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Chapter 2

NEW VISTAS IN UNDERSTANDING THE PATHOPHYSIOLOGY OF VALOSIN-CONTAINING PROTEIN (VCP)/P97 DISEASE

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ABSTRACT

Hereditary inclusion body myopathy associated with Paget's disease of bone and frontotemporal dementia (IBMPFD) is an increasingly recognized disorder caused by mutations in the Valosin Containing Protein (VCP)/ p97, an ubiquitin-dependent ATPase. VCP plays critical roles in ubiquitin proteasome system (UPS) mediated and autophagy associated protein degradation pathways. Varied phenotypes, including amyotrophic lateral sclerosis (ALS), cardiomyopathy, Parkinson's, myotonia, cataracts and anal

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incompetence are associated. VCP mutations have also been identified in 1-2% of familial ALS.

Immunohistochemistry studies implicate abnormal ubiquitin and TDP-43 inclusions and autophagy. In addition, mouse models developed by other researchers and our R155H VCP knock-in heterozygous mouse model demonstrates progressive weakness including muscle, brain and spinal cord pathology with increasing age. Genomic, muscle microarray, and myoblast studies in this important disorder has also enabled further understanding of the molecular pathophysiology involved in related disorders such as amyotrophic lateral sclerosis, frontotemporal lobar degeneration, Parkinson's, Huntington's, and other diseases. Such a comprehensive understanding could lead to novel therapeutic targets

INTRODUCTION

Inclusion Body Myopathy associated with Paget's disease of the bone and Frontotemporal Dementia (IBMPFD; OMIM 167320) is an important autosomal dominant multisystem disorder characterized by progressive weakness and atrophy of the skeletal muscle, early-onset Paget's disease of the bone and premature frontotemporal dementia (Kimonis, Kovach et al. 2000). This disease is caused by mutations in the Valosin Containing Protein (VCP)/p97 gene located on chromosome 9p13-p12 (Watts, Wymer et al. 2004). VCP's role as a regulator of protein degradation is also said to affect many basic cellular functions, such as, cell division, apoptosis, biogenesis of organelles, membrane integrity, phosphorylation, and cell signaling.

Mechanisms for the progressive proximal muscle weakness, as well as for the inclusion bodies and vacuole formation seen in the muscle fibers of patients are not well understood. To develop novel and effective treatments for this disease, it is necessary to clearly understand the molecular basis of the clinical manifestation. Immunohistological studies, biochemical analyses and genomic microarray expression analyses, in human as well as mouse tissues and myoblasts have been performed to unravel the molecular and pathophysiological mechanisms for the progressive proximal muscle weakness, inclusion body and vacuolar formation. Identification of TDP-43 as the major component of the ubiquitin-immunoreactive inclusions of VCP disease, frontotemporal lobar degeneration (FTLD), ALS and the varied phenotypes associated with VCP disease supports the hypothesis that VCP has a key role in many pathways leading to proteinopathies.

CLINICAL PHENOTYPES OF VALOSIN-CONTAINING PROTEIN (VCP)/P97 DISEASE

VCP disease is a progressive disease characterized by multiple phenotypic clinical features; however the key features are inclusion body myopathy, Paget's disease of bone, and frontotemporal dementia (Nalbandian, Donkervoort et al. 2011). To date there are more than 25 different missense mutations (Figure 1) of the VCP gene reported in more than 39 families worldwide.

VCP Inclusion Body Myopathy

Myopathy is present in 80-90% of affected individuals with an average age of onset in thirties to forties. Patients typically demonstrate progressive weakness and atrophy of the skeletal muscles of the pelvic and shoulder girdle muscles.

