Identification of an FHL1 protein complex containing ACTN1, ACTN4, and PDLIM1 using affinity purifications and MS-based protein–protein interaction analysis[†]

Parveen Sharma^{a,b,‡}, Thiruchelvi Shathasivam^{a,b,‡}, Vladimir Ignatchenko^c, Thomas Kislinger^{c,d}, and Anthony O. Gramolini^{a,b}

^aDepartment of Physiology, University of Toronto, Toronto, Ontario, Canada M5G 1L6. Fax: +1 416 978-8528; Tel: +1 416 978-5609

^bHeart and Stroke/Richard Lewar Centre of Cardiovascular Excellence, 150 College Street, Toronto, Ontario, Canada M5S 3E2

^cUniversity Health Network, Ontario Cancer Institute/Campbell Family Cancer Research Institute, Toronto, Ontario, Canada M5G 1L6. Fax: +1 416 581-7629; Tel: +1 416 581-7627

^dDepartment of Medical Biophysics, University of Toronto, Toronto, Ontario, Canada M5G 1L6

Abstract

Introduction—Four and a half LIM domains protein 1 (FHL1) is the most widely expressed member of the FHL family of proteins, consisting of four and a half highly conserved LIM domains. A multifunctional and integral role for FHL1 has been implicated in muscle development, structural maintenance, and signaling. To date, 27 FHL1 mutations have been identified that result in at least six different X-linked myopathies, with patients often presenting with cardiovascular complications. Since proteins assemble into dynamic complexes within the cell, FHL1 likely mediates its biological functions in conjunction with other proteins. Delineation of FHL1 interactions could provide insight into its regulatory functions.

Methods—We performed tandem affinity purification from human embryonic kidney 293 (HEK-293) cells to purify FHL1 and interacting proteins. To identify the potential interactors of FHL1 we performed a total of 9 different purifications from HEK-293 cells which included 3 experimental replicates for each biological condition: FHL1, tag control (DPYSL3), and negative control (empty vector). Purified samples were analyzed by liquid chromatography mass spectrometry (LC-MS). Potential interactors were then verified by immunoprecipitation from mouse heart ventricles and interactions visualized in adult cardiomyocytes using 3D fluorescence microscopy.

Results—We identified a total of 310 different proteins from all 9 purifications and by applying stringent filtering criteria we eliminated all proteins found in any of the controls and only allowed

[†]Electronic supplementary information (ESI) available: Table S1: 310 different proteins identified from all 9 purifications. Table S2: 138 identified in any of the three FHL1 purifications. See DOI: 10.1039/c0mb00235f

Correspondence to: Thomas Kislinger; Anthony O. Gramolini.

[‡]Equal contributions.

those that were detected in two or more bait purification. We identified 34 high confidence potential binding partners of FHL1. We then showed that FHL1 exists as part of a complex that binds with PDLIM1, GSN and ACTN1.

Introduction

Four and a half LIM domain protein 1 (FHL1) is a multifunctional protein, characterized by the tandem arrangement of four and a half highly conserved LIM domains.^{1,2} It belongs to a family of FHL proteins (FHL1–3, and 5 in humans) and is expressed primarily in skeletal and cardiac muscle, although FHL1 transcripts have been detected at relatively high abundance in the ovary, kidney, and lung.^{2–8} LIM domains are capable of interacting with other LIM domain proteins, forming homo- or heterodimers as well as associating with tyrosine-containing motifs, PDZ domains, ankyrin repeats, and helix–loop–helix domains.⁹ Multiple functions have been ascribed to full-length FHL1; in skeletal muscle FHL1 is involved with sarcomere assembly, muscle differentiation, growth, and in the biomechanical stress responses.^{10–12} These FHL1 functions have been associated with several signaling pathways, including integrin-mediated, mitogen-activated protein kinase, β -adrenergic receptor, G-protein coupled receptor, NFATc1, transforming growth factor β like, and estrogen receptor signaling pathways.^{11–18}

