DIFFERENTIALLY SPLICED ISOFORMS OF FAT1 ARE ASYMMETRICALLY DISTRIBUTED WITHIN MIGRATING CELLS

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Running title: Polarized distribution of FAT1 isoforms

INTRODUCTION

FAT cadherins are large ~500 kD transmembrane proteins with an extracellular domain containing 34 cadherin motifs and a cytoplasmic domain that harbors several protein interaction motifs (1). So far, Ena/VASP, Homer 3 and beta-catenin has been reported to interact with the FAT1 cytoplasmic domain (2-5). In drosophila, the orthologs of mammalian FAT1, dfat (fl) and dfat-like (ftl), have been implicated in the establishment of planar cell polarity and formation of tracheal epithelia, respectively (6,7). We and others have shown that mammalian FAT1 is localized along the leading edge, filopodial protrusions, and intercellular junctions (2,4). As demonstrated by knockdown experiments, mammalian FAT1 is essential for cellular polarization and directed cell migration (2,4,5). FAT1 regulates actin dynamics at least in part via a direct interaction with Ena/VASP proteins (2). Consistently, genetic inactivation of
FAT1 in mice leads to loss of podocyte foot processes in the kidney, which are a highly dynamic actin-based structures forming a specialized inter-cellular junction, termed slit-diaphragm (8-11).

In the present study, several novel splice isoforms of FAT1 were identified and characterized.

**EXPERIMENTAL PROCEDURES**

**Plasmid constructs**

FAT1(WT)mito encoded the fusion protein: N’-3xFlag-mouse FAT1 cytoplasmic domain (Genbank AY256848)-mitochondrial outer membrane targeting sequence from ActA-C’ in the backbone of p3xflag-CMV-10 as described (2). FAT1 deletion- and point mutants of this plasmid were generated using *Hind*III/*Xba*I sites by standard PCR-mutagenesis. Constructs FAT1(1-184)mito, FAT1(1-190)mito, and FAT1(1-204)mito, contained an additional artificially generated spacer (SYPYDLGTWLNIDGFHMS) between FAT1 and ActA. FAT1(+12)mito was generated using FAT1(WT)mito as template and the internal primers fwd 5’-gacaacgaaagcttggctgcctcgacttgagcaagccacgcgggtaccactgggataca-3’ and rev 5’-gccaagctttcgttgtcatcacaggactc-3’. FAT1(+32)mito and FAT1(+8TR)mito were generated by shuffling of subcloned RT-PCR products (see below) into *Bgl*II/*Kpn*I-digested FAT1(WT)mito. FAT1(+3)mito contained the peptide insert “177AAS” (Genbank acc.# AY256848) within the PTB-like motif and the internal primers fwd 5’-gacaacgaaagcttggctgcctcgacttgagcaagccacgcgggtaccactgggataca-3’ and rev 5’-gccaagctttcgttgtcatcacaggactc-3’. FAT1(+32)mito and FAT1(+8TR)mito were generated by shuffling of subcloned RT-PCR products (see below) into *Bgl*II/*Kpn*I-digested FAT1(WT)mito. FAT1(+3)mito contained the peptide insert “177AAS” (Genbank acc.# AY256848) within the PTB-like motif and the internal primers fwd 5’-gacaacgaaagcttggctgcctcgacttgagcaagccacgcgggtaccactgggataca-3’ and rev 5’-gccaagctttcgttgtcatcacaggactc-3’. FAT1(+32)mito and FAT1(+8TR)mito were generated by shuffling of subcloned RT-PCR products (see below) into *Bgl*II/*Kpn*I-digested FAT1(WT)mito. FAT1(+3)mito contained the peptide insert “177AAS” (Genbank acc.# AY256848) within the PTB-like motif and the internal primers fwd 5’-gacaacgaaagcttggctgcctcgacttgagcaagccacgcgggtaccactgggataca-3’ and rev 5’-gccaagctttcgttgtcatcacaggactc-3’. FAT1(+32)mito and FAT1(+8TR)mito were generated by shuffling of subcloned RT-PCR products (see below) into *Bgl*II/*Kpn*I-digested FAT1(WT)mito. FAT1(+3)mito contained the peptide insert “177AAS” (Genbank acc.# AY256848) within the PTB-like motif and the internal primers fwd 5’-gacaacgaaagcttggctgcctcgacttgagcaagccacgcgggtaccactgggataca-3’ and rev 5’-gccaagctttcgttgtcatcacaggactc-3’. FAT1(+32)mito and FAT1(+8TR)mito were generated by shuffling of subcloned RT-PCR products (see below) into *Bgl*II/*Kpn*I-digested FAT1(WT)mito. FAT1(+3)mito contained the peptide insert “177AAS” (Genbank acc.# AY256848) within the PTB-like motif and the internal primers fwd 5’-gacaacgaaagcttggctgcctcgacttgagcaagccacgcgggtaccactgggataca-3’ and rev 5’-gccaagctttcgttgtcatcacaggactc-3’.

Controls Dhs-mito and 2xFKBP-mito were generated by shuffling the cytoplasmic domain of murine Dachsous (~340 aa, from GenBank acc.# XM_194371) and of 2xFKBP (~220 aa, from pC2M-F2E, Ariad) 5’ of the mitochondrial outer membrane targeting sequence from ActA into p3xflag-CMV-10.

FAT1(WT)ΔCad encoded: N’-IgG leader sequence (MGWSCIILFLV ATATGVHSS)-FLAG-mouse full length FAT1 aa 3568-4590 (Genbank acc.# AY598447)-C’.

**RT-PCR from mouse tissues and cultured cells**

Total RNA was isolated from mouse tissues using Tri-Reagent® and from cultured cells using RNasey mini® columns (Qiagen, Hilden, Germany). Reverse transcription of 2-5 µg RNA, PCR protocols, and β-actin primers have been described (13). FAT1 primers were directed against mouse nucleotides 370-389 (fwd) and 561-580 (rev) (Genbank acc.# AY254868), and against corresponding nucleotide stretches of rat and human FAT1 sequences (Genbank acc.# AF100960 and NM_005245, respectively). Representative FAT1-PCR products were subcloned into pBlunt-PCR® (Invitrogen) and sequenced. For quantitative mRNA expression analysis, PCR bands were recorded using digital imaging (INTAS, Göttingen, Germany), and analyzed using Labimage software (Kapelan; www.labimaging.com).

