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## Pathogenic exon-trapping by SVA retrotransposon and rescue in Fukuyama muscular dystrophy

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### Abstract

Fukuyama muscular dystrophy (FCMD; MIM253800), one of the most common autosomal recessive disorders in Japan, was the first human disease found to result from ancestral insertion of a SINE-VNTR-*Alu* (SVA) retrotransposon into a causative gene<sup>1-3</sup>. In FCMD, the SVA insertion occurs in the 3'-untranslated region (UTR) of the *fukutin* gene. The pathogenic mechanism for

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**Author Contributions** M.T.-I., K.K., M.K., and T.T. designed the study. M.T.-I. performed the majority of experiments. K.K. developed a system to detect endogenous fukutin protein. M.K. performed biochemical analysis of VMO-injected mice. C.Y. produced the *fukutin* cDNA constructs for transfection experiments. K.M., T.O., and A.K. performed analyses of AON treatment in mice and various cell types. H.K., T.Y., and S.T. provided intellectual input. H.O.A., S.D., and R.K., provided patients' samples. M.T.-I., K.K., and T.T. wrote the paper.

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FCMD is unknown, and no effective clinical treatments exist. Here we show that aberrant mRNA splicing, induced by SVA exon-trapping, underlies the molecular pathogenesis of FCMD. Quantitative mRNA analysis pinpointed a region that was missing from transcripts in FCMD patients. This region spans part of the 3' end of the fukutin coding region, proximal part of the 3' UTR, and the SVA insertion. Correspondingly, *fukutin* mRNA transcripts in FCMD patients and SVA knock-in (KI) model mice were shorter than the expected length. Sequence analysis revealed an abnormal splicing event, provoked by a strong acceptor site in SVA and a rare alternative donor site in *fukutin* exon 10. The resulting product truncates the fukutin C-terminus and adds 129 amino acids encoded by the SVA. Introduction of antisense oligonucleotides (AONs) targeting the splice acceptor, the predicted exonic splicing enhancer, and the intronic splicing enhancer prevented pathogenic exon-trapping by SVA in FCMD patient cells and model mice, rescuing normal *fukutin* mRNA expression and protein production. AON treatment also restored fukutin functions, including *O*-glycosylation of  $\alpha$ -dystroglycan ( $\alpha$ -DG) and laminin binding by  $\alpha$ -DG. Moreover, we observe exon-trapping in other SVA insertions associated with disease (hypercholesterolemia<sup>4</sup>, neutral lipid storage disease<sup>5</sup>) and human-specific SVA insertion in a novel gene. Thus, although splicing into SVA is known<sup>6-8</sup>, we have discovered in human disease a role for SVA-mediated exon-trapping and demonstrated the promise of splicing modulation therapy as the first radical clinical treatment for FCMD and other SVA-mediated diseases.

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FCMD (incidence 1/34,000 births) shares phenotypic similarities with other severe muscular dystrophies, including muscle-eye-brain (MEB) disease and Walker-Warburg syndrome (WWS). All show deficiencies in *O*-glycosylation of  $\alpha$ -DG, an extracellular protein anchored on the plasma membrane. Insufficient *O*-glycosylation interferes with  $\alpha$ -DG's ability to interact with extracellular matrix proteins such as laminin<sup>9-10</sup>. For this reason, FCMD, MEB and WWS are categorized as “ $\alpha$ -dystroglycanopathies ( $\alpha$ -DGpathy)”<sup>10</sup>, and to date, no effective treatments exist for these conditions. SVA is a hominid-specific, composite non-coding retrotransposon that contains SINE (short interspersed sequence), VNTR (variable number of tandem repeat), and *Alu* sequences. It is still active in humans, polymorphic, and mobilized by the human LINE-1 *in trans*<sup>6,11-15</sup>.

In previous work, we showed that *fukutin* mRNA (10 exons, 7.4-kb and 6.4-kb cDNAs in size with two poly-A sites, 461-amino-acid protein with calculated molecular weight of 53.7 kDa) was not detectable by Northern blot analysis in FCMD patients carrying the SVA insertion<sup>2</sup>. To investigate the etiology of this decreased expression, we have now analyzed whole *fukutin* mRNA in lymphoblasts from FCMD patients using qRT-PCR. PCR products corresponding to the protein-coding region of *fukutin*, as well as those including sequences in the distal part of the 3' UTR (and thus downstream of the SVA insertion) were similar in abundance to those from an unaffected control (Fig. 1a). However, products located at sequence positions within the 3' UTR were markedly decreased relative to the control. From these results and along with previous reports of many 3' and 5' splice sites within SVA elements<sup>6-8</sup>, we hypothesized that abnormal splicing occurs somewhere between the end of the fukutin protein coding region and the SVA insertion.

