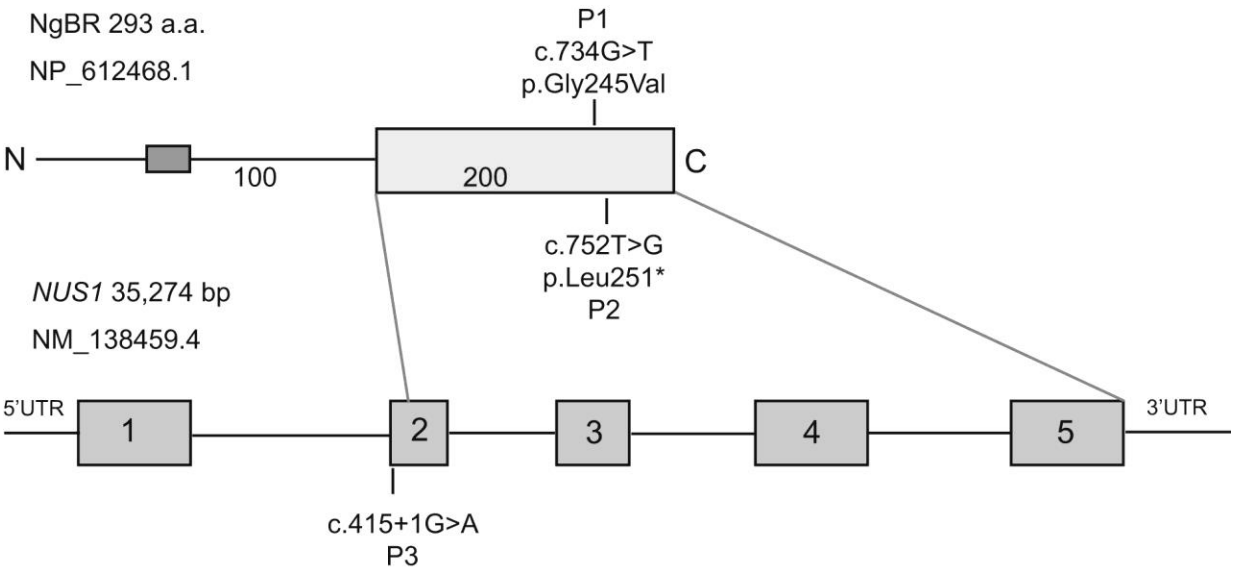
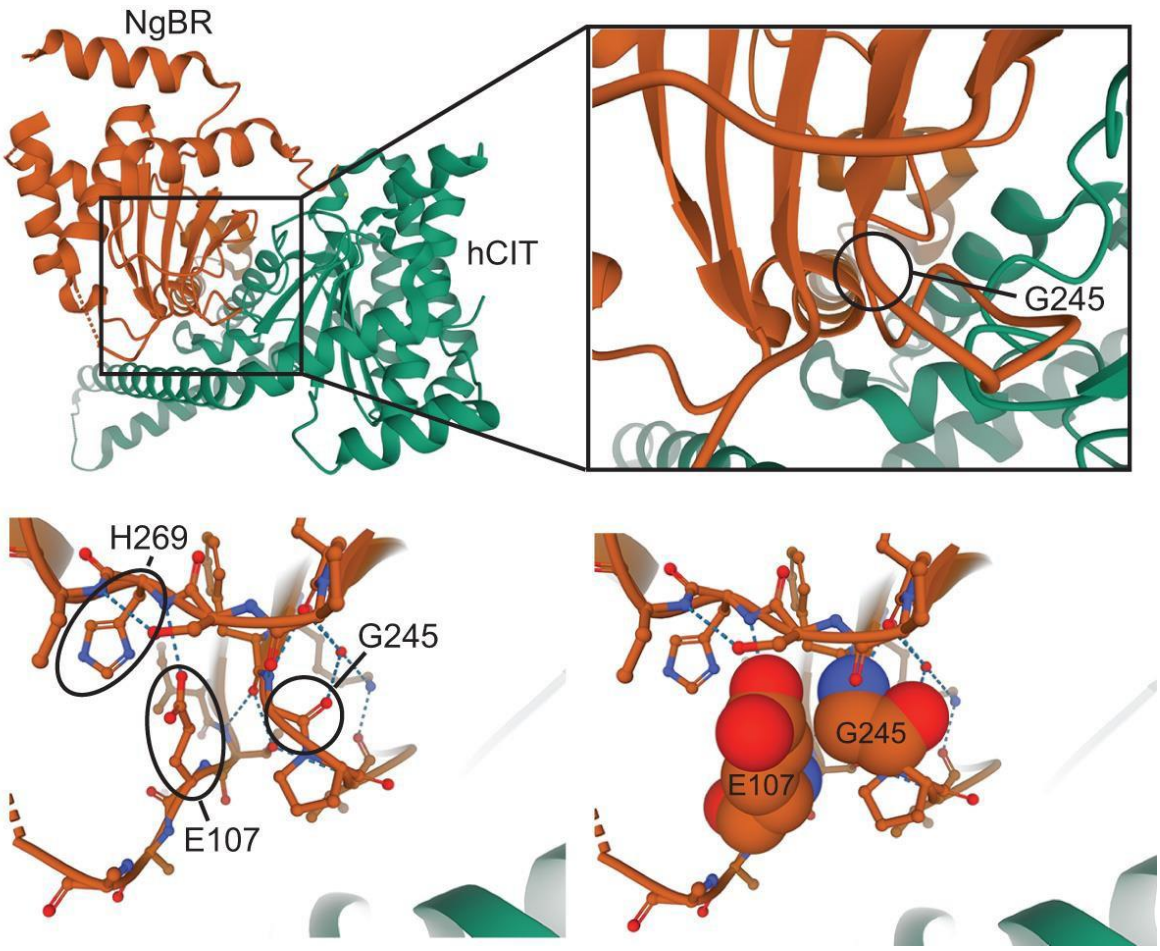
Supplemental Figure 1



