Published in final edited form as:

Am J Med Genet A. 2010 December ; 152A(12): 3157–3163. doi:10.1002/ajmg.a.33748.

HAX1 Mutations causing SCN and Neurological Disease Lead to Microstructural Abnormalities Revealed by Quantitative MRI

Kaan Boztug^{1,*}, Xiao-Qi Ding^{2,*}, Hans Hartmann³, Lena Ziesenitz¹, Alejandro A. Schäffer⁴, Jana Diestelhorst¹, Dietmar Pfeifer⁵, Giridharan Appaswamy¹, Sonja Kehbel², Thorsten Simon⁶, Abdullah Al Jefri⁷, Heinrich Lanfermann², and Christoph Klein^{1,#}

¹Department of Pediatric Hematology/Oncology, Hannover Medical School, Hannover, Germany

²Department of Neuroradiology, Hannover Medical School, Hannover, Germany

³Department of Pediatric Nephrology, Hepatology and Metabolic Diseases, Hannover Medical School, Hannover, Germany

⁴Computational Biology Branch, National Center for Biotechnology Information, National Institutes of Health, Department of Health and Human Services, Bethesda, MD, USA

⁵Department of Hematology/Oncology, Core Facility II Genomics, Freiburg University Medical Center, Freiburg, Germany

⁶Department of Pediatric Oncology and Hematology, Children's Hospital, University of Cologne, Cologne, Germany

⁷Department of Pediatric Hematology/Oncology, King Faisal Specialist Hospital and Research Center, Riyadh, Saudi Arabia

Abstract

Biallelic mutations in the gene encoding HCLS-associated protein X-1 (HAX1) cause autosomal recessive severe congenital neutropenia. Some of these patients display neurological abnormalities including developmental delay, cognitive impairment and/or epilepsy. Recent genotype-phenotype studies have shown that mutations in *HAX1* affecting transcripts A (NM_006118.3) and B (NM_001018837.1) cause the phenotype of SCN with neurological impairment, while mutations affecting isoform A but not B lead to SCN without neurological aberrations.

In this study, we identified a consanguineous family with two patients suffering from SCN and neurological disease caused by a novel, homozygous genomic deletion including exons 4–7 of the *HAX1* gene. Quantitative MRI analyses revealed general alterations in cerebral proton density in both of the patients, as well as in an additional unrelated patient with another *HAX1* mutation (Arg86X) known to be associated with neurological manifestations. This study provides first *in vivo* evidence of general neurodegeneration associated with HAX1 deficiency in SCN patients.

Introduction

Severe congenital neutropenia (SCN) is an inherited bone marrow failure disorder characterized by a paucity of mature neutrophils, increased susceptibility to bacterial and

[#]To whom correspondence should be addressed: Christoph Klein MD PhD, Department of Pediatric Hematology/Oncology, Medical School Hannover, Carl-Neuberg-Straße 1, D-30625 Hannover, Germany, Klein.Christoph@mh-hannover.de, Phone: +49-511-532-6711, Fax: +49-511-532-9120.

*These authors contributed equally to this work

fungal infections and increased risk of development of myelodysplastic syndrome (MDS) or acute myeloid leukemia (AML) [Welte et al., 2006].

Several monogenetic defects causing SCN have been identified, but for some patients no genetic explanation has been found (reviewed in [Boztug and Klein, 2009; Dale and Link, 2009]). The majority of patients with autosomal-dominant SCN bear mutations in the *ELANE* gene (formerly known as *ELA2*) [Dale et al., 2000]. Biallelic mutations in the gene *HAX1*, encoding the anti-apoptotic protein HAX1 [Suzuki et al., 1997] have been identified as a cause for autosomal-recessive SCN [Klein et al., 2007]. HAX1 is a ubiquitously expressed, predominantly mitochondrial protein with a critical role in stabilization of the mitochondrial membrane potential [Klein et al., 2007]. Similar to other genetic subgroups of SCN patients, HAX1-deficient patients have increased myeloid cell apoptosis [Boztug et al., 2009; Carlsson et al., 2004; Grenda et al., 2007; Köllner et al., 2006; Massullo et al., 2005]. HAX1 appears to have other functions whose relevance to SCN etiology is unclear; different functions may be carried out by distinct HAX1 protein isoforms encoded by the *HAX1* gene [Fadeel and Grzybowska 2009; Lees et al., 2008].

