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Neutral sphingomyelinase 2 deficiency is associated with lung anomalies similar to emphysema

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Abstract

Neutral sphingomyelinase 2 (nSMase2) upregulation was recently demonstrated to serve as a molecular link between smoke inhalation and emphysematous changes in lungs. Here we report that nSMase2 deficit impairs lung development in mice. We have shown previously that *fragilitas ossium* (*fro*) mice carry a mutation in the *Smpd3* gene, rendering nSMase2 catalytically inactive. Analysis of lung phenotype revealed that *fro* mice have abnormally enlarged alveoli and increased compliance of the respiratory system, similar to morphological and functional manifestations of emphysema. Analysis of sphingolipid content in *fro* lungs revealed a decreased level of C14:0 ceramide but no significant alterations in the levels of sphingosine or sphingosine-1-phosphate. Altogether, our data suggest that nSMase2 activity and ceramide level are critical for lung development and function. Based on our data, ceramide can no longer be viewed as a lipid solely detrimental to lung function.

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Conflict of interest: None.

Introduction

Mammalian lung development is an intricate process that requires precise regulation of a host of regulatory and structural proteins (Bonner et al. 2003; Mariani et al. 2002). While there are several genes whose involvement in pulmonary development have been well documented (Cardoso 2000; Warburton et al. 2000), new information continues to arrive from gene expression studies (Bonner et al. 2003; Mariani et al. 2002) and transgenic animal studies (Bridges and Weaver 2006). The abundance of cell types that undergo proliferation, differentiation, and apoptosis through lung development significantly complicates our understanding of the role of particular genes in this process. However, this understanding is of utmost clinical importance as it holds the key to the development of therapies for the structural and degenerative lung diseases.

The role of neutral sphingomyelinase 2 (nSMase2) in lung pathology was intensely studied during the past decade. nSMase2 is an enzyme that converts sphingomyelin to ceramide; on the cellular level, ceramides are known to regulate processes of apoptosis and differentiation (Hannun and Obeid 2008). Recently, activation of nSMase2 and an increase in ceramide level were reported in the lung in response to smoke inhalation (Filosto et al. 2011; Levy et al. 2009). These two facts and the additional finding that ceramide application causes emphysematous changes in lungs (Petrache et al. 2005) led to the understanding that nSMase2 activation and ceramide upregulation serve as molecular links between environmental challenges and emphysema development. Aside from emphysema, potential involvement of ceramides in cystic fibrosis (Hamai et al. 2009; Teichgraber et al. 2008; Wojewodka et al. 2011) and pulmonary edema (Goggel et al. 2004) was recently shown. However, the ceramide-producing enzymes affecting these lung disorders appear to be different. Whereas nSMase2 seems to be the key contributor to ceramide imbalance in emphysema (Filosto et al. 2011), acidic SMase (aSMase) is thought to be responsible for the derangement of ceramide level in cystic fibrosis (Guilbault et al. 2008; Teichgraber et al. 2008). These enzymes are localized to different sub-cellular compartments and likely govern different cellular pools of ceramide (Hannun and Obeid 2008).

Ceramide level is developmentally regulated in the lung (Longo et al. 1997). It was shown to increase with lung maturation, peaking in early adult lung (Longo et al. 1997). Alteration in both nSMase and aSMase levels were detected in development, contributing to the regulation of sphingomyelin–ceramide conversion (Longo et al. 1997). To further elucidate the role of nSMase2 and ceramide in lung development and function, we studied the lung phenotype of nSMase2-deficient mice, *fro/fro*. The mouse mutation *fragilitas ossium* (*fro*) was initially characterized by obvious skeletal anomalies (Guenet et al. 1981; Muriel et al. 1991; Sillence et al. 1993). We have shown that the mutation is caused by a ∼1.7 kb intragenic deletion within *Smpd3*, the gene encoding nSMase2 (Aubin et al. 2005). The deletion leads to the production of truncated nSMase2 lacking a critical amino acid residue in its catalytic site. We confirmed the loss of nSMase2 enzymatic activity in the brain and fibroblasts of *fro/fro* animals (Aubin et al. 2005).

