Supplemental Methods

Generation of Isoform Specific SEC23/SEC24 Antibodies

Isoform specific polyclonal antibodies were raised in rabbits against synthetic peptides corresponding to C-SKVPLTQATRGPQVQQPPPSNR (human SEC23A residues 207-228) and C-TKPAMPMQQARPAQPQEHPFASSR (human SEC23B residues 207-230). Antibodies were raised against MSQPGIPASGGAPAS(c) peptide for SEC24A and MSAPAGSSHPAASAR(c) for SEC24B. Antibodies were purified using the specific peptides coupled to Sulpholink™ (Pierce).

Immunoblotting using Tomato lectin

Cell lysates prepared from 0.5-1x10^6 erythroblasts were separated by SDS-polyacrylamide gel electrophoresis and transferred to PVDF membrane. For tomato lectin blots the equivalent of 2x10^4 cells was loaded per lane. For red cell ghosts and lysates, 0.2µg of ghosts and 10µg of lysate were loaded per well, respectively and transferred to PVDF membrane. For cultured cell lysates (t=0 to t=168h), the equivalent of 2x10^4 cells was loaded per lane. The PVDF membranes were blocked for 1 hour in 0.25% BSA in TBST (50mM Tris, 150mM NaCl, 0.1% (v/v) Tween20). Blots were incubated for 45 min with biotinylated tomato lectin (Vector Laboratories, used at 1µg/ml), washed three times in TBST and incubated 45 min with ExtrAvidin™ HRP (Sigma, used 1/8,000 in TBST). After washing three times for 5 min, the signal was detected using ECL™ detection reagent (GE Healthcare) and Amersham Hyperfilm ECL (GE Healthcare).

Immunofluorescence on matured cultured reticulocytes

Reticulocytes were isolated from the *in vitro* cultures by filtration using a 3mm polycarbonate insert (Nunc, Thermo Scientific) which removes the majority of nucleated cells and nuclei. The isolated reticulocytes were then left to mature for 72 hours in differentiation medium supplemented with 10% AB serum. Cells were fixed in 1% PFA and 0.0075% (v/v) Glutaraldehyde in PBSAG (PBS containing 1mg/ml BSA and 2mg/ml glucose) for 15min at room temperature. After 2 washes in PBSAG, the cells were cytospun onto coverslips coated with Cell-Tak (BD Bioscience) and permeabilised for 5 min in 0.05% TritonX100 in PBSAG. Cells were stained with primary antibodies against PDI and GPA followed by staining with a goat anti mouse Alexa 488 and a goat anti rabbit Alexa 594, all in PBSAG, and mounted in Mowiol. Images were taken using a Leica AOBS SP2 confocal microscope (63x/1.4 NA oil immersion lens) and processed using Adobe Photoshop 9.0.

Flow cytometry of peripheral blood red blood cells for PDI
Red blood cells from 4 control donors and 4 CDAII patients were fixed in 1% PFA and 0.0075% (v/v) Glutaraldehyde in PBSAG (PBS containing 1mg/ml BSA and 2mg/ml glucose) for 15min at room temperature. After 2 washes in PBSAG, the cells were permeabilised for 5 min in 0.05% TritonX100 in PBSAG and stained with the primary antibody against PDI or an IgG control antibody, followed by washes and staining

with an APC secondary antibody, all in PBSAG. The data was acquired using a MACsQuant Flow Cytometer (Miltenyi Biotech).

