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The cytoskeletal binding domain of band 3 is required for multiprotein complex formation and retention during erythropoiesis

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Abstract

Band 3 is the most abundant protein in the erythrocyte membrane and forms the core of a major multiprotein complex. The absence of band 3 in human erythrocytes has only been reported once, in the homozygous band 3 Coimbra patient. We use *in vitro* culture of erythroblasts derived from this patient, and separately shRNA mediated depletion of band 3, to investigate the development of a band 3 deficient erythrocyte membrane and to specifically assess the stability and retention of band 3 dependent proteins in the absence of this core protein during terminal erythroid differentiation. Further, using lentiviral transduction of N-terminally GFP tagged band 3, we demonstrate the ability to restore expression of band 3 to normal levels and to rescue key secondary protein deficiencies including GPA, protein 4.2, CD47 and Rh proteins arising from the absence of band 3 in this patient. By transducing band 3 deficient erythroblasts from this patient with band 3 mutants with absent or impaired ability to associate with the cytoskeleton we also demonstrate the importance of cytoskeletal connectivity for retention both of band 3 and of its associated dependent proteins within the reticulocyte membrane during the process of erythroblast enucleation.
Introduction

The bicarbonate/chloride exchanger band 3 is the most abundant membrane protein in the erythrocyte membrane where, alongside its transport function it performs a critical role in maintaining red cell structural integrity. Band 3 is an important component of at least two major multiprotein complexes, the ankyrin and junctional membrane complexes that serve as sites of vertical association between the plasma membrane and the underlying spectrin based cytoskeleton(1, 2). The N-terminal domain (1-359 amino acids) is responsible for the association with the cytoskeleton, possessing binding sites for ankyrin(3), protein 4.2(4), adducin(2) and protein 4.1(5) in addition to glycolytic enzymes(6, 7) and haemoglobin(8). The remaining C-terminal membrane domain (B3mem; 360-911 amino acids) possesses 12-14 transmembrane helices and performs a key role in regulating erythrocyte gas exchange by exchanging chloride and bicarbonate ions across the plasma membrane. The B3mem domain alone traffics efficiently to the plasma membrane when expressed in multiple cell types(9-12) but is not expressed endogenously in this form in vivo. Band 3 is also expressed as an N-terminally truncated isoform (kB3) in the alpha intercalated cells of the kidney where it is required for acid secretion(13, 14). The 65 amino acid kB3 N-terminal truncation removes the ankyrin binding site(15) but retains the ability to bind protein 4.2(11).

Specific mutations in the band 3 gene result in the erythrocyte diseases hereditary spherocytosis(16) or hereditary stomatocytosis and/or the kidney disease distal renal tubular acidosis(14, 17, 18). Hereditary spherocytosis (HS) is a form of haemolytic anaemia characterized by the presence of spherocytic erythrocytes with reduced size and increased fragility. Mutations in band 3 account for 10-20% of HS cases, are heterogeneously distributed throughout the protein structure and usually result in a decreased expression of band 3(19). The extent of this reduction and the mechanism by which it occurs varies according to the mutation but is believed to include mRNA and protein instability, or intracellular retention(20, 21).

Although band 3 null models have been artificially generated in both cattle(22) and mice(23), the naturally occurring complete absence of band 3 in humans has only been reported in one individual with the homozygous band 3 Coimbra mutation(24). Band 3 Coimbra is a V488M mutation in the fourth transmembrane helix of band 3 that in the heterozygous state results in typical mild HS and a partial reduction in band 3 expression (20%/2%). The homozygous patient presents with severe HS and dRTA, with almost complete absence of band 3 protein in erythrocytes(1). Absence of band 3 causes secondary erythrocyte membrane protein deficiencies, including the loss of protein 4.2 and severe reduction of glycophorin A and the Rh sub-complex proteins, that lead to the proposal that band 3 forms the core of a large macrocomplex(1). Whilst some progress has been made in our understanding of the assembly of erythrocyte membrane subcomplexes (band 3 and protein 4.2; Rh and RhAG) during erythropoiesis(25, 26), many details relating to the timing, hierarchy of protein-protein interactions and the role of cytoskeletal attachment and remodelling during membrane biogenesis remain to be determined. The in vitro culture of band 3 Coimbra patient cells presents a unique opportunity to study membrane protein complex assembly during erythroid membrane biogenesis in the absence of band 3, uncover the basis of secondary protein loss in this most definitive of HS cases and hence probe its role as a hub for establishing and maintaining dependent proteins’ interactions.
Methods

Antibodies

Antibodies are listed in the Supplemental Methods.

Flow Cytometry and FACS

Flow cytometry was performed using 1x10^5 cells stained for 30 minutes at 37°C with 5µg/ml Hoechst33342 (Sigma). Cells were labelled with extracellular primary antibodies for 30 minutes at 4°C and labelled with rat APC-conjugated anti-mouse IgG1 (Biolegend). Data was collected using a MacsQuant VYB cytometer and processed using FlowJo Version 7.2.5. For FACS, cultures were stained with Hoechst33342 (5µg/ml) at 37°C for 30 minutes then sorted using a BD Influx Cell Sorter; reticulocytes (Hoechst negative) and extruded nuclei (Hoechst positive, FSC low). GFPband 3 positive reticulocytes were obtained by sorting GFP-positive Hoechst-negative populations.