FHL1, an X-chromosome gene (Xq26),^{2,6} was recently identified to be causal for several different X-linked myopathies.^{19–22} A large subset of mutations was identified as causal for Emery–Dreifuss muscular dystrophy, characterized by the triad of joint contractures, muscular dystrophy, and cardiac dysfunctions.^{8,23} Furthermore, an X-linked recessive Emery–Dreifuss-like syndrome was also described, where patients presented with joint contractures and cardiovascular disorders but lacked muscle weakness or atrophy.²⁴ Differential FHL1 expression has also been shown in several cardiovascular disorders, including dilated and hypertrophic cardiomyopathy, atrial fibrillation, and pulmonary hypertension.^{15,25–30} We have previously generated a mouse model to recapitulate early onset dilated cardiomyopathy observed in humans and caused by an Arg-9-Cys conversion in phospholamban, an integral membrane phosphoprotein of the sarcoplasmic reticulum.^{25,31} From our comparative proteomic profiling of ventricular muscle tissue, FHL1 was identified as one of the top four most up-regulated proteins, with changes occurring early in disease.²⁵ Since differential expression could be detected before disease onset, with continued increase throughout disease, it was suggested FHL1 could participate in the transition of phenotype between early and late stages, and thus contribute to the development of cardiomyopathy.

Twenty-seven mutations are found in the FHL1 gene that are causal for six different myopathies, each presenting a combination of various protein aggregates, joint contractures, muscle atrophy/hypertrophy, and cardiovascular diseases, however, the molecular mechanism by which FHL1 contributes to the development and pathology of disease remains unknown. The diversity of FHL1s functional properties and the heterogeneity of its pathophenotypes may be mediated by the diversity of its interacting partners. Protein complexes comprise an important level of functional organization of a cells proteome, and contribute to the control and execution of cellular activities. Studying FHL1 protein interactions would thus provide insight into its functional capabilities and assist in

characterization of its regulation and regulatory roles. Here, we have tandem affinity purified tagged FHL1 constructs in order to determine protein–protein interactions of FHL1 from HEK-293 cells and then verified these interactions in mouse ventricle tissue. We show that FHL1 exists as part of a complex which includes PDZ and LIM domain protein 1 (PDLIM1), Gelsolin (GSN) and α -actinin (ACTN1).

Results

Purification of FHL1 containing protein complexes

In the present study, we expressed FHL1 fusion proteins with streptavidin binding proteins (SBP) and a calmodulin binding protein (CBP) peptide affinity tag (Fig. 1A). We used streptavidin and calmodulin protein immobilized on a sepharose resin for the co-purification of FHL1 and its interacting partners from mammalian cells (Fig. 1B and C). To distinguish technical artifacts inherent to the purification process, tag-control purifications were performed with an identically tagged protein, DPYSL3 (Fig. 1A). Negative control purifications were simultaneously conducted using cells transfected with an empty vector. The expression and purification of both tagged bait proteins (FHL1 and DPYSL3) from HEK-293 cells was verified by immunoblot analysis and Fig. 1B and C illustrate the efficiency of the applied TAP procedure. The presence of bait proteins in the flow-through following streptavidin/calmodulin bead binding was likely due to saturation of the volume of beads used. Although some bait protein was also lost during the subsequent washes, progressively less was lost with each additional wash and these were deemed necessary steps to improve the purity of the final product by removing nonspecific interacting proteins. Ultimately, in the final elution a significant amount of the expressed recombinant protein was purified and concentrated.

Since the aim of this study was to delineate FHL1 interactions and thereby determine binding partners of FHL1 we monitored the efficiency of bait protein being purified with putative binding partners by Coomassie staining. Fig. 1C shows that FHL1 was removed from a very complex protein mixture as a multitude of bands are visible in the flow through and that washing the beads removed non-specific and weak interactors which are the bands present in the washes. The protein mixture was eluted from the streptavidin beads and then incubated with the calmodulin beads and after this two-step purification FHL1 can be visualized as a band at 42 kDa (Fig. 1C) indicative of the molecular weight of the FHL1 fusion protein. A silver stain of the elution highlights that FHL1 was not purified in isolation as several other bands can be seen (Fig. 1D) representative of co-purified potential FHL1 interactors.

Identification of potential FHL1 interacting proteins

We performed a total of 9 different purifications from HEK-293 cells and analyzed them by mass spectrometry in order to identify the potential interactors of FHL1. These purifications included 3 experimental replicates for each biological condition: FHL1, tag control (DPYSL3), and a negative control (empty vector). For each biological condition, 3 purifications were performed to completion and eluted from a calmodulin resin, followed by in-solution trypsin digestion for MS analysis (see methods). The generated peptide spectra

were searched against the human IPI protein sequence database and only those proteins which were supported by at least two unique peptides per run were accepted for further consideration. When combined together, a total of 310 (Table S1, ESI[†]) different proteins were identified from all 9 purifications with 138 (Table S2, ESI[†]) identified in any of the three FHL1 purifications. In order to identify potential FHL1 interactors of high confidence, the subsets of identified proteins were filtered and compared according to Fig. 2. First, non-specific interactors were removed which included proteins found in any of the negative controls or tag control purifications and contaminating keratin proteins. Fig. 3A depicts the 310 co-purified and identified proteins *via* heatmap representation. Following the removal of the nonspecific interactors 80 proteins were identified as belonging uniquely to the FHL1 purifications (Fig. 3B). Next, to strengthen the confidence in potential interactors only those proteins detected in at least two FHL1 purification replicates were considered resulting in 34 potential binding partners.

