Graded Dorsal and Differential Gene Regulation in the *Drosophila* Embryo

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Graded Dorsal and Differential Gene Regulation in the *Drosophila* Embryo

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A gradient of Dorsal activity patterns the dorsoventral (DV) axis of the early *Drosophila melanogaster* embryo by controlling the expression of genes that delineate presumptive mesoderm, neuroectoderm, and dorsal ectoderm. The availability of the *Drosophila melanogaster* genome sequence has accelerated the study of embryonic DV patterning, enabling the use of systems-level approaches. As a result, our understanding of Dorsal-dependent gene regulation has expanded to encompass a collection of more than 50 genes and 30 *cis*-regulatory sequences. This information, which has been integrated into a spatiotemporal atlas of gene regulatory interactions, comprises one of the best-understood networks controlling any developmental process to date. In this article, we focus on how Dorsal controls differential gene expression and how recent studies have expanded our understanding of *Drosophila* embryonic development from the *cis*-regulatory level to that controlling morphogenesis of the embryo.

The classical definition of a morphogen requires that the protein be synthesized from a localized source to affect concentration-dependent outputs of gene expression. The *Drosophila* embryo is a specialized case because it is a syncytium at early stages, permitting the formation of transcription factor gradients, which regulate transcription in a graded, concentration-dependent manner. In the early *Drosophila* embryo, two maternally deposited transcription factors, Bicoid and Dorsal, specify the anteroposterior (AP) and dorsoventral (DV) axes, respectively. Different levels of these factors control the expression of distinct sets of genes. Here, we provide an overview of DV patterning of the early *Drosophila* embryo. We focus on the role of Dorsal and highlight the novel insights genome approaches have provided.

Initiation of the Dorsal Nuclear Gradient

Within the nuclei of early *Drosophila* embryos, the Dorsal transcription factor is present in a ventral-to-dorsal gradient (Figs. 1C,D and 2A) (Moussian and Roth 2005). The maternally supplied Dorsal transcript is distributed and translated uniformly throughout the embryo; however, activation of the Toll receptor is limited to ventral and ventrolateral regions of

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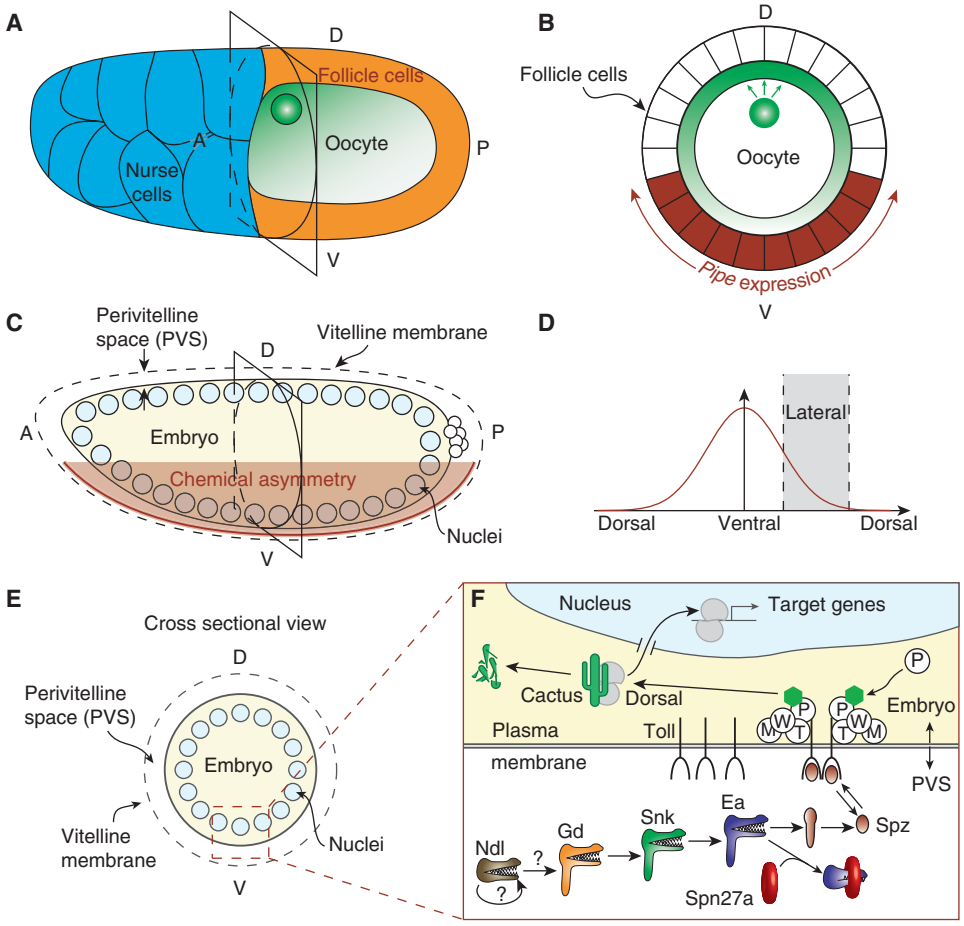

Figure 1. Overview of the ventral signaling pathway. (*A*) Schematic of St. 10 egg chamber. The oocyte nucleus is located at the dorsoanterior cortex. Gurken, which is locally translated, is present in a protein gradient (green). (*B*) Cross section of schematic of St. 10 egg chamber. Gurken signaling (green) represses *pipe* expression (brown) in the follicle cells. (*C*) Schematic of syncytial blastoderm embryo. The ventral follicle cells, which had expressed *pipe*, deposit an unknown "chemical asymmetry" into the perivitelline space. (*D*) The chemical asymmetry results in a ventral-to-dorsal signaling gradient. (*E*) Cross section of schematic of syncytial blastoderm embryo. The signaling gradient is initially established within the perivitelline space, a small extracellular space between the embryo and an outer vitelline membrane. (*F*) Illustration of ventral signaling pathway in the early embryo. In the perivitelline space (PVS), a protease cascade (Ndl, Gd, Snk, Ea) eventually activates Spz, the ligand for the Toll receptor. The serine protease inhibitor, Spn27a, inhibits the activity of Ea. Activated Spz transduces the signal into the embryo through Toll, causing the degradation of Cactus and the nuclear translocation of the transcription factor Dorsal. The roles of Tube (T), Pelle (P), Weckle (W), and Myd88 (M) are relatively unknown, but participate in a signaling complex at the cytoplasmic tail of Toll.

the embryo, and this causes the Dorsal protein to be translocated into the nucleus. The positional information guiding activation of Toll is initiated by the follicle cells surrounding the developing oocyte during Stage 10 of oogenesis (Fig. 1A,B) (Anderson 1998). At this stage, epidermal growth factor receptor (EGFR) signaling through the ligand Gurken limits expression of the gene *pipe* to the ventral-most follicle cells (Fig. 1B) (Schupbach 1987; Sen et al.

