Neutropenia-associated *ELANE* mutations disrupting translation initiation produce novel neutrophil elastase isoforms

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Abstract

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Pathology

Hereditary neutropenia usually results from heterozygous germline mutations of *ELANE*, encoding neutrophil elastase (NE). How mutations cause disease remains uncertain. Two hypotheses have been proposed. In one, *ELANE* mutations lead to mislocalization of NE. In the other, *ELANE* mutations disturb protein folding, inducing an unfolded protein response (UPR) in the endoplasmic reticulum (ER). Here we describe new types of mutations disrupting the translational start site. At first glance, they should block translation and are incompatible with either the mislocalization or misfolding hypotheses, which require mutant protein for pathogenicity. We find that start site mutations, instead, force translation from downstream, in-frame initiation codons, yielding amino-terminally truncated isoforms lacking ER-localizing (pre) and zymogen-maintaining (pro) sequences, yet retain essential catalytic residues. Patient-derived induced pluripotent stem cells (iPSC) recapitulate hematopoietic and molecular phenotypes. Expression of the amino-terminally deleted isoforms *in vitro* reduces myeloid cell clonogenic capacity. We define an internal ribosome entry site (IRES) within *ELANE* and demonstrate that adjacent mutations modulate IRES activity, independently of protein-coding sequence alterations. Some *ELANE* mutations therefore appear to cause neutropenia via production of amino-terminally deleted NE isoforms rather than by altering the coding sequence of the full-length protein.

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Chapter 1: Introduction.

Neutrophil granulocytes are the most abundant white blood cell in the body, accounting for 40-70 percent of all white blood cells with up $2x10^{11}$ cells produced each day.[1] Neutrophils are an important part of the innate immune system and are often the first immune cell to encounter a new pathogen. Neutrophils fight pathogens directly both through phagocytosis and through the use of a wide array of antimicrobial enzymes and indirectly through their recruitment of other immune cells towards a pathogen. They have a quick turnover once they enter the circulatory system, with an estimated half-life of 8 hours.[2] However, during an infection neutrophils migrate into tissues, become activated and can survive for several days.[3] Due to the short lived nature of neutrophils, constant production is required; without neutrophils simple pathogens can quickly develop into life threating infections.

Neutrophil development

Neutrophils are derived from hematopoietic stem cells (HSCs) located in the bone marrow. HSCs first divide into a common myeloid or common lymphoid progenitors. Myeloid progenitors then partially commit to the granulocyte linage by differentiating into myeloblasts which can differentiate into neutrophils through a process known as terminal granulocytopoiesis.[1] During terminal granulocytopoiesis, differentiating cells progress through several distinct intermediate cell stages (promyelocyte, myelocyte, metamyelocyte, band cell) and eventually end the process as neutrophils. The intermediates differ in the transcription factors activated and the antimicrobial proteins that are being produced (figure 1-1). The proteins are packaged into granules, which are small subcellular vesicles that come in 4 different types (azurophil, specific, gelatinase and secretory vesicles.)[4] Each type of granule contains a unique set of proteins that are released during different conditions.

During the myeloblast stage, the cell could still become a neutrophil or a monocyte. The transcription factors, CCAAT/enhancer binding protein α (C/EBPα) and growth factor independent-1 (GFI-1), drive the

cell towards the neutrophil lineage and promote the production of azurophil granules.[5,6] In the myelocyte and metamyelocyte, specific granules are produced along with high levels of the transcription factor C/EBPε.[7] In the final phase of granulocytopoiesis C/EBPε decreases while C/EBPβ, C/EBPγ, C/EBPδ, and C/EBPζ increase, transforming the cells into band cells and polymorphonuclear neutrophils, while producing gelatinase granules and secretory vesicles.[1,7]

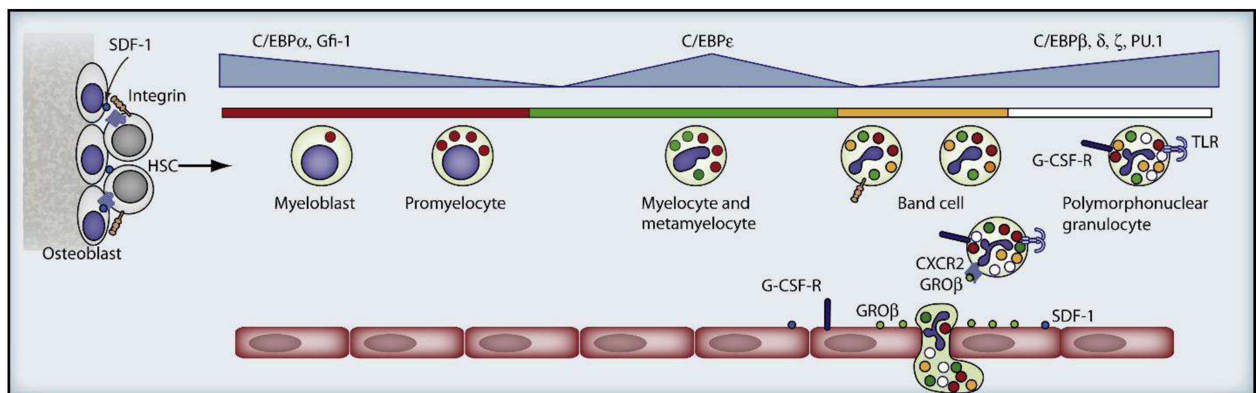

Figure 1-1 Granulocytopoiesis in the bone marrow

Stem cells localize to niches provided by osteoblasts and endothelial cells. The balance between PU.1 and C/EBPα and Gfi-1 expression determines the differentiation into the granulocytic or the monocytic pathways. The different subsets of granules (azurophil (red), specific granules (green), and gelatinase granules (yellow) and secretory vesicles (empty) are formed sequentially during maturation from promyelocytes, determined by the expression of transcription factors indicated on the top of the figure. Retention and release of cells is determined by the balance between CXCR4 (favoring retention) and CXCR2 (favoring release) and their ligands SDF-1, and KC and Groβ, respectively. G-CSF stimulates neutrophil release directly by effects on the neutrophil and indirectly by reducing the SDF-1 expression and enhancing the expression of Groβ on endothelial cells. Reprinted from Immunity, Volume 33 issue 5, Niels Borregaard, Neutrophils, from Marrow to Microbes, Page 657-670, Copyright 2010, with permission from Elsevier.

Antimicrobial activities of neutrophils

After development, neutrophils enter the blood stream and can be recruited into tissues by microbial molecules or by signals from within the tissue.[8] Once inside a tissue, neutrophils will migrate towards pathogens and sites of inflammation by chemotaxis. To identify bacteria they use toll-like receptor proteins to identify unique molecules that are present on pathogens, but not on host cells.[9] Once

identified, neutrophils have many mechanisms to kill pathogens, including phagocytosis, oxidative burst, antimicrobial proteins and neutrophil extracellular traps (NETs).

The neutrophil's primary method of destroying pathogens is to engulf the pathogen and internalize it through phagocytosis. Once the pathogen has been internalized into a phagosome, the pH of the vacuole is lowered by proton pumps, and superoxide is created by an NADPH oxidase complex. Protons combine with the superoxide to create hydrogen peroxide, which can then oxidize and destroy the pathogen. Because hydrogen peroxide is only mildly toxic, granules also fuse with the phagosome and release a variety of enzymatic proteins into the phagosome to assist in killing the pathogen. One particular protein, myeloperoxidase, increases the oxidative potential of hydrogen peroxide by converting it into hypochlorous acid, which is an even stronger oxidizer.[10] The pathogen is ultimately killed through oxidation of its cell wall and proteins.

Besides myeloperoxidase, the granules contain a wide variety of antimicrobial proteins. Most of these are proteases, such as neutrophil elastase, cathepsin G and proteinase 3 or defensins. Granules containing these proteins can either be fused with phagosomes, or they can be trafficked to outside the cell to attack pathogens that have not been phagocytized. They attack pathogens by cleaving pathogen proteins, including bacterial virulence factors.[11] Despite oxidation being the primary method of attacking pathogens, proteases are required to fight some infections. Mutations in C/EBPε result in a lack of azurophil and specific granules, but not secretory vacuoles that contain myeloperoxidase. Patients with those mutations are able to oxidize bacteria but have difficulty fighting some varieties of bacteria.[12]

The final method that neutrophils use to contain pathogens is neutrophil extracellular traps (NETs). NETs are formed when neutrophils go through a process called NETosis that kills the neutrophil and produces a dense array of chromatin and granular proteins that trap pathogens.[13,14] The exact process of NET formation is not fully understood but it does require the presence of granule proteins, like

myeloperoxidase and neutrophil elastase.[15] Depending on the pathogen the NETs are able to either kill the pathogen or inhibit its movement.[16] Despite being poorly understood, NET formation appears to be important for normal neutrophil antimicrobial function. Mice deficient in the histone modifying protein peptidylarginine deiminase 4 are not able to form NETs, and despite having otherwise normal neutrophils, are more susceptible to bacterial but not viral infection.[17,18]

Neutrophil homeostasis

Neutrophils are important for fighting infection, but overproduction and inappropriate activation of granular proteins can cause tissue damage. This occurs with some inflammatory diseases, such as rheumatoid arthritis and chronic obstructive pulmonary disease.[19] Therefore, careful regulation of the production and activity of neutrophils is required. The location of neutrophils is regulated by the protein CXCR4, which is a neutrophil surface protein that binds to stromal cell-derived factor 1 to retain the cell in the bone marrow. Expression of CXCR4 decreases as neutrophils mature, and when expression is low, neutrophils exit the bone marrow. While in the periphery, the amount of CXCR4 begins to increases over time. At the end of a neutrophil's life cycle, expression of CXRC4 is high, resulting in homing of the neutrophil back to the bone marrow.[20] In the bone marrow, macrophages phagocytose apoptotic neutrophils, which in turn stimulates the production of granulocyte colony stimulating factor (G-CSF).[21] G-CSF not only assists in the development of neutrophils, it also promotes the mobilization of mature neutrophils from the bone marrow. Through this process, macrophages both sequester toxic granule enzymes and begin the process of making new neutrophils to replace old neutrophils.

During an infection, homeostasis shifts in a process known as emergency granulopoiesis. Infections greatly increase the usage and destruction of neutrophils and, in response, the *de novo* production of neutrophils is upregulated. Macrophages,[22] hematopoietic stem cells,[23] and epithelial cells[24] all contain

proteins that recognize pathogens and upon stimulation, promote the production of cytokines that increase granulopoiesis in the bone marrow. These molecules, such as G-CSF, GM-CSF and IL-6, increase the production of neutrophils[25]. This neutrophil production uses a pathway independent from normal granulopoiesis, where C/EBPβ replaces C/EBPα.[26] C/EBPβ increases the rate of neutrophil production by inducing the self-proliferation of myeloid progenitors, as well as by promoting differentiation towards neutrophils.[27]

Definition and symptoms of neutropenia

Neutropenia is defined as neutrophil counts lower than 1000 per µL (ANC) in infants and 1500 per µL in adults.[28] Neutropenia is further subdivided into mild, moderate or severe neutropenia based on the exact counts, with severe being lower than 500 per µL.[29] One exception is individuals with benign ethnic neutropenia which has been linked to a polymorphism in the Duffy-Ag receptor for chemokines gene (*DARC*). Individuals with this mutation have a neutrophil count that falls into the neutropenic range, but are otherwise healthy.[30] Neutropenia can either be acute or chronic, with chronic neutropenia being defined as a neutropenia lasting longer than 3 months.[31]

Severe neutropenia greatly increases the risk of bacterial and fungal infection. Sites of infection are most commonly the mouth, skin or lungs but can also occur in the gastrointestinal tract. The pathogen that infects patients with chronic severe neutropenia is usually a common bacteria (streptococci, enterococci, pneumococci) or a common fungus (Candida or Aspergillus).[29]

Causes of neutropenia

There are many causes for neutropenia, and diagnosis of the condition is relatively common.[28] Neutropenia occurs when there is less than normal production of neutrophils, or greater than normal destruction of neutrophils. Neutropenia can be caused by an autoimmune or alloimmune antibody reaction which results in the premature destruction of neutrophils.[32,33] It can also be cancer induced, where cancer cells in the bone marrow limit neutrophil production and increase neutrophil destruction.[34] This is distinct from drug induced neutropenia that often occurs during the treatment of cancer with chemotherapy agents.[35] Other non-chemotherapy drugs, such as ticlopidine, clozapine, sulfasalazine, trimethoprim-sulfamethoxazole, and dipyrone may also cause neutropenia as a side effect.[28,36,37] The rarest cause of neutropenia is congenital neutropenia which greatly decreases the ability of the bone marrow to produce neutrophils.

Epidemiology and genetics of congenital neutropenia

Congenital neutropenia is a genetically complex disorder in which many different genes can be mutated. Each gene results in a different outcome with unique symptoms and attributes, but all share the common symptom of neutropenia. The genes are divided into two groups, those that involve only hematopoiesis and those that have extrahematopoietic manifestations. Mutations in *ELANE* account for the majority of cases of congenital neutropenia without extrahematopoietic manifestations.[38] Other mutated genes will be described in detail in chapter two.

The prevalence of congenital neutropenia is hard to calculate because of its rarity, and inconsistencies in how neutropenia is reported in different countries. However, current prevalence estimates range from 0.1 per million people to 9 per million people.[38] Another method of assessing how common a genetic disorder is an incidence at birth study, which tracks how many times the disease is observed and the birthrate within the study period. To date, two such studies have been done for congenital neutropenia.

