

Mutation of a new gene causes a unique form of Hermansky–Pudlak syndrome in a genetic isolate of central Puerto Rico

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Hermansky–Pudlak syndrome (HPS) is a rare autosomal recessive disorder characterized by oculocutaneous albinism and a storage pool deficiency due to an absence of platelet dense bodies^{1–3}. Lysosomal ceroid lipofuscinosis, pulmonary fibrosis and granulomatous colitis are occasional manifestations of the disease⁴. HPS occurs with frequency of one in 1800 in north-west Puerto Rico⁵ due to a founder effect⁶. Several non-Puerto Rican patients also have mutations in *HPS1*^{7,8}, which produces a protein of unknown function⁹. Another gene, *ADTB3A*, causes

HPS in the *pearl* mouse¹⁰ and in two brothers with HPS-2 (refs. 11,12). *ADTB3A* encodes a coat protein involved in vesicle formation^{3,13}, implicating HPS as a disorder of membrane trafficking. We sought to identify other HPS-causing genes^{7,8,14}. Using homozygosity mapping on pooled DNA of 6 families from central Puerto Rico, we localized a new HPS susceptibility gene to a 1.6-cM interval on chromosome 3q24. The gene, *HPS3*, has 17 exons, and a putative 113.7-kD product expected to reveal how new vesicles form in specialized cells. The

homozygous, disease-causing mutation is a large deletion and represents the second example of a founder mutation causing HPS on the small island of Puerto Rico. We also present an allele-specific assay for diagnosing individuals heterozygous or homozygous for this mutation.

In studying Puerto Rican families with HPS, we evaluated the possible existence of a genetic isolate of the disease in central Puerto Rico¹³ by examining six families (Fig. 1a) from Aibonito, Naranjito and Barranquitas, rural towns south of San Juan (Fig. 1b). We identified the 13 affected individuals available to us based upon their medical histories and clinical findings, including a bleeding diathesis, horizontal nystagmus, decreased vision and very mild pigment dilution of hair, skin and irides (Fig. 1c). We confirmed the diagnosis of HPS by demonstrating an absence of platelet dense bodies using wet-mount electron microscopy^{14,15} (Fig. 1c). All patients lacked the 16-bp duplication in *HPS1* (ref. 6).

