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Mutations in *HFE2* cause iron overload in chromosome 1q–linked juvenile hemochromatosis

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Juvenile hemochromatosis is an early-onset autosomal recessive disorder of iron overload resulting in cardiomyopathy, diabetes and hypogonadism that presents in the teens and early 20s (refs. 1,2). Juvenile hemochromatosis has previously been linked to the centromeric region of chromosome 1q (refs. 3–6), a region that is incomplete in the human genome assembly. Here we report the positional cloning of the locus associated with juvenile hemochromatosis and the identification of a new gene crucial to iron metabolism. We finely mapped the recombinant interval in families of Greek descent and identified multiple deleterious mutations in a transcription unit of previously unknown function (*LOC148738*), now called *HFE2*, whose protein product we call hemojuvelin. Analysis of Greek, Canadian and French families indicated that one mutation, the amino acid substitution G320V, was observed in all three populations and accounted for two-thirds of the mutations found. *HFE2* transcript expression was restricted to liver, heart and skeletal muscle, similar to that of hepcidin, a key protein implicated in iron metabolism[7–9]. Urinary hepcidin levels were depressed in individuals with juvenile hemochromatosis, suggesting that hemojuvelin is probably not the hepcidin receptor. Rather, *HFE2* seems to modulate hepcidin expression.

Two families with juvenile hemochromatosis not linked to 1q were recently found to have loss-of-function mutations in the gene encoding hepcidin[10]. Hepcidin is a small peptide hormone predominantly secreted by the liver[11], whose levels correlate inversely with rates of iron uptake in the gut and with the release of iron from macrophages[12,13] (**Fig. 1**). The clinical and biochemical phenotype of 1q-linked juvenile hemochromatosis is indistinguishable from that of hepcidin-deficient juvenile hemochromatosis, both having intestinal iron hyperabsorption leading to an early onset of severe iron overload associated with macrophages that do not load iron. This suggests that the more commonly mutated gene underlying 1q-linked juvenile hemochromatosis gene probably also functions in the hepcidin pathway.

To identify the gene associated with 1q-linked juvenile hemochromatosis, we collected samples from 12 unrelated families with juvenile hemochromatosis from Greece, Canada and France, 7 of whom were previously reported to be consistent with linkage to the juvenile hemochromatosis locus at 1q21 (*HFE2*; OMIM 602390). Only one family, JH7, is known to be consanguineous. Parents of all probands, where ascertained, were clinically and biochemically normal.

We verified absence of mutations of hepcidin in all 12 families and confirmed that juvenile hemochromatosis was consistent with linkage to 1q21 in these families by mapping a combination of publicly available markers and 18 new microsatellite markers identified from genomic sequence. Nine of the ten Greek families showed extended marker homozygosity in the 1q region (**Fig. 2**), consistent with linkage to a common gene as the chief determinant of juvenile hemochromatosis in this population. We reconstructed five different Greek haplotypes segregating in these families, one of which was observed repeatedly. Families JH4, JH8 and JH9 were each homozygous with respect to different haplotypes. The proband in family JH11 segregated alleles consistent with heterozygosity with respect to the common haplotype and a new haplotype.

We carried out multipoint linkage analysis to determine the statistical significance of the observed haplotype sharing and obtained a peak multipoint lod score of 4.05 in the shared segment for the Greek and

