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Epigenetic alterations by NuRD and PRC2 in the neonatal mouse cochlea

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Abstract

Mammalian cochlear supporting cells remain quiescent at postnatal ages and age-dependent changes in supporting cell proliferative capacity are evident. Ectopic *Atoh1* expression in neonatal supporting cells converts only a small percentage of these cells into hair cell-like cells. Despite tremendous potential for therapeutics, cellular reprogramming in the mammalian inner ear remains a slow inefficient process that requires weeks, with most cells failing to reprogram. Cellular reprogramming studies in other tissues have shown that epigenetic inhibitors can significantly improve reprogramming efficiency.

Very little is known about epigenetic regulation in the mammalian inner ear, and almost nothing is known about the histone modifications. Histone modifications are vital for proper transcriptional regulation, and aberrant histone modifications can cause defects in the regulation of genes required for normal tissue development and maintenance. Our data indicate that cofactors of repressive complexes such as NuRD and PRC2 are present in the neonatal organ of Corti. These NuRD cofactors are present throughout most of the organ of Corti from E18.5 until P4. By P6, these NuRD cofactors are mostly undetectable by immunofluorescence and completely lost by P7, but are detectable again at P8 and continue to be present through P21. The PRC2 enzymatic subunit, EZH2 is also highly present from E18.5 to P0 in the organ of Corti, but lost between P2 and P4. However, EZH2 staining is evident again throughout the organ of Corti by P6 and persists through P21. Our data provide evidence that HDACs, DNA methyltransferases, histone methyltransferases, and histone demethylases are expressed postnatally within the organ of Corti, and may be targets for drug inhibition to increase the capacity, speed, and efficiency of reprogramming a supporting cell into a hair cell.

Keywords

epigenetics; NuRD; PRC2; HDAC; KDM1A; EZH2; inner ear; cochlea

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1.1 Introduction

Cellular reprogramming studies have tremendous potential for therapeutic development, disease studies, and developmental processes (Soldner et al., 2009; Yamanaka, 2009). However, direct reprogramming through ectopic expression of defined transcription factors is a slow and inefficient process with most cells failing to reprogram (Huangfu et al., 2008; Mikkelsen et al., 2008). Additionally, the efficiency and yield of reprogrammed cells was shown to decline with increasing age and differentiation status of the donor cell (Hanna et al., 2010; Kim et al., 2010; Lister et al., 2011). Studies using induced pluripotent stem cells (iPSCs) have shown that inhibitors of epigenetic events such as DNA methylation, histone deacetylation, and histone methylation are able to improve reprogramming efficiency (Hanna et al., 2010; Huangfu et al., 2008; Kim et al., 2010; Lister et al., 2011; Mikkelsen et al., 2008).

In the auditory field, ectopic expression of the bHLH transcription factor, *Atoh1*, in neonatal cochlear supporting cells converts 6-11% of cochlear supporting cells into auditory hair cell-like cells that expressed multiple auditory hair cell markers and survive for at least 2 months *in vivo* (Kelly et al., 2012; Liu et al., 2012). However, the newly generated hair cell-like cells do not express the outer hair cell terminal differentiation marker *Prestin,* and lack mature hair cell morphology (Liu et al., 2012). Additionally, ectopic *Atoh1* expression at P30 is unable to convert supporting cells into hair cell-like cells (Kelly et al., 2012; Liu et al., 2012). These data suggest that cochlear supporting cells may lose their cellular plasticity and capacity for cellular reprogramming through epigenetic modifications that occur during early postnatal inner ear development (Walters and Zuo, 2013).

Epigenetics encompass a broad variety of biological processes but may best be described as a change in phenotype that is not caused by a change in DNA sequence. The two most well understood mechanisms of epigenetic alterations that lead to these phenotypic changes are DNA methylation and histone modifications. Posttranslational histone modifications alter the histones interaction with DNA and nuclear proteins. Histone H3 and H4 can be covalently modified at several sites, and modifications on specific lysine residues play a fundamental role in transcriptional regulation. The enzymes responsible for maintaining proper histone acetylation states include histone acetyltransferases (HATs) and histone deacetylases (HDACs), while the enzymes responsible for maintaining proper histone methylation status are histone methyltransferases and histone demethylases (Figure 1).

Class I HDACs, HDAC1 and HDAC2 are found in three distinct multiprotein complexes including the nucleosome remodeling and deacetylation (NuRD), CoREST, and Sin3 complexes (Cunliffe, 2008; Hayakawa and Nakayama, 2011). These complexes are highly conserved and function in distinct cellular processes. The NuRD complex is a transcriptional co-repressor essential for developmental transitions. The NuRD complex comprises at least two enzymatic subunits and several non-enzymatic subunits (Lai and Wade, 2011). Lysine-specific histone demethylase 1 (KDM1A), is also associated with the NuRD complex to demethylate di- and tri-methylated histone H3K4 (Wang et al., 2009). Histone deacetylation by the NuRD complex has been shown to recruit the polycomb repressive complex 2 (PRC2) to specific NuRD target genes (Reynolds et al., 2012). PRC2 is a transcriptional repressor complex required for gene silencing during multiple developmental processes. The histone methyltransferase activity of the PRC2 complex is done by the enzymatic subunit, enhancer of zeste homolog 2 (EZH2). EZH2 acts to repress transcription by di- and tri-methylating histone H3K27. Indicating that silencing of some NuRD target genes may require multiple repressive complexes for proper gene silencing during development.

Very little is known about epigenetic regulation in the mammalian inner ear. Although some DNA methylation studies in the mammalian inner ear have been reported (Mutai et al., 2009; Waldhaus et al., 2012), almost nothing is known about histone modifications that take place during mammalian inner ear development. Histone modifications are vital for proper transcriptional regulation, and aberrant histone modifications can cause defects in the regulation of genes required for normal tissue development and maintenance. A better understanding of the types of histone modifications made during mammalian inner ear development may provide information vital for future research toward complete cellular reprogramming and ultimately hearing regeneration. Here we report our comprehensive analysis of the distribution of several vital epigenetic factors involved in histone modification during late embryonic and early postnatal development of the auditory sensory epithelium (organ of Corti). Our results provide a framework for future studies of molecular mechanisms underlying epigenetic changes in the developing cochlea.

2.1 Material and Methods

2.2 Mice

FVB/NJ and C57BL/6J wild type mice were obtained from the Jackson Laboratory. Mice are housed with a 12/12h dark/light cycle and fed ad libitum. Each neonatal litter was divided into at least two neonatal time points, and a minimum of 3 littermates from at least 2 litters was assayed at each neonatal time point for both FVB/NJ and C57BL/6J to control for intra and inter litter variation. Timed pregnancies were established for both FVB/NJ and C57BL/6J wild type mice, and the morning of plug identification designated as E0.5. Embryos were collected following dam euthanization by CO_2 asphyxiation and hysterectomy, and washed briefly in PBS. All procedures were approved by St. Jude Children's Research Hospital Animal Care and Use Committee (ACUC).

2.3 Immunofluorescence

Embryonic (E18.5) and neonatal (P0-P7) ears from FVB/NJ and C57BL/6J wild type littermates were fixed in 4% paraformaldehyde overnight at 4°C. P8-P21 FVB/NJ and C57BL/6J wild type littermates were anesthetized with 250 mg/kg body weight tribromoethanol and perfusion fixed with 4% paraformaldehyde. P8-P21 ears were removed and placed in 4% paraformaldehyde overnight at 4°C, then incubated in 100mM EDTA for 2 days. All ears were treated with 30% sucrose protection overnight at 4°C, then flash frozen in TFM freezing medium (Triangle Biomedical Sciences, Durham, NC) for cryosectioning at 12μm. Following cryosectioning, sections were processed for immunofluorescence with antibodies against HDAC1 (1:500; Abcam, Cambridge, MA), HDAC2 (1:1000; Abcam), KDM1A (1:500; Abcam), CHD4 (1:200; Abcam), EZH2 (1:100; Cell Signaling, Danvers, MA), RCOR2 (1:200; Millipore, Temecula, CA), Histone H4ac (pan-acetylation) (1:1000; Active Motiff, Carlsbad, CA), and PVALB (1:500; Sigma, St. Louis, MO). Secondary antibodies were used at 1:200 and conjugated with Alexa 488, Alexa 568, Alexa 647 (Invitrogen, Carlsbad, CA) or HRP and then labeled with Tyramide Signal Amplification Kit (Vector Laboratories, Burlington, CA). Images were captured on a confocal microscope (Zeiss LSM 700 confocal microscope) using the Zen 2011 software then processed in LSM Image Browser and Photoshop CS3. Laser intensity was consistent between ages and samples.

