# Supplemental Data and Figures

# Altered Splicing from a Mutated Alternate Branch Point is Common in Severe Alpha-Spectrin Linked Inherited Anemia

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## Supplemental Data

*Two dimensional SDS-PAGE of erythrocyte membrane spectrin limited trypic digests*. A variant spectrin allele, α-spectrin$^{Bug\ Hill}$ ($\alpha^{BH}$), due to a missense mutation, A970D, associated with an acidic shift in isoelectric point of the αII domain peptides in 2-dimensional maps of limited trypic digests of membrane spectrin. The $\alpha^{BH}$ variant has been found in many, but not all, rHS patients. In many patients, the $\alpha^{BH}$ allele appears to be homozygous in peptide maps of spectrin tryptic digests, but the patients are heterozygous for the $\alpha^{BH}$ allele in genomic DNA. This discrepancy between protein and DNA analyses led to the hypothesis that the gene product of the non-$\alpha^{BH}$ spectrin allele is not assembled on the membrane, due to the presence of accumulation defective alleles, leading to apparent homozygosity of the $\alpha^{BH}$ allele.[1]

We observed similar findings in the rHS probands from kindreds 8, 9, 10, and 11. While probands exhibited absence or near-absence of normal spectrin αII domain peptides and apparent homozygosity for the $\alpha^{BH}$ allele, they were heterozygous for the $\alpha^{BH}$ allele in genomic DNA (Supplemental Table S1). Representative tryptic maps from patient 11 are shown in Supplemental Figure S3. These results confirm the previous observations and provide support for the hypothesis that the non-$\alpha^{BH}$ spectrin allele *in trans* is a production-defective allele.

*Molecular modeling.* Six missense mutations (5 novel) in 10 alleles were identified, with 4 located in the region where αβ-spectrin dimers self-association site into tetramers. These novel mutations were high conserved across species (Supplemental Figure S1) and were predicted to be deleterious by mutation prediction algorithms (Table 1). The stability of spectrin tetramer complexes affects erythrocyte membrane integrity because spectrin tetramer cross-linking of actin-based junctional complexes is a critical structural feature of the membrane skeleton. The effects of the α-spectrin mutations on tetramer structure and their likely functional consequences were evaluated using the structures of the univalent α-β tetramer complex,[2] closed spectrin dimers,[3,4] and the divalent tetramer complex.[5] Most previously characterized HE spectrin mutations mapped to the tetramer αβ interaction site have been shown to destabilize spectrin tetramers by directly perturbing the head-to-head tetramer contact interface.[2] Several mutations outside the tetramer have also been shown to stabilize closed spectrin dimers.[3] Because the pertinent dynamic spectrin interactions on the erythrocyte membrane are closed dimers ↔ open

dimers ↔ monovalent tetramer ↔ bivalent tetramer, over-stabilizing the structure of closed dimers shifts the overall equilibrium to the left and destabilizes spectrin tetramers.

Two of the mutations identified in the current study, R45G and L49R, are located in the contact site between the β17 and α0 motifs (Supplemental Figure 2), thereby directly destabilizing the tetramer interface, similar to other α0 mutations. Specifically, R45G is likely to very strongly destabilize tetramerization, because replacing the Arg with a Gly will remove a critical contact site between α and β-spectrin and form a substantial void in the αβ contact interface. Replacement of this Arg with a smaller residue was previously shown to destabilize tetramers because replacing the R45 with a Thr reduced the tetramerization $K_d$ by 35-fold, and an even smaller Ser side chain reduced binding below detectable levels.[5] As a Gly side chain is substantially smaller than a Ser, it is expected that this mutation will to be even more destabilizing than the Ser. Similarly, the L49R mutation is predicted to severely destabilize the tetramer. L49 is buried in a highly hydrophobic region in the tetramer contact interface,[2] where introduction of strong positive charge is expected to be very disruptive. The critical nature of interactions at this site is further demonstrated by the prior characterization of an L49F mutation that showed a 75-fold decrease in $K_d$ relative to wild type. Phe has a similar hydrophobicity to that of Leu but is much bulkier.[5]

