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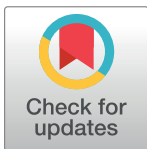
RESEARCH ARTICLE

# Myogenic differentiation of VCP disease-induced pluripotent stem cells: A novel platform for drug discovery

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## Abstract

Valosin Containing Protein (VCP) disease is an autosomal dominant multisystem proteinopathy caused by mutations in the *VCP* gene, and is primarily associated with progressive muscle weakness, including atrophy of the pelvic and shoulder girdle muscles. Currently, no treatments are available and cardiac and respiratory failures can lead to mortality at an early age. VCP is an AAA ATPase multifunction complex protein and mutations in the *VCP* gene resulting in disrupted autophagic clearance. Due to the rarity of the disease, the myopathic nature of the disorder, ethical and practical considerations, VCP disease muscle biopsies are difficult to obtain. Thus, disease-specific human induced pluripotent stem cells (hiPSCs) now provide a valuable resource for the research owing to their renewable and pluripotent nature. In the present study, we report the differentiation and characterization of a VCP disease-specific hiPSCs into precursors expressing myogenic markers including desmin, myogenic factor 5 (MYF5), myosin and heavy chain 2 (MYH2). VCP disease phenotype is characterized by high expression of TAR DNA Binding Protein-43 (TDP-43), ubiquitin (Ub), Light Chain 3-I/II protein (LC3-I/II), and p62/*SQSTM1* (p62) protein indicating disruption of the autophagy cascade. Treatment of hiPSC precursors with autophagy stimulators Rapamycin, Perifosine, or AT101 showed reduction in VCP pathology markers TDP-43, LC3-I/II and p62/*SQSTM1*. Conversely, autophagy inhibitors chloroquine had no beneficial effect, and Spautin-1 or MHY1485 had modest effects. Our results illustrate that hiPSC technology provide a useful platform for a rapid drug discovery and hence constitutes a bridge between clinical and bench research in VCP and related diseases.

## OPEN ACCESS

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## Introduction

Hereditary Inclusion Body Myopathy, Paget Disease of Bone, Frontotemporal Dementia (IBMPFD) and Amyotrophic Lateral Sclerosis (ALS), recently termed VCP-associated disease (VCP disease) is a multisystem disorder with a diverse collection of manifestations caused by

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mutations in the *valosin containing protein (VCP)* gene [1–4]. Inclusion body myopathy (IBM) is the most common feature present in up to 90% of affected individuals with an age of onset typically in their 30s. Patients typically demonstrate progressive weakness and atrophy of the skeletal muscles, generally starting with the pelvic and shoulder girdle muscles. Progressive muscle weakness typically advances to involve other limbs and respiratory muscles, resulting in patient mortality from cardiomyopathy or respiratory failure between approximately 40–60 years of age. Hallmarks of the muscle pathology include rimmed vacuoles, ubiquitin- and TDP-43-positive inclusions and increased autophagy markers such as Light Chain 3-I/II (LC3-I/II) and p62/SQSTM1. The second most common pathology is Paget's disease of the bone (PDB) which is observed in 49% of patients [3, 5, 6], typically with onset in their 30s to 40s. PDB is caused by excessive osteoclastic activity and increased bone turnover and susceptibility to deformities like bowing and fractures [7–9]. The third most common component of VCP disease is frontotemporal dementia (FTD), with an average age of onset of 54 years with an overall frequency of 33% [1, 5, 10]. Hallmarks of the muscle and brain pathology include inclusions of ubiquitin and TDP-43 also seen in other proteinopathies associated with FTD [11–13]. Additional pathology manifestations of VCP mutations include ALS in up to 15% of VCP disease patients, with Parkinson's disease (PD) [14] Alzheimer's disease (AD) [15, 16], and cardiomyopathy among other associated manifestations [5]. Interestingly VCP mutations also account for 3% of isolated familial ALS (fALS) [17, 18]. Over 40 VCP mutations have been identified worldwide in families from several parts of the world including Germany [19, 20], France [21], Austria [22], Italy [23], the United Kingdom [24], Australia [25], Korea [26], and the United States [1, 2, 5, 6, 27–31], with the R155H mutation present in more than 50% of affected individuals.

On a molecular level, VCP, a member of the type II AAA+ ATPase family, plays an important role in a plethora of cellular activities and recent studies have implicated the ubiquitin proteasome protein degradation pathway [32, 33], the autophagy cascade [5, 30, 33, 34], mitochondrial quality control [32, 35–37] and potentially other signaling pathways in the pathogenesis of VCP disease. *Sequestosome 1* (p62/SQSTM1) interacts with Light Chain 3 (LC3-I/II), which is an autophagic effector protein, to facilitate the process of autophagic uptake of aggregated proteins [5]. VCP is necessary to initiate the retro-translocation process for misfolded endoplasmic reticulum (ER) proteins; thus, mutations in the VCP gene result in defective ER associated protein degradation (ERAD) and ER stress responses [28]. Although VCP disease is a relatively rare disorder, exploration of its cellular and molecular mechanisms holds promise for explaining shared pathologies of more common proteinopathies, such as ALS, PD, and FTD.

Stem cells have revolutionized the field of human cell culture because they provide an immortal population of pluripotent cells, which can theoretically differentiate into any cell type [38–40]. Due to their renewable and pluripotent nature, stem cells also allow for the development of therapies for rare muscle conditions such as VCP disease, where tissue is sparse or difficult to access. In particular, patient-specific induced pluripotent stem cells (iPSCs) represent an excellent tool for modeling disease since they can be generated from adult somatic cells, thus, avoiding the ethical considerations involved with using embryonic stem cells. iPSCs will continue to be a sustainable method to model disease for gene therapies, drug therapies, and for transplantable stem cells for neuromuscular and related diseases [38, 41–45]. iPSC technology is already being utilized in other neurodegenerative diseases, including ALS [46], Duchenne muscular dystrophy (DMD) [47], PD [48], AD [49, 50], macular degeneration [51, 52], and type I diabetes mellitus [53, 54]. Therefore, creation and characterization of human derived VCP disease iPSCs has offered a novel therapeutic platform to investigate mechanisms of VCP disease and further the development of effective treatment [55]. Differentiating these

patient-derived iPSCs into a myogenic lineage has the promise to make significant contributions to our understanding of VCP disease [46, 47, 56–58]. The use of adult myogenic stem cells as a cell therapy for skeletal muscle regeneration has been attempted for decades, with only moderate success [40, 59–63]. However, several studies have recently reported development of more effective differentiation protocols with the use of skeletal muscle specific media and the induction of PAX3/7 with a retrovirus [47, 56, 64–69].

In this report, we differentiated patient-derived human induced pluripotent stem cells (hiPSC) into myogenic lineages to provide a useable resource to study the underlying pathological mechanisms of VCP disease and as a novel platform for a rapid drug screening. We showed a significant success rate of patient-derived and control iPSC differentiation into the myogenic lineages. Characterization of the VCP disease phenotype in our myogenic lineages revealed higher protein expression levels of classic VCP pathology including autophagy markers LC3-I/II, p62/SQSTM1, ubiquitin and upregulation and translocation of TDP-43 to the cytoplasm. We next screened the effects of various autophagy-modifying compounds to understand the molecular dysfunction in VCP pathology and as a therapeutic strategy for VCP-associated diseases. We observed significant improvement in pathology with autophagy inducers Rapamycin, Perifosine and AT101. Conversely chloroquine an autophagy inhibitor showed no benefit. Interestingly, Spautin-1 and MHY1485 showed a modest beneficial effect in preventing TDP-43 translocation from the nucleus to the cytoplasm. In summary, our study provides a unique and novel platform for investigating the underlying pathophysiology mechanisms of VCP disease and for rapid screening of new drugs that ameliorate autophagy dysfunction thus improving muscle integrity and/or slowing down the progression of muscle wasting in patients with VCP and related neurodegenerative diseases.

## Materials and methods

### Ethics statement

The University of California Irvine (Irvine, California) Institutional Review Board (IRB) (#2009–1005) approved this study including the consenting procedure. We explained the research to the participant, reviewed the consent form and provided an opportunity to ask questions in order to ensure that the subject understood the research. The subjects signed the approved consent form. Skin biopsy samples were submitted to Coriell repository and were used for the iPSCs generation. The control iPSCs were obtained from the University of Connecticut repository.

### Differentiation of iPSC into skeletal muscle lineage

VCP disease and control iPSCs were differentiated into skeletal muscle lineages using a modified protocol adapted from *Awaya et al.* (2012) [57]. Briefly, iPSCs were grown in mouse embryonic fibroblast (MEF) conditioned media (CM) supplemented with basic fibroblast growth factor (bFGF) (CM-bFGF) [70]. Once confluent, iPSCs were treated with 1 mg/mL collagenase in DMEM/F12 at 37°C for 5 min and removed from the plate by mechanical scraping. The cell clusters were then left floating in CM-bFGF for 7 days in a non-adhesive flask to form embryoid bodies (EBs). Next, the EBs were transferred to 0.2% gelatin-coated tissue culture plates in ITS medium (DMEM, 1X ITS-X (ThermoFisher Scientific, Carlsbad, CA), non-essential amino acids (ThermoFisher Scientific GlutamaxH supplement (ThermoFisher Scientific), and 100 mM 2-mercaptoethanol for 14 days to promote a mesenchymal lineage [57]. To encourage skeletal muscle differentiation, the medium was changed to skeletal muscle induction medium skIM (SkIM: high-glucose DMEM supplemented with 10% fetal calf serum (FCS; ThermoFisher Scientific), 5% horse serum (HS; Sigma, St. Louis, MO), non-essential



amino acids (Invitrogen), and 100 mM 2-mercaptoethanol for 7 days [57]. The differentiated cells were analyzed on days 7, 21, 49, 56, 63, and 70. Images of differentiation by differential interference contrast (DIC) microscopy were taken on days 0, 21, 50, 69. Primary myoblasts from the same patient or control subjects were used in this investigation for comparison of morphology.

## Flow cytometry

Differentiated myoblasts were phenotyped and sorted by flow cytometry (Sue and Bill Gross Stem Cell Institute, University of California-Irvine, Irvine, CA). Briefly, myogenic precursors at days 35 and 49 were dissociated with dissociation buffer (ThermoFisher Scientific) and stained with cell surface pluripotency marker, anti-CD34, and mesenchymal stem cell (MSC) markers anti-CD73, anti-CD105 and anti-CD29 (Life Technologies) and skeletal muscle marker, anti-CD56 (ThermoFisher Scientific) as well as isotype controls (BD Pharmingen). Dead cells were excluded by propidium iodide (Sigma-Aldrich) and samples were analyzed using FACSAria II and FACS Diva software (BD Biosciences, Franklin Lakes, NJ).

