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NBEAL2 is mutated in Gray Platelet Syndrome and is required for biogenesis of platelet alpha-granules

Meral Gunay-Aygun[1,2,*], **Tzipora C Falik-Zaccai**[3,4,*], **Thierry Vilboux**[1], **Yifat Zivony-Elboum**[3], **Fatma Gumruk**[5], **Mualla Cetin**[5], **Morad Khayat**[3], **Cornelius F Boerkoel**[1], **Nehama Kfir**[3], **Yan Huang**[1], **Dawn Maynard**[1], **Heidi Dorward**[1], **Katherine Berger**[1], **Robert Kleta**[1], **Yair Anikster**[6,7], **Mutlu Arat**[8], **Andrew S Freiberg**[9], **Beate E Kehrel**[10], **Kerstin Jurk**[10], **Pedro Cruz**[11], **Jim C Mullikin**[11], **James G White**[12], **Marjan Huizing**[1], and **William A Gahl**[1,2]

[1]Section on Human Biochemical Genetics, Medical Genetics Branch, National Human Genome Research Institute, National Institutes of Health, Bethesda, MD

[2]Office of Rare Diseases Research, Office of the Director, National Institutes of Health, Bethesda, MD

[3]Institute of Human Genetics, Western Galilee Hospital, Naharia, Israel

[4]Ruth and Bruce Rappaport Faculty of Medicine, Technion, Israel Institute of Technology, Haifa, Israel

[5]Pediatric Hematology Unit, Hacettepe University Children's Hospital, Ankara, Turkey

[6]Metabolic Disease Unit, Edmond and Lily Safra Children's Hospital, Sheba Medical Center, Tel Aviv, Israel

[7]Sackler Medical School, Tel Aviv, Israel

[8]Department of Hematology, Ankara University Faculty of Medicine, Ankara, Turkey

[9]Division of Pediatric Hematology/Oncology, PennState Hershey Children's Hospital, Hershey, PA

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Address correspondence to: Meral Gunay-Aygun, M.D., NHGRI, NIH, 10 Center Drive, Bldg 10, Rm 10C103, Bethesda, MD 20892, 301 594 4181, mgaygun@mail.nih.gov.

*These authors contributed equally.

Authorship Contributions:

M.G.-A. is the principal investigator of clinical trials NCT00069680 (Genetic Analysis of Gray Platelet Syndrome) and NCT00086476 (Investigations of Megakaryocytes from Patients with Abnormal Platelet Vesicles), M.G.-A wrote the manuscript, cultured megakaryocytes, M.G.-A, W.A.G. and T.C.F.-Z., designed and supervised research, M.G.-A, and T.C.F.-Z., and T.V., analyzed clinical and molecular data, T.C.F.-Z., is the principal investigator of the Israeli protocol "Clinical and Genetic Analysis of Gray Platelet Syndrome," W.A.G. is the principal investigator of clinical trial NCT00369421 (Diagnosis and Treatment of Patients With Inborn Errors of Metabolism), and accountable investigator of clinical trial NCT00069680 (Genetic Analysis of Gray Platelet Syndrome), M.G.-A., T.V., T.C.F.-Z., C.J.M., and M.H. supervised DNA sequencing, M.G.-A., T.V., Y. Z.-E., F.G., M.C., M.K., N.B., N.K., Y.H., K.B., R.K., and P.C., performed DNA sequencing, M.G.-A. T.C.F.-Z., F.G., M.C., M.K., N.K., R.K., Y.A., M.A., A.S.F., B.E.K., K.J., and J.G.W., recruited patients and provided clinical data, J.G.W., performed electron microscopy, D.M., performed proteomic analysis, D.H., cultured fibroblasts, T.C.F.-Z., T.V., Y. Z.-E., F.G., M.C., M.K., N.B., N.K., Y.H., D.M., H.D., K.B., R.K., Y.A., M.A., A.S.F., B.E.K., K.J., P.C., C.J.M., J.G.W., M.H., and W.A.G. participated in preparing the manuscript.

