Protein interaction screening identifies SH3RF1 as a new regulator of FAT1 protein levels

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ABSTRACT

Mutations and ectopic FAT1 cadherin expression are implicated in a broad spectrum of diseases ranging from developmental disorders to cancer. The regulation of FAT1 and its downstream signalling pathways remain incompletely understood. We hypothesized that identification of additional proteins interacting with the FAT1 cytoplasmic tail would further delineate its regulation and function. A yeast two-hybrid library screen carried out against the juxtamembrane region of the cytoplasmic tail of FAT1 identified the E3 ubiquitin-protein ligase SH3RF1 as the most frequently recovered protein-binding partner. Ablating SH3RF1 using siRNA increased cellular FAT1 protein levels and stabilized expression at the cell surface while overexpression of SH3RF1 reduced FAT1 levels. We conclude that SH3RF1 acts as a negative post-translational regulator of FAT1 levels.

Keywords
FAT1 cadherin; SH3RF1; E3 ubiquitin-ligase; yeast two-hybrid, protein-protein interaction; protein half-life

INTRODUCTION

Members of the Fat cadherin family are conserved from flies to vertebrates, with two members in *Drosophila*, ft and ft2 and four in humans, FAT1, FAT2, FAT3 and FAT4. All Fat genes encode large transmembrane receptors producing protein products of ~500-600kDa [1]. In humans, FAT1 was the first member of Fat cadherin family cloned [2] and as such, has been the most extensively studied. Early work using Fat1 knockout mice revealed a role in kidney development and a low penetrance of neurological and ophthalmological abnormalities [3]. Subsequent whole exome sequencing and homozygosity mapping in humans established that human *FAT1* mutations are also involved in glomerular nephropathy [4]. Similarly, defined single nucleotide polymorphisms (SNPs) of *FAT1* are linked to bipolar disorder [5] as well as autism [6] thereby establishing the relevance of FAT1 function to the homeostasis of these organ systems.

In addition to its role in developmental disorders, FAT1 has also been investigated in the context of cancer, in part due to early work classifying Drosophila *ft* as a tumor suppressor gene (TSG) [7]. *FAT1* was later found not to be the true ortholog of *ft*, but nevertheless a number of reports have shown loss, repression and inactivation of FAT1 occurs in certain cancers [8,9]. In contrast a number of cancer types display over-expression of FAT1 compared to their normal cell counterparts including acute leukemias [10], melanoma [11] and breast cancer [12]. Reconciling these apparent differences and its role in development will require a better contextual understanding of Fat1-regulated signaling pathways (reviewed in [1]).

To this end, lessons from *Drosophila* have indicated ft acts as an upstream receptor involved in controlling cell growth through the Hippo signalling pathway [13-16], and also lies upstream of the planar cell polarity pathway[17-20]. Vertebrate Fat1 is also linked to cell migration where it

localises to lamellopodia [21] and engages the actin cytoskeletal network through interaction with Ena/VASP proteins [22]. FAT1 can also regulate the Hippo pathway in neuronal differentiation [23] and Wnt-signalling by binding β-catenin [9]. Of note, high levels of FAT1 protects against TRAIL-induced apoptosis [24] and more recently cytoplasmic tail fragments of the Fat cadherins play an unexpected role controlling mitochondrial function [25-27]. Collectively these reports highlight the importance of the Fat1 cadherin, and in particular, the cytoplasmic tail as a central hub for activating downstream signalling pathways.

We therefore hypothesized that the discovery of additional novel interacting partners of the FAT1 cytoplasmic tail would allow for further dissection of the signalling functions of FAT1 in both known and unknown cellular contexts. Herein we report a novel interaction between the FAT1 cytoplasmic tail and SH3RF1 (also known as POSH) and provide clear evidence that SH3RF1 serves to modulate the cellular levels of FAT1.

MATERIALS AND METHODS

Antibodies, vectors and siRNA

Antibodies against SH3RF1 (H00057630, Abnova), V5 (Invitrogen), GAPDH and PP2A-Cα/β (ID6) (Santa Cruz) with in-house anti-FAT1 mAb and pAb used as previously described [11]. Gateway vectors and siRNA duplexes were purchased from Invitrogen and Shanghai-GenePharma, respectively.

Cell lines and Western blotting

Cell culture methods, preparation of whole cell lysates using NDE lysis buffer and Western blotting were as previously described [11]. Tris-Acetate Gels (NuPAGE®, Thermo Fisher Scientific) were used to resolve endogenous FAT1 in samples while Bis-Tris gels were employed for other analyses. Rabbit pAbs directed against the C-terminus of FAT1 were used for all immunoblotting experiments. Where indicated, bands were quantitated by densitometry using Fujifilm Multigauge v3.0 software.

Co-immunoprecipitation (coIP)

COS cells were transiently transfected with the pCS2+_FAT1 vector encoding the cytoplasmic domain (aa4203–4588) and pDEST40-SH3RF1 constructs using LipofectAMINE2000. Cells were lysed with IP buffer (50 mM Tris-HCl pH8.0, 200 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM DTT) and IP carried out as described [11]. For endogenous coIP in MDA-MB-231 cells, lysates were prepared using cadherin lysis buffer (1% NP40, 1% Triton X-100, 10mM HEPES (pH 7.4), 150mM NaCl, 2mM $CaCl_2$) before immunoprecipitation with a cocktail of anti-FAT1 mAb (NTD and CTD) (Fig. 3A) as previously described [11].

Yeast two-hybrid Screen

FAT1 (aa4210-4280) and FAT1 (aa4497-4588) 'bait' sequences were amplified by PCR with high fidelity, proof-reading DNA Polymerase (KOD Hot Start, Novagen) using gene-specific primers (Integrated DNA technologies) to add attB1 and attB2 sequence tags (Table S1) to opposing ends of the bait sequence. Yeast two-hybrid screening against a breast cancer cDNA library was performed as described [29].