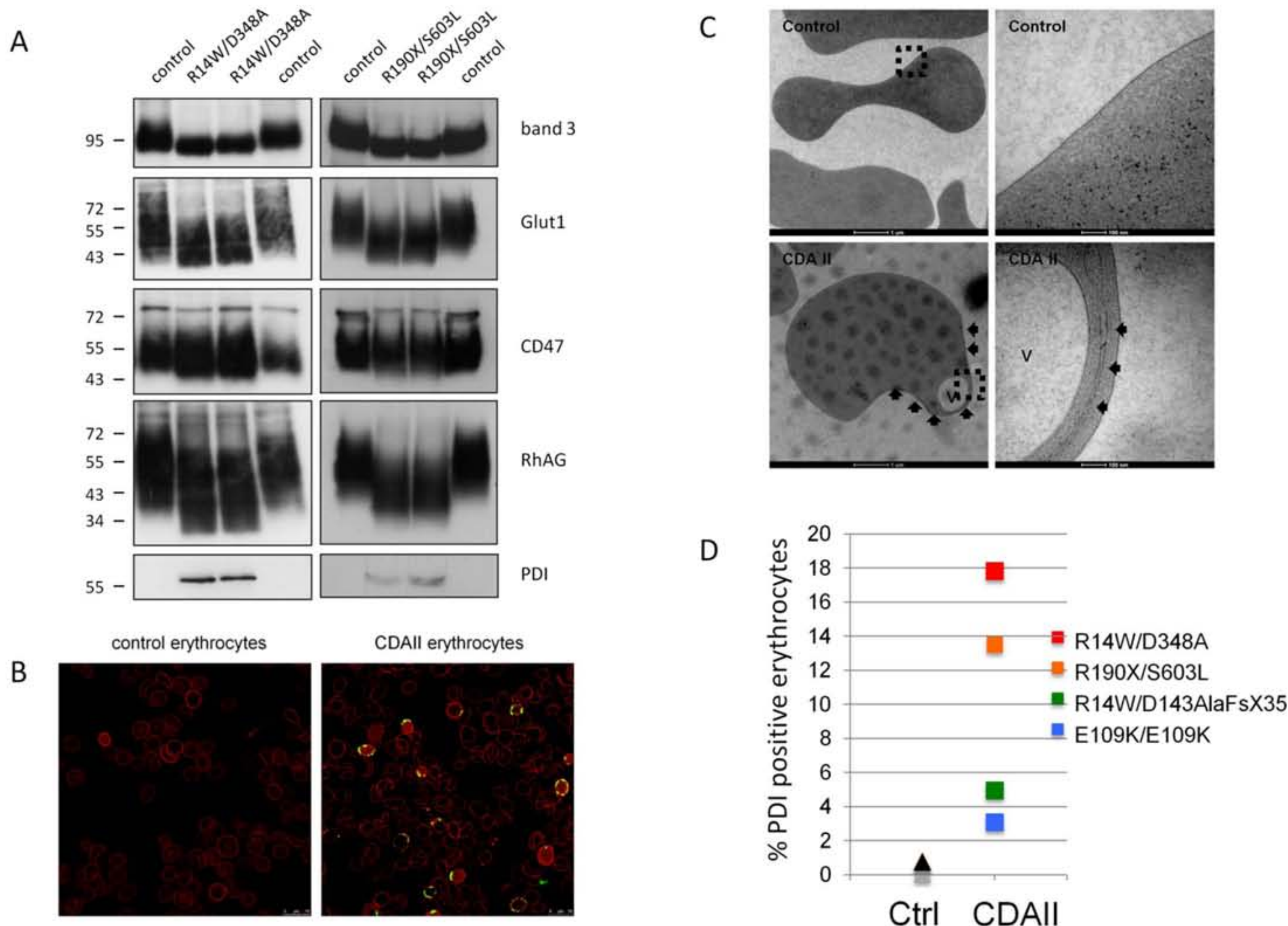
Transmission Electron Microscopy

1-2x10^6 cells were washed in 20% BSA (Sigma), pelleted and resuspended in minimal residual volume. 1µl cells were placed in a gold plated carrier (0.5 mm x 1.2 mm x 200 µm) and frozen in a Leica EM PACT2 high pressure freezer fitted with a rapid transfer system and stored in liquid nitrogen. Cells were prepared for epon embedding by freeze substitution in a Leica AFS2 apparatus as previously described (33). Sections were examined with a Tecnai™ 12 BioTWIN Transmission Electron Microscope (FEI™ Company).

