Mutations in XPR1 cause primary familial brain calcification associated with altered phosphate export

Andrea Legati1,34, Donatella Giovannini2–5,34, Gaël Nicolas6–8, Uriel López-Sánchez2–5, Beatriz Quintáns9,10, Joao C Jen2,34, Renee L Sears1,33, Eliana Marisa Ramos1, Elizabeth Speriti12,33, Maria-Jesúso Sobrido9,10, Angel Carracedo9,10, Cristina Castro-Fernández9,10, Stéphanie Cubizolle13, Brent I. Fogel12, Cyril Goizet14, Joanna C Jen2, Suppachok Kirdlarp15, Anthony E Lang6,17, Zosia Miedzybrodzka18, Witoon Mitarnun15, Martin Paucar19,20, Henry Paulson21, Jérémie Pariente22,23, Anne-Claire Richard6,7, Naomi S Salins24,25, Sheila A Simpson18, Pasquale Striano25, Per Svenningsson19,20, François Tison13, Vivek K Unni26, Olivier Vanacker27, Marja W Wessels28, Suppachok Wetchaphanphesat15, Michele Yang29, François Boller30, Dominique Camphion6,31, Didier Hannequin26,32, Marc Sitbon6,32, Daniel H Geschwind1,12, Jean-Luc Battini2–5 & Giovanni Coppola12

Primary familial brain calcification (PFBC) is a neurological disease characterized by calcium phosphate deposits in the basal ganglia and other brain regions and has thus far been associated with SLC20A2, PDGFB or PDGFRB mutations. We identified in multiple families with PFBC mutations in XPR1, a gene encoding a retroviral receptor with phosphate export function. These mutations alter phosphate export, implicating XPR1 and phosphate homeostasis in PFBC.

PFBC, also known as idiopathic basal ganglia calcification or Fahr’s disease, is a rare, clinically heterogeneous neurodegenerative disorder1. PFBC symptoms typically occur after the age of 40 years with progressive neuropsychiatric and movement disorders, although some individuals may remain asymptomatic. Clinical features include dystonia, parkinsonism, ataxia, psychosis, dementia, chorea and frontal-subcortical cognitive dysfunction. Bilateral calcifications of the basal ganglia are visualized on computed tomography (CT scans). PFBC is genetically heterogeneous, typically inherited in an autosomal dominant fashion. Causative mutations have been found in SLC20A2 (refs. 2,3), which encodes the phosphate transporter Pit2 (ref. 4), in PDGFRB5, which encodes platelet-derived growth factor receptor β (PDGFRβ), and in PDGFRB6, which encodes the PDGFRβ ligand. Altogether, mutations in these three genes account for 49% of families with PFBC in our cohort7.

We evaluated a North American family of Swedish ancestry with PFBC7 lacking mutations in SLC20A2, PDGFB and PDGFRB. Our analysis included 17 members (9 affected, 3 unaffected and 5 of unknown status; Supplementary Fig. 1a). When assessed, the clinical presentation consisted of dementia, speech impairment (slurred speech, palilalia), chorea and unsteady gait (Supplementary Table 1). CT scans of the brain showed extensive intracranial calcifications in basal ganglia extending to the cerebral cortex or cerebellum.

We performed exome sequencing in four affected family members (III-4, III-5, III-9 and IV-6) and in one unaffected family member (II-5). We identified a total of 83,848 variants in these 5 samples (Supplementary Fig. 1b). First, we filtered out variants present in dbSNP138 and present with frequency >1% in the 1000 Genomes Project and Exome Variant Server databases. We then focused on missense, splice-site, stop-gain or stop-loss, and frameshift variants. Seven variants (Supplementary Table 2) segregated with the disease in these 5 samples and were assessed in all 17 available family members using Sanger sequencing. Only one variant was present in all affected individuals and absent from unaffected family members (maximum logarithm of odds (LOD) score of 3.6):

Received 2 March; accepted 6 April; published online 4 May 2015; doi:10.1038/ng.3289

