

ORIGINAL ARTICLE

A Syndrome with Congenital Neutropenia and Mutations in *G6PC3*

Kaan Boztug, M.D., Giridharan Appaswamy, M.Sc., Angel Ashikov, Ph.D., Alejandro A. Schäffer, Ph.D., Ulrich Salzer, M.D., Jana Diestelhorst, B.Sc., Manuela Germeshausen, Ph.D., Gudrun Brandes, M.D., Jacqueline Lee-Gossler, M.Sc., Fatih Noyan, Ph.D., Anna-Katherina Gatzke, M.Sc., Milen Minkov, M.D., Ph.D., Johann Greil, M.D., Christian Kratz, M.D., Theoni Petropoulou, M.D., Isabelle Pellier, M.D., Christine Bellanné-Chantelot, Pharm.D., Ph.D., Nima Rezaei, M.D., Kirsten Mönkemöller, M.D., Noha Irani-Hakimeh, M.D., Hans Bakker, Ph.D., Rita Gerardy-Schahn, Ph.D., Cornelia Zeidler, M.D., Bodo Grimbacher, M.D., Karl Welte, M.D., and Christoph Klein, M.D., Ph.D.

ABSTRACT

BACKGROUND

The main features of severe congenital neutropenia are the onset of severe bacterial infections early in life, a paucity of mature neutrophils, and an increased risk of leukemia. In many patients, the genetic causes of severe congenital neutropenia are unknown.

METHODS

We performed genomewide genotyping and linkage analysis on two consanguineous pedigrees with a total of five children affected with severe congenital neutropenia. Candidate genes from the linkage interval were sequenced. Functional assays and reconstitution experiments were carried out.

RESULTS

All index patients were susceptible to bacterial infections and had very few mature neutrophils in the bone marrow; structural heart defects, urogenital abnormalities, and venous angiectasia on the trunk and extremities were additional features. Linkage analysis of the two index families yielded a combined multipoint lod score of 5.74 on a linkage interval on chromosome 17q21. Sequencing of *G6PC3*, the candidate gene encoding glucose-6-phosphatase, catalytic subunit 3, revealed a homozygous missense mutation in exon 6 that abolished the enzymatic activity of glucose-6-phosphatase in all affected children in the two families. The patients' neutrophils and fibroblasts had increased susceptibility to apoptosis. The myeloid cells showed evidence of increased endoplasmic reticulum stress and increased activity of glycogen synthase kinase 3 β (GSK-3 β). We identified seven additional, unrelated patients who had severe congenital neutropenia with syndromic features and distinct biallelic mutations in *G6PC3*.

CONCLUSIONS

Defective function of glucose-6-phosphatase, catalytic subunit 3, underlies a severe congenital neutropenia syndrome associated with cardiac and urogenital malformations.

From Hannover Medical School, Hannover, Germany (K.B., G.A., A.A., J.D., M.G., G.B., J.L.-G., F.N., A.-K.G., H.B., R.G.-S., C.Z., K.W., C. Klein); the National Center for Biotechnology Information, National Institutes of Health, Bethesda, MD (A.A.S.); the University Medical Center Freiburg, Freiburg, Germany (U.S.); St. Anna Children's Hospital, Vienna (M.M.); Children's Hospital, University of Heidelberg, Heidelberg, Germany (J.G.); the University of Freiburg, Freiburg, Germany (C. Kratz); Aghia Sophia Children's Hospital, University of Athens, Athens (T.P.); Centre Hospitalier Universitaire Angers, Angers, France (I.P.); Assistance Publique-Hôpitaux de Paris, Hôpital de la Pitié-Salpêtrière, Paris (C.B.-C.); the Immunology, Asthma, and Allergy Research Institute, Tehran University of Medical Sciences, Tehran, Iran (N.R.); Children's Hospital Amsterdamer Straße, Cologne, Germany (K.M.); Saint Georges University Hospital, Beirut, Lebanon (N.I.-H.); and Royal Free Hospital and University College London, London (B.G.). Address reprint requests to Dr. Klein at the Department of Pediatric Hematology and Oncology, Medical School Hannover, Carl-Neuberg-Straße 1, Hannover D-30625, Germany, or at klein.christoph@mh-hannover.de.

Drs. Appaswamy and Ashikov contributed equally to this article.

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SEVERE CONGENITAL NEUTROPENIA WAS described more than 50 years ago by Kostmann,^{1,2} and subsequently the disorder was found to consist of a heterogeneous group of diseases.^{3,4} In these syndromes, the neutropenia is associated with life-threatening bacterial infections early in life. Most patients respond to treatment with recombinant human granulocyte colony-stimulating factor (rhG-CSF), which increases neutrophil counts and decreases the frequency and severity of infections.⁵ Nonetheless, patients may remain at risk for both infectious complications and the development of clonal disorders of hematopoiesis, such as myelodysplastic syndrome and acute myeloid leukemia.⁶

Considerable progress has been made in identifying the molecular defects that cause congenital neutropenia.^{7,8} Many patients with severe congenital or cyclic neutropenia have a heterozygous mutation in *ELA2*, the gene encoding neutrophil elastase.⁹⁻¹¹ We recently identified homozygous mutations in *HAX1*, the gene encoding HCLS1-associated protein X1, in a subgroup of patients with autosomal recessive severe congenital neutropenia.¹² In addition, mutations in *WAS*, the gene encoding the Wiskott-Aldrich syndrome protein,^{13,14} and in *GFI1*, the gene encoding the growth factor independent 1 transcription-repressor protein,¹⁵ have been associated with a phenotype resembling Kostmann's syndrome. In many patients with congenital neutropenia, however, the underlying molecular cause remains unknown. Despite insights into the role of apoptosis in congenital neutropenia,^{12,16,17} the mechanisms of neutropenia and the risk of leukemia in patients with severe congenital neutropenia are incompletely understood. We report a syndrome consisting of severe congenital neutropenia, other congenital abnormalities, and biallelic mutations in *G6PC3*, the gene encoding glucose-6-phosphatase, catalytic subunit 3.

METHODS

PATIENTS AND CONTROLS

We took blood and bone marrow samples from patients and healthy volunteers with their written informed consent. The study was approved by the institutional review board of Hannover Medical School.

ANALYTICAL METHODS

We genotyped microsatellite markers in a whole-genome scan for a family with severe congenital neutropenia, with a pedigree identified as SCN-I. The equipment and protocols for genotyping have been described previously.¹² The genetic-linkage analysis was performed with the use of a combination of quantitative and qualitative syllogisms. The quantitative decisions were made with the use of lod scores and optimal recombination fractions computed with Superlink software.^{18,19} For calculation of lod scores, we modeled neutropenia as a fully penetrant autosomal recessive disease with no phenocopies and a disease-allele frequency of 0.001. The Marshfield map²⁰ was used to select usefully positioned markers for fine mapping. For details, see the Supplementary Appendix, available with the full text of this article at NEJM.org.

Exons and flanking intron-exon boundaries from candidate genes were amplified by polymerase chain reaction (PCR) and analyzed with the use of an ABI PRISM 3130 DNA Sequencer and DNA Sequencing Analysis software, version 3.4 (Applied Biosystems), and Sequencer, version 3.4.1 (Gene Codes Corporation). See the Supplementary Appendix for primer sequences and details of the restriction-length-polymorphism analysis of the frequency of the R253H mutation in healthy controls.

Promyelocytes were sorted by fluorescence-activated cell sorting, as described previously,¹⁷ with minor modifications. Analysis of the expression of the *G6PC3* and *HSPA5/BiP/Grp78* genes was performed with the use of a Roche LightCycler 2.0 (see the Supplementary Appendix).

The complete open reading frames of wild-type and mutant *G6PC3* were amplified by PCR and cloned in pYES-cup1 (modified from pYES-NT [Invitrogen]), as described by Ashikov et al.,²¹ and expressed in *Saccharomyces cerevisiae*. Centrifugation at 100,000×g produced a microsomal fraction that was assayed for hydrolysis of glucose-6-phosphate to glucose by the addition of ¹⁴C-glucose-6-phosphate (MP Biomedicals). Released ¹⁴C-glucose was separated from glucose-6-phosphate by anion exchange and measured in the eluate by liquid scintillation.

Whole-cell lysates from primary granulocytes were separated by sodium dodecyl sulfate-polyacrylamide-gel electrophoresis (SDS-PAGE), blot-

Table 1. Clinical and Molecular Findings in Patients with G6PC3 Deficiency.*

Patient No.	Sex	Race or Ethnic Group and Country of Origin†	Absolute Neutrophil Count‡ <i>per mm³</i>	Infections	Other Findings
1	M	Aramean, Turkey	60–246	Neonatal sepsis, otitis media	Type 2 atrial septal defect, cryptorchidism, prominent superficial venous pattern, intermittent thrombocytopenia, hepatosplenomegaly
2	F	Aramean, Turkey	54–240	Neonatal pneumonia and sepsis	Cor triatriatum, malformation of pulmonary veins, prominent superficial venous pattern, hepatosplenomegaly
3	F	Aramean, Turkey	0–61	Pneumonia, sepsis	Type 2 atrial septal defect, mitral insufficiency, prominent superficial venous pattern, intermittent thrombocytopenia, hepatosplenomegaly
4	M	Aramean, Turkey	0–322	Neonatal sepsis	Type 2 atrial septal defect, cryptorchidism, prominent superficial venous pattern
5	M	Aramean, Turkey	25–84	Neonatal sepsis	Prominent superficial venous pattern, intermittent thrombocytopenia
6	F	Turkish, Turkey	90–612	Pneumonia, sepsis	Type 2 atrial septal defect, pulmonary valve stenosis, panniculitis, prominent superficial venous pattern
7	F	White, Greece	30–1280¶	Perianal abscesses, recurrent urinary tract infections	Inner-ear hearing loss, prominent superficial venous pattern
8	F	White, Germany	75–210	Omphalitis, recurrent urinary tract infections, oral ulcers	Type 2 atrial septal defect, urachal fistula, microcephaly, prominent superficial venous pattern, intermittent thrombocytopenia
9	F	White, France	200–500	Upper and lower respiratory tract infections	Myopathy, prominent superficial venous pattern
10	M	White, Germany	0–3000	Urinary tract infection	Type 2 atrial septal defect, cryptorchidism, genital dysplasia, microcephaly, inner-ear hearing loss, prominent superficial venous pattern, intermittent thrombocytopenia
11	M	Persian, Iran	250–440	Neonatal sepsis, urinary tract infection	Type 2 atrial septal defect, patent ductus arteriosus
12	M	Arab, Lebanon	615–2000¶	Perianal abscesses, pneumonia, recurrent lower respiratory tract infections	Cryptorchidism, bilateral inguinal hernia, cleft palate

* G-CSF denotes granulocyte colony-stimulating factor.

† Race or ethnic group was self-assessed.

‡ Absolute neutrophil counts were determined before the start of treatment with G-CSF.

