DNA methylation and histone modifications are essential for regulation of stem cell formation and differentiation in zebrafish development

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Abstract

The complex processes necessary for embryogenesis require a gene regulatory network that is complex and systematic. Gene expression regulates development and organogenesis, but this process is altered and fine-tuned by epigenetic regulators that facilitate changes in the chromatin landscape. Epigenetic regulation of embryogenesis adjusts the chromatin structure by modifying both DNA through methylation and nucleosomes through posttranslational modifications of histone tails. The zebrafish is a well-characterized model organism that is a quintessential tool for studying developmental biology. With external fertilization, low cost and high fecundity, the zebrafish are an efficient tool for studying early developmental stages. Genetic manipulation can be performed *in vivo* resulting in quick identification of gene function. Large-scale genome analyses including RNA sequencing, chromatin immunoprecipitation and chromatin structure all are feasible in the zebrafish. In this review, we highlight the key events in zebrafish development where epigenetic regulation plays a critical role from the early stem cell stages through differentiation and organogenesis.

Key words: epigenetics; DNA methylation; zebrafish; development; stem cells; histone modifications

Embryonic development is an intricate process that requires a complex network of regulatory mechanisms to produce a viable organism. This progression requires coordinated gene expression, which is facilitated by both transcriptional regulators and epigenetic factors (Table 1). For the purposes of this review, we define epigenetics as the study of gene expression regulatory mechanisms that alter DNA structure, but not nucleotide sequences. The zebrafish is a well-established model organism for studying developmental biology. Although there are some small, but notable differences between the epigenetic regulation of mammals and zebrafish, the processes are largely well conserved, which solidifies the study of epigenetic regulation of zebrafish development as an ideal model [1]. In this review,

we highlight the key events in zebrafish development where epigenetic regulation plays a critical role.

Epigenetic regulation of stems cells in early development

In zebrafish embryogenesis, there are seven stages of early development: zygote, cleavage, blastula, gastrula, segmentation, pharyngula and hatching [2]. Postfertilization, there is a 3.3 h developmental window during which the zygote undergoes 10 rounds of cell division. In this window, during premidblastula transition (MBT) development, maternal and paternal genomes are remodeled via removal, exchange and deposition of DNA-

Table 1. Key players in DNA methylation and histone modification and their functions in zebrafish development

Developmental system	Epigenetic mechanism	Epigenetic factor	Reference
Hematopoietic development	DNA methylation	Dnmt1 and Dnmt3	[5]
		Tet2	[50] [51]
		Tet3	[50]
	Histone modification	Prc1 and Prc2	[37]
		Ezh2	[38]
		Hdac1	[39, 44]
		Hdac2	[44]
		Lsd1	[40, 44]
		DSIF and NELF	[45, 46]
		SWI/SNF	[53]
		NuRD	[33]
Cardiovascular system	Histone modification	Smyd4	[59]
		Smdy3 and Setd7	[61]
		Kdmbaa AND Kdm6bb	[67]
		Hdac	[68]
		Hdac5	[74]
Muscular system	Histone modification	Set7/9	[81]
		Smyd1a and Smyd1b	[85, 86]
Nervous system	DNA methylation	Dnmt1	[98]
		Dnmt3 and Ga9	[99]
		Tet	[173]
	Histone modification	Hdac1	[91]
		Hdac4	[149, 151]
		Smyd2a	[96]
		Kmt2a	[103]
		Knmt2d	[66]
		Kdm6	[66, 67]
		Kdm7	[97]
Digestive and endocrine system	DNA methylation	Dnmt1	[98, 109, 162, 171]
		Dnmt2	[111]
	Histone modification	Hdac1	[115, 174]
		Hdac3	[114, 117]
		Ezh2	[175]
		Jmjd3	[176]
Lymphatic	DNA methylation	Dnmt1	[124, 125]
		Tet	[129]
	Histone modification	Hdac1	[131, 135]
		Hdac3	[135]
		Lsd1	[137]
Skeletal	Histone modification	Hdac1	[174]
		Hdac4	[149, 151]
		Kat2a and Kat2b	[153]
		Moz	[152]
		Smyd3	[60]
		Prdm3 and Prdm16	[146]
		Phf8	[155]
Reproductive	DNA methylation	Dnmt1	[162]
		Dnmt3	[162]
		Dnmt3aa and Dnmt3bb	[110]
		Dnmt4	[110]
	Histone modification	Hdac1	[162]

and chromatin-associated proteins in preparation for embryonic transcription. Posttranslational marks, including methylation and acetylation of specific lysine (K) residues, are established on histones [3]. Genomes undergo global methylation changes to stabilize DNA methylation levels in the embryo to a state like those in somatic tissues [4]. A cascade of gene activation and inactivation events results in the establishment of gene regulatory networks necessary for embryonic development. Gene activation and inactivation events are mediated by

DNA methyltransferases (Dnmt), which catalyze the transfer of a methyl group to cytosine residues in DNA to form 5-methylcytosine (5mC) [5]. In zebrafish, there are eight Dnmt enzyme orthologues to mammalian DNMTs [6]. Dnmt1 maintains the methylated DNA created during replication [7]. Zebrafish Dnmt6 and Dnmt8 are related to mammalian DNMT3a, which carries out *de novo* methylation during development, whereas *dnmt3, dnmt5* and *dnmt8* are unique to teleosts [8, 9]. Other posttranslational marks include histone

modifications that are present around promoter and enhancer regions and aide in the regulation of gene expression. H3K9me3 and H3K27me3 are transcriptionally repressive, especially on methylated promoters, whereas transcriptionally permissive H3K4me3 on hypomethylated regions indicates the potential for gene expression following zygotic genome activation (ZGA) transition [10, 11].

At the establishment of MBT, zebrafish development is supported by proteins and RNAs stored in the egg cytoplasm. During the maternal to zygotic transition (MZT), the embryo begins regulation of the developmental program in a process called ZGA [12]. Maternal transcripts are degraded, and zygotic transcription provides proteins and microRNAs (miRNAs) to enhance maternal RNA clearance. Uhrf1, a multidomain protein that binds methylated cytosines, is required for Dnmt1 function, jointly repressing methylated prompter transcription by binding methylated CpG sites [13].

Histone modifications play a significant role during early zebrafish embryogenesis and have been identified as a prominent factor during ZGA [6]. The exposure of DNA for transcription or compaction for repression gene expression is regulated, in part, through histone modifications [14]. During MBT, the level of genes marked by two or more histone modification increases and developmental gain and loss of histone marks are more pronounced [15, 16]. At MBT, the maternal DNA methylome is maintained until the 16-cell stage when it is gradually degraded during cell divisions through passive DNA demethylation and *de novo* methylation, simultaneously [17]. Histone lysine modifications identify loci that are competent for transcriptional activation, thus only a subset of genes associated with monovalent or bivalent H3K4me3 or H3K27me3 modifications are actively transcribed [18] (Figure 1). During ZGA, DNA of many oocyte genes important to development is globally enriched with H3K4me3 and H3K27me3 with certain loci being demethylated during zygotic cleavage stages to a state almost identical to sperm chromatic patterns [19]. Specific regions throughout the genome are enriched by H3K36me3 and histone variant H2afv, becoming transcriptionally active and encoding for developmentally regulated transcription factors during embryonic axis formation, regional specification and organogenesis [20]. H2afv interacts with *dnmt1* to restrict DNA methylation during MZT and is believed to be essential for global DNA methylation reprogramming during early development [21]. H2afv and H3K4me1 act as placeholders in regions of DNA hypomethylation that resolve during ZGA into transcriptionally active or poised chromatin [22]. In zebrafish embryos carrying a mutation in h2afv, enhanced DNA methylation was observed during somitogenesis along with various multisystemic abnormalities.