1Department of Psychiatry, David Geffen School of Medicine, University of California, Los Angeles, Los Angeles, California, USA. 2Institut de Génétique Moléculaire de Montpellier, CNRS UMR 5535, Montpellier, France. 3Laboratory of Excellence GR-Ex, Paris, France. 4Laboratory of Excellence EpiGenMed, Montpellier, France. 5INSERM U1079, Institute for Research and Innovation in Biomedicine (IRIB), University of Rouen, Rouen, France. 6Department of Genetics, Center for Genome Sciences and Systems Biology, Washington University School of Medicine, St. Louis, Missouri, USA. 7Centre National de Référence pour les Malades Alzheimer Jeunes (CNR-MAJ), Rouen University Hospital, Rouen, France. 8Department of Genetics, Rouen University Hospital, Rouen, France. 9Fundación Pública Galega de Medicina Xenómica, Servizo Galego de Saúde (SERGAS), Instituto de Investigación Sanitaria (IDIS, Hospital Clínico Universitario), Santiago de Compostela, Spain. 10Grupo de Medicina Xenómica, Centro de Investigación Biomédica en Red de Enfermedades Raras (CIBERER, Universidad de Santiago de Compostela), Santiago de Compostela, Spain. 11Keizo Asami Laboratory, Federal University of Pernambuco, Recife, Brazil. 12Department of Neurology, David Geffen School of Medicine, University of California, Los Angeles, Los Angeles, California, USA. 13Neurology and Institute for Neurodegenerative Diseases, Bordeaux University Hospital and Bordeaux University, Bordeaux, France. 14Service de Génétique Médicale, Bordeaux Hospital University Center, Bordeaux, France. 15Division of Medicine, Buriram Hospital, Buriram, Thailand. 16Morton and Gloria Movement Disorders Clinic, Toronto Western Hospital, Toronto, Ontario, Canada. 17Edmond J. Safra Program in Parkinson’s Disease, Toronto Western Hospital, Toronto, Ontario, Canada. 18Medical Genetics Group, School of Medicine and Dentistry, University of Aberdeen, Aberdeen, UK. 19Translational Neuropharmacology, Clinical Neuroscience, Center for Molecular Medicine, Karolinska Institute, Stockholm, Sweden. 20Department of Neurology, Karolinska University Hospital Huddinge, Stockholm, Sweden. 21Department of Neurology, University of Michigan, Ann Arbor, Michigan, USA. 22INSERM, Imagerie Cérébrale et Handicaps Neurologiques, UMR 825, Pole Neurosciences, Centre Hospitalier Universitaire (CHU) Purpan, Toulouse, France. 23CHU de Toulouse, Université de Toulouse, Toulouse, France. 24Barrow Neurological Institute, Phoenix, Arizona, USA. 25Pediatric Neurology and Muscular Diseases Unit, Department of Neurosciences, Rehabilitation, Ophthalmology, Genetics, and Maternal and Child Health, University of Genoa ‘G. Gaslini’ Institute, Genoa, Italy. 26Department of Neurology, Oregon Health and Science University, Portland, Oregon, USA. 27Center for Medical Genetics, Ghent University Hospital, Ghent, Belgium. 28Department of Clinical Genetics, Erasmus Medical Center, Rotterdam, the Netherlands. 29Department of Pediatrics, Children’s Hospital Colorado and University of Colorado Denver, Aurora, Colorado, USA. 30Department of Neurology, George Washington University Medical School, Washington, DC, USA. 31Department of Research, Rowan University, Soledette-lès-Rouen, France. 32Department of Neurology, Rouen University Hospital, Rouen, France. 33Present addresses: Department of Genetics, Center for Genome Sciences and Systems Biology, Washington University School of Medicine, St. Louis, Missouri, USA (R.L.S.) and Kaiser Permanente Southern California Permanente Medical Group, Regional Reference Laboratories, Genetics Laboratory, Los Angeles, California, USA (E.S.). 34These authors contributed equally to this work. Correspondence should be addressed to J.-L.B. (jean-luc.battini@igmie.cnrs.fr) or G.C. (gcoppola@uc.edu).
Figure 1  Localization of the identified variants in the XPR1 protein and effect of p.Leu145Pro on protein expression and function. (a) Schematic of the XPR1 protein and the variants identified in this study. The SPX domain is in orange. (b) Efflux of inorganic $^{32}$P (P) in HEK293T cells transfected with control siRNA to luciferase (siLUC; lane 1), siRNA to XPR1 (siXPR1) alone (lane 2), or siRNA to XPR1 in combination with vector expressing HA-tagged wild-type (lane 3) or Leu145Pro (lane 4) human XPR1. Results are means ± s.e.m. in a representative experiment ($n = 3$). ** $P \leq 0.01$, *** $P \leq 0.001$ as tested against the luciferase-targeting siRNA control. (c) Immunoblot of HA-tagged XPR1 expression in cell lysates with an antibody to HA (top). β-actin was used as loading control (bottom). (d) Cell surface detection of XPR1 on HEK293T cells transfected as in b. Nonspecific staining with the secondary IgG antibody (filled histogram) and specific binding with the X-MLV receptor-binding domain (RBD) (unfilled histogram) are represented. Numbers indicate the specific mean fluorescence intensity of a representative experiment ($n = 3$). (e) Efflux of $^{32}$P in PBMCs collected from two control healthy donors (circles or squares) and two affected patients harboring the p.Leu145Pro alteration (triangles pointing up or down). Bars represent means ± s.e.m.; *** $P \leq 0.001$.