In Vitro Transcription and Translation and GST- Pull Down Assay

The TNT® T7 Quick coupled Rabbit Reticulocyte Transcription/Translation System (Promega, Madison, WI, USA) was used to label the FAT1 sequence (amino acids *4210-4280*) with [^{35}S]-methionine. Full length SH3RF1 was amplified from cDNA with a set of primers designed according to the human SH3RF1 cDNA sequence deposited in the GenBank™/EBI Data Bank (accession number GI: 89142742). SH3RF1 deletion mutants, ΔRING SH3RF1, (aa 1-11, 54-888), ΔRING SH3RF1 N TERM (aa1-11, 54-444), and ΔRING SH3RF1 C-TERM (aa 1-11, 260-888) were cloned into pDEST™40 plasmid using the Gateway® system (Invitrogen, Carlsbad, CA, USA).

Retroviral transductions

The pMIGII_SH3RF1 with the C-terminal V5 tag retrovirus was generated as described [30]. Stable MDA-MB-231 cells were generated by spinfection and GFP expressing cells sorted using FACS Aria II flow cytometer (BD) until >90% GFP-positive.

siRNA Transfection, and inhibition of protein translation and proteasome

Transient knockdown of SH3RF1 was carried out using pooled siRNA duplexes (Table S2). Cells were transfected with pooled SH3RF1 siRNA (#451, #455, #2107) or Neg Ctrl siRNA (with 150 pmol total siRNA: 50 pmol of each SH3RF1 or 150 pmol Neg Ctrl siRNA) using RNAi max

(Invitrogen) as per manufacturer's instructions. Cycloheximide and MG132 were purchased from Sigma, Lactacystin was purchased from Alexis Biochemicals. Cycloheximide (0.1 mM) was used to inhibit protein translation. MG132 (50 μM) and Lactacystin (10 μM) were for 2 hours prior to cell lysis.

Quantitative real time PCR (qPCR)

qPCR was carried out as described [10]. Briefly, total RNA was isolated using the RNA minispin kit (Illustra), quantitated using a Biophotometer (Eppendorf) and validated using an Agilent Bioanalyser (RIN >9 for all samples). cDNA synthesis was carried out with 500 ng total RNA using the Transcriptor High Fidelity cDNA Synthesis Kit (Roche) with random hexamers. Reverse transcription was carried out as follows: 10 min at 29 ºC, 60 min at 48ºC, then 5 min at 85ºC. Each qPCR reaction contained the following: 5μL cDNA (1:50 dilution), 6.25 μL 2X SensiFAST™ SYBR Hi-ROX (Bioline), 0.5 μL of each primer (10 μM; Table S3) for a final volume of 12.5 μL. Triplicate reactions were set up in a 96-well format with amplification reaction carried out using the Applied Biosystems 7500 real-time PCR machine using the following steps: Stage 1: 2 min at 50ºC, Stage 2: 10 min at 95ºC, Stage 3: 40 cycles of 15 sec at 95ºC followed by 1 min at 60ºC. FAT1 gene expression was normalized to the geometric mean of the reference genes (RPS18, NM_022551.2) and β-glucuronidase (GUSB, NM_000181.3) with the relative gene expression calculated using the 2^−(ddCt) method. Primer sequences are found within Table S3.

RESULTS

Yeast two-hybrid screening identifies novel FAT1 interacting partners

A yeast two-hybrid screen was undertaken to identify novel proteins interacting with the FAT1 cytoplasmic tail. To improve the possibility of identifying entirely novel interactions, two FAT1 'bait' constructs were designed that corresponded to the FAT1 juxta membrane (aa4210-4280) and carboxyl-terminal (aa4497-4588) regions respectively. The latter bait proved to be auto-activating in yeast and was omitted from the library screen (data not shown) (Fig. 1A). Applying the juxta-membrane bait of FAT1 to three independent library screens identified a total of 13 'prey' clones including SH3RF1, TRAF4, NOXA1, MT-ND1, SORBS2, and IGLL1 (Table 1). Of these candidates SH3RF1 was the most frequent 'prey' identified with a total of five independent cDNA clones recovered. The SH3RF1-FAT1 interaction was then further validated through second pass screening using a re-derived 'prey' against the $FAT1_{4210-4280}$ 'bait' and an unrelated 'bait', p53 as a control (Fig. 1B). Together these data provided strong evidence of a bona-fide SH3RF1-FAT1 in the context of the yeast two-hybrid system.

The two N-terminal SH3 domains of SH3RF1 interact with FAT1

The primary structure of the SH3RF1 protein consists of an N-terminal E3 ubiquitin ligase RING domain followed by four SH3 domains (Fig. 2A). *In silico* analysis of the FAT1 cytoplasmic tail identified a class II SH3 (PxxPxR) binding motif and moreover the in silico analysis predicted the first two SH3 domains of SH3RF1 are primarily involved in this interaction (Table 2). To test whether the SH3 domains of SH3RF1 were responsible for the interaction with FAT1, we utilised domain-deletion constructs of SH3RF1 in GST-pulldown assays. We confirmed that GST- $FAT1_{4210-4280}$ specifically bound full-length SH3RF1 in the assay and whilst the RING domain was dispensable for FAT1 binding, the N-terminal SH3 domains of SH3RF1 were absolutely required (Fig 2A).

Furthermore, the requirement for the N-terminal SH3 domains of SH3RF1 in the FAT1 interaction was confirmed using COS cells transiently co-transfected with epitope-tagged SH3RF1 truncation mutants (V5-SH3RF1$_{1\text{-}11,54\text{-}888}$ or V5-SH3RF1$_{1\text{-}11,260\text{-}888}$) together with a FAT1 intracellular fragment (ICF encompassing aa4203-4588) (Fig. 2B). Finally, the interaction between FAT1 and SH3RF1 was confirmed *in situ* using MDA-MB-231 where reciprocal co-immunoprecipitation was observed between endogenous FAT1 and SH3RF1 proteins (Fig. 2C).

