A Deep Intronic Mutation in the Ankyrin-1 Gene Causes Diminished Protein Expression Resulting in Hemolytic Anemia in Mice

Hua Huang*, PengXiang Zhao*, Kei Arimatsu§, Koichi Tabeta§, Kazuhisa Yamazaki†, Lara Krieg*†, Emily Fu*, Tian Zhang‡, Xin Du*

*Department of Medicine, University of California, San Diego, La Jolla, California 92093
§Division of Periodontology, Department of Oral Bioscience and †Division of Oral Science for Health Promotion, Laboratory of Periodontology and Immunology, Niigata University Graduate School of Medical and Dental Sciences, Niigata 951-8514, Japan
‡Department of Radiotherapy, Beijing ChaoYang Hospital, Capital Medical University, Beijing 100020, China

† Present address: Department of Pediatrics, Sanford Consortium for Regenerative Medicine, University of California, San Diego, La Jolla, California 92037
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Corresponding authors:

Primary: Xin Du; 9500 Gilman Drive, MC0716, La Jolla, CA 92093, USA; E-mail: xdu@ucsd.edu; Phone: (858)-822-3602;

Secondary: Tian Zhang; 8 GongTi-South Road, ChaoYang District, Beijing 100020, China; E-mail: timzt@126.com; Phone: (86)10-85231000
ABSTRACT

Linkage between transmembrane proteins and the spectrin-based cytoskeleton is necessary for membrane elasticity of red blood cells. Mutations of the proteins that mediate this linkage result in various types of hemolytic anemia. Here we report a novel N-ethyl-N-nitrosourea (ENU)-induced mutation of ankyrin-1, named *hema6*, which causes hereditary spherocytosis (HS) in mice through mild reduction of protein expression. The causal mutation was traced to a single nucleotide transition located deep into intron 13 of gene *Ank1*. *In vitro* minigene splicing assay revealed two abnormally spliced transcripts containing cryptic exons from fragments of *Ank1* intron 13. The inclusion of cryptic exons introduced a premature termination codon, which leads to nonsense-mediated decay of the mutant transcripts *in vivo*. Hence, in homozygous mice, only wild type ankyrin-1 is expressed, albeit at 70% of the level in wild type mice. Heterozygotes display a similar HS phenotype stemming from intermediate protein expression level, indicating the haploinsufficiency of the mutation. Weakened linkage between integral transmembrane protein, band 3 and underlying cytoskeleton was observed in mutant mice owing to reduced high-affinity binding sites provided by ankyrin-1. *Hema6* is the only known mouse mutant of *Ank1* allelic series that expresses full-length canonical ankyrin-1 at a reduced level, a fact that makes it particularly useful to study the functional impact of ankyrin-1 quantitative deficiency.

INTRODUCTION

Elasticity is a defining characteristic of the red blood cell (RBC) membrane, and depends upon maintenance of a high membrane surface area to volume ratio. Even
small reductions in surface area result in sequestration of RBCs from circulation and lysis. The ability of RBCs to maintain their membrane surface area is mediated by the underlying spectrin-based cytoskeleton, which is linked to and supports the lipid bilayer through numerous tethering sites (Bennett 1989; Bennett & Baines 2001). Two macromolecular complexes, the ankyrin complex and the 4.1R complex, provide the “vertical” linkages that tether plasma membrane and cytoskeleton (Mohandas & Gallagher 2008). In the ankyrin based complex, anchorage protein ankyrin-1 links spectrin tetramers to the cytoplasmic domains of the transmembrane proteins band 3 and RhAG (Bennett 1978; Bennett & Stenbuck 1979a; Bennett & Stenbuck 1980; Hargreaves et al. 1980), and comprises the key determinants of linkage between membrane and cytoskeleton. Deficiencies of the proteins involved in “vertical” interactions, including ankyrin-1, α and β spectrin, band 3, and protein 4.2, lead to unstable lipid bilayers that are prone to release as skeleton-free lipid vesicles, resulting in loss of membrane surface area and spherocytosis (Delaunay 2007; Mohandas & Gallagher 2008; Palek 1993).

Ankyrin-1 mutation is the most common cause of HS, accounting for approximately 35–65% cases in Northern European populations (Eber et al. 1996a; Gallagher 2005; Lanciotti et al. 1997). These mutations have been detected across the entire Ank1 gene. Missense and promoter mutations are common in recessive HS, while nonsense, frame shift, and splicing mutations mostly result in dominant HS; de novo ankyrin mutations occur with high frequency (Eber et al. 1996a; Gallagher 2005). The 210 kDa full-length ankyrin-1 protein contains three major functional domains: an N-terminal band 3-binding domain, a central spectrin-binding domain, and a C-terminal regulatory
domain containing a death domain motif (Bennett 1992; Peters & Lux 1993; Rubtsov & Lopina 2000). The regulatory domain modulates the affinities of both the spectrin- and band 3-binding domains for target proteins (Hall & Bennett 1987).

