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WDR1 Presence in the Songbird Basilar Papilla

Henry J. Adler^{1,3,*}, Elena Sanovich^{2,3}, Elizabeth F. Brittan-Powell^{2,3}, Kai Yan^{1,3}, and Robert J. Dooling^{2,3}

¹ Department of Biology, University of Maryland, College Park, MD 20742

² Department of Psychology, University of Maryland, College Park, MD 20742

³ Center of Comparative and Evolutionary Biology of Hearing, University of Maryland, College Park, MD 20742

Abstract

WD40 repeat 1 protein (WDR1) was first reported in the acoustically injured chicken inner ear, and bioinformatics revealed that WDR1 has numerous WD40 repeats, important for protein-protein interactions. It has significant homology to actin interacting protein 1 (Aip1) in several lower species such as yeast, roundworm, fruitfly and frog. Several studies have shown that Aip1 binds cofilin/actin depolymerizing factor, and that these interactions are pivotal for actin disassembly via actin filament severing and actin monomer capping. However, the role of WDR1 in auditory function has yet to be determined.

WDR1 is typically restricted to hair cells of the normal avian basilar papilla, but is redistributed towards supporting cells after acoustic overstimulation, suggesting that WDR1 may be involved in inner ear response to noise stress. One aim of the present study was to resolve the question as to whether stress factors, other than intense sound, could induce changes in WDR1 presence in the affected avian inner ear. Several techniques were used to assess WDR1 presence in the inner ears of songbird strains, including Belgian Waterslager (BW) canary, an avian strain with degenerative hearing loss thought to have a genetic basis. Reverse transcription, followed by polymerase chain reactions with WDR1-specific primers, confirmed WDR1 presence in the basilar papillae of adult BW, non-BW canaries, and zebra finches. Confocal microscopy examinations, following immunocytochemistry with anti-WDR1 antibody, localized WDR1 to the hair cell cytoplasm along the avian sensory epithelium. In addition, little, if any, staining by anti-WDR1 antibody was observed among supporting cells in the chicken or songbird ear.

The present observations confirm and extend the early findings of WDR1 localization in hair cells, but not in supporting cells, in the normal avian basilar papilla. However, unlike supporting cells in the acoustically damaged chicken basilar papilla, the inner ear of the BW canary showed little, if any, WDR1 up-regulation in supporting cells. This may be due to the fact that the BW canary

*Correspondence: Henry J. Adler, Ph.D., University of Maryland, 4212 Biology Psychology Building, College Park, MD 20742-0001, Telephone: 301-405-6882, FAX: 301-314-9358, hadler@umd.edu.

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already has established hearing loss and/or to the possibility that the mechanism(s) involved in BW hearing loss may not be related to WDR1.

Keywords

RT-PCR; immunocytochemistry; confocal microscopy; Belgian Waterslager canary; hearing; acoustic trauma; genetic hearing loss

1. INTRODUCTION

Structural changes in the auditory epithelium of either mammals or birds following intense sound exposure or ototoxic treatment have been well documented (see for review, Cotanche, 1999). Briefly, numerous hair cells are lost in the sensory epithelium regions most sensitive to the injurious stimulus (Cotanche, 1987a; Cruz et al., 1987; Corwin & Cotanche, 1988; Ryals & Rubel, 1988; Raphael, 1992; 1993; Dooling et al., 1997; 2006; Ryals et al., 1999; Dooling & Dent, 2001; Dooling et al., 2006). Hair cells that survive such an impact show substantial damage, including various stereocilia alterations (floppy, shortened, fused, elongated, missing, splattered) and shrunken apical surfaces (Saunders et al., 1985; Cotanche & Dopyera, 1990; Cotanche et al., 1991; Marsh et al., 1990; Raphael, 1993; Adler & Saunders, 1995; Dooling et al., 2006). The supporting cells that surround the surviving hair cells or missing hair cells display expanded apical surfaces (Cotanche & Dopyera, 1990; Cotanche et al., 1991; Marsh et al., 1990; Raphael, 1993; Adler & Saunders, 1995), and this expansion enables the affected supporting cells to occupy epithelial gaps produced by missing and/or shrinking hair cells. This event may be crucial because it may help preserve the ionic equilibrium among the three fluid compartments (scala vestibuli, media, and tympani) in the damaged inner ear (Poje et al., 1995; Saunders et al., 1996). At least in the bird inner ear, ototoxic (Epstein & Cotanche, 1995) or acoustic (Cotanche, 1987b; 1992; Adler et al., 1992; 1993; 1995a,b; Adler, 1996) insult affects the tectorial membrane and disrupts innervation patterns among lost or damaged hair cells (Wang & Raphael, 1996; Ofsie & Cotanche, 1996). Dark cells in the tegmentum vasculosum are also altered by intense sound (Ryals et al., 1995), and this change contributes in part to changes in the endocochlear potential, an important measure of the ionic equilibrium within the avian inner ear (Poje et al., 1995; Saunders et al., 1996).

The ability to recover from acoustic or ototoxic injury differs between birds and mammals. Hair cell loss leads to permanent hearing loss in the damaged mammalian organ of Corti. Conversely, new hair cells appear in the avian basilar papilla within four days after acoustic overstimulation or ototoxic treatment (Cotanche, 1987a; Cruz et al., 1987; Corwin & Cotanche, 1988; Ryals & Rubel, 1988; Raphael, 1992; 1993; Ryals et al., 1999; Dooling et al., 2006) and these cells become innervated and functional three days later (a total of seven days after insult; Wang & Raphael, 1996; Ofsie & Cotanche, 1996). The apical surfaces of surviving hair cells and supporting cells (Cotanche & Dopyera, 1990; Cotanche et al., 1991; Marsh et al., 1990; Raphael, 1993; Adler & Saunders, 1995) as well as dark cells in the tegmentum vasculosum (Ryals et al., 1995) return to near normal. The damaged tectorial membrane gains a new honeycombed pattern layer, at least partially restoring its shearing motion with the underlying hair cells in the region affected by noise (Cotanche, 1987b;

1992; Saunders et al., 1992; Adler et al., 1992; 1993; 1995a,b; Adler, 1996) or ototoxic drugs (Epstein & Cotanche, 1995). As a result of such repair, birds are able to regain most, if not all, of their lost hearing (see for review, Cotanche, 1999; Smolders, 1999; Dooling et al., 1997; 2006; Ryals et al., 1999; Dooling & Dent, 2001; Dooling et al., 2006). Thus, birds provide an important model for examining hair cell loss and regeneration following acoustic or ototoxic trauma.

Less common, but equally significant, is an avian model for hair cell loss and replacement following hereditary hearing loss. The only example of such a model is the Belgian Waterslager (BW) canary. Behavioral studies show that BW canaries have a profound hearing deficit at high frequencies (Okanoya & Dooling, 1985; 1987; 1990). Recent physiological studies using the auditory brainstem response show that the BW canary has nearly normal hearing sensitivity during the first week post-hatch, but this sensitivity starts to deteriorate during the second week and shows the functional deficit, similar to that of adult canaries, by one month of age (Brittan-Powell et al., 2002; Wright et al., 2004). The causes of such a functional deficit in BW canaries have been attributed to structural defects in the inner ear (Gleich et al., 1994; 1995; 1997 Gleich et al., 2001; Ryals & Dooling, 2002). For example, the BW canary ear displays approximately 30% hair cell loss along its sensory epithelium (Gleich et al., 1994) and a 12% reduction in afferent nerve fibers (Gleich et al., 2001). Hair cell regeneration is observed in the BW canary basilar papilla, but the rate of hair cell replacement is lower than that for hair cell loss (Gleich et al., 1997). Interestingly, exposure to intense sound induces a greater rate of hair cell regeneration in the BW canary, but the question as to how this positive change affects hearing loss in the BW canary remains to be resolved (Gleich et al., 1997). Last, but not the least, a recessive mutation located on the sex-linked (Z) chromosome may contribute to hair cell loss and its functional counterpart, hearing loss, in the BW canary (Wright et al., 2004).