Mice homozygous for a targeted deletion of *Hax1* suffer from severe neurodegeneration due to increased neuronal cell apoptosis [Chao et al., 2008]. Intriguingly, a fraction of HAX1-deficient patients, including descendants of the original Kostmann pedigree [Carlsson and Fasth 2001; Carlsson et al., 2008] also display neurological symptoms including cognitive impairment, severe neurodegeneration or epilepsy [Carlsson and Fasth 2001; Carlsson et al., 2008; Germeshausen et al. 2008; Ishikawa et al., 2008; Lanciotti et al., 2010; Matsubara et al., 2007; Rezaei et al., 2007]. Recently, it has become evident that human *HAX1* mutations affecting both isoform A (NM_006118.3) and isoform B (NM_001018837.1) lead to SCN with neurological involvement, while no obvious neurological abnormalities are found in patients in whom at least one *HAX1* allele carries a mutation affecting isoform A but not isoform B [Carlsson et al. 2008; Germeshausen et al. 2008].

An important stimulus for this study is that previous brain imaging studies [Carlsson and Fasth 2001; Carlsson et al., 2008; Germeshausen et al. 2008; Ishikawa et al., 2008; Lanciotti et al., 2010; Matsubara et al., 2007; Rezaei et al., 2007] did not identify any structural defects in HAX1-deficient patients, so the etiology of their neurological conditions has remained elusive. Here, we identified a consanguineous pedigree with two patients having SCN combined with a neurological phenotype, and we detected a homozygous genomic deletion in the *HAX1* gene. We applied newly established quantitative MRI methods that are sensitive to visually non-apparent microstructural changes [Ding et al., 2007] and could demonstrate alterations in proton density in the brain of these patients.

Materials and Methods

Subjects

All material from patients and healthy donors was obtained with informed assent/consent in accordance with the Declaration of Helsinki. This study was approved by the institutional review board at Hannover Medical School. Three patients, patient III-1 (P1, 28 year old female), patient III-8 (P2, 26 year old female), and one child patient (P3, 7 year old female) with *HAX1* mutations and neurological impairment, as well as three controls (two age-matched healthy adults C1 and C2, and a child with a *HAX1* mutation but no neurological impairment (CP3) were included in the MRI study. The ages indicated refer to the time when the patients were assessed at Hannover Medical School in 2008.

Genome-wide linkage analysis

We genotyped DNA samples from two affected individuals and ten unaffected individuals using the Affymetrix (Santa Clara, CA, USA) 250k *NspI* SNP mapping array, following the same procedures as in [Pfeifer et al., 2007] and recommended by Affymetrix.

The genotypes of the SNPs were called by the BRLMM (Bayesian Robust Linear Modeling and Mahalanobis distance) method (http://www.affymetrix.com/support/technical/whitepapers/brlmm_whitepaper.pdf), which is included in software provided by Affymetrix. This is supposed to be an improvement over the RLMM method proposed in [Rabbee and Speed 2006].

We used in-house software to identify large intervals where the two affected individuals are homozygous for the same genotypes at consecutive markers, and further evaluate whether the unaffected individuals have different genotypes for at least some markers in the interval [Glocker et al., 2009]. We computed LOD scores using the software FASTLINK version 4.1P [Cottingham et al. 1993; Lathrop et al. 1984; Schäffer et al. 1994] using markers of interest on chromosome 1. Neutropenia was modeled as a fully penetrant autosomal recessive disease with a disease allele probability of 0.001. The marriages of both pairs of carrier parents are known to be consanguineous (full genealogy not shown), so we hypothesize that the marriage of the grandparents was also consanguineous. As in previous studies [Hamada et al. 2002; Bohn et al., 2007], we added hypothetical ancestors to the pedigree to model the assumption that one ancestor common to all four carrier parents had the disease-associated mutation and haplotype. In this case we gave the four carriers a common grandparent. If the true common ancestor is more distant, as is likely, then any sharing of homozygous intervals between the patients P1 and P2 would be less likely and the LOD scores would be higher than what we report.

