Inducible expression of a disease-associated *ELANE* mutation impairs granulocytic differentiation, without eliciting an unfolded protein response

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ABSTRACT
Severe congenital neutropenia (SCN) is characterized by the near absence of neutrophils, rendering individuals with this disorder vulnerable to recurrent, life-threatening infections. A majority of SCN cases arises because germline mutations in the gene elastase, neutrophil expressed (ELANE), encoding the neutrophil granule serine protease, neutrophil elastase. Treatment with a high dose of granulocyte colony-stimulating factor (GCSF) increases neutrophil production and reduces infection risk. How ELANE mutations produce SCN remains unknown. The currently proposed mechanism is that ELANE mutations promote protein misfolding, resulting in endoplasmic reticulum (ER) stress and activation of the unfolded protein response (UPR) further triggering death of neutrophil precursors and resulting in neutropenia. Here, we studied the ELANE mutation p.G185R, often associated with greater clinical severity (e.g. decreased responsiveness to GCSF and increased leukemogenesis). Using an inducible expression system, we observed that this ELANE mutation diminishes enzymatic activity and granulocytic differentiation without significantly affecting cell proliferation, cell death, or UPR induction in murine myeloblast 32D and human promyelocytic NB4 cell lines. Impaired differentiation was associated with decreased expression of genes encoding critical hematopoietic transcription factors (Gfi1, Cebpd, Cebp, and Spi1), cell surface proteins (Csf3r and Gr1), and neutrophil granule proteins (Mpo and Elane). Altogether, these findings challenge the currently prevailing model that SCN results from mutant ELANE that triggers ER stress, UPR, and apoptosis.

INTRODUCTION
Severe congenital neutropenia (SCN) is a condition of extremely low absolute number of circulating neutrophils (<500/µl), which results in recurrent, life-threatening infections (1). An accumulation of granulocyte precursors (e.g., promyelocytes) is found in the bone marrow of affected individuals. Chronic administration of high dose granulocyte colony stimulating factor (GCSF) increases the number of neutrophils and prevents infections. GCSF (also known as CSF3) is an important cytokine promoting the survival, proliferation and differentiation of neutrophils and their
precursors (2), evidenced by severe neutropenia in mice with genetic ablation of Csf3 or its cognate receptor Csf3r. One major concern is that a subset of patients with SCN will acquire a mutation in CSF3R and develop myeloid malignancy. The leading cause of SCN are autosomally dominant mutations in ELANE, which encodes neutrophil elastase. The mechanism by which mutations in ELANE results in SCN remains elusive, and greater understanding of its role in the pathogenesis of SCN may identify the mechanism of action for GCSF and prevent the development of myeloid malignancies.

Previous studies have suggested that mutations in ELANE result in improper folding promoting endoplasmic reticulum stress (ER stress) and activation of the unfolded protein response (UPR) (3-5) and/or subcellular mislocalization (6,7) which may lead to either cell death or cell survival with an altered transcriptional profile and protein synthesis (8). By the promyelocyte stage of granulopoiesis, ELANE has become the most abundant protein, achieving millimolar concentration in neutrophils (9). Thus, ER stress could be sufficiently potent to cause cell death. Apoptosis induced by mutant neutrophil elastase expression can be responsible for reduced neutrophil counts (6,10,11). An alternate hypothesis is that mutated ELANE produces neutropenia through a block in differentiation. The block in terminal granulocytic differentiation may be attributed to aberrant transcription factor activity (12).

Progress towards understanding the pathogenesis of SCN has been hampered by lack of an animal model and technical challenges associated with patient-derived induced pluripotent stem cells. Here, we have used an inducible expression system of wild-type and mutant ELANE to investigate its biochemical and cellular properties. An inducible expression system avoids the problem of bias in selection of only surviving cells that express mutant ELANE. Furthermore, we used the murine myeloblast 32D and the human promyelocytic NB4 cell lines, both of which model granulocytic differentiation. We report here that mutant ELANE impaired granulocytic differentiation
without eliciting ER stress/UPR or apoptotic response.

