EXTENDED EXPERIMENTAL PROCEDURES

Materials.

Antibodies. Antibodies were obtained from the following sources: monoclonal anti-insulin antibody (ascites, clone K36AC10), antibodies against FLAG were purchased from Sigma (St. Louis, MO), rat monoclonal antibody against mouse proinsulin (against the peptide EVEDPQVAQLELGGC) was developed at the Scripps Antibody Production Core Facility, Rabbit polyclonal antibody against TMEM24 was obtained from Aviva Systems Biology (San Diego, CA), monoclonal antibodies to Ribophorin II (clone 3DI) was a gift from Dr. D. Meyer, University of California, Los Angeles, monoclonal antibody to GAPDH was from Ambion (Austin, TX), Rabbit polyclonal antibodies to HA epitope and GLUT2 were from Santa Cruz (Santa Cruz, CA) and mouse monoclonal antibodies (HA11) against HA epitope were from Covance (USA) and 12CA5 clone antibodies to HA epitope tag were purified in-house, monoclonal antibodies to Syntaxin 1A and polyclonal antibodies to PDI were obtained from Stressgen/Assay Designs (Ann Arbor, MI), monoclonal antibodies to EEA1 and γ-adaptin were from Transduction Laboratories (San Jose, CA), monoclonal antibodies to SNAP-25 were from Sternberger Monoclonals (SMI 81), monoclonal antibodies against ERGIC53 were obtained from Enzo Life Sciences, Guinea pig polyclonal antibodies to insulin, monoclonal antibodies against rat TGN38 (2F7.1) were obtained from Millipore (Temacula, CA) and Abcam (Cambridge, MA). Mitotracker Red CMXRos and monoclonal antibodies against V5 epitope were purchased from Invitrogen (Carlsbad, CA) and rabbit polyclonal antibodies to insulin were purchased from Cell Signalling (Danver, MA). Rabbit polyclonal antibodies to VAMP2 were from ABR now sold through Thermo Scientific (Rockford, IL).

Plasmids and cell lines. Full-length mouse cDNA clone of TMEM24 was obtained from Origene (Rockville, MD) and then subcloned into pcDNA 3.1 (+) with a FLAG tag at the

N-terminus and HA tag at the C-terminus (FG-TMEM24-HA) or only HA tag at the C-terminus (TMEM24-HA). Full length mouse cDNA clone for proinsulin was amplified from pancreatic islet cDNA library and cloned into pcDNA 3.1(+) with V5 tag at the C-terminus (Proinsulin-V5). Mouse fibroblast cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 25 mM glucose and supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin and 0.1 mg/ml streptomycin, mouse insulinoma cells (MIN6) were cultured in DMEM described above supplemented with 25μM β-mercaptoethanol, rat insulinoma (INS1-E) and RINm5F cells were grown in RPMI-1640 containing 11.1 mM glucose and supplemented with 10% FBS, 100 units/ml penicillin, 0.1 mg/ml streptomycin, 50 μM β-mercaptoethanol.

Insulin BIN methods.

Coupling of antibody to Protein G beads. 600 μl of protein G beads were washed with 50 mM sodium borate pH 8.2 and incubated with 300 μl of K36AC10 ascites (2.5 mg) or 2.5 mg of 18D2 or normal mouse IgG1 or rat IgG2c for 30 min at room temperature. Unbound antibodies were drained, beads were washed twice and then incubated with 16.0 mM of disuccinimidyl suberate (DSS) (Pierce 21655) dissolved in DMSO. After 1 h at room temperature, beads were drained and washed extensively with PBS. The reaction was terminated by washing the beads with 0.1 M ethanolamine, pH 8.0. After 10 min at room temperature, ethanolamine was drained and the uncrosslinked antibodies were removed by washing beads with 0.1M glycine, pH 2.2. Final wash was done with 50 mM sodium borate and the beads were then stored as 50% slurry in PBS containing 0.02% sodium azide.

Trypsin digestion of protein complexes for mass spectrometry. Acetone precipitated protein complexes were denatured with freshly prepared 8 M urea (Sigma, mass spec grade), reduced with 5mM TCEP (Pierce) and alkylated with 10 mM iodoacetamide. Urea was then diluted to 2 M, 1 mM $CaCl_2$ was added and each sample was digested with 2 μg of trypsin (Promega V5280) overnight at 37°C. Reaction was acidified by adding formic acid to a final concentration of 5%, samples were spun and supernatant was then subjected to LC/LC/MS/MS analysis using mass spectrometry as described in detail below.