## Treatments with autophagy-modifying agents

Day 50 myogenic progenitor patient and control cells were seeded onto gelatin-coated 4-well chamber slides or 6-well plates and cultured in sKiM media. Patient and control cells were either treated with autophagy inducers, Rapamycin (10  $\mu$ M) [71, 72], Perifosine (also known as KRX-0401) (80  $\mu$ M) [73] or AT101 (10  $\mu$ M) [74, 75] or autophagy inhibitors chloroquine (10  $\mu$ M) [76, 77], Spautin-1 (10  $\mu$ M) [78] or MHY1485 (2  $\mu$ M) [79] for 24 hours. Following treatment, immunocytochemical and Western blot analysis were performed and analyzed.

## Immunocytochemical analysis

Myogenic progenitor cells were seeded, at days 21 and 50 onto gelatin-coated 4-well chamber slides and cultured in sKiM media. Cells were washed with PBS then fixed in 4% paraformaldehyde (PFA) for 15 minutes, and permeabilized with Triton X-100 for ICC staining. To check for pluripotent markers, cells were stained with anti-Oct-3/4 and Nanog (Sigma-Aldrich, St. Louis, MO). To check for myogenic differentiation, both early (anti-MYF-5, desmin and anti-PAX-7) and late stage markers (anti-MyoD and anti-MYH2) were used. For VCP disease pathology analysis, cells were taken at day 50 and seeded as aforementioned. Both treated and untreated cells were then stained for 'classic' VCP pathology markers, anti-TDP-43, p62/SQSTM1 and LC3 (Abcam, Cambridge, MA).

## Western blot analysis

Protein samples from patient and control myogenic progenitors were extracted using RIPA buffer according to manufacturer's instructions (Thermo Scientific, Rockford, IL). Protein concentrations were identified using the Nanodrop technique according to the manufacturer's protocols. Equal amount of proteins from samples were run on Bis-Tris 4–12% NuPAGE gels using the Novex Mini Cell (Invitrogen Life Technologies, Carlsbad, CA). To confirm differentiation, both pluripotent markers (rabbit monoclonal anti-NANOG and rabbit monoclonal anti-Oct3/4) and myogenic markers (rabbit monoclonal anti-MYF-5, rabbit polyclonal anti-desmin, rabbit monoclonal anti-MyoG, rabbit polyclonal anti-MyoD and rabbit monoclonal anti-MYH2) were analyzed. To analyze expression levels of 'classic' VCP pathology, rabbit monoclonal anti-TDP-43, rabbit monoclonal anti-mTOR, rabbit monoclonal anti-LC3-I/II, rabbit monoclonal anti-p62/SQSTM1, and rabbit monoclonal anti-ubiquitin-specific

antibodies were used. Equal protein loading was confirmed by rabbit monoclonal anti-GAPDH antibody staining. All antibodies were purchased from Abcam. Cytoplasmic and nuclear protein fractions were extracted using NE-PER kit (Thermo Scientific). Densitometry was performed to quantitate the Western blot bands using Image J Program (National Institutes of Health, Bethesda, MD).

## Statistical analysis

Statistical analysis of densitometry data of three Western blot trials was performed using SPSS standard software (Version 13.0, Chicago, SPSS Inc.). Two-tailed student's *t* test was used to calculate *p* values.

## Results

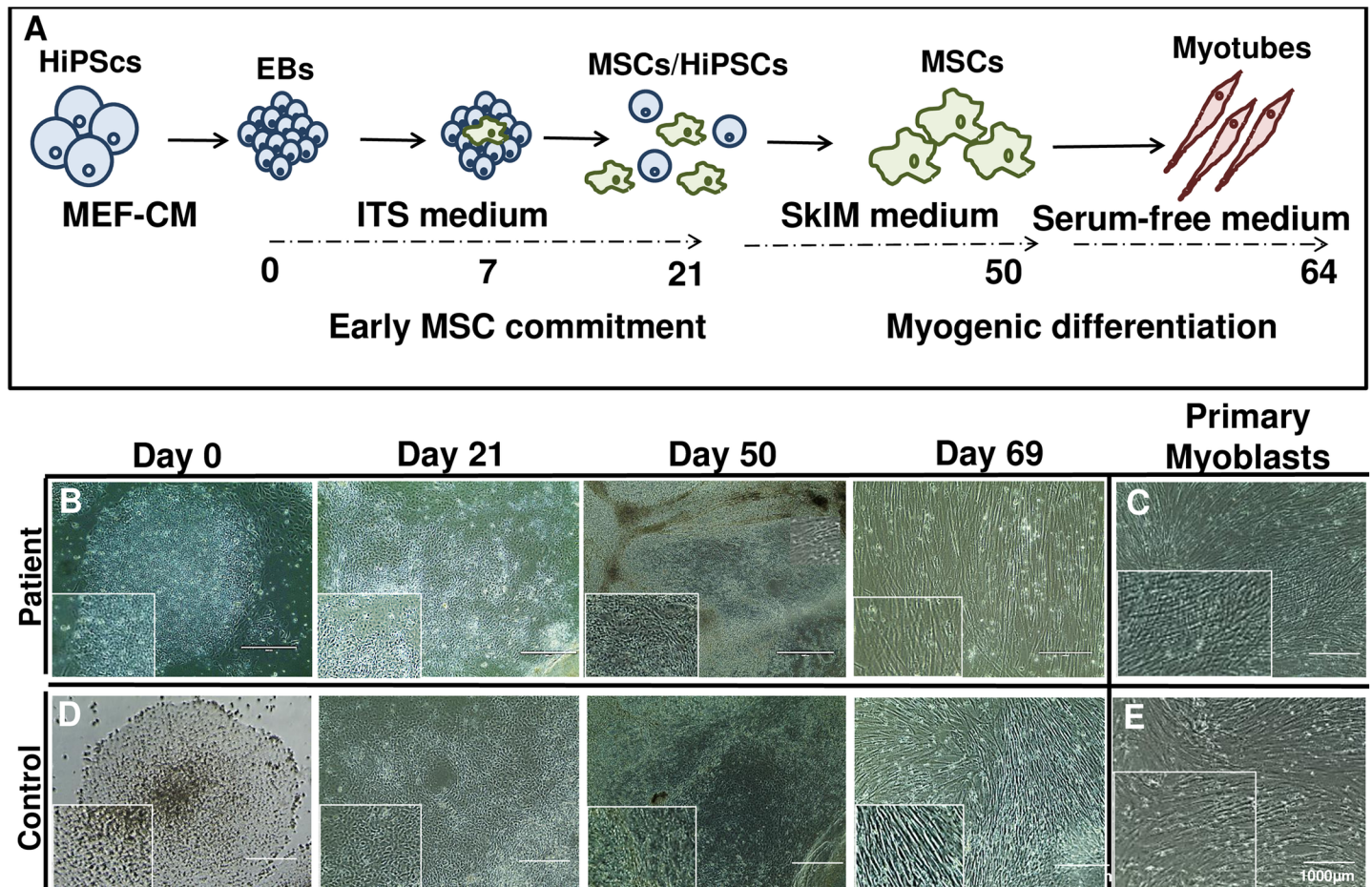
### Differentiation and validation of control and patient iPSC-derived myogenic lineage

Our goal in this study was to establish efficient differentiation of human iPSCs into a myogenic lineage to model VCP disease and related neuromuscular diseases. Additionally, we aimed to utilize these cells in subsequent drug screening studies. A schematic of human iPSC differentiation and maturation into myotubes is depicted in Fig 1A. We first cultured and imaged differentiation of iPSCs (Day 0–69) into a myogenic lineage in patient (Fig 1B) and control iPSC (Fig 1D) lines shown by differential interference contrast (DIC) microscopy. By day 69, these cells closely resembled primary myoblasts taken from the same patient (Fig 1C) or control myoblasts (Fig 1E).

Subsequently, myoblast lineages differentiated from patient-derived inducible pluripotent stem cells (iPSCs) were validated using both immunohistochemical (IHC) and Western blot methods. Staining with early myogenic precursor markers such as MYF-5, desmin, and Pax7 illustrated increased expression from day 21, and they are still expressed at day 50 (Fig 2A). Interestingly, late myogenic skeletal markers, such as MyoD and MYH2 were expressed by day 50, however, not detected at earlier time points (Fig 2B). Western blot analysis revealed the cells had lost expression of their pluripotent marker (Oct3/4 and Nanog) after day 21. Myogenic precursor markers (desmin, MYF5 and MyoD) begin to be expressed starting on day 21 and continue through day 50. Late myogenic markers (MyoG and MYH2) begin to be expressed last at day 50 (Fig 2E). Myogenic differentiation was then validated by FACS analysis using the pluripotent marker (CD34: 96.6% negative) and myoblast marker (CD56: 92.3% positive). Similarly, these cells were also positive for MSC markers (CD73, CD29 and CD105) (Fig 2F).

### Accumulation of autophagy markers in differentiated hiPSC myogenic lineage

The autophagy cascade whereby long-lived proteins are degraded is of critical importance in understanding the possible underlying mechanisms in VCP disease. We and others have previously shown that the autophagic pathway is disrupted in patients' myoblasts and in VCP<sup>R155H/+</sup> mouse models [30, 80, 81]. To determine the pathophysiological effects of VCP mutations on our differentiated disease myogenic lineages, we stained with mAbs specific to TDP-43, a hallmark of VCP pathology, mTOR, and autophagy markers LC3-I/II and p62/SQSTM1. Compared to the control myoblasts, VCP myoblasts showed increased cytoplasmic staining of TDP-43, (Fig 3A and 3B) and increased protein expression levels of the autophagic markers, LC3-I/II and p62/SQSTM1, thereby suggesting impaired degradation of the proteins involved in the autophagosome-lysosomal cascade (Fig 3A and 3B). Western blot analysis and densitometry



**Fig 1. Differentiation of patient and control VCP iPSC into myogenic lineages.** (A) Schematic of early hiPSCs and embryoid bodies commitment (Days 0–50) into early mesenchymal stem/stromal cells (MSC) and differentiation into myoblasts (Day 64). (B) Myogenic differentiation of human iPSC at Days 0, 21, 50, and 69 with (C) primary myoblast cells from a 57-year old patient diagnosed with IBMPFD. (D) Control derived myogenic precursors at Days 0, 21, 50, and 69 with (E) primary myoblast cells from age matched healthy control. Differential interference contrast (DIC) microscopy images of differentiated primary mature myoblasts from iPSCs. Scale: Bar = 1000 µm.

