Meinders, M., Shoemark, D. K., Dobbe, J. G. G., Streekstra, G. J., Frayne, J., & Toye, A. M. (2020). Expression and retention of thymidine phosphorylase in cultured reticulocytes as a novel treatment for MNGIE. *Molecular Therapy - Methods and Clinical Development, 17*, 822-830. https://doi.org/10.1016/j.omtm.2020.03.029

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Expression and Retention of Thymidine Phosphorylase in Cultured Reticulocytes as a Novel Treatment for MNGIE

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Mitochondrial neurogastrointestinal encephalomyopathy (MNGIE) is a rare autosomal metabolic disorder caused by thymidine phosphorylase (TP) deficiency. Successful therapeutic interventions for this disease rely on a means for efficient and long-lasting circulation of the TP enzyme. In this study we exploit lentiviral transduction of hematopoietic stem cells and an erythroid cell line (BEL-A) to generate reticulocytes that contain active TP. Significant loss of overexpressed TP during erythroid differentiation can be reduced by addition of the ubiquitination inhibitor MG132. However, the ubiquitination sites are located in the substrate binding site in human TP, and their removal abolished enzyme activity. Examination of the TP structure and mechanism suggested that these sites are only exposed in the absence of substrate. We show that supplementation of culture media with thymidine during differentiation reduces enzyme degradation, doubling the amount of TP retained in reticulocytes. This study provides proof of principle that therapeutic reticulocytes expressing TP can be generated in vitro and that ubiquitin-mediated degradation can be subverted through masking ubiquitination sites to ensure retention of human TP in reticulocytes following erythroid differentiation.

INTRODUCTION
Mitochondrial neurogastrointestinal encephalomyopathy (MNGIE) is an autosomal recessive metabolic disorder that usually manifests during early teens, with an average life expectancy of 37.5 years. The disease is very rare, with an estimated occurrence in the population of 1:1,000,000 but this may be an underestimate due to the varied clinical presentation, which includes severe gastrointestinal dysmotility, cachexia, peripheral neuropathy, diffuse leukoencephalopathy, and mitochondrial abnormalities.

MNGIE is caused by mutations in the TYMP gene, which encodes the thymidine phosphorylase (TP) enzyme. TP catalyzes the phosphorylation of thymidine (dTsd) and deoxyuridine (dUrD) to thymine or uridine, and 2-deoxy ribose 1-phosphate (2DR1P) in the cytosol. Here it plays a key role in pyrimidine salvage, recovering nucleosides after DNA/RNA degradation. Homozygous or compound heterozygous mutations in the TYMP gene cause a drastic reduction in protein expression or activity, which results in thymidine accumulation, and subsequently leads to an imbalanced intramitochondrial deoxy-nucleotide pool. This is thought to destabilize mitochondrial DNA by affecting mitochondrial DNA repair and replication, resulting in the broad variety of symptoms.

Although significant progress in the understanding on the molecular basis of the MNGIE has been made, we still lack an effective treatment. Currently, treatment is largely based on patient symptom management, which include nutritional supplements, prevention of infections, and pain relief. Research has focused on developing treatments to remove metabolites using hemodialysis, hematopoietic stem cell transplantation (HSCT), and TP enzyme replacement therapy. Although hemodialysis is beneficial, the effect is transient, and dialysis is required every 3 h. HSCT can restore expression of TP and improve the biochemical parameters, but transplantation has inherent risks, and achieving a suitable donor match can be challenging. A different method of increasing TP activity is the use of enzyme replacement therapy in platelets and red blood cells. Platelets naturally express high levels of TP, and platelet transfusion corrected the nucleoside imbalance in blood plasma. However, the improvement was temporary and multiple platelet transfusions per week are necessary for long-term improvements.

The most promising approach for enzyme replacement is the use of erythrocyte encapsulated TP (EETP). Erythrocytes do not normally express TP, but hypotonic hemolysis and isotonic resealing can be used to encapsulate Escherichia coli TP in autologous red blood
This has been successfully used in the clinic, achieving prolonged cessation of the MNGIE clinical phenotype by reducing plasma nucleoside levels. Although this method is promising, the methodology of encapsulation within erythrocytes using hypertonic lysis can compromise the lifespan of the erythrocytes, and patients can develop antibodies against the bacterial enzyme. Recently, progress has been made in the ex vivo culture of reticulocytes from CD34+ stem cells in vitro, with more than 10 mL of leuko-filtered packed reticulocytes produced using good manufacturing practice (GMP). This, and the recent development of an adult phenotype erythroid cell line (BEL-A), led us to investigate the possibility of using genetically modified reticulocytes as vehicles for TP transport as a novel therapy for MNGIE.