**Real-Time-RT-PCR from human biopsies**

Glomeruli from human biopsies were collected in the framework of the European Renal cDNA Bank consortium (ERCB), manually dissected, mRNA was prepared, reverse-transcribed, and real-time PCR was performed on a TaqMan ABI 7700 Sequence Detection System (Applied Biosystems, Weiterstadt, Germany) as described (14). The GAPDH PCR product and 18 S RNA served as internal standards, yielding comparable results (not shown). FAT1-specific primers (300 nM) and probes (100 nM) were: fwd 5’-
tccctgagctccttcca-3', rev 5'-cgcttggcatccaatctga-3', internal probe for FAT1(WT) 5'-cagtctctgacgctcctga-3', internal probe for FAT1(+12) 5'-aatgaatctttggctgctcctgacctca-3'. FAT1(+12)-specific primers for standard PCR-detection were: FAT1(+12)-5': fwd 5'-cagtctctgacgctcctga-3', rev 5'-gaggtcaggagcagccaaag-3'; FAT1(+12)-3': fwd 5'-ctttggctgctcctgacctc-3', rev 5'-cagtctctgacgctcctga-3'.

Antibodies
FAT1(+12)-specific polyclonal antiserum was raised in two rats and one guinea pig against a synthetic peptide “CNKEESLAA PDLKSPRGYH” N'-terminally fused to the antigenic protein KLH. Antiserum was centrifuged and used in dilutions of 1/50 for IF and of 1/500 for WB. Control preadsorption was done overnight at 4 °C (WB: 8 µg peptide/µl pure serum, IF: 800 µg peptide/µl pure serum). Other primary antibodies were used at indicated dilutions: previously described mouse α-FAT1(cyto) (2) (IF: 1/300 – 1/3000, WB: 1/5000), mouse α-Flag (M2, Sigma, 1/2000-3000). Secondary antibodies conjugated to fluorescence dyes Cy2 and Cy3 were from Jackson ImmunoResearch Laboratories, phalloidin-TRITC from Sigma and Mitotracker® red CMXRos from Molecular Probes.

Cell culture and IF
Cell lines were purchased from American Type Culture Collection: NRK-52E (#CRL-1571), RAT2 (#CRL-1764), COS-7 (#CRL-1651), HEK293T (#CRL-11268) and cultured in DMEM supplemented with 10% fetal calf serum and 200 U/ml of penicillin and streptomycin. A previously described immortalized podocyte cell line was cultured as described (15). Transfection was performed using Lipofectamin 2000™ (Invitrogen) for NRK-52E cells and using Fugene-6™ (Roche) or standard calcium phosphate method for COS-7 cells. IF studies were performed according to standard protocols. Briefly, cells grown on glass coverslips (collagen-coated for COS-7 cells) were fixed in 4% paraformaldehyde for 15 min and permeabilized with 0.2% Triton X-100 for 2 min (permeabilization omitted for experiments shown in Fig. 7B, C) or in methanol (-20 °C) for 15 min and subsequently blocked with 10% donkey serum. Nuclei were stained with Hoechst 33342 DNA-dye (1/10 000, 10 min.). Coverslips were mounted on glass slides with Mowiol® (Calbiochem/Merck). Images were acquired with a Leica DM-IRBE inverted microscope connected to a digital camera and Openlab® (Improvision) software or with a Leica DM-IRBE-TSP inverted confocal laser-scanning microscope connected to Leica confocal imaging software. Preparation for publication was done using Image J 1.33 U (NIH, USA) and Photoshop® (Adobe) software.

Western blot
For Western blot of recombinant FAT1-mito, COS-7 cell lysates were processed by 10 % SDS-PAGE, (10 µg/lane), transferred on nitrocellulose membranes and bands were visualized using standard ECL (Western Lightning Plus, Cat.# NEL105, Perkin Elmer). For detection of endogenous FAT1, NRK-52E cells were grown on dishes of 15 cm diameter and processed by 5 % SDS-PAGE, (50-100 µg lysate/lane; in equal amounts for each group in an experimental series). Experiments were performed at least three times and representative immunoblots are shown.

Time lapse microscopy
COS-7 cells grown on round coverslips were transfected with FAT1(WT)-mito for 36 h, stained with Mitotracker® red (1/500, 30 min), mounted on an air tight inverted chamber containing preconditioned medium and filmed at 37°C.

Mapping of the PTB-like motif
FAT1-mito constructs were transfected into mammalian cells and assessed for presence of mitochondrial redistribution into the cell periphery. For each construct at least 100 cells from three independent experiments were examined and classified.

RNAi
Knockdown of specific endogenous FAT1-isoforms using lentiviral transduction of small interfering hairpin RNA (shRNA) was performed.
as described previously (2). Two alternative FAT1(+12)-specific shRNA sequences were cloned into pLL3.7 (16): 12KD1: 5’-tgaatctttggctgctcctga ttcgt tcaggagcagccaaagattca ttttttc3’ or 12KD2: 5’-tggctgctcctgacctcagcaa ttcgt ttgctgaggtcaggagcagcca ttttttc3’ with identical results. All experiments were performed with 12KD1, now termed KD(+12). As control two point mutations were introduced into the shRNA hairpin sequence of 12KD1 (tggcagctcctgacg tcagcaa) (termed KD control).

KD(FAT1) lentivirus was described previously (2). Lentivirus was packaged in HEK-293T cells using a four-plasmid system (16) and used to transduce subconfluent NRK-52E cell with 90% transduction efficiency, as described (2).

Statistical analysis
Quantitative data from standard RT-PCR band analysis, real-time-PCR quantification, and morphometric cell evaluation were statistically analyzed using Prism software (www.GraphPad.com).

RESULTS
Alternative splicing of the FAT1 cytoplasmic domain
A comparison of the published mouse FAT1 mRNA sequences (Genbank acc.# AY256848 versus AJ250768) indicated the presence of a putative alternative splice site located at cytoplasmic nucleotide (nt) 529/530 (Fig. 1C, grey shaded sequences). Using RT-PCR, three splice inserts from mouse tissues or rat cell lines were cloned and sequenced. The corresponding exon-intron properties were established by comparative genomic sequence analysis (NC_000074.2). The mouse gene locus, mouse mRNA structure, and mouse mRNA/predicted protein sequences of the splice variants are shown in Fig. 1A-C, respectively. Three exons (cy1-3) encoded the previously described wild-type FAT1 cytoplasmic domain, FAT1(WT) (AY256848). Three alternatively spliced exons (A-C) were identified (Fig. 1A), which were invariably directly flanked by introns carrying the splice-donor-acceptor consensus sequence 5’ ag… …gt 3’ (data not shown). These exons were also present in the rat genome with 100 % nucleotide conservation (NC_005115) (not shown).

