A Novel Allele of Myosin VIIa Reveals a Critical Function for the C-Terminal FERM Domain for Melanosome Transport in Retinal Pigment Epithelial Cells

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Mutations in the head and tail domains of the motor protein myosin VIIa (*MYO7A*) cause deaf-blindness (Usher syndrome type 1B, USH1B) and nonsyndromic deafness (DFNB2, DFNA11). The head domain binds to F-actin and serves as the *MYO7A* motor domain, but little is known about the function of the tail domain. In a genetic screen, we have identified *polka* mice, which carry a mutation (c.5742 + 5G > A) that affects splicing of the *MYO7A* transcript and truncates the *MYO7A* tail domain at the C-terminal FERM domain. In the inner ear, expression of the truncated *MYO7A* protein is severely reduced, leading to defects in hair cell development. In retinal pigment epithelial (RPE) cells, the truncated *MYO7A* protein is expressed at comparative levels to wild-type protein but fails to associate with and transport melanosomes. We conclude that the C-terminal FERM domain of *MYO7A* is critical for melanosome transport in RPE cells. Our findings also suggest that *MYO7A* mutations can lead to tissue-specific effects on protein levels, which may explain why some mutations in *MYO7A* lead to deafness without retinal impairment.

Introduction

MYO7A consists of an N-terminal motor domain followed by a neck and tail domain (Chen et al., 1996). The motor domain enables movement of *MYO7A* on actin filaments. The tail domain interacts with vesicle-associated proteins such as Slac2-c/MyRIP suggesting that *MYO7A* might play a role in cargo transport (Kuroda and Fukuda, 2005; Soni et al., 2005; Klomp et al., 2007). Over 100 mutations have been identified in *MYO7A* that affect the head and tail domains and cause syndromic (USH1B) and nonsyndromic (DFNB2, DFNA11) deafness (<http://www.hgmd.cf.ac.uk/ac/gene.php?gene=MYO7A>). Mutations in the head domain are thought to affect *MYO7A* motor function, but little is known about the mechanisms by which mutations in the tail domain affect protein function.

Studies in mice have provided insights into the cellular mechanisms by which mutations in *MYO7A* cause disease. *Myo7a* mutations in *shaker-1* mice cause recessive deafness, vestibular dysfunction, and retinal abnormalities (Gibson et al., 1995; Liu et al., 1997). In the inner ear, *MYO7A* is expressed in mechanosensory hair cells and is required for hair bundle morphogenesis and mechanotransduction (Self et al., 1998; Kros et al., 2002). Within the retina, *MYO7A* localizes to the cilium of the photoreceptors, to the apical region of retinal pigment epithelial (RPE) cells, and to melanosomes within RPE cells (Wolfrum et al., 1998; El-Amraoui et al., 2002; Gibbs et al., 2004). In accordance with its expression pattern, *MYO7A* regulates opsin transport in photoreceptors and the phagocytosis of shed outer segments by RPEs (Liu et al., 1999; Gibbs et al., 2003). Melanosomes fail to localize to the apical processes in RPEs of *shaker-1* mice, indicating that *MYO7A* is required for movement and/or retention of melanosomes within the apical processes (Liu et al., 1998). Unlike the human patients, *shaker-1* mice do not show degeneration of photoreceptor cells, suggesting that the mice mimic only some aspects of the human disease. Nevertheless, mice provide currently the best available animal model for the human disease.

Here, we report a novel *Myo7a* allele termed *polka* that we isolated in a genetic screen in mice (Schwander et al., 2007). *Polka* mice carry a point mutation (c.5742 + 5G > A) in intron 42 that affects splicing and is predicted to truncate the tail domain of *MYO7A* at its C-terminal FERM domain. While the mutant *MYO7A* protein was expressed in the retina, little expression was