SH3RF1 regulates total and cell surface FAT1 protein levels

Collectively these results provide compelling evidence that SH3RF1 is a novel binding partner of FAT1. We therefore considered the functional consequences of this interaction. The presence of the RING domain in SH3RF1 demarks it as an E3 ubiquitin-protein ligase [31]. Indeed this activity has been demonstrated where SH3RF1 has been found to selectively mediate the ubiquitination and proteosomal-dependent degradation of target proteins [32-34]. This proposed that SH3RF1 could function to regulate FAT1 protein turnover. To test this we determined the impact of manipulating SH3RF1 levels on the expression of FAT1.

We first identified that the MDA-MB-231 breast cancer cell line expressed high levels of both FAT1 and SH3RF1. As previously determined for melanoma cells, FAT1 occurs in MDA-MB-231 cells both as full length (p500) and S1-processed (heterodimeric p430/p85) forms that can be distinguished using domain specific antibodies (Fig 3A) [11,35]. Subjecting the MDA-MB-231 cells to siRNA-mediated knockdown of SH3RF1 resulted in loss of SH3RF1 with a significant upregulation of FAT1 protein levels (Fig. 3B). This upregulation also led to a significant ~2.6 fold increase in cell surface expression of FAT1 as determined by cell surface biotinylation experiments. Here the amount of cell surface (biotinylated) FAT1 protein was significantly increased after

SH3RF1 knockdown with no concomitant change in processing of full length to the heterodimeric form of FAT1 (Fig. 3C). These results indicate that SH3RF1 can regulate total FAT1 levels including cell surface expression but does not regulate S1-cleavage of FAT1.

Reciprocal experiments were then conducted where SH3RF1 was transfected into the MDA-MB-231 cells. Recombinant expression of epitope-tagged SH3RF1 resulted in a reduction in FAT1 protein levels by ~30% (Fig 3D). Whilst this represents only a moderate decrease it is entirely consistent with the increase in recombinant SH3RF1 over endogenous levels. Indeed high level expression of recombinant SH3RF1 could not be achieved and we attribute this to SH3RF1 being able to regulate its own half-life [36]. This notion was supported by experiments using an SH3RF1 construct lacking the RING domain (ΔRING-SH3RF1; Fig. 3E). In comparison to full-length SH3RF1, high-level expression of ΔRING-SH3RF1 could readily be achieved (Fig. 3E). Moreover, consistent with experiments undertaken in the MDA-MB-231 cells (Fig. 3D), FAT1 expression levels were reduced by 30% after transfection of full-length SH3RF1. However overexpression of ΔRING-SH3RF1 did not significantly alter FAT1 levels (Fig. 3E), showing the RING domain of SH3RF1 is required to regulate FAT1 levels.

FAT1 has protein has an extremely short half life but SH3RF1 does not influence the rate of FAT1 protein decay

As a logical extension of this work we next analysed the effects of SH3RF1 on the stability of the FAT1 protein. To establish a baseline for this assay, we employed the cycloheximide chase assay to determine the half-life of the FAT1 protein. Unexpectedly, the half–life of FAT1 in MDA-MB-231 cells was found to ~2h with only 20% of original levels remaining after 8h (Fig. 4A). To confirm

this, the experiment was repeated in HEK293 where the half-life of FAT1 protein appeared even less than observed for MDA-MB-231 cells (Fig. 4A). In contrast, parallel blotting experiments with SH3RF1 showed it to be comparably long lived with relatively little diminishment of protein levels across the same time frame (Fig. 4A).

Given SH3RF1 is a known E3 ubiquitin-protein ligase and that manipulation of SH3RF1 can impact the levels of FAT1, it could be predicted that the half-life of FAT1 would be influenced by the levels of SH3RF1. To determine this, both MDA-MB-231 cells and HEK293 were pre-treated with SH3RF1 siRNA for 48h before conducting a 'chase' assay with the protein synthesis inhibitor cycloheximide. Consistent with prior experiments, baseline levels of FAT1 protein increased in both lines (~1.6-2 fold increase) after SH3RF1 levels were depleted using siRNA (Fig. 4B). Treatment with cycloheximide for 2h resulted in a reduction of FAT1 protein levels in both negative control (NC) siRNA and SH3RF1 siRNA treated cells. Notably, while the baseline levels of FAT1 protein are different for NC and SH3RF1 siRNA treatments, the relative rate of decay was not significantly altered. To ascertain if SH3RF1 levels affected the transcription and/or stability of FAT1 mRNA we undertook quantitative real-time PCR (qPCR) analyses (Fig. 4C). After 24h after siRNA depletion of SH3RF1 there was no significant increase in FAT1 mRNA in HEK293 cells although there was a small increase observed in MDA-MB-231 cells after depletion of SH3RF1. However given the ~2-3 fold increase in FAT1 protein levels observed after SH3RF1 depletion these data suggest that the major regulatory mechanism(s) involved are post-transcriptional in nature.

Further experiments in HEK293 treated with MG132 and lactacystin demonstrated these agents increased FAT1 protein levels, indicating involvement of the proteasome in FAT1 degradation. However, FAT1 levels still diminished after cycloheximide treatment in the presence of either

proteasomal inhibitor, likely indicating that non-proteasomal mechanisms are also involved in the half-life of the FAT1 protein (Fig. 4D).

DISCUSSION

In this report we sought to identify and functionally validate novel interacting partners of the FAT1 cytoplasmic tail. Screening of the juxta-membrane region against a breast cancer cDNA library identified six novel binding partners including SH3RF1 that was further investigated in detail. Notably other FAT1-interacting proteins were NADH dehydrogenase subunit 1 (MT-ND1), a key component of the mitochondrial respiratory chain and NADPH oxidase activator 1 (NOXA1), a component of the NADPH-oxidase complex that generates ROS. Significantly during the preparation of this manuscript, it was shown that fragments of the Fat1 cytoplasmic tail do translocate and directly regulate mitochondrial function [25,26]. Furthermore it was also recently shown that SORBS2 (ArgBP2) and SORBS1 (CAP/ponsin) also interact with the FAT1 cytoplasmic tail [37] providing independent evidence that these are bona fide protein-protein interactions. Moreover, it is notable that SORBS2 is suspected to be pathogenic factor in facioscapulohumeral muscular dystrophy (FSHD) [38], a disease also associated with compromised FAT1 expression in muscle cells [39]. Of the remaining binding partners identified, only immunoglobulin lambda and TRAF4 remain to be investigated in detail, but given the stringent nature of our screen we anticipate this work to yield interesting results.