§ Percentiles of height and weight were based on German reference data for white patients from Greece, Germany, and France and on Turkish reference data for other patients.

¶ During infectious episodes, an occasional increase in neutrophil counts was observed.

ted, and stained with antibodies against phospho-Mcl-1 (Ser159/Thr163), total glycogen synthase kinase 3 β (GSK-3 β), phospho-GSK-3 β (Ser9) (all from Cell Signaling Technology/New England Biolabs), Bip/Grp78 (BD Biosciences), and glyceralde-

hyde-3-phosphate dehydrogenase (GAPDH) (Santa Cruz Biotechnology) (see the Supplementary Appendix).

Bone marrow samples from patients and healthy control subjects were subjected to hypo-

Genotype	Therapy	Current Age	Current Height and Weight Percentiles§	
		yr	Height	Weight
c.[758G→A]+[758G→A], p.[Arg253His]+[Arg253His]	G-CSF, cardiac surgery	6	25–50	3–10
c.[758G→A]+[758G→A], p.[Arg253His]+[Arg253His]	G-CSF, cardiac surgery	3	25	10–25
c.[758G→A]+[758G→A], p.[Arg253His]+[Arg253His]	G-CSF, awaiting corrective cardiac procedure	11	10–25	10–25
c.[758G→A]+[758G→A], p.[Arg253His]+[Arg253His]	G-CSF, cardiac surgery	6	8 cm below 3rd percentile	1.3 kg below 3rd percentile
c.[758G→A]+[758G→A], p.[Arg253His]+[Arg253His]	G-CSF	4	3	1.0 kg below 3rd percentile
c.[554T→C]+[554T→C], p.[Leu185Pro]+[Leu185Pro]	G-CSF, awaiting corrective cardiac procedure	12	50	75
c.[141C→G]+[141C→G], p.[Tyr47X]+[Tyr47X]	G-CSF	13	25–50	50–75
c.[784G→C]+[784G→C], p.[Gly262Arg]+[Gly262Arg]	G-CSF, cardiac surgery	7	3–10	10
c.[677+1 G→A]+[829C→T], p.[?]+[Gln277X]	G-CSF	13	3–10	10
c.[778G→C]+[778G→C], p.[Gly260Arg]+[Gly260Arg]	G-CSF, cardiac surgery	17	4 cm below 3rd percentile	8 kg below 3rd percentile
c.[935dupT]+[935dupT], p.[Asn313fs]+[Asn313fs]	G-CSF, awaiting corrective cardiac procedure	10	10–25	10–25
c.[144C→A]+[144C→A], p.[Tyr48X]+[Tyr48X]	G-CSF	5	3–10	3–10

tonic lysis. Fixation and electron microscopy were performed as described previously.²²

Human *G6PC3* complementary DNA (cDNA) was cloned into a bicistronic retroviral vector (MMP)²³ containing murine *cd24* as a marker gene. RD114-pseudotyped retroviral particles were generated by tripartite transfection of MMP-based vectors together with the envelope plasmid and the packaging plasmid mPD.old.gag/pol into a human embryonic kidney-cell line (HEK293T). Trans-

duction of CD34+ cells and myeloid differentiation were performed as described previously.¹²

Apoptosis in peripheral-blood neutrophils or in cells that were differentiated into myeloid cells in vitro was induced with the use of tumor necrosis factor α (TNF- α) (50 ng per milliliter), thapsigargin (10 μ M), or tunicamycin (5 μ g per milliliter) (all from Sigma) and assessed by staining with annexin V (Invitrogen) and propidium iodide (Sigma). In fibroblasts, apoptosis was induced with

the use of 5 mM dithiothreitol (Roche). Caspase 3/7 activation was assessed as described previously¹² (see the Supplementary Appendix).

RESULTS

CLINICAL FINDINGS

Table 1 lists the main features of the five patients we studied. Patients 1 and 2, who were siblings born to consanguineous parents of Aramean descent, presented with neonatal sepsis. Their extended pedigree is denoted SCN-I (see Fig. 1 in the Supplementary Appendix). Their workup in the first year of life found severe neutropenia with a paucity of mature neutrophils in the peripheral blood and bone marrow. Bone marrow smears contained few granulocytes beyond the stage of promyelocytes or myelocytes (Fig. 1A and 1B, and Table 1 in the Supplementary Appendix). Erythrocyte counts were normal. Platelet counts in Patient 1 ranged from 73,000 to 425,000 per cubic millimeter, whereas Patient 2 had normal platelet counts. Both patients had unusually prominent subcutaneous veins, venous angiectasia, or both (Fig. 1C); Patient 1 had a type 2 atrial septal defect (Fig. 1D), and Patient 2 had cor triatriatum and hepatosplenomegaly. Genealogic investigations revealed that the SCN-I pedigree could be extended to include two additional sibships, each with one child affected by severe congenital neutropenia and type 2 atrial septal defect (Patients 3 and 4 in Fig. 1 in the Supplementary Appendix). We also identified a child with severe congenital neutropenia in a second consanguineous pedigree (SCN-II) from the same ethnic background (Patient 5 in Fig. 1 in the Supplementary Appendix). All patients received rhG-CSF, which resulted in an increase in neutrophil counts.

GENETIC STUDIES

Mutations in both *ELA2*¹⁰ and *HAX1*¹² were excluded in all five index patients. Linkage analysis provided statistical evidence that the gene of interest in SCN-I is located on chromosome 17q21 between D17S1299 (36.2 Mb, 62.0 cM) and D17S1290 (53.7 Mb, 82.0 cM) (Fig. 2A, and the Supplementary Appendix). We carried out a series of fine mapping steps in SCN-I and SCN-II and were able to genotype an additional 13 microsatellite markers between D17S1299 and D17S1290 in SCN-I and 11 of these 13 microsatellite markers in SCN-II. Table 2 in the Supplementary Appendix shows the

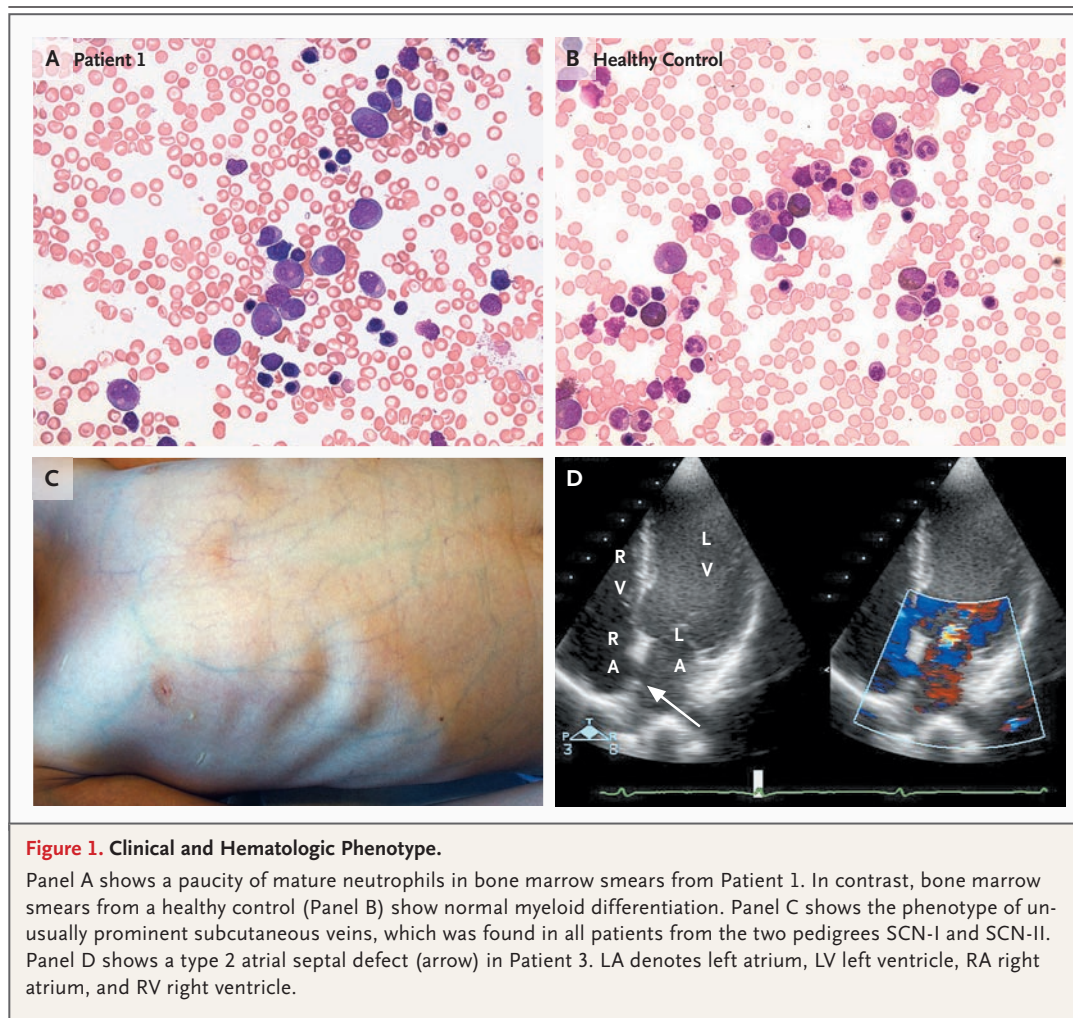
single-marker lod scores. On the assumption that the same gene is mutated in all five affected children, the maximal linkage interval spanned from D17S1789 (39.1 Mb, 63.1 cM) to D17S791 (42.2 Mb, 64.2 cM). With the use of D17S932, D17S950, and D17S806, the peak multipoint lod score in SCN-I alone was 4.98, and the peak two-pedigree multipoint lod score was 5.74.

Several candidate genes were identified in the SCN-I linkage interval (see Table 3 in the Supplementary Appendix). Of these, *G6PC3*, which encodes glucose-6-phosphatase, catalytic subunit 3, and is located in the narrowest possible linkage interval, was a plausible candidate, because abnormal glucose metabolism has been implicated in the neutropenia of type 1b glycogen storage disease.²⁴ DNA sequencing revealed a homozygous missense mutation in exon 6 of the *G6PC3* gene (c.G758A, p.R253H) (Fig. 2B). This mutation was found in all four affected children in SCN-I and in the one affected child in SCN-II. All parents were heterozygous for the mutation, a finding consistent with autosomal recessive inheritance of a germ-line missense mutation. According to restriction-site analysis, the R253H allele of the *G6PC3* gene was not found in 192 healthy central European persons. An *in silico* sequence analysis using the program Sorting Intolerant from Tolerant (SIFT)²⁵ calculated that the probability that this mutation is benign is 0.01. Analysis with the program PolyPhen²⁶ predicted that the R253H change probably interferes with the function of glucose-6-phosphatase. The wild-type protein is conserved in mammals, amphibians, bony fish, and insects.