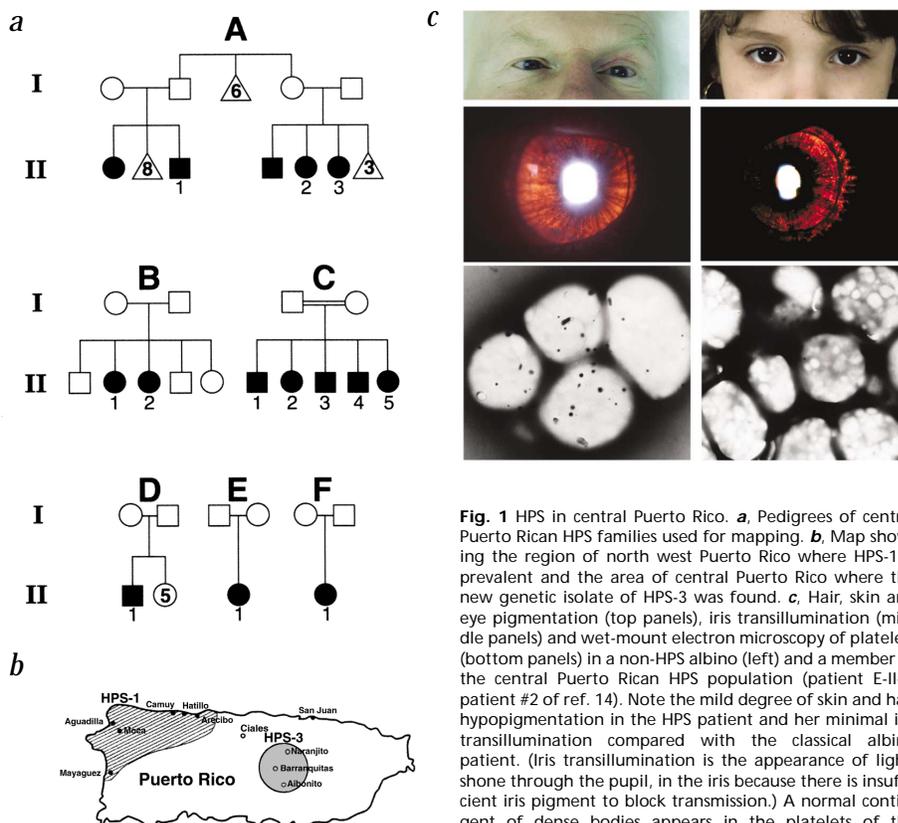
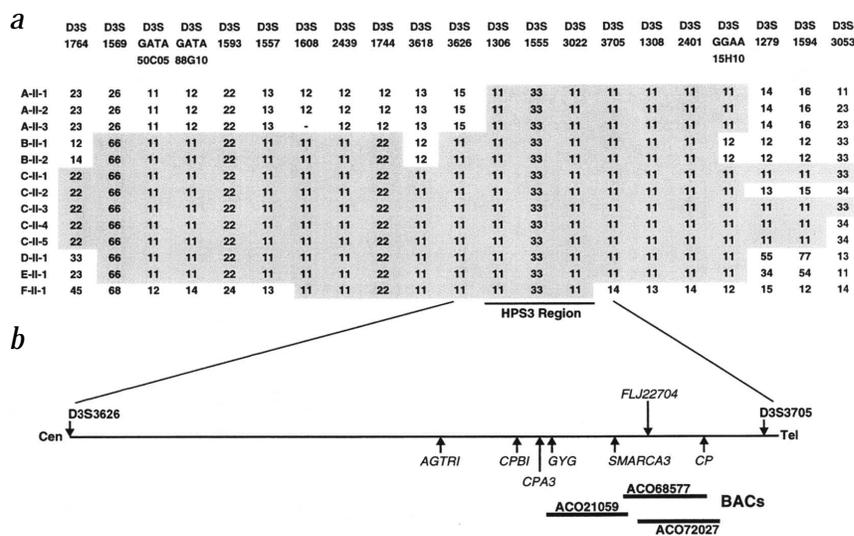


Fig. 1 HPS in central Puerto Rico. **a**, Pedigrees of central Puerto Rican HPS families used for mapping. **b**, Map showing the region of north west Puerto Rico where HPS-1 is prevalent and the area of central Puerto Rico where the new genetic isolate of HPS-3 was found. **c**, Hair, skin and eye pigmentation (top panels), iris transillumination (middle panels) and wet-mount electron microscopy of platelets (bottom panels) in a non-HPS albino (left) and a member of the central Puerto Rican HPS population (patient E-II-1, patient #2 of ref. 14). Note the mild degree of skin and hair hypopigmentation in the HPS patient and her minimal iris transillumination compared with the classical albino patient. (Iris transillumination is the appearance of light, shone through the pupil, in the iris because there is insufficient iris pigment to block transmission.) A normal contingent of dense bodies appears in the platelets of the non-HPS albino patient, whereas the HPS platelets lack dense bodies entirely.

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We carried out homozygosity mapping on pooled DNA from the 6 affected families using polymorphic markers, first concentrating on human chromosomes containing regions homologous to mouse loci to which the 14 known mouse mutants of HPS had been mapped¹⁶. We found no linkage to the *pale ear mouse/HPS1* locus (human chromosome 10q23), nor to the loci corresponding to the murine models ruby eye (10q24), gunmetal (14q11), mocha (19p13), ruby eye 2 (11p15/15q11), reduced pigment (19q13), pallid (15q11) or light ear (4p16). These studies excluded approximately two-thirds of the human genome before two markers in the region corresponding to the subtle gray locus¹⁷, *D3S1764* and *D3S1744*, showed homozygosity on pooled DNA from all affected individuals. We analyzed this region of chromosome 3q24 using 21 polymorphic markers. Contiguous homozygous markers defined an ancestral haplotype (Fig. 2a). All affected individuals exhibited an ancestral haplotype, while recombinations in five individuals caused a reduction in the length of the shared haplotype. These recombinations allowed us to tentatively narrow the critical region to the 1.6 cM interval between markers *D3S3626* and *D3S3705*.

