DOC2B promotes insulin sensitivity in mice via a novel KLC1-dependent mechanism in skeletal muscle

Jing Zhang[1,2], Eunjin Oh[1], Karla E. Merz[1], Arianne Aslamy[1,3], Rajakrishnan Veluthakal[1], Vishal A. Salunkhe[1], Miwon Ahn[1], Ragadeepthi Tunduguru[1,4] and Debbie C. Thurmond[1]

1. Department of Molecular and Cellular Endocrinology, Diabetes and Metabolism Research Institute, Beckman Research Institute of City of Hope, 1500 E. Duarte Road, Duarte, CA 91010, USA

2. Present address: Anwita Biosciences Inc, San Carlos, CA, USA

3. Department of Cellular and Integrative Physiology, Indiana University School of Medicine, Indianapolis, IN, USA

4. Department of Diabetes Complications and Metabolism, Diabetes and Metabolism Research Institute, Beckman Research Institute of City of Hope, Duarte, CA, USA

Karla E. Merz and Arianne Aslamy contributed equally to this study.

Table of Contents

ESM Methods

Materials, antibodies and plasmid constructs. Mouse anti-human DOC2B was purchased from Abnova (1:1000 dilution, Taipei, Taiwan, cat# 400008447-B01P). Rabbit anti-DOC2B, to detect the endogenous rat DOC2B protein in L6 myoblasts, was purchased from Proteintech (1:1000 dilution, Rosemont, IL, USA, cat# 20574-1-AP). Mouse anti-DOC2B, to detect the recombinant DOC2B protein in L6-GLUT4-myc myoblasts, was purchased from Biolegend (1:1000 dilution, San Diego, CA, USA, cat# 833201). Rabbit anti-green fluorescent protein (GFP) (1:5000 dilution) (cat# ab6556) and rabbit anti-KLC1 (1:1000 dilution, cat# ab187179) were purchased from Abcam (Cambridge, MA, USA). Rabbit anti-cMyc, goat anti-GLUT4 and donkey anti-goat-horseradish peroxidase (secondary antibody) were purchased from Santa Cruz Biotechnology (1:1000 dilution, Dallas, TX, USA). The goat anti-rabbit-horseradish peroxidase and anti-mouse-horseradish peroxidase secondary antibodies were purchased from Bio-Rad (Hercules, CA, USA). IRDye 700 goat anti-mouse IgG was purchased from LICOR (Lincon, NA, USA). All antibodies were validated using specific blocking peptide. Anti-GFP-conjugated agarose beads were purchased from Abcam (cat# ab69314) and anti-Myc-conjugated beads were from Thermo Fisher (cat# 20168, Waltham, MA, USA). The enhanced chemiluminescence (ECL) kit and ECL prime kit were purchased from Amersham Biosciences (Pittsburgh, PA, USA). Insulin (Humulin R) was obtained from Eli Lilly and Co. (Indianapolis, IN, USA).

The green fluorescent protein (GFP)-tagged *Doc2b-GFP* and *C2AB-GFP* plasmids were kindly provided by Dr. Uri Ashery (Tel-Aviv University, Tel-Aviv, Israel). Full-length rat *Doc2b* and *C2AB* (encoding residues 125–412) of rat *Doc2b* were cloned into the 5' *Kpn1* and 3' *BamH1* restriction sites of the pEGFP-N2 vector [1]. The backbone pEGFP-N2 plasmid was used as control. The *Doc2b-GFP-Y301* was generated using site-directed mutagenesis of the

Y301 to F301 (Genescript, Piscataway, NJ, USA). *hDOC2B-GFP* was constructed by inserting human *DOC2B* cDNA into the pEGFP-N2 plasmid and *hDOC2B-GFP-Y301F* was made by mutating Y301 to F301. *hDOC2B-GFP* and *hDOC2B-GFP-Y301F* were constructed by Genescript. *Ad-GFP*, *Ad-DOC2B-GFP* wildtype (WT) or *Ad-DOC2B-GFP-Y301F* were generated by inserting *GFP*, *hDOC2B-GFP* or *hDOC2B-GFP-Y301F* fusion genes into the viral vector pAd5CMVmpA, respectively. Adenoviral vectors were constructed and adenovirus amplified and purified by Viraquest (North Liberty, IA, USA). Adenovirus *Ad-DOC2B* alone was made similarly and virus packaged with GFP by Viraquest. The correct sequences of all the constructs were confirmed by sequencing.

Animals and *in vivo* experiments. The mice were housed in standard facilities and standard cages with filter tops at room temperature with a 06:00-18:00 day-night cycle. The mice were generated and maintained on the C57BL/6J genetic background and were fed with standard diet ad libitum except when fasted as indicated in the experiments. The mice were handled exclusively in biosafety cabinets to maintain SPF status.

