Transgenic expression of inclusion body myopathy associated mutant p97/VCP causes weakness and ubiquitinated protein inclusions in mice

Conrad C. Weihl1,2,*, Sara E. Miller1, Phyllis I. Hanson2 and Alan Pestronk1

1Department of Neurology and 2Department of Cell Biology and Physiology, Washington University School of Medicine, 660 S. Euclid Avenue, Saint Louis, MO 63110, USA

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Mutations in p97/VCP cause the autosomal-dominant, inherited syndrome inclusion body myopathy (IBM) associated with Paget’s disease of the bone and frontotemporal dementia (IBMPFD) (Watts, G.D., Wymer, J., Kovach, M.J., Mehta, S.G., Mumma, S., Darvish, D., Pestronk, A., Whyte, M.P. and Kimonis, V.E. (2004) Inclusion body myopathy associated with Paget disease of bone and frontotemporal dementia is caused by mutant valosin-containing protein. p97/VCP is a multi-functional protein with a role in the ubiquitin-proteasome system (UPS) (Wang, Q., Song, C. and Li, C.C. (2004) Molecular perspectives on p97-VCP: progress in understanding its structure and diverse biological functions. To understand how mutations in this protein lead to a myopathy, we generated several lines of transgenic mice expressing p97/VCP-WT (TgVCP-WT) or the most common IBMPFD mutant, p97/VCP R155H (TgVCP-RH), under a muscle-specific promoter. TgVCP-RH animals, but not controls, became progressively weaker in a dose-dependent manner starting at 6 months of age. Abnormal muscle pathology, which included coarse internal architecture, vacuolation and disorganized membrane morphology with reduced caveolin-3 expression at the sarcolemma developed coincident with the onset of weakness. These changes were not associated with alterations in sarcolemmal integrity as measured by muscle fiber uptake of Evan’s blue dye. Even before animals displayed measurable weakness, there was an increase in ubiquitin-containing protein inclusions and high-molecular-weight ubiquitinated proteins, markers of UPS dysfunction. We suggest that this early and persistent increase in ubiquitinated proteins induced by IBMPFD mutations in p97/VCP may ultimately lead to animal weakness and the observed muscle pathology. TgVCP-RH animals will be a valuable tool for understanding the pathogenesis of IBM and the role of the UPS in skeletal muscle.

INTRODUCTION

Inclusion body myopathies (IBMs) are a group of acquired and hereditary disabling muscle disorders with myopathic features, ‘rimmed vacuoles’ and accumulations of aggregated proteins (1). IBM associated with Paget’s disease of the bone (PDB) and frontotemporal dementia (FTD) (IBMPFD) is an autosomal-dominantly, inherited multisystem disorder because of mutations in the protein p97/VCP (2). The penetrance of each syndromic feature is variable with the most common initial presentation being weakness in 84% of patients, typically with onset in the fourth decade (2,3). PDB and FTD occur less frequently with a penetrance of 51 and 31% and mean onset of 42 and 55 years of age, respectively (2,3). Muscle pathology in IBMPFD patients has been described as a myopathy with rimmed vacuoles and no inflammation (4,5). Other features include p97/VCP containing ubiquitinated protein inclusions in both myonuclei and the sarcoplasm (4). Consistent with these findings, the central nervous system (CNS) pathology in IBMPFD includes intranuclear and cytoplasmic ubiquitinated inclusions in affected neurons (6,7).

