Diabetologia (2014) 57:2173–2182
DOI 10.1007/s00125-014-3312-y

ARTICLE

DOC2 isoforms play dual roles in insulin secretion and insulin-stimulated glucose uptake

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Received: 22 January 2014 / Accepted: 28 May 2014 / Published online: 9 July 2014

Abstract

Aims/hypothesis Glucose-stimulated insulin secretion (GSIS) and insulin-stimulated glucose uptake are processes that rely on regulated intracellular vesicle transport and vesicle fusion with the plasma membrane. DOC2A and DOC2B are calcium-sensitive proteins that were identified as key components of vesicle exocytosis in neurons. Our aim was to investigate the role of DOC2 isoforms in glucose homeostasis, insulin secretion and insulin action.

Methods DOC2 expression was measured by RT-PCR and western blotting. Body weight, glucose tolerance, insulin action and GSIS were assessed in wild-type (WT), *Doc2a*$^{-/-}$ (*Doc2a*KO), *Doc2b*$^{-/-}$ (*Doc2b*KO) and *Doc2a*$^{-/-}$/*Doc2b*$^{-/-}$ (*Doc2a/Doc2b*KO) mice in vivo. In vitro GSIS and glucose uptake were assessed in isolated tissues, and exocytotic proteins measured by western blotting. GLUT4 translocation was assessed by epifluorescence microscopy.

Results *Doc2b* mRNA was detected in all tissues tested, whereas *Doc2a* was only detected in islets and the brain. *Doc2a*KO and *Doc2b*KO mice had minor glucose intolerance, while *Doc2a/Doc2b*KO mice showed pronounced glucose intolerance. GSIS was markedly impaired in *Doc2a/Doc2b*KO mice in vivo, and in isolated *Doc2a/Doc2b*KO islets in vitro. In contrast, *Doc2b*KO mice had only subtle defects in insulin secretion in vivo. Insulin action was impaired to a similar degree in both *Doc2b*KO and *Doc2a/Doc2b*KO mice. In vitro insulin-stimulated glucose transport and GLUT4 vesicle fusion were defective in adipocytes derived from *Doc2b*KO mice. Surprisingly, insulin action was not altered in muscle isolated from DOC2-null mice.

Conclusions/interpretation Our study identifies a critical role for DOC2B in insulin-stimulated glucose uptake in adipocytes, and for the synergistic regulation of GSIS by DOC2A and DOC2B in beta cells.

Jia Li, James Cantley and James G. Burchfield contributed equally to this study.

Electronic supplementary material The online version of this article (doi:10.1007/s00125-014-3312-y) contains peer-reviewed but unedited supplementary material, which is available to authorised users.

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Keywords Adipocyte · Beta cell · Diabetes · DOC2 · Double C2 domain protein · Exocytosis · Glucose homeostasis · GLUT4 · Insulin action · Insulin secretion

Abbreviations

DOC2	Double C2 domain protein
*Doc2a*KO	*Doc2a*$^{-/-}$
*Doc2b*KO	*Doc2b*$^{-/-}$
DXA	Dual-energy x-ray absorptiometry
GSIS	Glucose-stimulated insulin secretion
ITT	Insulin tolerance test
$[Ca^{2+}]_i$	Intracellular calcium
MEFs	Mouse embryonic fibroblasts
PM	Plasma membrane
SNARE	Soluble N-ethylmaleimide-sensitive factor attachment protein receptor
WT	Wild-type

Introduction

Glucose homeostasis is maintained by insulin secretion from glucose-sensing pancreatic beta cells, coupled with glucose disposal by insulin-responsive tissues such as muscle and fat. Insulin resistance and defective insulin secretion result in glucose intolerance, hyperglycaemia and progression toward type 2 diabetes [1].

Vesicle exocytosis is a key process in both insulin secretion and insulin action. Beta cells sense glucose and secrete appropriate amounts of insulin using a coupling pathway involving glucose oxidation, ATP-sensitive potassium channel closure, membrane depolarisation and calcium influx [2]. Although the proximal steps in this coupling pathway are well defined, the manner and order in which distal components respond to elevated intracellular calcium ($[Ca^{2+}]_i$) and trigger insulin vesicle exocytosis are less clear. $[Ca^{2+}]_i$ elevation and oscillation induces vesicle movement and fusion with the plasma membrane (PM) via formation of a soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex involving v-SNAREs, t-SNAREs, synaptotagmins, Sec1/Munc18 proteins and other effectors [3, 4]. Many of these proteins play roles in insulin secretion, including VAMP2, VAMP3, syntaxin-1A, syntaxin-4, SNAP23, SNAP25, MUNC-18A and MUNC-18C [5]. Muscle and fat cells respond to insulin by triggering translocation of solute carrier family 2, facilitated glucose transporter member 4 (GLUT4) from intracellular vesicles to the PM to facilitate glucose uptake [6]. The proximal steps in this insulin signalling pathway are well characterised: once bound to its receptor, insulin initiates a signalling cascade involving activation of phosphatidylinositol 4,5-bisphosphate 3-kinase (PI3K) and AKT. However, the distal effectors governing vesicle movement and fusion are less clearly defined. Vesicle fusion is calcium dependent [7, 8] and involves an array of SNARE complex proteins [5].

Double C2 domain (DOC2) proteins are positive regulators of exocytosis that possess a similar structure to synaptotagmins, containing two C-terminal Ca^{2+}- and phospholipid-interacting domains [9–11]. Three DOC2 isoforms exist in mammals. DOC2A is predominantly expressed in the brain [9, 12], whereas DOC2B is ubiquitously expressed [10–12]; however, both show calcium-dependent affinity for phospholipids [9, 11]. Even within the brain, DOC2A expression is heterogeneous and restricted to areas such as the hippocampus, whereas DOC2B is more widely expressed [13]. A third isoform, DOC2C (also known as DOC2G), is thought to be calcium insensitive [14, 15], although its function is unclear. In neurons, DOC2A and DOC2B translocate to the PM in response to elevated $[Ca^{2+}]_i$ [15] and promote vesicle fusion [16]. DOC2 proteins interact with SNARE proteins and associated regulators, including MUNC-18 [12, 17, 18] and MUNC-13, via an N-terminal binding motif [19–21], and dynein [22] and the syntaxin-1–SNAP25 complex via a C-terminal C2B domain [16, 23].

