A Compound Heterozygous Mutation in Calpain 1 Identifies a New Genetic Cause for Spinal Muscular Atrophy Type 4 (SMA4)

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Spinal Muscular Atrophy (SMA) is a heterogeneous group of neuromuscular diseases characterized by degeneration of anterior horn cells of the spinal cord, leading to muscular atrophy and weakness. Although the major cause of SMA is autosomal recessive exon deletions or loss-of-function mutations of survival motor neuron 1 (SMN1) gene, next generation sequencing technologies are increasing the genetic heterogeneity of SMA. SMA type 4 (SMA4) is an adult onset, less severe form of SMA for which genetic and pathogenic causes remain elusive. Whole exome sequencing in a 30-year-old brother and sister with SMA4 identified a compound heterozygous mutation (p. G492R/p. F610C) in calpain-1 (CAPN1). Mutations in CAPN1 have been previously associated with cerebellar ataxia and hereditary spastic paraplegia. Using skin fibroblasts from a patient bearing the p. G492R/p. F610C mutation, we demonstrate reduced levels of CAPN1 protein and protease activity. Functional characterization of the SMA4 fibroblasts revealed no changes in SMN protein levels and subcellular distribution. Additional cellular pathways associated with SMA remain unaffected in the patient fibroblasts, highlighting the tissue specificity of CAPN1 dysfunction in SMA4 pathophysiology. This study provides genetic and functional evidence of CAPN1 as a novel gene for the SMA4 phenotype and expands the phenotype of CAPN1 mutation disorders.

Keywords: spinal muscular atrophy, calpain, patient fibroblasts, non-SMN1, β-catenin

INTRODUCTION

Spinal muscular atrophies (SMAs) are a clinically and genetically heterogeneous group of disorders in which the selective loss of spinal cord motor neurons, progressive muscle denervation, and skeletal muscle atrophy are the main pathological components (Crawford and Pardo, 1996). Survival of Motor Neuron 1 gene (SMN1, MIM#600354), located on chromosome 5q13.2 was initially identified as the childhood SMA causative gene (Lefebvre et al., 1995), soon after mapping this autosomal recessive disorder to chromosome 5q (Brzustowicz et al., 1990). The typical change is a deletion of at least exon 7 of SMN1 on both alleles, with 2–5% of cases having compound deletion and inactivating mutation on each allele (Prior et al., 1993).
Although the majority of SMN protein is translated from the telomeric SMN1 gene, expression of SMN in humans also relies on the SMN2 gene (MIM#601627), an almost identical centromeric copy of SMN1. The SMN2 copy has a C to T nucleotide substitution in exon 7 (Lorson et al., 1999). This single base change causes aberrant splicing and exclusion of exon 7 in SMN2 (Monani et al., 1999) which produces 90% expression of a truncated, less stable, and dysfunctional SMNΔ7 protein, leaving only 10% functional full-length SMN (Chang et al., 2004). The severity of dysfunction in SMA is inversely proportional to the number of copies of SMN2 and forms the basis for clinical classification of SMA subtypes (Lefebvre et al., 1997). Type 4 SMA (SMA4; MIM#271150) is the adult-onset form of SMA in which affected patients show 4 or more copies of SMN2. Patient diagnosis and initial symptoms usually present in the second or third decade of life starting with muscle weakness in the lower extremities. This muscle weakness progresses to affect independent walking and the upper limbs; in its mildest form walking can be achieved unaired and life expectancy remains unaffected (D’Amico et al., 2011).

Deletions and point mutations in SMN1 account for 96% of SMA patients, with 4% being unlinked to 5q13 (Wirth, 2000). The development of next generation sequencing technologies is increasing the number of genes associated with these non-5q SMA patients. These genes are involved in a variety of cellular and biological processes (Farrar and Kiernan, 2015) and research interest has focused on establishing a functional connection that links non-SMN-related and SMN-related spinal muscular atrophy (Soltic and Fuller, 2020). However, many of the other described causes of SMA are clinically dissimilar to SMN1 associated SMAS.

In this study, we present a family study of clinically typical phenotype SMA4 in which mutation analysis of the male proband and his affected sister was excluded for deletions and mutations in the SMN1 gene. Whole exome sequencing of family members identified a compound heterozygous mutation (p. G492R/p. F610C) within the calpain-1 (CAPN1) gene. Calpains are a calcium-activated cysteine family of proteases involved in numerous cellular processes, including myogenesis, muscle remodelling, and synaptic function (Nath et al., 1996; Campbell and Davies, 2012; Briz and Baudry, 2017). Loss-of-function mutations in CAPN1 are known to cause cerebellar ataxia (Wang et al., 2016) and hereditary spastic paraplegia (HSP) (Gan-Or et al., 2016) with the number of ataxia and/or pure HSP patients reporting CAPN1 mutations rapidly increasing (Mereaux et al., 2021). Interestingly, recent investigations described the role of CAPN1 regulating SMN (Wang et al., 2019) (de la Fuente et al., 2020) and a positive effect on SMA in vitro and in vivo models when treated with calpain inhibitors (de la Fuente et al., 2019). These findings suggest that both reduced and overactivated calpain can be associated with neurodegeneration and cause distinct clinical phenotypes. In the present work, using SMA4-derived skin fibroblasts bearing the p. G492R/p. F610C substitutions we demonstrate this compound heterozygous mutation reduces CAPN1 protein levels and protease activity. Our findings provide for the first time genetic evidence for CAPN1 as a novel non-5q-SMA causative gene.