CDA UPN	Molecular characterization	Sex	Age at diagnosis (yr)	Transfusions	Splenectomy (yr)	Hb (g/dL)	Retics (10^9/L)	Unconj. Bilirubin (mg/dL)	Serum Ferritin (μg/L)	Band3 hypoglycos.
0285/01	R14W/R554X	M	34	12/year until splenectomy	yes (38)	9.8	102	1.6	1170	yes
0284/01	R190X/S603L	F	30	3 in infancy	yes (24)	9.9	61	1.2	1791	yes
0267/01	R14W/D348A	F	9	1 at 6 month	yes (9)	9.7	104	0.6	66	yes
0280/01	E109K/E109K	F	24	2 occasional	No	9.5	105	2.5	457	yes
0262/01	R14W/D143AFsX35	F	41	3 in infancy	No	9.7	160	1.5	171	yes
Ref. values						12.2-16.7	24-84	<0.75	19-238	

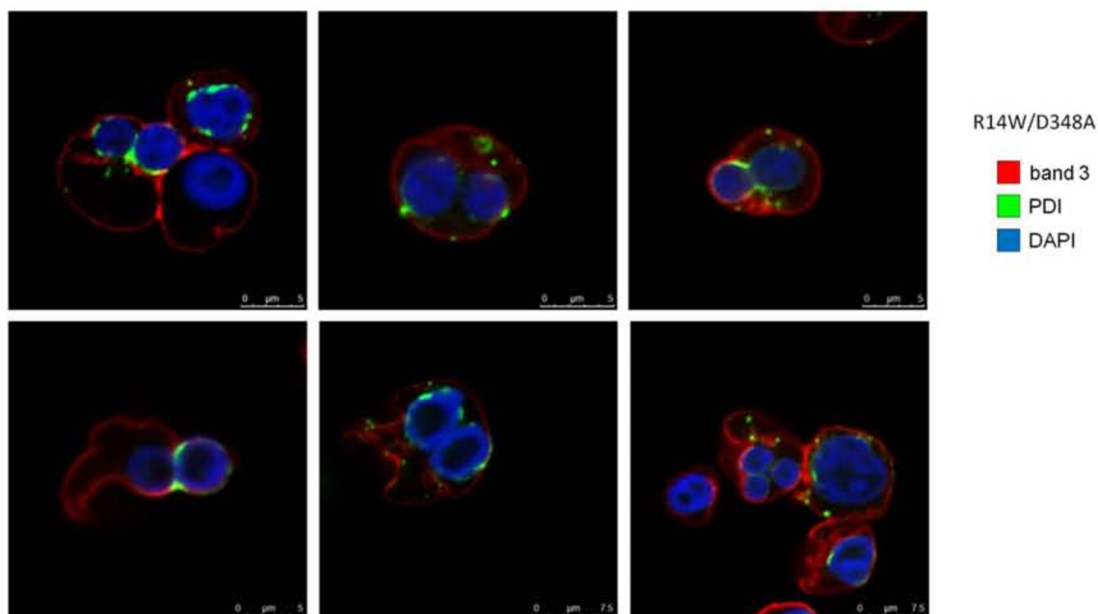
Supplemental Table 1 – Clinical and haematological data of the CDA II patients studied at the time of the diagnosis.