Elucidation of the pathogenesis of Ank1 mutations in HS has benefited from the analysis of ankyrin-1 mutant mice. Four mutant lines have been reported. In the normoblastosis mice (nb/nb), spontaneous deletion of a guanosine residue in exon 36 of Ank1 led to a frameshift and premature stop codon resulting in production of a truncated hypomorphic protein of 157 kDa (Birkenmeier et al. 2003). The truncated protein lacks the C-terminal regulatory domain, but maintains band 3- and spectrin-binding domains. In addition, while spectrin levels were reduced to 50% of wild type levels (Lux et al. 1979), expression of other integral membrane proteins was preserved. The Ank-1\textsuperscript{E1674} (RBC2), Ank1\textsuperscript{E924X}, and Ank1\textsuperscript{MRI23420} mutations were generated by random germline mutagenesis. Ank-1\textsuperscript{E1674} (RBC2) is a null mutation induced by ENU, leading to complete deletion of ankyrin-1 protein in homozygous animals, with a concomitant reduction in spectrin and protein 4.2. As a consequence, RBC2 red cells exhibited total disruption of membrane skeleton architecture, and the phenotype was manifested as a recessive trait of severe hemolytic HS in mice (Rank et al. 2009). Both Ank1\textsuperscript{E924X} and Ank1\textsuperscript{MRI23420} lines are hypomorphic mutations, resulting in truncated ankyrin-1 proteins lacking both spectrin-binding domain and C-terminal regulatory domain, while band 3-binding domain remains intact in Ank1\textsuperscript{E924X} (Hughes et al. 2011) or partially affected in Ank1\textsuperscript{MRI23420} (Greth et al. 2012).

Here we report the identification and characterization of a novel ENU-induced mutation in Ank1, named hema6. Both heterozygous and homozygous animals exhibit
characteristic features of hereditary spherocytosis, such as elevated RBC counts, low RBC mean corpuscular volume (MCV), increased osmotic fragility, and shortened RBC life span in vivo. The causal mutation resides deep in intron 13 of Ank1, causing incomplete alternative splicing of the transcript that preserved the production of wild type ankyrin-1 protein at a reduced level in homozygous animals. The aberrant splicing isoforms are predicted to encode a truncated protein containing two third of the band 3-binding domain. However, the mutant protein could not be detected in the ghost membrane and whole red blood cell lysate. Thus, hema6 mutation is distinct from other Ank1 mutants described previously. The analysis of these mice highlights the importance of optimal ankyrin-1 protein quantity in maintaining erythrocyte membrane stability.

METHODS

Mice:
Animals were generated on a pure C57BL/6J background, and were maintained under standard housing conditions and fed with laboratory rodent diet no. 5001 (LabDiet). Unless otherwise stated, 5-12 weeks old female and male mice were used in all experiments. All studies were approved by the Institutional Animal Care and Use Committee (IACUC) at University of California, San Diego.

Hematology analysis:
Whole blood was collected by submandibular bleeding into potassium EDTA-containing microvette® 100 tubes (Sarstedt) and analyzed on Scil abc automatic hematology
analyzer. Blood smear was stained with Wright-Giemsa stain. Reticulocytes count was analyzed with Retic-COUNT (Thiazole Orange) Reagent (BD biosciences). Serum erythropoietin was measured using Quantikine® Mouse/Rat Erythropoietin ELISA (R&D system, Minnesota). Serum total bilirubin concentration was measured using Total Bilirubin Reagent from Stanbio Laboratory, Texas, and following manufacturer’s protocol.

**Osmotic fragility test:**

Blood from 6-week-old mice was tested within 2 hours after collection. 1µl whole blood was mixed with 200µl NaCl gradients ranging from 0.3% to 0.9%, and then incubated at room temperature for 20 minutes. The mixture was centrifuged and the supernatant’s absorbance was measured at 540nm. The hemolysis percent was calculated for each solution and plotted against NaCl concentrations. The degree of lysis in 0.3% NaCl is considered to be 100% and 0% for 0.9% NaCl.

**Red cells in vivo survival assay:**

N-hydroxysuccinimide (NHS)-biotin (E-Z link; Thermo Scientific) was suspended in sterile saline at 4mg/ml and injected intravenously into wild type and hema6 mice, respectively, at a dose of 40mg/kg body weight. Animals were first bled 36 hours after injection, and then at 2-3 days interval in the first two weeks, followed by 3-4 days intervals in the third and fourth weeks, then once in the fifth week. 5-10µl of blood was sampled each time. Labeled cells were analyzed by flow cytometry after staining with
streptavidin phycoerythrin (PE). The percentage of biotinylated red blood cells over time reflects the survival of RBC from each genotype group.

**Electron Microscopy:**

Blood samples were fixed in 2.5% glutaraldehyde at room temperature, and washed in 0.1M phosphate with 5% sucrose. Aliquots of the cells were then placed on Thermanox coverslips and incubated at 4°C for 1hr to ensure attachment, followed by fixing in 2% osmium tetroxide. After extensive water wash followed by dehydration in graded ethanol series, the samples were processed through a critical point dryer (Tousimis autosamdri 815) and mounted onto scanning electron microscope (SEM) stubs with carbon tape. The stubs with attached coverslips were sputter coated with Iridium (EMS model 150TS) for subsequent examination and documentation on a Hitachi S-4800 SEM (Hitachi High Technologies America Inc., Pleasanton CA).