**MATERIALS AND METHODS**

**Research Guidelines and Regulations**

All research and cell culture procedures were conducted following written consent according to protocols approved by the Sydney Local Health District Human Ethics Review Committee, Concord Repatriation General Hospital, Sydney, Australia (reference number: HREC/11/CRGH/105). Informed consent for study participation was obtained from all patients and controls. All research was performed in accordance with relevant guidelines and regulations.

**Genetic Studies**

Prior to this study the proband was tested for the mutations in the SMN1 and SMN2 genes. Whole exome sequencing was outsourced to Macrogen (South Korea) as previously described (Drew et al., 2015). Sequence reads were mapped to the GRCh37/hg19 assembly. The mutations were validated with Sanger sequencing using BigDye Terminator Cycle Sequencing protocols at the ACRF Facility, Garvan Institute of Medical Research (Australia). Data filtering was performed using multiple publicly available databases including gnomAD (https://gnomad.broadinstitute.org/) (Karczewski et al., 2020), 1,000 Genomes (http://www.1000genomes.org/), and Exome Variant Server (https://evs.gs.washington.edu/EVS/). Evolutionary conservation of the CAPN1 p. G492 and p. F610 amino acid residues was assessed in silico using GERP (http://mendel.stanford.edu/sidowlab/downloads/gerp/index.html) (Davydov et al., 2010), phastCons (Siepel et al., 2005) and PhyloP (Pollard et al., 2010) (http://compgen.cshl.edu/phastweb/runtool.php). The predicted functional effect of the CAPN1 p. G492R and p. F610C mutations was assessed separately in silico using PolyPhen2 (http://genetics.bwh.harvard.edu/php2/) (Adzhubei et al., 2010), PROVEAN (http://provean.jcvi.org/seq_submit.php) (Choi et al., 2012), SIFT (https://sift.bii.a-star.edu.sg/www/SIFT_seq_submit2.html) (Sim et al., 2012), MutationAssessor (http://mutationassessor.org/r3/) (Reva et al., 2007), and MutationTaster2 (http://www.mutationtaster.org/) (Schwarz et al., 2014). Pathogenicity was interpreted using the American College of Medical Genetics and Genomics (ACMG) and the Association for Molecular Pathology (AMP) guidelines (Richards et al., 2015).

**Subjects, Cell Culture Maintenance and Growing Conditions**

Primary fibroblasts were cultured from a SMA4 patient and 3 neurologically normal skin biopsies and maintained with fibroblast cell culture medium (FDMEM): DMEM (Gibco, Life technologies) supplemented with 10% (v/v) fetal bovine serum (SAFC Biosciences), 1% (v/v) Penicillin Streptomycin (Gibco, Life technologies) and 1% (v/v) L-glutamine (Gibco, Life technologies) and maintained at 37°C in humidified air and 5% CO2.
Preparation of Protein From Cell Lysates

Fibroblast cells were cultured until confluence for preparation of cell lysates for western blot analysis and determining CAPN1 specific activity using RIPA buffer (150 mM NaCl, 1% (v/v) Triton X-100, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 50 mM Tris pH8.0, 1x Complete™ EDTA-free protease inhibitor cocktail tablet). Lysis buffer additionally contained 5 mM ethylenediamine (oxycarbethenitriol)tetraacetic acid (EGTA) and added 2 mM CaCl2 when indicated. Concentration of protein in the cells lysates was determined using the Pierce BCA Protein Assay Kit (ThermoScientific).

Western Blotting

Samples, SMA4 patient (proband II:2) and controls (n = 3), were prepared with 4x Laemmli sample buffer (BioRad) and 2-mercaptoethanol (Sigma) solution 9:1 (v/v). Cell lysates (15 µg) were size fractioned using mini-PROTEAN TGX Precast Gels (BioRad) and then transferred to an Immobilon®-P Polyvinylidene difluoride membrane (Sigma). Membranes were probed with the following primary antibodies: rabbit α-CAPN1 (CST), 1:1,500; mouse α-SMN (BD Transduction Laboratories), 1:2000; rabbit α-p-Akt (CST), 1:1,000; mouse α-Pan-Akt (CST), 1:2000; rabbit α-LC3B-II (CST) 1:1,000; rabbit α-β-actin (CST) 1:2000; rabbit α-caspase-3 (Abcam) 1:4,000 and rabbit α-β-actin (CST) 1:2000. Secondary antibodies used in this study include goat α-rabbit IgG HRP (Sigma), 1:10,000 and goat α-mouse IgG HRP (abcam) 1:5,000. Immobilon® Western Chemiluminescent HRP Substrate Reagent (Millipore) was added and protein bands were visualized using a ChemiDoc™ MP Imaging System (BioRad).

Immunohistochemistry

48 h before the experiment, 1.5 × 10^4 cells were plated onto optically clear bottom 96-well plates (CellCarrier-96, PerkinElmer). Cells were washed once using PBS and incubated with 1 µM TMRE (Thermofisher) in FDMEM for 45 min at 37°C. Cells were washed once using PBS and nuclei were stained with 1 µg/ml Hoechst in PBS for 10 min, followed by a final wash in PBS. 10 random images were taken using a Leica SP8 confocal microscope as indicated above. TMRE staining per cell was quantified using CellProfiler 4.0.6.

