Stomatin-deficient cryohydrocytosis results from mutations in SLC2A1: a novel form of GLUT1 deficiency syndrome

Joanna F Flatt1, Hélène Guizouarn2, Nicholas M Burton1,3, Franck Borgese2, Richard J Tomlinson4, Robert J Forsyth5, Stephen A Baldwin6, Bari E Levinson7, Philippe Quittet8, Patricia Aguilar-Martinez8, Jean Delaunay9, Gordon W Stewart10 and Lesley J Bruce1

1Bristol Institute for Transfusion Sciences, N.H.S. Blood & Transplant, UK; 2 Institut de Biologie du Développement et Cancer UMR6543, Université de Nice-CNRS, France; 3Department of Biochemistry, University of Bristol, UK; 4Honeylands Specialist Children’s Centre, Royal Devon and Exeter, UK; 5Institute of Neuroscience, Newcastle University, Newcastle, UK; 6Astbury Centre for Structural Molecular Biology, Institute of Membrane and Systems Biology, University of Leeds, UK; 7Department of Medicine, San Rafael Medical Center, San Rafael, USA; 8Laboratoire Central d'Hematologie et Hôpital Saint-Eloi, Montpellier, France; 9INERM U 779, Faculté de Médecine Paris-Sud, Univ Paris-Sud, Le Kremlin-Bicêtre, France; 10Department of Medicine, University College London, UK

Correspondence should be addressed to:
Lesley Bruce, Bristol Institute for Transfusion Sciences, N.H.S. Blood and Transplant, North Bristol Park, Filton, Bristol, BS34 7QH, UK.
email: lesley.bruce@nhsbt.nhs.uk
Tel: 44(0)117 9217550
FAX: 44(0)117 9125789

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Abstract

The hereditary stomatocytoses are a series of dominantly-inherited hemolytic anemias in which the permeability of the erythrocyte membrane to monovalent cations is pathologically increased. The causative mutations for some forms of hereditary stomatocytosis have been found in the transporter protein genes, RHAG and SLC4A1. Glut1 deficiency syndromes (glut1DS) result from mutations in SLC2A1, encoding glucose transporter 1 (glut1). Glut1 is the major glucose transporter in the mammalian blood-brain barrier and glut1DS are manifested by an array of neurological symptoms. We have previously reported two cases of stomatin-deficient cryohydrocytosis (sdCHC), a very rare form of stomatocytosis associated with a cold-induced cation leak, hemolytic anemia and hepatosplenomegaly but also with cataracts, seizures, mental retardation and movement disorder. We now show that sdCHC is associated with mutations in SLC2A1 that cause both loss of glucose transport and a cation leak, as shown by expression studies in Xenopus oocytes. Based on a 3D model of glut1, we propose potential mechanisms underlying the phenotypes of the two mutations found. We investigated the loss of stomatin during erythropoiesis and find this occurs during reticulocyte maturation and involves endocytosis. The molecular basis of the glut1DS, paroxysmal exercise-induced dyskinesia (PED) and sdCHC phenotypes are compared and discussed.
Introduction

The hereditary stomatocytoses (HSt) comprise a group of hemolytic anemias in which there is a deleterious increase in the diffusional permeability of the erythrocyte membrane to monovalent cations, which destabilises the osmotic balance (and therefore the volume control) of the cell. This “leak” is easily measured as the residual permeability of the erythrocyte membrane to potassium ions when the Na⁺K⁺ATPase and Na⁺K⁺2Cl⁻ cotransporter are inhibited with ouabain and bumetanide.¹ We have previously shown that most cases of cryohydrocytosis (CHC), in which the monovalent cation leak is increased at low temperature, results from amino acid substitutions in the membrane domain of band 3 (anion exchanger 1, SLC4A1).² More recently we have shown that overhydrated hereditary stomatocytosis (OHSt), characterized by a massive cation leak at 37°C and loss of stomatin, results from amino acid substitutions in the Rh-associated glycoprotein (RhAG).³ Band 3 and RhAG associate in the erythrocyte membrane to form a macrocomplex of proteins and may have complementary functions; band 3 is important for CO₂ transport, exchanging HCO₃⁻ ions for Cl⁻ ions and RhAG is a putative gas channel protein.⁴

In this paper we describe an extremely rare form of HSt known as stomatin-deficient CHC (sdCHC). The erythrocyte phenotype falls between CHC and OHSt. The erythrocytes have a large cation leak at low temperatures and are in this respect similar to CHC, but also lack stomatin like OHSt cells.⁵,⁶ Only two cases have been reported and in each case the condition is associated with a neurological disorder and cataracts.⁵ We have previously shown that sdCHC is not associated with mutations in either SLC4A1 or RHAG.⁷ We were prompted to investigate the glucose transporter 1 gene (SLC2A1) following two recent reports. The first showed that association of stomatin with the erythrocyte glucose transporter (glut1) switches glut1 from a glucose transporter to an L-dehydroascorbic acid (DHA) transporter.⁸ The second study showed that a patient with paroxysmal exertion-induced dyskinesia and a mutation in SLC2A1 also had cation leaky erythrocytes, although in this case the erythrocytes were echinocytic rather than stomatocytic.⁹

Paroxysmal exertion-induced dyskinesia (PED) is a mild form of the ever-increasing group of glut1 deficiency syndrome (glut1DS) diseases.¹⁰ In severe forms, there occur motor and mental developmental delay, seizures with infantile onset and movement disorder.¹⁰
The condition results from impaired glucose transport across the blood-brain barrier. Some symptoms of glut1DS can be ameliorated by a ketogenic diet which provides an alternative fuel for the brain. Our patients both suffered from seizures, developmental delay and movement disorders, a phenotype that was strikingly similar to severe glut1DS. However, the phenotype of our two patients was distinct from typical glut1DS or the previously reported PED with a cation leak. Erythrocytes from our patients lacked stomatin and were stomatocytic and both patients suffered with cataracts. Together our results describe a novel glut1DS phenotype.