FUNCTIONAL STUDIES

Enzymatic Activity of G6PC3 with the R253H Mutation

Wild-type *G6PC3* and *G6PC3* with the R253H mutation were expressed in *S. cerevisiae*. Microsomes were isolated from yeast transfected with wild-type *G6PC3* or *G6PC3* with the R253H mutation and assayed for phosphatase activity. Wild-type *G6PC3* hydrolyzed glucose-6-phosphate and the universal substrate p-nitrophenylphosphate (pNPP), as demonstrated by radioactive (Fig. 2C) and spectrometric (Fig. 3 in the Supplementary Appendix) assays, respectively. In contrast, the level of enzymatic activity of *G6PC3* with the R253H mutation did not exceed the level of phosphatase activity in yeast transfected with an empty vector.

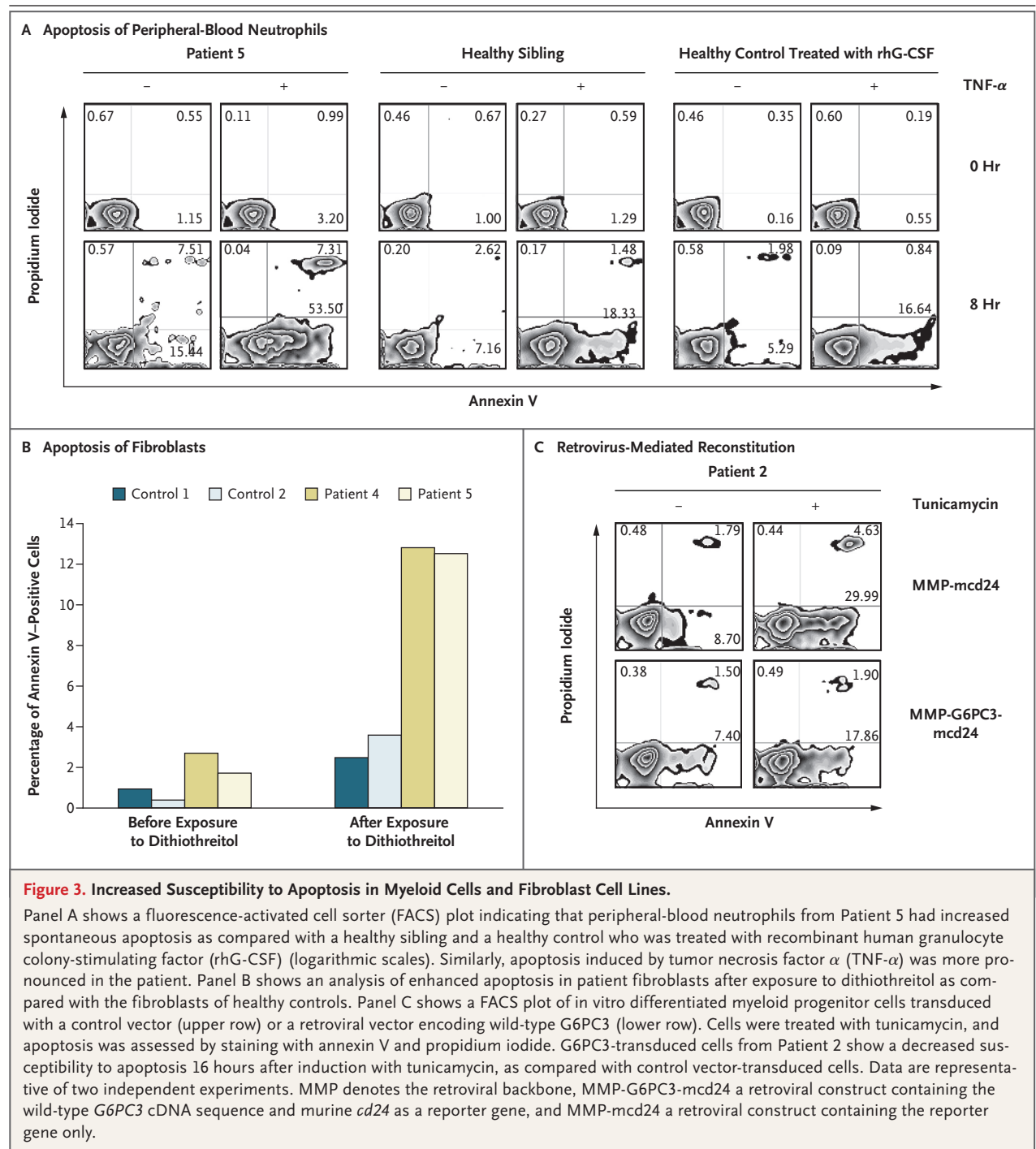


Apoptosis

Like neutrophils from patients with mutations in *ELA2*¹⁰ or *HAX1*,¹² peripheral-blood neutrophils from our patients had an increased rate of spontaneous apoptosis. Apoptosis of neutrophils was markedly accelerated after induction with TNF- α (Fig. 3A) or tunicamycin (data not shown), as assessed by annexin V staining and a test for activation of caspase 3/7, respectively (see Fig. 4 in the Supplementary Appendix). Since *G6PC3* is ubiquitously expressed and since the phenotype of our patients was not restricted to the hematopoietic system, we tested nonhematopoietic cells for susceptibility to apoptosis. Skin fibroblasts from patients with a deficiency of *G6PC3* had an increased susceptibility to apoptosis after dithiothreitol-induced stress to the endoplasmic reticulum (Fig. 3B).

To provide further evidence that this form of severe congenital neutropenia is caused by mutations in *G6PC3*, CD34+ hematopoietic stem cells from two patients were isolated and transduced with retroviral constructs containing either the wild-type *G6PC3* cDNA sequence and murine *cd24* as a reporter gene (MMP-*G6PC3*-mcd24) or the reporter gene only (MMP-mcd24). After in vitro differentiation in the presence of rhG-CSF and recombinant human granulocyte-macrophage colony-stimulating factor (rhGM-CSF), cells were exposed to tunicamycin to induce apoptosis and analyzed by flow cytometry by gating on mcd24-positive cells. In control-transduced cells from a patient, exposure to tunicamycin induced a high degree of apoptosis (30.0% of annexin V-positive cells and 4.6% of annexin V and propidium iodide-double-positive cells underwent apoptosis). By con-

Endoplasmic reticulum stress and the unfolded-protein response have been linked to the pathophysiology of aberrant organogenesis,²⁷ including structural heart defects²⁸ and congenital neutropenia



caused by mutations in *ELA2*.^{17,29} Transmission electron microscopy of bone marrow cells from four patients with *G6PC3* deficiency showed an enlarged rough endoplasmic reticulum in myeloid progenitor cells as compared with the rough endoplasmic reticulum in corresponding cells from a healthy subject (Fig. 4A and 4B, and Fig. 7 in the

Supplementary Appendix). This finding is consistent with increased endoplasmic reticulum stress. BiP messenger RNA (mRNA), a member of the chaperone family and another marker of endoplasmic reticulum stress, was measured by quantitative real-time PCR in bone marrow promyelocytes that had been isolated by flow cytometry.

The level of BiP mRNA was increased in promyelocytes from the two patients we tested as compared with that in promyelocytes from healthy subjects (Fig. 4C).

GSK-3 β

A signaling circuit linking glucose, GSK-3 β , and Mcl-1 has been previously established^{30,31} in which GSK-3 β controls glycogen metabolism, Wnt signaling, and apoptosis.³² Mcl-1, an antiapoptotic member of the Bcl-2 family, is involved in the maintenance of neutrophil viability.³³ GSK-3 β phosphorylates Mcl-1, thus facilitating its degradation in the proteasome.³⁰ We performed Western blot studies to estimate the levels of GSK-3 β , BiP, and Mcl-1 proteins in neutrophils exposed to tunicamycin, an agent that induces endoplasmic reticulum stress. Neutrophils from the two patients we examined had increased levels of BiP (Fig. 4E), an increase in the enzymatically active dephosphorylated form of GSK-3 β (Fig. 4D, and Fig. 8 in the Supplementary Appendix), and increased phosphorylation of Mcl-1 (Fig. 4E). To investigate whether intracellular glucose deprivation causes dephosphorylation of GSK-3 β , we inhibited glucose metabolism in neutrophils from two healthy subjects with the use of 2-deoxyglucose. Treatment with 2-deoxyglucose induced dephosphorylation of GSK-3 β (Fig. 4F) and increased apoptosis of neutrophils (Fig. 4G), whereas CD3-positive T lymphocytes were resistant to the effects of 2-deoxyglucose (Fig. 4G).

G6PC3 MUTATIONS IN OTHER PATIENTS

We assessed the frequency and variety of G6PC3 mutations in a cohort of patients with genetically unclassified severe congenital neutropenia. Of 104 such patients, 7 had distinct biallelic mutations in G6PC3 (Table 1). The mutations include nonsense mutations (Y47X and Y48X) that would abolish the function of the protein if the truncated mRNA was translated. The three other missense mutations were predicted to be deleterious to the protein by SIFT²² analysis, with probabilities of being benign of 0.03 for L185P, 0.00 for G262R, and 0.00 for G260R. None of these additional patients with G6PC3 mutations had mutations in ELA2 or HAX1, a finding suggesting that these three genetic defects are distinct variants of severe congenital neutropenia. None of the patients with G6PC3 deficiency had hypoglycemia or lactic acidosis (see Table 4 in the Supplementary Appendix), as is seen in glycogen storage disorders.

