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Epigenetic Regulation in Neural Crest Development

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Abstract

The neural crest is a migratory and multipotent cell population that plays a crucial many aspects of embryonic development. In all vertebrate embryos, these cells emerge from the dorsal neural tube then migrate long distances to different regions of the body, where they contribute to formation of many cell types and structures. These include much of the peripheral nervous system, craniofacial skeleton, smooth muscle, and pigmentation of the skin. The best-studied regulatory events guiding neural crest development are mediated by transcription factors and signaling molecules. In recent years, however, growing evidence supports an important role for epigenetic regulation as an additional mechanism for controlling the timing and level of gene expression at different stages of neural crest development. Here, we summarize the process of neural crest formation, with focus on the role of epigenetic regulation in neural crest specification, migration, and differentiation as well as in neural crest related birth defects and diseases.

Keywords

Neural Crest; Epigenetic; Development

Introduction

Neural crest cells are a population of multipotent stem/progenitor cells that are induced during gastrulation at the neural plate border, between the neural and non-neural ectoderm. By neurulation, definitive neural crest cells are specified as premigratory cells within the dorsal neural tube and initiate expression of typical neural crest markers like *FoxD3* and *Sox10*. They then emerge from the neural tube by undergoing an epithelial to mesenchymal transition (EMT) whereby they delaminate from the neuroepithelium, assume a mesenchymal morphology and migrate extensively to different parts of the body. After

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migration, they differentiate into numerous derivatives including neurons and glia of the peripheral nervous system, melanocytes, portions of the cardiac outflow tract, craniofacial bone and cartilage, and smooth muscle of major blood vessels (Bronner and LaBonne, 2012; Sauka-Spengler and Bronner-Fraser, 2008; Sauka-Spengler and Bronner, 2010). Understanding neural crest development is important because these cells are involved in a variety of birth defects, diseases and cancers, including cleft lip and palate, heart defects, Hirschprung's disease, melanoma and neurofibromatosis.

There is good evidence that transcriptional events are critical for many aspects of neural crest development. A neural crest gene regulatory network (GRN) (Meulemans and Bronner-Fraser, 2004) comprised of transcriptional and signaling events has been proposed to function in a feed-forward series of regulatory circuits (Betancur et al., 2010; Sauka-Spengler and Bronner-Fraser, 2006). This neural crest GRN appears to be highly conserved throughout vertebrates, including basal agnathans (Sauka-Spengler et al., 2007), suggesting that these regulatory mechanisms were in place before the divergence of jawed and jawless vertebrates, likely to the base of the origin of vertebrates at 550 million years ago.

In addition to transcriptional regulation, there is growing evidence to support roles for epigenetic regulation as critical for many aspects of neural crest development, most notably in controlling the timing of gene expression at different developmental stages. Here we discuss the critical role of epigenetic regulation during neural crest development and disease and some examples of how it impinges upon the neural crest GRN.

Overview of epigenetic regulation

Epigenetic modifications are defined as mechanisms that regulate gene expression without altering the underlying sequence of DNA (Bernstein et al., 2007). However, recent changes in the usage of the term have led to the suggestion that the requirement of heritability be dropped and that epigenetic events might better be defined as "the structural adaptation of chromosomal regions so as to register, signal or perpetuate altered activity states" (Bird, 2007).

Epigenetic modifiers can alter chromatin structure and genome function through different processes such as DNA modifications, histone modifications and variants, or can work as a complex to regulate higher-level chromatin conformation in an ATP-dependent manner. Depending on the specific type of regulator, the outcome can either lead to gene activation, in which the chromatin is relaxed and DNA is accessible to transcription factors, or to gene repression, where chromatin is tightly packed and inaccessible to transcriptional regulators.

Epigenetic modifiers, including "writers" and "erasers" that establish the epigenetic code (Fig. 1), are key regulators of developmental events and also aberrant marks associated with many types of cancers, and disease states (Portela and Esteller, 2010). Here, we focus on various epigenetic regulators that have been shown to play a role in neural crest development and neural crest related diseases (Table 1). The epigenetic machinery falls into the following groups: DNA methylation, histone methylation, histone acetylation, Polycomb repressive complex, ATP-dependent chromatin remodeling complex, and other regulators that work with the epigenetic machinery to regulate neural crest development. Different

types of DNA and histone modifications and family members of chromatin remodeling complexes have been reviewed recently in (Liu and Xiao, 2011).

Neural crest induction and specification

The process of neural crest formation is initiated by signaling events, mediated by ligands including BMPs, WNTs and FGFs that are secreted from neighboring tissues such as the neural and non-neural ectoderm as well as the underlying mesoderm. During gastrulation, these signals establish the neural plate border region and initiate neural crest induction (Basch and Bronner-Fraser, 2006; Heeg-Truesdell and LaBonne, 2004; Steventon et al., 2005; Stuhlmiller and Garcia-Castro, 2012). The neural plate border region has the competence to form not only neural crest cells but also other cell types such as placode cells and some central nervous system (CNS) cells. Signaling inputs in this region up-regulate a group of transcription factors called 'neural plate border specifier genes' including *Msx1/2*, *Pax3/7*, *Dlx5*, *AP2A*, *Gbx2* and *Zic1*. The collective and overlapping expression of these genes confers upon the neural plate border region the unique ability to form neural crest cells. However, among all the neural plate border specifiers, the *Pax3/7* genes, when combined to *Zic1*, are sufficient to activate a bona fide neural crest specification program (Basch et al., 2006; Hong and Saint-Jeannet, 2007; Milet et al., 2013; Monsoro-Burq et al., 2005; Sato et al., 2005).

