

Review

Chromatin Organization during *C. elegans* Early Development

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Abstract: Embryogenesis is characterized by dynamic chromatin remodeling and broad changes in chromosome architecture. These changes in chromatin organization are accompanied by transcriptional changes, which are crucial for the proper development of the embryo. Several independent mechanisms regulate this process of chromatin reorganization, including the segregation of chromatin into heterochromatin and euchromatin, deposition of active and repressive histone modifications, and the formation of 3D chromatin domains such as TADs and LADs. These changes in chromatin structure are directly linked to developmental milestones such as the loss of developmental plasticity and acquisition of terminally differentiated cell identities. In this review, we summarize these processes that underlie this chromatin reorganization and their impact on embryogenesis in the nematode *C. elegans*.

Keywords: chromatin; embryo development; histone modifications; dosage compensation; LADs; TADs

1. Introduction

Chromatin organization is a highly dynamic and precisely regulated process in the developing *C. elegans* embryo. After pronuclear fusion, the embryo exists in a totipotent state. As embryonic cells divide and differentiate, they establish cell-specific gene expression programs. This is accompanied by major remodeling of the chromatin structure. Several mechanisms are involved in this process. One of these is the formation of heterochromatin and euchromatin [1]. As embryogenesis progresses, the chromatin segregates into two distinct states: the actively transcribed euchromatin and the transcriptionally silent heterochromatin [1]. This occurs at specific points in the timeline of embryo development, and disruption of these events in embryogenesis results in functional consequences for the development of the embryo and the health of the adult worms. Formation of these chromatin states is also accompanied by the deposition of active and repressive histone marks that reinforce the appropriate transcriptional state for genomic loci [2,3]. Active histone modifications are generally associated with chromatin that have a higher rate of transcription, and conversely repressive histone modifications are generally associated with a silent chromatin state.

Spatial organization of chromatin within the nucleus is influenced by the establishment of topologically associating domains (TADs) and lamina-associating domains (LADs) on the chromatin. TADs are sub-megabase-scale chromatin structures that separate the genome into self-interacting domains [4,5]. As defined by interaction frequencies observed through Hi-C and multiplexed fluorescence *in situ* hybridization (FISH), DNA elements within a self-interacting domain share more interactions inside their domain compared to interactions with DNA elements outside their domains. LADs are heterochromatic genomic regions that are physically anchored to the nuclear lamina and they spatially sequester silent regions of the chromatin to the nuclear periphery [6]. Both these genomic structures regulate the organization of chromatin and are responsible,

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to some extent, for creating permissive and repressive environments for the transcription of appropriate genes in the appropriate context within embryogenesis.

For hermaphrodite embryos, there is the additional complication of dosage compensation. Dosage compensation in *C. elegans* is necessary to equalize the transcriptional output of the two hermaphrodite X chromosomes to that of the single male X chromosome [7]. It is a process that significantly alters the chromosome architecture on the X chromosome, its histone modification landscape and transcriptional output during embryo development. This review looks at the most recent evidence that explores the timeline of formation of key structural features on chromatin and the mechanisms that regulate the various aspects of chromatin architecture and organization throughout *C. elegans* early development.

2. Histone H3K9 Methylation-Mediated Heterochromatin Formation

There are broadly two types of chromatin inside an interphase nucleus, euchromatin and heterochromatin. Euchromatin refers to transcriptionally active “open” chromatin, and heterochromatin refers to transcriptionally inactive “closed” chromatin. As zygotic transcription in *C. elegans* is activated in 4-cell embryos [8], chromatin starts segregating into these two distinct forms from its previously uncondensed state. Heterochromatin is formed *de novo* as cells start differentiating and acquiring their specific cell identities [1], in a process that is precisely timed. Under transmission electron microscopy (TEM), heterochromatin appears as electron-dense regions (EDRs) inside the nucleus [9]. EDRs first appear in embryos during initiation of gastrulation at the 28-cell stage as numerous electron-dense puncta appear dispersed throughout the nucleus. As gastrulation progresses, these dense puncta begin coalescing into fewer but larger electron dense compartments throughout the nucleus. In late-stage embryos (>200 cells), large EDRs are clearly visible and are localized to the nuclear periphery [9].

In *C. elegans*, the most well-studied heterochromatin-associated repressive histone marks are H3K9me3 and H3K27me3. H3K9me3 is associated with, and required for the formation of repressive heterochromatin in many organisms [10–12]. H3K9me2/me3 histone marks are both dynamically regulated during early embryogenesis in *C. elegans* and their deposition on the chromosomes coincides with the timeline of EDR formation [9]. Histone H3K9me modifications promote the formation of heterochromatin through multiple mechanisms: they are responsible for the formation of active and inactive genomic compartments [13,14], compaction of individual chromosome regions [14] and recruitment of pathways that lead to the perinuclear anchoring of H3K9me2/me3-enriched genomic regions [13–18].

MET-2 is an H3K9 histone methyltransferase that is responsible for the deposition of H3K9me1/me2 marks [16]. In *met-2* mutant embryos, EDRs corresponding to heterochromatin start forming much later in embryogenesis [9]. EDR puncta only start appearing at the late gastrulation stage, and do not appear as dense as WT EDRs at this stage of development. In late-stage *met-2* mutant embryos, the EDRs coalesce to form larger territories, but these territories occupy a significantly smaller proportion of the nuclear volume than WT EDRs [9]. MET-2 is localized in the cytosol in early embryos and is transported inside the nucleus at the onset of gastrulation in 20–50 cell embryos by its co-factor LIN-65 [9,18]. Inducing premature nuclear accumulation of MET-2 using a nuclear localization signal (NLS) leads to a premature increase in H3K9me2 on the chromatin [9]. This suggests that heterochromatin could also form earlier in embryogenesis by inducing precocious nuclear accumulation of MET-2, but heterochromatin formation through visualizing EDRs has not been directly tested under these conditions. Similar to its *C. elegans* homolog, the mammalian homolog of MET-2, SETDB1, is known to promote heterochromatin formation through H3K9me3 [11].

MET-2 and its binding partners, LIN-65 and ARLE-14, form nuclear hubs during gastrulation of the embryo [9,18]. These hubs co-localize with H3K9me2 but exclude active marks such as H3K4me3 [9]. Loss of LIN-65 delays the deposition of repressive

H3K9me1/me2/me3 marks on the chromatin during development [9]. Loss of LIN-65 also destabilizes MET-2 and prevents the formation of MET-2 nuclear foci [9,18]. Loss of ARLE-14 delays only the deposition of H3K9me1/me2 without affecting MET-2 stability or nuclear hub formation. Interestingly, ARLE-14 does stabilize catalytically deficient MET-2 and strengthens its association with chromatin [19]. In *lin-65* mutants, repetitive DNA elements are significantly de-repressed [9,18]. In *arle-14* mutants, there is evidence for modest de-repression of repetitive elements in one study [9] but repetitive elements tested in another study did not show de-repression [18]. In sum, these indicate that heterochromatin formation may not be robust in *lin-65* mutants, and may also be affected in *arle-14* mutants. Furthermore, the LIN-65-mediated nuclear accumulation of MET-2 was found to be the rate-limiting step in the deposition of H3K9me2 on chromatin. Heterozygous *lin-65*+/− embryos with only one copy of *lin-65* had only half the levels of H3K9me2 in stage-matched gastrula embryos [9]. The nuclear accumulation of MET-2 at the gastrulation stage was also found to be controlled by the length of the S phase [20]. Increasing and decreasing the length of the S phase in early embryos by growing worms at different temperatures affected the rate of timing of MET-2 nuclear accumulation. Lower temperatures led to precocious nuclear accumulation of all three binding partners and growing worms at higher temperatures led to delayed accumulation [20].

