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A new murine model of Barth Syndrome neutropenia links TAFAZZIN deficiency to increased ER stress induced apoptosis

Running title

A new model of Barth syndrome neutropenia

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Key points

- ER-Hoxb8 conditional immortalization provides a system for the study of TAFAZZIN deficiency in myeloid progenitors and mature neutrophils
- Barth syndrome (i.e., TAFAZZIN-deficient) murine neutrophils appear to be more sensitive to apoptosis, possibly due to increased ER stress

Abstract

Barth syndrome is an inherited X-linked disorder that leads to cardiomyopathy, skeletal myopathy and neutropenia. These symptoms result from the loss of function of the enzyme TAFAZZIN, a transacylase located in the inner mitochondrial membrane that is responsible for the final steps of cardiolipin production. The link between defective cardiolipin maturation and neutropenia remains unclear. To address potential mechanisms of neutropenia, we examined myeloid progenitor development within the fetal liver of TAFAZZIN knock-out animals as well as within the adult bone marrow of wild-type recipients transplanted with TAFAZZIN-KO hematopoietic stem cells. We also used the ER-Hoxb8 system of conditional immortalization to establish a new murine model system for the *ex vivo* study of TAFAZZIN-deficient neutrophils. The TAFAZZIN-KO cells demonstrated the expected dramatic differences in cardiolipin maturation that result from a lack of TAFAZZIN enzyme activity. Contrary to our hypothesis, we did not identify any significant differences in neutrophil development or neutrophil function across a variety of assays including phagocytosis, and the production of cytokines or reactive oxygen species. However, transcriptomic analysis of the TAFAZZIN-deficient neutrophil progenitors demonstrated an upregulation of markers of endoplasmic reticulum stress and confirmatory testing demonstrated that the TAFAZZIN-deficient cells had increased sensitivity to certain ER-stress mediated and non-ER-stress mediated triggers of apoptosis. While the link between increased sensitivity to apoptosis and the variably penetrant neutropenia phenotype seen in some Barth syndrome patients remains to be clarified, our studies and new model system set a foundation for further investigation.

Introduction

Barth syndrome (BTHS) is a rare inherited X-linked disorder characterized by cardiomyopathy, skeletal muscle myopathy and neutropenia [1,2]. This disease arises following a loss-of-function mutation in the gene TAFAZZIN (*TAFAZZIN, which has also been previously annotated as TAZ*)[3]. TAFAZZIN is a mitochondrial enzyme that processes cardiolipin, a phospholipid that is found almost exclusively in the inner mitochondrial membrane [4]. The lack of normal TAFAZZIN protein activity abrogates the final step in cardiolipin maturation, resulting in reduced levels of cardiolipin with greater acyl chain diversity and greater saturation, as well as elevated levels of an intermediate, monolysocardiolipin, that contains 3 rather than 4 acyl chains. [5]

The clinical manifestation with the overall highest mortality is cardiomyopathy, which can progress to heart failure [6]. The link between TAFAZZIN-deficiency and myopathy has been explained by the high mitochondrial content of myocytes and by their dependence on efficient oxidative respiration. The components of the electron transport chain are co-localized with cardiolipin in the inner mitochondrial membrane, and their structural organization within the membrane lipid bilayer is dependent on mature cardiolipin, which is lacking in individuals with BTHS [7,8]. The role of cardiolipins within the mitochondria also goes beyond a structural one, with proposed roles in signaling, in modulating reactive oxygen species, and in mitochondrial membrane potential and apoptosis (reviewed in [9,10]).

In addition to cardiomyopathy, many individuals with BTHS suffer from recurrent episodes of neutropenia and life-threatening infections. The neutropenia has variable penetrance and is frequently intermittent. The mechanistic link between *TAFAZZIN* deficiency and neutropenia is not known [5,11,12]. Unlike cardiac and skeletal myocytes, neutrophils have relatively few mitochondria per cell and it was assumed that neutrophils rely on glycolysis for energy production though this assumption has more recently been challenged (reviewed in [13]). Within lymphoblasts derived from patients with BTHS, mitochondria display ultrastructural abnormalities, showing reduced cristae density, poorly aligned cristae, and fragmented cristae distribution [14]. However, information on proliferation, survival, or function of neutrophils in the BTHS patient is scarce. It is unclear if the underlying mechanism of neutropenia in BTHS is due to diminished production or increased clearance of mature neutrophils (e.g., increased apoptosis) [15–17].

Animal models of Barth syndrome have included zebrafish, drosophila and mouse and have been used primarily in the study of the cardiomyopathy and skeletal muscle defects [18–21]. One murine model of BTHS involves knockdown of TAFAZZIN by a doxycycline-inducible short hairpin RNA (shRNA) [21–23]. Hematologic abnormalities in this model have not characterized in depth, and the cardiac manifestations are mild and late in onset, likely due to residual TAFAZZIN expression.

More recently, a global TAFAZZIN-knockout (KO) mouse model (C57BL6/J) was developed which emulates many of the hallmark clinical presentations of BTHS [24]. Most of these mice die during the perinatal period, due to skeletal muscle weakness. Those few mice that survive into adulthood develop progressive cardiomyopathy and cardiac fibrosis. The number of circulating neutrophils in TAFAZZIN-KO mice that survive to adulthood (6-months) is reportedly lower than in wild-type (WT) mice ($p<0.01$, data found in Online figure V [24]).

Recent studies have pointed towards the importance of endoplasmic reticulum (ER) stress and the unfolded protein responses (UPR) in neutrophil survival, differentiation and function [19, 20, 21]. The expression of ER stress markers PERK and ATF6 are decreased during neutrophil differentiation in a stage-specific manner, suggesting that dysregulation of ER stress in neutrophils may affect the differentiation process. These authors linked mitochondrial ATP production and ER stress, showing that lower ATP supply from mitochondria promoted higher ER stress, which subsequently impaired protein folding and myeloid differentiation [19]. These data suggest a mechanistic link between mitochondrial defects, ER stress, and an UPR, ultimately resulting in decreased neutrophil survival and function.

