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SUPPLEMENTARY MATERIALS

DOC2b Enhances β-Cell Function Via A Novel Tyrosine Phosphorylation-Dependent Mechanism

Diti Chatterjee Bhowmick[1], Arianne Aslamy[2], Supriyo Bhattacharya[3], Eunjin Oh[1], Miwon Ahn[1], and Debbie C. Thurmond[1]

[1]Department of Molecular & Cellular Endocrinology, Diabetes and Metabolic Research Institute, Beckman Research Institute of City of Hope, Duarte, CA; [2]Department of Medicine, Cedars-Sinai Medical Center, West Hollywood, CA;[3]Department of Computational and Quantitative Medicine, City of Hope, Duarte, CA.

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Supplemental Table 1. Non-diabetic human islet donor information

ID#	Sex	Age	BMI	HbA1c%; mmol/mol	Used for
SAMN15314807	Male	27 Y	25.30	5.2; 33	Figure 1D
SAMN15337453	Male	26 Y	24.20	5.2; 33	Figure 1D
HU1206	Female	51 Y	25.16	5.6; 38	Figure 1D, 8A
HD1181	Male	28 Y	30.75	5.2; 33	Figure 6A
SAMN19470079	Male	39 Y	27.60	5.5; 37	Figure 6A
HU1221	Male	61 Y	29.34	5.9; 41	Figure 6A
SAMN13570019	Female	37 Y	24	5.2; 33	Figure 7A
SAMN14120450	Male	37 Y	31.9	5.5; 37	Figure 7A
HU1205	Female	42 Y	31.2	<5.7; <39	Figure 7A
SAMN17367763	Male	56 Y	33.0	5.3; 34	Figure 8A
SAMN18196260	Female	41 Y	28.0	5.3; 34	Figure 8A
SAMN21399152	Male	29 Y	22.9	5.3; 34	Suppl. Fig 5A
SAMN22814513	Male	26 Y	29.2	5.4; 36	Suppl. Fig 5A
SAMN22818629	Female	45 Y	21.7	5.5; 37	Suppl. Fig 5A
HU1232	Female	45Y	25.2	4.9; 30	Suppl. Fig 5A

Supplemental Table 2. Antibody information

#	Antibody Name	Vendor	Cat#	Dilution used
1.	Mouse anti-phosphotyrosine (pTyr) clone 4G10	EMD Millipore	05-321	1:1000
2.	Rabbit anti-DOC2b	In-house		1:1000
3.	Rabbit anti-phospho-Src^{Y416} ; (pSRC ^{Y416})	Cell Signaling Technology	2101S	1:1000
4.	Rabbit anti-GFP	Abcam	ab69314	1:1000
5.	Mouse anti-GFP	Takara Bio	632381	1:1000
6.	Rabbit anti-ezrin/radixin/moesin; (ERM)	Cell Signaling Technology	3142	1:1000
7.	Rabbit anti-phospho-ezrin (T567)/radixin (T564)/moesin (T558); (pERM)	Cell Signaling Technology	3141	1:1000
8.	Mouse anti-Radixin	Invitrogen	MA5-17245	1:1000
9.	Mouse anti-YES	BD Biosciences	BDB610375	1:200
10.	Rabbit anti-Syntaxin 4 (STX4)	In-house		1:5000
11.	Mouse anti-Tubulin	Sigma	T5168	1:20,000
12.	Mouse anti-GAPDH	Invitrogen	AM4300	1:20,000
13.	Mouse anti-Src	Cell Signaling	2110S	1:1000
14.	Rabbit anti FYN	Cell Signaling	4023S	1:1000
15.	Mouse anti-VAMP2	Synaptic Systems	104211	1:5000
16.	Rabbit anti-VAMP2	Abcam	Ab181869	1:2000

Supplemental Table 3. Potential DOC2B binding proteins identified by mass spectrometry

			Exclusive unique peptide count	
#	**Protein name**	**Alternate ID**	**DOC2b-GFP+Glucose**	**DOC2b-GFP+pV**
*	Double C2-like domain-containing protein beta	DOC2b	37	42
1	Protein unc-13 homolog A	Unc13a	15	4
2	Kinesin-1 heavy chain	Kif5b	36	46
3	Tropomodulin-2	Tmod2	27	28
4	Tropomodulin-3	Tmod3	25	20
5	Radixin	Rdx	20	25
6	Kinesin light chain 2	Klc2	19	14
7	Ezrin	Ezr	9	10
8	Moesin	Msn	9	11
9	Dynamin 1	Dnm1	4	4
10	Tyrosine-protein kinase Yes	Yes1	20	20

*DOC2B-GFP was successfully expressed and captured (positive control). Listing of DOC2b binding proteins captured by mass spectrometry include only those with >3 peptides under stimulated conditions (glucose, pV) and without peptides in GFP+Glucose/GFP+pV conditions.

Supplemental Information

Phosphoenrichment:

MIN6 β-cells were incubated in a serum and glucose-free medium as described for the insulin secretion assay, followed by glucose stimulation (20 mM, 2 min) or pervanadate treatment (0.5 mM, 5 min). For detection of phosphorylated proteins, the cells were washed twice with ice-cold 50 mM HEPES buffer (pH 7.0) and Phosphoprotein enrichment analysis was conducted using the Pierce Phosphoprotein Enrichment Kit (Cat# 9003) following the manufacturer's instructions.

Subcellular Fractionation:

Subcellular fractionation was performed at 4 °C using a previously reported protocol (1). Briefly, after glucose (20mM, 2 min) or pV treatment (0.5mM, 5 min) MIN6 β-cells were washed twice with cold HEPES buffer (50mM, pH 7.0) and harvested with 1 ml of homogenization buffer (20 mM Tris-HCl [pH 7.4], 0.5 mM EDTA, 0.5 mM EGTA, 250 mM sucrose, 1 mM DTT, 100 mM PMSF, and protease inhibitor cocktail tablets [ROCHE]). Cell lysates were disrupted by 10 strokes through a 27-gauge needle and centrifuged at 0.9 × g for 10 min. Postnuclear supernatants were centrifuged at 5500 ×g for 15 min, and the subsequent supernatant was centrifuged at 24,000 × g for 20 min to obtain the secretory granule fraction in the pellet. To isolate the PM fraction, the post-nuclear pellet was combined with 1 ml of Buffer A (10 mM Tris-HCl [pH 7.4], 0.25 M sucrose, 1 mM MgCl2) and 2 ml of Buffer B (10 mM Tris-HCl [pH 7.4], 2 M sucrose, 1mM MgCl2). The resulting mixture was overlaid with Buffer A and centrifuged at 113,000 × g for 1 h. The sucrose gradient achieved with Buffers A and B yielded an interface containing the PM fraction. This interface was collected, washed twice with homogenization buffer, and collected via

centrifugation at 6000 × g for 10 min. Both the PM and secretory granule pellets were resuspended in 1% Nonidet P-40 lysis buffer and stored at -80 °C until further analysis.

