Enhancer-promoter communication in *Drosophila* developmental gene transcription

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ABSTRACT Enhancers play an essential role in gene regulation by receiving cues from transcription factors and relaying these signals to modulate transcription from target promoters. Enhancer-promoter communications occur across large linear distances of the genome and with high specificity. The molecular mechanisms that underlie enhancer-mediated control of transcription remain unresolved. In this review, we focus on research in *Drosophila* uncovering the molecular mechanisms governing enhancer-promoter communication and discuss the current understanding of developmental gene regulation. The functions of protein acetylation, pausing of RNA polymerase II, transcriptional bursting, and the formation of nuclear hubs in the induction of tissue-specific programs of transcription during zygotic genome activation are considered.

KEYWORDS: enhancer, transcription, gene regulation, Pol II pausing, transcriptional bursting

Introduction

The ability to express different complements of genes underlies the capacity of cells carrying the same DNA genome to form diverse cell types during multicellular development. Transcription fundamentally depends on recruitment of the RNA polymerase II (Pol II) transcriptional machinery at promoter sequences, but regulation by non-coding enhancer sequences shape the magnitude and spatio-temporal dynamics of transcriptional activity (Shlyueva *et al*., 2014; Spitz and Furlong, 2012). Enhancers are essential cis-regulatory elements (CREs) that integrate regulatory signals from trans-acting transcription factors (TFs) into a transcriptional circuitry to drive cell type-specific programs of transcription from target promoters (Fig. 1). Enhancers are often located at large genomic distances from target promoters and must therefore communicate regulatory signals across the chromatin landscape that houses the DNA genome (Furlong and Levine, 2018). The genome is organized across multiple levels with chromosomes occupying territories within the nucleus, the segregation of chromatin into active and inactive compartments, topologically associating domains (TADs) with abundant internal interactions, including between enhancers and promoters (Jerkovic and Cavalli, 2021). Cofactors with enzymatic chromatin-modifying activities are often recruited by TFs to enhancers (Reiter *et al*., 2017) and it is increasingly recognized that modulation of chromatin, through histone posttranslational modifications (PTMs), the incorporation of histone variants, and DNA methylation, interplay with transcriptional states to ensure developmental gene regulation (Bannister and Kouzarides, 2011; Li *et al*., 2007). While enhancers are well defined as recruitment platforms for cell type-specific TFs with dense enrichment of TF binding sites (TFBS) (Shlyueva *et al*., 2014; Spitz and Furlong, 2012), mechanistic understanding of how enhancers communicate regulatory cues to promoters and modulate transcriptional activity is lacking (Catarnio and Stark, 2018; Furlong and Levine, 2018; Karr *et al*., 2022; Panigrahi and O’Malley, 2021).

*Drosophila melanogaster* (*Drosophila*) is a key model system for studying the molecular mechanisms underlying developmental gene regulation. During the first few hours of *Drosophila* embryogenesis, development progresses rapidly from a transcriptionally inert fertilized egg, through a series of 13 synchronous syncytial nuclear cycles (nc), to an embryo composed of ~6000 cells that undergoes zygotic genome activation (ZGA) and initiates cell type-

**Abbreviations used in this paper:** AIL, Autoinhibitory loop; AP, Anterior-posterior; CREs, Cis-regulatory elements; DI, Dorsal; DV, Dorsoventral; GRNs, Gene regulatory networks; GTFs, General transcription factors; H3K27ac, Histone H3 lysine 27 acetylation; H3K27me3, Histone H3 lysine 27 tri-methylation; nc, nuclear cycle; Pol II, RNA polymerase II; PTMs, Posttranslational modifications; TADs, Topologically associating domains; TF, Transcription factor; TFBS, TF binding sites; TSS, Transcription start site; ZGA, Zygotic genome activation.
specific transcriptional programs (Harrison and Eisen, 2015; Schulz and Harrison, 2019). ZGA is orchestrated by maternally supplied mRNAs and proteins which establish an elaborate gene regulatory landscape. Chromatin becomes accessible at specific CREs and differentially bound across cells by complements of TFs, cofactors and the transcriptional machinery, while chromatin states, with specific signatures of PTMs, form (Blythe and Wieschaus, 2016; Bozek et al., 2019; Calderon et al., 2022; Cusanovich et al., 2018; Koeenecke et al., 2016; Li et al., 2014; Liang et al., 2008; Reddington et al., 2020). ZGA occurs concomitantly with formation of a complex genome organization of TADs that may facilitate close proximity between enhancers and promoters. However, the role of TADs in gene expression and the mechanisms of enhancer-promoter interactions are unclear (Furlong and Levine, 2018; Ghavi-Helm et al., 2014; Hug et al., 2017; Ogiyama et al., 2018; Schoenfelder and Fraser, 2019).

Gene regulatory networks (GRNs) specify cell identities along the dorsoventral (DV) and anterior-posterior (AP) body axes to spatially coordinate formation of the germ layers and organize the future body plan (Ma et al., 2016; Stein and Stevens, 2014). These well-characterized Drosophila GRNs are valuable for investigating how gene regulatory mechanisms converge to produce cell-type specific transcriptional programs. The importance of better understanding the molecular mechanisms of enhancer function is underscored by the presence of disease-associated variants within non-coding sequences (Rickels and Shilatifard, 2018; Zaugg et al., 2022), the pervasiveness of mutations to chromatin-modifying proteins in cancer (Flavahan et al., 2017) and the relevance of transcriptional and chromatin alterations in aging (López-otín et al., 2017). In this review, we discuss current understanding of developmental gene regulation and particularly draw on research in Drosophila focused on uncovering the molecular mechanisms governing enhancer-promoter communication and enhancer-mediated control of transcription.

Enhancers operate in complex regulatory landscapes

Enhancer activity was initially demonstrated for a sequence element from the SV40 virus genome, capable of increasing the magnitude of transcription from the rabbit β-globin gene in HeLa cells when positioned more than 1 kb upstream or downstream from the transcription start site (TSS) (Banerji et al., 1981). Soon after, enhancer activity was detected for endogenous sequences from the eukaryotic genome (Banerji et al., 1983; Gillies et al., 1983). Enhancers have evolved to play a key role in orchestrating developmental transcriptional programs (Long et al., 2016; Shlyueva et al., 2014; Spitz and Furlong, 2012), vastly outnumbering protein-coding genes within eukaryotic genomes. The enhancer regulatory code is highly conserved across metazoans, with enhancer activities of many non-coding sequences preserved across distant species (Pennacchio et al., 2006; Wong et al., 2020). The functional hallmarks of enhancers include the capacity to modulate transcription in an orientation independent manner, operate at large genomic distances from the target promoter, and the ability to recapitulate their activity independent of the sequence context (reviewed in Shlyueva et al., 2014). In vivo reporter assay validation of sequences predicted to have enhancer activity remains essential for establishing functionality, and many candidate enhancers have not yet been validated (Kvon et al., 2014; Pennacchio et al., 2006; Shlyueva et al., 2014; Smith et al., 2023).

