Auditory hair cell defects as potential cause for sensorineural deafness in Wolf-Hirschhorn syndrome

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Abstract

WHSC1 is a histone methyltransferase (HMT) that catalyses the addition of methyl groups to lysine 36 on histone 3. In humans, WHSC1 haploinsufficiency is associated with all known cases of Wolf-Hirschhorn syndrome (WHS). The cardinal feature of WHS is a craniofacial dysmorphism, which is accompanied by sensorineural hearing loss in 15% of patients. Here, we show that WHSC1-deficient mice display craniofacial defects that overlap with WHS including cochlea anomalies. While auditory hair cells are specified normally, their stereocilia hair bundles required for sound perception, fail to develop the appropriate morphology. Furthermore, the orientation and cellular organisation of cochlear hair cells and their innervation are defective. These findings identify, for the first time, the likely cause of sensorineural hearing loss in WHS patients.
Introduction

Epigenetic processes, including histone modifications by specific enzymes, are crucial for controlling gene expression, but are often altered in various cancers and other disease conditions. One such histone modifying enzyme is Wolf-Hirschhorn syndrome candidate 1, \textit{WHSC1} (also known as \textit{NSD2} and \textit{MMSET}), mutations in which cause cancer and the human disorder, Wolf-Hirschhorn syndrome (WHS; OMIM 194190). WHS is a rare condition with an estimated prevalence of 1/20,000 to 1/50,000 births and a female predilection of 2:1 (Battaglia et al., 1999; Bergemann et al., 2005; Maas et al., 2008). It is caused by the deletion of two Wolf-Hirschhorn syndrome critical regions – WHSCR-1 (Wright et al., 1997) and WHSCR-2 (Zollino et al., 2003). WHSCR-1 is located ~2Mb near the end of chromosome 4p16.3 and contains two genes in close proximity, \textit{WHSC1} and \textit{WHSC2} (Wright et al., 1997). WHSCR-2 is located between 1.4 and 1.9Mb near the end of 4p16.3 and the locus contains a number of genes, including fibroblast growth factor receptor 3 (\textit{FGFR3}), leucine zipper-EF-hand containing transmembrane protein 1 (\textit{LETM1}), Msh homeobox 1 (\textit{MSX1}) and \textit{WHSC1} (Dimmer et al., 2008; Simon and Bergemann, 2008; Zollino et al., 2003). Although most of the deletions are \textit{de novo}, approximately 20% of the cases arise from unbalanced chromosome translocation t(4;8)(p16;23) inherited from either parent, probably as a result of gene duplication (Bergemann et al., 2005). \textit{WHSC1} is considered the top candidate gene, as it is consistently haploinsufficient (as part of a larger deletion) in all known cases of WHS (Bergemann et al., 2005; Van Buggenhout et al., 2004).

\textit{WHSC1} functions as a histone methyltransferase (HMT) to regulate gene expression in both embryonic and adult tissue (Brito et al., 2009; Martinez-Garcia et al., 2011). However, while the activity of \textit{WHSC1} is controversial, the consensus is that it catalyses methylation of lysine 36 residues on histone 3 (H3K36me), when presented with nucleosomes, the main components of chromatin (Li et al., 2009; Marango et al., 2008; Wagner and Carpenter, 2012). Like all other H3K36-specific HMTs identified thus far, \textit{WHSC1} contains the catalytic SET domain (Wagner and Carpenter, 2012). It also contains the chromatin binding domain, proline-tryptophan-tryptophan-proline (PWWP), which interacts with H3K36me, a plant homeodomain (PHD), and a high-mobility group (HMG) DNA binding domain (Wang and Carpenter, 2012). The HMG domain of \textit{WHSC1} can interact with the DNA-binding domain of the androgen receptor (AR) and in the presence of
the ligand, enhances AR-mediated transcriptional activation, thereby implicating WHSC1 in the promotion of prostate carcinogenesis (Kang et al., 2009).