Characterization of the 34 potential FHL1 interacting proteins

A Gene Ontology (GO) Term enrichment analysis was performed to determine which molecular functions and biological processes were overrepresented (Fig. 4A and B). Gene names corresponding to the 34 proteins found in multiple FHL1 purifications were inputted for pathway analysis into the Gene Ontology For Functional analysis (GOFFA) software tool which is the primary biological interpretation tool using Gene Ontology (GO) in ArrayTrack <http://www.fda.gov/ScienceResearch/BioinformaticsTools/Arraytrack>.³⁶ Terms associated with cell structural organization and development, and organization of and binding to actin cytoskeletal components were particularly enriched within the potential interactor proteins ($p < 0.05$, $n = 5$, $E > 2$) (Fig. 4A and B). These terms were comparable to the GO-terms associated with FHL1.

The online STRING 8.3 database (<http://string.embl.de/>) was then used to identify any previously described interactions amongst FHL1 and the 34 potential interactors, from human sources. We found that the most overrepresented network contained 14 proteins found in our subset of 34 proteins and was found to be involved in the regulation of the actin cytoskeleton (Fig. 5). Smaller protein clusters were mapped to metabolic processes and nucleic acid binding (Fig. 5).

Validation of interactions

FHL1 has previously been shown to be upregulated during muscle development^{12,37} where major cytoskeletal remodeling occurs. With this in mind and the elucidation of the large protein interaction network involved in the regulation of the actin cytoskeleton (Fig. 5) found in our subset of 34 proteins we were interested in validating FHL1 interactions with proteins found within this network.

Protein–protein interactions between LIM domains have previously been demonstrated and shown that LIM domains can form homo- and hetero-dimerization.⁹ For this reason we

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chose to pursue from our potential interactors (Fig. 5) PDLIM1, a protein which also contains a LIM domain. The cDNA for PDLIM1 was cloned into the pEF-DEST51Gateway vector which contains alternative dual tags V5 and 6× histidine tags. To investigate the binding of FHL1 to PDLIM1 cell lysates from HEK-293 cells transfected with FHL–CBP/SBP tags and PDLIM1–V5/6His tagged we subjected to immunoprecipitation experiments using either the anti-CBP antibody (FHL1) or the anti-V5 antibody (PDLIM1). We found that FHL1 co-immunoprecipitated with PDLIM1 (Fig. 6A) and in addition the reciprocal immunoprecipitation using the anti-V5 antibody for PDLIM1 co-immunoprecipitated with FHL1 (Fig. 6B). Neither protein was detected in the A/G resin alone or the immunoglobulin control IPs (Fig. 6A).

In order to determine whether FHL1 and PDLIM1 form a complex *in vivo* we performed an immunoprecipitation from both wild type (Fig. 6C) and transgenic R9C cardiac ventricle lysates (Fig. 6D). Immunoprecipitation using anti-FHL1 antibody co-immunoprecipitated PDLIM1 from both wild type and R9C cardiac lysates shows that the complex exists not only in wild type heart but also in the disease model. Further immunoblot analysis of the immunoprecipitation determined that FHL1 not only binds to PDLIM1 but precipitates Gelsolin (GSN) and α -actinin (ACTN1) (Fig. 6C and D), two proteins also isolated in our mass spectrometry analysis (Fig. 5).

Immunofluorescence verification of complex formation

To further verify a complex formation between FHL1 and PDLIM1, ACTN1 and GSN we carried out immunofluorescence on dissociated adult mouse cardiac cells. Fig. 7A shows that FHL1 co-localizes with PDLIM1, ACTN1 and GSN, further supporting the existence of a complex. A detailed three dimensional (3D) volume analysis (Fig. 7B) was constructed from sequential z-sections of cells assembled using the Imaris software (version 7.1.1, Bitplane AG, Zurich, Switzerland) which allowed the calculation of co-localization statistics using Imaris software. This showed that FHL1 co-localized 22% with PDLIM with a Pearson coefficient of 0.44, 30% co-localization with GSN with a Pearson coefficient of 0.5 and 44% co-localisation with ACTN1 with a Person coefficient of 0.6.