**Red cell ghost preparation, SDS-PAGE and Western blot analysis:**

Approximately 500µl blood was collected by cardiac puncture into K₃EDTA containing tubes, and sedimented at 1000xg in 5mM NaPi (pH 7.4), 150mM NaCl solution with 0.75% Dextran T-500 at 4°C. Packed red cells were washed 4 times in ice-cold phosphate-buffered saline supplemented with 10mM glucose, and then lysed by ice-cold Mg²⁺ lysis buffer (5mM NaPi, pH 7.4, 1mM MgCl₂, 1mM dithiothreitol, and protease inhibitor cocktail). Ghosts were pelleted by centrifugation at 39,000xg. RBC ghosts or packed red cells were resuspended in Laemmli sample buffer, and subjected to SDS-PAGE using 4-15% gradient gels. After electrophoresis, proteins were
either stained with Coomassie blue R250 or transferred to nitrocellulose membrane for Western blotting. The membranes were incubated with individual primary antibodies as indicated and visualized using SuperSignal® West Pico Chemiluminescent reagents (Thermo Scientific). Antibody against full-length ankyrin-1 protein was a gift from Velia Fowler; P89 antibody specifically targeted to the N-terminus of ankyrin-1 was kindly provided by Connie Birkenmeier; band 3, protein 4.2 and β-spectrin antibodies were generously provided by Xiuli An; and actin (1-19) was purchased from Santa Cruz Biotechnology. Film was scanned and bands were quantified with ImageJ.

**Detergent extraction of band 3 from red cell ghost:**
As previously described (Gauthier *et al.* 2011), 50µl packed ghosts were mixed with 150µl PBS containing various concentrations of Triton X-100, and incubated at 4°C for 30 minutes. The cell suspension was then centrifuged at 21,000xg for 20 minutes at 4°C, and the supernatant was subjected to SDS-PAGE followed by western blot analysis using antibody against band 3.

**Measurement of serum iron levels:**
Serum was mixed with an acid-precipitating/reducing solution (0.6M trichloroacetic acid, 0.4M sodium thioglycollate, 2M HCl). The samples were then centrifuged and the supernatant was collected from each sample and added to the chromagen bathophenanthroline (1.5M sodium acetate, 0.5mM bathophenanthroline disulfonic salt). The optical density of each sample was measured at 535 nm. Iron standards (Thermo Scientific) ranging from 31.25 to 500 µg/dL were assayed simultaneously.
**Histology and Tissue iron staining:**

Spleen sections from wild type and *hema6* mice were stained with hematoxylin and eosin. Iron staining was performed with standard Prussian blue staining procedure. Histology images were acquired using Zeiss Axio Observer fluorescent microscope, and Zen pro 2011 image capture system.

**Genetic mapping and whole genome sequencing:**

Genetic mapping was accomplished by bulk segregation analysis as previously described (Xia et al. 2010), using a panel of 127 single nucleotide polymorphisms across the genome. Once a critical region was established by genetic mapping, candidate genes within the region were sequenced.

Whole genome sequencing of a homozygous *hema6* mouse was performed on Illumina HiSeq2000 system with Next Generation Sequencing core facility at The Scripps Research Institute.

**In vitro pre-mRNA splicing assay:**

Ankyrin-1 mRNA processing was analyzed using ankyrin minigene assay (Cooper 2005). *Ank1* exons 11-16 were amplified from genomic DNAs of wild-type and *hema6* homozygous mice, respectively, using primers 5’ GTCTGTGGGTGCTTGTTGGTGCT 3’ and 5’ TAGCCAGAAGCAGGTCTGGGAGC 3’. The amplified PCR products were cloned into vector pcDNA3.1/V5-His-TOPO (Invitrogen, CA). HEK293 cells transiently transfected with minigene plasmid were harvested 48 hours post transfection, total RNA
was prepared, and RT-PCR was performed using primers corresponding to T7 promoter/priming site and BHG-Reverse priming site on the vector. Amplification products were subcloned and sequenced.

**Quantitative real-time RT-PCR:**
Bone marrow total RNA was extracted using TRIzol® reagent (Invitrogen, CA) and 1 µg total RNA was reverse transcribed by RETROscript® First Strand Synthesis kit (Applied Biosystems, CA) using random decamers. Quantitative PCR was carried out on a StepOnePlus™ Real-Time PCR system (Applied Biosystems, CA). The primers corresponding to sequences in exons 25 and 26 of *Ank1* (5′CCGTTGTGATCCGATCTGAAG3′; 5′CACAGGGCTAATGTTGTCTGAG3′) were used. *Gapdh* (5′GGTCATCATCTCCGCCCCTTCTGC3′; 5′GAGTGGGAGTTGCTGTTGAAGTCG3′) was used as the invariant control.

**Statistical analysis:**
Statistical significance was evaluated using the unpaired Student’s *t* test, and differences with *P*<0.05 were considered statistically significant. GraphPad Prism® 5 was used for statistical evaluation.

