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REVIEW

Dynamic patterning by morphogens illuminated by cis-regulatory studies

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ABSTRACT

Morphogen concentration changes in space as well as over time during development. However, how these dynamics are interpreted by cells to specify fate is not well understood. Here, we focus on two morphogens: the maternal transcription factors Bicoid and Dorsal, which directly regulate target genes to pattern *Drosophila* embryos. The actions of these factors at enhancers has been thoroughly dissected and provides a rich platform for understanding direct input by morphogens and their changing roles over time. Importantly, Bicoid and Dorsal do not work alone; we also discuss additional inputs that work with morphogens to control spatiotemporal gene expression in embryos.

KEY WORDS: *Drosophila melanogaster*, Bicoid (Bcd), Dorsal (Dl), Morphogen, Thresholds, Cis-regulatory mechanism, Gradient, Chromatin, Repressor, Activator, Enhancer, Embryonic patterning

INTRODUCTION

Morphogens are molecules distributed in a graded manner in order to provide positional cues for cell fate specification during development (Rogers and Schier, 2011; Wolpert, 1996). In general, morphogens are proteins that are produced from localized sources and transported in the extracellular space toward neighboring cells. Once the gradients are formed in this way, the receiving cells interpret their respective position within the tissue by sensing the concentration of morphogens. In response, cells activate different sets of target genes, thus initiating the patterning processes and ultimately defining cell fate. This phenomenon is observed for a number of morphogens acting in various organisms, such as sonic hedgehog (Shh) in chick neural tube patterning (Briscoe et al., 2001), Wingless (Wg) in *Drosophila* appendage development (Neumann and Cohen, 1997), squint in zebrafish germ layer patterning (Chen and Schier, 2001) and activin in *Xenopus* mesoderm and ectoderm induction (McDowell and Gurdon, 1999). Owing to the importance of morphogens for patterning, dissecting the molecular mechanisms by which they control gene activation has been an intense field of study for decades.

One of the core issues investigated with regards to morphogens is the mechanism by which their concentrations are translated into gene expression outputs. One of the earliest models of patterning proposed that differences in target gene spatial expression domains are directly related to morphogen concentration (Wolpert, 1969) (Fig. 1A). In this threshold-dependent model, target gene transcription is initiated only when the morphogen concentration is present above a certain level, whereas transcription is not initiated in domains where the morphogen concentration is present below this level. In this manner, gradients of morphogens regulate patterning in a field of cells by differentially controlling gene expression in response to the concentration change present in space.

In addition, recent studies have shed light on the importance of time, both duration and timing of exposure to morphogens, for target gene expression (Rogers and Schier, 2011; Sagner and Briscoe, 2017). Target gene expression is influenced by morphogen input as well as by the current gene expression state of the receiving cells. Responses to a given morphogen will differ between receiving cells with different extant gene expression programs. For example, during dorsal-ventral (DV) patterning in gastrulating zebrafish embryos, cell fate is specified in a temporally progressive manner due to changes in target tissue sensitivity to BMP signaling inputs. BMP signaling during early gastrulation supports patterning of anterior ventrolateral domains, whereas later the same signal promotes patterning of posterior ventrolateral domains (Tucker et al., 2008). Despite accumulating data supporting temporal specificity of morphogens in development, it remains unclear whether changes in levels of morphogens over time (i.e. morphogen dynamics) generally control dynamics in target gene expression directly. Target gene dynamics may relate instead to a function of the underlying gene regulatory networks (i.e. an indirect response). The highly context-specific response highlights the importance of understanding how time can influence the effects of morphogens on target genes.

In *Drosophila* embryos, two morphogens, Dorsal (Dl) and Bicoid (Bcd), control patterning along two body axes: the dorsal-DV and anterior-posterior (AP), respectively. How Dl and Bcd morphogens control patterning across the developing embryo has been and remains an active area of investigation. These specific morphogens are unusual because they act directly in nuclei before cellularization of the *Drosophila* embryo has occurred; therefore, the responses of their target genes do not rely on extensive intracellular signaling cascades. For this reason, and because *Drosophila* embryos develop very quickly (morphogen-mediated patterning occurs within only a few hours), it is often assumed that increases in morphogen concentration correlate directly with expression of target genes. In this Review, we compare what is known about Bcd- and Dl-dependent patterning mechanisms, with a particular emphasis on the functions of these morphogens over time. We also discuss what is known about additional inputs that contribute to spatiotemporal regulation of target gene expression. Modeling efforts aimed at understanding Dl and Bcd functions have been highlighted in recent reviews (Huang and Saunders, 2020; Schloop et al., 2020) and are therefore not discussed here, but suggest that morphogen concentration alone is not sufficient to predict target gene expression and reinforce the view that additional inputs are important.

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Development (2021) 148, dev196113. doi:10.1242/dev.196113

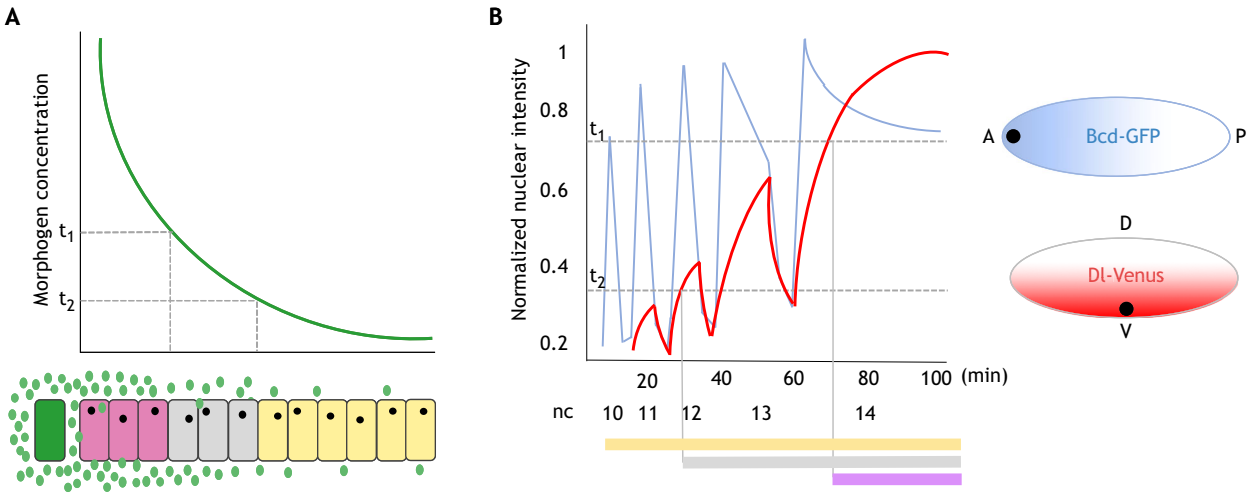

Fig. 1. Comparison of Bicoid and Dorsal morphogens in space and over time in *Drosophila* embryos. (A) The French flag model for patterning (Wolpert, 1969) includes morphogen production from a source (green) along with its diffusion to neighboring cells, generating a morphogen gradient. A cell exposed to morphogen concentration above threshold 1 (t_1) adopts one cell fate (pink), whereas cells at a distance adopt distinct cell fates (gray versus yellow) in response to lower morphogen concentrations above or below threshold 2 (t_2). (B) A conceptual representation of the AP patterning morphogen Bicoid (Bcd; blue) and the DV patterning morphogen Dorsal (Dl; red) from nuclear cycle (nc) 10 to 14. Fluorescence intensity was measured by monitoring Bcd-GFP or Dl-Venus. For Bcd-GFP, the measurements were taken at a single nucleus located 10% of the way along the AP axis. For Dl-Venus, the measurements were taken at the ventral-most region. The intensities were normalized to the maximum value (Gregor et al., 2007; Reeves et al., 2012). Scheme representing gradients of Bcd along the AP axis (blue) and Dl along the DV axis (red) in *Drosophila* blastoderm embryos, and a hypothetical target gene threshold responses graph. A, anterior pole; P, posterior pole; D, dorsal region; V, ventral region.