Erythroblast Culture

Peripheral blood mononuclear cells were isolated from platelet apheresis blood waste (NHSBT, Bristol) from healthy donors and homozygous V488M patient with written informed consent for research use in accordance with the Declaration of Helsinki and approved by Local Research Ethics Committee (REC 12/SW/0199). The culture systems used have been reported previously(27, 28) and are outlined in Supplemental Methods.

Lentiviral Constructs

XLG3-GFP vector was modified to include SalI and MluI enzyme sites downstream of GFP. The cDNAs for band 3, band 3 membrane domain and kidney band 3 were amplified by PCR from respective BSXG1 plasmids(12, 14) using primers incorporating SalI and MluI and ligated into the modified XLG3-GFP vector. pLKO.1 shRNA plasmids were designed by the Broad Institute and purchased from Open Biosystems.

Lentiviral transduction of erythroblasts

For XLG3-GFPB3, XLG3-GFPB3mem or XLG3-GFPkB3 rescue experiments, concentrated virus was added to erythroblasts on Day 9 with 8µg/ml polybrene (Sigma). After 24 hours, cells were washed with PBS and resuspended in expansion medium (Supplemental Methods). For knockdown experiments CD34^+ cell derived erythroblasts were transduced on Day 3 with concentrated virus in the presence of 8µg/ml polybrene. After 24 hours, cells were washed with PBS and resuspended in expansion medium containing puromycin (1µg/ml) for at least 72 hours but removed during differentiation.
Results

Band 3 Coimbra is expressed at low levels during erythropoiesis but is not present at the plasma membrane

Although band 3 is absent from the membranes of band 3 Coimbra erythrocytes, it is unknown whether this mutant protein is actually expressed in erythroblasts during terminal differentiation. To answer this, erythroblasts derived from peripheral blood mononuclear cells of the band 3 Coimbra patient were expanded and differentiated in parallel to those derived from healthy donors. Figure 1 shows that only low levels of band 3 V488M mutant is synthesized during erythropoiesis as judged by immunoblotting (<1%), this protein exhibits a barely detectable diffuse intracellular localization. Surface expression of band 3, as assessed by flow cytometry was less than 1% at all stages of erythropoiesis (Figure 2A). Expression levels of the band 3 binding protein 4.2, mimic the relative expression of band 3 mutant from the onset of its expression, consistent with the known early dependence of protein 4.2 on band 3 (25, 29).

Secondary protein loss due to the absence of band 3 occurs early during erythropoiesis

To determine the stage of secondary protein loss in the absence of band 3, surface expression of major erythrocyte membrane proteins was monitored throughout terminal differentiation in both band 3 Coimbra patient and healthy donor erythroblast cultures (Figure 2A). The expression of the band 3 chaperone protein glycophorin A, which in healthy donors closely mimics that of band 3, is reduced throughout terminal differentiation of erythroblasts derived from the band 3 Coimbra patient with a reticulocyte expression of 38.6% compared to control. The Wrb antigen, a dual epitope formed at the interface of band 3 and glycophorin A was largely absent, at 3.1% in reticulocytes (Figure 2B).

The Rh proteins and RhAG exhibit an increase in their surface expression within the first 48 hours of differentiation, albeit at a reduced total surface level compared to controls. However, no further increase in Rh protein expression occurs in band 3 Coimbra patient erythroblasts after 48hrs. This results in an accumulating relative deficiency as terminal differentiation progresses, culminating in a relative expression level of 39% for Rh in reticulocytes (compared to 33% in erythrocytes (1)). Unexpectedly RhAG expression, which is reduced to 15% in band 3 Coimbra erythrocytes, was not dramatically reduced in its surface expression relative to control during erythropoiesis, and was expressed at an average of 65% of healthy donor levels in reticulocytes. CD47, another component of the Rh sub-complex that is expressed prior to terminal differentiation displays a progressive reduction in surface expression relative to control, manifesting approximately 48 hours into terminal differentiation. Surface levels on reticulocytes were 34% of wild type levels (compared to 15% in erythrocytes(1)). CD44 expression levels in patient and healthy donor erythroblasts are closely matched prior to enucleation, but increases to 134% in patient reticulocytes relative to controls, consistent with erythrocyte levels(1). Glycophorin C, which is not dependent upon band 3 for its expression or stability is unaltered in its expression relative to control throughout erythropoiesis.

shRNA mediated depletion of band 3 recapitulates the patient phenotypes

To confirm that the secondary deficiencies observed in band 3 Coimbra erythroblasts and reticulocytes are due specifically to the deficiency in band 3, shRNAs were used to deplete expression of band 3 in erythroblasts. Figure 3A-B shows western blots and flow cytometry data illustrating band 3 expression levels for reticulocytes differentiated from erythroblasts expressing two independent band 3 shRNAs (95% and 75% band 3 knockdowns respectively) compared with a non-targeting shRNA control (scramble). Expression of band 3 dependent erythrocyte proteins including
glycophorin A and Rh complex components was heavily reduced, recapitulating the alterations observed in band 3 Coimbra erythroblasts throughout differentiation (Figure 3C-D).