RESULTS

Mutant ELANE exhibited reduced activity and altered subcellular localization. To investigate the effect of ELANE mutations on myeloid cell growth, we deployed a doxycycline-inducible model of ELANE expression in 32D and NB4 cells. In this model we induced the expression of wtELANE or ELANE G185R. The G185R mutation is one of the more clinically deleterious ones causing SCN, requiring greater GCSF dose and associated with a higher risk of transformation to myeloid malignancy (16). Induction of ELANE expression occurred in a dose-dependent manner with maximal expression observed at 1 µg/ml in 32D and NB4 cells (Figure 1A,B). The dose of 1 µg/ml doxycycline was used for all subsequent experiments. The induction of ELANE using 1 µg/ml doxycycline was comparable to the amount of ELANE present in human neutrophils (Supplementary Figure S1). We next evaluated the enzymatic activity of mutant ELANE. Mutant ELANE demonstrated loss of enzymatic activity (Figure 2A). Immunofluorescence studies indicated a diffuse localization of mutant ELANE as compared to a granular expression of wtELANE upon both GCSF and IL3 treatment (Figure 2B). Thus we observe that mutant ELANE G185R is enzymatically inactive and its localization is different compared to wtELANE.

Inducible expression of mutant ELANE did not affect proliferation, viability, and apoptosis. To determine the effect of mutant ELANE expression on proliferation, 32D cells were treated with 100 ng/ml GCSF +/- doxycycline (Dox). Cell counting was performed using trypan blue to exclude counting of dead cells. Doxycycline treatment of 32D cells did not affect cell growth, survival, or differentiation (Supplementary Figure S2 A-D). Similar observation in cell growth and viability were noted upon doxycycline treatment of NB4 cells (Supplementary Figure S3 A-C). Treatment with GCSF or ATRA for six days revealed no effect of mutant ELANE expression on cell proliferation, and viability in 32D or NB4 cells respectively (Figure 3A,B). Cell cycle analysis in 32D cells further shows no differences in cell cycle initiation and progression in 32D
cells upon expression of either wt or mutant ELANE (Supplementary Figure S4), supporting the observations of no effect on cell growth. The expression of mutant ELANE did not elicit cell death or apoptosis determined using Annexin V and propidium iodide staining by flow cytometry in 32D or NB4 cells (Figure 3C,D). The findings were supported by western blot analysis for intra-cellular anti-apoptotic (Mcl1) and pro-apoptotic (BIM, Caspase-3, and PARP1) mediators that did not demonstrate any differences between wtELANE and mutant ELANE G185R induction in 32D cells (Figure 3E). Altogether, these data demonstrate that inducible expression of mutant ELANE did not inhibit proliferation or promote cell death.

**Mutant ELANE failed to trigger the ER stress/UPR.** Induction of UPR has been proposed in several studies to explain the pathogenesis of SCN associated with mutant ELANE (4,5,17,18). Interestingly, the mouse Elane G193X knock-in strain required concomitant administration of bortezomib, which inhibits proteasomal activity and produces ER stress to induce UPR, to generate neutropenia (18). Using our inducible system, we measured time-dependent changes in UPR markers. Doxycycline was added to 32D cells growing in murine IL3 to induce ELANE expression, and cells were harvested at 0, 4, 8, and 24 h for mRNA extraction and qPCR analysis. There was no statistically significant upregulation of the UPR markers Hspa5, Atf4, Atf6, Gadd34, Edem1, and Xbp1 splicing following induction of mutant ELANE vs wtELANE (Figure 4A). Thapsigargin, an inducer of UPR, and bortezomib were used as positive controls (Figure 4B). There was no significant increase in UPR markers ATF4, ATF6, HSPA5, DDIT3, GADD34, and XBP1 splicing upon induction of mutant ELANE in NB4 cells (Figure 4C). Altogether, these data demonstrate that mutant ELANE did not mediate ER stress/UPR.

