Sachs et al., http://www.jcb.org/cgi/content/full/jcb.200603073/DC1

Supplemental materials and methods

Generation of *Cd151* knockout mice

A BAC clone comprising exons 1–9 of *Cd151* was isolated from a 129S6/SvEvTac library (RPCI-21; Invitrogen). An 8.8-kb fragment of genomic *Cd151* was cloned in three steps into pFlexible, which is a generic targeting vector containing the selectable marker *puroΔtk* and loxP and frt recombination sites (van der Weyden et al., 2005) using sequence-specific primers containing restriction site tags. Fragment *Cd151* I was amplified with *Pwo* polymerase using primers P4 plus P5. Primers for the amplification of *Cd151* II and III were P6 plus P7 and P8 plus P9, respectively. After linearization with PmeI and NotI, 80 µg of the target construct were electroporated into 129/Ola-derived embryonic stem cells. Colonies resistant to 3.3 µM puromycin were screened for the desired homologous recombination by Southern blotting using a 5′-specific probe designed with primers P10 and P11. The *puroΔtk* cassette flanked by *frt* sites was removed by transient transfection of pFLPe (Rodriguez et al., 2000). Colonies resistant to 5 µM ganciclovir were selected, and exons 2–4 of *Cd151* were subsequently deleted by transient transfection of a Cre-expression plasmid pOG231 (Fig. S1, A and B; O'Gorman and Wahl, 1997). One recombinant ES cell clone harboring the *Cd151*-null allele was injected into mouse C57BL/6 blastocysts, which were transferred to mothers of the same strain. The chimeric male offspring was mated with FVB/N females. Agouti coat–colored offspring was screened for the absence of exons 2–4 by PCR analysis of tail DNA with primers P1–P3 (Fig. S1 C). Heterozygous mice were intercrossed and littermates were analyzed. The absence of CD151 was verified by immunoblotting lysates of $Cd151^{-/-}$ mouse embryonic fibroblasts (Fig. S1 D), which were prepared from embryos at 13.5-d post coitum. Table S1 shows *Cd151* sequence-specific primers with restriction site tags used for cloning of *Cd151* fragments.

Generation of podocyte-specific *Itga3* knockout mice

$Itga3^{fl/fl}$ mice, which were generated by flanking exon one of the integrin α3 gene with two loxP sites, were crossed with 2.5P-Cre transgenic mice (Moeller et al., 2003) and *Cd151*-null mice to produce animals with a podocyte-specific deletion of the α3 subunit, alone or in combination with the deletion of *Cd151*, respectively. All animal experiments were carried out with approval from the relevant institutional animal ethics committees.

Immunoblotting

Mouse embryonic fibroblasts were lysed in Nonidet P-40 lysis buffer (1% [vol/vol] Nonidet P-40, 100 mM NaCl, 20 mM Tris-HCl, pH 7.5, and 4 mM EDTA) containing a cocktail of protease inhibitors (Sigma-Aldrich). Lysates were clarified by centrifugation at 20,000 *g* for 20 minutes at 4°C. Aliquots of cell lysates containing equal amounts of proteins were subjected to SDS-PAGE on a 12% polyacrylamide gel under nonreducing conditions, followed by transfer to Immobilon PVDF membranes (Millipore). The membranes were blocked and blots were subsequently developed with the indicated antibodies using the ECL detection kit (GE Healthcare) according to the manufacturer's protocol.

ABR measurements

ABR measurements were performed in a soundproof room with low reverberation. Needle electrodes were placed on M1 and M2 (left and right mastoids) and referred to the frontocentral midline (vertex, Cz) to record the auditory-evoked potentials. A ground electrode was placed halfway on the tail of the mice. Interelectrode impedances were measured before and after

Table S1. Primers used in this study

Primer number	Sequence (5′ → 3′)	Restriction sites
P1	GTCTGACCACCCTATTCATTGTC	–
P2	GCATGCTGCCTCACTGAAAGC	–
P3	GGATGCCAGCAATGCTCTCCA	–
P4	*GGCGCGCCCCTCGGAATTTGCATTTCAGG*	AscI
P5	*GGCGCGCCGGATCC*AAGAGACAATCAGCAGACTGGATGTA	BamHI–AscI
P6	*CCTTAATTAA*GGCTTAGGTTGATGGATGTG	PacI
P7	*CCTTAATTAAA*GTTCTGAAGCTCGTGTCTG	PacI
P8	*CCTGCAGGGATATC*ATGAAGGAGCTAGGCAGCCCCAGA	EcoRV–SbfI
P9	*CCTGCAGGG*TCGTGCTATGTTCAAACTGAGCT	SbfI
P10	GTATACTGTGTACTCCTTGGCTC	–
P11	AGAGGTCCAGAACTGAGGTC	–

Cd151 sequence-specific primers with restriction site tags (italic) used for cloning of Cd151 fragments into pFlexible (P4–P9), for generating the 5′-Southern blot probe (P10 and P11), and for PCR analysis of mouse tail DNA (P1–P3).