Cloned mRNA variant FAT1(+12) contained exon C alone (36 nt, Fig. 1B), encoding 12 additional amino acids (aa) in frame (Fig. 1C; deposited under GenBank acc.# DQ320124 (mouse) and DQ320127 (rat)). Cloned mRNA variant FAT1(+32) contained exons B and C (96 nt, Fig. 1B) encoding 32 additional aa in frame (Fig. 1C; DQ320125). Cloned mRNA variant FAT1(+8TR) contained exons A-C (149 nt, Fig. 1B) which led to the premature termination of the predicted protein after just 8 aa resulting in a truncation of the FAT1 cytoplasmic domain immediately after the splice site (Fig 1C; DQ320126).

The data was corroborated by mRNA sequence submitted to the database (17) and by genomic sequence-derived exon-prediction (all numbers are GenBank acc.#). Mouse FAT1(+12): (AJ250768, AY598446, XM_885736); mouse FAT(+32): (AY598445, XM_896394); mouse FAT(+8TR): (AY598444, XM_896357; rat FAT(+12): L41684). Database analysis for human alternative splice isoforms revealed an ortholog of mouse FAT1(+12) (AY598439), showing 100 % nucleotide insert identity with the mouse and rat sequences, and of a truncated form similar to mouse FAT1(+8TR) (AY598440). The zebrafish genome contained a sequence corresponding to mouse FAT1 exon B that was also flanked by the intron splice consensus sequence 5’ ag… …gt 3’ (data not shown).

FAT1 isoform expression was investigated in various mouse tissues (Fig. 1D). FAT1(WT) mRNA was detected in all tissues. FAT1(+32) and (+8TR) were expressed specifically within the central nervous system where they predominated over FAT1(WT). FAT1(+12) was detected in highest amounts in lung, and in trace amounts in all other tissues except brain (Fig. 1D’, brighter exposure of D).

Generation of an antiserum specific for alternatively spliced FAT1 isoforms
A splice insert-specific antiserum α-FAT1(+12) was raised in rat and guinea pig against the common additional 12 aa splice insert present in variants FAT1(+12) and FAT1(+32) (Fig. 2A, red). Both α-FAT1(+12) antisera derived from
rat or guinea pig yielded identical results with immunoblotting or immunofluorescence (Fig. 2B-F'). For all experiments the guinea pig antiserum was used except for Fig. 3A-A'', where the rat antiserum was used. The novel antiserum was compared against a previously described antiserum α-FAT1(cyto) (2) that is directed against the entire FAT1 cytoplasmic domain and detects all FAT1 isoforms (Fig. 2A', green). To characterize the antiserum, the cytoplasmic domain of either wild-type or spliced FAT1 (termed FAT1(WT)mito and FAT1(+12)mito, respectively, schematic in Fig. 5A) was targeted ectopically to the mitochondrial outer leaflets in COS-7 cells (2). Double-immunofluorescent staining using both antisera showed that α-FAT1(cyto) recognized both FAT1mito isoforms, as predicted (Fig. 2B, C; green fluorescence), while α-FAT1(+12) recognized exclusively FAT1(+12)mito transfected cells (Fig. 2B', C'; red fluorescence), indicating the antibody's specificity for the splice-insert. Of note, FAT1(WT)mito-transfected mitochondria were displaced towards the cellular leading edges, as described previously (2), but FAT1(+12)mito-expressing mitochondria remained in a perinuclear position (see below). Immunoblotting of lysates from FAT1mito-transfected cells confirmed the specificity of α-FAT1(+12) antisera. Pre-adsorption of α-FAT1(+12) with the immunizing peptide abolished the signal (Fig. 2D).

The capacity of antisera to detect endogenous full-length FAT1 was assessed. Lysates from non-transfected NRK-52E cells were immunoblotted with either α-FAT1(cyto) or α-FAT1(+12), each yielding a band of the predicted size of ~500 kDa (2) (Fig. 2E).

Differential subcellular localization of endogenous FAT1 isoforms
To investigate the potential functional characteristics of FAT1 isoforms, immunofluorescent double-stainings of various cell lines were performed. Immunofluorescent staining of early confluent native NRK-52E cells with α-FAT1(+12) revealed a signal at intercellular junctions and the nucleus (Fig. 2F) which could be abolished by antigen-preabsorption (Fig. 2F').

A striking differential subcellular distribution of FAT1 isoforms was observed in subconfluent migrating NRK-52E cells: α-FAT1(cyto) staining was observed along cellular leading edges as well as along intercellular junctions, and in a proportion of nuclei (Fig. 3A; green fluorescence), as described previously (2,4). By contrast, α-FAT1(+12) staining was completely absent at leading edges. α-FAT1(+12) staining was only detected along intercellular contacts and within the nuclei (Fig. 3A'; red fluorescence). A similar subcellular distribution of FAT1 isoforms could be observed in RAT2 and COS-7 cells (not shown).

It was verified by RT-PCR that cultured NRK-52E cells expressed both FAT1(WT) and (+12) mRNAs (Fig. 3B). Because FAT1 is essential for glomerular epithelial cells of the kidney in-vivo (8), the expression of FAT1(WT) and (+12) mRNAs was evaluated in a murine conditionally immortalized podocyte cell line (15) (Fig. 3C-E''). The subcellular localization of FAT1 isoforms in this podocyte cell line and in NRK-52E cells was identical (Fig. 3D-E''). These data indicate that FAT1(WT) was the only isoform localized along the leading edge of lamellipodial protrusions, while spliced FAT1(+12) was confined to intercellular junctions.

Splicing of endogenous FAT1 is regulated
Since alternative splicing occurred in a tissue-specific fashion in the mouse (Fig. 1D, D'), it was investigated if expression of FAT1 isoforms was differentially regulated under different culture conditions in NRK-52E cells. RT-PCR of proliferating subconfluent NRK-52E cells expressed predominantly FAT1(WT) mRNA, while quiescent cultures grown to confluence for 72 h predominantly expressed FAT1(+12) (Fig. 4A). Quantitative analysis of digitally analyzed band intensities revealed a statistically significant shift by a factor of 2.2 from a relative predominance of FAT1(WT) to a relative predominance of FAT1(+12) between proliferating and quiescent cells (p<0.002; Fig. 4A). These data were corroborated at the protein level by immunoblotting of cellular lysates derived from subconfluent, early confluent or quiescent NRK-52E cells. Again, a significant shift toward the expression FAT1(+12) was observed (n=3; Fig. 4B). In addition, down-
regulation of total FAT1 protein in confluent cells was observed as reported previously (4). Subcellular FAT1 isoform expression was analyzed by immunofluorescence. Consistent with the above described data, early confluent cultures expressed both FAT1(WT) and (+12) at intercellular junctions (Fig. 4C-C’’), while in quiescent cells a down-regulation of FAT1(WT) was observed. After prolonged culture, both isoforms were eventually down-regulated (Fig. 4D-D’’).