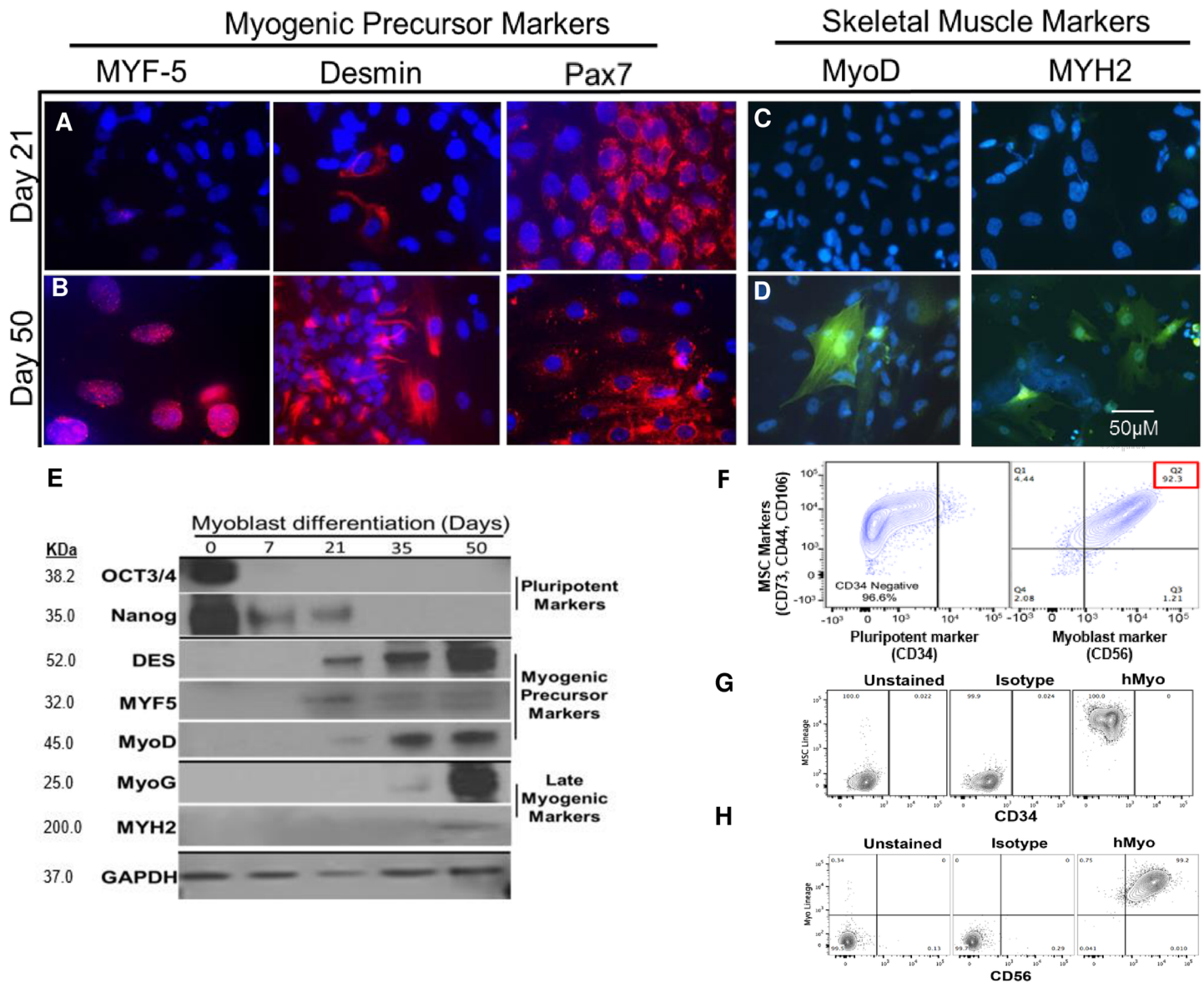
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analysis of Western blot data relative to GAPDH, confirmed these results with significant increased TDP-43 ( $p < 0.001$ , both day 21 and 50), LC3I/II ( $p < 0.005$  on day 21,  $p < 0.001$  on day 50), p62/SQSTM1 ( $p < 0.001$ , both day 21 and 50) and ubiquitin ( $p < 0.001$ , both day 21 and 50) (Fig 3C and 3D) as well as the cytoplasmic mislocalization of TDP-43 in VCP myoblasts (Fig 3E).

### Autophagy modifiers and VCP's interplay in the autophagy cascade

Several studies showed that the autophagy cascade is highly dysregulated in VCP disease [30, 82–84]. However, the underlying mechanisms of such dysregulation remain to be fully elucidated. We, therefore, decided to utilize our differentiated patient-derived hiPSCs together with several autophagy inhibitors and activators that interact as various different points in the autophagy cascade (Fig 4A) to gain insight in the pathogenesis and explore if autophagy modulation could ameliorate VCP disease pathology. These modifiers and the location of their impact within the autophagy cascade are illustrated in Fig 4A.



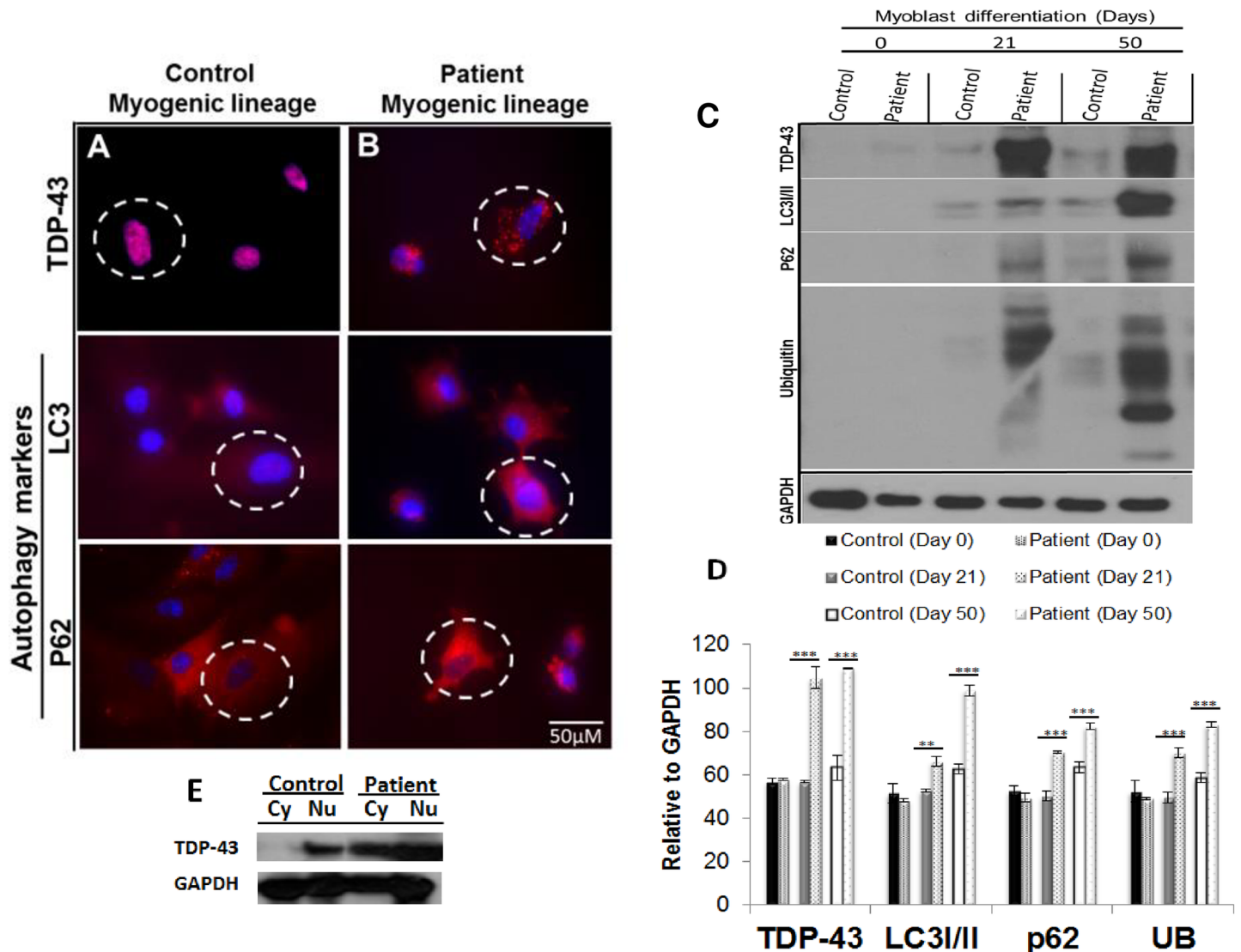


**Fig 2. Validation of myogenic differentiation in patient and control-derived iPSCs.** Human iPSC from a 57-year old patient diagnosed with VCP disease-derived myogenic precursors were stained at Day 21 and Day 50 with (A-B) MYF-5, desmin, and Pax7; (C-D) MyoD and MYH2. Representative merged overlay images of stained iPSC with DAPI. Scale: Bar = 50  $\mu$ M. (E) Western blot analysis of myoblast differentiation markers at Day 0, 7, 21, 35, and 50 with anti-Oct3/4, Nanog, desmin, MYF5, MyoD, MyoG and MYH2. GAPDH was used as a positive loading control. (F) FACS analysis of iPSC-derived MSCs with pluripotent marker (CD34) and myoblast marker (CD56). (G) CD34 isotype control. (H) CD65 isotype control.

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## Drug screenings with autophagy-modifiers

