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A Time of Change: Dynamics of Chromatin and Transcriptional Regulation During Nuclear Programming in Early *Drosophila* Development

ANN BOIJA AND MATTIAS MANNERVIK*

Department of Molecular Biosciences, The Wenner-Gren Institute, Stockholm University, Stockholm, Sweden

SUMMARY

In order for a new organism to form, the genomes of the highly specialized egg and sperm need to be reprogrammed into a totipotent state that is capable of generating all of the cell types that comprise an organism. This reprogramming occurs by erasing chromatin modifications, leaving the cells in a naïve state, followed by the induction of specialized programming events. Pioneer factors bind to the genome prior to zygotic genome activation, followed by acetylation of histones and further chromatin specialization by the addition of methylation marks later during differentiation. Genome-wide approaches have provided insight into the genomic and epigenomic regulation of gene expression during development, providing a new perspective on the process of cell specification and differentiation. In this review, we discuss how distal DNA and core promoter elements, RNA polymerase pausing, transcription factors, and co-regulators interact to shape the chromatin landscape and direct tissue-specific expression patterns during embryo development, focusing on the well-characterized *Drosophila* embryo.

[D]evelopmental determinants orchestrate differential chromatin states, providing new insights into the link between epigenetics and developmental patterning.

*Corresponding author:
Department of Molecular Biosciences
The Wenner-Gren Institute
Stockholm University
SE-10691 Stockholm, Sweden.
E-mail: mattias.mannervik@su.se

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INTRODUCTION

One fundamental question in biology is how cells with identical DNA sequence become different cell types and form distinct tissues that, together, comprise an entire organism. Developmental biologists have been trying to answer this question by various approaches, initially using transplantation experiments, followed by genetic and molecular methods, and more recently by genome-wide studies. The precise control of tissue-specific gene expression is central to cell differentiation and development. Gene regulation must be highly dynamic in order to switch genes on and off when needed during development and to respond to specific environmental cues.

Covalent modifications of histones is one dynamic method of transcriptional regulation. The discovery that acetylation of histones is associated with highly transcribed genes was made over 50 years ago (Allfrey

Abbreviations: CBP, CREB-binding protein; ChIP, chromatin immunoprecipitation; H3K#[ac/me], histone 3 lysine # [acetylated/methylated]; Pol II, RNA Polymerase II; P-TEFb, positive-transcription elongation factor b; ZGA, zygotic genome activation

et al., 1964). Since then, genome-wide mapping has been performed for various histone modifications in a wide range of organisms during different stages of development (Liu et al., 2005; Wang et al., 2008; Negre et al., 2011; Roudier et al., 2011). Like histone acetylation, trimethylation of lysines 4 and 36 in histone H3 (H3K4me3 and H3K36me3) is linked to gene activation, whereas methylation of H3 lysines 9 and 27 (H3K9me3 and H3K27me3) is associated with heterochromatin formation and transcriptional silencing (Kouzarides, 2007). H3K4 mono-methylation (H3K4me1) is a mark of cis-regulatory DNA regions (enhancers) (Smith and Shilatifard, 2014). There are also variants of the four canonical histones —H2A, H2B, H3, and H4—that are associated with different chromatin activities (Weber and Henikoff, 2014).

Genes are controlled by activators and repressors that bind to DNA and recruit transcriptional co-regulators (Mannervik, 2014; Fig. 1). Activators tend to be broadly distributed while repressors are spatially limited. The master regulatory unit of transcription is the enhancer or cis-regulatory module where transcription factors bind in different combinations, resulting in various spatio-temporal gene expression patterns (Levine, 2010). Ultimately, transcription factors control the recruitment and release of RNA Polymerase II (Pol II) from the promoter (Fig. 1).

Genome sequencing, bioinformatic techniques that scan the entire genome for clusters of transcription factor binding sites, and the binding profiles of transcription factors and histone modifications achieved with genome-wide chromatin immunoprecipitation (ChIP) studies (ChIP–chip and ChIP-sequencing) have provided new insights into gene regulatory networks. For example, of the transcription factors analyzed, most are detectably bound to at least several thousand genomic regions—but how many of these are biologically relevant? A transgenic reporter-gene assay in *Drosophila* embryos compared the expression driven by regulatory regions with high versus low occupancy of the transcription factor Krüppel (Fisher et al., 2012). Regions bound by high levels of Krüppel *in vivo* drove expression of the reporter gene during early development, whereas most regions with low levels of Krüppel binding did not. Thus, most of the lowest-occupancy interactions of transcription factors with DNA are unlikely to play a biologically significant role in regulating transcription. Instead, a model of continuous transcription networks has been proposed, where most animal transcription factors each bind over a quantitative series of DNA occupancy levels, spanning both functional and non-functional DNA binding events (Biggin, 2011).

Embryonic pattern formation, cell specification, and their underlying developmental pathways are highly conserved and often proceed using similar molecular mechanisms in different species (Wolpert, 1994). The early *Drosophila melanogaster* embryo constitutes a very well characterized system to study gene regulation *in vivo*. We will therefore focus on the genomic and epigenomic regulation of gene expression during development in *Drosophila,* drawing parallels to other systems as appropriate.

