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13. SUPPLEMENTARY NOTES

14. ABSTRACT
A very severe inherited bone marrow failure (BMF) syndrome is caused by mutations in the endosomal-lysosomal vacuolar protein sorting regulator VPS45.1-3 Missense mutations in two highly conserved residues (T224N and E238K) of VPS45 were identified in several consanguineous families and additional families with the same and novel VPS45 mutations have since been identified in Israel and the U.S.4-6. All have severe congenital neutropenia (SCN) with neutrophil and platelet dysfunction, as well as myelofibrosis leading to multilineage BMF.

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1. INTRODUCTION:

A very severe inherited bone marrow failure (BMF) syndrome is caused by mutations in the endosomal-lysosomal vacuolar protein sorting regulator VPS45.[1-3] Missense mutations in two highly conserved residues (T224N and E238K) of VPS45 were identified in several consanguineous families and additional families with the same and novel VPS45 mutations have since been identified in Israel and the U.S.[4-6]. All have severe congenital neutropenia (SCN) with neutrophil and platelet dysfunction, as well as myelofibrosis leading to multilineage BMF. *We hypothesize that aberrant conformation of the mutant VPS45 protein leads to abnormal granule assembly and/or transport, with consequent apoptosis and cellular dysfunction in myeloid cells and megakaryocytes.* Our project uses interdisciplinary studies in cellular and mouse models to elucidate the previously unexplored molecular mechanisms for this novel form of BMF caused by VPS45 mutations, while gaining insights into other congenital BMF syndromes caused by defective vesicular transport and granules. Because VPS45 has heretofore only been studied in the regulation of generic endosomal-lysosomal transport,[17,47] this "experiment of nature" also opens new avenues of investigation into its function in assembly and transport of specialized exocytic granules, cell death and human disease.

2. KEYWORDS:

Apoptosis
Bone marrow failure
Endoplasmic reticulum stress
Exocytosis
Granule biogenesis
Hematopoiesis
Megakaryocytes
Membrane fusion
Mouse embryonic stem cells
Mouse models
Myelofibrosis
Neutropenia
Neutrophil function
Sec1/Munc-18 (SM) family proteins
Severe congenital neutropenia
SNARE proteins
VPS45

3. ACCOMPLISHMENTS

- **What were the major goals of the project?**

Our original Specific Aims were:

1. Determine the *molecular* consequences of VPS45 mutations in neutrophils derived from mouse embryonic stem (ES) cells. We have introduced knockout and T224N mutations by CRISPR/Cas9 mutagenesis of ES cells, which are being differentiated *in vitro* along the myeloid lineage to mature neutrophils.[40] We will test the "intracellular traffic jam" hypothesis by examining the effects of pathogenic VPS45 mutations on SNARE assembly, fusion and granule biogenesis, using assays developed and currently used in the Munson lab,[20,41,42] adapted to ES cell-derived neutrophils.

2. Determine the *functional* consequences of VPS45 dysfunction in neutrophils derived from mouse ES cells. We will investigate whether abnormalities in the biogenesis and fusion of exocytic granules lead to cellular defects associated with BMF and diminished host defense. We will focus on apoptosis, and its predicted trigger, endoplasmic reticulum (ER) stress, as well as phagocytic defects related to granule dysfunction, using assays developed and/or regularly used by the Newburger lab.[43-46]

3. Test the functional and *physiological* consequences of VPS45 dysfunction in mouse models of VPS45-related BMF. Chimeric mice incorporating ES cells from Aim 1 are being bred to establish colonies of T224N missense as well as frameshift (haploinsufficient and knockout) mutant mice. We will determine whether VPS45 pathogenic mutations or haploinsufficiency reproduce the human syndrome of BMF, myelofibrosis, and neutrophil dysfunction, and begin to dissect the mechanisms underlying the *in vivo* phenotype.

In addition, we are taking the opportunity to investigate new human VPS45 mutations as they are identified.

- **What was accomplished under these goals?**

Novel human VPS45 mutations. We collaborated with physicians in Oklahoma to analyze and model, in silico, a novel P468L mutation (Fig. 1),4 that is being incorporated into our planned studies. We are also studying the molecular and cellular phenotype of a Boston patient with a heterozygous P253L mutation and a mild phenotype. That mutation maps to a different part of the protein, which may be involved in interactions with the vesicle SNARE.