a c.434T>C transition in the mammalian XPR1 gene (NM_0047363.3; encoding xenotropic and polytropic retrovirus receptor 1), predicted to result in a deleterious p.Leu145Pro alteration at a highly conserved residue within the SPX domain shared by SYG1/PHO81/XPR1 proteins$^5$ (Fig. 1a) and absent from repositories of sequence variation, including the Exome Aggregation Consortium (ExAC) database.

Further sequencing of XPR1 in 86 additional sporadic and familial cases identified the same p.Leu145Pro variant in two affected individuals from a family of French descent and five additional missense variants. Pedigree analyses and the segregation patterns of variants surrounding XPR1 suggested that the two families carrying the p.Leu145Pro variant were not related (Online Methods). Three other variants, p.Ser136Asn, p.Leu140Pro and p.Leu218Ser (Fig. 1a, Table 1, Supplementary Fig. 2 and Supplementary Table 3), all located in the SPX domain or in its vicinity and predicted to be damaging, were absent from variation repositories. The p.Lys53Arg variant, also absent from variation databases, is predicted to be non-damaging. The p.Ile575Val variant has a minor allele frequency of 0.068% in ExAC, interchanges two hydrophobic residues in a transmembrane domain and is likely to represent a rare polymorphism. None of these variants were found in two in-house series (126 French and 161 North American controls of European ancestry screened with exome sequencing and targeted resequencing, respectively).