Supplemental Figure 1



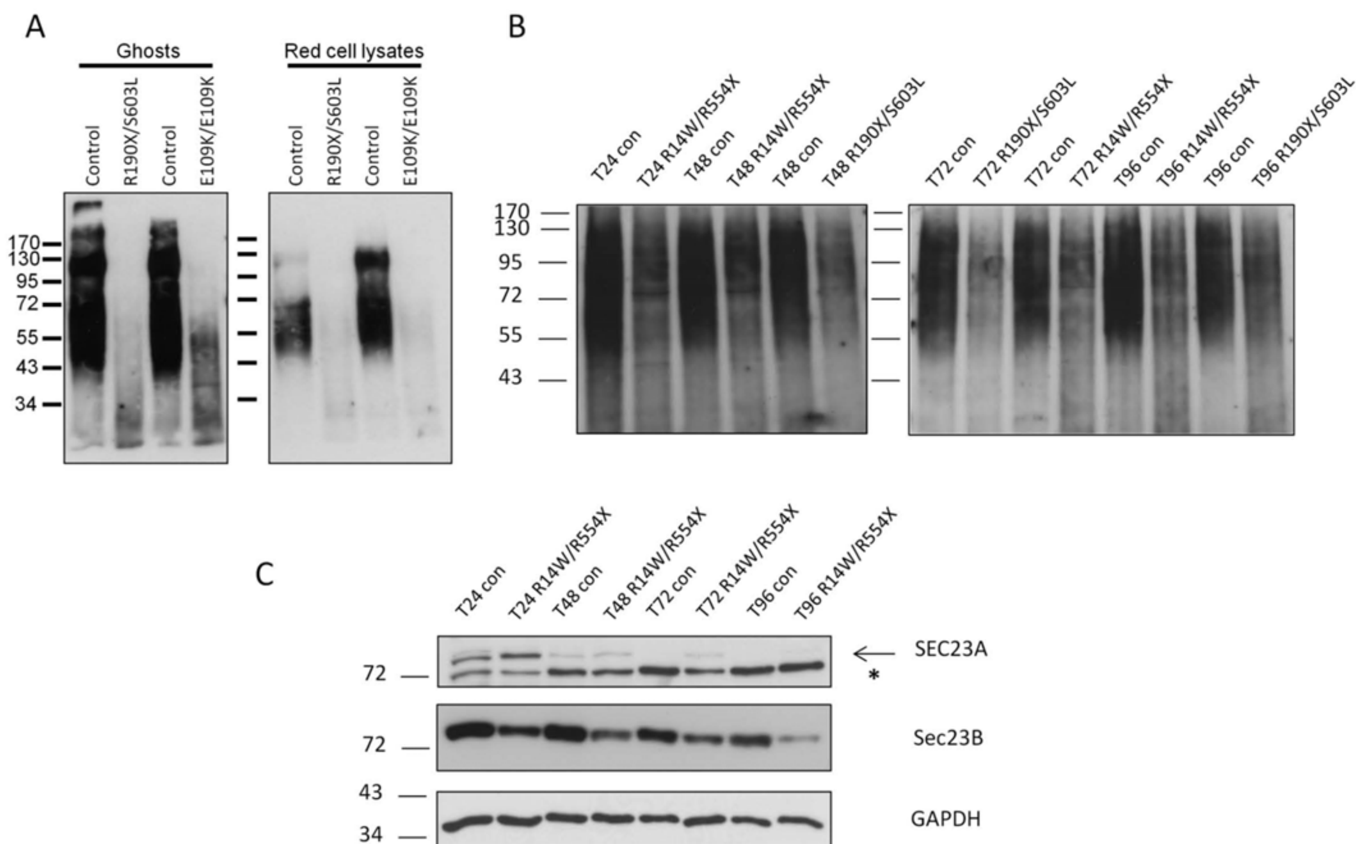
Supplemental Figure 1. CDAII patient erythrocytes display characteristic phenotypes.
A) Hypoglycosylation of erythrocyte membrane proteins in erythrocyte ghosts prepared from 2 unrelated CDAII patients and healthy donor controls in which proteins were separated by SDS PAGE and immunoblotted with antibodies specific for band 3, Glut1, RhAG, CD47. Positive PDI staining in CDAII patient ghost preparations indicates the presence of ER remnants in the membrane of these erythrocytes. **B)** Erythrocytes isolated from the peripheral blood of control or CDAII patient (R14W/D348A) were fixed in acrolein, permeabilised with Triton X-100 and stained with antibodies specific for band 3 (red) and PDI (green). PDI staining indicative of ER remnants in the erythrocyte membrane is present in a subset of CDAII patient but not healthy donor erythrocytes. **C)** Transmission electron micrographs of CDAII patient (R14W/D348A) erythrocytes demonstrate the presence of a second membrane (lower panels) approximately 50 nm beneath the plasma membrane (arrowheads). Panels on the right are high magnification images of the boxed regions in the left panels. Frequently, large vacuoles are localised to double membrane sites (V), with a gap of 50 nm between the vacuole membrane and the "extra" membrane. D) Graph shows the percentage of PDI positive red blood cells in the peripheral blood of 4 control donors and 4 CDAII patients detected by flow cytometry (the values for the 4 control donors are 0.76, 0.79, 0.48, 0.08 and for the indicated 4 CDAII patients are 17.8, 13.5, 4.9 and 3.1).

