SUPPLEMENTARY APPENDIX

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

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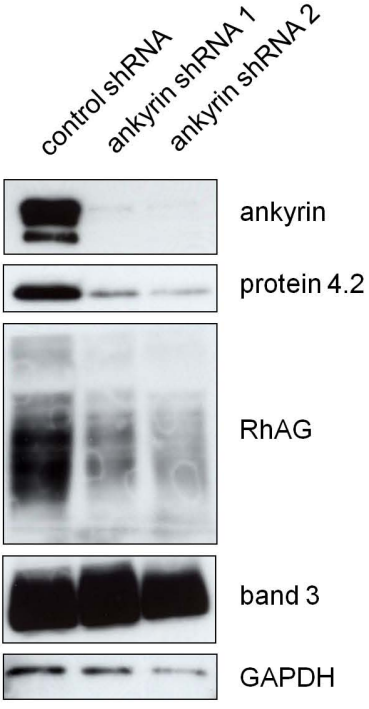
Supplemental Information

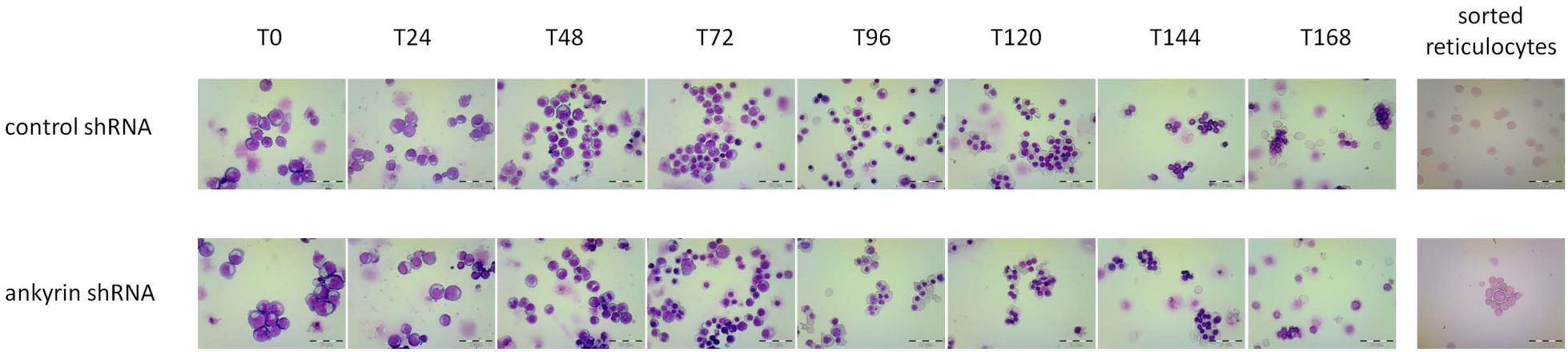
Ankyrin patients

These patients have heterozygous ANK1 mutations that affect diverse regions thoughout the ankyrin molecule; p.(Leu115Pro) and p.(Leu324Pro) concern mutations in the N-terminal domain that binds to band 3, p.(Glu876fs) affects the central spectrin binding domain, aberrant splicing due to the c.4105-1G>A mutation may affect both the spectrin binding domain as well as the C-terminal (regulatory) domain, p.(Thr1734fs) is located in the C terminal regulatory domain whereas the p.(Arg1682*) nonsense mutation is predicted to lead to a truncated ankyrin protein that lacks part of C-terminal domain.

Supplemental Figure 1

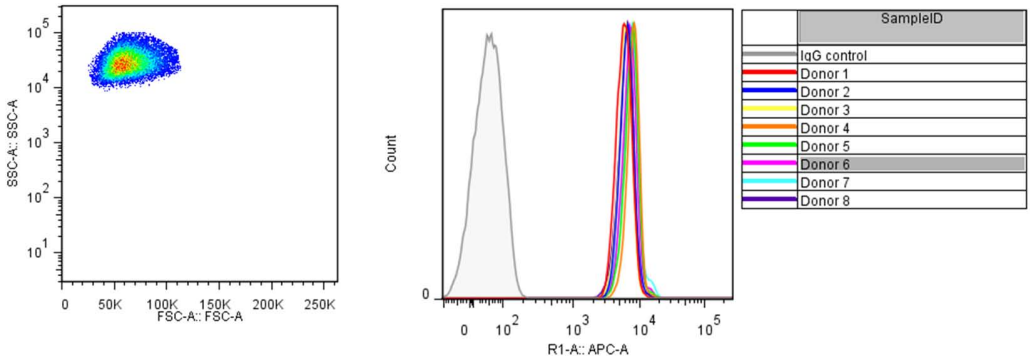


Supplemental Figure 1 Ankyrin knockdown in human *in vitro* culture shows reproducible effects with multiple hairpins. 1x 10^6 orthochromatic erythroblasts (T96) expressing non targeting scramble control shRNA or shRNAs targeting ANK1 were lysed, proteins separated by SDS PAGE and immunoblotted with mouse monoclonal antibodies specific for ankyrin (BRIC274), protein 4.2 (BRIC273), band 3 (BRIC170) or rabbit antibodies against RhAG (in house) and GAPDH (Santa Cruz).