In these patients we have found two novel mutations in the \textit{SLC2A1} gene: a GGC to GAC substitution in codon 286 resulting in the substitution of a glycine residue with an aspartic acid residue at position 286 (Gly286Asp) and the deletion of three nucleotides (ATC) resulting in the deletion of amino acid Ile435 or Ile436. We have expressed the mutant glut1 proteins in Xenopus oocytes and shown that both mutant proteins leak cations, while neither transports glucose. We have modeled the effect of these mutations on glut1. Using confocal microscopy we have investigated the loss of stomatin during erythropoiesis in sdCHC and OHSt and find this occurs predominantly during reticulocyte maturation and involves endocytosis.
Patients and Methods

Patients

Stomatin-deficient cryohydrocytosis patients: Both patients have been described previously.\textsuperscript{5,6} Patient sdCHC(A) was reported as patient D-II-2, and patient sdCHC(B) as patient E-II-1 in Fricke et al\textsuperscript{5}. Both patients have stomatin-deficient cryohydrocytosis (OMIM 608885; http://www.ncbi.nlm.nih.gov) with leaky erythrocytes (Na\textsuperscript{+} and K\textsuperscript{+} transport rates increased by about ten fold at 4°C) resulting in moderate hemolytic anemia with periodic hemolytic crises.\textsuperscript{5} Hypoglycorrhachia (low cerebrospinal fluid glucose concentration) has not been tested for in these patients.

Overhydrated hereditary stomatocytosis patients: Patient OHSt(A) was described previously.\textsuperscript{3} Patient OHSt(B) has the same phenotype and mutation in \textit{RHAG} (Phe65Ser) as patient OHSt(A) (Unpublished results, G.Stewart & L.Bruce, 25.05.2011)

Glut1DS patients: Both patients had classical glut1 deficiency syndrome (glut1DS) (OMIM 606777: http://www.ncbi.nlm.nih.gov) with hypoglycorrhachia. Patients Glut1DS(A) and Glut1DS(B) were reported as patients 26 and 18 in Leen et al\textsuperscript{12}. Glut1DS(A) presented early with seizures, myoclonic jerks and developmental delay. Since diagnosis at 6 years his condition has been reasonably well controlled by a ketogenic diet and ethosuximide and he is now in special education for moderate learning difficulties. Glut1DS(B) presented with abnormal eye movements at 6 months followed shortly by onset of seizures and global development delay. He later developed microcephaly and four limb spasticity with severe learning difficulties.

All samples were sent by the referring clinicians for diagnostic analysis with the informed consent of the patients or their families in accordance with the Declaration of Helsinki. This work was approved by the National Research Review Committee.

\textit{DNA sequencing analysis}

Genomic DNA was isolated from blood samples. The coding regions of exons 1 to 10 of human \textit{SLC2A1} were amplified by PCR, using exon specific primers, and the DNA sequenced as described previously.\textsuperscript{2} DNA sequencing was used to analyze exons 6 and 10 of \textit{SLC2A1} from all available family members and 35 unrelated controls.

\textit{Erythrocyte membrane protein analysis}

Preparation of erythrocyte membranes, SDS-PAGE and Western blotting of membrane proteins were carried out as previously described.\textsuperscript{4} Treatment with peptide N-glycosidase F was as described.\textsuperscript{13} Only the sdCHC(A) sample was available for study and was typed
as Rh-type ‘rr’. As the quantity of a number of erythrocyte proteins alters with Rh phenotype, Rh ‘rr’ controls were used. Protein concentration was estimated using the Bradford assay and equal amounts (typically 5 or 10 μg) of ghosts loaded per track of each gel. The rabbit polyclonal antibodies to glut1 (raised against the C-terminal sequence of human glut1 (residues 477-492) and stomatin, and the mouse monoclonal antibodies BRIC5 (anti-CD58) and BRIC221 (anti-Lu) were used as previously described. Western blots were analyzed by semi-quantitative scanning densitometry using the Kodak MI software (Carestream Health, Inc. Rochester, NY).

Culture of mononuclear cells

Human mononuclear cells were isolated from buffy coats and cultured based on the method of Leberbauer et al, with modifications, using serum-free StemSpan Expansion Medium (SFEM, Stemcell Technologies, London, UK) supplemented with stem cell factor, insulin-like growth factor 1 and interleukin-3 (SCF, 100 ng/ml; IGF-1, 40 ng/ml; IL-3, 1 ng/ml; R&D Systems, Abingdon, UK), erythropoietin (EPO, 3 U/ml, Roche, Lewes, UK), dexamethasone (dex, 1 mM; Sigma-Aldrich, Dorset, UK) and low-density lipoprotein (LDL, 1 μL/ml; Calbiochem, Nottingham, UK). Cells were seeded at 10 x 10^6/ml on day 0 (phase 1) and were reseeded then maintained at a concentration of 1 x 10^5/ml from day 5 onwards (phase 2) in vented T25 Falcon flasks (Becton Dickinson, Oxford, UK) in 5% CO2 at 37°C. Cell cultures were differentiated (phase 3) using SFEM supplemented with holotransferrin (1 mg/ml; R&D Systems, Abingdon, UK) and AB serum (3 % v/v), EPO (10 U/ml), insulin (10 ng/ml) and 3,5,3'-triiodo-L-thyronine (T3, 1 μM) from Sigma-Aldrich, Dorset, UK.

Confocal microscopy

Early stage cells were seeded on 0.01% (w/v) poly-L-lysine (Sigma-Aldrich, Dorset, UK) coated cover slips (0.5 x 10^6 cells per coverslip) and incubated 1 h at 37°C in 5% CO2. Cells were fixed with 3% formaldehyde (TAAB, Aldermaston, UK) for 20 minutes and permeabilized with 0.05% (w/v) digitonin (Sigma-Aldrich, Dorset, UK) for 5 minutes. Antibody application and imaging was done as described. Late stage cells/reticulocytes were seeded as above but fixed in 1% formaldehyde, and permeabilized in 0.05% (w/v) saponin (Sigma-Aldrich, Dorset, UK) with subsequent washes and incubations including 0.005% (w/v) saponin. Mature erythrocytes were seeded as above, incubated 20 min at RT, then fixed in 1% formaldehyde plus 0.0075% glutaraldehyde and permeabilized in 0.1% (w/v) Triton-X100 (Sigma-Aldrich, Dorset, UK). Primary antibodies used were: rabbit
polyclonal anti-glut1 (ref 14), mouse monoclonal anti-stomatin (GARP50, ref 18) and rabbit polyclonal anti-transferrin receptor (Abcam, Cambridge, UK). Goat anti-mouse Alexafluor 488 or goat anti-rabbit Alexafluor 546 secondary antibodies were used. Samples were imaged using 40 x oil-immersion lenses on a Leica DMI6000 B inverted microscope with phase contrast connected to a Leica TCS-SP5 confocal laser scanning microscope (Leica, Wetzlar, Germany). Images were obtained using Leica LAS software and subsequently processed using Adobe Photoshop (Adobe, San Jose, CA).