During neurulation, neural plate border circuitry activates a set of transcription factors called the 'neural crest specifier genes' in the dorsal neural tube. These include genes like *AP2*, *n-Myc*, *Id*, *Snail2*, *FoxD3*, *Ets-1*, *Sox8/9/10*, with some differences in the timing of their initial expression (Khudyakov and Bronner-Fraser, 2009). These factors function to maintain multipotency, promote their epithelial-to-mesenchymal transition (EMT), initiate delamination and migration, while also affecting cell proliferation and survival. A bona fide neural crest cell is first recognizable by the expression of transcription factors such as *FoxD3*, *Sox9*, *Snail2*, and *Sox10*, which are expressed in the dorsal neural tube and/or newly delaminated neural crest cells, depending upon the species. These genes regulate downstream effector genes to promote EMT and migration, at which point the neural crest cells become an identifiable population of multipotent- migratory stem-like cells (Barembaum and Bronner-Fraser, 2005; Gammill and Bronner-Fraser, 2003; Sauka-Spengler and Bronner-Fraser, 2008).

Neural crest EMT and migration

During the epithelial to mesenchymal transition process, neural crest cells alter cell junctions, adhesive properties and morphology to acquire cell motility, which enables them to migrate long distances to their final destinations. For example, they switch from expression of cadherins characteristic of epithelial cells to cadherins of more mesenchymal character and lose tight junctions while establishing gap junctions. Neural crest specifier genes like *Snail2* and *FoxD3* regulate downstream genes to facilitate this process. As a result, N-Cad and Cad6B are down-regulated and Cad7 is up-regulated, along with an N-cad to E-cad switch and modulation of gap junction proteins and integrins (Kerosuo and Bronner-Fraser 2012; Strobl-Mazzulla and Bronner 2012a; Rogers et al., 2013). At this

point, neural crest cells become a distinct group of mesenchymal cells that delaminate from the neuroepithelium and migrate out of the dorsal neural tube.

During their migration, neural crest cells interact with each other and with their environment via signaling receptors such as Neuropilins, Robo and Eph receptors, which respond to ligands that guide them to specific destinations or restrict them from certain territories (Betancur et al., 2010; Sauka-Spengler and Bronner-Fraser, 2008).

At the end of migration, the expression of most neural crest specifier genes is down-regulated. However, expression of some factors like *Sox10* and *FoxD3* remain on in a subset of cells and contribute to terminal differentiation (Kelsh, 2006). Depending on the axial location and time of emigration, neural crest cells give rise to a wide variety of derivatives such as peripheral neurons and glia, craniofacial skeleton, cartilage derivatives, and melanocytes. (Betancur et al., 2010; Le Douarin, 1982).

DNA methylation in the neural crest

Methylation of the fifth position of cytosine (5-methylcytosine, 5mC) is a highly conserved epigenetic modification of DNA found in most plant and animal models (Law and Jacobsen, 2010) and has a profound impact on genome stability and gene expression (Jaenisch and Bird, 2003; Smith and Meissner, 2013). During development, epigenetic repression, via DNA methylation, is one of the most common ways to shut down alternative pathways during cell type specification and lineage commitment (Cedar and Bergman, 2008). DNA methylation also has been implicated in genome imprinting and inactivation of the silent X chromosome (Ooi et al., 2009).

Although the DNA methylation pattern in somatic cells is stably maintained, genome-wide DNA methylation is erased at specific developmental stages such as in preimplantation embryos (Mayer et al., 2000; Oswald et al., 2000; Sasaki and Matsui, 2008). Global DNA demethylation is important for reprogramming cells in early embryos to enable reacquisition of pluripotency (Mayer et al., 2000). However, a unifying mechanistic understanding of active DNA demethylation has only been realized recently. After fertilization, the repressive parental 5mC marks are erased by iterative oxidation by TET proteins to generate oxidized cytosine bases known as 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC) and 5-carboxycytosine (5caC) (Kriaucionis and Heintz, 2009; Tahiliani et al., 2009). As development proceeds, new DNA methylation marks are established to gradually restrict the cell's potential (Borgel et al., 2010; Mayer et al., 2000; Reik, 2007). In mammalian cells, 60–90% of the cytosine residues are methylated in the context of CpG dinucleotides (Gardiner-Garden and Frommer, 1987; Namihira et al., 2004). However, recent work suggests that non-CpG methylation is relatively abundant in oocytes, pluripotent embryonic stem cells and mature neurons (Lister et al., 2013; Lister et al., 2009; Shirane et al., 2013; Xie et al., 2012); to date, the function of mammalian non-CpG methylation remains unclear.

This process is mediated by the family of DNA methyltransferases: DNMT1, DNMT3A and 3B. These enzymes catalyze the transfer of a methyl group on cytosine using S-adenosyl-L-methionine as a donor (Turek-Plewa and Jagodzinski, 2005), thereby inhibiting interaction

of some proteins with DNA, while facilitating the binding of others (Jaenisch and Bird, 2003; Smith and Meissner, 2013).

