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NBEAL2 is mutated in gray platelet syndrome and is required for biogenesis of platelet α-granules

Meral Gunay-Aygun[1,2,13], Tzipora C Falik-Zaccai[3,4,13], 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] & William A Gahl[1,2]

Gray platelet syndrome (GPS) is an autosomal recessive bleeding disorder that is characterized by large platelets that lack α-granules. Here we show that mutations in *NBEAL2* (neurobeachin-like 2), which encodes a BEACH/ARM/WD40 domain protein, cause GPS and that megakaryocytes and platelets from individuals with GPS 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.

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, hemostasis and wound healing[1,2], whereas platelet dense (δ) granules contain small, nonprotein molecules, such as calcium, serotonin, ADP, ATP and pyrophosphate, that promote platelet aggregation[1,3–5]. Bleeding disorders that arise from defects in platelet granules constitute the platelet storage pool diseases (SPDs) and include isolated δ-granule deficiency (δ-SPD), combined α- and δ-SPD and isolated α-granule deficiency[3,4,6] (GPS; MIM139090). Platelets from individuals with GPS are large and appear gray under light microscopy[5–7] (**Fig. 1a**,**b**); the diagnosis is confirmed by electron microscopy 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 rarely severe[6]. GPS is also associated with myelofibrosis (**Fig. 1e**,**f**) and splenomegaly as a consequence of myelofibrosis[5–7]. The basis of the myelofibrosis remains unknown, but constitutive release of platelet-derived growth factor and other profibrotic substances from megakaryocytes into the bone marrow may be involved[5].

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

By genome-wide linkage analysis and homozygosity mapping of 25 individuals with GPS from 14 unrelated families, we previously mapped the GPS disease locus to a 9.4-megabase interval on chromosome 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 individuals with GPS had mutations in *NBEAL2* , including five missense, three nonsense, four frameshifting and three consensus splice-site mutations (**Table 1**, **Fig. 1g** and **Supplementary Figs. 1** and **2**). None of these variants was found in the 1000 Genomes Database (see URLs), 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 had compound heterozygous mutations.

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

Individuals from families GPS-6 and GPS-8 showed intrafamilial variability. Although each is homozygous for splice mutation c.1296+5G>C, the subject from family GPS-6 has mild coagulopathy, and the subject from family GPS-8 has severe

[1]Section on Human Biochemical Genetics, Medical Genetics Branch, National Human Genome Research Institute, US National Institutes of Health, Bethesda, Maryland, USA. [2]Office of Rare Diseases Research, Office of the Director, US National Institutes of Health, Bethesda, Maryland, USA. [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, Penn State Hershey Children's Hospital, Hershey, Pennsylvania. [10]Department of Anaesthesiology and Intensive Care, Experimental and Clinical Haemostasis, University Hospital Münster, Münster, Germany. [11]US National Institutes of Health (NIH) Intramural Sequencing Center, NIH, Bethesda, Maryland, USA. [12]Department of Laboratory Medicine, University of Minnesota, Minneapolis, Minnesota, USA. [13]These authors contributed equally to this work. Correspondence should be addressed to M.G.-A. (mgaygun@mail.nih.gov).

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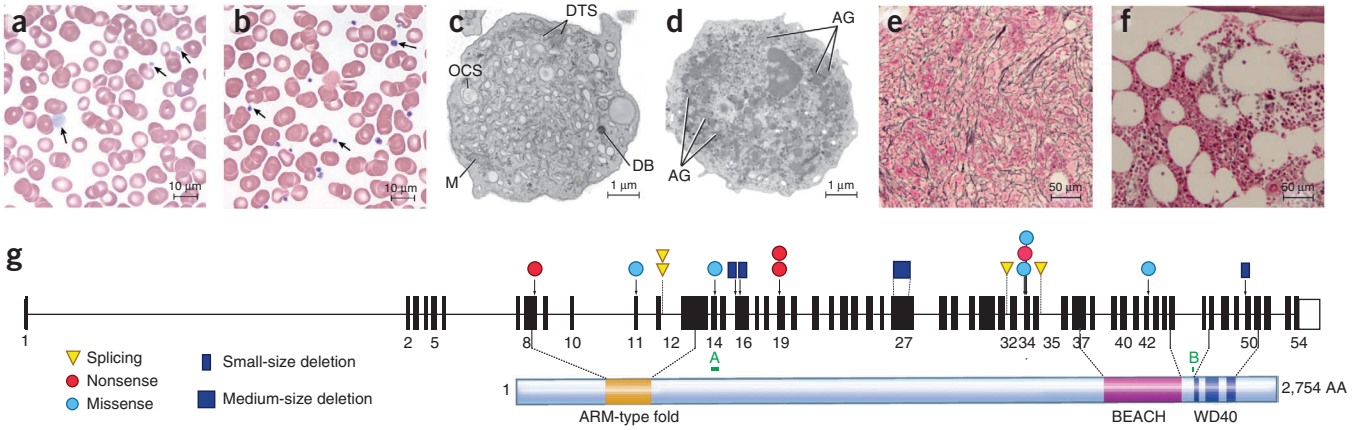