We then performed long range RT-PCR using primers that flank the region corresponding to decreased expression. In FCMD patients, we detected a single 3-kb PCR product, which is

shorter than the 5-kb product seen in the normal control (Fig. 1b). This observation was consistent in several tissue types from FCMD patients (Supplementary Fig. 1). PCR from genomic DNA produced an 8-kb product in FCMD patients, as compared with a 5-kb product in the control (Fig. 1b). Sequence analysis of the 3-kb product from FCMD cDNA revealed a splicing event (Supplementary Fig 2). This event generates a new donor-side breakpoint within the final coding exon (exon 10), located 116 bp upstream from the authentic stop codon. A rare alternative donor site at that position is activated and trapped by an alternative acceptor site located within the inserted SVA, creating an additional and aberrant exonic sequence (exon 11) (Figure 1c). The acceptor-side breakpoint is located 274 bp downstream from the start of the SVA insertion, between ag and TC (Figure 1c). The acceptor site has not been described in the previous reports of SVA splicing<sup>6,7</sup>. This location is preceded by a pyrimidine rich stretch, the SVA (TCTCCC)<sub>41</sub> hexamer at the 5' end of the SVA element, with a possible favorable branch point. Predicted exonic splicing enhancer (ESE) sites occur around 70 bp downstream from the new acceptor site. We confirmed that the aberrant splicing event can be abolished by replacing AG with GG at the acceptor junction in cultured cells transfected with a *fukutin* construct carrying SVA insertion (Supplementary Fig. 3). *Fukutin* expression was not altered by cycloheximide (CHX) treatment, indicating that the transcript was not subject to nonsense-mediated mRNA decay (NMD), possibly because this exon-trapping occurred within the last exon, and the new stop codon exists downstream of the new last exon-exon junction (Supplementary Fig. 4).

We have recently generated knock-in (KI) mice that carry a humanized *fukutin* exon 10, which either includes (Hp allele) or does not include (Hn allele) the SVA insertion, and bred these strains with heterozygous *fukutin* knockout mice to obtain compound heterozygotes (Hp/-)<sup>16</sup>. KI mice that are homozygous (Hp/Hp) and compound heterozygous (Hp/-) are representative of the human FCMD alleles. These mice exhibit hypoglycosylation of  $\alpha$ -DG in skeletal muscle, which is the most significant characteristic in  $\alpha$ -DGpathy<sup>16</sup>. Quantitative RT-PCR in various tissues from Hp/Hp mice revealed an aberrant splicing pattern identical to that seen in human patients (Supplementary Fig. 5). Northern blot analysis detected abnormally spliced *fukutin* mRNA species at the expected sizes of 5.6 and 4.6 kb in FCMD patients, whereas the normal *fukutin* mRNAs appeared at 7.4 and 6.4 kb (Fig. 1d and Methods). We replicated these results in the KI model mice (Fig. 1e and Supplementary Fig. 6a). The consistent observations between human FCMD patients and KI model mice lead us to conclude that a splicing abnormality underlies the pathogenesis of FCMD.

Abnormal splicing excises the authentic stop codon and produces another stop codon located 388 bp downstream from the 5' side of the new exon 11 (Fig. 1c). The predicted protein lacks the C-terminal 38 amino acids of *fukutin*, instead containing 129 amino acids derived from the SVA sequence (Supplementary Fig. 7). Endogenous *fukutin* is scarce and difficult to detect; however, we were able to identify both normal and aberrant forms of the protein in human and mouse using immunoprecipitation followed by Western blot analysis. The abnormal *fukutin* protein in FCMD displayed the predicted mobility shift (Fig. 2a-c and Supplementary Fig. 6b).

We introduced normal and aberrantly spliced *fukutin* cDNA constructs into mammalian cell lines. Whereas normal *fukutin* localized to the Golgi apparatus, the aberrantly spliced

fukutin protein is displaced completely from the Golgi to the endoplasmic reticulum (ER) (Fig. 2d and Supplementary Fig. 8). Further examination showed that a *fukutin* construct lacking the C-terminal 38 amino acids also mislocalized to the ER (Fig. 2d and Supplementary Fig. 8), suggesting that the C-terminal domain of fukutin is important for localization to the Golgi. Thus, impairment of this domain may lead to fukutin dysfunction in FCMD. The mislocalization is unlikely to be toxic because FCMD is an autosomal recessive disease and heterozygous carriers of the SVA insertion have no symptoms.

We next tested if exon-trapping occurs in other diseases with SVA insertion<sup>6</sup>. In a patient with autosomal recessive hypercholesterolemia (ARH), a 2.6-kb SVA is inserted within intron 1 of the *LDLRAP1* gene<sup>4</sup>. A patient with lipid storage disease with subclinical myopathy (NLSDM) also has a 1.9-kb SVA insertion in exon 3 of the *PNPLA2* gene<sup>5</sup>. We found abnormally spliced products induced by SVA exon-trapping in these patients' fibroblast (Fig. 1f left and middle panels, Supplementary Fig. 9 and 10, and Supplementary Table 1). CHX treatment to fibroblasts from these patients increased expression of the genes (Supplementary Fig. 9a and 10a), suggesting that the SVA-trapped transcripts are likely to be subjected to NMD<sup>6,17</sup>. In a search for the exact same events using the same acceptor site as FCMD in the human genome, we located two expressed sequence tags (ESTs) on human chromosome 4 (DA436529 and DA060755) that represent a spliced transcript induced by an SVA element. We found exonization in a human-specific insertion of SVA (AB627340) into a small gene (Fig. 1f right panel and Supplementary Fig. 11). The human-specific exon-trapping of SVA in the small gene might influence human evolution and development.