MicroRNAs (miRNAs) are small non-noncoding RNAs (ncRNAs) that control gene expression through regulatory mechanisms including messenger RNA (mRNA) deadenylation, degradation and translation suppression [23]. miR-430 is transcribed at ZGA and is instrumental in the removal of approximately 400 maternal mRNAs for the activation of zygotic genomes [24, 25]. The miR-430 family promotes the degradation of Smarca2, a chromatin remodeler, and promotes the formation of heterochromatin, tightly compacted DNA, following the ZGA clearance of maternal transcripts and the absence of H2K9me3 until the 1000-cell stage [26] . It is one of the earliest and most transcribed regions following ZGA [26, 27]. miR-430 also acts in a capacity to optimize Nodal signaling by inhibiting the expression of 'lefty,' a Nodal antagonist [28]. Nodal signaling then upregulates miR-206 expression during zebrafish gastrulation to modulate gastrulation movements [29].
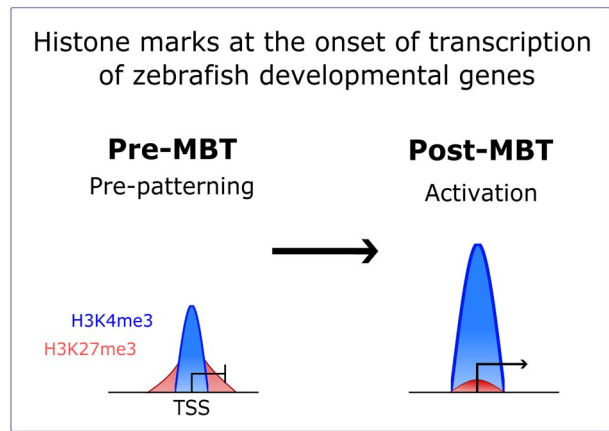

Figure 1. Histone modifications of H3K4me3 and H3K27me3 during MBT development.

Histone methyltransferase (HMT) *setdb2* also regulates convergence and extension movements necessary to form the zebrafish body plan in gastrulation [30]. *Setdb2* catalysis of H3K9me3 restricts dorsal formation and regulates left–right asymmetry, affecting notochord, somite and midline formation [30]. Protein arginine methyltransferases are responsible for arginine methylation in both histone and non-histone proteins. The knockdown of *prmt1* has been shown to result in delayed growth, shortened body length, tail deformation, cardiac edema and decreased H4R3 methylation [31]. As a developmental regulator, *prmt1* can be detected in embryos from the zygotic period until early larval stages. Recent reviews from Akdogan *et al.* [32] and Horsfield [33] provide further exploration of early zebrafish embryogenesis.

Epigenetic regulation of hematopoietic development

Zebrafish hematopoiesis begins with a primitive wave that generates erythroid cells, macrophages and megakaryocytes. The precursors for primitive hematopoiesis emerge during the 1–5 somite stage in the anterior lateral mesoderm (ALM) and posterior lateral mesoderm (PLM) [34]. Precursor cells expressing *scl*, *lmo2* and *gata1* develop into primitive erythroid cells within the PLM. At 15 hours postfertilization (hpf), these cells migrate toward the trunk midline to form the intermediate cell mass and begin to express embryonic globin [35]. Primitive macrophages are generated from the segment of the ALM at the rostral blood island. The nucleosome remodeling and deacetylase (NuRD) complexes play critical roles in several steps of hematopoiesis [36, 37]. A component of the NuRD complex, metastasis-associated protein 3 (Mta3-NuRD) is essential for the regulatory hierarchy of primitive hematopoiesis [38]. Histone deacetylases (HDACs) are important for the deacetylation of core histones in chromatin, to repress transcription by removing acetyl groups from lysine residues of histones H3 and H4. The inhibition of NuRD activity through depletion of Mta3 or HDAC inhibitors was shown to abolish primitive hematopoietic lineages and cause abnormal angiogenesis, whereas overexpression of NuRD components enhanced the expression of *scl* and *lmo2* in zebrafish embryos [38]. The loss of *smarca1* also had devastating effects on the expression of *scl, gata1* and *β-globin e3*, indicating its role in early hematopoiesis [39]. Similarly, *chrac1*, *actr2b* and *hdac9b* knockdown resulted in *gata1* and *β-globin e3*
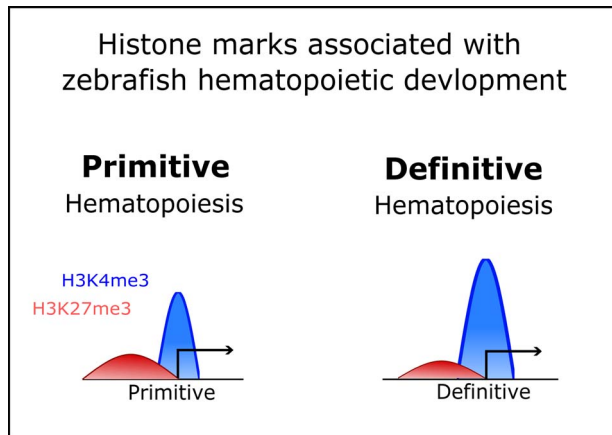
Figure 2. Histone modifications of H3K4me3 and H3K27me3 during primitive and definitive hematopoietic development.

reduction, indicating their role in erythroid progenitor formation from the mesoderm [39].

Definitive hematopoiesis begins around 24 hpf with the development of erythro-myeloid progenitors and hematopoietic stem cells (HSCs), the most thoroughly characterized tissue-specific stem cells in zebrafish development. During the endothelial-to-hematopoietic transition (EHT) a subset of endothelial cells lining the aorta gonad mesonephros (AGM) region acquire multilineage and long-term repopulating potential and bud as HSCs into the circulation [40, 41]. The EHT undergoes tight epigenetic regulation in the form of DNA and RNA methylation, histone modification and chromatic remodeling.

Principle polycomb repressive complexes (PRCs) have been shown to be key regulators of gene expression during hematopoietic development. Prc1 is one of the earliest known epigenetic regulators of HSC development, as an inhibitor of hemogenic endothelial cell specification [42]. Prc1 works in coordination with Ring-type E3 ubiquitin transferase (Ring1b) of Prc2 to repress histone H2AK119 ubiquitination. A component of Prc2, enhancer of zeste homolog 2 (Ezh2), silences gene transcription through H3K27me3. Ezh2 also functions independently of Prc2 as a circadian clock regulator that enhances zebrafish primitive and definitive hematopoiesis [43].

During definitive hematopoiesis, Hdac activity regains critical importance as *hdac1* acts downstream of Notch to promote HSC formation in the AGM [44]. As a component of the CoREST repressive complex, Hdacs regulate hemogenic endothelial cells through deacetylation of H3K56 and H4K16 [45]. Lysine-specific demethylase1 (Lsd1) is responsible for mono- or di-methylation of H3K4 or H3K9 and is believed to share a cooperative function with growth factor independence 1a (Gfi1a) and Gfi1b, a zinc finger repressor that is required for HSC maintenance, neutrophil generation and lymphoid development [45–49] (Figure 2). Definitive erythroid and megakaryocyte generation is regulated by Gfi1b [49]. Gfi1 complexes catalyze serial histone modifications by recruiting Lsd1 and CoREST via SNAG domains and tethering them to their targets [49].