XPR1 is a cell surface multipass membrane protein initially identified as the mammalian receptor for xenotropic murine leukemia viruses (X-MLV)$^9,10$. It contains an N-terminal SPX domain (Fig. 1a) that is also found in several yeast and plant proteins involved in phosphate homeostasis$^{11,12}$. We have recently shown that XPR1 mediates phosphate export$^1$, a function that is highly conserved across evolution$^{13,14}$. We tested all the newly identified XPR1 variants in a complementation assay for phosphate efflux in human cells$^9$, wherein phosphate efflux decreased after the introduction of XPR1-targeting small interfering RNA (siRNA) but was restored by the expression of wild-type or mutant XPR1 (Fig. 1b). We found that Leu145Pro mutant XPR1 neither reestablished phosphate efflux nor served as the receptor for X-MLV during infection (Fig. 1b and Supplementary Fig. 3). Consistent with this observation, phosphate efflux was also impaired in the peripheral blood mononuclear cells (PBMCs) isolated from the two patients harboring the p.Leu145Pro alteration that we tested (Fig. 1c). This substitution affected cell surface exposure of XPR1 on HEK293T cells, as monitored by flow cytometry with an XPR1 ligand (XRBD) derived from the X-MLV envelope glycoprotein$^9$, although the expression levels of Leu145Pro XPR1 remained substantial (Fig. 1c,d). Remarkably, expression of Leu145Pro XPR1 specifically decreased the phosphate efflux of endogenous XPR1 (Fig. 1b), whereas we observed no effect on the expression of the phosphate importers PiT1 and PiT2 or on phosphate uptake (Supplementary Fig. 3), supporting a trans dominant-negative effect of the Leu145Pro mutant on wild-type XPR1. In contrast, the four other mutants were present at the plasma membrane and served as potent retrolaviral receptors (Supplementary Fig. 4 and Supplementary Table 4). The three variants p.Ser136Asn, p.Leu140Pro and p.Leu218Ser all affected XPR1 function.

Table 1 Rare XPR1 variants identified in the index proband and a follow-up cohort of 86 index cases

| Genomic position | cDNA   | Protein           | PolyPhen-2 function prediction | SIFT function prediction | MutationTaster function prediction | dbSNP ID | 1000 Genomes frequency | NHLBI EVS frequency | ExAC allelic frequency | GERP      |
|------------------|--------|-------------------|-------------------------------|--------------------------|-----------------------------------|----------|------------------------|---------------------|-----------------------|-----------|
| Chr. 1: 180,756,925 | c.158A>G | p.Lys53Arg        | Benign                        | Tolerated                | Disease causing                   | –        | Absent                 | Absent              | Absent                | 5         |
| Chr. 1: 180,772,707 | c.407G>A | p.Ser136Asn       | Probably damaging              | Damaging                | Disease causing                   | –        | Absent                 | Absent              | Absent                | 5.93      |
| Chr. 1: 180,772,719 | c.419T>C | p.Leu140Pro       | Probably damaging              | Damaging                | Disease causing                   | –        | Absent                 | Absent              | Absent                | 5.93      |
| Chr. 1: 180,772,734 | c.434T>C | p.Leu145Pro       | Probably damaging              | Damaging                | Disease causing                   | –        | Absent                 | Absent              | Absent                | 5.93      |
| Chr. 1: 180,775,665 | c.653T>C | p.Leu218Ser       | Probably damaging              | Damaging                | Disease causing                   | –        | Absent                 | Absent              | Absent                | 5.15      |
| Chr. 1: 180,842,993 | c.1723A>G | p.Ile575Val       | Benign                        | Tolerated                | Disease causing                   | rs147941113 | 0.040%      | 0.054%              | 83/121,330 (0.068%) | 2.09      |

NHLBI EVS, National Heart, Lung, and Blood Institute Exome Variant Server; ExAC, Exome Aggregation Consortium database (accessed February 2015). Variants in XPR1 currently explain about 5.5% of cases in the French cohort and 2.5% of cases in the North American cohort. Thus, XPR1 mutations are less common than SLC20A2 and PDGFRA mutations but are more common than PDGFB mutations.
activity to various degrees, despite normal expression of the three phosphate transporters PiT1, PiT2 and XPR1 (Supplementary Fig. 4). Expression of XPR1 with the predicted non-damaging p.Lys53Arg substitution restored phosphate efflux to wild-type levels, making the causative role of this variant in PFBc uncertain.

After SLC20A2, XPR1 is the second PFBC-associated gene to encode a phosphate transporter. The presence of PFBC-causing mutations in SLC20A2 (PiT2) suggests that inhibition of phosphate uptake may lead to deposition of calcium phosphate in the vascular extracellular matrix. In contrast, inhibition of phosphate export, associated with the XPR1 mutations, is expected to increase the intracellular phosphate concentration. Therefore, XPR1 mutation-mediated calcium phosphate precipitation is likely to occur intracellularly, as is characteristic of osteoblasts during bone mineralization.

