 to 25  $\mu$ W and detected in a 597-660 nm spectral window. To image His2av-iRFP, the 700 nm laser  
593 line was set to 10% and detected in a 700-799 nm spectral window. In all channels, detection was  
594 performed using the counting mode of the HyD detectors.

595 All movies were taken at ~50% along the anterior-posterior axis of the embryo.

#### 596 **4.4 ParB experiment fly crosses and microscopy**

597 We created flies with and without functional Dorsal expressing ParB2-eGFP maternally driven by  
598 the *nanos* promoter and MCP-mCherry driven by two copies of a minimal *nanos* enhancer to label  
599 our locus DNA and nascent mRNA, respectively. In addition, we added a parS sequence followed by  
600 a 400 bp spacer (created with SiteOut, *Estrada et al. (2016)*) to our DBS\_6.23 enhancer. We then  
601 crossed male flies containing parS-DBS\_6.23-MS2 to *yw; ParB2-eGFP; eNosx2-MCP-mCherry*; + females  
602 to create embryos that have our locus of interest labeled with eGFP colocalized with transcriptional  
603 loci in the MCP-mCherry channel (Fig. 4A and B).

604 After mounting embryos using the protocol described above in Section 4.3, we used the sequen-  
605 tial scanning mode on the Leica SP8 confocal microscope to eliminate bleedthrough from eGFP  
606 into the mCherry channel, and imaged at approximately 20 s per stack, half the rate used in other  
607 imaging experiments in this study.

#### 608 **4.5 Image and time-series analysis**

609 Image analysis was performed in Matlab using the custom pipeline described in *Garcia et al. (2013)*  
610 and *Lammers et al. (2020)* (this pipeline can be found in the [mRNA Dynamics Github repository](#)).  
611 Image segmentation was also aided by the Trainable Weka Segmentation plugin in Fiji (*Witten et al.,*  
612 *2016; Arganda-Carreras et al., 2017*). Further analysis of time-series and other data were likewise  
613 performed in Matlab. Movies for publication were made in Fiji (*Schneider et al., 2012; Schindelin*  
614 *et al., 2012*).

#### 615 **4.6 Measuring Dorsal-mVenus concentration**

616 Dorsal-mVenus concentration was calculated as in (Fig. S5). As shown in the figure, we measured  
617 the average mVenus fluorescence intensity in a circle of 2  $\mu$ m radius at the center of the nucleus in  
618 every z-slice of each nucleus. This results in a z-profile of fluorescence values covering the nucleus  
619 itself and the cytoplasm below and above it. The reported concentration corresponds to the value  
620 at the middle z-plane of each nucleus. To find this plane, we fit a parabola to the fluorescence  
621 z-profile. We use as the nuclear concentration the fluorescence value at the plane corresponding to  
622 the fitted parabola's vertex (Fig. S5B). We then plotted this value over time and selected a single time  
623 point for each trace corresponding to the middle of each nucleus's observed trajectory (Fig. S5B).  
624 To determine the background fluorescence in the mVenus channel we imaged flies with the same  
625 genotype as 2x Dorsal except for the Dorsal-Venus fusions. We calculated the average nuclear  
626 fluorescence in the mVenus channel across nuclear cycle 12 and subtracted this value from our  
627 Dorsal-Venus measurements.

#### 628 **4.7 Curve fitting and parameter inference**

629 Curve fitting and parameter inference were performed in Matlab using the [MCMCSTAT Matlab](#)  
630 [package](#) using the DRAM Markov Chain Monte Carlo algorithm (*Haario et al., 2006*). For simplicity,  
631 uniform priors were assumed throughout.

### 632 **5 Acknowledgments**

633 We thank Greg Reeves for providing the Dorsal-mVenus and *d<sup>l</sup>* fly lines. We also thank Francois  
634 Payre and Philippe Valenti for sharing a ParB2-eGFP plasmid and a 2xIntB2 (aka *parS*) plasmid.

635 We would like to thank Rob Phillips, Jane Kondev, and members of the Garcia lab for their helpful  
636 feedback on the manuscript.

637 H.G.G was supported by the Burroughs Wellcome Fund Career Award at the Scientific Interface,  
638 the Sloan Research Foundation, the Human Frontiers Science Program, the Searle Scholars Program,  
639 the Shurl and Kay Curci Foundation, the Hellman Foundation, the NIH Director's New Innovator  
640 Award (DP2 OD024541-01), and an NSF CAREER Award (1652236). AR was supported by NSF GRFP  
641 (DGE 1752814).

## 642 **6 Biological material**

<b>Plasmids</b>	
<b>Name (hyperlinked to Benchling)</b>	<b>Function</b>
<a href="#">pIB-1Dg-evePr-MS2v5-LacZ-Tub3UTR</a>	DBS_6.23-MS2 reporter
<a href="#">pIB-1DgS-MS2v5-LacZ-Tub3UTR</a>	DBS_5.81-MS2 reporter
<a href="#">pIB-1DgW-MS2v5-LacZ-Tub3UTR</a>	DBS_5.39-MS2 reporter
<a href="#">pIB-1DgAW-MS2v5-LacZ-Tub3UTR</a>	DBS_5.13-MS2 reporter
<a href="#">pIB-1DgSVW-MS2v5-LacZ-Tub3UTR</a>	DBS_4.8-MS2 reporter
<a href="#">pIB-1DgVW-MS2v5-LacZ-Tub3UTR</a>	DBS_4.73-MS2 reporter
<a href="#">pIB-1DgVW-MS2v5-LacZ-Tub3UTR</a>	DBS_4.29-MS2 reporter
<a href="#">pIB-2xIntB2-Neutral400-1Dg-MS2v5-LacZ-Tub3UTR</a>	DBS_6.23-MS2 reporter with two ParB2 binding sites (note that 2xIntB2 is termed a parS sequence in the main text)
<a href="#">DI-mVenus-dsRed</a>	Donor plasmid for Dorsal-mVenus CRISPR knock-in fusion
<a href="#">pU6-DlgRNA1</a>	Synthetic guide RNA for Dorsal-mVenus CRISPR knock-in fusion
<a href="#">pBPhi-eNosx2-pTrans-NoNLS-MCP-mCherry-tub3'UTR</a>	Maternally deposited MCP-mCherry
<a href="#">pCasper4-His2Av-iRFP</a>	Histone2Av fusion to infrared RFP (His-iRFP)
<a href="#">pCasper4-Pnos-NoNLS-MCP-mCherry-TUB3'UTR</a>	Maternally deposited MCP-mCherry
<a href="#">pCasper-pNos-NoNLS-ParB2-GFP-TUB3'UTR</a>	ParB-eGFP

**Table 1.** List of plasmids used to create the transgenic fly lines used in this study.

Fly lines	
Genotype	Usage
<i>yw; ParB2-eGFP; eNosx2-MCP-mCherry; +</i>	Label reporter DNA and nascent RNA
<i>yw; Dorsal-mVenus, pNos-MCP-mCherry; pNos-MCP-mCherry, His2Av-iRFP</i>	Females to visualize Dorsal protein, label nascent RNA, label nuclei
<i>yw; Dorsal-mVenus, pNos-MCP-mCherry; Dorsal-mVenus, pNos-MCP-mCherry, His2Av-iRFP</i>	Females to visualize Dorsal protein, label nascent RNA, label nuclei
<i>yw; dl<sup>1</sup>, pNos-MCP-mCherry; pNos-MCP-mCherry, His2Av-iRFP</i>	Females to label nascent mRNA and label nuclei in embryos lacking Dorsal protein
<i>yw; 1Dg(11); +</i>	Males carrying the DBS_6.23-MS2 reporter
<i>yw; 1DS(2); +</i>	Males carrying the DBS_5.81-MS2 reporter
<i>yw; 1DgW(2); +</i>	Males carrying the DBS_5.39-MS2 reporter
<i>yw; 1DgAW(3); +</i>	Males carrying the DBS_5.13-MS2 reporter
<i>yw; 1DgSVW(2); +</i>	Males carrying the DBS_4.8-MS2 reporter
<i>yw; 1DgVWV(3); +</i>	Males carrying the DBS_4.73-MS2 reporter
<i>yw; 1DgVW); +</i>	Males carrying the DBS_4.29-MS2 reporter
<i>yw; 2xIntB2-1Dg(4)(5)(6); +</i>	Males carrying the DBS_6.23-MS2 reporter with two ParB2 binding sites (note that 2xIntB2 is termed <i>parS</i> in the main text and in figures)

**Table 2.** List of fly lines used in this study and their experimental usage

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## 897 Supplementary information

### 898 S1 Appendix

#### 899 S1.1 Calculating the fraction of active loci and the transcriptional onset time by 900 solving the kinetic barrier model

We describe here in detail the method we used to solve kinetic barrier model presented in Section 2.1 and Figure 2A. The problem posed in Figure 5A, namely the time evolution of the probability of nuclei occupying a discrete number of consecutive states, can be described by the following system of linear differential equations (also known as the 'master equation')

$$\frac{d\bar{P}}{dt} = \mathbf{K}(t)\bar{P}, \quad (S1)$$

901 where  $\bar{P}$  is a column vector containing the probability as a function of time of each of the states  
902 that the system can be in.  $\mathbf{K}$  corresponds to the transition rate matrix containing the rates that  
903 dictate the passage from each OFF state to the next and to the final ON state.