RESULTS

Endogenous TP Expression in Erythroid Progenitors, Reticulocytes, and Erythrocytes

Before exploring the possibility of genetically modifying reticulocytes to express TP, we first confirmed the baseline endogenous expression and TP activity levels in isolated hematopoietic CD34+ stem cells, standard donor-derived reticulocytes, and erythrocytes. Expression was assessed by flow cytometry on fixed and permeabilized cells, and TP activity was determined on cell lysates using a spectrophotometer-based assay whereby the difference in absorbance levels of thymine, in the presence of TP, after 60 min at 37°C was measured. TP activity was measured in 1 x 10^6 in vitro-cultured red blood cells.

Endogenous TP expression and activity in erythroid cells differentiates in vitro from CD34+ hematopoietic stem cells and native peripheral blood reticulocytes and no TP expression or activity in red blood cells (Figures 1A–1C). Freshly isolated platelets from peripheral blood were included as a positive control (Figures 1B and 1C).
expression in an expanding erythroid cell line, BEL-A, which has the capacity to differentiate into reticulocytes, which are comparable to in vitro-cultured reticulocytes.17 The advantage of also using BEL-A cells is that these provide a sustainable source of cells that can be genetically modified and stored frozen with the modification maintained indefinitely, whereas CD34+-derived cultures are finite and need to be reinitiated each time. Expanding BEL-A cells (which are comparable to proerythroblast erythroblasts) have a low endogenous TP expression (Figures 1G and 1H), and the BEL-A-derived reticulocytes exhibit a very low TP expression (Figure 1H), comparable to CD34+-derived cultured reticulocytes (Figure 1E) and in vivo-isolated reticulocytes (Figure 1B).

**Exogenous Overexpression of TP in In Vitro CD34⁺ Reticulocytes and BEL-A-Derived Reticulocytes Using Lentivirus**

Cultured erythroid progenitors expressing TP (cTP) and expanding BEL-A cells expressing TP (bTP) were created by stably transducing the cells with lentivirus-expressing TP cDNA. Subclones were created from the polyclonal bTP population by blind single cell sorting using fluorescence-activated cell sorting (FACS). Day 7 cTP (proerythroblast) cells and expanding bTP (proerythroblast) cells exhibited a 25- and 45-fold increase, respectively, in TP enzyme expression by flow cytometry compared to endogenous expression of TP in untransduced (UT) proerythroblast cells (Figures 2A and 2B).

The cTP cells were differentiated and TP expression was measured at the indicated days by flow cytometry (Figure 2A) and by western blot expression in cTP and bTP reticulocytes is observed to be 6- and 12-fold increased relative to UT proerythroblast levels, a striking decline of expression is observed during terminal differentiation. The activity assay confirmed the presence of active enzyme in cTP proerythroblasts with an approximate concentration of 1.2 × 10⁻⁸ U/cell (Figure 2D). Subsequently, samples were taken during differentiation at the indicated time points, and activity was measured. We observed an approximately 3-fold reduction of activity in cTP-reticulocytes compared to cTP proerythroblasts, which have a final concentration of 4.4 × 10⁻⁹ U/cell. A 3-fold reduced expression was also observed using flow cytometry between cTP proerythroblasts and cTP reticulocytes.