Six genes, *AGTR1*, *CPB1*, *CPA3*, *GYG*, *SMARCA3* and *CP*, are located within the central portion of the critical region (Fig. 2b). As none of these encodes a protein likely to be involved in vesicle formation, genes we focused on the remainder of the critical region. Two genomic contigs, NT_005540 and NT_005616, covered the entire critical region except for approximately 50 kb. We searched these contigs for candidate genes or EST clusters. In the region between *SMARCA3* and *CP* (Fig. 2b), the Sanger Center Ensembl database revealed two predicted genes designated, *ENSG00000071786* and *ENSG00000071785*. The latter gene sequence corresponds to a cDNA, FLJ22704 (gi 10439197), which has 1,984 bp spanning 7 predicted exons and a poly(A) tail.

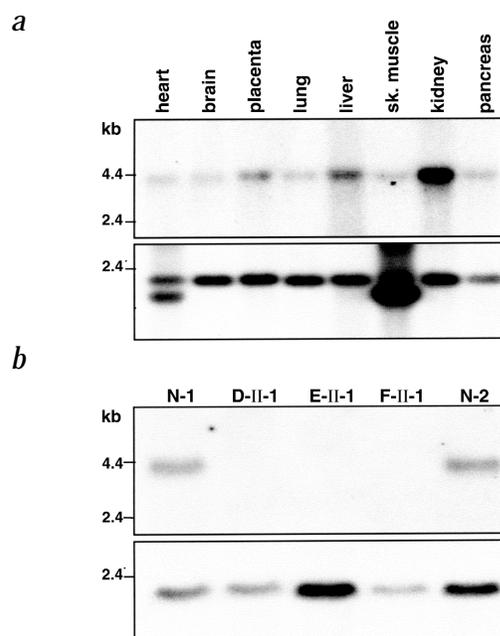
We used primers designed according to the sequence of clone FLJ22704 to amplify control cDNA by PCR, but cDNA from central Puerto Rican patients produced no band (data not shown).

Fig. 3 Northern-blot analyses using a probe to a portion of *HPS3* cDNA. **a**, The probe hybridized to a 4.4-kb band of all tissues tested on this multiple tissue northern blot. Expression appeared greatest in kidney, with strong bands in liver and placenta. The lower panel shows the band for β -actin, which served as a control for RNA loading. **b**, A 4.4-kb band of RNA from normal fibroblasts hybridized with the FLJ22704 probe (lanes 1,5), whereas no band appeared using RNA from fibroblasts of three central Puerto Rican HPS patients D-II-1, E-II-1, and F-II-1 (lanes 2-4).

Fig. 2 Homozygosity mapping of HPS-3. **a**, Genotypes of 13 central Puerto Rican individuals with HPS-3 showing homozygosity for chromosome 3q24 markers. Historic recombinations with markers *D3S3705* (in individual F-II-1) and *D3S3626* (family A) allow definition to the critical region harboring the HPS-3 locus. **b**, Genetic map of the HPS-3 critical region, containing genes indicated by arrows and based upon the NCBI human genome draft. The three BACs contain unordered sequence and cover the region in which *HPS3* resides.