DRB sensitivity-inducing factors (DSIF) and negative elongation factor (NELF) complex disruption have been shown to diminish runx1+ cells in the zebrafish AGM [50]. DSIF and NELF cause RNA Polymerase II (Pol II) to pause 30–50 nucleotides downstream of transcription start sites (TSS) that affect chromatic accessibility at hematopoietic genes [51]. The loss of Pol II pausing resulted in the misregulation of TGFβ and IFN-γ, pathways known to be involved in HSC formation [52, 53]. The inhibition of Pol II pausing through DSIF or NELF disruption was shown to cause premature elongation, upregulation of TGF-β genes, loss of IFN-γ gene expression and decreased chromatin accessibility [50].

As hematopoietic development continues, Dnmt1 ensures the maintenance of methylation through DNA replication, whereas Dnmt3 methylates unmodified genomic regions [5]. The ten–eleven translocation (Tet) proteins Tet1, Tet2 and Tet3, a family of cytosine dioxygenases, alter DNA methylation by converting 5mC to 5-methylcytosine and then 5-formylcytosine and 5-carboxylcytosine, facilitating methylation [54]. Tet2 and Tet3 contribute to the early formation of HSCs by localizing to the AGM. The loss of *tet2* expression inhibits erythropoiesis, whereas the double knockout of both *tet2* and *tet3* results in the loss of *runx1* marker staining in zebrafish embryos during HSC specification at 36 hpf [55, 56]. In the AGM, Tet2 and Tet3 are required for Notch signaling and can be rescued by restoring the expression of the gata2b/scl/runx1 network.

Progenitors derived from HSCs migrate to the caudal hematopoietic tissue (CHT) where they proliferate and undergo massive differentiation. Cells eventually migrate to the thymus and the kidney where they begin for lymphopoiesis and adult hematopoiesis, respectively. Recent studies have shown a requirement for Dnmts in the late steps of the EHT hierarchy. *Dnmt1*-deficient zebrafish exhibited reduced HSC proliferation in the CHT, leading to reduced myeloid and lymphoid cell [42]. The loss of methylation of *cebpa* CpG islands leads to de-repression of promoter activity that is required for definitive hematopoiesis [57]. Hematopoietic differentiation is also regulated by switch/sucrose non-fermentable (SWI/SNF) chromatin remodeling factors. Smarcd2, a component of the SWI/SNF complex in HSCs, is required for neutrophil granulocyte differentiation and B cell development [58].

Epigenetic regulation of cardiovascular development

Vertebrates acquire their three-dimensional (3D) heart structure during embryogenesis. In zebrafish, cardiovascular development begins with precardiac cell specification at 5 hpf in the anterior plate of the mesoderm. High-resolution live imaging results suggest that during heart development, zebrafish undergo two distinct phases of cardiomyocyte differentiation [59–62]. Epigenetic regulation of the cardiovascular system is critical in the regulation of gene expression necessary for development [63] (Figure 3).

HMTs play key roles in cardiovascular development. The SET and MYND domain containing (SMYD) family of histone lysine methyltransferases has been recognized as indispensable for proper heart development [64]. The knockdown of several *smyd* genes led to cardiac abnormalities and defects [64–67]. The knockdown of *smyd3*, a H3K4 methyltransferase, in zebrafish embryos showed abnormal cardiac looping at 48 hpf [65, 68]. Further analysis presented a downregulation of transcription factors, *amhc*, *vmhc* and *cmlc2,* which are crucial to early cardiomyocyte differentiation [66].

Another HMT, SET domain containing methyltransferase 7 (Setd7) (also known as set7/9), has been hypothesized to work alongside Smyd3 specifically in cardiac development. Setd7 only methylates non-methylated H3K4 (H3K4me), which is further methylated by Smyd3 to become H3K4me2 [66]. The transcripts
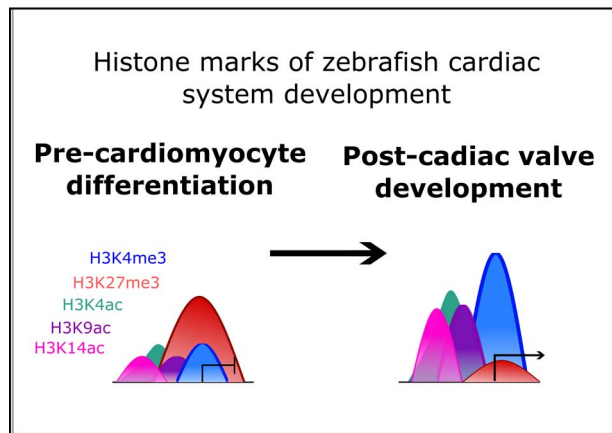
Figure 3. Histone modifications of H3K4me3, H3K27me3, H3K4ac, H3K9ac and H3K14ac during cardiac system development.

of both *smyd3* and *setd7* are abundantly expressed within the developing heart of zebrafish at 36 to 48 hpf [65–67]. During this time, zebrafish undergo a second round of cardiomyocyte differentiation, known as the second heart field (SHF). A cardiogenic population in the SHF transitions into myocardium that is specific to arterial pole development, whereas the atrium and ventricle become distinguishable [62, 69]. The knockdown of *smyd3* and *setd7* displayed no obvious morphological abnormalities in early development but led to later cardiac-specific developmental abnormalities including edema [66]. Both Smyd3 and Setd7 are expressed within the atrial and ventricular region. The abnormalities in the morphants suggest an important zygotic role in zebrafish cardiac development for this H3K4 methyltransferase [65–67]. Cardiomyocyte markers did not abrogate with a mutant *smyd3* or *setd7*, which suggests a redundant function in early cardiac development, but their ubiquitous expressions may be essential in regulating histone modifications for all cell types [66]. Together, Smyd3 and Setd7 present a synergetic function in zebrafish development by regulating H3K4 methylation.

Unlike Smyd3/Setd7, *smyd4* transcripts are specifically enriched in the developing heart and blood vessels and in the developing ventricles [64]. *smyd4* mutants have defects in patterning and looping, decreased proliferation in ventricles, and a reduction in acetylation of H3K4, H3K9 and H3K14 [64]. SMYD4 has been shown to interact with HDAC1 in a mouse cardiomyocyte cell line, suggesting that an impairment in smyd4 affects Hdac1 function. This is supported by previous studies showing Hdac1 and Hdac2 aid in maintaining cardiomyocyte function [64, 70]. The knockdown of smyd4 significantly reduced H3K4me2 and H3K4me3 while increasing H3K4me [64]. These biochemical approaches to understand smyd4 have coined its epigenetic regulation as a methyltransferase and a negative regulator of Hdac1 [64].

Another SET domain-containing HMT, Lysine(K)-specific methyltransferase 2d (Kmtd2), plays a role in conferring H3K4me3 marks during cardiac looping [71]. Kmt2d associates with Lysine(K)-specific demethylase 6a (Kdm6a), a histone demethylase responsible for the removal of H3K27me3 repressive marks on genes associated with cardiomyocyte differentiation [71, 72]. Kdm6ba and Kdm6bb promote trabecular growth during cardiac development [72]. Kdm6ba and Kdm6bb promote cardiomyocyte proliferation by decreasing H3K27me3 levels suggesting that H3K27me3 marks are substantial throughout

cardiac cell types and may be regulated by cell-type-specific demethylases like KDMs [67].

The development of the atrioventricular (AV) canal starts with myocardial expression of *bmp4*, *tbx2b* and *vcana* with and endocardial expression of *notch1b*, *has2* and *neuregulin* [60, 62, 73]. Tet-dependent activation of Activin-A, an activator of the *bmp4* pathway, is necessary to facilitate the attachment of proepicardial cells to the AV canal-associated regions of the heart [54]. Of the four classes of Hdacs (I-IV), class I members have been associated with regulating cardiomyocyte differentiation in zebrafish and mammals [70, 73–75]. Kim *et al*. [73] treated zebrafish with trichostatin A (TSA) to inhibit class I and II Hdacs and observed aberrant linear heart tube formation. Levels of H3K9ac and H3K18ac increased, but there was no effect on heart chamber-specific cells [66, 70, 73, 75, 76]. In contrast, the expressions of *bmp4*, *tbx2b*, *notch1b* and *NFATc1* were significantly reduced upon Hdac inhibition [73, 75, 76]. These studies show that Hdacs are required to regulate histone acetylation during cardiac valve formation.

