Approaches for the study of epigenetic modifications in the inner ear and related tissues

Bradley J. Waters[1] and **Brandon C. Cox**[2]

[1]Departments of Neurobiology and Anatomical Sciences, and of Otolaryngology and Communicative Sciences, University of Mississippi Medical Center, Jackson, MS, 39216

[2]Departments of Pharmacology and Surgery, Division of Otolaryngology, Southern Illinois University School of Medicine, Springfield, IL 62711

Abstract

DNA methylation and histone modifications such as methylation, acetylation, and phosphorylation, are two types of epigenetic modifications that alter gene expression. These additions to DNA regulatory elements or to the tails of histones can be inherited or can also occur *de novo*. Since epigenetic modifications can have significant effects on various processes at both the cellular and organismal level, there has been a rapid increase in research on this topic throughout all fields of biology in recent years. However, epigenetic research is relativity new for the inner ear field, likely due to the limited number of cells present and their quiescent nature. Here, we provide an overview of methods used to detect DNA methylation and histone modifications with a focus on those that have been validated for use with limited cell numbers and a discussion of the strengths and limitations for each. We also provide examples for how these methods have been used to investigate the epigenetic landscape in the inner ear and related tissues.

Keywords

DNA methylation; histone methylation; histone acetylation; histone phosphorylation

Introduction

The term epigenetics refers to changes in gene expression which can be mitotically or meiotically heritable, but do not arise from changes to actual DNA sequences. Example mechanisms of epigenetic regulation include DNA modifications, chromatin structural dynamics, histone modifications, microRNAs, and non-coding RNAs. In addition to being heritable, epigenetic changes can occur *de novo* in response to environmental factors, intra-

and extra-cellular signaling, normal cellular processes such as development or aging, and pathological conditions. Epigenetic changes can have significant effects on numerous cellular and organismal processes including, but not limited to: transcription, replication, recombination, cell cycle entry and progression, stemness/multipotency, differentiation, damage repair, metabolism, survival, migration, morphology/polarity, and physiology (Chao and D'Amore, 2008; Feinberg, 2007; Greer and Shi, 2012). As such, there has been a dramatic increase in research focused on epigenetic modifications and epigenetic regulation of gene expression in all fields of biology.

The present review is focused on the most common DNA modification, methylation, and the best characterized histone modifications: acetylation, methylation, and phosphorylation. For DNA methylation, methyl groups are added by DNA methyltransferases (DNMTs), most commonly at cytosine residues that are followed by a guanine residue (or CpG dinucleotides). This results in decreased gene expression by either recruiting proteins that condense the chromatin or by preventing transcription factors from binding (Bird, 2002; Bird and Wolffe, 1999). DNA methylation is relatively stable compared to histone modifications and therefore more likely to be heritable. However DNA demethylation can occur passively by successive rounds of cell division in the absence of DNMTs, or actively by ten-eleven translocation (TET) enzymes (Kohli and Zhang, 2013).

Histone proteins, which form complexes that wind and unwind DNA into condensed or open states, can accept post-translational modifications on their N-terminal tails, resulting in either increased or decreased gene expression depending on the modification, its location, and the cellular context. While the majority of histone modification studies have focused on histone proteins H3 and H4, histone proteins H1, H2A, and H2B, as well as several of the known histone variants (e.g. H2AX), can also be modified. Among the better studied modifications are histone acetylation, methylation, and phosphorylation. Histone acetylation occurs when histone acetyltransferases (HATs) catalyze the transfer of an acetyl group from acetyl coenzyme A to conserved lysine residues. This removes lysine's positive charge, decreasing the affinity between DNA and histones and opening the chromatin. In general, this allows transcription factors and polymerases easier access to promoter regions to enhance gene expression. Histone deacetylates (HDACs) remove acetyl groups, which promotes chromatin condensation and generally decreases transcription (Eberharter and Becker, 2002).

Methylation of histones can occur at basic amino acid residues (lysine, arginine, and histidine). However, methylation of histidine appears to be rare and the methylation of arginine and lysine residues are therefore more commonly studied (Greer and Shi, 2012). To date, several methyltransferases have been characterized for their ability to methylate various residues on histone tails. These generally belong to the families of SET-domain containing proteins, DOT1-like proteins, and the protein arginine N-methyltransferases (PRMTs). The first two methylate lysine residues while the latter methylates arginines. Demethylation is also accomplished by a number of enzymes, some known and some unknown. While arginine demethylases are not well elucidated, a number of lysine demethylases that act on histone tails have been characterized. The majority of these belong to two families: the amine oxidases and the jumonji C (JmjC)-domain-containing

dioxygenases (Biswas and Rao, 2018; Greer and Shi, 2012). It is important to note that many of these enzymes are not specific to histones, but can methylate or demethylate other proteins as well (Carlson and Gozani, 2016) Thus, experiments that knockout or inhibit the activity of specific enzymes that alter the methylation status of histones may reveal pleiotropic effects.

Unlike histone acetylation, which has the general feature of accessible chromatin and increased transcriptional activity, histone methylation is more complex. The site of methylation, as well as the number of methyl groups added at an individual residue, and whether the methyl groups are symmetric or asymmetric, can have opposing effects on chromatin condensation and transcription. Perhaps the three most well-studied histone modifications are methylation of lysines 4, 9, and 27 on histone protein H3 (H3K4, H3K9, and H3K27, respectively). Trimethylation of H3K4 (H3K4me3) is generally associated with increased transcriptional activity, while H3K9me2, H3K9me3, and H3K27me3 are associated with transcriptional repression. While the simple example of these three methylation sites illustrates how the outcome is dependent upon which residue is methylated, it is also known that the number of methyl groups on an individual residue can have opposing effects on transcription. For example, while H3K9me2 and H3K9me3 are generally repressive, H3K9 monomethylation (H3K9me) is generally associated with open chromatin and active transcription (Barski et al., 2007). In addition, even some modifications thought to be acting in one way, can, in certain cases, act in the opposite manner. H3K4me3, which is generally associated with transcriptional activation, has been shown to repress transcription under certain circumstances (Shi et al., 2006). While the effects of histone methylations are admittedly complex, this complexity suggests that histone modifications may work in a coordinated fashion. The combination of methylations, acetylations, and/or other modifications at multiple sites, rather than just the methylation status of one specific residue, may be what determines the overall effect on chromatin structure and transcriptional activity (Bernstein et al., 2006).

Although acetylation and methylation are the most heavily studied histone modifications, there are a number of residues on histone proteins that can be phosphorylated, including serines, threonines, tyrosines, and histidines. While the list of kinases and phosphatases shown to phosphorylate or dephosphorylate histone residues is too long to describe here (see Rossetto et al., 2012), there are also many writer and eraser enzymes that have yet to be identified. Also, similar to what is seen with the enzymes that methylate and demethylate histone residues, the enzymes that affect histone phosphorylation are generally promiscuous and exert enzymatic activity on other proteins as well (Baek, 2011). While less is known about the effects of phosphorylation at many histone sites, certain phosphorylations have been associated with proliferation or cell death. Figure 1 summarizes the residues on histone proteins H1, H2A, H2AX,H2B, H3, and H4 that are known to be acetylated, methylated, or phosphorylated and, if known, the general effects of these modifications.

Despite the rapid pace of advancement in epigenetic studies across biology, epigenetic research in the inner ear is relatively new. The few epigenetic studies that have been conducted using cells from the inner ear or related tissues have shown that DNA methylation and histone modifications play critical roles in development, aging, hair cell (HC) survival,

response to insult, and HC regeneration (reviewed in Doetzlhofer and Avraham, 2017). However based on studies from other organs and cell types, it is likely that changes to the epigenetic landscape will affect nearly every cellular process and every cell type within the inner ear. Understanding these epigenetic changes will be critical to developing therapies to treat or prevent hearing loss. Yet, researchers studying the inner ear and associated tissues must face the added challenges of tissue inaccessibility, small cell populations, and heterogeneity of cell types. These can be significant obstacles as most of the techniques available for epigenetic research were developed using cell lines and are routinely carried out with millions, if not billions, of homogeneous cells in each sample. In stark contrast to this, the mouse cochlea, a common model for the study of audition, is encased in bone, contains less than 4,000 HCs on average (Ehret and Frankenreiter, 1977), and exhibits a high rate of cell death upon dissociation. Furthermore, mechanosensory organs like the organ of Corti are comprised of several different cell types with differing morphologies, functions, and transcriptomes. It is likely, therefore, that each cell type possesses unique epigenomic features as well. Thus, any attempts to investigate epigenetic modifications within or between subsets of cochlear cell types (e.g. HC vs. supporting cell (SC), or apical vs. basal turns of the cochlea) not only require cell sorting, but result in a further reduction in the number of viable cells that can be obtained for study. Furthermore, cells in the mammalian inner ear are largely quiescent which has required much additional effort to generate cell culture models (Chang et al., 2015; Koehler and Hashino, 2014; Walters et al., 2016). While some cell culture models have been developed, it is important to consider that they are still largely experimental and require primary tissue comparisons to be thoroughly vetted, especially for epigenetic modifications which can differ dramatically *in vitro* as compared to *in vivo* (Nestor et al., 2015). In recent years, however, technological advances have made it possible to examine epigenetic modifications from limited cell numbers, and in some cases within single cells. It is therefore now possible to investigate the epigenetic landscape in cells from *in vivo* inner ear tissues (or other related models) using a variety of different tools. Here, we provide an overview of several of these techniques, with a focus on those that have been validated for use with single cells or at least with limited cell numbers, and where applicable, provide examples for how these methods have been used by auditory and vestibular researchers. As one more point of caution, however, it is important to note that while these techniques have been demonstrated to work at the single cell level or with only hundreds or thousands of isolated cells in other systems, there are likely to be challenges in adopting some of these techniques to inner ear or related tissues. This is due not only to the small numbers of cells that can be isolated, but also the heterogeneity of the tissues and challenges faced in enriching for a single cell type. As there are stark differences in epigenetic features across different cell types, the importance of enriching for homogeneous cell populations cannot be understated. Data from many of these techniques with material isolated from heterogeneous populations of cells can be difficult to interpret as a method for assigning each detected modification to one cell type or another is currently lacking. Even homogeneous cell populations often do not reveal wholly conserved epigenetic features, but exhibit their own variability. Thus, it is critical for many of the experimental approaches outlined here that highly enriched cell populations be used, or, where possible, isolated single cells.

