Inclusion Body Myopathy - Paget Bone Disease - Frontotemporal Dementia Syndrome Caused by Mutated Valosin Containing Protein.

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Key Words:
Ubiquitination, Limb-girdle muscular dystrophy, Osteoclast, AAA ATPase, CDC48, p97

Inclusion body myopathy associated with Paget disease of bone and frontotemporal dementia (IBMPFD) is a dominant progressive disorder and maps to chromosome 9p21.1-p12. We investigated thirteen chromosome 9 linked families with IBMPFD using a candidate gene approach. Subsequently, six missense mutations within the Valosin Containing Protein (VCP) gene (a member of the AAA-ATPase superfamily) were found exclusively in all 61 affected individuals. Haplotype analysis indicated that descent from two founders in two separate American kindreds accounted for most patients. It is evident that mutations in the gene coding for VCP occur in all affected individuals in 13 families with the phenotype of IBMPFD. VCP is associated with a variety of cellular activities, including the control of cell cycle, membrane fusion, and the ubiquitin-proteasome degradation pathway. Identification of VCP as the gene causing IBMPFD has important implications for other inclusion body diseases including myopathies, dementias and Paget disease of bone, as it may define a new common pathological ubiquitin-based pathway.

Hereditary inclusion body myopathy (h-IBM) associated with Paget disease of bone (PDB) and frontotemporal dementia (FTD) - IBMPFD - is a rare, complex and ultimately lethal, autosomal dominant disorder (MIM 605382)[1]. IBMPFD features adult-onset proximal and distal muscle weakness (clinically resembling limb girdle muscular dystrophy), early-onset PDB in most cases, and “premature” FTD[2]. The disorder maps to chromosome 9p21-p12, but the genetic basis is previously unknown.

Within 13 families (12 from the United States and 1 from Canada, **Fig. 1** and **Supplementary Fig. 1**), 82% of patients had myopathy, 49% had PDB, and 30% had early-onset FTD. The mean age of presentation was 42 years for both IBM and PDB, whereas FTD typically presented at age 53 years. In IBMPFD myopathic muscle and Pagetic osteoclasts, inclusions appear similar - suggesting disruption within the same pathological pathway.

Haplotype analysis of the IBMPFD families identified two ancestral, disease-associated haplotypes, distinguishing families 1, 3, 7, and 16 (Group A) of English/American origin from families 2 and 5 (Group B) of German/English origin (**Table 1**). The predominant IBMPFD haplotype of Group A includes a core haplotype flanked by D9S1118 and D9S234 (6.47cM, 35.1Mb), probably transmitted from a shared ancestor (“founder”). However, it is unknown whether this individual emigrated to the United States or if the VCP mutation originated in the USA and then radiated.

Using a candidate gene approach, we first excluded *GNE* (UDP-N-acetylglucosamine 2-epimerase/N-acetylmannosamine kinase), which causes IBM2 or Nonaka myopathy and sialuria,[3,4] and then several additional genes[5]. Subsequently, we identified six missense mutations (**Table 1, Supplementary Fig. 2a-e**) within VCP (NM_007126) also called CDC48 or p97 (a member of the AAA-ATPase super family - *ATPase Associated with a variety of cellular Activities*)[6]. Families 1, 3, 4, 7, 10, 15 and 16 share a 464 G > A (R155H) change in exon 5, whereas family 11 also has an alteration at base 464 but involving a G > C (R155P) change. Families 2 and 5 have an alteration at the first base of the same codon 463 C > T (R155C). Family 6 has a transition mutation 695 C > A (A232E) in exon 6. Family 9 has a base change in exon 3 at 283 C > G (R95G), whereas family 13 features a change at base 572 that is G > C (R191Q) in exon 5. The group A haplotype shares the 464 G > A (R155H) mutation and Group B share the 463 C > T (R155C) mutation. In families 4, 10 and 15 (who have the same *VCP* mutation as Group A, but unique haplotypes), their mutations probably arose independently from Group A. Families 6, 9, 11 and 13 do not share haplotypes and their *VCP* mutations are unique, therefore arising independently. Nevertheless, 10 of the 13 IBMPFD families have an amino acid change at codon 155 in *VCP*, which seems therefore, to be a mutation “hot spot”.

Hence, identification of 6 distinctive cosegregating missense mutations within the gene encoding VCP identifies this as the genetic basis for *IBMPFD*.

Immunohistochemistry of normal muscle sections with a polyclonal anti-VCP antibody showed staining of endomysial vessels, lipofuscin accumulations and to a mild degree, muscle fiber cytoplasm within muscle fibers (**Fig. 2a**). However, in sections of sporadic inclusion body myositis (s-IBM) muscle showed VCP staining localized to debris within inclusions and vacuoles (**Fig. 2b***)*. Interestingly, there was significant staining of VCP within inflammatory cells at the focal invasion sites of muscle fibers (**Fig. 2c**). VCP is up regulated in regenerating muscle fibers of s-IBM (**Fig. 2d**). In IBMPFD, VCP was localized in large or small rounded aggregates in scattered muscle fibers (**Fig. 2e, f**), including those with no clear vacuoles or other morphological changes. Thus, VCP is commonly present in aggregates from IBMPFD and s-IBM muscle, although the predominant pattern of localization for VCP differs between IBMPFD and s-IBM.