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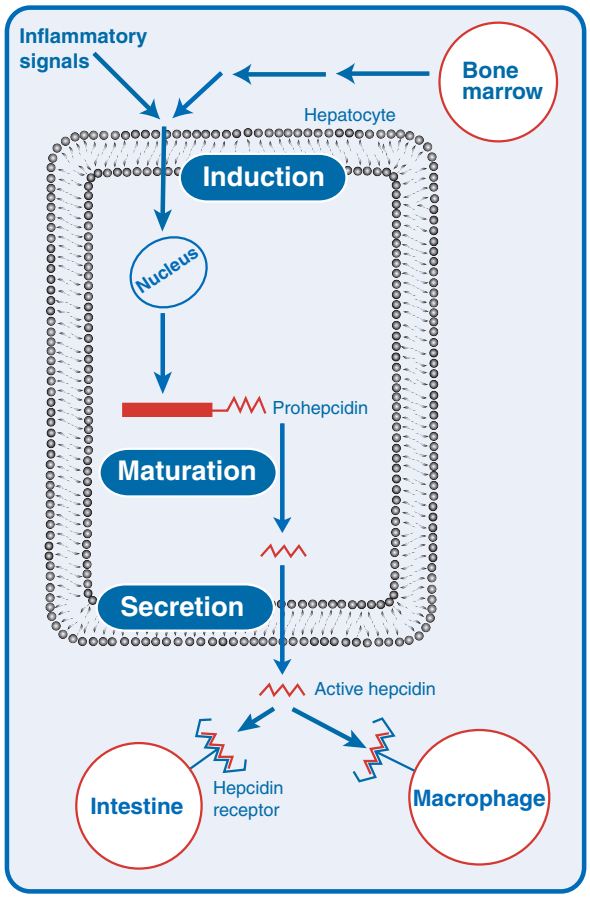

Figure 1 Hepcidin modulates iron transport from enterocytes and macrophages. Hepcidin production is modulated by inflammatory signals, iron levels and signals from the bone marrow (erythroid drive). Hepcidin is initially produced as prohepcidin (84 amino acids), which is processed by cleavage to the putatively active form of 25 amino acids. Elevated levels of hepcidin prevent iron uptake from the intestine and iron release from macrophages.

Canadian families combined. The April 2003 genome sequence assembly (build 33) contains numerous gaps and duplications, but we were able to estimate the size of the linkage interval and define the linkage boundaries on the basis of existing sequence contigs. Recombinant events placed outer boundaries at CA3AL590452 and CA3AL359207.

We next embarked on a positional cloning effort. According to our interpretation of the genome assembly, the region of ~1.7 Mb associated with juvenile hemochromatosis contains 21 RefSeq annotated genes. In the course of sequencing these genes, we identified multiple mutations in one particular gene in the minimal recombinant interval (**Table 1**). This gene corresponds to anonymous transcript *LOC148738* in RefSeq, although we predicted a slightly more complex gene structure from available cDNA and expressed-sequence tag (EST) evidence (**Fig. 3a**). The observed mutations include four missense mutations in residues that are highly conserved in evolution (**Table 1** and **Fig. 3b**), a premature termination mutation and a frameshift mutation. We detected six different mutations accounting for all 24 alleles in the ten Greek families, one Canadian family and one French family. None of the mutations was observed in over 500 control chromosomes. The mutations cosegregated completely with the juvenile hemochromatosis phenotype, and results of microsatellite-based haplotype analysis were consistent with recessive inheritance and full penetrance. We observed one common mutation, the G320V missense variant, in the seven Greek families who share the common Greek haplotype and in Canadian and French families.

We predict that hemojuvelin is transcribed from a gene of 4,265 bp into a full-length transcript with five spliced isoforms (**Fig. 3a**). The putative full-length protein from the longest transcript (transcript 1) is 426 amino acids; the occurrence of this transcript in humans has been confirmed experimentally by RT-PCR and sequencing of a novel cDNA clone. Hemojuvelin contains multiple protein motifs (**Fig. 3a**) consistent with a function as a membrane-bound receptor or secreted polypeptide hormone. Orthologs of human hemojuvelin are found in mouse, rat and zebrafish (**Fig. 3b**). Sequence comparison shows that human hemojuvelin is >85% identical to the mammalian orthologs

