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DEVELOPMENT AND DISEASE

Characterization of zebrafish *merlot/chablis* as non-mammalian vertebrate models for severe congenital anemia due to protein 4.1 deficiency

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SUMMARY

The red blood cell membrane skeleton is an elaborate and organized network of structural proteins that interacts with the lipid bilayer and transmembrane proteins to maintain red blood cell morphology, membrane deformability and mechanical stability. A crucial component of red blood cell membrane skeleton is the erythroid specific protein 4.1R, which anchors the spectrin-actin based cytoskeleton to the plasma membrane. Qualitative and quantitative defects in protein 4.1R result in congenital red cell membrane disorders characterized by reduced cellular deformability and abnormal cell morphology. The zebrafish mutants *merlot* (*mot*) and *chablis* (*cha*) exhibit severe hemolytic anemia characterized by abnormal cell morphology and increased osmotic fragility. The phenotypic analysis of *merlot* indicates severe hemolysis of mutant red blood cells, consistent with the observed cardiomegaly, splenomegaly, elevated bilirubin levels and erythroid hyperplasia in the kidneys. The result of electron microscopic analysis demonstrates that *mot* red blood cells have membrane abnormalities and exhibit a severe loss of cortical membrane organization. Using positional cloning techniques and a candidate gene approach, we demonstrate that *merlot* and *chablis* are allelic and encode the zebrafish erythroid specific protein 4.1R. We show that mutant cDNAs from both alleles harbor nonsense point mutations, resulting in premature stop codons. This work presents *merlot*/*chablis* as the first characterized non-mammalian vertebrate models of hereditary anemia due to a defect in protein 4.1R integrity.

Key words: Zebrafish, Erythroid protein 4.1, Hereditary elliptocytosis, Congenital anemia, *merlot*, *chablis*, Marginal band

INTRODUCTION

In order for red blood cells to flow through the microcirculation without fragmentation and loss of membrane integrity, they must possess a remarkable property known as cellular deformability (Weed, 1970; Mohandas et al., 1979), which is determined by three elements: cytoplasmic viscosity, cellular geometry and the material property of the membrane (Mohandas and Chasis, 1993). The red cell membrane is composed of the lipid bilayer, integral transmembrane proteins and a network of structural proteins that form the membrane skeleton (Gallagher et al., 1998). The major proteins of the membrane skeleton are α and β spectrins, actin, ankyrin, and protein 4.1. Interactions between these proteins form a protein network (Liu et al., 1987) that is vertically attached to the lipid bilayer by binding to the cytoplasmic domain of the integral membrane proteins, band 3 and glycophorin C. These protein interactions are known to be essential determinants of red cell morphology, deformability and mechanical stability (Mohandas and Chasis, 1993). A vast body of clinical research points to the fact that qualitative or quantitative disruption of these protein-protein interactions due to mutations in the membrane proteins results in defective structure and function of red cell membrane, leading to congenital anemias (Bossi and Russo, 1996; Palek and Sahr, 1992; Tse and Lux, 1999; Palek, 1987).

Erythrocyte protein 4.1 (band 4.1 or 4.1R) is a multifunctional structural protein in the red cell membrane skeleton whose interaction with both transmembrane and cytoskeletal proteins plays an indispensable role in maintaining red cell morphology, membrane deformability and mechanical stability (Yawata et al., 1997; Conboy, 1993). Human protein 4.1 was first identified in the erythroid cell membrane skeleton (Conboy et al., 1986a), and contains four distinct structural and functional domains with molecular weights of 30, 16, 10 and 22/24 kDa (Leto and Marchesi, 1984). Through its spectrin-

binding domain at the 10 kDa domain (Correas et al., 1986), protein 4.1R accelerates and stabilizes the interaction between spectrin and actin filaments (Ohanian et al., 1984). This ternary complex is essential for maintaining the red cells mechanical stability (Lorenzo et al., 1994). Red cells that are completely deficient in 4.1R or lack the spectrin-binding domain of 4.1R have abnormal elliptical cell morphology and fragile membrane (Tchernia et al., 1981; Marchesi et al., 1990). Protein 4.1R also plays a crucial role in anchoring the spectrin-actin framework to the overlaying lipid membrane. Via its N-terminal 30 kDa membrane-binding domain, protein 4.1R interacts with the cytoplasmic domains of the membrane proteins band 3 (Pasternack et al., 1985), glycophorin C (Hemming et al., 1995; Marfatia et al., 1995), p55 (Chang and Low, 2001; Alloisio et al., 1993; Nunomura et al., 2000) and CD44 (Nunomura et al., 1997). Calmodulin also binds to this domain and modulates protein 4.1R interactions with its binding partners (Tanaka et al., 1991; Lombardo and Low, 1994; Nunomura et al., 2000). The presence of the evolutionarily conserved N-terminal membrane-binding domain (also called the FERM domain: 4.1-Ezrin-Radixin-Moesin) (Chishti et al., 1998; Hoover and Bryant, 2000; Arpin et al., 1994) is a common feature of a diverse group of band 4.1 related proteins such as 4.1G, 4.1N and 4.1B in vertebrates (Peters et al., 1998), and the putative 4.1R homolog in *Drosophila*, also known as *coracle* (Fehon et al., 1994).

Although the process of erythropoiesis in mammals and nonmammalian vertebrates is essentially the same, there are structural and morphological differences between mature red cells. The erythroid precursors of nonmammalian vertebrates, similar to their counterparts in mammals, are spherical and contain a round nucleus with open chromatin and basophilic cytoplasm. However, terminally differentiated and mature red cells of other vertebrates are nucleated and elliptical, while mammals have anucleated red blood cells with round, biconcave morphology. During differentiation, the cytoskeleton of red cells undergoes extensive reorganization that results in their final morphology (Wickrema et al., 1994). The elliptical morphology of red cells of nonmammalian vertebrates is due to their structure of cytoskeletal system, which in addition to the membrane skeleton contains intermediate filaments and an enveloping layer of microtubules called the marginal band (Cohen, 1991; Cohen et al., 1998). The extent of interaction between the marginal band and the protein network of membrane skeleton in red blood cells is yet to be characterized.

The availability of induced mutations that affect different aspects of hematopoiesis is one of the advantages of the zebrafish system. The zebrafish mutants *merlot* and *chablis* are members of a group of zebrafish mutants that have normal onset of primitive hematopoiesis followed by a decrease in the number of circulating red blood cells at subsequent stages of larval development (Ransom et al., 1996). One member of this group, *riesling*, has been characterized as having a mutation in β-spectrin (Liao et al., 2000).

In this work, we present the characterization of *merlot*/*chablis* phenotype and identification of zebrafish protein 4.1R as the mutated gene in *merlot* and *chablis*. We also present *merlot*/*chablis* as genetic models to study the role of protein 4.1R in morphogenesis and terminal maturation of nucleated red blood cells.

MATERIALS AND METHODS

Zebrafish strains and maintenance

Both alleles of *merlot* (*mot*^{tu275} and *mot*^{tm303c}) and of *chablis* (*cha*^{tu242e} and *cha*^{tu245}) were generated in a large-scale chemical mutagenesis screen (Haffter et al., 1996; Driever et al., 1996). *mot*^{tu275} was maintained on Tübingen (Tü) background, whereas *mot*^{tm303c} was maintained on standard AB background. Both *chablis* alleles were maintained on AB genetic background. Polymorphic strains for meiotic mapping, SJD and Darjeeling were generous gifts of S.L. Johnson.

