Severe Ankyrin-R deficiency results in impaired surface retention and lysosomal degradation of RhAG in human erythroblasts

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ABSTRACT

Ankyrin-R provides a key link between band 3 and the spectrin cytoskeleton that helps to maintain the highly specialized erythrocyte biconcave shape. Ankyrin deficiency results in fragile spherocytic erythrocytes with reduced band 3 and protein 4.2 expression. We use *in vitro* differentiation of erythroblasts transduced with shRNAs targeting ANK1 to generate erythroblasts and reticulocytes with a novel ankyrin-R 'near null' human phenotype with less than 5% of normal ankyrin expression. Using this model, we demonstrate that absence of ankyrin negatively impacts the reticulocyte expression of a variety of proteins, including band 3, glycophorin A, spectrin, adducin and, more strikingly, protein 4.2, CD44, CD47 and Rh/RhAG. Loss of band 3, which fails to form tetrameric complexes in the absence of ankyrin, alongside GPA, occurs due to reduced retention within the reticulocyte membrane during erythroblast enucleation. However, loss of RhAG is temporally and mechanistically distinct, occurring predominantly as a result of instability at the plasma membrane and lysosomal degradation prior to enucleation. Loss of Rh/RhAG was identified as common to erythrocytes with naturally occurring ankyrin deficiency and demonstrated to occur prior to enucleation in cultures of erythroblasts from a hereditary spherocytosis patient with severe ankyrin deficiency but not in those exhibiting milder reductions in expression. The identification of prominently reduced surface expression of Rh/RhAG in combination with direct evaluation of ankyrin expression using flow cytometry provides an efficient and rapid approach for the categorization of hereditary spherocytosis arising from ankyrin deficiency.

Introduction

The ankyrins are a family of structural adaptor proteins that link membrane proteins to the cytoskeleton in a diverse range of cell types where they play a critical role in the formation and organization of physiologically important membrane proteins within specialized membrane microdomains.[1] Canonical ankyrins have a modular

domain organization with an N-terminal membrane binding domain containing 24 ANK repeats (1-24), followed by a central spectrin binding domain, a death domain and a C-terminal regulatory domain.[2] Erythrocyte ankyrin (or ankyrin-R), alongside protein 4.2, links the tetrameric band 3-based multiprotein complex to the underlying spectrin cytoskeleton *via* associations with both the D2 and D3-4 regions of the ankyrin membrane binding domain.[3] Ankyrin-R can also associate with other erythrocyte membrane proteins, including CD44,[4] Na^+K^+ATPase[5] and RhAG.[6] RhAG is a 50kDa glycosylated NH_3 and CO_2 membrane transport protein[7,8] that together with the Rh proteins RhCe and RhD forms the core of the Rh subcomplex that also contains CD47, LW and GPB.[9] Evidence supporting the interaction between RhAG and ankyrin includes a drop in RhAG expression in the *nb/nb* mouse with ankyrin deficiency, an interaction of the C-terminal tail of RhAG and the D2 subdomain of the ankyrin membrane binding domain *in vitro*, and the presence of the RhAG mutation D399A, shown to abrogate direct ankyrin binding *in vitro* in a patient with Rh_{null} syndrome.[6]

In humans, mutations in the ankyrin-R gene are responsible for 50% of hereditary spherocytosis (HS) cases,[10,11] a form of hemolytic anemia affecting 1 in 2000 people of Northern European ancestry.[12] Although mutations in the *ANK1* gene that result in hereditary spherocytosis are relatively prevalent, such mutations are most commonly heterozygous in nature and encode an array of complex alterations that include substitutions within specific binding sites and the generation of novel spliced or truncated protein products in addition to partial protein deficiency. This heterogeneity complicates the identification of secondary protein deficiencies that result from reduced ankyrin expression in human red blood cells and the corresponding insight into the role that this protein plays in the generation of the unique erythrocyte membrane cytoskeletal architecture. Reduced expression of the ankyrin binding proteins band 3 and protein 4.2 has been noted in these patients,[13-15] and in murine models of ankyrin deficient HS, secondary protein deficiencies of GPA, protein 4.2 and Rh, but not band 3 or GPC, were observed.[16] However, more extensive characterization of the membrane protein composition in humans, including the effect on RhAG and other putative ankyrin associated proteins, is so far lacking.

A more detailed assessment of the importance and role that ankyrin-R protein expression plays in directing the development and composition of the human erythrocyte membrane is complicated by the absence of a naturally occurring null phenotype for this protein. In this study, we employ lentiviral shRNA transduction of primary erythroid cells and demonstrate the ability to deplete ankyrin-R expression to a level considerably lower than that observed *in vivo*.[17] These cells were used to address the influence that ankyrin deficiency exerts on the membrane composition of the erythrocyte and developing erythroblast membrane, and to gain mechanistic insight regarding the loss of dependent proteins in its absence. The observed reduction in the plasma membrane expression of the core Rh complex components Rh and RhAG was confirmed on red blood cells from patients with HS due to mutations in *ANK1*. Furthermore, we propose a flow cytometry based strategy for easy identification of HS erythrocytes with ankyrin deficiency that could be used to direct diagnoses of HS molecular genetic defects.

Methods

Donor and patient blood

Platelet apheresis waste blood (NHSBT, Bristol, UK) from healthy donors or peripheral blood from patients previously diagnosed with HS because of a mutation in *ANK1* were obtained with written informed consent for research use in accordance with the Declaration of Helsinki and approved by Bristol Research Ethics Committee Centre reference 12/SW/0199, as previously described.[18]

Erythroid cell culture and lentiviral transduction

Briefly, $CD34^+$ cells were purified from peripheral blood mononuclear cells using Magnetic Activated Cell Sorting (Miltenyi Biotec) following the manufacturer's instructions, cultured for three days in Stemspan (Stem Cell Technologies) supplemented with SCF (100 ng/mL), Epo (2 U/mL, Bristol Royal Infirmary, UK), dexamethasone (1 μM, Sigma), IGF-1 (40 ng/mL, R&D Systems), cholesterol-rich lipids (40 μg/mL, Sigma) and 1 ng/mL IL-3 (R&D Systems): Phase 1. After three days, 50 μL concentrated lentivirus was added per $1x10^6$ cells in expansion medium - IMDMExp (Phase 2), as previously described,[18] supplemented with 8 μg/mL polybrene. After 20 h, the cells were washed 3 times in PBS and plated in fresh IMDMExp. Erythroblasts were further expanded and differentiated (Phase 3), as previously described.[18] Lentiviral transduction of erythroblasts was performed as previously described using pLKO.1 vector with puromycin selection. Broad Institute Repository shRNA sequences TRCN0000083720 (shRNA 1) and TRCN0000083721 (shRNA 2) targeting ANK1 were used in preliminary experiments and TRCN0000083720 used unless otherwise stated.