Glucose induces DOC2B translocation to the PM in cultured beta cells [24], and insulin secretion is directly proportional to DOC2B levels [18, 24]. DOC2B is expressed in 3T3-L1 adipocytes [18] and translocates to the PM in response to insulin, where it acts as a calcium-sensitive modulator of GLUT4 vesicle fusion and glucose uptake [25] by facilitating membrane curvature [26]. Moreover, DOC2B overexpression enhances and its depletion inhibits insulin-stimulated glucose uptake by 3T3-L1 adipocytes [25]. A recent study reported that mice with a global deletion of *Doc2b* are glucose intolerant, with defective glucose-stimulated insulin secretion (GSIS) and impaired insulin-stimulated glucose uptake into muscle [27]. However, the role of DOC2B in adipocyte insulin action was not assessed. In contrast to the emerging literature supporting a role for DOC2B in insulin secretion and action, the function of DOC2A in metabolic tissues has not yet been investigated. Although DOC2A is predominantly expressed in the brain, DOC2A protein and mRNA have been detected in both Min6 beta cells and primary mouse islets [24]. Therefore, the aim of our study was to investigate the role of both DOC2A and DOC2B isoforms in glucose homeostasis in vivo, and in insulin secretion and insulin action ex vivo, using *Doc2a*$^{-/-}$ (*Doc2a*KO), *Doc2b*$^{-/-}$ (*Doc2b*KO) and *Doc2a*$^{-/-}$/*Doc2b*$^{-/-}$ (*Doc2a*/*Doc2b*KO) mice.

Methods

Materials See electronic supplementary material (ESM) Methods for details of reagents, media and antibodies.

Mice *Doc2a*KO and *Doc2b*KO mice were provided by M. Verhage and A. J. A. Groffen [13, 28] and maintained on a

C57Bl/6 genetic background. Mice were group housed, with food and water provided ad libitum. Mice were anaesthetised with isoflurane and killed by cervical dislocation prior to tissue collection. Mouse husbandry, care and experiments were performed in accordance with the Australian National Health and Medical Research Council guidelines, and local institutional guidelines. Ethical approval was granted to D. E. James by the Garvan/St. Vincent's Hospital animal ethics committee.

In vivo assessment of glucose homeostasis Male mice were studied from 16 weeks of age. Adiposity (% body fat) was quantified by dual-energy x-ray absorptiometry (DXA) scanning. Glucose tolerance testing was performed by i.p. glucose injection (2 g/kg) at 09:00 hours, following a 16 h fast. Insulin tolerance testing was performed by i.p. insulin injection (0.75 U/kg) at 14:00 hours, following a 6 h fast. Blood glucose was measured via tail-tip bleeds using an Accu-Chek glucometer (Roche, Indianapolis, IN, USA). GSIS was assessed, following a 16 h fast, by i.v. (tail vein) glucose injection (1 g/kg), with subsequent blood sampling from the tail tip for insulin ELISA (Crystalchem, Downers Grove, IL, USA). Plasma NEFAs were measured by enzymatic assay (Wako Diagnostics, Moutainview, CA, USA).

Islet isolation, in vitro insulin secretion, and redox and glucose oxidation assays Islet isolation, culture and insulin secretion assays were performed as previously described [29]. Insulin was assayed by RIA (Millipore, Billerica, MA, USA) or ELISA. For details of islet redox and glucose oxidation assays, see ESM Methods.

Pancreatic histology Pancreases were excised, formalin fixed and immunostained as previously described [30]. Imaging was performed using a Leica DM6000B microscope with LAS Power Mosaic. Probability maps were generated in ilastik v1.0 [31], with insulin-positive areas (beta cells) and total pancreatic areas of sections quantified using ImageJ [32].

In vitro glucose uptake assays In vitro insulin-stimulated glucose uptake was measured in muscle and fat explants from mice using radiolabelled tracers (see ESM Methods).

Transfection and culture of MEF-derived adipocytes Preparation, culture, differentiation and transfection of adipocytes derived from mouse embryonic fibroblasts (MEFs) were performed as previously described [33]. Transfection was performed using 5 μg pHluorin-GLUT4-tdTomato plasmid DNA. Electroporated adipocytes were seeded onto Matrigel-coated coverslips (BD Biosciences, Franklin Lakes, NJ, USA) and maintained in Dulbecco's modified Eagle medium containing 10% FCS and 20 mmol/l GlutaMAX (Life Technologies, Carlsbad, CA, USA) for 48 h. Adipocytes were serum starved for 2 h before imaging. Experiments were performed at 37°C in Krebs–Ringer phosphate buffer containing 2% BSA and 100 nmol/l insulin, as indicated. Fluorescence image analysis was performed as previously described [34].

Statistical analysis Data were analysed using GraphPad Prism 6 (GraphPad Software, La Jolla, CA, USA) and presented as the mean ± SEM. The AUC was calculated using the trapezoidal method. Differences between groups were tested using two-tailed unpaired Student's t tests for simple comparisons, or one-way ANOVA with Tukey's multiple comparisons test. A p value of <0.05 was considered statistically significant. Array data analysis is described in the ESM Methods.

