ABSTRACT: Inclusion-body myopathy with Paget's disease and frontotemporal dementia (IBMPFD) is a disease of muscle, bone, and brain that results from mutations in the gene encoding valosin-containing protein (VCP). The mechanism of disease resulting from *VCP* mutations is unknown. Previous studies of VCP localization in normal human muscle samples have found a capillary and perinuclear distribution, but not a nuclear localization. Here we demonstrate that VCP is present in both myonuclei and endothelial cell nuclei in normal human muscle tissue. The immunodetection of VCP varies with acetone or paraformaldehyde fixation. Within the nucleus, VCP associates with the nucleolar protein fibrillarin and Werner syndrome protein (Wrnp) in normal and IBMPFD muscle. In patients with inclusion-body myositis (IBM), normal nuclear localization is present and some rimmed vacuoles are lined with VCP. These findings suggest that impairment in the nuclear function of VCP might contribute to the muscle pathology occurring in IBMPFD.

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NUCLEAR LOCALIZATION OF VALOSIN-CONTAINING PROTEIN IN NORMAL MUSCLE AND MUSCLE AFFECTED BY INCLUSION-BODY MYOSITIS

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Mutations in the gene encoding valosin-containing protein (VCP) are associated with the clinical syndrome of inclusion-body myopathy with Paget's disease and frontotemporal dementia (IBMPFD).[18] VCP has a range of reported functions, including the assembly of nuclear membranes,[10,15] but the specific mechanism that results in tissue pathology in IBMPFD is unknown. The location of VCP has been studied in several mammalian cell culture lines, where it is present in the cytoplasm and nucleus.[17] VCP in normal human muscle tissue has previously been reported as present in small capillaries around muscle fibers[9,18] and near muscle myonuclei,[9] but not within nuclei despite the presence of a nuclear localization signal in its N-terminus.[17] Here we demonstrate a nuclear localization of VCP in normal human muscle tissue, inclusion-body myositis (IBM), and muscle tissue with the most common IBMPFD-associated VCP mutation. Within myonuclei, VCP associates with fibrillarin and Werner syndrome protein.

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Abbreviations: ABR, Affinity BioReagents; CD31, cluster of differentiation 31; DAB, 3,3′-diaminobenzidine tetrahydrochloride; DAPI, 4′6-diamidino-2-phenylindole; IBM, inclusion-body myositis; IBMPFD, inclusion-body myopathy with Paget's disease and frontotemporal dementia; PFA, paraformaldehyde; TDP-43, TAR DNA binding protein 43; VCP, valosin-containing protein; Wrnp, Werner syndrome protein

Key words: IBM; IBMPFD; inclusion-body myopathy with Paget's disease and frontotemporal dementia; inclusion-body myositis; muscle nucleus; valosin-containing protein; Werner syndrome protein

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MATERIALS AND METHODS

Muscle Tissue. Muscle biopsy samples were obtained from five subjects with no evidence of a neuromuscular disease, by clinical evaluation, laboratory studies, and muscle histopathology, and classified as normal. Muscle biopsy samples were also obtained from four subjects with definite IBM (according to European Neuromuscular Centre criteria[1]) and from three subjects with IBMPFD with the most common R155H mutation who were

undergoing diagnostic muscle biopsies. Our Institutional Review Board approved these studies.

Immunohistochemistry and Microscopy. Muscle samples were cryostat-sectioned at 10 μm at –23°C and placed on glass slides. Slides were dipped into acetone at room temperature and then frozen at –20°C for subsequent studies. Primary antibodies used were a monoclonal anti-VCP (cat. no. MA3-004, clone 5, isotype IgG2a; ABR-Affinity BioReagents, Golden, Colorado) and a rabbit polyclonal anti-VCP (kind gift of Dr Chou-Chi Li, National Cancer Institute, Frederick, Maryland). Cryostat sections were fixed in either cold (5°C) 4% paraformaldehyde (PFA) for 5 min and then soaked consecutively in cold (5°C) 0.05 M Tris buffer, pH 7.6, and room temperature Tris buffer, or were fixed in cold acetone ($-10° \pm 5°$C) for 5 min and soaked in Tris buffer at room temperature. All tissue sections were then moved to Tris buffer supplemented with 4% porcine serum for immunohistochemistry.

