CHAPTER ONE

Lighting up the central dogma for predictive developmental biology

Hernan G. Garcia[a,b,c,d,*], Augusto Berrocal[a], Yang Joon Kim[c], Gabriella Martini[a], Jiaxi Zhao[b]

[a]Department of Molecular and Cell Biology, University of California at Berkeley, Berkeley, CA, United States
[b]Department of Physics, University of California at Berkeley, Berkeley, CA, United States
[c]Biophysics Graduate Group, University of California at Berkeley, Berkeley, CA, United States
[d]Quantitative Biosciences-QB3, University of California at Berkeley, Berkeley, CA, United States
[*]Corresponding author: e-mail address: hggarcia@berkeley.edu

Contents

Abstract

Although the last 30 years have witnessed the mapping of the wiring diagrams of the gene regulatory networks that dictate cell fate and animal body plans, specific understanding building on such network diagrams that shows how DNA regulatory regions control gene expression lags far behind. These networks have yet to yield the predictive power necessary to, for example, calculate how the concentration dynamics of input transcription factors and DNA regulatory sequence prescribes output patterns of gene expression that, in turn, determine body plans themselves. Here, we argue that reaching a predictive understanding of developmental decision-making calls for an interplay between theory and experiment aimed at revealing how the regulation of the processes of the central dogma dictate network connections and how network topology guides cells toward their ultimate developmental fate. To make this possible, it is crucial to break free from the snapshot-based understanding of embryonic development

Current Topics in Developmental Biology, Volume 137
ISSN 0070-2153
https://doi.org/10.1016/bs.ctdb.2019.10.010

facilitated by fixed-tissue approaches and embrace new technologies that capture the dynamics of developmental decision-making at the single cell level, in living embryos.

1. Introduction

A ubiquitous mystery in nature is how a single cell develops into a multicellular organism. One of the great achievements of genetics and new genome-wide technologies over the last few decades has been the identification of the regulatory molecules that underlie developmental programs. This work has revealed that animal body plans are determined by the action of activators and repressors connected in complex gene regulatory networks. One of the best-studied regulatory networks drives segmentation of the early embryo of the fruit fly *Drosophila melanogaster*. As shown in Fig. 1A, decades of concerted effort have uncovered the identities of the regulatory molecules that determine fly body segments as well as the network connections between these molecules (reviewed in Carroll, Grenier, & Weatherbee, 2001; Davidson, 2006; Peter & Davidson, 2015). However, the amassed data about regulatory proteins and network connections has been mostly descriptive and has not been accompanied by parallel successes in predictively understanding cellular decision-making in developing embryos. To be concrete, is it possible to predict developmental phenotypes from network diagrams? Clearly, if we were to change the names of the genes in the fruit fly segmentation network (Fig. 1B), the network connections—the topology—would be insufficient to predict that this particular network would result in a fly.

The central thesis of this article is that achieving predictive understanding of developmental decision-making requires a two-pronged approach. First, each arrow in these networks must be endowed with molecular and quantitative information that makes it possible to predict how the genome dictates *developmental* input-output functions: the functions relating output protein levels of a gene to the concentrations of its input transcription factors (Fig. 2A). That is, how do the number, placement, and affinity of transcription-factor binding sites within enhancers establish the relation between input transcription-factor concentration and output transcription? How is mRNA processed as it forms a cytoplasmic pattern? How is this mRNA pattern translated into a protein pattern that can be subject to further post-translational regulation and that can ultimately feed back into the

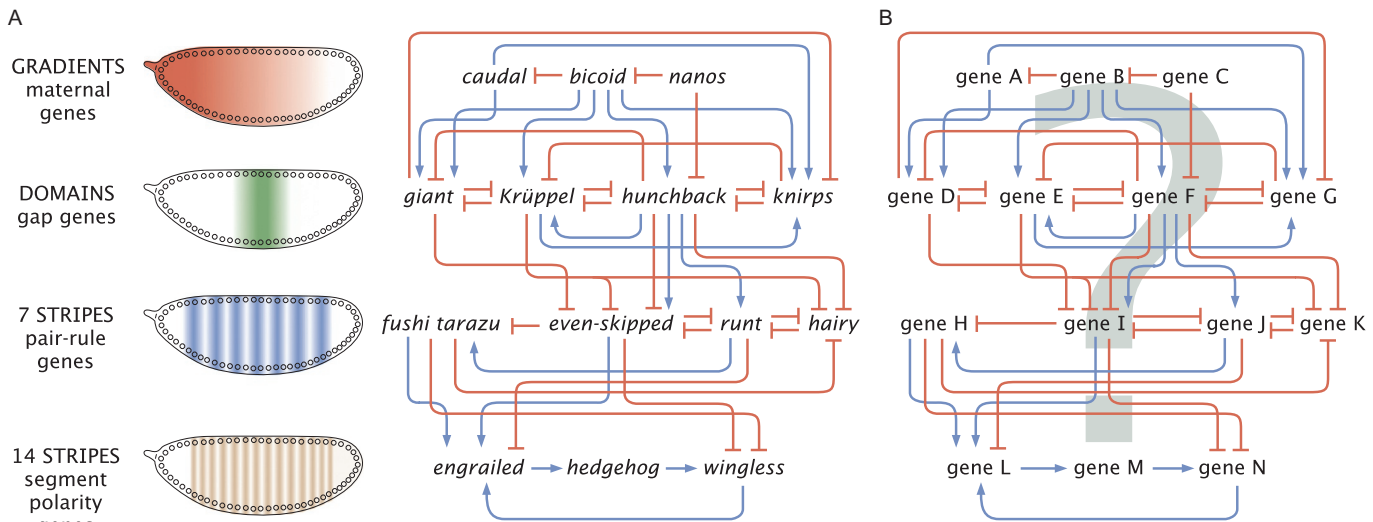

Fig. 1 Gene regulatory networks driving development. (A) Current state of the art in mapping the network that drives segmentation in the fruit fly *Drosophila melanogaster*. (B) Close examination of a network with the same topology, but with different genetic actors, reveals that this description is insufficient to predict that this network will lead to the emergence of a segmented embryo. *Adapted from Carroll, S.B., Grenier, J.K., & Weatherbee, S.D. (2001). From DNA to diversity: Molecular genetics and the evolution of animal design (Malden, Mass: Blackwell Science); Edgar, B.A., Odell, G.M., & Schubiger, G. (1989). A genetic switch, based on negative regulation, sharpens stripes in Drosophila embryos.* Developmental Genetics 10*, 124–142; Jaeger, J. (2011). The gap gene network.* Cellular and Molecular Life Sciences 68*, 243–274; von Dassow, G., Meir, E., Munro, E.M., & Odell, G.M. (2000). The segment polarity network is a robust developmental module.* Nature, 406*, 188–192.*

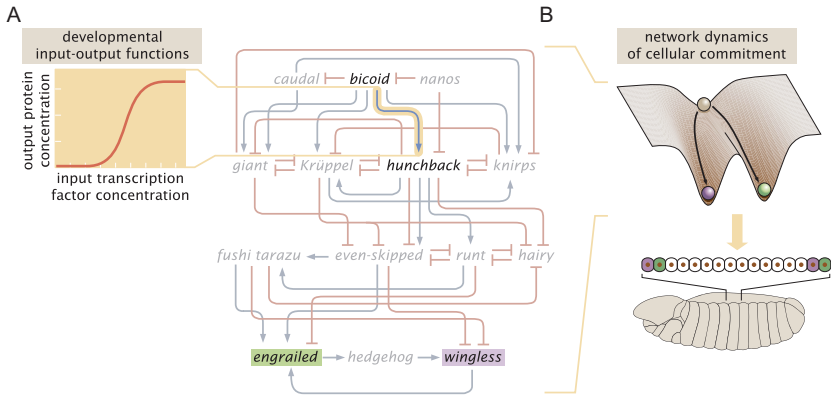

Fig. 2 A two-pronged approach to achieving predictive understanding of developmental decision-making. (A) A quantitative and predictive description of gene regulatory networks in development demands uncovering how the regulation of the processes of the central dogma prescribe developmental input-output functions represented by network arrows. (B) Arrows define the network topology which drives cells within an embryo into discrete and stable developmental states. In this example, the topology defines two distinct developmental trajectories that lead to cells expressing *engrailed or wingless,* and to the adoption of distinct fates that establish body segments in the fly.

network? Second, we must reveal how these network connections conspire together to drive cells into discrete and stable gene expression states that in turn commit these cells to their ultimate developmental fates (Fig. 2B).

In order to achieve this hefty goal of predictive developmental biology, here we argue that developmental biologists urgently require new theoretical models that make precise and falsifiable predictions. Further, we posit that, in order to test the predictions from these models, developmental biology needs to break free from the static view of development shaped by widespread fixed-tissue techniques and establish new technologies that reveal the regulation of the processes of the central dogma at the single-cell level as developmental programs are deployed in real time. Here we focus on the specific case study of the segmentation of the early embryo of the fruit fly. This review is therefore not encyclopedic, and falls short of an exhaustive scholarly survey of the many exciting contributions to quantitative developmental biology in the recent literature. Rather, by focusing on a representative case study, we propose a concrete framework for establishing a quantitative and predictive developmental biology writ large that can be adopted by developmental biologists and biophysicists working on any organism.

2. Turning the fruit fly *Drosophila melanogaster* into a substrate for predictive developmental biology

Drosophila is already a workhorse for developmental biology and genetics research. The fruit fly is also an ideal substrate for realizing predictive developmental biology. The fly network in Fig. 1A shows that many of its fundamental parts (regulatory molecules and connections) have already been identified. The picture that emerges is one of a network that is simple enough that its regulatory interactions could be enumerated, yet intricate enough that it captures the essence of more "complex" developmental processes. As a result, even though some regulatory interactions might remain unmapped, the fly segmentation network offers a unique opportunity to uncover the fundamental and quantitative rules behind developmental decision-making.

What is required to quantitatively predict and control developmental outcomes from regulatory parameters in the fruit fly—and ultimately any organism? The bacterial *lac* operon showcases how to build a predictive understanding of cellular decision-making. Over the last 15 years, theoretical models of this operon, such as those shown in Fig. 3A, have precisely predicted the mean rate of transcription and its cell-to-cell variability as a function of regulatory architecture invoking only a handful of free parameters (Fig. 3B, reviewed in Phillips et al., 2019).

Clearly, flies differ from bacteria; even the architecture of the transcriptional process is different. While bacterial transcription only requires a handful of molecules to be present at the promoter, eukaryotic transcriptional initiation requires the assembly of vast macromolecular complexes such as the preinitiation complex, plus regulatory steps to evict nucleosomes from the DNA to render it accessible to transcription factors (reviewed by Fuda, Ardehali, & Lis, 2009). Similar complexity exists in every step of the central dogma in eukaryotes, from splicing, to transcriptional termination, to translation, to post-translational modifications (Alberts, 2015).

However, the challenges of quantitatively dissecting development go beyond the combinatorial complexity stemming from the numerous molecular machines involved in the processes of the central dogma in eukaryotes. Biology textbooks are dominated by snapshots of static gene-expression patterns. Thus, a large part of our understanding of developmental biology rests on the *assumption* that developmental dynamics can be easily inferred from these static pictures. However, development is a highly dynamic

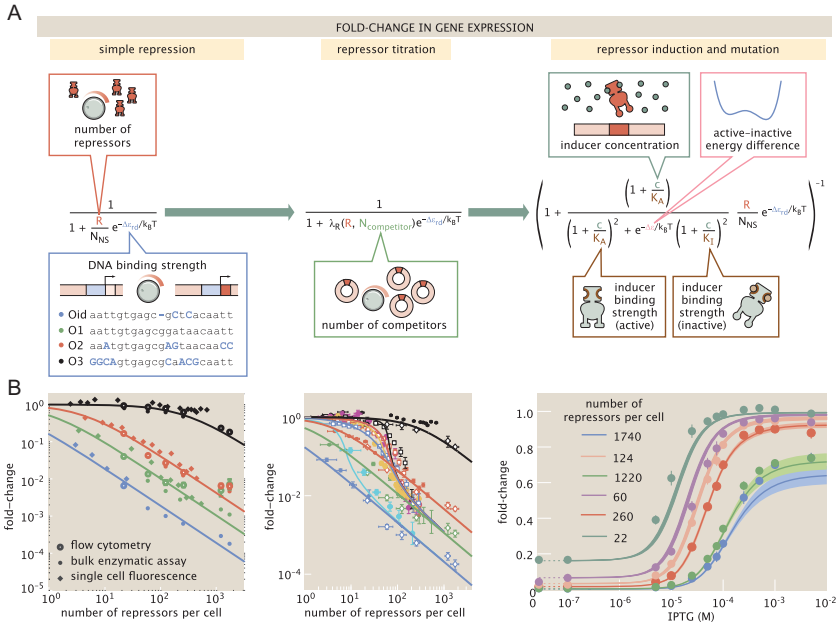

Fig. 3 Unraveling regulation of the *lac* operon through theory and experiment. (A) Examples of theoretical predictions for the fold-change in gene expression of simple repression by Lac repressor (defined as the ratio of gene expression level in the presence of repressor to the gene expression level in in the absence of repressor) for increasingly complex regulatory situations. Each step relies on the parameters learned in the previous iteration. (B) Parameter-free or one-parameter fits to the data demonstrate that simple repression is predictive. *Adapted from* Phillips, R., Belliveau, N.M., Chure, G., Garcia, H.G., Razo-Mejia, M., & Scholes C. (2019). Figure 1 theory meets figure 2 experiments in the study of gene expression, Annual Review of Biophysics, 48, 121–163.

process: choreographed gene expression patterns are rapidly deployed in space and time, and can exist for periods of time as short as 15 min (Bothma et al., 2014). Thus, predicting developmental biology of *Drosophila* not only calls for theoretical frameworks with predictive power, but also demands experimental technologies that reveal how the processes of the central dogma are regulated in real time as single cells commit to their fates and development unfolds.