Results

Doc2 expression in metabolic tissues Using RT-PCR, we found that *Doc2b* was expressed in all tissues tested, including the brain, heart, liver, islets, muscle and fat (Fig. 1a). In contrast, *Doc2a* expression was detected only in the brain and islets (Fig. 1b), consistent with previous reports [9, 12, 24]. *Doc2b* expression was undetectable in *Doc2b*KO and *Doc2a/Doc2b*KO mice. *Doc2a* expression was undetectable in *Doc2a/Doc2b*KO mice, but was comparable with wild-type (WT) in *Doc2b*KO mice (Fig. 1b). To confirm the efficient ablation of DOC2 proteins, we performed western blotting of brain and fat lysates using an antibody that recognises both DOC2A and DOC2B isoforms. A DOC2 protein band was detected in WT fat but absent in fat samples from both *Doc2b*KO and *Doc2a/Doc2b*KO mice (Fig. 1c), indicating the efficient deletion of *Doc2b*. Conversely, intensity of a DOC2 protein band detected in WT brain was reduced by *Doc2b* deletion and strongly reduced by deletion of both *Doc2a* and *Doc2b* (Fig. 1c), thus confirming *Doc2a* deletion. The residual band seen in *Doc2a/Doc2b*KO mice is probably DOC2C.

To investigate whether *DOC2* genes are expressed in humans, we analysed a published human beta cell RNA-seq data set [35] and found *DOC2A* expression to be sevenfold greater than *DOC2B* (Fig. 1d). We found a similar pattern in our analysis of a published human beta cell gene expression array [36], which also revealed that *DOC2A* and *DOC2B* mRNAs were nonsignificantly altered in type 2 diabetes (ESM Data file). Finally, *DOC2B* expression was detected in human visceral adipose tissue biopsies [37] by gene expression array (R. Chaudhuri and D. E. James, unpublished observations). These data reveal that mice and humans have similar DOC2 expression patterns.

Impaired glucose tolerance in Doc2bKO and Doc2a/Doc2bKO mice Body weight was not altered by the deletion

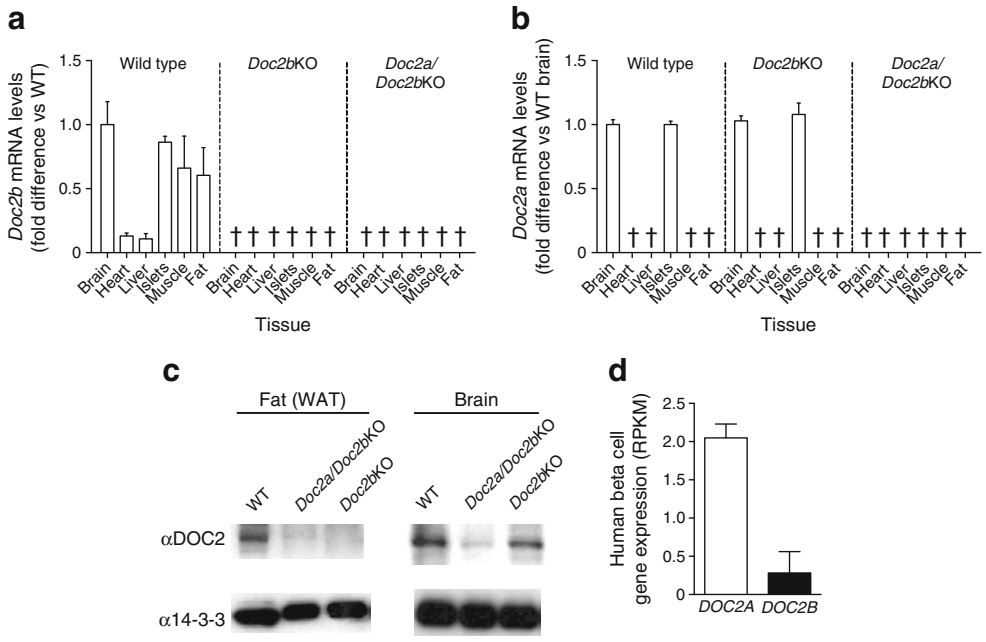

Fig. 1 Tissue expression of (**a**) *Doc2b* and (**b**) *Doc2a* mRNA, quantified by RT-PCR and normalised to WT levels; $n=3$. † Indicates transcript was undetectable. (**c**) Western blotting of epididymal fat and brain lysates to confirm the presence and deletion of DOC2 isoforms (~46 kDa). (**d**) DOC2 isoform expression in human beta cells, plotted as reads per kb of transcript per million mapped reads (RPKM) from published RNA-seq data [35]. WAT, white adipose tissue

of DOC2 isoforms (Fig. 2a). However, by DXA scanning, we detected a significant increase in adiposity in both *Doc2b*KO and *Doc2a/Doc2b*KO mice relative to controls (Fig. 2b). Body fat was similar in *Doc2b*KO and *Doc2a/Doc2b*KO mice (Fig. 2b), suggesting that DOC2B, rather than DOC2A, plays a role in regulating adiposity.

To determine the effect of loss of DOC2 isoforms on whole-body glucose homeostasis, we performed i.p. glucose