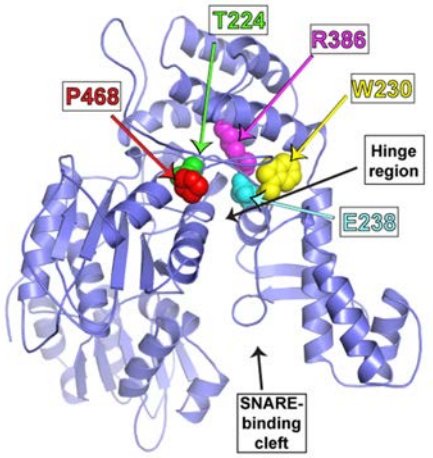

Fig. 1. Disease-related mutants mapped onto a model for VPS45. Human VPS45 mutations all map to the hinge region and are hypothesized to alter VPS45 conformation, resulting in abnormal SNARE binding.

Mouse ES cell models of VPS45 mutations.

Mouse Vps45 mutant ES cells provide an *in vitro* model of disease. Neutrophils derived from ES cells model normal myeloid differentiation and function, including granule formation, exocytosis, and apoptosis.[59-61] These pluripotent cells also offer the future capability of investigating the effects of VPS45 mutations on other lineages. Working with the UMass Mutagenesis and Transgenic Animal Cores, we produced VPS45 monoallelic T224N and indel/frameshift, as well as one compound biallelic mutant line with T224N and indel/frameshift mutations. ES cell clones with indel/frameshift mutations show 40-70% decreased expression of VPS45 protein, while the T224N/indel compound heterozygous mutant clonal line with ~50% protein expression (Fig. 2) models humanT224N homozygous mutant cells with similarly decreased protein content.[1,2] Rabenosyn-5 protein levels are concomitantly decreased. Neutrophils derived from the biallelic VPS45 mutant ES cells show decreased side scatter (Fig. 3), which measures secretory granule content, and decreased expression of ß1-integrin CD11b, relative to those derived from parental wild type ES cells.

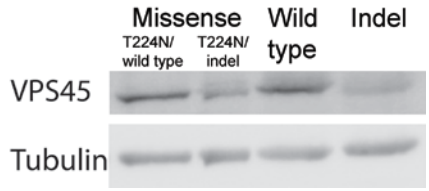

Fig. 2. Western blot of protein extracts from ES cells with the indicated gene editing.

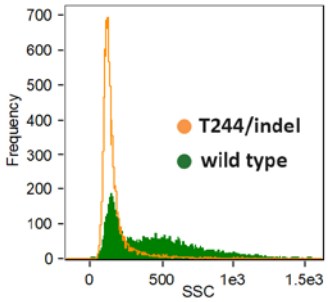

Fig. 3. Side scatter (SSC) by flow cytometry in ES-derived neutrophils.

Mouse models of VPS45 mutations

We generated Vps45 mutant mice, modeling the T224N and E238K human mutations. The former was derived by blastocyst injections of our CRISPR/Cas9-edited ES cells; the latter, by direct oocyte injection of CRISPR/Cas9-gRNA-donor DNA complexes. Two colonies of T224N mutant mice are being bred: the first, for generation of homozygous, heterozygous, and littermate wild type control mice for immediate analysis; the second for back-crosses. The latter, after at least 4 generations to eliminate off-target editing sites, will be used for definitive experiments in the proposed project. Homozygous knock-in mutants are being born in Mendelian ratios, and both males and females are fertile.

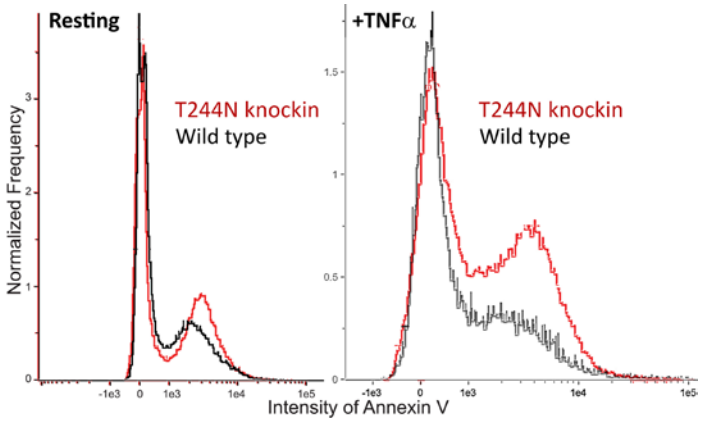

Fig. 4. Early apoptosis. Flow cytometry of annexin V binding in resting and TNFα (100 U/1 hr)-stimulated neutrophils from wild type and homozygous T244N knock-in mice.

