SHORT COMMUNICATION

Hepatic Primary and Secondary Cholesterol Deposition and Damage in Niemann-Pick Disease

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Niemann-Pick C disease is a neurovisceral disorder caused by mutations in the *NPC* gene that result in systemic accumulation of intracellular cholesterol. Although neurodegeneration defines the disease's severity, in most patients it is preceded by hepatic complications such as cholestatic jaundice or hepatomegaly. To analyze the contribution of the hepatic disease in Niemann-Pick C disease progression and to evaluate the degree of primary and secondary hepatic damage, we generated a transgenic mouse with liver-selective expression of NPC1 from embryonic stages. Hepatic NPC1 re-expression did not ameliorate the onset and progression of neurodegeneration of the NPC1-null animal. However, the mice showed reduced hepatomegaly and dramatic, although not complete, reduction of hepatic cholesterol and serum bile salts, bilirubin, and transaminase levels. Therefore, hepatic primary and secondary cholesterol deposition and damage occur simultaneously during Niemann-Pick C disease progression. (*Am J Pathol* 2016; 186: 517–523; <http://dx.doi.org/10.1016/j.ajpath.2015.12.002>)

Niemann-Pick C disease (NP-C) is a neurovisceral lipid storage disorder affecting 1 in 120,000 live births.¹ NP-C is transmitted in an autosomal-recessive manner by mutations in *NPC1* (95% of cases) or *NPC2* genes. The NPC proteins mediate cholesterol efflux from endosomes and NP-C is characterized by systemic accumulation of unesterified cholesterol and other lipids in lysosomes.

The clinical spectrum of NP-C is highly variable, from a neonatal fatal disorder to adult-onset neurodegeneration. Although neurodegeneration commonly defines NP-C severity, it often is preceded by visceral complications such as cholestatic jaundice and hepatosplenomegaly.^{1–3} The hepatic disease is especially severe in perinatal and infantile periods; patients with fetal hydrops survive for only a few days and 10% of the neonates with cholestatic icterus die after 3 to 6 months.

The current view is that hepatic and neuronal diseases progress independently in NP-C.⁴ A mouse with specific loss of neuronal NPC1 recapitulates the neurodegeneration of the NPC1-null animal.^{5,6} However, although specific

neuronal NPC1 re-expression in NPC1-null mice increases survival, neurologic defects still are observed.^{7,8} Thus, it is likely that neuronal NPC1 delays pathogenesis, however, glial and visceral NPC1 is needed for complete rescue.⁴

Two transgenic mice were used to argue about the involvement of the hepatic disease in NP-C.⁴ An *Npc1* rescue using a prion-promoter, expressing NPC1 primarily but not exclusively in neurons,⁹ generated a mouse with

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high levels of neuronal NPC1 and low levels of hepatic NPC1. The animals presented with liver histiocytosis but not detectable neurodegeneration, suggesting that neuronal NPC1 ameliorates neurodegeneration even in the presence of hepatic damage. The second strategy used a Tet-inducible *Npc1* activated in several visceral tissues but not in neurons. Visceral NPC1 did not ameliorate neurodegeneration.¹⁰ However, in this study NPC1 re-expression was not liver-specific, hepatic NPC1 levels were not controlled, and NPC1 was not expressed during embryogenesis. Because the most aggressive visceral disease occurs in neonates and *Npc1* is required during embryogenesis,¹¹ embryonic NPC1 re-expression is necessary to unequivocally define causality of hepatic NPC1 in the complex clinical spectrum of NP-C. Likewise, a single case of hepatic transplantation in an NP-C patient was reported.¹² After an initial period of stabilization, progression of neurologic symptoms and recurrence of storage material in the transplanted liver were observed, suggesting primary and secondary hepatic damage. To analyze the contribution of hepatic disease in NP-C progression and the sources of primary and secondary hepatic damage, we generated a transgenic mouse with liver-selective expression of NPC1 from embryonic stages.