3. Lighting up the central dogma to assign quantitative and predictive meaning to arrows

Arrows in network diagrams encode *developmental* input–output functions that predict how the concentrations of input transcription factors

determine output protein levels (Fig. 4A). These input-output functions, which are the fundamental unit of any quantitative description of developmental programs, arise from the regulation of each step of the central dogma from transcriptional initiation, to mRNA processing, to translation and post-translational modifications (Fig. 4B). There is a specific input-output function for each specific step of the central dogma.

Over the last 40 years, a plethora of theoretical models have sought to predict *transcriptional* input-output functions: how the concentration of input activators and repressors and the arrangement of their binding sites on regulatory DNA dictate the output rate of transcription (Fig. 4C) (Ackers, Johnson, & Shea, 1982; Bintu et al., 2005a, 2005b; Buchler, Gerland, & Hwa, 2003; Estrada, Wong, DePace, & Gunawardena, 2016; Fakhouri et al., 2010; Gregor, Tank, Wieschaus, & Bialek, 2007; Hammar et al., 2014; He, Samee, Blatti, & Sinha, 2010; Kanodia et al., 2012; Li, Cesbron, Oehler, Brunner, & Hofer, 2018; Samee et al., 2015; Sayal, Dresch, Pushel, Taylor, & Arnosti, 2016; Scholes, DePace, & Sanchez, 2017; Segal, Raveh-Sadka, Schroeder, Unnerstall, & Gaul, 2008; Sherman & Cohen, 2012; Vilar & Leibler, 2003; von Hippel, Revzin, Gross, & Wang, 1974). However, despite this wide repertoire of models, only recently did the technology necessary to directly measure transcriptional input-output functions in development become available.

3.1 Lighting up transcriptional dynamics

For years, the state of the art for directly measuring transcriptional activity in developing embryos consisted of fixed-tissue techniques such as *in situ* hybridization, fluorescence *in situ* hybridization (FISH) or single-molecule FISH (Lawrence, Singer, & Marselle, 1989; Raj, Peskin, Tranchina, Vargas, & Tyagi, 2006; Singer & Ward, 1982; Tautz & Pfeifle, 1989). In these techniques, mRNA is labeled such that sites of nascent transcript formation appear as puncta in each nucleus (O'Farrell, Edgar, Lakich, & Lehner, 1989; Zenklusen, Larson, & Singer, 2008). The signal, often fluorescence, emitted by these puncta reports on the number of mRNA molecules being actively transcribed. These technologies have been applied to many biological questions, such as the molecular nature of transcription in development and how mitosis inhibits transcription (Boettiger & Levine, 2013; Bothma, Magliocco, & Levine, 2011; Little, Tikhonov, & Gregor, 2013; Shermoen & O'Farrell, 1991; Zoller, Little, & Gregor, 2018).

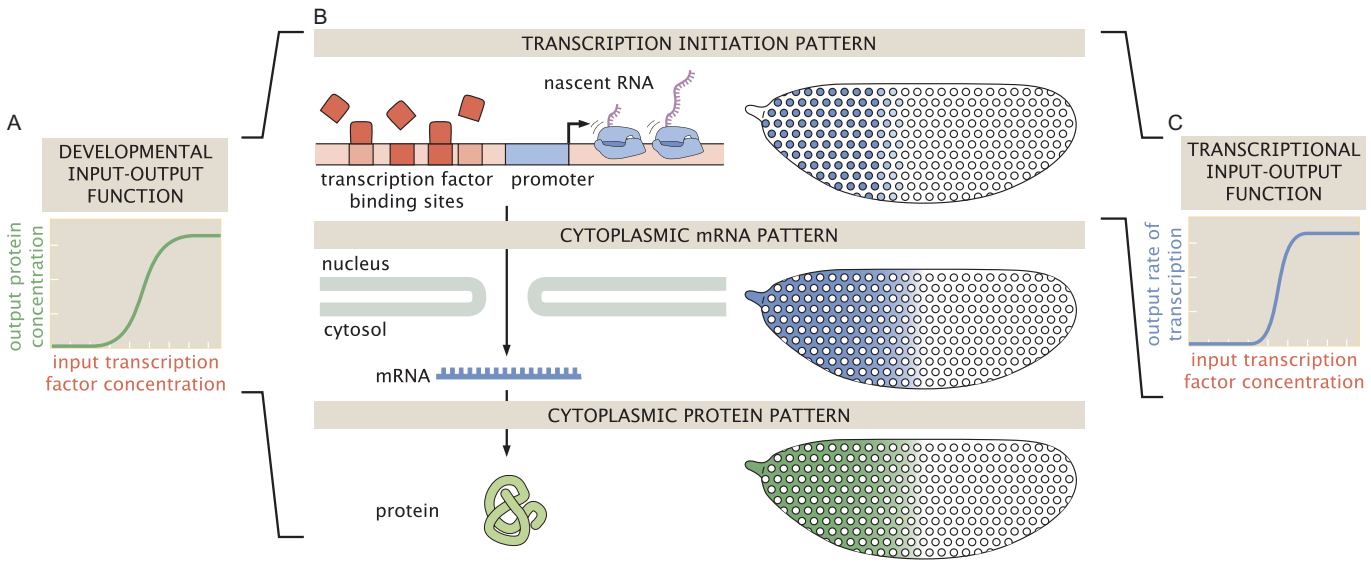

Fig. 4 Regulation along the central dogma. (A) Arrows in gene regulatory networks encode developmental input-output functions that predict output protein concentration as a function of input transcription-factor concentration. (B) Developmental input-output functions are the result of the regulation of multiple steps of the central dogma. (C) Transcriptional input-output functions predict output transcriptional activity as a function of input transcription-factor concentration.

However, the reliance on fixed tissue in these techniques casts doubt on their suitability for measuring dynamical developmental input-output functions; using dead, fixed embryos yields stop-motion "movies" for which each frame requires a new embryo (Dubuis, Samanta, & Gregor, 2013; Poustelnikova, Pisarev, Blagov, Samsonova, & Reinitz, 2004). To measure the output transcription rate of a cell (Fig. 4C), the expression status of a single cell needs to be measured for at least two time points. But since fixed-tissue techniques necessarily only access one time point, they cannot enable the dialog between theory and experiment advocated for in this article. As a result, previous works were constrained to assuming that transcription is in steady-state such that transcriptional dynamics remain largely unaltered during nuclear cycles (Fakhouri et al., 2010; Little et al., 2013; Park et al., 2019; Sayal et al., 2016; Xu, Sepulveda, Figard, Sokac, & Golding, 2015; Xu, Skinner, Sokac, & Golding, 2016; Zoller et al., 2018).

Recently, this critical limitation was overcome by adapting the MS2 system (Bertrand et al., 1998) to measure the instantaneous content of nascent RNA of a specific gene in single cells of a living, developing embryo (Garcia et al., 2013; Lucas et al., 2013). This MS2 system, and its sister PP7 system (Chao, Patskovsky, Almo, & Singer, 2008; Larson, Zenklusen, Wu, Chao, & Singer, 2011), integrate a repetitive DNA sequence into a gene's untranslated region. Upon transcription, the MS2 sequence folds and forms a loop. These mRNA loops are bound by a maternally provided mRNA binding protein fused to GFP (Fig. 5A). As a result, sites of nascent transcript formation become visible as fluorescent puncta due to the localization of active RNA polymerase II molecules to the tagged gene; these puncta are easily visualized using laser scanning two-photon or confocal microscopy, or using light-sheet microscopy (Fig. 5B). Using single-molecule mRNA FISH, the fluorescence value corresponding to each punctum can be converted into an absolute number of polymerase II molecules actively transcribing the gene as a function of time and position along the embryo (Fig. 5C) (Garcia et al., 2013). The result is the first-ever dynamical measurement of transcription in single cells within a living multicellular organism (Bothma & Levine, 2013).

This new ability to interrogate transcriptional activity in real time during development has unveiled new insights into the fundamental and dynamical nature of developmental processes. Here we showcase a few examples:

i. The gene-expression patterns that dictate cellular fate commitment are much more short-lived than previously thought (Bothma et al., 2014; Lammers et al., 2018).

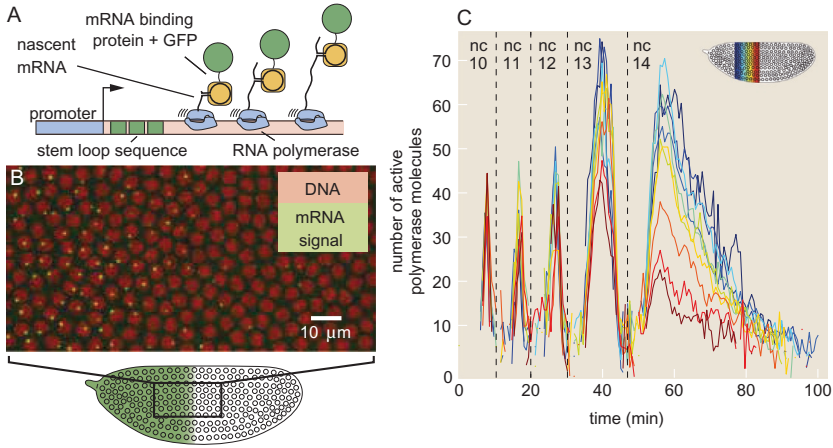

Fig. 5 Accessing transcriptional dynamics in live fly embryos. (A) Repeats of the MS2 sequence are added to a gene that, when transcribed, folds into a stem loop that is recognized by an mRNA binding protein fused to GFP; fluorescence is proportional to transcriptional activity. (B) Typical field of view showing sites of transcription in single nuclei for a reporter of the step-like *hunchback* expression pattern. (C) Number of actively transcribing RNA polymerase II molecules as a function of time for different positions along the embryo's axis. (nc: nuclear cycle.). *Adapted from Garcia, H.G., Tikhonov, M., Lin, A., & Gregor, T. (2013). Quantitative imaging of transcription in living Drosophila embryos links polymerase activity to patterning.* Current Biology, 23, *2140–2145.*

- **ii.** The processes by which enhancers coordinate their activities with each other and with promoters can be directly visualized (Bothma et al., 2015; Chen et al., 2018; El-Sherif & Levine, 2016; Lim, Heist, Levine, & Fukaya, 2018; Scholes, Biette, Harden, & DePace, 2019).
- **iii.** Transcription-factor concentration is read out to generate precise output patterns (Ferraro et al., 2016; Lim, Levine, & Yamazaki, 2017; Tran et al., 2018).
- **iv.** Mitosis and pioneer transcription factors dictate the transcriptional dynamics of embryos (Dufourt et al., 2018; Esposito, Lim, Guessous, Falahati, & Levine, 2016; Yamada et al., 2019).
- **v.** The real-time elongation rate of RNA polymerase II can be captured and quantified (Fukaya, Lim, & Levine, 2017; Garcia et al., 2013).

All of these insights afforded by MS2 will make it possible to rewrite biology textbooks by capturing the processes of cellular commitment in real time and by dramatically overturning or significantly complementing our previous knowledge stemming from fixed-embryos techniques.

Perhaps one of the most captivating outcomes of the tagging of early fly developmental genes with MS2 and PP7 has been the confirmation of the

long-suspected presence of transcriptional bursts in development (Little et al., 2013; Pare et al., 2009) *via* their real-time visualization (Bothma et al., 2014). As shown in Fig. 6A and B, the existence of these bursts indicates that the rate of transcriptional initiation is non-zero only during transient, but stochastic, periods of time (Bothma et al., 2014; Chubb, Trcek, Shenoy, & Singer, 2006; Golding, Paulsson, Zawilski, & Cox, 2005; Yunger, Rosenfeld, Garini, & Shav-Tal, 2010). These live-imaging techniques have made it possible to determine the ubiquity of transcriptional bursting in development and to start revealing their molecular control mechanisms (Berrocal, Lammers, Garcia, & Eisen, 2018; Bothma et al., 2014; Desponds et al., 2016; Falo-Sanjuan, Lammers, Garcia, & Bray, 2019; Fukaya, Lim, & Levine, 2016; Lammers et al., 2018; Lim, Fukaya, Heist, & Levine, 2018).

