Mouse fukutin deletion recapitulates dystroglycanopathy phenotypic variability and impairs dystroglycan processing downstream of *O*-mannose phosphorylation.

Aaron M Beedle[1,2], Amy J Turner[1], Yoshiaki Saito[1], John D Lueck[1], Steven J Foltz[2], Marisa J Fortunato[2], Patricia M Nienaber[1] and Kevin P Campbell[1*]

Supplementary Materials

Supplementary Methods:

Generation of a Novel Dystroglycanopathy Mouse Model

Fukutin targeting construct.

A floxed fukutin (*Fktn*) targeting construct was generated from *Fktn* genomic fragments and assembled with a 5' LoxP + flirted PGK-neo cassette and a 3' LoxP sequence flanking exon 2, with intron 1 and intron 2 sequences for up- and downstream homology arms, respectively (Suppl. Fig. 1A). Briefly, *Fktn* fragments were amplified by PCR from mouse 129sve ES cell genomic DNA using Herculase DNA polymerase (Stratagene) (for PCR primers, see Suppl. Table 1). A blunted exon 2 fragment was inserted upstream of loxP in subcloning vector pBYloxP and intron 2 was inserted downstream of loxP via XhoI/SalI-KpnI. The exon2/loxP/intron2 fragment was inserted into pBY49 downstream of loxP/frt/PGKneo/frt with KpnI-BamHI. Lastly, intron 1 was inserted upstream of the floxed *Fktn* exon 2 via NotI-XhoI/SalI. The completed targeting vector was linearized for electroporation into 129sve ES cells by the U. Iowa Gene Targeting Facility. pBYloxP and pBY49 were gifts from Baoli Yang (University of Iowa, Iowa City, IA).

Supplementary Table 1: Fukutin Targeting Construct Cloning Primers

Fktn intron 1 F	aaaaaaaaacgcggccgcTTACCATCACCCTGTCTACC
Fktn intron 1 R	aaactcgAGGAGAGCACCATGGCAAGAAG
Fktn exon 2 F	TTCTTGCCATGGTGCTCTCC
Fktn exon 2 R	aaagaattcACTGACTCCAGCCCAGTATC
Fktn intron 2 F	tttctcgagGGTTGGAGTCGTAGCAGTG
Fktn intron 2 R	aaaggtaccGTGTTATCAGCAAGGGGAAG

Floxed fukutin mouse line.

Two lines of ES cells with targeted homologous recombination at the *Fktn* chromosome 4 locus were identified by PCR screening (cell lines DT101 and DT736; see Suppl. Table 2 for screening primers). ES cells were injected into mouse C57BL/6J blastocysts using standard protocols (U. Iowa Gene Targeting Facility); germline transmission of the floxed *Fktn* allele was confirmed by PCR genotyping for one strain (Suppl. Table 2). $Fktn^{L/+}$ mice were bred to the FLPeR strain (JAX #003946) on a C57BL/6J background to remove the neomycin selection cassette. Due to a high frequency of mosaicism (>99%) in heterozygotes, mice were bred to FLPe homozygosity to achieve germline PGK-neo removal. FLPe recombination was confirmed by genotyping PCR for the presence of the FLPe recombined band and the absence of the neo band (Suppl. Table 2). Following neo cassette removal, the FLPe gene was bred out of the floxed

fukutin line. $Fktn^{L/+}$ or $Fktn^{L/L}$ mice at C57BL/6J backcross 4 or 5 were used to generate knockouts for experiments.

A fukutin null allele was also generated from the floxed fukutin strain. $Fktn^{L/+}$ mice were bred to $ZP3^{cre/+}$ (JAX #003651). Female $Fktn^{L/+}$, $ZP3^{cre/+}$ progeny were bred to C57BL/6J males to generate $Fktn^{+/-}$, $ZP3^{cre/+}$ pups with germline deletion of floxed *Fktn* exon 2. The ZP3 cre allele was subsequently bred out of the colony. The fukutin null allele strain was maintained by heterozygote breeding ($Fktn^{+/-}$ x $Fktn^{+/-}$); no $Fktn^{-/-}$ pups were ever born from these crosses demonstrating embryonic lethality of germline fukutin disruption (Suppl. Fig. 1D).

Supplementary Table 2: Screening and Genotyping Primers

Fktn ES upstream screen F	CCAGCTCCGTGTTCAGTTTGTATTT
Fktn ES upstream screen R	GTGAGACGTGCTACTTCCATTTGTC
Fktn ES downstream screen F	TCATAGCCTGAAGAACGAGATCAGC
Fktn ES downstream screen R	TAAGCCTGCACAGATAAAGCTGGAA
Fktn Flox F	GAAGTCAAGATACTGGGCTG
Fktn Flox R	ATGACAACGTTCCCCTCAAG
Fktn FLPe recombination F	TCTTTAGTCAGCGTGGTCAGAA
Fktn FLPe recombination R	AGCTTGGGGGGATCCTAGGAA
Fktn Cre recombination F	TCTTTAGTCAGCGTGGTCAGAA
Fktn Cre recombination R	GCAAGGGCCAAGAGGATATCTA
Cre F (Lewandoski et al., 1997)	TGATGAGGTTCGCAAGAACC
Cre R (Lewandoski et al., 1997)	CCATGAGTGAACGAACCTGG
TgMCK-Fktn F	CTTTAGACAAAAGGTTTTGCCCTCC
TgMCK-Fktn R	TCCCTTTGGATTTTGATGAACC

Fukutin conditional knockout mice.

For whole animal inducible fukutin deletion, Tamoxifen-cre mice (JAX strain #004682) were bred to fukutin null mice and then crossed into the floxed strain for inducible knockout mouse ($Tg^{cre\text{-}Esr1}$, $Fktn^{L/-}$, iKO) generation: $Tg^{cre\text{-}Esr1}$, $Fktn^{+/-}$x $Fktn^{L/L}$ or $Tg^{cre\text{-}Esr1}$/$Fktn^{+/-}$ x $Fktn^{L/+}$. Fktn iKO mice were at backcross 5 (Fig. 1, Supp. Fig. 2) or backcross 6 (Suppl. Fig.2) on C57BL/6J. Whole animal fukutin deletion was achieved by two doses of tamoxifen (0.2 mg/kg body weight, Sigma) delivered by gavage at 6 weeks of age.

For muscle specific fukutin deletion, MCK-cre and myf5-cre mouse lines were used (1,2). Early skeletal muscle specific Cre recombinase expression (E8) was achieved by myf5-cre, which turns on during myoblast proliferation to initiate *Fktn* gene excision in knockout mice ($Myf5^{cre/+}$, $Fktn^{L/L}$ = Myf5-cre/Fktn KO; breeding $Myf5^{cre/+}$/$Fktn^{L/+}$ x $Fktn^{L/L}$ of $Fktn^{L/+}$). Mice with glycosylation defect in striated, differentiated muscle initiated at E17 ($MCK^{cre/+}$,$Fktn^{L/L}$ = MCK-cre/Fktn KO) were generated by breeding $Fktn^{L/+}$/$MCK^{cre/+}$ x $Fktn^{L/L}$ or $Fktn^{L/+}$. All Myf5-cre/Fktn and MCK-cre/Fktn study mice were at backcross 4 on C57BL/6J.

Mice were genotyped using standard protocols; oligonucleotide primers are listed in Supplementary Table 2.

Generation of Early Muscle Fukutin Knockout Mice with Fukutin Re-expression

Transgenic mice with fukutin expression in striated muscle.

A mouse fukutin cDNA fragment with optimized Kozak initiation sequence was cloned from BC017539 (Open Biosystems). Site-directed mutagenesis was used to correct a mutation in the original IMAGE clone, leucine at amino acid 38, to match proline 38 encoded by the mouse fukutin genomic sequence. The fukutin fragment was assembled in pBSSKII with the MCK promotor/enhancer region and the SV40 polyadenylation sequence (Suppl. Fig. 4A; pBSSKII-MCK was a gift from Jeff Chamberlain (University of Washington, Seattle, WA)). The transgene was liberated using SpeI and KpnI for microinjection into B6SJL mouse embryos by the University of Iowa Transgenic Animal Facility. Nine of twelve founders had stable germline transmission of the MCK-fukutin transgene; however, one line was discontinued due to poor breeding.

