Supplemental Materials for "Unique sphingomyelin patches are targets of a beta cell specific antibody" by Amol Kavishwar, Zdravka Medarova, Anna Moore

Detergent treatments

RinM5f were treated with different detergents to dissolve IC2 antigen. Ten million cells were suspended in 10 ml of detergent solution (saponin, Triton X100, NP-40, Tween 20, Tween 40, IGEPAL, CHAPS or OBG dissolved to a final concentration of 2% in PBS) containing protease inhibitor cocktail (Pierce) and vortexed vigorously and kept on reciprocal mixer at 4^0C. After 1 hr the tubes were centrifuged at 10,000 g and the supernatant was separated. Pellet was resuspended again in detergent solution and the process was repeated two more times. All the supernatants were pooled and both the pellet and the supernatant were tested for presence of antigen.

Gel Electrophoresis and immunoblotting

For electrophoresis, RinM5f cell pellet was directly suspended in Laemmli sample buffer (Bio-Rad) with or without 5% BME and heated in boiling water bath for 5 min. Tubes were centrifuged at 10,000g for 10 min and supernatant was loaded (50 μg protein/well) in 12% acrylamide gels (Invitrogen). After electrophoresis, proteins were transferred onto PVDF or nitrocellulose membrane and immunoblotted with IC2 antibody.

Crosslinking with DTSSP

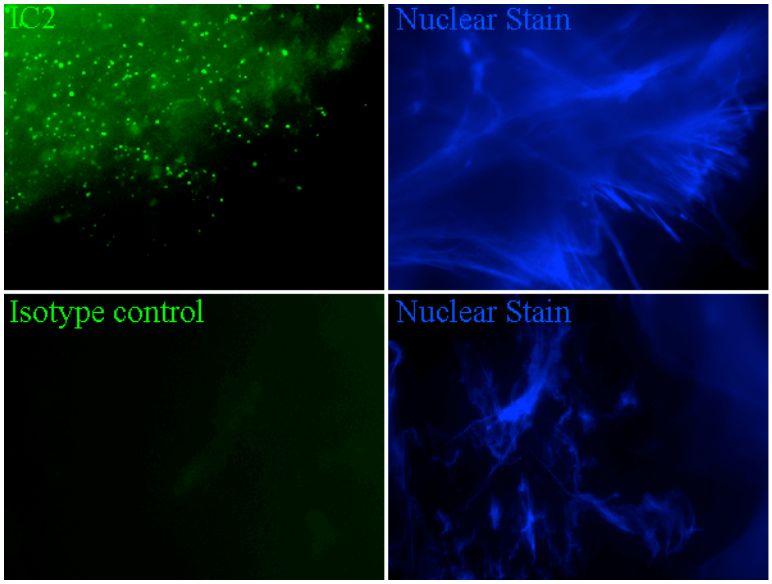
Water-soluble, cell impermeable, thiol-cleavable crosslinker 3,3´-dithiobis[sulfosuccinimidylpropionate] (DTSSP) was used (Pierce) to covalently crosslink IC2 with its antigen on surface of RinM5f cells as described by manufacturer. Essentially, cells grown on tissue culture plates were washed with cell culture medium and incubated with IC2 (1 μg/ml, diluted in culture medium) at 4^0C for 2 hrs. Unbound antibody was washed off with PBS and crosslinking was performed with DTSSP diluted in PBS (2mM/L) for 2 hrs at 4^0C. Crosslinking reaction was stopped by addition of Tris buffer and cells were collected by scrapping culture plate and suspended in Laemmli sample buffer, boiled for 5 min and then diluted 10 fold with 1% triton X 100 in 10 mM Tris buffer pH 7.4 such that final concentration of SDS was less than 0.1%. Immunoprecipitation was performed by adding beads coupled with anti-Rat(IgM) antibody. IC2-antigen complex was eluted from beads by changing pH and the complex was resolved on 12% SDS-PAGE gel followed by either silver staining of gel or transfer to membrane followed by immunoblotting with anti-Rat(IgM)-HRP antibody.