VCP mutations in families 1-7, 9-11, 13, 15, and 16 cluster in the N-terminal CDC48 domain (**Fig. 3a**), which is involved in ubiquitin binding[7,8]. This highly structured N-domain forms two distinct regions[9]. VCP is highly conserved among species and the amino acid residues mutated in IBMPFD are conserved in the higher mammals (**Fig. 3b**). VCP forms a homohexamer where the D1/D2 domains bind in a head-to-tail ring[9] allowing the N-terminal domain to undergo conformational changes without affecting the stability of the homohexamer ring structure. VCP missense mutations causing IBMPFD disrupt the double ψ barrel (R95G: family 9), the four-stranded β barrel (R155C/H/P: families 1-5,7,10,11,15), or the flexible linker (R191Q: family 13). Hence, the affected ubiquitin-binding domain may impair N-terminal domain binding of specific partner proteins. The family 6 mutation (A232E), within the α5 helix of the a/b sub-domain of the first AAA-ATPase domain (D1), is potentially more deleterious because the D1 domain provides the protein's main catalytic activity essential for hexamer formation[10]. In fact, affected individuals in family 6 have fractures and PDB at an earlier age and the myopathy seems especially aggressive.

VCP has been associated with several distinct and crucial cell protein pathways[11]; namely cell cycle, homotypic membrane fusion, nuclear envelope reconstruction, postmitotic Golgi reassembly, DNA damage response, suppressor of apoptosis, and ubiquitin-dependent protein degradation[12-18]. VCP also binds to expanded poly-glutamine (poly-Q) protein aggregates[18,19]. The poly-Q binding domain of human VCP maps to amino acid residues 142-200, which encompasses a region of the N-domain and linker (N domain to D1) that contains two of the mutations we identified[18]. A *Drosophila* VCP (ter94) loss-of-function mutant has been identified as a dominant suppressor of expanded poly-Q induced neuronal degeneration[20]. The suppressive effects of the loss-of-function mutant did not appear to result from inhibition of poly-Q aggregate formation, but from the degree of VCP loss-of-function. This suggests that a gene dosage response for VCP expression is crucial to its function in expanded poly-glutamine (poly-Q) induced neuronal degeneration. To further support this, in transgenic *Drosophila*, where VCP levels were elevated, severe apoptotic cell death was induced, whereas homozygous VCP loss-of-function mutants were embryonic lethal[20].

VCP is an essential gene important for the cell cycle and apoptosis pathways, neither of which appear to be disrupted in IBMPFD, since affected individuals are obviously viable. Clues concerning the nature of the mutations we identified in VCP can be drawn from pathways that have been implicated in other aggresome-associated degenerative disorders, which all involve protein quality control and the ubiquitin protein degradation pathways[21-24]. There are a number of independent studies supporting the fact that disruption of a specific function of VCP leads to inclusion body formation: 1) Experiments identifying the involvement of VCP in ERAD have shown that dysfunction of VCP causes vacuole and inclusion body formation, ultimately leading

to cell death[18,19,25]. 2) VCP has been found to interact directly with polyubiquitinated proteins[18-20]. 3) VCP has been identified as co-localizing with ubiquitin-containing nuclear inclusions in the cerebral cortex from a number of neuronal degenerative disorders involving protein quality control and the ubiquitin protein degradation pathways, such as Huntington, Alzheimer, Creutzfeldt-Jakob, and Parkinson disease (in particular the Lewy bodies) as well as motor neuron disease with dementia[26]. 4) Interestingly, mutations clustering in the ubiquitin-binding domain of sequestosome 1 (*SQSTM1, p62)*[27,28] cause autosomal dominant Paget disease of the bone (PDB3). We propose that mutations in *VCP,* like *SQSTM1,* cause PDB by compromising ubiquitin-binding and are targeting similar cellular pathways or proteins. Furthermore, p62 has been shown to co-localize with inclusion bodies in a number of degenerative disorders[22,23]. Thus, it seems likely that IBMPFD is a new member of the aggresome-associated disorders, and that mutations in VCP have identified a new link in the pathway that leads to aggresome formation. Since IBMPFD is a dominant progressive syndrome it is likely that the mutations we have identified are relatively subtle and that aging, oxidative stress and ER stress define a threshold whereby the IBMPFD phenotype is subsequently manifest. Rather than the mutations disrupting a normal function of VCP, they could add new toxic gain-of-functions that results in new VCP actions. Alternatively, the mutation could be a dominant negative that disrupts normal hexamer formation of the VCP protein.