Flow cytometry and FACS

Flow cytometry and FACS on cultured erythroid cells and erythrocytes was performed as previously described.[18] For assessment of ankyrin levels by flow cytometry, donor and patient erythrocytes were fixed with 1% paraformaldehyde + 0.0075% glutaraldehyde in PBS supplemented with 1 mg/mL BSA (PBSAG), 2 mg/mL glucose for 10 min at room temperature, permeabilized with 0.05% Triton X-100 for 2 min, centrifuged for 3 min at 500 G, washed once in PBSAG, re-suspended in PBSAG containing 4% BSA, and stained sequentially with BRIC272 (anti-ankyrin) and APC-conjugated polyclonal anti-mouse IgG (Biolegend), as described.[18] Pronase epitope recovery experiments were performed as previously described.[19] A list of antibodies used in this study is provided in the *Online Supplementary Table S1*. Data were acquired on a MACSQuant flow cytometer and analyzed using FlowJo v.7.6.5 (FlowJo).

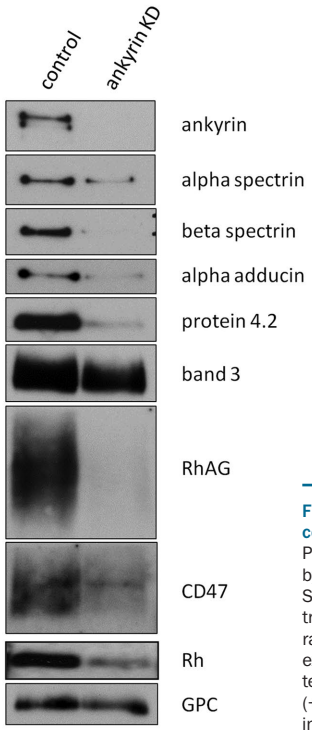
Blue Native PAGE

Detergent extraction using 0.5% C12E8 in 5 mM phosphate buffer containing 2 mM PMSF and 1% protease inhibitor cocktail (Calbiochem) was performed on $2x10^6$ T120 erythroblasts per sample for 10 min on ice. Samples were centrifuged at 150,000 G for 30 min at 4°C and protein complexes from the extract separated using the Novex Native PAGE BisTris Blue Native polyacrylamide gel electrophoresis system (Thermo Fisher Scientific). Samples were run on 4%-16% gels with final sample concentration of 0.0625% Coomassie G and light blue cathode buffer-and transferred to PVDF membrane according to the manufacturer's instructions.

Inhibition of lysosomal degradation

A combination of 10 μg/mL leupeptin, 10 μg/mL pepstatin A, 10 μg/mL E64d or DMSO alone was added to non-targeting con-

A



B

Protein	Antibody	ANK KD Mean Surface Expression (% of control)
band 3	BRIC71	59.0 ± 0.4
band 3	BRIC200	61.1 ± 2.4
Wrb	BRIC13	65.5 ± 1.9
GPA	BRIC256	68.0 ± 1.8
CD47	BRIC32	31.0 ± 1.6
Rh	BRIC69	12.0 ± 1.0
RhAG	LA1818	22.7 ± 2.2
CD44	BRIC222	53.4 ± 5.6
GPC	BRIC4	70.1 ± 1.4
CD99	12E7	124.2 ± 10.4

Figure 1. shRNA mediated knockdown of ankyrin results in a novel reticulocyte phenotype with almost complete absence of ankyrin expression accompanied by widespread secondary protein deficiencies. (A) Purified populations of ankyrin knockdown and control reticulocytes were obtained from *in vitro* cultures by FACS sorting of the Hoechst negative population. 5 x 10^5 reticulocytes were lysed, proteins separated by SDS-PAGE and immunoblotted with mouse monoclonal antibodies specific for ankyrin (BRIC274), β spectrin (BRAC65), α spectrin (BRIC172), α adducin (Santa Cruz), band 3 (BRIC170) protein 4.2 (BRIC273) or rabbit antibodies against RhAG, CD47 or GPC (in house). Data are representative of three independent experiments. (B) Table showing ankyrin knockdown reticulocyte mean surface expression of indicated proteins relative to reticulocytes derived from erythroblasts expressing a non-targeting control shRNA. Means (+ standard error) are derived from 3 independent cultures using average flow cytometry mean fluorescent intensities (MFI) of reticulocyte populations from three time points per culture.

trol or ankyrin knockdown basophilic erythroblasts (T24) for 24 h in culture, then fixed for immunofluorescence or counted and processed for SDS PAGE and immunoblotting.

Immunofluorescence

Cells were fixed for 15 min in 1% PFA + 0.0075% glutaraldehyde in PBSAG, allowed to adhere to 1% PEI coated coverslips, then labeled and imaged as previously described[19] using a Leica SP5 AOBS confocal laser scanning microscope with 100x oil-immersion objective (NA 1.4).

Results

Ankyrin is required for normal expression of multiple erythroid membrane and cytoskeletal proteins

To understand the essential role ankyrin plays in the assembly and maintenance of the human erythrocyte membrane, ankyrin-R was knocked down using shRNA lentivirally tranduced into $CD34^+$ cells isolated from healthy donor peripheral blood and then these cells were expanded and differentiated *in vitro*. Ankyrin knockdown of up to 95% was achieved and maintained in reticulocytes differentiated from erythroblasts expressing two independent shRNAs (*Online Supplementary Figure S1*). Analysis of protein expression by immunoblotting of total cell lysates derived from equal numbers of sorted ankyrin knockdown and control reticulocytes revealed reductions in the expression of cytoskeletal proteins spectrin and adducin, the membrane proteins CD47, CD44 and band 3 and strikingly large reductions in expression of protein 4.2 and RhAG (Figure 1A). Reductions in the reticulocyte expression of band 3 and GPA, and to a greater extent CD47, Rh, RhAG and CD44, were also observed by flow cytometry (Figure 1B).