**Membrane protein sorting during enucleation is disrupted in the absence of band 3**

Since the absence of band 3 impacts on multiple membrane proteins including GPA which has previously been used successfully to obtain separate reticulocyte and extruded nuclei populations (28), cells were instead stained with Hoechst and then separated based on FSC gates as indicated in Figure 4A. Reticulocyte and extruded nuclei (pyrenocyte) populations obtained using this strategy were sorted by FACS for verification of population purity and found to be comparable to populations sorted using GPA/Hoechst or GPC/Hoechst in healthy donors.

By double labelling cell populations with Hoechst together with antibodies recognising extracellular epitopes of proteins of interest, ratios were derived for the partitioning of specific proteins between the reticulocyte and pyrenocyte plasma membrane during enucleation. Figures 4B-C illustrate that in addition to the decrease in surface expression of proteins including GPA, CD47 and Rh and RhAG prior to enucleation of band 3 Coimbra erythroblasts, the residual pool of these proteins also displays a decrease in retention within the reticulocyte plasma membrane during erythroblast enucleation. Thus mis-sorting during enucleation due to the absence of band 3 contributes to the overall reduction in expression relative to control, however the proportion of protein loss during enucleation for the majority of dependent proteins is minor compared to the loss due to altered plasma membrane stability prior to enucleation. No effect on partitioning was observed for GPC, which resides in the junctional complex and is not known to be dependent on band 3. CD44 exhibited increased retention within the reticulocyte membrane in the absence of band 3, demonstrating that the increased relative expression observed in band 3 deficient erythrocytes and reticulocytes occurs during enucleation.

**Exogenous expression of GFP-tagged wild type band 3 in homozygous band 3 Coimbra erythroblasts can completely restore surface expression of key erythrocyte membrane proteins**

To determine whether secondary protein deficiencies resulting from the absence of plasma membrane band 3 during erythropoiesis could be rescued, band 3 Coimbra patient erythroblasts were lentivirally transduced with a plasmid expressing GFP-tagged band 3 (GFPB3). An N-terminal GFP tag did not affect delivery to the plasma membrane in K562 cells (30). When expressed in normal erythroblasts we observed the expected plasma membrane localization and accumulation (data not shown). Supplementary Figure 2A shows flow cytometry data demonstrating surface expression of GFPB3 in band 3 Coimbra reticulocytes. Transduction efficiency achieved using the lentiviral GFPB3 vector was up to 97%. The rescue of band 3 surface expression in the patient reticulocytes varied between transductions and within the GFP positive population displaying an average of 54% (BRIC71) or 41% (BRIC200) of healthy donor reticulocyte band 3 levels (Supplemental Figure 2A). The patient reticulocytes expressing the highest levels of the GFPB3 vector exhibited the same level of band 3 as healthy donor reticulocytes (Supplementary Figure 2A-right panel), illustrating for the first time the complete rescue of band 3 expression within a population of band 3 null patient cells. Importantly, introduction of GFPB3 into patient erythroblasts rescued the expression of GPA, CD47, Rh and RhAG in reticulocytes. The degree of rescue directly correlated with the expression level of the GFPB3 in these cells (Supplemental 2B and Figure 5B).

**V488M band 3 surface expression is not rescued by association with wild type band 3**

To determine whether the introduction of GFPB3 rescued surface expression of the band 3 Coimbra protein (V488M band 3), surface immunoprecipitations using the extracellular monoclonal band 3
antibody BRIC6 were performed on sorted GFP positive populations of transduced band 3 Coimbra orthochromatic erythroblasts. Supplementary Figure 2C shows that BRIC6 immunoprecipitates the ~135kDa GFPB3 from the surface of these cells but fails to precipitate any 95kDa V488M mutant band 3 protein, indicating that the V488M mutant cannot be rescued by heterodimerisation with wild type protein. GFP-tagged V488M band 3 could not be immunoprecipitated by BRIC6 when transduced into healthy donor erythroblasts (data not shown). These data indicate that expression of the band 3 in heterozygous V488M is accounted for by expression of protein from the wild type allele alone and not by wild type rescue of the mutant protein.