Although MS2 and PP7 made it possible to directly confirm the existence of transcriptional bursts in development, their precise quantitative characterization presents challenges: note that neither MS2 nor PP7 actually report on the rate of transcriptional initiation. An actively transcribing RNA polymerase molecule remains loaded on the DNA, and contributes to the overall fluorescence signal, until transcription terminates (Fig. 6A and B). As a result, the signal from MS2 and PP7 reports on the integrated transcriptional activity over a time window corresponding to the dwell time of RNA polymerase on the gene (given by the time to elongate the mRNA and to terminate transcription). Thus, output fluorescence is not directly related to the instantaneous promoter state and is instead the *convolution* of the promoter activity over a time window (Fig. 6B).

Recently, this fundamental limitation has been circumvented by various strategies. First, by focusing on promoter-enhancer interactions that rarely produce transcriptional bursts, the manual identification and measurement of the properties of these bursts was achieved (Fukaya et al., 2016). Second, by examining the autocorrelation of the output fluorescence signal (Coulon et al., 2014; Coulon & Larson, 2016; Larson et al., 2011), average bursting dynamics (such as the burst size, frequency, and amplitude) were revealed for a wider range of promoter dynamics than those accessible by manual analysis (Desponds et al., 2016). Finally, many computational tools have been recently developed to infer the most likely bursting state of a promoter in a single cell (Bronstein, Zechner, & Koeppl, 2015; Corrigan, Tunnacliffe, Cannon, & Chubb, 2016; Featherstone et al., 2016; Hey et al., 2015; Lammers et al., 2018; Molina et al., 2013; Suter et al., 2011; Zechner, Unger, Pelet, Peter, & Koeppl, 2014; Zoller, Nicolas,

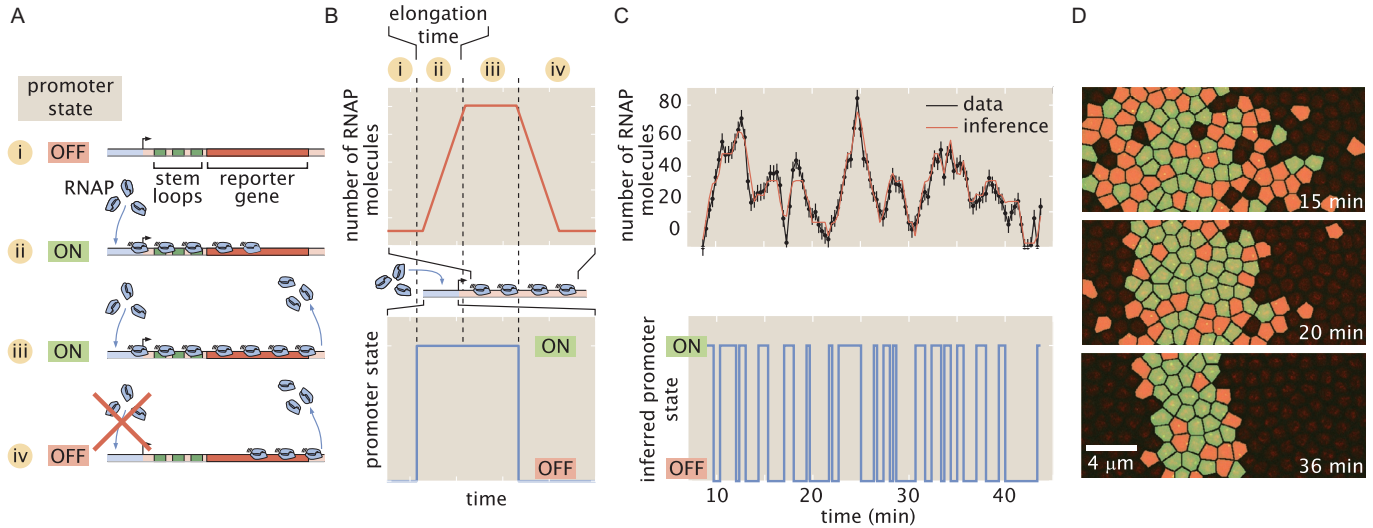

Fig. 6 Relation between MS2 fluorescence and instantaneous transcriptional activity. (A) Loading of RNA polymerase, and transcript elongation and termination as a gene is transiently turned on. (B) These discrete changes in promoter state are convolved with the elongation and termination times, resulting in a trapezoidal-like modulation of the number of RNA polymerase molecules on the reporter, as indicated by MS2 fluorescence. (C) Inference of promoter state from MS2 data using Hidden Markov models. (D) Inference of promoter states for cells expressing a transcriptional reporter of stripe 2 of the *even-skipped* gene. *Panels (C and D) Adapted from Lammers, N.C., Galstyan, V., Reimer, A., Medin, S.A., Wiggins, C.H., & Garcia, H.G. (2018). Multimodal transcriptional control of pattern formation in embryonic development.* bioRxiv, 335919.

Molina, & Naef, 2015). For example, techniques based on Hidden Markov Models enable queries of the instantaneous transcriptional activity of an individual promoter within a single cell as development progresses (Fig. 6C). Thus, novel computational approaches are opening a direct window into the molecular mechanisms of transcription factors by extracting promoter-switching kinetics and correlating these kinetics with the concentrations of input transcription factors (Lammers et al., 2018).

As with any technology to shed light on the inner workings of cells, it is also important to be aware of the potential caveats associated with the implementation of MS2 in development. First, even though single mRNA molecules can be visualized as they are transcribed in bacteria and yeast (Golding et al., 2005; Larson et al., 2011), the signal-to-background present in embryos only allows for the detection of the fluorescence signal of, at the most, three mRNA molecules as they are being transcribed (Garcia et al., 2013). This low signal-to-background stems primarily from the thick optical sections afforded by widespread laser-scanning confocal microscopes which are much wider than the site of transcription and hence capture much of the free mRNA binding protein-GFP fusion in the nucleoplasm. The introduction of new microscopy modalities with higher axial resolution such as lattice light-sheet microscopy (Chen et al., 2014) could enable the single-molecule detection of mRNA molecules as they are being transcribed in an embryo. Further, doubts have been cast on whether the presence of MS2 loops in a transcript affect its stability (Garcia & Parker, 2015; Golding & Cox, 2004; Haimovich et al., 2016; Heinrich, Sidler, Azzalin, & Weis, 2017; Kim, Vieira, Kim, Kesawat, & Park, 2019). While effects on mRNA stability are probably irrelevant if MS2 is used as a reporter of transcriptional activity, these effects could certainly confound downstream measurements of mRNA export and processing, and affect the reliable operation of endogenous genes. New MS2 sequences are actively being developed to circumvent these limitations (Tutucci et al., 2018). In addition, since intronic RNA is rapidly processed during transcription (Coulon et al., 2014), inserting MS2 loops inside introns could prove a reliable strategy to tag endogenous genes without affecting the life cycle of their mRNA molecules. Even if no introns are present in a gene, synthetic introns can be introduced in order to realize this labeling strategy (Bothma, Norstad, Alamos, & Garcia, 2018).

3.2 Lighting up protein dynamics and transcriptional input-output functions

Despite these encouraging breakthroughs in measuring output transcriptional dynamics in real time, biologists have until recently lacked the

technology to measure the fast dynamics of translation and degradation of the *input* transcription factors (Fig. 4). Although engineered fluorescent proteins such as GFP have chromophore maturation half-times as low as 6 min *in vitro* or in cultured cells (Nagai et al., 2002), maturation half-times increase to >30 min in embryos of developmental biology workhorses such as frogs, zebrafish, and flies (Little, Tkacik, Kneeland, Wieschaus, & Gregor, 2011). These time scales are much slower than many of the key processes in development. For example, the fruit fly transcription factor Fushi tarazu has a half-life of 8 min (Bothma et al., 2018; Kellerman, Mattson, & Duncan, 1990), and the Hes proteins that drive segmentation in vertebrates have a half-life of ~20 min (Hirata et al., 2004; Schroter et al., 2008). Thus, in many developmental contexts, by the time GFP fusions become fluorescent, the developmental processes these fusions are supposed to report on are already over. This fundamental limitation has prevented developmental biologists from following the central dogma with high spatiotemporal resolution and, more specifically, has made it impossible to measure input transcription-factor concentration dynamics in transcriptional input-output functions.

To enable the real-time measurement of input transcription-factor dynamics over the fast-paced process of development, and to circumvent the confounding effects of fluorescent protein maturation kinetics, the nanobody-based LlamaTag was recently introduced to light up protein concentration dynamics (Bothma et al., 2018). Here, nanobodies, single-chain antibodies raised in llamas against GFP or mCherry variants, are fused to a transcription factor of interest. In parallel, the fluorescent protein is provided maternally such that when development begins, this protein is already mature and uniformly distributed throughout the embryo. Upon translation of the transcription-factor fusion in the cytoplasm, the LlamaTag binds the free fluorescent protein. This complex is translocated into the nucleus *via* the transcription factor's nuclear localization signal, resulting in an enrichment of nuclear fluorescence that directly reports on the nuclear concentration of the complex. Thus, by leveraging localization of fluorescence proteins rather than the (more common) synthesis of new proteins, this technology becomes insensitive to fluorescent-protein maturation (Fig. 7A and B). LlamaTags have already made it possible to correlate bursts in transcriptional activity with bursts in protein concentration, to measure protein degradation, and to reveal the diffusion-mediated coupling between neighboring nuclei that can drive pattern formation in the fly syncytium (Bothma et al., 2018). Excitingly, these tags have also made it possible to quantify

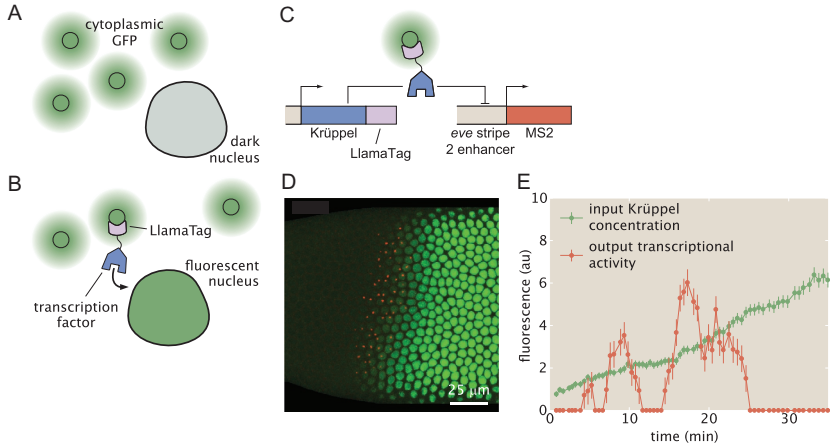

Fig. 7 Beating the fluorescent protein maturation speed limit with LlamaTags. (A) GFP expressed in the cytoplasm is (B) bound by a fusion of a LlamaTag to a transcription factor of interest. The increase in nuclear fluorescence upon translocation of the fusion to the nucleus reports transcription-factor concentration. (C) Combination of LlamaTag and MS2 tagging to simultaneously measure Krüppel repressor concentration and *even-skipped* (*eve*) stripe 2 transcriptional activity. (D) Snapshot of a fly embryo expressing Krüppel-LlamaTag and reporting on *eve* transcriptional activity using MS2 27 min into nuclear cycle 14. (E) Measured input and output dynamics in a nucleus within the stripe. *Panels (D and E) Adapted from Bothma, J.P., Norstad, M.R., Alamos, S., & Garcia, H.G. (2018). LlamaTags: A versatile tool to image transcription factor dynamics in live embryos.* Cell, 173*, 1810–1822.*

transcriptional input-output functions at the single-cell level by enabling real-time measurement of instantaneous input transcription factor concentration and output transcriptional activity (Fig. 7C–E).

Just like regular fusions of transcription factors to fluorescent proteins, LlamaTag fusions can affect endogenous protein function. Further, these tags are limited to tagging proteins that undergo translocation after translation, such as transcription factors, and cannot report on the concentration dynamics of proteins that remain in the cytoplasm to perform their function. However, due to the nascent nature of LlamaTags, the full set of potential caveats associated with these tags, and of possible solutions to those caveats, is yet to be revealed as this technique is adopted by developmental biologists.

3.3 Wiring up the synthetic embryo

For the first time, developmental biologists are positioned to directly measure transcriptional input-output functions that capture rapid modulations in

the concentration dynamics of input transcription factors and the resulting output transcriptional activity. A crucial next step is to identify regulatory architectures amenable to theoretical modeling that can be attacked with this new arsenal of tools.