Mass Spectrometry (Link et al., 1999). Protease-digested immune complexes were subjected to LC/LC/MS/MS analysis (Swanson et al., 2009; Washburn et al., 2001). A three phase microcapillary column was constructed by first packing a Reverse Phase (RP) column containing 10 cm of 3 μm of Aqua C18 slurry (125A°, Phenomenex) into a 100μm fused silica capillary which had been previously pulled to obtain a 5μm diameter tip using a Sutter Instruments laser puller (Sutter Manufacturing, Novato, CA). Methanol was then passed through to finish packing and then the column was equilibrated with Buffer A (5% acetonitrile/ 0.1% formic acid). Next, a 250 μm silica capillary was packed with 3.5 cm of 5 μm of strong cation exchange resin (Partisphere, Whatman), followed by another 4 cm of 5μm Aqua C18 (125A°, Phenomenex). The column was then equilibrated with Buffer A for 30 min before the peptide mixture was loaded onto the 5 μm Aqua C18 end of the column using a high pressure cell. After the peptide mixture is loaded, the column was equilibrated with buffer A and connected to the RP column using a filtered union (Upstate scientific). The loaded and washed split column (desalting column–filter union–analytical column) is placed in line with Agilent 1100 quaternary HPLC and the flow rate was set to 0.15 ml/min from the controller to get a targeted flow rate at the tip to be 200-300 nL/min. The samples were analyzed using a modified 4-step

separation using buffer A (5% acetonitrile/0.1% formic acid), buffer B (80% acetonitrile/0.1% formic acid) and buffer C (500 mM ammonium acetate/5% acetonitrile/0.1% formic acid). Step 1 consisted of 70 min gradient of Buffer B from 0-70%. Step 2-3 had 3 min of 100% Buffer A, 2 min of Buffer C (40% for step 2 and 100% for step 3), a 5 min gradient from 0-15% Buffer B and a 92 min gradient from 15-70% Buffer B. The final step consisted of 3 min of 100% Buffer A, 2 min of 90% Buffer C and 10% Buffer B, a 5 min 0-15% gradient of Buffer B and a 92 min 15-70% gradient of Buffer B. This buffer profile was chosen as it reduced the time required to analyze each sample and did not compromise the number of peptides identified upon comparison with running profiles with increased number of steps. As peptides are separated and eluted from the microcapillary column, they were electrosprayed directly into an LTQ 2-dimensional ion trap mass spectrometer (ThermoFinnigan, Plato Alto, CA) with the application of a distal 2.4kV Spray voltage. A cycle of one full scan mass spectrum (400-1400 m/z) followed by 8 data dependent MS/MS spectra at 35% normalized collision energy was repeated continuously throughout each step of the multidimensional separation. Application of mass spectrometer scans functions and HPLC solvent gradients are controlled by the Xcalibur data system.

Analysis of mass spectrometry data. MS/MS spectra were analyzed using the following software analysis protocol. Poor quality spectra were removed from the dataset using an automated spectral quality assessment algorithm (Bern et al., 2004). Tandem mass spectra generated in each run were searched against a mouse protein database (EBI-IPI_mouse_3.30_06-28-2007_con_reveresed.fasta) using SEQUEST^{TM} algorithm (Eng et al., 1994). Database search results were filtered, sorted and displayed using DTASelect program (Cociorva et al., 2007). DTASelect 2.0 uses a linear discriminant analysis to dynamically set Xcorr and DeltaCN thresholds for the entire dataset to

