Published in final edited form as:
Hematol Oncol Clin North Am. 2013 February ; 27(1): 19–41. doi:10.1016/j.hoc.2012.10.004.

ELANE Mutations in Cyclic and Severe Congenital Neutropenia —Genetics and Pathophysiology

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Abstract

There are two main forms of hereditary neutropenia: cyclic and severe congenital neutropenia (SCN). Cyclic neutropenia is an autosomal dominant disorder in which neutrophil counts fluctuate between nearly normal levels and close to zero with 21-day periodicity. In contrast, SCN, also known as Kostmann syndrome, consists of chronic and profound neutropenia, with a characteristic promyelocytic maturation arrest in the bone marrow. Unlike cyclic neutropenia, SCN displays frequent acquisition of somatic mutations in the gene, *CSF3R*, encoding the Granulocyte Colony-Stimulating Factor Receptor (G-CSFR), and a strong predisposition to developing myelodysplasia (MDS) and/or acute myeloid leukemia (AML). Cyclic neutropenia is caused by heterozygous mutations in the gene, *ELANE* (formerly known as *ELA2*), encoding the neutrophil granule serine protease, neutrophil elastase. SCN is genetically heterogeneous, but it is most frequently associated with *ELANE* mutations. While some of the different missense mutations in *ELANE* exhibit phenotype-genotype correlation, the same mutations are sometimes found in patients with either form of inherited neutropenia. The mutations lead to production of a mutant polypeptide, but no common biochemical abnormality, including effects on proteolysis, has been identified. Two non-mutually exclusive theories have been advanced to explain how the mutations might produce neutropenia. The mislocalization hypothesis states that mutations within neutrophil elastase or involving other proteins responsible for its intracellular trafficking cause neutrophil elastase to accumulate in inappropriate subcellular compartments. The misfolding hypothesis proposes that mutations prevent the protein from properly folding, thereby inducing the stress response pathway within the endoplasmic reticulum (ER). We discuss how the mutations themselves provide clues into pathogenesis, describe supporting and contradictory observations for both theories, and highlight outstanding questions relating to pathophysiology of neutropenia.

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Keywords

Cyclic neutropenia; Severe congenital neutropenia; *ELANE*; Neutrophil elastase; Granulocyte-colony stimulating factor (G-CSF)

Neutropenia

Normal neutrophil counts in peripheral blood typically range between 1500 to 8500 cells/μl after the age of one year[1], although they vary between individuals from different parts of the world and at different ages. "Benign ethnic neutropenia" has been used to describe the normally lower neutrophil levels observed in certain populations, including African-Americans and some Middle Eastern groups[2]. Within individuals, neutrophil counts fluctuate in response to environmental and host factors[3] as well as circadian cycles[4].

Heritability of neutrophil counts

Total white blood cell counts, which are largely reflective of the number of neutrophils, are moderately heritable[5]. The Duffy Antigen Receptor for Chemokine (*DARC*) gene, is at least partly responsible for the lower neutrophil counts observed in individuals of African ancestry[6–8] and is associated with resistance to malaria[7]. Several recent genome-wide association studies (GWAS) [9–11] confirmed association of neutrophil counts in people of African descent with single nucleotide polymorphisms in linkage disequilibrium with *DARC* on chromosome 1q and also identified a locus on chromosome 17q among individuals of European descent, in addition to other genetic regions. (It may be coincidental, but it is interesting that *HAX1* and *G6PC3*, whose mutations cause SCN, as described below, are located in proximity to the regions identified in GWAS on chromosomes 1q (nearby *DARC*) and 17q, respectively. However, the area of chromosome 19p where *ELANE* is located has not emerged in GWAS of neutrophil counts.)

Mendelian forms of neutropenia

In contrast to the genetically influenced benign variation in neutrophil numbers arising in the general population are distinct, so-called "Mendelian" neutropenic disorders exhibiting highly penetrant single gene autosomal dominant, autosomal recessive, or X-linked inheritance.

Historically, there have been two primary classes of hereditary neutropenia. The first is cyclic neutropenia (sometimes referred to as cyclic hematopoiesis), in which neutrophil counts oscillates with approximately 21-day periodicity, fluctuating between nearly normal levels and a nadir lasting several days that often reaches zero[12]. Early descriptions of cyclic neutropenia date to the first half of the twentieth century[13, 14]. The periodicity of the neutrophil cycling is exemplified by an unusual case[15] in which a child with acute lymphoblastic leukemia received an allogeneic bone marrow transplant from her sister, who, along with other members of the family, was afflicted with cyclic neutropenia. The recipient was cured of leukemia, but developed cyclic neutropenia, which she had not inherited at birth. Remarkably, the neutrophil counts for the two sisters subsequently cycled synchronously on the same days of the calendar. Monocytes also cycle in these patients, but their counts typically do so in a phase that is opposite to that of neutrophils. Because monocyte counts are typically much lower than neutrophils, the cycling is also less apparent, probably due to greater variation in sampling errors with serial blood counts. Nearly all cases of cyclic neutropenia are autosomal dominant or represent *de novo* autosomal dominant mutations in *ELANE*, which encodes neutrophil elastase[16].

Description of the second major type of hereditary neutropenia is generally attributed to Kostmann, who in 1956 described a now eponymous syndrome of non-cyclical "infantile agranulocytosis" observed among families residing in a remote area of northern Sweden[17–19]. Individuals with this disorder demonstrate non-cyclical severe neutropenia with a characteristic arrest of granulocytic differentiation at the promyelocyte stage evident upon bone marrow examination[20]. While a substantial portion of the disorder now most often referred to as SCN is the result of allelic, heterozygous (and therefore dominantly-acting) mutations in *ELANE* (that sometimes overlap with the mutations observed in cyclic neutropenia)[16], it is now known that SCN represents a genetically heterogeneous group of disorders[21]. The mutated genes include those encoding the HAX1, G6PC3, WAS, GFI1, STK4, and tafazzin proteins (Table 1).

