Dido mutations trigger perinatal death and generate brain abnormalities and behavioral alterations in surviving adult mice

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Nearly all vertebrate cells have a single cilium protruding from their surface. This threadlike organelle, once considered vestigial, is now seen as a pivotal element for detection of extracellular signals that trigger crucial morphogenetic pathways. We recently proposed a role for Dido3, the main product of the death inducer-obliterator (dido) gene, in histone deacetylase 6 delivery to the primary cilium (Sánchez de Diego A, et al. (2014) Nat Commun 5:3500). Here we used mice that express truncated forms of Dido proteins to determine the link with cilia-associated disorders. We describe dido mutant mice with high incidence of perinatal lethality and distinct neurodevelopmental, morphogenetic, and metabolic alterations. The anatomical abnormalities were related to brain and orofacial development, consistent with the known roles of primary cilia in brain patterning, hydrocephalus incidence, and cleft palate. Mutant mice that reached adulthood showed reduced life expectancy, brain malformations including hippocampal hypoplasia and agenesis of corpus callosum, as well as neuromuscular and behavioral alterations. These mice can be considered a model for the study of ciliopathies and provide information for assessing diagnosis and therapy of genetic disorders linked to the deregulation of primary cilia.

Significance

The primary cilium is an organelle protruding from most postmitotic vertebrate cells. A growing body of data supports the crucial role of primary cilium in developmental signaling pathways. Recent studies describe the main stages in ciliogenesis at the morphological level and components of some of the mechanisms involved, including the selective acetylation of tubulin. How this acetylation is modulated in cilia nonetheless remains poorly understood. Here we show that the death inducer-obliterator (dido) gene product, which regulates histone deacetylase 6 deacetylase activity, is necessary for orofacial development in the mouse embryo and influences brain patterning and neuromuscular activity. Mice deficient in dido function present neonatal mortality and various ciliopathies including cleft palate and hydrocephalus, as well as hippocampal and commissural dysplasia.

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although they developed a myelodysplasia/myeloproliferative-like syndrome (MDS/MPD) (17).

We also generated a dido mouse mutant that lacks exon 16, which encodes the Dido3 isoform-specific 1080 C-terminal amino acids. This didoΔNt allele is lethal in homozygosis at Thelier embryonic stage ts10−11 and is associated with DNA damage, apoptosis, and growth arrest during embryonic stem (ES) cell differentiation in vitro (18). The didoΔNt allele rescued embryonic lethality of the didoΔCt mutation as well as the ability of dido3ΔAc mutant mouse ES cells to differentiate (18). During these studies, we identified a synthetic phenotype in the didoΔNtΔCt two double heterozygotes that drive severe perinatal lethality. Here we describe this phenotype, which identifies an important role for the dido gene in craniofacial development. The few didoΔNtΔCt mice that reached adulthood had metabolic and neuromuscular alterations that highlight dido involvement in many physiological functions, including behavioral abnormalities.

Results

Dido Function is Necessary in Vivo for Primary Cilium Control. We previously reported the ability of the didoΔNt allele to rescue the embryonic lethality of the dido3ΔAc mutation (18). In addition, we described dissociation between Dido function in stem cell differentiation and its known role in the SAC, centrosome amplification, and cytokinesis (17, 19).

We also previously identified the role of Dido3 in the control of cilium size. To analyze the phenotype of the didoΔNt allele-rescued embryonic lethality of the didoΔCt mutation in vivo, we crossed didoΔNt/+ × didoΔCt/+ mice. To test the relevance of normal Dido activity in cilia development and maintenance, we quantified primary cilia in the hippocampal granular cell layer of adult mouse brain. Adenyl cyclase III (ACIII) is a prominent cilary marker (20); to compare brain sections from 3-mo-old wild type (WT) and didoΔNtΔCt/+ mice, we used anti-ACIII staining and confoc microscopy (Fig. 1 A and B). The percentage of ciliated cells was reduced in didoΔNtΔCt/+ mice (Fig. 1C), although mean cilium length did not differ significantly (Fig. ID). Distribution of cilium length is altered in didoΔNtΔCt brains, with increased frequency of longer and shorter cilia (Fig. 1E and Fig. S2).