One study in Canada found a rate of 15.9 cases per million births[39], the other study in Sweden found a rate of 10 cases per million births.[40]

Treatments and outcomes of congenital neutropenia

Recurrent infections are the most life threatening outcome of congenital neutropenia. In the 1950's when congenital neutropenia was first recognized as a disorder, most patients died from infection.[38,41] With the use of antibiotics, life threating infections became less common and more patients survived.[42] The most recent treatment, granulocyte colony stimulating factor (G-CSF) improves neutrophil counts and lessens infections even further. However not all patients respond equally to G-CSF and lethal infections still occur.[43,44] Despite the improvements in neutrophil counts, oral infections that are severe enough to result in tooth loss still frequently occur. This has been hypothesized to be due to differences in neutrophil granule content in those patients.[45]

Patients with congenital neutropenia also have a significantly increased risk of developing myeloid leukemia.[46] The risk of leukemia is linked to the administration of G-CSF, although leukemia has been reported in patients not taking G-CSF. 21% of individuals that have been on G-CSF therapy for 10 years develop leukemia. The risk also appears to be dose dependent. In one study, people who responded poorly to G-CSF therapy, and who were typically on a higher dose, had a 41 % chance of developing leukiemia.[44] In another study, 78 % of patients that developed leukemia had mutations in the G-CSF receptor gene.[47] Despite the risks of infection and leukemia, overall survival is 90% up to 20 years old and 80% up to 30 years old.[38]

Chapter 2: *ELANE* Mutations in Cyclic and Severe Congenital Neutropenia—Genetics and Pathophysiology

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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.

Keywords

Cyclic neutropenia

Severe congenital neutropenia

ELANE

Neutrophil elastase

Granulocyte-colony stimulating factor (G-CSF)

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.

Neutropenia

Normal neutrophil counts in peripheral blood typically range between 1500 to 8500 cells/µl after the age of one year[48], 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[49]. Within individuals, neutrophil counts fluctuate in response to environmental and host factors[50] as well as circadian cycles[51].

Heritability of neutrophil counts

Total white blood cell counts, which are largely reflective of the number of neutrophils, are moderately heritable[52]. The Duffy Antigen Receptor for Chemokine (*DARC*) gene, is at least partly responsible for the lower neutrophil counts observed in individuals of African ancestry[30,53,54] and is associated with resistance to malaria[53]. Several recent genome-wide association studies (GWAS) [55-57] 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[58]. Early descriptions of cyclic neutropenia date to the first half of the twentieth century[59,60]. The periodicity of the neutrophil cycling is exemplified by an unusual case[61] 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[62].

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[41,63,64]. 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[65]. 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)[62], it is now known that SCN represents a genetically heterogeneous group of disorders[66]. The mutated genes include those encoding the HAX1, G6PC3, WAS, GFI1, STK4, and tafazzin proteins (Table 2-1).

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[29]. For example, *HAX1*-associated SCN is frequently accompanied by neurodevelopmental complications, including decreased cognitive function and seizures[67]. Individuals with *G6PC3* mutations frequently have congenital heart disease and other clinical features[68]. Patients with mutations in *GFI1* display a range of alterations in lymphocyte numbers or function[69,70]. 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[71]. 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[72].

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)[73-75]. In one series, the cumulative incidence of MDS/AML was 31%[75], and while leukemia is anecdotally observed with cyclic neutropenia[76], 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[77]. 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[78]. (Although typically encountered as somatic mutations, there is at least one case report of SCN arising from germline CSF3R mutation[79].) 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[73], 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[80].

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[81]. Neutrophils from SCN patients are deficient in antimicrobial peptides, including α-defensins[82]. Reduced abundance of transcripts encoding other neutrophil granule components is also reported[83,84]. 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[85]. 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[86,87]. 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[88].

Osteoporosis[89] and other bone mineralization defects[90,91] are seen in SCN, although they may be a side effect of treatment with rhG-CSF[92,93]. G-CSF administration reduces bone mineral density by activating osteoclasts[94] and inhibiting osteoblasts[95]. 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[96]. 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[97]. 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[96].

We next evaluated *ELANE* as a candidate gene in 27 SCN unrelated cases[98]. 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*[99]. 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[100], 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[101]. 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[62,102]. 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[103,104]. 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[62], 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[105]. 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[106]. 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[107]. 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[108]. Arguing against that possibility, though, is the previously discussed case of another patient with two *cis ELANE* mutations[101]; 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[109].

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[110], 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[103,111,112] was that it must mean that *ELANE* mutations are, by themselves, not causative of neutropenia[111]. 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[113,114]. 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,[66,104,112]). 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[96]. Since then, hundreds of patients with either

cyclic neutropenia or SCN who have *ELANE* mutations have been identified[102,115,116]—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[117], 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[118]. 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[119] 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[62]. 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[102,120]. 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[97]. 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[121]; 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[11] as substrates, including the outer membrane protein of *E. coli*[122], 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)[123] and stromal cell-derived factor-1α (SDF-1α, also referred to as CXCL12)[124], which serves as a chemoattractant for lymphocytes, monocytes, and dendritic cells. Interestingly, inactivation of SDF-1α in mice leads to deficient myelopoiesis[125] and inactivation of its receptor, CXCR4, is the cause of the WHIM syndrome (warts, hypogammaglobulinemia, infections, and myelokathexis)[126]. 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[127-129].

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[130]. 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)[131].

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[132]. As for post-translational modifications, neutrophil elastase contains two sites of N-glycosylation[97].

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[133]. 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[134]. As with other serine proteases, there is a triad of catalytic residues (S195, D102, and H57) forming a "charge relay" system[97]. 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[135]. 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[136]. Among other serpins, monocyte neutrophil elastase (now referred to as SerpinB1) appears to account for a substantial portion of physiologic inhibition of neutrophil elastase[137]. 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[138]. "Canonical inhibitors", including elafin[139] and secretory leukocyte protease inhibitor[140] 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[141]. Similarly, mice genetically deficient in *Hax1* are also not neutropenic, but instead exhibit lymphocytopenia and neuronal cell death[142]. 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[143,144]. Notably, introduction of human SCN patient-associated mutations into murine *Gfi1* coding sequences, and forced expression in murine bone marrow progenitors blocks granulopoiesis[145]; 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[68], *G6pc3* deficient mice had previously been found to be neutropenic[146].

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[147,148]. A common element to some models involves disturbance of a feedback loop[149]. Abnormal responses to G-CSF or accelerated cell loss through apoptosis affecting the hematopoietic stem cell may be one example of an autoregulatory loop[150]. A feedback model we favor[62] 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[151,152], 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[153]. 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[128]. 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[154]. Among mutations retaining proteolytic activity, there is no measurable difference in sensitivity to α1-protease inhibitor[154]. There is similarly lack of consistency as to whether mutations affect glycosylation[155]. Theoretical modeling of the mutations has also not identified likely perturbations common to known mutations[119]. 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[156-159], as well as being detected within the nucleus[70,160-165]. Although we at one time proposed that neutrophil elastase might be an integral transmembrane protein[166], in light of other evidence, it seems more likely that neutrophil elastase attaches to the plasma membrane via electrostatic interactions[97], in particular, with sulfate-containing proteoglycans[167]. 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[168,169].

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[166]. In humans, mutation of *AP3B1* produces Hermansky Pudlak syndrome type 2[170].

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[171], 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[172].

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[166,173]. Further, neutrophil elastase in neutrophils from dogs affected with canine cyclic neutropenia is not fully proteolytically processed[173]. A yeast two-hybrid system used for testing potential AP3 cargo protein interactions reveals a potential association between neutrophil elastase and AP3[55]. 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[55] as well as neutrophils obtained from patients with *ELANE* mutations[155] 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 Chédiak-Higashi syndrome. Humans with Chédiak-Higashi syndrome also have partial albinism and commonly neutropenia[174]. It is caused by mutations in the *LYST* gene, which encodes a protein regulating lysosomal trafficking[175]. 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[176,177]. (As far as we aware, there has been no study examining if neutrophil elastase is aberrantly localized in human Chédiak-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, Chédiak-Higashi syndrome has been described in at least six species[178], and, while mice with Chédiak-Higashi syndrome are not neutropenic, at least one other species—cats—in

addition to humans, is[179]. 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[180]. As noted previously, gene targeted mice deficient in Gfi1 were initially reported as having neutropenia[143,144]. 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[69,70,100]. 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[69,181] 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[52]. 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[69]). 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[136]. Link and colleagues[116], as well as Kollner et al.[155], 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[182]. 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[183]. 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[184]. Notably, many patients with

Wolcott-Rallison syndrome exhibit neutropenia[185]. 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[186] 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[155], 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[102] 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[187], and several of the genes causing SCN can induce cell death[66].

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[115]. 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.

Tables

Table 2-1. 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

Figures

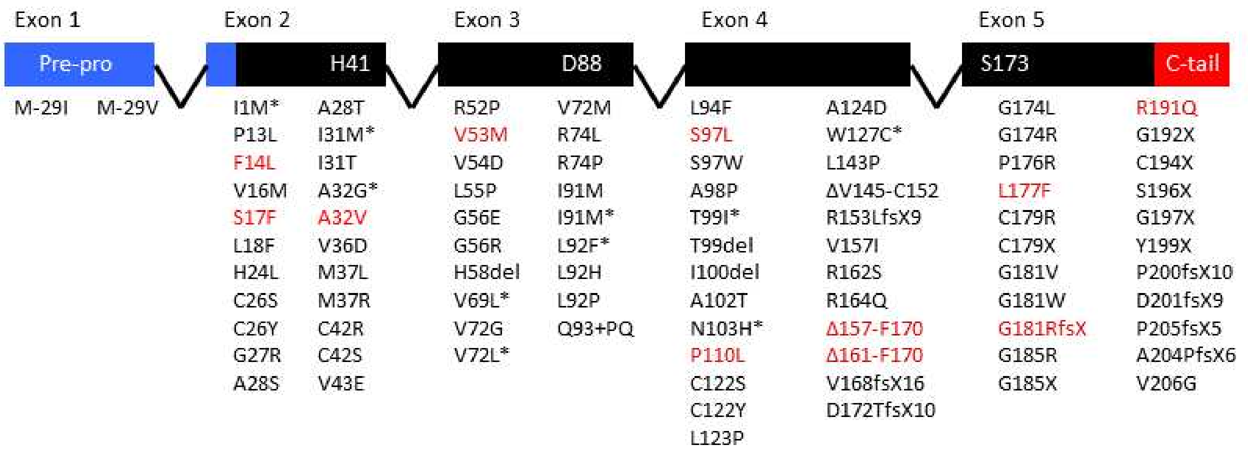

Figure 2-1. Schematic of mutations of ELANE associated with SCN and cyclic neutropenia. Amino acids listed in white represent the catalytic triad active site. Mutations in red are primarily associated with cyclic neutropenia but some have also been reported in SCN.

Chapter 3: Neutropenia-associated ELANE Mutations Disrupting Translation Initiation Produce Novel Neutrophil Elastase Isoforms

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Neutropenia-associated *ELANE* mutations disrupting translation initiation produce novel neutrophil elastase isoforms

Short Title: *ELANE* TRANSLATION INITIATION MUTATIONS

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Key Points

- *ELANE* mutations in the first codon and Kozak sequence yield amino-terminally truncated neutrophil elastase lacking pre and pro sequences.
- The study implies that sometimes neutrophil elastase coding sequence changes are incidental and noncoding *ELANE* variants are pathogenic.

Abstract

Hereditary neutropenia usually results from heterozygous germline mutations of *ELANE*, encoding neutrophil elastase (NE). How mutations cause disease remains uncertain. Two hypotheses have been proposed. In one, *ELANE* mutations lead to mislocalization of NE. In the other, *ELANE* mutations disturb protein folding, inducing an unfolded protein response (UPR) in the endoplasmic reticulum (ER). Here we describe new types of mutations disrupting the translational start site. At first glance, they should block translation and are incompatible with either the mislocalization or misfolding hypotheses, which require mutant protein for pathogenicity. We find that start site mutations, instead, force translation from downstream, in-frame initiation codons, yielding amino-terminally truncated isoforms lacking ER-localizing (pre) and zymogen-maintaining (pro) sequences, yet retain essential catalytic residues. Patient-derived induced pluripotent stem cells (iPSC) recapitulate hematopoietic and molecular phenotypes. Expression of the amino-terminally deleted isoforms *in vitro* reduces myeloid cell clonogenic capacity. We define an internal ribosome entry site (IRES) within *ELANE* and demonstrate that adjacent mutations modulate IRES activity, independently of protein-coding sequence alterations. Some *ELANE* mutations therefore appear to cause neutropenia via production of amino-terminally deleted NE isoforms rather than by altering the coding sequence of the full-length protein.

Introduction

There are two main forms of hereditary neutropenia. In cyclic neutropenia, neutrophil counts oscillate with 21-day periodicity.[188] In severe congenital neutropenia (SCN), neutrophil counts are statically low, promyelocytic maturation arrest occurs in the bone marrow, and disease often progresses to myelodysplasia or acute myeloid leukemia (AML).[188] Heterozygous mutation of *ELANE* causes almost all cases of cyclic neutropenia[189] and the majority of SCN.[98] Because neutropenia is often lethal, germline mutations frequently arise *de novo*.[188] Additional genes causing SCN include *HAX1*,[105] *GFI1*,[190] *G6PC3*,[68] and others.[191]

ELANE encodes the neutrophil granule serine protease, neutrophil elastase (NE).[97] While it is unclear how *ELANE* mutations cause neutropenia, nearly all of its myriad mutations are either amino acid missense substitutions, small insertions or deletions preserving translational reading frame, or carboxyl-terminal chain-terminating mutations escaping nonsense-mediated decay.[188,192] The mutational spectrum would seem to exclude haploinsufficiency as a mechanism, because mutations predicting absence of protein have not yet been reported.