According to their function, the DNMTs can be categorized into two main groups with DNMT1 representing the maintenance methyltransferase and DNMT3A and 3B acting as *de novo* methyltransferases. DNMT1 preferably attacks the newly synthesized strand of DNA during chromatin replication, repairing and maintaining the pattern of methylation according to the parent strand (Chen and Li, 2006). The *de novo* methyltransferases (DNMT3A and 3B) are the major players in tissue specific regulation during development, establishing the initial CpG methylation pattern. Their activities can be modulated by a catalytically inactive family member, DNMT3L (Jurkowska et al., 2011; Siddique et al., 2012; Wienholz et al., 2010).

The de novo DNMTs recognize CpGs and newly methylate DNA by transferring a methyl group to cytosine residues (Cheng and Blumenthal, 2008). Such methylation of CpG sites, generally located at the promoter region of a gene, is thought to inhibit gene expression, as shown in cancer and stem cells (Altun et al., 2010; Miranda and Jones, 2007; Momparler and Bovenzi, 2000; Suzuki and Bird, 2008). DNMT3A and its paralog DNMT3B have been shown to be vital for normal mammalian development and play important roles in disease (Ehrlich et al., 2008; Jaenisch and Bird, 2003; Linhart et al., 2007; Yan et al., 2011). For example, *Dnmt3A* homozygous knockout mice die several weeks after birth, and *Dntm3B* homozygous knockout embryos have rostral neural tube defects and growth impairment (Okano et al., 1999), suggesting a very important role of these proteins during development.

In the chick embryo, *Dnmt3A* is predominantly expressed in the neural crest territory and its loss of function results in down regulation/loss of neural crest specifier genes *Sox10, Snail2, FoxD3,* and the expansion of neural genes *Sox2* and *Sox3* into the neural crest territory (Hu et al., 2012). Intriguingly, *Dnmt3A* plays an early function in repressing the neural genes *Sox2* and *Sox3* in the presumptive neural crest region, and this down-regulation of neural genes in the dorsal neural fold is a prerequisite to activate neural crest specifier genes (Hu et al., 2012).

Mutations in human *Dnmt3B* are found in ICF (immunodeficiency-centromeric instability-facial anomalies) syndrome, comprised of facial abnormalities such as widened nasal bridge and hypotelorism, neurological dysfunction and other related defects (Ehrlich et al., 2008; Jin et al., 2008). These defects are consistent with an important role for DNMT3B in neural crest development. In zebrafish, DNMT3B and histone methyltransferase G9a cooperate to regulate neurogenesis through Lef1 and play a critical role in forming the precursors of craniofacial structures, brain and retina (Rai et al., 2010). In human embryonic stem cells, knockdown of *Dnmt3B* accelerates neural and neural crest differentiation and increases the expression of neural crest specifier genes (*Pax3, Pax7, FoxD3, Sox10* and *Snail2*) (Martins-Taylor et al., 2012).

Dnmt3B expression is significantly up-regulated during neural crest induction in chicken embryos (Adams et al., 2008). In contrast to the human syndrome and stem cell studies, however, conditional knock-down of *Dnmt3B* in the mouse neural crest using Wnt1- or

Sox10-cre does not produce an apparent craniofacial phenotype (Jacques-Fricke et al., 2012). Wnt-1-Cre driven knock-out of DNMT3B exhibits only mild migration defects of dispersed Sox10 positive cells, that recover during cranial gangliogenesis (Jacques-Fricke et al., 2012). One possibility for the difference between these findings in different species is that the neural crest defect obtained in *Dnmt3B* mutant mice may be due to a requirement for this protein earlier during neural crest development. Alternatively, this could reflect differences between species, or may indicate that the primary function of DNMT3B is in non-neural crest tissue.

Histone modifications

Histone proteins associate with compacting DNA strands and organize them into structural components called nucleosomes. Each nucleosome contains eight histones: two of each of the core histones H2A, H2B, H3 and H4 form octameric structures called nucleosome cores around which DNA is wrapped with unstructured tails (Gibney and Nolan, 2010). The core histone proteins are highly conserved throughout evolution and their tails are subject to post-translational modifications such as methylation, acetylation, deacetylation, phosphorylation, ubiquitination, and sumoylation (Berger, 2007; Kouzarides, 2007), with modification on histone H3 being one of the best described to date (Fig. 1). New histone marks and new types of histone modifications continue to be discovered (Tan et al., 2011). Among these modifications, histone methylation and acetylation are currently the best studied in neural crest cells and play an essential role in neural crest development.

Histone methylation is associated with both active and repressive transcription (Kouzarides, 2007). Histone methyltransferases add methylation marks whereas histone demethylases remove them. H3K4me3 (Histone 3 Lysine 4 trimethylation) established by Trithorax group proteins is indicative of transcriptionally permissive chromatin states, and is mostly found in the promoter regions of genes (Akkers et al., 2009; Barski et al., 2007; Cheung et al., 2010; Pan et al., 2007). H3K36me3 is associated with euchromatic regions that are associated with active transcription and primarily found in gene bodies. In contrast, H3K27me3 catalyzed by Polycomb repressive complex (Schwartz et al., 2006; Simon and Kingston, 2009; Swigut and Wysocka, 2007; Tolhuis et al., 2006), and H3K9me3 catalyzed by G9a methyltransferase (Allan et al., 2012; Nielsen et al., 2001; Shi et al., 2003) are associated with transcriptional repression.