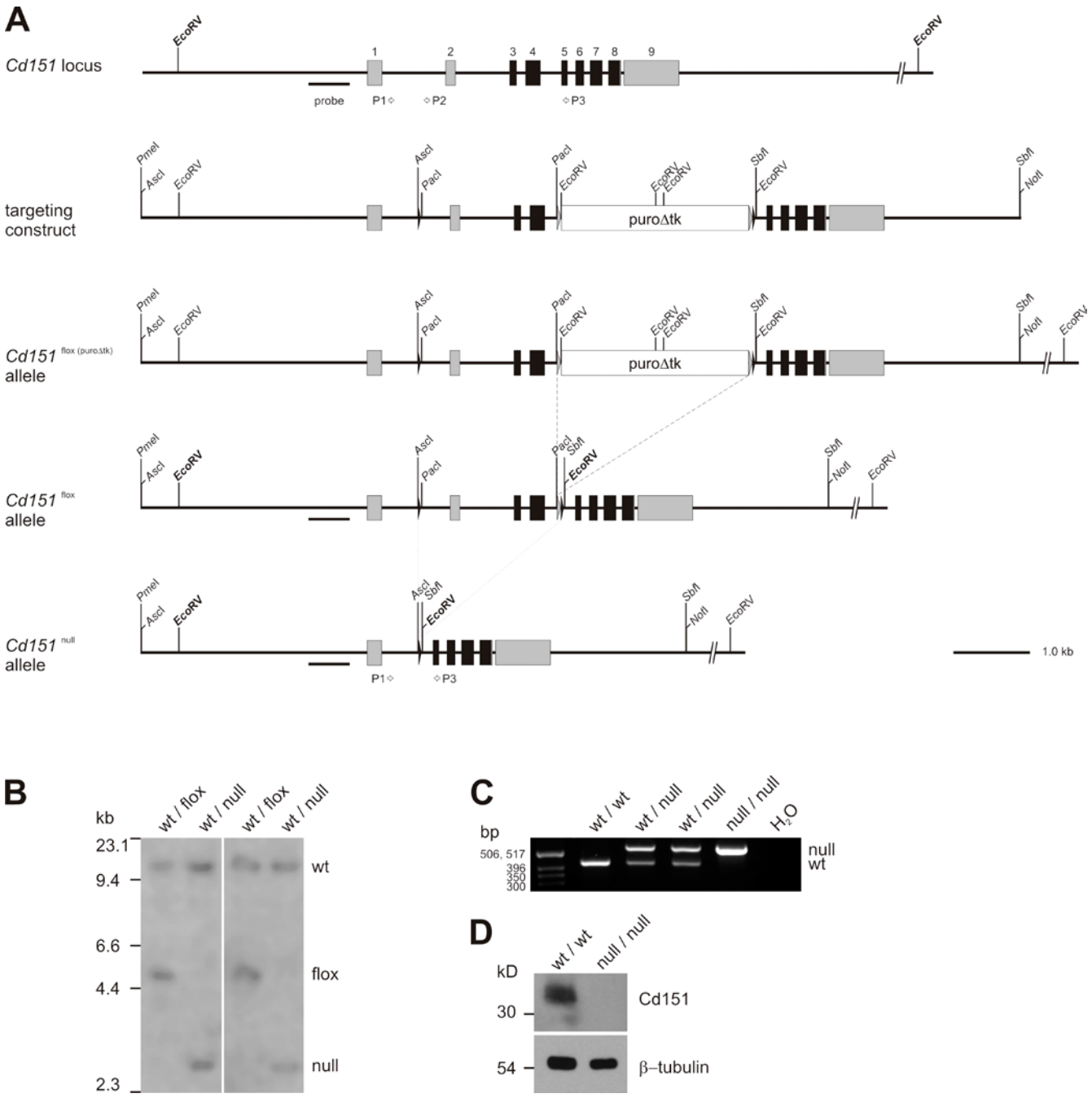
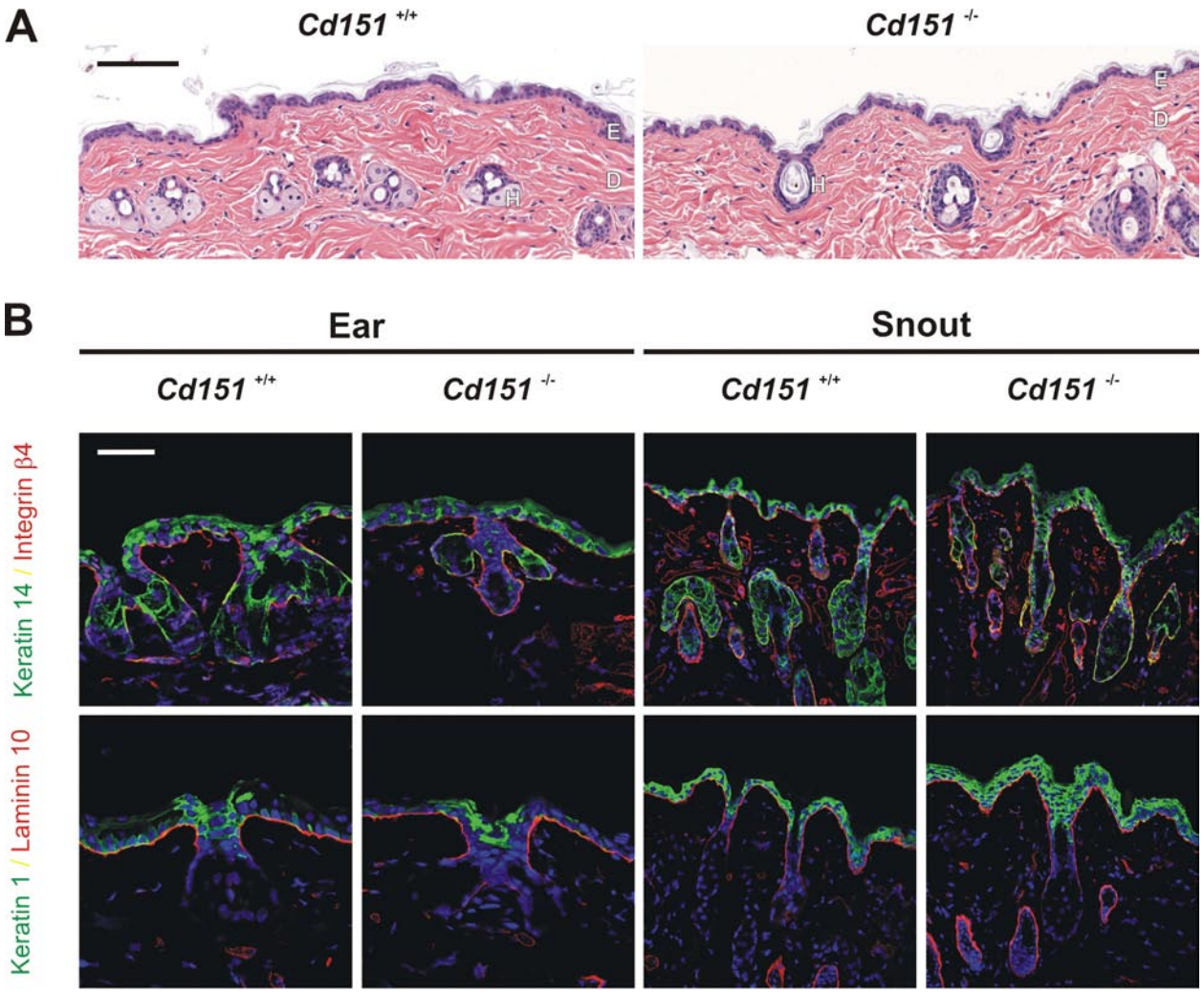