To test if splicing of FAT1 is also regulated in-vivo, FAT1 isoform expression was investigated by quantitative real-time RT-PCR in dissected glomeruli from human kidney biopsies. Within the glomerulus, FAT1 is predominantly expressed by podocytes (18). Similar to cultured podocytes, both FAT1(WT) and (+12) isoforms were detected in-vivo (Fig. 4E, F). In normal glomeruli, a relative predominance of FAT1(WT) over FAT1(+12) was observed (Fig. 4G). This ratio was changed by a factor of 1.9 ± 0.1 to a relative predominance of FAT1(+12) in minimal change disease (MCD), membranous glomerulonephritis (MGN), and focal-segmental glomerulosclerosis (FSGS) (p < 0.05 for each group, Fig. 4G).

A PTB-like motif targets FAT1(WT) towards the leading edge and can be inactivated by alternative splicing

The differential subcellular distribution of FAT1 splice isoforms indicated that this effect was mediated by a specific protein-protein interaction that was regulated by alternative splicing. Similar to endogenous FAT1(WT), ectopic expression of the FAT1(WT) cytoplasmic domain on mitochondrial outer leaflets (schematic in Fig. 5A) resulted in polarized mitochondrial redistribution towards the cellular leading edge (Fig. 2B-B’’; Fig. 5B-B’’, H), as described previously (2). Mitochondria are normally distributed in a perinuclear fashion close to the Golgi apparatus, as shown in untransfected cells in Fig. 5B’, cell 1. Mitochondrial targeting of control proteins (i.e. the cytoplasmic domain of protocadherin Dachsous, Fig. 5C, or two repeats of the FK506-binding protein, Fig. 5D) did not result in mitochondrial redistribution, indicating that mitochondrial redistribution was a characteristic intrinsic to the wild-type FAT1 cytoplasmic domain. Thus, mitochondrial redistribution was a useful assay to map the domain that mediates the unique subcellular distribution of FAT1(WT) towards the leading edge. Various mutants of FAT1-mito including all splice variants (+12), (+32), and (+8TR) were tested (Fig. 6A; splice site indicated by vertical bar). A quantitative grading system for the extent of distribution towards the leading edge was established and transfected cells were scored into one of three classes of either perinuclear (0 %), incomplete (<50 %) or complete (>50 %) mitochondrial redistribution towards the leading edge, respectively (exemplary cells are shown in Fig. 5E-L). Equal protein expression of each mutant was verified by immunoblotting (Fig. 5M). The statistical analysis for each mutant is shown in Fig. 6B (n=100 cells; 3 independent experiments each). FAT1(WT)mito mediated complete mitochondrial redistribution towards the leading edge in 90% of the cells, while mitochondrial distribution remained unchanged in cells transfected with any of the splice variants (+12) and (+32) similar to controls (Dhs and 2xFKBP). As predicted, FAT1(+8TR)mito was not targeted to mitochondria owing to the premature stop codon, resulting in its diffuse cytoplasmic distribution (Fig. 5K). These data show that polarized redistribution of FAT1 was abolished in all spliced FAT1 isoforms.

To map the essential amino acids mediating redistribution of FAT1(WT), C-terminal and N-terminal deletion constructs of FAT1(WT)mito were analyzed (Fig. 6A, B): (1-220), (1-204), (1-190; Fig. 5G), (1-184; Fig. 5E), (1-176), (202-385), (177-385), (174-385), (139-285), (Δ160-188; Fig. 5L). The minimal sequence necessary for redistribution of FAT1(WT) was mapped to cytoplasmic aa 174-190 (DDNGYWDTSWPSVP) as indicated by a black horizontal bar in Fig. 6C. Alignment for consensus sequence homology of mouse, chicken and zebrafish FAT1 together with mammalian FAT3 identified a conserved domain at aa 158-204, as indicated by a box in Fig. 6A and a grey bar in Fig. 6C. Of note, the identified motif was the only motif of vertebrate FAT1 that was conserved in its *drosophila* homologue *fil*, suggesting an important evolutionary conserved function (Fig. 6D).
The alternative splice site was located immediately within the identified minimal motif separating 4 highly conserved amino acids \(175\text{DNxYH}\) similar to a phosphotyrosine-binding (PTB) motif (Fig. 6C; black vertical line indicating the splice site). Indeed, when Y178 was mutated to glutamic acid (Y178E) mitochondrial redistribution was totally abolished (Fig. 6A, B). Substitution of Y178 to phenylalanine (F), a residue resembling a tyrosine residue that cannot be phosphorylated, had no effect (Y178F, Fig. 6A, B). In agreement with this, no evidence for tyrosine-phosphorylation of the wild-type FAT1 cytoplasmic domain was observed (not shown). Mutation of a second conserved tyrosine residue Y200 (to both E and F) had no effect (not shown). These findings support the notion that \(175\text{DNxYH}\) represents a minimal protein-protein interaction core motif that is not regulated by tyrosine-phosphorylation. Indeed, the identified motif shared characteristics with the Disabled1 (Dab1)-PTB-binding motif of \(C.\) \(e\)\(l\)\(e\)\(g\)\(a\)\(n\)s protein kinase C 3 (PKC3) \((\text{xxDNxxFHxx})\) which does not require phosphorylation of the essential tyrosine residue (19,20).

To investigate if the motif is inactivated by insertion of an irrelevant sequence or by insertion of a specific inactivating sequence, three additional FAT1-mito constructs were generated. Insertion of only 3 irrelevant amino acids (AAS) at the splice site \(\text{FAT1(}+\text{3)mito}\) significantly reduced mitochondrial redistribution while insertion of longer irrelevant fragments e.g. of 98 aa \(\text{FAT1(}+\text{98)mito}\) totally abolished redistribution (Fig. 6A, B). Furthermore, the functionality of the splice isoform \(\text{FAT1(}+\text{12)mito}\) could be rescued by insertion of the last three aa (DDN) derived from the N’-terminal half of the FAT1 cytoplasmic domain (see Fig. 6C) next to the essential tyrosine Y178 reconstituting the essential core sequence of the PTB-like motif \((\text{sequence: } \ldots_{172}\text{DDN} \text{eslaapdskpr} \ldots_{179}\text{DDGYHWD} \ldots\), Fig. 6A, B). These experiments show that the splice site was located at a functionally critical position. It was concluded that splice inserts \(+12\) and \(+32\) sterically disrupted a PTB-like motif.

Association of cellular process-formation and FAT1(WT)

As shown in Fig. 5B and H, the identified PTB-like motif mediated redistribution of recombinant FAT1(WT) in a polarized fashion towards the cellular leading edge. To verify this finding, movements of FAT1(WT)mito decorated mitochondria were observed for 12 hours using time-lapse video microscopy in transiently transfected COS-7 cells (supplemental movie S1; green cell, transfected with FAT1(WT)mito; red cell, non-transfected control). FAT1(WT)mito-decorated mitochondria were rearranged into a highly dynamic fashion into cellular processes towards the leading edge of migrating cells. The majority of FAT1-decorated mitochondria were redistributed into the direction of the overall cellular movement. Redistribution of FAT1(WT)mito-decorated mitochondria was closely associated with polarized process formation.

