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Stepwise progression of embryonic patterning

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Abstract

It is long established that the graded distribution of Dorsal transcription factor influences spatial domains of gene expression along the dorsoventral axis of *Drosophila melanogaster* embryos. However, the more recent realization that Dorsal levels also change in time raises the question of whether these dynamics are instructive. Here, an overview of dorsoventral axis patterning is provided focusing on new insights identified through quantitative analysis of temporal changes in Dorsal target gene expression from one nuclear cycle to the next (‘steps’). Possible roles for the step-wise progression of this patterning program are discussed including (i) tight, temporal regulation of signaling pathway activation, (ii) control of gene expression cohorts, and (iii) to ensure irreversibility of the patterning and cell fate specification process.

Keywords

Dorsal transcription factor; *Drosophila* embryo; dorsal-ventral patterning; dynamics; morphogen gradients; spatiotemporal gene expression

Transcription factor dynamics regulate target gene expression

Subdividing the embryo into distinct domains of gene expression by combinatorial control of transcription factors is an important function of regulatory networks acting in early embryos including those of *Drosophila* [1–5]. These early patterning events influence the activation of signaling pathways to support tissue differentiation and also control cell movements required for the generation of a multilayered embryo; the developmental actions that encompass gastrulation [6, 7]. To study these events at the transcriptional level in *Drosophila* embryos, previous studies of early zygotic gene expression have considered one or two time-points spanning the first four hours of early embryo development [8–11], and yet recent studies suggest gene expression patterns change on the order of minutes rather than hours [e.g. 12, 13, 14]. Furthermore, only recently has it come to light that transcription factors in the early embryo exhibit changes in levels over time [15–18]. At least in part these dynamics relate to the fast nuclear divisions that encompass *Drosophila* early embryonic

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development and result in oscillatory inputs to target genes. Transcription factor dynamics appear to be a general mechanism of regulating gene expression [19, 20] and highlight the need to study temporal regulation of developmental gene expression as a complement to previous studies of embryonic patterning in *Drosophila*, which have focused on spatial control of gene expression [21–23].

The Dorsal transcription factor is dynamic as are its target genes

In the *Drosophila* embryo, the pivotal transcription factor, Dorsal, is present in a nuclear-cytoplasmic gradient along the dorsoventral (DV) axis that instructs differential gene expression, yet the establishment of this morphogen gradient is atypical [24–26]. *dl* transcripts are maternally deposited and uniformly distributed [27, 28]. The protein, however, is present in a nuclear gradient through differential activation of the upstream receptor, Toll [29]. Thereby, this gradient does not result from localized expression of Dorsal protein but, instead, involves a nuclear-cytoplasmic shift in levels of this factor along the DV axis as regulated by Toll receptor signaling [30–32]. Dorsal acts as activator of transcription to support the expression of target genes in ventral and lateral regions of the embryo as well as repressor of transcription to limit the expression of a subset of target genes to dorsal regions [33–35]. In this manner, more than fifty genes are differentially expressed along the DV axis [21, 36]. High levels of nuclear-localized Dorsal in ventral regions specify the mesoderm, whereas lower levels of nuclear Dorsal in lateral regions specify the neurogenic ectoderm [37, 38]. The prevailing model in the field had been that the changes in levels of Dorsal in space, along the DV axis, is important for establishing different domains of gene expression.

However, more recent studies have identified that Dorsal levels also change in time [17, 39], raising the question of whether and how temporal changes of this factor impact gene expression. How the nuclear distribution of Dorsal gives rise to precise gene expression patterns was recently investigated using live *in vivo* imaging and quantitative analysis. It was revealed that the Dorsal transcription factor gradient is highly dynamic, increasing in levels over time, and not achieving steady state until Dorsal levels plummet at gastrulation [13]. Up to this point during the first three hours of development, levels of this factor build within nuclei, from one nuclear cycle to the next such that by cellularization a ~3-fold increase is realized compared to previous nuclear cycles. In addition, Dorsal levels oscillate with each and every nuclear cycle, dropping rapidly as nuclei divide and Dorsal escapes into the cytoplasm. Following nuclear division, import of Dorsal back into the nucleus is relatively slow leading to a gradual increase. This relatively slow import of Dorsal into the nucleus compared with other transcription factors acting at this time such as Bicoid, for example, likely relates to the requirement of Toll-mediated signaling to mediate entry of Dorsal to the nuclei and explains why levels of Dorsal increase as the length of nuclear cycles increases (Box 1) [40]. In contrast, the nuclear distribution of the Bicoid transcription factor stabilizes relatively quickly within every nuclear cycle and, moreover, stays relatively constant from one nuclear cycle to the next [16]. The observation that the Dorsal morphogen gradient changes in time, within as well as between nuclear cycles, suggests time impacts gene regulatory network activation.

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The levels of Dorsal transcription factor almost double from one nuclear cycle to the next, approximately every 10 minutes [13]. How might a factor act as morphogen, to control spatial patterning, if its levels constantly change? One possibility is that these transcription factor dynamics also induce unappreciated gene expression dynamics. By close analysis of the expression associated with four Dorsal target genes within precisely-staged, fixed embryos, two distinct temporal trends were found associated with targets [13]. Expression of the gene *short-gastrulation* (*sog*) [41] was found to be 'plastic' (dynamic), with levels changing constantly both upwards and downwards in time. For *sog*, it appears possible to turn gene expression on/off in time, presumably, in response to changing levels of Dorsal above/below an activation threshold when nuclear concentration oscillates between syncytial divisions. In contrast, other genes expressed along the DV axis, also Dorsal targets, such as *snail* (*sna*) [42, 43] exhibit more of a 'ratchet' (monotonic) effect in that levels continue to steadily increase and expression domains never refine to narrower patterns once established despite changes in Dorsal. This "ratchet effect" is similar to the target response of another morphogen, Activin, important for patterning in *Xenopus* [44]. Thus, this preliminary analysis of four genes expressed along the DV axis in the *Drosophila* embryo identified two different temporal responses: dynamic (e.g. *sog*) versus monotonic (e.g. *sna*) [13]. However, as only a small number of targets were examined, it was not possible to distinguish whether these temporal changes were gene-specific responses or general network-wide trends. Furthermore, these dynamics may relate to differences in mRNA stability of transcripts or other post-transcriptional effects that have been little studied in the early embryo in relation to zygotic transcripts.

A temporally fine-scale, quantitative assay of gene expression provides insights into stepwise activation of *Drosophila* embryogenesis

An assay of gene expression dynamics was performed recently using NanoString nCounter technology (Box 2) to measure the levels of expression for ~70 genes in the early *Drosophila* embryo, focusing on those expressed along the DV axis and providing further insight into the dynamics of genes expressed in the early embryo [45, 46]. Ten time points spanning nuclear cycles (NC) 10 through 14 and also including gastrulation were investigated through assay of gene expression within individual, carefully-staged *Drosophila* embryos (Figure 1, Key Figure). Nuclear cycle 14 was divided into four time points, 14A–14D, providing data from before (14A), during (14B and 14C), and after (14D) cellularization. In this analysis, the data suggested that tight temporal regulation of gene expression is key in the activation of the zygotic gene regulatory network and important for a properly developing embryo.