Over the last three decades, a great deal of research has focused on the role of transcription factor binding sites in transcriptional input-output functions (Chen, Xu, Mei, Yu, & Small, 2012; Crocker et al., 2015; Crocker, Ilsley, & Stern, 2016; Driever, Thoma, & Nusslein-Volhard, 1989; Fakhouri et al., 2010; Hare, Peterson, Iyer, Meier, & Eisen, 2008; Harrison, Li, Kaplan, Botchan, & Eisen, 2011; Jiang & Levine, 1993; Park et al., 2019; Sayal et al., 2016; Small, Blair, & Levine, 1992; Stathopoulos & Levine, 2005; Swanson, Evans, & Barolo, 2010). Often, complex gene-regulatory regions featuring dozens of binding sites for several transcription factors are dissected *via* systematic deletions of these sites. Such approaches have revolutionized our understanding of the spatial control of developmental patterning, as exemplified by the famed dissection of the regulatory logic of the enhancer that regulates stripe 2 of the *even-skipped* gene, which revealed how activators and repressors work together to create precise gene expression patterns in the fly embryo (Arnosti, Barolo, Levine, & Small, 1996; Small et al., 1992; Small, Kraut, Hoey, Warrior, & Levine, 1991).

Nonetheless, these approaches often face an insurmountable barrier when moving from the qualitative realm to a quantitative understanding that makes it possible to predict transcriptional input-output functions. Complex regulatory architectures, by definition, demand complex theoretical models that in turn are plagued by a plethora of unknown regulatory parameters. Consider the *hunchback* P2 enhancer, perhaps one of the simplest and most studied regulatory architectures in all of development (Driever et al., 1989; Margolis et al., 1995; Park et al., 2019; Perry, Bothma, Luu, & Levine, 2012). The Bicoid activator can bind at least six sites in this enhancer (Driever et al., 1989). Predicting the transcriptional input-output function of *hunchback* P2 activation by Bicoid using even simple models based on equilibrium statistical mechanics demands previous knowledge of at least 27 parameters (Fig. 8; Garcia, Brewster, and Phillips (2016), see also Garcia, Kondev, Orme, Theriot, and Phillips (2007) for an introduction to statistical mechanics for life scientists). This number only grows as assumptions regarding equilibrium are relaxed (Estrada et al., 2016). Inferring these parameters from the measurement of a transcriptional input-output function is both a massive computational and—more critically—conceptual challenge (Garcia et al., 2016).

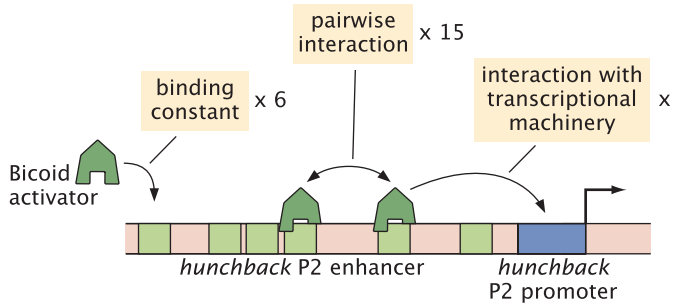

Fig. 8 Combinatorial complexity of endogenous gene regulatory regions. The *hunchback* P2 enhancer is bound by at least six Bicoid activators to regulate *hunchback*. A simple model featuring only binding energies, pairwise interactions between bound activators, and pairwise interactions between each activator and the transcriptional machinery would demand the fitting of 27 unknown parameters.

Synthetic biology could empower our dissection of developmental enhancers. Inspired by work in bacteria, an alternative to fitting complex theoretical models to complex experimental architectures in development is to *bend nature to understand it* (Garcia et al., 2016; Phillips et al., 2019). Specifically, building synthetic enhancers bearing only one binding site for an activator such as Dorsal or Bicoid dramatically reduces regulatory complexity. To be concrete, we consider an activator that is distributed in an exponential gradient along the embryo (Fig. 9A) resulting in a step-like output pattern of gene expression. As shown in Fig. 9B, a thermodynamic model describing this simple regulatory architecture has only two free parameters: the binding affinity of the activator to the DNA (K_d) and a parameter that captures the strength with which a bound activator drives gene expression (r_{AP}) and that depends on the distance between the activator binding site and the promoter. Thus, by measuring the height and position of the activator-driven developmental boundary, these two key parameters can be obtained.

This synthetic approach offers an opportunity to iteratively embrace regulatory complexity. Specifically, consider the case where the complexity of the synthetic enhancer is increased by adding a second activator binding site (Fig. 9C). If we rely on the parameters obtained in the previous iteration (Fig. 9B), and if we assume only pair-wise interactions between bound transcription factors, then only one new unknown parameter emerges. This new parameter, ω_{AA}, characterizes protein-protein interactions that lead to cooperativity and to the sharpening of the boundary. Thus, by harnessing the knowledge obtained in previous iterations, each successive iteration

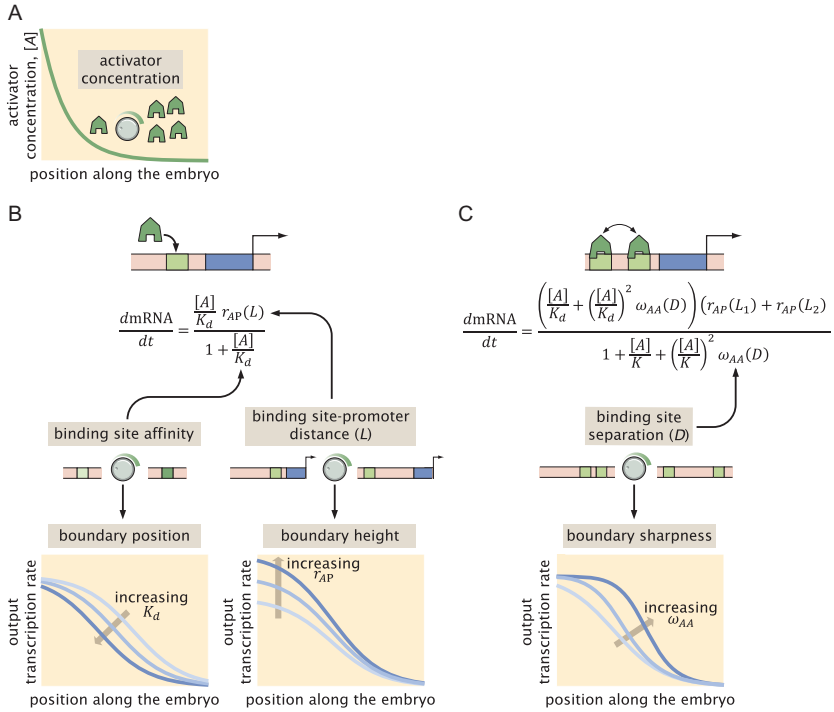

Fig. 9 A synthetic approach to uncovering the governing equations of gene regulatory regions in development. (A) Exponential activator concentration profile along the embryo assumed for this illustrative example. (B, C) Equations, regulatory parameters, and developmental patterns for synthetic enhancers containing (B) one or (C) two activator binding sites. (B) A reporter construct with a single activator binding site drives a step-like pattern whose boundary position is determined only by the binding site affinity (K_d), and whose boundary height is governed by the interaction between the activator and the transcriptional machinery (r_{AP}). (C) Adding a second activator binding site introduces only one new free parameter accounting for activator-activator interactions (ω_{AA}). This parameter controls boundary sharpness. For simplicity, we do not account for the existence of a basal rate of transcription. However, the addition of this parameter to the model would not modify the overall synthetic strategy significantly.

of this synthetic approach only requires the fitting of one or two new parameters. After multiple iterations, the synthetic architectures converge onto endogenous ones—accompanied by increasingly complex, but still predictive, theoretical models.

We speculate that this approach could be used to dissect complex regulatory architectures featuring multiple activators and repressors. Crucially, the key components of synthetic dissection already exist: multiple examples of minimal regulatory architectures featuring binding sites of transcription

factors such as Bicoid, Dorsal, Giant, Snail, and Twist have been shown to drive detectable levels of gene expression (Burz & Hanes, 2001; Burz, Rivera-Pomar, Jackle, & Hanes, 1998; Driever et al., 1989; Erceg et al., 2014; Fakhouri et al., 2010; Hanes, Riddihough, Ish-Horowicz, & Brent, 1994; Jiang & Levine, 1993; Lebrecht et al., 2005; Ma, Yuan, Diepold, Scarborough, & Ma, 1996; Park et al., 2019; Ronchi, Treisman, Dostatni, Struhl, & Desplan, 1993; Sayal et al., 2016; Simpson-Brose, Treisman, & Desplan, 1994; Szymanski & Levine, 1995). Recent work has demonstrated the feasibility of this synthetic approach to testing theoretical models of transcriptional regulation in development in the context of activation by Dorsal and Twist, repression by Giant and Snail, and for synthetic transcription factors (Crocker et al., 2016; Fakhouri et al., 2010; Sayal et al., 2016).

3.4 Predicting the central dogma beyond transcription

So far, we have concerned ourselves with the prediction and measurement of transcriptional input-output functions. However, it is important to keep in mind that the information encoded by each arrow in cartoons of gene regulatory networks accounts for multiple steps along the central dogma (Fig. 4B). From chromatin accessibility to alternative splicing to post-translational modifications, seemingly simple arrows capture multiple molecular steps, all of which can be subject to regulation—calling, once again, for an interplay between theory and experiment to uncover the governing equations corresponding to each regulatory step. However, despite huge leaps in genomics (Goodwin, McPherson, & McCombie, 2016; Koboldt, Steinberg, Larson, Wilson, & Mardis, 2013; Shlyueva, Stampfel, & Stark, 2014), technologies to measure chromatin accessibility and modifications, protein binding to the DNA, promoter-enhancer interactions, mRNA processing, translational regulation, and post-translational modifications in single cells within living embryos have lagged behind (Buenrostro, Wu, Chang, & Greenleaf, 2015; Matera & Wang, 2014; Mayer, Landry, & Churchman, 2017; Park, 2009).

First, chromatin must be accessible for transcription factors to bind DNA. However, technology to reveal this accessibility or the epigenetic state of histones in the vicinity of a binding site has been mostly limited to genome-wide or fixed-tissue approaches (Blythe & Wieschaus, 2016; Boettiger et al., 2016; Cusanovich et al., 2018; Haines & Eisen, 2018; Li, Burkhardt, Gross, & Weissman, 2014). The recent development of genetically encoded modification-specific intracellular antibodies (mintbodies)

that bind chromatin with specific modifications such as H3K9 acetylation and H4K20 methylation (Sato et al., 2013, 2016), as well as split-luciferase probes to image H3K9 and H3K27 methylation (Sekar, Foygel, Gelovani, & Paulmurugan, 2015), will enable concrete progress in the real-time monitoring of chromatin state in development at the single-cell level.

New imaging technologies and improved fluorescent probes have made it possible to image individual transcription factors as they bind DNA inside living fly embryos (Chen et al., 2014; Mir et al., 2017; Tsai et al., 2017). These measurements have revealed that, while transcription factors appear to spend no more than a few seconds bound to DNA, their binding throughout the nucleus is not uniform: hubs or domains of increased local concentration (and of increased binding frequency) have been found for both Bicoid and Ultrabithorax. While some of these regions of increased binding probability may depend on the pioneer transcription factor Zelda (Mir et al., 2017), their functional role remains unclear. To make progress toward a molecular understanding of how genes read out transcription-factor concentration, it will be necessary to directly correlate this binding with output transcriptional activity—which is only now becoming possible in single cells (Cho et al., 2016; Chong et al., 2018; Donovan et al., 2019; Li et al., 2019), and for which feasibility in live embryos was recently demonstrated (Mir et al., 2018).

Most developmental enhancers do not reside in the vicinity of their target promoter; they are supposed to loop or to translocate over vast distances of DNA in order to carry out their regulatory function (for a recent review on the subject, see Furlong & Levine, 2018). Recently, enhancer position and promoter activity were simultaneously visualized in the early fly embryo in the context of DNA looping (Chen et al., 2018) and transvection (Lim, Heist, et al., 2018). These works demonstrated that bringing enhancers and promoters in close proximity is necessary but not sufficient to activate transcription. These results, plus speculation about larger structures involved in transcriptional regulation (Mir et al., 2017, 2018; Tsai et al., 2017), and reports that stable promoter-enhancer contacts might not be needed for transcriptional activation (Alexander et al., 2019; Benabdallah et al., 2019; Gu et al., 2018) suggest that the classical paradigm of direct contact between enhancers and promoters may have to be revisited.

Regulation does not cease after transcription initiation: the rate of mRNA elongation can be under regulatory control, and fly embryos process mRNA through splicing as well as RNA polymerase II pausing and termination to achieve precise and rapid development (Bentley, 2014;

Core & Adelman, 2019; Larschan et al., 2011; Richard & Manley, 2009). While current genome-wide techniques have been powerful for revealing correlations among large sets of genes, visualizing mRNA processing could shed further light on the role of this processing in development. By combining MS2 and PP7 to label different parts of the same nascent RNA in the human β-globin gene, the life history of an mRNA was revealed as it underwent transcription, splicing, and termination (Coulon et al., 2014). This approach is being adopted in the fly embryo to, for example, determine transcript elongation rates (Fukaya et al., 2017).