To test if FAT1 was also quantitatively associated with the formation of cellular processes, a partial full-length clone of wild-type FAT1 lacking the N-terminal 34 cadherin repeats was expressed within the cellular plasma membrane \((\text{FAT1(}WT)\text{ΔCadh})\) (Fig. 7A). As a control, a clone lacking the identified PTB-like motif was generated \((\text{FAT1(}Δ160-188)\text{ΔCadh}; \text{see Fig. 6A, C for sequence})\). Both constructs efficiently localized to the plasma membrane as shown by indirect immunofluorescence staining of transiently transfected COS-7 cells omitting the permeabilization step: Staining for the extracellular FLAG-epitope yielded a signal (green), while co-staining for the intracellular FAT1-cytoplasmic domain by \(\alpha\)-FAT1(cyto) (red) was negative (Fig. 7B, C). Importantly, FAT1(WT)ΔCadh was localized towards the cellular leading edge and into cellular processes similar to FAT1(WT)mito (Fig. 7B). This polarized redistribution was clearly abolished in the mutated clone, FAT1(Δ160-188)ΔCadh (Fig. 7C) indicating that FAT1(WT)ΔCadh was not enriched as a result of cellular artifacts.

A quantitative analysis of the number of cell protrusions per cell transfected with either FAT1(WT)ΔCadh or FAT1(Δ160-188)ΔCadh was performed. Strikingly, the majority of FAT1(WT)ΔCadh transfected cells displayed
numerous cellular protrusions that were enriched with FAT1(WT)ΔCadh at their tips (representative cells are shown in Fig. 7D, E). In contrast, cells transfected with FAT1(Δ160-188)ΔCadh consistently exhibited a rounded phenotype (Fig. 7F). Statistical analysis revealed that 95% of cells transfected with FAT1(Δ160-188)ΔCadh lacked protrusions, while 40% of FAT1(WT)ΔCadh-transfected cells exhibited one to two and another 35% exhibited three or more protrusions (p<0.001; Fig. 7G). On average, cells transfected with FAT1(WT)ΔCadh exhibited 2 long protrusions per cell, while cells transfected with FAT1(Δ160-188)ΔCadh exhibited none (p<0.001; Fig. 7H).

The FAT1(WT) isoform is sufficient for directed cell migration

The increased number of cellular protrusions in FAT1(WT)ΔCadh transfected cells suggested a differential role of the FAT1 splice isoforms in cell migration. To test this hypothesis, the expression of FAT1 splice isoforms was selectively silenced using RNA interference (16). NRK-52E cells were transduced with a lentivirus (KD(+12)) expressing a shRNA sequence specific for the splice inserts common only to the FAT1(+12) and (+32) isoforms. Expression of FAT1 isoforms was selectively silenced as demonstrated by immunoblotting of cellular lysates and by RT-PCR of total RNA extracts (Fig. 8A,B). Splice specific knockdown did not affect expression of wild-type FAT1. Knockdown of all FAT1 isoforms was achieved using a previously described lentivirus (KD(FAT1)) (2). These results also confirmed the specificity of the α-FAT1(+12) antiserum. NRK-52E cell monolayers were transduced with control virus (KD control), or virus specific for FAT1 splice isoforms (KD(+12)) or for total FAT1 (KD(FAT1)) and subjected to a wound assay. Lentiviral transduction efficiency was adjusted to 90% transduced cells, verified by co-expression of a viral eGFP reporter gene (2). Ten hours after wounding, wound closure was significantly impaired in FAT1 deficient cells (KD(FAT1)) compared to controls (KD control), as described previously (2,4). In contrast, wound closure was improved in cells that only expressed wild-type FAT1 (KD(+12)) compared to controls by a factor of 1.7 (mean remaining wound diameter of 106 μm vs. 180 μm). These results confirm that only the wild-type FAT1 isoform at the cellular leading edge is essential for directed cell migration.

DISCUSSION

In this study, three alternative splice isoforms of FAT1 with important functional differences from wild-type FAT1 were identified. The data show that endogenous FAT1(WT) represents the isoform through which FAT1 mediates functions of directed cell migration at the leading edge that have been previously described (2). This notion is supported by the fact that only FAT1(WT) was associated with cellular process formation and was essential for directed cell migration in a wound healing assay. In addition, FAT1(WT) was the predominant isoform in migrating cells in vitro and in healthy glomeruli with intact podocyte foot processes in vivo. Furthermore, cells are able to modulate their FAT1(WT) to FAT1(+12) expression ratios in response to extracellular signals (quiescent versus migratory). The isoform-specific antisera described in this work will therefore be an important tool to study specific FAT1 functions.

The association of endogenous FAT1(WT) with cellular leading edges was mapped to a novel PTB-like motif (175DNxYH179). This motif was functionally inactivated in alternatively spliced FAT1 isoforms. As shown by insertion of irrelevant sequences into the splice site, this mechanism was sterical in nature rather than dependent on a specific additional sequence. Consequently, FAT1(+12) and (+32) are predicted to be identical in function, although it cannot be ruled out that the sequence exerts other functions not detected in our analysis. Of note, the PTB-like motif and its surrounding region was highly conserved from drosophila ftl to mammals, suggesting its importance for FAT1 function, and supporting the notion that ftl is the true ortholog of mammalian FAT1 (6). It remains to be determined whether the PTB-like motif directly mediates targeting of FAT1(WT) to the leading edge, e.g. by vesicle transport. Alternatively, the identified PTB-motif may be implicated in the transduction of cellular polarization signals via asymmetrical enrichment
of wild-type FAT1 along the cellular leading edge. Indeed, asymmetrical enrichment in “cortical platforms” (21) as seen with FAT1(WT) is an established mode of transducing polarity signals in directed cell migration (22-27). Major events regulating directed cell migration are actin reorganization and polarized capture of microtubuli (21). Recombinant FAT1 was redistributed into similar locations as the cytosolic microtubular bridging protein or kinesin-transported cargos suggesting that the PTB-like motif interacts at the leading edge with the microtubular system (25,28-31).

At the nucleotide level, a striking evolutionary sequence conservation of the splice inserts including their surrounding introns was observed. This suggests the presence of important regulatory sequences for the splicing machinery. In a recent study in human patients, the C-terminus of the FAT1 gene has been shown to harbor alleles linked to bipolar disease (32). In the present study, mRNAs encoding spliced isoforms FAT1(+32) and (+8TR) were expressed exclusively within the CNS. One of the SNPs associated with bipolar disease lies within the PTB-like motif at S167 but does not result in a change of the protein sequence (TCC to TCT; rs1298865)(32). However, such silent point mutations may influence mRNA processing (33). Susceptibility to bipolar disease may be conferred by non-coding mutations that change the ratio between the FAT1 splice isoforms.

