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Closing a Small Gap in Our Understanding of Excitation-Contraction Coupling: JSR90/JFP90 is Junctophilin 1.

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***Abstract*— Development of a panel of monoclonal antibodies against triad junctional membranes provided a powerful tool for the characterisation of multiple proteins participating in excitation-contraction coupling (ECC), including the ryanodine receptor (RyR) calcium channel, dihydropyridine receptor voltage-sensor and linker protein triadin. Another component of ECC, termed the 90 kDa junctional foot protein (JFP90/JSR90) was identified with an antibody clone, mAb VF1c. This protein is predominantly expressed in skeletal muscle, is most abundant in fast-twitch fibres and is phosphorylated by an endogenous kinase. Additional studies indicated that it forms a supramolecular complex with the RyR and is upregulated in muscles from aged humans. Despite its potential importance, the molecular identity of JFP90 was not determined at the time. In the current study, the identity of JFP90/JSR90 was deduced by immunoprecipitation with mAb VF1c and proteomic analysis of the major proteins isolated. Using this approach, it was determined that JFP90 is junctophilin 1, a triad-enriched protein that links the sarcoplasmic reticulum to the t-tubules, thereby contributing to ECC. Consolidated with data from previous publications, the current study reveals novel properties of junctophilin 1, in particular that it is selectively upregulated in the soleus muscle, but not quadriceps, of aged mice.**

***Keywords* — excitation-contraction coupling, 90 kDa junctional foot protein (JFP90/JSR90), junctophilin 1; ageing.**

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I. Introduction

In skeletal muscle, communication between voltage-sensing dihydropyridine receptors (DHPR) in the t-tubular system and ryanodine receptor (RyR) calcium release channels in the terminal cisternae of the sarcoplasmic reticulum (TC-SR) is critical for excitation-contraction coupling (ECC), *for review see* [1]. Triad junctions represent couplings between the t-tubular and the TC-SR membranes and are highly enriched in proteins participating in ECC. A milestone in the understanding of the biochemistry of this process was the generation of a panel of monoclonal antibodies by immunisation of mice with purified triad junction membranes. Antibodies produced using this approach proved invaluable for the characterisation of multiple proteins involved in ECC, including the DHPR [2], RyR [3] and the junctional glycoprotein triadin [4].

One clone from this library, monoclonal antibody (mAb) VF1c, recognises a component termed the 90 kDa junctional foot protein (JFP90) or the 90 kDa junctional SR protein (JSR90). This membrane protein is highly enriched in the triad junctions, is phosphorylated by an endogenous kinase, is exclusively detected in skeletal muscle and not in other tissues, and is most abundant in type 2 fast-twitch fibres [5]. Expression of JFP90 is markedly reduced in canine muscles treated by chronic low-frequency electrical stimulation, used as a model of fast-slow fibre type transition [6]. Chemical cross-linking studies suggested that JFP90 forms a supramolecular complex with the RyR and so, is potentially involved in the regulation or organisation of ECC [7]. Using mAb VF1c to probe immunoblots of vastus lateralis samples from male humans ranging in age from 47 to 73 years old, it was qualitatively demonstrated that JFP90 is upregulated in aged muscles [8]. Since the efficiency of ECC is reduced in flexor digitorum brevis myofibres from old (20-22 month) compared with young (3-6 months) mice [9], increased JFP90 expression might participate in the pathology of sarcopenia, or might be a compensatory response to it. Given the public health impact of age-related declines in muscle performance [10], understanding the roles of JFP90 in skeletal muscle is of scientific and medical merit.

A major limitation in understanding the function of JFP90 has been the failure to deduce its molecular identity. In the current study, in order to address this deficiency, mAb VF1c was used to immunoprecipitate JFP90 from solubilised rabbit back muscle membranes. Proteomic analyses of two major proteins isolated, of apparent molecular weight 90 kDa and 68 kDa, indicated that they are full-length and truncated forms of junctophilin 1 (JPH1), a triad junction-enriched SR membrane protein that links TC-SR to the t-tubular system [11, 12]. Epitope mapping indicated that mAb VF1c binds to a region between residues 369 and 460 of 662 amino acid protein. These findings are consistent with the notion that JPH1 is cleaved by endogenous calcium-dependent proteases, or calpains [13].

