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Genetic Heterogeneity in Severe Congenital Neutropenia: How Many Aberrant Pathways Can Kill a Neutrophil?

Alejandro A. Schäffer[1] and **Christoph Klein**[2]

[1]Computational Biology Branch, National Center for Biotechnology Information, National Institutes of Health, Department of Health and Human Services, Bethesda, MD 20894 USA

[2]Department of Pediatric Hematology/Oncology, Hannover Medical School, Carl Neuberg Strasse 1, 30625 Hannover, Germany

Abstract

Purpose of review—Severe congenital neutropenia (SCN) is a primary immunodeficiency in which lack of neutrophils causes inadequate innate immune host response to bacterial infections. SCN occurs with sporadic, autosomal dominant (AD), autosomal recessive (AR), and X-linked recessive (XLR) inheritance, as well as in a variety of multi-system syndromes. A principal stimulus for this review is the identification of novel genetic defects and pathophysiological insights into the role of neutrophil apoptosis.

Recent findings—Identification of mutations in HAX1 in autosomal recessive SCN (Kostmann disease), large epidemiological study estimating the risk of progression from SCN to leukemia, better understanding of how heterozygous mutations in neutrophil elastase (*ELA2*) cause SCN, molecular characterization of a novel syndromic form of SCN called p14 deficiency, and new animal models for several syndromic forms of SCN.

Summary—We consider the numerous genes mutated in SCN, many attempts to make animal models of SCN, and results from both human and mouse studies investigating the molecular mechanisms of neutrophil apoptosis. Investigations of how SCN genes and apoptosis pathways are connected should lead to better understanding of the pathogenesis of neutropenia and apoptosis pathways relevant to many cell types.

Keywords

severe congenital neutropenia; neutrophils; genetics; apoptosis; primary immune deficiencies

Introduction

This review is about severe congenital neutropenia (SCN), a primary immunodeficiency in which lack of neutrophils causes susceptibility to infections. Neutrophils are leukocytes in the innate immune system [1] that help their host fight infections using secreted molecules toxic to microbes, proteases to cut microbial proteins, and phagocytosis to ingest and degrade dying microbes [2]. Neutrophils are classified along with basophils and eosinophils as “granulocytes” because they possess interior granules including “primary” or “azurophilic granules” that generally sequester, and when needed secrete microbicidal peptides and proteinases such as cathepsin G, neutrophil elastase, and proteinase 3 [2]. Neutrophil “secondary” or “specific”

Correspondence author: Christoph Klein MD PhD, Department of Pediatric Hematology/Oncology, Hannover Medical School, Carl Neuberg Strasse 1, D30625 Hannover, Germany, Phone + 49-511-532-6718, Fax +49-511-532-9120, Email E-mail: klein.christoph@mh-hannover.de.

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granules contain lactoferrin, transcobalamin II, lysozyme, lipocalin, and some membrane proteins [2]. Neutrophils have a high turnover, since they are produced at a rate exceeding 10^6 cells/min/kg [3] and have an estimated half-life of under 12 hours in healthy, uninfected individuals [4,5]. When the host is fighting an infection, neutrophils die even more quickly [1,6]. Neutrophils die by apoptosis rather than necrosis, thus limiting tissue damage in the host [7]. In addition, a peculiar mechanism by which neutrophils launch “neutrophil extracellular traps” to kill bacteria and trigger their own suicide has recently been detected [8••].

Neutropenia definition

Neutropenia refers to the condition of having an inadequate number of neutrophils. Neutropenia is diagnosed by measuring the absolute neutrophil count (ANC) in peripheral blood and defined as an ANC below a commonly-used threshold such as 1500/μl; severe neutropenia is typically diagnosed when the ANC is below 500/μl, while some clinicians prefer the threshold 200/μl [9]. However, these thresholds were derived mostly by testing white individuals, and members of other ethnicities may have lower, yet protective ANC [9]. For the purpose of fighting infections what matters more are the microbicidal functions of neutrophils and the capability to rapidly ramp up the production of neutrophils in the bone marrow and their release into the bloodstream.

In adults, neutropenia is most commonly an acquired condition. Neutropenia is commonly seen in cancer patients being treated with chemotherapy that indiscriminately kills rapidly proliferating cells or in association with autoimmune disorders. In this review, we focus on severe congenital neutropenia (SCN), in which low ANC is observed from birth and it remains low, unless the patient is treated. The incidence of SCN has been estimated at 1/200,000 [3], but more epidemiological studies are needed.

Neutropenia treatment

SCN was almost always fatal in infancy until effective supportive treatment regimens became available. These include powerful antibiotics and recombinant human granulocyte colony stimulating factor (G-CSF) [10,11]. G-CSF both stimulates the production of more neutrophils [4,12] and delays their apoptosis [13,14]. The vast majority of SCN patients respond to G-CSF, with ANC increased toward the normal range and a concurrent reduction in infections [15, 16••]. Some patients also respond to a related hematopoietic growth factor, granulocyte-macrophage colony stimulating factor (GM-CSF) [e.g., 17], but some do not [18]. A recent in vitro study confirmed that G-CSF is more effective than GM-CSF at delaying neutrophil apoptosis and preserving the neutrophil chemotactic response to some cytokines; for example, G-CSF preserves the chemotactic response to interleukin 8, but GM-CSF does not [19•].

Although G-CSF keeps many SCN patients alive and functioning into adulthood [20], a recent study estimated that SCN patients die from sepsis at an elevated rate of 0.9% per year [16••]. Prolonged survival with SCN is associated with a substantially increased risk of acute myeloid leukemia (AML)[21]. The same study estimated the 10-year incidence of AML or related myelodysplastic syndrome at 21% [16••]. The occurrence of AML has been associated with somatic mutations in the gene *CSF3R*, which encodes the receptor for G-CSF [22–24]. Mermel et al. [25•] suggested that these somatic mutations are leukemogenic because they eliminate signaling to Src family kinases Hck and Lyn, which are negative regulators of granulopoiesis. A recent study found *CSF3R* mutations in newly diagnosed SCN patients who had not yet started on G-CSF treatment [24]. While this suggests that G-CSF treatment does not initiate the leukemogenesis, Sloand et al. [26•] suggested that G-CSF helps the progression to leukemia by favoring the proliferation of cells with a monosomy of chromosome 7 over healthy cells disomic for that human chromosome.