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Figure 1. Auditory function in *polka* mice. **A**, Auditory brainstem response (ABR). Representative examples of click-evoked ABR waveforms for a C57BL/6J wild-type and a *polka* homozygous mutant mouse at different sound intensities (in decibels). ABR waves I–IV are indicated for recordings obtained from a wild-type mouse. The *polka* mutant mouse failed to respond to all tested sound intensities (0–90 dB). **B**, Average auditory thresholds for 2-month-old mice (wild type $n = 4$, *polka* $n = 9$; the mean $\pm$ SD). **C**, Representative DPOAE response spectra for a wild-type (top trace) and a *polka* mouse (bottom trace) at a single stimulus condition (median primary frequency = 16 kHz). Note the cubic distortion product ($2f_1 - f_2$), which is absent in recordings with *polka* mice at 60 dB. **D**, Cubic distortion product levels as a function of stimulus intensity at 10 kHz from wild-type and *polka* mice. No response was observed in *polka* mice at all intensity levels (wild type $n = 4$, *polka* $n = 9$; mean $\pm$ SD). **E**, DPOAE thresholds were highly elevated at all frequencies analyzed (wild type $n = 4$, *polka* $n = 9$; mean $\pm$ SD). Primary frequencies were maintained at an f_2/f_1 ratio of 1.22, and L_1 was equal to L_2 .

observed in the inner ear. Hair bundles in the inner ear showed morphological defects that likely caused the deafness phenotype of *polka* mice. In the retina, the mutant MYO7A protein was still localized to the apical processes of RPE cells, but associated less well with melanosomes, which were mislocalized. We conclude that the C-terminal FERM domain of MYO7A is important for association with and transport of melanosomes. In addition, our findings show that a point mutation in a gene can differentially affect its expression in the inner ear and retina, a finding that might be relevant to understanding disease mechanisms associated with mutations in *Myo7a*.

Materials and Methods

ENU mutagenesis screen and evaluation of inner ear function. The ENU mutagenesis protocol and primary phenotypic screen has been described previously (Reijmers et al., 2006; Schwander et al., 2007). The measurement of auditory brainstem responses (ABRs), distortion product otoacoustic emissions (DPOAEs), and vestibular function followed our published procedures (Schwander et al., 2007).

Linkage analysis and DNA sequencing. Linkage analysis using single nucleotide polymorphism (SNP) markers was performed as described previously (Wiltshire et al., 2003; Schwander et al., 2007). Affected *polka* mice were bred with 129S1/SvImJ mice. The F_1 offspring were intercrossed to obtain F_2 mice and tail DNA was prepared for linkage mapping using SNPs markers (Wiltshire et al., 2003). Map Manager QTX (Manly et al., 2001) was used to calculate logarithm of the odds (LOD) scores and perform interval mapping. Exons and exon–intron boundaries of genes in the mapped intervals were sequenced. Primers for PCR amplification and DNA sequencing were designed with Primer 3 software (MIT). DNA

numbering is based on *Myo7a* cDNA (NM_008663) and starts with +1 as the A of the ATG initiation methionine.

Histology, immunohistochemistry, and scanning electron microscopy. Staining of sections and TUNEL staining were performed as described previously (Fariñas et al., 1996; Müller et al., 1997; Gibbs et al., 2004). Whole-mount staining and scanning electron microscopy of cochlear sensory epithelia were performed as described previously (Senften et al., 2006; Schwander et al., 2007).

Immunoelectron microscopy and gold particle quantitation. For immunoelectron microscopy, eyecups were fixed by immersion in 0.25% glutaraldehyde, 4% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.4. Samples were embedded in LR White (EMS). Ultrathin sections (70 nm) were etched with saturated sodium periodate and blocked with 4% bovine serum albumin (BSA) in antibody buffer (1% BSA + 1% Tween 20) for 1 h. The sections were then incubated with primary antibodies (anti-MYO7A and anti-MYRIP) overnight at 4°C. After washing, samples were incubated with goat anti-rabbit IgG conjugated to 12 nm gold (EMS) for 1 h. Finally sections were stained with uranyl acetate and lead citrate. Sections that were not incubated with the primary antibody or *shaker-1* sections (*sh1*^{4626SB}) (Rinchik and Carpenter, 1999) were processed at the same time and used as negative controls. Immunolabeling density was determined by counting gold particles in ultrathin sections. Images were taken randomly along the RPE, 25 per condition, and analyzed. Gold particles were considered to be associated with the melanosome membrane when located at a maximum of 30 nm of the membrane of the organelle, as described previously (Klomp et al., 2007). Section area was determined with ImageJ software.