The current investigation also found that JPH1 was upregulated in soleus muscle from old (25 month) compared with adult (12 month) mice, but its expression levels were indistinguishable between the two age groups in quadriceps. Overall, this work links previous biochemical data about JFP90 to JPH1, highlighting unexplored avenues for exploration of the biology of this protein. For example, JFP90 (and therefore JPH1) is phosphorylated by an endogenous kinase, but the mechanisms and functional consequences of this have not been investigated to date. 'Filling-the-gaps' approaches to consolidation of scientific knowledge, such as that presented here, are likely to become increasingly important in the near future, given the vast and expanding quantity of data that are available via the internet and other resources. Part of this work has been presented in oral form at the Royal Academy of Medicine in Ireland (RAMI), Biomedical Section Meeting at University College Cork, 20th June 2013.

II. Methods

2.1. Materials

Protease inhibitors, materials for SDS-PAGE, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate (CHAPS, ≥98%), anti-smooth muscle myosin mAb hSM-V, horse-radish peroxidase conjugated secondary antibodies and general reagents were from Sigma-Aldrich Ireland Ltd. (Arklow, Co. Wicklow, Ireland). Dynal Protein G paramagnetic beads and a rabbit polyclonal antibody (pAb anti-JP1) generated against a C-terminal peptide from JPH1 were purchased from Bio-Sciences Limited (Dun Laoghaire, Co. Dublin, Ireland). An antibody recognising JFP90, mAb VF1c, was obtained from Novus Biologicals, (Littleton, USA).

2.2. Animals

All animal procedures were carried out in accordance with local procedures, approved by the appropriate ethics committees. Experiments on the effects of ageing on muscle expression of JFP90 were performed in accordance with UK Home Office Guidelines under the Animals (Scientific Procedures) Act 1986. Male C57Bl/6 mice were housed in a temperature controlled environment with a 12 h light-dark cycle and were fed *ad libitum* on a standard laboratory chow. Animals were maintained to an age of either 12 months ('adult') or 25 months ('old'), then were initially anesthetised using 100 μL of Dormitor (0.15 mg/mL) and Ketaset (20 mg/mL), prior to sacrifice using cervical dislocation.

2.3. Preparation of tissue lysates and microsomal membranes

For the preparation of lysates, tissue samples were quickly excised and flash-frozen in liquid nitrogen. They were subsequently thawed in 10 volumes per wet weight of modified RIPA buffer containing 150mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 200mM NaF with freshly added protease inhibitors (1 μg/mL aprotinin, leupeptin, pepstatin A and 0.5 mM phenylmethylsulfonyl fluoride (PMSF)) and homogenised on ice with 3 by 30 s bursts of an Ultraturrex disperser, fitted with a T-25 probe. Insoluble material was removed via centrifugation at 12,000 g for 30 min at 4˚C. Microsomal membranes, enriched in SR, were prepared from rabbit back muscle by differential ultracentrifugation, as described previously [14]. All samples were divided into 0.5 mL aliquots, flash-frozen in liquid nitrogen and stored at -80°C until use.

2.4. Solubilisation, immunoprecipitation and proteomics

Rabbit skeletal muscle microsomes (4 mg protein/reaction) were solubilised in 2 mL of 2% CHAPS, 0.5 M NaCl, 0.5 mM PMSF, 20 mM Tris-HCl pH7.4 for 1h on a shaking platform at room temperature (18-22°C). Samples were then diluted in 8 mL/reaction of ice-cold 0.5 mM PMSF, 20 mM Tris-HCl pH7.4, prior to being centrifuged to clear insoluble material (113,000 g, Beckman Type 65 rotor for 1 h, 4°C). Supernatants were incubated with 50 μL of Dynal Protein G paramagnetic beads pre-bound to 5 μL of either mAb VF1c or mAb hSM-V (anti-smooth muscle myosin, as an isotype matched negative control) ascites fluid, according to the manufacturer's instructions. Following incubation for 1h at room temperature on a shaking platform, immune complexes bound to paramagnetic beads were collected using a magnetic separator and were resuspended in 1 mL of ice-cold 0.1% CHAPS, 0.5 mM PMSF, 20 mM Tris-HCl pH7.4. Following another three wash steps, paramagnetic beads were resuspended in 50 μL of 2 x concentrated sample buffer (1.6 % SDS, 8% glycerol, 0.04 % bromophenol blue, 4 mM dithiothreitol, 50 mM Tris-HCl pH6.8) and were heated to 95°C for 5 min prior to separation on 7.5% SDS-PAGE minigels. Following staining with Coomassie R250, major bands in the mAb VF1c immunoprecipitate were excised and analysed using a commercial MALDI-MS and MS/MS protein identification service (Proteomics service, Technology Facility, Department of Biology, University of York, UK). Parallel blots were transferred to polyvinylidene fluoride (PVDF) prior to immunostaining with mAb VF1c, as described in *Section 2.6*. Identification of potential PEST motifs in target proteins was determined using 'epestfind' software at (http://emboss.bioinformatics.nl/cgi-bin/emboss/epestfind/). PEST motifs target proteins for degradation by calcium-dependent proteases, or calpains [15]. A PEST score

of greater than +5 indicated that the potential motif was considered 'of interest'.

