Award Number: W81XWH-13-1-0066

TITLE: Translational Control in Bone Marrow Failure

PRINCIPAL INVESTIGATOR: Marshall S. Horwitz

CONTRACTING ORGANIZATION: University of Washington
Seattle, WA  98195-9472

REPORT DATE: April 2014

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland  21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and
should not be construed as an official Department of the Army position, policy or decision
unless so designated by other documentation.
1. REPORT DATE
April 2014

2. REPORT TYPE
Annual

3. DATES COVERED
1 May 2013 - 30 Apr 2014

4. TITLE AND SUBTITLE
Translational Control in Bone Marrow Failure

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-13-1-0066

5c. PROGRAM ELEMENT NUMBER

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

6. AUTHOR(S)
Marshall S. Horwitz

E-Mail: horwitz@uw.edu

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Washington
SEATTLE WA 98195-0001

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)
USAMRMC

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
Severe congenital neutropenia (SCN) is an inherited bone marrow failure syndrome most often resulting from autosomal dominant or de novo transmission of heterozygous mutations in the gene, ELANE, encoding neutrophil elastase. Other causes of SCN include autosomal recessive inheritance of HAX1 mutations. The purpose of this research is to understand how mutations in both ELANE and HAX1 lead to neutropenia, in order to gain further understanding into normal homeostatic regulation of granulopoiesis and how it is disrupted in a variety of bone marrow failure syndromes. Based on identification of a new class of mutations in ELANE that disrupt the translational start site and recent findings that HAX1 may be an RNA-binding protein, we have hypothesized that ELANE has the potential to encode amino-terminally truncated polypeptides initiating from internal translational start sites and that mutations in either ELANE or HAX1 may deregulate normal translational control of ELANE. In scope, we have proposed a series of experiments to be performed in cell culture systems to identify RNA sequences bound by HAX1, to determine if ELANE mutations influence HAX1 binding to ELANE mRNA and influence use of alternate translational start sites, and to biochemically characterize amino-terminally truncated neutrophil elastase. After the first year of funding we have preliminary identified mRNA sequences to which HAX1 protein may bind. More significantly, in a published study, using both cultured cells as well as SCN patient-derived induced pluripotent stem cells (iPSC), we have determined that mutations disrupting the translation initiation site of ELANE can lead to production of amino-terminally truncated polypeptides. One or more of the aberrantly translated forms of neutrophil elastase mislocalizes within the cell and retains proteolytic activity, thereby suggesting a potential mechanism for how mutations throughout the length of neutrophil elastase may prove pathogenic.

15. SUBJECT TERMS
Neutropenia, bone marrow failure, neutrophil elastase, ELANE, HAX1, alternate translation, induced pluripotent stem cells (iPSC)

16. SECURITY CLASSIFICATION OF:

<table>
<thead>
<tr>
<th>a. REPORT</th>
<th>b. ABSTRACT</th>
<th>c. THIS PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>U</td>
<td>U</td>
<td>U</td>
</tr>
</tbody>
</table>

17. LIMITATION OF ABSTRACT
UU

18. NUMBER OF PAGES
38

19a. NAME OF RESPONSIBLE PERSON
USAMRMC

19b. TELEPHONE NUMBER (include area code)
# Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Introduction</td>
<td>2</td>
</tr>
<tr>
<td>Body</td>
<td>3</td>
</tr>
<tr>
<td>Key Research Accomplishments</td>
<td>8</td>
</tr>
<tr>
<td>Reportable Outcomes</td>
<td>9</td>
</tr>
<tr>
<td>Conclusion</td>
<td>10</td>
</tr>
<tr>
<td>References</td>
<td>NA</td>
</tr>
<tr>
<td>Appendices</td>
<td>11</td>
</tr>
</tbody>
</table>
Introduction

The overall goal of this research is to understand how mutations in the genes \textit{ELANE}, encoding neutrophil elastase, and \textit{HAX1}, encoding HCLS1 associated protein X-1 (HAX1), cause hereditary forms of neutropenia. Previously, competing hypotheses have posited that mutant forms of neutrophil elastase either subcellularly mislocalize or misfold, whereas the specific role of \textit{HAX1} has been less clear. Our recent identification of a subgroup of patients with \textit{ELANE} translational start site mutations has challenged both hypotheses because, at first glance, such mutations are not expected to result in translation of protein. We have offered a new, unified hypothesis in which we propose that \textit{ELANE} may encode a set of polypeptides with variable in-frame amino-terminal initiation sites whose production is ordinarily suppressed by \textit{HAX1} and that mutations in either gene may lead to their pathogenic production. To test this hypothesis, we have proposed three aims designed to, first, determine \textit{HAX1} binding specificity to \textit{ELANE} mRNA; second, identify the range of mutations within \textit{ELANE} that may contribute to aberrant translation initiation; and, third, characterize the biochemical properties of amino-terminally truncated neutrophil elastase polypeptides.

We have developed a protocol for the specific co-immunoprecipitation of HAX1-mRNA complexes, as shown in the figure below. (A) Cells are lysed in the presence of RNAse inhibitor and bound to antibody-coated beads. (B) This western blot developed with anti-HAX1 shows specific enrichment for HAX1 protein in the eluent compared to IgG control serum. (C) Total RNA co-precipitated is shown, as measured using an Agilent Bioanalyzer. Note specific enrichment for recovered total RNA using anti-HAX1 compared to IgG control. (D) Bioanalyzer elution profile shows integrity of input RNA (demonstrated by microRNAs at ~25 sec peak and later eluting 18S/28S RNA peaks). Chromatograms of elution products show no dominant species of RNA (though microRNAs are still present with relative abundance), indicating that there is a heterogeneous pool of bound RNA.

1b. Perform SOLiD sequencing of immunoprecipitated mRNA (months 1-6).