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[10]Department of Anaesthesiology and Intensive Care, Experimental and Clinical Haemostasis, University Hospital Münster, Münster, Germany

[11]NIH Intramural Sequencing Center, NIH, Bethesda, MD

[12]Department of Laboratory Medicine, University of Minnesota, Minneapolis, MN

Abstract

Gray Platelet Syndrome (GPS) is an autosomal recessive bleeding disorder with large platelets that lack α-granules. We found that mutations of *NBEAL2* (neurobeachin-like 2), encoding a BEACH/ARM/WD40 domain protein, cause GPS. We demonstrated that human megakaryocytes and platelets express a unique combination of *NBEAL2* transcripts. Proteomic analysis of sucrose-gradient subcellular fractions of platelets indicated that *NBEAL2* localizes to the dense tubular system (endoplasmic reticulum) in platelets.

Keywords

Gray platelet syndrome; NBEAL2; neurobeachin; platelet α-granules; organelle biogenesis

Platelets are organelle-rich cells that transport granule-bound compounds to tissues throughout the body. Platelet α-granules, the most abundant platelet organelles, store large proteins that, when released, promote platelet adhesiveness, haemostasis and wound healing[1,2], while platelet dense (δ) granules contain small, non-protein molecules such as calcium, serotonin, adenosine diphosphate, adenosine triphosphate, and pyrophosphate that promote platelet aggregation[1,3,4,5]. Bleeding disorders arising from defective platelet granules constitute the platelet storage pool diseases (SPD) and include isolated δ -granule deficiency (delta-SPD), combined α and δ -SPD and isolated α -granule deficiency (GPS; OMIM #139090)[3,4,6]. GPS platelets are large and appear gray on light microscopy (Fig. 1a-b)[5-7]; the diagnosis is confirmed by electron microscopy (EM) showing absent or markedly reduced α-granules in platelets[8](Fig. 1c-d) and in megakaryocytes[9], although both platelets and megakaryocytes have rudimentary α-granule precursors[10].

Clinical manifestations of GPS are usually mild to moderate and infrequently severe[6]. GPS is also associated with myelofibrosis (Fig. 1e-f) and splenomegaly as a consequence of myelofibrosis[5-7] The basis of myelofibrosis remains unknown, but constitutive release of platelet-derived growth factor and other pro-fibrotic substances from megakaryocytes into the bone marrow may be involved[5].

Some platelet α-granule constituents are passively (e.g., immunoglobulins, albumin) or actively (e.g., fibrinogen) taken up from the plasma by receptor-mediated endocytosis; others are synthesized in megakaryocytes (e.g., platelet factor 4, β-thromboglobulin) and trafficked to the organelle[3]. In GPS, proteins synthesized in megakaryocytes are markedly reduced, while endocytosed α-granule constituents are less affected[3]. This suggests that GPS megakaryocytes fail to pack their endogeneously synthesized secretory proteins into developing α-granules.

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By genome-wide linkage analysis and homozygosity mapping of 25 GPS patients from 14 unrelated families, we previously mapped the GPS disease locus to a 9.4 megabase interval on 3p21.1-22.1 that includes 197 protein coding genes[6]. Initial whole exome and Sanger dideoxy sequencing revealed no mutations in any of these genes, but subsequent Sanger dideoxy sequencing of exons not previously covered did reveal mutations in a single gene. In fact, 15 unrelated GPS patients exhibited *NBEAL2* (ENSG00000160796) mutations, including 5 missense, 3 nonsense, 4 frameshifting and 3 consensus splice site mutations (Table 1, Fig. 1g, Supplementary Fig. 1 and 2). None of these variants was found in the 1000 Genomes Database (http://www.1000genomes.org/), which contained 629 genomes as of February 2011, or in 100 ethnically matched control individuals. The 14 affected individuals with identity by descent had homozygous mutations (Table 1) whereas those without identity by descent exhibited compound heterozygous mutations.