FCMD alleles of the *fukutin* gene contain a fully intact protein coding sequence, raising the possibility that FCMD could be treated by restoring translation of the full-length protein through splicing modulation with AONs. To identify promising target sequences in various cell lines, we produced 25-mer 2'-O-methyl phosphoramidite (2'OMePS) AONs targeted to the acceptor (A1-A3), donor (D1-D5) and ESE sites (E1-E4) in *fukutin* pre-mRNA (Supplementary Fig. 12). We introduced the AONs into various cell types and assessed the recovery of normal processing and restoration of the authentic stop codon (Fig. 3a). Cells with A3 and E3 showed strong suppression of SVA-derived splicing. The greatest recovery of *fukutin* mRNA, to levels of more than 40% of the normal control, was achieved with a combination of A3, E3, and D5 (AED) (Fig. 3a). The D5 sequence overlaps with a predicted ISE site within the aberrant intronic sequence; in normal *fukutin*, this sequence resides in exon 10 (Supplementary Fig. 12).

We injected octa-guanidine morpholino oligonucleotide (Vivo-Morpholino, VMO)<sup>18</sup> AED cocktail locally into skeletal muscle of KI mice and evaluated the therapeutic effect calculating the percent recovery of normally processed mRNA. In the AED-treated tibialis anterior (TA) and gastrocnemius (GC) of Hp/Hp and Hp/- mice, the amount of corrected *fukutin* mRNA increased significantly relative to mice treated with control VMO (Fig. 3b and Supplementary Fig. 13). We assessed fukutin protein recovery in injected skeletal muscle tissue from Hp/Hp mice. Consistent with the significant increase of restored normal mRNA, normal fukutin protein was rescued (Fig. 4a). We examined  $\alpha$ -DG glycosylation in AED-treated Hp/- mice. Deficiently glycosylated  $\alpha$ -DG, at the predicted smaller size, was reduced in abundance, whereas normal-sized  $\alpha$ -DG increased following AED treatment

(Fig. 4b). The signal intensity for glycosylated  $\alpha$ -DG was clearly increased, and a shift in the  $\alpha$ -DG core was observed, indicating that the rescued fukutin is functional. Laminin overlay assays revealed a marked increase in  $\alpha$ -DG laminin-binding ability, indicating that  $\alpha$ -DG function also is recovered (Fig. 4b). We next tested systemic AED treatment by intravenous injection for Hp<sup>-/-</sup> mice. This treatment also showed the recovery of normally glycosylated  $\alpha$ -DG in AED-treated mice (Fig. 4c).

We administered the VMO AED cocktail to human lymphoblasts and myotubes. As in KI mice, we observed successful correction of the splicing abnormality. The corrected *fukutin* mRNA was restored to 50% or more of the levels seen in normal controls (Fig. 3c). We believe this to be sufficient recovery, considering that unaffected FCMD carriers have only 50% of normal *fukutin* mRNA. Finally, we tested recovery of the fukutin protein and the glycosylation of  $\alpha$ -DG in FCMD patients' cells. Not only was normal fukutin protein expression significantly rescued in AED-treated lymphoblasts (Fig. 4d), we observed recovery of normally glycosylated  $\alpha$ -DG in AED-treated myotubes (Fig. 4e). Immunofluorescence staining also showed immensely increased glycosylated  $\alpha$ -DG (Fig. 4f). A laminin clustering assay showed increased laminin clustering ability, which is characteristically absent in  $\alpha$ -DGpathy<sup>19</sup> (Fig. 4f). These data show that AED treatment effectively rescues normal fukutin, confirming our observation of abnormal *fukutin* splicing and raising the possibility of splicing modulation therapy as the first treatment for FCMD. To treat neuronal migration disorder of FCMD, prenatal treatment may be necessary, but it is currently difficult for ethical and technical reasons. Nevertheless, improving even only the muscular symptom would greatly ameliorate quality of life of the patients as well as the patients' families.

