Int. J. Dev. Biol. 68: 169-188 (2024)
https://doi.org/10.1387/ijdb.230218gh

THE INTERNATIONAL JOURNAL OF DEVELOPMENTAL BIOLOGY
www.intjdevbiol.com

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 (Catarino 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.

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Submitted: 20 October, 2023; Accepted: 16 January, 2024; Published online: 3 June, 2024.

ISSN: Online 1696-3547, Print 0214-6282

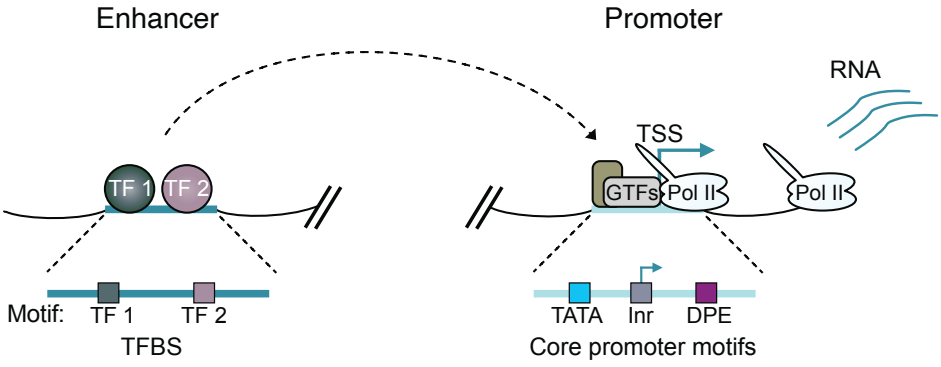

Fig. 1. Enhancers and promoters integrate regulatory signals from transcription factors (TFs) to the genome to drive transcription from promoters. The schematic depicts the core circuitry of enhancers and promoters, highlighting transcription factor binding sites (TFBS) that recruit TFs to enhancers. Additionally, core promoter motifs, such as the TATA box, initiator element (Inr), and downstream promoter element (DPE), are shown. These motifs play a crucial role in recruiting general transcription factors (GTFs) and the RNA Polymerase II (Pol II) machinery to promoters, to initiate the transcription process.

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; Koenecke *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*., 2013). 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-Regi *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). Polycomb response elements (PREs), the recruitment sites of Polycomb group (PcG) 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; Nien *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; Creyghton *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; Xi *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 distinguish 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