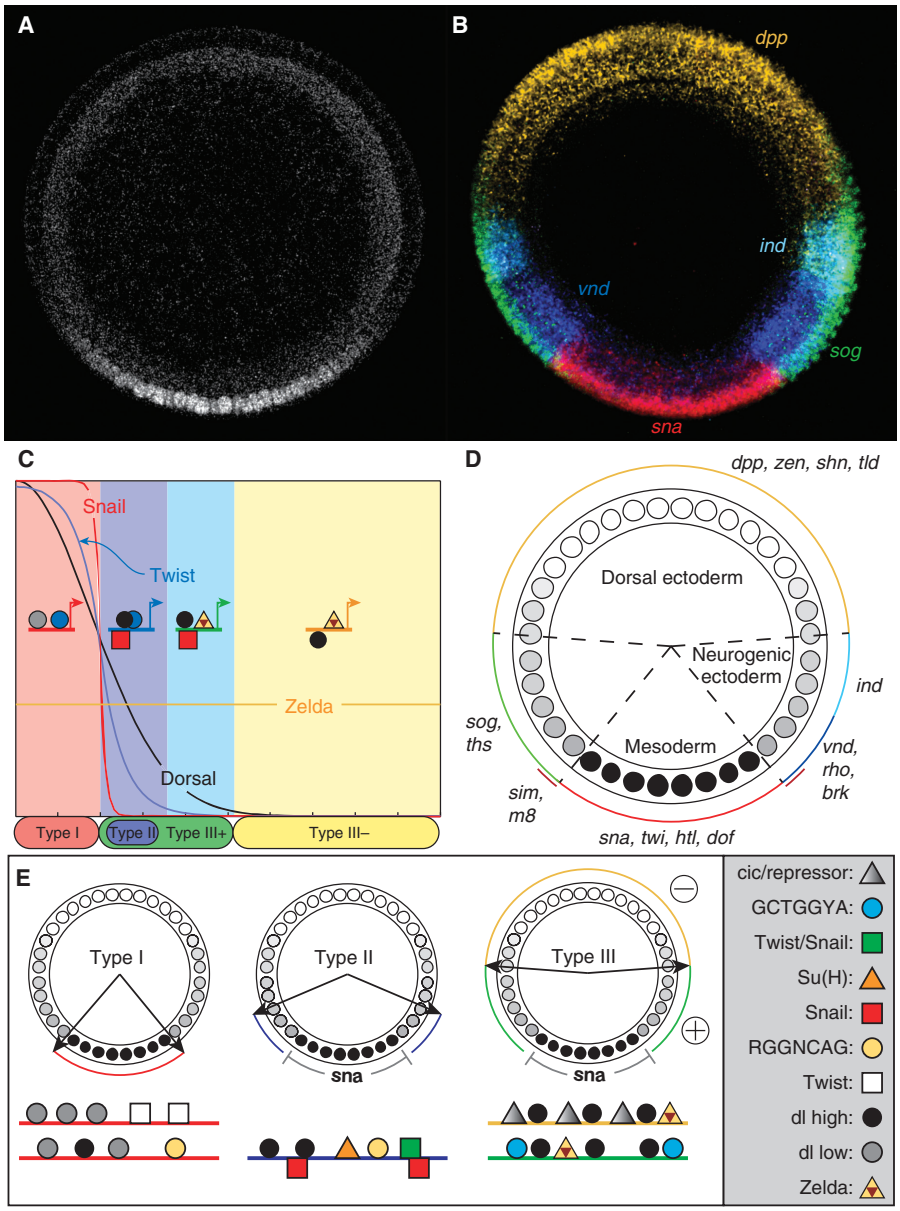

Figure 2. Illustration of early embryonic fate map. (*A*) Cross section of Stage 5 *Drosophila* embryo, fluorescently stained with an α-Dorsal antibody. (*B*) Cross section of Stage 5 *Drosophila* embryo, fluorescently stained by in situ hybridization to detect Dorsal target gene transcripts *dpp*, *ind*, *vnd*, *sog*, and *sna*. (*C*) Dorsal and Twist cooperate to specify both Type I and Type II Dorsal target genes. Dorsal functions together with Zelda to support expression of Type III (+ and −) target genes (See legend in part E). (*D*) Schematic of fate map. The Dorsal nuclear gradient divides the embryo into three main subtissues: mesoderm, neurogenic ectoderm, and dorsal ectoderm. The neurogenic ectoderm can be further divided into ventral and dorsal halves. (*E*) Groupings of Dorsal target genes. Type I genes are expressed in the ventral-most portion of the embryo, where Dorsal nuclear levels are the highest. Type II genes have dorsal borders in the middle of the neurogenic ectoderm. These genes are also repressed by Snail. Type III genes have their dorsal (+) or ventral (−) borders at roughly 50% DV axis, and contain sites for both Dorsal binding as well as a uniformly expressed activator, such as Zelda.

1998). *pipe* encodes a protein sharing homology with vertebrate heparan sulfate 2-O-sulfotransferases, but likely functions independent of heparan (Zhu et al. 2005).

As oogenesis proceeds, the *pipe*-expressing follicle cells deposit an unknown "chemical asymmetry" into the eggshell, which spatially regulates an extracellular protease cascade, comprised of four proteases: Nudel (Ndl), Gastrulation-defective (Gd), Snake (Snk), and Easter (Ea) (Fig. 1F) (Smith and DeLotto 1994; Sen et al. 1998; LeMosy et al. 2001; Peri et al. 2002). The protease reactions occur in the perivitelline space and culminate in the processing of Spätzle (Spz), the protein ligand for Toll (Fig. 1C–F) (Roth 1993; Morisato 2001; Zhu et al. 2005). In addition to the protease cascade that ensures localized activation of Spz in ventral regions, the gradient of active Spz is further shaped through the action of a serpin (serine-protease inhibitor), Spn27A (Hashimoto et al. 2003; Ligoxygakis et al. 2003).

