Supplemental Figures to Maritzen et al. *'Role of the vesicular chloride transporter ClC-3 in neuroendocrine tissue'*

Figure S1.

Characterization of a new ClC-3 specific antibody

A Western blots of kidney membranes (30 µg/lane) from WT and ClC-3 KO mice to test the specificity of the new anti-ClC-3 antibodies in comparison to the 3A4 antibody described previously (Stobrawa et al., 2001). Sera obtained from two rabbits (1035, 1036, indicated above) that had been immunized with a mixture of two ClC-3 peptides were purified against either peptide (C3/03A or C3/03B) as indicated. When purified against the C3/03A peptide, either serum recognized a band of the correct size (indicated by arrow) in WT, but not KO kidney extract. Purification against peptide C3/03B gave antibodies that reacted only poorly in Western blots. Therefore, sera purified against peptide A were used in further studies. ***B-G*** Test of the new antibody (#1036 serum purified against peptide A in KO-controlled immunohistochemistry. Stained WT and KO paraffin-embedded sections of perfusion-fixed brain (hippocampal CA3 region) ***B***, anterior pituitary ***C***, posterior pituitary ***D***, pancreatic islet ***E***, adrenal gland ***F*** and thyroid ***G*** . Scale bars: 25 µm.

Figure S2.

Strong expression of ClC-3 in retina (*A*) and in pancreatic islets (*B*)

Aa Strong ClC-3 staining is observed in the inner and outer plexiform layer which is also positive for synaptophysin. ***Ab*** shows the inset in right panel of ***Aa*** magnified. ***Ac*** no ClC-3 staining is detected in *Clcn3*$^{-/-}$ retina, demonstrating the specificity of the antibody. Scale bar: 100 µm. (OS: outer layer of photoreceptors, ONL: outer nuclear layer, OPL: outer plexiform layer, INL: inner nuclear layer, IPL: inner plexiform layer, GCL: ganglion cell layer). ***B*** ClC-3 is expressed in all secretory cell types of pancreatic islets, as shown by co-staining for insulin (labels β-cells) ***Ba***, glucagon (labels α-cells) ***Bb***, somatostatin (labels δ-cells) ***Bc***, and pancreatic polypeptide (labels PP cells) ***Bd***.

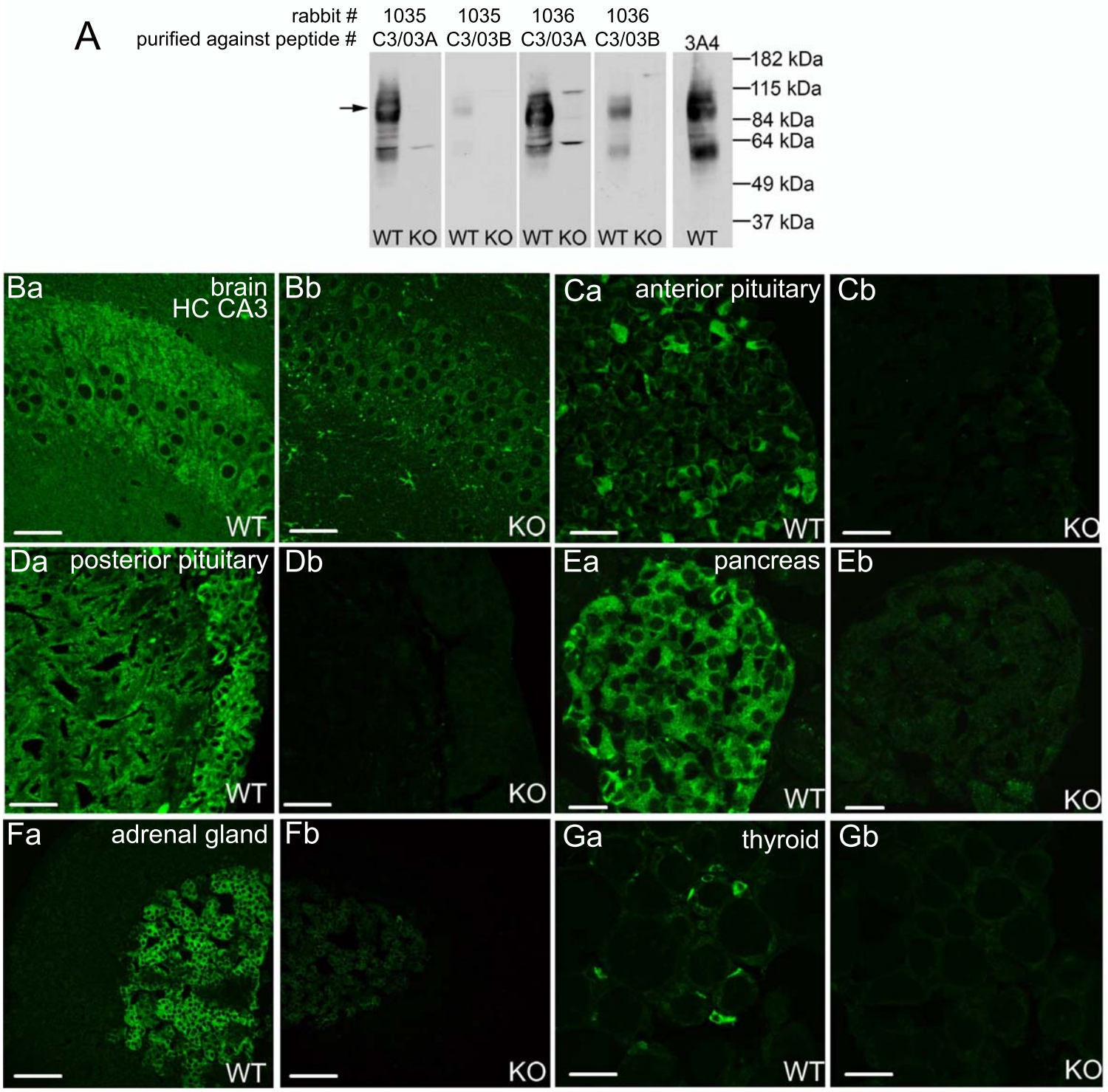
Figure S3.

