

# Modeling *TCIRG1* Neutropenia by Utilizing Patient Derived Induced Pluripotent Stem Cells

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## Abstract

Congenital neutropenia is characterized by a reduced neutrophil count, decreased innate immunity and increased susceptibility to recurrent infections. While congenital neutropenia has various genetic causes, recent studies have linked *TCIRG1* mutations to this condition. *TCIRG1*, a key component of the vacuolar ATPase (V-ATPase) complex, is essential for osteoclast function, but its role in hematopoiesis remains unclear. We previously identified heterozygous *TCIRG1* mutations, including R736S, R736C, R736P, and E722D, in individuals with congenital neutropenia. However, the mechanism by which these mutations lead to impaired granulopoiesis remains unknown.

To investigate the functional consequences of *TCIRG1* mutations, we generated induced pluripotent stem cells (iPSCs) from affected individuals and healthy controls. Using *in vitro* differentiation protocols, we assessed hematopoietic progenitor formation, proliferation, survival, and neutrophil differentiation. We observed significant defects in myeloid differentiation and increased cell death in patient-derived iPSC lines. CRISPR/Cas9-mediated correction of the R736C mutation restored normal neutrophil differentiation, confirming its pathogenic role. Immunofluorescence analysis revealed reduced expression and altered intracellular localization of the *TCIRG1* protein, characterized by a more diffuse cytosolic distribution in the mutant cell lines.

Our findings suggest that *TCIRG1* mutations impair neutrophil development, likely through structural and functional disruption of the V-ATPase complex. This study provides new insights into the molecular basis of *TCIRG1*-associated neutropenia and highlights potential avenues for therapeutic intervention.

**Keywords:** *TCIRG1*, Neutropenia, Granulopoiesis, Neutrophils, iPSCs, CRISPR/Cas9, V-ATPase

## Introduction

Neutropenia, characterized by an abnormally low neutrophil count, is a hematological disorder that increases susceptibility to recurrent infections [1,2]. While neutropenia has diverse genetic and acquired causes, recent studies have implicated mutations in the *TCIRG1* (T-cell immune regulator 1) gene in a subset of cases [3-5]. *TCIRG1* is best known for its role in osteoclast function and bone resorption, with loss-of-function

mutations linked to autosomal recessive osteopetrosis (ARO) [6-10]. However, emerging evidence suggests that *TCIRG1* mutations can also impair neutrophil development and survival, leading to congenital neutropenia.

Despite its established role in bone homeostasis, the precise mechanism by which *TCIRG1* dysfunction results in neutropenia remains unclear. We previously identified a novel heterozygous missense mutation, R736S, in *TCIRG1*,

as the cause of severe congenital neutropenia (SCN) in a large multigenerational family [3]. Additionally, we found a statistically significant correlation between lower absolute neutrophil counts (ANC) and rare *TCIRG1* missense variants in a population-based genome screening cohort [4]. More recently, we identified five other unrelated patients and families with congenital neutropenia harboring heterozygous E722D, R736C and R736P mutations. Independently, Shinwari *et al.* identified a novel V52L mutation in *TCIRG1*, also associated with a neutropenic phenotype [5].

The Genome Aggregation Database (gnomAD) contains extensive data on heterozygous *TCIRG1* variants in the general population, most of whom are healthy and exhibit no hematological abnormalities. However, why specific heterozygous *TCIRG1* mutations at R736 and E722 lead to neutropenia remains unknown.

In this study, we showed that repair of the R736C mutation with CRISPR/Cas9 editing of patient derived induced pluripotent stem cells (iPSCs) corrected the defect in myelopoiesis, and modeled *TCIRG1*-associated neutropenia using patient derived iPSCs, and characterized the hematopoietic abnormalities through *in vitro* differentiation studies.