achieve a user-specified false positive rate (5% in this analysis). Spectral count information stored in DTASelect-filter.txt files for different runs were extracted and compared using Perl script dtarray2.pl (Kaschani et al., 2009). Resulting dtarray2.txt files from six independent immunoprecipitation and MS analysis runs for control and insulin samples; three separate samples of proinsulin immunoprecipitation and normal IgG were loaded into Excel (Microsoft Corp., Redmond, WA) and used for final analysis. Spectral counts were reported as corresponding to particular proteins (identified by IPI ids) by the mass spectrometry software pipeline. The proteins identified were mapped to mouse genes using conversions available from the IPI (Kersey et al., 2004). Proteins that either did not map to an Entrez gene ID or were annotated as pseudogenes, were eliminated from further analysis. Proteins that may bind non-specifically and appear in the experiment were determined through the control runs. In order to be considered for further analysis, a protein had to be identified in the insulin and/or proinsulin IP replicates at least twice (counted separately for insulin and proinsulin IPs) at no fewer than 2x the maximum spectral counts observed for that protein in any control, or appear in at least one replicate with no fewer than 5x the maximum spectral counts observed for that protein in any control and at least 20 spectral counts. After this background filtration, spectral counts were normalized separately for each replicate experiment by dividing by the total number of spectral counts assigned to proteins in that replicate experiment. The normalized spectral counts thus quantify the fraction of the defined interactome for each replicate experiment. P values for assessing the likelihood of a real difference between the insulin and proinsulin IPs were calculated with Student's t-test, using only non-zero replicates and corrected for multiple testing using Benjamini-Hochberg method. In cases where the insulin IP had two or more non-zero values and the proinsulin IP did not, the variance of the proinsulin IP was conservatively estimated as equal to 2x the variance of the insulin IP so that a p value could be estimated; the converse was done

where the insulin IP did not have two non-zero samples but the proinsulin IP did. High confidence proteins were identified by IPI accession number and were then mapped to genes (NCBI Entrez IDs) using conversions available on the IPI website (www.ebi.ac.uk/IPI/). We also analyzed the data using SAINT (Significance Analysis of Interactome) that is an advanced approach for statistical analysis of interaction data from affinity purification-mass spectrometry experiments using label free quantitation (Choi et al., 2011). As SAINT is based on probabilistic scoring, it eliminated some of the previously established interactors of insulin whereas our criteria for filtration retained these interactors. Hence, our filtration was used to derive the final set of proteins. Log_2 graph was plotted using Excel Macro (Microsoft Corp., Redmond, WA) and the heatmap was generated using R (http://www.R-project.org). Comparison of our interactome with the two other proteomic datasets (Schrimpe-Rutledge et al., 2012; Waanders et al., 2009) was done using the published data. For comparison of gene expression datasets (Mahdi et al., 2012; Marselli et al., 2010) complete dataset was retrieved from http://www.ncbi.nlm.nih.gov/geo with the series number GSE41762 and GSE20966 respectively, and was further analyzed with the GEO2R tool.

Network building. Protein-protein interactions have largely been characterized for human proteins as compared to mouse proteins. To facilitate network analyses, we first converted the mouse proteins to human proteins using orthology. Human orthologs of mouse proteins were found by using the OrthoMCL program, version 1.4 (Li et al., 2003). Using sequence similarity, OrthoMCL is able to distinguish between in-paralogs and out-paralogs. In cases where multiple human proteins are in-paralogs of one or more mouse proteins (meaning that gene duplication occurred after the last common ancestor), all human in-paralogs are considered equivalent to all mouse in-paralogs. The large network of secretory proteins was constructed by taking all of the human orthologs

and searching the PINA (Wu et al., 2009) binary interactions database, downloaded locally, for all interconnections between these proteins. The proteins were then linked with edges according to this dataset. The Gene Ontology (GO) (Ashburner et al., 2000) cellular compartment annotations for each human protein were used to locate the proteins in subcellular compartments (or the degradation process); some compartment localizations were changed when examination of relevant data and publications suggested a better localization or helped distinguish between multiple localizations suggested by GO. The previous insulin granule proteomics studies data are a compilation of the data from previous proteomic analyses (Suckale and Solimena, 2010). The smaller networks for proteins along the secretory pathway were constructed with the same resources as above, but allowing for proteins that do not appear in the current mass spectrometry experiments to be added to the networks when they have one or more interactions with proteins that do appear in the current experiments. In some cases, specifically for PCSK2, CPN1, SCGN, and COPB2, interactions amongst interactors of the starting protein were included to expand the networks; these are termed two-hop networks (because we take two hops away from the starting protein to complete the network).

Other protocols.