DNA extraction, PCR and sequencing

DNA of study participants was isolated by using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) or the Peqlab Gold Tissue DNA kit (Peqlab, Erlangen, Germany) according to the manufacturer's instructions. The coding region of *HAX1* (ENSG00000143575) was amplified using the following PCR conditions: initial denaturation at 95°C for 5 min, 35 cycles of 95°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min, and final elongation at 72°C for 10 min. Amplicons were purified using the QIAquick Gel extraction kit (Qiagen, Hilden, Germany) and sequenced with the ABI PRISM BigDye Terminator cycle sequencing ready reaction kit v3.1 (Applied Biosystems, Darmstadt, Germany). The primers (all purchased from MWG Operon, Ebersberg, Germany) used for amplification and sequencing of exons 1–2 have been described previously [Klein et al., 2007]; primers for exons 3–7 were as follows: Exon 3 forward GGGTTGGTGGGTGAAATAAA, Exon 3 reverse ACCGCACCTAGCCAGTC, Exon 4–5 forward GTTTTGGAGCTCGGGAGTAG, Exon 4–5 reverse TGCTCAGCAGTTTCTTTACACAG, Exon 6–7 forward GAACGAATGCCCTGAAACAG and Exon 6–7 reverse AATAAGGAGAAGGCAGAGGACC. Sequencing was performed using an ABI Prism 3130 DNA Sequencer (Applied Biosystems, Darmstadt, Germany); the sequence reads were analyzed with the DNA Sequencing Analysis software version 3.4 (Applied Biosystems).

Long-range polymerase chain reaction (PCR)

A long-range PCR protocol was established to detect the genomic deletion in the patients. Primers used were: HAX1 long range fw: TTCCCCTCACCCCTAAGACT; HAX1 long range rev: CTCCAGCCTGTGTGACAGAA. This PCR reaction should yield a PCR product of 5,936bp size in individuals without a genomic deletion contained in this region. PCR

conditions were as follows: 1 cycle initial denaturation 94°C for 5 min, 11 cycles of 94°C for 20 sec, 63.5°C for 30 sec and 68°C for 2.5 min and 20 cycles of 94°C for 20 sec, 60°C for 30 sec and 68°C for 2.5 min plus 10 sec per cycle, followed by final elongation at 68°C for 10 min.

Protein blot analysis

Western blot analyses were performed according to standard protocols. Primary antibodies used were anti-HAX1 (BD Transduction Laboratories, Germany) and anti-GAPDH (Santa Cruz Biotech, Germany) for loading control. HRP-conjugated anti-mouse (BD Transduction Laboratories) secondary antibody was detected using a chemiluminescent substrate (Pierce Technology/Thermo Fisher Scientific, Bonn, Germany). Images were captured on a Chemidoc XRS Imaging System (Bio-Rad, Munich, Germany).

MRI measurements

All MRI studies were carried out on a 1.5 Tesla MR system (Avanto, Siemens, Erlangen, Germany) with a standard head coil. The routinely applied MRI protocol consisted of a sagittal T1-weighted 3D-MPRAGE sequence, a transversal proton/T2-weighted turbo spin echo (TSE) sequence that acquires images at three different echo times (TR/TE1/TE2/TE3 = 3610/13/71/130ms), a transversal FLARE sequence, a coronal T2-weighted TSE, one single-shot spin-echo echo-planar imaging sequences (EPI) with diffusion gradients in three orthogonal directions ($b = 0, 500$ and 1000 s/mm^2) for diffusion-weighted imaging (DWI). The total scan time was about 25 min. MRI morphological findings were evaluated by two experienced neuroradiologists. **Parameter mapping:** Both relative proton density (PD = the proton density in patient in ratio the proton density of an aqueous phantom in %) and T2 relaxation time (T2) maps were obtained as described earlier [Ding et al. 2008]. Apparent diffusion coefficient (ADC) maps were obtained on-line from the DWI data via the software integrated into the scanner hardware. **Regions of interest analysis:** For each individual, numeric values of the T2, PD, ADC and FA of white matter (WM) and gray matter (GM) were measured in 16 pre-defined brain areas selected as regions of interest (ROIs) on corresponding parameter maps, and the values were compared between patients and matched controls by using a standard paired t-test. All ROIs were placed with reference to anatomic landmarks in order to exclude partial volume effect, and drawn manually as a square of $20 \pm 2 \text{ mm}^2$. Six white matter ROIs were chosen within bilateral frontal (fWM) and parieto-occipital white matter (pWM), and at genu (gCC) and splenium (sCC) of the corpus callosum. Ten gray matter ROIs were drawn bilaterally within caudate nucleus (CN), pallidum (Pal), putamen (Put), thalamus, (Thal) and occipital gray matter (GM). The numeric measurements of PD, T2, and ADC values in ROIs were carried out by using the free software ImageJ [Girish and Vijayalakshmi 2004]. Graphic plots of the measured PD, T2, and FA values and t-test were carried out with the software ORIGIN (Microcal Software, Inc., Piscataway, NJ, USA).