**Mutant ELANE impaired granulocytic differentiation.** We next determined the effect of mutant ELANE expression on GCSF-induced differentiation in 32D cells. The cells were treated with 100 ng/ml GCSF +/- 1 µg/ml of doxycycline (Dox). At day 6, cells were harvested, cytospun, and stained using Wright-Giemsa type staining to determine differentiation status based on
morphology. We observed cells expressing mutant \textit{ELANE} were more frequently undifferentiated (Figure 5A). Differential counts were performed on 200 cells and were grouped into three stages representing progenitors (myeloblasts, promyelocytes), intermediate granulocytes (myelocytes and metamyelocytes) and mature granulocytes (bands and segmented) (19). Blinded differential cell counting showed a statistically significant increase in progenitor cells when induced with doxycycline to express mutant \textit{ELANE} (Figure 5B). We next assessed gene expression of granulopoietic transcription factors (\textit{Cebpa, Cebpb, Cebp\textsubscript{d}, Cebp\textsubscript{e}, Gfi1, Spi1}) and other markers of granulocyte differentiation (\textit{Mpo, Elane, Ltf, Csf3r, and Gr1}) using qPCR. Normalizing the expression of these markers to that of \textit{wtELANE}, we observed that cells expressing mutant \textit{ELANE} had, in case of multiple genes, 50\% reduced expression (Figure 6A-B). However, \textit{Cebpa} and \textit{Cebpb} did not show a mutant \textit{ELANE} dependent suppression of gene expression (Figure 6C). These are similar to a report of SCN patients’ neutrophils, which even after GCSF mediated differentiation lacked antimicrobial activity due to defects in granule protein expression and reduced MPO and ELANE (6,17,20). The doxycycline inducible system for 32D \textit{wtELANE} and \textit{ELANE G185R} cells showed similar efficacy of expression (Supplementary Figure S5)

\textbf{DISCUSSION}

Granulocytic development is a prodigious process (10\textsuperscript{11} cells/day) divided into multiple stages, tightly regulated by a small set of ligand-receptor reactions and transcription factors (21). SCN is characterized by profound deficiency of neutrophils with a production arrest at the promyelocyte stage. This stage of neutrophil production is marked by formation of primary granules consisting of myeloperoxidase, neutrophil elastase, cathepsin G, and proteinase 3. The association of SCN and promyelocytic arrest in individuals with mono-allelic mutations of \textit{ELANE} demonstrates that granulopoiesis is central to SCN pathogenesis (22). While the \textit{Elane\textsuperscript{-/-}} or the \textit{Elane G193X} knock-in mouse strains do not display neutropenia (at least in the absence of further protease inhibition in the latter model), impaired granulopoiesis occurs in human...
patient-derived iPSCs and ectopic expression of wtELANE rescues neutrophils production (4).

The mechanism by which ELANE mutations cause SCN remains poorly understood. A prevailing explanation is that as one of the most abundant proteins in the promyelocyte, mutant ELANE triggers ER stress and cell death (4,5,17). This is supported by the observation that a single dose of bortezomib elicits neutropenia in the mouse ELANE G193X knock-in strain (17). The fact that a common side effect of therapeutic bortezomib is neutropenia, even when normally administered in patients with wtELANE, exposes limitations of this mouse model. Alternative explanations, which are not as well supported by experimental data, include i) mislocalization of ELANE causes cell death, 2) protease activity of ELANE degrades a critical factor for differentiation and survival, and 3) mutant ELANE blocks differentiation without causing cell death.

Experimental data are limited by the paucity of experimental models. The use of transient expression in cell lines, as performed here, has significant limitations. Nevertheless, as noted, the mouse model is also imperfect. Patient-derived iPSC involves careful culturing conditions. Myeloid and non-hematopoietic cell lines have relied on stable transfectants, which have shortcoming of studying those cells that survive expression of the mutation.