In particular, it was found that not all time points during early embryonic development are equal in terms of changing gene expression. While maternal genes are constantly being degraded and zygotic genes are constantly being expressed during the blastoderm stage, the average fold-change in expression between various time points can differ greatly. Both the greatest increase in transcription and decrease in abundance occur during the first part of NC 14 (i.e. the transition from NC 14A to 14B). In fact, the rapid increase in transcription seen at this stage is over four times higher than the increase later in NC 14 (i.e. between NC 14C and 14D) less than 30 minutes later. This drastic difference may relate to Dorsal

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transcription factor dynamics. Prior to NC 14, nuclei divide too rapidly to allow Dorsal to build to high levels. Also, some active transcription may be aborted at every division due to the limited time available [47–49]. This transition at the beginning of NC 14 is the first time in development that both Dorsal nuclear import and transcription can proceed uninterrupted for over 15 minutes. There are also more zygotic transcription factors present at the start of NC 14 as the result of their transcription and translation into functioning proteins during the previous nuclear cycles. These factors combine to make the short time period of around 15 minutes the most transcriptionally active during the blastoderm stage. By mid NC 14 (i.e. NC 14C), many genes have reached a steady state of abundance, and while there are more transcripts present than 30 minutes before during the period of rapid transcription, the overall change is the lowest of any time point studied. This steady state and period of relatively little change occurs just after Dorsal reaches its own maximum concentration in nuclei and ceases increasing. It is not coincidental, therefore, that the expression rate of genes that rely so closely on Dorsal match the nuclear concentration dynamics of Dorsal itself.

Another benefit of the fine time scale quantitative profile provided by NanoString experiments is the ability to observe and dissect sub-circuits within the overall developmental Gene Regulatory Network (GRN). One of the most common sub-circuits found in GRN topologies is the feed forward loop, where an initial activator works cooperatively with one of its own targets to further activate more genes [50, 51]. A key property of feed forward loops is that the activating effects of individual components are additive or synergistic, and that each input alone is unable to activate target genes at full strength [52]. An example of a feed forward loop in the *Drosophila* developmental GRN is found in the mesoderm, where Dorsal first activates Twist, and then Dorsal and Twist together activate many other mesoderm genes (Figure 2) [2, 43]. Since Twist has been shown to also activate mesoderm genes in the *Drosophila* embryo, it is a prime candidate for investigation and use in dissecting such network circuitry [9, 42, 53, 54].

The additive nature of feed forward loops can be observed by comparing the dynamics of Dorsal-Twist cooperative activation in wild type embryos to the activating ability of Dorsal alone in *twi*- flies. Using NanoString, it can be observed that during the blastoderm stage between NCs 10 and 13, the expression of mesoderm genes slowly and steadily increases at every nuclear cycle, but then undergoes a very rapid increase starting at NC 14 until a steady state in transcript levels is reached. This bimodal profile may relate to temporal increases in Dorsal levels and/or to the additive effect of a second factor joining a feed forward loop (Figure 2). When *twi* is mutated so it can no longer bind to DNA and mutant embryos are assayed by NanoString, the rapid increase in transcription usually observed in NC 14 does not occur, and the slower rate of transcription observed in NCs 10–13 is maintained [45]. This difference in transcription rate demonstrates the additive nature of feed forward loops; at NC 14, Dorsal alone is able to activate its targets at a moderate level, but the input of Twist is able to provide an additional boost transcription that is added to the input of Dorsal to support high level expression. While a role for Twist in supporting expression of genes in the early embryo has been appreciated, using the NanoString to quantify levels of expression in individual, staged embryos illuminated the temporal role for Twist in supporting expression of genes, specifically, at NC 14 [53].

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Possible roles for step-wise progression of embryonic gene expression programs

Moving forward an important goal in the field is to understand the role of dynamics of gene expression in supporting proper embryonic development [12, 14, 39, 55, 56]. The recent quantitative analysis of gene expression in *Drosophila* embryos has highlighted activation of genes expressed along the DV axis occurs in a step-wise manner [13, 45]. We contend this step-wise activation program is instrumental for DV patterning and suggest three ideas regarding its roles, below.

Activation of signaling pathways

Cell-cell signaling is not thought to broadly impact DV patterning until cellularization at the 14th nuclear cycle, when cells form, as before this point the embryo develops as a syncytium in which nuclei are not separated from each other by cell membranes [2, 57]. It is, presumably, for this reason that genes requiring input from Notch or EGFR signaling such as *single-minded* (*sim*) and *intermediate neuroblasts defective* (*ind*), respectively, exhibit delayed expression that coincides with cellularization [58, 59]. However, recent studies have found that nuclei become compartmentalized before cellularization is complete [60] suggesting that cell-cell signaling may be possible earlier.

The progressive activation of the DV patterning GRN in the early *Drosophila* embryo may promote activation of signaling pathways in a step-wise manner. It is appreciated that subdivision of the embryo into distinct domains of expression, through patterning, is necessary to set-up activation of signaling pathways through differential expression of receptors and ligands. However, findings that signaling pathway components are expressed before NC14, some as early as NC10, suggests that activation of signaling may occur as a step-wise progression influenced by the gene network program to impact activation and/or levels of signaling. Studies in other systems have provided evidence that “fold-change” may trigger signaling activation rather than a particular threshold level of ligand; arguing that step-wise activation of signaling may be important [61, 62]. Furthermore, in such a system, the temporal presentation of ligands may be more influential than absolute levels in supporting signaling pathway activation, supporting the recent view that concentration-dependence is not pivotal to the action of morphogens [1, 23].

Control of gene expression cohorts

Another finding from the NanoString study is that, while early embryogenesis is a dynamic time in general, there are stages of rapid coordinated changes in gene expression. For example, a gene cohort of Dorsal targets expressed in the mesoderm exhibit a gradual increase in abundance between NC 10 and 13, but then all exhibit a rapid and coordinated increase in transcription rate as NC14 begins (Figure 3). This coordinated increase occurs at the same time for all six mesoderm genes included in the NanoString study (*twi, mes3, sna, hbr, NetA*, and *htl*), and coincides with the time of high dynamic change between NCs 14A and 14B. In contrast, target genes of Bicoid expressed along the anterior-posterior (AP) axis, such as *hb* and *otd*, show no signs of a coordinated increase in expression between NCs 14A and 14B, or any other time point [45]. The AP targets of Bicoid increase gradually during

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the time course without a rapid change in expression strength. This is likely due to the relatively stable levels of Bicoid found along the AP axis during early embryogenesis.

A second group of six genes expressed in the dorsal ectoderm as targets of the TGF-β pathway [63] behaved in a somewhat different way compared to the mesoderm genes (Figure 3). Like the group of mesoderm genes, the transcription of all six TGF-β target genes is also coordinated temporally. Unlike the mesoderm genes that all behave similarly, two classes of TGF-β target genes were uncovered based on different kinetics of expression at the onset of NC14. One set exhibited slow and steady transcription whereas the other exhibited rapid expression. Despite these differences between mesoderm genes and TGF-β targets, the temporal coregulation of different groups of genes reinforces the idea that coordinated and precise timing of transcription is a key feature of the early GRN and has been observed in other systems [64]. Furthermore, identification of additional gene expression cohorts such as these will facilitate approaches aimed to identify shared regulatory motifs in enhancers and promoters that support shared dynamics.

An additional difference is uncovered between the mesoderm and TGF-β target genes when the number of transcripts per cell is calculated instead of overall number of transcripts per embryo. When the overall number of transcripts for each group is divided by the number of cells expressing each gene, the mesoderm genes are maintained in a rank-order of abundance through the entire time course, while TGF-β target genes are expressed in a very similar number of transcripts per cell. A possible explanation for the persistent differences in expression per cell for mesoderm genes compared to the similar levels of expression for TGF-β targets is their position in the GRN. The mesoderm genes are some of the first zygotic genes to be activated in the network, while the TGF-β target genes are at the output level of a signaling pathway at the end of the pre-gastrulation network. It may be important to maintain different levels of gene expression early in developmental pathways in order to activate or repress targets in varying ways, while genes at the output level of signaling pathways are programmed to be expressed in similar levels to each other as the signaling pathway integrates changing inputs into a stable output.