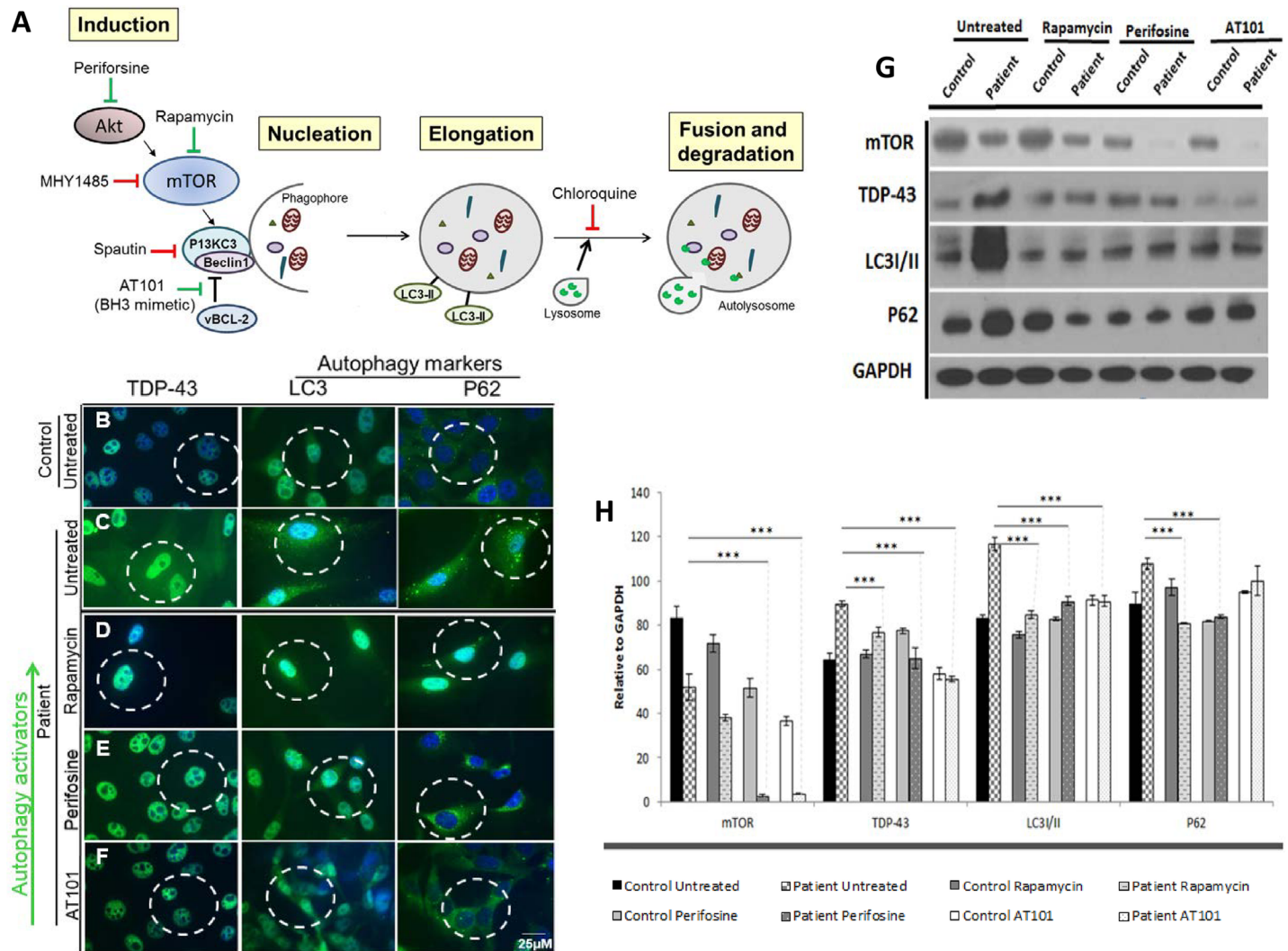
To explore the effects of autophagy-modifying drugs on the iPSC-derived patient VCP myoblasts, we treated them with autophagy stimulators Rapamycin, Perifosine, and AT101 (Fig 4) and autophagy inhibitors chloroquine, Spautin-1, and MHY1485 (Fig 5). These autophagy modifiers were selected to help understand the pathogenesis of VCP disease because they target the autophagy cascade and its intermediates at various locations (Fig 4A). Rapamycin, a key modulator of the mammalian Target of Rapamycin (mTOR) pathway has shown neuroprotection properties in several neurodegenerative diseases, including Alzheimer's disease,



**Fig 3. Characterization of autophagy signaling cascade in control and patient VCP iPSC-derived myoblast lineages.** Differentiated (A) control and (B) iPSC-derived myoblast lineages were immunostained with TDP-43, LC3, and p62/SQSTM1. Representative merged overlay images of stained iPSC with DAPI. Scale: Bar = 50  $\mu$ M. (C) Western blot analysis of iPSC-derived control and patient myoblasts with anti-TDP-43, LC3/II, p62/SQSTM1, and ubiquitin. GAPDH was used as a positive loading control. (D) Densitometry analyses confirmed these Western blot results. Statistical significance is denoted by \* $p < 0.05$ , \*\* $p < 0.005$  and \*\*\* $p < 0.001$ . (E) Western blot analysis of cytoplasmic (Cy) and nuclear (Nu) fractions of iPSC-derived control and patient myoblasts with anti-TDP-43.

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Parkinson’s disease, Huntington’s disease and spinocerebellar ataxia type 3 [71, 72, 85]. Rapamycin functions by inhibiting mTOR whose activation inhibits autophagy [86]. The accumulation of damaged proteins and the failure of autophagy clearing is a hallmark of VCP-associated diseases; therefore, we hypothesized that Rapamycin treatment may show some benefit. Here, we treated our cells with 10  $\mu$ M Rapamycin for 24 hours and demonstrate significant reduction in the ‘classic’ VCP pathology markers TDP-43, LC3 and p62/SQSTM1, while mTOR activity was slightly diminished in VCP when compared to untreated patient samples (Fig 4B–4D). These results were confirmed by Western blot and densitometry (Fig 4G and 4H). Perifosine inhibits mTOR signaling through a different mechanism than classical mTOR inhibitors such as Rapamycin. Perifosine is an

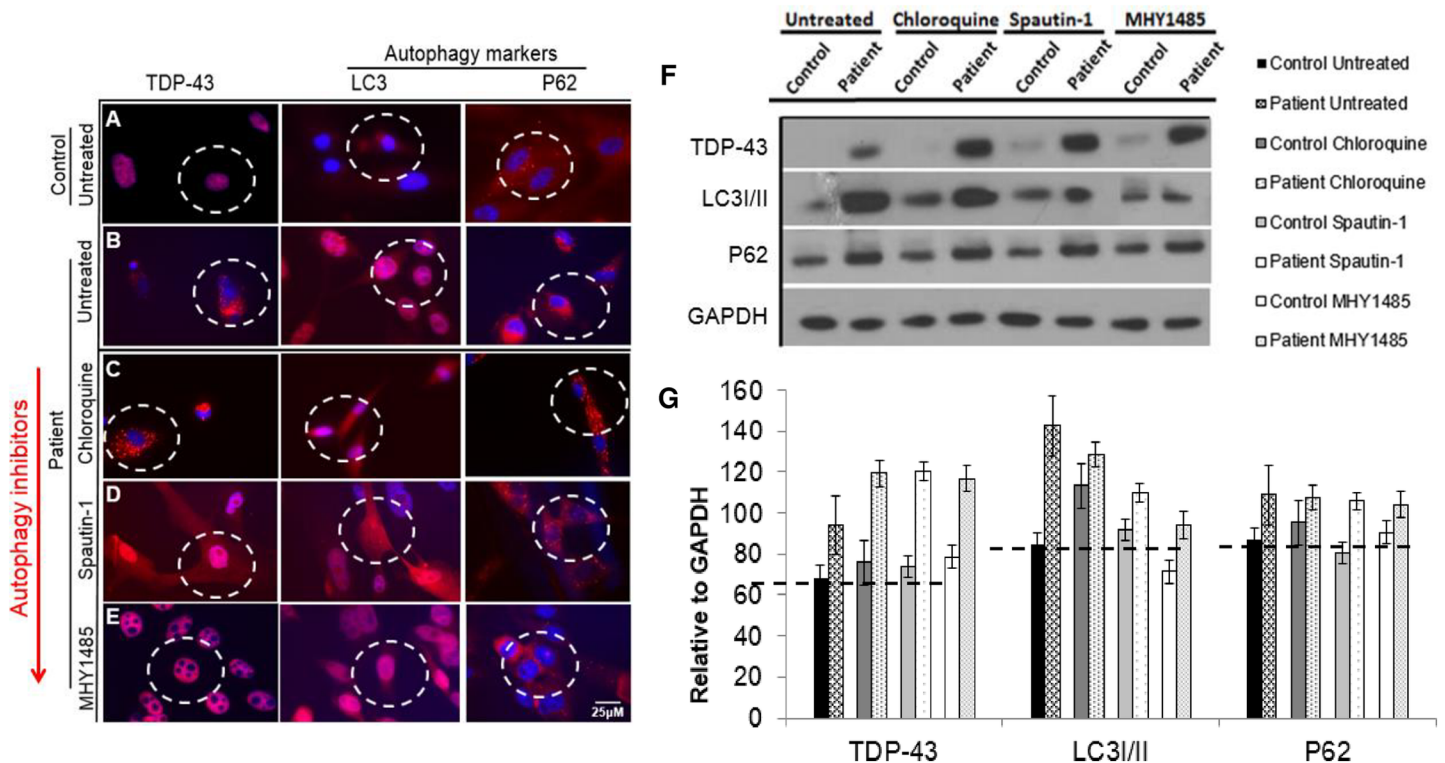


**Fig 4. Drug screening with autophagy inducers Rapamycin, Periforsine and AT101 in patient VCP iPSC-derived myogenic lineages.** (A) Schematic of intervention with autophagy modifying agents. Green arrows show the active location of autophagy activators Rapamycin, Periforsine and AT101. Red arrows show the active location of autophagy inhibitors chloroquine, Spautin-1 and MHY1485. VCP is indicated to have an interactive role with Akt and as a chaperone protein with ubiquitin. (B) Untreated differentiated control and (C) untreated patient derived myogenic lineages. Patient derived myogenic lineage were treated with either (D) Rapamycin (10 μM), (E) Periforsine (80 μM) or (F) AT101 (10 μM) for 24 hours. Subsequently, cells were stained with TDP-43, LC3 or p62/SQSTM1 antibodies. Representative merged overlay images of stained iPSC with DAPI. Scale: Bar = 25 μm. White dotted lines represent areas of increased or decreased expressions. (G) Western blot analysis of iPSC-derived control (C) and patient (P) myoblasts with mTOR, TDP-43, LC3I/II and p62/SQSTM1. GAPDH was used as a positive loading control. (H) Densitometry analyses of the Western blot. Black dotted line indicates expression over baseline control sample. Statistical significance is denoted by \*p<0.05, \*\*p<0.005 and \*\*\*p<0.001.

<https://doi.org/10.1371/journal.pone.0176919.g004>

alkylphospholipid, which induces cell cycle arrest and apoptosis through the inhibition of the serine-threonine protein kinase (Akt) also known as protein kinase B [73]. Several publications have shown that Akt and VCP interact and that VCP is a target of Akt signaling [87, 88], therefore we hypothesized that inhibition of Akt may be beneficial for VCP disease. In this report, we examined the effects of Periforsine by treating our cells (80 μM) and showed reduction in the “classic” VCP pathology markers TDP-43, LC3 and p62/SQSTM1 when compared to untreated patient samples (Fig 4B, 4C and 4E). These results were confirmed by Western blot and densitometry with p<0.001 (Fig 4G and 4H).





**Fig 5. Drug screening with autophagy inhibitors chloroquine, Spautin-1, and MYH1485 shows in patient VCP iPSC-derived myoblast lineages.** (A) Untreated differentiated control and (B) untreated patient derived myogenic lineages. Patient derived myogenic lineage were treated with either (C) chloroquine (10  $\mu$ M), (D) Spautin-1 (10  $\mu$ M) or (E) MYH1485 (2  $\mu$ M) for 24 hours. Subsequently, cells were stained with TDP-43, LC3 or p62/SQSTM1 antibodies. Representative merged overlay images of stained iPSC with DAPI. Scale: Bar = 25  $\mu$ m. White dotted lines represent areas of increased or decreased expressions. (F) Western blot analysis of iPSC-derived control and patient myoblasts probed against TDP-43, LC3-I/II, and p62/SQSTM1 antibodies. GAPDH was used as a positive loading control. (G) Densitometry analyses from Western blot. Black dotted line indicates expression over baseline control sample.

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Furthermore, we examined the effects of AT101, an orally available and well-tolerated natural BH3-mimetic that activates Bax and also induces mitochondrial Smac release in our *in vitro* model [75]. Here, we treated our cells with 10  $\mu$ M AT101 and show reduction in VCP pathology marker TDP-43 and less so autophagy markers LC3 and p62/SQSTM1 when compared to untreated patients ( $p < 0.001$ ) (Fig 4B, 4C, 4F, 4G and 4H).