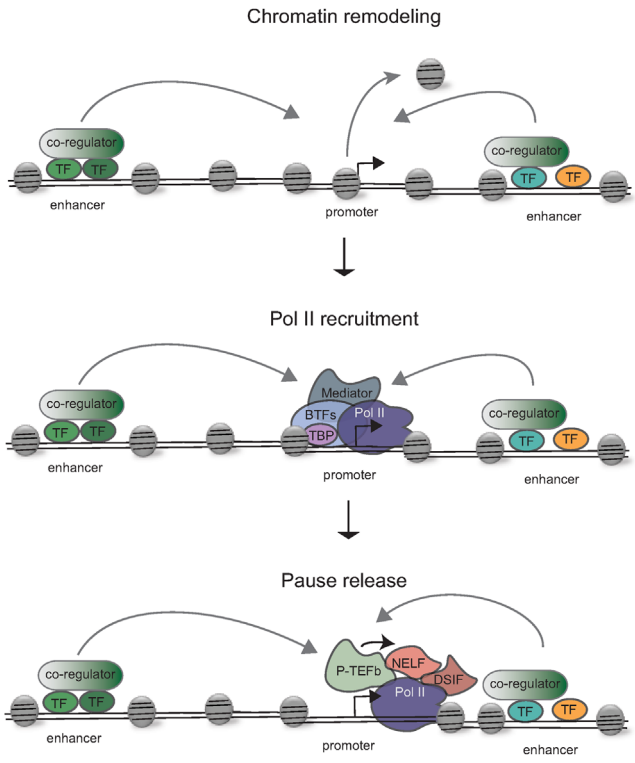

Figure 1. Gene activation requires chromatin remodeling, Pol II recruitment, and release from the promoter. Activation of gene expression in metazoans is regulated by transcription factors (TF) that bind to cis-regulatory DNA sequences (enhancers). Transcription factors interact with co-regulators to remodel chromatin, making the promoter accessible to pre-initiation complex assembly. This is initiated by basal transcription factors (BTFs), including the TATA-box binding protein (TBP), that recognize specific core promoter elements. These basal transcription factors position Pol II over the transcription start site. The Mediator complex bridges enhancer-bound transcription factors with Pol II, facilitating polymerase recruitment to the promoter. On many genes, transcription initiation is followed by Pol II pausing approximately 50 bp downstream of the transcription start site. This promoter-proximal pausing involves the negative elongation factors NELF and DSIF. Pol II is released into productive elongation by P-TEFb kinase-dependent phosphorylation of NELF, DSIF, and Pol II itself.

FERTILIZATION—MERGING OF TWO HAPLOID GENOMES FROM HIGHLY SPECIALIZED CELLS

In animals, a new individual is created by the fusion of two highly specialized cells, the sperm and the egg. These haploid cells (the gametes) are derived from germ cells and undergo two separate and complex differentiation processes, oogenesis, and spermatogenesis, which require a major chromatin reorganization (Albert and Peters, 2009; Burton and Torres-Padilla, 2010). In *Drosophila*, histone modifications have an important function in preparing oocyte chromatin for meiosis, which results in a very condensed chromatin structure and almost no transcription (Iovino, 2014). During spermatogenesis, the histones first become hyperacetylated and then substituted by protamines (Rathke et al., 2014), again resulting in a highly

compact chromatin from which there is no transcription (Fig. 2).

The egg is activated in conjunction with fertilization. In *Drosophila*, this is achieved by release of the oocyte from the ovary into the oviduct, resulting in an increase in intracellular calcium levels (Heifetz et al., 2001; Horner and Wolfner, 2008a; Sartain and Wolfner, 2013; Kaneuchi et al., 2015). As a consequence of fertilization meiotic arrest of the oocyte is released; haploid female and male pronuclei are formed; and maternal mRNAs and proteins become activated or repressed. Interestingly, a large fraction of proteins involved in chromatin modification and organization are translationally upregulated upon egg activation, including the histone demethylases KDM5 (Little imaginal discs, Lid), KDM4A, and KDM4B (Kronja et al., 2014). Therefore, an important task during egg activation is to prepare the newly formed zygotic genome for subsequent transcriptional activation.

REPROGRAMMING OF GERM CELL EPIGENOMES

Completion of meiosis in the fertilized egg results in a haploid female pronucleus that can combine with the haploid genome from the sperm. For this to occur, the sperm nucleus has to undergo a major change from the highly condensed protamine-based state (Horner and Wolfner, 2008b), which involves the replacement of the sperm-specific protamines with histones and histone variants from maternal stores (Fig. 2). This results in chromatin decondensation and formation of a maternally derived nuclear envelope (Poccia and Collas, 1997). Further decondensation leads to a male pronucleus similar in size to the female pronucleus. In *Drosophila,* the histone chaperones NAP-1, NLP, nucleophosmin, and TAP/p32 may participate in the removal of protamines (Emelyanov et al., 2014); histone chaperone HIRA incorporates the histone variant H3.3 in cooperation with the Yemanuclein protein (Loppin et al., 2005; Orsi et al., 2013), and the chromatin remodeler CHD1 is required to facilitate chromatin decondensation of the male pronucleus (Konev et al., 2007). The resulting paternal chromatin is thus replete with the histone variant H3.3 whereas the maternal chromatin consists of the canonical histone H3. Fusion of these decondensed male and female pronuclei forms the zygotic nucleus.