SET-25 is another H3K9 histone methyltransferase that is responsible for the deposition of H3K9me3. In *set-25* mutants, there is a complete loss of heterochromatin, as visualized by EDRs [20]. The nucleus throughout gastrulation appears as uniform and devoid of EDRs as it does post-fertilization. Even in >200 cell embryos, there is no detectable formation of heterochromatin [20]. Despite this, *set-25* mutant embryos are viable and the embryos are able to terminate developmental plasticity, suggesting that heterochromatin formation is not required for initiating cellular differentiation [20]. *met-2* mutants lack virtually all embryonic H3K9me1/me2 but still have deposition of H3K9me3, albeit at significantly reduced levels [9]. This is because SET-25 can be recruited to genomic loci by two independent mechanisms, a MET-2-dependent pathway which targets repetitive elements and satellite repeats and a NRDE-3-dependent pathway which targets transposons and insertions [21,22]. In the absence of *met-2*, H3K9me3 can still be formed *de novo* through the NRDE-3-dependent pathway [21] and, thus, the reduced heterochromatin that is present in *met-2* mutants can be attributed to this pathway.

3. Other Mechanisms of Heterochromatin Formation

Unlike in *Drosophila* or humans, where H3K9me3 and H3K27me3 are enriched on different chromosomal regions, H3K9me3, H3K27me3 and H3K23me3 tend to co-occur on stable heterochromatin in *C. elegans* [23–26]. The role of H3K27me3 is relatively well-characterized in embryos. In contrast to H3K9me2/me3, which are dynamically regulated throughout embryonic development, H3K27me3 is inherited in the embryos from modifications already present on both the oocyte and the sperm [27,28]. In germ cells, the H3K27me3 mark is deposited by the polycomb repressive complex 2 (PRC2), composed of MES-2, MES-3 and MES-6 [29]. PRC2 enables the *de novo* formation of H3K27me3 during larval development of germ cells [27].

In the embryonic stages, H3K27me3 inherited from parental germ cells is maintained on chromosomes through the first few rounds of cell divisions. During embryogenesis, this mark is enriched over genomic loci that were silent in their parental germlines [28]. Existing H3K27me3 is then propagated during successive embryonic cell divisions by the PRC2 complex [28,30]. The MES-2-mediated propagation of H3K27me3 on chromatin in embryos undergoing gastrulation is important for chromatin compaction at this stage in development. Extrachromosomal arrays and endogenous chromatin loci were both found to be physically de-compacted in 100-cell embryos with a *mes-2* mutation compared to WT, as measured by immunofluorescence experiments [30]. This phenotype was accompanied by a decrease in H3K27me3 deposition [30].

Some demethylases and interactors of the PRC2 complex also regulate the deposition of H3K27me3 on the chromatin. UTX-1 is an H3K27me3 demethylase that is broadly expressed in embryos and is responsible for regulating the levels of H3K27me2/me3 [31]. Three KDM6 demethylase family members, JMJD-3.1, JMJD-3.2 and JMJD-3.3, present in *C. elegans* embryos function redundantly with each other to demethylate H3K27me3 as well [31]. The PRC2 complex can be targeted to genomic loci during embryogenesis by LET-418 [32], the *C. elegans* Mi2 homolog that is a component of the nucleosome remodeling and deacetylase (NuRD) complex critically responsible for embryonic development in mammals [33–35]. In the absence of LET-418, H3K27me3 is specifically reduced at LET-418 target genes [32]. JMJD-1.2 is another demethylase that regulates three heterochromatin marks, H3K9me2, H3K23me2 and H3K27me2, present in embryos and was found to protect DNA in early embryos from replication stress [36], though the mechanism for this has not been elucidated. Some other less studied repressive histone marks present on heterochromatin during embryogenesis include H3K23me2 [24] and H3K56me3 [37]. In addition to H3K23 methylation, other H3K23 modifications are also widely present in embryos, as measured by mass spectrometry [24], though their roles in development, if any, are less known.

Some histone variants are also known to play important roles in facilitating the process of heterochromatin formation by enabling the deposition of repressive histone modifications. In metazoans, both H3 and H3.3 are expressed at various stages of development and the presence of these different histone variants potentiates the deposition of specific histone modifications. In the case of H3.3, active histone marks such as H3K4me3 and H3K36me2 are catalyzed preferentially [38–42]. In contrast, the canonical H3 favors the deposition of repressive histone marks associated with heterochromatin such as H3K9me2/me3 and H3K27me2/me3 [39]. In early embryogenesis from 2-cell to 50-cell stages, cells inside the embryos are enriched in H3.3 and have very low levels of H3 and therefore H3K9me. This is a holdover from germ cells where H3.3 is maintained at a high level in both oocytes and sperm [42]. Class I and Class II H3 genes are specific gene clusters that produce H3 and have distinct patterns of expression and regulation during embryogenesis [42]. Class I and Class II H3 begin accumulating on the chromatin at the 2-cell stage and at the onset of gastrulation, respectively, and H3 slowly starts replacing H3.3 and allows the gradual accumulation of H3K9me2/3 [42]. At the late embryo stages, H3 is highly enriched on the entire embryo, except for the P lineage germ cells that retain H3.3 enrichment [42]. This process is conserved in mammals, where H3.3 inherited from germ cells is replaced with other histone H3 variants [43].

Another important player in regulating heterochromatin is linker histone H1, transcribed from *his-24* [44], which promotes the deposition of repressive histone marks on the chromatin during embryogenesis [45]. HIS-24 is cytoplasmic in both the maternal and paternal germ cells, and immediately after fertilization is rapidly translocated to the nucleus where it associates with the chromatin at the pronucleus stage [45]. HIS-24 continues to be associated with the chromatin through embryogenesis in all cells, with the exception of the Z2 and Z3 primordial germ cells (PGCs) [45]. This mechanism of germ cell cytoplasmic retention and rapid nuclear translocation is lost in mutants for *mes-2*, *mes-3*, *mes-4*, *mes-6* and *sir-2.1*, suggesting the involvement of these proteins in this pathway [45]. *his-24* mutant worms were unable to silence extrachromosomal transgenes due to a loss of H3K9me2 repressive mark and aberrant gain of H3K4me3 active mark [44,45]. A *his-24* mutation also exacerbated the defect in H3K27me3 deposition on the chromosomes of germ cells in *mes-3* mutants [45]. Taken together, these results suggest that linker histone H1 may promote the accumulation of repressive histone marks in early embryogenesis, though the mechanism and the potential involvement of the PRC2 complex components remain unclear. In addition to *his-24*, there are seven other linker histone variants in *C. elegans*, *hil-1* through *hil-7*. These additional variants do not have any known roles in embryogenesis [46].