Although indistinguishable in their degree of neutropenia compared to SCN caused by *ELANE* mutations, the various autosomal recessive or X-linked forms of SCN display non-hematologic clinical features depending upon the responsible gene[22]. For example, *HAX1*-associated SCN is frequently accompanied by neurodevelopmental complications, including decreased cognitive function and seizures[23]. Individuals with *G6PC3* mutations frequently have congenital heart disease and other clinical features[24]. Patients with mutations in *GFI1* display a range of alterations in lymphocyte numbers or function[25, 26]. In contrast, patients with either SCN or cyclic neutropenia resulting from *ELANE* mutations lack associated syndromic features not immediately relatable to disturbance of neutrophil numbers or function, presumably because expression of *ELANE*, in contrast to the case for *HAX1* or *G6PC3* and other genes, is mainly restricted to myeloid progenitor cells[27]. Recently, though, neutrophil elastase was found to be expressed in smooth muscle cells derived from neointimal lesions in mice and humans with pulmonary vascular disease[28].

An important clinical feature of SCN that largely differentiates it from cyclic neutropenia is the risk for disease progression to myelodysplasia (MDS) and/or acute myeloid leukemia (AML)[29–31]. In one series, the cumulative incidence of MDS/AML was 31%[31], and while leukemia is anecdotally observed with cyclic neutropenia[32], its occurrence is uncommon. Leukemic transformation arising with hereditary bone marrow failure syndromes is well-described and not unique to SCN; it is found at similar frequency, for example, in Shwachman-Diamond syndrome[33]. However, a unique feature of leukemic progression in SCN is the strong (although not completely invariant) association with acquired mutations of the gene, *CSF3R*, encoding the G-CSF Receptor[34]. (Although typically encountered as somatic mutations, there is at least one case report of SCN arising from germline CSF3R mutation[35].) While this is the subject of another chapter in this issue, there are concerns that treatment with recombinant human granulocyte colony-stimulating factor (rhG-CSF) elevates risk for malignant transformation in SCN[29], particularly in light of the strong association with somatic mutations of the G-CSF Receptor gene and the demonstration in mice that SCN-associated G-CSFR mutations confer clonal dominance only with administration of exogenous G-CSF[36].

Functional deficiency of neutrophils in addition to neutropenia

An often overlooked property of cyclic neutropenia and SCN is that there are likely functional deficiencies of the neutrophils that may contribute to the risk for infection even in addition to the neutropenia observed in such individuals. Electron microscopy of myeloid progenitor cells from SCN patients reveals ultrastructural abnormalities in the formation of neutrophil primary granules containing the hydrolytic enzymes responsible for effecting antimicrobial defense[37]. Neutrophils from SCN patients are deficient in antimicrobial peptides, including α-defensins[38]. Reduced abundance of transcripts encoding other neutrophil granule components is also reported[39, 40]. A largely successful form of treatment

of cyclic neutropenia and SCN involves administration of rh-GCSF, which increases neutrophil counts in most subjects to within the normal range. However, neutrophils recovered from the peripheral blood from treated SCN patients display abnormalities in the maturation of granules, and the neutrophils are functionally deficient in antimicrobial activity against fungal and bacterial pathogens[41]. This may be reflected in the types of organisms that are encountered by individuals with cyclic neutropenia and SCN, who may exhibit vulnerability to uncommon pathogens[42, 43]. On the other hand, the phenomenon is not necessarily a unique feature of SCN. When neutrophils are collected from CD34+ cells from peripheral blood from healthy donors then expanded *ex vivo* in the presence of a cytokine cocktail including G-CSF, the resulting neutrophils similarly exhibit reduced quantities of granule components, including neutrophil elastase, as well as limited bactericidal activity[44].

Osteoporosis[45] and other bone mineralization defects[46, 47] are seen in SCN, although they may be a side effect of treatment with rhG-CSF[48, 49]. G-CSF administration reduces bone mineral density by activating osteoclasts[50] and inhibiting osteoblasts[51]. Bone remodeling could conceivably also be secondary to effects of dysregulated neutrophil production and/or activation in the bone marrow microenvironment.

Discovery of mutations in ELANE in cyclic neutropenia and severe congenital neutropenia

In the late 1990's, our group in Seattle performed genome-wide analysis for genetic linkage in 13 families with multiple generations of individuals affected with cyclic neutropenia, using the then-current technology of microsatellite markers, distributed with mean separation of approximately 10 cM genetic distance[52]. In every family, we observed genetic linkage to markers mapping near the distal terminus of the short arm of chromosome 19, where obvious candidate genes included a cluster of three paralogous serine chymotryptic-type proteases, *ELANE* (then known as *ELA2*) encoding neutrophil elastase; *PRTN3* encoding proteinase 3, which is the target of "c-ANCA" (cytoplasmic antineutrophil cytoplasmic antibodies) autoantibodies associated with the rheumatologic disorder Wegener's granulomatosis; and *NAZC* encoding azurocidin, in which the catalytic serine has disappeared, resulting in a protein that no longer displays proteolytic activity. It is worth noting that these proteins display antimicrobial activity distinct from their enzymatic activity[53]. After sequencing this cluster of genes, we detected seven different heterozygous mutations in *ELANE* that segregated with cyclic neutropenia in each of the 13 families we studied[52].

