LETTER TO JMG

Espin gene (*ESPN*) mutations associated with autosomal dominant hearing loss cause defects in microvillar elongation or organisation

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J Med Genet 2006;**43**:157–161. doi: 10.1136/jmg.2005.032086

Background: Espins are actin bundling proteins present in hair cell stereocilia. A recessive mutation in the espin gene (*Espn*) has been detected in the jerker mouse and causes deafness, vestibular dysfunction, and hair cell degeneration. More recently mutations in the human espin gene (*ESPN*) have been described in two families affected by autosomal recessive hearing loss and vestibular areflexia.

Objective: To report the identification of four additional *ESPN* mutations (S719R, D744N, R774Q, and delK848) in patients affected by autosomal dominant hearing loss without vestibular involvement.

Results: To determine whether the mutated *ESPN* alleles affected the biological activity of the corresponding espin proteins in vivo, their ability to target and elongate the parallel actin bundles of brush border microvilli was investigated in transfected LLC-PK1-CL4 epithelial cells. For three mutated alleles clear abnormalities in microvillar length or distribution were obtained.

Conclusions: The results further strengthen the causative role of the espin gene in non-syndromic hearing loss and add new insights into espin structure and function.

Espins are a family of multifunctional actin cytoskeletal regulatory proteins. They are able to influence the organisation, dimensions, dynamics, and signalling capabilities of the actin-filament-rich, microvillus type specialisations that mediate sensory transduction in various mechanosensory and chemosensory cells.[1] They are associated with the parallel actin bundles of hair cell stereocilia and are the target of mutations that cause deafness and vestibular dysfunction in mice (jerker) and humans (DFNB36).[2 3] One striking illustration of espin activity in vivo is the ability to cause a dramatic, concentration dependent lengthening of brush border microvilli and their parallel actin bundles in transfected LLC-PK1-CL4 epithelial cells.[4] The espin COOH-terminal peptide, which contains the 116 amino acid actin bundling module,[5] is required for this microvillar lengthening activity.[4] This ability to increase microvillar length in transfected epithelial cells has led to the hypothesis that espins play an essential role in determining the length of hair cell stereocilia.[4] In support of this hypothesis, espin protein level is positively correlated with stereocilium length,[4] and abnormally short hair cell stereocilia are observed in homozygous jerker mice which lack espin protein.[2] A human deafness recessive locus DFNB36 has recently been mapped to chromosome 1p36.3 in two consanguineous families affected by deafness and vestibular areflexia.[3]

The phenotype segregates with mutations in *ESPN*, which encodes espin. These findings establish espin as an essential protein for hearing and vestibular function in humans. Here we describe the identification of new functionally abnormal *ESPN* alleles associated with dominant forms of hearing loss without vestibular involvement.

METHODS

We analysed 450 subjects for mutations in the espin gene. Inclusion criteria were: absence of the most common mutations within the *GJB2* gene; sensorineural hearing loss; and normal tympanometric evaluation.[6] In all cases, vestibular data were obtained by clinical examination and routine vestibular tests (one or more of the following tests: caloric, rotatory, optokinetic, swinging torsion, statokinesimetric, and vestibulo-vegetative). The series includes cases with a variable degree of hearing loss, ranging from mild to profound, and with a variable age of onset, from congenital to late onset. Familial records were available in most cases. The majority of patients came from central and southern Italy (200), while 140 were from Spain, 54 from Belgium, and 50 from Israel. After informed consent, peripheral blood was obtained from all subjects, and DNA was isolated from blood leucocytes using standard methods.

Thirteen different primer pairs were designed in order to amplify the 13 coding exons of the *ESPN* gene, including the splice sites (the polymerase chain reaction (PCR) primer sequences and conditions are available upon request). All amplicons were screened by denaturing high performance liquid chromatography (DHPLC) on a WAVE® nucleic acid fragment analysis system HSM (Transgenomic Inc, Santa Clara, California, USA) according to the protocols supplied. DHPLC data analysis was based on a subjective comparison of sample and reference chromatograms. PCR products showing an abnormal chromatographic profile were directly sequenced on an automated sequencer (ABI 3100; Perkin Elmer, Norwalk, Connecticut, USA).