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Genetics and etiology of SCN

Neutropenia, as measured by low ANC, could be the consequence of: a) a defect in granulopoiesis b) a defect in moving the neutrophils from the bone marrow to the bloodstream or c) excessive apoptosis. Identifying genes mutated in the germline of SCN patients and studying the normal function of the encoded proteins is one strategy to recognize novel mechanisms of neutrophil homeostasis and/or function. Two stimuli for this review are the discoveries of mutations in the gene *HAX1* in some cases of nonsyndromic autosomal recessive neutropenia [27••] and the molecular characterization of a new neutropenia syndrome caused by a homozygous mutation in the gene p14 [28••]. Because HAX1 protein was known to be a negative regulator of apoptosis [29], the first of these findings has brought increased attention to the possibility that excess apoptosis is a central mechanism in the etiology of SCN [30]. Genetic heterogeneity and the role of apoptosis in the etiology of SCN are themes of this review. From this perspective, central questions in the molecular biology of SCN include:

1. Which genes mutated in SCN lead to excessive apoptosis of neutrophils?
2. In which apoptosis pathways do the encoded proteins participate?

In the next section, we explain the genetic underpinnings of these two questions by reviewing the molecular etiologies of syndromic and nonsyndromic SCN. Grenda and Link [31•] give a lengthier review of this topic. In this article, we define syndromic SCN as those clinical phenotypes that include a non-immunological aspect, while nonsyndromic forms have the phenotype confined to the immune response. We only use the childhood phenotype (e.g., not including progression to leukemia) to make this distinction. “Recent advances” are formally defined as those not published in print before 1 January 2006.

Molecular Causes of Syndromic and Nonsyndromic Severe Congenital Neutropenia

Both syndromic and non-syndromic SCN can occur in autosomal dominant, autosomal recessive, X-linked recessive, or sporadic forms. Table 1 summarizes genes currently known to be mutated in monogenic syndromic and non-syndromic SCN. A substantial fraction of non-syndromic SCN cases have no gene mutation identified, but quantifying this has been confusing because of clinical and geographic biases in ascertainment.

A variety of monogenic, multi-system syndromes including neutropenia as a symptom have been reported. New, rare neutropenia syndromes continue to be discovered [28••,49•]. We list many as “miscellaneous” because there are no known connections between the functions of the mutated genes. One exception to this confusing heterogeneity is a category of four syndromes (Hermansky-Pudlak syndrome type 2, Griscelli syndrome type 2, Chediak-Higashi syndrome, and the recently discovered p14 deficiency) that combine neutrophil dysfunction with hypopigmentation.

Autosomal dominant non-syndromic SCN

The most commonly mutated gene in non-syndromic SCN is *ELA2*, which codes for neutrophil elastase, a protease contained in the azurophilic granules [50•]. Heterozygous mutations in *ELA2* were discovered first in a related disorder called cyclic neutropenia, in which the ANC cycles from low to normal levels over a period of approximately 21 days [36]. Cyclic neutropenia is inherited as an autosomal dominant trait, and most cases appear to be associated with heterozygous mutations of *ELA2* [50•]. Subsequent to the discovery of *ELA2* mutations in cyclic neutropenia, *ELA2* mutations were also found in sporadic and familial cases of SCN [32]. To date, over 40 *ELA2* mutations have been found, including some which are associated with both phenotypes [50•].

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Before the discovery of *ELA2* mutations, dominant inheritance of SCN had been occasionally documented [e.g., 51], but was controversial. Recently, a striking example of dominant inheritance was reported where an anonymous sperm donor fathered five neutropenic children all of whom inherited the S97L mutation [52•]. It remains mysterious how the sperm donor escaped clinical attention, but one possibility is that he is mosaic for the mutation.

ELA2 was a plausible candidate gene for neutropenia because it encodes a known neutrophil protein, but both the complete function of neutrophil elastase and the mechanism of dominance are not fully understood. Neutrophil elastase cleaves bacterial proteins as one infection fighting mechanism. Neutrophil elastase may also bind to and cleave host proteins. One candidate for this role is N2N, a member of the Notch family of proteins, which has been shown to be cleaved by ELA2 in vitro [53]. Other candidates are G-CSF and G-CSFR [54]. Li and Horwitz [55] showed that a variety of heterozygous mutations in *ELA2* act in a truly dominant negative fashion, inhibiting the enzymatic activity of the protein encoded by the wild type allele. ELA2 with a disease-associated G185R mutation mis-localizes and accelerates apoptosis of differentiating early myeloid cells [56].

Two recent studies have added considerable insight to these earlier findings. Thusberg and Vihinen [57•] used 30 bioinformatics tools to predict, in silico, the effects of 32 missense mutations in *ELA2*. They found that mutations could: affect a conserved residue, change the electrostatic surface potential, affect critical contacts, cause the protein to become disordered, change the protein structure in other ways, but that there was no single cause that could explain all mutations. This finding is consistent with in vitro results of Köllner et al. [58•] showing that several different missense mutations could trigger the unfolded protein response causing the neutrophil with mutant ELA2 to die via apoptosis. We explore the apoptosis theme in a later section. The difficulty in understanding the mechanism of action of *ELA2* mutations stems from the lack of an adequate animal model, as discussed in the next section.

Another rare cause of autosomal dominant SCN is heterozygous mutations in the zinc finger domain of the transcription factor *GFI1* [33]. The original report showed that GFI1 protein binds to *ELA2* DNA and speculated that repression of ELA2 is related to the neutropenia.

