Dysfunction of *GRAP*, encoding the GRB2-related adaptor protein, is linked to sensorineural hearing loss

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We have identified a *GRAP* variant (c.311A>T; p.Gln104Leu) cosegregating with autosomal recessive nonsyndromic deafness in two unrelated families. *GRAP* encodes a member of the highly conserved growth factor receptor-bound protein 2 (GRB2)/Sem-5/drk family of proteins, which are involved in Ras signaling; however, the function of the growth factor receptor-bound protein 2 (GRB2)-related adaptor protein (GRAP) in the auditory system is not known. Here, we show that, in mouse, *Grap* is expressed in the inner ear and the protein localizes to the neuronal fibers innervating cochlear and utricular auditory hair cells. Downstream of receptor kinase (*drk*), the *Drosophila* homolog of human *GRAP*, is expressed in Johnston's organ (JO), the fly hearing organ, and the loss of *drk* in JO causes scolopidium abnormalities. *drk* mutant flies present deficits in negative geotaxis behavior, which can be suppressed by human wild-type but not mutant GRAP. Furthermore, drk specifically colocalizes with synapsin at synapses, suggesting a potential role of such adaptor proteins in regulating actin cytoskeleton dynamics in the nervous system. Our findings establish a causative link between *GRAP* mutation and nonsyndromic deafness and suggest a function of GRAP/drk in hearing.

adaptor proteins | drk | *Drosophila* | GRAP | nonsyndromic hearing loss

Hearing loss (HL) is the most common sensory disorder with an incidence of 1–3 in 1,000 births (1). Genetic factors show high heterogeneity and are responsible for more than half of the cases with congenital HL (2). It is estimated that 70% of genetic HL is nonsyndromic sensorineural HL and mostly shows an autosomal recessive inheritance pattern (1, 3). The identification of DNA variants causing HL is rapidly advancing due to technological developments in genome sequencing. However, delineating causality for a novel rare variant in a gene not previously associated with HL is difficult and often requires functional studies in model organisms.

The mouse has been shown to be an excellent model organism to study human deafness due to the anatomic and physiologic similarities of their auditory systems with those of humans (4). Recent studies of *Drosophila*'s hearing organ, Johnston's organ (JO), highlight the molecular and genetic conservations of the mechanosensory transduction in flies and vertebrates (5, 6). Several key genes first identified in *Drosophila*, such as *atonal*, *crinkled*, and *nanchung*, that are required for the specification or function of auditory cell types in the JO are also important for the normal development or function of the vertebrate ear (5). In addition, 20% of *Drosophila* auditory-associated genes have human homologs and are implicated in hearing disorders (7).

In this paper, via exome and genome sequencing of over 60 consanguineous multiplex families with autosomal recessive nonsyndromic HL, which are negative for variants in known deafness genes (details of families are given in *SI Appendix*, Table S1), we have identified a *GRAP* variant in two unrelated Turkish families. We show that *Grap* is expressed in the inner ear of mice during the development and in adults. Moreover, Grap localizes in the neuronal fibers that innervate the auditory hair cells. To provide evidence for the causality of the variant, we established a loss-of-*Drosophila* ortholog (downstream of receptor kinase, drk) model, which recapitulates aspects of the human phenotype. In addition, our functional studies of growth factor receptor-bound protein 2 (Grb2)-related adaptor protein (GRAP) in vivo suggest a causal link between mutant *GRAP* and deafness.

Results

Affected Individuals Diagnosed with Nonsyndromic Profound Deafness. Four affected individuals from two consanguineous families of Turkish origin (Fig. 1*A*) showed congenital profound sensorineural hearing loss (Fig. 1*B*). Audiograms of one unaffected member from each family were normal (Fig. 1*B*). The remainder of the physical examinations were normal. In the affected individuals, there was no history of pre- or postnatal exposure to

Significance

Specialized cells in the inner ear translate sound into electrical signals for hearing, which are transferred to the brain through the cochlear nerve. Many of the molecular components of the inner ear are currently unknown. This paper uses a genetic approach to identify *GRAP* as a gene mutated in human deafness. In mice, Grap is present at the neuronal fibers innervating the auditory hair cells. The *Drosophila* homolog of the human Grb2-related adaptor protein (GRAP), drk, localizes to the synaptic terminals of neurons, and its disruption leads to hearing organ abnormalities associated with defects in locomotor behavior. We conclude that GRAP/drk plays an indispensable role in hearing.