Results

We identified a consanguineous Turkish family with two affected children (Fig. 1a). Patient III-1 had been diagnosed with SCN at age 19 after relocation to Germany and has been treated using recombinant G-CSF since the diagnosis was established. Since the age of 3, a global developmental delay became obvious. The patient has impaired motor function without evidence of spasticity or ataxia or peripheral neuropathy, mental retardation, combined conductive and inner ear hearing loss and severe epilepsy, which has required medication with valproic acid. At age 28, clinical neurological examination showed clumsiness, peroneal weakness and reduced vibration sensitivity suggesting additional peripheral neuropathy. There were no signs of ataxia or spasticity.

Patient III-8 was diagnosed with SCN at age 5 and has been treated with G-CSF since age 9. This patient showed a motor developmental delay (ability to walk at 2 years of age), an inner ear hearing loss and mental retardation. At age 26, clinical neurological examination was normal except for clumsiness. There were no signs of ataxia or spasticity. Overall, the neurological phenotype in patient III-1 is considerably more severe than in III-8.

Affymetrix 250K SNP-chip analysis revealed a homozygous 1.28Mb interval spanning 151.7Mb-153.0Mb on chromosome 1 (human genome build 36) in which the affected individuals are homozygous for the same haplotype and the other twelve genotyped individuals are not (Fig. 1a). The next largest interval meeting those qualitative two criteria for perfect segregation spans approximately 0.6Mb. The largest perfect interval also caught our attention because it includes the gene *HAX1*. Using the SNPs rs4845578 (located at 152.096Mb) and rs11265607 (152.624Mb) and marker allele probabilities of 0.5 gives a two-marker LOD score of 2.6; using the highest (and hence most cautious) of the HapMap probabilities boosts the score to 3.1.

We sequenced *HAX1* in both index patients and could not identify any mutations in exons 1–3. However, we could not amplify exons 4–7 in repeated PCR reactions and thus speculated whether a homozygous genomic deletion may underlie this observation. Using primers that bind upstream of exon 1 and downstream of exon 7, respectively, we could show that both of the affected patients have a genomic deletion of size approximately 2.0kbp (Fig. 1b). Western blot analysis showed complete absence of HAX1 protein (Fig. 1c). DNA sequencing analysis of these products provided the precise location of the genomic deletion, which is 2,042bp in size (Fig. 1d). The entire deletion is located between two adjacent SNPs on the Affymetrix chip, and hence cannot be detected from the SNP genotype calls.

To assess in more detail the neurological disease in these patients, we performed 12 channel EEG. In both patients, this showed abnormal background activity with increased Beta activity and Theta activity during wakefulness which was attributed to sedation. In patient III-1, the EEG was pathologic with irregular frontal spike-wave paroxysms during sleep stage 1 (Fig. 2a). While a slight to moderate cerebellar atrophy was apparent in patient III-1, we could not detect overt changes in patient III-8 with conventional MRI (Fig. 2b). However, the results of quantitative MRI measurements revealed significant differences between the patients and the controls: A general reduction of cerebral proton density in patients was found both in white matter (27% in P1 and 10% in P2, $p < 0.01$) and gray matter (30% in P1 and 14% in P2, $p < 0.01$). A slightly prolonged T2 relaxation time was also found in patients P1 (~4%) and P2 (~6%), but not significant ($p > 0.01$). No significant differences between the patients and the controls were found using the ADC measurements.