Chloroquine is a lysosomotropic agent that prevents endosomal acidification [89]. Chloroquine inhibits autophagy as it raises the lysosomal pH, which leads to inhibition of both fusion of autophagosome with lysosome and lysosomal protein degradation [90, 91]. To uncover if chloroquine could reduce VCP pathology by inhibiting the formation of autolysosome, thought to be the “classic” vacuole associated with VCP disease, we treated our cells with 10  $\mu$ M of chloroquine. Herein, we show chloroquine has no effect on VCP pathology markers TDP-43, LC3 and p62/SQSTM1 when compared to untreated patient myoblasts (Fig 5A–5C). These results were confirmed by Western blot and densitometry (Fig 5F and 5G). Spautin-1 inhibits the activity of two ubiquitin-specific peptidases, USP10 and USP13, causing an increase in proteasomal degradation of class III PI3 kinase complexes, which have been shown to regulate autophagy [78]. To uncover if Spautin-1 could reduce VCP pathology by inhibiting autophagy downstream of Akt and mTOR we treated our cells with 10  $\mu$ M Spautin-1. We found Spautin-1 had no effect on VCP pathology markers TDP-43, LC3 and p62/SQSTM1 when compared to the untreated patient myoblasts (Fig 5A, 5B and 5D). These results were confirmed by Western blot and



densitometry (Fig 5F and 5G). MHY1485 is an mTOR activator that potently inhibits autophagy by suppression of fusion between autophagosomes and lysosomes [79]. To uncover if MHY1485 could reduce VCP pathology by inhibiting autophagy by inducing mTOR we treated our cells with 2 $\mu$ M MHY1485. In this report, we show MHY1485 has no effect on VCP pathology markers LC3 and p62/SQSTM1 when compared to untreated patient myoblasts (Fig 5A, 5B and 5E), however, TDP-43 was seen to be expressed correctly in the nucleus (Fig 5A, 5B and 5E). Although, spatial expression of TDP-43 was corrected, interestingly Western blot revealed its overexpression in our patient cells treated with MHY1485 (Fig 5F and 5G). LC3 and p62/SQSTM1 overexpression levels were confirmed by Western blot and densitometry (Fig 5F and 5G).

## Discussion

Human induced pluripotent stem cells (hiPSCs) represent a versatile model system for studying diseases that affect a number of organs. When given the proper stimuli, hiPSCs can be differentiated into a number of desired cell types and tissues. Moreover, the consistency, expandability, and purity of the hiPSCs provide a valuable tool to screen and test drugs *in vitro*. Therefore, developing robust iPSC models of both rare and common disorders is an excellent option for those diseases requiring poorly accessible and/or limited availability tissue samples. Due to the pleiotropic nature of VCP disease, we recently established iPSC lines to elucidate the pathobiology and cellular and molecular mechanisms underlying this disease [55]. In the present study, we report the differentiation of VCP disease-specific hiPSCs into a myogenic lineage for the discovery of the underlying molecular mechanisms and the development of a drug-screening assay offering the significant possibility to intervene in the early stages of the disease. Ultimately, knowledge of the cellular and molecular signaling pathways affected by VCP mutations provides future promise in the development, assessment, and clinical application of pharmacological and gene therapies to prevent or slow down the progression of VCP disease.

We previously reported differentiation and characterization of VCP patient hiPSCs into a neural lineage [55]. These differentiated neural cells showed all the typical hallmarks of VCP pathology, including increased p62/SQSTM1, LC3-I/II and TDP-43. Inclusion body myopathy (IBM) is the most common feature present in 80–90% of affected VCP patients [5, 31]. Typically, the progressive muscle weakness rapidly advances resulting in patient mortality from cardiomyopathy or respiratory failure between approximately 40–60 years of age. The differentiation of human iPSCs into skeletal muscle cells has been challenging with methods ranging from serial media changes to viral infection of myogenic genes such as Pax7 [47, 64, 68]. Of note, an interesting observation we made was that staining with early myogenic precursor markers such as MYF-5, desmin, and Pax7 illustrated increased expression from Day 21, and they are still expressed at Day 50. Late myogenic markers (skeletal markers), such as MyoD and MYH2 were expressed by Day 50, however, not observed at earlier time points. However, a literature search suggested that Pax7 was expressed in the cytoplasm during different cell cycle stages and development [92, 93]. We hypothesize that these cells, aptly named myogenic lineage cells are not fully differentiated yet. However, we do believe they are suitable for our purpose of drug screening as they do display ‘classic’ VCP-associated disease pathology features. The reduced desmin organization and reduced MyoD expression are likely due to these cells not reaching full maturity.

To the best of our knowledge, this is the first article to fully differentiate patient-derived hiPSCs into a myogenic lineage modeling VCP-associated myopathy. These cells are 92% positive for CD56+ (myogenic markers) and mesenchymal stem cell markers (MSC+). Notably, we

used these myogenic lineages to characterize the phenotypical features observed in humans. VCP disease muscle pathology is characterized by the presence of rimmed vacuoles, ubiquitin and TDP-43 positive inclusions and increased autophagy markers p62/SQSTM1 and LC3 indicating a potentially dysfunctional autophagy pathway. Autophagy plays an important role in degrading defective organelles. Recent studies have shown that p62/SQSTM1 interacts with the autophagic effector protein LC3-I/II to mediate the autophagic uptake of aggregated proteins. Previous researchers have shown that expression of VCP disease mutant proteins results from autophagosome accumulation and that these autophagosomes fail to mature into autophagolysosomes and degrade LC3; indicating autophagy is impaired in VCP disease [94]. Thus, we characterized the autophagy cascade and observed that the differentiated VCP myogenic lineage demonstrated a significant increase in the expression of p62/SQSTM1, LC3-I/II, and ubiquitin in comparison with the control myogenic lineage. There was also translocation of TDP-43 from the nucleus to the cytoplasm, another hallmark of VCP pathology thus suggesting that this patient derived myogenic lineage displays the typical of VCP disease pathology.

In the present study, we also examined the development of a rapid drug-screening assay to understand the true interactions of the underlying molecular mechanisms of VCP disease and how to target them in hopes to discover potential treatments for VCP disease. Our work as well as work from others indicating dysfunctional autophagy as the instigator of VCP muscle pathology led us to use our drug screening assay to target the autophagy pathway with potent inhibitors and activators [5, 30, 33, 83, 94].

Firstly, we investigated the activation of autophagy by treating our differentiated cells with autophagy stimulators Rapamycin, Perifosine, or AT101 [71–73, 75]. Rapamycin a key modulator of the mammalian Target of Rapamycin (mTOR) pathway has shown significant promise and neuroprotection in Alzheimer's disease, Parkinson's disease, Huntington's disease and spinocerebellar ataxia type 3 [71, 72, 85]. Rapamycin associates with mTORs intracellular receptor FKBP12 [95]. The FKBP12-rapamycin complex binds directly to the FKBP12-Rapamycin Binding (FRB) domain of mTOR, inhibiting its activity [95]. mTOR activation inhibits autophagy, however it simultaneously stimulates protein synthesis and cell growth which can result in accumulations of damaged proteins and organelles [86]. The accumulation of damaged proteins and the failure of autophagy clearing is a 'hallmark' of VCP disease and we hypothesized that Rapamycin treatment may ameliorate the dysfunctional autophagy cascade as it is upstream of VCP involvement in autophagy. Treatment with 10  $\mu$ M of Rapamycin for 24 hours showed a reduction in the "classic" VCP pathology markers TDP-43, LC3 and p62/SQSTM1 when compared to untreated patient, mirroring untreated control. Several publications have now shown that serine-threonine protein kinase (Akt) also known as protein kinase B and VCP interact and that VCP is a target of Akt signaling. Vandermoere *et al.* (2006) identified VCP as an essential target of Akt signaling and demonstrated that Akt and VCP co-immunoprecipitated and co-localized under Akt activation in MCF-7 breast cancer cells. In addition, they identified Ser-351, Ser-745, and Ser-747 as Akt phosphorylation sites on VCP via site-directed mutagenesis [87, 88]. We, therefore, hypothesized that inhibition of Akt may be beneficial for VCP disease. Perifosine is an alkylphospholipid which induces cell cycle arrest and apoptosis through the inhibition of Akt [73]. mTOR is a target for Akt, the activation of which suppresses autophagy. In the event of ER stress such as in VCP disease, mTOR is innately downgraded to suppress protein production. All treatments with autophagy activators result in further reduction in mTOR, we hypothesize that by mechanistically reducing protein production mTOR inhibitors can improve VCP disease pathology. Perifosine has also been shown to exhibit anti-cancer properties, in bladder cancer, hepatocellular carcinoma and lung cancer [73, 96–98]. Treatment with 80  $\mu$ M Perifosine showed reduction in the "classic" VCP pathology markers TDP-43, LC3I/II and p62/SQSTM1 when compared to untreated patient,

mirroring the untreated controls. Our last autophagy inducer, AT101, is an orally available and well-tolerated natural BH3-mimetic that activates Bax and also induces mitochondrial Smac release [75]. AT101 has shown anti-tumor activity as a single agent and in combination with standard anticancer drugs in a variety of tumor models [74]. We hypothesize that as AT101 works downstream of both Rapamycin and Perifosine, it may be more effective in ameliorating VCP pathology. We found treatment with 10  $\mu$ M AT101 showed a reduction in VCP pathology markers TDP-43, LC3 and p62/*SQSTM1* when compared to untreated patient, mirroring untreated control. The results from these drug studies suggest that autophagy modulation, in particular autophagy activation agents, may be beneficial for patients suffering from VCP disease myopathy.

Secondly, we investigated the inhibition of autophagy by treating our differentiated cells with autophagy inhibitors chloroquine, Spautin-1 and MHY1485. Chloroquine is a lysosomotropic agent that prevents endosomal acidification [89]. Chloroquine inhibits autophagy by raising the lysosomal pH, which leads to inhibition of both fusion of autophagosome with lysosome and lysosomal protein degradation [90, 91]. Herein, we demonstrate chloroquine has no effect on VCP pathology markers TDP-43, LC3 and p62/*SQSTM1* when compared to untreated patient. Our second treatment was with Spautin-1 which promotes the degradation of Vps34 PI3 kinase complexes by inhibiting two ubiquitin specific peptidases, USP10 and USP13, which target the Beclin1 subunit of Vps34 complexes. Since USP10 mediates the de-ubiquitination of p53, regulating de-ubiquitination activity of USP10 and USP13 by Beclin1 provides a mechanism for Beclin1 to control the levels of p53. By this mechanism, Spautin-1 increased cancer cell death in the setting of nutrient deprivation when autophagy would normally act as a survival mechanism in these metabolically stressed cells [78]. We discovered treatment with Spautin-1 (10  $\mu$ M for 24 hours) had no effect on VCP pathology markers TDP-43, LC3 and p62/*SQSTM1* when compared to untreated patient. Lastly we tested MHY1485, which is an mTOR activator that potentially also inhibits autophagy by suppression of fusion between autophagosomes and lysosomes [79]. To uncover if MHY1485 could reduce VCP pathology, by inhibiting autophagy, by inducing mTOR, we treated our cells with MHY1485. We hypothesized that it could have a negative effect, as previous studies showed MHY1485 leads to the accumulation of LC3-I/II and enlargement of the autophagosomes in a dose- and time- dependent manner [79]. Interestingly, we demonstrated that treatment with MHY1485 (2  $\mu$ M for 24 hours) had a modest beneficial effect on VCP pathology with a decrease in markers LC3 and p62. Also, TDP-43 was observed to be expressed in the correct location (nucleus), mirroring our control cells. However, although spatial expression of TDP-43 was corrected, Western blot revealed it is still overexpressed in our patient cells treated with MHY1485. Future studies with varying MHY1485 doses and treatment times will be needed to uncover if MHY1485 could be beneficial for VCP patients. Future in-depth studies into the autophagy and mitophagy cascades, cell survival and animal studies to investigate the effects of these drugs on muscle function and structure need to be completed.

