

## Report

# Recurrent Mutation of the Gene Encoding sequestosome 1 (*SQSTM1/p62*) in Paget Disease of Bone

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Paget disease of bone (PDB) is a common disorder characterized by focal and disorganized increases of bone turnover. Genetic factors are important in the pathogenesis of PDB. We and others recently mapped the third locus associated with the disorder, *PDB3*, at 5q35-qter. In the present study, by use of 24 French Canadian families and 112 unrelated subjects with PDB, the *PDB3* locus was confined to ~300 kb. Within this interval, two disease-related haplotype signatures were observed in 11 families and 18 unrelated patients. This region encoded the ubiquitin-binding protein sequestosome 1 (*SQSTM1/p62*), which is a candidate gene for PDB because of its association with the NF- $\kappa$ B pathway. Screening *SQSTM1/p62* for mutations led to the identification of a recurrent nonconservative change (P392L) flanking the ubiquitin-associated domain (UBA) (position 394–440) of the protein that was not present in 291 control individuals. Our data demonstrate that two independent mutational events at the same position in *SQSTM1/p62* caused PDB in a high proportion of French Canadian patients.

Paget disease of bone (PDB [MIM 167250]) is a chronic disease of the skeleton that affects up to 2%–3% of the population aged >40 years (Siris and Canfield 1990; Klein and Norman 1995). The disorder is characterized by focal areas of increased and disorganized bone turnover, leading to bone pain, deformity, pathological fracture, neurological complications, and an increased risk of osteosarcoma (Hamdy 1995; Kanis 1998).

Genetic factors play an important role in the pathogenesis of PDB. The disease often segregates as an autosomal dominant trait manifesting genetic heterogeneity and incomplete penetrance (Haslam et al. 1998; Nance et al. 2000; Good et al. 2001; Hocking et al. 2001; Laurin et al. 2001; Leach et al. 2001). The disorder was initially linked to susceptibility loci at chromosomes 6p21.3 (*PDB1*) and 18q21-22 (*PDB2*) (Fotino et al. 1977; Tilyard et al. 1982; Cody et al. 1997; Haslam et al. 1998). *PDB2* also contains the gene responsible for familial ex-

pansile osteolysis (FEO [MIM174810]) (Hughes et al. 1994), a rare bone dysplasia with some similarities to PDB. However, the involvement of *PDB1* and *PDB2* may be restricted to only a few families, since linkage analyses excluded these two loci in the vast majority of the pagetic kindreds subsequently studied (Breannan Moore and Hoffman 1988; Hocking et al. 2000; Nance et al. 2000; Good et al. 2001; Laurin et al. 2001).

The primary cellular defect of PDB seems to reside in the osteoclasts. Within the pagetic lesion, osteoclasts are large, multinucleated, and overactive and contain paramyxovirus-like inclusions (Rebel et al. 1974; Mills and Singer 1976; Howatson and Fornasier 1982). Osteoclast formation and activation involve the receptor activator of NF- $\kappa$ B (RANK) pathway, a tumor-necrosis factor (TNF) family member (Hsu et al. 1999). Activating mutations of the *TNFRSF11A* gene encoding this receptor have been found to be responsible for FEO and rare cases of early-onset familial PDB (Hughes et al. 2000). Targeted disruption of several components of this signaling pathway in the mouse, including *RANK*, *RANK ligand*, *TRAF6*, and *NF- $\kappa$ B1/NF- $\kappa$ B2*, affects osteoclast differentiation and/or activity leading to osteopetrosis (reviewed by McLean and Olsen [2001]).

Exploiting a genomewide-scan approach and large French Canadian families, we recently mapped two new