Drosophila morphogens Bicoid and Dorsal: gradient formation and dynamics

Upon fertilization, *Drosophila* embryos undergo 13 rounds of rapid DNA replication and mitoses in the absence of cytokinesis. These divisions, referred to as nuclear cycles, occur in a syncytium in which there are no cellular membranes and the embryo is essentially one cell. The first nine nuclear cycles last about 8 min each; whereas starting at nuclear cycle (nc) 10, the duration lengthens. At this point, widespread zygotic gene expression is initiated. It is at this early stage of the developmental process of the *Drosophila* embryo that patterning along the AP and DV body axes is established (reviewed by Stathopoulos and Newcomb, 2020). Two maternally deposited morphogens, Bcd and Dl, are key factors that orchestrate this patterning process.

Bicoid: a morphogen that controls patterning along the AP axis

Maternally expressed *bcd* mRNA contains localization sequences that result in the concentration of transcripts at the anterior pole of *Drosophila* embryos. Upon fertilization, translation of these localized transcripts results in a gradient of protein along the AP axis (Fig. 1B) (Driever and Nüsslein-Volhard, 1988). Synthesis in the anterior pole is followed by diffusion, as well as protein degradation, to extend the range and adapt the shape of the Bcd gradient, respectively (Durrieu et al., 2018; Gregor et al., 2007; Little et al., 2011). Once the gradient forms, Bcd, a homeodomain transcription factor (TF), binds to specific DNA sequence motifs, and differentially activates genes along the AP axis (Struhl et al., 1989). Bcd can affect target gene expression even in posterior regions, where the gradient is more diffuse, by forming local hubs within nuclei that are concentrated enough to facilitate Bcd binding to enhancers (Mir et al., 2017). Furthermore, despite the short time that Bcd molecules are bound to DNA (Mir et al., 2018), this input is translated into accurate and fast gene expression decisions at target enhancers that occur on the timescale of a few minutes (Desponds et al., 2020). Together, these mechanisms that regulate the distribution of Bcd in the embryo and its interaction with target enhancers determine the ability of this morphogen to differentially affect gene expression across the AP axis.

Although most studies of Bcd target gene expression are focused on nc14, nuclear Bcd is observed as early as nc6 and exhibits spatiotemporal dynamics (reviewed by Huang and Saunders, 2020; Little et al., 2011). Bcd levels rise quickly within nuclei at the onset of each nuclear cycle (Fig. 1B). Furthermore, although peak levels increase slightly from one nuclear cycle to the next, compared with Dl dynamics (discussed below) Bcd changes are relatively small when measured from the perspective of a single nucleus at the anterior of the embryo (Fig. 1B) (Gregor et al., 2007; reviewed by Sandler et al., 2018). In addition, studies of specific target genes, such as *hunchback* (*hb*), have suggested that Bcd input becomes dispensable by late nc14. At this point, the expression boundaries of many targets, including *hb, knirps* (*kni*) and *Krüppel* (*Kr*), are supported by cross-regulation of gap genes that function as repressors (Jaeger et al., 2004; Liu et al., 2013; Manu et al., 2009).

These particular studies have suggested that early nc14 is the pivotal time when Bcd provides positional information for patterning (Liu et al., 2013). However, another recent study has shown that removal of Bcd at any time between nc10 and gastrulation causes a loss of anterior-most embryonic structures (Huang et al., 2017). Whereas integration of Bcd over an ∼1.5 h period is required for a subset of target genes expressed in anterior cells, cells located more posteriorly only require Bcd input for a short time to establish the correct fates. In this way, cell fates along the AP axis are determined from posterior to anterior and may facilitate boundary formation, because nuclei that have reached their final fates may no longer be influenced by fluctuations in local concentration of Bcd (reviewed by Huang and Saunders, 2020). These results support the view that there is not one particular time point at which Bcd acts, but rather that it acts at different times depending on the AP localization of expression of its targets.

Development (2021) 148, dev196113. doi:10.1242/dev.196113

Dorsal: a morphogen that controls patterning along the DV axis

dl mRNA, like *bcd*, is maternally deposited; unlike *bcd*, *dl* mRNA and protein that is synthesized upon fertilization are uniformly distributed in precellular embryos (Anderson and Nüsslein-Volhard, 1984; Roth et al., 1989). Dl cannot enter the nucleus on its own, however, and the regulated transport of protein into ventral nuclei establishes a gradient that allows Dl to function as a morphogen to support DV axis patterning (Roth et al., 1989; Rushlow et al., 1989; Steward, 1989). Toll signaling pathway activation regulates this nuclear translocation of Dl protein. In the absence of Toll signaling pathway activation, Dl is bound by the IκB homolog Cactus and is therefore sequestered in the cytoplasm (Belvin and Anderson, 1996). Cactus facilitates diffusion, or shuttling, of Dl within the syncytial blastoderm by forming a Dl/Cactus complex (Carrell et al., 2017). This mechanism also helps to ensure that patterning is robust to changes in Dl morphogen concentration (Al Asafen et al., 2020). Activation of the Toll receptor leads to a downstream intracellular cascade that ultimately results in phosphorylation and subsequent degradation of Cactus. The Toll receptor is ubiquitously expressed throughout embryos; however, its ligand Spätzle is activated in a graded manner along the DV axis within the extracellular space, such that highest Toll receptor activation occurs in ventral regions (reviewed by Reeves and Stathopoulos, 2009). In this way, Dl is freed from the Dl/Cactus complex in a ventrally biased manner and is then imported into nuclei, where it activates over 50 genes (Belvin et al., 1995; Stathopoulos et al., 2002). The result of Toll signaling is a nuclear-cytoplasmic Dl gradient with the highest levels of nuclear Dl in ventral regions and progressively lower levels dorsally (Fig. 1B).

In contrast to the fast nuclear import displayed by Bcd at the onset of each nuclear cycle, levels of nuclear Dl rise slowly throughout the nuclear cycle. This difference likely relates to Toll-dependent signaling being required for Dl nuclear import, whereas Bcd is free to enter nuclei immediately once they reform after each division. Nuclear Dl levels rise during each nuclear cycle, plummet upon nuclear envelope breakdown at division and are slowly re-established upon nuclei reformation following division (Reeves et al., 2012). In addition, Dl levels rise between nuclear cycles, never reaching a steady state; such that, from the perspective of a nucleus located in ventral regions, input exhibits a saw-tooth trend (Fig. 1B).

Cis-regulatory interpretation of morphogen concentration and importance of additional inputs

The dynamics associated with the morphogens Bcd and Dl suggest that both provide not only positional cues, but also impart temporal information toward target gene expression during the *Drosophila* body patterning processes. Enhancer sequences provide information about the cis-regulatory logic of inputs that function to support gene expression outputs and provide insight into the mechanisms of action of these morphogens.