To test whether thymidine and thymine are able to cross the intact reticulocyte cell membrane, and whether TP is active in the intracellular environment of reticulocytes, an activity assay was performed on whole cells. Thymidine was added to the media of 1 × 10⁶ cells at 37°C, and subsequently the concentration of thymine was measured in the supernatant after 1, 3, 6, and 24 h of incubation. Lysed cTP reticulocytes were used as a control, which were incubated with thymidine for 1 h (Figure 2E). As expected, the increase in thymine production is slower in whole-cell supernatants, compared to lysed cells, but after a 24-h incubation period, a comparable concentration of thymine was measured in the supernatant compared to lysed cells. This indicates that intact cells expressing TP can phosphorylate thymidine, and thymidine and thymine are able to cross the cell membrane.
The cTP cells are morphologically comparable to UT cells by cytospin during differentiation (Figure S2A), and we confirmed by confocal microscopy that TP is localized in the cytoplasm in reticulocytes (Figure S2B). Expression of essential membrane proteins was measured on unmodified control and cTP reticulocytes, and no differences were observed (Figure S2C). The deformability and size of the cTP-derived reticulocytes was measured using an automated rheoscope cell analyzer (ARCA). This showed that the deformability of cTP reticulocytes is comparable to that of the unmodified control reticulocytes and that they have a comparable size (Figure S2D).

TP Is Degraded by the Ubiquitin Degradation Pathway in Erythroblasts

The substantial loss of TP enzyme expression during differentiation suggested to us that TP is likely being actively degraded during terminal differentiation. Ubiquitination and lysosomal degradation are the main cellular degradation pathways during erythropoiesis. To test whether exogenous TP degradation during differentiation is due to ubiquitination or lysosomal degradation, we subjected the day 14 (orthochromatic erythrocytes) cTP cells to the proteasome inhibitor MG132, or a lysosomal degradation inhibitor, leupeptin, during culture. TP expression was first measured on day 14 and then 24 h after incubation with MG132, leupeptin, or the vehicle control. Figure 3A shows that application of MG132 inhibited degradation, whereas leupeptin and the vehicle control had no effect. This demonstrates that ubiquitination during erythroid differentiation is a significant cause of human TP protein degradation.

Human and mouse TP proteins are 81% identical, and sequence comparison confirmed that two known ubiquitination sites located at residues 115 and 221 in the mouse TP enzyme are conserved in the human TP structure. Examination of the structures suggested to us that both conserved lysines are an integral part of the thymidine binding site, and therefore the alteration of these important residues could abolish TP activity or stability because the active site is altered (see Figure 3B). The two lysine residues in human TP were exchanged for arginine residues to preserve the active site structure but remove the ubiquitination sites, making TP-mutant (mut). This enzyme was expressed in both day 3 in vitro cultured erythrocytes and expanding BEL-A cells (bTP-mut), and the cells subsequently differentiated. The TP-mut expression levels achieved were comparable to day 6 cTP and expanding bTP cells (see Figures 4A and 4B), but no TP activity was detected in the cTP-mut reticulocyte (Figure 4C). This supports our conjecture that the mutations...
compromise the enzyme activity and stability. Therefore, either the enzyme active site would need to be reengineered to retain activity but remove ubiquitin sites, or an alternate means of disrupting TP ubiquitination is required.

**TP Degradation Is Reduced by Thymidine Supplementation**

After further examination of the molecular human TP structure we observed that the two ubiquitination sites that correspond with murine TP would only be available for ubiquitination in the absence of substrate. We therefore hypothesized that supplementation of the culture media with TP enzyme substrate thymidine would result in a closed TP structure, reducing its degradation by obscuring access to the lysine’s ubiquitination sites in the active site. However, previous reports have shown that the addition of thymidine can arrest the cell cycle and also inhibit cell growth in K562 cells at a concentration of 1 mM. To determine whether increased thymidine concentrations in our culture media could prevent TP degradation, media were daily supplemented with 0.5 mM thymidine starting at different times during differentiation. We confirmed an increase in cell death and inhibition of differentiation when thymidine was added early during in vitro culture, e.g., on day 0 of differentiation (data not shown). To circumvent this, 0.5 mM thymidine supplementation was added daily during differentiation, from day 14 (polychromatic erythroblast stage) in cTP, which is approximately the point when overexpressed human TP is observed to be degraded. A growth curve confirmed that the addition of thymidine from day 14 did not affect cell growth (Figure 5A). This maneuver doubled TP enzyme abundance in cTP reticulocytes (Figure 5B) compared to cells produced using the standard differentiation media, which was not supplemented with thymidine. The enzyme activity assay for the thymidine supplemented cTP was not significantly different from the untreated cTP, and we presume that this is due to the cells already containing excess thymidine before lysis, which distorts the assay. As an additional control we also added a known TP inhibitor (TPI), and we show that this significantly reduces the TP activity (Figure 5C).