The pathogenic mechanisms leading to muscle weakness in IBM, and IBMPFD in particular, are unclear, but may relate...
to the accumulation and aggregation of undegraded proteins. In the case of IBM, protein aggregates have been shown to contain β-amyloid, hyperphosphorylated tau and ubiquitin, proteins known to accumulate in other aggregate diseases (1). One hypothesis is the pathogenic pathway in IBM that leads to muscle damage relates to a dysfunction in ubiquitin-proteasome system (UPS)-mediated protein degradation resulting in the accumulation of aggregated proteins (8,9). p97/VCP belongs to the AAA (ATPases associated with a variety of cellular activities) family of proteins. The cellular function of p97/VCP is complex because it is involved in diverse cellular processes including protein degradation, organelle biogenesis and cell-cycle regulation (10). One cellular function of p97/VCP is as regulator of the UPS. p97/VCP associates with several pathway components, including polyubiquitinated proteins, 26S proteasome subunits and a variety of E3 ubiquitin ligases (11–13). Depletion of p97/VCP from cell extracts leads to an accumulation of cytosolic polyubiquitinated undegraded proteins (11). In addition to the degradation of cytosolic proteins, p97/VCP is involved in the degradation of at least a subset of improperly folded proteins from the membrane and lumen of the endoplasmic reticulum (ER), a process known as endoplasmic-reticulum-associated degradation (ERAD) (14). The expression of dominant negative p97/VCP in cultured cells impairs the degradation of ERAD substrates and leads to extensive ER-derived vacuolation (15). IBMPFD mutant p97/VCP expression leads to an increase in ubiquitinated protein aggregates in cultured C2C12 myoblasts (16). In addition, degradation of an ERAD substrate is slowed in these cells (16). Depletion of p97/VCP from the membrane and lumen of the endoplasmic reticulum (ER), a process known as endoplasmic-reticulum-associated degradation (ERAD) (14). The expression of dominant negative p97/VCP in cultured cells impairs the degradation of ERAD substrates and leads to extensive ER-derived vacuolation (15).

RESULTS

Generation of mice overexpressing p97/VCP-WT and p97/VCP-R155H in skeletal muscle

We modeled the muscle phenotype of IBMPFD in mice by expressing the human IBMPFD R155H mutant transgene in the presence of normal endogenous murine p97/VCP under the regulation of a muscle creatine kinase (MCK) promoter. The R155H mutation is the most common IBMPFD mutation in p97/VCP (R155H) in mouse muscle. These animals recapitulate some of the key phenotypic and skeletal muscle features of IBMPFD and open the way to a more detailed study of the underlying molecular and pathogenic mechanisms in IBM.

TgVCP-RH mice are weak and have disease-associated pathology

After breeding to the F3 generation with C57/B6 mice, a 1:1 Mendelian inheritance ratio was observed for all strains [17:18 (positive:negative) and 15:16 (+:−) for WT1 and WT2 strains, respectively, and 15:15 (+:+) and 26:26 (+:−) for strains RH9 and RH12, respectively, suggesting that transgene expression does not affect the pattern of inheritance. Control, TgVCP-WT and TgVCP-RH animals were similar in size and weight and had no differences in mortality up to 15 months of age. To see if these animals had evidence of muscle weakness, we measured mouse forelimb grip strength using a well-characterized system (20). Grip strength testing consists of five separate measurements using a trapeze bar attached to a force transducer that records peak generated
force (Stoelting, Wood Dale, IL). Mice instinctively grab the bar with their forepaws and continue to hold when being pulled backwards by the tail, releasing only when unable to maintain grip. The resulting measurement is recorded and the three highest measurements are averaged to give the strength score. Normal animals can generate ~400–500 g of force (20). Strength testing was performed on TgVCP-WT, TgVCP-RH and non-transgenic littermate control animals. TgVCP-RH animals were weak compared with control and TgVCP-WT lines. Weakness progressively worsened from 6 to 10 months with 10-month-old animals generating one-half the force of age-matched controls (Fig. 1E). Interestingly, TgVCP-RH strain TgVCP-RH12 which expresses higher levels of mutant protein (see Fig. 1A) becomes weaker earlier than TgVCP-RH9 which expresses less mutant protein. These data suggest that there may be a dose-related effect of mutant transgene expression. There was no evidence of weakness at 3 months of age in any of the animal lines.

Given that TgVCP-RH animals were weak between 6 and 10 months of age, we elected to examine the histopathologic changes in their skeletal muscle. We performed routine histochemical analysis on mouse skeletal muscle sections from biceps, triceps, quadriceps/hamstrings, soleus/gastrocnemius and diaphragm. In addition to myopathic features which include abnormal variation in muscle fiber size and an increase in endomysial connective tissue, the most distinctive feature of IBM muscle pathology is rimmed vacuoles seen by hematoxylin and eosin (H&E) or modified Gomori trichrome (mGT) stains. Although our TgVCP-RH animal skeletal muscle sections stained with H&E did not show classic rimmed vacuoles, they did show prominent small linear basophilic ‘rimmed cracks’ in the sarcoplasm and subsarcolemmal regions at both 6 and 10 months of age (Fig. 2B and C) that were not present in control or TgVCP-WT animals (Fig. 2A). Staining of similar muscle sections with mGT demonstrated disordered internal architecture with blue staining inclusions and linear red rimmed cracks between myofibrils in TgVCP-RH animals (Fig. 2E). These changes were not present in age-matched TgVCP-WT (Fig. 2D) or control animals. TgVCP-RH animals also demonstrated abnormal variation in muscle fiber size and an increase in endomysial connective tissue that was more severe in the diaphragm and most prominent after 12 months of age (compare Fig. 2F with G). Sarcoplasmic and occasionally myonuclear congo red positive staining was seen at 15 months of age in only one of the characterized TgVCP-RH lines (RH12) and was again most prominent in the diaphragm (Fig. 2H). No congo red staining was seen in TgVCP-WT or control animals at similar time points.