We next evaluated *ELANE* as a candidate gene in 27 SCN unrelated cases[54]. In 21 of the 27 cases, we detected one of 15 different heterozygous *ELANE* mutations. Many of the cases occurred sporadically, but when DNA samples from other affected individuals in the family were available, in nearly all cases when there was evidence of parent-to-child transmission, consistent with autosomal dominant forms of inheritance, the same *ELANE* mutation was detected in all members of a pedigree. For the most part, *ELANE* mutations occurring in SCN patients are distinct from those found in patients with cyclic neutropenia. Nevertheless, at times the same particular mutation is found in individuals with decidedly clinically distinct forms of neutropenia, i.e. cyclic versus chronic. There are several possible explanations. First of all, the clinical evaluation of cyclic neutropenia can be challenging, as observing cycles requires frequent serial blood counts. Oscillation patterns may exhibit irregularity, may disappear entirely at times, and are influenced by therapy, particularly rhG-CSF treatment which tends to shorten the periodicity of cycling and increase the amplitude of the cycles, without abrogating cycling completely. Some individuals may cycle but exhibit low peak amplitudes resulting in a classification of SCN. Another possibility is that

the clinical assignment was made in light of knowledge of the mutation, so genotype-phenotype correlations could conceivably be biased as a result of knowledge of the particular mutation. Finally, there is some recent evidence to suggest that there could be modifier genes and that the modifiers might themselves include variants of *ELANE*, *HAX1*, and *G6PC3*[55]. In particular, two individuals with SCN who appeared to be heterozygous for a pathogenic mutation in *ELANE* were additionally heterozygous for either *HAX1* or *G6PC3* mutations. (Ordinarily, *HAX1* and *G6PC3* act recessively, and homozygous mutation is felt to be required to produce the SCN phenotype.) Another patient was a compound heterozygote for two different pathogenic *HAX1* mutations but was also heterozygous for a *G6PC3* mutation. Finally, one other SCN patient was heterozygous for both *G6PC3* and *HAX1* mutations. It is worth emphasizing that although recessive forms of SCN are rare, consideration of Hardy-Weinberg equilibrium indicates that carriers are markedly more common, raising the possibility that heterozygosity for *HAX1* or *G6PC3* is an incidental finding in these cases. In another study[56], two SCN patients had novel variants in *GFI1*, which can also be a cause of SCN (see below). In yet another study, an SCN patient was found to have two *ELANE* amino acid missense substitution mutations (V53M and V69M), both occurring in *cis* on the maternally inherited allele[57]. The mother however, exhibited only one of the variants (V69M, which has not apparently been seen before in either cases or controls) and was asymptomatic with normal neutrophil counts. (There was also a third variant within an intron whose parental inheritance, if any, was uncertain.) Thus, V53M must have arisen *de novo*. Interestingly, V53M had only previously been found in patients with cyclic neutropenia[16, 58]. The authors therefore concluded that the combination of V69M, which alone had no effect on granulopoiesis (as found in the mother), in combination with the V53M mutation, ordinarily producing cyclic neutropenia, yielded SCN with a particularly severe clinical course, including invasive pulmonary mycosis and development of MDS with progression to AML at age 8 years.

Another suggestion of modifier genes comes from an unusual case in which a healthy sperm donor fathered (at least) eight neutropenic children with six different women[59, 60]. Seven of the eight children had SCN whereas one of the children demonstrated evidence of neutrophil cycling. The authors interpreted this observation as indicating phenotype determination by modifying genes, given that all the affected children inherited the same paternal allele. (Sanger dideoxy DNA sequence analysis of the father's sperm was consistent with mosaic representation of the *ELANE* mutation. See further discussion of genetic mosaicism below.)

As noted, *de novo* mutation of *ELANE* is fairly common[16], more so among SCN than cyclic neutropenia cases. In fact, the occurrence of *de novo ELANE* mutation complicated the search for autosomal recessive forms of SCN. There are individuals with SCN residing in Sweden who are descendants of the cases first reported by Kostmann more than 50 years ago. The gene responsible for autosomal recessive SCN in this population, *HAX1*, was mapped by linkage analysis and positionally cloned by making use of this Swedish family as well as other families from ethnically isolated populations in the Middle East[61]. Four members of Kostmann's original family with SCN were studied. While three were found to be homozygous for the same *HAX1* mutation, one individual whose disease was thought clinically indistinguishable from that of his affected siblings (and therefore was attributed to the same ancestral gene) evidently lacked the segregating *HAX1* mutation and was reported to have a new *ELANE* mutation not present in his parents[62]. This is not a genetically surprising phenomenon, given that disorders with reduced reproductive fitness—noting that severe infectious complications, as well as the risk for leukemic progression, severely shorten life—must necessarily arise from new mutations, because affected individuals are less likely to survive to reproductive age. Nevertheless, it is possible that *ELANE* may represent a hotspot or new germline mutation. We have reported several cases where there have been two *de novo* substitutions occurring in *cis* on the same allele of *ELANE*,

representing a phenomenon that has only been extremely rarely reported for other disorders[63]. In the two cases we described where the origin of the mutation could be determined, the mutations had arisen on paternally-inherited alleles. This could possibly reflect vulnerability of this locus to mutational phenomenon, or, by analogy to study of *de novo FGFR3* mutations causing achondroplasia and other new mutations showing paternal bias, it could reflect selective advantage during male germ cell formation[64]. Arguing against that possibility, though, is the previously discussed case of another patient with two *cis ELANE* mutations[57]; one was *de novo* and one was inherited, but both came from the mother.

The phenomenon of *de novo* mutation leads to discussion of the significance of germline mosaic *ELANE* mutations. It is now well recognized that in a variety of autosomal dominant disorders, unaffected individuals lacking evidence of the mutation in DNA obtained from peripheral blood or epithelial or other cells obtained by buccal swab or skin biopsy may be a parent to multiple children exhibiting the disorder and sharing a common mutation (the aforementioned sperm donor being one such example). In such cases, the “transmitting” parent is understood to harbor the mutation in only a fraction of the cells required to exhibit symptoms in the relevant tissue (such as bone marrow for neutropenic disorders) yet sufficiently populates the germline so that its inheritance follows Mendelian expectations[65].