Protein extraction from tissues and cell lines. Tissues were dissected and collected from adult female C57BL/6 mice and homogenized in PBS (Phosphate Buffered Saline) with protease inhibitors (Sigma) and 1 mM PMSF. After incubation on ice for 10-15 min, supernatants were collected and protein concentration was determined using Pierce BCA determination kit and 10 μg of protein was loaded for each tissue. For cell lines, lysates were prepared by incubating cells in buffer containing 50 mM Tris pH 7.4, 150

mM NaCl and 1% TX-100 on ice for 20-30 min. Cell lysates were clarified by centrifugation and protein concentration was determined by Pierce BCA kit and 20 μg of protein was loaded per lane. Proteins separated on 10% SDS-PAGE were transferred to nitrocellulose membranes and blotted with anti-TMEM24 antibody (1:2000, Aviva Systems Biology). MIN6 or COS1 cells were co-transfected with pcDNA, TMEM24-HA and proinsulin-V5 using lipofectmaine 2000. Cells were washed and lysed after 48 hours post-transfection in 1%TX-100 containing lysis buffer. Cell lysates were clarified and incubated with HA antibodies (12CA5) overnight at 4ºC. Protein G beads were added and incubated for 3-4 hours, beads were then pelleted, washed with lysis buffer and bound proteins were eluted in 50mMTris and 1%SDS at 100ºC for 5 min. Eluted samples were separated on 4-12% MES gels and analyzed by western blotting using Rabbit HA antibodies or mouse monoclonal V5 antibodies. Human islets were obtained from the National Diseases Research Interchange with approval from the Institutional Review Board. About 1000 islet equivalents (IEQ) were lysed in 200μl of buffer containing 1% TX-100 on ice for 30 min followed by sonication at 10% power for 20 sec. The lysate was then centrifuged at 10,000 x g for 10 min and ~ 50 IEQ were loaded on SDS-PAGE. Mouse islets were isolated as described below and the lysate was prepared similarly to that of human islets.

Isolation and immunoprecipitation from mouse islets. Pancreata from C57BL/6J-Tyrc-2J/J were distended using 0.2 mg/ml of collagenase P (Roche) at various regions, then isolated and further digested with 2-3 ml of 2 mg/ml of collagenase P per pancreata in a 37°C water bath with intermittent shaking for 32 min. Digestion was terminated when pieces of pancreas were no longer visible. Hanks Balanced Salt Solution (HBSS) containing 10 mM Hepes and 0.1% BSA (medium A) was added and further incubated on ice for 10-12 min. The top part of the media was aspirated to remove acinar cells and

fat. This process was repeated 3 times and then the islet-containing media was passed through a 500 μm mesh to remove any undigested material. The filtrate was then centrifuged at 400 x g for 2 min to collect the islet-containing pellet which was then resuspended in 5 ml of HBSS containing 10 mM Hepes and 10% FBS (medium B) per pancreata and underlaid with 5 ml of Histopaque 1077 (Sigma). The gradient was then centrifuged at 800 x g for 20 min without a brake and the interface was collected and washed with ten times the volume, 3 times. The islet-containing pellet was resuspended in RPMI-1640 containing Pen/Strep, 11.1 mM glucose, 50 mM β-mercaptoethanol and primocin. Islets were handpicked under a dissection microscope and used for further analyses. Islets were recovered overnight after isolation in RPMI media and ~180 islets lysed in a small volume of lysis buffer and immunoprecipitation carried out using the same protocol used for MIN6 cells described in main text experimental procedures.

In vivo cross-linking of MIN6 cells. Cells were washed with PBS and incubated with 1 mM DSP (Pierce) solution at room temperature for 30 min. DSP was then quenched by adding 20 mM Tris pH 7.5 (final concentration) followed by incubation at room temperature for 15 min. Cells were then washed with 50 mM Tris pH 7.5 and 150 mM NaCl twice, scraped and lysed in 50 mM Tris pH 7.5, 150 mM NaCl and 1% TX-100 in the presence of protease inhibitors.