Supplemental Figure 2



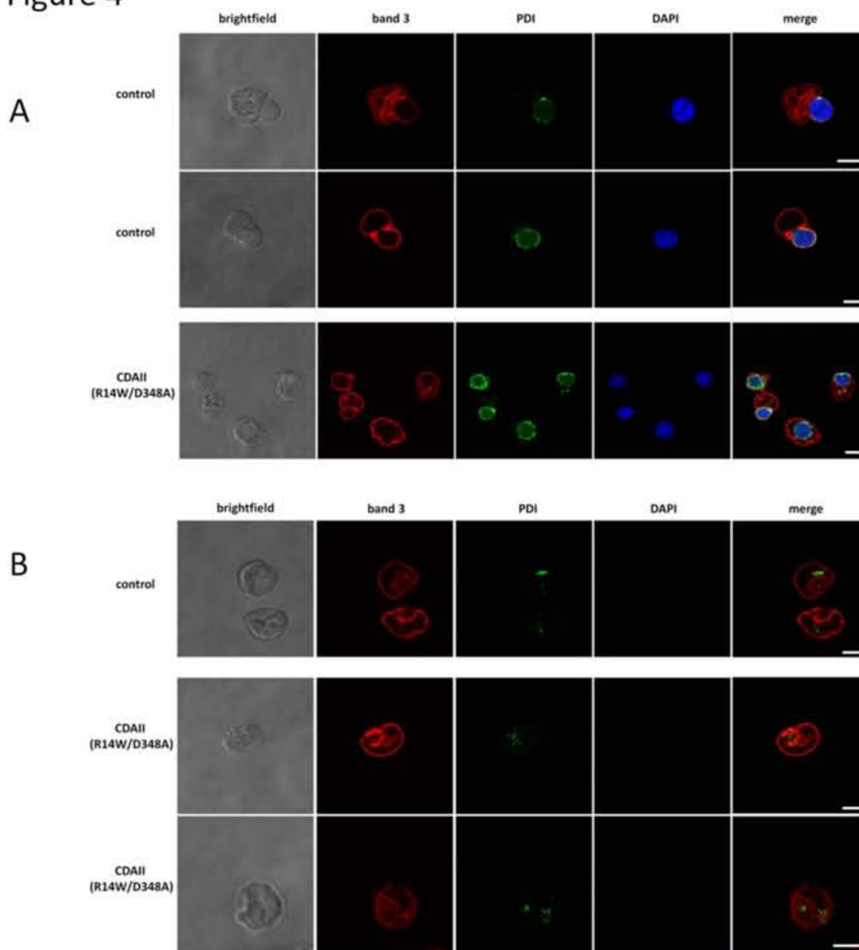
Supplemental Figure 2. Multinuclear orthochromatic erythroblasts can be observed in culture. Confocal images illustrating the presence of multinuclear orthochromatic erythroblasts from patient R14W/D348A generated by *in vitro* culture. Cells were fixed in acrolein, permeabilised and stained with antibodies specific for band 3 (red), PDI (green) and DAPI (blue). Scale bars are indicated on individual images.