Multiple sections were studied to determine optimal fixation methods, antibody concentrations, primary antibody incubation duration, and secondary antibody conditions. Sections (PFA fixed) were incubated for 1 h with monoclonal anti-VCP (ABR, 1:50,000 dilution) and subsequently incubated for 30 min with horseradish peroxidase–labeled polymer conjugated to goat antimouse immunoglobulins (ImmunoVision Technologies, Daly City, California). Polyclonal anti-VCP (1:5,000) was incubated with PFA-fixed sections for 2 h. Sections were then incubated consecutively for 30 min each with swine antirabbit immunoglobulins (1:80 dilution; DakoCytomation, Carpinteria, California) and peroxidase-rabbit anti-peroxidase complex (PAP, 1:100 dilution, DakoCytomation), or for 30 min with horseradish peroxidase–labeled polymer conjugated to goat antirabbit immunoglobulins (ImmunoVision Technologies). Antibody localization was effected by using a peroxidase reaction with 3,3′-diaminobenzidine tetrahydrochloride (DAB) as the chromogen. Sections were counterstained with methyl green.

For comparison of PFA-fixed and acetone-fixed sections, the polyclonal anti-VCP (1:2,000 dilution) incubations were carried out for 70 min. This was followed by consecutive 40-min incubations with biotin-labeled swine antirabbit antibodies and horseradish peroxidase–conjugated streptavidin (LSAB+ System; DakoCytomation). Antibody localization utilized DAB and sections were counterstained with methyl green.

Control studies were performed substituting IgG2a isotype control antibody for the ABR antibody at the same immunoglobulin concentration, while normal rabbit serum served as control for the polyclonal antibody, and Tris buffer served as control for endogenous peroxidase.

Acetone-fixed and PFA-fixed cryostat sections were employed for the detection of Werner syndrome protein (Wrnp). Incubations were carried out with rabbit polyclonal anti-Wrnp (Abcam, Cambridge, Massachusetts) at various dilutions (1:100 to 1:10,000) and incubation times (1.5–15 h) followed by horseradish peroxidase–labeled polymer conjugated to goat antirabbit immunoglobulins (30-min or 1-h incubation; ImmunoVision Technologies). Staining (DAB, methyl green counterstain) was detected over a range of conditions.

Immunofluorescence studies were carried out with polyclonal anti-VCP (1:5,000 dilution, 1-h or 2-h incubation), and monoclonal antibodies to dystrophin (clone Dy8/6C5, 1:50 dilution, 1-h incubation; Novocastra/Vision Biosystems, Norwell, Massachusetts), CD31 (clone JC/70A, 1:25 dilution, 1-h incubation; DakoCytomation), and fibrillarin (clone mAbcam 18380, 1:50 dilution, 1-h incubation; Abcam). Secondary antibodies were Alexa Fluor 488–labeled goat antirabbit immunoglobulins (1:400 dilution, 1-h incubation) and AlexaFluor 555–labeled goat antimouse immunoglobulins (1:400 dilution, 1-h incubation; Molecular Probes/Invitrogen, Carlsbad, California), respectively. Subsequently, nuclei were stained with DAPI [1 μg/ml in phosphate-buffered saline (PBS)] for 1 min. For dual staining the monoclonal and polyclonal antibody pairs could be incubated sequentially or in admixture (each at their correct dilution) followed by incubation with the secondary antibodies sequentially or in admixture (each at 1:400 dilution).

In a similar fashion, immunofluorescence studies were carried out with monoclonal anti-VCP (1:30,000 dilution, 2-h incubation) and polyclonal anti-fibrillarin (1:100 dilution, 1-h incubation; Abcam) or 2 h when in admixture with monoclonal anti-VCP. Secondary antibodies were AlexaFluor 488–labeled goat antimouse immunoglobulins (1:400 dilution, 1-h incubation) and AlexaFluor 555 goat antirabbit immunoglobulins (1:400 dilution, 1-h incubation) alone or in admixture. Also, studies were carried out with a mix of monoclonal anti-VCP (1:30,000 dilution) and rabbit polyclonal