Downstream of the Spz-mediated activation of Toll, intracellular signaling occurs through the action of several maternal factors. These proteins, which include Weckle, Myd88, Tube, and Pelle, function together to facilitate the degradation of Cactus, a cytoplasmic tethering protein, thereby releasing Dorsal from cytoplasmic retention (Fig. 1F) (Hecht and Anderson 1993; Belvin et al. 1995; Grosshans et al. 1999; Sun et al. 2004; Chen et al. 2006). With the exception of Weckle, this intracellular signaling module is well conserved in vertebrates in which the homologs are involved in regulation of the immune response (Belvin and Anderson 1996). In addition to freeing Dorsal from Cactus, Toll signaling likely potentiates Dorsal nuclear translocation in a Cactus-independent manner, as even in *cactus* mutants, Dorsal is not fully nuclear on the dorsal side of the embryo (Bergmann et al. 1996). However, despite the lack of Toll-mediated signal on the dorsal side of the embryo, recent in vivo imaging studies using a Dorsal-GFP fusion protein show a constant shuttling of the protein between the cytoplasm and the nuclei, including the dorsal-most nuclei (DeLotto et al. 2007). Therefore, it appears that exclusion of Dorsal from nuclei is not achieved by simply preventing Dorsal nuclear import, but by a balance between slow import and rapid export.

Differential Expression of Dorsal Target Genes

The gradient of nuclear-localized Dorsal regulates a number of genes in a concentration-dependent manner (Fig. 2B,D) (reviewed in Stathopoulos and Levine 2004). High levels of nuclear Dorsal present in ventral regions activate genes such as *twist* (*twi*) and *snail* (*sna*), which are required for specification of the mesoderm (Simpson 1983; Thisse et al. 1987; Jiang et al. 1991; Pan et al. 1991; Ray et al. 1991; Ip et al. 1992a). Intermediate levels of nuclear Dorsal, present in ventrolateral regions, induce the expression of genes such as *rhomboid* (*rho*) and *ventral neuroblasts defective* (*vnd*), which are important for specification of the neurogenic ectoderm (Bier et al. 1990; Ip et al. 1992a; Jimenez et al. 1995; Stathopoulos et al. 2002). Low levels of nuclear Dorsal activate genes such as *short-gastrulation* (*sog*) and *thisbe* (*ths*) in broad lateral domains across the embryo (Markstein et al. 2002; Stathopoulos et al. 2002). These genes are required for patterning the dorsal ectoderm, amnioserosa, and dorsal mesoderm (Francois et al. 1994; Stathopoulos et al. 2004).

Dorsal functions as both an activator of transcription to induce gene expression and a repressor to keep genes silenced (Jiang et al. 1992; Dubnicoff et al. 1997). The same low levels of Dorsal that activate genes in lateral regions of the embryo also mediate repression of certain targets, such as *decapentaplegic* (*dpp*), *tolloid* (*tld*), and *zerknüllt* (*zen*), thereby limiting their expression to regions where nuclear Dorsal is absent (Ip et al. 1991; Huang et al. 1993; Kirov et al. 1994). These genes are required for proper patterning of the dorsal ectoderm and amnioserosa, which develops in the dorsal-most regions of the embryo (Rushlow and Levine 1990; Ferguson and

Anderson 1992b; Ferguson and Anderson 1992a).

Different levels of activated Toll direct graded Dorsal-dependent gene expression outputs. Characterization of several Toll alleles (Anderson et al. 1985) supports this view (e.g., Stathopoulos et al. 2002). Dominant mutations in the Toll gene ($Toll^{10B}$), which presumably constitutively activate the receptor (Schneider et al. 1991), result in ubiquitous activation of genes such as *twi* and *sna* that are normally only expressed in ventral regions of the embryo. Concomitantly, genes such as *rho*, *sog*, or *dpp* are repressed. Specific recessive alleles ($Toll^{rm9}$ and $Toll^{rm10}$), which are presumed to result in a partially active receptor (Schneider et al. 1991), direct ubiquitous activation of lower-response genes such as *rho* and *sog*, whereas genes such as *sna* and *dpp* are absent. In the absence of Toll-mediated signaling, genes such as *dpp* and *zen* are ubiquitously expressed, as no nuclear Dorsal is present and therefore genes activated by Dorsal fail to be expressed (e.g., *sna*, *vnd*, etc.).

Perhaps the most compelling evidence that the levels of activated Toll can instruct distinct target gene expression profiles stems from ectopic expression experiments. These studies demonstrated that the DV axis can be reoriented such that genes normally expressed along the DV axis are instead expressed along the AP axis (Huang et al. 1997). This reorientation of axes was accomplished through the ectopic expression of an $anterior^{HIGH}$-$posterior^{LOW}$ gradient of constitutively activated Toll receptor. This gradient of activated Toll receptor was sufficient to support differential Dorsal-target gene expression. Thus, like activin-dependent receptor signaling in *Xenopus* (Dyson and Gurdon 1998), it appears that cells in the early embryo are responsive to different levels of activated Toll receptor. Although the levels of nuclear Dorsal that result from differential activation of the Toll receptor have not been quantified, taken together, these results suggest that differential activation of the Toll receptor regulates the level of nuclear Dorsal along the DV circumference, which in turn determines gene expression output.