In contrast to the above two mutants, M70R is outside the direct tetramerization binding site (Supplemental Figure 2A) suggesting that another mechanism is involved in destabilization of tetramers. When this mutation is mapped onto a divalent tetramer structure (Supplemental Figure 2B), direct contact with a loop on the laterally associated chain is observed where it contacts P335 and S336 (Supplemental Figure 2C, left panel). Replacement of Met with an Arg both replaces an uncharged moderately hydrophilic residue with a strong positive charge and causes a steric clash with the interacting residues on the laterally associated chain, as illustrated by the overlap of side chains (Supplemental Figure 2C, right panel). Although no mutations that disrupt the inter-strand interface have been characterized biochemically, this mutation is likely to only mildly affect tetramer stability. Moderate structural disruption of lateral association of strands is unlikely to have any effect on the association of the first head-to-head association (see Supplemental Figure 2A), and the difference in affinity between univalent and bivalent head-to-head tetramer formation is known to be small. Another possibility is that the R49 mutation might affect the closed-open dimer equilibrium, analogous to the L260P mutation (2). However,

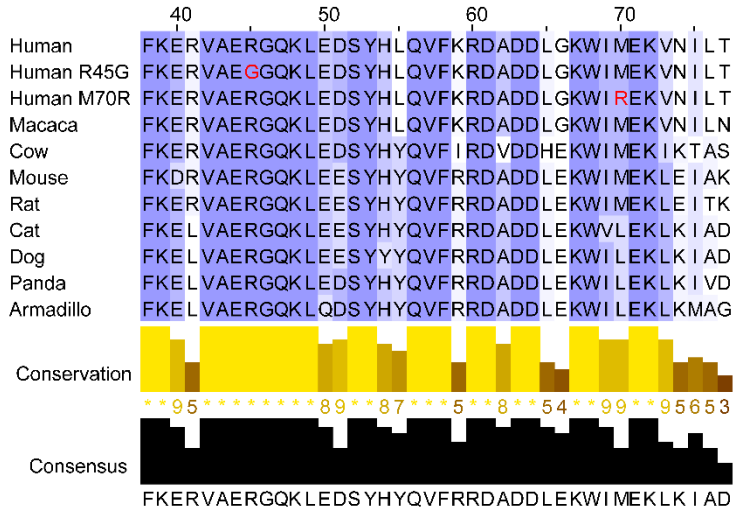
as shown in Supplemental Figure 2D, this mutation is not located in a contact site or hinge region and is unlikely to affect the closed dimer binding affinity. Furthermore, unlike the Pro at residue 260, Arg typically does not destabilize helices; therefore, a direct structural destabilization of the protein fold is unlikely.

In summary, mapping these three α-spectrin mutations onto known spectrin dimer and tetramer structures strongly suggests that R45G and L49R directly and severely destabilize tetramers by perturbing the αβ head-to-head contact interface. In contrast, M70R does not affect either the direct tetramer αβ contact region or closed dimers, but it appears to destabilize tetramers by perturbing lateral association of the two strands in the bivalent tetramer.

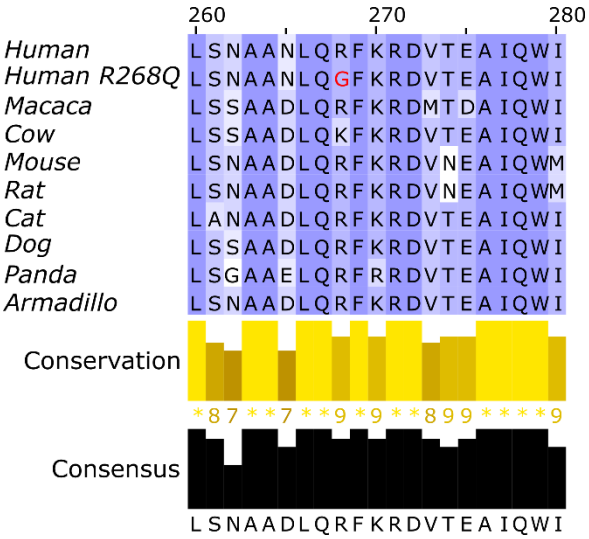
## Supplemental Figures

***Supplemental Figure S1. Conservation of α-spectrin missense mutations associated with recessive hereditary spherocytosis and hereditary yropoikilocytosis.*** Computational analyses revealed the amino acids with rHS- and HPP-associated missense mutations exhibit high degrees of conservation across species. Panel A shows R45G and M70R. Panel B shows R268Q. Panel C shows E346G.