904 For  $n$  OFF states followed by a ON state connected by irreversible transitions with a rate of  $k(t)$ ,  
905 Equation S1 can be written as

$$\begin{bmatrix} \frac{dP(OFF_1,t)}{dt} \\ \frac{dP(OFF_2,t)}{dt} \\ \dots \\ \frac{dP(OFF_n,t)}{dt} \\ \frac{dP(ON,t)}{dt} \end{bmatrix} = \begin{bmatrix} -k(t) & 0 & \dots & 0 & 0 \\ k(t) & -k(t) & \dots & 0 & 0 \\ \dots & \dots & \dots & \dots & \dots \\ 0 & 0 & \dots & -k(t) & 0 \\ 0 & 0 & \dots & k(t) & 0 \end{bmatrix} \times \begin{bmatrix} P(OFF_1,t) \\ P(OFF_2,t) \\ \dots \\ P(OFF_n,t) \\ P(ON,t) \end{bmatrix}, \quad (S2)$$

906 where  $P(s, t)$  indicates the probability of the system being in state  $s$  at time  $t$ .

907 As described in Section 2.4, the transition rate matrix,  $\mathbf{K}$ , is a function of time as a consequence  
908 of the assumption that the transition rate between states,  $k$ , depends on the time-varying Dorsal  
909 concentration. In our model,  $k$  is given by

$$k(t) = c \cdot \frac{\frac{[D](t)}{K_D}}{1 + \frac{[D](t)}{K_D}}, \quad (S3)$$

911 where  $K_D$  is the Dorsal binding dissociation constant and  $c$  is a rate constant. If  $k$  were a constant,  
912 then the system of equations describing transcriptional dynamics could be solved analytically.  
913 However, because  $k(t)$  depends on the empirical Dorsal-mVenus fluorescence dynamics, which  
914 does not have a concrete functional form, solving the system in Equation S2 becomes analytically  
915 intractable. Thus, in order to obtain the probability of each state as a function of time,  $\bar{P}$ , and  
916 calculate the fraction of active loci and the mean transcription onset times, we solve the system in  
917 Equation S2 numerically for a given number of  $n$  OFF states. Specifically, at each time step  $dt$ , we  
918 calculated how the probability of each state changes with respect to the previous time step.

919 To calculate  $P(s, t)$  we need to consider the previous time step  $t - 1$  and take into account three  
920 possible scenarios:

- 921 1. Loci that were already in state  $s$  at time  $t - 1$  and stay in this state at time  $t$ .
- 922 2. Loci that were in state  $s - 1$  at  $t - 1$  that transition into state  $s$  at time  $t$ .
- 923 3. Loci that were in state  $s$  at time  $t - 1$  that leave this state by transitioning to the next state  $s + 1$   
924 at time  $t$ .

925 The likelihood of a locus jumping from one state to the next at time  $t$  during an arbitrarily small  
926 time window of  $dt$  is given by the transition rate  $k(t) \times dt$ . As a result, the probability of the promoter  
927 locus being in state  $s$  at time  $t$  can be calculated as

$$\underbrace{P(s,t)}_{\text{Probability of state } s \text{ at time } t} = \underbrace{P(s,t-1)}_{\text{being in state } s \text{ at } t-1} + \underbrace{k(t)dtP(s-1,t-1)}_{\text{enter from state } s-1} - \underbrace{k(t)dtP(s,t-1)}_{\text{leave for state } s+1}. \quad (S4)$$

928 It is clear that, for  $s = 1$ ,  $P(s - 1, t - 1) = 0$ , since there is not a previous state from which loci can  
 929 enter the first OFF state. Similarly, since promoters cannot leave the final ON state once they have  
 930 entered it,  $P(n + 2, t - 1) = 0$  for  $n$  OFF states.

To obtain the fraction of active loci, we initialize the system to  $P(s = 1, t = 0) = 1$  and calculate  $P(s = n + 1, t = T/dt)$ , where  $T$  is the duration of the transcriptional window such that

$$\text{Fraction of active loci} = P(n + 1, T/dt). \quad (S5)$$

To obtain the mean transcriptional onset time, we calculate the expected value  $\mathbb{E}[\text{onset}]$  of the time to reach the final  $n + 1$  state before the end of the transcriptional time window at  $t = T$ . From the definition of expected value,

$$\mathbb{E}[\text{onset}] = \sum_{i=1}^{i=T} t_i \times p_i, \quad (S6)$$

where  $t_i$  indicates a given onset time and  $p_i$  the probability of loci having that specific onset time. Note that the sum only runs until the end of the transcription time window  $T$ , as loci that will remain inactive for the duration of the nuclear cycle should not be considered in our calculation of the mean transcriptional onset time. This means that  $p_i$  is a normalized probability, calculated only amongst loci that turn on before time  $T$  such that

$$\sum_{i=1}^{i=T} p_i = 1. \quad (S7)$$

In terms of the system described in Equation S4, the probability  $p_i$  of loci reaching the ON state  $n+1$  at time  $t_i$  is

$$\text{Probability of loci to turn on at time } t_i = P(n + 1, t_i) - P(n + 1, t_i - 1). \quad (S8)$$

And the normalized probability  $p_i$  of loci reaching the ON state  $n+1$  at time  $t_i$  among loci that reach it before  $T$  is

$$\text{Probability of loci to turn on at time } t_i \text{ (normalized)} = p_i = \frac{P(n + 1, t_i) - P(n + 1, t_i - 1)}{\sum_{i=1}^{i=T} [P(n + 1, t_i) - P(n + 1, t_i - 1)]}. \quad (S9)$$

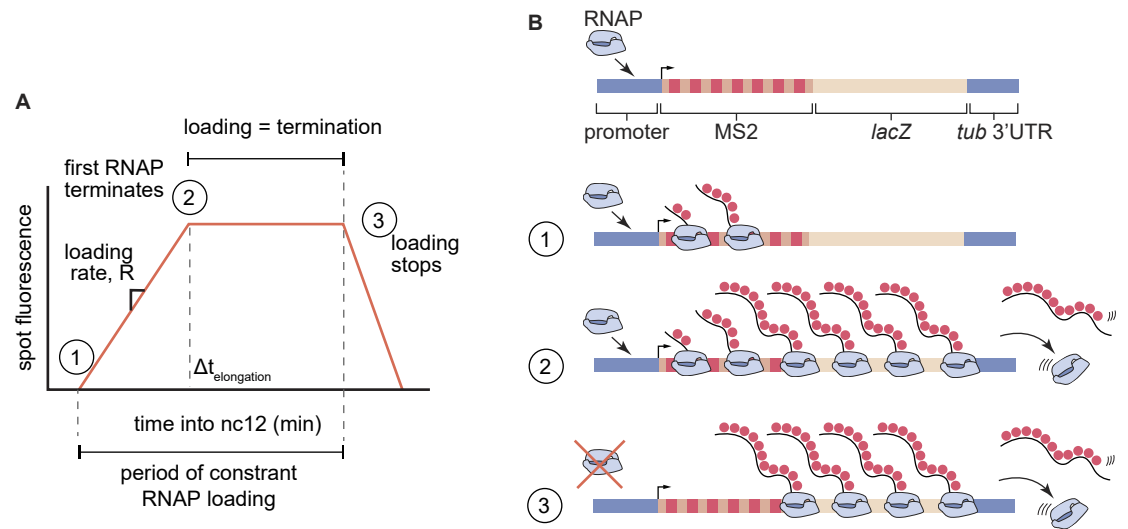
Replacing  $p_i$  in Equation S6 with its definition in Equation S9, we arrive at the formula for the mean transcriptional onset time

$$\text{Mean transcriptional onset time} = \mathbb{E}[\text{onset}] = \sum_{i=0}^{i=T} t_i \frac{P(n + 1, t_i) - P(n + 1, t_i - 1)}{\sum_{i=1}^{i=T} [P(n + 1, t_i) - P(n + 1, t_i - 1)]}. \quad (S10)$$

931 Note that the solutions for the fraction of active loci (Eqn. S5) and their mean transcription onset time  
 932 (Eqn. S9) ultimately depend on the Dorsal concentration over time  $[DI](t)$  as they determine  $P(t, n)$ .  
 933 Hence, to generate predictions that can be directly compared to our live-imaging measurements,  
 934 we need to solve these equations accounting for the Dorsal-mVenus fluorescence dynamics that  
 935 determine  $[DI](t)$ .