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| Marker         | Family or individual |    |       |       |    |    |    |       |       |  |
|----------------|----------------------|----|-------|-------|----|----|----|-------|-------|--|
|                | Common (7 families)  | DD | UN096 | UN054 | PT | CT | GE | UN070 | UN010 |  |
| NL7            | 1                    | 1  | 1-2   | 3-3   | 11 | 3  | 8  | 3-11  | 3     |  |
| NL5            | 3                    | 4  | 3-8   | 1-6   | 9  | 6  | 3  | 3-9   | 6-4   |  |
| <i>D5S2073</i> | 12                   | 12 | 12-8  | 12-1  | 2  | 2  | 2  | 2-8   | 2-6   |  |
| SNP1(916)      | T                    | T  | T     | T     | C  | C  | C  | C-T   | C     |  |
| SNP2(976)      | A                    | A  | A     | A     | G  | G  | G  | G-A   | G     |  |
| 1215C→T        | T                    | T  | T     | T     | T  | T  | T  | T     | T     |  |
| NL9            | 2                    | 2  | 2-1   | 2-1   | 3  | 3  | 3  | 3     | 3     |  |
| NL27           | 10                   | 10 | 10-1  | 10-5  | 8  | 8  | 8  | 8-2   | 8-6   |  |
| NL25           | 7                    | 7  | 7     | 7-1   | 4  | 4  | 6  | 4-2   | 4-7   |  |
| <i>D5S408</i>  | 3                    | 3  | 2     | 2-4   | 2  | 2  | 4  | 2-8   | 2-10  |  |
| NL3            | 2                    | 2  | 2-7   | 2-5   | 2  | 2  | 5  | 2     | 1-7   |  |
| NL18           | 6                    | 6  | 8-4   | 8-2   | 2  | 2  | 11 | 2-8   | 8-4   |  |
| NL6            | 3                    | 3  | 3     | 3-5   | 3  | 3  | 3  | 3     | 3     |  |
| <i>D5S2006</i> | 1                    | 1  | 3-5   | 4-2   | 2  | 4  | 3  | 4-2   | 4-2   |  |

**Figure 1** Haplotype analysis in French Canadian families with PDB (common, DD, PT, CT, and GE families) and allele combinations of informative sporadic cases (UN096, UN054, UN070, and UN010). Markers are indicated on the left, with the 1215C→T mutation and two informative SNPs at position 916 and 976 in the coding region of *SQSTM1/p62*. The marker order is based on haplotype analysis of 24 families (379 individuals), allowing the lower level of recombination. Shared haplotypes are represented by vertical black bars (PDB3H1) or are boxed (PDB3H2). Because haplotypes of sporadic cases could not be determined, both alleles for heterozygotic markers are depicted.

PDB-causing genes, at 5q35-qter (*PDB3*) and 5q31 (*PDB4*) (Laurin et al. 2001). Using a different set of families, Hocking et al. also described a candidate locus at chromosome 5q35 (Hocking et al. 2001). We report here the confinement of the *PDB3* locus to ~300 kb and the identification of a recurrent mutation in *SQSTM1/p62*, a gene also involved in the NF-κB signaling pathway.

Blood samples were obtained from 479 family members, 112 sporadic patients, and 205 individuals from the general population (108 men and 97 women, mean age 62 years, residing within a 50-km radius of Quebec City, contacted at random, and almost all French Canadian). All affected individuals displayed the typical phenotype for adult-onset PDB, with characteristic radiographic and scintigraphic abnormalities. A detailed clinical description of the families and clinical procedures has been presented elsewhere (Laurin et al. 2001). A full set of pedigree drawings for families included in the present study is also available at the CHUL Research Center Web site.

We previously reported that one founder disease haplotype cosegregated with Paget disease in eight families showing linkage at *PDB3*. One ancestral recombination event in one of these families restricted the disease interval to within 6 cM. To further refine this interval, we developed eight new microsatellite markers, using the Human Genome Working Draft sequence, and performed saturation mapping. We searched for (CA)<sub>n</sub> and (TG)<sub>n</sub> repeats on sequences of BAC clones included within the contig

covering the 6-cM interval. Flanking primers were designed by use of Primer 3 software (Primer 3 Software Distribution Web site). The more-informative markers were retained. The sequences of these primers are available at the CHUL Research Center Web site. Purified PCR products were pooled and genotyped on a 377 ABI sequencer. Data were analyzed with GENOTYPER 2.5 (ABI). Haplotypes were constructed by use of SIMWALK 2.8 (Sobel and Lange 1996). This analysis strengthened the inference of a common founder haplotype in the eight families originally linked at *PDB3*. This haplotype was named PDB3H1 (fig. 1, alleles within black vertical bars). These new markers allowed us, by means of the DD family, to position the disease interval between NL5 and the telomere (fig. 1).