To verify that the quantitative MRI alterations were a general feature of *HAX1* deficiency with neurological impairment, we had the opportunity to investigate an unrelated, 7-year old *HAX1*-deficient patient (P3) with a mutation in *HAX1* (homozygous c.C258T, p.Arg86X) that is known to lead to neurological involvement [Carlsson et al., 2008; Germeshausen et al. 2008]. When compared to an age-matched *HAX1*-deficient patient (CP3) with a mutation that affects exclusively transcript variant A and thus does not lead to a neurological phenotype (Trp44X), we found similar results as mentioned above: reduction of proton density both in cerebral white matter (9%, $p < 0.01$) and gray matter (11%, $p < 0.01$) were measured in P3, as well as prolonged T2 relaxation time (14% in WM, 7% in GM) in the absence of overt morphological abnormalities.

Discussion

In this study, we identified a consanguineous family with two affected family members suffering from HAX1 deficiency associated with neurological disorder. We could identify a novel genomic deletion including exons 4–7 as the genomic cause of this disease. Interestingly, two copies of a 27-nucleotide sequence occur at the boundaries of the deletion, which can form a hairpin structure and thus explain the occurrence of nonallelic homologous recombination [Inoue and Lupski, 2002]. Our study is in line with the finding that *HAX1* mutations affecting both transcripts A and B are associated with SCN and a neurological phenotype [Carlsson et al., 2008; Germeshausen et al. 2008]. Moreover, the observation that the specific neurological phenotype and degree of impairment differs in both patients suggests that additional factors, genetic or environmental, may influence the neurological phenotype.

As described above, the patients in this study showed inconspicuous findings in conventional MRI, except unspecific cerebellar atrophy in P1. However, we were interested to analyze whether there are visually invisible microstructural alterations in the patients when compared to age-matched controls. Using quantitative MRI measurements we found that in both adult patients (P1 and P2), a general reduction of proton density (PD) was evident, with nonsignificant increase of T2 relaxation. PD is known to be sensitive to certain MRI-visible pathological changes. For example, increased PD was observed in tumor [Just and Thelen, 1988], or in stroke after the insult [Gideon et al., 1999]. By contrast, little has been reported about alterations of PD in normal appearing brain tissue of patients with neurological diseases. An increase of PD was found in diet-treated phenylketonuria patients, which was suggested to be induced by increased brain cell-packing density [Ding et al., 2002]. In line with this report, we suggest that reduced PD in our patients are being caused by reduction of the neuronal cell density, consequently more free water and thus slightly increased T2 relaxation time [Ding et al., 2004]. Furthermore, these microstructural brain changes appear to correlate with the extent of neurological impairment, as the most severe alterations were seen in the patient with the most severe neurological phenotype (P1). An interesting observation is that in these patients the variations of PD were more pronounced in gray matter than in white matter, which implies that the pathological changes may be more pronounced in different areas of the brain.

This is the first study identifying measurable alterations in the brain of HAX1-deficient patients with neurological impairment, which may have important implications for the clinical care of these patients. Our method may also be relevant to patients with other syndromes in which clinically obvious neurological impairment is not accompanied by changes in conventional MRI.

Acknowledgments

We thank the families and patients for support of our studies and all clinical and nursing staff as well as the Severe Chronic Neutropenia International Registry (SCNIR) at Hannover Medical School for great support. This research was supported by grants from the Clotten Stiftung (CK), and the Fritz-Thyssen Foundation (KB and CK). This research was also supported by the Intramural Research Program of the NIH, NLM (AAS). K.B. is supported by a fellowship from the Else-Kröner-Fresenius-Stiftung.