Genetic mapping and linkage analysis

A polymorphic mapping strain was generated by crossing heterozygote *mot*^{tu275} with the polymorphic WIK strain. Embryos were collected from pair mating of Tü/WIK heterozygotes and phenotyped at 96 hours post fertilization (hpf) for anemia. Genomic DNA extraction from individual embryos and bulk segregant analysis (BSA) was performed as described (Talbot and Schier, 1999; Zhang et al., 1998). Simple sequence-length polymorphism (SSLP) markers (Shimoda et al., 1999; Knapik et al., 1998) used for BSA were selected from the MGH web server (http://zebrafish.mgh.harvard.edu) and purchased from Research Genetics (Huntsville, AL). Diploid mutant *cha* and wild-type embryos were collected from AB/Darjeeling and AB/SJD heterozygotes. Genome-wide scanning on BSA pooled DNA was used for linkage analysis. Close microsatellite markers, z11376 and z13511, were found to flank the *cha* locus.

Selection of candidate gene and isolation of cDNA

Single wild-type and mutant embryos were genotyped with SSLP markers z25218, z10036 and z25278, and the genetic locus defined by them was searched for cloned ESTs (WUZGR, http://zfish.wustl.edu). Two cDNA clones, Fc37c08 and Fb70c02, were identified as potential candidate genes and were purchased from Incyte Genomics (St Louis, MO). Fc37c08 was a partial clone of 2 kb and Fb70c02 was a full-length 5 kb sequence of the cDNA encoding for a protein homologous to human erythroid protein 4.1. Total RNA from adult tissue (blood and kidney) and single wild-type and mutant (both *mot* alleles) embryos was extracted by Trizol (Gibco-BRL, Rockville, MD). The first strand cDNA was synthesized using the SuperScript First-Strand Synthesis System for RT-PCR (Gibco-BRL) following the manufacturer's protocol. The oligo (dT) primed first strand cDNA was subjected to PCR amplification using the Expand Long Template PCR System, (Roche, Indianapolis, IN) with a 5′- GATCATTGCCGGACATGTAAA-3′ forward primer and a 5′-TGTAAGCGGGTGAAATAAGCT-3′ reverse primer. The amplified full-length 5Kb cDNA fragment of the zebrafish erythroid protein *4.1R* was cloned into the PCR 2.1 TOPO TA vector (Invitrogen, Carlsbad, CA) and sequenced (primer sequences available upon request).

The *cha* locus was independently mapped near SSLP markers, z11376 and z13511, which allowed the identification of a candidate EST, Fb70c02, within this genetic interval. A large P1 artificial chromosome (PAC) clone, 215:J16, was isolated by hybridization with the Fb70c02 EST clone. The termini of PAC 215:J16 were sequenced to derive simple sequence conformational polymorphic (SSCP) primers (forward, 5′-TCGTCGCTCAGTCATCAAGTAACA-3′; reverse, 5′-TGACATGAACCTTCGCTTCCC-3′) for analyzing embryos that were genetic recombinants with markers, z11376 and z13511.

Mutation and linkage analysis using allele-specific primers

To confirm the mutation, RT-PCR on total RNA from several 24 hpf mutant embryos was performed and the PCR products were sequenced. For the *mot*^{tu275} allele, a forward primer (P10; 5′-

GTGACAACAGAGGAGATCCAA-3′) and a reverse primer (P14; 5′-AACACCTCAACAGCCGAACC-3′) were used to amplify a 630 nucleotide fragment that contained the point mutation. For *mot*^{tm303c}, the forward primer P10 was used with a reverse primer (P4; 5′-CGATTCAGCCTTTTCTCTCTA-3′) to amplify a 1.4 kb fragment containing the point mutation. Linkage analysis with allele-specific primers (Bottema and Sommer, 1993; Newton et al., 1989) was used to confirm the linkage of the anemic phenotype to the genomic sequence. PCR was performed on genomic DNA extracted from adult heterozygotes and 96 hpf mutant (*mot*^{tu275}) and wild-type embryos. A common forward primer (P10) was used with wild-type-specific reverse primer (WtR; 5′-ACCTCAACAGCTGGACCTCG-3′) or *mot*^{tu275}-specific reverse primer (MutR; 5′-ACCTCAACAGCTGGACCTCA-3′) (point mutation G to A is underlined) to amplify ~550 bp fragment of genomic DNA. PCR conditions were optimized so that the primer pair P10 and WtR only amplified wild-type DNA, whereas the primer pair P10 and MutR only amplified mutant DNA. For confirmation of the *cha*^{tu242e} and *cha*^{tu245} nonsense mutation, genomic DNA was amplified with primers (forward, 5′-GATGTGGAGGACGACTGGTTTATC-3′; reverse, 5′-CTTCGTCTCTGGTACTGTTATCTGTTC-3′). The PCR product was analyzed by allele-specific oligonucleotide hybridization (Farr et al., 1988) with either wild-type (5′-TTTTTAGTGCRAGGTCCRG-3′) or mutant (5′-TTTTTAGTGTRAGGTCCRG-3′) oligonucleotide.

Truncation mutation analysis

The full-length 5 kb cDNA fragments from wild-type and mutant (*mot*^{tu275}) fish were cloned into PCR 2.1 TOPO TA vector (Invitrogen) and were subjected to in vitro transcription and translation using the TNT T7 reticulocyte lysate system (Promega, Madison, WI). Synthesized wild-type and mutant proteins were labeled by including [^{35}S] methionine in the reaction, and were resolved by a 10% SDS-PAGE, which was dried and exposed to autoradiographic film.

In situ hybridization

Whole-mount in situ hybridization with digoxigenin-labeled RNA probes was performed on 24 hpf wild-type and mutant embryos (both *mot* alleles) as described (Westerfield, 1993) with some modifications. A fragment of zebrafish *P4.1R* cDNA (from –97 to +1575 bp) was amplified by RT-PCR from wild-type adult blood, subcloned into PCRII vector (Invitrogen), and used as a template for generating sense and antisense RNA probes. The full-length cDNA clone, Fb70c02, was also used as a template for probe synthesis. The construct was digested by *Kpn*I and the RNA probe spanning the 3′UTR and poly-A tail was synthesized using SP6 RNA polymerase. A probe for *gata1* was generated as described elsewhere (Long et al., 1997). cDNAs encoding for embryonic globins were isolated from our embryonic blood-specific cDNA library. Briefly, inserts were amplified by PCR using flanking vector primers containing T7 RNA polymerase site. The PCR products were used as templates to generate probes.

Blood analysis, tissue preparation and *o*-dianisidine staining

Adult fish were anesthetized in a solution of 0.05% tricaine (Sigma) and peripheral blood was drawn by cardiac puncture using heparinized pulled micro needles. Embryonic blood was collected by cutting the tail of embryos in a PBS solution containing tricaine, sodium citrate and albumin. Collected embryonic blood cells were spun onto a glass slide at 750 rpm for 3 minutes using a Cytospin 2 (Shandon, Pittsburgh, PA). Blood smears were stained with Wright-Giemsa method following the manufacturer's instruction (Sigma). The red blood cell number of wild-type and mutant adult fish was determined using a Neubauer hemocytometer. Hemoglobin was measured using Drabkin's reagent (Sigma). Exactly 2 μl of blood was suspended in 1 ml of Drabkin's reagent and incubated at room temperature for 10 minutes. The optical density was then measured at a wavelength of 540 nm using a Smart Spec 3000 spectrophotometer (BioRad,