The regulation of translation is also widespread in gene regulatory networks. For example, Bicoid represses Caudal translation (Dubnau & Struhl, 1996; Niessing, Blanke, & Jackle, 2002; Rivera-Pomar, Niessing, Schmidt-Ott, Gehring, & Jackle, 1996), while Nanos downregulates Hunchback post-transcriptionally, through either a decrease in translation or an increase in mRNA degradation (Cho et al., 2006; Irish, Lehmann, & Akam, 1989; Murata & Wharton, 1995; Struhl, 1989; Wang & Lehmann, 1991; Wharton & Struhl, 1991). However, we know much less about how translation is regulated at the single mRNA level than we know about the details of transcription. For example, is the translation of specific mRNA molecules downregulated by decreasing the peptide elongation rate of all ribosomes, or by decreasing the fraction of mRNA molecules that are translated? These questions and others can be answered by implementing recently developed reporters for measuring the first round of translation (Halstead et al., 2015), and by examining the translational dynamics of individual mRNA molecules at the single-cell level (Morisaki et al., 2016; Wang, Han, Zhou, & Zhuang, 2016; Wu, Eliscovich, Yoon, & Singer, 2016; Yan, Hoek, Vale, & Tanenbaum, 2016).

Finally, many developmental decisions are mediated by the post-translational modification of proteins. Regulation *via* protein phosphorylation is ubiquitous in development (for a review on this subject see, for example, Peter & Davidson, 2015; Ubersax & Ferrell, 2007). Antibodies cannot always distinguish between phosphorylated and non-phosphorylated protein forms, which hinders our ability to determine how signaling dynamics dictate development. When phosphorylation drives the nuclear localization of a transcription factor, such as for the transcription factor Capicua (Grimm et al., 2012), tracking its nuclear localization using a fusion to a fluorescent protein or a LlamaTag directly reports on the protein's signaling state. New sensors reveal kinase and phosphatase activity without requiring modulation of the cellular localization of their substrates; novel *kinase*

translocation reporters can be engineered to become targets of a particular signaling pathway (Kudo et al., 2018; Oldach & Zhang, 2014; Regot, Hughey, Bajar, Carrasco, & Covert, 2014). Upon phosphorylation by the kinase of interest, these sensors change their fluorescence or are translocated to the nucleus, where they report on signaling activity. Implementing these technologies in the embryo could open the door to systematic dissection, at the single-cell level, of the signaling cascades that underlie protein post-translational modifications during development.

Technology is already available to quantify the flow of information along each step of the central dogma in real time and at the single-cell level, as highlighted by the various approaches showcased above. Thus, the main challenge ahead is not one of technology development, but one of implementing these technologies in developing embryos. The new and exciting data generated by these rising technologies must be matched with new theoretical models that draw us closer to a quantitative and predictive understanding of how the regulation of the processes of the central dogma impact cellular decision-making.

4. Developmental programs as dynamical systems

So far, we have focused on new technologies and theoretical approaches that enable the predictive dissection of the input-output functions encoded by each arrow in gene regulatory networks (Fig. 2A). However, predictive understanding of the parts that make a network does not guarantee understanding of how those arrows work together to realize developmental programs. It has been repeatedly hypothesized that the ultimate developmental fate of each cell arises from the trajectory of the gene-expression state of a cell as it traverses the regulatory landscape shaped by the network topology, the patterns of connections between network elements (Fig. 2B, reviewed in Jaeger, Manu, & Reinitz, 2012; Jaeger & Monk, 2014).

By borrowing tools from dynamical systems theory, multiple teams have attempted to describe how network topology prescribes these developmental trajectories. While some of these works have sought to model multiple layers of the network simultaneously, others have focused on isolated network motifs, such as the widespread mutual repression regulatory architecture (Edgar et al., 1989; Gursky et al., 2011; Jaeger, Blagov, et al., 2004; Jaeger, Surkova, et al., 2004; Lopes, Vieira, Holloway, Bisch, & Spirov, 2008; Manu et al., 2009; Papatsenko & Levine, 2011; von Dassow et al., 2000;

Von Dassow & Odell, 2002). Using gene expression data from fixed embryos sorted into temporal classes, these studies have, for instance, revealed how gene expression domains shift along the embryo as development progresses (Jaeger, Surkova, et al., 2004), and how multiple arrows work together to "lock" individual cells into specific developmental fates (Papatsenko & Levine, 2011).

These investigations have been complemented by the realization that the landscape shaped by these arrows is not static. For example, temporal changes in the concentration of transcription factors such as that of the Bicoid activator over development can propagate through the network, effectively modulating the network's topology and impacting cellular, and therefore embryonic, phenotype (Verd et al., 2018; Verd, Crombach, & Jaeger, 2017; Verd, Monk, & Jaeger, 2019). We urgently require theoretical tools to deal with such *non-autonomous dynamical systems*, where network parameters are modulated in time. Further, to test the predictions of these models, it will be necessary to simultaneously visualize the transcriptional activity and protein products of multiple genes in single cells as these networks are deployed. Currently, it is possible to simultaneously image only one input transcription factor and the transcriptional activity of one of its target genes (Bothma et al., 2018). This limitation to multiplexing underscores the need for new fluorescent probes with a large repertoire of spectral ranges, as well as advances in microscopy techniques that make it possible to spectrally resolve these different probes.

5. Toward quantitative and predictive developmental biology

The experimental technologies and theoretical approaches reviewed in this article are the means to the ultimate goal of a predictive understanding of developmental decision-making. Demanding a quantitative and predictive understanding of biological phenomena sharpens our questions and makes our inquiries more sensitive to inconsistencies that may reveal new biological insights that would have remained hidden from qualitative approaches (Cohen, 2004; Garcia, Sanchez, Kuhlman, Kondev, & Phillips, 2010). However, in our opinion, the discovery of new molecular players does not constitute a guiding objective in and of itself (Phillips, 2015). Even in the absence of new discoveries, we would like to define successful physical biology of embryonic development as the demonstration that developmental programs can be predictive, much as it has been shown

in the context of gene regulatory programs in bacteria (Garcia et al., 2016; Phillips et al., 2019).

Although such predictive understanding calls for a quantitative view of how all the processes of the central dogma are regulated in development, the topics covered in this article have been vastly biased toward the regulation of transcriptional initiation. We believe that this bias reflects the state of the art in the field, as it is now possible to monitor transcriptional initiation and the concentration dynamics of the transcription factors that direct this initiation in real time during development. However, new technologies, some of which were briefly reviewed here, enable real-time, single-cell, high-precision, *in vivo* measurements of other steps of the central dogma. We therefore envision that, as these technologies are unleashed to unravel development, they will yield the dialog between theory and experiment that has been a defining factor in our understanding of the regulation of transcriptional initiation.

Of course, we must not forget that development transcends regulation of the central dogma! Ultimately, expression patterns arising from gene regulatory networks drive the morphogenic movements that bring about tissue growth and biological shape, and these movements further determine, in turn, gene expression patterns (for reviews on the subject, see Chan, Heisenberg, & Hiiragi, 2017; Gilmour, Rembold, & Leptin, 2017; Mammoto, Mammoto, & Ingber, 2012; Totaro, Panciera, & Piccolo, 2018). The capacity to measure and manipulate actomyosin networks is now making it possible to relate the activity of these regulatory networks to the massive cellular rearrangements that characterize morphogenesis and to control them synthetically (Campas, 2016; Farrell, Weitz, Magnasco, & Zallen, 2017; Guglielmi, Barry, Huber, & De Renzis, 2015; He, Martin, & Wieschaus, 2016; Kale et al., 2018; Martin, Kaschube, & Wieschaus, 2009; Streichan, Lefebvre, Noll, Wieschaus, & Shraiman, 2018). These new measurements and allied theoretical and computational approaches promise to close the gap between our understanding of morphogen gradients and our understanding of morphogenesis.

Finally, although this review limited its scope to the fruit fly, no one species holds all the keys to predictively understanding development. A key challenge will be to demonstrate that the strategies put forth here can also reveal the physical biology of embryos of other developmental biology workhorses such as worms, fish, and mice. Excitingly, the real-time visualization of transcription and mRNA processing was recently achieved in all three of these model organisms (Campbell, Chao, Singer, & Marlow, 2015;

Hadzhiev et al., 2019; Lee, Shin, & Kimble, 2019; Lionnet et al., 2011). Thus, the technologies discussed in this review article are ushering in a new era in developmental biology in which the focus on spatial, almost static, control of developmental programs is being replaced by a dynamical view that embraces the quantitative spatiotemporal control of development (Berrocal et al., 2018; Bothma & Levine, 2013). This new language will empower the discourse between theory and experiment that will revolutionize our ability to predict—and ultimately manipulate—developmental programs at will.

Acknowledgments

The authors are grateful to Jack Bateman, Jacques Bothma, James Briscoe, Leigh Harris, Thomas Lecuit, Mustafa Mir, Rob Phillips, Clarissa Scholes, Stephen Small and Michael Stadler for discussions and/or comments on this manuscript. However, the opinions and point of view expressed here are the authors' and those acknowledged above should not be blamed for these views. HGG was supported by the Burroughs Wellcome Fund Career Award at the Scientific Interface, the Sloan Research Foundation, the Human Frontiers Science Program, the Searle Scholars Program, the Shurl & Kay Curci Foundation, the Hellman Foundation, the NIH Director's New Innovator Award (DP2 OD024541-01), and an NSF CAREER Award (1652236). Y.J.K. was supported by Korea Foundation for Advanced Studies. G.M. was supported by an NIH NRSA Training Grant (T32 HG 00047) and by an NSF Graduate Fellowship. A.B. was supported by the University of California Institute for Mexico and the United States (UC MEXUS). Finally, the references cited throughout the text are meant as a guide to the reader to the literature, and do not attempt to provide a scholarly assessment of the whole field of fly development or of developmental biology writ large.

References

Ackers, G. K., Johnson, A. D., & Shea, M. A. (1982). Quantitative model for gene regulation by lambda phage repressor. *Proceedings of the National Academy of Sciences of the United States of America*, *79*, 1129–1133.

Alberts, B. (2015). *Molecular biology of the cell* (6th ed.). New York, NY: Garland Science, Taylor and Francis Group.

Alexander, J. M., Guan, J., Li, B., Maliskova, L., Song, M., Shen, Y., et al. (2019). Live-cell imaging reveals enhancer-dependent Sox2 transcription in the absence of enhancer proximity. *eLife*, *8*, e41769.

Arnosti, D. N., Barolo, S., Levine, M., & Small, S. (1996). The eve stripe 2 enhancer employs multiple modes of transcriptional synergy. *Development*, *122*, 205–214.

Benabdallah, N. S., Williamson, I., Illingworth, R. S., Kane, L., Boyle, S., Sengupta, D., et al. (2019). Decreased enhancer-promoter proximity accompanying enhancer activation. *Molecular Cell*, *76*(3), 473–484.e477.

Bentley, D. L. (2014). Coupling mRNA processing with transcription in time and space. *Nature Reviews. Genetics*, *15*, 163–175.

Berrocal, A., Lammers, N. C., Garcia, H. G., & Eisen, M. B. (2018). Kinetic sculpting of the seven stripes of the Drosophila even-skipped gene. *bioRxiv*, 335901.

Bertrand, E., Chartrand, P., Schaefer, M., Shenoy, S. M., Singer, R. H., & Long, R. M. (1998). Localization of ASH1 mRNA particles in living yeast. *Molecular Cell*, *2*, 437–445.

Bintu, L., Buchler, N. E., Garcia, H. G., Gerland, U., Hwa, T., Kondev, J., et al. (2005a). Transcriptional regulation by the numbers: Applications. *Current Opinion in Genetics & Development*, *15*, 125–135.

Bintu, L., Buchler, N. E., Garcia, H. G., Gerland, U., Hwa, T., Kondev, J., et al. (2005b). Transcriptional regulation by the numbers: Models. *Current Opinion in Genetics & Development*, *15*, 116–124.

Blythe, S. A., & Wieschaus, E. F. (2016). Establishment and maintenance of heritable chromatin structure during early Drosophila embryogenesis. *eLife*, *5*, e20148.

Boettiger, A. N., Bintu, B., Moffitt, J. R., Wang, S., Beliveau, B. J., Fudenberg, G., et al. (2016). Super-resolution imaging reveals distinct chromatin folding for different epigenetic states. *Nature*, *529*, 418–422.

Boettiger, A. N., & Levine, M. (2013). Rapid transcription Fosters coordinate snail expression in the Drosophila embryo. *Cell Reports*, *3*(1), 8–15.

Bothma, J. P., Garcia, H. G., Esposito, E., Schlissel, G., Gregor, T., & Levine, M. (2014). Dynamic regulation of eve stripe 2 expression reveals transcriptional bursts in living Drosophila embryos. *Proceedings of the National Academy of Sciences of the United States of America*, *111*, 10598–10603.

Bothma, J. P., Garcia, H. G., Ng, S., Perry, M. W., Gregor, T., & Levine, M. (2015). Enhancer additivity and non-additivity are determined by enhancer strength in the Drosophila embryo. *eLife*, *4*, e07956.

Bothma, J., & Levine, M. (2013). Development: Lights, camera, action—The Drosophila embryo goes live! *Current Biology*, *23*, R965.

Bothma, J. P., Magliocco, J., & Levine, M. (2011). The snail repressor inhibits release, not elongation, of paused Pol II in the Drosophila embryo. *Current Biology*, *21*, 1571–1577.