Confocal Microscopy:

MIN6 cells plated onto glass coverslips were incubated in serum and glucose free MKRBB for 2 h and treated for 5 min with 0.1 mM pV and with or without 20 μM SU6656. The cells were immediately fixed and permeabilized in 4% paraformaldehyde plus 0.1% Triton X-100 for 10 min at room temperature. The fixed and permeabilized cells were blocked in a 1% BSA, 5% donkey serum solution for 1 h at room temperature followed by incubation with rabbit anti-phospho-SFK Y^{416} (1:100, cat# 2101S, Cell Signaling Technology) or YES kinase (1:50, cat# BDB610375) overnight at 4 °C. The cells were then washed 3 times with PBS and incubated with an Alexa Fluor 647 secondary antibody (Invitrogen) at 1:300 or Alexa Fluor 488 secondary antibody (Invitrogen) at 1:100 for 1 h at room temperature. The cells were washed 3 times with PBS; during the second wash, DAPI was added to stain nuclei. Coverslips were mounted onto the slides using Vectashield for confocal fluorescence microscopy. Fluorescent images from at least five fields for each condition were captured using a Zeiss 700 confocal microscope (single-channel scanning, 20X objective). ImageJ (National Institutes of Health) was used to prepare the final images.

Human islet perifusion:

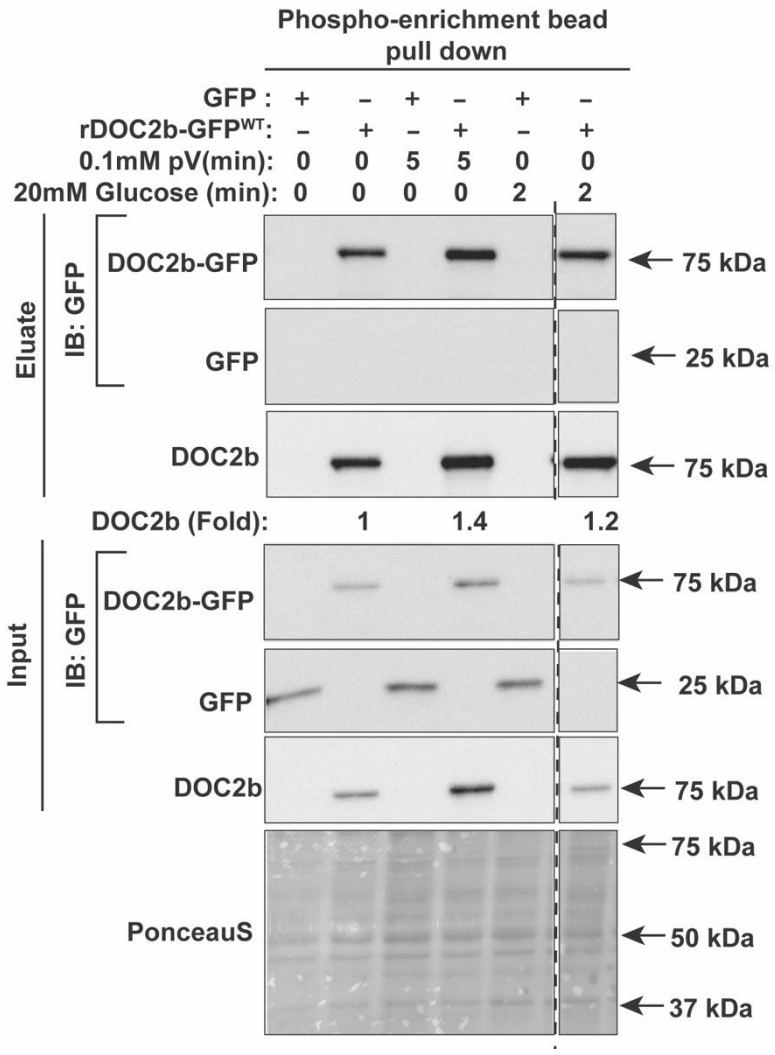
Human islets were obtained through the Integrated Islet Distribution Program. Human islets were allowed to recover in CMRL medium for 2 hours, and then handpicked. Islets were immediately transduced (multiplicity of infection 100) with Adenovirus: Ad. GFP or Ad.rDOC2b-GFP^{WT} or Ad.rDOC2b-GFP^{Y301F} for perifusion analysis as described (2, 3). Insulin content from donor islets

were solubilized in Nonidet P-40 lysis buffer. Insulin was quantitated by human insulin ELISA kit (Mercodia, Cat. # 10-1113-01).