The most frequent interaction was between FAT1 and SH3RF1 and this was validated *in vitro* showing that binding to FAT1 involved the N-terminal SH3 domains of SH3RF1, most likely through a class II SH3 (PxxPxR) binding motif located within the juxta-membrane region of FAT1. SH3RF1 (also known as POSH) is a multi-functional protein composed of four SH3 domains and a single RING (Really Interesting New Gene) domain at the N-terminus. The majority of research understanding the function SH3RF1 has been within the central nervous system where it can regulate

neuronal cell migration by localizing activated Rac1 and F-actin assembly [40] and induce axon outgrowth and increase neuronal process length [41,42]. SH3RF1 over expression can also induce apoptosis in neuronal cells by regulating the JNK pathway [36]. However, our SH3RF1 over-expression experiments in MDA-MB-231 did not lead to apoptosis and therefore suggests different roles for SH3RF1 in different murine and human cellular settings. Indeed some reports have indicated high SH3RF1 levels can be protective with knockdown of SH3RF1 sensitizing prostate cancer cell lines in TRAIL-induced apoptosis [43] and human RASF cells to Fas-mediated apoptosis [44].

SH3RF1 can also regulate its own half-life in a RING-dependent manner via ubiquitination [36]. Indeed, RING-domain is the hallmark of E3-ubiquitin ligases [31] and several ubiquitination substrates for SH3RF1 have been identified. SH3RF1 can increase the levels of level of Herp at the endoplasmic reticulum through ubiquitination and thereby regulate calcium homeostasis[33]. Similarly, SH3RF1 ubiquitination activity aids virion release by modulating the activity and location of Gag[45] and ALG2-interacting protein X (ALIX) [46]. SH3RF1-mediated ubiquitination has also been demonstrated to play a functional role in the cell surface expression of receptors including ROMK1, a component of ROMK potassium channels [34] and STIM1, a synaptic membrane protein expressed by hippocampal neurons [47]. Given these precedents we therefore hypothesized that FAT1 protein levels could also be regulated by SH3RF1.

Down-regulation of SH3RF1 by siRNA caused a significant increase in the level FAT1 protein in breast cancer cells and HEK293 cells concomitant with an increase in cell surface expression. Conversely, over expression of SH3RF1 led to a decrease in FAT1 protein, albeit to a lesser extent than the effects observed with reciprocal SH3RF1 depletion. However given the

difficulty encountered in achieving high level expression of SH3RF1, the reciprocal changes observed are consistent with the notion that SH3RF1 regulates cellular levels of FAT1. However, unlike the exemplary reports where SH3RF1 regulated the translocation of ROMKI [34] and STIM1 [47] to or from the plasma membrane, SH3RF1 impacted the total cellular pool of FAT1. Our experiments also establish that the RING domain of SH3RF1 is necessary to modulate FAT1 protein levels implying that SH3RF1 ubiquitin ligase activity is involved.

Having established that SH3RF1 could regulate FAT1 protein levels we then investigated the rate of protein decay and found quite unexpectedly that FAT1 has a very short half-life of less than 2 hours. Previous studies of E-cadherin, a dynamic component of adherens junctions, have a half-life between 5-10 hours [48]. Considering the core protein mass of FAT1 at ~500 kDa, 5 times the size of E-cadherin, the <2 hour half-life of FAT1 is remarkable. The SH3RF1-mediated ubiquitination of ROMK1 channels involves the ubiquitin-proteasome system [34] and we therefore investigated how SH3RF1 influenced the half-life of FAT1. Depletion of SH3RF1 did not alter the rate of FAT1 protein decay, an observation that held for both cell lines tested. This data argues against a simplified role for SH3RF1 being the only protein involved in the ubiquitin-mediated decay of FAT1 through either proteasomal and/or non-proteasomal (e.g. lysosomal) mechanisms. Rather there are likely to be more complex processes at play. For example, it has been shown for E-cadherin that the E3-ubiquitin-ligase Hakai binds to it transmembrane proximal region where it can promote either endocytosis/recycling or alternatively destruction [49,50]. Taken together, the short-half life and the potential multiplex role in the regulation of FAT1 protein levels has significant implications for understanding the variety of FAT1 signaling functions necessary for tissue homeostasis. In conclusion, we have demonstrated that SH3RF1 and FAT1 interact *in vivo* with functional assays, providing clear evidence that SH3RF1 serves to modulate cellular levels of FAT1. These findings

further illustrate that the functional significance of protein-protein interactions is often context dependent and this report will aid our understanding on the role FAT1 plays in a diverse range of pathologies.

REFERENCES

1 Sadeqzadeh E, de Bock CE & Thorne RF (2013) Sleeping Giants: Emerging Roles for the Fat Cadherins in Health and Disease. *Med Res Rev*.

2 Dunne J, Hanby AM, Poulsom R, Jones TA, Sheer D, Chin WG, Da SM, Zhao Q, Beverley PC & Owen MJ (1995) Molecular cloning and tissue expression of FAT, the human homologue of the Drosophila fat gene that is located on chromosome 4q34-q35 and encodes a putative adhesion molecule. *Genomics* **30**, 207–223.

3 Ciani L, Patel A, Allen ND & ffrench-Constant C (2003) Mice lacking the giant protocadherin mFAT1 exhibit renal slit junction abnormalities and a partially penetrant cyclopia and anophthalmia phenotype. *Molecular and Cellular Biology* **23**, 3575–3582.