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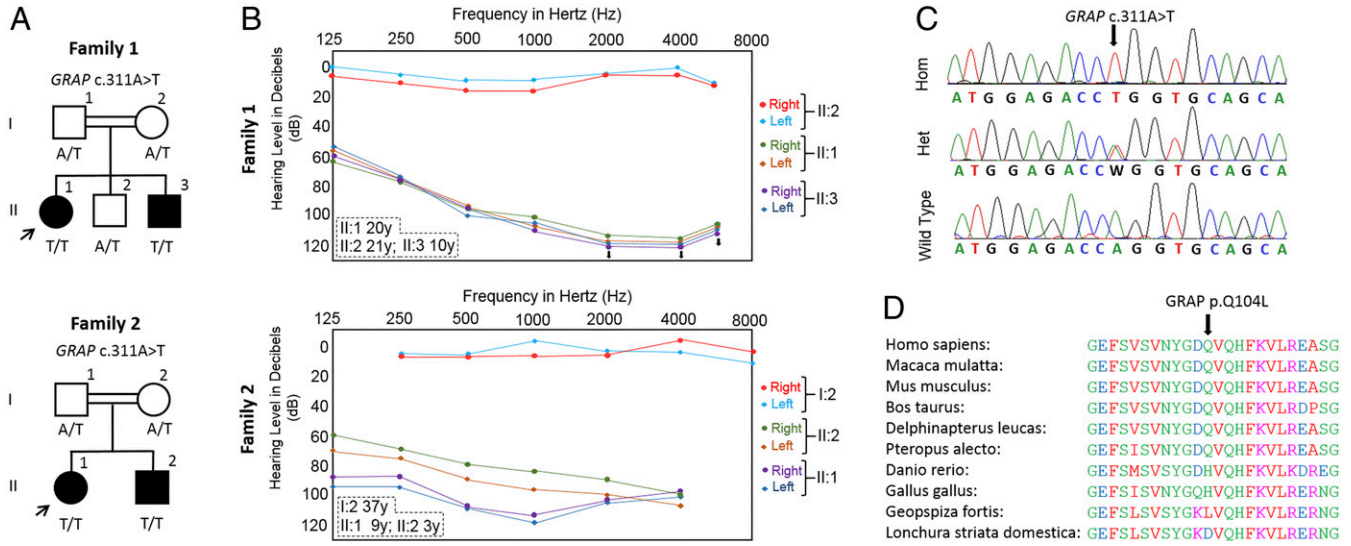

Fig. 1. Nonsyndromic profound deafness is diagnosed in affected individuals who are homozygous for a *GRAP* variant. (*A*) Pedigrees and segregation of the *GRAP* c.311A>T variant in families 1 and 2. (*B*) Hearing thresholds obtained from pure tone audiograms of the affected individuals showing severe to profound HL. Unaffected individuals display normal hearing. Ages indicated are at the time of the audiograms. (*C*) Electropherograms showing the identified variant. The wild-type (WT) traces are from an unrelated individual. Hom, homozygous mutant, Het, heterozygous mutant. (*D*) Amino acid sequences of the partial SH2 domain of GRAP in different species. Amino acid Gln104 (Q104) is highly conserved in mammals.

ototoxic medications, head or sound trauma, neonatal jaundice, premature birth, intrauterine infections, or perinatal hypoxia. They all had normal gross motor development without balance problems, vertigo, dizziness, or nystagmus. Tandem walking was normal, and the Romberg test was negative.

Exome and Genome Sequencing Identifies a *GRAP* Variant. The coverages of targeted regions in the exome data for the 10-fold read depth were 87% and 86%, and the average read depths were 50× and 52× in the probands of family 1 and family 2, respectively. Genome sequencing showed average sequencing read depths of 66× and 44×; coverages of, at least, 4× were 96.1% and 99.7% of the genome in the probands of family 1 and family 2, respectively. The analysis of data did not reveal a single nucleotide polymorphism, indel, or copy number variant in any of the previously recognized genes for nonsyndromic HL (https://hereditaryhearingloss.org) or syndromic HL (OMIM; www.omim.org). After applying the filtering criteria specified under the *Materials and Methods* section, we identified a variant mapping to a run of homozygosity (*SI Appendix*, Tables S2 and S3), chr17:18,927,685 (Hg19) and NM_006613.3: c.311A>T (p.Gln104Leu) in *GRAP* in both probands. Sanger sequencing showed that the variant cosegregates with deafness as an autosomal recessive trait in both families (Fig. 1 *A* and *C*). The variant has been seen in only 1 of 242,154 alleles in the gnomAD database (allele freq: 0.000004129) and is absent from over 500 Turkish exomes in our in-house database. Exons and intron–exon boundaries of homozygous runs in both samples are well covered with exome and genome sequencing (*SI Appendix*, Table S3). The variant affects a conserved residue (GERP: 4.45), and multiple in silico analysis tools predict it as damaging (*SI Appendix*, Table S4).

Screening of the variant in 690 unrelated Turkish probands with nonsyndromic HL did not show a positive sample. A two point logarithm of the odds score of 3.7364 was calculated for the two families between the identified variant and the phenotype assuming autozygosity. Shared ancestry of the variant encompassing ∼1.9 Mb was demonstrated via flanking single nucleotide variant genotypes (*SI Appendix*, Table S5).

Sanger sequencing of complementary DNA (cDNA) covering exon-exon boundaries obtained from a saliva sample of a proband did not show abnormal splicing caused by the *GRAP* c.311A>T variant (*SI Appendix*, Fig. S1; the primer sequences used are available in *SI Appendix*, Table S6).