Materials and Methods

Construction of the Targeting Vectors

The conditional allele is based on the knockout-first strategy.¹³ The final targeting vector was assembled from two component plasmids using a Gateway-based switching technology^{14,15}: the intermediate targeting vector, which is gene specific and contains the homology arms and the attachment site right (attR)1 and attR2 Gateway sites, and gene targeting element plasmid or the LIL2 plasmid, including a promoterless selection cassette flanked by attachment site left (attL)1 and attL2 Gateway sites (Figure 1A). For the intermediate targeting vector two consecutive recombining steps are required: insertion of an attR1/attR2 zeo-pheS Gateway element into the B6 mouse bacterial artificial chromosome clone containing the *Npc1* gene (between exons 1 and 2), and subcloning the modified region of genomic DNA into a Gateway-adapted plasmid backbone. The final targeting vector was assembled *in vitro* in a two-part Gateway reaction. As a result, the *Npc1* gene at intron 1 contains a removable floxed cassette with an acceptor splicing site and a polyadenylation signal that interrupts transcription rendering a NPC1-null animal (referred to hereafter as *Npc1*^{fl/fl}).

Embryonic Stem Cell Targeting

To obtain heterozygous G4 (F1 129S/B6) mouse embryonic stem cells, final targeting constructs were transfected by electroporation using standard procedures. After electroporation, cells were plated in the absence of G418 for 24 hours, and then grown in the presence of 20 µg/mL G418 during 1 week. On day 7, cells were trypsinized and seeded,

and colonies were picked in duplicate and placed onto 96-well culture plates. For large-scale screening, one plate was subjected to long-range PCR using primers GF1 (forward, 5'-GGTTGGGTGCTCCGCCGTTG-3') and LAR3 (reverse, 5'-CACAACGGGTCTTCTGTAGTCC-3'), whereas the other plate was used to expand the positive clones. Southern blot analysis was performed as a secondary screen. Genomic DNA from embryonic stem cells was digested with EcoRI and probed with a 0.57-kb neo probe, expecting a 7.2-kb fragment for the knock-out allele.

Generation of NPC1-Null Mice and Genotyping Analysis

Heterozygous *Npc1*^{fl/+} embryonic stem cell clones were injected into the C57BL/6J blastocysts, selecting a chimeric mouse that transmitted the floxed allele through the germline. Background purification was obtained by a speed congenics system¹⁶ through successive interbreeding with C57BL/6J mice. Genomic DNA extraction and PCR genotyping was performed using a commercially available Accustart II Mouse Genotyping Kit (Quanta Bioscience, Gaithersburg, MD). The knock-out alleles were detected using primers up forward, 5'-TCCTTGGCGGTCGGTCTCC-TTA-3'; up reverse, 5'-CCTTCGCTCAGAGGACCCCA-GT-3'; and neomycin resistance, 5'-CGGAATCGTTT-TCCGGG-3'); resulting in a 477-bp product from the wild-type allele and a 735-bp product from the targeted allele.

Generation of the Mice with Specific Re-Expression of Hepatic NPC1

Mice expressing Cre-recombinase under the control of the albumin promoter and the α -fetoprotein enhancer (Afp-Alb-Cre) [strain name: B6; SJL-Tg (Alb1-cre) 1Khk/Mmmh, stock number: 029876-MU; Mutant Mouse Regional Resource Centers, University of Missouri, MO] were interbred with *Npc1*^{fl/+} mice. This transgenic mouse contains a random insertion of the transgene [Tg (Alb1-cre) 1Khk]. The transgene is formed by the coding sequence of the Cre-recombinase fused to a 2-kb fragment of the mouse albumin promoter and a 4.6-kb fragment of the mouse α -fetoprotein enhancer. Cre recombinase recognizes the *loxP* sites resulting in excision of the floxed cassette and allowing the re-expression of the *Npc1* gene in the liver. Genotyping of *Npc1*^{fl/+} Afp-Alb-Cre^{+/-} was performed by PCR using genomic DNA. For the detection of Afp-Alb-Cre the following primers were used: CRE5 (forward, 5'-GCGGCATGGTGCAAGTTGAAT-3') and CRE3 (reverse, 5'-CGTTCACCGGCATCAACGTTT-3').