Human espin 3A cDNA was obtained by reverse transcription PCR from human testis RNA (BD Biosciences, Clontech, Palo Alto, California, USA). Mutations were introduced by PCR. All cDNAs were checked by DNA sequence analysis before introduction into the *SmaI* site of the pEGFP-C2 vector for transfection studies in differentiated LLC-PK1-CL4 epithelial cells.[4] Briefly, cells were cultured at 37°C in minimum essential medium alpha medium (with L-glutamine, without nucleosides) supplemented with 10% fetal bovine serum (Invitrogen, San Diego, California, USA) and 100 U/ml penicillin and streptomycin. Cells cultured on glass cover slips for 8 to 10 days (~75% confluency) were transfected for four hours with a fixed amount of plasmid DNA using Lipofectamine (Invitrogen) and examined 20 to 24 hours later. Cells were fixed with 2% paraformaldehyde,

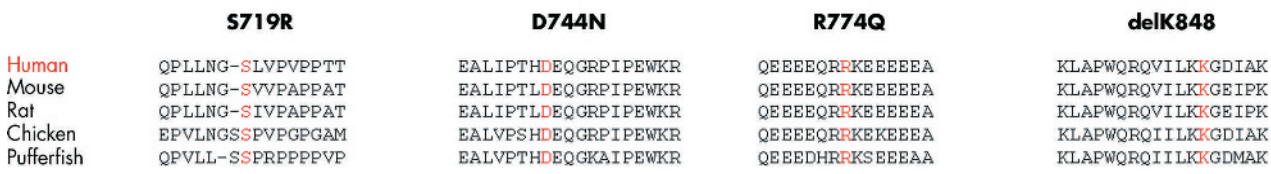

Figure 1 Amino acid sequence alignments for the peptides that contain the newly identified *ESPN* mutations. Alignments were undertaken using ClustalX and WU-Blast 2. The mutated residues are shown in red. Note their conservation across species.

treated briefly with 0.1% Triton X-100, labelled with Texas Red–phalloidin (Molecular Probes, Eugene, Oregon, USA) to detect F-actin, and mounted in 5% *n*-propylgallate, 90% glycerol. Transfected cells were identified by green fluorescent protein (GFP) fluorescence; 0.5 µm confocal z sections were collected at room temperature using a confocal microscope and a 100× 1.4 NA oil immersion objective. LSM510 imaging software was used to generate stacks from which measurements of microvillar length were made. Images were saved in TIF format, transferred to Photoshop (Adobe Systems), assembled into composites, and converted to CMYK colour format with minor adjustments of brightness and contrast. Antibody to purified recombinant rat espin 2B, previously called rat Purkinje cell espin 1 (GenBank accession number AF540946), was prepared in rabbits and affinity purified on columns of rat espin 2B-sepharose 4B. This antibody has been used extensively to detect espin isoforms on western blots and by immunocytochemistry.[1]

Western blotting was carried out using the ECL method (Amersham Biosciences, Amersham, UK) on extracts prepared from replicate dishes by boiling (or heating at 100°C) for three minutes in SDS gel sample buffer.[4]

RESULTS

Mutational screening of the *ESPN* gene by DHPLC and sequencing of 450 subjects from different countries allowed us to identify one deletion and three missense mutations that were associated with autosomal dominant hearing loss (table 1). The first such mutation to be identified was an A→C nucleotide change at position 2155. This replaces a conserved serine residue at position 719 with an arginine (S719R) (fig 1). (The numbering schemes used are those of prototypical espin (GenBank accession number NM_031475), which is referred to as espin 1 in the revised nomenclature for espin isoforms[1].) This mutation was detected in a small Italian kindred, in which two affected individuals in two generations show a dominant form of progressive sensorineural hearing impairment that starts in the second decade and leads to a mild to moderate hearing loss in the fourth decade (high frequencies mainly involved). No vestibular involvement was detected using standard vestibular tests. Normal members of the family (two additional subjects) were completely negative for the presence of the mutation.