Mutations distribute throughout NE, and effects on biochemical properties such as proteolytic activity, serpin inhibition, and glycosylation appear inconsistent.[155,192,193] Two theories about how mutations affect NE have been proposed: In one, mutant NE is mistrafficked, while, in the other, mutant NE misfolds, activating an unfolded protein response (UPR) in the endoplasmic reticulum (ER).

With respect to the mistrafficking hypothesis, NE is stored in lysosome-like granules but also distributes to the plasma membrane and nucleus.[97] Some *ELANE* mutations are reported to disturb NE trafficking, both *in vitro*[120,194] and *in vivo*[155] (though other studies have not found mislocalization).[192] Furthermore, mutations in the gene encoding the lysosomal transporter protein AP3B1, which is involved in trafficking NE[194], are responsible for the neutropenic disorders Hermansky-Pudlak syndrome type 2[195] and canine

cyclic neutropenia,[194] and, at least in dogs, NE is mislocalized.[173] Chédiak -Higashi syndrome, caused by mutations in a different lysosomal trafficking protein (LYST), may also cause neutropenia,[174] and in a mouse model of the disorder, NE is mistrafficked.[176] Finally, mutations in other genes involved in lysosomal trafficking, including *VPS13B*[196] and *VPS45*,[197,198] also cause neutropenia.

Regarding the misfolding hypothesis, when certain *ELANE* mutations are expressed *in vitro*, UPR markers, including BiP, XBP1, and GRP78, are upregulated.[155,182] A supportive observation involves Wolcott-Rallison syndrome, which, in addition to other features, includes neutropenia and is caused by mutations in *EIF2AK3*, encoding PERK kinase, which functions as a sensor of ER stress.[199] Gene-targeted mice carrying a neutropenia-associated *Elane* mutation develop neutropenia when ER degradation is blocked with the proteasome inhibitor bortezomib, resulting in high levels of ER stress.[183]

Here we describe a new category of *ELANE* mutations disrupting the translation initiation codon or the immediately adjacent Kozak sequence that does not easily fit with either the mislocalization or misfolding hypothesis. Because they might not be expected to produce a protein, these mutations would seem contrary to the concept that a mutant polypeptide causes disease. The aim of the present study is to determine the molecular effects of mutations involving *ELANE*'s initiation codon.

Materials and Methods

Mutational analysis

Sanger DNA sequencing of *ELANE* from PCR-amplified peripheral blood genomic DNA was performed as described.[189] Research was approved by the University of Washington Institutional Review Board, and participants gave written informed consent.

iPSC generation

Peripheral blood mononuclear cells were transduced using lentiviral vectors containing *Oct4*, *Sox2*, *Klf4*, and *c-Myc*. Details in Supplementary Materials.

Cell culture

Cell lines were purchased from ATCC. U937 and HL-60 were cultured in RPMI 1610 (Life Technologies, Grand Island, NY). HeLa and RBL-1 were cultured in DMEM (Life Technologies). Media was supplemented with 10% fetal bovine serum containing 100 units/mL penicillin and 100 µg/mL streptomycin.

IRES experiments

ELANE segments were PCR-amplified from genomic DNA and inserted into a bicistronic (PRF) vector (gift from Dr. Vincent Mauro, Scripps Research Institute), containing firefly and *Renilla* Luciferase. A construct containing the triplicated active region was made by separating 3 tandem repeats with a 9-basepair oligonucleotide derived from a non-IRES region from the mouse β-globin gene[200] (Supplementary Table 1). Vectors were transfected into HeLa cells using Fugene HD (Promega, Madison,WI). Cells were harvested after 24 hours. Luciferase assays were performed using the Dual-Luciferase Reporter Assay System (Promega) on a Synergy 4 plate reader (BioTek, Winooski, VT). Firefly activity was normalized to *Renilla* activity.

Western blotting

Chicken IgY raised against the NE carboxyl-terminus was used for western blots, as described.[165]

Clonogenic capacity assay

Wildtype and mutant *ELANE* vectors were generated by inserting *ELANE* into pIRES2-ZsGreen (Clonetech, Mountain View, CA) obtained from previously described vectors.[193] U937 cells were

transfected with the Amaxa Nucleofector (Lonza, Basel, Switzerland) Kit C, 2 µg of DNA, and program W-01. 24 hours post-transfection, single cells were sorted into 96-well plates using a FACSAria II (BD, San Jose, CA), gated on ZsGreen fluorescent protein expression. Two weeks later, plates were scored for wells containing ≥ 20 cells.

ER stress and apoptosis assay

HL-60 cells were transfected with the same vectors as the clonogenic capacity assay, with 1.25 % DMSO.[201] ZsGreen-positive cells were sorted with a FACSAria II, and RNA isolated using RNeasy Plus Mini Kits (Qiagen, Germantown, MD). RNA was reverse-transcribed with Super Script II RT (Life Technologies). *HSPA5* (Hs00607129_gH) expression, normalized to *ACTB* (4352935E), was quantified using TaqMan (Life Technologies) assays on an Applied Biosystems 7300 real-time PCR instrument. ER stress was induced in control cells with 2 µg/mL tunicamycin for 16 hours. For apoptosis assays, transfected HL-60 cells were incubated for 24 hours, then serum-starved for 16 hours. Apoptosis was assessed with the Annexin V-PE Apoptosis Detection Kit 1 (BD Pharmingen) per manufacturer instructions on a FACSCanto II, gating ZsGreen-positive cells.

Proteolytic activity assay

Proteolytic activity was assessed as described,[193] following 16-hour incubation.

Statistical Methods

Comparisons between groups employed Student's two-tailed t-test.

Results

Mutations disrupting the *ELANE* translational start site

An SCN-associated *ELANE* mutation, with A>G substitution at the first position of the ATG methionine translation initiation codon (Table 1), was reported previously by the French Neutropenia Register.[102]

Neither parent was affected or possessed the mutation, indicating it arose *de novo*. As an isolated case, it remained uncertain whether the mutation was causative. Since then, the proband has had an affected child inheriting the mutation, and we have observed another *de novo* occurrence in an unrelated patient in the French Register. The same mutation, again *de novo*, has subsequently been described in two additional, unrelated patients,[192,202] including one unresponsive to recombinant human granulocyte colony stimulating factor therapy.[202] Recently, additional SCN patients with mutations of the second (T>G) and third (G>C) positions of the ATG methionine initiation codon have been described[192] and, in the case of the former, also seen in the French Register (Table 1).

We report here for the first time, a different mutation (G>A) at the third position of the ATG initiation codon arising *de novo* in an SCN patient, whose affected child also inherited the mutation (Table 1 and Supplementary Figure 3-1A). We additionally describe for the first time, a cyclic neutropenia patient with mutation (A>T) of the conserved -3 position of the Kozak translation initiation sequence, where a purine, as found in wildtype *ELANE*, is strongly preferred[203] (Table 1 and Supplementary Figure 3-1B).

In sum, there are 9 unrelated probands with neutropenia comprising 5 different translation start mutations (Table 1). No other *ELANE* coding sequence variants are observed among these patients. These variants have not been described in 6,503 individuals in the NHLBI exome sequence project (http://evs.gs.washington.edu), or cataloged in 1000 Genomes[204], dbSNP[117] (version 135), or among hundreds of controls[192]. Because they are exclusively found in patients with neutropenia who lack other causative mutations and often arise *de novo* yet can be autosomally dominantly transmitted, we conclude that they are causal. Among a total of 191 different known *ELANE* mutations,[192] all, except for these, are predicted to result in translation of correspondingly mutated NE protein. A closer look at this class of mutations is warranted.

ELANE start site mutations activate translation from downstream initiation codons

We hypothesized that instead of preventing translation of NE, mutations of *ELANE*'s translational start site may, instead, lead to translation commencing from internal methionine ATG codons. There are three downstream, in-frame ATG codons that could potentially be used as start sites when the canonical initiation codon is mutated (Figure 3-1A). (There are also two out-of-frame initiation codons. One is followed immediately afterward by a stop codon, and the other is terminated after 9 codons.)

To test this possibility, we transfected *ELANE* cDNA containing mutations involving the initiation codon and the Kozak sequence in RBL-1 cells, which are conventionally employed for the study of NE because they lack endogenous NE yet correctly target it to granules,[205] and performed a western blot with an antibody detecting a carboxyl-terminal epitope. When the initiation codon is mutated from ATG to either ATA or GTG, shortened forms of NE are evident (Figure 3-2, c.3G>A, c.1A>G). When the highly conserved nucleotide in the Kozak sequence 3 nucleotides upstream from the initiation codon is mutated from A to T, then the shorter proteins are produced along with the wildtype protein (Figure 3-2, c.-3A>T). To verify that shortened forms of NE represent translation initiation from alternate sites and are not due to post-translational processing, expression vectors were generated containing just the open reading frames (ORFs) representing all in-frame methionine codons (wildtype compared to ATG2, ATG3, and ATG4). Correspondingly shorter isoforms were also produced when the region upstream from each internal in-frame ATG was deleted, with the exception that there was no detectable expression from ATG4 ORF (Figure 3-2).

We tested for the presence of shorter NE isoforms in patient-derived samples. Bone marrow was unavailable on patients with start site mutations. Western blot performed on peripheral blood was uninterpretable due to neutropenia (not shown). We therefore generated induced pluripotent stem cells (iPSC) from a patient with the c.1A>G mutation (Supplementary Figure 2), which showed normal

karyotype, pluripotent stem cell marker expression, and ability to differentiate into all three germ layers. Upon hematopoietic differentiation, compared to control, there was no significant difference in the level of $CD34^+$ cells, but subsequent myeloid differentiation demonstrated fewer bands and neutrophils; increased promyelocytes, myelocytes, metamyelocytes; more monocytes; and increased apoptosis of $CD34^-/CD33^+$ cells (Supplementary Figure 3-3A-3D). (The extent of apoptosis, however, may fall short of explaining cytopenias and could be an epiphenomenon.) Western blot of $CD34^-/CD13^+/CD11b^{low}/CD15^{++}$ myeloid cells derived from iPSC (Supplementary Figure 3-3E) confirmed reduced abundance of full-length NE (as expected with heterozygosity for the mutation, where wildtype is also present) and uniquely increased abundance of at least one shorter isoform. Additional higher molecular weight products, as also seen in RBL-1 cells, were detected. Isoform sizes in iPSC appear shifted upward slightly, compared to RBL-1 cells, suggesting differences in post-translational modification. Experiments with iPSC therefore corroborate phenotype and downstream initiation of translation.

Defining an *ELANE* internal ribosome entry site (IRES)

Figure 3-2 shows that translation normally initiating from the canonical ATG1 start site in wildtype *ELANE* does not lead to translation of polypeptides from internal ORFs. However, the ATG2 ORF expression vector yields a range of shorter polypeptides corresponding to translation initiation from ATG2, ATG3, and ATG4. We reasoned that there could be an internal ribosome entry site (IRES) situated between ATG2 and ATG3. IRES sequences are regions of mRNA that recruit the ribosome to sites adjacent to internal ATG codons and permit internal initiation of translation in a cap-independent manner.[206]

To confirm the presence of an IRES and exclude the possibility that the cap site is still used for ribosome entry when the canonical initiation codon is mutated, we introduced a termination codon (c.83C>A=p.S-2X) between ATG1 and the first internal methionine codon, ATG2, in *cis* with the Kozak sequence mutation (c.-3A>T). The shorter isoforms are present in greater abundance while polypeptides initiating

from ATG1 terminate prior to the carboxyl terminus and are consequently no longer detected by antibody to the carboxyl-terminus (Supplementary Figure 3-4).

One IRES, found in mouse *Nkx6-2*, exhibits reverse complementarity to 18S rRNA, and mutations disturbing basepairing between *Nkx6-2* mRNA and 18S rRNA disrupt IRES activity.[207] We scrutinized *ELANE* mRNA and found it indeed contains a region of reverse complementarity to 18S rRNA just downstream from ATG2, spanning the region of complementarity to 18S rRNA defined in *Nkx6-2* (Figure 3-1B). In computer simulations where random sequences the same length as the putative IRES were generated from *ELANE* mRNA and compared to 18S rRNA, sequence complementarity was determined to be highly non-random ($p=1.3\times10^{-6}$) (Supplementary Figure 5).

We therefore evaluated whether the region of 18S rRNA complementarity possesses IRES activity. We used a test vector containing distinguishable tandem, Luciferase (*Renilla* and firefly) reporters. The potential IRES was inserted in-between the two Luciferase genes. The upstream *Renilla* Luciferase contains a Kozak consensus sequence needed for translation initiation; however, translation of the downstream firefly Luciferase requires that the inserted sequences possess IRES activity. We tested several overlapping segments from within the region of *ELANE* exhibiting complementarity to 18S rRNA for IRES activity in transfected HeLa cells. Region 1 showed a 2.5-fold increase in IRES activity, but regions 2 and 3 did not exhibit IRES activity, compared to vector alone (Figure 3-3A), possibly due to presence of additional start codons within those regions. Additionally, a 5'-UTR of similar length from an arbitrary gene (*KLHDC8B*) lacked IRES activity (not shown). To further verify that region 1 contained an IRES, we evaluated its activity when it was triplicated in head-to-tail sequence. IRES activity was increased by ~6-fold, compared to vector alone (Figure 3-3B), suggesting that IRES activity is not adventitious.