Irreversibility of the embryonic patterning process

Another factor contributing to the irreversible nature of the step-wise activation of the GRN is syncytial nuclear division leading to increasingly stronger pulses of nuclear Dorsal. Beginning at NC 10, when nuclei migrate to the periphery of the embryo, nuclei in the ventral portions of the embryo are exposed to the highest concentrations of Dorsal and begin transcribing early mesoderm-determining transcription factors such as *twist* (*twi*) and *sna* [42, 65, 66]. Although the first few nuclear cycles during the syncytial blastoderm stage are brief, around 10 minutes each, the short length of many early transcription factors allows them to be fully transcribed before nuclear division aborts active transcription. This brief pulse of transcription supplies mature transcripts to allow for the translation of full-length and functional proteins, able to either activate additional mesoderm genes in the case of *twi* or repress the expression of neurogenic ectoderm genes in the case of *sna*. The first active transcription factors set into motion cascades of activation and repression, with each

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subsequent nuclear cycle being accompanied by higher concentrations of Dorsal leading to the presence of even more early transcription factor gene products.

Each nuclear cycle can be thought of as a developmental step, leading nuclei or cells down a one-way trajectory towards their ultimate fate. GRN activation is a natural consequence of early Dorsal-mediated expression of the first transcription factors. The rapid nature of syncytial nuclear divisions combined with ever-increasing concentrations of Dorsal ensures that regulatory states established early in development are robustly transmitted and engrained in nuclei during subsequent nuclear divisions. By the time cellularization occurs in the middle of NC 14, the previous rounds of nuclear division have set up a situation where the cells have no choice but to follow the path laid out for them, and the rapid onset of intracellular signaling pathways only serves to further cement these fates. On the other hand, Bicoid levels do not change as dramatically. It is possible that Bicoid is required only early in the AP patterning GRN, to set a chain of events in motion that relies more heavily on duration of Bicoid signal than absolute concentration in nuclei. Dorsal may remain continuously necessary as its concentration increases: supporting early patterning as well as late patterning events, up to when its levels plummet at gastrulation. An extreme extrapolation of these ideas is that Bicoid is permissive whereas Dorsal is instructive.

Key challenges in studying spatiotemporal regulation of gene expression programs

As discussed above, dynamic gene expression likely relates to proper timing of signaling pathway activation and also the step-wise progression of gene expression programs helps support irreversibility of the process. However, we have only scratched the surface in understanding how dynamic gene expression within this gene network is controlled at a mechanistic level. Below we comment on three areas of future research that will provide insight and better understanding.

Roles of additional transcription factors, both ubiquitous and spatially localized, in controlling temporal gene expression programs

It is clear that the ubiquitous zinc-finger transcription factor Zelda is very important for supporting early zygotic expression in the early *Drosophila* embryo [67, 68]. However, other factors also contribute to timing of gene expression. STAT92E, another ubiquitous factor, broadly influences early zygotic transcription, as does the Grainy head transcriptional activator, exemplified by its support of *ind* expression [69–71]. It is likely that a number of ubiquitous activators including Zelda, STAT92E, and Grainy head impact patterning and that these factors may exhibit different timing of action. Also, as discussed above for Dorsal, transcription factors known primarily for their roles in supporting spatial patterning may also regulate timing of gene expression. Lastly, globally-acting repressors likely function to counterbalance this activation, to regulate spatial [72] as well as temporal expression. Understanding how these factors, ubiquitous or spatially-localized, collectively influence timing of gene expression programs is an important area of future research. Additionally, synthetic reporter constructs combining transcription factor binding sites [e.g. Dorsal, Zelda, and the early transcriptional repressor Suppressor of Hairless, Su(H)] have begun to examine

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the relationship between number and organization of binding sites, ‘cis-regulatory logic’ or ‘grammar’, to spatial regulation of expression [72–74]. Another promising future direction is to study how combinatorial control and organization of sites relates to timing and levels of gene expression [75–77].

Coordinate action of cis-regulatory modules and role in supporting gene expression dynamics

Transcription factors, for the most part, act on cis-regulatory modules (CRMs), and to understand how timing of gene expression is regulated a better understanding of how CRMs cooperate to support gene expression must be acquired. Recent studies have found that multiple cis-regulatory modules are often associated with genes and are co-acting [78–81]. Some CRMs work concurrently to control spatial domains and levels of expression [82, 83], whereas others work sequentially to control the changing expression of genes in time [84]. These insights lead directly to the question of how multiple CRMs coordinate in space and time. Recent studies have identified autoregulatory feedback as the mechanism regulating the switch from an early-acting CRM to a later-acting CRM at the *brinker* gene locus, which regulates the spatiotemporal expression of this gene [84]. Therefore, understanding of both (i) spatiotemporal inputs (i.e. transcription factor dynamics) as well as (ii) CRMs acting and their coordinate action is required to understand how temporal gene expression is controlled.

Role of post-transcriptional regulation in temporal gene expression and gene functions

In an analysis of spatiotemporal profiles for the genes *sog* and *sna*, evidence was obtained that *sog* transcripts are degraded at the transition from NC 13 to NC 14 whereas *sna* transcripts are retained [13]. A simple explanation is that the short timeframe of NC 13, under 15 minutes, is not long enough to support transcription of long genes and therefore nascent transcripts that do not reach maturity are degraded [48, 49]. However, an alternate possibility (not mutually exclusive) is that post-transcriptional mechanisms influence the abundance and stability of zygotic transcripts present in the embryo [85–87]. An exciting future direction would be to uncover how post-transcriptional regulation factors into the timing of developmental progression and, specifically, to uncover how it influences DV patterning and signaling pathway activation.

Concluding remarks

In summary, recent studies highlight the need to consider the dorsal-ventral gene regulatory network as a step-wise process in which the status of the system (i.e. gene expression) is assayed with fine temporal resolution. Use of the NanoString technology has supported generation of a time-series from carefully staged, individual *Drosophila* embryo fixed samples [45]. From these data, dynamic trends within gene regulatory networks can be inferred such as identification of gene expression cohorts and specific, temporal roles for transcription factors [64]. Furthermore, imaging transcripts directly and dynamically, in living embryos over time, is a complementary approach that is also able to assay dynamics of nascent transcripts associated with a single gene [88–90]. Identifying technologies that make it possible to assay expression levels for tens of genes *in vivo* live would be an exciting future frontier [91]. The ultimate goal is to attain a complete understanding of the control of

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spatiotemporal gene expression, how it results from the action of transcription factors on one or more cis-regulatory modules. Model organisms are an excellent choice for such system level analyses aimed at deciphering regulatory logic that can help us better understand GRNs acting in humans. As more GRN studies emerge, it is becoming clear that a common set of subcircuit designs is used [92, 93]. Additional trends, or even differences, may emerge from more comparative studies.