We then exploited a haplotype signature strategy to find ancestral haplotypes shared by affected individuals. The French Canadian population, which frequently shows founder effects, is particularly well suited for such a procedure (Scriver 2001). Genotyping of 112 unrelated patients revealed that 8 of them harbored part of the PDB3H1 haplotype for several contiguous markers. A second signature was also detected in 10 patients with sporadic PDB, and its counterpart haplotype was observed in 10 affected members of three additional kindreds. This haplotype was named “PDB3H2” (fig. 1, boxed alleles). In one of these three families, three of eight patients did not share any of the disease-related haplotypes, suggesting the presence either of a second

disease gene, located at a different *PDB* locus, or of nongenetic (environmental) phenocopies (see pedigree CT at the CHUL Research Center Web site). As is depicted in figure 1, four patients with sporadic PDB who carried short portions of either of the two haplotype signatures and the pedigree GE haplotype allowed us to confine the *PDB3* locus within an interval of ~300 kb, between markers NL5 and NL25.

We searched the Unigene and the LocusLink databases, from the National Center for Biotechnology Information (NCBI) server, for genes and ESTs mapping within the *PDB3* locus. Particular interest was put on those genes mapping within the reduced interval (fig. 2A, B, and C). Two of these were *sequestosome 1* (*SQSTM1/p62*), which mediates intracellular signaling through the IL-1/TNF pathways towards NF- $\kappa$ B (Sanz et al. 1999, 2000) and *MAPK9*, a potential component of the RANK signaling pathway. On the basis of the association between these pathways and osteoclast differentiation/activation, we thoroughly screened these two genes for mutations. We did not find any sequence variation within the *MAPK9* gene.

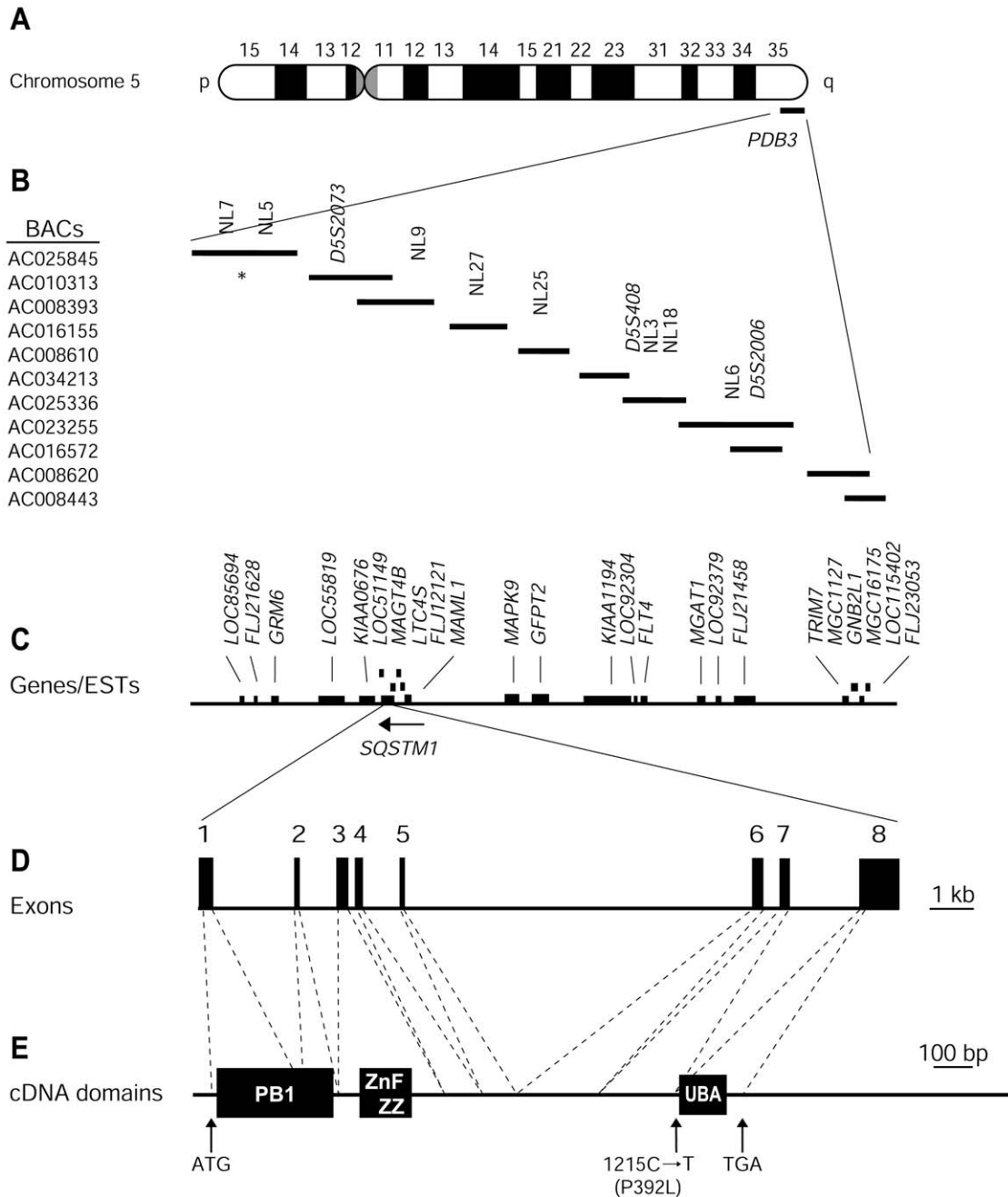
The 2,870-nt *SQSTM1/p62* transcript is contained within eight exons distributed over a 16-kb genomic segment (fig. 2D). Exon 1 encodes the methionine start codon at position 41, whereas exon 8 encompasses the stop codon at position 1363 and the entire 3' UTR (fig. 2D and 2E). We initially tested five affected members of the DD and TH families, who carried the PDB3H1 haplotype, as well as two unaffected individuals >54 years old that we investigated. One of these five symptomatic individuals, TH-143, was homozygous for PDB3H1 (Laurin et al. 2001). This homozygote was a member of a severely affected nucleus, and his phenotype was similar to that of his heterozygotic sibs. We designed primer pairs for amplification of the eight coding exons of *SQSTM1/p62* and at least 30 nt of each of the neighboring intronic sequences. Amplification products were purified and sequenced on a 3700 ABI sequencer using the Big Dye Deoxy Terminator Cycle Sequencing kit (ABI). Both strands were analyzed using STADEN package 1.1 (Staden 1996).