Within the renal glomerulus, upregulation of the FAT1(+12) splice isoform was associated with diseases that go along with heavy protein-loss into the urine, namely minimal change disease, membranous glomerulonephritis, and focal-segmental glomerulosclerosis. These glomerulopathies are associated with loss of the complicated structure of the actin cytoskeleton (termed foot process effacement) and formation of adherens junctions. No glomerular leukocyte infiltration or other glomerular cell proliferation occurs in minimal change disease and membranous glomerulonephritis excluding artifacts from other cell types. Our data therefore suggest that FAT1 isoform expression is also regulated in-vivo and that loss of podocyte foot processes is associated with a relative increase in expression of the junction-associated isoform FAT1(+12). These observations are of potential diagnostic or even therapeutic value.
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ABBREVIATIONS
aa = amino acids, Dhs, protocadherin dachsous; FAT1(WT), FAT1 wild type isoform; FKBP, FK506-binding protein; ftl, drosophila fat-like; ft, drosophila fat; IF, immunofluorescence; KD, knockdown; nt, nucleotides; PTB, phosphotyrosine-binding; shRNA, small hairpin RNA.

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FIGURE LEGENDS

Fig. 1. Alternative splicing of the FAT1 cytoplasmic domain

A. Schematic of the mouse gene locus of the FAT1 cytoplasmic domain encoded by three major exons (cy1-3) and the identified alternatively spliced exons (A-C). B, schematic of murine FAT1 mRNA splice forms of the cytoplasmic domain: wild-type (WT) and the alternative splice variants FAT1(+12), FAT1(+32), and FAT1(+8TR). Exons A-C are shown as white, grey, and black boxes, respectively. Cloning was performed by RT-PCR using primers positioned as indicated by black arrows. C, protein and mRNA sequence alignment of the identified alternatively spliced mouse FAT1 isoforms. Splice inserts are marked by a box. A published wild-type FAT1(WT) and splice isoform FAT1(+12) were identified from the database (in grey). FAT1 isoforms and Genbank Acc.# are indicated on the left. Corresponding sequence positions within these sequences are indicated on the right. D, FAT1 splice isoform-specific RT-PCR from mouse tissues (n = 3) with corresponding β-actin PCR-products from non-saturating reactions. In controls, reverse transcriptase or the cDNA template were omitted (RT-, H2O). FAT1(+32) and FAT1(+8TR) were detected exclusively in brain. FAT1(+12) message was highest in lung tissues, but was also expressed in all peripheral tissues (longer exposure is shown in D’).

Fig. 2. Characterization of a splice-insert specific antiserum, α-FAT1(+12)

Schematic of antibody epitopes. A, antiserum α-FAT1(+12) was directed specifically against the splice insert “ESLAAPDLSKPR” present in isoforms FAT1(+12) and FAT1(+32). A’, α-FAT1(cyto) was directed against all FAT1 isoforms. B, the wild-type epitope of the recombinant FAT1(WT) cytoplasmic domain overexpressed on mitochondrial outer leaflets of COS-7 cells (FAT1(WT)mito) was detected by α-FAT1(cyto), but not by α-FAT1(+12) (B’, merge in B’’). FAT1(WT)mito-loaded mitochondria were redistributed towards the leading edge (arrowheads). C, FAT1(+12)mito was detected by α-FAT1(cyto), as well as α-FAT1(+12) antiserum (C’, merge in C’’). FAT1(+12)mito-loaded mitochondria remained in a perinuclear localization (arrows). D, lysates from FAT1(WT)mito and FAT1(+12)mito expressing cells immunoblotted with α-FAT1(cyto) detected ~80 kD bands, as predicted (left lanes). α-FAT1(+12) detected exclusively FAT1(+12)mito (middle lanes). The signal was abolished by peptide preadsorption of α-FAT1(+12) (right lanes). E-F, specific detection of endogenous FAT1(+12) by α-FAT1(+12) in NRK-52E cells. Using immunoblot analysis (E), both antisera detected a ~500 kD band specific for endogenous FAT1. Using indirect immunofluorescence, α-FAT1(+12) revealed junctional staining in NRK-52E cells (F) that was abolished by antigen preadsorption (F’).

Fig. 3. Differential subcellular distribution of FAT1 splice isoforms

A-A’’. Immunofluorescent staining of subconfluent NRK-52E cells with α-FAT1(cyto), green, and α-FAT1(+12), red. α-FAT1(cyto) stained both leading edges (arrowheads) and intercellular junctions (A, arrows). In contrast, α-FAT1(+12)-staining was strictly confined to intercellular junctions (A’). Overlay of both channels demonstrated co-staining along intercellular junctions (A’’). B-C, mRNA expression of FAT1(WT) and FAT1(+12) was detected in NRK-52E and RAT2 cell lines, as well as in a murine podocyte cell line (PCL). Corresponding β-actin PCR-products from non-saturating reactions were used to control for equal loading. As controls, the reverse transcriptase or the cDNA template were omitted (RT-, H2O). D-E’’, the differential subcellular localization of FAT1 isoforms was confirmed in a murine podocyte cell line (double-staining with α-FAT1(cyto), green, and α-FAT1(+12), red).