In fact, documentation of such “germline mosaicism” has allowed for drawing some interesting conclusions about how *ELANE* mutations cause neutropenia. In the first instance in which germline mosaicism was reported[66], an unaffected father was found in peripheral blood from his DNA to demonstrate the same *ELANE* mutation as present in his affected child. Closer inspection revealed that the mutation was detectable in about half of his lymphocytes but less than 10% of neutrophils. Individual hematopoietic colonies obtained from peripheral blood were either heterozygous for the mutation or were homozygous wild type. One suggested explanation for this and other demonstrated or likely cases of *ELANE* mosaicism[59, 67, 68] was that it must mean that *ELANE* mutations are, by themselves, not causative of neutropenia[67]. We believe however that a more probable—and provocative—conclusion is just the opposite: that neutrophil progenitors bearing the mutation do not eventuate in mature neutrophils yet are unable to block differentiation of wild type cells[69, 70]. These observations implicate cell autonomous phenomenon rather than involvement of paracrine effects, at least with respect to how mutations in *ELANE* lead to SCN, if not also cyclic neutropenia.

ELANE gene mutations

It is first of all worth noting that the pathogenic role of *ELANE* mutations in hereditary forms of neutropenia has been repeatedly called into question (for example,[21, 60, 68]). We find this baffling. Families with cyclic neutropenia or SCN exhibit clear-cut multigenerational patterns of inheritance consistent with single gene autosomal dominant transmission. The initial linkage analysis identified just a single locus in 13/13 pedigrees with cyclic neutropenia, and *ELANE* mutations, some different and some recurrent yet appearing on different ancestral haplotypes (meaning that they arose independently within the population), were detected in all 13 families[52]. Since then, hundreds of patients with either cyclic neutropenia or SCN who have *ELANE* mutations have been identified[58, 71, 72]—to list but just a few of the many cases reported in the literature. Multiple examples of *de novo* mutation of *ELANE*, which is generally accepted as evidence of causality—particularly when examining only a single gene, are now known. Cases of germline mosaicism are also consistent with a causative role. Commercial genetic testing has become routine. Finally, as whole genome or whole exome sequencing becomes increasingly common, and databases of common variants are compiled by sequencing

thousands of controls[73], other causative genes can be excluded and the absence of the observed *ELANE* mutations from control populations can be confirmed. We assert that there are few other genes where the evidence of causality is so strong.

The nature of the mutations observed in *ELANE* also provides clues about their pathogenicity

ELANE contains five exons. All known *ELANE* chain-terminating mutations (nonsense or frameshift mutations leading to altered reading frames with incorrect translation of an out-of-frame stop codon) occur in the fifth and final exon. (Chain-terminating mutations within *ELANE* are, for the most part, confined to SCN patients and are not observed with cyclic neutropenia.) While chain-terminating mutations occurring within internal exons generally produce transcripts that are selectively removed prior to translation through the process of nonsense mediated decay and therefore do not produce an abbreviated polypeptide, mutations occurring in the final exon are typically exempted from such cellular quality control mechanisms and do actually lead to peptides truncated at the carboxyl terminus[74]. This important observation leads to two significant conclusions: First, haploinsufficiency of neutrophil elastase is unlikely to be causative of the disorder, otherwise chain-terminating mutations or whole gene deletions, which have never been observed, should be expected in the first four exons. Second, the carboxyl terminal portion of the polypeptide likely contributes functionally to prevent neutropenia (as discussed below, potentially a binding site for AP3, involved in intracellular trafficking of neutrophil elastase).

Initially, shortly following discovery of *ELANE* mutations in cyclic neutropenia and SCN, there appeared to be possible clustering patterns related to either the lineal or tertiary distribution of mutations with respect to gene and protein structure, respectively, that predicted their occurrence within either subtype of inherited neutropenia. Potential correlation between mutation location and phenotype has substantially weakened[75] as the number of unique mutations has grown as genetic analysis of *ELANE* has become increasingly common in the evaluation of early childhood neutropenia. Mutations, typically single base missense substitutions or small in-frame indels or splice site mutations producing in-frame deletion or insertion of a few amino acid residues, are now found distributed throughout the length of the gene, rendering any clear pattern, if one actually exists, less obvious (Fig. 1). It has become difficult to maintain an accurate database of the various mutations now described because of the plurality of research and commercial labs performing testing. Still, some general observations regarding the nature of the mutations hold. The splice donor site at intron 4 is perhaps the most frequent site at which mutations occur[16]. Base substitutions at the first, third, or fifth position of the intron force utilization of a cryptic splice donor site 30 nucleotides upstream of the canonical site, leading to deletion of ten amino acid residues from within a region of the protein containing the catalytic site (Δ161-F170). These mutations are nearly exclusively found in individuals with cyclic neutropenia and are the overwhelmingly most common mutations, with respect to their common effect on protein coding, in cases of cyclic neutropenia. In addition to the observation of chain-terminating mutations in the final exon being nearly exclusively found among patients with SCN, a fairly commonly seen mutation, G815R, seems to confer a particularly severe clinical course[58, 76]. Individuals with this mutation tend to have lower neutrophil counts, are refractory to rhG-CSF treatment, and appear to progress to MDS and/or AML at high frequency.

Biochemistry of neutrophil elastase

ELANE encodes neutrophil elastase, a monomeric approximately 30 kDa glycoprotein[53]. As noted, neutrophil elastase is closely related to azurocidin and proteinase 3, which are

encoded by adjacent genes in the same cluster on chromosome 19p. All three enzymes are also closely related to cathepsin G, whose gene is found on a different chromosome (14q). All four proteins are major component of neutrophil azurophilic granules.

Neutrophil elastase takes its name from the fact that the connective tissue protein elastin was among the first of its known substrates[77]; however, neutrophil elastase claims a large number of different proteins among its substrates. Its ability to accept virulence factors from a variety of Gram-negative bacterial species[78] as substrates, including the outer membrane protein of *E. coli*[79], contributes to its antimicrobial activity.