X-linked recessive SCN

X-linked recessive SCN was associated with an activating missense mutation in *WASP* (Wiskott-Aldrich syndrome protein), initially in a single pedigree [34]. Recently, two more male SCN patients with mutations in *WASP* were reported, confirming the initial finding [35•]. As its name indicates, WASP was originally found to be mutated in a different immunodeficiency, Wiskott-Aldrich syndrome, for which the known mutations generally eliminate or reduce the protein activity. The hallmark symptoms of Wiskott-Aldrich syndrome are excessive bleeding secondary to (micro-) thrombocytopenia, recurrent infections, and eczema. A milder phenotype is seen in a condition termed X-linked thrombocytopenia (reviewed in [59]).

Autosomal recessive SCN

In contrast to dominant SCN, recessive inheritance in SCN was established over 50 years ago by Kostmann [60] who published a large pedigree with many cases and proved the inheritance mode by statistical tests. However, no gene for autosomal recessive SCN had been found until recently. One discovery that stimulates this review is that homozygous nonsense mutations in the gene *HAX1* explain some cases of autosomal recessive SCN [27••], including surviving descendants of the original SCN pedigree published by Kostmann. A substantial fraction of recessive non-syndromic SCN cases do not yet have an associated mutation [27••, unpublished observation].

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The full name of HAX1 is "HS1 interacting protein 1" because HAX1 was first discovered via a yeast two-hybrid screen with HS1, which is a member of the Src family of kinases and plays roles in proliferation and apoptosis of lymphoid cells [61]. The 'X' comes from the "X-Gal colony filter assay", used in the yeast two-hybrid experiment. HAX1 was shown to localize to the mitochondria, and this was a useful clue to make *HAX1* a candidate gene for SCN. In SCN patients with two null mutations of HAX1, it is presumed that the HS1-HAX1 protein interaction cannot take place, but how this relates to the SCN phenotype is unclear. More pertinent to the SCN phenotype are the results showing that HAX1 is an anti-apoptotic protein that is cleaved by Omi/HTRA2 [29], as part of the mitochondrial apoptosis pathway, covered in a later section. This function of HAX1 suggested that patients with mutations should have a loss of mitochondrial membrane potential and consequent excessive neutrophil apoptosis, and this hypothesis was confirmed [27••].

Syndromes that combine SCN or neutrophil dysfunction with hypopigmentation

Some of the granules in neutrophils also belong to a more general class of lysosome-related organelles, sometime called "secretory lysosomes". Other cell types with secretory lysosomes include: cytotoxic T lymphocytes, platelets, and melanocytes [62]. The secretory lysosomes serve to sequester, transport, and secrete substances such as pigment and microbicidal peptides that could be toxic to the cell if released at the wrong time and place. When secretory lysosome proteins are defective, the phenotypic consequence may be a multi-system disorder affecting different cell types. When the syndrome affects melanocytes, the phenotypic consequence is hypopigmentation and that symptom is more obvious to clinicians, but less dangerous to the patient than any associated leukocyte symptoms. All known secretory lysosome defect syndromes are purely recessive. Here we focus on four such syndromes that combine hypopigmentation and either SCN or another neutrophil dysfunction: Hermansky-Pudlak syndrome (HPS), type 2; Griscelli syndrome (GS), type 2; Chediak-Higashi syndrome (CHS); and the recently described p14 deficiency.

The original syndrome described by Hermansky and Pudlak [63] consists primarily of hypopigmentation and prolonged bleeding times due to defective platelet granules. There are 8 known human genes each of which is mutated in a different recessive form of HPS, and these forms are numbered HPS1-HPS8 according to the chronological order of gene discovery. Only HPS2, which is caused by a mutation in the gene *AP3B1*, includes a neutrophil dysfunction. The dysfunction was originally characterized as neutropenia [38], but recent work extends this phenotype. The AP3B1 protein is part of a protein transport complex called AP3. Neutrophil dysfunction has not been associated with mutations in other members of the complex. It is unknown what AP3 cargo is most relevant to neutrophil count or function, but Benson et al. [64] suggested that neutrophil elastase is an AP3 cargo in both dogs and humans. Support for this hypothesis comes from the observation that when G185R mutant ELA2 mis-localizes, the AP3 complex also mis-localizes [56]. Recent findings on HPS2 include: 1) extension of the phenotype to include dysfunction of other cell types such as NK cells, NKT cells and some antigen presenting cells [65,66]; 2) description of a patient whose phenotype also included hemophagocytic lymphohistiocytosis and whose genotype also included a heterozygous mutation in *RAB27A*, the gene mutated in GS2 [67]; and 3) positive resolution of a conjecture of Huizing et al. [68] that the affected members of a pedigree described by Kotzot et al. [69] have a form of HPS2 and not a different hypopigmentation-SCN syndrome [66].

GS2 is also characterized by albinism and (intermittent) neutropenia. Unlike HPS2, the platelet granules in GS2 patients look normal, but the platelet count may be reduced [70]. GS2 patients are susceptible to infections both because of the neutropenia and defective function of cytotoxic lymphocytes. The protein deficient in GS2, RAB27A is part of a three-protein complex including myosin VA and melanophilin. Deficiency of either of the other two proteins is also

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associated with hypopigmentation, but is not associated with any immune dysfunction. Neutrophils in GS2 patients appear to be defective in bacterial killing [71]. Recent progress includes the discovery that RAB27A plays a role in the release of myeloperoxidase (MPO) from azurophilic granules of neutrophils [72•], and this could explain the functional defect in GS2 neutrophils.

CHS is characterized by hypopigmentation, a bleeding disorder, immunodeficiency partly due to neutropenia and partly due to defective natural killer cells [31•,73–75]. The most severely affected CHS patients also have a progressive neurological disease [31•]. In CHS, neutrophils and other secretory granule cells have mis-shaped granules with inclusion bodies [31•] In neutrophils, these defective granules are now known to be the azurophilic granules, essential for bacterial killing [76]. As noted in Table 1, some CHS patients have double mutations in the gene *LYST*. Karim et al. [77] showed both that there is a genotype-phenotype correlation among those CHS patients with LYST mutations, and that some CHS patients do not have *LYST* mutations. No second CHS gene or genetic locus has been found.