4 Gee HY, Sadowski CE, Aggarwal PK, Porath JD, Yakulov TA, Schueler M, Lovric S, Ashraf S, Braun DA, Halbritter J, Fang H, Airik R, Vega-Warner V, Cho KJ, Chan TA, Morris LGT, ffrench-Constant C, Allen N, McNeill H, Büscher R, Kyrieleis H, Wallot M, Gaspert A, Kistler T, Milford DV, Saleem MA, Keng WT, Alexander SI, Valentini RP, Licht C, Teh JC, Bogdanovic R, Koziell A, Bierzynska A, Soliman NA, Otto EA, Lifton RP, Holzman LB, Sibinga NES, Walz G, Tufro A & Hildebrandt F (2016) FAT1 mutations cause a glomerulotubular nephropathy. *Nat Commun* **7**, 10822.

5 Blair IP, Chetcuti AF, Badenhop RF, Scimone A, Moses MJ, Adams LJ, Craddock N, Green E, Kirov G, Owen MJ, Kwok JBJ, Donald JA, Mitchell PB & Schofield PR (2006) Positional cloning, association analysis and expression studies provide convergent evidence that the cadherin gene FAT contains a bipolar disorder susceptibility allele. *Molecular Psychiatry* **11**, 372–383.

6 Neale BM, Kou Y, Liu L, Ma'ayan A, Samocha KE, Sabo A, Lin C-F, Stevens C, Wang L-S, Makarov V, Polak P, Yoon S, Maguire J, Crawford EL, Campbell NG, Geller ET, Valladares O, Schafer C, Liu H, Zhao T, Cai G, Lihm J, Dannenfelser R, Jabado O, Peralta Z, Nagaswamy U, Muzny D, Reid JG, Newsham I, Wu Y, Lewis L, Han Y, Voight BF, Lim E, Rossin E, Kirby A, Flannick J, Fromer M, Shakir K, Fennell T, Garimella K, Banks E, Poplin R, Gabriel S, DePristo M, Wimbish JR, Boone BE, Levy SE, Betancur C, Sunyaev S, Boerwinkle E, Buxbaum JD, Cook EH Jr, Devlin B, Gibbs RA, Roeder K, Schellenberg GD, Sutcliffe JS & Daly MJ (2012) Patterns and rates of exonic de novo mutations in autism spectrum disorders. *Nature* **485**, 242–245.

7 Mahoney PA, Weber U, Onofrechuk P, Biessmann H, Bryant PJ & Goodman CS (1991) The fat tumor suppressor gene in Drosophila encodes a novel member of the cadherin gene superfamily. *Cell* **67**, 853–868.

8 Nakaya K, Yamagata HD, Arita N, Nakashiro K-I, Nose M, Miki T & Hamakawa H (2007) Identification of homozygous deletions of tumor suppressor gene FAT in oral cancer using CGH-array. *Oncogene* **26**, 5300–5308.

9 Morris LGT, Kaufman AM, Gong Y, Ramaswami D, Walsh LA, Turcan S, Eng S, Kannan K, Zou Y, Peng L, Banuchi VE, Paty P, Zeng Z, Vakiani E, Solit D, Singh B, Ganly I, Liau L, Cloughesy TC, Mischel PS, Mellinghoff IK & Chan TA (2013) Recurrent somatic mutation of FAT1 in multiple human cancers leads to aberrant Wnt activation. *Nat. Genet.* **45**, 253–261.

10 de Bock CE, Ardjmand A, Molloy TJ, Bone SM, Johnstone D, Campbell DM, Shipman KL, Yeadon TM, Holst J, Spanevello MD, Nelmes G, Catchpoole DR, Lincz LF, Boyd AW, Burns GF & Thorne RF (2012) The Fat1 cadherin is overexpressed and an independent prognostic factor for survival in paired diagnosis-relapse samples of precursor B-cell acute lymphoblastic leukemia. *Leukemia* **26**, 918–926.