2.4 RNA Isolation and Real-Time PCR

Each neonatal litter was divided into at least two neonatal time points, and a minimum of 3 littermates from at least 2 litters was assayed at each neonatal time point for both FVB/NJ and C57BL/6J. Pups were euthanized by decapitation, ears removed by gross dissection, placed in ice cold HBSS, and then microdissected for the organ of Corti. Organ of Corti

RNA was isolated using the RNAqueous-Micro RNA Isolation Kit (Ambion, Austin, TX). Isolated RNA was treated with DNase I prior to cDNA synthesis. cDNA was generated using Superscript First-Strand cDNA Synthesis system for RT-PCR (Invitrogen) with random primers.

Relative expression levels were assayed utilizing TaqMan Gene Expression Master Mix and TaqMan probes (Applied Biosystems, Foster City, CA) for *Hdac1, Hdac2, Ezh2, Chd4, Kdm1a, Rcor2, Prestin, Atoh1, mTert, Dnmt3a, Dnmt3b*, 1*8S, GAPDH*, and *Actb*. Reactions were run in triplicate in an Eppendorf Realplex^2 Mastercycler System. The level of *18S, GAPDH*, and *Actb* were used as internal controls and were run as a multiplex reaction with each assayed gene. The difference in C_T between the assayed gene and *18S, GAPDH*, and *Actb* for any given sample was defined as $\Delta C_{T(X)}$. The difference in $\Delta C_{T(x)}$ between two samples was defined as $\Delta\Delta C_{T(X)}$, which represents a relative difference in expression of the assayed gene. The fold change of the assayed gene relative to *18S, GAPDH*, and *Actb* was defined as $2^{-\Delta\Delta CT}$ (Livak and Schmittgen, 2001). DataAssist software (Applied Biosystems) was used for statistical analysis and to confirm $\Delta C_{T(X)}$ calculation.

3.1 Results

3.2 Histone deacetylases are present in the neonatal organ of Corti

Histone deacetylation plays a vital role in cell cycle regulation, cellular differentiation, and tissue development in mammals. *Hdac1* and *Hdac2* are typically co-expressed and often show functional redundancy in many tissues and cultured cell lines (LeBoeuf et al., 2010; Wilting et al., 2010; Yamaguchi et al., 2010). HDAC1 and HDAC2 play a crucial role in cellular proliferation and differentiation by regulating the expression of genes such as *Cdkn1a* (*p21), Cdkn1b* (*p27), Cdkn2a (p16)*, and *Trp53* (LeBoeuf et al., 2010; Wilting et al., 2010; Yamaguchi et al., 2010). Since mammalian cochlear supporting cells remain quiescent at postnatal ages and age-dependent changes in supporting cell proliferative capacity are evident (White et al., 2006), we analyzed late embryonic and postnatal wild type organ of Corti for the presence of histone deacetylases, HDAC1 and HDAC2, by immunofluorescence. Since staining for HDAC1 and HDAC2 was identical for each age in wild type mice, HDAC1 and HDAC2 will be referred to as HDAC1/2. We found that at E18.5 (FVB *n=6*; B6 *n=6*) HDAC1/2 are broadly found throughout the organ of Corti including the OHCs, IHC, DCs, OPC, IPC, IPh, LER, and GER (Figure 2A and A'). At P0 (FVB *n=8*; B6 *n=6*), HDAC1/2 remain present in the OHCs, IHC, DCs, OP, LER, GER but is variably present in the IPC and IPh (Figure 2B and B'). At P2 (FVB *n=8*; B6 *n=6*) and P4 (FVB *n=8*; B6 *n=6*), HDAC1/2 appear reduced but present in the OHCs, IHC, DCs, OPC, GER and is variably present in the IPC, IPh, and LER (Figure 2C, C', D and D'). However by P6 (FVB *n=8*; B6 *n=6*), HDAC1/2 are largely reduced but present in the GER and variably present in IHC, IPC, and IPh (Figure 2E and E'), while by P7 (FVB *n=8*; B6 *n=6*) HDAC1/2 staining is no longer apparent within the organ of Corti (Figure 2F and F'). Surprisingly, HDAC1/2 reappear in the organ of Corti in the OHCs, IHC, DCs, OPC, IPC, and IPh at P8 (FVB *n=6*; B6 *n=6*) (Figure 2G and G'), and continues to be present from P9-P14 (data not shown) and past hearing onset at P21 (FVB *n=6*; B6 *n=6*) (Figure 2H and H').

To determine whether HDAC1/2 affect histone acetylation levels, we analyzed late embryonic and postnatal wild type organ of Corti for the presence of histone H4 acetylation using antibodies against histone H4 pan-acetylation (H4ac). We found an inverse correlation between HDAC1/2 and H4ac in the organ of Corti. HDAC1/2 staining steadily decreases from E18.5 to P7, while H4ac levels increase from E18.5 to P7 (FVB *n=8*; B6 *n=6*) in the organ of Corti (Figure 3A-F'). While at P8 when HDAC1/2 reappear in the organ of Corti, H4ac at P8 appears reduced (FVB *n=6*; B6 *n=6*) (Figure 3G and G') and remains low through P21 (FVB *n=6*; B6 *n=6*) in the organ of Corti (Figure 3H and H').

Since the half-life of a protein can widely vary within a cell from minutes to several days, we analyzed *Hdac1* and *Hdac2* mRNA levels in the micro-dissected neonatal organ of Corti by quantitative real-time PCR (qPCR) using Taqman probes. *Hdac1* and *Hdac2* mRNA levels were analyzed at P0, P1, P2, P3, P4, P5, P6, P7, and P21 (for each time point FVB *n=6*; B6 *n=6*), values are presented as relative to P0 (each time point FVB *n=6*; B6 *n=6*). We found that *Hdac1* and *Hdac2* follow a similar pattern of expression within the neonatal organ of Corti. *Hdac1* and *Hdac2* mRNA levels steadily decrease from P0 to P6, then at P7 and P21, both *Hdac1* and *Hdac2* mRNA levels increase approximately 4 fold compared to the P0 time point (Figure 2I and J). The apparent spike in *Hdac1* and *Hdac2* mRNA levels precedes HDAC1/2 protein detection by 1 day in the organ of Corti, suggesting that HDAC1 and HDAC2 may be subject to post-transcriptional and/or translational regulation (Spriggs et al., 2010; Vogel and Marcotte, 2012).These data together with the immunofluorescence data indicates that both *Hdac1* and *Hdac2* are expressed and may have a functional role during postnatal organ of Corti maturation.

3.3 Neonatal organ of Corti expresses lysine-specific histone demethylase 1 (KDM1A)

KDM1A is a lysine-specific histone demethylase that acts to repress gene transcription by removing methylation sites from H3K4me1/2 (Wang et al., 2007; Wang et al., 2009). However, KDM1A typically functions with additional co-factors such as HDAC1 and HDAC2 in repressive complexes including the Co-REST and NuRD complexes to control transcription factor access to target genes by altering chromatin structure thereby regulating gene transcription (Wang et al., 2007; Wang et al., 2009). To test whether KDM1A is present in a similar pattern to HDAC1/2, we analyzed wild type organ of Corti for the presence of KDM1A by immunofluorescence. We found that at E18.5 (FVB *n=6*; B6 *n=6*) KDM1A is present throughout the organ of Corti including the OHCs, IHC, DCs, OPC, IPC, IPh, LER, and GER (Figure 4A and A'). At P0 (FVB *n=8*; B6 *n=6*), KDM1A is retained throughout the organ of Corti including the OHCs, IHC, DCs, OPC, IPC, IPh, LER, and GER (Figure 4B and B'). At P2 (FVB *n=8*; B6 *n=6*), KDM1A appears reduced but present in the OHCs, DCs, OPC, IPC, IPh, GER and is variably present in the IHC and LER (Figure 4C and C'). By P4 (FVB *n=8*; B6 *n=6*), remains present in the OHC, IPh, GER and is variably present in DC, OPC, IPC, and IHC (Figure 4D and D'). At P6 (FVB *n=8*; B6 *n=6*), KDM1A is greatly reduced but remains present in the GER and appears variably in IPC and IPh (Figure 4E and E'). By P7 (FVB *n=8*; B6 *n=6*), KDM1A staining is no longer apparent within the organ of Corti (Figure 4F and F'). Similar to HDAC1/2 staining, KDM1A is apparent again at P8 (FVB *n=6*; B6 *n=6*) in the OHCs, IHC, DCs, OPC, IPC, and IPh (Figure 4G and G'), and remains detectable in the organ of Corti from P9 to P14 (data not shown) and beyond hearing onset at P21 (FVB *n=6*; B6 *n=6*) (Figure 4H and H").