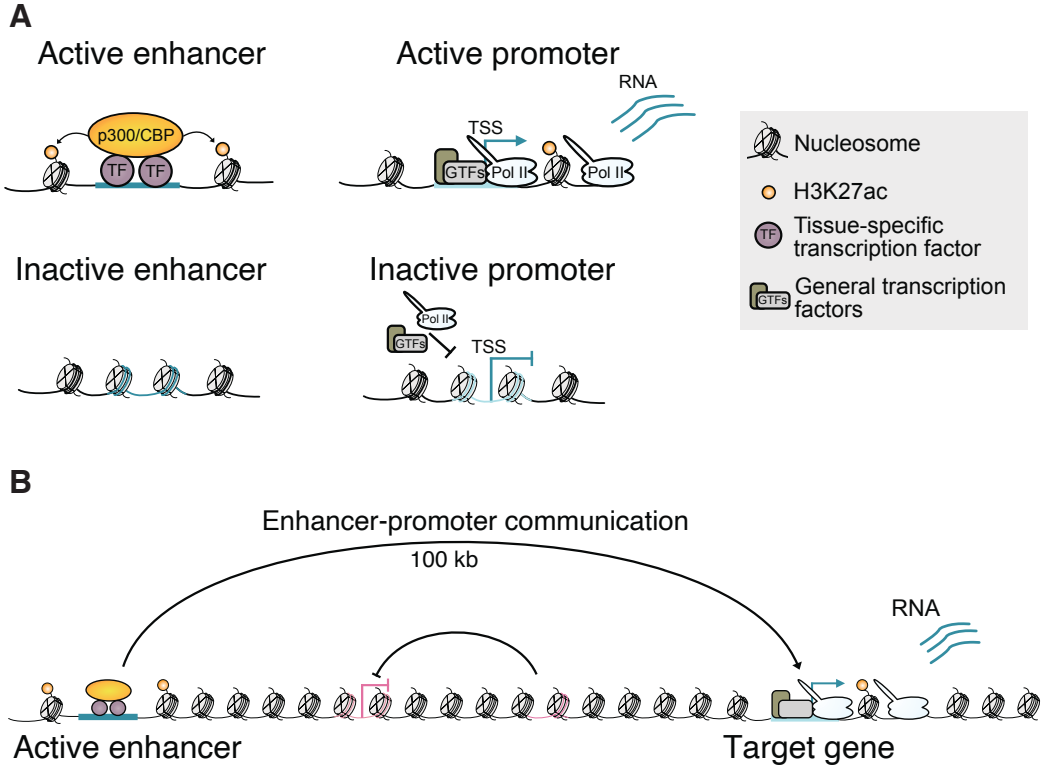

Fig. 2. Enhancers operate across chromatin to influence transcription from target promoters. (A) Schematic showing active and inactive enhancer and promoter chromatin states. **(B)** Communication between enhancers and promoters often occurs over large genomic distances with specificity, enabling these 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*., 2008a; 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; Irizarry 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* (*tld*) 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; Hamaratoglu *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; Koenecke *et al*., 2016; Koenecke *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; Karaiskos *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 (Boija and Mannervik, 2016; Ing-Simmons *et al*., 2021; Koenecke *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.