Complementation tests and genotyping. Heterozygous *polka* mice were crossed with homozygous *sh1*^{4626SB} mice, which carry a predicted *Myo7a*-null allele (Mburu et al., 1997; Rinchik and Carpenter, 1999). Auditory thresholds were determined by ABR tests. Genotyping was performed by PCR using a set of primers that flank the *polka* mutation in the *Myo7a* gene: forward primer, 535f 5'-GGTCTGCAGAAGTTGAGTG-3', and reverse primer 535r 5'-AAGCTTGTGCTGCCATGTACC-3'. PCR fragments were purified and digested with BstYI to give a 150 bp product in homozygous mutants, and 300 bp and 150 bp products in heterozygous littermates. For the *Myo7a*^{4626SB} mutation genotyping was performed as described previously (Holme and Steel, 2002).

RT-PCR and quantitative PCR. RNA was isolated from cochleas and eyes by using Trizol (Invitrogen), according to the manufacturer's instructions. RNA concentration was determined using Nanodrop. cDNA was synthesized from 400 ng of RNA with Superscript III reverse transcriptase (Invitrogen) and oligo(dT) primers. RT-PCR analysis for the splicing of *Myo7a* transcripts was performed with primers exon41f 5'-CATAAGACTACCCAGATCTTC-3', exon42f 5'-GGCTGCTGCTCAAGTCTTC-3', exon42r 5'-GAAGACTTGAGCAGCAGCC-3', and exon43r 5'-GAAATCATTCTCTGGGACGC-3'. Gene expression was assessed by quantitative PCR by using gene-specific primers and SYBR green (Applied Biosystems) in a PTC-200 thermal cycler (Bio-Rad) coupled to a Chromo4 real-time PCR detection system (Bio-Rad). *Myo7a* mRNA expression data were normalized by using *cadherin 23* (*Cdh23*) and *otofelin* (*Otof*), as well as the housekeeping genes *36B4* and *GAPDH*, as reference genes. The primer sequences recognizing *Myo7a* were as follows: forward primer, myo7a_5431f1 5'-ATCCTCTGCCTCATGTTCAG-3', reverse primer, myo7a_5594r1 5'-CGGGGAAGTAGACCTT-

GTGGA-3'; for cadherin 23: forward primer, *cdh23_6157f3* 5'-GCCACCTGTTTCATCACTATC-3', reverse primer, *cdh23_6260r3* 5'-TGGCTGTGACTTGAAGGACTG-3'; for otoferlin: forward primer, *otof_4059f3* 5'-GGAAGAGAAGGAAGAGATGGAAAG-3', reverse primer, *otof_4143r3* 5'-GGGCTCTGGTTTTCTTCTTTTC-3'.

Results

Analysis of auditory and vestibular function in *polka* mice

We have previously described a forward genetics screen in mice aimed at identifying recessive deafness traits (Schwander et al., 2007). One of the lines from the screen, termed *polka*, showed prominent circling behavior and performed poorly in forced swim tests, indicative of vestibular dysfunction. *Polka* mice also failed to show an acoustic startle response (ASR) (Schwander et al., 2007). As defects in the ASR can be caused by auditory defects, general defects in the nervous system or altered motor function, we next tested *polka* mice for auditory function by evaluating their ABR (Zheng et al., 1999). To determine auditory thresholds we applied broadband click stimuli to 2-month-old mice starting at 90 dB and then decreasing in intensity. In *polka* mice ABR thresholds were highly elevated (>90 dB) when compared to wild-type C57BL/6J mice (Fig. 1A,B), suggesting that the auditory phenotype can be attributed to impaired hair cell or neuronal function. To study hair cell function, we measured the DPOAEs. In wild-type mice, DPOAEs were dependent on the stimulus intensity at a given frequency, but were not detectable in the mutants, as shown in a plot of DPOAE level versus stimulus level at the mean primary frequency of 10 kHz (Fig. 1C,D). Similar observations were made at all of the frequencies analyzed (6–28 kHz) (Fig. 1E), indicating that the function of outer hair cells was impaired across the entire analyzed frequency spectrum.

Polka is a novel allele of the *Myo7a* gene

As part of the original genetic screen we performed heritability testing and demonstrated that *polka* mice, which were derived on a C57BL/6J background, inherit their deafness/balance phenotype recessively (Schwander et al., 2007). To map and positionally clone the affected gene, we outcrossed affected *polka* mice to 129S1/SvImJ mice. The resulting offspring was intercrossed to obtain F₂ mice for ABR phenotyping, tail DNA preparation, and SNP mapping. Consistent with a nonlethal recessive trait, we found ~21% of 190 F₂ animals analyzed to be affected (Schwander et al., 2007).