cis-Regulatory Control of DV Axis Patterning

Classical genetic screens identified 10 genes that function in the early embryo to control DV patterning (*sna*, *twi*, *single-minded* [*sim*], *m8*, *brinker* [*brk*], *rho*, *sog*, *tld*, *zen*, and *dpp*) (reviewed in Stathopoulos and Levine, 2004; Klambt et al. 1989; Bier et al. 1990; Leptin and Grunewald 1990; Nambu et al. 1990; Rushlow and Levine 1990; Kosman et al. 1991; Ferguson and Anderson 1992b; Ferguson and Anderson 1992a; Jimenez et al. 1995; Jazwinska et al. 1999). Early experiments to dissect the logic of how Dorsal controls the expression of these genes involved the use of highly laborious methods to identify the genomic sequence controlling target-gene expression. These *cis*-regulatory sequences are commonly defined as noncoding genomic sequences able to drive expression of a *lacZ* reporter in a manner similar to the endogenous patterns of genes. This requires that the reporter mimics both spatial and temporal aspects of the endogenous gene. To identify the respective *cis*-regulatory sequences for five of these Dorsal target genes (i.e., *sna*, *rho*, *tld*, *dpp*, and *zen*) "pre-genome sequencing," genomic walks and associated promoter analysis using reporter genes were necessary (Ip et al. 1991; Jiang et al. 1991; Ip et al. 1992b; Huang et al. 1993; Kirov et al. 1994; Stathopoulos and Levine 2002a). Nevertheless, this small sampling offered the first insights into the general mechanisms used to control patterning of the DV axis by Dorsal, and these still stand today.

First, classical studies based on the analysis of *cis*-regulatory sequences showed that Dorsal can function as either an activator or a repressor to control gene expression along the DV axis (Jiang et al. 1993). Context-dependent interactions were proposed as the mechanism controlling whether Dorsal functions as a repressor; specifically, those resulting from cooperative interactions with DNA-binding proteins occupying associated AT-rich sequences (Kirov et al. 1993). For example, the Torso receptor tyrosine kinase signaling pathway, which is activated at the poles,

selectively masks the ability of Dorsal to function as a transcriptional repressor at these positions (Rusch and Levine 1994). Torso signaling modulates the ability of Capicua (Cic), Cut, and Dri to function, proteins that influence the ability of Dorsal to function as a transcriptional repressor (Valentine et al. 1998; Jimenez et al. 2000). More recently, the activation and repression activities of Dorsal have been uncoupled, opening the way for a detailed assessment of these functions in vivo (Ratnaparkhi et al. 2006).

Combinatorial interactions between Dorsal and other transcription factors were found to be important in particular regions of the embryo. For instance, synergistic DNA binding between Dorsal and Twist, a bHLH transcription factor, permits gene expression in more lateral regions of the embryo where neither Dorsal nor Twist is capable of inducing gene expression independently (Gonzalez-Crespo and Levine 1993; Jiang and Levine 1993; Stathopoulos and Levine 2002b). Furthermore, transcriptional repressors function to refine the expression domains produced by activators. For instance, to restrict *rho* expression to ventrolateral stripes in the embryo, Dorsal and Twist activation is antagonized in ventral regions by the Snail repressor, resulting in the lateral stripe pattern of gene expression exhibited by these genes (Kosman et al. 1991; Ip et al. 1992a).

Lastly, the affinity of Dorsal binding sites within *cis*-regulatory sequences was found to influence the domain of target-gene expression (Jiang and Levine 1993). A *twi cis*-regulatory sequence, which normally directs expression to ventral regions of the embryo, exhibited a dorsally expanded expression domain (i.e., the ventrolateral domain) when the low affinity Dorsal binding sites were mutated to high-affinity ones.

In summary, despite the limited nature of this initial set of *cis*-regulatory sequences, Dorsal-mediated patterning was observed to follow three principles: (1) Dorsal functions as a context-dependent activator or repressor; (2) combinatorial regulation between Dorsal and other transcription factors affects transcriptional outputs; and (3) the binding affinity of Dorsal binding sites can influence the spatial extent of gene regulation. Following publication of the *Drosophila* genome, bioinformatic studies revealed these principles to be general.

THE DORSAL GENE REGULATORY NETWORK: INITIAL INSIGHTS ACQUIRED FROM THE GENOMIC SEQUENCE

The availability of the sequenced genome of *Drosophila melanogaster* in 2000 facilitated the analysis of Dorsal-dependent gene expression on a whole-genome scale (Adams et al. 2000). Newly developed bioinformatic approaches permitted scanning the entire genome for clusters of transcription-factor binding sites in a matter of seconds (Berman et al. 2002; Markstein et al. 2002). Furthermore, microarrays containing probes for predicted open reading frames were made commercially available and included many previously uncharacterized genes. Using a microarray-based approach, many additional Dorsal-dependent genes were identified (Stathopoulos et al. 2002), bringing the estimated total number of genes regulated by Dorsal to about 50. Surprisingly, all genes differentially expressed along the DV axis could be categorized into six basic patterns of gene expression (Fig. 2D). Before such a large-scale analysis, it was unclear, with only 10 known target genes, whether common boundaries of gene expression existed or if genes might be expressed in many different domains along the DV axis. Bioinformatic approaches rapidly identified an additional 20 *cis*-regulatory sequences for genes expressed along the DV axis. This increased the total fivefold from the five found by the highly laborious methods in the preceding 10-year period, and this number is constantly growing as more and more *cis*-regulatory sequences are characterized (reviewed in Markstein et al. 2002; Stathopoulos et al. 2002; Markstein et al. 2004; Stathopoulos and Levine 2004; Stathopoulos et al. 2004; Stathopoulos and Levine 2005b).

Classification of DV Patterns

Based on their expression patterns along the DV axis, this first set of 25 *cis*-regulatory sequences were categorized into four classes: ventral (Type I), ventrolateral (Type II), broad lateral (Type III+), and dorsal (Type III−) domains (Fig. 2E) (Stathopoulos and Levine 2004). Analysis of multiple Dorsal-dependent *cis*-regulatory sequences for coexpressed genes led to generalizations and "combinatorial codes" for which transcription factor binding sites control particular expression patterns. For instance, in addition to the presence of Dorsal sites in all the classes, the motif GCTGGYA was identified in enhancers that direct expression in a broad lateral stripe. In contrast, CACATGT and RGGNCAG motifs were identified in enhancers supporting expression in ventrolateral stripes (Stathopoulos et al. 2002; Markstein et al. 2004). The factors that bind to the GCTGGYA and RGGNCAG sites remain unidentified, whereas both Twist and Snail have been shown to bind the CACATGT site (Ip et al. 1992a).