Methods for Detection of DNA Methylation

Perhaps the first technique to gain prominence for the investigation of epigenetic modifications is the use of sodium bisulfite to convert unmethylated cytosine residues at CpG dinucleotides to uracil residues (Frommer et al., 1992). Since methylated cytosines are protected from deamination by sodium bisulfite, direct comparison of bisulfite-treated and untreated DNA allows the localization of methylated cytosines. Depending on the scientific question and resources at hand, bisulfite treated DNA can be analyzed by PCR methods or by direct sequencing (Frommer et al., 1992; Li and Tollefsbol, 2011). PCR methods are generally useful when one gene or site within the genome, or at most a handful of sites, are of interest. Methylation-specific primers (MPs), designed to complement sites containing CpG dinucleotides, readily amplify methylated sites in bisulfite treated DNA (where CpG sequences are preserved), but are less efficient at amplifying these sites when they are unmethylated (i.e. unprotected from bisulfite and therefore converted into UpG sequences). Unmethylated-primers (UPs) are also generated and designed to cover the same sequences containing the CpG dinucleotides, but their sequences complement UpG residues at these sites. Thus comparisons using both sets of primers allows detection of cytosine methylation. For example, if the CpG sites are methylated, there should be robust amplification of bisulfite treated DNA with the MPs, but not with the UPs. If the site(s) of interest is unmethylated, then the UPs should give robust amplification and the MPs should be less efficient (Herman et al., 1996).

Nested methylation specific PCR (nested-MSP) is a modified version of this technique developed to detect methylation in small quantities of DNA (approximately 10ng). In nested-MSP, two rounds of PCR are used after bisulfite treatment. In the first round of PCR, primers that amplify independent of DNA methylation are designed to flank potential methylation sites in genes of interest. Unmethylated CpG residues will be converted to UpG residues by the bisulfite treatment and therefore amplified as TpG residues in the first round of PCR. These amplicons then undergo a second round of PCR with MPs and UPs that are nested within the first amplicon. Again there will be robust amplification of methylated CpGs with MPs, whereas the TpG residues generated from unmethylated CpGs will be amplified by the UPs (Scher et al., 2012).

Another PCR-based approach relies on thermal cycling coupled with a fluorometer to detect differences in melting temperature based on CpG vs. UpG content. For this method, primers are designed specifically to bracket, but not compliment, potential CpG sites that could be methylated. Following bisulfite treatment, methylated cytosine residues are amplified as such, whereas unmethylated cytosines, which were converted to uracils by the bisulfite treatment, are amplified as thymidines. The resulting amplicons will therefore have greater or lesser CpG content, respectively, which will result in different melting temperatures. The methylated samples with greater CpG content will melt at a higher temperature than the unmethylated samples with lesser CpG content (Guldberg et al., 2002). This type of analysis can be easily conducted using most commercially available qPCR cyclers and analysis software. Although we are not aware of any studies applying these techniques to single cells, amplification by PCR means that DNA methylation using bisulfite treatment can in theory

be applied to the small numbers of cells typically found in auditory and vestibular organs, and should only require similar amounts of input as in typical real-time qPCR experiments.

Though simple, cost effective, and easy to carry out in most labs, PCR analyses of bisulfite treated DNA have two primary limitations. First, as the output of these experiments is binary (presence or absence of a band after a certain number of cycles, or higher vs. lower melting temperature), it can be difficult to determine how many methyl groups are present if more than one CpG dinucleotide is contained within the sequence being interrogated. Second, these methods are not suited to interrogate large portions of the genome, but rather are best for examining only one or a few DNA sequences at a time. The former limitation can be overcome by sequencing the PCR amplicons obtained in an approach termed bisulfite sequencing PCR, which allows the methylation status of individual CpG dinucleotides to be obtained (Li and Tollefsbol, 2011). However, to query large portions of the genome, one can employ whole-genome bisulfite sequencing (WGBS) or Bisulfite sequencing (BS-seq) which can most simply be described as the application of high-throughput sequencing methods to bisulfite treated DNA. In this method, DNA is isolated from the cells of interest, treated with bisulfite, and then cleaved into readable fragments which are ligated to adapters and sequenced (Clark et al., 2017, 1994; Cokus et al., 2008; Eckhardt et al., 2006; Smallwood et al., 2014). In this way, the whole genome can be sequenced and compared to reference genomes, as well as to sequences from untreated DNA. This allows for detection of (theoretically) all of the methylated sites within a cell's genome. These methods are best performed in purified or homogeneous populations of cells as it is difficult to interpret or assign intermediate levels of methylation in heterogeneous populations.

Another approach that uses high-throughput sequencing to interrogate the whole genome for methylated cytosine residues is methylated DNA immunoprecipitation sequencing (MeDIP-seq) which relies on antibodies that recognize and bind specifically to methylated DNA. In this approach, DNA is extracted, fragmented, and immunoprecipitated using methylcytosine-specific antibodies. The antibodies are then removed by reverse crosslinking, the fragment ends are repaired and ligated to adapters, and then sequenced (Taiwo et al., 2012; Weber et al., 2005). If the starting quantity of DNA is a limiting factor (e.g. from a small number of cells obtained from a cochlea), MeDIP can also be followed by real-time qPCR for analysis to examine select genes rather than looking at the entire genome (Tsaliki et al., 2012).

Other options for interrogating global DNA methylation are amplification of intermethylated sites (AIMS) and comparative methylation hybridization (CMH), which rely on restriction endonucleases that are methylation sensitive (HpaII, SmaI) and insensitive (MspI, XmaI) to cut the DNA differentially. In AIMS, the digested DNA is ligated to an adapter, amplified by PCR, and fractionated on a gel for comparison between experimental and control samples (Frigola et al., 2002). While this approach generates a footprint for the overall methylation across the genome, it does not provide the locations or sequences of the methylated regions. However, partial sequences can be obtained by extracting bands of interest from the resulting gel and sequencing them. Also, while a methylated DNA footprint allows for quantification of the number of sites that are methylated, the frequency of methylation at a site across the cell population is difficult to quantitate or compare across groups with this

method. In contrast to AIMS, CMH uses microarrays or high-throughput sequencing to assess the methylation status at sites recognized by the endonucleases and therefore can provide quantification of frequency within a cell population, as well as location and sequence information for these sites across the genome (Nelson, 2008). It is important to keep in mind that all high-throughput sequencing based approaches do not guarantee 100% coverage of the genome, particularly in samples with low input (e.g. single cells or low cell number) or in heterogeneous samples (e.g. anything other than single cells or clonal cells, the latter of which may still be heterogeneous in terms of DNA methylation). In addition, sites that are not uniformly methylated across most or all of the cells may not rise above the signal-to-noise ratio to be detected. Also, endonuclease based methods are limited by the sequence specificity of the endonucleases, cutting only at CCGG and/or CCCGGG sequences, but ignoring CpG sites not flanked by other C's and G's.

High performance liquid chromatography (HPLC) or liquid chromatography coupled with mass spectrometry (LC-MS) are also sensitive methods for measuring DNA methylation and may also be used to interrogate small numbers of cells (Armstrong et al., 2011; Ehrich et al., 2005; Lin et al., 2016). While these methods can provide an assessment of the global level of methylation in cell populations, they do not easily allow for localization of the methylated sites or quantification at single-loci resolution. In addition, HPLC and mass spectrometry require specialized equipment and technical expertise that may limit accessibility. In contrast, PCR based methods are more economical and more likely available in most labs that study the inner ear and related tissues. Table 1 summarizes the utility and caveats for each of these methods used to investigate DNA methylation.