Using tail DNA from nine affected and four unaffected F₂ animals (26 meiotic events), we ran SNP arrays as previously described (Wiltshire et al., 2003; Schwander et al., 2007). The mutation in *polka* mice mapped to a 27 MB interval on chromo-

Figure 2. SNP mapping of the mutation in *polka* mice. **A**, DNA from nine affected mice (af) and four unaffected mice (uf) was analyzed for SNP markers on chromosome 7, which are listed in the first column and indicate the megabase position (68.363–103.844). The mouse identification numbers and phenotypes are indicated in the two top rows. The genotype for each marker is given as follows: B, homozygous C57BL/6J genotype; C, homozygous BALB/cByJ; H, heterozygous; and blanks indicate SNPs for which the data collection was incomplete. On the right is a diagram of mouse chromosome 7 showing the interval to which the auditory/vestibular phenotype mapped with the relative locations of three genes reported within the interval. **B**, Sequence chromatograph from representative unaffected (wt) and affected (*polka* $-/-$) mice at the 3'-end of exon 42 of *Myo7a*. A G-to-A transition (c.5742 + 5G > A) that was found only in affected mice is boxed. The point mutation creates a BstYI restriction site, facilitating genotyping of the mice. **C**, An agarose gel picture shows the resolved fragments from BstYI RFLP/PCR analysis on genomic DNA of a wild-type, and a homozygous *polka* mouse (wild type, 300 bp; and mutant, 150 bp). **D**, Complementation analysis. Homozygous *polka* mice were crossed with heterozygous *sh1*^{4626SB} mice. Note that in the offspring only mice that were compound heterozygotes for the *polka* and *sh1*^{4626SB} alleles showed elevated auditory thresholds.

somes (Fig. 2A). We next sequenced the exons and exon–intron boundaries of all annotated and predicted genes in the interval, including the *Myo7a* gene, which has previously been linked to deafness in mice and humans (Gibson et al., 1995; Weil et al., 1995). Sequencing of all exons of *Myo7a* from multiple affected and unaffected control mice did not reveal any point mutations. However, a G-to-A transition (c.5742 + 5G > A, reference sequence: NM_008663) was present at the fifth position of intron 42, and uniquely homozygous in mice that displayed the deafness phenotype (Fig. 2B) (112 mice analyzed). The point mutation was confirmed by restriction analysis (Fig. 2B,C). No mutation was found in any other gene in the interval (data not shown).

To confirm that the point mutation in the *Myo7a* gene caused the deafness phenotype, we performed complementation tests with *shaker-1* mice (*sh1*^{4626SB}) (Fig. 2D) (Rinchik et al., 1990; Rinchik and Carpenter, 1999). The *Myo7a*^{4626SB} mutation intro-

Figure 3. RT-PCR analysis of mRNA from the *Myo7a* locus. **A**, Predicted donor splice site motif and splice score. **B**, RT-PCR analysis of the *Myo7a* transcripts obtained from the inner ear (P5) from wild-type and *polka* mice. PCR analysis of genomic DNA from wild-type mice was included as a control. Note that fragments including the *polka* mutation (Ex42/43, Ex41/43) were ~50 bp larger than fragments amplified from wild-type mRNA. **C**, Sequence determination of *Myo7a* PCR products generated from mutant mice. A splicing defect induced the retention of the first 49 bp of intron 42. **D**, Domain structure of MYO7A. The *polka* mutation creates a frameshift and introduces a premature stop codon, which is predicted to truncate the protein within the FERM₂ domain.

duces a stop codon near the 5' end, which likely results in a functional null allele (Hasson et al., 1997; Mburu et al., 1997). We crossed homozygous *polka* mice with heterozygous *sh1*^{4626SB} mice, genotyped the offspring, and determined auditory thresholds by measuring ABRs. Mice that were compound heterozygotes for the *polka* and *sh1*^{4626SB} alleles tested deaf, while littermates carrying one *polka* allele and one wild-type allele showed normal auditory thresholds (Fig. 2D). Heterozygous *sh1*^{4626SB} mice also had normal hearing function (data not shown). We conclude that the point mutation in the *Myo7a* gene is responsible for the deafness phenotype in *polka* mice.