	JH3-201		JH5-202		JH6-205		JH7-201		JH10-201		JH12-201		JH11-201		JH4-203		JH8-202		JH9-202	
D1S252	104	92	102	102	96	96	104	104	92	92	92	92	92	104	92	102	ND	*102*	92	92
D1S2696	167	167	167	165	ND	*165*	*167*	*165*	167	167	165	165	167	167	161	175	171	173	171	169
CA3AL590452	251	249	249	251	255	249	249	247	249	249	ND	ND	ND	ND	249	251	249	249	251	249
CA1AL355505	264	264	264	264	264	264	264	264	264	264	*264*	*264*	264	268	264	264	264	264	264	264
GGAA1AL355505	297	297	297	297	297	297	297	297	297	297	*297*	297	293	293	293	293	293	293	297	297
CA1AL160282	195	195	195	195	195	195	195	195	195	195	195	195	195	195	195	195	193	193	195	195
D1S2344	255	255	255	255	255	255	255	255	255	255	255	255	255	253	255	255	259	259	253	253
GATA1AL160282	380	380	380	380	380	380	380	380	380	380	363	363	380	384	363	363	384	384	363	363
D1S442	270	270	270	270	270	270	270	270	270	270	ND	ND	270	270	278	278	270	270	272	272
CA1AL445591	377	377	377	377	377	377	377	377	377	377	ND	*377*	377	379	377	377	379	379	379	379
CA3AL359207	407	407	407	407	407	407	407	409	407	407	ND	*407*	407	407	419	419	409	409	419	419
CA2AL359207	221	221	221	221	221	221	221	221	221	221	221	221	221	225	221	221	221	221	221	221
CA1AL359207	256	256	256	256	256	256	256	242	256	256	ND	ND	256	242	246	246	242	242	246	246
CA2AL590667	196	196	196	196	196	196	196	184	196	196	ND	ND	196	184	194	194	196	196	184	184
CA1AL590667	238	238	238	238	*238*	*238*	238	244	238	238	ND	ND	238	244	238	238	238	238	244	244
CA2AL391904	208	208	208	208	*208*	*208*	208	208	208	208	*208*	ND	208	206	208	208	208	208	208	208
CA1AL391904	235	235	235	235	*239*	*239*	235	237	235	235	ND	ND	235	237	235	235	235	235	237	237
CA1AL356378	176	178	176	176	*176*	*176*	176	170	176	176	176	176	176	170	174	174	180	180	184	184
CA1AL596177	289	289	289	289	289	289	289	277	289	289	ND	ND	289	277	289	289	289	289	285	285
D1S2612	170	170	170	170	170	170	170	170	170	170	170	170	170	182	180	180	174	174	180	180
CA1AL591493	398	398	402	402	*398*	*398*	398	398	400	396	ND	ND	396	398	398	398	402	402	398	398
CA1AL358073	176	176	164	164	176	176	176	164	174	176	*176*	*176*	176	176	176	176	172	172	176	176
D1S3466	201	201	205	205	201	201	201	209	201	197	197	201	205	197	197	197	197	197	205	205
D1S498	191	191	195	195	191	191	191	189	193	191	193	191	195	197	195	195	199	193	191	195
D1S2347	291	291	272	272	291	291	291	285	283	283	ND	ND	274	272	272	272	272	272	272	272
D1S2343	242	242	256	256	260	260	260	256	258	242	*256*	ND	258	256	258	258	256	242	256	256
D1S2635	157	151	161	157	154	159	152	157	157	163	161	154	157	152	159	152	161	159	159	154

Figure 2 Genetic analysis of kindreds affected with juvenile hemochromatosis. Haplotype data in ten Greek families with juvenile hemochromatosis. Genotypes are shown for 27 informative markers from the 1p13–q23 genetic interval in the indicated individuals, with each of the consensus haplotypes shaded in a different color. Markers designated 'D1S' are described in build 33 of the human genome, and newly generated microsatellite markers are designated by repeat type and human genomic clone accession numbers. ND indicates genotypes that were not determined. Alleles of uncertain phase are underlined, inferred alleles are italicized and alleles observed most frequently in 56 Greek control chromosomes are shown in bold. Marker order is based on a revised interpretation of the April 2003 build 33 of the human genome assembly. The red bar indicates the critical interval associated with juvenile hemochromatosis.