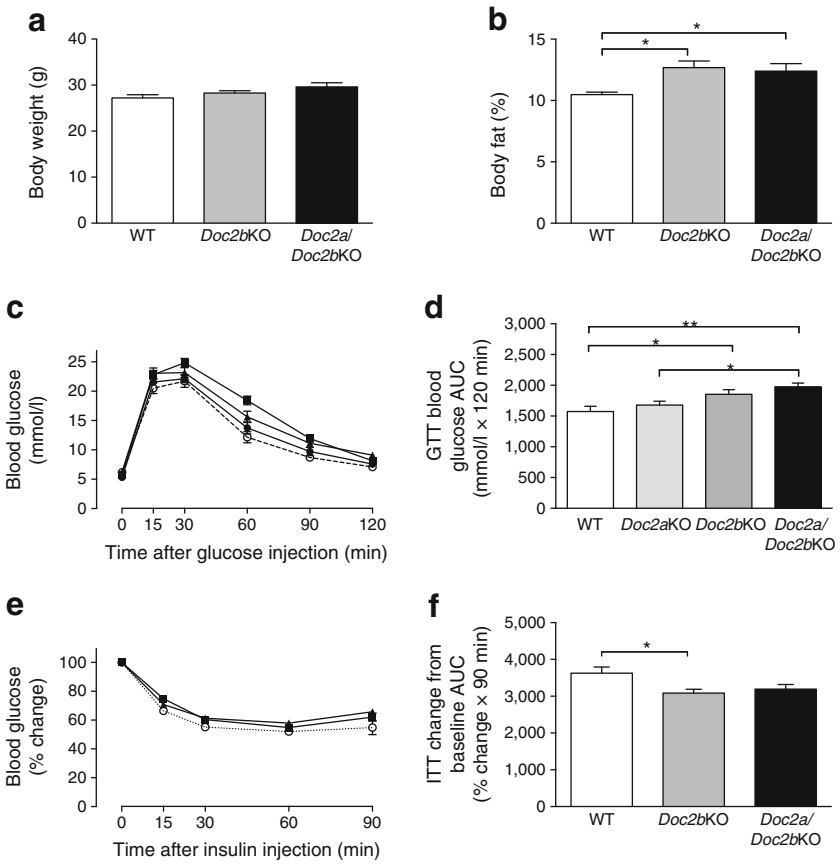

Fig. 2 Deletion of *Doc2* isoforms impairs glucose homeostasis. (**a**) Body weight of 16-week-old male mice; $n=6$. (**b**) Body fat measured by DXA; $n=6$. (**c**, **d**) Glucose tolerance test (i.p. glucose, 2 g/kg) following a 16 h fast; $n=7$–8. (**e**, **f**) ITT (i.p. insulin, 0.75 U/kg) following a 6 h fast; $n=7$. Effect of genotype was assessed by ANOVA. $^*p<0.05$, $^{**}p<0.01$. Open circle, WT; closed circle, *Doc2a*KO; triangle, *Doc2b*KO; square, *Doc2a/Doc2b*KO

tolerance tests. *Doc2b*KO mice showed mild, but significant, glucose intolerance with a larger glucose excursion than controls (Fig. 2c, d), consistent with a previous study [27]. In contrast, *Doc2a*KO mice showed a nonsignificant impairment in glucose tolerance (Fig. 2c, d). Deletion of both isoforms caused pronounced glucose intolerance (Fig. 2c, d), indicating that DOC2A and DOC2B play synergistic roles in regulating glucose homeostasis. Fasting blood glucose levels were comparable in *Doc2b*KO (6.26±0.24 mmol/l), *Doc2a/Doc2b*KO (5.74±0.14 mmol/l) and WT (6.18±0.31 mmol/l) mice, but slightly reduced in *Doc2a*KO mice (5.31±0.19 mmol/l; p=0.042 vs WT by Student's t test).

Next, we subjected these mouse models to an i.p. insulin tolerance test (ITT), which revealed that *Doc2b*KO mice have a slight, but significant, impairment in insulin action relative to controls (Fig. 2e, f). The similar insulin action between *Doc2b*KO and *Doc2a/Doc2b*KO mice suggests that DOC2B, but not DOC2A, is involved in the insulin response.

Impaired GSIS in Doc2-null mice Glucose tolerance is tightly coupled to GSIS. Therefore, we sought to investigate whether beta cell dysfunction underpins glucose intolerance in DOC2-null mice. Fasting insulin levels were not altered between WT, *Doc2a*KO, *Doc2b*KO and *Doc2a/Doc2b*KO mice (Fig. 3a), indicating that DOC2 isoforms do not regulate basal insulin exocytosis. We detected biphasic GSIS in all genotypes in vivo (Fig. 3b–d). GSIS in *Doc2a*KO mice was comparable to WT, although with a trend toward decreased second-phase secretion. *Doc2b*KO mice had intact first-phase GSIS, but impaired second-phase GSIS, whereas *Doc2a/Doc2b*KO mice showed pronounced defects in both phases (Fig. 3b–d). As GSIS is regulated by systemic factors, as well as beta cell-intrinsic mechanisms, it is plausible that deletion of *DOC2* genes in non-islet tissues is responsible for the defective GSIS in vivo. Therefore, we tested the intrinsic insulin secretory responses in islets isolated from DOC2-null mice in vitro. Basal insulin secretion and KCl-triggered insulin secretion were similar among WT, *Doc2b*KO and *Doc2a/Doc2b*KO genotypes (Fig. 3e), confirming that loss of DOC2 isoforms does not alter insulin vesicle exocytosis under basal or elevated $[Ca^{2+}]_i$ conditions per se. The GSIS response to a physiological glucose dose (11 mmol/l) was significantly decreased (by 30%) in *Doc2a/Doc2b*KO mice, yet unaltered by *Doc2b* deletion alone, relative to control islets (Fig. 3e). Islet insulin content did not change with genotype (Fig. 3f). Preliminary data from *Doc2a*KO islets showed normal GSIS (11 mmol/l glucose; *Doc2a*KO islets 102.2%±8.3% change from WT control; n=3). These data reveal that deletion of *Doc2a* or *Doc2b* alone has a relatively minor effect on insulin secretion, whereas deletion of both isoforms specifically impairs GSIS,