**RESULTS**

*Hema6*, an ENU-induced mutant phenotype of hereditary spherocytosis with mild hemolysis:
In an effort to identify proteins with non-redundant function in erythropoiesis, we established a genetic screen for abnormal red cell production and survival in mutant mice generated by large-scale ENU mutagenesis (Arnold et al. 2012; Krieg et al. 2011). The genetic screen employs simple hematological measurements of red cell indices, and has identified genetic defects resulting in hemolysis, hemoglobinopathy and iron-deficiency anemia (Krieg et al. 2011) (and unpublished data). The index Hema6 mouse was detected in the screen by its reduced MCV and mean corpuscular hemoglobin (MCH), but elevated counts of RBCs. Tests for heritability indicated that the hema6 phenotype is transmitted as a dominant trait (Table 1). Platelet counts and the percentages of different leukocyte populations were comparable between hema6 and wild type mice, indicating a restricted defect in the erythroid lineage. Peripheral blood smears showed microcytosis and spherocytosis of hema6 RBCs (Figure 1A), and SEM highlighted morphologic changes in the erythrocytes from homozygous mice with stomatocytes, spherocytes, and membrane blebbing (Figure 1B). In view of the elevated RBC counts, we examined erythrocyte production and destruction in hema6 mice. Serum erythropoietin level was elevated in hema6 at 8 weeks of age (Figure 1C), indicating an increased erythropoietic activity that possibly accounted for the increased number of mature red cells; consistently, mild reticulocytosis was observed in hema6 mice (Figure 1D). Because increased erythropoietic activity may be a compensatory response to premature destruction of red cells, we evaluated the survival of mature erythrocytes in hema6 mice. Both homozygous and heterozygous hema6 mice exhibit elevated total bilirubin concentration in the serum, indicating increased hemolysis (Figure 1E). The survival of
erythrocytes in vivo was assessed by labeling mature red cells with biotin and followed the percentile changes of biotinylated RBCs over time. The erythrocyte half-life in hema6 homozygous mice was approximately 18 days compared with 28 days in the wild type controls (Figure 1F). The spleens of hema6 mice were slightly enlarged (97 ± 22 mg in homozygous mutant versus 71 ± 8 mg in wild type mice), and histology revealed a certain degree of effacement of the normal splenic architecture indicative of extramedullary erythropoiesis (Figure 1G). Iron was excessively deposited in the spleens of hema6 homozygotes, indicating increased sequestration and destruction of abnormal RBCs (Figure 1H). Liver iron store was comparable between hema6 mice and wild type controls even at 16 weeks of age (data not shown).

A characteristic feature of spherocytosis is increased red cell osmotic fragility owing to the decreased surface-to-volume ratio as a result of membrane loss. Consistent with the morphologic changes observed in hema6 mice, both heterozygous and homozygous hema6 RBCs exhibited increased osmotic fragility compared to that in wild type mice (Figure 2).

Identification of a novel Ank1 mutation in hema6 mice.

To map the hema6 phenotype, we crossed homozygous mice to wild type C57BL/10J mice, and the resultant F1 hybrid mice were intercrossed to generate F2 mice for mapping. Thirteen F2 mice displaying anemia (mice with lowest MCV were used) and 14 mice without anemia were used for genetic mapping by bulk segregation analysis, and linkage was observed on chromosome 8 (Figure 3A). Genotyping of individual mice for the C57BL/6J and C57BL/10J alleles of the markers on chromosome 8 confined the
critical region containing the *hema6* locus to a 13 Mb interval containing 139 annotated genes (NCBI M37) (Figure 3B, Table S1). Within the critical region, gene *Ank1* encoding erythroid ankyrin-1, whose mutations are the most common cause of typical, autosomal dominant hereditary spherocytosis (Eber *et al.* 1996b; Gallagher 2005; Lux *et al.* 1990), represents a promising candidate. We directly sequenced the genomic locus of *Ank1* in the index *hema6* mouse. Indeed, a homozygous T to C transition was identified in intron13 (Figure 3C), being 791 nucleotides away from exon13 and 209 nucleotides to exon14; the mutation is named *Ank1* IVS13+209T>C, in accordance with the systematic names in nomenclature for mutations in human (Gallagher 2005).

To further confirm IVS13+209T>C is the causative mutation, whole genome sequencing of a homozygous *hema6* mouse using Illumina HiSeq2000 system was performed. 74% of exonic sequences genome wide was covered at ≥ 4X coverage. As expected, IVS13+209T>C mutation was detected with 15 high-quality sequencing reads as homozygous variant. Separate genotyping of 60 mice consisting of 22 homozygotes, 31 heterozygotes and 7 wild type controls confirmed the concordance between *Ank1*\textsuperscript{hema6} genotypes and manifested phenotypes. Therefore, *Ank1* IVS13+209T>C is the causative mutation for the hemolytic hereditary spherocytosis in *hema6* mice.