During neurulation, the H3K9me3 mark is abundant in dorsal compared with ventral neural tube cells, reflecting clear differences in the epigenetic background of neural crest versus neural progenitor cells (Fig. 2). Histone demethylases such as members of the Jumonji family can revert H3K9me3 and H3K36me3 (Tan et al., 2008). Accordingly, *JmjD2A* (also known as *Kdm4A*) is the first epigenetic gene discovered to regulate neural crest specification via modulating H3K9me3 of neural crest genes (Strobl-Mazzulla et al., 2010). *JmjD2A* is expressed in the neural crest forming territory during specification. Moreover, knocking down *JmjD2A* causes dramatic loss of neural crest specifier genes such as *Sox10, Snail2,* and *FoxD3. In vivo* ChIP assays reveal direct interaction of JmjD2A with *Sox10* and *Snail2* promoter regions that are occupied by H3K9me3 (Strobl-Mazzulla et al., 2010).

Thus, JmjD2A is required to demethylate histones occupying the promoters at the proper time and place to allow neural crest specification to occur.

Consistent with the importance of histone demethylases in development, patients with mutations in the histone demethylase PHF8, a JmjC domain containing protein, have craniofacial deformities. PHF8 is capable of demethylating the repressive marks H4K20me1 and H3K9me1 around the transcription start site to activate transcription. In zebrafish, PHF8 directly regulates homeodomain transcription factor *MsxB* during cranial facial development especially in the lower jaw (Phillips et al., 2006; Qi et al., 2010). This transcriptional regulator has been previously implicated in regulation of neural crest development in many vertebrate models (Maxson and Ishii, 2008; Monsoro-Burq et al., 2005; Phillips et al., 2006; Takahashi et al., 2001).

Histone acetylation is associated with active transcription and histone de-acetylation silences transcription (Jenuwein and Allis, 2001). HATs and HDACs are two classes of enzymes that antagonize each other (Shahbazian and Grunstein, 2007). HATs (histone acetyltransferases) transfer acetyl groups to lysines, and their binding is correlated with active transcription (Carrozza et al., 2003; Shahbazian and Grunstein, 2007). Acetylation neutralizes the charge of lysine residues and weakens their interactions with negatively charged DNA, allowing the chromatin structure to open up, thus increasing accessibility to transcription factors (Ekwall, 2005; Wang et al., 2009). HATs have also been identified as co-transcriptional activators (Roth et al., 2001; Yang, 2004). In contrast, HDACs (histone deacetylases) de-acetylate lysine residues and one of their major functions is to remove acetyl groups added by HATs (Wang et al., 2002). As a result, chromatin is reset to its tightly packed state (Hsieh et al., 2004). As a consequence, HDACs have been identified as transcriptional co-repressors (Kadosh and Struhl, 1997; Rundlett et al., 1998).

Epigenetic annotation such as histone acetylation is closely associated with enhancer activity and is a new, powerful tool to identify neural crest cis-regulatory regions together with conserved regulatory regions. For example, H3K27ac is associated with active enhancers (Bonn et al., 2012; Cotney et al., 2012; Creyghton et al., 2010; Heintzman et al., 2009; Rada-Iglesias et al., 2011). In human neural crest cells derived from hESC, a tour de force ChIP-seq study using antibodies to several histone marks reveals that active enhancer regions are enriched with H3K27ac and H3K4me1 while lacking H3K4me3 (Rada-Iglesias et al., 2012). Neural crest enhancer elements that are conserved between human and chicken are both enriched for H3K27ac. Moreover, the finding that a TFAP2A, together with nuclear receptors NR2F1 and NR2F2, leads to the establishment of a transcriptionally permissive enhancer chromatin states, opens the possibility to identify new important genes involved in neural crest development (Rada-Iglesias et al., 2011). Many have now been identified as a result of these studies and it will be interesting to monitor their functional characterization.

In neural crest development, the HDAC inhibitor Trichostatin A (TSA) promotes trunk neural crest cell specification (Murko et al., 2013). In chick, *in ovo* treatment with the inhibitor TSA induces neural crest markers *Bmp4*, *Pax3*, *Sox9* and *Sox10*, and dysregulates the proper timing of expression of cadherins, such as *Cad6B* and *N-cad*, resulting in premature loss of epithelial characteristics.

The HDAC repression complex also plays an essential role in regulating neural crest migration. Premigratory neural crest cells from the dorsal neural tube undergo an epithelial to mesenchymal transition to gain migratory properties and travel to distant parts of the body. The transcriptional repressor Snail2 has been reported to directly repress transcription of the adhesion molecule *Cad6B* in premigratory neural crest cells (Hatta et al., 1987; Nakagawa and Takeichi, 1995; Taneyhill et al., 2007). Epigenetic regulation has been shown to play a critical underlying molecular role in this repression (Strobl-Mazzulla and Bronner, 2012). Interaction between an adaptor protein, PHD12, and Snail2 recruits the repressive complex Sin3A/HDAC to the *Cad6B* promoter region. As a result, *Cad6B* transcription is repressed via histone deacetylation. Thus, the dual coordination between epigenetic regulators, such as PHD12, and transcription factors, such as Snail2, is required to cooperatively regulate the process of neural crest EMT (Strobl-Mazzulla and Bronner, 2012).