## 936 S1.2 Relating MS2 signal to the statistical mechanical model

937 In order to understand how the maximum MCP-mCherry fluorescence of a locus relates to the  
 938 average RNAP loading rate, a model for the fluorescence trajectory during a nuclear cycle is required.  
 939 We start by assuming that RNAP molecules begin loading at a time  $t_0$  into the nuclear cycle and  
 940 continue to load at a constant rate proportional to  $R$ , as shown in Equation 2 ( $R = R_{max} \cdot p_{bound}$ ) and  
 941 step (1) in Figure S1. The observed signal increases linearly until the first polymerase terminates  
 942 transcription. At this point, the signal plateaus at the value  $f_{max}$  because polymerase molecules  
 943 continue to be loaded onto the gene at a constant rate while simultaneously terminating at the  
 944 same rate at the end of the gene (Fig. S1, step (2)). We note that, in this model, initiation halts at



**Figure S1. Trapezoid model of transcription dynamics during early embryonic nuclear cycles in *Drosophila*.** (A) Depiction of a piece-wise linear approximation to average measured fluorescence of loci as a function of time during nuclear cycle 12. In step (1), RNAP molecules are loaded on to the gene at an average constant rate,  $R$ . After the first RNAP terminates transcription at time  $\Delta t_{\text{elongation}}$ , initiation and termination balance each other out, leading to a constant fluorescence value (step (2)). In step (3), initiation ends, causing the observed fluorescence to monotonically decrease. (B) Schematic of the RNAP loading behavior at each step in (A).

945 step (3), leading to a decrease in fluorescence as elongating polymerases finish transcribing. Note  
946 that this step is not accounted for in any analyses or models in this study.

947 Given this model, the maximum fluorescence observed in a trace is given by

$$f_{\max} \approx \alpha \cdot R \cdot \Delta t_{\text{elongation}}, \quad (\text{S11})$$

948 , where  $R$  is the loading defined in Equation 2, and  $\alpha$  is the instantaneous fluorescence per mRNA  
949 molecule that we estimate in Section S1.3. As a result, the maximum fluorescence is proportional  
950 to the loading rate, namely

$$f_{\max} \propto \alpha \cdot R. \quad (\text{S12})$$

951 Thus, we now have an expression for  $f_{\max}$  that enables us to relate our measurements to the  
952 thermodynamic model's prediction for  $R$ , the RNAP loading rate (Fig. 2E).

### 953 S1.3 MS2 Calibration

954 To estimate the fluorescence detection threshold in our system, we calibrated the MCP-mCherry  
955 signal to single molecule fluorescence *in situ* hybridization (smFISH) data from Garcia et al. (2013).  
956 This calibration is based on the fact that, to produce one mRNA molecule, RNAP has to spend a  
957 defined amount of time on the reporter thus contributing to the integrated spot fluorescence. We  
958 define  $\alpha$  as the fluorescence of one RNAP molecule bearing a labeled nascent RNA and  $\Delta t_{\text{elongation}}$   
959 as the time RNAP spends on the reporter gene to synthesize one mRNA molecule (Fig. S2A). Then, the  
960 integrated spot fluorescence corresponding to the production of one mRNA molecule,  $\beta$ , is

$$\beta(a.u. \times \text{min} \times \text{molecule}^{-1}) = \alpha(a.u. \times \text{molecule}^{-1}) \times \Delta t_{\text{elongation}}(\text{min}). \quad (\text{S13})$$

961 From the definition of  $\beta$  above, it follows that the integrated fluorescence of a spot over time  
962 corresponds to the total number of mRNA molecules produced by that locus in that period (Fig. S2A).  
963 Using smFISH, Garcia et al. (2013) measured the mean number of mRNA molecules produced per  
964 nucleus by a P2P-MS2 reporter transgene during nuclear cycle 13 as a function of anterior-posterior  
965 position (Fig. S2B). To compare these data with the measurements obtained from our imaging



966 setup, we imaged the same reporter using 2x Dorsal flies and calculated the mean integrated spot  
967 fluorescence across all nuclei as a function of position along the anterior-posterior axis (Fig. S2B). We  
968 plotted these two measurements against each other and fitted the data to a line going through the  
969 origin (Fig. S2C). The slope of this line indicates  $\beta$ , the integrated spot fluorescence corresponding  
970 to a single produced mRNA molecule.

971 With this fluorescence calibration factor in hand, we can now estimate  $\alpha$ , the spot fluorescence  
972 corresponding to a single RNAP molecule attached to one nascent mRNA molecule with 24 MS2  
973 loops. We can estimate  $\Delta t_{\text{elongation}}$  by invoking the elongation rate of RNAP in the fly embryo,  $v_{\text{elong}}$ ,  
974 and the length of our reporter,  $L$ , such that

$$\Delta t_{\text{elongation}} = \frac{L}{v_{\text{elong}}}. \quad (\text{S14})$$

975 Using this expression for  $\Delta t_{\text{elongation}}$ , we can solve for  $\alpha$  in Equation S13 to obtain the fluorescence of  
976 a single RNAP molecule given by

$$\alpha = \frac{\beta \times v_{\text{elong}}}{L}. \quad (\text{S15})$$

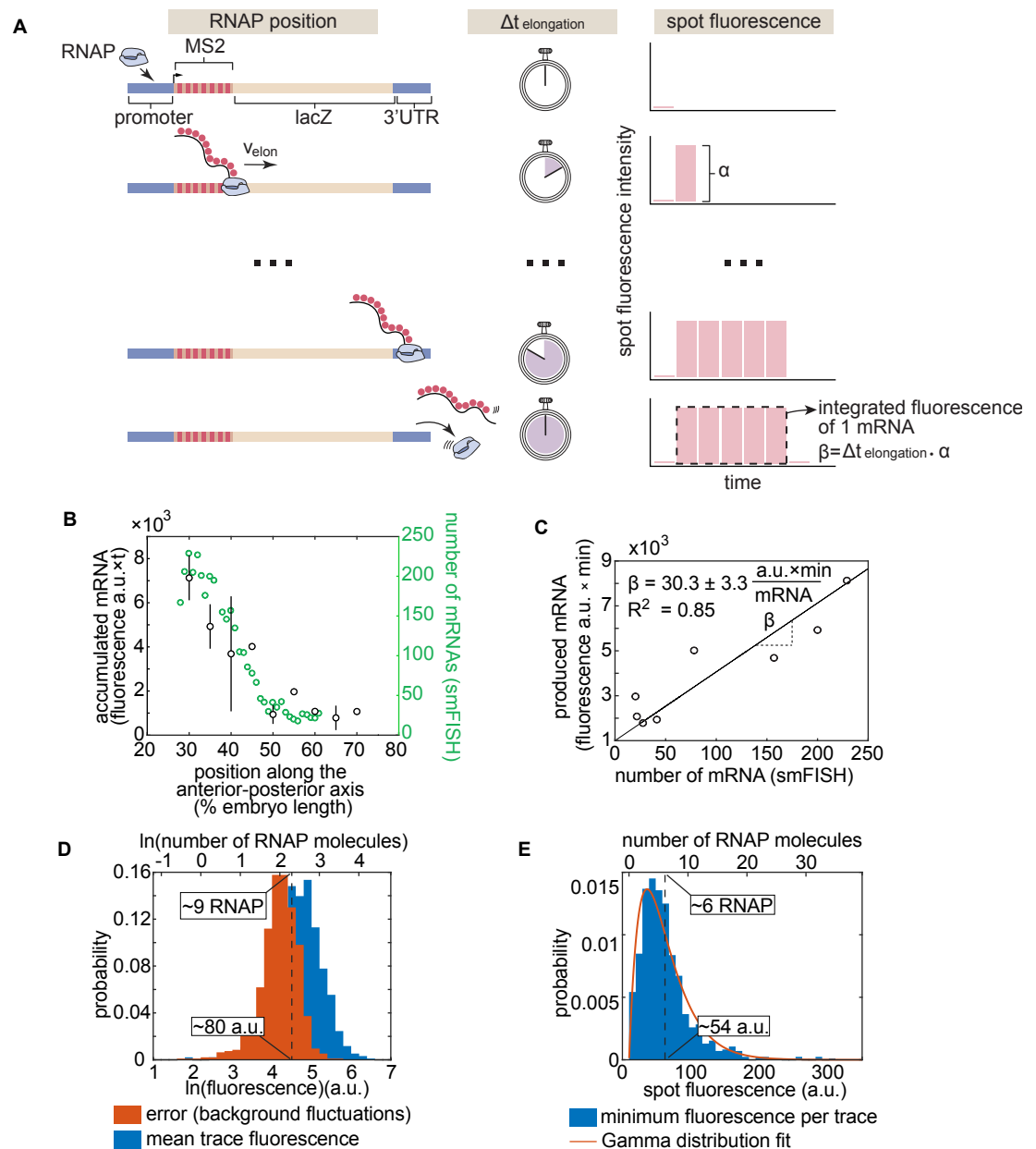
977 We next replace  $L$  by the length of our reporter transgene, 5.2 kbp. In addition we replace  $v_{\text{elong}}$  by a  
978 previously experimentally measured value of  $1.5 \pm 0.14$  kbp/min (*Garcia et al., 2013*), and  $\beta$  by the  
979 calibration factor shown in Figure S2C. We then arrive at