Intronic mutation can create cryptic splice site that competes with the normal splice sites during RNA processing, resulting in mature mRNA with improperly spliced intron sequences. In order to determine whether *Ank1* IVS13+209T>C is associated with alternative pre-mRNA splicing, we performed RT-PCR with total RNA extracted from bone marrow using primers in exons 13 and 14. As expected, the RT-PCR product of RNA isolated from wild type C57BL/6J mice yielded an normal cDNA fragment of 400


bp. However, three cDNA fragments were reverse-transcribed from RNA isolated from *hema6* homozygotes: one major segment corresponding to the size of the normal cDNA fragment and two additional fragments of higher molecular weight, but in very low abundance. It suggests that they may be aberrant transcripts resulted from alternative splicing (Figure 4A). Therefore, we further analyzed Ank1 mRNA processing in *hema6* mice using ankyrin minigene in an *in vitro* functional splicing assay. An Ank1 minigene corresponding to sequences spanning exons 11-16 were prepared from wild type and *hema6* homozygous mice, and transfected into HEK293 cells, respectively. The pre-mRNA splicing of the minigene was evaluated by analysis of the resulted transcripts. RT-PCR products from HEK293 cells transfected with the wild type minigene showed one specific band with the expected size, indicating correct mRNA splicing from exons 11 to 16; whereas Ank1*<sup>hema6</sup>* minigene yielded a correct spliced transcript and the same 2 splicing isoforms as identified in bone marrow RNA, containing cryptic exons derived from intron 13 sequences (Figure S1). The long splicing isoform contained a cryptic exon of 317nt resulted from the utilization of a preexisting 3’ splice site that locates 108nt upstream of Ank1 IVS13+209T>C mutation; the short splicing isoform incorporated two cryptic exons defined by using aforementioned 3’ splice site and two other preexisting 5’ and 3’ splice sites at downstream of Ank1 IVS13+209T>C mutation (Figure 4B and Figure S2). Both mutant transcripts would introduce an in-frame stop codon resulting in the premature truncation of protein ankyrin-1, with addition of 8 aberrant amino acids after residue 501. However, we were unable to detect the full-length aberrant mRNA, indicating nonsense-mediated decay of the mutant transcripts.
Ank1<sup>hema6</sup> mutation causes reduction in quantity of ankyrin-1 protein, resulting in weakened interaction between RBC membrane and cytoskeleton.

The normally spliced Ank1 transcript is dominant in homozygous hema6 mice, indicating that mRNA processing through the cryptic splice sites activated by Ank1 IVS13+209T>C mutation was less efficient. However, their competition for the use of splicing machinery may reduce the yield of normal Ank1 transcript. We, therefore, further quantified Ank1 mRNA levels in erythroid progenitors from bone marrow in wild type and hema6 mice by quantitative real-time PCR. Overall, Ank1 transcript was significantly reduced in both heterozygous and homozygous hema6 mice compared to that in wild type mice. Ank1 mRNA level is consistently higher in homozygous hema6 mice than that in heterozygotes, although their difference is not significant (Figure 5A).

We next examined the consequence of Ank1 IVS13+209T>C mutation on ankyrin-1 protein expression. RBC ghost membranes were prepared from wild type C57BL/6J and hema6 homozygous mice, and the protein content was separated on a 4-15% SDS-PAGE gel and stained with Coomassie blue R250 (Figure 5B). The overall staining pattern of the ghost membrane appeared to be identical between wild type and hema6 homozygous mice, so was the relative intensity of bands representing major RBC membrane and skeleton proteins, with slightly “thinner” appearance at ankyrin-1 area in hema6 homozygotes. We further quantified the levels of ankyrin-1 by western blot analysis using an antibody against the full-length ankyrin protein purified from human erythrocytes. Normal ankyrin-1 protein of 210 kDa was readily detected in both wild type and hema6 mice, but the protein levels were reduced to approximately 81% and 70% of wild type level in Ank1<sup>hema6/+</sup> and Ank1<sup>hema6/hema6</sup> ghosts, respectively, when normalized.
by the actin level (Figure 5C). The predicted truncated ankyrin-1\textsuperscript{hema6} is approximately 55 kDa, with complete deletion of β-spectrin binding domain, C-terminal regulatory domain, and one third of membrane-binding domain. Presumably, it should exist as a cytosolic protein. We conducted immunoblotting on whole blood lysates from wild type, Ank1\textsuperscript{hema6/+}, and Ank1\textsuperscript{hema6/hema6} mice with antibody that we used previously to detect the full-length ankyrin-1 protein. Only a 210 kDa band, corresponding to the full-length protein could be detected in all samples. We reason that the epitope(s) recognized by the antibody raised against full-length protein may not exist anymore in the truncated protein. We further attempted to detect the truncated protein by P89 antibody, which is specifically targeted the N-terminus (amino acid 183-191) of ankyrin-1 protein. Similarly, other than the 210 kDa band, no band at approximately 55 kDa could be observed in ghost membrane and whole blood lysate samples from Ank1\textsuperscript{hema6/+} and Ank1\textsuperscript{hema6/hema6} mice; moreover, the western blot didn’t show any other bands distinct in hema6 homozygous and heterozygous mice from that in wild type mice (data not shown). These results are consistent with the prediction that the mutant transcripts are subjected to degradation by nonsense mediated decay pathway, and wild type ankyrin-1 is the only form of protein expressed in hema6 red cells.

Within ankyrin based multiprotein complex, ankyrin-1 attaches β-spectrin tetramers to the cytoplasmic domain of band 3, thereby provide high-affinity linkage between the plasma membrane and underlying skeletal network. We further evaluated β-spectrin, protein 4.2 and band 3 expression levels in Ank1\textsuperscript{hema6/hema6} red cells by western blot analysis and eosin-5′-maleimide staining, respectively. Hema6 homozygous membranes contained comparable levels of β-spectrin and protein 4.2 relative to
C57BL/6J controls (data not shown), but showed 80% of wild type level in band 3 expression (Figure S3).