In numerous attempts to identify molecular pathways involved in inner ear response to stress, Lomax and colleagues performed several techniques (including differential display; subtractive hybridization; and gene microarrays) to develop gene expression profiles in both the peripheral and central auditory systems in birds (Gong et al., 1996; Lomax et al., 2000; 2001; Warner et al., 2003) and mammals (Lomax et al., 2000; 2001; Cho et al., 2004; Holt et al., 2005). These profiles have identified genes whose expression increases (or even decreases) in either the avian basilar papilla or mammalian organ of Corti as well as their central counterparts following trauma, whether it be acoustic, ototoxic or otherwise (Gong et al., 1996; Lomax et al., 2000; 2001; Warner et al., 2003; Cho et al., 2004; Holt et al., 2005). One of the first genes identified as up-regulated in the acoustically damaged avian inner ear was WDR1 (Adler et al., 1999), encoding a protein with numerous WD40 repeats, motifs important for protein-protein interactions. Chicken WDR1 not only has 86% identity to both human WDR1 and mouse Wdr1 (Adler et al., 1999) but also demonstrates significant sequence identity to a protein called *actin-interacting protein 1* (Aip1) in numerous species such as *Dictyostelium discoideum* (Aizawa et al., 1999; Konzok et al., 1999), *Physarum polycephalum* (Shimada et al., 1992; Matsumoto et al., 1998), *Caenorhabditis elegans* (Ono, 2001; Mohri & Ono, 2003), *Saccharomyces cerevisiae* (Amberg et al., 1995; Iida & Yahara, 1999), *Xenopus laevis* (Okada et al., 1999; 2002), *Arabidopsis thaliana* (Allwood et

al., 2002), and *Drosophila melanogaster* (Ren et al., 2007). These homologs indicate that WDR1 is well conserved and may perform a critical cellular function (Adler et al., 1999).

What adds extra significance to the role of WDR1 in cellular function is its potential relationship with cofilin/actin depolymerizing factor (ADF), an important component in the highly regulated process of actin polymerization and depolymerization. This process is essential for many cellular activities, including (but not limited to) proliferation, transport, and migration. Even though WDR1 has been shown to co-localize with actin and cofilin in hair cells in the chicken auditory epithelium (Oh et al., 2002), it remains to be seen whether or not chicken or mammalian WDR1 interacts with actin or cofilin/ADF. Nonetheless, several studies have confirmed binding of Aip1 to cofilin/ADF in yeast (Rodal et al., 1999) and *Xenopus* (Okada et al., 1999; 2002), and this type of interaction has implications for actin severing and capping. For example, both *Dictyostelium* and *Xenopus* Aip1 improves the ability of cofilin to cleave actin filaments (*Dictyostelium* - Aizawa et al., 1999; Balcer et al., 2003; *Xenopus* - Okada et al., 2002), because Aip1 caps the barbed ends of the actin filaments, thus reducing the concentration of ends capable of adding actin monomers (Okada et al., 2002; Ono, 2003).

In addition to its localization to hair cells in both the normal and acoustically injured avian inner ear, WDR1 was shown to be present in supporting cells in the bird basilar papilla only after exposure to intense sound, and this presence was limited to the lesion area (Oh et al., 2002). WDR1 was also co-localized with actin and cofilin among the same cells (Oh et al., 2002). Furthermore, Northern blot analysis with a WDR1 probe on both normal and damaged chicken basilar papilla RNAs revealed higher WDR1 expression levels in the sound-exposed inner ear than in the unaffected basilar papilla (Adler et al., 1999). The sound-induced changes in WDR1 localization and expression suggest that WDR1 may be involved in inner ear response to stress. Exactly what WDR1 does in auditory function and/or inner ear response to stress remains to be determined, but we speculate that WDR1 co-localization with cofilin among hair cells and supporting cells following acoustic overstimulation or any other damaging factor (e.g., genetic mutations or ototoxic drugs) may contribute to actin reorganization among these cells.

In the present study we expanded our previous analysis of WDR1 expression and localization in the chicken inner ear (Adler et al., 1999; Oh et al., 2002) by including songbirds: two canary strains, one of which has a profound hearing loss at high frequencies (BW canary), and the zebra finch. Using chicken as positive control for WDR1 presence, we performed reverse transcription, followed by polymerase chain reactions (RT-PCR) with WDR1-specific primers, to confirm that WDR1 was expressed in the inner ears of all avian species. We also developed a WDR1-specific antibody and used it for immunocytochemistry on avian basilar papilla whole mounts, followed by confocal microscopy examinations on the sensory epithelium. These methods confirmed and extended the early findings of WDR1 presence in the avian inner ear (Adler et al., 1999; Lomax et al., 2001; Oh et al., 2002) and WDR1 localization in hair cells (Oh et al., 2002).

2. MATERIALS AND METHODS

2.1. Bioinformatics

To provide updates on WDR1, SMART analysis [<http://smart.embl-heidelberg.de> (Schultz et al., 1998; Letunic et al., 2004)] was performed to detect different putative motifs such as WD40 repeats. Chicken WDR1 cDNA was also analyzed against two zebra finch brain EST databases: [http://titan.biotech.uiuc.edu/cgi-bin/ESTWebsite/estima_start?seqSet=songbird (Dr. David Clayton); for further details, see <http://songbirdgenome.org>].

2.2. Subjects

Three avian species [domestic chickens (*Gallus gallus*, 5 – 7 days post-hatching), adult canaries [*Serinus canaria* with two strains, Belgian Waterslager (BW) and non-BW (e.g., Gloster)], and adult zebra finches (*Taeniopygia guttata*)] were used in the present study. The animals and experiments described below were approved by the Institutional Committee on the Use and Care of Animals at the University of Maryland.

2.3. ABR testing

The auditory brainstem response (ABR) technique was used to determine hearing sensitivity prior to either molecular biology or confocal studies for both strains of canaries and compared to pre-existing data for these birds (Brittan-Powell et al., 2002; Wright et al., 2004). Briefly, birds were sedated with an intramuscular injection of ketamine (25–50 mg/kg) and diazepam (2 mg/kg). Standard platinum alloy electrodes (Grass F-E2; West Warwick, RI) were placed under the skin high at the vertex, directly behind the right ear canal, and the ground electrode behind the canal of the ear contralateral to stimulation. A JBL Professional Series speaker (Model 2105H, James B Lansing Sounds Inc) was placed 30 cm from the bird's right ear and played tone trains to the birds. Each tone train consisted of nine individual tone bursts (5 ms in duration; 1 ms rise/fall COS^2) with 20 ms inter stimulus interval for a total stimulus duration of 235 ms. All trains were presented at a rate of 4/s, sampled at 25 kHz and resulted in 200–300 averages. The stimulus presentation, ABR acquisition, equipment control, and data management were coordinated using a Tucker-Davis Technologies (TDT; Gainesville, FL, USA) System 3 (for further details on setup and procedure, see Brittan-Powell et al., 2005). ABR threshold was defined for each subject as the intensity 2.5 dB below the lowest stimulus level at which a response could be visually detected.

2.4. Reverse Transcription – Polymerase Chain Reactions (RT-PCR)

Four neonatal chicks, two BW canaries, three Gloster canaries, and four zebra finches were sacrificed via halothane anesthesia and decapitation. Their basilar papillae were removed, and RNA was isolated, according to RNeasy protocol (QIAGEN Inc., Valencia, CA). Five μg of RNA from each avian group were reverse transcribed to cDNA, according to Superscript II protocol (Invitrogen Corp., Carlsbad, CA). cDNA was then subjected to PCR with AmpliTaq Gold (Perkin Elmer, Wellesley, MA) – one-time activation at 94°C, followed by 35 cycles of denaturation at 94°C, annealing at 52°C, and extension at 72°C. A pair of oligonucleotides (forward primer 5'-TTGCCTGGACTGAAGACAG-3'; reverse

primer 5'-CTGTTCCCATCAGGAGAAAATC-3') flanking chicken WDR1 nucleotide (nt) positions 493–778 were used for PCR. PCR products were electrophoresed in 1.2% agarose gel, gel purified (according to Gel Extraction Protocol, QIAGEN), and processed for sequencing at the University of Maryland DNA Sequencing Core. DNA sequences were subjected to BLAST searches for matches with any known DNAs (Altschul et al., 1997).