Interactions between morphogen transcription factors and pioneer factors initiate gene activation during embryonic patterning

Enhancers are short regions of DNA that control transcription of genes, presumably, by making direct contact with gene promoters (reviewed by Furlong and Levine, 2018). In general, enhancers are DNA sequences of a few hundred base pairs long (average ∼500 bp) located in *cis* to target genes, and include short, specific TF-binding sites. Enhancer activity is context dependent and perturbing its action can lead to developmental defects and disease (Bhatia et al., 2013; Lupiáñez et al., 2015; Uslu et al., 2014).

During early *Drosophila* development, TFs distributed in gradients serve as morphogens to support cell fate specification by controlling enhancer activity. Quantitative *in vivo* measurements of animal transcription, including recent innovations in live imaging, are widely used to extend understanding of morphogen responses (Ferraro et al., 2016). Specific, quantitative features of gene transcription, such as precision, accuracy, robustness, plasticity and stability can now be reliably assayed (Bentovim et al., 2017). For example, a study of the *hb* proximal enhancer, a 750 bp region regulated by Bcd, has demonstrated that this enhancer, together with its associated promoter, P2, is sufficient to properly capture the spatiotemporal dynamics of establishment of the *hb* pattern (Lucas et al., 2018). However, in this particular case, mathematical modeling suggested that the six Bcd-binding sites present in this enhancer sequence are insufficient to support expression that is as steep as the *hb* gene expression pattern. Additional input has been invoked to explain this discrepancy, because either additional cryptic, possibly low-affinity, Bcd-binding sites and/or other TFs distributed in gradients could also affect the delineation of the boundary. For example, maternal Hb protein is also present in an anterior-to-posterior gradient, and supports zygotic *hb* expression by facilitating expression at a lower Bicoid concentration threshold and by making activation faster (Porcher et al., 2010; Simpson-Brose et al., 1994). Although Bcd activates more than 40 target genes to initiate AP patterning in an apparently concentration-dependent manner, it is recognized that input from other factors is necessary to support proper target gene expression (reviewed in in Briscoe and Small, 2015).

In particular, during early embryonic development, enhancer regions must become free of nucleosomes, in order to be accessible to binding of morphogen TFs. This process is supported by a particular class of TFs called pioneer factors. Pioneers are the first sequence-specific DNA-binding factors to engage target sites within chromatin during development. A pioneer factor can bind to target enhancers in ‘closed’ or inaccessible chromatin and facilitate chromatin remodeling processes, which allows other TFs to subsequently bind enhancers to control target gene activity (Zaret and Carroll, 2011). At the early blastoderm stage, maternally deposited Zelda (Zld) acts as a crucial pioneer factor to increase chromatin accessibility by supporting local depletion of nucleosomes with the effect being dependent on the number and position of Zld motifs (Li et al., 2014; Schulz et al., 2015; Sun et al., 2015). Furthermore, Zld functions as a global activator to initiate zygotic gene expression, including early body axis patterning genes in embryos (Harrison et al., 2011; Nien et al., 2011).

Zld associates with enhancer sequences for patterning genes that are subsequently also bound by Bcd and Dl. A recent study has examined occupancy of Zld and Dl on DNA throughout the genome with fine temporal resolution, revealing that although Zld binding can be detected as early as nc8, little to no Dl binding is observed at that time (Li and Eisen, 2018 preprint). These results suggest that Zld input precedes that of Dl and possibly potentiates Dl binding at target gene enhancers. In *zld* mutants, chromatin accessibility at enhancers decreases, and Bcd or Dl binding is significantly reduced (Li and Eisen, 2018 preprint; Xu et al., 2014). Thus, Bcd- or Dl-dependent enhancers lose their activity when *zld* is absent. Similarly, when the number of Zld-binding sites in enhancers of target genes is reduced, reporter expression can be delayed and exhibits domain shifts (Yamada et al., 2019). In contrast, addition of more Zld-binding sites to enhancers can result in precocious expression (Bosch and Bosch, 2006; Foo et al., 2014) or expansion of the expression domains (Ozdemir et al., 2014). Furthermore,

Development (2021) 148, dev196113. doi:10.1242/dev.196113

introducing Zld-binding sites into an inactive enhancer can convert it into a morphogen-dependent responsive enhancer (Foo et al., 2014; Xu et al., 2014). Zld does not remain detectably associated with mitotic chromosomes, although sub-nuclear dynamic hubs form where transient Zld binding likely occurs (Dufourt et al., 2018). It is possible that Zld potentiates transcriptional outputs by the Dl and Bcd morphogens by modulating the nuclear environment to support their ability to exist in hubs (Mir et al., 2018; Yamada et al., 2019), by influencing the activity of specific promoters (Ling et al., 2019) and/or by supporting poised RNA polymerase at target genes pre-activation (Boija and Mannervik, 2016; Koenecke et al., 2017). Taken together, these studies indicate that Zld likely functions by priming the genome to potentiate morphogens to activate target gene enhancers in space but also by influencing gene expression timing.

Although, to date, no studies have demonstrated whether Dl can directly regulate chromatin accessibility, a recent study using ATAC-seq has analyzed the ability of Bcd to support chromatin accessibility (Buenrostro et al., 2015; Hannon et al., 2017). Chromatin accessibility associated with particular Bcd target genes active along the AP axis is sensitive to levels of this morphogen (Hannon et al., 2017). In particular, the results are consistent with a model in which Bcd operates at high concentrations at the anterior of embryos to establish chromatin accessibility at target sites. When levels of Bcd are artificially increased, enhancers associated with some Bcd target genes most sensitive to Bcd levels exhibit increases in chromatin accessibility. These results suggest that high levels of Bcd promote remodeling of chromatin structure.

Relationship of affinity of binding sites for Dl and Bcd within target gene enhancers to threshold outputs

Once enhancer regions of target genes become accessible, morphogens also play a role in promoting activation of transcription to support the patterning process. As discussed above, in the threshold-dependent model, morphogen concentration provides positional information to drive cells to differentiate into distinct fates by activating domain-specific genes. In the context of *Drosophila* DV patterning, three Dl-dependent thresholds have been characterized, high, intermediate and low (Fig. 2A), that correspond to three categories of genes: types I, II and III expressed in distinct domains (reviewed by Reeves and Stathopoulos, 2009). Type I genes are expressed in the ventral region of the embryo, where the highest levels of Dl are established due to Toll signaling. In this region, presumptive mesodermal target genes including *snail* (*sna*) and *twist* (*twi*) are activated. In the ventrolateral region of the embryo, intermediate levels of Dl activate type II genes, including *ventral nervous system defective* (*vnd*) and *vein* (*vn*), which specify the presumptive neurogenic ectoderm. Type III genes are active in more dorsal regions and have two modes of expression in response to low levels of morphogen: those activated by low Dl that are present in lateral regions, such as *short gastrulation* (*sog*); and those repressed by low Dl with expression limited to dorsal regions, such as *decapentaplegic* (*dpp*). These subsets of type III genes support neurogenic ectoderm and dorsal ectoderm fates, respectively (Fig. 2A). Furthermore, mutant backgrounds with uniformly high, intermediate or low levels of Dl exhibit broad expression of either type I, II or III genes, respectively; these genotypes facilitated the identification of over 50 Dl target genes and miRNAs using gene expression profiling (Biemar et al., 2006; Stathopoulos et al., 2002).