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References

1. Briscoe J, Small S. Morphogen rules: design principles of gradient-mediated embryo patterning. Development. 2015; 142:3996–4009. [PubMed: 26628090]
2. Stathopoulos A, Levine M. Genomic regulatory networks and animal development. Dev Cell. 2005; 9:449–462. [PubMed: 16198288]
3. Davidson EH, Levine MS. Properties of developmental gene regulatory networks. Proc Natl Acad Sci U S A. 2008; 105:20063–20066. [PubMed: 19104053]
4. Zinzen RP, et al. Combinatorial binding predicts spatio-temporal cis-regulatory activity. Nature. 2009; 462:65–70. [PubMed: 19890324]
5. Carmena A, et al. Combinatorial signaling codes for the progressive determination of cell fates in the Drosophila embryonic mesoderm. Gene Dev. 1998; 12:3910–3922. [PubMed: 9869644]
6. Stathopoulos A, Levine M. Whole-genome analysis of Drosophila gastrulation. Curr Opin Genet Dev. 2004; 14:477–484. [PubMed: 15380237]
7. Lee JY, et al. Wnt/Frizzled signaling controls C-elegans gastrulation by activating actomyosin contractility. Current Biology. 2006; 16:1986–1997. [PubMed: 17055977]
8. Zeitlinger J, et al. Whole-genome ChIP-chip analysis of Dorsal, Twist, and Snail suggests integration of diverse patterning processes in the Drosophila embryo. Genes Dev. 2007; 21:385–390. [PubMed: 17322397]
9. Ozdemir A, et al. High resolution mapping of Twist to DNA in Drosophila embryos: Efficient functional analysis and evolutionary conservation. Genome research. 2011; 21:566–577. [PubMed: 21383317]
10. MacArthur S, et al. Developmental roles of 21 Drosophila transcription factors are determined by quantitative differences in binding to an overlapping set of thousands of genomic regions. Genome Biol. 2009; 10:R80. [PubMed: 19627575]
11. Graveley BR, et al. The developmental transcriptome of Drosophila melanogaster. Nature. 2011; 471:473–479. [PubMed: 21179090]
12. Lott SE, et al. Noncanonical compensation of zygotic X transcription in early Drosophila melanogaster development revealed through single-embryo RNA-seq. PLoS Biol. 2011; 9:e1000590. [PubMed: 21346796]
13. Reeves GT, et al. Dorsal-ventral gene expression in the Drosophila embryo reflects the dynamics and precision of the dorsal nuclear gradient. Dev Cell. 2012; 22:544–557. [PubMed: 22342544]
14. Ali-Murthy Z, et al. An essential role for zygotic expression in the pre-cellular Drosophila embryo. PLoS Genet. 2013; 9:e1003428. [PubMed: 23593026]
15. Shvartsman SY, et al. Dynamics of maternal morphogen gradients in Drosophila. Curr Opin Genet Dev. 2008; 18:342–347. [PubMed: 18602472]
16. Gregor T, et al. Stability and nuclear dynamics of the bicoid morphogen gradient. Cell. 2007; 130:141–152. [PubMed: 17632061]

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17. Liberman LM, et al. Quantitative imaging of the Dorsal nuclear gradient reveals limitations to threshold-dependent patterning in Drosophila. Proc Natl Acad Sci U S A. 2009; 106:22317–22322. [PubMed: 20018754]
18. Kanodia JS, et al. Dynamics of the Dorsal morphogen gradient. Proc Natl Acad Sci U S A. 2009; 106:21707–21712. [PubMed: 19996178]
19. Purvis JE, Lahav G. Encoding and decoding cellular information through signaling dynamics. Cell. 2013; 152:945–956. [PubMed: 23452846]
20. Levine JH, et al. Functional roles of pulsing in genetic circuits. Science. 2013; 342:1193–1200. [PubMed: 24311681]
21. Stathopoulos A, et al. Whole-genome analysis of dorsal-ventral patterning in the Drosophila embryo. Cell. 2002; 111:687–701. [PubMed: 12464180]
22. Chen H, et al. A system of repressor gradients spatially organizes the boundaries of Bicoid-dependent target genes. Cell. 2012; 149:618–629. [PubMed: 22541432]
23. Wolpert L. Positional Information and Pattern Formation. Curr Top Dev Biol. 2016; 117:597–608. [PubMed: 26970003]
24. Moussian B, Roth S. Dorsoventral axis formation in the Drosophila embryo–shaping and transducing a morphogen gradient. Curr Biol. 2005; 15:R887–899. [PubMed: 16271864]
25. Stein DS, Stevens LM. Maternal control of the Drosophila dorsal-ventral body axis. Wiley Interdiscip Rev Dev Biol. 2014; 3:301–330. [PubMed: 25124754]
26. Rogers KW, Schier AF. Morphogen gradients: from generation to interpretation. Annual review of cell and developmental biology. 2011; 27:377–407.
27. Anderson KV, Nusslein-Volhard C. Information for the dorsal–ventral pattern of the Drosophila embryo is stored as maternal mRNA. Nature. 1984; 311:223–227. [PubMed: 6434989]
28. Simpson P. Maternal-zygotic gene interactions during formation of the dorsoventral pattern in Drosophila embryos. Genetics. 1983; 105:615–632. [PubMed: 17246169]
29. Anderson KV, et al. Establishment of dorsal-ventral polarity in the Drosophila embryo: the induction of polarity by the Toll gene product. Cell. 1985; 42:791–798. [PubMed: 3931919]
30. Roth S, et al. A Gradient of Nuclear-Localization of the Dorsal Protein Determines Dorsoventral Pattern in the Drosophila Embryo. Cell. 1989; 59:1189–1202. [PubMed: 2688897]
31. Steward R. Relocalization of the Dorsal Protein from the Cytoplasm to the Nucleus Correlates with Its Function. Cell. 1989; 59:1179–1188. [PubMed: 2598266]
32. Rushlow CA, et al. The graded distribution of the dorsal morphogen is initiated by selective nuclear transport in Drosophila. Cell. 1989; 59:1165–1177. [PubMed: 2598265]
33. Stathopoulos A, Levine M. Dorsal gradient networks in the Drosophila embryo. Dev Biol. 2002; 246:57–67. [PubMed: 12027434]
34. Ray RP, et al. The control of cell fate along the dorsal-ventral axis of the Drosophila embryo. Development. 1991; 113:35–54. [PubMed: 1765005]
35. Jiang J, et al. Individual dorsal morphogen binding sites mediate activation and repression in the Drosophila embryo. EMBO J. 1992; 11:3147–3154. [PubMed: 1322296]
36. Biemar F, et al. Comprehensive identification of Drosophila dorsal-ventral patterning genes using a whole-genome tiling array. Proc Natl Acad Sci U S A. 2006; 103:12763–12768. [PubMed: 16908844]
37. Chopra VS, Levine M. Combinatorial patterning mechanisms in the Drosophila embryo. Brief Funct Genomic Proteomic. 2009; 8:243–249. [PubMed: 19651703]
38. Reeves, GT.; Stathopoulos, A. Graded Dorsal and Differential Gene Regulation in the *Drosophila* Embryo. In: Briscoe, J., et al., editors. Perspectives on Generation and Interpretation of Morphogen Gradients. Cold Spring Harbor Laboratory Press; 2009.
39. Rushlow CA, Shvartsman SY. Temporal dynamics, spatial range, and transcriptional interpretation of the Dorsal morphogen gradient. Curr Opin Genet Dev. 2012; 22:542–546. [PubMed: 22981910]
40. Belvin MP, Anderson KV. A conserved signaling pathway: the Drosophila toll-dorsal pathway. Annual review of cell and developmental biology. 1996; 12:393–416.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