Later in development, HDACs play important roles in regulating downstream neural crest differentiation. HDAC1 has distinct spatial and temporal functions in neural crest-derived cells in zebrafish as it is involved in both melanophore specification and craniofacial cartilage development. HDAC1 mutant zebrafish embryos exhibit a severe reduction in the number of melanoblasts expressing MITFa, a critical transcription factor for melanoblast development, and retain prolonged *FoxD3* expression in neural crest cells. FoxD3 physically interacts with the MITFa promoter and reducing *FoxD3* expression in HDAC1 mutants partially rescues the melanoblast defects. Thus, during normal melanogenesis, HDAC1 is required to repress *FoxD3* expression that in turn de-represses MITFa to allow melanophore specification, migration and differentiation (Ignatius et al., 2008). In addition, HDAC1 is also involved in neural crest derived craniofacial and peripheral neuron development (Ignatius et al., 2013). Craniofacial cartilage defects are observed in mutant HDAC1 zebrafish in which fewer branchial arch precursors marked by *hoxb3a*, *dlx2*, and *dlx3* are specified and chondrocyte precursors fail to differentiate. The differentiation of enteric and dorsal root ganglion neurons in the posterior gut and tail are also disrupted in these mutant embryos. Interestingly, sympathetic neurons precursors can successfully undergo generic neuronal differentiation but fail to become noradrenergic (Ignatius et al., 2013). Overall, HDAC1 is required for distinct developmental processes and its activity is present in a broad range of cell types during neural crest derived differentiation.

In zebrafish craniofacial morphogenesis, embryos treated with HDAC4 morpholino exhibit loss of cranial neural crest derived palatal skeletal precursor cells and this later results in defects in the developing palate including cleft plate and a shortened face (DeLaurier et al., 2012). In human development, HDAC4 is also highly associated with neural crest related diseases and syndromes. Haploinsufficiency of HDAC4 is associated with brachydactyly mental retardation syndrome with features such as craniofacial and skeletal abnormalities (Williams et al., 2010). In addition, high throughput SNP analysis has linked HDAC4 with nonsyndromic oral clefts, a common birth defect closely related to neural crest development (Park et al., 2006). Moreover, infants exposed to valproic acid (VPA, an HDAC inhibitor), an anticonvulsant and mood-stabilizing drug, during pregnancy have an increased risk of

neural crest and neural tube related malformations including cleft lip and palate, and cardiovascular defects (Alsdorf and Wyszynski, 2005; Wyszynski et al., 2005).

Other HDACs important for neural crest development include HDAC3 and 8. HDAC3 is crucial for the regulation of smooth muscle differentiation and cardiac outflow tract formation during cardiac neural crest development in mouse (Singh et al., 2011). Similarly, conditional deletion of HDAC8, driven by Wnt1-Cre in mice, results in loss of specific cranial skeletal elements. HDAC8 epigenetically controls skull morphogenesis in neural crest-derived cells by repressing homeobox transcription factors *Otx2* and *Lhx1* (Haberland et al., 2009).

Although histone deacetylation was originally thought to function by silencing genes via local compaction of the chromatin structure, it is now clear that HDACs also can activate or maintain the active state (Wang et al., 2009) by acting in concert with HATs. Therefore, the level of histone acetylation and the presence of HDACs at a specific gene locus do not necessarily correlate with the activity status of the gene. For example, a recent study in mice has demonstrated that both HDAC1 and HDAC2 direct the specification of neural crest cells into peripheral glia (Jacob et al., 2014) by binding to the promoter region of the transcription factor Pax3 and activating its expression. In turn, Pax3 is required to maintain *Sox10* expression levels necessary to trigger expression of the fatty acid binding protein 7 (Fabp7), one of the early determinants necessary for the neural crest differentiation into Schwann cell precursors and satellite glia. Moreover, HDAC1/2 also bind to and activate the promoter region of myelin protein P0, necessary for the peripheral glial differentiation. Consistent with these observations, deletion of HDAC1/2 in mouse neural crest cells leads to depletion of satellite glia and Schwann cell precursors in dorsal root ganglia and peripheral nerves.

In summary, HDACs execute important functions in the control of both enhancer activity and promoter regions of transcription factors to regulate gene expression. Their activity affects numerous aspects of neural crest development ranging from specification to migration and differentiation.

Polycomb repressive complex

The polycomb repressive complexes (PRC) epigenetically silence the transcription of their target g dependent manner. PRC1 and PRC2 are both involved in the differentiation of neural crest-derived craniofacial structures. During chondrogenesis and skeleton formation, Hox genes are normally turned off in the cranial neural crest. EZH2, enhancer of zeste homolog 2, is one of the four core subunits of PRC2. Conditional knockout of *Ezh2* in pre-migratory neural crest cells in mice leads to de-repression of *Hox* genes in cranial neural crest cells, which in turn suppresses osteochondrogeneis and prevents craniofacial cartilage and bone formation (Schwarz et al., 2014). Similarly, Ring1b/Rnf2 (the single E3 ubiquitin ligase) in the PRC1 complex regulates cranial neural crest differentiation into chondrocytes (van der Velden et al., 2013). Zebrafish *Ring1b* mutants lack cranial cartilage, bone and musculature due to the inability of cartilage precursors to differentiate into chondrocytes. Interestingly, H3K27me3 and PRC proteins are reduced at the promoter regions of neural

crest specifier genes upon knock-down of DNMT3B in hESCs (Martins-Taylor et al., 2012) suggesting cross-regulation between these epigenetic regulators.