Recently, the interaction between Pdk2 and Hdac5 was found to be critical in heart remodeling [77, 78]. Hdac5 is regulated by protein kinase D2 (PKD), a homologous protein of the PKD family [77, 79, 80]. The mutation bungee inhibits endocardial Notch signaling due to a missense mutation in zebrafish *pkd2* [79]. Notch signaling allows cells to undergo epithelial-to-mesenchymal transformation (EMT), important for valve formation. This is the result of a missense mutation in zebrafish pkd2 [79]. *Pkd2* mutants are unable to phosphorylate Hdac5 leading to the downregulated expression of target genes like Krüppel-like factor (KLF) 2a and 4a, transcription factors essential for heart valve formation [79, 81]. Together, this work suggests an indispensable interaction between Pkd2, Hdac5 and KLF during cardiovascular development.

Epigenetic regulation of muscle development

Embryonic myogenesis in zebrafish is influenced by the regulation of transcription factors, signaling pathways and post-transcriptional modifications. Myogenesis begins in the paraxial mesoderm where myogenic progenitors appear in the somite stage during segmentation at 10 hpf [82]. Myoblasts and satellite cells commit to myogenic lineages and differentiate into mature myofibers [82, 83]. Myogenic regulator factors and signaling pathways, such as Wingless related integration site (WNT), bone morphogenetic protein (BMP) and sonic hedgehog (shh), play a crucial role the programming of muscular development [82]. Transcriptional regulation through histone acetylation and methylation orchestrates the terminal differentiation of muscle cells (Figure 4).

The MyoD family is a master regulator of the myogenic lineage and differentiation of skeletal muscle cells [84]. MyoD interacts with HMTs, suggesting a significant role for epigenetic regulation in muscle cell differentiation [85]. As methylation patterns of H3K4 globally correlate with gene transcriptional levels, *set7/9* has increased expression during myoblast differentiation [86, 87]. Tao *et al*. [86] demonstrated a knockdown of *set7/9* decreased H3K4me1, which led to repressed expression of myogenic differentiation genes and impairment of skeletal muscle differentiation. Further exploration indicated a direct interaction between Set7/9 and MyoD at two different domains to initiate chromatin remodeling [86]. These results demonstrate an essential role for set7/9 H3K4 methylation in myocyte differentiation.
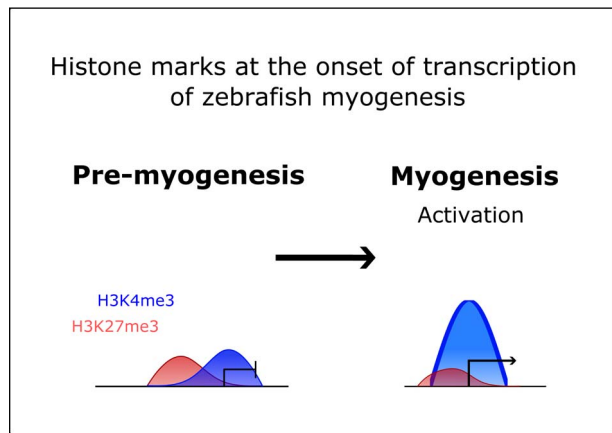
Figure 4. Histone modifications of H3K4me3 and H3K27me3 during the activation of zebrafish muscle development.

Figure 5. Histone modifications of H3K36me3 and H3K27me2 during the activation of zebrafish neural tube development.

Sarcomere assembly occurs through the process of myofibrillogenesis, which is essential for differentiation of muscle cells [88, 89]. In skeletal and cardiac muscle, Smyd1b aids in proper filament assemble during myofibrillogenesis [88–90]. Previous studies show embryonic lethality in *Smyd1b* mutant mice at embryonic day 10.5 due to cardiac muscle deficiencies [88]. A disruption of myofibril organization of skeletal and cardiac muscle correlated with *smyd1b* mutant zebrafish embryos [91]. Even though its function in histone methylation is not well understood, Smyd1b methylates H3 at K4 but not K9, suggesting a role in transcriptional activation [90–92]. Li *et al.* [91] demonstrated the upregulation of myosin chaperones expressed in muscle cells upon smyd1b knockdown, which caused complete disorganization among sarcomeric structures. Mutant *smyd1b* zebrafish have decreased myosin methylation, suggesting Smyd1b is a muscle-specific methyltransferase [91].

Epigenetic regulation of nervous system development

Neurogenesis in zebrafish begins as the neuroectoderm is derived by the mesodermal layer during gastrulation [93]. Factors such as *sox2*, and other members of the Sox family, are secreted and induction of neural progenitors begins [93, 94]. Undifferentiated neural progenitor cells follow a sequence of specification and differentiation to generate multiple neuronal and glial cell types. Epigenetic regulating factors are involved in coordinating the path of neural progenitor differentiation (Figure 5). Alternations to these epigenetic modulators have led to several developmental disorders that suggest a role of chromatin remodeling in neurogenesis [93, 94].

Promotion of neuronal specification and CNS patterning was associated with 18 hdac-regulating genes [95]. Zebrafish Hdac1 is required for specification and patterning of neurons and maintenance of vertebrate neurogenesis through the repression of Notch target genes [96, 97]. The impairment of proneural genes and neuronal specification occurs upon the knockdown of *hdac1* [96, 98]. *hdac1* mutants fail to maintain hedgehog signaling (shh) communication with hindbrain precursor cells and show impaired retinal cell differentiation into neurons and glial cells [97, 98]. Hdac1 was revealed to interact with the ascending and descending (Add) protein. In the absence of *add*, retinal cells show continual proliferation and do not switch to neuron differentiation [98]. This suggests that the interaction between Hdac1
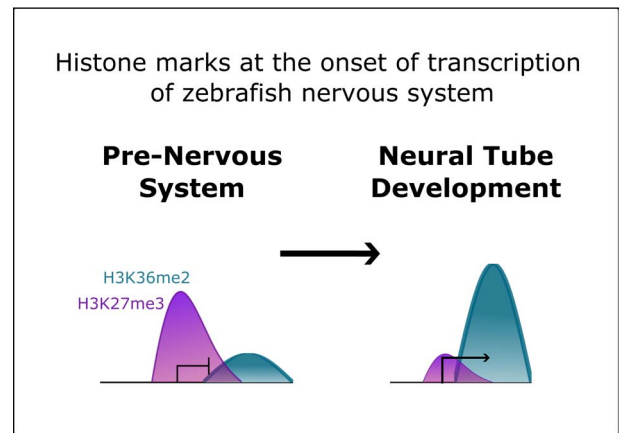
and Add and the shh pathway may regulate proliferation in retinal cells to aid in development of the nervous system [96–99]. Smyd2 is also involved in heart and brain tissue through activation of transcription via H3K36 dimethylation [100, 101]. Smyd2 was also shown to interact with Hdac1 and the Sin3A complex, suggesting an intricate mechanism of epigenetic regulation of the nervous system [101].