Skeletal muscle tissue from patients with IBM and IBMPFD has prominent ubiquitin containing inclusions of both sarcoplasmic and myonuclear (1,4). We looked for this in our TgVCP-RH animals using the FK2 antibody that recognizes ubiquitinated proteins. FK2 immunofluorescence revealed prominent ubiquitinated protein inclusions that were sarcoplasmic, subsarcolemmal and intramyonuclear in skeletal muscle tissue from 6-month-old TgVCP-RH animals (Fig. 2J). Age-matched control and TgVCP-WT animals demonstrated FK2 immunoreactivity that was less intense around myonuclei (Fig. 2I).

**Figure 1.** Generation of TgVCP-WT and TgVCP-RH transgenic mouse lines. (A) Total quadriceps lysates from 3-month-old control (Non-trg), TgVCP-WT (wtTrg1 and wtTrg2) and TgVCP-RH (mutTrg9, mutTrg6 and mutTrg12) animals were immunoblotted for p97/VCP. Control lanes represent the amount of endogenous p97/VCP. All transgenic lines had an increase in p97/VCP protein levels. Notably wtTrg2 and mutTrg9 transgenic lines have comparable levels of protein and were used in subsequent experiments. Actin loading control for each sample is shown below. (B–D) Dual immunofluorescence of 3-month-old quadriceps muscle from (B) control, (C) TgVCP-WT and (D) TgVCP-RH animals using anti-p97/VCP (red) and anti-collagen IV (green) antibodies. Endogenous murine p97/VCP is predominantly localized to endomysial connective tissue with a low level in the sarcoplasm. Both TgVCP-WT and TgVCP-RH animals have an increase in sarcoplasmic p97/VCP immunofluorescence that does not occur in the endomysium. White bar is 25 μm. (E) Quantitative grip strength testing in grams, of control, TgVCP-WT (WT1, WT2) and TgVCP-RH (RH9, RH12) at 3, 6 and 10 months of age. TgVCP-RH12 animals have statistically significant weakness beginning at 6 months that is worsened by 10 months of age and TgVCP-RH9 animals develop statistically significant weakness at 10 months of age. At least five animals from each transgenic line for each time point was measured. *P-value of <0.05; **P-value of <0.005.
IBMPFD and IBM patient skeletal muscle has p97/VCP containing inclusions (2,4,19). We looked for this in our transgenic animals using two different antibodies to p97/VCP. No similar change was seen in any of the animal lines.

Longitudinal toluidine blue semi-thin plastic-embedded sections of 6-month-old TgVCP-RH skeletal muscle showed intermyofibril vacuoles consistent with sarcotubular dilation that was not present in age-matched control or TgVCP-WT animals (compare Fig. 3A with 4A). Higher resolution analysis by transmission EM highlighted disruption between myofibrils in TgVCP-RH animal skeletal muscle that was not evident in TgVCP-WT animals (compare Fig. 3B with C). Other features seen in TgVCP-RH muscle included sarcotubular dilatation that was in continuity with myeloid bodies and vacuoles containing proteinaceous debris (Fig. 3D and E). In addition, there were prominent autophagosomes in TgVCP-RH muscle (Fig. 3D).