Perinatal Lethality in didoΔNtΔCt Mice. To analyze the phenotype of the didoΔNt allele-rescued embryonic lethality of the didoΔCt mutation in vivo, we crossed didoΔNt/+ × didoΔCt/+ mice. After a 1-y follow-up of the colony, didoΔNtΔCt/+; didoΔNtΔCt, didoΔCtΔCt, and WT genotypes appeared at frequencies of 2.0, 37.2, 30.7, and 30.7%, respectively (Fig. 2), indicating selective loss of many double-heterozygous mice (P < 10−5). Genotype analysis at various days postcoitum (dpc) of embryos in a didoΔNtΔCt × didoΔCt/+ cross showed expected Mendelian frequencies up to birth (Fig. 2B), but most didoΔNtΔCt mice died shortly thereafter.

Neonatal death after 24 h postpartum is usually associated with inability to suckle rather than with respiratory failure or homeostatic deficiencies (21). We found no gross morphological alterations or hemorrhage in didoΔNtΔCt pups, nor were there obvious breathing defects in most didoΔNtΔCt × didoΔCt/+ litterers during the first 24 h after birth, although some didoΔNtΔCt mice showed cyanosis by days 1 and 2 after birth.

Another cause of perinatal lethality is linked to macroautophagy defects (22). As a cilium-associated protein, Dido3 is potentially linked to the autophagosome (23); knockout mice for another cilium-associated protein, autophagy-related 5 (Atg5), show shortened primary cilia (24). We tested the dido/autophagosome relationship by monitoring microtubule-associated protein 1 light chain 3B (LC3B). Cytosolic LC3B is processed for autophagosome formation; Atg5 and Atg16 activate cytosolic LC3B-1, which is phospholipid conjugated and forms membrane-bound, autophagosome-associated LC3B-II. We studied WT and didoΔNtΔCt embryos obtained by cesarean section at 19 dpc (full term). At time 0 and after 6 h in starvation conditions, heart protein was isolated and analyzed in Western blot for LC3BII expression. We found no differences between WT and mutant embryos at any time (Fig. 3A). Adult primary fibroblasts and EBV-immortalized murine embryonic fibroblasts (MEFs) cultured in starvation medium showed no LC3B processing defect, either in primary lung (Fig. 3B) or in immortalized fibroblasts (Fig. 3C).

DidoΔNtΔCt pups showed no size differences compared with WT or heterozygous littermates at birth (Fig. 4A), whereas those that survived >24 h showed reduced body size, suggesting a nutrition defect (Fig. 4B). Mice were inspected visually through the semitransparent skin; from ~12 h postpartum onwards all didoΔNtΔCt mice showed little or no milk in the stomach (Fig. 4A and B), which was diluted and air filled (Fig. 4C). A possible glucose mobilization deficiency was ruled out by glycemia measurement and by estimation of glycogenolysis activity by RT-PCR of hepatic glucose-6-phosphatase (Fig. S3).

The few didoΔNtΔCt mice that reached adulthood showed no external morphological abnormalities, except that size and weight were approximately one-half to two-thirds that of WT or heterozygous littermates (Fig. 4D), and life expectancy at birth was <1 y (Fig. 4E). Adults were infertile and showed limited cachexia. Hemograms, blood biochemistry profiles, and proteoglycans of didoΔNtΔCt mice showed normal values, with only slight hypoproteinemia compared with those of littermates (Fig. S4).

Histochemical studies showed anemia in some individuals as assessed by the Turnbull blue reaction (Fig. 4F), and visual examination showed differences in body fat accumulation (Fig. 4G), implicating metabolic problems. Various nonrecurring tumors were occasionally found in necropsies, which suggests nonspecific activity that facilitates tumor growth.

Delayed Closure of the Palate Primordium During didoΔNtΔCt Embryo Development. The snout of some didoΔNtΔCt fetuses was shorter than the normal control (Fig. 4G). Immunofluorescence labeling of the palate primordium revealed a significant delay in closure of the didoΔNtΔCt mutants compared with controls (Fig. 4H).