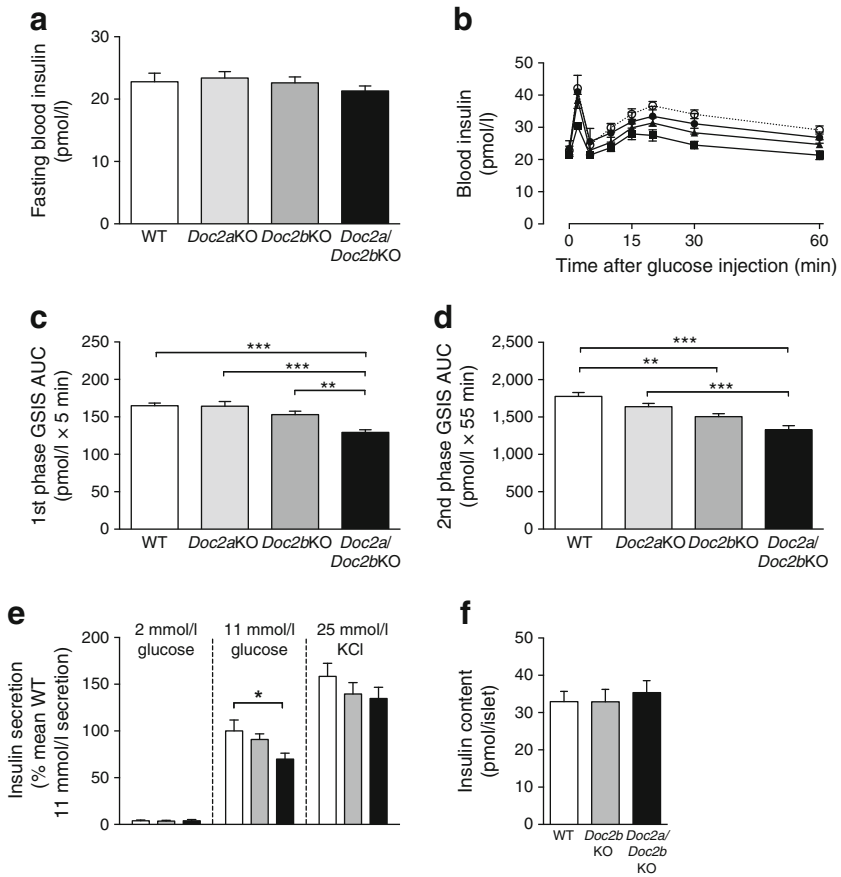

Fig. 3 Defective GSIS in DOC2-null mice. (**a**) Fasting blood insulin levels were unaltered by deletion of DOC2 isoforms; n=6–9. (**b**–**d**) GSIS assessed in vivo (i.v. glucose, 1 g/kg) following a 16 h fast: (**c**) first phase, 0–5 min; (**d**) second phase, 5–60 min; GSIS quantified as AUC; n=6–9. In vitro GSIS and KCl-induced (**e**) insulin secretion and (**f**) insulin content, assessed using isolated islets; n=12–13. Genotype effect was tested by ANOVA. *p<0.05, **p<0.01, ***p<0.001. Open circle/white bar, WT; closed circle/light grey bar, *Doc2a*KO; triangle/dark grey bar, *Doc2b*KO; square/black bar, *Doc2a/Doc2b*KO

suggesting that DOC2A and DOC2B have synergistic roles in beta cell function.

Normal islet glucose metabolism, SNARE protein levels and beta cell mass in DOC2-null islets We next considered other aspects of beta cell function that could be indirectly affected by DOC2 deletion and thus contribute to the phenotype. First, we undertook experiments to determine whether loss of DOC2 isoforms impairs beta cell glucose metabolism. In response to glucose stimulation, the autofluorescent signals from the pyridine nucleotide NAD(P)H and flavoprotein FAD^{2+} are increased and decreased, respectively [38], reflecting the chemical reduction of these intermediates. In response to glucose, the NAD(P)H:FAD^{2+} ratio rose to an equivalent level in both WT and *Doc2a/Doc2b*KO islets (Fig. 4a, b). In addition, the glucose oxidation rate, assessed by measuring $^{14}CO_2$ production from [U-^{14}C]glucose, was equivalent in WT and *Doc2a/Doc2b*KO islets (Fig. 4c), confirming that normal glucose-driven metabolic flux is not dependent on DOC2. Next, western blotting of islet lysates revealed that levels of the SNARE complex proteins syntaxin-1A, MUNC-18A, MUNC-18C and SNAP25 were unaltered by deletion of *Doc2a* or *Doc2b* (Fig. 4d). Finally, we performed a histological analysis of pancreatic sections from DOC2 mutant mice, which revealed normal islet structure and insulin staining in the absence of *Doc2a* and *Doc2b* (Fig. 4e), consistent with the normal islet insulin content (Fig. 3f). Furthermore, the percentage of the pancreas composed of insulin-containing beta cells was nonsignificantly altered in *Doc2a*/*Doc2b*KO mice (Fig. 4f). These results indicate that DOC2 proteins do not play a role in beta cell glucose metabolism, SNARE complex protein levels or the regulation of beta cell mass.

Reduced glucose uptake in adipose tissue, but not skeletal muscle, from Doc2bKO mice In vitro insulin-stimulated 2-deoxy[^{3}H]glucose uptake was similar in isolated muscle from WT, *Doc2b*KO and *Doc2a/Doc2b*KO mice (Fig. 5a, b). This is consistent with the relatively modest defect in the ITT response, since glucose uptake is largely driven by muscle insulin action. In contrast, insulin-stimulated 2-deoxy[^{3}H]glucose uptake into epididymal adipose tissue explants was significantly reduced (by 33%) by *Doc2b* deletion (Fig. 5c), whereas basal 2-deoxy[^{3}H]glucose uptake was unaffected by genotype. These data demonstrate that DOC2B is a positive regulator of adipocyte insulin action. Adipose tissue from both *Doc2b*KO and *Doc2a/Doc2b*KO mice displayed a similar degree of insulin resistance (Fig. 5c), suggesting that DOC2A does not play a direct role in intrinsic adipocyte function. These data are consistent with the lack of *Doc2a* expression in fat (Fig. 1b). Overall, these data suggest that the small defect in whole-body insulin action observed in the absence of *Doc2b* (Fig. 2e, f) is caused by impaired insulin-stimulated glucose uptake by adipose tissue. The small magnitude of this defect at the whole-body level is consistent with the quantitatively minor role played by adipose tissue in insulin-driven whole-body glucose clearance. To assess another facet of adipocyte insulin action, we measured plasma NEFA levels during an ITT. NEFA suppression by insulin was unaffected by *Doc2b* deletion (Fig. 5d), indicating that the adipose tissue defect in *Doc2b*KO mice is confined to insulin-stimulated glucose uptake.