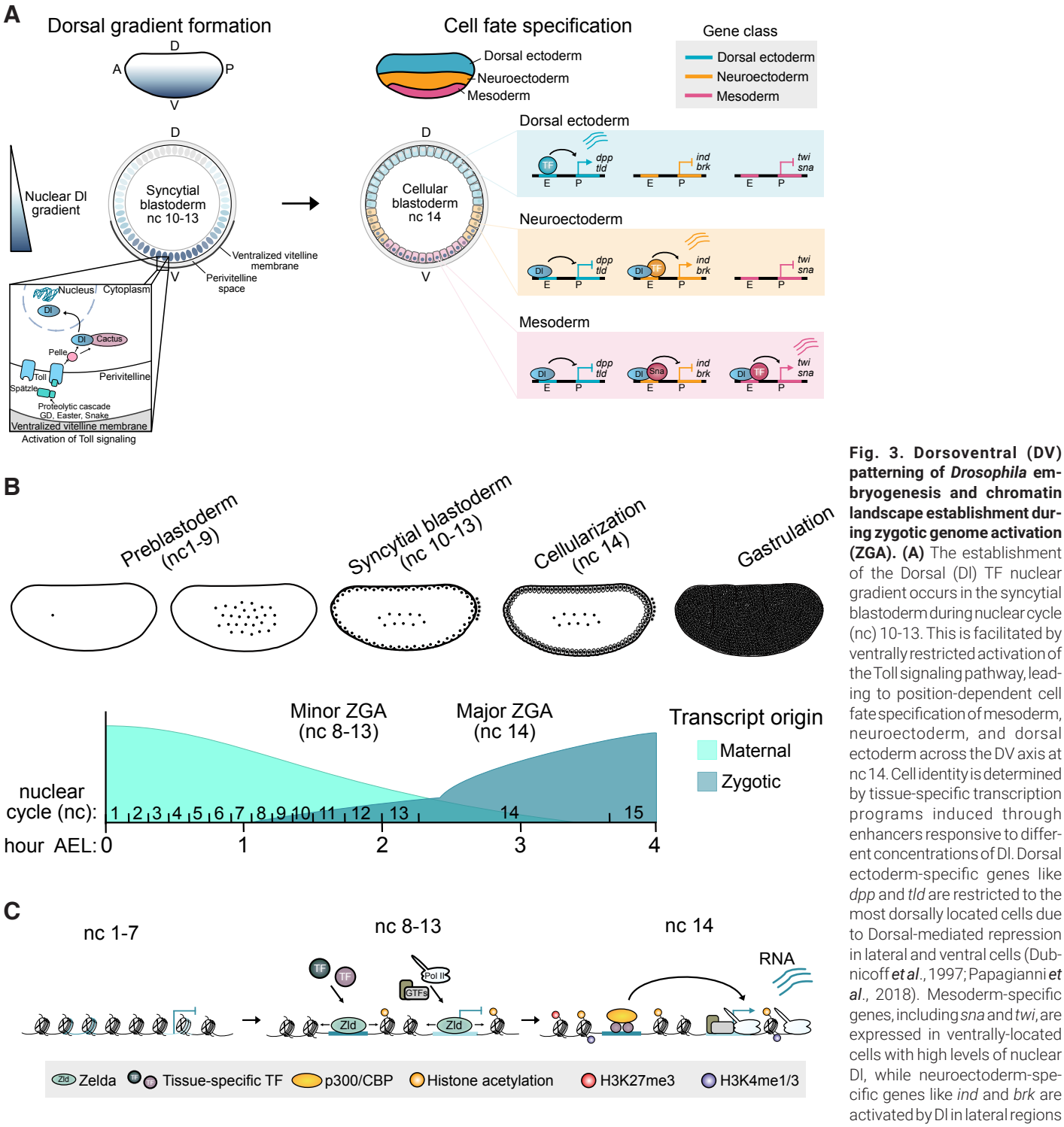

Fig. 3. Dorsoventral (DV) patterning of *Drosophila* embryogenesis and chromatin landscape establishment during zygotic genome activation (ZGA). (A) The establishment of the Dorsal (Dl) TF nuclear gradient occurs in the syncytial blastoderm during nuclear cycle (nc) 10-13. This is facilitated by ventrally 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 tissue-specific 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 Brindle, 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.

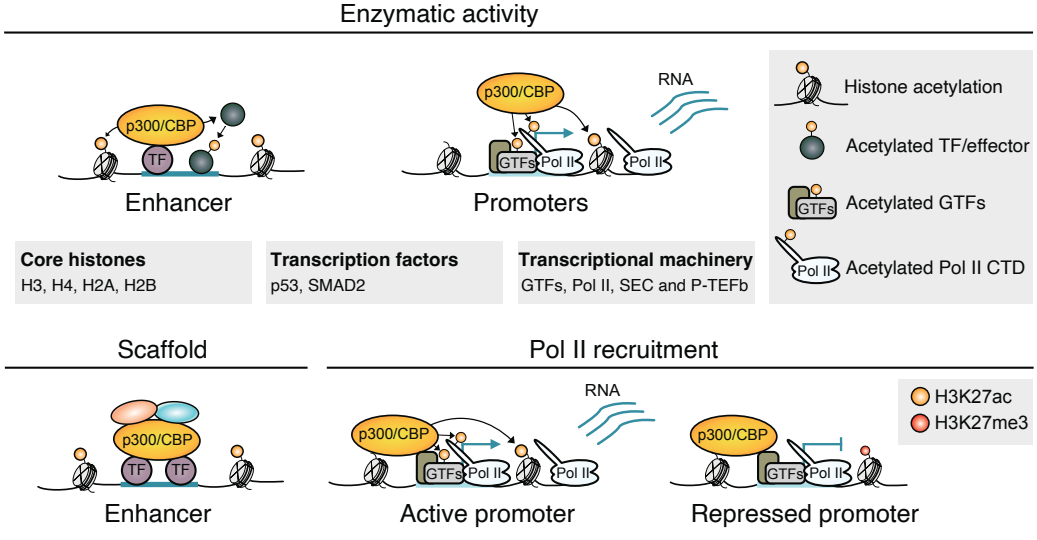

Fig. 4. Functions of p300/CBP in gene regulation. The chromatin-modifier p300/CBP regulates transcription through enzymatic acetyltransferase activity, but also has non-enzymatic activities. p300/CBP acetylates lysine residues on all four core histones, including H3K27ac to mark active enhancers and 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; Rada-Iglesias *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-κB (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).