Fig. 1. Quantification of primary cilia in the adult hippocampal granular cell layer. (A and B) Immunofluorescent visualization of ACIII-positive primary cilia (green) of WT (A) and didoΔNtΔCt (B) mice (z-projection of confocal stack; DAPI-stained nuclei in blue) (Scale bar, 30 μm.) (C) didoΔNtΔCt mutant genotype is associated with a lower percentage of ciliated cells. Cilia were counted manually in 30 microscopy fields of 5-μm-thick brain sections from two WT and two didoΔNtΔCt mice. Individual values are shown; means are calculated for each genotype. Unpaired Student's t test with Welch's correction, **P < 0.01. (D and E) Length of individual cilia for WT (n = 62) and didoΔNtΔCt mice (n = 54) were determined (Materials and Methods and Fig. S2). (D) The median value and the 50th (boxes) and 10th–90th percentile range (whiskers) are shown. Student's t test indicates no significant differences. (E) Distribution of cilium length in didoΔNtΔCt brain is different from WT. Snedecor's F distribution, P = 0.01.
than in WT mice (Fig. 5A). We dissected the oral cavity of dido^{ΔNi/ΔCi} mutant F1 pups at 19.5 dpc (full-term pregnancy) to expose tongue, throat, and palate. The secondary palate was 15% shorter in dido^{ΔNi/ΔCi} than in WT mice (Fig. 5B and C), in agreement with the consistent lack of ruga 7b (the last-formed ruga in normal development), indicative of abnormal palate growth. The few double-heterozygous dido^{ΔNi/ΔCi} mice that survived to adulthood showed normal palatal rugae number and palate size (Fig. S5), again suggesting defective palate development as a cause of perinatal death.

To determine the origin of the craniofacial and palate defects in newborn dido^{ΔNi/ΔCi} mice, we analyzed palate features in dido^{ΔNi/ΔCi} F1 embryos. At 15.5 dpc, the palatal shelves had already fused in WT embryos, whereas the mouth roof in dido^{ΔNi/ΔCi} embryos remained open, with some degree of variability at this age (Fig. 5D). No cleft palate defects were observed in dido^{ΔNi/ΔCi} neonates, however, suggesting that the mutation causes delayed growth that gives rise to a shorter, defectively ossified palate.

Brain and Neurobehavioral Abnormalities in Surviving Adult dido^{ΔNi/ΔCi} Mice. Previous reports of dido expression in the CNS, in embryo brain (www.emouseatlas.org/emage/; EMAGE:2631) and adult cerebellum (Allen Mouse Brain Atlas, mouse.brain-map.org/, experiment 69262318), and in gray matter of the spinal cord (Allen Mouse Spinal Cord Atlas, mouse.brain-map.org/, experiment 100014937), prompted us to analyze the effect of dido mutations on CNS development and neuron function. Histological examination of the brain showed enlarged ventricles with proportional reduction of caudoputamen and lateral striatum, agenesis of the corpus callosum, and hippocampus dysplasia (Fig. 6A).

Whereas WT mice displayed a normal grasping reflex, dido^{ΔNi/ΔCi} mice normally stretched their forelimits toward the wires and gripped them effectively, but tucked in their hind limbs and maintained them clasped.

Spontaneous locomotor activity of the mice was measured in a chamber equipped with infrared sensors. There was no notable difference in horizontal activity, defined as exploratory movements around and across the base of the chamber (Fig. 6B); in contrast, dido^{ΔNi/ΔCi} mice showed reduced vertical activity (Fig. 6C), which assesses exploratory movements while standing upright, usually on the walls of the chamber. Pawprint tests along a covered straight path showed no marked gait or stride differences between dido^{ΔNi/ΔCi} and WT mice. To further test sensorimotor performance, we used a thermal plantar test to assess adult dido^{ΔNi/ΔCi} and WT mice. Latency to paw withdrawal following plantar heating with an infrared beam was similar in the two mouse groups (Fig. 6D); results were similar at different heat potencies, as well as after hand or foot stimulus. The results on the whole suggested mild distal motor neuropathy.