Membrane preparation and extraction of TMEM24. Six confluent 15 cm dishes of MIN6 cells were washed with PBS twice and cells were detached from the dishes by incubating with PBS containing 5 mM EDTA for 10 min at 37°C. Cells were centrifuged, resuspended in 20 mM Tris pH 7.4 containing 0.05% DNase (Roche) and protease inhibitor tablet (Roche) and lysed by passing through cell cracker five times. Lysate was precleared by spinning at 1000 x g for 10 min and the supernatant was collected and further spun at 100,000 x g for 1 h at 4°C to collect the crude membrane pellet. The

membrane pellet was resuspended in 100 mM Tris pH 7.4 and 40μl of sample were incubated with 10μl of either buffer alone or buffer containing 5 M NaCl, 5 M Urea, 0.5 M Na_2CO_3, 5% TX-100 or 5% SDS on ice for 45 min. The sample was centrifuged at 100,000 x g for 1 h, sample buffer was added to the pellet and supernatant, and samples were analyzed by SDS-PAGE and Western blotting using antibodies to TMEM24 (1:2000), Ribophorin (1:3500) and GAPDH (1:1000).

Immunofluorescence microscopy. Mouse fibroblasts, grown on coverslips in DMEM plus 10% FBS plus Pen/Strep, were transiently transfected with a FLAG-TMEM24-HA construct using lipofectamine 2000. After 24h cells were fixed with 4% paraformaldehyde (PFA) for 15 min at room temperature and then washed with PBS three times. Permeabilization was performed using 0.3% TX-100 to permeabilize all membranes or with 3 μg/ml of digitonin to selectively permeabilize the plasma membrane for 20 min at 4°C. Cells were then washed with PBS three times and blocked with 10% goat serum for 1 h at room temperature. This was followed by incubation with primary antibodies and then with secondary antibodies for 1 h at room temperature. After four washes with PBS, cells were incubated with PBS containing 1 μg/ml of DAPI (Sigma) for ten min and coverslips were mounted on glass slides with a drop of Fluoromount G (EM Sciences). For colocalization analyses, insulinoma cells were grown on coverslips or 4-well Permanox slides (LabTek) and then fixed with 4% Paraformaldehyde (PFA) and permeabilized with 0.2% TX-100 at 4°C for 10 min. Cells were then blocked with 10% goat serum for 30 min at room temperature and then stained with various primary antibodies for 1 h at room temperature, subsequently washed with PBS and then stained with secondary antibodies. After 1 h at room temperature, cells were washed with PBS, incubated with PBS containing 1 μg/ ml of DAPI for 10 min and coverslips were placed with a drop of Fluoromount G. For blocking with antigenic peptide, the primary antibody

was incubated at room temperature for 2.5 h with or without the antigenic peptide (Aviva Systems Biology, AAPP27877) at 1:1 ratio of antibody to peptide. This mixture was then used as the primary antibody for immunofluorescence analyses and all other steps were performed as described above.

Immunohistochemistry of cryosections. Mice were first perfused with PBS and then 4% PFA, pancreata was isolated and incubated in 4% PFA overnight. Tissue was then washed in PBS and incubated with sucrose-PBS gradient each time until it sank to the bottom (5% sucrose for 1 h, 10% sucrose for 1 h, 20% sucrose overnight and 30% sucrose for 1 h). Tissue was then placed in a Tissue-Tek cryomold containing OCT embedding compound and frozen on dry ice bath. Cryosections were cut to 10 mm thick sections and slides were stored at -80°C. Before staining, slides were brought to room temperature for 30 minutes. Sections were washed with PBS three times and then blocked with 10% goat serum in PBS containing 0.2% TX-100 for 30 min. Sections were incubated with primary antibody in 5% goat serum overnight at 4°C, washed and then incubated with secondary antibody for 2 h at room temperature. Sections were washed, incubated with 5 μg/ml of DAPI, mounted with a drop of Fluoromount G and examined using a confocal microscope.