**Preparation of mutant constructs and expression in Xenopus laevis oocytes**

The cDNA clone, pBS/KS-glut1, was prepared by inserting a BamHI fragment containing the entire coding region of glut1 from the original pSGT-glut1 clone into the BamHI site of pBluescript II KS (+). We found that this original clone coded for phenylalanine at position 152. Amino acid 152 is now known to be a leucine residue (Human Genome project; http://www.ncbi.nlm.nih.gov/genome/guide/human/). Therefore we first corrected the pBS/KS-glut1 clone by substituting Phe152 with Leu152 using the Quikchange II mutagenesis kit (Strategene, Amsterdam Zuidoost, The Netherlands) using pBS/KS-glut1 as a template and the primers:

- Phe152Leu sense: tcaccacagcctcttcggtgctgcc
- Phe152Leu antisense: gggccccaggaagagcttggtgta

The amino acid substitution Gly286 to Asp286 and the deletion of amino acid Ile435 or Ile436 (one ‘TCA’ was deleted from the sequence) were made using the Quikchange II mutagenesis kit (Strategene, Amsterdam Zuidoost, The Netherlands) using pBS/KS-glut1corrected as a template and the primers:

- Gly286Asp sense: cccagcagctgctgacatcaacgctgtctt
- Gly286Asp antisense: aagacagcctgacatcagcacagcttggtg
- ΔIle435 sense: acgtgtgtgctccctacgtctctcactcactgtg
- ΔIle435 antisense: cacagtgtagatgacagctgtagggaccacacagt

The sequences of the final constructs were confirmed by automated sequencing (ABI PRISM 3100 Genetic Analyzer Automatic Sequencer (Applied Biosystems, Warrington, UK)). The methods used for the preparation of cRNA, expression in oocytes and measurement of Na+ and K+ content by flame photometry have been described. The method used for the measurement of oocyte Li+ influx has been described.
**Immunoblotting of expressed protein in oocytes**

Oocyte membranes were prepared as described previously\textsuperscript{21}, and separated on a 10% SDS-PAGE polyacrylamide gel as described.\textsuperscript{3} Western blots were probed with the rabbit polyclonal anti-glut1 antibody\textsuperscript{14} (1:40,000).

**Deoxy-D-glucose uptake measurements in oocytes**

Oocytes dedicated to glucose uptake measurements and membrane cryo-sectioning were injected with 20ng cRNA and incubated for 48-72 h at 18°C in OR3 medium with composition in mM: CaCl\textsubscript{2}, 0.74; MgCl\textsubscript{2}, 0.58; MgSO\textsubscript{4}, 0.48; KCl, 3.1; KH\textsubscript{2}PO\textsubscript{4}, 0.26; NaCl, 81; Na\textsubscript{2}HPO\textsubscript{4}, 0.79; D+ galactose, 2.9; sodium pyruvate, 2.9; penicillin 100 U/ml; streptomycin 100 µg/ml. The oocytes were then transferred to Modified Barth’s Solution (MBS) with composition in mM: NaCl, 88; KCl, 1.0; NaHCO\textsubscript{3}, 2.4; HEPES, pH 7.6, 10; CaCl\textsubscript{2}, 0.41; Ca(NO\textsubscript{3})\textsubscript{2}, 0.33; MgSO\textsubscript{4}, 0.82; sodium pyruvate, 2.5; penicillin, 20 IU/ml; streptomycin, 0.02 mg/ml. Transfer was more than 18 h prior to the glucose uptake experiment to allow equilibration to the medium change. Oocytes (5 per condition) were incubated in MBS or MBS containing phloretin (100 µM) and cytochalasin B (50 µM) for 20 min at RT and then incubated for 20 min (within the linear phase of glucose influx) with gentle agitation in 2-[\textsuperscript{3}H(G)]-deoxy-D-glucose solution (25 µM 2-deoxy-D-glucose, 0.5 µCi/ml in MBS), then washed 3 times with stop solution (100 µM phloretin in PBS, ice cold). Oocytes were solubilised in 1% sodium dodecyl sulphate solution (SDS) and radioactivity measured using a scintillation counter.

**Cryo-sectioning and immunohistochemical staining of oocytes**

Glut1 expression at the plasma membrane of oocytes was confirmed by immunofluorescence microscopy as described previously.\textsuperscript{22} Permeabilization and staining of oocytes were carried out as described previously\textsuperscript{22} but using an anti-glut1 rabbit polyclonal antibody\textsuperscript{14} (1:5000 in 4% BSA) and goat anti-rabbit Alexafluor 546-conjugated secondary antibody (1:500).

**Structural modeling of the mutant glut1 proteins**

The crystallographically-determined structure of the *Escherichia coli* glycerol-3-phosphate transporter (PDB ID 1pw4; ref 23) was used as a template to build homology models of the wild-type and mutant glut1 proteins using the MODELLER software\textsuperscript{24} and the NCBI reference sequence NP_006507.2 which contains a leucine at position 152. Twenty models were generated for each of the wild-type and mutant glut1 proteins. The large
unaligned loop regions (residues Glu43-Thr62, Pro208-Met252 and Gln469-Val492) were further optimised using the MODELLER ‘dope_loopmodel’ routine. One hundred models were generated for each loop and scored with the DOPE function. Ten models for each isoform with the best overall DOPE scores were superposed and visually inspected. The three models (wild-type, Gly286Asp and △Ile435) with the best overall DOPE scores were selected for further analysis. The quality of each model was assessed with MOLPROBITY.