Analysis of DNA Methylation in the Inner Ear (and Related Tissues)

Bisulfite sequencing PCR was used on DNA from cochlear extracts to examine methylation in two regions of interest in the promoter of *Gap Junction Beta 2* or *GJB2* which encodes Connexin 26 (Lin et al., 2017; Wu et al., 2014). Connexin 26 is a component of the gap junction channels found in SCs of the sensory epithelium and in fibrocytes of the spiral ligament and spiral limbus. These channels recycle potassium ions from the base of HCs to the endolymph and play an important role in homeostasis (Kikuchi et al., 2000). Mutations in *GJB2* are found in up to 50% of patients with non-syndromic sensorineural hearing loss (Kenneson et al., 2002), making it the most common deafness-related gene. In a model of intrauterine hypoxia, Lin et al., (2017) used bisulfite sequencing PCR and found increased methylation of CpG sites in the *GJB2* promoter of cochlear cells from 8 week old rats that were exposed to hypoxic conditions for 14 days *in utero*. This was accompanied by a ~40% decrease in Connexin 26 mRNA and protein expression, as well as significant hearing loss. A second study used a model of age-related hearing loss where rats were chronically treated with D-galactose which causes mitochondrial damage and increased formation of reactive oxygen species in the cochlea (Chen et al., 2011; Zhong et al., 2011). These rats had hypermethylation of CpG sites in the *GJB2* promoter of cochlear cells at 10 months of age, as detected by bisulfite sequencing PCR, along with a 22–34% decrease in Connexin 26 mRNA and protein expression (Wu et al., 2014). Thus similar to congenital loss of function mutations in *GJB2*, hypermethylation of the *GJB2* promoter can also decrease expression of Connexin 26 and cause hearing loss. Of note, both studies used DNA isolated from the

whole cochlea and therefore it is unknown whether there are cell-type specific differences in the methylation of the *GJB2* promoter between SCs and fibrocytes or among SC subtypes. Since bisulfite sequencing PCR was used, these studies could only assess select DNA sequences (i.e., two sites within the *GJB2* promoter); therefore the methylation status of other genes in these models remains unknown.

A combination of nested-MSP and MeDIP-qPCR was used to investigate changes in the methylation of the promoter regions for three genes using a cell line created from mouse utricle sensory epithelial cells (MUCs) (Zhou and Hu, 2016). These cells expressed several HC-specific genes after treatment with 5-azacytidine, a compound which inhibits DNMT activity and therefore decreases DNA methylation (Chen et al., 2013). After bisulfite treatment, nested-MSP was performed on the promoter regions of *Atoh1*, *Pou4f3*, and *Cdh1* (which encode the proteins ATOH1, POU4F3, and E-cadherin, respectively) and the brightness of the bands resulting from the PCR reactions using MPs vs. UPs were compared. MUCs treated with 5-azacytidine had significantly decreased amounts of methylated DNA in the promoter regions of *Atoh1* and *Pou4f3*, with minimal change in the promoter of *Cdh1*. Using the more quantitative method of MeDIP-qPCR, the authors found a ~80% decrease in the methylation of *Atoh1* and *Pou4f3* promoters and no significant change in the methylation of the *Cdh1* promoter. Experiments run in parallel found increases in the mRNA for each of these genes (Zhou and Hu, 2016). Similar to bisulfite sequencing PCR, both nested-MSP and MeDIP-qPCR used in this study only allowed the analysis of selected genes of interest. If the authors had instead used MeDIP-seq, they could have identified nearly all of the genes in MUCs with methylation changes after 5-azacytidine treatment in an unbiased manner. However as with any method that includes sequencing, MeDIP-seq is significantly more costly than MeDIP-qPCR and can require isolation and purification of greater starting quantities of DNA.

WGBS was used to generate genome-wide maps of DNA methylation at the single nucleotide resolution during key stages of inner ear maturation. Yizhar-Barnea et al., (2018) isolated DNA from the sensory epithelium of the mouse cochlea at embryonic day (E) 16.5, postnatal day (P) 0, and P22 and subjected the samples to WGBS. Though the DNA came from both sensory and nonsensory cells in the organ of Corti, and therefore neither the methylation patterns nor their dynamics could be assigned to specific cell types, several findings still emerged from the data. First, significantly more DNA methylation changes (both methylation and demethylation) occurred between P0 and P22 when cochlear maturation occurs, compared to E16.5 to P0 when cells are differentiating. Second, while assaying DNA methylation is often considered a means for identifying genomic regions where transcription is repressed, identifying unmethylated or low-methylated regions can suggest putative promoters and enhancers. From the data obtained in this study, many such potential transcription factor binding sites were identified, and, when mapped against available transcriptomic data, potential regulation sites for several genes known to be important in cochlear development and function were confirmed. In addition, novel findings included the identification of the transcription factor *Bach2* and the HIF1 signaling pathway as potentially important factors in cochlear maturation. Finally, the authors used *in silica* tools to map the low methylated regions identified in the mouse genome to their orthologous sequence locations in the human genome. This mapping suggested numerous putative

enhancers or promoters of genes that may be involved in human deafness, and the authors validated one of these sites using CRISPR-on technology (Cheng et al., 2013) which revealed that a low methylated region located between the known deafness genes *GJB2* and *GJB6* did not regulate the expression of the more proximal *GJB2*, but did have a pronounced effect on activating transcription of the more distal *GJB6*. This study very nicely highlights the wealth of data that can be obtained using WGBS, and the hypotheses that can be generated from such data, such as putative enhancer and promoter sequences, putative candidate genes that are likely to be expressed or repressed at different developmental stages or under different treatment conditions, and transcription factors and signaling pathways that may play a role in the cells and conditions being investigated. However, it is critical to remember that the predictions generated by WGBS and subsequent *in silica* analyses must be validated due not only to the size of the datasets generated, but also because the predictions may be flawed, and because not all unmethylated DNA is actively transcribed.

Mutai et al., (2009) used a modified version of AIMS combined with bisulfite sequencing PCR to investigate DNA methylation changes that occur in the sensory epithelium of rat cochleae before and after hearing onset. Using AIMS, comparison of the PCR products between P1 and P14 samples revealed a band specific to the P1 samples that was observed in repeated experiments. After this fragment was purified, re-amplified, and sequenced, it was identified as a potential regulatory element for the transcription factor *Pou3f3*. Bisulfite sequencing qPCR was then used to assess the methylation status of the *Pou3f3* regulatory element at the two ages, where increased methylation was observed in the P14 samples along with a ~40% decrease in *Pou3f3* mRNA. The use of AIMS in this study allowed a non-biased approach to identify *Pou3f3* which had not previously been studied in the cochlea. The authors demonstrated that *Pou3f3* is expressed in SCs and mesenchymal cells of the spiral ligament, spiral limbus, and Reissner's membrane. Since no phenotype was observed in cochleae of *Pou3f3* knock mice and the cells expressing *Pou3f3* (i.e. cells of the greater epithelial ridge and mesenchyme) are known to decrease in number between P1 and P14, one caveat of this data which may affect its interpretation is that the P14 samples included more Pou3f3-negative cells than at P1. Since Pou3f3-negative cells are expected to have increased methylation of *Pou3f3* (because this gene is not expressed), the methylation status of *Pou3f3* in Pou3f3-positive cells at P14 remains unclear. This highlights one of the limitations of using heterogeneous tissue for epigenetic studies.

Two studies used bisulfite PCR followed by mass spectrometry to investigate changes in the methylation status of the regulatory elements for specific genes using inner ear tissue. Waldhaus et al., (2012) investigated methylation of promoter and enhancer regions of *Sox2* in otospheres, derived from the postnatal cochlea. *Sox2* is considered a pluripotency factor in embryonic and neural stem cells (Masui et al., 2007; Suh et al., 2007; Takahashi and Yamanaka, 2006). Yet in the inner ear it promotes the formation of prosensory progenitor cells (Kiernan et al., 2005) and neurons in the cochleovestibular ganglion (Steevens et al., 2017), and is required for the differentiation of HCs (Puligilla and Kelley, 2017) and SCs (Dabdoub et al., 2008) at later ages. Waldhaus et al., (2012) used a method called EpiTYPER where DNA is bisulfite treated, then amplified by PCR using primers that are amended with a T7 promoter. Following PCR, the reverse strands of the amplicons are transcribed using T7 polymerase and subsequently, the RNA molecules are cut into

fragments using RNaseA. Fragments derived from methylated CpG islands will retain CG dinucleotide sequences in their RNA, whereas unmethylated CpG islands will be transcribed as CA nucleotides which have a 16 Da lower mass than CG nucleotides. The RNA fragments are analyzed with matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry which, for each cohort of RNA fragments, will yield two peaks 16 Da apart. The ratio of the signal intensities of these two peaks provides the ratio of methylated to unmethylated CpGs. Data from such an EpiTYPER experiment showed that the *Sox2* promoter had similar levels of methylation in otospheres as in embryonic and neural stem cells, yet two enhancer regions, *SRR1* and *SRR2*, had increased methylation in the otospheres compared to the other cell types. Next methylation of the otic-specific enhancers, *NOP1* and *NOP2*, were examined in cells from E13.5, P4, and P21 cochleae where all four enhancer regions showed increased methylation with age. Interestingly when otospheres were grown under differentiating conditions or were treated with epidermal growth factor (EGF), the methylation of *NOP1* and *NOP2* was similar to P21 cochlear cells. In another study, Xia et al., (2015) used EpiTYPER to examine methylation of the *Notch1* promoter in cochleae from neonatal and adult mice. *Notch1* is a receptor that mediates Notch signaling, a pathway known to regulate HC differentiation and regeneration (Kelley, 2007; McGovern et al., 2018). Studies have shown that Notch signaling is downregulated in mouse cochleae during the first postnatal week through an unknown mechanism (Hartman et al., 2007; Maass et al., 2015; Murata et al., 2006). Xia et al., (2015) investigated methylation changes to the *Notch1* promoter as a possible explanation for its decreased expression. DNA extracted from the auditory epithelium of P0, P4, P8, and P16 mice had a progressive increase in methylation of the *Notch1* promoter with age, while *Notch1* mRNA expression decreased over the same time period. The same experiments were performed in adult mice where HCs were killed by the aminoglycoside kanamycin. There was no change in the methylation of the *Notch1* promoter in samples with HC damage compared to controls, yet there was a small increase in *Notch1* mRNA expression. The authors concluded that *Notch1* expression changes after HC damage are not regulated by DNA methylation. The combination of bisulfite PCR with mass spectrometry in these two examples allowed the measurement of methylation changes at specific DNA sequences which would not have been possible if mass spectrometry had been used alone. However mass spectrometry alone could have provided an assessment of global methylation changes across the genome.