**DISCUSSION**

This study has demonstrated for the first time that exogenous human TP can be overexpressed using lentivirus transduction of the TP cDNA in CD34+ hematopoietic cells and in an erythroid line, BEL-A. These cells were subsequently expanded and differentiated to reticulocytes with confirmed retention of TP. We have also demonstrated that although it is possible to express TP in in vitro-cultured reticulocytes, the enzyme is degraded by ubiquitination during differentiation, limiting the abundance of TP per reticulocyte. Ubiquitination is a necessary process during differentiation to remove non-erythroid and non-essential proteins, and therefore this presents a significant barrier to the expression of exogenous proteins for therapeutic purposes during red blood cell development. To circumvent this problem, we propose that a first step for overexpressing proteins is the removal of known or putative ubiquitination sites that are identified in the sequence of exogenous proteins, to prevent loss during degradation. For enzymes where the ubiquitination site is an integral part of the substrate binding domain such as in the case of TP, an alternative strategy is needed, as an alteration of the ubiquitination motif within the active site can disrupt activity. Our novel observation shown here for TP is that inclusion of the TP enzyme substrate thymidine increases the abundance of the TP in reticulocytes, likely by masking the enzyme ubiquitin sites. Although thymidine is known to be toxic to cells, we show that this can be achieved by supplementing the culture medium with substrate (in this case thymidine) during the late stages of terminal differentiation. Furthermore, since patients have a concentration of >3 μmol/L of thymidine in their blood,
this should maintain TP in reticulocytes while they mature to red blood cells.

A clinical trial in a MNGIE patient conducted by Bax and colleagues\(^30\) successfully encapsulated a therapeutic dose of 51 U of \(E.~coli\)-produced TP in \(10^{10}\) erythrocytes, whereby 1 U of TP is defined as the activity required to convert 1.0 \(\mu\)mol of thymidine to thymine per minute. Our cTP-cultured erythroblasts had an average expression of TP corresponding to 84 U in \(10^{10}\) cells. The expression level decreases during differentiation and results in an activity corresponding to 44 U in \(10^{10}\) reticulocytes. We show that TP expression can be doubled in reticulocytes by daily addition of thymidine in the culture medium during the late stages of differentiation. Although the addition of thymidine did prevent degradation, we acknowledge that TP will still be partially lost in the cytosol surrounding the nucleus during enucleation, and some loss may also occur during the process of reticulocyte maturation when cells reduce in size.\(^31\)

The advantage of the use of \textit{in vitro}-cultured reticulocytes modified genetically to express and retain TP is that these cells overproduce the enzyme during normal differentiation and have not been subjected to a damaging hypotonic lysis, which is required for the encapsulation of TP in red blood cells. In addition, since the cultured reticulocytes are newly synthesized nascent red blood cells, they are expected to have a normal red blood cell lifespan of up to 120 days, potentially reducing the number of transfusions needed for patients compared to using donated blood derived red blood cells that is a mixture of ages.

The utility of \textit{in vivo} animal models to assess survival and efficacy of transfused human red blood cells is limited by the short time frame (48–72 h) in which these cells are able to circulate, even where immunocompromised (macrophage-depleted NSG mice) mouse models have been employed,\(^32\) and although a mouse model that recapitulates the MNGIE phenotype has been previously described,\(^33\) such a model does not exist on a NSG background. As such, there is currently no way to pre-assess the survival or efficiency of excess thymidine removal from sera by modified red blood cells in circulation. Nevertheless, data derived from a single injection using autologous \textit{in vitro}-cultured reticulocytes suggest that these cultured reticulocytes have a half-life of 28 ± 2 days, which was comparable to a normal red blood cell transfusion.\(^34\) Therefore, the use of modified \textit{in vitro}-cultured reticulocytes with TP could decrease the frequency of transfusions needed, which would reduce the risk of transfusion-related symptoms. Another advantage of using \textit{in vitro}-cultured TP reticulocytes is that we have used unmodified human TP, which is unlikely to induce an immune response.