Statistics
Statistical significance was calculated using the two-tailed, unpaired Student’s t test.
Results

DNA analysis

DNA analysis of the SLC2A1 gene in patient sdCHC(A) showed a GGC to GAC point mutation in codon 286 leading to Gly286Asp amino acid substitution. In patient sdCHC(B), we found a three nucleotide deletion (ATC) leading to the deletion of either Ile435 or Ile436 (YVFIIFTVLL to YVFIFTVLL) (Figure 1). The Gly286Asp substitution was not found in the parents or two siblings of patient sdCHC(A). Relatives of patient sdCHC(B) were not available for study. Both the Gly286Asp substitution and the Ile435 deletion are presumed to be spontaneous de novo mutations. Neither mutation was found in 35 normal control DNAs. These are novel glut1 mutations and differ from those reported in other glut1DS or PED patients (Figure 1). The SLC2A family of proteins is highly similar across all 13 isoforms (Suppl. Figure 1). SLC2A1 is highly conserved across all species (Suppl. Figure 2). Ile435 and Ile436 are almost completely conserved across all the species examined (Suppl. Figure 2), and Gly286 is one of only 24 residues in the glucose transporter family that are completely conserved across all species and isoforms (Suppl. Figures 1 & 2).

Glut1 modeling

We examined the structural basis for the cation leak and loss of glucose transport activity caused by the Gly286Asp and △Ile435 mutations using our refined glut1 models (Figure 2A). Full details of the MOLPROBITY analysis of all glut1 models are given in Table 1. Our model of wild-type glut1 predicts that Gly286 lies adjacent to the putative glucose transport pathway (Figure 2B). This is consistent with the findings of Salas-Burgos et al., who predicted that Gly286 would form part of an exofacial glucose binding site. Our model of Gly286Asp glut1 predicts the potential formation of a novel salt bridge between Asp286 and Lys38, which lies on the N-terminal membrane span (TM1) (Figure 2B). This salt bridge would probably reduce the conformational mobility of the protein in this key region, explaining the lack of glucose transport in this mutant. Even in the absence of salt bridge formation, the Gly286Asp mutation is likely to be deleterious for glucose transport. Hruz and Mueckler showed that the more conservative mutation of Gly286 to cysteine reduced activity by 75%, possibly due to the proximity of Gly286 to residues Gln282 and Gln283, which have been postulated to form hydrogen bonds to glucose.
The ΔIle435 mutation removes a single amino acid from the C-terminal membrane span (TM12) which forms extensive contacts with TM7. All of the residues in TM12 are tolerant to substitution with cysteine, consistent with this helix having no direct role in formation of the transport pathway. However, residues Y432VFII in the N-terminal, extracellular end of TM12 are PCMBS-sensitive when mutated to cysteine, suggesting that at some stage of the transport cycle these residues are exposed to extracellular solvent. Other models of glut1 suggest Y432VFII may even be permanently solvent-exposed.

The change in sequence register caused by the ΔIle435 mutation results in residues C-terminal of Ile436 on TM12 (437 – 458, see Figure 1 and 2C) shifting to the i-1 position on the α-helix, relative to their position in the wild-type protein. The homology model of ΔIle435 glut1 predicts that between residues Phe437 and Thr448 these changes could be easily accommodated with the existing protein fold. However, the side-chain of Tyr449 is too bulky to fit in the small pocket formed by the close packing of TM9 and TM12 in this region (Figure 2C). In order for the overall fold to be maintained Tyr449 is forced to adopt an energetically unfavourable side-chain conformation. Therefore, the mutation may be accommodated by larger, longer-range conformational changes which would be difficult to predict accurately.

Erythrocyte membrane protein analysis

Immunoblotting analysis of stomatin-deficient CHC erythrocyte membranes (sdCHC(A)) confirmed the reduction of stomatin, as described previously, and showed that most other major membrane proteins – band 3, RhAG, CD47, glycophorin A, glycophorin B, protein 4.2, protein 4.1, p55, flotillin 1, CD44, CD59, DAF, AQP1, LW were present in normal amounts (data not shown). Two adhesion proteins, the Lutheran blood group glycoprotein (Lu, basal cell adhesion molecule (BCAM), CD239) and the lymphocyte function associated protein 3 (LFA-3, CD58) were increased in sdCHC(A) membranes (Figure 3B). This increase has been noted before in other hemolytic anemias but the cause is not known (ref 4; Bruce LJ unpublished results). Immunoblotting analysis showed the glut1 protein from sdCHC(A) erythrocytes was expressed at normal levels compared to control glut1 (Figure 3A). Semi-quantitative scanning densitometry analysis of the deglycosylated bands showed that glut1 protein in sdCHC(A) was 101% that of control. By contrast, immunoblotting of erythrocyte membranes from two glut1DS patients showed that they have reduced glut1 levels but normal stomatin levels (Figure 3A). Semi-
quantitative scanning densitometry analysis of the deglycosylated bands showed that glut1 protein in both glut1DS(A) and glut1DS(B) was about 63% that of control. These results suggest that whereas the glut1DS erythrocytes only express wild type glut1, the sdCHC erythrocytes express both the wild type and the mutant glut1 proteins.

Deoxy-D-glucose (3-OMG) transport in oocytes
Uptake of deoxy-D-glucose was measured in Xenopus oocytes. Two days after cRNA injection (or three days after, inset Figure 4A) the water-injected oocytes showed minimal deoxy-D-glucose uptake whereas wild-type glut1 (glut1-WT) oocytes showed a large uptake (~63 pmol/oocyte/hour). Oocytes expressing the glut1-Gly286Asp or glut1-\(\Delta\)Ile435 mutants showed minimal uptake (~12 pmol/oocyte/hour) and oocytes co-expressing mutant and glut1-WT showed about 50% of glut1-WT uptake (~39 pmol/oocyte/hour) (Figure 4A). In a different experiment, uptake of deoxy-D-glucose was measured in Xenopus oocytes with and without the glucose transporter inhibitors phloretin (100 μM) and cytochalasin B (50 μM). In the absence of inhibitors, there was no significant difference between the deoxy-D-glucose uptake in water injected oocytes and in oocytes expressing the glut1-Gly286Asp or glut1-\(\Delta\)Ile435 mutants, suggesting that the mutant proteins do not transport glucose (Figure 4B). As before, oocytes co-expressing mutant and glut1-WT showed about 50% of glut1-WT uptake (Figure 4B). In the presence of inhibitors deoxy-D-glucose uptake was reduced to about the same level in all oocytes. Uptake of 3-O-methyl-D-glucose (3-OMG) was also measured and oocytes expressing the glut1-Gly286Asp or glut1-\(\Delta\)Ile435 mutants showed minimal 3-OMG uptake (Suppl. Figure 3). Immunocytochemistry of oocyte slices showed that all glut1 constructs were expressed in the oocyte membrane (Suppl. Figure 4).