4. Developmental Regulation of Active Chromatin

After fertilization and pronuclear fusion, the chromosomes exist in a decondensed state until the onset of the gastrula stage where they begin partitioning into active and inactive compartments. Prior to the onset of gastrulation, many active epigenetic marks inherited from parental germ cells are present on the chromosomes. These include methylation marks such as H3K4me2 [47] and H3K79me2 [48], and acetylation marks such as H2BK12ac, H3K23ac, H3K27ac, H4K16ac, H4K5ac, H4K8ac, and H4K12ac [48]. After the first few rounds of DNA replication, the previous parent-of-origin specific distribution of histone marks on the chromatin is completely altered. Acetylation marks are largely equalized over all the chromosomes [48]. The well-studied H3K4me2/me3 marks, in contrast, show more dynamic regulation in early embryos.

H3K4me3 is enriched at transcription start sites (TSS) of active genes, whereas H3K4me2 tends to be uniformly distributed over the gene body [49]. At the resolution of microscopy, H3K4me2/me3 are present uniformly and at relatively high levels in early 2–4-cell embryos, except for the X chromosome where it is depleted in embryos [50–52]. At the 8-cell stage, H3K4me3 begins being enriched on some blastomeres in a lineage-specific manner. H3K4me3 is completely lost in the germline blastomere, and some of the somatic blastomeres [50]. In contrast, it is highly enriched on the AB descendants at eight cells and remains enriched in 80-cell embryos in all the cells of the AB lineage [50]. The AB blastomere is formed from the first cell division in the *C. elegans* embryo. The AB lineage gives rise to the nervous system, the hypodermis and about half of the pharyngeal tissues [53].

The *C. elegans* mixed-lineage leukemia (MLL) complex, which has methyltransferase activity targeting H3K4 and demethylase activity targeting H3K27, is responsible for this lineage-specific regulation [50,54,55]. Different components of the MLL complex are differentially required for H3K4 methylation at different developmental stages. The two important components of the complex required in embryos are SET-2 and ASH-2. SET-2 is the *C. elegans* homologue of mammalian SET-1, which is a core member of the mammalian MLL complex. ASH-2, which was initially suggested to be a core member of the *C. elegans* MLL complex [54], was later found to be dispensable for MLL activity at specific stages of development [56], indicating it is an ancillary binding partner of the MLL complex rather than a core component. During embryonic development, both ASH-2 and SET-2 are required for H3K4me3 deposition, but ASH-2 also functions in a SET-2-independent pathway for H3K4me2 deposition [56]. SET-2 is specifically required for accumulation of H3K4me2 in PGCs at later stages of embryogenesis [56]. Another member of the MLL complex, SET-16, also has H3K4 methyltransferase activity *in vitro* [54,56]. However, depletion of SET-16 causes embryonic lethality [57], making it difficult to elucidate its potential role in regulating H3K4me during development. UTX-1, an H3K27me2/me3 demethylase, forms a complex with SET-16 and its expression is required for SET-16 expression and vice versa [31], suggesting cooperation between pathways regulating active and repressive histone marks during embryogenesis.

HTZ-1 is a histone H2A variant that is required for proper embryo development [58]. HTZ-1 is maternally loaded into embryos and incorporated into chromatin, starting at the 4-cell stage. Maternally loaded HTZ-1 mRNA is actively converted into protein, and the levels of HTZ-1 increase with the progress of embryogenesis [58]. It is enriched on the chromatin upstream of a subset of developmentally important actively transcribed genes at their TSS, where it regulates transcription by RNA PolII [58]. Depletion of HTZ-1 in embryos results in developmental defects, such as embryonic lethality and early larval arrest, likely due to mis-expression of these developmental regulators [58]. The various relevant histone variants and their roles in embryogenesis have been summarized in Table 1.

Table 1. Histone variants, associated PTMs and functions in embryogenesis.

Histone Variant	Histone Marks	Active/Repressive	Localization and Function	Ref
H3	H3K9me2/me3, H3K27me2/me3	Repressive	Canonical histone H3 that favors the deposition of repressive histone marks. Depleted from chromatin in early embryos. Transcribed in embryos from 2-cell stage and accumulates on chromatin throughout early embryogenesis on all cells except P-lineage cells. Promotes termination of developmental plasticity.	[39,42]
H3.3	H3K4me3, H3K36me2	Active	Histone H3 variant that favors the deposition of active histone marks. Inherited from germ cells in early embryos in 2-cell to 50-cell stage. Depleted from chromatin during embryo development, except in P-lineage cells.	[38,39,41,42]
H1.1	H3K9me2	Repressive	Linker histone H1 that promotes the accumulation of repressive histone marks. Rapidly translocated from the cytoplasm into the nucleus after fertilization. Associated with chromatin in all embryonic cells except Z2 and Z3 PGCs. Promotes the silencing of heterochromatic loci.	[44,45]
HTZ-1		Active	Histone H2A variant enriched upstream of transcribed genes required for development where it influences PolII engagement. Incorporated into chromatin starting at 4-cell stage and required for appropriate embryonic development.	[58]

5. Antagonism between Repressive and Active Histone Marks

Active and repressive histone marks tend to, with some exceptions, be enriched on mutually exclusive genomic regions in *C. elegans*. The histone methyltransferases that are responsible for regulating the deposition of these marks frequently interact and antagonize each other to establish the epigenetic landscape of the *C. elegans* early embryo. The MLL complex, with well-characterized interactions with both active and repressive histone post-translational modifications (PTMs), is one of the prime examples of this balancing act. The MLL complex-mediated H3K4me2/me3 deposition sustains transcription at its target loci in embryos [2]. In addition, the MLL complex member SET-16 also associates with H3K27me2/me3 demethylase UTX-1 [31]. Despite this interaction, the two marks can be toggled independently in embryos [54]. *utx-1* mutants exhibit significant embryonic lethality, which may suggest that the demethylase activity is important for embryogenesis. However, mutant embryos with catalytically deficient UTX-1 which cannot remove H3K27me2/me3, are viable and develop into healthy adults. In fact, rather than its demethylase activity, it is the interaction between UTX-1 and SET-16 that seems to be crucial for embryo development [31].