The *Npc1*^{fl/+} Afp-Alb-Cre^{+/-} mice were intercrossed, rendering the following: i) *Npc1*^{fl/fl}, ii) *Npc1*^{+/+} [referred to hereafter as the wild-type (WT)], iii) *Npc1*^{fl/fl} Afp-Alb-Cre^{+/-} and *Npc1*^{fl/fl} Afp-Alb-Cre^{+/+} (referred to hereafter as Cre-*Npc1*^{fl/fl}), and iv) *Npc1*^{+/+} Afp-Alb-Cre^{+/-} and *Npc1*^{+/+} Afp-Alb-Cre^{+/+} (referred to hereafter as the Cre-WT). The mice were genotyped using the UF, UR, NeoR, CRE5, and

Figure 1 Construction of gateway targeting vectors to restore NPC1 hepatic expression. **A:** DNA recombination occurs between site-specific attachment sites left and right, attL and attR, respectively. The attL1/attL2 sites of the L1L2 plasmid allow recombinational cloning of the LacZ/neo element with the attR1/attR2 sites of the intermediate targeting vector that contains a negative selection marker (pheS) inserted between exon 1 and exon 2 of the *Npc1* gene. Upon recombination, the final targeting vector contains the LacZ/neo cassette flanked by loxP-sites inserted into the *npc1* intron. **B:** After homologous recombination, the targeting cassette is inserted into the genome of embryonic stem cells, disrupting *Npc1* gene function. Subsequent exposure to liver-specific Cre recombinase deletes the LacZ/neo cassette, restoring gene expression in liver (see *Materials and Methods*). **C:** PCR genotyping of the mice used in this study. **Upper panel:** PCR reaction using the primers shown in panels A and B; UF, UR, and NeoR produce either a 735-bp product from the floxed *Npc1* truncated allele (*Npc1^{fl/fl}*) or a 477 bp from the WT allele. **Lower panel:** The 232-bp Cre-specific PCR product is detected using CRE5 and CRE3 primers. **D and E:** NPC1 levels in liver or brain of 2-day-old (E) and 9-week-old (D) animals measured by Western blot analysis. *n* = 3. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; WT, wild-type.

CRE3 primers in two PCR reactions (Figure 1C). All animals received humane care in compliance with institutional guidelines regulated by the European Community. The experimental protocols used were approved by the Animal Care Committee of the Hospital Clinic—Universidad de Barcelona. All mice were housed in groups in cages with a 12-hour light/dark cycle. Food and water were available *ad libitum* and the weight of the animals (Figure 2) was determined weekly after genotyping.

Tissue Homogenization

Liver and brain tissue were homogenized using the Bullet Blender (Next Advance, Cambridge, MA) at 6000 × *g* for 5 minutes at 4°C in homogenization buffer (10 mmol/L Tris, pH 7.5, 150 mmol/L NaCl, 5 mmol/L EDTA) and a mixture of protease inhibitors. Protein concentration was quantified using the BCA Protein Reagent Assay kit (Pierce Chemical Co., Rockford, IL).

Western Blot Analysis

Protein lysates were subjected to SDS–polyacrylamide gel electrophoresis performed in 7.5% polyacrylamide. Proteins were transferred electrophoretically to Immobilon-P Transfer Membranes (Merck Millipore Chemicals and Life Science GesmbH, Madrid, Spain). Membranes were blocked and incubated with a 1:1000 dilution of anti-NPC1 (Abcam, Cambridge, UK) or 1:1000 dilution of anti-glyceraldehyde-3-phosphate dehydrogenase (GenScript USA, Inc., Piscataway, NJ). Membranes were washed and incubated with peroxidase-conjugated secondary antibodies (1:3000; Amersham-Pharmacia, GE Healthcare Europe GmbH, Barcelona, Spain) and developed with ECL reagent (Biological Industries Israel Beit-Haemek Ltd., Kibbutz Beit Haemek, Israel) on Kodak X-OMAT film (Kodak, Rochester, NY). Densitometric analysis was performed using ImageJ version 1.45 (NIH, Bethesda, MD; <http://imagej.nih.gov/ij>).