FORMATION OF TOTIPOTENT NUCLEI

The first nuclear divisions generate totipotent nuclei in the syncytial blastoderm (Fig. 2). Early transplantation experiments of preblastula- and gastrula-stage nuclei, which have started to differentiate, into unfertilized eggs demonstrated that the nuclei could be reprogrammed to totipotent cells that developed to the larval stage (Illmensee, 1968,1973). Later experiments generated adult *Drosophila* by a similar nuclear transplantation experiment, wherein preblastoderm-stage nuclei were injected into activated haploid eggs (Haigh et al., 2005). Together, these studies indicate that, as in vertebrates (Gurdon and Wilmut, 2011), the oocyte has the capacity to reprogram somatic cells into a totipotent state. Therefore, maternal factors have to be involved in reprogramming. Such reprogramming is very inefficient in both vertebrates and in *Drosophila*, however, as around 97% of *Drosophila* recipients die during embryogenesis (Haigh et al., 2005). Although mechanical damage to the egg during transplantation could be part of the explanation, it is possible that an inability to completely reprogram epigenetic information contributes to the low efficiency.

EMERGENCE OF HETEROCHROMATIN DURING BLASTODERM FORMATION

Two types of chromatin—late replicating, largely transcriptionally silent heterochromatin and early replicating, transcriptionally active euchromatin—are readily distinguished in eukaryotic cell nuclei (Fig. 3). Constitutive heterochromatin forms in all cells and silences repetitive DNA elements at centromeres, telomeres, and along the Y-chromosome, whereas facultative heterochromatin stably silences expression of cell-type specific genes and one of the two X-chromosomes in female mammals (Beisel and Paro, 2011). Constitutive heterochromatin, however, is absent from cleavage-stage *Drosophila* embryos and only becomes cytologically visible at the blastoderm stage (around 2-hr-old embryos) (Vlassova et al., 1991). Heterochromatin-mediated gene silencing is initiated about an hour later, at the time of gastrulation (Lu et al., 1998), suggesting that heterochromatin gradually matures into a functional state (Fig. 2).

Constitutive heterochromatin consists of H3K9me2/me3 and heterochromatin protein 1a (HP1a). Surprisingly, maternally contributed H3K9 methylase Su(var) 3−9 and HP1a do not establish heterochromatin until the blastoderm stage (Vlassova et al., 1991), implying that their activity is regulated. For example, HP1a becomes phosphorylated at the time of heterochromatin assembly (Eissenberg et al., 1994). Small RNAs of the piRNA class may also be involved since depleting the maternal contribution of Piwi affects heterochromatic silencing and the binding of HP1a to pericentric heterochromatin (Gu and Elgin, 2013). The H3K4me2 demethylase KDM1 (LSD1, Su(var) 3−3) helps define the border between euchromatin and heterochromatin by preventing the spread of H3K4me2 and H3K9 acetylation into heterochromatin in blastoderm-stage embryos (Rudolph et al., 2007).

Facultative heterochromatin generated by Polycomb silencing and H3K27me3 is also not established until the blastoderm stage. Using a temperature-sensitive allele of *extra sex combs* (*esc*), which is part of Polycomb repressive complex 2 (PRC2) that methylates H3K27, it was shown that the homeotic transformation resulting from a failure to repress *Hox* gene expression only occurs from the end of the blastoderm stage until the end of the germ-band stage (Struhl and Brower, 1982). Similarly, using a LexA-Polycomb fusion protein to target Polycomb repression to a reporter gene showed that, despite maternal expression of