MYO15A is a known nonsyndromic HL gene located ∼900 kb away from the *GRAP* c.311A>T variant. Whole genome sequencing did not identify a potentially causative variant within *MYO15A* (*SI Appendix*, Table S7).

The p.Gln104Leu Variant Most Likely Alters Interactions of GRAP with Its Cellular Partners. *GRAP* encodes a protein consisting of a central Src homology 2 (SH2) domain flanked by two Src homology 3 (SH3) domains (8). The SH2 domain is required for interacting with phosphotyrosine-containing sites of the activated receptors and cytoplasmic proteins (8). The p.Gln104Leu variant is located within the SH2 domain of GRAP. We modeled the atomic structure of its SH2 domain in a complex with a tyrosine-phosphorylated (pY) peptide harboring the pYVNV sequence (*SI Appendix*, Fig. S2). The Gln104 and the preceding Asp103 residues are both located close to the peptide-binding pocket, although they do not interact directly with pY peptide residues. This suggests that p.Gln104Leu is unlikely to completely disrupt interactions of the SH2 domain of GRAP with its cellular partners and that it may be involved in attuning these interactions.

The conservation at GRAP residue 104 extends only through mammals (Fig. 1*D*). In many birds, such as finches, the AspGln dyad located at positions 103/104 in human GRAP is altered to residues, such as LysAsp (Bengalese finch; *Lonchura striata domestica*) and LysLeu (medium ground finch; *Geospiza fortis*). As anatomy and physiology (such as the audible sound frequency range) of the cochlea between mammals and finches are considerably different, it is quite conceivable that the p.Gln104Leu variant may lead to more serious consequences in the context of GRAP signaling in humans than its naturally occurring variants in finches.

***Grap* Is Expressed in the Mouse Inner Ear.** Human (NP_006604.1) and mouse (NP_082093.1) GRAP/Grap are composed of 217 amino acids sharing 92% identity. Total RNA was isolated from tissues of WT animals at E17.5 and P15. Reverse transcription polymerase chain reaction (RT-PCR) using specific primer pairs (*SI Appendix*, Table S6) produced a unique band of 166 bp corresponding to the WT *Grap* messenger RNA in all tested tissues. *Gapdh* expression was utilized as the control (Fig. 2*A*).

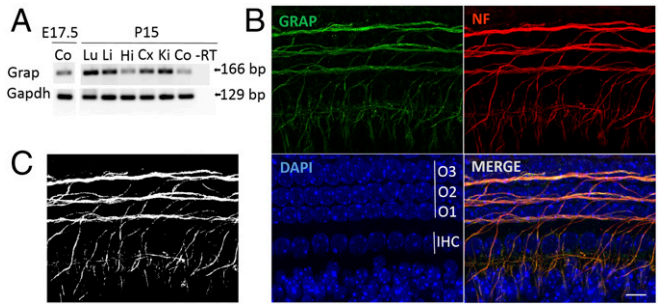

Fig. 2. *Grap* is expressed in the mouse inner ear. (*A*) RT-PCR of *Grap* expression in different tissues at embryonic 17.5 and postnatal day 15, in different mouse tissues. *Gapdh* expression was used as the control. Co, cochlea; Cx, cortex; Hi, hippocampus; K_i, kidney; Li, liver; Lu, lung. (*B*) Representative z-stack projection of P_0 whole mount cochlea stained for Grap (green). The red signals correspond to the neurofilament (NF) heavy chain that stains the spiral ganglion neuron (SGN) innervation of hair cells. Cell nuclei are counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (blue). Note the localization of Grap at the length of the nerves. (Scale bar: 10 μm.) (*C*) Black and white image indicates colocalization of Grap and NF as a result of the colocalization highlighter plugin (ImageJ).

Both the Shield (https://shield.hms.harvard.edu/) and gEAR databases (https://umgear.org/) show low levels of *Grap* expression in the inner and outer auditory hair cells. The gEAR database shows that *Grap* might be expressed also in the utricle. To evaluate the localization of Grap in the inner ear, we first validated an anti-GRAP antibody (*SI Appendix*, Fig. S3) and then utilized it for immunofluorescence performed in whole mount cochleae from P_0 WT mice as well as a cross section of the inner ear (Fig. 2*B* and *SI Appendix*, Figs. S4 and S5). Immunostaining shows that Grap is localized in the SGN fibers innervating auditory hair cells, both inner and outer, as well as the utricular hair cells (Fig. 2*B* and *SI Appendix*, Figs. S4 and S5). An antibody recognizing the neurofilament heavy chain was utilized to counterstain hair cell innervation (Fig. 2 *B* and *C*).

drk, the *Drosophila* Homolog of GRAP, Is Expressed in the *Drosophila* Hearing Organ, JO. GRAP and GRB2, the mammalian homologs of *Drosophila* drk and *Caenorhabditis elegans* Sem-5, belong to the same protein family and share identical protein architectures (SH3-SH2-SH3) (9, 10). In humans, GRAP and GRB2 have distinct expression patterns and possibly functions (8, 11). Bioinformatics analysis of amino acid sequences suggests that human GRAP and *Drosophila* drk share 54% identity and 68% similarity (Fig. 3 *A* and *B* and *SI Appendix*, Fig. S6).