Figure 1 Cellular studies of GPS. (**a**) Light microscopy of a peripheral blood smear of the patient from family GPS-14 showing pale gray platelets (arrows), some larger than normal. (**b**) Normal, darkly stained platelets (arrows). (**c**) Transmission electron microscopy of thin sections of a platelet from GPS-13 showing the 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 showing myelofibrosis (black strands). (**f**) Normal bone marrow without fibrosis. Scale bars, 10 µm (**a**,**b**), 1 µm (**c**,**d**) and 50 µm (**e**,**f**); magnification ×200. (**g**) Schematic representation of *NBEAL2* with mutations indicated. The NBEAL2-001 isoform 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.

coagulopathy[6]. We found similar effects in other subjects; the severity of coagulopathy, myelofibrosis and splenomegaly did not correlate with the type or location of the *NBEAL2* mutation (**Table 1** and **Fig. 1g**).

Human *NBEAL2* is predicted to produce 15 mRNA transcripts, of which 7 would be protein coding (**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 Ensembl isoforms NBEAL2-001, NBEAL2-201/003, NBEAL2-203/004 and NBEAL2-202 was expressed in megakaryocytes and platelets (**Supplementary Table 1**).

Antibodies to NBEAL2 are not commercially available. To explore the subcellular localization of NBEAL2 protein, we performed proteomic analysis on sucrose-gradient subcellular fractions from normal platelets and identified two tryptic peptides from NBEAL2 by mass spectrometry (**Fig. 1g** and **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 electron microscopic analysis[2] that probably originated from the dense tubular system. In fact, fraction 4 was enriched in markers for the dense tubular system and endoplasmic reticulum (data not shown).

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

Table 1 Mutations in *NBEAL2* in individuals with GPS

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

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

Future studies of megakaryocytes from individuals with GPS might shed light on the pathogenesis of myelofibrosis in GPS.

It is not known how the absence of NBEAL2 function in megakaryocytes results in defective α-granule biogenesis. However, NBEAL2 belongs to the family of proteins that contain 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, MIM214500), 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 is predicted to interact with WDFY3 (WD repeat and FYVE domain-containing 3), as well as DLL1 and jagged 1, which are involved in hematopoiesis, according to an analysis with Gene Network Central (see URLs). In addition, WDFY3 itself interacts with CHS1. DLL1 is the human homolog of the notch Delta ligand, and jagged 1 is the ligand for the receptor notch 1. These predicted protein-protein interactions are entirely based on computational predictions; future experiments will determine their accuracy. Understanding the function of NBEAL2 will probably lead to the discovery of new pathways of organelle formation and maturation.

URLs. 1000 Genomes Project Database, http://www.1000genomes.org/; http://www.sabiosciences.com/genenetwork/genenetworkcentral.php.

Accession codes. *NBEAL2*, Ensembl ENSG00000160796; NBEAL2-001 isoform, Ensembl ENST00000450053.

Note: Supplementary information is available on the Nature Genetics website.

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AUTHOR 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 and cultured megakaryocytes; M.G.-A., W.A.G. and T.C.F.-Z. designed and supervised research; M.G.-A., 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., J.C.M., C.F.B. and M.H. supervised DNA sequencing; M.G.-A., T.V., Y.Z.-E., F.G., M.C., M.K., C.F.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; H.D. cultured fibroblasts; T.C.F.-Z., T.V., Y.Z.-E., F.G., M.C., M.K., C.F.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., J.C.M., J.G.W., M.H. and W.A.G. participated in preparing the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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