Neutrophil elastase is also involved in the processing of cytokines, chemokines, and growth factors, including tumor necrosis factor alpha (TNF-α)[80] and stromal cell-derived factor-1α (SDF-1α, also referred to as CXCL12)[81], which serves as a chemoattractant for lymphocytes, monocytes, and dendritic cells. Interestingly, inactivation of SDF-1α in mice leads to deficient myelopoiesis[82] and inactivation of its receptor, CXCR4, is the cause of the WHIM syndrome (warts, hypogammaglobulinemia, infections, and myelokathexis)[83]. In WHIM syndrome, peripheral neutropenia is a consequence of myelokathexis, the retention of neutrophils within the bone marrow. Of unknown relevance to SCN, neutrophil elastase also cleaves G-CSF and its receptor[84–86].

Neutrophil elastase is synthesized as a 267 amino acid residue inactive zymogen. It is post-translationally processed to first remove a 27 residue “pre” signal sequence required for intracellular membrane insertion and ultimately extracellular secretion[87]. Then a “pro” peptide of just two amino acids is removed from the amino terminus by dipeptidyl peptidase I (DPPI, also known as cathepsin C)[88]. Neutrophil elastase also contains a carboxyl terminal propeptide of 20 residues that is cleaved by an as-yet unidentified protease. Fully processed mature neutrophil elastase contains 218 amino acid residues. Unlike other serine proteases, such as trypsin, where proteolytic conversion from zymogen to active enzyme yields two intertwined polypeptides, the processed portions of neutrophil elastase are not found to associate with the resulting mature single protein chain[89]. As for post-translational modifications, neutrophil elastase contains two sites of N-glycosylation[53].

Interestingly, deficiency of DPPI, responsible for cleaving the two residue propeptide of neutrophil elastase, causes an autosomal recessive human disorder, Papillon-Lefevre syndrome, consisting of skin and periodontal disease[90]. Individuals with Papillon-Lefevre syndrome therefore fail to appropriately excise the carboxyl terminal propeptide domain from neutrophil elastase, proteinase 3, cathepsin G, and azurocidin, but yet are not described as neutropenic.

Neutrophil elastase’s tertiary structure is comprised of two β-barrels, containing six antiparallel β-sheets connected through a linker segment, and a carboxyl-terminal α-helical domain[91]. As with other serine proteases, there is a triad of catalytic residues (S195, D102, and H57) forming a “charge relay” system[53]. Initially, the histidine is deprotonated by the carboxylate side chain of aspartate. The histidine, in turn, deprotonates serine. The proton originally bound to the serine hydroxyl group is transferred to the amino group in the substrate’s peptide bond, allowing the histidine to accept a proton from water, which then attacks the acyl enzyme intermediate, leading to reformation of the enzyme.

Neutrophil elastase and related chymotryptic serine proteases have several endogenous inhibitors. Most prominent perhaps are the serpins (serine proteinase inhibitors), of which the most well-characterized is α1-protease inhibitor (also known as α1-anti-trypsin, but which may play a more significant role in inhibiting neutrophil elastase). Human genetic deficiency of α1-protease inhibitor leads to early onset of emphysematous lung disease, resulting from unopposed neutrophil elastase-mediated destruction of pulmonary elastic

fibers and other connective tissues[92]. Certain genetic variants of α1-protease inhibitor can also cause cirrhosis due to their ability to misfold and form protein aggregates which prove toxic to hepatocytes[93]. Among other serpins, monocyte neutrophil elastase (now referred to as SerpinB1) appears to account for a substantial portion of physiologic inhibition of neutrophil elastase[94]. Serpins achieve inhibition of neutrophil elastase and other serine proteases through an irreversible suicide substrate mechanism in which the targeted enzyme cleaves the serpin and generates a covalently bound inhibitory complex[95]. "Canonical inhibitors", including elafin[96] and secretory leukocyte protease inhibitor[97] form another group of endogenous inhibitors of neutrophil elastase and related serine proteases. Although encoded by different gene families, they share a canonical formation of their inhibitory protease binding loop.

Failure of mouse genetic models to recapitulate neutropenia

A factor hampering the understanding of the mechanism whereby human mutations result in neutropenia is the lack of correspondence to human phenotypes in mouse genetic models. Gene targeting of human *ELANE* mutations at orthologous positions in murine *Elane* fail to produce aberrant granulopoiesis[98]. Similarly, mice genetically deficient in *Hax1* are also not neutropenic, but instead exhibit lymphocytopenia and neuronal cell death[99]. There appear then to be species-specific differences in granulopoiesis. However, not all mouse models are failures in this regard. The impetus for screening *GFI1* as a candidate gene for human SCN (see below) came from observations of neutropenia in *Gfi1* knockout mice[100, 101]. Notably, introduction of human SCN patient-associated mutations into murine *Gfi1* coding sequences, and forced expression in murine bone marrow progenitors blocks granulopoiesis[102]; providing the first biological proof in an animal model for a human neutropenia-associated mutation. Finally, although *G6PC3* was identified as an SCN gene using a genome-wide linkage strategy[24], *G6pc3* deficient mice had previously been found to be neutropenic[103].