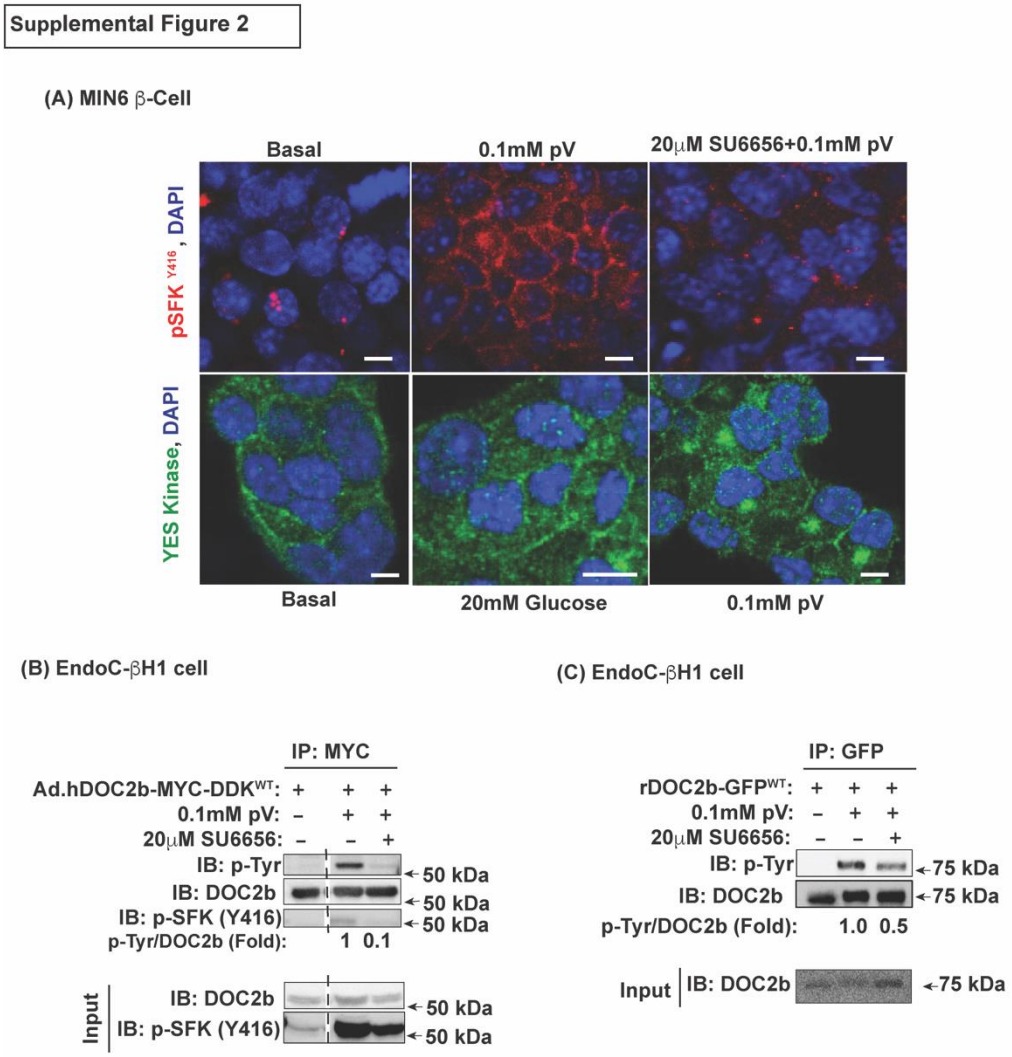
Reference

1. Yoder, S. M., Dineen, S. L., Wang, Z., and Thurmond, D. C. (2014) YES, a Src family kinase, is a proximal glucose-specific activator of cell division cycle control protein 42 (Cdc42) in pancreatic islet beta cells. *J Biol Chem* **289**, 11476-11487
2. Spurlin, B. A., and Thurmond, D. C. (2006) Syntaxin 4 facilitates biphasic glucose-stimulated insulin secretion from pancreatic beta-cells. *Mol Endocrinol* **20**, 183-193
3. Oh, E., Stull, N. D., Mirmira, R. G., and Thurmond, D. C. (2014) Syntaxin 4 up-regulation increases efficiency of insulin release in pancreatic islets from humans with and without type 2 diabetes mellitus. *J Clin Endocrinol Metab* **99**, E866-870

Supplemental Figure 1

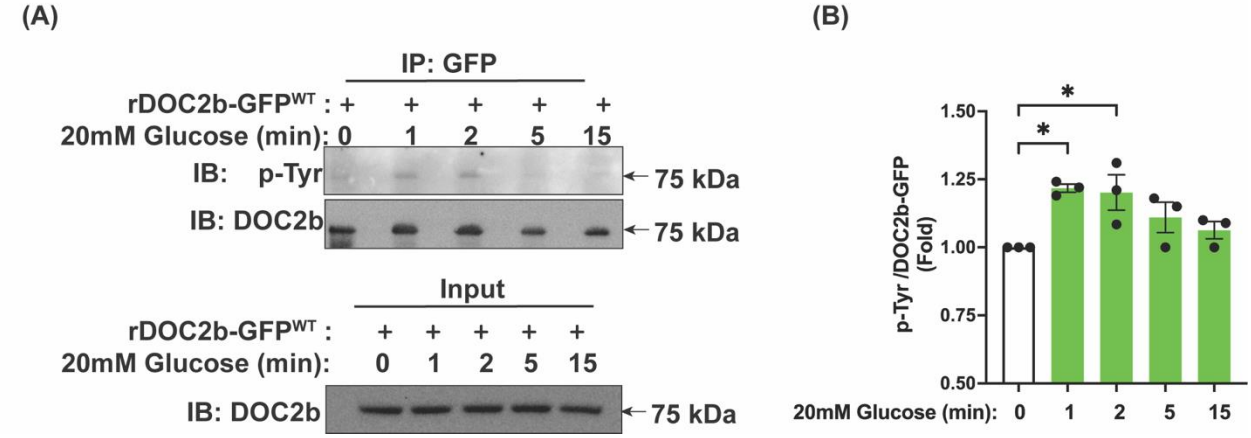


Suppl. Figure 1. Phosphoprotein enrichment shows increased DOC2b phosphorylation after glucose stimulation and pervanadate (pV) treatment. Representative western blots showing rDOC2B-GFP^{WT} phosphorylation in MIN6 β-cells after glucose (20 mM, 2 min) or pervanadate (pV; 0.1 mM, 5 min) treatment evaluated using a phosphorylated protein enrichment column followed by immunoblot (IB) analysis. Vertical lines denote splicing of lanes from within the same gel exposure. Ponceau S staining shows gel loading. Data shown are representative of three independent experiments.