**Differences in α-Tubulin Deacetylation of the Sciatic Nerve in dido^{ΔNi/ΔCi} Mutant Mice.** Sciatic nerves were dissected from thighb of dido^{ΔNi/ΔCi} and WT mice of ages between 7 and 15 mo. A sample of each nerve was processed for histological study and another used to prepare whole protein extracts. Axon number and average myelin thickness were determined (Fig. S6); no differences were found in axon density or myelin integrity (Fig. 6E). Relative amounts of total and acetylated α-tubulin were evaluated by Western blot (Fig. S7). In age-matched mice, we found a significant difference in the amount of acetylated α-tubulin normalized to total amounts; each dido^{ΔNi/ΔCi} mouse had lower levels of acetylated α-tubulin than an age-matched WT littermate (Fig. 6F). No significant differences were observed in total α- + β-tubulin levels between mutant and WT mice.

**Discussion.** Dido3 is a key determinant of cilium size (14), and Dido3 mutations cause chromosome segregation defects, meiosis prophase alterations, and stem cell differentiation blockage (17, 19, 26, 27). Misregulation of the dido gene is linked to increased incidence of genomic instability, myelodysplasia, and myeloproliferation, melanoma, and infertility (13, 17, 26, 28). Elucidating the mechanisms that govern the complex activities could help clarify the role of Dido in promoting stem cell differentiation, cilia size, tumorigenesis and, as shown here, life expectancy, brain development, and neural behavior alterations.

Homozygous dido^{ΔNi/ΔCi}, heterozygous dido^{ΔNi/+}, and dido^{ΔCi/+} mice are viable, although mice of the first two genotypes occasionally develop MDS/MPD. We anticipated dido^{ΔNi} complementation of the severe early embryonic lethality associated with the dido^{ΔCi} allele in homozygosis (18), but low dido^{ΔNi/ΔCi} frequency in weaned offspring suggested complementation deficiencies. This lack of complementation affects cilogenesis in vivo, as noted by abnormal cilium size and numbers in the adult brain.
Normal mice pups gain 50–70% body weight from birth to day 2 (29), which highlights the importance of efficient nutrition in the first 48 h. We thus infer that the primary cause of death of dido<sup>ΔNi/ΔCt</sup> pups is an inability to feed adequately, although the metabolic anomalies detected in adult mutants could contribute to lethality. Inadequate feeding could be due to the mechanics of suckling itself, but also to deficient recognition or processing of pheromonal and/or complex odor signals (30). Although primary suckling itself, but also to deficient recognition or processing of pheromonal and/or complex odor signals (30). Although primary

for pheromone detection (31), we suspect that the inability of dido<sup>ΔNi/ΔCt</sup> mice to feed is a consequence of their craniofacial abnormalities or/and neurological behavior defects.

Palatal shelves grow horizontally and fuse to each other by 15.5 dpc (32). At this age, dido<sup>ΔNi/ΔCt</sup> fetuses showed a marked delay in this process, albeit with considerable individual variation. Because of its frequency in man, the genetic basis of cleft palate has been studied extensively. Although the list of genes with a role in this condition is long (33), to our knowledge it does not include dido. Major alterations in Dido proteins might be too deleterious, as for the homozygous dido<sup>ΔCt</sup> condition in mice, and only specific allelic combinations on a given genetic background would result in this ciliopathy. It is also possible that dido does not primarily drive human cleft palate-like syndromes, but mediates the effects of other genes. Alterations in BMP are among those most often found in orofacial malformations (34); Braig and Bosserhoff (13) reported that dido is a notable mediator of BMP downstream effects. The dido gene is a target of the BMP-dependent Smad family of transcriptional activators, which are implicated directly in experimental cleft palate induced by all-trans retinoic acid.

