Short Communication

Development of a Rab9 Transgenic Mouse and Its Ability to Increase the Lifespan of a Murine Model of Niemann-Pick Type C Disease

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Niemann-Pick, type C (NP-C) disease is an autosomal recessive neurovisceral storage disorder in which cholesterol and sphingolipids accumulate. There is no specific treatment for this disease, which is characterized by progressive neurological deterioration, sometimes accompanied by hepatosplenomegaly. We and others have shown that overexpression of certain Rab GTPases corrects defective membrane trafficking and reduces lipid storage in cultured NP-C fibroblasts. Here, we tested the possibility that Rab protein overexpression might also have beneficial effects *in vivo* using a murine model of NP-C. We first generated several lines of transgenic mice that ubiquitously overexpress Rab9 up to ~30-fold more than endogenous levels and found that the transgene expression had no obvious effects on fertility, behavior, or lifespan in normal mice. These transgenic strains were then crossed with NP-C mutant mice to produce NP-C homozygous recessive mice with and without the Rab9 transgene. Life expectancy of the NPC1 homozygous recessive animals was extended up to 22% depending on gender and the transgenic strain that was used. Histological studies and lipid analysis of brain sections indicated that the NP-C mice carrying the Rab9 transgene had dramatically reduced storage of GM₂ and GM₃ gangliosides relative to NP-C animals lacking the transgene. These results demonstrate that

Rab9 overexpression has the potential to reduce stored lipids and prolong lifespan *in vivo*. (Am J Pathol 2009, 174:14–20; DOI: 10.2353/ajpath.2009.080660)

Niemann-Pick, type C (NP-C) is an autosomal recessive, neurodegenerative disease that is characterized by massive accumulation of cholesterol in peripheral tissues and glycosphingolipids in the brain. In humans, most cases of NP-C arise from mutations in *NPC1* that encodes a large membrane protein with multiple transmembrane domains homologous to the sterol-sensing domains found in Patched, HMG-CoA reductase, and SCAP.^{1–3} Current therapeutic approaches for NP-C that are being explored using animal models include substrate reduction therapy to reduce the biosynthesis of stored glycosphingolipids,^{4–6} allopregnanolone therapy that seeks to correct the reduced neurosteroid levels seen in NP-C animals,⁷ and treatment with a synthetic oxysterol that activates genes involved in cholesterol breakdown and removal.⁸

We previously showed that overexpression of the small GTPase, Rab9, in cultured cells results in correction of lipid-trafficking defects associated with NP-C and significantly reduces lipid accumulation.^{9–11} Similar results have been obtained by overexpression of Rabs 4, 7, or 8 in cultured fibroblasts,^{12–14} or Rab9 in NP-C mouse neurons.¹¹ Although the underlying mechanism for this correction is not completely understood, several studies

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have shown that elevated endosomal cholesterol interferes with the GDP dissociation inhibitor extraction of Rab proteins from endosomal membranes.^{13,15,16} Overexpression of Rab proteins may thus be sufficient to stimulate the intracellular transport that is otherwise blocked by the stored lipids. In the present study, we sought to test whether Rab overexpression might also have a beneficial effect *in vivo*. We developed strains of transgenic mice that ubiquitously overexpress Rab9 and then crossed these transgenic mouse strains into a mouse model of NP-C disease. We show that the overexpression of Rab9 increased the average lifespan by up to ~22% and reduced some symptoms associated with this disease, suggesting that stimulation of intracellular transport might have therapeutic potential.

Materials and Methods

All procedures involving mice were performed in accordance with the recommendations and approval of the Institutional Animal Care Use Committee of the Mayo Clinic and Foundation. Mice were weaned at postnatal day 21 and fed a standard chow diet. BALB/cNcr-Npc1^{m1N/+} breeding stock were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained as heterozygotes. Homozygous NP-C mutant mice are referred to throughout as NPC^{mut/mut} mice.

Generation of Rab9 Transgenic Mice

HA-tagged human Rab9 (I.M.A.G.E. Consortium) was inserted into the pCAGGS vector (generously provided by J. Miyazaki, Osaka University, Osaka, Japan), which contains the chicken actin promoter that is widely used for ubiquitous expression of proteins in transgenic mice.¹⁷ DNA containing the promoter and the Rab9 transgene was then purified for microinjection into fertilized eggs. Transgenic mice were generated by the Mayo Clinic Transgenic Core by pronuclear microinjection of the foreign DNA fragments into one-cell-stage mouse embryos from FVB, C57BL/6J mice. Microinjected embryos were then transferred into surrogate mothers and progeny carrying the transgene were bred to establish the transgenic mouse lines. From this procedure we obtained ~100 adult candidate mice. Tail biopsies were then assessed for proper integration of the transgene by polymerase chain reaction (PCR) and five transgenic strains (designated as strains 1, 100, 400, 500, and 800) were selected and further characterized (see text).