We analyzed the wild type neonatal organ of Corti by qPCR using TaqMan probes for the mRNA expression levels *Kdm1a* (each time point FVB *n=6*; B6 *n=6*). Similar to *Hdac1* and *Hdac2* mRNA levels, *Kdm1a* expression levels decrease from P0 to P6 (Figure 4I). *Kdm1a* expression at both P7 and P21 increases by approximately 3 fold compared to the P0 time point (Figure 4I), which coincides with *Hdac1* and *Hdac2* expression levels at P7 and P21. The KDM1A immunofluorescence and qPCR data taken together with the HDAC1 and HDAC2 data suggests that these epigenetic factors may have a combinatorial role in regulating maturation of the postnatal organ of Corti.

3.4 The NuRD complex is present within the neonatal inner ear

The NuRD complex is required for lineage-specific gene expression by regulating the chromatin state through histone modifications and chromatin remodeling (Lai and Wade, 2011; Reynolds et al., 2012; Wang et al., 2009). The core component of the NuRD complex is the ATP-dependent chromatin remodeling protein, chromodomain helicase DNA binding

protein 4 (CHD4) (Lai and Wade, 2011). To test whether CHD4 is present in the neonatal organ of Corti, we analyzed wild type organ of Corti for the presence of CHD4 by immunofluorescence. We found that at E18.5 (FVB *n=8*; B6 *n=6*) CHD4 is present in organ of Corti including the OHCs, IHC, DCs, OPC, IPC, IPh, LER, and GER (Figure 5A and A'). From P0 (FVB *n=8*; B6 *n=6*) to P2 (FVB *n=8*; B6 *n=6*), CHD4 continues to be detected in the OHCs, IHC, DCs, OPC, IPC, IPh, GER and is variably detectable in the LER (Figure 5B-C'). While at P4 (FVB *n=8*; B6 *n=6*), CHD4 appears reduced but present in the OHCs, IHC, DCs, OPC, IPC, IPh, GER and remains variably detectable in the LER (Figure 5D and D'). By P6 (FVB *n=8*; B6 *n=6*), CHD4 is only found in the IHC and is undetectable within the organ of Corti by P7 (FVB *n=8*; B6 *n=6*) (Figure 5E-F'). CHD4 is apparent again in the organ of Corti in the OHCs, IHC, DCs, OPC, IPC, and IPh at P8 (FVB *n=6*; B6 *n=6*) (Figure 5G and G'), and continues to be present from P9-P14 (data not shown) and past hearing onset at P21 (FVB *n=6*; B6 *n=6*) (Figure 5H and H').

To determine the temporal expression pattern of *Chd4*, we analyzed *Chd4* mRNA levels in the micro-dissected neonatal organ of Corti by qPCR using Taqman probes (each time point FVB *n=6*; B6 *n=6*). We found a similar pattern of *Chd4* mRNA expression within the neonatal organ of Corti as was detected for *Hdac1, Hdac2,* and *Kdm1a*, with expression levels steadily decreasing from P0 to P6, then at P7 and P21 expression is increased approximately 3 fold compared to the P0 time point (Figure 5I). Altogether, our data suggest that the NuRD complex functions to regulate proper temporal and cell-type specific gene expression within the organ of Corti to achieve terminal cellular differentiation during postnatal development.

Since HDAC1, HDAC2, and KDM1A are known to be co-factors in both the CoREST and NuRD repressive complexes (Lai and Wade, 2011; LeBoeuf et al., 2010; Qureshi et al., 2010; Reynolds et al., 2012; Wang et al., 2007; Wang et al., 2009), we analyzed wild type organ of Corti for the presence of the core component of these repressive complexes by immunofluorescence and qPCR (FVB *n=8*; B6 *n=6*). The core component of the murine CoREST complex is RCOR2. We were unable to detect the presence or mRNA expression of RCOR2 by immunofluorescence or qPCR, indicating that the CoREST complex is unlikely to have a functional role during neonatal organ of Corti maturation.

3.5 PRC2 is present in the neonatal cochlea and may function in combination with NuRD

Precise control of gene expression is vital for both cellular differentiation and development. During development lineage-specific genes require a precise temporal and spatial pattern of expression, while the expression of genes such as pluripotency genes must be repressed. Achieving proper gene expression during developmental transitions requires multiple levels of gene regulation from transcription factor availability to the chromatin state. NuRD has been shown to recruit additional repressive complexes such as PRC2 to specific target genes (Reynolds et al., 2012). Similar to NuRD, PRC2 is essential for proper developmental transitions. The enzymatic subunit of the PRC2 repressive complex is the histone methyltransferase, EZH2. To test whether PRC2 may work in conjunction with NuRD during neonatal organ of Corti maturation, we examined wild type organ of Corti for the presence of EZH2 by immunofluorescence. We found that at E18.5 (FVB *n=6*; B6 *n=6*) EZH2 can be detected throughout the organ of Corti including OHCs, IHC, DCs, OPC, IPC, IPh, LER, and GER (Figure 6A and A'). EZH2 continues to be present in the OHCs, IHC, DCs, OPC, IPC, IPh, GER, and variably in the LER at P0 (FVB *n=8*; B6 *n=6*) (Figure 6B and B'). However, by P2 (FVB *n=8*; B6 *n=6*) EZH2 is no longer detected within the organ of Corti and remains absent through P4 (FVB *n=8*; B6 *n=6*) (Figure 6C-D'). Then at P6 (FVB *n=8*; B6 *n=6*), EZH2 is found present again within IHC, DCs, OPC, IPC, IPh, LER, and GER (Figure 6E and E'). By P7 (FVB *n=8*; B6 *n=6*), EZH2 is once again found throughout the organ of Corti including OHCs, IHC, DCs, OPC, IPC, IPh, LER, and GER

(Figure 6F and F'). EZH2 remains detectable in the organ of Corti at P8 (FVB *n=6*; B6 *n=6*) (Figure 6G and G'), and is still apparent from P9-P14 (data not shown) and past hearing onset at P21 (FVB *n=6*; B6 *n=6*) (Figure 6H and H').

The temporal expression pattern of *Ezh2* was measured by qPCR using Taqman probes in micro-dissected neonatal organ of Corti (each time point FVB *n=6*; B6 *n=6*). *Ezh2* expression rapidly declines from P0 to P1, expression remains very low until P5 at which point *Ezh2* expression increases approximately 5 fold compared to P0 time point (Figure 6I). From P5 until P7, *Ezh2* expression increases with approximately an 8 fold increase in expression at P7 and 3 fold increase at P21 compared to the P0 time point. The transient temporal pattern of HDAC1, HDAC2, KDM1A, CHD4, and EZH2 staining and mRNA levels suggests that both NuRD and PRC2 may be required for specific developmental transitions within the neonatal organ of Corti and may function alone or in combination at lineage-specific target genes.