Rat *Doc2b* cDNA was subcloned into the 5' *Pme1* and 3' *BamH1* restriction sites in the pTRE-IRES-nGFP vector provided by Drs. Solomon Afelik and Jan Jenssen (Cleveland Clinic, Cleveland, OH) and microinjected into B6 oocytes to produce a TRE-*Doc2b*$^{-/+}$ founder. Skeletal muscle-specific muscle creatine kinase (*Mck*) promoter reverse tetracycline transactivator (*Mck*-rtTA$^{-/+}$) mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). TRE-*Doc2b*$^{-/+}$ offspring crossed to *Mck*-rtTA$^{-/+}$ mice produced Doxycycline (Dox)-inducible skeletal muscle-selective expression in the double transgenic offspring (skm*Doc2b*-dTg) mice. Male and female B6 skm*Doc2b*-dTg mice were administered Dox-treated (2 mg/ml) or standard drinking

water for 3 weeks to induce DOC2B overexpression prior to phenotyping assessment. High-fat diet (HFD) containing 60% of calories from fat, with or without Dox, (cat# S6470 and S3282, respectively, Bio-Serv, Flemington, NJ, USA), was fed to 7-8-week-old male mice for 4-5 weeks to induce insulin resistance prior to phenotyping and body composition assessment.

Age-matched and genotype-matched transgenic or control mice were randomly sorted into Dox-treated or untreated groups. The mice were anesthetized using isoflurane for organ harvest. The EchoMRI (cat# 600-00120, EchoMRI, Houston, TX, USA) and metabolic caging studies (cat# 994640-LM-008, TSE Systems Inc, Chesterfield, MO, USA) were carried out in the Comprehensive Metabolic Phenotyping Core at City of Hope. Animals were maintained in ventilated cages with a 12 h light/dark cycle, with access to food and water *ad libitum* under protocols approved by the City of Hope or Indiana University School of Medicine Institutional Animal Care and Use Committees and in accordance with Guidelines for the Use and Care of Laboratory Animals, as well as all other applicable local and national regulations.

RNA extraction and quantitative real-time PCR analysis. Muscle was isolated and pulverized using a polytron homogenizer. RNA was extracted using the TriReagent according to the manufacturer's protocol (MilliporeSigma, St. Louis, MO, USA). Synthesis of cDNA was performed using the iScript cDNA synthesis kit, as described by the manufacturer (Bio-Rad, Hercules, CA, USA). cDNA was amplified by PCR using the iQ SYBR Green Supermix (Bio-Rad). Rat *Doc2b* and *Hprt* were amplified using primers that were previously described [2, 3]. Real-time PCR was carried out for 40 cycles using the iCycler (Bio-Rad). Each cycle was run at 95°C for 15 s, 60°C for 30 s and 72°C for 30 s. RNA-free samples were used as negative controls

and did not produce amplification. Melting curve and gel analyses (sizing and sequencing) verified single products of the appropriate size.

Cell-surface GLUT4-myc detection. Briefly, L6-GLUT4-myc myoblasts were incubated in serum-free medium for 40 min prior to adding insulin (100 nM) for 20 min at 37 °C. The cells were washed three times with ice-cold PBS and then fixed with 4% (wt/vol) paraformaldehyde in PBS for 20 min at room temperature, blocked in Odyssey blocking buffer (LI-COR Biosciences, Lincoln, NE, USA) for 1 h at room temperature, and incubated with mouse anti-Myc (1:100) overnight at 4 °C. The cells were extensively washed with PBS and then incubated with infrared-conjugated goat anti-mouse secondary antibody for 1 h at room temperature. The immunofluorescence intensity was quantified using the Odyssey CLx infrared imaging system (LI-COR Biosciences), and the data were normalized to SYTO 60 (Invitrogen, Carlsbad, CA, USA), a red fluorescent nucleic acid stain.

2-deoxyglucose uptake. L6-GLUT4-myc myoblasts were seeded at $1x10^4$ cells/ml in differentiation medium (MEM-alpha medium containing 2% FBS (vol/vol)) in 24-well plates. Myotubes will form after 7 days. The myotubes were then transduced with the *hDOC2B-GFP* recombinant adenoviruses. Medium was changed at 12 hr after transduction. Forty-eight hours after viral addition, L6-GLUT4-myc myotubes were preincubated in serum-free FCB buffer (125 mmol/l NaCl, 5 mmol/l KCl, 1.8 mmol/l $CaCl_2$, 2.6 mmol/l $MgSO_4$, 25 mmol/l HEPES, 2 mmol/l pyruvate, and 2% (wt/vol) BSA) for 40 min. Then, the cells were stimulated with or without 100 nmol/l insulin for 20 min, and 2-deoxy-glucose uptake was determined by

measuring 2-deoxy-d-[2,6 ^{3}H] glucose incorporation. The results were normalized to protein content.

