Kidney failure in mice lacking the tetraspanin CD151

Norman Sachs,1 Maaike Kreft,1 Marius A. van den Bergh Weerman,2 Andy J. Beynon,3 Theo A. Peters,3 Jan J. Weening,2 and Arnoud Sonnenberg1

1Division of Cell Biology, The Netherlands Cancer Institute, 1066 CX Amsterdam, Netherlands
2Department of Pathology, Academic Medical Center, University of Amsterdam, 1105 AZ Amsterdam, Netherlands
3Department of Otorhinolaryngology, Radboud University Nijmegen Medical Center, 6500 HC Nijmegen, Netherlands

The tetraspanin CD151 is a cell-surface molecule known for its strong lateral interaction with the laminin-binding integrin α3β1. Patients with a nonsense mutation in CD151 display end-stage kidney failure associated with regional skin blistering and sensorineural deafness, and mice lacking the integrin α3 subunit die neonatally because of severe abnormalities in the lung and kidney epithelia. We report the generation of CD151-null mice that recapitulate the renal pathology of human patients, i.e., with age they develop massive proteinuria caused by focal glomerulosclerosis, disorganization of the glomerular basement membrane, and tubular cystic dilation. However, neither skin integrity nor hearing ability are impaired in the Cd151-null mice. Furthermore, we generated podocyte-specific conditional knockout mice for the integrin α3 subunit that show renal defects similar to those in the CD151 knockout mice. Our results support the hypothesis that CD151 plays a key role in strengthening α3β1-mediated adhesion in podocytes.

Introduction

Tetraspanins form a family of small proteins that are expressed in virtually all cell types and tissues (Boucheix and Rubinstein, 2001; Hemler, 2005). They consist of short intracellular termini, four transmembrane domains, and one small and one large extracellular loop that contain two highly conserved cysteine motifs. Tetraspanins oligomerize into tetraspanin-enriched microdomains, in which they associate with integrins, Ig superfamily members, growth factor receptors, and proteoglycans. Tetraspanin-enriched microdomains modulate diverse cellular activities, such as adhesion strengthening, migration, signal transduction, and proliferation (Hemler, 2005). The importance of proper tetraspanin function is demonstrated by several human diseases; distinct mutations in A15 (TSPAN7) and RDS (TSPAN22) cause X-linked mental retardation and retinal dystrophy, respectively (Travis et al., 1989; Zemni et al., 2000). A nonsense mutation in CD151 (TSPAN24) leads to end-stage hereditary nephropathy associated with pretibial epidermolysis bullosa and sensorineural deafness (Karamatic Crew et al., 2004).

N. Sachs and M. Kreft contributed equally to this paper.
Correspondence to Arnoud Sonnenberg: a.sonnenberg@nki.nl

Abbreviations used in this paper: ABR, auditory brainstem response; FP, foot process; GBM, glomerular basement membrane; GESD, glomerular epithelial slit diaphragm; pAb, polyclonal antibody.
The online version of this article contains supplemental material.

CD151 is expressed in epithelia, endothelia, muscle cells, renal glomeruli, proximal and distal tubules, Schwann cells, platelets, and dendritic cells (Sincock et al., 1997; Sterk et al., 2002). Extensive biochemical studies have shown that CD151 is the primary tetraspanin associated with the laminin-binding integrins α3β1, α6β1, α6β4, and α7β1 (Sterk et al., 2000, 2002; Boucheix and Rubinstein, 2001; Hemler, 2005). The interaction of CD151 with α3β1 is particularly strong and occurs at high stoichiometry (Yauch et al., 1998).

Integrins are αβ heterodimeric cell surface proteins that dynamically link the extracellular matrix and/or adjacent cells to the intracellular cytoskeleton (van der Flier and Sonnenberg, 2001; Hynes, 2002). In epithelial cells, the integrins α3β1 and α6β4 are mainly present in the basolateral compartment, where they bind to the basement membrane component laminin-5. Although much more severe, the phenotypes associated with mutations in Itga3, Itga6, and Itgb4 share features with the phenotype of patients with truncated CD151, indicating that the CD151–α3β1 and CD151–α6β4 heterotrimers are functionally important. Mice that lack the β4 subunit suffer from extensive detachment of the epidermis, and patients without functional α6β4 display junctional epidermolysis bullosa (Borradori and Sonnenberg, 1999; Uitto and Pulkkinen, 2001). Mice without α3 exhibit the mild skin blistering associated with ruptured basement membranes and die shortly after birth because of...
severe abnormalities in the epithelia of lung and kidney (Kreidberg et al., 1996; DiPersio et al. 1997).