3.6 Repressive histone modifications correspond with altered gene expression

During development, the expression of lineage-specific genes must be up-regulated, while genes required for maintaining cellular plasticity and proliferation need to be down-regulated in a precise temporal pattern. NuRD and PRC2 are required during these developmental transitions for proper gene regulation. Genes such as *telomerase reverse transcriptase*(*mTert*) are known to be highly expressed in cells that have greater capacity for proliferation, *mTert* then becomes increasingly down-regulated during differentiation as cells become more committed to a cellular lineage (Armstrong et al., 2000). We found by qPCR using Taqman probes that during neonatal organ of Corti maturation *mTert* expression steadily decreases between P0 and P7 (each time point FVB *n=6*; B6 *n=6*) (Figure 7A), which correlates with previous work showing loss of postnatal proliferative capacity *in vivo* (Ruben, 1967). We also analyzed by qPCR using Taqman probes the expression of *Atoh1*, which is thought to be one of the earliest determinants of hair cell fate. Although *Atoh1* expression is highest in the embryonic inner ear (Driver et al., 2013), we analyzed *Atoh1* expression levels by qPCR during neonatal maturation (each time point FVB *n=6*; B6 *n=6*). We found that *Atoh1* mRNA levels continuously decline from P0 to P7 (Figure 7B), with P7 *Atoh1* levels being nearly undetectable. To determine whether lineage-specific genes become up-regulated during neonatal inner ear maturation, we analyzed by qPCR using Taqman probes mRNA expression levels of the outer hair cell-specific gene, *Prestin,* by qPCR. *Prestin* expression levels were not consistently detectable in the neonatal organ of Corti until P3, thus expression levels are relative to P3 instead of P0 as described above. *Prestin* mRNA levels steadily increase from P3 to P6, then at P7 *Prestin* expression increases by approximately 17 fold compared to the P3 time point (each time point FVB *n=6*; B6 *n=6*) (Figure 7C).

Age-related DNA methylation studies have found a positive correlation between aging and increased DNA methylation, which results in gene silencing (Hernandez et al., 2011; Maegawa et al., 2010). Studies in the mammalian inner ear show a positive correlation between increased DNA methylation on *Sox2* enhancers and supporting cell differentiation and loss of stemness (Waldhaus et al., 2012). NuRD binding specificity relies upon the methyl binding properties of their methyl-CpG binding domain protein subunits, MBD2 and MBD3 (Lai and Wade, 2011). DNA methylation plays an important role during development and changes in the DNA methylation pattern serve as a key epigenetic process in transcriptional regulation (Jurkowska et al., 2011). Two DNA methyltransferase enzymes, DNMT3A and DNMT3B, are responsible for establishing *de novo* DNA methylation patterns during development (Jurkowska et al., 2011). Additionally, NuRD and PRC2 were shown to recruit DNMTs to target genes to stabilize the silencing of target genes (Cai et al., 2013; Vire et al., 2006). To determine whether *Dnmt3a* and *Dnmt3b* are expressed during

neonatal organ of Corti maturation, we measured the expression levels of *Dnmt3a* and *Dnmt3b* by qPCR using Taqman probes (each time point FVB *n=6*; B6 *n=6*). We found that *Dnmt3a* and *Dnmt3b* have a relatively low level of expression from P0 to P4, then both *Dnmt3a* and *Dnmt3b* expression increases approximately 7 fold by P5, and an approximate 35-40 fold increase in expression by P7 compared to the P0 time point (Figure 7D). These data taken together with the NuRD and PRC2 data suggest that a key developmental transition occurs in the organ of Corti after the first week of postnatal development, and that this developmental transition requires multiple levels of epigenetic regulation.

4.1 Discussion

Epigenetic events such as histone modifications and DNA methylation are essential for proper development. Developmental patterns of epigenetic events in the mammalian organ of Corti may help elucidate targets toward developing new therapeutics in the treatment of hearing loss and hearing regeneration. Our data indicates that cofactors of repressive complexes such as NuRD and PRC2 are present in both FVB/NJ and C57BL/6J wild type organ of Corti during the first postnatal week. HDAC1, HDAC2, KDM1A, and CHD4 are all highly present in OHCs, IHC, DCs, OPC, IPC, IPh, LER, and GER just prior to birth at E18.5. These NuRD cofactors remain present in the OHCs, IHC, DCs, OPC, IPC, IPh, and GER from P0 until P4. At P6, these cofactors of the NuRD complex are mostly undetectable by immunofluorescence within the organ of Corti, completely lost by P7, returns at P8, and remain present through hearing onset at P21. Surprisingly, there was no discernible difference in the expression or staining pattern between the two wild type strains tested, which suggests that these epigenetic factors are fundamentally required for postnatal organ of Corti maturation.

Although some variability in the presence of HDAC1, HDAC2, KDM1A, and CHD4 exists, the variability in staining may be explained by the location within the organ of Corti, basal to apical. Immunofluorescence for HDAC1, HDAC2, KDM1A, and CHD4 was lost progressively within the organ of Corti from base to apex. During development, the organ of Corti matures in a similar pattern (base to apex) (Ruben, 1967), which may explain some of the variability seen in the HDAC1, HDAC2, KDM1A, and CHD4 staining pattern for specific cell types. Differential rates of protein degradation between cell types within the organ of Corti may also contribute to variability in the staining pattern, since immunofluorescence visualization is dependent upon a threshold level of protein availability. However, the large degree of overlap in HDAC1, HDAC2, KDM1A, and CHD4 staining that exists both temporally and spatially indicates that the NuRD repressive complex functions to help regulate proper gene expression during postnatal inner ear development.

The disparity between mRNA and protein detection at P7 for components of the NuRD complex is somewhat confounding. However, producing too little or too much of a single subunit of a protein complex can compromise the proper assembly of the entire complex. Many regulatory mechanisms occur after mRNAs are produced to precisely time proper complex assembly including cap-dependent translation initiation, ribosomal entry, and mRNA sequestration by miRNAs and RNA binding proteins (Spriggs et al., 2010; Vogel and Marcotte, 2012). Recent work has shown that mRNA from the myogenic determination gene *Myf5* is sequestered by *miR-31* in mRNP granules to precisely regulate cellular differentiation. Altogether these data suggest that the precise regulation of both terminal cellular differentiation and NuRD complex assembly may contribute to the 1 day discordance between mRNA expression levels and protein detection.

During development, NuRD may recruit additional repressive complexes such as PRC2 to specific target genes (Reynolds et al., 2012). Our data show that the PRC2 enzymatic subunit, EZH2, is present at E18.5 and P0 in a similar pattern of expression as the NuRD subunit, HDAC1, HDAC2, KDM1A, and CHD4. Interestingly, EZH2 is not detectable from P2 to P4 in the organ of Corti but returns throughout the organ of Corti by P6 and remains present through P21. These data suggest that NuRD and PRC2 may have a combinatorial role for regulating gene expression at specific developmental time points, but NuRD and PRC2 are differentially present within the organ of Corti from P2 to P7. These data together suggest that although NuRD may recruit PRC2 to specific target genes during postnatal inner development, both NuRD and PRC2 also have independent functional roles for regulating the chromatin state.

Histone modifications made by NuRD and PRC2 are typically repressive marks that silence the expression of specific-target genes during cellular transitions including cell fate specification, cellular differentiation, and tissue development (Hernandez et al., 2011; Lande-Diner et al., 2007; LeBoeuf et al., 2010; Maegawa et al., 2010; McCabe et al., 2012; Miranda and Jones, 2007; Wang et al., 2007; Wang et al., 2009; Yamaguchi et al., 2010). Genes required for proliferation (*mTert*) and cell fate specification (*Atoh1*) were increasingly down regulated between P0 and P7 in the organ of Corti, which is consistent with reports related to organ of Corti quiescence and maturation during neonatal development. However, these developmental time points also correlate with increased expression of genes such as *Prestin, Dnmt3a*, and *Dnmt3b* which are required for terminal cellular differentiation. Although NuRD and PRC2 are unlikely to directly regulate genes whose expression is elevated, the increased expression of these genes corresponds with the role of NuRD and PRC2 in terminal cellular differentiation during mammalian tissue development.