A more direct example of antagonism is the exclusion of active marks on heterochromatin through the formation of MET-2 nuclear foci on chromatin. In addition to the established role of MET-2 in H3K9me-mediated heterochromatin formation, catalytically deficient *met-2* mutants (*met-2 CD*) that are unable to catalyze the methylation of H3K9 are still able to maintain some of their function in regulating heterochromatin, suggesting that MET-2 has an H3K9me-independent role during embryogenesis [19]. Catalytically inactive *met-2* only has a partial effect on the de-repression of heterochromatin genes, where 28% of genes de-repressed in *met-2* null mutants remained repressed in *met-2 CD* mutants [19]. Evidence from ChIP-seq suggests that the formation of MET-2 nuclear foci themselves, without catalyzing H3K9me1/me2 formation, are important for excluding the active histone marks H3K9ac and H3K27ac at *met-2* target genomic loci [19]. *met-2 CD* mutants do not gain active marks on *met-2* target genomic loci, which prevents most of these loci from being de-repressed even in the absence of the repressive H3K9me histone marks [19]. MET-2 co-factors LIN-65 and ARLE-14 are important for forming and stabilizing these MET-2 foci and strengthening their association with the chromatin [19]. While the mechanism behind foci formation is not known, MET-2 and LIN-65 both contain disordered domains [9,18,19], which suggestss that liquid–liquid phase separation may be a contributor [59,60].

Antagonism between MES-4-mediated H3K36me3 and PRC2-mediated H3K27me3 is important for maintaining appropriate patterns of gene expression in germ cells [28].

Early embryos inherit both the active H3K36me3 and repressive H3K27me3 from germ cells, where these marks occupy mutually exclusive genomic loci [28]. In these early embryos, germline-expressed genes carried H3K36me3 whereas soma-specific genes carried H3K27me3 [28]. The loss of MES-4 and its associated H3K36me3 mark led to the acquisition of repressive H3K27me3 on germline-specific genes in embryos. Other genomic loci carrying MES-4-independent H3K36me3 did not acquire any repressive marks. This evidence shows that MES-4 activity on germline-specific genes repels the activity of the repressive MES-2/3/6 complex [28]. Histone modifications covered in this review as well as their associated functions have been summarized in Table 2.

Table 2. Histone modifications and their functions during embryogenesis.

Histone Modification	Histone Methyl/Acetyl Transferase	Histone Demethylase/Deacetylase	Active/Repressive	Function	Ref
H3K9me2	<i>met-2</i>	<i>jmjd-1.2</i>	Repressive	Dynamically increases during gastrulation. Repressive mark enriched on heterochromatin. High levels of H3K9me2 promotes developmental plasticity.	[9,14,19,20,25,36,61]
H3K9me3	<i>set-25</i>		Repressive	Dynamically increases during gastrulation. Repressive mark enriched on heterochromatin. Linked to heterochromatin formation, tethering of the chromatin to nuclear periphery, TAD formation and establishment of dosage compensation on the hermaphrodite X chromosome.	[14,16,18,20,21,25,62–64]
H3K27me3	<i>mes-2</i>	<i>utx-1, jmjd-3.1, jmjd-3.2, jmjd-3.3</i>	Repressive	Inherited from maternal and paternal germ cells and dynamically enriched in embryonic cells in a lineage-specific manner. Repressive mark enriched on heterochromatin and LADs. Promotes termination of developmental plasticity.	[25,27–30,62,65]
H3K23me2	<i>set-32</i>	<i>jmjd-1.2</i>	Repressive	Repressive mark enriched on heterochromatin.	[24,25,36]
H4K20me1/me2	<i>set-4</i> deposits H4K20me2	<i>dpy-21</i> converts H4K20me2 to H4K20me1 on the X chromosome	Repressive	H4K20me1 is selectively enriched on the X chromosome by <i>dpy-21</i> . Repressive mark required for the establishment of dosage compensation in hermaphrodite embryos.	[66–70]
H3K4me2	<i>ash-2, set-16</i>		Active	Active mark inherited from paternal and maternal germ cells, generally enriched over gene bodies where it permits transcription at genomic loci. Enriched uniformly in all cells in early embryos, enriched specifically on PGCs in late embryos.	[2,47,50–52,54,56,57]
H3K4me3	<i>ash-2, set-2, set-16</i>		Active	Active mark inherited from paternal and maternal germ cells, generally enriched at TSS where it permits transcription at genomic loci. Enriched uniformly in all cells in early embryos. Enriched in a lineage-specific manner starting at the eight-cell stage.	[2,49–52,54,57]
H3K36me3	<i>mes-4, met-1</i>		Active	Active mark enriched on euchromatin. Required for expression of germline-specific genes in early embryos.	[17,28]
H3K9ac			Active	Active mark excluded from heterochromatin by MET-2 nuclear foci.	[19]
H3K27ac			Active	Active mark excluded from heterochromatin by MET-2 nuclear foci.	[19]
H4K16ac			Active	Active mark inherited from maternal germ cells and selectively depleted from the X chromosome.	[69,70]
H3K56me3			Repressive	Repressive mark enriched on heterochromatin.	[37]
H3K79me2			Active	Active mark inherited from paternal and maternal germ cells. Depleted on chromatin in 1–4 cell embryos, then enriched after 16-cell stage.	[48]

6. Spatial Organization of Chromatin inside the Nucleus

As the embryos differentiate and heterochromatin is formed, the chromatin is dramatically reorganized spatially inside the embryonic nuclei. This reorganization segregates active chromatin from silent chromatin, and this process is primarily driven by pathways that anchor heterochromatic regions to the nuclear periphery where they are relatively inaccessible to transcriptional machinery. The specific regions of chromatin that are sequestered to the nuclear lamina are also called lamina-associated domains (LADs) [6]. In *C. elegans*, LADs occur on all the distal regions of autosomes, called chromosome “arms”, and only the left distal arm on the X chromosome [65]. LADs are enriched in repetitive sequences and transcriptionally inactive genes, and they tend to have deposition of H3K27me3 and H3K9me2/me3 repressive marks [16,25,62,65].

LADs are tethered to the inner nuclear lamina and were first defined in *C. elegans* by their association with LEM-2, an inner membrane transmembrane protein [65]. LEM-2 ChIP-seq experiments show that the regions of heterochromatin that directly associate with the lamina, called LEM-2 subdomains, are punctuated by gaps. These gaps in LEM-2 subdomains are more frequent and bigger in size as they move further away from the chromosome arms and are enriched in transcriptionally active genes. Inactive genes in embryonic LADs, and specifically ones that occur on LEM-2 subdomains that interact directly with the nuclear lamina, tend to remain transcriptionally silent through development [65].