In the renal glomerulus, podocytes are anchored to the glomerular basement membrane (GBM) via α3β1 and dystroglycans (Mundel and Shankland, 2002). The interdigitating foot processes (FPs) of podocytes are connected by glomerular epithelial slit diaphragms (GESDs) consisting of nephrin, podocin, P-cadherin, and other proteins, which are linked directly or indirectly to the cytoskeleton. Both disturbed podocyte–GBM anchoring and podocyte–podocyte interaction at the level of GESDs lead to the loss of FPs, a dysfunctional filtration barrier, and, ultimately, to glomerulosclerosis and renal failure (Pavenstädt et al., 2003).

We report the generation of knockout mice for Cd151 that show severe renal failure caused by progressive abnormalities of the GBM, loss of podocyte FPs, glomerulosclerosis, and cystic tubular dilation. Furthermore, we show that mice with a targeted deletion of the α3 subunit in podocytes have a similar, although more severe, phenotype.

Results and discussion

Cd151-null mice were generated (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200603073/DC1), born at the expected Mendelian ratio (0.28 (+/+); 0.49 (+/-); 0.23 (-/-), n = 86), and appeared to be healthy and normal at first observation. However, urine analysis of Cd151−/− mice by SDS-PAGE revealed that all mice developed proteinuria before 3 wk of age, which is indicative of kidney dysfunction (Fig. 1 A). Both the onset and the degree of proteinuria were variable. Nevertheless, all knockout mice had to be killed before the age of 9 mo because of substantial loss of body weight. A quantitative immunoblot of urinary albumin showed high levels of this protein in Cd151−/− mice that increased with age and reached a plateau at 6 wk (3 mo follow up; unpublished data). In contrast, only traces of albumin were present in the urine of wild-type and Cd151+/− mice (Fig. 1, B and C). As expected, the severity of the renal pathology among Cd151−/− littermates also varied considerably (Fig. 2, A and B), and there were animals with mildly or severely affected kidneys in the same litter. Histological examination of the mildly affected kidneys showed focal glomerulosclerosis, and interstitial fibrosis and inflammation (Fig. 2, D and G). The GBM of some capillary loops was abnormally thick and formed extensive spikes (Fig. 2 J). EM revealed that the GBM was laminated and that FPs in contact with the abnormal GBM were effaced (Fig. 3, A–C). On the vascular side, the endothelium was swollen and fenestrations were occasionally lost (Fig. 3, B and C). Severely affected kidneys were contracted and their capsules granulated because of cortical degeneration (Fig. 2 B). Light microscopy showed extensive glomerulosclerosis in several stages, tuft adhesions to Bowman’s capsule with extracapillary cell proliferation and fibrosis, and marked expansion of the mesangial matrix. Proximal tubuli were either dilated and contained PAS-positive protein casts or they displayed degeneration of varying severity (Fig. 2, E and H). Furthermore, we observed periglomerular fibrosis and focal interstitial inflammation in close proximity to interlobular blood vessels (unpublished data). Silver staining showed the glomeruli to be segmentally or globally sclerosed with extensive deposits of basement membrane components (Fig. 2 K), an observation that could be confirmed by ultrastructural analysis (unpublished data). To investigate at what age GBM defects occur, we subjected kidneys of newborn and 1-wk-old Cd151−/− mice to EM. Although many capillary loops of newborn Cd151−/− mice already have a laminated GBM, it seems that spike formation does not occur until the mice are 1-wk-old (unpublished data). Together, these observations suggest that the mild abnormalities found in some of the mice represent early stages of the severe phenotype in the other mice. Tubular changes may be secondary to massive glomerular protein leakage, but may also reflect dysfunction of the tubules themselves.