A genome-wide computational search also identified an additional set of sequences, denoted the "TAGteam" (CAGGTAG and slight variations of this sequence), which is present in *cis*-regulatory elements of many genes expressed at the onset of zygotic transcription (ten Bosch et al. 2006; De Renzis et al. 2007). This sequence is recognized by a ubiquitous, maternally provided transcription factor, Zelda, and is present in several Dorsal-dependent enhancers, most especially genes of the Type III+/− classes, such as *sog*, *dpp*, and *zen* (Liang et al. 2008; Liberman and Stathopoulos 2009). Moreover it appears that Zelda is required for correct spatial patterning of these genes: In Zelda mutants, expression of the Type III− target genes *dpp* and *zen* disappear, whereas the Type III+ gene *sog* diminishes and acquires the characteristics of a Type II pattern (Liang et al. 2008).

It is interesting to note that three of the known *cis*-regulatory sequences—those directing expression of *sim*, *m8*, and *intermediate neuroblasts defective* (*ind*) genes—drive expression in domains that are somewhat different from any of the others (Kasai et al. 1998; Stathopoulos and Levine 2005b; Zinzen et al. 2006a). However, the enhancers for *sim* and *m8* contain many transcription factor binding sites also found in Type II enhancers. This suggests that these responses should be similar, yet *sim* and *m8* are only expressed in stripes a single cell wide, compared with *vnd*, *brk*, and *rho*, which are expressed in stripes 5–7 cells wide (Fig. 2D,E). It is likely that Notch signaling is required for activation of *sim* and *m8*, which would explain their more refined expression domain (Morel and Schweisguth 2000; Cowden and Levine 2002). Furthermore, recent analysis of the *ind* enhancer has suggested that this pattern is actually similar to the Type III+ responses, such as *sog* and *ths*, in terms of activation potential. However, the *ind* gene is refined by the action of localized activators (i.e., downstream of EGFR signaling) and repressors. Thus, to refine *ind*, Vnd functions in ventrolateral regions of the embryo, and an unknown repressor presumably functions in dorsal and/or dorsolateral regions of the embryo. Together, these repressors restrict *ind* to its lateral expression domain (Fig. 2B,D,E) (Cowden and Levine 2003; Stathopoulos and Levine 2005b).

In other words, although there are apparently six different gene-expression patterns, it seems that only three distinct activation "thresholds" exist (Types I, II, and III), and the diversity of patterns is generated by repressors or additional requirements for activation acting downstream of Dorsal (Fig. 2E). Together, these three activation thresholds delineate distinct gene-expression boundaries and thus subdivide the embryo into four domains (Types I, II, and III+/−). From this analysis and the previous studies, the following generalizations can be made (Fig. 2C,E): First, Twist and Dorsal function in a synergistic manner to regulate expression of both Type I and Type II target genes. Type II genes are distinguished from Type I genes because, generally, they are repressed by Sna and require higher-affinity Dorsal sites or stronger Dorsal/Twist