In summary, this study has provided the first proof of principle demonstration of the enhancement of \textit{ex vivo}-produced reticulocytes, from either CD34\(^+\) cells or an erythroid line, by engineering the cells during red cell development to overexpress TP as a novel cellular therapy treatment for MNGIE. The CD34\(^+\)-derived engineered reticulocytes could be made autologous or allogeneic, and because these are red blood cells that have lost their nuclei, there is no anticipated regulatory barrier to using lentivirus on the cells. Given that GMP-compliant production of reticulocytes is now feasible for small volumes in a process amenable to modification by overexpressing enzymes, and a proof-of-principle autologous transfusion into a volunteer has been conducted and further allogenic trials are planned,\(^35,36\) the enhancement of reticulocyte function through lentivirus transduction of erythroid precursors may represent an early therapeutic use of \textit{in vitro}-cultured blood in the clinic. We acknowledge that although the culture efficiency of the BEL-A cells and scalability need to be improved and a suitable GMP-compatible erythroid line needs to be produced, the production of erythroid lines containing overexpressed therapeutic proteins represents a more convenient and sustainable way to make a future therapeutic reticulocyte product for patients.\(^37\) Furthermore, when combined with gene editing approaches using CRISPR-Cas9, these cell lines can be enhanced further to produce an engineered therapeutic product designed to be compatible to a broader range of patients.

**Figure 5. TP Expression Is Significantly Increased by Supplemeting the Culturing Media with Thymidine**

The expression TP in cTP and cells during differentiation was increased by supplementing the culture media with 0.5 mM thymidine every 24 h from day 14 (polychromatic stage) to cTP cells. (A) Growth curve shows the fold increase in cell number during differentiation with the absence or addition of 0.5 mM thymidine. (B) Expression of TP was measured by flow cytometry using 1 × 10\(^5\) fixed and permeabilized reticulocytes from cultures either untreated in standard media or treated with media supplemented by thymidine (\(N = 4 \pm \text{SEM})\). (C) Specific TP activity was confirmed by an activity assay where we used a thymidine phosphorylase inhibitor (TPI) as a negative control.
MATERIALS AND METHODS

Antibodies
Monoclonal TP antibody (clone P-GF.44C) was used at 1:10 dilution (Thermo Scientific, Gillingham, UK). For membrane protein analysis, 1:1 (v/v) diluted GPC (BRIC4), CD47 (BRIC32), Rh (BRIC69), Band3 (BRIC71), GPA (BRIC256), and RhAG (LA1818) (IBGRL Reagents, Bristol, UK) were used. Secondary antibodies used were allophycocyanin (APC)-conjugated monoclonal anti-mouse immunoglobulin G (IgG)1 or polyclonal anti-IgG (BioLegend, London, UK) or Alexa Fluor 647-anti-human (Jackson ImmunoResearch Laboratories, Cambridgeshire, UK) and used at 1:50 (v/v). For confocal imaging, a polyclonal GPC C-terminal antibody at 1:50 was used (in-house). Secondary antibodies used were goat anti-rabbit-Alexa Fluor 594 (Invitrogen, Massachusetts, USA) and goat anti-mouse-Alexa Fluor 488 (Invitrogen).

BEL-A Cell Culture
BEL-A cells were cultured as previously described.17 In brief, cells were maintained in expansion medium (StemSpan serum-free expansion medium [SFEM; STEMCELL Technologies, Cambridge, UK]) supplemented with 50 ng/mL stem cell factor [SCF; Miltenyi Biotec, Bisley, UK], 3 U/mL erythropoietin [EPO; Roche, Welwyn Garden City, UK], 1 μM dexamethasone [Sigma-Aldrich, Poole, UK], and 1 μg/mL doxycycline [Sigma-Aldrich]) at 1–3 × 10^5 cells/mL. Complete medium changes were performed every 48 h. Differentiation was induced as previously described: cells were seeded at 1.5 × 10^5/mL in differentiation medium (Isocove’s modified Dulbecco’s medium [IMDM], Source BioScience, Nottingham, UK) containing 3% (v/v) AB serum (Sigma-Aldrich), 2 mg/mL human serum albumin (HSA) (Irvine Scientific, Newtown Mount Kennedy, Ireland), 10 μg/mL insulin (Sigma-Aldrich), 3 U/mL heparin (Sigma-Aldrich), 500 μg/mL transferrin (Sanquin Blood Supply, the Netherlands), and 3 U/mL EPO (Roche, Welwyn Garden City, UK) supplemented with 1 ng/mL interleukin (IL)-3 (R&D Systems, Abingdon, UK), 10 ng/mL SCF, and 1 μg/mL doxycycline. After 2 days, cells were resuspended at 3 × 10^5/mL in fresh medium. On differentiation day 4, cells were resuspended at 5 × 10^5/mL in fresh medium without doxycycline. On differentiation day 6, a complete media change was performed, and cells were resuspended at 1 × 10^6/mL. On day 8, cells were transferred to differentiation medium (containing no SCF, IL-3, or doxycycline) and maintained at 1 × 10^6/mL with complete medium changes every 2 days until day 12.17,36