Table 1 Genetic and clinical information of families with mutations in *HFE2*

Individual	Origin	Number of affected individuals in family	Age at onset	Age at diagnosis	Serum ferritin ($\mu g\ l^{-1}$)	Transferrin saturation (%)	Hypo-gonadism	Arthro-pathy	Skin pigmentation	Glucose intolerance	Heart disease	Hepatic fibrosis	Mutation status	Effect on coding sequence
JH1-301	Canada	3	7	7	339	94	–	–	+	–	–	+	Compound heterozygous	I222N, G320V
JH3-201	Greece	1	21	25	2,283	100	+	+	+	–	–	+	Homozygous	G320V
JH4-203	Greece	1	39	49	4,127	90	+	+	+	–	–	+	Homozygous	I281T
JH5-201	Greece	2	32	39	3,553	100	+	+	+	+	+	+	Homozygous	G320V
JH6-205	Greece	2	25	32	2,500	100	+	+	+	+	+	+	Homozygous	G320V
JH7-201	Greece	3	20	21	NA	100	+	–	+	–	–	NA	Homozygous	G320V
JH8-202	Greece	1	26	33	5,900	98	+	–	+	–	–	+	Homozygous	C361fsX366
JH9-201	Greece	2	28	33	1,125	80	+	+	–	+	–	+	Homozygous	G99V
JH10-201	Greece	1	21	25	5,250	100	+	–	–	–	–	+	Homozygous	G320V
JH11-201	Greece	1	33	37	731	100	–	–	+	–	–	–	Compound heterozygous	G320V, R326X
JH12-201	Greece	1	29	31	2,254	100	+	–	–	–	–	NA	Homozygous	G320V
JH13-301	France	1	16	23	7,125	83	+	+	+	+	+	+	Homozygous	G320V

+, present; –, absent; NA, information not available.

and ~45% identical to the fish ortholog. The hemojuvelin isoform of 426 amino acids also shares considerable sequence similarity with the repulsive guidance molecule[14] (RGM or RGMA) of human (48% identity) and chicken (46% identity; **Fig. 3b**). In humans there is a third RGM-like protein, RGMB, whose biological function is currently unknown.

We examined *HFE2* expression in 16 human tissue types by probing northern blots with a probe from exon 4 and detected substantial expression in adult and fetal liver, heart and skeletal muscle (**Fig. 4**). The primary RNA observed in these tissues migrated at about 2.2 kb, consistent with full-length transcript 1 in **Figure 3a**. After reprobing the same blots for hepcidin, we detected strong expression in adult and fetal liver only. We later detected expression of hepcidin in a heart-specific northern blot.

We measured hepcidin peptide levels in urine samples from a subset of Greek individuals with juvenile hemochromatosis. Deleterious mutations of hemojuvelin reduce hepcidin levels despite iron overload, which normally induces hepcidin expression[15]. Hepcidin levels were consistently depressed in the individuals with juvenile hemochromatosis: homozygous affected individuals from five different families had 5–11 ng mg^{-1} creatinine compared with 14–165 ng mg^{-1} creatinine in four heterozygous unaffected carriers and 10–100 ng mg^{-1} creatinine in unrelated controls. In one individual who did not have hemochromatosis who had an infection at the time of measurement, urine hepcidin level was very high (1,024 ng mg^{-1} creatinine), as expected. These results suggest that HFE2 acts as a modulator of hepcidin expression, although it is not possible to distinguish a pretranscriptional from a post-transcriptional or even post-translational role for HFE2 in the absence of liver biopsies to measure hepcidin mRNA levels. In adult-onset hereditary hemochromatosis[16] and *Hfe* knockout mice[17,18], hepcidin levels are inappropriately low for the degree of iron overload. Thus, we believe that juvenile hemochromatosis and adult-onset hereditary hemochromatosis are on the same biochemical and phenotypic spectrum, with juvenile hemochromatosis representing the more severe, earlier-onset phenotype with absent (or very low) hepcidin, and adult-onset hemochromatosis manifesting later in life with only partial deficiency for hepcidin[18] (**Fig. 5**). The direct result of this hepcidin deficiency is that in both adult-onset hemochromatosis and juvenile hemochromatosis there is intestinal iron hyperabsorption. The excessive iron uptake in juvenile hemochromatosis is greater than that seen in adult-onset hereditary hemochromatosis, reflecting the lower levels of hepcidin associated with juvenile hemochromatosis and culminating in an earlier onset of a more acute phenotype.