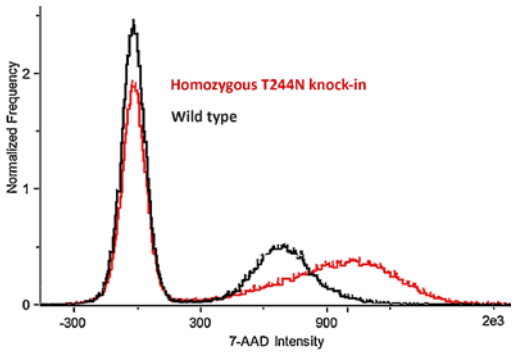

Fig. 5. Late apoptosis. Flow cytometry of 7AAD signal in resting neutrophils from wild type and T244N knock-in mice.

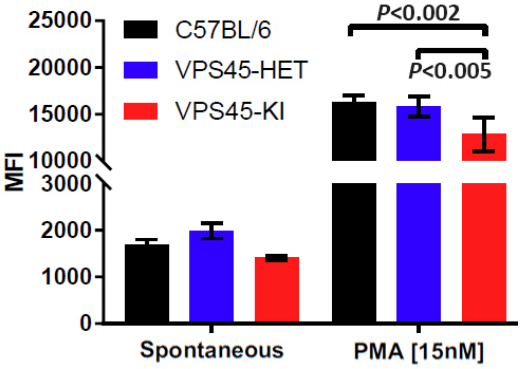

Fig. 6. Phagocyte oxidase activity measured as mean fluorescence intensity (MFI) of resting or PMA-stimulated DHR oxidation in purified bone marrow neutrophils of wild type and T244N mutant knock-in heterozygous and homozygous mice.

Direct oocyte injections of Cas9/gRNA plasmid and ssDNA template led to recent births of homozygous (one male, one female) and heterozygous (one male, one female) E238K mutants, as well as indel/frameshift heterozygotes, including one compound heterozygote with E238K mutation on the other allele. The indels, both occurring at the gRNA targeting site, are a 7 bp deletion resulting in a termination codon 2 amino acids downstream, and a C insertion resulting in termination after 8 amino acids. Notably, the homozygous knock-in mutants and the double heterozygote are viable; none are yet old enough to test phenotype or fertility. Back-crossing will be necessary both for elimination of off-target sites and for sorting out the mosaicism common after editing by oocyte injection. We will use mice with VPS45 knockout indel mutations to provide haploinsufficient controls for low VPS45 protein levels in the knock-in mutants, as well as homozygous knockouts to test phenotype and function if viable.

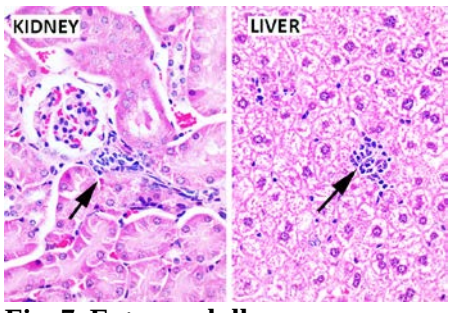

Fig. 7. Extramedullary hematopoiesis. Small pockets (arrows) evident in H&E sections of kidney and liver.

<u>Mutant neutrophils show functional defects</u>. Neutrophils harvested from homozygous VPS45 T244N mutant mice, compared to those from wild type littermates, show elevated rates of baseline and TNF-induced apoptosis, demonstrated by both annexin-V binding (Fig. 4) and AAD7 uptake (Fig. 5), indicating increases in both early and late stages of apoptosis. The neutrophils also have significantly decreased NADPH oxidase activity (Fig. 6).