One molecular model proposed to explain threshold responses is that Dl-binding site affinity sets the Dl level required for enhancer activation (reviewed by Hong et al., 2008b; Reeves and Stathopoulos, 2009). In general, ventrally expressed genes (i.e. type I) have enhancers with low-affinity Dl-binding sites, so it follows that their activation requires high Dl levels. In contrast, ventrolaterally expressed genes (i.e. type II) generally have enhancers with high-affinity Dl-binding sites, so their activation is possible at lower levels of Dl (Fig. 3A). A study of 18 enhancers active along the DV axis found evidence that affinity of Dl-binding sites correlates with DV position of gene expression (Papatsenko

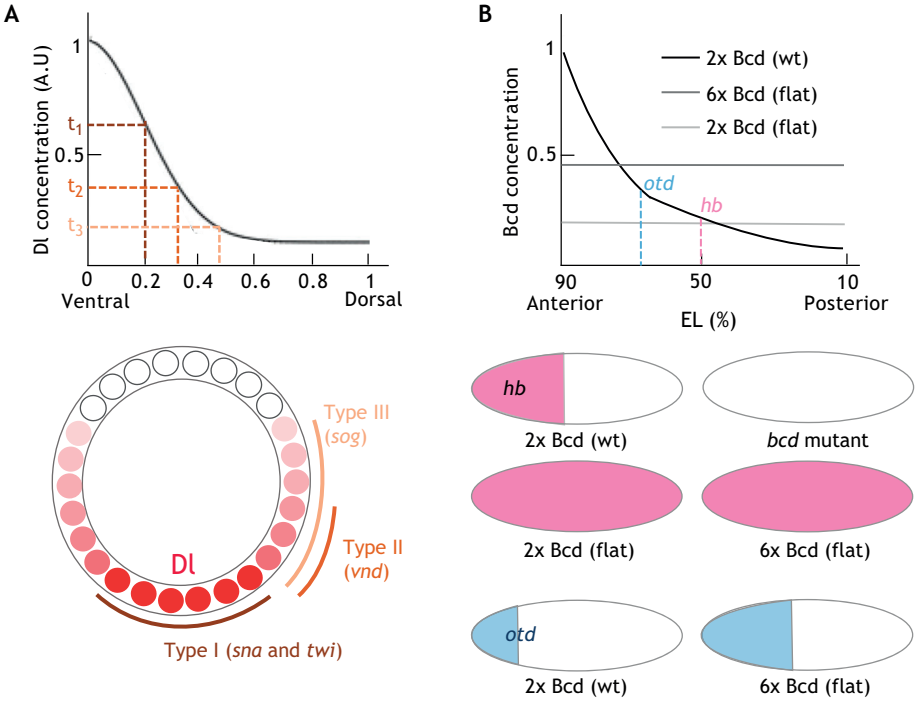

Fig. 2. Morphogen target genes are activated in a threshold-dependent manner. (A) Dl threshold-dependent gene activation along the DV axis of *Drosophila* embryos. The graph shows how three different Dl thresholds (t_1, t_2 and t_3) establish three gene expression domains along the DV axis. Schematic of an embryo cross-section showing three gene expression domains (types I-III) dictated by threshold-dependent responses to graded nuclear Dl levels (red). (B) A set of Bcd target genes is controlled by Bcd in a threshold-dependent manner. In wild-type embryos with two copies of *bcd*, Bcd exists in a graded manner along the AP axis [2x BCD (wt), black line] and also supports *hb* expression (pink) at the anterior of embryos. In a *bcd* mutant, *hb* expression is lost. When the Bcd gradient is flattened by assay in specific mutant backgrounds, resulting in intermediate [6x BCD (flat), mid-gray line] or low [2x BCD (flat), light-gray line] levels of Bcd throughout the embryo, *hb* expression expands to the posterior pole region. Another Bcd-target gene, *otd* (blue), also is expressed at the anterior in wild-type embryos, but 6x BCD embryos exhibit only posterior expansion of *otd,* not the ubiquitous expression seen for *hb* (Ochoa-Espinosa et al., 2009). EL, egg length.

Development (2021) 148, dev196113. doi:10.1242/dev.196113

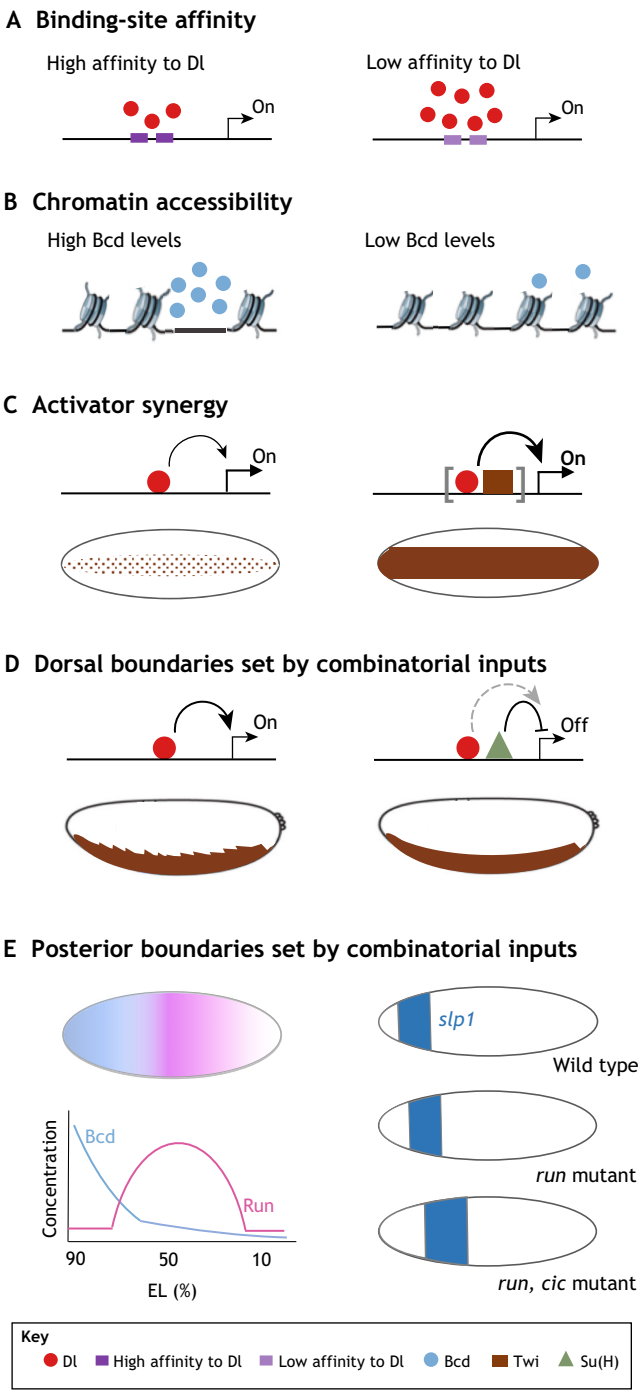

Fig. 3. Factors influencing threshold outputs. (A) Affinity of Dl-binding sites (high affinity, dark purple; low affinity, light purple) on target enhancer regions dictates Dl threshold levels (red). (B) Regulation of chromatin accessibility by Bcd (blue). In nuclei with a high Bcd concentration, chromatin is more accessible compared with the low Bcd concentration domain. (C) Cooperative inputs of Dl and its activator Twi (brown rectangle) synergistically support target gene expression (shown in brown). (D) Dorsal boundaries of DV patterning genes are correctly set by a repressive input, e.g. from Su(H) (green triangle), which acts to limit Dl-dependent activation (Ozdemir et al., 2014). Without repressive Su(H) input, the boundaries of *sna* (brown) are imprecise. In comparison, combinatorial input from Dl and Su(H) supports precise *sna* dorsal boundaries with clear on/off domains of expression. (E) Posterior boundaries of AP patterning genes are controlled by Run (pink). In the *run* mutant, *slp1* expression (blue) slightly expands posteriorly, and expands even more posteriorly in a *run* and *cic* double mutant. EL, egg length.

and Levine, 2005). Since this analysis was conducted, many additional enhancers active along the DV axis have been characterized (Ozdemir et al., 2011; Zeitlinger et al., 2007), including a number of secondary (shadow) enhancers associated with genes (Hong et al., 2008a), bringing the current enhancer tally closer to 50. As these additional enhancers have yet to be thoroughly analyzed with respect to TF binding, it remains to be seen whether Dl-binding site affinity does indeed correlate with position of target gene boundaries along the DV axis in general.