JO is the component of the *Drosophila* auditory system required for sensing gravity, wind flow, and near-field sound (12, 13). To examine the function of the *Drosophila* homolog of GRAP in hearing, we first examined the distribution of drk in JO. There are around 200 scolopidia suspended within JO; these are the functional units of hearing and share evolutionarily conserved mechanosensory transduction mechanisms with vertebrate hair cells (7) (Fig. 3 *C* and *D*). Immunostaining of JO cryosections reveals the expression pattern of drk in scolopidia, including mechanosensory neurons, scolopale cells, and cap cells (Fig. 3 *E* and *F*).

drk is distributed at the cell bodies and neurites of JO neurons (Fig. 3*F*); however, the localization of drk at the neuronal terminals has not been examined. drk was first identified through a genetic screen for modifiers of signaling mediated by the protein

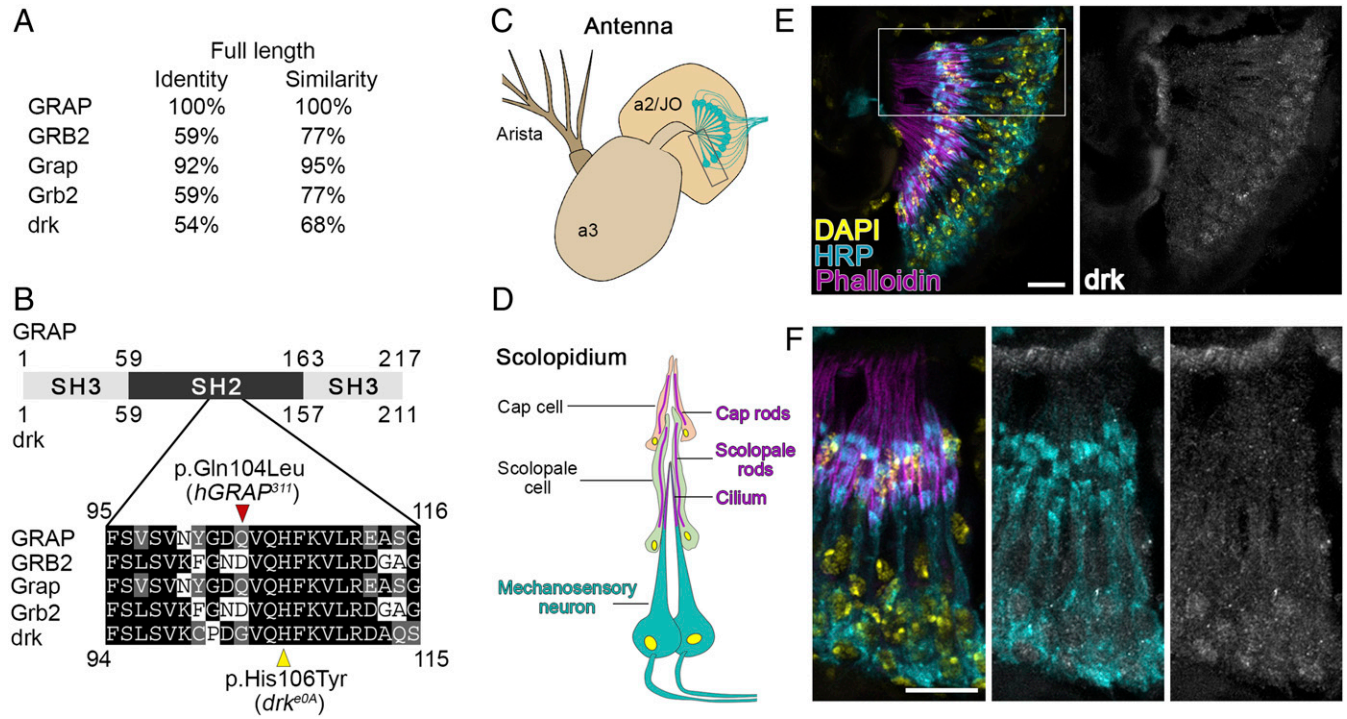

Fig. 3. drk is the *Drosophila* homolog of human GRAP and is expressed in the scolopidia. (*A*) Homology of human, mouse, and *Drosophila* adaptor proteins GRAP/GRB2/drk scored by full-length amino acid identity and similarity. (*B*) Diagram illustrating the protein structure of GRAP and drk. Sequence alignment of a partial SH2 domain shows evolutionary conservation (the black background indicating identical amino acid and the gray background indicating a similar amino acid). The red arrow head indicates the mutation identified in humans with nonsyndromic HL. The yellow arrow head indicates a *Drosophila* mutant allele. (*C*) Schematic shows *Drosophila* antenna. Mechanosensory neurons (labeled with cyan) are suspended within the JO, which is the A2 segment of the fly antenna. (*D*) Schematic shows the organization of one scolopidium (boxed area in *C*). (*E*) Confocal micrographs show the expression pattern of drk in the JO. DAPI labels nuclei, horseradish peroxidase (HRP) labels neuronal membranes, and phalloidin labels cap rods, scolopale rods, and actin bundles in the cilium. (*F*) Confocal micrographs of the high magnification of scolopidia (boxed area in *E*). (Scale bars: *E* and *F*, 10 μm.)