References

- Bohn G, Allroth A, Brandes G, Thiel J, Glocker E, Schäffer AA, Rathinam C, Taub N, Teis D, Zeidler C, Dewey RA, Geffers R, Buer J, Huber LA, Welte K, Grimbacher B, Klein C. A novel human primary immunodeficiency syndrome caused by deficiency of the endosomal adaptor protein p14. *Nature Med.* 2007; 13:38–45. [PubMed: 17195838]

- Boztug K, Appaswamy G, Ashikov A, Schäffer AA, Salzer U, Diestelhorst J, Germeshausen M, Brandes G, Lee-Gossler J, Noyan F, Gatzke AK, Minkov M, Greil J, Kratz C, Petropoulou T, Pellier I, Bellanné-Chantelot C, Rezaei N, Mönkemöller K, Irani-Hakimeh N, Bakker H, Gerardy-Schahn R, Zeidler C, Grimbacher B, Welte K, Klein C. A syndrome with congenital neutropenia and mutations in *G6PC3*. *N Engl J Med*. 2009; 360:32–43. [PubMed: 19118303]
- Boztug K, Klein C. Novel genetic etiologies of severe congenital neutropenia. *Curr Opin Immunol*. 2009; 21:472–480. [PubMed: 19782549]
- Carlsson G, Aprikyan AAG, Tehranchi R, Dale DC, Porwit A, Hellström-Lindberg E, Palmblad J, Henter J-I, Fadeel B. Kostmann syndrome: severe congenital neutropenia associated with defective expression of Bcl-2, constitutive mitochondrial release of cytochrome *c*, and excessive apoptosis of myeloid progenitor cells. *Blood*. 2004; 103:3355–3361. [PubMed: 14764541]
- Carlsson G, Fasth A. Infantile genetic agranulocytosis, morbus Kostmann: presentation of six cases from the original "Kostmann family" and a review. *Acta Paediatr*. 2001; 90:757–764. [PubMed: 11519978]
- Carlsson G, van't Hooft I, Melin M, Entesarian M, Laurencikas E, Nennesmo I, Trebińska A, Grzybowska E, Palmblad J, Dahl N, Nordenskjöld M, Fadeel B, Henter J-I. Central nervous system involvement in severe congenital neutropenia: neurological and neuropsychological abnormalities associated with specific *HAX1* mutations. *J Intern Med*. 2008; 264:388–400. [PubMed: 18513342]
- Chao J-R, Parganas E, Boyd K, Hong CY, Opferman JT, Ihle JN. Hax1-mediated processing of HtrA2 by Parl allows survival of lymphocytes and neurons. *Nature*. 2008; 452:98–102. [PubMed: 18288109]
- Cottingham RW Jr, Idury RM, Schäffer AA. Faster sequential genetic linkage computations. *Am J Hum Genet*. 1993; 53:252–263. [PubMed: 8317490]
- Dale DC, Link DC. The many causes of severe congenital neutropenia. *N Engl J Med*. 2009; 360:3–5. [PubMed: 19118300]
- Dale DC, Person RE, Bolyard AA, Aprikyan AG, Bos C, Bonilla MA, Boxer LA, Kannourakis G, Zeidler C, Welte K, Benson KF, Horwitz M. Mutations in the gene encoding neutrophil elastase in congenital and cyclic neutropenia. *Blood*. 2000; 96:2317–2322. [PubMed: 11001877]
- Ding X-Q, Fiehler J, Kohlschütter B, Wittkugel O, Grzyska U, Zeumer H, Ullrich K. MRI abnormalities in normal-appearing brain tissue of treated adult PKU patients. *J Magn Reson Imaging*. 2008; 27:998–1004. [PubMed: 18425822]
- Ding X-Q, Kucinski T, Wittkugel O, Goebell E, Grzyska U, Görg M, Kohlschütter A, Zeumer H. Normal brain maturation characterized with age-related T2 relaxation times: an attempt to develop a quantitative imaging measure for clinical use. *Invest Radiol*. 2004; 39:740–746. [PubMed: 15550835]
- Fadeel B, Grzybowska E. HAX-1: a multifunctional protein with emerging roles in human disease. *Biochim Biophys Acta*. 2009; 1790:1139–1148. [PubMed: 19524642]
- Germeshausen M, Grudzien M, Zeidler C, Abdollahpour H, Yetgin S, Rezaei N, Ballmaier M, Grimbacher B, Welte K, Klein C. Novel *HAX1* mutations in patients with severe congenital neutropenia reveal isoform-dependent genotype-phenotype associations. *Blood*. 2008; 111:4954–4957. [PubMed: 18337561]
- Gideon P, Rosenbaum S, Sperling B, Petersen P. MR-visible brain water content in human acute stroke. *Magn Reson Imaging*. 1999; 17:301–304. [PubMed: 10215486]
- Girish V, Vijayalakshmi A. Affordable image analysis using NIH Image/ImageJ. *Indian J Cancer*. 2004; 41:47. [PubMed: 15105580]
- Glocker E-O, Hennigs A, Nabavi M, Schäffer AA, Woellner C, Salzer U, Pfeifer D, Veelken H, Warnatz K, Tahami F, Jamal S, Manguiat A, Rezaei N, Amirzargar AA, Plebani A, Hanneschläger N, Gross O, Ruland J, Grimbacher B. A homozygous *CARD9* mutation in a family with susceptibility to fungal infections. *N Engl J Med*. 2009; 361:1727–1735. [PubMed: 19864672]
- Grenda DS, Murakami M, Ghatak J, Xia J, Boxer LA, Dale D, Dinanuer MC, Link DC. Mutations of the *ELA2* gene found in patients with severe congenital neutropenia induce the unfolded protein response and cellular apoptosis. *Blood*. 2007; 110:4179–4187. [PubMed: 17761833]