WHS is a contiguous gene syndrome in which the deletion size varies among affected individuals, with larger deletions resulting in more severe phenotypes. Prognosis thus depends on the diagnosis: most severe cases are stillborn; ~35% die within two years, and those who survive into adulthood only make slow but steady progress in growth (Shannon et al., 2001; Zollino et al., 2003). The major features of the syndrome include a distinctive craniofacial appearance (broad, flat nasal bridge, prominent glabella, short philtrum, micrognathia, and ocular hypertelorism), short stature due to growth retardation and global developmental delay, intellectual disability, and seizures. Speech problems, genitourinary abnormalities and other craniofacial manifestations such as proptosis, cleft palate, cleft lip and defective dentition are also common (Battaglia et al., 1999, 2001; 2008; Bergemann et al., 2005; Maas et al., 2008; Shannon et al., 2001; Tachdjian et al., 1992; Van Borsel et al., 2004; Verbrugge et al., 2009; Zollino et al., 2008). The deletion of WHSC1 is associated with many characteristic WHS features, including the distinctive facial appearance (Bergemann et al., 2005; Van Buggenhout et al., 2004). WHSC1 mouse mutant phenotypes resemble some WHS phenotypic features in human, including developmental delay, growth retardation, heart, midline and craniofacial defects (Nimura et al., 2009). While heterozygous mice are viable and show varying degrees of the WHS phenotype, homozygous mice show more severe phenotype and die shortly after birth (Nimura et al., 2009). Mouse knockout studies associate LETM1 deletion with seizures and abnormal neuronal activity (Zollino et al., 2003; 2008), while dental and cleft abnormalities may be due to loss of MSX1 function (Nieminen et al., 2003).

The syndrome is also characterised by otological manifestations such as poorly formed ears (microtia), nystagmus, preauricular cysts or fistula (pits), epicanthal folds, low-set ears, otitis media, and hearing loss (Battaglia et al., 1999, 2001; 2008; Bergemann et al., 2005; Chen et al., 2011; Maas et al., 2008; Shannon et al., 2001; Tachdjian et al., 1992; Van Borsel et al., 2004; Verbrugge et al., 2009; Zollino et al., 2008). Yet, the causative gene (or genes) for hearing loss in WHS has not been identified. Deletion of FGFR3 may contribute to hearing loss as FGFR3−/− mice are deaf due to disorganisation of cochlear supporting cells and possibly innervation of the outer hair cells (Colvin et al., 1996; Hayashi et al., 2007; Mansour et al., 2009, 2013; Puligilla et al., 2007). While hearing loss in WHS patients is mostly conductive, and often secondary to chronic otitis media (Battaglia et al., 1999; 2008;
Lesperance et al., 1998; Ulualp et al., 2004), clinical studies also identified a number of cases with sensorineural hearing loss, both with and without conductive hearing loss (Battaglia and Carey, 1999; Lesperance et al., 1995; 1998; Simon and Bergemann, 2008). However, the molecular and cellular mechanisms responsible for sensorineural defects are not known.

In this study, we examine the inner ears of WHSC1 deficient mice and describe for the first time defects in auditory hair cells of the cochlea associated with loss of WHSC1. Our observations provide new insights into the role of histone modifying enzymes in cochlear development and into the pathophysiology of sensorineural hearing loss in WHS patients.

Materials and Methods

WHSC1 mice

The breeding and generation of WHSC1 mutant mice are described in Nimura et al (2009).

Histology and skeletal preparation

Embryos for histological analysis were decalcified in 10% EDTA/0.1M Tris-HCL (pH8.0) for two weeks at 4°C. This was followed by dehydration in an ascending ethanol series and embryos were processed for paraffin wax sectioning (8μm). Picro-Sirius red and alcian blue trichrome staining (Puchtler et al., 1973) was performed on the sections. Skeletal preparation was performed as described in Rice et al. (2003).

In-situ hybridisation (ISH) and immunohistochemistry (IHC)

The inner ear was fixed in 4% para-formaldehyde (PFA) in phosphate buffered saline (PBS) overnight at 4°C, the cochlea and vestibule were dissected in DEPC-treated PBS. ISH was performed using Dig-labelled ATOH1 riboprobe (gift from MA Basson). Antibodies used were rabbit MYO7A (1:1000, Proteus), rabbit acetylated α-tubulin (1:500, Abnova), rabbit
p75NTR (1:500, Abnova), detected with anti-rabbit AlexaFluor 488 or 568 (1:1000, Invitrogen), mouse NF-M (1:100, Invitrogen), detected with anti-mouse AlexaFluor 488 or 568 (1:1000, Invitrogen) and AlexaFluor 488 or 568 Phalloidin (1:1000, Invitrogen).

Microscopy and imaging

Images were captured using a SMZ1500 stereo microscope (Nikon), an Axiovert 200M (Zeiss) compound microscope and a TCS SP5 confocal (Leica) microscope. Figure panels were assembled in Photoshop CS (Adobe).