Discussion

FHL1 is a LIM-only protein, characterized by the tandem arrangement of four and a half LIM domains which function as a modular protein-binding interface to mediate protein–protein interactions. To date, 27 mutations have been identified in the human *FHL1* gene resulting in a multitude of muscular dystrophies. Our current understanding of FHL1 is suggestive of important functions ranging from developmental organization, muscle force transmission, and even a role in cell migration.

In this study we purified FHL1 using affinity purification tags to elucidate potential FHL1 binding partners. Using mass spectrometry and stringent protein filtering criteria we identified 34 high confidence interactors of FHL1. GO-term analysis of these proteins revealed many were involved in biological processes which were rationally plausible for FHL1 involvement, such as organization and binding of the actin cytoskeleton, motility, intracellular transport, cell development and regulation, and muscle processes. Potential

interacting proteins could be mapped to network pathways involving regulation of the actin cytoskeleton, cell–cell signaling, fatty acid metabolism, MAPK and Wnt signaling, and focal adhesion, tight junction, and integrin signaling. Then, using the online protein database STRING 8.3 we mapped 14 out of 34 proteins which included the PDZ and LIM domains containing protein PDLIM1, GSN and ACTN1 to an existing evidence-based protein interaction network. We investigated several interactions further and demonstrated by co-immunoprecipitation and 2D and 3D immunofluorescence that FHL1 interacts as part of a complex with PDLIM1, GSN and ACTN1 (Fig. 8).

α -Actinin-1

α -Actinins are ubiquitously expressed actin-filament cross-linking proteins, belonging to a highly conserved family of actin-binding proteins, the spectrin superfamily: ACTN1 belongs to the calcium sensitive class of actinin isoforms and is crucial to the extensive actin stress-fiber subcellular structure.³⁸ Stress fibers consist of bundles of actin filaments, approximately 10–30 in number, held together by the crosslinking protein ACTN1 and randomly oriented.³⁹ In muscle systems, the contractile sarcomeric structures are deemed the equivalent of the actin stress fiber network in non-muscle cells.^{38,39}

Interestingly, FHL1 was previously identified as a component of the stress sensor complex at the sarcomeric I-band, where it interacts with the MAPK cascade components, Raf1, MEK2, and ERK2, at the N2B region of titin in cardiomyocytes.¹¹ Though the precise mechanisms were not elucidated, FHL1 was suggested to mediate a connection between the muscle stretch sensor and downstream responses by titin and MAPK components.¹¹ Similarly, in human epithelial cells, ACTN1 specifically, as opposed to non-muscle ACTN4, was associated with deformation, strain-induced cytoskeletal signaling.⁴⁰ Altogether, an interaction between ACTN1 and FHL1 is likely a critical coupling in regulating the actin-based stress fiber structure and function given the complex protein interactions that FHL1 appears to be able to mediate.

Gelsolin

Gelsolin was identified here as an interactor of FHL1.⁴¹ Within the cell, the multifunctional cytoplasmic GSN has been best described for its role in regulating turnover of the actin filament.^{41,42} Structurally, the cytoplasmic GSN is composed of six GSN-like domains and exists in a globular conformation in the absence of calcium. In the presence of calcium, the structure is relaxed allowing for the formation of a calcium binding domain coordinated by actin and the fourth GSN-like domain. In its active form, GSN binds to and severs two adjacent actin filaments, with GSN remaining bound to the newly formed barbed ends of one of the resulting shorter filaments.^{41,42}

Considering the actin filament remodeling properties of GSN, the phenotype of myoblast cells overexpressing FHL1, and the overrepresented biological GO term amongst the group of 34 potential interactors, FHL1 may participate in cell motility functions together with GSN. Since multiple factors contribute to the general properties of the cytoskeleton, including filament length, flexibility, concentration and presence of crosslinks, proteins capable of modifying any of these parameters are potential candidates for regulating cellular

morphology and functions, such as cell shape and motility.⁴¹ Both GSN and FHL1 demonstrated co-localization in HEK-293 cells in this study and previously along actin stress fibers.^{30,42}