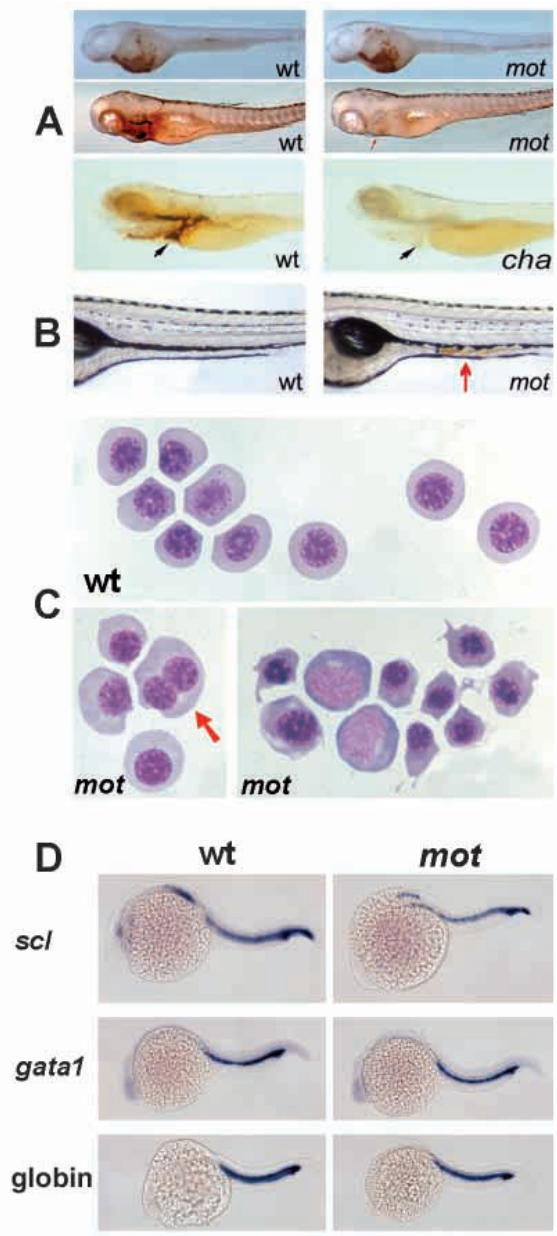

Fig. 1. Characterization of the embryonic phenotype in *mot/cha*. (A) Whole-mount *o*-dianisidine staining of wild-type and *mot/cha* embryos; (top) at 48 hpf, wild-type and *mot* embryos have a similar number of blood cells; (middle) at 96 hpf, *mot* embryos lack circulating blood cells (arrow); (bottom) wild-type and *cha* embryos at 72 hpf are stained with *o*-dianisidine for hemoglobin in the cardiac sinus (arrows). (B) After the onset of anemia, excretion of bile pigments in *mot* embryos is noticeable (arrow) and continues for several days. (C) Wright-Giemsa staining of circulating red cells collected at 48 hpf from wild-type and *mot* embryos reveals the presence of cells with abnormal morphology in the *mot* embryos. Wild-type cells are spherical with round, open nuclei, but *mot* contains binucleated cells (arrow), and morphologically abnormal cells with condensed nuclei and spiculated membranes. (D) Whole-mount RNA in situ analysis of wild-type and *mot* embryos with the hematopoietic genes, *scl*, *gata1* and globin reveals comparable levels of transcript expression in wild-type and *mot* embryos at 24 hpf, indicating that erythropoiesis in *mot* fish is not interrupted. Lateral views are illustrated with anterior towards the left and dorsal towards the top.

Hercules, CA). Red blood cell indices were determined using a GEN®S Coulter Counter (Miami, FL). Kidney and spleen smears from adult wild-type and mutant fish were prepared as described (Long et al., 2000). Analysis of hemoglobin expression in embryos was performed using *o*-dianisidine, as described previously (Detrich et al., 1995).

Osmotic fragility

Freshly drawn blood from wild-type and homozygous adult fish was washed three times in 0.9% NaCl and suspended in saline to ~10% packed cell volume. Cell suspension (5 μl) was added to each of 150 μl solutions that contained 0.900%, 0.800%, 0.700%, 0.600%, 0.500%, 0.475%, 0.450%, 0.425%, 0.400%, 0.300% and 0.200% of NaCl (wt/vol). Cells were incubated at room temperature for 15 minutes, and then centrifuged for 3 minutes at 420 ***g***. The percent hemolysis in the supernatant was measured at 540 nm with the spectrophotometer.

Electron microscopy and TUNEL assay

For scanning electron microscopy, blood was drawn from adult wild-type and homozygous mutant fish and washed three times in cold saline. Blood cells were crosslinked in buffered glutaraldehyde and post fixed in osmium tetroxide. Dehydrated cells were rinsed in hexamethyldisilazane, sputter-coated with gold and examined by a Cambridge 360 Scanning Electron Microscope (Leo Electron Microscopy, Cambridge, UK). For transmission electron microscopy, freshly drawn blood cells from wild-type and *mot* fish were crosslinked and dehydrated as above, embedded in Epon, and 60 nm sections stained with uranyl acetate and lead citrate were examined with a JEOL JEM-100CX electron microscope (JEOL, Ltd. Tokyo, Japan). ApopTag (Intergen, NY) kit was used for detection of apoptotic cells in peripheral blood, kidney and spleen.

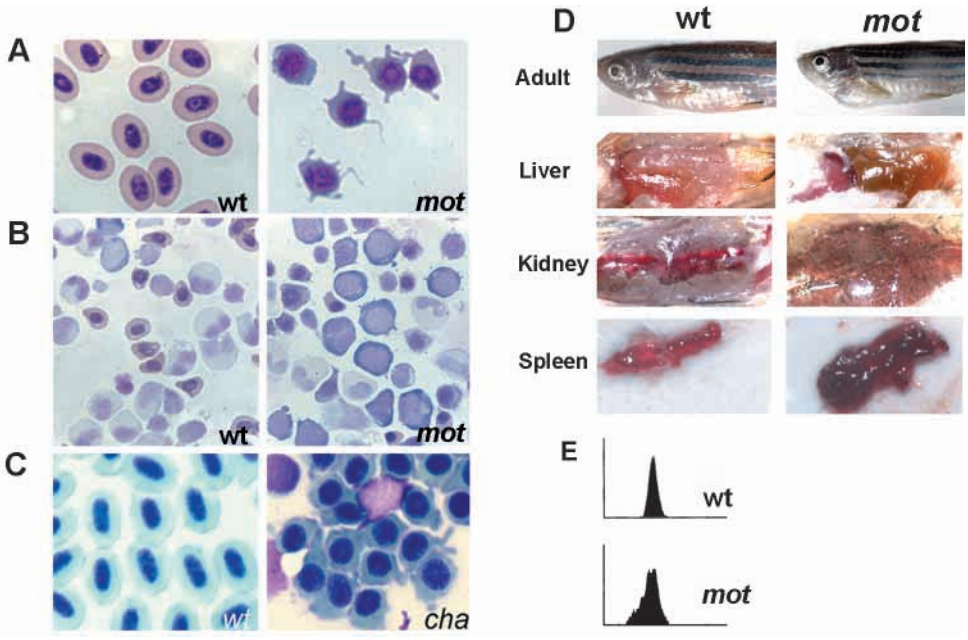

Fig. 2. Hematological analysis and gross anatomy of adult *mot*. (A) Wild-type red blood cells are terminally differentiated with elliptical morphology, condensed nuclei and hemoglobin-filled cytoplasm. Blood cells collected from adult *mot* fish exhibit a maturation arrest at the late basophilic erythroblast stage and striking membrane abnormalities. (B) Wright-Giemsa staining of a tissue preparation of the wild-type and mutant fish kidneys reveals an erythroid hyperproliferation in the *mot* fish with a drastically decreased myeloid/erythroid ratio. A few cells at the early proerythroblast stage also show membrane spiculation. (C) Peripheral blood cells from wild-type zebrafish are compared with concentrated peripheral blood cells from adult *cha* fish, which show abnormal morphology and differentiation arrest. (D) Gross anatomy of *mot* fish shows a dilated cardiac chamber compared with wild type. An icteric liver, greatly enlarged kidney and splenomegaly are always present in the adult *mot* fish. (E) Mean cell volume of red blood cells of wild-type and mutant fish, as measured by an automated Coulter Gen S instrument, shows that wild-type cells have a uniformly distributed volume, whereas *mot* cells show a significant variation in size. The anisocytosis in *mot* cells is due to random membrane fragmentation of *mot* cells.