Cell counts and measurements

The total number of hair cells was counted on photographs of immunostained cochleae. For counts by cochlear region, the cochlear length was divided into equal basal, middle and apical portions, and the total number of hair cells was counted in a 200µm field. Cochlear length was determined using Fiji (ImageJ) and drawing a line from the base of the cochlea to the apex. Statistical comparisons were made using unpaired, two-tailed Student’s t-tests.

Results

Craniofacial manifestation of WHS clinical features in WHSC1<sup>−/−</sup> mice

The characteristic craniofacial dysmorphism of WHS patients is associated with haploinsufficiency of the WHSC1 gene (Bergemann et al., 2005; Van Buggenhout et al., 2004) and replicated in WHSC1 mutant mice (Nimura et al., 2009). Here, we confirm the published phenotypes. WHSC1<sup>−/−</sup> mutants are developmentally delayed and are smaller in size compared to control or heterozygous littermates (Nimura et al., 2009). Close examination of the craniofacial structures reveals a decrease in the size of WHSC1<sup>−/−</sup> mutant heads by 22% (1230±9 µm, n=3 vs. 1577±4 µm, n=3; p=4x10<sup>7</sup>, Fig.1A-D) and a temporal protrusion of the skull (Fig.S1B, C, E, F, arrowheads) that also characterises WHS patients. Furthermore,
WHSC1−/− mice have open eyelids, with widely spaced protruding eyes compared to WHSC1+/+ or WHSC1+/- littermates (Fig.1A, B; not shown). The protruding eye phenotype is also observed in some WHS patients with hypertelorism (Bergemann et al., 2005; Van Buggenhout et al., 2004). About 15% of WHS patients exhibit sensorineural hearing loss (Toriello and Smith, 2013), although the cause remains unknown. To determine whether sensorineural hearing loss is associated with WHSC1 deletion, we analysed inner ear development in WHSC1 mutant mice. These mutants do not survive to postnatal day 10 (P10) and the number of viable WHSC1−/− embryos are greatly reduced from late embryonic stages (Nimura et al., 2009). We therefore focused on E16.5 and E18.5, when the inner ear is fully formed and sensory hair cells are at different stages of differentiation and maturation (Chen and Segil, 1999). WHSC1 mutants form pits in the outer ear (Fig.1E, F, arrow), similar to phenotypes observed in WHS patients presenting with preauricular cysts/pits and/or epicanthal folds (Bergemann et al., 2005; Van Buggenhout et al., 2004). Sections reveal the loss or underdevelopment (i.e., microtia) of the pinnae that results in pit formation (Fig.1G, H green dashed line; Fig.S2). The middle ear bones and the inner ear are largely normal, as is the eustachian tube and ear canal, even though they appear slightly constricted (Fig.1G, H; Fig.S2, dotted lines). Collectively, our observations confirm that WHSC1 mutants phenocopy WHS characteristics attributed to the deletion of WHSC1 including craniofacial abnormalities, external ear and eye anomalies.

WHSC1 is not required for the formation of hair cells

Although WHSC1 mutant inner ears are smaller, their gross morphology is normal. We next sought to determine whether the cellular anatomy of the cochlear and vestibular structures is affected in WHSC1 mutants. The transcription factor ATOH1 is expressed in cells committed to the hair cell lineage and functions upstream of the differentiation marker MYO7A (Bermingham et al., 1999; Chen and Segil, 1999). ATOH1 is expressed normally in vestibular (not shown) and in auditory hair cells in both WHSC1−/− and WHSC1+/- inner ears (Fig.2A, B; not shown). The size of the cochlea was slightly, but not significantly shorter in heterozygous animals (3264±41μm vs. 3276±65μm; n=3, p=0.8), but significantly decreased by 22% (2541±113μm vs. 3276±65μm; n=3, p=0.002) in homozygous mutants (Fig.2E), consistent with a global reduction in the size of the WHSC1−/− mutants. However, the number of cochlear turns is unaffected (Fig.2A, B) and the expression of MYO7A is initiated normally (Fig.2C,
D), suggesting that **WHSC1** is not required for cochlear duct elongation or the formation and differentiation of hair cells.