Table 1 Mutations and polymorphisms identified in *ESPN* (GenBank accession number NM_031475)

Exon	Nucleotide	Protein
Mutation		
EX10	A2155C	S719R
EX10	G2230A	D744N
EX10	G2321A	R774Q
EX13	2541-2543delAAG	delK848
Polymorphism		
EX2	C390U	Silent
EX3	C564G	Silent
EX3	A591G	Silent
EX3	G627A	Silent
EX5	G909C	Silent
EX8	T1476C	Silent

A G→A nucleotide change was detected at position 2230, leading to an amino acid substitution from aspartic acid to aspargine at position 744 (D744N). This aspartic acid residue is highly conserved across species (fig 1). The mutation was detected in an Italian patient affected by severe bilateral sensorineural hearing loss involving all frequencies, without vestibular involvement. Although there was a family history of hearing loss, no additional genetic and instrumental tests could be carried out in other family members.

A G→A nucleotide change affecting position 2321 was detected in a sporadic Italian case. This change leads to substitution of a highly conserved arginine residue with glutamine at position 774 (R774Q) (fig 1). The patient is affected by late onset (age 42) mild bilateral sensorineural hearing loss involving all frequencies but mainly the high ones.

Finally, the sequence from a Spanish sporadic case showed a three nucleotide deletion (2541-2543delAAG), leading to the loss of a lysine residue at position 848 in the COOH-terminal peptide (delK848). This patient (now aged 4) has severe bilateral sensorineural hearing impairment without vestibular involvement. Independent analysis of the proband sample confirmed the presence of this mutated allele, which affects a residue that is highly conserved across species (fig 1) and must be catalogued as a de novo alteration.

Two hundred chromosomes of individuals from the same geographical areas as the patients were examined for the presence of the mutant alleles described above. None of the changes was detected in the control samples. We also detected six synonymous substitutions (table 1) in the cohort of hearing subjects. As these latter changes do not create putative cryptic splice sites or did not segregate with deafness (data not shown), they have been excluded as causative mutations for deafness in our cohort of patients.

To determine whether the espin gene mutations associated with autosomal dominant sensorineural hearing loss had a discernible effect on espin function in vivo, we compared the effects of the wild type and mutated espin proteins on the microvilli of transiently transfected LLC-PK1-CL4 epithelial cells.[4] When maintained as confluent islands for 8 to 10 days in culture, LLC-PK1-CL4 epithelial cells invariably express uniform brush border microvilli 1–1.5 µm in length.[4 7 8] When expressed exogenously in these cells by transient transfection, wild type espins are efficiently targeted to the microvilli and bring about a dramatic elongation of the microvilli and the parallel actin bundle scaffold at their core.[4] This activity, which is believed to stem directly from espin cross links in the core actin bundle, could explain the positive correlation noted between espin protein level and stereocilium length and the shortening of hair cell stereocilia observed in the espin deficient homozygous jerker mice.[2 4]