tyrosine kinase Sevenless, which is required for the proper development of the *Drosophila* compound eye (14). We thus examined the localization of drk at lamina synapses where photoreceptor cells and lamina neurons make synaptic contacts. The repetitive lamina cartridge has a well-defined structure that has been extensively exploited to characterize the cellular actions of proteins at synapses (15). Through the colabeling of several synaptic markers, we found that, at the presynaptic terminals, drk partially overlaps with the active zone associated cytoskeletal matrix protein Bruchpilot (Brp) and has a distinct expression pattern from synaptic vesicle protein cysteine string protein (CSP) (Fig. 4 *A* and *C*). Moreover, drk has a specific localization that highly overlaps with synapsin, a presynaptic phosphoprotein that associates with vesicles and the cytoskeleton, and regulates synaptic vesicle clustering and plasticity (16) (Fig. 4 *B* and *C*).

Mutations in *drk* Lead to Hearing Loss in *Drosophila*. Several *drk* mutant alleles, including ethyl methanesulfonate (EMS)-induced point mutations (drk^{e0A}, drk^{6}, and drk^{14-7}) and deletion (drk^{Δ}), have been previously identified (7). Specifically, the drk^{e0A} allele possesses a point mutation in the invariable residue (p.His106Tyr) in the SH2 domain, adjacent to the p.Gln104Leu mutation in GRAP, which affects both the phosphotyrosine binding and the localization of drk (10). Complementation analysis shows 100% adult lethality of the homozygous mutants and various survival defects of different compound heterozygous mutant animals (Fig. 5*A*), suggesting that drk is essential for development and survival. To examine the impact of *drk* mutations on the JO function, we first used negative geotaxis analysis to determine the level of gravity sensing, balance, and coordination (13, 17). Since compound heterozygous drk^{6}/drk^{14-7} (♂/♀) have a 56% survival rate (Fig. 5*A*), we first tested the negative geotaxis performance in surviving drk^{6}/drk^{14-7} adults and found severe locomotor deficits in these flies compared with WT flies, suggesting defects in gravity sensing and/or neuromuscular system dysfunction (Fig. 5*B*). To overcome the lethality of homozygous flies and to determine the function of *drk* in the JO, we generated mosaic animals (*eyFLP*; drk^{Δ}) with a homozygous deletion of *drk* in the antenna and the eye but heterozygous in the rest of the body through flippase/flippase recognition target (Flp/FRT)-mediated mitotic recombination using the *eyFLP* system (18). We observed severe locomotor deficits in mosaic flies suggesting strong defects in gravity sensing as the main reason for the climbing defects because the JO is homozygous for drk^{Δ} whereas the body (neuromuscular tissue) is heterozygous (Fig. 5*B*). Next, we examined the cellular morphology of scolopidia in two compound heterozygous flies including drk^{6}/drk^{14-7} and drk^{6}/drk^{e0A} as well as the mosaic flies *eyFLP*; drk^{Δ}. We observed disorganized scolopidia in the mutant flies as revealed by phalloidin staining, which labels cap rods, scolopale rods, and the actin bundles within the cilia of the mechanosensory neurons (19) (Figs. 3*D* and 5*C*, and *SI Appendix*, Fig. S7). The JO mechanosensory neurons project their axons into the brain and largely innervate the antennal mechanosensory motor center (AMMC), the primary processing region for auditory input (20). To visualize the effects of the loss of drk in JO neurons, we used mosaic analysis with a repressible cell marker (MARCM) technique (21) to label the JO neuron clones that are homozygous for *drk* deletion ($drk^{\Delta/\Delta}$). Compared with isogenized control, we observed significantly reduced AMMC area, indicating a significant loss of $drk^{\Delta/\Delta}$ sensory nerve terminals in the brain (Fig. 5 *D* and *E*). These results suggest that drk is required for functional and morphological integrities of the scolopidia, sensory neurons, and the AMMC brain neuropil.