Validation of fukutin transgenic mice

The eight TgMCKfktn mouse lines were categorized for fukutin expression by Western blot on skeletal muscle total microsomes (custom Rbt 257). Fukutin was undetectable in one strain and weakly detectable in three strains. An additional three strains showed moderate fukutin signals while the final strain strongly expressed the transgene protein. Our antibody was not suitable for immunofluorescence, so transgenic fukutin localization could not be determined. However, no abnormalities were detected in muscle histology or dystroglycan glycosylation. Two lines, one with moderate fukutin expression (47539-2), and one with strong expression (47274-1), were selected for colony maintenance. The TgMCKfktn 47539-2 line (C57BL/6J BC2) was used for breeding Tg x Myf5-Fktn mice.

Transgenic x knockout mice

TgMCKfktn 47539-2 mice (backcross 2, C57BL/6J) were mated to $Fktn^{L/+}$ mice (backcross 4, C57BL/6J). This Tg x Fktn colony was maintained by breeding $TgMCKFktn^{Tg/+}$, $Fktn^{L/+}$ mice to pure $Fktn^{L/L}$ mice. $TgMCKFktn^{Tg/+}$, $Fktn^{L/L}$ mice were bred to $Myf5^{cre/+}$, $Fktn^{L/+}$ mice to generate knockout mice expressing the fukutin transgene ($TgMCKFktn^{Tg/+}$, $Myf5^{cre/+}$, $Fktn^{L/L}$ = Tg x KO). Tg x KO, KO and Tg littermates were analyzed as described for the muscle-specific fukutin knockout colonies.

Treadmill/Exercise Protocol

Mice were challenged with horizontal running using a 4 lane mouse treadmill (Accuscan). Mice were placed on the treadmill with a 0.3 Amp shock platform and allowed to warm-up for 5 minutes at 3 meters/min. The running protocol followed with 5 min at 10m/min, 5 min at 15m/min, 5 min at 20m/min and up to 15 min at 25m/min (30 min maximum run time). Mice were removed from the treadmill at any point in the protocol if they reached "exhaustion". This was defined as resting on the shock platform for at least 10 seconds. Arterial tail bleeds were taken at 2 hr post-exercise to obtain "exercised" serum creatine kinase measurements.

Cardiotoxin Muscle Damage and eMHC Fiber Area Analysis

Mice (16 to 17 wks old) were anesthetized with isoflurane and lower hindlimbs were shaved. Calf muscles were injected three timeswith 25 μl of vehicle (left calf) or 25 μl of 10 μM cardiotoxin (CTX, right calf, Accurate Chemical & Scientific Corp) in 0.9% sterile saline. Mice

were monitored for recovery from anesthetic and returned to housing. Mice were euthanized for saline and CTX-treated tissue collection 7 days or 4 weeks post-injection.

Muscle regeneration was assessed using saline and CTX-treated cryosections stained for embryonic myosin heavy chain (eMHC) antibody (Developmental Studies Hybridoma Bank). Adjacent pictures were taken to collect images of all eMHC-positive(eMHC+) fibers (20x magnification; Olympus X71 microscope). eMHC+ fibers were analyzed with Image-Pro Express (v6.3, MediaCybernetics) as follows: 1) the calibration scale was set to the image scale bar; 2) images were adjusted to optimize contrast; 3) each eMHC+ fiber was selected using the measurement polygon tool (wand or trace);and 4) autocalculated area measurements were collected for each eMHC+ fiber and exported for graphing/analysis. Individual fiber areas for all eMHC+ cells in each cryosections were pooled according to genotype (littermate, KO; 2 mice per group) and treatment (saline left leg or CTX right leg). The variation of eMHC+ fiber areas was nonparametric (D'Agostino& Pearson omnibus normality test failed) so statistical significance between genotypes was measured using a Mann-Whitney two-tailed T-test (GraphPad Prism). Saline-treated littermates were not tested because they had no active regeneration (3 or fewer eMHC+ fibers per group).

Bin size for plotting the frequency distribution of eMHC+ fiber areas was estimated using the equation $h = (3.5\sigma)/(n^{1/3})$, where h is the optimized bin width, σ is the standard deviation, and n is the number of observations (3); h=64and 75μm^2 for the smallest and largest CTX treatment groups. Therefore, all eMHC+ fiber area measurements were binned from 0 to 75μm^2, 75 to 150μm^2, 150 to 225μm^2, 225 to 300μm^2, 300 to 375μm^2, 375 to 450μm^2 and >450μm^2. The number of measurements per bin was counted in each group and normalized to the total number of eMHC+ observations for the specific group and plotted (GraphPad Prism). Analysis of eMHC+ fibers from untreated 20 week old myf5-Cre/Fktn (E8) and MCK-Cre/Fktn (E17) KO iliopsoas muscles was performed identically.

Biochemical Analyses

Dystroglycan phosphate bond cleavage by aqueous hydrofluoric acid (HFaq) has been described (4). Briefly, PHOS-bead void fractions (without phosphate buffer) were dried by vacuum concentration at 45°C. Samples and HFaq (Sigma) were precooled on ice. 100µL ice cold HFaq (HF+) or ddH_2O (HF-) was added using appropriate safety controls for HF use. Samples were mixed gently and incubated overnight at 4°C. HFaq was removed from cold samples under a stream of N_2 gas. Samples were washed with ddH_2O and all samples (HF+ and HF-) were redried by vacuum concentration at 45°C. Proteins were resuspended in 5xLSB for SDS-PAGE.

For total microsome preparation ($TgMCK^{Fktn}$ mice), homogenized skeletal muscle (buffer: 20mM $NaH_2PO_4 \cdot H_2O$, 20mM $Na_4P_2O_7 \cdot 10H_2O$, 1mM $MgCl_2$, 0.303M sucrose, 0.5mM EDTA, pH7.1 + PIs) was spun for 23 min at 14,000g. Supernatants were centrifuged for 37 min at 142,000g. Microsome pellet was resuspended in Buffer I (20mM Tris pH7.0, 0.303M sucrose + PIs).

RT-PCR

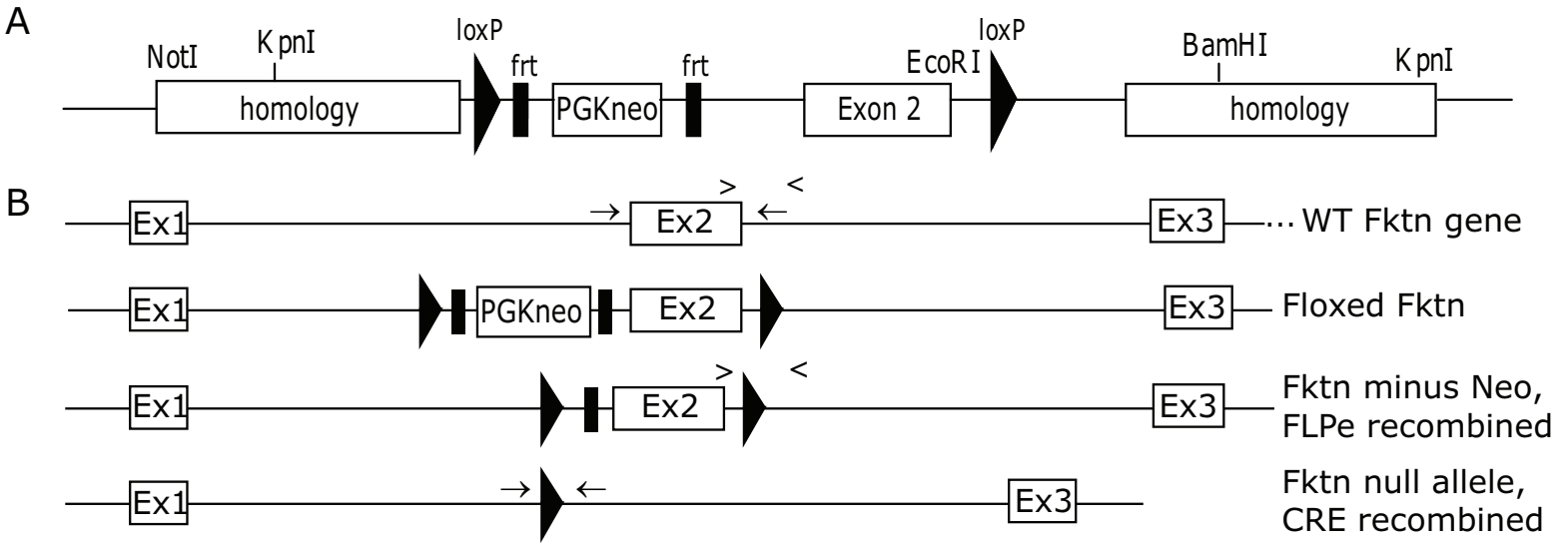
Primers for RT-PCR are shown in Supplementary Table 3.