In cultured fibroblasts, overexpression of GSN resulted in increased motility, whereas osteoclasts isolated from GSN null mice exhibited decreased motility.⁴³ These osteoclasts failed to form podosomes which are cell adhesion structures and affected their normal and osteopontin-induced motile properties.⁴⁴ Furthermore, delayed retraction of filopodia from neuronal growth cones from GSN null mice was also observed. Neuronal growth cones are highly motile structures, and since the formation of filopodia which is mainly dependent on adseverin function appeared unaffected, GSN could be involved in this cellular retraction process.⁴⁴ Similarly, in $\alpha 5\beta 1$ -integrin activated myoblasts, overexpression of FHL1 promoted cell spreading and migration while inhibiting cell adhesion.¹⁴ In differentiated muscle cells, overexpression of FHL1 resulted in significant branching with multiple major cytoplasmic protrusions from the cell body. Our data suggest that GSN and FHL1 are possibly components of the normal, regulated, cell motility processes, as opposed to pathological migration. In accordance with this, both GSN and FHL1 have been detected repeatedly downregulated in a variety of tumors, particularly in invasive disease.^{45–50}

PDZ–LIM protein 1

Of the potential FHL1 interactors identified in this study, PDLIM1 appears to be the least characterized. However, based on knowledge obtained from other PDZ–LIM proteins, the relationship PDLIM1 maintains with FHL1 is possibly as an adaptor protein. PDLIM1 belongs to the family of PDZ–LIM proteins, whereby each member possesses an N-terminal PDZ domain and one to three LIM domains at the C-terminal. PDLIM1 contains only one LIM domain, but both the PDZ and LIM domains are common multifunctional protein–protein interaction motifs.^{51,52} In non-muscular cells, PDLIM1 has been localized to stress fibers *via* interaction with ACTN1 by its PDZ domain.^{51,52} Moreover, PDLIM1 is actually essential for the assembly of stress fibers as well for the maturation of focal complexes into focal adhesions. As determined from a trophoblast-derived choriocarcinoma cell line, RNAi mediated suppression of PDLIM1 resulted in a loss of stress fibers, with localization of actin filaments and α -actinin at the cells periphery.⁵³ Studies from resting platelets suggested PDLIM1 and ACTN1 actually formed a complex in the cytosol first, before translocating to the F-actin rich cytoskeleton.⁵¹

Many PDZ–LIM proteins function as adaptor proteins mediating a connection between signaling molecules and the actin cytoskeleton, usually binding kinases at their LIM domains.^{51,53} For instance, Cypher binds ACTN2 at the Z-line *via* its PDZ domain, while simultaneously recruiting protein kinase C *via* its LIM domain.⁵⁴ Similarly, another member, Enigma interacts with β -tropomyosin through its PDZ domain and with receptor tyrosine kinases at its LIM domain.⁵⁵ When co-expressed in U2OS osteosarcoma cells, PDLIM1 itself interacts with the normally nuclear Clik1 kinase *via* its LIM domain, and causes relocalization to the actin stress fibers.⁵⁶ Thus, PDLIM1 might also target FHL1 to the actin stress fibers, possibly *via* a LIM–LIM domains interaction. LIM–LIM domain interactions have been described, for instance, between two LIM only proteins such as muscle LIM

protein and CRP.⁵⁷ LIM–LIM domain interactions likely also mediate the interaction between FHL1 and FHL2, since both proteins possess only LIM domains.⁵⁸ Furthermore, PDLIM1 may even be responsible for shuttling FHL1 out of the nucleus, similar to Clik1.⁵⁶ PDLIM1 and FHL1 co-IF studies performed for this project demonstrated nuclear colocalization in HEK-293 cells and C2C12 myoblasts. However, in differentiated myotubes, FHL1 is absent from the nucleus.⁵⁹ Since the FHL1A isoform lacks a known nuclear export signal, an alternative mechanism must exist to ensure its relocalization from the nucleus upon cell differentiation. Perhaps PDLIM1 acts to target FHL1 to the sarcomeric and cytoskeletal elements.

Conclusion

FHL1 is a LIM-only protein, containing a tandem arrangement of four and a half LIM domains, and is expressed predominantly in skeletal muscle. Multiple functions have been attributed to FHL1, for instance, sarcomere assembly, cytoskeletal remodeling, biomechanical stress response, muscle hypertrophy, and transcriptional regulation. In addition, integrin-mediated, MAPK, β -AR, and GPCR signaling pathways are representative of some of the pathways associated with FHL1. The diversity of FHL1's functional properties may be mediated by the diversity of its interacting partners. Given the multifaceted nature of FHL1 and its association with a variety of disease conditions, understanding its cellular roles, protein functions, and protein interactions will certainly offer insight into the pathomechanism of muscle function and disease.