Here we investigated the pathogenesis of neutropenia in TAFAZZIN-KO mice. We took advantage of our established ER-Hoxb8 system of conditional immortalization to generate granulocyte-monocyte progenitors from TAFAZZIN-KO and WT murine fetal liver hematopoietic progenitors [22]. These progenitors are capable of unlimited culture and expansion ex vivo as well as synchronous differentiation to mature and functional neutrophils upon inactivation of the ER-Hoxb8 protein. Overall, our in vitro and in vivo findings point towards a mechanism of ER stress and apoptosis underlying the phenotype of neutropenia in patients with Barth syndrome.

Methods

Detailed methods are provided in the **Supplemental Materials**.

Results

TAFAZZIN deficient mice demonstrate normal fetal liver hematopoietic development

We hypothesized that the loss of TAFAZZIN may result in a defect in myelopoiesis that would be exacerbated during periods of stress or infectious challenge. Unfortunately, the majority of TAFAZZIN-knockout ($TAFAZZIN^{null/Y}$; abbreviated in the figures as TAZ-KO) mice do not survive into adulthood, preventing a straightforward analysis of adult hematopoiesis.[18]

In the developing embryo, blood development occurs in the fetal liver (beginning around E11.5) before the hematopoietic stem cells (HSCs) migrate to bone marrow.[23] To investigate whether the TAFAZZIN deficiency results in impaired neutrophil development, we characterized the myeloid progenitors within the hematopoietic compartment of TAFAZZIN-KO and wild-type (TAFAZZIN$^{WT/Y}$; abbreviated WT) fetal livers at five time points (E14 to E16) along embryonic development (**Figure 1B**). Using timed pregnancies, fetal livers were dissected and dissociated into single-cell suspensions for antibody staining and flow cytometry (the gating strategy is indicated in **Figure 1A**) [25]. Of note, the percent of lineage negative, KIT^{POS} cells was much higher in the fetal liver hematopoietic cells as compared to adult murine bone marrow. PCR based genotyping distinguished WT from TAFAZZIN-KO embryos (**Figure S1A**) and distinguished male from female embryos (**Figure S1B**).

Analysis of the TAFAZZIN-deficient E14 and E14.5 embryos suggested a lower percentage of granulocyte monocyte progenitors (GMPs) as compared to their WT littermates (**Figure 1C**), though statistical power is limited by the limited number of embryos per litter and the lack of multiple replicates at each time point. This difference was abrogated at the E15, E15.5 and E16 time points where the distribution of myeloid progenitors was strikingly similar between KO and WT embryos (**Figure 1B**). Overall, these experiments suggest that the lack of TAFAZZIN activity may result in slightly delayed, but ultimately normal myeloid progenitor development.

TAFAZZIN deficiency does not impact adult hematopoiesis

The majority of TAFAZZIN-KO animals die in the perinatal stage making an analysis of the adult bone marrow compartment challenging. To investigate the developmental potential of TAFAZZIN-deficient hematopoietic stem cells (HSCs) and myeloid progenitors we employed a strategy of fetal liver stem cell transplantation [26,27].

This experiment was performed twice, and the results presented in **Figures 2 and S2**. In the first experiment, E15 embryos (2 WT male and 4 TAFAZZIN-KO male) were genotyped, pooled and transplanted into wild-type recipients (**Figure 2A**). Here the fetal liver hematopoietic cells are marked with CD45.2 while the lethally irradiated, wild-type, recipients are the congenic CD45.1^{STEM} strain [28]. CD45.1^{STEM} helper bone marrow was also provided given the limited number of fetal liver cells.

The contribution of the donor WT and TAFAZZIN-KO cells remained stable over 12-weeks post-transplant as demonstrated by peripheral blood sampling (**Figure 2B**). The percentage of CD45.2 white blood cells was on average 79% and 63% in the WT fetal liver into WT recipients and TAFAZZIN-KO fetal liver into WT recipients transplants respectively. The stability of the %CD45.2 over time suggested that the TAFAZZIN-KO cells had similar reconstitution potential in the hematopoietic system. It is possible that we simply transplanted fewer hematopoietic stem cells, and

also possible that the TAFAZZIN-KO cells had slightly lower engraftment potential. The mice were considered to have stable reconstitution at 16-weeks, establishing a cohort of mice specifically lacking TAFAZZIN in their hematopoietic system.

At approximately 16 weeks, analysis of the complete blood counts (CBC) of these transplanted mice consistently demonstrated leukopenia (**Figure 2C**) in the setting of normal peripheral red blood cell and platelet counts. Flow cytometric analysis of the peripheral blood cells, with a focus on cells derived from the transplanted fetal liver cells ($CD45.2^{+}$) revealed fewer white blood cells across the spectrum of B, T and myeloid cells without the specific loss of any compartment (**Figure 2D,** gating strategy **Figure S2D**). Analysis of the bone marrow compartment was performed by flow cytometry (**Figure 2E,** gating strategy **Figure S2E**); no differences were detected within the bone marrow mature or progenitor populations when comparing mice transplanted with WT or TAFAZZIN-KO fetal liver cells.

In the second experiment, E15.5 embryos (4 WT males and 1 TAFAZZIN-KO male) were genotyped, pooled and transplanted. Analysis of reconstitution (**Figure S2A**), complete blood counts (**Figure S2B**), and flow cytometric characterization of peripheral blood leukocytes (**Figure S2C**) was entirely consistent with our first experiment. The degree of fetal liver cell (CD45.2) reconstitution at 16-weeks was slightly less in the TAFAZZIN-KO transplants (89%) as compared to the WT transplants (95%), again suggesting simple technical variability or a mild defect in engraftment. The bone marrow compartment of these mice was not assayed, as the mice were used for the *in vivo* phagocytosis experiments described below.

Overall, these experiments point towards a mild and consistent leukopenia in the mice transplanted with the TAFAZZIN-KO fetal liver cells. This leukopenia manifests as a mild decrease across lineages, including peripheral B-cells, T-cells, myeloid cells, and neutrophils.

