

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

George Papanikolaou<sup>1</sup>, Mark E Samuels<sup>2</sup>, Erwin H Ludwig<sup>2</sup>, Marcia L E MacDonald<sup>2</sup>, Patrick L Franchini<sup>2</sup>, Marie-Pierre Dubé<sup>3</sup>, Lisa Andres<sup>2</sup>, Julie MacFarlane<sup>2</sup>, Nikos Sakellaropoulos<sup>1</sup>, Marianna Politou<sup>1</sup>, Elizabeta Nemeth<sup>4</sup>, Jay Thompson<sup>2</sup>, Jenni K Risler<sup>2</sup>, Catherine Zaborowska<sup>2</sup>, Ryan Babakaiff<sup>2</sup>, Christopher C Radomski<sup>2</sup>, Terry D Pape<sup>2</sup>, Owen Davidas<sup>2</sup>, John Christakis<sup>5</sup>, Pierre Brissot<sup>6</sup>, Gillian Lockitch<sup>7</sup>, Tomas Ganz<sup>4</sup>, Michael R Hayden<sup>2,8</sup> & Y Paul Goldberg<sup>2,8</sup>

**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<sup>7–9</sup>. 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<sup>10</sup>. Hepcidin is a small peptide hormone predominantly secreted by the liver<sup>11</sup>, whose levels correlate inversely with rates of iron uptake in the gut and with the release of iron from macrophages<sup>12,13</sup>

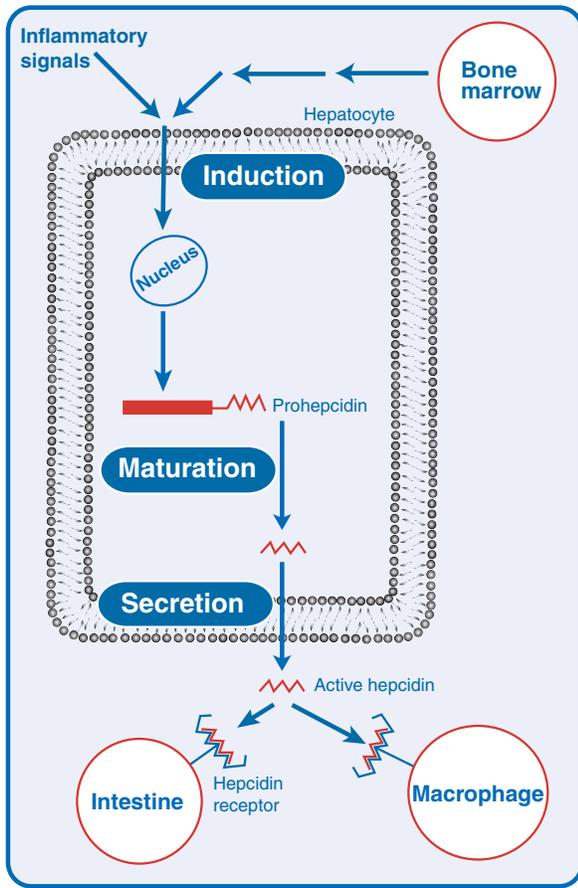
(Fig. 1). The clinical and biochemical phenotype of 1q-linked juvenile hemochromatosis is indistinguishable from that of hepcidin-deficient juvenile hemochromatosis, both having intestinal iron hyperabsorption leading to an early onset of severe iron overload associated with macrophages that do not load 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

<sup>1</sup>First Department of Internal Medicine, National and Kapodistrian University of Athens, School of Medicine, Laikon General Hospital, Athens 11527, Greece. <sup>2</sup>Xenon Genetics, Burnaby, British Columbia V5G 4W8, Canada. <sup>3</sup>Xenon Genetics Research, Montreal, Quebec H3A 1L2, Canada. <sup>4</sup>Department of Medicine, University of California Los Angeles, Los Angeles, California 90095, USA. <sup>5</sup>Department of Hematology, Theagenio Cancer Center, Thessaloniki 54007, Greece. <sup>6</sup>Service Des Maladies du Foie & INSERM U-522, University of Rennes, Rennes 35033, France. <sup>7</sup>Department of Pathology & Laboratory Medicine, University of British Columbia, Vancouver, British Columbia V6H 3V4, Canada. <sup>8</sup>Department of Medical Genetics, University of British Columbia, Vancouver, British Columbia V6H 3N1, Canada. Correspondence should be addressed to M.R.H. (mrh@cmmt.ubc.ca).