Retrotransposons account for nearly half of the human genome<sup>20</sup>. Increased numbers of reports have highlighted positive and negative contributions of retrotransposons to human health and disease<sup>21,22</sup>. In addition to being the causative factor for FCMD, ARH, and NLSDM, SVA insertions have also been implicated in hereditary elliptocytosis, X-linked agammaglobulinemia, neurofibromatosis type 2, and X-linked dystonia-parkinsonism<sup>12,23-26</sup>. It has been suggested that SVA insertions cause such diseases through genomic deletion, reduced mRNA expression, or skipping of neighboring exons<sup>17,22</sup>. Recently, SVA splicing has been suggested to generate variation within and across species by activating functional 3' splice sites within SVAs across human genome, controlling gene transcription, creating alternative splicing by exon-trapping, or inducing premature stop codons, and was experimentally demonstrated<sup>6</sup>. Our findings underscore the importance of SVA functions in human disease and support the possibility of radical treatment against SVA-induced disease by splicing modulation therapy. AONs have become one of the most promising and practical candidate chemicals for splicing modulation therapy in cancer<sup>27</sup>, infectious diseases<sup>28</sup> and Duchenne muscular dystrophy<sup>29,30</sup>. In demonstrating the ability of AONs to rescue fukutin function in FCMD, we introduce a novel clinical role for AONs in treating FCMD and other SVA-mediated diseases, while providing new insights about the influence of SVAs on human evolution, development, and disease.

## Methods

### Antisense oligonucleotides

Twenty-five-mer 2'OMePS (GeneDesign and Invitrogen) and VMO oligonucleotides (GeneTools) were designed to target potential splice-modulating sequences of SVA-inserted *fukutin*, including a splicing acceptor site, a splicing donor site, ESEs, and ISEs as follows: A1, CCGTGGGAAGGAGACTGTGGAGGGAG; A2, GGAGACCGTGGAAGGAGACTGTGGA; A3, AGAGGGAGACCGTGGAAGGAGACTG; E1, CACCGTCCAGCCTTGGCTCGGCATC; E2, CTGCAGTGAGCCGAGATGGCAGCAG; E3, GAGGCAGGAGAATCAGGCAGGGAGG; E4, GAAAACCAGTGAGGCGTAGCAGGCT; D1, CAGGTCTTACCATAGTTGGCTTCAA; D2, CAGGAATCTTCCAGGTCTTACCATA; D3, GAGCGCTTCCAGTCCCACGTCTTTA; D4, TCCATTGGGTTGCACATTGGGAGGA; D5, CATCCCACTCAGAAATAGGCCAGAT; DYS, GGCCAAACCTCGGCTTACCTGAAAT<sup>31</sup>. U (uracil) was used instead of T (thymine) for the synthesis of 2'O-MePS oligos. Target sequences are shown in Supplementary Fig. 12. ESE sites were predicted by ESEfinder 3.0 (<http://rulai.cshl.edu/cgi-bin/tools/ESE3/ese finder.cgi>), and ISE sites were predicted by ACESCAN2 (<http://genes.mit.edu/acescan2/index.html>). AONs were solubilized in sterile distilled water.

### Animals and cells

All mouse experimental protocols were approved by the Ethics Review Committees for Animal Experimentation at Osaka University Graduate School of Medicine and Kobe University Graduate School of Medicine. FCMD KI model mice and the mouse nomenclature have been described previously<sup>16</sup>. The transgenic alleles containing normal and SVA-inserted human exon 10 were named Hn and Hp, respectively: Hp/Hp is homozygous for the SVA allele; Hn/Hn is homozygous for the normal allele; Hp/+ and Hp/- are SVA carriers and compound heterozygotes for the SVA and knock-out alleles, respectively. The ages of mice used in experiments varied from two to six months. The mouse ES cell line carrying the SVA-inserted human genomic *fukutin* exon 10 was generated from Hp/Hp mice. The ES cell line carrying a *fukutin* knock-out allele (KO) has been described previously<sup>32</sup>. The commercially available mouse ES cell line AB2.2 was used as a control. Human lymphoblasts were obtained from FCMD patients with homozygous SVA insertions and unaffected individuals. Human primary myoblasts were derived from muscle biopsies from FCMD and unaffected patients. Human primary fibroblasts were obtained by skin biopsy from ARH and NLSM patients. Human autopsy brain samples were obtained from FCMD (fetus and 34-year-old) and DMD patients (34-year-old). Chimpanzee brain sample was kindly provided by GAIN (Great Ape Information Network, Japan). Human brain RNA was purchased from Clontech. All clinical samples were used with the approval of Human Ethics Review Committees of Osaka University Graduate School of Medicine and Kobe University Graduate School of Medicine.

## Myoblast differentiation

Myoblast cells were maintained at 37 °C and 5% CO<sub>2</sub> in DMEM medium plus 20% fetal bovine serum, 2.5 ng/ml of basic fibroblast growth factor (Sigma), and a 0.5% penicillin-streptomycin-amphotericinB mix (Wako). Myotubes were obtained from confluent myoblast cultures following 10 to 14 days of serum deprivation and replacement with 2% FBS.