Phosphate import and export are interdependent functions that regulate intracellular phosphate homeostasis. However, it is not yet known whether XPR1 and PiT2 coregulate each other and/or are regulated by common factors. The PFBC-associated PDGFRB and PDGFB proteins, known to modulate phosphate transport, may also function as regulators of XPR1 and PiT2 levels in the brain.

Phosphate transport might also alter XPR1-dependent phosphate export in areas of the brain where phosphate transporters are expressed.

Five of the six XPR1 variants described in this study are clustered in the cytoplasmic N-terminal portion of XPR1 (Fig. 1a), and four lie in the SPX domain, which appears to be dispensable for intrinsic phosphate export activity.

A role of the SPX domain in protein trafficking may explain the retention of the Leu145Pro XPR1 mutant in cells.

Interestingly, the corresponding substitution disrupts a dileucine motif known to be involved in protein endocytosis and plasma membrane trafficking that is highly conserved across evolution (Supplementary Fig. 5). The other mutants with impaired phosphate efflux activity were efficiently localized to the plasma membrane, suggesting function(s) other than trafficking for the SPX domain. Notably, the SPX domain was shown to modulate intracellular cyclic AMP (cAMP) levels, presumably through interactions with G protein β subunits, although its role in phosphate regulation remains to be elucidated.

XPR1 is actively expressed in neuronal stem cells and human brain, and Xpr1 is expressed in several regions of the mouse brain (Allen Mouse Brain Atlas database; see URLs). Direct involvement of XPR1 in phosphate export and its expression pattern in brain support its role in cerebral phosphate homeostasis. These results identify XPR1 as a new gene associated with PFBC and provide new insights into the role of phosphate homeostasis in PFBC etiology.

ACKNOWLEDGMENTS

We acknowledge and thank all of the participants and families for their valuable contributions to our study; our clinical and laboratory members.

J. DeVogel and the University of California Los Angeles (UCLA) Neuroscience Genomics Core, J. Toucham and J. Laval for their assistance and constant support; and the National Heart, Lung, and Blood Institute (NHLBI) GO Exome Sequencing Project and its ongoing studies, which produced and provided exome variant calls for comparison: the Lung GO Sequencing Project (HL-102923), the Women’s Health Initiative (WHI) Sequencing Project (HL-102924), the Broad GO Sequencing Project (HL-102925), the Seattle GO Sequencing Project (HL-102926) and the Heart GO Sequencing Project (HL-103010). We are also indebted to the Montpellier Rio Imaging (MRI) platform for flow cytometry experiments. This work was funded by the US National Institutes of Health/National Institute of Neurological Disorders and Stroke (R01NS040752 to D.H.G.), by Association Française contre les Myopathies (AFM) and Ligue Nationale contre le Cancer (Comité de l’Hérault; to J.-L.B.), and by Fondation pour la Recherche Médicale (FRM) and a FEDER European Union Languedoc-Roussillon grant (Transportome; to M.S.). We also acknowledge the support of the National Institute of Neurological Disorders and Stroke Informatics Center for Neurogenetics and Neurogenomics (PNSNS02691). D.G. was supported by FRM, Institut National du Cancer (INCA) and Labex GR-Ex (ANR-11-LABX-0051) fellowships, and U.L.-S. was supported by a Labex EpiGenMed (ANR-10-LABX-0212-01) fellowship. Labex is funded by the ‘Investissements d’Avenir’ of the French National Research Agency. J.-L.B. and M.S. were supported by INSERM. M.-J.S. and B.Q. are supported by the Fondo de Investigación Sanitaria, grant PI12/00742; INNOPHARMA project MINECO-USEC; and FEDER funds. M.-J.S. and B.Q. hold research contracts from the Institute of Health Carlos III–SERGAS. J.R.M.O. acknowledges funding from FACEPE (APQ 1831-4.01-12) and CNpq (475756/2013-7; 480255/2013-3; B.R.F. is funded by the US National Institutes of Health grants K08HM086297 (National Institute of Mental Health) and R01NS8029409 (National Institute of Neurological Disorders and Stroke).