Chromatin restricts enhancer accessibility

The communication of regulatory signals occurs across chromatin, and genomic approaches to predict sequences with enhancer activity based on DNA and chromatin features have been useful for identifying enhancers genome-wide and inferring cell-type specific enhancer activities (Encode Project Consortium, 2012; Negre et al., 2011; The modEncode Consortium et al., 2010). Active enhancers are characterized by accessible chromatin depleted of nucleosomes to allow for DNA binding by TFs. Genome-wide profiling has revealed that chromatin accessibility is dynamically regulated across cell types and correlates with TF binding (Fig. 2A) (Boyle et al., 2008; Li et al., 2011; Pique-Regí et al., 2011; Shlyueva et al., 2014; Spitz and Furlong, 2012; Thurman et al., 2012). Elevated chromatin accessibility is also a feature of other CREs. At active promoters, nucleosome depletion is important for assembly of the transcriptional machinery (Cairns, 2009) and Pol II may compete with nucleosomes to maintain an accessible state (Fig. 2A) (Core and Adelman, 2019; Gilchrist et al., 2010; Levine, 2011). Polcomb response elements (PREs), the recruitment sites of Polycomb group (PoG) proteins that are responsible for transcriptional silencing of developmental genes across eukaryotes (Schuettengruber et al., 2017), also exhibit low nucleosome density (Hunt et al., 2022; Mito et al., 2007; Schuettengruber et al., 2009; Schwartz et al., 2006).

A major function of housing the genome in chromatin is to prevent spurious transcription (Kornberg and Lorch, 2020). Therefore, factors capable of selectively producing nucleosome-depleted regions play a key role in gene regulation. A subset of TFs, known as pioneer factors, have the capacity to initiate the opening of
compact chromatin (Balsalobre and Drouin, 2022; Spitz and Furlong, 2012). Pioneer factors can interact with nucleosomal DNA and recruit chromatin remodelers to deplete nucleosomes at these sites, providing DNA access for non-pioneer TFs. In this manner, the maternally supplied pioneer factor Zelda (Zld) selectively establishes chromatin accessibility at important CREs for ZGA in Drosophila (Harrison et al., 2011; Liang et al., 2008; Ning et al., 2011; Schulz and Harrison, 2019; Sun et al., 2015). Zld orchestration of ZGA is supported by several other TFs with pioneer-like activities, including the mitotic bookmarker GAGA factor (GAF) (Bellec et al., 2022; Gaskill et al., 2021), Odd-paired (Opa) (Koromila et al., 2020; Soluri et al., 2020) and CLAMP (Duan et al., 2021).

**Active enhancers are marked by H3K27 acetylation**

Specific signatures of histone PTMs are important markers for identifying enhancers and predicting enhancer activity states genome-wide (Shlyueva et al., 2014). Acetylation of histone H3 lysine 27 (H3K27ac) is detected at nucleosomes flanking enhancers, and its enrichment correlates with transcriptional activity from associated genes (Fig. 2A), allowing the mark to distinguish active from inactive or poised enhancers (Bonn et al., 2012; Creighton et al., 2010; Heintzman et al., 2009; Rada-Iglesias et al., 2011). Occupancy of p300/CBP, the histone acetyltransferases responsible for depositing H3K27ac (Tie et al., 2009), has also been used to predict enhancers (Heintzman et al., 2007; Visel et al., 2009; Xie et al., 2007). Nevertheless, p300/CBP recruitment and acetyltransferase activity can be uncoupled because strong occupancy also occurs at hypoacetylated regions enriched for the repressive PcG mark trimethylated H3K27 (H3K27me3) (Holmqvist et al., 2012; Hunt et al., 2022; Philip et al., 2015; Rada-Iglesias et al., 2011). H3K27ac marks both active enhancers and promoters, but mono- and tri-methylation of H3K4 distinguishes enhancers and promoters, respectively (Heintzman et al., 2007; Rada-Iglesias et al., 2011; Shlyueva et al., 2014). Whether histone acetylation plays a direct causative role in gene activation remains largely unclear (Henikoff and Shilatifard, 2011; Millán-Zambrano et al., 2022). Histone acetylation may indirectly influence transcription by supporting the recruitment of effector proteins that recognize acetylated lysines, such as the coactivator bromodomain-containing protein 4 (BRD4) (Dey et al., 2003; Fujisawa and Filippakopoulos, 2017; Millán-Zambrano et al., 2022).

Distinct motif compositions and chromatin features shape the specificities of enhancers and promoters, influencing cofactor compatibilities (Haberle et al., 2019; Neumayr et al., 2022). Different chromatin remodelers are necessary for developmental and housekeeping transcriptional programs in Drosophila cells (Hendy et al., 2022). Remarkably, enhancers can operate long-range, spanning hundreds of kilobases (kb) from their target promoter, often bypassing intervening promoters (Fig. 2B). This implies a topological change in 3D organization, involving chromatin fiber folding, to confer specificity and allow proximity or physical contact for enhancer-promoter communication. However, while enhancers are well-defined compositionally by the TFBS they encode and specific chromatin features (Shlyueva et al., 2014; Spitz and Furlong, 2012), how signals are communicated from enhancers to target promoters, modulating transcriptional activity, remains unclear (Catarino and Stark, 2018; Furlong and Levine, 2018; Karr et al., 2022; Panigrahi and O’Malley, 2021).

**Enhancer-mediated control of Drosophila developmental transcription**

Studies of Drosophila embryonic patterning have unveiled how enhancers coordinate developmental gene expression (Irizarry and Stathopoulos, 2021; Small and Arnosti, 2020). The precise stripes of pair-rule gene expression across the AP axis of the early embryo are driven by multiple enhancers, each encoding different combinations of TFBS (Levine, 2010; Small and Arnosti, 2020; Small et al., 1992; Stanojevic et al., 1991). These enhancers act additively to form the overall expression pattern. The involvement of multiple enhancers with partially redundant activity, known as

**Fig. 2. Enhancers operate across chromatin to influence transcription from target promoters. (A)** Schematic showing active and inactive enhancer and promoter chromatin states. **(B)** Communication between enhancers and promoters often occurs over large genomic distances with specificity, enabling these long-range interactions to bypass intervening enhancers and promoters.
shadow enhancers, in directing gene expression patterns may contribute to phenotypic robustness (Cannavò et al., 2016; Frankel et al., 2010; Hong et al., 2008b; Osterwalder et al., 2018; Perry et al., 2010). *Drosophila* embryogenesis has been a key model for systematically characterizing non-coding sequences with developmental enhancer activity in vivo (Kvon et al., 2014). Despite its gene-dense nature relative to mammalian genomes, long-range interactions are widespread in the *Drosophila* genome. Many enhancer-promoter interactions occur at distances of more than 10 kb, with the majority spanning at least several kb. This separation still requires a topological mechanism to provide the necessary physical proximity for enhancer-bound TFs and cofactors to influence the transcriptional machinery at the promoter (Furlong and Levine, 2018; Ghavi-Helm et al., 2014; Hou et al., 2012; Hug et al., 2017; Sexton et al., 2012). The extensive validation of enhancers and widespread embryonic formation of enhancer-promoter interactions make *Drosophila* embryogenesis well suited for dissecting the mechanisms of enhancer-mediated gene activation.