Schematic overview of NgBR protein and *NUS1* gene structure

Supplemental Figure 2



Crystal structure of the *NUS1* and *DHDDS* gene products. X-ray crystal structure (PDB: 6W2L) of NgBR and hCIT suggests NgBR with p.Gly245Val variant likely destabilizes the interactions among the three ß-sheets that converge near that residue. Gly245 is proximal to Glu107 and His269 that share an ionic interaction and hydrogen bond between the side chains and backbone, respectively. Alteration of Gly245 residue to Val245 in NgBR is predicted to push the Gly107 residue away, disrupting multiple interactions with its neighboring residues. Destabilization of the three ß-sheets would be anticipated, either impairing the folding of p.Gly245Val variant of NgBR or making the protein more vulnerable to proteolysis. The destabilization of this region might also alter the association of NgBR with the cis-isoprenyltransferase subunit (hCIT). Hydrogen bonds are shown as blue dotted line and ionic bond is not shown.

Supplemental Table 1- Clinical features in the patients within this study

Features	Patient 1	Patient 2	Patient 3
Gender	Female	Female	Male
Age at evaluation	12 years	30 years	3 years
NUS1 variant	c.734G>T; p.Gly245Val	c.752T>G p.Leu251*	c.415+1G>A
Inheritance	De novo	De novo	De novo
Speech delay	Mild	Moderate	Moderate-severe
Motor delay	No	No	Mild-moderate
Intellectual disability	Borderline	Moderate	Unknown
Seizures	Generalized tonic-clonic; absence	Absence, grand mal	Generalized tonic-clonic (febrile); myoclonic; complex partial
Age at seizure onset	2 years	4 years	2years
Age at tremor onset	3 years	22 years	2 years
Ataxia	Mild	Slight	Uncertain