2.5. Generation of anti-WDR1 Antibodies

Polyclonal antibodies were generated against two distinct synthetic peptides. One of them was identical to the first 20 amino acids (aa) in the amino terminus of *Homo sapiens* WDR1 (MPYEIKKVFASLPQVERGVVS; 100% identity to chicken WDR1 aa positions 3–22). This peptide was longer by eight amino acids than that used for Oh and colleagues (Oh et al., 2002) for the purpose of enhancing antigenicity. The second peptide was DRNNPSKPLHVIKGHSK, identical to *Homo sapiens* WDR1 aa positions 305–321 (and 82% identical to chicken WDR1 aa positions 307–323). This highly antigenic region occupies part of the sixth WD40 repeat of vertebrate WDR1 (Adler et al., 1999; also see Table 1 in this paper). In order to raise the antibodies, the peptides were coupled to KHL, a carrier protein, via a cysteine bond (Princeton Biomolecules, Columbus, OH).

Four rabbits (PB738, PB739, PB740 and PB741) were pre-bled to provide preimmune serum. After the initial serum collection, two of the rabbits (PB738 and PB739) each received an injection of the MPYE peptide for every other week until the 12th week, while the other two (PB740 and PB741) had the DRNN peptide at the same time. These animals were bled six times, beginning at 8 weeks after the initial peptide injection and every 2 weeks thereafter. After the final bleed, antibodies (Ab) were affinity purified (Covance Research Products Inc., Denver, PA), yielding a final concentration of 1.10 mg/mL (PB738), 1.19 mg/mL (PB739), 1.66 mg/mL (PB740) and 1.86 mg/mL (PB741).

2.6. Enzyme-linked immunosorbent assays (ELISAs)

The reactivity and specificity of the immune sera to each synthetic peptide were confirmed by analyzing the ability of dilutions of sera to detect the peptide using ELISAs. ELISA analysis of the new antibodies indicated that the rabbits responded very well to their immunogens. The optimal Ab concentration against the peptide at 50% titration was shown to be 1:100,000 (data not shown).

2.7. Western Blot Analysis

Ten neonatal chicks were anesthetized with halothane and decapitated. All basilar papillae, along with one piece of chicken brain and one piece of chicken muscle, were quickly excised and frozen in dry ice. The three tissues were homogenized in RIPA buffer (3 μ L/mg of wet weight tissue), briefly sonicated, and centrifuged at $14,000 \times g$ for 10 minutes at 4°C. Protein concentrations of the extracts were measured, using Coomassie Blue reagent (Pierce Biotechnology, Rockford, IL).

Approximately 20 μ g protein lysate per tissue were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were electrophoretically transferred from the SDS-PAGE gel to a PVDF Western blotting membrane (Invitrogen).

The membrane was blocked with TBST (Tris buffered saline with 0.1% Tween-20) with 3% bovine serum albumin (BSA) for 1 hour, incubated with anti-WDR1 Ab (1:1,000) in TBST with 1% BSA overnight on a shaker at 4°C, treated with 1:5,000 alkaline phosphatase-conjugated goat anti-rabbit Ab (Pierce Biotechnology) in TBST with 1% BSA for 1 hour at RT, washed in TBST, and reacted with a chemiluminescence agent (ECF substrate; Amersham Biosciences, Buckinghamshire, UK). After the membrane was dried, it was viewed under ultraviolet light.

To validate the specificity of the primary antibody, 1:1,000 anti-WDR1 Ab (1:1,000) and 1:100 blocking peptide (against which the Ab was raised) were pre-incubated together in TBST with 1% BSA at RT for two hours, prior to PVDF membrane staining by anti-WDR1 Ab with or without blocking peptide.

2.8. Immunocytochemistry

Ten chickens, 10 BW canaries, four non-BW canaries, and two zebra finches were sacrificed with halothane anesthesia, followed by decapitation. The temporal bones were removed, dissected to expose the basilar papillae, and were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) for 30 – 60 minutes at room temperature (RT). The time from sacrifice to the onset of fixation lasted approximately 10 minutes per bird. After fixation, the basilar papillae were dissected free of their surroundings, washed in PBS, and were permeabilized in 0.3% Triton X-100 in Tris-HCl for 30 min. Immunocytochemistry was performed before and after Ab affinity purification. Before anti-WDR1 Ab was purified, the specimens were blocked first in 2% dry fat-free milk and then in 10% normal goat serum (NGS; Jackson Immunoresearch Laboratories, West Grove, PA) in TBS (Tris-buffered saline) for 30 min each at RT. The ears were immunostained overnight at 4°C in 1:1,000 anti-WDR1 Ab in TBS with 1% NGS. Following Tris-HCl washes, the specimens were labeled again in 1:1,000 fluorescein-conjugated phalloidin (Molecular Probes, Eugene, OR) and 1:1,000 Alexa Fluor 633-conjugated goat anti-rabbit Ab (Invitrogen) in TBS with 1% NGS. Immunostaining in the avian inner ear with anti-WDR1 Ab was compared to that with preimmune rabbit serum or with only secondary antibody (e.g., Alexa Fluor 633-conjugated goat anti-rabbit Ab).

After affinity purification of anti-WDR1 Ab, several modifications were made. First, the buffered saline used for washes and Ab dilutions was PBS, not TBS. Secondly, the ears were blocked in 10% normal donkey serum (NDS), instead of dry fat-free milk and NGS, in part because NDS might provide better blocking than NGS or milk, leading to less non-specific staining. Thirdly, the secondary antibody used was 1:1,000 Cy5-conjugated F(ab')₂ fragment donkey anti-rabbit Ab (Jackson Immunoresearch) in PBS with 1% NDS. Finally, two negative controls were used: labeling with only the secondary antibody (e.g., Cy5-conjugated donkey anti-rabbit Ab) and staining with anti-WDR1 antibody blocked by peptide identical to the WDR1 region against which the antibody was generated. To produce a negative control with the blocking peptide, 1:1,000 anti-WDR1 Ab and 1:100 peptide were pre-incubated together in PBS with 1% NDS for 2 hrs at RT before anti-WDR1 immunostaining in the bird basilar papilla.

After buffered saline washes, the stained ears were mounted with Prolong AntiFade medium (Molecular Probes) on slides, cover-slipped, and examined with a Zeiss 510 confocal microscope (Carl Zeiss International, Thornwood, NY).

3. RESULTS

3.1. Bioinformatics updates on avian and mammalian WDR1

We originally stated that WDR1 contained nine WD40 repeats (Adler et al., 1999). However, SMART analysis (Schultz et al., 1998; Letunic et al., 2006) on chicken WDR1, human WDR1, and mouse Wdr1 yielded 11 putative WD40 repeats (Table 1). Also, SMART analysis on human WDR1 indicated one region of low complexity at aa positions 413–424, but similar analyses on mouse Wdr1 and chicken WDR1 did not reveal such a region. Recently, the 3-D structure of yeast (Voegtli et al., 2003) and roundworm (Mohri et al., 2004) WDR1 homolog, Aip1, exhibited 14 WD40 repeats. The sequence of some WD40 repeats is so degenerate that they cannot be detected by computer analysis (Voegtli et al., 2003).

In addition, the zebra finch brain ESTIMA analysis (Clayton) showed 93% identity between chicken WDR1 nucleotide positions 1075–1779 and zebra finch brain EST SB020001000F05 (GENBANK Accession # CK301354). This strong identity suggested that the zebra finch genome contains the gene encoding WDR1. This gene being present in songbird brain supports and extends the findings of WDR1 expression in avian brain via Northern (Adler et al., 1999) and Western blot analyses (Fig. 2, present study).

3.2. ABR testing

All birds showed ABR thresholds that were similar to previous studies (Brittan-Powell et al., 2002; Wright et al., 2004). In particular, BW canaries exhibited elevated ABR thresholds across all frequencies (0.5–8 kHz), particularly at high frequencies, as compared to those obtained from the Gloster (non-BW) canaries (data not shown).