Total RNA from three technical replicates of co-immunoprecipitation using both HAX1 antibodies and IgG control were converted to cDNA and prepared for next-generation sequencing using the ABI SOLiD Whole Transcriptome Analysis Kit. Mapping of raw sequence reads to consensus coding sequence (CCDS) genes was performed using ABI’s Bioscope software pipeline.

1c. Preliminary biocomputational analysis of sequence data, including mapping to reference genome (months 7-10).

Preliminary analysis was performed from the full mapping set that had ≥5× coverage for each of the sequenced HAX1 libraries. Sequences mapping to several genes were identified where there was relative enrichment for HAX1 co-immunoprecipitated RNA compared to IgG co-immunoprecipitated RNA.
**EPHB2** (Ephrin Receptor): 16× vs IgG, 13.5× vs total RNA. **EPHB2** has been shown to be involved in cell motility. Neutropenic Gfi1-deficient mice also show an increase in **EPHB2**.

**ASTN1** (Astrotactin): 5.4× vs IgG, 8× vs total RNA. Astrotactin is a neuronal adhesion molecule required for glial-guided migration of young postmitotic neuroblasts in cortical regions of developing brain, including cerebrum, hippocampus, cerebellum, and olfactory bulb. These are similar areas to where apoptosis is seen in Hax1-deficient mice, noting that congenital neutropenia patients with HAX1 mutations can demonstrate neurological involvement.

**CASP9** (Caspase 9): 4.6× vs IgG, 2.5× vs total RNA. HAX1 has been shown to protect cells from apoptosis in a Caspase 9 inhibitory fashion.

**FAF1** (FAS associated factor 1): 2.5× vs IgG, 4× vs total RNA. FAF1 enhances FAS-mediated apoptosis. FAS-mediated apoptosis is implicated in neutropenia and the neutrophil life cycle.

1d. **Gene ontogeny and pathway characterization of identified mRNA species (months 11-13).**

These studies are being initiated, but will benefit from additional sequence data.

1e. **Consensus binding site determination (months 14-16).**

These studies are planned. Additional sequence data are required to determine a meaningful consensus.

1f. **Troubleshoot sequencing failures, allow time for repetition and alternate approaches (months 17-24).**

Although time is reserved later in the project period for the trouble-shooting phase, one issue preliminarily identified concerns lack of read-depth in the experiments we performed on the ABI SOLiD sequencing platform, vis-à-vis improvements in next-generation DNA sequencing technology utilizing other platforms, primarily those from Illumina.

**Task 2. Determine if ELANE mutations, particularly synonymous ELANE mutations, affect HAX1 binding and influence cryptic start site utilization.**

Many of the experiments for Tasks 2 and 3 have been completed and published in Tidwell et al. Neutropenia-associated ELANE mutations disrupting translation initiation produce novel neutrophil elastase isoforms Blood 2014;123:562-569 (appended). Specifics sections of this publication with respect to sub-tasks are identified below.

2a. **Prepare cDNA expression constructs for various mutations and express in RBL cells (months 1-36).**

cDNA constructs corresponding to the following mutations have been generated and tested in RBL cells: wild type, c.-3A>T, c.1A>G, C.3G>A, V16M, S17F, L18P, and G185R. Their use is reported in Figs. 2-5 and supplemental Figs. S4 and S6-S10 of Tidwell et al. with respect to additional sub-tasks, as noted in detail below. Construction of additional vectors corresponding to mutations distributed elsewhere will proceed throughout the duration of the proposed project.
2b. Prepare cDNA constructs for use in yeast 3-hybrid assay (months 1-6).


2c. Western blots to assay for internal start site utilization in transfected RBL cells (months 6-12).

Several of the mutations have been tested as published in Tidwell et al. (Figs. 2 and S4). Although not initially planned, there have been significant improvements and opportunities utilizing induced pluripotent stem cell (iPSC) technology since the time of the original submission. Through collaborations with other investigators at Cincinnati Children’s Hospital and elsewhere, we successfully established several patient-derived iPSC cell lines containing defined mutations. Significantly, we were able to demonstrate alternative translational start site utilization in both transfected RBL cells and, more importantly, patient-derived iPSC (Figs. S2-3 of Tidwell et al.). These additional studies in iPSC extend and support our findings and corroborate our hypothesis that mutations disrupting the canonical start site of ELANE lead to production of amino-terminally truncated neutrophil elastase polypeptides and suggest a more general context for how mutations elsewhere in the protein may prove pathogenic (Fig. 6, as noted in Discussion, of Tidwell et al.).

2d. Prepare vectors containing mutations for IRES test assay (months 3-9).

Bi-cistronic IRES test vectors containing mRNA sequences corresponding to NM-001972.2 nucleotides 171-233 (Region 1), 42-233 (Region 2), and 1-233 (Region 3) have been prepared and tested, as shown in Fig. 3A of Tidwell et al. Additionally, mutations corresponding to p.V16M, p.S17F, and p.L18P have been introduced into a an IRES test vector containing the 171-233 region (Fig. 3B of Tidwell et al.).

2e. Develop IRES test assay in HeLa cells (months 9-12).

A HeLa cell IRES test assay was successfully developed (Fig. 3 of Tidwell et al.). The findings are reported in the paper:

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 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 (Fig. 3B of Tidwell et al.), suggesting that IRES activity is not adventitious.

As noted in our proposal, ELANE mutations frequently locate to the putative IRES. 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 (Fig. 3B of Tidwell et al.). Two of the mutations (p.V16M and p.S17F) markedly increased activity, compared to empty vector. When transfected into RBL cells (Fig. S4 of Tidwell et al.),
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.

2f. Perform yeast 3-hybrid assays with mutant constructs (months 15-36).

These experiments remain planned for later stages of the project.

2g. Develop HAX1 RNAi assay in HL60 cells (months 20-36).

These experiments remain planned for later stages of the project.

Task 3. Characterize the biochemical properties of internally-translated neutrophil elastase polypeptides (months 14-36).

Even though these studies had not been planned to start until later stages of the project, we initiated them earlier than anticipated, in part because of rapid developments enabled by the use of patient-derived iPSC. Many of these experiments have been published in Tidwell et al., as noted below.