Calpain Assay

The hydrolysis of the fluorogenic substrate Succ-LLVY-AMC (Abcam) by calpains in patient and control fibroblast lysates was performed in a reaction buffer containing 25 mM Tris-HCl, 145 mM NaCl (pH 7.4) and 0.5 mM EGTA in the absence or presence of calcium as previously described (Chelko et al., 2021) with some modifications. To specifically determine CAPN1 activity, these experiments were performed in the presence of 1 µM PD151746 (C11), a recently developed calpain inhibitor showing high specificity for CAPN1 (calpain-1: Kᵢ = 260 nM; calpain-2: Kᵢ = 5.33 µM). The reaction was initiated by adding, 5 mM CaCl2 (3 mM free Ca²⁺) and the 100 µM Suc-Leu-Tyr-AMC enzyme to each sample containing 100 µg protein, continuing at 30°C for 45 min. The fluorescence of 7-amino-4-methylcoumarin (Ex 380 nm/Em 450 nm) was monitored every 30s using an EnSpire™ Multimode Plate Reader (PerkinElmer). The rate of hydrolysis (increase in fluorescence/second) was determined from the linear portion of the curve. CAPN1 activity was estimated by subtracting the rate of hydrolysis in the presence of 1 µM C11 from the value obtained in the absence of calpain inhibitors. Free calcium concentrations were calculated using the website maxchelator.stanford.edu/CaEGTA-TS.htm.

Molecular Modelling

The PyMOL program (Schrodinger) was used to localise the p. F610 and p. G492 residues into the ribbon structure diagram of the Rattus norvegicus CAPN1 protein (PDB:3BOW). The impact of the variants on CAPN1 protein stability was modelled and predicted using the PremPS online tool (https://lllab.jysw.suda.edu.cn/research/PremPS/). In silico modelling changes in protein-protein interaction between calpain2 and calpastatin (PDB:3BOW) were predicted using mCSM-PPI2 (http://biosig.unimelb.edu.au/mcsm_ppi2/).

Statistical Analysis

For the statistical analysis, three independent experiments under the same conditions were performed and 2-way ANOVA followed by Tukey’s post hoc test used to assess the significance of the results. The data are expressed as mean ± SEM. The following statistical thresholds have been applied throughout the study: *p < 0.05; **p < 0.01; ***p < 0.001, ****p < 0.0001.
**FIGURE 1** | SMA4 causative p. G492R/p. F610C compound het mutation in CAPN1. (A) Pedigree of SMA4 family. Squares indicate male, circles indicate females. Solid symbols denote affected individuals and open symbols unaffected individuals. Carriers are represented with internal dots. Asterisk indicates individuals in which whole exomes sequencing was performed and the black arrow shows the proband. (B) Sanger sequencing confirms the nucleotide changes c.1474G > A (p.G492R) and c.1829T > G (p.F610C) in the two affected siblings (II:2 and II:3). (C) The substituted amino acid residues (p. G492 and p. F610) are located within highly conserved functional domains of the CAPN1 protein. (D) CAPN1 secondary structure displaying the protease domain (yellow) and the C2 domain-like (C2L, orange) and penta-EF-hand (PEF, red) Ca²⁺ binding domains bearing the p. G492R and p. F610C mutations respectively. Affected residues located in the 3D ribbon structure of CAPN1 using PyMOL and the *Rattus norvegicus* CAPN1 crystalized structure (PDB:1QXP). (E) The impact of the p. G492R and p. F610C missense mutations in the protein stability (ΔΔG) of CAPN1 predicted using the PremPS online tool. For clarity, the ribbon was hidden and only the non-covalent interactions affected by the substitutions are displayed. Dotted lines represent hydrophobic (blue), polar (light blue), hydrogen bonds (violet) and Van der Waals (green) interactions in the wild-type and mutant structures. Positive ΔΔG predicts a reduction in the stability of the resulted protein.
RESULTS

Clinical and Electrodiagnostic Findings
This research examines a family study of SMA4 in which the male proband presented in 2011 aged 36, onset of symptoms was uncertain but older than 2 years. The presenting symptoms were of increasing painless weakness, without sensory loss, propensity to falls, plus cramps in upper and lower limbs following usage. The affected family members have now been followed for 10 years. The clinical findings on presentation were of normal cranial nerves, symmetrical proximal > distal limb weakness, with disproportionate weakness of triceps and quadriceps. There was shoulder girdle, latissimus dorsi, triceps and quadriceps wasting and a waddling gait. Strength in the fingers and toes was normal without muscle hypertrophy. MRC scores are presented in Supplementary Table S1. Fasciculations were visible in proximal upper and lower limbs. There was no action nor rest tremor. The reflexes were depressed at triceps, absent knee jerks, other limb reflexes present, normal coordination, sensation and sphincter function. The proband’s full sister was first seen aged 34, but reported difficulty getting off the floor from age 21. Clinical findings were essentially the same. The proband’s half-brother, parents and son were clinically normal (Figure 1A).