This screening revealed a C→T transversion at position 1215 in *SQSTM1/p62* exon 8 in all five affected individuals (fig. 3). This change caused the substitution of proline 392 to a leucine (P392L). Sequencing of exon 8 in the eight families sharing the PDB3H1 disease haplotype demonstrated that the P392L variant was present in all affected individuals carrying the disease haplotype and therefore cosegregated with PDB. Genomic sequence analysis of exon 8 in 86 nonaffected spouses and 205 individuals from the general population revealed no sequence variations at this position, indicating that this sequence change was unlikely to be a single-nucleotide polymorphism.

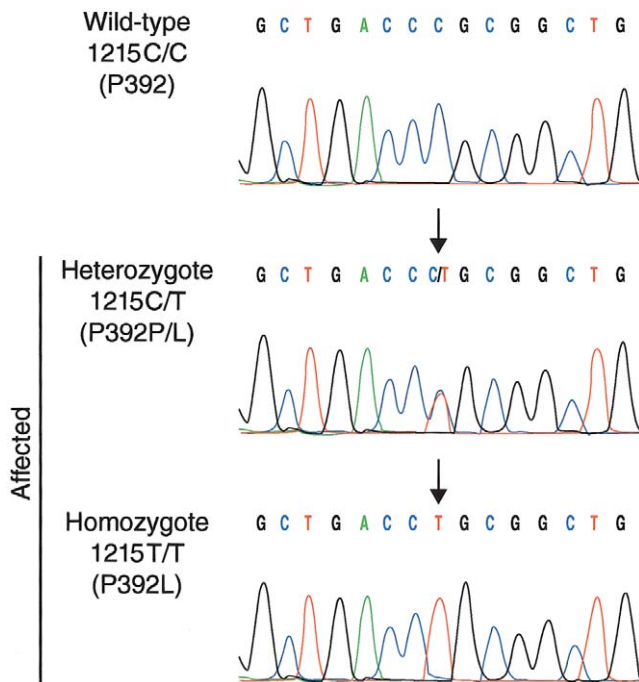
To further test this association between *SQSTM1/p62* and PDB, we screened for mutations among 112 sporadic cases and the remaining 16 families. The same 1215C→T substitution was detected in 10 affected individuals from three additional families and in 18 patients with sporadic PDB, all of them harboring one of the two haplotypic signatures described above. The *SQSTM1/p62*<sup>P392L</sup> mutation was thus found in 18 (16%) of the 112 sporadic cases and in 11 (46%) of the 24 families tested. We did not find additional disease-causing mutations. We did, however, identify five SNPs: 380C→T (A117V), in exon 3; 862G→C (Q274E), in exon 6; 916C→T (E292E), in exon 6; 976G→A (R312R), in exon 6; and 994C→A (S318S), in exon 6. Frequencies of these five SNPs were similar between affected and unaffected subjects. None of these SNPs had alleles associated with the mutation. However, as is depicted in fig. 1, two of these SNPs, 916C→T and 928G→A, showed distinct alleles cosegregating with the PDB3H1 and PDB3H2 disease haplotypes. These two SNPs were therefore used as proximal markers to further dissociate the two disease haplotypes. For instance, the PDB3H1 haplotype harbored a T and an A at positions 916 and 976, respectively, whereas the PDB3H2 signature displayed a C and a G at these two positions.