Recently a new rare syndrome combining neutropenia and hypopigmentation was characterized in a single family. This syndrome is tentatively called "p14 deficiency" because it is caused by a homozygous point mutation in the 3' untranslated region of the gene that encodes p14 [28••]. The patients do have a small amount of p14 and this likely explains why they can live, while p14-knockout mice are not viable [78•]. Other symptoms of p14 deficiency include short stature, hypogammaglobulinemia and reduced numbers of B cell subsets, and defective function of cytotoxic T cells, which contribute to a general immunodeficiency [28••]. p14 is an endosomal adaptor protein that acts as a scaffold when binding to MP1, enabling MP1 to participate in the ERK signaling cascade [79,80]. As for GS2, and CHS, neutrophils in p14-deficient patients are defective in bacterial killing [28••].

Miscellaneous syndromes that include neutropenia as a symptom

WHIM syndrome derives its name from the combination of symptoms: warts, hypogammaglobulinemia, infections and myelokathexis [81]. "Myelokathexis" means that neutrophils are not released properly from the bone marrow to the blood stream. Myelokathexis occurs also as a non-syndromic form of neutropenia [82], but no mutated genes have been found for non-syndromic myelokathexis. In the Introduction, we suggested that inadequate release of neutrophils to the blood stream might be a problem separate from granulopoiesis or apoptosis, but Aprikyan et al. [83] showed that myelokathexis is associated with excessive apoptosis and under-expression of the apoptosis inhibitor *bcl-x*. The hypogammaglobulinemia in WHIM syndrome is mild [81].Since neutrophils and immunoglobulins mainly protect against bacterial infections, it is surprising that infection with human papillomavirus (HPV) is common in WHIM syndrome, although immunity to other viral pathogens is normal [31•].

WHIM syndrome is unique among the syndromes we discuss in that inheritance of most cases is autosomal dominant [43,81]. These cases are usually associated with a heterozygous activating mutation in the C-terminal section of the chemokine receptor gene *CXCR4* [43]. No mutated gene has been found for sporadic or recessive cases of WHIM syndrome. The molecular pathogenesis appears to be due to damaged signaling between stromal-derived factor 1 (SDF-1) and CXCR4 [31•]. Three recent findings strengthen and clarify this hypothesis: 1) in an in vitro assay, addition of extra SDF-1 to WHIM patient cells eliminates excess apoptosis [84•]; 2) in a mouse model, G-CSF (known to be effective for WHIM syndrome patients) downregulates *CXCR4* expression and this stimulates release of neutrophils into the bloodstream [85•]; 3) human $CD34^+$ cells transduced with mutant CXCR4 showed increased engraftment into bone marrow associated with increased apoptosis upon transplantation into NOD/SCID mice [86•].

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Cohen et al. [87] described an autosomal recessive syndrome including obesity, hypotonia, mental deficiency, craniofacial anomalies, large incisors, limb anomalies, and spinal anomalies. Norio et al. [88] observed that in a majority of Finnish Cohen syndrome patients the phenotype also included chorioretinal dystrophy and SCN. A clinical consensus to address the phenotypic heterogeneity and including neutropenia as a primary symptom can be found in [89]. Despite the phenotypic heterogeneity, Cohen syndrome is genetically homogeneous . all patients have a double mutation of *VPS13B* (a.k.a. *COH1*) [44]. The human protein VPS13B is homologous to a yeast protein VPS13, which has a function in protein sorting and intracellular trafficking. However, there is no detailed explanation of how VPS13B deficiency causes the symptoms of Cohen syndrome, including the neutropenia.

Shwachman-Diamond syndrome (SDS) is autosomal recessive and is characterized by pancreatic insufficiency, failure to gain weight, skeletal abnormalities, and bone marrow dysfunction [90,91]. Most, but not all SDS patients have neutropenia [92]. Progression to leukemia is common [93]. The majority of SDS patients have two distinct mutations in the gene named *SBDS* [45,94]. A major recent advance was the characterization of the function of the orthologous yeast protein SDO1 (SBDS ortholog 1). SDO1 participates in a complex that facilitates release of Tif6 from pre-60S ribosomes in the process of their maturation [95••]. Understanding of the proteins in this complex led Menne et al. [95••] to propose that *EFTUD1* (elongation factor Tu GTP binding domain containing 1), which is the human ortholog of yeast *EFL1* (elongation factor like 1) may be the gene mutated in SDS patients who do not have *SBDS* mutations.

Glycogen storage disease (GSD) type I comprises at least two variants of an autosomal recessive syndrome that share the symptoms of glycogen accumulation in the liver leading to hypoglycemia, hepatomegaly, growth retardation, and other metabolic problems such as lactic aciduria. The disease is due to difficulty in converting glycogen to glucose. Type Ib was initially distinguished from type Ia by a metabolic test showing that patients with the much more common type Ia [96] have deficient activity of the enzyme glucose-6-phosphatase, while patients with type Ib have adequate glucose-6-phosphatase activity [97]. It was shown later that type Ib differs from type Ia in including neutropenia as a common symptom [98]. The gene mutated in GSD Ib is now called *SLC37A4* and it encodes a protein that transports glucose 6-phosphate from the cytosol to the endoplasmic reticulum (glucose-6-phosphate-translocase) [31•,37]. The cellular reason why neutropenia occurs in GSD Ib but not GSD Ia was elucidated via knockout mouse models, as explained in the next section. Why glucose and glycolysis may be more important to neutrophils than some other cell types is discussed in the section on apoptosis.