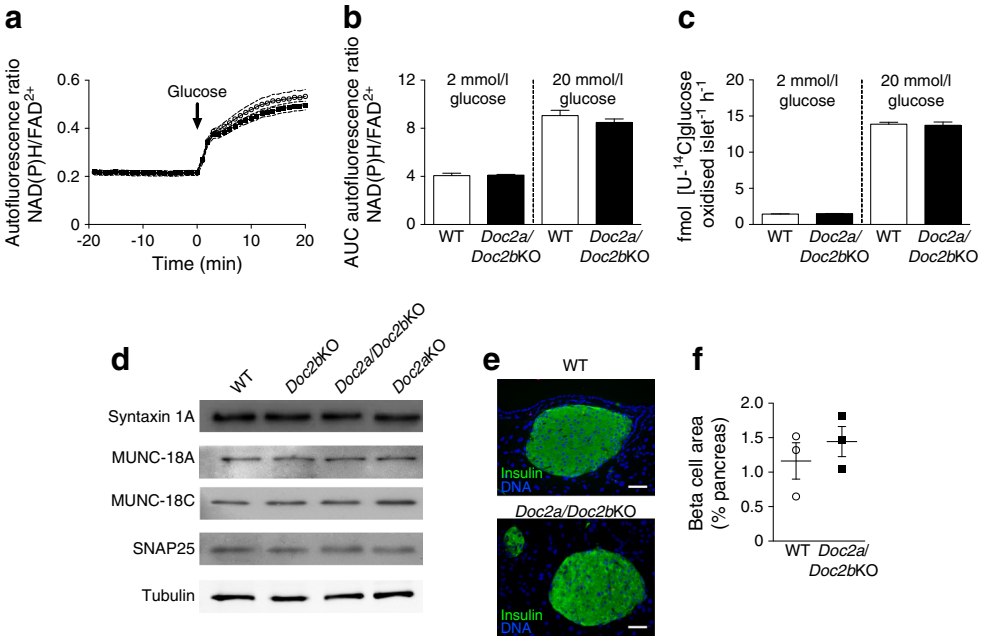

Fig. 4 Normal islet glucose metabolism, SNARE protein levels and beta cell mass in DOC2-null mice. (**a**, **b**) In vitro islet imaging of NAD(P)H and FAD^{2+} autofluorescence; n=5. (**c**) Oxidation of [U-^{14}C]glucose to $^{14}CO_2$ in islets. (**d**) Western blot quantification of proteins involved in beta cell exocytosis in islet lysates. (**e**) Pancreatic sections stained for insulin (green) and DNA (DAPI; blue); scale bar is 50 μm. (**f**) Quantification of the pancreatic area composed of insulin-positive beta cells; n=3, NS by Student's t test. Circle, WT; square, *Doc2a/Doc2b*KO

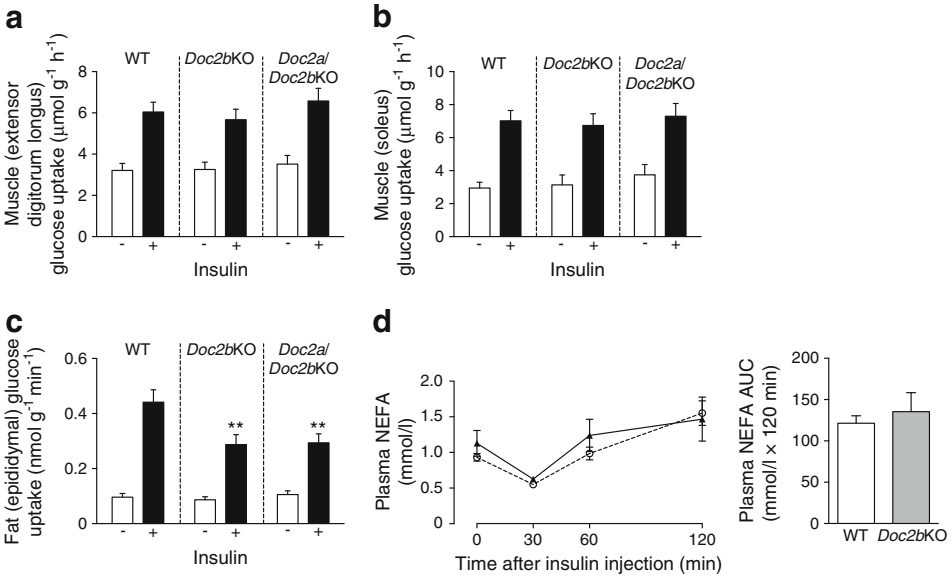

Fig. 5 Impaired insulin-stimulated glucose uptake in adipocytes from *Doc2b*KO mice. In vitro insulin-stimulated glucose uptake was assessed in (**a**, **b**) isolated muscle (n=11–13) and (**c**) epididymal fat (n=21–24). (**d**) In vivo insulin suppression of plasma NEFA (i.p. insulin 0.75 U/kg) following a 6 h fast (n=3). Effect of genotype assessed by (**d**) Student's t test (AUC) or (**a**–**c**) ANOVA. **p<0.01 vs WT insulin. Circle, WT; triangle, *Doc2b*KO