Here, we deployed human and murine myeloid cell lines with an inducible expression system of human ELANE. We found that mutated ELANE did not affect survival, viability, and proliferation. Mutated ELANE impaired granulocytic differentiation at the promyelocyte stage. Furthermore, mutated ELANE demonstrated absent enzymatic activity and did not elicit a strong UPR. This is consistent with previous studies where ELANE G185R did not induce UPR (6,10). In addition, we also observe a diffused expression of ELANE G185R as compared to a localized expression of wtELANE, which corroborates other reports of mutant ELANE mislocalisation (4,6,7). However, our findings do not allude to invariable observation of apoptosis due to mutant ELANE expression, reported previously.
(4,6,7,10,17). We thus observe a mutant ELANE dependent impaired granulocytic differentiation in 32D cells. This is attributed to suppression of gene expression of transcription factors involved on granulocytic differentiation. Mutant ELANE by its mislocalization may perturb the transcriptional regulation of granulopoiesis. One possible mechanism for impaired differentiation is defective GCSF/GCSF Receptor signaling. Wild-type neutrophil elastase has been shown to degrade both GCSF and its cognate receptor (23). However, enzymatic activity was abrogated. Other aberrant functions could impair terminal granulocytic differentiation. Since patients with deletion of the ELANE locus do not display neutropenia (24), mutant ELANE’s acting as a scaffolding protein could sequester a critical mediator of differentiation.

SCN is a life-threatening disease, most frequently caused by dominantly acting mutations in ELANE, which encodes a neutrophil serine protease. Current dogma holds that the neutropenia results from excess deaths due to mutations that cause a misfolding and an enhanced UPR. Our studies with an inducible expression system for mutant ELANE suggests a new explanation of impaired differentiation with only modest UPR effects.

**MATERIALS AND METHODS**

**Cell lines and culture conditions.** The factor-dependent murine myeloblast cell line, 32D cl3 and human promyelocytic leukemic NB4 cell line harboring the t(15;17) were used. 32D cells were grown in IMDM (ThermoFisher Scientific, Waltham, MA) supplemented with 10% fetal bovine serum (FBS, Gemini BioProducts, West Sacramento, CA), 1% PenStrep (Thermo Fisher Scientific), 2 ng/ml murine IL3 (Peprotech, Rocky Hill, NJ), 1X Glutamax (Thermo Fisher Scientific). NB4 were cultured in RPMI (Corning Inc, Corning, NY) supplemented with 10% FBS, 1% PenStrep and 1X Glutamax. All cell lines were grown at 37°C, 5% CO₂. Doxycycline-inducible expression of ELANE was performed using plnducer20 lentiviral vector system (13) and introduced into 32D and NB4 cells by lentiviral transduction. Lentiviral particles were made as described elsewhere (14). The plnducer 21 containing either wild-type (wt) or mutant ELANE G185R were
cloned employing Gateway cloning. The original source of the *ELANE* inserts were from the pCS2+ vector which were generated as previously described (15). To induce expression of *ELANE*, media was further supplemented with 1 µg/ml of doxycycline hyclate (Sigma Aldrich, St Louis, MO), unless otherwise specified. Media was replenished with fresh doxycycline every 48 h. 32D cells were differentiated using 100 ng/ml GCSF (Amgen Inc. Thousand Oaks, CA). All trans-retinoic acid (ATRA, Sigma Aldrich) treatment of NB4 cells was achieved by adding ATRA (in 0.1% (v/v) DMSO) at a final concentration of 1 µM. Cell count and viability was performed using trypan blue exclusion. Cells were stained with 0.4% trypan blue (Thermo Fisher Scientific) and counted manually using a hemocytometer or the Luna II automated cell counter (Logos Biosystems, Annandale, VA). Cells stained with trypan blue were counted as dead and those that excluded the dye were counted as live. Live cell counts were used to report cell number, and percentage of live cell over total cells was used to report viability. To determine granulocytic differentiation using changes in cellular morphology, cells were subjected to cytocentrifugation at a speed of 500 rpm for 5 min. Samples were subsequently fixed and stained using the PROTOCOL Hema 3 staining kit (Fisher Scientific, Hampton, NH), which is comparable to Wright-Giemsa staining. Slides were analyzed and pictures obtained using the Axion microscope (Zeiss, White Plains, NY).