Expression of intracellular CLC proteins in β-cell lines, and subcellular localization of ClC-3 in Rinm5F cells and in primary β-cells

Aa Western blot of insulin-secreting cell lines for vesicular CLC proteins and marker proteins. 30 µg of INS-1E and Rinm5F membrane preparations were separated by SDS-PAGE, transferred to nitrocellulose and probed with antibodies directed against ClC-3 to ClC-7, carboxypeptidase E (CPE; marker for LDCVs), synaptophysin (SLMV marker) and actin as loading control. All intracellular CLC proteins could be detected in these cells. ***Ab,c*** Subcellular fraction of Rinm5F cells. Fractionation was carried out as described for INS-1E cells (Fig. 6). 18 fractions were collected from top to bottom and analyzed by Western blotting for the presence of ClC-3, -5, -6 and -7. Synaptophysin immunoreactivity indicates the presence of SLMVs. LDCVs were detected by an insulin ELISA ***Ac***. Like in INS-1E cells (Fig. 6), the ClC-3 signal peaks together with synaptophysin in light fractions (7-9). The bulk of insulin was found in fractions 13-14 (a.u., arbitrary units).

B, subcellular localization of ClC-3 in primary β-cells. After performing sucrose density gradient centrifugations of primary β-cell homogenates, 18 fractions were collected starting from the top and analyzed by Western blotting with antibodies against carboxypeptidase E (LDCV marker) ***Ba***, rab4 (endosomal marker) ***Bb***, synaptophysin (SLMV marker) ***Bc*** and ClC-3 ***Bd***. The distribution of secretory granules was also ascertained by determining the insulin concentration of the fractions ***Be***. Lysosomes were identified by measuring the activity of the lysosomal enzyme β-hexosaminidase ***Bf***. Protein content was measured by BCA kit ***Bg***. Secretory granules peak in a heavier portion of the gradient (1.2 – 1.5 M sucrose, fractions 11 – 15) than the smaller endosomes and SLMVs (0.7 – 0.9 M sucrose, fractions 5 – 9). ClC-3 was found to co-purify mainly with the lighter vesicles. Fractions 1 – 4 correspond to the “load” of the gradient and contain soluble proteins, including proteins leaked out of disrupted vesicles.