2.5. Epitope mapping of mAb VF1c

In order to determine the site of interaction of mAb VF1c with JPH1, cDNA constructs encoding overlapping fragments of this protein were heterologously expressed in yeast [16], using protocols essentially the same as those described previously for ankyrin-1 [17]. The JPH1 clones corresponded to the following amino acid residues of human JPH1: JP1A: 1-233, JP1B: 232-369, JP1C:369-571, JP1D: 141-278, JP1G: 229-460, JP1L: 154-278, JP1M: 229-550, JP1Delta: 154-635. These proteins were resolved on 10% SDS-PAGE minigels, transferred to PVDF and immunostained with mAb VF1c or pAb JP1 as described in *Section 2.6*. To further define the mAb VF1c epitope within the region identified by screening yeast fusion proteins, peptide arrays of overlapping 18-mers were generated, essentially as described previously [18].

2.6. General protein methods

Protein concentrations were determined using the Bradford assay [19], using bovine serum albumin as a standard. Quantities of protein loaded onto SDS-PAGE minigels are noted in the figure legends. Western transfer and immunostaining of proteins with mAb VF1c (1:1500 dilution) or pAb JP1 (1:1000) were performed as described previously [20]. Following immunostaining, PVDF blots were stained with Coomassie R250 as a protein loading control, essentially as described by Welinder and Ekblad [21].

2.7. Statistical methods

For quantification of protein expression, images of immunostained or Coomassie R250 stained PVDF membranes were captured using a gel documentation system and exported as JPEG files. The densities of immunoreactive bands, or of total protein in each lane, were determined using NIH ImageJ software (http://rsbweb.nih.gov/ij/). The density of each immunoreactive band was divided by the total protein density in that lane and the mean values of these ratios in tissue from young animals was taken as 100%, for comparisons with corresponding tissues from old mice. These mean normalised band densities were compared using one-way ANOVA with *post hoc* Tukey's tests, taking a p value of less than 0.05 as significant.

III. RESULTS

3.1. JFP90/JSR90 is Junctophilin-1

In order to determine the primary structure of JFP90/JSR90, microsomal membranes from rabbit back muscle were solubilised and immunoprecipitated using mAb VF1c. Several proteins were immunoprecipitated by mAb VF1c, but not by an isotype-matched negative control mAb, *Fig. 1, 'Coomassie R250'*. These include proteins with apparent molecular weights of >300 kDa, 170 kDa, 111 kDa, 90 kDa, 68 kDa and 22 kDa. Of these proteins, only the 90 kDa ('Band I') and 68 kDa ('Band II') were immunostained with mAb VF1c on a parallel western blot, *Fig. 1*, 'mAb VF1c'. Immunoprecipitation facilitated major enrichment of the mAb VF1c interacting proteins, since these represented minor components of solubilised muscle microsomes, which were not detectable in the solubilised material by immunoblot under these conditions. This purification step permitted proteomic identification of JFP90/JSR90 using a commercial service. Both Band I and Band II were good matches to rabbit junctophilin-1 (JPH1), in terms of MOWSE scores (230 and 116) and coverage, *Fig. 2C*. The bands had three JPH1 peptides in common (residues 84-91, 145-156 and 304-313), but Band I contained an additional, more C-terminal peptide (558-573). This suggests that Band II might represent the product of proteolytic cleavage of full-length JPH1, Band I. Since JPH1 is reported to be cleaved by calcium-dependent proteases in rat skeletal muscle [13], the presence of calpain cleavage sites, or PEST motifs, in rabbit JPH1 was examined using epestfind software. This identified one strong potential PEST motif, with a score of +12.35 (the threshold value is +5), between residues 458 and 471, *Fig. 2C*. Calpain cleavage of 72 kDa full-length JPH1 at this site would generate products of 50 kDa and 22 kDa. Although these calculated molecular weights do not match those determined experimentally (90 kDa full-length, 68 kDa product), possibly because of the highly basic amino acid composition (predicted isoelectric point of full-length JPH1= 9.4; note the anomalous migration of JPH1 fusion proteins in *Fig. 2A* and *2B*), the 22 kDa shift in molecular weight is as predicted, *Fig. 2C*. This also raises the possibility that the minor 22 kDa protein immunoprecipitated by mAb VF1c is the C-terminal calpain cleavage product of JPH1. Attempts to verify this by western blotting of the mAb VF1c immunoprecipitates with an anti-JPH1 antibody raised against a C-terminal peptide were unsuccessful, due to low signal-to-noise ratios on these blots (*not shown*).