The ER-Hoxb8 system of conditional immortalization establishes TAFAZZIN deficient GMPs

The ER-Hoxb8 system was first described in 2006 and has been adopted by more than 125 laboratories worldwide as a model for the study of myeloid progenitors and their mature progeny cells including neutrophils, monocytes, eosinophils, basophils and dendritic cells [29]. The retrovirally-delivered ER-Hoxb8 protein is constitutively expressed but active only in the presence of supra-physiologic concentrations (0.5 µM) of beta-estradiol (E2). While active, ER-Hoxb8 arrests myeloid differentiation at the GMP stage of development, allowing for their continuous self-renewal and propagation *ex vivo*. Upon removal of E2, which inactivates the ER-Hoxb8 protein, the cells undergo synchronous and terminal differentiation to mature neutrophils over approximately 4-days.

Following transduction of wild-type and TAFAZZIN-KO fetal liver cells, we generated conditionally immortalized GMP populations and then single-cell clones in the presence of stem cell factor (SCF) and E2 (**Figure 3A**). The genotype of the clones was confirmed by PCR and immunoblotting confirmed the lack of TAFAZZIN protein expression (**Figure 3B**). The proliferation rate varied slightly from clone to clone, though overall the TAFAZZIN-KO GMPs proliferated more slowly than the WT GMPs. Both WT and TAFAZZIN-KO GMPs exhibited normal morphologic differentiation to mature neutrophils as evidence by Wright-Giemsa staining of Cytospin™ preparations 4-days out of E2 (**Figure 3C and Figure S3A**). Both WT and TAFAZZIN-KO GMPs exhibited the expected acquisition of CD11b and GR1 cell-surface staining upon withdrawal of E2 and differentiation (**Figure 3D**). Multiple WT and TAFAZZIN-KO clones were analyzed for mitochondrial content using parallel methods of quantitative RT-PCR (**Figure S3B**) and MitoTracker™ staining (**Figure S3C**). The lack of TAFAZZIN did not lead to a significant change in the mitochondrial content of the GMP clones.

On very rare occasions, TAFAZZIN-KO animals survive the perinatal period into adulthood. The unexpected availability of a TAFAZZIN-KO adult mouse and wild-type male littermate control permitted the isolation and morphologic analysis of Cytospin™ preparations by Wright-Giemsa staining of primary bone marrow neutrophils (**Figure S3E**). The appearance of the primary neutrophils was similar to the ER-Hoxb8 derived neutrophils, and in neither setting did the lack of TAFAZZIN change the morphologic appearance of the cells.

The loss of TAFAZZIN results in ultrastructural changes within the mitochondria

Transmission electron microscopy was performed on wild-type and TAFAZZIN-KO GMPs and mature neutrophils. Within the progenitors, mitochondrial size appeared equal but mitochondrial cristae were more distinct in the WT cells and spiral structures more prevalent in the TAFAZZIN-KO cells (**Figure 3E and S4A**). Intriguingly, the TAFAZZIN-KO mitochondria were often entirely encircled by rough endoplasmic reticulum (**Figure 3E and S4C**). These same findings persisted in the mature neutrophils (**Figure 3E and S4B**).

The loss of TAFAZZIN in myeloid progenitors results in the expected increase in monolysocardiolpin

The ER-Hoxb8 WT and TAFAZZIN-KO GMPs provided an unlimited source of homogenous progenitors for lipodomic analysis following methanol-chloroform extraction from four wild-type and four TAFAZZIN-KO clones. The total pool of cardiolipin was significantly reduced in the TAFAZZIN-KO cells with the expected shift towards the accumulation of monolysocardiolipin (MLCL) (**Figure 4A**). When looking at specific species of cardiolipins and enumerating the number of

unsaturations (e.g., double bonds) within the four cardiolipin lipid tails, there was a clear distinction with the expected complete lack of highly-unsaturated cardiolipins in the TAFAZZIN-KO cells (**Figure 4B**), consistent with published findings [30].

The loss of TAFAZZIN does not affect neutrophil phagocytosis or cytokine production

The terminally differentiated neutrophil has myriad effector functions for efficient pathogen clearance including phagocytosis, cytokine production, and the release of antimicrobial proteins. The ER-Hoxb8 GMPs were differentiated *ex vivo* and experiments performed 4-days following removal of E2. Mature neutrophils were incubated with fluorescently labeled BioParticles Staphylococcus *aureus* (S. *aureus*) and Escherichia *coli* (E. *coli*) to assess phagocytic capacity. Imaging flow cytometry (ImageStream®) confirmed the process of phagocytosis by demonstrating that bacteria were truly internalized as compared to stuck to the extracellular membrane (**Figure 4C**). The capacity for phagocytosis of both S. *aureus* and E. *coli* *in vitro* was strikingly similar across multiple WT and TAFAZZIN-KO clones (**Figure 4D**).

To examine phagocytosis *in vivo* we took advantage of wild-type mice that had received hematopoietic stem cell transplantations from both WT and TAFAZZIN-KO fetal livers, as described above (**Figure 2A**). At 20-weeks post-transplant, >85% of their hematopoietic cells were derived from the transplanted fetal liver cells (CD45.2) and could be distinguished from host (CD45.1) on the basis of CD45 staining. These mice received a single intraperitoneal injection of fluorescently labeled E. *coli* BioParticles to stimulate neutrophil recruitment and phagocytosis within the peritoneal cavity. At 3-hours post-injection there was no difference in the degree of recruitment (data not shown) or in the degree of *in vivo* phagocytosis (**Figure 4E**) between WT and TAFAZZIN-KO neutrophils.

In similar fashion to the *in vitro* phagocytosis assays described above, ER-Hoxb8 neutrophils were stimulated with lipopolysaccharide (LPS) as a strong TLR4 agonist mimicking infection with gram-negative rod bacteria. Using ELISA-based assays, we quantified the production and release of tumor necrosis factor alpha (TNF-α), neutrophil elastase (ELA2), and neutrophil gelatinase associated lipocalcin (NGAL). Similar to our phagocytosis assays, there were no significant differences between WT and TAFAZZIN-KO clones in their ability to produce TNF-α, ELA2 or NGAL (**Figure 4F**). In addition, there were no significant differences in the ability of mature neutrophils to migrate across a semi-permeable transwell membrane in response to LPS stimulation (**Figure S3D**).