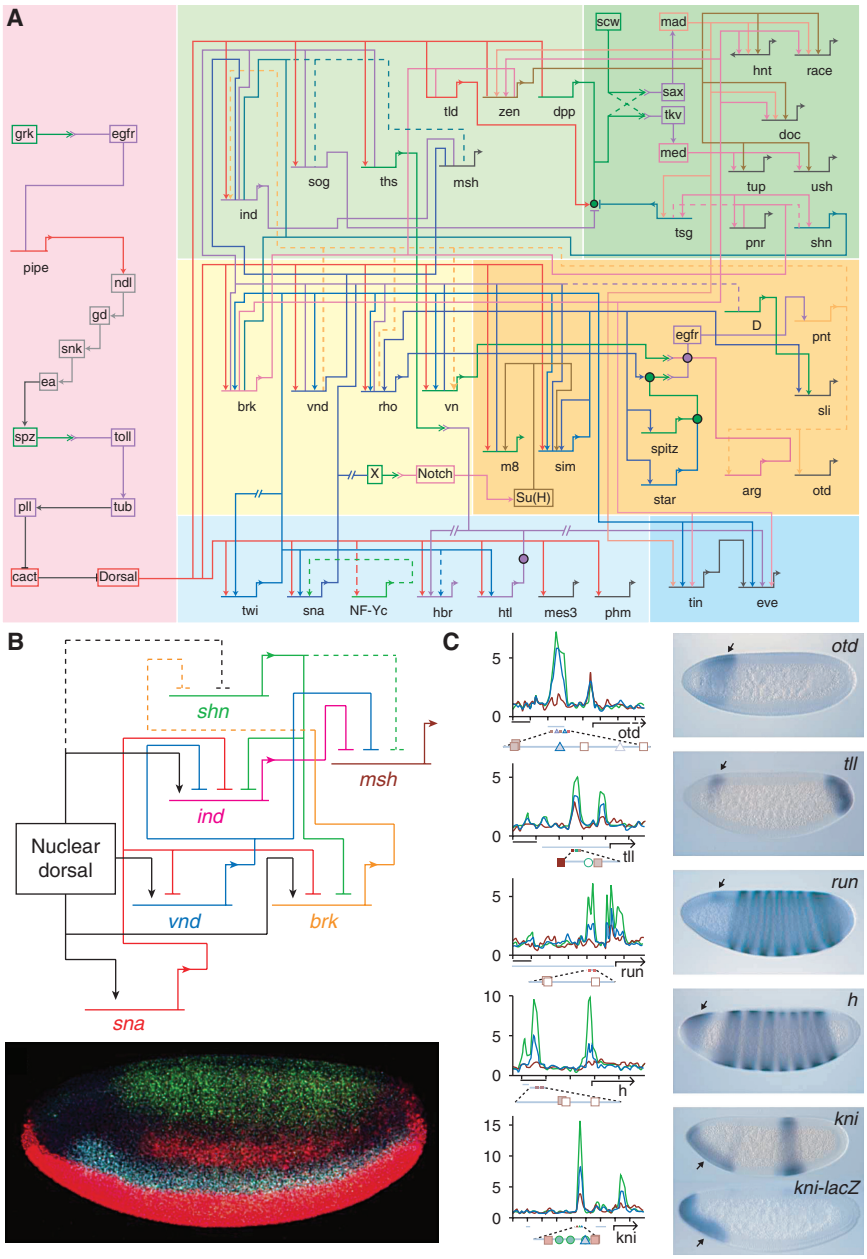

Figure 3. Fruits of genomic approaches. (*A*) Dorsal gene regulatory network. The number of known Dorsal target genes increased roughly fivefold, from 10 to 50, through the use of genomic approaches. Careful study of the interactions among these genes allows for the construction of a network diagram. (Reprinted, with permission, from Levine and Davidson 2005, ©National Academy of Sciences.) (*B*) Network of repressors. Dorsal activity along the dorsoventral (DV) axis initiates a cascade of repressors (*upper* panel). Whole-mount embryo (*lower* panel) depicts the spatial organization of such genes by in situ hybridization using riboprobes to detect transcripts, with *sna* (red) on the ventral side, *vnd* (cyan) in the ventral neurogenic ectoderm (*brk* not shown), *ind* (dim, red) just dorsal of *vnd*, and *schnurri* (*shn*, green) in the dorsal ectoderm. *msh* (not shown) is expressed in a narrow stripe just dorsal to *ind*. Dashed connections are only hypothesized. (*Continued*)

synergy (Jiang and Levine 1993; Papatsenko and Levine 2005). Second, gene expression in dorsolateral regions of the embryos (Type III+) requires the function of both Dorsal and Zelda, while in dorsal regions (Type III−), Dorsal activity must be essentially absent but Zelda is still required (Liang et al. 2008; Liberman and Stathopoulos 2009). Further analysis of *cis*-regulatory region architecture suggested that the organization of sites relative to each other, in particular of the relationship between Dorsal and Twist transcription factors, may be important to support expression in given domains (Jiang and Levine 1993; Erives and Levine 2004; Papatsenko and Levine 2005; Zinzen et al. 2006b).

Insights into the *cis*-Regulatory Mechanisms Controlling Dorsal Target Gene Expression: Tiling Arrays and ChIP Techniques

Tiling arrays, in which probes are designed to span the entire genomic sequence, typically at ~100 base-pair intervals, facilitated an assay of all sequences including the noncoding regions that were absent from previous open-reading-frame restricted arrays. In addition to presenting an unbiased platform useful for gene expression studies, the tiling arrays also allow whole-genome chromatin immunoprecipitation (ChIP) experiments (i.e., ChIP-chip) that assay in vivo occupancy of transcription factor binding.

Tiling arrays were used in expression-based screening to identify transcripts that are differentially expressed along the DV axis. Besides increasing the number of genes exhibiting Dorsal-dependent regulation from approximately 50 predicted by standard chip hybridizations (Stathopoulos et al. 2002) to approximately 100 (Biemar et al. 2006), the tiling arrays also identified specific, differentially expressed splice products (e.g., *bunched*) and miRNAs (mir1 and mir9a), that could not be distinguished with standard arrays containing probes designed only to assay predicted gene products.

Experiments to identify *cis*-regulatory regions using ChIP-chip methodology were able to identify in vivo binding site occupancy for Dorsal, Twist, and Snail transcription factors (Sandmann et al. 2007; Zeitlinger et al. 2007). Hundreds of newly predicted *cis*-regulatory regions were found throughout the genome, based on the assumption that occupancy of a factor on the DNA is associated with changes in gene expression observed for nearby genes. Five novel Dorsal-binding (Zeitlinger et al. 2007) and six novel Twist-binding *cis*-regulatory sequences (Sandmann et al. 2007) were confirmed. These studies also suggested extensive cross talk between DV and AP patterning (Fig. 3C) (Zeitlinger et al. 2007). Furthermore, other ChIP-chip analyses conducted in the early *Drosophila* embryo showed that the occupancy of genomic sites by most transcription factors is extensive (Li et al. 2008), the suggestion being that much of the binding identified by ChIP analyses is not necessarily required for the spatial patterns of gene expression. Future studies will be required to confirm how many occupied sites produce a functional output for each of the transcription factors in question. Nevertheless, ChIP-chip analyses represent a significant advance over pure in silico bioinformatic approaches in the identification of *cis*-regulatory sequences.

Biological Insights into Patterning: The Gene Regulatory Network

The regulatory interactions responsible for patterning the early *Drosophila* embryo are summarized in a gene-regulatory network, a circuit

Figure 3. (*Continued*). It remains to be determined whether *shn* or another repressor functions in dorsal regions. (Image of embryo modified, with permission, from Stathopoulos and Levine 2005a, ©Elsevier.) (*C*) Cross talk between the Dorsal and AP patterning networks. ChIP-chip analyses with Dorsal, Snail, and Twist antibodies reveal strong binding peaks (*left*) for one or more of these proteins in several AP patterning genes. In situ hybridizations of reporter gene expression in whole-mount embryos (*right*) reveal DV asymmetries in these genes. (Reprinted, with permission, from Zeitlinger et al. 2007.)

diagram that describes all the genetic and *cis*-regulatory interactions uncovered to date (Fig. 3A) (Levine and Davidson 2005; Stathopoulos and Levine 2005a). The information depicted includes genetic interactions based on mutant analysis, *cis*-regulatory information, and ectopic expression experiments. The *cis*-regulatory sequences serve as a platform by which information is processed in particular cells and a decision made to either express or silence a gene. Arrows at the end of lines symbolize binding to *cis*-regulatory regions by activators, whereas blunt ends symbolize repressors binding to *cis*-regulatory regions.

Analysis of this network reveals that the *Drosophila* embryo uses different mechanisms to establish domains of expression. Feed-forward loops, such as the requirement of the Dorsal-target gene, Twist, to turn on the repressor Snail, function as timing mechanisms that ensure that genes are expressed in proper sequence. In addition, patterning is initiated when the embryo itself is a syncytium, an unusual aspect of *Drosophila* embryogenesis. The common cytoplasm of the *Drosophila* syncytium permits the formation of transcription-factor gradients, which can directly act on gene-expression outputs. This stands in contrast to patterning in a cellularized environment, which relies heavily on cell–cell communication coupled to signal transduction (Fig. 3B). Clearly, some signaling is active within the syncytium as Toll signaling controls nuclear import of Dorsal, but it is unclear how many other signaling pathways are also functioning. In contrast, it has been shown that several signaling pathways become active immediately following cellularization.