3a. Prepare cDNA expression constructs for each of the internally translated polypeptides for transfection into RBL cells (months 14-17).

We prepared cDNA constructs isolating each of the internal ORFs and transfected them into RBL cells (i.e. Fig. 2 of Tidwell et al., right panel, as demonstration of their expression). Moreover, we were fortunate in that we derived iPSC from a patient with the c.1A>G mutation, who therefore expresses ORFs corresponding to ATG2, ATG3, and ATG4 but not ATG1 (Figs. S2-3 of Tidwell et al.).

3b. Determine if internally translated polypeptides are catalytically active when transfected into RBL cells (months 18-24).

To determine if amino-terminally truncated forms of neutrophil elastase 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 (Figs. 5A and S6 of Tidwell et al.).

3c. Determine the subcellular localization of internally translated polypeptides by immunofluorescent staining within transfected RBL cells (months 24-26).

To determine if amino-terminally truncated forms of neutrophil elastase 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 (Fig. S10 of Tidwell et al.).

3d. Determine subcellular localization of internally translated polypeptides based on subcellular fractionation (months 27-29).

These experiments remain planned and will be performed at later stages of the project.

3e. Determine if internally translated polypeptides induce expression of ER or other cellular stress markers (months 30-33).

We assessed whether amino-terminally truncated forms of neutrophil elastase 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 (Fig. 5B of Tidwell et al.), whereas control cells transfected with wildtype ELANE and stimulated with tunicamycin, which induces ER stress, showed a 12× increase in HSPA5 expression (Fig. S7 of Tidwell et al.). We conclude that mutations altering the start site of translation may not require that an ER stress response occur, in order to prove pathogenic. This observation is in agreement with our proposed model (Fig. 6 of Tidwell et al.) that a stress response caused by other mutations may activate alternative translation, which are the true effectors of pathogenesis.

3f. Evaluate for apoptotic effects of transfected internally translated polypeptides (months 33-36).

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 neutrophil elastase. There was no increase in apoptosis as observed by annexin V binding (Figs. 5C and S8 of Tidwell et al.). Additionally, RT-PCR assays revealed no increase in expression across a panel of apoptosis-related genes (Figs. S9 of Tidwell et al.). The fact that apoptosis was evident in iPSC (Fig. S3 of Tidwell et al.), however, exposes limitations of HL-60 cells.

3g. Develop a flow-sorting based clonogenic assay to evaluate for toxic effects on cell growth of internally translated polypeptides upon transfection of U937 cells (months 33-36).

To determine if expression of neutrophil elastase 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. 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 (Fig. 4 of Tidwell et al.). 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.

Of even greater relevance, the establishment of patient-derived iPSC allowed us to determine whether alternative start site utilization affected hematopoietic colony formation in vitro. 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 (Figs. S3A-3D of Tidwell et al.).

Task 4. Data will be analyzed and a report shall be prepared, concurrent with the overall aim to report findings in the peer-revised biomedical literature, in order to allow accessibility to physicians, scientists, patients, and the general public.

As noted above, some of the tasks have been completed and are now published in Tidwell et al. (PubMed PMID 24184683), which received an accompanying commentary (Borregaard. Severe congenital neutropenia: new lane for ELANE. Blood. 2014;123:462-3).
Key Research Accomplishments

- Demonstration that $ELANE$ mutations in the first codon and Kozak sequence yield amino-terminally truncated neutrophil elastase lacking pre and pro sequences that lead to aberrant subcellular localization and that may retain catalytic activity.

- The study implies that sometimes neutrophil elastase coding sequence changes are incidental and noncoding $ELANE$ variants are pathogenic. This finding may have broad applicability to interpretation of synonymous variants identified through genome-wide association studies (GWAS) and other contemporary population screens searching for new disease-causing genes.

- Used an induced pluripotent stem cells (iPSC) model of $ELANE$ mutations to recapitulate clinical defects in hematopoiesis with accompanying in vitro demonstration of alternate translational start site utilization.

- Provided further experimental evidence for the role of HAX1 in regulating $ELANE$ translation.
Reportable Outcomes

Manuscripts


Development of cell lines, tissue or serum repositories

Generated ELANE-mutant induced pluripotent stem cells (iPSC).
Conclusions

Neutrophils are the first line of innate immune defense against bacterial and fungal infections. Neutropenia can occur in a variety of contexts, including as a primary complication of chemotherapy for solid tumors, such as breast cancer, with acquired hematopoietic malignant disorders such as myelodysplasia, and in a variety of other acquired and heritable bone marrow failure syndromes. Understanding the molecular genetic basis of hereditary neutropenia will elucidate mechanisms of normal and pathological hematopoiesis and conceivably could eventually result in new forms of therapy for neutropenia.

Our studies have defined a complex translational regulatory mechanism governing production of neutrophil elastase, encoded by the gene \textit{ELANE}, whose mutations are responsible for the most common form of hereditary neutropenia. Our findings challenge preconceptions about the enzyme’s processing and subcellular trafficking. Our results imply that mutations not directly altering protein coding sequence may be still be consequential and serve as an example for interpreting other potentially synonymous mutations discovered in other genetic studies, including in particular genome-wide association studies (GWAS) for common disease.