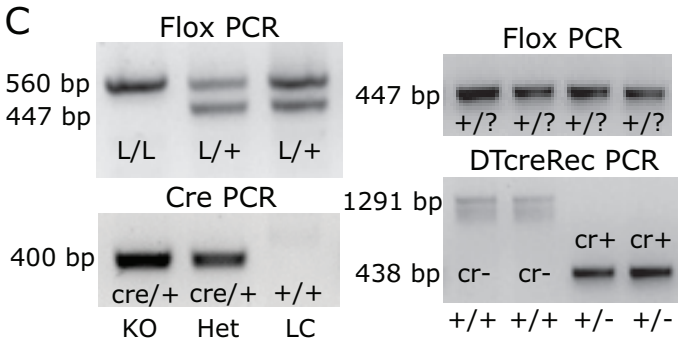
Supplementary Table 3: RT-PCR primers

RT-PCR Fktn exon 1 F	GCCTCCGCCGCAGCTCTA
RT-PCR Fktn exon 4 R	CGGGCCTTGAGAAGCATTTTT

References

1. Bruning JC, Michael MD, Winnay JN, et al. A muscle-specific insulin receptor knockout exhibits features of the metabolic syndrome of NIDDM without altering glucose tolerance. *Mol Cell.* Nov 1998;2(5):559-569.
2. Tallquist MD, Weismann KE, Hellstrom M, Soriano P. Early myotome specification regulates PDGFA expression and axial skeleton development. *Development.* Dec 2000;127(23):5059-5070.
3. Scott DW. On optimal and data-based histograms. *Biometrika*. 1979; 66(3):605-610.
4. Yoshida-Moriguchi T, Yu L, Stalnaker SH, et al. O-mannosyl phosphorylation of alpha-dystroglycan is required for laminin binding. *Science.* Jan 1 2010;327(5961):88-92.





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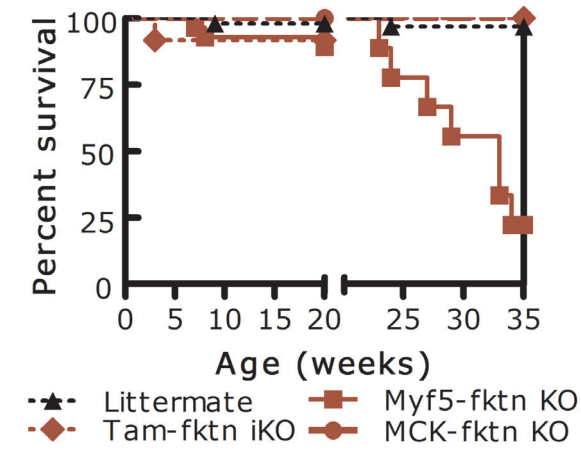
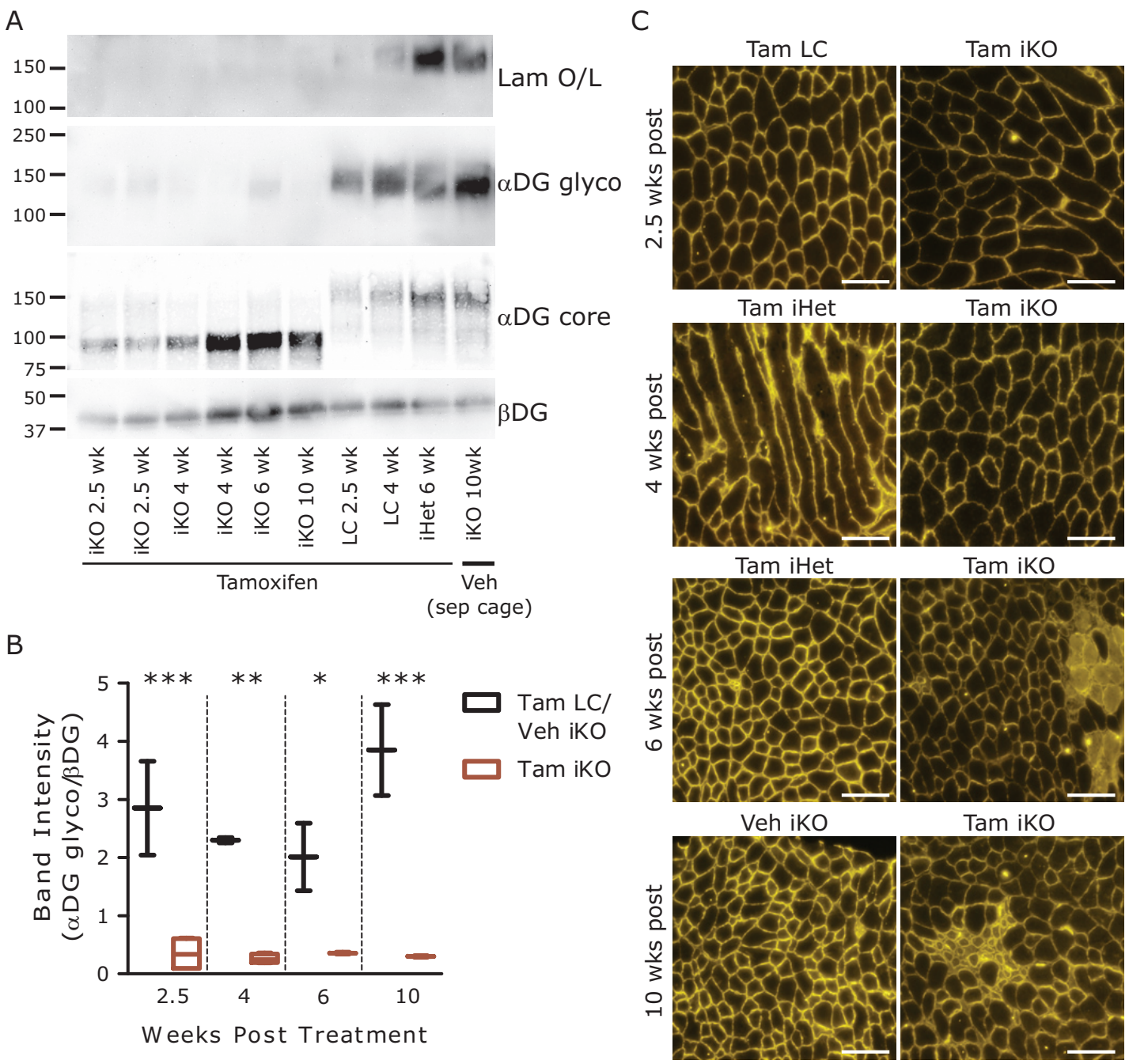
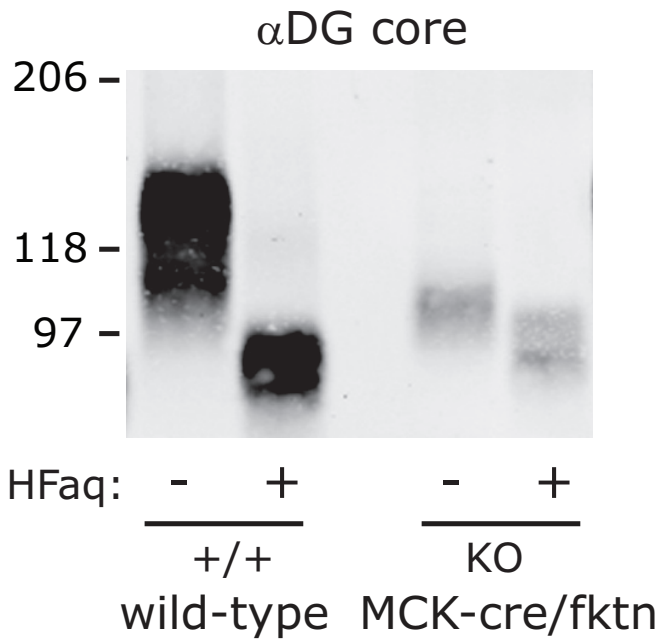
Colony breeding	genotype	% Exp	% Act	n
Fktn +/- x +/-	-/-	25	0***	95
Fktn L/+ x L/+	L/L	25	16*	126
Tam-cre, Fktn +/- x Fktn L/L	iKO	25	23	93
MCK-cre, Fktn L/+ x Fktn L/L	KO	25	20**	141
Myf5-cre, Fktn L/+ x Fktn L/L	KO	25	18**	173