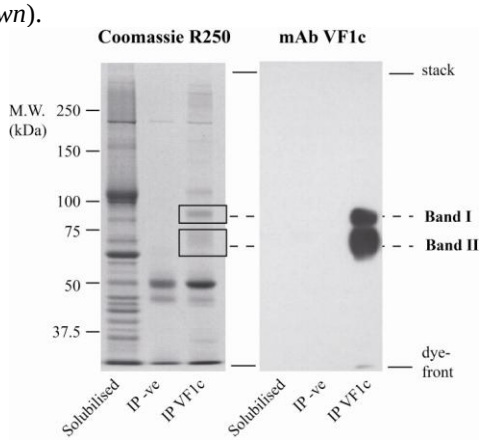

Fig. 1. Immunoprecipitation and proteomics of JFP90 reveals that it is JPH1. Rabbit back muscle microsomes were CHAPS-solubilised and immunoprecipitated with either mAb VF1c (anti-JFP90, 'IP VF1c) or an isotype-matched negative control ('IP –ve'). Immunoprecipitates and solubilised starting material were then resolved on 7.5% SDS-PAGE minigels and either stained with Coomassie R250, or transferred to PVDF membranes by western blotting and immunostained with mAb VF1c. Immunoreactive protein bands ('Band I' and 'II') were excised from the Coomassie stained gels for protein identification. Data shown are representative of three separate experiments.

3.2 Mapping of the epitope for mAb VF1c.

To map the location of the epitope for mAb VF1c within the primary structure of JPH1, thereby confirming that JFP90 and this protein are identical, overlapping segments of this protein were overexpressed in yeast and probed with the antibody on western blots. The anti-JFP90 antibody was strongly immunoreactive with fusion proteins corresponding to residues 229-460, 229-550 and 154-635 of human JPH1, but also recognised a polypeptide spanning amino acid 369-571, *Fig. 2A*. This indicates that mAb VF1c recognises a segment spanning residues 369-460 of human JPH1, corresponding to a divergent/α-helical region within this protein that has been postulated to play a role in determining the width of the gap between the t-tubule and SR [12].

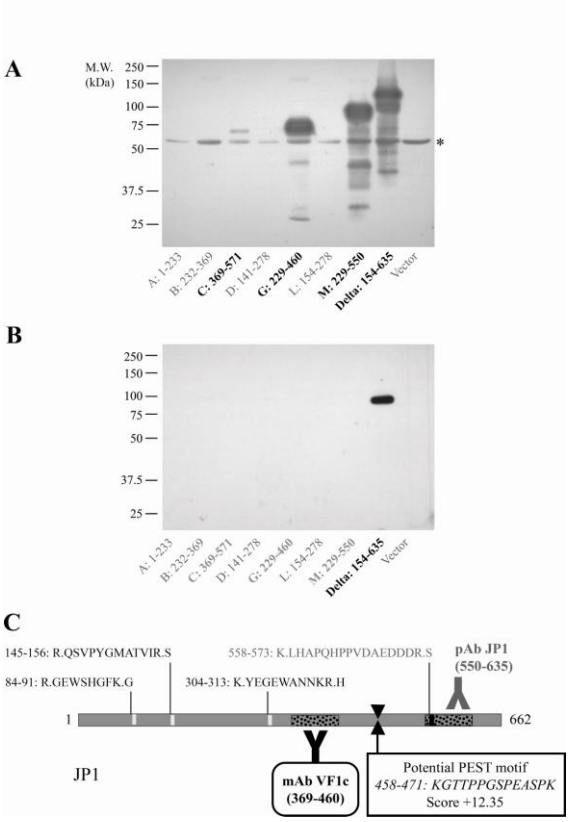

Fig. 2. The region from residues 369-460 of human JPH1 contains the epitope of mAb VF1c. *A.* Fragments of human JPH1 were overexpressed in yeast, resolved by SDS-PAGE and immunoblotted with mAb VF1c. The asterisk (*) indicates a protein present in yeast that was detected in the 'Vector' only transfected sample. *B.* A parallel blot was probed with a commercial rabbit antiserum directed against a C-terminal peptide from JPH1. *C.* Diagram summarising the relative positions of peptides derived from immunoprecipitated JFP90 (match from Band I only in grey font), the positions of the epitopes of mAb VF1c and anti-JP1 antiserum and the location of a potential calpain cleavage site, or PEST motif.