Crosses Between NP-C and Rab9 Transgenic Mice (see Supplemental Figure S1 at <http://ajp.amjpathol.org>)

Because mice homozygous for the NPC1 mutation are not fertile, we first crossed the Rab9 transgenic animals (Rab9^{TG+/TG-}, NPC^{WT/WT}) with NPC1 heterozygotes (NPC^{WT/mut}). Offspring that were heterozygous for the transgene and for the NP-C mutation (Rab9^{TG+/TG-},

NPC^{WT/mut}) were identified by PCR of tail snips. These animals were then crossed with NPC1 heterozygotes to obtain offspring that were homozygous for the NP-C mutation and heterozygous for the Rab9 transgene (Rab9^{TG+/TG-}, NPC^{mut/mut}), as well as NP-C disease control animals (Rab9^{TG-/TG-}, NPC^{mut/mut}). NPC^{WT/WT} animals $\pm$ Rab9^{TG} were also used in some experiments.

Genotyping

One hundred ng of total DNA, isolated from tail biopsies as described,¹⁸ were added to the reaction mixture [49 μ l consisting of 10 $\times$ PCR buffer (5 μ l) (Denville Scientific Inc., South Plainfield, NJ), 10 mmol/L dNTPs (1 μ l), 1 μ l each of forward and reverse primers (25 μ mol/L stock solutions; primers synthesized by IDT, Coralville, IA) for NPC-1 (forward: 5'-GGTGCTGGACAGCCAAGTA-3'; reverse: 5'-GATGGTCTGTTCTCCCATG-3') or Rab9 (forward: 5'-GTACCCATACGATGTTCCGG-3'; reverse: 5'-GGCTTTCGGTGAAGATTGACTG-3'), TaqDNA polymerase (1 U) (Denville Scientific Inc.) and dH₂O (41 μ l)] and subjected to PCR. PCR conditions consisted of 95°C $\times$ 5 minutes, 94°C $\times$ 1 minute, 55°C $\times$ 2 minutes, and 72°C $\times$ 2 minutes, for 30 cycles and then 72°C $\times$ 10 minutes. PCR products were resolved on a 1% agarose gel by electrophoresis.

Western Blotting, Immunohistochemistry, and Lipid Analysis

Brain and liver samples from Rab9^{TG+/-} founder mice and from NPC^{mut/mut} mice expressing the Rab9 transgene were harvested and homogenized in 50 mmol/L Tris, pH 7.4, 25 mmol/L KCl, 0.25 mol/L sucrose with protease inhibitors. To visualize the HA-tagged Rab9 transgene, total brain and liver proteins (5 μ g/lane) were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membranes, incubated with monoclonal anti-Rab9 Ab (diluted 1:1000, catalog no. R5404; Sigma Chemical Co., St. Louis, MO) followed by goat anti-mouse horseradish peroxidase (GE Health Care, Piscataway, NJ), and detected by chemiluminescence. To evaluate the levels of endogenous Rab9 protein, control animals that did not express the Rab9 transgene were processed as above, but using 20 μ g of protein/lane. β -Actin levels on the same blots were detected using an anti- β -actin antibody (diluted 1:1000, catalog no. 4967; Cell Signaling Technologies Inc., Danvers, MA).

For immunostaining and lipid analysis of GM₂ and GM₃ gangliosides, 70- or 80-day-old mice obtained from crosses of NP-C mice with Rab9 transgenic strain 500 or 800 were placed under deep anesthesia and perfused with saline. One cerebral hemisphere was immersion-fixed in 4% paraformaldehyde overnight at 4°C and subsequently immunostained for GM₂ and GM₃ gangliosides as described.¹⁹ In some experiments, one half of the brain (including cerebrum and cerebellum) was homogenized and used for lipid extraction and thin-layer chromatographic analysis essentially as described.^{6,20}