Methods for Detection of Histone Modifications

Several methods have been developed for the study of histone modifications, some focusing on the proteins themselves and others on the DNA located in close proximity to histones that have been modified. LC-MS used alone can provide a snapshot of the relative frequency for all of the histone modifications (e.g. acetylation, methylation, phosphorylation, sumoylation, and ubiquitination) simultaneously. When coupled with peptide fragmentation or proteomics approaches, LC-MS can provide more precise information including the frequency of a specific modification at a single amino acid location on a histone protein (e.g. the percent of H3 proteins in a given sample that are acetylated at lysine 27), as well as grouping information (e.g. how many of those lysine 27 residues are mono- vs. di- vs. tri-methylated) (Önder et al., 2015). When combined with chromatin immunoprecipitation (ChIP) or

chromatin proteomics approaches, regionally localized histones (i.e. those in a promoter or enhancer of interest) can also be assessed (Byrum et al., 2012; Waldrip et al., 2014; Wang et al., 2013).

For labs that do not have access to mass spectrometry equipment, reagents, and expertise, histone modifications can also be investigated by immunofluorescence, ChIP-qPCR, or ChIP-seq. For example, antibodies specific to methylation, dimethylation, or trimethylation of lysine 9 on histone H3 (H3K9me, H3K9me2, and H3K9me3, respectively) allow investigators not only the ability to visualize the relative prevalence of those histone modifications in cells and tissues of interest, but also to immunoprecipitate all of the histones that are methylated at H3K9 from a sample, and do so with specificity based on the number of methyl groups present. In recent years, there has been a vast expansion of commercially available antibodies against nearly all of the known modified residues on histone tails. These antibodies are useful for immunofluorescence to provide initial characterizations of the relative prevalence of specific histone modifications in a tissue (e.g. Layman et al., 2013). However, immunostaining as an approach for making direct comparisons of histone modifications across cell types, time points, or treatment conditions can be difficult because fluorescent intensities across images of immunostained samples can be variable and differences that are not global, but only occur on a limited number of histones per cell, may be too subtle to be detected. However, if single-cell resolution is not required, western blotting, ELISAs, or a bioanalyzer can be used to obtain relative quantification of the frequency of certain histone modifications from pools of cells or whole tissues (Dai et al., 2013; Wen et al., 2015). Additionally, with the increased use and improvement of super-resolution imaging techniques, image analyses of immunostained histone modifications may prove more useful in the near future. In the meanwhile, ChIP methods are the most common method for investigating histone modifications as they allow for more direct quantification. ChIP using an antibody against a specific histone modification of interest can be coupled with PCR or sequencing, or with mass spectrometry as aforementioned. In recent years, several modified ChIP protocols such as μChIP (Gilfillan et al., 2012) have been developed for use with small numbers of cells and in some cases, single cells (Rotem et al., 2015). In ChIP approaches, an antibody raised to bind selectively against a specific histone modification (e.g. anti-H3K9me2) is applied to nuclear lysates containing sheared DNA and DNA-binding proteins including the histones. After the antibodies bind, the antibody/histone complexes are immunoprecipitated, bringing the DNA in those nucleosomes along with it. The antibodies and histones are then removed by reverse cross-linking and primers specific to a region of interest can be used in PCR or qPCR to obtain relative quantification for the frequency of the histone modification of interest at the specific region of interest (Milne et al., 2009). To obtain a more global picture of the relative frequency of a particular modification (e.g. how much of the genome is associated with H3K9me2), the immunoprecipitated complexes can be quantified by mass spectrometry (Byrum et al., 2012; Waldrip et al., 2014; Wang et al., 2013). Finally, to determine not only how many histones in the genome are modified in a certain way, but also to identify the genomic sequences closest to those histones, the DNA obtained after reverse crosslinking can be adapted and sequenced using a next generation platform (i.e., ChIP-seq). In addition to its utility in identifying genomic regions associated with various histone marks, ChIP-seq

using antibodies against certain well-described histone modifications can also, by proxy, point researchers in the direction of genes that may be activated or silenced under certain conditions (Klisch et al., 2011). For example, H3K4me3 is largely associated with active promoters of genes (Barski et al., 2007), so by performing ChIP-seq with an anti-H3K4me3 antibody, one can identify large cohorts of genes that are likely being actively transcribed. However, it is important to note that no single histone modification has been shown to correlate 100% with either activation or repression of gene expression, so ChIP-seq experiments like this are generally best for generating hypotheses about genes that may be activated or repressed with subsequent studies required to test those hypotheses directly. An additional approach that may be useful for interrogating histone modifications and the role(s) of histone modifying enzymes is DNA adenine methyltransferase identification sequencing (DamID-seq) (Steensel and Henikoff, 2000). By fusing histone writer or eraser proteins (e.g. a HAT or HDAC) to *E coli* DNA adenine methyltransferase, regions of the genome where the enzyme of interest is interacting with histones can be marked by the methylation of nearby adenines. The genomic positions of these sites can then be assayed by DpnI endonuclease treatment, which cleaves only at methylated adenines in GATC sequences (Kehat et al., 2011; Tolhuis et al., 2006). The resulting fragments can then be sequenced and mapped back to an intact reference genome sequence.

ChIP-seq is currently the most widely used method for assessing the prevalence of, as well as the genes most likely affected by, histone modifications. This wealth of information is certainly a strength of the approach and no doubt contributes to its swift and widespread adoption. However, it is important to consider that ChIP based approaches rely on antibodies which can have issues with specificity (e.g. off target binding) or sensitivity (i.e. incomplete coverage at rarer sites due to reduced binding kinetics). In contrast to this, the strength of DamID and LC-MS approaches are that they do not require antibodies and therefore may be more useful for targeted investigation of relatively rare histone modifications. In such instances, LC-MS would be useful for assessing the prevalence of a rare histone modification, while DamID-seq would be more useful for identifying genes affected by rare modifications. Table 2 summarizes the utility and caveats for each of these methods used to investigate histone modifications.

Analysis of Histone Modifications in the Inner Ear (and Related Tissues)

There are several recent studies where immunofluorescence was used to study histone modifications in the inner ear and related tissues. Two studies used antibodies generated against acetylated histone residues and image analysis to measure the effect of gentamycin-induced or noise-induced HC damage on histone acetylation (Chen et al., 2009, 2016). Using cochlear explants treated with gentamycin, Chen et al., (2009) found a decrease in H2A, H2B, H3, and H4 acetylation over time as HCs began to die. Yet there was no change in histone acetylation in neighboring Hensen cells, a subtype of SCs. In adult mice exposed to high intensity noise that induces permanent threshold shifts, there was a decrease in the H3K9ac marks in outer HCs located in the basal turn of the cochlea and in marginal cells of the stria, yet no change in H3K9ac was detected in the outer HCs of apical and middle turns or in spiral ganglion neurons (SGNs) (Chen et al., 2016). Another study examined changes in both acetylation and methylation of H3K9 by immunostaining and confocal microscopy.

In this report, H3K9ac was readily detectable in the SGN and in the organs of Corti from young mice, but not detectable in aged mice. By contrast, H3K9me2 was detectable in cells in aged cochleae, particularly in the basal turns where HC loss was evident, but H3K9me2 was not readily detected in young cochleae (Watanabe and Bloch, 2013). These results led the authors to speculate that more genes may be epigenetically silenced with increasing age and that increased levels of H3K9me2 may presage HC death. Consistent with this idea, Yu et al., (2013) also using immunostaining and western blotting, demonstrated increased levels of H3K9me2 in response to a number of ototoxic insults (cisplatin, copper, ultraviolet light, and neomycin) in organotypic cochlear cultures. The benefit of studies such as these is that they can demonstrate readily detectable levels of several acetylated and methylated histone residues in numerous cell types in the cochlea, and provide initial comparisons of histone acetylation and methylation status between control and ototoxic conditions and/or between neighboring cells within the same samples. However, as mentioned above, the caveats to immunofluorescent approaches such as this, is that they are only semi-quantitative, and do not reveal the specific regions of the genome where histones are modified. Nor can they provide high resolution information about the dynamics of acetylation/deacetylation or methylation/demethylation across the entire population of histones in a given cell or pool of cells. Specifically, studies of this type only allow for the determination of a net effect (e.g. overall decrease of H3K9ac immunoreactivity), but it is likely that, within any given cell, histone proteins in different parts of the genome are being modified differently (e.g. acetylation of H3K9 in some nucleosomes and deacetylation at other locations). Therefore, immunostaining and western blotting reveal only large net effects, but do not provide information about which genes are being silenced and which genes may be being activated in response to a particular stimulus.