Deficient GLUT4 exocytosis in Doc2bKO adipocytes To investigate whether the reduced glucose uptake in adipose tissue is associated with deficient GLUT4 trafficking, MEFs were isolated from WT and *Doc2b*KO mice, differentiated into adipocytes and transfected with a dual-colour GLUT4 probe comprising pHluorin inserted into the first exofacial loop of GLUT4 and tdTomato at the C terminus [34]. The signal from pHluorin is sensitive to changes in GLUT4 localisation: it is low in intracellular compartments and high when exposed to the extracellular environment. In contrast, tdTomato fluorescence is insensitive to changes in GLUT4 localisation and thus provides a direct measure of total probe levels per cell. Thus, the pHluorin:tdTomato ratio provides an accurate indication of surface GLUT4 levels relative to total GLUT4. In WT adipocytes, insulin induced a robust increase in cell surface GLUT4, which was markedly disrupted by *Doc2b* deletion (Fig. 6a). These data reveal that adipocyte DOC2B protein expression is essential for normal insulin-stimulated GLUT4 exocytosis.

Insulin signalling and SNARE complex proteins are intact in DOC2-null adipocytes Using epididymal adipose tissue lysates, we measured levels of the insulin signalling proteins AKT and AS160/TBC1D4, along with SNARE complex components syntaxin-4A, MUNC-18C and VAMP2. VAMP2 protein levels were modestly enhanced in *Doc2b*KO adipose tissue, whereas levels of all other proteins were equivalent in WT, *Doc2b*KO and *Doc2a/Doc2b*KO adipose tissue (Fig. 6b). Therefore, the absence of DOC2B does not disrupt insulin signalling or the levels of GLUT4 or exocytosis proteins, consistent with a direct role for DOC2B in GLUT4 vesicle fusion.

Discussion

In this study, we used mice with *Doc2a*, *Doc2b*, or *Doc2a* and *Doc2b* deletions to provide physiological and biochemical evidence that DOC2 proteins play an important role in glucose homeostasis.

Deletion of both *Doc2a* and *Doc2b*, but not of either isoform alone, resulted in impaired biphasic GSIS in vivo and GSIS in vitro. This suggests that both DOC2 isoforms play synergistic roles in regulating beta cell function, with the presence of at least one isoform being necessary for adequate GSIS. We show that islets, similar to the brain, express both *Doc2a* and *Doc2b* isoforms, whereas most tissues only express *Doc2b*. As DOC2A is less sensitive than DOC2B to calcium [15], the presence of both isoforms may facilitate the appropriate secretory responses across the wide range of $[Ca^{2+}]_i$ that occurs in beta cells and neurons. In contrast, the higher calcium sensitivity of DOC2B [15] may facilitate efficient insulin action in cells in which fluctuations in $[Ca^{2+}]_i$ are relatively small. In such cells, DOC2B alone probably plays a role in regulated trafficking events, for example GLUT4 translocation in adipocytes. Similar types of regulated trafficking pathways have been documented in other cell types, for example renal water transport [39] and ion channel translocation in epithelial cells [40]. It will therefore be interesting to determine whether DOC2B plays a universal role in such pathways.

GSIS was significantly impaired in the absence of both DOC2A and DOC2B, although it was not completely abolished: GSIS was detectable in both *Doc2a/Doc2b*KO mice and isolated islets. Similarly, a previous study in neurons found that 50% of calcium-sensitive exocytotic events persisted after *Doc2a* and *Doc2b* gene deletion [13]. Therefore, the likely role of DOC2 proteins in beta cells is to

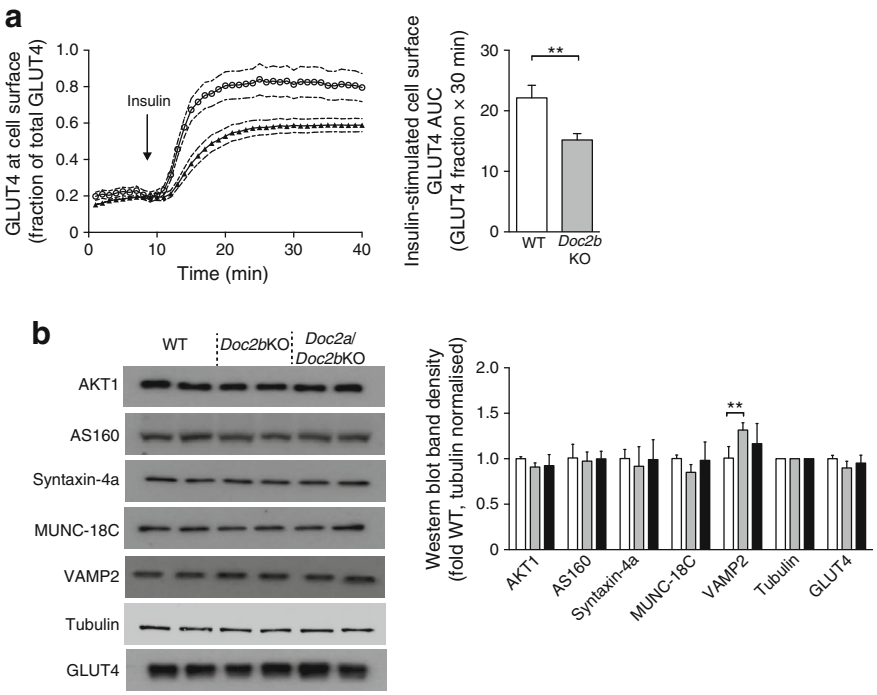

Fig. 6 Impaired GLUT4 exocytosis in *Doc2b*KO adipocytes. (**a**) Epifluorescence microscopy, showing GLUT4 cell membrane localisation in adipocytes derived from WT and *Doc2b*KO embryonic fibroblasts; n=17–18. (**b**) Western blot analysis of proteins involved in adipocyte insulin signalling and exocytosis using epididymal fat lysates; GLUT4, n=2; other proteins, n=4; mean ± SD. Genotype effect assessed by (**a**) Student's t test (AUC) or (**b**) ANOVA. **p<0.01. Circle/white bar, WT; triangle/grey bar, *Doc2b*KO; black bar, *Doc2a/Doc2b*KO

increase vesicle release probability and augment the secretory response to glucose.