In this study we have shown that nSMase2 inactivation in *fro* mice leads to the spontaneous development of a lung disorder, manifesting as abnormal enlargement of airspaces and

increased compliance of the respiratory system. We have also shown that *fro* mice exhibit lower ceramide levels with no significant changes in the levels of sphingosine or S1P. We conclude that ceramide deficiency rather than an alteration in another bioactive sphingolipid level is responsible for the lung phenotype of *fro*.

Materials and methods

Mice

Mouse breeding and experimental procedures were approved by GHSU IACUC. Since the initial discovery of the *fro* mutation in an outbred stock (Guenet et al. 1981), it has been maintained by breeding heterozygous (+/*fro*) mice together. This breeding scheme led to the emergence of an uncharacterized genetic background, FRA/Pas. To avoid postnatal lethality associated with *fro* maintenance on FRA/Pas background, *fro* was transferred to C57BL/6N and C3H/HeN (Charles River, Wilmington, MA) background by repeated backcrosses (>10) of heterozygous (+/*fro*) mice. Resulting congenic strains (referred to as B and H) were maintained by breeding heterozygous mice together.

Mice genotyping

Conditions for genomic DNA preparation, PCR amplification, and DNA separation by electrophoresis on agarose gels were described previously (Poirier et al. 2002). The mice were genotyped by PCR with the following primers: 5′-GGGACGACGTCTGCCTCAGG-3′; 5′-TTAGAGGTCCCAACCACAGG-3′; and 5′-CCCAGGTGCTGGGCAGAAGG-3′. With these primers it is possible to amplify specific wild-type (145 bp) and mutant (189 bp) DNA fragments.

Lung morphometry

Six-week-old FRA/Pas male littermates were anesthetized using pentobarbital (75 mg/kg body weight). Chest cavities were opened and lungs were inflated by instillation of 4 % paraformaldehyde. Lungs were extracted, fixed with paraformaldehyde overnight, and embedded in paraffin; then 5-μm-thick sections were stained with hematoxylin and eosin. Assessment of the mean linear intercept (Lm) and alveolar destructive index (DI) was done as described previously (Robbesom et al. 2003).

Assessment of respiratory mechanics

Ten-week-old +/+, +/*fro*, and *fro*/*fro* littermates were anesthetized with pentobarbital and tracheostomized; a metal tracheal cannula (1.2 mm internal diameter) was inserted and tied firmly into place. Mice were then connected to a FlexiVent ventilator (SCIREQ, Montreal, Quebec, Canada). Compliance of the respiratory system was measured as described previously (Dimitropoulou et al. 2009).

Ceramide, sphingosine, and sphingosine-1-phosphate levels

Calvaria were collected from newborn mice. One-day-old +/*fro* and *fro*/*fro* males were killed by decapitation. Skin and brain were removed from the skull and the remaining soft tissues were detached with a scalpel. The calvaria were snap-frozen in liquid nitrogen.

Lungs were collected from 10-week-old mice. The +/+, +/*fro*, and *fro*/*fro* male littermates were anesthetized and their chests were opened. After sectioning the inferior vena cava and nicking the left atrium, 5 ml of PBS was injected through the right ventricle to flush out blood from the lungs. The lungs were then excised and snap-frozen in liquid nitrogen.

Total lipids were extracted and the sphingolipids were analyzed with a qualitative and quantitative assay via combined liquid chromatography–tandem mass spectrometry (LC–MS/MS) as previously described (Berdyshev et al. 2006).

Statistical analysis

Data from each group (+/+, +/*fro*, and *fro*/*fro*) were tested for normality using the Shapiro–Wilk method and then analyzed with the *t* test or parametric ANOVA test followed by Tukey's multiple comparison post-hoc test. Analysis was done using Origin 8 software (OriginLab, Northampton, MA, USA) or PRISM 4 software (GraphPad Software, San Diego, CA, USA).

Results

nSMase2 deficiency results in a lung disorder

Evaluation of lung phenotype in nSMase2-deficient animals revealed unexpected morphological anomalies in mutant mice lungs (Fig. 1). The lung tissue sections from 6-week-old male *fro*/*fro* mice showed a dramatic increase in distal airspace size. To quantitate differences in alveolarization, the mean linear intercept (Lm) of mutant, heterozygous, and wild-type mice was evaluated. This measurement assesses the distance between alveolar septa across the lung; therefore, fewer septa will yield a larger Lm value. Analysis of Lm in *fro*/*fro* lungs demonstrated a 70 % increase compared to the wild-type lungs (Fig. 2a). Analysis of alveolar DI revealed a significant increase in the number of alveoli manifesting emphysematous changes among the three genotypes (Fig. 2b).