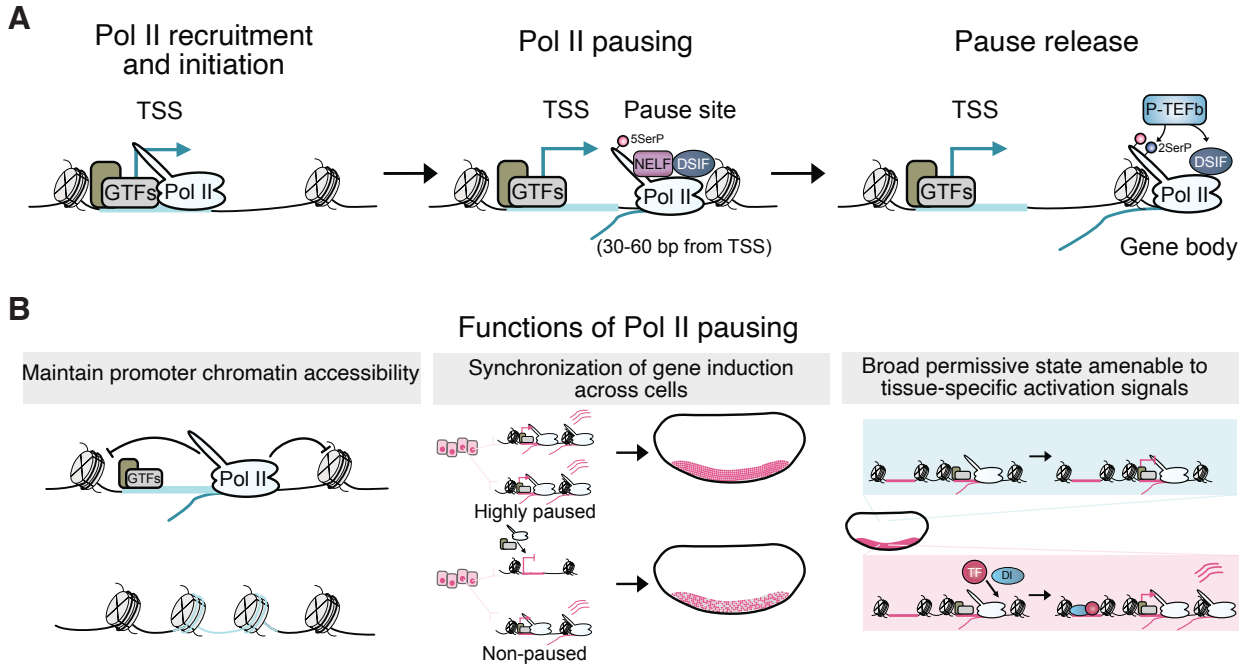

Fig. 5. Pol II pausing and its functions during *Drosophila* embryogenesis. (A) Schematic depicting the steps in the transcriptional cycle, showing the recruitment of Pol II, promoter-proximal Pol II pausing, and release of Pol II into productive elongation. **(B)** Proposed functions of Pol II pausing in gene regulation during development include aiding in repelling nucleosomes from promoter DNA to maintain accessible promoter chromatin (Gilchrist ***et al***., 2010). Pol II pausing is also suggested to synchronize gene induction across cells of a tissue (Boettiger and Levine, 2009; Day ***et al***., 2016; Gaertner and Zeitlinger, 2014; Lagha ***et al***., 2013; Levine, 2011; Ramalingam ***et al***., 2021; Saunders ***et al***., 2013). Additionally, pausing may provide a broadly permissive promoter state that is receptive to tissue-specific signals triggering pause release and gene induction (Gaertner and Zeitlinger, 2014).

Consistent with serving a more fundamental and widespread role, pausing been shown to repel nucleosomes at promoters to maintain nucleosome depletion and ensure promoter DNA is kept accessible for efficient cycles of Pol II recruitment and transcription (Fig. 5B) (Core and Adelman, 2019; Gaertner and Zeitlinger, 2014; Gilchrist *et al*., 2010; Levine, 2011). Pausing may also help synchronize the induction of transcription across tissues and reduce transcriptional noise by attenuating variation in Pol II recruitment between cells (Boettiger and Levine, 2009; Day *et al*., 2016; Gaertner and Zeitlinger, 2014; Lagha *et al*., 2013; Levine, 2011; Ramalingam *et al*., 2021; Saunders *et al*., 2013). Highly stable paused Pol II at the pause site can inhibit new initiation events from taking place, potentially controlling the refractory period between rounds of transcription (Shao and Zeitlinger, 2017).

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 pauses 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). Bursts have 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 TFIID 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

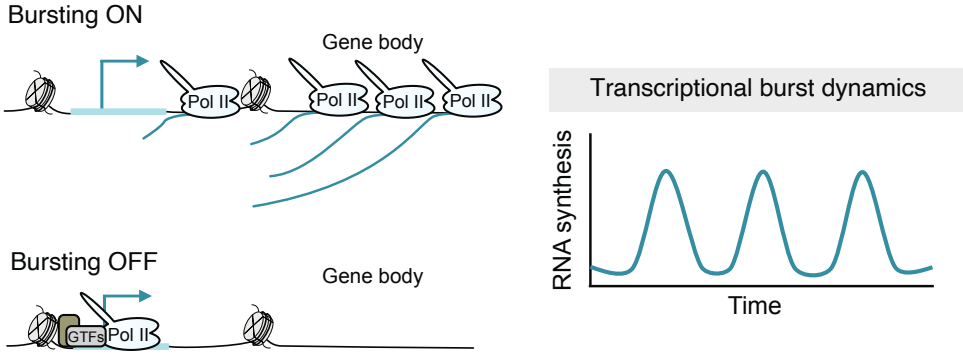

Fig. 6. Gene induction often occurs through bursts of transcription. Schematic of a gene in bursting ON and OFF configurations and a depiction of waves of RNA synthesis resulting from alternations between the ON and OFF states over time.