An age-related decline in proliferative and reprogramming capacity in the mammalian cochlea has been shown both *in vitro* and *in vivo* (Driver et al., 2013; Izumikawa et al., 2005; Kelly et al., 2012; Liu et al., 2012; Malgrange et al., 2002; Oshima et al., 2007; Ruben, 1967; Sinkkonen et al., 2011; Waldhaus et al., 2012; Zheng and Gao, 2000). *In vivo* studies of hair cell regeneration found that both transient and irreversible induction of ectopic *Atoh1* in supporting cells during the first postnatal week, leads to the formation of hair cell-like cells (Kelly et al., 2012; Liu et al., 2012). However, induction of *Atoh1* at later postnatal ages (P8-P14) showed a dramatic decrease in the capacity of supporting cells to form hair cell-like cells, and by P30, induction of *Atoh1* no longer leads to the formation of hair cell-like cells (Kelly et al., 2012; Liu et al., 2012). These data are consistent with our findings that during the first postnatal/neonatal week, epigenetic alterations are occurring within the organ of Corti. The presence of repressive complexes such as NuRD and PRC2 suggests that silencing histone marks may change the chromatin state such that transcription factors no longer have adequate access to target genes required for supporting cell to hair cell conversion. Additionally, these data correlate with our gene expression profiles showing a dramatic increase in *Hdac1, Hdac2, Kdm1a, Chd4, Ezh2, Dnmt3a*, and *Dnmt3b* mRNA levels at P7. These gene products are all associated with a global repression of gene transcription (Cheng et al., 2003; McCabe et al., 2012; Palmieri et al., 2009; Reynolds et al., 2012) and likely play a role in altering the reprogramming capacity of the mammalian cochlea.

Cellular reprogramming has extraordinary potential as a therapeutic for hearing loss. Epigenetic effects on cellular reprogramming have been characterized in iPSCs. Similar to cellular reprogramming in the inner ear, reprogramming somatic cells to a pluripotent state is a long inefficient process with most cells failing to reprogram (Cohen and Melton, 2011; Hanna et al., 2010; Huangfu et al., 2008; Kim et al., 2010; Li et al., 2009; Mikkelsen et al.,

2008; Yamanaka, 2009). Studies have found that under normal reprogramming condition (transcription factor induction) somatic cells induced to become iPSCs retain the epigenetic memory of their donor cell (Cohen and Melton, 2011; Hanna et al., 2010; Huangfu et al., 2008; Kim et al., 2010; Li et al., 2009; Mikkelsen et al., 2008; Yamanaka, 2009). The epigenetic memory of the donor cell greatly influences the iPSCs capacity to become other cell types but iPSCs do retain their ability to be easily converted back to the original donor cell type (Cohen and Melton, 2011; Hanna et al., 2010; Huangfu et al., 2008; Kim et al., 2010; Li et al., 2009; Mikkelsen et al., 2008; Yamanaka, 2009). The speed and efficiency of cellular reprogramming to an iPSC state can be enhanced by epigenetic inhibitor treatment. Donor cells treated with inhibitors against HDACs, DNA methyltransferases, histone methyltransferases and/or histone demethylases have a faster more efficient reprogramming capacity (Huangfu et al., 2008; Kim et al., 2010; Papp and Plath, 2011). Our data provides evidence that HDACs (*Hdac1* and *Hdac2*), DNA methyltransferases (*Dnmt3a* and *Dnmt3b*), histone methyltransferases (*Ezh2*), and histone demethylases (*Kdm1a*) are expressed postnatally within the organ of Corti, and may be targets for drug inhibition to increase the capacity, speed, and efficiency of reprogramming a supporting cell into a hair cell.

Acknowledgments

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Highlights

1. Histone deacetylases are present in the neonatal organ of Corti
2. Neonatal organ of Corti expresses lysine-specific histone demethylase 1 (KDM1A)
3. The NuRD complex is present within the neonatal inner ear
4. PRC2 is present in the neonatal cochlea and may function in combination with NuRD
5. Repressive histone modifications correspond with altered gene expression

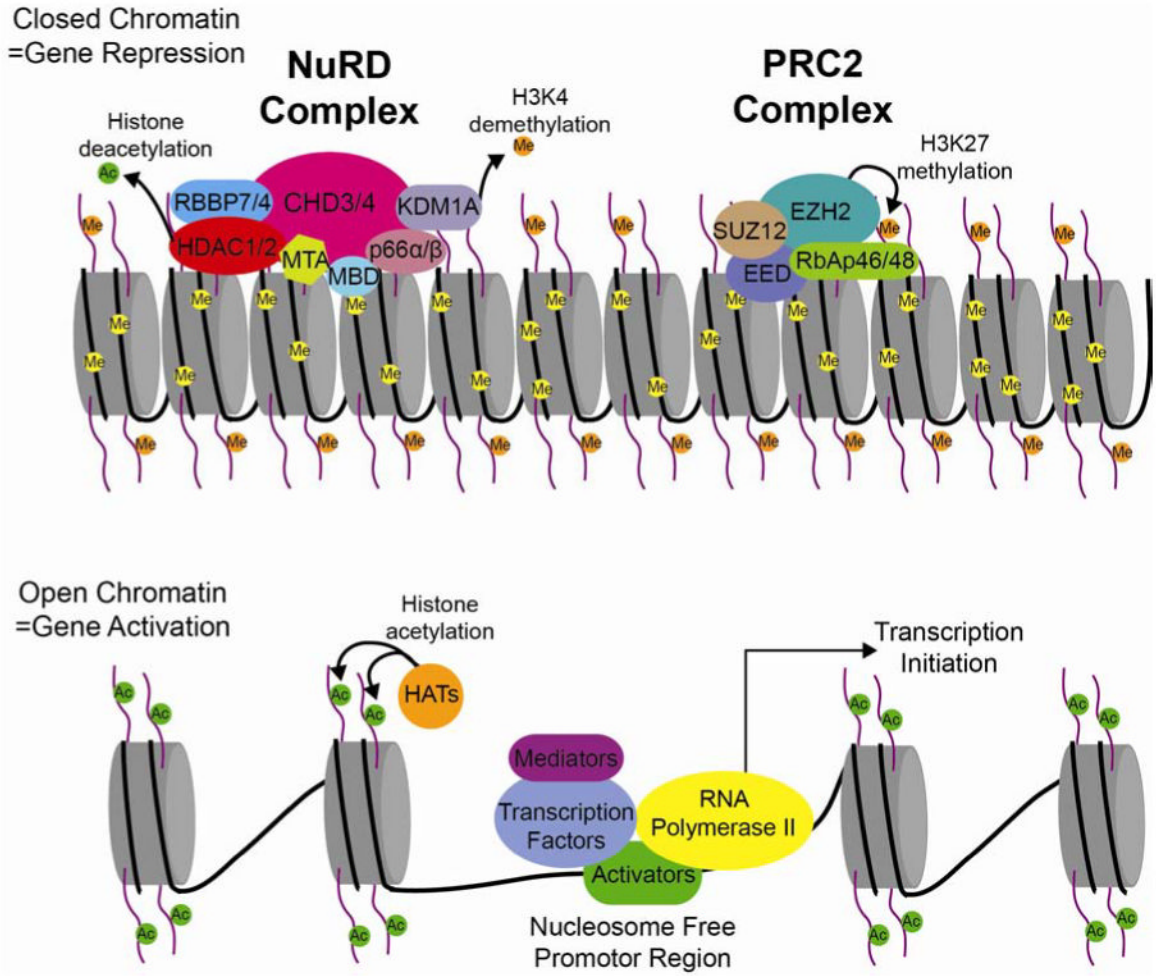

Figure 1.

Cartoon diagram depicting the nucleosome dense, compacted chromatin associated with gene repression (upper portion), compared to the open more relaxed chromatin of an actively transcribed gene (bottom portion). The NuRD and PRC2 complexes modify the chromatin structure through histone modifications to initiate and maintain gene repression.