Fig. S1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84 kDa
64 kDa
49 kDa
37 kDa
WT KO WT KO WT KO WT KO WT
Ba brain HC CA3
WT
Bb
KO
Ca anterior pituitary
WT
Cb
KO
Da posterior pituitary
WT
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Fa adrenal gland
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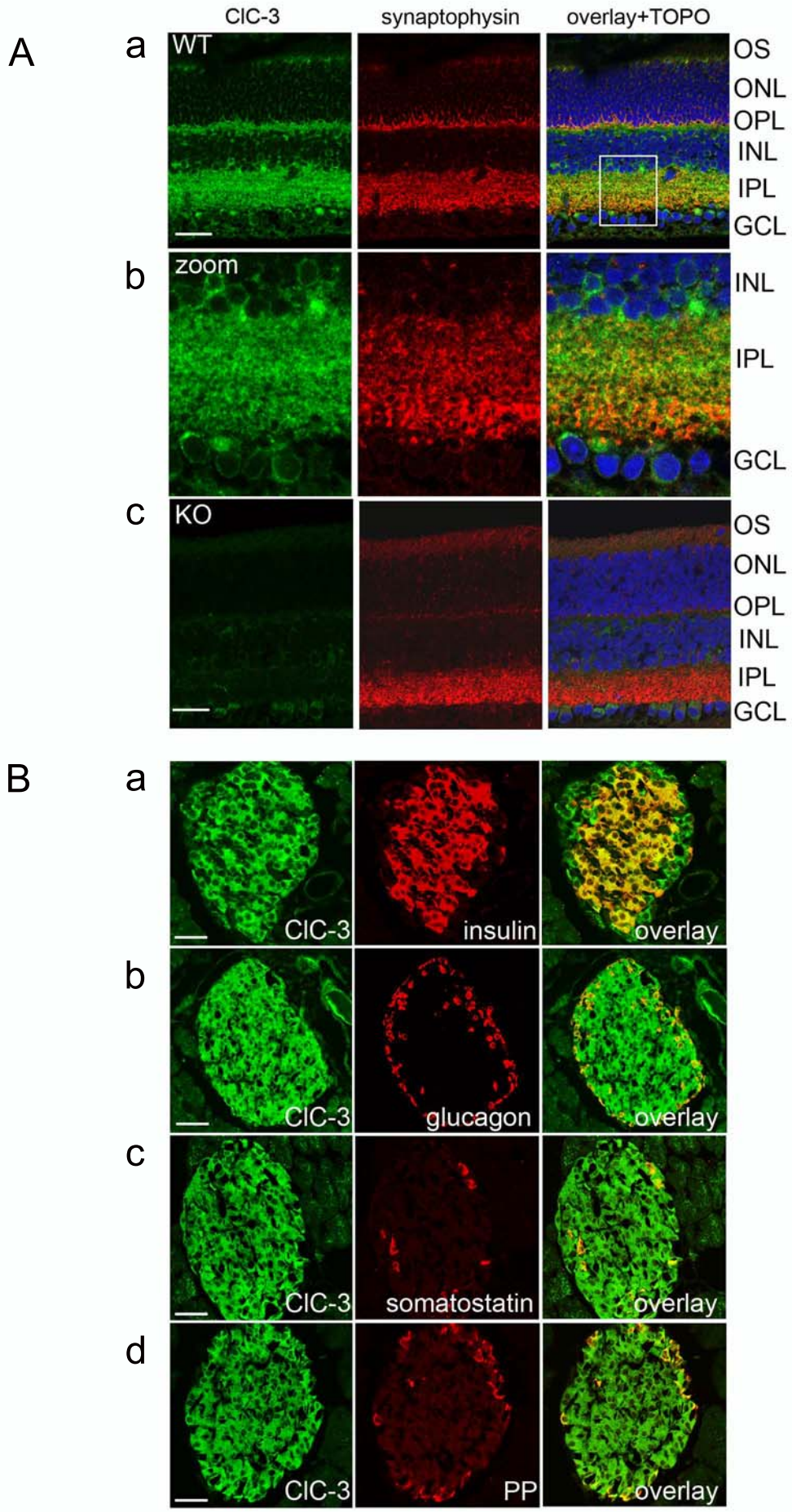
Ga thyroid
WT
Gb
KO