Supplemental Table 2 - Technical details of exome sequencing

	Individual 1	Individual 2	Individual 3
Sequencing laboratory	Greenwood Genetic Center, USA	GeneDx, USA	GeneDx, USA
Sequencing type	Trio exome sequencing (Sanger sequencing of *NUS1* in proband and parents)	Trio exome sequencing (Sanger sequencing of *NUS1* in proband and parents)	Trio exome sequencing (Sanger sequencing of *NUS1* in proband and parents)
Capture and library construction	Agilent SureSelect Clinical Research Exome	Proprietary capture (GeneDx)	Proprietary capture (GeneDx)
Sequencing platform	Illumina NextSeq500	Illumina NovaSeq	Illumina NovaSeq
Average depth of targeted bases	~207X	~88X	~154X
Percentage of bases covered >10x	~97%	~98.4	~98.9%

Standard laboratory procedures were utilized for DNA isolation, library preparation, sequencing, and variant filtration. The *NUS1* transcript used in this study was NM_138459.4.

Supplemental Table 3 – in silico prediction scores of variants

Software	Variant & Score	Classification comment
PolyPhen2 (HumVar score)	c.734G>T (p.Gly245Val) : 1 c.752T>G (p.Leu251*): c.415+1G>A:	Damaging N/A N/A
PROVEAN	c.734G>T (p.Gly245Val) : -8.57 c.752T>G (p.Leu251*): c.415+1G>A:	Deleterious N/A N/A
MutationTaster	c.734G>T (p.Gly245Val) : 1 c.752T>G (p.Leu251*): c.415+1G>A:	Disease causing Disease causing Disease causing
GERP++	c.734G>T (p.Gly245Val) : 5.55 c.752T>G (p.Leu251*):5.55 c.415+1G>A: 4.56	Highly constrained Highly Constrained Constrained
PhastCons100 (vertebrate)	c.734G>T (p.Gly245Val) : 1 c.752T>G (p.Leu251*):1 c.415+1G>A: 1	Conserved Conserved Conserved

Summary of *in silico* predictor algorithms. These variants are registered in the ClinGen allele registry under the following ID numbers: CA365537346 (c.734G>T), CA365536583 (c.415+1G>A), and CA365537384 (c.752T>G).

Materials and Methods

Reagents

4-methylumbellyferyl-β-D-glucopyranoside (M3633) or 4-methylumbellyferyl-β-D-galactopyranoside (M1633), or filipin (F4764) was purchased from Sigma Aldrich. Anti-LAMP2 antibody (H4B4) was obtained from Developmental Studies Hybridoma Bank (DSHB). Anti-ICAM1 antibody (sc-8439) was purchased from Santa Cruz Biotechnology, HRP conjugated β-actin antibody (ab20276) and anti-NgBR antibody (ab168351) were purchased from Abcam. Anti-NPC2 antibody was a gift from Dr. Peter Lobel (CABM, Rutgers University). Anti-HDAC1 antibody was purchased from Proteintech (10197-1-AP). Protease inhibitor cocktail (88666) and Lipofectamine^{TM} 2000 were purchased from Thermo Scientific. Nogo B receptor (*NUS1*) (NM_138459) Human ORF Clone (RC224928) was purchased from Origene.

Zebrafish Strains and Husbandry

Animals were maintained according to standard protocols. Wild type zebrafish (TL, AB) strains were obtained from the Zebrafish International Resource Center (ZIRC, Eugene, OR). The hsp701:Lamp1-RFP transgenic line was provided by Dr. Michel Bagnat (Duke University) [1]. Embryonic staging was performed according to established criteria [2]. In some cases 0.003% 1-phenyl 2-thiourea (PTU) was added to block pigmentation.

Cell culture

Skin fibroblasts (WT, P1 and P2) and HeLa cells were cultured in DMEM media containing 10% fetal bovine serum (FBS, Benchmark) and penicillin (100 IU/mL)/streptomycin (100 µg/mL, Media Tech) in a 5% CO_2 atmosphere, 37^{o}C humid incubator.