Dido also regulates expression of integrin αV (13), which probably has a role in correct precursor cell migration from the neural crest of the developing embryo toward the zone that becomes the jaw primordium. Mouse dido expression correlates negatively with body weight and jaw length in several strains (35, 36). Further work is needed to determine how Dido mutations act to produce the defects in mouse palate development, as well as the mechanisms by which dido mediates or prompts action in palate development by molecules such as BMP or integrin αV. Specific focus on human Dido gene status in studies of cleft palate etiology will help to elucidate its clinical relevance.

The few surviving adult dido<sup>ΔNi/ΔCt</sup> mice showed hindlimb dyskinesia. As clamping behavior is common in mouse models of various human central and peripheral neuromuscular patholo-

gies, we analyzed the possibility of a neuromuscular system disorder in dido<sup>ΔNi/ΔCt</sup> mice. Equilibrium and neuromuscular tests (tightrope walking, rotarod running, or grip strength) did not indicate consistent differences, arguably due to the disparity in body weight between mice of each genotype. It is unclear whether the reduced vertical activity observed in dido<sup>ΔNi/ΔCt</sup> mice is the result of peripheral neuropathy, a muscular defect, or both, but it coincides with clamping behavior to imply spinal and/ or hindlimb neuromuscular disease. If so, it would be essentially a

Fig. 4. Phenotypic alterations associated with dido<sup>ΔNT/ΔCT</sup>. (A) At 0.5 dpp, mutant pups were similar in size to WT littermates, but their stomachs contained little or no milk. (B) At 1.5–2 dpp, the size of surviving mutant pups was approximately two-thirds that of WT littermates, with very little milk in the stomach. Grid lines = 2 mm. (C) dido<sup>ΔNT/ΔCT</sup> mutant pups showed accumulated air in the stomach. A peritoneal incision to expose the stomach shows depletion of ferrous iron (II) storage in spleen of dido<sup>ΔNT/ΔCT</sup> mice. (Inset; Scale bar, 5 mm.) (D) Reduced body size was maintained throughout life. At 13–17 mo of age, weight of dido<sup>ΔNT/ΔCT</sup> mice was about one-half that of littermates, whereas dido<sup>ΔACt</sup> and dido<sup>ΔNT</sup> showed no size differences compared with WT mice. (E) Life expectancy was greatly reduced for dido<sup>ΔNT/ΔCT</sup> mice, again with no effect for dido<sup>ΔACt</sup> and dido<sup>ΔNT</sup> genotypes. (F) Turnbull’s blue stain shows depletion of ferrous iron (II) storage in spleen of dido<sup>ΔNT/ΔCT</sup> compared with WT mice. (Scale bar, 100 μm.) Original magnification, 6x. (G) Abdominal fat tissue (arrows) is virtually absent in 9-mo-old female dido<sup>ΔNT/ΔCT</sup>.

Fig. 5. Craniofacial defects in dido<sup>ΔNT/ΔCT</sup> neonates. (A) dido<sup>ΔNT/ΔCT</sup> mice showed altered craniofacial development at birth, with a shortened development compared with WT littermates. Representative images of both genotypes are shown, with a false-color merged image for comparison. (B and C) Palate was fully closed at birth, but the distance between raege 2 and 8 was 15% shorter in dido<sup>ΔNT/ΔCT</sup> than in WT pups (WT, n = 4; dido<sup>ΔNT/ΔCT</sup>, n = 2; Student’s t test P = 0.0016). (D) Delayed palate closure in dido<sup>ΔNT/ΔCT</sup> embryos. At 15.5 dpc, embryos were extracted and the lower jaw and tongue removed to expose the developing palate. The secondary palate was fully closed in WT embryos (Left), whereas dido<sup>ΔNT/ΔCT</sup> embryos showed varying degrees of delay in palate closure, with partial (Center) or almost no (Right) horizontal growth of palatal halves (arrows) toward the midline at the time analyzed (n = 8). (White scale bar, 5 mm.)
Further study, which will also identify factors other than increased tubulin deacetylation that affect the distal neuropathy in dido<sup>−/−</sup> mice. Whereas the limited survival of these mice to adulthood poses difficulties in their use, we are currently generating conditional dido mutants, which will be valuable for identifying the many roles of this gene.