Barth syndrome is an X-linked recessive syndrome. Symptoms include: dilated cardiomyopathy, skeletal myopathy, carnitine deficiency, 3-methylglutaconic aciduria and neutropenia [99,100]. Two decades after its initial characterization, survival for Barth syndrome patients had improved dramatically due to recognition and treatment of the cardiac defects and the neutropenia [101]. Barth syndrome patients were found to have a deficiency of cardiolipin [101], which is a protein uniquely synthesized in the inner membrane of mitochondria [102]. The mutated gene, now called *TAZ*, was identified by positional cloning [46]. Female carriers of *TAZ* mutations have skewed X inactivation selecting against the chromosome with the mutated gene, which explains why they have no reported phenotype [103].

Cartilage hair hypoplasia was originally characterized by short stature and unusually kinked hair [104]. Susceptibility to viral infections and severe chickenpox are mentioned in the original report, but immunodeficiency was not considered a defining clinical finding [104]. Later studies recognized neutropenia as a recurrent finding and it was estimated that ¼ of Finnish

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CHH patients have neutropenia [105]. Cartilage hair hypoplasia (CHH) like Cohen syndrome is a rare recessive syndrome seen most commonly in the Amish and Finnish populations [104,105]. Another feature in common with Cohen syndrome is that there is substantial phenotypic variability, but locus homogeneity. All CHH patients have a double mutation in the RNA coding gene RMRP, which is transcribed to be a subunit of the RNAse mitochondrial RNA processing complex [47]. The phenotypic variability may be due to allelic heterogeneity, since some patients have two "mild" mutations, some more severely affected patients have one "mild" mutation and one null mutation, and no CHH patients have two null mutations [31•]. The neutropenia in CHH appears to be due to a defect in granulopoiesis, not migration or apoptosis [31•].

Pearson's syndrome combines the symptoms of anemia, neutropenia, thrombocytopenia, pancreatic insufficiency, and a vacuolization of erythroid and myeloid precursor cells in the bone marrow [106]. Pearson's syndrome is unique among the diseases discussed here in that it is caused by deletions in the mitochondrial genome [48]. Just as for *ELA2* mutations in SCN, these deletions are usually new, and could occur in mitosis leading to germline mosaicism [31•].

Attempts to Make Animal Models

Animal models of SCN could provide opportunities to test the effects of specific infections and to do invasive experiments at a molecular level. Since neutrophils are part of the innate immune system, one might hope that neutrophil development and function would be similar in mice and humans. Non-mammalian models could be relevant since neutrophils exist in bony fish; similar cells, called "heterophils" exist in birds and reptiles [107]. Moreover, cells with phagocytic capability of neutrophils exist in many invertebrates; of historical note, Metchnikoff won the 1908 Nobel Prize for his studies of phagocytosis in starfish. Recently, fish and fruitfly models of Barth syndrome have been developed, but neither study examined neutrophils or other phagocytic cells [108,109]. Despite the deep evolutionary history of neutrophil-like cells, the development of animal models may be hampered by lineage-related specificity in the granule contents, perhaps due to co-evolution of vertebrate hosts and their common bacterial pathogens [1,107]. Attempts to make animal models, especially mouse models of human monogenic SCN, have yielded mixed results.

Table 2 summarizes some models for the disorders discussed in the previous section, and a few other models such as *Csf3−/−* and *Csf3r−/−* mice that are neutropenic and are relevant to human SCN because *Csf3* codes for mouse G-CSF and *Csf3r* codes for its receptor. The mouse models that best correspond to the human SCN phenotype are for some of the miscellaneous neutropenia syndromes such as: WHIM syndrome, Chediak-Higashi syndrome, and glycogen storage disease (GSD) Ib using respectively a xenotransplant model [86•], a natural mouse model [40], and engineered mice with a single gene knocked out unconditionally [119] or conditionally [120]. In the case of GSD Ib, the neutropenia documented in a recently developed mouse knockout of *G6pc3* [116] helps explain why GSD Ib includes neutropenia as a symptom, but GSD Ia does not. GSD Ia is caused by mutations in *G6PC,* which encodes glucose-6 phosphatase α, while the GSDIb gene, SLC37A4 encodes the glucose-6 phosphatase transporter. These two proteins interact in glycogen to glucose conversion in the liver and kidney, and deficiency of either one causes an equivalent GSD I phenotype in those cells. In neutrophils, the glucose-6 phosphatase transporter is present, but G6PC is replaced by a paralogous protein G6PC3 [116]. Therefore, Cheung et al. tested and confirmed the hypothesis that in neutrophils, knocking out either *Slc37a4* or *G6pc3* causes an equivalent neutropenic phenotype [116,120].

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Another gene for which knockout mouse models were helpful to understand a human form of SCN is *Gfi1*. That *Gfi1−/−* mice are severely neutropenic [114,115], led Person et al. to sequence *GFI1* in human patients and find heterozygous point mutations [33]. The phenotype of humans with heterozygous *GFI1* mutations is much milder than the knockout mice, but heterozygous *Gfi1+/−* mice are not neutropenic [114]. To our knowledge, no knock-in models of the point mutations in *GFI1* have been attempted.

Human SCN-associated mutations in *GFI1*, *WASP*, or *ELA2* appear not to be functionally null, and this has been especially problematic in the case of *ELA2*, the most commonly mutated gene in human nonsyndromic SCN. *Ela2* knockout mice have normal ANC, but poor neutrophil function [112]. The latter finding is consistent with a role for neutrophil elastase in bacterial killing [2,50•]. The former finding is consistent with the fact that no humans have been reported with a phenotype SCN and a genotype including mutations in both alleles of *ELA2*. Grenda et al. [113] engineered a mouse with a heterozygous *Ela2* mutation knocked in, but the mice do not have neutropenia. Thus, there is no animal model well-suited to studying heterozygous *ELA2* mutations.