$$\alpha = \frac{30.3 \frac{\text{a.u.} \cdot \text{min}}{\text{RNAP}} \times 1.5 \frac{\text{kbp}}{\text{min}}}{5.2 \text{ kbp}} = 8.837 \text{ a.u. per molecule}. \quad (\text{S16})$$

980 Note that  $v_{\text{elong}}$  and  $\beta$  have an associated error that leads to uncertainty in the calculation of  $\alpha$ .  
981 Propagating these errors results in an uncertainty of 0.046 a.u. per RNAP, or approximately 14%.  
982 This uncertainty should be viewed as an underestimate since, for example, we are not accounting  
983 for embryo-to-embryo variability in the accumulated mRNA measured by microscopy or smFISH.

984 Using this calibration factor, we can now determine the detection threshold of our experimental  
985 setup in terms of absolute number of RNAP molecules. One way of determining this threshold is by  
986 comparing the mean fluorescence of the dimmest spots with the magnitude of their corresponding  
987 background fluctuations. If these values overlap, then it is not possible to determine with certainty  
988 whether a spot correspond to actual signal or to background. This approach reveals a detection  
989 threshold of  $\approx 80$  a.u. or  $\approx 9$  RNAP molecules (Fig. S2D). A second strategy to determining the  
990 detection threshold is looking at the fluorescence of the dimmest detected spots. Their average  
991 fluorescence indicates the value under which no reliable detection is possible. This analysis reveals  
992 a detection limit of  $\approx 54$  a.u. or  $\approx 6$  RNAP molecules (Fig. S2E). These values for our detection limit  
993 using MCP-mCherry are on the order of twice the limit determined for similar experiments that  
994 used MCP-eGFP or PCP-eGFP (*Garcia et al., 2013; Alamos et al., 2020*), most likely due to mCherry  
995 being a dimmer fluorophore than eGFP (*Lambert, 2019*).

996 Finally, in the main text (Section 2.5), we estimated the maximum fluorescence corresponding to  
997 the basal level of RNAP molecules on our reporter constructs (Section 2.5). We include here details  
998 of the calculation. Since the length of the coding region of our reporter constructs is 5.2 kbp, and  
999 the footprint of RNA Polymerase II is 40 bp (*Selby et al., 1997*), 130 RNAPs can fit on the gene at any  
1000 given time. Since we estimate the maximum fluorescence corresponding to basal transcription to  
1001 be  $\approx 20$  RNAP molecules (Section 2.5), the reporter is  $20/130 \approx 15\%$  saturated by RNAPs.



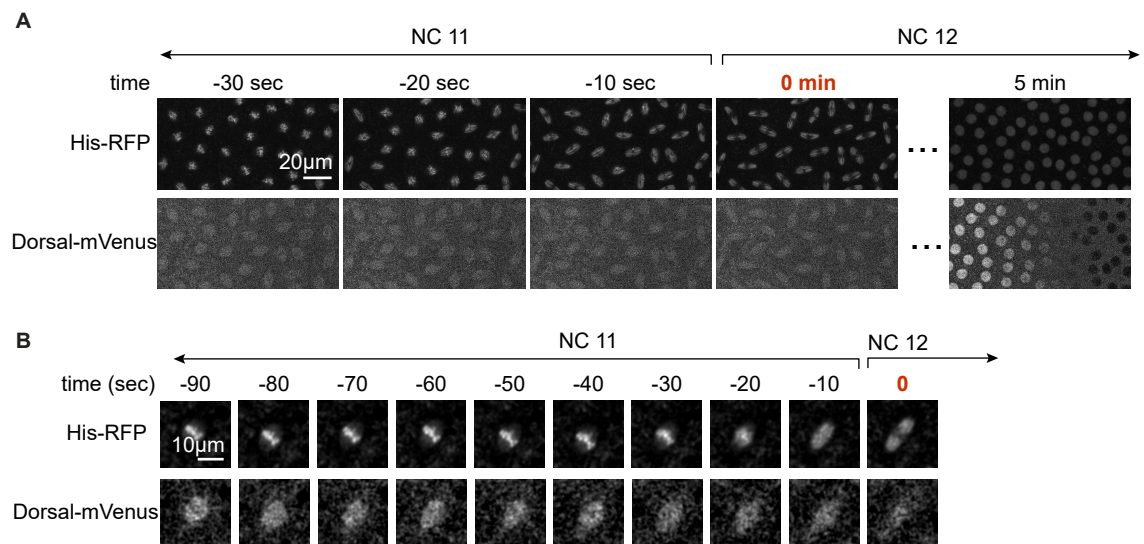
**Figure S2. Absolute calibration of MS2 using single molecule FISH.** (A) Schematic showing that the integrated spot fluorescence corresponding to the production of one mRNA,  $\beta$ , is equal to the fluorescence of a single RNAP molecule,  $\alpha$ , multiplied by the time it spends on the gene,  $\Delta t_{\text{elongation}}$ . (B) Mean accumulated mRNA per nucleus (in nuclear cycle 13) based on the integrated MS2 fluorescence of P2P-MS2 employing the imaging conditions used for our reporter data ( $N=6$  embryos) compared to the number of mRNA molecules per nucleus produced in nuclear cycle 13 as reported by single molecule FISH by Garcia *et al.* (2013). (C) Scatter plot showing data from (B) corresponding to the same anterior-posterior bin. The solid line shows the best linear fit to all data points. The slope error corresponds to the standard error of the fit. The error in the fluorescence per RNAP is the propagated standard error taking the errors in elongation rate and calibration slope into account as described in this section's text. (D) Histograms of mean trace fluorescence in all particles across all experiments and the error in the fluorescence of these particles as reported by fluctuations in the fluorescence background. Because the spot fluorescence was obtained by integrating over three slices, the corresponding error was propagated by multiplying the error from one slice (using the method described in (Garcia *et al.*, 2013)) by  $\sqrt{3}$ . The dashed line indicates the center of where the two distributions overlap, suggesting a detection limit of approximately 9 RNAP molecules. (E) Histogram of the minimum spot fluorescence per trace across all experiments. The dashed line indicates the mean of the distribution, suggesting a detection limit of approximately 6 RNAP molecules. Note that in (D) and (E) the top x-axis is expressed in terms of absolute number of RNAP molecules using the calibration from (C). A best fit to a Gamma distribution is shown in red for ease of visualization.

#### 1002 **S1.4 Measuring transcriptional onset times**

1003 We measured the time at which each locus turns on by determining the first time point where a spot  
 1004 was detected. To make this possible, we needed a reliable way to estimate  $t = 0$  which corresponded  
 1005 to the beginning of the nuclear cycle.