Figure 2 Characterization of Cre- $Npc1^{fl/fl}$ mice. **A:** Age of death of females (left) and males (right) of $Npc1^{fl/fl}$ (black bars) and Cre- $Npc1^{fl/fl}$ (Cre, white bars). **B** and **C:** Body weight of females (**B**) and males (**C**) in $Npc1^{fl/fl}$, Cre- $Npc1^{fl/fl}$, WT, and CRE-WT. **D** and **E:** Sagittal brain sections of 9-week-old WT, $Npc1^{fl/fl}$, and Cre- $Npc1^{fl/fl}$ (Cre) stained with Cresyl violet (**D**) or calbindin-D28k antibody (**E**). **E:** Cerebellar lobules II, III, and IV. **Arrowheads:** Purkinje cells. **F:** Liver sections were stained with hematoxylin and eosin. The central vein (asterisk) and lipid-loaded hepatocytes are shown (arrows). **Bottom row** contains images enlarged from the boxed area in the corresponding panel in the top row (**E** and **F**). $n = 10$ (**A–C**); $n = 3$ (**F**). Scale bars: 0.25 cm (**D**); 1 mm and 250 μ m (**E**); 100 and 25 μ m (**F**). WT, wild-type.

Serum Biochemical Analysis

Serum was collected after centrifugation of blood samples at $6000 \times g$ for 1.5 minutes at 4°C in Serum Heparin Separator Tubes (Becton Dickinson, Franklin Lakes, NJ). Serum parameters were determined by the Laboratorio Core facility of Hospital Clínic i Provincial de Barcelona. Briefly, aspartate aminotransferase and alanine aminotransferase were quantified by enzymatic reactions according to the International Federation of Clinical Chemistry method without pyridoxal

phosphate. Lactate dehydrogenase was determined by using the pyruvate-to-lactate conversion method kit (Germany Roche Diagnostics GmbH, Nonnenwald, Germany), and high-density lipoprotein cholesterol was measured using a commercial immunoturbidimetric method (Wako Chemicals, Neuss, Germany). Total bilirubin and direct bilirubin levels were quantified by the vanadate oxidation method (total bilirubin and direct bilirubin; Advia Chemistry Systems; Siemens AG, Munich, Germany). Biliary acids were determined by enzymatic reaction and Thio-NADH production (Spinreact,

Figure 3 Hepatic study of 9-week-old Cre-Npc1^{fl/fl} mice. The results are expressed as the ratio between the values of Npc1^{fl/fl} versus WT (black bars) or Cre-Npc1^{fl/fl} versus Cre-WT (white bars). The dashed line indicates the value at which there were no differences with the WT. Absolute values corresponding to WT are included in parentheses (see *Materials and Methods*). **A**: Percentage of body weight that corresponds to the weight of the liver (liver weight/body weight × 100). **B** and **C**: Hepatic levels of cholesterol (**B**) and triacylglycerol (**C**). **D–H**: Serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), and high-density lipoprotein (HDL). *n* = 5 (**A–H**). **P* < 0.05, ***P* < 0.01 by *t*-test of matched WT.

Girona, Spain). All of the assays were measured on the Siemens Advia 1800 analyzer. The results are expressed as the ratio between the values obtained in Npc1^{fl/fl} or Cre-Npc1^{fl/fl} mice with respect to the corresponding WT animal (WT and Cre-WT, respectively). Thus, a value of 1 corresponds to normality and it is indicated with a line in the graphs of *Figure 3*. At least five mice were analyzed for each parameter. The absolute value of each parameter corresponding to WT (females and males) is indicated at the bottom of the graph. The statistical significance corresponds to the *t*-test between the values obtained in Npc1^{fl/fl} versus Cre-Npc1^{fl/fl}.

Liver Cholesterol and Triacylglycerol Determination

Triacylglycerol content in liver homogenates was determined by an enzymatic method using the Triglyceride Detection Kit (BioSystems, Barcelona, Spain). Hepatic cholesterol was quantified by a fluorometric method using the Amplex Red Cholesterol Assay Kit (Life Technologies, Carlsbad, CA). Both methods were used according to the manufacturer's instructions. The results are expressed as the ratio between the value obtained in Npc1^{fl/fl} or Cre-Npc1^{fl/fl} mice with respect to the corresponding WT animal

(WT and Cre-WT, respectively). At least five mice were analyzed for each parameter and the statistical significance was calculated with the *t*-test comparing Npc1^{fl/fl} versus Cre-Npc1^{fl/fl}.