Calcium sensors that control insulin secretion have long been sought. Synaptotagmin-7 contributes to beta cell calcium sensing [41], but other regulatory calcium sensors have yet to be identified [42]. DOC2A and DOC2B are calcium sensitive, and our current study implicates these proteins in beta cell calcium sensing. As both glucose and KCl increase $[Ca^{2+}]_i$, one would expect DOC2 proteins to be involved in insulin secretion induced by both stimuli. However, *Doc2a/Doc2b*KO islets showed defective GSIS and normal KCl-induced insulin secretion. As glucose activates metabolic pathways to induce heterogeneous $[Ca^{2+}]_i$ oscillations [43], whereas KCl chemically depolarises the PM to induce a $[Ca^{2+}]_i$ spike, DOC2 proteins may not be necessary for calcium-triggered insulin secretion per se. They may instead respond to a glucose-specific signal in beta cells to augment GSIS. This signal may be glucose-regulated $[Ca^{2+}]_i$ oscillations, $[Ca^{2+}]_i$ hotspots, or an alternative second messenger such as cAMP or diacylglycerol, both of which exhibit increased concentration close to the beta cell PM in response to glucose [44, 45]. In support of the latter possibility, diacylglycerols are thought to increase the association of DOC2 with the MUNC13-1 vesicle transport regulatory protein [19]; disrupting this interaction inhibits neurotransmitter release in rat neurons [20]. MUNC13-1 is expressed in islets and positively regulates GSIS [46–48]. It is therefore likely that MUNC13-1 and DOC2 isoforms act in concert to promote insulin granule exocytosis.

In contrast to our study, there was a recent report of defective biphasic GSIS and defective KCl-triggered insulin secretion in islets from *Doc2b*KO mice [27]. Curiously, we only observed a subtle defect in second-phase GSIS in vivo in *Doc2b*KO mice, and failed to observe a significant defect in insulin secretion from isolated *Doc2b*KO islets. An earlier study demonstrated that GSIS was impaired in DOC2B-depleted Min6 cells, although KCl-triggered insulin release was normal [24]. While the explanation for these contradictory data remains to be established, our study clearly demonstrates that loss of both DOC2 isoforms severely impairs GSIS and glucose tolerance. Therefore, these studies collectively indicate an important role for DOC2 in beta cells.

In adipocytes, we found DOC2B to be a critical, positive regulator of insulin-stimulated GLUT4 translocation to the PM and of glucose uptake. As calcium has been implicated in GLUT4 exocytosis [7, 8] our current study, taken together with others [25, 26], suggests that DOC2B may be the calcium sensor that regulates GLUT4 trafficking in adipocytes. Intriguingly, we were unable to identify a role for DOC2B in insulin-stimulated glucose uptake in skeletal muscle. This contradicts another study [27], but is consistent with our observation of only subtle defects in glucose tolerance and in vivo insulin action in *Doc2b*KO mice. Interestingly, the major difference between WT and *Doc2b*KO mice in the Ramalingam et al study [27] was observed during the latter phase of the ITT, whereas the initial slope of the ITT (0–30 min) did not differ between WT and *Doc2b*KO animals. As the early phase of an ITT is principally governed by peripheral insulin action, whereas the latter phase is influenced by counter-regulatory responses, these data suggest that muscle insulin sensitivity was also largely intact in these *Doc2b*KO

animals. The ability of DOC2B to regulate GLUT4 translocation in adipocytes, but not in muscle, may indicate that differences in $[Ca^{2+}]_i$ and calcium dynamics between these cell types [49] require different sets of calcium-sensitive proteins. One question that deserves further investigation is whether DOC2C fulfils the same role in muscle GLUT4 trafficking as DOC2B does in adipocytes.

Finally, we observed a small but significant increase in body fat accompanying *Doc2b* deletion, suggesting that DOC2B may play an additional role in the central or peripheral control of adiposity. Targeted experiments are therefore required to identify the underlying DOC2B-regulated homeostatic pathways responsible for this phenotype.

In conclusion, our study has identified and characterised a novel synergistic role for DOC2A and DOC2B in regulating insulin secretion, and for DOC2B in regulating GLUT4 vesicle exocytosis in adipocytes, thereby revealing dual roles for DOC2 isoforms in regulating glucose homeostasis.

Acknowledgements We thank T. Südhof (Stanford University, CA, USA) for providing antibodies. We thank M. van de Bunt and A. Gloyn (University of Oxford, UK) for assistance with RNA-seq data analysis.

Funding This work was supported by a National Health and Medical Research Council of Australia (NHMRC) program grant (DEJ) and project grant (JC), and by the Chinese Scholarship Council (JL). DEJ is an NHMRC Senior Principal Research Fellow.

Duality of interest The authors declare they have no duality of interest associated with this manuscript.

Contribution statement DEJ conceived the studies. JC wrote the manuscript, with critical input from all authors. DEJ, JC, JL, JGB and JS designed experiments and interpreted data. JC, JL, JGB, JS, CCM, PTW, HP and RC designed experiments, and acquired and analysed data. MV and AJAG contributed to the acquisition and interpretation of data. All authors approved the final manuscript. DEJ is the guarantor of this work.

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