Hypotheses for pathogenicity: general considerations

The distinctive neutrophil count oscillations in cyclic neutropenia have attracted considerable attention. Theoretical models of varying levels of mathematical complexity have been proposed[104, 105]. A common element to some models involves disturbance of a feedback loop[106]. Abnormal responses to G-CSF or accelerated cell loss through apoptosis affecting the hematopoietic stem cell may be one example of an autoregulatory loop[107]. A feedback model we favor[16] supposes that mature neutrophils elaborate an inhibitor of myelopoiesis whose concentration depends upon the numbers of neutrophils present. In normal operation, if peripheral destruction of neutrophils in response to infection, inflammation, or other stress leads to their consumption, then low levels of the inhibitor will allow myelopoiesis to proceed. Once levels of neutrophils have adequately risen, the inhibitor would presumably dampen further production. If there were a perturbation in this circuit such that the hypothesized mediator (or the pathways upon which it acted) had its "gain" set at too high a level, then neutrophil production would be overly inhibited—but only for a while because the inhibitor's synthesis is itself dependent on neutrophil production. One can imagine how this would lead to a cyclical pattern.

In fact, there is support for such a "chalone" model, as it was once termed[108, 109], that precedes the discovery of the role of mutations in neutrophil elastase in hereditary forms of neutropenia. A search for molecules possessing predicted inhibitory capacity lead to purification of a neutrophil membrane fraction[110]. The active component within the fraction could be suppressed with chemical inhibitors of neutrophil elastase, yielding the hypothesis that neutrophil elastase was itself the chalone responsible for governing steady state levels of

neutrophils[85]. While it is attractive to hypothesize that a defective chalone could yield an overly sensitive feedback circuit, a potential problem lies in the observation of an absence of neutropenia among individuals who are genetically mosaic for neutrophil elastase mutations (as discussed above). In this special circumstance, the presence of the mutation in some myeloid precursors is insufficient to impair production of neutrophils from progenitor cells lacking the mutation. While the observation would seem to rule out the possibility that neutrophil elastase could be acting as a diffusible factor, it does not exclude the potential for it having a more local, if not completely cell autonomous effect, within the bone marrow microenvironment. Nevertheless, as details of such a model necessarily remain conjectural, it is best, in our opinion, to work toward pathophysiologic explanation of inherited neutropenia by building upon observations about the consequences of the mutations.

Lack of biochemical consistency

The mutations have varied effects on measurable biochemical activities of neutrophil elastase. While most reduce or abrogate biochemical activity, a few of the reported mutations lead to apparently fully functional enzyme[111]. Among mutations retaining proteolytic activity, there is no measurable difference in sensitivity to α1-protease inhibitor[111]. There is similarly lack of consistency as to whether mutations affect glycosylation[112]. Theoretical modeling of the mutations has also not identified likely perturbations common to known mutations[75]. No simple hypothesis based on consistent biochemical properties is therefore evident as a likely explanation for how the mutations lead to disease.

Mislocalization hypothesis

In addition to being localized within neutrophil granules, neutrophil elastase is secreted and is found on the cell surface[113–116], as well as being detected within the nucleus[26, 117–122]. Although we at one time proposed that neutrophil elastase might be an integral transmembrane protein[123], in light of other evidence, it seems more likely that neutrophil elastase attaches to the plasma membrane via electrostatic interactions[53], in particular, with sulfate-containing proteoglycans[124]. We initially proposed that mislocalization of mutant neutrophil elastase might contribute to disease pathogenesis based on several observations. Foremost among them was the molecular genetic elucidation of a similar disease in dogs, canine cyclic neutropenia[125, 126].

Despite their seeming similarities, there are phenotypic differences between human and canine cyclic neutropenia. While the human disorder is autosomal dominant, the canine disease is autosomal recessive. The cycle length in dogs is between 10–12 days, instead of the 21 day periodicity observed in humans. The canine disorder is largely confined to the collie breed, where it also results in characteristic coat color dilution, giving rise to its common name, “gray collie syndrome”. Most importantly, the disorders are due to mutations in two different genes in the two different species. *ELANE* is intact in collies; instead homozygous mutations are found in *AP3B1* encoding the beta subunit of the adapter protein 3 complex[123]. In humans, mutation of *AP3B1* produces Hermansky Pudlak syndrome type 2[127].

Hermansky Pudlak syndromes are genetically heterogeneous disorders typically consisting of partial albinism and platelet granule deficiencies leading to a bleeding diathesis. Among at least nine known types of human disease (and an even larger number of murine types of this disorder), only type 2 is associated with neutropenia. Although there are not many known human cases, none of those have been described as having cyclical neutropenia; instead their neutrophil counts appear to be chronically low. Another distinguishing feature in comparison to human SCN arising from *ELANE* mutations is that no patients with

Hermansky Pudlak syndrome type 2 (or dogs with canine cyclic neutropenia) have been reported as developing MDS or AML.

In spite of the fact that canine cyclic neutropenia actually proves to be an animal model for a different human disease, it may nevertheless offer some insight into the pathogenesis of human cyclic neutropenia. AP3 is involved in the trafficking of cargo proteins from the trans-Golgi network to lysosomes[128], which, in neutrophils consist of granules. Among its well characterized cargo proteins is tyrosine hydroxylase, which fails to appropriately localize within melanosomes. The affected melanosomes may be thought of as a sort of specialized lysosomal compartment within melanocytes and account for the pigmentary phenotype in Hermansky Pudlak syndrome type 2[129].

We entertained the hypothesis that neutrophil elastase could serve as an AP3 cargo protein. Support for this hypothesis comes from the fact that in AP3-deficienct dogs with canine cyclic neutropenia, neutrophil elastase's distribution is altered as measured by immunofluorescent localization patterns within the cell and also by biochemical fractionation[123, 130]. Further, neutrophil elastase in neutrophils from dogs affected with canine cyclic neutropenia is not fully proteolytically processed[130]. A yeast two-hybrid system used for testing potential AP3 cargo protein interactions reveals a potential association between neutrophil elastase and AP3[9]. Importantly, the region of neutrophil elastase responsible for this interaction is within the processed carboxyl terminus, which is recurrently deleted in chain-terminating SCN mutations. Moreover, analysis in cultured cells[9] as well as neutrophils obtained from patients with *ELANE* mutations[112] reveals that mutant neutrophil elastase is mislocalized within the cell, again as evidenced by immunofluorescent staining patterns and biochemical fractionation.