**Drosophila** **dorsal-ventral patterning**

Position-dependent transcriptional programs define cell identities along the *Drosophila* embryonic DV axis in response to a nuclear gradient of the maternally-supplied Rel family TF Dorsal, peaking ventrally and progressively decaying dorsally (Fig. 3A) (Hong et al., 2008b; trizarry and Stathopoulos, 2021; Stathopoulos and Levine, 2002; Stein and Stevens, 2014). A cascade of maternal effect genes relays DV polarity to the syncytial blastoderm through ventrally restricted activation of Toll signaling, releasing Dorsal from an inactive cytoplasmic complex to enter nuclei (Belvin et al., 1995; Roth et al., 1989; Rushlow et al., 1989; Steward, 1987, 1989). High nuclear Dorsal ventrally activates mesoderm-specific genes like twist (twi) and snail (sna) to form mesoderm, while intermediate and low levels of nuclear Dorsal laterally induce neuroectoderm genes, including intermediate neuroblasts defective (ind) and brinker (brk), directing neuroectoderm formation. Sna, with the CtBP and Ebi corepressors, represses neuroectoderm-specific genes in the mesoderm (Fig. 3A) (Nibu et al., 1998; Qi et al., 2008). Dorsally located cells lacking nuclear Dorsal activate genes like decapentaplegic (dpp) and tolloid (tid) for dorsal ectoderm patterning. Besides acting as a transcriptional activator for mesoderm- and neuroectoderm-specific genes, Dorsal, along with the transcriptional repressor Capicua and corepressor Groucho, confines the expression of dorsal ectoderm-specific genes to dorsally located cells (Dubnicoff et al., 1997; Papagianni et al., 2018). Dpp/BMP signaling further defines cell types along the dorsal ectoderm (Ashe et al., 2000; Ferguson and Anderson, 1992; Hamaratoglou et al., 2014). The Dorsal gradient forms during the first 90 minutes of embryogenesis and is active between nc 10-14 (Liberman et al., 2009; Reeves et al., 2012). It is during this critical developmental window that the TF cue is received by enhancers of the DV GRN and communicated to modulate transcription from the promoters of DV-regulated genes.

More than 100 DV-regulated genes, many encoding developmental regulators, and 200-400 enhancers have been identified. Initial interrogation of the DV GRN involved genetic analyses (reviewed in Stein and Stevens, 2014), followed by genomic approaches including bioinformatics mapping of Dorsal binding sites and whole-genome microarray analysis of the transcriptome and Dorsal occupancy in mutant embryos with uniform DV cell fates (Biemar et al., 2006; Markstein et al., 2002; Stathopoulos et al., 2002; Zeitlinger et al., 2007b). Recently, next generation sequencing methods have captured spatially- and temporally-resolved transcriptional and epigenomic landscapes during *Drosophila* embryogenesis (Blythe and Wieschaus, 2016; Bozek et al., 2019; Chen et al., 2013; Holmqvist et al., 2012; Hunt et al., 2024; Ing-Simmons et al., 2021; Koenecce et al., 2016; Koenecce et al., 2017; Li et al., 2014; Lott et al., 2011), including at the single-cell scale (Calderon et al., 2022; Cusanovich et al., 2018; Hunt et al., 2024; Ing-Simmons et al., 2021; Karaikos et al., 2017; Reddington et al., 2020).

The threshold-dependent model proposes that spatially-regulated expression along the DV axis is achieved by integration of the Dorsal TF signal at enhancers responding to different Dorsal levels, determined by the affinity and organization of the binding sites they encode (Reeves and Stathopoulos, 2009; Rusch and Levine, 1996; Stathopoulos and Levine, 2002, 2004). While Dorsal initiates DV patterning, and enhancer binding site affinity correlates with positional expression (Papatsenko and Levine, 2005), precise expression domains of DV-regulated genes involve Dorsal acting in concert with other tissue-specific TFs, repressors and cofactors (Holmqvist et al., 2012; Kosman et al., 1991; Mannervik et al., 1999; Zeitlinger et al., 2007b). DV patterning constitutes one of the best-characterized GRNs in nature and has been a major model system for illuminating enhancer-mediated control of developmental transcription.