In sum, we are gratified to quote from a commentary that accompanied our paper (Tidwell et al; Blood 2014;123:562-569), from distinguished scientist Dr. Niels Borregaard at the University of Copenhagen: “Although this paper concerns a small fraction of patients with a rare disorder, the study opens a new path for understanding how genetic defects affect cellular differentiation that may be relevant to other diseases, both congenital and acquired, not the least of which include the myelodysplastic syndromes and acute myeloid leukemia. (Borregard. Blood 2014;123:462-463)”
Neutropenia-associated *ELANE* mutations disrupting translation initiation produce novel neutrophil elastase isoforms


Updated information and services can be found at:
http://bloodjournal.hematologylibrary.org/content/123/4/562.full.html

Articles on similar topics can be found in the following Blood collections
Phagocytes, Granulocytes, and Myelopoiesis (453 articles)

Information about reproducing this article in parts or in its entirety may be found online at:
http://bloodjournal.hematologylibrary.org/site/misc/rights.xhtml#repub_requests

Information about ordering reprints may be found online at:
http://bloodjournal.hematologylibrary.org/site/misc/rights.xhtml#reprints

Information about subscriptions and ASH membership may be found online at:
http://bloodjournal.hematologylibrary.org/site/subscriptions/index.xhtml
PHAGOCYTES, GRANULOCYTES, AND MYELOPOIESIS

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

Timothy Tidwell,1 Jeremy Wechsler,1 Ramesh C. Nayak,2 Lisa Trump,2 Stephen J. Salipante,3 Jerry C. Cheng,4 Jean Donadieu,5 Taly Glaubach,6 Seth J. Corey,6 H. Leighton Grimes,2,7 Carolyn Lutzko,2,8,9 Jose A. Cancelas,2,10 and Marshall S. Horwitz1

1Department of Pathology, University of Washington School of Medicine, Seattle, WA; 2Division of Experimental Hematology and Cancer Biology, Cincinnati Children's Hospital Medical Center, Cincinnati, OH; 3Departments of Laboratory Medicine and Genome Sciences, University of Washington School of Medicine, Seattle, WA; 4Kaiser Permanente, Los Angeles Medical Center and David Geffen School of Medicine at UCLA, Los Angeles, CA; 5French Severe Chronic Neutropenia Registry, Hospital Trousseau, Paris, France; 6Department of Pediatrics, Northwestern University Feinberg School of Medicine, Chicago, IL; 7Division of Immunobiology, Cincinnati Children's Hospital Medical Center, Cincinnati, OH; 8Division of Regenerative Medicine and Cellular Therapies, Hoxworth Blood Center, University of Cincinnati College of Medicine, Cincinnati, OH; 9Translational Core Laboratories, Division of Experimental Hematology and Cancer Biology, Cincinnati Children's Hospital Medical Center, Cincinnati, OH; and 10Research Division, Hoxworth Blood Center, University of Cincinnati College of Medicine, Cincinnati, OH

Hereditary neutropenia is usually caused by heterozygous germline mutations in the ELANE gene encoding neutrophil elastase (NE). How mutations cause disease remains uncertain, but 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 in the endoplasmic reticulum (ER). In this study, we describe new types of mutations that disrupt 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 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 the production of amino-terminally deleted NE isoforms rather than by altering the coding sequence of the full-length protein. (Blood. 2014;123(4):562-569)

Introduction

There are two main types of hereditary neutropenia: cyclic neutropenia and severe congenital neutropenia (SCN). In cyclic neutropenia, neutrophil counts oscillate with 21-day periodicity.1 In SCN, neutrophil counts are statically low, promyelocytic maturation arrest occurs in the bone marrow, and disease often progresses to myelodysplasia or acute myeloid leukemia.1 Heterozygous mutation of ELANE causes almost all cases of cyclic neutropenia2 and the majority of SCN.3 Because neutropenia is often lethal, germline mutations frequently arise de novo.1 Additional genes causing SCN include HAX1,4 GFI1,5 G6PC3,6,7 and others.2 ELANE encodes the neutrophil granule serine protease, neutrophil elastase (NE).3 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.1,9 The mutational spectrum would seem to exclude haploinsufficiency as a mechanism, because mutations predicting an 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.9-11 Two theories on 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). Relevant to the mistrafficking hypothesis, NE is stored in lysosome-like granules, but distributes to the plasma membrane...
and nucleus. Some ELANE mutations are reported to disturb NE trafficking, both in vitro and in vivo (though other studies have not found mislocalization). Furthermore, mutations in the gene encoding the lysosomal transporter protein AP3B1, which is involved in trafficking NE, are responsible for the neutropenic disorders Hermansy-Pudlak syndrome type 2 and canine cyclic neutropenia, and, at least in dogs, NE appears to be mislocalized. Chédiak-Higashi syndrome, caused by mutations in a different lysosomal trafficking protein, may also cause neutropenia, and in a mouse model of the disorder, NE appears to be mistrafficked. Finally, mutations in other genes involved in lysosomal trafficking, including VPS13B and VPS45, also cause neutropenia.

With the misfolding hypothesis, when certain ELANE mutations are expressed in vitro, UPR markers including binding immunoglobulin protein, XBP1, and GRP78 are upregulated. A supportive observation involves Wolcott-Rallison syndrome, which, in addition to other features, includes neutropenia and is caused by mutations in protein kinase RNA-like ER kinase (PERK) which functions as a sensor of ER stress. Gene-targeted mice carrying a neutropenia-associated ELANE mutation develop neutropenia when ER degradation is blocked with the proteasome inhibitor borzomycin, resulting in high levels of ER stress.

Here, we describe a new category of ELANE mutation disrupting the translation initiation codon or the immediately adjacent Kozak sequence that does not easily fit with either the mislocalization or misfolding hypotheses. Because they might not 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 polymerase chain reaction (PCR)-amplified peripheral blood genomic DNA was performed as described. Research was approved by the University of Washington Institutional Review Board, and participants gave written informed consent in accordance with the Declaration of Helsinki.

Induced pluripotent stem cell (IPSC) generation

Peripheral blood mononuclear cells were transduced using lentiviral vectors containing Oct4, Sox2, Klf4, and c-Myc. See details in supplemental Materials and methods section on the Blood Web site.