**Western blotting.** Cells were lysed using radioimmunoprecipitation assay buffer (RIPA buffer) supplemented with 1X protease inhibitor cocktail, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM sodium orthovanadate (all EMD Millipore, Burlington, MA). The lysates were quantified for protein concentration using BCA assay (Pierce Thermo Fisher). Finally, the proteins were denatured with 1X laemmlii buffer (Biorad, Hercules, CA), containing β-mercaptoethanol. Lysates were then electrophoresed on Polyacrylamide Mini-gel and transferred onto nitrocellulose membranes (Biorad). Membranes were subsequently blocked with Tris-buffered saline (TBS, Fisher Scientific), 0.1% Tween-20 (Sigma Aldrich), containing 5% non-fat dry milk (Bio-Rad). Primary antibodies
(Supplementary Table 1A) were applied in TBST with 5% non-fat dry milk/TBST overnight at 4°C, followed by incubation with a horseradish peroxidase-conjugated secondary antibody (Rockland Immunochemicals, Gilbertsville, PA) dissolved in TBST solution with 5% non-fat dry milk for 1 h at room temperature and addition of ECL™ start Western Blotting detection reagent (GE healthcare, Chicago, IL). The immunoblots were visualized on a photographic film (Agfa Healthcare, Belgium) developed using SRX-101a developer (Konica Medical corp, Wayne, NJ). Band intensities were quantified using ImageJ software (National Institute of Health, Bethesda MD).

**Apoptosis analysis.** Flow cytometry was used to determine apoptosis using Annexin V-propidium iodide staining. Treated cells were stained using Alexa Fluor 488 Annexin V and propidium iodide as per the manufacturers protocol (Dead Cell Apoptosis Kit, ThermoFischer Scientific). Data for all flow cytometry experiments were acquired using a BD LSRFortessa cytometer (BD Biosciences, Franklin Lakes, NJ). The data were analysed using FlowJo v10.5.3 (FlowJo LLC, Ashland, OR). Overnight treatment with 1µM Camptothecin (Sigma Aldrich) was used as positive control.

**Cell Cycle analysis.** After overnight serum and cytokine starvation to induce G1 arrest, IL3 and FBS containing media was freshly added and then, on indicated time points cells were collected, resuspended in PBS (Fisher Scientific), and ice-cold 70% ethanol (Fisher Scientific) was added dropwise while mildly vortexing. After removing ethanol, cells were washed with 0.3% Tween-20 (Fisher Scientific) in PBS and then resuspended in DNA staining buffer (PBS/PI 50 µg/ml, RNase A 100 µg/ml. After 15 minutes incubation at 37°C, PI signal was acquired on BD LSRFortessa flow cytometer. Diploid (G1/G0), and tetraploid (G2/M) cells were quantified applying Dean-Jett-Fox model using FlowJo v10.5.3 (FlowJo LLC).

**RNA isolation and qPCR-based gene expression analysis.** Total RNA was isolated from the harvested cells using TRIzol reagent (ThermoFisher) according to the manufacturer's instructions. Reverse transcription was
performed by iScript II cDNA synthesis kit (BioRad) which involves propriety blend of Oligo dTs and random hexamers. cDNA was used for real time quantitative PCR (qPCR) to determine gene expression using custom designed primers (Supplementary Table 2). qPCR was performed using PowerUp SYBR Green Master Mix (Applied Biosystems, Foster City, CA) on a StepOne Plus system (ThermoFisher). Gene expression analysis was performed using the ΔΔC_T method, with Actin/ACTIN as the reference gene, time 0 as reference sample to determine normalized expression.