CD34+ Cell Culture
The donated blood from apheresis waste was provided with written informed consent for research use given in accordance with the Declaration of Helsinki from National Health Service Blood and Transplant, Filton, Bristol, United Kingdom). The studies on erythropoiesis and research protocols were reviewed and approved by the Bristol Research Ethics committee (REC Number 12/SW/0199). As previously described,17 CD34+ hematopoietic stem cells were isolated from human blood donor mononuclear cells or from thawed cryopreserved cord blood units by magnetic bead separation according to the manufacturer’s instructions (Miltenyi Biotec). CD34+ cells were grown at a density of 2 × 10^5 cells/mL using a base medium consisting of IMDM (Source BioScience) containing 3% (v/v) AB serum, 2 mg/mL HSA, 10 μg/mL insulin, 3 U/mL heparin, 500 μg/mL transferrin, and 3 U/mL erythropoietin. In the first stage (days 0–10) this was supplemented with 10 ng/mL SCF and 1 ng/mL IL-3, and in the second stage (days 11–13) with 10 ng/mL SCF. In the final stage to day 19, only the base medium was used. 10 μM leupeptin (Sigma-Aldrich) or 5 μM MG132 (Sigma-Aldrich) or vehicle was added at day 14 to 1 × 10^6 differentiating erythroblasts for 24 h at 37°C, 5% CO2.

Lentiviral Transduction
A human TP cDNA sequence was ordered and cloned in the XLG3 vector by GenScript (Leiden, the Netherlands). The original TP sequence was mutated at sites 115 and 221 from a lysine to arginine, thereby creating the TP-mut lentivirus as prepared according to previously published protocols.37 For transduction of BEL-A and CD34+ hematopoietic cells, virus was added to 2 × 10^5 cells in 2 mL of medium in the presence of 8 μg/mL Polybrene for 24 h. Cells were washed three times and resuspended in fresh medium.

Flow Cytometry and FACS
For flow cytometry on undifferentiated BEL-A cells, 1 × 10^5 cells were fixed in 1% paraformaldehyde and 0.0075% glutaraldehyde, permeabilized with 0.1% Triton X-100, resuspended in PBSAG (PBS + 1 mg/mL bovine serum albumin [BSA], 2 mg/mL glucose) + 1% BSA, and labeled with primary antibody for 30 min at 4°C. Cells were washed in PBSAG, incubated for 30 min at 4°C with appropriate APC-conjugated secondary antibody, and washed, and data were acquired on a MACSQuant VYB analyzer using a plate reader. Reticulocytes were identified by gating on a Hoechst-negative population. For FACS sorting of cells, a BD Influx cell sorter was used to isolate single clones by sorting the propidium iodide-negative population into 96-well plates.

TP Activity Assay
1 × 10^6 cells were resuspended in lysis buffer (50 mM Tris-HCl [pH 7.2], 1% [w/v] Triton X-100, 2 mM phenylmethylsulfonyl fluoride [PMSF], 0.02% [v/v] 2-mercaptoethanol). The lysate was centrifuged at 16,000 × g for 30 min at 4°C and then 176 mM thymidine and 5 × TP reaction buffer (0.5 M Tris-arsenate [pH 6.5]) was added to the supernatant. The controls used were lysis buffer alone or lysis buffer containing known concentrations of purified TP protein (Sigma-Aldrich). The reaction was incubated at 37°C for 60 min and then terminated by addition of 0.3 M NaOH. The absorbance was measured using a spectrophotometer at 299 nm and compared to the TP enzyme standard concentration curve.38

Cytospin Staining
1 × 10^6 cells were cytopspun onto glass slides, fixed in methanol, and stained with May-Grünwald-Giemsa stains according to the manufacturer’s protocol. Images were taken with a Leica DM750
microscope coupled to an Olympus U-TVO.5XC-3 camera using a ×40 lens and processed using Adobe Photoshop 9.0 (Adobe Systems).