Additional evidence in support of the threshold model comes from genes whose boundaries of expression along the DV axis shift over time in concert with Dl gradient dynamics (Reeves et al., 2012). For example, *sna* is expressed in the ventral region of embryos by nc13, at which point it can be deduced that the Dl concentration has risen above the threshold required to support its activation. Subsequently, during early nc14, as Dl levels continue to increase, *sna* expression dorsally expands, indicating that the level of Dl necessary to support its expression changes spatially over time (Reeves et al., 2012). Presumably, *sna* exhibits a real-time response to dynamic morphogen input.

However, some studies reveal additional complexities that highlight the limitations of the threshold model alone for explaining morphogen-dependent gene expression. For example, *twist* (*twi*) is an early Dl target gene that encodes a bHLH TF that functions in a presumed feed-forward mechanism along with Dl to support ventral gene expression (Kosman et al., 1991). Many enhancers associated with genes expressed along the DV axis are bound by both Dorsal and Twist (Zeitlinger et al., 2007). When Twi is ectopically expressed at high levels, the Dl threshold responses are spatially reversed (Stathopoulos and Levine, 2002), suggesting that, although Dl threshold responses exist, they also likely receive input from other factors and can be influenced by Twi levels in particular. Furthermore, as many of the target genes of Dl are co-regulated by multiple enhancers, the affinity of Dl-binding sites to one enhancer may not be a good predictor of the role of Dl in the context of multi-enhancer cis-regulatory systems, as discussed below.

Similar to the DV patterning target genes, a set of genes expressed along the AP axis directly respond to Bcd in a concentration-dependent manner (Driever et al., 1989). In embryos in which the gradient has been flattened using specific genetic backgrounds that produce uniform Bcd levels across the embryo, AP target genes *hb*, *Kr* and *giant* (*gt*) are expressed in an on/off fashion (Fig. 2B) (Ochoa-Espinosa et al., 2009). Like ubiquitous expression of Dl target genes in mutant backgrounds that contain one level of Dl (e.g. Stathopoulos et al., 2002), the genes *hb* and *gt* are responsive to specific levels of Bcd and are expressed broadly in embryos that contain low or intermediate levels of Bcd throughout, respectively (Ochoa-Espinosa et al., 2009). In contrast, the genes *orthodenticle* (*otd*), *empty spiracles* (*ems*) and *buttonhead* (*btd*) are not ubiquitously expressed in embryos with flattened Bcd gradients but instead exhibit shifts only in the positions of their posterior boundaries of expression (Ochoa-Espinosa et al., 2009) (Fig. 2B). This result suggests that *otd*, *ems* and *btd* boundaries are positioned by other mechanisms, not simply in response to Bcd levels. However, a more recent study has suggested that the approach of flattening of the Bcd gradient using mutants does not completely flatten Bcd levels and that a shallow gradient is still present. This other group, using a transgenic approach to flatten the Bcd gradient, has found that some target genes (e.g. *btd*) respond with all or none responses to Bcd levels, whereas others (e.g. *kni*) exhibit only a shift (Hannon et al., 2017). Taken together, the results suggest that, similar to Dl target genes, some AP patterning genes

Development (2021) 148, dev196113. doi:10.1242/dev.196113

are activated and positioned in a Bcd concentration-dependent manner, but others are not; it is certain that additional factors also play roles during the patterning process along the AP axis.

In contrast to a preliminary analysis of a subset of Dl target enhancers (Papatsenko and Levine, 2005), an analysis of Bcd target gene enhancers has found little correlation between the affinity of binding sites within target gene enhancers and the expression domains of many AP-patterning genes. This finding does not support a model in which Bcd threshold response relates simply to binding-site affinity (Briscoe and Small, 2015; Ochoa-Espinosa et al., 2005; Segal et al., 2008). Surprisingly, however, chromatin accessibility of Bcd target genes is sensitive to Bcd concentration and suggests that gene expression may be threshold dependent, even if the affinity of specific binding sites does not always follow a straightforward correlation (Fig. 3B) (Hannon et al., 2017). It is possible that not only the binding-site affinity, but also the length of exposure to a morphogen, can influence threshold outputs.

Combinatorial regulation by multiple factors in the establishment of expression boundaries along the AP and DV axes

While Dl is a pivotal input to target genes expressed in ventral regions along the DV axis, these genes also receive input from an early Dl target gene, Twi (Ozdemir et al., 2011; Sandmann et al., 2007), as noted above. During DV axis patterning, boundaries of ventral target genes are defined by synergistic interactions between Dl and Twi (Szymanski and Levine, 1995). For example, a 57 bp sequence within the *twi* proximal element (*twi*_PE) enhancer, which is located adjacent to the promoter, has two low-affinity Dl-binding sites and drives expression within a 12- to 14-cell width ventral domain (Jiang and Levine, 1993). The dorsal boundaries of the expression supported by this element are expanded to encompass a domain of 20 cells in width upon addition of two Twi-binding sites (E-box sequences) to the *twi*_PE sequence (Fig. 3C).

In addition to synergistic input by morphogens and other activators, repressors are also crucial regulators of the spatial limits of target gene expression. The Dl gradient establishes the initial expression pattern, but the early domains of expression of Dl target genes are, in general, broader than the final patterns. Dl target genes expressed in the presumptive neurogenic ectoderm are repressed by Sna to specify the ventral boundaries of these stripes of gene expression (Ip et al., 1992; e.g. Kosman et al., 1991). Sna repressor is expressed in ventral regions and functions by inhibiting initiation, not release, of paused RNA Polymerase II (Bothma et al., 2011). Positioning of the dorsal boundaries of Dl target genes requires additional repressive inputs, including those from the maternal proteins Suppressor of Hairless [Su(H)] (Ozdemir et al., 2014; Schweisguth and Posakony, 1992) and Capicua (Cic) (Ajuria et al., 2011; Garcia and Stathopoulos, 2011). In the case of *sna*, the Su(H) TF sets the dorsal boundary through its binding at the *sna* distal enhancer (Fig. 3D). When the Su(H)-binding sites are mutated in this enhancer, expression is expanded dorsally. Other Dl target genes (e.g. *sog*) also exhibit dorsally expanded expression in *Su(H)* mutants, suggesting a more general role for this factor (Ozdemir et al., 2014).