In normal mouse red cells, about 40% band 3 molecules are laterally immobilized, and 80% are rotationally immobile or rotate slowly as the result of their association with the cytoskeleton through either high- or low-affinity interactions with ankyrin-1 (Rubtsov & Lopina 2000; Yi et al. 1997). We hypothesized that reduced quantity of ankyrin-1 would weaken the association of band 3 with cytoskeleton. To evaluate the functional consequence of Ank1<sup>hema6</sup> mutation, we performed detergent extraction of band 3 from ghost membrane using Triton X-100. Band 3 was more easily to be extracted from hema6 ghost membrane than wild type membrane (Figure 6). Thus, fewer band 3 remained in the membrane of Ank1<sup>hema6/hema6</sup> red cells than in normal red cells after detergent extraction, indicating weakened linkage of red cell membrane to the cytoskeleton.

**DISCUSSION**

Hereditary spherocytosis is a common hemolytic anemia affecting all ethnic groups; however, its clinical severity varies a great deal from mild to severe outcome. Ankyrin mutations are the most common mutations associated with HS, resulted from qualitative, and/or quantitative abnormalities in ankyrin-1 protein. Here, we describe a germline mouse mutant, named hema6 that develops mild HS due to a deep intronic mutation in ankyrin-1 encoding gene Ank1 resulting in decreased expression of normal ankyrin-1 protein.
Two alternatively spliced transcripts were generated in *hema6* by the utilization of cryptic splice sites in intron 13 activated by *Ank1* IVS13+209T>C mutation. Pre-mRNA splicing is a highly regulated process assuring the precise excision of introns and ordered array of exons. In addition to the canonical splice site sequences and branch point, exonic splicing enhancers (ESEs) or silencers (ESSs) also plays important role in the regulation of alternative splicing events, as well as in the definition of constitutive exons (Goren *et al.* 2006; Sironi *et al.* 2004). Subtle changes (e.g. point mutations) in the intronic sequences may enable pseudo exons splicing competent through modulating these elements. Based on ESEfinder and RESCUE_ESE analyses (Cartegni *et al.* 2003; Fairbrother *et al.* 2004), the pathological substitution of C for T in *Ank1* intron 13 created a novel exonic splicing enhancer motif AA\text{CAACCGT}, which likely induced the inclusion of cryptic exons encompassing the mutation (Figure S2). Furthermore, the splice site strength of the cryptic 5’ splice site located 13nt downstream of *Ank1* IVS13+209T>C was enhanced in *Ank1\text{hema6/hema6}* compared to that in wild type *Ank1* (donor strength score 0.38 in wild type versus 0.68 in *hema6*), according to the splice site prediction algorithm SplicePort (Dogan *et al.* 2007). It is worth mentioning that an exonic splicing silencer motif TTTGGGCA located 12nt upstream of *Ank1* IVS13+209T>C mutation was identified by both Sironi *et al.* and Fas_ESS algorithms (Sironi *et al.* 2004; Wang *et al.* 2004). Although the actual impact of the mutation on this ESS element remains to be determined, it is tantalizing to speculate that binding of SR proteins to the novel ESE motif created by the nucleotide transition may pose potential steric hindrance to the binding of hnRNP proteins to ESS, thereby compromising its repression on exonic splicing. Emerging evidences have
shown that deep intronic mutations causing subtle changes in pseudo exon sequences can activate their inclusion in mature transcripts and cause genetic diseases, e.g. malignancies and cancers in humans. This highlights the importance of screening for deep intronic mutations in cancer patients, and opens the possibility of antisense gene therapy for the treatment of cancer and other diseases caused by this class of mutation. 

*Hema6* erythrocytes underwent premature hemolysis in the spleen, but iron store in the liver was essentially not changed compared to age-matched wild type controls. A modest elevation of erythropoietin level, reticulocytosis and extramedullary erythropoiesis in the spleen were observed in *hema6* mice at 8 weeks of age, indicating a compensatory erythropoietic response to hemolysis. Excessive iron released by premature destruction of red cells could be all utilized to meet the demand of increased erythropoiesis, and therefore doesn’t cause apparent iron loading as seen in severe case of hemolytic anemia. Consistently, hematocrit and hemoglobin levels were well maintained in *hema6* mice over long period of time in life (> 6 months). Interestingly, we observed hemolytic anemia worsened in some *hema6* homozygous mice at older age, where a reduction in RBCs number and hemoglobin level became apparent at 7 months old (Figure S4). Whether the increased hemolysis is due to slow-down of marrow response or further decreased synthesis of ankyrin-1 is in need of further investigation.