Histone H3K9 methylation is required for the peripheral anchoring of heterochromatin. MET-2 and SET-25 function redundantly to position heterochromatic arrays at the periphery in early embryos in an H3K9me2/me3-dependent mechanism [16,18]. Heterochromatin anchoring is completely lost in *met-2; set-25* double mutants [16,18]. However, there are differing reports of the effect of H3K9 HMT single mutants in regulating anchoring of heterochromatic arrays compared to endogenous heterochromatin. In the study by Towbin et. al. (2012), *met-2* mutants did not show any defect in heterochromatic array anchoring but *set-25* mutants showed a partial defect [16]. LEM-2 ChIP experiments in the study by Delaney et. al. (2019) found complete ablation of anchoring of endogenous heterochromatin in *met-2* mutants, but no effect in *set-25* [18]. MET-2 and SET-25 can independently methylate certain targets [21], which can be used to explain some of these differences. SET-25 was shown to be able to methylate heterochromatic arrays independently of MET-2 [21], which can explain the absence of de-tethering phenotype in *met-2* mutants in Towbin et. al. (2012). However, on endogenous targets *set-25* requires *met-2* for methylation, which is corroborated by experiments from Delaney et. al. (2019). Delaney et. al. (2019) also found LIN-65, a co-factor of MET-2, to be required to maintain perinuclear anchoring in early embryos [18]. CEC-4 is a chromodomain protein that binds to H3K9me deposited by MET-2 and SET-25 and anchors the H3K9 methylated chromatin to the nuclear lamina [63,64]. CEC-4 is required for peripheral anchoring of heterochromatin in early embryos, but not at later stages of development [64]. Mechanisms of tethering differ between species [71], but the requirement of H3K9me2 seems to be a conserved feature seen in several model organisms [72,73].

MRG-1 regulates perinuclear anchoring in larval cells and is also active during embryogenesis [17]. In *mrg-1* mutant embryos, heterochromatic arrays are de-compacted but maintain their association with the nuclear lamina due to the presence of CEC-4-mediated anchoring [17]. Perinuclear anchoring is lost in both embryonic and larval stages in *cec-4; mrg-1* double mutants [17]. The formation of LADs in embryos also requires balancing active and repressive chromatin marks. In this process, MRG-1 plays a central role where it prevents CBP-1-mediated active H3K27ac mark from spreading to silent regions of the genome [17]. MRG-1 associates with euchromatic regions of the genome, where it is thought to bind to the active H3K36me2/me3 marks. In *C. elegans* and other metazoans, it also forms a complex with HAT and HDAC complexes [74–76]. CBP-1 is a co-factor for several transcription factors and promotes several pathways of somatic cell differentiation during embryogenesis [77]. In Cabianca et. al. (2019), the authors

quantified the localization and de-compaction of heterochromatic foci formed by integrated arrays to show that the loss of MRG-1-mediated sequestering of CBP-1 to euchromatic loci led to de-tethering from the nuclear lamina and de-compaction of condensed chromatin. ChIP-qPCR data also showed the gain of H3K27ac active mark on heterochromatin. MET-1 and MES-4-mediated deposition of H3K36me2/me3 marks on euchromatin is also required for proper tethering of heterochromatin [17]. Cabianca et. al. (2019) suggests that this may be through the MRG-1 pathway as MRG-1 is thought to be able to bind these histone marks, though the exact mechanism remains unclear [17]. Exogenous heterochromatic arrays tested for peripheral anchoring in early embryos showed de-repression in H3K9me mutants, but also in mutants for several other histone modification pathways that do not affect peripheral localization of these arrays [16]. Additionally, *cec-4* mutants did not show de-repression of heterochromatic genes [64]. Together, this suggests that independent pathways of gene repression through histone modifications and CEC-4-mediated peripheral anchoring are active in early embryogenesis.

Telomeres present at the ends of chromosomes are also peripherally localized inside the nucleus. The peripheral enrichment of telomeric regions is evident from 20–50 cell embryos, and increases with the progression of embryogenesis [78]. The pathways regulating telomeric positioning in embryos are independent and distinct from those regulating sub-telomeric heterochromatin. Telomere localization in embryos is dependent on SUN-1, GEI-17 and the shelterin protein POT-1. In the larval stages of development, there is evidence of additional redundant pathways as telomeric regions localize to the periphery in L1 larvae, even in the absence of embryonic tethering pathways [78]. POT-1 associates directly with telomeric DNA and directs their association to the periphery in a SUN-1-dependent manner. GEI-17, a SUMO E3 ligase, was also found to be required for this function [78]. However, while the sumoylation of proteins in this pathway is well characterized in yeast [79], its role in *C. elegans* remains to be elucidated. Both pathways regulating the spatial organization of DNA during embryo development have been summarized in Figure 1.

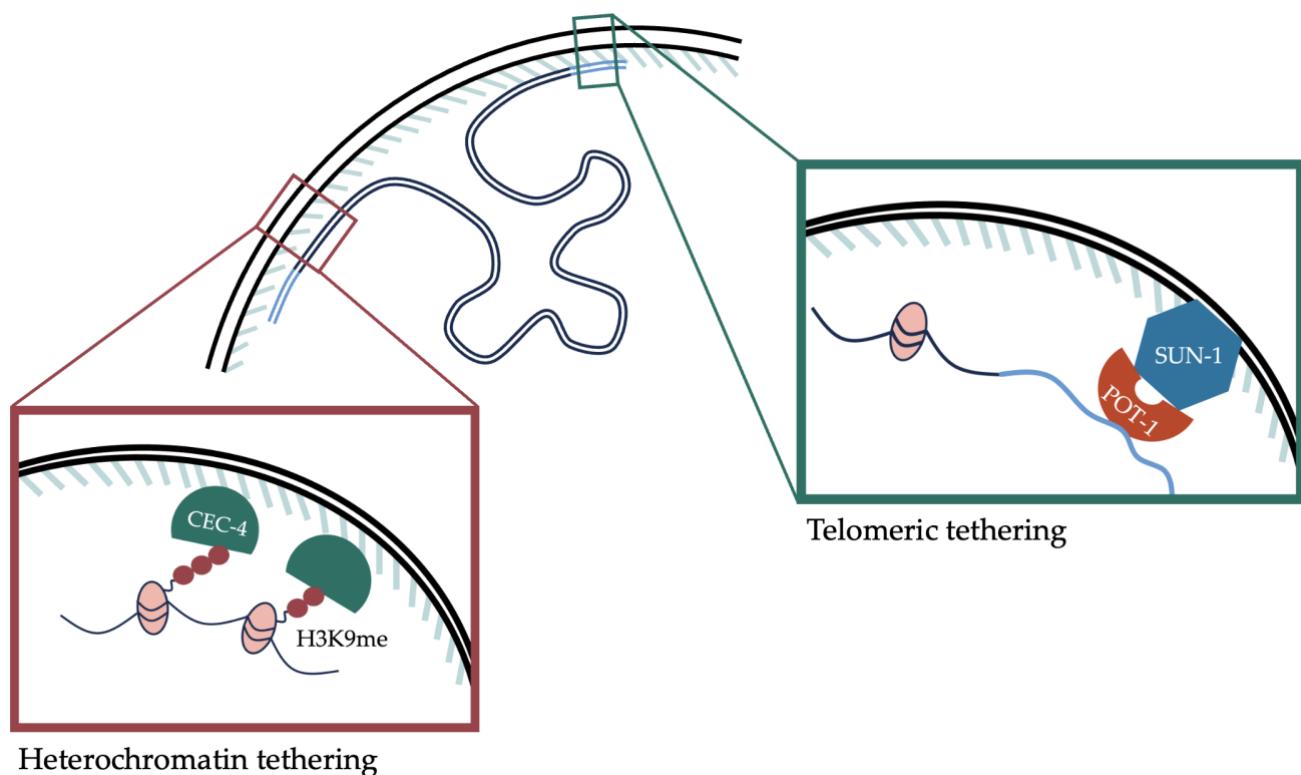


Figure 1. Schematic representation of tethering pathways for heterochromatin and telomeric chromatin.