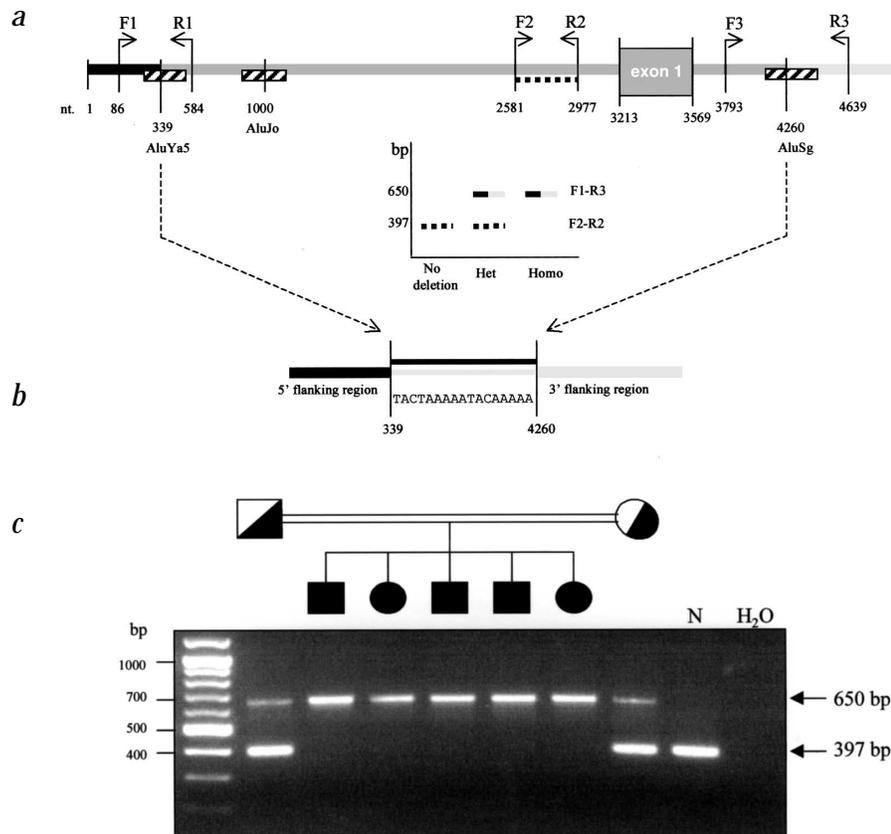
Northern-blot analysis using a 1,225-bp FLJ22704 cDNA probe revealed a 4.4-kb message in several human tissues (Fig. 3a) and in normal fibroblasts, but no message in fibroblasts obtained from three patients (Fig. 3b).

Using patients' genomic DNA as a template, we amplified the seven exons of the predicted gene and found no mutated sequence. This suggested that we had identified only a portion of the complete gene, particularly as the FLJ22704 probe gave a 4.4-kb transcript whereas the cDNA was only 2.0 kb in size. As we concluded the 3' end of the FLJ22704 clone has a poly(A) tail, the missing sequence was 5' to our cDNA. We designed a forward primer at the 3' end of *ENSG00000071786* and a reverse primer at the 5' end of the first exon of FLJ22704. With normal cDNA as a template, we amplified a fragment of approximately 1.3 kb, indicating that *ENSG00000071785* and *ENSG00000071786* are parts of the same gene. We continued our expansion with 5'-RACE and searched for human ESTs, linking together five that overlap (gi 7142976, gi 12600915, gi 12771035, gi 12669922 and gi 12951337) before we found a stop codon preceding the start site of our gene.



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Fig. 4 Structure of the *HPS3* deletion and multiplex PCR amplification assay for its presence or absence. (Nucleotide numbering is according to GenBank accession #AF375663). **a**, Schematic basis of the 3904-bp deletion. The deletion (shaded line) contains exon 1 (shaded box) and is flanked by normal-sequence 5' (black line) and 3' (light shaded) regions. The deletion breakpoint occurs within two *Alu* repeats (crossed boxes), *AluYa5* and *AluSg*. F1, F2 and F3 refer to forward primer sequences, R1, R2 and R3 to reverse primer sequences. **b**, The deletion breakpoint occurs somewhere within the 17-bp region shared by the two *Alu* repeats. PCR amplification across the deletion yields a 650-bp fragment. Two primers within the deleted area (F2 and R2) are used to amplify the DNA of patients lacking the deletion; the product is a 397-bp fragment. The inset shows the band produced for homozygous normal, heterozygous deleted and homozygous deleted individuals. **c**, Multiplex PCR in family C. PCR amplification products indicate the presence (650-bp fragment) or absence (397-bp fragment) of the 3,904-bp deletion. Lane 1, molecular weight markers; lane 2, father, heterozygous for the deletion; lanes 3–7, affected children (CII, 1–5), homozygous for the deletion; lane 8, mother, heterozygous for the deletion; lane 9, normal control, lacking the deletion; lane 10, no-DNA control.