Muscle weakness progresses to involve other limb and respiratory muscles, with death from cardiac or respiratory failure typically in the 50s-60s. The diagnosis of IBMPFD muscle disease is based on clinical features, and skeletal muscle histology which demonstrates rimmed vacuoles and sarcoplasmic inclusion bodies immunoreactive for ubiquitin and TDP-43 (Kimonis, Kovach et al. 2000; Watts, Thorne et al. 2003) (Weihl, Miller et al. 2008). Serum creatinine kinase concentration is usually normal to mildly elevated. Electromyogram (EMG) shows myopathic, and frequently mixed myopathic and neuropathic changes. Ultrastructural examinations of patient' muscle have shown atrophic and vacuolated muscle fibers containing abundant nuclear and cytoplasmic, paired helical filaments (PHF) with congophilia, accumulation of phosphorylated tau, and ApoE, as well as excessive beta-amyloid precursor protein epitopes.

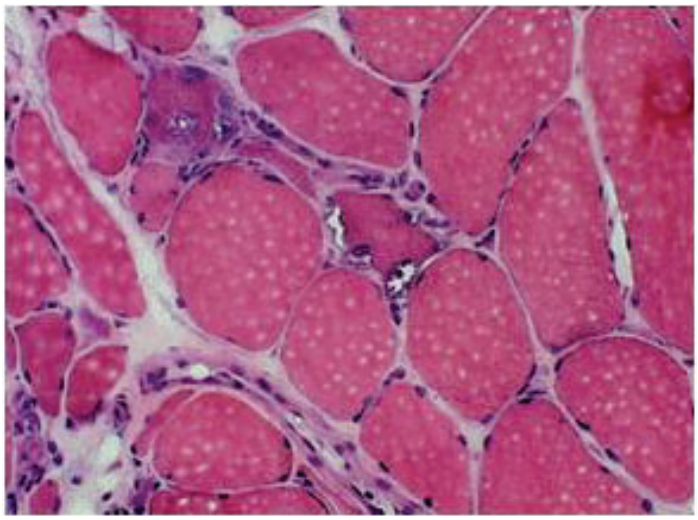

Figure 1. Muscle biopsy analysis in a 54-year-old gentleman with VCP-associated inclusion body myopathy. H and E staining illustrating the myopathic features including variability in fiber size due to atrophy and hypertrophy (diameter 5-160 microns), angulated fibers, and numerous atrophic fibers containing one or more, slit-like rimmed vacuoles.

Paget's Disease of the Bone (PDB)

PDB is observed in approximately 50% of patients (Kimonis, Kovach et al. 2000; Watts, Thorne et al. 2003), typically with an early onset in the thirties. PDB is caused by excessive osteoclastic activity and increased bone turnover and susceptibility to deformities like bowing and fractures. It involves focal areas of increased bone turnover that typically lead to spine and/or hip pain, and localized enlargement and deformity of the long bones. Skeletal radiographs reveal diagnostic changes of coarse trabeculation, cortical thickening and spotty sclerosis in the skull, pelvis, spine and scapula that later becomes widespread (Farpour, Tehranzadeh et al. 2011).

The diagnosis of PDB is based on elevated serum alkaline phosphatase, urine concentrations of collagen degradation markers pyridinoline (PYD) and deoxypyridinoline (DPD), and diagnostic skeletal radiographs and/or radionuclide scans. These findings are typically present in presymptomatic individuals 10 to 15 years before the diagnosis of PDB can be made (Kimonis, Fulchiero et al. 2008). Pagetoid osteoclasts contain nuclear paired helical filaments.

Paget disease of bone is responsive to treatment with bisphosphonates such as Zoledronic acid, which are potent suppressors of osteoclastic activity (Siris, Weinstein et al. 1996; Ralston, Galwey et al. 2005), and this treatment may be potentially useful in preventing PDB in individuals at risk of VCP-associated PDB.