Western Blot Analyses

For cells: Cells grown at 80~90% confluent were lysed on plate by scraping in RIPA lysis buffer containing 50 mM Tris-HCl buffer pH 8.0, 150 mM NaCl, 1.0 % NP-40, 0.1 % SDS, 0.5 % sodium deoxycholate, and protease inhibitor cocktail and collected in an Eppendorf tubes on ice. After vigorous vortexing for 20 seconds, the tube was incubated on ice for 30 min before centrifugation at 20,000 g for 10 min. The supernatant was saved and protein concentration determined using the BCA Protein Assay Kit (Thermo Fisher Scientific). Lysates were resolved by SDS-PAGE and immunoblotting performed using anti-LAMP2 antibody (1:2,000), anti-ICAM1 antibody (1:500), anti-NgBR antibody (1:1,000), anti-NPC2 antibody (1:2,000), anti-HDAC1 antibody (1:2,000), followed by secondary antibody incubation (typically anti-mouse-HRP or anti-rabbit-HRP, 1hr, room temperature). HRP-conjugated anti-β actin antibody was typically used at a 1:10,000 dilution. Immunoblot images were obtained using a ChemiDoc™ imager (BioRad).

Filipin Staining

For cells: Cells were plated on a gelatin coated cover slip in 12 well dish at 40~50 % confluence. After 1 day, cells were washed two times with DPBS (containing Ca^{2+} and Mg^{2+} ion) and then fixed for 1hr in 3.7 % formaldehyde in DPBS. The resulting fixed cells were washed 3 times for 5 min with DPBS and then treated with 0.05 mg/mL of filipin in DPBS containing 10 % FBS for 1 h. The resulting cells were washed 3 times for 10 min with DPBS. Stained cells were imaged with a 60x OIL immersion objective (N.A.1.4) on an Olympus FV3000 laser scanning confocal microscope. Maximum

intensity projections were generated using the Image J software (NIH). Images were processed using Adobe PhotoShop (CS6).

Glycosidase activity assay

Fibroblast cells (WT, P1 or P2) grown 80~90 % confluent were harvested by trypsinization Cells were washed with DPBS and then collected in Eppendorf tubes. After brief centrifugation and removal of supernatant, the resulting cell pellets were flash frozen in a dry ice bath and kept in -20 °C until being analyzed. For analysis, cell pellets in the Eppendorf tubes were lysed by sonication with DPBS containing Ca^{2+}, Mg^{2+}, protease inhibitor cocktail, and 0.1 % Triton X-100, followed by incubation on ice for 30 min. After centrifugation at 20,000 g for 10 min, the resulting supernatant was saved and protein concentration was determined by BCA assay. Protein concentration of each lysate was adjusted to 0.5mg/mL for glycosidase activity assay by dilution with the lysis buffer. 4-methylumbellyferyl-β-D-glucopyranoside (14.5 mM in DMSO) or 4-methylumbellyferyl-β-D-galactopyranoside (14.5 mM in DMSO) were diluted to 290 µM with 0.4 M NaOAc buffer (pH 4.5). Glycosidase reactions were done by mixing each lysate (25 µL, 0.5 mg/mL) with one of the 4-MU-glycopyraniside (25 µL, 290 µM) in a 96 well black plate and followed incubation at 37 °C for 1 h. Quenching of reaction was done by adding 200 µL of 0.1 M glycine buffer (pH 10.4). Fluorescence was measured (ex/em = 355 nm/460 nm) with Cytation™ 5 Cell Imaging Multi-Mode Reader (BioTek).