41. Francois V, et al. Dorsal-ventral patterning of the Drosophila embryo depends on a putative negative growth factor encoded by the short gastrulation gene. Genes Dev. 1994; 8:2602–2616. [PubMed: 7958919]
42. Kosman D, et al. Establishment of the mesoderm-neuroectoderm boundary in the Drosophila embryo. Science. 1991; 254:118–122. [PubMed: 1925551]
43. Ip YT, et al. dorsal-twist interactions establish snail expression in the presumptive mesoderm of the Drosophila embryo. Genes Dev. 1992; 6:1518–1530. [PubMed: 1644293]
44. Gurdon JB, et al. Cells' perception of position in a concentration gradient. Cell. 1998; 95:159–162. [PubMed: 9790523]
45. Sandler JE, Stathopoulos A. Quantitative Single-Embryo Profile of Drosophila Genome Activation and the Dorsal-Ventral Patterning Network. Genetics. 2016
46. Geiss GK, et al. Direct multiplexed measurement of gene expression with color-coded probe pairs. Nat Biotechnol. 2008; 26:317–325. [PubMed: 18278033]
47. O'Farrell PH. Developmental biology. Big genes and little genes and deadlines for transcription. Nature. 1992; 359:366–367. [PubMed: 1406945]
48. Shermoen AW, O'Farrell PH. Progression of the cell cycle through mitosis leads to abortion of nascent transcripts. Cell. 1991; 67:303–310. [PubMed: 1680567]
49. Lee MT, et al. Zygotic genome activation during the maternal-to-zygotic transition. Annual review of cell and developmental biology. 2014; 30:581–613.
50. Davidson EH, et al. A genomic regulatory network for development. Science. 2002; 295:1669–1678. [PubMed: 11872831]
51. Mangan S, Alon U. Structure and function of the feed-forward loop network motif. P Natl Acad Sci USA. 2003; 100:11980–11985.
52. de-Leon SBT, Davidson EH. Modeling the dynamics of transcriptional gene regulatory networks for animal development. Developmental Biology. 2009; 325:317–328. [PubMed: 19028486]
53. Sandmann T, et al. A core transcriptional network for early mesoderm development in Drosophila melanogaster. Genes Dev. 2007; 21:436–449. [PubMed: 17322403]
54. Seher TC, et al. Analysis and reconstitution of the genetic cascade controlling early mesoderm morphogenesis in the Drosophila embryo. Mech Dev. 2007; 124:167–179. [PubMed: 17267182]
55. Wu H, et al. Temporal and spatial dynamics of scaling-specific features of a gene regulatory network in Drosophila. Nat Commun. 2015; 6:10031. [PubMed: 26644070]
56. Manu, et al. Canalization of gene expression and domain shifts in the Drosophila blastoderm by dynamical attractors. PLoS Comput Biol. 2009; 5:e1000303. [PubMed: 19282965]
57. Perrimon N, et al. Signaling mechanisms controlling cell fate and embryonic patterning. Cold Spring Harb Perspect Biol. 2012; 4:a005975. [PubMed: 22855721]
58. Morel V, Schweisguth F. Repression by suppressor of hairless and activation by Notch are required to define a single row of single-minded expressing cells in the Drosophila embryo. Genes Dev. 2000; 14:377–388. [PubMed: 10673509]
59. Lim B, et al. Kinetics of gene derepression by ERK signaling. Proc Natl Acad Sci U S A. 2013; 110:10330–10335. [PubMed: 23733957]
60. Mavrakis M, et al. Cells within a cell: Insights into cellular architecture and polarization from the organization of the early fly embryo. Commun Integr Biol. 2009; 2:313–314. [PubMed: 19721875]
61. Shoval O, et al. Fold-change detection and scalar symmetry of sensory input fields. P Natl Acad Sci USA. 2010; 107:15995–16000.
62. Goentoro L, et al. The Incoherent Feedforward Loop Can Provide Fold-Change Detection in Gene Regulation. Mol Cell. 2009; 36:894–899. [PubMed: 20005851]
63. Ashe HL, et al. Dpp signaling thresholds in the dorsal ectoderm of the Drosophila embryo. Development (Cambridge). 2000; 127:3305–3312.
64. Dubrulle J, et al. Response to Nodal morphogen gradient is determined by the kinetics of target gene induction. Elife. 2015; 4
65. Leptin M. twist and snail as positive and negative regulators during Drosophila mesoderm development. Genes Dev. 1991; 5:1568–1576. [PubMed: 1884999]

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66. Alberga A, et al. The snail gene required for mesoderm formation in Drosophila is expressed dynamically in derivatives of all three germ layers. Development. 1991; 111:983–992. [PubMed: 1879366]
67. Liang HL, et al. The zinc-finger protein Zelda is a key activator of the early zygotic genome in Drosophila. Nature. 2008; 456:400–403. [PubMed: 18931655]
68. Nien CY, et al. Temporal coordination of gene networks by Zelda in the early Drosophila embryo. PLoS Genet. 2011; 7:e1002339. [PubMed: 22028675]
69. Tsurumi A, et al. STAT is an essential activator of the zygotic genome in the early Drosophila embryo. PLoS Genet. 2011; 7:e1002086. [PubMed: 21637778]
70. Harrison MM, et al. Grainyhead and Zelda compete for binding to the promoters of the earliest-expressed Drosophila genes. Dev Biol. 2010; 345:248–255. [PubMed: 20599892]
71. Garcia M, Stathopoulos A. Lateral gene expression in Drosophila early embryos is supported by Grainyhead-mediated activation and tiers of dorsally-localized repression. PLoS One. 2011; 6:e29172. [PubMed: 22216201]
72. Ozdemir A, et al. Su(H)-Mediated Repression Positions Gene Boundaries along the Dorsal-Ventral Axis of Drosophila Embryos. Dev Cell. 2014; 31:100–113. [PubMed: 25313963]
73. Jiang J, Levine M. Binding affinities and cooperative interactions with bHLH activators delimit threshold responses to the dorsal gradient morphogen. Cell. 1993; 72:741–752. [PubMed: 8453668]
74. Liberman LM, Stathopoulos A. Design flexibility in cis-regulatory control of gene expression: synthetic and comparative evidence. Dev Biol. 2009; 327:578–589. [PubMed: 19135437]
75. Erives A, Levine M. Coordinate enhancers share common organizational features in the Drosophila genome. Proc Natl Acad Sci U S A. 2004; 101:3851–3856. [PubMed: 15026577]
76. Farley EK, et al. Suboptimization of developmental enhancers. Science. 2015; 350:325–328. [PubMed: 26472909]
77. Crocker J, et al. Evolution acts on enhancer organization to fine-tune gradient threshold readouts. PLoS Biol. 2008; 6:e263. [PubMed: 18986212]
78. Perry MW, et al. Evolution of insect dorsoventral patterning mechanisms. Cold Spring Harb Symp Quant Biol. 2009; 74:275–279. [PubMed: 19843594]
79. Hong JW, et al. Shadow Enhancers as a Source of Evolutionary Novelty. Science. 2008; 321:1314. [PubMed: 18772429]
80. Barolo S. Shadow enhancers: frequently asked questions about distributed cis-regulatory information and enhancer redundancy. Bioessays. 2012; 34:135–141. [PubMed: 22083793]
81. Staller MV, et al. Shadow enhancers enable Hunchback bifunctionality in the Drosophila embryo. Proc Natl Acad Sci U S A. 2015; 112:785–790. [PubMed: 25564665]
82. Perry MW, et al. Shadow enhancers foster robustness of Drosophila gastrulation. Curr Biol. 2010; 20:1562–1567. [PubMed: 20797865]
83. Dunipace L, et al. Complex interactions between cis-regulatory modules in native conformation are critical for Drosophila snail expression. Development. 2011; 138:4075–4084. [PubMed: 21813571]
84. Dunipace L, et al. Autoregulatory Feedback Controls Sequential Action of cis-Regulatory Modules at the brinker Locus. Dev Cell. 2013; 26:536–543. [PubMed: 24044892]
85. Kuersten S, Goodwin EB. The power of the 3′ UTR: translational control and development. Nat Rev Genet. 2003; 4:626–637. [PubMed: 12897774]
86. Semotok JL, Lipshitz HD. Regulation and function of maternal mRNA destabilization during early Drosophila development. Differentiation. 2007; 75:482–506. [PubMed: 17509066]
87. Lasko P. Posttranscriptional regulation in Drosophila oocytes and early embryos. Wiley Interdiscip Rev RNA. 2011; 2:408–416. [PubMed: 21957026]
88. Lucas T, et al. Live imaging of bicoid-dependent transcription in Drosophila embryos. Curr Biol. 2013; 23:2135–2139. [PubMed: 24139736]
89. Garcia HG, et al. Quantitative imaging of transcription in living Drosophila embryos links polymerase activity to patterning. Curr Biol. 2013; 23:2140–2145. [PubMed: 24139738]