The *polka* mutation affects splicing of the *Myo7a* transcript

The G-to-A transition in *Myo7a* disrupts the 3' end of the U1 snRNP binding site (Fig. 3A) (Blencowe, 2000). Applying the statistical Shapiro–Senapathy splicing algorithm (Senapathy et al., 1990), splice scores of 0.82 and 0.88 result for the mutant and the wild-type variant, respectively. The lower splice score indicates a lowered binding affinity of the mutated splice site with the corresponding U1 snRNP, which could result in either exon skipping or cryptic splice site activation (Krawczak et al., 2007). To examine the effect of the mutation on the splicing of *Myo7a* transcripts, we performed reverse-transcription PCR (RT-PCR) amplification on total RNA isolated from vestibular sensory patches from wild-type and *polka* mice. Amplification with primers targeting exon 42 and 43, which are located 5' and 3' of the mutated intron 42, identified a novel *Myo7a* mRNA species in mutant samples, which was ~50 bp larger than the wild-type transcript (Fig. 3B). Subcloning and DNA sequencing of the amplified products revealed a 49 bp insert between exons 42 and 43 in *polka* mice (Fig. 3C). The sequence of the insert completely matched part of intron 42 indicating that the mutation in *polka* mice led to

the utilization of a cryptic splice site in intron 42 (GA/GTGGGT) generating an altered transcript (Myo7a⁴²⁺⁴⁹) that includes intronic sequences. The MYO7A C-terminal tail contains two FERM domains (Fig. 3D) (Chen et al., 1996). Based on our sequencing data we predicted that the *polka* mutation leads to premature truncation of MYO7A after the first 56 aa of the C-terminal FERM domain (referred to in the following as FERM₂) and the addition of a 33-aa-long aberrant C-terminal peptide (Fig. 3D).

Defective hair bundle morphology and melanosome localization in *polka* mice

Myo7a mutations in *shaker-1* mice lead to defects in hair bundle development in the inner ear and to defects in the localization of melanosomes to the apical process of RPE (Liu et al., 1998; Self et al., 1998). To determine whether the *polka* mutation caused similar phenotypic defects, we first stained cochlear sensory epithelia from heterozygous and homozygous *polka* mice as whole mounts with phalloidin to label F-actin in stereocilia (Fig. 4). In *polka* mice, stereociliary bundles were disorganized in all four rows of hair cells. Fragmented bundles with defective morphology were present that contained a

small number of short stereocilia (Fig. 4A,B, arrows), and some bundles failed to develop a clear polarity in the apical hair cell surface (Fig. 4B, asterisk). These findings were confirmed by scanning electron microscopy (Fig. 4C–H). Bundles were smaller, many of the stereocilia were short and malformed (Fig. 4D,H, arrows), and polarity defects were evident (Fig. 4D,H, asterisk).

Next we analyzed retinal sections from *polka* mice (Fig. 5). At the light microscopic level, 2-month-old *polka* mice revealed no obvious structural defects in the retina, including the photoreceptors. However, unlike in wild-type mice, melanosomes did not localize to the apical processes of RPE cells (Fig. 5B, arrowheads). We conclude that the *polka* mutation leads to similar phenotypic manifestations in the ear and retina as observed in *shaker-1* mice.

MYO7A expression is affected in a tissue-specific manner

Several *shaker-1* mutations map to the myosin motor domain and are thought to affect both motor function and MYO7A protein levels (Hasson et al., 1997; Mburu et al., 1997). As the mutation in *polka* mice maps to the tail domain, we hypothesized that it might affect hair cells and RPE cells by a different mechanism. As one possibility, truncation of the FERM₂ domain might affect protein function in cargo transport. However, the mutation might also lead to instability of the RNA or protein thereby affecting MYO7A levels. To distinguish between these possibilities, we compared the expression of the MYO7A transcript and protein in wild-type and *polka* mice.

Murine MYO7A is a 250 kDa polypeptide that is highly expressed in the cochlea, retina, testis, lung, and kidney (Hasson et al., 1995). To analyze MYO7A protein expression, we performed Western blots on tissue extracts from wild-type and mutant mice

Figure 4. Analysis of hair bundle morphology. **A, B**, Images of phalloidin-stained whole mounts from the middle turn of the cochlea of heterozygous (**A**) and homozygous (**B**) *polka* mice at postnatal day 5 (P5). In *polka* mice morphological changes were evident such as splaying of stereocilia (arrows) and cell polarity defects (asterisk). **C–H**, Scanning electron micrographs of cochlear whole mounts of P5 heterozygous (**C, E, G**) and homozygous (**D, F, H**) *polka* mice. Note that hair bundles were smaller and fragmented in homozygous mutants (arrows). Scale bars: **A, B**, 8 μ m; **C, D**, 5 μ m; **E–H**, 2 μ m.