Ankyrin is required for membrane protein retention during human erythroblast enucleation

Mislocalization of protein during enucleation has previously been suggested to account for protein loss based on confocal immunofluorescent imaging of nb/nb mouse erythroblasts undergoing enucleation.[20] By staining differentiating ankyrin deficient erythroblasts grown in our human *in vitro* culture system with Hoechst and using a recently published flow cytometry gating strategy,[18] protein partitioning profiles were determined for localization of specific membrane proteins within the plasma membrane of the reticulocyte and pyrenocyte (the extruded condensed nucleus encased by thin layer of cytoplasm and plasma membrane) derived from non-targeting control and ankyrin knockdown erythroblasts, respectively. The bar graphs in Figure 2A demonstrate that in the ankyrin deficient cultures there is an increase in the proportion of the ankyrin-R associated complex proteins band 3, GPA, RhAG, Rh and CD47 partitioning to the plasma membrane surrounding the extruded nucleus at this stage of differentiation. Interestingly, reduced reticulocyte plasma membrane retention was also observed for the junctional complex component GPC, as well as CD99, indicating that the effects of severe ankyrin deficiency extend beyond established ankyrin associated proteins. To confirm the mis-sorting of band 3 and GPA to the pyrenocyte during enucleation, sorted populations of reticulocytes and pyrenocytes were lysed and separated by SDS-PAGE. Figure 2B confirms the increased amount of GPA and band 3 in the pyrenocytes extruded from the ankyrin deficient erythroblasts relative to those from control erythroblasts. The remaining cytoskeletal adaptor protein 4.2 (the expression of which is reduced with severe depletion of ankyrin expression) is retained within the reticulocyte

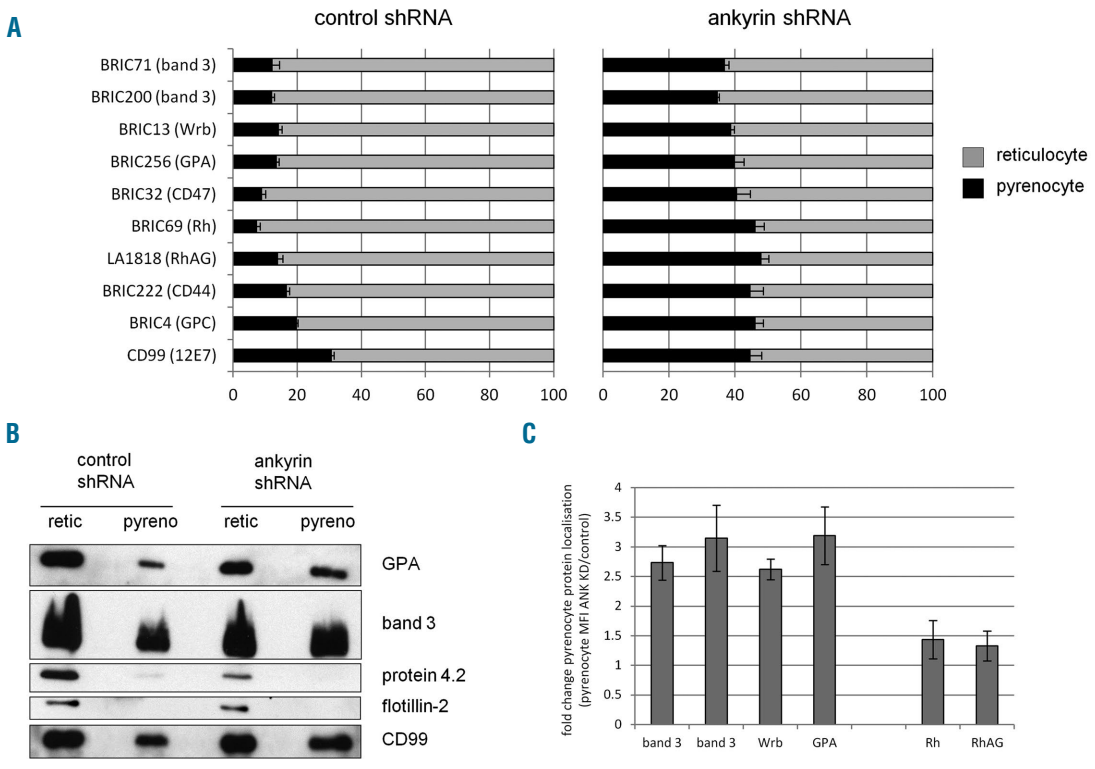


Figure 2. Mislocalization to the pyrenocyte plasma membrane during enucleation accounts for reduced reticulocyte expression of band 3/GPA but not Rh/RhAG in the absence of ankyrin. (A) Graphical representation of protein partitioning profiles between plasma membranes of reticulocytes and extruded nuclei (pyrenocytes) of indicated proteins from control and ankyrin knockdown cultures during enucleation. Partitioning data are presented as percentages derived from mean fluorescent intensities for each antibody on the pyrenocyte and reticulocyte populations divided by the total intensity for the two populations % nucleus = [MFI nucleus/(MFI nucleus + MFI reticulocyte)]x 100 and % reticulocyte = [MFI reticulocyte/(MFI reticulocyte + MFI nucleus)]x100. (B) $5x10^5$ reticulocytes and extruded nuclei from control and ankyrin shRNA transduced cultures collected by FACS were lysed (with addition of 1 μL Omnicleave for nuclei populations), separated by SDS-PAGE and immunoblotted with antibodies specific for indicated proteins. (C) Bar chart illustrating differences in gross levels of indicated proteins on pyrenocytes extruded from control or ankyrin knockdown erythroblasts. Fold change = MFI nuclei from ankyrin knockdown/MFI nuclei from control. In all cases means (+ standard error) are derived from 3 independent cultures using average mean fluorescent intensities (MFI) of populations from three time points *per* culture.

membrane and the membrane protein flotillin maintains the partitioning profile observed in healthy donor[21] and non-targeting shRNA expressing cells. Similar experiments conducted using protein 4.2 knockdown cells did not result in reduced reticulocyte expression of Rh, RhAG or CD44 (*data not shown*), demonstrating that these effects are not an indirect consequence of the secondary protein 4.2 expression deficiency that occurs in response to ankyrin knockdown.

Importantly, although severe ankyrin-R deficiency results in an increase in the proportion of Rh and RhAG lost with the extruded pyrenocyte during enucleation as a percentage of total levels, absolute levels of these two proteins on the pyrenocytes derived from ankyrin knockdown erythroblasts are only minimally increased relative to control (Figure 2C). This observation highlights the fact that, although impaired reticulocyte retention of RhAG during erythroblast enucleation does occur in response to severe depletion of ankyrin, the majority of RhAG protein loss due to ankyrin-R deficiency must occur prior to enucleation.