Boundaries of AP patterning genes are also set by combinatorial input from activators, pioneer factors, as well as repressors. Bcd-Hb cooperativity is important for activation of a subset of Bcd target genes (Porcher et al., 2010; Simpson-Brose et al., 1994). In particular, maternal and zygotic Hb is required for specification of anterior structures (Simpson-Brose et al., 1994). Alternatively, Zld pioneer factor has been shown to support activation of enhancers in response to low concentrations of Bcd in the posterior of embryos (Xu et al., 2014). Repressors also support AP patterning. For example, Runt (Run) TF is expressed in the embryonic trunk and excluded from the poles, and acts as a repressor to limit the posterior boundaries of particular Bcd-dependent target genes, such as *otd* (Chen et al., 2012; Gergen and Butler, 1988). Similarly, enhancers of *ems*, *sloppy paired 1* (*slp1*) and *sloppy paired 2* (*slp2*) also exhibit a posterior shift in the *run* mutant. At these enhancer regions, high-affinity Run-binding sites are enriched, and their mutation results in a posterior shift of the expression patterns of the target gene. Furthermore, posterior shifts become more severe when the enhancers are expressed in embryos mutant for multiple repressors, such as Run, Cic and the gap protein Kr (Chen et al., 2012; Löhr et al., 2009) (Fig. 3E).

Taken together, the studies described in this section expand our understanding of how morphogens support expression of patterning genes. Although these transcription factors can activate target gene enhancers in a concentration-dependent manner, input from morphogens alone is usually not sufficient to explain many aspects of target gene expression responses. Input from additional effectors at enhancers is key, where synergistic activation contributes to specificity and robustness of target gene response. Pioneer TFs are required to move nucleosomes so that morphogens and these other inputs can act. This combinatorial regulation by multiple factors modifies the concentration-dependent responses to morphogen gradients and sets the final expression domain of the target genes. Furthermore, multiple repressors delineate boundaries of genes expressed along both the DV and AP axes, and these factors can also influence timing of gene expression. Broadly expressed repressors Run and Su(H) can also act to influence the timing of enhancer action, possibly acting as a counterbalance to pioneer activators, such as Zld (Koromila and Stathopoulos, 2017). In this way, gradients of morphogens play a crucial role in activating expression of target genes across fields of cells such as the DV and AP axes in a concentration-dependent manner, but this regulation occurs in the context of multiple other nuanced inputs that ensure precise and robust outputs in space and time.

Morphogen inputs to multiple co-acting enhancers can vary

The *Drosophila* embryo has been instrumental as a model system for demonstrating that gene expression patterns are supported through coordinate action of enhancer sequences. Some cis-regulatory systems are composed of multiple, distinct enhancers that function in an apparently additive manner to generate gene expression patterns. For example, five enhancers combine to support the expression of seven stripes of expression of the gene *even skipped* (*eve*) (Fujioka et al., 1999; Small et al., 1992) (Fig. 4D). Even though these enhancers support predominantly different patterns, surprisingly, they also can interact; for example, deletion of the *eve* stripe 1 enhancer leads to precocious and expanded expression of *eve* stripe 2 (Lim et al., 2018), possibly due to sharing of repressor input.

In contrast, it also has become clear that some genes are regulated by several enhancers that support similar spatiotemporal activity (Barolo, 2012; Hong et al., 2008a; McGregor et al., 2007). This is true even in the short period of time before embryos undergo gastrulation. Owing to the similarity in expression output, the first enhancer identified at a locus was called the ‘primary enhancer’ and the subsequent enhancers that exhibited similar spatio-temporal expression were named ‘shadow enhancers’. It was initially thought that the functions of *Drosophila* shadow enhancers, typically located at a distance from the promoter, are redundant to counterpart

Development (2021) 148, dev196113. doi:10.1242/dev.196113

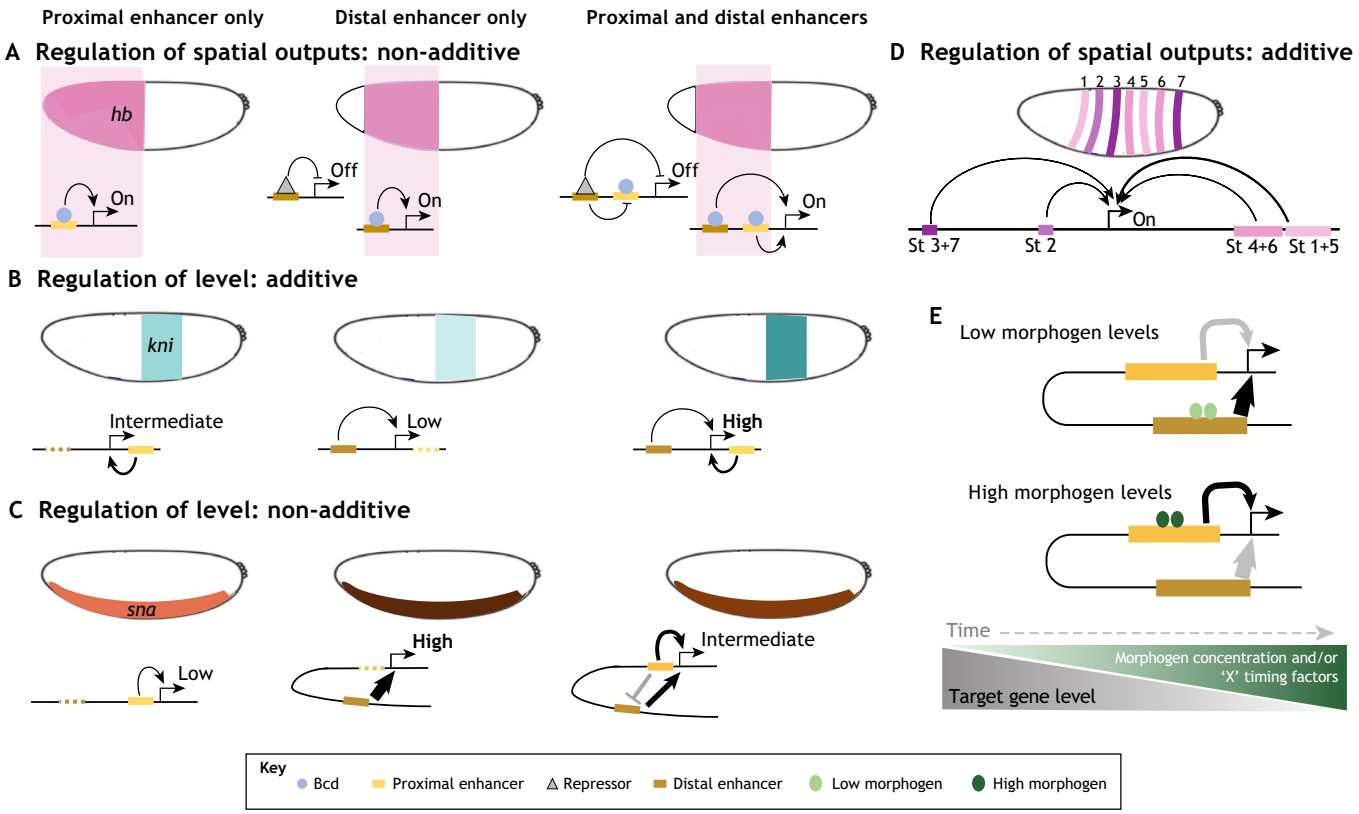

Fig. 4. Multiple enhancers coordinate to support precise gene expression outputs. (A) The proximal (yellow) and distal (gold) enhancers that are bound by Bcd to drive *hb* gene expression (pink) are active in similar domains, but the distal enhancer expression, specifically, has a repressive input that acts in a dominant fashion (Perry et al., 2012). (B) *kni* levels (teal) are supported by both proximal and distal enhancers, and each enhancer equally contributes to support *kni* levels (Bothma et al., 2015). (C) Proximal and distal enhancers at the *sna* locus act together to regulate levels of expression (brown) in a non-additive (or sub-additive) manner (Bothma et al., 2015; Dunipace et al., 2011). This response presumably relates to dominant repression by the proximal enhancer that acts to limit the distal enhancer activity. (D) *eve* expression (purple shades) is driven by multiple enhancers that support expression in predominantly distinct domains (reviewed by Borok et al., 2010). St, stripe. (E) Schematic showing how morphogen concentration may differentially control individual enhancer action over time in response to rising morphogen levels, as associated with Dorsal, and/or through input from timing factors that provide temporal information ('X').

primary enhancers, as is commonly the case in vertebrates (Xiong et al., 2002). More recently, it has been suggested that the *Drosophila* shadow enhancers that act to support patterning in embryos have similar, but not identical, functions compared with the associated primary enhancers (Dunipace et al., 2011; Dunipace et al., 2019; El-Sherif and Levine, 2016; Perry et al., 2011). Contributions from multiple enhancers to a single target gene complicate the issue of how concentration-dependent input from morphogens and regulation by other transcription factors translate into observed gene expression outputs.