Although peripheral blood counts in the mutant mice remain normal at age 30 weeks and bone marrow shows no overt fibrosis, pockets of extramedullary hematopoiesis have appeared in kidney (an unusual facet of the human phenotype) and liver (Fig. 7).

Autophagy requires vesicle fusion and proper endolysosomal trafficking,[118,119] and is closely associated not only with NADPH oxidase activity and phagocytosis,[120-124] but also with apoptosis.[125] In yeast, SNARE proteins required for macroautophagy[128] include Tlg2,[79] the yeast homolog of the VPS45 binding partner syntaxin-16. Autophagy and apoptosis are in many ways competing processes, as autophagy is primarily a cell survival mechanism that provides nutrients by recycling of intracellular organelles and macromolecules. [80,126,127] More specifically, autophagy blocks the induction of apoptosis, and apoptosis-associated caspase activation shuts off the autophagic process.[125] However, the complex interplay of autophagy and apoptosis also includes both delay and enhancement of caspase activation,[126] so enhanced autophagy can also be a pro-apoptotic process.

A standard screening assay measures the processing of microtubule-associated protein 1A/1B-light chain 3 (LC3) to LC3-phosphatidylethanolamine conjugate (LC3-II.[132,133] A pilot experiment suggests an increase in the ratio of LC3-II to LC3-I in homozygous T224N mouse neutrophils (Fig. 8). Further mechanistic experiments will be carried out with the help of Dr. Eric Baehrecke, a leader in the field.[127,128,132,134]

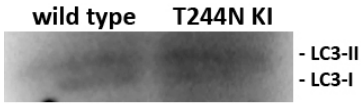

Fig. 8. Western blot of LC3 in neutrophils from wild type and homozygous T244N knock-in (KI) mice.

- **What opportunities for training and professional development has the project provided?**

Nothing to Report

- **How were the results disseminated to communities of interest?**

Presentation to UMass hematopoiesis research group in February, 2018. National meeting presentations and publications planned when more data are available.

- **What do you plan to do during the next reporting period to accomplish the goals?**

Continue studies described in the original application.

4. IMPACT

It is still too early in the study to assess its impact.

5. CHANGES/PROBLEMS

- Changes in approach and reasons for change

The lack of neutropenia in the T224N mice makes some of the proposed studies difficult. We will monitor the potentially more severe E238K knockin mutation for neutropenia but also consider breeding with Mcl1 conditional mutants to diminish the high level of anti-apoptotic activity in mice and bring out the neutropenia phenotype.

- Actual or anticipated problems or delays and actions or plans to resolve them

None

- Changes that had a significant impact on expenditures

None

- Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

None

6. PRODUCTS:

Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

- What individuals have worked on the project?

Name:	Peter E. Newburger, MD
Project Role:	P.I.
Researcher Identifier (e.g. ORCID ID):	0000-0002-8615-673X
Nearest person month worked:	2
Contribution to Project:	Responsible for overall direction of the project, planning of experiments, interpretation of results, and communication of findings. Also overall fiscal management and communication

	with the DoD. He directly oversees the cell biology and animal experiments.
Funding Support:	N/A
Name:	Mary Munson, PhD
Project Role:	Co-PI
Researcher Identifier (e.g. ORCID ID):	0000-0003-2297-8053
Nearest person month worked:	2
Contribution to Project:	Shares with Dr. Newburger the overall direction of the project, planning of experiments, interpretation of results, and communication of findings. She is directly responsible for the biochemical aspects of the project
Funding Support:	N/A
Name:	Kimberly Ng, MD
Project Role:	Post-doctoral fellow
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	1
Contribution to Project:	Responsible for ES cell culture and transfection, transgenic mouse genotyping and phenotyping, and mouse colony maintenance.
Funding Support:	N/A
Name:	Zhiqing Zhu, MS
Project Role:	Research associate
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	4
Contribution to Project:	Responsible for transgenic mouse genotyping and phenotyping, and mouse colony maintenance.
Funding Support:	N/A

Name:	Kristyn Norris
Project Role:	Graduate research assistant
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	12
Contribution to Project:	Responsible for molecular and cell biology experiments, including molecular and biochemical analyses of protein-protein interactions and phagocyte biology
Funding Support:	N/A

- Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

No

- What other organizations were involved as partners?

None

8. SPECIAL REPORTING REQUIREMENTS

N/A

9. APPENDICES

NONE