The N-terminal cytoplasmic domain is required for multiprotein complex stabilisation

Band 3 forms vertical linkages between the plasma membrane and the cytoskeleton through protein associations mediated via its cytoplasmic N-terminus. However, the membrane domain of band 3 also represents a significant site of interaction in itself that could alone potentially rescue secondary protein deficiencies upon reintroduction to band 3 null erythroblasts. To explore the hypothesis that band 3 association with the cytoskeleton is required for the stability of band 3 interacting/dependent proteins during erythropoiesis and enucleation, band 3 Coimbra patient erythroblasts were transduced with lentivirus expressing GFPB3, band 3 membrane domain (GFPB3mem; lacking the N-terminus) or GFP-tagged kidney band 3 (GFPkB3), which binds protein 4.2 but not ankyrin. Figure 5A shows that expression of key proteins reduced in band 3 Coimbra reticulocytes that are normally associated with band 3 in the ankyrin/band 3 tetramer based multiprotein complex are significantly rescued by expression of GFPB3. In contrast, reticulocytes expressing GFPB3mem, with the exception of a small increase in GPA, exhibited no increase in surface expression of these proteins compared to untransduced patient reticulocytes. This is true even when samples are matched for GFP intensity to account for the differential expression and retention of band 3 post-enucleation. The expression level of GFPB3 within these cells correlated with the degree of rescue of secondary protein deficiencies as illustrated in Figure 5B which demonstrates a positive correlation between GFP intensity and surface expression of GPA and Rh in particular. Expression of GFPkB3 resulted in a partial rescue of CD47 (Figure 5C), which correlates with the partial rescue of protein 4.2 observed using GFPkB3 compared to the high level of rescue achieved by GFPB3 and the complete absence of protein 4.2 expression in band 3 Coimbra reticulocytes expressing GFPB3mem (Figure 5D). Rh protein expression is also rescued by expression of GFPB3 in band 3 Coimbra patient reticulocytes, but not by the introduction of either GFPB3mem or GFPkB3. Interestingly, rescued expression of RhAG was only observed in patient reticulocytes expressing the highest levels of full length GFPB3 (Figure 5B) and was not rescued by expression of either GFPB3mem or GFPkB3.

To explore in detail the expression of GFPB3 and the N-terminally truncated mutant proteins, surface protein expression profiles were acquired throughout differentiation for patient erythroblasts transduced with GFPB3, GFPB3mem and GFPkB3 alongside untransduced patient and healthy donor erythroblasts. Figure 6A shows that expression of GFPB3 results in a partial rescue of band 3 associated proteins including CD47, Rh and RhAG that is not observed by expression of GFPB3mem. Rescue of CD47 expression by GFPB3 is evident from an early stage of differentiation, consistent with the previously reported establishment of dependence of this protein on protein 4.2 in basophilic erythroblasts(25) but for other proteins is most evident following the transition from orthochromatic erythroblasts to reticulocytes coincident with the point of enucleation. Interestingly, this figure also demonstrates the observed consistent higher expression of GFPB3mem compared to GFPB3. Despite this high expression, which peaks at the late polychromatic/early orthochromatic stages, no rescue of secondary protein deficiencies occurring in the band 3 Coimbra patient was observed using GFPB3mem. In fact, the majority of these proteins exhibited reduced expression relative to
untransduced patient erythroblasts. This was accompanied by a higher level of cell death (unpublished observation), which may indicate that GFPB3mem expression at such a high level is toxic to erythroblasts. Importantly, transduction of band 3 Coimbra erythroblasts with the cytoskeleton binding compromised GFPkB3 (which expresses at a lower level) also fails to rescue expression of the majority band 3 dependent proteins without reduction in protein expression relative to untransduced patient erythroblasts illustrating that it is inability to bind the cytoskeleton that accounts for the absence of rescue of band 3 associated protein expression by GFPB3mem. These data are confirmed by a separate experiment in which GFPB3mem is expressed at a lower level and no gross reduction in surface protein expression levels relative to untransduced patient erythroblasts is observed (Supplemental Figure 2).

**Cytoskeletal binding via the N-terminus is important but not absolutely required for reticulocyte membrane retention of band 3**

Cytoskeletal attachment has long been proposed as the predominant factor governing retention of intrinsic membrane proteins during enucleation however empirical evidence in support of this presumption, particularly in humans is lacking. By expressing a truncated band 3 without capacity to bind the cytoskeleton in the absence of full length wild type protein, transduced band 3 Coimbra erythroblasts provide a model that enables the assessment not only of the importance of this attachment for retention of band 3 itself but also the retention of proteins for which band 3 provides an indirect cytoskeletal linkage.

Figure 6B shows enucleation protein partitioning ratios for GFPB3, GFPB3mem and GFPkB3 in band 3 Coimbra cells. Importantly, GFPB3 partitions with a similar ratio (5% loss with the nucleus) to endogenous band 3 in healthy donors (5-8%). In contrast, 38% of the non-cytoskeletal binding GFPB3mem domain and a lower 27% of GFPkB3 is lost with the plasma membrane surrounding the nucleus during enucleation (Figure 6C). Gross surface expression of band 3 within the extruded nuclei population are shown in Figure 6D, which illustrates that whilst average band 3 surface expression across the population of nuclei extruded from erythroblasts is lower for patient cells rescued with GFPB3 than for the truncated mutants, a proportion of these nuclei contain much higher levels of band 3. We hypothesize that these nuclei derive from erythroblasts with the highest GFPB3 expression. On this basis alongside its increased mobility, it is likely that the level of protein expressed in erythroblasts prior to enucleation may contribute partly to the increased partitioning of GFPB3mem to the nucleus compared to GFPkB3, which is similarly compromised in its capacity to bind ankyrin but expresses at a lower level in orthochromatic erythroblasts. Flow cytometry performed on cytoskeleton shells of Triton X-100 extracted band 3 Coimbra erythroblasts showed complete absence of GFP signal for shells derived from cells expressing GFPB3mem demonstrating its complete extraction in contrast to those from erythroblasts expressing GFPB3 and validating the absence of cytoskeletal association of GFPB3mem prior to enucleation (data not shown).