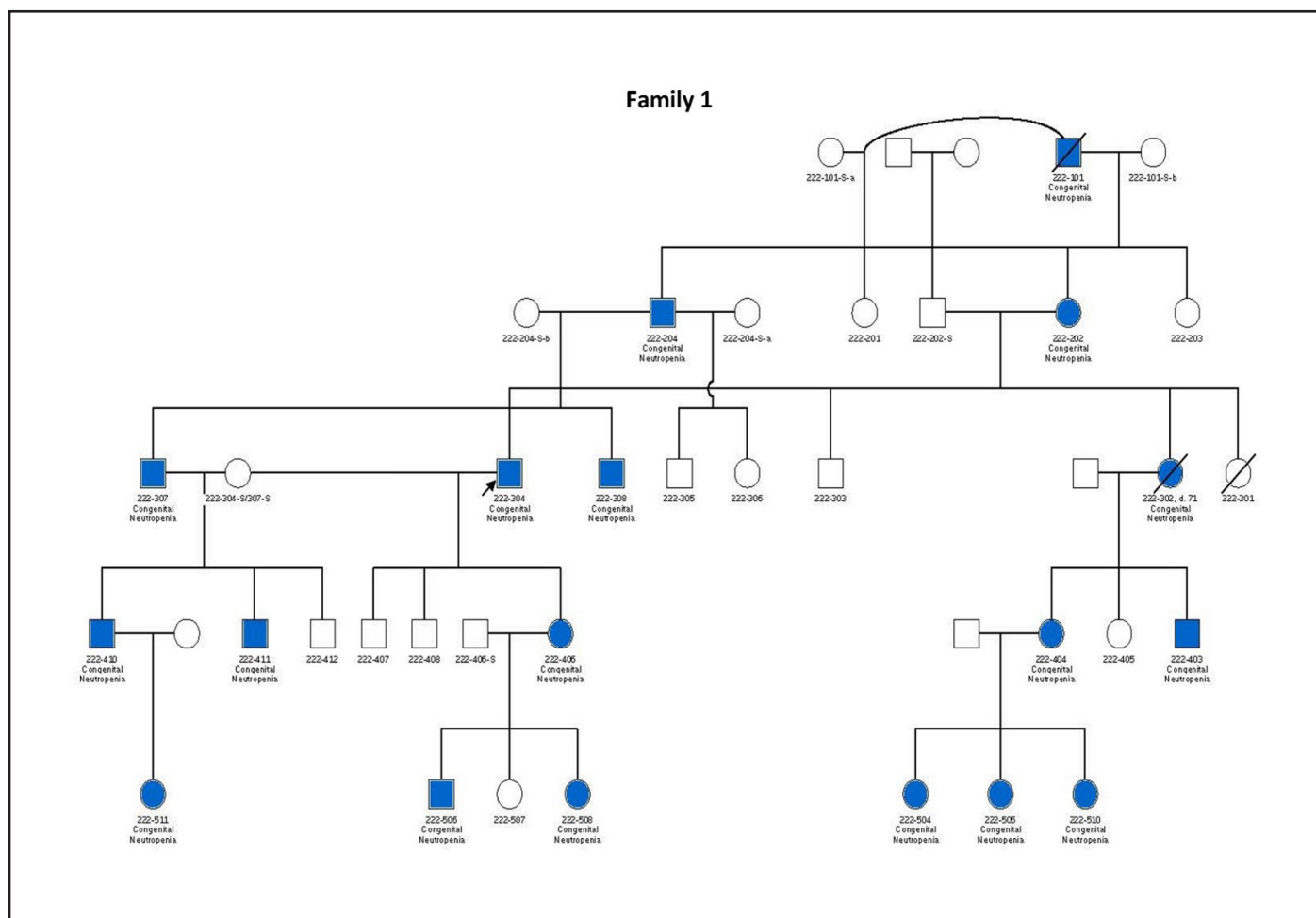
## Materials and Methods

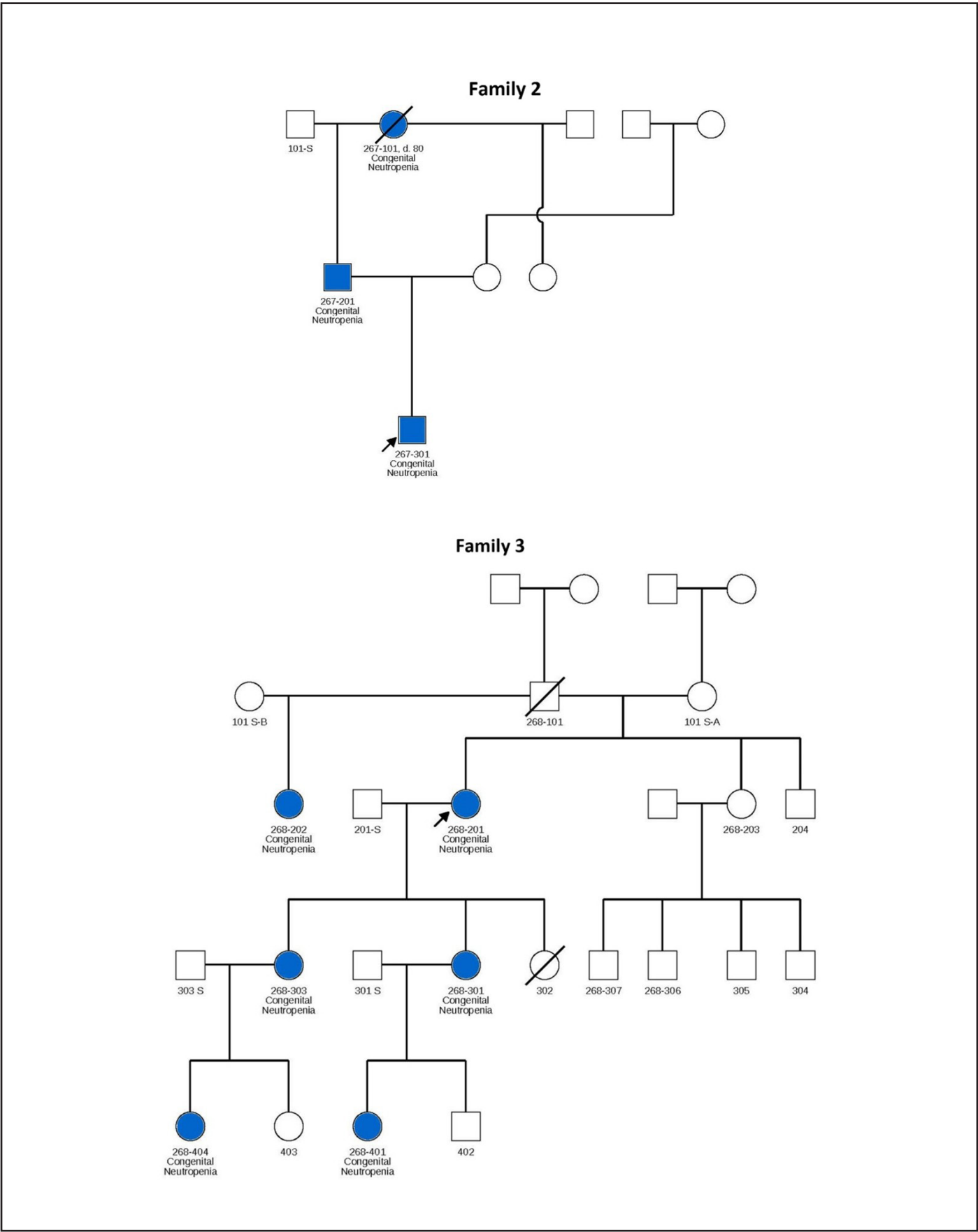
### Patients

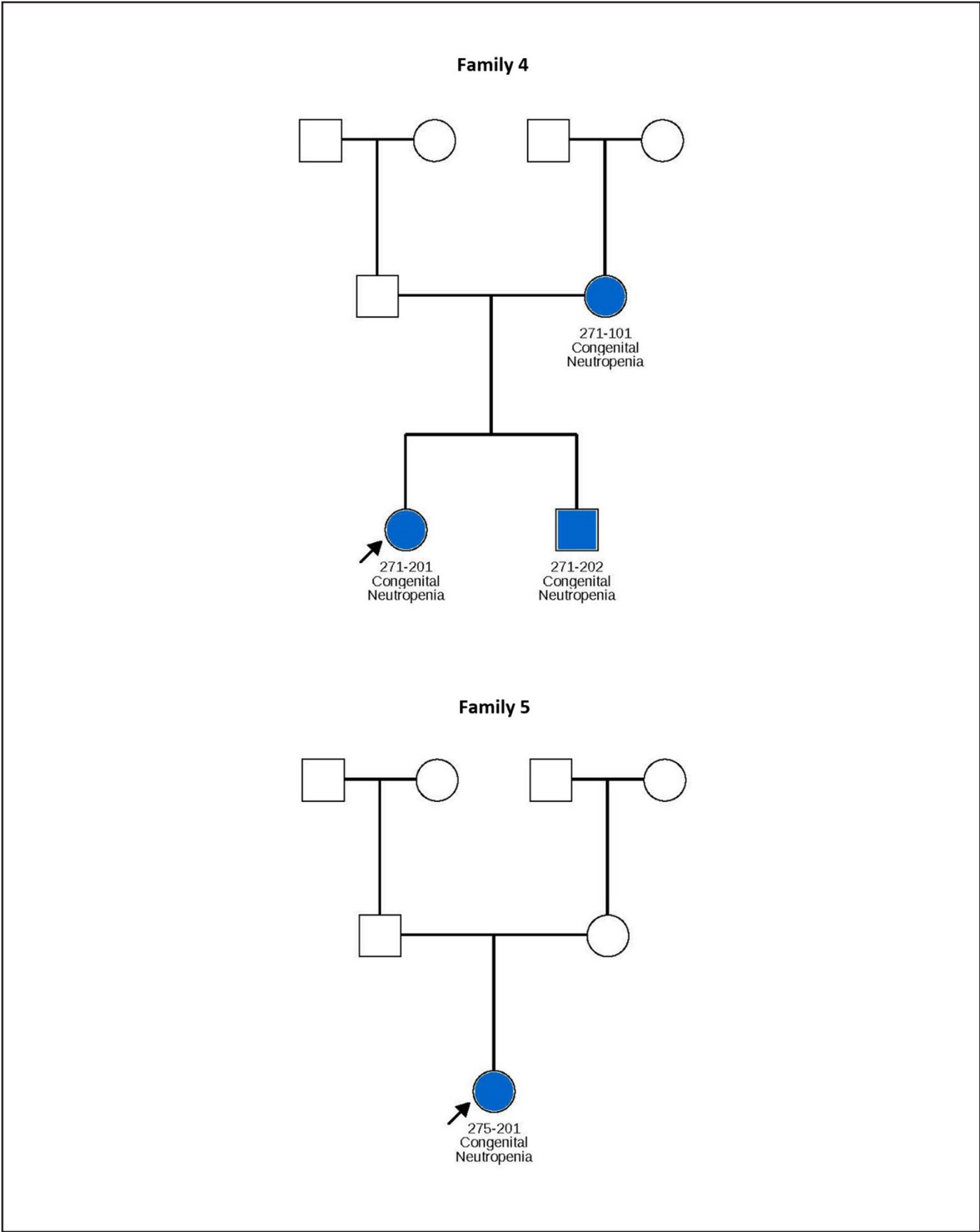
Clinical information and biological materials for the study were obtained with informed consent in compliance with University of Washington regulations. All procedures were conducted in accordance with ethical standards and approved protocols to ensure the protection of participant rights and confidentiality.