In addition to cartilage differentiation, defects in PRC genes are associated with neural crest related diseases and syndromes. *Aebp2*, a component of the PRC2, is expressed in the neural crest territory (Kim et al., 2009). Heterozygous mouse mutants of *Aebp2* gene present phenotypes similar to human patients with Hirschsprung's disease and Waardenburg syndrome. Both disorders are caused by migratory and developmental defects in neural crest cells (Ahola et al., 2009; Inoue et al., 2002; Kim et al., 2011). Expression levels of key neural crest genes are affected in *Aebp2* heterozyous mutants. In particular, *Sox10* is consistently down-regulated, similar to the reduced SOX10 dosage frequently observed on Waardenburg syndrome type 4 human desease. It is possible, that *Aebp2* misregulation is responsible for Hirschsprung's disease and Waardenburg syndrome via improper epigenetic regulation of the neural crest genes (Kim et al., 2011).

ATP-dependent chromatin remodelers

The ATP-dependent chromatin remodeling complexes such as SWI/SNF, ISWI, and CHD regulate gene expression by changing the position or structure of higher order chromatin in an ATP-dependent manner. They create nucleosome-free regions to facilitate access of DNA to transcription factors and regulatory proteins (Kwon and Wagner, 2007; Wu et al., 2009). CHD7, an ATP-dependent chromatin domain helicase DNA-binding domain member, cooperates with PBAF (SWI/SNF chromatin remodeling complex (Muchardt and Yaniv, 2001)) to promote neural crest specification in hESC induced to become neural crest cells. CHD7 activates core neural crest transcriptional circuitry genes including *Sox9* and *Twist* through directly regulating their enhancer regions (Bajpai et al., 2010). Sixty-seven percent of patients with CHARGE syndrome (a rare genetic disorder) have CHD7 mutations (Zentner et al., 2010). CHD7 impairment in Xenopus embryos recapitulates major CHARGE syndrome features such as craniofacial malformations, peripheral nervous system abnormalities and heart defects (Bajpai et al., 2010). Consistent with this, CHD7 deficient mice exhibit craniofacial abnormalities (Bosman et al., 2005; Hurd et al., 2007; Layman et al., 2009). Taken together, these data suggest that CHARGE syndrome is a result of CHD7 malfunction in early neural crest development. In addition, in zebrafish, Brg1, a member of the SWI/SNF chromatin-remodeling complex, plays an important role in neural crest induction, possibly via regulating the promoter region of *Snail2* (Eroglu et al., 2006).

WSFT, Williams syndrome transcription factor, is a major subunit of two distinct ATP-dependent chromatin remodeling complexes: WINAC and WICH (Barnett and Krebs, 2011). It is one of the genes associated with Williams syndrome, a developmental disorder in which patients have defects in neural crest derived tissues. These include facial abnormalities, heart defects, and neural problems, among other abnormalities. In Xenopus embryos, WSFT is expressed in the migratory neural crest and branchial arches. Knockdown of WSFT perturbs *Snail* and *Snail2* expression in the branchial arches. There are severe defects in neural crest migration and maintenance, while neural crest induction is unaffected (Barnett et al., 2012). In mice, WSFT heterozygotes exhibit cardiovascular abnormalities that phenocopy Williams syndrome patients (Yoshimura et al., 2009). Taken together, these

data suggest that malfunction of WSFT during neural crest development is a major contributor to Williams syndrome.

Other epigenetic regulators

The reduced folate carrier (RFC) is a membrane-bound receptor involved in folate uptake by cells. Mice lacking RFC1 develop multiple defects including neural tube, craniofacial and heart abnormalities (Gelineau-van Waes et al., 2008). In Xenopus, XRFC is expressed exclusively in the neural crest domain and its morpholino-mediated knockdown down-regulates neural crest markers such as *Zic1, Snail2,* and *FoxD3* (Li et al., 2011). As a result, *Twist1* positive neural crest cells fail to migrate ventrally and embryos exhibit similar phenotypes to those observed in mice. In animal cap assays, knock-down of RFC reduces the levels of H3K4me1 and H3K4me3. Over expressing lysine transferase hMLL1 in XRFC MO treated embryos fully rescues *Zic1* and *FoxD3* expression and partially rescues *Snail2* and *Twist1* expression (Li et al., 2011). Taken together, these data suggest that an RFC mediated folate metabolic pathway controls neural crest development through epigenetic mechanisms.

Leo1 is a component of the Polymerase-Associated Factor (PAF1) complex associated with chromatin remodeling and gene regulation (He et al., 2004; Krogan et al., 2003; Simic et al., 2003). In zebrafish mutants with truncated Leo1 protein, there is reduced expression of *Crestin, Gch2,* and *Miftfa* in neural crest derived cells (Nguyen et al., 2010). As a consequence, mutants have phenotypes such as reduced numbers of melanocyte, craniofacial cartilage, and glial cells. It is interesting to speculate that Leo 1 may be essential for neural crest differentiation, possibly through epigenetic regulations.