Fig. 4. Differential regulation of FAT1 isoform expression

A-D, FAT1 isoform expression in native NRK-52E cells. A, RT-PCR analysis of subconfluent and confluent cells. Lower panel: Equal amounts of mRNA were used for each non-saturating RT-PCR reaction as verified by β-actin product intensities. As controls, the reverse transcriptase or the cDNA-template were omitted (not shown). The FAT1(WT) mRNA isoform was more prevalent in
subconfluent cells while FAT1(+12) mRNA was more prevalent in confluent cells. Upper panel: The expression-ratio of digitally analyzed band-intensities of FAT1(WT) and FAT1(+12) isoforms was calculated for each experiment. Relative FAT1(WT) mRNA expression was down-regulated by a factor of 2.2 in comparison to FAT1(+12) (n=5 each, data are means ± SD, p<0.002, unpaired two-sided t-test). B, FAT1 isoform expression was assessed by immunoblotting in subconfluent, early confluent and confluent cells using α-FAT1(cyto) and α-FAT1(+12) (n=3). While overall expression of FAT1 isoforms decreased with the degree of confluency, the relative expression of FAT1(+12) protein increased. Equal protein load was controlled by Ponceau-staining (lower panel). C-C**, immunofluorescent staining of subconfluent (not shown) and early confluent cells showed junctional co-staining with both α-FAT1(cyto) and α-FAT1(+12) in early confluent cells. D-D**, in confluent cells, staining for FAT1 was lost with both antisera as shown by RT-PCR and immunoblotting. E, RT-PCR analysis of dissected glomeruli from human kidney biopsies showed expression of both FAT1 isoforms. F, expression of FAT1(+12) was confirmed by two different specific primer pairs amplifying a segment at the 5′ and the 3′ side of the alternative splice site, respectively. G, using quantitative real-time PCR, glomeruli from human biopsies of healthy (control) and diseased kidneys were analyzed (MCD: minimal change disease, MGN: membranous glomerulonephritis, FSGS: focal-segmental-glomerulosclerosis). In diseases, the relative amount of alternatively spliced FAT1(+12) was predominant over FAT1(WT), while in control kidneys, FAT1(WT) was predominant. Relative FAT1(WT) expression was lowered by a factor of 1.8 (MCD), 1.9 (MGN), and 2.0 (FSGS), respectively, when compared to control (dot = one experiment; horizontal bar = mean; p<0.05 for control vs. each disease; one-sided ANOVA, post hoc Bonferroni comparison).

**Fig. 5. Polarized subcellular redistribution of FAT1 mutants**
A. The FAT1 cytoplasmic domain was targeted to mitochondria outer leaflets using a C-terminal signal sequence derived from ActA. B-B**, when overexpressed in COS-7 cells, FAT1(WT)mito-decorated mitochondria accumulated along the cell periphery close to the plasma membrane within the tips of cellular protrusions (2). Overexpressed FAT1(WT)mito was detected using α-FLAG antiserum (B), mitochondria were detected using MitoTracker®-red (B'). Endogenous perinuclear cytoplasmic distribution of mitochondria can be observed in non-transfected cells (1). C-D, as controls, the cytoplasmic domains of dachsous (Dhs) or 2xFKBP (two repeats of FK506-binding protein) were expressed on mitochondrial outer leaflets (α-FLAG staining). No mitochondrial redistribution was observed in these cells. E, similarly, no redistribution was mediated by the FAT1 cytoplasmic domain truncated at aa 184 (α-FLAG staining). C-E, representative cells without mitochondrial rearrangement (0%). Two cells with incomplete or complete mitochondrial redistribution are shown in F (<50%, α-FAT1(cyto)) and G (>50%, α-FLAG). FAT1(1-190)mito was sufficient to mediate complete mitochondrial redistribution (G), while a truncated construct just 6 aa shorter was without effect (E, α-FLAG).
H-L, NRK-52E cells transfected with various FAT1mito mutants subjected to a wound assay (red, f-actin; green, α-FLAG staining; blue, DNA). FAT1(WT)mito was redistributed towards the leading edge (H, arrowheads), while the splice variants (+12) and (+32) as well as a mutant lacking the identified motif (Δ160-188) show normal mitochondrial distribution (arrows). K, FAT1(+8TR) was expressed diffusely in the cytoplasm as a result of a stop codon cleaving off the C-terminal mitochondrial targeting peptide. M, similar protein expression levels of FAT1(WT)mito, FAT1(+12)mito and FAT1(Δ160-188)mito were verified by α-FLAG immunoblotting.

**Fig. 6. Identification of a novel PTB-like motif within the FAT1 cytoplasmic domain**
A. Schematic of FAT1-mito mutants used to map the minimal motif necessary for redistribution of FAT1 towards the cellular leading edge. The identified conserved motif (aa 158-204) is indicated by a box, separated by a bar to mark the splice site at aa 176/177. B, semi-quantitative evaluation of mitochondrial redistribution. Three categories of mitochondrial redistribution were defined as indicated
in the legend. C, amino acid sequence of the identified motif shown as pretty plot alignment of FAT1 cytoplasmic domains (Genbank acc. #: AAP82173, XP_420679, AAO15696, AAU00738, respectively). Consensus sequences (boxes), alternative splice site (vertical bar), entire (grey horizontal bar), and minimal motif (black horizontal bar) necessary for polarized redistribution are indicated. D, bestfit alignment of mouse FAT1 and Drosophila melanogaster ftl cytoplasmic domains (Genbank acc. #: AAP82173 and AAF49078, respectively). Exclusively, the identified motif was conserved. Identity and low similarity are indicated by vertical lines and dots, respectively.

Fig. 7. Expression of FAT1 cytoplasmic domain at the plasma membrane is associated with cell protrusions
A. Schematic of FAT1(WT)ΔCadh targeting the FAT1 cytoplasmic domain to the plasma membrane. B-C, immunofluorescent staining of non-permeabilized COS-7 cells transiently transfected with FAT1(WT)ΔCadh and FAT1(Δ160-188)ΔCadh, respectively (α-FLAG, green; α-FAT1(cyto), red; nuclei, blue). B, overexpressed FAT1(WT)ΔCadh was redistributed into cellular protrusions, similar to FAT1(WT)mito (arrowheads). C, FAT1(Δ160-188)ΔCadh, lacking the PTB-like motif, localized in a diffuse punctate pattern. D-F, α-FLAG-staining of permeabilized cells transfected with FAT1(WT)ΔCadh or FAT1(Δ160-188)ΔCadh, respectively. The majority of FAT1(WT)ΔCadh expressing cells exhibited cellular protrusions that were enriched with FAT1(WT)ΔCadh (D). Other cells displayed multiple long extensions that contained FAT1(WT)ΔCadh near the tip (E). F, by contrast, overexpression of FAT1(Δ160-188)ΔCadh was associated with a rounded cell shape. G, statistical analysis of the percentage of cells with 0, 1-2, or 3 and more protrusions for FAT1(WT)ΔCadh or FAT1(Δ160-188)ΔCadh (n=30 cells each, data are means, p<0.001, χ-square-test). H, alternative analysis evaluating the average number of cell protrusions per group (data are means ± SD, p<0.001, two-sided Mann-Whitney-test).

Fig. 8. The FAT1(WT) isoform is sufficient for efficient wound closure
NRK-52E cells were transduced with control lentivirus (KD control), or virus containing an shRNA template specific for either FAT1(+12) (KD(+12)), or all FAT1 isoforms (KD(FAT1)). A: Specific knockdown of FAT1 splice isoforms is shown in SDS-PAGE of cellular lysates immunoblotted with α-FAT1(cyto) or α-FAT1(+12) shows preservation of both isoforms in controls and selective FAT1(+12)-deficiency in KD(+12) cells. Deficiency of all FAT1 isoforms was observed in KD(FAT1) transduced cells. Lower panel: Ponceau’s staining demonstrating equal protein load of each lane. B: Splice isoform-specific suppression of mRNA expression detected by RT-PCR. Identical amounts of mRNA were used as demonstrated by RT-PCR of endogenous β-actin. C: Wound closure is affected by FAT1 splice isoforms. Mean wound diameters of FAT1(+12) deficient cells were smaller compared to control cells (106 µm vs. 180 µm). In contrast, knockdown of all FAT1 isoforms was associated with significantly increased mean wound diameters (445 µm). N = 5 wounds from 3 paired experiments. Means were calculated from four diameter-measurements within each 2 cm wound using analySIS® software (Soft imaging system, Muenster, Germany). Data are means ± SEM; ANOVA, post hoc Bonferroni-analysis.