## RNA isolation, RT-PCR, qRT-PCR, and sequencing

To inhibit NMD, CHX (100 µg/ml) (Sigma) was added to the culture medium 24 hours prior to RNA isolation. For RT-PCR and for qRT-PCR, total RNA was extracted using the RNeasy Plus Mini kit (Qiagen), and cDNA was obtained using the Superscript III One-step RT-PCR system (Invitrogen) with random primers, following the manufacturer's instructions. SYBR Pre-mix Ex Taq (Takara) was used for qRT-PCR, and expression values were normalized to *gapdh* as an internal control for mRNA quantity. Data were obtained from triplicate experiments. To detect abnormally-spliced RT-PCR products from FCMD, ARH, and NLSDM patients, and from human brain *AB627340* cDNA, long range PCR was performed using LA Taq with LA Taq Buffer II (Takara), adding dimethyl sulfoxide and 7-deaza-dGTP (Roche). The RT-PCR products were directly sequenced (FCMD and NLSDM), or cloned with the TOPO TA Cloning Kit (Invitrogen) before sequencing (ARH and *AB627340*). To calculate the expression ratio in Fig. 1a, Supplementary Figs. 4, 5, 9, 10, and 13 the value in the mutant sample was divided by the value in the normal sample, as measured by qRT-PCR. To identify AON target sequences, we designed three primers to distinguish recovered transcripts from unrecovered transcripts by AON treatment (Fig. 3a). Similarly, we designed three primers to compare expression amount of SVA-trapped to SVA-untrapped transcripts of the *AB627340* gene (Supplementary Fig. 11a). One primer on SVA in Fig. 3a and Supplementary Fig. 11a was within *Alu*-like domain, and the sequence was 5'-GAAAACCAGTGAGGCGTAGC-3'. To calculate the percent recovery of normal mRNA processing in Figs. 3b, 3c, and Supplementary Fig. 13, the value of treated sample was divided by that of normal samples, as measured by qRT-PCR at sequence position 1341, where the authentic stop codon resides. Primer sequences for qRT-PCR and RT-PCR are available upon request.

## Northern blot analysis

Previous attempts to detect *fukutin* mRNA in FCMD patients by Northern blot analysis have been unsuccessful<sup>2</sup>, likely because the predicted mRNA sequence is the same size as abundant ribosomal RNA. Moreover, the tertiary structure of *fukutin* mRNA is presumably complicated due to the immensely GC-rich SVA sequence. Therefore, we performed Northern blot analysis of FCMD and control mRNA after treatment to remove abundant ribosomal RNA and strong denaturation to untangle the *fukutin* transcript. Total RNA (1 mg) was extracted from human lymphoblasts, mouse ES cells, mouse brain, and mouse skeletal muscle using TRIzol (Invitrogen). Oligotex-dT30<Super> (Takara) was used to extract more than 3 µg of poly-A RNA. Ribosomal RNA was removed using Ribo-Minus (Invitrogen). Stronger denaturation of RNA was achieved by incubating polyA-RNA samples with a combination of 0.8 M glyoxal and 50 % DMSO in 10 mM sodium phosphate buffer (pH 7.0) for 60 minutes at 55 °C. Three-µg poly-A RNA was loaded on the agarose

gel. A *fukutin* cDNA clone covering the *fukutin* coding sequence was <sup>32</sup>P-labeled and used as a probe.

### cDNA expression constructs

The normal *fukutin* cDNA encodes full-length *fukutin* protein. The spliced *fukutin* construct encodes abnormal *fukutin*, as shown in Supplementary Fig. 7. The truncated *fukutin* construct lacks the C-terminal 38 amino acids. All constructs encoded FLAG epitope tags fused to the C-terminus of the expressed protein.

### Cell transfection

HeLa S3 cells and C2C12 cells were transfected with normal *fukutin* construct, spliced *fukutin* construct, and truncated *fukutin* construct using FuGENE 6 (Roche). *Fukutin* localization was determined using immunocytochemistry two days after transfection. For transfection of AONs, 2'OMePS were introduced into various cell lines, including mouse ES cells, human myoblasts and human lymphoblasts, using Lipofectin (Invitrogen).

### Detection of endogenous *fukutin* protein

The polyclonal rabbit anti-*fukutin* antibody RY213 recognizes the peptide CLKIESKDPRLDGIDS, and the polyclonal goat-anti-*fukutin* antibody 106G2 recognizes full-length *fukutin* protein lacking the N-terminal hydrophobic domain. Endogenous *fukutin* was detected via immunoprecipitation using 106G2, from cell or tissue lysates containing 5 to 10 mg of total protein in lysis buffer (1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 20 mM Tris-Cl, pH 7.5, and 150 mM NaCl), followed by Western blot analysis using affinity-purified RY213.

### Immunofluorescence and Western blot analysis

Cells were washed and fixed with 4% paraformaldehyde in PBS. The following primary antibodies were used: anti-GM130 (monoclonal, BD Bioscience), anti-KDEL (monoclonal, Stressgen), anti-FLAG (rabbit polyclonal, MBL), anti- $\alpha$ -DG (monoclonal, I1H6C4 and VIA4-1, Millipore), and anti-laminin (rabbit polyclonal, Sigma). To stain nuclei, 4',6-diamidino-2-phenylindole (DAPI, Sigma) was added to the secondary antibody solution at a final concentration of 1 ng/ml. Cells were observed under fluorescence confocal microscopy (Carl Zeiss). Western blot analysis and laminin overlay assays were performed as described previously<sup>16</sup>.

