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Supplement

CD47 surface stability is sensitive to actin disruption prior to inclusion within the band 3 macrocomplex

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Supplementary Figure S1

Figure S1. K562 cells and SH-SY5Y cells express CD47 isoform 2 and isoforms 3 and 4, but CD47 isoforms 3 and 4 were only detected at the surface of SH-SY5Y cells. (A) Cells were lysed and separated by SDS-PAGE (10^6 cells/lane). Western blots were then probed with anti-CD47out1 (Total), anti-CD47 isoform 2 or anti-CD47 isoforms 3 and 4 (protein band indicated by *) (n=2). Total cell, and cell surface immunoprecipitations followed by internal immunoprecipitations, were carried out on 5 - 10 x 10^6 K562 cells (B) and SH-SY5Y cells (C). The eluates were separated by SDS-PAGE against K562 and SH-SY5Y cell lysates (10^6 cells/lane). Western blots were then probed with anti-CD47 isoform 2 or anti-CD47 isoforms 3 and 4 (n=2).
Supplementary Figure S2.

A

CD47 (BRIC32)

Time during differentiation (hours)

CD44 (BRIC222)

MFI

Scramble

Protein 4.2 KD

B

Scramble

Protein 4.2 KD

C

Membrane protein expression (Average MFI expression as a % of the Scramble control ± SEM)

Band 3

Protein 4.2

CD44

CD47

GAPDH

Scramble

Protein 4.2 KD
Figure S2 The hallmarks of protein 4.2 deficiency are recapitulated following shRNA-mediated knockdown of protein 4.2 via lentiviral transduction. Erythroblasts were transduced with lentiviral pLKO.1 protein 4.2 shRNA1, or a scramble shRNA control, as detailed in the methods. (A and C) 1.5 x 10⁶ protein 4.2 deficient and scramble control erythroblasts were incubated with Hoechst for 45 minutes at 37°C, to separate erythroblasts from reticulocytes and nuclei, and fixed in 1% PFA and 0.0075% glutaraldehyde²⁴ prior to labelling with BRIC32 (CD47) or BRIC222 (CD44) for 30 minutes at 4°C. The cells were then incubated with an APC-conjugated monoclonal rat anti-mouse IgG1 secondary, and membrane protein expression was assessed by flow cytometry. (A) Every 24 hours during differentiation expression of CD47 and CD44 on erythroblasts was assessed by flow cytometry (n=3 for each time point). (B) Filtered reticulocytes generated from in vitro cultured protein 4.2 deficient erythroblasts compared to Scramble shRNA control erythroblast, were lysed and separated by SDS-PAGE (1.8 x 10⁵ reticulocytes/lane). Western blots were probed with rabbit anti-C terminal band 3 antibody (YNTU), BRIC273 (anti-protein 4.2), BRIC222 (anti-CD44), anti-CD47 (CD47out1) and anti-GAPDH as a loading control (n=2). (C) Average protein expression of CD47 and CD44 on reticulocytes between 96-144 hours post-differentiation (Average MFI as a % of the scramble control ± SEM (n=3 at each time point), *** p ≤ 0.001; ** p ≤ 0.01 using the Students T test). Emissions from 10,000 events were detected using a MACSQuant Analyser 10 flow cytometer and data was analysed using FlowJo version 10.
Supplementary Figure S3

**Figure S3. Inhibition of CD47 endocytosis in K562 cells.** 1 x 10⁶ K562 cells were incubated with MiTMAB (30µM), Dynasore (80µM), Pitstop 2 (30µM), Sucrose (15mM) or a DMSO control for 20 minutes at 37°C. K562 cells were then stained with (A) BRIC32 (anti-CD47), or (B) P5D2 (anti-β1 Integrin) followed by APC-conjugated monoclonal IgG1 anti-mouse secondary antibody, before being assessed by flow cytometry (Average MFI as a % of the DMSO controls ± SD (n=4); *** p ≤ 0.001; ** p ≤ 0.01, * p ≤ 0.05 using the Students T test). 10,000 events were detected using a MACSQuant Analyser 10 flow cytometer and data was analysed using FlowJo version 10.
**Supplemental Table 1. Summary of the proteomic profile of peptides detected following BRIC126 immunoprecipitations (IP) in K562 cells, EXP, T0 and T48 erythroblasts, and mature erythrocytes (RBCs).** The total BRIC126 IP peptides and IgG peptides are shown alongside the ratio total/IgG. Clean total (Tot) peptides are underlined and bold. * Indicates no ratio due to absence of IgG control peptides. Peptides detected in the BRIC126 IP that are less than 2-fold enriched, compared to the isotype control (IgG) are highlighted in grey.

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