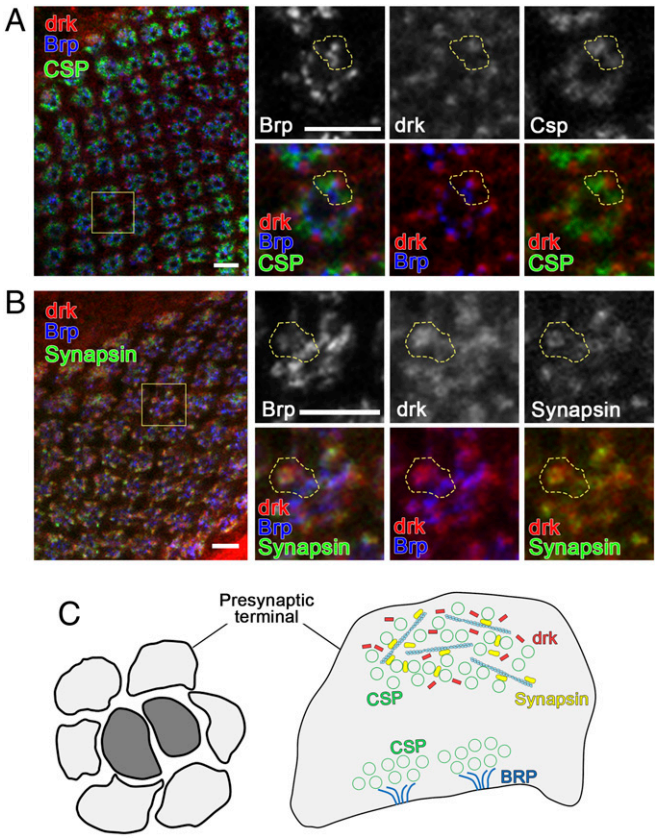

Fig. 4. drk colocalizes with synapsin at lamina synapses. (*A* and *B*) Confocal micrographs show the localization of drk within lamina synapses. Brp labels the active zone structure. CSP labels synaptic vesicles (*A*), and synapsin (*B*) associates with vesicles and the cytoskeleton within presynaptic terminals. (Scale bars: 5 μm.) (*C*) Schematic shows the localization of synaptic proteins within lamina presynaptic terminals.

***$GRAP^{311}$* Mutant Protein Shows a Loss-of-GRAP Function When Expressed in Vivo.** To gain insight into the causative nature of the human mutant GRAP in vivo, we generated transgenic flies carrying WT (*$GRAP^{WT}$*) and mutant (*$GRAP^{311}$*) human *GRAP*: *UAS-$GRAP^{WT}$-HA* or *UAS-$GRAP^{311}$-HA*. *GRAP* was overexpressed using a pan-neuronal driver *C155-GAL4*, and the GRAP overexpression had no effects on the overall morphology of fly brains (*SI Appendix*, Fig. S8*A*). Surprisingly, we found an upregulation of the endogenous drk when overexpressing GRAP in the nervous system (*SI Appendix*, Fig. S8*A*). Importantly, overexpression of *$GRAP^{311}$* induced a significantly higher level of drk compared with that of *$GRAP^{WT}$* in both the mushroom body and the antenna lobe (*SI Appendix*, Fig. S8*B*). In addition, our negative geotaxis analysis suggests that both drk and *$GRAP^{WT}$* overexpression significantly reduces the negative geotaxis performance of the flies, whereas overexpression of *$GRAP^{311}$* does not affect the negative geotaxis behavior (*SI Appendix*, Fig. S8*C*). These data suggest a potential genetic interaction between human *GRAP* and *Drosophila drk*, a tight regulation of *drk*/*GRAP* expression in neurons, and a difference in protein function between $GRAP^{WT}$ and $GRAP^{311}$ in vivo.

Next, we expressed *drk*, *$GRAP^{WT}$*, and *$GRAP^{311}$* in the mosaic animals using the pan-neuronal driver *elav-GAL4* to test whether expressing *drk*/*GRAP* transgenes could rescue the negative geotaxis deficits. As shown in Fig. 5*B*, expressing *drk* or *$GRAP^{WT}$* significantly improves the negative geotaxis performance of the mosaic flies (mean pass rate of 48.8% for the *drk* group and 18% for the *$GRAP^{WT}$* group). However, *$GRAP^{311}$* expression has no effect on the negative geotaxis behavior of mosaic animals (mean pass rate of 5.5%). These data suggest a functional homology between drk and $GRAP^{WT}$ and the deleterious consequence of *$GRAP^{311}$*.

Discussion

In this paper, we present a *GRAP* variant (c.311A>T; p.Gln104Leu; *$GRAP^{311}$*) that is associated with nonsyndromic HL in humans. We