### Mutagenesis analysis

We made the four *fukutin* constructs: pHn, human normal *fukutin* construct consisting of exon 2-9 cDNA and genomic normal exon 10; pHp, patient *fukutin* construct consisting of exon 2-9 cDNA and genomic patient exon 10 with SVA insertion; pSpl, patient *fukutin* construct pHp which lacks the abnormally-spliced region; pAcc, patient *fukutin* construct pHp with AG to GG replacement at the acceptor site within SVA sequence. These constructs were transfected into HeLa S3 cells using Effectene (Qiagen). After extraction of poly-A RNA by Oligotex, Northern blot analysis was performed using 2  $\mu$ g of poly-A RNA for each sample with stronger denaturation mentioned above.

### AON treatment of FCMD model mice

For intramuscular injection, a percutaneous injection of cardiotoxin (10  $\mu$ M) (Latoxan) was performed into TA (0.3 nmol) and calf (0.7 nmol) of Hp/+, Hp/–, Hp/Hp, and Hn/Hn mice on Day 0 (n=3 for each genotype). On Days 1, 4, and 7, VMO (400 mg/Kg) solubilized in sterile distilled water was injected. AED and Dys were administered to the left legs and the right legs, respectively. For systemic injection, an intraperitoneal injection of Butorphanol tartrate (5 mg/Kg) (Bristol-Myers Squibb) was performed on Day 0. VMO (20 mg/Kg) solubilized in 5 % glucose solution was administered by intravenous injection via tail vein on Days 1 and 7 (n=4 for Hp/–, n=2 for Hp/+). Mice were sacrificed on Day 21, and total RNA or protein lysate was isolated from each tissue for further analyses of *fukutin* mRNA expression, fukutin protein translation, and glycosylation of  $\alpha$ -DG.

### AON treatment of human patient cell lines

For protein analysis, VMO cocktails (AED and Dys) were introduced into FCMD lymphoblasts and normal control lymphoblasts at a final concentration of 2.5  $\mu$ M in culture medium using a Gene Pulser II Electroporator (0.25-kV voltage, 960- $\mu$ F capacitance, with 0.4 cm gene pulser cuvettes, giving a time-constant readout of ~40 msec) (Bio-Rad) (n=2). For glycosylation analysis, VMO cocktails (AED and Dys) were introduced into FCMD patient myoblasts and normal control cells by direct addition to the culture medium at a final concentration of 4  $\mu$ M (n=2). Following incubation for 48 hours, cells were collected, and total RNA or protein lysate was isolated.

### Laminin clustering assay

The AED cocktail was introduced into myotubes by direct addition to the culture medium at a total concentration of 4  $\mu$ M following a medium change on Day 2. On Days 10 to 14, mouse EHS laminin-1 (Sigma) was added with fresh medium at a concentration of 1.0 nM and incubated for 30 minutes, followed by immunocytochemistry.

### SVA sequence analysis

SVA sequence was aligned to the SVA reference sequence present in Repbase (<http://www.girinst.org/repbase/update/index.html>)<sup>33</sup> and the location on the SVA reference of the splicing acceptor and donor sites in SVA was determined.