**Loss of WHSC1 function disrupts cochlear hair cell organisation**

Although **ATOH1** is expressed normally and hair cells differentiate along the length of the cochlea, we noticed a stronger and wider expression domain of **ATOH1** at the basal and middle turns of the cochlea in mutants (Fig.3A, B), prompting us to investigate the hair cell arrangement in more detail. Normally, cochlear hair cells are arranged in organised rows, with one row of inner hair cells (IHCs) and three rows of outer hair cells (OHCs) (Fig.3C). At E16.5, this organisation is already observed in the basal and mid-basal turns of the cochlea, while mid-apical and apical (except the apical tip) hair cells become organised by E18.5, as these are the last to mature, albeit the first to exit the cell cycle (Chen and Segil, 1999; Matei et al., 2005). Instead of four discernible rows, the mutant cochlea shows multiple (and somewhat ambiguous) hair cell rows (Fig.3C-F; n=4/5). While it is possible that this arrangement is due to a developmental delay, this is unlikely because we observe the same phenotype at E18.5 (Fig.4A-D; n=14/14): there are extra IHC and/or OHC rows, ranging from the normal 4 rows to an abnormal 6 rows in the basal and middle regions of **WHSC1**-/− cochlea (Fig.4C, D; n=14/14). A small proportion of heterozygous **WHSC1**+/− cochlea (20%) show the same hair cell phenotype, (n=8/40; Fig.S4G, I, arrowheads). In the mutant cochlea, the total number of hair cells does not significantly differ compared to wild type controls (3382±5 vs. 3385±2; n=3) or heterozygous littermates (3385±6 vs. 3385±2; n=3; Fig.4E), suggesting that it is the failure of hair cell arrangement along the length of the cochlear duct that ultimately results in their inappropriate accumulation in some regions. In support of this notion, the last set of differentiating hair cells in the apical turn appear more sparsely distributed, probably due to reduced numbers in this region (Fig.3A, B). This observation is confirmed at E18.5, where **MYO7A**+ hair cells are strongly reduced in the apical tip of the mutant cochlea when compared to controls (Fig.S3C, D). In particular, the number of OHCs are significantly reduced in the mutant apex (62±2 vs. 91±3; n=5, p=0.001), with a corresponding increase in the base (94±4μm vs. 78±2μm; n=5, p=<0.001; Fig.4F). The number of IHCs is less affected, with a slight but significant increase in the middle (27±0.7 vs. 26±0.6; n=5, p=0.03; Fig.4F). **WHSC1**+/− do not show any statistically significant changes as the phenotype is more subtle, but there are visibly more OHCs in the base (80±3 vs. 78±2;
n=5, p=0.2) and middle (89±3 vs. 85±5; n=5, p=0.3) turns of the cochlea (Fig.4F, Fig.5G, I, arrowheads). Thus, WHSC1 function is required as hair cells begin to align and arrange themselves into distinct rows along the length of the cochlea.

**WHSC1 mutants show abnormal hair cell shape and size but normal pillar cells**

To investigate the hair cell phenotype further we analysed E18.5 cochlea immunostained for MYO7A by confocal microscopy. We find a striking difference in size and shape between mutant and wildtype cochlear hair cells. While all hair cells appear similar in size in \( WHSC1^{+/+} \) cochlea, their size varies from normal to small in \( WHSC1^{-/-} \) mice (Fig.4A, B), and to a lesser extent in \( WHSC1^{+-} \) (Fig.5G). These differences seem to affect the intercellular space: in mutant mice some hair cells make contact with many other cells (without any intercellular gaps) whereas other cells (especially IHCs) are more spaced out, not establishing any contacts at all (Fig.5A-F). This hair cell disorganisation is also observed in sections (Fig.5G-J, arrowheads), where extra cells appear to be squeezed between existing cells (in this case OHCs, Fig.5H, J, asterisk). The inner pillar cells are present and appear to be normal (Fig.S4), although one inner pillar cell was missing in \( WHSC1^{+/} \) cochlea (Fig.S4I, J, arrow; n=1/13).

**Innervation is disrupted in \( WHSC1^{+/} \) and \( WHSC1^{-/-} \) cochleae**

As sensorineural hearing loss can also be caused by abnormalities in the bipolar spiral ganglia neurons that innervate hair cells, we performed immunolabelling for neurofilament (NF) to detect neuronal fibres. Spiral ganglion projections to IHCs appear to be normal but more fibres seem to target the OHCs (Fig.5G-J; Fig.6). In \( WHSC1^{+/} \) cochleae, the majority of fibres terminate on the IHCs, while some cross the tunnel of Corti, turn towards the base and form three rows of spiral fibres (Fig.6A-D). This arrangement of fibres is disrupted in both \( WHSC1^{+/} \) (Fig.6E-H; n=3/25) and \( WHSC1^{-/-} \) (Fig.6I-L; n=7/11) cochleae. Together these findings suggest that WHSC1 function is required for the organisation of spiral fibres, in addition to hair cell arrangement.
Stereocilia formation is severely perturbed in WHSC1 mutant cochlea