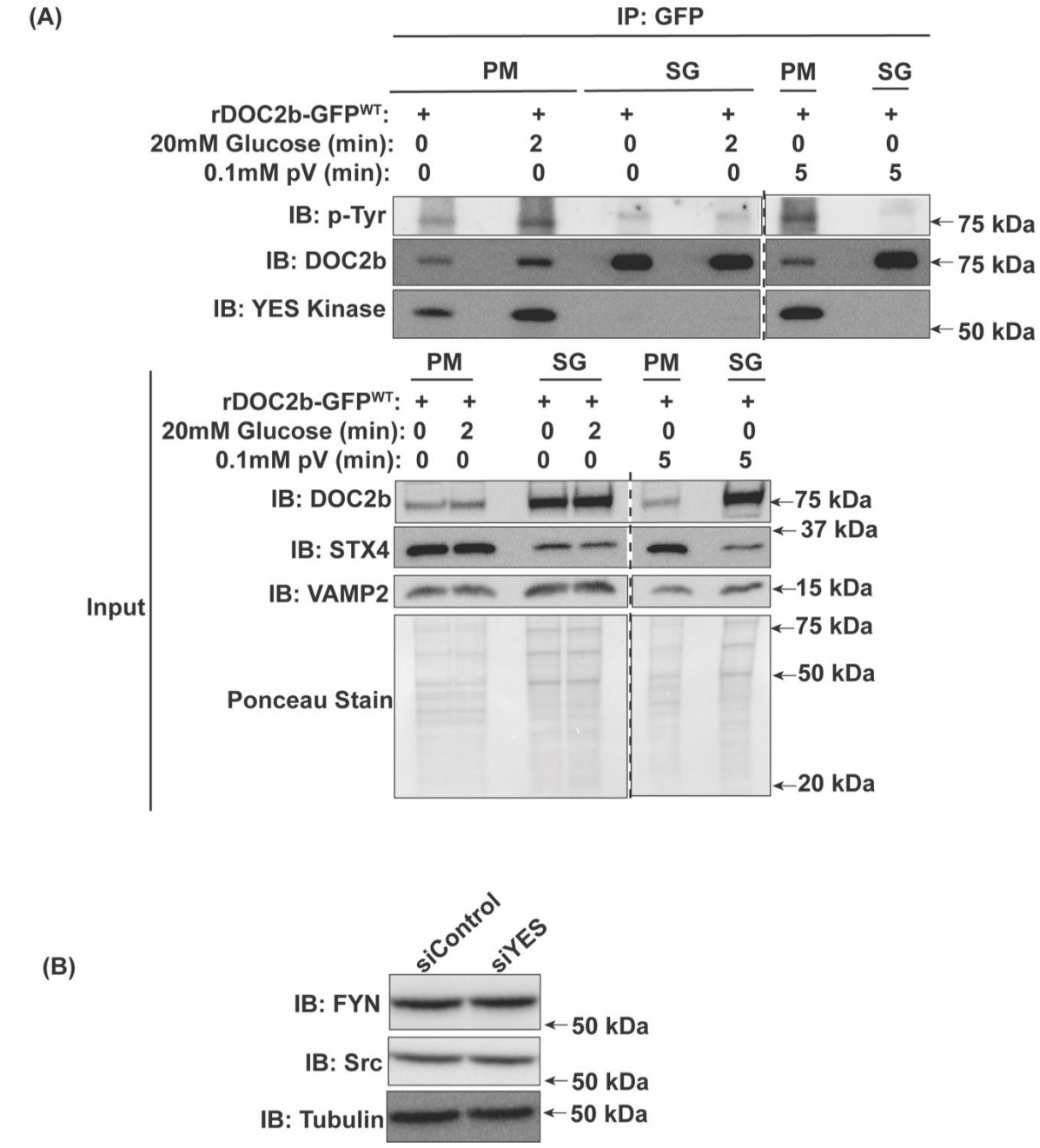
Suppl. Figure 2. Src Family Kinase activity is required for the pervanadate-induced tyrosine phosphorylation of DOC2b in human EndoC-βH1 cells. (A) Representative confocal micrographs of MIN6 β-cells treated with vehicle or 0.1 mM pervanadate (pV; 5 min) with or without 20 μM SU6656 (2h), or with 20 mM glucose (2 min) and immunostained for pSFK^{Y416}(red) or YES kinase (green). DAPI was used for nuclear staining (blue). Scale bar = 10 μm. (B) Representative immunoblots (IB) of human EndoC-βH1 cells transduced with Ad.hDOC2b-Myc-DDK^{WT}, treated with vehicle or 0.1 mM pervanadate (pV; 5 min) with or without 20 μM SU6656 (1h) followed by immunoprecipitation (IP) and IB analysis. (C) Representative IB of human EndoC-βH1 cells transduced with Ad.rDOC2b-GFP^{WT} and treated with vehicle or 0.1 mM pV (5 min) with or without 20 μM SU6656 (1h) followed by IP and IB analysis. Vertical lines denote splicing of lanes from within the same gel exposure. Data are representative of two independent experiments.

Supplemental Figure 3



Suppl. Figure 3. Time course of glucose-stimulated tyrosine phosphorylation of DOC2b in β-cells. (A): Representative immunoblot (IB) images of MIN6 β-cells transfected with DOC2b-GFP^{WT} plasmid and treated with 20 mM glucose for the designated time followed by immunoprecipitation (IP) and IB analysis. Input shows similar DOC2b protein content in each IP reaction. (B): Quantification of the IBs from three independent experiments. Data are shown as mean ± SEM; *$p<0.05$.