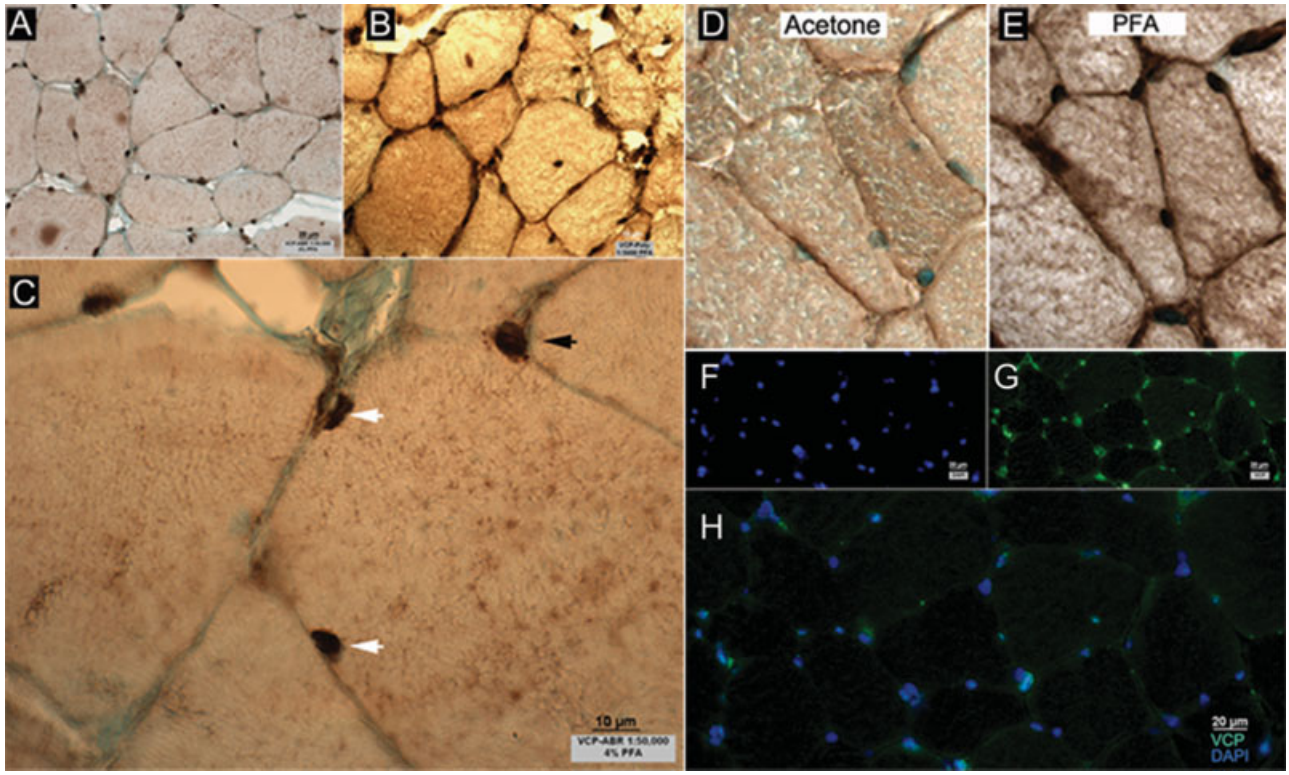

FIGURE 1. Nuclear localization of valosin-containing protein (VCP) in normal muscle. **(A–C)** Light immunohistochemistry. Structures stained at the subsarcolemmal border have the typical shape and location of myonuclei. **(A)** Monoclonal anti-VCP and **(B)** polyclonal anti-VCP staining of nuclear-like structures. The polyclonal anti-VCP antibody additionally stains the spaces between myofibers. **(C)** Higher magnification of monoclonal anti-VCP; white arrows highlight elongated subsarcolemmal structures characteristic of myonuclei and the black arrow highlights a location more typical of capillaries. **(D,E)** Nuclear staining with polyclonal anti-VCP is less apparent when tissue is fixed in acetone than with paraformaldehyde. **(D)** Acetone fixation. Nuclei show methyl green counterstaining but no VCP immunoreactivity. **(E)** Paraformaldehyde fixation. Adjacent 10-μm sections of the region corresponding to **D** of normal muscle show prominent nuclear staining. Other than fixation, identical conditions were used for each section, with a polyclonal anti-VCP antibody titer of 1:2,000. **(F–H)** Nuclear localization of VCP in normal muscle. VCP-ABR antibody. **(F,G)** True-color fluorescent images of DAPI and VCP immunoreactivity. **(H)** Digitally superimposed images **F** and **G** show colocalization of VCP (white speckling of blue nuclei) in 96% of the nuclei.

anti-Werner syndrome protein, Wrnp (1:100 to 1:5,000 dilution; Abcam) with 3–5-h incubation followed by a 60–80-min incubation with the labeled secondary antibodies admixture. Tris buffer and normal rabbit serum at the same immunoglobulin concentration as the polyclonal anti-Wrnp were used as negative controls.