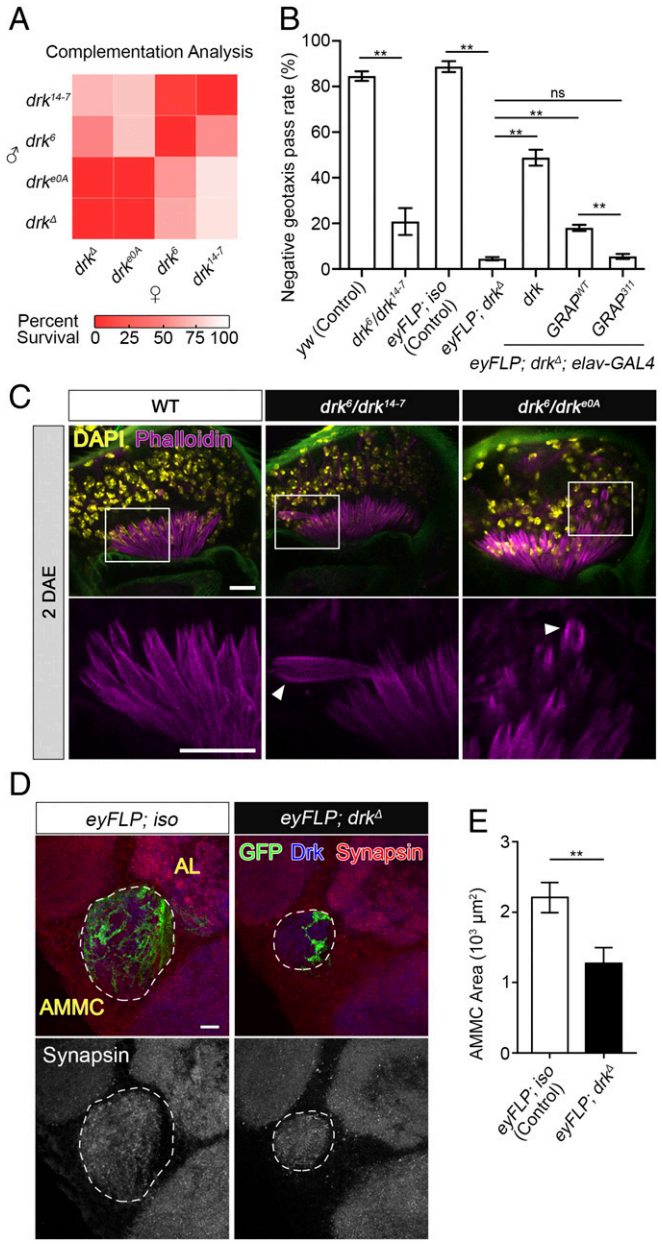

Fig. 5. *drk* mutations cause the morphological abnormalities of the scolopidia and locomotor deficits that can be suppressed by $GRAP^{WT}$ but not $GRAP^{311}$ expression. (*A*) Heat map shows percent survival of *drk* compound heterozygous flies that survive to adults. drk^{14-7}, drk^{6}, and drk^{e0A} are EMS-induced mutant alleles and drk^{Δ} is a deletion allele. (*B*) Negative geotaxis performance [mean ± standard error of the mean; each data point obtained from a group of 9 or 10 individuals, n =5 for drk^{6}/drk^{14-7} group (due to low survival rate) and n = 10 for all of the rest of the groups (>100 flies per genotype tested)] of female flies at 2 days after eclosion (DAE). (*C*) Confocal micrographs show the morphology of actin bundles in the scolopidium. Both drk^{6}/drk^{14-7} and drk^{6}/drk^{e0A} flies have disorganized phalloidin staining patterns (the white arrowheads). (*D*) Mosaic analysis with a repressible cell marker analysis of AMMC. Green fluorescent protein (GFP) marks the WT patches in *eyFLP; iso* group, and $drk^{\Delta/\Delta}$ patches in *eyFLP; drk*$^{\Delta}$ group. (*E*) Quantification of the AMMC area outlined in *D* (mean ± standard deviation; n = 3). (Scale bars: 10 µm.) One-way analysis of variance post hoc Tukey test; $^{**}P < 0.01$. ns, not significant.

further carried out functional studies of *GRAP* in vivo to provide evidence for its function in hearing. Studies in mice and *Drosophila* show that orthologs of *GRAP* are expressed in the hearing organ of each species. Furthermore, mutations in the *Drosophila* ortholog cause locomotor deficits and morphological abnormality of the scolopidia and sensory neurons in the JO, the *Drosophila* organ for hearing and balance. Importantly, *Drosophila* drk and human GRAP are functionally homologous as expressions of WT but not mutant human GRAP cDNA that can rescue the sensory phenotype in the drk mutant flies. These results establish a necessary role of GRAP and its orthologs in hearing and the deleterious nature of the $GRAP^{311}$ variant.

GRAP was cloned as a cytoplasmic signaling protein that possesses the same structural arrangement as Grb2 (8). The SH2 domains directly recognize phosphotyrosine-containing sites on activated receptor protein kinases (RTKs) and cytoplasmic proteins. It has been suggested that the adaptor proteins Grb2/Sem-5/drk provide a functional link between RTKs and activation of Ras signaling (10, 17, 18). The SH3 domains, particularly those located on the amino-terminal side of Grb2/Sem-5/drk, bind the C-terminal tail of the Sos protein (8). Similarly, Grap binds to Sos through its amino-terminal SH3 domain (8). The stable association of Grb2/drk with the Ras guanine nucleotide exchange factor, Sos, and membrane localization of the Grb2/drk-Sos complex results in the activation of Ras (10, 14, 22–24). Grap is known to be expressed in lymphocytes and complexed with other proteins, including Sos, upon T-cell activation (8, 11). A mutant mouse model deficient of Grap shows an augmented mitogenic response of lymphocytes; ectopic expression of Grap leads to an interruption of signal transmission from the Ras-Erk pathway into the nucleus. This suggests a negative regulatory role of Grap in mediating mitogenic responses of lymphocytes (25). In human B cells, GRB2 and GRAP amplify signaling by the stimulated B-cell receptors and connect them to the activation of the Ras-controlled Erk-MAP kinase pathway (26).