Co-immunoprecipitation and immunoblotting. L6-GLUT4-myc myoblasts were transfected with *Doc2b-GFP* or the control pEGF-N2 plasmid DNA, and 48 h later were incubated in serum-free medium for 40 min and stimulated with 100 nmol/l insulin at various time points. Cells were harvested into 1% (wt/vol) NP-40 lysis buffer for preparation as cleared detergent cell lysates. Lysates (2.5 mg protein) were immediately used in immunoprecipitation reactions by incubation with 30 μl of rabbit polyclonal anti-GFP antibody conjugated to Sepharose beads overnight. Co-immunoprecipitation from WT mouse skeletal muscle tissue was performed similarly, using 4 mg tissue homogenate combined with anti-myc-conjugated beads (Thermo Fisher, Waltham, MA and cat# 20168). The immunoprecipitated proteins were washed three times with lysis buffer and boiled in Laemmli sample buffer containing fresh dithiothreitol (DTT). The eluted proteins were resolved by 10% (wt/vol) SDS-PAGE and were subject to either mass spectrometry analysis by the Mass Spectrometry and Proteomics Core at City of Hope, or they were transferred to PVDF or nitrocellulose membranes for immunoblotting. Ponceau S staining was used to evaluate protein loading. Proteins in cell lysates were resolved using 10–12% (wt/vol) SDS-PAGE and transferred to PVDF membranes for immunoblotting. Immunoreactive bands were visualized using ECL, ECL Prime, or Supersignal Femto reagents and imaged using a BioRad Chemi-Doc Touch gel documentation system.

Subcellular fractionation of skeletal muscle. Tg and Wt littermate female mice (4-6 months old) were fasted for 16 h (18:00-10:00), the transgenic mice were given an intraperitoneal

injection of 21 U of Humulin R per kg of body weight or the same volume of saline as a control. After 30 min, the mice were killed, and the gastrocnemius muscles were rapidly dissected and trimmed of fat and connective tissues and immediately placed in ice cold homogenization buffer (20 mmol/l HEPES, pH 7.4, 250 mmol/l sucrose, 1 mmol/l EDTA, 1 mmol/l phenylmethylsulphonyl fluoride and Complete O proteinase inhibitor at 1 tablet/10 ml buffer) and homogenized with a Polytron PT-10 homogenizer 3 times in 10 s bursts. Homogenates were centrifuged at 2000 x g for 5 min at 4 °C, and supernatant then centrifuged at 9000 x g for 20 min at 4 °C. That supernatant was subsequently centrifuged at 180,000 x g for 90 min. Pellets containing t-tubule and sarcolemmal membrane fractions (5 µg protein each) were resolved by 10% (wt/vol) SDS-PAGE for subsequent immunoblotting for GLUT4.

References

[1] Groffen AJ, Brian EC, Dudok JJ, Kampmeijer J, Toonen RF, Verhage M (2004) Ca(2+)-induced recruitment of the secretory vesicle protein DOC2B to the target membrane. J Biol Chem 279: 23740-23747

[2] Weghofer M, Karlic H, Haslberger A (2001) Quantitative analysis of immune-mediated stimulation of tumor necrosis factor-alpha in macrophages measured at the level of mRNA and protein synthesis. Ann Hematol 80: 733-736

[3] Motyl KJ, Botolin S, Irwin R, et al. (2009) Bone inflammation and altered gene expression with type I diabetes early onset. J Cell Physiol 218: 575-583

ESM Table 1. Non-diabetic and type 2 diabetic human skeletal muscle donor information.

NDRI#	Age	Sex	BMI	Condition	Muscle group	Experimental Use
ND09702	52	M	22.8	ND	Right leg	protein
ND09704	68	M	30.3	ND	Right leg	protein, mRNA
ND09743	46	M	34.9	ND	Right leg	protein, mRNA
ND09744	62	M	28.2	ND	N/A	protein
ND09749	49	F	21.6	ND	Quadriceps	protein, mRNA
ND09703	68	F	29.9	T2D	Right quadriceps	protein, mRNA
ND09705	65	M	33.8	T2D	Left quadriceps	protein
ND09706	45	M	44.5	T2D	Left quadriceps	protein, mRNA
ND09754	67	M	32.3	T2D	Quadriceps	protein, mRNA
ND10105	53	F	28	T2D	Left quadriceps	protein, mRNA
ND13347	78	M	26.4	ND	Right quadriceps	protein
ND13230	68	F	40.2	ND	Left quadriceps	protein
ND13216	54	M	31.3	ND	Right quadriceps	protein
ND13214	74	M	32.1	T2D	Left quadriceps	protein
ND13363	59	M	41.5	T2D	Right quadriceps	protein
ND13239	58	M	50.3	T2D	Right quadriceps	protein

ND: non-diabetic; T2D: type 2 diabetic; N/A: detailed information not available.

ESM Table 2. Sequences of the primers and probes used for quantification of transcript levels.