Bothma, J. P., Norstad, M. R., Alamos, S., & Garcia, H. G. (2018). LlamaTags: A versatile tool to image transcription factor dynamics in live embryos. *Cell*, *173*, 1810–1822.

Bronstein, L., Zechner, C., & Koeppl, H. (2015). Bayesian inference of reaction kinetics from single-cell recordings across a heterogeneous cell population. *Methods (San Diego, Calif)*, *85*, 22–35.

Buchler, N. E., Gerland, U., & Hwa, T. (2003). On schemes of combinatorial transcription logic. *Proceedings of the National Academy of Sciences of the United States of America*, *100*, 5136–5141.

Buenrostro, J. D., Wu, B., Chang, H. Y., & Greenleaf, W. J. (2015). ATAC-seq: A method for assaying chromatin accessibility genome-wide. *Current Protocols in Molecular Biology*, *109*, 21–29.

Burz, D. S., & Hanes, S. D. (2001). Isolation of mutations that disrupt cooperative DNA binding by the Drosophila bicoid protein. *Journal of Molecular Biology*, *305*, 219–230.

Burz, B. S., Rivera-Pomar, R., Jackle, H., & Hanes, S. D. (1998). Cooperative DNA-binding by Bicoid provides a mechanism for threshold-dependent gene activation in the Drosophila embryo. *The EMBO Journal*, *17*, 5998–6009.

Campas, O. (2016). A toolbox to explore the mechanics of living embryonic tissues. *Seminars in Cell & Developmental Biology*, *55*, 119–130.

Campbell, P. D., Chao, J. A., Singer, R. H., & Marlow, F. L. (2015). Dynamic visualization of transcription and RNA subcellular localization in zebrafish. *Development*, *142*, 1368–1374.

Carroll, S. B., Grenier, J. K., & Weatherbee, S. D. (2001). *From DNA to diversity: Molecular genetics and the evolution of animal design*. Malden, Mass: Blackwell Science.

Chan, C. J., Heisenberg, C. P., & Hiiragi, T. (2017). Coordination of morphogenesis and cell-fate specification in development. *Current Biology*, *27*, R1024–R1035.

Chao, J. A., Patskovsky, Y., Almo, S. C., & Singer, R. H. (2008). Structural basis for the coevolution of a viral RNA-protein complex. *Nature Structural & Molecular Biology*, *15*(1), 103–105.
Chen, B. C., Legant, W. R., Wang, K., Shao, L., Milkie, D. E., Davidson, M. W., et al. (2014). Lattice light-sheet microscopy: Imaging molecules to embryos at high spatiotemporal resolution. *Science*, *346*, 1257998.
Chen, H., Levo, M., Barinov, L., Fujioka, M., Jaynes, J. B., & Gregor, T. (2018). Dynamic interplay between enhancer-promoter topology and gene activity. *Nature Genetics*, *50*, 1296–1303.
Chen, H., Xu, Z., Mei, C., Yu, D., & Small, S. (2012). A system of repressor gradients spatially organizes the boundaries of bicoid-dependent target genes. *Cell*, *149*, 618–629.
Cho, P. F., Gamberi, C., Cho-Park, Y. A., Cho-Park, I. B., Lasko, P., & Sonenberg, N. (2006). Cap-dependent translational inhibition establishes two opposing morphogen gradients in Drosophila embryos. *Current Biology*, *16*, 2035–2041.
Cho, W. K., Jayanth, N., English, B. P., Inoue, T., Andrews, J. O., Conway, W., et al. (2016). RNA Polymerase II cluster dynamics predict mRNA output in living cells. *eLife*, *5*, e13617.
Chong, S., Dugast-Darzacq, C., Liu, Z., Dong, P., Dailey, G. M., Cattoglio, C., et al. (2018). Imaging dynamic and selective low-complexity domain interactions that control gene transcription. *Science*, *361*(6400), pii: eaar2555.
Chubb, J. R., Trcek, T., Shenoy, S. M., & Singer, R. H. (2006). Transcriptional pulsing of a developmental gene. *Current Biology*, *16*, 1018–1025.
Cohen, J. E. (2004). Mathematics is biology's next microscope, only better; biology is mathematics' next physics, only better. *PLoS Biology*, *2*, e439.
Core, L., & Adelman, K. (2019). Promoter-proximal pausing of RNA polymerase II: A nexus of gene regulation. *Genes & Development*, *33*, 960–982.
Corrigan, A. M., Tunnacliffe, E., Cannon, D., & Chubb, J. R. (2016). A continuum model of transcriptional bursting. *eLife*, *5*, e13051.
Coulon, A., Ferguson, M. L., de Turris, V., Palangat, M., Chow, C. C., & Larson, D. R. (2014). Kinetic competition during the transcription cycle results in stochastic RNA processing. *eLife*, *3*, e03939.
Coulon, A., & Larson, D. R. (2016). Fluctuation analysis: Dissecting transcriptional kinetics with signal theory. *Methods in Enzymology*, *572*, 159–191.
Crocker, J., Abe, N., Rinaldi, L., McGregor, A. P., Frankel, N., Wang, S., et al. (2015). Low affinity binding site clusters confer hox specificity and regulatory robustness. *Cell*, *160*, 191–203.
Crocker, J., Ilsley, G. R., & Stern, D. L. (2016). Quantitatively predictable control of Drosophila transcriptional enhancers in vivo with engineered transcription factors. *Nature Genetics*, *48*, 292–298.
Cusanovich, D. A., Reddington, J. P., Garfield, D. A., Daza, R. M., Aghamirzaie, D., Marco-Ferreres, R., et al. (2018). The cis-regulatory dynamics of embryonic development at single-cell resolution. *Nature*, *555*, 538–542.
Davidson, E. H. (2006). *The regulatory genome: Gene regulatory networks in development and evolution*. Burlington, MA; San Diego: Academic.
Desponds, J., Tran, H., Ferraro, T., Lucas, T., Perez Romero, C., Guillou, A., et al. (2016). Precision of readout at the hunchback gene: Analyzing short transcription time traces in living fly embryos. *PLoS Computational Biology*, *12,* e1005256.
Donovan, B. T., Huynh, A., Ball, D. A., Patel, H. P., Poirier, M. G., Larson, D. R., et al. (2019). Live-cell imaging reveals the interplay between transcription factors, nucleosomes, and bursting. *The EMBO Journal*, *38,* e100809.
Driever, W., Thoma, G., & Nusslein-Volhard, C. (1989). Determination of spatial domains of zygotic gene expression in the Drosophila embryo by the affinity of binding sites for the bicoid morphogen. *Nature*, *340*, 363–367.

Dubnau, J., & Struhl, G. (1996). RNA recognition and translational regulation by a homeodomain protein. *Nature*, *379*, 694–699.
Dubuis, J. O., Samanta, R., & Gregor, T. (2013). Accurate measurements of dynamics and reproducibility in small genetic networks. *Molecular Systems Biology*, *9*, 639, [Electronic Resource].
Dufourt, J., Trullo, A., Hunter, J., Fernandez, C., Lazaro, J., Dejean, M., et al. (2018). Temporal control of gene expression by the pioneer factor Zelda through transient interactions in hubs. *Nature Communications*, *9*, 5194.
Edgar, B. A., Odell, G. M., & Schubiger, G. (1989). A genetic switch, based on negative regulation, sharpens stripes in Drosophila embryos. *Developmental Genetics*, *10*, 124–142.
El-Sherif, E., & Levine, M. (2016). Shadow enhancers mediate dynamic shifts of gap gene expression in the Drosophila embryo. *Current Biology*, *26*, 1164–1169.
Erceg, J., Saunders, T. E., Girardot, C., Devos, D. P., Hufnagel, L., & Furlong, E. E. (2014). Subtle changes in motif positioning cause tissue-specific effects on robustness of an enhancer's activity. *PLoS Genetics*, *10,* e1004060.
Esposito, E., Lim, B., Guessous, G., Falahati, H., & Levine, M. (2016). Mitosis-associated repression in development. *Genes Development*, *30*, 1503–1508.
Estrada, J., Wong, F., DePace, A., & Gunawardena, J. (2016). Information integration and energy expenditure in gene regulation. *Cell*, *166*, 234–244.
Fakhouri, W. D., Ay, A., Sayal, R., Dresch, J., Dayringer, E., & Arnosti, D. N. (2010). Deciphering a transcriptional regulatory code: Modeling short-range repression in the Drosophila embryo. *Molecular Systems Biology*, *6*, 341, [electronic resource].
Falo-Sanjuan, J., Lammers, N. C., Garcia, H. G., & Bray, S. (2019). Enhancer priming enables fast and sustained transcriptional responses to Notch signaling. *Developmental Cell*, *50*, 411–425.
Farrell, D. L., Weitz, O., Magnasco, M. O., & Zallen, J. A. (2017). SEGGA: A toolset for rapid automated analysis of epithelial cell polarity and dynamics. *Development*, *144*, 1725–1734.
Featherstone, K., Hey, K., Momiji, H., McNamara, A. V., Patist, A. L., Woodburn, J., et al. (2016). Spatially coordinated dynamic gene transcription in living pituitary tissue. *eLife*, *5,* e08494.
Ferraro, T., Esposito, E., Mancini, L., Ng, S., Lucas, T., Coppey, M., et al. (2016). Transcriptional memory in the Drosophila embryo. *Current Biology*, *26*, 212–218.
Fuda, N. J., Ardehali, M. B., & Lis, J. T. (2009). Defining mechanisms that regulate RNA polymerase II transcription in vivo. *Nature*, *461*, 186–192.
Fukaya, T., Lim, B., & Levine, M. (2016). Enhancer control of transcriptional bursting. *Cell*, *166*, 358–368.
Fukaya, T., Lim, B., & Levine, M. (2017). Rapid rates of Pol II elongation in the Drosophila embryo. *Current Biology*, *27*, 1387–1391.
Furlong, E. E. M., & Levine, M. (2018). Developmental enhancers and chromosome topology. *Science*, *361*, 1341–1345.
Garcia, H. G., Brewster, R. C., & Phillips, R. (2016). Using synthetic biology to make cells tomorrow's test tubes. *Integrative Biology (Camb)*, *8*, 431–450.
Garcia, H. G., Kondev, J., Orme, N., Theriot, J. A., & Phillips, R. (2007). A first exposure to statistical mechanics for life scientists. *arXiv*. arXiv preprint arXiv: 0708.1899.
Garcia, J. F., & Parker, R. (2015). MS2 coat proteins bound to yeast mRNAs block 5' to 3' degradation and trap mRNA decay products: implications for the localization of mRNAs by MS2-MCP system. *RNA (New York, NY)*, *21*, 1393–1395.
Garcia, H. G., Sanchez, A., Kuhlman, T., Kondev, J., & Phillips, R. (2010). Transcription by the numbers redux: Experiments and calculations that surprise. *Trends in Cell Biology*, *20*, 723–733.