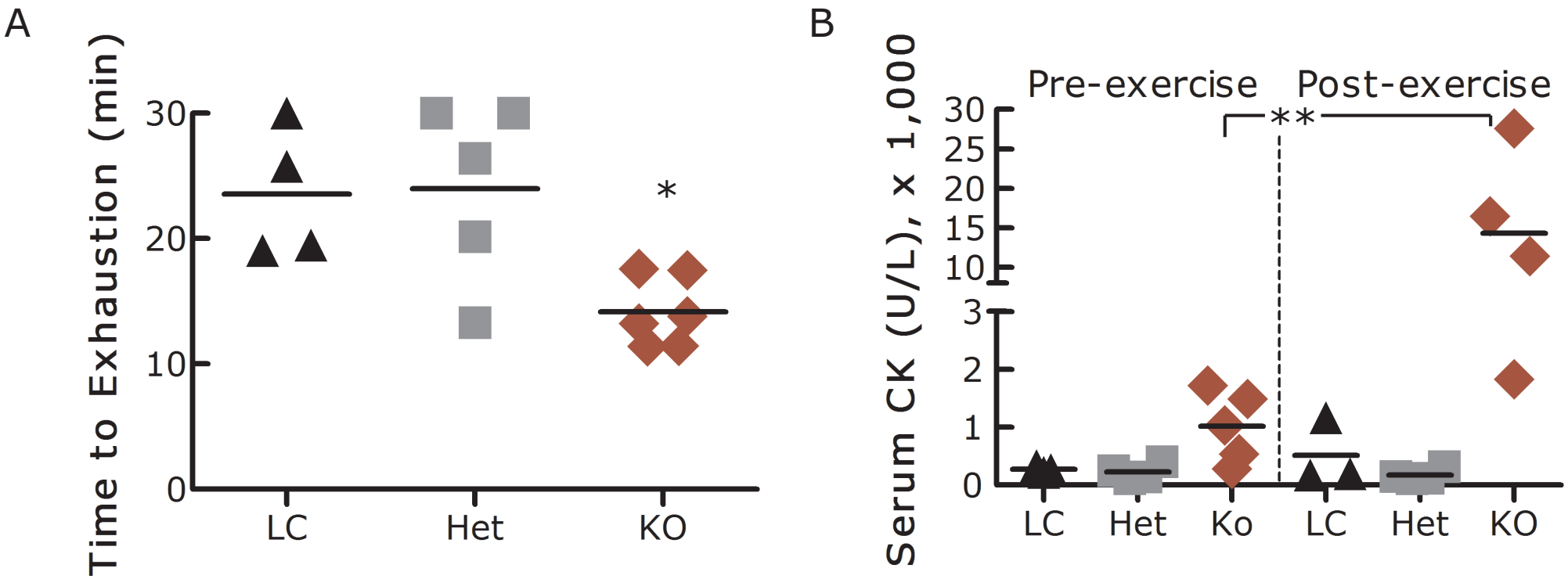
Supplementary Figure 1: Conditional fukutin knockout mice. A, A schematic view of the fukutin targeting construct for ES cell recombination. The floxed region contains a flirted neo cassette followed by Fcmd exon 2. Flanking regions of introns 1 and 2 were added to facilitate homologous recombination. B, Wild-type and targeted alleles are shown. The initial targeted gene included the entire floxed region with neo cassette (Floxed Fktn). The flirted neo cassette was removed by flippase recombination (Fktn minus Neo). A strain with germline removal of exon 2 was also generated to create a fukutin null allele (Fktn null allele). Open arrowheads indicate primer sites for floxed genotyping; arrows indicate primer sites for detection of the null cre recombined allele. C, Sample genotyping results show wild-type (+), floxed (L), and null (-, cr+) fukutin alleles and presence (cre) or absence of cre (+). D, Breeding statistics for pure floxed fukutin (minus neo), fukutin null allele, and conditional knockout colonies are shown. % Exp is the percentage expected from Mendelian inheritance; % Act is the actual percentage of mice with the genotype in the colony; n is the number of pups used for the analysis. Chi square tests indicate that inheritance in the floxed fukutin (X^2=8.39, P<0.05), fukutin null (X^2=27.94, p<0.001), and muscle specific knockout colonies (MCK-cre/Fktn X^2=15.05, p<0.002; Myf5-cre/Fktn X^2=13.93, p<0.005) is not Mendelian (Tam-cre/Fktn X^2=2.09, p>0.5). E, An informal survival curve based on post hoc analysis of deaths or required humane ethanasias of experimental mice. In attempts to age mice to 20 wks, 1 littermate and 3 Myf5-fktn knockout mice died (littermate n=45; Myf5-Fktn KO n=28; MCK-Fktn KO n=19; Tam-Fktn iKO n=13; left side). In attempts to age mice past 20 wks, Myf5-Fktn KO mice had very poor survival with most mice dying in the 20-30 wk age range (n=9). While fewer mice were aged from other colonies, deaths were rare (littermates, n=23; MCK-Fktn KO, n=5;Tam-Fktn iKO, n=4).



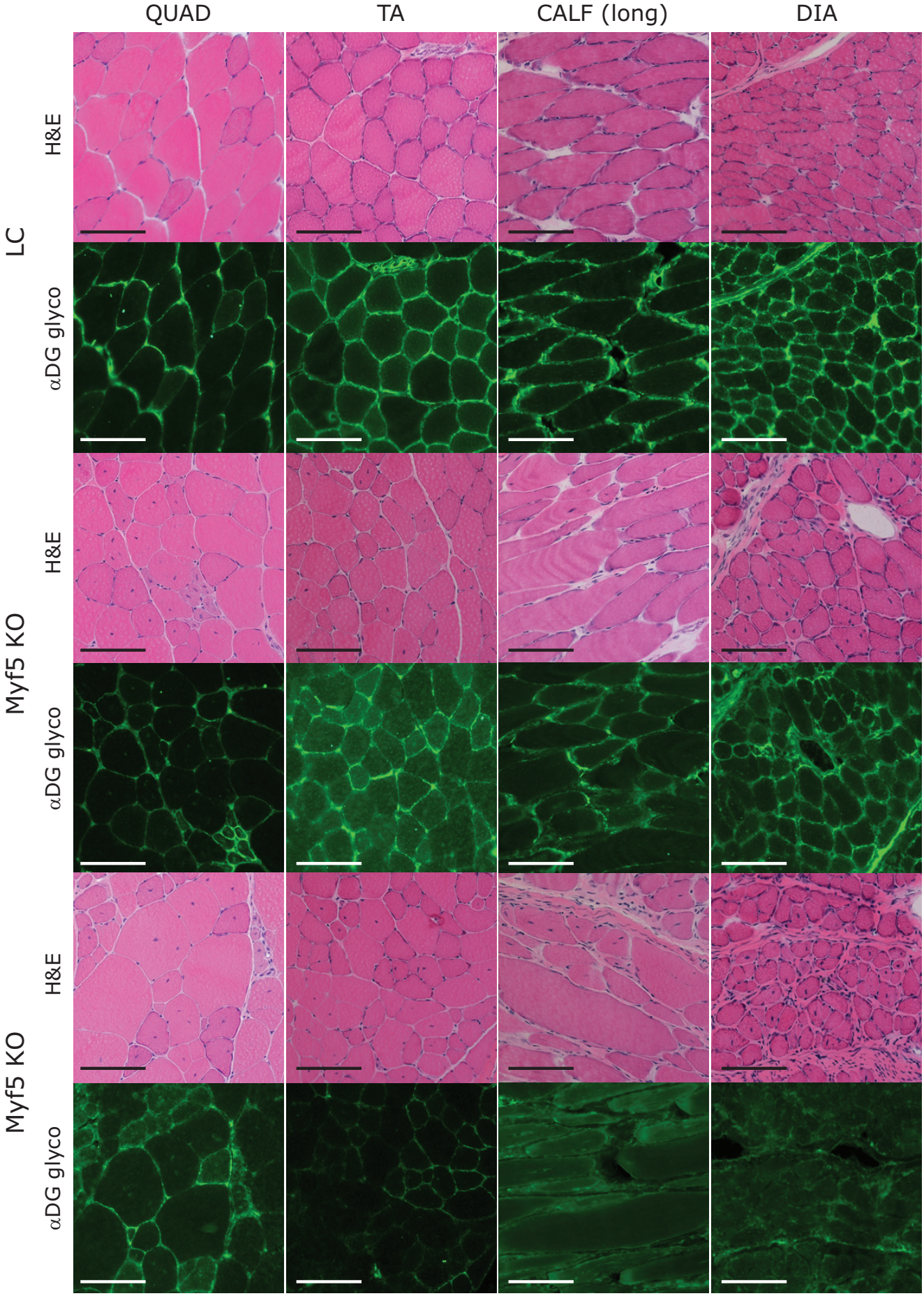
Supplementary Figure 2. Inducible, whole animal fukutin deletion disrupts dystroglycan glycosylation as early as 2.5 weeks post-tamoxifen. Tamoxifen inducible Fktn KO mice ($Tam^{cre+}/Fktn^{L/-}$, iKO; $Tam^{cre+}/Fktn^{L/+}$, iHet; $Tam^{wt}/Fktn^{L/+}$, LC) were treated twice by oral gavage with tamoxifen or vehicle (veh). In this study, vehicle- and tamoxifen-treated littermates were separated for 7 days beginning just prior to the first oral gavage to prevent tamoxifen exposure of vehicle-treated mice. A, Western blots of WGA enriched hindlimb skeletal muscle at various weeks post treatment. Dystroglycan functional glycosylation and molecular mass were measured by laminin overlay (Lam O/L), glyco specific antibody IIH6 (αDG glyco) and core protein antibody Gt20 (αDG core). Abnormal dystroglycan glycosylation was evident at all time points tested (2.5 to 10 weeks post treatment). B, Western blot band densitometry was used to determine the ratio of glycosylated αDG to βDG. Glyco αDG was significantly reduced in tamoxifen treated iKO WGA enriched muscle at all time points tested (one-way ANOVA, Bonferroni's multiple comparisons test; 2.5wk Tam LC vs Tam iKO, $p<0.001$; 4wk Tam LC vs Tam iKO, $p=0.001$ to 0.01; 6wk Tam iHet vs Tam iKO, $p=0.01$ to 0.05; 10wk Veh iKO vs Tam iKO, $p<0.001$). C, Glycosylated αDG is variably reduced, but still detectable by immunofluorescence in iliopsoas muscle at 2.5 to 10 weeks post-tamoxifen. 20x magnification, scale bars indicate 100μm. Six and 10 wk post-treatment groups were first dosed at 6 weeks old; colony BC6 C57BL/6J. Two and a half and 4 wk post-treatment groups were dosed at 14 and 17, or 14 and 28 weeks old, respectively; colony BC5 C57BL/6J.