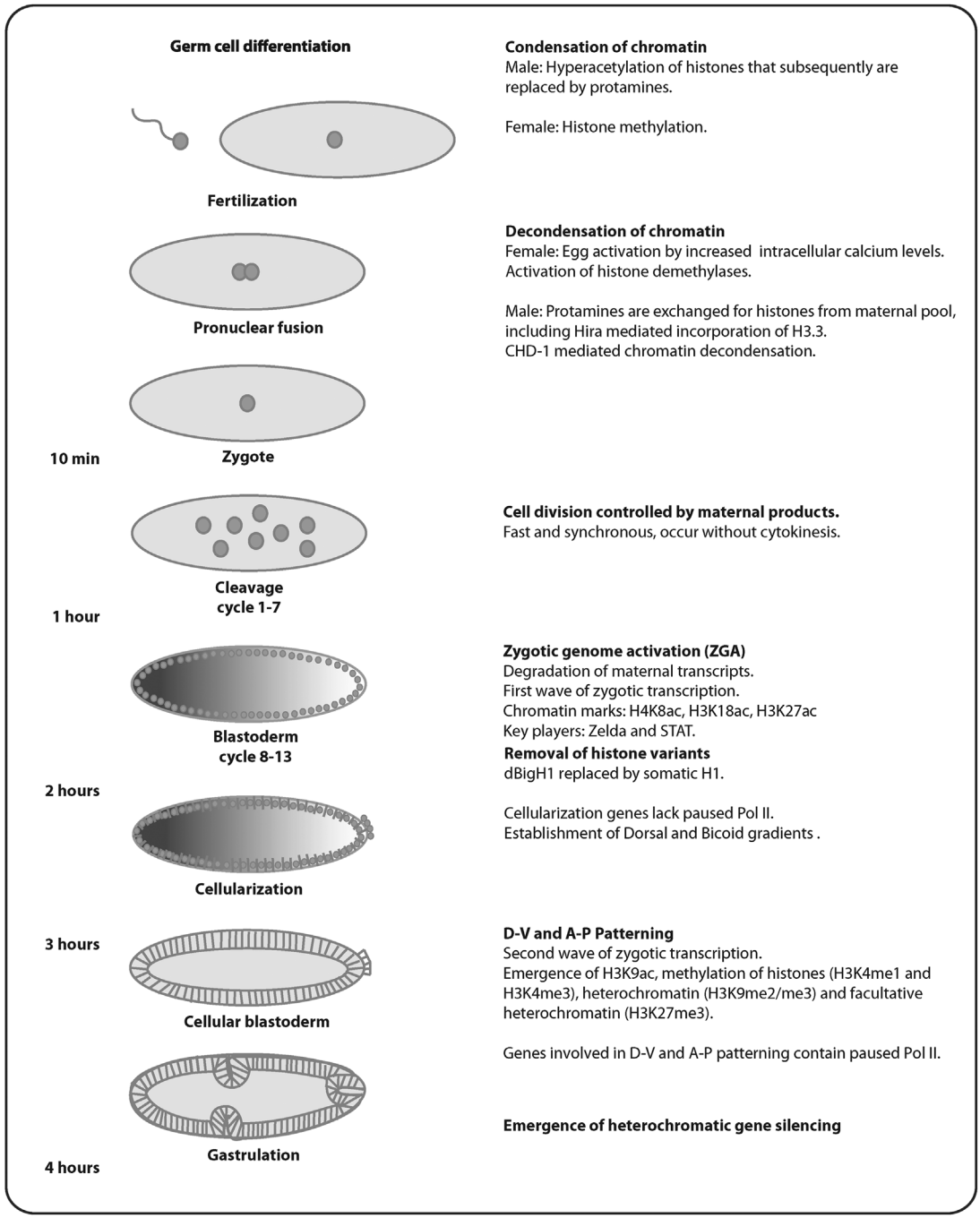

Figure 2. Nuclear programming involves changes in chromatin state and the onset of zygotic transcription. Terminally differentiated germ cells, the sperm and the egg, are transcriptionally silenced due to highly condensed chromatin containing protamines instead of histones (sperm) or to histone methylation (egg). Egg activation and fertilization leads to translational activation of histone demethylases along with the exchange of protamines for histones and histone variants in the male pronucleus. During the first nuclear divisions, transcription is silent. A first wave of ZGA, at cycle 8, is accompanied by acetylation of some histone residues and replacement of linker histone dBigH1 for somatic H1. The pioneer transcription factor Zelda facilitates binding of other transcription factors, including the dorsal–ventral (D–V) and anterior–posterior (A–P) morphogens Dorsal and Bicoid, respectively. At the major second wave of ZGA, histone methylation marks become established and many patterning genes are regulated by release of paused Pol II from the promoter. Heterochromatic gene silencing does not occur until after gastrulation.

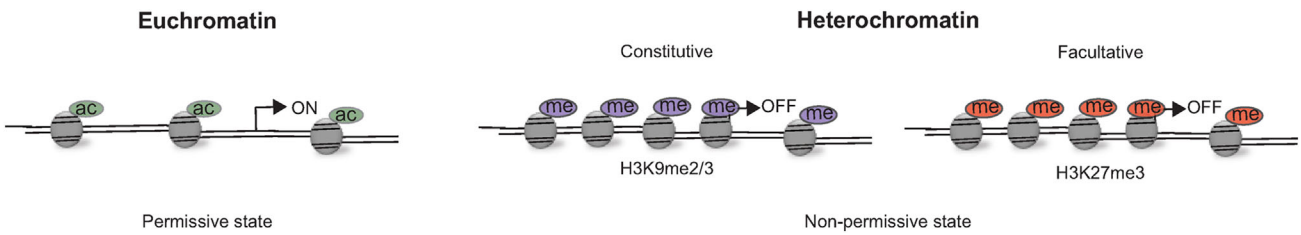

Figure 3. Eukaryotic genomes are embedded in euchromatin and heterochromatin. Euchromatin is the less-condensed form of chromatin that is accessible for regulatory proteins and RNA polymerase. It permits active transcription of genes, and is often associated with histone acetylation. Heterochromatin, on the other hand, is a more tightly packed form of chromatin that is characterized by histone methylation, leading to a non-permissive state for regulatory factors and gene transcription. There are two types of heterochromatin: Constitutive heterochromatin forms at repetitive DNA elements and consists of H3K9me2/3, whereas facultative heterochromatin silences gene expression in a cell-type specific manner and is characterized by H3K27me3.

the fusion protein, silencing cannot occur prior to the blastoderm stage (Poux et al., 2001). The timing of Polycomb-associated H3K27me3 accumulation correlates with its known role in regulating developmental genes in a cell-type specific manner (Boyer et al., 2006; Lee et al., 2006; Schwartz et al., 2006).