Target cDNA	Forward primer written 5' to 3' sense	Reverse primer written 5' to 3' antisense
Genotyping primers		
Doc2b	GGCAGAGGACAAGTCCCTGG	GGTCGACGGCGCTATTCAGATCC
DOC2B real-time PCR primers		
Rat Doc2b	CCA GCA AGG CAA ATA AGC TC	GTT GGG TTT CAG CTT CTT CA
Mouse Doc2b	CCAGCAAGGCAAATAAGCTC	TTGGGCTTCAGCTTCTTCA
Human DOC2B	CCAGTAAGGCAAATAAGCTC	TTGGGTTTCAGCTTCTTCA
Rat Gapdh	GACATGCCGCCTGGAGAAAC	GACATGCCGCCTGGAGAAAC
Mouse Hprt	TCAACGGGGGACATAAAAGT	CCAGTGTCAATTATATCTTCAACAATC
Rat Hprt	GGTCCATTCCTATGACTGTAGATTTT	CAATCAAGACGTTCTTTCCAGTT
Human HPRT	TATGGCGACCCGCAGCCCT	CATCTCGAGCAAGACGTTCAG
Sequencing primers		
CMV forward	CGCAAATGGGCGGTAGGCGTG 3	
EGFP-N		CGT CGC CGT CCA GCT CGA CCA
BGH-R		TAGAAGGCACAGTCGAGG

ESM Table 3. Fasting serum analytes from Dox(-) control and Dox(+) Skm*Doc2b*-dTg mice.

		Dox(-) control	Dox(+) Tg
Female	Insulin (pmol/l)	16.6 ± 10.2	9.5 ± 3.0
	Glucagon (ng/l)	152.1 ± 21.5	220.8 ± 44.6
	Cholesterol (mmol/l)	2.2 ± 0.1	2.4 ± 0.1
	NEFA (mEq/L)	1.0 ± 0.1	1.2 ± 0.1
Male	Insulin (pmol/l)	36.2 ± 13.2	35.1 ± 16.4
	Glucagon (ng/l)	80.7 ± 9.8	99.6 ± 14.2
	Cholesterol (mmol/l)	3.3 ± 0.2	3.2 ± 0.2
	NEFA (mEq/L)	1.0 ± 0.1	1.2 ± 0.1

Data represent the average ± SEM; no significant differences were detected. Serum was collected from 16 h-fasted Dox(-) and Dox(+) Skm*Doc2b*-dTg male and female mice at 5-6 months of age (n=5-7). NEFA: non-esterified fatty acids.

ESM Table 4. Tissue and body weights of Dox(-) control and Dox(+) Skm*Doc2b*-dTg mice.