To investigate whether the progressive GBM abnormalities are correlated with glomerular injury, we stained for tenascin-C and fibulin-2. Both proteins have been shown to be up-regulated in response to glomerular and vascular lesions (Assad et al., 1993; Wada et al., 2001). We observed an increased glomerular...
expression of tenascin-C in both mildly and severely affected kidneys, but fibulin-2 was up-regulated only in the latter (Fig. 4, A–C). Whereas fibulin-2 is important for the migration of smooth muscle cells, tenascin-C regulates migration of a variety of cell types, including fibroblasts (Hsia and Schwarzbauer, 2005; Strom et al., 2006). The finding that tenascin-C is already present in the early stages of the disease suggests that fibrosis precedes the pathology of the vasculature. Consistent with the ultrastructural finding that the GBM of Cd151−/− mice is thickened, staining for nidogen and laminin-10 revealed an abnormally strong presence of these GBM components in peripheral capillary loops and extracapillary spaces (Fig. 4, K–M and P–R). To exclude an arrest of the normal developmental switch from α1.α1.α2 (IV) collagen to the α3.α4.α5 (IV) and α5.α5.α6 (IV) collagen networks, as seen in the X-linked form of Alport’s syndrome (Kalluri et al., 1997), we stained for all six chains of collagen IV. The results showed the mature collagen IV pattern to be present in all glomeruli (unpublished data). Only the α2 (IV) chain was strongly up-regulated in podocytes of the severely affected kidneys (Fig. 4 W). We also checked the integrity of the filtration barrier by staining for the GESD components podocin and nephrin (Fig. 4, P–R and U–W). Both proteins appeared to be down-regulated in the mildly affected kidneys, and are almost absent in the severely affected kidneys, demonstrating a complete loss of GESD architecture (Fig. 4, R and W). Together, these results support the hypothesis that in Cd151-null mice disorganization of the GBM precedes the effacement of FPs and the loss of GESDs. Patients with a nonsense mutation in CD151 (Karamatic Crew et al., 2004) also have epidermolysis bullosa and deafness, but neither of these was observed in the Cd151−/− mice by histological and immunofluorescent analysis and measurements of auditory brainstem responses (ABR), respectively (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200603073/DC1).

The highly stoichiometric binding of CD151 to the α3β1 integrin, and the fact that mice lacking the α3 subunit show severe renal defects, led us to hypothesize that the absence of CD151–α3β1 complexes is responsible for the renal pathology seen in our Cd151-null mice. In Itga3 knockout mice, glomerular capillary branching is impaired, leading to a decreased number of capillary loops. Furthermore, podocytes fail to form normal FPs and lose lateral cell junctions (Kreidberg et al., 1996). To study the deletion of Itga3 after glomerular capillary branching, and to investigate possible similarities with the phenotype in our Cd151-null mice, we generated conditional Itga3 knockout mice and crossed them with 2.5P-Cre mice that express the Cre recombinase under the control of the human podacin promoter (Moeller et al., 2003). As shown by immunofluorescence, α3 is, indeed, almost absent in the glomeruli of these 2.5P-Cre; Itga3−/− mice (Fig. 4, I and J), indicating that this integrin subunit is mainly expressed by differentiated podocytes. 2.5P-Cre; Itga3−/− mice show massive proteinuria starting within the first week of age (Fig. 1, A and B),
knockout mice. Itga3; Cd151 much less prominent in the 2.5P-Cre; Itga3−/− mice, but not 2.5P-Cre; Itga3+/−, mice.

Itga3−/− mice exhibit severe structural or functional renal abnormalities (Fig. 1 A, Fig. 3 D, and Fig. 4); neither do 6-wk-old compound heterozygotes (2.5P-Cre; Itga3+/−) mice. Structurally, the milky, discolored kidneys develop abdominal edema when 5–6 wk old and, subsequently, have to be killed. Notably, sclerosis of the glomeruli, up-regulation of collagen type IV, and down-regulation of GESD proteins podocin and nephrin (Fig. 4).

As shown by ultrastructural and immunofluorescent analysis, electron micrographs reveal GBM abnormalities and FP effacement in kidneys of mildly affected Cd151−/− and 2.5P-Cre; Itga3+/−, but not 2.5P-Cre; Itga3+/−, mice (A) Glomeruli of Cd151−/− mice (3-mo-old) show abnormal capillary loops characterized by GBM thickening and loss of the regular pattern of podocyte FPs. (B) Peripheral glomerular capillary with a lumenal thrombocyte and loss of fenestrations (arrowhead). The GBM is irregularly thickened and shows protrusions toward the capsular space (*). Podocyte FPs are partially lost (arrows). (C) Irregular GBM showing lamination, thickening, and protrusions (*). The endothelium is swollen and fenestrations are partially lost (arrowheads), as are podocyte FPs (arrows). Glomeruli of 6-wk-old 2.5P-Cre; Itga3−/+ mice show abnormalities (D), whereas all glomeruli of 2.5P-Cre; Itga3−/+ mice exhibit severe structural changes (E and F). (E) Abnormally thickened GBM with protrusions throughout the glomerulus. (F) Podocyte FPs are completely lost (arrows) and GBM protrusions (*) are present along the entire length of the capillary loops. Solid lines point to normal structures (GBM and FPs), whereas dotted lines indicate abnormal thickening of the GBM and effacement of FPs. Bc, Bowman’s capsule; Cs, capsular space; E, erythrocyte; Ec, endothelial cell; Fp, foot process; Lb, lamina basalis (GBM); Pdc, podocyte; T, thrombocyte; V, vascular lumen. Bars: (A, D, and E) 10 μm; (B, C, and F) 2 μm.