The complete gene, *HPS3*, consists of 3,921 bp and 17 exons. The translation start site is at position 141 and the stop site at position 3,155 of the cDNA. The genomic organization, including intron/exon boundaries, is given in Web Table A, along with primers for amplifying each exon.

To determine the mutation in central Puerto Rican patients, we sequenced exons 2–17 and found them to be normal. Exon 1 did not, however, amplify, suggesting that it might be deleted. We therefore amplified approximately 500-bp fragments of patients' genomic DNA both downstream and upstream of exon 1, using primer pairs closer and closer to each other until no product was obtained (which indicated that at least one of the primers was identical or complementary to sequence within the deleted segment). We then amplified across the deleted segment and sequenced the product. Comparison with the sequence of BAC AC021059 revealed that the deletion is flanked by two *Alu* repeats of the SINE family, *AluYa5* and *AluSg* (Fig. 4a). The deletion consists of a 3,904-bp region encompassing all of exon 1, roughly 673 bases of intron 1 and approximately 2,874 bases upstream of exon 1.

We could not determine the exact deletion breakpoint because it occurred within a 17-bp stretch of nucleotides shared completely by *AluYa5* (nt 225–535) and *AluSg* (nt 4129–4410; Fig. 4b). Using selected primers flanking the deletion (F1 and R3; Fig. 4a), we amplified a 650-bp fragment. Combined with amplification of a 397-bp fragment within the deleted area (using primers F2 and R2; Fig. 4a), this provides the basis for a multiplex PCR amplification assay to detect the presence of zero, one or two deletion alleles in any DNA sample (Fig. 4b). An example is presented for family C (Fig. 4c).

The protein HPS3 is predicted to consist of 1,004 amino acids, and have a molecular weight of 113.7 kD and a pI of 6.01. We think that it may be cytosolic because it has no predicted

transmembrane regions and no glycosylation sites. It is predicted to be 43% α -helix, 19% extended strand, 30% random coil and 7% β -turn. It has no homology to any functional proteins, including those associated with known mouse models of HPS, with yeast vesicle proteins for sorting¹⁸ or with other gene products involved in vesicle formation. The protein encoded by *HPS3* does, however, contain a potential clathrin-binding motif¹⁹ at residues 172–176, two consensus dileucine signals²⁰ and 12 tyrosine-based sorting signals²¹ for targeting to vesicles of lysosomal lineage (Fig. 5). It also contains a possible endoplasmic reticulum membrane retention signal (KKPL) at residues 1000–1003; such dilysine motifs exist in the carboxy-terminal regions of type Ia membrane proteins that traffic between the endoplasmic reticulum and the Golgi apparatus²². A peroxisomal matrix targeting signal (RLD-SQHSHL) is of questionable significance as it is located in the middle of the HPS3 protein and this PTS2 motif usually functions in the amino-terminus of peroxisomal matrix proteins²³.

HPS3 is the third gene known to cause HPS when mutated³, and we call the associated disease HPS-3. We are currently determining the extent of HPS-3 among our non-Puerto Rican HPS patients with apparently intact *HPS1* and *ADTB3A*^{7,8}. HPS-3 among central Puerto Ricans seems milder than HPS-1, which is associated with fatal pulmonary fibrosis⁴, although patients with HPS-1 exhibit enormous clinical variability^{2,3,24,25}. HPS-2, caused by mutations in *ADTB3A*¹¹, presents as a disorder of intermediate severity¹². It will be of critical interest to determine the mechanism by which the HPS-3 protein assists in the formation of melanosomes and dense bodies.