G.N., A.-C.R., D.H. and D.C. are supported by INSERM, the University Hospital of Rouen and the French CNR-MAI.

AUTHOR CONTRIBUTIONS

M.S., D.H.G., J.-L.B. and G.C. designed the study. A.L., D.G., G.N. and U.L.-S. designed and performed experiments. A.L., D.G., M.P., A.M., N.S., S.C.-F., S.C., Á.C., M.-J.S., Á.C., B.L.F., C.G., J.C.J., S.K., A.E.L., Z.M., W.M., M.P., H.P., J.R.M.O., E.S., M.-J.S., T.H., G.N. and B.Q. contributed to data collection and analysis. D.G. was supported by FRM, Institut National du Cancer (INCA) and Labex GR-Ex (ANR-11-LABX-0051) fellowships, and U.L.-S. was supported by a Labex EpiGenMed (ANR-10-LABX-0212-01) fellowship. Labex is funded by the ‘Investissements d’Avenir’ of the French National Research Agency. J.-L.B. and M.S. were supported by INSERM. M.-J.S. and B.Q. are supported by the Fondo de Investigación Sanitaria, grant PI12/00742; INNOPHARMA project MINECO-USEC; and FEDER funds. M.-J.S. and B.Q. hold research contracts from the Institute of Health Carlos III–SERGAS. J.R.M.O. acknowledges funding from FACEPE (APQ 1831-4.01-12) and CNpq (475756/2013-7; 480255/2013-3; B.R.F. is funded by the US National Institutes of Health grants K08HM086297 (National Institute of Mental Health) and R01NS8029409 (National Institute of Neurological Disorders and Stroke).

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

URLs. Allen Mouse Brain Atlas database, http://mousbrain-map.org/; TOP2 transmembrane protein display software, http://www.sacs.ucsf.edu/TOP2/; dbSNP database, http://www.ncbi.nlm.nih.gov/SNP/; 1000 Genomes Project, http://www.1000genomes.org/; Exome Variant Server, http://evs.gs.washington.edu/EVS/; Exome Aggregation Consortium (ExAC), http://exac.broadinstitute.org/.

BRIEF COMMUNICATIONS

© 2015 Nature America, Inc. All rights reserved.

ACTIVITY

nature genetics | volume 47 | number 6 | june 2015

METHODOLOGY

Methods and any associated references are available in the online version of the paper.

npg © 2015 Nature America, Inc. All rights reserved.
ONLINE METHODS

Patients. Patient enrollment. Seventeen individuals from one family were enrolled in this study through the University of California Los Angeles (UCLA) Medical Center after providing informed consent. The clinical features of this family were described in a previous report. An additional 86 cases self-defined as Caucasian with a clinical diagnosis of idiopathic brain calcification (either sporadic or with family history of brain calcifications) and with no mutations in SLC20A2, PDGFRA and PDGFRB were recruited at collaborating institutions. Some of these individuals were included in previous clinical or genetic studies (47 of them were recruited using inclusion criteria as previously described). The study was approved by the UCLA Institutional Review Board. Patients gave informed, written consent for genetic analyses. Medical history was obtained and neurological examinations were performed for all probands and additional family members. Serum calcium and parathormone levels were assayed to exclude calcium dysregulation and other metabolic disorders that would cause brain calcifications unrelated to PFBC.

Neuroimaging. Head CT scans were performed as part of the diagnostic workup or were reviewed for the presence of calcifications or other brain abnormalities. Subjects with CT scans positive for calcifications were given an affected disease status, whereas patients with a negative CT scan who were >50 years of age and remained asymptomatic until their death were assigned an unaffected disease status. Subjects whose CT scans were negative but who were under the age of 50 years and subjects whose CT scan results were not available were classified as having unknown disease status.