- Hamada T, McLean WHI, Ramsay M, Ashton GHS, Nanda A, Jenkins T, Edelstein I, South AP, Bleck O, Wessagowit V, Mallipeddi R, Orchard GE, Wan H, Dopping-Hepenstal PJC, Mellerio JE, Whittcock NV, Munro CS, van Steensel MAM, Steijlen PM, Ni J, Zhang L, Hashimoto T, Eady RAJ, McGrath JA. Lipoid proteinosis maps to 1q21 and is caused by mutations in the extracellular matrix protein 1 gene (*ECM1*). *Human Molec Genet.* 2002; 11:833–840. [PubMed: 11929856]
- Inoue K, Lupski JR. Molecular mechanisms for genomic disorders. *Annu Rev Genomics Hum Genet.* 2002; 3:199–242. [PubMed: 12142364]
- Ishikawa N, Okada S, Miki M, Shirao K, Kihara H, Tsumura M, Nakamura K, Kawaguchi H, Ohtsubo M, Yasunaga S, Matsubara K, Sako M, Hara J, Shiohara M, Kojima S, Sato T, Takihara Y, Kobayashi M. Neurodevelopmental abnormalities associated with severe congenital neutropenia due to the R86X mutation in the *HAX1* gene. *J Med Genet.* 2008; 45:802–807. [PubMed: 18611981]
- Just M, Thelen M. Tissue characterization with T1, T2 and proton density values: results in 160 patients with brain tumors. *Radiology.* 1988; 169:779–785. [PubMed: 3187000]
- Klein C, Grudzien M, Appaswamy G, Germeshausen M, Sandrock I, Schäffer AA, Rathinam C, Boztug K, Schwitzer B, Rezaei N, Bohn G, Melin M, Carlsson G, Fadeel B, Dahl N, Palmblad J, Henter J-I, Zeidler C, Grimbacher B, Welte K. *HAX1* deficiency causes autosomal recessive severe congenital neutropenia (Kostmann disease). *Nat Genet.* 2007; 39:86–92. [PubMed: 17187068]
- Köllner I, Sodeik B, Schreek S, Heyn H, von Neuhoff N, Germeshausen M, Zeidler C, Krüger M, Schlegelberger B, Welte K, Beger C. Mutations in neutrophil elastase causing congenital neutropenia lead to cytoplasmic protein accumulation and induction of the unfolded protein response. *Blood.* 2006; 108:493–500. [PubMed: 16551967]
- Lanciotti M, Indaco S, Bonanomi S, Coliva T, Mastrodicasa E, Caridi G, Calvillo M, Dufour C. Novel *HAX1* gene mutations associated to neurodevelopment abnormalities in two Italian patients with severe congenital neutropenia. *Haematologica.* 2010; 95:168–169. [PubMed: 20065084]
- Lathrop GM, Lalouel J-M, Julier C, Ott J. Strategies for multilocus analysis in humans. *Proc. Nat. Acad. Sci. USA.* 1984; 81:3443–3446. [PubMed: 6587361]
- Lees DM, Hart IR, Marshall JF. Existence of multiple isoforms of HS1-associated protein X-1 in murine and human tissues. *J Mol Biol.* 2008; 379:645–655. [PubMed: 18472110]
- Massullo P, Druhan LJ, Bunnell BA, Hunter MG, Robinson JM, Marsh CB, Avalos BR. Aberrant subcellular targeting of the G185R neutrophil elastase mutant associated with severe congenital neutropenia induces premature apoptosis of differentiating promyelocytes. *Blood.* 2005; 105:3397–3404. [PubMed: 15657182]
- Matsubara K, Imai K, Okada S, Miki M, Ishikawa N, Tsumura M, Kato T, Ohara O, Nonoyama S, Kobayashi M. Severe developmental delay and epilepsy in a Japanese patient with severe congenital neutropenia due to *HAX1* deficiency. *Haematologica.* 2007; 92:e123–e125. [PubMed: 18055975]
- Pfeifer D, Woellner C, Petersen A, Pietrogrande MC, Franco JL, Yeganeh M, Ehl S, Matamoros N, Sprecher E, Puck JM, Veelken H, Grimbacher B. The hyper-IgE syndrome is not caused by a microdeletion syndrome. *Immunogenetics.* 2007; 59:913–926. [PubMed: 18000661]
- Rabbee N, Speed TP. A genotype calling algorithm for Affymetrix SNP arrays. *Bioinformatics.* 2006; 22:7–12. [PubMed: 16267090]
- Rezaei N, Chavoshzadeh Z, Alaei OR, Sandrock I, Klein C. Association of *HAX1* deficiency with neurological disorder. *Neuropediatrics.* 2007; 38:261–263. [PubMed: 18330843]
- Schäffer AA, Gupta SK, Shriram K, Cottingham RW Jr. Avoiding recomputation in linkage analysis. *Human Hered.* 1994; 44:225–237.
- Suzuki Y, Demoliere C, Kitamura D, Takeshita H, Deuschle U, Watanabe T. HAX-1, a novel intracellular protein, localized on mitochondria, directly associates with HS1, a substrate of Src family tyrosine kinases. *J Immunol.* 1997; 158:2736–2744. [PubMed: 9058808]
- Welte K, Zeidler C, Dale DC. Severe congenital neutropenia. *Semin Hematol.* 2006; 43:189–195. [PubMed: 16822461]