In thirteen IBMPFD families only four amino acid residues (three in the N-terminal domain and one in the D1 domain) are mutated in VCP, suggesting either a mutation hot-spot in the N-domain or that VCP has such tight operational constraints that other types of mutation elsewhere are lethal. Indeed, homozygous loss-of-function mutants in *Drosophila* were embryonic lethal[20] and could explain the lack of a knockout mouse model for VCP. Our current findings identify VCP as having a new and crucial role in aggresome disease, particularly in the seemingly unrelated tissues affected in IBMPFD, and indicate that VCP has more specialized functions remaining to be characterized.

PATIENTS AND METHODS

Clinical Evaluations

Written consent from each subject was approved by the Springfield, IL Committee for Research Involving Human Subjects, and by Children's Hospital, Boston, MA. Volunteers were all over age 18 years because IBMPFD manifests in adults. IBMPFD muscle phenotype is of variable severity, and mild asymmetry characterize the muscle weakness of IBMPFD[1,2]. Myopathic features include variation in muscle fiber size, mildly increased endomysial connective tissue, and large focal regions of "myopathic grouping" commonly seen in h-IBM2. Rimmed vacuolar inclusions were noted in approximately 35% of muscle biopsy specimens analyzed in the 13 families. Electron microscopy (EM) from IBMPFD biopsies showed atrophic and vacuolated muscle fibers containing abundant nuclear and cytoplasmic, paired helical filaments (PHF) with congophilia, accumulations of phosphorylated *tau*, apolipoprotein E (ApoE), and excessive β-amyloid precursor protein epitopes[1,2].

Similarly, EM of PDB osteoclasts in 4 affected individuals from IBMPFD family 11 identified seemingly characteristic nuclear and cytoplasmic PHF inclusions[29]

Chromosomal Mapping

Peripheral blood DNA was extracted using the PureGene DNA isolation kit (Gentra Systems, Inc., Minneapolis, MN). IBMPFD linkage to chromosome 9p21.1-p12 was known in 4 families,[2] and confirmed in the 9 new kindreds. The disease haplotype was constructed for each family to identify the critical locus.[2]

Assessments of Candidate Genes

A candidate approach involved genes prioritized and selected by their expression patterns and putative functions. Sequences provided by the Human Genome Project were identified containing Genethon markers that mapped to the disease region. Each sequence was then assessed using the National Center for Biotechnology Information BLAST search program (http://www.ncbi.nlm.nih.gov/BLAST/) to determine the exon/intron structures of candidate genes – including the gene encoding the Valosin Containing Protein (VCP) (MIM #601023).

Mutation Analysis of the VCP Gene

Mutation analysis of the VCP gene (NM_007126) initially involved two affected individuals from each of the 13 families. Non-affected individuals and unrelated relatives served as controls. PCR primers for genomic DNA were designed to include at least 50bp of intron sequence, from either side of the exon, for all 17 exons. Sequences longer than 1000 bp were divided into multiple, overlapping segments for amplification. PCR products were gel purified using the Gel Extraction Kit (Qiagen, Valencia, CA), and sequenced with an ABI 377 sequencer, using a dRhodamine terminator cycle sequencing kit (Applied BioSystems Inc., Foster City, CA). Sequence and trace file comparisons were carried out using Lasergene 99 software (DNAStar Inc., Madison, WI). We then screened > 180 additional control chromosomes by denaturing high-performance liquid chromatography (dHPLC) (Transgenomic, Inc. Omaha, NE) and by restriction digests (see below) for any base changes in exons 3, 5 and 6, where mutations were identified, to rule out the possibility that the base changes were common polymorphisms. No base changes were detected in these control chromosomes.

Cosegregation Studies

Restriction site mapping from PCR amplified exons was utilized to confirm that the mutation cosegregated with disease in the families containing mutation 695 C > A (family 6) which destroys an *Mfe*I site, 283 C > G (family 9) destroys an *RSA*I restriction site and 464 C > T (family 11) which creates a *BSSK*I restriction site. Cosegregation for mutations 463 C > T, 464 G > A, and 572 G > C was confirmed using dHPLC in a blinded study of 79 individuals from 7 families.

Immunohistochemistry

To determine the presence of VCP in postmortem sections from normal, IBM and IBMPFD muscle sections were subjected to immunohistochemistry with anti-VCP polyclonal antibody. Immunohistochemistry was performed as described previously[2]. The immune reactivity was detected by light microscopy using horseradish peroxidase.