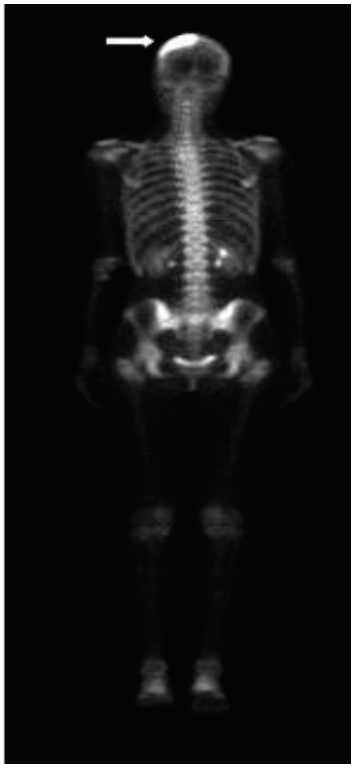

Figure 2. Detection of Paget disease by Technetium bone scan in a 30-year old female with myopathy and headache. An arrow indicates Paget disease of the right parietal skull bone.

Frontotemporal Dementia (FTD)

Premature FTD is a degenerative condition that affects the frontal and temporal lobes of the brain that control reasoning, personality, movement, speech, social graces, and language. In VCP disease, premature FTD is observed in 30% of patients with an average age of onset in the mid-fifties, and is characterized by dysnomia, dyscalculia, relative preservation of memory, and during the later stages, auditory comprehension deficits for even one-step commands, alexia, and agraphia (Kimonis, Kovach et al. 2000; Watts, Thorne et al. 2003). Diagnosis of FTD requires detailed neuro-psychological testing. Neuropathologic features include frontal and temporal lobar atrophy, neuron loss, gliosis, and ubiquitin and TAR DNA-binding nuclear protein-43 (TDP-43)-positive intranuclear inclusions. TDP-43, encoded by the TARDBP gene, has been identified as the major pathological protein of FTLD with ubiquitin-immunoreactive inclusions (FTLD-U) with or without ALS and in sporadic ALS

(Cairns, Bigio et al. 2007; van der Zee, Pirici et al. 2009) indicating common pathogenic pathways. Individuals die from progressive muscle weakness and cardiac and respiratory failure typically in their fifties and sixties, the clinical course being more rapid in the presence of central nervous system degeneration.

Histological studies of the brain neuropathological changes in patients with VCP mutations revealed a novel pattern of ubiquitin pathology characterized by ubiquitin-positive neuronal intranuclear inclusions and dystrophic neurites. The ubiquitin pathology was abundant in the neocortex, less robust in limbic and subcortical nuclei, and absent in the dentate gyrus. Only rare inclusions were detected with antibodies to VCP and there was no biochemical alteration in the VCP protein (Forman, Mackenzie et al. 2006). TDP-43 was identified as a major disease protein in the ubiquitin-positive inclusions of sporadic and familial frontotemporal lobar degeneration with ubiquitin-positive inclusions (FTLD-U) (Neumann, Mackenzie et al. 2007). As a result of this work frontotemporal dementia associated with VCP is now classified along with other FTD disorders (Cairns, Bigio et al. 2007) (Liscic, Grinberg et al. 2008). Recently, mutations in the TARDBP gene in familial and sporadic ALS have been reported which demonstrate that abnormal TDP-43 alone is sufficient to cause neurodegeneration. Thus, our work in the FTD associated with IBMPFD has lent new insights into the common pathogenesis of a spectrum of disorders called TDP-43 proteinopathies including: FTLD-U, ALS, and VCP disease. It is anticipated that these discoveries and a revised nosology of FTLD will contribute toward an accurate diagnosis, and the development of new diagnostic tests and therapies.

Amyotropic Lateral Sclerosis (ALS)

ALS is associated with fibrillations, spasticity, hyperreflexia, fasciculations, and electrophysiological evidence of motor neuron involvement, such as denervation and reinnervation with bulbar signs. Recently Johnson et al. performed whole exome sequencing in 78 familial ALS samples from 210 cases of unrelated families and identified VCP mutations in 1-2% of samples (Johnson, Mandrioli et al. 2010).