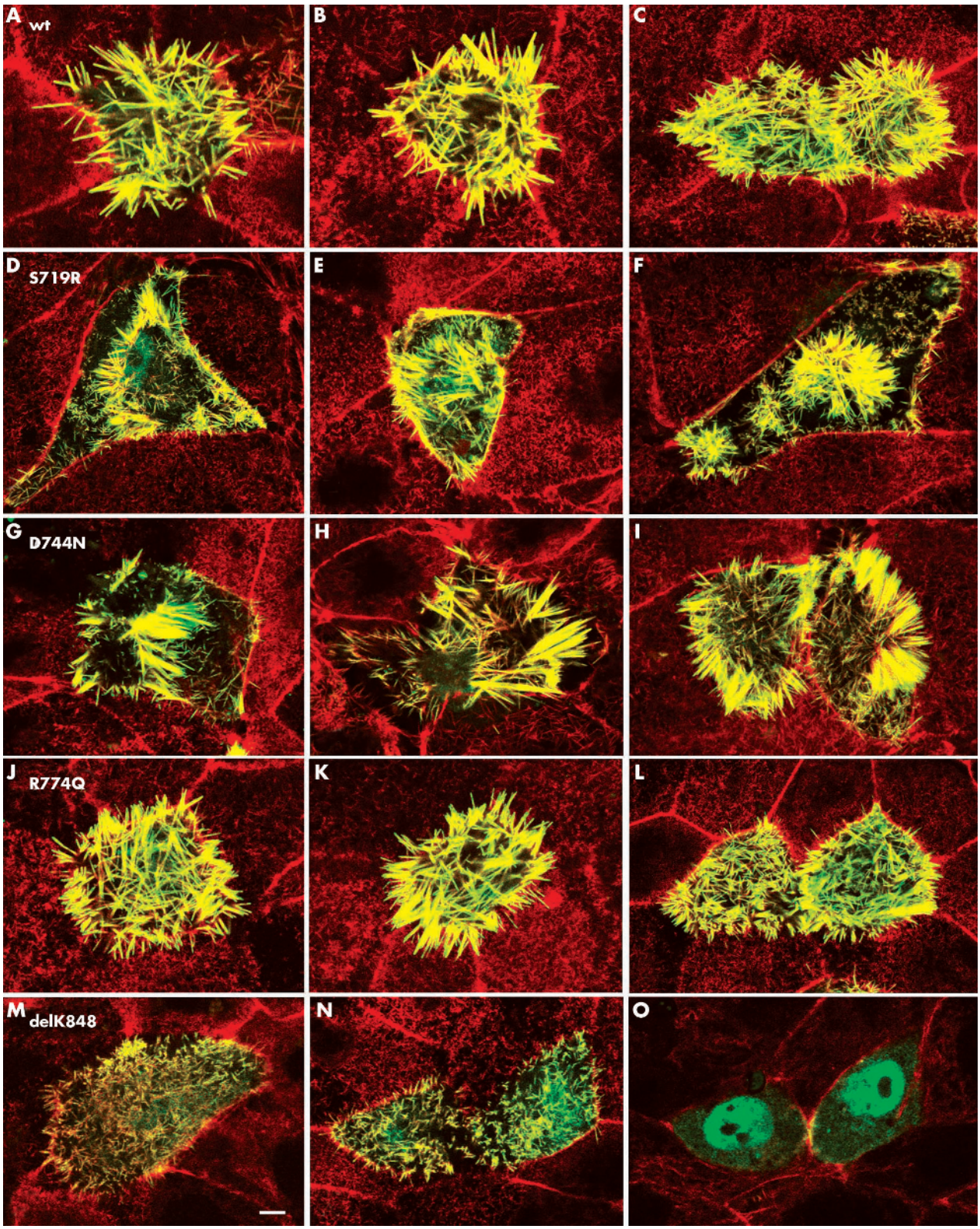

Figure 2 Functional comparisons of wt and mutated human espin 3A using the LLC-PK1-CL4 epithelial cell transfection model. Differentiated LLC-PK1-CL4 epithelial cells were transfected with GFP-wt human espin 3A or the designated mutated construct, labeled for F-actin with Texas Red-phalloidlin and examined by confocal microscopy. Multiple examples are shown. A-C, wt. D-F, S719R. G-I, D744N. J-L, R774Q. M-O, delK848. All images are of the apical (microvillar) surface of the monolayer, except O, which is a z-section through the middle of the cells in N highlighting the nuclear accumulation of the delK848 construct. The F-actin-rich junctional complexes adjointing the apical-lateral boundaries of neighboring cells are often evident as red lines. Note that the wt construct (A-C) is colocalized (yellow) in microvilli that are much longer than the brush border microvilli (red) of surrounding control (untransfected) cells. The R774Q (J-L) is indistinguishable from wt. The S719R (D-F) and D744N (G-I) constructs are targeted to microvilli and cause microvillar elongation, but the ong microvilli frequently appear in irregular patches that occupy only a small percentage of the apical surface. The delK848 construct (M-O) is severely impaired in microvillar elongation and shows abnormally high accumulation in the nucleus (O). The objects within the nucleus that exclude the GFP-delK848 construct are nucleoli. Bar, 5 μm.

After 8 to 10 days in culture, LLC-PK1-CL4 cells were transfected with plasmids encoding GFP tagged wild type or mutated human espin 3A under the control of the cytomegalovirus promoter. Espin 3A is the major espin isoform found in cochlear hair cells.[1] Between 20 and 24 hours after transfection, the cells were fixed, labelled with Texas Red-phalloidin to localise F-actin, and imaged by cofocal microscopy. The wild type GFP-human espin 3A was found to be highly concentrated in the microvilli and caused about a fivefold elongation of the microvilli, from 1.33 (0.04) μm, the length in untransfected control cells, to 6.28 (0.09) μm (mean (SEM), n = 167 to 189 microvilli, 14 to 17 cells) (fig 2A–C). The long microvilli of the transfected cells appear yellow because the GFP-espin is colocalised with the microvillar F-actin bundles, which are brightly labelled with the Texas Red–phalloidin. The construct bearing the R774Q mutation gave results that were not significantly different from those obtained with the wild type (length = 6.2 (0.1) μm; n = 153 microvilli, 14 cells) (fig 