Rhesus protein complex deficiency as a result of a loss of ankyrin occurs prior to erythroblast enucleation

To investigate the stage at which RhAG and other proteins are lost prior to enucleation in response to severe depletion of ankyrin-R expression, erythroblasts derived from $CD34^+$ cells transduced with non-targeting or ankyrin-R targeting shRNA were expanded and differentiated *in vitro* and protein expression examined at 24-h intervals by immunoblotting and flow cytometry. Representative cytospin images charting the morphological differentiation of control and ankyrin deficient erythroblasts are shown in *Online Supplementary Figure S2*. Figure 3A demonstrates that in the almost complete absence of ankyrin-R the cytoskeletal adaptor protein 4.2, which associates with band 3 early during erythropoiesis[19] and is rapidly degraded in its absence, is strikingly reduced in its expression relative to non-targeting control vector transduced erythroblasts from the onset of terminal differentiation.

Surface expression of both band 3 and GPA was maintained at normal levels until the enucleation stage (Figure 3B). In contrast, a relative reduction in the expression of the Rh complex proteins Rh, RhAG and CD47 is observed from an early stage of differentiation, which in the case of Rh/RhAG becomes more pronounced as these proteins accumulate at the plasma membrane in the control erythroblasts. The expression profile and levels of GPC in erythroblasts transduced with ankyrin targeting shRNA was similar to control, although a reduction in the reticulocytes was observed by flow cytometry. Interestingly, the

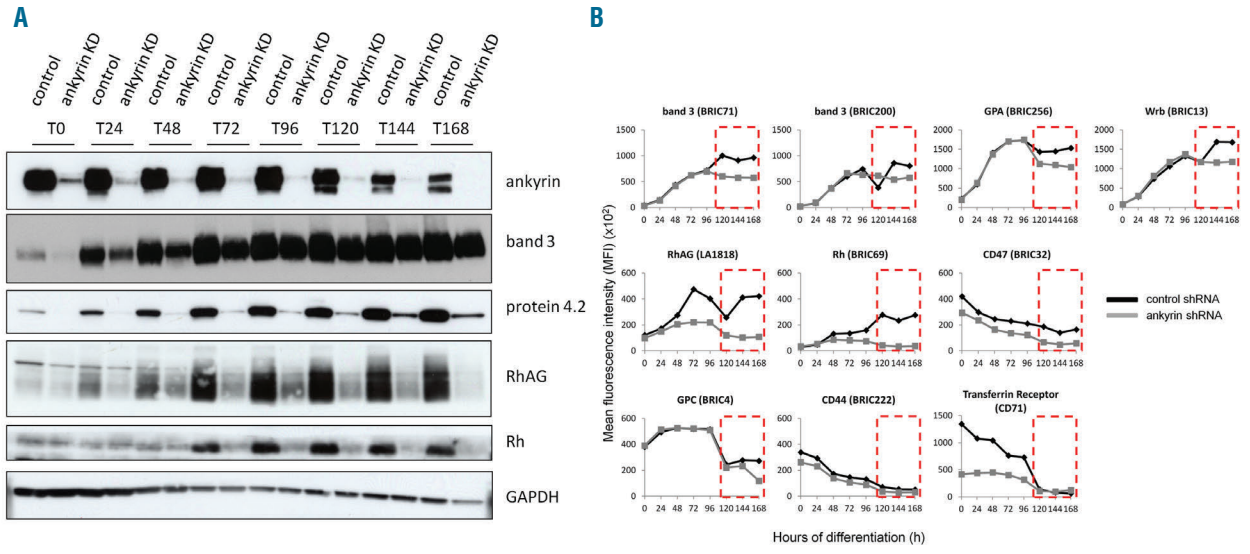


Figure 3. Severe depletion of ankyrin impacts expression of protein 4.2, Rh and RhAG prior to enucleation. (A) 5x10^5 differentiating erythroblasts expressing scramble control shRNA (Control) or ankyrin shRNA (Ankyrin KD) were removed from culture at 24-h intervals throughout differentiation, lysed and proteins separated by SDS-PAGE. Lysates were immunoblotted with antibodies specific for ankyrin (BRIC274), protein 4.2 (BRIC273), band 3 (BRIC170) or rabbit antibodies against RhAG (in house) and GAPDH (Santa Cruz). (B) Surface expression of indicated proteins was monitored on differentiating erythroblasts expressing non-targeting control or ANK1 shRNA at 24-h intervals using the indicated monoclonal antibodies as detailed in the *Online Supplementary Table S1*. The dashed box highlights the switch from gating on erythroblasts to reticulocytes post enucleation. All data shown are representative of three independent experiments.

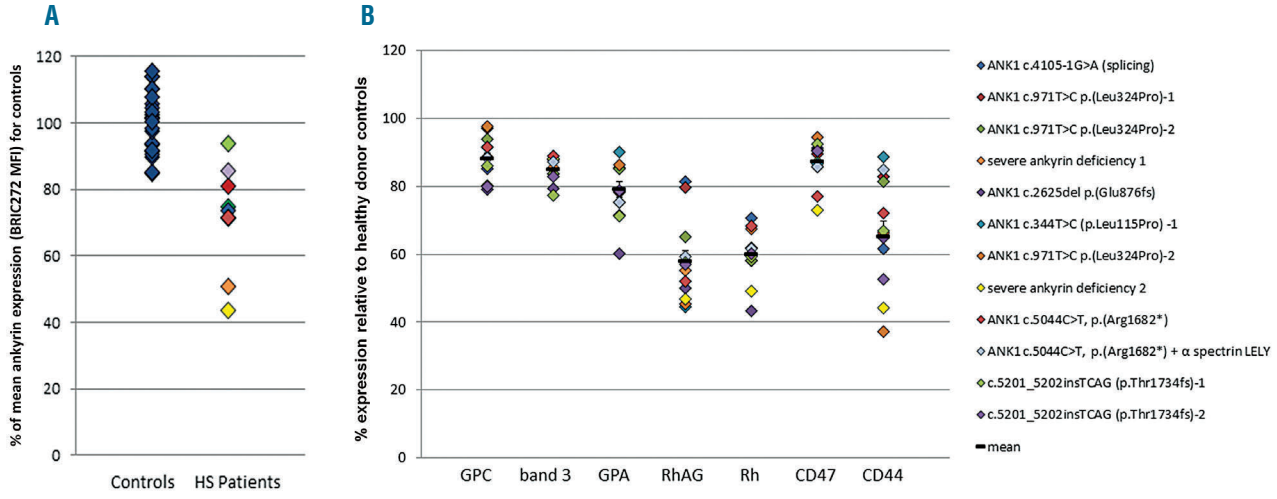
loss of ankyrin also caused a pronounced reduction in the steady state levels of transferrin receptor throughout terminal differentiation.