Immunostaining of the Rab9 transgene in the brain of NPC^{mut/mut} animals was performed by the Mayo Tissue and Cell Molecular Analysis Core Facility. Mouse brains of 50-day-old animals were collected and fixed as described above. The samples were then embedded in paraffin and 4- μ m sections were prepared. Sections were deparaffinized by three washes in xylene and rehydrated in a series of decreasing concentrations of alcohol. The slides were then placed in preheated (37°C) 1 mmol/L ethylenediaminetetraacetic acid, pH 8.0, for 30 minutes, cooled to room temperature in the same buffer for 5 minutes, and then rinsed for 5 minutes in running distilled water. Endogenous peroxide was blocked with 3% H₂O₂ for 5 minutes. Samples were then washed and incubated with an anti-HA Rat mAb (clone 3F10 used at 1:1000; Roche Applied Science, Indianapolis, IN) for 1 hour, washed, and further incubated with the secondary antibody (Rat-on-Mouse Polymer detection kit; Biocare Medical, Concord, CA) for 30 minutes. The sections were then washed, incubated in 3,3-diaminobenzidine for 5 minutes, counterstained with modified Schmidts' hematoxylin for 5 minutes, rinsed under tap water, and mounted with an aqueous mounting media.

Lifespan Studies

Animals were housed in standard cages and conditions. Rab9^{TG+/-} NPC^{WT/mut} (separate breeders for each strain) and Rab9^{TG-/-} NPC^{WT/mut} mice were crossed (see Supplemental Figure S1 at <http://ajp.amjpathol.org>). The date of birth of each litter was noted. Pups were genotyped by PCR and weaned at 3 weeks. Only Rab9^{TG+/-} NPC^{mut/mut} and Rab9^{TG-/-} NPC^{mut/mut} were kept. We then monitored the lifespan of Rab9^{TG+/-} NPC^{mut/mut} mice versus Rab9^{TG-/-} NPC^{mut/mut} mice obtained from crosses within the same strain, thus assuring that the backgrounds of the animals being compared were as close as possible. It was not possible to exclusively use littermates for these studies because of the small number of required genotypes occurring in each litter. Beginning at 5 weeks, NPC^{mut/mut} mice were monitored daily for date of death. Mortality date was recorded when mice were found dead or judged to be in severe distress (eg, could not stand or exhibited paralysis of hind limbs). In the latter case, the moribund animals were euthanized according to Institutional Animal Care and Use Committee guidelines.

Results and Discussion

We first generated transgenic mice (C57BL/6 background) that expressed HA-tagged human Rab9 ubiquitously and constitutively under the control of the chicken actin promoter (see Materials and Methods). We selected five founder mice that were positive for the Rab9 transgene (TG) by PCR. These animals were bred with normal C57BL/6 mice to create five separate strains of Rab9 transgenic mice. Each strain was perpetuated by breeding Rab9^{TG} heterozygotes with normal mice. The five strains varied in their level of Rab9 overexpression (up to 30-fold higher than endogenous Rab9) and the Rab9

transgene was detected in all tissues tested (liver, brain, kidney, skin) for each strain; however, the relative distribution in different tissues varied among the different transgenic strains. For example, strain 1 exhibited high Rab9^{TG} levels in liver, but an intermediate level in brain, whereas strain 500 had the highest levels of Rab9^{TG} in both brain and liver (Figure 1A). This variation is seen more clearly in Figure 1B, in which the amount of Rab9^{TG} in each transgenic strain was normalized to the level of endogenous Rab9 in the liver. No negative effects of the Rab9^{TG} on breeding, longevity, or behavior of the mice were observed. In addition, Rab9^{TG} mouse tissues were normal on examination for gross pathology.

Each Rab9 transgenic strain was then crossed with animals from our NPC1 (BALB/c background) breeding colony. This required multiple crosses because mice homozygous for the NPC1 mutant allele are not fertile and must be maintained as heterozygotes. Thus the initial crosses were between a transgenic mouse (Rab9^{TG+/-} NPC^{WT/WT}) and an NPC heterozygote (Rab9^{TG-/-} NPC^{WT/mut}). Offspring were screened by PCR of tailsnips to identify NPC heterozygous animals expressing the Rab9 transgene (ie, Rab9^{TG+/-} NPC^{WT/mut}). Those animals were then backcrossed with the NPC heterozygous breeding stock, and their offspring were screened for animals homozygous for the NPC mutant allele, with (Rab9^{TG+/-} NPC^{mut/mut}) or without (Rab9^{TG-/-} NPC^{mut/mut}) the Rab9 transgene (see Supplemental Figure S1 at <http://ajp.amjpathol.org>).