All missense variants alter conserved amino acids and have high pathogenicity prediction scores (Supplemetary Fig. 1p). Splice donor site mutations c.1296+5G>A, c.5301+1G>A result in use of cryptic intronic donor sites demonstrated in blood mRNA, and c. 5720+5G>A is predicted to obliterate a splice donor site (Supplementary Fig. 1).

Patients from families GPS-6 and GPS-8 illustrated interfamilial variability. Although each is homozygous for splice mutation c.1296+5G>C, the patient from family GPS-6 has mild coagulopathy and the patient from family GPS-8 has severe coagulopathy[6]. Similar findings were observed for other GPS patients; the severity of coagulopathy, myelofibrosis and splenomegaly did not correlate with the type or location of the *NBEAL2* mutation (Table 1, Fig. 1g).

Human *NBEAL2* is predicted to produce 15 different mRNA transcripts, of which 7 would be protein coding (Ensembl) (Supplementary Fig. 3a). We designed cDNA primers to differentially amplify all protein encoding *NBEAL2* transcripts (Supplementary Fig. 3) and tested RNA from a variety of human hematopoietic cells and other tissues. A unique combination of transcripts encoding NBEAL2-001, 201/003, 203/004 and 202 was expressed in megakaryocytes and platelets (Supplementary Table 1).

Antibodies to NBEAL2 are not available. To explore the subcellular localization of NBEAL2 protein, we performed proteomic analysis on sucrose-gradient subcellular fractions from normal platelets and identified 2 tryptic peptides from the protein NBEAL2 using mass spectrometry (Fig. 1g, Supplementary Fig. 4). These peptide sequences, contained within NBEAL2 transcripts 001, 003 and 201, were found in platelet subcellular fraction 4, which contained small membrane structures on EM analysis[2] that likely originated from the dense tubular system. In fact, fraction 4 was enriched in dense tubular system and ER markers (data not shown).

The fibrotic nature of the GPS bone marrow prevented us from obtaining sufficient bone marrow for ex-vivo expansion of GPS megakaryocytes, the only cell type that expresses the GPS phenotype of defective α-granule biogenesis. Our previous microarray data in GPS fibroblasts showed overexpression of fibronectin[11], essential for proplatelet formation in cultured megakaryocytes[12] and critical for megakaryocyte-matrix interactions[13]. Future

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studies investigating GPS megakaryocytes might shed light on the pathogenesis of the myelofibrosis in GPS.

How absence of NBEAL2 function in megakaryocytes results in defective α-granule biogenesis remains unknown. However, NBEAL2 belongs to the family of proteins that contain an ARM, BEACH (beige and Chediak-Higashi syndrome) and WD40 domains, highly conserved regions that are crucial for protein-protein interactions, membrane dynamics and vesicle trafficking[14]. Another such protein is CHS1, which is defective in Chediak-Higashi disease (CHD, OMIM #214500), a disorder of immunodeficiency, platelet dense granule defects, partial albinism, and enlarged lysosomes or lysosome-related organelles in hematopoietic cells and melanocytes[4,15]. The precise cell biological defects in GPS and CHD remain unknown, but both diseases involve large proteins and impaired formation and trafficking of intracellular vesicles. NBEAL2 protein is predicted to interact with WDFY3 (WD repeat and FYVE domain containing 3), which itself interacts with CHS1, and with DLL1 and jagged 1 (http://www.sabiosciences.com/genenetwork/genenetworkcentral.php), known to have roles in hematopoiesis. DLL1 is the human homolog of the Notch Delta ligand and jagged 1 is the ligand for the receptor notch 1. These protein-protein interactions are entirely based on computational predictions; future experiments will determine their accuracy. Understanding NBEAL2 function will likely lead to the discovery of novel pathways of organelle formation and maturation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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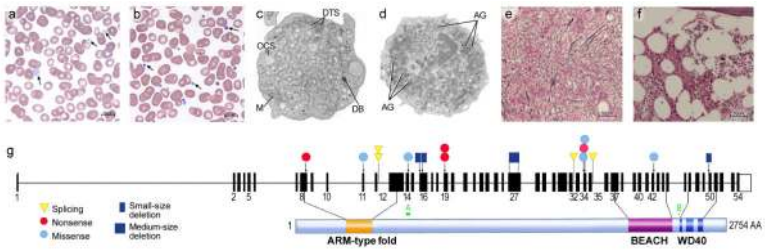