7. Emergence of Topologically Associating Domains (TADs)

Topologically associating domains (TADs) are sub-megabase-scale 3D chromatin structures that are a conserved feature of chromatin organization [4,5]. TADs are composed of chromatin domains that have higher frequencies of interaction within the DNA sequences inside the domain and have relatively low interaction frequencies with DNA sequences in other domains. These interactions that define TADs are highly stochastic and occur at low absolute frequencies [76,80–82], but these small interactions are higher than expected by probability and specifically regulated by transcriptional states and epigenetic landscapes. Most TADs are demarcated by boundaries that isolate neighboring domains [83–85]. In other organisms, CTCF proteins mark these TAD boundaries and act as transcriptional insulators. However, *C. elegans* does not have a CTCF ortholog, and no other protein that may perform a similar function has been identified [86]. Eigenvector deconvolution of TAD domains is commonly used to categorize TADs into two compartments: the transcriptionally active “A” compartment and the silent “B” compartment [83].

Recent advancements in chromosome conformation capture technologies such as Hi-C have enabled us to study the formation of these local 3D structures and global TAD distribution through development. Using Hi-C in mixed-stage *C. elegans* embryos, strong TADs were found to be present on the X chromosome [87]. TADs on the autosomes are also present at this stage, although the boundaries are significantly weaker. TADs are more regularly spaced on the X chromosome compared to the autosomes and resemble mammalian TADs. TAD formation on the X is dependent on the dosage compensation complex (DCC). In DCC mutants, previously isolated TAD domains have increased contact frequencies with each other, and clearly demarcated TAD boundaries are lost [68,87]. The role of TAD formation in hermaphrodite dosage compensation is explored in more detail later in this review.

Methylation of histone H3K9 by MET-2 and SET-25 also affects TAD formation in embryos [14]. TADs are significantly weaker and less insulated in *met-2*; *set-25* mutants that have lost H3K9me2/me3 repressive marks. *cec-4* mutants also exhibited weaker TADs, though the effect was much less substantial than in *met-2*; *set-25* [14], suggesting that both H3K9me deposition and perinuclear anchoring are independently contributing to TAD formation during embryogenesis. This weakening of TADs and compartments leads to some genes being mis-segregated into the wrong compartment, i.e., genes previously in an active A compartment being mis-segregated into an inactive B compartment; however, this does not seem to lead to any significant changes in gene expression [14].

Hi-C analysis in late-stage embryos showed that the autosomes assume a conformation where the two arms of an autosome form into inactive B compartments, and the middle transcriptionally active regions forms the active A compartment [87]. The two B compartments have high interaction frequency within their domains but also with each other. Another study used chromosome tracing with a multiplexed FISH approach to study the onset of TAD and compartment formation on *C. elegans* autosomes during early embryonic development [15]. This approach relies on FISH probes spanning 100kb genomic windows within known TADs to capture the 3D conformation of individual chromosomes in single cells. The results showed that the conventional conformation of the autosomes is seen to first arise at the gastrulation stage during embryogenesis [15]. These conventional compartments are not present in autosomes in early embryos (2–25 cells), where the two B compartments are isolated from each other and separated spatially by the middle A compartment in an unconventional “barbell” conformation. Though the B compartments are separated in early embryos, local folding and increased contact frequency within the individual B compartments was evident even in these early embryos [15]. This indicates that formation of compartments is a very early step in embryogenesis.

This study also showed that the CEC-4-mediated anchoring of heterochromatin induces stretching of the chromosomes in early embryos. CEC-4 is involved in the perinuclear anchoring of heterochromatin domains through H3K9me [64] and this function of CEC-4 was found to be an important mediator of compartment size and overall chromosome compaction [15]. Using the multiplexed FISH approach, *cec-4* mutant early embryos seem to have more compacted chromosomes due to the loss of stretching. This is in contrast to older embryos and adults, where heterochromatic arrays and the endogenous X chromosome are de-compacted due to the loss of CEC-4 [17,63,64].

8. Epigenetic Modifications and the Loss of Developmental Plasticity

Most somatic cells progressively lose developmental plasticity after the gastrula stage and acquire their terminal identities at the end of gastrulation, which occurs around 6 h after fertilization [88–93]. The reorganization of chromatin from its uncompartmentalized state into euchromatin and heterochromatin at the onset of gastrulation coincides with this loss of developmental plasticity in *C. elegans* [20,30,88].

In *C. elegans*, MET-2 and SET-25 both regulate the timing of developmental plasticity loss and differentiation. In mammals, loss of the MET-2 homolog SETDB1 leads to the loss of cellular differentiation and aids in somatic cellular reprogramming [94]. In the context of cellular reprogramming, H3K9me3 was found to block TFs from accessing broad chromatin domains whose expression was necessary to induce pluripotency [95]. In *C. elegans*, the loss of developmental plasticity was tested directly in *met-2* and *set-25* single mutant embryos in a cell fate challenge assay, where they ectopically express a transcription factor, *hlh-1*, that promotes the differentiation of embryonic cells to body wall muscle cells [91]. Ectopic expression of *hlh-1* before the loss of developmental plasticity promotes the differentiation of all embryonic cells into body wall muscle cells [91]. In this assay, MET-2 was found to promote the loss of developmental plasticity, as significantly more *met-2* mutant embryos retained their developmental plasticity at the 100-cell stage compared to WT [20]. In contrast, SET-25 was found to inhibit loss of plasticity as *set-25* mutant embryos were less plastic than WT at 100 cells [20]. These contrasting effects of *met-2* and *set-25* in embryos suggests that lower levels of H3K9me3, a phenotype present in both mutants, is not driving the loss of plasticity. Instead, the regulation of plasticity is likely through H3K9me2 as this mark is completely lost in *met-2*-mutant embryos but is modestly increased in *set-25* mutants [20]. This differentiation assay provides evidence that *met-2* and *set-25* both regulate developmental plasticity, likely through their modulation of H3K9me2 and its subsequent effects on the timing of heterochromatin formation, where the mis-regulation of the histone mark alters the timing of the onset of heterochromatin formation [20].

The mammalian homologs of MET-2 and SET-25 are SETDB1 and EHMT2, respectively. Loss of these methyltransferases during mammalian embryogenesis results in embryonic lethality [96,97]. However, unlike the mammalian homologs, mutant *C. elegans* embryos that are null for *met-2* and *set-25* have developmental delays and lower viability than WT but are still able to develop into adult worms [19,20]. Both these mutants are still able to eventually lose developmental plasticity and begin differentiating, indicating that there may be other regulators of plasticity at later stages of development. *set-25* mutants are particularly striking in their complete inability to form heterochromatin even at the late-embryonic 200-cell stage [20]. Even with a complete loss of heterochromatin at these stages, the mutant worms are in fact less plastic than WT [20]. Additionally, RNA-seq experiments have shown that disruption of H3K9me causes gene-specific changes by modulating transcription factor activity [61]. These gene expression changes were independent of chromatin compaction, and were enriched in tissue-specific transcriptional programs [61]. These results suggest that in *C. elegans*, heterochromatin formation and the loss of plasticity can be decoupled.