Methylation and demethylation of histone targets is also critical for nervous system development. JmjC domain-containing protein, Kdm7, demethylation of mono- and dimethylated H3K9 and H3K27 led to the expression of key components in embryonic neural tube development [102]. The inhibition of Kdm7 zebrafish orthologues resulted in developmental brain defects, suggesting Kdm7s function to remove silencing marks on chromatin during nervous system development [102]. The disruption of lysine methyltransferase 2A, kmt2a, results in a disruption of neural progenitor proliferation, early neuron differentiation and impaired gliogenesis [103]. These studies demonstrate the importance of histone methylation in nervous system development.

Uhrf1 interacts with Dnmt1 and is required for DNA methylation in zebrafish. The absence of either Uhrf1 of Dnmt1 catalytic function results in a wave of apoptosis in the epithelial layer, followed by apoptosis and unraveling of secondary lens fibers [104]. Angileri *et al.* [105] further demonstrated a requirement for *dnmt1* in the maintenance of retinal stem cell homeostasis, cell cycle progression and incorporation of ciliary marginal zone-derived cells into the retina. Tet2 and Tet3 are also critical regulators of retinal cell differentiation and morphogenesis where their loss of function results in defects in retinogenesis, failed retinal cell differentiation and failed terminal morphogenesis of retinal ganglion cells and photoreceptors [106]. Although Dnmt1 is known for its role in terminal differentiation of organ-specific tissue, Dnmt3 acts as a *de novo* methyltransferase that aids in particular methylation patterns specific to neurogenesis early in development [107]. Morpholino knockdown of *dnmt3* results in significant brain and retinal defects in zebrafish [16, 107]. This is consistent with work from Rai *et al.* [107], which identified the absence of the neurogenesis regulator *lef1* rescued *dmnt3* morphants. Further studies showed Dnmt3 associates with g9a, an H3K9 methyltransferase, to regulate the activity of Lef1 during neurogenesis [107]. Knockdowns of both *dnmt3* and *g9a* led to a decrease in methylated H3K9, as well as upregulation in *lef1*. This
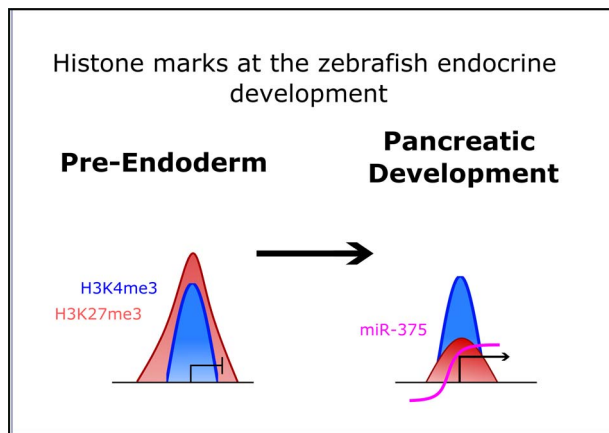
Figure 6. Histone modifications of H3K4me3, H3K27me3 and miR-375 during the activation of zebrafish endocrine development.
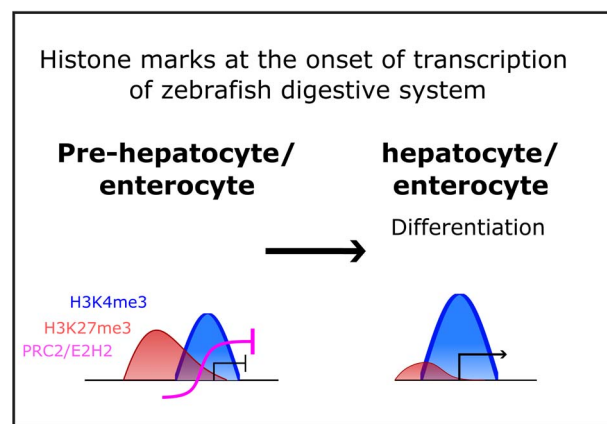

Figure 7. Histone modifications of H3K4me3, H3K27me3 and PRC2 during the activation of zebrafish digestive system differentiation and development.

Epigenetic regulation of the digestive and endocrine development

Embryonic stem cells (ESCs) maintain a bivalent chromatin profile to balance self-renewal and differentiation. The formation of the mesendodermal lineage is established by the end of gastrulation, about 10 hpf [2]. Nodal signaling initiates development of the endoderm from the dorsal and lateral cells of the blastoderm margin of the late blastula-stage embryo, which will give rise to the thyroid, pancreas, liver, thymus and gallbladder [108]. Repressive H3K27me3 and activating H3K4me3 allow for rapid activation of promotors that regulate lineage development [109].

Specification of the zebrafish liver and pancreas progenitors are defined during the beginning stages of gut formation (~18 hpf) [110]. Pancreatic progenitors evaginate from the endodermal tube at 24 hpf to form the dorsal pancreatic bud [111]. The endocrine pancreas develops from the dorsal side by the aggregation of endocrine islet [112]. Histone demethylases (HDMs) Kdm6a (Utx) and Kdm6b (Jmjd3) remove H3K27me3, initiating differentiation of the endoderm [113] (Figure 6). Hdac acetylation was shown to play a role in regulating pancreatic cell fate decision from multipotent pancreatic progenitor cells [114]. Kloosterman et al. [115] found that miR-375 plays a key role in pancreatic islet development and inhibition of the miR-375 resulted in dispersed islet cells following embryonic development. By 52 hpf the dorsal bud is encapsulated by the ventral bud which gives rise to acinar and duct cells essential for mature exocrine pancreatic development [116]. Exocrine pancreas development and gut differentiation require DNA methylation by Dnmt1 for the survival of acinar cells in the pancreas, but not for pancreatic duct or endocrine cell formation [16, 111, 117]. In mutant dmnt1 zebrafish, development of the pancreas was impaired after 84 hpf [118]. This suggests that dnmt1 is essential for acinar cell survival by maintaining methylation patterns, but expendable for all other pancreatic development [16, 116, 117].

The specification of the liver on the ventral side follows pancreatic specification [111]. This requires interaction between the foregut endoderm and the later plate mesoderm where hepatoblasts differentiate into mature hepatocytes and biliary cells [108]. Through analysis of dnmt and hdac mutant zebrafish embryos, dnmt3aa and dmnt4 were found to be expressed in the liver and pancreas at 72 hpf [119]. Morpholino knockdown of dnmt2 led to differentiation defects in the liver [120]. Under normal conditions, l-fabp and transferrin-α expression begins at 48 hpf and continues past 80 hpf. In the absence of dnmt2, embryos showed little to no expression of these late markers [120, 121]. The dnmt2 mutation only caused a slight reduction in early differentiation markers foxa3 and gata6 expression [111, 120]. A derivative of dnmt2 was able to rescue late differentiation in the liver demonstrating the role of dnmt2 in late differentiation of liver development [120]. uhrf1 mutants did not show liver bud expansion due to hepatocyte apoptosis that can be attributed to dnmt1 repression [122].

Hdacs have also been implicated in embryonic zebrafish liver development [73, 123, 124]. Hdac1 specifically mediates the hepatic fates from the zebrafish foregut endoderm marking it as a requirement for endocrine differentiation [124]. hdac1 depletion affected liver size, promoted the formation of ectopic endocrine tissue and has been shown to play a role in liver specification and differentiation and exocrine pancreatic specification [125]. The disruption of hdac1 activity between 14 and 18 hpf resulted in little to no specification of hepatoblasts, as well as defects in foregut tissue expansion and extra-hepatopancreatic duct formation [124]. Hdac3 plays a role in liver formation as a suppressor of growth differentiation factor 11 (gdf11) [123, 126]. gdf11 knockdown resulted in rescue of small liver phenotypes [123]. Overexpression of hdac3 caused liver enlargement suggesting a specific role for hdac3 in the differentiation of endoderm cells to hepatoblasts [123]. It is important to note that the Wnt/β-catenin pathway is important to liver specification and morphogenesis [127]. HDAC inhibitors have shown to alter the gene expression of this pathway resulting in defects of liver formation in zebrafish [117, 127]. These findings suggest that epigenetic modifications of factors by acetylation/deacetylation aid in liver organogenesis (Figure 7). Furthermore, liver-specific transcription factors hhes, foxa3 and prox1 were absent in zebrafish treated with HDAC inhibitors valproic acid and TSA [123].