Immunofluorescence and confocal microscopy

Blood smears from adult wild-type and homozygous *mot* fish were prepared as described above. Cells were extracted in 0.2% Triton X-100 in phosphate-buffered saline (PBS) for 30 minutes, fixed in 4% paraformaldehyde for 20 minutes at room temperature, and then post fixed in methanol for 10 minutes at –20°C. Cells were incubated in 1% bovine serum albumin (BSA)/PBS for 1 hour, incubated with anti-tubulin monoclonal antibody (Sigma) in 1%BSA/PBS for 1 hour, washed three times in PBS, incubated with Cye3-conjugated anti-mouse IgG antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) for 1 hour, washed 3 times in PBS and counterstained with DAPI.

Rescue experiment and microinjection

A zebrafish protein 4.1-GFP fusion construct was generated by RT-PCR. Two primers, rescF (5′-AAGCTTCCCGCTTTTGCAGATCA-3′) and rescR (5′-GGTACCTCGAGCAAATATTTCT-3′), were used to amplify the full-length *P4.1* cDNA and remove the stop codon. The PCR product was cloned in frame into the green fluorescent protein (GFP) expression vector pEGFP-N3 (Clontech). The integrity of the construct was verified by restriction mapping and sequencing. Fertilized eggs from a cross of *mot*^{tu275} homozygotes were dechorionated and injected at the one- to two-cell stage with P4.1-GFP construct (150 ng/μl) as described (Meng et al., 1999). Injected embryos were incubated at 28°C, examined for GFP expression, phenotyped and stained with *o*-dianisidine at 96 hpf.

GenBank Accession number

AY124488 for full-length zebrafish P4.1R cDNA and protein sequence.

RESULTS

Characterization of *mot* and *cha* embryos

Both recessive alleles of *mot* and *cha* mutants were recovered in a large-scale screen of ENU-induced mutagenesis (Weinstein et al., 1996; Haffter et al., 1996). The *mot* and *cha* phenotypes are characterized by the onset of a severe anemia at 96 hpf. Homozygote embryos develop normally for the first 2-3 days, but as they reach 4-days of age, there is a significant decrease in the number of circulating erythroid

Table 1. Hematological parameters of wild-type and *mot* –/– fish

Genotype	Wild type (*n*=5)	*mot/mot* (*n*=5)
Red blood cell levels ($\times 10^6/\mu l$)	3.64±0.25	0.80±0.04
Hemoglobin (optical density at 540 nm)	0.109±0.018	0.014±0.001

The reduced red blood cell count and hemoglobin level of *mot* fish are indicative of severe anemia.
Values are mean±s.e.m.

cells (Fig. 1A). Accompanied by erythroid destruction, there is an excretion of bile pigments visible as a yellow-orange stripe in the gastrointestinal tract of the mutant embryos, which is suggestive of accelerated hemolysis of embryonic red cells (Fig. 1B). Analysis of Wright-Giemsa stained embryonic blood at 48 hpf showed marked morphological differences between wild-type and mutant red cells (Fig. 1C). Wild-type erythroid cells are spherical with basophilic cytoplasm and a round nucleus with open chromatin. Examination of *mot* peripheral blood smears revealed the presence of morphologically abnormal cells with spiculated membranes, as well as binucleated cells. After 96 hpf, wild-type blood cells essentially conform to the mature elliptical erythroid morphology with condensed nucleus and hemoglobin-filled cytoplasm. At this stage, some mutant embryos have only 50-100 circulating blood cells, compared with ~1000-3000 circulating red cells in wild-type embryos. Wright-Giemsa stained pooled cells from several mutant embryos revealed immature cells with pyknotic nuclei, little cytoplasm and abnormal membrane projections (data not shown).

In order to further examine the effect of the *mot* mutation on primitive erythropoiesis, we performed whole-mount in situ hybridization on 24 hpf wild-type and *mot* fish. We used *scl*, *gata1* and embryonic globins as markers of hematopoietic stem cells, early and terminally differentiated red blood cells, respectively. The results, illustrated in Fig. 1D, revealed normal expression level of these genes in *mot* fish. These observations suggest that the process of primitive erythropoiesis is uninterrupted in *mot* embryos and that the anemia is due to accelerated hemolysis of abnormal cells.

Hematological and pathological analysis of adult *mot* and *cha* fish

About 5-10% of homozygous embryos could be raised to adulthood with frequent feeding and ample supply of oxygenated water. *mot* adult fish suffer from growth retardation and appear pale. Moreover, cardiomegaly is observed in most adult fish, predominantly in males (Fig. 2). Analysis of peripheral blood from adult homozygotes showed evidence of severe anemia. Red blood cell count and hemoglobin levels were significantly decreased in *mot* fish (Table 1). Despite a severe reduction in the number of red cells and hemoglobin concentration, adult *mot* are viable, fertile and appear to exhibit normal fish behavior. Examination of *mot* peripheral blood revealed microcytic cells that exhibit differentiation arrest at the basophilic erythroblast stage with severe abnormal membrane morphology, as seen in Fig. 2A. About 1% binucleated cells were also detected in blood smears of *mot* fish, whereas in wild type no binucleated cells were observed

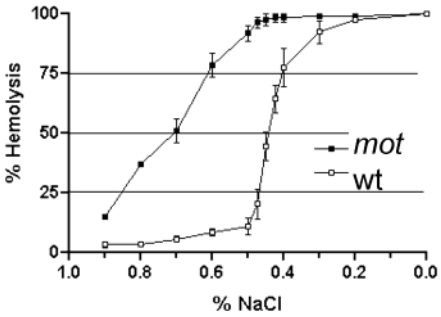

Fig. 3. Osmotic fragility of wild-type and *mot* red blood cells. Red cells of five wild-type and three mutant fish were subjected to the osmotic fragility test (results represent means±s.e.m.). Wild-type cells exhibit a sigmoid curve when exposed to hypotonic solutions. The red blood cells from *mot* fish are extremely sensitive to osmotic stress and show an increased fragility.

(data not shown). Examination of stained kidney smears revealed a marked increased in the number of erythroid progenitors in *mot* mutant (Fig. 2B), indicating a reactive hematopoiesis. Evidence of membrane abnormalities was also noted in a few proerythroblasts, suggesting that abnormal membrane structure may have an impact in the early stages of erythroid differentiation. When adult *mot* fish were dissected (Fig. 2D), prominent features included enlarged gall bladder and spleen, as well as an increased concentration of bilirubin in the internal organs (liver, GI tract). Hypercellular and enlarged kidneys (the site of definitive hematopoiesis in adult fish) were also observed in homozygous fish, resulting from a compensatory mechanism for excess hemolysis and reduced survival of erythroid cells.

Similarly, a small number of homozygous *cha* embryos could be raised to juvenile and adult stages. The gross physical features of *cha* adults are identical to those observed for other zebrafish mutants with severe anemia. Examination of the peripheral blood smear reveals both quantitative and qualitative defects with differentiation arrest at the late erythroblast stage (Fig. 2C). Enlarged and hypercellular kidney and spleen were notable in *cha* adults, consistent with reactive hematopoiesis (data not shown).

These data indicate that *mot* fish suffer from a severe and partially compensated hemolytic anemia with symptoms similar to hemolytic anemia in humans. The enlarged gall bladder is especially intriguing because such enlargement usually occurs in humans in many cases of hereditary anemia because of obstructive bilirubin gallstones.