Together, these results suggest that TAFAZZIN is dispensable for the terminal neutrophil effector functions of phagocytosis, cytokine production and antimicrobial granule release.

The loss of TAFAZZIN does not affect neutrophil production of reactive oxygen species (ROS)

Neutrophils generate a variety of reactive oxygen species (ROS) for pathogen killing including superoxide (O_2^-) and hydrogen peroxide (H_2O_2). **Figure 5A** outlines some of the commonly understood ROS pathways as well as the reagents used to measure and to distinguish the different species. Using the ER-Hoxb8 mature neutrophils, we compared oxygen consumption, superoxide generation and hydrogen peroxide generation between the WT and TAFAZZIN-KO cells.

Neutrophils were stimulated by phorbol myristate acetate (PMA), an activator of protein kinase C, and oxygen consumption monitored over time using the the Seahorse XF platform; there was no significant difference when comparing WT and TAFAZZIN-KO cells (**Figure 5B**).

Neutrophils were stimulated with PMA as well as the chemotactic peptide formyl-methionyl-leucyl-phenylalanine (fMLP), or the multivalent peptide poly-arginine-glycine-aspartate (poly-RGD). Extracellular superoxide anion production was measured over time using a technique of cytochrome C reduction and the area under the curve (AUC) was calculated as a measure of total superoxide production (**Figure 5C**). There were no differences in superoxide production of neutrophils when comparing WT and TAFAZZIN-KO clones.

The cell-permeant dye H_2DCFDA (fluorescein 2', 7'-dichlorodihydrofluorescein diacetate) can be converted intracellularly to the fluorescent molecule DCF (2', 7'-dichlorofluorescein) in the presence of intracellular H_2O_2. Thus, the accumulation of DCF, and of green fluorescence can be monitored by flow cytometry as a surrogate of intracellular H_2O_2. WT and TAFAZZIN-KO neutrophils responded similarly to the three stimuli of PMA, fMLP and RGD in terms of H_2O_2 production and DCF fluorescence (**Figure 5D**).

Wild-type and TAFAZZIN-KO GMPs and mature neutrophils were assayed under homeostatic conditions to measure the amount of mitochondrial superoxide using the dye indicator MitoSOX. There was no significant difference between the two genotypes in terms of the amount of measurable mitochondrial superoxide (**Figure 5E**).

Together, these results suggest that TAFAZZIN is dispensable for the generation of ROS in response to multiple stimuli within the context of the mature neutrophil.

The loss of TAFAZZIN does not affect mitochondrial complex assembly

Given the role of mature cardiolipin in the organization of the inner mitochondrial membrane and the close relationship with complexes and supercomplexes of the electron transport chain [31–34], we examined the effect of TAFAZZIN deficiency on complex assembly in our model GMP cell lines. Following the isolation of purified mitochondrial, a system of BN-PAGE (Blue native polyacrylamide

gel electrophoresis) was used to separate the high molecular weight complexes under non-denaturing conditions [35–37]. These were stained with Coomassie Brilliant Blue G-250 (**Figure 6A**) and quantification done on the LI-COR scanner as Coomassie can be readily imaged in the 700 nm channel (**Figure 6B**). A comparison of three WT and three TAFAZZIN-KO clones did not reveal significant differences in mitochondrial complex assembly in our model GMPs. Higher molecular weight supercomplexes were not readily visible in the mitochondrial isolated from our neutrophil progenitors. Overall, these results are fairly consistent with previous results in wild-type and Barth syndrome human lymphoblasts, where the RNAi knockdown of TAFAZZIN in the wild-type lymphoblasts did not lead to changes in the mitochondrial complexes [37].

Gene expression analysis suggests increased endoplasmic reticulum (ER) stress

Given the functional similarity of neutrophils in our assays, we turned to a transcriptomic comparison (RNA-sequencing) of ER-Hoxb8 GMPs. The lack of TAFAZZIN did not lead to widespread transcriptomic changes (**Figure 6C**). Furthermore, there were no obvious and significant changes at the pathway level that resulted from the loss of TAFAZZIN (e.g., Gene ontology, DAVID, GSEA, data not shown). In manually curating the gene expression data, we noted that TAFAZZIN was the gene most differentially expressed (as would be expected) and that housekeeping genes such as beta-actin (ACTB) were unaffected by the loss of TAFAZZIN expression (**Figure 6D**).

One of the most differentially expressed genes was the transcription factor DDIT3 (DNA Damage Inducible Transcript 3), also known as CHOP (C/EBP homologous protein) (**Figure 6E**). Given the role of CHOP as a terminal effector of endoplasmic reticulum (ER) stress [38], we examined the expression of other canonical ER stress pathway genes and found significant changes in the expression of ATF4 and ERN1 as well as apoptosis pathway genes BCL2 (**Figure 6E**). Notably, many of the other genes in the ER stress and apoptotic pathways did not show significant differences at the transcriptomic level.

Gene expression data is publicly available in GEO (GSE151006) and the complete list of CPM, logFC, and FDR can be found in **Table 1**.

TAFAZZIN deficient myeloid progenitors are more sensitive to apoptosis and ER stress *in vitro*

Given the gene expression differences, we wished to assay the sensitivity of the WT and TAFAZZIN-KO ER-Hoxb8 GMPs to triggers of apoptosis. Cells were exposed to an inhibitor of BCL2 (ABT199), an inhibitor of MCL1 (AMG176), and a trigger of ER stress (thapsigargin). Thapsigargin is an inhibitor of the sarcoplasmic endoplasmic reticulum Ca2+ ATPase (SERCA) which leads to ER

stress and activation of the unfolded protein response and ultimately cell death. Viability was assayed at 24-hours. The TAFAZZIN-KO cells showed increased sensitivity to apoptosis following inhibition of BCL2, but not MCL1, and were slightly more sensitive to thapsigargin (**Figure 7A**). Given the subtle nature of the differences, these assays were repeated on multiple clones and on five to six different experimental days to account for any technical differences; the data in **Figure 7A** is displayed without error bars for the sake of clarity (the same data with standard deviation error bars is presented in **Figure S5A**).