2J–L). In contrast, constructs bearing the S719R mutation or the D744N mutation made long microvilli, as long as or longer than wild type, but they often appeared in clumps that occupied only a relatively small percentage of the apical cell surface (fig 2D–I). A simple quantification of the behaviour of these latter two mutations revealed an eight- to 10-fold increase in the number of cells showing such a disorganised microvillar phenotype relative to wild type: while only 2–3% of cells transfected with wild type human espin 3A showed disorganised long microvilli, 25–30% of cells transfected with the D744N or S719R construct showed the defect (n = 540–710 transfected cells for each construct in three independent experiments). The remainder of the cells transfected with the S719R or D744N construct (70–75%) looked similar to those transfected with wild type. Finally, the construct bearing the delK848 mutation was severely impaired in microvillar elongation (fig 2M, 2N). The delK848 construct caused only a 1.5-fold elongation, from 1.33 (0.04) μm to 2.02 (0.04) μm (n = 136 microvilli, 13 cells), compared with the nearly fivefold elongation observed with wild type. In fact, compared with the other constructs, the delK848 construct appeared to be less efficiently targeted to microvilli and commonly showed higher levels of accumulation in the nucleus. This trend, which was often noticeable as a green haze beneath the microvilli (fig 2N), was seen to better advantage in z sections positioned below the microvilli (fig 2O). Western blots of the transfected cells labelled with affinity purified espin antibody showed a single major band at the expected molecular mass (~64 kDa = ~29 kDa for GFP and linker + ~35 kDa for human espin 3A) for each construct, and indicated that there were no major quantitative differences in the levels of the wild type and mutated proteins in the transfected cells that could account for their differing effects on the microvilli (data not shown).

DISCUSSION

Espins are associated with the parallel actin bundles of hair cell stereocilia and are the target of mutations that cause deafness and vestibular dysfunction in mice and non-syndromic recessive hearing loss and vestibular areflexia in humans.[2 3] Here, we report that mutations in *ESPN* also cause dominant forms of hearing loss without vestibular signs. Moreover, using the LLC-PK1-CL4 transfection model, we detected in vivo functional deficits for three of the new mutations that could affect the dimensions or organisation of hair cell stereocilia.