**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.

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

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.

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	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	380 380	363 363	380 384	363 363	384 384
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	208 208	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 ( $\text{g l}^{-1}$ )	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<sup>14</sup> (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.

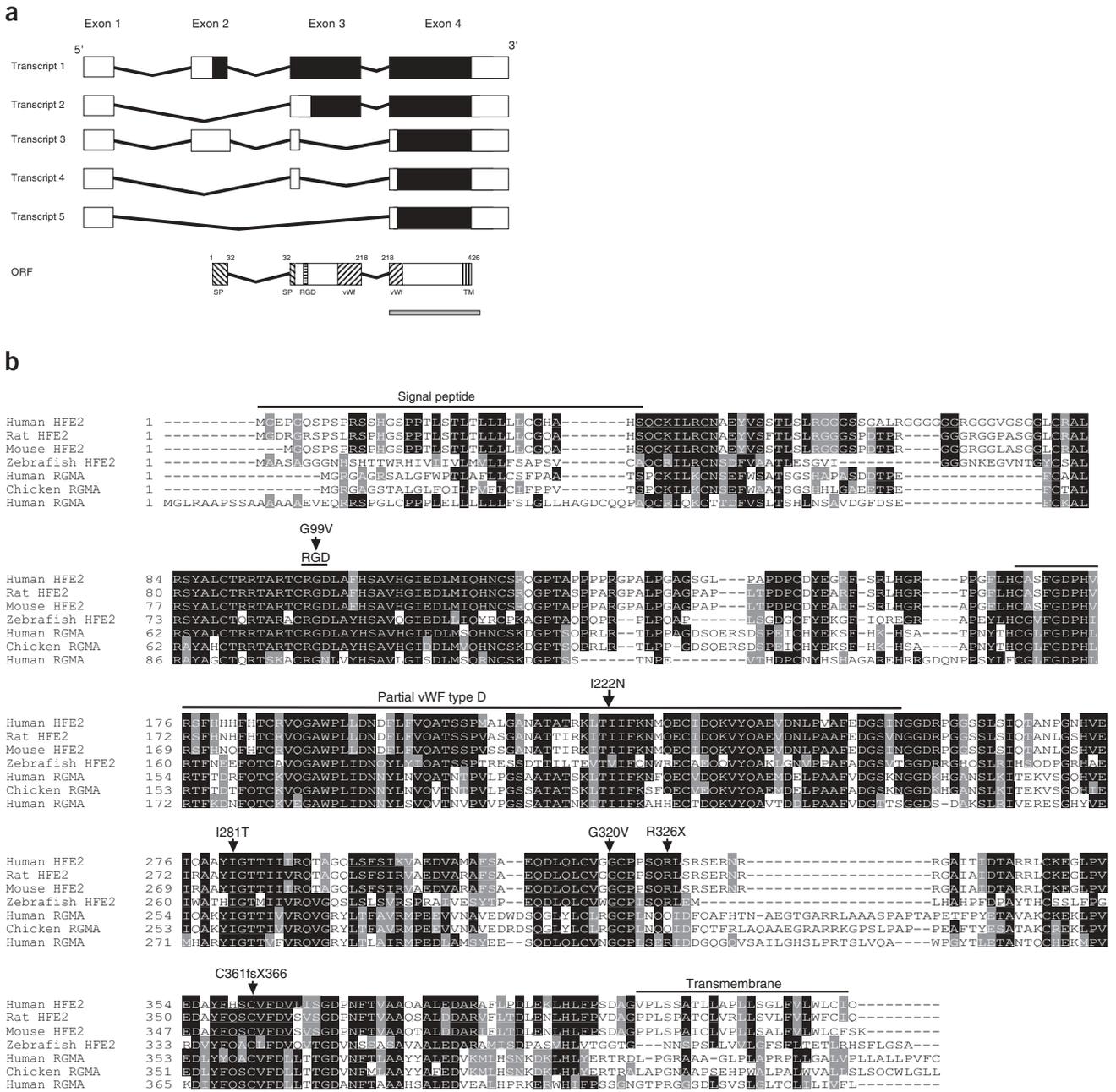
We measured hepcidin peptide levels in urine samples from a subset of Greek individuals with juvenile hemochromatosis. Deleterious mutations of hemojuvelin reduce hepcidin levels despite iron overload, which normally induces hepcidin expression<sup>15</sup>. Hepcidin levels were consistently depressed in the individuals with juvenile hemochromatosis: homozygous affected individuals from five different families had 5–11 ng mg<sup>-1</sup> creatinine compared with 14–165 ng mg<sup>-1</sup> creatinine in four heterozygous unaffected carriers and 10–100 ng mg<sup>-1</sup> 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<sup>-1</sup> 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<sup>16</sup> and *Hfe* knockout mice<sup>17,18</sup>, 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<sup>18</sup> (**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<sup>8</sup>. In contrast, in both other animal models<sup>19</sup> and human diseases<sup>20</sup>, 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)<sup>21</sup>. Anemia of inflammation is an acquired disorder, seen in individuals with various conditions including infection, malignancy and chronic inflammation<sup>22</sup>. It is characterized by a retention of iron by macrophages and decreased intestinal iron absorption, which leads to reduced iron availability for erythropoiesis<sup>21,23</sup>.