Supplemental Figure 3



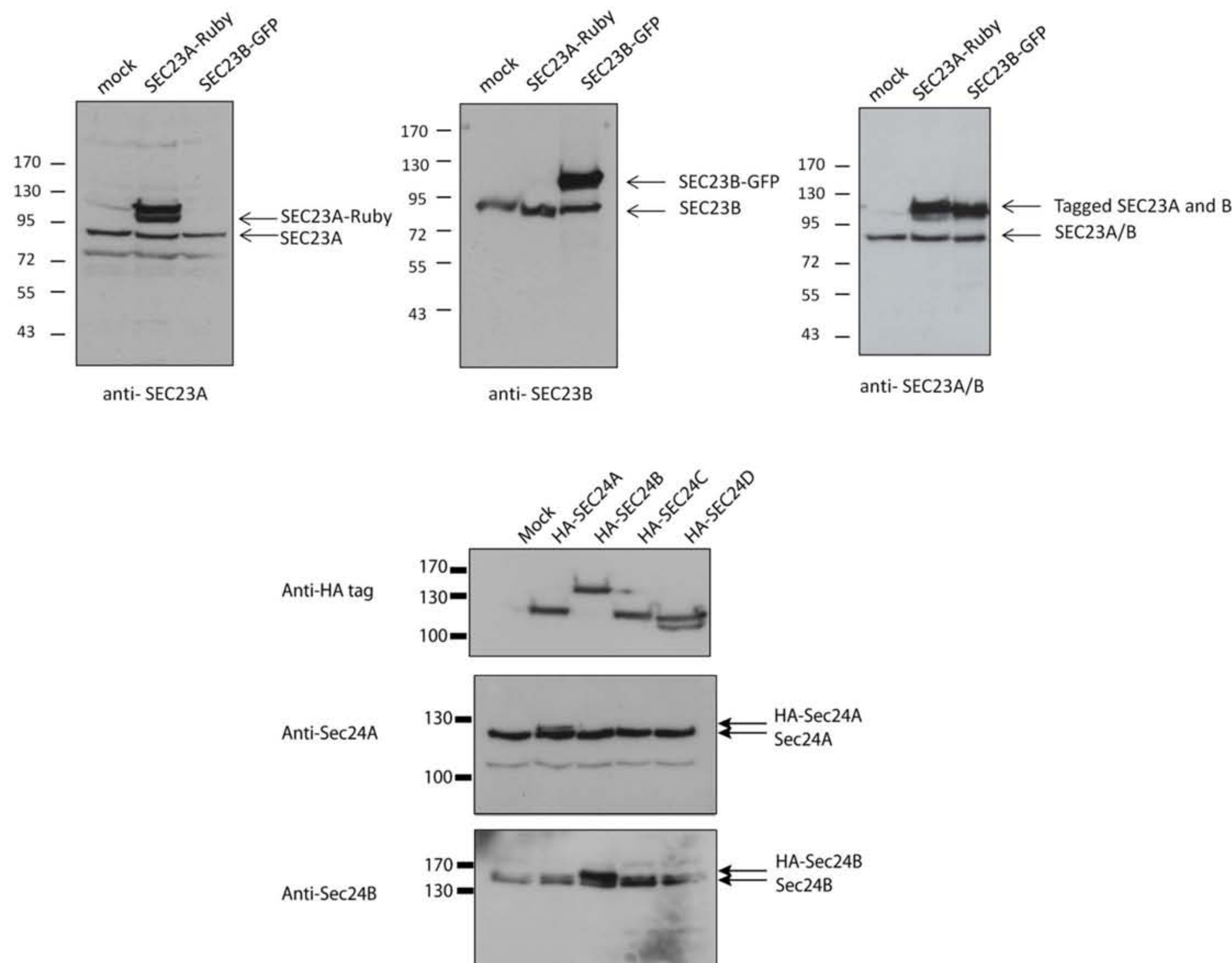
Supplemental Figure 3. Differences between normal and CDAII patient erythrocytes and erythroblasts. A) Proteins from healthy donor or indicated CDAII patient erythrocyte ghosts or whole cell lysates were separated by SDS PAGE and immunoblotted using tomato lectin as outlined in the Supplementary methods. Tomato lectin binds complex glycans and the difference between control and CDAII samples can be observed using this technique. B) $2x10^4$ erythroblasts from normal control or CDAII patient (R14W/R554X and R190X/S603L as indicated) *in vitro* erythroblast cultures were lysed, proteins separated by SDS PAGE and detected using tomato lectin. Alteration of tomato lectin binding can be seen from 24-48 hours in the CDAII patient erythroblasts. C) $5x10^5$ erythroblasts from the R14W/R554X CDAII patient at the indicated time points were lysed, proteins separated by SDS PAGE and immunoblotted with rabbit antibodies to SEC23B, SEC23A, and GAPDH. Arrow indicates SEC23A band, * highlights antibody cross reactivity with an unrelated protein.