Hair cell stereocilia are critical for their function as mechanosensory cells and to transmit sound information to the auditory nuclei in the brain stem (Yoshida and Liberman, 1999). Stereocilia develop on the apical surface of each hair cell and they can be visualised by Phalloidin to label F-actin polymers. Before morphogenesis of the hair bundle stereocilia, a specialised primary cilium, the kinocilium, visualised by acetylated α-tubulin, forms at the apical surface of each hair cell. As the kinocilium moves towards the cell periphery, actin stereocilia begin to form around it into distinct morphology (inverted ‘V’ in OHCs and ‘C’ in IHCs). This arrangement of the hair bundle stereocilia is a manifestation of planar cell polarity (PCP; Ezan and Montcouquiol, 2013; Goodrich and Strutt, 2011; Sienknecht et al., 2014). At E18.5, hair cells in the base of the cochlea already show appropriate stereocilia morphology in control animals: they are aligned and orientated in the same direction (Fig.7A-C; Fig.S4A). While kinocilia and stereocilia are present in WHSC1+/− and WHSC1−/− mutants, they exhibit abnormal shape, length and orientation (Fig.7D-J; Fig.S4G, M). Frequently, stereociliary hair bundles appear to be localised around the cell periphery. In homozygous WHSC1−/− mice a substantial proportion of outer and inner hair cells lacks kinocilia or stereocilia (Fig.7D-J; Fig.S4G, M; asterisk). Taken together, our data suggest that WHSC1 is required during hair cell differentiation as the cells begin to organise themselves into distinct rows and form stereocilia, orientated in one direction.

Discussion

WHS patients show varying phenotypes including sensorineural and conductive hearing loss in approximately 40% of patients (Battaglia et al., 2008). To elucidate the possible cause of sensory defects, we analysed the inner ear of the WHSC1 mouse model for WHS. Our results reveal an important role for WHSC1 in auditory hair cell development, particularly during cellular organisation and stereocilia morphogenesis, and in hair cell innervation. Furthermore, the results provide new insights into the epigenetic regulation of hair cell polarity. WHS patients commonly present with otological manifestations such as preauricular and/or auricular abnormalities, and of them, almost half have chronic otitis media with effusion.
In addition, some patients also display sensorineural hearing loss, and a smaller percentage still, may have missing OHCs in areas within the basal region of the organ of Corti, but only in one ear (Ulualp et al., 2004). In the organ of Corti of WHSCI\(^{-/-}\) mice, IHCs and OHCs are missing within the apical turn in both ears. We provide evidence that this is not due to the lack of hair cell differentiation. Rather, we find that the hair cells fail to arrange appropriately along the length of the cochlea, resulting in their accumulation in the basal and medial turns. We also show that hair cell innervation is disrupted, which may contribute to sensorineural hearing loss. Our data identify haploinsufficiency of WHSCI as a potential cause of sensorineural hearing loss in WHS patients.

**WHSCI and PCP signalling**

Disorganisation of hair cells is a hallmark of defects in planar cell polarity (Ezan and Montcouquiol, 2013; Goodrich and Strutt, 2011; Sienknecht et al., 2014). Hair cells display both an extrinsic and intrinsic level of polarity, i.e. they are arranged in an organised pattern along the cochlea and stereocilia form at their apical surface (Ezan and Montcouquiol, 2013; Sienknecht et al., 2014; Tarchini et al., 2013). During its development, the cochlear epithelium elongates via convergent-extension movements, a mechanism controlled by core PCP signalling mediated through the RAC-JNK pathway. Defects in the core PCP components such as VANGL, result in a shortened cochlear duct and the failure of hair cells to organise appropriately, including the orientation (but not the formation) of stereociliary bundles (Ezan and Montcouquiol, 2013; Sienknecht et al., 2014; Torban et al., 2008). Mutations in a parallel pathway, the FAT signalling pathway, also results in hair cell defects, and VANGL2 and FAT4 genetically interact (Ezan and Montcouquiol, 2013; Saburi et al., 2008; Sienknecht et al., 2014). We observe the WHSCI mutant phenotype at the same time when hair cells respond to these extrinsic tissue polarity cues to organise themselves. Moreover, hair cells interpret these cues to drive cell-intrinsic polarity by positioning the basal body appropriately, which ultimately leads to an organised stereociliary hair bundle. This step is independent of core PCP genes, but involves RAC-PAK signalling, with LIS1 regulating localised RAC-PAK signalling, and maintaining stereocilia bundles during both fetal and postnatal development (Sipe et al., 2013). Conditional inactivation of LIS1 hair cells (LIS1\(^{-/}\)KO) affects their organisation into distinctive rows, cell shape and size, and stereocilia formation, while the cochlea length is normal (Sipe et al., 2013). Thus, the WHSCI\(^{-/-}\)
phenotype described here is remarkably similar to $LIS1^{cKO}$, but does not correlate with defects observed in core PCP mutants. While hair cell bundles are determined by positioning of the basal body, the organised rows of hair cells are regulated by a family of cell adhesion molecules called NECTINS (Togashi et al., 2011). NECTIN1, -2 and -3 are localised at the cell-cell contacts between hair cells, and the cellular organisation defects in $LIS1^{cKO}$ organ of Corti is likely to be caused by impaired NECTIN-mediated cell adhesion (Sipe et al., 2013; Fukuda et al 2014). Loss of $MYO7A$ in mice also results in stereocilia defects (Self et al., 1998), however, in both $LIS1$ and $WHSC1$ mutants, $MYO7A$ is expressed normally. Thus, the strong correlation of $WHSC1$ and $LIS1$ mutant phenotypes suggests that both may function in the same pathway. Future experiments will need to address the epistatic relationship between $LIS1$ and $WHSC1$, and the question of whether $WHSC1$ transcriptionally regulates $LIS1$ through histone modification.