To validate the findings of our ER-Hoxb8 GMPs, primary fetal liver hematopoietic cells from the litters of three timed pregnancies were also exposed to ABT199 and viability assayed at 24-hours. Consistent with the data in the ER-Hoxb8 cell lines, the primary fetal liver TAFAZZIN-KO cells also demonstrated a significantly increased sensitivity to apoptosis (**Figure S5B**).

TAFAZZIN deficient neutrophils are more sensitive to ER stress *in vivo*

Recognizing the limitations of *in vitro* assays, we wished to assess the sensitivity of mature neutrophils to ER stress *in vivo*. We took advantage of our system of fetal liver transplantation (**Figure 2A**) to reconstitute adult mice with a TAFAZZIN-deficient hematopoietic compartment. This experiment was performed twice. In the first iteration, mice were exposed to a single dose of thapsigargin delivered by intraperitoneal injection, and the protein expression of CHOP in peripheral blood neutrophils was assayed by intracellular antibody staining three hours after injection (**Figure 7B**, gating strategy shown in **Figure S5C**). The increase in CHOP expression was more pronounced in TAFAZZIN-deficient neutrophils, suggesting an increased propensity towards apoptosis following a trigger of ER stress.

In the second experiment, the protein expression of an expanded number of ER stress markers was evaluated (**Figure 7C**). Consistent with the first experiment, the TAFAZZIN-deficient neutrophils again appeared to have an increased propensity to undergo apoptosis following treatment with thapsigargin, as evidenced by the increased expression of CHOP in addition to ATF4, ATF6 and BiP. In this second experiment, the lack of difference within the lymphoid population of peripheral blood cells was highly suggestive that this propensity towards apoptosis was limited to cells of the myeloid compartment.

Together, these data suggest that the loss of TAFAZZIN expression results in increased sensitivity to ER stress-induced apoptosis both in myeloid progenitors when assayed *in vitro* and in mature circulating neutrophils *in vivo*.

Discussion

In this study we took advantage of a TAFAZZIN-KO mouse and combined *in vivo* studies with *in vitro* studies using the ER-Hoxb8 system of conditional immortalization to assess the effect of TAFAZZIN-deficiency on myeloid progenitors and mature neutrophils. The strengths of our study include the analysis of embryonic hematopoietic development in parallel with the analysis of adult hematopoiesis using a transplantation model. Furthermore, we were successful in the generation of ER-Hoxb8 GMPs from both wild-type and TAFAZZIN-KO fetal liver cells, providing an inexhaustible source of progenitors and mature neutrophils for mechanistic studies, and a useful and practical reagent for the field. Where possible, data generated in the context of the ER-Hoxb8 model system was validated using primary cells, though the paucity of cells (in most cases available from the embryonic fetal liver) made this challenging and spoke to the practical advantage of the ER-Hoxb8 lines.

The neutropenia of Barth syndrome can be pleiotropic with almost 90% of patients affected to some degree though individual patients can have periods of neutropenia alternating with normal neutrophil counts [6,39]. The molecular basis of the neutropenia has been elusive and difficult to replicate in model systems [16]. The original description of Barth syndrome reported histologic and electron microscopic analyses of patient bone marrow and concluded that “a maturation arrest was observed at the myelocyte stage… myeloblasts, promyelocytes and myelocytes had abnormal mitochondria” [1]. Functional studies of patient neutrophils, including phagocytosis, oxygen consumption and chemotaxis were normal [1]. Later, neutrophils isolated from patients demonstrated increased Annexin-V binding *in vitro* in the absence of other markers of apoptosis [15]. In these patients, colony formation (CFU-GM) from peripheral blood mononuclear cells (PBMCs) in methylcellulose media demonstrated the same number of colonies as controls, arguing against a developmental defect [15].

Another group used shRNA-mediated knockdown of TAFAZZIN in human acute myeloid leukemia cells lines (e.g., HL60 and U937) and similarly demonstrated increased Annexin-V binding. This was accompanied by changes in mitochondrial membrane potential as well as leaking of mitochondrial cytochrome c, activation of caspase-3 and increased apoptosis [17]. These changes seemed specific to the myeloid leukemia cell lines as TAFAZZIN knockdown in lymphoid cell lines had minimal to no effect [17].

The first murine model of Barth syndrome was based on a doxycycline-inducible transgenic short hairpin RNA for the knockdown of TAFAZZIN [40,41]. Though the data was not shown, the authors reported neutropenia in 50% of TAFAZZIN-KO animals (2 months old) as well as 20% of WT control animals. However, they attributed this to the confounding variable of doxycycline (administered to the mice via drinking water or chow), which can decrease circulating neutrophil numbers in mice [42].

In this historical context, our results are quite consistent with what has been published. We were expecting a more dramatic functional phenotype under the hypothesis that our system was one of complete lack of TAFAZZIN activity (due to the murine genetic knockout) rather than a knock-down (shRNA) or potential low level residual TAFAZZIN activity (e.g., hypomorphic mutation) that can be seen in patient samples. The lack of deficit does not appear to be due to a compensatory mechanism, in the sense that there are striking differences in the cardiolipin content and makeup of wild-type and TAFAZZIN-KO myeloid progenitors.

In our transplantation model, where TAFAZZIN-KO bone marrow was transplanted into WT recipient mice, might the lack of *in vivo* hematopoietic phenotype be due to the compensatory presence of intact TAFAZZIN within the stromal and endothelial cells of the bone marrow? This question is made more relevant by the observation that mitochondria can be transferred between hematopoietic and bone marrow stromal cells, particularly in the context of leukemia or other inflammatory settings [43–45]. Cell-to-cell mitochondrial transfer has not been well-characterized in populations of normal GMPs or mature neutrophils, so we suspect that this is less likely to be a confounding variable in our system.