Neuropsychology showed normal nerve conduction study findings with normal compound muscle action potential amplitudes, distal motor latencies and motor nerve conduction velocities of the median, ulnar, tibial and peroneal nerves, and normal sensory studies of the median, ulnar and sural nerves. Electromyography in 2011 of the proband of a limited selection of limb muscles consistently showed some active features on insertion at rest, and a chronic neurogenic reinnervation pattern on activation with increased motor unit amplitudes and polyphasia, more proximally than in the more distal tibialis anterior. A high isolated firing rate (early recruitment) was observed on activation with decreased interference pattern at full activation. Details are provided in Supplementary Table S2. Quantitative EMG was not performed and electrophysiology has not been repeated in recent years.

Creatine kinase level was 466 U/ml (normal range 22–198 U/ml). Deltoid muscle biopsy showed grouped atrophic fibres of both fibre types consistent with chronic neurogenic changes. MRI spine and a lumbar puncture were non-informative or normal.

Genetic Analysis
Clinical testing of the SMN1 and SMN2 in the proband excluded mutations in the SMN1 gene. Subsequent whole exome sequencing of the proband and his sister identified a compound heterozygous mutation involving two rare missense variants in the CAPN1 gene. The variants were located in exon 13 and exon 18 (NM_001198868): c.1474G > A p. G492R [chr11: 64,974,054 (hg19)] and c.1829T > G p. F610C [chr11:64,977,354 (hg19)] respectively. Family segregation analysis showed the unaffected father and mother were carriers of the p. F610C and p. G492R mutation respectively. The unaffected half-brother of the proband was a carrier of p. F610C (Figure 1A).

The mutations were confirmed by Sanger sequencing for all individuals (Figure 1B). Assuming linkage equilibrium, the probability of two SNPs occurring together in an individual can be calculated by multiplying the minor allele frequencies (MAF) of the SNPs (Slatkin, 2008). The reported MAF of c.1474G > A (rs17883283) is 0.001 and the reported MAF of c.1829T > G (rs200876514) is 0.0003, and therefore the possibility of them co-occurring in an individual is 0.00003% (3/10, 000, 000 individuals). Furthermore, the probability of two affected siblings inheriting c.1474G > A and c.1829T > G from their carrier mother and father respectively is 6.25% (1/16). This statistical unlikelihood of the results described here provides strong genetic evidence for the involvement of compound heterozygous CAPN1 mutations in SMA4.

The amino acid residues p. G492 and p. F610 are located within highly conserved regions of the CAPN1 protein (Figure 1C), and evolutionary constraint of these residues was further supported by in silico analysis (Supplementary Table S1). Additional computational tools further predicted a damaging effect of p. G492R and p. F610C on CAPN1 function (Supplementary Table S3). Although most HSP-causative missense mutations are located in the protease domain of CAPN1 (Lai et al., 2020), they can be found across the 3 functional domains of the protein. The p. G492 and p. F610 amino acid residues are in the C2-like domain and the penta-EF-hand domain (PEF) respectively, far from the CAPN1 active site (p.C115), and not located near each other in the 3D structure of the protein (Figure 1D). In silico modelling the impact of the p. G492R and p. F610C mutations using the PremPS online tool predicts a change in the free energy of folding (ΔΔG) of +1.38 kcal/mol and +1.64 kcal/mol respectively, suggesting pathogenicity of these substitutions through decreased stability of CAPN1 (Figure 1E). According to the variant pathogenicity guidelines determined by ACMG-AMP, both the p. G492R and p. F610C variant can be separately classified as likely pathogenic (PM1, PM2, PP2, PP3).

SMA4 Patient Fibroblasts Show Reduced CAPN1 Protein Levels and CAPN1 Activity
Mutations in CAPN1 causing HSP lead to a reduction of CAPN1 protein expression and are associated with diminished calpain protease activity in patient-derived cells (Wang et al., 2016). Recent research however associates overactivated calpain-1 in the pathogenesis of SMA, suggesting the utility of calpain inhibition in SMA therapy (de la Fuente et al., 2019; de la Fuente et al., 2020). To investigate the effect the p. G492R/p. F610C compound heterozygous mutation has on CAPN1, skin fibroblasts from the SMA4 patient (II:2) and 3 gender/age matched neurologically normal controls were cultured. Protein levels of CAPN1 in fibroblasts lysates were examined by western blot (Figure 2A) and showed a reduction in CAPN1 expression in the SMA4-derived cells when compared to the three control lines. Quantification of these bands (Figure 2B) demonstrated the patient cells have 35% less calpain-1 than control fibroblasts (kSMA4 = 0.88 versus kCtrls = 1.23 densitometry units). Calpain activity in fibroblasts lysates was determined measuring the
hydrolysis rate of the fluorescent substrate Succ-LLVY-AMC in the presence of 3 mM free Ca\(^{2+}\). To specifically assess CAPN1 activity, these experiments were performed in the presence of saturating (20 µM) and low concentration (1 µM) PD151746 (C1I), a calpain inhibitor showing high specificity for CAPN1 (Figure 2C). Our experiments showed the SMA4 patient cells have reduced CAPN1-specific protease activity (% of Succ-LLVY-AMC hydrolysis rate in the presence of 3 mM free Ca\(^{2+}\) by the value obtained in the presence of 1 µM C1I) when compared to all control lines (x\(_{\text{SMA4}}\) = 12% versus x\(_{\text{Ctrls}}\) = 22% of CAPN1-specific protease activity, Figure 2D).