**Contribution of WHSC1 and FGFR3 deletion to deafness in WHS patients**

Amongst the genes found in the WHSCR-2 is $FGFR3$, which when deleted in mice, results in profoundly deaf animals (Colvin et al., 1996; Puligilla et al., 2007). However, while hair cells in $FGFR3^{-/-}$ mice are normal, a subset of supporting cells, which are crucial for hair cell function, are defective. Specifically, inner and outer pillar cells, although present, fail to differentiate (Colvin et al., 1996; Hayashi et al., 2007; Puligilla et al., 2007). In addition, the sensory epithelium is wider, with an extra row of OHCs in the apex of the cochlea, accompanied by an increase in the number of Deiters’ cells (Hayashi et al., 2007). Inner pillar cells show limited p75$^{NTR}$ expression (Puligilla et al., 2007), but it is unclear whether these cells are completely absent or transformed into another cell type. In the $FGFR3$ mouse model for Muenke syndrome ($FGFR3^{p244R/+}$), Deiters’ cells transform into pillar cells (Mansour et al., 2009) and these two types of supporting cells can reversibly switch fates in an FGF-dependent manner (Mansour et al., 2013). In addition to supporting cell defects, the outer hair cells and their innervation by spiral ganglia fibres are also disrupted (Puligilla et al., 2007). Thus, FGF signalling via $FGFR3$ regulates the fate decision between two populations of supporting cells (Colvin et al., 1996; Hayashi et al., 2007; Mansour et al., 2009; 2013; Puligilla et al., 2007), influencing hair cell function indirectly. Therefore, $FGFR3$, like $WHSC1$, may contribute to sensorineural hearing loss in WHS patients (Battaglia and Carey, 1999). However, the process by which they cause hearing impairment differs considerably,
although remarkably, in both FGFR3 and WHSC1 mutants, the spiral fibres fail to organise into distinct rows.

WHS patients present with variable phenotypes. It is likely that deletion of both WHSC1 and FGFR3 contributes to sensorineural hearing loss in severe cases with large deletions in the critical region. However, hearing defects in patients with smaller deletions are likely to be associated with WHSC1 haploinsufficiency alone, since this is observed in every known case of WHS, while FGFR3 deletion is not. The incidence of sensorineural hearing loss in WHS is approximately 15% out of 40% of patients that present with hearing loss (Battaglia et al., 2008; Toriello and Smith, 2013). Interestingly, our results reveal that only 20% of the heterozygous WHSC1+/− cochlea show a hair cell phenotype suggesting that these mice model the human syndrome quite accurately. Furthermore, it suggests that more patients with conductive hearing loss may also exhibit sensorineural hearing loss than currently identified. As with all syndromes with intellectual disabilities, prognosis is often better if hearing loss can be diagnosed as early as possible. The identification of WHSC1 as an important histone modifier whose activity is necessary for stereocilia development opens a new route to our understanding of cochlea biology and hearing loss.