Cation transport in oocytes
The mutant glut1 proteins were expressed in Xenopus oocytes and cation leak measured. Normal levels of the intracellular cations Na\(^+\) and K\(^+\) were found in Xenopus oocytes expressing non-injected (NI) or glut1-WT, whereas the levels of these cations were altered in oocytes expressing glut1-Gly286Asp or glut1-\(\Delta\)Ile435 (Figure 5A). Li\(^+\) influx was minimal in oocytes expressing NI or glut1-WT, whereas expression of glut1-Gly286Asp or glut1-\(\Delta\)Ile435 increased Li\(^+\) influx (Figure 5B). All glut1 constructs were shown to be expressed in the oocyte (Inset Figure 5B).
Confocal imaging
The expression of glut1 and stomatin were imaged in early and late erythroblasts cultured from mononuclear cells (isolated from peripheral blood) and in peripheral blood from control, sdCHC(A) and OHSt(A) samples. Stomatin and glut1 are expressed at the plasma membrane of early erythroblasts from both control and sdCHC cells (Figure 6A). Stomatin was still present and colocalised with glut1 in the plasma membrane of both control and sdCHC cells at the reticulocyte stage (Figure 6B). However sdCHC reticulocytes also showed more internal stomatin than controls, appearing in vesicles or as an aggregate at the reticulocyte/nuclear junction (Figure 6B, 7). Control peripheral blood erythrocytes showed colocalisation of stomatin and glut1 at the membrane in all cells, whereas stomatin was only present in a minority of sdCHC erythrocytes, probably the reticulocytes or immature erythrocytes (Figure 6C). These results suggest that in sdCHC cells stomatin is lost late, during reticulocyte maturation. This result was unexpected since in a previous study we had shown that stomatin was lost from OHSt(A) cells during early erythropoiesis.36 In order to make a direct comparison between the two stomatin-deficient cell types, we cultured OHSt(A) erythroblasts under the same conditions used for the sdCHC cell culture. We found that glut1 was present in the internal compartments and at the plasma membrane in the early erythroblasts from both control and OHSt(A) cells (Figure 6D). However, stomatin remained intracellular in these early OHSt(A) cells (Figure 6D). This result confirms our previous study36 and shows that the timing and mechanism of stomatin loss differs between these OHSt(A) and sdCHC cells. It was not possible to culture these OHSt(A) erythroblasts to the reticulocyte stage however peripheral blood erythrocytes from this patient3 showed some limited expression of stomatin in a few cells (Figure 6E), showing that some stomatin does manage to move to the plasma membrane in these cells. Using a different culture system (see Suppl data) we successfully cultured a second OHSt sample (OHSt(B)) through to the reticulocyte stage. OHSt(B) cells gave an expression profile that was somewhere between sdCHC and OHSt(A). Some OHSt(B) cells expressed stomatin in the intracellular compartments, but the cells also showed weak expression of stomatin at the plasma membrane of early erythroblasts and expression of stomatin in the reticulocyte membrane (Suppl data, Figure 5).

Together these results suggested that stomatin may be involved in the removal of misfolded or obsolete proteins from the maturing reticulocyte. To further characterise the
internal stomatin staining in control and sdCHC reticulocytes we therefore co-stained with various internal markers. No colocalisation was found with anti-LAMP2 (a lysosomal marker) or anti-CD63 (a late endosome marker) (data not shown) but some colocalisation was found, more markedly in the sdCHC reticulocytes, with anti-CD71 which binds to the transferrin receptor (an endosomal marker that is lost during reticulocyte maturation) (Figure 7). Further studies are planned to clarify the role of stomatin in reticulocyte maturation.
Discussion

This paper describes the mutations in two unrelated patients with a novel syndrome of cation-leaky stomatocytic hemolysis, cataracts, developmental neurological delay and seizures. We have shown that these sdCHC patients are heterozygous for different mutations in \textit{SLC2A1}, which codes for glut1, a glucose transporter. Many mutations in the \textit{SLC2A1} gene have been associated with glut1 deficiency syndrome (glut1DS) (some of which are shown in Figure 1). Glut1DS is a condition where haploinsufficiency for glut1 restricts the availability of glucose in the central nervous system (CNS) causing hypoglycorrhachia, early-onset epilepsy resistant to anticonvulsants, slowing of head growth, developmental delay, and a complex movement disorder.\textsuperscript{26} These mutations are normally heterozygous and often code for an unstable mRNA or protein, leading to a 50% loss of glut1 protein (as seen in Figure 3). Total loss of glut1 activity is lethal as shown in the mouse model for glut1DS.\textsuperscript{37} Hemolytic anemia has not previously been reported in these classic glut1DS patients, suggesting that haploinsufficiency of glut1 is not critical in erythrocytes. Loss of glut1 activity in other tissues similarly may not be critical, or may be compensated for by alternative glucose transporters.

Milder forms of glut1DS are probably caused by mutations in \textit{SLC2A1} that simply reduce the rate of glucose transport. One such sub-type of glut1DS is familial paroxysmal exercise-induced dyskinesia (PED) with epilepsy, where the involuntary dystonic, choreoathetotic and ballistic movements occur after prolonged exercise and affect only the exercised limbs (mutations shown in Figure 1).\textsuperscript{30} It has been suggested that the severity of the phenotype of glut1DS correlates with the type of mutation.\textsuperscript{38} To expand on this theory, we would hypothesise that the phenotype reflects both the amount of glut1 protein expressed, the activity of the glut1 and whether the expressed protein is misfolded so that it leaks cations. In Table 2 we compare what is known of the different phenotypes of glut1DS and PED with or without anemia. Our results suggest that (i) haploinsufficiency for glut1, or expression of transport defective glut1, causes neurological symptoms, but is asymptomatic in the lens or erythrocyte, that (ii) expression of cation-leaky mutant glut1 in the erythrocyte membrane causes hemolytic anemia and that (iii) expression of cation-leaky, mutant glut1 in the lens epithelium may cause cataracts (see below).