11 Sadeqzadeh E, de Bock CE, Zhang XD, Shipman KL, Scott NM, Song C, Yeadon T, Oliveira CS,

Jin B, Hersey P, Boyd AW, Burns GF & Thorne RF (2011) Dual processing of FAT1 cadherin protein by human melanoma cells generates distinct protein products. *J. Biol. Chem.* **286**, 28181–28191.
12 Kwaepila N, Burns G & Leong AS-Y (2006) Immunohistological localisation of human FAT1 (hFAT) protein in 326 breast cancers. Does this adhesion molecule have a role in pathogenesis? *Pathology* **38**, 125–131.
13 Bennett FC & Harvey KF (2006) Fat cadherin modulates organ size in Drosophila via the Salvador/Warts/Hippo signaling pathway. *Curr. Biol.* **16**, 2101–2110.
14 Silva E, Tsatskis Y, Gardano L, Tapon N & McNeill H (2006) The tumor-suppressor gene fat controls tissue growth upstream of expanded in the hippo signaling pathway. *Curr. Biol.* **16**, 2081–2089.
15 Cho E, Feng Y, Rauskolb C, Maitra S, Fehon R & Irvine KD (2006) Delineation of a Fat tumor suppressor pathway. *Nat. Genet.* **38**, 1142–1150.
16 Willecke M, Hamaratoglu F, Kango-Singh M, Udan R, Chen C-L, Tao C, Zhang X & Halder G (2006) The fat cadherin acts through the hippo tumor-suppressor pathway to regulate tissue size. *Curr. Biol.* **16**, 2090–2100.
17 Fanto M, Clayton L, Meredith J, Hardiman K, Charroux B, Kerridge S & McNeill H (2003) The tumor-suppressor and cell adhesion molecule Fat controls planar polarity via physical interactions with Atrophin, a transcriptional co-repressor. *Development* **130**, 763–774.
18 Yang C-H, Axelrod JD & Simon MA (2002) Regulation of Frizzled by fat-like cadherins during planar polarity signaling in the Drosophila compound eye. *Cell* **108**, 675–688.
19 Brittle AL, Repiso A, Casal J, Lawrence PA & Strutt D (2010) Four-jointed modulates growth and planar polarity by reducing the affinity of dachsous for fat. *Curr. Biol.* **20**, 803–810.
20 Ishikawa HO, Takeuchi H, Haltiwanger RS & Irvine KD (2008) Four-jointed is a Golgi kinase that phosphorylates a subset of cadherin domains. *Science* **321**, 401–404.
21 Tanoue T & Takeichi M (2004) Mammalian Fat1 cadherin regulates actin dynamics and cell-cell contact. *J. Cell Biol.* **165**, 517–528.
22 Moeller MJ, Soofi A, Braun GS, Li X, Watzl C, Kriz W & Holzman LB (2004) Protocadherin FAT1 binds Ena/VASP proteins and is necessary for actin dynamics and cell polarization. *EMBO J.* **23**, 3769–3779.
23 Ahmed AF, de Bock CE, Lincz LF, Pundavela J, Zouikr I, Sontag E, Hondermarck H & Thorne RF (2015) FAT1 cadherin acts upstream of Hippo signalling through TAZ to regulate neuronal differentiation. *Cellular and Molecular Life Sciences*, 1–17.
24 Kranz D & Boutros M (2014) A synthetic lethal screen identifies FAT1 as an antagonist of caspase-8 in extrinsic apoptosis. *EMBO J.* **33**, 181–197.
25 Cao LL, Riascos-Bernal DF, Chinnasamy P, Dunaway CM, Hou R, Pujato MA, O'Rourke BP, Miskolci V, Guo L, Hodgson L, Fiser A & Sibinga NES (2016) Control of mitochondrial function and cell growth by the atypical cadherin Fat1. *Nature*.
26 de Bock CE & Thorne RF (2016) Cell biology: A mitochondrial brake on vascular repair. *Nature*.
27 Sing A, Tsatskis Y, Fabian L, Hester I, Rosenfeld R, Serricchio M, Yau N, Bietenhader M, Shanbhag R, Jurisicova A, Brill JA, McQuibban GA & McNeill H (2014) The atypical cadherin fat directly regulates mitochondrial function and metabolic state. *Cell* **158**, 1293–1308.
28 Fam SR, Paquet M, Castleberry AM, Oller H, Lee CJ, Traynelis SF, Smith Y, Yun CC & Hall RA (2005) P2Y1 receptor signaling is controlled by interaction with the PDZ scaffold NHERF-2. *Proc. Natl. Acad. Sci. U.S.A.* **102**, 8042–8047.
29 De Bock CE, Lin Z, Mekkawy AH, Byrne JA & Wang Y (2010) Interaction between urokinase receptor and heat shock protein MRJ enhances cell adhesion. *Int. J. Oncol.* **36**, 1155–1163.
30 Fock E-L, Yan F, Pan S & Chong BH (2008) NF-E2-mediated enhancement of megakaryocytic

differentiation and platelet production in vitro and in vivo. *Experimental Hematology* **36**, 78–92.
31 Deshaies RJ & Joazeiro CAP (2009) RING domain E3 ubiquitin ligases. *Annu. Rev. Biochem.* **78**, 399–434.
32 Kim G-H, Park E, Kong Y-Y & Han J-K (2006) Novel function of POSH, a JNK scaffold, as an E3 ubiquitin ligase for the Hrs stability on early endosomes. *Cell. Signal.* **18**, 553–563.
33 Tuvia S, Taglicht D, Erez O, Alroy I, Alchanati I, Bicoviski V, Dori-Bachash M, Ben-Avraham D & Reiss Y (2007) The ubiquitin E3 ligase POSH regulates calcium homeostasis through spatial control of Herp. *J. Cell Biol.* **177**, 51–61.
34 Lin D-H, Yue P, Pan C-Y, Sun P, Zhang X, Han Z, Roos M, Caplan M, Giebisch G & Wang W-H (2009) POSH stimulates the ubiquitination and the clathrin-independent endocytosis of ROMK1 channels. *Journal of Biological Chemistry* **284**, 29614–29624.
35 Sadeqzadeh E, de Bock CE, Wojtalewicz N, Holt JE, Smith ND, Dun MD, Schwarte-Waldhoff I & Thorne RF (2014) Furin processing dictates ectodomain shedding of human FAT1 cadherin. *Exp. Cell Res.* **323**, 41–55.
36 Xu Z, Kukekov NV & Greene LA (2003) POSH acts as a scaffold for a multiprotein complex that mediates JNK activation in apoptosis. *EMBO J.* **22**, 252–261.
37 Braun GS, Kuszka A, Dau C, Kriz W & Moeller MJ (2016) Interaction of atypical cadherin Fat1 with SoHo adaptor proteins CAP/ponsin and ArgBP2. *Biochem. Biophys. Res. Commun.* **472**, 88–94.
38 Robin JD, Ludlow AT, Batten K, Gaillard M-C, Stadler G, Magdinier F, Wright WE & Shay JW (2015) SORBS2 transcription is activated by telomere position effect-over long distance upon telomere shortening in muscle cells from patients with facioscapulohumeral dystrophy. *Genome Res.* **25**, 1781–1790.
39 Mariot V, Roche S, Hourdé C, Portilho D, Sacconi S, Puppo F, Duguez S, Rameau P, Caruso N, Delezoide A-L, Desnuelle C, Bessières B, Collardeau S, Feasson L, Maisonobe T, Magdinier F, Helmbacher F, Butler-Browne G, Mouly V & Dumonceaux J (2015) Correlation between low FAT1 expression and early affected muscle in facioscapulohumeral muscular dystrophy. *Ann Neurol.* **78**, 387–400.
40 Yang T, Sun Y, Zhang F, Zhu Y, Shi L, Li H & Xu Z (2012) POSH localizes activated Rac1 to control the formation of cytoplasmic dilation of the leading process and neuronal migration. *Cell Rep* **2**, 640–651.
41 Taylor J, Chung K-H, Figueroa C, Zurawski J, Dickson HM, Brace EJ, Avery AW, Turner DL & Vojtek AB (2008) The scaffold protein POSH regulates axon outgrowth. *Mol. Biol. Cell* **19**, 5181–5192.
42 Dickson HM, Zurawski J, Zhang H, Turner DL & Vojtek AB (2010) POSH is an intracellular signal transducer for the axon outgrowth inhibitor Nogo66. *J. Neurosci.* **30**, 13319–13325.
43 Christian PA, Fiandalo MV & Schwarze SR (2011) Possible role of death receptor-mediated apoptosis by the E3 ubiquitin ligases Siah2 and POSH. *Mol. Cancer* **10**, 57.
44 Tsuda M, Kawaida R, Kobayashi K, Shinagawa A, Sawada T, Yamada R, Yamamoto K & Aigaki T (2010) POSH promotes cell survival in Drosophila and in human RASF cells. *FEBS Lett.* **584**, 4689–4694.
45 Alroy I, Tuvia S, Greener T, Gordon D, Barr HM, Taglicht D, Mandil-Levin R, Ben-Avraham D, Konforty D, Nir A, Levius O, Bicoviski V, Dori M, Cohen S, Yaar L, Erez O, Propheta-Meiran O, Koskas M, Caspi-Bachar E, Alchanati I, Sela-Brown A, Moskowitz H, Tessmer U, Schubert U & Reiss Y (2005) The trans-Golgi network-associated human ubiquitin-protein ligase POSH is essential for HIV type 1 production. *Proc. Natl. Acad. Sci. U.S.A.* **102**, 1478–1483.
46 Votteler J, Iavnilovitch E, Fingrut O, Shemesh V, Taglicht D, Erez O, Sörgel S, Walther T, Bannert N, Schubert U & Reiss Y (2009) Exploring the functional interaction between POSH