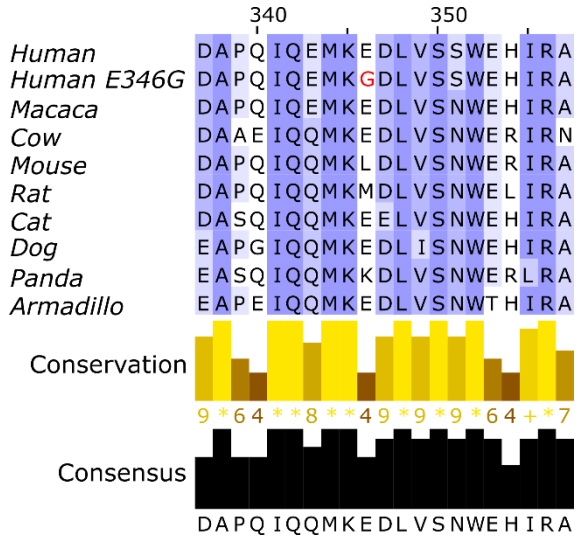
A



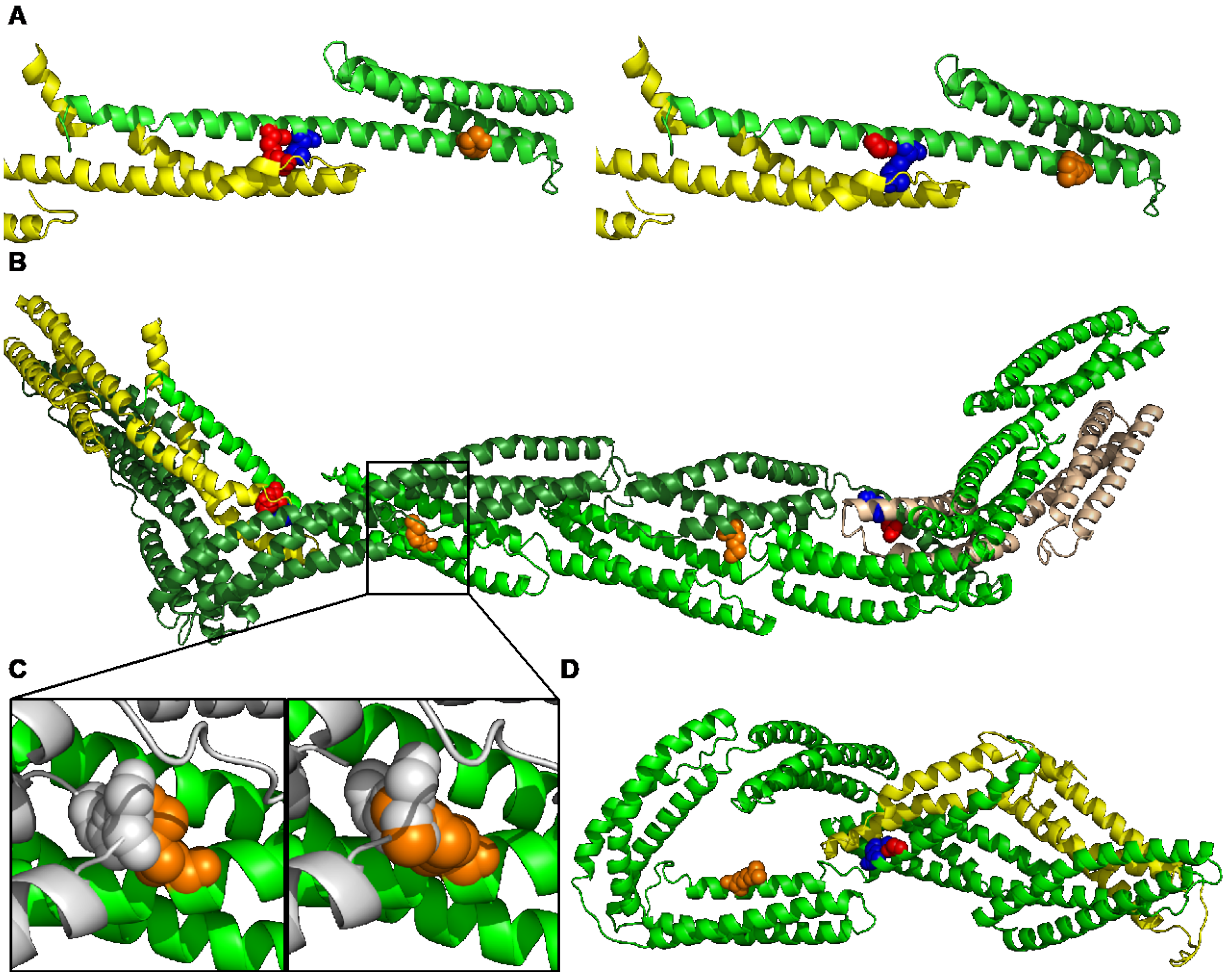

B



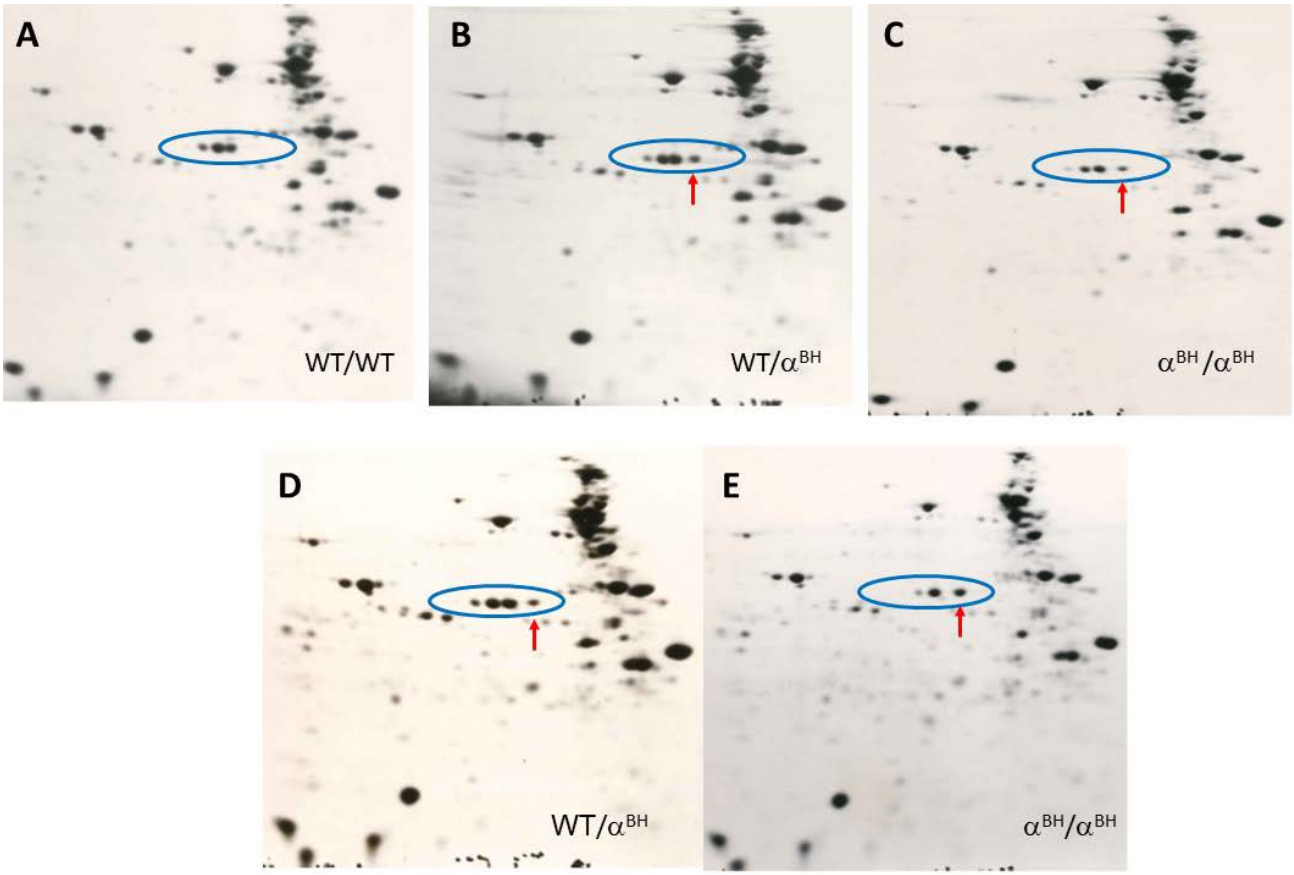

C



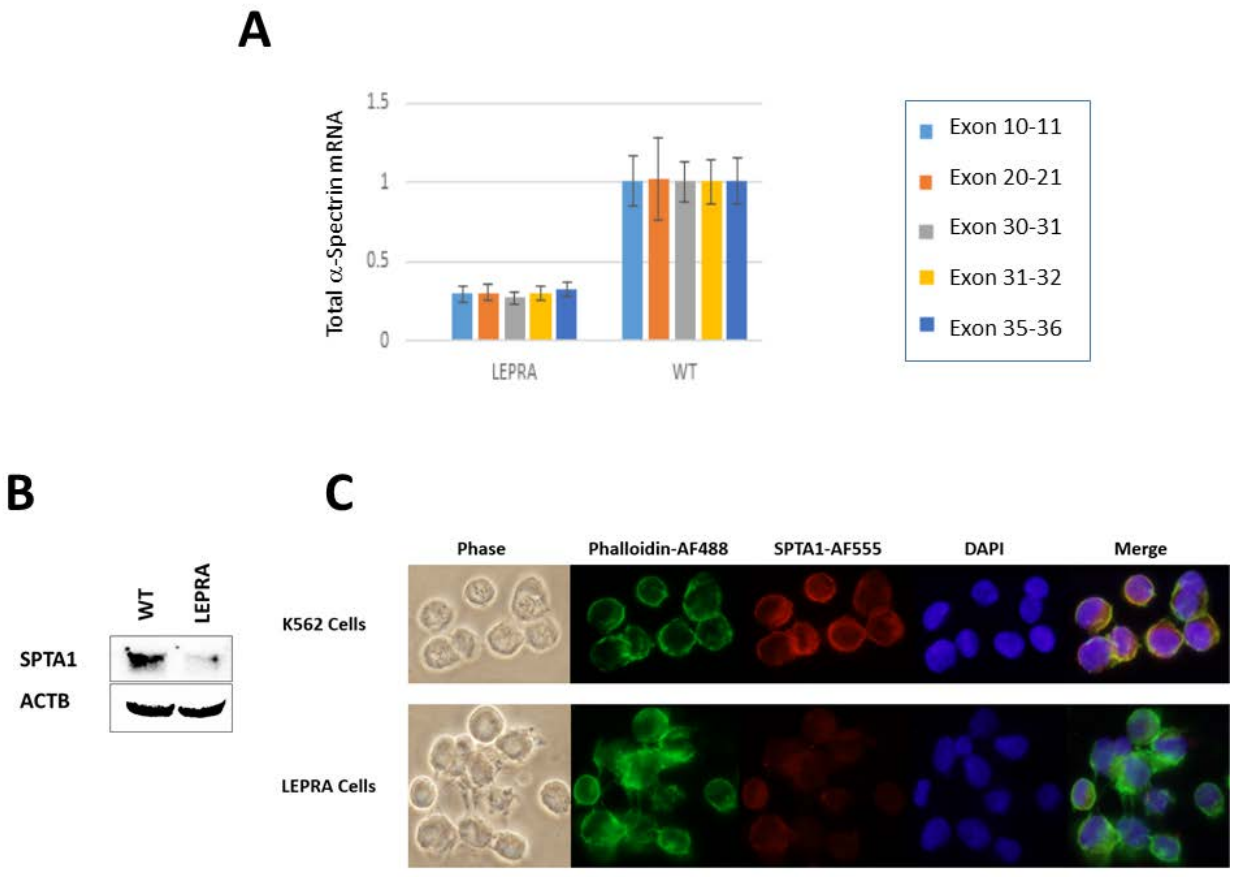
***Supplemental Figure S2. Molecular modeling. Impact of the R45G. L49R, and M70R α-spectrin mutations on structural stability and interactions.*** In panels A, B, and D the polypeptide chain backbones are shown in green for α-spectrin and in yellow for β-spectrin; side chains of wild type and mutated residues are highlighted using red (R45G), blue (L49R) and orange (M70R) spheres. A. A model of the univalent head-to-head tetramer association highlighting wild-type (left panel) and corresponding mutant residues (right panel). B. A model of the bivalent head-to-head tetramer association and lateral association of the two strands in the tetramer region. C. *Left* panel – a zoomed image highlighting the wild type residue M70 with the laterally associated α-spectrin chain shown in gray and residues from this chain that contact M70 shown using gray spheres (P335 and S336); *right panel* – the same image showing the R79 mutation, which overlaps the laterally associated chain. D. A spectrin dimer in the closed dimer conformation showing the three mutations.