ACTIVATION OF THE ZYGOTIC GENOME: A CHANGE IN THE CHROMATIN LANDSCAPE

Maternal mRNA and proteins that are deposited in the fertilized egg ensure the initial control of metazoan embryo development. During this first phase, the zygotic genome is transcriptionally inactive. The onset of zygotic gene expression occurs after a species-specific number of mitotic cycles, during a process known as the ''maternal-to-zygotic transition.'' This is a conserved process in metazoans, and includes the degradation of maternal transcripts that are substituted by zygotic transcripts (Tadros and Lipshitz, 2009). Zygotic genome activation (ZGA) occurs in two distinct waves: a minor wave that activates only a few tens of genes followed by a major wave that activates several hundreds of genes. This transcriptional programming results in drastic morphological changes (Lee et al., 2014).

In *Drosophila melanogaster,* the first 13 cell-division cycles occur without cytokinesis, resulting in a syncytium (Fig. 2). Maternal-contributed products control the initial, very fast and synchronous 7 cycles. The onset of ZGA occurs during cycle 8, while the nuclei begin to migrate to the embryo cortex. The first nuclei arrive at the posterior cortex during cycle 9, where they are incorporated into the pole cells, which become the future germ cells. Somatic cell precursors, on the other hand, arrive at the cortex during cycle 10, where they divide four more times before being surrounded by plasma membrane, thereby forming a cellular blastoderm, a process known as cellularization (Sullivan and Theurkauf, 1995). The histone variant, embryonic linker histone H1 dBigH1, is abundant before cellularization, and is replaced by the somatic histone H1 at the time of ZGA; in the absence of dBigH1, ZGA occurs prematurely (Perez-Montero et al., 2013). The onset of the second, major wave of zygotic transcription occurs at cycle 14.

A recent study showed that the two waves of zygotic transcription are accompanied by two distinct groups of chromatin marks (Li et al., 2014). Acetylation of H4K8, H3K18, and H3K27 are the first chromatin marks associated with zygotic transcription, accumulate starting at cycle 8, and increase through cycle 14. By contrast, the chromatin marks H3K9ac, H3K4me1, H3K4me3, H3K36me3, and H3K27me3 are absent during this initial transcriptional burst; they instead accumulate during the second wave of transcription (Chen et al., 2013; Li et al., 2014). This temporal distinction agrees with bulk analysis of histone modifications during *Drosophila* embryogenesis, which revealed that detectable levels of H3K27me3 could only be observed after 4 hr of development and that the levels increase with time (Tie et al., 2009). By contrast the higher levels of H3K27ac present during early stages declined with time (Tie et al., 2009). Therefore, exit from a naïve cell state occurs through two distinct chromatin changes: acetylation of the chromatin followed by methylation (Fig. 4).

PRE-PATTERNING BY UBIQUITOUSLY EXPRESSED PIONEER FACTORS INVOLVED IN ZGA

Maternal transcription factors expressed ubiquitously have been shown to play a key role in in the process of ZGA. One factor that is needed for both waves of ZGA is Zelda (Zinc-finger early *Drosophila* activator) (Liang et al., 2008), which binds to target genes prior to the onset of ZGA (Liang et al., 2008; Harrison et al., 2011; Nien et al., 2011). Zelda recognizes the TAGteam motif implicated in the activation of pre-blastoderm transcripts by several studies (ten Bosch et al., 2006; De Renzis et al., 2007; Li et al., 2008; Liang et al., 2008). The TAGteam motif is also enriched in highly occupied target (HOT) regions, where many transcription factors bind even in the absence of their canonical recognition motifs (Li et al., 2008; MacArthur et al., 2009; Roy et al., 2010; Kvon et al., 2012; Satija and Bradley, 2012). Zelda has been shown to augment transcription and to collaborate with transcription factors involved in embryo patterning, such as Dorsal and Bicoid