All of the newly described mutations affect residues conserved across species and were never found in normal chromosomes. Three of four of the mutations—D744N, R774Q, and delK848—mapped to the distal COOH-terminal peptide that includes the actin bundling module.[5] This peptide, which is shared among all known espin isoforms,[1] is necessary and sufficient for potent actin bundling activity in vitro[5] and for microvillar elongation activity in vivo.[4]

The delK848 mutation severely impairs microvillar elongation and causes abnormally high levels of espin protein to accumulate in the nucleus. This mutation is located in the COOH-terminal part of the actin bundling module, in a region believed to encompass one of its two F-actin binding sites.[5] In fact, the 13 and 19 amino acid COOH-terminal parts of the protein that include the homologous lysine residue in rat espins are known to be required for actin bundling[5] and microvillar elongation activity,[4] respectively. Thus our functional data on this mutated allele are in agreement with the results of previous studies. Moreover, the severity of the functional deficit noted with the delK848 mutation in the LLC-PK1-CL4 transfection model is strongly correlated with the severe phenotype of the corresponding patient, who had bilateral severe sensorineural hearing loss with a very early onset in childhood. The basis for the increased nuclear accumulation of the delK848 construct is not clear, but this result raises the intriguing possibility that the dominant effect of this mutation is exerted in the nucleus.

Two of the mutated alleles, D744N and S719R, cause a novel defect in microvillar organisation in the LLC-PK1-CL4 transfection model. The D744N mutation maps to the actin bundling module, whereas the S719R mutation maps to an upstream location, which is currently without a known function. Although both of these mutated constructs appear capable of elongating microvilli-like wild type espin, they also cause a marked increase in the frequency of transfected cells showing long microvilli confined to small patches of apical surface. This peculiar behaviour is noted only rarely in cells transfected with wild type espins and has not been detected previously for espin constructs bearing a wide variety of different truncation or deletion mutations.[4] This defect in microvillar organisation could reflect aberrant espin targeting or a dominant negative effect of the mutated espin on the other organising elements at the apical end of the epithelial cell. Patients carrying these alleles have moderate to severe bilateral hearing loss.

Finally, the R774Q mutation causes no obvious problems in the LLC-PK1-CL4 transfection model. This mutation was detected in a sporadic case with a mild form of hearing loss and a late onset (over age 40). Some possible conclusions can be drawn from this case, considering both functional and clinical data: first, the mutation is causative but its effect is too small to be detected in our functional assay; second, the mutated allele has a small contribution per se on the phenotype but interacts as a modifier with the genetic background contributing to the phenotype itself; third, the mutation, even if affecting an highly conserved residue and never being present in normal controls, is truly a neutral variant and not a causative allele.

In conclusion, the data presented here show an association between *ESPN* mutations and dominant forms of hearing loss. The detrimental effects of these mutations noted in the LLC-PK1-CL4 transfection model provide new insight into espin structure and function and suggest that the patients carrying these mutated alleles have defects in the elongation or organisation of their hair cell stereocilia.

ACKNOWLEDGEMENTS

The work was supported by grants from: Telethon Foundation and CNR (to PG); the National Institutes of Health (R01 DC004314 to JRB); DURSI and the ''Redes Tematica se Investigacion Cooperativa of the Fondo de Investigaciones Sanitarias'' (G03/203) (to XE). EB is supported by a fellowship from the Generalitat de Catalunya (Departament d'Universitats, Recerca i Societat de la Informació, DURSI), 2003-FI066). We thank Dr Patricia A Loomis for her help on

confocal microscopy, and Dr Karen Avraham and Dr Guy Van Camp for providing DNA samples and manuscript revision.

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Conflicts of interest: none declared

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Received 15 February 2005
Revised version received 24 May 2005
Accepted for publication 25 May 2005

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