Loss of function of hepcidin in mice also leads to severe iron overload, mimicking the biochemical and clinical phenotype of juvenile hemochromatosis[8]. In contrast, in both other animal models[19] and human diseases[20], overexpression of hepcidin leads to macrophage iron retention and an iron-deficient phenotype typical of the iron disturbances found in anemia of inflammation (also called anemia of chronic disease)[21]. Anemia of inflammation is an acquired disorder, seen in individuals with various conditions including infection, malignancy and chronic inflammation[22]. It is characterized by a retention of iron by macrophages and decreased intestinal iron absorption, which leads to reduced iron availability for erythropoesis[21,23].

Consistent with the proposed role of hepcidin in the pathogenesis of anemia of inflammation, the defects in iron absorption and reuse in anemia of inflammation are accompanied by elevated urinary (and presumably serum) hepcidin levels. Existing therapy for anemia of inflammation is mainly targeted to treating the underlying disorder, with no efficacious treatment specifically directed to amelioration of the iron deficiency. Therapeutics that mimic the juvenile hemochromatosis phenotype (hepcidin deficiency) will serve to reduce hepcidin levels and thereby treat the opposite phenotype of anemia of inflammation (hepcidin excess). Thus, hemojuvelin represents a new therapeutic target for the treatment of anemia of inflammation.

Recent reports that adult-onset hereditary hemochromatosis can result from digenic inheritance with compound heterozygosity with respect to *HFE* and *HAMP* (encoding hepcidin) and that mutations in *HAMP* can contribute to the severity of adult-onset hereditary hemochromatosis[24] suggest that modulation of other genes in the hepcidin pathway may also predispose to the adult phenotype. Therefore, we are currently exploring the role of hemojuvelin in modulating the onset and severity of adult-onset hemochromatosis. The identification of hemojuvelin presents new therapeutic and diagnostic opportunities for the management of iron-related disorders.