Figure S1. **Targeting strategy and molecular analysis of recombinant embryonic stem cells and *Cd151* knockout mice.** (A) *Cd151* gene structure, targeting construct, and different *Cd151* mutant alleles. Numbered gray and black boxes represent noncoding and coding exons, respectively. Gray and black triangles mark frt and loxP sites. *Cd151* fragments I, II, and III, along with their base pair positions and restriction sites used to generate the targeting construct, are indicated. Shown are the locations of EcoRV cleavage sites (bold), along with a hybridizing probe and primers (arrows) that were used for the analysis of the different mutant alleles by southern blotting and PCR, respectively. Dashed and dotted lines indicate the FLPe- and Cre-specific recombination events, respectively. (B) Southern blot analysis of four independently targeted ES clones before (wild-type/flox) and after (wild-type/null) Cre-mediated recombination. Embryonic stem cell DNA was digested with EcoRV, subjected to agarose gel electrophoresis, and transferred to nitrocellulose. 14.0-, 5.0-, and 3.2-kb fragments corresponding to wild-type, floxed, and null alleles, respectively, were detected by hybridization with a radiolabeled *Cd151* genomic probe. (C) PCR analysis of genomic DNA from wild-type, heterozygous, and knockout mice using primers P1–P3. (D) Immunoblot analysis for the presence of Cd151 in whole cell lysates of MEFs isolated from wild-type and knockout mice.