1006 Typically, fluorescently labeled histone is used to determine the timing of anaphase (*Garcia*  
 1007 *et al., 2013*). However, only a small fraction of our embryos had measurable levels of visible  
 1008 Histone-iRFP, most likely due to embryo-to-embryo variability and the low density of DNA in the  
 1009 nucleus in nuclear cycle 12 (compared to later nuclear cycles when His-iRFP is more visible). When  
 1010 the Histone-iRFP signal was insufficient to determine anaphase, we relied on the Dorsal-mVenus  
 1011 channel. As we describe below, just like Histone-iRFP, the nuclear Dorsal fluorescence also shows a  
 1012 characteristic pattern during mitosis.

1013 To precisely determine which features of the Dorsal-mVenus channel to use for mitosis timing,  
 1014 we imaged Dorsal-mVenus and Histone-RFP—which, as opposed to Histone-iRFP, can be consistently  
 1015 detected—simultaneously (Fig.S3). This exercise showed that the edges of nuclei become less well  
 1016 defined as they enter mitosis and then elongate at the beginning of anaphase (Fig. S3). In this way,  
 1017 we could identify precise anaphase frames in movies with no visible Histone-iRFP. Despite using this  
 1018 method, we still estimate that there may be a 2-3 frame error (i.e. 20-30 s) in our determination of  
 1019 anaphase. Thus, this error is  $< 20\%$  of the measured period of transcriptional activity within nuclear  
 1020 cycle 12 ( $\sim 3$  min).



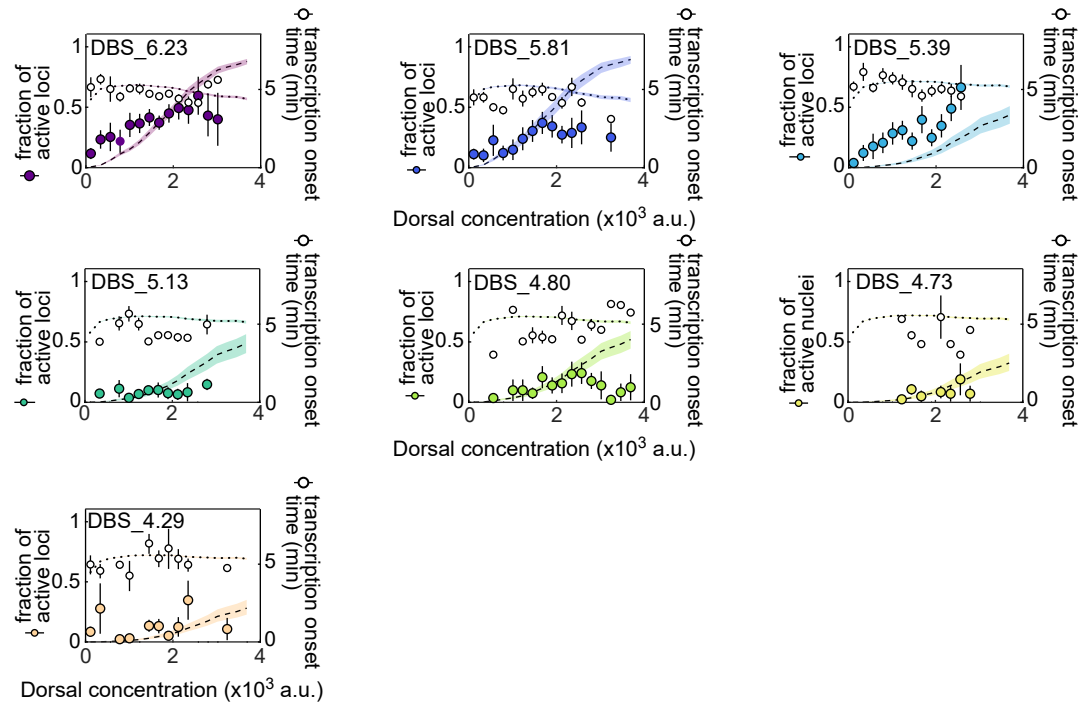
**Figure S3. Using the Dorsal-mVenus channel to determine the timing of mitosis. (A)** Visual comparison of nuclei in the field of view of Histone-RFP and Dorsal-mVenus channels during nuclear division. **(B)** Same as (A), but zoomed into a single nucleus. In (A) and (B),  $t = 0$  min in red text corresponds to anaphase.

#### 1021 **S1.5 Kinetic barrier fits with a different functional form of the transition rate $k$**

1022 In the main text, we hypothesize that the transition rate between OFF states and between the last  
 1023 OFF state and the ON state is proportional to Dorsal occupancy (Eqn. 1). Here, we show that another  
 1024 functional form for  $k$  in the kinetic barrier model can only partially recapitulate the fraction of active  
 1025 loci and transcriptional onset times for each of our enhancers. This functional form is motivated  
 1026 by the idea that Dorsal could catalyze a change in the promoter (e.g. opening of chromatin) in  
 1027 a manner dependent on the speed of its first occurrence of binding rather than its equilibrium  
 1028 occupancy. Specifically, inspired by (*Eck et al., 2020*), we posit that

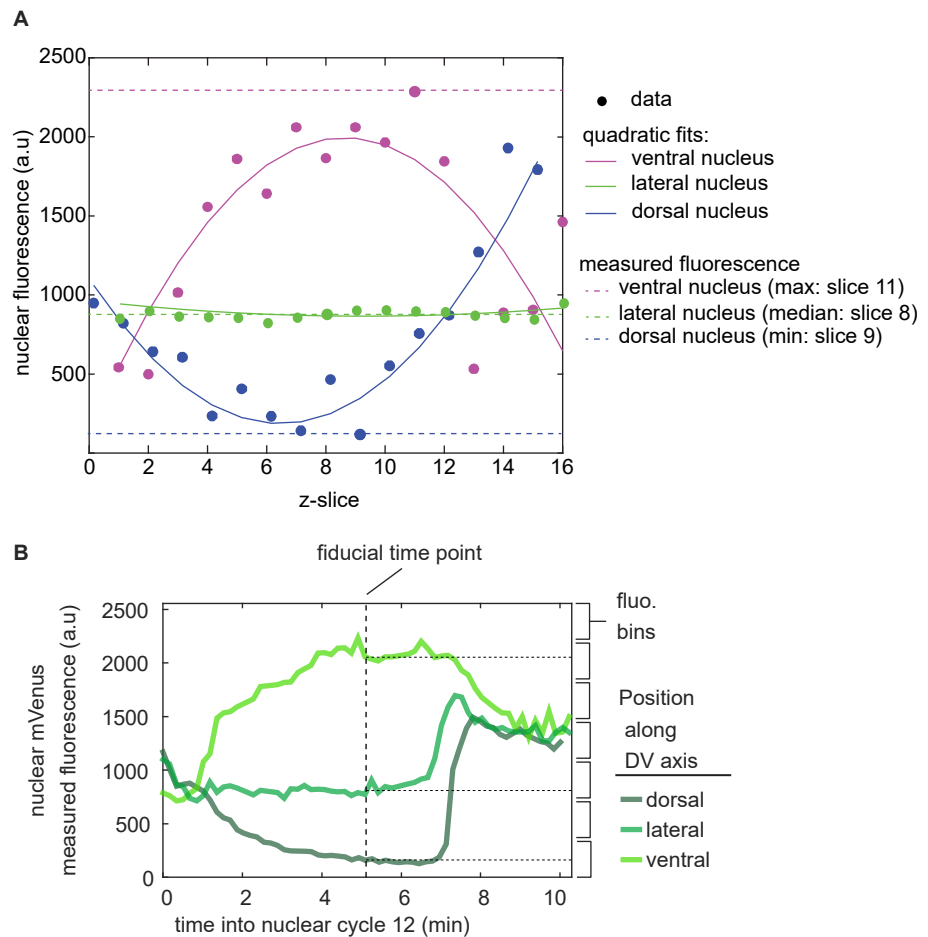
$$k = c \cdot [DI]. \quad (S17)$$

1029 In this alternate model, we assume that the Dorsal binding site affinity dependence is wrapped  
 1030 up into the  $c$  parameter. Thus, we fit each enhancer using a distinct value of  $c$ . As can be seen in  
 1031 Figure S4, this alternate model cannot fit the data as well as when  $k$  is assumed to be proportional  
 1032 to the Dorsal occupancy as described in the main text and in Figure 5. Specifically, this functional  
 1033 form is less capable of recapitulating the saturation plateau of the fraction of active loci at high  
 1034 Dorsal concentrations.