Differential Expression of Signaling-pathway Components Along the DV Axis Controls Patterning and Cell Movement

Roughly half of the estimated Dorsal target genes encode signaling molecules. These genes are essential for the localized activation of FGF (fibroblast growth factor), EGFR, and TGF (transforming growth factor)-β signaling pathways in the mesoderm, neurogenic ectoderm, and dorsal ectoderm of pregastrula embryos, respectively (Fig. 4A) (reviewed in Stathopoulos and Levine 2004). For example, localized TGF-β signaling in the dorsal ectoderm depends on Dorsal-regulated silencer elements that are associated with three target genes, *dpp*, *tld*, and *zen*, as well as restricted expression of *sog* and *brk* in ventrolateral regions of the embryo. Through spatial restriction of multiple signaling pathway components, Dorsal ultimately controls not only further patterning, as in the case of TGF-β, but also cell movements and subsequent differentiation events, as in the case of FGF signaling.

The FGF receptor (FGFR), Heartless (Htl), and its two ligands—Pyramus (Pyr) and Thisbe (Ths)—are all Dorsal targets (Stathopoulos et al. 2004). Htl is one of only two FGFRs present in *Drosophila* (reviewed in Szebenyi and Fallon 1999), and controls mesoderm migration during gastrulation (Beiman et al. 1996; Gisselbrecht et al. 1996). *htl* expression requires peak levels of nuclear Dorsal and/or Twist (Figs. 2D and 4A) (Stathopoulos et al. 2004). *pyr* and *ths* were first discovered in microarray screens designed to identify genes expressed along DV axis (Stathopoulos et al. 2002). The *pyr* and *ths* genes, which are linked on the genome, encode related FGF ligands and show dynamic patterns of gene expression in the embryo (Stathopoulos et al. 2004; Gryzik and Müller 2004). The early expression of the *ths* gene is Dorsal-dependent and it is induced by even the lowest levels of nuclear Dorsal present in dorsolateral regions of the embryo (Figs. 2D and 4A) (Stathopoulos et al. 2004). After internalization of presumptive mesoderm cells (i.e., invagination), cells containing the FGFR are free to contact cells expressing the ligands, enabling activation of the FGF signaling pathway.

In general, cell movements are orchestrated during gastrulation through the action of signaling pathways, which become active after cellularization. Twist and Snail transcription factors play an instrumental role in controlling invagination and ventral furrow formation by regulated expression of the genes *folded-gastrulation*, *concertina*, and *T48* (Parks

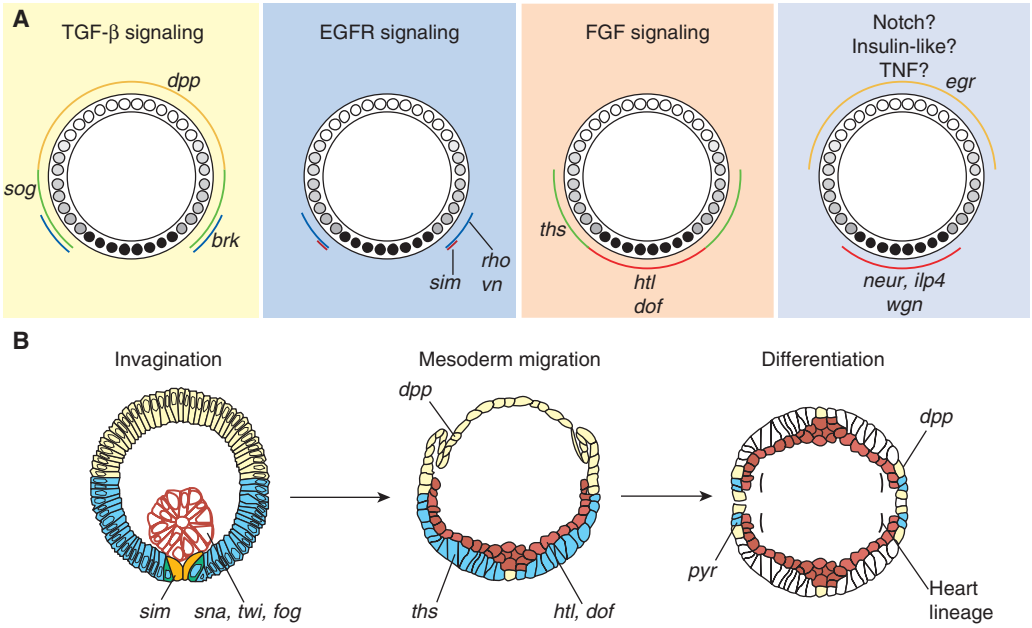

Figure 4. Dorsal target genes are integral components of signaling pathways that function to control gastrulation and differentiation. (*A*) Dorsal regulates the activation of the TGF-β, EGFR, and FGF signaling pathways by spatial regulation of pathway components. Dorsal target genes also include components of Notch, Insulin-like, TNF, and Wnt (not shown) pathways. Whether this results in regulated activation remains to be determined. (Modified, with permission, from Stathopoulos and Levine 2004, ©Elsevier.) (*B*) Shown are cross sections through an embryo with the indicated expression patterns of specific genes involved in the respective processes: invagination (the invaginated mesoderm has formed a tube [red] and ectodermal cells are at the surface [blue and yellow]), mesoderm migration (the ectoderm forms a surface on which the mesoderm migrates), and differentiation of the cardiac mesoderm (dorsal somatic lineages including heart precursors are induced when the mesoderm contacts Dpp-expressing cells). Expression of *ths* (Blue), *htl* and *downstream of Fgf* (*dof*) (Red), and *dpp* (Yellow). (Reprinted, with permission, from Stathopoulos and Levine 2004, ©Elsevier.)

and Wieschaus 1991; Dawes-Hoang et al. 2005; Sandmann et al. 2007). This results in an epithelial-to-mesenchymal transition (EMT) of the presumptive mesodermal cells located in the interior of the embryo. These cells then proceed to migrate along the ectodermal cells toward dorsal regions of the embryo (Fig. 4B). This migration is required for subsequent differentiation of the mesoderm into distinct cell types such as cardiac, dorsal somatic, and visceral mesoderm cell lineages. FGF signaling likely functions at multiple steps during the migration to control the directed movement of cells (McMahon et al. 2008; Kadam et al. 2009). As a result of this coordinated migration, once mesoderm cells reach the dorsal ectoderm, multiple signaling inputs control which differentiation programs are adopted. For instance, in addition to FGF signaling, TGF-β signaling is also required to induce mesoderm cells that reach the dorsal-most regions of the ectoderm to adopt cardial and dorsal somatic mesoderm lineage choices (Fig. 4B).