**Figure 2.** TgVCP-RH mice have IBM-associated disease pathology. Unless stated in the text, there was no qualitative difference between TgVCP-WT (WT1 or WT2) strains or TgVCP-RH (RH9 or RH12) strains, and a representative micrographs of skeletal muscle from TgVCP-WT or TgVCP-RH animals is shown. Diaphragmatic muscle from (A, D, F) TgVCP-WT or (B, C, E, G, H) TgVCP-RH (B, I, J) at 6 months, (C–E) 10 months or (F and G) 12 months of age. Frozen muscle sections are stained (A–C) with hematoxlin and eosin and (D and E) modified gomori-trichrome while (F and G) are semi-thin plastic-embedded sections stained with toluidine blue. White arrows demonstrate basophilic (B and C) or red (E) rimmed cracks. Black arrows (G) highlight variability in muscle fiber size characteristic of a myopathy. (H) Congo red staining visualized by Texas red filters demonstrates positive staining of fibers both in the sarcoplasm and myonuclei of 15-month-old TgVCP-RH12 animal skeletal muscle. Panels I and J are frozen tissue sections of quadriceps skeletal muscle from 6-month-old TgVCP-WT (I) and TgVCP-RH animals immunostained for ubiquitinated proteins with FK2 antibody. Closed white arrow highlights large sarcoplasmic and open white arrow highlights myonuclear congo red (H) or ubiquitinated protein (J) inclusions. Black line at bottom of each figure is 25 μm.

TgVCP-RH mice have disorganized sarcolemmal structure

In TgVCP-RH animals, 6 months of age or older sarcolemmal structure was markedly abnormal with prominent invaginations and protrusions that were not apparent in control or TgVCP-WT animals. This pathology was seen best on longitudinal toluidine blue plastic-embedded sections (compare Fig. 4A with B). These changes prompted us to explore the localization of proteins normally found in the sarcolemma. Consistent with IBMPFD patient skeletal muscle, there was normal sarcolemmal localization of dystrophin, α2-laminin and α-sarcoglycan (5) in TgVCP-WT and TgVCP-RH animals (Fig. 4C–H). However, abnormal sarcolemmal morphology is still apparent in TgVCP-RH animals’ skeletal muscle sections (Fig. 4 D, F and H). In contrast, immunostaining for caveolin-3 was reduced on the sarcolemma and increased in the sarcoplasm of TgVCP-RH but not control or TgVCP-WT animals (compare Fig. 5A with B). Immunoblots showed that total amounts of caveolin-3 protein in muscle were unchanged in TgVCP-RH animals compared to control and TgVCP-WT animals (Fig. 5C). To determine whether the changes in sarcolemmal structure were associated with changes in sarcolemmal membrane integrity, we injected 9-month-old control, TgVCP-WT and TgVCP-RH animals with Evan’s blue dye (EBD). EBD associates with albumin and is taken up into muscle cells when the sarcolemma is damaged (21). There was little EBD dye present in myofibers from control, TgVCP-WT and TgVCP-RH quadriceps muscle (Fig. 5D–F). The increase in EBD around muscle fibers in the TgVCP-RH tissues is consistent with the increased connective tissue seen in TgVCP-RH animals (22).

Ubiquitinated protein accumulation precedes muscle weakness and IBM pathology in TgVCP-RH animals

At time points earlier than 6 months of age, there was no evidence of animal weakness or prominent histopathologic changes in skeletal muscle from TgVCP-RH, TgVCP-WT or control animals. In order to identify a marker of disease that might precede these symptomatic changes, we elected
to look at FK2 immunoreactivity in 3 months old TgVCP-RH animals. There was an increase in FK2 immunoreactivity in these animals. The increase in FK2 immunoreactivity became more pronounced as the animals aged (Fig. 6C). The FK2 immunoreactivity appeared to be more prominent in myonuclei in 3-month-old animals and was both sarcoplasmic and myonuclear in older animals. Interestingly, there was an age-dependent increase in ubiquitinated proteins in both control and TgVCP-WT animals (Fig. 6A and B). Western blots of skeletal muscle lysates using the FK2 antibody showed a parallel increase in high-molecular-weight ubiquitinated proteins that was evident at time points as early as 30 days and persisted at 10 months of age (Fig. 6D and E for histogram of the densitometry three independent results).

DISCUSSION

We generated a transgenic animal model using skeletal muscle-specific expression of IBMPFD mutant p97/VCP. These animals develop progressive weakness, vacuolation, sarcolemmal pathology and congo red positive and ubiquitinated inclusions. Importantly, an abnormal level of ubiquitinated proteins in affected mouse skeletal muscle is detectable at time points as early as 1 month of age prior to animal weakness and other pathology.