Rh/RhAG expression levels are prominently reduced in a panel of hereditary spherocytosis patients with confirmed ankyrin mutations

To determine whether the secondary membrane protein deficiencies identified in our *in vitro* derived ankyrin near null model are also observed in naturally occurring ankyrin deficient HS patients, we obtained a blood sample from a variety of patients with confirmed or suspected ankyrin mutations/deficiency. Although mutations in the gene encoding ankyrin-R (ANK1) are known to be responsible for HS in approximately 50% of cases, direct quantification of ankyrin deficiencies in the erythrocytes of these patients is notoriously laborious.[17] To facilitate the assessment of the level of ankyrin deficiency in patient erythrocytes, we developed a protocol for the detection of ankyrin by flow cytometry using the monoclonal antibody BRIC272. The epitope that BRIC272 recognizes on ankyrin-R is not known but we have shown that it does not recognize the ANK90 binding domain (*data not shown*). Figure 4A and *Online Supplementary Figure S3* demonstrate the variability in ankyrin expression within the healthy donor population as assessed using this technique which is consistent with that previously reported by radioactive immunoassay.[17] This figure also shows expression of ankyrin relative to mean healthy donor controls in a random selection of patients available to us, and provides quantitative data that defines several mild and two severe deficiencies within this cohort.

Expression of major membrane proteins was assessed in these patients (Figure 4B). Note that despite the fact that ANK1 mutations (where known – additional details on the mutations are provided in the *Online Supplementary Appendix*) affect different regions of the ankyrin protein, a consistent significant reduction was observed in the surface expression of Rh and RhAG, as identified in our *in vitro* generated ankyrin knockdowns. This Rh/RhAG alteration is consistently prominent compared to the smaller reductions in expression of other proteins that reside within the ankyrin associated multiprotein complex including band 3 and GPA. No significant difference in the degree of RhAG reduction was observed between patients with mild and severe ankyrin deficiencies. In several patients, reduced expression of the ankyrin binding protein CD44 was also observed, although in others expression of this protein was decreased to only a mild degree. In contrast, erythrocytes from HS patients with differing clinical severity resulting from defined mutations in the genes encoding band 3 and protein 4.2 display increased or unaltered CD44 expression, respectively[22-25] (*Online Supplementary Table S2*).

To determine whether loss of Rh/RhAG also occurs prior to enucleation in naturally occurring ankyrin deficient patients, peripheral blood samples were obtained from a small number of ankyrin-R deficient HS patients with a range of clinical severities. Erythroblasts were expanded and differentiated in order to produce a population containing nucleated orthochromatic erythroblasts and reticulocytes. Consistent with the ankyrin-R knockdown results, Figure 4C demonstrates a substantially reduced surface expression of Rh/RhAG in orthochromatic erythroblasts of the more severely ankyrin deficient patient (approx. 43% normal ankyrin-R) prior to enucleation that is enhanced to only a minor degree by additional loss during enucleation. Surprisingly, despite obvious reductions in the expression of RhAG in the erythrocytes provided from mildly ankyrin deficient HS patients (Figure 4B), reductions in expression of this protein were not observed in the orthochromatic erythroblasts or (and to



C

PATIENT	Rh (BRIC69)		RhAG (LA1818)		band 3 (BRIC71)	
	ortho	retic	ortho	retic	ortho	retic
Severe ankyrin deficiency 2	55.8%	51.5%	63.0%	49.1%	87.8%	81.4%
L115P-1	74.9%	70.7%	104.5%	87.8%	105.7%	87.9%
R1682*	105.9%	93.4%	105.3%	86.0%	102.1%	100.2%
L324P-1	66.9%	70.1%	103.4%	92.0%	101.3%	101.4%

Figure 4. Reduced erythrocyte surface expression of Rh/RhAG is common to hereditary spherocytosis patients with ankyrin deficiency. Erythrocytes isolated from peripheral blood of healthy donors and patients diagnosed with hereditary spherocytosis arising from ankyrin mutations or deficiency were fixed and permeabilized where necessary, as detailed in Materials and Methods. Expression of (A) ankyrin or (B) indicated proteins was measured by flow cytometry and expressed relative to healthy donor control erythrocytes treated in the same way for each experiment. Data are presented as mean fluorescent intensity relative to healthy donor control for each antibody and patient. (C) Erythroblasts derived from peripheral blood samples of severe and mildly ankyrin deficient hereditary spherocytosis patients were differentiated to generate orthochromatic erythroblasts and reticulocytes. Surface expression of indicated proteins in orthochromatic erythroblast and reticulocyte populations across three consecutive days were assessed by flow cytometry and mean data presented as mean fluorescent intensity relative to healthy donor controls cultured in parallel.

only a mild degree) in cultured reticulocytes from these patients.

Severe ankyrin-R deficiency abrogates tetrameric band 3 macrocomplex formation

Ankyrin can simultaneously bind to two dimers of band 3 *via* two distinct and co-operative sites located in the D2 and D3-4 domains forming the band 3 tetramer based core for a major membrane cytoskeletal multiprotein complex. We hypothesized that the dramatic reduction in surface expression of Rh complex components but not band 3 under conditions of severe ankyrin deficiency prior to enucleation indicated a requirement for band 3 tetramer formation for stable surface expression of these proteins that would be disrupted in the absence of ankyrin. Using Blue Native PAGE electrophoresis of C12E8 detergent extracts of healthy donor erythroblasts, we show for the first time using this approach that it is possible to distinguish two separate complexes containing band 3 (Figure 5A). Based on co-migration, the upper complex likely contains band 3, ankyrin, protein 4.2 and RhAG, and has a molecular weight consistent with the classical ankyrin associated band 3 tetrameric multiprotein complex. The second band 3, containing complex of lower molecular weight, likely represents the dimeric population of band 3. This population may be partially comprised of band 3 dissociated from tetrameric complexes during detergent extraction; however, immunoblotting with a GLUT1 specific antibody identified this protein to be present specifically within the region of the gel corresponding with dimeric band 3 and completely absent from that overlapping with the ankyrin associated tetrameric band 3 complex. This represents a novel demonstration that, despite its reported association with band 3,[26] GLUT1 does not reside within the ankyrin associated complex of proteins in healthy donor erythroblast membranes. Figure 5B shows that detergent extracts from non-targeting control shRNA transduced erythroblasts have both the higher molecular weight (tetrameric) and smaller complexes of band 3 (two representative immunoblots shown for band 3), whereas in the severe ankyrin knockdown erythroblasts extracts only the lower molecular weight band 3 complex is detected.