ELANE mutations activating the IRES

It is worth noting that *ELANE* mutations frequently locate to the putative IRES.[188,192] We tested 3 of these mutations (p.V16M, p.S17F, and p.L18P) for effect upon IRES activity, using the dual Luciferase reporter containing triplicated *ELANE* IRES (Figure 3-3B). Two of the mutations (p.V16M and p.S17F) markedly increased activity, compared to empty vector. When transfected into RBL-1 cells (Supplementary Figure 4), p.V16M produces one of the shorter isoforms also seen with the c.-3A>T mutation. Results for S17F were inconclusive because there was limited expression of even the wildtype isoform, which would also be consistent with the possibility that S17F disrupts translation from the canonical start site. Further study is needed to determine the nature of polypeptides produced by these mutations, optimal sequence for IRES function, and how mutations within *ELANE* mRNA may modulate IRES activity.

Effect of amino-terminally truncated NE on clonogenic capacity

To determine if expression of NE polypeptides initiating from internal translational start sites are harmful to cells, we expressed cDNAs containing internal ORFs in U937 promonocytes, performing a previously described “clonogenic capacity” assay, developed to study neutropenia-associated *ELANE* mutation induction of the UPR.[182] Three different vectors were used in order to isolate the different ORFs and determine their effects; in addition to expressing the ATG2 ORF and the ATG3 ORF, we also evaluated the ATG>GTG mutation, which produces all 3 internal ORFs. (We did not test ORF4 individually, because its production seems to require upstream sequences and, in isolation, does not produce detectable protein.) All of the vectors expressing shortened forms of NE reduced clonogenic capacity (Figure 3-4). When comparing the shorter isoforms of NE to each other, the GTG mutation, which leads to production of all 3 internal ORFs, had a greater effect on inhibiting clonogenic capacity than did the ATG2 ORF by itself.

Cellular and biochemical properties of amino-terminally truncated NE

To determine if amino-terminally truncated forms of NE retain enzymatic activity, we tested their ability to cleave the NE-specific substrate MeO-Suc-Ala-Ala-Pro-Val-pNA following expression in transfected RBL-1 cells. We found that the c.1A>G mutation retained minimal, yet statistically significant, residual activity, but that isolated ORFs corresponding to the second and third ATGs lacked activity (Figure 3-5A, Supplementary Figure 6).

We assessed whether amino-terminally truncated forms of NE induce ER stress by measuring expression of *HSPA5*, which codes for the protein BiP. We found no increase in *HSPA5* in HL-60 cells transfected with *ELANE* mutations altering the translational start site (Figure 3-5B), whereas control cells transfected with wildtype *ELANE* and stimulated with tunicamycin, which induces ER stress,[182] showed a 12-fold increase in *HSPA5* expression (Supplementary Figure 7).

We tested whether amino-terminally truncated forms of NE induce apoptosis in HL-60 cells transfected with vectors expressing either wildtype or amino-terminally truncated forms of NE. There was no increase in apoptosis as observed by annexin V binding (Figure 3-5C and Supplementary Figure 8). Additionally, RT-PCR assays revealed no increase in expression across a panel of apoptosis-related genes (Supplementary Figure 9). The fact that apoptosis was evident in iPSC exposes limitations of HL-60 cells.

To determine if amino-terminally truncated forms of NE subcellularly mislocalize, we transfected RBL-1 cells with the c.1A>G and c.3G>A mutations. Compared to granular cytoplasmic distribution of the wildtype, the shorter polypeptides appear to localize to nuclei (Supplementary Figure 10).

Discussion

Somatic and germline mutations of translational start sites are reported for several human disease-associated genes, where it is speculated that, instead of abrogating translation, they may force

utilization of downstream initiation codons.[208,209] Nevertheless, in only just a few instances has the phenomenon actually been demonstrated. One example involves acquired mutations of the hematopoietic transcription factor GATA1 associated with transient myeloproliferative disorder and megakaryoblastic leukemia of Down syndrome.[210] Start codon mutations or, more often, proximal chain-terminating mutations just downstream from the initiation codon, lead to production of a shorter GATA1 variant commencing from a second initiation codon, which lacks the activation domain yet retains ability to bind DNA and cofactors.[211,212] Similar mutations producing similar effects occur in the transcription factor C/EBPα in AML.[213] Another example involves germline mutation of *TPO*, encoding thrombopoietin, in hereditary thrombocytopenia, disrupting a splice-site, which leads to deletion of the canonical start site with initiation shifting downstream to the next in-frame ATG codon.[214] Here, we show that neutropenia-associated germline mutations disrupting the initial ATG codon and adjacent non-coding Kozak translation initiation sequence of *ELANE* lead to production of amino-terminally truncated isoforms of NE initiating translation from downstream, in-frame methionine ATG codons.

Potential alternate translation start sites are predicted for as many as 12% of human genes.[215] Furthermore, potential alternate start sites are evolutionarily conserved and are utilized in many cases.[216-218]

One of the hypothesized roles of alternate translation start sites is to alter amino terminal localization signals and ultimately protein location; 30% of proteins derived from potential alternate start sites are predicted to have different subcellular localization.[215] One example is Neuropeptide Y, which has two translation start sites. The protein produced from the first initiation codon is targeted to secretory vesicles whereas the protein initiating downstream locates to mitochondria.[219] We show that, at least *in vitro*, pathogenic isoforms of NE resulting from start site mutations similarly become mislocalized when truncated at the amino terminus, probably because the shorter isoforms lack the ER-localizing signal sequence.

We demonstrate IRES activity for a region of *ELANE* between the second and third internal ATG codons. Based on prior studies of *Nkx6-2*, we suspect this activity is due to direct ribosomal binding mediated by *ELANE* mRNA sequence complementarity to 18S rRNA.[220] This IRES permits production of both the ATG2 and ATG3 ORF NE polypeptides from a single mRNA when the initial ATG codon is mutated. It is less clear if this IRES normally initiates translation when the initial ATG codon or flanking Kozak sequence are not altered. Another interesting observation is that mutations of this region enhance activity despite reducing complementarity to 18S rRNA compared to wildtype. In previous studies, it was found that the optimum length of complementarity to 18S rRNA was 7 nucleotides, and any sequence longer or shorter decreased IRES activity.[221]

It is likely that other coding sequences within *ELANE* mRNA contribute to translational regulation directly either by influencing binding to the ribosome or other translation factors or indirectly by affecting mRNA folding. If so, some coding sequence *ELANE* mutations may favor production of internally-translated isoforms, in which case, protein sequence changes could prove incidental and noncontributory to pathogenesis. Conversely, *ELANE* variants involving synonymous codon substitutions that do not alter coding sequence may nevertheless prove pathogenic because they disturb the translational start site, IRES, or other mRNA sequences regulating translation from internal start sites in *ELANE*. In our own genetic testing of neutropenic patients, we have detected novel synonymous *ELANE* variants but have not previously designated them as deleterious.[188]

ELANE mutations inactivating the ATG initiation codon are found in SCN patients and lead to absence of the full-length protein with expression of amino-terminally truncated isoforms not seen with the wildtype allele. However, the Kozak sequence mutation occurs in a patient with cyclic neutropenia and results in production of both full-length protein and, compared to SCN patients, reduced levels of the amino-terminally truncated isoforms, suggesting that less expression of the shortened isoforms correlates with what might be considered milder disease.

The shortened isoforms reduce clonogenic capacity in an assay of myeloid proliferation. Cells transfected with the shorter forms of NE did not exhibit increases in ER stress when compared to wildtype. It is not surprising that the mutations do not induce ER stress since they are predicted to lack the signal sequence necessary to translocate to the ER. However, the presence of a “smear” of high molecular weight products evident in western blots in Figure 3-2 and Supplementary Figure 3-3E, suggests that the shortened forms of NE may still misfold and aggregate, albeit not in the ER. It is possible that aggregated NE may sequester transcription factors. ORFs 2 and 3 are additionally predicted to lack the pro zymogen activation sequence whose removal is required for catalytic function, yet they retain the catalytic triad of histidine, aspartate, and serine necessary for proteolytic activity, raising the possibility that they are mature and possess enzymatic activity immediately following translational synthesis. We found that mutation disrupting the canonical translation start site retained only minimal proteolytic activity, yet it is possible that even residual activity could be damaging if not properly compartmentalized.

In evaluating the relevance of this new category of mutations disrupting the translational start site, it is unlikely that all of the numerous *ELANE* mutations would disrupt translational regulation of mRNA and lead to production of internally-translated ORFs. Nevertheless, we speculate that there may be a mechanism through which conventional mutations altering the primary sequence of NE may lead to production of the internally-translated isoforms via protein misfolding.

The presence of shorter isoforms of *ELANE* generated by an IRES actually fits with both of the current models of how mutations may cause disease (Figure 3-6). If the misfolding hypothesis held true, the shorter isoforms could be produced by the IRES through a *trans* mechanism. In times of stress, protein misfolding within the ER activates PERK kinase, which phosphorylates EIF2,[222] resulting in a preference for IRES-mediated translation over normal cap-dependent translation.[223] Our observations also fit well with the mislocalization hypothesis. Since the shortened isoforms do not contain a pro peptide signal sequence, they are unlikely to correctly transit through the ER, where they would ordinarily become

glycosylated. Previously, we demonstrated that when sites of asparagine-linked glycosylation in NE are mutated, NE accumulates in the nucleus.[165] In fact, the shorter isoforms appear to localize to the nucleus in RBL-1 cells, congruent with the theory that they do not enter the ER and are not glycosylated. Aberrant localization may contribute to pathogenesis, but further research is needed to establish if this is also true in human neutrophils and what link it may have to disease.

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Authorship

Contribution: T.T., J.W., and M.S.H. conceived and designed the study. H.L.G., C.L., and J.A.C designed iPSC studies. T.T., J.W., S.J.C., H.L.G., C.L., J.A.C. and M.S.H. analyzed and interpreted the data. T.T. and M.S.H. wrote the paper. J.W. performed isoform expression and western blot experiments (except for iPSC); R.C.N. and L.T. performed iPSC studies; S.J.S. performed immunolocalization studies; T.T. performed all other studies, with assistance of J.W. S.J.C., T.G., J.C.C., and J.D. ascertained and clinically evaluated patients described here.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

Tables

Table 3-1. ELANE translational start site mutations

			Kozak Sequence						
cDNA (NM_001972.2)	**No. Probands**	**Phenotype**	a	c	c	A	T	G	**Reference**
c.-3A>T	1	Cyclic neutropenia	t						This report
c.1A>G	4	SCN				g			Refs. 9, 27, 28, this report
c.2T>G	2	SCN					g		Ref. 9, this report
c.3G>C	1	SCN						c	Ref. 9
c.3G>A	1	SCN						a	This report

Figures

A

Exon 1 Exon 2 Exon 3 Exon 4 Exon 5

B

V16M S17F L18P

A U C

GUGUCCCUGCAGCUGCGCGGAGG *ELANE* nt 171-193

CAAAGGGCCUUCGACGGGCCGCC 18S rRNA nt 1146-1124

Figure 3-1. Location of downstream in-frame ATG codons in ELANE mRNA and IRES structure. (A) Schematic of *ELANE* mRNA. In-frame downstream ATG codons that may be used as initiation start sites are marked with an *. (B) Potential *ELANE* IRES showing complementarity to 18S rRNA. Nucleotide substitutions introduced by p.V16M, p.S17F, and p.L18P mutations are marked.

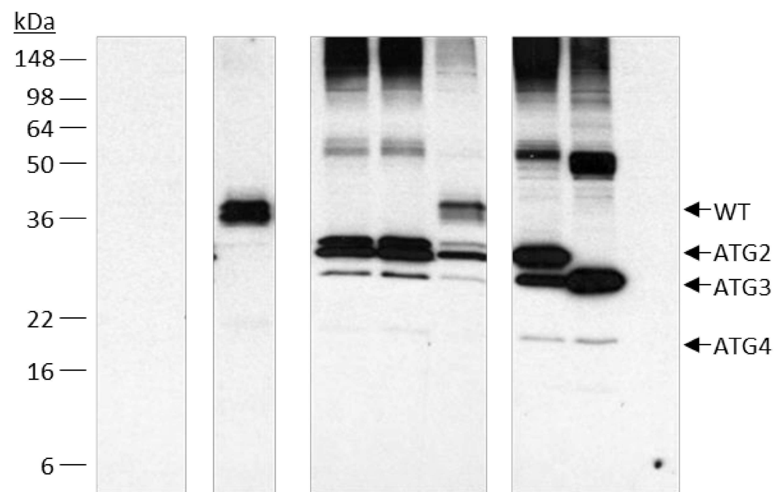


Figure 3-2. Western blot of RBL-1 cells transfected with ELANE vectors containing translational start site mutations, using an antibody to the carboxyl terminus of NE. Mutations of the canonical methionine initiation codon (c.3G>A and c.1A>G) and the non-coding Kozak sequence (c.−3A>T) lead to expression of shorter isoforms of *ELANE*. When the upstream region is removed and just the ORF that corresponds to each ATG codon is expressed, separated isoforms are identifiable (ATG2 ORF, ATG3 ORF, ATG 4 ORF). On the right, molecular weight markers (in kDa) are shown. On the left, arrows point to NE isoforms.