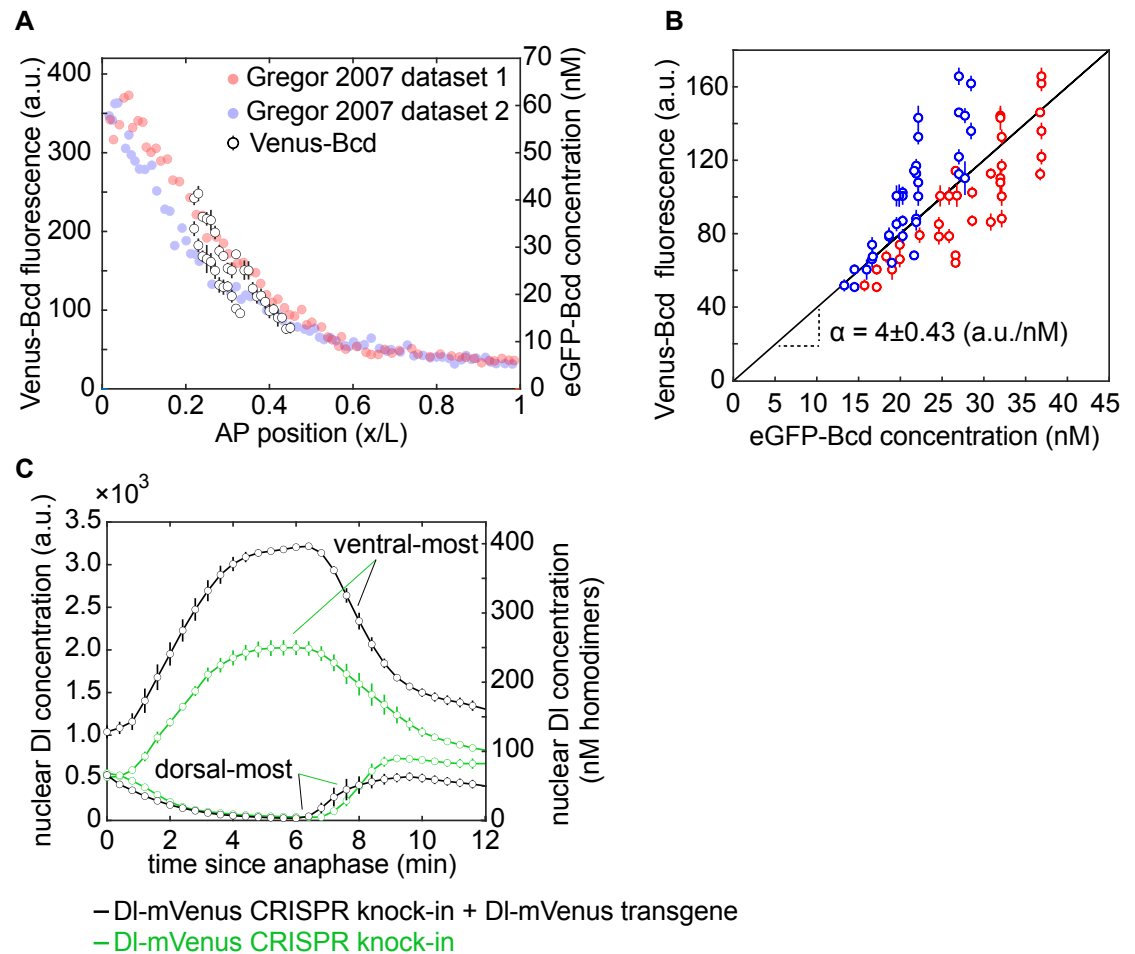


**Figure S4. Fits to kinetic barrier model using  $k = c \cdot [DI]$ .** Data and model fits for the fraction of active loci (left y-axis) and mean transcription onset time (right y-axis) for each enhancer. Empty black circles correspond to the experimentally observed mean transcription onset time. Filled colored circles correspond to experimentally observed mean fraction of active loci. Error bars on observations correspond to the standard error of the mean. Fitted curves are represented as black dashed lines (fraction of active loci) and black dotted lines (mean transcription onset times), which correspond to predictions using median parameter values from the joint posterior distribution. Colored shaded areas indicate the 25%-75% credible interval.

1035 **S2 Supplementary figures**

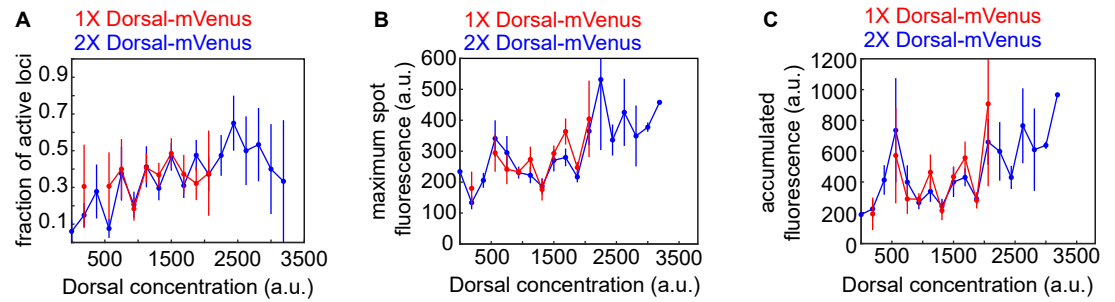


**Figure S5. Measuring Dorsal-mVenus nuclear fluorescence across the dorsoventral axis. (A)** In each frame, the Dorsal-mVenus fluorescence is measured in each z-slice across nuclei. This creates a series of fluorescence values as a function of z-slice (filled circles). z-slices at the top and the bottom correspond to cytoplasmic fluorescence. Thus, in ventral nuclei, the brightest slice is the z-slice corresponding to the best estimate of the true nuclear fluorescence (magenta circles). On the other hand, dorsal nuclei have a lower Dorsal concentration than the cytoplasm, so the darkest slice is a better estimate of the true Dorsal concentration (blue circles). In lateral nuclei, the nuclear fluorescence is similar to that of the cytoplasm (green circles). To identify which z-slice to use for nuclear fluorescence calculations, we fit the fluorescence,  $f$ , over z-slices,  $z$ , to a quadratic equation,  $f = az^2 + bz$ , where  $a$  and  $b$  are the coefficients of this quadratic equation. Then, we use the value of  $a$  to determine whether the nucleus is ventral ( $a < -0.5$ ), lateral ( $-0.5 < a < 0.5$ ), or dorsal ( $a > 0.5$ ). Next, in ventral nuclei, we take the brightest z-slice as the Dorsal-mVenus fluorescence of that frame (dashed horizontal magenta line). In lateral nuclei, we take the median of fluorescence values over z-slices (dashed horizontal green line). In dorsal nuclei, we take the darkest z-slice as the respective frame's Dorsal-mVenus fluorescence (dashed horizontal blue line). **(B)** Representative time traces of nuclear Dorsal-mVenus fluorescence. To calculate transcriptional activity as a function of Dorsal protein, we sort nuclei into Dorsal concentration bins based on the Dorsal-mVenus fluorescence at a single fiducial time point halfway through the respective lifetime of each nucleus.