Cell culture

Human leukemic monocyte lymphoma (U937) and human promyelocytic leukemia (HL-60) cell lines were purchased from ATCC and cultured in RPMI 1610 (Life Technologies, Grand Island, NY). Henrietta Lacks (HeLa) and rat basophil leukemia 1 (RBL-1) cells were cultured in Dulbecco’s modified eagle medium (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, The Scripps Research Institute, La Jolla, CA), containing firefly and Renilla luciferase. A construct containing the triplicated active region was made by separating 3 tandem repeats with a 9-bp oligonucleotide derived from a non–internal ribosome entry site (IRES) region from the mouse β-globin gene (supplemental 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 microplate 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.

Clonogenic capacity assay

Wild-type and mutant ELANE vectors were generated by inserting ELANE into pRSRE2-ZsGreen (Clonetech, Mountain View, CA) obtained from previously described vectors. U937 cells were transfected with the Amaxa Nucleofector Kit C (Lonza, Basel, Switzerland), 2 μg of DNA, and program W-01. After 24 hours posttransfection, single cells were sorted into 96 well plates using a FACSAria II (BD Biosciences, 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% dimethylsulfoxide. ZsGreen-positive cells were sorted with a FACSARIA IL and RNA was isolated using RNeasy Plus Mini Kits (Qiagen, Germantown, MD). RNA was reverse-transcribed with SuperScript II reverse transcriptase (Life Technologies). Heat-shock 70-kDa protein 5 (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, and 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, following 16-hour incubation.

Statistical methods

Comparisons between groups employed Student 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 Registry. 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 Registry. The same mutation, again de novo, has subsequently been described in two additional, unrelated patients, including one unresponsive to recombinant human granulocyte colony stimulating factor therapy. 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, and, in the case of the former, was 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 supplemental Figure 1A). Also for the first time, we describe 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 wild-type ELANE, is strongly preferred29 (Table 1 and supplemental Figure 1B).

In summary, there were 9 unrelated probands with neutropenia comprising 5 different translation start mutations (Table 1). No other ELANE-coding sequence variants were observed among these patients. These variants have not been described in 6503 individuals in the National Heart, Lung, and Blood Institute’s Exome Sequencing Project (http://evs.gs.washington.edu), cataloged in 1000 Genomes,30 dbSNP31 (version 135), or among hundreds of controls.9 Since these variants were exclusively found in patients with neutropenia who lacked other causative mutations, and often arose de novo, but can be autosomally dominantly transmitted, we conclude that they are causal. Among a total of 191 different known ELANE mutations,9 all, except for these, were 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 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 complementary DNA (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, they correctly target it to granules),32 and performed a western blot with an antibody detecting a carboxyl-terminal epitope. When the initiation codon was mutated from ATG to either ATA or GTG, shortened forms of NE were evident (Figure 2, c.3G>A, c.1A>G). When the highly conserved nucleotide in the Kozak sequence 3 nucleotides upstream from the initiation codon was mutated from A to T, shorter proteins were produced along with the wild-type protein (Figure 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 (wild-type compared with 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 2).

We tested for the presence of shorter NE isoforms in patient-derived samples. Bone marrow was unavailable in patients with start-site mutations. Western blot performed on peripheral blood could not be interpreted due to neutropenia (not shown). We, therefore, generated iPSC from a patient with the c.1A>G mutation (supplemental Figure 2), which showed normal karyotype, pluriptotent stem-cell marker expression, and the ability to differentiate into all three germ layers. Compared with control, upon hematopoietic differentiation, there was no significant difference in the level of CD34+ cells, but subsequent myeloid differentiation demonstrated the following: fewer bands and neutrophils; increased promyelocytes, myelocytes, and metamyelocytes; more monocytes; and increased apoptosis of CD34+/CD33+ cells (supplemental Figure 3A-D). (The extent of apoptosis, however, may fall short of explaining cytopenias and could be an epiphenomenon.) Western blot of CD34+/CD13+/CD11blow/CD15+ myeloid cells derived from iPSC (supplemental Figure 3E) confirmed reduced abundance of full-length NE (as expected with heterozygosity for the mutation, where wild-type is also present) and uniquely increased abundance of at least one shorter isoform. Additional higher molecular weight products, as seen in RBL-1 cells, were detected. Isoform sizes in iPSC appeared to be slightly shifted upward, compared with RBL-1 cells, suggesting differences in posttranslational modification. Experiments with iPSC, therefore, corroborate phenotype and downstream initiation of translation.

**Defining an ELANE IRES**

Figure 2 shows that translation normally initiating from the canonical ATG1 start site in wild-type 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...
ELANE translation initiation mutations

That could be an IRES situated between ATG2 and ATG3. IRES sequences are regions of messenger RNA (mRNA) that recruit the ribosome to sites adjacent to internal ATG codons and permit internal initiation of translation in a cap-independent manner.33

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>G) and the noncoding Kozak sequence (c.-3A>T) lead to expression of shorter isoforms of ELANE. When the upstream region is removed and only the ORF that corresponds to each ATG codon is expressed, separated isoforms are identifiable (ATG2 ORF, ATG3 ORF, and ATG 4 ORF). Molecular weight markers (in kDa; right) and NE isoforms (arrows; left) are shown.

Effect of amino-terminally truncated NE on clonogenic capacity

To determine if the 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.21 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 possibly due to the presence of additional start codons within those regions. Additionally, a 5′-UTR of similar length from an arbitrary gene (KLHDCCB) lacked IRES activity (not shown). To further verify that region 1 contained an IRES, we evaluated its activity when it was triplicated in a head-to-tail sequence. IRES activity was increased by 6.9-fold, compared with the vector alone (Figure 3B), suggesting that IRES activity is not adventitious.

ELANE mutations activating the IRES

It should be noted that ELANE mutations frequently locate to the putative IRES.1,9 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 3B). Two of the mutations (p.V16M and p.S17F) markedly increased activity, compared with the empty vector. When transfected into RBL-1 cells (supplemental Figure 4), p.V16M produced 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 wild-type 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.