Experimental procedures

Cloning and dual affinity tagging

The human cDNA clones for FHL1 and dihydropyrimidinase-like 3 (DPYSL3) were purchased from OriGene (catalog numbers TC119225 and TC119256 respectively). DPYSL3 was used as a tag control for tandem affinity purification experiments considering its lack of actin- and cytoskeleton-associated GO terms, and availability in our lab. PCR amplification was performed to remove stop codons and introduce novel EcoRI and SalI restriction sites at the 5' and 3' ends of the sequences respectively. The following primers were used for PCR: AAGAATTCTGGGCACCATGGCGGAGAAG (FHL1 forward), GTCGACCAGC TTTTGGCACAGTC (FHL1 reverse), AAGAATTCACCG CCACCATGTCCTACC (DPYSL3 forward), and GT CGACACTCAGA GATGTGATATTAGAAC (DPYSL3 reverse). Amplified PCR products absent of their stop codons were first inserted into pCR2.1-TOPO vectors (Invitrogen) by following the manufacturer's instructions. Cloning was then performed using a standard digestion and ligation protocol, utilizing EcoRI and SalI restriction enzymes (Fermentas) into the InterPlay TAP vectors (Stratagene) which contain the two C-terminal affinity tags: streptavidin binding peptide (SBP) and calmodulin binding peptide (CBP). All constructs were sequenced for validation using generic T3 Reverse and T7 Forward primers.

The human cDNA for PDLIM1 was obtained from the Human ORFeome Collection (version 1, Open Biosystems) and cloned into the pEF-DEST51 Gateway destination vector (Invitrogen) containing the V5 epitope and 6 $\times$ His (V5/6 $\times$ His) tags using LR ClonaseTM II

enzyme mix (Invitrogen). To confirm insertion of the cDNA, nucleotide sequencing was performed using conventional BGH Reverse and T7 Forward primers.

HEK-293 cell culture

HEK-293 cells (ATCC) were cultured in Dulbecco's modified Eagle's media H21 (Princess Margaret Hospital, Toronto) supplemented with 10% fetal calf serum and 1% non-essential amino acids. The culture and transfection of HEK-293 cells has been described in previous publications.^{32,33}

Tandem affinity purification

Purification of dual affinity tagged proteins was performed from HEK-293 cells. 48 h following transfection, HEK-293 cells were harvested with an ice cold solution of 1× PBS (137 mM NaCl, 10 mM phosphate, 2.7 mM KCl, pH of 7.4) supplemented with 5 mM EDTA, and centrifuged (4100 rpm, 5 min, 4 °C). The cells were then washed once with ice cold 1× PBS and centrifuged (4100 rpm, 5 min, 4 °C). The InterPlay Mammalian TAP Purification Kit (Stratagene) was then used, with slight modifications. First, to prepare a protein extract, the pelleted cells were resuspended in 2 ml of Lysis Buffer (Stratagene). The cells were then subjected to three successive rounds of freeze-thawing (10 min each), followed by dounce homogenization on ice (30 strokes with a loose pestle) and 1 h incubation on ice with occasional vortexing. To remove cell debris, the lysate was then centrifuged at 8000 rpm for 20 min at 4 °C. The resulting protein lysate was then incubated with 50 µl of the washed streptavidin resin for 2 h at 4 °C. Proteins bound to the streptavidin beads were then separated from the flow-through by centrifugation (3800 rpm, 5 min, 4 °C) and the beads washed three times in 1 ml of streptavidin binding buffer (Stratagene). Bound proteins were eluted with 100 µl of ice cold streptavidin elution buffer (Stratagene) for 1 h. To further purify the tagged protein complexes, the eluted sample was then incubated with the washed calmodulin beads as per manufacturer's protocol, but rotated overnight at 4 °C. The calmodulin beads with bound protein were then washed three times with 1 ml of calmodulin binding buffer (Stratagene). Purified protein complexes were then eluted with two successive 1 h elutions with 100 µl of ice cold Calmodulin Elution Buffer (Stratagene) at 4 °C.