Purification of lentivirus. HEK293T cells at ~60% confluency on a 15 cm dish were transfected with 24 μg of shRNA construct, 15.6 μg of RRE, 6 μg of REV and 8.4 μg of VSV-G using 162 μl of Fugene 6 (Roche) according to the manufacture's instruction for transfection. Cells were changed to fresh DMEM+10%FBS+ Pen/Strep after 24 h and supernatants containing virus were collected at 24, 48 and 72 h post media change. Virus supernatants collected from three 15 cm dishes were pooled and passed through 0.45 μm Millipore MF filters with addition of polybrene (Sigma) to a final concentration of

5 μg/ml. To concentrate the virus, viral supernatants were centrifuged at 50,000 x g for 2 h at room temperature. At the end of the spin the supernatant was discarded and pellet was resuspended in HBSS (14175-095 Invitrogen) supplemented with 0.5 mM (0.1 g/L) $MgCl_2$, 0.4 mM (0.1g/L) $MgSO_4$, 1 mM (0.14g/L) $CaCl_2$. Virus was aliquoted and stored at -80◦C until used. The amount of virus used for knockdown was experimentally optimized for cell lines with each virus preparation.

Pulse chase analysis. MIN6 cells were plated at $8x10^5$ cells in 35 mm dishes and infected with 20 μl of concentrated scrambled or TMEM24 shRNA virus. After 72 h of infection, cells were washed with PBS twice and starved with DMEM (-Met, -Cys) for 1 h at 37°C. Cells were incubated for 30 min with 100 μCi of ^{35}S (Amersham) to pulse and chase was carried out with complete medium for 0, 20, 40 or 80 min at 37°C. Cells were then lysed with buffer containing 1% TX-100 on ice for 30 min and lysates were precleared at 12,000 rpm for 20 min. One sixth of the lysate was removed for western blot analysis to determine the levels of TMEM24 knockdown. The remaining samples were adjusted to equal protein concentrations and immunoprecipitated from each time point with 1 μl of mouse monoclonal antibodies (K36AC10) for 2-3 h at 4°C followed by addition of 30 μl of Protein G (Amersham, 50% slurry) beads at 4°C overnight. Beads were washed twice with lysis buffer and bound proteins were eluted with SDS-PAGE sample buffer. Half of the eluate was separated by running on 10-20% SDS-PAGE, transferred to nitrocellulose paper and exposed to autoradiography.

Pseudoislet culture - MIN6 cells were plated at $8x10^5$ cells in a 6-well dish and infected with 10 ml of concentrated lentivirus. After 48 hours, cells were trypsinized and reseeded at $2x10^5$ cells/well of ultra-low cluster plate from Costar (3471) (Kelly et al., 2011). Three

days later, cells were incubated in MIN6 media containing 4 mM glucose overnight. Secretion assays, ELISA and western blots were carried out as described.

ELISA - INS-1 cells were plated at $8x10^5$ cells/ well in a 6-well plate in RPMI-1640 containing 10% FBS, pen/strep and 11.1 mM glucose. After 24 h, infected cells with 6 μl of scrambled or TMEM24 shRNA viruses and changed to medium containing 4 mM glucose after 48 h of infection. On the day of ELISA, cells were washed with KRB buffer (15 mM Hepes pH 7.4, 120 mM NaCl, 1 mM $MgCl_2$, 24 mM $NaHCO_3$, 2.5 mM $CaCl_2$, 0.1% BSA) and incubated with basal KRB buffer containing 2.8 mM glucose and 5 mM KCl. After 1 h at 37°C, cells were incubated with either basal medium, KRB with 25 mM glucose or KRB with 35 mM KCl for 1 h at 37°C. Supernatants were collected and spun at 2000 rpm for 10-15 min to eliminate floating cells. Cells were then washed with PBS and lysed with 1% TX-100 containing lysis buffer on ice for 40 min.

Calcium Imaging. MIN6 cells were plated on 12mm coverslips and infected with either scrambled or shTMEM24 lentivirus for four days followed by overnight incubation with low glucose media. Cells were then incubated with Fura-2 AM (Molecular Probes) in Basal KRB buffer containing 10% Pluronic acid solution for 30 minutes. After washing cells with basal buffer, they were transferred to a temperature controlled microscopic stage and perfused with buffer containing 25mM glucose or 35mM KCl using a perfusion system at 5ml/min. Images were collected on a Zeiss Axiovert 200M, 20X and single cell fluorescence was measured using Sutter DG-4 illuminator switched between 340 and 380 nm excitation for Fura-2 ratiometric quantitation. Images were acquired with a Hamamatsu Orca-ER using MetaFluor software. Results are presented as average of the number of cells selected for each condition from separate experiments.