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

Figure 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; and Region 3: nt 1-233 (NM_001972.2). (B) Activity from 3 tandem repeats of nt 171-193 of ELANE mRNA. Only region 1 showed an increase in IRES activity (Figure 3B). Two of the mutations (p.V16M and p.S17F) markedly increased activity, compared with the empty vector. When transfected into RBL-1 cells (supplemental Figure 4), p.V16M produced 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 wild-type 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.
HSPA5 induce ER stress by measuring expression of that isolated ORFs corresponding to the second and third ATGs with wild-type translational start site (Figure 5B), whereas control cells transfected retained minimal, yet statistically significant residual activity, but that isolated ORFs corresponding to the second and third ATGs lacked activity (Figure 5A and supplemental Figure 6).

We assessed whether amino-terminally truncated forms of NE induce ER stress by measuring expression of HSPA5, which codes for the binding immunoglobulin protein. We found no increase in HSPA5 in HL-60 cells transfected with ELANE mutations altering the translational start site (Figure 5B), whereas control cells transfected with wild-type ELANE and stimulated with tuinumycin, which induces ER stress, showed a 12-fold increase in HSPA5 expression (supplemental Figure 7).

We also tested whether amino-terminally truncated forms of NE induce apoptosis in HL-60 cells transfected with vectors expressing either wild-type or amino-terminally truncated forms of NE. There was no increase in apoptosis as observed by Annexin V binding (Figure 5C and supplemental Figure 8). Additionally, reverse transcription (transcriptase)-PCR assays revealed no increase in expression across a panel of apoptosis-related genes (supplemental Figure 9). The fact that apoptosis was evident in iPSC exposes the 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 with granular cytoplasmic distribution of the wild-type, the shorter polypeptides appeared to localize to nuclei (supplemental 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. However, the demonstration of this phenomenon has been rare. One example involved acquired mutations of the hematopoietic transcription factor GATA1 associated with transient myeloproliferative disorder and megakaryoblastic leukemia of Down syndrome. Start codon mutations or, more often, proximal chain-terminating mutations just downstream from the initiation codon, lead to the production of a shorter GATA1 variant commencing from a second initiation codon, which lacks the activation domain yet retains the ability to bind DNA and cofactors. Similar mutations producing similar effects occur in the transcription factor C/EBPα in acute myeloid leukemia. Another example involved germline mutation of TPO, encoding thrombopoietin, in hereditary thrombocytopenia, and disrupting a splice-site, which leads to deletion of the canonical start site with initiation shifting downstream to the next in-frame ATG codon.

Here, we show that neutropenia-associated germline mutations disrupt the initial ATG codon, and adjacent noncoding Kazok 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. Furthermore, potential alternate start sites are evolutionarily conserved and are used in many cases.

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. One example is neutrophil 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. We have shown 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.

**Figure 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. A total of 384 wells were counted for each group during each experiment. Data shown are mean ± standard deviation of 3 independent experiments. *P < .05 vs WT; **P < .005 vs WT; †P = .05 vs c.1A>G.

**Figure 5. Properties of amino-terminally truncated NE.** (A) Mutant forms of NE are unable to cleave MeO-Suc-Ala-Ala-Pro-Val-pNA, an 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. (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 deviations of 2 independent experiments are shown. *P < .05 vs GFP.
We demonstrated IRES activity for a region of ELANE between the second and third internal ATG codons. Based on prior studies of Nkx6-2, we suspected this activity was due to direct ribosomal binding mediated by ELANE mRNA sequence complementarity to 18S rRNA. This IRES permits the 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 is not altered. Another interesting observation was that mutations of this region enhanced activity despite reducing complementarity to 18S rRNA compared with wild-type. In previous studies, it was found that the optimum length of complementarity to 18S rRNA was 7 nucleotides, and any sequence longer or shorter than that, decreased IRES activity.

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 neverthless 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.

ELANE mutations inactivating the ATG initiation codon are found in SCN patients and lead to the absence of full-length protein with expression of amino-terminally truncated isoforms not seen with the wild-type allele. However, the Kozak sequence mutation occurs in patients with cyclic neutropenia and results in the production of both full-length protein, and compared with 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 reduced 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 with wild-type. It is not surprising that the mutations did not induce ER stress since they were 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 2 and supplemental Figure 3E, suggested 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 retained the catalytic triad of histidine, aspartate, and serine necessary for proteolytic activity, raising the possibility that they were mature and possessed 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 alter the primary sequence of NE, which may lead to the production of internally-translated isoforms via protein misfolding.

The presence of shorter isoforms of ELANE generated by an IRES, actually fits with the two current models on how mutations may cause disease (Figure 6). If the misfolding hypothesis holds 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, which phosphorylates eukaryotic translation initiation factor 2, resulting in a preference for IRES-mediated translation over normal cap-dependent translation. Our observations also fit well with the mislocalization hypothesis. Since the shortened isoforms do not contain a propeptide 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. 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.

Acknowledgments

This work was supported by research grants from the National Institutes of Health (R01DK58161) and the US Department of Defense (BM120152).
References


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\(^1\) and cultured on murine embryonic fibroblasts. 20-30 days after transduction, iPSC-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 iPSC 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.\(^2\) 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.

References


## PCR Primers for pIRES2 vectors:

<table>
<thead>
<tr>
<th>Primer Type</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT forward</td>
<td>CAAGCAAGCGCAGGCCATCTCGGAATTCCAGCCCCACCATGACCCTCGGCCGCT</td>
</tr>
<tr>
<td>GTG forward</td>
<td>CAAGCAAGCGCAGGCCATCTCGGAATTCCAGCCCCACCGTGACCCTCGGCCGCT</td>
</tr>
<tr>
<td>ATG2 ORF forward</td>
<td>CAAGCAAGCGCAGGCCATCTCGGAATTCCAGCCCCACCATGGTGTCCCTGCA</td>
</tr>
<tr>
<td>ATG3 ORF forward</td>
<td>CAAGCAAGCGCAGGCCATCTCGGAATTCCAGCCCCACCATGTCGGCCGCGCA</td>
</tr>
<tr>
<td>Reverse primer A</td>
<td>ACGTACGTGATGAGAGCATAGAAGGTAGGATCCTCAGTGGTCTCTGCTGGCC</td>
</tr>
</tbody>
</table>