RhAG loss in the absence of ankyrin occurs via lysosomal degradation

Reduced expression of RhAG in the absence or severe depletion of ankyrin could be accounted for by impaired delivery or a reduced stability at the plasma membrane. To explore the molecular mechanism that underlies reduced RhAG expression in the absence of ankyrin,

pronase surface epitope recovery experiments were performed to assess the delivery efficiency of RhAG. Figure 6A shows that, following pronase treatment of control and ankyrin shRNA transduced erythroblasts, reappearance of the LA1818 epitope after a 1-h recovery period occurs to a similar degree in both control and ankyrin knockdown erythroblasts, indicating an equivalent level of delivery of RhAG to the plasma membrane in the presence and absence of ankyrin. Contrastingly, over a longer 2-h recovery time period, RhAG surface levels are reduced in the erythroblasts lacking ankyrin relative to control erythroblasts, suggesting an altered stability within the plasma membrane in the absence of ankyrin-R. In contrast, reappearance of band 3 BRIC6 epitopes following pronase treatment is increased after 1 h in ankyrin knockdown erythroblasts but not significantly different to control after 2-h recovery, possibly indicating a more rapid delivery of band 3 to the plasma membrane in the absence of ankyrin mediated band 3 tetramers. Confocal imaging of immunofluorescently labeled RhAG in ankyrin knockdown erythroblasts identified weak plasma membrane labeling of RhAG together with isolated intracellular clusters of RhAG labeling that co-localize with the lysosomal protein LAMP1, indicating localization of RhAG in a lysosomal compartment, which also appears enlarged/aggregated in these cells (Figure 6B). To confirm that reduced RhAG expression in ankyrin deficient erythroblasts results from lysosomal degradation of destabilized plasma membrane RhAG, cells were treated with a combination of lysosomal hydrolase inhibitors leupeptin, pepstatin A and E64d. Figures 6C and D show that treatment of ankyrin knockdown erythroblasts with this combination of lysosomal inhibitors results in an accumulation of RhAG that can be observed by immunofluorescence and immunoblotting of total cell lysates, demonstrating that loss of ankyrin causes lysosomal degradation of RhAG due to instability at the plasma membrane. Of note, we were unable to inhibit degradation of protein 4.2 in ankyrin knockdown erythroblasts using either lysosomal (leupeptin, pepstatin A, E64d), proteosomal (MG132) or calpain (calpain inhibitor I) inhibitors (*data not shown*). Protein 4.2 appears to be rapidly degraded in the absence of ankyrin, but in the presence of band 3 by an as yet undefined mechanism.

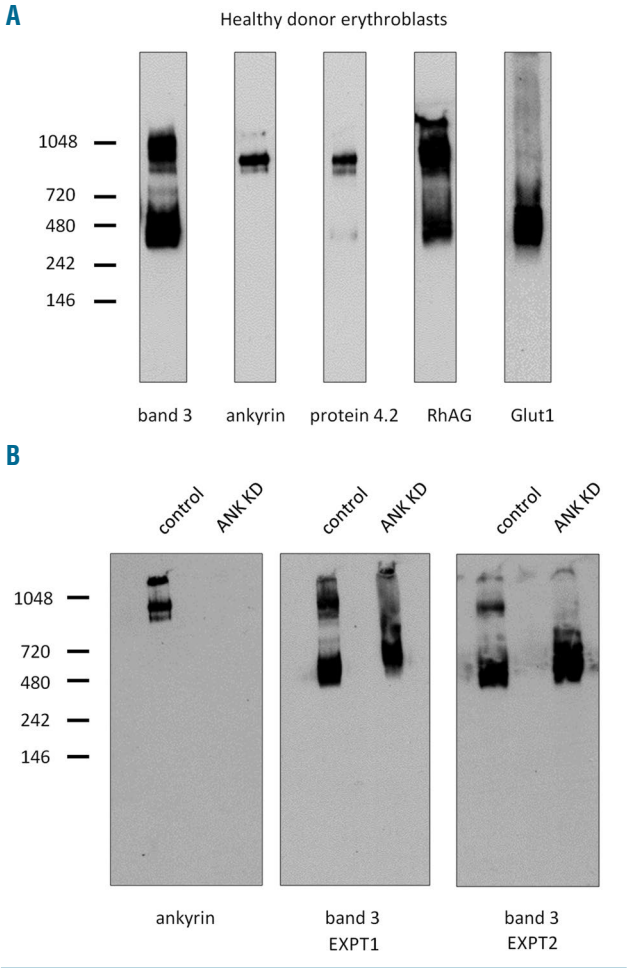


Figure 5. Ankyrin depletion disrupts formation of band 3 tetramers. C12E8 detergent extracts of healthy donor (A) or cells expressing non-targeting control or ankyrin shRNAs (B) removed from culture after 120 h of differentiation were separated by Blue Native PAGE. Protein complexes were immunoblotted using mouse monoclonal antibodies specific for ankyrin (BRIC274), protein 4.2 (BRIC273), or rabbit antibodies against band 3, RhAG or GLUT1 (in house). Two independent examples of separation of the two band 3 complex populations are shown in Figure 4B. Molecular weights are indicated in kDa.

Discussion

Using shRNA mediated knockdown in primary erythroblasts prior to terminal erythroid differentiation we have demonstrated the ability to deplete ankyrin-R expression by up to 95% generating an effective novel near null reticulocyte phenotype. As would be predicted, the reduction of ankyrin expression predominantly impacts the surface expression of membrane proteins assigned to the band 3 tetramer based erythrocyte multiprotein complex. The greatest impact of ankyrin deficiency is not on band 3 itself, however, but on the stability of the Rhesus protein sub complex (RhAG, Rh in particular), whether mediated by shRNA or by natural ankyrin genetic mutations. Importantly, reduced surface expression of the core Rh subcomplex components Rh and RhAG precedes the loss of the more extensively studied ankyrin binding protein band 3. We also demonstrate that absence of ankyrin during erythropoiesis abrogates the ability of band 3 to form the larger of two band 3-containing macrocomplexes (likely the band 3 tetrameric complex) detected by Blue Native PAGE. Protein 4.2, which fails to express at any stage of erythropoiesis in the absence of band 3 (but in the presence of ankyrin),[18] is severely depleted in the absence of ankyrin at a stage of erythroid differentiation at which band 3 surface expression is unaltered, highlighting a reliance of protein 4.2 on the ankyrin mediated tetrameric organization specifically of band 3 for its stable expression. Interestingly severe depletion of ankyrin also impacts upon the reticulocyte expression of α/β spectrin and α adducin, highlighting the importance of this protein for stable maintenance of the wider erythroid cytoskeletal architecture.