**Interplay between enhancer chromatin states and tissue-specific transcription in *Drosophila***

During early *Drosophila* embryogenesis, chromatin rapidly changes in the lead-up to ZGA, transitioning from a presumed highly condensed and hypoacetylated state to a heterogeneous landscape of distinct chromatin states with selective accessibility and the deposition of histone PTMs at specific genomic regions (Fig. 3 B,C) (Blythe and Wieschaus, 2016; Bozek et al., 2019; Harrison et al., 2011; Hunt et al., 2024; Li et al., 2014; Schulz and Harrison, 2019). Histone acetylation by p300/CBP accumulates at important enhancers and promoters associated with Zld activity (Harrison et al., 2011; Hunt et al., 2024; Li et al., 2014). H3K27ac is enriched at DV enhancers and promoters, and differential H3K27ac enrichment between DV cell types has been used to predict DV-regulated enhancers (Boja and Mannervik, 2016; Ing-Simmons et al., 2021; Koenecce et al., 2016). Integrating multiple indicators of the epigenome (H3K27ac and p300/CBP enrichment and chromatin accessibility) predicted DV-regulated enhancers more accurately than each could individually (Hunt et al., 2024). Interestingly, the strength of this tissue-specific enhancer chromatin state, consisting of elevated H3K27ac, p300/CBP, and chromatin accessibility, was highly predictive of the magnitude of tissue-specific transcription from associated DV-regulated genes. The DV promoter chromatin state, as defined by these epigenomic markers, was less tissue-specific and therefore not as effective at predicting differential transcription as the enhancer. Nevertheless, while these studies have uncovered chromatin features that strongly correlate with transcriptional states, much remains ambiguous regarding functional involvement.
Fig. 3. Dorsoventral (DV) patterning of Drosophila embryogenesis and chromatin landscape establishment during zygotic genome activation (ZGA). (A) The establishment of the Dorsal (Dl) TF nuclear gradient occurs in the syncytial blastoderm during nuclear cycle (nc) 10-13. This is facilitated by ventrally restricted activation of the Toll signaling pathway, leading to position-dependent cell fate specification of mesoderm, neuroectoderm, and dorsal ectoderm across the DV axis at nc 14. Cell identity is determined by tissue-specific transcription programs induced through enhancers responsive to different concentrations of Dl. Dorsal ectoderm-specific genes like dpp and tld are restricted to the most dorsally located cells due to Dorsal-mediated repression in lateral and ventral cells (Dubnicoff et al., 1997; Papagianni et al., 2018). Mesoderm-specific genes, including sna and twi, are expressed in ventrally-located cells with high levels of nuclear Dl, while neuroectoderm-specific genes like ind and brk are activated by Dl in lateral regions but not ventrally due to repression by Sna with the CtBP and Ebi corepressors (Nibu et al., 1998; Qi et al., 2008). Robust induction of the transcriptional programs patterning the DV axis depends on Dl acting in concert with other tissue-specific TFs, repressors, and cofactors. (B) A schematic of the stages of early Drosophila embryogenesis, illustrating nuclei (represented by black dots) dividing through rapid nuclear cycles. Nuclear divisions occur first within the center of the embryo in the preblastoderm stages (nc 1-9) before nuclei migrate to the periphery during the syncytial blastoderm stages (nc 10-13). At the periphery, nuclei cellularize to form the cellular blastoderm (nc 14) before undergoing gastrulation. The exchange of transcriptional control from maternal to zygotic during the minor and major waves of ZGA is also depicted. The hours after egg laying (AEL) are indicated across the trajectory and the corresponding nuclear cycles shown. (C) Changes to the chromatin landscape at an enhancer and promoter that become transcriptionally active at nc 14. In the lead-up to transcriptional induction, regulatory sequences are made accessible by the pioneer factor Zelda, facilitating the recruitment of TFs and the transcriptional machinery. Chromatin states that are linked to transcription, such as the H3K27ac histone modification, also begin to accumulate.
Functions of histone acetylation in transcription

Whether histone acetylation plays an instructive role in gene activation is unclear (Henikoff and Shilatifard, 2011; Millán-Zambrano et al., 2022). Early in vitro studies noted that histone acetylation suppressed the intrinsic inhibitory effect of histones on transcription from chromatinized DNA templates (Allfrey et al., 1964). This effect is understood to be mediated by acetylation-induced alterations to nucleosomal electrostatic interactions, leading to decompaction and increased amenability for transcription (Fenley et al., 2010). However, histone tail PTMs are considered unlikely to impact nucleosome dynamics and may instead influence transcription indirectly by providing a binding platform for effector proteins (Millán-Zambrano et al., 2022). Consistently, the coactivator BRD4 is recruited to Drosophila DV enhancers and promoters in a tissuespecific manner correlating with H3K27ac and transcriptional activation (Hunt et al., 2024). H3K27ac was also recently observed to enhance cooperative binding at human OCT4-pioneered sites (Sinha et al., 2023). Zelda binding at H3K27ac-marked DV and non-DV enhancers before ZGA in Drosophila (Hunt et al., 2024; Li et al., 2014) raises speculation about a similar association in the early embryo.

Acetylation of non-histone proteins

p300/CBP, like many other chromatin-modifiers, is a multifunctional protein with enzymatic and non-enzymatic activities, complicating the attribution of observed effects to specific functions (Fig. 4) (Bedford and Brindley, 2012; Dorighi et al., 2017; Hunt et al., 2022; Morgan and Shilatifard, 2020; Rickels et al., 2017). In addition to acetylating histones, the acetyltransferase activity of p300/CBP targets non-histone proteins, such as TFs, coactivators, and effectors of important signaling pathways (Weinert et al., 2018).

Acetylation can influence protein function in diverse ways, including affecting protein stability, localization, protein-protein interactions, and DNA-binding ability (Spange et al., 2009). p300/CBP is equipped with a bromodomain and various other protein-protein interaction domains that may mediate non-enzymatic functions (Dancy and Cole, 2015). These functions could include acting as a scaffold to facilitate the assembly of protein complexes (Fig. 4) (Chan and La Thangue, 2001).

p300/CBP is also found at promoters, where it interacts with the Pol II transcriptional machinery (Boija et al., 2017; Cho et al., 1998; Heintzman et al., 2007). At promoters, p300/CBP may influence transcription through enzymatic and non-enzymatic functions (Fig. 4) (Boija et al., 2017; Narita et al., 2021), aiding in the formation of the pre-initiation complex (PIC) via interactions with TFIIB (Kwok et al., 1994) and modulating Pol II promoter release, potentially by acetylating the transcriptional machinery and the +1 nucleosome (Boija et al., 2017; Narita et al., 2021; Schröder et al., 2013; Stasevich et al., 2014). The presence of p300/CBP and the Pol II transcriptional machinery at promoters of genes devoid of acetylation and productive transcription suggests its different functions can be partitioned (Hunt et al., 2022).

The accumulation of p300/CBP histone acetylation around ZGA is conserved in flies, zebrafish and mice (Bogdanović et al., 2012; Dahl et al., 2016; Li et al., 2014; Schulz and Harrison, 2019). Modulation of p300 acetyltransferase activity has been reported to disrupt ZGA in zebrafish (Chan et al., 2019; Miao et al., 2022; Sato et al., 2019). Interestingly, although p300/CBP is essential for ZGA in Drosophila, ZGA can occur in embryos with catalytically inactive p300/CBP (Ciabrelli et al., 2023). Moreover, p300/CBP protein depletion, but not acetyltransferase activity inhibition, compromises chromatin accessibility (Hogg et al., 2021; Hunt et al., 2022; Vannam et al., 2021), further highlighting the crucial non-enzymatic roles of p300/CBP activities in gene regulation.