Garcia, H. G., Tikhonov, M., Lin, A., & Gregor, T. (2013). Quantitative imaging of transcription in living Drosophila embryos links polymerase activity to patterning. *Current Biology*, *23*, 2140–2145.
Gilmour, D., Rembold, M., & Leptin, M. (2017). From morphogen to morphogenesis and back. *Nature*, *541*, 311–320.
Golding, I., & Cox, E. C. (2004). RNA dynamics in live Escherichia coli cells. *Proceedings of the National Academy of Sciences of the United States of America*, *101*, 11310–11315.
Golding, I., Paulsson, J., Zawilski, S. M., & Cox, E. C. (2005). Real-time kinetics of gene activity in individual bacteria. *Cell*, *123*, 1025–1036.
Goodwin, S., McPherson, J. D., & McCombie, W. R. (2016). Coming of age: Ten years of next-generation sequencing technologies. *Nature Reviews. Genetics*, *17*, 333–351.
Gregor, T., Tank, D. W., Wieschaus, E. F., & Bialek, W. (2007). Probing the limits to positional information. *Cell*, *130*, 153–164.
Grimm, O., Sanchez Zini, V., Kim, Y., Casanova, J., Shvartsman, S. Y., & Wieschaus, E. (2012). Torso RTK controls Capicua degradation by changing its subcellular localization. *Development*, *139*, 3962–3968.
Gu, B., Swigut, T., Spencley, A., Bauer, M. R., Chung, M., Meyer, T., et al. (2018). Transcription-coupled changes in nuclear mobility of mammalian cis-regulatory elements. *Science*, *359*, 1050–1055.
Guglielmi, G., Barry, J. D., Huber, W., & De Renzis, S. (2015). An optogenetic method to modulate cell contractility during tissue morphogenesis. *Developmental Cell*, *35*, 646–660.
Gursky, V. V., Panok, L., Myasnikova, E. M., Manu, Samsonova, M. G., Reinitz, J., et al. (2011). Mechanisms of gap gene expression canalization in the Drosophila blastoderm. *BMC Systems Biology*, *5*, 118.
Hadzhiev, Y., Qureshi, H. K., Wheatley, L., Cooper, L., Jasiulewicz, A., Van Nguyen, H., et al. (2019). A cell cycle-coordinated polymerase II transcription compartment encompasses gene expression before global genome activation. *Nature Communications*, *10*, 691.
Haimovich, G., Zabezhinsky, D., Haas, B., Slobodin, B., Purushothaman, P., Fan, L., et al. (2016). Use of the MS2 aptamer and coat protein for RNA localization in yeast: A response to "MS2 coat proteins bound to yeast mRNAs block 5' to 3' degradation and trap mRNA decay products: implications for the localization of mRNAs by MS2-MCP system" *RNA (New York, NY)*, *22*, 660–666.
Haines, J. E., & Eisen, M. B. (2018). Patterns of chromatin accessibility along the anterior-posterior axis in the early Drosophila embryo. *PLoS Genetics*, *14*, e1007367.
Halstead, J. M., Lionnet, T., Wilbertz, J. H., Wippich, F., Ephrussi, A., Singer, R. H., et al. (2015). Translation. An RNA biosensor for imaging the first round of translation from single cells to living animals. *Science*, *347*, 1367–1671.
Hammar, P., Wallden, M., Fange, D., Persson, F., Baltekin, O., Ullman, G., et al. (2014). Direct measurement of transcription factor dissociation excludes a simple operator occupancy model for gene regulation. *Nature Genetics*, *46*, 405–408.
Hanes, S. D., Riddihough, G., Ish-Horowicz, D., & Brent, R. (1994). Specific DNA recognition and intersite spacing are critical for action of the bicoid morphogen. *Molecular and Cellular Biology*, *14*, 3364–3375.
Hare, E. E., Peterson, B. K., Iyer, V. N., Meier, R., & Eisen, M. B. (2008). Sepsid even-skipped enhancers are functionally conserved in Drosophila despite lack of sequence conservation. *PLoS Genetics*, *4*, e1000106.
Harrison, M. M., Li, X. Y., Kaplan, T., Botchan, M. R., & Eisen, M. B. (2011). Zelda binding in the early Drosophila melanogaster embryo marks regions subsequently activated at the maternal-to-zygotic transition. *PLoS Genetics*, *7*, e1002266.
He, B., Martin, A., & Wieschaus, E. (2016). Flow-dependent myosin recruitment during Drosophila cellularization requires zygotic dunk activity. *Development*, *143*, 2417–2430.

He, X., Samee, M. A., Blatti, C., & Sinha, S. (2010). Thermodynamics-based models of transcriptional regulation by enhancers: The roles of synergistic activation, cooperative binding and short-range repression. *PLoS Computational Biology*, *6*, 1–15.

Heinrich, S., Sidler, C. L., Azzalin, C. M., & Weis, K. (2017). Stem-loop RNA labeling can affect nuclear and cytoplasmic mRNA processing. *RNA (New York, NY)*, *23*, 134–141.

Hey, K. L., Momiji, H., Featherstone, K., Davis, J. R., White, M. R., Rand, D. A., et al. (2015). A stochastic transcriptional switch model for single cell imaging data. *Biostatistics*, *16*, 655–669.

Hirata, H., Bessho, Y., Kokubu, H., Masamizu, Y., Yamada, S., Lewis, J., et al. (2004). Instability of Hes7 protein is crucial for the somite segmentation clock. *Nature Genetics*, *36*, 750–754.

Irish, V., Lehmann, R., & Akam, M. (1989). The Drosophila posterior-group gene nanos functions by repressing hunchback activity. *Nature*, *338*, 646–648.

Jaeger, J., Blagov, M., Kosman, D., Kozlov, K. N., Manu, Myasnikova, E., et al. (2004). Dynamical analysis of regulatory interactions in the gap gene system of Drosophila melanogaster. *Genetics*, *167*, 1721–1737.

Jaeger, J., Manu, & Reinitz, J. (2012). Drosophila blastoderm patterning. *Current Opinion in Genetics & Development*, *22*, 533–541.

Jaeger, J., & Monk, N. (2014). Bioattractors: Dynamical systems theory and the evolution of regulatory processes. *The Journal of Physiology*, *592*, 2267–2281.

Jaeger, J., Surkova, S., Blagov, M., Janssens, H., Kosman, D., Kozlov, K. N., et al. (2004). Dynamic control of positional information in the early Drosophila embryo. *Nature*, *430*, 368–371.

Jiang, J., & Levine, M. (1993). Binding affinities and cooperative interactions with bHLH activators delimit threshold responses to the dorsal gradient morphogen. *Cell*, *72*, 741–752.

Kale, G. R., Yang, X., Philippe, J. M., Mani, M., Lenne, P. F., & Lecuit, T. (2018). Distinct contributions of tensile and shear stress on E-cadherin levels during morphogenesis. *Nature Communications*, *9*, 5021.

Kanodia, J. S., Liang, H. L., Kim, Y., Lim, B., Zhan, M., Lu, H., et al. (2012). Pattern formation by graded and uniform signals in the early Drosophila embryo. *Biophysical Journal*, *102*, 427–433.

Kellerman, K. A., Mattson, D. M., & Duncan, I. (1990). Mutations affecting the stability of the fushi tarazu protein of Drosophila. *Genes Development*, *4*, 1936–1950.

Kim, S. H., Vieira, M., Kim, H. J., Kesawat, M. S., & Park, H. Y. (2019). MS2 labeling of endogenous Beta-Actin mRNA does not result in stabilization of degradation intermediates. *Molecules and Cells*, *42*, 356–362.

Koboldt, D. C., Steinberg, K. M., Larson, D. E., Wilson, R. K., & Mardis, E. R. (2013). The next-generation sequencing revolution and its impact on genomics. *Cell*, *155*, 27–38.

Kudo, T., Jeknic, S., Macklin, D. N., Akhter, S., Hughey, J. J., Regot, S., et al. (2018). Live-cell measurements of kinase activity in single cells using translocation reporters. *Nature Protocols*, *13*, 155–169.

Lammers, N. C., Galstyan, V., Reimer, A., Medin, S. A., Wiggins, C. H., & Garcia, H. G. (2018). Multimodal transcriptional control of pattern formation in embryonic development. *bioRxiv*, 335919.

Larschan, E., Bishop, E. P., Kharchenko, P. V., Core, L. J., Lis, J. T., Park, P. J., et al. (2011). X chromosome dosage compensation via enhanced transcriptional elongation in Drosophila. *Nature*, *471*, 115–118.

Larson, D. R., Zenklusen, D., Wu, B., Chao, J. A., & Singer, R. H. (2011). Real-time observation of transcription initiation and elongation on an endogenous yeast gene. *Science*, *332*, 475–478.

Lawrence, J. B., Singer, R. H., & Marselle, L. M. (1989). Highly localized tracks of specific transcripts within interphase nuclei visualized by in situ hybridization. *Cell*, *57*, 493–502.
Lebrecht, D., Foehr, M., Smith, E., Lopes, F. J., Vanario-Alonso, C. E., Reinitz, J., et al. (2005). Bicoid cooperative DNA binding is critical for embryonic patterning in Drosophila. *Proceedings of the National Academy of Sciences of the United States of America*, *102*, 13176–13181.
Lee, C., Shin, H., & Kimble, J. (2019). Dynamics of Notch-dependent transcriptional bursting in its native context. *bioRxiv, Developmental Cell*, *50*, 426–435.
Li, G. W., Burkhardt, D., Gross, C., & Weissman, J. S. (2014). Quantifying absolute protein synthesis rates reveals principles underlying allocation of cellular resources. *Cell*, *157*, 624–635.
Li, C., Cesbron, F., Oehler, M., Brunner, M., & Hofer, T. (2018). Frequency modulation of transcriptional bursting enables sensitive and rapid gene regulation. *Cell Systems*, *6,* 409-423 e411.
Li, J., Dong, A., Saydaminova, K., Chang, H., Wang, G., Ochiai, H., et al. (2019). Single-molecule nanoscopy elucidates RNA polymerase II transcription at single genes in live cells. *Cell*, *178*, 491–506.
Lim, B., Fukaya, T., Heist, T., & Levine, M. (2018). Temporal dynamics of pair-rule stripes in living Drosophila embryos. *Proceedings of the National Academy of Sciences of the United States of America*, *115*, 8376–8381.
Lim, B., Heist, T., Levine, M., & Fukaya, T. (2018). Visualization of transvection in living drosophila embryos. *Molecular Cell*, *70,* 287-296 e286.
Lim, B., Levine, M., & Yamazaki, Y. (2017). Transcriptional pre-patterning of Drosophila gastrulation. *Current Biology*, *27*, 286–290.
Lionnet, T., Czaplinski, K., Darzacq, X., Shav-Tal, Y., Wells, A. L., Chao, J. A., et al. (2011). A transgenic mouse for in vivo detection of endogenous labeled mRNA. *Nature Methods*, *8*, 165–170.
Little, S. C., Tikhonov, M., & Gregor, T. (2013). Precise developmental gene expression arises from globally stochastic transcriptional activity. *Cell*, *154*, 789–800.
Little, S. C., Tkacik, G., Kneeland, T. B., Wieschaus, E. F., & Gregor, T. (2011). The formation of the Bicoid morphogen gradient requires protein movement from anteriorly localized mRNA. *PLoS Biology*, *9*, e1000596.
Lopes, F. J., Vieira, F. M., Holloway, D. M., Bisch, P. M., & Spirov, A. V. (2008). Spatial bistability generates hunchback expression sharpness in the Drosophila embryo. *PLoS Computational Biology*, *4*, e1000184.
Lucas, T., Ferraro, T., Roelens, B., De Las Heras Chanes, J., Walczak, A. M., Coppey, M., et al. (2013). Live imaging of bicoid-dependent transcription in Drosophila embryos. *Current Biology*, *23*, 2135–2139.
Ma, X., Yuan, D., Diepold, K., Scarborough, T., & Ma, J. (1996). The Drosophila morphogenetic protein Bicoid binds DNA cooperatively. *Development*, *122*, 1195–1206.
Mammoto, A., Mammoto, T., & Ingber, D. E. (2012). Mechanosensitive mechanisms in transcriptional regulation. *Journal of Cell Science*, *125*, 3061–3073.
Manu, Surkova, S., Spirov, A. V., Gursky, V. V., Janssens, H., Kim, A. R., et al. (2009). Canalization of gene expression and domain shifts in the Drosophila blastoderm by dynamical attractors. *PLoS Computational Biology*, *5*, e1000303.
Margolis, J. S., Borowsky, M. L., Steingrimsson, E., Shim, C. W., Lengyel, J. A., & Posakony, J. W. (1995). Posterior stripe expression of hunchback is driven from two promoters by a common enhancer element. *Development*, *121*, 3067–3077.
Martin, A. C., Kaschube, M., & Wieschaus, E. F. (2009). Pulsed contractions of an actin-myosin network drive apical constriction. *Nature*, *457*, 495–499.
Matera, A. G., & Wang, Z. (2014). A day in the life of the spliceosome. *Nature Reviews. Molecular Cell Biology*, *15*, 108–121.

Mayer, A., Landry, H. M., & Churchman, L. S. (2017). Pause & go: From the discovery of RNA polymerase pausing to its functional implications. *Current Opinion in Cell Biology*, *46*, 72–80.

Mir, M., Reimer, A., Haines, J. E., Li, X. Y., Stadler, M., Garcia, H., et al. (2017). Dense Bicoid hubs accentuate binding along the morphogen gradient. *Genes & Development*, *31*, 1784–1794.

Mir, M., Stadler, M. R., Ortiz, S. A., Hannon, C. E., Harrison, M. M., Darzacq, X., et al. (2018). Dynamic multifactor hubs interact transiently with sites of active transcription in Drosophila embryos. *eLife*, *7*, e40497.

Molina, N., Suter, D. M., Cannavo, R., Zoller, B., Gotic, I., & Naef, F. (2013). Stimulus-induced modulation of transcriptional bursting in a single mammalian gene. *Proceedings of the National Academy of Sciences of the United States of America*, *110*, 20563–20568.

Morisaki, T., Lyon, K., DeLuca, K. F., DeLuca, J. G., English, B. P., Zhang, Z., et al. (2016). Real-time quantification of single RNA translation dynamics in living cells. *Science*, *352*, 1425–1429.

Murata, Y., & Wharton, R. P. (1995). Binding of pumilio to maternal hunchback mRNA is required for posterior patterning in Drosophila embryos. *Cell*, *80*, 747–756.

Nagai, T., Ibata, K., Park, E. S., Kubota, M., Mikoshiba, K., & Miyawaki, A. (2002). A variant of yellow fluorescent protein with fast and efficient maturation for cell-biological applications. *Nature Biotechnology*, *20*, 87–90.

Niessing, D., Blanke, S., & Jackle, H. (2002). Bicoid associates with the 5'-cap-bound complex of caudal mRNA and represses translation. *Genes & Development*, *16*, 2576–2582.

O'Farrell, P. H., Edgar, B. A., Lakich, D., & Lehner, C. F. (1989). Directing cell division during development. *Science*, *246*, 635–640.

Oldach, L., & Zhang, J. (2014). Genetically encoded fluorescent biosensors for live-cell visualization of protein phosphorylation. *Chemistry & Biology*, *21*, 186–197.