Complexity of WHSC1 function

The variability of the defects observed in WHS patients and in WHSC1+/− mice may be due to the interaction of WHSC1 with different proteins. During heart development, WHSC1 interacts with and negatively modulates the transcriptional activity of NKX2.5, an important heart determinant (Nimura et al., 2009). WHSC1 also interacts with β-CATENIN and transcriptionally regulates the cell cycle regulator CCND1 through H3K36 trimethylation (Toyokawa et al 2011). More recently, BRD4 and P-TEFb were identified as WHSC1 interacting partners, and together facilitate transcriptional elongation (Sarai et al., 2013). Furthermore, WHSC1 and HIRA, a histone H3.3-specific chaperone, interact to prolong H3.3 incorporation into activated genes (Sarai et al., 2013). In addition to histone methylation, WHSC1 promotes recruitment of NF-κB and p300 complexes to the promoters of NF-κB target genes, resulting in increased transcription of target genes including WHSC1 itself (Yang et al 2012). Finally, WHSC1 is phosphorylated in response to DNA double-strand breaks (Matsuoka et al., 2007; Pei et al., 2011), which mediates its recruitment to sites of DNA damage where it dimethylates H4K20 to recruit 53BP1 and promote DNA repair.
(Hajdu et al., 2011; Pei et al., 2011). This identifies WHSC1 as a component of the DNA damage response (DDR) machinery, and it has been proposed that the features of WHS attributed to \( WHSC1 \) function arise as a result of a defective DDR pathway (Hajdu et al., 2011). Whether this holds true for the cochlea remains to be determined as the specific interacting protein for WHSC1 in the organ of Corti has not been identified. However, because of the specific defects in \( WHSC1^{-/-} \) inner ears, potential candidates are components of the PCP machinery.

**Conclusion**

In summary, WHSC1 mutant mice provide a good model for understanding the causes of sensorineural hearing loss in WHS patients. We show that loss of WHSC1 function leads to the failure of hair cell organisation and of stereocilia formation in the cochlea, as well as innervation defects. Importantly, about 20% of heterozygous mice show a similar phenotype, matching closely with the incidence of sensorineural hearing loss observed in WHS affected individuals. This is particularly exciting since epigenetic modifications like histone methylation are reversible and therefore good drug targets.

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**Competing interests**

The authors declare no competing financial interests.
Author contributions

MA and AS designed the experimental concepts, KU generated the mouse embryos, MA performed the experiments and analysed the data, MA and AS wrote the manuscript.

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References


**Figures**

**Figure 1: Craniofacial phenotype of WHSCI mutant mice**

A-F: E16.5 wildtype and mutant heads and transverse sections stained for Picro-Sirius red trichrome (G, H). *WHSCI*<sup>-/-</sup> mice have the characteristic broad, flat nasal bridge, prominent glabella, short philtrum, and micrognathia observed in WHS patients as well as microtia and hypertelorism. The middle and inner ear morphology are normal. Scale bars: 500μm (A,F); 200μm (G,H). Arrowhead = eustachian tube, black asterisk = ear canal, yellow asterisk = middle ear bones, iec = inner ear capsule, v = vestibule, c = cochlea.
Figure 2: Development of cochlear and vestibular hair cells

A, B: E16.5 wildtype and mutant cochlea revealing ATOH1 expression. C, D: Immunolabelling of mutant cochlea and vestibule for MYO7A (green) counterstained with Hoechst to label nuclei (blue). Auditory and vestibular hair cell development is normal in WHSC1<sup>−/−</sup> mice. E: E18.5 cochleae stained with Hoechst. Scale bars: 200μm (A-D); 500μm (E). l = lateral crista, a = anterior crista, u = utricle, s = saccule.
Figure 3: Cochlear hair cells fail to arrange into organised rows

A, B: Low power images of E16.5 cochlea showing *ATOH1* expression. C-F: high power images of E16.5 cochlear hair cells showing normal expression of *ATOH1* but failure to arrange into organised rows. Scale bars: 200μm (A, B); 25μm (C-F). OHC = outer hair cell, IHC = inner hair cell.
Figure 4: Extra hair cells in the basal and middle regions of the cochlea

A-D: E18.5 wildtype and mutant organ of Corti immunostained for MYO7A (shown for basal turn of the cochlea). The extra rows of hair cells result from the disorganised arrangement of the hair cells. E: Quantification of the total number of hair cells in cochleae of WHSC1+/+, WHSC1+/− and WHSC1−/− littermates showed no significant difference (n=3, p=0.4). F: Quantification of the total number of IHCs and OHCs in 200μm region of base, middle and apical turns of the cochleae in heterozygous and homozygous mutant and control littermates (n=5). Note the significant increase in the number of IHCs in the middle (p=<0.05) and OHCs in the base (p=<0.0005), with a concomitant decrease of OHCs in the apex (p=<0.005) of WHSC1−/− cochlea. Error bars are SEM. Scale bars: 25μm.
Figure 5: Auditory hair cells are disorganised in WHSC1<sup>−/−</sup> mice