The lack of a functional deficit may be due to the observation that mature neutrophils have relatively few mitochondria (on a per cell basis) and even those mitochondria are quite small [46] (in comparison to the very high number of large mitochondria seen in the cardiac myocytes). Still, we were surprised that there did not appear to be a difference in the production of ROS, which is felt to be dependent on the efficient function of the mitochondrial electron transport chain complexes. Our mitochondrial supercomplex data, based on Blue Native PAGE, did not show any obvious changes in supercomplex assembly in the absence of TAFAZZIN. Our transmission electron micrographs of TAFAZZIN deficient neutrophils demonstrated the known ultrastructural differences as well as a previously undescribed tendency of the rough endoplasmic reticulum to completely encircle the TAFAZZIN-deficient mitochondria.

Neutrophil mitochondria have been reported to play a limited role in supporting ATP production, but a more important role in apoptosis and cell death [47]. These findings led us to investigate the sensitivity to apoptosis in cells lacking TAFAZZIN. We demonstrated that the lack of TAFAZZIN leads to an increased sensitivity to BCL2 inhibition and to ER stress triggered by the chemical thapsigargin. The variable presentation of neutropenia in patients with Barth syndrome is likely due to the pleiotropic effects of other modifying genes, possibly due to the variability of residual TAFAZZIN enzyme activity, and certainly influenced by the nuanced differences in how patients respond to periods of stress. It is not clear how environmental, nutritional, or infectious stresses, may be affecting the pool of circulating neutrophils in certain patients with Barth Syndrome and may be contributing to susceptibility to apoptosis and therefore to neutropenia.

The link between TAFAZZIN deficiency and increased sensitivity to the ER stress induced apoptosis is unknown. The communication between ER and mitochondria and its role in regulating critical biological functions and apoptosis has been described [47,48] and our electron micrographs clearly demonstrate physical contact between these organelles. The ER–mitochondrial contacts allow for the transport of metabolites, such as lipids, between the ER and mitochondria. In fact, cardiolipin is synthesized from phosphatidyl acid (PA) that is transported from the ER to the mitochondrial inner membrane [49]. It is possible that the depletion of CL or the accumulation of monolysoCL that results from TAFAZZIN deficiency may disrupt the normal lipid crosstalk between the ER and mitochondria, leading to an accumulation of undesired lipid species in the ER. Dysregulation of lipid metabolism is known to activate the ER stress and apoptotic signaling pathways [50], pointing towards a potential mechanism to explain why TAFAZZIN deficiency results in an increased sensitivity to apoptosis.

Patients with Barth syndrome and neutropenia are typically treated with granulocyte colony stimulating factor (G-CSF) to boost their circulating neutrophil count, with most patients responding to G-CSF therapy [39]. G-CSF is known to inhibit neutrophil apoptosis *in vitro* and *in vivo* [51,52]. Overall, the clinical response to G-CSF in patients with Barth syndrome, possibly counteracting a predisposition towards apoptosis, is thus consistent with our model data.

We hope that these studies, and the generation of ER-Hoxb8 myeloid progenitors, will set the foundation for further mechanistic studies and help point towards therapeutic strategies to address Barth syndrome neutropenia. While this is a very rare disease, these model systems will also be useful in addressing fundamental questions of how loss of TAFAZZIN and changes in mitochondrial-specific cardiolipin composition impact myeloid development, function, and survival, possibly linked to other sub-cellular systems including protein trafficking through the endoplasmic reticulum.

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Data Sharing Statement

Further information and requests for reagents may be directed to the corresponding author D.B.S. (dbsykes@mgh.harvard.edu).

Author Contributions

The authors confirm contribution to the paper as follows: study conception and design: DBS, JS; data collection: DBS, JS, JM, TB, JE, NA, SW, SFT, DRK; analysis and interpretation of results: DBS, JS, JM, TB, JE, NVG, MC, RS, SFT, DRK, SW, WP; writing original draft: DBS, JS, TB; manuscript review and editing DBS, JS, TB, NVG, JM, NA, WP. All authors reviewed the results and approved the final version of the manuscript.

Conflict of Interest Disclosures

JS is currently employed by Biogen and declares no competing interests with this work performed at the Massachusetts General Hospital. DBS is a co-founder and holds equity in Clear Creek Bio, is a consultant and holds equity in SAFI Biosolutions, and is a consultant for Keros Therapeutics. WTP and SW hold intellectual property in TAFAZZIN gene therapy. WTP is on the Barth Syndrome Foundation Scientific and Medical Advisory Board. Other authors have no relevant disclosures.

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Figure Legends

Figure 1. TAFAZZIN is dispensable for fetal liver myelopoiesis. (A) Flow cytometry gating strategy for fetal liver myeloid progenitor populations. Common myeloid progenitors (CMP), megakaryocyte-erythrocyte progenitors (MEP), and granulocyte-macrophage progenitors (GMP) were identified based on CKIT, SCA1, CD16/32 and CD34 staining. (B) Myeloid progenitors were enumerated in the WT or TAFAZZIN-KO fetal livers harvested at embryonic days 14, 14.5, 15, 15.5, and 16. The numbers of fetal livers included in the analysis is indicated. (C) A focus on the frequency of GMPs, combined from the other panels, within the fetal livers of WT or TAFAZZIN-KO embryos shows as significant difference only at E14. Error bars indicate the mean ± SD. T-tests were performed. *P< .05. Other results are not statistically significant.