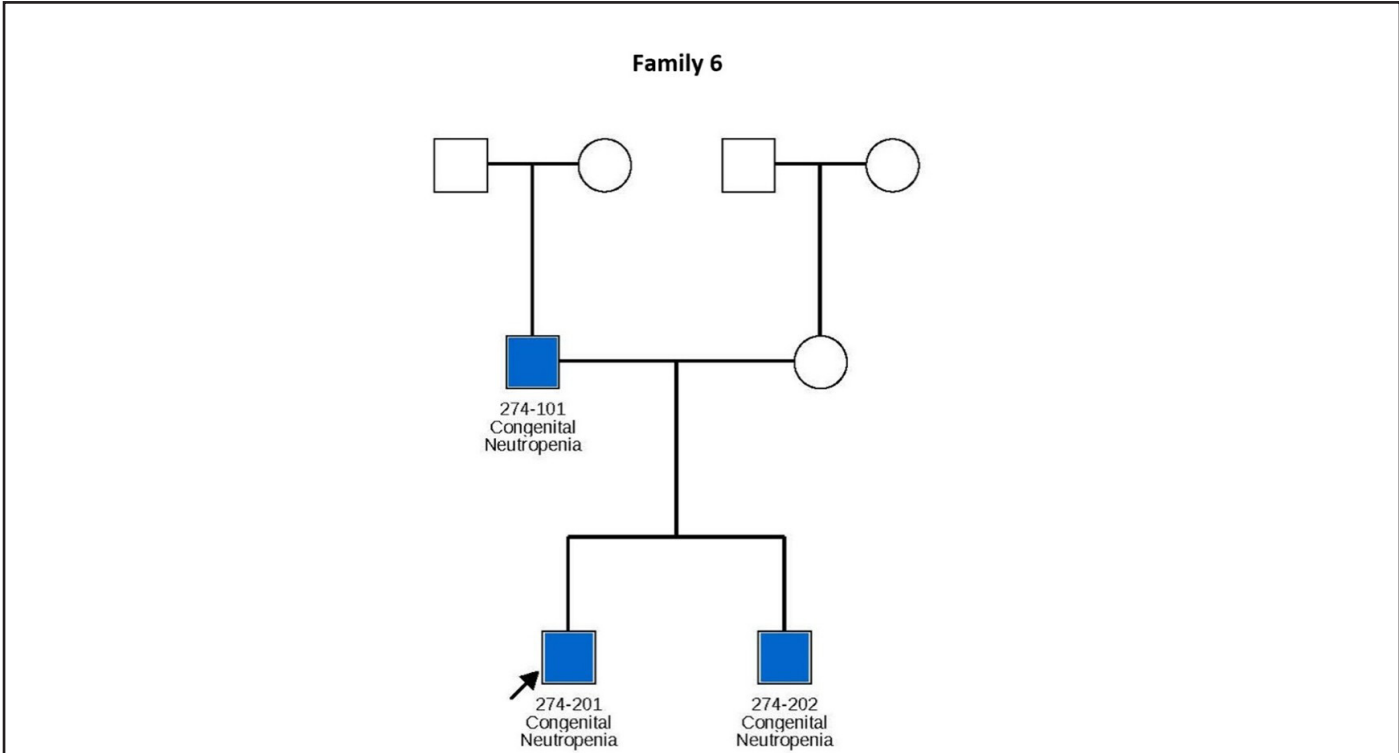
In each family, the *TCIRG1* mutation shows perfect segregation, with every individual carrying the mutation exhibiting clinical manifestations consistent with the neutropenia phenotype.

In this study, we analyzed cells derived from patients 307 and 411 from Family 1, hereafter referred to as patients 1 and 2, respectively, and 101 and 201 from Family 2 hereafter referred to as patients 3 and 4, respectively (see pedigrees in **Figure 1**). Patients 307 and 411 from Family 1 are severely neutropenic and have experienced recurrent severe infections, including mouth ulcers, skin infections, and perirectal and liver abscesses. Similarly, patients 201 and 301 from Family 2 exhibit severe neutropenia. Notably, Patient 301, a 5-year-









**Figure 1. Pedigrees of six families carrying *TCIRG1* mutations, showing inheritance patterns and affected individuals.** Subjects shown in blue are positive for the *TCIRG1* mutation and exhibit a neutropenia phenotype. Subjects shown in white are either negative for the *TCIRG1* mutation, not known to present any clinical symptoms, or lack available information on clinical presentation and mutational analysis. Arrows indicate the index patient in each family, through whom the rest of the family was identified and subsequently studied.

old boy, has absolute neutrophil counts (ANCs) consistently below  $0.2 \times 10^9/L$  even when appearing clinically well, but he remains highly susceptible to infections, including severe gingivitis and mouth ulcers. His father (Patient 201, age 53) also has persistent neutropenia with ANCs typically below  $0.5 \times 10^9/L$ . Interestingly, Patient 101, despite carrying the same mutation, presents with milder neutropenia.

Patient 201 from Family 3 is severely neutropenic, with ANCs

typically ranging between  $0.2\text{--}0.3 \times 10^9/L$ . Her two daughters, patients 301 and 303, are also neutropenic, with ANCs usually below  $0.5 \times 10^9/L$ , and suffer from recurrent mouth sores, sinusitis, and episodes of pneumonia. In Family 4, most affected members display relatively milder neutropenia, with ANCs ranging from  $1.0$  to  $5.4 \times 10^9/L$ , though chronic mouth ulcers are common. Patient 201 from this family has more severe neutropenia and significant health complications (see **Table 1**).

Table 1. Clinical and genetic characteristics of patients from 6 families with <i>TCIRG1</i> mutations. The table summarizes the absolute neutrophil count (ANC) ranges, observed clinical problems, and the identified <i>TCIRG1</i> mutations for each family. The clinical problems observed in each family are described above and are consistent with the typical phenotype associated with congenital neutropenia.			
Family #	Family ANC Range ( $\times 10^9/L$ )	Family Clinical Problems	<i>TCIRG1</i> Mutation
1	0.054 - 2.232	Mouth ulcers, skin infections, oral abscesses, perirectal abscesses, liver abscesses	R736S
2	0.056 - 8.500	Gingivitis, mouth ulcers, otitis media, tonsillitis, pneumonia	R736C
3	0.160 - 0.900	Mouth ulcers, oral abscess, bronchitis, sinusitis, otitis media, pneumonia, sepsis (hospitalized)	R736C
4	0.043 - 5.400	Growth failure, skin infections, gingivitis, multiple febrile episodes requiring hospitalization, otitis media, rectal ulcer, tuberculosis	E722D
5	0.200 - 1.000	Mouth ulcers, recurrent urinary tract infection, skin infection, mastitis complicated by abscess	R736C
6	0.100 - 1.600	Pneumonia resulting in hospitalization	R736P

Patient 201 from Family 5 carries the *TCIRG1* R736C mutation, which appears to have arisen *de novo*. She exhibits mouth ulcers, recurrent urinary tract infections, and mastitis complicated by abscess formation, with ANC's typically below  $0.5 \times 10^9/L$ . Finally, affected members of Family 6 generally present with mild to moderate neutropenia, with ANC's ranging from  $0.1$  to  $1.6 \times 10^9/L$ , and have experienced multiple hospitalizations due to pneumonia.

### iPSC reprogramming

Patient-derived skin fibroblasts (patients 1, 2, and 4) and whole blood (Patient 3) were collected with informed consent in accordance with institutional ethical guidelines (IRB# 00001762). Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood using Ficoll density gradient centrifugation and cultured in complete PBMC medium (StemPro-34 SFM, Cat. no 10639011) supplemented with StemPro-34 Nutrient Supplement (Thermo Fisher Scientific, Cat. no 10641) for 4–7 days. Both fibroblasts and PBMCs were reprogrammed into iPSC using CytoTune-iPS 2.0 Sendai Reprogramming Kit following the manufacturer's recommendation (Thermo Fisher, Cat.no A16517). Cells were transduced at the recommended multiplicity of infection (MOI) per manufacturer's guidelines and incubated at 37°C, 5% CO<sub>2</sub> for 24 hours, after which the media was replaced and cells were maintained in expansion medium (StemPro + cytokines for PBMC and DMEM + 10% FBS for fibroblasts). On day 3 post-transfection, the PBMCs were transferred to irradiated mouse embryonic fibroblast (MEF) feeder layers and cultured in StemPro media without cytokines; the media was switched to DMEM/F12 medium supplemented with sodium butyrate (0.1mM) and SAHA (50nM) on day 7. On day 7 post-transduction, fibroblast cells were transferred to irradiated MEF in DMEM/F12 medium supplemented with sodium butyrate (0.1mM) and SAHA (50nM). Emerging iPSC colonies were identified between days 18–21, manually picked under a stereomicroscope, and transferred to fresh irradiated MEF plates. Once iPSC lines were established, they were adapted to feeder-free conditions on Matrigel-coated plates in mTeSR-Plus (STEMCELL Technologies, Cat. No 100-0276) and maintained for at least three passages with daily media changes and routine passaging using Accutase.