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90. Bothma JP, et al. Dynamic regulation of eve stripe 2 expression reveals transcriptional bursts in living Drosophila embryos. Proc Natl Acad Sci U S A. 2014; 111:10598–10603. [PubMed: 24994903]
91. Dean KM, Palmer AE. Advances in fluorescence labeling strategies for dynamic cellular imaging. Nat Chem Biol. 2014; 10:512–523. [PubMed: 24937069]
92. Davidson EH. Emerging properties of animal gene regulatory networks. Nature. 2010; 468:911–920. [PubMed: 21164479]
93. Alon U. Network motifs: theory and experimental approaches. Nat Rev Genet. 2007; 8:450–461. [PubMed: 17510665]
94. Hansen KD, et al. Biases in Illumina transcriptome sequencing caused by random hexamer priming. Nucleic Acids Res. 2010; 38
95. Roberts A, et al. Improving RNA-Seq expression estimates by correcting for fragment bias. Genome Biology. 2011; 12

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Box 1

Case Studies in Transcription Factor Localization and Concentration

Two prominent transcription factors active early in *Drosophila* development are Dorsal and Bicoid. The nuclear concentration, gradients, and embryonic localization of both transcription factors have been characterized, and present a contrast in nuclear import strategies [13, 16]. Both are imported into nuclei during syncytial nuclear cycles, but the dynamics and import rate are different between the two (Figure I). While Bicoid undergoes a rapid uptake, it also undergoes a decrease in concentration before nuclear division, indicating an overshoot and reduction in concentration to a lower steady state. Nuclear cycles 10–12 are too short to reach this overshoot and reduction, but nuclear cycles 13 and 14 show this characteristic, with the concentration of Bicoid stabilizing before mitosis, when it then drops to low levels before being imported again. Dorsal, on the other hand, undergoes a slower increase to maximum levels at each nuclear cycle, with no overshoot. While Dorsal never reaches a steady state during early nuclear cycles, the concentration of Dorsal begins to level off during nuclear cycle 13 and finally achieves a steady state during nuclear cycle 14, demonstrating a different import mechanism than that of Bicoid. Both Bicoid and Dorsal leave nuclei at very similar rates and times between nuclear cycles, indicating that export is likely due to rapid diffusion of the transcription factors when the nuclear envelope breaks down during mitosis.

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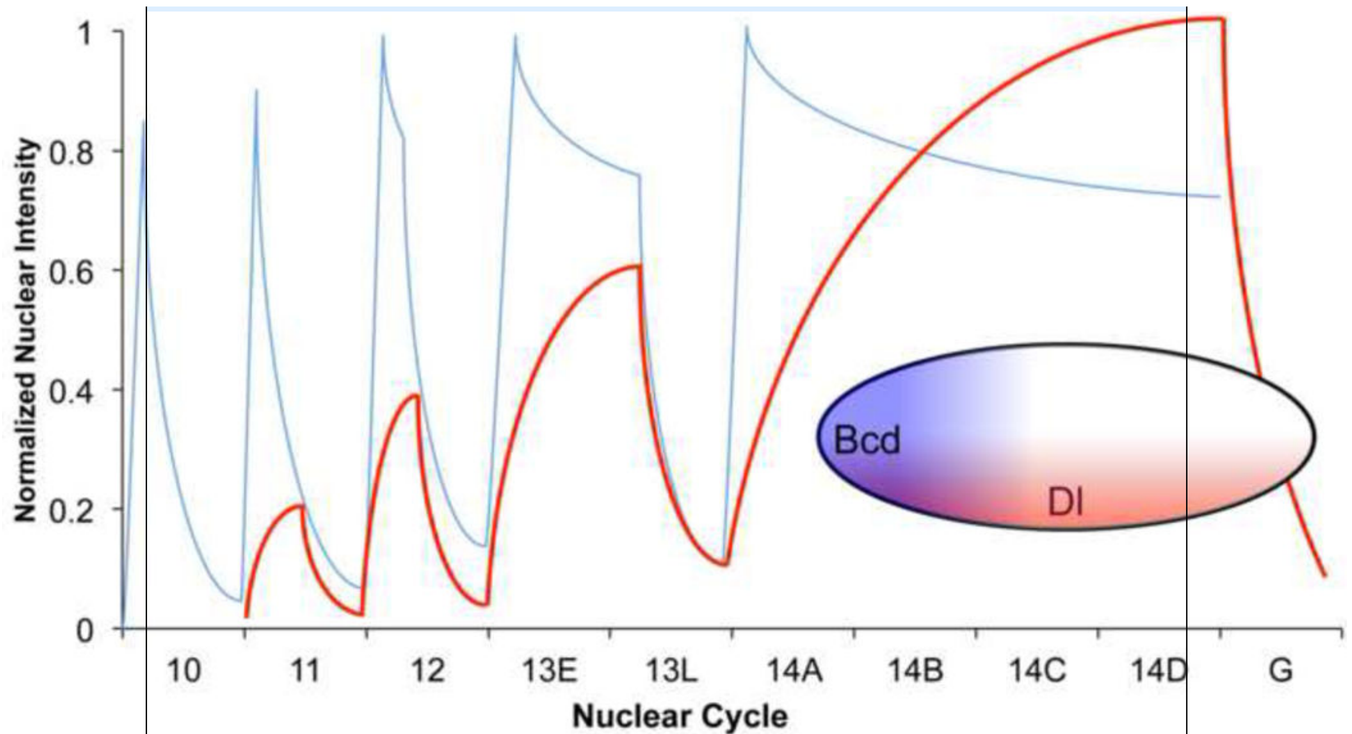

Figure I. Bicoid and Dorsal Dynamics - Comparison of Nuclear Levels

A conceptual representation of the concentration of transcription factors Bicoid (blue) and Dorsal (Dl; red) in nuclei during late nuclear cycles based on data from previous studies [13, 16]. Measurements were obtained by monitoring live fluorescent intensity of a bcd-GFP or dl-Venus fusion molecules from a single nucleus at 10% along the AP axis for Bicoid or ventral most position for Dorsal. Nuclear intensity is normalized to the maximum for each transcription factor and overlaid. Inset is an illustration of a *Drosophila* embryo with transcription factor concentration gradients for Bicoid and Dorsal.

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Box 2

Explanation of NanoString Technology

The recently developed NanoString technology directly detects and counts RNA targets by binding fluorescently labeled single stranded RNA probes to mRNA of interest. There are two RNA probes for each mRNA counted; a capture probe and a reporter probe. Each probe hybridizes to 50bp of target mRNA, and both probes target directly adjacent sequences, totaling 100bp (Figure II). The reporter probe is also bound by a series of fluorescently-labeled single stranded RNAs in a specific barcode pattern that is different for each mRNA target being detected. This way, each mRNA target has a unique fluorescent barcode bound by the reporter probe. The capture probe is conjugated to a biotin bead, so that when both probes are hybridized to the target mRNA, the new mRNA-probe complex will bind to a streptavidin-coated imaging slide. Probe hybridization takes place at 65° C for ~18 hours in a standard benchtop thermal cycler, after which the mRNA-probe complex is bound to an imaging cartridge and linearized with an electric current in the robotic nCounter Prep Station. The cartridge is transferred to the nCounter Digital Analyzer where the cartridge is scanned and each fluorescent barcode is imaged and counted.

This method of detecting and counting mRNA molecules has several advantages over methods such as qPCR or RNAseq. There is no reverse transcription, target amplification, or fragmentation involved in the NanoString protocol. All three of these steps have been shown to introduce bias in coverage or quantification of targets [94, 95]. The addition of external RNA control spike-ins to samples analyzed on the nCounter further enhances the quantitative nature of the system, allowing for absolute quantification of all targets [45].