**Neutrophil elastase activity.** Fluorometric analysis of neutrophil elastase activity was performed using the kit (Sigma Aldrich). Briefly, 32D cells with inducible expression of wt and G185R ELANE were treated with or without doxycycline for 48 hours. This was followed by treatment of the cell pellets according to manufacturer’s instructions. The readings were taken in 96-well black optical plates (ThermoFisher) using Gen5 plate reader (BioTek Instruments, St. Winooski, VT) for enzyme kinetics over a period of 40 minutes, every 5 minute interval. Purified neutrophil elastase activity and its inhibition with specific inhibitor Sivelestat (Sigma Aldrich), served as positive and negative control.

**Immunofluorescence.** Commercially purchased human neutrophils (Astarte Biologics, Bothell, WA) and 32D cells with inducible expression of wt and G185R ELANE were harvested following 48 h treatment of GCSF or IL3. A concentration of 500,000 cells/mL was collected for cytocpin and subsequently fixed with 4% formaldehyde in PBS, permeabilized with 0.5% Triton X-100 in DPBS, and blocked with 3% BSA using the Image-iT Fixation/Permeabilization Kit (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. Following three washes in 1X PBS, the cells were incubated with Alexa Fluor 594 Conjugated ELANE (E9C9L) XP Rabbit mAb (Cell Signaling Technology, Danvers, MA) diluted 1:100 in 3% BSA for 1 h at 37°C. The cells were washed three times with 1X PBS and nuclei were counterstained with 1 µg/mL DAPI for 30 min at room temperature. Slides were then mounted and imaged with a Leica.
SP8 confocal microscope (Leica Microsystems, Buffalo Grove IL).

Statistical Analysis and Data Availability. Appropriate statistical analysis (indicated in figure legends) were performed using GraphPad Prism 8.0 software (GraphPad, La Jolla, CA). All the data are contained in the manuscript.

Conflict of Interests
The authors declare no conflict of interests.
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FIGURE LEGENDS

Figure 1. Inducible expression of wt and G185R ELANE. Western blot and quantitation of blots as mean relative intensity ± SD (n = 2), showing induction of human ELANE (wt & G185R) upon doxycycline treatment of A) 32D cells and B) NB4 cells at indicated concentration. Mean relative intensity was calculated relative to Actin. Statistical significances calculated using two way ANOVA and Bonferonni multiple comparison post test show no significant differences between induction of wt or G185R ELANE.

Figure 2. Enzymatic activity and localization of wt and G185R ELANE. A) Neutrophil elastase activity profile of ELANE (wt & G185R) expressing 32D cells over indicated time period using fluorometric quantification. 32D Parental cells were used as control. Data from two independent experiments performed in triplicates are represented as mean ± SD. Statistical significance were computed using Two way ANOVA followed by multiple comparison Bonferonni post test (* = p<0.05). B) Immunoflorescence staining of 32D cells with Alexa Fluor 594 conjugated anti-ELANE Ab and DAPI upon indicated treatment for 48 h.

Figure 3. Effect of wt and G185R ELANE on proliferation, viability, and apoptosis. Trypan blue exclusion based cell count and viability analysis of A) 32D cells expressing (wtELANE & G185R) upon GCSF treatment (100 ng/ml). B) NB4 cells upon ELANE (wt & G185R) induction in ATRA (1 µM) treatment. Data are presented as mean ± SD (n = 3) C) AnnexinV-PI based apoptosis analysis of ELANE (wt & G185R) expressing 32D cells with IL3 (2 ng/ml) and GCSF (100 ng/ml) treatment for 3 days. This assay is the representative of 3 independent experiments. D) Flow cytometry based apoptosis analysis of NB4 cells with or without ATRA (1 µM) treatment for 3 days, representative of 3 independent experiments E) Immunoblotting was performed to determine the effect of ELANE (wt & G185R) expression on indicated pro and anti-apoptotic markers. Quantitation was performed using ImageJ. Data are represented as mean relative intensity normalized to Day 0 ± SD, n = 3. Relative intensity for cleaved Caspase 3 and PARP1 were determined with respect to total Caspase 3 (uncleaved and cleaved) and total PARP1 (cleaved and uncleaved), respectively. Relative levels of BIM and Mcl1 were
determined with respect to ACTIN. Statistical significances calculated using two way ANOVA and Bonferonni multiple comparison post test show no significant differences between induction of wt or G185R ELANE.