Enucleation protein partitioning ratios obtained for band 3 dependent proteins reinforce the relevance of the capacity of band 3 to associate with the cytoskeleton, with the retention of band 3 based multiprotein complex proteins GPA, Rh, CD47 in the reticulocyte membrane rescued to wild type levels by reintroduction of full length GFPB3 but not GFPB3mem. The increased loss of CD47 during enucleation in the absence of band 3 was partially rescued by GFPkB3, which also rescued protein 4.2 expression, but not by GFPB3mem which does not bind protein 4.2. Neither Rh nor RhAG were rescued by kB3 during enucleation, reinforcing the particular dependence of CD47 on the presence of protein 4.2 as observed previously(29, 31, 32).
Discussion

This work has demonstrated by the study of in vitro culture of erythroblasts derived from the unique homozygous V488M ‘band 3 null’ patient and reproduced by shRNA mediated depletion of band 3 in erythroblasts of healthy donors, the importance of band 3 for stable surface expression of multiple dependent proteins (GPA, CD47, Rh, protein 4.2) throughout erythropoiesis. Reductions in the relative plasma membrane expression of these proteins are conspicuous from the onset of erythropoiesis, accrue predominantly throughout the remainder of terminal differentiation and are compounded by additional loss during enucleation. Interestingly, throughout erythropoiesis, RhAG expression, whilst reduced relative to healthy donors, was maintained at a significantly higher level than that previously reported for mature band 3 Coimbra erythrocytes. In contrast, Rh, which associates closely with RhAG is depressed in its expression to a much greater degree throughout terminal differentiation. We cannot exclude the possibility that a larger gross reduction in RhAG expression is partially masked by increased availability of the LA1818 epitope, however an alternative explanation is that in the absence of band 3, RhAG can be stabilized in the erythroblast plasma membrane by direct binding to the cytoskeleton or indirectly through the formation of alternative sub-complexes that may be subsequently disrupted as the reticulocyte matures. In fact, it is very likely that in the absence of band 3, further loss of band 3 dependent proteins occurs during remodelling of the reticulocyte to generate mature erythrocytes since the relative expression levels are still higher than observed in the patients erythrocytes.

We also show here that secondary protein loss can be efficiently rescued by lentiviral expression of a GFP-tagged full-length band 3 in the patient erythroblasts, with a direct correlation observed between the expression of the tagged band 3 and the degree of rescue of secondary deficiencies. Band 3mem, which lacks the capacity to associate directly with the cytoskeleton, partitions to the membrane surrounding the extruded nucleus at a much greater level than full length band 3 and is incapable of rescuing either the relative drop in expression of band 3 dependent proteins in erythroblasts or the reduced reticulocyte retention of these proteins during enucleation. Expression of kB3 in patient erythroblasts results in a partial rescue of protein 4.2 and its dependent protein CD47. Although a large proportion of this protein is also lost during enucleation compared to band 3, enhanced retention of kB3 in the reticulocyte membrane is observed compared to band 3mem. This supports a weak association of kB3 with the cytoskeleton that is likely accounted for by the retention of protein 4.2 binding.

The capacity of band 3 to associate with the cytoskeleton clearly influences the degree to which it is retained in the partitioning of membrane and associated proteins during erythroblast enucleation. However, erythrocytes are known to contain mobile pools of band 3 without apparent cytoskeletal constraint. One explanation for the presence of this pool could be detachment from the cytoskeleton post enucleation during reticulocyte remodelling. Our data shows that, despite an increased loss of band 3mem relative to wild type band 3 with the plasma membrane surrounding the nucleus, the majority of band 3 that is unable to bind the cytoskeleton is still retained in the reticulocyte membrane during enucleation suggesting that cytoskeletal association is not the sole determinant of band 3 retention in the reticulocyte membrane.

In summary, we present data here that uncovers the molecular basis of secondary protein loss in a unique and definitive case of severe hereditary spherocytosis. We show that loss of proteins associated either directly with band 3 or indirectly as part of band 3 centred multiprotein complexes can be accounted for, in its absence, by a combination of reduced surface expression in the early stages of erythropoiesis and inefficient retention within the nascent reticulocyte membrane during
enucleation. This work demonstrates the role of band 3 as a hub for assembly or stabilisation of proteins within the ankyrin associated band 3 tetramer based complex at an early stage of terminal erythroid differentiation and illustrates the importance of cytoskeletal connectivity via its N-terminus for efficient retention of associated proteins within the reticulocyte membrane during enucleation. Finally, we demonstrate the feasibility of manipulating band 3 expression in primary erythroblasts both using shRNA and by exogenous expression of GFP-tagged band 3 and mutants thereof in a unique band 3 null patient environment.