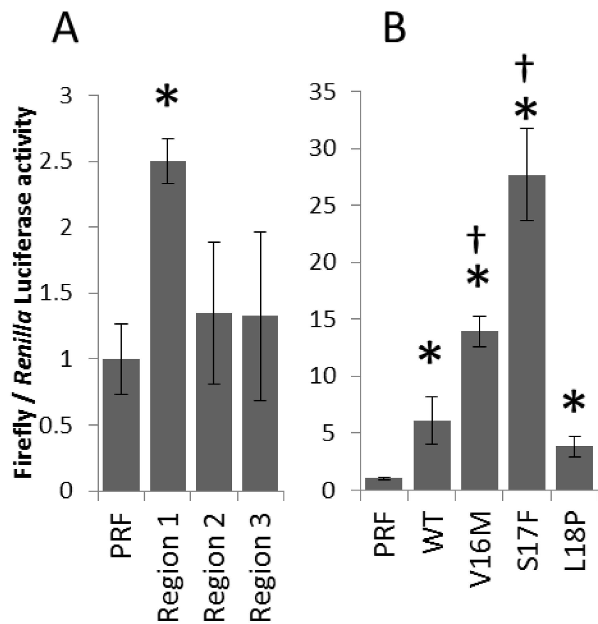


Figure 3-3. IRES activity from fragments of ELANE mRNA. (A) Activity from 3 overlapping regions of *ELANE* mRNA. Only region 1 showed an increase in IRES activity. Region 1: nt 171-233, Region 2: nt 42-233, Region 3: nt 1-233 (NM_001972.2). (B) Activity from 3 tandem repeats of nt 171-193 of *ELANE*, either wildtype (WT) or mutant sequence. *$p < 0.05$ vs. PRF, $^{\dagger}p < 0.05$ vs. WT. Data shown are mean ± standard deviation of 3 independent experiments.

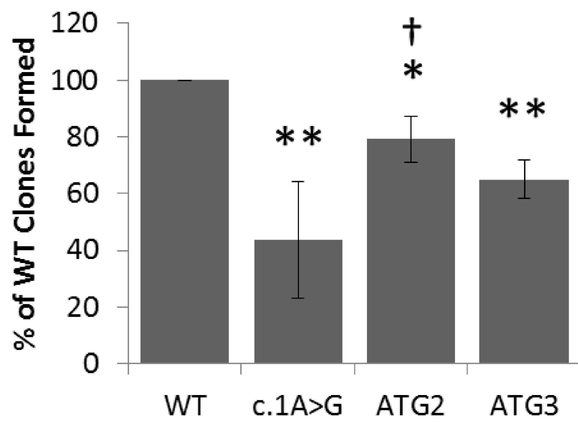
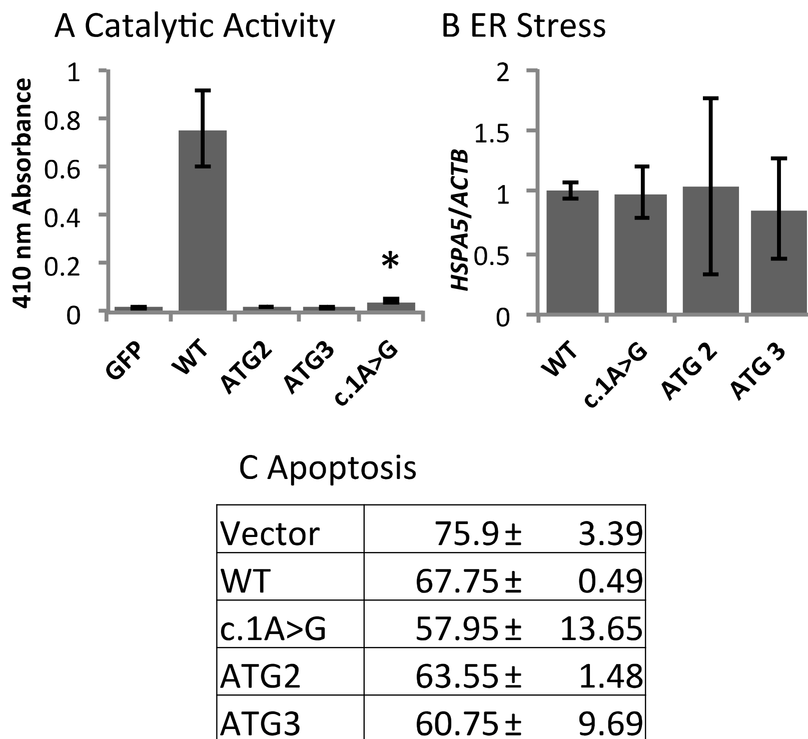


Figure 3-4. Clonogenic capacity of U937 cells transfected with ELANE vectors. Cells transfected with c.1A>G, ATG2 ORF, and ATG 3 ORF all formed significantly fewer clones. 384 wells were counted for each group during each experiment. Data shown are mean ± standard deviation of 3 independent experiments. $*p < 0.05$ vs. WT, $**p < 0.005$ vs. WT, $^{\dagger}p = 0.05$ vs. c.1A>G.



Vector	75.9 ±	3.39
WT	67.75 ±	0.49
c.1A>G	57.95 ±	13.65
ATG2	63.55 ±	1.48
ATG3	60.75 ±	9.69

Figure 3-5. Properties of amino-terminally truncated NE. (A) Mutant forms of NE are unable to cleave MeO-Suc-Ala-Ala-Pro-Val-pNA, a NE specific substrate after a 16 hour incubation, except for c.1A>G which showed minimal residual activity. Data shown are mean ± standard deviation of 3 independent experiments. *P < 0.05 vs. GFP. (B) No significant difference in ER stress was observed, as measured by *HSPA5* expression. Data shown are mean ± standard deviation of 3 independent experiments. (C) There is no observed increase in apoptosis, as measured by Annexin V staining. Mean ± standard deviation of 2 independent experiments are shown.

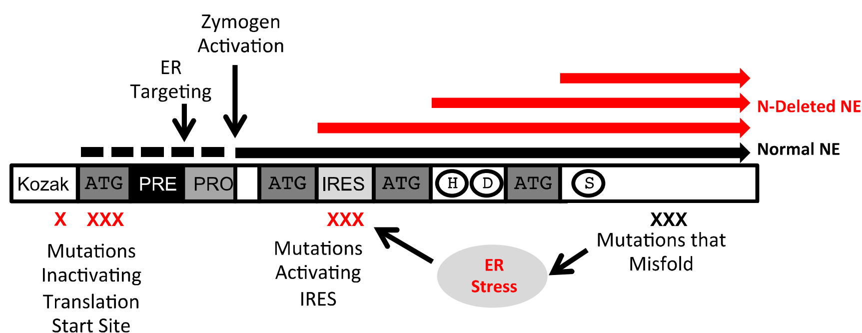


Figure 3-6. Congruence with current disease hypotheses. A schematic of *ELANE* is shown. Mutations that inactivate the translation initiation codon and the Kozak sequence force translation to initiate from downstream, in-frame ATG codons, resulting in amino-terminally truncated NE (NE) that lacks an ER-localizing pre signal sequence as well as a pro zymogen sequence ordinarily restraining proteolytic activity prior to its removal. A second group of mutations may activate an IRES, also leading to translation of the internal ORFs. A third type of mutation, we speculate, may cause protein misfolding which activates an ER stress response and promotes IRES utilization, thereby indirectly also leading to translation of the internal ORFs. Catalytic triad of his, asp, ser residues is shown. Dotted line shows amino terminal sequences ordinarily cleaved from the mature enzyme.

Appendix I: supplementary materials for chapter 3.

Supplementary materials and methods for iPSC generation and differentiation

Peripheral blood mononuclear cells from an SCN patient and normal control was transduced using lentiviral vectors containing *Oct4*, *Sox2*, *Klf4*, and *c-Myc*[224] and cultured on murine embryonic fibroblasts. 20-30 days after transduction, iPS-like colonies were picked, expanded, and transferred to Matrigel (BD Biosciences) and mTeSR1 (Stem Cell Technologies, Vancouver, BC, Canada) media for 20-30 passages. Characterization of iPS lines was done by flow cytometry for SSEA-1, SSEA-4, Tra-1-60, Tra-1-81, and CD9 markers. All lines retained a normal karyotype throughout culture and retained *ELANE* mutations as determined through Sanger sequencing. Directed differentiation of iPS lines into ectoderm, mesoderm, and endoderm at passage 20-30 suggests the cell lines are able to differentiate into all three germ layers.

Hematopoietic differentiation was carried out using methods adapted from Gandre-Babbe *et al.*[225] Briefly, $5x10^5$ cells were plated on growth factor-reduced Matrigel (BD Biosciences) and cultured for 24 hours in hES media supplemented with 10 mM ROCK inhibitor (Y27632, Millipore, Billerica, MA). After 24 hours, media was changed to RPMI supplemented with L-glutamine, ascorbic acid, MTG, BMP4 (5 ng/ml), CHIR99021 (930 ng/ml) and VEGF (50 ng/ml). Twenty-four hours after media change, CHIR99021 was removed from the culture medium and bFGF added at 20 ng/ml. On day 3, medium was changed to StemPro 34 (Life Technologies) containing BMP4 (5ng/ml), bFGF (20ng/ml), and VEGF (50 ng/ml). On days 4-5, cells were cultured in StemPro34 containing VEGF (15 ng/ml) and bFGF (5ng/ml). On day 6, media was changed to serum free SFD media with VEGF (50 ng/ml), Flt3L (5 ng/ml), SCF (50 ng/ml), and bFGF (50 ng/ml). For days 7-10, cells were cultured in SFD with VEGF (50ng/ml), Flt3L (5ng/ml), SCF (50ng/ml), TPO (50 ng/ml), and IL-6 (10 ng/ml). Non-adherent cells were collected from the media on days 8-10 and expanded in SFD media containing IL-3 (10ng/ml), GM-CSF (10 ng/ml), and SCF (50 ng/ml). To differentiate into neutrophils, G-CSF (50-1,000 ng/ml) was added to the previous medium for 5 days. Flow cytometry

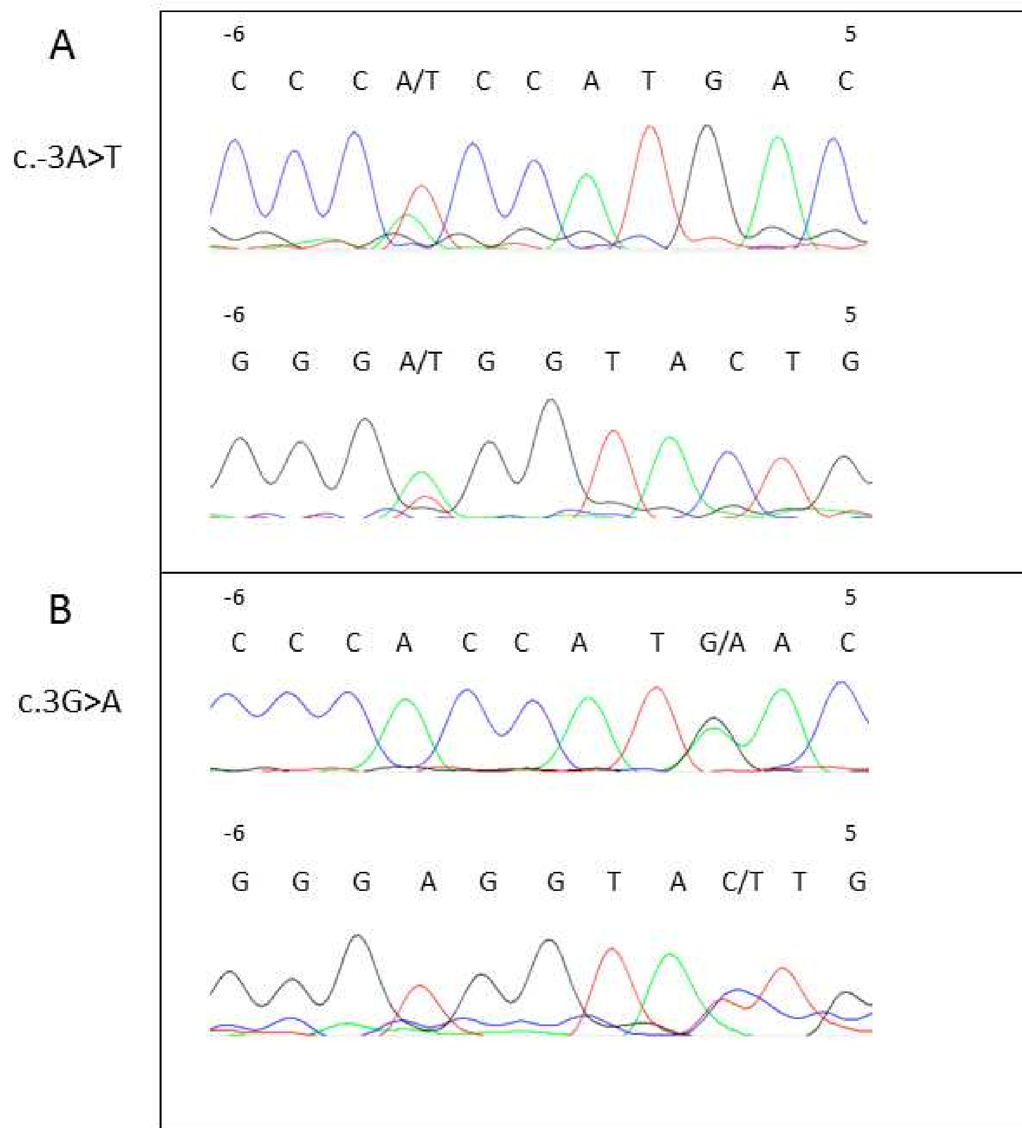
analysis was performed using specific antibodies against human (h) CD45 (APC-Cy7), CD34 (PECy7), CD33 (PE) and against Annexin V (FITC) for apoptosis analysis.