Protease treatment

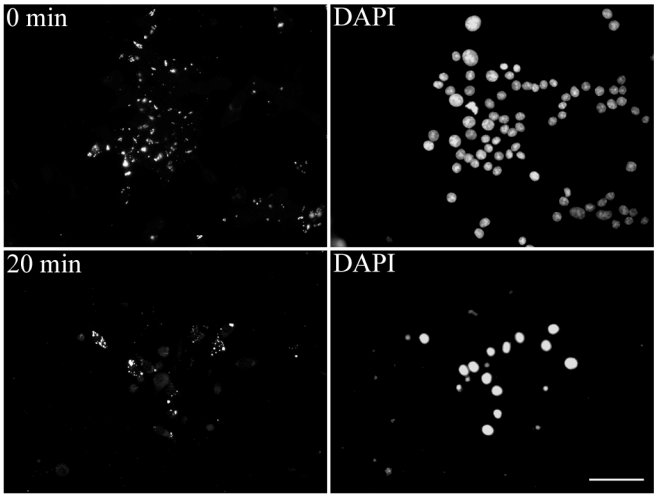
Ins1E cells grown on coverslips were fixed with 4% formaldehyde for 5 min at room temperature followed by extensive washing to remove traces of formaldehyde and then treated with trypsin (0.025%) or pronase (0.1%) for 5, 10 or 20 min at room temperature. Incubations of over 20 min resulted in detachment of cells. Following digestion, cells were immuno-stained with IC2, as described earlier and observed under fluorescence microscope. Untreated cells served as control.

Immunostaining of paraffin-embedded tissue sections

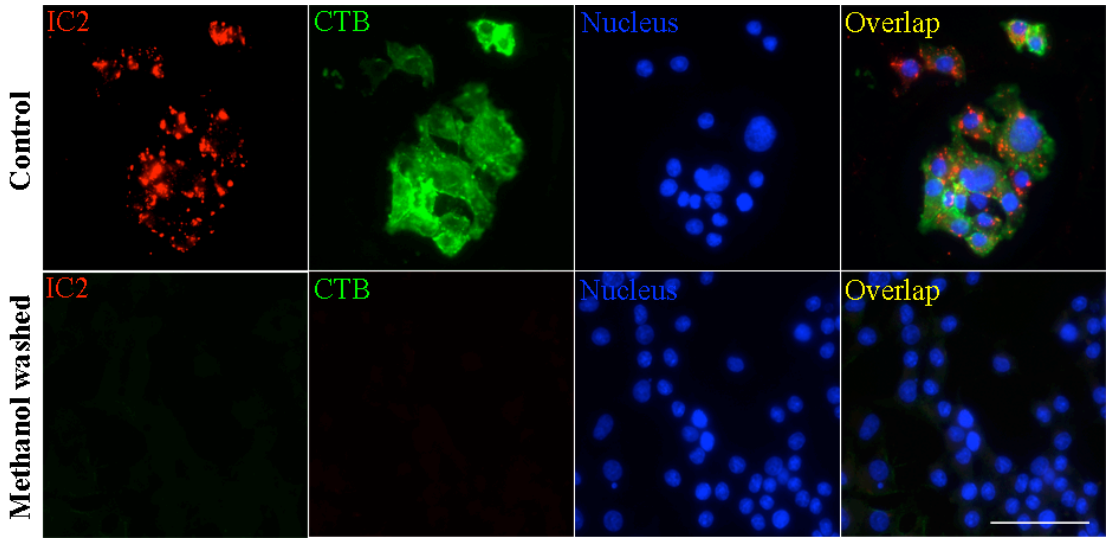
Pieces of pancreatic tissue were fixed in 4% formaldehyde and dehydrated by passing through series of progressively concentrated ethanol bath and cleared by three changes of xylene. The sections were then dipped in molten paraffin and solidified. After trimming paraffin block, 5 μm thick sections were cut and collected on slides. Sections on slide were de-parafinized by dipping in xylene followed by dipping in ethanol and then washing with distilled water to rehydrate the section and then stained with IC2 antibody as described earlier.



Supplementary figure 1. **IC2-antigen resists extraction with Triton X 100** RinM5f cells were extracted with Triton X 100 and the insoluble fraction was stained with IC2. Isotype control antibody served as control.

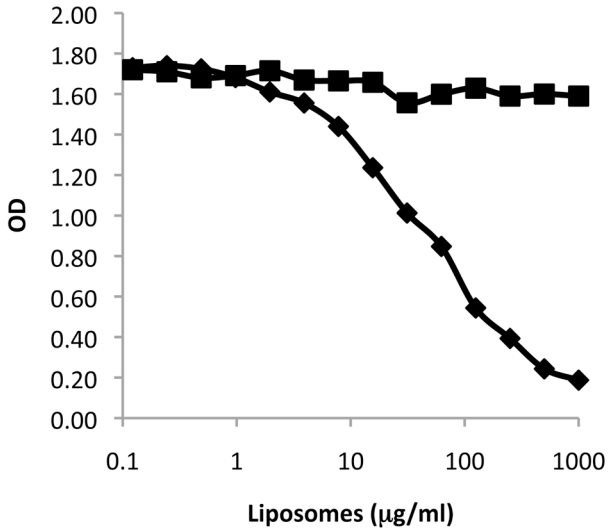


Supplementary figure 2. **IC2-antigen resists trypsin digestion** Cells were digested with trypsin for up to 20 min and then stained with IC2.