Morphologic Analysis

Mice were euthanized by decapitation at 9 weeks of age. Immediately after decapitation, the liver, spleen, and brain were removed. Brains were halved sagittally and the hemispheres were processed for histology or protein extraction. For histology, liver and brain tissues from 9-week-old animals were fixed by immersion in 4% paraformaldehyde solution in 0.1 mol/L sodium phosphate, pH 7.2, for 96 hours. After fixation, liver tissues were paraffin-embedded, sectioned (2 μm), and stained with hematoxylin and eosin. Brain tissues were cryoprotected with 30% sucrose in phosphate-buffered saline with 0.02% sodium azide, and frozen in dry-ice-cooled 2-methylbutane. Serial sagittal sections (30 μm thick) were cut on a cryostat and collected as free-floating sections. Some were stained with cresyl violet as previously described¹⁷ and others were processed for immunohistochemistry. After treatment with H₂O₂ (0.3% in phosphate-buffered saline, 10%

methanol) for 15 minutes and blocking with 5% normal horse serum and 0.2% bovine serum albumin for 2 hours, sections were incubated with anti-calbindin D-28k (1:10,000; Swant, Marly, Switzerland) antibody overnight at 4°C. After washing, sections were incubated with biotinylated secondary antibodies (ABC kit; Pierce, Tattenhall, UK) and then with avidin-biotin complex. Finally, they were developed with 0.05% diaminobenzidine 0.02% H₂O₂.

Results

We generated a transgenic mouse with liver-selective NPC1 expression from embryonic stages^{18,19} to precisely define the extent in which hepatic disease participates in NP-C and evaluate the degree of primary and secondary hepatic damage in NP-C. The strategy (Figure 1) followed three steps: i) generations of mice with the *Npc1* gene containing a removable floxed cassette arresting *Npc1* transcription (*Npc1*^{fl/fl}; see *Materials and Methods*), ii) crossing *Npc1*^{fl/+} and Afp-Alb-Cre^{+/-} mice and selecting for Afp-Alb-Cre^{+/-} *Npc1*^{fl/+} progeny, and iii) back-crossing Afp-Alb-Cre^{+/-} *Npc1*^{fl/+} to generate Afp-Alb-Cre^{+/-} *Npc1*^{fl/fl} or Afp-Alb-Cre^{+/+} *Npc1*^{fl/fl} progeny (Cre-*Npc1*^{fl/fl}; see *Materials and Methods*).²⁰ The Cre-*Npc1*^{fl/fl} mouse expresses Cre-recombinase under the albumin promoter and the α -feto-protein enhancer (hepatocyte-specific), removing the floxed cassette to allow *npc1* transcription. In the Cre-*Npc1*^{fl/fl} mice after 9 weeks the protein levels in the liver were similar to wild-type animals (WT and Cre-WT); however, NPC1 was not expressed in brain (Figure 1D) and other tissues (data not shown). The WT and the Cre-WT mice did not show differences in weight (Figure 2) or in the rest of parameters analyzed in this study. Because differences between the sexes in hepatic cholesterol homeostasis have been proposed, we analyzed females and males separately. The Cre-expression pattern during embryogenesis (embryonic day 15.5) has been shown previously,^{18,19} and NPC1 already was detected in the liver of newborn Cre-*Npc1*^{fl/fl} mice (2 days) (Figure 1E). Densitometric quantitation of the Western blot analysis suggested that hepatic NPC1 levels in the Cre-*Npc1*^{fl/fl} were approximately 60% of the levels in WT mice (WT, 1.99 $\pm$ 0.89 arbitrary units; *Npc1*^{fl/fl}, 1.22 $\pm$ 0.64 arbitrary units; *n* = 3).

When compared with WT, *Npc1*^{fl/fl} and Cre-*Npc1*^{fl/fl} mice showed delayed development, weight loss, and signs of ataxia after 8 weeks and died after 10 weeks (Figure 2, A–C). In both strains, the anatomic analysis showed the characteristic loss of Purkinje cells in the cerebellum,²¹ especially in lobules II to IV (Figure 2, D and E). Thus, re-expression of hepatic NPC1 does not ameliorate progression of the fatal neurodegeneration occurring in NPC1-null mice.

