

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

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Supplemental Methods

Antibodies

Monoclonal mouse antibodies were BRIC 4 (GPC), BRIC13 (Wrb), 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 cytopins

Cells (1×10^5) were cytopun 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].

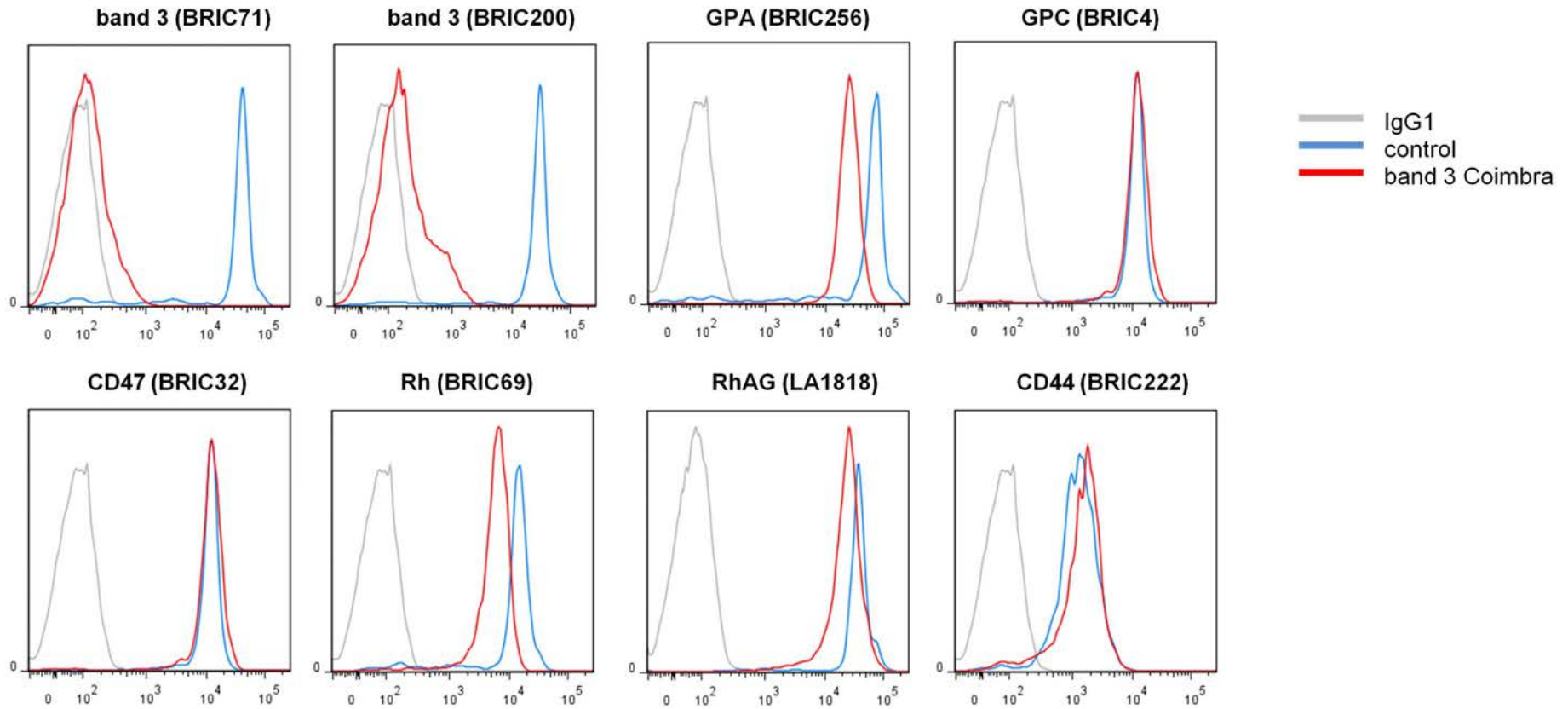
Immunofluorescence

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

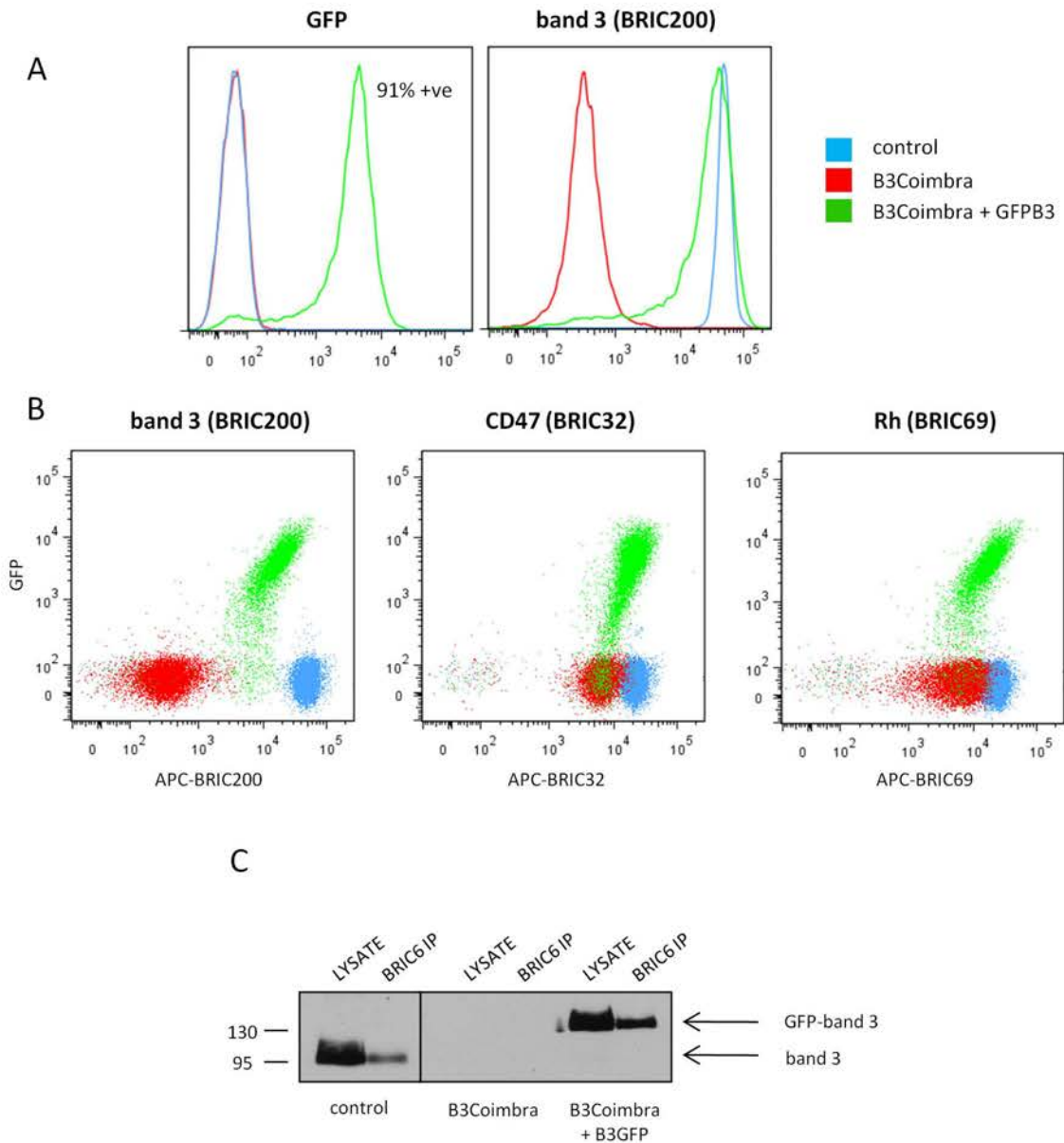
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3. van den Akker, E., et al., *Investigating the key membrane protein changes during in vitro erythropoiesis of protein 4.2 (-) cells (mutations Chartres 1 and 2)*. Haematologica, 2010. **95**(8): p. 1278-86.
4. Satchwell, T.J., et al., *Critical band 3 multiprotein complex interactions establish early during human erythropoiesis*. Blood, 2011.

Supplemental Figure 1



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



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 5×10^6 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 immunoblotted 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.