Osmotic fragility test is a sensitive test for detection of cells with altered tolerance to osmotically induced stress. Although in humans increased osmotic fragility is characteristic of hereditary spherocytosis, we hypothesized that as the membrane structure of red blood cells in *mot* fish is abnormal, as seen on peripheral blood smears, the osmotic fragility test may be informative. Our results indicated that *mot* cells were, indeed, profoundly susceptible to osmotic stress (Fig. 3). The salt concentration in which 50% of wild-type cells undergo hemolysis is significantly lower (0.425%) compared with mutant cells (0.700%). This means that, compared with wild-type cells, *mot* cells are considerably more fragile in hypotonic solutions.

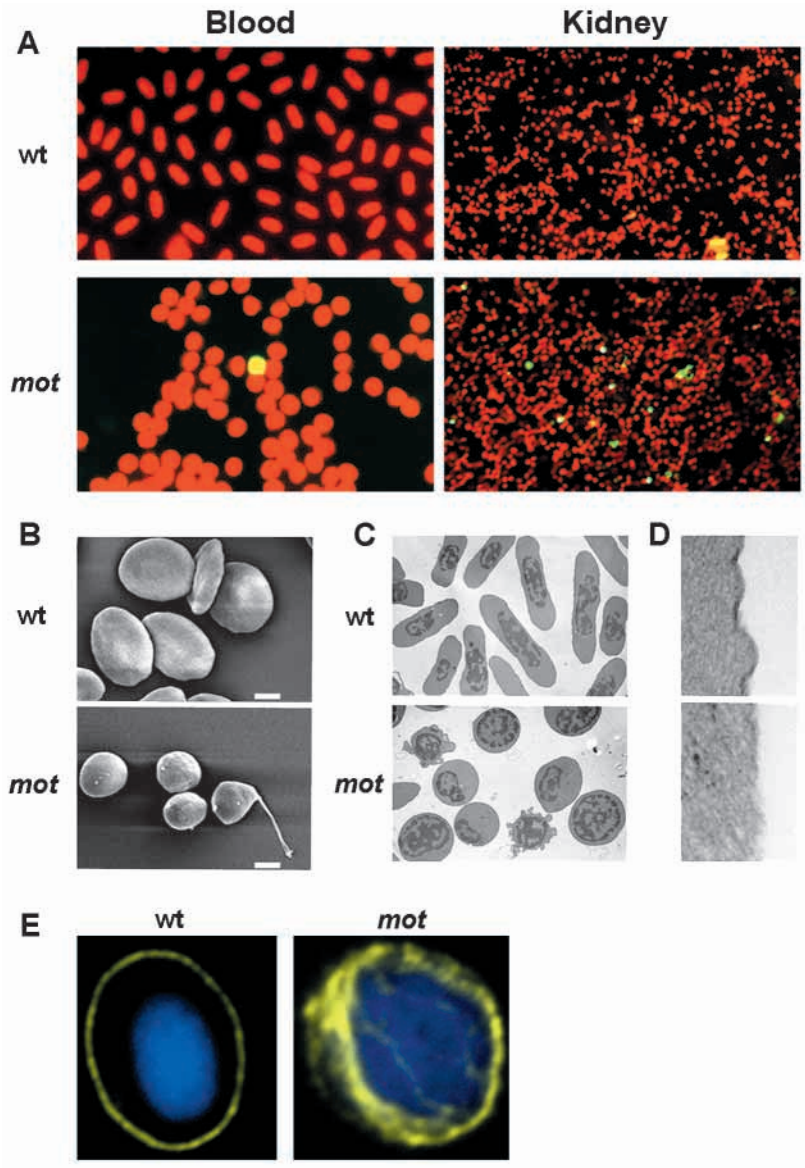

Fig. 4. Apoptosis and analysis of red cell membrane and marginal band. (A) A fluorescent confocal TUNEL assay on peripheral blood and kidney from wild-type and *mot* cells reveals a significant number of apoptotic cells (green) in the kidneys of *mot*, whereas peripheral blood of mutant fish showed a few cells undergoing apoptosis. (B) Scanning electron micrographs show wild-type cells with biconcave elliptical morphology. Red cells from *mot* fish appear microspherocytic, with abnormal membrane pitting and projections. (C,D) Transmission EM analysis reveals elliptical wild-type cells with elongated nuclei and compact, organized cortical membranes. The spherical *mot* cells with spiculated membranes lack a sharply packed and organized cortical membrane. (E) A confocal immunofluorescence analysis detects the localization of microtubules in a marginal band in wild-type red cells (green), whereas in mutant cells the microtubules exhibit a diffuse localization. Scale bar: in B, 2.5 μm.

TUNEL assay, electron microscope analysis and detection of marginal band

The results of TdT-mediated dUTP nick-end labeling (TUNEL) assay showed evidence of extensive apoptosis in kidneys (~5%) and to a lesser degree in the peripheral blood (about 1%) of *merlot* fish (Fig. 4A). In wild-type fish, we did not detect any apoptotic cells in the peripheral blood, and in the kidneys less that 1% of the cells were apoptotic. This result was consistent with previous data indicating that hemolysis of nucleated red blood cells is accompanied by DNA fragmentation (Orkin and Weiss, 1999; Liao et al., 2000). The increased apoptosis in erythroid progenitors in the kidneys suggest that the membrane lesion in *mot* cells is an early event during erythroid differentiation. The peripheral blood of *mot* fish contained slightly, but significantly, more apoptotic cells than the wild-type. Although splenomegaly was a prominent feature in *mot*, we did not detect a significant difference in the number of apoptotic cells between wild-type and mutant fish in the spleen (data not shown). This was not unexpected, as sequestration of abnormal cells in the spleen is a phagocytic-mediated phenomenon and may not be detected by our methods.

In order to study the ultrastructural membrane abnormalities in *mot* cells, we performed scanning and transmission electron microscopic analyses. Fig. 4B,C illustrates the results of SEM and TEM, respectively. Examination of red cells with SEM revealed that wild-type cells have an elliptical morphology with smooth membranes, whereas *mot* cells are spherical, microcytic and have profound membrane abnormalities, such as surface pitting and membrane projections. Upon analysis of blood cell cross sections with transmission EM, we observed a severe loss of the cortical membrane organization and integrity in mutant cells (Fig. 4D). The results of the ultrastructural analysis were consistent with our previous observations of stained blood smears and confirmed our hypothesis that abnormal morphology and fragmentation of *mot* cells is due to loss of membrane integrity, organization and stability.

We attempted to investigate whether the formation of marginal band of microtubules is compromised in *mot* cells. Immunohistochemistry analysis showed (Fig. 4E) that in wild-type red blood cells microtubules are confined to a marginal band at the long axis of cells. By contrast, *mot* cells exhibit a diffuse pattern of microtubule filaments in different planes of the cell, indicating that the absence of protein 4.1 in the red cell membrane cytoskeleton affects the proper assembly of microtubules into a marginal band at the periphery of red blood cells.

Based on phenotypical, hematological and molecular analysis of the zebrafish *mot*, we hypothesized that the defect in *mot* gene is intrinsic to red cells, and that the *mot* is a structural component of erythroid cell membrane. Based on this hypothesis and the results of positional cloning experiments, we searched the available cloned and mapped EST databases for possible candidate genes.