Fig. 4. Functions of p300/CBP in gene regulation. The chromatin-modifier p300/CBP regulates transcription through enzymatic acetyltransferase activity, but also has non-enzymatic activities. p300/CBP acetylates lysine residues on all four core histones, including H3K27ac to mark active enhancers and promoters (Feller et al., 2015; Weinert et al., 2018), but also acetylates diverse non-histone proteins, including TFs, transcriptional regulators, and effectors of signaling pathways (Weinert et al., 2018). p300/CBP also enzymatically targets important components of the transcriptional machinery at promoters, including GTFs, Pol II, the super elongation complex (SEC) and P-TEFb, which alongside acetylation of the +1 nucleosome, may stimulate Pol II release from the promoter into productive transcription (Boija et al., 2017; Narita et al., 2021; Schröder et al., 2013; Stasevich et al., 2014). Non-enzymatic activities of p300/CBP in gene regulation could include scaffolding to help bring together TFs and other transcriptional regulators into large assemblies at target loci (Chan and La Thangue, 2001) and supporting assembly of the transcriptional machinery at promoters by interacting with TFIIB (Kwok et al., 1994). p300/CBP support of Pol II recruitment also occurs at the promoters of certain genes in repressive, H3K27me3-enriched chromatin, where productive transcription is lacking (Hunt et al., 2022).
Targeting H3K27 directly by replacing it with non-modifiable residues (H3K27A/R) does not compromise gene activation in Drosophila and mouse embryonic stem cells (mESCs), indicating that p300/CBP functions aside from acetylating H3K27 are important (Leatham-Jensen et al., 2019; McKay et al., 2015; Pengelly et al., 2013; Sankar et al., 2022; Zhang et al., 2020). p300/CBP is proposed to act as an 'acetyl spray' that targets lysines across the tails of histones (Feller et al., 2015; Weinert et al., 2018). Supporting this spray-like activity, histone H2B tail acetylation by p300/CBP can also predict active enhancers (Narita et al., 2023). It is possible that acetylation of neighboring histone tail residues confers functional redundancy or is a by-product of enzymatic activity directed at non-histone proteins, which also affects residues on local histones. The acetyltransferase activity of p300/CBP is tightly regulated by a lysine-rich autoinhibitory loop (AIL) that, in a deacetylated state, prevents substrate access to the active site (Thompson et al., 2004). Dimerization of TFs and binding of enhancer RNAs (eRNAs) are proposed to trigger displacement of the p300/CBP AIL, allowing for enzymatic activity to occur (Bose et al., 2017; Ortega et al., 2018). The Drosophila Pcg protein Pc and its mammalian CBX orthologs directly interact with the p300/CBP AIL to block acetyltransferase activity, potentially preventing turnover from transcriptionally repressive to active states (Tie et al., 2016). This aligns with the detection of p300/CBP at hypoacetylated sites (Holmqvist et al., 2012; Hunt et al., 2022; Philip et al., 2015; Radigleias et al., 2011) and supports localized multi-step enzymatic activation of p300/CBP that is uncoupled from chromatin recruitment. The transcription of DV-regulated genes anti-correlates with H3K27me3, suggesting that local enzymatic inhibition of CBP by PcGs may help ensure tissue-specific transcription (Hunt et al., 2024; Koenecke et al., 2017). CBP binding across cell types of the DV axis indicates the potential for distinct recruitment and enzymatic activity state dynamics at enhancers and promoters, where enhancer occupancy is highly tissue-specific and correlates with tissue-specific TF presence, H3K27ac, and transcription (Hunt et al., 2024). In contrast, CBP promoter binding is less tissue-specific, with residual levels persisting at promoters in inactive states.

An intriguing mechanism for enhancer-promoter communication, termed the TF activity gradient (TAG) model, proposes that acetylated TFs produced by enhancer-localized enzymatic p300/CBP activity diffuse to target promoters in close proximity, where they increase the transcriptional output (Karr et al., 2022). In this model, histone deacetylases (HDAC), chromatin modifiers that also target non-histone proteins (Glozak et al., 2005), deacetylate TFs to spatially limit the diffusing acetylated TF signal. Indeed, HDACs are recruited to active promoters in metazoans, where they may attenuate acetylated TF signals (Filion et al., 2010; Rincon-Arano et al., 2012; Wang et al., 2008). Concentrations of p300/CBP and other coactivators appear to be limiting in cells, with the number of molecules being far outnumbered by HDACs (Gillespie et al., 2020). In Drosophila cell culture, pharmacological HDAC inhibition rapidly elevates histone acetylation levels at active promoters and correlates with Pol II promoter release (Vaid et al., 2020). These findings support HDAC-mediated attenuation of acetylated TF signals as they diffuse from enhancers and the maintenance of the majority of nuclear p300/CBP in an inactive state (Karr et al., 2022).

Considering the uncertainty surrounding the direct role of histone acetylation in transcription, there is significant focus on identifying factors that undergo function-altering acetylation. This criteria may be met by the positive transcription elongation factor-b complex (P-TEFB), which stimulates Pol II elongation across gene bodies to produce transcripts (Fujinaga et al., 2023; Jonkers and Lis, 2015) and is recruited to promoters by DNA-binding TFs, including NF-kB (Barboric et al., 2001; Danko et al., 2013), and BRD4 (Dey et al., 2003; Jang et al., 2005; Yang et al., 2005). Both BRD4 and P-TEFB are recruited to Drosophila DV enhancers and promoters in a tissue-specific manner (Hunt et al., 2024) and undergo p300/CBP-dependent acetylation in mammalian cells (Weinert et al., 2018). Acetylation of P-TEFB marks its active state, free from inactive sequestration within the 7SK small nuclear ribonucleoprotein (7SK snRNP) complex (Cho et al., 2009). In summary, several observations from enhancer-driven transcription during DV patterning align with a model of enhancer-promoter communication where p300/CBP actively participates. Drosophila embryogenesis provides a conducive in vivo setting for delineating the genomic distribution of both enzymatically active and inactive p300/CBP. Further investigations can focus on unravelling the regulatory mechanisms governing the loading BRD4 and P-TEFB at promoters by enhancers, and establishing whether their acetylation occurs in a functionally significant manner.

How do enhancers influence transcriptional activity at promoters?

When enhancer-promoter communication occurs, signals of activation must be integrated into the transcriptional circuitry by modulating the activity of the Pol II transcriptional machinery. Transcription is a complex, multi-step process, and post-recruitment control of Pol II entry into elongation has emerged as a major rate-limiting checkpoint that controls the transcriptional output (Core and Adelman, 2019; Jonkers and Lis, 2015). At many metazoan genes, Pol II efficiently initiates transcription but pauses in the promoter-proximal region, typically 30-60 bp downstream of the TSS (Fig. 5A). This pausing of Pol II is highly stable, with most half-lives at the pause site ranging between 5-15 minutes, providing a window of time for regulatory signals from enhancers to exert their influence on the transcriptional cycle (Henriques et al., 2013; Jonkers et al., 2014; Shao and Zeitlinger, 2017). The release of Pol II from the pause site into productive elongation is tightly controlled by factors that either maintain pausing or stimulate promoter escape (Core and Adelman, 2019). P-TEFB is understood to promote pause release by phosphorylating specific residues on the Pol II C-terminal domain (CTD) and counteracting factors involved in maintaining pausing, such as SpT5, although the precise mechanism leading to elongation is not yet fully understood (Fujinaga et al., 2023; Price, 2000; Yamada et al., 2006).