***Supplemental Figure S3. Maps of limited tryptic digests of erythrocyte membrane spectrin***. Two-dimensional SDS-PAGE with isoelectric focusing was performed on limited tryptic digests of erythrocyte spectrin from normal controls and $\alpha^{BH}$ patients and family members. Fractionation from left to right is by isoelectric focusing, and from top to bottom by SDS-PAGE. A. Normal control (WT/WT). The three normal spectrin αII domain peptides are circled. B. Mother of proband #11. In panel B, a novel peptide representing the $\alpha^{BH}$ allele (blue arrow) is present in the clinically normal, heterozygous mother (WT/$\alpha^{BH}$) of patient #11. C. Proband #11. In Panel C, there is near complete absence of one of the normal αII peptides, with a large amount of the novel peptide representing the $\alpha^{BH}$ allele (blue arrow), appearing at the protein level as homozygous $\alpha^{BH}/\alpha^{BH}$. Genetic studies revealed the proband was a $\alpha^{BH}$-$\alpha^{LEPRA}$ heterozygote in *trans* to a splicing mutation in IVS 37. D. Mother of proband #6. a novel peptide representing the $\alpha^{BH}$ allele (blue arrow) is present in the clinically normal, heterozygous mother (WT/$\alpha^{BH}$) of patient #6. E. Panel E. Proband #6. In Panel E, there is complete absence of one of the normal αII peptides, with a large amount of the novel peptide representing the $\alpha^{BH}$ allele (blue arrow), appearing at the protein level as homozygous $\alpha^{BH}/\alpha^{BH}$. Genetic studies revealed the proband was homozygous $\alpha^{BH}$-$\alpha^{LEPRA}/\alpha^{BH}$-$\alpha^{LEPRA}$.



***Supplemental Figure S4. CRISPR/Cas9 Gene editing/creation of a homozygous $\alpha^{LEPRA}$ K562 cell line***. K562 cells were rendered homozygous for the $\alpha^{LEPRA}$ allele as described in Supplemental Methods. Panel A. Quantitative reverse-transcriptase PCR of the α-spectrin gene was performed on RNA extracted from wild type (WT) and $\alpha^{LEPRA}$ K562 cells. Due to the length of the *SPTA1* mRNA transcript, primer pairs spanning intron-exon boundaries were utilized to assess spectrin transcripts across the *SPTA1* locus. B. Western blot analysis of α-spectrin and actin in WT and homozygous $\alpha^{LEPRA}$ K562 cells. C. Immunofluorescene of WT and $\alpha^{LEPRA}$ K562 cells. From left to right, the panels show phase contrast microscopy, AF488-conjugated phalloidin, AF555-conjugated α-spectrin antibody, DAPI, and an image with all three merged. As expected, $\alpha^{LEPRA}$ K562 cells had less α-spectrin message, protein and immunofluorescence than wild type K562 cells.