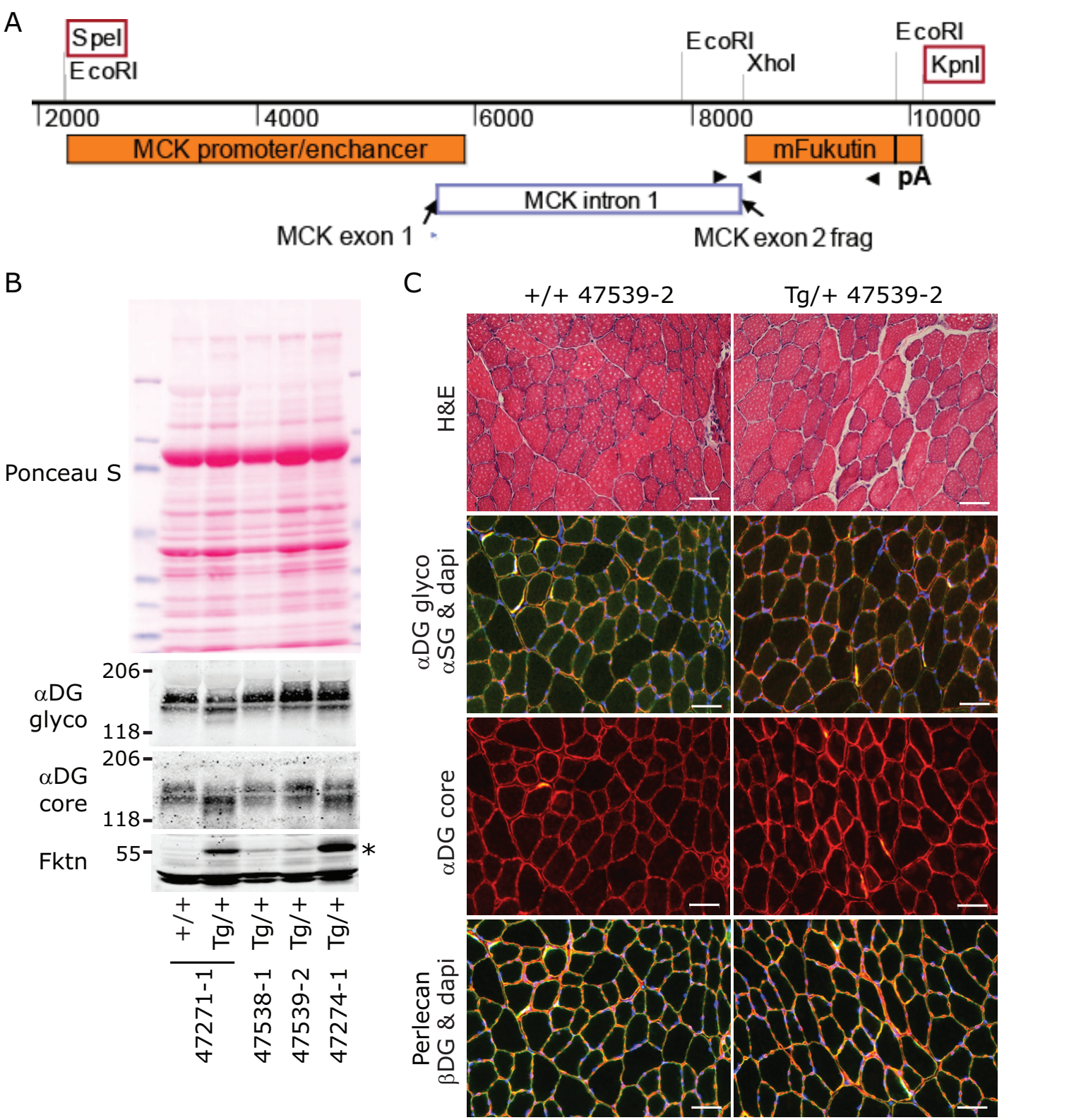
Supplementary Figure 3: Aqueous hydrofluoric acid treatment of PHOS-bead void αDG. WGA enriched solubilized skeletal muscle was applied to PHOS-beads (see Fig. 2). The PHOS-bead void fraction was collected and treated overnight with cold aqueous hydrofluoric acid (HFaq, +) or sham control (-). Samples were separated by SDS-PAGE and Western blotted with the αDG core antibody. Both hypoglycosylated αDG from MCK-cre/Fktn knockout mice and wild-type αDG have reduced molecular weight with HFaq treatment indicating that a phosphate bond has been cleaved. Numbers indicate molecular weight in kDa.