Corollary support for this hypothesis comes from similar observations in Chediak-Higashi syndrome. Humans with Chediak-Higashi syndrome also have partial albinism and commonly neutropenia[131]. It is caused by mutations in the *LYST* gene, which encodes a protein regulating lysosomal trafficking[132]. Corresponding mutation of *Lyst* in mice is responsible for the beige strain. Beige mice are deficient in neutrophil chemotaxis and bactericidal activity; though they are not neutropenic, significantly, neutrophil elastase is aberrantly subcellularly localized in beige mice[133, 134]. (As far as we aware, there has been no study examining if neutrophil elastase is aberrantly localized in human Chediak-Higashi syndrome.) It thus seems that mislocalization of neutrophil elastase either via mutation of neutrophil elastase itself or in proteins regulating its lysosomal transport can produce neutropenia, at least in humans. (Oddly enough, Chediak-Higashi syndrome has been described in at least six species[135], and, while mice with Chediak-Higashi syndrome are not neutropenic, at least one other species—cats—in addition to humans, is[136]. These observations support the view that there are species-specific differences in granulopoiesis that allow for different neutropenic phenotypes in the presence of identical genetic defects.)

Another gene responsible for SCN, albeit rarely, but offering potential support for the mislocalization hypothesis, is *GFI1*, encoding a transcription factor involved in maintenance of hematopoietic stem cells[137]. As noted previously, gene targeted mice deficient in Gfi1 were initially reported as having neutropenia[100, 101]. More precisely, in addition to lymphopenia and other lymphocyte abnormalities, the mice failed to produce mature neutrophils and monocytes in the peripheral blood, but instead exhibited scant numbers of cells demonstrating an intermediary phenotype. Screening *GFI1* as a candidate gene in otherwise unexplained cases of SCN has led to the identification of occasional mutations in this gene[25, 26, 56]. The human phenotype closely resembles the mouse phenotype in that the peripheral neutrophils exhibit an immature morphology, as well as lymphocyte abnormalities consistent with those observed in mice. Myeloid progenitor cells exhibit

deficiencies in the appearance of granulocytes in colony formation assays. Intriguingly, although *GFI1* targets many genes for transcriptional regulation, both repression and activation, *ELANE* is a target of its transcriptional repression[25, 138] and there are elevated levels of *ELANE* and its translated product, neutrophil elastase, in people and mice deficient in Gfi1. One possibility is simply that over-expression of neutrophil elastase overwhelms normal intracellular trafficking pathways and leads to its accumulation in cellular compartments where it is not ordinarily found (or overwhelms ER folding pathways, as described below).

(One case involving germline mutation of *GFI1* is quite remarkable[5]. A young man developed cyclic neutropenia as an adult and was found to have two *cis de novo GFI1* mutations (both of the mutations described in different patients in another report[25]). Neutrophil elastase appeared mislocalized from the granules to the nucleus of his neutrophils. Moreover, he exhibited T lymphocyte immunity to proteinase 3 and neutrophil elastase, and autoimmune destruction of his neutrophils was felt to be the cause of his cylic neutropenia.)

The mislocalization hypothesis, however, suffers from the finding that not all neutrophil elastase mutations have demonstrable effects upon the protein's subcellular localization; moreover, there are not necessarily clean divisions between the alternate destinations of the enzyme (ER retention, accumulation at or near the cell surface, excessive granular deposition, and possibly nuclear presence) and the phenotype (cyclic neutropenia versus SCN) associated with particular mutations.

Unfolded protein hypothesis

An alternative hypothesis for the pathogenic effects of the various neutrophil elastase mutations takes inspiration from how mutations in one of its inhibitors, α1-protease inhibitor, produce hepatotoxicity[93]. Link and colleagues[72], as well as Kollner et al.[112], have posited that the mutations cause the nascent polypeptide to misfold, thereby inducing a stress response, largely coordinated within the ER, which leads to apoptosis. Indeed, there is substantial support for this hypothesis in cell models of the disorder in which particular mutations are expressed in cultured cells and found to induce markers of ER stress response, including expression of BiP/GRP78 and splicing of XBP1 mRNA[139]. Additional support for this hypothesis derives from study of transgenic mice carrying a targeted mutation of *Elane* (G193X) found in human SCN, which produces a truncated polypeptide[140]. As with other mouse models, the mice initially failed to yield a neutropenic phenotype. However, treatment with the proteasome inhibitor bortezomib, which among other effects results in inhibition of ER-associated degradation pathways, did evoke a neutropenic phenotype. A supportive observation from human genetics involves Wolcott-Rallison syndrome. That disorder is caused by mutations in *EIF2AK3*, a kinase for translation initiation factor-2, which functions as a proximal sensor of ER stress. Disruption of *EIF2AK3* produces ER stress in pancreatic β-islet cells, thereby eventuating in early-onset diabetes mellitus[141]. Notably, many patients with Wolcott-Rallison syndrome exhibit neutropenia[142]. When mice containing the G193X allele of *Elane* were crossed with *Eif2ak3*-deficient mice, there was however no neutropenia or other apparent effects upon granulopoiesis. While this may not be entirely surprising in light of the failure of most mouse genetic models to recapitulate a neutropenic phenotype corresponding to the equivalent human disorder, it does raise the possibility that non-specific effects of bortezomib's chemical inhibition of the proteasome[143] might be contributing to neutropenia in these experiments. An additional concern is that, as with the other hypotheses advanced to explain how *ELANE* mutations eventuate in neutropenia, not all mutations are capable of consistently experimentally evoking the unfolded protein response. The unfolded protein response hypothesis has

additionally yet to offer insight into distinguishing between how different *ELANE* mutations might produce either cyclic neutropenia or SCN, based on the properties of the mutant protein. Finally, it should be emphasized that the mislocalization hypothesis and the unfolded protein hypothesis are not mutually exclusive. Indeed, in the earliest report of induction of the unfolded protein response by Kollner and colleagues[112], it was felt that aberrant cytoplasmic localization was associated with, if not required, for induction of the stress response.