Parkinson's Disease (PD)

We have recently reported Parkinson's, mild myopathy, rare rimmed vacuoles and early signs of FTD in the proband in a VCP family with an R159C mutation (Chan, Le et al. 2012). Recently, his cousin has been diagnosed with PD (unpublished report).

Other reports in the literature indicate that PD is a well defined feature of VCP disease (Spina, Van Laar et al. 2008). VCP however is not a common cause of idiopathic PD. Majounie et al. (2012) (Majounie, Traynor et al. 2012) screened the VCP gene in a large cohort of 768 late-onset sporadic and familial PD cases (average age at onset, 70 years), and identified a I27V (c.468A_G, p.) variant in one case, which had been previously reported to be potentially pathogenic by Rohrer et al., (2011) (Rohrer, Warren et al. 2011). A 3-D model analysis of p.I27V showed that amino acid I27 lies close to the cluster of a known pathogenic mutation and that a change to valine may affect interaction with neighboring monomers in the VCP hexamer configuration. However, this p.I27V variant was also present in two

neurologically normal individuals from the Coriell repository raising questions about its pathogenicity.

EMERGING FUNCTIONS OF THE VCP/P97

Molecular Studies of IBMPFD localized the gene to human chromosome 9p21.1-p12 (Kovach, Waggoner et al. 2001), and later the gene responsible for the disease was identified as VCP (Valosin Containing Protein) (Watts, Wymer et al. 2004), which is the only gene thus far known to be associated with this triad. VCP is highly conserved in evolution, and belongs to the family of type II AAA (ATPases associated with a variety of cellular activities) having two ATPase domains (D1 and D2) (Confalonieri and Duguet 1995) (Neuwald, Aravind et al. 1999) (Ogura and Wilkinson 2001) (Patel and Latterich 1998) (Zwickl and Baumeister 1999) (Wang, Song et al. 2004), two linker domains (L1 and L2), as well as the N-terminal- and C-terminal domains (Figure 3).

VCP has been reported to be involved in several cellular activities including homotypic membrane fusion, transcription activation, nuclear envelope reconstruction, post-mitotic organelle reassembly, cell cycle control and apoptosis (Rabouille, Kondo et al. 1998) (Hetzer, Meyer et al. 2001) (Rabinovich, Kerem et al. 2002).