Figure 1. Cellular studies of gray platelet syndrome

(a) Light microscopy of a peripheral blood smear of the patient from family GPS-14 showing pale gray platelets (arrows), some larger than normal. (**b**) Normal, darkly stained platelets (arrows). (**c**) Transmission electron microscopy of thin sections of a platelet from GPS-13 showing absence of α-granules and abundant channels of the open canalicular system (OCS). DTS: Dense tubule system; DB: Dense body; M: Mitochondrion. (**d**) Normal platelet with α-granules (AG). (**e**) Reticulin staining of bone marrow of the patient from family GPS-4 displaying myelofibrosis (black strands). **(f)** Normal bone marrow without fibrosis. Scale bar indicates 10 μm (a, b) and 50 μm (e,f), (magnification x200) **(g)** Schematic representation of *NBEAL2* gene (ENSG00000160796) with mutations indicated. The NBEAL2-001 isoform (ENST00000450053) is depicted with its BEACH, WD40 and ARM-type fold domains. Small green bars, labeled A and B, represent two NBEAL2 peptide fragments identified by mass spectrometry. A: WGSPTSLEGELGAVAIFHEALQATALR; B: AFFAEVVSDGVPLVLALVPHR.

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Table 1

Mutations in *NBEAL2* (ENSG00000160796) in patients with Gray Platelet Syndrome*

Family No.	Ethicity	Severity of Bleeding	Mutations			
			cDNA	Protein	Exon/ Intron	Mutation State
GPS-1	Muslim Bedouins	Moderate	c.2701C>T	p.Arg901X	19	Homozygous
GPS-2	Mennonite	Severe	c.881C>G	p.Ser294X	8	Homozygous
GPS-3	Caucasian (French)	Moderate	c.1163T>C	p.Leu388Pro	11	Homozygous
GPS-4	Caucasian (Turkish)	Severe	c.5720+5G>A		Intron 35	Homozygous
GPS-5	Caucasian (Turkish)	Mild	c.5515C>T	p.Arg1839Cys	34	Homozygous
GPS-6	Caucasian (Turkish)	Mild	c.1296+5G>C		Intron 12	Homozygous
GPS-7	Caucasian (German)	Severe	c.2257_2260delGCCC	p.Ala753SerfsX65	16	Homozygous
GPS-8	Caucasian	Severe	c.1296+5G>C		Intron 12	Homozygous
GPS-9	Caucasian (Turkish)	Severe	c.3819_4174del356	p.Val1274GlyfsX32	27	Homozygous
GPS-10	African American	NA	c.2029T>A	p.Trp677Arg	14	Homozygous
GPS-11	Caucasian	Moderate	c.7604delG	p.Gly2535ValfsX5	50	Homozygous
GPS-12	Caucasian	Severe	c.5505T>G	p.Tyr1835X	34	Heterozygous
GPS-13	Caucasian	Moderate	c.2701C>T	p.Arg901X	19	Compound heterozygous
			c.6787C>T	p.His2263Tyr	42	
GPS-14	African American	Mild	c.2156delT	p.Phe719SerfsX100	16	Compound heterozygous
			c.5497G>A	p.Glu1833Lys	34	
GPS-15	Hispanic (Mexican)	Mild	c.5301+1G>A		Intron 32	Homozygous

*See also Supplementary Fig. 1. Extensive clinical and mapping data on families GPS-1 to GPS-14 were previously reported[6]. GPS-1 to GPS-11[6] and GPS-15 (Supplementary Fig. 2) exhibited identity by descent. In GPS-12, only 1 heterozygous mutation was identified and no tissue was available for *NBEAL2* mRNA analysis. NA: not available.