We first examined the tissue distribution of the Rab9 transgene in 88- to 113-day-old NPC^{mut/mut} animals and found that although the levels of transgene overexpression in the brain were very similar in the NPC^{mut/mut} animals and in the corresponding founder strains, the levels of expression in the liver in several strains were much lower than in the founders (Figure 1A). For example, in the founders from strain 500 the transgene expression was ~30-fold higher than endogenous Rab9 in the liver, whereas in the Rab9^{TG+/-} NPC^{mut/mut} animals, the levels of the transgene in the liver were similar to that of endogenous Rab9 (Figure 1, A and B). One possible explanation for reduced expression of Rab9^{TG} in livers of the experimental animals compared to the founder strains is that there is an effect of the NP-C disease state on expression of the transgene (eg, there is known to be considerable hepatocyte damage in the NP-C liver²¹). Thus, we investigated Rab9^{TG} expression in livers from Rab9^{TG+/-} NPC^{WT/WT} mice of the 500 strain that were products of the same crosses as the Rab9^{TG+/-} NPC^{mut/mut} experimental animals (ie, had identical genetic backgrounds). We found that these animals had high Rab9 transgene expression in the livers (Figure 1C), much like the founder mice (Figure 1A). Thus, we conclude that the lower Rab9 transgene expression observed in the livers of NPC^{mut/mut} mice of some strains is a result of an interaction of the NP-C disease state and Rab9 transgene expression. We also used immunohistochemistry to verify the expression of the HA-tagged Rab9 transgene in brain sections from 7-week-old Rab9^{TG+/-} NPC^{mut/mut} animals (strain 500) and readily detected the Rab9 transgene in the brain (Figure 1D). The HA-Rab9 transgene was expressed

Figure 1. Expression of the Rab9 transgene in founder mice and in Rab9^{TG}+/- NPC^{mut/mut} mice. **A** and **B**: Brain and liver samples from founder and NPC^{mut/mut} animals from each of the Rab9 transgenic strains at 6 weeks of age were harvested and blotted with an antibody that recognizes both the HA-tagged human Rab9, and endogenous mouse Rab9. Five μ g of protein per lane were used to visualize the HA-tagged Rab9 transgene, whereas 20 μ g of protein per lane were required for reliable detection of endogenous Rab9 (see Materials and Methods for details). Samples were also blotted for β -actin to demonstrate equal loading. **B**: Bar graphs show the Rab9 transgene levels for transgenic founders ($n = 3$) and Rab9^{TG}+/- NPC^{mut/mut} ($n = 3$) mice (age, 10 weeks) in brain (red) relative to the signal for endogenous Rab9 in the liver (green). **C**: Western blotting for Rab9 of liver and brain homogenates (5 μ g) from 4-week-old Rab9^{TG}+/- NPC^{WT/WT} and Rab9^{TG}+/- NPC^{mut/mut} mice (strain 500), as well as Rab9^{TG}- controls (20 μ g). **D**: Immunostaining of the Rab9 transgene in the brain of NPC^{mut/mut} animals at 7 weeks. **Left**: Brain sections from 50-day-old animals of the indicated genotypes were stained with a rat anti-HA Ab to detect the Rab9 transgene. Brown staining indicates the presence of the expressed Rab9 transgene. **Right**: Enlargement of the cerebellum from the **left** panel. **Left** (low mag) panels in D, $\times 2.3$. **Right** (high mag) panels in D, $\times 7$.

throughout the brain and was particularly enriched in the cerebellum (Figure 1D).

The NPC^{mut/mut} mice, with or without the Rab9 transgene, were next examined with respect to several parameters that are affected as the mutant mice age. i) Activation of microglia. Microglia are resident immune cells of the central nervous system that secrete potentially neurotoxic molecules such as tumor necrosis factor- α on activation. Inappropriate activation of microglia has been implicated in several neurodegenerative disorders including NP-C disease, and it has been suggested that lipid storage may cause microglial dysfunction.^{22,23} To examine the possibility that Rab9 expression has an effect on the activation of microglia, brain sections from the cerebellum were stained with a microglial-specific antibody, Iba1.²⁴ In agreement with previous reports, the NPC^{mut/mut} sample showed a large accumulation of microglia with the typical amoeboid morphology characteristic of activated cells, whereas only ramified (resting)

microglia were seen in NPC^{WT/WT} animals.^{4,22,25} However, there was no obvious difference in NPC^{mut/mut} mice with or without expression of the Rab9 transgene in either 7- or 10-week-old animals (data not shown). ii) Weight loss. NP-C mice typically begin to lose weight 6 to 7 weeks after birth. To determine whether the Rab9 transgene had an effect on this parameter, we weighed mutant animals weekly throughout a 10- to 11-week period (Figure 2A). NP-C mice that were Rab9^{TG}-negative began to lose weight at weeks 5 to 6, whereas Rab9^{TG}-positive animals showed a trend toward increased weight retention. This was particularly evident for male offspring produced from NP-C animals crossed with transgenic strain 500 (Figure 2A). A similar trend was observed for other strains, but the effects were smaller (see Supplemental Figure S2 at <http://ajp.amjpathol.org>). At present we have no explanation for why the retardation in weight loss was more pronounced for males than females. iii) Lipid storage in the cerebral cortex. One hallmark of NP-C mice is