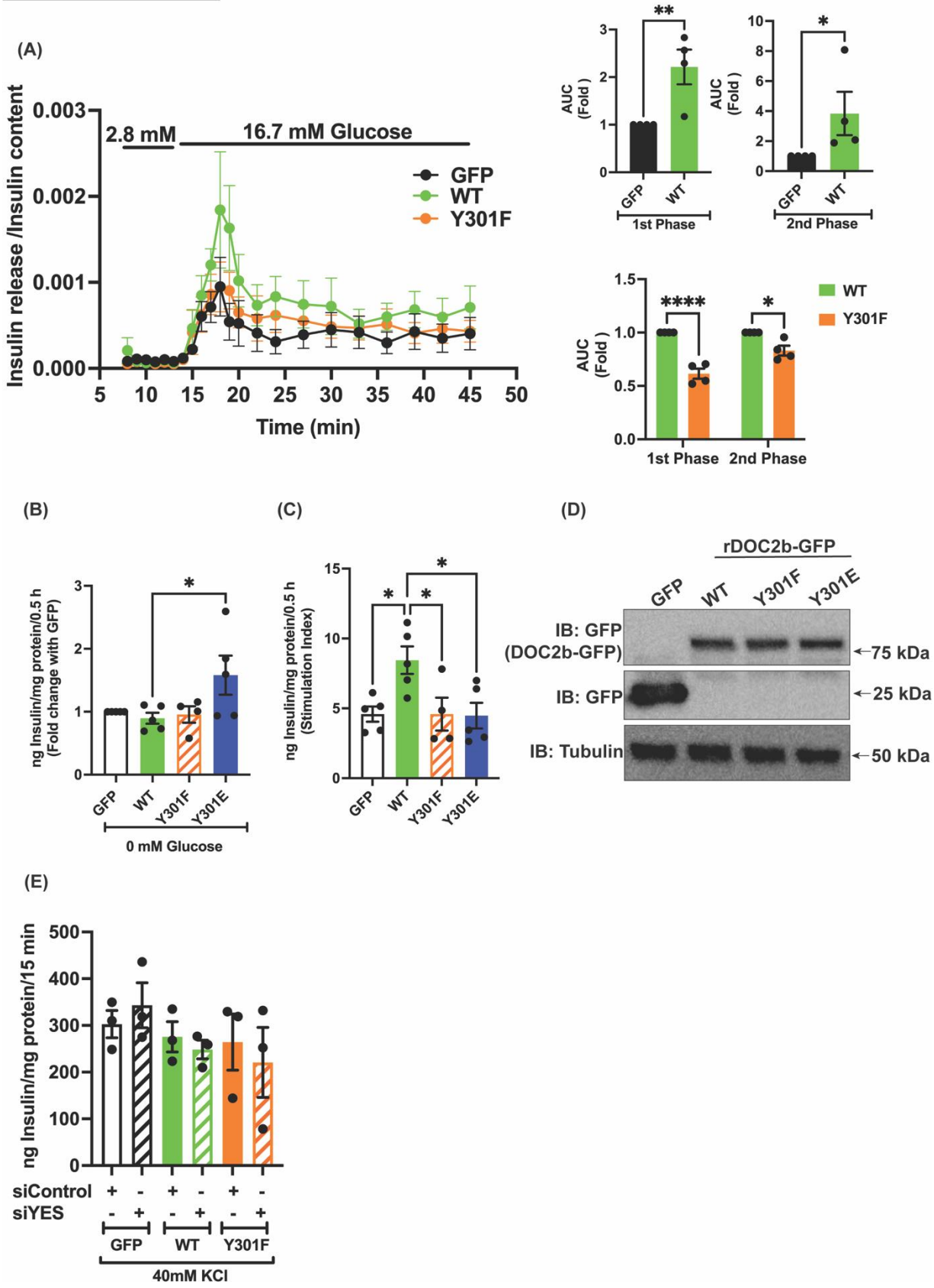
Supplemental Figure 4



Suppl. Figure 4. The tyrosine phosphorylated pool of exogenously expressed DOC2b resides at the plasma membrane together with the YES kinase in MIN6 β-cells. (A) Representative immunoblots (IB) of plasma membrane (PM) and secretory granule (SG) fractions of MIN6 β-cells transfected with rDOC2b-GFP^{WT} plasmids and treated with 20 mM glucose or 0.1 mM pV for the designated time followed by immunoprecipitation (IP) and IB analysis. Anti-STX4 and anti-VAMP2 (Abcam) antibodies were used to validate the integrity of the PM and SG fractions

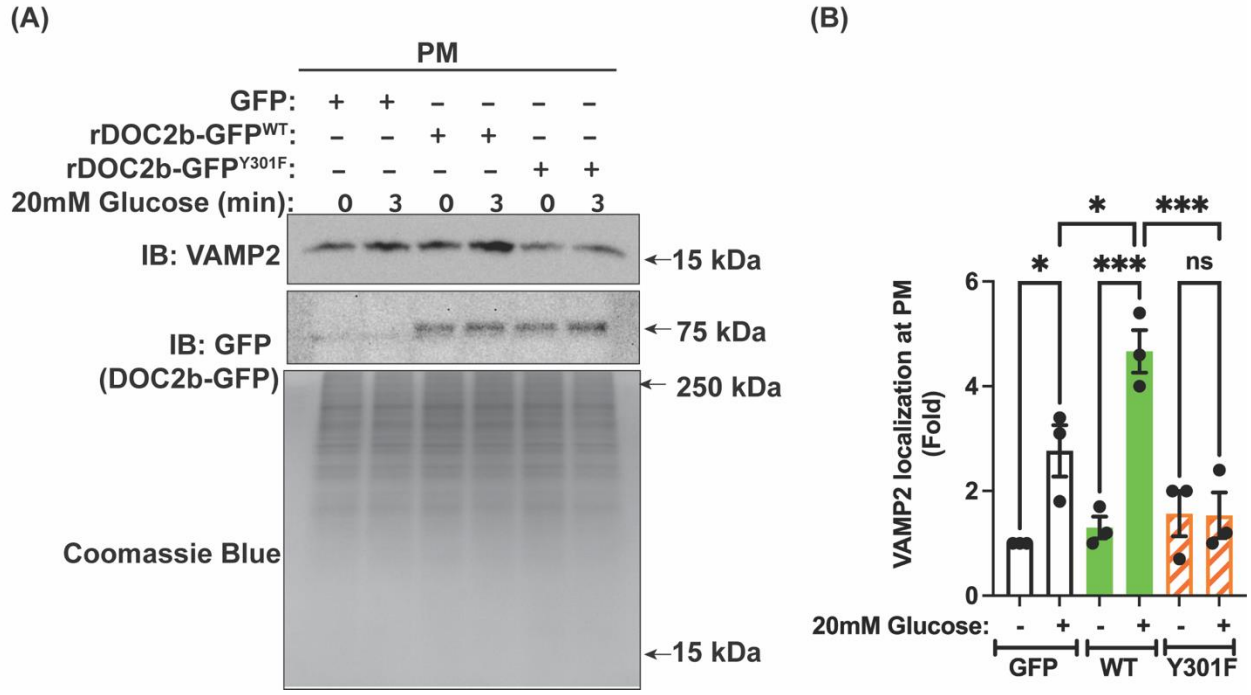
respectively. Ponceau S staining was used to assess gel loading in input lysate analysis. Vertical lines denote splicing of lanes from within the same gel exposure. (B) Representative IB of MIN6 β-cells transfected with siControl or siYES oligonucleotides for 48 h. Data are representative of three independent experiments.