Immunofluorescence
5 × 10^6 cells were fixed in suspension in 1% paraformaldehyde and 0.0075% glutaraldehyde and washed three times in PBSB Glycine before being cytospun onto coated coverslips. Cells were then permeabilized with 0.05% Triton X-100 for 5 min at room temperature and then blocked in PBS-4% BSA for 45 min, incubated with primary antibodies in PBS-4% BSA for 1 h, washed with PBS, and incubated for 1 h with the appropriate secondary antibodies. Coverslips were washed and mounted on microscope slides using Mowiol (Calbiochem, San Diego, USA) containing 2.5% (w/v) DABCO antifade reagent (Sigma-Aldrich). Confocal images were taken using a Leica SPE single-channel confocal laser scanning microscope attached to a Leica DMi8 inverted epifluorescence microscope.

SDS-PAGE and Western Blotting
1 × 10^6 cells were taken at designated time points during differentiation and pelleted, snap-frozen, and stored at −80°C. Pellets were lysed for 10 min on ice in lysis buffer (20 mM Tris-HCl [pH 8.0], 137 mM NaCl, 10 mM EDTA, 100 mM NaF, 1% [v/v] Nonidet P-40, 10% [v/v] glycerol, 10 mM Na_2VO_4, 2 mM PMSF, and protease inhibitors [Calbiochem]). Equal numbers of lysed cells were loaded and separated by SDS-PAGE and then immunoblotted.

Reticulocyte Deformability Measurements Using ARCA
1 × 10^6 reticulocytes were resuspended in 200 μL of polyvinylpyrrolidone (PVP) solution (viscosity, 28.1; Mechatronics Instruments, the Netherlands). Samples were assayed in an ARCA (automated rheo-cell analyser) assay, which consists of a plate-to-plate optical shearing stage (model CSS450) mounted on a Linkam imaging microscope and cell analyser,39 which consists of a plate-to-plate optical shearing stage (model CSS450) mounted on a Linkam imaging microscope and cell analyser, which allowed the analysis and pelleting, snap-frozen, and stored at −80°C. Samples were assayed in an ARCA (automated rheo-cell analyser) assay, which consists of a plate-to-plate optical shearing stage (model CSS450) mounted on a Linkam imaging microscope and cell analyser, which provided details of the mouse ubiquitination sites (https://omictools.com/C2106) was used to align human and mouse sequences. Protein structures were visualized, and images were produced with UCSF Chimera software (https://www.cgl.ucsf.edu/chimera/).

SUPPLEMENTAL INFORMATION
Supplemental Information can be found online at https://doi.org/10.1016/j.omtm.2020.03.029.

AUTHOR CONTRIBUTIONS
Experiments were conceived and designed by M.M. and A.M.T. M.M. and D.S. carried out the experiments, performed the analysis and prepared the figures. J.F. provided the BEL-A cell line. J.G.G.D., G.J.S. and J.F. edited the manuscript. A.M.T. supervised the project and edited the manuscript. All authors read and approved the manuscript.

ACKNOWLEDGMENTS
We thank Dr. Tim Satchwell for critical reading of the manuscript. We also thank Sanquin Blood Supply (the Netherlands) for the supply of human transferrin. This work was funded by BrisSynBio via the BBSRC/EPSRC Synthetic Biology Research Centre (BB/L01386X/1) and also the Defence Science and Technology Laboratory (DSTL), along with funding from NHS Blood and Transplant (NHSBT) R&D (WP15-05) and NIHR Blood and Transplant Research Unit (NIHR BTRU) in Red Cell Products (IS-BTU-1214-10032). This manuscript presents independent research funded in part by the National Institute for Health Research (NIHR). The views expressed are those of the authors and not necessarily those of the NHS, the NIHR, or the Department of Health and Social Care.

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