Several epigenetic regulators have been identified to play a role in intestinal development. Proper formation of the gut epithelium is dependent on the expression of uhrf1 for the maintenance of methylation marks. Zebrafish embryos carrying a mutation in uhrf1 have reduced epithelial barrier function. Loss of methylation on the tnfa promoter results in enhanced tumor necrosis factor expression, intestinal epithelial cell shedding and apoptosis [128]. The loss of both uhrf1 and dnmt1 has

been shown to disrupt intestinal smooth muscle cell formation, further implicating the importance of DNA methylation in intestinal development [129]. *Ezh2*, a mediator of repressive H3K27 marks, is required for proper intestinal and liver formation. Ezh2 mutants showed significant impairment in intestinal development and decreased expression of the intestinal marker *fab2* and hepatic marker, *fabp10a* [130].

Epigenetic regulation of lymphatic system development

Development of the zebrafish lymphatic system begins with definitive hematopoiesis. Definitive HSCs produce lymphoid cells through expression of *rag1, rag2, ikaros, lck* and *gata3* [131]. The zebrafish kidney functions like mammalian bone marrow, providing a niche for HSPCs and production of myeloid, erythroid, thromboid and lymphoid lineages, whereas the thymus produces mature T cells into adulthood [132, 133]. B cells develop in the pancreas and have been reported at 3.5 day postfertilization (dpf). The zebrafish lymphatic system diverges from the mammalian lymphatic system in lacking lymph nodes as well as Peyer's patches and geminal centers, creating some controversy as to the site where B cell continue maturation [134].

Epigenetic regulation plays a vital role in both T and B cell differentiation and antibody response [135]. Specifically, DNA methylation play a vital role in T cell development as evidenced in *dmnt1* mutant zebrafish. The *dmnt1* mutation resulted in a severe reduction in T cell development in the thymus at 5 dpf. *in situ* hybridization and quantitative polymerase chain reaction (qPCR) also verified an impairment in B and T cell development [136]. Iwanami *et al.* [137] noted reduced levels of *lck*, *zap70* and *rag1* associated with T cell development in embryonic *dmnt1* mutant zebrafish. In adults, there was a consistent reduction of lymphocytes in the kidney marrow; however, erythroid and myeloid cells were not affected, indicating a possible necessity for *dmnt1* in T cell specification [137].

Further differentiation of T cells into subgroups is also mediated by DNA methylation. The methylation of CpGs at specific sites in the promoters of various genes in T cells is believed to be mechanisms for T cell differentiation into T cell subsets. *Dnmt1* methylation of the *foxp3* promoter CpG is believed to drive T cells toward T_{reg} differentiation [138]. *foxp3* expressing T cells, zT_{reg}, function similarly to mammalian T_{regs}. zT_{regs} were shown to rapidly infiltrate damaged spinal cords, retinas and hearts to produce organ-specific proregenerative factors to stimulate regenerative precursor cell proliferation [139]. The differentiation of naïve $CD4^+$ Th cells into Th1 and Th2 effector cells is mediated by the cytokine environment [140]. Th1 cells mediate cellular immunity, whereas Th2 cells instigate humoral immune responses. IL-12 is recognized as a key Th1 cell differentiation driver, whereas IL-4 causes Th2 effector cell generation [141].

The Tet family of enzymes plays a role in Th1 and Th2 differentiation. Tet2 has previously been shown to selectively repress IL-6 in innate myeloid cells to resolve inflammation [140]. Yang *et al.* has shown that the knockdown of Tet1 or Tet3 in zebrafish resulted in lost expression of IL-4 and IL-13A as well as blocked Th2 cell differentiation. Conversely, *Ifn-γ*, *Irf1*, *tbet* and *stat1*, all Th1-related genes, were dramatically upregulated, showing that TET1 and TET3 are essential for Th2-type immunity [140].

Hdacs have also been implicated in T and B cell development. During HSC emergence, Hdac1 is recruited to the *erk* promoter by SMAD1/5 and represses *erk1/2* expression by deacetylating H3K9 and H3K27 [142] (Figure 8). HDACs have also been identified
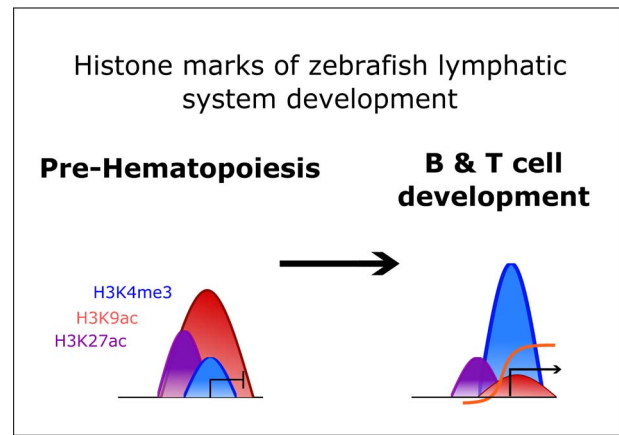

Figure 8. Histone modifications of H3K4me3, H3K9ac and H3K27ac during the activation of zebrafish lymphatic system development.

in the development of the pronephric and metanephric kidney [143]. *Rnal* progenitor cells are specified within the mesoderm at 12 hpf. At 36 hpf, the progenitor cells epithelialize, the nephron is patterned, and with blood supply delivery, the pronephros becomes the functional larval kidney [144]. The treatment of 2 hpf zebrafish embryos with Hdac inhibitors led to increased renal progenitor cell numbers through lowering the threshold of retinoic acid required for transcription activation [145]. Kidney field expansion compromises kidney function into adulthood due to renal progenitor cell hyperplasia, highlighting the necessity for tight Hdac regulation in early zebrafish embryonic development for the development of functional pronephros. High Hdac activity in the metanephric kidney is critical for promoting cell survival and for kidney organogenesis. Hdac1 and Hdac3 form complexes with Nuclear receptor corepressor 2 (Nor2) that are critical for HSC emergence and T cell development in zebrafish. *nor2* morpholino knockdown resulted in the absence of T cells in the thymus at 4 dpf [146].

Environmental regulation of the zebrafish immune system has been studied to investigate innate and adaptive immunity [147]. The introduction of pathogens and microbes to zebrafish colonies causes histone modifications, DNA methylation changes and alterations of miRNA in immune tissues. Microbial priming followed by treatment with TSA or pargyline, Hdac inhibitors, or Lsd1, a H3K4 demethylase, resulted in chromatin modifications of H3K9ac and H3K4me3 in the promoter regions of *il1b*, *il12a* and *tnfα* [148]. Exposure to chronic disease-causing pathogenic mycobacteria (E11) has been shown to cause downregulation of histone family members when compared with exposure to an acute disease-causing strain (Mma20), and HDAC 9b was upregulated during both infections. During mycobacterial infection, *tnfα*, *il1b* and TLR2 induced MMP9, a tissue-remodeling gene that also requires histone acetyltransferase p300 and chromatin modifications for induction [149, 150].