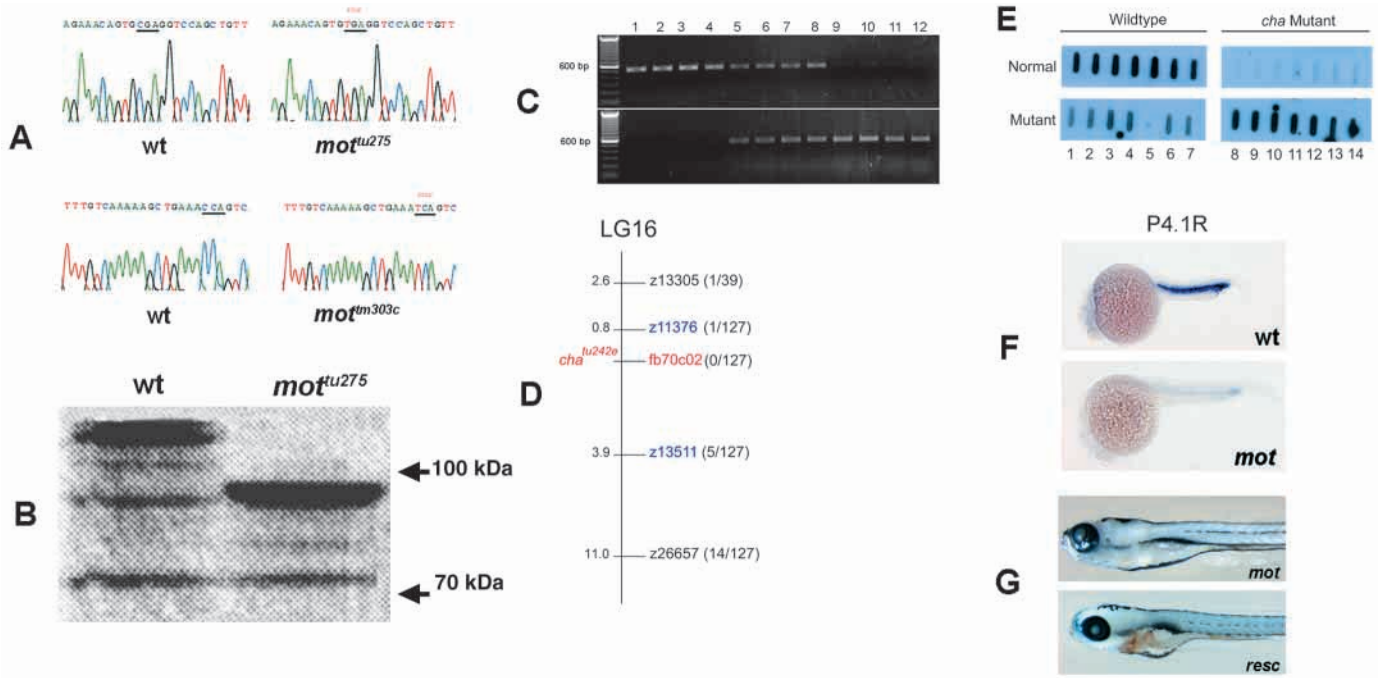

Fig. 5. Identification of protein 4.1R as the mutated gene in *mot/cha*. (A) cDNA sequence comparison of wild-type and both alleles of *mot* shows two nonsense point mutations in the *mot* alleles (point mutations are underlined). The DNA sequence of *mot*^{tm303c} and its corresponding wild-type sequence shown here are in the 3′to 5′ orientation. (B) A protein truncation test shows the truncated translation product of *mot*^{tu275} cDNA compared with that of wild-type cDNA. (C) Linkage analysis via allele specific PCR primers on genomic DNA from wild-type (lanes 1-4), heterozygous (lanes 5-8) and *mot* (lanes 9-12). The wild-type primer, top, amplifies wild-type and heterozygous DNA, whereas the mutant primer, bottom, amplifies DNA from heterozygous and *mot* embryos. (D) Genetic map of the *cha* locus on LG16. EST Fb70c02 showed no genetic recombinants from the *cha* locus in 127 informative animals. (E) Allele-specific oligonucleotide hybridization for wild-type and mutant protein 4.1R sequences. Wild-type siblings (lanes 1-7) and mutant siblings (lanes 8-14) show complete linkage with either normal or mutant protein 4.1R alleles, respectively. (F) *P4.1R* in situ hybridization shows an expression pattern restricted to the hematopoietic intermediate cell mass. Wild-type 24 hpf embryos exhibit high-level expression of the *P4.1R* transcript, whereas the level of transcript expression is greatly reduced in *mot* embryos. (G) At 96 hpf, *mot* embryos that were injected with *P4.1R*/GFP construct are partially rescued and have a higher number of circulating red cells compared with *mot* controls.

Positional cloning of *mot* and *cha* with a candidate gene approach

We used bulk segregant analysis to map the *mot* gene on linkage 16. Because the available genetic markers flanked a sizable physical distance, chromosomal walking was not feasible and we decided to approach the problem by examining cloned ESTs. As we had already hypothesized that the mutation in *mot* is likely to affect red blood cells membrane integrity, it was fortunate that we located the EST encoding the zebrafish protein 4.1. DNA sequence analysis from wild-type and both *mot*^{tu275} and *mot*^{tm303c} alleles, revealed nonsense point mutations in the *mot* gene causing premature stop codons that would cause a truncated protein 4.1. In *mot*^{tu275} we detected a C-to-T transition, whereas in *mot*^{tm303c} a G-to-A transition caused premature termination codons at +2349 and +3036 nucleotides, respectively (Fig. 5A). Both mutations occurred in a GC-rich region of the cDNA, and are upstream from the spectrin-binding domain (SBD) of *P4.1R*, generating a truncated and dysfunctional protein that lacks the SBD. In order to confirm that point mutation would result in premature translation termination, we performed a protein truncation test using cloned wild-type and *mot*^{tu275} cDNAs as templates, which detected a truncated protein product from the mutant allele (Fig. 5B). We repeated the RT-PCR and sequencing on several mutant embryos from different batches to confirm our results. In order to determine if the point mutation is linked to the mutant phenotype, we performed linkage analysis by allele-specific PCR on genomic DNA extracted from heterozygous adult, wild-type and *mot*^{tu275} embryos. PCR analysis of 100 embryos did not show any recombination, indicating a close linkage between the point mutation in *4.1R* and *mot*^{tu275} phenotype. A representation of PCR products is shown in Fig. 5C.

Using genome-wide scanning for linkage analysis, the *cha* gene was independently mapped to LG16 (Fig. 5D). Two SSLP markers, z11376 and z13511, defined a close genetic interval for the *cha* locus. Within this interval, a candidate EST was identified, Fb70c02, which encoded the full-length sequence for protein 4.1. Analysis of 3′UTR sequences from EST Fb70c02 was not informative for meiotic mapping; therefore, a PAC clone, 215:J16, for the corresponding genomic region was isolated. Polymorphic SSCP primers from this PAC clone demonstrated no genetic recombinants out of 127 informative *cha*^{tu242e} animals. Sequence analysis of both *cha* mutant alleles revealed the identical nonsense mutation as *mot*^{tu275}, namely a C-to-T transition at nucleotide +2349 that results in premature translational termination. To confirm that the protein 4.1 mutation was linked to the *cha* phenotype, individual embryos

Fig. 6. Amino acid sequence of zebrafish protein 4.1. The BLAST application was used to perform a pair-wise amino acid alignment of protein 4.1 in human and zebrafish, and to identify putative functional domains of zebrafish P4.1. Amino acids 1-330 (yellow) correspond to the N-terminal FERM domain; 400-1400 correspond to the 16 kDa domain; 1400-1513 (red) correspond to the spectrin binding domain; and the last 21 amino acids correspond to the 20 kDa domain of human protein 4.1. The four tandem repeats starting at amino acid 780 and ending at 1106 are indicated in green and blue.

from a *cha* heterozygous mating were sorted by anemic phenotype and genotyped by allele-specific oligonucleotide hybridization (Fig. 5E). Wild-type sibling embryos were either heterozygous or homozygous for the normal allele; by contrast, all *cha* mutant embryos were homozygous for the mutant allele. Pair-wise mating between *mot* and *cha* heterozygous fish failed to complement the anemic phenotype (data not shown), confirming that *mot* and *cha* have the same genetic defect in protein 4.1R.