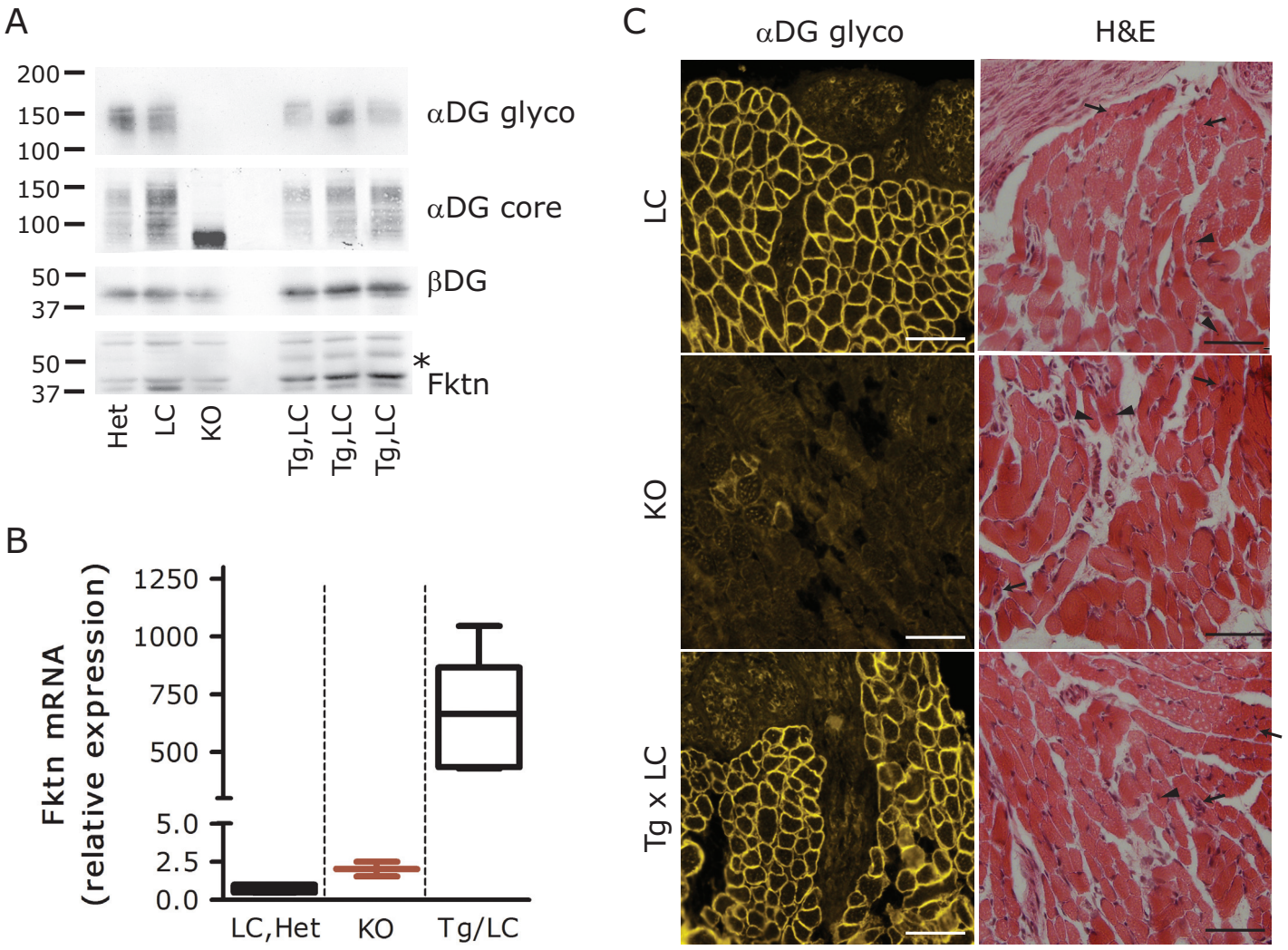
Supplementary Figure 4: Exercise sensitivity of MCK-cre/Fktn mice with fukutin deletion at embryonic day 17 in differentiating muscle. A) MCK-cre/Fktn knockout mice and littermates were run in an exercise protocol on a horizontal treadmill until exhaustion (warmup: 3 m/min, 5 min; run: 10 m/min for 5 min, 15 m/min for 5 min, 20 m/min for 5 min, 25 m/min for 15 min (maximum run time of 30 min)). Run times were recorded and plotted according to genotype. Comparison of LC vs KO and Het vs KO was significant (p=0.01 to 0.05), LC vs Het was not significant (one-way ANOVA, Tukey post-test). B) When possible, serum creatine kinase activity was measured 24 hr pre-run and 2 hr post-run to assess exercise-induced muscle damage. Black triangles show littermate controls, grey squares indicate heterozygotes, and red diamonds denote knockout mice from the MCK-cre/Fktn mouse line. We compared pre-exercise vs post-exercise for each genotype (one-way ANOVA, Bonferroni post-test of selected pairs). Exercise increased serum CK in MKC-cre/Fktn KO mice (p=0.001 to 0.01), but not in Het or LC mice.



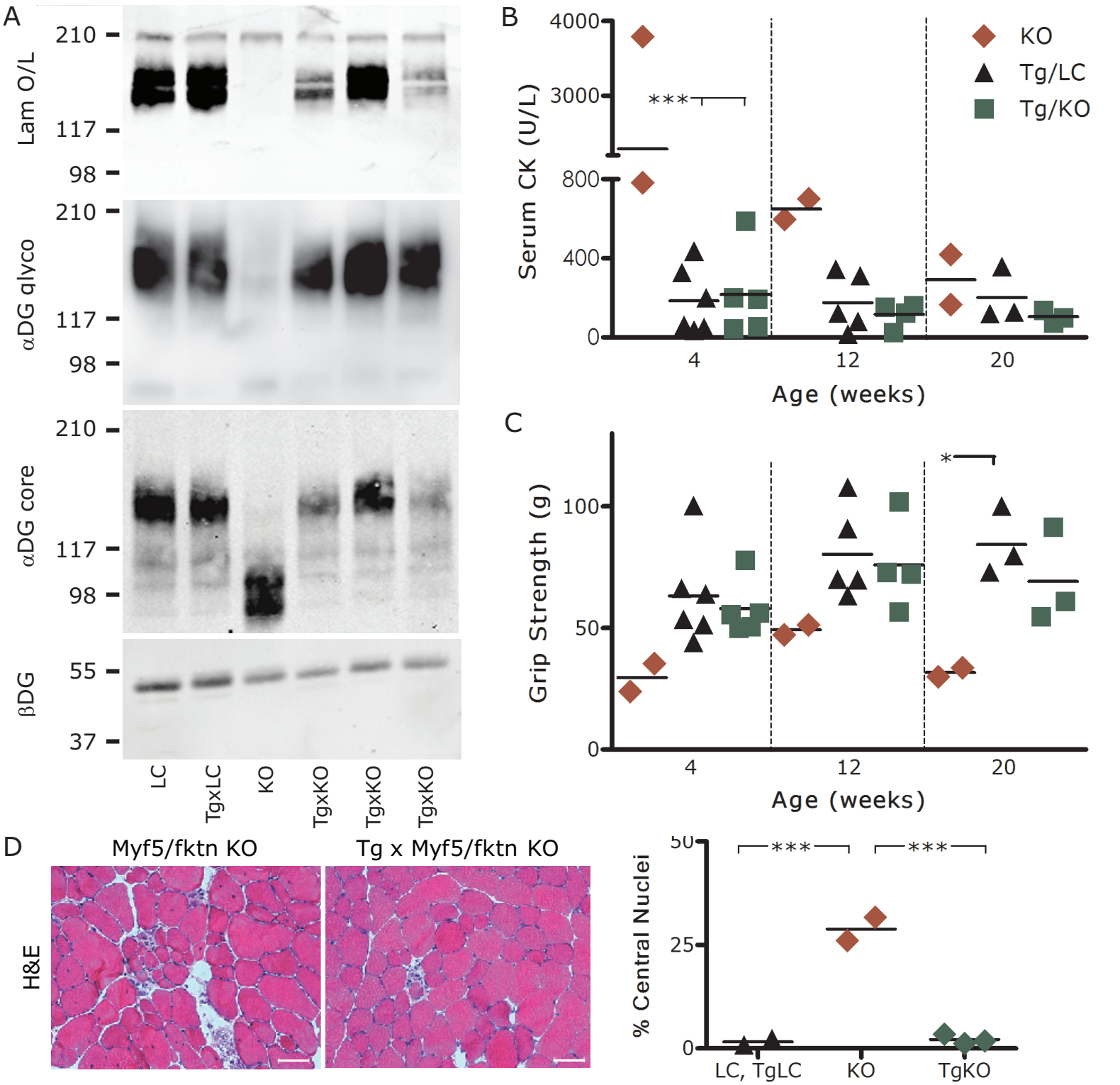
Supplementary Figure 5. Fukutin deletion initiated by Myf5-cre at embryonic day 8 causes dystrophy in all skeletal muscles tested. Histological analyses of quadriceps (Quad), tibialis anterior (TA), calf (in longtitudinal section), and diaphragm (DIA) muscles from 20 wk old littermate (LC) and knockout (KO) mice show distrophic features (H&E) and patchy or absent staining with IIH6 αDG glyco antibody. Tissues are from the same mice as in Fig. 4A; 20x magnification, scale bar indicates 100μm.