In attempts to further define the epitope of mAb VF1c, an array of overlapping 18-mer peptides was constructed that corresponded to this region. However, signals detected by mAb VF1c on such arrays did not qualitatively differ from those probed with secondary antibody alone, *Fig. 3*, suggesting that this antibody might recognise an epitope that is dependent on higher order structure within the protein. As anticipated, a C-terminally directed commercial anti-JPH1 antiserum only bound to a fusion protein containing the C-terminus of this protein, 'Delta:154-635', *Fig. 2B.* The relative positions of the mAb VF1c and anti-JP1 are shown in the diagram in *Fig. 2C*, along with the peptide sequences deduced from proteomic analyses and the potential calpain cleavage site.

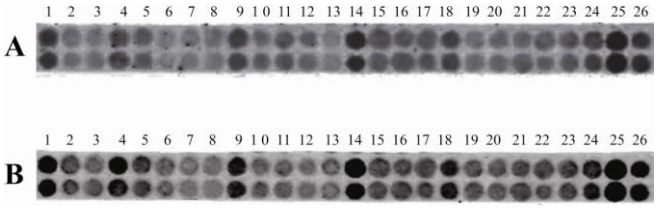

Fig. 3. Mapping of the mAb VF1c epitope using peptide arrays. Overlapping 18-mer peptide spots (1-26) covering residues 369-460 of rabbit junctophilin 1 (NCBI Accession Number: NP_001075465.1) were synthesised in duplicate on two arrays. One array, A, was probed with a 1:500 dilution of mAb VF1c, followed by washing then a 1:2500 of LiCor goat anti-mouse IgG IRDye 680LT conjugated secondary antibody. A parallel array, B, was probed with secondary antibody only. Immunoreactive spots were detected using a LiCor Odyssey scanner. Data are representative of two separate experiments. The sequences of peptides are available on request.

3.3. During ageing, junctophilin-1 is significantly upregulated in mouse soleus but not in quadriceps.

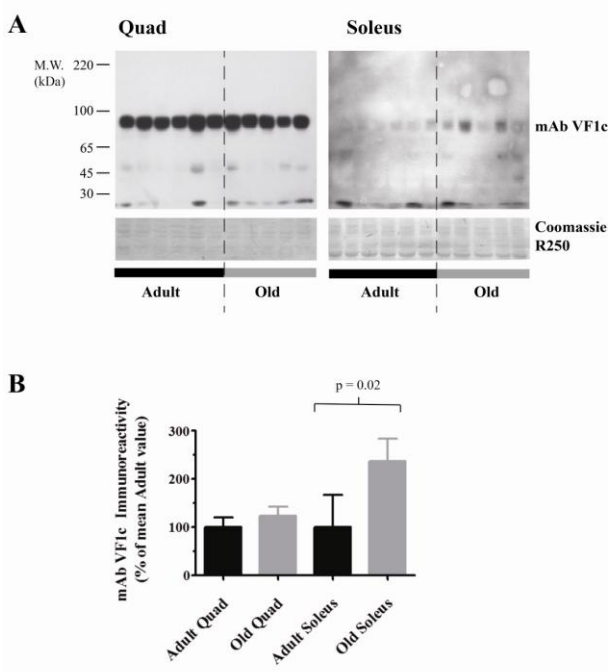

Fig. 4. JP1 is upregulated in soleus, but not quadriceps, muscle from old mice. *A.* Immunoblots of lysates (30 µg protein/lane) from 'Adult' (12 months, n = 6) or 'Old' (25 month, n =5) mouse quadriceps ('Quad') or soleus muscle, probed using mAb VF1c. Each blot was post stained with Coomassie R250 to estimate protein loading. *B.* Band densities from the immunoblots in *Panel A* were normalised to protein loading and expressed relative to the Adult group of animals for each muscle type. JPH1 protein expression was significantly upregulated in the soleus of old mice ($p = 0.02$ by one-way ANOVA and Tukey's *post-hoc* test), but not in quadriceps. Data are representative of two technical replicates.