In order to examine the spatial expression pattern of P4.1R, we used several riboprobes generated from different domains of *P4.1* to perform whole-mount in situ hybridization. The results revealed that *mot* gene expression is erythroid-specific and restricted to the intermediate cell mass (Fig. 5F). In both alleles of *mot* (data not shown for mot^{tm303c}), a drastically decreased level of *P4.1R* transcripts was detected, which is possibly due to nonsense-mediated mRNA decay (Culbertson, 1999; Ruiz-Echevarria et al., 1998).

We examined the level of two proteins that P4.1R interacts, band 3 and β-spectrin, in erythroid lysates by western analysis from *mot*, *mot* heterozygous and wild-type siblings; no differences were detected in the level of band 3 and β-spectrin protein (data not shown).

The cloned protein *4.1* cDNA contains multiple open reading frames with the longest containing 4605 nucleotides, encoding a 1535-amino acid protein with a calculated molecular weight of 180 kDa. The deduced amino acid sequence of zebrafish P4.1R derived from the nucleotide sequence was subjected to a homology search using the BLAST program at NCBI. We used ExPASY proteomics tools (http://ca.expasy.org/) to analyze zebrafish P4.1R for conserved domains and prediction of protein-sorting signals and localization sites, which revealed several putative phosphorylation and glycosylation sites along with several bipartite nuclear localization signals. Amino acid sequence comparison of zebrafish P4.1R with human P4.1R showed an identity of 58% (73% similarity) in the N-terminal FERM domain and a 41% identity (54% similarity) in the SBD (data not shown). Structural domains of zebrafish protein 4.1 include an N-terminal membrane binding domain of ~330 amino acids, ~1000 amino acids with a novel sequence, the spectrin binding domain of 100 amino acids, followed by 22 amino acids at the C terminus (Fig. 6). The organization of structural domains of zebrafish protein 4.1 is different from those of mammalian protein 4.1. In mammalian 4.1R, there is a short 16 kDa domain that is flanked by the FERM domain and the spectrin-binding domain. In zebrafish 4.1R, this domain is composed of approximately 1000 amino acids. We searched various databases for known homology to this region, but it appeared that this was a novel sequence with no known homologous sequence. One interesting aspect of this domain is the presence of a sequence of about 90 amino acids that repeats four times in a tandem fashion, starting at position 780 and ending at 1105. A BLAST search did not detect any known peptides with significant homology with this domain.

Although structural domains of human and zebrafish protein 4.1 are divergent in some regards, zebrafish protein 4.1 contains the putative functional elements found in human 4.1R. The high degree of sequence similarity in the membrane and spectrin-binding domains, along with the erythroid-restricted expression pattern of the zebrafish protein, is indicative of a functional conservation of P4.1R in zebrafish. Comparison, alignment and phylogenetic analysis of the deduced amino acid sequence of zebrafish P4.1R, human, mouse, *Xenopus* and *Drosophila* P4.1R proteins revealed an evolutionary conservation of the FERM domain in these proteins (Fig. 7).

P4.1-GFP construct and transgenic rescue

In order to confirm further that *4.1R* is the mutated gene in *mot* mutants, transgenic rescue experiments were performed. We generated a GFP fusion construct by cloning the wild-type zebrafish *P4.1R* cDNA into pEGFP-N3 expression vector, which was then injected into mutant embryos collected from mating mot^{tu275} homozygotes. At 24 and 48 hpf, the expression of GFP was examined using an FITC filter, which showed the expected mosaicism that is seen in F_0 transgenic embryos (Wang et al., 1998) (data not shown). Injected and control mutant embryos were allowed to grow for 96 hours. At that time, all uninjected embryos showed signs of anemia, whereas partial rescue of the anemia was observed in about 10% of the injected embryos. *o*-Dianisidine staining showed that hemoglobin expression was considerably greater in the rescued embryos than in the uninjected controls (Fig. 5G). These results confirmed that protein *4.1R* is the mutated gene responsible for the *mot* phenotype.

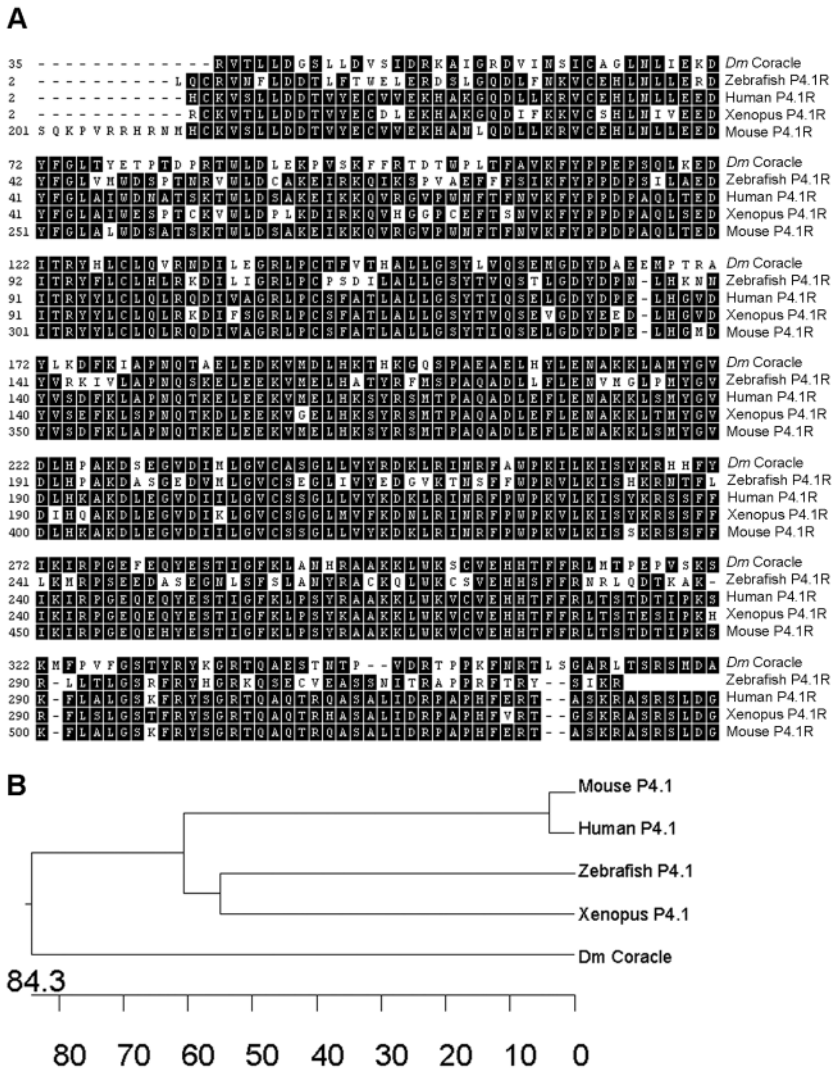

Fig. 7. Amino acid alignment and phylogenetic tree of P4.1R family members. (A) Alignment of the conserved FERM domain in P4.1R proteins from zebrafish, human, mouse, *Xenopus* and *Drosophila* (*Dm coracle*). Shaded blocks indicate similar and identical amino acids. (B) A phylogenetic tree of the protein P4.1R amino acid sequences reveals an evolutionary conservation between members of P4.1R superfamily. The MegAlign application in DNAStar software was used for alignment and construction of the phylogenetic tree.