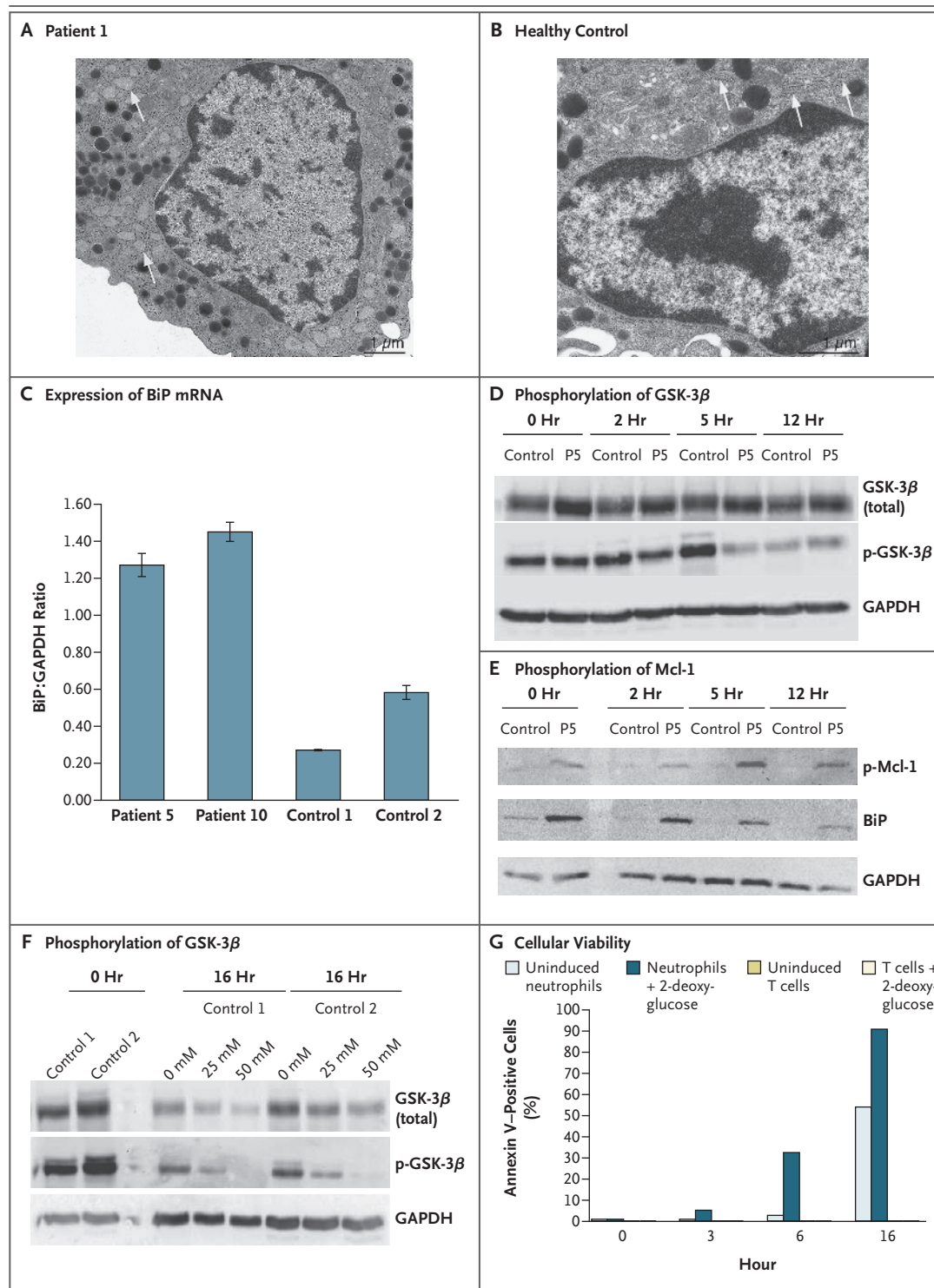
Figure 4 (facing page). Pathophysiologic Consequences of G6PC3 Deficiency.

Panel A is a transmission electron micrograph showing the aberrantly enlarged rough endoplasmic reticulum (arrows) in a myeloid progenitor cell of a patient with G6PC3 deficiency. Panel B shows the rough endoplasmic reticulum (arrows) in a cell from a healthy subject. Panel C illustrates increased endoplasmic reticulum stress in patients with G6PC3 deficiency, as documented by increased expression levels of BiP messenger RNA in purified promyelocytes. Error bars indicate the standard deviation. GAPDH denotes glyceraldehyde-3-phosphate dehydrogenase. Panel D is a Western blot showing an increase in the enzymatically active, dephosphorylated form of glycogen synthase kinase 3 β (GSK-3 β) in Patient 5 (P5) after induction with tunicamycin (p-GSK-3 β denotes phosphorylated GSK-3 β). Panel E shows increased phosphorylation of Mcl-1 and increased levels of BiP in neutrophils from Patient 5 (P5) with G6PC3 deficiency at different time points after induction with tunicamycin (p-Mcl-1 denotes phosphorylated Mcl-1). Panel F shows 2-deoxyglucose-induced dephosphorylation of GSK-3 β in two healthy donors. Panel G shows that healthy neutrophils, but not CD3-positive T cells, are susceptible to apoptosis on pharmacologic glucose depletion with the use of 2-deoxyglucose. Some values are too small to appear clearly.

A clinical review of all 12 patients with G6PC3 deficiency found variation in clinical features. Of the 12 patients, 8 had various cardiac malformations and 10 had a phenotype of unusually prominent subcutaneous veins, venous angiectasia, or both (Fig. 1, and Fig. 2 in the Supplementary Appendix). Five patients had urogenital malformations, including cryptorchidism and urachal fistula (the urachus is a channel between the bladder of the fetus and the allantois). Two patients had inner-ear hearing loss, and two had delayed growth but no dysmorphic features (Table 1).

DISCUSSION

We have described a congenital neutropenia syndrome with biallelic mutations in G6PC3. Like patients with mutations in HAX1¹² or ELA2,¹⁶ patients with G6PC3 deficiency lacked mature neutrophils in the bone marrow and had increased susceptibility to apoptosis in peripheral neutrophils. Of 12 patients, 8 had structural heart defects (e.g., type 2 atrial septal defect, cor triatriatum, or pulmonary stenosis) and 5 had urogenital defects (e.g., cryptorchidism or urachal fistula). In most patients, an atypical, increased visibility of the superficial veins, angiectasia, or both was prominent. The spectrum of developmental aberrations may



depend on factors other than the mutant *G6PC3*. Perhaps increased susceptibility to apoptosis also affects cardiac or urogenital development in patients with *G6PC3* deficiency. There is clinical variation in other neutropenia syndromes, such as Co-

hen syndrome and cartilage-hair hypoplasia, even though the two syndromes are genetically homogeneous.⁸ All patients in our cohort had a response to treatment with rhG-CSF, and to date no patient has had a clonal hematopoietic disorder.

Three human genes mediating glucose-6-phosphatase activity have been discovered: *G6PC1*, *G6PC2*, and *G6PC3*. *G6PC1*, the classic glucose-6-phosphatase in the liver, kidney, and small intestine, catalyzes the hydrolysis of glucose-6-phosphate, an essential step in the gluconeogenic and glycogenolytic pathways. Patients without *G6PC1* activity have type Ia glycogen storage disease.³⁴ *G6PC2* is expressed only in pancreatic islet cells^{35,36} and may be involved in glucose-dependent insulin secretion by controlling free glucose levels.³⁷ Statistically significant associations between non-coding polymorphisms in or near *G6PC2* and the level of glucose after an overnight fast have been shown.^{38,39} In contrast to *G6PC1* and *G6PC2*, *G6PC3* is ubiquitously expressed.^{40,41} Glucose-6-phosphate is transported to the endoplasmic reticulum by a glucose-6-phosphate transporter (*G6PT*).⁴² The stoichiometry and topologic relationships between the catalytic subunits of glucose-6-phosphatase and *G6PT* are unclear, but they do have a functional link.⁴²⁻⁴⁴ The complex formed between *G6PT* and *G6PC1* (and perhaps also the complex between *G6PT* and *G6PC2*) appears to maintain normoglycemia. Our data show that *G6PC3* is needed to maintain neutrophil viability and suggest an important role for glucose in the homeostasis of human neutrophils.

Cheung et al. recently described the phenotype of *g6pc3*-deficient mice that was generated by gene targeting.⁴⁵ These mice had neutropenia and neutrophil dysfunction; another group had previously shown that murine *g6pc3* deficiency results in lowered plasma cholesterol and elevated glucagon levels.⁴⁶ We could not identify any consistent aberration in neutrophil function or any metabolic aberrations in patients with *G6PC3* deficiency. The underlying mechanism of increased apoptosis of neutrophils in the absence of *G6PC3* involves increased endoplasmic reticulum stress, which is usually seen in cases of deficient protein folding in the endoplasmic reticulum. In an attempt to counteract potentially toxic effects that can ensue from abnormally folded proteins, cells initiate a rescue program that, if ineffective, leads to apoptosis.⁴⁷ We have provided evidence that *GSK-3 β* , a key enzyme that regulates cellular differentiation and apoptosis,³² is implicated in this pathway. In the absence of intracellular glucose, *GSK-3 β* is activated and thus can phosphorylate the antiapoptotic molecule Mcl-1, thereby mediating its degradation.^{30,31} Neutrophils from patients with *G6PC3* deficiency have higher levels of nonphos-

phorylated *GSK-3 β* and phosphorylated Mcl-1 than do neutrophils from unaffected people, a finding suggesting that a decrease in antiapoptotic Mcl-1 accounts for increased apoptosis in *G6PC3*-deficient neutrophils. These alterations may be in part responsible for the phenotype of *G6PC3* deficiency.

Although we cannot rule out the possibility that additional mechanisms could contribute to the increase in apoptosis in *G6PC3*-deficient neutrophils, our data suggest that *G6PC3* acts by a pathway involving *GSK-3 β* to maintain the viability of neutrophils. Evidence of increased endoplasmic reticulum stress has previously been reported in patients with mutations in *ELA2*,^{17,29} and premature apoptosis of neutrophils is known to cause the phenotype of congenital neutropenia.^{12,16,17} Thus, *G6PC3* deficiency is another example of how increased apoptosis of neutrophil granulocytes can cause congenital neutropenia.

Five arguments support our claim that *G6PC3* deficiency causes neutropenia. Distinct biallelic *G6PC3* mutations were found in two pedigrees and seven singleton patients with congenital neutropenia; sequence analysis predicted that all four missense mutations are likely to affect the function of *G6PC3*; expression of wild-type *G6PC3* and *G6PC3* with the R253H mutation in yeast showed that the R253H mutation abrogates enzymatic activity; *g6pc3*^{-/-} knockout mice have neutropenia and increased myeloid-cell apoptosis; and the susceptibility to apoptosis in *G6PC3*-deficient myeloid cells was reduced by retroviral transfer of the wild-type *G6PC3* gene.

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Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

Supplement to: Boztug K, Appaswamy G, Ashikov A, et al. A syndrome with congenital neutropenia and mutations in *G6PC3*. N Engl J Med 2009;360:32-43.

Supplementary Methods

Patients and controls

The first pedigree of Aramean descent has four affected individuals and is referred to as SCN-I; a second Aramean pedigree has one affected individual and is referred to as SCN-II. After we identified the mutated gene in SCN-I and SCN-II, we sequenced that gene in the DNA from an additional 104 SCN patients without known mutations in *ELA2* or *HAX1*.

Genome-wide linkage analysis

We list 5 sets of “principles” or “rules of inference” that we used in our linkage analysis here, so that we may refer to them succinctly in **Supplementary Results**. There were some subtleties in this linkage analysis and it seems useful to write explicitly what principles justify our reasoning. Also, it is useful to distinguish which inferences are based on LOD score evidence, and which inferences are not.

1. *Marker order principle*. All markers are in the same order in every individual (chromosome). This principle is implicit in virtually every published linkage analysis, but our study happened to land in a region of chromosome 17 that includes a ~900kb inversion polymorphism¹. Therefore, it is not surprising that several markers are tied for position on the Marshfield map, one marker (D17S930) is out of order, and the set of available microsatellite markers is sparse. During an intermediate stage of the study, we considered the possibility that different individuals in SCN-I may have at least one of their copies of chromosome 17 containing the minor allele of the inversion polymorphism, but it turned out that the mutated gene is above (lower Mb position) the sometimes inverted segment, so we did not need to determine the marker order of each individual within the possibly inverted segment.