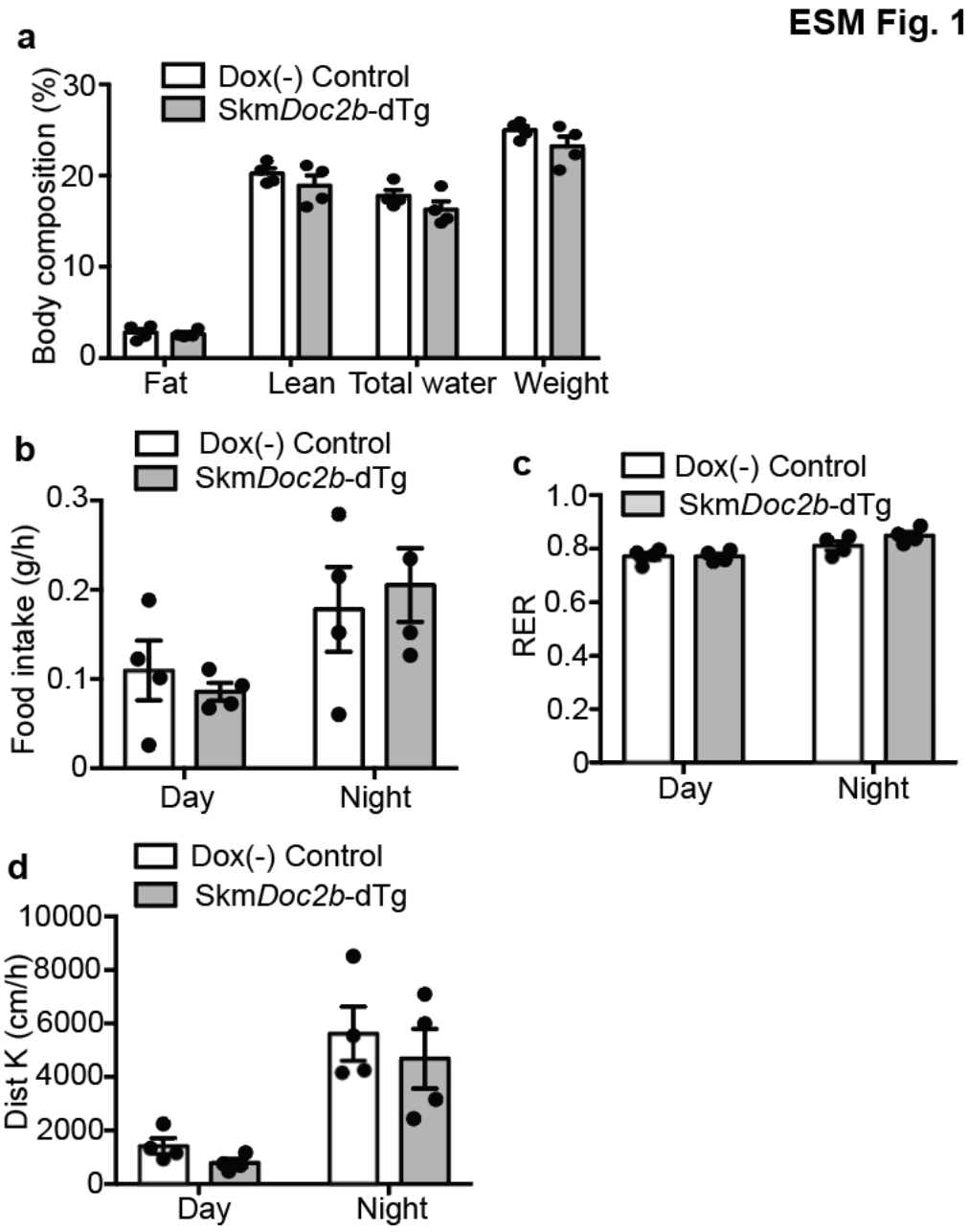
		Dox(-) control	Dox(+) Tg
Female	Body weight (g)	20.8 ± 1.3	20.6 ± 0.6
	Tissue (% of body weight)		
	Brain	2.08 ± 0.15	2.05 ± 0.12
	Muscle	1.26 ± 0.04	1.15 ± 0.04
	Fat	0.75 ± 0.12	1.03 ± 0.22
	Spleen	0.37 ± 0.06	0.25 ± 0.03
	Lungs	0.70 ± 0.02	0.83 ± 0.05
	Liver	4.26 ± 0.28	4.41 ± 0.21
	Kidney	1.42 ± 0.05	1.59 ± 0.06
	Heart	0.59 ± 0.39	0.56 ± 0.03
	Pancreas	0.53 ± 0.37	0.63 ± 0.10
Male	Body weight (g)	28.2 ± 0.4	26.5 ± 1
	Tissue (% body weight)		
	Brain	1.50 ± 0.09	1.59 ± 0.05
	Muscle	1.26 ± 0.05	1.17 ± 0.06
	Fat	1.53 ± 0.24	1.38 ± 0.20
	Spleen	0.23 ± 0.01	0.20 ± 0.01
	Lungs	0.68 ± 0.05	0.64 ± 0.02
	Liver	4.34 ± 0.13	3.96 ± 0.21
	Kidney	1.42 ± 0.03	1.47 ± 0.04
	Heart	0.54 ± 0.03	0.53 ± 0.02
	Pancreas	0.61 ± 0.04	0.52 ± 0.03

Data represent the average ± SEM. Weights are from female and male Dox(-) and Dox(+) Skm*Doc2b*-dTg mice at 4–6 months of age (n = 5-7). Muscle: hindlimb muscles from both legs, fat: both epigonadal fat pads and kidney: both kidneys. No statistically significant differences were detected.