The increase in Lm and alveolar destruction indicated that nSMase2-deficient mice developed a lung disorder morphologically similar to emphysema. To confirm the nature of the pathological changes, we subjected *fro* mice to pulmonary function tests. Performance of the tests with the FlexiVent ventilator required animals to reach a certain weight and age. To generate *fro*/*fro*, +/*fro*, and +/+ animals for this analysis, we first had to overcome the early mortality problem that exists in the original FRA/Pas strain. Most of the *fro*/*fro* mice maintained on the FRA/Pas background died within a week after birth (Sillence et al. 1993). To circumvent this problem, we generated two congenic strains carrying the *fro* mutation on C57BL/6N (B strain) or C3H/HeN (H strain) background. Although perinatal mortality was still observed on the BB or HH background (Table 1), most of the *fro*/*fro* offspring on the mixed B × H background survived. This background was chosen to generate mice for future experiments. We mated heterozygous females from the B strain with heterozygous males from the H strain (cross B × H); all the mice from this cross had a similar hybrid genetic background. All first-generation hybrid B × H *fro*/*fro* mice exhibited the limb deformities identical to those described in the original FRA/Pas strain (Guenet et al. 1981; Muriel et al. 1991; Sillence et al. 1993) and the anomalies in lung morphology described in Fig. 1.

Assessment of lung functional parameters in 10-week-old +/+, +/*fro*, and *fro*/*fro* mice (Fig. 2c) revealed that nSMase2 deficiency significantly increases compliance. In males, the compliance of both heterozygous and homozygous mutant groups was significantly different from the compliance of wild-type mice. In females, there was no significant difference between the wild-type and heterozygous animals; however, mice homozygous for the mutation had a higher compliance than wild-type animals.

nSMase2 deficiency affects the level of C14:0 ceramide

To link the nSMase2 product ceramide to the observed lung phenotype and to further characterize molecular aspects of nSMase2 deficiency, we measured changes in certain ceramide subspecies in the most affected tissues of *fro*/*fro* mice, namely, lung and bone. LC–MS/MS analysis of lung extracts revealed a significant difference between wild-type and *fro*/*fro* mice in C14:0 ceramide only (Fig. 3a, b). Analysis of bone extracts from mutant mice confirmed that the C14:0 ceramide subspecies was among the most affected (Figs. 3c, 4). As ceramide is known to be metabolized to sphingosine and S1P (Hannun and Obeid 2008), we also assessed the level of these lipids in nSMase2-deficient mice. We failed to detect significant changes in sphingosine and sphingosine-1-phosphate in lung or bone (Fig. 5). These data suggest that changes in the cer-amide C14:0 level rather than changes in the sphingosine or S1P level are responsible for the lung and bone phenotypes observed in *fro*/*fro* mice.

Discussion

Assessment of morphological and functional changes in the lungs of nSMase2-deficient mice led us to the conclusion that these mice develop a lung alveolarization disorder similar to the morphological and physiological manifestations of emphysema. These findings were unexpected, as earlier data linked nSMase2 overexpression and ceramide production to emphysema development (Filosto et al. 2011). To verify that ceramide deficit is the culprit of an abnormal lung phenotype, we undertook an extensive LC–MS/MS analysis of the affected lipids. Since the effects of the ceramide downstream metabolite S1P are opposing the effects of ceramide (Hannun and Obeid 2008), we carefully assessed whether S1P alterations can be responsible for the *fro* lung abnormalities. Analysis of sphingolipid content showed no effect of nSMase2 deficiency on sphingosine or S1P levels This unexpected result could be explained by some compensatory mechanisms; activation/inhibition of other sphingolipid metabolism enzymes (Supplementary Fig. 1) could indeed mitigate the effects of nSMase2 deficiency on sphingosine and S1P levels. Altogether, these data unequivocally link ceramide deficit to the lung development disorder observed in nSMase2-deficient mice.