There are, however, instances where immunostaining of histone modifications can reveal relatively straightforward information regarding critical cellular processes. This is particularly true for certain histone residues that become widely phosphorylated during proliferation or cell death. For example, phosphorylation of serine 10 on histone H3 (H3S10ph) has been shown to be important for M phase of the cell cycle, and the detection of H3S10ph by immunostaining can be used to identify cells that were in M phase at the time of fixation. Indeed, several inner ear or related papers have used anti-H3S10ph antibodies in this manner (Cox et al., 2014; Huh et al., 2015; Li et al., 2017; Liu et al., 2012; Mantela et al., 2005; Okano et al., 2011; Pan et al., 2013; Raft et al., 2014; Sulg et al., 2010; Urness et al., 2015; Walters et al., 2014; Weber et al., 2008). While useful, it is important to note that H3S10ph has also been shown to play a role in DNA damage/repair and cell death, where, for example, H3S10ph may be increased in response to cisplatin (Park and Kim, 2012), a chemotherapeutic agent known to kill HCs. This is an important factor to consider when using H3S10ph as a marker for cell cycle, and other methods should be used in conjunction to confirm proliferation and/or rule out cell death. Another important phosphorylated site that may be detectable using immunostaining is phosphorylation of serine 139 on the H2A variant, H2AX. Indeed, H2AXS139 phosphorylation (aka γ-H2AX) occurs in a rapid and robust manner near double strand breaks in the DNA, and is essential for recruiting repair complexes, as well as arresting cell cycle progression (Podhorecka et al., 2010). Since double strand breaks are a hallmark of apoptosis (Didenko and Hornsby,

1996), immunostaining for γ-H2AX is often used as a marker for apoptotic cells, and several investigations of inner ear and related tissues have utilized anti-γ-H2AX staining for this purpose (Hansen et al., 2008; Laos et al., 2014; Slattery et al., 2014; Sulg et al., 2010; Yue et al., 2013).

ChIP-qPCR was recently used to probe histone modifications in the regulatory regions of *Atoh1* during embryonic and postnatal cochlear maturation. *Atoh1* is a transcription factor that is necessary for HC differentiation (Bermingham, 1999; Woods et al., 2004). Its expression follows a bell-shaped curve where prosensory cells initiate expression between E13.5–14.5, followed by downregulation after HC differentiation between E17.5 and P6 (Driver et al., 2013; Kelley, 2006; Maass et al., 2015; Woods et al., 2004). Stojanova et al., (2016) used fluorescent activated cell sorting (FACS) to purify either prosensory cells, HCs, or SCs and then performed μCHIP-qPCR with H3K27me3, H3K4me3, H3K9me3, or H3K9ac antibodies using ~25,000 cells per reaction. Three regions in the *Atoh1* locus contained H3K27me3 and H3K4me3 bivalent marks in prosensory cells from E14.5 cochlea. These histone modifications are known to maintain genes in a "poised", but not expressed state (Bernstein et al., 2006). In contrast, in HCs at E17.5, H3K27me3 and H3K4me3 bivalent marks were dramatically reduced. In addition, there was an increase in the H3K9ac marks on the *Atoh1* locus at E17.5 which generally promotes gene expression (Karmodiya et al., 2012). These epigenetic marks corresponded with a ~50 fold increase in *Atoh1* expression between E14.5 and E17.5 and SCs at P1 maintained the H3K27me3 and H3K4me3 bivalent marks observed in prosensory cells. When comparing FACS-purified HCs from E17.5 and P6 cochlea, a time period when *Atoh1* expression is downregulated, H3K9ac marks decreased and there was an increase in H3K9me3, an epigenetic modification associated with gene repression (Rea et al., 2000). Another study (Abdolazimi et al., 2016) used the same methods to investigate histone acetylation changes in the *Atoh1* locus after neonatal cochlear explants were treated with the gamma-secretase inhibitor DAPT, which stimulates the conversion of SCs into HCs and activates *Atoh1* expression (Doetzlhofer et al., 2009; Golden et al., 2015; Korrapati et al., 2013; W. Li et al., 2015; Maass et al., 2015). Abdolazimi et al., (2016) found that 24 hours after DAPT treatment, FACS-purified SCs had increased H3K9ac marks in the *Atoh1* locus. The combination of FACS and μCHIP-qPCR used in these studies provided a focused analysis of the time-dependent and cell-type specific changes to the epigenetic landscape of *Atoh1*. However only one gene was investigated here and changes in histone modifications may be occurring in many other genes as well, which could have been elucidated if ChIP-seq or DamID-seq were used.

In a recent paper, ChIP-seq and ChIP-qPCR were utilized to identify candidate genes important in the differentiation of immortalized multipotent otic progenitor (iMOP) cells into SGN-like cells in culture (Song et al., 2017). iMOP cells are an immortalized cell line derived from Sox2-expressing cells from ~E12 mouse otocysts that have been transduced with retrovirus to express *c-Myc* and are maintained in a proliferative state by supplementation with basic fibroblast growth factor (bFGF). Upon removal of bFGF, iMOP cells can differentiate into HC-like cells, SC-like cells, and SGN-like cells (Kwan et al., 2015). Interestingly, when the proneural gene *Neurog1* is ectopically expressed in proliferating iMOP cells, it promotes further proliferation, but in differentiating iMOP cells,

Neurog1 promotes differentiation into SGN-like cells which are non-mitotic. To identify the genes involved in this switch from proliferation to differentiation in iMOP cells, Song et al (2017) performed ChIP-seq using antibodies against H3K4me3, H3K27me3, and RNA polymerase II. Antibodies against H3K4me3 and RNA polymerase II should immunoprecipitate promoter regions in activated genes, while antibodies against H3K27me3 are more likely to precipitate regions that are repressed (Barski et al., 2007). Since iMOP cells in culture are a heterogeneous mixture of cells that may be either proliferative, non-differentiating or differentiating, non-proliferative, the authors hypothesized that regions of DNA immunoprecipitated by both the marks for transcriptional activation and repression would be critical in the shift from proliferation to differentiation and therefore critical to the process of SGN-like cell differentiation. The ChIP-seq data identified *Cdk2* and *Neurod1* as potential regulators of iMOP cell proliferation and differentiation, respectively. ChIP-qPCR using antibodies against H3K9ac or H3K9me3 and primers targeted to the promoters of *Cdk2* and *Neurod1* further supported the hypothesis that *Cdk2* promoters were more likely to be active in proliferating cells, while *Neurod1* promoters were more likely to be active in differentiated cells. Specifically, amplification of H3K9ac immunoprecipitated DNA yielded more *Cdk2* promoter sequences in proliferating iMOP cells as compared to differentiated SGN-like cells, and this pattern was reversed when *Neurod1* promoter sequences were amplified. When antibodies against the repressive mark H3K9me3 were used to immunoprecipitate the DNA, more *Cdk2* sequences were amplified in the differentiated SGN-like cells than in the proliferating iMOP cells, while more *Neurod1* promoter sequences were amplified in the proliferating iMOPs than in the SGN-like cells. These experiments, combined with others in the paper, strongly suggest that *Neurog1* can promote *Cdk2* to enhance proliferation in the bFGF condition, and promote *Neurod1* to enhance SGN-like cell differentiation when bFGF is absent. Furthermore, the ChIP-qPCR data suggest that it may be the status of H3K9 at the *Cdk2* and *Neurod1* promoters that acts as the switch to determine whether *Neurog1* will promote *Cdk2* and proliferation or *Neurod1* and differentiation. The use of ChIP-seq in this study highlights its utility for identifying promoters and genes that are differentially regulated under diverse conditions, while ChIP-qPCR was used to support and extend these results.

Methods for the Manipulation of Epigenetic Writers and Erasers

While detection of changes in DNA methylation or histone modifications during development, after a drug treatment, or during disease progression is informative, these epigenetic changes can also be intentionally altered using pharmacological agents or genetic tools. For example DNMT inhibitors such as 5-azacytidine and 5-aza-2-deoycytidine have been used with MUC cells where they caused decreased methylation in promoter regions of the genes investigated (Zhou and Hu, 2016, 2015). HAT inhibitors such as curcumin and HDAC inhibitors such as valproic acid, trichostatin A, sodium butyrate, and suberoylanilide hydroxamic acid (SAHA) will prevent or remove histone acetylation, respectively. Curcumin was used in cochlear explant cultures where it reduced the numbers of HCs and decreased expression of *Atoh1* mRNA, which corresponded with decreased H3K9ac marks in the *Atoh1* locus (Stojanova et al., 2016). Many studies have shown that HDAC inhibitors can have a protective effect against noise-induced (Chen et al., 2016; Yang et al., 2017) or

ototoxic drug-induced (Chen et al., 2009; Drottar et al., 2006; Layman et al., 2015; Wang et al., 2015; Yang et al., 2017) HC damage and hearing loss. These pharmacological agents have a broad and global effect, altering epigenetic changes across the genome.