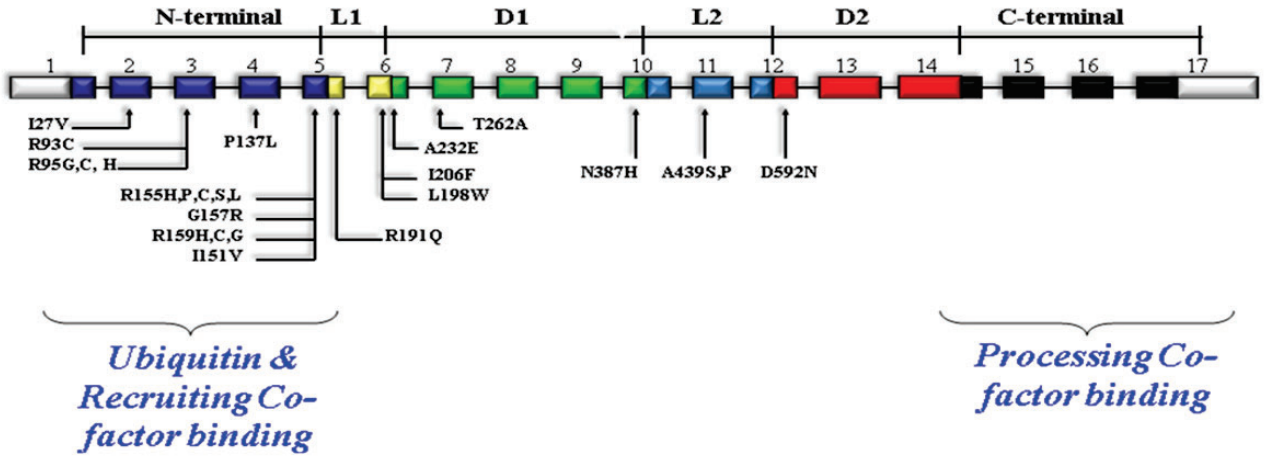

Figure 3. Functional domains and disease mutations in VCP. Arrows indicate the locations of mutations relative to the exon-intron structure where exons are numbered 1-17 **(**Watts, Wymer et al. 2004**).**

Most of these activities are related to its suggested main function, endoplasmic reticulum associated degradation of proteins (ERAD), which is capable of destroying both integral membrane proteins and luminal proteins. This activity functions as a quality control for newly synthesized polypeptides, which selectively eliminates aberrant proteins in the secretory pathway (Jarosch, Geiss-Friedlander et al. 2002). VCP forms homohexamers and binds to several different adapter proteins, enabling VCP to target specific substrates for degradation (Kondo, Rabouille et al. 1997) (Meyer, Shorter et al. 2000).

DISEASE MUTATIONS OF VCP

Disease mutations cluster in the CDC48 domain located in the N-terminus of the protein, which is involved in ubiquitin binding and protein-protein interactions (Dai and Li 2001) (Rape, Hoppe et al. 2001) (Figure 3). Most of the mutated residues causing IBMPFD are adjacent and potentially interact with each other, suggesting that these residues may have a similar and specific function within the VCP homohexamer. The most common R155H mutation has been shown to have a normal hexameric structure (Weihl, Dalal et al. 2006) and elevated ATPase activity in transfected cells (Kakizuka 2008). Interestingly Niwa et al. (2012) reported that ATPase levels were the highest with the two mutations associated with the most severe clinical phenotype (A232E and R155C) (Niwa, Ewens et al. 2012). Disease mutations were observed to interfere with the binding activities of interacting proteins (Fernandez-Saiz and Buchberger 2010; Ritz, Vuk et al. 2011), a factor that may be key in the VCP disease pathogenic mechanisms.

Prior to the identification of VCP gene mutation in IBMPFD, VCP was only indirectly implicated in the pathogenesis of neurodegenerative diseases. Specifically, VCP was found in a small proportion of the pathological lesions in AD (senile plaques), PD (Lewy bodies), ALS (MND inclusions), and poly-glutamine repeat diseases (intranuclear inclusions) (Hirabayashi, Inoue et al. 2001) (Mizuno, Hori et al. 2003).

Experimentally, TER94, the Drosophila homolog of VCP, has been shown to modulate poly-glutamine-induced neurodegeneration (Higashiyama, Hirose et al. 2002). Finally, investigations into endoplasmic reticulum-associated degradation indicate that dysfunction of VCP caused vacuole and inclusion body formation, thereby leading to cell death (Hirabayashi, Inoue et al. 2001) (Dai and Li 2001) (Weihl, Dalal et al. 2006).

At the moment more than 25 disease mutations have been identified (Figure 3). Mutations were found to cluster, thus, potentially defining a domain of the VCP having a critical role in skeletal muscle, bone cell and brain function. In particular, we have identified a mutation hot spot at the amino acid residue 155 (R155H/P/C/S).

To date, we have recruited over 30 families from several parts of the world since reporting our first family in 2000 (Kimonis, Kovach et al. 2000). Clinical findings have been reported by our group (Kimonis, Kovach et al. 2000) (Kovach, Waggoner et al. 2001) (Watts, Wymer et al. 2004) (Schroder, Watts et al. 2005) (Kimonis, Mehta et al. 2008) (Kimonis, Fulchiero et al. 2008).