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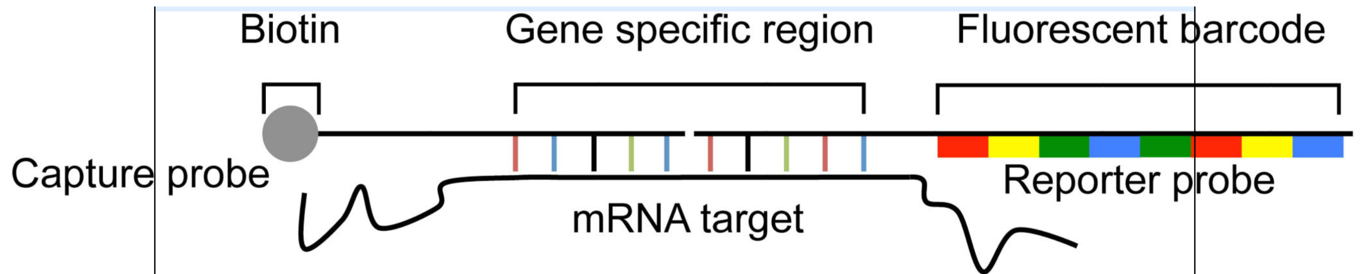

Figure II. NanoString set-up for identification of individual mRNA targets

A representation of NanoString probes hybridized to a target mRNA molecule. Both probes anneal to contiguous 50bp regions of the mRNA molecule. The reporter probe contains a target-specific fluorescent barcode, and the capture probe is conjugated to biotin for binding to a streptavidin-coated imaging cartridge.

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Outstanding Questions Box

While mRNA profiling experiments highlight temporal building of the gene expression program, a role for this step-wise progression is not completely clear, leading to several unanswered questions.

- Is this step-wise progression important for regulating the timing of signaling pathway activation and/or robustness? If so, what purpose does this serve? Does it support irreversibility of the patterning process? Studies in other systems have provided evidence that "fold-change" may trigger signaling activation rather than a particular threshold level of ligand, arguing that step-wise activation of signaling may be important.
- How is the GRN initiated? In *Drosophila*, nuclei populate the center of the embryo until NC 9 and only migrate to the periphery at NC 10. This makes their identification and staging by live imaging difficult. A key challenge is to overcome this physical barrier in order to provide insight into initiation of this GRN program.

How is step-wise progression controlled at the cis-regulatory level?

- Do other ubiquitous activators function together to control timing of gene expression in the early embryo? Analysis of the organization of sites within CRMs could help to determine how these relate to timing and levels of gene expression.
- Do CRMs act in sync with particular nuclear cycles and if so, how is this regulated? Is there a temporal hand-off from one enhancer to the next?
- Does post-transcriptional regulation play a role in the regulation of zygotic gene expression? If so, what how does this impact function of genes within this patterning network?

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Trends Box

- The Dorsal transcription factor exhibits changes in levels over time, raising the question of how this morphogen controls patterning of the *Drosophila* embryo when so dynamic.
- Spatiotemporal examination of a small number of Dorsal target genes had shown gene expression is dynamic as well, and this study was recently expanded using NanoString technology to provide a temporal analysis of ~70 genes in the *Drosophila* early embryo.
- Quantitative, fine-scale temporal data for levels of transcripts in the early *Drosophila* embryo has helped clearly define temporal roles for transcription factors and identified gene expression cohorts, to provide new insight into the step-wise progression of the dorsoventral patterning gene regulatory network.

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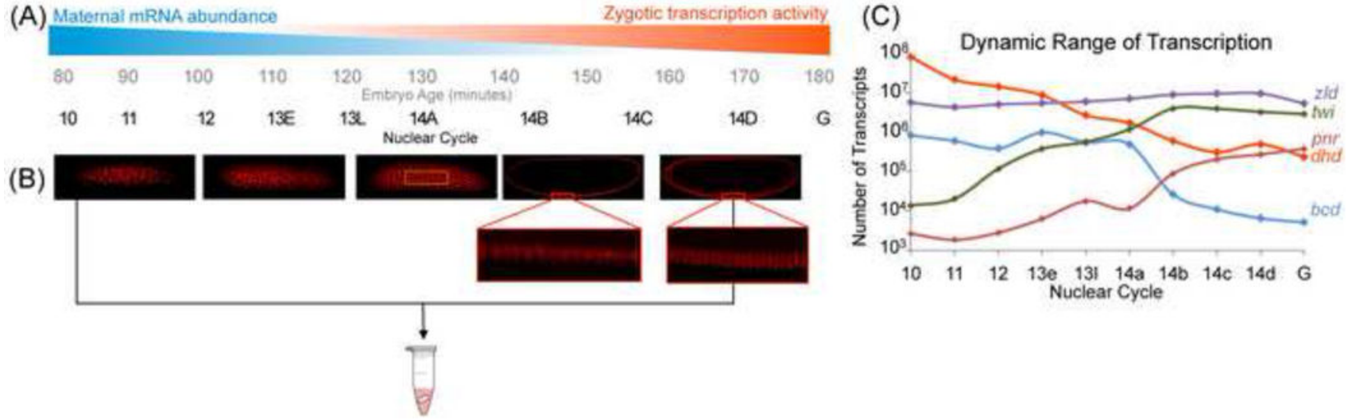

Figure 1, Key Figure. Timeline of Embryonic Development and Dynamic Range of Gene Expression

(A) A timeline of early embryonic development in *Drosophila*. Maternal transcripts deposited during oogenesis are degraded while zygotic transcripts increase in abundance as the genome is first activated. The embryo age in minutes after egg laying (grey text) and corresponding nuclear cycle (black text) are aligned. (B) Nuclear density increases as nuclear cycles progress until nuclear cycle 14, then nuclei elongate until gastrulation, as shown in expanded images. Microfuge tube: individual embryos were selected at specific time points for analysis and to create a developmental time course. (C) mRNA abundance is highly variable and dynamic between different genes at the same time point and for the same gene at different time points. Different gene counts can vary by over four orders of magnitude simultaneously (*dhd* vs. *pnr* NC 10) or the same gene can vary by over 200- in around an hour (*dhd, twi*, and *pnr*). Figure from [45]; permission to reproduce granted by the Genetics Society of America.