Figure 1.
(A) Pedigree from the consanguineous family described in this study. Patients III-1 and III-8 were diagnosed with severe congenital neutropenia with neurological impairment. (B) Long-range PCR demonstrating a genomic deletion in the *HAX1* locus of approximately 2kb in size. (C) Western blot analysis showing complete absence of HAX1 protein in patient III-1, similar to another HAX1-deficient patient with a W44X mutation in *HAX1* and in contrast to a healthy control. (D, E) Chromatograms showing the precise location of the genomic deletion identified in patients III-1 (P1) and III-8 (P2) compared to a healthy control. A 27 nucleotide repeat is normally present in intron 3 of the *HAX1* gene and downstream of exon 7. However, as illustrated, the genomic locus of HAX1 in the patients is altered and contains

only one occurrence of the 27 nucleotide sequence. This observation suggests formation of a hairpin between the repeat structures as the molecular basis underlying the genomic deletion that arose once in an ancestor of these patients.

Figure 2.

(A) Abnormal EEG in patient III-1 showing irregular frontal spike-wave paroxysms during sleep stage 1. (B) T2-weighted MRI images of the HAX1-deficient patients with neurological disease in this study (P1-P3) and the corresponding, age-matched controls (C1, C2 and CP3). No abnormalities were apparent on these MRI scans except a moderate cerebellar atrophy in P1. (C) and (D) Proton density was significantly decreased in the three patients when compared to the age-matched controls in different areas in the gray matter (C) and white matter (D).