Conclusions

Many of the most common human birth defects are related to abnormal neural crest development. Neural crest malformation can lead to craniofacial defects like cleft lip and palate, heart septation defects, and agangliogenesis of the colon (Jiang et al., 2006; Tennyson et al., 1986; Youn et al., 2003). In addition, neural crest cells are involved in a variety of diseases and syndromes such as Hirschsprung's disease (HSCR), Wardensburg syndrome (WS), CHARGE syndrome and Williams Syndrome (Ahola et al., 2009; Bajpai et al., 2010; Inoue et al., 2002; Kim et al., 2011; Yoshimura et al., 2009). Although some of these syndromes are based in transcriptional or metabolic events, others like CHARGE syndrome clearly involve epigenetic factors.

Thus, in addition to transcriptional regulators, epigenetic modifiers serve as important inputs that are crucial for proper neural crest development. At a given time point, epigenetic modifiers control aspects of neural crest development in a spatially and temporally specific manner. They are capable of communicating at both the promoter and enhancer regions to render DNA as accessible state for transcription, poise an enhancer region for future activation, or remove active marks to turn off transcription. Often, these regulators cross-talk and work together to achieve their goals. For example, DNA methyltransferases often work with histone methyltransferases to shut down genes and histone demethylases read and

remove repressive marks along with histone acetylases to activate transcription at the appropriate time. Each cell lineage is marked with different combinations of epigenetic modifications at a given developmental time point. Uncovering these marks in neural crest cells will be critical for understanding both neural crest development and related diseases.

Epigenetic machinery also works closely with transcription factors and lineage specific genes to achieve tissue specific regulation. Most of the interactions between epigenetic regulators and downstream transcriptional effectors reviewed here are correlative. Only a few relationships have been proven to be direct. Future work must dissect the molecular mechanisms underlying each predicted interaction and identify new genes and regulatory circuits acting during neural crest development. The tools to accomplish this goal--high throughput technologies such as Chip-seq and RNA-seq combined with in vivo perturbation analysis and genome editing—are now in place. It will be of great value to expand our understanding of events underlying neural crest formation and gene regulatory interactions at the mechanistic level, from both a transcriptional and epigenetic vantage point.

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Highlights

- Epigenetic contributions during neural crest development
- Role of Histone and DNA modifiers in developing nervous system
- Involvement of epigenetic modifications in neural crest related diseases

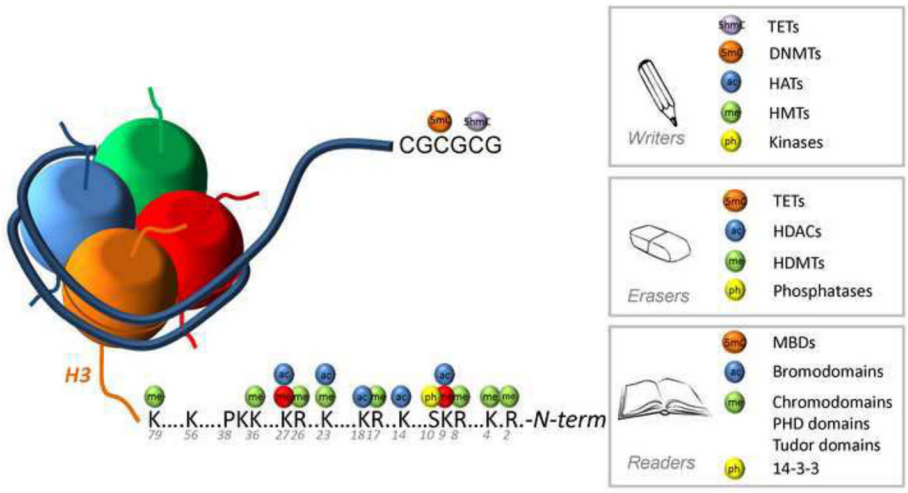

Figure 1.

Schematic diagram of the different epigenetic marks identified on histone H3 and DNA and their respective “writer”, “eraser” and “reader” proteins. Histone methylations on red and green are associated with transcriptional repression and activation, respectively. TETs, Ten-Eleven translocation enzymes; DNMTs, DNA methyltransferases; HATs, histone acetyltransferases; HDACs, histone deacetylases; HMTs, histone methyltransferases; and HDMTs, histone demethylases.