Kimonis et al. (2005, 2007, 2008 (Kimonis and Watts 2005; Kimonis and Watts 2007; Kimonis, Mehta et al. 2008)) summarized the various diagnoses provided by their physicians which included limb girdle muscular dystrophy (LGMD), facioscapular muscular dystrophy, scapuloperoneal muscular dystrophy, and amyotrophic lateral sclerosis (ALS) (Kimonis and Watts 2005; Kimonis and Watts 2007; Kimonis, Mehta et al. 2008). As a result of the original reports (Kimonis, Kovach et al. 2000; Kovach, Waggoner et al. 2001; Watts, Wymer et al. 2004; Schroder, Watts et al. 2005; Kimonis, Fulchiero et al. 2008; Kimonis, Mehta et al. 2008) and as a result of the Genetests resource (www.genetests.org) (Kimonis and Watts 2007) and CLIA clinical testing for the VCP gene in the Mitomed laboratory (http://mitomed.bio.uci.edu/bin/ view/Mitomed/MITOMEDClinicalLaboratory), several more patients have been diagnosed. Since reporting our first family in 2000 (Kimonis, Kovach et al. 2000) families are now being reported from several parts of the world: Germany

(Schroder, Watts et al. 2005; Djamshidian, Schaefer et al. 2009), France (Guyant-Marechal, Laquerriere et al. 2006), Austria (Haubenberger, Bittner et al. 2005), Italy (Bersano, Del Bo et al. 2007; Viassolo, Previtali et al. 2008), UK (Miller, Jackson et al. 2009), other families from the US (Spina, Van Laar et al. 2008), Australia (Kumar, Needham et al. 2010) and by our group (Watts, Thomasova et al. 2007). To date we have recruited more than 50 families from several parts of the world and 30 of our families harbor 16 VCP mutations reported.

ApoE as a Modifier Gene

Mehta et al (2007) (Mehta, Watts et al. 2007) investigated ApoE as a potential modifier gene to account for the observed variation in FTD expression in IBMPFD. The epsilon-4 allele of ApoE (ApoE4) is known to be a risk factor for AD, and it has been implicated in other dementias including diffuse Lewy body disease (Griggs, Askanas et al. 1995) and vascular dementia. The status of ApoE in sporadic frontotemporal lobar degeneration and other subtypes remains controversial (Askanas and Engel 1998). Microtubule associated protein tau (MAP-tau) haplotype was also investigated because of its described modifier effect in frontotemporal lobar degeneration, and possibly in AD as well. ApoE4 was found to be strongly associated with the presence of dementia in IBMPFD, but no significant association with the common H1-tau haplotype was observed (Mehta, Watts et al. 2007).

Studies of the Cellular Pathology in VCP Disease

To elucidate pathological cascades resulting in muscle weakness in IBMPFD patients, human primary myoblast cells isolated from patients and control subjects were analyzed (Vesa, Su et al. 2009). Mutant cell lines expressed either the most common VCP mutation R155H or a novel R155S mutation revealed that patient myoblasts accumulated large vacuoles that fused with lysosomes. Examination of patients' primary myoblasts demonstrate that defective cell fusion processes and terminal differentiation to myotubes from myoblasts is abnormal (Vesa, Su et al. 2009), 10-20% of the wild-type cells showed multinucleated myotubes, whereas mutant cells were never seen to have more than two nuclei.

Autophagy is a process that degrades cytoplasmic components within vesicles which deliver the contents to the lysosome/vacuole for degradation. LC3-I is an 18 kDa cytosolic form and LC3-II is a 16 kDa membrane bound form that is enriched in the autophagic vacuole fraction. The conversion from LC3-I to LC3-II can be used as a sensitive marker for distinguishing autophagy in mammalian cells. The LC3 staining of mutant cell lysates showed a significantly increased LC3-II expression when compared to the wild-type cell lines suggesting disrupted autophagy. Mutant cell lines also showed increased apoptosis, which was demonstrated by increased Caspase-3 activity and TUNEL-staining in mutant cells when compared to wild type cell lines.