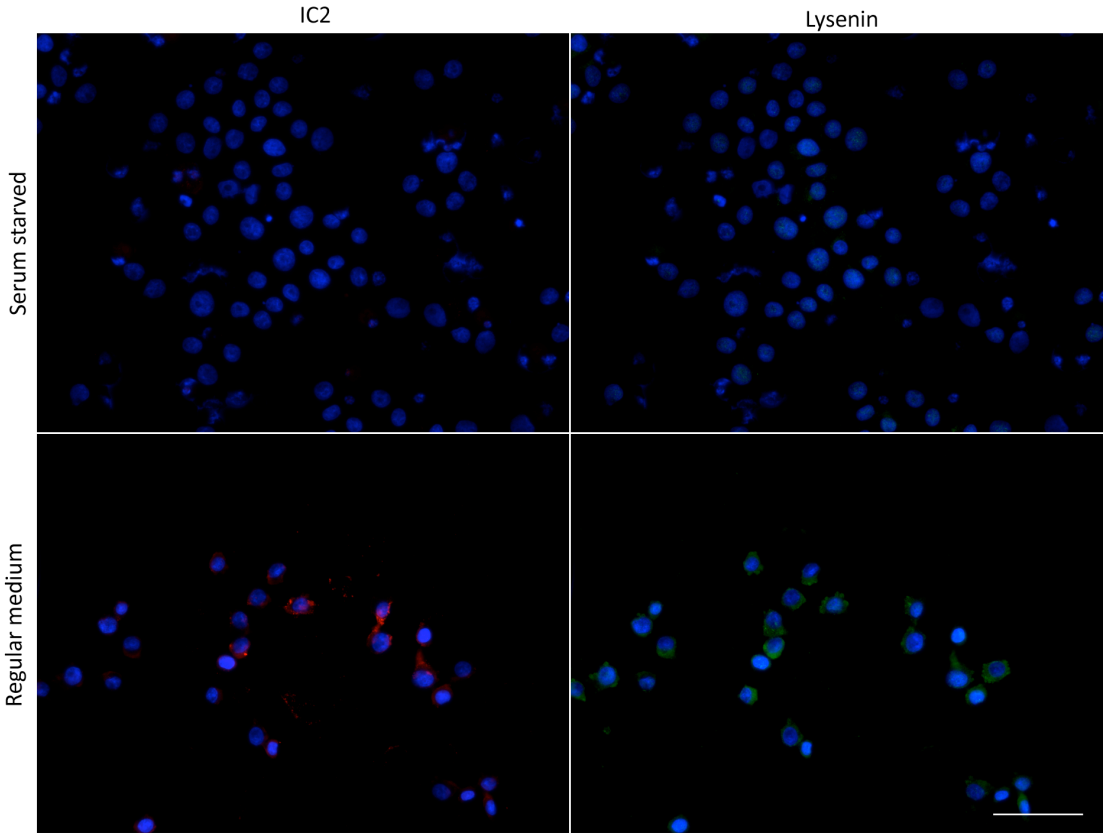


Supplementary figure 3. **IC2-antigen is soluble in methanol** RinM5f grown on glass coverslips were washed with methanol and stained with IC2 and CTB. Cells not washed in methanol served as control.

Supplementary figure 4. **Immunostaining of TLC plate confirm presence of IC2 antigen** IC2-antigen purified from TLC plate was again subjected another round of TLC using a solvent system known to separated out other lipids, particularly PC from SM and then immunostained with IC2.



Supplementary figure 5. **Competition ELISA** Lipids extracted from RinM5f were coated on ELISA plate. Lipid vesicle made of SM (diamond) competed for binding with IC2 and resulted in loss of IC2 binding as the concentration of SM increased. At the same time lipid vesicles made of PC (square) did not compete and no resultant loss of signal was observed.



Supplementary figure 6. **Serum starvation down regulates SM** Cells serum starved for 24 hrs were stained with IC2. Cells grown in complete medium acted as control. Bar = 50µm.