The occurrence of a founder mutation in *HPS3* causing HPS in central Puerto Rico is remarkable in view of the existence of another HPS-causing founder mutation in north-west Puerto



Fig. 5. Amino acid sequence of the HPS3 protein, consisting of 1,004 amino acids. The clathrin binding motif (LLDFE, aa 172–176) and endoplasmic reticulum membrane retention signal (KKPL, aa 1000–1003) are boxed in dark. Twelve tyrosine sorting motifs are shaded in gray and two dileucine-based sorting motifs are within open boxes. A peroxisomal matrix targeting signal is in italics and underlined.

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1  MVQLYNLHPPFGSQVVPCKLEPDRFCGGGRDALFVAAGCKVEAFVAGQELCQPRCAFST
61  LGRVLRLAYSEAGDYLVVAIEEKNKATFLRAYVNRNRKRTENSRCVIRMIGHNVGPPFSKA
121  FRDQMYIIEEMPLSEAPLCISCCPVKGDLLVGTNKLVLFLSLKYQIINEEFSLLDFERSLI
181  IHIDNITPVEVSVFCVGVAVVMSDLEVLIVKLESGPKNGERVHHHPKHTNRRIRRTTEEGIS
241  NEISQLESDDFVICQKPLELLGEKSEQSLVSTLESTGLADEKRKYSHFQHLLYRRRAPD
301  ISSYVLSDDIKLHSLQLLPIYQGTGSLTSDGKNLSQEKELLSLFCFFSLPHVGYLYMVVKS
361  VELMSVYQYPEKSSQAVLTPQFLHVIITSNLQCFTVRCSSAAAAREEDPYMDTTLKACPPV
421  SMDVICALRIQLFVIGLKAICHFKNHIILLTKAEPEAIPERRQSPKRLLSRKDTSVKIKIPP
481  VAEAGWNLYIVNTISPVQLYKEMVDYSNTYKTVKQSCIHLLSEAHLLVRAALMDASQLE
541  PGEKAELLEAFKESCGHLDGDCYSRLDSQHSHTLLPYYKMSSGLSMAEVLARTDWTVEDGLQ
601  KYERGLIFYINHSLYENLDEELNEELAAKVVQMFYVAEPKQVPHILCSPSMKNINPLTAM
661  SYLRKLDTSGFSSILVTLTKAAVALKMGDLDMHRNEMKSHSEMKLVCGFLEPRLLQQR
721  KGQIVPTELALHLKETQPGLLVASVLGLQKNNKIGIEEADSFVKVCAKDEDTIPQLLVD
781  FWEAQLVACLPDVVVLQELFFFKLTSQYIWRLSKRQPPDTTPLRTSEDLINACSHYGLIYYPW
841  VHVVISSDSLADKNYTEDLSKLQSLICGSPFDIASIIPFLEPLSEDTIAGLSVHVLCRTR
901  LKEYEQCIDILLERCPEAVIPYANHELKEENRTLWKKLLPELCQRIKCGGEKYQLYLSS
961  LKETLSIVAVELELKDFMNVLPEDGTATFFLPYLLYCSRKKPLLT

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Rico⁵. We attempted to determine when and where the *HPS3* founder mutation arose. Using the method of Luria and Delbruck²⁶, we estimated that the deletion occurred in central Puerto Rico in approximately 1880–1890 (see Methods). At that time, the ancestors of three of our HPS-3 families (B, E and F) emigrated from the town of Ciales (Fig. 1b) to the towns of Aibonito, Barranquitas and Naranjito because of harsh economic conditions in Ciales. Each of the three families also traces its ancestry to one individual, Calixto Rivera, who brought his relatives to Aibonito and the surrounding area to deforest his land for tobacco growing²⁷. It is possible that a member of this family, emigrating to this mountainous, isolated region of central Puerto Rico, carried the founding mutation.