2. *LOD score principles*. Single-marker or multipoint LOD scores well over 3 give statistical proof of linkage. When the LOD score is maximized at a recombination fraction $\theta=0$, the most likely explanation of the data is that there are no recombinants. The exact LOD score threshold one can use to declare linkage is unimportant in this study because we have some markers with LOD scores over 4 and multipoint scores are higher (see also², Chapter 4).

3. *Recombinant principles*. Obligate recombinants are indicated by a LOD score of $-\infty$ at a recombination fraction of $\theta = 0$, and these can be used to define *absolute* boundaries of a linkage interval. When the LOD score at $\theta = 0$, is not $-\infty$, but is also not maximum, then this implies that either there is a recombination or more than the minimum number of disease-associated alleles need to enter the pedigree to explain the data, and one may therefore

conclude that a marker for which the LOD score is maximized at $\theta > 0$ defines a *likely* boundary of a linkage interval. (see also ², Chapter 4)

4. *Marker homozygosity principles.* Due to the consanguinity in SCN-I and SCN-II, we expected all the affected individuals to have homozygous marker genotypes at markers near the disease gene ³. However, the critical subtlety is that it is not necessary for all 5 affected individuals (4 in SCN-I and 1 in SCN-II) to be homozygous for the same allele to have the LOD score maximized at $\theta = 0$. One reason is that we cannot connect SCN-II to SCN-I, so the disease-associated allele in SCN-II could be different. A more subtle reason is that affected individual SCN-I/P4 does *not* have a known ancestor in common with SCN-I/P1, SCN-I/P2, and SCN-I/P3. Therefore, in any explanation of the data to suggest genetic linkage, a disease associated allele has to enter SCN-I in at least 2 different founders, and the standard definition of pedigree likelihood ² does not favor scenarios in which the 2 disease-associated alleles are identical. Therefore, we may (and did) observe markers at which the LOD score in SCN-I is maximized at $\theta=0$ and SCN-I/P1, SCN-I/P2, and SCN-I/P3 are all homozygous for one allele, while SCN-I/P4 is homozygous for a different allele. There are known consanguineous disease pedigrees that violate the marker homozygosity principles in various ways (see e.g. ^{4, 5}); therefore, the homozygosity principle should not be considered as decisive as the recombinant principles.

5. *Founder principle.* Even though we could not find a common ancestor for all five affected individuals, or even all four affected individuals in SCN-I, we expected that there was such a founder. Therefore, markers at which all affected individuals are homozygous for the same allele are preferred to other markers, and conversely markers at which different affected individuals are homozygous for different alleles define possible linkage interval boundaries. The extent to which the all affecteds with the same allele scenario is preferred cannot be quantified, so we avoid the use of the adjective “likely”, and this principle should be considered the weakest of the 5.

Amplification and sequence analysis of the *G6PC3* gene

The primers used for amplification of all exons of the human *G6PC3* gene were as follows: exon 1 forward: acgtgcctgcaacagtgg, exon 1 reverse: gaaggcccttgagacagttg, exon 2 forward: agtactctgtgtcctgcccg, exon 2 reverse: agaggctggctccttggtg, exons 3-4 forward: gctcttctcagccaggaaag, exons 3-4 reverse: ggagcgatgggaagatcc, exon 5 forward: ttctcttgccaagctgcac, exon 5 reverse: aatgaaccagagacacaccag, exon 6.1 forward: caaaacagaacatgggagtgg, exon 6.1 reverse: ggaaaggaaaggaggcac, exon 6.2 forward:

cctcaagtacaccctctggc, exon 6.2 reverse: agactactggagcagcctgg. PCR conditions were as follows: 1 cycle initial denaturation 95°C for 5 min and 30 cycles of 94°C for 30 sec, 60°C for 30 sec and 72°C for 1 min, followed by final elongation at 72°C for 10 min.

Analysis of the mutation c. G758A (p. R253H) in healthy controls was done by restriction fragment length polymorphism analysis using the enzyme Tsp45I. The nucleotide exchange c. G758A leads to the loss of a recognition site for Tsp45I in the PCR product generated with *G6PC3* primers exon 6.1 forward and exon 6.1 reverse. Therefore, after complete digestion with Tsp45I, homozygous wildtype individuals showed two DNA fragments (180bp and 310 bp), whereas in homozygous mutated individuals the PCR product of 490bp remained intact. Heterozygous individuals showed three bands (180bp, 310bp and 490bp) after enzymatic cleavage by Tsp45I.

The Genbank Id for *G6PC3* mRNA is NM_138387, and the corresponding protein sequence has id NP_612396; sequence positions given throughout the manuscript and Supplementary Information refer to these sequences.

Isolation of early myeloid progenitors from bone marrow

Whole bone marrow from patients and controls was subject to hypotonic lysis. Cells were stained using anti-CD9-APC (BD Biosciences), anti-CD14-PC7, anti-CD15-PE and anti-CD16-FITC (all from Beckman Coulter). Promyelocytes were sorted on a FACS Aria as CD9⁺CD14⁻CD15⁺CD16^{low} cells, as described previously⁶. Cytospin preparations were performed routinely and confirmed purity of promyelocytes of >85%.

Real-Time PCR analysis

Gene expression analysis was performed on a Lightcycler 2.0 (Roche) using a standard protocol. Primers used were: *G6PC3* fw: gtgtggctctggatcacctt; *G6PC3* rev: cctgtgatcatgcagtggtcc; *HSPA5/Bip/Grp78* fw: tgggaggtgtcatgaccaaac; *HSPA5/Bip/Grp78* rev: caggtgtcaggcgattctgg; *GAPDH* fw: ttcaccaccatggagaagc; *GAPDH* rev: tgatggcatgcactgtggc.

Determination of enzymatic activity of wildtype and mutant G6PC3

The complete open reading frames of wild type and mutant *G6PC3* were PCR amplified and cloned in pYES-cup1 (modified from pYES-NT (Invitrogen) as described in⁷) with a C-terminal His tag. Three independently transformed *Saccharomyces cerevisiae* colonies were selected for each construct including an empty vector control. The 100,000 g microsomal fractions were isolated as published⁷ except that microsomes were resuspended

in 10 mM 3-(N-morpholino) propane sulfonic acid (MOPS), 0.8 M sorbitol pH7 (400 µl / gram of cells). Equal expression was monitored by Western blot using anti penta-His. Fifty µl of microsomes were preincubated for 10 minutes on ice in 100 mM MOPS pH 6.5 in a total volume of 70 µl containing either 0.1% saponin or 0.5% Triton X-100. The reaction in which glucose-6-phosphate (G6P) is hydrolyzed to glucose was started by addition of [¹⁴C]G6P (MP Biomedicals) to a final concentration of 10 mM and transfer to 37°C for 10 min. To dissolve microsomes completely to allow separation by anion exchange, the Triton X concentration of both was brought to 1%. The product was separated over 1-X8 resin (200-400 mesh, acetate form, Biorad) retaining [¹⁴C]G6P. Free [¹⁴C]glucose was measured in the eluate by liquid scintillation. P-nitrophenylphosphate (pNPP) assays were done similarly, except that 10 µl of vesicles were used per assay and that production of pNP was monitored continuously by photospectrometry at 405 nm.

Immunoblot analyses

After incubation with the primary antibody, staining with HRP-conjugated goat anti-mouse antibody (BD Biosciences) was performed and blots were developed using a Chemiluminescence Kit (Pierce, Bonn, Germany) and images were captured on a Chemidok XRS Imaging System (Bio-Rad, Munich, Germany).

ER stress in peripheral neutrophils was induced using tunicamycin (1.5 µg/ml; Sigma) Intracellular glucose deprivation was performed using 2-deoxyglucose at 25 or 50mM (Sigma-Aldrich), respectively.

Apoptosis assays

Peripheral blood neutrophils were isolated and exposed to TNF-α (50 ng/ml), thapsigargin (10µM) or tunicamycin (5 µg/ml; all from Sigma) to induce apoptosis. At different timepoints after induction of apoptosis, cells were stained with Annexin-V (Invitrogen) and propidium iodide (Sigma) and analyzed by flow cytometry. Caspase 3/7 activation was assessed using a commercially available kit as described previously⁸.

Apoptosis assays in fibroblast cell lines from patients and controls were performed after 24hr serum deprivation and subsequent induction of apoptosis using 5mM dithiothreitol (Roche) for 24 hours.

For apoptosis assays in *in vitro* differentiated myeloid cells, cells were harvested 6 days after induction of differentiation and starved of cytokines for 24 hours. Transduced cells were differentiated from non-transduced cells using an anti-mCD24-PE-Cy5 antibody (eBiosciences).

Flow cytometry data were analyzed using FlowJo software version 5.7.2 for Windows.

Functional neutrophil assays

Primary granulocytes were purified by Ficoll (Amersham Biosciences) density gradient centrifugation followed by hypotonic lysis, yielding a purity >95% as assessed by Giemsa-stained cytopsin preparations. Bacterial lysis capacity was assessed using a lactose permease-deficient *E. coli* strain (ML-35) as previously published^{9, 10}. Respiratory burst was assayed using commercially available dichlorodihydrofluorescein-BSA immune complexes (OxyburstTM assay, Invitrogen).

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Supplementary Results

To identify the underlying mutation of this previously unrecognized syndromal variant of SCN, we performed genetic linkage analysis. However, we could not trace back the SCN-I pedigree to a single founder couple, and we could not connect SCN-II to SCN-I. Therefore, the genetic linkage analysis was done under variable assumptions about which individuals are homozygous for the same mutation (see **Supplementary Methods**). After a putative linkage region was established in SCN-I, the pedigree SCN-II was included in the fine mapping process. A coarse genome scan of SCN-I suggested only regions on chromosomes 3 and 17, with multipoint LOD scores over 1. The region on chromosome 3 had a higher LOD score using genome scan markers only, but was associated with the affected individuals preferentially having a compound heterozygous genotype. Due to the homozygosity principles (see **Supplementary Methods**), we preferred the region on chromosome 17 bounded by markers D17S1299 (36.2Mb, 62.0cM) and D17S1290 (53.7Mb, 82.0cM), as each marker had two out of four affected individuals homozygous for the same allele, three out of four affected individuals were homozygous at one or both of the markers, and none of the unaffected individuals were homozygous at either marker (**Fig. 2a**.)

Fine mapping and LOD score computations established genetic linkage to the region, via the LOD score principles, with 4 consecutive markers showing single-marker scores over 3 in SCN-I alone, and 3 of these showing single-marker scores over 4 when combining SCN-I and SCN-II. Multipoint linkage analysis strengthened the results; for example, using D17S932, D17S950, and D17S806, the peak multipoint LOD score in SCN-I alone was 4.98, and the peak two-pedigree multipoint LOD score was 5.74.