## Summary and conclusions

Herein, we report the successful differentiation of VCP patient specific hiPSCs into myoblasts exhibiting phenotype and dysfunction characteristics of typical VCP disease autophagy and thus providing a novel platform for a rapid drug screening. Evaluation of the ameliorative effects of several autophagy modifiers using these cells as a drug-screening platform revealed that some autophagy modulators may hold promise for VCP disease myopathy. The VCP hiPSC model system offers a unique platform in understanding the underlying pathophysiology molecular mechanisms and vistas in improving muscle integrity and/or slowing down the progression of muscle wasting in patients with VCP and related neurodegenerative diseases.

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## Author Contributions

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## References

1. Watts GD, Wymer J, Kovach MJ, Mehta SG, Mumm S, Darvish D, et al. Inclusion body myopathy associated with Paget disease of bone and frontotemporal dementia is caused by mutant valosin-containing protein. *Nature Genet.* 2004; 36(4):377–81. <https://doi.org/10.1038/ng1332> PMID: 15034582
2. Kimonis VE, Watts GD. Autosomal dominant inclusion body myopathy, Paget disease of bone, and frontotemporal dementia. *Alzheimer disease and associated disorders.* 2005; 19 Suppl 1:S44–7.
3. Kimonis VE, Kovach MJ, Waggoner B, Leal S, Salam A, Rimer L, et al. Clinical and molecular studies in a unique family with autosomal dominant limb-girdle muscular dystrophy and Paget disease of bone. *Genet Med.* 2000; 2(4):232–41. <https://doi.org/10.1097/00125817-200007000-00006> PMID: 11252708
4. Kimonis VE, Fulchiero E, Vesa J, Watts G. VCP disease associated with myopathy, Paget disease of bone and frontotemporal dementia: review of a unique disorder. *Biochimica et Biophysica Acta.* 2008; 1782(12):744–8. <https://doi.org/10.1016/j.bbadis.2008.09.003> PMID: 18845250
5. Nalbandian A, Donkervoort S, Dec E, Badadani M, Katheria V, Rana P, et al. The multiple faces of valosin-containing protein-associated diseases: inclusion body myopathy with Paget's disease of bone, frontotemporal dementia, and amyotrophic lateral sclerosis. *J Mol Neurosci.* 2011; 45(3):522–31. <https://doi.org/10.1007/s12031-011-9627-y> PMID: 21892620
6. Kimonis VE, Mehta SG, Fulchiero EC, Thomasova D, Pasquali M, Boycott K, et al. Clinical studies in familial VCP myopathy associated with Paget disease of bone and frontotemporal dementia. *Am J Med Genet.* 2008; 146A(6):745–57. <https://doi.org/10.1002/ajmg.a.31862> PMID: 18260132
7. Ralston SH, Langston AL, Reid IR. Pathogenesis and management of Paget's disease of bone. *Lancet.* 2008; 372(9633):155–63. [https://doi.org/10.1016/S0140-6736\(08\)61035-1](https://doi.org/10.1016/S0140-6736(08)61035-1) PMID: 18620951
8. Ralston SH. Pathogenesis of Paget's disease of bone. *Bone.* 2008; 43(5):819–25. <https://doi.org/10.1016/j.bone.2008.06.015> PMID: 18672105
9. Mirra JM, Brien EW, Tehranzadeh J. Paget's disease of bone: review with emphasis on radiologic features, Part I. *Skeletal radiology.* 1995; 24(3):163–71. PMID: 7610408

10. Kimonis V, Donkervoort S, Watts G. Inclusion Body Myopathy Associated with Paget Disease of Bone and/or Frontotemporal Dementia. *Gene Reviews*. University of Washington, Seattle. 2007 [updated 2011];1993–2017.
11. Rosso SM, Kamphorst W, de Graaf B, Willemsen R, Ravid R, Niermeijer MF, et al. Familial frontotemporal dementia with ubiquitin-positive inclusions is linked to chromosome 17q21-22. *Brain*. 2001; 124 (Pt 10):1948–57. PMID: [11571213](https://pubmed.ncbi.nlm.nih.gov/11571213/)
12. Neumann M, Mackenzie IR, Cairns NJ, Boyer PJ, Markesbery WR, Smith CD, et al. TDP-43 in the ubiquitin pathology of frontotemporal dementia with VCP gene mutations. *J Neuropathol Exp Neurol*. 2007; 66(2):152–7. <https://doi.org/10.1097/nen.0b013e31803020b9> PMID: [17279000](https://pubmed.ncbi.nlm.nih.gov/17279000/)
13. Forman MS, Mackenzie IR, Cairns NJ, Swanson E, Boyer PJ, Drachman DA, et al. Novel ubiquitin neuropathology in frontotemporal dementia with valosin-containing protein gene mutations. *J Neuropathol Exp Neurol*. 2006; 65(6):571–81. PMID: [16783167](https://pubmed.ncbi.nlm.nih.gov/16783167/)
14. Majounie E, Traynor BJ, Chio A, Restagno G, Mandrioli J, Benatar M, et al. Mutational analysis of the VCP gene in Parkinson's disease. *Neurobiol Aging*. 2012; 33(1):209 e1–2.
15. Nacmias B, Piaceri I, Bagnoli S, Tedde A, Piacentini S, Sorbi S. Genetics of Alzheimer's Disease and Frontotemporal Dementia. *Curr Mol Med*. 2014; 14(8):993–1000. <https://doi.org/10.2174/1566524014666141010152143> PMID: [25323872](https://pubmed.ncbi.nlm.nih.gov/25323872/)
16. Shamirian S, Nalbandian A, Khare M, Castellani R, Kim R, Kimonis VE. Early-onset Alzheimers and cortical vision impairment in a woman with valosin-containing protein disease associated with 2 APOE epsilon4/APOE epsilon4 genotype. *Alzheimer disease and associated disorders*. 2015; 29(1):90–3. <https://doi.org/10.1097/WAD.0b013e318298e54f> PMID: [23715207](https://pubmed.ncbi.nlm.nih.gov/23715207/)
17. Williams KL, Solski JA, Nicholson GA, Blair IP. Mutation analysis of VCP in familial and sporadic amyotrophic lateral sclerosis. *Neurobiol Aging*. 2012; 33(7):1488.e15–1488.e16.
18. Van Langenhove T, Van der Zee J, Van Broeckhoven C. The molecular basis of the frontotemporal lobar degeneration-amyotrophic lateral sclerosis spectrum. *Ann Med*. 2012; 44(8):817–28. <https://doi.org/10.3109/07853890.2012.665471> PMID: [22420316](https://pubmed.ncbi.nlm.nih.gov/22420316/)
19. Djamshidian A, Schaefer J, Haubenberger D, Stogmann E, Zimprich F, Auff E, et al. A novel mutation in the VCP gene (G157R) in a German family with inclusion-body myopathy with Paget disease of bone and frontotemporal dementia. *Muscle & nerve*. 2009; 39(3):389–91.
20. Schroder R, Watts GD, Mehta SG, Evert BO, Broich P, Fliessbach K, et al. Mutant valosin-containing protein causes a novel type of frontotemporal dementia. *Annals of neurology*. 2005; 57(3):457–61. <https://doi.org/10.1002/ana.20407> PMID: [15732117](https://pubmed.ncbi.nlm.nih.gov/15732117/)
21. Stojkovic T, Hammouda el H, Richard P, Lopez de Munain A, Ruiz-Martinez J, Gonzalez PC, et al. Clinical outcome in 19 French and Spanish patients with valosin-containing protein myopathy associated with Paget's disease of bone and frontotemporal dementia. *Neuromuscul Disord*. 2009; 19(5):316–23. <https://doi.org/10.1016/j.nmd.2009.02.012> PMID: [19364651](https://pubmed.ncbi.nlm.nih.gov/19364651/)
22. Haubenberger D, Bittner RE, Rauch-Shorny S, Zimprich F, Mannhalter C, Wagner L, et al. Inclusion body myopathy and Paget disease is linked to a novel mutation in the VCP gene. *Neurology*. 2005; 65 (8):1304–5. <https://doi.org/10.1212/01.wnl.0000180407.15369.92> PMID: [16247064](https://pubmed.ncbi.nlm.nih.gov/16247064/)
23. Bersano A, Del Bo R, Lamperti C, Ghezzi S, Fagioli G, Fortunato F, et al. Inclusion body myopathy and frontotemporal dementia caused by a novel VCP mutation. *Neurobiol Aging*. 2009; 30(5):752–8. <https://doi.org/10.1016/j.neurobiolaging.2007.08.009> PMID: [17889967](https://pubmed.ncbi.nlm.nih.gov/17889967/)
24. Miller TD, Jackson AP, Barresi R, Smart CM, Eugenicos M, Summers D, et al. Inclusion body myopathy with Paget disease and frontotemporal dementia (IBMPFD): clinical features including sphincter disturbance in a large pedigree. *J Neurol Neurosurg Psychiatry*. 2009; 80(5):583–4. <https://doi.org/10.1136/jnnp.2008.148676> PMID: [19372299](https://pubmed.ncbi.nlm.nih.gov/19372299/)
25. Kumar KR, Needham M, Mina K, Davis M, Brewer J, Staples C, et al. Two Australian families with inclusion-body myopathy, Paget's disease of bone and frontotemporal dementia: novel clinical and genetic findings. *Neuromuscul Disord*. 2010; 20(5):330–4. <https://doi.org/10.1016/j.nmd.2010.03.002> PMID: [20335036](https://pubmed.ncbi.nlm.nih.gov/20335036/)
26. Kim EJ, Park YE, Kim DS, Ahn BY, Kim HS, Chang YH, et al. Inclusion body myopathy with Paget disease of bone and frontotemporal dementia linked to VCP p.Arg155Cys in a Korean family. *Arch Neurol*. 2011; 68(6):787–96. <https://doi.org/10.1001/archneurol.2010.376> PMID: [21320982](https://pubmed.ncbi.nlm.nih.gov/21320982/)
27. Wehl CC, Pestronk A, Kimonis VE. Valosin-containing protein disease: inclusion body myopathy with Paget's disease of the bone and fronto-temporal dementia. *Neuromuscul Disord*. 2009; 19(5):308–15. <https://doi.org/10.1016/j.nmd.2009.01.009> PMID: [19380227](https://pubmed.ncbi.nlm.nih.gov/19380227/)
28. Watts GD, Thorne M, Kovach MJ, Pestronk A, Kimonis VE. Clinical and genetic heterogeneity in chromosome 9p associated hereditary inclusion body myopathy: exclusion of GNE and three other candidate genes. *Neuromuscul Disord*. 2003; 13(7–8):559–67. PMID: [12921793](https://pubmed.ncbi.nlm.nih.gov/12921793/)