Promoter-proximal Pol II pausing at developmental genes

Drosophila early embryogenesis has played a pivotal role in elucidating the role of pausing in the coordination of tissue-specific transcription during development. Pausing was initially proposed as a mechanism to prime inducible genes for rapid signal-responsive transcriptional induction (Gilmour and Lis, 1986; Levine, 2011; Rougvie and Lis, 1988), but genome-wide mapping of Pol II occupancy subsequently uncovered the prevalence of pausing at developmental genes across metazoans (Core et al., 2008; Muse et al., 2007; Nechaev et al., 2010; Zeitlinger et al., 2007a).
Consistent with serving a more fundamental and widespread role, pausing has been proposed to constitute a broadly permissive state across cell types that is amenable to restricted signals of gene induction (Fig. 5B) (Gaertner and Zeitlinger, 2014). While the majority of paused genes are transcriptionally active, Pol II is recruited and paused at developmental promoters in inactive states, including Dv-regulated genes during early embryogenesis (Hunt et al., 2024; Zeitlinger et al., 2007a). Pausing also occurs at the promoters of many H3K27me3-enriched PcG repressed developmental genes in mammalian embryonic stem cells (Bernstein et al., 2006; Kanhere et al., 2010; Stock et al., 2007) and Drosophila (Enderle et al., 2011; Hunt et al., 2022). Therefore, pausing appears to be a major control point of the transcriptional cycle where activating and repressing signals converge. Paused Pol II can persist at genes that have been downregulated, marking their prior activation (Gaertner et al., 2012). The presence of paused Pol II at DV-regulated genes in both active and inactive tissues seems to result from its recruitment before the establishment of the Dorsal gradient in largely transcriptionally quiescent naïve cells. These promoters may be pioneered with accessibility at this early stage of development by Zelda and are highly enriched for core promoter elements capable of recruiting the transcriptional machinery (Blythe and Wieschaus, 2016; Harrison et al., 2011; Hunt et al., 2024). These findings support tissue-specific transcription in the early embryo involving the establishment of a paused state broadly, rendering cells receptive to spatially restricted developmental cues. In the case of DV patterning, the developmental cue is tissue-specific enhancer activation by Dorsal, which may modulate BRD4 and P-TEFb activity through p300/CBP-catalyzed acetylation to promote pause release. Drosophila AP patterning genes also recruit paused Pol II in naïve cells, and mammalian Pol II is pre-loaded at promoters before ZGA (Liu et al., 2020), suggesting a conserved process for developmental gene induction.

Recently, the role of the Integrator complex in the regulation of Pol II pause release to control productive transcriptional elongation has been characterized in mammals (Fianu et al., 2021; Stein et al., 2022; Vervoort et al., 2021; Wang et al., 2023). Integrator drives premature termination through two enzymatic activities: an endonuclease that cleaves nascent RNA and a protein phosphatase that removes stimulatory phosphorylation associated with Pol II pause release and productive elongation (reviewed in Wagner et al., 2023). Targeted and rapid protein depletion has revealed that promoter H3K4me3 recruits the integrator complex subunit 11 (INTS11) endonuclease (Wang et al., 2023). Analyzing H3K4 methylation in Drosophila embryos indicates that DV enhancers acquire H3K4me1 at ZGA in both active (with H3K27ac) and uninduced (without H3K27ac) states, while both H3K4me1 and H3K4me3 are present at DV promoters (Koenecke et al., 2017; Li et al., 2014). It
would be interesting to apply the DV model system to distinguish cell type-specific differences in the deposition of H3K4 methylation and functionally interrogate the relationship between tissue-specific chromatin features and pause release during development.

**Transcription occurs in bursts that are modulated during developmental gene regulation**

Transcription occurs in stochastic bursts of RNA synthesis, resulting from alternation between active and inactive states (Fig. 6) (Leyes Porello et al., 2023; Rodriguez and Larson, 2020; Tunnacliffe and Chubb, 2020). The discontinuous nature of transcription was first observed in *Drosophila* embryo chromosome spreads by electron microscopy in the late 1970s (Miller and McKnight, 1979). Since then, the ability to study gene bursting has been spurred by the development of highly sensitive microscopy and genomics techniques that have been leveraged to explore transcriptional dynamics at the single-cell scale. *Drosophila* provides a major model system for studying gene bursting in vivo during development. Bursting has been visualized for genes of interest in early *Drosophila* embryos by single-molecule RNA FISH (smFISH) and captured live in real-time using the MS2/MCP system (Leyes Porello et al., 2023). Gene bursting dynamics have also been inferred transcriptome-wide from single-cell RNA-seq (scRNA-seq) data (Hunt et al., 2024; Jiang et al., 2017; Kim and Marioni, 2013; Larsson et al., 2019).

Several models, differing in the number of promoter activity states accommodated, have been applied to describe transcriptional bursts (Rodriguez and Larson, 2020; Tunnacliffe and Chubb, 2020). A two-state model, where promoters fluctuate between active/ON and inactive/OFF configurations, has been widely adopted to infer transcriptional dynamics (Berrocal et al., 2020; Larsson et al., 2019; Peccoud and Ycart, 1995; Raj et al., 2006; Zoller et al., 2018). Bursting has been characterized by parameters including frequency and size (number of RNA molecules produced per burst) that can be inferred from this model. However, the failure to accommodate intermediary promoter activity states, such as pausing, means the two-state model may not always satisfactorily capture promoter dynamics (Tunnacliffe and Chubb, 2020). As a result, multistate models have been proposed to better explain the transcriptional activity of some genes (Bartman et al., 2019; Corrigan et al., 2016; Neuert et al., 2013; Pimmett et al., 2021; Suter et al., 2011). While these experimental techniques and modelling approaches have helped establish that gene burstiness is a highly evolutionarily conserved feature of transcription, a major focus has been to reveal the molecular determinants that modulate bursting dynamics.