While *cec-4*-mutant worms are healthy and appear WT, they respond uniquely to cell fate challenge assays. The authors used the same cell fate challenge assay described

earlier, which uses the TF *hs:hlh-1* to promote differentiation of mid-stage embryos into muscle cells. A significant fraction of *cec-4* mutant embryos, which lack the peripheral anchoring of heterochromatin, continued to differentiate into normal embryos and hatched into L1s despite widespread expression of the transcription factor [64]. The authors reported that these hatched L1s appeared abnormal and had a low survival rate [64]. The authors suggest that this could be due to the failure of *cec-4* mutants to stabilize the ectopic differentiation program initiated by *hlh-1* [64]. However, several H3K9me pathway mutants tested in this assay also exhibit changes in developmental plasticity during embryogenesis [20], suggesting an alternative hypothesis that *cec-4* mutant embryos could be less developmentally plastic.

Similar to H3K9me2, PRC2-mediated deposition of repressive H3K27me3 mark by EZH2 was also found to be required for the reprogramming and establishment of pluripotency [98]. In mammals, both of these marks are required for normal embryonic development, and mutant embryos lacking the methyltransferases for these repressive marks die early in gestation [94,96,98]. In *C. elegans*, PRC2 complex member MES-2, which is required for H3K27me2/me3 [29], promotes the transition from a developmentally plastic state to differentiated embryos [30]. Using the cell fate challenge assay, loss of *mes-2* was found to increase the number of plastic cells in early 100-cell embryos. There were also significant changes in the transcriptome for developmental regulators of cell fate and differentiation for both *mes-2* and *mes-3* mutants, which suggested that the PRC2 complex is required for promoting a differentiated cell fate [30].

Another important mechanism involved in the loss of developmental plasticity at the gastrula stage is the loss of histone variant H3.3 inherited from the oocyte, and the enrichment of canonical H3 in the developing embryo, which facilitates the deposition of repressive histone marks on the chromatin that are linked to the process of heterochromatin formation [42]. *C. elegans* mutants that lacked the ability to enrich H3 in somatic cells had a defect in timely loss of developmental plasticity. This was tested in a cell fate challenge assay using ectopic expression of the TF *che-1*, which promotes differentiation into neuronal cell fate. Without the incorporation of H3 and its associated H3K9me2/me3 and H3K27me2/me3 repressive marks, embryos maintained developmental plasticity much later into embryogenesis [42].

9. Establishment of Dosage Compensation on the X Chromosome

The process of dosage compensation is unique for the *C. elegans* sex chromosomes compared to other organisms. Expression of transcripts from the two X chromosomes of hermaphrodite worms are repressed by half to match that of the single male X chromosome [7]. Establishment of dosage compensation and repression of the X chromosomes involves chromatin reorganization during early embryogenesis and its onset coincides with gastrulation and the loss of pluripotency [70,99]. This process is initiated by X signal elements such as *sex-1*, *sex-2*, *ceh-39* and *fox-1*, and autosomal signal elements such as *sea-1* and *sea-2*, that are transcribed from the sex chromosome and autosomes respectively [100–106]. The ratio of X:A signal elements determines whether XOL-1 is activated or repressed during early embryogenesis [107]. XOL-1 is the master regulator of sex determination and dosage compensation in *C. elegans* [107]. In hermaphrodites with two X chromosomes, the X-signal elements are able to repress XOL-1 which then promotes hermaphrodite sex development pathways as well as the initiation of dosage compensation on the X chromosomes [104].

The dosage compensation complex (DCC) consisting of a condensin I^{DC} and several accessory proteins is responsible for carrying out the process of dosage compensation [108,109]. Condensin I^{DC} is a *C. elegans*-specific ortholog of the conserved metazoan condensin complexes [109]. DPY-27, a core component of the condensin I^{DC}, is visibly enriched on the X chromosome shortly after the onset of gastrulation in 30–50 cell embryos [70,110]. The DCC loads onto binding sites on the X chromosomes and spreads along the entire chromosome [111–113]. Loading of condensin I^{DC} on the X chromosome alone is

sufficient to initiate the process of chromosome compaction through chromatin looping [87,114], which leads to global gene repression on the X [115] and is thought to lead to TAD formation [87,114].

The loading of the DCC onto the X initiates the process of dosage compensation, which also involves the formation of TADs on the X [87,116]. In dosage compensation (DC)-deficient embryos, TAD boundaries and compartment formation are weaker [68,87]. DCC recruitment sites called *rex* sites were found to mark strong TAD boundaries on the X [87,114]. Removing *rex* sites abolished these TAD boundaries and conversely the introduction of a new *rex* site on the X created a novel TAD boundary [114,116]. However, abolishing almost all of the TAD structure on the X chromosomes in embryos did not significantly affect gene expression due to dosage compensation, suggesting that TAD formation is not the major driver of DCC-mediated repression [116].

Dosage compensation, as measured by RNA-seq on staged embryo populations, was not seen to be completely established during embryogenesis [66]. This was tested by measuring the transcriptional output from X chromosomes of hermaphrodites compared to samples of mixed hermaphrodites and males. In all the embryo stages sampled in the study, the hermaphrodite X chromosomes were upregulated when normalized against X chromosomes from mixed-sex embryos [66]. Even though condensin I^{DC} loading on the X occurs shortly after the onset of gastrulation, establishment of dosage compensation cannot be accurately measured by RNA-seq due to the presence of mRNAs already transcribed in the embryo before this stage. However, in early embryos, there is evidence for the partial dosage compensation of zygotic genes that are newly transcribed in embryos after zygotic gene activation at the 4-cell stage. Depletion of *dpy-27* by RNAi in early- and mixed-stage embryos resulted in the significant de-repression of X chromosomes over WT at those stages, suggesting that the DCC is actively establishing dosage compensation throughout embryogenesis [66].

In addition to chromatin looping by the condensin I^{DC}, several members of the DCC are either directly involved in or recruit other proteins that carry out ancillary mechanisms that reinforce X chromosome compaction, gene repression and tethering of the X chromosomes to the nuclear lamina [63,69,117]. One of these mechanisms is mediated by H4K20me1, a repressive histone mark that becomes enriched on the X chromosomes in hermaphrodites during embryogenesis [66,67]. DPY-21, one of the members of the DCC, is a H4K20 demethylase that is responsible for this X-specific enrichment of H4K20me1 [68]. Deposition of H4K20me1 and its enrichment on the X chromosomes is initiated in bean stage embryos, though enrichment only peaks later in late 3-fold embryos [66,67,70]. RNA-seq experiments on *dpy-21* mutants suggests that DPY-21 is required for the establishment of dosage compensation, and that loss of *dpy-21* has a more pronounced effect on X expression during mid to late embryogenesis [66].