**SMA4 Patient Fibroblasts Show No Changes in SMN1 Protein Levels or SMN1 Subcellular Distribution**

In approximately 4% of SMA patients, there is not a direct genetic causative link with SMN1 (Wirth, 2000). Increasing evidence suggest however there is a functional association between these additional genes and SMN-related spinal muscular atrophy (Soltic and Fuller, 2020). SMN is a known proteolytic target of endogenous calpains (Walker et al., 2008) (Fuentes et al., 2010) and recent investigations demonstrate CAPN1 regulate SMN levels in vitro (Wang et al., 2019) and in vivo (de la Fuente et al., 2020). To determine if reduced CAPN1 protein and activity shown in the SMA4 patient cells (Figure 2) affects the intracellular levels and/or the subcellular distribution of SMN, western blot analysis and immunofluorescence experiments were performed (Figure 3). Cleavage of full length (FL) SMN (38 KDa) by calpains produces an N-terminal 28 KDa fragment distinctly detectable by immunoblotting. Protein lysates from the SMA4 patient and three controls were blotted using an anti-SMN antibody. Western blot analysis shows there is not a significant change in the levels of the FL SMN and/or cleaved 28 KDa fragment between affected and control lines (Figure 3A). Quantification of these bands (FL and FL/cleaved SMN) confirms the small variations observed don’t reached statistically significant differences across the three control lines utilised in this study (FL: x\(_{\text{SMA4}}\) = 1.09 versus x\(_{\text{Ctrls}}\) = 1.17 densitometry units; FL/cleaved SMN: x\(_{\text{SMA4}}\) = 0.55 versus x\(_{\text{Ctrls}}\) = 0.74 densitometry units; Figure 3A).
FIGURE 3 | SMN protein levels subcellular distribution in SMA4 patient fibroblasts show no changes in SMN1 protein levels or SMN1 subcellular distribution. (A) Levels of SMN protein determined by western blot analysis in lysates from a SMA4 patient and control fibroblasts. β-actin was used as a loading control in these experiments. High exposition of the membrane blots allow visualization of both the full length (FL) SMN (38 KDa) and the cleaved SMN fragment (28 KDa). (A) The amount of FL SMN and FL SMN/cleaved SMN was determined and shown in bar graphs as the mean ± SEM for each cell line. (B) Immunofluorescence analysis in control and SMA4-derived fibroblasts detects SMN (green) distribution in cytoplasmic and within the nuclei (blue) localising to subnuclear structures (gemins). Boxed areas are enlarged to allow visualization of cells showing no gemins (green arrowhead) and nuclei with SMN-gemins (white arrowhead). (B) Quantification of the SMN cytoplasmic expression (integrated intensity) within each cell. Violin plot shows the full distribution of all data points acquired (n > 250 cells). (B) Incidence of SMN-gemins shown in bar graphs (as the mean ± SEM from 3 separate experiments for each cell line) and distributed in 3 separated groups, including cells showing no gemins (no pattern), cells showing 1 or 2 SMN-gemins (lined pattern) and nuclei in which 3 or more SMN gemins were detected (clear bar). (C) Representative example of the in-house developed CellProfiler pipeline to allow segmentation of the cytoplasm and nuclear gemins in the immunofluorescence experiments. (C) Representative cartoon showing subcellular expression of SMN in skin fibroblasts highlighting a similar distribution in control and SMA4-derived cells.
FIGURE 4 | Effect of the CAPN1 compound heterozygous mutation on intracellular pathways affected in spinal muscular atrophy. (A) Protein levels of the autophagy marker LC3B (LC3B-II, 14 KDa and LC3B-I, 16 KDa) determined by western blot analysis in lysates from SMA4 patient (proband II:2) compared to controls fibroblasts. β-actin was used as a loading control in these experiments. (A') The expression of LC3B type I (LC3B-I) and LC3B type II (LC3B-II) was determined and shown in bar graphs as the mean ± SEM from 3 independent experiments. (B) Autophagy occurrence visualised by confocal microscopy using an anti-LC3B antibody (green) in cells stained with phalloidin (violet). (B') Quantification of the LC3B cytoplasmic expression within each cell. Violin plot shows the full distribution of all data points (Continued)
SMN is expressed both in the cytoplasm and in the nuclei, where it localises to subnuclear bodies called gems (Liu et al., 1997). To assess whether the p. G492R/p.F610C compound heterozygous mutation has any effect on the intracellular localisation of SMN, immunofluorescence staining was performed (Figure 3B) and images segmented into nuclear and cytoplasmic compartments to allow the precise subcellular quantification of SMN (Figure 3C). Levels of cytoplasmic SMN were comparable between SMA4 fibroblasts and control cells and no changes were detected in the integrated density quantified in the cytoplasmic compartment (cytoplasmic SMN: $x_{SMA4} = 303.5$ versus $x_{Ctrls} = 302.27$, SD$_{Ctrls} = 12.92$ integrated intensity; Figure 3B). The appearance of SMN nuclei gems was classified in three categories that included nuclei showing no gems, nuclei showing either 1 or 2, or cells displaying 3 or more SMN gems. Detailed quantification following these criteria confirmed the visual observation that no changes are detected in the SMA4-derived cells when compared to control fibroblasts (0 gems: $x_{SMA4} = 57.48\%$ versus $x_{Ctrls} = 59.33\%$; 1–2 gems: $x_{SMA4} = 24.00\%$ versus $x_{Ctrls} = 27.87\%$; >3 gems: $x_{SMA4} = 2.91\%$ versus $x_{Ctrls} = 4.59\%$ of all cells; Figure 3B).