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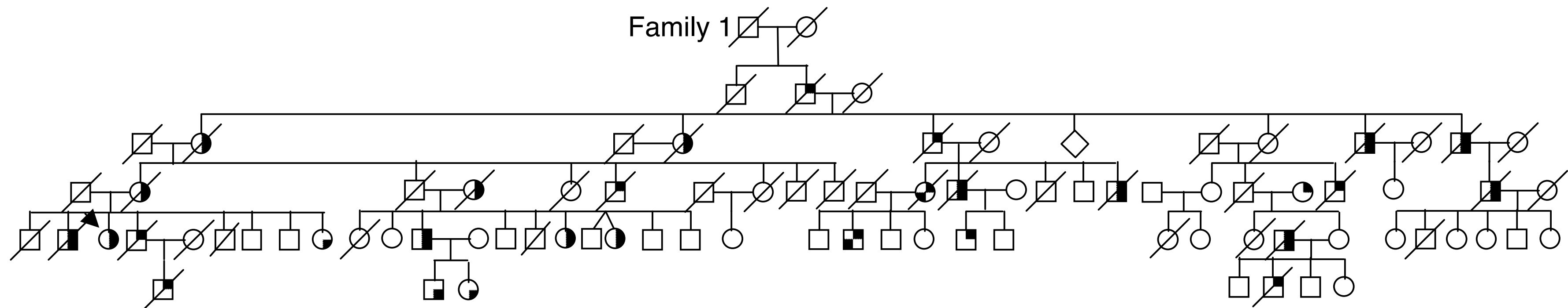
Table 1. Haplotypes for the IBMPFD locus on chromosome 9p21-q21.11.

	Inter Marker Distances													
cM	1.08				0.53				0.72	0.79	2.27	1.07		Σ 6.47cM
Kb	398.1	849.6	448.1	575.1	865.4	647.5	833.8	432.7	409.3	419.6	1073.5	28163.5		Σ 35.1Mb
Family	D9S1118	D9S304	D9S165	D9S1878	D9S1805	VCP*	D9S163	D9S1804	D9S1791	D9S50	D9S1874	D9S2148	D9S234	
1	14	13	12	9	10	464 G>A (5) R155H	1	4	13	15	18	17	10	**Group A**
3	14	13	12	9	10	464 G>A (5) R155H	1	4	13	15	18	17	10	
7	14	13	12	9	10	464 G>A (5) R155H	1	4	13	15	18	17	12	
16	14	13	12	9	10	464 G>A (5) R155H	1	4	13	15	18	17	12	
2	14	5	10	17	10	463 C>T (5) R155C	4	4	7	13	21	7/23	12	**Group B**
5	20	5	10	17	10	463 C>T (5) R155C	4	4	9	13	16	9	–	
4	14	17	13	5	9	464 G>A (5) R155H	4	9	11	13	22	7	6	**Unique Haplotype**
6	–	13	11	18	7	695 C>A (6) A232E	3	9	8	21	4	7	–	
9	14	3	12	9	12	283 C>G (3) R95G	5	4	10	13	18	7	10	
10	–	–	–	–	–	464 G>A (5) R155H	–	–	–	–	–	–	–	
11	20	9	12	9	7	464 G>C (5) R155P	4	4	8	14	21	13	18	
13	20	5	10	9	10	572 G>A (5) R191Q	4	4	7	13	21	9	16	
15	–	16	9	16	5	464 G>A (5) R155H	14	2	12	12	9	15	–	

The Group A haplotype was 14-13-12-9-10-1-4-13-15-18-17 for markers D9S1118, D9S304, D9S165, D9S1878, D9S1805, D9S163, D9S1804, D9S1791, D9S50, D9S1874 and D9S2148, respectively. The Group B haplotype contained the 5-10-17-10-4-4 core haplotype at markers D9S304, D9S165, D9S1878, D9S1805, D9S163 and D9S1804, respectively. The minimal shared haplotype (D9S304 to D9S1804) represented a physical distance of approximately 3.6 Mb. Note family 10 is too small for haplotype analysis to determine the disease haplotype, but does not share a haplotype with other families. Families are assigned an arbitrary number and therefore are not sequential. *Base numbers relative to ATG start.

Figure 1. Pedigrees of Thirteen Families with Inclusion Body Myopathy Associated with Paget Disease of the Bone and Frontotemporal Dementia.



Squares indicate male family members, and circles female family members. Arrows indicate probands, and symbols with a slash indicate deceased family members.