A Zeiss Axioimager with an Apotome optical sectioning device and Axiovision software (Carl Zeiss, Oberkochen, Germany) were used for 0.4-μm optical sections and 3D reconstructions.

RESULTS

Myonuclear and Endothelial Cell Nuclear Localization of VCP in Normal Human Skeletal Muscle. By light microscopy, both the polyclonal anti-VCP and monoclonal ABR anti-VCP antibodies stained structures that had the typical appearance and location of myonuclei, as well as structures in the typical location of capillaries (Fig. 1A–C). Weaker diffuse cytoplasmic staining was present as well, sometimes in focal accumulations typical of artifact often seen in muscle immunohistochemistry. The nuclear localization with the polyclonal VCP antibody was less apparent with acetone fixation than with PFA fixation (Fig. 1D,E). In PFA-fixed sections, both the ABR monoclonal antibody and the rabbit polyclonal antibody showed the same pattern of nuclear staining, but the polyclonal antibody additionally appeared to react to connective tissue around myofibers and within blood vessel walls. No staining was present with control IgG2a nonimmune isotype matched (for the ABR monoclonal antibody) antibodies or in Tris control sections in which the primary antibody was omitted.

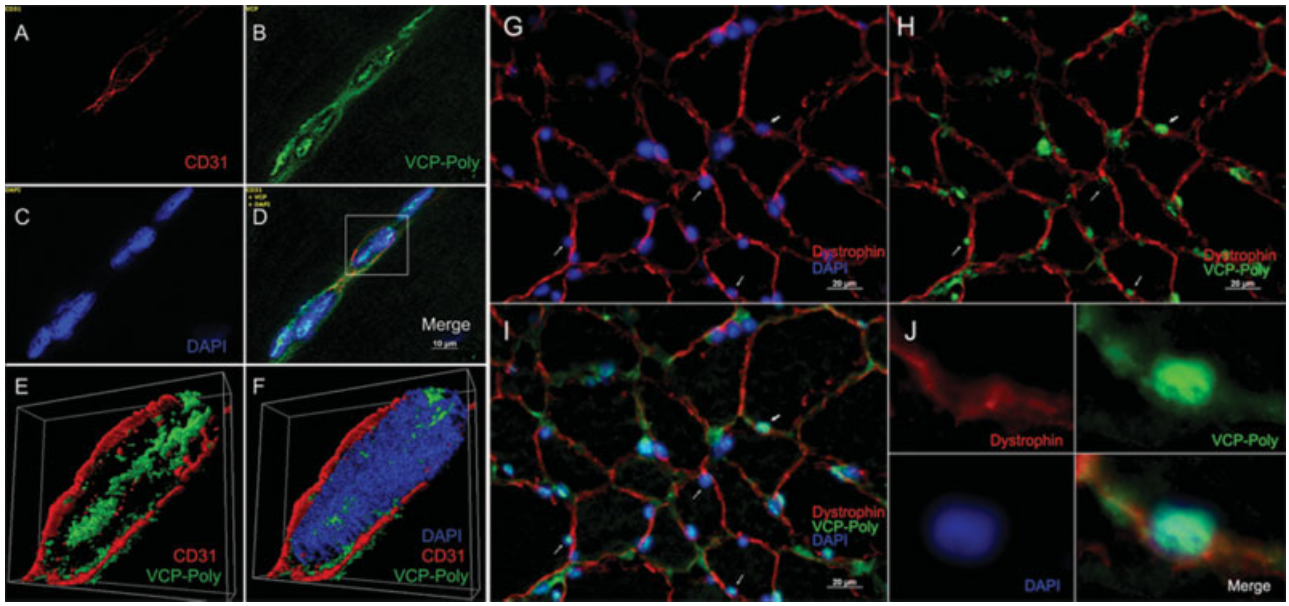

FIGURE 2. Both endothelial cell nuclei and myonuclei contain VCP. **(A–F)** Endothelial cell nuclei contain VCP. Triple immunofluorescence and optical sectioning microscopy. **(A)** CD31 is used to identify endothelium, **(B)** rabbit polyclonal anti-VCP, and **(C)** DAPI to identify nuclei. **(D)** Merged view, showing VCP within nuclei that lie within a blood vessel. **(E,F)** Region boxed in **D** further studied. Ten 0.4-μm optical sections were imaged and used for 3D reconstruction, demonstrating unambiguously the intravascular location of intranuclear VCP. Note that, as in the light immunohistochemistry sections, polyclonal VCP antibody is reactive against both endothelial cytoplasmic and nuclear antigens. **(G–J)** Triple immunofluorescence study with anti-dystrophin, polyclonal anti-VCP, and DAPI demonstrate multiple VCP-positive nuclei internal to myofibers in subsarcolemmal locations, confirming them as myonuclei (arrows). **(G)** Dystrophin and DAPI show nuclei internal to myofibers. **(H)** Dystrophin and VCP show VCP internal to nuclei. **(I)** Combined dystrophin, VCP, and DAPI show most nuclei contain VCP. **(J)** Magnified view of one myonucleus (thick arrow in **I**) shows the VCP-positive nucleus to be internal to the dystrophin stained sarcolemmal membrane.