## Primers and sequences for PRF vector

<table>
<thead>
<tr>
<th>Region</th>
<th>Primer Type</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>ELANE Region 1 F</td>
<td>GCGCgaattcGTGTCCCTGCAGCT</td>
</tr>
<tr>
<td></td>
<td>ELANE Region 2 F</td>
<td>GTTTgaattCCTCCCGCAGCTGCGCGGAGGACACg</td>
</tr>
<tr>
<td></td>
<td>ELANE Region 3 F</td>
<td>GTCTgaattcGCACGGAGGGGACAG</td>
</tr>
<tr>
<td></td>
<td>ELANE R</td>
<td>GTCTTcatggGACGAAGTTGGGC</td>
</tr>
</tbody>
</table>

## Ligated sequences

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>ELANE 3rp b 1 +</td>
<td>[phos]aattgGTGTCCCTGCAGCTGCGCGGAGGttgtgacatGTGTCCCTGCAGCT</td>
</tr>
<tr>
<td>ELANE 3rp b 1 -</td>
<td>[phos]AGGGACACatgtgagaaCCTCCCGCAGCTGCGCGGAGGACACg</td>
</tr>
<tr>
<td>ELANE 3rp b 2 +</td>
<td>[phos]GCGCGGAGGttgtgacatGTGTCCCTGCAGCTGCGCGGAGGc</td>
</tr>
<tr>
<td>ELANE 3rp b 2 -</td>
<td>[phos]catggCCTCCCGCAGCTGCGCGGAGGACACatgtagaaCCTCCCGCAGCTGC</td>
</tr>
<tr>
<td>v16m 3rp 1 +</td>
<td>[phos]aatttcGTTCCTGCAGCTGCGCGGAGGttgtgacatGTGTCCCTGCAGCT</td>
</tr>
<tr>
<td>v16m 3rp 1 -</td>
<td>[phos]AGGGACACatgtgagaaCCTCCCGCAGCTGCGCGGAGGACACg</td>
</tr>
<tr>
<td>v16m 3rp 2 +</td>
<td>[phos]GCGCGGAGGttgtgacatGTTCCTGCAGCTGCGCGGAGGc</td>
</tr>
<tr>
<td>v16m 3rp 2 -</td>
<td>[phos]catggCCTCCCGCAGCTGCGCGGAGGACACatgtgagaaCCTCCCGCAGCTGC</td>
</tr>
<tr>
<td>s17f part 1 F</td>
<td>[phos]aattgGTGTCCTGCAGCTGCGCGGAGGttgtgacatGTGTTCCTGCA</td>
</tr>
<tr>
<td>s17f part 1 R</td>
<td>[phos]AGGAcACatgtgagaaCCTCCCGCAGCTGCGCGGAGGACACg</td>
</tr>
<tr>
<td>s17f part 2 F</td>
<td>[phos]GCGCGGAGGttgtgacatGTGTTCCTGCA</td>
</tr>
<tr>
<td>s17f part 2 R</td>
<td>[phos]catggCCTCCCGCAGCTGCGCGGAGGACACatgtgagaaCCTCCCGCAGCTGC</td>
</tr>
<tr>
<td>I18p part 1 F</td>
<td>[phos]aattgGTGTCCTGGcGCAGCTGCGCGGAGGttgtgacatGTGTCCcGCAGCTGCGCGGAGGc</td>
</tr>
<tr>
<td>I18p part 1 R</td>
<td>[phos]GGGACACatgtgagaaCCTCCCGCAGCTGCGCGGAGGACACg</td>
</tr>
<tr>
<td>I18p part 2 F</td>
<td>[phos]GCGCGGAGGttgtgacatGTGTCCTGGcGCAGCTGCGCGGAGGc</td>
</tr>
<tr>
<td>I18p part 2 R</td>
<td>[phos]catggCCTCCCGCAGCTGCGCGGAGGACACatgtgagaaCCTCCCGCAGCTGC</td>
</tr>
</tbody>
</table>