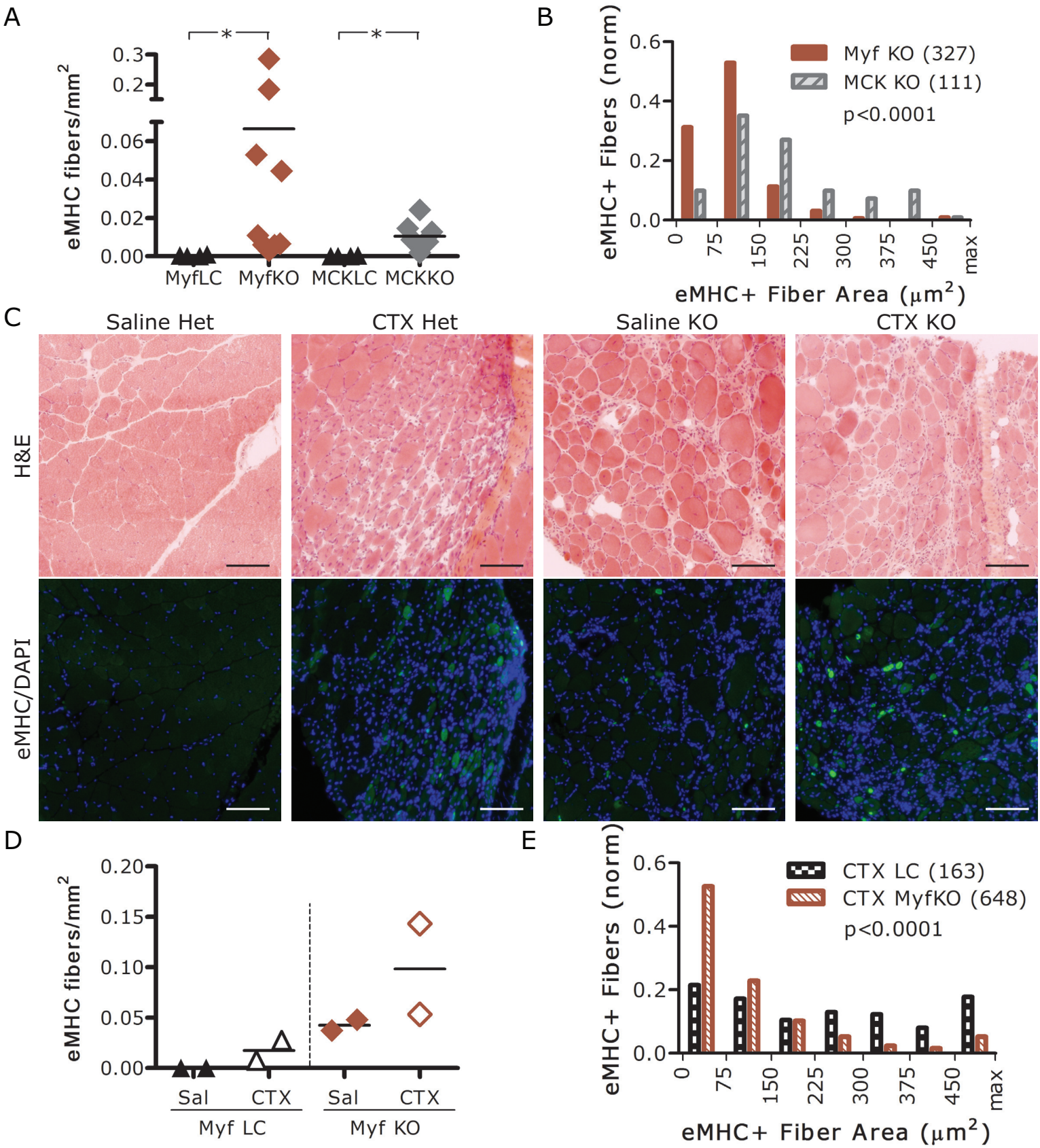
Supplementary Figure 6: A novel transgenic strain with fukutin expression initiated at embryonic day 17 in differentiating muscle, TgMCK-Fktn. A) Schematic of the transgenic fukutin construct design driven by the mouse muscle creatine kinase (MCK) promoter. B) Skeletal muscle total microsomes of littermate (+/+) and transgene expressing (Tg/+) mice from different founder strains were Western blotted for glycosylated αDG, αDG core protein (αDG core, αDG glyco) and fukutin (Fktn, Rbt 257); the Ponceau S stained blot is shown for sample loading. Fukutin protein was strongly detected in 47271-1 and 47274-1 strains, weakly detected in 47538-1 and 47539-2 strains. Molecular weights are shown in kDa. C) H&E and immunofluorescence staining of 10 week old iliopsoas muscles from littermate or Tg/+ mice of the 47539-2 TgMCK-Fktn line for αDG (αDG core, αDG glyco), βDG, α-sarcoglycan (α-SG) and dapi nuclear stain. No differences were detected between +/+ and Tg/+ mice of any strain (other strains not shown); 20x magnification, 50μm bar.



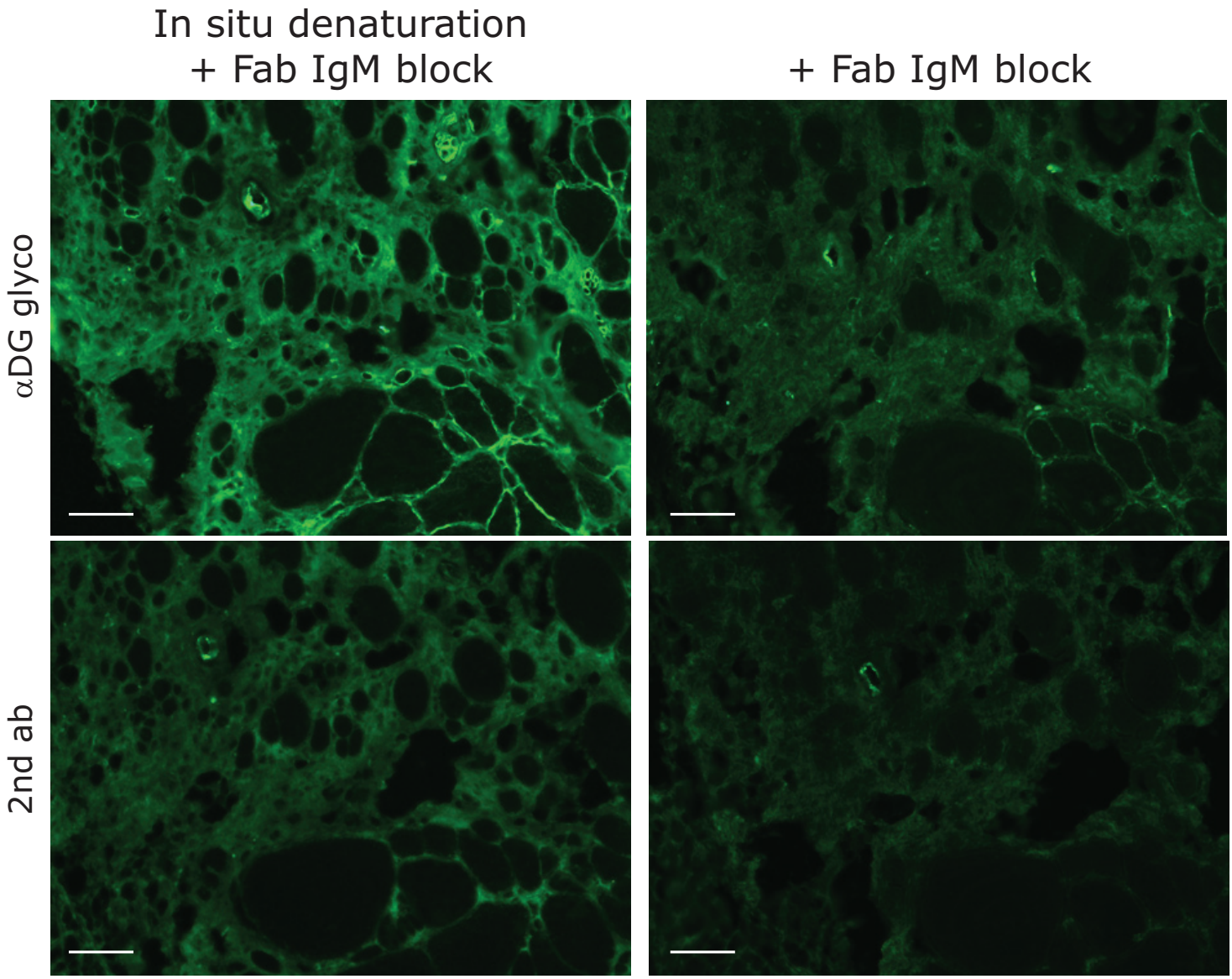
Supplementary Figure 7. Fukutin transgene expression (TgMCK-Fktn) precedes the onset of overt necrosis in Myf^{cre+}/Fktn$^{L/L}$ mice. Postnatal day 8 (PD8) pups from the Tg-MCK-Fktn x Myf5-cre/Fktn colony were analyzed for transgene expression and dystroglycanopathy. No transgene expressing KO mice (Tg/KO) were present in the litters examined. However, littermate (LC, het), transgene expressing littermate (Tg/LC) and Myf5-cre KO (KO) mice were obtained. A, Skeletal muscle total microsomes were tested for α-dystroglycan glycosylation (αDG glyco) and core protein (αDG core), β-dystroglycan (βDG), and recombinant fukutin (Fktn, Rbt 257). αDG glycosylation and molecular weight was reduced in KO mice and fukutin protein was increased in Tg/LC mice (asterisk) at PD8. Numbers indicate molecular weight in kDa. B, Fukutin transcript expression (endogenous or transgene) was detected by quantitative PCR of calf muscle RNA from PD8 pups. Fktn mRNA was normalized to housekeeping gene Oaz1 in triplicate for each sample (dCt). Individual pup dCt values were normalized to a littermate control sample (ddCt) and converted from log phase to determine the ratio of gene expression relative to littermate control (relative expression). At PD8, Fukutin mRNA is elevated more than 600-fold in transgenic mice (Tg/LC) compared to endogenous expression (LC,het, n=2; KO, n=1; Tg/LC, n=3; technical replicates=2). C, αDG glycosylation (αDG glyco) was disrupted in iliopsoas muscle of KO mice at PD8. However, groups of small fibers (arrows) and centrally nucleated fibers (arrowheads) were detected in muscle from all genotypes suggesting that these features are indicative of the pup age rather than evidence of accrual of dystrophic features at this time point (H&E staining). 40x magnification, scale bar indicates 50μm. Note, in this model, skeletal muscle Fktn gene knockout is initiated at embryonic day 8 (E8), and muscle Fktn transgene expression is initiated at E17.