Exome analysis. Genomic DNA was extracted from peripheral blood and fragmented by sonication, using the Covaris acoustic disruptor (E210, Covaris) to achieve an average fragment size of 200 bp. We used 1 µg of DNA from each family member for PCR to identify single nucleotide polymorphisms (SNPs) in the proband of the French family who also carried the c.434T>C (p.Leu145Pro) variant. We obtained data back to the 1750s in one branch of the French family (mother of the proband, known to be affected) and could not find any common ancestry with the North American family of Swedish descent. Furthermore, we compared exome sequencing data from all affected and unaffected individuals from the North American family in XPR1 and the surrounding genes on chromosome 1 and identified three variants (one downstream and two upstream of XPR1) that were present in the affected individual and absent in the unaffected individual: rs7536561 (529 kb from the XPR1 variant), rs79485039 (113 kb from the XPR1 variant) and rs3747958 (125 kb from the XPR1 variant). We sequenced these SNPs in the proband of the French family who also carried the c.434T>C (p.Leu145Pro) mutation and found reference sequences for all of them. This analysis supports the hypothesis that the mutation occurred on different haplotypes in the two families.

Cells. HEK293T (human embryonic kidney) and CHO hamster cells were cultured in DMEM supplemented with 10% FBS (Invitrogen) and non-essential amino acids in a 5% CO2 incubator at 37 °C under humid atmosphere. For phosphate-free incubations, cells were grown in phosphate-free DMEM (ThermoFisher) with 10% charcoal-stripped FBS (Biological Industries). Cell lines in this study were free from mycoplasma contamination, as determined by monthly testing.

PBMCs were isolated from peripheral blood collected in the presence of heparin from both healthy donors and patients who had signed informed consent for research purposes. Blood samples were subjected to density gradient separation on Histopaque-1077 (1:1 ratio; Sigma-Aldrich) and centrifuged 24 h after blood collection. After centrifugation, the PBMC layer was collected and washed in DMEM before evaluation of phosphate efflux.

Plasmids and siRNAs. The mutations encoding p.Leu145Pro, p.Lys53Arg, p.Ser164Asn, p.Leu140Pro and p.Leu218Ser were generated by site-directed mutagenesis using recombinant PCR (details are available upon request). Sequences encoding HA-tagged versions of human XPR1 were introduced into both the pCHIX expression vector and the pLXSN retroviral vector. The siRNA sequences (Integrated DNA Technologies) targeting the 3′ UTR of human XPR1 were as follows: 5′-GGAAUUCAGCAGCAUCUCU-3′ and 5′-GCAACUUCACGCAUGUAUATT-3′. siRNA directed against the firefly luciferase gene was used as a control. HEK293T cells grown on six-well plates coated with poly(o-lysine) were transfected with 50 pmol of siRNA per well using the calcium phosphate method.

Phosphate flux in human cells. Phosphate uptake and efflux from HEK293T cell monolayers, transfected 2 d before assays, were measured as previously described. Briefly, for uptake measurements, cells were incubated for
were detected with horseradish peroxidase (HRP)-conjugated anti-mouse or anti-rat antibodies (1:5,000 dilution; SouthernBiotech) and visualized with the Pierce ECL western blotting substrate (Thermo Scientific), according to the manufacturer’s protocol.

Immunoblotting. Whole-cell extracts (15 μg) were separated by 12% SDS-PAGE under reducing conditions, transferred to PVDF membranes and probed with antibodies against HA (3F10, Roche Applied Science; 1:5,000 dilution) or β-actin (A5441, Sigma-Aldrich; 1:5,000 dilution). Proteins of interest were detected with horseradish peroxidase (HRP)-conjugated anti-mouse or anti-rat antibodies (1:5,000 dilution; SouthernBiotech) and visualized with the Pierce ECL western blotting substrate (Thermo Scientific), according to the manufacturer’s protocol.