Supplementary Table 1. PCR primers and sequences used for vector creation.
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 $1.3 \times 10^{-6}$. 
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.
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.
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.

```python
from numpy import random
import matplotlib.pyplot as plt

elanemRNA = 'GCACGAGGAGGAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGA4
```
```
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()
Severe congenital neutropenia: new lane for ELANE

Niels Borregaard
Severe congenital neutropenia: new lane for \textit{ELANE}

Niels Borregaard

In this issue of \textit{Blood}, Tidwell et al\textsuperscript{1} demonstrate that mutations in the start codon (protein synthesis is initiated at the codon ATG) of neutrophil elastase (\textit{ELANE}) result in the production of N-terminally truncated elastase, which mislocates to the nucleus and results in severe congenital neutropenia (SCN).

SCN is a rare condition with a serious impact on heath and quality of life. Although treatment with granulocyte colony-stimulating factor (G-CSF) largely restores circulating neutrophil counts and effectively reduces infections, the condition carries a risk of progression to acute myeloid leukemia of 2.3\% per 10 years.\textsuperscript{2}

A major breakthrough in understanding the genetic background of SCN and its more benign relative, cyclic neutropenia, was the discovery by Marshall Horwitz and David Dale and colleagues that virtually all forms of cyclic neutropenia, and most forms of autosomal-dominant SCN, which covers about 70\% of the cases, are caused by mutations in the coding region of \textit{ELANE}, the gene for neutrophil elastase.\textsuperscript{3,4} one of the 4 serine proteases localized to azurophil granules of neutrophils along with the commonly used neutrophil marker, myeloperoxidase. These seminal papers were presented before the era of exome sequencing and were the result of meticulous studies of pedigrees of patients that made it possible to narrow down the gene defect to chromosome 19p13.3 and then verify that mutations were present in \textit{ELANE}.

Why should mutations in the gene for elastase cause such problems? Although it is still not evident why \textit{ELANE} mutations result in cyclic neutropenia, it is more easily comprehended that mutations in \textit{ELANE}, one of the genes most abundantly expressed in promyelocytes,\textsuperscript{5} may perturb the further development of these cells when transcription of such mutated \textit{ELANE} is at its peak: but how? Several mechanisms have been offered. One explains elastase as a transmembrane protein with SCN mutations preventing its sorting to granules, resulting in routing to the plasma membrane, where it may cause havoc.\textsuperscript{6} Another argues that mutations result in misfolding of elastase, which exceeds the capacity of the endoplasmic reticulum for corrections and induces an unfolded protein response leading to death of the cells,\textsuperscript{7} much akin to the necrosis of liver cells in severe forms of \(\alpha-1\)-antitrypsin deficiency.\textsuperscript{8} Why G-CSF should ameliorate this is not quite evident, but it is perhaps the result of reduction in production time and hence the amount of protein synthesized at the promyelocyte stage before other transcription factors take over and shut down the production of the offending misfolded protein.

In this issue of \textit{Blood}, a novel mechanism is put forward and supported by several lines of evidence.\textsuperscript{1} The authors identified 8 cases of SCN where the mutations of \textit{ELANE} affect 1 of the 3 nucleotides of the canonical translation initiation codon, ATG, which puts methionine on as the first amino acid when protein synthesis starts in all species. When this is mutated, the translational machinery will skip this site for initiation of translation and search for alternative ATG sites further downstream that satisfy the minimal requirements for initiation of translation, and such are indeed present in \textit{ELANE} but alas result in shortened forms of elastase (see figure). Importantly, the part that is skipped codes for the signal peptide that guides proteins into the endoplasmic reticulum and eventually into granules. Instead, these N-terminally truncated forms are produced as cytosolic proteins. The presence of active proteases in the cytosol could certainly be expected to elicit apoptosis, but these truncated elastase forms are barely active and do not induce apoptosis. Instead, they stick to the nuclei of the cells. Although the authors demonstrate that such mutations negatively affect the proliferation of cells, it is still an open question whether the truncated forms by virtue of their mislocation and charge can block access of transcription factors to the nucleus necessary for further differentiation and hence explain the block of differentiation so characteristic of this condition.

These mutations are found only in a small minority of patients, but other \textit{ELANE} mutations, located in the vicinity of the alternative translation initiation sites, make these more palatable for ribosomes to start translation at such internal ribosomal entry sites and putatively result in truncated elastase and SCN, even when the traditional start site is not mutated. Although this paper concerns a small fraction of patients with a rare disorder, the study opens a new path for understanding how genetic defects affect cellular differentiation that may be relevant to other diseases, both congenital and acquired, not the least of which include the myelodysplastic syndromes and acute myeloid leukemia.

\textbf{Conflict-of-interest disclosure:} The author declares no competing financial interests.

\textit{Blood}, 23 January 2014 • Volume 123, Number 4

\textit{From bloodjournal.hematologylibrary.org at UNIVERSITY OF WASHINGTON on April 21, 2014. For personal use only.}
Each immunoglobulin G (IgG) molecule contains 2 oligosaccharide groups linked to asparagine residues at the 297 positions of the Fc domain. Each glycan usually consists of a complex heptasaccharide core containing N-acetylglucosamine (GlcNAc) and mannose to which variable numbers of galactose, fucose, sialic acid, and sometimes bisecting GlcNAc residues are attached (see figure). It is now well established that the character of these glycans can critically influence immunoglobulin function, particularly by modulating affinity for Fcγ receptors. One of these posttranslational modifications, the addition of a fucose residue in α1,6 linkage to the first GlcNAc of the oligosaccharide core (“core fucosylation”), modulates the affinity of IgG Fc for the FcγRIII receptor expressed on natural killer cells, macrophages, neutrophils, and other cells. IgG molecules lacking a core-fucose residue bind more tightly to FcγRIII and exhibit enhanced cellular immune function, for example, are more effective in antibody-dependent cellular cytotoxicity. The molecular basis for this effect was recently characterized by Ferrara and coworkers; the potential advantage of using monoclonal antibodies lacking a core-fucose residue in cancer chemotherapy is currently under investigation. Up to 30% of IgG molecules in normal human serum lack a core-fucose residue, but how core fucosylation is regulated, and the extent to which it influences the severity of antibody-mediated human disease, are poorly understood.

NAIT, a significant cause of morbidity and mortality in newborns, is caused by maternal antibodies specific for the antigen 1a (HPA-1a). The antigen against which these antibodies are most often directed is designated “HPA-1a.” In a woman sensitized to HPA-1a and carrying a fetus at risk for NAIT, a tool capable of predicting NAIT severity could be extremely helpful in optimizing prenatal and perinatal management. Various studies have shown that serologic measurement of antibody potency alone is not sufficient for this purpose.

In this issue of Blood, Kapur et al describe studies in which HPA-1a antibodies were isolated from serum of 48 women sensitized to HPA-1a who gave birth to an infant with NAIT. The isolated immunoglobulins were digested with trypsin and subjected to nano liquid-chromatography tandem mass spectrometry (liquid-chromatography mass spectrometry (LC-MS/MS)) to identify the immunoglobulin G (IgG) molecules that bind to the HPA-1a antigen. A representative serum IgG from one of these women was digested with trypsin and subjected to mass spectroscopic analysis. The resulting glycan-bound peptides in the tryptic digest were analyzed for the presence of core fucose and fucose in α1,6 linkage to the first GlcNAc residue in the oligosaccharide core. The results showed that the antibodies isolated from these women had a higher affinity for HPA-1a than the antibodies that were not isolated from these women. The authors conclude that these data suggest that core fucosylation may be a factor in the pathogenesis of NAIT and that the use of core fucosylated antibodies may be more effective than fucosylated antibodies in promoting phagocytosis of antibody-coated platelets.