Supplemental Figure 5



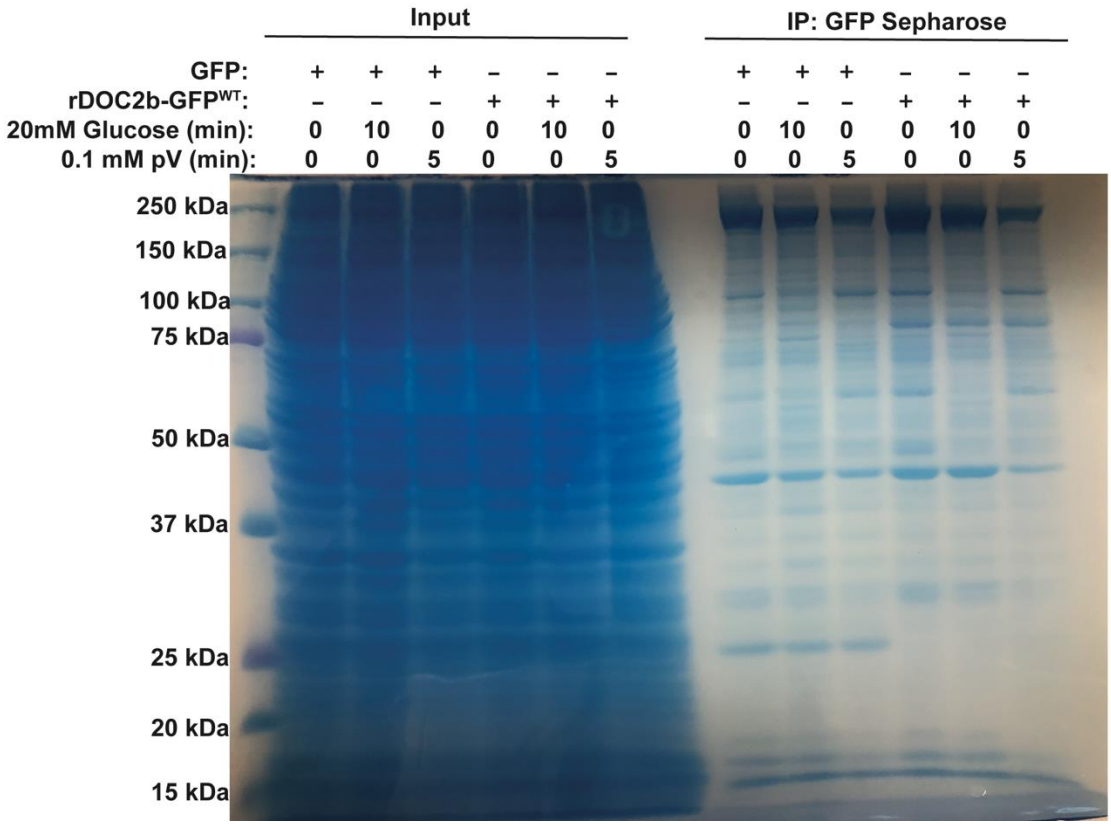
Suppl. Figure 5. Y301 phosphorylation is important for DOC2b-mediated boosting of biphasic GSIS in β-cells. (A) Perifusion traces from of human islets obtained from four independent non-diabetic donors. Upon arrival from the islet centers, healthy human islets were sized and sorted into three equal groups for immediate transduction with control Ad.GFP or rDOC2b-GFP$^{WT/Y301F}$ adenoviruses. 48 h later the islets were perifused in parallel chambers with 2.8 mM glucose, then stimulated with 16.7 mM glucose as depicted. Insulin was quantified using a human insulin ELISA kit (Mercodia, Cat. # 10-1113-01). AUC analysis was calculated for each donor islet phase of secretion and normalized to Ad.GFP or Ad.rDOC2b-GFP^{WT} as indicated in the bar graphs. (B) Basal insulin release of MIN6 β-cells transfected with GFP/rDOC2b-GFP^{WT}/rDOC2b-GFP^{Y301E}/ rDOC2b-GFP^{Y301F}. (C) Stimulation index (SI) from GSIS assay of MIN6 β-cells transfected with GFP/rDOC2b-GFP^{WT}/rDOC2b-GFP^{Y301E}/ rDOC2b-GFP^{Y301F}. (D) Representative immunoblot (IB) images of MIN6 β-cells transfected with GFP/rDOC2b-GFP^{WT}/rDOC2b-GFP^{Y301E} /rDOC2b-GFP^{Y301F}. Data are representative of 4-5 independent experiments. (E) KCl-stimulated insulin secretion from MIN6 β-cells transfected with siControl/siYES together with GFP/ rDOC2b-GFP^{WT}/rDOC2b-GFP^{Y301F}. KCl stimulation was performed for 15 min. Insulin was quantified using a Mouse Insulin ELISA (Alpco, Salem, NH, cat# 80-INSMSH-E01). GSIS/KSIS data represent 3-5 independent experiments using different cell passages. Data are shown as mean ± SEM; *$p<0.05$; **$p<0.002$; ****$p<0.0001$.