Flow cytometry. Cell surface expression of phosphate transporters was monitored on HEK293T cells with soluble ligands derived from the RBD of different Env proteins. XRBD, koala retrovirus (KoRV) RBD (KoRBD) and amphotropic-MLV RBD (ARBD) were used to detect XPR1, PiT1 and PiT2, respectively13,28. Binding assays were carried out as previously described 13. Briefly, 5 × 10^5 cells were resuspended in 200 μl of PBA (PBS with 2% FBS and 0.1% sodium azide) containing the proper RBD, incubated for 30 min at 37 °C, washed twice with PBA and incubated for 20 min at 4 °C with Alexa Fluor 488–conjugated anti-mouse IgG1 antibodies (1:500 dilution; Invitrogen). Cells were immediately analyzed on a FACSCalibur instrument (Becton Dickinson), and data analysis was performed using FlowJo software.

Virus production. LAPSN viral vectors were produced from 2 × 10^6 HEK293T cells in 10-cm dishes cotransfected using the calcium phosphate method with an MLV-based LAPSN retroviral vector carrying the alkaline phosphatase reporter gene29 (10 μg), the MLV Gag-Pol expression vector (pC57GPBEB; 5 μg20, and expression vectors for either vesicular stomatitis virus glycoprotein (VSV-G) or the X-MLV envelope (Env) glycoprotein (5 μg). Virion-containing media were collected 2 d later, filtered through a 0.45-μm (pore size) filter and stored at −80 °C before use. LXSN viral vectors carrying wild-type or mutant XPR1 were produced under the same conditions, except that the LAPSN retroviral vector was replaced by the various XPR1 LXSN vectors.

Viral infection and G418 selection. CHO cells stably expressing XPR1 constructs were generated by transducing CHO cells with the pL(XPR1)SN, pL(XPR1K53R)SN, pL(XPR1S136N)SN, pL(XPR1L140P)SN, pL(XPR1L145P)SN and pL(XPR1L218S)SN vectors or with empty pLXSN vector and selecting cells the next day with medium containing 1.5 mg/ml G418 (active fraction). G418-resistant clones were pooled after 2 weeks of selection before further experiments.

CHO cells (2 × 10^5) stably expressing wild-type XPR1 or the Lys53Arg, Ser136Asn, Leu140Pro, Leu145Pro or Leu218Ser mutants from the MLV-based LXSN retroviral vector were plated in 12-well plates and infected the following day with serial dilutions of replication-defective LAPSN retroviral vector pseudotyped with the X-MLV Env or VSV-G glycoprotein. Cells were stained 2 d later for alkaline phosphatase expression, as previously described 29, and data analysis was performed using FlowJo software.

Statistical analysis. The Student’s t test in GraphPad Prism 5 software was used to calculate P values, and the following convention was used: *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001; ****P ≤ 0.0001.

21. Nicolas, G. et al. Brain 136, 3395–3407 (2013).
22. Adzhubei, I.A. et al. Nat. Methods 7, 248–249 (2010).
23. Ng, P.C. & Henikoff, S. Genome Res. 11, 863–874 (2001).
24. Schwarz, J.M., Rödelsperger, C., Schuelke, M. & Seelow, D. Nat. Methods 7, 575–576 (2010).
25. Cooper, G.M. et al. Genome Res. 15, 901–913 (2005).
26. Manel, N. et al. Cell 115, 449–459 (2003).
27. Miller, A.D. & Rosman, G.J. Biotechniques 7, 980–982 (1989).
28. Petit, V. et al. Lab. Invest. 93, 611–621 (2013).
29. Miller, D.G., Edwards, R.H. & Miller, A.D. Proc. Natl. Acad. Sci. USA 91, 78–82 (1994).
30. Lassaux, A., Sitbon, M. & Battini, J.-L. J. Virol. 79, 6560–6564 (2005).