### CRISPR/Cas9 genome editing of iPSCs

CRISPR/Cas9 genome editing was used to generate the *TCIRG1* S733F mutation in iPSCs derived from a healthy donor (WTC-11, Coriell, #GM25256). The same approach was employed to correct the R736C mutation in patient-derived iPSCs (Patient 4). Cells were electroporated with a Cas9 ribonucleoprotein (RNP) complex, consisting of Cas9 (0.3  $\mu$ M, Sigma, Cat.No CAS9PROT) and gRNA (1.5  $\mu$ M, Synthego, CCGCCTCCTACCTGCGCCTG for S733F knock-in and CCGCCTCCTACCTGTGCCTG for R736C repair), along with a single-stranded DNA (ssDNA) donor template (2  $\mu$ M, IDT) for

homology-directed repair (HDR) (TCATGCACCAGGCCATCCA CACCATCGAGTTCTGCCTGGGCTGCGCTCCAACACCGCCTTCT ACCTGCGCCTGTGGGGCCCTGAGCCTGGCCCCACGCCGTGAG TGACCTGGCCACCGACG for S733F knock-in and TCATGCACC AGGCCATCCACACCATCGAGTTCTGCCTGGGCTGCGTCTC CAACACCGCCTCTACCTGCGCCTGTGGGGCCCTGAGCCTGGCC CAGCCCCGTGAGTGACCTGGCCACCGACG for R736C repair). Electroporation was conducted using an Amaxa nucleofector (Human Stem Cell Kit 2) in the presence of a ROCK inhibitor to enhance cell survival and recovery post-transfection. To further optimize editing efficiency, cells were treated with HDR Enhancer v2 (IDT).

Following electroporation, cells were cultured in mTeSR Plus media (STEMCELL Technologies, Cat. No 100-0276). Individual iPSC colonies were manually hand-picked, expanded, and transferred into 96-well plates for further processing. Genomic DNA was extracted from each clone using QuickExtract (Epicentre, Cat. No QE09050), and a nested PCR strategy was employed to amplify the target region, ensuring high specificity and sensitivity for detecting edits. Purified PCR products were treated with EXO-SAP (ThermoFisher, Cat No 78205) to remove excess primers and nucleotides before being submitted for Sanger sequencing (Genewiz). Sequence analysis was then performed to confirm successful editing (**Supplementary Figure 1**).

### iPSC hematopoietic differentiation

iPSCs were differentiated into hematopoietic progenitors using STEMdiff Hematopoietic Kit (STEMCELL Technologies, Cat No 05310) following the manufacturer's protocol. Briefly, iPSCs were maintained in feeder-free conditions before initiating differentiation. Colonies were dissociated into "embryonic body" aggregates and seeded in 12 well plates (STEMCELL Technologies, Cat No 200-0624) in hematopoietic differentiation medium, supplemented with provided cytokines and growth factors to promote hematopoietic lineage specification. On day 12 the floating cells were harvested and differentiation efficiency was assessed by flow cytometry for CD34 (Biolegend Cat.no 343510) and CD45 (Biolegend Cat.no 368508) expression.

### Myeloid differentiation

The resultant CD34<sup>+</sup> cells were allowed to recover for 3 days in CD34<sup>+</sup> expansion media and were subjected to a differentiation protocol as previously described [11,12]. In brief, HSCs were cultured for 7 days in RPMI (Cat.no 11875093, Gibco™) supplemented with 1% Glutamax (Cat.no 35050061, Gibco™), 10% FBS (Cat.no 04-001-1A, Biological Industries), 5 ng/ml IL-3 (Cat.no 200-03), SCF (Cat.no 300-07), GM-CSF (Cat.no 300-03) & 10 ng/ml G-CSF (Cat.no 300-23), all from PeproTech, for proliferation and myeloid progenitor differentiation followed by a 7-day culture in RPMI, 1% Glutamax, 10% FBS, 1% Penn Strep (Cat.no 03-031-1B, Biological Industries), 10 ng/ml G-CSF for neutrophil differentiation and maturation.



## Cytospin staining

$8 \times 10^4$  cells at day 14 of differentiation were spun onto Cytoslide microscope slides (ThermoScientific, Cat No 5991056) using Cytospin 4 low speed cytocentrifuge (Thermo Scientific) and stained with Kwik-Diff staining system (Shandon, Cat. No 9990700) according to manufacturer's recommendations. Microphotographs were taken on LEITZ LABORLUX S polarizing light microscope at 400X magnification using Nikon DSLR digital camera.

## Flow cytometry

Myeloid maturation of CD34<sup>+</sup> cells was analyzed at day 14 of the differentiation by flow cytometry utilizing antibodies characterizing the neutrophilic lineage. CD66b anti-human; Pacific Blue (Cat No. 305112, Biolegend), CD14 anti-human; APC (Cat No. 130-110-520), CD11b anti-human; APC (Cat No. 130-110-554) and CD15 anti-human; Pacific Blue (Cat No. 130-113-488) all from Miltenyi Biotec, unless indicated otherwise, were used. Cell debris was gated out by using a zombie yellow viability kit (Biolegend, 423103).

## Immunofluorescence analysis and TCIRG1 intracellular localization

Induced pluripotent stem cell (iPSC)-derived CD34<sup>+</sup> hematopoietic progenitor cells were differentiated toward the myeloid lineage using a standard differentiation protocol as previously described [11,12]. Differentiation was carried out under optimized culture conditions for 14 days, with regular media changes to support myeloid lineage commitment.

At the end of the differentiation period, cells were harvested, spun onto slides and fixed with 4% paraformaldehyde for immunofluorescence staining. Cells were permeabilized with 0.2% Triton X-100, then blocked with 5% normal goat serum. Slides were incubated with rabbit anti-human TCIRG1