each measurement (<8 kOhm). Click stimuli of 100 μs and tone burst stimuli of 8, 16, and 32 kHz (1 ms rise/fall, 3 ms plateau time) were presented in a sound field by placing the loudspeakers 5 cm in front of each ear. The loudness levels at the position of the ear were measured and calibrated with a Bruel and Kjaer 2203 sound pressure level (SPL) meter. All thresholds were corrected afterwards for the soundfield setup. Before the measurements were performed, the mice were i.p. in-



C

Group	Stimulus			
	Click	8kHz	16kHz	32kHz
Cd151 $^{+/+}$ (n=6)	23.8 ± 3.3	10.8 ± 4.5	12.5 ± 3.1	35.0 ± 5.6
Cd151 $^{+/-}$ (n=5)	22.0 ± 5.3	14.0 ± 7.3	8.0 ± 8.7	22.5 ± 4.3
Cd151 $^{-/-}$ (n=7)	22.3 ± 3.7	7.9 ± 6.1	5.0 ± 6.4	27.9 ± 9.2

Figure S2. **Normal skin morphology and normal inner ear function in *Cd151*$^{-/-}$ mice.** (A) No differences in skin organization between hematoxylin and eosin–stained back skin samples of 8-mo-old wild-type and *Cd151*$^{-/-}$ mice. (B) Immunofluorescence analysis of ear and snout skin samples of *Cd151*$^{+/+}$ and *Cd151*$^{-/-}$ mice. The respective proteins are shown in green and red, yielding yellow upon colocalization. Nuclei are counterstained with TOPRO (blue). Both keratin 1 and 14 are normally localized in the suprabasal and basal epidermal layers. The epidermal–dermal border is not disrupted in *Cd151*$^{-/-}$ mice, as basal keratinocytes nicely express β4 and are anchored to the basement membrane (laminin 10). (C) Mean absolute ABR thresholds and SEM in decibel sound pressure level for three 12-wk-old groups of mice (*Cd151*$^{+/+}$, *Cd151*$^{+/-}$, and *Cd151*$^{-/-}$). Data show no statistically significant different hearing thresholds between the three groups (analysis of variance, $P > 0.05$). E, epidermis; D, dermis; H, hair follicle. Bars, 50 μm

jected with 200 mg/kg ketamine anesthetic. Stimuli were presented with a fixed stimulation rate of 32 Hz and a standard auditory evoked potential recording system (Synergy; Oxford Instruments) was used to record the ABRs. The analysis time was set at 15 ms from the onset of the click, with a 1.5-ms prestimulus time to assess baseline levels. The recorded electroencephalograph signals were high-pass filtered at 100 Hz and low-pass filtered at 3 kHz; an automatic artifact rejection and a 60-Hz notch filter were used to avoid electromyograph or external noise. Auditory brainstem responses were obtained from

both contra- and ipsilateral stimulation sites. The electroencephalograph signals were averaged for different stimulation levels according to standard audiometrical top-down procedures, starting at 90 dB (SPL), uncorrected for the soundfield. Peaks were identified according to the Jewett and Williston nomenclature (Jewett and Williston, 1971). The auditory hearing threshold was defined as the lowest level (in dB SPL) at which at least one reproducible peak was visually recognized in the responses obtained from the ipsilateral measured ear. Between-group ABR threshold differences of click- and high-frequency tone bursts were determined and analyzed for the control, *Cd151*$^{-/-}$, and *Cd151*$^{+/-}$ mice using analysis of variance by the Bonferroni statistics (SPSS version 12.0.1; SPSS, Inc.).

References

Jewett, D.L., and J.S. Williston. 1971. Auditory-evoked far fields averaged from the scalp of humans. *Brain*. 94:681–696.

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