Notably, transgenic expression of p97/VCP-R155H failed to generate classic rimmed vacuoles but instead had evidence of rimmed cracks. We elected to look at skeletal muscle pathology coincident with and before the onset of animal weakness. This allowed us to correlate changes in muscle pathology with disease progression in our animals. An additional reason is that aged inbred mouse strains have skeletal muscle pathology...
such as tubular aggregates and mitochondrial changes that may confound the pathologic examination of older ages (23). It may be that rimmed vacuole formation occurs at time points later than analyzed in our study and may explain the variability and occasional rarity of rimmed vacuoles seen in IBMPFD patient biopsies (4). Moreover, it is intriguing that animal weakness can occur with ubiquitinated protein accumulation in the absence of rimmed vacuoles, suggesting that UPS dysfunction is the principal cause of muscle weakness in these animals.

It is unclear how the accumulation of ubiquitinated proteins leads to skeletal muscle dysfunction. It may be that undegraded proteins are intrinsically toxic to muscle fibers, and as ubiquitinated proteins accumulate over time, disordered internal architecture, vacuolation, sarcolemmal changes and weakness ensue. It has been suggested that perturbations in the UPS may be involved in the pathogenesis of IBM (8). This speculation was based on immunohistochemical analysis of acquired and hereditary forms of IBM in which ubiquitinated protein inclusions are a prominent feature (24). The identification of mutations in p97/VCP, a protein essential for UPS function in IBMPFD patients, further supports the concept that aberrant protein degradation through the UPS is pathogenic in skeletal muscle and leads to IBM. The CNS changes in IBMPFD also suggest involvement of the UPS. The dementia in IBMPFD is accompanied by ubiquitin-positive cytoplasmic and nuclear inclusions similar to those in the human IBMPFD myopathies and our TgVCP-RH animal skeletal muscle (4,6,7; and unpublished observations).

Our animal model demonstrated intermyofiber vacuolation. Ultrastructural analysis suggests that vacuolation may be sarcomembranous in origin. Dominant negative p97/VCP expression in cultured cells causes extensive ER-derived vacuolation (15), however the role of p97/VCP in the extensively organized skeletal muscle sarcotubular system is unknown. IBMPFD mutants in cell culture cause dilation of the ER network as well (16). These changes may relate to impairment in p97/VCP’s role in ERAD (14). It is interesting that the loss of Trim32, an E3 ligase important in the ubiquitination of skeletal muscle-specific substrates, causes sarcotubular swelling in patients with LGMD2H (25). In addition, loss of sil1, an ER resident co-chaperone essential to ERAD, leads to ubiquitinated protein accumulation in cells and a rimmed vacuole myopathy (26).

We and others have described sarcoplasmic accumulations of p97/VCP in IBMPFD patient skeletal muscle (2,4,19). In addition, our previous study found that transient overexpression in cell culture of IBMPFD mutant p97/VCP containing a C-terminal GFP tag led to the presence of cytoplasmic,
Figure 6. TgVCP-RH mice have an increase in ubiquitinated proteins. Immunostaining for ubiquitinated proteins using the FK2 antibody of quadriceps muscle from 3-, 6- or 9-month-old (A) control, (B) TgVCP-WT, and (C) TgVCP-RH animals. Note the increase in ubiquitinated protein inclusions that are sarcoplasmic and nuclear. Inset box highlights an FK2 lined rimmed vacuole. Line at the bottom is 25 μm. (D) Immunoblot of quadriceps muscle lysates from control, TgVCP-WT (WT1) and TgVCP-RH (RH9 and RH12) with the FK2 antibody. Note the increase in high-molecular-weight ubiquitinated proteins (HMW-Ub) in TgVCP-RH muscle lysates. In order to visualize a quantitative difference in the HMW-Ub smear, a lighter exposure of the 10-month time point is shown as well. Myosin loading control is below blot. (E) Densitometric quantitation of immunoblots developed with ECL substrate from three independent experiments is shown. Error bars represent standard deviation and * denotes P-value < 0.01 when compared to control animals of the same age.
endoplasmic-rireticulum-associated p97/VCP inclusions (16). This is in contrast to our current data in which we see few p97/VCP inclusions in skeletal muscle from our transgenic mouse VCP. It may be that transient overexpression of GFP-tagged IBMPFD mutants in cell culture exaggerates a propensity for these mutants to aggregate that is not detected as readily in our transgenic animals.