SDS-PAGE

Immunoblot analyses were performed using standard SDS-PAGE, immunodetection, and chemiluminescent procedures. Blots were probed with the following antibodies: anti-CBP tag (ICL), anti-V5 tag (Invitrogen), FHL1 (Aviva Systems Biology), PDLIM1 (Sigma), DPYSL3 (Chemicon), GSN (BD Transduction Laboratories), ACTN1 (Santa Cruz). Silver staining of proteins was performed using standard protocols.³⁴

Immunofluorescence

Adult cardiomyocytes were dissociated from wild type mouse ventricle. Ventricle tissue was first washed with Hanks solution (136 mM NaCl, 4.16 mM NaHCO₃, 0.34 mM NaH₂PO₄, 5.36 mM KCl, 0.44 mM KH₂PO₄, 5.55 mM dextrose, 5 mM Hepes) to remove excess blood and then cut into 4–6 pieces and incubated in Hanks solution containing 1 mg ml⁻¹

collagenase overnight at room temperature under very gentle shaking. The tissue was then transferred into dissociation solution (Hanks solution with 10 mM taurine, 0.1 mM EGTA, 10 mM BDM, 1 mg ml⁻¹ BSA, 1 mg ml⁻¹ collagenase) and a magnetic stirbar added to the tube and rotated very gently at 37 °C for 5 min to dissociate cells. This step was repeated 4 times to dissociate all the cells from the ventricle tissue. Once dissociated the cells were washed in PBS and then fixed for 30 min in 2% PFA at 4 °C. For immunofluorescence non-specific interactions were first suppressed with 5% horse serum (HS) in permeabilization buffer (0.2% Tween-20, 0.5% Triton X-100 in PBS pH 7.0) for 30 min and then samples were incubated with primary antibodies in permeabilization buffer overnight at 4 °C. Samples were then washed 3 times with PBS and incubated with either Alexa Fluor 488 or Alexa Fluor 633. 2D images were collected by using a LeicaDMIRBE inverted microscope equipped with a Leica TCS SP laser scanning confocal system. Spectra for Alexa Fluor 488 were collected by excitation at λ 488 nm and emission collected between λ 490 nm– λ 510 nm; images for Alexa Fluor 633 were collected by excitation at λ 633 nm and emission between λ 640 nm– λ 670 nm. 3D images were collected by using a Quorum Angstrom Grid Axiovert 200M inverted structured illumination microscope system. For 3D Region of Interest (ROI) analyses, sequential sections of stained cells were acquired for 3D image reconstruction and colocalization measurements. A 3D volume was constructed from sequential z-sections of cells assembled in a 3D volume using Imaris software (version 7.1.1, Bitplane AG, Zurich, Switzerland). Colocalization statistics were calculated using Imaris software.

Mass spectrometry sample preparation

Purified samples were subjected to an in-solution tryptic digestion prior to mass spectrometry analysis. In-solution trypsin digestion was performed on eluates from the calmodulin resin by the addition of 25 ng μ l⁻¹ of trypsin (Promega) and 27 mM of CaCl₂ and then rotated overnight at 37 °C. Samples were then subjected to solid-phase extraction using OMIX C-18 pipette tips (Varian Inc.) to purify the peptides from potential contaminants, according to the manufacturer's instructions. Eluted peptides were vacuum concentrated and stored at –80 °C until use.

Mass spectrometry

Mass spectrometry experiments were carried out on a LTQ linear ion-trap mass spectrometer (Thermo Fisher Scientific) equipped with a nano-electrospray source (Proxeon Biosystems, Denmark). A 75 μ m inner diameter fused silica capillary (InnovaQuartz, USA) was pulled to a fine tip using a P-2000 laser puller (Sutter Instruments) and packed with ~7 cm of Jupiter 4 μ Proteo 90 Å C₁₂ reverse phase resin (Phenomenex, USA). Samples were loaded manually on separate columns using an in-house pressure vessel. Peptides were eluted from the microcapillary columns using a 2-h gradient, using a Thermo Surveyor HPLC system and electrosprayed directly into the MS. The MS functions and the HPLC solvent gradients were controlled by the Xcalibur data system (Thermo Fisher Scientific).

Protein identification

Raw MS files were converted to *m/z* XML using ReAdW and searched by a locally installed version of X!Tandem against a human IPI (International Protein Index; <http://>

www.ebi.ac.uk/IPI) protein sequence database (created August 2010). To estimate and minimize our false positive rate the protein sequence database also contained every IPI protein sequence in its reversed amino acid orientation (*target-decoy strategy*) as recently described.³⁵ A rigorous peptide quality control strategy was applied to effectively minimize false positive identifications, as recently described.³⁵ For the presented study, we have set the value of total reverse spectra to total forward spectra to 0.5%, resulting in a low number of decoy sequences in the final output. Only peptides matching these criteria were accepted to generate the final list of identified proteins, with further criteria set to select only those proteins identified with a minimum of two unique peptides. All data will be available online (<https://proteomecommons.org/tranche/>).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