**Interrogating Intracellular Pathways Affected in Spinal Muscular Atrophy in SMA4-Derived Fibroblasts**

Cellular and molecular pathways leading to motor neuron degeneration in SMN-related and non-5q SMAs are not yet fully understood. To evaluate the occurrence of intracellular pathways that might be triggered by decreased CAPN1 activity (Figure 2D) and had been previously associated with in vitro models of SMA, we investigated the incidence of autophagy mitochondrial function and apoptosis in SMA4 fibroblasts.

Changes in the autophagosome formation and in the autophagy flux have been reported in SMA (Garcera et al., 2013) (Piras et al., 2017) and modulation of endogenous calpain associated with increased SMN availability through regulation of autophagy (Periyakaruppillai et al., 2016). To investigate autophagy in SMA4-derived cells we determined the expression of the autophagy marker LC3B by westernblot and immunofluorescence analysis (Figures 4A,B). Westernblot analysis indicated that the amount of LC3B-I and LC3B-II in the patient cells was within the normal range shown in the control fibroblasts (LC3B-I: $x_{SMA4} = 0.81$ versus $x_{Ctrls} = 0.93$, SD$_{Ctrls} = 0.21$; LC3B-II: $x_{SMA4} = 1.73$ versus $x_{Ctrls} = 1.88$, SD$_{Ctrls} = 0.33$ densitometry units Figure 4A’). Accordingly, quantification of the immunofluorescent staining shows no changes in the levels of cytosolic LC3B positive puncta between the SMA4 patient fibroblasts and controls (Figure 4B). Although recent data indicates a tissue-specific regulation in SMA of the autophagy process (Sansa et al., 2021b), our data suggest that the CAPN1 compound heterozygous mutation doesn’t impact the autophagy process in SMA4-derived cells.

Mitochondrial dysfunction has been extensively associated with SMA pathophysiology and it is evident across the clinical spectrum of SMA [reviewed at (James et al., 2021)]. Interestingly, relocation of CAPN1 into the mitochondria disrupts ATP synthase-α protein (ATP5A1) and ATP synthase activity (Ni et al., 2016) and recent research describes CAPN1-induced cleavage of mitochondrial-bound apoptosis-inducing factor (AIF) (Chelko et al., 2021). In this study we utilised the production of cellular ATP (Figure 4C), which main production source is the oxidative phosphorylation at the mitochondria, and the mitochondrial membrane potential marker TMRE (Figure 4D), that reflects the functional status and viability of mitochondria, as indicators of the mitochondrial health in SMA4-derived fibroblasts. Our data indicates a good correlation between ATP levels and membrane potential within each cell line (Figure 4C and Figure 4D). These parameters in the SMA4 fibroblasts are within the normal range captured by the neurologically normal control cells (ATP levels: $x_{SMA4} = 2.11 \times 10^5$ versus $x_{Ctrls} = 1.95 \times 10^5$, SD$_{Ctrls} = 0.39 \times 10^5$ luminescence arbitrary units; TMRE: $x_{SMA4} = 193.3$ versus $x_{Ctrls} = 215.77$, SD$_{Ctrls} = 41.54$ integrated intensity) suggesting the CAPN1 mutation does not impair mitochondrial function in our model.

The antiapoptotic role of SMN (Anderton et al., 2013) and the activation of apoptotic processes through the PI3K-Akt neuronal survival pathway has been established in SMA (Sansa et al., 2021a) and has been associated with CAPN1 loss of function in cerebellar ataxia (Wang et al., 2016). We interrogated this pathway in SMA4 fibroblasts by westernblot analysis using anti phospho-Akt Ser473 (pAkt) and anti caspase-3 antibodies. Our experiments showed no statistically significant difference in the levels of pAkt when normalised against the total levels of Akt (pan Akt) or in the levels of caspase-3 in the SMA4 cells when compared to control fibroblasts (pAkt/pan Akt: $x_{SMA4} = 0.40$ versus $x_{Ctrls} = 0.42$, SD$_{Ctrls} = 0.01$; caspase-3: $x_{SMA4} = 0.84$ versus $x_{Ctrls} = 1.06$, SD$_{Ctrls} = 0.20$ densitometry units). This data suggests that the p. G492R/p. F610C compound heterozygous

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**FIGURE 4** | acquired (n > 250 cells). (C) ATP production was measured using the ATPliTe assay kit. Arbitrary luminescence units (ALU) are shown for each experimental group from data obtained for 3 independent experiments. (D) Mitochondria membrane potential was assessed in live cells using 1 μM TMRE (green), 1 μM FCCP (carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone) was used as a positive control to uncouple mitochondrial oxidative phosphorylation. (D) Quantification of the TMRE staining (integrated intensity) for untreated (solid bars) and FCCP treated (lined pattern) cells was calculated for 10 random images and represented using bar graphs as the mean ± SEM from 3 separate experiments for each cell line. (E) Protein levels of the PI3K-Akt neuronal survival pathway protein p-Akt (relative to total pan Akt) and the proapoptotic caspase-3 determined by western blot analysis. β-actin was used as a loading control in these experiments. (E) The expression of p-Akt and caspase-3 was determined and shown in bar graphs as the mean ± SEM from 3 independent experiments.
mutation does not induce apoptosis through the absence of the pro-survival Akt pathway.