Among the analyzed ceramide subspecies, the C14:0 ceramide level was the most affected by nSMase2 deficiency. The fact that C14:0 ceramide was one of the least abundant subspecies increased the likelihood of a signaling role assigned to this ceramide. Earlier it was suggested (Hannun and Obeid 2008) that levels of bioactive lipids determine their mechanisms of action. Whereas trace lipids like S1P can modulate the action of high-affinity receptors, bulk lipids most likely exhibit their action via nonspecific effects such as those on

the lipid bilayer properties. Thus, the specificity of target interaction decreases with an increase of lipid concentration. The fact that in affected tissues C14:0 ceramide concentration was even lower than the concentration of the well-known signaling molecule S1P implies that ceramide C14:0 may have a very specific mode of action elicited via high-affinity targets.

Although the observed effect of nSMase2 deficiency is most likely elicited early in development and therefore may not reflect the role of nSMase2 in adulthood, nSMase2 and its product ceramide can no longer be viewed as solely deleterious to lung function (Filosto et al. 2011; Petrache et al. 2005).

The fact that the effect of *fro* mutation on lung development was semidominant in males and recessive in females suggests that gender can modulate the severity of abnormalities caused by nSMase2 deficiency. Notably, the gender effect on emphysema development has also been reported in human patients. Although certain controversy exists, some studies show that among smokers, males are more likely to develop emphysema than females (Camp et al. 2009) and manifest a higher concentration of inter-leukin 6 (de Torres et al. 2011). Further investigation into the role of hormonal or sex chromosome-related mechanisms in *fro* lung disorder manifestation may aid the understanding of gender-specific differences in emphysema development.

In conclusion, our data reveal a previously unknown role of nSMase2 and ceramide in lung development. Whereas earlier data linked ceramide increase to lung impairment, our findings show that a relatively modest decrease in lung ceramide level results in a serious developmental abnormality. Since variations in the physiological level of ceramide seem to be of extreme importance to pulmonary development and function, more studies are necessary for the development of new ceramide-centered therapies for alleviating degenerative lung diseases or inducing lung regeneration.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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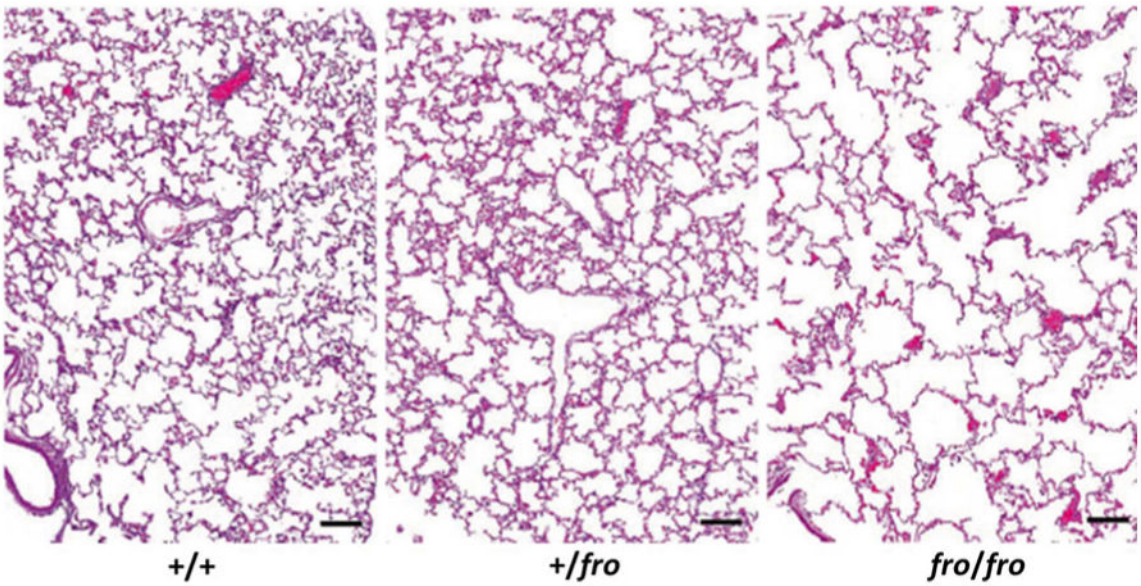

Fig. 1.