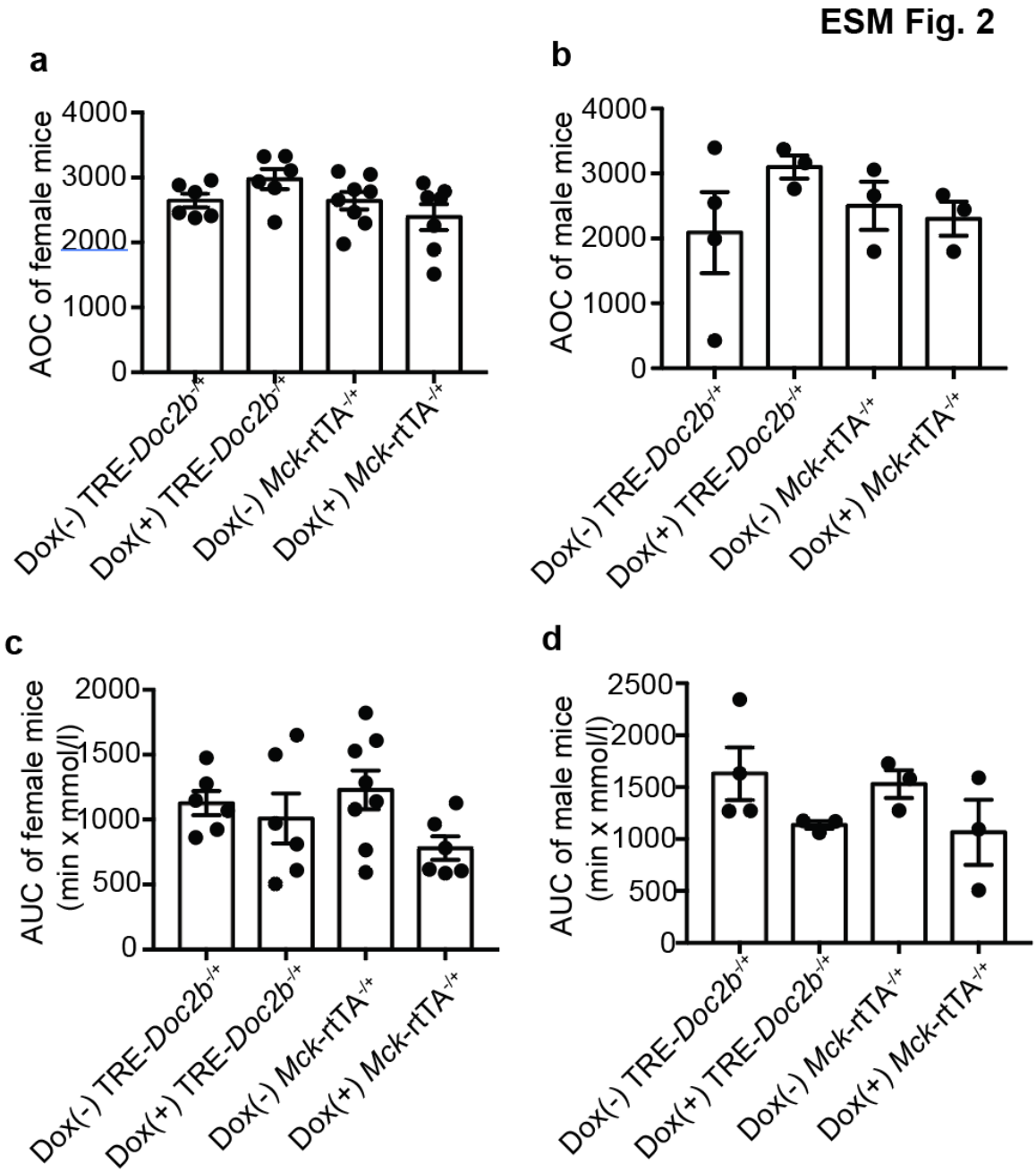
ESM Table 5. Potential DOC2B binding proteins identified by mass spectrometry.

#	Identified Proteins	Accession Number	Molecular Mass (kDa)	Exclusive Unique Peptide Count			
				GFP	DOC2B-GFP	DOC2B-GFP+Ins	DOC2B-GFP+pV
1	78 kDa glucose-regulated protein precursor	NP_037215.1	72	0	0	12	17
2	kinesin light chain 1	XP_006240687.1	68	0	3	6	7
3	filamin-C	NP_001178791.1	291	0	0	6	6
4	heat shock protein 105 kDa	NP_001011901.1	96	0	0	5	5
5	moesin	NP_110490.1	68	0	0	4	6
6	coatomer subunit alpha	NP_001128012.1	138	0	0	4	9
7	fermitin family homolog 2	NP_001011915.1 (+2)	78	0	0	4	4
8	synaptopodin 2-like protein isoform 1	NP_001292067.1 (+1)	103	0	0	5	6
9	filamin-B	NP_001100758.1	275	0	0	6	5
10	trifunctional enzyme subunit alpha, mitochondrial precursor	NP_570839.2	83	0	0	3	4
11	splicing factor, proline- and glutamine-rich	NP_001020442.1	75	0	0	3	3
13	PREDICTED: coatomer subunit beta isoform X1	XP_008757882.1	107	0	0	3	4
14	40S ribosomal protein S2	NP_114026.3	31	0	0	4	4
*	double C2-like domain-containing protein beta	NP_112404.1	46	0	14	16	19

*: Identification of DOC2B indicates that DOC2B-GFP was successfully expressed and bound to the beads.