Each erythrocyte contains approximately $10^6$ band 3 molecules, assembled into $4 \times 10^5$ intramembrane particles comprised primarily of dimers, whereas ankyrin is present in roughly $10^5$ copies per cell, corresponding to a limiting stoichiometry of approximately one ankyrin for four band 3 proteins (Bennett & Stenbuck 1979b; Nigg & Cherry 1980). It is now believed that band 3 tetramers represent the predominant ankyrin binding
partners on the red cell membrane (Michaely & Bennett 1995; Thevenin & Low 1990). Accordingly, ~30% reduction in ankyrin-1 in hema6 homozygous mice would result in ~1.2 x 10^5 (roughly 12%) band 3 molecules free of high-affinity attachment to the underlying cytoskeleton, and shed off with unstable lipid vesicles. Band 3 expression was consistently preserved at about 80% of normal value in hema6 homozygotes. The concordance between quantitative reduction of ankyrin-1 protein and phenotypic severity (i.e. reduction in MCV) as witnessed in Ank1^hema6/+ and Ank1^hema6/hema6 mice further highlighted the importance of adequate ankyrin protein level in stabilizing membrane and maintaining favorable surface area in erythrocytes.

In contrast to existing ankyrin-null or hypomorphic mouse mutants, hema6 is unique in that only wild type ankyrin protein was produced in mutant erythrocytes at a modestly reduced level. This defect closely parallels some mild human HS cases, such as Ankyrin Napoli and Bugey (del Giudice et al. 1996; Morle et al. 1997), in which ~24% reduction in overall ankyrin-1 protein level was observed. These patients showed moderate hemolytic anemia at admission and the anemia was compensated during follow-up examination. Hema6 therefore represents a new model for the trial of preclinical therapeutic interventions for the treatment of HS patients with ankyrin-1 deficiency. Furthermore, it was reported that nb/nb, Ank-1^1674 and Ank1^MR123420 mice are resistant to malaria infection (Greth et al. 2012; Rank et al. 2009; Shear et al. 1991). However, the contribution of ankyrin-1 to the resistance remains controversial. In nb/nb and Ank1^MR123420 mice, it is difficult to evaluate the involvement of wild type ankyrin-1 in the parasite infection because of the existence of truncated ankyrin proteins. In Ank-1^1674 null mice, severe anemia in the homozygous animals precludes them from the
test. In this regard, *hema6* provides a valuable model to evaluate the role of ankyrin-1 in malaria parasites entry and maturation inside the red cells.

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**Literature Cited**


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Table 1. Red blood cell indices of wild type, heterozygous and homozygous hema6 mice

<table>
<thead>
<tr>
<th>Strain</th>
<th>RBC (10^6/µl)</th>
<th>HGB (g/dL)</th>
<th>HCT(%)</th>
<th>MCV(fl)</th>
<th>MCH(pg)</th>
<th>MCHC (g/dL)</th>
<th>RDW(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6J</td>
<td>9.2±0.3</td>
<td>14.1±0.4</td>
<td>43.2±1.8</td>
<td>47.5±0.5</td>
<td>15.4±0.2</td>
<td>32.6±0.3</td>
<td>16.6±1.0</td>
</tr>
<tr>
<td>hema6/+</td>
<td>10.3±0.6**</td>
<td>14.6±0.7</td>
<td>43.9±2.6</td>
<td>42.2±0.4**</td>
<td>14.2±0.4**</td>
<td>33.4±0.8*</td>
<td>16.0±0.7</td>
</tr>
<tr>
<td>hema6/hema6</td>
<td>10.2±0.5**</td>
<td>14.2±0.4</td>
<td>41.5±1.7</td>
<td>40.8±0.8**</td>
<td>13.9±0.4**</td>
<td>34.2±1.1**</td>
<td>16.2±0.6</td>
</tr>
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</table>

Automated complete blood counts (CBC) were obtained for 6 wild type mice, 5 hema6 (+/-) and 9 hema6 (-/-) mice at 6 weeks of age, both male and female mice were used. Results are presented as mean ± SD. * P< 0.05, ** P< 0.01, Student’s t test. Comparisons of hema6/+ and hema6/hema6 with wild type mice were made, and the statistical significance was shown in the table.

RBC: red blood cell; HGB: hemoglobin; HCT: hematocrit; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; RDW: red cell distribution width.
FIGURE LEGEND

Figure 1. Hematological phenotypes of hema6 mice. (A) RBCs exhibit spherocytosis when examined by Wright-Giemsa staining of blood smears. Image was taken at 40X magnifications. (B) SEM highlighted the morphologic changes in the erythrocytes from hema6 mouse. (C) Serum erythropoietin level. (D) Reticulocytes count. (E) Serum bilirubin level. (F) Shortened erythrocyte life span in hema6 mice. Red cells were labeled with biotin in vivo by injecting mice with biotinylation reagent N-hydroxysuccinimide-biotin at 6 weeks of age. The survival of red cells was followed by measurement of the percentage of labeled erythrocytes by FACS. In (C-F), measurements were done in mice at 8 weeks of age, n=3 for wild type control, n=4 for hema6 homozygotes, data is expressed as mean ± SD. Asterisks denote the level of statistical significance (two-tailed Student's t test) between hema6 (-/-) and C57BL/6J mice. *P < 0.05; **P < 0.01; ***P < 0.001. In order to show the maximum difference between wild type and hema6 mice, data on heterozygotes are not shown. (G) Splenic histology from C57BL/6J and hema6 homozygous mice. Spleens were isolated from wild-type or hema6 homozygotes and stained for hematoxylin and eosin. (H) High levels of iron deposition in hema6 (-/-) mice, assessed by Prussian blue staining of spleen sections. Hema6 mice have much more iron-positive cells (blue straining) relative to C57BL/6J controls. In both G and H, sections are shown at 10X magnification.