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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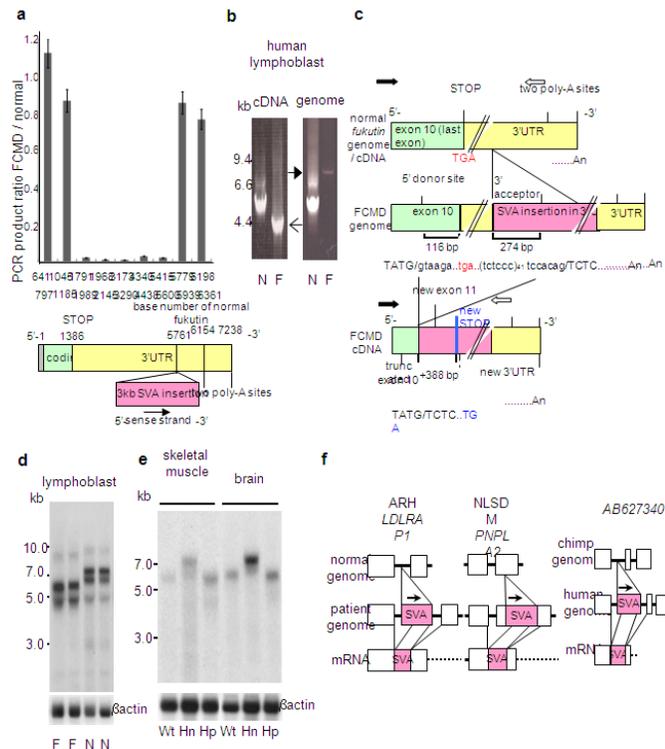
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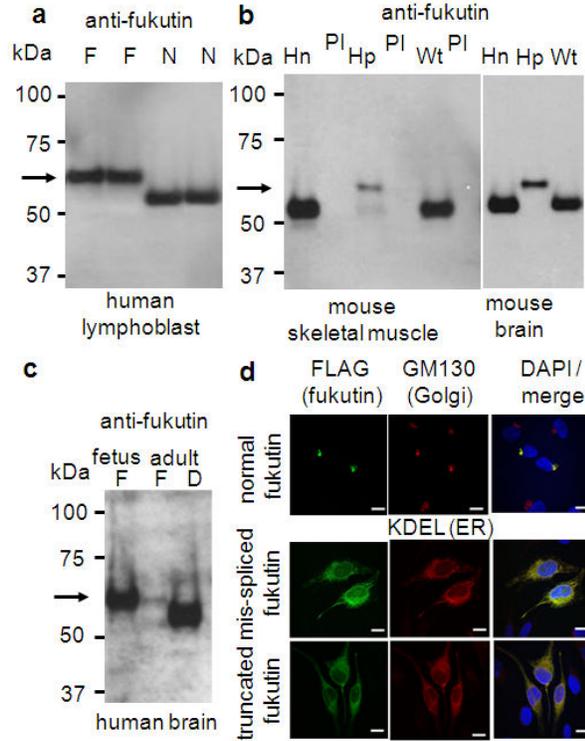
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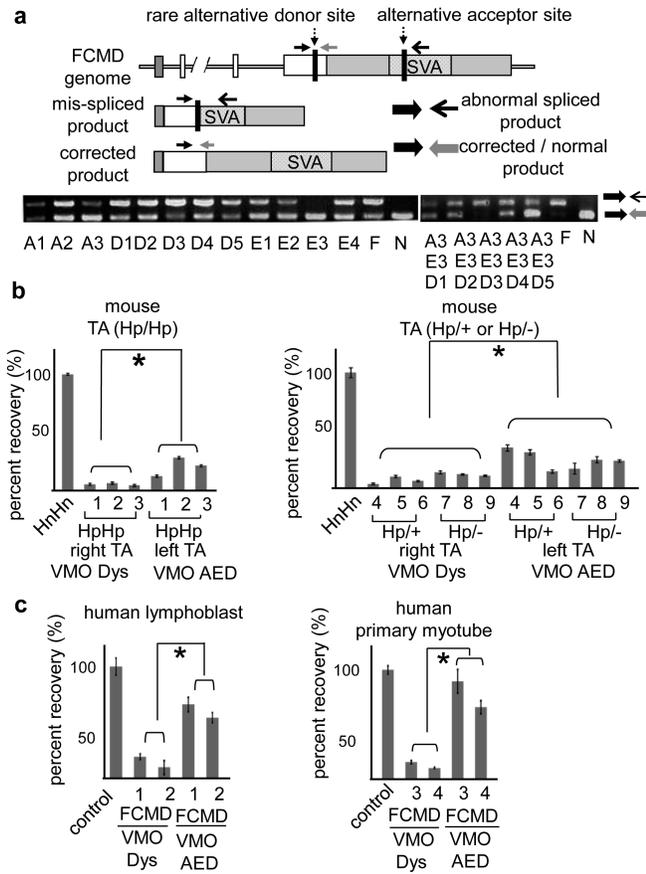
**Figure 1. An SVA retrotransposal insertion induces abnormal splicing in FCMD**

**a**, Expression analysis of various regions of *fukutin* mRNA in lymphoblasts. Gray bar, the ratio of RT-PCR product in FCMD patients relative to the normal control; Numbers on the X axis, nucleotide positions of both forward and reverse primers in *fukutin*. Error bars, s.e.m. **b**, Long range PCR using primers flanking the expression-decreasing area (nucleotide position 1061 to 5941) detected a 3-kb PCR product in FCMD lymphoblast cDNA (open arrow) and 8-kb product in FCMD genomic DNA (closed arrow). In the normal control, cDNA and genomic DNA both showed 5-kb PCR products. The 8-kb band was weak probably because VNTR region of SVA is GC-rich (82%). **c**, Schematic representation of genomic DNA and cDNA in FCMD. Black and white arrows, forward and reverse sequencing primers. The intronic sequence in FCMD is indicated in lower case. The authentic stop codon is colored in red, and the new stop codon is colored in blue. **d**, **e**, Northern blot analysis of *fukutin* in human lymphoblasts (**d**) and model mice (**e**). F, FCMD; N, normal control. The wild-type mouse *fukutin* mRNA was detected at a size of 6.1 kb. Both skeletal muscle (left) and brain (right) showed smaller, abnormal bands (open arrows) in Hp/Hp mice. Wt, wild type; Hn, Hn/Hn mice; Hp, Hp/Hp mice. **f**, Schematic representation of genomic DNA and cDNA in ARH (*LDLRAP1*, left), NLSM (*PNPLA2*, middle), and human (*AB627340*, right).