nSMase2 deficit causes enlargement of alveoli. Representative lung cross sections from 6-week-old nSMase2-deficient (*fro*/*fro*), heterozygous (+/*fro*), and wild-type (+/+) mice stained with hematoxylin and eosin. *Scale bars* = 100 μm

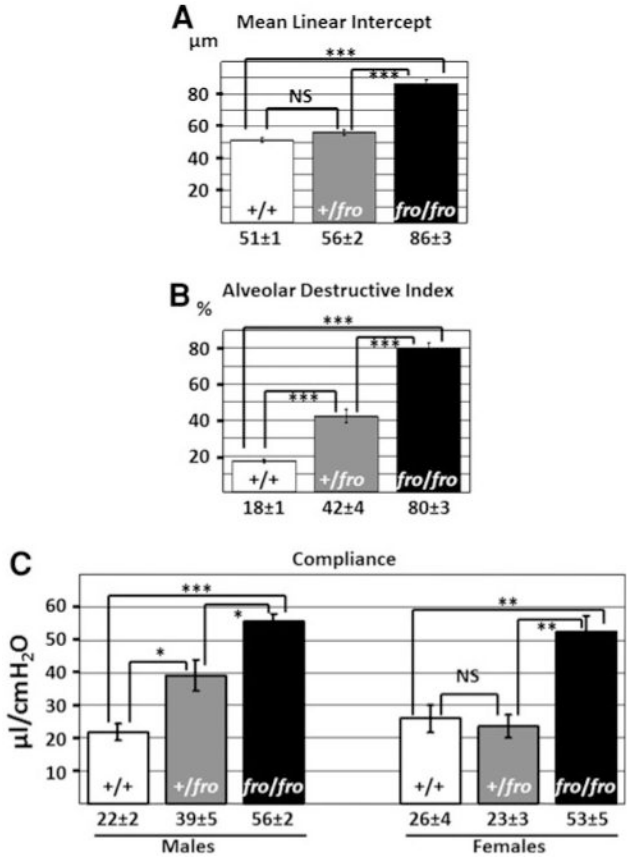

Fig. 2.

Lung phenotype observed in *fro* mice is consistent with emphysema manifestation. The mean linear intercept (**a**) and alveolar destructive index (**b**) are presented as mean ± SEM. **c** Respiratory compliance of 10-week-old +/+, +/*fro*, and *fro*/*fro* littermates from the B × H cross assessed with FlexiVent apparatus is presented as mean ± SEM. The data were analyzed with ANOVA and Tukey post-hoc. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

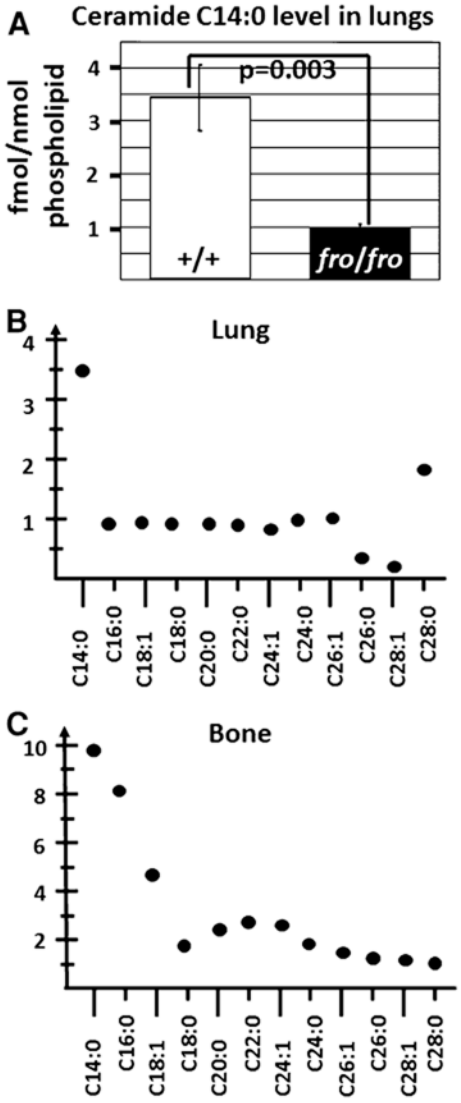

Fig. 3.