Supplemental Table S1. Clinical and Laboratory Data

| Patient # | Diagnosis | Age at Referral/Sex | Hematocrit (%) | Reticulocyte Count (%) | Splenectomy | Spectrin Content | $\alpha^{BH}$ allele Protein/DNA | Comment |
|---|---|---|---|---|---|---|---|---|
| 8 | rHS | 10mo/M | 20-25% | 1-2% | No | 65% | Homozygous/ Heterozygote | Multiple transfusions |
| 9 | rHS | 7yo/M | 15-20% | 18-20% | Yes | 53% | Homozygous/ Heterozygote | Microspherocytosis after splenectomy |
| 10 | rHS | 3 yo/M | 21-24% | 9-12% | No | 70% | Homozygous/ Heterozygote | |
| 11 | rHS | 2yo/M | 21-26% | 8-18% | No | 57% | Homozygous/ Heterozygote | Marked microspherocytosis |
| 12 | HPP | 6yo/M | 31% | 10% | Yes | 76% | Homozygous/ Heterozygote | Father normal, mother HE |
| 13 | HPP | 28 yo/F | 34% | 8% | No | 82% | Homozygous/ Heterozygote | Mother normal, father unknown |
| 14 | HPP | 2 yo/M | 24.8-26.8 | 6-7.4% | No | 78% | Homozygous/ Heterozygote | Mother normal, father HE |

Supplemental Table S2. Transfusion Dependent Patients

| Patient # | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 |
|---|---|---|---|---|---|---|---|---|---|---|
| Sex | F | M | F | F | M | F | F | F | M | M |
| Ethnicity | Hawaiian /mixed | African American | White, non-Hispanic | White, non-Hispanic | White, non-Hispanic | Asian, White, non-Hispanic | Asian | White, non-Hispanic | White, non-Hispanic | Hispanic |
| Age at referral | 16 mo | 2 mo | 7 yo | 14 yo | 26 mo | 3yr 3 mo | 10 yo | 2yr 6mo | 6y 4mo | 6 mo |
| Working diagnosis | Unknown HA | DBA variant | Non-immune HA | DBA variant | Congenital HA | HA, ?HS | Unknown HA | ?HS | ?HS | Unknown HA |
| Family history | None | None | None | None | Father, anemia | Mother neonatal hyper-bilirubinemia | Yes, sibling died of same mutations | Father, HS | None | None |
| In utero anemia | Yes | Yes | No | No | No | No | Yes | Yes | No | No |
| In utero transfusion | Yes | Yes | No | No | No | No | Yes | Yes | No | No |
| Neonatal anemia | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | No | Yes |
| Neonatal Hyperbili-rubinemia | Yes | Yes | Yes | Yes | No | Yes | Yes | Yes | No | Yes |
| Neonatal transfusion | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | No | No |
| Exchange transfusion | No | Yes | No | No | No | No | Yes | No | No | No |
| Splenomegaly | Yes | Yes | Yes | Yes | No | Yes | Yes | Yes | No | Yes |
| Bone marrow | Dyserythro-poiesis multi-nucleate precursors | Dys-erythro-poiesis | Erythroid hyper-plasia increased iron | Erythroid hyper-plasia, bi-nucleate pre-cursors | No dyserythro poiesis | Increased erythro-poiesis | NA | Normo-cellular few hemo-phagocytic cells | Erythroid hyperplasia, iron overload | Erythroid hyper-plasia |
| Iron overload | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | No |
| Highest ferritin (ng/ml) | 2887 | 4724 | 930 | 4179 | 1592 | NA | 2446 | 1530 | 2558 | 243 |

Supplemental Table S2. Transfusion Dependent Patients (continued)

| **Patient #** | **15** | **16** | **17** | **18** | **19** | **20** | **21** | **22** | **23** | **24** |
|---|---|---|---|---|---|---|---|---|---|---|
| **Treatment** | | | | | | | | | | |
| Transfusions | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| Central venous catheter | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| Steroids | Yes | No | No | Yes | Yes | No | No | No | Yes | No |
| Chelation | Yes | Yes | Yes | Yes | Yes | No | Yes | Yes | Yes | No |
| Splenectomy | Yes | No | No | Yes | Yes* | No | Yes | Yes | Yes | No |
| HSC transplant | No | No | No | Yes | No | No | Yes | Yes | No | No |

Abbreviations: HA: hemolytic anemia; DBA: Diamond Blackfan Anemia; HS: hereditary spherocytosis; NA: not available.
*Partial splenectomy.

**Supplemental References**


1. Tse, W.T. *et al.* Amino-acid substitution in alpha-spectrin commonly coinherited with nondominant hereditary spherocytosis. *Am J Hematol* **54**, 233-41 (1997).
2. Ipsaro, J.J. *et al.* Crystal structure and functional interpretation of the erythrocyte spectrin tetramerization domain complex. *Blood* **115**, 4843-52 (2010).
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