In contrast, the histology of the liver showed an almost normal morphology and, in contrast to *Npc1*^{fl/fl}, few Cre-*Npc1*^{fl/fl} hepatocytes accumulated intracellular lipids (Figure 2F). Furthermore, the Cre-*Npc1*^{fl/fl} mice showed a normal hepatic index, highly reduced—although not

completely—hepatic cholesterol levels, and hepatic triglyceride levels similar to WT (Figure 3, A–C). In contrast, the characteristic splenomegaly associated with NP-C was not corrected after hepatic re-expression of NPC1 ($\times 1.65$ in *Npc1*^{fl/fl} and $\times 1.76$ in Cre-*Npc1*^{fl/fl}; *n* = 5). The hepatic damage—estimated by serum transaminase, bilirubin, and bile acid levels—was highly but not entirely reduced, suggesting secondary causes of liver disease (Figure 3, D–F). Similarly, cytotoxicity (serum lactate dehydrogenase) was drastically, although not completely, recovered in Cre-*Npc1*^{fl/fl} mice (Figure 3G), suggesting that although most of the tissue damage recovers after re-expression of hepatic NPC1, cytotoxicity persists in Cre-*Npc1*^{fl/fl} mice. Finally, we took advantage of Cre-*Npc1*^{fl/fl} to further analyze the intriguing observation that NP-C patients show low levels of circulating high-density lipoprotein cholesterol, which correlates inversely with NP-C severity and the age of death.²² Indeed, the *Npc1*^{fl/fl} mice showed a significant reduction of high-density lipoprotein cholesterol, although it was not corrected in Cre-*Npc1*^{fl/fl} animals (Figure 3H). Thus, we speculate that low high-density lipoprotein cholesterol in NP-C patients is not caused by hepatic malfunctioning but through impaired cholesterol uptake by the lipoprotein in peripheral tissues.

Discussion

We have generated the first NPC1-null mice with liver-selective NPC1 re-expression from embryonic stages. Now, the Cre-*Npc1*^{fl/fl} mice can be used in neurologic studies to eliminate those symptoms arising from hepatic disease and, as shown here, secondary to the severity of the neurodegeneration in NPC1-null animals. We show that most of the hepatic damage in NP-C is primary and largely is rescued by re-expression of hepatic-NPC1. However, hepatic cholesterol, serum transaminase, and serum bilirubin levels remain slightly increased in Cre-*Npc1*^{fl/fl} mice, indicating the existence of extrahepatic factors that could explain the recurrence of liver damage after transplantation.¹²

Neuronal re-expression of NPC1 does not completely recover neurodegeneration in NPC1-null animals,^{7,8} suggesting that glial and visceral NPC1 are needed for rescue. The mouse generated here now can be crossed with strains expressing Cre-recombinase under tissue-specific promoters to generate a mouse with NPC1 expression in one or various cell types. These studies undoubtedly will define causes of primary and secondary neuronal toxicity, a central question for understanding the complex clinical spectrum of NP-C and essential for designing more efficient therapeutic approaches.