3.3. Sequence similarity of WDR1 among avian species

RT-PCR analysis of four avian groups with the two chicken WDR1 – specific primers (see Methods) yielded a single 286 base-pair (bp) fragment from each group (Fig. 1). After the band was sequenced, BLAST searches matched this band to a *WDR1* DNA sequence from nt positions 493 to 778. Further analysis on the 286-bp sequence (and its corresponding amino acid sequence) of the two canary strains and zebra finch revealed high identity to the same region in chicken at both nucleotide and amino acid levels (Table 2). In conclusion, RT-PCR verified WDR1 presence in the inner ears of both chicken and songbirds.

3.4. Western blot analysis

In order to test specificity of four anti-WDR1 antibodies (PB738, PB739, PB740, PB741), several rounds of Western blotting were performed on chicken tissue extracts. The first round of Western blotting took place without blocking peptide, and only PB741 yielded a 67 kDa polypeptide (consistent with the predicted molecular weight of WDR1) in both brain and ear (data not shown). The next round of Western blotting with PB741 was performed

with and without blocking peptide on chicken ear, brain, and muscle. Again, this antibody detected a strong expression of a polypeptide at 67 kDa in all the three tissues (Fig. 2). Also, muscle showed an intense second polypeptide band at 51 kDa (Fig. 2). All these bands disappeared when the blocking peptide was used (Fig. 2). Hence, PB741 was chosen as the anti-WDR1 antibody for immunocytochemistry on avian inner ears.

3.5. WDR1 localization in hair cells

To confirm that WDR1 is localized to hair cells in the avian inner ear, we examined the avian basilar papilla by immunofluorescence with anti-WDR1 Ab and phalloidin, the latter of which binds actin and visualizes the actin filaments of cells in the sensory epithelium. The actin content is found in hair cell stereocilia (Figs. 3 – 5), hair cell cuticular plates (Fig. 5), and supporting cell microvilli (Figs. 3 – 5). This actin staining has confirmed numerous findings elsewhere (e.g., Raphael, 1992; 1993), and its purpose in the present paper was to facilitate WDR1 localization by anti-WDR1 Ab along the sensory epithelium.

Figures 3 and 4 provide surface views of the sensory epithelium of the avian inner ear before and after anti-WDR1 Ab was purified, respectively. In Figure 3, top (A–C) and bottom (G–I) panels represent the chicken and BW canary basilar papillae incubated with anti-WDR1 Ab, respectively, whereas middle panels (D–F) display a chicken inner ear stained with preimmune serum. Anti-WDR1 Ab (Fig. 3B & H), but not preimmune serum (Fig. 3E), labeled hair cell luminal surfaces. Little, if any, WDR1 labeling was observed among supporting cells (Fig. 3 – 5). While the figures were obtained from the middle regions of the BW and chicken basilar papillae, similar observations were made throughout the auditory sensory epithelia of the BW, non-BW canary, zebra finch and chicken (data not shown).

Figure 4 presents the BW canary auditory epithelium at both hair cell surface (Row A) and nuclear (Row B) levels. These rows represent ears incubated with Cy5-conjugated donkey anti rabbit Ab alone (left), anti-WDR1 Ab blocked by peptide (middle), and anti-WDR1 Ab alone (right). Anti-WDR1 Ab alone stained hair cell surfaces and cytoplasm at surface and nuclear levels, respectively (Fig. 4, right), while neither secondary antibody alone (Fig. 4, left) nor anti-WDR1 Ab blocked by peptide (Fig. 4, middle) evinced much staining among hair cells or supporting cells at both surface and nuclear levels. At the nuclear level with anti-WDR1 Ab alone, the labeled cytoplasm encapsulated the mostly unlabeled nucleus (Fig. 4, right). An intensely bright spot was observed in the relative middle of the nucleus (Fig. 4, right). When either secondary antibody alone (Fig. 4, left) or anti-WDR1 Ab blocked by peptide (Fig. 4, middle) was used, the spot did not appear. This observation held for the non-BW canary (data not shown), but chicken basilar papillae incubated with anti-WDR1 Ab failed to display such a spot (see below).

Fortuitous sections offered side views of several basilar papillae in chicken and BW canaries (Fig. 5). In Fig. 5A & C, chicken and BW canary inner ears were incubated with anti-WDR1 Ab, respectively, while another chicken basilar papilla was stained with Cy5-conjugated donkey anti-rabbit Ab alone (Fig. 5B). Secondary Ab alone revealed little, if any, labeling (Fig. 5B), while the hair cell cytoplasm of both chicken and BW canary basilar papillae were stained by anti-WDR1 Ab (Fig. 5A, C). Again, a bright and round spot occupied the middle of each relatively unlabeled nucleus in BW canary (Fig. 5C), but not in chicken (Fig.

5A). In conclusion, anti-WDR1 Ab consistently localized WDR1 to hair cells in the auditory epithelium of both chicken and songbirds (Figs. 3 – 5).

4. DISCUSSION

In addition to verifying WDR1 presence in the avian inner ear via RT-PCR, the present study used whole-mount preparations on avian basilar papillae, utilized confocal microscopy to localize WDR1 in the basilar papilla, and confirmed and extended previous findings (Oh et al., 2002), which used cryosections and fluorescent microscopy. Even though both techniques enabled different views of similar tissues at different magnifications, both produced several clear views of hair cells stained by anti-WDR1 Ab at different angles, and led to the same conclusion that WDR1 is localized to hair cells in the avian inner ear.

4.1. Western blotting in chicken muscle

In addition to the 67 kDa band, Western blotting on chicken muscle with anti-WDR1 antibody yielded a second major band at 51 kDa (Fig. 2). This polypeptide was similar in size to the 51 kDa band observed in rat pheochromocytoma cells (Shin et al., 2004). This might represent one or more versions of WDR1: an uncharacterized WDR1 isoform, alternative splicing, and/or post-translational modifications of the translated peptide. The different possibilities necessitate further studies on WDR1 presence in chicken muscle, especially because actin plays a major role in muscle motion.

4.2. WDR1 localization to a spot in the middle of hair cell nucleus

It is interesting that anti-WDR1 Ab illuminated a spot in the middle of many hair cell nuclei in the songbird (Figs. 4 Right & 5C), but not chicken (Fig. 5A), basilar papilla. This difference in spot presence may be due to technical issues such as animal variation in the fixation or immunostaining quality. Another potential explanation is species differences. For example, it is possible that hair cells may produce more actin turnover in the songbird inner ear than in the chicken. In addition, the observation of such spots in the hair cell nuclei in both the BW and non-BW canary inner ear suggested that stress (at least by genetic mutations) may not contribute to spot appearances in hair cell nuclei. One might wonder if ototoxic drugs themselves could induce increased WDR1 activity among hair cell nuclei in the chicken inner ear, but anti-WDR1 Ab failed to illuminate spots in the middle of surviving hair cell nuclei in the chicken basilar papilla following gentamicin treatment (Adler, unpublished observations).

The size, shape, and location of the bright spot in the middle of hair cell nuclei appear to be compatible with those of hair cell nucleoli observed elsewhere (e.g., Cohen & Fermin, 1978). However, further studies (for example, Hoescht or DAPI staining) are needed to identify the spot.