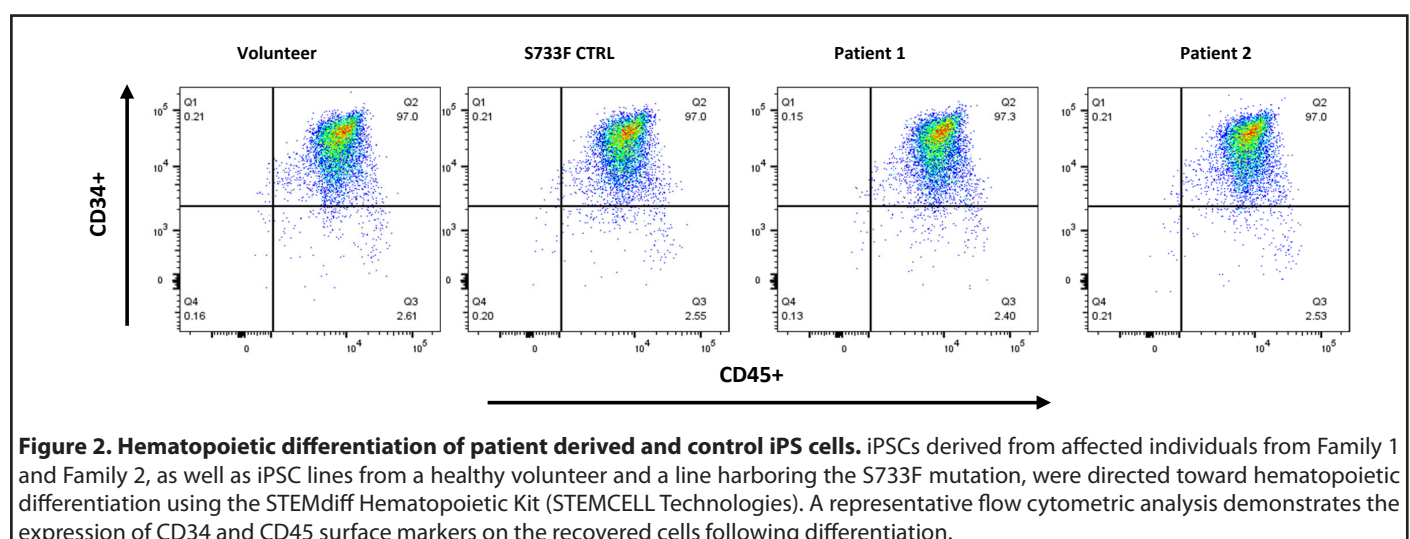
(1:100 dilution; Abcam, ab224654) and mouse anti-human myeloperoxidase (MPO) (1:20 dilution; R&D Systems, MAB3174) primary antibodies for 48-72 hours at 4°C in a humidified chamber, washed and then incubated for 40 minutes at room temperature with Alexa Fluor 647 conjugated polyclonal goat anti-rabbit IgG H&L (1:200 dilution; Abcam, ab150079) and Alexa Fluor 488 conjugated polyclonal goat anti-mouse IgG H&L (1:200 dilution; Abcam, ab150113) secondary antibodies. Cells were washed, then mounted using ProLong Gold with DAPI (Invitrogen, P36935) and visualized at the Keck Imaging Center (University of Washington) using a Leica DMI6000 widefield immunofluorescence microscope and 40x water immersion objective.

## Results

### iPSCs hematopoietic differentiation

To understand the specifics of the mutations in the positions R736 and E722, we have also created another mutant iPSC line harboring heterozygous S733F mutation utilizing CRISPR/Cas9 technology. We selected this particular variant due to its proximity to R736 site and its high prevalence in the healthy population. Individuals with heterozygous variant of S733F are healthy and do not have any hematological abnormalities. Homozygous S733F mutations are causing osteopetrosis. We are using this cell line along with 2 healthy volunteer lines for comparative studies along with cell lines from the affected families.

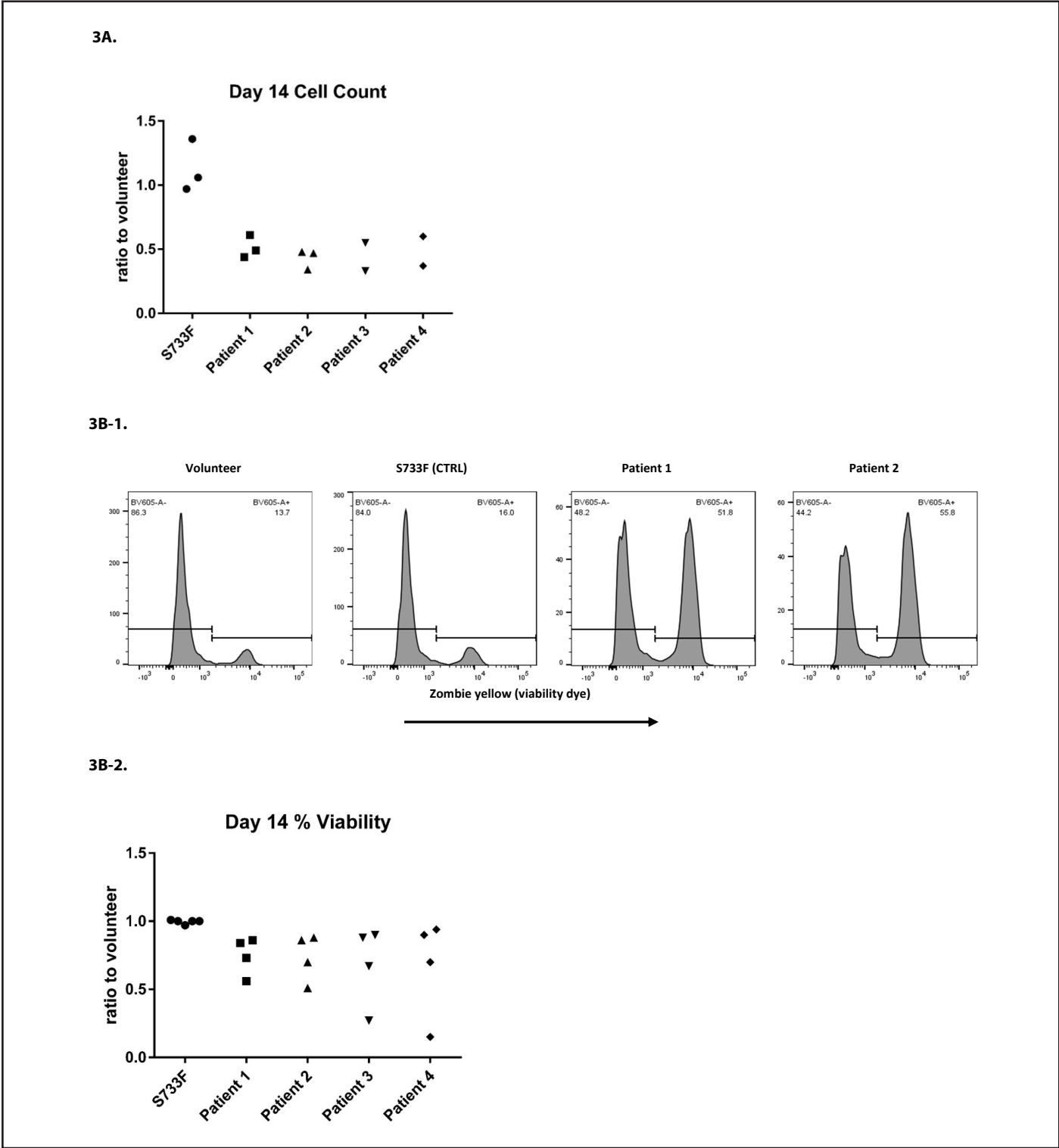
Four iPSC lines from the Family 1 and 2 harboring R736S and R736C mutations, as well as iPSC lines derived from a healthy volunteer and the line harboring S733F mutation were pushed towards the hematopoietic differentiation utilizing STEMdiff Hematopoietic Kit (STEMCELL Technologies). All lines generated comparable CD34<sup>+</sup>/CD45<sup>+</sup> myeloid progenitor cell populations (**Figure 2**).