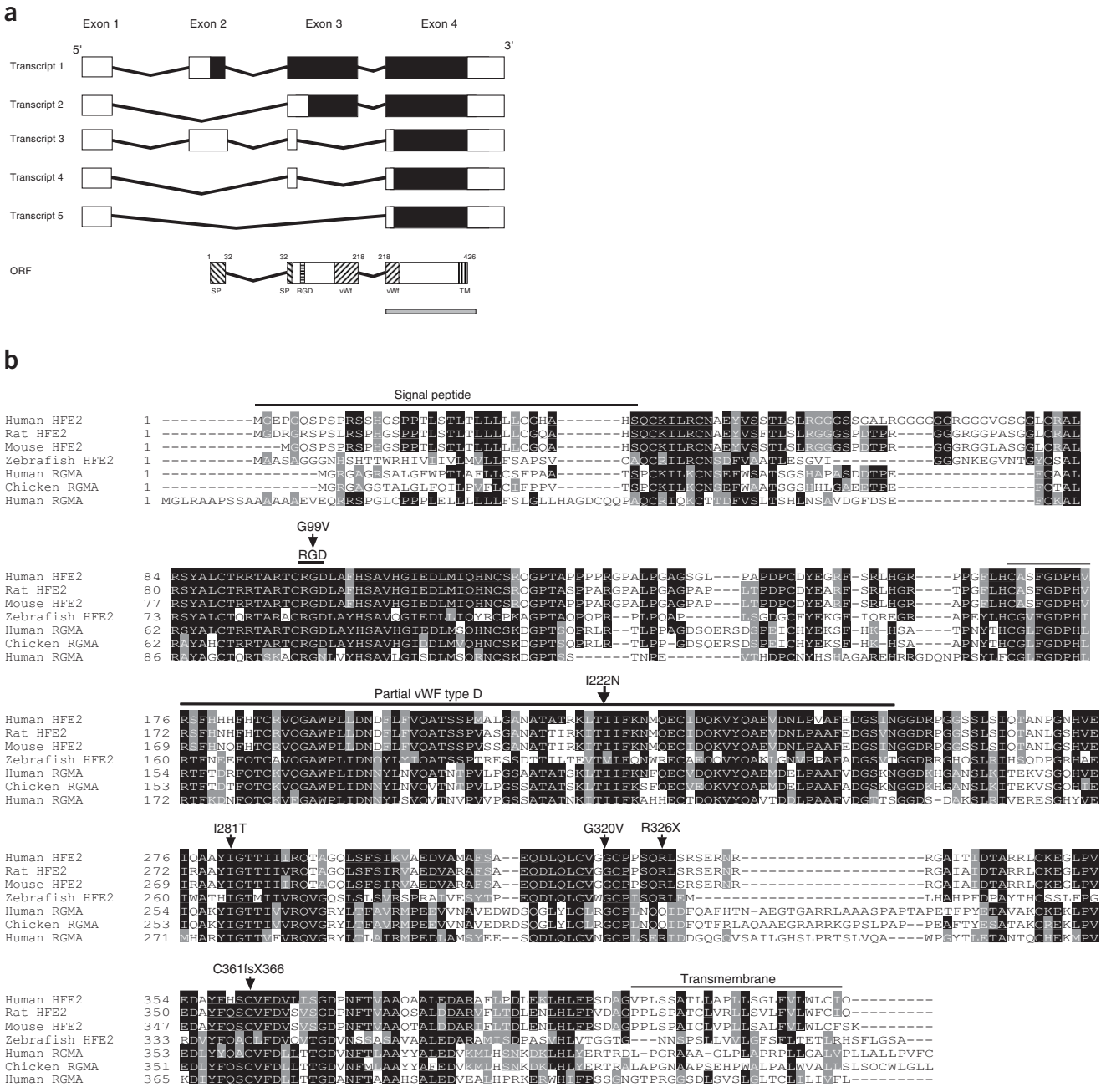

Figure 3 *HFE2* gene structure and bioinformatics analysis. (**a**) Transcript 1 was determined from sequencing a novel RT-PCR cDNA clone from human liver RNA. Alternatively spliced transcripts 2–5 were based on occurrence in EST or cDNA clones in public databases and RT-PCR experiments. Each of the five putative transcripts of *HFE2* may be translated into a polypeptide. Transcripts 3, 4 and 5 generate the same protein; hence, there are three hemojuvelin isoforms of 426, 313 or 200 amino acids. Exon 2 was predicted in Ensembl for human *HFE2* based on a rat cDNA clone containing this exon (incorrectly annotated as human but 100% identical with rat genomic sequence). Additional mouse ESTs, conservation of the exon in human genomic sequence and a novel human cDNA clone verified the coding region of transcript 1. Untranslated sequence is colored white, translated sequence black. Below the transcripts is shown a version of the longest open reading frame (ORF) with protein domains parsed across the exons and codon numbers given at splice junctions (SP, signal peptide; RGD, tri-amino acid motif; vWf, partial von Willebrandt factor; TM, transmembrane). Gray horizontal bar at bottom indicates northern-blot probe. (**b**) Multiple sequence alignment of HFE2 (hemojuvelin) with orthologs of mouse, rat and zebrafish and paralogs of human and chicken. The longest cDNA sequence (transcript 1) and its predicted protein sequence were used as the basis for sequence numbering beginning from the putative initiating methionine. Above sequences, protein functional domains are shown as horizontal bars. Amino acid changes in individuals with juvenile hemochromatosis are indicated by arrows.

METHODS

Selection of study subjects. All samples used in this study were collected with informed consent and approved for study by institutional review boards and ethics committees at all affiliated institutions. Families JH3–JH7 were previously reported[5] as families 1–5, respectively; families JH8 and JH9 were also previously reported[4] as families 1 and 2, respectively. Diagnoses of affected individuals in these families were previously reported[4,25]. We diagnosed additional probands with juvenile hemochromatosis based on early presentation with disease-related clinical complications, including hypogonadotrophic hypogonadism, heart disease and skin pigmentation, along with testing of transferrin saturation, serum ferritin levels and hepatic siderosis (**Table 1**). Families JH11 and JH13 consist of individual probands. Families JH3, JH4 and JH5 originated in a small area in southwestern Greece; families JH6 and JH7 originated in a mountain area of central Greece; and family JH10 originated from northwestern and northeastern Greece (maternal and paternal sides, respectively). Families JH8, JH9, JH11 and JH12 also live in Greece, and family JH13 lives in France. Family JH1 lives in Canada and is of European origin. Consanguinity has been documented only in family JH7 (marriage between first cousins). Control DNAs included at least 90 DNAs from each of three different sources: Greek, northern European and the Coriell polymorphism discovery resource containing multiple ethnicities.