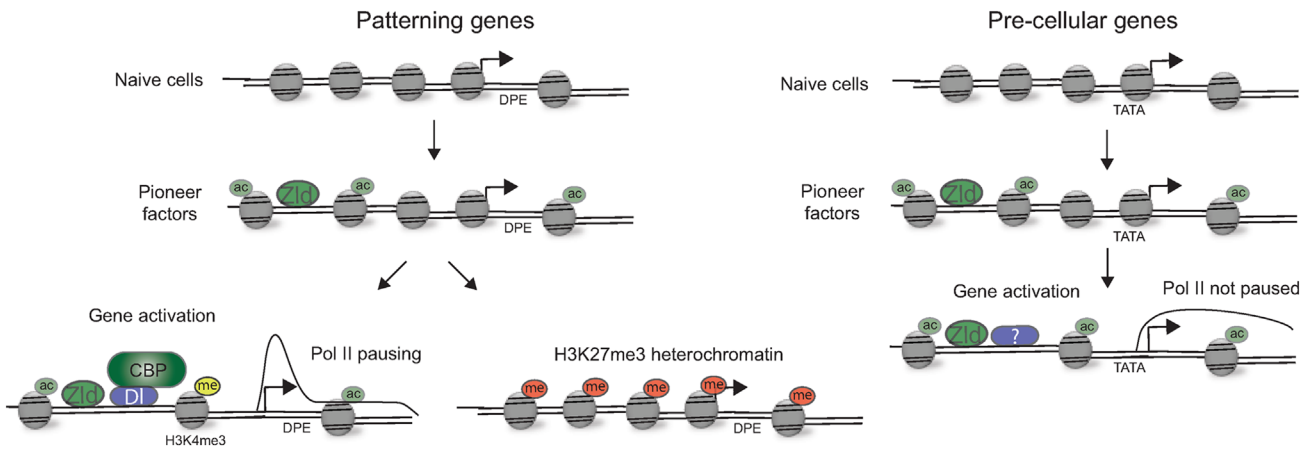

Figure 4. A changing chromatin landscape during cell specification and differentiation. The transition from naïve cells to specified and then differentiated cells is initiated by the pioneer transcription factor Zelda (Zld), which can overcome the steric constrains of chromatin. Zelda facilitates the binding of other factors, such as Dorsal (Dl), to genes involved in embryonic patterning. Dorsal recruits the cofactor CBP, resulting in gene activation. During development, a gene is dressed with different chromatin states, depending on which tissue the cell will form. Binding of pioneer factors is accompanied by histone acetylation, which is followed by a chromatin state with additional histone acetylation as well as H3K4me3 at the promoter of fully activated genes. H3K27me3 heterochromatin keeps pattern genes silent in tissues where they are not expressed. Transcription of many patterning genes is regulated by Pol II pausing, and is associated with specific core promoter motifs, for example the downstream promoter element (DPE). By contrast, many pre-cellular genes are rapidly transcribed, not paused, and associated with distinct core promoter elements, such as the TATA motif.

(Reeves and Stathopoulos, 2009; Kanodia et al., 2012; Xu et al., 2014).

Chromatin provides a steric constraint on how transcription factors can bind DNA. Pioneer transcription factors, however, can overcome such constraints, enabling them to engage chromatin that is not accessible by other types of transcription factors. The interaction of pioneer factors with chromatin facilitates binding of other transcription factors, cofactors, and chromatin-modifying, and remodeling enzymes, culminating in gene activation (Iwafuchi-Doi and Zaret, 2014). Zelda binding increases DNA accessibility and facilitates the binding of Dorsal and Bicoid to target enhancers (Foo et al., 2014; Xu et al., 2014). Furthermore, DNA accessibility correlates with the number of Zelda binding sites, which sets the threshold for responding to the Dorsal gradient (Foo et al., 2014). These studies show that Zelda shares many characteristics with pioneer factors during ZGA.

Homologs of Zelda have not yet been identified in organisms other than insects. Until recently, it was unclear if a specific factor also primed the earliest zygotic genes for activation in other organisms. Two recent studies in zebrafish, however, identified the pluripotency-inducing factors Pou5f1 (a homolog of mammalian Oct4), Nanog, and Sox19b (a Sox2 homolog) on target sites before the onset of zygotic transcription (Lee et al., 2013; Leichsenring et al., 2013). They were further shown to be important for activation of the earliest zygotic genes. These factors act as pioneering factors during the reprogramming of differentiated cells into induced pluripotent stem cells (iPS; Soufi et al., 2012), and may play a similar role during ZGA in zebrafish (Lee et al., 2014). Thus, Pou5f1, Nanog, and Sox19b perform a similar function to Zelda in priming early genes for activation. Reciprocally, this observation raises the possibility that Zelda contributes to the pluripotency of early *Drosophila* embryos.

How transcription is triggered at ZGA remains to be characterized. In what way specific transcriptional regulators, epigenetic control, and recruitment of Pol II convene to turn genes on is still an open question. Whereas around 550 promoters are bound by Pol II in cycle 12, Pol II can be detected at approximately 3000 promoters in cycle 13 (Blythe and Wieschaus, 2015). Interestingly, Pol II at promoters newly bound in cycle 13 pauses downstream of the transcription start site and do not resume transcription elongation until cycle 14. Pol II binding to these promoters is necessary for the association of replication protein A to the genome, which stalls DNA replication at transcriptionally engaged promoters (Blythe and Wieschaus, 2015). This recruitment process activates a DNA replication checkpoint in cycle 13, thereby lengthening the cell cycle by 50% compared to the previous cycle. Zelda mainly affects the binding of Pol II to promoters that are already occupied in cycle 12, and has little effect on the genes newly bound by Pol II in cycle 13 (Blythe and Wieschaus, 2015).

The transducer of the JAK/STAT pathway, STAT92E, has been shown to activate the transcription of many early genes in cooperation with Zelda. The STAT-binding motif is enriched in promoters of early zygotic genes, and embryos lacking maternal STAT show significant downregulation of early zygotically expressed genes (Tsurumi et al., 2011). By combining available transcriptome and ChIP-sequencing data of different transcription factors

and histone modifications at the time of ZGA, one bioinformatic study revealed that genes that are up-regulated during ZGA are enriched in binding motifs for Zelda, Tramtrack, and GAGA-factor (GAF) (Darbo et al., 2013). Furthermore, predicted enhancers of genes activated at ZGA are bound by GAF and the histone acetyltransferase CREB-binding protein (CBP), and exhibit open chromatin regions.

Collectively, these studies demonstrate that ubiquitously expressed factors may act as pioneers to pre-pattern the genome. Moreover, they are important for the onset of the massive transcription that occurs during the maternal-to-zygotic transition (Fig. 4).