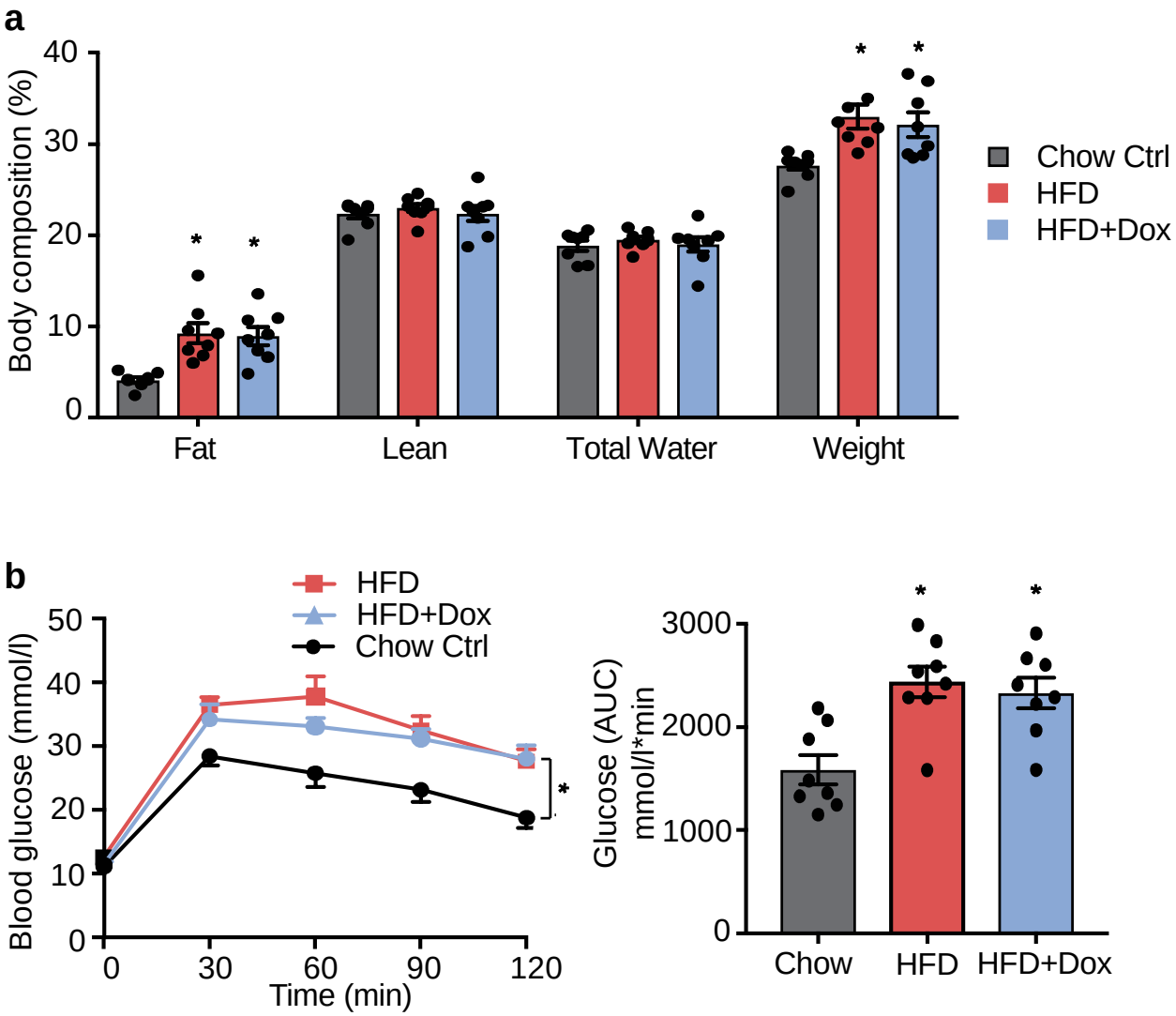


ESM Fig. 1. (a) Body composition measured by Eco-MRI. (b-d) Metabolic caging studies, wherein the data represent the average ± SEM for four pairs of 6-month-old female mice: food intake (b), energy expenditure (RER) (c), and physical activity represented by the distance K (Dist K). (d). n=4; no significant differences were observed between the Dox(-) and Dox(+) Skm*Doc2b*-dTg groups.



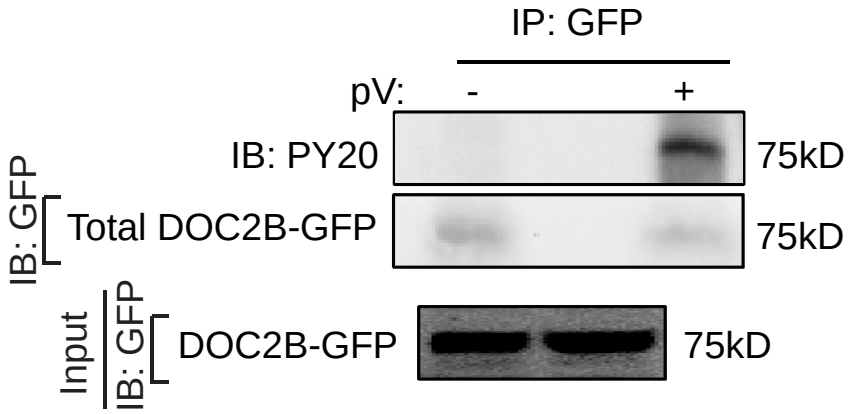
ESM Fig. 2. IPITT and IPGTT assays in control mice. Area over the curve (AOC) calculated based on results from IPITT assay in both female (a) and male (b) control TRE-*Doc2b*$^{-/+}$ and *Mck*-rtTA$^{-/+}$ mice with and without Dox induction. N=3-8. No significant differences were detected among the groups.