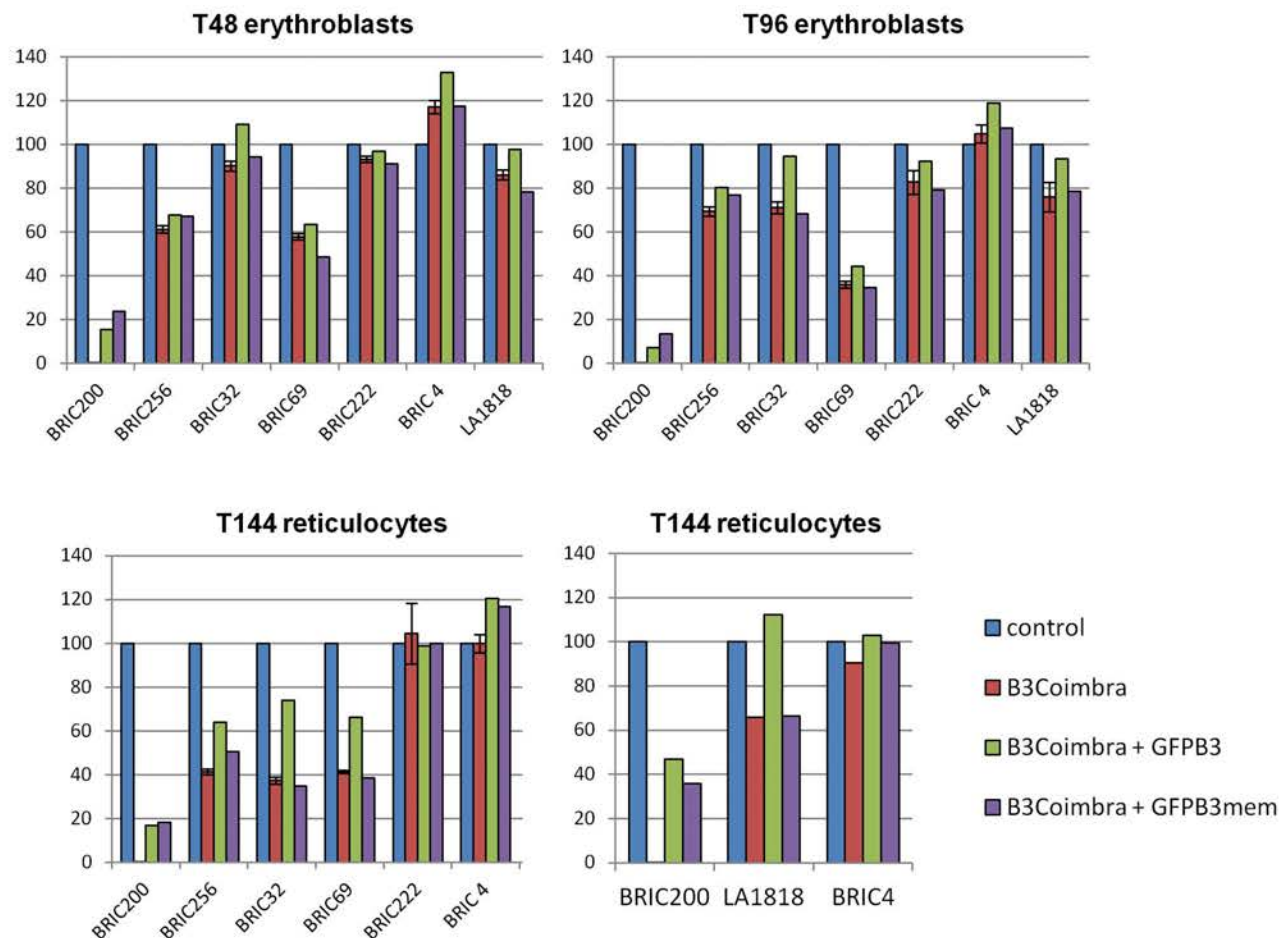
Supplemental Figure 2. Expression of band 3 in band 3 Coimbra reticulocytes can be rescued to wild type levels by lentiviral transduction of patient erythroblasts with N-terminally GFP tagged band 3. A) Representative histogram illustrating transduction efficiency of GFP band 3 in band 3 Coimbra reticulocytes (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 5×10^6 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 immunoblotted 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

Protein	Antibody	band 3 Coimbra (% of control)	band 3 Coimbra + GFPB3 (% of control)	band 3 Coimbra + GFPB3mem (% of control)	band 3 Coimbra + GFPkB3 (% of control)
band 3	BRIC71	0.3 ± 0.1	53.8 ± 4.8	41.5 ± 3.5	27.0 ± 9.9
band 3	BRIC200	0.2 ± 0.2	41.4 ± 8.1	31.8 ± 4.5	19.5 ± 10.2
Wrb	BRIC13	3.1 ± 0.3	67.5 ± 2.7	41.8 ± 6.9	36.5 ± 2.8
GPA	BRIC256	38.6 ± 3.6	72.8 ± 1.5	47.8 ± 3.4	45.5 ± 2.3
CD47	BRIC32	33.6 ± 2.9	86.9 ± 7.4	29.3 ± 2.0	44.5 ± 0.4
Rh	BRIC69	39.2 ± 2.3	69.2 ± 4.7	35.5 ± 2.7	39.5 ± 2.2
RhAG	LA1818	65.4 ± 4.6	101.1 ± 11.0	59.8 ± 6.6	72.6 ± 6.5
CD44	BRIC222	133.7 ± 6.8	104.5 ± 6.6	115.5 ± 12.4	126.1 ± 15.4
GPC	BRIC4	94.9 ± 6.3	104.0 ± 8.4	96.4 ± 13.4	89.3 ± 10.0

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



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 \pm 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.