Epigenetic regulation of skeletal development

The skeletal system in zebrafish is composed of chondroblasts, chondrocytes, osteoblasts and osteocytes, which form and maintain cranial, axial and appendicular skeletal elements [151, 152]. Although bone formation does not begin until 4–5 dpf, BMPs promote mesenchymal cells, early on to differentiate into chondrocytes and osteoblasts [151, 152]. Studies have shown DNA methylation and histone acetylation and methylation play

a key role in chondrocyte and osteocyte differentiation [152, 153]. Blocking key epigenetic regulators impairs terminal skeletal development in zebrafish embryos.

Craniofacial skeletal development requires widespread communication through multiple cell types and tissues [151]. Cranial neural crest (CNC) cells undergo EMT to differentiate into chondrocyte and osteoblast lineages [151, 154]. The positive regulatory domain (PRDM) family has been characterized as an important regulator of the EMT [155, 156]. During craniofacial development, the absence of prdm3 and prdm16 led to hypoplasia and other cartilage defects [156, 157]. A combinatorial loss of function analysis of *prdm1a*, *prdm3* and *prdm16* led to extreme defects in cartilage elements, with partial loss of jaw joints and a cleft ethmoid plate [157]. The loss of *prdm3* and *prdm16* reduced levels of H3K9me/me3 and H3K4me3 [157]. The interactions between Prdm3 and Prdm16 with H3K9me act to repress gene expression, whereas H3K4me3 aids in activating gene expression [155, 157, 158]. These results show marks lost in the absence of *prdm3* and *prdm16* lead to a defect in craniofacial structures specifically derived from CNC cell populations, highlighting their essential role in skeleton formation and proper CNC cell development.

Hdac1 expression migrates to the branchial arches, fin buds and hindbrain at 36–48 hpf [159]. Reduced *hdac1* expression results in loss of craniofacial cartilage [159]. The initiation of cartilage ossification begins around 60–72 hpf. Craniofacial bones and cartilage undergo specific differentiation of CNC cells to form skeletal elements [151, 154]. HDAC4, a class II HDAC, is important for maturation and initiation of endochondral ossification in mice [160, 161]. In zebrafish, *hdac4* is expressed in migratory CNC cells during embryogenesis [162]. Through an interaction with Mef2, Hdac4 also controls ossification levels in endochondral bone [161]. A frameshift mutation of *hdac4* causes an upregulation of ossification markers, as well as increased ossification in the ventral pharyngeal cartilage in zebrafish [160, 162, 163]. The frameshift eliminates the interaction between Mef2c and Hdac4 while increasing the expression of *runx2a* and *runx2b* at 4 dpf [160]. Morpholino knockdown of *hdac4* led to a reduced number of CNC cells near the eye that contributed to a deformed palatal skeleton phenotype [162]. These suggest an epigenetic role for deacetylation in skeletal patterning.

Histone acetyltransferase, Kat2a and Kat2b are widely expressed through zebrafish embryogenesis, specifically in craniofacial development. A double knockout of *kat2a* and *kat2b* resulted in undeveloped or shortened cartilage components and craniofacial skeleton along with a decrease in bone-specific markers and a loss in H3K9 acetylation [164]. Interaction between Kat2a, Kat2b and H3K9 suggests the roles of these histone acetyltransferases as essential for differentiation of craniofacial cartilage [164, 165].

*phf*8 is expressed in the head and jaw region up from 1 to 3 dpf. Craniofacial and brain defects in zebrafish have been linked to Phf8 that has been identified to demethylate H4K20me1, H3K9me1/2 and H3K27me2 [166]. Although phf8 is located near over 7000 TSS, it is dependent on H3K4me3 binding to positively regulate gene expression. Morpholino knockdown of *phf*8 resulted in an absence of the lower jaw [166]. *phf*8 mutant embryos showed decreased demethylase activity leading to increases in H3K20me1 and H3K9me1/2 as well as pharyngeal arch defects that were significantly rescued with a catalytically inactive pfh8 [166]. These results suggest an involvement of Phf8 in regulating histone methylation in zebrafish skeletal development.

Epigenetic regulation of reproductive development

The zebrafish gonads develop from the dorsolateral lining of the peritoneal cavity. During gastrulation, primordial germ cells transverse to the germinal epithelium of the ovaries and develop into gametes [167]. All juvenile zebrafish develop an ovary. In females, the ovary continues to develop while males undergo a transitional intersexual phase to form differentiated testis 21–42 dpf [168]. The reproductive processes in zebrafish are regulated by interactions between steroid hormones along the hypothalamic-pituitary-gonad axis and steroidogenesis of gonad tissue [169]. The hypothalamus releases gonadotropin-releasing hormone that stimulates the pituitary gland to release gonadotropin hormones follicle-stimulating hormone and luteinizing hormone (LH). Gonadotropin hormones then modulate the reproductive process through the production of sex hormones 17β-estradiol (E2) and testosterone (T) [170]. Sex determination is controlled by the molecular events associated with sex determining regions in the Y chromosome, leading to sexual plasticity and the proposal of polygenic sex determination mechanisms [171, 172]. Epigenetic regulation of the zebrafish reproductive system is still poorly understood; however, it is believed that epigenetic mechanisms play a role in sexual differentiation sensitivity to environmental cues, including temperature and oxygen saturation [173]. In female zebrafish, the hypomethylated *esr1* promoter results in higher expression levels in the liver when compared to males. *dnmt1, dnmt3* and *hdac1* were over-expressed in the zebrafish ovary when compared with the testis. Additionally, *dnmt1* and *dnmt3* transcription in the testis was strongly correlated with global DNA methylation [173]. To date, DNA methylation, histone modifications and ncRNAs have been credited with having the potential to regulate the expression of reproductive genes [174].

Insight about epigenetic regulation of the zebrafish reproductive system can be gleaned from toxicology studies investigating the reproductive effects of drugs that are classed as endocrine disruptor compounds (EDC). The treatment of zebrafish embryos with propiconazole, a triazole fungicide that reduces total cholesterol, lipoprotein lipase and fatty acid synthesis, was shown to disrupt fecundity and fertilization in zebrafish populations [175]. Reproductive ability regulated by the hypothallus-pituitary-gonad-liver was disrupted due to steroidogenic pathway inhibition and increased *dmnt1* and *dmnt3bb.2* in the female ovary [176]. In the testes, the EDC initiated a decrease in global DNA methylation that resulted in decreased fertilization [175]. In the hypothalamus-pituitary-thyroid axis of zebrafish, the follicle is the functional units of the teleost thyroid and is composed of endoderm-derived thyrocytes. The adult zebrafish thyroid consists of individual follicles of variable shape and diameter lying between the first gill arch and the bulbus arteriosus, along the ventral aorta [177]. These follicles produce thyroid hormones (THs) triiodothyronine (T3) and thyroxine (T4) [178]. Tris(1,3-dichloro-2-propyl) phosphate, a known Dnmt inhibitor, causes hypomethylation of CpG islands in intragenic regions, leading to TH dysregulation and reproduction defects [179].