Tables

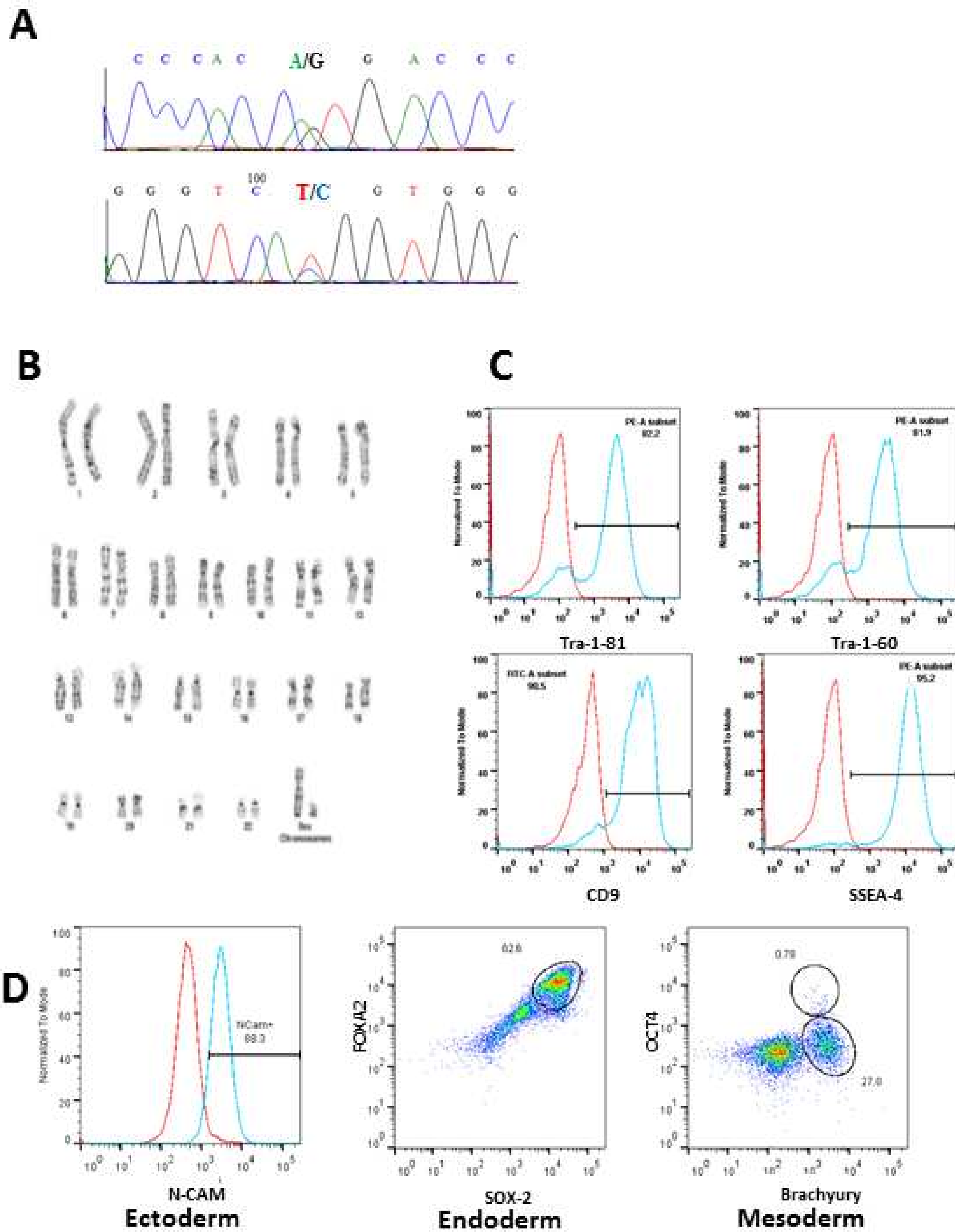
Supplementary Table 1. PCR primers and sequences used for vector creation.

PCR Primers	
for pIRES2 vectors:	
WT forward	CAAGCAAGCGCAGGCCATCTCGGAATTCCAGCCCCACCATGACCCTCGGCCGC
GTG forward	CAAGCAAGCGCAGGCCATCTCGGAATTCCAGCCCCACCGTGACCCTCGGCCGC
ATG2 ORF forward	CAAGCAAGCGCAGGCCATCTCGGAATTCCAGCCCCACCATGGTGTCCCTGCAGCTG
ATG3 ORF forward	CAAGCAAGCGCAGGCCATCTCGGAATTCCAGCCCCACCATGTCGGCCGCGCACTGC
Reverse primer	ACGTACGTGATGAGAGCAGTAGAGGATCCTCAGTGGGTCCTGCTGGCC
Primers and sequences for PRF vector	
ELANE Region 1 F	GCGCgaattcGTGTCCCTGCAGCT
ELANE Region 2 F	GTTTgaattcACCCTCGGCCGCCG
ELANE Region 3 F	GTCTgaattcGCACGGAGGGGCAG
ELANE R	GTCTccatggGACGAAGTTGGGCG
Ligated sequences	
ELANE 3rp b 1 +	[phos]aattcGTGTCCCTGCAGCTGCGCGGAGGttctgacatGTGTCCCTGCAGCT
ELANE 3rp b 1 -	[phos]AGGGACACatgtcagaaCCTCCGCGCAGCTGCAGGGACACg
ELANE 3rp b 2 +	[phos]GCGCGGAGGttctgacatGTGTCCCTGCAGCTGCGCGGAGGc
ELANE 3rp b 2 -	[phos]catggCCTCCGCGCAGCTGCAGGGACACatgtcagaaCCTCCGCGCAGCTGC
v16m 3rp 1 +	[phos]aattcaTGTCCCTGCAGCTGCGCGGAGGttctgacataTGTCCCTGCAGCT
v16m 3rp 1 -	[phos]AGGGACAtatgtcagaaCCTCCGCGCAGCTGCAGGGACAtg
v16m 3rp 2 +	[phos]GCGCGGAGGttctgacataTGTCCCTGCAGCTGCGCGGAGGc
v16m 3rp 2 -	[phos]catggCCTCCGCGCAGCTGCAGGGACAtatgtcagaaCCTCCGCGCAGCTGC
s17f part 1 F	[phos]aattcGTGTtCCTGCAGCTGCGCGGAGGttctgacatGTGTtCCTGCAGCT
s17f part 1 R	[phos]AGGaACACatgtcagaaCCTCCGCGCAGCTGCAGGaACACg
s17f part 2 F	[phos]GCGCGGAGGttctgacatGTGTtCCTGCAGCTGCGCGGAGGc
s17f part 2 R	[phos]catggCCTCCGCGCAGCTGCAGGaACACatgtcagaaCCTCCGCGCAGCTGC
l18p part 1 F	[phos]aattcGTGTCCCcGCAGCTGCGCGGAGGttctgacatGTGTCCCcGCAGCT
l18p part 1 R	[phos]gGGGACACatgtcagaaCCTCCGCGCAGCTGCgGGGACACg
l18p part 2 F	[phos]GCGCGGAGGttctgacatGTGTCCCcGCAGCTGCGCGGAGGc
l18p part 2 R	[phos]catggCCTCCGCGCAGCTGCgGGGACACatgtcagaaCCTCCGCGCAGCTGC

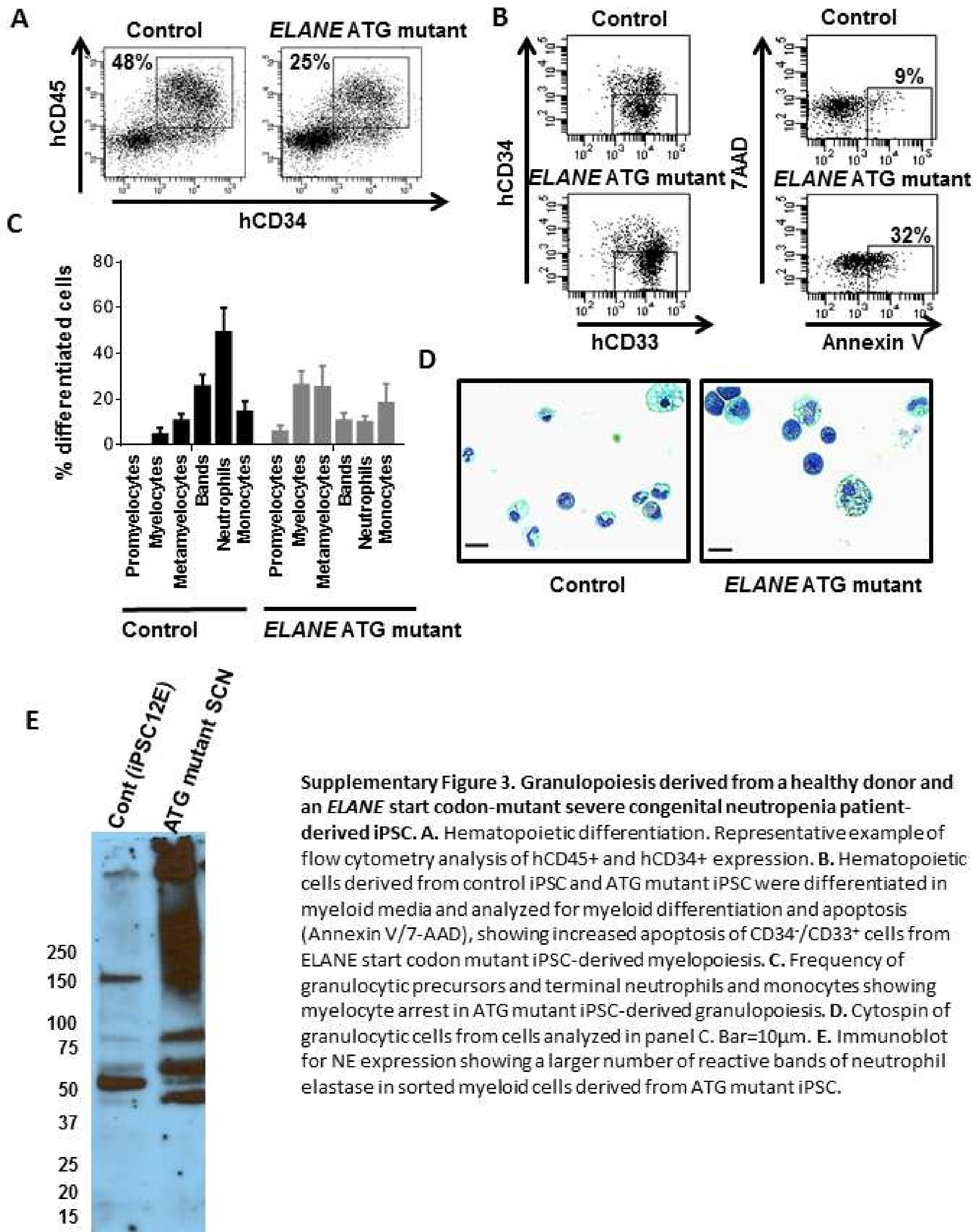
Figures



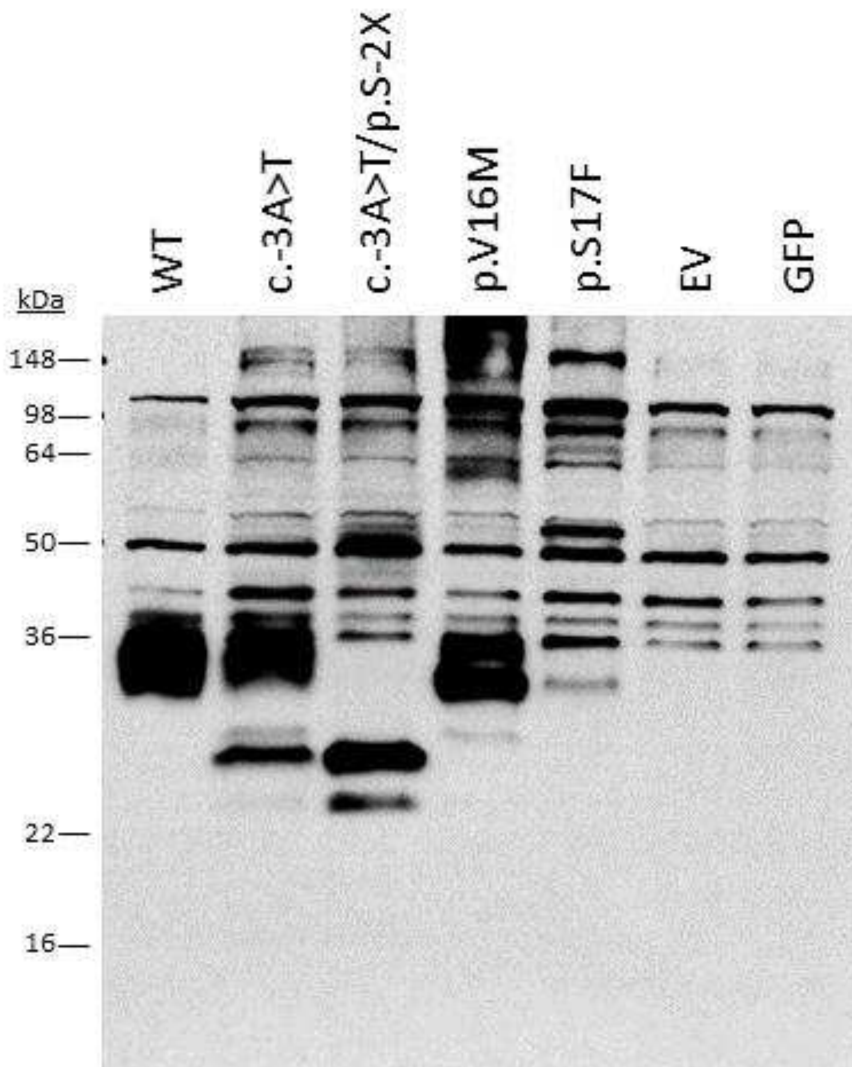
Supplementary Figure 1. **Electropherogram from Sanger DNA sequencing of bases -6 to 5 from exon 1 of *ELANE* from congenital neutropenia patients**. Forward and reverse sequencing was performed for both patients, and the reverse sequence was flipped horizontally to better align with the forward sequence A. A heterozygous mutation (A>T) was observed at the c.-3 position. B. A heterozygous mutation (G>A) was observed at position c.3.