p97/VCP has a clear role in the UPS and associates with several pathway components including polyubiquitinated proteins. Currently, it is unclear whether IBMPFD mutations in p97/VCP disrupt its normal function in this pathway and result in a loss of function or perhaps mutations confer a toxic gain of an unrelated function leading to weakness. However, current evidence suggests that these mutations may confer a gain of function. In particular, previous studies demonstrate that IBMPFD mutant p97/VCP has normal ATPase activity and normal binding to three essential co-factors, Ufd1, Npl4 and ataxin-3 (4,16). Our data demonstrate worsening skeletal muscle weakness with higher expression of IBMPFD mutant p97/VCP, suggesting that IBMPFD mutant p97/VCP may behave in a gain of function manner. Moreover, the highest expressing transgenic line, TgVCP-RH12, was the only line that demonstrated congophilic amyloid inclusions (although at 15 months of age).

The prominent sarcolemmal membrane abnormalities in our mouse model have not been previously described in aggregate myopathies. Interestingly, these changes happen in the absence of fiber necrosis and are not associated with an alteration in membrane permeability as assessed by EBD uptake. This is consistent with skeletal muscle biopsies from IBMPFD patients which do not show prominent muscle fiber degeneration and have modestly elevated serum creatine kinase (2,4,5). Whether the sarcolemmal changes are associated with alterations in membrane polarization and calcium release remains to be determined, but has been seen in other mouse models of IBM (27).

The mislocalization of caveolin-3 in TgVCP-RH animals is associated with the sarcolemmal changes and temporally correlate with animal weakness. Why these changes occur in association with the accumulation of ubiquitinated proteins is unclear? Mutations in caveolin-3 cause a variety of skeletal muscle disorders including LGMD1C, a distal myopathy and rippling muscle disease (28). The mislocalization of caveolin-3 and structural changes in sarcolemmal membranes could play a role in the weakness in our TgVCP-RH mice. Patients with IBMPFD have a clinical picture with both proximal and distal muscle weakness and overlap the phenotypic spectrum of caveolinopathies (4,5). In addition, a similar mislocalization of caveolin-3 is seen in IBMPFD patient muscle biopsies (unpublished observations). Dysregulation of the UPS has been implicated in caveolin-3-associated muscle disease. In particular, caveolin-3 has been identified as an ERAD substrate and disease-causing mutations in caveolin-3 cause the protein to aggregate, sequester normal caveolin-3 and mislocalize to the Golgi (29). These changes are corrected upon proteasome inhibition (29). Although a direct interaction between caveolin-3 and p97/VCP has not been demonstrated, it is conceivable that p97/VCP regulation of ERAD substrates, such as caveolin-3, causes the sarcolemmal membrane changes in our transgenic animals.

In summary, we find that transgenic expression of IBMPFD mutant p97/VCP results in an increase in ubiquitinated proteins in skeletal muscle. The accumulation of these ubiquitinated proteins precedes other skeletal muscle pathology such as vacuolation and sarcolemmal membrane changes, which instead appear to coincide with the onset of measurable animal weakness. We suggest that the accumulation of ubiquitinated proteins over time results in skeletal muscle weakness and myopathic pathology. The study of IBMPFD muscle disease using our transgenic mouse model will lend insight into this unique disorder and should also shed light on other diseases with UPS dysfunction and prominent ubiquitin pathology.

**MATERIALS AND METHODS**

**Transgenic animal construction**

Human p97/VCP cDNA was obtained from Drs Virginia Kimonis and Giles Watts. The R155H mutation was generated using QuickChange site-directed mutagenesis (Stratagene) and confirmed by direct DNA sequencing. Both p97/VCP-WT and p97/VCP-R155H cDNAs were subcloned directly into a 1256 MCK-CAT transgenic targeting vector (obtained from Dr Stephen Hauschka, University of Washington) using a Seamless Cloning kit (Stratagene). The promoter and coding sequence were confirmed by DNA sequence analysis. A linear fragment containing the MCK-p97/VCP sequence was isolated by digesting the targeting vector with HindIII and KpnI and subsequent gel purification. This fragment was sent to the mouse genetics core facility at Washington University for transgenic animal production. Animals were screened for transgene insertion using PCR amplification of tail DNA. Animals were bred to C57/B6 (Jackson Laboratories) to at least the F3 generation for phenotypic analysis. Control animals were non-transgenic littersmates.