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<sup>24</sup> 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 hemoujuvelin 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 (hemoujuvelin) 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<sup>5</sup> as families 1–5, respectively; families JH8 and JH9 were also previously reported<sup>4</sup> as families 1 and 2, respectively. Diagnoses of affected individuals in these families were previously reported<sup>4,25</sup>. 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<sup>26</sup>.

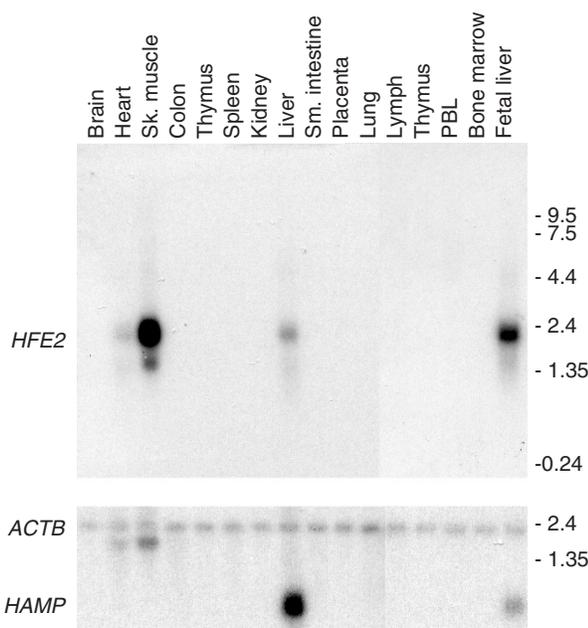
**Linkage and haplotype analysis.** We estimated allele frequencies from 16 untransmitted haplotypes from the ten Greek pedigrees and from 40 genotyped

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.

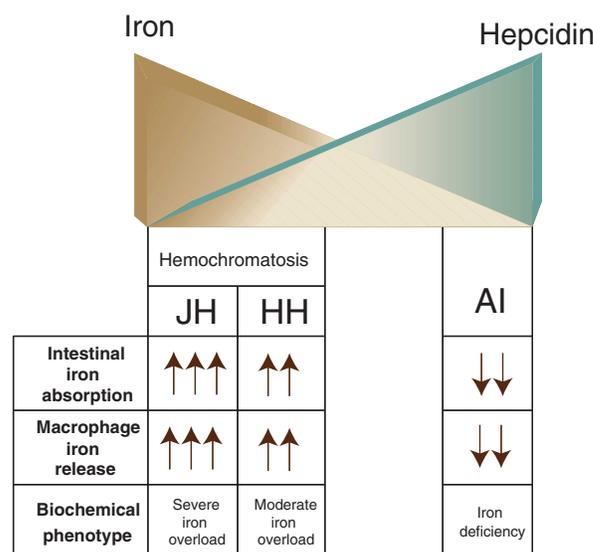
**Mutation detection.** We designed primers to amplify coding sequences for genes present in the defined 1q interval. The process of primer design involved the identification of candidate genes and their respective exons in Ensembl, automated primer design for the exons using Primer3 and validation of 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' I 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<sup>27,28</sup> 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 <sup>32</sup>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<sup>29</sup> 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  $\beta$ -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<sup>15</sup> 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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