Because Cd151-null mice can form normal GBMs with a regular FP pattern (Fig. 3 A) and expression of both α3 and α6 in the glomeruli and tubules appeared to be normal (Fig. 4, F–H), we suggest that the function of α3 in development is not affected by the absence of CD151. Instead, we propose that reduced integrin α3β1-mediated adhesion is the main cause of the phenotype observed in our Cd151−/− mice. The filtration of plasma exerts considerable mechanical stress on the filtration barrier. Podocyte FPs, thus, have to withstand substantial mechanical forces. Indeed, CD151 appears to be involved in adhesion strengthening because adhesions mediated by CD151 (CD151−/−) complexes tolerate stronger mechanical forces than those mediated by α6β1 alone (Lammerding et al., 2003). A similar effect has been suggested for CD151−/− mice (Nishiuchi et al., 2005). Thus, FPs without CD151 might not be able to withstand prolonged transcapillary pressure, a phenomenon that is also responsible for the renal manifestations in the Alport’s syndrome after several years of life in patients with an abnormal GBM caused by collagen type IV mutations (Kalluri et al., 1997). Epithelial cells may become partially detached, leading to a compensatory production of new basement membrane components. As a result, the GBM thickens and spikes are formed, as has also been described in membranous nephropathy (Minto et al., 1998). Correct reassembly of basement membranes upon injury is impaired in the skin of Cd151−/− mice (Cowin et al., 2006). If the absence of CD151 is similarly important for GBM repair, this vicious circle would indeed result in glomerular malfunction.
glomerulosclerosis precedes the loss of GESD components, leading to the aforementioned renal pathology.

The assumption that CD151 is important for maintaining glomerular architecture upon mechanical stress is in accordance with the observation that glomeruli develop normally and that abnormalities only occur after several weeks or months. Furthermore, it explains differences in the rate of progression among littermates, as the degree of intraglomerular hydrostatic pressure depends on several genetic and epigenetic factors. Differences in genetic background might also explain why Cd151-null mice that have been previously described did not show renal failure as observed in our mice (Wright et al., 2004). In conclusion, we show that the renal manifestations in our mice lacking CD151 are similar to those in patients with a mutated CD151. Our data support in vitro studies pointing to a role of CD151–α3β1 complexes have an essential function in vivo.
Materials and methods

Urination analysis
Urine from mice younger than 3-week-old were collected by applying gentle dorsal pressure to the caudal area of the animal. Older mice were placed in metabolic cages for 24 h. Samples were then analyzed by SDS-PAGE, followed by Coomassie Brilliant blue staining, or by competitive ELISA using the Alburell M kit that was obtained from Exxon.

Histological analysis
Sections of kidneys were prepared, fixed for 1 d in EAF (ethanol, acetic acid, and formaldehyde), and stained with PASD, HE, or Jones’ methenamine silver. Images were taken with PL APO objectives (10×<0.25 NA, 40×<0.95 NA, and 63×1.4 NA oil; Carl Zeiss MicroImaging, Inc.) on an Axioskop S100/AxioCam HR color system using AxioVision 4 software (Carl Zeiss MicroImaging, Inc.).

Ultrastructural analysis
After fixation in Karnovsky buffer for 48 h, the material was post-fixed with 1% osmic tetroxide, the tissue samples were block-stained with 1% uranyl acetate, dehydrated in dimethoxypropane, and embedded in epoxyresin LX112. LM sections were stained with toluidine blue. EM sections were stained with tannic acid, uranyl acetate, and lead citrate, and then examined using a transmission electron microscope (Philips CM10; FEI). Images were acquired using a digital transmission EM camera (Morada 10–12; Soft Imaging System) using Research Assistant software (RvC).