Chronologically, we first established genetic linkage in SCN-I alone with a LOD score over 3 at marker D17S806 (43.2Mb, 66.9Mb). At that time, the genotyped markers defining the maximal linkage interval boundaries, via the first recombinant principle (see Methods), were still D17S1299 and D17S1290. However, on the lower side, D17S787 (50.6Mb, 75.0cM) gave a better boundary, using the second recombinant principle that the LOD score should be maximized at $\theta=0$ in the minimal linkage interval. The LOD score for D17S787 is maximized at $\theta=0.06 > 0$ because the affected individual SCN-I/P4 (**Fig. 2a**) has a heterozygous genotype at D17S787, forcing more than the minimum number of disease-associated alleles to enter the pedigree.

Using the annotated human genome build 36, we determined that there were 258 genes with mRNA evidence between D17S1299 and D17S787. We continued fine mapping, and also commenced sequencing some of these 258 genes that appeared to be promising

functional candidates to be mutated in SCN. Because the two types of experiments were done in parallel, some of the genes sequenced were later shown to lie outside the most preferred subinterval for linkage; however, none of the sequenced genes lie outside the maximal interval defined solely by obligate recombinants.

In attempt to further shrink the preferred linkage interval, we made inferences from the fine mapping genotypes (syllogisms are explained in **Supplementary Methods**). Using only the first principle of recombinants on SCN-I only, the maximal linkage interval was reduced on both sides to being bounded by D17S1789 (39.1Mb, 63.1cM) and D17S941 (47.2Mb, 71.9cM), and this subinterval contained 145 genes. SCN-II yielded positive LOD scores in the upper part of this interval, but negative scores in the lower part due to an apparent crossover between markers D17S950 (40.6Mb, 63.6cM) and D17S791 (42.2Mb, 64.2cM). As explained above, we expected that SCN-I and SCN-II would have the same gene mutated. Using this assumption and the LOD score and homozygosity principles, the combined likely linkage interval was shrunk to being bounded by D17S1789 (39.1Mb, 63.1cM) and D17S791 (42.2Mb, 64.2cM), which contained only 55 genes. Within that 55-gene interval, at marker D17S950 (40.6Mb, 63.6cM), the affected individuals SCN-I/P1, SCN-I/P2, and SCN-I/P3 are homozygous for the 174bp allele, while the affected individuals SCN-I/P4 and SCN-II/P5 are homozygous for the 184bp allele (**Fig. 2a**). Moreover, at marker D17S934 (40.4Mb, 63.6cM), all four affected individuals in SCN-I are homozygous for the 105bp allele, while SCN-II/3 is homozygous for the 109bp allele. Therefore, by the founder principle (see Methods), we reasoned that the preferred linkage interval could be shrunk to being bounded by D17S1789 (39.1Mb, 63.1cM) and (40.4Mb, 63.6cM), which contained only 36 genes. Within this interval, we had two genotyped markers D17S930 (40.1Mb, 64.2cM (misplaced)) and D17S1861 (40.2Mb, 63.6cM) at which all 5 affected individuals were homozygous for the same allele and the LOD score was maximized at $\theta=0$. The mutated gene is one of the 36 genes in the preferred linkage interval.

Supplementary Table 1. Bone marrow differential from patients P1, P2, P4 and P5 before start of G-CSF treatment.

	P1	P2	P4	P5
Age at time of bone marrow aspiration	1 month	1 month	1 month	2 months
Blasts	0.5%	0%	2%	1%
Promyelocytes	8.5%	3%	9%	25%
Myelocytes	1.5%	4.5%	3%	2%
Metamyelocytes	0%	0%	0%	2%
Band neutrophil granulocytes	0%	0%	0%	0%
Segmented neutrophil granulocytes	0%	0%	0%	0%
Eosinophilic granulocytes	0%	0%	1%	0%
Basophilic granulocytes	0%	0%	0%	0%
Monocytes	10%	7.5%	11%	9%
Lymphocytes	23%	19.5%	40.5%	13%
Erythroid progenitors	56.5%	65.5%	33.5%	47%

Supplementary Table 2. Single-marker LOD scores between markers D17S1299 and D17S1290.

	Family	$\theta = 0.0$	$\theta = 0.05$	$\theta = 0.10$	$\theta = 0.15$	Best θ	Best Score
D17S1299 36.2Mb,62.0cM	SCN-I	$-\infty$	-0.12	0.10	0.11	0.13	0.12
D17S932 38.5Mb,63.1cM	SCN-I SCN-II Total	$-\infty$	0.73	0.79	0.71	0.09	0.71
D17S1789 39.1Mb,63.1cM	SCN-I SCN-II Total	$-\infty$ 0.68 $-\infty$	1.31 0.60 1.90	1.21 0.48 1.69	1.00 0.37 1.37	0.05 0.00 0.07	1.31 0.68 1.91
D17S930 40.1Mb,64.2cM	SCN-I SCN-II Total	1.78 0.33 2.11	1.52 0.26 1.78	1.26 0.21 1.47	1.01 0.16 1.17	0.00 0.00 0.00	1.78 0.33 2.11
D17S1861 40.2Mb,63.6cM	SCN-I SCN-II Total	3.63 0.89 4.52	3.18 0.75 3.93	2.72 0.62 3.34	2.27 0.49 2.77	0.00 0.00 0.00	3.63 0.89 4.52
D17S934 40.4Mb,63.6cM	SCN-I SCN-II Total	3.56 0.78 4.35	3.07 0.64 3.71	2.58 0.51 3.09	2.10 0.39 2.48	0.00 0.00 0.00	3.56 0.78 4.35
D17S950 40.6Mb,63.6cM	SCN-I SCN-II Total	3.18 0.93 4.11	2.73 0.79 3.52	2.30 0.65 2.95	1.90 0.52 2.42	0.00 0.00 0.00	3.18 0.93 4.11
D17S791 42.2Mb,64.2cM	SCN-I SCN-II Total	3.59 -2.00 1.59	3.10 -0.29 2.81	2.62 -0.09 2.53	2.15 -0.01 2.14	0.00 0.27 0.04	3.59 0.03 2.84
D17S806 43.2Mb,66.9cM	SCN-I SCN-II Total	3.86 -1.78 2.08	3.40 -0.51 2.89	2.94 -0.29 2.64	2.48 -0.18 2.29	0.00 0.50 0.04	3.86 0.00 2.90
D17S1827 44.0Mb,66.9cM	SCN-I SCN-II Total	2.05 -1.90 -0.46	1.73 -0.60 1.13	1.42 -0.35 1.07	1.12 -0.22 0.91	0.00 0.50 0.06	2.15 0.00 1.14
D17S943 45.2Mb,68.4cM	SCN-I SCN-II Total	1.44 -1.90 -0.46	1.27 -0.60 0.67	1.07 -0.35 0.72	0.87 -0.22 0.66	0.00 0.50 0.09	1.44 0.00 0.73
D17S1820 47.1Mb,71.9cM	SCN-I	1.84	1.63	1.41	1.19	0.00	1.84
D17S941 47.2Mb,71.9cM	SCN-I SCN-II Total	$-\infty$ -2.07 $-\infty$	1.58 -0.74 0.84	1.51 -0.45 1.06	1.32 -0.30 1.03	0.06 0.50 0.11	1.58 0.00 1.06
D17S787 50.6Mb,75.0cM	SCN-I	0.10	1.50	1.45	1.28	0.06	1.51
D17S1290 53.7Mb,82.0cM	SCN-I	$-\infty$	0.69	0.93	0.92	0.12	0.94

Supplementary Table 3. List of functional candidate genes on chromosome 17q21 which were sequenced without evidence of mutations leading to altered protein sequences. The genes are listed in their order along chromosome 17. Before embarking on the positional search, we sequenced *HAX1* and *ELA2* to rule out mutations in these previously established neutropenia genes.

Sequenced gene	GenBank accession number
<i>CD300LG</i>	NM_145273
<i>TMEM101</i>	NM_032376
<i>LSM12</i>	NM_152344
<i>ASB16</i>	NM_080863
<i>UBTF</i>	NM_014233
<i>SLC25A39</i>	NM_016016
<i>FZD2</i>	NM_001466
<i>DBF4B</i>	NM_145663
<i>NMT1</i>	NM_021079
<i>IMP5</i>	NM_175882
<i>CDC27</i>	NM_001256
<i>PHB</i>	NM_002634

Supplementary Table 4. Glucose, Lactate and Pyruvate levels in peripheral blood from G6PC3-deficient patients.

	P1	P2	P3	P5	Normal values
Fasting glucose [mmol/L]	4.2	4.2	4.9	4.0	3.9 – 5.5
Lactate [mmol/L]	0.88	0.97	1.41	0.98	< 2.5
Pyruvate [μ mol/L]	49	61	91	n.d.	
Lactate/Pyruvate ratio	18	16	15	n.d.	< 25

n.d., not done

Supplementary Figure 1. Full pedigree diagram of SCN-I.

Supplementary Figure 2. Skin phenotype of G6PC3-deficient patients.

Supplementary Figure 3. Defective enzymatic activity of G6PC3^{R253H} using the universal substrate pNPP.

This panel shows activity of the same three constructs as depicted in **Fig. 2c** using the universal substrate p-Nitrophenylphosphate (pNPP). Presented is the continuous pNP production over time in the presence of saponin (solid lines) and triton-X-100 (dotted lines). Green: wild type, Red: R253H, Blue: empty vector. Data represents the average of three independent microsomal preparations.

Supplementary Figure 4. Increased apoptosis in peripheral neutrophils from P10 and increased Caspase 3/7 activation.

Panel A shows increased spontaneous and TNF- α or Thapsigargin-induced neutrophil apoptosis in patient P10, as compared to a healthy control. Panel B shows increased caspase 3/7 activation (x axis in FACS histogram) upon induction of apoptosis in neutrophils from patients P2 and P3, as compared to a healthy donor with (HD1) or without G-CSF supplementation (HD2), respectively.

Supplementary Figure 5. Decreased tunicamycin-induced apoptosis in G6PC3-transduced myeloid cells from patients P2 and P4 at timepoints 16hr and 24hr post induction. Note that the data for P2 at 16 hours post induction are also shown as FACS plots in **Fig. 3c**.