and ALIX and the relevance to HIV-1 release. *BMC Biochem.* **10**, 12.
47 Keil JM, Shen Z, Briggs SP & Patrick GN (2010) Regulation of STIM1 and SOCE by the ubiquitin-proteasome system (UPS). *PLoS ONE* **5**, e13465.
48 Bendinelli P, Maroni P, Matteucci E & Desiderio MA (2015) HGF and TGFβ1 differently influenced Wwox regulatory function on Twist program for mesenchymal-epithelial transition in bone metastatic versus parental breast carcinoma cells. *Mol. Cancer* **14**, 112.
49 Pece S & Gutkind JS (2002) E-cadherin and Hakai: signalling, remodeling or destruction? *Nat. Cell Biol.* **4**, E72–4.
50 Fujita Y, Krause G, Scheffner M, Zechner D, Leddy HEM, Behrens J, Sommer T & Birchmeier W (2002) Hakai, a c-Cbl-like protein, ubiquitinates and induces endocytosis of the E-cadherin complex. *Nat. Cell Biol.* **4**, 222–231.

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AUTHOR CONTRIBUTIONS

CDB, RFT, MH, KS, SA and MDD performed experiments. CDB, TJM, KMM, HH, RFT analyzed and interpreted data. CDB, RFT wrote the manuscript. All authors reviewed the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

ETHICS STATEMENT

All procedures for the use of human cell lines were approved by the human ethics committee of the University of Newcastle, Australia (H-506-0607).

FIGURE LEGENDS

Figure 1. Yeast two hybrid screen of the FAT1 cytoplasmic tail identifies a novel protein interaction with SH3RF1.

A, Schematic of the FAT1 cytoplasmic tail highlighting the two regions selected for 'baits' in the yeast two-hybrid screen. The central FC1/FC2 regions (amino acids 4258-4498) are indicated. The terminal end 'bait' was found to be auto-activating in the absence of any prey interaction and therefore not used for any subsequent screening. **B**, Secondary confirmation from the second pass mating screen for activation of the *HIS3* and *ADE2* reporter genes of SH3RF1 with $FAT1_{4210-4280}$ bait. PJ69-4α yeast transformed to express either SH3RF1 or Large T antigen were streaked onto the appropriate media plates and then the $FAT1_{4210-4280}$ yeast was then streaked in a perpendicular direction. Growth was compared to the positive control of the known interaction between the Large T antigen and the p53 protein. Growth occurred on the -Leu/-Trp dropout media plate indicating successful generation of diploid yeast. No growth occurred on the SD minimal media base plate indicating no contamination by wild-type yeast. There was yeast growth on both the -Leu/-Trp/-His and -Leu/-Trp/-Ade dropout media plates indicating activation of both *HIS3* and *ADE2* reporter genes respectively indicating a positive interaction between FAT1 and SH3RF1.

Figure 2. GST pull downs and co-immunoprecipitation confirms FAT1 interaction with SH3RF1

A, *In vitro* translated deletion constructs of SH3RF1 were incubated with GST-FAT1(aa4210-4280) with specific binding observed for full length SH3RF1 and the N-terminal construct compared to 10% input controls. When the two N-terminal SH3 domains are removed, there was no observable binding. Deletion of the N-terminal RING domain did not affect binding between FAT1 and

SH3RF1. **B**, Reciprocal co-immunoprecipitation between the cytoplasmic tail of FAT1 (aa4210-4588) and V5-tagged SH3RF1 after ectopic expression in COS cells. No association occurred when the two N-terminal SH3 domains of SH3RF1 were removed, reconciling with the GST-pulldown assay. **C**, Co-immunoprecipitation assay shows reciprocal binding between endogenous FAT1 and SH3RF1 in MDA-MB-231 cell lysates.

Figure 3. SH3RF1 regulates FAT1 protein levels.