**Authorship and Disclosures**

Contribution: TJS conceived and designed experiments, performed experiments, wrote and edited the manuscript; BRH performed experiments and AJB contributed to developing methodology; MLR managed patient care and provided patient samples. BRH, AJB and MLR all read and edited the final manuscript. AMT was the principal investigator, conceived and designed experiments, wrote and edited the manuscript.

Conflict of Interest Disclosure: The authors declare no competing financial interests

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Figure 1. The V488M band 3 mutant (band 3 Coimbra) is expressed at very low levels during erythropoiesis but does not traffic to the plasma membrane. A) Erythroblasts derived from healthy donor and homozygous band 3 Coimbra patient cultures at indicated timepoints were fixed and labelled with a monoclonal antibody for band 3 (BRIC170) and a rabbit polyclonal to GPA, stained with DAPI and imaged using confocal microscopy. This confirms that the band 3 Coimbra protein is weakly expressed in the patient erythroblasts and does not localise to the plasma membrane unlike normal band 3. By 144 hours the band 3 Coimbra expression is not detectable by immunofluorescence imaging. Panels were acquired using a Leica SP5 AOBS confocal laser scanning microscope using a 100x oil-immersion objective (N.A 1.4). Scale bar represents 5 µm. B) 5×10^5 differentiating erythroblasts derived from the peripheral blood of healthy donor control or band 3 Coimbra patient were removed from culture at 24h intervals, cells were lysed, proteins separated by SDS-PAGE and immunoblotted with monoclonal or antibodies to band 3 N-terminus (BRIC170), protein 4.2 (BRIC273) and rabbit polyclonal antibodies to band 3 C-terminus and GAPDH.

Figure 2. Reduced surface expression of known band 3 associated proteins in the absence of band 3 is evident throughout erythropoiesis. A) Surface expression of indicated proteins was monitored on differentiating erythroblasts from healthy donor control and band 3 Coimbra patient cultures at 24 hour intervals using the indicated monoclonal antibodies as detailed in Supplemental Methods. The dashed box highlights the switch from gating on erythroblasts to reticulocytes post enucleation. Note that antibody induced agglutination prevented collection of T144 control cells within this specific experiment, but the average RhAG expression of band 3 Coimbra reticulocytes relative to control across additional cultures is shown in Figure 2B. B) Table showing mean band 3 Coimbra reticulocyte surface expression of indicated proteins relative to healthy donor control. Means (± standard error) are derived from 3 independent cultures using average mean fluorescent intensities (MFI) of reticulocyte populations from multiple timepoints where possible. Representative histograms to derive the reticulocyte data are shown in Supplemental Figure 1.

Figure 3. shRNA mediated knockdown of band 3 recapitulates secondary protein deficiencies observed in band 3 Coimbra erythroid cultures. A) 2.5×10^5 FACS sorted reticulocytes derived from erythroblasts transduced with shRNAs targeting band 3 or a non-targeting scramble control were lysed, proteins separated by SDS PAGE and immunoblotted with antibodies to band 3 (BRIC170) and actin. B) Flow cytometry histograms illustrating efficiency of band 3 knockdown in reticulocytes expressing two independent shRNAs. C) Surface expression of indicated proteins was monitored on differentiating erythroblasts expressing 2 independent band 3 shRNAs and a non-targeting control at 24 hour intervals using the indicated monoclonal antibodies as detailed in Supplemental Methods. The dashed box highlights the point at which gating was switched from erythroblasts to reticulocytes post enucleation. D) Table shows mean surface expression of indicated proteins on reticulocytes derived from band 3 knockdown erythroid cultures relative to the non-targeting scramble control. Means (± standard error) for each shRNA are derived from 2 independent cultures using average mean fluorescent intensities (MFI) of reticulocyte populations from 3 consecutive timepoints.

Figure 4. Reticulocyte retention of band 3 associated proteins during erythroblast enucleation is reduced in the absence of band 3. A) Gating strategy for separation of reticulocytes, erythroblasts and extruded nuclei using forward scatter and fluorescent intensity of DNA (Hoechst) was validated by FACS sorting of indicated populations. Representative cytopsins from sorted populations are shown together with Hoechst/antibody dual labelling for GPA and GPC illustrating the validity of this gating approach compared to a previously published method. B) Graphical representation of protein
partitioning profiles between reticulocytes and nuclei of indicated proteins from healthy donor and band 3 Coimbra patient cultures. C) Table showing protein partitioning values for indicated proteins. Means (± standard error) are derived from 3 independent cultures using average mean fluorescent intensities (MFI) of reticulocyte and nuclei populations from multiple timepoints where possible.