Global Microarray Analysis of Human Muscle

Since VCP disease mutations may cause disturbances in common signaling pathways, Nalbandian et al. (2012) performed the first investigation to identify the key molecular mediators and signaling cascades in skeletal muscle by global gene microarray (Nalbandian, Ghimbovschi et al. 2012). The hope was that understanding the dysregulated genes and molecular mechanisms underlying VCP disease would help identify novel therapeutic targets. Microarrays reveal gene expression profiles of human tissues and provide valuable insight into molecular pathways involved in the pathogenesis or abnormally regulated in disease. Expression profiles using human genome microarray technology in vastus lateralis muscles from patients and their first degree relatives identified

1868 ($p<0.01$) and 261 ($p<0.001$) probe sets that were differentially expressed in the patients' muscle.

The global microarray analysis revealed dysregulation mainly of the genes involved in the actin cytoskeleton cascade, involving several critical growth factor receptors, such as FGFR2 and EGFR; and disruption of the autophagy pathway genes, namely ATG4A, mTOR, and FoxO3 and genes in the lysosomal pathway. The molecular signaling intermediates involved in these pathways are known to be important for the crosstalk between protein breakdown and synthesis in IBMPFD disease. Understanding these key mediators will prove useful for development of novel therapeutic strategies for patients with VCP disease.

Table 1. Gene pathways as classified by KEGG and BIOCARTA. Pathways enriched with dysregualated genes in VCP-associated patients (Nalbandian, Ghimbovschi et al. 2012)

Pathways <0.05	Pathway	Number of genes	EASE Score
KEGG_PATHWAY	Regulation of actin cytoskeleton	29	2.00E-03
KEGG_PATHWAY	ErbB signaling pathway	12	2.50E-02
KEGG_PATHWAY	Cancer	8	3.50E-02
KEGG_PATHWAY	Regulation of autophagy	3	4.00E-02
KEGG_PATHWAY	Lysosome	15	4.80E-02
BIOCARTA PATHWAY	Mechanism of Protein Import into the Nucleus	5	7.90E-03
BIOCARTA PATHWAY	Agrin in Postsynaptic Differentiation	5	1.00E-02
BIOCARTA PATHWAY	Erk1/Erk2 Mapk Signaling pathway	4	5.10E-02

VCP Disease Mouse Models

To be able to study the in vivo effects of the VCP mutations, as well as to understand the pathogenesis of VCP disease, mouse models have been developed and have proven to be very promising. Both human and mouse VCP proteins consist of 806 amino acids, and the mouse protein differs by only one amino acid residue at position 684 when compared to the human

protein. In addition to a functional VCP gene, the mouse genome has also a non-functional pseudogene (Hoyle, Tan et al. 1997) (Muller, Meyer et al. 1999). The targeted deletion of VCP by Cre-loxP technology was reported to result in early embryonic lethality (Muller, Deinhardt et al. 2007) suggesting an important role for the intact VCP expression in embryonic development. In contrast heterozygous mice lacking one VCP allele were indistinguishable from their wild-type littermates. On the other hand, transgenic mice over-expressing the most common human VCP mutation (R155H) under the regulation of a muscle creatine kinase promoter became progressively weaker in a dose-dependent manner starting at 6 months of age (Weihl, Miller et al. 2007). These mutant mice showed muscle pathology including coarse internal architecture, vacuolization and disorganized membrane morphology. Also the level of ubiquitinated protein inclusions was increased, even before animals displayed measurable muscle weakness. Custer et al. (Custer, Neumann et al. 2010) generated transgenic mice, ubiquitously over-expressing mutant forms of VCP which exhibited characteristic inclusion body myopathy with muscle weakness and presence of blue-rimmed vacuoles. Paget-like bone disease with focal lytic and sclerotic regions was seen. Widespread TDP-43 brain pathology and abnormalities in behavioral testing were observed (Weihl, Miller et al. 2007; Custer, Neumann et al. 2010).