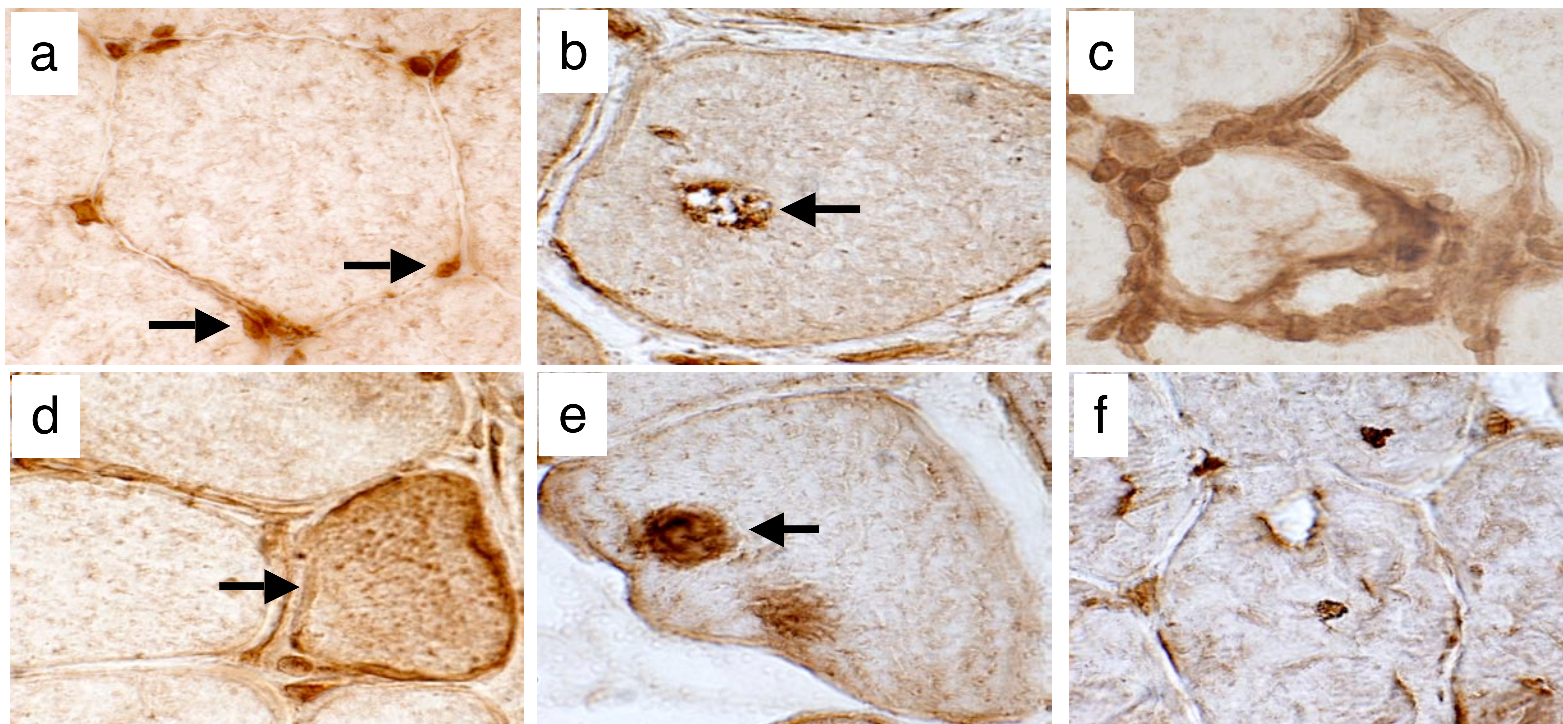
represents inclusion body myopathy.

represents Paget disease of the bone.

represents frontotemporal dementia.

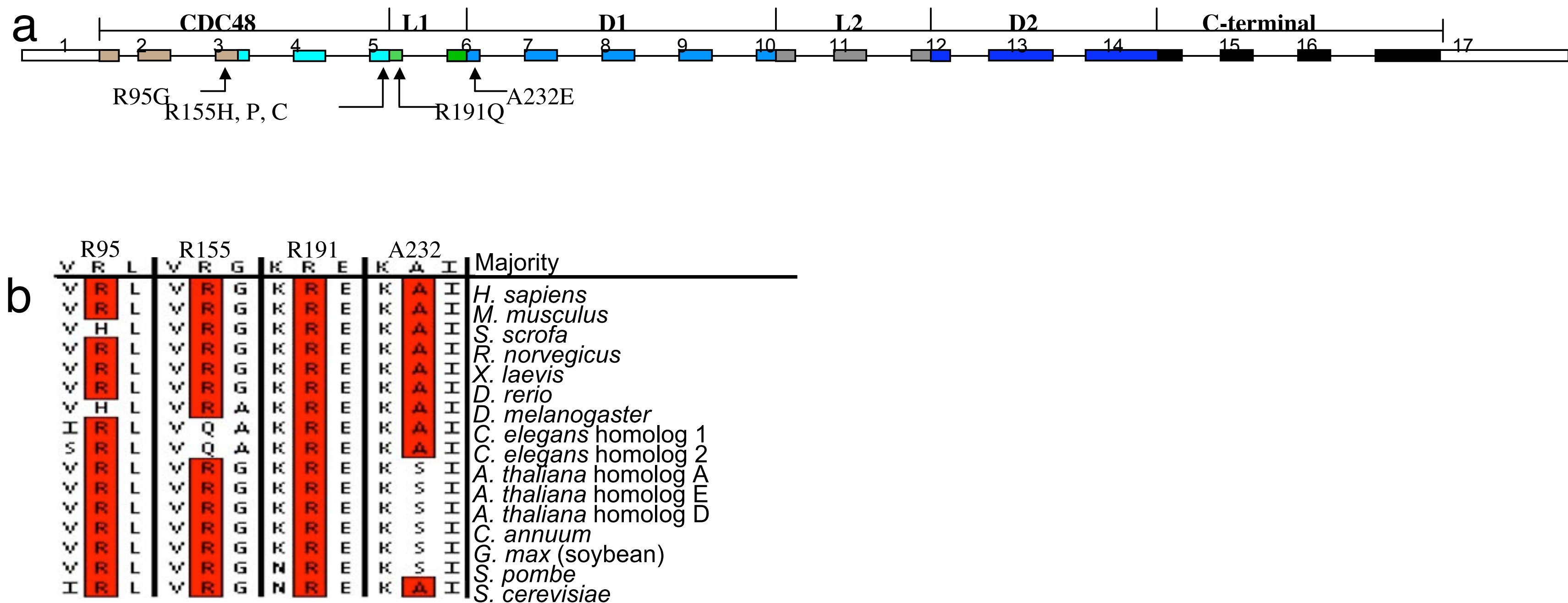
Only clinically diagnosed family members are shown without ages due to confidentiality issues.