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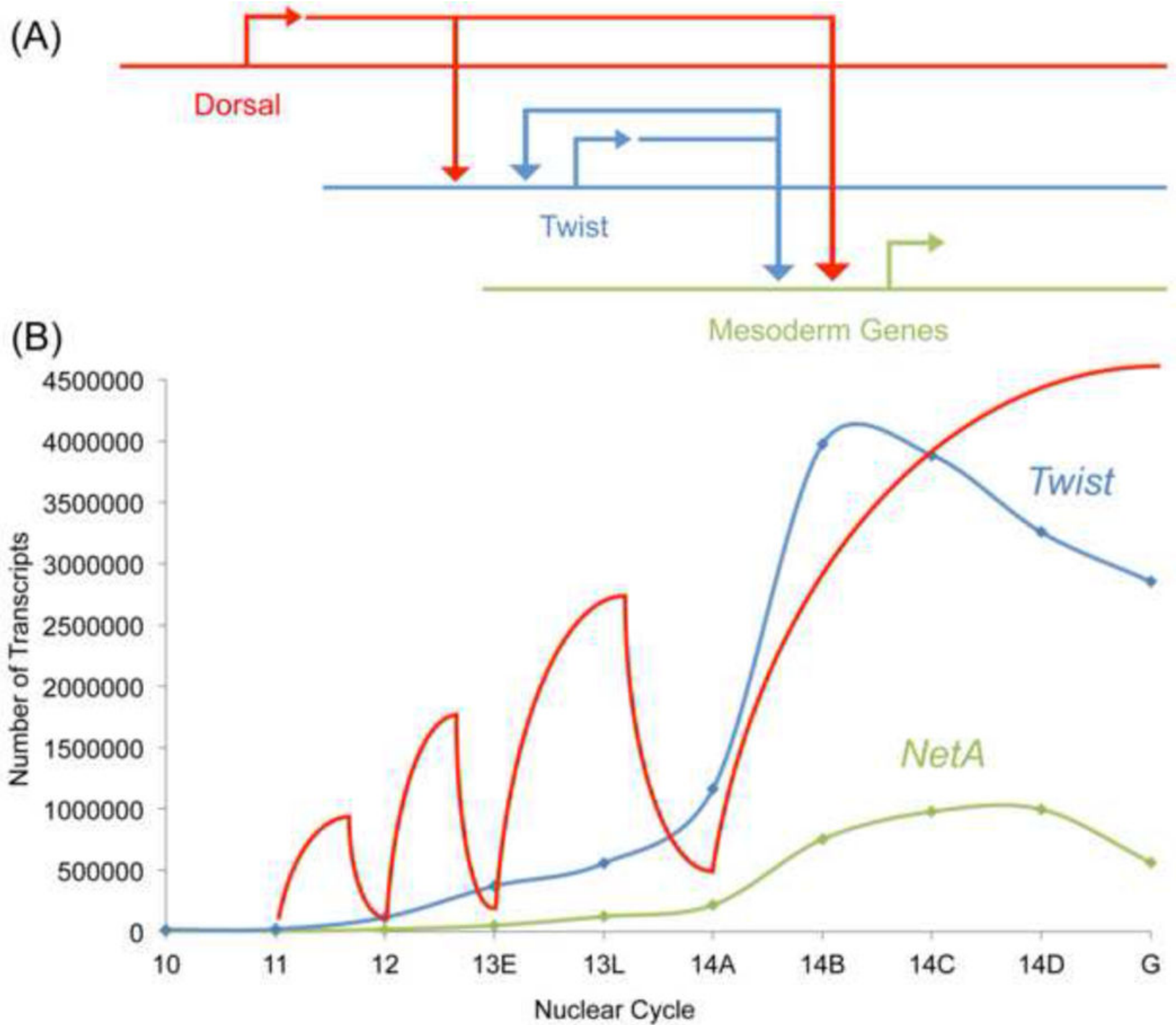

Figure 2. Dynamics of the Maternal to Zygotic Transition: Dorsal/Twist Feed Forward Loop

(A) A schematic of the early gene regulatory network architecture of the mesoderm showing the feed forward loop between Dorsal, Twist, and the rest of the mesoderm genes. Length of line for each network component corresponds to the nuclear cycle of activation and detectable presence on the plot below. (B) Transcriptional activity of *twi* (blue) and downstream mesoderm gene *NetA* (green) overlaid with a representation of the Dorsal concentration (red) in ventral nuclei.

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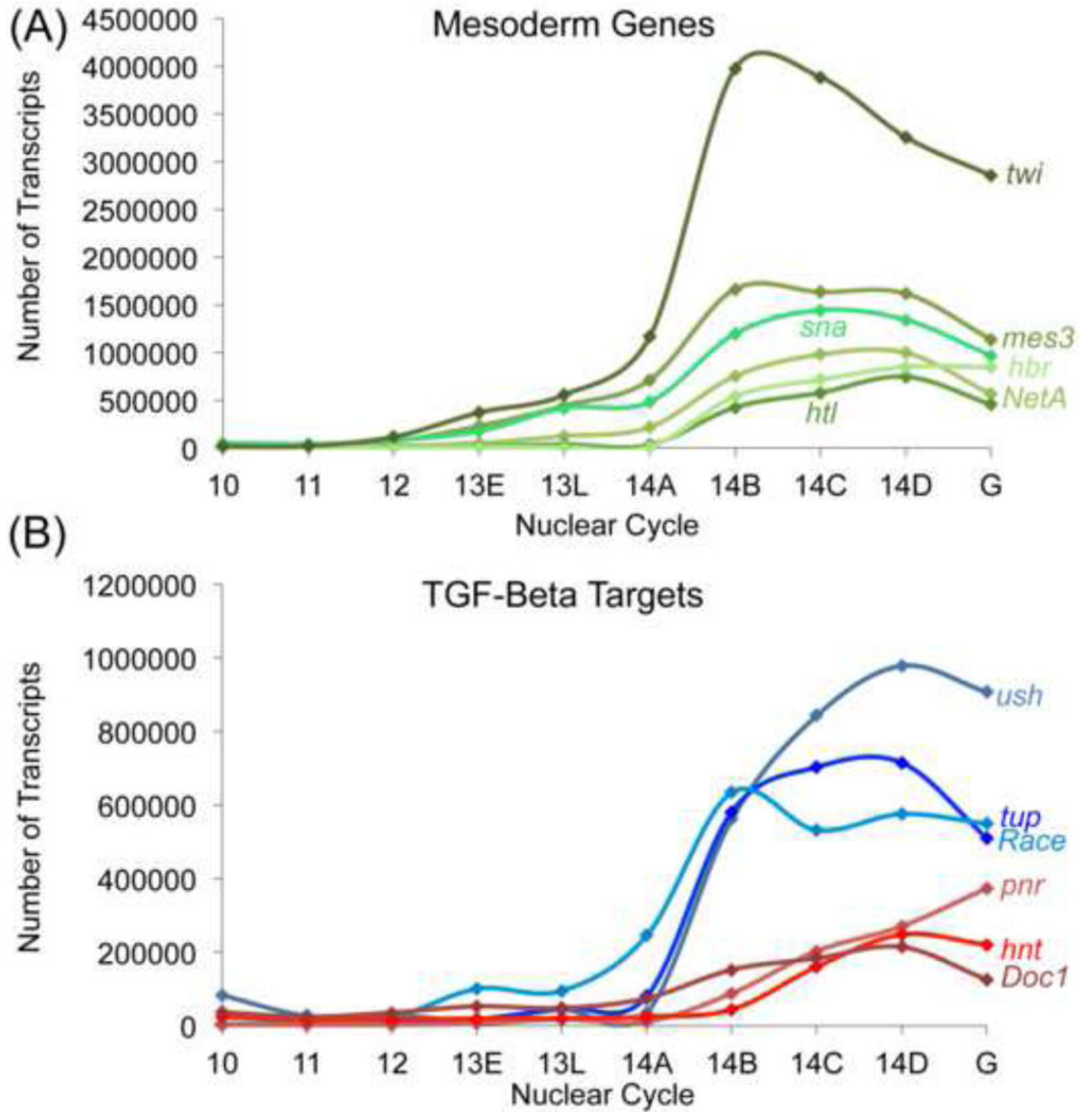

Figure 3. Timing of Gene Cohort Expression

(A) Genes expressed in the mesoderm are regulated as a cohort, with two coordinated phases of activation. Early coordinated activation begins at NC 12, while robust coordinated activation begins at NC 14A for all genes examined (*mes3*, *sna*, *NetA, hbr, htl*) except for *twi* that is upregulated much faster and likely serves as input to other mesodermally-expressed genes together with Dorsal. (B) A cohort of genes expressed at NC 14A as targets of the TGF-β pathway in the dorsal ectoderm. The genes are temporally co-regulated, but diverge in their transcription rates. Genes *ush, tup*, and *Race* are transcribed quickly and

reach a steady state, while genes *pnr, hnt*, and *Doc1* are transcribed moderately. Figure adapted from [45]; permission to reproduce granted by the Genetics Society of America.

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