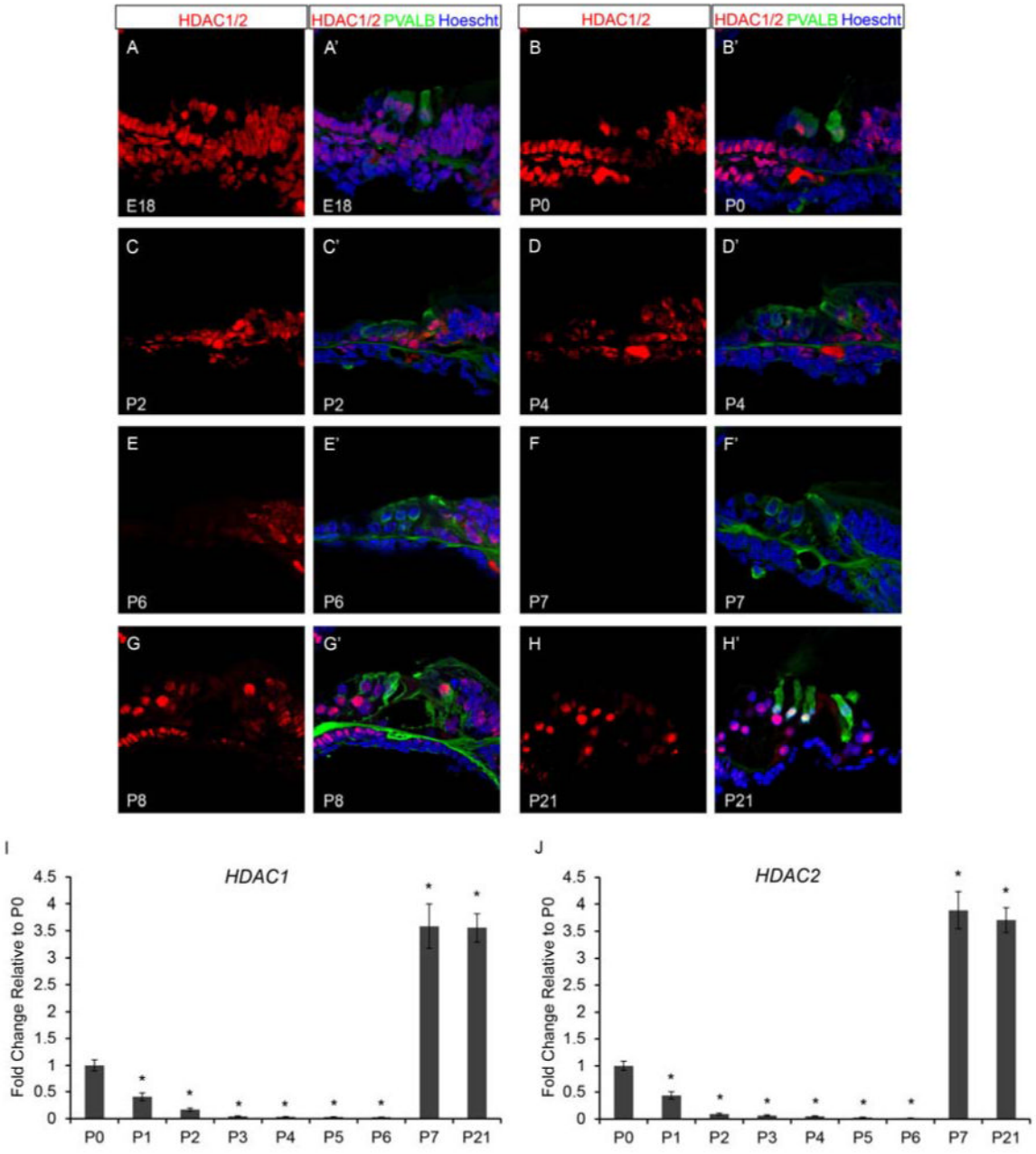

Figure 2.

Neonatal murine organ of Corti expresses histone deacetylases, HDAC1 and HDAC2. Immunofluorescence of wild type neonatal mouse cochlea was performed using antibodies against HDAC1 and 2 (red), Parvalbumin (PVALB) (green), and Hoechst (blue). Taqman gene expression assays were done on microdissected organ of Corti. Expression levels are relative to endogenous gene controls *18S, GAPDH*, and *Actb*. Fold changes are shown relative to P0. (A & A') HDAC1/2 are found throughout the organ of Corti at E18.5. (B & B') HDAC1/2 are still present in a majority of cell types in the organ of Corti at P0. (C-D') Staining for HDAC1/2 appear reduced at P2 and P4 compared to E18.5 and P0. (E & E') P6 organ of Corti retains some HDAC1/2 label in the OPCs, IPCs, IPhs, and IHCs. (F & F') HDAC1/2 staining is no longer apparent in the organ of Corti by P7. (G-H') HDAC1/2

reappears in the organ of Corti by P8 and continues to be detected at P21 (I) Expression of *Hdac1* and *Hdac2* are very similar at each time point analyzed. *Hdac1* and *Hdac2* expression steadily decreases from P0 to P6, then at P7 and P21 expression is increased compared to the P0 time point. All representative images were taken from the middle turn of the cochlea. **P<0.05* by Data Assist Software.

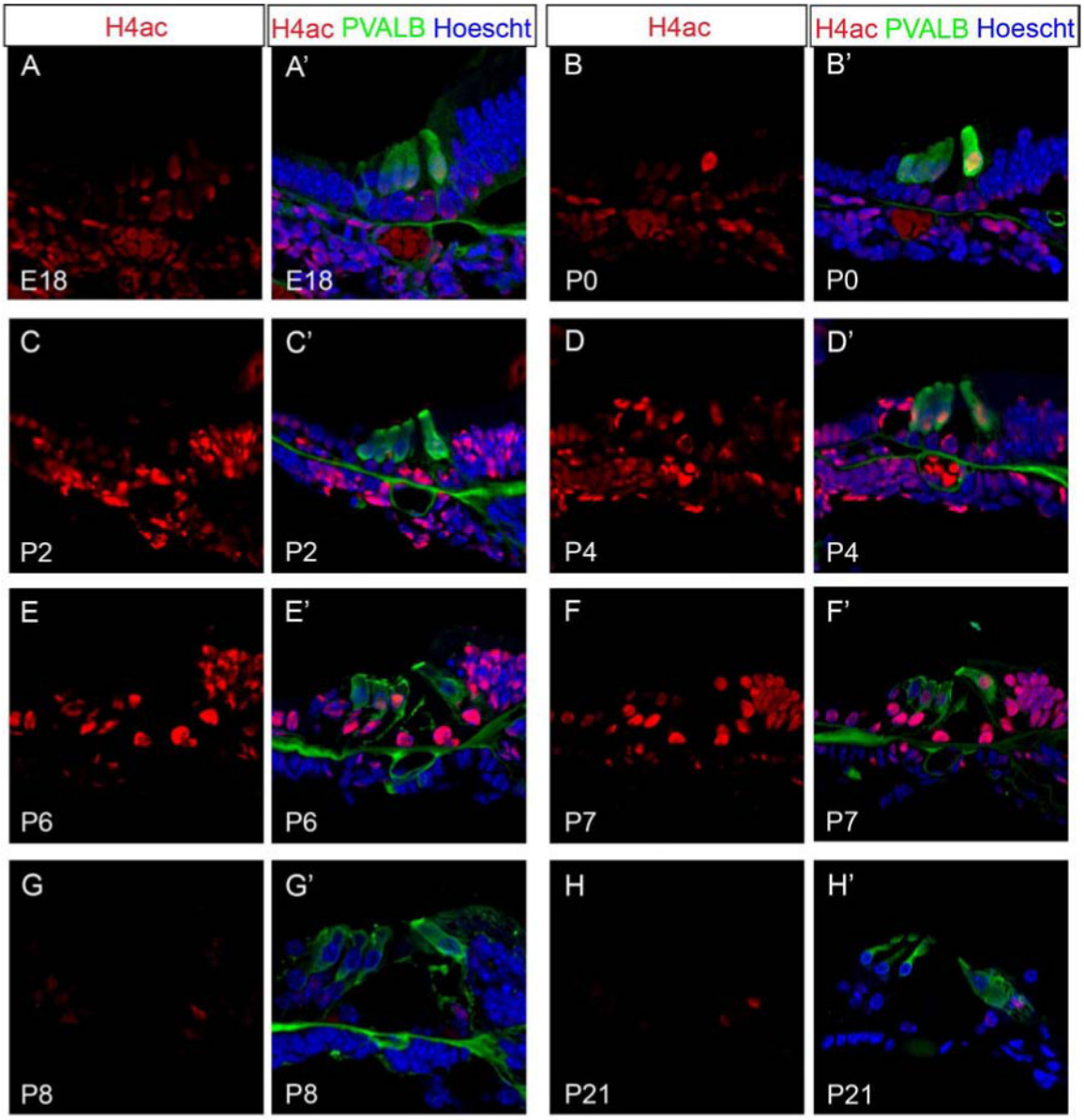

Figure 3.