β-catenin as a Possible Molecular Crosstalk Between CAPN1, SMN and Non-5q SMA Genes

Multiomics studies are providing new insights into the pathomechanisms underlying SMA and identifying new molecular targets and potential therapeutic options (Meijboom et al., 2021). Within the set of proteins that appear differentially expressed in the synapse and spinal cords from mouse SMA models, β-catenin shows a 400% upregulation that is confirmed in muscle biopsies from SMA patients. Importantly, the list of proteins differentially expressed in SMA is significantly enriched in β-catenin targets, suggesting β-catenin may present a major feature of neuromuscular pathology in SMA (Wishart et al., 2014). When we compared the data set of proteins differentially expressed in these
SMA models to a published list of direct targets of the protease activity of CAPNI (Shinkai-Ouchi et al., 2016), β-catenin is the only common protein we identified (Figure 5A). Pathway analysis shows a strong link between all SMA genes and CTNNB1, the gene that encodes β-catenin, with CAPNI clustering with CTNNB1, LMNA, AR, UB1A and TFG (Figure 5B, green spheres) and GARS and SMN1 being at the edge of a second cluster (Figure 5B, blue spheres) connected with the first one by UB1A (shown by the dotted line). Reduced CAPNI activity shown in SMA4 fibroblasts (Figure 5D) may lead to accumulation of β-catenin in the patient cells. Western blot analysis however indicates the levels of β-catenin in the SMA4 fibroblasts are within the range shown by the three control lines used in this study (xSMAs = 1.09 versus xCtrls = 1.09, SDCtrls = 0.19 densitometry units; Figure 5C). In cells, β-catenin is mostly expressed in the nuclei where it regulates gene expression (Behrens et al., 1996) (Molenaar et al., 1996) and at the plasma membrane associated with E-cadherin regulating cell adhesion (Yap et al., 1997). Immunofluorescence staining confirms localisation of β-catenin in these locations (Figure 5D) and quantification of nuclear and cytoplasmic expression indicates β-catenin levels are comparable between SMA4 fibroblasts and control cells in these compartments (Nuclear β-catenin: xSMAs = 225.50 versus xCtrls = 224.60, SDCtrls = 22.34; cytoplasmic β-catenin: xSMAs = 862.30 versus xCtrls = 834.83, SDCtrls = 80.28 integrated intensity; Figure 5D). The role of the Wnt/β-catenin signalling as a common molecular pathway in which SMN and non-5q SMAs may converge has recently been suggested (Sotic and Fuller, 2020). Our experiments using skin fibroblasts however suggests this association may be tissue-specific and further experiments using patient-derived motor neurons may provide further evidence of the functional association between CAPNI, β-catenin and degeneration of motor neurons in SMA4 patients.

DISCUSSION

Over 96% of SMA patients receive a genetic diagnosis based on loss of function deletions or mutations on the SMN1 gene. These cases represent the more severe SMA types 1, 2 and generally type 3 cases, in which a prompt and early intervention is needed to minimise the irreversible and devastating clinical consequences following motor neuron loss. Research interest has focused accordingly in identifying venues to increase cellular availability of SMN protein. SMA research has pioneered gene-targeted therapy with currently 3 SMN-targeted therapies (SMN2 splicing modifiers and SMN1 gene replacement) approved by the FDA/EMA and a larger number are in clinical development (Chaytow et al., 2021).

Approximately 4% of SMA patients are not genetically linked to 5q13 and, although these cases belong to the less severe SMA types 3 and 4 or in many cases have a somewhat different phenotype, there is an urgent need for developing SMN-independent therapies to address motor neuron degeneration in these patients. In this regard, next generation sequencing technologies are increasing the number of genes associated with these non-5q SMAs and are therefore providing new pieces of evidence to identify a functional connection among the cellular and biological processes affected by these mutations. In this work we identified CAPNI as a new genetic cause for SMA type 4. The two affected siblings bear a compound heterozygous mutation (c.1474G > A and c.1829T > G) inherited from their unaffected father and mother, respectively. Importantly, the proband’s half-brother bearing the c.1474G > A substitution is clinically normal, providing strong genetic evidence for the involvement of CAPNI mutations in the current absence of additional SMA patients/families bearing mutations in this gene.

Calpains are evolutionarily conserved and widely expressed Ca2+-activated cysteine proteases and increasing evidence support their role in neuronal remodelling and neurodegeneration. The activity of calpains is tightly regulated and both reduced protease activity and abnormal activation can have deleterious effects, leading to impaired and promiscuous cleavage of various targets, respectively (Metwally et al., 2021). In this regard, previously identified CAPNI mutations are a known genetic cause for cerebellar ataxia (Wang et al., 2016) and hereditary spastic paraplegia (HSP) (Gan-Or et al., 2016). In these cases, functional evidence points to CAPNI loss of function as the underlying pathomechanism. Interestingly, until our study there had not been genetic evidence linking calpain function with SMA development, although recent investigations have described the role of CAPNI regulating SMN protein (Wang et al., 2019) (de la Fuente et al., 2020), suggesting the use of calpain inhibitors as a therapeutic strategy for SMA treatment (de la Fuente et al., 2019).