Supplemental Figure 4



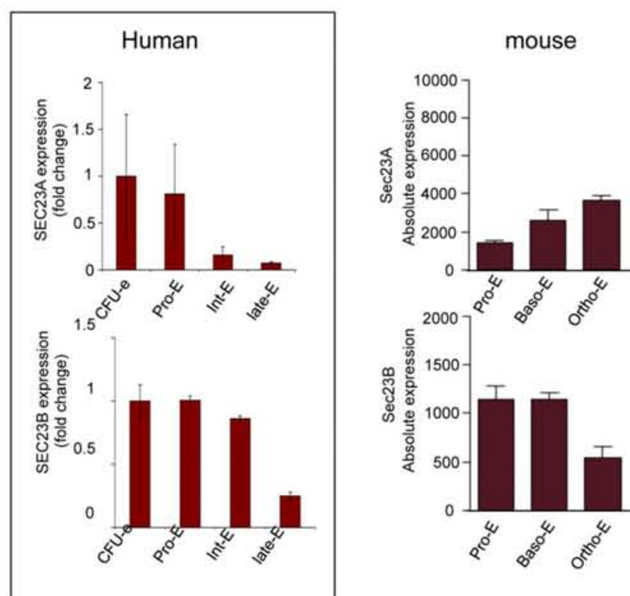
Supplemental Figure 4. Normal clearance of the bulk of ER protein PDI occurs during erythroblast enucleation in CDAII. Confocal images of acrolein fixed enucleating erythroblasts and reticulocytes stained with a rabbit antibody to band 3, monoclonal PDI antibody and DAPI demonstrate **A)** the loss of the bulk of PDI during enucleation of control erythroblasts (top and middle panels) and CDAII patient (bottom panel) and **B)** PDI positively labelled remnants can be observed in both control (top panel) and patient (middle and lower panel) nascent reticulocytes.

Supplemental Figure 5



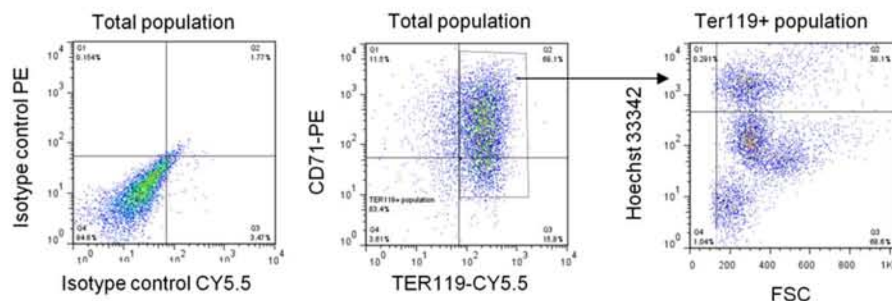
Supplemental Figure 5. Validation of SEC23 and SEC24 antibody isoform specificity. SEC23 isoform specificity of antibodies generated in house to SEC23A, SEC23B was confirmed by expression of GFP tagged SEC23B or Ruby tagged SEC23A in HEK293T cells. Cells were lysed and probed with purified rabbit polyclonal antibodies to SEC23A and SEC23B respectively or a commercial SEC23 antibody that detects both isoforms (Sigma). SEC24 isoform specificity of antibodies generated in house to SEC24A and SEC24B was confirmed by expression of HA tagged SEC24 isoforms in HEK293T cells. Cells were lysed and probed with purified rabbit polyclonal antibodies to SEC24A and SEC24B respectively. Figure demonstrates detection of endogenous and tagged specific SEC23 and SEC24 isoforms for each antibody without cross reactivity with the other isoforms.