(Fig. 6A). A 250 kDa protein was detected in extracts from brain, kidney, eye and ear in wild-type mice. In eye and brain extracts of homozygous mutant mice, the band for MYO7A was of similar intensity as in wild type, but it was shifted to a slightly smaller size. In eye extracts from heterozygous mutants two bands of the predicted size of full-length and mutant MYO7A could be distinguished (Fig. 6B). These findings support the interpretation of our sequencing results, and indicate that as a consequence of aberrant splicing a truncated MYO7A protein is expressed in the brain and eye of *polka* mice. Quantification of expression levels using Western blots from eye extracts confirmed that MYO7A expression levels in the retina were not reduced (Fig. 6C). In contrast, MYO7A expression levels were strongly reduced in extracts from mutant kidney and cochlea (Fig. 6A, C). We conclude that the *polka* mutation leads to tissue-specific effects in the expression of MYO7A.

To further define the mechanisms that caused tissue-dependent instability of mutant MYO7A, we compared mRNA levels of *Myo7a* in eye and ear tissues from *polka* and wild-type mice by quantitative RT-PCR analysis. Expression of *Myo7a* transcripts

Figure 5. Defects in melanosome localization. **A, B**, Retinal semithin sections from 6-month-old mice. *Polka* mice showed no signs of retinal degeneration (**A**). Melanosomes failed to localize in apical processes of mutant RPE cells (**B**, arrowheads). OS, Photoreceptor outer segments; IS, photoreceptor inner segments; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; Scale bars: **A, B**, 10 μ m.

Figure 6. MYO7A protein and mRNA expression. **A**, Western blot analysis revealed a slightly reduced molecular weight for MYO7A in brain and eye extracts from mutant mice, while its expression was drastically reduced in kidney and cochlea. **B**, Wild-type and mutant MYO7A were detected as a double band in eye extracts from heterozygous *polka* mice. An additional smaller band of unknown origin was identified only in extracts from heterozygous mice (asterisk). **C**, Densitometry of Western blots. **D–F**, Real-time RT-PCR analysis. mRNA levels of *Myo7a* in ear and eye were quantified in relation to otoferrin (*Otof*) (**D**), 36B4 (**E**), and cadherin 23 (*Cdh23*) (**F**). Levels of *Myo7a* were drastically decreased in ears, but not in eyes of *polka* mice. Data are expressed relative to levels of *Myo7a* transcripts in wild type [set as equal to 1; mean $\pm$ SEM of 4 animals (*polka*) and 3 animals (wild type) per group].

Figure 7. MYO7A expression in hair cells. **A**, Cochlear whole mounts of heterozygous and homozygous *polka* mice at P5 were stained with phalloidin (green) to reveal hair cell stereocilia and an antibody against MYO7A (red). MYO7A expression was detected in outer and inner hair cells of heterozygous mice but was absent in the mutants. **B**, At higher magnification, MYO7A was localized to the cuticular plate and toward tips of stereocilia in heterozygous mice. In mutant hair cells, MYO7A could no longer be detected. Occasionally, very low amounts of protein could be visualized at tips of stereocilia in some hair cells (arrows). Scale bars: **A**, **B**, 8 μ m.

Figure 8. MYO7A expression in the retina. **A**, **B**, Retinal cryosections from wild-type and *polka* mutant mice were stained with antibodies against MYO7A (red) and with phalloidin (green) to visualize F-actin (**A**). In **B**, high-magnification views of stainings for MYO7A (red) are shown. Expression of MYO7A in the apical RPE of *polka* mice was still detectable but not as well confined to the apical region of the cells compared to wild type (**B**, arrows). Scale bars: **A**, **B**, 10 μ m.

was strongly decreased in ear tissue of mutant mice when compared with wild type (Fig. 6D–F). In contrast, no significant difference in the levels of *Myo7a* was observed in the eye (Fig. 6D–F), suggesting that mutant mRNAs in the ear but not the retina are degraded by the nonsense-mediated decay pathway (Isken and Maquat, 2008).