Studies of the molecular determinants of bursts have linked enhancers to transcriptional control through modulation of gene burst frequencies (Fukaya et al., 2016; Walters et al., 1995). Chromatin features, such as differential histone acetylation at enhancers, have been found to correlate with the burst frequency of target genes (Larsson et al., 2019; Nicolas et al., 2018). The burst size of genes shows a strong association with the occurrence of specific core promoter motifs, suggesting that it is largely sequence-encoded at promoters (Hornung et al., 2012; Larsson et al., 2019). Inferring burst kinetics in *Drosophila* embryonic DV tissue scRNA-seq data corroborates that enhancer-mediated control of burst frequencies and promoter-encoded burst sizes underlie developmental gene induction in vivo (Hunt et al., 2024). DV genes are characterized by a low burst frequency and the capacity for strong bursts. The high burst size capacity may derive from an overrepresentation of the core promoter motifs TATA and INR that bind TFIIID to trigger PIC assembly (Haberle and Stark, 2018; Joo et al., 2017), but could possibly also be influenced by pausing (Hunt et al., 2024; Pimmett et al., 2021; Tantale et al., 2021). Notably, differential promoter loading of P-TEFb between DV tissues correlates with burst size, implicating the pause release circuitry in the induction of strong bursts (Hunt et al., 2024). The tissue-specific active chromatin state found at DV enhancers correlates strongly with elevated burst frequencies at target promoters but is also well correlated with burst size increases for genes whose induction does not involve significant changes in burst frequency. While the molecular determinants that modulate bursts are being revealed, much remains unclear about the mechanism of enhancer-promoter communication that allows burst induction to be signaled.

**Enhancer-promoter communication across the chromatin landscape**

Enhancer-promoter communications occur in complex regulatory environments where they must overcome the challenges of genomic distance and specificity when surrounded by non-target enhancers and promoters. These communications occur within the genomic organization of TADs (Dixon et al., 2012; Sexton et al., 2012), with boundaries harboring insulator activities that prevent unintended interactions between enhancers and promoters in different TADs (Jerkovic and Cavalli, 2021). Similar to their mammalian counterparts, *Drosophila* TAD boundaries are enriched in DNA binding motifs for insulator proteins (Hou et al., 2012; Hug et al., 2017; Jiang et al., 2009; Kaushal et al., 2022; Sexton et al., 2012; Stadler et al., 2017; Ulianov et al., 2016). Techniques that capture chromosome conformation have revealed the emergence of *Drosophila* TADs and focal contacts between enhancers and promoters in parallel with ZGA (Fig. 7A) (Ghavi-Helm et al., 2014; Hug et al., 2017; Ogiyama et al., 2018). In addition to containing motifs for insulator proteins, early forming
Drosophila TAD boundaries have specific signatures of histone modifications and can be enriched for Pol II and Zld (Hug et al., 2017). TADs form independently of zygotic transcription, but insulation at some boundaries is compromised by transcriptional inhibition and depletion of Zld.

In mammals and Drosophila, there is disparity between the loss of TAD insulation when insulator proteins are perturbed and the modest effects on ensuing gene expression (Cavalheiro et al., 2023; Kaushal et al., 2021; Nora et al., 2017; Rao et al., 2017; Schwarzet al., 2017). Disruption of TAD boundaries in some instances have been demonstrated to rewire genome organization, leading to ectopic enhancer-promoter interactions that impact gene expression (Franke et al., 2016; Lupiáñez et al., 2015; Nora et al., 2012). While TADs are reported to be largely invariant between cell types (Dixon et al., 2015; Dixon et al., 2012; Nora et al., 2012), cell type-specific TADs have been detected, and intra-TAD interactions are reported to be dynamically regulated (Bonev et al., 2017; Chathoth and Zabet, 2019; Kragesteen et al., 2018; Le Dily et al., 2014; Mateo et al., 2019). A class of CREs known as tethering elements has been shown to facilitate intra-TAD enhancer-promoter and promoter-promoter interactions in Drosophila (Batut et al., 2022; Calhoun et al., 2002). Moreover, GAF-mediated pairing of tethering elements may help ensure CRE selectivity (Batut et al., 2022; Li et al., 2023). Promoter-promoter contacts between promoter-proximal tethering elements have been shown to underlie the transcriptional coupling of genomically distant functionally-related genes with similar spatio-temporal expression dynamics driven by shared enhancers (Levo et al., 2022). Some interactions between enhancers and promoters also occur across TADs (Batut et al., 2022; Balasubramanian et al., 2024; Yokoshi et al., 2020).

While TADs are proposed to produce insulated genomic domains within which enhancers and promoters interact, the mechanisms behind these interactions remain unclear (Furlong and Levine, 2018). Chromatin looping is proposed to bring genomically distant enhancers and promoters into contact (Fig. 7B). The formation of chromatin loops may involve physical interactions between enhancer and promoter-bound architectural proteins, such as CTCF, and TFs with stable or transient proximity required for transcriptional activation (Fig. 7C). Both population-
Based chromosome conformation methods and imaging have observed enhancers and promoters in close physical proximity (Cruz-Molina et al., 2017; Espinola et al., 2021; Ghavi-Helm et al., 2014; Hsieh et al., 2020; Ing-Simmons et al., 2021; Jin et al., 2013; Krietenstein et al., 2020; Li et al., 2012; Sanyal et al., 2012). Generally, enhancer-promoter proximity correlates with gene activity (Bonev et al., 2017; Ghavi-Helm et al., 2014; Mateo et al., 2019; Rao et al., 2014; Sanyal et al., 2012), and forced looping can induce transcription of some genes (Bartman et al., 2016; Deng et al., 2012; Deng et al., 2014). However, certain studies have detected no correlation, or even an anti-correlation, between enhancer-promoter proximity and gene activity (Alexander et al., 2019; Benabdallah et al., 2019; Chen et al., 2018; Heist et al., 2019). The model of dedicated and stable looping between an enhancer and its target promoter is challenged by the capacity of an enhancer to simultaneously drive bursts of transcription from flanking genes and to co-activate reporter genes in cis and in trans through the phenomenon of transversion in Drosophila embryos (Fukaya et al., 2016; Lim et al., 2018).

An alternative model of enhancer-promoter communication, not reliant on direct looping, is the TF activity gradient (TAG), proposing that acetylated TFs produced by enhancer-localized enzymatic p300/CBP activity diffuse to target promoters in close proximity to activate transcription (Fig. 7C) (Karr et al., 2022). In this model, histone deacetylases (HDACs), chromatin modifiers that also enzymatically target non-histone proteins (Glozak et al., 2005), deacetylate TFs to spatially limit the diffusing acetylated TF signal. HDACs are recruited to active promoters in metazoans, where they may attenuate acetylated TF signals (Filion et al., 2010; Rimcon-Arano et al., 2012; Wang et al., 2008). The quantity of molecules from coactivators such as p300/CBP appears to be limited in cells, being far surpassed by HDACs (Gillespie et al., 2020). Consistently, pharmacological HDAC inhibition in Drosophila cells rapidly elevates histone acetylation levels at active promoters and correlates with Pol II promoter release (Vaid et al., 2020). Overall, the relationship between enhancer-promoter proximity and the transfer of regulatory cues that lead to activation remains unresolved.