ESM Fig. 3



ESM Fig. 3. HFD induced obesity and glucose tolerance in the skm*Doc2b*-dTg mice. (a) Body composition in Chow-fed control (Ctrl) and HFD-fed non- or Dox-induced mice as measured by Eco-MRI. (b) Intraperitoneal glucose tolerance test and area under the curve (AUC) analysis in Chow Ctrl, HFD and HFD+Dox mice. * $p<0.05$ as compared to the Chow Ctrl group.

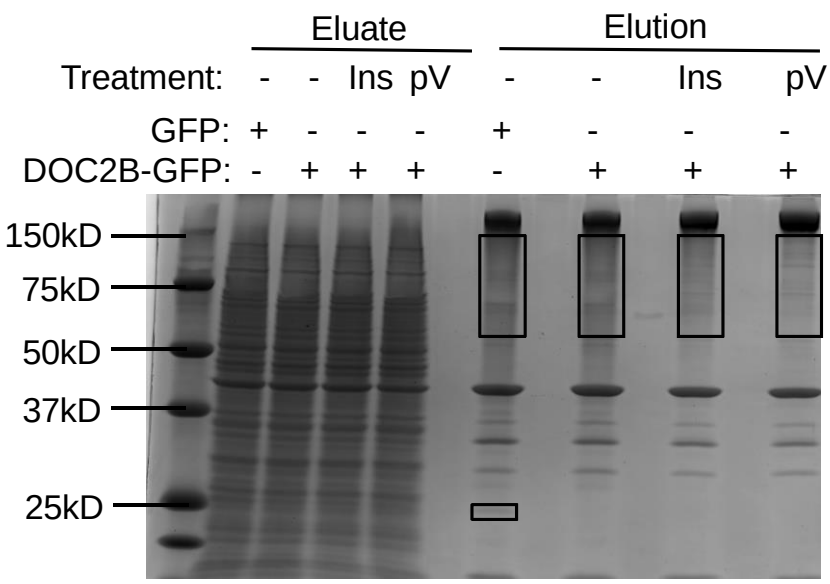
ESM Fig. 4



ESM Fig. 4. Detecting DOC2B phosphorylation upon pervanadate (pV) treatment using anti-PY20 antibody. L6 cells overexpressing DOC2B-GFP were treated with freshly prepared pV for 5 minutes, and the resulting cell lysates were coimmunoprecipitated using anti-GFP sepharose beads. The beads were boiled and eluted proteins resolved by SDS-PAGE for immunoblot detection (IB) using anti-PY20 and -GFP antibodies; data shown are representative of two independent experiments.

ESM Fig. 5

a



b

XP_006240687.1 (100%), 64,696.9 Da
PREDICTED: kinesin light chain 1 isoform X4 [Rattus norvegicus]
6 exclusive unique peptides, 6 exclusive unique spectra, 7 total spectra, 69/568 amino acids (12% coverage)

MHDNMSTMVY EHNSILQSLL LGLSEAQVMM RDELANTQQK ISPSEDKDSD AAQQGGYEIP EDLEKTSGHD **ALAIR**EKTLG KRALEIREKV YYQRALEIYQ ETLYKEILTR KDGSSFGEYG **EAAETLEEAA** SRESLNVDVV RQQQPRRR

MKEEKLEKLT ETLKCLKKDD ALSNHLNAVE LQKSEQSVAQ SSKEPLDDLF ARLRTLHNLV HPDVATMLNI R**DHPAVAATL** LGKDHPDVAK T**KLGPDDPNV** AHERFGSVD GWYKACKVDS **LR**SRKQGLDN KYESGPDGGE

QDEIISKTKQ ESNLVEEKSS SEKQKLRAQV LEEEKKHLEF PNDEDDPGQG IQYASQGRYE LALVYRDQNK **NNLAVLYGK**R QLNNLALLCQ **AK**TKNNLASC DENKPIWMHA PTVTTTLKNL VHKQR**VAEVL** EVSMSVEWNG

VIQGLEALKN MIRKSLEMLE RRLCQENQWL MNQLKKYDDD IQQQHSSAAA VAVPLCKQAL **YKDAANLLND** GKYKEAEPLC NQGKYEEVEY YLKQGKFKQA EERECKGKQ GALYRRQGK**F** **NDPENVEK**RR GTRRASLCGK

ESM Fig. 5. Mass spectrometry to identify the potential interacting partners of DOC2B. (a) Gel image to demonstrate the protein profiles from coimmunoprecipitation experiments using GFP-antibody-conjugated sepharose beads. The boxed areas, which show the largest differences among the lanes, were cut out for mass spectrometry analysis. (b) The highlighted peptides show the sequence coverage of KLC1 molecules by 6 exclusive unique spectra, which indicate 100% possibility that the identified molecule was KLC1.