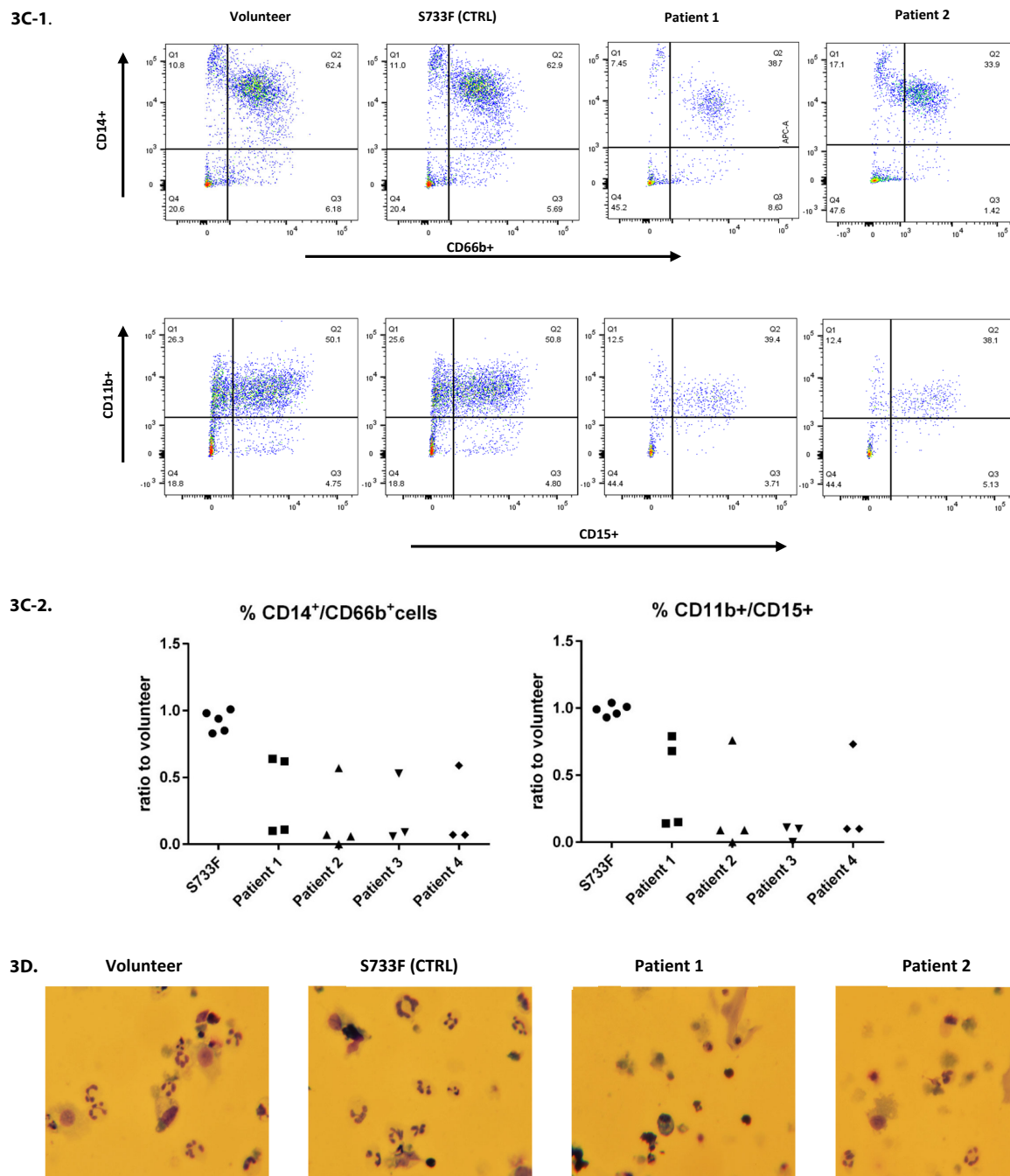
**Myeloid differentiation: proliferation, cell survival, and neutrophil formation**

The iPSC derived CD34<sup>+</sup> cells were further pushed towards the myeloid differentiation using the protocol published recently by our group [11,12]. After the 14 days of differentiation there was a sizable drop in cell proliferation in

all patient cell lines (**Figure 3A**). There was also a noticeable cell death in patient cell lines (**Figures 3B-1, 3B-2, and 3D**). The myeloid differentiation in the patients' cells, measured by flow cytometry and assessed with surface markers CD66b, CD14, CD11b, CD15, was substantially impaired (**Figures 3C-1 and 3C-2**).







**Figure 3. Proliferation, survival and differentiation of iPSC derived CD34+ hematopoietic cells.** iPSC derived CD34+ cells pushed towards the myeloid differentiation using the standard protocol previously described [11,12]. **A.** Cell proliferation was evaluated at day 14 of differentiation by using Moxi V automated cell counter and the number of live cells recorded. For each individual experiment, the total number of patient cells was divided by the percentage measured from normal volunteer (control) cells, and this ratio was plotted. Data from four patients over three experiments. **B.** Cells after 14 days of differentiation were evaluated using Zombie Yellow (Biolegend) cell viability dye and utilizing flow cytometry. **B1:** Representative histograms from experiments are shown. **B2:** For each individual experiment, the % viability of patient cells was divided by the percentage measured from normal volunteer (control) cells, and this ratio was plotted. Data from four patients over four experiments. **C.** Cells after 14 days of differentiation were stained with CD66b, CD11b, CD14 and CD15 surface markers and analyzed by flow cytometry. **C1:** Representative histograms from experiments are shown. **C2:** For each individual experiment, the percentage of patient cells expressing a mature phenotype was divided by the percentage measured from normal volunteer (control) cells, and this ratio was plotted. Data from four patients over four experiments. **D.** Cell cytopins stained with Kwik-Diff (eosin/methylene blue) were imaged using a Nikon digital camera. Cell differentiation was evaluated at 400x magnification by light microscope.

## CRISPR/Cas9 assisted repair of R736C mutation

To validate the causative effect of *TCIRG1* mutations on the maturation, survival, and proliferation of hematopoietic CD34<sup>+</sup> cells in our cell lines, we conducted a "reverse genetics" experiment. Using CRISPR/Cas9 gene editing, we repaired the R736C mutation in iPSCs derived from Patient 4 (#201 of Family 2). We then performed hematopoietic and subsequent myeloid differentiation experiments with this edited cell line, comparing the results to those from a healthy volunteer and an equally manipulated but unedited R736C-mutant cell line from the same patient. The mutation-repaired cell line exhibited normal differentiation and survival characteristics, similar to the volunteer-derived cell lines (**Figure 4**).

## TCIRG1 protein intracellular localization

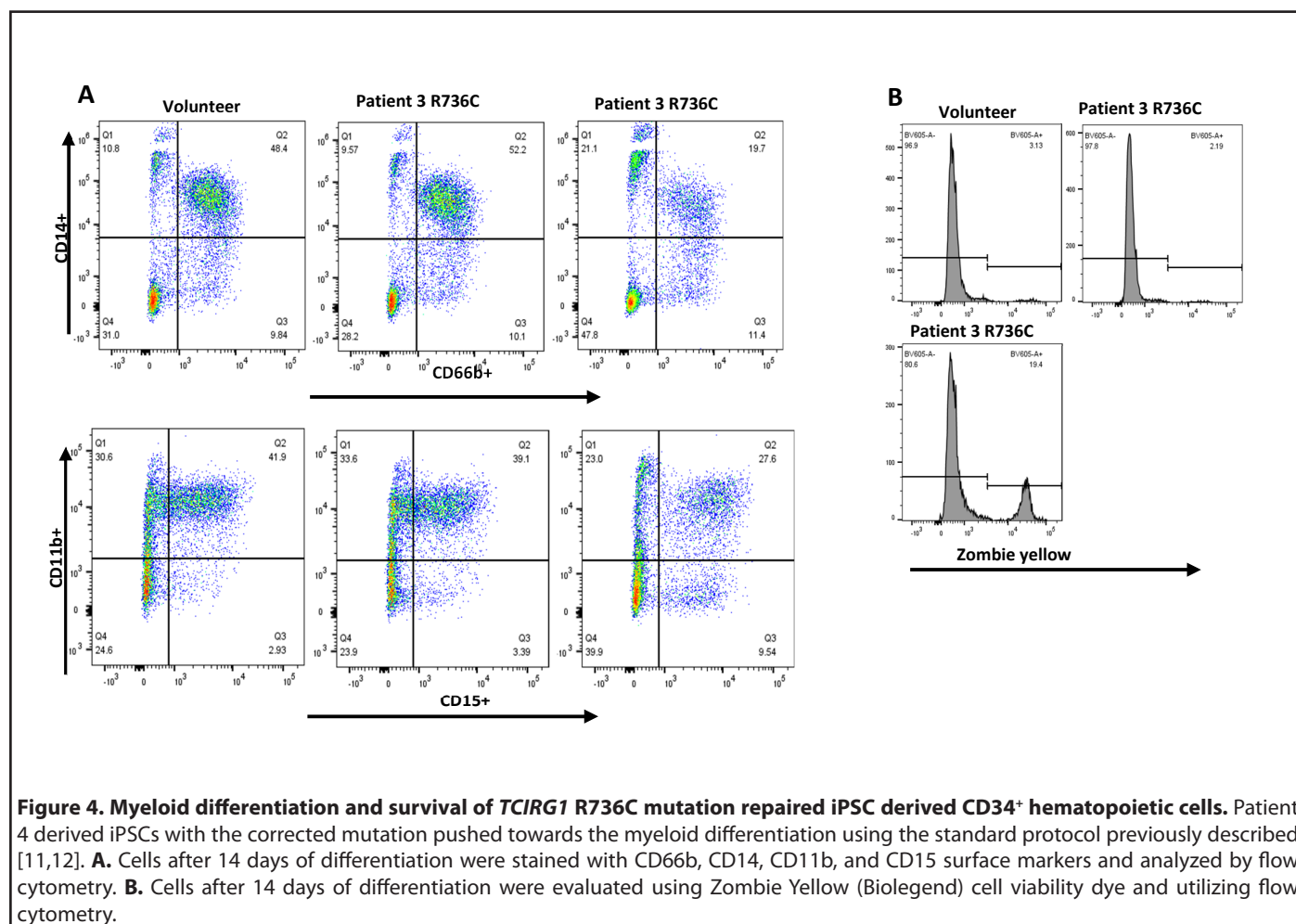
We performed TCIRG1 protein intracellular localization experiments of iPSC differentiated granulocytes utilizing immunostaining and confocal fluorescence microscopy.