A, Schematic representation of full length FAT1 (p500) and hetero-dimeric form of FAT1 (p430 + p85) generated after a furin-mediated S1 cleavage event [35]. Both forms are expressed at the cell surface. The location of the different antibodies and their epitopes used in immunoprecipitation and immunoblotting are indicated. **B,** Western blotting for FAT1 using antibodies directed against the C-terminal domain after transient siRNA mediated knockdown of SH3RF1 in MDA-MB-231 cells. Blotting against SH3RF1 indicated a 90% decrease in protein levels with a concomitant >2 fold increase in FAT1 compared to control Neg Ctrl siRNA treatment. The C-terminal FAT1 antibody detects high MW FAT1 (p500) and p85, one part of the FAT1 heterodimer formed post-translationally via S1-cleavage. **C,** Cell surface biotinylation of MDA-MB-231 cells and immunoprecipitation of FAT1. Using Neutravidin-HRP to reveal surface proteins showed increased amounts of FAT1 at the cell surface FAT1 after SH3RF1 knockdown. Azide treatment followed by Western blotting against FAT1 confirmed FAT1 levels recovered after SH3RF1 knockdown. Depletion of SH3RF1 did not affect FAT1 heterodimer formation since the relative amounts of uncleaved (p500) and cleaved forms (p430 and p85) remained constant. **D,** Stable expression of V5-tagged SH3RF1 in MDA-MB-231 cells leads to a ~30% decrease in FAT1 expression. **E,** Transient

transfection of full length SH3RF1 and the ΔRING-domain SH3RF1 mutant in HEK293 cells reveals the RING domain is essential for the ability of SH3RF1 to decrease FAT1 protein levels.

Figure 4. FAT1 protein has a 2-hour half-life but depletion of SH3RF1 does not decrease the rate of FAT1 protein decay.

A, HEK293 and MDA-MB-231 cells were treated with cycloheximide and chased for 2-8h. Western blotting of cell lysates showed FAT1 to have a protein half-life of 2h for both cell lines while SH3RF1 levels were relatively unchanged ('Fraction FAT1 remaining' represents densitometry of p500 bands normalised to 0h). **B,** Transient siRNA knockdown of SH3RF1 lead to increased FAT1 protein in both HEK293 and MDA-MB-231 cells. Treatment with cycloheximide for 2h decreased FAT1 protein levels by a ~50% in both control and SH3RF1 siRNA samples indicating SH3RF1 does not affect the rate of FAT1 protein decay. **C,** qPCR assay measuring relative transcript levels in control versus SH3RF1 siRNA-treated cells in HEK293 and MDA-MB-231 cells. **D,** Proteasomal inhibition in HEK293 cells using either MG132 or Lactacystin for 2h increases FAT1 levels but addition of cycloheximide for the second hour of the experiment showed that FAT1 is still subject to degradation when the proteasome is inhibited.

TABLES

Table 1. Identification of FAT1$_{4210-4280}$ interacting prey proteins. Individual clones that grew after second pass screening for an interaction with the FAT1$_{4210-4280}$ juxta-membrane region had the interacting 'prey' sequences amplified by PCR, gel purified and then identified through automated Sanger sequencing and BLAST analysis. For clone C6, the prey PCR yielded two products and these were sequenced separately.

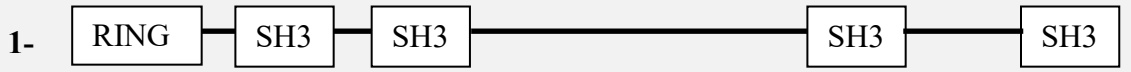
Clone ID	**BLAST result of nucleotide sequence**	**Alignment with amino acid sequence (coding region)**
A1	Homo *sapiens* SH3 domain containing ring finger 1 (SH3RF1) GI: 89142742	44aa-343aa
B17	Homo *sapiens* TNF receptor-associated factor 4 (TRAF4), GI: 118402591	55aa-367aa
B22	Homo *sapiens* TNF receptor-associated factor 4 (TRAF4), GI: 118402591	263aa-477aa
B23	Homo *sapiens* NADPH oxidase activator 1 (NOXA1), GI: 74759404	224aa-476aa
B36	Homo *sapiens* TNF receptor-associated factor 4 (TRAF4), GI: 118402591	263aa-477aa
C2	Homo *sapiens* SH3 domain containing ring	49aa-343aa

	finger 1 (SH3RF1) GI: 89142742	
C3	Homo *sapiens* SH3 domain containing ring finger 1 (SH3RF1), GI: 89142742	49aa-341aa
C4	Homo *sapiens* NADH dehydrogenase subunit 1, GI: 145967962	6aa-318aa
C5	Homo *sapiens* SH3 domain containing ring finger 1 (SH3RF1), GI: 89142742	49aa-314aa
C6-1	Homo *sapiens* sorbin and SH3 domain containing 2 (SORBS2), GI: 77404349	329aa-613aa
C6-2	Homo *sapiens* immunoglobulin lambda locus (IGLL1) GI: 23110977	1aa-233aa
C7	Homo *sapiens* SH3 domain containing ring finger 1 (SH3RF1), GI: 89142742	49aa-306aa
C8	Homo *sapiens* NADH dehydrogenase subunit 1,GI: 145967962	6aa-259aa

Table 2: *In silico* SH3 interaction prediction between FAT1 and SH3RF1 using bioinformatic algorithm SH3-Hunter

FAT1 Bait Sequence: (aa4210-4280) Class II SH3 Motif (PxxPxR)	KHQAEPKDKH LGPATAFLQR PYFDSKLNKN IYSDIP**PQVP VR**PISYTPSI PSDSRNNLDR NSFEGSAIPE

SH3RF1 Schematic



SH3RF1 SH3 Domain (Uni Prot: Q7Z6J0)	**Score**	**Sensitivity**	**Precision**
SH3RF1: SH3_#1 aa134-193	0.965	57%	70%
SH3RF1: SH3_#2 aa196-259	0.977	57%	70%
SH3RF1: SH3_#3 aa445-556	-	-	-
SH3RF1: SH3_#4 aa829-888	-	-	-