Because of the light microscopic appearance of VCP immunoreactive structures that had the typical shape and location of myonuclei, we performed double-fluorescent immunohistochemistry, which confirmed that VCP staining localized with the nuclear stain DAPI (Fig. 1F–H). To further confirm the location of these nuclei, we performed immunofluorescent studies with thin 0.4-μm optical sections using optical sectioning microscopy and 3D reconstructions of VCP combined with dystrophin, to outline the muscle sarcolemmal membrane, and with CD31, to outline vessel endothelium, both with DAPI. These studies demonstrated VCP within myonuclei and endothelial cell nuclei (Fig. 2).

Nuclear and Perinucleolar Localization of VCP in Myonuclei and Endothelial Cell Nuclei. The specific location of VCP within human myonuclei is of interest, as localization to nucleoli has been noted in some mammalian cell lines.[17] We examined the relationship of VCP to the nucleolar protein fibrillarin. VCP typically localized to regions around nucleolar fibrillarin, often leaving a "pocket" for fibrillarin (Fig. 3). This arrangement was particularly evident with 3D reconstructions of optical sections (Fig. 3I–L).

Overlapping Localization of Werner Syndrome Protein with VCP. The Werner syndrome protein (Wrnp) is a DNA helicase. Mutations in Wrnp result in a multisystem disease likened to premature aging. Previous reports have demonstrated physical interaction between VCP and Wrnp in cell culture.[12] We accordingly looked at Wrnp in myonuclei and its location with respect to VCP. Light immunohistochemistry demonstrated punctate and infrequently diffuse nuclear localization of Wrnp in muscle (Fig. 4). Triple immunofluorescent studies showed small deposits of Wrnp typically colocalized with larger regions of VCP (Supplementary Fig. 1). Unlike with fibrillarin, there appeared to be complete overlap of Wrnp-containing regions with a portion of a VCP region.

Normal Nuclear Localization of VCP in Human Mutant R155H VCP IBMPFD Muscle. Immunohistochemistry of muscle from three patients with IBMPFD and R155H VCP mutation showed intact nuclear staining (Fig. 5).