Figure 2. Staining of normal and diseased human muscle with polyclonal anti-VCP antibody



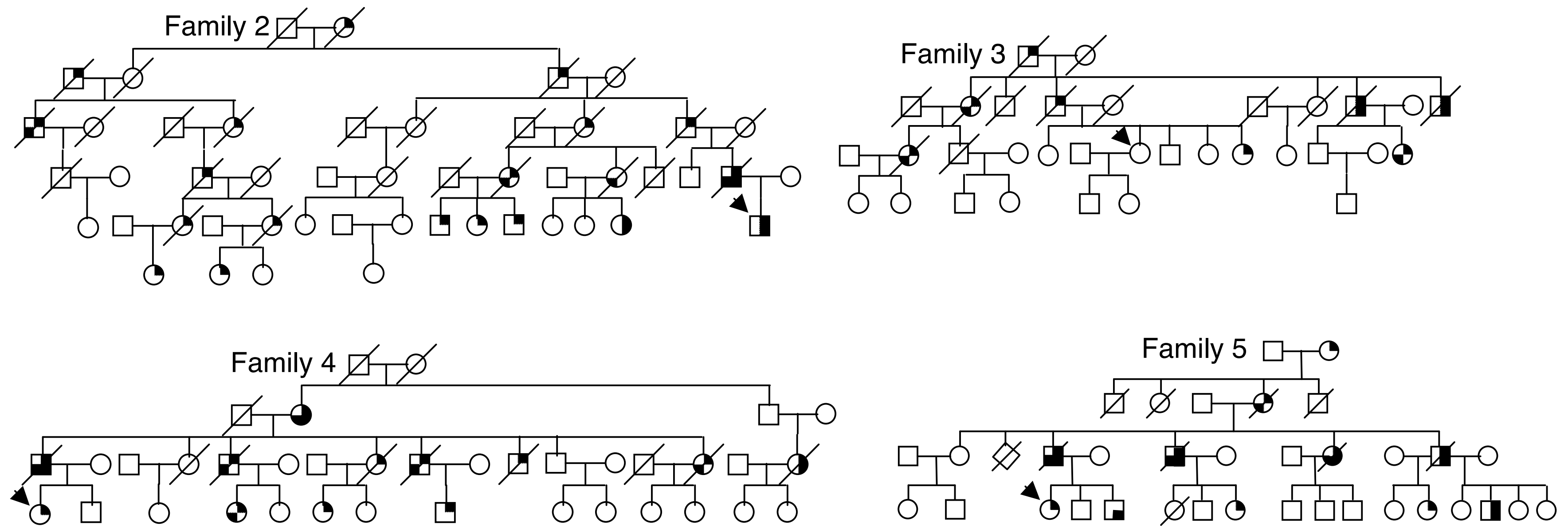
(**a**) Normal muscle. VCP is prominently located in small endomysial capillaries (right arrow). In muscle fibers VCP accumulates with lipofuscin granules (left arrow) at the periphery and more diffusely, at low levels, in the cytoplasm. (**b**) Sporadic inclusion body myositis (s-IBM): VCP is present in material in a vacuole (arrow) and in small accumulations in the muscle fiber cytoplasm. (**c**) s-IBM: VCP is strongly stained in endomysial inflammatory cells surrounding a muscle fiber. (**d**) s-IBM: VCP is up regulated in regenerating muscle fibers. (**e**) IBMPFD: Large focal inclusion (arrow) within muscle fiber contains VCP. (**f**) IBMPFD: Multiple small foci are present within a muscle fiber. (Magnification x540)

Figure 3. Mutations in the Valosin Containing Gene (*VCP*) in Patients with Inclusion Body Myopathy Associated with Paget Disease of the Bone and Frontotemporal Dementia.