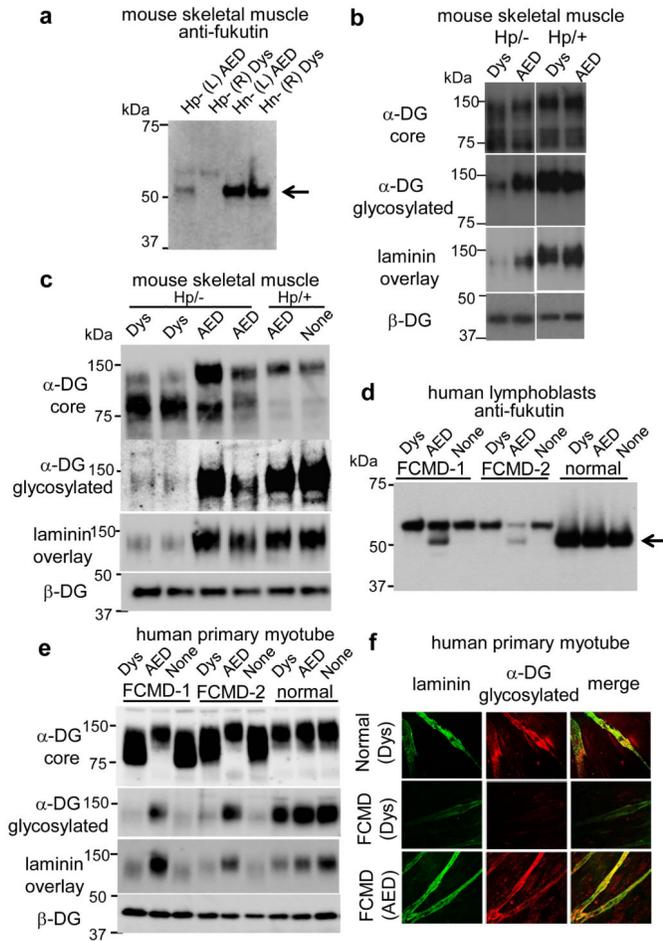
**Figure 2. Abnormal fukutin protein in FCMD**

**a-c**, Immunoprecipitation analysis of fukutin protein in human lymphoblasts (**a**), both skeletal muscle and brain tissues from Hp/Hp mice (**b**), and brain tissue from FCMD patients (**c**). Closed arrow, abnormal fukutin. N, normal sample; F, FCMD patient sample. Hn, Hn/Hn mice; Hp, Hp/Hp mice; PI, preimmune serum. D, Duchenne muscular dystrophy (DMD) patient. **d**, The subcellular localization of fukutin. Top, normal fukutin; middle, mis-spliced fukutin; bottom, truncated fukutin. Stained with anti-FLAG (Left, to detect fukutin), anti-GM130 (middle, Golgi marker, top) and anti-KDEL (ER marker, middle and bottom), and merge (right, with DAPI stain). Scale bar, 10  $\mu$ m.



**Figure 3. AON cocktail rescues normal *fukutin* mRNA**

**a**, RT-PCR diagram of three primers designed to assess normal *fukutin* mRNA recovery (upper). Black closed arrow, a common forward primer located on *fukutin* coding region; black open arrow, a reverse primer to detect the abnormal RT-PCR product (161 bp); gray closed arrow, the other reverse primer to detect the restored normal RT-PCR product (129 bp). The effect on Hp/Hp ES cells treated with each single or a cocktail of AONs (lower). F, FCMD; N, normal sample. **b**, Rescue from abnormal splicing in VMO-treated in Hp/Hp mice and Hp/+ mice. Local injection of AED cocktail into TA (n=3). Dys, a negative control. **c**, Rescue from abnormal splicing in VMO-treated human FCMD lymphoblasts (left, n=2) and myotubes (right, n=2). The Y axis shows the percent recovery of normal mRNA (\* p < 0.01 by Student's t-test). Error bars, s.e.m.



**Figure 4. AON cocktail treatment rescues normal fukutin protein and functional  $\alpha$ -DG**

**a, d**, Immunoprecipitation analysis of fukutin protein after local treatment with VMO (AED) in FCMD model mice (**a**) and human FCMD lymphoblasts (**d**). Arrow, normal fukutin protein. L, left TA; R, right TA. Dys, negative control. **b, c, e**, TA muscle after local (**b**) or systemic (**c**) AED treatment and human FCMD lymphoblasts treated with the AED (**e**) were analyzed by Western blot using antibodies against  $\alpha$ -DG core protein (top panel) and glycosylated  $\alpha$ -DG (second), and by a laminin overlay assay (third). Bottom,  $\beta$ -DG (internal control). **f**, Laminin clustering assay. Left, anti-laminin; middle, anti-glycosylated  $\alpha$ -DG; right, merged images. Upper, normal myotubes treated with control VMO; middle, FCMD patient myotubes treated with control VMO; bottom, FCMD patient myotubes treated with AED.