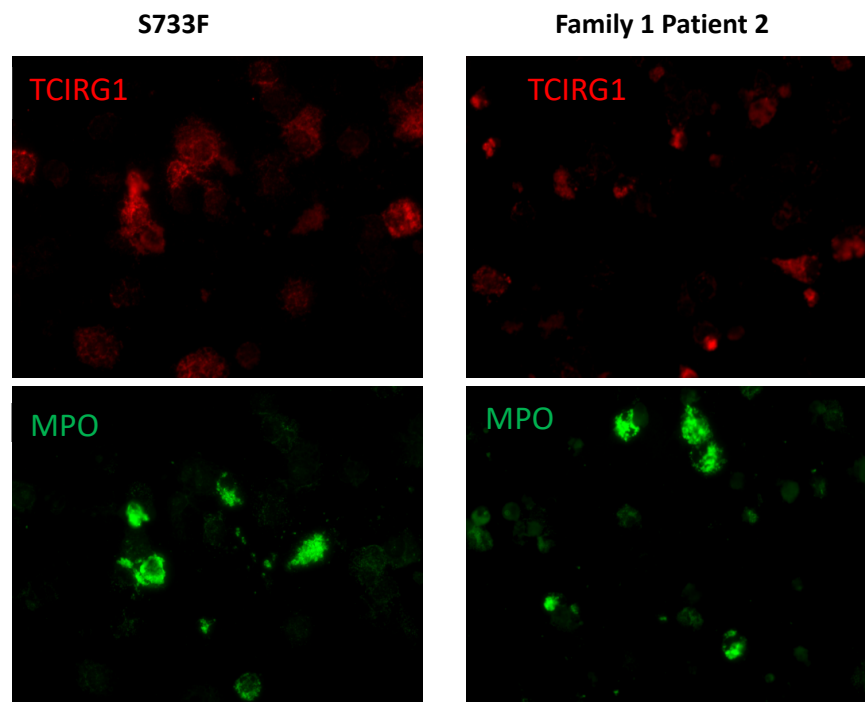
In these experiments, we used 4 patient derived cell lines from the families 1 and 2, as well as "benign" S733F cell lines and healthy volunteer. In order to correlate TCIRG1 localization in

the cells, we also performed myeloperoxidase (MPO) staining, an abundantly expressed neutrophil protein localized in the primary granules. These studies revealed diffused and unstructured intracellular localization and decreased expression of TCIRG1 protein throughout the cytosol in all 4 mutant cell lines. TCIRG1 staining in both control cell lines (volunteer and S733F) retained granularity, with localization in the subcellular granules. MPO staining was unaltered in all cell lines (**Figure 5**).

## Discussion

In this study, we utilized patient-derived induced pluripotent stem cells (iPSCs) to model *TCIRG1*-associated neutropenia and elucidate the mechanistic consequences of *TCIRG1* mutations on myeloid differentiation. Our findings provide strong evidence that heterozygous missense mutations at the R736 and E722 positions impair neutrophil development, survival and maturation. Through *in vitro* differentiation studies and CRISPR/Cas9-mediated gene correction, we demonstrated that the observed hematopoietic defects are directly attributable to these mutations, supporting their pathogenic role in congenital neutropenia.





**Figure 5. *TCIRG1* and MPO immunostaining of iPSC derived granulocytes** (representative slides of Patient 2 from Family 1 and S733F CTRL cell line). iPSC derived CD34<sup>+</sup> cells pushed towards the myeloid differentiation using the standard protocol previously described [11,12]. Cells after 14 days of differentiation were stained with anti-*TCIRG1* (Abcam, ab224654) and anti-MPO antibodies (R&D Systems, MAB3174) and evaluated under the immunofluorescence confocal microscope.

Our data reveal that patient-derived iPSCs with *TCIRG1* mutations exhibit significantly reduced proliferation and increased cell death during myeloid differentiation. These defects were accompanied by a marked reduction in neutrophil-specific markers (CD66b, CD14, CD15, and CD11b) and impaired granulocytic maturation, as observed through flow cytometry and cytospin analyses. Importantly, genome editing to correct the R736C mutation restored normal neutrophil differentiation, confirming that this mutation is causative.

Our immunofluorescence studies revealed an altered intracellular localization of *TCIRG1* in patient-derived cells, suggesting that these mutations disrupt its proper trafficking or retention within cellular compartments. The effect of these mutations on the protein structure is predicted to be “probably damaging” based on Polyphen-2 SNP data. These findings align with prior research demonstrating that mutations in *TCIRG1* can compromise osteoclast activity and, perhaps, an as-yet unidentified mechanism involved in the neutrophil maturation process.

Interestingly, despite the presence of numerous heterozygous *TCIRG1* variants in the general population, only mutations affecting residues R736 and E722 have been associated with neutropenia—this is in contrast to hundreds of other known

heterozygous *TCIRG1* mutations that have not been reported to cause this phenotype.

*TCIRG1* is located at 11q13, consists of 20 exons and encodes subunit a3, a 116-kD component (OC116), of V-ATPases. These are vacuolar proton pumps which mediate H<sup>+</sup> transport through intracellular compartments and organelles of eukaryotic cells, including the phagocytic vacuoles of neutrophils. V-ATPase is composed of two main functional domains: peripheral V1 and membrane associated V0. Both domains consist of multiple subunits; a3 is the largest subunit in V0 domain and has trans-membrane localization. *TCIRG1* is highly expressed in myeloid progenitors, osteoclasts and is essential for bone resorption [6-8].

Our understanding of *TCIRG1* and how it may cause neutropenia is incomplete. Biallelic loss-of-function mutations of *TCIRG1* are the major cause of infantile malignant osteopetrosis (IMO) (OMIM 259700), a lethal autosomal recessive disorder with secondary hematological and neurological abnormalities [9,10,13]. Loss of *TCIRG1* leads to impaired acidification at the ruffle border interface between osteoclasts and bone, which results in impaired bone resorption [14,15]. Osteoclasts regulate osteoblasts, a cell population implicated in hematopoietic stem cell maintenance [16,17] and mobilization of hematopoietic

progenitor cells [18]. Studies of RNA profiling of murine osteoblasts indicate that *TCIRG1* is expressed in this cell population [19]. Thus, it is possible that R736S, R736C, R736P and E722D *TCIRG1* mutations, observed in the neutropenic patients, may indirectly disrupt granulopoiesis by altering the bone marrow microenvironment. Of particular relevance to SCN, RNA expression profiling of granulocytic precursors from healthy donors suggests that *TCIRG1* is highly expressed in promyelocytes (Human Protein Atlas). Thus, these variants may also directly act on granulocytic precursors to impair their differentiation.