Figure 2. TAFAZZIN deficiency leads to a mild peripheral leukopenia in adult mice. (A) Schematic outlining the approach of transplanting fetal liver cells from WT or TAFAZZIN-KO donors (CD45.2) into wild-type recipient mice (CD45.1). CD45.1 and CD45.2 staining of leukocytes in the peripheral blood quantifies the degree and contribution of engraftment. (B) The frequency of CD45.2 donor cells, both WT and TAFAZZIN-KO, in the peripheral blood remains stable following transplantation. (C) Complete blood counts of the peripheral blood demonstrate a mild leukopenia in mice transplanted with TAFAZZIN-KO fetal liver cells. (D) Flow cytometry was used to enumerate specific populations within the peripheral blood, with a focus on the transplanted CD45.2 cells. The lack of TAFAZZIN results in significantly fewer B-cells, T-cells, myeloid cells, and neutrophils. (E) Flow cytometric analysis of mature and progenitor populations within the bone marrow did not reveal any differences between mice reconstituted with WT or TAFAZZIN-KO fetal liver cells. Error bars indicate the mean ± SD. T-tests were performed. **P < .01; ***P < .001; ****P < .0001. *NS*=not statistically significant.

Figure 3. Conditionally immortalized GMPs generated from WT and TAFAZZIN-KO fetal liver hematopoietic progenitors. (A) Schematic outlining the generation of conditionally immortalized WT and TAFAZZIN-KO GMPs via the retroviral expression of the ER-Hoxb8 fusion protein, resulting in unlimited *ex vivo* expansion of progenitors in the presence of estrogen. (B) Immunoblotting confirms the absence of TAFAZZIN expression in the TAFAZZIN-KO GMPs using two different antibodies; β-actin and COX-IV are included as housekeeping controls. (C) Wright-Giemsa staining of WT and TAFAZZIN-KO mature neutrophils. (D) Cell surface staining of CD11b (Mac1) and GR1 (Ly6G/C) was assayed by flow cytometry in the GMPs (Day 0) and as the cells differentiated out of estrogen to mature neutrophils (Day 3 and 5). There was no apparent difference between WT and TAFAZZIN-KO

clones. (E) Transmission electron microscopy of WT and TAFAZZIN-KO GMPs demonstrated more distinct mitochondrial cristae in the WT cells (black arrows, upper), with spiral cristae structures more prevalent in the KO (white arrow, lower). In many cases, the mitochondria within the TAFAZZIN-KO cells was encircled by RER (black arrows, lower). Scale bar: 500 nm.

Figure 4. TAFAZZIN deficiency results in cardiolipin changes but does not affect neutrophil function. (A) Quantification of cardiolipin and monolysocardiolipin content in WT and TAFAZZIN-KO GMPs. Total CL and MLCL values are normalized to the average of WT cells. (B) The degree of unsaturation within the cardiolipin acyl chains was compared between WT and TAFAZZIN-KO cells. The average of the 4 clones is displayed, demonstrating the expected decrease in # of unsaturations in the TAFAZZIN-KO cells. (C) Imaging flow cytometry was used to confirm phagocytosis of FITC-labeled BioParticles within the WT or TAFAZZIN-KO mature neutrophils. The intracellular *S. aureus* or *E. coli* BioParticles (green) can be quantified separated from the extracellular particles simply adherent to the cell's surface. CD11b (blue) was used to highlight the membrane and DAPI (red) the nuclei of the neutrophils. (D) The degree of *in vitro* phagocytosis was quantified by flow cytometry across multiple WT and TAFAZZIN-KO clones. This assay was performed on three independent occasions. (E) *In vivo* analysis of phagocytosis was performed in a limited number of adult mice (CD45.1) that had been transplanted with WT or TAFAZZIN-KO fetal liver hematopoietic cells (CD45.2) as described in Figure 2A. Mice received an intraperitoneal injection of BioParticles and peritoneal fluid was collected at 3-hours for flow cytometric analysis. This experiment was performed twice and data from both experiments combined. (F) The amount of TNF-alpha, neutrophil gelatinase (NGAL) and neutrophil elastase (ELA2) secreted from LPS-stimulated (4-hours, 10 ng/ml) mature neutrophils was quantified by ELISA. This experiment was performed twice. Error bars indicate the mean ± SD. T-tests were performed. ***$P < .001$. *NS*=not statistically significant.

Figure 5. The loss of TAFAZZIN does not affect the production of neutrophil reactive oxygen species. (A) Schematic outlining commonly understood ROS pathways as well as reagents used to quantify and distinguish the different species within neutrophils. Microbial pathogens can be recognized and engulfed by neutrophils. In the process of phagocytosis, neutrophils activate membrane-associated NADPH oxidase to generate a powerful oxidative burse (measured by the Seahorse assay). During this oxidative burst, oxygen is consumed generating ROS including superoxide (O_2•−) (measured by the Cytochrome C assay) and hydrogen peroxide (H_2O_2) (measured by the H_2DCF assay). The mitochondria are also an important source of ROS, in particular, O_2•− (measured by the MitoSOX reagent). (B) The oxygen consumption rate (OCR) was measured within the XF Neutrophil Activation

Assay. Cells were stimulated with phorbol 12-myristate 13-acetate (PMA, 100 ng/ml) and OCR monitored over 240 minutes. This experiment was performed twice. (C) WT and TAFAZZIN-KO neutrophils were stimulated with PMA, fMLP, or RGD and the production of superoxide monitored over 60 minutes. The total superoxide (area under the curve) is displayed. This experiment was performed three times. (D) WT and TAFAZZIN-KO neutrophils were stimulated with PMA, fMLP, or RGD and the conversion of H_2DCF to its fluorescent product was quantified by flow cytometry and displayed as mean fluorescence intensity (MFI). Unstimulated cells are displayed as a negative control. This experiment was performed three times. (E) Mitochondrial ROS was quantified by MitoSOX staining and flow cytometry in both GMPs and mature neutrophils. Data is displayed the MFI normalized to the WT clones. This experiment was performed twice (GMP) and once (neutrophils). Error bars indicate the mean ± SD. T-tests were performed. *NS*=not statistically significant.