Dominant repression of multiple enhancers promotes dynamic shifts during patterning

During patterning, accumulating evidence suggests that refinement of the expression domain is predominantly controlled by distal ('shadow') enhancers (Dunipace et al., 2011; Perry et al., 2011). At the *sna* locus, the proximal enhancer supports a slightly expanded domain, whereas the distal enhancer supports a domain similar to that supported by the full *sna* locus (Dunipace et al., 2011). This is because the distal enhancer is responsive to input from repressors (Ozdemir et al., 2014). Furthermore, the distal enhancer limits the proximal enhancer activity in the region where the gene is repressed. In this manner, the distal enhancer can dominantly affect the final expression domain of the gene (Fig. 4A). It is possible that distal enhancers are associated, more generally, with dominant repression inputs and this allows genes to be silenced conditionally only when the enhancer is active.

Non-additive expression output is not only a feature of genes expressed along the DV axis, but is also a crucial mode of regulation in genes expressed along the AP axis. For example, the proximal and distal enhancers at the *hb* locus share similar spatial and temporal activity in the anterior region in early embryos. However, unlike the proximal enhancer, the distal enhancer does not support expression at the anterior pole. At the anterior pole, Torso signaling represses *hb* expression, and only the distal enhancer is responsive to input from Torso signaling. To set the *hb* anterior boundary, the distal enhancer interferes with the proximal enhancer activity, resulting in *hb* repression at the anterior pole (Fig. 4A) (Perry et al., 2011). Furthermore, non-additive effects are also observed in the cis-regulatory systems associated with other genes expressed along the AP axis, including *kni* and *Kr* (El-Sherif and Levine, 2016). In this way, coordinate input from multiple enhancers is necessary to set correct boundaries for a number of AP genes, even in the cases where individual enhancer outputs appear similar (Barolo and Levine, 1997; El-Sherif and Levine, 2016; Perry et al., 2011).

In addition, enhancer interactions not only help to refine spatial patterns, but can also function to modulate levels of gene expression. For example, some genes, such as *kni, sna* and *Kr*, require balanced input from two enhancers (Bothma et al., 2015; Scholes et al., 2019). In the case of *kni*, two enhancers act in an

Development (2021) 148, dev196113. doi:10.1242/dev.196113

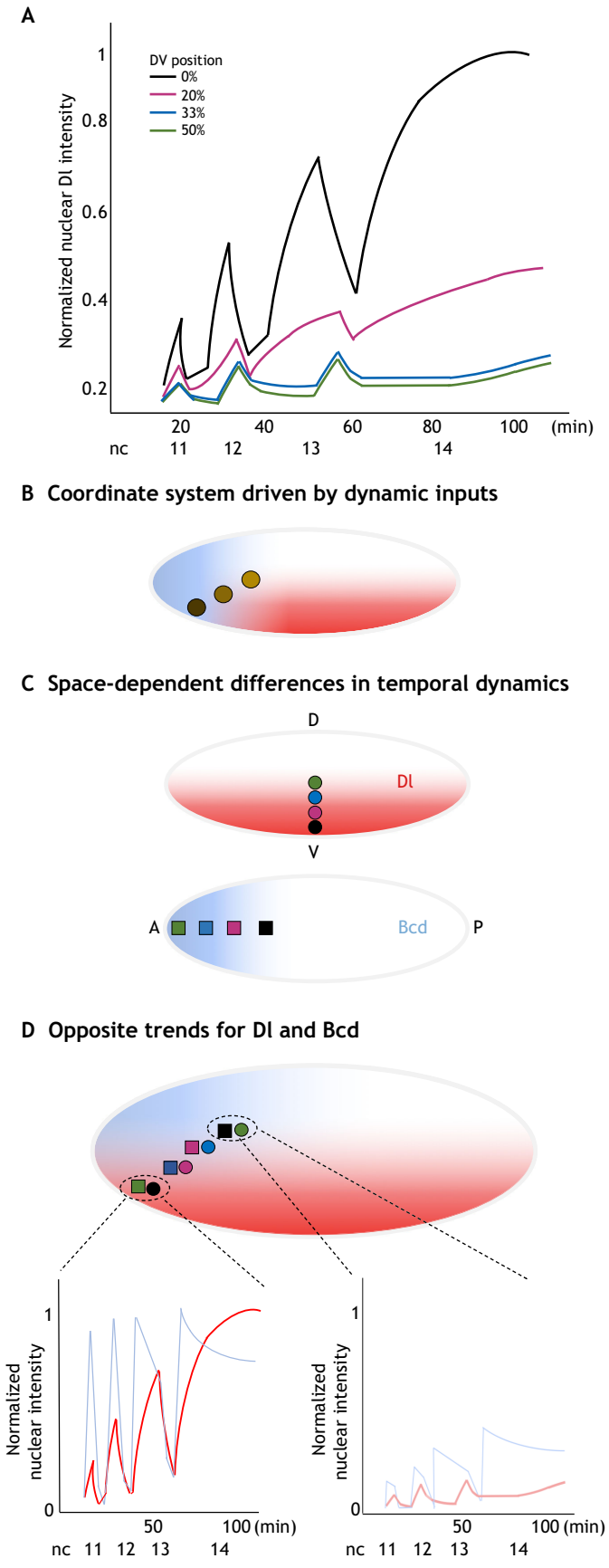

Fig. 5. Morphogen dynamics in time and space. (A) A representation of morphogen Dorsal (Dl) nuclear levels at four different positions along the DV axis from nuclear cycle (nc) 11 to 14. Fluorescence intensity was measured by monitoring Dl-Venus (Reeves et al., 2012). (B) Nuclei (brown) in different positions along the DV or AP axes exhibit different levels of Dl (red) and Bcd (blue) morphogens. (C) Dynamics of morphogen inputs over time may vary along the respective axes. This is measured for Dl (colored circles along the DV axis, top, correspond to A) and hypothesized for Bcd, which may relate to increased protein production over time or to diffusion. (D) Dual input by two morphogens exhibiting opposite dynamic trends. Nuclei along the DV axis exhibit progressively smaller changes in levels of nuclear Dl from one nuclear cycle to the next (see A), whereas it is hypothesized that Bcd exhibits the opposite trend over time such that a nucleus located more posteriorly may have a larger relative increase in Bcd levels compared with a more anterior nucleus (compare graphs).