4.3. Was there any WDR1 localization among supporting cells in the BW canary inner ear?

Oh and colleagues reported that WDR1 was not localized to the supporting cells in the normal chicken basilar papilla. However, such localization was observed among supporting cells in the sensory epithelium impacted by intense sound (Oh et al., 2002). This raised a

question as to whether or not WDR1 would be up-regulated in the supporting cells in the BW canary inner ear because the genetic mutation in this strain leads to hair cell loss and subsequent hearing loss (Gleich et al., 1994; 1995; 1997; Wright et al., 2004). Here, Figures 4 and 5 show clear separation among hair cells by anti-WDR1 Ab staining but little, if any, labeling in the supporting cells of the BW canary basilar papilla. These observations suggest several possible scenarios. One of them is that the mechanism(s) leading to BW hearing loss may not involve WDR1. A second possibility is that genetic mutations may not be stressful enough to induce WDR1 localization among supporting cells in the BW canary ear. Last, but not least, BW canaries may have already established hearing loss, and the effect of genetic mutations on WDR1 localization may have become negligent by then. At one month posthatching, young BW canaries exhibit elevated auditory thresholds that are equal to those of adult BW canaries (Brittan-Powell et al., 2002; Wright et al., 2004). Since all the BW canaries used in the present study were adult (one year of age or older), they may have gone past the time during which supporting cells might express WDR1 during the maturation of hearing loss in the developing BW canary. Hence, these birds displayed little, if any, WDR1 labeling among supporting cells in their inner ears. This calls for further examination on WDR1 presence in the BW canary inner ear from hatching to one month of age.

4.4. What did WDR1 bioinformatics and localization tell about WDR1 role in hearing?

As mentioned earlier, several studies have suggested a function for WDR1 in actin dynamics, especially actin depolymerization. To help determine WDR1 function, our SMART analysis (Letunic et al., 2006; Schultz et al., 1998) on mammalian and avian WDR1 revealed 11 putative WD40 repeats. However, it is most likely incomplete, because it may have failed to detect 'hidden' WD40 repeats (Voegtli et al., 2003). This 'hidden' problem has been resolved, at least in part, by x-ray crystallography studies on yeast (Voegtli et al., 2003) and roundworm (Mohri et al., 2004) Aip1. These studies revealed a highly resolved structure of two connected seven-bladed β -propellers, totaling 14 WD40 regions and resembling an open clamshell (Voegtli et al., 2003; Mohri et al., 2004).

The Aip1 crystal structure has implications for the role of WDR1 in actin dynamics. Genetic manipulations on Aip1/WDR1 have emphasized the importance of Aip1/WDR1 in regulating actin disassembly. Point mutations in yeast Aip1 have disrupted its ability to bind actin or cofilin, altering the process of actin disassembly via severing and capping (Rodal et al., 1999; Mohri et al., 2004; 2006; Clark et al., 2006; Okada et al., 2006). Consequently, these mutations have located two actin binding sites, one of which resides in the amino-terminal β -propeller and the other in the carboxy-terminal β -propeller (Mohri et al., 2004; 2006; Clark et al., 2006; Okada et al., 2006). The mutations also have identified one cofilin/ADF binding site, which is located in the cleft between the two propellers and is also flanked by the actin binding sites (Clark et al., 2006; Clark & Amberg, 2007).

Molecular manipulations in Aip1/WDR1 from different species have affected a variety of cellular activities, all of which involve actin dynamics. DAip1, a *Dictyostelium* homolog of Aip1/WDR1, was localized to cellular regions enriched in filamentous actin, and its null mutations have been shown to impair several cellular functions such as endocytosis, cytokinesis and motility (Konzok et al., 1999; Gerisch et al., 2004). RNA interference

inhibition of Aip1 has caused defects in cell surface morphology in *Drosophila*, due to cortical F-actin accumulation (Rogers et al., 2003) as well as poor leaf and plant growth in *Arabidopsis* (Ketelaar et al., 2004). Even null mutations can be lethal in several species. While null mutations could cause defects in actin organization, but not death, in yeast (Rodal et al., 1999; Clark et al., 2006; Okada et al., 2006) and in slime molds (Konzok et al., 1999; Gerisch et al., 2004), similar mutations have been fatal to *Drosophila* (Ren et al., 2007) and mouse (Kile et al., 2007) during embryonic development, indicating that WDR1/Aip1 is an essential gene in at least two species.

Despite the fact that Wdr1/Aip1 null mutations cause death in mouse embryos (Kile et al., 2007), studies on mammalian WDR1 have begun to determine how WDR1 mutations could affect cell function in mammals. Mice heterozygous for Wdr1 defects (created by a point mutation) displayed both blood clotting disorder (involving megakaryocytes) and autoinflammatory disease (involving neutrophils) before dying at age of 3 – 6 months (Kile et al., 2007). This establishes the necessity for Wdr1 in actin dynamics critical for megakaryocyte and neutrophil development and function (Kile et al., 2007). Studies via murine caspace-11 null mutations and Wdr1/Aip1 knockdowns as well as immunoprecipitation between caspace-11 and Aip1 showed that caspace-11, a protein important for cytokine secretion and apoptosis during inflammatory response, binds Wdr1/Aip1 (Li et al., 2007). This interaction enhances cell migration, both by facilitating actin filament severing and by promoting actin depolymerization (Li et al., 2007). The murine Wdr1/Aip1 studies stress the significance of WDR1/Wdr1/Aip1 in the well-being of not only mice, but also humans, especially when it could concern Alport syndrome, a hereditary human disease with deafness, kidney problems and blood clotting disorder. It would be interesting to observe whether or not WDR1/Aip1 mutations could affect mammalian hearing.

In previous studies, we exhibited increases in WDR1 expression (Adler et al., 1999) as well as WDR1-cofilin co-localization in supporting cells (Oh et al., 2002) in the avian basilar papilla following acoustic overstimulation. These changes suggest a role for WDR1 in inner ear response to stress as well as its potential contributions to actin reorganization among hair cells and supporting cells in the injured basilar papilla region. We speculate that sound-induced actin reorganization may aid the affected supporting cells in expanding their apical surfaces, closing gaps created by missing hair cells in order to preserve the ionic equilibrium, at least between scalae media and tympani. Another possibility is that actin reorganization may facilitate supporting cell de-differentiation prior to processes [i.e., supporting cell mitosis (Corwin & Cotanche, 1988; Ryals & Rubel, 1988; Raphael, 1992) and/or direct transdifferentiation (Adler & Raphael, 1996; Baird et al., 1996; Roberson et al., 1996; Adler et al., 1997; Steyger et al., 1997)] leading to hair cell regeneration. It is also possible that actin reorganization among hair cells shrinks their bodies (at least at the apical surface) following exposure to intense sound, and this mechanism may aid their survival, self-repair, and/or death. However, as mentioned above, WDR1 was localized to hair cells, but not supporting cells, in the inner ear of the adult BW canary. This observation failed to answer the question as to whether or not a source of inner ear injury, other than intense sound, could increase WDR1 expression among supporting cells, and calls for further

studies on WDR1 localization during the progress of hearing loss in the developing BW canary and on actin reorganization among hair cells and supporting cells in the same bird inner ear.

4.5. Summary

Our RT-PCR and immunocytochemistry data on songbird inner ears confirmed and extended our finding that WDR1 is localized to hair cells in the avian auditory sensory epithelium (Adler et al., 1999; Oh et al., 2002). However, the question remains as to whether genetic mutations could induce changes in WDR1 localization among supporting cells, and necessitates further studies on WDR1 presence in the bird basilar papilla affected by one or more sources of damage, other than intense sound.