On a molecular level, the *HPS3* deletion probably arose by homologous recombination across two *Alu* repeats. In the human genome, *Alu* repeats number approximately one million and occur on average every 4 kb. They are 70–98% conserved and approximately 300 bp long, with stretches of similar 120-bp and 150-bp regions separated by a short (A)-rich area²⁸. The *HPS3* deletion occurred between an *AluSg* and an *AluYa5* repeat; not all humans have both repeats.

Amplification of normal genomic DNA across the 3' deletion breakpoint, using primers F3 and R3 (Fig. 4a), yielded the expected fragment containing the *AluSg* repeat sequence. However, amplification across the 5' breakpoint, using primers F1 and R1 (Fig. 4a), gave two fragments of size 169 bp and 498 bp, respectively. Sequencing revealed that these fragments differed by the insertion of an *AluYa5* repeat. Not only was the normal template DNA heterozygous for the *AluYa5* repeat, but the repeat sequence was also present in BAC AC021059 but absent from BAC AC068577. Furthermore, the *AluYa5* repeat was present in approximately 22% of 36 alleles tested, whether from Puerto Rican or non-Puerto Rican individuals. *AluYa5* repeats were inserted into the human genome relatively recently²⁹. It seems that the central Puerto Rican deletion arose in the (A)-rich region of an *AluYa5* repeat (Fig. 4b) that the founder individual happened to carry.

The location of *HPS3* on chromosome 3q24, which is homologous to the region of mouse chromosome 3 implicated for the subtle gray mutant suggests that the two genes may be homologous. Initial investigations indicate, however, that the subtle gray mouse¹⁷ has *HPS3* mRNA of normal size and quantity (data not shown). This suggests that *HPS3* is not related to subtle gray mutation despite mapping to the same general region. Definitive resolution of this issue awaits complete analysis of the subtle gray gene sequence and that of other mouse models.

Methods

Genotyping. We collected buccal DNA samples from affected individuals and unaffected first-degree relatives after informed consent under a protocol

approved by the NICHD Institutional Review Board and the Institutional Review Board of the University of Puerto Rico-Medical Sciences Campus. We prepared three DNA pools: affected, obligate heterozygotes (parents and offspring of affected individuals) and unaffected (normal and unrelated Puerto Rican individuals from central Puerto Rico).

We used pooled DNA prepared by mixing an equal volume of each sample into one tube as templates for amplification with a panel of 387 microsatellite polymorphic markers having average heterozygosity of 76% and average spacing of 10 cM between markers (Verson 8 panel, CHLC Human Screening Set, Research Genetics). Those markers which showed a reduction in the number of alleles in the affected pool compared with the control pool were verified by typing of individual family members and by analysis of other nearby markers. We typed genetic markers on the LI-COR 4200S using 25-cm polyacrylamide gels. We obtained primer sequences for genetic markers from Genome DataBase and modified them so that one primer of each pair was tailed with either M13F or M13R. We amplified DNA samples (1 µl) in 5 µl reactions containing 0.1 pmol of each of the forward and reverse primers, 0.16 pmol of the corresponding fluorescently labeled M13 primer, 0.2 units of AmpliTaq (Perkin-Elmer) in PCR buffer containing 2 mM MgCl₂ and 100 µM of each of the dNTPs. The amplification profile consisted of an initial denaturation for 2 min at 94 °C followed by 30 cycles of 94 °C for 30 s, 55 °C for 75 s, 72 °C for 30 s and a final extension for 5 min at 72 °C (V. Sheffield, pers. comm.).

cDNA amplification. The primers used to demonstrate that *ENSG00000071785* and *ENSG00000071786* are part of *HPS3* are: forward 5'-GTAATGAAATTTACAGCTTGAGTCAG-3' (cDNA nt 859–885); reverse 5'-GTCAC^TAAGATCGATGAAAACCCAG-3' (nt 2170–2146). These primers amplified a 1.32-kb fragment. We used Human Kidney Marathon-Ready cDNA (Clontech) to confirm the 5' sequence of human *HPS3*. Primers used in this study were, in the first round, Adaptor Primer 1 (Clontech) and 5'-GGTCTCCTTTCACAGGGCAACAGG-3' (nt 582–559); nested PCR, in the second round, Adaptor Primer 2 (Clontech) and 5'-CTGAGGCTTTGCTG AATGGTCCC-3' (nt 505–482). PCR conditions for both amplification steps were as specified in the manufacturer's protocol.