### Gene activation in invariant chromosome conformations

During Drosophila embryogenesis, enhancer-promoter proximities are established before transcriptional activation (Espinola et al., 2021; Ghavi-Helm et al., 2014). Strikingly, despite substantial differences in chromatin state and transcriptional activity, DV loci do not markedly change in chromatin configuration between DV tissues (Ing-Simmons et al., 2021). High-resolution imaging of CRE interactions within the dorsocross (doc) TAD, which encompasses three co-expressed dorsal ectoderm-specific genes, revealed that multiple enhancers and promoters coalesce in close proximity independently of cell fate (Espinola et al., 2021). Similarly, anterior-posterior regulated genes display a consistent chromatin configuration in both anterior and posterior halves of early Drosophila embryos, despite their distinct transcriptional states (Stadler et al., 2017). These observations suggest that a preformed topology with close proximity between enhancers and promoters might be a crucial prerequisite for gene activity, but additional signals are necessary to initiate transcription. Interestingly, promoters associated with preformed interactions are enriched for paused Pol II, further implying their priming for activity in response to the required regulatory cue (Ghavi-Helm et al., 2014).

### Nuclear hubs and phase separation in gene activation

Observations of close proximity between potentially multiple enhancers and promoters are consistent with the concept of nuclear hubs or transcription factories, where high concentrations of TFs, cofactors, and the Pol II machinery drive robust bursts of gene expression (Fig. 7D) (Furlong and Levine, 2018; Iborra et al., 1996; Lim and Levine, 2021). Zelda forms sub-nuclear hubs of high concentration within which frequent transient binding events occur that potentiate gene expression during Drosophila embryonic patterning (Dufourt et al., 2018; Mir et al., 2018). Notably, the size of enhancer TF clusters of the anterior-posterior patterning morphogen Bicoid correlates with the burst size of target reporter genes (Kawasaki and Fukaya, 2023).

The process of liquid-liquid phase separation (LLPS), where dense cooperative assemblies of proteins form liquid-like condensates or droplets, has been proposed to explain how high sub-nuclear concentrations of transcriptional components might be achieved (Hnisz et al., 2017). Condensate formation is thought to be driven by multivalent interactions through intrinsically disordered regions (IDRs). Liquid-like condensate formation has been associated with the transcriptional capacity of several coactivators (Boja et al., 2018; Cho et al., 2018; Sabari et al., 2018) and Pol II (Boehning et al., 2018; Cho et al., 2018; Guo et al., 2019; Lu et al., 2018), which have consistently been detected in nuclear clusters by various methods (Cisse et al., 2013; Cook, 1999; Pownall et al., 2023). p300/CBP nuclear condensates are linked to enhanced HAT activity at active genomic sites (Ma et al., 2021) but also the sequestration of HAT inactive condensates in H3K27me3-enriched chromatin (Zhang et al., 2021).

Condensates are also influenced by DNA sequence, with enhancer elements dense in TF recruitment motifs supporting condensate formation in vitro (Shrinivas et al., 2019). While IDR interactions may underlie TF and Pol II clustering (Chong et al., 2018), much debate concerns whether cluster formation in vivo is attributed to LLPS and what relevance this may pertain to transcription (Alberti et al., 2019; McSwiggen et al., 2019). Multivalent clustering has been shown to enhance the transcription activation strength of synthetic TFs, but liquid-like droplet formation did not (Trojanowski et al., 2022).

The formation of localized clusters of TFs, cofactors and the Pol II machinery into hubs at enhancers and promoters in close proximity presents a plausible organization for gene regulatory circuitry. Interestingly, RNAs associated with transcription initiation stimulate condensate formation, while RNAs produced during bursts of elongation have been linked to condensate dissociation (Henninger et al., 2021). This observation aligns with Pol II cluster dynamics observed through chromatin expansion microscopy, where enhancer-promoter interactions occur transiently, and bursts of transcriptional elongation are associated with their separation, termed the “kiss and kick” model (Pownall et al., 2023). At present, what constitutes the transmissible enhancer signal is unclear.
Conclusion

_Drosophila_ embryogenesis has served as a key model system for functionally dissecting developmental gene regulation. The rapid induction of cell type-specific transcriptional programs at ZGA well positions the _Drosophila_ embryo for studying the molecular mechanisms governing how enhancers communicate regulatory cues to promoters and induce transcription. Leading up to ZGA, an elaborate genome organization forms with differential chromatin accessibility, TF binding, and histone PTM deposition, along with the establishment of proximity between enhancers and promoters. The interaction of these different layers of genome regulation to direct transcription across space and time remains unresolved. A major goal is to obtain a mechanistic understanding of how regulatory information is transferred between enhancers and promoters and what the communicable signal may be. It is striking that chromosome conformation is similar in different _Dv_ cell types, despite their major differences in chromatin and transcriptional states. This suggests a preformed topology may be a prerequisite, but not the molecular trigger, of enhancer-promoter communication. Such an organization could facilitate multi-step regulation of developmental transcription, as seen in the DV GRN. Here, within a pre-established organizational architecture of enhancer-promoter proximity, TFs and cofactors may cluster at enhancers in a cell type-specific manner to initiate bursts of transcription from promoters primed with paused Pol II (Fig. 8). The DV GRN is well-positioned for future efforts to functionally interrogate potential mediators of communication, such as rapid optogenetic depletion of p300/CBP or effectors it may modify, like BRD4 or P-TEFb, as achieved in the early embryo for other factors (Huang et al., 2017; McDaniel et al., 2019; Singh et al., 2022).

A key aspect of elucidating enhancer-promoter communication is pinpointing the steps in the transcriptional cycle where regulatory influence is integrated. The DV GRN suggests that tissue-specific transcription is driven by post-recruitment control of the transition of Pol II from pausing to elongating. The recruitment of paused Pol II across cells in the early embryo suggests the widespread adoption of a promoter state conducive to tissue-specific signals. Enhancer-driven transcriptional induction, achieved through control of pause release, is consistent with modulation of the pause release machinery being a key output of enhancer activation. _Drosophila_ embryogenesis is primed to continue to play a pivotal role in unravelling these unknowns.

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Conflicts of interest

The authors declare that they have no conflicts of interest.

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Fig. 8. Developmental gene induction during _Drosophila_ developmental patterning. The current understanding of _Drosophila_ DV gene regulation suggests a multi-step process in the activation of DV-regulated genes. Chromatin is made accessible by Zelda at key enhancers and promoters. Following this, Pol II pausing is established, and enhancer-promoter proximities form across cells prior to ZGA (Hunt et al., 2024). Tissue-specific signals, in the form of the Dorsal (Dl) TF gradient and other tissue-specific TFs, lead to the formation of an active chromatin state. The communication of this signal to target promoters occurs via an unknown mechanism, ultimately inducing the release of paused Pol II from target promoters.