(**a**) Functional domains and mutations of the VCP gene. Arrows indicate the places where mutants occur relative to the exon-intron structure, where the exons are numbered 1 to 17. The relative position of N-domain (CDC48, grey and cyan), flexible linker (L1, green), first AAA ATPase domain (D1, blue), linker region (L2, dark grey), second AAA ATPase domain (D2, dark blue) and C-domain (C-terminal, black) are indicated, whereas the 5' and 3' UTRs are represented in white. (**b**) Species conservation of amino acid residues mutated in IBMPFD, where the red highlight indicates identical residues.

Supplementary Figure 1. Pedigrees of Thirteen Families with Inclusion Body Myopathy Associated with Paget Disease of the Bone and Frontotemporal Dementia.



Squares indicate male family members, and circles female family members. Arrows indicate probands, and symbols with a slash indicate deceased family members.

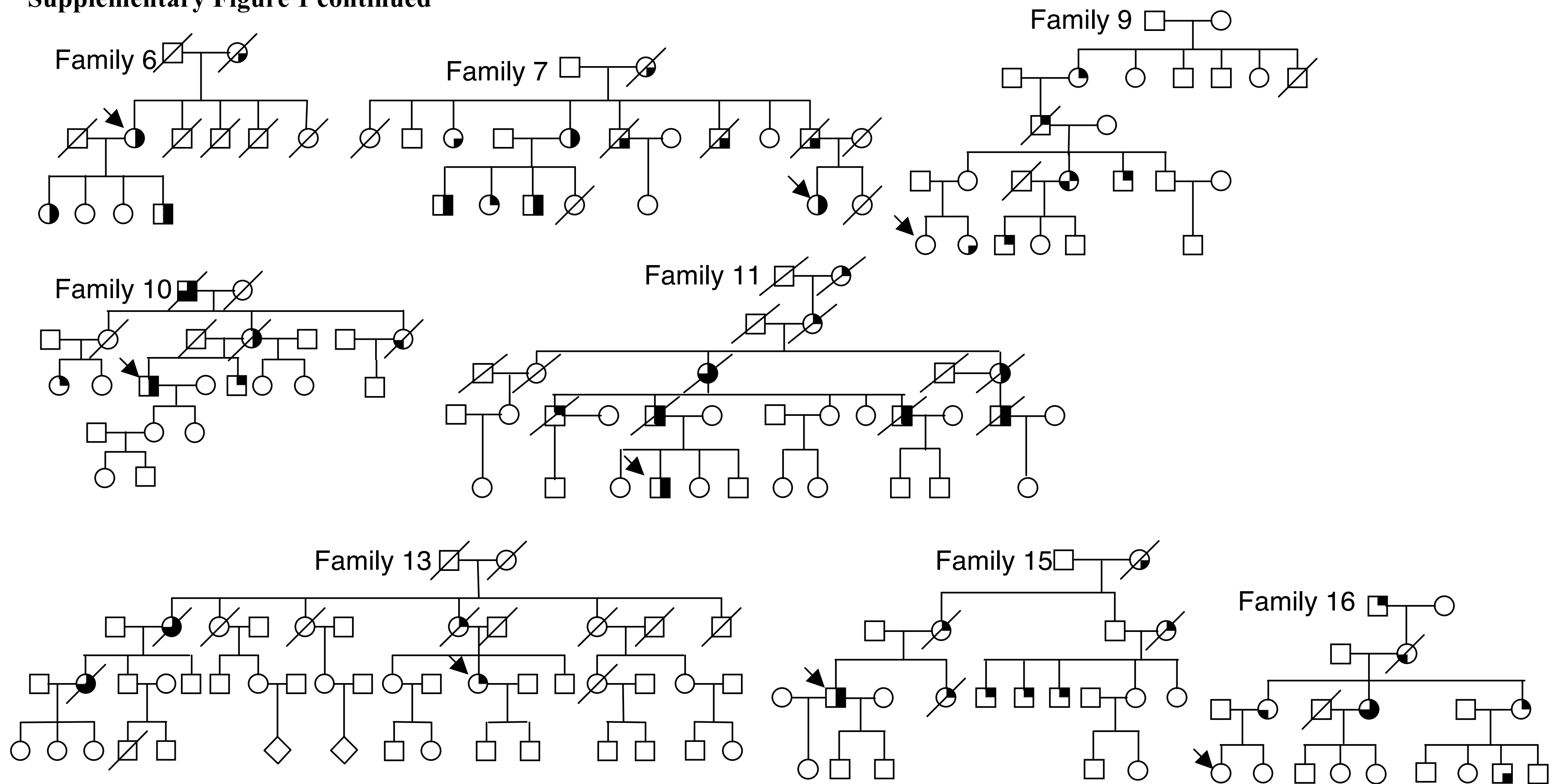
◳ ◔ represents inclusion body myopathy.

◲ ◒ represents Paget disease of the bone.

◱ ◓ represents frontotemporal dementia.

Only clinically diagnosed family members are shown without ages due to confidentiality issues.

Supplementary Figure 1 continued



Squares indicate male family members, and circles female family members. Arrows indicate probands, and symbols with a slash indicate deceased family members.