Yu et al., (2013) used the compounds BIX01294 or UNC0638 to pharmacologically inhibit G9a/GLP methyltransferase. This in turn reduced H3K9me2 levels in HCs in cochlear explants and, *in vivo*, promoted HC survival against neomycin-induced ototoxicity. Similarly, pharmacological inhibition of lysine specific demethylase 1 (LSD1) using the monoamine oxidase inhibitor 2-PCPA has been shown to cause increased histone methylation and apoptosis in zebrafish neuromasts during development (He et al., 2013). Interestingly, however, two similar studies using pharmacological inhibitors of LSD1 suggested that histone methylation may actually prevent apoptotic death of HCs in response to neomycin treatment (He et al., 2015) and of SGNs in response to cisplatin treatment (Li et al., 2015). Genetic disruption studies, as well as more detailed mapping of histone methylation in response to toxic insults, are likely needed to determine whether there may be any generalizable aspects of these histone marks on cell survival, or if the effects of methylation at H3K4 and/or H3K9 are regulating different populations of genes in different cell types. In addition to influencing cell survival, histone methylation has been implicated in proliferation where LSD1 activity appears to be necessary for proliferation during development and HC regeneration in zebrafish neuromasts. Again, pharmacological inhibition of LSD1 by 2-PCPA was used and resulted in an increase in H3K4me2 immunostaining which was correlated with decreased proliferation and decreased HC regeneration (He et al., 2016, 2013).

Others have opted for a genetic approach. For example, morpholino based knockdown of *Dnmt3a*, a DNA methylating enzyme, resulted in a significant reduction in the size of the otic placode and reduced expression of several otic-specific genes (*Pax2, Gbx2, Sox10*, and *Soho1*) in developing chicken embryos. In contrast, the expression patterns of several other otic-specific genes (*Otx2, Sox2*, and *Sox3*) were unaffected by *Dnmt3a* knockdown (Roellig and Bronner, 2016). Interestingly, this report suggests that DNA methylation is necessary for the ___activation___ of certain genes. While several possible mechanisms could explain this such as: 1) repression of repressor proteins leading to activation of downstream targets, 2) repression of genes in cells of one domain causing activation of genes in cells in an adjoining domain via cell signaling, or 3) DNA methylation may not always lead to transcriptional repression, but may block access of repressive complexes to DNA thereby promoting gene expression. These data are again an important reminder that one cannot presume to ascribe an all-up or all-down characteristic to any epigenetic modification.

Methods to Assess Chromatin Conformation

In epigenetic studies, sometimes it is not the modifications to the DNA or the histones that are important, but rather a determination of how accessible the DNA in a particular region(s) of interest may be at different times or under different experimental conditions. Several techniques have been developed that allow for the assessment of chromatin structure. Perhaps the most prominent method is the assay for transposase accessible chromatin sequencing, or ATAC-seq (Buenrostro et al., 2013). ATAC-seq works by treating DNA with

an engineered transposase enzyme that has high cutting and ligating activity on accessible DNA; that is, DNA which is not protected by being tightly condensed around histone complexes. Treatment with the hyperactive Tn5 transposase not only cuts accessible DNA, but also simultaneously ligates adapters for high throughput sequencing. Sequenced reads from single cells then provide a map of regions where the chromatin is in an open conformation. When using pools of cells, sequence read numbers positively correlate with the probability of a site being open across the cell population. However, it is again important to note that data from heterogeneous cell populations will not be as robust as data from sorted or clonal cell populations. To avert this potential problem, ATAC-seq can be conducted on single cells (Buenrostro et al., 2015), suggesting that it is amenable to the study of auditory and vestibular tissues. Indeed, ATAC-seq has already been used to study developing mouse otocysts and cochleae (Gálvez et al., 2017). Although the authors were only interested in the regulation of a single gene, *Atoh1*, ATAC-seq provided whole genome maps that identified the regions that were more accessible at E10.5 versus E14.5. These two timepoints were chosen because at E10.5 *Atoh1* is repressed by *Neurog1* during neurogenesis and at E14.5 there is autoactivation of *Atoh1* by ATOH1 binding to its 3' enhancer during HC differentiation. ATAC-seq was also run on E14.5 cochleae that were treated with the gamma-secretase inhibitor LY411575 which causes upregulation of *Atoh1* via the inhibition of Notch signaling. While there did not appear to be any differences in accessibility of the *Atoh1* promoter or enhancer regions across these three different samples, the ATAC-seq data did reveal that the A region of the 3' enhancer remained inaccessible in all three groups, while the B region of the enhancer was open, suggesting that the B region of the enhancer is critical for regulating *Atoh1* expression in the developing cochlea. Subsequent validation experiments showed that the enhancer B region is the primary driver for *Atoh1* positive feedback and a critical site for *Neurog1*-mediated repression of *Atoh1*.

DNase-seq was a predecessor to ATAC-seq and is still a viable alternative to ATAC-seq, or can be conducted in parallel to ATAC-seq for increased robustness of data. DNase-seq operates on a similar premise in which condensed chromatin is protected from nuclease activity and therefore accessible chromatin can be digested into small fragments that are isolated and sequenced (Jin et al., 2015; Song and Crawford, 2010). Chromosome conformation capture methods (3C, 4C, 5C, Hi-C, and capture-C) are additional useful approaches for assaying chromatin accessibility (Belton et al., 2012; Dekker et al., 2002). Although these methods are primarily used for detecting regions of chromatin that interact across distances (e.g. distal promoters and enhancers), they can also reveal regions where there is a large degree of condensed DNA or DNA brought into close proximity by histone modifications. These methods work by crosslinking closely appositioned DNA/protein complexes with formaldehyde. The DNA is then digested by restriction enzymes into fragments that undergo proximity ligation (so that non-complexed DNA is not ligated), and reverse cross-linking then linearizes the resulting hybrid DNA. In chromosome conformation capture (3C, see Dekker et al., 2002), ligations of close proximity DNA are measured by qPCR where a single set of primers can be designed to interrogate whether two non-sequential regions of the genome interact, or a matrix of primer pairs across numerous qPCR reactions can be used to interrogate multiples of such hypothetical close proximity relationships. In chromosome conformation capture-on-chip (4C, see Simonis et al., 2006),

microarrays or next-gen sequencing (4C-seq) are used rather than qPCR to assess close proximity interactions of DNA across the genome. To accomplish this, the 3C protocol is followed up to and including ligation and reverse crosslinking, after which an additional restriction digest is performed and then an additional ligation to create circular DNA constructs. Primer and PCR amplification is again employed, but in this approach, the circular DNA allows for both primers to be designed to be complementary to sequences in only one of the DNA fragments (rather than requiring one primer to bind in each of the two ligated fragments). The amplicons are then identified and quantified by microarray or sequencing. With the 4C approach it is therefore possible to query all of the regions of DNA that interact with one region of interest. In chromosome conformation capture carbon copy (5C, see Dostie et al., 2006), the 3C library is generated by fixation, digestion, ligation, and reverse crosslinking, and then a "carbon copy" is made by annealing forward and reverse oriented oligonucleotides that are designed to contain sequences corresponding to the 3' ends of the restriction fragments and with universal (T7 or T3) tails for amplification. When these 5C oligos anneal next to each other they can be ligated by Taq ligase to create unique oligonucleotides containing sequences that map to both interacting pieces. These new fragments (the 5C library) can then be amplified by PCR using universal primers and can then be assessed by microarray or sequencing. Due to the requirements for specially designed oligos, 5C does not provide complete coverage of the genome, but it does allow for a less biased, higher throughput approach than 3C for interrogating many regions of the DNA and many of the interacting regions. Hi-C (see Lieberman-Aiden et al., 2009) was the first "all versus all" approach derived from 3C and works by introducing biotinylated nucleotides into the 5' overhangs of the digested DNA prior to the proximity ligation step during the construction of the 3C library. After this, the DNA is subjected to reverse cross-linking, purification, and shearing and then a biotin pull down enriches for ligated fragments which are then sequenced. Hi-C thereby provides an unbiased approach to interrogate the whole genome for interacting regions of DNA and the data can be used to generate three dimensional maps of chromatin structure. In a related protocol called ChIA-PET (chromatin interaction analysis by paired end tag sequencing, (see Fullwood et al., 2009) regions of DNA that are in close proximity, as well as DNA-binding proteins are fixed with formaldehyde, the chromatin is sheared and antibodies against proteins of interest (e.g. histone subunits) are immunoprecipitated to enrich for DNA associated with those proteins and the DNA associated with that DNA. Closely interacting DNA then undergoes proximity ligation and paired-end-tagging followed by sequencing and data analysis. Although the authors are not aware of studies with inner ear or related tissues that used these approaches, single cell Hi-C (Nagano et al., 2015) and Hi-ChIP, a low sample input version of ChIA-PET (Mumbach et al., 2016), have been established and may prove useful for such experiments. Table 3 summarizes the utility and caveats for each of these methods used to investigate chromatin structure.