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References

1. Vanier MT: Niemann-Pick disease type C. *Orphanet J Rare Dis* 2010, 5:16
2. Alobaidy H: Recent advances in the diagnosis and treatment of niemann-pick disease type C in children: a guide to early diagnosis for the general pediatrician. *Int J Pediatr* 2015, 2015:816593
3. Mengel E, Klunemann HH, Lourenco CM, Hendriksz CJ, Sedel F, Walterfang M, Kolb SA: Niemann-Pick disease type C symptomatology: an expert-based clinical description. *Orphanet J Rare Dis* 2013, 8:166
4. Lopez ME, Scott MP: Genetic dissection of a cell-autonomous neurodegenerative disorder: lessons learned from mouse models of Niemann-Pick disease type C. *Dis Model Mech* 2013, 6:1089–1100
5. Elrick MJ, Pacheco CD, Yu T, Dadgar N, Shakkottai VG, Ware C, Paulson HL, Lieberman AP: Conditional Niemann-Pick C mice demonstrate cell autonomous Purkinje cell neurodegeneration. *Hum Mol Genet* 2010, 19:837–847
6. Yu T, Shakkottai VG, Chung C, Lieberman AP: Temporal and cell-specific deletion establishes that neuronal *Npc1* deficiency is sufficient to mediate neurodegeneration. *Hum Mol Genet* 2011, 20:4440–4451
7. Borbon I, Totenhagen J, Fiorenza MT, Canterini S, Ke W, Trouard T, Erickson RP: Niemann-Pick C1 mice, a model of “juvenile Alzheimer’s disease”, with normal gene expression in neurons and fibrillary astrocytes show long term survival and delayed neurodegeneration. *J Alzheimers Dis* 2012, 30:875–887
8. Erickson RP: Current controversies in Niemann-Pick C1 disease: steroids or gangliosides; neurons or neurons and glia. *J Appl Genet* 2013, 54:215–224
9. Loftus SK, Erickson RP, Walkley SU, Bryant MA, Incao A, Heidenreich RA, Pavan WJ: Rescue of neurodegeneration in Niemann-Pick C mice by a prion-promoter-driven *Npc1* cDNA transgene. *Hum Mol Genet* 2002, 11:3107–3114
10. Lopez ME, Klein AD, Dimbil UJ, Scott MP: Anatomically defined neuron-based rescue of neurodegenerative Niemann-Pick type C disorder. *J Neurosci* 2011, 31:4367–4378
11. Schwend T, Loucks EJ, Snyder D, Ahlgren SC: Requirement of *Npc1* and availability of cholesterol for early embryonic cell movements in zebrafish. *J Lipid Res* 2011, 52:1328–1344
12. Gartner JC Jr, Bergman I, Malatack JJ, Zitelli BJ, Jaffe R, Watkins JB, Shaw BW, Iwatsuki S, Starzl TE: Progression of neurovisceral storage disease with supranuclear ophthalmoplegia following orthotopic liver transplantation. *Pediatrics* 1986, 77:104–106
13. Testa G, Schaft J, van der Hoeven F, Glaser S, Anastassiadis K, Zhang Y, Hermann T, Stremmel W, Stewart AF: A reliable lacZ expression reporter cassette for multipurpose, knockout-first alleles. *Genesis* 2004, 38:151–158
14. Hartley JL, Temple GF, Brasch MA: DNA cloning using in vitro site-specific recombination. *Genome Res* 2000, 10:1788–1795
15. Skarnes WC, Rosen B, West AP, Koutourakis M, Bushell W, Iyer V, Mujica AO, Thomas M, Harrow J, Cox T, Jackson D, Severin J, Biggs P, Fu J, Nefedov M, de Jong PJ, Stewart AF, Bradley A: A conditional knockout resource for the genome-wide study of mouse gene function. *Nature* 2011, 474:337–342
16. Markel P, Shu P, Ebeling C, Carlson GA, Nagle DL, Smutko JS, Moore KJ: Theoretical and empirical issues for marker-assisted breeding of congenic mouse strains. *Nat Genet* 1997, 17: 280–284
17. Giralt A, Saavedra A, Carreton O, Xifro X, Alberch J, Perez-Navarro E: Increased PKA signaling disrupts recognition memory and spatial memory: role in Huntington’s disease. *Hum Mol Genet* 2011, 20:4232–4247
18. Parviz F, Li J, Kaestner KH, Duncan SA: Generation of a conditionally null allele of *hnf4alpha*. *Genesis* 2002, 32:130–133
19. Krupczak-Hollis K, Wang X, Kalinichenko VV, Gusarova GA, Wang IC, Dennewitz MB, Yoder HM, Kiyokawa H, Kaestner KH, Costa RH: The mouse Forkhead Box m1 transcription factor is essential for hepatoblast mitosis and development of intrahepatic bile ducts and vessels during liver morphogenesis. *Dev Biol* 2004, 276: 74–88
20. Kellendonk C, Opherk C, Anlag K, Schutz G, Tronche F: Hepatocyte-specific expression of Cre recombinase. *Genesis* 2000, 26: 151–153
21. Sarna JR, Larouche M, Marzban H, Sillitoe RV, Rancourt DE, Hawkes R: Patterned Purkinje cell degeneration in mouse models of Niemann-Pick type C disease. *J Comp Neurol* 2003, 456: 279–291
22. Garver WS, Jelinek D, Meaney FJ, Flynn J, Pettit KM, Shepherd G, Heidenreich RA, Vockley CM, Castro G, Francis GA: The National Niemann-Pick Type C1 Disease Database: correlation of lipid profiles, mutations, and biochemical phenotypes. *J Lipid Res* 2010, 51: 406–415