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Abbreviations

WDR1	WD40 repeat 1
Aip1	actin interacting protein 1
BW	Belgian Waterslager
ADF	actin depolymerizing factor
RT-PCR	reverse transcription – polymerase chain reactions
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
nt	nucleotides
aa	amino acids
bp	base pairs
Ab	antibodies
RT	room temperature
BSA	bovine serum albumin
NGS	normal goat serum
NDS	normal donkey serum
TBS	Tris buffered saline
PBS	phosphate buffered saline
ABR	auditory brainstem response
kHz	kiloHertz
kDa	kiloDaltons

st stereocilia
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References

- Adler HJ, Kenealy JFX, DeDio RM, Saunders JC. Threshold shift, hair cell loss, and hair cell bundle stiffness following exposure to 120 and 125 dB pure tones in the neonatal chick. *Acta Otolaryngol* (Stockh). 1992; 112:444–454. [PubMed: 1441985]
- Adler HJ, Poje CP, Saunders JC. Recovery of auditory function and structure in the chick after two intense pure tone exposures. *Hear Res*. 1993; 71:214–224. [PubMed: 8113139]
- Adler HJ, Saunders JC. Hair cell regeneration in the chick following two exposures to intense pure tone. *J Neurocytology*. 1995; 24:111–116.
- Adler HJ, Mantooth J, Raphael Y. Comparative analysis of patch lesions in the chick inner ear following acoustic overstimulation: optical vs. scanning electron microscopy. *Scan Microsc*. 1995a; 9:825–831.
- Adler HJ, Niemiec AJ, Moody DB, Raphael Y. Tectorial membrane regeneration in acoustically damaged birds: an immunocytochemical technique. *Hear Res*. 1995b; 86:43–46. [PubMed: 8567420]
- Adler HJ. Tectorial membrane regeneration in the adult quail following multiple acoustic overstimulations. *Audiol Neurotol*. 1996; 1:65–79. [PubMed: 9390791]
- Adler HJ, Raphael Y. New hair cells arise from supporting cell conversion in the acoustically damaged chick inner ear. *Neurosci Letters*. 1996; 205:17–20.
- Adler HJ, Komeda M, Raphael Y. Further evidence for supporting cell conversion in the damaged avian basilar papilla. *Int J Devel Neurosci*. 1997; 15:375–385. [PubMed: 9263020]
- Adler HJ, Winnicki RS, Gong T-WL, Lomax MI. A gene upregulated in the acoustically damaged chick basilar papilla encodes a novel WD40 repeat protein. *Genomics*. 1999; 56:59–69. [PubMed: 10036186]
- Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res*. 1997; 25:3389–3402. [PubMed: 9254694]
- Aizawa H, Katadae M, Maruya M, Sameshima M, Murakami-Murofushi K, Yahara I. Hyperosmotic stress-induced reorganization of actin bundles in Dictyostelium cells over-expressing cofilin. *Genes to Cells*. 1999; 4:311–324. [PubMed: 10421841]
- Allwood EG, Anthony RG, Smertenko AP, Reichelt S, Drobak BK, Doonan JH, Weeds AG, Hussey PJ. Regulation of the pollen-specific actin-polymerizing factor LIADF1. *Plant Cell*. 2002; 14:2915–2927. [PubMed: 12417710]
- Amberg DC, Basart E, Botstein D. Defining protein interactions with yeast action in vivo. *Struct Biol*. 1995; 2:28–35.
- Baird RA, Steyger PS, Schuff N. Mitotic and non-mitotic mechanisms of hair cell regeneration in the bullfrog. *Ann NY Acad Sci*. 1996; 781:59–70. [PubMed: 8694449]
- Balcer HI, Goodman AL, Rodal AA, Smith E, Kugler J, Heuser JE, Goode BL. Coordinated regulation of actin filament turnover by a high-molecular-weight Srv2/CAP complex, cofilin, profilin, and Aip1. *Curr Bio*. 2003; 13:2159–2169. [PubMed: 14680631]
- Brittan-Powell EF, Dooling RJ, Wright T, Mundinger PC, Ryals BM. Development of auditory sensitivity in Belgian Waterslager (BW) canaries. *Abstr Assoc Res Otolaryngol*. 2002; 25:947.
- Brittan-Powell EF, Lohr B, Hahn DC, Dooling RJ. Auditory brainstem responses in the Eastern Screech Owl: an estimate of auditory thresholds. *J Acoust Soc Am*. 2005; 118:314–321. [PubMed: 16119351]
- Cho Y, Gong TW, Kanicki A, Altschuler RA, Lomax MI. Noise overstimulation induces immediate early genes in the rat cochlea. *Brain Res Mol Brain Res*. 2004; 130:134–148. [PubMed: 15519684]

- Clark MG, Amberg DC. Biochemical and genetic analyses provide insight into the structural and mechanistic properties of actin filament disassembly by the Aip1p-cofilin complex in *Saccharomyces cerevisiae*. *Genetics*. 2007; 176:1527–1539. [PubMed: 17483419]
- Clark MG, Teply J, Haarer BK, Viggiano SC, Sept D, Amberg DC. A genetic dissection of Aip1p's interactions leads to a model for Aip1p-cofilin cooperative activities. *Mol Biol Cell*. 2006; 17:1971–1984. [PubMed: 16421248]
- Cohen GM, Fermin CD. The development of hair cells in the embryonic chick's basilar papilla. *Acta Otolaryngol*. 1978; 86:342–358. [PubMed: 716857]
- Corwin JT, Cotanche DA. Regeneration of sensory hair cells after acoustic trauma. *Science*. 1988; 240:1772–1774. [PubMed: 3381100]
- Cotanche DA. Regeneration of hair cell stereociliary bundles in the chick cochlea following severe acoustic trauma. *Hear Res*. 1987a; 30:181–195. [PubMed: 3680064]
- Cotanche DA. Regeneration of the tectorial membrane in the chick cochlea following severe acoustic trauma. *Hear Res*. 1987b; 30:197–206. [PubMed: 3680065]
- Cotanche DA. Video-enhanced DIC images of the noise-damaged and regenerated chick tectorial membrane. *Exp Neurol*. 1992; 115:23–26. [PubMed: 1728568]
- Cotanche DA. Structural recovery from sound and aminoglycoside damage in the avian cochlea. *Audiol Neurotol*. 1999; 4:271–285. [PubMed: 10516388]
- Cotanche DA, Dopyera CE. Hair cell and supporting cell response to acoustic trauma in the chick cochlea. *Hear Res*. 1990; 46:29–40. [PubMed: 2380125]
- Cotanche DA, Petrell A, Picard DA. Structural reorganization of hair cells and supporting cells during noise damage, recovery and regeneration in the chick cochlea. *Ciba Found Symp*. 1991; 160:131–150. [PubMed: 1752160]
- Cruz RM, Lambert PR, Rubel EW. Light microscopic evidence of hair cell regeneration after gentamicin toxicity in chick cochlea. *Arch Otolaryngol Head Neck Surg*. 1987; 113:1058–1062.
- Dooling RJ, Dent ML. New studies on hair cell regeneration in birds. *Acoust Sci & Tech*. 2001; 22:93–99.
- Dooling RJ, Ryals BM, Manabe K. Recovery of hearing and vocal behavior after hair cell regeneration. *Proc Natl Acad Sci USA*. 1997; 94:14206–14210. [PubMed: 9391178]
- Dooling RJ, Ryals BM, Dent ML, Reid TL. Perception of complex sounds in budgerigars (*Melopsittacus undulatus*) with temporary hearing loss. *J Acoust Soc Am*. 2006; 119:2524–2532. [PubMed: 16642864]
- Epstein JE, Cotanche DA. Secretion of a new basal layer of tectorial membrane following gentamicin-induced hair cell loss. *Hear Res*. 1995; 90:31–43. [PubMed: 8975003]
- Gerisch G, Faix J, Kohler J, Muller-Taubenberger A. Actin-binding proteins required for reliable chromosome segregation in mitosis. *Cell Motil Cytoskeleton*. 2004; 57:18–25. [PubMed: 14648554]
- Gleich O, Dooling RJ, Manley GA. Inner-ear abnormalities and their functional consequences in Belgian Waterslager canaries (*Serinus canarius*). *Hear Res*. 1994; 79:123–136. [PubMed: 7806475]
- Gleich O, Klump GM, Dooling RJ. Peripheral basis for the auditory deficit in Belgian Waterslager canaries (*Serinus canarius*). *Hear Res*. 1995; 82:100–108. [PubMed: 7744706]
- Gleich O, Dooling RJ, Presson JC. Evidence for supporting cell proliferation and hair cell differentiation in the basilar papilla of adult Belgian Waterslager canaries (*Serinus canarius*). *J Comp Neurol*. 1997; 377:5–14. [PubMed: 8986868]
- Gong TL, Hegeman AD, Shin JJ, Adler HJ, Raphael Y, Lomax MI. Identification of genes expressed after noise exposure in the chick basilar papilla. *Hear Res*. 1996; 96:20–32. [PubMed: 8817303]
- Holt AG, Asako M, Lomax CA, MacDonald JW, Tong L, Lomax MI, Altschuler RA. Deafness-related plasticity in the inferior colliculus: gene expression profiling following removal of peripheral activity. *J Neurochem*. 2005; 93:1069–1086. [PubMed: 15934929]
- Iida K, Yahara I. Cooperation of two actin-binding proteins, cofilin and Aip1, in *Saccharomyces cerevisiae*. *Genes to Cells*. 1999; 4:21–32. [PubMed: 10231390]