Supplementary figure
Fig. S1.Time-lapse video-microscopy of COS-7 cells transiently transfected with FAT1(WT)mito
Mitochondria were fluorescently stained by MitoTracker® red. Exposures of 250-fold magnification were taken every 15 minutes over 12 hours. Transfected cells were identified by co-transfected eGFP at the end of the sequence and were retrospectively labeled in green throughout the entire sequence for better recognition. As a control, a non-transfected cell was highlighted in red.
Fig. 1

A gene locus of the mouse FAT1 cytopl. domain

exons: cy1 cy2 A B C cy3

3' 138 53 60 36 629 5'

bp length

1 kb

B mRNA

mFAT1(WT) mRNA

mFAT1(+12) mRNA

mFAT1(+32) mRNA

A B C

mFAT1(+8TR) mRNA

C

mFAT1 (WT) AAF8217 S C D D N

mFAT1 (+12) CAB65271 S C D D H

mFAT1 (+32) ABC59054 S C D D H

mFAT1 (+8TR) ABC59055 S C D D H

mFAT1 (WT) AY255648 tcttgtgatgacaat

mFAT1 (+12) AJ255768 tcttgtgatgacaat

mFAT1 (+32) DQ320124 tcttgtgatgacaat

mFAT1 (+8TR) DQ320125 tcttgtgatgacaat

mFAT1 (+12) DQ320126 tcttgtgatgacaat

mFAT1 (+32) DQ320122 tcttgtgatgacaat

mFAT1 (+8TR) DQ320123 tcttgtgatgacaat

D

mFAT1(+8TR) mFAT1(+32) mFAT1(+12) mFAT1(WT)

bp 307 247 211 211

actin

D' mFAT1(+12) mFAT1(WT)

bp 247 211

long liver kidney cortex ileum

bp 448 307 247 211

hepa tumor lung liver kidney cortex ileum RT QO

bp 307 247 211 211

actin

bp 448 307 247 211

hepa tumor lung liver kidney cortex ileum RT QO

bp 307 247 211 211

actin

bp 448 307 247 211

hepa tumor lung liver kidney cortex ileum RT QO
Fig. 2

A  FAT1(+12) or (+32) isoforms

B  FAT1(WT)mito

C  FAT1(+12)mito

D  lysate: +12 WT +12 WT +12 WT

E  lysate: NRK-52E

F  α-FAT1(+12) (preadsorbed)
Fig. 4

A

|   | subconfluent | confluent |
|---|--------------|-----------|
| FAT1 (+12) | 247 bp      | 211 bp    |
| FAT1 (WT)  | 115 bp      | 79 bp     |
| β-actin    | 69 bp       | 66 bp     |

B

|   | subconfluent | confluent |
|---|--------------|-----------|
| FAT1 (+12) | 500 kD      | 500 kD    |
| FAT1 (cyto)| 175 kD      |           |

C

C' C''

D

D' D''

E

|   | flanking primers |
|---|------------------|
| FAT1 (+12) | 115 bp |
| FAT1 (WT)  | 79 bp  |

F

|   | 5' | 3' |
|---|----|----|
| FAT1 (+12) | 69 bp | 66 bp |

G

|   | control | MCD | MGN | FS/SGS |
|---|---------|-----|-----|--------|
| ratio of (WT)/(+12) | [Graph] | [Graph] | [Graph] | [Graph] |
Fig. 5

A

FLAG  ActA-mito
Cytodomain  mitochondrial
FAT1-mito

B  B'  B''

α-FLAG  FAT1(WT)mito  mitochondria
merge

C  D  E'  F  G

Dhe-mito  2xFKBP-mito  FAT1(1-184)mito  FAT1(WT)mito  FAT1(1-190)mito

H  I  J  K  L

FAT1(WT)mito  FAT1(+12)mito  FAT1(+32)mito  FAT1(+8TR)mito  FAT1(Δ160-188)mito

M

175 kDa  +12  Δ160-188
Fig. 6

A  FAT1 cytoplasmic domain mutants

| amino acid | 1 | 158 | 204 | 385 |
|------------|---|-----|-----|-----|
| FAT1 (WT)  |   |     |     |     |
| (+12)      |   |     |     |     |
| (+32)      |   |     |     |     |
| (+4TR)     |   |     |     |     |
| (1-220)    |   |     |     |     |
| (1-204)    |   |     |     |     |
| (1-190)    |   |     |     |     |
| (1-184)    |   |     |     |     |
| (1-176)    |   |     |     |     |
| (202-385)  |   |     |     |     |
| (177-385)  |   |     |     |     |
| (174-385)  |   |     |     |     |
| (139-285)  |   |     |     |     |
| (180-188)  |   |     |     |     |
| (Y178E)    |   |     |     |     |
| (Y178F)    |   |     |     |     |
| (+3)       |   |     |     |     |
| (+68)      |   |     |     |     |
| (+12+DDN)  |   |     |     |     |
| Dhs        |   |     |     |     |
| 2xFKBP     |   |     |     |     |

B  % cells with mito. redistribution

| % cells with mito. redistribution |
|-----------------------------------|
| 0  | 25 | 50 | 75 | 100 |

Legend:
- complete redistribution (>50% of mitochondria)
- incomplete redistribution (<50%)
- perinuclear distribution

C  amino acid

| mouse FAT1 | 139 | 148 | 156 | 157 | 158 | 159 | 160 | 161 | 162 |
|------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| chicken FAT1 |     |     |     |     |     |     |     |     |     |
| zebrafish FAT1 |     |     |     |     |     |     |     |     |     |
| mouse FAT3 |     |     |     |     |     |     |     |     |     |

D  amino acid

| mouse FAT1 | 154 | 174 | 176 | 179 | 194 | 198 | 200 | 204 | 205 |
|------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| chicken FAT1 |     |     |     |     |     |     |     |     |     |
| drosophila |     |     |     |     |     |     |     |     |     |

Legend:
- complete redistribution (>50% of mitochondria)
- incomplete redistribution (<50%)
- perinuclear distribution
Differentially spliced isoforms of FAT1 are asymmetrically distributed within migrating cells

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