Fig. S2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glucagon
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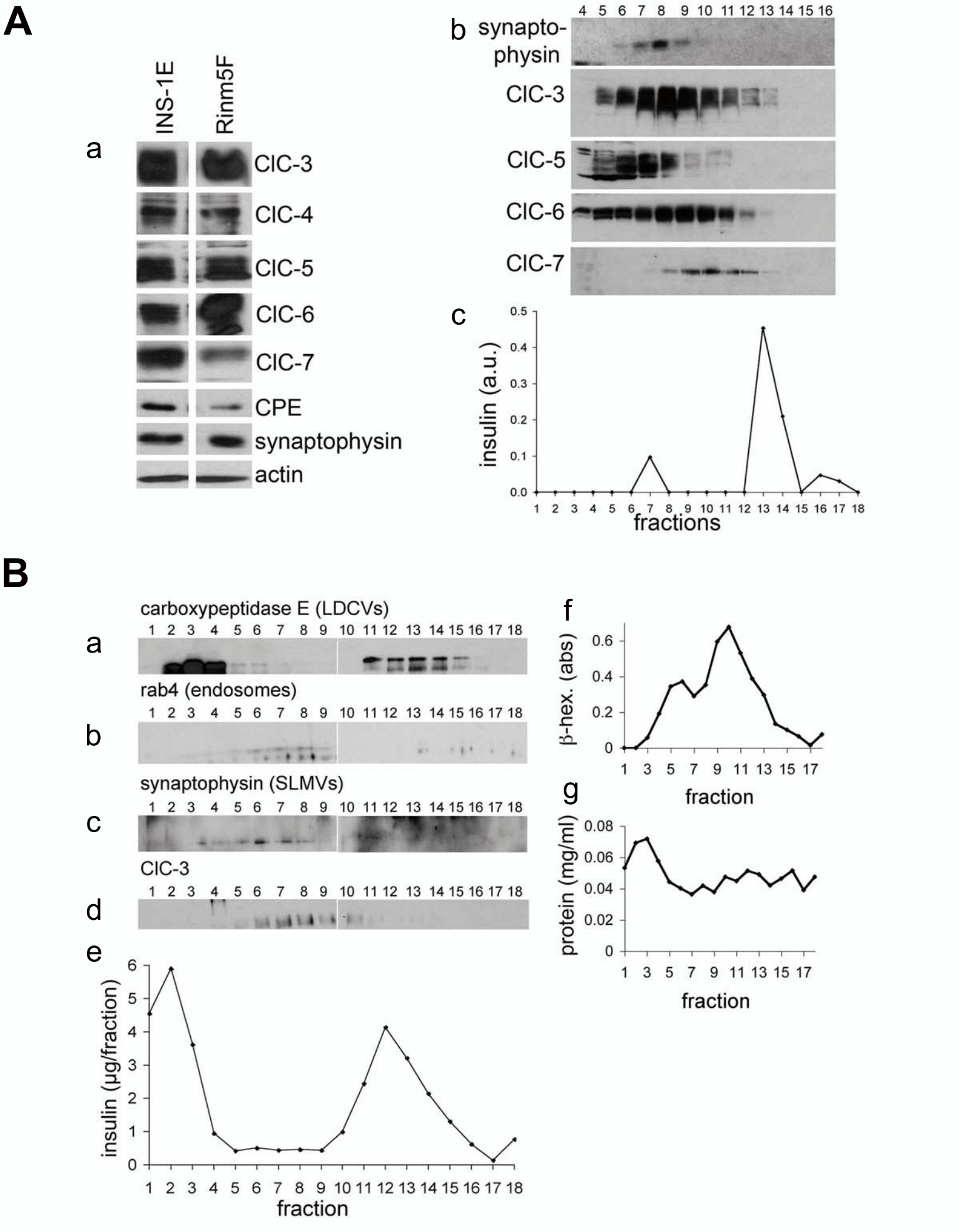
Fig. S3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ClC-4
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actin
b
synapto-physin
ClC-3
ClC-5
ClC-6
ClC-7
4 5 6 7 8 9 10 11 12 13 14 15 16
c
insulin (a.u.)
fractions
B
a
carboxypeptidase E (LDCVs)
b
rab4 (endosomes)
c
synaptophysin (SLMVs)
d
ClC-3
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
e
insulin (µg/fraction)
fraction
f
β-hex. (abs)
fraction
g
protein (mg/ml)
fraction