- Ketelaar T, Allwood EG, Anthony R, Voigt B, Menzel D, Hussey PJ. The actin-interacting protein AIP1 is essential for actin organization and plant development. *Curr Biol*. 2004; 14:145–149. [PubMed: 14738737]
- Kile BT, Panopoulos AD, Stirzaker RA, Hacking DF, Tahtamouni LH, Willson TA, Mielke LA, Henley KJ, Zhang J-G, Wicks IP, Stevenson WS, Nurden P, Watowich SS, Justice MJ. Mutations in the cofilin partner Aip1/Wdr1 cause autoinflammatory disease and macrothrombocytopenia. *Blood*. 2007; 110:2371–2380. [PubMed: 17515402]
- Konzok A, Weber I, Simmeth E, Hacker U, Maniak M, Müller-Taubenberger A. DAip1, a *Dictyostelium* homologue of the yeast actin-interacting protein 1, is involved in endocytosis, cytokinesis, and motility. *J Cell Biol*. 1999; 146:453–464. [PubMed: 10427097]
- Letunic I, Copley RR, Pils B, Pinkert S, Schultz J, Bork P. SMART 5: domains in the context of genomes and networks. *Nucleic Acids Res*. 2006; 34:D257–260. [PubMed: 16381859]
- Li J, Brieher WM, Scimone ML, Kang SJ, Zhu H, Yin H, von Andrian UH, Mitchison T, Yuan J. Caspace-11 regulates cell migration by promoting Aip1-cofilin-mediated actin polymerization. *Nature Cell Biol*. 2007; 9:276–286. [PubMed: 17293856]
- Lomax MI, Huang L, Cho Y, Gong TL, Altschuler RA. Differential display and gene arrays to examine auditory plasticity. *Hear Res*. 2000; 147:293–302. [PubMed: 10962193]
- Lomax MI, Gong T-WL, Cho Y, Huang L, Oh S-H, Adler HJ, Raphael Y, Altschuler RA. Differential gene expression following noise trauma in birds and mammals. *Noise Health*. 2001; 3:19–35. [PubMed: 12689446]
- Marsh RR, Xu LR, Moy JP, Saunders JC. Recovery of the basilar papilla following intense sound exposure in the chick. *Hear Res*. 1990; 46:229–237. [PubMed: 2394635]
- Matsumoto S, Ogawa M, Kasakura T, Shimada Y, Mitsui M, Maruya M, Isohata M, Yahara I, Murakami-Murofushi K. A novel 66-kDa stress protein, p66, associated with the process of cyst formation of *Physarum polycephalum* is a *Physarum* homologue of a yeast actin-interacting protein, AIP1. *J Biochem*. 1998; 124:326–331. [PubMed: 9685722]
- Mohri K, Ono S. Actin filament disassembling activity of *Caenorhabditis elegans* actin-interacting protein 1 (UNC-78) is dependent on filament binding by a specific ADF/cofilin isoform. *J Cell Sci*. 2003; 116:4107–4118. [PubMed: 12953066]
- Mohri K, Ono K, Yu R, Yamashiro S, Ono S. Enhancement of actin-depolymerizing factor/cofilin-dependent actin disassembly by actin-interacting protein 1 is required for organized actin filament assembly in the *Caenorhabditis elegans* body wall muscle. *Mol Biol Cell*. 2006; 17:2190–2199. [PubMed: 16525019]
- Mohri K, Vorobiev S, Federov AA, Almo SC, Ono S. Identification of functional residues on *Caenorhabditis elegans* actin-interacting protein 1 (UNC-78) for disassembly of actin depolymerizing factor/cofilin-bound actin filaments. *J Biol Chem*. 2004; 279:31697–31707. [PubMed: 15150269]
- Neer EJ, Schmidt CJ, Nambudripad R, Smith TF. The ancient regulatory-protein family of WD-repeat proteins. *Nature*. 1994; 371:297–300. [PubMed: 8090199]
- Ofsie MS, Cotanche DA. Distribution of nerve fibers in the basilar papilla of normal and sound-damaged chick cochleae. *J Comp Neurol*. 1996; 370:281–294. [PubMed: 8799856]
- Oh S-H, Adler HJ, Raphael Y, Lomax MI. WDR1 colocalizes with ADF and actin in the normal and noise-damaged chick cochlea. *J Comp Neurol*. 2002; 448:399–409. [PubMed: 12115702]
- Okada K, Blanchoin L, Abe H, Chen H, Pollard TD, Bamburg JR. *Xenopus* actin-interacting protein 1 (XAip1) enhances cofilin fragmentation of filaments by capping filament ends. *J Biol Chem*. 2002; 277:43011–43016. [PubMed: 12055192]
- Okada K, Obinata T, Abe H. XAIP1: a *Xenopus* homologue of yeast actin interacting protein 1 (AIP1), which induces disassembly of actin filaments cooperatively with ADF/cofilin family proteins. *J Cell Sci*. 1999; 112:1553–1565. [PubMed: 10212149]
- Okada K, Ravi H, Smith EM, Goode BL. Aip1 and cofilin promote rapid turnover of yeast actin patches and cables: a coordinated mechanism for severing and capping filaments. *Mol Biol Cell*. 2006; 17:2855–2868. [PubMed: 16611742]
- Okanoya K, Dooling RJ. Colony differences in auditory thresholds in the canary (*Serinus canarius*). *J Acoust Soc Am*. 1985; 78:1170–1176. [PubMed: 4056211]