**Figure 4. Induction of G185R ELANE did not enhance UPR markers.** A) 32D cells expressing wt and G185R ELANE were analyzed by qPCR for expression of indicated UPR markers over time period of 24 h. Data are presented as mean ± SD (n = 3). B) qPCR to determine expression of UPR genes *Atf6*, *Hspa5*, *Ddit3* in 32D parent cells treated with either thapsigargin or bortezomib for 0, 2, 4, 6, and 8 h. Data are presented as mean ± SD (n = 3). C) NB4 cells expressing wt and G185R ELANE were analyzed by qPCR for expression of indicated UPR markers (n = 3, mean ± SD). Statistical significances computed using two way ANOVA followed by multiple comparison post test using Bonferonni method show no significant differences between wt and G185R ELANE expressing cells.

**Figure 5. G185R ELANE impaired morphological differentiation.** A) Wright-Geimsa based morphological analysis of cytospin immobilized 32D parental cells and ones expressing ELANE (wt & G185R). For quantitation, cells were classified into three different stages of differentiation shown by different colored arrows: Brown arrow - myeloblasts/promyelocytes (MB/PM); Green arrow - myelocytes/metamyelocytes (M/MM); Red arrow – bands/segmented (B/S) B) Graphical description of differential cells count of 32D parental, and 32D cells expressing wtELANE and G185R. Differential counts are indicated as bar charts. Data are represented as mean ± SD (n = 3). Statistical significances were computed using student’s t-test (* = p<0.05, ns = non significant).

**Figure 6. G185R ELANE decreased granulocytic differentiation inducers and markers.** A) Differentiation markers, Elane, Mpo, Ltf, Csf3r and Gr-1, B) Differentiation transcription factors, Gfi1, Spi1, Cebpd, and Cebpe and C) Cebpa and Cebpe, in 32D cells expressing ELANE (wt & G185R) treated with 100 ng/ml GCSF + 1 µg/ml doxycycline for 6 days. The expression levels for each day in cells expressing mutant ELANE are normalized to expression on respective day in cells expressing wtELANE.
The data are represented as mean ± SD of n = 4. Statistical significance were computed using Two way ANOVA followed by multiple comparison post test using Bonferroni method ( * = p<.05, ** = p<.01, *** = p<.001, **** = p<.0001).
Figure 5

A

Parent  wtELANE  G185R

B

% Cells

MB/PM: Myeloblast/Promyelocytes
M/MM: Myelocytes/Metamyelocytes
B/S: Bands and segments

< 0.05  ns
FIGURE 6

A

| Gene | Days | Relative expression |
|------|------|---------------------|
| Elane | 0 2 4 6 | 1.0 0.5 0.2 0.1 |
| Mpo  | 0 2 4 6 | 1.0 0.5 0.2 0.1 |
| Ltf  | 0 2 4 6 | 1.0 0.5 0.2 0.1 |
| Csf1r | 0 2 4 6 | 1.0 0.5 0.2 0.1 |
| Grl  | 0 2 4 6 | 1.0 0.5 0.2 0.1 |

B

| Gene | Days | Relative expression |
|------|------|---------------------|
| Gfl1 | 0 2 4 6 | 1.0 0.5 0.2 0.1 |
| Spi1 | 0 2 4 6 | 1.0 0.5 0.2 0.1 |
| CebpA | 0 2 4 6 | 1.0 0.5 0.2 0.1 |
| Cebpd | 0 2 4 6 | 1.0 0.5 0.2 0.1 |

C

| Gene | Days | Relative expression |
|------|------|---------------------|
| CebpA | 0 2 4 6 | 1.0 0.5 0.2 0.1 |
| Cebpb | 0 2 4 6 | 1.0 0.5 0.2 0.1 |
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