Figure 2. Effect of Rab9 overexpression on symptoms of NP-C mice. **A:** Retardation of weight loss. Male mice obtained from crosses of NP-C mice with Rab9 transgenic mice (strain 500) were weighed weekly. NPC^{mut/mut} animals began to lose weight at weeks 6 to 7, whereas the presence of the Rab9 transgene retarded the weight loss. *n* = 5, 10, and 7 for Rab9^{TG-} NPC^{WT/WT}, Rab9^{TG+} NPC^{mut/mut}, and Rab9^{TG-} NPC^{mut/mut} mice, respectively. Values were significantly different in two-tailed *t*-tests (*P* < 0.05) between Rab9^{TG+} NPC^{mut/mut} and Rab9^{TG-} NPC^{mut/mut} mice at all points after 4 weeks. Similar results were obtained using the other transgenic strains and are shown in Supplemental Figure S2 at <http://ajp.amjpatbol.org>. **B:** Rab9 transgene expression reduces ganglioside accumulation in the brain. Representative examples of cortex from normal, Rab9^{TG-} NPC^{mut/mut}, and Rab9^{TG+} NPC^{mut/mut} mice immunostained for GM₂ and GM₃ gangliosides are shown in **B**. Note the reduction in ganglioside staining in NPC^{mut/mut} mice expressing the Rab9 transgene. Two additional experiments gave similar results (data not shown). Similar results to those shown in **B** were also obtained using NPC^{mut/mut} mice expressing the Rab9^{TG} from founder strain 800 (see Supplemental Figure S3 at <http://ajp.amjpatbol.org>). Original magnification: ×35. **C:** Gangliosides were extracted from 80-day-old NPC^{mut/mut} mice ± Rab9 transgene (strain 500), separated by thin-layer chromatography, and quantified by image analysis after staining the gangliosides with resorcinol.⁶ Bar graph shows the reduction in GM₃ (45%) and GM₂ (32%) levels in NPC^{mut/mut} mice expressing the Rab9 transgene, relative to the levels of GM₃ and GM₂ in control, NPC^{mut/mut} mice without the transgene. Values are the mean of two experiments.

the storage of gangliosides and cholesterol in the brain.^{1,19,26} To test the effect of Rab9 overexpression on this storage, we first performed ganglioside immunocy-

Figure 3. Effect of Rab9 overexpression on lifespan of NP-C mice. **A:** Kaplan-Meier survival plots for NPC^{mut/mut} mice without or with expression of the Rab9 (strain 500) transgene (*P* < 0.0001). Kaplan-Meier plots for other strains are shown in Supplemental Figure S4 at <http://ajp.amjpatbol.org>. **B:** Bar graphs showing percent increase in lifespan based on the difference in lifespan of NPC^{mut/mut} animals ± Rab9^{TG} expression. All values were significant except for strain 400. Student's *t*-test was used for calculating statistical significance. *n* values for each group are as follows: all animals, Rab9^{TG+} = 28, Rab9^{TG-} = 31; females, Rab9^{TG+} = 11, Rab9^{TG-} = 21; males, Rab9^{TG+} = 17, Rab9^{TG-} = 10.

tochemistry on sections of the cerebral cortex obtained from wild-type mice and NPC^{mut/mut} mice ± Rab9^{TG} expression.¹⁹ All samples were from 80-day-old male mice obtained from crosses with transgenic strain 500 because these gave the greatest increase in longevity (see below). Rab9^{TG} overexpression decreased the levels of GM₂ and GM₃ gangliosides in the cerebral cortex as assessed qualitatively by immunostaining of three sets of animals of the indicated genotypes (Figure 2B). Gangliosides were also extracted from the whole brain of the mice and analyzed by thin-layer chromatography using resorcinol, a ganglioside-specific stain (Figure 2C).²⁰ In agreement with the results obtained by immunostaining, we found the highest levels of GM₂ and GM₃ storage in NPC^{mut/mut} animals and a >30% reduction in this storage in NPC^{mut/mut} mice expressing the Rab9 transgene. No storage of these gangliosides was seen in NPC^{WT/WT} brain extracts (data not shown) as previously reported.⁶ We also found that expression of the Rab9^{TG} from strain