Papatsenko, D., & Levine, M. (2011). The Drosophila gap gene network is composed of two parallel toggle switches. *PLoS One*, *6*, e21145.

Pare, A., Lemons, D., Kosman, D., Beaver, W., Freund, Y., & McGinnis, W. (2009). Visualization of individual Scr mRNAs during Drosophila embryogenesis yields evidence for transcriptional bursting. *Current Biology*, *19*, 2037–2042.

Park, P. J. (2009). ChIP-seq: Advantages and challenges of a maturing technology. *Nature Reviews. Genetics*, *10*, 669–680.

Park, J., Estrada, J., Johnson, G., Vincent, B. J., Ricci-Tam, C., Bragdon, M. D., Shulgina, Y., Cha, A., Wunderlich, Z., Gunawardena, J., & DePace, A. H. (2019). Dissecting the sharp response of a canonical developmental enhancer reveals multiple sources of cooperativity. *eLife*, *8*, e41266.

Perry, M. W., Bothma, J. P., Luu, R. D., & Levine, M. (2012). Precision of hunchback expression in the Drosophila embryo. *Current Biology*, *22*, 2247–2252.

Peter, I. S., & Davidson, E. H. (2015). *Genomic control process: Development and evolution*. London, UK; San Diego, CA, USA: Academic Press is an imprint of Elsevier.

Phillips, R. (2015). Theory in biology: Figure 1 or figure 7? *Trends in Cell Biology*.

Phillips, R., Belliveau, N. M., Chure, G., Garcia, H. G., Razo-Mejia, M., & Scholes, C. (2019). Figure 1 theory meets figure 2 experiments in the study of gene expression. *Annual Review of Biophysics*, *48*, 121–163.

Poustelnikova, E., Pisarev, A., Blagov, M., Samsonova, M., & Reinitz, J. (2004). A database for management of gene expression data in situ. *Bioinformatics*, *20*, 2212–2221.

Raj, A., Peskin, C. S., Tranchina, D., Vargas, D. Y., & Tyagi, S. (2006). Stochastic mRNA synthesis in mammalian cells. *PLoS Biology*, *4*, e309.

Regot, S., Hughey, J. J., Bajar, B. T., Carrasco, S., & Covert, M. W. (2014). High-sensitivity measurements of multiple kinase activities in live single cells. *Cell*, *157*, 1724–1734.

Richard, P., & Manley, J. L. (2009). Transcription termination by nuclear RNA polymerases. *Genes & Development*, *23*, 1247–1269.
Rivera-Pomar, R., Niessing, D., Schmidt-Ott, U., Gehring, W. J., & Jackle, H. (1996). RNA binding and translational suppression by bicoid. *Nature*, *379*, 746–749.
Ronchi, E., Treisman, J., Dostatni, N., Struhl, G., & Desplan, C. (1993). Down-regulation of the Drosophila morphogen bicoid by the torso receptor-mediated signal transduction cascade. *Cell*, *74*, 347–355.
Samee, M. A., Lim, B., Samper, N., Lu, H., Rushlow, C. A., Jimenez, G., et al. (2015). A systematic ensemble approach to thermodynamic modeling of gene expression from sequence data. *Cell Systems*, *1*, 396–407.
Sato, Y., Kujirai, T., Arai, R., Asakawa, H., Ohtsuki, C., Horikoshi, N., et al. (2016). A genetically encoded probe for live-cell imaging of H4K20 monomethylation. *Journal of Molecular Biology*, *428*, 3885–3902.
Sato, Y., Mukai, M., Ueda, J., Muraki, M., Stasevich, T. J., Horikoshi, N., et al. (2013). Genetically encoded system to track histone modification in vivo. *Scientific Reports*, *3*, 2436.
Sayal, R., Dresch, J. M., Pushel, I., Taylor, B. R., & Arnosti, D. N. (2016). Quantitative perturbation-based analysis of gene expression predicts enhancer activity in early Drosophila embryo. *eLife*, *5*.
Scholes, C., Biette, K. M., Harden, T. T., & DePace, A. H. (2019). Signal integration by shadow enhancers and enhancer duplications varies across the Drosophila embryo. *Cell Reports*, *26,* 2407-2418 e2405.
Scholes, C., DePace, A. H., & Sanchez, A. (2017). Combinatorial gene regulation through kinetic control of the transcription cycle. *Cell Systems*, *4,* 97-108 e109.
Schroter, C., Herrgen, L., Cardona, A., Brouhard, G. J., Feldman, B., & Oates, A. C. (2008). Dynamics of zebrafish somitogenesis. *Developmental Dynamics: An Official Publication of the American Association of the Anatomists*, *237*, 545–553.
Segal, E., Raveh-Sadka, T., Schroeder, M., Unnerstall, U., & Gaul, U. (2008). Predicting expression patterns from regulatory sequence in Drosophila segmentation. *Nature*, *451*, 535–540.
Sekar, T. V., Foygel, K., Gelovani, J. G., & Paulmurugan, R. (2015). Genetically encoded molecular biosensors to image histone methylation in living animals. *Analytical Chemistry*, *87*, 892–899.
Sherman, M. S., & Cohen, B. A. (2012). Thermodynamic state ensemble models of cis-Regulation. *PLoS Computational Biology*, *8*, e1002407.
Shermoen, A. W., & O'Farrell, P. H. (1991). Progression of the cell cycle through mitosis leads to abortion of nascent transcripts. *Cell*, *67*, 303–310.
Shlyueva, D., Stampfel, G., & Stark, A. (2014). Transcriptional enhancers: From properties to genome-wide predictions. *Nature Reviews. Genetics*, *15*, 272–286.
Simpson-Brose, M., Treisman, J., & Desplan, C. (1994). Synergy between the hunchback and bicoid morphogens is required for anterior patterning in Drosophila. *Cell*, *78*, 855–865.
Singer, R. H., & Ward, D. C. (1982). Actin gene expression visualized in chicken muscle tissue culture by using in situ hybridization with a biotinated nucleotide analog. *Proceedings of the National Academy of Sciences of the United States of America*, *79*, 7331–7335.
Small, S., Blair, A., & Levine, M. (1992). Regulation of *even-skipped* stripe 2 in the *Drosophila* embryo. *The EMBO Journal*, *11*, 4047–4057.
Small, S., Kraut, R., Hoey, T., Warrior, R., & Levine, M. (1991). Transcriptional regulation of a pair-rule stripe in Drosophila. *Genes & Development*, *5*, 827–839.
Stathopoulos, A., & Levine, M. (2005). Localized repressors delineate the neurogenic ectoderm in the early Drosophila embryo. *Developmental Biology*, *280*, 482–493.

Streichan, S. J., Lefebvre, M. F., Noll, N., Wieschaus, E. F., & Shraiman, B. I. (2018). Global morphogenetic flow is accurately predicted by the spatial distribution of myosin motors. *eLife*, *7*, e27454.

Struhl, G. (1989). Differing strategies for organizing anterior and posterior body pattern in Drosophila embryos. *Nature*, *338*, 741–744.

Suter, D. M., Molina, N., Gatfield, D., Schneider, K., Schibler, U., & Naef, F. (2011). Mammalian genes are transcribed with widely different bursting kinetics. *Science*, *332*, 472–474.

Swanson, C. I., Evans, N. C., & Barolo, S. (2010). Structural rules and complex regulatory circuitry constrain expression of a Notch- and EGFR-regulated eye enhancer. *Developmental Cell*, *18*, 359–370.

Szymanski, P., & Levine, M. (1995). Multiple modes of dorsal-bHLH transcriptional synergy in the Drosophila embryo. *The EMBO Journal*, *14*, 2229–2238.

Tautz, D., & Pfeifle, C. (1989). A non-radioactive in situ hybridization method for the localization of specific RNAs in Drosophila embryos reveals translational control of the segmentation gene hunchback. *Chromosoma*, *98*, 81–85.

Totaro, A., Panciera, T., & Piccolo, S. (2018). YAP/TAZ upstream signals and downstream responses. *Nature Cell Biology*, *20*, 888–899.

Tran, H., Desponds, J., Perez Romero, C. A., Coppey, M., Fradin, C., Dostatni, N., et al. (2018). Precision in a rush: Trade-offs between reproducibility and steepness of the hunchback expression pattern. *PLoS Computational Biology*, *14*, e1006513.

Tsai, A., Muthusamy, A. K., Alves, M. R., Lavis, L. D., Singer, R. H., Stern, D. L., et al. (2017). Nuclear microenvironments modulate transcription from low-affinity enhancers. *eLife*, *6*.

Tutucci, E., Vera, M., Biswas, J., Garcia, J., Parker, R., & Singer, R. H. (2018). An improved MS2 system for accurate reporting of the mRNA life cycle. *Nature Methods*, *15*, 81–89.

Ubersax, J. A., & Ferrell, J. E., Jr. (2007). Mechanisms of specificity in protein phosphorylation. *Nature Reviews. Molecular Cell Biology*, *8*, 530–541.

Verd, B., Clark, E., Wotton, K. R., Janssens, H., Jimenez-Guri, E., Crombach, A., et al. (2018). A damped oscillator imposes temporal order on posterior gap gene expression in Drosophila. *PLoS Biology*, *16*, e2003174.

Verd, B., Crombach, A., & Jaeger, J. (2017). Dynamic maternal gradients control timing and shift-rates for Drosophila gap gene expression. *PLoS Computational Biology*, *13*, e1005285.

Verd, B., Monk, N. A., & Jaeger, J. (2019). Modularity, criticality, and evolvability of a developmental gene regulatory network. *eLife*, *8*.

Vilar, J. M., & Leibler, S. (2003). DNA looping and physical constraints on transcription regulation. *Journal of Molecular Biology*, *331*, 981–989.

von Dassow, G., Meir, E., Munro, E. M., & Odell, G. M. (2000). The segment polarity network is a robust developmental module. *Nature*, *406*, 188–192.

Von Dassow, G., & Odell, G. M. (2002). Design and constraints of the Drosophila segment polarity module: Robust spatial patterning emerges from intertwined cell state switches. *The Journal of Experimental Zoology*, *294*, 179–215.

von Hippel, P. H., Revzin, A., Gross, C. A., & Wang, A. C. (1974). Non-specific DNA binding of genome regulating proteins as a biological control mechanism: I. The lac operon: Equilibrium aspects. *Proceedings of the National Academy of Sciences of the United States of America*, *71*, 4808–4812.

Wang, C., Han, B., Zhou, R., & Zhuang, X. (2016). Real-time imaging of translation on single mRNA transcripts in live cells. *Cell*, *165*, 990–1001.

Wang, C., & Lehmann, R. (1991). Nanos is the localized posterior determinant in Drosophila. *Cell*, *66*, 637–647.

Wharton, R. P., & Struhl, G. (1991). RNA regulatory elements mediate control of Drosophila body pattern by the posterior morphogen nanos. *Cell*, *67*, 955–967.

Wu, B., Eliscovich, C., Yoon, Y. J., & Singer, R. H. (2016). Translation dynamics of single mRNAs in live cells and neurons. *Science*, *352*, 1430–1435.
Xu, H., Sepulveda, L. A., Figard, L., Sokac, A. M., & Golding, I. (2015). Combining protein and mRNA quantification to decipher transcriptional regulation. *Nature Methods*, *12*, 739–742.
Xu, H., Skinner, S. O., Sokac, A. M., & Golding, I. (2016). Stochastic kinetics of Nascent RNA. *Physical Review Letters*, *117*, 128101.
Yamada, S., Whitney, P. H., Huang, S. K., Eck, E. C., Garcia, H. G., & Rushlow, C. A. (2019). The Drosophila pioneer factor Zelda modulates the nuclear microenvironment of a dorsal target enhancer to potentiate transcriptional output. *Current Biology*, *29,* 1387-1393 e1385.
Yan, X., Hoek, T. A., Vale, R. D., & Tanenbaum, M. E. (2016). Dynamics of translation of single mRNA molecules in vivo. *Cell*, *165*, 976–989.
Yunger, S., Rosenfeld, L., Garini, Y., & Shav-Tal, Y. (2010). Single-allele analysis of transcription kinetics in living mammalian cells. *Nature Methods*, 7, 631–633.
Zechner, C., Unger, M., Pelet, S., Peter, M., & Koeppl, H. (2014). Scalable inference of heterogeneous reaction kinetics from pooled single-cell recordings. *Nature Methods*, *11*, 197–202.
Zenklusen, D., Larson, D. R., & Singer, R. H. (2008). Single-RNA counting reveals alternative modes of gene expression in yeast. *Nature Structural & Molecular Biology*, *15*, 1263–1271.
Zoller, B., Little, S. C., & Gregor, T. (2018). Diverse spatial expression patterns emerge from unified kinetics of transcriptional bursting. *Cell*, *175,* 835-847 e825.
Zoller, B., Nicolas, D., Molina, N., & Naef, F. (2015). Structure of silent transcription intervals and noise characteristics of mammalian genes. *Molecular Systems Biology [Electronic Resource]*, *11*, 823.