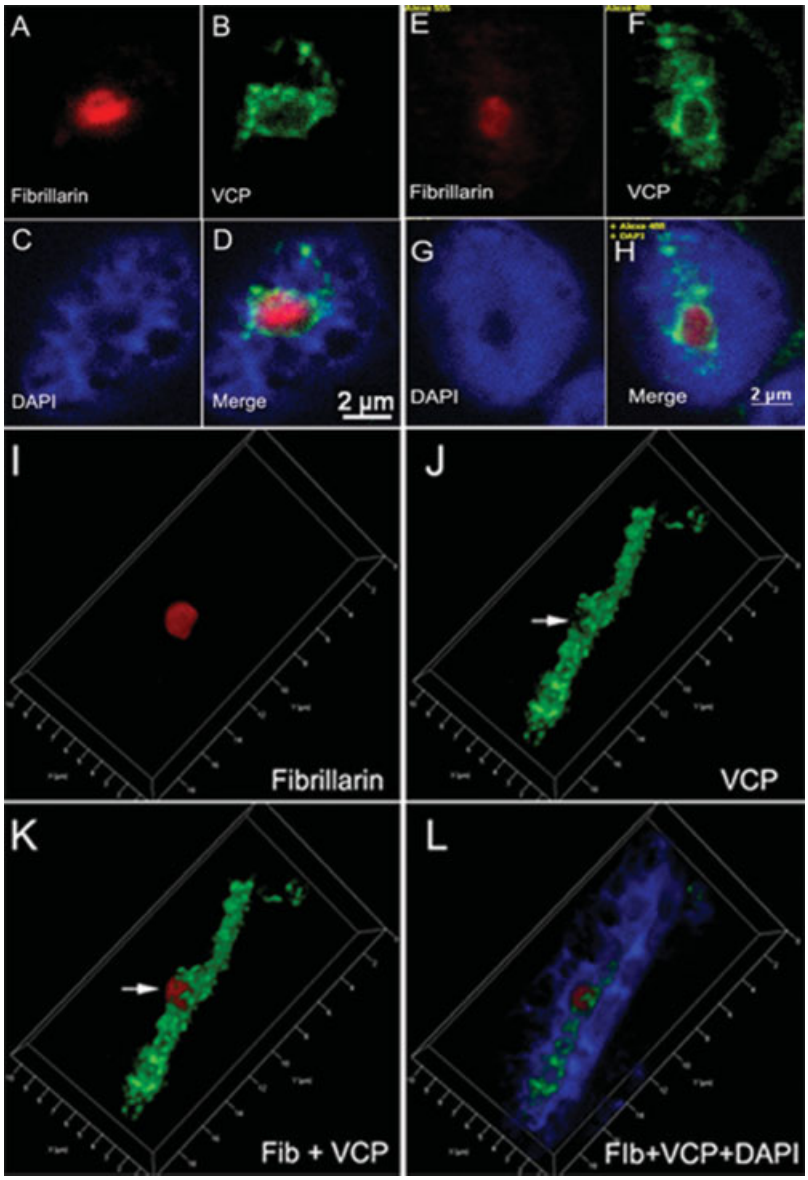

FIGURE 3. Perinucleolar distribution of VCP. The relationship of VCP to the nucleolar protein fibrillarin within three distinct myonuclei. **(A–D)** One nucleus, and **(E–H)** another nucleus imaged with 0.4-μm optical sections. VCP is largely perinucleolar, leaving a "pocket" (in **B** and **F**) for the fibrillarin-rich portion of the nucleolus. **(I–L)** 3D reconstruction of 0.4-μm optical sections through a myonucleus shows a linear arrangement of VCP, with a cleft (arrow in **J**) with a distinct space for the nucleolar protein fibrillarin's localization. Polyclonal anti-VCP and monoclonal anti-fibrillarin antibodies used.

Immunofluorescent studies of VCP with fibrillarin and DAPI in IBMPFD muscle showed VCP within nuclei and with variable sometimes overlapping relationship to fibrillarin (Supplementary Fig. 2). Unlike the normal muscle, no perinucleolar VCP pockets were seen; the significance of this result is uncertain. Studies of Wrnp's relationship to VCP showed no difference compared to normal.

VCP Nuclear and Rimmed Vacuole Localization in IBM. VCP was present in IBM myonuclei, definitively identified by their internal location away from the sarcolemmal membrane (Fig. 6A–C). Vacuoles were sometimes lined with VCP (Fig. 6D). As in normal muscle, nucleolar fibrillarin overlapped with a portion of VCP (Supplementary Fig. 3).

DISCUSSION

VCP has not previously been recognized within nuclei in normal human muscle tissue. Instead, it has been reported within capillaries but not to a more specific endothelial compartment.[9,18] In muscle

from one patient with IBMPFD, VCP has been reported in nuclei.[11] Although that finding was considered abnormal, the findings in normal muscle were not reported. Here we demonstrate a nuclear localization within normal myonuclei and furthermore demonstrate a more precise localization within capillaries that includes the endothelial cell nuclei. In IBMPFD with the R155H mutation, nuclear localization is not disrupted and the specific sublocalization of VCP appears normal. In IBM the nuclear localization is present and some vacuoles are lined with VCP.