It remains to be determined whether the phenotype associated with dido mutations is related not only to primary, nonmotile cilia regulation but is also modified by effects on motile cilia, which could account for phenotypic characteristics such as infertility or adult hydrocephalus.

The previously demonstrated role of Dido in the control of cilium length, based on its interaction with HDAC6, might be the basis for understanding the brain alterations observed in dido<sup>−/−</sup> mice. Lack of Atat1, whose action is opposite that of HDAC6, leads to hydrocephalus and hippocampal dysplasia (40), brain alterations similar to those associated with the dido<sup>−/−</sup> genotype. Although Atat1 and HDAC6 are respectively the major α-tubulin acetyltransferase (41) and deacetylase (40) in mice, atat1<sup>−/−</sup> and hdac6<sup>−/−</sup> mice are viable and fertile (42). This is not the case for dido<sup>−/−</sup> mice (43). Mitochondrial dysfunction in dido<sup>−/−</sup> mice was predicted to have early embryonic lethality, which suggests that Dido3 function is not restricted to the control of α-tubulin acetylation/deacetylation or that of other HDAC6 targets.

In a number of cases, dido mutation shows a distinct manifestation of cilia defects during brain development, namely agenesis of corpus callosum. It is tempting to speculate on a relationship of this gene with autism, as this aplasia is a distinctive characteristic of both the human condition (43) and its murine model (44). Of particular interest in human patients are subtelomeric microdeletions involving band 2q13.33, where the Dido gene is located. In a study of six subjects with this deletion (45), only one showed a thin corpus callosum and autistic disorders (46); this subject differed from others with normal MRI in the deletion of just 12 loci. These loci did not include Dido, but the breakpoint mapped only ~60 kb downstream of the Dido transcription unit. This is compatible with neotomere formation that could affect control of Dido expression, supporting the hypothesis of Dido involvement in human autistic behavior.

In summary, we describe various neurodevelopmental and morphogenetic phenotypes associated with murine dido mutations. Our model will help to understand the pathogenic mechanisms of some genetic or epigenetic disorders and could improve diagnosis and choice of treatment.

**Materials and Methods**

**Mice.** Heterozygous dido<sup>+</sup> mice were generated as described (17, 18) and maintained on a mixed genetic background (62.5% Sv129, 37.5% C57). Double heterozygous dido<sup>+</sup> offspring were obtained from heterozygous parents, and sex-matched WT littermates were used as controls. Mice were handled according to national and European Union guidelines, and experiments were approved by the Comité Ético de Experimentación Animal, Centro Nacional de Biotecnología, Consejo Superior de Investigaciones Científicas.

**Histology.** Mouse brains were fixed in PBS/4% (wt/vol) paraformaldehyde (PFA; 24 h) and cryoprotected in PBS/30% (wt/vol) sucrose (~48 h). Trimmed brains (cut along the longitudinal axis) were embedded in OCT (Sakura). Floating sections (30 μm) were stained in 0.1% cresyl violet in 1% acetic acid (5 min).

For cilia analysis, brains were fixed in PBS/4% PFA, 24 h, and paraffin embedded. Sections (5 μm thick) were stained with rabbit anti-rat ACIII antibody (1:500; Santa Cruz) and goat anti-rabbit Alexa-488 (BD Systems); cell nuclei were DAPI stained. Confocal microscopy was performed using an IX81 microscope (Olympus). For 3D reconstruction and cilium measurement, we used Imaris 7 software.

For Turnbull staining, 4-μm-thick paraffin sections were hydrated, placed in 0.06 N potassium ferricyanide staining solution (1 h), washed in 1% acetic acid, and counterstained with nuclear-fast red (5 min). Sciatric nerve portions (7–8 mm long) were collected. Proximal (next to spinal segments L3–6) and distal segments (~1 mm) were used for histochemistry; central segments were processed for protein analysis. Cryosections (10 μm) were stained with 0.5% toluidine blue.
Autophagy Assay. Protagomer (2.5 mg Depo-Provera; Upjohn) was administered to pregnant females at 17.5 and 18.5 dpc. Fetuses were extracted by cesarean section at 19.5 dpc and placed in humidified chamber (30 °C). Heart samples were extracted at time 0 and 4 h.