Additionally, a DCC-independent mechanism also contributes to shaping the chromatin of the X chromosomes of early embryos. The active histone mark H4K16ac is depleted on the X chromosome in all stages of embryogenesis, starting at 4-cell embryos [69,70]. The H4K16ac mark is inherited in the embryo from germ cells, where it is depleted from the X chromosome. The depletion of H4K16ac in embryos precedes the loading of the DCC, and at this stage it is dependent on MES proteins MES-2, MES-3 and MES-4, which are components of the PRC2 complex [70]. After DCC loading during early gastrulation, the depletion becomes DCC-dependent [69,70]. In *mes-2* mutants, DPY-27 loading onto the X chromosomes is delayed compared to WT. In *mes-2* and *mes-4* mutants, enrichment of H4K20me1 on the X chromosome is also delayed [70]. The delay in onset of dosage compensation in *mes-2/mes-4* mutants is likely related to their function in regulating the expression of early zygotic genes such as *sdc-2* that are important for the initiation of dosage compensation. The timeline of dosage compensation and other major mechanisms of embryonic chromatin reorganization are summarized in Figure 2.

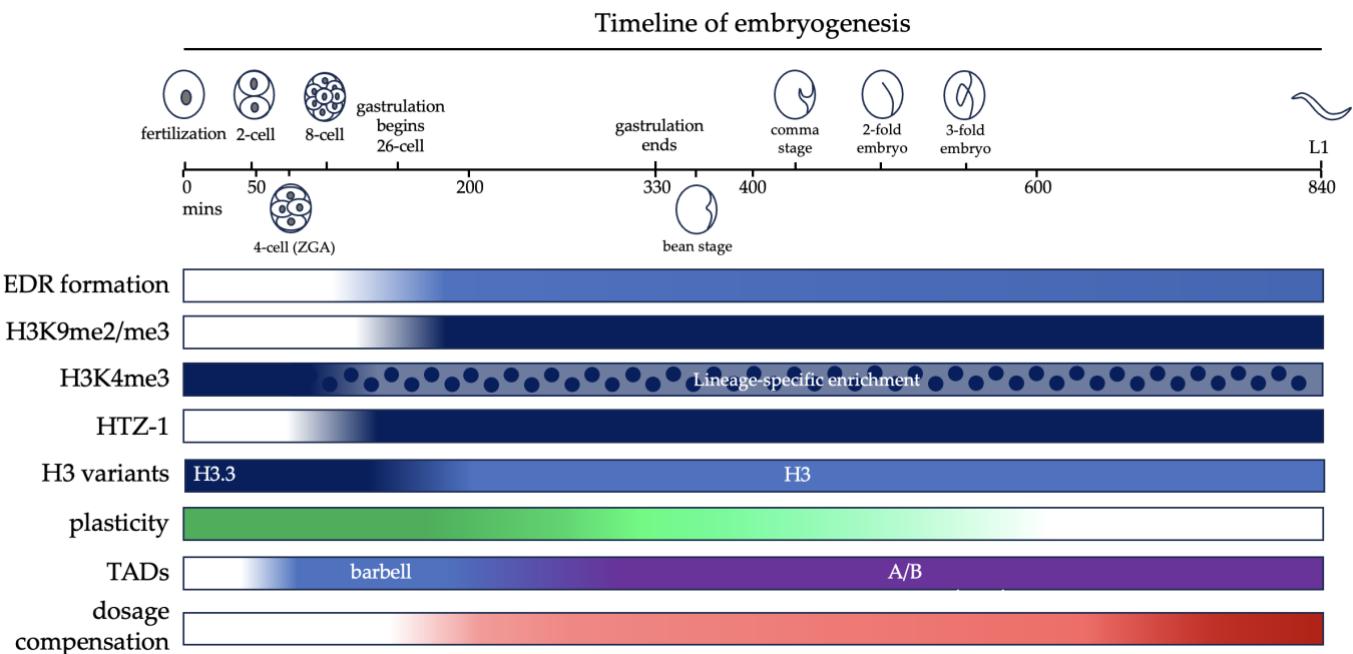


Figure 2. Timeline of chromatin reorganization during embryogenesis.

10. Conclusions and Future Directions

The chromatin landscape is dynamically regulated in *C. elegans* early embryogenesis. Separation of the chromatin into heterochromatin and euchromatin is one of the early steps of embryogenesis. Evidence suggests that *de novo* heterochromatin formation, which is mediated by the deposition of repressive histone marks, may be important for regulating the precise timing of termination of developmental plasticity and onset of cell differentiation programs in early embryos. However, while heterochromatin formation does regulate the timing of these processes, it is dispensable for differentiation and the acquisition of terminal cell fate. The precise mechanisms of how deposition of repressive histone marks leads to heterochromatin formation and consequently how heterochromatin formation may influence the loss of developmental plasticity remain to be elucidated.

The pathways that are responsible for the tethering of heterochromatin to the nuclear lamina play important roles in maintaining appropriate transcriptional silencing of those genomic regions at specific stages of embryonic development. While tethering is dependent on H3K9me deposition, evidence shows that deposition of repressive histone modifications and anchoring by tethering pathways contribute independently to transcriptional silencing of heterochromatin. Loss of proteins that mediate this peripheral anchoring of heterochromatin during embryogenesis seem to also affect the robustness of cell fate and differentiation pathways. The mechanisms behind how these pathways are being influenced by lamina tethering of silent domains, and whether these mechanisms overlap with the influence of heterochromatin formation on plasticity, is an interesting question for further research.

Initiation of dosage compensation on the hermaphrodite X chromosomes is an important process that contributes to chromatin organization during embryogenesis. This process involves many mechanisms that are also known to modulate autosome architecture during embryo development. Loading of the DCC onto the X chromosome at the onset of gastrulation is followed by chromosome compaction and a reduction in transcription through pathways of chromatin condensation by condensin I^{DC}, as well as the deposition of repressive histone modifications, depletion of active histone modifications and peripheral tethering to the nuclear lamina. While the onset of termination of plasticity coincides with the first detectable loading of the DCC

components onto the X chromosome and both processes involve PRC2-mediated histone modifications, whether these two pathways are directly related has not yet been tested.

Recent advances in chromosome conformation capture technologies have allowed us to observe the highly dynamic process of TAD formation during embryogenesis. It has also allowed the study of some of the mechanisms that regulate these processes in embryos. However, the functional significance of TAD formation at these stages is still an open question. From studies in other model organisms and in *C. elegans* adult tissues, it is evident that the disruption of the TAD structures does not necessarily lead to altered transcription. Rather, the relationship between the transcriptional state of the chromatin and TAD structures is complex and the transcriptional state may be governed by several redundant pathways. With this background, the importance and functional relevance of TAD structure and TAD formation during embryogenesis requires further examination.

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