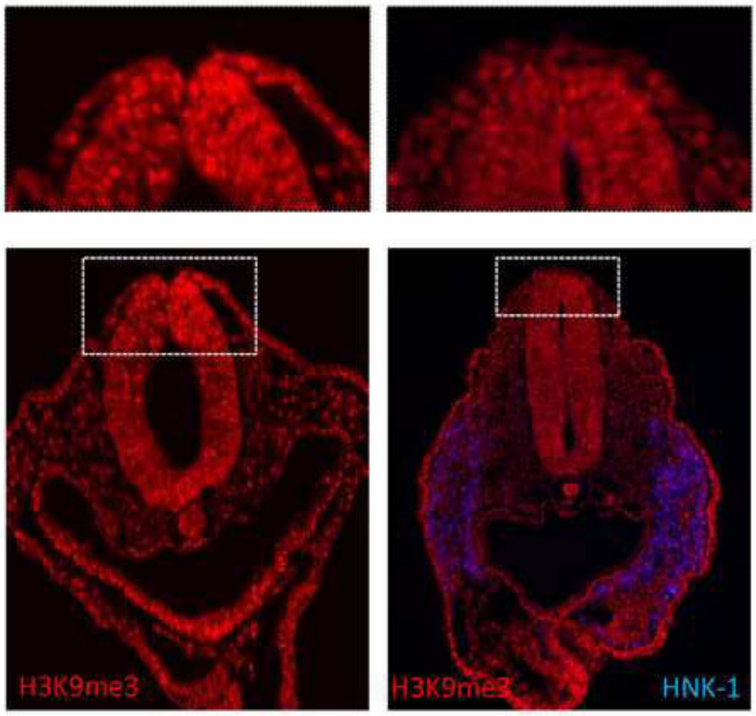

Figure 2.

Transverse section through a chick embryo stained with an antibody to the H3K9me3 mark (red) illustrates variation in the abundance of the mark between premigratory neural crest, at the dorsal aspect of the neural tube, and the ventral neural tube progenitors. After neural crest migration, evidenced by the HNK-1 marker (in blue), none of those highly abundant H3K9me3 positive cells are observed on the entire neural tube (unpublished data).

Table 1

Summary of epigenetic regulation in neural crest development

Summary of epigenetic regulations in neural crest specification, EMT, migration, differentiation, and neural crest related diseases and syndromes. ON/OFF column indicates whether the epigenetic regulator acts to turn transcription of its downstream target gene(s) on or off. Targets column represents direct and/or indirect downstream targets. Role in neural crest (NC) development column characterizes the specific timing during neural crest development, specific neural crest derived structures, and diseases/defects in which the regulator is known to play a role.

Stage of NC Development	Gene Name	Epigenetic Mark	On/ Off	Downstream Targets	Role in NC development	Citations
Neural Crest Specification						
	DNMT3A	DNA methylation targeting CpG island	OFF	Sox2/3, SoxE, Snail2, FoxD3	neural to neural crest transition	Hu et al. 2012
	JmjD2A	histone deacetylase targeting H3K9me3	ON	Sox10, Snail2	neural crest specification	Strobl-Mazzulla et al. 2010
	CHD7	ATP-dependent chromatin remodeler	ON	Sox9, Twist	neural crest specification	Eroglu et al. 2006
	Brg1	chromatin remodeler	ON	Snail2	neural crest induction	Eroglu et al. 2006
	TSA	HDAC inhibitor	ON	Bmp4, Pax3, Sox9, Sox10	trunk crest specification	Murko et al. 2013
Neural Crest EMT and migration						
	PHD12	histone deacetylase complex member	OFF	Cad6B	neural crest EMT	Strobl-Mazzulla and Marianne Bronner, 2012
	HDAC8		OFF		facial skeleton	
	RFC	co-factor of hMLL1 lysine methyltransferase targeting H3K4me	ON	Zic1, Snail2, FoxD3	dorsal to ventral migration	Li et al. 2011
Neural Crest Differentiation						
	PHF8	histone demethylase targeting H4K20me1 and H3K9me1	ON	MSXB	cranial facial jaw development	Phillips et al. 2006; Qi et al. 2010
	HDAC1	removes acetylation	OFF	FoxD3, MITFa, hoxb3a, dlx2, dlx3	melanoblast development; craniofacial and peripheral neuron development;	Ignatius et al. 2008; Ignatius et al., 2013.
	HDAC3	removes acetylation	OFF		smooth muscle and cardia outflow tract	Singh et al. 2011
	HDAC8	removes acetylation	OFF	Otx2, Lhx1	cranial differentiation into skull	Haberland et al. 2009

Stage of NC Development	Gene Name	Epigenetic Mark	On/ Off	Downstream Targets	Role in NC development	Citations
	DNMT3B	DNA methylation	OFF	Lef1	craniofacial structures, brain and retina development	Rai et al. 2010
	HDAC4	removes acetylation	OFF		palatal skeleton formation	DeLaurier et al. 2012
	G9a	histone methylation	OFF	Lef1	craniofacial structures, brain and retina development	Rai et al. 2010
	VPA	HDAC inhibitor	ON		neural tube defects, cleft lip and palate, and cardiovascular defects	Alsdorf and Wyszynski 2005; Wyszynski et al. 2005
	Ring1b/Rnf 2	polycomb repressive complex 1	OFF		craniofacial chondrocytes differentiation	Velden et al, 2013
	Ezh2	polycomb repressive complex 2	OFF	Hox genes	craniofacial skeleton formation	Schwarz et al., 2014
Syndromes and Diseases						
	WSTF	ATP-dependent chromatin remodeler	ON	Snail, Snail2	Williams Syndrome	Barnett et al. 2012, Yoshimura et al. 2009
	Aebp2	polycomb repression associated with H3K27me3	OFF	Sox10, Pax3, Snail2	Hirschsprung's disease and Waardenburg Syndrome	Kim et al. 2011
	CHD7	ATP-dependent chromatin remodeler	ON	Sox9, Twist	CHARGE sindrome	Eroglu et al. 2006
	HDAC4	removes acetylation	OFF		brachydactyly mental retardation syndrome, oral clefts	Williams et al. 2010; Park et al. 2006