Antibodies
Rat mAbs used in this study were 4G6 against laminin-10 (provided by L. Sorokin, University of Münster, Münster, Germany), GoH3 against α6, MB1.2 against β1 (a gift from B.M. Chan, University of Western Ontario, London, Canada), LAT-2 against tenascin-C (van der Flier et al., 1997), and 346-11A against β4 (Abcam). Y. Sado (Shigei Medical Research Institute, Yamada, Japan) provided the rat mAbs H11, H22, H31, RH42, M54, and B66 against the mouse collagen IV chains α1, α2, α3, α4, α5, and α6, respectively. Rabbit polyclonal antibodies (pAbs) directed against mouse nidogen and mouse fibulin-2 were generous gifts from T. Sasaki (Max Planck Institute for Biochemistry, Munich, Germany); pAbs against nephronectin and podocin were from H. Holthöfer (University of Helsinki, Helsinki, Finland), and C. Antignac (Cochin Biomedical Research Institute, Paris, France). The pAbs against keratin 14 and 1 were purchased from BabCO. Immunization of New Zealand rabbits with the cytoplasmic tail of human α3A fused to GST and the peptide CKNKDTMVVKYHGSGHEVSSAVDKLQGEFH coupled to KLH (Pierce Chemical Co.) yielded pAbs 141742 against α3A and 140190 against CD151, respectively. Texas red- and FITC-conjugated secondary antibodies were purchased from Invitrogen.

Immunofluorescence microscopy
Tissues from adult mice were collected and embedded in cryoprotectant (Tissue-tek O.C.T.; Sakura Finetek Europe). Cryosections were prepared, fixed in cold acetone, blocked with 10% BSA in PBS, and incubated for 45 min with primary antibodies undiluted (LAT-2, GoH3, MB1.2, 4G6) or diluted 1:2 (H22), 1:50 (346-11A), 1:100 (anti-fibulin-2, anto-podoacin, anti-nidogen, and 141742), 1:250 (anti-nidogen), and 1:300 (anti-keratin 1 and 14), followed by incubation with secondary antibodies diluted 1:200 for 45 min. Samples were analyzed at 37°C using a 63×1.4 AF-CH2 PL APO CS objective on a TCS SP2 AOBS confocal microscope (both from Leica). Images were acquired using LCS 2.3.1 (Leica) and processed using CorelDRAW Graphics Suite 12 (Corel).

Online supplemental material
Fig. S1 shows the targeting strategy and molecular analysis of recombinant embryonic stem cells and Cd151 knockout mice. Fig. S2 shows the histology and immunofluorescence analysis of keratins 1 and 14 on back skin samples from wild-type and Cd151−/− mice, as well as auditory hearing thresholds of wild-type, Cd151−/−, and Cd151−/− mice upon click and tone burst stimuli, as determined by ABR measurements. The Supplemental materials and methods describes the generation of transgenic mice, immunoblotting, and ABR measurements. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200603073/DC1.

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References
the gene responsible for retinal degeneration slow (rds). Nature. 338:70–73.


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 pFLoPe (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
Itga3fl/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

<table>
<thead>
<tr>
<th>Primer number</th>
<th>Sequence (5′ → 3′)</th>
<th>Restriction sites</th>
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<tbody>
<tr>
<td>P1</td>
<td>GTCTGACCACCCATCATTGCT</td>
<td>–</td>
</tr>
<tr>
<td>P2</td>
<td>GCGATGCCTCCTCATGAAACG</td>
<td>–</td>
</tr>
<tr>
<td>P3</td>
<td>GCATCCCGCAATGCTCTCCA</td>
<td>–</td>
</tr>
<tr>
<td>P4</td>
<td>GCCGCCGCCCTCAGAATTGCAATTG</td>
<td>AscI</td>
</tr>
<tr>
<td>P5</td>
<td>GGCAGCGGCCGCTCCAGAATGCTCTCCA</td>
<td>BamHI–Ascl</td>
</tr>
<tr>
<td>P6</td>
<td>CCTAAATAGGCTGGTATATTGCTTG</td>
<td>PacI</td>
</tr>
<tr>
<td>P7</td>
<td>CCTAAATAGGCTGGTATATTGCTTG</td>
<td>PacI</td>
</tr>
<tr>
<td>P8</td>
<td>CCTGACGGGATATCATGAGAAGGCTAGTCGACTCATGAAATGCT</td>
<td>EcoRV–SbfI</td>
</tr>
<tr>
<td>P9</td>
<td>CCTGACGGGATATCATGAGAAGGCTAGTCGACTCATGAAATGCT</td>
<td>SbfI</td>
</tr>
<tr>
<td>P10</td>
<td>GTATACTGGTATCCTTGGTCT</td>
<td>–</td>
</tr>
<tr>
<td>P11</td>
<td>AGAGTCCGAAATTGAGGCT</td>
<td>–</td>
</tr>
</tbody>
</table>

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-
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 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