Supplementary Figure 6. Assessment of neutrophil function in G6PC3-deficient patients

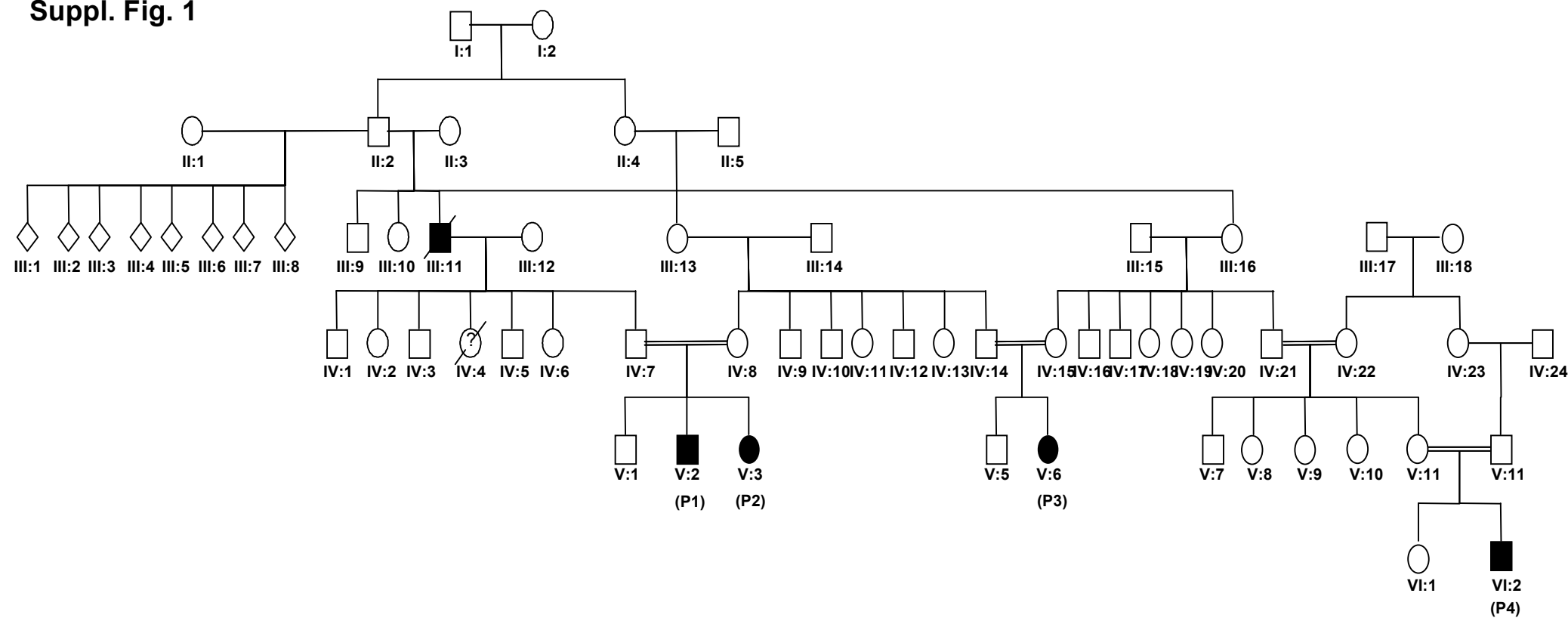
Panel A shows intact *E. coli* killing activity of patients (P2, P3) in comparison to healthy donors. Panel B shows normal respiratory burst formation in neutrophils from patients P1 and P3.

Supplementary Figure 7. Electron microscopy in myeloid progenitor cells from patients P2, P3 and P10.

Myeloid cells from Patients P2, P3 and P10 show the same abnormal phenotype of an enlarged endoplasmic reticulum (ER) indicative of enhanced ER stress as P1 (**Fig. 4**).

Supplementary Figure 8. Western Blot analysis of GSK3 β , Mcl1 and Bip expression in neutrophils from an additional G6PC3-deficient patient (P3).

Suppl. Fig. 1



Suppl. Fig. 2

P1



P1



P2



P3



P3



P6



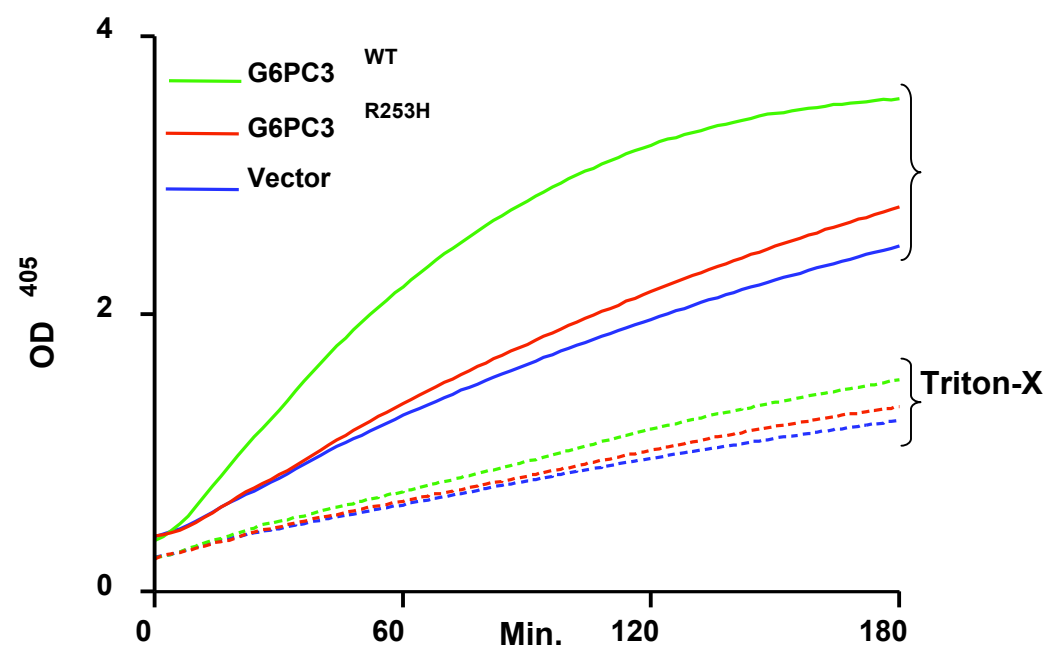
P6

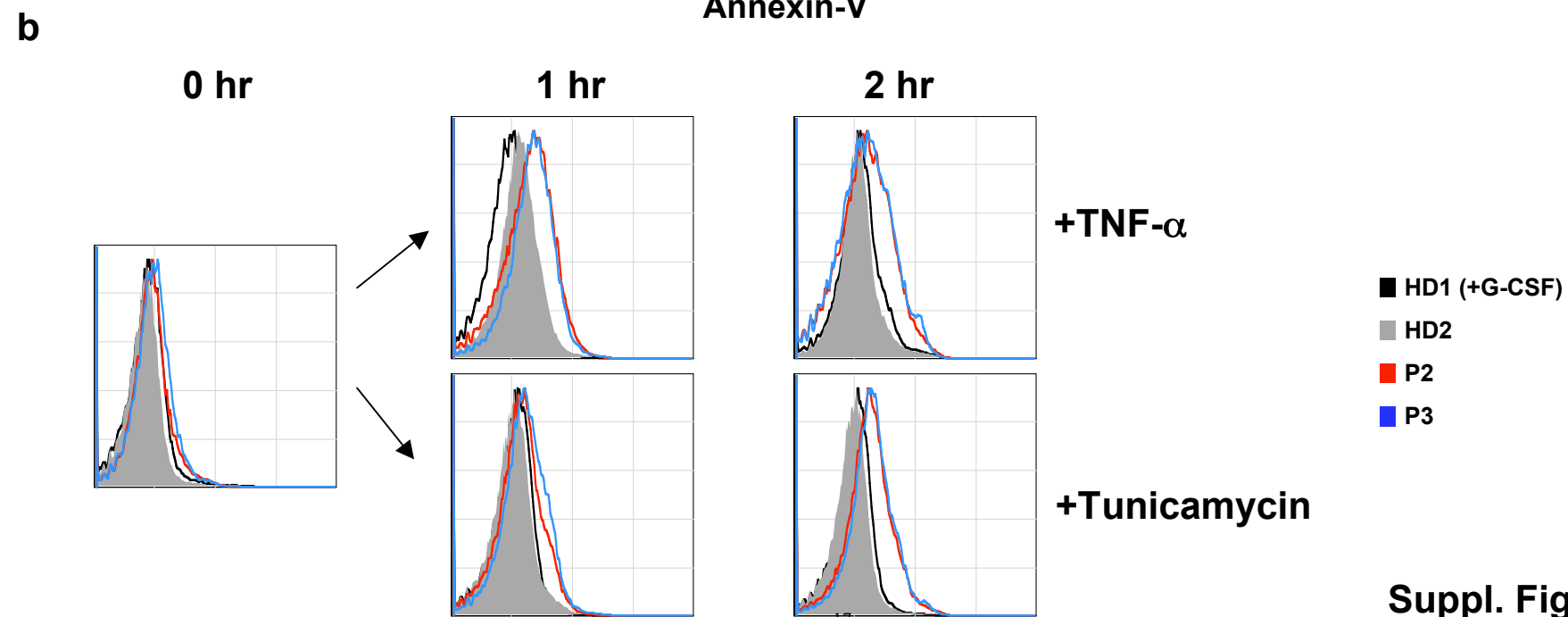
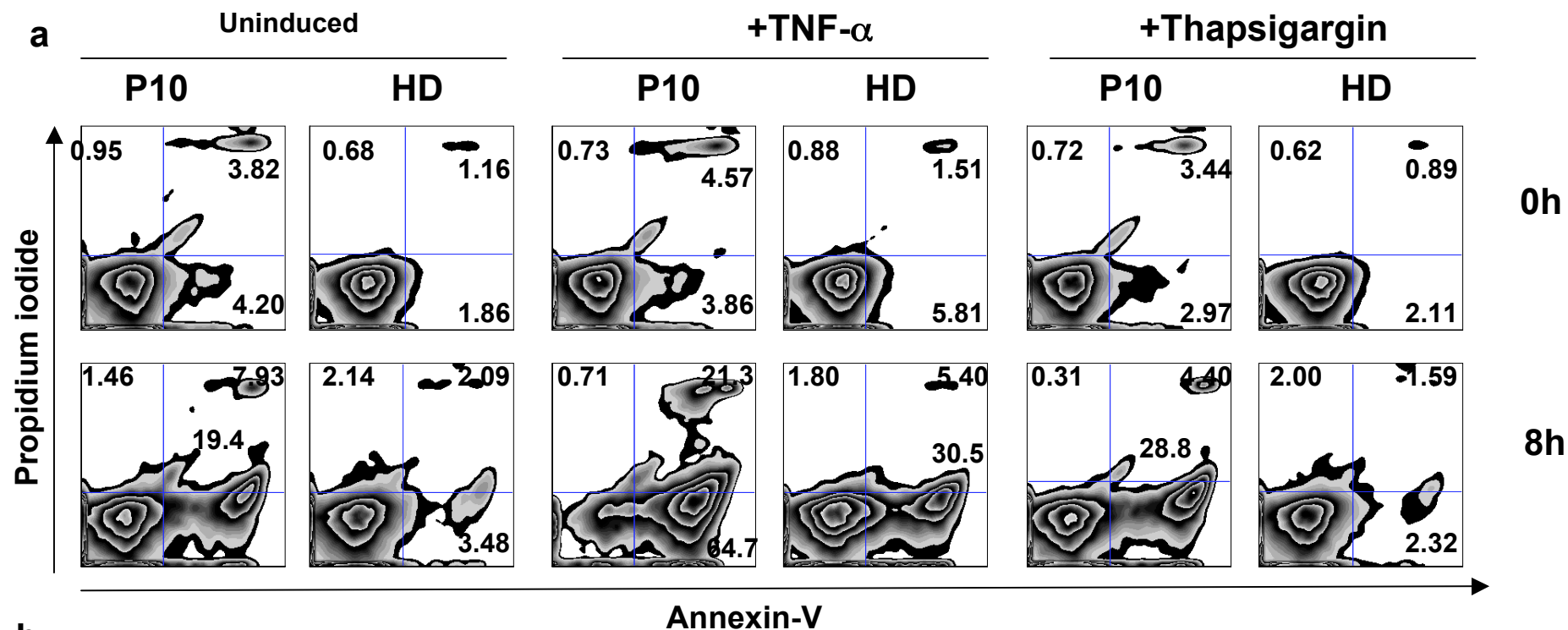