The extent to which glut1 is expressed in erythrocytes has not always been investigated in glut1DS. We show here that in two cases of classic glut1DS the amount of glut1 in the
erythrocyte membrane is reduced to about 60% of normal (Figure 3). In the previous study of PED with anemia the amount of glut1 in the erythrocyte membrane was not measured, although expression and trafficking of the three mutant proteins (△QQLS, G314S and A275T) in Xenopus oocytes was normal. In our case of glut1DS with hemolytic anemia (sdCHC(A)) we show that the mutant protein is expressed (Figure 3) and leaks cations (Figure 5) but displays very little glucose transport activity (Figure 4).

**Erythrocyte cation leak**

Previous cation-leaky erythrocyte conditions have been shown to be caused by mutations in other large multi-spanning membrane proteins. Non-stomatin-deficient CHC results from mutations in $SLC4A1^{239-41}$ and OHSt from mutations in $RHAG^3$. Here we show that the cation leak in sdCHC results from mutations in $SLC2A1$. Together these results show that large multspanning proteins have the capacity to leak cations if they are both misfolded and present in the plasma membrane. They may do this simply by disrupting the lipid membrane or by allowing cations to pass through the mutant transport channel.

**Erythrocyte morphology**

The difference in erythrocyte morphology between the previous study of PED with hemolytic anemia and our patient with sdCHC may be due to the permeability of the erythrocytes to Ca$^{2+}$ ions. In the study of PED with hemolytic anemia the mutant glut1 (△QQLS) was shown to cause a leak of Na$^+$, K$^+$ and Ca$^{2+}$ ions when expressed in oocytes. Leak of Ca$^{2+}$ ions can activate the Gardos channel in erythrocytes, causing loss of KCl, dehydration and echinocytosis. In the present study permeability to Ca$^{2+}$ ions, Gardos channel activity or phosphatidyl-serine exposure were not tested but it was shown that the mutant glut1 proteins (Gly286Asp; △Ile435) caused a leak of Na$^+$ and K$^+$ ions when expressed in oocytes. This leak in erythrocytes causes an increase in mean cell volume and stomatocytosis.

**Cataracts**

A further significant difference between the sdCHC patients and the classic glut1DS patients is the occurrence of lens cataract. To our knowledge glut1DS and PED have not been associated with cataracts, so it is unlikely that the cataracts in our patients are caused by lack of glucose in the lens. Presumably the functionally-haploid status of glut1 transport in these patients is sufficient to maintain lens metabolism. In our patients, it can
be argued that the cataract may be due to Na\(^+\) and K\(^+\) leakage through the lens epithelium. Glut1 is expressed in the lens epithelium, the coating around the anterior pole of the lens.\(^{42}\) This epithelium has a role in the control of the water content of lens: it enables a convective flow within the interior of the lens that is thought to provide nourishment to the fibre cells of the interior, which is avascular.\(^ {43}\) Abnormalities in ion and water transport mechanisms have previously been suggested to be important in cataract formation.\(^ {44,45}\) We would hypothesise that cataracts are formed in the sdCHC patients because the mutant (misfolded) glut1 protein is expressed in the lens epithelium where it leaks cations and undermines this convective microcirculatory system.

**Stomatin loss**

Stomatin, also known as band 7.2b, is a monotopic, oligomeric, lipid raft-associated protein involved in membrane organisation, cholesterol-dependent regulatory processes and possibly regulation of ion channels.\(^ {46}\) Stomatin is deficient in both stomatin-deficient cryohydrocytosis (sdCHC) and overhydrated hereditary stomatocytosis (OHSt), both conditions that feature extreme erythrocyte cation leaks, however the reason for the stomatin depletion in these conditions is not known. Stomatin is known to associate with glut1 (ref 47), decreasing its affinity for glucose\(^ {8}\) or targeting glut1 to lipid rafts on glucose deprivation.\(^ {48}\) Previously we hypothesised that the loss of stomatin in these very leaky, energy depleted erythrocytes occurred to allow maximum glucose transport.\(^3\)

We have studied the loss of stomatin in OHSt and sdCHC erythroblasts during erythropoiesis in order to make a direct comparison between the two cell types. We found that stomatin expression at the plasma membrane of early and late erythroblasts varied sdCHC>OHSt(B)>OHSt(A). Thus stomatin-deficient red cells display a spectrum of expression levels during erythropoiesis suggesting that stomatin has a complex role in these variant cells. In sdCHC and OHSt(B) reticulocytes some of the stomatin appeared intracellular, in fairly large vesicles or aggregates. Staining with various internal markers showed some colocalisation of stomatin with anti-CD71 which binds to the transferrin receptor, an endosomal marker lost during reticulocyte maturation (Figure 7). Membrane stomatin expression in mature erythrocytes occurred in only a few sdCHC and OHSt cells and these may represent reticulocytes (Figure 6C,E and Suppl data Figure 5). So it is likely that stomatin is lost in the final stages of reticulocyte maturation in the circulation and involves endocytosis. The exact role of stomatin in the erythrocyte membrane is not
known, but together these results suggest that stomatin may be involved in the removal of misfolded or obsolete proteins from the maturing reticulocyte. Interestingly, a recent study of low potassium dog erythrocytes (which lack both Na⁺K⁺ATPase and stomatin) showed that stomatin was lost, together with the Na⁺K⁺ATPase, at the reticulocyte maturation stage⁴⁹ and stomatin has been shown previously to be associated with exosomes from human reticulocytes.⁵⁰

In conclusion, we have described here a new syndrome of glut1DS with hemolytic anemia and cataracts, also known as stomatin-deficient cryohydrocytosis (sdCHC). Glut1DS can be alleviated by a ketogenic diet²⁶ and this diet may benefit our sdCHC patients. We have shown that our patients are not only haploinsufficient for glucose transport but that their cells almost certainly express the mutant glut1 proteins causing further problems. In erythrocytes the mutant glut1 is associated with a cation leak causing stomatocytosis and hemolysis, leading to anemia and splenomegaly. In the CNS, although the primary problem is insufficient functional glut1, the cation leak may exacerbate the neurological symptoms. In the lens epithelium the cation leak may disrupt the microcirculatory system, causing cataracts. Our data resolve the molecular cause of stomatin-deficient cryohydrocytosis and suggest a novel mechanism of cataract formation.
Acknowledgements

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Author contributions

Contribution: JFF: DNA and protein analysis, deoxy-D-glucose transport, expression studies in *Xenopus laevis* oocytes, confocal microscopy; HG, FB: Cation transport and western blots in *Xenopus laevis* oocytes; NMB: Modeling studies; RJT, RJF, BEL, PQ & PAM: Reexamination of the patients and provision of samples; SAB: Provision of glut1 clone and antibody; JD: Provision of patient samples and manuscript preparation; GWS: Provision of patient samples and manuscript preparation; LJB: Research design, protein analysis, manuscript preparation.