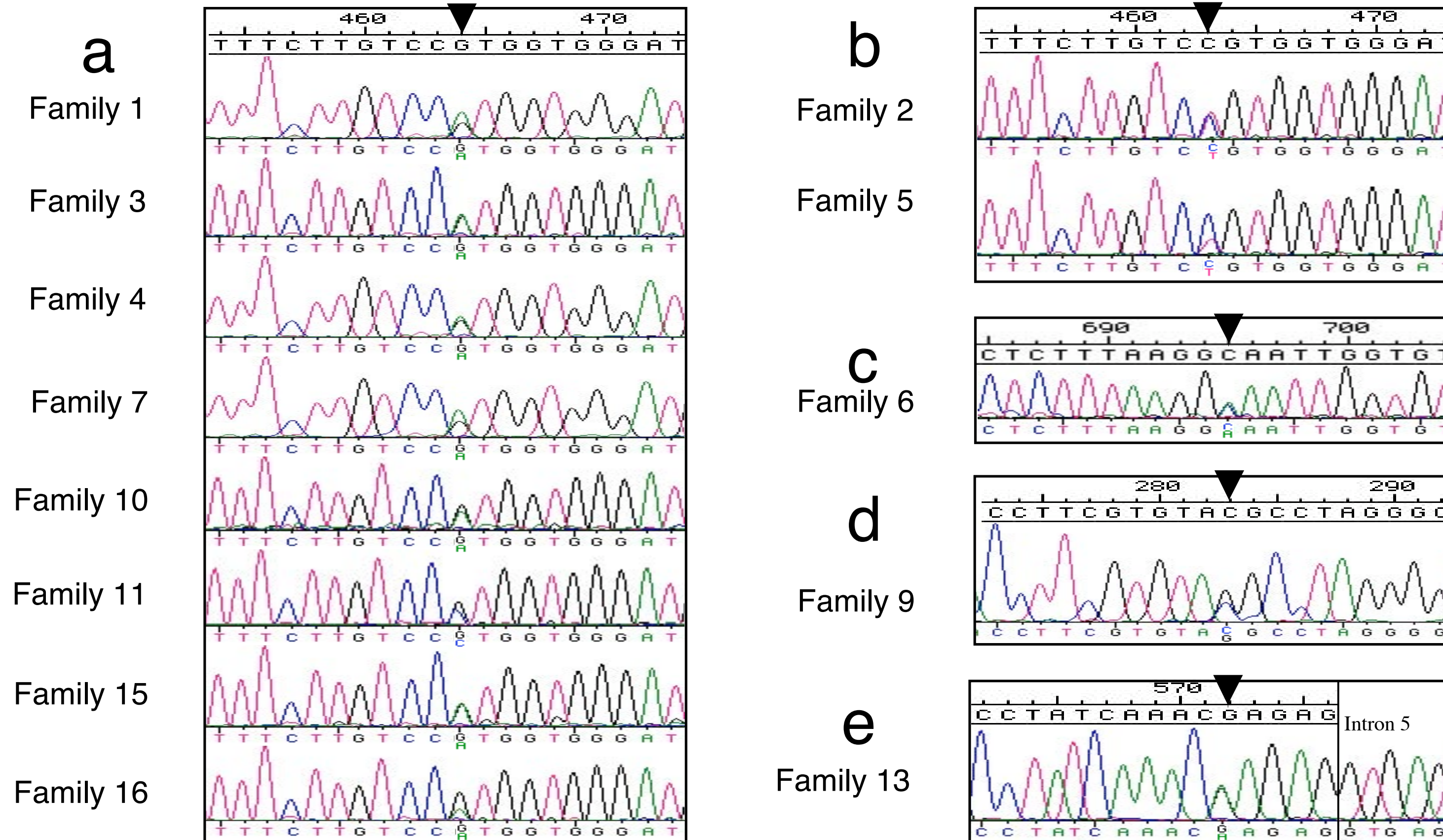
◰ ◴ represents inclusion body myopathy.

◲ ◶ represents Paget disease of the bone.

◱ ◵ represents frontotemporal dementia.

Only clinically diagnosed family members are shown without ages due to confidentiality issues.

Supplementary Figure 2. Mutations in the Valosin Containing Gene (*VCP*) in Patients with Inclusion Body Myopathy Associated with Paget Disease of the Bone and Frontotemporal Dementia.



Sequencing chromatograms of genomic DNA from patients are shown. Since IBMPFD is dominant, all chromatograms show two overlapping peaks at the same locus (arrow) denoting heterozygous mutations (**a** to **e**). None of these mutations were detected in > 90 control DNA samples (180 alleles). Further, from the 13 IBMPFD families, 61 affected and 62 unaffected individuals, respectively, were tested for the *VCP* mutations. Mutations were found in all of the affected but none of the unaffected individuals and demonstrated 100% cosegregation with the disease phenotype.