**Figure 5.** The N-terminal cytoskeletal binding domain of band 3 is required for rescue of band 3 associated protein expression in band 3 Coimbra reticulocytes. A) Bar chart illustrating reticulocyte surface expression level of indicated proteins relative to healthy donor control for untransduced band 3 Coimbra patient reticulocytes and GFP positive patient reticulocytes derived from erythroblasts rescued with GFP tagged band 3, band 3 membrane domain and kidney band 3 respectively. Means (± standard error) are derived from 3 independent cultures using average mean fluorescent intensities (MFI) of reticulocyte populations from multiple timepoints where possible. For GFPkB3 rescue experiments, data are derived from 2 independent cultures. Values used are provided as a table in Supplemental Figure 1. B) Dot plots illustrating correlation between reticulocyte expression of GFP tagged band 3 (GFP intensity) and expression of band 3 associated proteins (x axis with indicated antibodies) in healthy donor reticulocytes (blue), untransduced band 3 Coimbra patient reticulocytes (red), patient cells rescued with full length band 3 (green) or with band 3 membrane domain only (purple). Cells were fixed with 1% paraformaldehyde + 0.0075% glutaraldehyde prior to labelling to reduce antibody induced cell clustering and gated using Hoechst to exclude nucleated cells. C) Dot plots showing rescue of CD47 in band 3 Coimbra reticulocytes by expression of GFP tagged band 3 (right and left panels), a partial rescue with GFPkB3 (left panel) but not GFP-tagged band 3 membrane domain (right panel). D) Reticulocytes were FACS sorted based on Hoechst negativity and GFP positivity where appropriate. 2.5×10^5 cells were lysed, proteins separated by SDS PAGE and immunoblotted with monoclonal antibodies to band 3 (BRIC170), protein 4.2 (BRIC273) and ankyrin (BRIC274). Note the absence of protein 4.2 in untransduced band 3 Coimbra cells, rescue of protein 4.2 expression with GFPB3 but not GFPB3mem and a partial rescue with GFPkB3.

**Figure 6** Efficient reticulocyte retention of band 3 and associated membrane proteins is dependent on the cytoskeleton binding N-terminal domain during erythroblast enucleation. A) Surface expression of indicated proteins was monitored on differentiating erythroblasts from healthy donor control, band 3 Coimbra patient cultures and GFP positive populations from respective rescue experiments commencing 48 hours post differentiation at 24 hour intervals using monoclonal antibodies as detailed in Supplemental Methods. The dashed box highlights the switch from gating on erythroblasts to reticulocytes post enucleation. B) Table showing protein partitioning profiles between plasma membranes of extruded nuclei and reticulocytes for indicated proteins and cultures. Means (± standard error) are derived from 3 independent cultures for healthy donor, band 3 Coimbra patient and patient cells rescued with GFPB3 or GFPB3mem and 2 independent cultures for patient cells rescued with GFPkB3 using average mean fluorescent intensities (MFI) of reticulocyte and nuclei populations from multiple timepoints where possible. Asterisks indicate statistically significant differences (P<0.05 Students T Test) between patient and control or between B3GFP or B3memGFP transduced and untransduced patient cells respectively. Statistical evaluation of GFPkB3 transduced cells was not performed due to n=2. Note that amalgamated protein partitioning data for control and untransduced band 3 Coimbra nuclei and reticulocytes were initially presented in Figure 4C are repeated as a reference for comparison here. C) Graphical representation of protein partitioning profiles between reticulocytes and nuclei for band 3 (BRIC200), GPC (BRIC4) and CD47 (BRIC32) derived from healthy donor, untransduced band 3 Coimbra patient and transduced band 3 Coimbra patient erythroblasts as indicated. D) Offset histogram showing gross surface levels of band 3 within extruded
nuclei populations of healthy donors, band 3 Coimbra and respective GFPband 3 rescue erythroid cultures.
Figure 2

A

![Graphs showing mean fluorescent intensity over differentiation timepoints for different proteins and cell lines.](image)

B

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<th>B3Coimbra Mean Surface Expression (% of control)</th>
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Figure 3

A

B

C

D

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Supplemental Methods

Antibodies

Monoclonal mouse antibodies were BRIC 4 (GPC), BRIC13 (Wrβ), BRIC69 (Rh), BRIC71 (band 3), BRIC200 (band 3), BRIC222 (CD44), BRIC256 (GPA), LA1818 (RhAG), BRIC170 (band 3), BRIC6 (band 3), BRIC273 (protein 4.2), BRIC274 (ankyrin) (all IBGRL, NHSBT Filton, Bristol). Rabbit polyclonal antibodies used were against band 3, GPA (in house), and GAPDH (Santa Cruz). Secondary antibodies used were goat anti–mouse-Alexa 488/594 and goat anti-rabbit-Alexa 488/594 (Invitrogen), APC conjugated rat anti-mouse IgG1 (Biolegend), HRP-conjugated swine anti-rabbit and rabbit anti-mouse (Dako).