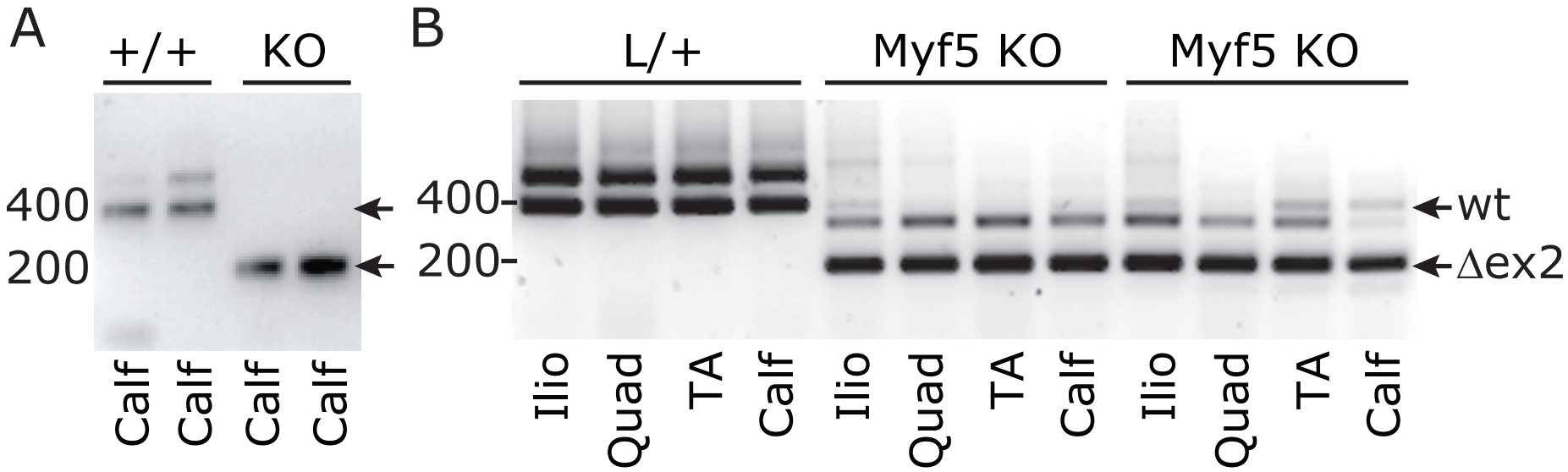
Supplementary Figure 8: Transgenic fukutin gene replacement in muscle at at embryonic day 17 (E17) prevents dystrophic disease in Myf5-cre/Fktn E8 knockout (KO) mice. A) WGA-enriched muscle was prepared as in Fig. 2 from TgMCK-Fktn x Myf5-cre/Fktn mice. Laminin overlay (Lam O/L), αDG protein and glycosylation (αDG core, αDG glyco) and βDG are shown in KO mice with the fukutin transgene (Tg x KO); littermate control (LC); Transgenic LC (Tg x LC); and Myf5-cre/Fktn KO (KO). Molecular weights are shown in kDa. B, C) Serum creatine kinase (CK) and forelimb grip strength measured as in Fig 4. Transgene expressing KO mice (Tg/KO, green squares) were not different from transgenic LC mice (Tg/LC, black triangles). KO littermates (red diamond) are shown for comparison (*** $p<0.001$ serum CK 4 wk KO vs. Tg/LC or Tg/KO; * $p=0.01$-0.05, grip strength 20 wk KO vs. Tg/LC; all other age-matched pairs $p>0.05$; Bonferroni post-test). D) H&E staining of 20 wk iliopsoas from KO mice with or without the transgene (left; 20x magnification, 50μm scale bar) and centrally nucleated fibers (% CN, right). CN is not different between Tg/KO and Tg/LC mice (LC/TgLC vs. TgKO $p>0.05$; ***LC/TgLC or TgKO vs. KO $p<0.001$; Tukey post-test).



Supplementary Figure 9: Embryonic myosin heavy chain (eMHC) variation in myf5-cre (E8) KO mice. A,B, Analysis of eMHC positive fibers in 20wk iliopsoas from littermate and KO mice of myf5-cre/Fktn (E8) and MCK-cre/FKTN (E17) mice (LC n=4; KO n=8, both groups). Both myf5 and MCK KO mice show a dystrophic increase in eMHC fibers (LC vs KO, p= 0.01-0.05), but there are more eMHC fibers in severe myf5 KO mice (A). eMHC fibers are smaller in myf5 KO than MCK KO mice (B, p<0.0001). C,D,E, Calf muscle 7 days post-cardiotoxin (CTX) injury in littermate and myf5-cre KO mice. Myf5 KO muscle can induce a regenerative response (C,D, increased eMHC with CTX treatment; 20x, 100μm bar), but eMHC fiber size is smaller in the KO than littermate mice (E, p<0.0001 n=2 mice per group). The total pooled number of eMHC fibers in the area analyses are indicated in parentheses.



Supplementary Figure 10: In situ denaturation for antigen retrieval prior to immunofluorescence may enhance background fluorescence and the apparent sarcolemmal αDG signal of dystrophic muscle. Myf5-cre/Fktn knockout muscle (iliopsoas, 20 wk) was processed for immunofluorescence with or without an in situ protein denaturation to improve signal detection. Slides were then incubated with mouse IgM F(ab) fragment to block signal from endogenous mouse antibodies in the section prior to incubation with antibody IIH6 to recognize glycosylated αDG (αDG glyco). Denaturation enhanced the glycosylated αDG signal but also increased background autofluorescence. The culmination of these effects likely causes some overestimation of αDG glycosylation in knockout mouse sections. Primary antibody was omitted as an experimental control (2nd ab). 20x magnification, 50μm scale bar.



Supplementary Figure 11: Analysis of fukutin exon 2 deletion. Primers in exons 1 and 4 were used to amplify the Fktn exon 2 region by RT-PCR from 20 wk old (A) or 27 wk old (B) muscle RNA for littermate (+/+ or L/+) or Myf5-cre/Fktn embryonic day 8 (E8) skeletal muscle knockout (KO) mice. Expected band sizes are 407 bp for wild-type transcripts (wt) and 209 bp for transcripts with exon 2 deleted (Δex2). Alternatively spliced bands were also amplified in some samples. Notably, the wild-type transcript was absent from most KO muscle samples. Detection of residual wild-type mRNA in a few KO muscles indicates a low level of incomplete cre excision or, more likely, detection of non-muscle-derived fukutin transcript (e.g. peripheral nerve). Abbreviations are: iliopsoas, ilio; quadriceps, quad; tibialis anterior, TA; gastrocnemius + soleus, calf.