Interestingly, while reduced levels of a truncated MYO7A protein were still expressed in the inner ear of *polka* mice, the protein was no longer detectable in stereocilia (Fig. 7A, B), suggesting that the residual protein was confined to the cell body. We next determined the expression and localization of the truncated MYO7A protein in the retina of *polka* mice by immunohistochemistry. At the light microscopic level, MYO7A was still localized to the apical processes of RPE cells, although the signal appeared more diffuse and was more widespread (Fig. 8A, B), indicative of changes of MYO7A distribution within RPE cells.

Previous studies have provided evidence that the exophilin, Slac2-c/MYRIP, binds to a domain in MYO7A that includes the FERM₂ domain, which is affected by the *polka* mutation. Furthermore, Slac2-c/MYRIP links RAB27A on melanosomes to MYO7A (El-Amraoui et al., 2002; Fukuda and Kuroda, 2002; Kuroda and Fukuda, 2005; Klomp et al., 2007). The functional significance of these interactions has remained unclear. We hypothesized that these interactions might be important for melanosome transport into the apical processes of RPE cells, and that this process might be affected in *polka* mice. We therefore determined the subcellular distribution of MYO7A and Slac2-c/MYRIP in

RPEs by immunoelectron microscopy (Fig. 9). Quantification of immunogold showed that total levels of MYO7A and MYRIP were unaffected in RPE cells and in photoreceptor cilia of mutant mice (Fig. 9; supplemental Fig. 1, available at www.jneurosci.org as supplemental material; and data not shown), consistent with the Western blot data. However, the fraction of MYO7A associated with melanosomes was significantly reduced in mutants compared to wild type, and the number of melanosomes without MYO7A was significantly increased. In parallel, MYO7A levels in the apical region of RPE cells were reduced, and in the basal region increased, indicating that MYO7A was redistributed in the mutants. The data suggest that the FERM₂ domain is required for the recruitment of MYO7A to melanosomes (Fig. 9A, B, E). In contrast, the density of Slac2-c/MYRIP on melanosomes was unaffected (Fig. 9C–E). As Slac2-c/MYRIP also binds to the melanosome associated protein RAB27A, our findings suggest that Slac2-c/MYRIP is recruited independently of MYO7A to melanosomes. However, disruption of the interaction between Slac2-c/MYRIP and MYO7A likely explains the defect in the transport of melanosomes into the apical processes of RPEs.

Discussion

We describe here the *polka* mouse line, which carries a point mutation in *Myo7a* that sheds light on the function of its tail domain. The *polka* mutation led to aberrant splicing of *Myo7a* transcripts that affected its stability in the inner ear. As a consequence, the MYO7A protein was no longer expressed in the stereocilia of hair cells, leading to defects in hair bundle development. In contrast, in the retina of *polka* mice MYO7A protein with a truncation in the FERM₂ domain were expressed at similar

Figure 9. Subcellular localization of MYO7A and MYRIP in the RPE. **A–D**, Immunoelectron microscopy on wild-type (**A**, **C**) and mutant (**B**, **D**) RPE from retinal sections labeled with anti-MYO7A and anti-MYRIP antibody and 15 nm protein A–gold. Gold particles have been circled. **E**, Quantification of the immunolocalization data. Gold particles were counted in ultrathin sections. Twenty-five images were analyzed randomly for each condition and animal. Gold particles were considered to be associated with the melanosome membrane when located at a maximum of 30 nm from the membrane of the organelle, as described previously (Klomp et al., 2007). Values are mean $\pm$ SEM ($n = 3$ wild-type and mutant animals). Scale bar, 100 nm.

levels as full-length protein in wild-type mice. As a consequence, melanosome transport in RPE cells was affected. We conclude that the FERM₂ domain of MYO7A is required for cargo transport into the apical processes of RPE cells. Our findings also show that a *Myo7a* point mutation can differentially affect gene expression in the inner ear and retina. The latter findings might explain why some mutations in MYO7A in humans only affect hearing function, while others affect both hearing and vision.