DISCUSSION

The zebrafish *mot*/*cha* mutants and congenital anemia

The zebrafish *mot* and *cha* mutants suffer from a severe congenital hemolytic anemia because of the loss of red cell membrane deformability and integrity. We used positional cloning techniques with a candidate gene approach to demonstrate that *mot* and *cha* are allelic and encode the erythroid-specific isoform of protein 4.1R, a crucial component of the red blood cell membrane skeleton. Linkage analysis and rescue experiments provided additional confirmation that the molecular defect in protein 4.1R is the underlying cause of the anemic phenotype in *mot* fish. Sequence analysis of *4.1R* cDNA from both alleles of *mot* and *cha* revealed two different nonsense point mutations resulting in premature stop codons at amino acids 784 and 1012. Both mutations occur before the crucial spectrin-binding domain of 4.1R, rendering the translated protein 4.1R dysfunctional. However, severely reduced levels of *4.1R* transcripts, as detected by whole-mount RNA in situ hybridization of *mot* embryos, implies that mutant *4.1R* transcripts are subjected to RNA surveillance and degraded because of nonsense-mediated RNA decay (Frischmeyer and Dietz, 1999; Culbertson, 1999; Ruiz-Echevarria et al., 1998). As a result, it is highly likely that no protein 4.1R is translated in mutant cells, and therefore, both alleles of *mot* and *cha* represent 4.1R null phenotypes.

The *mot* and *cha* mutations were originally thought to be different based on genetic non-complementation data (Ransom et al., 1996). However, the finding that *mot* and *cha* are allelic and encode the same genetic defect in our present study most likely reflects an error in the previous complementation data. An alternative possibility for the same mutation for both *cha* alleles and mot^{tu275} would be mutability 'hot spot' of this locus.

Although hereditary elliptocytosis caused by dysfunctional or lack of 4.1R has been reported in mammals, this is the first report in a non-mammalian vertebrate animal model. Human mutations in erythroid-specific protein 4.1R generate two groups of molecular abnormalities. The first group causes partial or complete deficiency of P4.1 in red cells, whereas the second group disrupts the erythroid-specific spectrin-binding domain of P4.1 (Lorenzo et al., 1994; Conboy et al., 1993; Alloisio et al., 1981; McGuire et al., 1988; Conboy et al., 1986b; Alloisio et al., 1982; Tchernia et al., 1981). The pathological consequences of both defects are the presence of mild to severe anemia known as hereditary elliptocytosis (HE). The molecular defect, as well as pathological symptoms in the zebrafish *mot* is similar to hereditary elliptocytosis (HE) in mice and humans. Human red cells deficient in protein 4.1R are characterized by abnormal elliptical morphology, reduced red cell deformability, increased red cell membrane fragmentation and disrupted skeletal network (Tchernia et al., 1981; Yawata et al., 1997). Analysis of peripheral blood showed abnormal membrane spiculation and fragmentation in *mot* cells, indicative of cytoskeletal fragility and membrane loss. This progressive loss of redundant membrane in *mot* cells results in microcytic and spherocytic cells, and generates fragile red cells with a decreased surface area to volume ratio.

Recently, a mouse knockout of protein 4.1R was generated to study the role of 4.1R in erythroid and nonerythroid cells (Shi et al., 1999). Protein 4.1 null homozygote mice suffer from a moderate hemolytic anemia, with red blood cells exhibiting abnormal morphology and increased membrane fragility.

These mice are viable and reach adulthood, which is in contrast to *mot*/*cha* fish, where only 5-10% of homozygote embryos survive to adulthood. Interestingly, a majority of hematopoietic mutations in zebrafish are embryonic lethal and are characterized by a mild to severe reduction in the number of circulating red cells at 2-4 days of development (Ransom et al., 1996). It appears that abnormal embryonic red cells in zebrafish mutants undergo an accelerated clearance from circulation before the onset of definitive hematopoiesis. The circulation in zebrafish starts shortly after 24 hpf and exposes red cells to the mechanical and chemical stress of microcirculation, which may be efficient enough to sensitize abnormal cells to early destruction.

Examination of peripheral blood from adult *mot* fish demonstrated morphologically abnormal cells with an apparent differentiation arrest at the late erythroblast stage. We propose two hypotheses that may explain these findings. Icteric internal organs and the enlarged spleen in adult *mot* fish are indicative of a severe extravascular hemolysis. Presence of immature red cells in the peripheral blood may be a consequence of premature release of basophilic erythroblasts from the kidneys in response to increased cell destruction of more mature cells. The assembly of protein 4.1 into the membrane cytoskeleton for the final stabilization of the red cell membrane skeleton occurs late during erythroid maturation (Lazarides and Woods, 1989; Lazarides, 1987). As normal function and survival of circulating mature red cells depend on their cellular deformability and structural integrity (Weed, 1970), the more mature red cells with accumulated membrane lesions are selectively sequestered by the spleen. This results in a left shift in erythroid maturation, which is seen as a maturation arrest in the peripheral blood. Another hypothesis is the arrest in erythroid maturation may be related to ineffective erythropoiesis. Cytological examination of the kidneys revealed a profound erythroid hyperplasia. However, the number of immature erythroid progenitors was much higher than more mature cells, suggestive of a maturation arrest and ineffective erythropoiesis. The presence of apoptotic erythroid cells in the kidneys of *mot* fish is consistent with recent data describing the correlation of apoptosis of erythroid progenitors and ineffective erythropoiesis in several human anemias (Pootrakul et al., 2000; Mathias et al., 2000). Further studies may provide insights into the role of protein 4.1R in maturation of erythroid cells in zebrafish.

Role of zebrafish protein 4.1 in erythroid morphogenesis

Terminal maturation of erythroid cells involves the development of anucleated erythrocyte from nucleated proerythroblast and is associated with dynamic changes in the membrane and cytoskeletal organization and composition (Woods and Lazarides, 1988; Bennett, 1985; Wickrema et al., 1994; Woods et al., 1986). The correlations of abnormal red cell morphology with genetic defects in membrane cytoskeletal proteins are well established (Delaunay et al., 1996; Davies and Lux, 1989; Palek, 1987; Mohandas and Gascard, 1999). In non-mammalian vertebrates, terminal maturation entails the genesis of an ellipsoid nucleated red cell from its spheroid precursor. Extensive in vitro analyses of the red cells of non-mammalian vertebrates show that their elliptical morphology is partly due to the presence of marginal band of microtubules (Winckler and Solomon, 1991; Cohen et al., 1998; Cohen, 1991). However, owing to the lack of a genetic model to analyze and elucidate the morphogenic mechanism of vertebrate red cells in vivo, the extent and nature of interactions between the marginal band and other cytoskeletal proteins and their possible role in erythroid maturation and morphogenesis is not defined. The profound abnormal morphology and membrane instability of *mot* cells, and evidence of an abnormal formation of the marginal band support the crucial role of zebrafish protein 4.1R in maintaining the integrity of the red blood cell membrane. Characterization of hereditary spherocytosis in zebrafish *riesling*, resulting from defective erythroid β spectrin, also demonstrated that the aggregation of microtubule filaments into a marginal band is compromised in mutant red cells (Liao et al., 2000). These results, along with evidence of the interaction of the marginal band with ERM proteins and F-actin (Correas et al., 1986; Birgbauer and Solomon, 1989), provide strong evidence that multifunctional protein elements of the membrane skeleton that form multi-protein complexes are key components in determining and sustaining the nucleated red cell morphology.

In this report, we have presented the zebrafish mutant *mot* as a genetic model for hereditary anemia because of the abnormal structure of the erythroid specific protein 4.1R. Over the past few years, characterization of zebrafish mutants with defects in hematopoiesis has established the zebrafish as a useful genetic model to study hematopoiesis in higher vertebrates. The zebrafish mutant *merlot* provides an excellent animal model with which to explore protein 4.1R structure and function further in nucleated erythroid cells.

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