29. Watts GD, Thomasova D, Ramdeen SK, Fulchiero EC, Mehta SG, Drachman DA, et al. Novel VCP mutations in inclusion body myopathy associated with Paget disease of bone and frontotemporal dementia. *Clin Genet*. 2007; 72(5):420–6. <https://doi.org/10.1111/j.1399-0004.2007.00887.x> PMID: 17935506
30. Vesa J, Su H, Watts GD, Krause S, Walter MC, Martin B, et al. Valosin containing protein associated inclusion body myopathy: abnormal vacuolization, autophagy and cell fusion in myoblasts. *Neuromuscul Disord*. 2009; 19(11):766–72. <https://doi.org/10.1016/j.nmd.2009.08.003> PMID: 19828315
31. Mehta SG, Khare M, Ramani R, Watts GD, Simon M, Osann KE, et al. Genotype-phenotype studies of VCP-associated inclusion body myopathy with Paget disease of bone and/or frontotemporal dementia. *Clin Genet*. 2013; 83(5):422–31. <https://doi.org/10.1111/cge.12000> PMID: 22909335
32. Braun RJ, Zischka H. Mechanisms of Cdc48/VCP-mediated cell death: from yeast apoptosis to human disease. *Biochimica et biophysica acta*. 2008; 1783(7):1418–35. <https://doi.org/10.1016/j.bbamcr.2008.01.015> PMID: 18284922
33. Meyer H, Bug M, Bremer S. Emerging functions of the VCP/p97 AAA-ATPase in the ubiquitin system. *Nat Cell Biol*. 2012; 14(2):117–23. <https://doi.org/10.1038/ncb2407> PMID: 22298039
34. Fujita E, Kouroku Y, Isoai A, Kumagai H, Misutani A, Matsuda C, et al. Two endoplasmic reticulum-associated degradation (ERAD) systems for the novel variant of the mutant dysferlin: ubiquitin/proteasome ERAD(I) and autophagy/lysosome ERAD(II). *Hum Mol Genet*. 2007; 16(6):618–29. <https://doi.org/10.1093/hmg/ddm002> PMID: 17331981
35. Kim NC, Tresse E, Kolaitis RM, Molliex A, Thomas RE, Alami NH, et al. VCP is essential for mitochondrial quality control by PINK1/Parkin and this function is impaired by VCP mutations. *Neuron*. 2013; 78(1):65–80. <https://doi.org/10.1016/j.neuron.2013.02.029> PMID: 23498974
36. Braun RJ, Zischka H, Madeo F, Eisenberg T, Wissing S, Buttner S, et al. Crucial mitochondrial impairment upon CDC48 mutation in apoptotic yeast. *The Journal of biological chemistry*. 2006; 281(35):25757–67. <https://doi.org/10.1074/jbc.M513699200> PMID: 16822868
37. Bartolome F, Wu HC, Burchell VS, Preza E, Wray S, Mahoney CJ, et al. Pathogenic VCP mutations induce mitochondrial uncoupling and reduced ATP levels. *Neuron*. 2013; 78(1):57–64. <https://doi.org/10.1016/j.neuron.2013.02.028> PMID: 23498975
38. Angelos MG, Kaufman DS. Pluripotent stem cell applications for regenerative medicine. *Curr Opin Organ Transplant*. 2015; 20(6):663–70. <https://doi.org/10.1097/MOT.0000000000000244> PMID: 26536430
39. Abramson S, Miller RG, Phillips RA. The identification in adult bone marrow of pluripotent and restricted stem cells of the myeloid and lymphoid systems. *The Journal of experimental medicine*. 1977; 145(6):1567–79. PMID: 140917
40. Moharil J, Lei P, Tian J, Gaile DP, Andreadis ST. Lentivirus Live Cell Array for Quantitative Assessment of Gene and Pathway Activation during Myogenic Differentiation of Mesenchymal Stem Cells. *PLoS ONE*. 2015; 10(10):e0141365. <https://doi.org/10.1371/journal.pone.0141365> PMID: 26505747
41. Tzatzalos E, Abilez OJ, Shukla P, Wu JC. Engineered heart tissues and induced pluripotent stem cells: Macro- and microstructures for disease modeling, drug screening, and translational studies. *Adv Drug Deliv Rev*. 2015.
42. Haston KM, Finkbeiner S. Clinical Trials in a Dish: The Potential of Pluripotent Stem Cells to Develop Therapies for Neurodegenerative Diseases. *Annu Rev Pharmacol Toxicol*. 2015.
43. Grimm FA, Iwata Y, Sirenko O, Bittner M, Rusyn I. High-Content Assay Multiplexing for Toxicity Screening in Induced Pluripotent Stem Cell-Derived Cardiomyocytes and Hepatocytes. *Assay Drug Dev Technol*. 2015.
44. Young-Pearse TL, Morrow EM. Modeling developmental neuropsychiatric disorders with iPSC technology: challenges and opportunities. *Curr Opin Neurobiol*. 2015; 36:66–73. <https://doi.org/10.1016/j.conb.2015.10.006> PMID: 26517284
45. Stoccoa G, Lanzib G, Yuec F, Gilianib S, Sasakic K, Tommasinid A, et al. Patients' induced pluripotent stem cells to model drug induced adverse events: a role in predicting thiopurine induced pancreatitis? *Curr Drug Metab*. 2015.
46. de Boer AS, Eggan K. A perspective on stem cell modeling of Amyotrophic Lateral Sclerosis. *Cell cycle*. 2015:0.
47. Darabi R, Arpke RW, Irion S, Dimos JT, Grskovic M, Kyba M, et al. Human ES- and iPS-derived myogenic progenitors restore DYSTROPHIN and improve contractility upon transplantation in dystrophic mice. *Cell Stem Cell*. 2012; 10(5):610–9. <https://doi.org/10.1016/j.stem.2012.02.015> PMID: 22560081
48. Takahashi J. [Cell replacement therapy for Parkinson's disease using iPSC cells]. *Rinsho Shinkeigaku*. 2013; 53(11):1009–12. PMID: 24291862

49. Okano H, Yamanaka S. iPS cell technologies: significance and applications to CNS regeneration and disease. *Mol Brain*. 2014; 7:22. <https://doi.org/10.1186/1756-6606-7-22> PMID: 24685317
50. Nieweg K, Andreyeva A, van Stegen B, Tanriover G, Gottmann K. Alzheimer's disease-related amyloid-beta induces synaptotoxicity in human iPS cell-derived neurons. *Cell Death Dis*. 2015; 6:e1709. <https://doi.org/10.1038/cddis.2015.72> PMID: 25837485
51. Singh R, Shen W, Kuai D, Martin JM, Guo X, Smith MA, et al. iPS cell modeling of Best disease: insights into the pathophysiology of an inherited macular degeneration. *Human molecular genetics*. 2013; 22(3):593–607. <https://doi.org/10.1093/hmg/ddt469> PMID: 23139242
52. Singh R, Kuai D, Guziewicz KE, Meyer J, Wilson M, Lu J, et al. Pharmacological Modulation of Photoreceptor Outer Segment Degradation in a Human iPS Cell Model of Inherited Macular Degeneration. *Mol Ther*. 2015.
53. Raikwar SP, Kim EM, Sivitz WI, Allamargot C, Thedens DR, Zavazava N. Human iPS cell-derived insulin producing cells form vascularized organoids under the kidney capsules of diabetic mice. *PLoS ONE*. 2015; 10(1):e0116582. <https://doi.org/10.1371/journal.pone.0116582> PMID: 25629318
54. Maehr R. iPS cells in type 1 diabetes research and treatment. *Clin Pharmacol Ther*. 2011; 89(5):750–3. <https://doi.org/10.1038/clpt.2011.1> PMID: 21430661
55. Dec E FD, Nalbandian A, Gargus M, Katheria V, Rana P, Ibrahim A, Hatch M, Lan M, Llewellyn KJ, Keirstead H and Kimonis VE. Disease-Specific Induced Pluripotent Stem Cell Modeling: Insights into the Pathophysiology of Valosin Containing Protein (VCP) Disease. *Journal of Stem Cell Research & Therapy*. 2014; 4(2).
56. Mizuno Y, Chang H, Umeda K, Niwa A, Iwasa T, Awaya T, et al. Generation of skeletal muscle stem/progenitor cells from murine induced pluripotent stem cells. *FASEB J*. 2010; 24(7):2245–53. <https://doi.org/10.1096/fj.09-137174> PMID: 20181939
57. Lal H, Emmett-Oglesby MW. Behavioral analogues of anxiety. *Animal models*. *Neuropharmacology*. 1983; 22(12B):1423–41. PMID: 6142426
58. Roca I, Requena J, Edel MJ, Alvarez-Palomo AB. Myogenic Precursors from iPS Cells for Skeletal Muscle Cell Replacement Therapy. *J Clin Med*. 2015; 4(2):243–59. <https://doi.org/10.3390/jcm4020243> PMID: 26239126
59. Meola G, Velicogna M, Brigato C, Pizzul S, Rotondo G, Scarlato G. Growth and differentiation of myogenic clones from adult human muscle cell cultures. *Eur J Basic Appl Histochem*. 1991; 35(3):219–31. PMID: 1932334
60. Kaufman SJ, George-Weinstein M, Foster RF. In vitro development of precursor cells in the myogenic lineage. *Dev Biol*. 1991; 146(1):228–38. PMID: 2060703
61. Quinn LS, Holtzer H, Nameroff M. Generation of chick skeletal muscle cells in groups of 16 from stem cells. *Nature*. 1985; 313(6004):692–4. PMID: 3974704
62. Schultz E, Jaryszak DL. Effects of skeletal muscle regeneration on the proliferation potential of satellite cells. *Mech Ageing Dev*. 1985; 30(1):63–72. PMID: 3999814
63. Bischoff R, Holtzer H. Mitosis and the processes of differentiation of myogenic cells in vitro. *The Journal of cell biology*. 1969; 41(1):188–200. PMID: 5775784
64. Shelton M, Kocharyan A, Liu J, Skerjanc IS, Stanford WL. Robust generation and expansion of skeletal muscle progenitors and myocytes from human pluripotent stem cells. *Methods*. 2015.
65. Chal J, Oginuma M, Al Tanoury Z, Gobert B, Sumara O, Hick A, et al. Differentiation of pluripotent stem cells to muscle fiber to model Duchenne muscular dystrophy. *Nature biotechnology*. 2015; 33(9):962–9. <https://doi.org/10.1038/nbt.3297> PMID: 26237517
66. Salani S, Donadoni C, Rizzo F, Bresolin N, Comi GP, Corti S. Generation of skeletal muscle cells from embryonic and induced pluripotent stem cells as an in vitro model and for therapy of muscular dystrophies. *Journal of cellular and molecular medicine*. 2012; 16(7):1353–64. <https://doi.org/10.1111/j.1582-4934.2011.01498.x> PMID: 22129481
67. Borchin B, Chen J, Barberi T. Derivation and FACS-mediated purification of PAX3+/PAX7+ skeletal muscle precursors from human pluripotent stem cells. *Stem cell reports*. 2013; 1(6):620–31. <https://doi.org/10.1016/j.stemcr.2013.10.007> PMID: 24371814
68. Abujarour R, Valamehr B. Generation of skeletal muscle cells from pluripotent stem cells: advances and challenges. *Front Cell Dev Biol*. 2015; 3:29. <https://doi.org/10.3389/fcell.2015.00029> PMID: 26029693
69. Awaya T, Kato T, Mizuno Y, Chang H, Niwa A, Umeda K, et al. Selective development of myogenic mesenchymal cells from human embryonic and induced pluripotent stem cells. *PLoS ONE*. 2012; 7(12):e51638. <https://doi.org/10.1371/journal.pone.0051638> PMID: 23236522