Other studies have shown that *TCIRG1* through alternative splicing and usage of an alternative initiation codon in exon 7 gives rise to the membrane protein TIRC7 demonstrated to play an essential role for the immune response [20]. There is evidence that more than two transcript variants of *TCIRG1* are expressed in human tissues (e.g., heart, liver, kidney, lung, pancreas) [21]. Studies in mice have shown that homozygous mutation at R740S (analogous to human R736 position) is lethal in mice and heterozygous mutation of the V-ATPase  $\alpha 3$  subunit R740S causes dominant negative osteopetrosis [14]. RNA interference to silence TIRC7, Ap6i +/- mice, reduced inflammatory responses in a mouse model of periodontal disease and decreased osteoclasts and monocytes [22]. These reports do not comment on granulocytopoiesis. Studies in yeast have shown that missense mutations in R735 (analog for the human R736 position) disrupt the functional integrity of vacuolar V-ATPase and thereby disrupt proton pumping, a selective effect of this specific mutation [23,24]. These studies infer that these missense mutations at R736 may act in a dominant fashion. Neutropenia with mutations R736S, R736C, and R736P occur at this exact location. Although direct structural data on *TCIRG1* is lacking, arginine at position 736 and glutamic acid at position 722 lie outside of the ATPase's V0 domain which is responsible for proton translocation and are evolutionarily highly conserved [24]. We further investigated the roles of R736 and E722 by examining high-resolution cryo-EM structures of a homologous yeast V-ATPase (PDB: 6C6L, 6M0R) [25,26]. These structural data, along with accompanying molecular dynamics simulations, demonstrate that the guanidino group of R736 and the carboxylic acid group of E722 are key components of the proton transport pathway that underlies ATP hydrolysis-driven proton pumping by the V-ATPase. Substituting R736 with residues such as cysteine, proline, or serine—which lack guanidino groups—or replacing E722 with an aspartate residue, would be expected to disrupt proton transport and thereby impair V-ATPase activity in neutrophils. Indeed, even a conservative substitution of lysine at the equivalent R736 position in yeast has been shown to abolish function [24]. Although the structural consequences of these mutations are well understood, the connection between impaired proton transport and the clinical phenotype of congenital neutropenia observed in patients heterozygous for R736 or E722 mutations remains to be fully elucidated. Finally, mutations at S733 are not predicted to interfere with proton

transport based on the proposed mechanism [26], consistent with the benign nature of the S733F variant in the general population and supporting its use as a negative control in this study.

### Tissue-specific effects of *TCIRG1* mutations

One of the most intriguing aspects of *TCIRG1*-associated neutropenia is the apparent lack of defects in other tissues, despite the gene's well-established role in osteoclast function and expression in other tissues. This suggests that neutrophils may have a unique dependence on *TCIRG1*-mediated V-ATPase activity, possibly due to their reliance on vesicular trafficking for granule formation and intracellular signaling. Recent publications continue to reveal the increasingly multifaceted roles of neutrophils, including their involvement in tumor microenvironment homeostasis, wound healing, and other processes beyond their traditional function in host defense [27-29]. Unlike osteoclasts, which exhibit redundancy in acidification mechanisms [30], neutrophil precursors may be uniquely vulnerable to disruptions in *TCIRG1* function, leading to impaired differentiation and survival.

Another possibility is that alternative compensatory pathways exist in non-hematopoietic tissues, allowing them to circumvent the effects of *TCIRG1* mutations. In neutrophils, however, these compensatory mechanisms may be absent or insufficient to maintain proper V-ATPase function, leading to selective lineage defects. It is well-known that during maturation, neutrophils lose some key organelles, like mitochondria, Golgi apparatus and have very few ribosomes [31,32]. Further research into the tissue-specific expression patterns and regulatory networks governing *TCIRG1* function will be crucial in unraveling these mechanisms.

### Hypothesis: Gain-of-function effects in *TCIRG1* neutropenia

An alternative hypothesis is that the molecular mechanism of *TCIRG1*-associated neutropenia is not due to a simple loss-of-function, but rather a gain-of-function effect caused by the specific mutations at R736 and E722. These mutations may induce aberrant intracellular trafficking of *TCIRG1*, leading to its mis-localization and subsequent disruption of normal vesicular pathways. Our experiments on the intracellular localization of the *TCIRG1* protein support this hypothesis. This could trigger an unfolded protein response (UPR) and promote apoptosis in neutrophil progenitors, selectively impairing their differentiation and survival. Given that neutrophils have a highly specialized and dynamic vesicular system required for granule formation and antimicrobial function, even minor disruptions in trafficking may have profound effects on their development. Further studies are needed to explore whether these mutations interfere with protein degradation pathways, vesicle fusion, or cellular stress responses in neutrophil precursors.



Similar gain-of-function effects causing UPR and apoptosis have been described in other autosomal dominant genetic diseases. For instance, mutations in *ELANE*, which encodes neutrophil elastase, lead to severe congenital neutropenia (SCN) through the accumulation of misfolded protein and ER stress, resulting in increased cell death of neutrophil precursors [33,34]. Similarly, autosomal dominant mutations in *SOD1* associated with amyotrophic lateral sclerosis (ALS) induce toxic protein aggregation, causing oxidative stress and neuronal apoptosis [35,36]. Another example is hereditary transthyretin amyloidosis, where misfolded transthyretin proteins aggregate and cause cytotoxicity in peripheral nerves and heart [37]. These parallels suggest that the heterozygous mutations in *TCIRG1* at R736 and E722 may also induce a pathogenic gain-of-function effect through disruption of protein homeostasis, leading to neutrophil-specific cellular stress and apoptosis.

### Clinical implications and future directions

The identification of *TCIRG1* mutations as a genetic cause of congenital neutropenia has important clinical implications for diagnosis and patient management. The severity of neutropenia and associated risk of infectious complications varies within the six families, suggesting that, future studies should investigate potential genetic modifiers that may influence disease severity. Additionally, therapeutic strategies aimed at restoring V-ATPase function, either through targeted small molecules or gene correction approaches, warrant further exploration.

As the unfolded protein response and apoptosis appear to play a central role in *TCIRG1*-associated neutropenia, experimental therapeutic approaches targeting these pathways should be explored. Apoptosis inhibitors such as caspase inhibitors (e.g., Emricasan) or BH3 mimetics (e.g., Venetoclax) may provide protection against excessive cell death in neutrophil progenitors [38,39]. Additionally, pharmacological UPR modulators such as tauroursodeoxycholic acid (TUDCA) or salubrinal, which help alleviate ER stress, could be investigated as potential interventions to rescue neutrophil differentiation and survival [40-43]. Future studies should evaluate the efficacy of these compounds in patient-derived cell models to determine their potential therapeutic benefit.

Our study also raises intriguing questions regarding the broader role of *TCIRG1* in hematopoiesis. While traditionally associated with osteoclast function, our data suggests that some *TCIRG1* mutations are critical for neutrophil survival. Further research is needed to define the specific cellular pathways disrupted by these *TCIRG1* mutations and determine whether these findings extend to other hematopoietic lineages.

### Conclusion

In summary, our study provides compelling evidence that

some heterozygous *TCIRG1* mutations disrupt myeloid differentiation, survival and maturation of neutrophils. By leveraging patient-derived iPSCs and genome editing, we have established a mechanistic link between *TCIRG1* dysfunction and congenital neutropenia. These findings not only enhance our understanding of neutrophil development but also pave the way for potential therapeutic interventions in *TCIRG1*-associated hematological disorders.

### Conflicts of Interest

The authors have no conflicts of interest and there are no commercial sponsors.

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