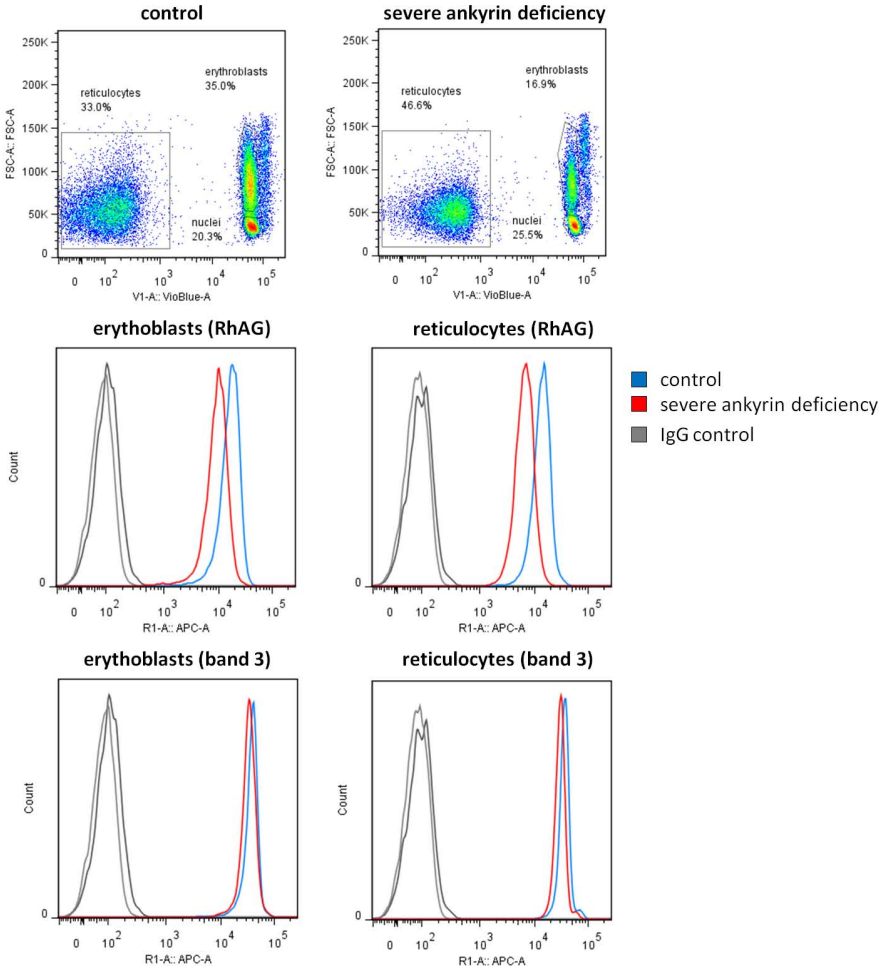
Supplemental Figure 2 – Severely deficient ankyrin erythroblasts exhibit normal morphological progression during differentiation. Cytospins depicting morphological stages of erythroid differentiation of control and ankyrin knockdown erythroblasts

Supplemental Figure 3



Supplemental Figure 3 – Variability in ankyrin expression within healthy donors as measured by flow cytometry using BRIC272

Supplemental Figure 4



Supplemental Figure 4 – Severely ankyrin deficient patients exhibit RhAG loss prior to erythroblast enucleation. Gating strategy for selection of reticulocytes and orthochromatic erythroblasts in control and ankyrin deficient patients together with flow cytometry histograms demonstrating RhAG (LA1818) and band 3 (BRIC71) expression in erythroblasts and reticulocytes.

Supplemental Table 1

Protein	**Antibody**	Source
band 3	BRIC71	IBGRL
band 3	BRIC200	IBGRL
Wrb	BRIC13	IBGRL
GPA	BRIC256	IBGRL
CD47	BRIC32	IBGRL
Rh	BRIC69	IBGRL
RhAG	LA1818	IBGRL
CD44	BRIC222	IBGRL
GPC	BRIC4	IBGRL
ankyrin	BRIC274	IBGRL
ankyrin	BRIC272	IBGRL
α spectrin	BRIC172	IBGRL
β spectrin	BRAC65	IBGRL
band 3	BRIC170	IBGRL
GPA	BRIC163	IBGRL
CD71	DF1513	Santa Cruz
CD99	12E7	George Banting
RhAG	rabbit polyclonal	in house
CD47	rabbit polyclonal	in house
band 3	rabbit polyclonal	in house
GLUT1	rabbit polyclonal	in house
Rh	rabbit polyclonal	in house
LAMP1	rabbit polyclonal	in house
GPC	rabbit polyclonal	in house
GAPDH	rabbit polyclonal	Santa Cruz
Flotillin 2	rabbit polyclonal	Cell Signalling

Supplemental Table 1 List of antibodies used in this study

Supplemental Table 2

Protein	Antibody	4.2 null (% of control)	B3 S667F hetero (% of control)	B3 M663K de novo hetero (% of control)
GPC	BRIC4	106.7	110.9	105.7
band 3	BRIC71	78.1	71.5	76.2
GPA	BRIC256	92.5	94.6	88.3
RhAG	LA1818	96.5	98.5	86.2
Rh	BRIC69	83.6	86.5	78.8
CD47	BRIC32	19.9	98.2	90.6
CD44	BRIC222	465.5	103.4	93.9

Supplemental Table 2 Table showing percentage expression of indicated proteins relative to healthy donor controls for hereditary spherocytosis patients with mutations in band 3 as indicated or absence of protein 4.2