THE ROLE OF POL II PAUSING DURING TRANSCRIPTIONAL REGULATION OF DEVELOPMENTAL GENES

Transcription factors bind DNA regulatory elements to control recruitment and release of Pol II from the promoter (Fig. 1). Pol II recruitment and release of a paused polymerase downstream of the transcription start site constitute two major checkpoints during transcription. Approximately 50 bp downstream of the transcription start site, Pol II is held in a paused state by the negative elongation factors NELF and DSIF. Release from pausing is mediated by positive-transcription elongation factor b (P-TEFb) kinase, which phosphorylates these factors as well as the carboxy-terminal domain of Pol II, allowing it to proceed into elongation (Peterlin and Price, 2006; Gilmour, 2009). Inhibiting P-TEFb activity with flavopiridol affects practically all gene expression in metazoans (Henriques et al., 2013; Jonkers et al., 2014). Notably, active, non-paused genes are most sensitive to P-TEFb inhibition, demonstrating a continuous requirement for the efficient release of Pol II into productive elongation (Henriques et al., 2013).

Understanding the regulation of transcription elongation has gained increasing interest in the last decade, and Pol II pausing has been shown to be wide-spread in *Drosophila* and mammalian genomes (Adelman and Lis, 2012). Indeed, many developmentally regulated genes are associated with paused polymerase (Zeitlinger et al., 2007). The recent high-resolution technology of global run-on sequencing (GRO-seq), which maps nascent RNAs produced by transcriptionally engaged polymerases, has been used to record Pol II occupancy across the genome (Core et al., 2008). For example, mapping of genome-wide Pol II distribution by GRO-seq identified polymerase pausing as a wide-spread mechanism for regulating genes involved in axis patterning in the early *Drosophila* embryo (Saunders et al., 2013). Among paused genes, the rate-limiting step is often pause release—although some paused genes are still regulated by recruitment of Pol II to the promoter. Most patterning genes, including the majority of gap genes, pair-rule genes, segment polarity genes, as well as genes involved in dorsal–ventral patterning, were found to be regulated by pause release (Saunders et al., 2013). One function for Pol II pausing could be to facilitate synchronous gene activation in a group of cells during development (Boettiger and Levine, 2009).

In *Drosophila*, pre-cellular genes activated at the first wave of ZGA, including genes involved in cellularization, do not show Pol II pausing, and display a distinct set of core promoter elements compared to genes activated later during development (Chen et al., 2013). The composition of pre-cellular genes is often short and intronless to meet the need for rapid transcription during the short cell cycles (Rothe et al., 1992). A subset of non-paused, pre-cellular genes are the most sensitive to P-TEFb knockdown, both in terms of gene expression changes and morphological phenotypes (Dahlberg et al., 2015). Similar gene expression changes and morphological phenotypes were observed upon knockdown of Mediator subunits, indicating that the Mediator co-activator complex and P-TEFb collaborate in transcriptional regulation *in vivo* (Dahlberg et al., 2015).

Transcription involves assembly of a pre-initiation complex consisting of basal transcription factors and Pol II at the core promoter (Fig. 1). Different sets of core promoter elements have been identified, depending on the regulatory mechanism of transcription, recruitment, or pause release. Promoters of genes regulated by recruitment are enriched in the TATA-box motif, whereas genes that are or will become paused at some time point are enriched for the GAGA-motif and Pause button (Chen et al., 2013). Thus, the nature of the core promoter influences Pol II pausing, which can affect the synchrony of gene activation in a group of cells. Indeed, substitution of the highly paused *snail* core promoter with less-paused promoters resulted in stochastic activation of *snail* expression and increased variability of mesoderm invagination (Lagha et al., 2013).

Composition of the core promoter can also influence the expression level of genes (Juven-Gershon et al., 2006). Some enhancers have a preference for either TATA promoters or promoters with a downstream core promoter element (DPE) (Ohtsuki et al., 1998; Butler and Kadonaga 2001). The transcription factor Caudal prefers DPE-containing promoters (Juven-Gershon et al., 2008), and Dorsal target genes are enriched for DPE motifs (Zehavi et al., 2014). These two core promoter types are regulated by different means: TATA-binding protein (TBP) is required for transcription of TATA promoters, but interferes with DPE promoter transcription, which instead relies on the TBP-related factor TRF2 (Hsu et al., 2008; Kedmi et al., 2014).

Whereas developmentally regulated genes are cell-type specific, ubiquitously expressed housekeeping genes are expressed in many different cell types. Recent studies showed that enhancer-promoter specificity contributes to this difference in expression profile (Zabidi et al., 2015). Core promoter motifs differ between genes with different function, and many enhancers exhibit specificity to one of these two types of promoters. Two transcription factors, Dref and GAGA-factor, were identified that separate housekeeping from developmental transcriptional programs (Zabidi et al., 2015). Thus, the composition of core promoter motifs further elaborate transcriptional control during

development. In summary, many developmental genes tend to be regulated at the step of pause release and these promoters are associated with specific core promoter elements (Fig. 4).

PATTERNING THE EMBRYO INTO DISTINCT CELL TYPES

Patterning of the *Drosophila* embryo occurs by positional information established by maternal factors. The initial transition from naïve cells to differentiated cells is mediated by gradients of two transcription factors: Dorsal establishes the dorsal–ventral axis of the embryo while Bicoid determines the anterior–posterior axis (St Johnston and Nusslein-Volhard, 1992; Fig. 2).