A similarly discouraging situation exists for the hypopigmentation+neutropenia syndromes Hermansky-Pudlak syndrome, type 2 and Griscelli syndrome, type 2, even though some affected humans appear to have double null mutations in the genes *AP3B1* [68] and *RAB27A* [39]. *Ashen* mice, which have a natural homozygous null mutation in *Rab27a*, have a pigment defect, but no apparent immunodeficiency [122]. *Pearl* and *mocha* mice, natural mutants which have different components of the AP3 complex disrupted, also have a pigment defect, but no apparent immunodeficiency [e.g., 121]. AP3B1-deficient gray collies have a pigment phenotype and cyclic neutropenia, but not SCN [64].

Finally, we consider prospects for mouse models of HAX1 deficiency and p14 deficiency. The p14 situation appears challenging since the only known human mutation is in the 3' UTR and affected individuals have low levels of p14 protein [28••]. A constitutive knockout of the orthologous mouse gene is not viable [78•]. Since some amount of p14 seems necessary for development of many tissues, a strategy such as reducing expression levels by siRNA may be more effective than modifying the *p14* locus in the germline. For HAX1, all three known human mutations lead to premature stop codons in translation [27••], giving hope that a knockout of mouse *Hax1* could be relevant. Indeed, a *Hax1−/−* mouse was developed independently of and in parallel with the *HAX1* human gene hunt, but its phenotype does not recapitulate the phenotype of human SCN patients [J. Ihle, personal communication].

Apoptosis and Severe Congenital Neutropenia

The recent discovery of HAX1 mutations in SCN [27••] combined with the previous knowledge that HAX1 is an anti-apoptotic protein in the intrinsic/mitochondrial pathway of apoptosis [29] increases interest in the role of mitochondria in neutrophil apoptosis [123•]. The recent discovery of neutrophil extracellular traps [8••] emphasizes the need to study specific cell death pathways in neutrophils, which may not exist or work the same way in other cell types. There are two core molecular pathways for apoptosis. The "extrinsic" or "death receptor-dependent" pathway is initiated by signals outside the cell and a critical step is signaling via a transmembrane protein with a death receptor domain to caspase 8 inside the cell. The "intrinsic" or "mitochondrial" pathway is initiated by signals inside the cell and a critical step is release of cytochrome c from the mitochondria. The two pathways share the final step of signaling via caspase 3, but otherwise are mostly disjoint.

Neutrophils may be an attractive cell type in which to study apoptosis because the high turnover under normal physiological conditions means that there are lots of apoptosis events to observe and slight perturbations in apoptosis pathway signaling could lead to neutrophil phenotypes

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that would be hard to observe in longer-lived cells. Therefore, Table 3 summarizes some molecules whose apoptotic roles have been studied in neutrophils specifically. Many of these proteins, including HAX1, participate in the intrinsic/mitochondrial pathway.

The apparent importance of the mitochondrial pathway in neutrophils may be surprising because neutrophil mitochondria are few in number and hard to see [123•,124]. In many cell types, mitochondria play at least three important roles: housing the mitochondrial genome, generating energy in the form of ATP, and controlling apoptosis via the intrinsic pathway. The observation of mitochondrial genome deletions in Pearson's syndrome [48] suggests that the mitochondrial genome is essential to normal neutrophil homeostasis. Whether any proteins encoded by the mitochondrial genome are essential to control neutrophil apoptosis has not been studied, to our knowledge. Several studies suggested that ATP production in neutrophil mitochondria is unimportant because neutrophils get energy from glucose via glycolysis, which is necessary in the microbe-infested, hypoxic micro-environments where neutrophils do their killing [123•,124,139]. The reliance on glycolysis over mitochondrial respiration is seen also in eosinophils [140]. The importance of glucose to neutrophils can be understood via the phenotypes of human GSD Ib (Table 1) and mouse knockouts of *Slc37a4* and *G6pc3* (Table 2). Also in contrast to other cell types, neutrophil mitochondria may have neutrophil-specific roles in phagocytosis and chemotaxis [141].

Increased apoptosis in neutrophils, as for other cell types can be detected for example, by staining for annexin V binding to phosphatidylserine or by measuring mRNA or protein levels of caspase 3. However, these methods do not identify the pathway by which the apoptotic signal cascade reached caspase 3, and this explains some of the question marks in the Pathway column of Table 3. To distinguish mitochondrial pathway apoptosis one can stain with JC-1 and observe the loss of potential, denoted by $\Delta\psi_m$, in the mitochondrial inner membranes [e.g., 27••,141]. Increased mitochondrial apoptosis was seen in HAX1 deficiency [27••] and GSD Ib [132]. Increased apoptosis by other or unknown mechanisms has been seen in WHIM syndrome or models thereof [84•,86•] and dominant or cyclic SCN associated with *ELA2* mutations [58•, 130]. In contrast, *TAZ* mutations do not cause increased neutrophil apoptosis [142].

For most of the other genes listed in Table 1, apoptosis has not been studied in neutrophils from affected individuals. One intriguing gene in this category is *GFI1*. Person et al. [33] hypothesized that the transcription factor GFI1 is relevant to neutrophils via an interaction with the DNA for *ELA2*. However, GFI1 participates in the transcription of other genes, and Nakazawa et al. [143•] recently showed that the domain of GFI1 mutated in SCN plays a role in the repression of pro-apoptotic *Bax*, suggesting that *GFI1* mutations cause SCN via increased apoptosis, unrelated to ELA2 levels.