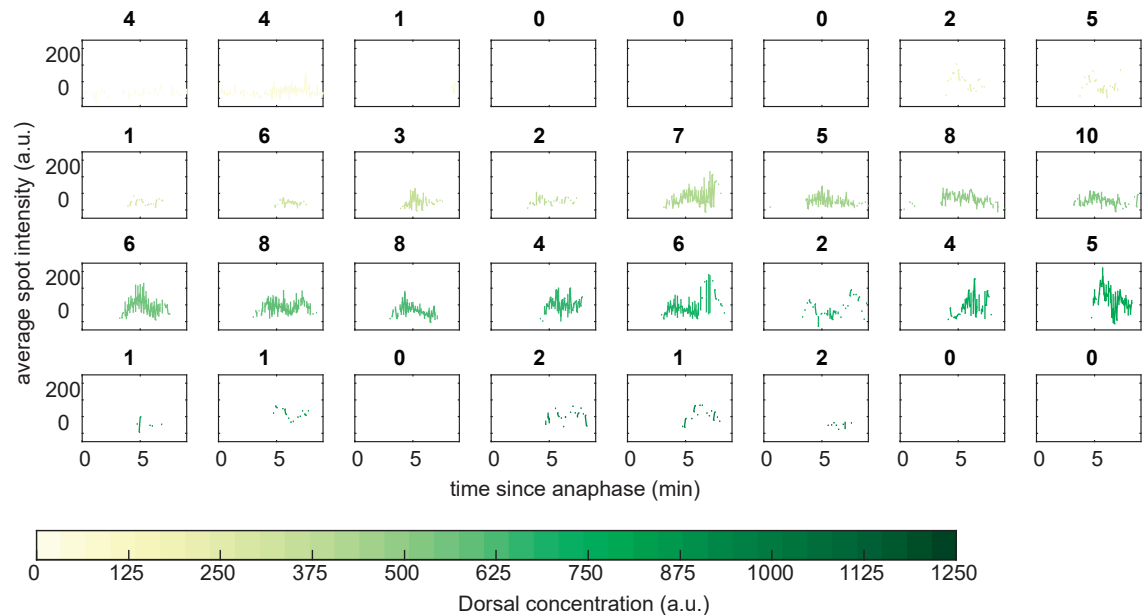


**Figure S6. Absolute calibration of Dorsal-mVenus fluorescence using Venus-Bicoid and previously measured eGFP-Bicoid concentration.** (A) Three embryos derived from *yw;Venus-Bicoid;BcdE1* homozygous mothers were imaged in nuclear cycle 14 using the imaging conditions of our MS2 experiments. The nuclear fluorescence was calculated 15 min into nuclear cycle 14 for cross-comparison with absolute eGFP-Bicoid concentration measurements from Figure 2B of *Gregor et al. (2007)*. We compare the fluorescence values of Venus-Bicoid to the absolute concentration of eGFP-Bicoid along the anterior-posterior axis of the embryo. (B) Plot of Venus-Bicoid fluorescence as a function of eGFP-Bicoid fluorescence. Each data point corresponds to the mean  $\pm$  standard deviation of the fluorescence of all nuclei belonging to the same 1% spatial window along the anterior-posterior axis. These data were compared to two different absolute measurements of eGFP-Bicoid, shown in red and blue. Linear fit was performed assuming no intercept term since we are estimating a proportionality constant. The slope's error ( $\alpha$ ) corresponds to the 95% confidence interval. (C) Mean and SEM of the Dorsal nuclear concentration in the ventral-most and dorsal-most nuclei across four embryos. 1x and 2x correspond to embryos from homozygous females containing one or two Dorsal-mVenus alleles, respectively. The right y-axis shows the concentration of Dorsal homodimers assuming 6 fluorescence a.u. per mVenus molecule based on (A) and (B).

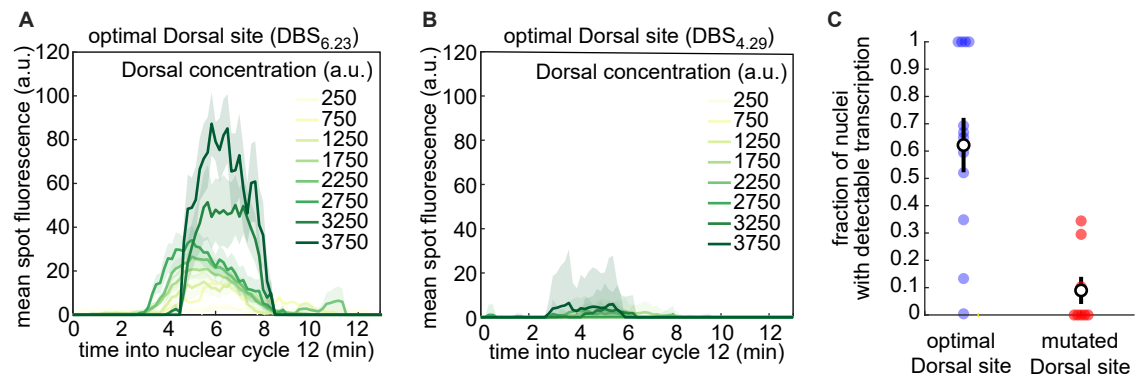




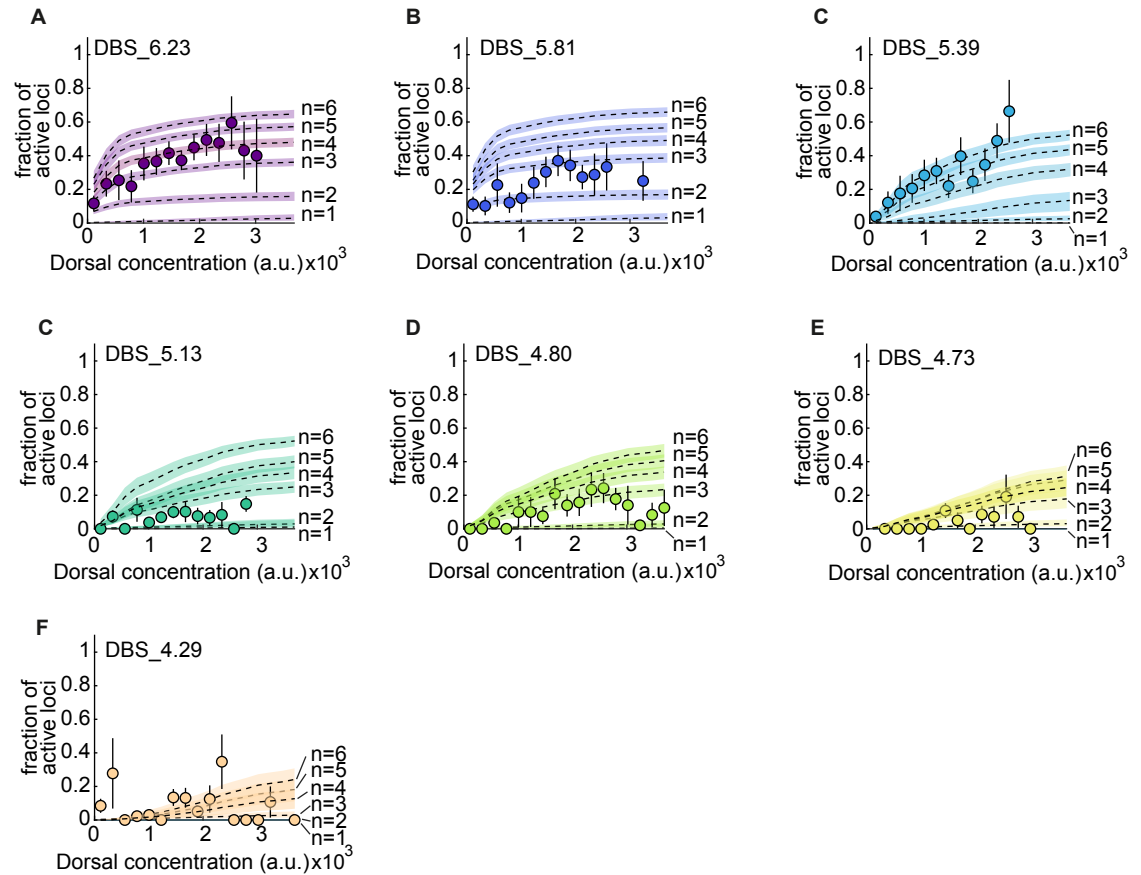
**Figure S7. Comparing the activity of the Dorsal-mVenus transgene to that of two copies of Dorsal-mVenus provided by a transgene plus a CRISPR knock-in.** For the DBS\_6.23 reporter construct, we imaged embryos laid by two different mothers. 1x mothers (red) carry *dll*<sup>1</sup> (a null Dorsal allele) and a Dorsal-mVenus transgene created by *Reeves et al. (2012)*. 2x mothers (blue) carry a Dorsal-mVenus CRISPR knock-in and the aforementioned Dorsal-mVenus transgene. Nuclei from these different mothers were binned according to their mVenus fluorescence and different activity metrics were measured for each bin. The two Dorsal-mVenus populations are not different within error such that it is valid to treat embryos laid by these different mothers as equivalent. (Error bars correspond to the standard error across at least three embryos per Dorsal-mVenus fluorescence bin.)