CONCLUDING REMARKS

Much work has been focused on the study of the Dorsal patterning network. Classical genetic studies have uncovered several Dorsal target genes, as well as many of the components of the upstream pathway. Conventional promoter analysis methods using reporter genes have

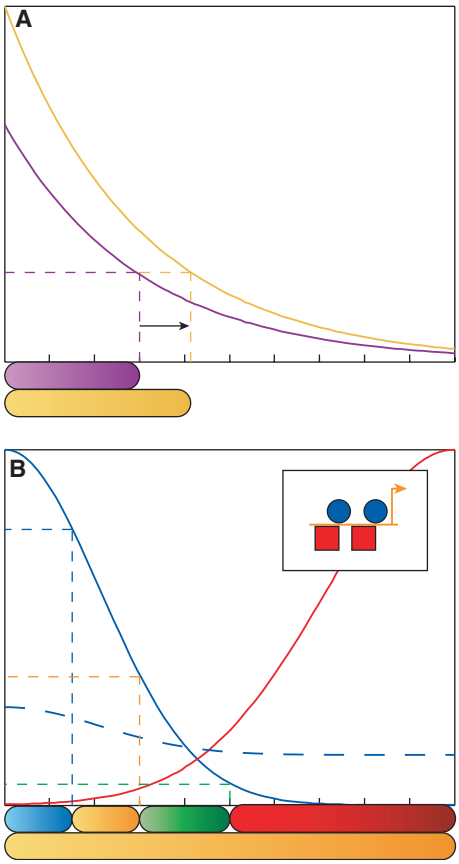

Figure 5. Revisiting the morphogen gradient model. (*A*) Depiction of classical morphogen gradient (purple). Horizontal dashed line denotes the concentration threshold that defines one cell fate boundary (shown below the graph). Gold curve denotes the same morphogen simulated with a 50% increase in morphogen production. The classical morphogen model predicts that the cell fate boundary, in response, will also shift by roughly 50% (arrow). This is an unacceptably sensitive system, and does not comport with experimental evidence. (*B*) Illustration of the affect of multiple inputs to *cis*-regulatory modules. In this hypothetical tissue, a primary morphogen (blue) initiates expression of several target genes (blue, orange, green, red) within the tissue. A secondary morphogen (red) is expressed in the red cells, but is repressed in the rest of the tissue by the primary morphogen, and acts as a repressor to other target genes (*Inset*). Consider a case in which the primary morphogen is present in a shallow gradient (dotted blue), at a concentration above green threshold, yet below the orange threshold, so that no secondary morphogen is present. The classical morphogen gradient model would predict all cells to turn green. However, because the secondary morphogen also serves as input to the target genes (and is not present in this case), it is possible that instead the orange gene is ubiquitously expressed.

revealed the inputs and minimal enhancers that regulate several Dorsal target genes. Genome-level studies have increased this knowledge fivefold, from the standpoint of numbers of target genes and numbers of enhancers known. ChIP-chip studies have uncovered multiple enhancer sites and cross talk between AP and DV systems. All of these data together reveal several layers of regulation in this system, including multiple inputs to each of the Dorsal target genes (see Figs. 2C and 3B).

The existence of this regulation is not surprising, as physical arguments show that a lone morphogen is not sufficient to explain developmental patterning (Lander 2007; Jaeger et al. 2008). In particular, a single-morphogen model fails to account for the remarkable insensitivity of developmental patterns with respect to many natural and experimental perturbations to the system (see Fig. 5A). Therefore, any viable model of morphogen-mediated patterning must include multiple levels of regulation on target gene expression (see Figs. 2 and 3).

However, this is simply a *necessary* condition for developmental reliability. It remains to be seen whether the abundance of multiple inputs and feedback regulation seen in the Dorsal patterning system, and in morphogen systems in general, is *sufficient* to explain the exquisite precision of threshold outputs and the remarkable reliability seen in tissue patterning. Systems-level analyses of patterning mechanisms present an opportunity to address this question.

For example, tissue-level modeling has shown that the robustness of a pattern can generally be improved with negative feedback regulation (Eldar et al. 2003; Reeves et al. 2005). In the Dorsal system, negative feedback

occurs through zygotic expression of WntD (Ganguly et al. 2005; Gordon et al. 2005), and perhaps Cactus as well (Araujo and Bier 2000). In another example involving the Bicoid patterning system, a recent study of gradient interpretation directly shows that absolute concentration thresholds of Bicoid are not the only determinate factors in this system, likely because there are multiple spatially dependent inputs to Bicoid target genes (see hypothetical scenario in Fig. 5B) (Ochoa-Espinosa et al. 2009). Is this also true for the Dorsal patterning system? This is likely to be the case in light of the current evidence, which shows that multiple inputs exist to control the expression of genes along the DV axis (Figs. 2 and 3) and that compensatory mechanisms may support expression in mutant backgrounds that show decreased Dorsal levels (Stathopoulos and Levine 2002b). This would imply that the absolute levels of Dorsal are less important than the sum total of several factors present within nuclei, integrated in a complex manner at each individual enhancer site (see Fig. 5B). Thus, the key to differential gene expression is combinatorial regulation through the interaction of multiple transcription factors. This underscores the need for a thorough understanding of the network of gene regulatory interactions that supports expression of genes.

Answering systems-level questions regarding the formation and effects of morphogen gradients will require integrating data from a wide variety of time, length, and concentration scales, ranging from the *cis*-regulatory code to tissue-wide signaling interactions. Subfields of traditional and post-genomic experimental biology, quantitative microscopy, and computational biology will each exert significant influence on future studies of morphogen gradients.

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