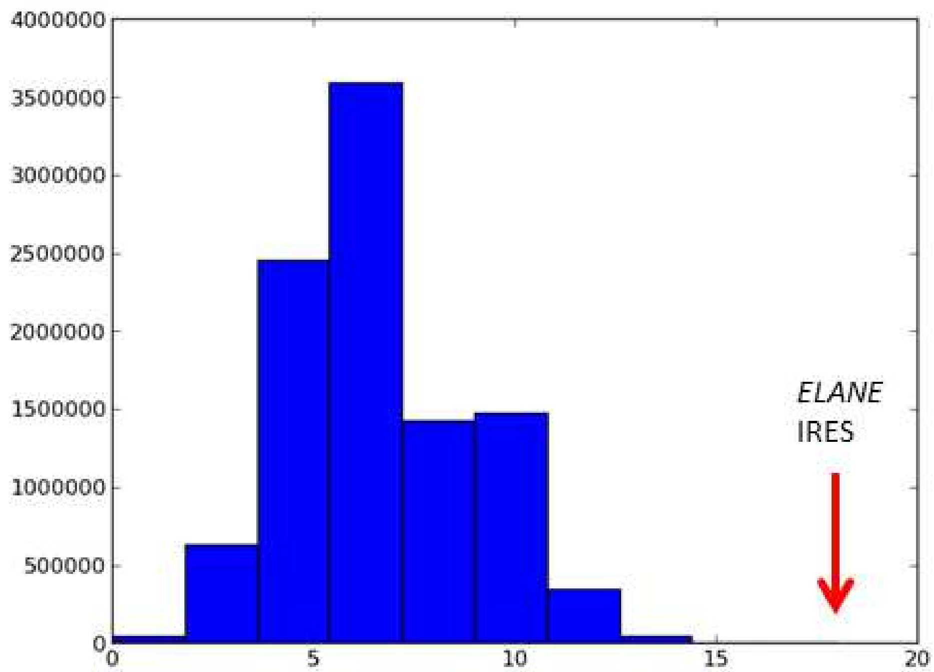
Supplementary Figure 2. Characterization of SCN iPSC line 110. A. Forward (top) and reverse (bottom) sequencing was performed for another patient with heterozygote c.1A>G mutation. **B.** The iPSC line maintained a normal karyotype . **C.** iPSC 110 was tested for flow cytometry at passages 5-25 and expressed markers consistent with pluripotency Tra-1-60, Tra-1-81, CD9, and SSEA-4. **D.** SCN iPSC were differentiated into endoderm, ectoderm, and mesoderm lineages and stained for markers of each lineage. Ectoderm: Ncam; Endoderm: FOXA2, SOX2; Mesoderm: Brachyury.



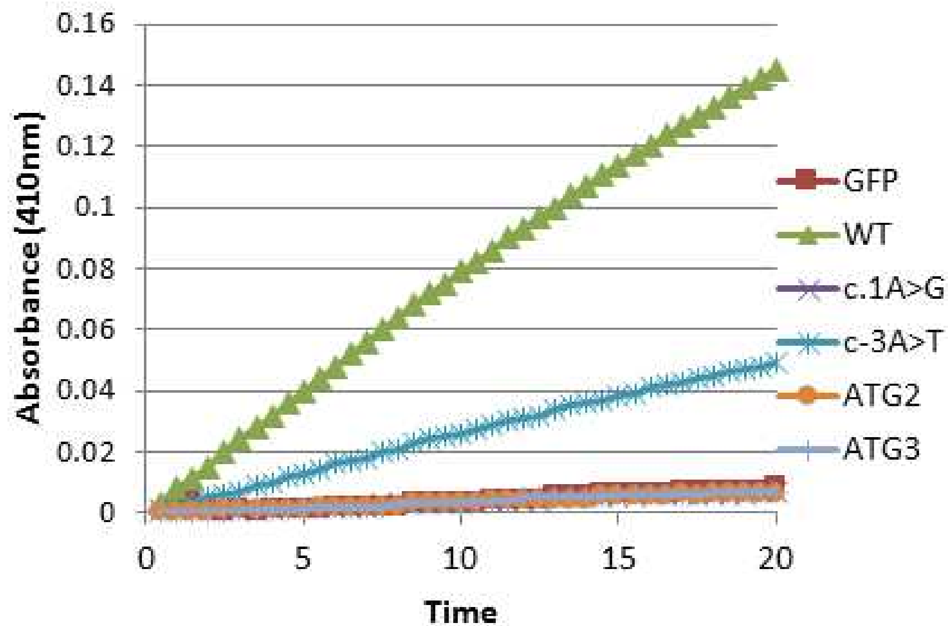
Supplementary Figure 3. Granulopoiesis derived from a healthy donor and an *ELANE* start codon-mutant severe congenital neutropenia patient-derived iPSC. A. Hematopoietic differentiation. Representative example of flow cytometry analysis of hCD45+ and hCD34+ expression. **B.** Hematopoietic cells derived from control iPSC and ATG mutant iPSC were differentiated in myeloid media and analyzed for myeloid differentiation and apoptosis (Annexin V/7-AAD), showing increased apoptosis of $CD34^-$/$CD33^+$ cells from ELANE start codon mutant iPSC-derived myelopoiesis. **C.** Frequency of granulocytic precursors and terminal neutrophils and monocytes showing myelocyte arrest in ATG mutant iPSC-derived granulopoiesis. **D.** Cytospin of granulocytic cells from cells analyzed in panel C. Bar=10µm. **E.** Immunoblot for NE expression showing a larger number of reactive bands of neutrophil elastase in sorted myeloid cells derived from ATG mutant iPSC.



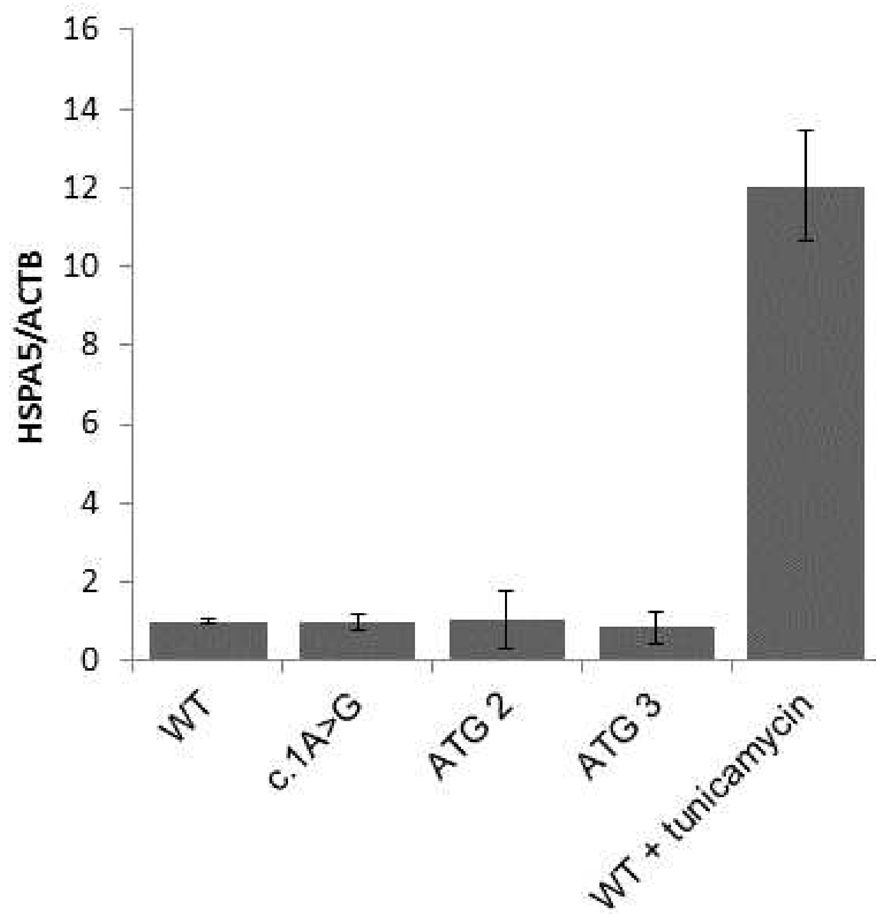
Supplementary Figure 4. Translational effects of various *ELANE* mutations in RBL-1 cells. For p.S-2X (28 amino acids from translation start site and -2 residues before amino terminus of fully processed protein following pre-pro cleavage), a stop codon was generated by a single nucleotide substitution (c.83C>A) in the c.-3A>T vector. V16M and S17F were generated from the WT *ELANE* vector. p.S-2X still produced the shorter isoforms representing ATG2 ORF and ATG3 ORF despite not producing the WT protein because of the premature termination codon between ATG1 and ATG2. V16M produced a protein of shorter length than WT and which aligns with a band produced by the c.-3A>T vector. S17F did not produce any additional bands but demonstrates limited expression in RBL-1 cells, rendering it uncertain as to whether it is capable of yielding shorter isoforms. Background bands from empty vector (EV) and an expression vector for green fluorescent protein (GFP) are present in this series of experiments.



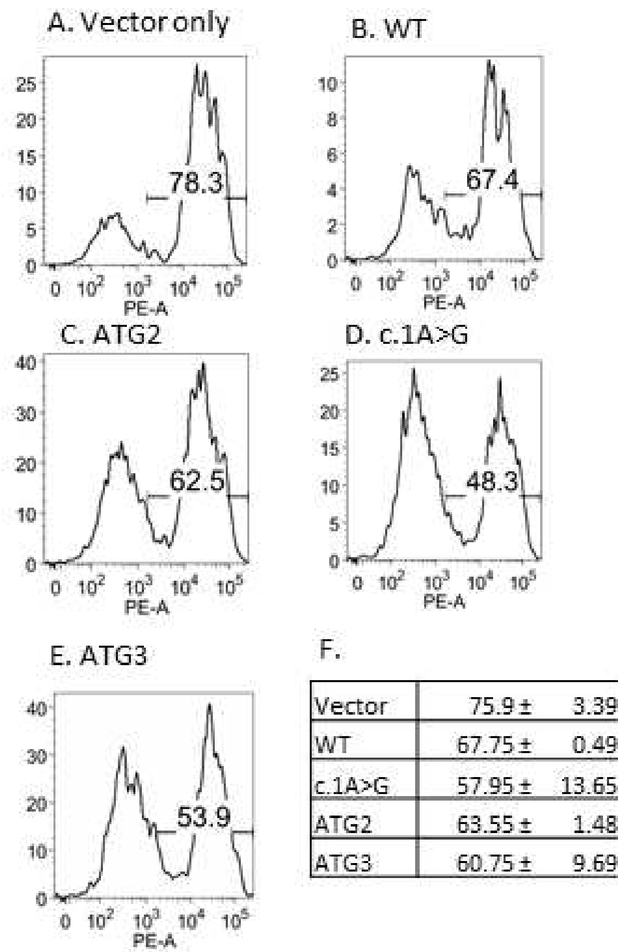
Supplementary Figure 5. Histogram of the number of complementary bases between 10 million random sequences and the 18S rRNA. Out of the ten million random sequences generated, only 13 were of equal or greater complementarity (18 basepairs) as the potential IRES in *ELANE,* giving a probability that the sequence exhibits complementarity due to chance of $1.3E^{-6}$.



Supplementary Figure 6. Catalytic activity of various forms of *ELANE* measured by cleavage of MeO-Suc-Ala-Ala-Pro-Val-pNA. RBL-1 cells were electroporated with 30 µg *ELANE* vector (Ref. 11) and 5 µg PRF vector using a Gene Pulser (Bio-Rad, Hercules, CA) at 250 V and 500 µF. Cells were lysed in luciferase passive lysis buffer, and elastase catalytic activity assay was performed as described (Ref. 11) , using a Synergy 4 plate reader. Time points were collected every minute for 20 minutes and then a final time point 16 hours later. Only WT neutrophil elastase produced significant catalytic activity. c.-3A>T produced activity due to the presence of a small amount of WT NE. Samples that included only shortened forms of NE (c.1A>G, ATG2, and ATG3) did not demonstrate catalytic activity exceeding that of the GFP vector. Data shown is from a single experiment and is representative of multiple experiments.