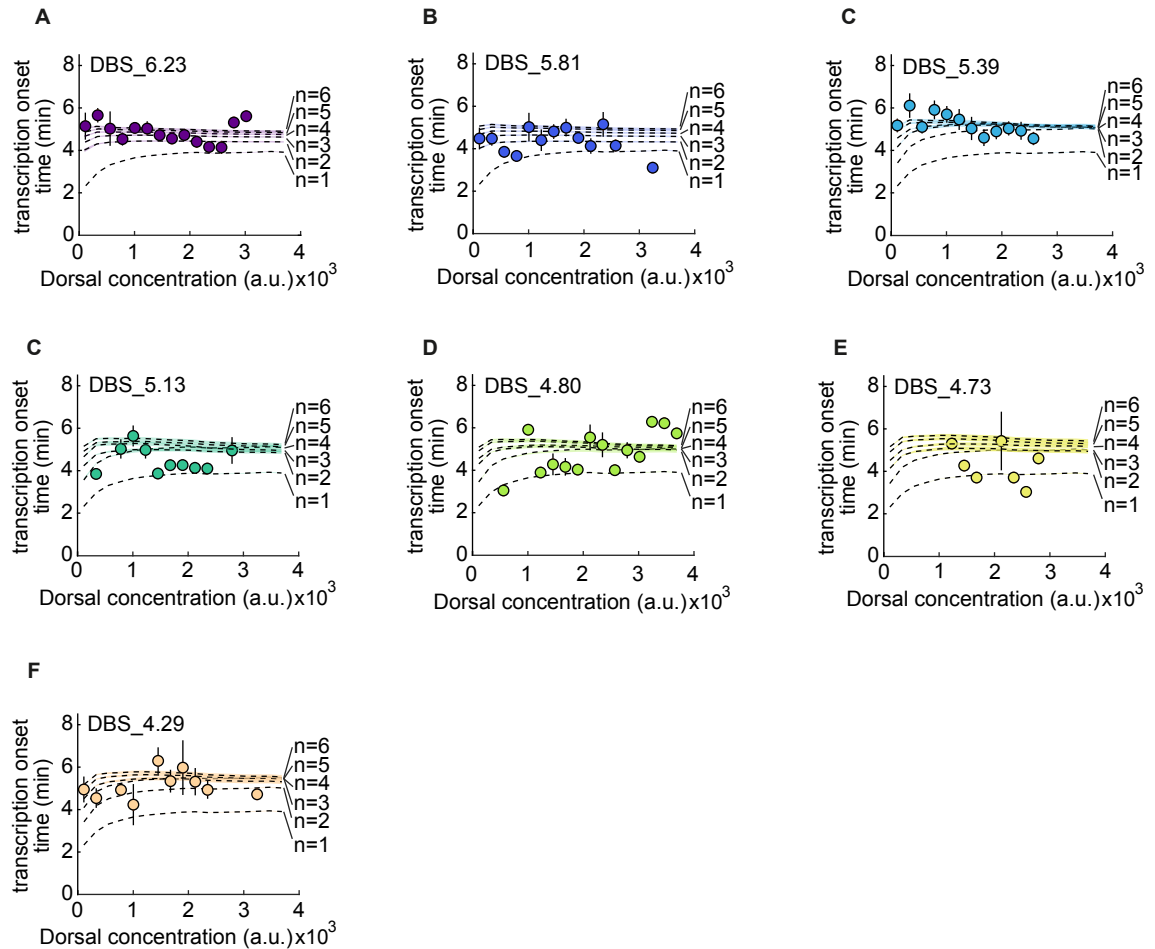
**Figure S8. Mean DBS\_6.23 transcription spot intensity over time.** Mean spot intensity from DBS\_6.23 transcription spots over time. Each plot corresponds to a different Dorsal-mVenus concentration as indicated by the legend. The nature of the data makes it challenging to estimate the RNAP loading rate by fitting a line to the initial rise of fluorescence. Bold letters above each plot indicate the number of particles included in each bin.



**Figure S9. Transcription driven by a minimal Dorsal synthetic enhancer with a mutated Dorsal binding site.** (A,B) Mean fluorescence over time across all loci in the field of view from an embryo carrying a minimal synthetic enhancer with a (A) single optimal and (B) a mutated Dorsal binding site. (C) Fraction of nuclei in which we detected a transcription spot at any time during the duration of nuclear cycle 12 in nuclei exposed to high Dorsal concentration (2600–3200 a.u.) within the field of view. Filled circles correspond to individual embryos. Black circles show the mean across all embryos. Shaded areas in (A) and (B) and error bars in (C) correspond to the standard error of the mean.



**Figure S10. Fits of the kinetic barrier model to the fraction of active nuclei using different numbers of transitions,  $n$ .** (A) Mean fraction of active loci as a function of Dorsal concentration in the DBS\_6.23 enhancer. Dashed lines show model fits using different number of OFF states  $n = 1, 2, 3, 4, 5,$  and  $6$ , corresponding to predictions using median parameter values from the joint posterior distribution. Fits are performed simultaneously across all enhancers with the value of  $c$  being shared and the value of  $K_D$  being allowed to vary across enhancers. The shaded areas indicate the 25%-75% credible interval. (B-F) Same as (A) for the rest of minimal synthetic enhancers. Error bars in (A)-(F) correspond to the SEM taken over  $N > 3$  embryos containing 3 or more nuclei in a given bin.



**Figure S11. Fits of the kinetic barrier model to the transcription onset times using different numbers of transitions,  $n$ .** (A) Mean transcription onset time as a function of Dorsal concentration in the DBS\_6.23 enhancer. Dashed lines show model fits using different number of OFF states  $n = 1, 2, 3, 4, 5,$  and  $6$ , corresponding to predictions using median parameter values from the joint posterior distribution. Fits are performed simultaneously across all enhancers with the value of  $c$  being shared and the value of  $K_D$  being allowed to vary across enhancers. The shaded areas indicate the 25%-75% credible interval. (B-F) Same as (A) for the rest of minimal synthetic enhancers. Error bars in (A)-(F) correspond to the SEM taken over  $N > 3$  embryos containing 3 or more nuclei in a given bin.

### 1036 S3 Supplementary tables

Parameter	Mean	Std. Dev.
$c$ ( $\text{min}^{-1}$ )	0.55	0.037
$K_D(\text{DBS\_6.23})$	210	85
$K_D(\text{DBS\_5.81})$	150	52
$K_D(\text{DBS\_5.39})$	980	450
$K_D(\text{DBS\_5.13})$	870	360
$K_D(\text{DBS\_4.80})$	870	340
$K_D(\text{DBS\_4.73})$	$1.5 \times 10^3$	680
$K_D(\text{DBS\_4.29})$	$3.7 \times 10^3$	$3.1 \times 10^3$

**Table S1.** Inferred parameters from kinetic barrier model fits in Figure 5. Each  $K_D$  has units of a.u..

Parameter	Mean	Std. Dev.
$R_{max}$	510	190
$K_D(\text{DBS\_6.23})$	$6.3 \times 10^3$	$5.5 \times 10^3$
$K_D(\text{DBS\_5.81})$	$5.4 \times 10^4$	$2.6 \times 10^4$
$K_D(\text{DBS\_5.39})$	$4.3 \times 10^4$	$2.7 \times 10^4$
$K_D(\text{DBS\_5.13})$	$3.9 \times 10^4$	$2.7 \times 10^4$
$K_D(\text{DBS\_4.80})$	$6.7 \times 10^4$	$2.2 \times 10^4$
$K_D(\text{DBS\_4.73})$	$6.6 \times 10^4$	$2.3 \times 10^4$
$K_D(\text{DBS\_4.29})$	$6.8 \times 10^4$	$2.2 \times 10^4$
$\omega$	14	23
$P/K_p$	0.65	0.23

**Table S2.** Inferred parameters from fits of the thermodynamic model to the RNAP loading rates measured in Figure 6.  $R_{max}$  and  $K_D$  each have units of a.u., while the remaining parameters are unitless.

### 1037 S4 Supplementary videos

1038 For better quality of visualization, we recommend downloading these videos.

- 1039 • **Video S1. DBS\_6.23 confocal movie.** Confocal microscopy movie taken on the ventral side of  
1040 a developing fly embryo ( $yw$ ;  $MCP\text{-}mCherry$ ,  $DI\text{-}mVenus(\text{CRISPR}) / \text{DBS\_6.23-MS2}$ ;  $MCP\text{-}mCherry$ ,  
1041  $DI\text{-}mVenus$ ,  $His\text{-}iRFP / +$ ) during nuclear cycle 12. Left: Dorsal- $mVenus$ ; Right:  $MCP\text{-}mCherry$ .
- 1042 • **Video S2. ParB experiment confocal movie.** Confocal microscopy movie taken on the  
1043 ventrolateral side of a developing fly embryo ( $yw$ ;  $ParB\text{-}eGFP$ ,  $MCP\text{-}mCherry / \text{intB2-DBS\_6.23-}$   
1044  $MS2$ ;  $+$ ) during nuclear cycle 12. Left:  $ParB\text{-}eGFP$ ; Right:  $MCP\text{-}mCherry$ .