**A-F:** E18.5 wildtype and mutant organ of Corti immunostained for MYO7A (green). Differences in cell shape and size affect the arrangement of hair cells. **G-J:** MYO7A and neurofilament (red) staining in sections show more SGN fibres towards outer hair cells in WHSC1<sup>−/−</sup> mice; nuclei stained with Hoechst (blue). Scale bars: 10μm (A-F, I, J); 30μm (G, H). Panels (I, J) show higher magnifications of the white boxed regions in G and H, respectively. Arrowheads = hair cells; asterisk = hair cell squeezed in between other hair cells. SGN = spiral ganglia neurons, Mid = middle region of the cochlea.
Figure 6: Organisation of spiral fibres is disrupted in *WHSC1* mutant cochleae

Basal region of E18.5 wildtype and mutant organ of Corti immunostained for neurofilament (red), phalloidin (green) and Hoechst (blue). The innervations pattern appears normal as spiral ganglia fibres project towards their hair cell targets (A, E, I). However, while fibres crossing the TC turn basally and fasciculate to form three distinct rows in wildtype (C, D), they fail to fasciculate in heterozygous and homozygous mutants (G, H, K, L). Note the orderly arrangement of similarly shaped and sized hair cells in wildtype (B) versus disorganised arrangement in mutant (F, J) cochleae. Scale bars: 30μm (A, E, I); 10μm (B-D, F-H, J-L). TC = tunnel of Corti; S1-S3 = spiral fibre rows; O1-O4 = outer hair cell rows; IHC = inner hair cells.
**Figure 7: Stereocilia formation is perturbed in WHSCI⁻/⁻ hair cells**

**A-I:** Basal region of E18.5 wildtype and mutant organ of Corti labelled with acetylated α-tubulin to visualise kinocilia (red) and phalloidin (green) to reveal stereocilia. Kinocilia and stereocilia formation is abnormal in the mutant cochlea. **J:** quantification and schematic representation of the hair bundle defects in wildtype (n=10), heterozygous (n=10) and homozygous (n=10) animals. In mutants, the kinocilia (black dots) and stereocilia (purple) are either absent or have largely abnormal orientation. Those that do have a kinocilia can either have no stereociliary hair bundles or these bundles are localised around the cell perimeter. Scale bars: 10μm. Asterisk: D = central position of kinocilia rather than peripheral; G = no kinocilium, Arrowheads = outer hair cell kinocilia, Arrows = inner hair cell kinocilia.
Translational Impact

Clinical issue

Wolf-Hirschhorn syndrome (WHS) is a rare genetic disorder in humans that causes severe growth retardation, seizures and characteristic craniofacial defects. Individuals can also present with heart defects, cleft lip and/or palate, hearing impairment, and eye anomalies. WHS is caused by the partial deletion of the short arm of chromosome 4, which harbours two overlapping critical regions (WHSCR-1 and WHSCR-2) consisting of multiple genes. Phenotypic variability and severity of the syndrome in affected individuals is determined by the extent of the deletion in these regions. The only gene common to both critical regions is WHSC1, which is also deleted in every known case of WHS, and thus is considered to be the best candidate causative gene for the syndrome. WHSC1 is an enzyme that plays essential roles in cell cycle regulation, transcription and DNA repair via epigenetic modifications. The WHSC1 mutant mouse is amongst the few animal models developed for WHS. However, the contribution of individual genes within the WHS critical regions to different phenotypes often cannot be established firmly.

Results

In WHS patients, the causes of sensorineural deafness have so far not been established. Using a mouse model, we show here that loss of WHSC1 function leads to characteristic sensory hair cell defects in the inner ear. WHSC1 is required specifically for the organised distribution of cochlear hair cells and of hair bundles. Thus, these findings provide a possible explanation for sensorineural hearing loss in human WHS patients.

Implications and future directions

WHSC1 is a methyltransferase, which regulates gene expression by modifying histone tails, specifically H3K36. Our findings suggest that this activity is crucial for the arrangement of cochlear hair cells and their stereocilia. Since epigenetic modifications by WHSC1 are reversible, they are excellent targets for drug therapy in WHS patients.