Previous studies have provided insights into the mechanisms by which mutations in *Myo7a* can cause disease, largely focusing on different alleles of *Myo7a* that affect its motor domain. While mRNA levels were not affected in mice carrying mutations in the MYO7A motor domain, protein levels were drastically reduced, leading to the suggestion that motor-domain mutations destabi-

lize MYO7A (Hasson et al., 1997). In addition, some USH1 mutations in the MYO7A head domain also affect motor function of recombinant human MYO7A, suggesting that defects in the interaction of MYO7A with actin also contribute to the disease (Watanabe et al., 2008).

Our findings now show that pathological changes in *polka* mice are caused by different mechanisms. The mutation in *polka* mice inactivates the exon 42 splice donor site in *Myo7a* and activates a cryptic splice-donor within the intron flanked by exon 42 and 43. Therefore a *Myo7a* transcript is generated with a 49 base pair intronic insert that introduces a premature stop codon. The abundance of the aberrantly spliced transcript is drastically reduced in the cochlea, likely as a consequence of nonsense-mediated mRNA decay (Isken and Maquat, 2008). MYO7A protein is also no longer detectable in stereocilia leading to defects in hair bundle development. In contrast, *Myo7a* transcripts escaped nonsense-mediated decay in the retina leading to the expression of a truncated MYO7A protein at similar levels as in wild-type mice. However, our findings provide evidence that the truncated protein is functionally impaired. Despite the presence of the truncated MYO7A protein in the apical processes of RPE cells, *polka* mice show defects in melanosome organization indistinguishable from the *shaker-1* phenotype (Liu et al., 1998; Gibbs et al., 2004).

Mutant MYO7A may be misfolded or compromised in its motor function as studies with myosin V have shown that the tail domain regulates the function of its motor domain (Trybus et al., 1999; Homma et al., 2000; Wang et al., 2000; Thirumurugan et al., 2006). However, we think that this possibility is unlikely because mutant MYO7A is properly targeted to the apical processes of RPE, which would likely not occur with a misfolded protein or a protein without motor function. Instead, we favor the alternative hypothesis that MYO7A is unable to bind and transport cargo. Consistent with this model, the exophilin Slac2-c/MyRIP is thought to function as a linker protein between RAB27A on melanosomes and MYO7A (Fukuda and Kuroda, 2002; Kuroda and Fukuda, 2005; Klomp et al., 2007). In yeast two-hybrid and in *in vitro* assays Slac2-c/MyRIP can bind to the C-terminal 464 aa of MYO7A that contain the FERM₂ domain (El-Amraoui et al., 2002). Therefore, disruption of the FERM₂ domain might affect interactions with melanosomes, which is consistent with our immunoelectron microscopy studies that revealed reduced association of MYO7A with melanosomes in RPEs of *polka* mice.

Interestingly, *polka* mice like previously described *Myo7a* alleles fail to show clear signs of retinal degeneration, highlighting the fact that melanosome localization is not critical for retinal viability (Liu et al., 1998). In fact, most, if not all mouse models of USH1 do not reproduce the retinal degeneration phenotype of USH1 patients (Williams, 2008). A recent study suggests that defective photoreceptor function might cause retinal degeneration in USH1B (Jacobson et al., 2008). The reason why genetic lesions in *polka* and *shaker-1* alleles do not reproduce the photoreceptor degeneration found in USH1B patients remains obscure. Possibilities have been discussed previously (Liu et al., 1998; Lillo et al., 2003), but it should be noted that many mouse models of retinal degeneration, as well as other neurodegenerative disorders, mimic the pathological changes observed in humans only incompletely. Of interest, however, is the recent demonstration that mutations in the FERM₂ domain of MYO7A can lead to USH1B in humans (Jaijo et al., 2006; Riazuddin et al.,

2008). Similar to the allele identified in *polka* mice, the FERM₂ mutation (c.5856G > A) affects the last nucleotide in exon 42 and has been predicted to influence splicing of the *myo7a* transcript (Jaijo et al., 2006). Based on our studies, it seems likely that such mutations in the FERM₂ domain destabilized the *myo7a* transcript in the inner ear thereby causing defects in hair bundle development. In contrast, MYO7A protein in the retina might be functionally impaired leading to visual defects in the affected patients. As disease causing mutations have been mapped to several structural domains within the complex MYO7A tail region, it will be important to generate in the future mouse models that specifically target other structural domains besides the FERM₂ domain to define their function as well as pathogenesis mechanisms that lead to visual and auditory impairment.

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