800 reduced ganglioside storage in the cerebral cortex and hippocampus in 70-day-old NPC^{mut/mut} mice (see Supplemental Figure S3 at <http://ajp.amjpathol.org>). Thus, Rab9^{TG} expression significantly reduced ganglioside storage in the brains of NP-C mice. In contrast, we could detect no effect of Rab9^{TG} expression on filipin staining of unesterified cholesterol in brain sections obtained from NPC^{mut/mut} animals (data not shown).

Finally, we evaluated the effect of the Rab9 transgene on the longevity of the NP-C mutant mice by comparing the lifespan of animals homozygous for the NP-C mutant allele and expressing the Rab9 transgene to that in Rab9-negative animals obtained from the same strain. This assured that the backgrounds of the animals being compared were identical. This was important because the Rab9 transgenic founder strains were produced in a different background than the available NP-C mouse model (see above). Survival data for NP-C mice ($\pm$ Rab9^{TG} expression) were obtained for each transgenic strain and are displayed as Kaplan-Meier plots, which give the percentage of animals surviving at each day after birth. Survival curves and percent increase in lifespan for males, females, and both combined are shown in Figure 3, A and B, and Supplemental Figure S4 at <http://ajp.amjpathol.org>. All strains exhibited a significant increase in lifespan when the Rab9 transgene was expressed, with the largest increase ($\sim$ 22%) seen for male mice (Rab9^{TG+/-} NPC^{mut/mut}) obtained from crosses with transgenic strain 500 (Figure 3A). Taken together, the results in Figures 2 and 3 demonstrate that expression of the Rab9 transgene reduced some of the symptoms seen in the NP-C mouse model and significantly extended the lifespan of these animals. The observation that lifespan was significantly extended in each of the five Rab9 transgenic strains suggests that life extension was attributable to Rab9 overexpression rather than interference with an unrelated gene during insertion of the transgene into the mouse DNA, because the Rab9 transgene is likely to be inserted at a different point in each mouse strain.

Our previous *in vitro* studies using cultured NP-C fibroblasts and neurons showed that overexpression of Rab4, -7, or -9 corrected aberrant membrane trafficking and reduced lipid storage in cultured NP-C cells.^{9,10,13} Similar results have been demonstrated by other laboratories.^{14,27} In the present study we extended this work to an animal model of NP-C disease and show that Rab9 overexpression can have a beneficial effect *in vivo*. Although modest, the effect of overexpression of Rab9 on life extension was similar to results obtained with substrate reduction therapy using Miglustat.⁶ In addition, there are a number of considerations that might lead to further improvement of this approach. First, it is unknown whether the transgenic Rab9 is fully active in the mouse, since previous studies demonstrated that cholesterol can interfere with Rab9 function.^{12,15} In addition, we found that the level of Rab9 overexpression was greatly reduced in the liver (Figure 1C), raising the possibility that some of the protein could be inactivated or degraded at a critical time during disease progression. These effects might be overcome using an inducible expression system so that the Rab9 transgene could be activated at a

particular age and for a length of time chosen by the investigator. Second, the overexpression of other Rabs (eg, Rab4, -7, or -8) that control other pathways of intracellular transport and that have been shown to be effective in ameliorating the NP-C phenotype *in vitro* may be more effective than Rab9 *in vivo*.^{9,13,14} Third, targeting specific cell types such as the Purkinje cells for overexpression might give better results than ubiquitous overexpression of Rab9 as used in the current study. However, to fully develop the therapeutic potential of modulating membrane transport, future studies will need to concentrate on small molecule screens to identify compounds that can stimulate the intracellular transport machinery similar to Rab overexpression. Fourth, recent studies in treating NP-C *in vivo* have used multiple approaches with considerable success.⁸ Thus, combined therapy using Rab overexpression and another approach such as substrate reduction may have a synergistic effect on outcome of this treatment. Finally, we note that the Rab9 transgenic mice developed in the current study may have other important applications beyond NP-C. These include studies of secretion in the intestine and/or liver, possible application to other lysosomal storage diseases, *in vivo* studies of the regulation of Rab9 expression and activity, and a source of various murine cell types with high levels of Rab9 expression.

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