An in vivo RNA interference screen has found a potential role for drk in actin filament organization; however, the underlying mechanisms remain unclear (17). Our data provide evidence for drk in actin organization, which may relate to the dysfunction of scolopidia in drk mutant JO. In addition, GRAP/drk may be involved in the neural development of the hearing organ. Neurotrophins play a critical role in neural development, regulating differentiation, neurite extension, target innervation, and survival (27). Brain derived neurotrophic factor (BDNF) is a neurotrophin known to influence neurons in the inner ear via high-affinity RTKs in the cochlea (28). Mice deficient in BDNF exhibit reduced cochlear neuronal populations especially in the apical turn (29–31). Mice null for TrkB, a RTK utilized by BDNF, are reported to lose 15–20% of spiral ganglion neurons (30, 31). Inhibition of Ras in spiral ganglion explants treated with BDNF eliminates the BDNF-induced increase in spiral ganglion neurite number (32). Given the role of GRAP connecting RTKs to Ras signaling, its expression in mice in the neurons innervating hair cells and disruption of mechanosensory neurons with reduced AMMC size in *Drosophila* mutants, it is possible that GRAP plays a role in the development of neural connections of the hearing organ through the modification of Ras signaling. Our observation of the drk and GRAP overexpression phenotypes suggests the tight regulation of this RTK-GRAP-Ras signaling pathway where too much or too little GRAP results in sensory dysfunction. To further support an important role of GRAP in hearing, mutations in the gene coding for SOS1, a protein that is known to interact with GRAP, as well as in other members of the Ras signaling lead to Noonan and related syndromes that include sensorineural HL as a finding (33–35).

In conclusion, our paper unveils a necessary role of GRAP in hearing. Its localization to the neuronal tissue of the hearing organ in multiple species suggests a conserved role in the transmission of electrical signals of hearing to the brain.

Materials and Methods

More details of *Materials and Methods* are in the *SI Appendix*.

Human Subjects. This paper was approved by the University of Miami Institutional Review Board and the Ankara University Medical School Ethics Committee (Turkey). A signed informed-consent form was obtained from each participant or, in the case of a minor, from the parents. Detailed past medical histories and thorough physical examinations including otoscopy and ophthalmoscopy revealed no abnormalities, and electrocardiograms were unremarkable.

DNA Sequencing and Bioinformatics. Exome and genome sequencing were performed on the probands of each family by using a previously published protocol (36, 37). Variants were filtered with minor allele frequency thresholds of 0.005 for recessive and 0.0005 for dominant variants as recommended (38). We used GERP > 2 for conservation (39). We also filtered variants by using the criteria of the combined annotation-dependent depletion (CADD) score > 20 (40), the deep annotation-dependent neural network (DANN) score > 0.95 (41), and they were damaging for Provean (provean.jcvi.org/index.php) and FATHMM (fathmm.biocompute.org.uk/) for missense variants. XHMM and Conifer were used for the copy number variant (CNV) detection with exome data (42); CNVs were called using a CNVnator with genome data (43). Sanger sequencing was used for confirmation and cosegregation of the variant. Homozygous runs were detected from exome data and were used during the filtering of variants (*SI Appendix*, Tables S2 and S3).

Mouse Studies. WT C57BL/6 mice were bred and maintained at the University of Miami. All procedures were approved by the University of Miami Institutional Animal Care and followed the National Institutes of Health (NIH) Guidelines, "Using Animals in Intramural Research" (44). Cochlear expression of *Grap* was checked in embryos of 17.5 dpc and P15 via RT-PCR. Immunofluoresence was performed on P_0 using a goat anti-GRAP polyclonal antibody (ab9703; Abcam) as the primary antibody.

***Drosophila* Studies.** The following fly strains were used in the studies: drk^{14-7} (27622), drk^{6} (27623), drk^{e0A} (5691), *actin-GAL4, FRT42Diso*, *eyFLP*; *FRT42D*, w^{+}, and cl^{-}, obtained from Bloomington *Drosophila* Stock Center; $drk^{\Delta P24}$ (named as drk^{Δ} in this paper) from E. Skoulakis's laboratory. All *drk* alleles were normalized to the w^{-} background and balanced with the *CyO, KrGFP* chromosome. For mosaic analysis, drk^{Δ} was recombined with the *FRT42Diso* chromosome. Climbing behavior was measured as previously described (45). For immunofluorescence studies in *Drosophila*, the following primary and secondary antibodies were used in this paper: anti-drk (from E. Skoulakis's laboratory), anti-Brp (DSHB, AB_2314866), anti-Csp (DSHB AB_528183), anti-synapsin (DSHB, AB_2313867), Cy5 conjugated anti-HRP (123175021; Jackson ImmunoLab), Alexa 546 conjugated phalloidin (A22283; ThermoFisher Scientific), and Cy5 conjugated anti-HRP and secondary antibodies conjugated to Alexa 488/568/647 (ThermoFisher Scientific).

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