70. Zhou YP, Rochat A, Hatzfeld A, Peiffer I, Barbet R, Hatzfeld J, et al. [bFGF-stimulated MEF-conditioned medium is capable of maintaining human embryonic stem cells]. *Fen Zi Xi Bao Sheng Wu Xue Bao*. 2009; 42(3–4):193–9. PMID: [19697701](#)
71. Siddiqui A, Bhaumik D, Chinta SJ, Rane A, Rajagopalan S, Lieu CA, et al. Mitochondrial Quality Control via the PGC1alpha-TFEB Signaling Pathway Is Compromised by Parkin Q311X Mutation But Independently Restored by Rapamycin. *J Neurosci*. 2015; 35(37):12833–44. <https://doi.org/10.1523/JNEUROSCI.0109-15.2015> PMID: [26377470](#)
72. Bove J, Martinez-Vicente M, Vila M. Fighting neurodegeneration with rapamycin: mechanistic insights. *Nature reviews Neuroscience*. 2011; 12(8):437–52. <https://doi.org/10.1038/nrn3068> PMID: [21772323](#)
73. Richardson PG, Eng C, Kolesar J, Hideshima T, Anderson KC. Perifosine, an oral, anti-cancer agent and inhibitor of the Akt pathway: mechanistic actions, pharmacodynamics, pharmacokinetics, and clinical activity. *Expert Opin Drug Metab Toxicol*. 2012; 8(5):623–33. <https://doi.org/10.1517/17425255.2012.681376> PMID: [22512706](#)
74. Balakrishnan K, Burger JA, Wierda WG, Gandhi V. AT-101 induces apoptosis in CLL B cells and overcomes stromal cell-mediated Mcl-1 induction and drug resistance. *Blood*. 2009; 113(1):149–53. <https://doi.org/10.1182/blood-2008-02-138560> PMID: [18836097](#)
75. Mei H, Lin Z, Wang Y, Wu G, Song Y. Autophagy inhibition enhances pan-Bcl-2 inhibitor AT-101-induced apoptosis in non-small cell lung cancer. *Neoplasma*. 2014; 61(2):186–92. [https://doi.org/10.4149/neo\\_2014\\_024](https://doi.org/10.4149/neo_2014_024) PMID: [24299314](#)
76. Moore BR, Page-Sharp M, Stoney JR, Ilett KF, Jago JD, Batty KT. Pharmacokinetics, pharmacodynamics, and allometric scaling of chloroquine in a murine malaria model. *Antimicrob Agents Chemother*. 2011; 55(8):3899–907. <https://doi.org/10.1128/AAC.00067-11> PMID: [21646487](#)
77. Zirin J, Nieuwenhuis J, Perrimon N. Role of autophagy in glycogen breakdown and its relevance to chloroquine myopathy. *PLoS Biol*. 2013; 11(11):e1001708. <https://doi.org/10.1371/journal.pbio.1001708> PMID: [24265594](#)
78. Liu J, Xia H, Kim M, Xu L, Li Y, Zhang L, et al. Beclin1 controls the levels of p53 by regulating the deubiquitination activity of USP10 and USP13. *Cell*. 2011; 147(1):223–34. <https://doi.org/10.1016/j.cell.2011.08.037> PMID: [21962518](#)
79. Choi YJ, Park YJ, Park JY, Jeong HO, Kim DH, Ha YM, et al. Inhibitory effect of mTOR activator MHY1485 on autophagy: suppression of lysosomal fusion. *PLoS ONE*. 2012; 7(8):e43418. <https://doi.org/10.1371/journal.pone.0043418> PMID: [22927967](#)
80. Badadani M, Nalbandian A, Watts GD, Vesa J, Kitazawa M, Su H, et al. VCP associated inclusion body myopathy and paget disease of bone knock-in mouse model exhibits tissue pathology typical of human disease. *PLoS ONE*. 2010; 5(10).
81. Nalbandian A, Llewellyn KJ, Badadani M, Yin HZ, Nguyen C, Katheria V, et al. A progressive translational mouse model of human valosin-containing protein disease: the VCP(R155H/+) mouse. *Muscle & Nerve*. 2013; 47(2):260–70.
82. Nalbandian A, Ghimbovschi S, Radom-Aizik S, Dec E, Vesa J, Martin B, et al. Global gene profiling of VCP-associated inclusion body myopathy. *Clin Transl Sci*. 2012; 5(3):226–34. <https://doi.org/10.1111/j.1752-8062.2012.00407.x> PMID: [22686199](#)
83. Tresse E, Salomons FA, Vesa J, Bott LC, Kimonis V, Yao TP, et al. VCP/p97 is essential for maturation of ubiquitin-containing autophagosomes and this function is impaired by mutations that cause IBMPFD. *Autophagy*. 2010; 6(2):217–27. PMID: [20104022](#)
84. Ju JS, Weihl CC. Inclusion body myopathy, Paget's disease of the bone and fronto-temporal dementia: a disorder of autophagy. *Human molecular genetics*. 19(R1):R38–45. <https://doi.org/10.1093/hmg/ddq157> PMID: [20410287](#)
85. Cai Z, Yan LJ. Rapamycin, Autophagy, and Alzheimer's Disease. *J Biochem Pharmacol Res*. 2013; 1(2):84–90. PMID: [23826514](#)
86. Proud CG. Amino acids and mTOR signalling in anabolic function. *Biochemical Society transactions*. 2007; 35(Pt 5):1187–90. <https://doi.org/10.1042/BST0351187> PMID: [17956308](#)
87. Klein JB, Barati MT, Wu R, Gozal D, Sachleben LR Jr., Kausar H, et al. Akt-mediated valosin-containing protein 97 phosphorylation regulates its association with ubiquitinated proteins. *The Journal of biological chemistry*. 2005; 280(36):31870–81. <https://doi.org/10.1074/jbc.M501802200> PMID: [16027165](#)
88. Vandermoere F, El Yazidi-Belkoura I, Slomianny C, Demont Y, Bidaux G, Adriaenssens E, et al. The valosin-containing protein (VCP) is a target of Akt signaling required for cell survival. *The Journal of biological chemistry*. 2006; 281(20):14307–13. <https://doi.org/10.1074/jbc.M510003200> PMID: [16551632](#)
89. Steinman RM, Mellman IS, Muller WA, Cohn ZA. Endocytosis and the recycling of plasma membrane. *The Journal of cell biology*. 1983; 96(1):1–27. PMID: [6298247](#)

90. Liang X, Tang J, Liang Y, Jin R, Cai X. Suppression of autophagy by chloroquine sensitizes 5-fluorouracil-mediated cell death in gallbladder carcinoma cells. *Cell Biosci.* 2014; 4(1):10. <https://doi.org/10.1186/2045-3701-4-10> PMID: 24581180
91. Shintani T, Klionsky DJ. Autophagy in health and disease: a double-edged sword. *Science (New York, NY.* 2004; 306(5698):990–5.
92. Day K, Paterson B, Yablonka-Reuveni Z. A distinct profile of myogenic regulatory factor detection within Pax7+ cells at S phase supports a unique role of Myf5 during posthatch chicken myogenesis. *Dev Dyn.* 2009; 238(4):1001–9. <https://doi.org/10.1002/dvdy.21903> PMID: 19301399
93. Knappe S, Zammit PS, Knight RD. A population of Pax7-expressing muscle progenitor cells show differential responses to muscle injury dependent on developmental stage and injury extent. *Frontiers in aging neuroscience.* 2015; 7:161. <https://doi.org/10.3389/fnagi.2015.00161> PMID: 26379543
94. Ju JS, Fuentealba RA, Miller SE, Jackson E, Piwnicka-Worms D, Baloh RH, et al. Valosin-containing protein (VCP) is required for autophagy and is disrupted in VCP disease. *The Journal of cell biology.* 2009; 187(6):875–88. <https://doi.org/10.1083/jcb.200908115> PMID: 20008565
95. Huang S, Bjornsti MA, Houghton PJ. Rapamycins: mechanism of action and cellular resistance. *Cancer Biol Ther.* 2003; 2(3):222–32. PMID: 12878853
96. Zhang J, Hong Y, Shen J. Combination treatment with perifosine and MEK-162 demonstrates synergism against lung cancer cells in vitro and in vivo. *Tumour Biol.* 2015; 36(7):5699–706. <https://doi.org/10.1007/s13277-015-3244-2> PMID: 25697899
97. Kim MN, Ro SW, Kim do Y, Kim da Y, Cho KJ, Park JH, et al. Efficacy of perifosine alone and in combination with sorafenib in an HrasG12V plus shp53 transgenic mouse model of hepatocellular carcinoma. *Cancer chemotherapy and pharmacology.* 2015; 76(2):257–67. <https://doi.org/10.1007/s00280-015-2787-7> PMID: 26037205
98. Amantini C, Morelli MB, Santoni M, Soriani A, Cardinali C, Farfariello V, et al. Sorafenib induces cathepsin B-mediated apoptosis of bladder cancer cells by regulating the Akt/PTEN pathway. The Akt inhibitor, perifosine, enhances the sorafenib-induced cytotoxicity against bladder cancer cells. *Oncoscience.* 2015; 2(4):395–409. <https://doi.org/10.18632/oncoscience.147> PMID: 26097873