A previous study that employed immunofluorescent staining of enucleating ankyrin deficient nb/nb murine erythroblasts attributed reduced expression of band 3, GPA and RhAG on the erythrocytes from this mouse to reduced retention of these proteins within the reticulocyte membrane during enucleation.[20] The data presented here illustrate that, whilst band 3 and GPA do display increased partitioning to the pyrenocyte plasma membrane during

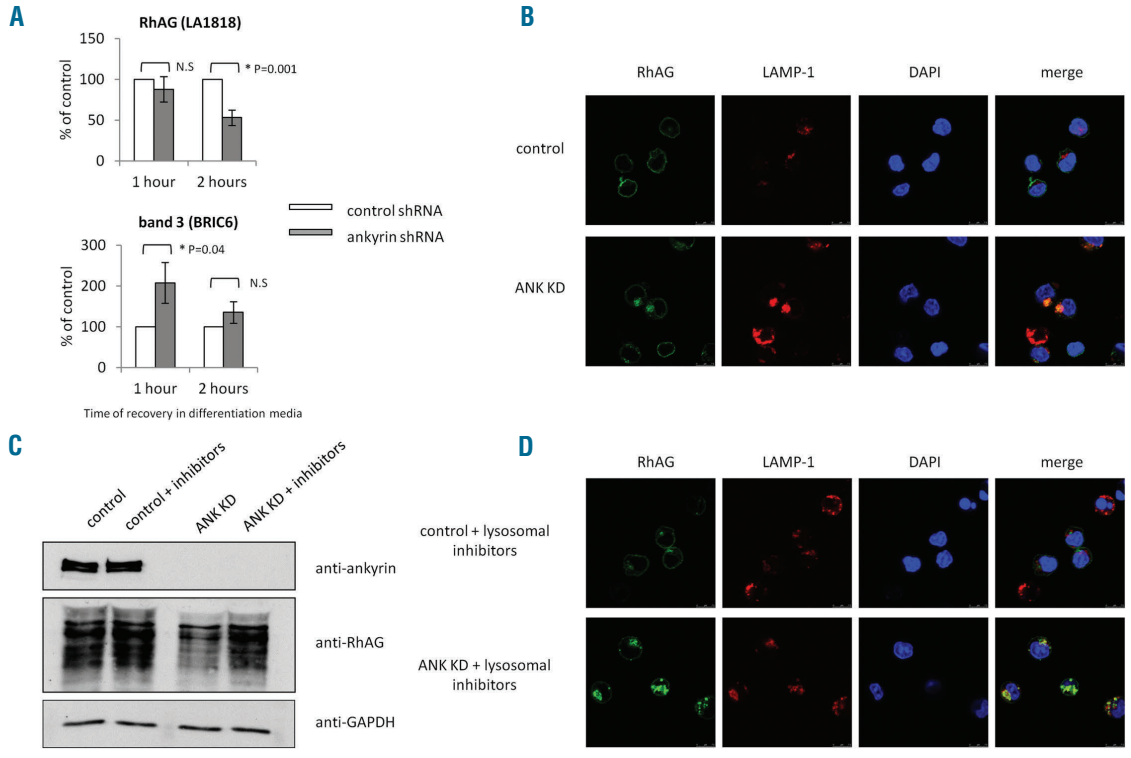


Figure 6. Ankyrin depletion results in lysosomal degradation of RhAG in differentiating erythroblasts. Differentiating erythroblasts (at T72) were removed from culture, washed and re-suspended in 500 μg/mL pronase for 30 min at 37°C. Cells were washed in PBS and transferred into pre-warmed differentiation media. Reappearance of LA1818 and BRIC6 epitopes was assessed at indicated times. (A) Histograms showing mean reappearance of LA1818 and BRIC6 epitopes in ankyrin knockdown erythroblasts as assessed by flow cytometry after 1 h and 2 h recovery as a % of control = (MFI ankyrin knockdown/MFI control)x 100. Error bars represent standard error of the mean derived from data from 4 independent cultures at T72. Statistical significance was assessed using Student's *t*-test. (B) Differentiating erythroblasts at T48 from control and ankyrin knockdown cultures were fixed, immunolabeled with antibodies specific for RhAG (LA1818) and LAMP-1 and stained with DAPI. Images were acquired using a Leica SP5 AOBS confocal laser scanning microscope using a 100x oil-immersion objective (N.A 1.4). (C) Differentiating basophilic erythroblasts from control and ankyrin knockdown cultures were treated with a combination of lysosomal inhibitors as outlined in the Methods section. After 24 h treatment, $5x10^5$ cells were lysed, proteins separated by SDS PAGE and immunoblotted with antibodies specific for ankyrin (BRIC274), RhAG (in house) and GAPDH. Immunoblots are representative of three independent experiments. (D) Differentiating erythroblasts from control and ankyrin knockdown cultures treated with lysosomal inhibitors were fixed, immunolabeled with antibodies specific for RhAG (LA1818) and LAMP-1 and stained with DAPI. Images were acquired using a Leica SP5 AOBS confocal laser scanning microscope using a 100x oil-immersion objective (N.A 1.4).

human erythroblast enucleation, accounting for their reduced reticulocyte expression in the absence of ankyrin, the contribution of this mechanism to reduced reticulocyte RhAG expression is only minor. Intriguingly, we demonstrate that loss of the bulk of the core Rh subcomplex components, Rh and RhAG in the absence of ankyrin is temporally distinct from that of band 3/GPA, and in the case of RhAG, occurs *via* a distinct mechanism involving lysosomal degradation of unstable protein internalized post delivery to the plasma membrane during erythroblast differentiation. This mechanism of RhAG turnover likely also accounts for the loss of RhAG reported in Rhnull phenotype resulting from the p.(Asp399Ala) RhAG mutation within the putative ankyrin binding site,[6] and is also consistent with the surprising stability of RhAG in the absence of band 3 during the early stages of terminal differentiation.[18] Interestingly, ankyrin 'near null' reticulocytes exhibit a slightly greater reduction in the surface expression of Rh than that of RhAG. A population of RhAG is known to be capable of existing within the erythrocyte membrane independently of Rh.[6] One possible explanation for this discrepancy in the levels of the Rh core components is that it is accounted for by a population of RhAG that is independent of both Rh and ankyrin, and achieves membrane stability *via* interaction with non-tetrameric complex band 3. Importantly, Rh/RhAG but not band 3/GPA were also found to be dramatically decreased in the pre-enucleation orthochromatic erythroblasts of a naturally occurring HS patient with a 57% deficiency in ankyrin expression, demonstrating the relevance of observations made using our 'near null' model to understanding secondary protein loss in ankyrin deficient HS patients. The presence of RhAG at close to wild-type levels on orthochromatic erythroblasts and reticulocytes of mildly ankyrin deficient HS patients in spite of reduced erythrocyte expression is surprising. This observation highlights the difficulty in uncovering the basis of secondary protein loss in patients with missense ANK1 mutations that may retain misfolded or truncated protein products with potentially unpredictable effects, and suggests that additional protein loss must occur during reticulocyte remodeling and membrane shedding within the circulation of these patients. The fact that mild ankyrin deficiency appears to be well tolerated (at least in the early stages of erythroid differentiation), suggests that, initially, ankyrin exists in excess of that required for band 3 tetramer and Rh subcomplex stabilization. Nevertheless, the observation that the contribution of different mechanisms of secondary protein loss (pre and post enucleation) differs according to the degree of ankyrin deficiency in