Histone H4 pan-acetylation staining appears inversely correlated with HDAC1/2 staining during postnatal inner ear development. Immunofluorescence of wild type postnatal mouse organ of Corti was performed using antibodies against H4ac (red), PVALB (green), and Hoechst (blue). Confocal laser intensity and gain were kept consistent between samples and ages. (A-H') H4ac staining increases from E18.5 to P7, while at P8 H4ac staining is reduced and remains low through P21 in the organ of Corti. All representative images were taken from the middle turn of the cochlea.

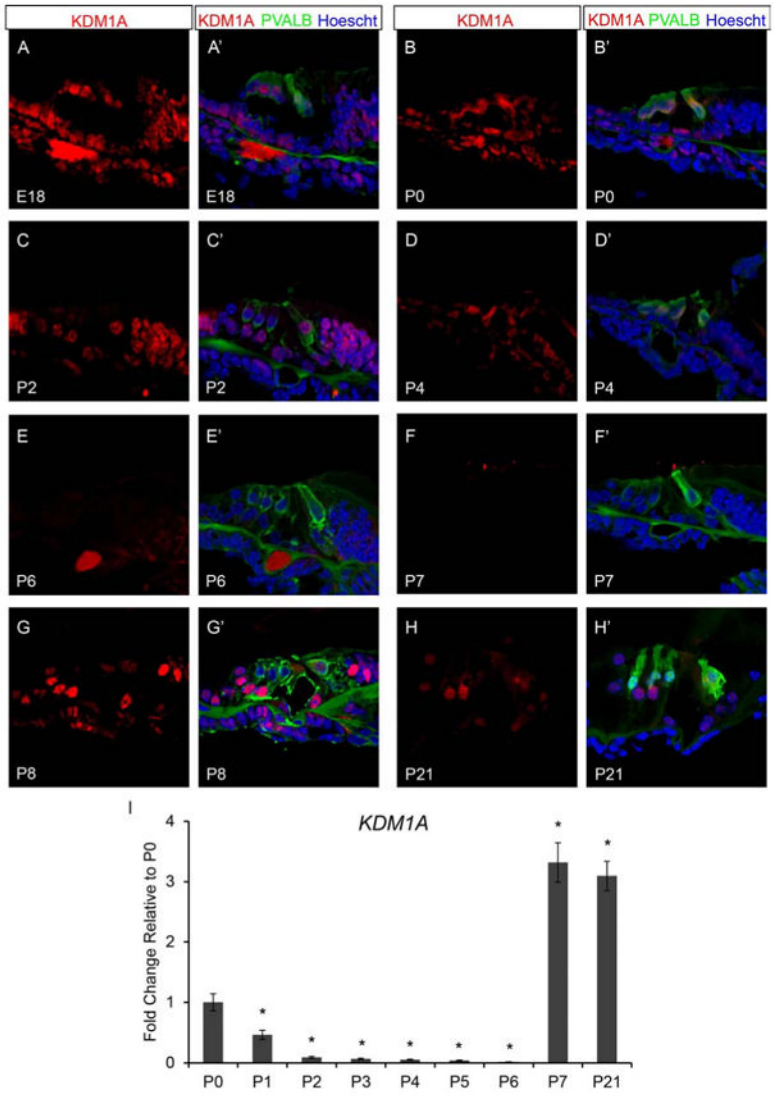

Figure 4.

Histone demethylase, KDM1A is detected in the neonatal mouse cochlea. Immunofluorescence of wild type neonatal mouse organ of Corti was performed using antibodies against KDM1A (red), PVALB (green), and Hoechst (blue). Taqman gene expression assays were done on microdissected organ of Corti. Expression levels are relative to endogenous gene controls *18S, GAPDH*, and *Actb*. Fold changes are shown relative to P0. (A & A') KDM1A are found throughout the organ of Corti at E18.5. (B & B') KDM1A is still present in most cell types in organ of Corti at P0. (C & C') Staining is present but less apparent in the organ of Corti by P2. (D & D') Staining for KDM1A is variable in IHCs, DCs, OPCs, and IPCs but is retained in OHCs, IPhs, and GER cells at P4. (E-F') Very little KDM1A label remains apparent in the organ of Corti at P6, and is undetectable by P7. (G-H') KDM1A is detected again in the organ of Corti by P8 and continues to be present through P21. (I) *Kdm1a* expression levels steadily decrease from P0 to P6, but is increased at P7 and P21 compared to the P0 time point. All representative images were taken from the middle turn of the cochlea. **P<0.05* by Data Assist Software.

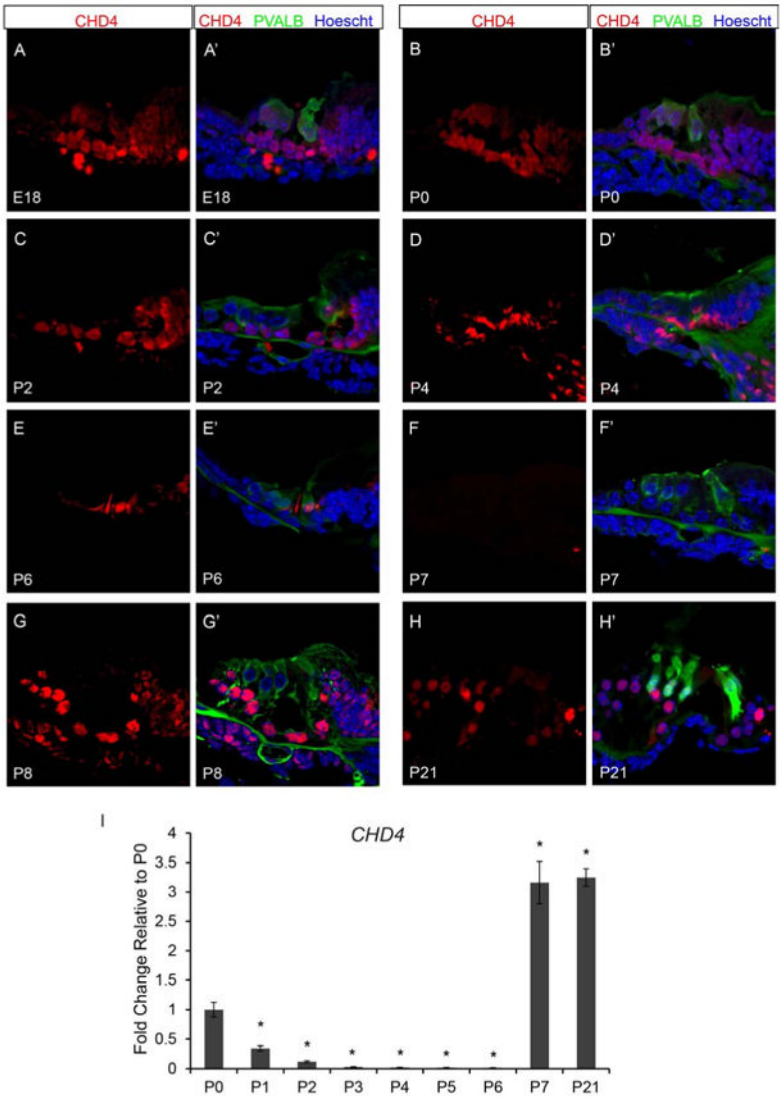

Figure 5.

The core subunit of the NuRD repressive complex, CHD4, is detected in a similar pattern of expression as HDAC1, HDAC2, and KDM1A. Immunofluorescence of wild type organ of Corti was performed using antibodies against CHD4 (red), PVALB (green), and Hoechst (blue). Taqman gene expression assays were done on microdissected organ of Corti. Expression levels are relative to endogenous gene controls *18S, GAPDH*, and *Actb*. Fold changes are shown relative to P0. (A & A') CHD4 is highly present throughout the organ of Corti at E18.5. (B-C') From P0 to P2, CHD4 continues to be detected in the vast majority of cells in the organ of Corti. (D & D') While at P4, CHD4 continues to be detected throughout the organ of Corti but appears reduced in intensity. (E & F') By P6, CHD4 staining is only found in the IHCs of the organ of Corti, and is no longer apparent by P7. (G-H') KDM1A is detected again in the organ of Corti by P8 and continues to be present through P21. (I) Similar to *Hdac1, Hdac2*, and *Kdm1a, Chd4* mRNA levels steadily decrease in expression from P0 to P6, and then at P7 and P21 expression is increased compared to the P0 time point.. All representative images were taken from the middle turn of the cochlea. **P<0.05* by Data Assist Software.