Badadani et al. reported the first knock-in mouse model heterozygous for the most common VCP R155H mutation and the neomycin cassette (Badadani, Nalbandian et al. 2010). The heterozygous mice were shown to express the mutant VCP allele, and they are viable and fertile. Muscle strength and rotarod performance of mutant mice were decreased when compared to their wild-type littermates. Quadriceps muscle from knock-in mice showed variation in fiber size and accumulated rimmed vacuoles (Figure 4). Electron microscopy of quadriceps muscles demonstrated disorganized structures and vacuolization of muscle fibers further confirming the muscle pathology of the generated knock-in mice. Additionally, mitochondrial abnormalities were seen in the mutant muscle (Badadani, Nalbandian et al. 2010). The knock-in mouse model also demonstrates progressive accumulation of TDP-43, ubiquitin, and LC3 in muscle and brain.

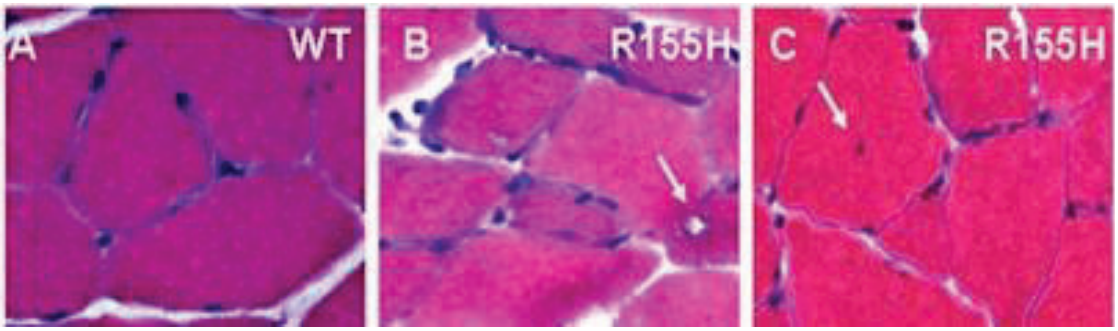

Figure 4. Histological analysis in the WT and $VCP^{R155H/+}$ mouse muscle. (A–C) Quadriceps muscles from 9-10 month-old wild-type and $VCP^{R155H/+}$ knock-in mice were analyzed by H &E staining. (B) An enlarged vacuole in the mutant tissue is shown by white arrows and (C) centrally located nuclei are revealed in the mutant mice shown by white arrows (Badadani, Nalbandian et al. 2010).

These results indicate that the phenotype of the generated transgenic mouse models and the knock-in model resembles VCP-associated disease pathology and can be used for pre-clinical treatment studies.

SUMMARY

In summary, VCP is at the crossroads of many cellular functions. VCP cooperates with diverse partner proteins to help process ubiquitin-labeled proteins for degradation and recycling by the proteasome. Other cellular functions for VCP include a role in protecting cells from protein stress by its effects on autophagy, endosomal sorting, regulation of protein degradation at the outer mitochondrial membrane, and key chromatin-associated processes ensuring genome stability during proliferation. Meyer et al. (2012) (Meyer, Bug et al. 2012) recently comprehensively reviewed the emerging functions of the VCP/p97. Thus, it is not surprising that VCP disease is a progressive multisystem disease which involves so many organ systems potential as a result of disruption of different mechanisms.

The transgenic and $VCP^{R155H/+}$ knock-in heterozygous mice are excellent models for the disease since the mice demonstrate progressive weakness and other hallmarks of VCP disease. Understanding the pathogenesis of this disease has implications for finding potential therapeutic targets in other related ubiquitin and progressive TDP proteinopathies.

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