Another challenge to the unfolded protein hypothesis is to explain why it is that only mutations in neutrophil elastase are found in human neutropenia. Conceivably, mutations of the closely related proteinase 3 or azurocidin, whose genes lie adjacent to *ELANE* and are similarly prominent granule components, have not been detected. However, to date, no mutations of these genes have been reported.

Challenges posed by translation initiation mutations

Our lab and others[58] have found SCN patients who have mutations in the initiator methionine codon at the first translated residue encoded by *ELANE*. At first glance, such mutations are problematic in light of other hypotheses. These alleles should not produce a polypeptide and therefore contradict observations based on an absence of gene deletion mutations suggesting that haploinsufficiency of neutrophil elastase causes neutropenia. Moreover, they are incompatible with both the mislocalization and the misfolding hypotheses. (If there is no mutant protein to be made, then how can it mislocalize or misfold?) Our preliminary studies indicate that these mutations force translation from downstream internal initiation codons, which would otherwise encode internal methionine residues, and produce a polypeptide truncated at the amino-terminus. We find that some of these internally translated polypeptides are also intracellularly mislocalized. (Our preliminary studies show it accumulating in the nucleus.) It is more difficult to imagine how these peptides could invoke the ER stress response, since their effect is to delete the signal sequence required for targeting to the ER. Further study of this unusual class of mutations is likely to be informative.

Cellular consequences of the mutations

Whatever the biochemical consequence of mutant neutrophil elastase, it must somehow translate into a failure of neutrophil maturation. Only a few explanations for neutropenia are tenable: neutrophil progenitors can stop proliferating, they can die, or they can differentiate into an alternate fate. The last possibility is intriguing in light of the reciprocal relationship between neutrophil and monocyte counts in both cyclic neutropenia and SCN. With respect to cell death, intriguingly, some of the earliest electron microscopy studies of cyclic neutropenia demonstrated aberrant promyelocyte granule formation accompanied by autophagy[144], and several of the genes causing SCN can induce cell death[21].

Multiple outstanding questions remain: What accounts for how mutations in the same gene, *ELANE*, can produce two different forms of neutropenia (cyclic vs SCN) and why, for that matter, is cycling present at all? Importantly, how does pharmacologic administration of rh-GCSF improve granulocyte counts? Another important question that is far from being answered are why does MDS and AML develop in SCN but usually not in cyclic neutropenia? A recent report of multi-step evolution of acute myeloid leukemia from SCN with an *ELANE* mutation over a 17 year period involved the acquisition of five distinct *CSF3R* mutations, with three of them disappearing[71]. Compared to other leukemia-predisposing bone marrow failure syndromes, why are mutations in the gene encoding the G-CSF Receptor such a common feature in SCN? And, does pharmacologic administration of rh-GCSF promote clonal outgrowth of the mutated receptor? No doubt that answers to

such questions, should and when they come, will be broadly relevant to both normal and malignant hematopoiesis.

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Key Points

- Heterozygous mutations in the gene, *ELANE*, encoding neutrophil elastase cause cyclic neutropenia and are the most common cause of severe congenital neutropenia.
- Although many mutations are known, they all result in a translated polypeptide indicating that it is not merely haploinsufficiency of the enzyme that is responsible for neutropenia.
- Two competing but not mutually exclusive hypotheses suggest that protein mislocalization or protein misfolding may contribute to pathophysiology.

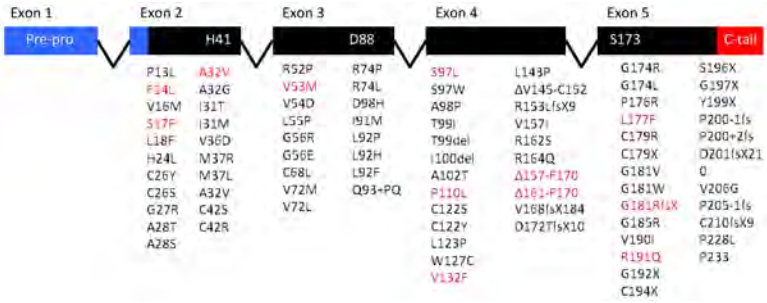

Figure 1.

Schematic of mutations of *ELANE* associated with SCN and cyclic neutropenia. Amino acids listed in white represent the catalytic triad active site. Mutations in red are primarily associated with cyclic neutropenia but some have also been reported in SCN.

Table 1

Genetic Causes of Human Neutropenia

Disease	Affected Gene	Manner of Inheritance	Syndromic
Cyclic Neutropenia	*ELANE*	AD	No
Severe Chronic Neutropenia	*ELANE*	AD	No
Severe Chronic Neutropenia	*CSF3R*	AD	No
Severe Chronic Neutropenia	*HAX1*	AR	Yes, neurodevelopmental features
Severe Chronic Neutropenia	*G6PC3*	AR	Yes, congenital heart disease and other features
Severe Chronic Neutropenia	*GFI1*	AR	Yes, lymphocyte abnormalities
Severe Chronic Neutropenia	*STK4*	AR	Yes, lymphopenia, congenital heart disease
Neutropenia	*SBDS*	AR	Yes, Shwachman-Diamond syndrome
Neutropenia	*RMRP*	AR	Yes, cartilage-hair hypoplasia syndrome
Neutropenia	*SLC37A4*	AR	Yes, glycogen storage disease 1b
Severe Chronic Neutropenia	*WAS*	X-linked	No (different from Wiskott Aldrich syndrome)
Severe Chronic Neutropenia	*TAZ*	X-linked	Yes, Barth syndrome