Figure 6. Transcriptomic analysis implicates genes associated with endoplasmic reticulum stress induced apoptosis. (A) Blue native polyacrylamide gel electrophoresis (BN-PAGE) of purified mitochondrial from three WT ER-Hoxb8 clones and three TAFAZZIN-KO clones. The gel is Coomassie stained. The five mitochondrial supercomplexes are highlighted on the right. (B) Following quantification of the mitochondrial supercomplexes, the relative contributions of each supercomplex within each clone depicted. (C) Heat map representation of gene expression from WT (n=6) and TAFAZZIN-KO (n=5) GMP clones. (D) TAFAZZIN is the most highly differentially expressed gene. (E) The count per million (CPM) are displayed for select genes involved in mediated ER stress and apoptosis. The false discovery rate (FDR) is displayed with each gene.

Figure 7. TAFAZZIN deficient myeloid progenitors are more sensitive to apoptosis and ER stress signals. (A) Viability was assessed *in vitro* (CellTiter Glo®) at 24-hours after exposure to ABT199, AMG176, or Thapsigargin. This assay was performed on >5 different days with multiple clones; the total N is displayed on each graph. The same data with error bars is displayed in Figure S5A. A two-way analysis of variance (ANOVA) was used to determine the statistical difference between viability curves. (B) *In vivo* CHOP protein expression following thapsigargin treatment was assayed by intracellular antibody staining on peripheral blood neutrophils from adult mice transplanted and reconstituted with WT or TAFAZZIN-KO fetal liver hematopoietic cells as described in Figure 2A. CHOP expression was quantified as mean fluorescence intensity (MFI) by flow cytometry. (C) The above experiment was repeated on additional mice to quantify CHOP as well as other intracellular proteins. The expression of CHOP and BiP in the peripheral blood circulating myeloid populations following treatment with thapsigargin demonstrated a trend towards increase in the TAFAZZIN-KO

animals. T-tests were performed, and *p* values are indicated on the plots. All other results are not statistically significant.

Figure S1. Validation of WT- and TAFAZZIN-KO genotypes by polymerase chain reaction (PCR) genotyping. (A) Two WT and two TAFAZZIN-KO clones are displayed to highlight the published genotyping strategy. A 2% agarose gel was used to resolve the PCR products. (B) An example of the expected results from the gender genotyping PCR are displayed to highlight how to distinguish female and male embryos.

Figure S2. Analysis of adult hematopoiesis in the setting of TAFAZZIN deficiency: Trial #2. (A) The frequency of CD45.2 donor cells, both WT and TAFAZZIN-KO, in the peripheral blood remains stable following transplantation out to 16-weeks. (B) Complete blood counts of the peripheral blood demonstrate a mild leukopenia in mice transplanted with TAFAZZIN-KO fetal liver cells. Hematocrit and platelet counts are not altered in the setting of TAFAZZIN deficiency. (C) Flow cytometry was used to enumerate specific populations within the peripheral blood, with a focus on the transplanted CD45.2 cells. The lack of TAFAZZIN results in significantly fewer B-cells, T-cells, myeloid cells, and neutrophils. (D) Flow cytometry gating strategy for identifying mature cells in the peripheral blood. (E) Flow cytometry gating statregy for identifying myeloid progenitors in the bone marrow. Error bars indicate the mean ± SD. T-tests were performed. ***P* < .01; ****P* < .001; *****P* < .0001. *NS*=not statistically significant.

Figure S3. The lack of TAFAZZIN does not affect morphology, mitochondrial content, or transwell migration. (A) Wright-Giemsa staining and 100x oil light micrographs of WT and TAFAZZIN-KO GMP progenitors [(+)E2] and mature neutrophils [(-)E2 4-days]. (B) Mitochondria were quantified by comparing copies of mitochondrial DNA to genomic DNA by quantitative RT-PCR. This experiment was performed three times and the data was combined and is displayed. (C) Analysis of mitochondrial mass using Mitotracker staining and quantification by flow cytometry was performed in both GMPs and mature neutrophils and normalized to the wild-type clones. (D) Neutrophil migration towards LPS (100 ng/ml) was assayed by transwell migration. (E) Wright-Giemsa staining and 100x oil light micrographs of WT and TAFAZZIN-KO primary adult bone marrow neutrophils. Error bars indicate the mean ± SD. T-tests were performed. *NS*=not statistically significant.

Figure S4. The lack of TAFAZZIN results in ultrastructural changes in mitochondria. (A) WT and TAFAZZIN-KO progenitors. Mitochondria cristae tended to be more distinct in the WT (black arrows, upper right), with spiral structures more prevalent in the KO (white arrow, lower right). Although not specific to the KO, mitochondria tended to be encircled by rough endoplasmic reticulum (RER, black arrows, lower right). The density of the mitochondria lumen was greater in the WT. Scale bars: left panels 2 um; right panels 500 nm. (B) WT and TAFAZZIN-KO mature neutrophils. The size of the mitochondria was approximately equal, with well-defined cristae more commonly resolved in the WT neutrophils (black arrows, upper right). Spiral structures with the lumen of the mitochondria were occasionally noted in WT but were considerably more prevalent in the KO neutrophils (black arrows, lower right). Autophagasomes were present in both groups but were considerably more prevalent in the KO (white arrows, lower right). Scale bars: left panels 2 um; right panels 500 nm. (C) TAFAZZIN-KO progenitors. Mitochondria were commonly encircled by RER (black arrows). Spiral structures within the lumen of the mitochondria were common (white arrows). Scale bar = 500 nm.

Figure S5. TAFAZZIN deficient myeloid progenitors are more sensitive to apoptosis and ER stress signals. (A) ER-Hoxb8 GMP were set up in 96-well plate format and viability was assessed *in vitro* (CellTiter Glo®) at 24-hours after exposure to ABT199, AMG176, or Thapsigargin. This assay was performed on >5 different days with multiple clones; the total N is displayed on each graph. This same data without error bars is displayed in Figure 7A. A two-way analysis of variance (ANOVA) was used to determine the statistical difference between viability curves. (B) Primary fetal liver hematopoietic cells from the embryos derived from three timed pregnancy litters were set up in 96-well plate format and viability was assessed *in vitro* (CellTiter Glo®) at 24-hours after exposure to ABT199. (C) Flow cytometry gating strategy for identification of the CD45.2 lymphoid and myeloid cells within the peripheral blood.