GSD 1b is one of the more appealing SCN syndromes to study because of the good match between the phenotypes seen in the human disease and animal models (Table 2). As Kuijpers et al. [132] observed excess apoptosis in cells from human patients, Cheung et al. [116] observed excess apoptosis in neutrophils of mice deficient in *G6pc3*. However, they arrived at possibly divergent conclusions about the pathways that are used in the apoptosis. Kuijpers et al observed translocation of Bax to the mitochondria, which is considered one hallmark of mitochondrial pathway apoptosis. Cheung et al. used Annexin V staining and caspase 3 assays, which cannot determine the pathway, but they speculate that the apoptosis arises due to the lack of glucose, which may cause stress to the endoplasmic reticulum [116]. This possibility is interesting because stress in the endoplasmic reticulum is what causes the unfolded protein response (UPR). Increased UPR has been demonstrated in mutant ELA2 cells [58•]. The unfolded protein response is a danger signal, not an inherent apoptosis signal, which can arise due to a variety of problems in the cellular environment, including low glucose [144]. It has been suggested that the low glucose is problematic in any cell because it interferes with N-

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linked glycosylation [144]. In neutrophils, low glucose may be an even more severe problem because of the reliance on glycolysis for energy.

In summary, the molecular analysis of patients with SCN has shown that several genes may lead to increased apoptosis of neutrophils, yet the exact pathways of apoptosis remain elusive. Furthermore, we do not yet fully understand how these mutated genes connect separate pathways of cell death.

Discussion

SCN is an exception to two paradigms in molecular medicine:

1. Identification of molecular causes of genetic diseases precedes the development of effective treatment, and detection of different mutated genes in different patients will lead to individualized treatments (sometimes called "pharmacogenomics").
2. When functions and genes are clearly conserved from fish or mice to humans, disrupting the orthologous genes in model organisms will lead to comparable phenotypes.

Table 1 lists 14 genes that can be mutated in a phenotype that includes neutropenia, yet there are few known connections between these genes or the proteins they encode. The genetic heterogeneity in SCN is so extreme that one affected descendant in the Kostmann family has an *ELA2* mutation [145], while the rest have a shared homozygous *HAX1* mutation [27••].

Identification of the genes mutated in human neutropenia could aid in the engineering and analysis of animal models to understand what the encoded proteins do normally, and what pathways are disrupted when the proteins are missing or mutated. For understanding some neutropenia genes and proteins, such as SBDS, studies in lower organisms such as yeast can be very useful [87••]. Since neutrophils are phagocytic cells and part of the more primitive innate immune system, one might hope for good correspondence between the phenotypes of mice and humans with orthologous mutations. Most encouraging are the excellent phenotypic correspondences between mice and humans with defects in glucose metabolism leading to either nonsyndromic SCN or glycogen storage type Ib. Discouraging is the discordance of phenotypes between mice and humans with heterozygous mutations of *ELA*2 or homozygous mutations of AP3B1.

The discrepancy between human and mouse models is also made more surprising by recent findings that increasingly implicate excessive apoptosis as a proximal cause of SCN. It remains to be explained why the apoptosis pathways affected in humans behave differently in mice. Another apoptosis-related neutrophil mystery is: Why does expedited apoptosis when neutrophils kill microbes lead to increased neutrophil production, but mutant-ELA2-associated apoptosis and HAX1-deficiency-associated apoptosis do not?

Possible explanations for these conundrums might be found by studying differentiation pathways in neutrophil development. For example, Skokowa et al. recently reported that the transcription factor *LEF1* (lymphoid enhancer-binding factor 1) has low levels of expression in SCN and that restoration of expression levels of *LEF1* leads to improved granulopoiesis in vitro [146••]. LEF1 controls expression of C/EBPα, an important transcription factor governing myelopoiesis. Moreover, *LEF1* can be speculatively connected to the transcription of several genes mutated in SCN; for example, the promoter of *ELA2* has a binding site for *LEF1* [147]. If the targets of LEF1 protein or the timing of *LEF1* expression differ between mice and humans this could help explain the phenotypic discrepancies.

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Another possible partial explanation for these conundrums is that the ANC is determined by poorly understood signaling pathways more so than by apoptosis pathways. Mathematical modeling suggests that cyclic neutropenia is related to a defect in one or more feedback loops [148]. The facts that mutations in *ELA2* can cause either SCN or cyclic neutropenia and AP3B1 deficiency causes cyclic neutropenia in dogs, suggest that feedback loops defective in cyclic neutropenia may be more severely damaged in and relevant to SCN. If G-CSF acts by amplifying a signaling pathway in which the mechanism of apoptosis is irrelevant, as modeled for example in [149], then this could explain why patients with so many different genetic causes of SCN benefit from G-CSF. Identifying the participants in such a signaling pathway could be aided by finding what genes are mutated in those SCN patients who do not respond to G-CSF. However, keeping those patients alive is a clinical challenge.

In sum, severe congenital neutropenia can be caused by defects in a variety of single genes and pathways. Surprisingly, many of these mutated genes, such as the recently identified *HAX1* [27••] and *p14* [28••], are expressed in a variety of cell types, not just neutrophils or just leukocytes or just immunity-related cells. The neutrophil specificity of the resulting phenotypes appears to be due to the high turnover and short lifespan of neutrophils, making them more sensitive to defective pathways of proliferation and especially apoptosis. Therefore, laboratory studies of naturally occurring human forms of SCN and engineered animal models are yielding new general molecular insights into birth and death of both neutrophils and other types of cells.

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Table 1

Genes mutated in human syndromic and non-syndromic congenital neutropenias, not including some genes mutated in combined immunodeficiencies. The references given here are to papers that identified the initially discovered mutation(s). Other papers describing the clinical phenotypes and additional mutations are cited in the text. Inheritance: AD= autosomal dominant; AR = autosomal recessive; XLR= X-linked recessive; M = mitochondrial. Gene symbols are the current HUGO Gene Nomenclature Committee-approved symbols with the exception of p14 that has no approved symbols; therefore, gene symbols may differ from gene names used in the cited papers.