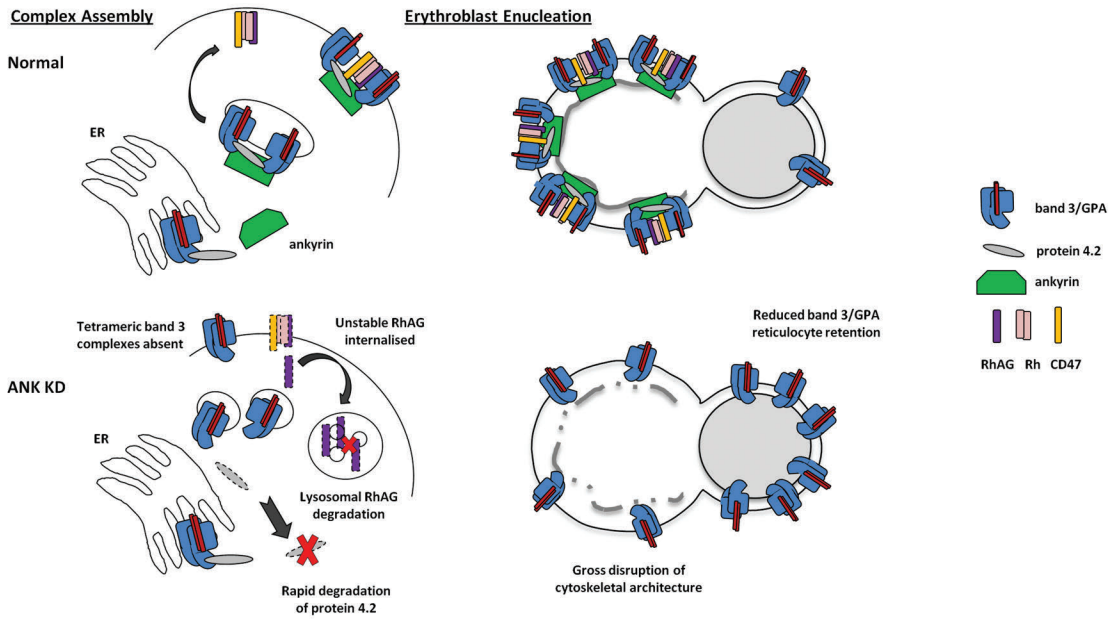


Figure 7. Summary model for impaired complex assembly and loss of proteins in the absence of ankyrin during erythropoiesis. Under normal conditions, band 3 chaperoned by GPA, associates with protein 4.2 in an early intracellular compartment. Ankyrin mediates formation of band 3 tetramers which in turn stabilize plasma membrane expression of the core Rh proteins, Rh and RhAG leading to the formation of a band 3 'macrocomplex'. In the absence of ankyrin, band 3 fails to form a tetrameric complex, protein 4.2 is destabilized and degraded, RhAG, lacking the stabilization provided by association with tetrameric band 3 and/or ankyrin is rapidly internalized post delivery to the plasma membrane and degraded *via* the lysosomes. Dimeric band 3 maintains its association with GPA and is expressed at normal levels within the plasma membrane for the majority of erythroid differentiation. During erythroblast enucleation, gross disruptions in cytoskeletal architecture allied to impaired band 3 connectivity to this structure in the absence of ankyrin result in increased partitioning of band 3/GPA to the pyrenocyte plasma membrane and reduced reticulocyte expression of these proteins.

erythroid cells highlights important features for consideration of the role of ankyrin in membrane protein stability and remodeling at multiple stages in the ultimate development of a mature erythrocyte.

These data expand our understanding of the assembly of multiprotein complexes that occurs as part of erythroid membrane biogenesis during normal erythropoiesis, reveal the role of the cytoskeletal adaptor protein ankyrin-R, and describe the mechanism by which different associated proteins are lost during erythropoiesis. We propose the model summarized in Figure 7 for the assembly of the ankyrin associated multiprotein complex in which plasma membrane expression of Rh/RhAG is reliant upon stabilization by ankyrin. This likely occurs both indirectly through the formation of band 3 tetramers (further supported by the inability of the band 3 membrane domain to rescue reticulocyte expression of RhAG in an otherwise band 3 null environment)[18] and through low affinity direct association of the C-terminus of RhAG with ankyrin, as previously suggested.[6] Absence or severe reduction of ankyrin-R abrogates the formation of band 3 tetramers, resulting in expression of dimeric band 3 only, which results in the loss of protein 4.2 and the rapid internalization and lysosomal degradation of RhAG in differentiating erythroblasts post initial delivery to the plasma membrane. Dimeric band 3 and associated GPA are retained within the plasma membrane throughout the initial stages of erythropoiesis, but display increased partitioning to the pyrenocyte plasma membrane in the absence of cytoskeletal association *via* ankyrin. Reduced reticulocyte expression of other proteins reported here without established associations with ankyrin likely results both from reduced cytoskeletal association and increased loss of plasma membrane during enucleation and subsequent remodeling brought about by gross disruption of membrane cytoskeletal integrity in the absence of ankyrin.

Finally, mutations in the *ANK1* gene are responsible for approximately 50% of HS cases in Europe and North America; however, current methods for identification and quantification of ankyrin deficiency are laborious and time consuming.[17] In addition to the identification of consistently prominent reductions in the expression of the core Rh subcomplex proteins Rh and RhAG across a range of HS patients with ANK1 mutations, we report the application of a monoclonal anti-ankyrin antibody BRIC272 for the quantification of ankyrin expression using intracellular flow cytometry of permeabilized erythrocytes. We, therefore, propose that monitoring expression of Rh/RhAG and ankyrin levels directly by flow cytometry in patient's erythrocytes provides a convenient screen for rapid preliminary identification and categorization of HS arising from mutations in ANK1.

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