additive way; generating higher levels of *kni* expression than is produced by either enhancer alone (Fig. 4B), whereas the distal and proximal *sna* enhancers act in a non-additive (sub-additive) manner to support proper levels of endogenous *sna* gene expression (Bothma et al., 2015; Dunipace et al., 2011) (Fig. 4C). Although each *sna* enhancer is responsive to Dl levels during early DV patterning (Irizarry et al., 2020), these two enhancers support *sna* with different strengths: the distal enhancer drives high expression levels ('strong enhancer'), whereas the proximal enhancer supports low expression levels ('weak enhancer') (Bothma et al., 2015; Dunipace et al., 2011). Presumably, frequent interactions between *sna* distal enhancer and the *sna* promoter mediate RNA polymerase II binding and release at the maximal level, so additional input from the weak proximal enhancer does not further increase *sna* overall levels (Bothma et al., 2015). Instead, when the proximal enhancer is deleted, *sna* levels increase relative to when both enhancers are present. In contrast, when the distal enhancer is deleted, *sna* levels decrease in comparison with when both enhancers are present. Thus, these results suggest that the proximal enhancer attenuates the activity of the distal enhancer and the action of both enhancers is required to support correct *sna* levels (Fig. 4C) (Dunipace et al., 2011). Similar studies at the *Kr* locus have also identified enhancers that act in a sub-additive way to support *Kr* expression (see Scholes et al., 2019). However, the molecular mechanism by which the proximal enhancer limits the distal enhancer activity is unknown. Perhaps these co-acting enhancers respond to different morphogen levels or with temporally distinct activities to support dynamic regulation of gene expression levels (Fig. 4E).

It is clear that the *cis*-regulatory logic inherent in target gene enhancer sequences serves to interpret morphogen inputs to support spatial as well as temporal-regulated outputs (Datta et al., 2018; Koromila et al., 2020; Yuh et al., 1998). Using diverse mechanisms, co-acting enhancer pairs likely act to support precise and accurate gene expression (Bentovim et al., 2017), even when the dose of Bcd and Dl morphogens varies in response to genetic and environmental conditions.

Conclusions

In summary, by dissecting the role of morphogen inputs directly as well as the ability of cis-regulatory modules to work in a coordinate manner, the field is making progress towards understanding the process of patterning. As highlighted above, significant similarities exist between the mechanisms of target gene activation mediated by the two morphogens Bcd and Dl: combinatorial activation, boundary positioning by repressors and contribution from multiple/shadow enhancers (Fig. 5B). However, several differences suggest these morphogens have distinct attributes.

For example, the differential dynamics associated with morphogens likely impact temporal processing of target gene threshold responses. Interestingly, Dl and Bcd appear to have opposite spatiotemporal trends. At the ventral-most region of the

embryo where peak Dl levels are present, a nucleus experiences a relatively large increase in nuclear levels of Dl morphogen from one nuclear cycle to the next (e.g. 0% DV position) (Fig. 5A), whereas nuclei located at a distance along the DV axis perceive a smaller increase (e.g. 50% DV position) (Fig. 5A). Therefore, along the DV axis, ventral nuclei (see plot for trace) see the largest change in Dl exposure over time, and nuclei located along the DV axis experience a decreasing differential the more dorsal their position in the embryo (Fig. 5C). By contrast, Bcd exhibits the opposite trend (Fig. 5C). Nuclei positioned more posteriorly are likely exposed to a relatively larger differential in Bcd levels during patterning, owing to increased Bcd protein production over time, as well as to diffusion of Bcd from the anterior (Fig. 5D). These different dynamics likely impact target gene threshold responses; for example, by influencing transcriptional bursting (Sanchez and Golding, 2013). Despite the variety of cis-regulatory elements driving gap gene expression downstream of Bcd, these genes exhibit similar bursting kinetics (Zoller et al., 2018). However, bursting frequency associated with individual enhancers has been shown to vary (Fukaya et al., 2016). Whereas the bursting parameters regulated by Bcd and Dl morphogens remain unclear, the BMP morphogen gradient has been shown to control burst frequency by differentially regulating promoter activation rate (Hoppe et al., 2020). In addition to further study of the influence of Bcd and Dl on bursting kinetics, recent advances in single-cell sequencing coupled to ATAC-seq analyses, as well as the use of optogenetic approaches to inactivate these TFs with temporal precision, should also provide further insight into Bcd and Dl morphogen responses.

In addition, absolute morphogen levels may not necessarily act only to regulate the spatial domain of target gene expression. In the case of Dl at the late blastoderm stage, many target genes maintain constant expression domains, whereas Dl levels continue to increase. During this late stage of the patterning process, Dl may no longer impact patterning of some target genes because other transcription factors, activators and/or repressors have taken over this role. A recent study of temporal regulation of *sna* expression identified that the Dl target Twi can suffice to activate late expression through one particular enhancer, *sna_Distal,* and serves as a molecular example of the phenomenon known as hysteresis (Huang et al., 2017; Irizarry et al., 2020). In various contexts, hysteresis, a behavior of systems that depends on memory, has been proposed as a mechanism to support robust patterning under inherent noisiness during development (Balaskas et al., 2012; Bollenbach et al., 2008; Manu et al., 2009). It will be of interest to determine whether these maternal transcription factors play any role in gene regulation after cellularization, when their nuclear gradients are still building, but both Dl and Bcd have been shown to be expendable for the expression of particular target genes (i.e. *sna* and *Kr*, respectively) (Huang et al., 2017; Irizarry et al., 2020).

Furthermore, how scaling of gene expression patterns along body axes is controlled is not completely understood. Several studies have focused on how scaling across the AP axis is achieved by deposition of varying amounts of *bcd* mRNA into eggs that is dependent on their volume (Cheung et al., 2011). This maternally derived size-dependent information can propagate in space and time due to the dynamics of the gene regulatory network (Wu et al., 2015). In DV patterning, the shape of the Dl nuclear gradient scales with size of the DV axis but, surprisingly, only a subset of target genes scale correspondingly (Chahda et al., 2013; Garcia et al., 2013). Furthermore, how morphogen input affects scaling of target genes is not completely clear. However, studies of Bcd input to *hb* gene expression have suggested that Bcd provides input to early-acting enhancers and, subsequently, other Bcd-independent late-acting enhancers take over (Liu and Ma, 2013). It has been suggested that this 'hand-off' mechanism allows a window of opportunity during which *hb* gene expression can benefit from early Bcd gradient input in order to process gradient properties, including information pertaining to scaling. Whether scaling impacts one or more enhancers acting in cis-regulatory systems is an interesting issue that remains to be investigated.

Patterning requires that cells integrate inputs from graded morphogens as well as from a variety of other transcription factors present as a result of the extant gene regulatory network. Furthermore, the complex enhancer properties described above (i.e. enhancer dominance, long-range interactions and hysteresis/memory) likely include spatiotemporal control mechanisms present in many metazoan genes and not just limited to patterning outputs (Katikala et al., 2013). Nevertheless, patterning by morphogens in early *Drosophila* embryos provides an excellent system with which to explore how spatiotemporal gene expression is controlled, and thereby provide insight into developmental gene expression more broadly. For example, stage-specific changes in responsiveness of target genes to morphogen levels is also observed in other *Drosophila* developmental processes, as well as in vertebrate systems (Balaskas et al., 2012; Nahmad and Stathopoulos, 2009). Cells become desensitized with increased exposure to morphogen input over time; furthermore, hysteresis allows the cells to maintain proper identities regardless of fluctuation of morphogen input (Balaskas et al., 2012; Dessaud et al., 2010). It will be of interest to determine whether specific enhancer action(s) supports these and other examples of gene expression dynamics described across developmental systems.

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Competing interests
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