Supplemental Figure 6



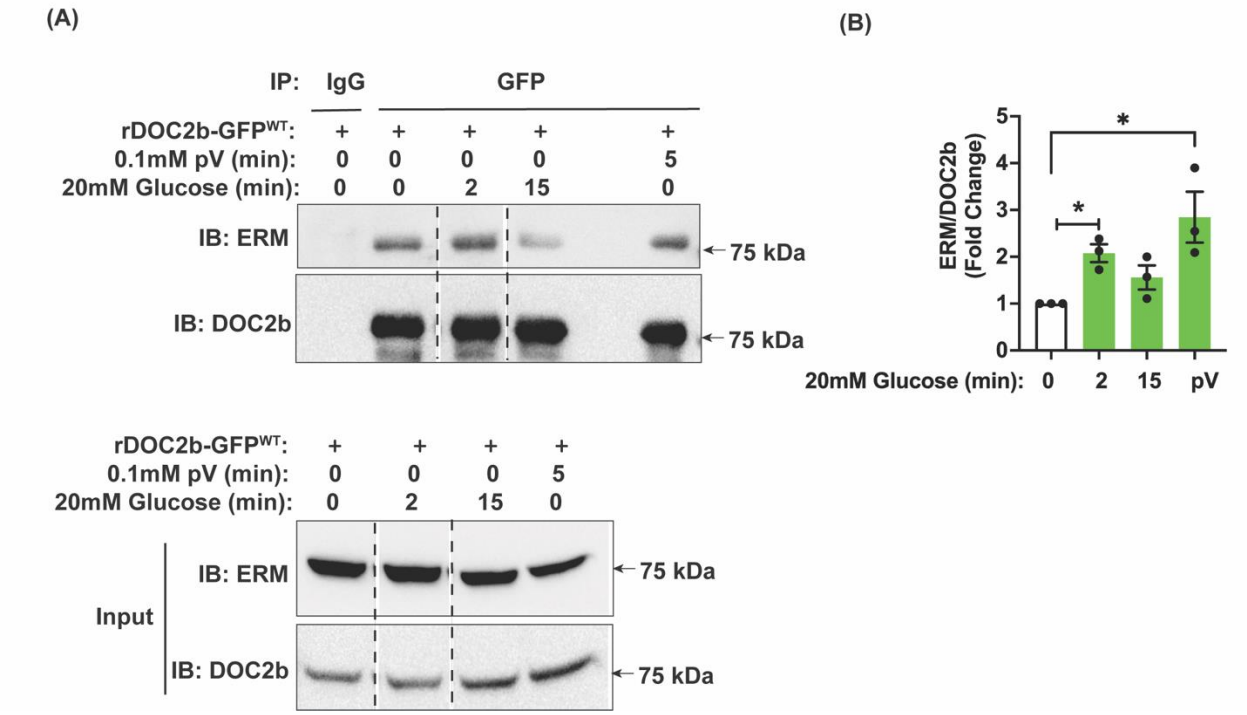
Suppl. Figure 6. Y301 phosphorylation is required for DOC2b's beneficial action on glucose stimulated ISG granule abundance at the PM of β-cells. (A) Representative western blot images of plasma membrane (PM) enriched fractions of MIN6 β-cells transduced with adenoviruses: GFP/rDOC2b-GFP^{WT}/rDOC2b-GFP^{Y301F}. 48 h following transduction the cells were treated with or without 20mM glucose for 3 min followed by subcellular fractionation and immunoblot analysis (IB). 15% SDS-PAGE and 4 µg of PM fraction protein was used to evaluate VAMP2 localization. Coomassie blue staining was used to determine the loading in the western blot (15% SDS-PAGE). (B) Quantification of the IBs from three independent experiments. Data are shown as mean ± SEM; *p<0.05, ***p<0.001.

Supplemental Figure 7



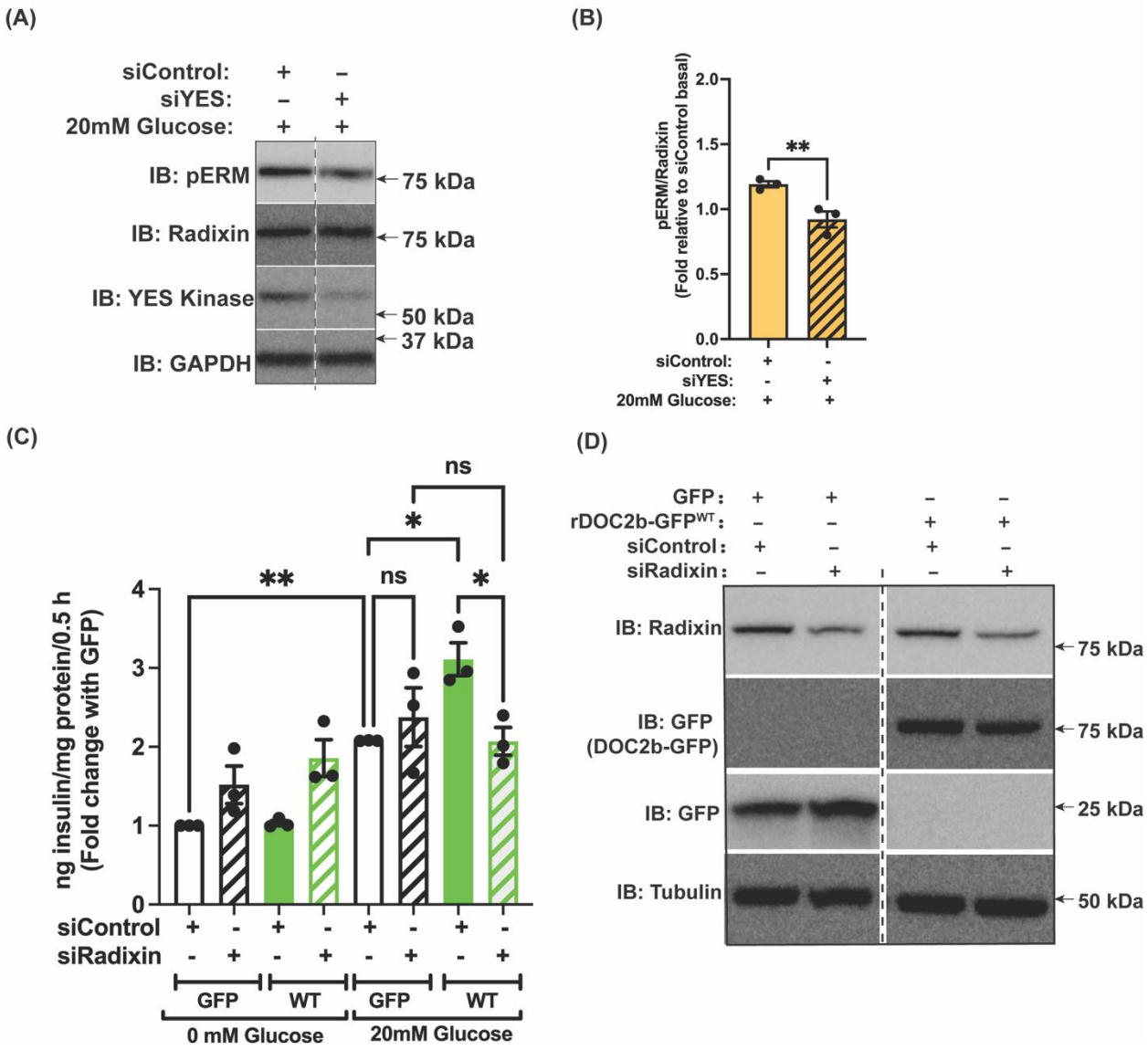
Suppl. Figure 7. Representative gel image of MIN6 β-cells transfected with either GFP or rDOC2b-GFP^{WT} and subsequently treated with or without glucose (20 mM) or pervanadate (0.1 mM), lysates prepared and used for immunoprecipitation (IP). The gel was stained with SIMPLY BLUE SAFE stain and imaged to demonstrate the protein profiles from immunoprecipitation (IP) experiments. Proteins spanning 15-250 kDa were dissected out for mass spectrometry analysis.