Bicoid mRNA is localized to the anterior pole of the oocyte and is translated upon egg deposition, resulting in a high concentration of Bicoid protein in the anterior pole with progressively lower levels towards the posterior pole (Berleth et al., 1988; Driever and Nusslein-Volhard, 1988b). High levels of Bicoid mediate the formation of the most anterior structures; intermediate levels the head structures; and low levels the thoracic and anterior abdominal structures. Changing the level of Bicoid protein results in a shift in positional identity, arguing that Bicoid acts as a morphogen that instructs cell fate in a manner dependent on its concentration (Driever and Nusslein-Volhard, 1988a). More recent results, however, argue against a strict Bicoid morphogen hypothesis: first, the shift in position of target genes when Bicoid levels are modified is less than would be expected from the threshold model (Houchmandzadeh et al., 2002). Second, Bicoid can activate genes at a lower concentration than present at the boundaries of target-gene expression in wild-type embryos, indicating that Bicoid is present in excess at all positions (Ochoa-Espinosa et al., 2009). Third, there is no evident correlation between the quality of Bicoid binding motifs and the position of target-gene expression along the anterior–posterior axis (Ochoa-Espinosa et al., 2005). Finally, analysis of Bicoid-dependent regulatory elements revealed that their expression boundaries are primarily positioned by repressive gradients that antagonize Bicoid-mediated activation (Chen et al., 2012). Comparison of active and inactive regulatory elements containing Bicoid-binding motifs showed that the TAGteam motif bound by Zelda was enriched at active enhancers. Furthermore, inserting Zelda sites into inactive Bicoid-bound enhancers converted some of them to Bicoid-responsive enhancers (Xu et al., 2014). These studies emphasize the importance of combinatorial binding of activators and repressors to enhancer regions for proper gene control.

Dorsal activates genes in a concentration-dependent manner, establishing three distinct thresholds of gene activity depending on the enhancer composition of the target genes (Chopra and Levine, 2009). Type I enhancers contain poor Dorsal sites and only respond to high levels of Dorsal in the mesoderm. Type II enhancers contains a fixed arrangement of Dorsal and Twist sites that mediate expression of target genes in the neuroectoderm. Finally, type III enhancers possess optimal Dorsal binding sites, such that the genes that are controlled by them respond to low levels of Dorsal. Dorsal can function as an activator as well as a repressor. The ability of Dorsal to act as a repressor involves its interaction with DNA-binding proteins that occupy AT-rich sequences flanking the Dorsal site, and results in the recruitment of the Groucho co-repressor (Ratnaparkhi et al., 2006).

Comparing the genome-wide occupancy of the CBP co-activator in the early embryo with 40 different transcription factors revealed its preferential binding to sequences bound by Dorsal (Holmqvist et al., 2012). In embryos lacking Dorsal protein, CBP showed a preference for binding to sequences occupied by Smad proteins that pattern the dorsal ectoderm in response to signaling by the transforming growth factor beta family member Dpp (Holmqvist et al., 2012). Consistent with this finding, dorsal–ventral patterning and Dpp signaling is impaired in CBP hypomorphic embryos (Waltzer and Bienz, 1999; Lilja et al., 2003). Thus, the CBP co-activator has a prominent role in dorsal–ventral patterning and less pronounced effects on anterior–posterior patterning in the embryo.

Investigating the transition of naïve embryonic cells into three different cell types, we found that cell specification is accompanied by the establishment of different chromatin states on individual genes among the cell types (Boija and Mannervik, submitted). Dorsal recruits CBP to mediate histone acetylation in the neuroectoderm, whereas repression by the Snail protein results in histone hypoacetylation in the mesoderm. In the dorsal ectoderm, Polycomb-mediated repression and H3K27me3 is acquired (Boija and Mannervik, submitted). Therefore, developmental determinants orchestrate differential chromatin states, providing new insights into the link between epigenetics and developmental patterning.

PERSPECTIVES

During the course of differentiation, there is a dramatic change in the chromatin landscape. For a new organism to be formed, chromatin marks of the highly specialized germ cells need to be erased for the two pronuclei to fuse and form a totipotent cell. Pioneer factors initiate the exit from the naïve cell state, which is accompanied first by histone acetylation and then by methylation. Patterning the embryo into different cell types involves transcription factors that bind to enhancers and recruit co-regulators, thereby establishing distinct chromatin states.

Although genome-wide data have positively impacted our understanding of transcriptional regulation, many mechanistic aspects still remain to be tested at the gene level *in vivo*. When is transcription-factor binding functional? How are combinations of factors used to regulate genes in different tissues? How do transcription factors and co-regulators cooperate to achieve gene regulation? How is Pol II pausing controlled? Is histone modification a cause or consequence of gene regulation? With the development of new methodologies—including live imaging and quantitative measurements of transcription *in vivo* (Gregor et al., 2014), a histone gene-

replacement system (Gunesdogan et al., 2010), and CRISPR/Cas9 technology to manipulate endogenous gene expression (Harrison et al., 2014)—the *Drosophila* embryo promises to remain a rich source for understanding the transcriptional mechanisms that underlie cell specification and differentiation.

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