P10



Suppl. Fig. 3

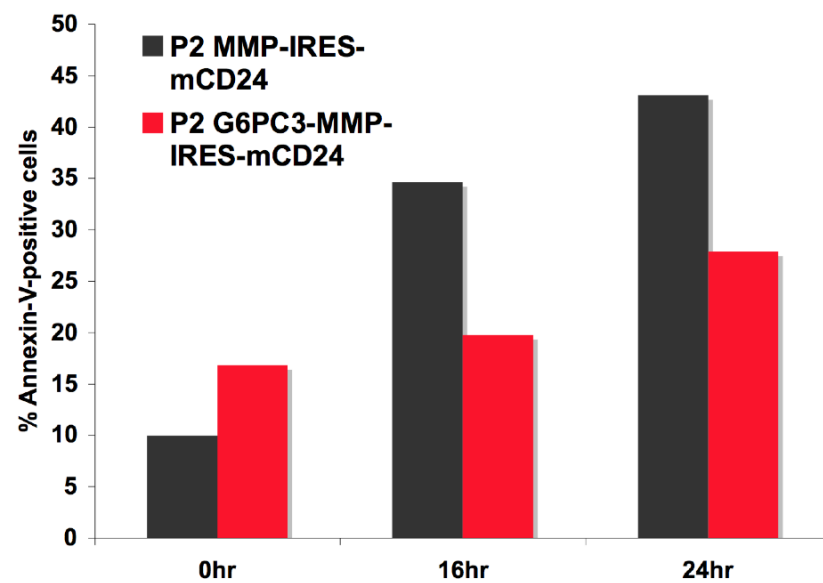




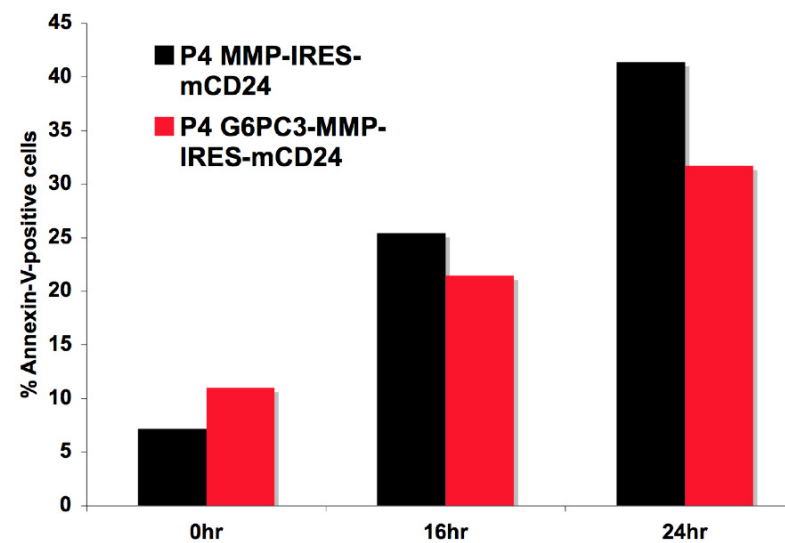
Suppl. Fig. 4

Suppl. Fig. 5

a

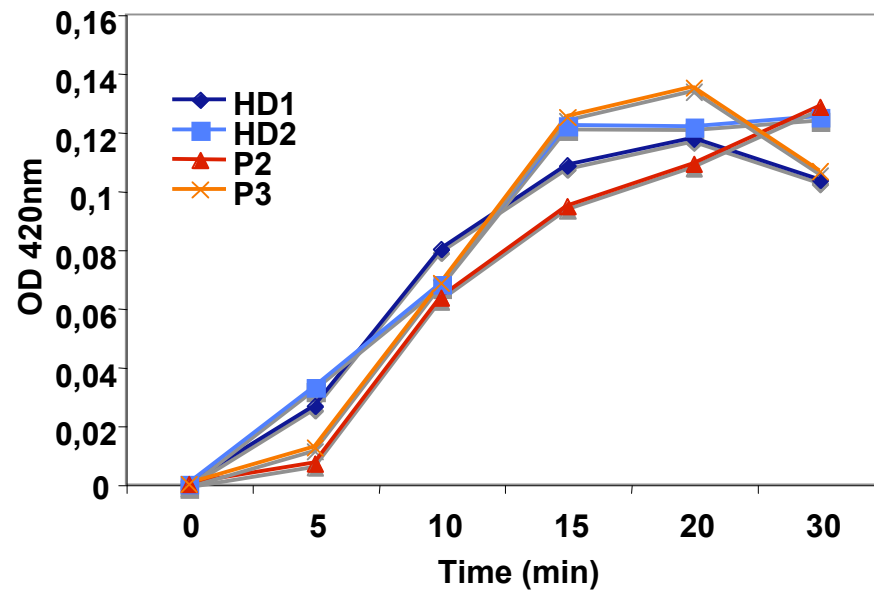


b

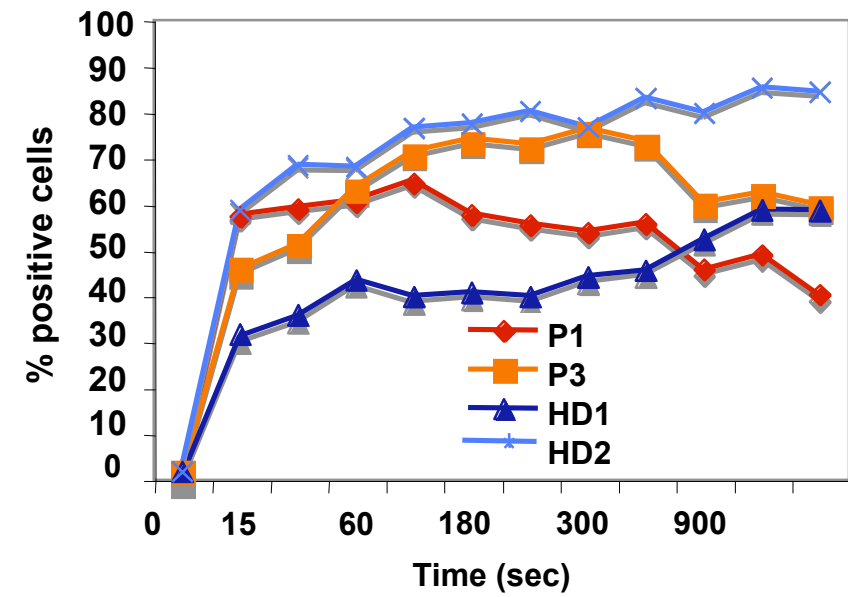


Suppl. Fig. 6

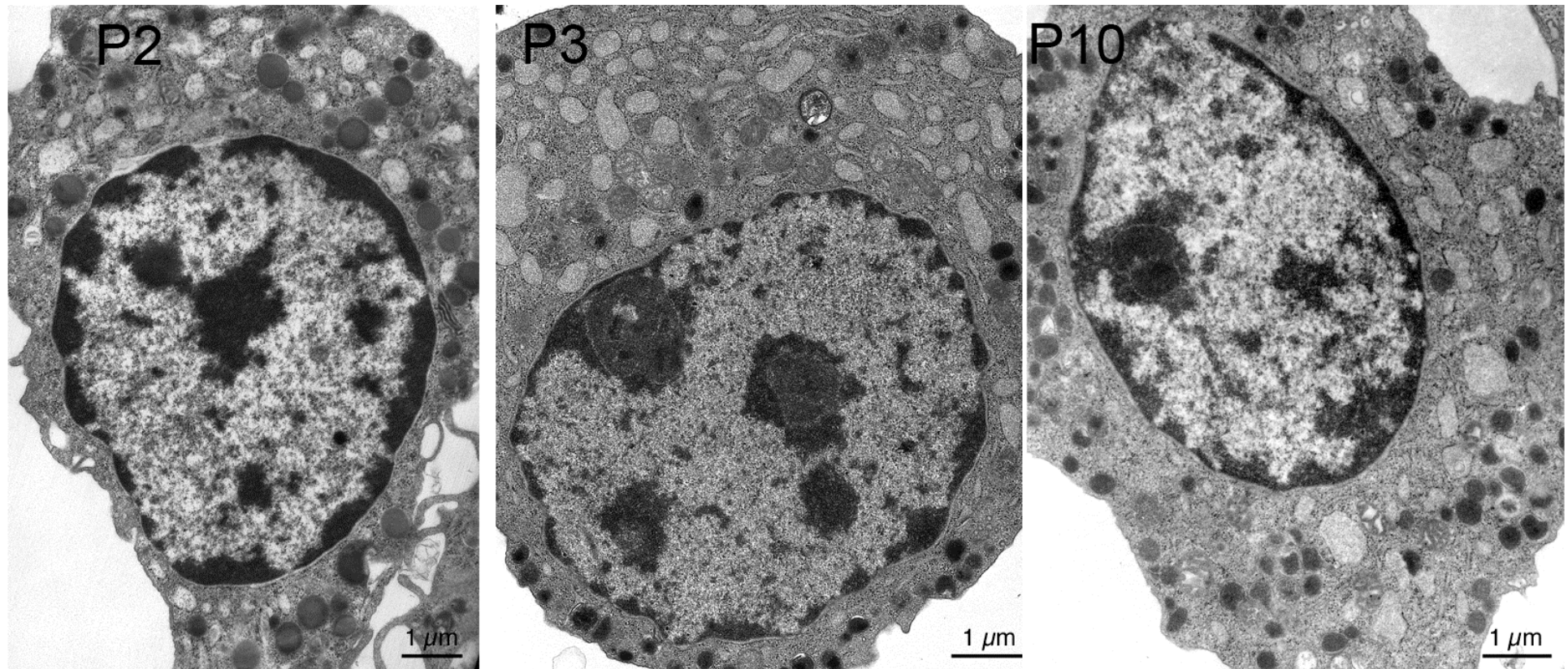
a



b



Suppl. Fig. 7



Suppl. Fig. 8