Several possibilities exist for the lack of recognition of nuclear localization in the two previous reports[9,18] describing immunohistochemical studies for VCP in normal human muscle samples. First, the method of tissue fixation affects immunodetection of nuclear VCP. In our studies, fixation with acetone showed little nuclear localization with polyclonal VCP, whereas PFA fixation readily showed nuclear staining (Fig. 1D,E). The failure of some nuclear antigens to demonstrate immunoreactivity with acetone fixation has previously been recognized in other tissues.[2,7] Acetone fixation could alternatively result in extraction from the nuclei of certain proteins. The type of fixation used in those earlier studies is not reported or referenced in either of the two previous publications.[9,18] Second, capillary staining recognized previously was not further localized, as we have done, to nuclei within capillaries. Lastly, as myonuclei are typically located just under the sarcolemmal membrane, there may be uncertainty in distinguishing them from capillaries located just external to the sarcolemma. The use of immunofluorescence with dystrophin staining of the sarcolemma, as we have done, is particularly helpful in this regard.

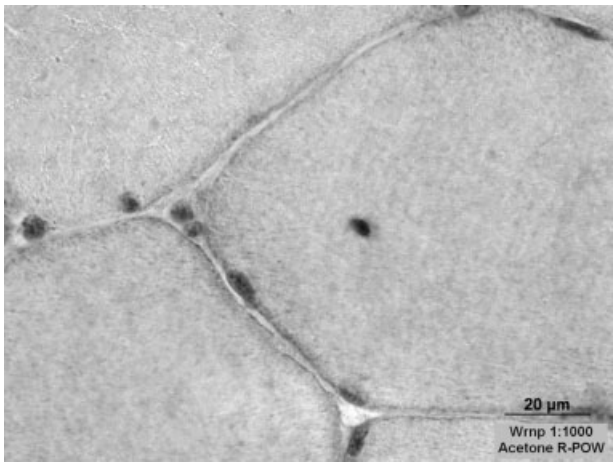

FIGURE 4. Werner syndrome protein in normal muscle tissue nuclei. Grayscale image.

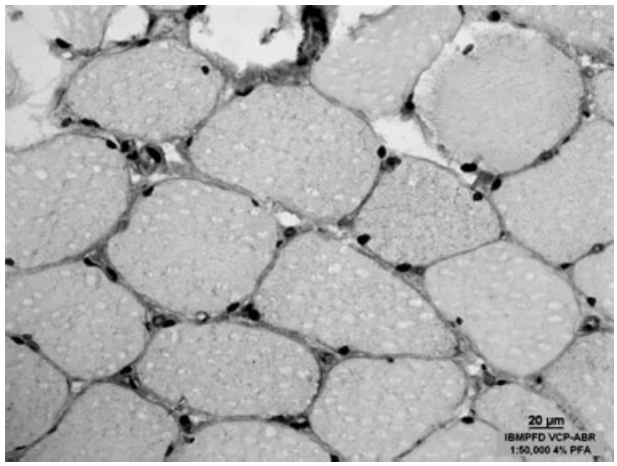

FIGURE 5. Intact nuclear VCP in IBMPFD R155H VCP mutant muscle. Grayscale image.

Although early studies did not find nuclear localization of VCP despite a putative N-terminal nuclear localization signal,[14] subsequent studies demonstrated nuclear localization in a variety of transfected cell lines[13,17,19] and confirmed through mutation studies a nuclear localization signal.[17] Two patterns of VCP nuclear fluorescence have been reported in cell lines: a punctuate pattern throughout the nucleus and several foci of intense fluorescent signal colocalizing with the nucleolar protein fibrillarin.[17] In normal human skeletal muscle, we find that VCP tends to be restricted to regions around, rather than overlapping with, the fibrillarin-rich portion of the nucleolus. In three patients with mutant VCP, overlap of fibrillarin within a larger region of VCP was seen more frequently. VCP has also been found to interact physically with Wrnp,[12] a DNA helicase in which mutations produce a premature aging syndrome. We found uniformly that Wrnp colocalized to a region overlapping with VCP.

A prominent aspect of muscle pathology in patients with VCP mutations is the presence of rimmed vacuoles. Rimmed vacuoles in two other diseases, IBM and oculopharyngeal muscular dystrophy, occur in association with nuclear pathology.[3–5,8] The localization of VCP to myonuclei shown here suggests that some muscle pathology in IBMPFD may result from a disturbance of the nuclear function of VCP, which includes the maintenance and assembly of nuclear membrane.[10,15] In this regard, nuclear pathology consisting of ubiquitinated nuclear inclusions[6] and TAR DNA binding protein 43 (TDP-43) nuclear inclusions[16] have been described in IBMPFD neurons, and a hypothesis has been suggested that