Preparation of WT and mutant NUS1 (p.Gly245Val) expression vector

A pcDNA3.1 clone of human WT-NUS1 DNA was purchased from Origene. The G245V variant was introduced by site directed mutagenesis using the Stratagene System. Primers for mutagenesis were:

Forward – TGATTTAGTATTGAAGTTCGTTCCTGTGGACAGCACATTAG

Reverse - CTAATGTGCTGTCCACAGGAACGAACTTCAATACTAAATCA

Transfection of HeLa cells with WT or mutant (p.Gly245Val) NUS1

70 % confluent HeLa cells in 6 well dish were treated with each DNA of WT-NUS1 (3.0µg), mutant NUS1(G245V, 3.0 µg) or GFP (3.0 µg) as control in pCDNA3.1 (+) Hygro with Lipofectamine 2000 (9 µL) in 1mL of OptiMEM. After 4 hours, media was replaced to normal culture media (DMEM containing 10% FBS). After 24 h, cells were lysed by scraping on plate as described above. The resulting lysate were further analyzed by SDS-PAGE and immunoblotting. For filipin staining the transfected cells at 24 h were replated on a coverslip in 12 well dish and incubated for additional 24 h before staining.

Quantification of total dolichol and polyprenol lipids

Extraction of metabolites and liquid chromatography combined with high-resolution mass spectrometry (LC-HRMS) analyses was performed as we previously described [3]. Briefly, 1 mL cold methanol was added to each sample and three consecutive extractions with 2 mL of hexane followed by pulse-vortex for 1 min and 10 min sonication in an ultrasonic bath. The upper phases were combined, dried under nitrogen and stored at - 80°C until LC-HRMS analysis. For LC-HRMS analysis, samples were resuspended in 100 µL of methanol/acetonitrile/2-propanol (60:25:15, v/v) pulse vortex 5 times and sonicate for 10 min. Analysis of polyisoprenoids was performed on an ionKey/MS system composed of an ACQUITY UPLC M-Class, the ionKey source, and an iKey HSS T3, 100 Å, 1.8 µm (particle size), 150 µm × 100 mm column coupled to a

SYNAPT G2-Si mass spectrometer (Waters Corporation, Milford, MA, USA). Isoprenoid separation was accomplished as described previously [4]. For unlabeled samples, peak identification, alignment, normalization, and statistical treatment of the data was performed using Progenesis QI software (Nonlinear Dynamics, Waters Corporation, Milford, MA, USA). The area of the analyte (metabolite) peak was normalized to the total cell protein. The total levels of polyprenol and dolichol were calculated from at least three independent biological replicates by adding the response of each detected metabolite and is expressed as a percentage of the total levels in the WT.

Real-time quantitative PCR

Total RNA was extracted from cell pellet using GenElute Mammalian Total RNA Miniprep Kit (Sigma) and quantified on the NanoDrop One system (Thermo Scientific). Isolated RNA was subsequently treated with TURBO DNA-free™ Kit (Cat# AM1907, Invitrogen™) and cDNA generated using the qScript cDNA Synthesis Kit (Quanta bio). RT-qPCR assays were performed with forward (GCCATTTGGCAGTGAAGGTG) and reverse (CTTCTGGGCTACTAACTGGCA) primers. All methods were in accordance with MIQE standards [5]. Target gene Cq values were normalized to RPL4 transcript abundance. For analyses of *nus1* WT and variant allele expression, NUS1 transcripts were amplified using primers flanking the missense variant (Forward: GAGCTGCTCAGGACTTTTGC) (Reverse: GGCTGCATATTGACGAAGGG). Products were cloned using the TOPO TA kit and sequenced.

Antisense morpholino injections

Morpholino knockdown of *nus1* was performed and assessed as previously described [6]. Briefly, a previously validated translation-blocking morpholino (5′-

ACACCATCTCATACAGCGAAGCCAT-3′) was purchased from Gene Tools, LLC (Eugene. OR). The degree of morpholino knockdown was assayed by Western blot using the anti-NgBR antibody (see below) following injection of 0.3-1.0µM reagent into 1-cell stage embryos.

Software and statistical analyses: In cases where numerical or quantitative data was generated standard deviations and either a Student's t-test or the Dunnett's test was used to assess statistical significance. Data was processed on GraphPad Prism (Version 7.0a). In cases where staining patterns were assessed visually, representative embryos are shown and the number of animals from multiple experimental samples that resembled those pictured was calculated. Embryo gender is not established until later in development and as such is not a relevant consideration for these studies.

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