- Okanoya K, Dooling RJ. Strain differences in auditory thresholds in the canary (*Serinus canarius*). *J Comp Psychol*. 1987; 101:213–215. [PubMed: 3608427]
- Okanoya K, Dooling RJ, Downing JD. Hearing and vocalizations in hybrid Waterslager-Roller canaries (*Serinus canarius*). *Hear Res*. 1990; 46:271–275. [PubMed: 2394637]
- Ono S. The *Caenorhabditis elegans* unc-78 gene encodes a homologue of actin-interacting protein 1 required for organized assembly of muscle actin filaments. *J Cell Biol*. 2001; 152:1313–1319. [PubMed: 11257131]
- Ono S. Regulation of actin filament dynamics by actin depolymerizing factor/cofilin and actin-interacting protein 1: new blades for twisted filaments. *Biochem*. 2003; 42:13363–13370. [PubMed: 14621980]
- Poje CP, Sewell DA, Saunders JC. The effects of exposure to intense sound on the DC endocochlear potential in the chick. *Hear Res*. 1995; 82:197–204. [PubMed: 7775285]
- Raphael Y. Evidence for supporting cell mitosis in response to acoustic trauma in the avian inner ear. *J Neurocytol*. 1992; 21:663–671. [PubMed: 1403011]
- Raphael Y. Reorganization of the chick basilar papilla after acoustic trauma. *J Comp Neurol*. 1993; 330:521–532. [PubMed: 8320342]
- Ren N, Charlton J, Adler PN. The flare gene, which encodes the AIP1 protein of *Drosophila*, functions to regulate F-actin disassembly in pupal epidermal cells. *Genetics*. 2007; 176:2223–2234. [PubMed: 17565945]
- Roberson DW, Kreig CS, Rubel EW. Light microscopic evidence that direct transdifferentiation gives rise to new hair cells in regenerating avian auditory epithelium. *Aud Neurosci*. 1996; 2:195–205.
- Rodal AA, Tetreault JW, Lappalainen P, Drubin DG, Amberg DC. Aip1p interacts with cofilin to disassemble actin filaments. *J Cell Biol*. 1999; 145:1251–1264. [PubMed: 10366597]
- Rogers SL, Wiedemann U, Stuurman N, Vale RD. Molecular requirements for actin-based lamella formation in *Drosophila* S2 cells. *J Cell Biol*. 2003; 162:1079–1088. [PubMed: 12975351]
- Ryals BM, Rubel EW. Hair cell regeneration after acoustic trauma in adult *Coturnix* quail. *Science*. 1988; 240:1774–1776. [PubMed: 3381101]
- Ryals BM, Dooling RJ, Westbrook E, Dent ML, MacKenzie A, Larsen ON. Avian species differences in susceptibility to noise exposure. *Hear Res*. 1999; 131:71–88. [PubMed: 10355606]
- Ryals BM, Stalford MD, Lambert PR, Westbrook EW. Recovery of noise-induced changes in the dark cells of the quail tegmentum vasculosum. *Hear Res*. 1995; 83:51–61. [PubMed: 7607991]
- Saunders JC, Adler HJ, Pugliano FA. The structural and functional aspects of hair cell regeneration in the chick as a result of exposure to intense sound. *Exp Neurol*. 1992; 115:13–17. [PubMed: 1728559]
- Saunders, JC.; Doan, DE.; Cohen, YE.; Adler, HJ.; Poje, CP. Recent observations on the recovery of structure and function in the sound damaged chick ear. In: Salvi, RJ.; Henderson, D.; Colletti, V.; Fiorini, F., editors. *Auditory System Plasticity and Regeneration*. New York: Thieme Medical Publishers; 1996. p. 62-83.
- Schultz J, Milpetz F, Bork P, Ponting CP. SMART, a simple modular architecture research tool: identification of signaling domains. *Proc Natl Acad Sci USA*. 1998; 95:5857–5864. [PubMed: 9600884]
- Shimada Y, Kasakura T, Yokota M, Miyata Y, Murofushi H, Sakai H, Yahara I, Murakami-Murofushi K. Expression of a 66-kD heat shock protein associated with the process of cyst formation of a true slime mold, *Physarum polycephalum*. *Cell Struct Funct*. 1992; 17:301–309.
- Shin DH, Lee E, Chung YH, Mun GH, Park J, Lomax MI, Oh SH. Subcellular localization of WD40 repeat 1 protein in PC12 rat pheochromocytoma cells. *Neurosci Lett*. 2004; 367:399–403. [PubMed: 15337274]
- Smolders JW. Functional recovery in the avian ear after hair cell regeneration. *Audiol Neurotol*. 1999; 4:286–302. [PubMed: 10516389]
- Sondek J, Bohm A, Lambright DG, Hamm HE, Sigler PB. Crystal structure of a G_A protein $\beta\gamma$ dimer at 2.1 resolution. *Nature*. 1996; 379:369–374. [PubMed: 8552196]
- Steyger PS, Burton M, Hawkins JR, Schuff NR, Baird RA. Calbindin and parvalbumin are early markers of non-mitotically regenerating hair cells in the bullfrog vestibular otolith organs. *Int J Devel Neurosci*. 1997; 15:417–432. [PubMed: 9263023]

- Voegtli WC, Madrona AY, Wilson DK. The structure of Aip1p, a WD repeat protein that regulates cofilin-mediated actin depolymerization. *J Biol Chem.* 2003; 278:34373–34379. [PubMed: 12807914]
- Wall MA, Coleman DE, Lee E, Iñiguez-Lluhi JA, Posner BA, Gilman AG, Sprang SR. The structure of the G protein heterotrimer $G_{i\alpha 1}\beta_1\gamma_2$. *Cell.* 1995; 83:1047–1058. [PubMed: 8521505]
- Wang Y, Raphael Y. Re-innervation patterns of chick auditory sensory epithelium after acoustic overstimulation. *Hear Res.* 1996; 97:11–18. [PubMed: 8844182]
- Warner SJ, Hutson MR, Oh SH, Gerlach-Bank LM, Lomax MI, Barald KF. Expression of ZIC genes in the development of the chick inner ear and nervous system. *Dev Dyn.* 2003; 226:702–712. [PubMed: 12666207]
- Wright TF, Brittan-Powell EF, Dooling RJ, Munding PC. Sex-linked inheritance of hearing and song in the Belgian Waterslager canary. *Proc R Soc Lond B (Suppl).* 2004; 271:S409–S412.

Figure 1.

Gel electrophoresis of PCR products from four avian groups. Each +RT product depicts a band with a length of 286 nucleotides, while the –RT products lack a band. For –RT products, RNA was not reverse transcribed, because water, instead of Superscript II, was used. Thus, the –RT products act as negative control. The presence and absence of the 286-nt product in the +RT and –RT specimens, respectively, indicate successful PCR of cDNA transcribed from RNA from the ears themselves, not genomic DNA.

Figure 2.

Western blotting on chicken brain, muscle, and basilar papilla with purified PB741 (left) and PB741 with blocking peptide (right). PB741 alone yields a band of 67 kDa in all three tissue lanes, equaling the estimated molecular weight of WDR1. The muscle lane with PB741 depicts another strong band at 51 kDa. All of these bands essentially disappear following incubation with PB741 and blocking peptide, signifying the specificity of PB741.

Figure 3.

Immunolocalization of WDR1 and actin in whole mounts of chicken and BW canary basilar papilla. Fluorescent micrographs of chicken (A – F) and BW canary (G – I) basilar papillae following double immunostaining with phalloidin (green) and anti-WDR1 Ab (PB741; red) or preimmune rabbit serum. Panel I: the merging of phalloidin (panel G) with anti-WDR1 (panel H) labels in the BW ear; Panels C and F: similar merging in the chicken ear incubated with anti-WDR1 (A & B) and similar ear tissue with preimmune rabbit serum (D & E), respectively. Phalloidin labels hair cell stereocilia (st) and supporting cell microvilli (arrowheads; A, D, G), whereas anti-WDR1 Ab stains the hair cell cytoplasm (asterisks; B & H), including its apical surface. Preimmune rabbit serum labels few, if any, components (E). In conclusion, both chicken (C) and BW canary (I) inner ears present similar WDR1 labeling patterns.

Figure 4.

Fluorescent micrographs of BW canary basilar papilla after double immunostaining with phalloidin (green; supporting cell microvilli [arrowheads] and hair cell stereocilia [st]) and affinity purified PB741 (red). Two negative controls were used – one without anti-WDR1 Ab (left) and the other with anti-WDR1 Ab blocked by peptide (center). Both these controls show little, if any, red staining, while PB741 alone labels among hair cell cytoplasm (asterisks) at both hair cell surface (A) and nucleus (B) levels (right). Also, PB741 alone stains spots in the middle of hair cell nuclei (n) at the hair cell nucleus level (right).

Figure 5.

Side views of avian basilar papillae following double immunolabeling with affinity purified PB741 and phalloidin. While phalloidin (green) locates hair cell stereocilia (st) and cuticular plates (cp) in both chicken (A, B) and BW (C) canary inner ears, hair cell cytoplasm (asterisks) among chicken (A) and BW canary (C) basilar papillae exhibit red labeling by PB741, combined with fluorescent dye conjugated secondary antibody. This secondary antibody alone yields little, if any, red staining among hair cells in the chicken inner ear (B). Also, PB741 illuminates round spots in hair cell nuclei (n) in the basilar papilla of the BW canary, but not chicken. Anti-WDR1 Ab staining among hair cell cytoplasm and nuclei has been observed in the inner ears of the non-BW canary and zebra finch (data not shown).

Table 1

WD40 Regions in Chicken WDR1

WD40 Region	Amino Acid Position (Adler et al., 1999)	Amino Acid Position (present)
1	58 – 88	49 – 88
2	93 – 136	93 – 136
3	146 – 177	137 – 177
4	189 – 219	180 – 219
5	234 – 264	222 – 264
6	320 – 352	311 – 352
7	365 – 394	356 – 394
8	--	436 – 475
9	--	481 – 519
10	532 – 562	523 – 562
11	575 – 605	566 – 605

Table 2

DNA and Amino Acid Identity to Chicken WDR1

Avian Strain	DNA Identity (nt 493 – 778)	Amino Acid Identity (aa 111 – 204)
Gloster Canary	94%	99%
BW Canary	94%	99%
Zebra Finch	95%	100%