Immortalized (19) or primary fibroblasts (obtained from 3-mo-old mice by mechanical disaggregation and trypsin digestion) were cultured in rich medium (DMEM + 10% FBS). At second passage, cells were plated at 75% confluence, cultured overnight, washed twice with HBSS, and incubated in starvation (HBSS) or rich medium (4 h). Cells and tissue samples were lysed in cold PBS, 1% SDS with protease inhibitors. Equalized samples were fractionated in NuPAGE 12% Bis-Tris gels (Life Technologies) and transferred to Typhoon-8800 nitrocellulose membranes (General Electric). LC3B I/II was detected with rabbit polyclonal antibody ab51520 (Abcam), anti-rabbit IgG-HRP (Dako), and Amersham-ECL (General Electric). Images were captured with X-ray film or a Proxima 2700 device (Isogen). Autophagy density was analyzed with Adobe Photoshop CS5.

Behavioral Tests. Spontaneous locomotor activity was assessed in a flat 40×40 cm Perspex cage with two sets of IR emitter/sensor arrays for automatic monitoring of horizontal and vertical activity (Ugo Basile). Each mouse was tested for 5 min on 3 different days, and scores were averaged.

Supporting Information

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Mouse Genomic region Chromosome 2 H4; 2

Fig. S1. (Left) Genetic map of the murine dido gene, showing location of 16 exons, three messages generated by differential splicing and coding regions, and the two deletions studied here. (Right) Scheme of the three protein isoforms and the artificial deletions used in this study.

Fig. S2. Representative image of cilia measurement. The 3D reconstructions of confocal images were analyzed. Only cilia fully enclosed in the volume (A and B) were included. Cilia with one end outside the acquired planes (C) were discarded.
Fig. S3. Glucose metabolism in starved neonates. After a 4-h starvation, blood glucose levels were determined (A) and glycogenolysis (B) was monitored in liver cDNA samples by RT-PCR of glucose-6-phosphatase (G6PC) and normalized to β-actin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Student’s t test showed no significant differences between WT and didoΔNT/ΔCT groups. Relative quantification (RQ) was calculated as $2^{-\Delta\Delta C_{T}}$ on the mean WT values. Primers are as follows: GAPDH, 5′ CCACACACCATCCAGGA and 5′GCACATCTCAGCAGCCG; β-actin, 5′ GCCACACCCCTCTACAATG and 5′ TGATGGCTACGTACATGGCTG; and G6PC, 5′ CAAGATGACGTTCAACAC and 5′ ATGGTCACCTCTACCTAC.

Fig. S4. Hematology values for didoΔNT/ΔCT and WT mice (mean ± SD, n = 3 + 3). (A) Erythrocyte series. MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; RDW-SD, red blood cell distribution width. (B) White cell series. (C) Biochemical profile. A/G, albumin/globulin ratio.

Fig. S5. Representative image of adult palate in surviving didoΔNT/ΔCT mice. A complete set of rugae is observed.
Fig. S6. Myelin thickness determination. Frozen sections of proximal and distal parts of sciatic nerves from four *didoΔNT/ΔCT* and six WT mice were toluidine blue stained (A). (Yellow scale bar, 50 μm.) We selected 20 axons per sample by superimposing a grid on the image. Outer (A) and inner (a) areas of each myelin sheet were calculated with Photoshop CS5 after manual outlining of perimeters. Mean thickness (t) was calculated by the formula $t = (A/\pi)^{1/2} - (a/\pi)^{1/2}$. Axon density (B) was calculated for the full nerve section by manual counting. Individual values are given for proximal (P) and distal (D) sections, and mean ± SD. (C) Mean ± SD myelin thickness in individual samples, in arbitrary units. There were no significant differences.

Fig. S7. Acetylated tubulin determination. Western blots of right and left sciatic nerve protein extracts from WT and *didoΔNT/ΔCT* (d) mice.