Qualitative data from another laboratory indicated that JFP90, demonstrated to be JPH1 in the current work, is upregulated with ageing in human vastus lateralis muscle [8]. To examine this further, expression levels of JPH1 protein from quadriceps or soleus muscle from 'adult' and 'old' mice were assessed by western blotting using mAb VF1c. Qualitatively, mouse quadriceps ('Quad') muscle expresses greater quantities of JPH1 than soleus ('Soleus'), *Fig. 4A*. There was an apparent upregulation of JPH1 in old animals in the soleus, but not in the quadriceps. To quantify alterations in JPH1 protein expression with ageing, band densities were normalised to total protein by *post hoc* Coomassie R250 staining of PVDF membranes. This demonstrated that soleus muscle from old mice expressed significantly greater levels of JPH1 protein than adult animals ($240 \pm 50\%$ ($n = 5$, mean $\pm$ standard error) versus 100 ± 30 % ($n = 6$); $p = 0.02$), whereas its abundance was not significantly altered in the quadriceps, *Fig. 4B*.

IV. Discussion

The data presented here strongly support the notion that JFP90, a protein whose molecular identity was unknown, is JPH1. This permits unification of data relating to JFP90 to that on JPH1. For example, JFP90 and therefore JPH1, is upregulated with age in human vastus lateralis muscle [8]. A study published by Brooks and Faulkner in 1988 [22] revealed that the contractile properties of soleus fibres were preserved between adult (9-10 months) and old (25 month) mice, whereas extensor digitorum longus (EDL) fibres from aged animals displayed significant reductions in specific maximum tetanic force. In particular, ageing was associated with a prolongation of isometric twitch duration and a leftward shift in the frequency-force relationship in soleus, but not in EDL muscles. The authors speculated that these compensatory changes in soleus fibres could be related to alterations in Ca^{2+}-handling. JPH1 plays a key role in muscle Ca^{2+} signalling, linking the DHPR voltage-sensor to other ECC components [16]. Transgenic mice lacking JPH1 expression exhibit no suckling and early postnatal death, decreased formation of triad junctions and reduced electrically evoked force production in muscle fibres [23]. Furthermore, shRNA-mediated downregulation of both JPH1 and JPH2 in C2C12 cell myotubes resulted in decreased Ca^{2+} transients in response to the RyR agonist caffeine and aberrant Ca^{2+}-induced Ca^{2+}-release, leading to chronic Ca^{2+}-store depletion [24]. Decreased JPH1 protein expression is also associated with early deficits in strength that occur within three days of eccentric exercise [25]. In addition, reduced JPH1 levels have been linked with muscle weakness that occurs in the murine *mdx* model of muscular dystrophy [13]. Together with the current findings, these studies suggest that upregulation of JPH1 in soleus muscle from aged mice represents a compensatory mechanism, to enhance triad junction density, sarcoplasmic reticulum Ca^{2+}-loading and force production [8]. It was unexpected that JPH1 was not detectably altered in mouse quadriceps, given that this muscle group contains the vastus lateralis, in which this protein is upregulated with ageing in humans. This distinction could relate to differences in muscle physiology between mice and humans, or to the other muscle groups present in the quadriceps.

Other aspects of JPH1 biology unveiled by its relationship to JFP90 include that it is phosphorylated by an endogenous kinase in skeletal muscle [5]. This indicates that JPH1 function might be modulated by phosphorylation, an aspect that awaits exploration. In different muscle types, JFP90 has been detected as either a single major protein (for example, mouse quadriceps, *Fig. 3A*), or a doublet (rabbit back muscle, *Fig. 1*), *also see references* [6] *and* [7]. Given that JPH1 can be cleaved by calpains [13] at a site within the α-helical linker region, proteolysis might serve as another mechanism for tissue- and activity-dependent regulation of this protein.

This project was initiated by expertise-based identification of a gap in the knowledge of ECC, namely the molecular identity of JFP90. Similar approaches will be particularly timely, given the quantities of scientific data and literature that are currently available via internet accessible databases. Such data mining and integration methods are amenable to automation, which has already been implemented to identify knowledge gaps in diverse fields, including in the characterisation of red algal evolution [26] and in construction of protein phosphorylation networks [27]. Application of related strategies to different areas is likely to open fruitful avenues of scientific discovery.

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