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; Schwarzer *et 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 in mammals (Franke *et al*., 2016; Lupiáñez *et al*., 2015; Nora *et al*., 2012). However, the depletion of architectural proteins understood to mediate contacts and the dramatically rearranged genome organization in *Drosophila* balancer chromosomes do not majorly affect enhancer-promoter communications and transcription (Ghavi-Helm *et al*., 2019; Hsieh *et al*., 2022). 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-

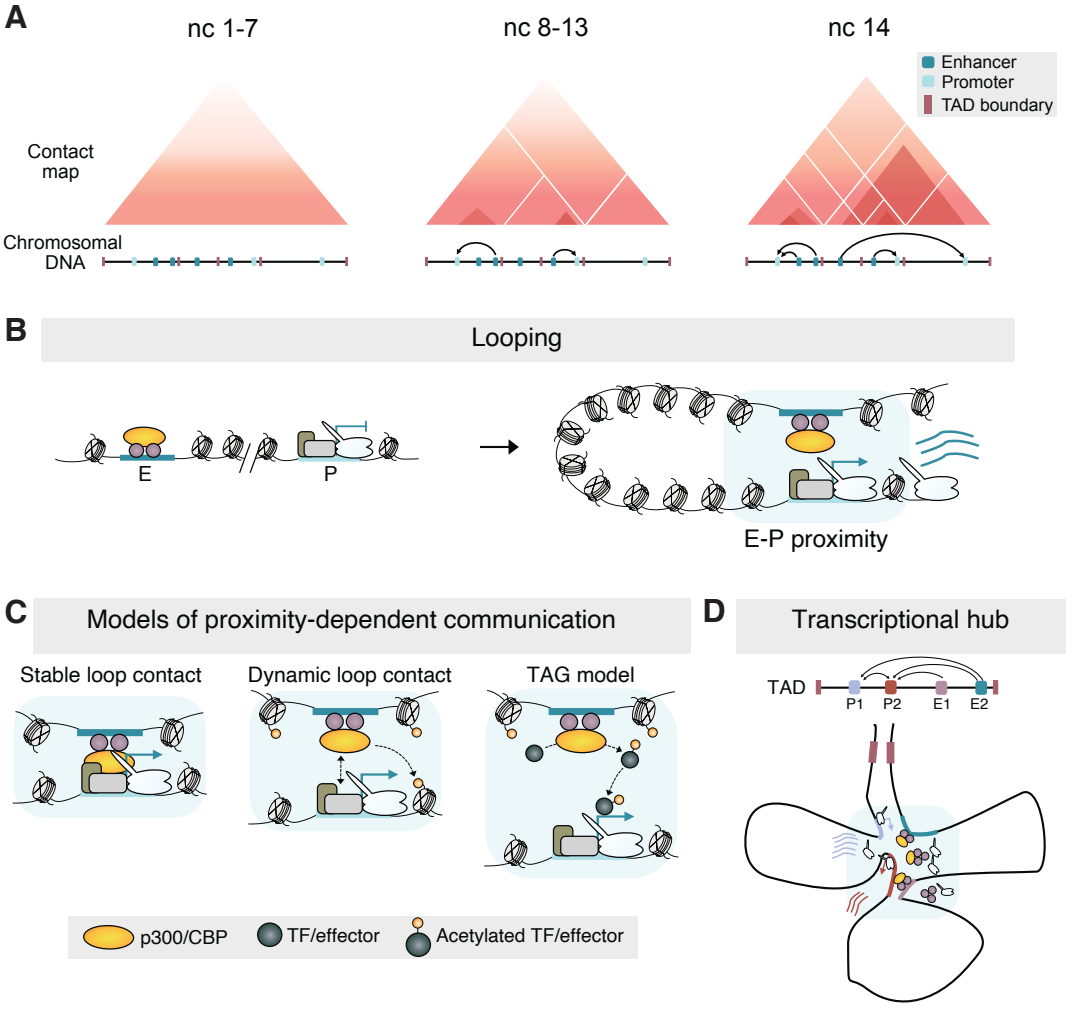

Fig. 7. 3D genome organization and models of enhancer-promoter communication. (A) Schematic of genome organization during early *Drosophila* embryogenesis, showing the formation of TADs and the establishment of enhancer-promoter contacts. **(B)** Model presenting the looping topological conformation that brings enhancers and promoters into proximity. **(C)** Models illustrating proximity-dependent enhancer-promoter communication, showing stable and transient direct enhancer-promoter contacts mediated by physical interactions between enhancer- and promoter-bound factors. Dynamic interactions may transmit signals to promoters via the deposition of histone PTMs at promoter chromatin by enhancer-bound chromatin factors. The transcription factor activity gradient (TAG) model suggests an enhancer-localized activation signal, such as p300/CBP-mediated acetylation of TFs or transcriptional effector