Erythroblast Culture

Peripheral blood mononuclear cells were isolated from platelet apheresis blood waste (NHSBT, Bristol) from healthy donors and homozygous V488M patient with written informed consent for research use in accordance with the Declaration of Helsinki and approved by local Research Ethics Committee (REC 12/SW/0199). Erythroblasts were expanded and differentiated as described previously [1, 2] using IMDM (Source Biosciences) supplemented with BSA (0.01g/ml) for initial expansion. For knockdown experiments, CD34+ cells were pooled from 4 separate platelet apheresis blood waste cones using a CD34+ magnetic isolation kit (Miltenyi Biotech) according to the manufacturer’s protocol, expanded for 3 days in Stemspan (Stem Cell technologies) before culture in erythroblast expansion and differentiation medium as described in [2].

SDS PAGE and Western Blotting

Erythroblasts and reticulocytes were lysed using buffer containing 20mM Tris-HCl pH 8.0, 137mM NaCl, 10mM EDTA, 100mM NaF, 1% (v/v) Nonidet P-40, 0.5% (w/v) deoxycholate, 0.1% SDS (w/v), 10% (v/v) glycerol, 10mM Na3VO4, 2mM PMSF, 1% (v/v) protease inhibitor cocktail set V (Calbiochem). Proteins were separated using SDS PAGE and immunoblotted as described previously [2-4].

Staining of cytospins

Cells (1 × 10⁵) were cytospun 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 a Pixera Penguin 600CL camera using a 40× lens and processed using Adobe Photoshop 9.0 (Adobe Systems).

Immunoprecipitations

BRIC6 surface immunoprecipitations were performed using cell numbers indicated in specific figure legends as described previously [4].

Immunofluorescence
Erythroblasts were fixed in suspension using either 0.5% acrolein or 1% PFA as indicated in the figure legends. Immunolabelling and confocal microscopy were conducted as described previously [2, 4].

Supplemental References

Supplemental Figure 1. Proteins normally associated with band 3 are reduced in their surface expression on in vitro cultured reticulocytes derived from the band 3 Coimbra patient relative to healthy donor controls. Representative histograms illustrating surface expression of indicated proteins from reticulocyte population taken 144 hours post induction of differentiation used for the generation of Figures 2A. Due to the absence of this timepoint for RhAG (LA1818) in this figure, the above histogram was derived from an alternative representative experiment at T144.
Supplemental Figure 2. Expression of band 3 in band 3 Coimbra reticulocytes can be rescued to wild type levels by lentiviral transduction of patient erythroblasts with N-terminally GFP tagged band 3. A) Representative histogram illustrating transduction efficiency of GFP band 3 in band 3 Coimbra reticulocytes (left panel) and histogram illustrating surface expression of band 3 in band 3 Coimbra reticulocytes derived from untransduced and GFP band 3 transduced erythroblasts compared to healthy donor reticulocytes (right panel). B) Dot plots demonstrating correlation between band 3 expression (GFP intensity) and degree of rescue of secondary protein deficiency in band 3 Coimbra erythroblasts C) BRIC 6 cell surface immunoprecipitations were conducted using 5x10^6 FACS sorted orthochromatic erythroblasts from band 3 Coimbra GFPB3 positive, untransduced band 3 Coimbra and untransduced healthy donor cultures. Proteins were eluted, separated by SDS PAGE and immunblotted with a rabbit C-terminal polyclonal antibody to band 3. Note that in band 3 Coimbra erythroblasts rescued with GFPB3, the 135kDa GFPB3 is present but no 95kDa endogenous band 3 Coimbra protein is immunoprecipitated from the cell surface.
# Supplemental Figure 3

<table>
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<th>band 3 Coimbra + GFPB3mem (% of control)</th>
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**Supplemental Figure 3. The N-terminal cytoskeletal binding domain of band 3 is required for rescue of band 3 associated protein expression in band 3 Coimbra reticulocytes.** Table showing data used to generate Figure 6A. Means (± standard error) are derived from 3 independent cultures using average mean fluorescent intensities (MFI) of reticulocyte populations from multiple timepoints where possible. For GFPkB3 rescue experiments, data are derived from 2 independent cultures.
Supplemental Figure 4. Inability of GFPB3mem to rescue is not the result of cellular toxicity. Bar charts illustrating expression of proteins with indicated antibodies in band 3 Coimbra patient erythroblasts and reticulocytes rescued with GFPB3 or GFPB3mem relative to healthy donor control cells at the same stage. Note the much lower expression of GFPB3mem at T48 and T96 in this experiment compared to the data presented in Figure 6A. At this lower level, surface expression of proteins in cells transduced with GFPB3mem matches that of untransduced band 3 Coimbra cells highlighting the inability of this mutant protein to rescue expression of band 3 associated proteins compared to GFPB3 in the absence of cellular toxicity induced by super expression of GFPB3mem. B3Coimbra data are derived from the mean +/- SEM of labelling for untransduced patient erythroblasts together with GFP negative populations from patient erythroblasts transduced with GFPB3 or GFPB3mem. Due to absence of the healthy donor reticulocyte sample labelled with LA1818 (RhAG), data from a further rescue experiment is included for this protein with corresponding BRIC200 (band 3) and BRIC4 (GPC) labelling data for reference.