Conflict of interest disclosure: The authors have no conflict of interest to declare.
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16. Van den Akker E, Satchwell TJ, Pellegrin S, Daniels G, Toye A. The majority of the in vitro expansion potential resides in CD34(-) cells, outweighing the contribution of CD34(+) cells and significantly increasing the erythroblast yield from peripheral blood samples. *Haematologica.* 2010;95(9):1594-1598.


Table 1  MOLPROBITY analysis of glut1 homology models

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>Gly286Asp</th>
<th>△Ile435</th>
<th>1SUK</th>
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<tbody>
<tr>
<td><strong>Clashscore</strong></td>
<td>16.58 (49th)</td>
<td>17.65 (44th)</td>
<td>15.54 (55th)</td>
<td>75.37 (0th)</td>
</tr>
<tr>
<td>(percentile)</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Poor rotamers (%)</td>
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<td>1.52</td>
<td>2.54</td>
<td>5.58</td>
</tr>
<tr>
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<td>0.00</td>
<td>0.00</td>
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<tr>
<td>Ramachandran favored (%)</td>
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<td>96.36</td>
<td>97.00</td>
<td>88.98</td>
</tr>
<tr>
<td>Cβ deviations &gt;0.25Å</td>
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<td>21</td>
<td>17</td>
<td>27</td>
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<tr>
<td>MolProbity score*</td>
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<td>2.13 (64th)</td>
<td>2.18 (60th)</td>
<td>3.49 (2nd)</td>
</tr>
<tr>
<td>(percentile§)</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Residues with bad bonds (%)</td>
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<td>0.00</td>
<td>0.00</td>
<td>1.42</td>
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<tr>
<td>Residues with bad angles (%)</td>
<td>0.21</td>
<td>0.43</td>
<td>0.21</td>
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</table>

Note: The table includes details of the MOLPROBITY analysis of our glut1 homology models (WT, wild-type glut1; Gly286Asp glut1; △Ile435 glut1) and of a previously published glut1 model (PDB ID 1SUK, ref 23). 1SUK is consistent with practically all biochemical and mutagenesis data but contains significant deviations from ideal geometry when analysed by state-of-the-art validation software. MOLPROBITY\(^{24}\) ranks 1SUK in the 2\(^{nd}\) percentile of crystallographic structures determined at approximately 2 Å resolution (where the 100\(^{th}\) percentile is the best). In contrast, our refined model of wild-type glut1 is ranked in the 55\(^{th}\) percentile.

* Clashscore is the number of serious steric overlaps (> 0.4 Å) per 1000 atoms.
† 100\(^{th}\) percentile is the best among structures of comparable resolution; 0\(^{th}\) percentile is the worst (N=715, 2.00Å ± 0.25Å).
‡ MolProbity score is defined as: 0.42574*log(1+clashscore) + 0.32996*log(1+max(0,pctRotOut-1)) + 0.24979*log(1+max(0,100-pctRamaFavored-2)) + 0.5.
§ 100\(^{th}\) percentile is the best among structures of comparable resolution; 0\(^{th}\) percentile is the worst (N=12522, 2.00Å ± 0.25Å).
<table>
<thead>
<tr>
<th>Condition</th>
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<th>PED</th>
<th>PED + anemia</th>
<th>sdCHC + anemia</th>
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<td>n.k.</td>
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<td>mild</td>
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<td>yes</td>
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<td>yes</td>
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<td>no</td>
<td>yes</td>
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<td>n.k.</td>
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<td>yes</td>
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<td>no</td>
<td>yes</td>
<td>no</td>
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<td>normal?</td>
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<td>Cataracts</td>
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<td>no</td>
<td>no</td>
<td>yes</td>
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<td>no</td>
<td>possibly</td>
</tr>
<tr>
<td>Cation leaky glut1 in lens epithelium</td>
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<td>no</td>
<td>no</td>
<td>probably</td>
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<td>Cation leaky glut1 in red cell membrane</td>
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<td>no</td>
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</tbody>
</table>

Abbreviations: n.k., not known; GlutDS, glut1 deficiency syndrome; PED: paroxysmal exercise-induced dyskinesia; sdCHC, stomatin-deficient cryohydrocytosis.
**Figure Legends**

**Figure 1. Schematic diagram of glut1.**
A schematic diagram of glut1 is shown, the membrane spans based on the model shown in Figure 2A. Mutations (reading frame shifts, sites of insertions or substitutions) associated with glut1-deficiency syndrome (DS) highlighted in blue. Glut1-DS is also associated with splice site mutations and deletions (not shown). Mutations associated with paroxysmal exertion induced dyskinesia (PED) highlighted in light green. Deletion of “QQLS” associated with PED and echinocytic anemia highlighted in dark green. Mutations associated with stomatin-deficient cryohydrocytosis highlighted in red. The 24 amino acids with a bold outline are totally conserved residues across all glucose transporter isoforms and all species (Suppl. Figures 1 & 2).

**Figure 2. Homology model of glut1.**
A) **Structural model of wild-type glut1.** Cartoon representation of glut1 model coloured from blue at the N-terminus to red at the C-terminus with transmembrane helices numbered 1-12. Left panel shows the view from outside the cell; right panel shows view from within the membrane.

B) **Structural model of Gly286Asp mutation.** Wild-type (left) and Gly286Asp (right) homology models are displayed as Cα ribbon coloured from blue at the N-terminus to red at the C-terminus. The Cα atom of Gly286 is shown as a grey sphere and side-chains of Lys38 and Asp286 are displayed as sticks coloured by atom type. The putative novel salt-bridge between Lys38 and Asp286 is shown as a dashed yellow line.

C) **Structural model of △Ile435 mutation.** Homology model of △Ile435 glut1 is displayed as Cα ribbon. Left panel shows the view from outside the cell; right panel shows view from within the membrane. Region of the protein between Ile435 and the C-terminus is coloured orange. The side-chain of Tyr449 (numbered as in the wild-type protein) is shown as sticks in two conformations. The high-energy conformation observed in the final, refined model is shown in grey; for illustrative purposes the closest energetically-favourable side-chain conformation is shown in red. Adoption of this conformation would cause extensive steric clashes with the residues of transmembrane helix 9.