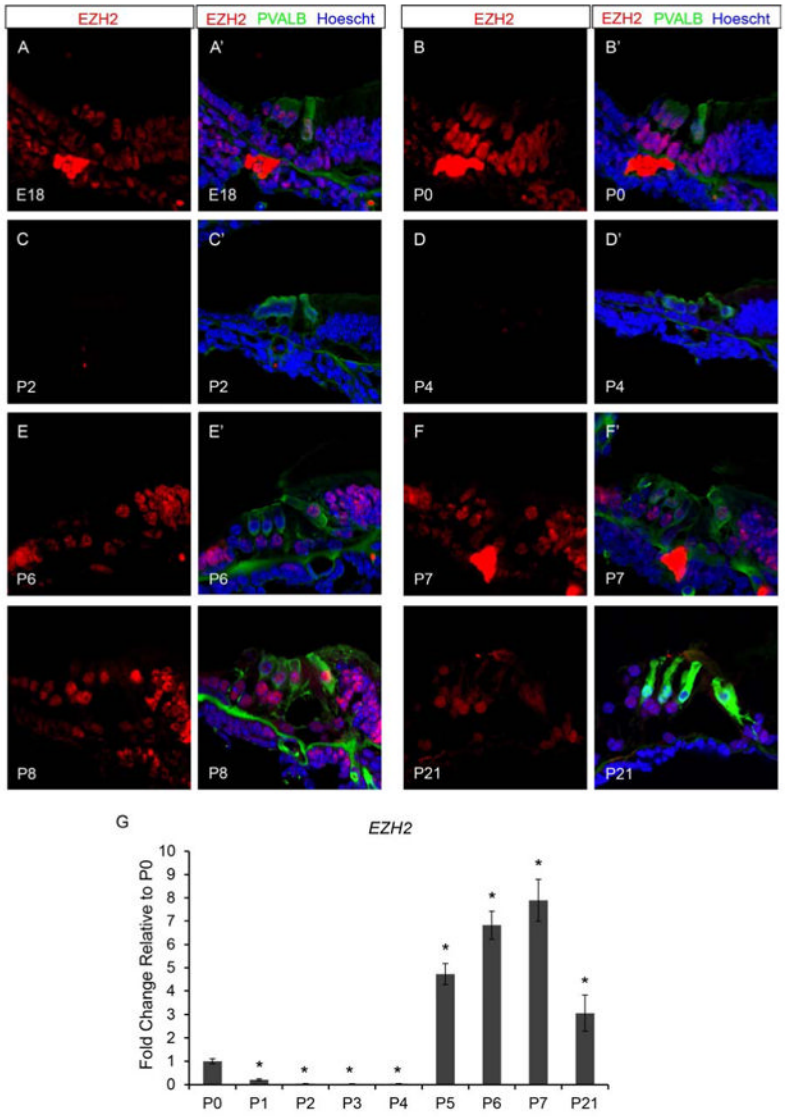

Figure 6.

Neonatal murine organ of Corti expresses the PRC2 enzymatic subunit, the histone methyltransferase, EZH2. Immunofluorescence of wild type neonatal mouse cochlea was performed using antibodies against EZH2 (red), PVALB (green), and Hoechst (blue). Taqman gene expression assays were done on microdissected organ of Corti. Expression levels are relative to endogenous gene controls *18S, GAPDH*, and *Actb*. Fold changes are shown relative to P0. (A-B') EZH2 is detected throughout the organ of Corti at both E18.5 and P0 time points. (C-D') Staining for CHD4 is no longer apparent in the organ of Corti from P2 to P4. (E & E') At P6, EZH2 staining is detected again in a majority of cell types in the organ of Corti. (F & H') EZH2 is present throughout the organ of Corti by P7 and remains detectable from P8 to P21. (I) *Ezh2* expression rapidly declines from P0 to P1, and expression remains very low through P4. From P5-P7 and at P21, *Ezh2* expression is increased in the organ of Corti compared to the P0 time point. All representative images were taken from the middle turn of the cochlea. **P<0.05* by Data Assist Software.

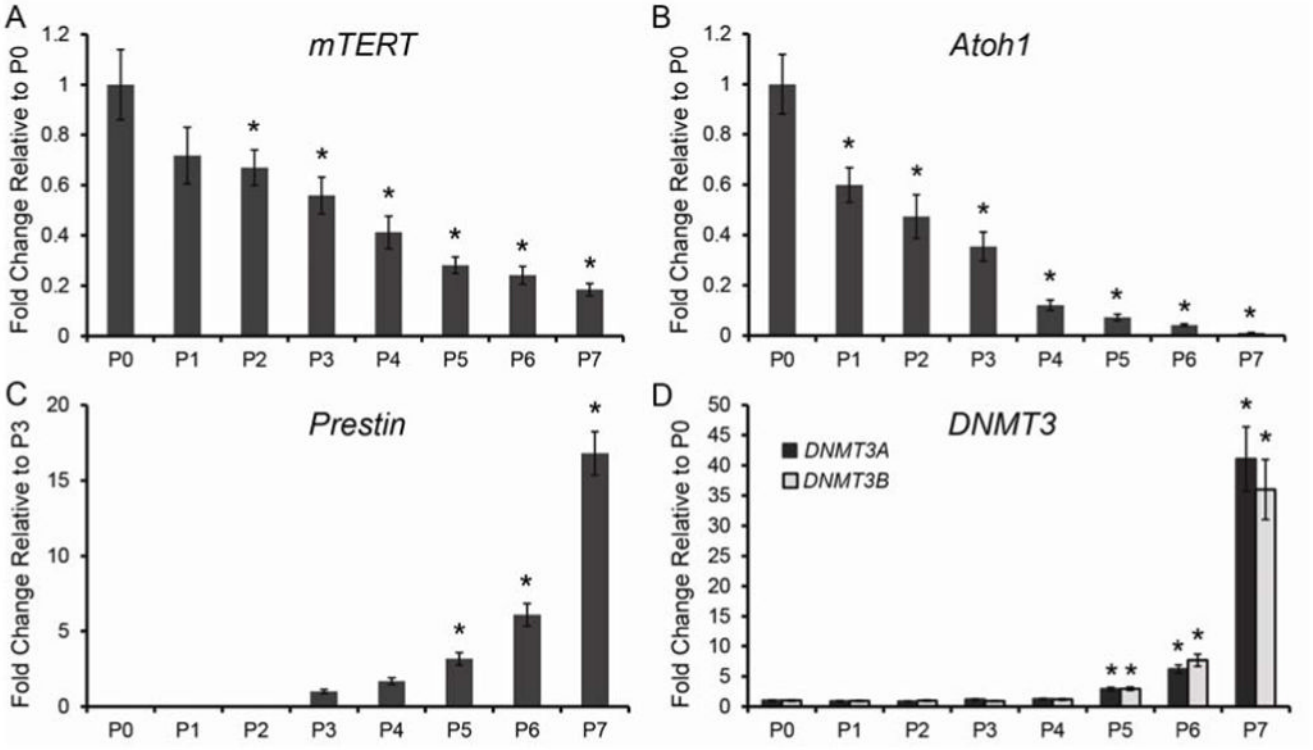

Figure 7.

Organ of Corti maturation coincides with alterations gene during neonatal development. TaqMan gene expression assays were done on microdissected organ of Corti. Expression levels are relative to endogenous gene controls *18S, GAPDH*, and *Actb*. Fold changes are shown relative to P0 for all genes except *Prestin* which is relative to the P3 time point. (A and B) Expression of both *mTert* and *Atoh1* steadily decrease from P0 to P7. (C) *Prestin* expression was not readily detectable in the organ of Corti until P3, and then *Prestin* expression levels continue to increase from P4 to P7. (D) *Dnmt3a* and *Dnmt3b* have low levels of expression between P0 and P6, and then expression of *Dnmt3a* and *Dnmt3b* increases dramatically at P7. **P< 0.05* by Data Assist Software.