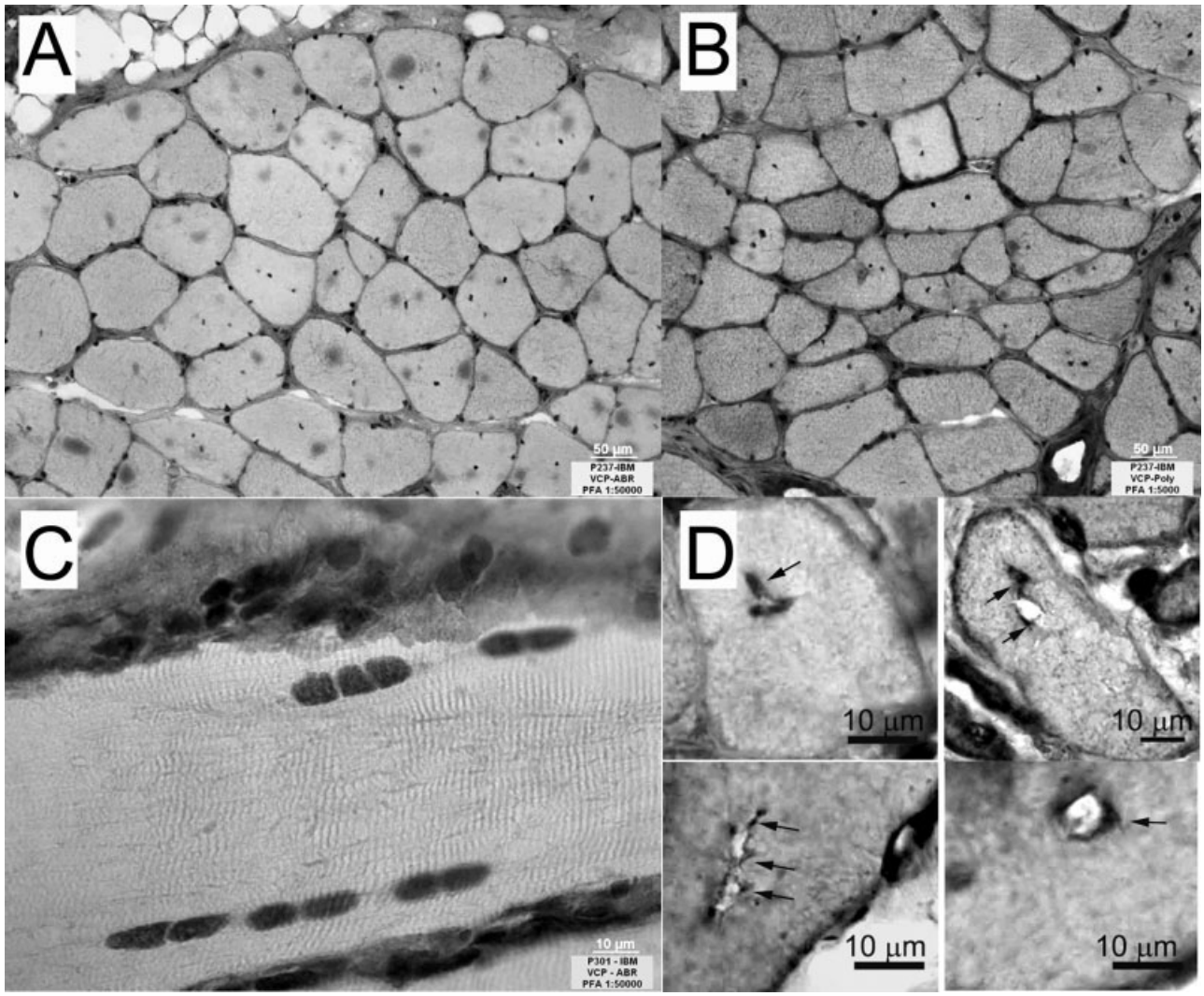

FIGURE 6. VCP staining in IBM muscle. Immunoreactivity of IBM myonuclei with both the **(A)** ABR-VCP antibody and **(B)** the polyclonal VCP antibody. **(C)** Longitudinal sections showing myonuclei as defined by their position inside the sarcolemmal membrane. **(D)** Vacuoles staining with VCP at their periphery (arrows). Panels **A** and **B** from Patient P237; Panel **C** from Patient P301; Panel **D** from Patient P170. Grayscale image.

VCP gene mutations result in impaired degradation of TDP-43.[16]

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