FHL1 purification from HEK-293 cells. (A) Schematic diagram of CBP and SBP tagged bait protein FHL1 and negative control protein DPYSL3. (B) Tagged FHL1 was transiently transfected into HEK-293 cells and purified using calmodulin beads which bind to the CBP tag and streptavidin beads which bind to the SBP tag. Western blot monitors FHL1 protein (probed with anti-FHL1 antibody) and DPYSL3 (probed with anti-DPYSL3 antibody) through the purification process. The flow through shows that not all the protein binds to the beads probably due to saturation of the beads, and that some protein is lost during the washes but this was deemed necessary in order to get a purified complex and remove non-specific interactors. A significant band showing both proteins are retained in the final elution. (C) Coomassie staining of gel in (B) showing that FHL1 one is not isolated alone but alongside other bands which indicate potential interactors. (D) Silver stain of final elution highlights other bands which co-purify with FHL1 and represent potential putative binding partners. Arrow indicates FHL1 at 42 kDa.

Fig. 2.

Workflow of data filtering of mass spectrometry purification data. Multiple purification experiments from transiently transfected HEK-293 cells identified a total of 310 proteins in 9 mass spectrometry runs. To filter the data we first selected those proteins identified uniquely in FHL1 purifications by removing non-specific interactors including keratins and proteins found in the DPYSL3 tag-controls or negative empty vector control. Next, only those proteins detected in ≥ 2 FHL1 datasets were retained to identify high confidence potential binding partners of FHL1. The online STRING 8.3 database (<http://string.embl.de/>) was then used to identify any previously described interactions amongst FHL1 and the 34 potential interactors.

Fig. 3. Proteins identified by mass spectrometry from FHL1–SBP/CBP purifications. (A) Heatmap representations of proteins identified by mass spectrometry in all tandem affinity purified samples from HEK-293 cells. Color intensities depict total spectral counts. (B) FHL1 MS datasets filtered to remove proteins found in either negative (empty-vector) or tag control (DPYSL3) purifications. Heatmap represents proteins detected only in FHL1 purifications.

Fig. 4.

Analysis of overrepresented GO-terms found within the 34 potential FHL1 interactors. GO-term analysis of the 34 potential interactors of FHL1 was performed using GOFFA. (A) Graphical representation of enriched GO molecular functions ($p < 0.01$, $n = 5$, $E < 2$). (B) Graphical representation of enriched GO biological processes ($p < 0.01$, $n = 5$, $E > 2$).

Fig. 5. Known interactions among FHL1 and the 34 potential interactors (evidence based). Protein–protein interaction network depicting previously defined interactions amongst the group of FHL1 and 34 potential interactors. Interactions were identified using the online STRING 8.3 database, from human sources.

Fig. 6.

Validation of potential interactors of FHL1. (A) HEK-293 cells were transiently transfected with FHL1–CBP/SBP and PDLIM1–V5/6His and immunoprecipitated using the anti-CBP antibody. Immunoblot detection verified FHL1 co-immunoprecipitated with PDLIM1 detected using the anti-V5 antibody. (B) HEK-293 cells were transiently transfected with PDLIM1–V5/6His and FHL1–CBP/SBP and immunoprecipitated using the V5 antibody. Immunoblot detection verified PDLIM1 co-immunoprecipitated with FHL1 detected using the anti-CBP antibody. (C) Endogenous immunoprecipitation from wild type mouse ventricle using anti-FHL1 antibody co-immunoprecipitated with PDLIM1, GSN and ACTN1. (D) Endogenous immunoprecipitation from R9C mouse ventricle using anti-FHL1 antibody co-immunoprecipitated with PDLIM1, GSN and ACTN1.

Fig. 7.

Immunofluorescence of FHL1 shows co-localization with PDLIM1, GSN and ACTN1. (A) 2D immunofluorescence in adult ventricle cardiomyocytes shows FHL1 co-localized with PDLIM1, GSN and ACTN1. (B) 3D immunofluorescence of FHL1 shows immunofluorescence of FHL1 in adult cardiomyocytes co-localized with PDLIM1 with 22% of dataset co-localization with a Pearson coefficient of 0.44, co-localization with GSN showed 30% of dataset co-localization with a Pearson coefficient of 0.5 and co-localization with ACTN1 with 44% of dataset co-localization with a Pearson coefficient of 0.6.

Fig. 8.

Schematic of potential FHL1 interaction complex. Potential FHL1 complex based on this study and protein–protein interaction network identified using the online STRING 8.3 database, from human sources. Dashed lines indicate protein interactions confirmed in this study, solid lines indicate previously defined interactions.