**Figure 3. Erythrocyte membrane protein analysis.**
Erythrocyte membranes were separated on 10% Laemmli gels and immunoblotted using antibodies as shown. Loading: C1, C2, controls 1 and 2. P indicates proband (sdCHC(A))
except where labeled as glut1DS patient A or B. A) The glut1 protein is heavily glycosylated (seen as a broad 50-100 kDa band). Scanning densitometry analysis of the deglycosylated glut1 band (labeled ‘PNGase treated’) showed normal amounts of glut1 in the sdCHC patient and reduced amounts of glut1 (by ~ 40%) in the glut1DS patients. A protein 4.2 loading control is shown beneath each blot. The reduction in stomatin has been shown previously and is characteristic of sdCHC. Stomatin was present in normal amounts in the glut1DS patients. B) Both CD58 and Lutheran protein were increased in the sdCHC sample. An actin loading control is shown beneath each blot.

Figure 4. Glucose uptake into Xenopus laevis oocytes expressing wild-type or mutant glut1.

Oocytes were injected with 20 ng of WT or mutant glut1 (or 10 ng WT and 10 ng mutant where coexpressed). Oocytes expressing WT or mutant glut1 were assayed 2 days post injection (data are means of 4 replicates of 5 oocytes, +/- s.e.m. (n=4)). *P <0.05, **P <0.01, ***P <0.001.

A) Deoxy-D-glucose uptake in Xenopus laevis oocytes. Inset: continuing the same experiment with oocytes assayed 3 days post injection (data are means of 4 replicates of 5 oocytes, +/- s.e.m. (n=4)).

B) Inhibition of deoxy-D-glucose uptake in Xenopus laevis oocytes. Oocytes were incubated in MBS or MBS containing phloretin (100 μM) and cytochalasin B (50 μM) for 20 min at RT before addition of [3H]-deoxy-D-glucose (data are means of 4 replicates of 5 oocytes, +/- s.e.m. (n=4)).

Figure 5. Cation leak in Xenopus laevis oocytes expressing wild-type or mutant glut1.

A) Intracellular sodium and potassium ion concentrations were measured by flame photometry on washed, extracted oocytes (3 replicates of 5 oocytes per condition) after 72 hours’ incubation at 19°C in MBS containing ouabain (0.5 mM) and bumetanide (5µM). Data, expressed in µmol/g of dry weight (µmol/g d.w.), are the mean values for four separate experiments, +/- s.e.m. (n=12). *P <0.05, **P <0.01, ***P <0.001.

B) Li⁺ influx (as a surrogate for Na⁺) was measured two days post injection. Oocytes (7 per condition) were incubated for 2 hours at 19°C in medium where NaCl was substituted by LiNO₃ in the presence of ouabain (0.5 mM) and bumetanide (5µM). Li⁺ content in each
oocyte extract was measured by atomic absorption spectrometry with a Perkin Elmer AAS 3110 (Perkin Elmer SAS, Courtaboeuf, France). The graph shows the data from seven repeat experiments. To normalise the data, in each individual experiment NI was taken as 100% and the Li uptake in wild-type, glut1-G286D and glut1-\(\Delta I435\) was expressed as a percentage of NI. The seven values were then averaged and plotted, +/- s.e.m. (n=7). *P <0.05, **P <0.01, ***P <0.001. Inset: Glut1 expression levels in *Xenopus laevis* oocytes were assessed by immunoblotting using an antibody to the C-terminal region of glut1.

**Figure 6. Confocal imaging of cultured erythroblasts**
A) Confocal imaging of cultured early stage sdCHC and control erythroblasts probed with anti-glut1 (red) and anti-stomatin (green).
B) Confocal imaging of cultured late stage sdCHC and control reticulocytes probed with anti-glut1 (red) and anti-stomatin (green).
C) Confocal imaging of peripheral blood from sdCHC and control probed with anti-glut1 (red) and anti-stomatin (green).
D) Confocal images of cultured early stage OHSt(A)\(^3\) erythroblasts probed with anti-glut1 (red) and anti-stomatin (green).
E) Confocal images of peripheral blood from OHSt(A)\(^3\) probed with anti-glut1 (red) and anti-stomatin (green).

**Figure 7. Colocalisation of stomatin with endosomal marker CD71.**
Confocal imaging of cultured late stage control and sdCHC reticulocytes probed with anti-stomatin (green) and anti-CD71 (transferrin receptor, red). The z-stack of sdCHC and control reticulocytes was examined and the number of internal vesicles in which stomatin appeared, or TfR appeared, or in which the proteins colocalised, was noted. On average control reticulocytes contained 3 stomatin positive vesicles per cell (n=55 cells examined), whereas sdCHC reticulocytes contained 6 stomatin positive vesicles per cell (n=27 cells examined). In control cells ~ 30% of the stomatin positive vesicles colocalised with transferrin receptor. In sdCHC cells ~ 60% of the stomatin positive vesicles colocalised with transferrin receptor.
Figure 3

A)

- Glut1 - sdCHC(A) C1 C1 P P C2 C2
- Glut1 - Glut1DS(A) C1 C1 P1 P1 C2 C2
- Glut1 - Glut1DS(B) C1 C1 P2 P2 C2 C2
- Stomatin - sdCHC(A) C1 C1 P P C2 C2
- Stomatin - Glut1DS(A) C1 C1 P1 P1 C2 C2
- Stomatin - Glut1DS(B) C1 C1 P2 P2 C2 C2

PNGase treated
Protein 4.2

B)

- CD58 - sdCHC(A) C1 C1 P P C2 C2
- Lu - sdCHC(A) C1 C1 P P C2 C2

actin
Figure 4

A) deoxy-glucose uptake (pmol/oocyte/hour)

B) deoxy-glucose uptake (pmol/oocyte/hour)
Figure 5

A) 

B)
Figure 6

A) Early

B) Late

C) Red Cells

D) Early

E) Red Cells
Figure 7

Control

sdCHC

Stomatin  Transferrin Receptor  merge
Stomatin-deficient cryohydrocytosis results from mutations in SLC2A1: a novel form of GLUT1 deficiency syndrome