Supplemental Figure 6

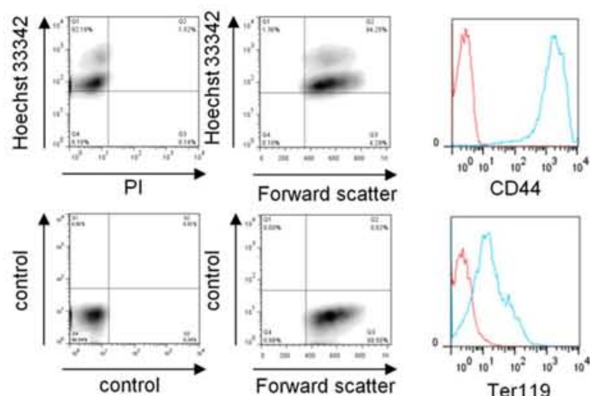


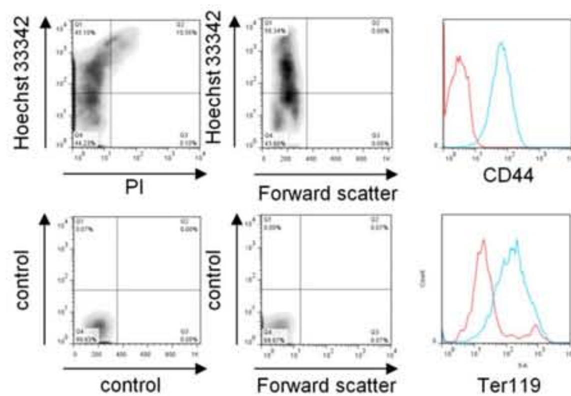
Supplemental Figure 6. Differential mRNA expression of SEC23A and SEC23B during human and mouse erythropoiesis. The mRNA expression profiles of SEC23A and SEC23B mRNA was taken from global gene expression studies of adult mouse primary definitive erythroid cells sorted from bone marrow (Kingsley et al., 2012 Blood) and human erythroid cells produced during erythropoiesis (Merryweather Clarke et al, 2011 Blood). The data represents the average expression and standard deviation of a minimum of three independent experiments. Where applicable data from multiple annotations of the same gene were pooled. The human data is represented as a fold change compared to the CFU-E stage (left panel) and the mouse data as absolute probeset values (right panel). These data show that expression of SEC23A in mouse is maintained during mouse erythropoiesis but is significantly decreased during human erythropoiesis.

Supplemental Figure 7



Fetal liver cells





Supplemental Figure 7 Il3 erythroid cell line and fetal liver derived erythroblast differentiation. Il3 erythroblasts were differentiated as indicated in Materials and Methods. **A)** After 96 hours the CD71/GPA pattern (middle dot plot) and the enucleation rate (right dot plot; using the cell permeable Hoechst 33342) were analyzed by flow cytometry. Isotypic controls are indicated in the dot plot on the left. Note the presence of CD71 and Hoechst low cells in the middle panel and right panel indicative of terminally differentiated enucleated reticulocytes. **B)** Fetal liver erythroblasts were differentiated as indicated in material and methods. Enucleation and CD44/GPA expression was analyzed using flow cytometry. Note the forward scatter (size) reduction, the appearance of Hoechst negative cells (enucleation), a sharp reduction in CD44 expression and the increase in GPA expression upon comparing the 72 hours differentiated fetal liver cells (right panels) to the 0 hours differentiating fetal liver cells (left panel).