nSMase2 deficit affects ceramide levels. The levels of ceramide subspecies in lungs of 10-week-old mice (**a, b**) and calvaria of newborn mice (**c**) were assessed with LC–MS/MS. **a** Ceramide C14:0 level is presented as mean ± SEM; *p* value from *t* test is indicated. **b, c** The ratio of ceramide content between unaffected mice [+/+ lungs (**b**) and +/*fro* bone (**c**)] and mutant mice (*fro*/*fro*)

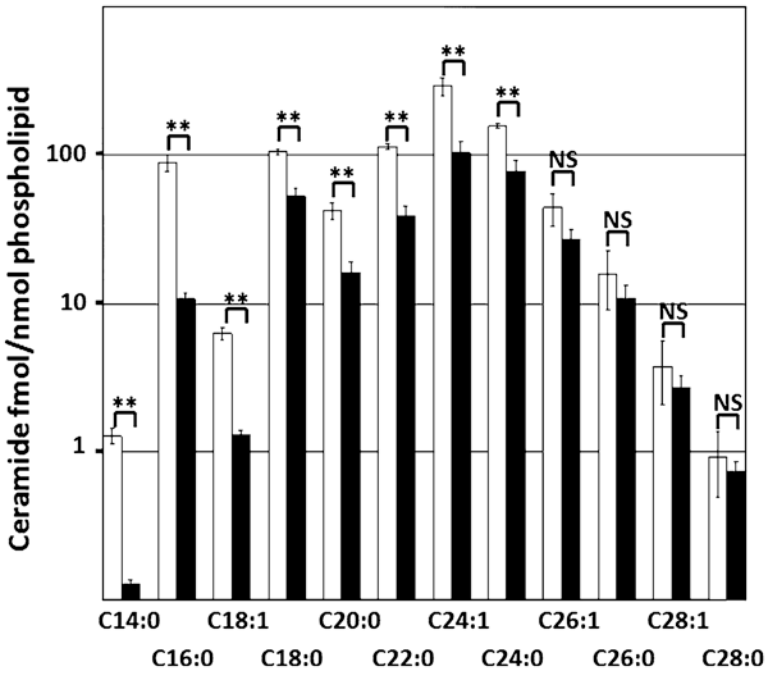

Fig. 4.

nSMase2 deficit affects ceramide content in calvaria. Calvaria were chosen for analysis as these bones contain only osteoblasts, the bone-forming cells. Calvaria was analyzed in *fro*/*fro* newborn mice because skeletal dysplasia was the most obvious at birth and tended to fade away in older mice. Comparisons were done between *fro*/*fro* (*black bars*) and +/*fro* (*open bars*) mice as +/*fro* mice do not manifest skeletal phenotype. Levels of ceramide subspecies were assessed with LC–MS/MS and presented as mean ± SEM; results of *t* test analysis are shown as $^{**}p < 0.01$ and NS = $p > 0.05$

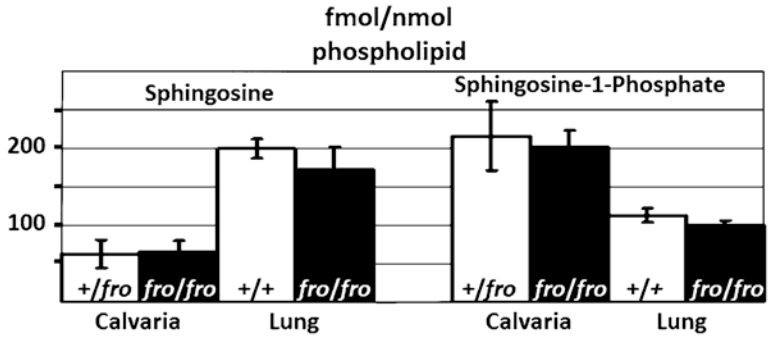

Fig. 5.

nSMase2 deficit does not affect sphingosine or sphingosine-1-phosphate levels. Sphingosine and sphingosine-1-phosphate levels in *fro*/*fro* tissues were compared against the sphingolipid levels in unaffected tissues (+/*fro* for calvaria and +/+ for lung) using *t* test

Table 1

Survival rate of *fro/fro* mice at weaning

FRA/Pas	B strain	H strain	B × H hybrid
10 % (Sillence et al. 1993)	35 % (Khavandgar et al. 2011)	57 % (*n* = 14)	88 % (*n* = 17)