Figure 2. Increased osmotic fragility of hema6 RBCs. 1µl of fresh blood was added to 0.2ml of a series of hypotonic solutions with NaCl content ranging from 0.3% to 0.9%. The degree of lysis in 0.3% of NaCl is considered to be 100% and 0% for 0.9% of NaCl.
n=3 for both wild type and hema6 heterozygous mice and n=4 for homozygous hema6 mice, data was expressed as mean ± SD. Asterisks denote the level of statistical significance (Student's t test) between hema6 (-/-) and C57BL/6J mice (in red), or hema6 (+/-) and C57BL/6J mice (in blue). *P < 0.05; ***P < 0.001.

Figure 3. Genetic mapping and positional cloning of phenotypic mutation in hema6 mice. (A) Genetic linkage on chromosome 8 was established by whole-genome SNPs analysis using 27 mice (13 anemic, 14 non-anemic mice). (B) Results of genotyping individual mice for the C57BL/6J and C57BL/10J alleles of the markers on chromosome 8. The number under each column represents the number of F2 mice with the indicated genotypes. The critical region was refined to 13 Mb, between markers B10HEMA60006 and B10HEMA60009. (C) A single nucleotide transition (T>C) was detected in the intron 13 of Ank1 gene.

Figure 4. Splicing analyses of Ank1hema6 mRNA transcript. (A) Reverse transcription of Ank1 mRNA in erythroid progenitors from bone marrow in wild type and homozygous hema6 mice. A forward primer located in Ank1 exon 13 and a reverse primer in exon 14 were used. The transcript structure corresponding to the amplified fragment is shown on the right. (B) Schematic illustration of the splice isoforms resulting from the mutated Ank1hema6 allele. Original exons 13 and 14 are represented by open frame, whereas cryptic exons are shaded in grey (not at scale). The mutated nucleotide residing in the middle of intron 13 is highlight in yellow and shown in bold red font. All 5’ and 3’ splice sites that were activated and utilized to generate different splicing forms within intron 13
are shown along the line, and the in-frame premature stop codon is indicated in italic red fonts. The sizes of removed introns and cryptic exons in 2 splice isoforms are annotated in the figure.

**Figure 5.** \(\text{Ank1}^{\text{hema6}}\) mutation causes decreased ankyrin-1 protein expression on the red cell membrane skeleton. (A) Wild type \(\text{Ank1}\) mRNA level was reduced in \(\text{hema6}\) mice. Total RNA was isolated from bone marrow in both heterozygous and homozygous \(\text{hema6}\) mice, as well as sex- and age-matched C57BL/6J control mice. \(\text{Ank1}\) expression was assessed by quantitative RT-PCR with GAPDH transcript as endogenous control. \(n=4\) for all groups of mice. Data is expressed as mean ± SD. (B) Coomassie blue-stained SDS-PAGE of ghost membrane proteins. (C) Immunoblot of ghost membrane proteins with antibodies against ankyrin-1 and actin, respectively (upper panel). The protein levels of ankyrin-1 were quantitated by densitometry (lower panel). Result shown here represents three independent experiments, and data is expressed as mean ± SD. \(n=3\) for all groups.* \(P<0.05\), ** \(P<0.01\), *** \(P<0.001\).

**Figure 6.** Band 3 extractability from the red cell membrane skeleton in wild type and \(\text{hema6}\) mice. Ghost membrane proteins were extracted with Triton X-100 at indicated concentration, and proteins released into the supernatant were analyzed by SDS-PAGE and probed with antibody against band 3. Result shown here is representative for at least three independent experiments.
Figure 1
Figure 3

A

LOD Score

Chromosome

B

<table>
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<tr>
<th>Marker</th>
<th>Position (Mb)</th>
<th>Mutants</th>
<th>Wild types</th>
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<tr>
<td>B10HEMA60002</td>
<td>7.7</td>
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<td>B10HEMA60006</td>
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<td>B10HEMA60009</td>
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</tr>
<tr>
<td>B10HEMA60011</td>
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Mice number

- C57BL/6J/C57BL/6J
- C57BL/6J/C57BL/10J
- C57BL/10J/C57BL/10J

C

5' T A G C A A T A A C C G T G A 3'

C57BL/6J

5' T A G C A A C A A C C G T G A 3'

hema6 (-/-)
Figure 4

A

DNA ladder

wt

Ank1

B

Long isoform

Short isoform

wt

Figure 4
Figure 5
<table>
<thead>
<tr>
<th>Ghosts</th>
<th>wt</th>
<th>Ank&lt;sup&gt;1&lt;/sup&gt;&lt;sub&gt;hem&lt;sub&gt;6/hem&lt;sub&gt;6&lt;/sub&gt;&lt;/sub&gt;</th>
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</thead>
<tbody>
<tr>
<td>Triton X-100</td>
<td>0%</td>
<td>0%</td>
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![Figure 6](image_url)