Supplemental Figure 8



Suppl. Figure 8. Time course of glucose-stimulated tyrosine phosphorylation of DOC2b in β-cells. (A) Representative immunoblots (IB) of MIN6 β-cells transfected with rDOC2b-GFP^{WT} and treated with 20 mM glucose or 0.1 mM pervanadate (pV) for the designated times followed by immunoprecipitation (IP) and IB analysis. Vertical lines denote splicing of lanes from within the same gel exposure. (B) Quantification of IB from three independent experiments. Data are shown as mean ± SEM; *$p<0.05$.

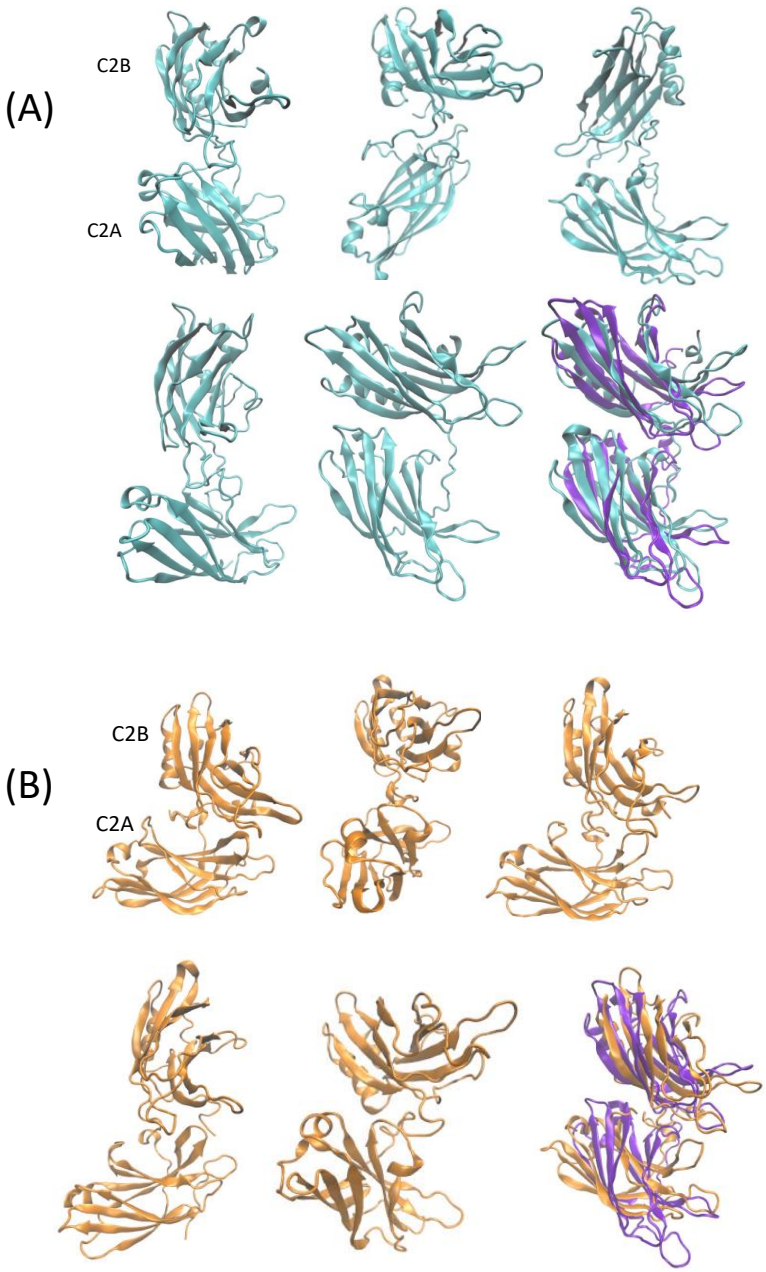
Supplemental Figure 9



Suppl. Figure 9. YES kinase and Radixin are required for DOC2b-mediated boosting of GSIS in β-cells. (A) Representative immunoblot (IB) images of MIN6 β-cells transfected with siControl/siYES kinase for 48 h and stimulated with 20 mM glucose for 10 min. Data are representative of 3 independent experiments using different cell passages. (B) Quantification of IB from three independent experiments. Data are shown as mean ± SEM; *p<0.05. (C) GSIS of MIN6 β-cells transfected with siControl/siRadixin together with GFP/ rDOC2b-GFP^{WT}. 48 h following transfection, GSIS was performed for 30 min. Insulin was quantified using a Mouse Insulin ELISA (Alpco, Salem, NH, cat# 80-INSMSH-E01). (D) Representative IB of MIN6 β-cells transfected with siControl/siRadixin together with GFP/ rDOC2b-GFP^{WT}. Data are

representative of 3 independent experiments. Anti-GAPDH/tubulin antibodies were used as loading controls. Data are shown as mean ± SEM; *$p<0.05$; **$p<0.002$, ns= not significant.

Supplemental Figure 10



Suppl. Figure 10. Example DOC2B conformations sampled during MD shown as representative structures from the clustering of MD trajectories. (A) human, (B) rat. The conformations representing the alpha-fold structures are shown on the bottom right in each sub-panel. The alpha-fold structure (in violet) is overlaid for comparison.