like P-TEFb, diffuses to the target promoter in close proximity to induce transcription (Karr *et al*., 2022). **(D)** The transcriptional hub model for gene activation proposes the clustering of multiple enhancers and promoters in proximity. Within this hub environment, a pool of high concentrations of TFs, cofactors, and Pol II collectively drives robust activation (Furlong and Levine, 2018).

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 transvection 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; Rincon-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 conformation 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 conformation 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 (Boija *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.

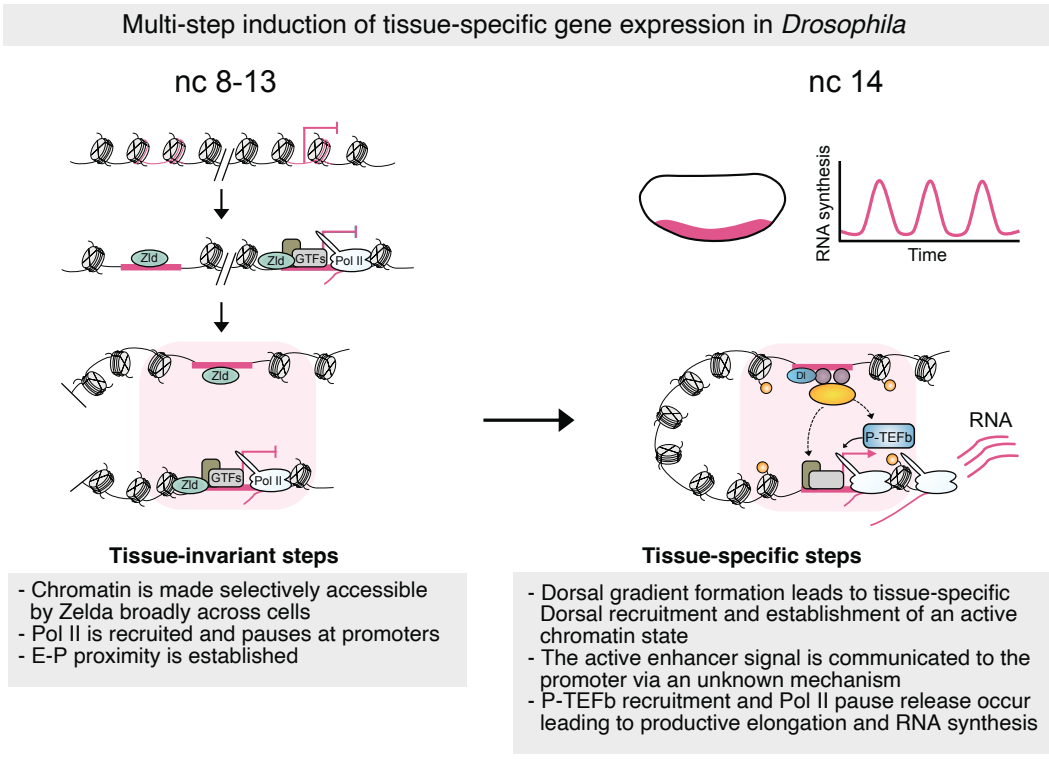

Fig. 8. Developmental gene induction during *Drosophila* developmental patterning. The current understanding of *Drosophila* DV gene regulation suggests a multi-step process in the activation of DV-regulated genes. Chromatin is made accessible by Zelda at key enhancers and promoters. Following this, 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.

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.

Acknowledgments

We thank members of the Mannervik lab for helpful discussions. The work was funded through the Swedish Research Council (Vetenskapsrådet) to M.M.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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