The 1215C→T transversion in exon 8 resulted in the substitution of a cyclic amino acid proline residue at position 392 for a leucine. This residue was conserved in the mouse and rat homologues, Osi (oxidative stress induced) (GenBank accession number NM\_011018) and Zip (PKC-zeta-interacting protein) (GenBank accession number Y08355), respectively. The P392 residue being located within the C-terminal end of the protein flanking the ubiquitin associated domain (UBA) (Hofmann and Bucher 1996; Schultz et al. 1998; Wilkinson et al. 2001) at position 394–440 could thus be of particular importance for the conformation and/or function of this region.

Cosegregation of the P392L variant with the phenotype, its absence in 86 spouses and 205 individuals from the general population, the nonconservative nature of the amino acid substitution, and the conservation of the UBA domain, including the P392 residue, among other species (mouse and rat) provided support for the idea that this amino acid change was causing PDB. Except for homozygote TH-143 (fig. 3), pagetic individuals carrying the P392L variant were all heterozygotes, confirming the dominant mode of action of this mutation in a high proportion of our French Canadian patients. As 13 of the 24 families investigated did not harbor any mutation in the *SQSTM1/p62* coding sequence, it is likely that other disease gene(s) are also associated with PDB, in agreement with the well-established genetic heterogeneity of the disorder (Haslam et al. 1998; Hocking et al. 2001; Laurin et al. 2001; Good et al. 2002). Indeed,



**Figure 2** Physical map of the *PDB3* locus at 5q35-qter and genomic organization of *SQSTM1/p62*. We retrieved a contig of candidate BACs, overlapping the *PDB* interval from the Human Genome Project Working Draft (Golden Path). We localized Généthon microsatellite markers (Dib et al. 1996), present within this interval, using an ePCR approach (Schuler 1998). Genotyping additional markers using the same approach confirmed order of the BAC clones. *A*, Chromosome 5. *B*, BAC contig of the *PDB3* linkage region and localization of microsatellite markers. Eight new markers were developed for this study; NL5 and NL25 defined the 300-kb *PDB* critical interval. The asterisk (\*) indicates the NL7–NL5 subcontig being in a different order relative to the Golden Path (August 2001 release). *C*, Genes present within the *PDB3* linkage region. *D*, Genomic structure of *SQSTM1/p62*. Exons are represented as boxes and intronic sequences as thin bars. *E*, cDNA and mutation localization. The segments encoding the Phox and Bem1p domain (PB1), zinc finger ZZ, and UBA domains (Schultz et al. 1998; SMART Web site) are shown by black boxes.



**Figure 3** *SQSTM1/p62* mutation P392L. Sequence electropherogram of a wild-type unaffected subject (*top panel*) and heterozygous or homozygous patients (*middle and lower panels*). Nucleotide and predicted amino acid changes are indicated on the left. The arrows point to the mutation. The nucleotide sequences of primers annealing to exon 8—flanking intronic DNA used for amplification and sequencing of exon 8 were as follows: forward, 5'-CACTGTGGCCTGTGAG-GAC-3', and reverse, 5'-CAGTGAGCCTTGGGTCTCG-3'. A full description of the primers used for amplification and sequencing is available at the CHUL Research Center Web site.

we recently mapped 2 of these 13 families to a fourth locus for the disorder, *PDB4*, at 5q31 (Laurin et al. 2001), and there is evidence for three other loci associated with the disorder: *PDB5* at 2q36, *PDB6* at 10p13, and a locus at 18q23 (Hocking et al. 2001; Good et al. 2002).