Sequencing and sequence analyses. We performed automated sequencing on a Beckman CEQ 2000, using the CEQ Dye Terminator Cycle Sequencing kit according to the manufacturer's protocols (Beckman Coulter). We used BLAST analysis for sequence homology searches, made available through the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/BLAST/>). We also searched for unknown candidate gene sequences using Project Ensembl of the Sanger Centre, at <http://www.ensembl.org/>. We analyzed the protein sequence using the ISREC search-tools (Swiss Institute for Experimental Cancer Research,



<http://www.isrec.isb-sib.ch/>), the Baylor College of Medicine Search Launcher (<http://searchlauncher.bcm.tmc.edu/>), and the PSORT program from the Human Genome Center, IMS, University of Tokyo, Japan (<http://psort.nibb.ac.jp>).

Mutation screening. Using genomic DNA from leukocytes or cultured fibroblasts as template, we performed PCR amplification of each exon for subsequent sequencing. Web Table A gives the primer sets for each exon. In general, we amplified 200–400 ng of genomic DNA using standard PCR procedures³⁰, with an annealing temperature for all primer pairs of 58 °C. The PCR products were electrophoresed in 1% agarose and stained with ethidium bromide. For detection of the deletion mutation, we used the standard mutation screening described above, with two sets of primers, F1 (gDNA nt 86–106, 5′-GGTGTGTTTGTAGAGATGCAGA-3′) and R3 (nt 4616–4638, 5′-GCATAGCCACCAGCTTTTGCAACG-3′), and F2 (nt 2581–2604, 5′-CGTGAACCCACGTTGAGATGTC-3′) and R2 (nt 2954–2977, 5′-CGTTCTGACAATTCATCATCTATC-3′). In addition, we used primer F3 (nt 3793–3815, 5′-GACGCTGACATCTCTTCGCTTG-3′), for amplification of the 3′ breakpoint.

Northern-blot analysis. We purchased multiple tissue northern (MNT) filters from Clontech. We isolated fibroblast mRNA and prepared and probed northern blots as described¹⁴. The probe was human *HPS3* cDNA, random-primer labeled with α [³²P]-dCTP (DuPont/New England Nuclear) and prepared by PCR amplification of normal cDNA using forward primer 5′-GAATTAGCAGCAAAAGTGGTTCA-3′ (nt 2013–2035) and reverse primer 5′-TCCTTACTTGTACTTTGGCATC-3′ (nt 3233–3210). We probed the same set of filters with *ACTB* (β -actin).

Determination of age of founder mutation. In general, the size of a conserved haplotype surrounding a mutation provides an estimate of the age of the mutation. For our group of patients, the ancestral haplotype encompassed at least 11 cM. If the Puerto Rican population of *HPS-3* patients descended from a single founder, and the population expanded at a constant exponential rate, the Luria–Delbruck method²⁶ estimates the number of generations (g) that have passed since the mutation was first introduced. That is, the proportion of chromosomes carrying the mutation in which recombination with the conserved haplotype has occurred equals $1 - e^{-g\theta}$, where θ is the recombination fraction in Morgans. From our data, we estimate that recombination with the ancestral chromosome has occurred in approximately 44% of the cases. Solving for g , the introduction of the mutation to the central Puerto Rican population occurred approximately 5.3 generations, or 110–120 years, ago.

GenBank accession numbers. Sequence data for *HPS3* cDNA, AY033141; genomic sequence of *HPS3*, AF375663.

Note: supplementary information is available on the Nature Genetics web site (http://genetics.nature.com/supplementary_info/).

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