Disease	Inheritance	Mutated Gene	Reference(s)	comments
Severe Congenital Neutropenia (SCN)	AD	*ELA2*	[32]	Mostly missense mutations
SCN	AD	*GFII*	[33]	Missense mutations in the zinc finger domain
SCN	XLR	*WAS*	[34,35•]	Missense mutations that lead to constitutive activation
SCN	AR	*HAX1*	[27••]	
Cyclic neutropenia	AD	*ELA2*	[36]	Mutations mostly different from those causing SCN
Glycogen storage disease type Ib	AR	*SLC37A4*	[37]	
Hermansky-Pudlak syndrome, type 2	AR	*AP3B1*	[38]	Other types of Hermansky-Pudlak syndrome do not include neutropenia
Griscelli syndrome, type 2	AR	*RAB27A*	[39]	Other types of Griscelli syndrome do not include neutropenia
Chediak-Higashi syndrome	AR	*LYST*	[40–42]	
p14 deficiency	AR	*p14*	[28••]	
WHIM syndrome	AD	*CXCR4*	[43]	Mutations truncate the cytoplasmic tail of the protein
Cohen syndrome	AR	*VPS13B*	[44]	
Shwachman-Diamond syndrome	AR	*SBDS*	[45]	Most patients have two compound heterozygous mutations
Barth syndrome	XLR	*TAZ*	[46]	
Cartilage hair hypoplasia	AR	RMRP	[47]	This is an RNA gene that does not code for a protein.
Pearson's syndrome	M	Mitochondrial deletion	[48]	Mutations in mitochondrial DNA

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Table 2

Animal models used to study the pathogenesis of the diseases listed in Table 1. This Table includes both naturally occurring and engineered mutants. This Table includes animals with mutations in genes orthologous to those in Table 1 as well as some animals with mutations in genes relevant to neutrophil development and apoptosis.

Organism	Gene mutation	Reference	Phenotypic comments
Mouse	*Csf3* null	[110]	Neutropenia, but ANC > 0
Mouse	*Csf3r* null	[111]	Neutropenia, but ANC > 0; expedited apoptosis
Mouse	*Ela2* null	[112]	Mice have poor neutrophil function, but normal neutrophil counts
Mouse	*Ela2* heterozygous knockin of V72M	[113]	Neutrophil count and function appear normal
Mouse	*Gfi1* null	[114,115]	Severe immunodeficiency, including neutropenia; heterozygote mice have no phenotype
Mouse	*G6pc3* null	[116]	Neutropenia
Mouse	*A1-a* (homolog of human *BCL2A1*) null	[117]	Neutrophils have enhanced apoptosis
Mouse	*Mcl1* conditional null in macrophages and neutrophils	[118•]	Neutrophils have excess apoptosis, but macrophages do not.
Mouse	*Slc37a4* null	[119]	Hypoglycemia, hyperlipidemia, neutropenia (similar to human GSDIb)
Mouse	*Slc37a4* knockout+transplant to bone marrow	[120]	Defects in neutrophil number and function
Dog (collies)	*AP3B1* null	[64]	Gray coat color and cyclic neutropenia
Mouse	*Ap3b1* null	[121]	This mouse is called *pearl* due to the hypopigmentation similar to that in human HPS2, but there is no mention of neutropenia or any immunodeficiency
Mouse	*Rab27a* null	[122]	The mouse is called *ashen* due to the hyopigmentation similar to that in human Griscelli syndromes, but there is no mention of neutropenia or any immunodeficiency
Mouse	*Lyst* null?	[40]	Name is *beige* and it resembles human CHS
Mouse	*p14* null	[78•]	Embryonic lethal
Mouse	Truncated *Cxcr4* xenotransplantion of human cells into NOD/ SCID mice	[86•]	Myelokathexis
Yeast	*SDO1/YLR022C* (homolog of SBDS)	[95••]	Deficiency in pre-60S ribosome maturation
Zebrafish	*TAZ* knockdown	[109]	Neutrophils not investigated
Drosophila	TAZ ortholog null	[110]	Phagocytic cells not investigated

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Table 3

Proteins related to apoptosis in neutrophils for which at least one neutrophil-specific study has been published. The pathway could be Intrinsic (a.k.a. mitochondrial), Extrinsic (a.k.a. death receptor), Both (meaning both Intrinsic or Extrinsic), or UPR (meaning unfolded protein response, whose relationship to the Intrinsic and Extrinsic pathways is uncertain). The human/mouse column records whether any of the studies cited in the references column were done on human cells or mouse cells. As suggested by the text associated with Table 2, inferences about pathways of human neutrophil apoptosis that rely on studies done exclusively on mouse cells should be made with extreme caution.

Protein	Pro or Anti(-apoptotic)	Pathway	Reference(s)	Mouse or Human
A1-a	Anti	Intrinsic ?	[117]	Mouse
Bax	Pro (when translocated to the mitochondria)	Intrinsic	[124–127]	Both
Bcl-x	Anti	Intrinsic	[83,118•,125]	Both
Bcl-2	Anti	Intrinsic	[30,118•]	Both
Bid	Pro	Intrinsic	[127,128]	Human
Bim	Pro	Intrinsic ?	[129]	Mouse
Calpain-1	Pro	Intrinsic	[126]	Human
Caspase 3	Pro	Both	[e.g., 14,116,126]	Both
CXCR4	Mutations enhance apoptotis	?	[84•]; (see [86•] for a partly contradictory study on a transplant model)	Human
ELA2	Mutations enhance apoptosis	UPR	[54,58•,130]	Human
G-CSF	Anti	Intrinsic (only?)	[14,19•,111,127]	Both
GM-CSF	Anti	?	[19•,131]	Both
G6PC3	Anti	UPR?	[116]	Mouse
G6PT (encoded by *SLC37A4*)	Anti	Intrinsic (only?)	[132]	Human
HAX1	Anti	Intrinsic	[27••]	Human
Mcl-1	Anti	Intrinsic	[118•,128]	Both
Omi/HTRA2	Pro, but see [133] for a more nuanced perspective	Intrinsic	[124]	Human
SBDS	Mutations may enhance apoptosis	Extrinsic	[134] contradicted by [135]	Human
Smac/DIABLO	Pro, but see [133] for a more nuanced perspective	Intrinsic	[124,126]	Human
TNF-α	Pro	Both	[124,136–138]	Both