Markers and genotyping. We used commercially available markers (ABI) for genotyping in the 1q21 interval. We designed an additional 18 custom markers using existing sequence obtained from GenBank for fine-mapping. We carried out radiation hybrid mapping on selected microsatellite markers or other sequence-tagged sites using the TNG hybrid panel (Research Genetics) to resolve contig order. We carried out genotyping on an Applied Biosystems Prism 3100 Genetic Analyzer running Genemapper software. We verified mendelian inheritance of alleles for all markers using the PedCheck program[26].

Linkage and haplotype analysis. We estimated allele frequencies from 16 untransmitted haplotypes from the ten Greek pedigrees and from 40 genotyped control Greek individuals. We carried out multipoint linkage analysis with Genehunter using an inheritance model with 0.99 penetrance, 0.000005 phenocopy rate and a recessive disease allele frequency of 0.01. We determined haplotypes using Genehunter.

Mutation detection. We designed primers to amplify coding sequences for genes present in the defined 1q interval. The process of primer design involved the identification of candidate genes and their respective exons in Ensembl, automated primer design for the exons using Primer3 and validation of the primers using e-PCR. We amplified PCR products using standard PCR conditions with Qiagen *Taq* polymerase on a Peltier Thermal Cycler (MJ Research, PTC-225). We treated PCR products with 4 units exonuclease and 4 units shrimp alkaline phosphatase for 2–16 h and used 5 μl for sequencing. We carried out sequencing using BigDye Terminator on an ABI 3700 sequencer (ABI) and sequence analysis and mutation detection using the Phred/Phrap/Consed/Polyphred[27,28] or Sequencher software suites. We designed additional primers to amplify and sequence all published and predicted exons of *LOC148738*, including the 5′ and 3′ untranslated regions and the 500-bp presumptive sequence upstream of the first exon. All primer sequences are available on request.

Northern-blot analysis. We purchased Clontech northern blots and probed them with ^{32}P-labeled probes. We generated substrates for probes from purified, PCR-amplified products from genomic DNA for *LOC148738* and *HAMP* and from manufacturer's supplied reagents for actin according to manufacturer's instructions. RNA for RT-PCR was either purchased from Clontech or Biochain or prepared from tissues using the Qiagen RNeasy Protect Midi kit. We prepared single-strand cDNA using the Invitrogen SuperScript First-Strand Synthesis for RT-PCR kit according to the manufacturer's instructions.

Bioinformatics. We carried out all pairwise sequence comparisons using BLAST 2 Sequences[29] with the following parameters: program, blastp; matrix, BLOSUM62; open gap penalty, 11; extension gap penalty, 1; gap_x_dropoff, 50; word size, 3; expect, 10. We aligned sequences using ClustalX.

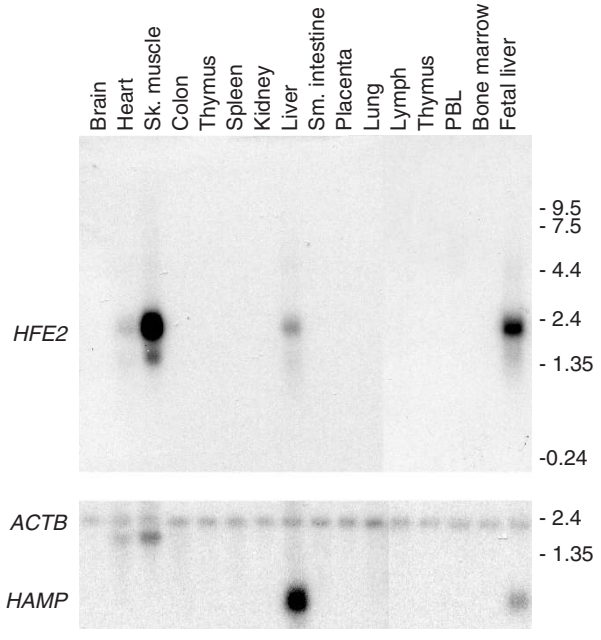

Figure 4 Tissue expression of hemojuvelin. Northern blots of human tissues were probed with sequences encompassing exon 4 of *HFE2* and then reprobed with probes for hepcidin (*HAMP*) and β-actin (*ACTB*). The *ACTB* probe highlights a second isoform specific to skeletal muscle and heart, in addition to the ubiquitous transcript in these tissues. Sizes are relative to lane standards. PBL, peripheral blood lymphocytes.