**Skeletal muscle immunoblot**

Freshly isolated skeletal muscle tissue was flash-frozen in liquid nitrogen and stored at −80°C. Frozen tissue was added to 1× skeletal muscle sample buffer [4% SDS, 125 mM Tris pH 8.8, 40% glycerol, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 100 mM dithiothreitol (DTT)] and homogenized on ice with a mortar and pestle and then briefly sonicated with a Branson Sonifier with 50% cycle with an output of six on ice for 5 min. The lysate was then spun at 16 000g for 10 min and supernatant was collected. Equivalent amounts of protein lysates were added to each lane after the addition of 2× Laemmli buffer and boiling for 5 min. Immunoblots were performed as previously described (16). The following antibodies were used: mouse anti-p97 (BD Biosciences), rabbit anti-caveolin-3 (ABcam), FK2 (biomol), mouse anti-actin (Sigma).

**Skeletal muscle histochemistry and immunohistochemistry**

Freshly isolated skeletal muscle was mounted using tragacanth gum and quick frozen in liquid-nitrogen-cooled isopentane. Samples were stored at −80°C until sectioning. Frozen
biopsy samples were sectioned into 7 μm thick sections. Skeletal muscle was harvested from biceps, triceps, quadriceps/hamstrings, soleus/gastrocnemius, diaphragm and axial muscle. Histochemistry and immunohistochemistry were performed as previously described (2,5). Congo red staining was visualized using a Texas red filter set as previously described for skeletal muscle tissue (30). In addition to the antibodies used above for immunoblots, rabbit polyclonal anti-p97/VCP (obtained from Virginia Kimonis) rabbit anti-collagen IV (ABcam), mouse monoclonal anti-dystrophin (Dys-1) (ABcam), mouse monoclonal anti-α2-laminin (ABcam), and mouse monoclonal anti-α-sarcoglycan (ABcam) were used. In the case of FK2 immunohistochemistry, fixed muscle sections were incubated in 1 M KCl, 30 mM HEPES, 65 mM PIPES, 10 mM EDTA, 2 mM MgCl2, pH 6.9 for 1 h at room temperature to remove soluble proteins prior to immunolabeling as has been described previously for immunohistochemical detection of aggregate prone proteins (31).

For thin-section electron microscopy, freshly isolated skeletal muscle was placed in Karnovsky’s fixative overnight. Fixed tissue was embedded, sectioned and stained according to standard procedures.

Quantitative grip strength measurement

Strength testing consisted of five separate measurements using a trapeze bar attached to a force transducer that recorded peak generated force (Stoelting, WoodDale, IL). Mice instinctively grab the bar with their forepaws and continue to hold while being pulled backwards by the tail, releasing only when unable to maintain grip. The resulting measurement was recorded, and the three highest measurements were averaged to give the strength score. For each time point and strain, at least five animals were used. P-values were determined by paired Student’s t-test.

Evan’s blue dye injection

To evaluate muscle cell membrane integrity, EBD (10 mg/ml in phosphate-buffered saline) was injected intraperitoneally into mice (0.1 ml/10 g body weight) as described previously (21). The mice were killed 12–16 h after injection, and their muscles were sectioned and examined under a fluorescence microscope for uptake of dye.

Densitometric analysis

Autoradiographs of immunoblots from three independent experiments were scanned using an Epson 636 Expression Scanner. Densitometry of high-molecular-weight ubiquitinated proteins (HMW-Ub) was analyzed using Scion Image analysis software (Scion Corporation, Frederick, ML, USA) and normalized to the myosin loading standard, so as not to bias samples with more or less protein. For each experimental condition, the relative change in HMW-Ub from TgVCP-WT or TgVCP-RH muscle lysates was determined by comparing it with the amount of HMW-Ub in control muscle lysates from the same experiment. The average amount of HMW-Ub present in control muscle lysates was arbitrarily set to 1. The plotted histogram represents the average change in HMW-Ub from three independent experiments. Error bars represent standard deviation and P-values were determined by paired Student’s t-test.

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Conflict of Interest statement. None declared.

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