Conclusion

This review is far from exhaustive, both in regard to the tools and approaches presented, as well as to the epigenetics studies that have been undertaken in the inner ear and related tissues. For example, there are many protein complexes that regulate gene expression that

are recruited to histones by the modifications that have been discussed. One such example CHD7, a chromodomain helicase that is recruited to enhancer regions of DNA and positively correlates with transcription factor recruitment and gene activation, is recruited to the DNA by H3K4me (Schnetz et al., 2010). As mutations in CHD7 are a known cause of CHARGE syndrome, which can result in hearing loss and vestibular dysfunction, there are a number of articles that have examined the role of CHD7 in the development and function of auditory and vestibular tissues (Choo et al., 2017; Green et al., 2014; Micucci et al., 2014; Yao et al., 2018). Review of these and other articles pertaining to histone related proteins are beyond the scope of this already extensive review. However, the pharmacological and genetic methods described above for the manipulation of histone writers and erasers are similar to the types of approaches one would most likely use to pursue questions about the functions of histone related proteins like CHD7. Furthermore, acetylation, methylation, and phosphorylation represent only a fraction of the types of modifications that can occur on histone residues. Indeed, histones, like many other proteins, can be ubiquinated (Weake and Workman, 2008), sumoylated (Shiio and Eisenman, 2003), glycosylated (Sakabe et al., 2010), ribosylated (Hottiger, 2015), biotinylated, citrullinated, crotonylated, succinylated, and the list goes on (Xu et al., 2014). This suggests that there is still much left to explore regarding the roles of histone modifications, not only in the inner ear, but more broadly as well. Many of the tools outlined above for the study of histone acetylation and methylation can be repurposed for the study of these modifications as well. Genetic and pharmacological manipulations of the writer and eraser enzymes may also provide valuable insights into the functions of various histone modifications in many cellular processes important in the development, proliferation, differentiation, survival, and function of the cells in the inner ear and related tissues.

Acknowledgements

Abbreviations:

AIMS	amplification of intermethylated sites
ATAC-seq	assay for transposase accessible chromatin sequencing
bFGF	basic fibroblast growth factor
BS-seq	bisulfite sequencing
ChIA-PET	chromatin interaction analysis by paired end tag sequencing
ChIP	chromatin immunoprecipitation
CMH	comparative methylation hybridization
CpG	cytosine residue followed by a guanine residue

DamID-seq	DNA adenine methyltransferase identification sequencing
DNMT	DNA methyltransferase
E	embryonic day
EGF	epidermal growth factor
FACS	fluorescent activated cell sorting
HAT	histone acetyltransferase
HDAC	histone deacetylate
HCs	hair cells
HPLC	high performance liquid chromatography
iMOP	immortalized multipotent otic progenitor
JmjC	jumonji C
LC-MS	liquid chromatography coupled with mass spectrometry
LSD1	lysine specific demethylase 1
MALDI-TOF	matrix-assisted laser desorption ionization time-of-flight
MeDIP-seq	methylated DNA immunoprecipitation sequencing
MP	methylation-specific primer
MUCs	mouse utricle sensory epithelial cells
nested-MSP	nested methylation specific PCR
P	postnatal day
PRMT	protein arginine N-methyltransferase
SAHA	suberoylanilide hydroxamic acid
SCs	supporting cells
SGN	spiral ganglion neuron
TET	ten-eleven translocation
UP	unmethylated primers
UpG	uracil residue followed by a guanine residue
WGBS	whole-genome bisulfite sequencing

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Highlights

- Low cell numbers or other challenges have hindered inner ear epigenetic research

- Recent methodological advances provide the means for overcoming such challenges

- DNA methylation, histone modifications, and chromatin accessibility can be assayed

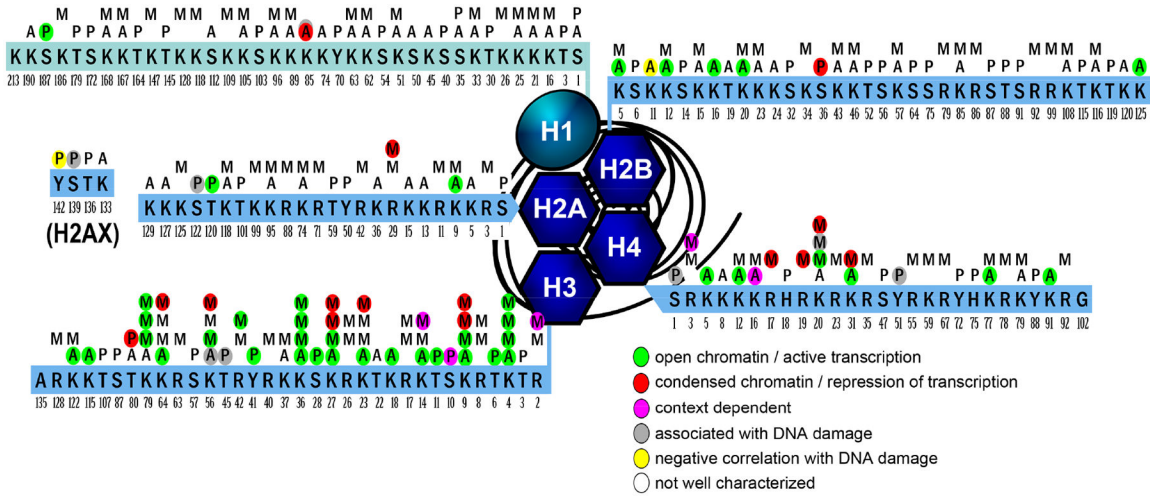- Methods and examples of epigenetic studies of hearing and balance are presented

Figure 1:

Post-translational modifications of histones. Sites known to undergo acetylation (A), methylation (M), and/or phosphorylation (P) are shown for the four canonical histone core proteins (H2A, H2B, H3, and H4, blue hexagons) as well as for the linker histone H1 (teal oval) and the histone variant H2AX (which extends beyond the 129 amino acids of the canonical H2A subunit). Modifications for which the function has been described are color coded: green labels marks most often linked to chromatin openness or active transcription and red labels marks most often associated with chromatin condensation and transcriptional silencing. While it is possible that all of the modifications (including those shown as red or green) may act in a context dependent manner, those labeled with magenta have roughly equal numbers of reports suggesting both actions. Modifications in gray have been described as primarily occurring in close proximity to double strand breaks or other DNA damage, while those coded yellow are markedly absent when there is DNA damage in close proximity. For those modifications that are not assigned a color, relatively little is known about their function. It is important to note that many more residues that could potentially be modified exist on these histone proteins, but only those where an acetylation, methylation, or phosphorylation have been experimentally demonstrated are shown, except for the N' terminal amino acids which have been included on each tail to indicate their overall length. Additionally, residues known to be modified in other ways (e.g. ubiquitination or ribosylation) have been excluded, as have histone variants other than H2AX. Methylation states are represented such that a single methylation mark ("M") denotes a residue that has been shown to be methylated, but differences between mono-, di-, or tri-methylation have not been described. For those residues with 2 or 3 "M" marks, monomethylation is represented by the bottom "M" closest to the peptide sequence, trimethylation is in the uppermost position, and dimethylation is in the middle. Finally, it is also important to note that color coding of functions is based on either currently accepted general function, or, in some cases, on limited publications that exist for a particular modification. Therefore, one cannot assume the represented function will occur in all cases, especially for modifications that have recently been characterized. As an example of this, Shi et al., 2006 demonstrated a silencing function for H3K4me3, however, there are many more reports which show

H3K4me3 as a mark of active transcription which is its more generally accepted function, so it is shown as green in the figure.

Table 1:

Methods for Investigating DNA methylation

Approach	Utility	Caveats	Examples from Inner Ear or Related Tissues
Bisulfite PCR with high resolution melt analysis	Rapid, cost-efficient method to determine if a specific region or multiple regions, of interest are methylated.	1. Not useful for querying many regions of interest or for coverage of the whole genome. 2. Binary output (Y/N) may fail to reveal multiple methylation sites in a given region and does allow for comparison of methylation levels across experimental groups.	N/A
Bisulfite sequencing PCR, Nested-MSP	Rapid, cost-efficient methods to determine if a specific region or multiple regions of interest are methylated or may have multiple sites of methylation.	1. Not useful for querying many regions of interest or for coverage of the whole genome.	Mutai et al., 2009 Wu et al., 2014 Lin et al., 2017
EpiTyper (bisulfite PCR, transcription, MALDI-TOF MS)	Useful for determining the ratio of methylated vs unmethylated status of a particular region or several regions of the genome from a pool of cells or tissue of interest	1. Not ideal for whole genome interrogation 2. Can be costly 3. Requires expertise and equipment for MALDI-TOF mass spectrometry	Waldhaus et al., 2012 Xia et al., 2015
BS-seq, WGBS	Useful for mapping methylation sites across the whole genome. If coupled with single cell isolation (scBS-seq) can detect monoallelic methylation. Allows for quantification and therefore comparison across experimental groups.	1. Can be costly. 2. May require additional bioinformatic tools or other expertise with next generation sequencing and subsequent data analysis	Yizhar-Barnea et al., 2018
MeDIP-qPCR	Rapid, cost-effective method for determining whether there is methylated DNA in a specific genomic region or in a handful of regions of interest and how those levels may compare across experimental groups	1. Not ideal for many regions of interest or whole genome interrogation 2. Does not provide absolute quantification, only relative comparison across groups	Zhou and Hu, 2016
MeDIP-seq	Useful for mapping methylation sites across the whole genome and providing quantitation and comparison across experimental groups.	1. Can be costly. 2. May require additional bioinformatics and next generation sequencing expertise. 3. Single cell MeDIP is not yet well-established and cell sorting to obtain homogenous populations is therefore critical.	N/A
AIMS	A versatile approach that offers similar benefits to BS-PCR, but can be used to provide information about global rather than local levels of DNA methylation. PCR amplification instead of sequencing results in cost savings. Gel extraction and sequencing of bands of interest can provide spatial localization of methylated sites of interest within the genome. DNA methylome footprints can be compared across samples.	1. Use of sequence-specific endonucleases limits detection and biases the results toward sequences that are recognized by the endonuclease used. 2. Amplification rather than sequencing prevents localization of methylation sites across the genome. 3. Comparisons across groups are limited to quantitation of the number of sites that are overwhelmingly methylated within a sample. Frequencies of methylation at specific regions within a cell population cannot be determined or compared using this method.	Mutai et al., 2009
CMH	Provides site-specific methylation information across the genome. Use of microarrays instead of next-gen sequencing provides cost savings. Unlike AIMS, frequencies of methylation in a cell population can be determined and compared across samples.	1. Genome coverage by microarray is limited compared to direct sequencing. 2. Sequence-specific endonucleases limit detection of all methylated sites similar to AIMS.	N/A
LC-MS	Highly sensitive for detecting global levels of DNA methylation and does not require special enzymes or pretreatment.	1. Can be costly. 2. Requires specialized equipment and expertise in mass spectrometry. 3. Does not provide location-specific information, only global levels of DNA methylation in a given sample.	N/A

Approach	Utility	Caveats	Examples from Inner Ear or Related Tissues
Genetic or pharmacological manipulation of DNMTs or TETs	Can uncover important processes and genes that are regulated by DNA methylation. Genetic manipulations can be designed to be cell-type specific obviating the need for cell sorting.	1. Disruption of an enzyme may be fatal or, if not, may lead to complex and overlapping phenotypes due to targeting multiple loci. 2. Partiality of enzymes means coverage is not likely to be global. 3. Redundancy of enzymes means that genes that are regulated by methylation may be missed. 4. Context dependent divalence of enzymes to methylate or demethylate substrates may lead to further complexity of phenotypic results. 5. Potential off-target effects of pharmacological compounds may confound results.	Chen et al., 2013 Zhou & Hu, 2015 Zhou & Hu, 2016 Roellig & Bronner, 2016

NOTE for Table 1: All of the techniques listed above with the exception of "genetic or pharmacological manipulation of DNMTs or TETs" are ideally performed using homogeneous or sorted cell populations or isolated single cells. Cellular heterogeneity can introduce a large degree of noise into the data and result in incorrect estimations of the frequencies and locations of DNA methylation in the genomes of cell types of interest.

Table 2:

Methods for Investigating Histone Modifications

Approach	Utility	Caveats	Examples from Inner Ear or Related Tissues
LC-MS	Can provide information about all of the modifications present on all of the histone proteins that are present in a sample. Can be used to compare global levels of histone modifications across experimental groups.	1. Does not link individual histones and their modifications to specific locations in the genome. 2. Additional experiments are necessary to directly link changes in modifications to any phenotype. 3. Can be costly, require special equipment, and require additional expertise in mass spectrometry.	N/A
Immunofluorescence, Western blotting	Economical, fast, and easily implemented in most laboratories. Can provide initial information and allow for correlation of various cellular processes with certain modifications that are present at high levels.	1. Variability can result from differences in fixation, section thickness, age of sample, age of antibody, antibody promiscuity, and a number of other factors making direct quantification difficult and perhaps less reliable than other methods. 2. Requires finding and validating reliable antibodies.	Mantela et al., 2005 Hansen et al., 2008 Weber et al., 2008 Chen et al. 2009 Sulg et al., 2010 Okano et al., 2011 Liu et al., 2012 Pan et al., 2013 Watanabe & Bloch 2013 Yu et al., 2013 Yue et al., 2013 Cox et al., 2014 Laos et al., 2014 Raft et al., 2014 Slattery et al., 2014 Walters et al., 2014 Huh et al., 2015 Urness et al., 2015 Chen et al., 2016 Li et al., 2017
ChlP-PCR or ChIP qPCR	Cost effective method that allows for the determination of individual histone modification at small numbers of genomic sites of interest. If the PCR is quantitative, comparisons can be made across experimental groups.	1. Is limited to the investigation of one, or at most a few, histone modifications of interest. 2. Is dependent on finding a reliable antibody against the modification of interest. 3. Only suitable for genes of interest as it does not provide genome-level information.	Abdolazimi et al., 2016 Stojanova et al., 2016 Song et al. 2017
ChlP-seq	Allows for the identification of all genomic regions that are bound to histones that have the modification of interest. Can be used to compare global levels of histone modifications across experimental groups.	1. Is dependent on finding a reliable antibody with good binding kinetics. 2. Can be costly and may require additional bioinformatics and next generation sequencing expertise.	Song et al., 2017
DamlD-seq	Requires low input of DNA. Provides whole genome sequence data including all locations of the genome where the histone modifying enzyme of interest was in close proximity to the DNA. Depending on the approach used, it is possible to generate cell type-specific expression of the adenine methyl-transferase-conjugated enzyme thus limiting the need for cell sorting.	1. Requires knowledge of the histone modifying enzyme of interest. 2. Requires genetic editing approaches to fuse adenine methyltransferase to the histone modifying enzyme of interest. 3. If the histone modifying enzyme has multiple roles involving DNA, the resulting data may include sites not related to the histone modification of interest. 4. May require additional bioinformatics and next generation sequencing expertise	N/A

Approach	Utility	Caveats	Examples from Inner Ear or Related Tissues
Genetic or pharmacological manipulation of writer and eraser enzymes	Can uncover important processes and genes that are regulated by a histone modification of interest (or by a set of histone modifications). Genetic manipulation can be designed to be cell type specific obviating the need for cell sorting.	1. Overlap of function across a variety of histone writing and erasing enzymes as well as promiscuous activity of these enzymes on other proteins may yield complex phenotypes and can make it difficult to tie phenotypes directly to the histone modification being tested. 2. Potential off-target effects of pharmacological compounds may confound results.	Drottar et al., 2006 Stojanova et al., 2009 He et al., 2013 Yu et al., 2013 He et al., 2015 Layman et al., 2015 Li et al., 2015 Wang et al., 2015 Chen et al., 2016 He et al., 2016 Stojanova et al., 2016 Yang et al., 2017

NOTE for Table 2: All of the techniques listed above with the exception of "DamiID-seq" and "Genetic or pharmacological manipulation of writer and eraser enzymes" are ideally performed with sorted or homogeneous populations of cells or isolated single cells. Cellular heterogeneity can introduce a large degree of noise into the data resulting in incorrect estimations of the frequency and locations of histone modifications in the cell types of interest.

Table 3:

Methods for Investigating Chromatin Structure

Approach	Utility	Caveats	Examples from Inner Ear or Related Tissues
ATAC-seq	Provides genome-level data revealing the probabilities of finding any given region in an open or closed state across a cell population.	1. Can be costly 2. May require additional bioinformatics and next generation sequencing expertise.	Galvez et al., 2017
DNase-seq	Provides genome-level data revealing the probabilities of finding any given region in an open or closed state across a cell population.	1. Can be technically challenging as it requires determination of optimal DNase concentration and duration of treatment which is variable across sample types. 2. May require pooling of samples to achieve the required numbers of cells. 3. Can be costly 4. May require additional bioinformatics and next generation sequencing expertise.	N/A
Chromatin capture techniques (3C, 4C, 5C, Hi-C)	Provides structural information about chromatin from cells or tissues of interest. Is particularly useful for discovery of distal promoters and enhancers. Can provide indirect information about the level to which DNA is condensed or open.	1. Can be costly 2. May require additional expertise, including bioinformatic assistance to map ligated sequences to two or more disparate locations in the genome. 3. ATAC-seq and DNase-seq provide more direct measures of chromatin accessibility.	N/A
ChIA-PET, Hi-ChIP	Highly useful for investigating the role of specific DNA interacting proteins and their effects on chromatin structure.	1. Can be costly 2. May require additional expertise, including bioinformatic assistance to map ligated sequences to two or more disparate locations in the genome. 3. May require pooling of cells as the ChIP step reduces the amount of input material obtained per cell.	N/A

NOTE for Table 3: All of the techniques listed above are ideally performed with sorted or homogeneous populations of cells or isolated single cells. Cellular heterogeneity can introduce a large degree of noise into the data resulting in incorrect estimations of the frequencies of chromatin interactions or the degree to which DNA in certain regions is accessible in a given cell type.