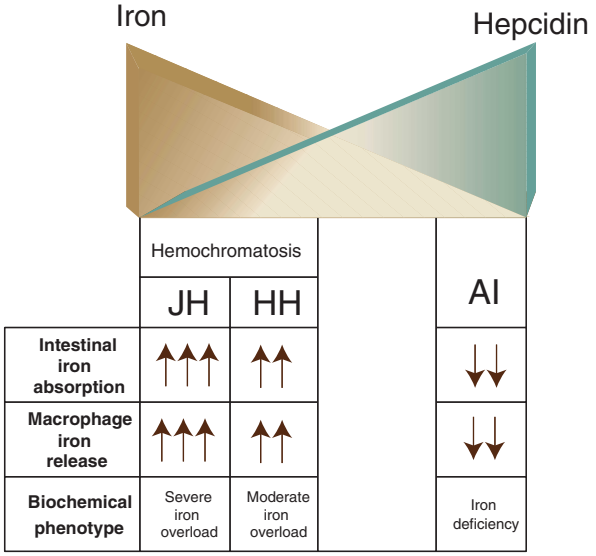

Figure 5 Anemia of inflammation and hemochromatosis represent opposite ends of the phenotypic spectrum of iron-related disorders. Anemia of inflammation (AI) is characterized by high levels of hepcidin, which leads to iron deficiency and iron-rich macrophages. In contrast, in hemochromatosis, hepcidin levels are low with enhanced intestinal absorption and whole-body iron overload. Macrophages in hemochromatosis are iron-depleted. Juvenile hemochromatosis (JH) and adult-onset hereditary hemochromatosis (HH) both show iron overload with iron-depleted macrophages, but the phenotype is more severe in juvenile hemochromatosis.

We identified orthologs of human hemojuvelin in mouse (although protein coding potential annotated in the database does not correspond to full-length open reading frame of the actual sequence), rat and zebrafish (identified by a sequence similarity search of genes predicted by Genscan, gene structure based on genomic sequence traces and supporting ESTs). We identified paralogs of hemojuvelin in human (RGM or RGMA, RGMB) and chicken (RGM) from Blast comparison to GenBank.

Urinary hepcidin assay. Urinary creatinine concentrations were measured by UCLA Clinical Laboratories. Cationic peptides were extracted from urine using CM Macroprep (BioRad), eluted with 5% acetic acid, lyophilized and resuspended in 0.01% acetic acid. Urinary hepcidin concentrations were determined by immunodot assay. Briefly, we analyzed urine extracts equivalent to 0.1–4 mg of creatinine along with 0.6–40 ng hepcidin standards on dot blots on Immobilon P membrane (Millipore). We detected hepcidin on the blots using rabbit antibody to human hepcidin[15] with goat antibody to rabbit horseradish peroxidase as second antibody. We developed the blots by the chemiluminescent detection method (SuperSignal West Pico Chemiluminescent Substrate, Pierce) and quantified them with the Chemidoc cooled camera running Quantity One software (BioRad). Using this assay, we determined the normal range of urinary hepcidin to be 10–100 ng per mg creatinine (data not shown).

GenBank accession numbers. Translated portion of *HFE2* transcript 1, based on a novel sequenced cDNA clone, AY372521; predicted human *HFE2* transcripts 1–5, BK001575–BK001578 and BC017926, respectively; predicted zebrafish *HFE2* translated portion, BK001579. The mouse *HFE2* ortholog sequence was inferred with modifications from NM_027126; rat *HFE2* ortholog was inferred with modifications from AK098165 (annotated as human but identified as rat by comparison with genomic sequences); zebrafish *HFE2* was inferred with modifications from AI437181 and BG985666.

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COMPETING INTERESTS STATEMENT

The authors declare competing financial interests (see the *Nature Genetics* website for details).

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