In the liver of zebrafish, estrogens regulate the expression of vitellogenin (*vtg*) and zona radiata proteins (*zrp*), important proteins for oocyte development that are over-expressed in the female zebrafish [180]. Vitellogenin production is also regulated by the methylation levels of the 5′ flanking region of *vitellogenin I* in the liver [181]. Vitellogenesis, the production of yolky eggs in oviparous species, involves the transport of gene products
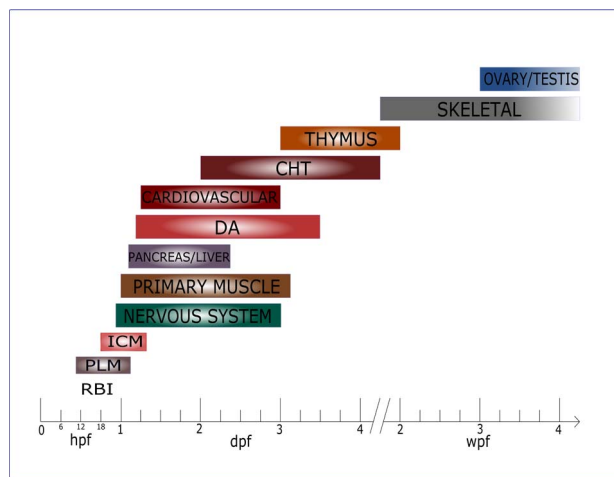
Figure 9. Timeline of key elements in zebrafish development from prehatching to adulthood. The period of organogenesis including the development of the liver, muscles, thymus, skeleton and ovaries/testes as well as major organ systems is included in order to development. Developmental stages are shown in hours postfertilization, day postfertilization and weeks postfertilization. Note that skeletal and ovary/testes development continues beyond the scope of this timeline.

from the liver to the ovary [181]. The process of oogenesis is then regulated by estrogen receptors (*esr1, esr2a*), steroidogenic acute regulatory protein (*star*) and progesterone receptors (*pgrmc1, pgrmc3*). Another important gene for oocyte maturation and germinal vesicle breakdown processes is LH/choriogonadotropin receptor *lhcgr*. The treatment of zebrafish with bisphenol A (BPA) down-regulated oocyte maturation-promoting signals [26]. BPA treatment resulted in reduced H3K27me3 in *fshr* and *star* regions and reduced H2K4me3 in *fshr* and *lhcgr* regions in the ovaries. There was also a decrease in H3K27me3 in the TSS region causing a decrease in *lhcrg* expression in ovaries [26]. Laing *et al*. [182] found that BPA treatment resulted in *dnmt1* disruption, leading to a significant reduction in DNA methylation in the testes and ovaries and to reduced reproductive ability. Bisphenol S (BPS) treatment increased 17β-estradiol in males and female zebrafish and decreased testosterone production in males [169]. Upregulation of ovarian aromatase (*cyp19a*) via decreased DNA methylation in the 5′ flanking region of the gene was also observed [183]. Conversely, elevated temperatures have been shown to increase *cyp19a1a* promoter DNA methylation and decrease gene expression, resulting in the masculinization of female zebrafish [171].

Evolutionary duplication of epigenetic regulators in development

It is important to point out the several duplication events that have occurred in the zebrafish genome throughout evolution. Over time, it has been suggested that 20% of duplicated gene pairs have remained within the genome and have diverged into their own function [184]. We conducted a reverse genetic screen with 425 human chromatin factors in zebrafish to identify epigenetic regulators involved in hematopoiesis [39]. Many chromatin factors identified in blood formation had duplicate gene pairs but were found to be tissue specific in relation to HSC development [39]. The mechanism underlying duplicate divergence is still poorly understood but studies have shown a link to DNA

methylation and epigenetic silencing being essential for proper function of duplicate genes.

DNA methylation has been recognized as indispensable for the maintenance and divergence of duplicate genes. Following the genome duplication event, *de novo dnmt3a* (*dnmt3aa* and *b*) and *dnmt3b* (*dnmt3ba, dnmt3bb.1, dnmt3bb.2* and *dnmt3bb.3*) arose in zebrafish which became a significant characteristic in the teleost pedigree [185, 186]. During embryogenesis, both *dnmt3a* and *dnmt3b* are vital for the rise of proper methylation patterns that become stable in cells once differentiated [107, 185]. Although *dnmt3aa* and *dnmt3ab* show highly conserved, similar ubiquitous expression patterns during zebrafish development, the *dnmt3b* genes are recognized as subfunctional, as they are distributed through specific tissue development [107, 185]. Although both homologs of *dnmt3* have similar sequences, a loss of *dnmt3bb.3* showed prominent developmental abnormalities specifically in the brain and retina [107, 120, 185]. In contrast, *dnmt3aa* and *dnmt3ab* were found to be expressed in all tissue types signifying a more conserved function for this gene and a more target-specific function for *dnmt3b* paralogues [185].

Moreover, *ezh1* gene arose from *ezh2* gene duplication. Although *ezh2* plays a role during zebrafish embryogenesis, *ezh1* has a more significant role further along in development [187–189]. At 24 hpf, *ezh1* is ubiquitously expressed but becomes more tissue specific around 48 hpf. qPCR conducted by Völkel *et al*. [187] showed that between 24 hpf and 5 dpf *ezh2* expression starts to decrease, whereas *ezh1* expression starts to rise. As this could be the result of subfunctionalization of *ezh1* over time, studies in mice and ESCs show that the PRC2-Ezh1 complex contributes to Ezh2 mutants which is why H3K27me2/3 and H3K27 methylation is not fully eradicated [188, 189] . Although PRC2-Ezh1 complex does not have a high HMT activity, a loss of function analysis of *ezh1,* during zebrafish embryogenesis, results in an increase of *ezh2* resulting in the lack of disruption in H3K27me2/3 levels [187–189]. This suggests a redundant function of *ezh1* during development with zygotic *ezh2* present. When *ezh2* is knockdown, maternal *ezh1* contributes to embryonic development in zebrafish but survival is only abundant until 14 dpf [187].

The function of duplicate genes has been widely studied in zebrafish and epigenetic silencing via methylation has shown to play a significant role in altering expression patterns throughout these events [190]. As several duplications have occurred throughout evolution, a study conducted by Zhong *et al*. [190] showed that promoter regions of newer duplicated genes were methylated, whereas older duplicates were unmethylated. This suggests epigenetic silencing during embryogenesis is essential for proper growth and against 'pseudogenization' [191]. Further exploration suggests that this silencing persuades duplicate genes toward contrasting functions [190, 191]. Mechanisms underlying the variance of duplicate genes are still largely unexplored.

Concluding remarks

The zebrafish is a wonderful model for understanding epigenetic regulation of development (Figure 9). DNA methylation, histone modifications and ncRNAs all contribute to ensuring proper formation of the mature zebrafish organism. In this review, we have highlighted many of the critical epigenetic marks and epigenetic regulators of zebrafish development. These processes facilitate regulation of gene expression at the transcriptional and translational levels. It is important to point out that this

regulation is established by proper spatial organization of chromosomes [192]. At the start of ZGA, the 3D organization of the genome is lost and proper zebrafish development relies heavily on histone modifications to keenly repress and active transcription [192, 193]. Chromosome begins to form compartments A and B that separate active and inactive chromatin, further subdivide into topologically associating domains [192, 193]. Studies in other organisms showed the disruption of these compartments resulted in increased oncogene activation, misexpression of transcription factors, and developmental disorders suggesting a strong link between 3D chromatin organization and gene expression [192, 194–196].

Although there are several systems where much remain to be uncovered, particularly with epigenetic regulation of the lymphatic and reproductive systems. The field is rapidly changing, and the improvements in resolution for epigenetic tools like chromatin immunoprecipitation-sequencing, assay for transposase accessible chromatin-sequencing, cleavage under targets and release using nuclease and Hi-capture sequencing (Hi-C) will continue to advance our understanding of the importance of epigenetic regulation of zebrafish development [197–200]. These advances not only enhance our understanding of zebrafish developmental biology, but also influence our knowledge of higher-level organismal development, including humans.

Key points

- Numerous epigenetic and genetic factors function together to regulate stem cell maintenance and differentiation.
- All terminally differentiated cells originate from a pool of specific precursors regulated by DNA methylation and histone modifications.
- Hdac1 and DNA methyltransferases are major epigenetic regulators of several differentiation pathways required for organogenesis

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