Haplotype analysis by means of intragenic SNPs was useful in determination of the origin of the mutation and the genetic relatedness of the patients carrying it. Indeed, we found that the P392L mutation, associated with two distinct haplotypes, probably originated from two independent events. Since two different intragenic SNPs haplotypes were identified, our data do not support a very ancient common founder for this mutation. This mutation, occurring at a hypermutable CpG dinucleotide sequence (Green et al. 1990; Koeberl et al. 1990), might have arisen by deamination of a methylated cytosine. For other disorders, several authors already suggested that such mutational hotspots may explain recurrent mutations (Glaser et al. 1999; Rizzo et al. 1999; Aksentijevich et al. 2001; Lund et al. 2001). Additional

mutational events may also be found in *SQSTM1/p62*, since one other study showed linkage between pagetic families of different ethnic origins and markers at *PDB3* (Hocking et al. 2001).

It has been proposed that *SQSTM1/p62* selectively interacts with TRAF6 and is thus an important intermediary in interleukin-1 (IL-1) and TNF $\alpha$  signaling toward NF- $\kappa$ B activation (Sanz et al. 1999, 2000). TRAF6 interacts with RANK, a member of the TNF receptor family. Its binding is essential for RANK-induced NF- $\kappa$ B activation (Darnay et al. 1999), and TRAF6-deficient mice exhibit severe osteopetrosis and are defective in osteoclast formation because of impaired signaling induced by RANK (Naito et al. 1999). The functional importance of *SQSTM1/p62* in NF- $\kappa$ B activation has been further highlighted by the observation that its depletion severely abrogated NF- $\kappa$ B activation through the TNF $\alpha$  or IL-1 pathways (Sanz et al. 1999, 2000). The precise role of *SQSTM1/p62* remains to be elucidated, but our data suggest that *SQSTM1/p62* may be involved in signaling pathways that control osteoclast activity, differentiation or survival. Further studies in both transgenic animals and cell-culture systems are required to understand the cell biology of this disease and the effect of the *SQSTM1/p62*<sup>P392L</sup> mutation.

The present study emphasized the importance of heredity in the pathogenesis of adult-onset PDB. Indeed, the *SQSTM1/p62*<sup>P392L</sup> mutation was causing, respectively, 16% and 46% of sporadic and familial cases of the disorder in the population tested. As two distinct disease-related haplotypes, encompassing two intragenic SNPs, shared the same *SQSTM1/p62*<sup>P392L</sup> mutation generated by two independent events, our data suggest that this recurrent variation may be frequently associated with PDB in other white populations.

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## Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

CHUL Research Center Web site, <http://www.crchul.ulaval.ca/public/articles/Laurin2002a.htm> (for pedigree drawings of families included in this study and for sequences of primers)

GenBank, <http://www.ncbi.nlm.nih.gov/Genbank/> (for BAC clone RP11-281O15 [accession number AC025845], CTB-77M18 [accession number AC010313], CTC-241N9, [accession number AC008393], CTD-2376K3, [accession number AC016155], RP11-101O23 [accession number AC008610], CTC-573N18 [accession number AC 034213], RP11-17A5 [accession number AC025255], CTB-22L19 [accession number AC016572], CTB-14A14 [accession number AC008620], and CTC-338M12 [accession number AC008443], for human *SQSTM1/p62* cDNA [accession number NM\_003900], and mouse homologue *Osi* [accession number NM\_011018], rat homologue *ZIP* [accession number Y08355])

G en thon, <http://www.genethon.fr> (for the reference genetic map)

Human Genome Project Working Draft at UCSC, <http://genome.ucsc.edu/> (August 2001 release)

NCBI Human Genome Resources, <http://www.ncbi.nlm.nih.gov/genome/guide/human/> (for annotated sequences and identification of candidate genes)

Online Mendelian Inheritance in Man (OMIM), <http://www.ncbi.nlm.nih.gov/Omim/> (for PDB [MIM 167250], *PDB1* [MIM 167250], *PDB2* [MIM 602080], *PDB3* [MIM 606262], *PDB4* [MIM 606263], FEO [MIM 174810], and *TNFRSF11A* [MIM 603499])

Primer 3 Software Distribution Web site, [http://www-genome.wi.mit.edu/genome\\_software/other/primer3.html](http://www-genome.wi.mit.edu/genome_software/other/primer3.html) (for Primer 3 software)

SMART, <http://smart.embl-heidelberg.de/> (for prediction of PB1, ZZ, and UBA domains)

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