We therefore sought to determine whether the compound heterozygous mutation in CAPNI leads to loss of function of the resulting protein or to increased protease activity. Our experiments demonstrate a 35% reduction in the CAPNI protein levels in the SMA4-derived fibroblasts. Accordingly, CAPNI activity in the patient cells accounts for 12% of total Ca2+ activated protease activity versus a 22% reported for the control lines. While this suggests the p. G492R/p. F610C mutation reduces CAPNI activity, this may only be a direct consequence of the reduced availability of CAPNI in the SMA4 cells, associated with the reduced stability of the mutant CAPNI predicted by in silico modelling (Figure 1F). Importantly, our current experimental approach can’t exclude the hypothesis of increased protease activity of the mutant CAPNI in motor neurons from the SMA patient. Tissue specificity of calpain activity has recently been demonstrated (de la Fuente et al., 2020). De la Fuente et al. (2020) showed opposite calpain-1 activity profiles in SMA fibroblasts (downregulation) and SMA-derived iPSC motor neurons (increased protease activity), although calpain-1 protein levels were reduced in both tissues. Damaging calpain overactivation has been associated with the pathogenic reduction of the natural endogenous calpain inhibitor calpastatin in vivo models of Alzheimer’s disease (Rao et al., 2014), amyotrophic lateral sclerosis (Rao et al., 2016) and Huntington’s disease (Hu et al., 2021). Although merely speculative, the 3D structure of Rattus norvegicus calpain-2 (CANP2) in association with calpastatin (Hanna et al., 2008) provides a plausible hypothesis for the potential impact the p. G492R/p. F610C compound heterozygous mutation may have on CAPNI regulation by its endogenous inhibitor. Calpastatin binds as an extended polypeptide over the surface of calpains, contacting specific hydrophobic areas of the enzyme (Figure 6A). The α-helix from the N-terminal side of calpastatin passes through the calpain’s PEF Ca2+ binding domain, establishing a hydrophobic bond with the F610 residue (F597 in CANP2, Figure 6B). This contact stabilises the interaction between
calpain and calpastatin and computational prediction of the protein-protein affinity between the F597C mutant and the inhibitor estimates a significant reduction in the stability of the resulting complex ($\Delta \Delta G = -1.43 \text{ kcal/mol}$). Although the amino acid G492 (G479 in CANP2) is not in direct contact with calpastatin in this model, in silico analysis also predicts a reduction in the affinity between the inhibitor and the mutant calpain ($\Delta \Delta G = -0.37 \text{ kcal/mol}$), possibly due to changes in the overall stability of the beta sheet domain in which G492 is located (Figure 1F). Altogether, the p. G492R/p. F610C compound heterozygous mutation may lead to a reduction in the affinity of the calpain inhibitor calpastatin for CAPN1, leading to increased calpain activity in patients and supporting the hypothesis of using calpain inhibitors as a therapeutic strategy in SMA (de la Fuente et al., 2019).}

Our experiments using patient fibroblasts have not provided definitive information regarding which cellular and molecular pathways may be affected by the SMA4 causative CAPN1 mutation. Autophagic processes, mitochondrial function and apoptotic pathways appeared unaltered in the SMA4 cells. Additionally, protein levels and intracellular distribution of $\beta$-catenin, a target of the protease activity of calpain-1 suggested as a molecular crosstalk between SMN and non-5q SMAs (Soltic and Fuller, 2020) that establishes functional association with a number of SMA genes (Figure 5B), remain normal in the SMA4 derived fibroblasts. Patient fibroblasts have proven to be a suitable model to investigate pathomechanisms in neurodegenerative diseases (Auburger et al., 2012), however the precise pathways activated by genetic mutations can be overlooked when not using complementary approaches, including spinal cord motor neurons and in vivo models (Juneja et al., 2019). In SMA, this has been recently illustrated by the vastly different autophagy profile between muscle, motor neurons and skin fibroblasts from a SMA patient (Sansa et al., 2021b). Further studies using neuronal in vitro and in vivo models bearing the p. G492R/p. F610C compound heterozygous mutation will allow describing the precise pathomechanisms leading to motor neuron loss in SMA. Our research provides another example of neurodegenerative diseases caused by mutations in calpain genes and increases the genetic heterogeneity of non-5q SMAs, adding CAPN1 to the list of spinal muscular atrophy causative genes.

**DATA AVAILABILITY STATEMENT**

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding authors.

**ETHICS STATEMENT**

The studies involving human participants were reviewed and approved by Sydney Local Health District Human Ethics Review Committee, Concord Repatriation General Hospital, Sydney, Australia (reference number HREC/11/CRGH/105). The patients/participants provided their written informed consent to participate in this study.

**AUTHOR CONTRIBUTIONS**

Conceptualization: P-SG, KM; Methodology: P-SG, EM, and AA; Investigation: P-SG, KM; Resources: VS, KM, MK, and RS; Data
curation: P-SG, EM, and AA; Writing–original draft: P-SG; Writing–review and editing: P-SG, EM, AA, GB, VS, KM, MK, RS, and KM; Supervision: P-SG, KM; Project administration: KM; Funding acquisition: KM.

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SUPPLEMENTARY MATERIAL
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CAPN1 Mutation Causes SMA4