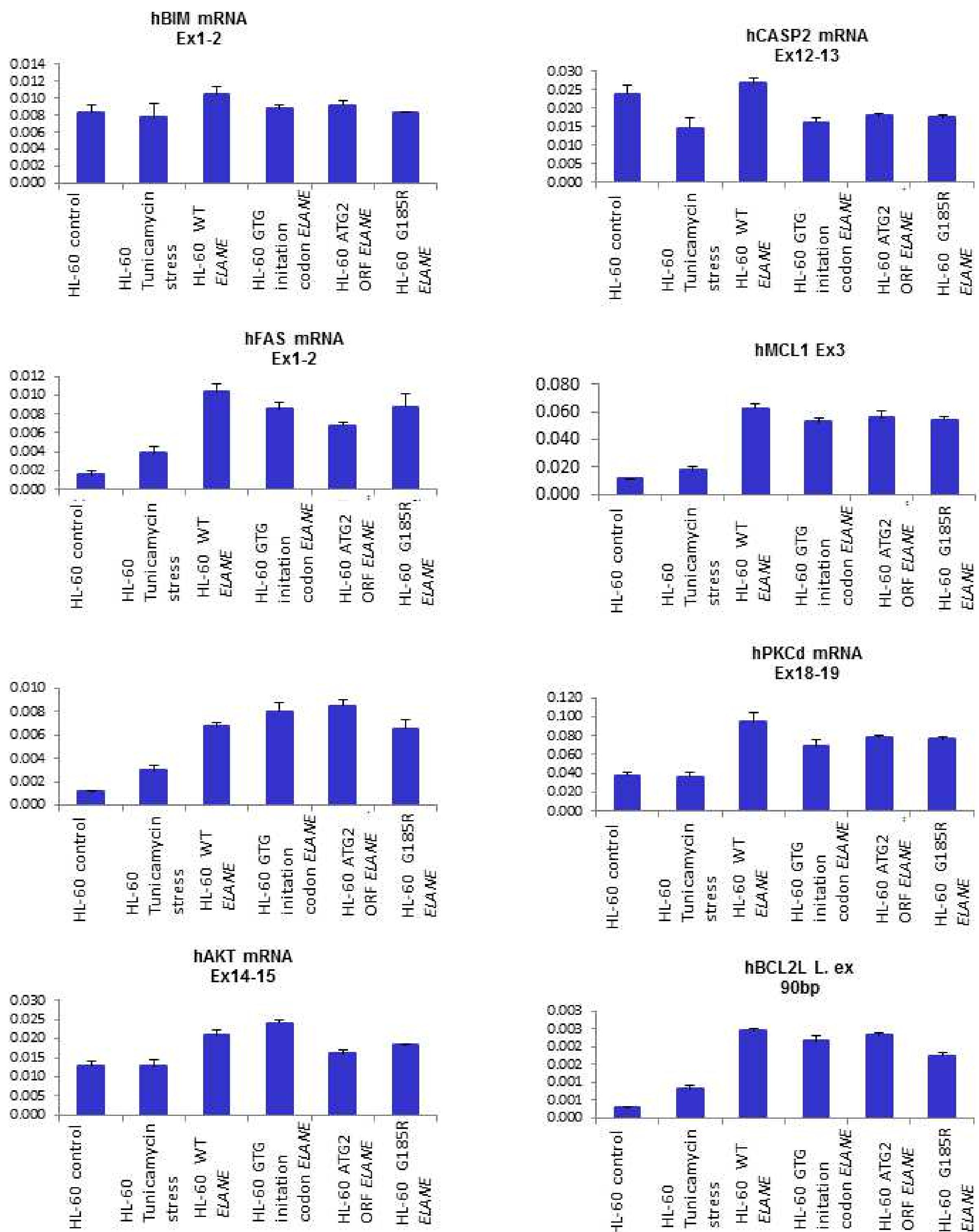


Supplementary Figure 7. Control experiment for *HSPA5* activation. HL-60 cells transfected with wild type (WT) *ELANE* were stimulated with tunicamycin, which greatly increased *HSPA5* expression. Data shown are from 3 independent experiments. Mean ± standard deviation.

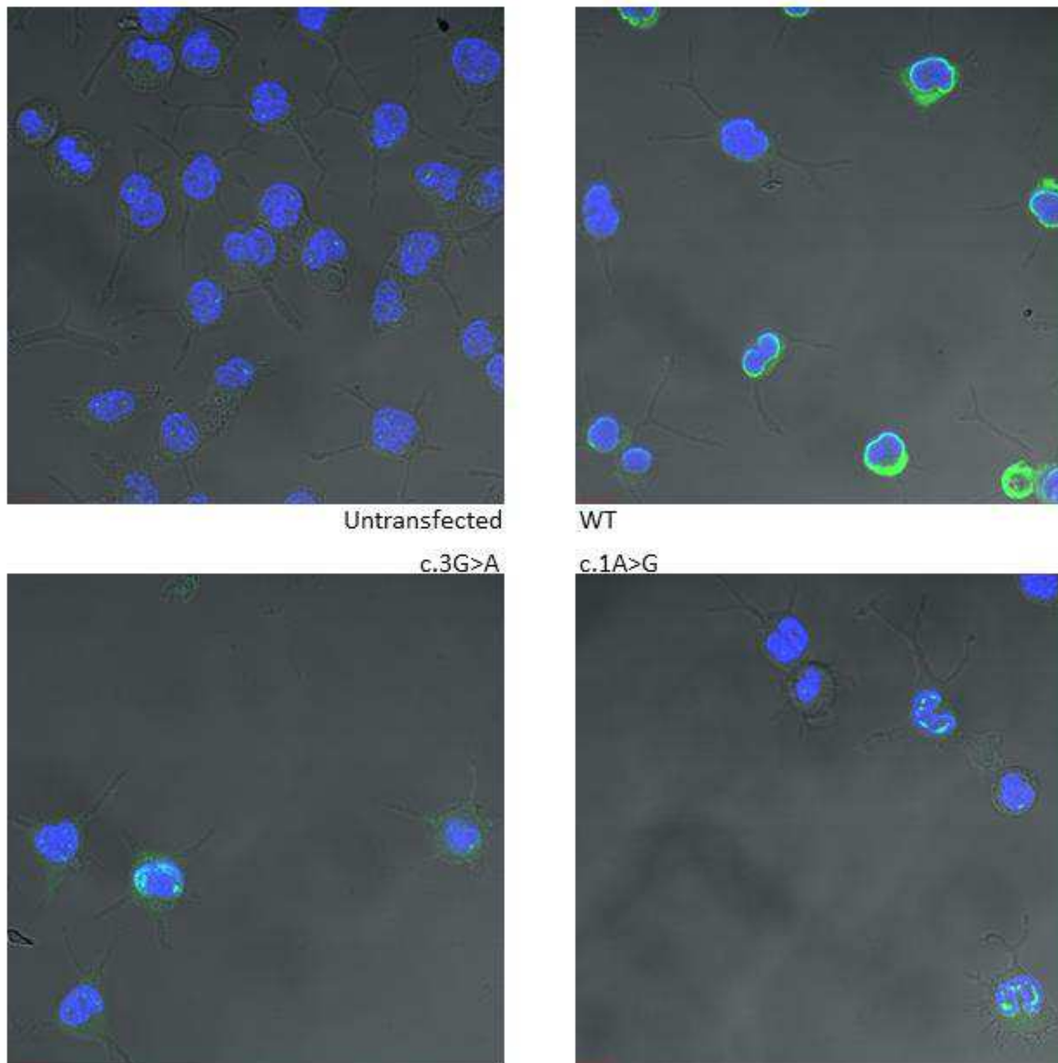


Vector	75.9 ±	3.39
WT	67.75 ±	0.49
c.1A>G	57.95 ±	13.65
ATG2	63.55 ±	1.48
ATG3	60.75 ±	9.69

Supplementary Figure 8. Flow Cytometry histograms of HL-60 cells transfected with *ELANE* vectors stained with PE-conjugated annexin V. A-E. representative data of annexin V positive cells from a single experiment. All of the mutants show less apoptosis (C-E) than vector alone (A) or WT *ELANE* (B). F. Table of mean ± standard deviation for 2 independent experiments.



Supplementary Figure 9. PCR panel of apoptosis related genes. RNA extracted from HL-60 cells transfected with *ELANE* vectors was used for RT-PCR of various apoptosis related genes.



Supplementary Figure 10. Confocal photomicrographs of RBL-1 cells transfected with WT and mutant forms of Neutrophil Elastase. Transfection of RBL cells and immunofluorescent imaging was performed as described (Ref. 11) with the following modifications: The secondary antibody was fluorescein-conjugated. Cells were additionally simultaneously stained for HAX1 using 40 µL/mL of mouse monoclonal anti-HAX1 clone 52 antibody (BD) detected with 5µL/mL Alexa fluor 555 goat anti-mouse secondary antibody (Life Technologies). Imaging was performed on a Zeiss LSM 510 confocal microscope at the Fred Hutchinson Cancer Research Center, using a Plan-Apochromat 63×/1.4 Oil DIC1 objective. Images were viewed and scale bars inserted with Zeiss LSM Image Browser Version 4,2,0,121. Views shown are planar. Alexa fluor channel was turned off. DIC brightness was adjusted within Zeiss LSM Image Browser. Scan Zoom varied between images, and sizes of images were enlarged/reduced to achieve similar magnifications and accordingly cropped within Microsoft PowerPoint for presentation. Scale bar (red, lower left) is 10 µm.

Programing supplement

Program Supplement 1. Program for simulation of random nucleotides pulled from the *ELANE* mRNA compared for complementarity with nucleotides 1146-1124 of the 18S rRNA. All simulations were run on the Enthought Canopy Python distribution (version 1.0.1.1189) with NumPy (version 1.7.1) and MatLibPlot (version 1.2.1) packages installed. Ten million random sequences of the same length as the potential *ELANE* IRES were generated using the *ELANE* mRNA as a nucleotide pool without replacement. The random sequences were then checked against bases 1146-1124 of the 18S rRNA for complementarity, and the total number of complementary bases was stored and plotted on a histogram.

```
from numpy import random
import matplotlib.pyplot as plot
elanemRNA='GCACGGAGGGGCAGAGACCCCGGAGCCCCAGCCCCACCATGACCCTCGGCCGCCGACTCGCGTGTCT\
TTTCCTCGCCTGTGTCCTGCCGGCCTTGCTGCTGGGGGGCACCGCGCTGGCCTCGGAGATTGTGGGGGGCCGGCGAGC\
GCGGCCCCACGCGTGGCCCTTCATGGTGTCCCTGCAGCTGCGCGGAGGCCACTTCTGCGGCGCCACCCTGATTGCGCC\
CAACTTCGTCATGTCGGCCGCGCACTGCGTGGCGAATGTAAACGTCCGCGCGGTGCGGGTGGTCCTGGGAGCCCATAAC\
CTCTCGCGGCGGGAGCCCACCCGGCAGGTGTTCGCCGTGCAGCGCATCTTCGAAAACGGCTACGACCCCGTAAACTTGC\
TCAACGACATCGTGATTCTCCAGCTCAACGGGTCGGCCACCATCAACGCCAACGTGCAGGTGGCCCAGCTGCCGGCTCA\
GGGACGCCGCCTGGGCAACGGGGTGCAGTGCCTGGCCATGGGCTGGGGCCTTCTGGGCAGGAACCGTGGGATCGCCAG\
CGTCCTGCAGGAGCTCAACGTGACGGTGGTGACGTCCCTCTGCCGTCGCAGCAACGTCTGCACTCTCGTGAGGGGCCGG\
CAGGCCGGCGTCTGTTTCGGGGACTCCGGCAGCCCCTTGGTCTGCAACGGGCTAATCCACGGAATTGCCTCCTTCGTCC\
GGGGAGGCTGCGCCTCAGGGCTCTACCCCGATGCCTTTGCCCCGGTGGCACAGTTTGTAAACTGGATCGACTCTATCAT\
CCAACGCTCCGAGGACAACCCCTGTCCCCACCCCCGGGACCCGGACCCGGCCAGCAGGACCCACTGAGAAGGGCTGCCC\
GGGTCACCTCAGCTGCCCACACCCACACTCTCCAGCATCTGGCACAATAAACATTCTCTGTTTTGTAGAAAAAAAAAAA\
AAAAA'
elaneIRES =  'guguccugcagcugcgcggagg'
rRNA=        'caaagggccuucgacgggccgcc'
def pickRandomSequence(sourceseq,length):
    "picks a random sequence of given length pulled from the\
sourceseq without replacement"
    array=[]
    L=len(sourceseq)
    for i in range(L):
        array.append(sourceseq[i])
    randomArray = random.choice(array,length,False)
    randomSeq=''
    for i in range(len(randomArray)):
        randomSeq +=randomArray[i]
    return randomSeq
def complementCount(sequence1,sequence2):
    "given two DNA sequences,counts the number of complementary base pairs"
    count=0
    if len(sequence1) != len(sequence2):
        return -1
    L = len(sequence1)
    seq1 = sequence1.lower()
    seq2 = sequence2.lower()
```

```
    for i in range(L):
        if seq1[i] == 'a'and seq2[i] =='t':
            count+=1
            continue
        elif seq1[i] == 't'and seq2[i] =='a':
            count+=1
            continue
        elif seq1[i] == 'g'and seq2[i] =='c':
            count+=1
            continue
        elif seq1[i] == 'c'and seq2[i] =='g':
            count+=1
            continue
        elif seq1[i] == 'u'and seq2[i] =='a':
            count+=1
            continue
        elif seq1[i] == 'a'and seq2[i] =='u':
            count+=1
            continue
    return count
resultsbin=[]
trialcount = 10000000
actualCompNumber = complementCount(elaneIRES,rRNA)
for i in range(trialcount):
    x= pickRandomSequence(elanemRNA,len(elaneIRES))
    result = complementCount(x,rRNA)
    resultsbin.append(result)
resultsbin.sort() #sorts from low to high
try: #tries to find index of actual comp count in the results array
    P=(trialcount-(resultsbin.index(actualCompNumber)))/float(trialcount)
except ValueError:
    P=0
#number of times the random sequence is more complementary then the actual
#divided by number of random sequences
print P
plot.hist(resultsbin)
plot.show()
```

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