SUPPLEMENTAL FIGURE LEGENDS

Figure S1. Insulin Biosynthetic Interaction network (insulin BIN) (**A**) 500 μg of MIN6, INS-1 or human islet lysates were immunoprecipitated with anti-proinsulin antibody conjugated to beads at 4°C for 5 hrs. Beads were washed and bound proteins were eluted and loaded on 4-12% MES gel. Western blotting was carried out with guinea pig anti-insulin antibody that recognizes both proinsulin (*) and insulin (arrowhead). (**B**) MIN6 interactome dataset was compared with two other human islet proteomic studies (Schrimpe-Rutledge et al., 2012; Waanders et al., 2009) and gene expression datasets that compared normal vs. type 2 diabetes populations (Mahdi et al., 2012; Marselli et al., 2010). Percentage overlap was computed by analyzing the number of proteins in MIN6 interactome that appeared in these other datasets (**C**) Protein G, Proinsulin or Insulin immunoprecipitates from MIN6 cells were probed with antibodies to the indicated proteins identified in the interactome (**D**) Secretory components of insulin BIN are arranged as nodes (ovals or hexagons), in which the shade of the node represents the mean of normalized spectral counts associated with a particular node calculated from all replicates of insulin and proinsulin samples; green represents the highest and red the lowest normalized spectral count. Nodes for proteins identified in previous proteomic studies of insulin secretory granules are shown as hexagons (Brunner et al., 2007; Hickey et al., 2009). Straight grey lines represent the edges in the network that show direct or indirect interaction with insulin, black straight lines represent interactions between nodes in the interactome based on PINA and blue lines connect insulin to proteins that have described function in insulin folding, trafficking or secretion or an association with type I or type II diabetic conditions.

Figure S2. Subnetworks of proteins in the interactome. Subnetworks for proteins involved in (**A**) insulin trafficking (COPB1) (**B** and **C**) processing (CPN1 and PCSK2) and

(**D** and **E**) granule maturation (Rab 27a and VAPA/B) are derived using the protein interaction database and literature. Color and shape schemes for nodes are as described in **Figure S1**.

Figure S3. Sequence alignment of TMEM24. (**A**) Domain diagram of TMEM24. TMEM24 contains a transmembrane domain (TM), a predicted C2 domain, a degenerate C1-like domain and a phosphatidyl serine binding region that includes the degenerate C1 domain and the region between C1 and C2 domains (**B**) Protein sequences of TMEM24 from mouse (NP_082185.2), rat (NP_001011996), human (NP_055622.3), chimpanzee (XP_508807) and zebra fish (NP_001070851.1) were compared using CLUSTAL W. **Key**: *, indicates the residues which are identical in all sequences; : (colon), indicates conserved substitutions; . (point) indicates semi-conserved substitutions; no key means no sequence similarity. Conservation in sequence is also depicted by the bar graph below the alignment. The transmembrane region predicted by TMHMM (Krogh et al., 2001) is indicated by a solid line. The first box corresponding to amino acids 288-412 of both human and mouse proteins shows homology to a C2 domain and the second box shows a region with weak similarity to phorbol ester and diacylglycerol binding C1-like domain (**C**) Table shows pairwise scores calculated as the number of identities in the best alignment, divided by the number of compared residues.

Figure S4. TMEM24 is a type I membrane protein. (**A**) Crude membrane fractions were made from MIN6 cells and proteins were extracted with either buffer alone or 0.1M Na_2CO_3 or 1 M urea or 1% TX-100 or 1% SDS. Pellet and supernatant fractions were Western blotted with antibodies against TMEM24, ribophorin or GAPDH. (**B**) Mouse fibroblasts were transfected with TMEM24 tagged with FLAG epitope tag at the N-terminus and HA epitope tag at the C-terminus. Cells were then fixed, permeabilized

with either 3 µg/ml digitonin or with 0.3% TX-100 and stained with antibodies against FLAG and HA followed by Alexa fluor conjugated secondary antibodies. PDI, an ER luminal protein was used as a control to monitor permeabilization of membranes. Scale bar; 5 µm.

Figure S5. Subcellular localization of TMEM24. INS-1 cells were fixed, permeabilized and costained with antibodies against TMEM24 protein (green) and different markers of (**A**) subcellular organelles (red) or (**B**) SNARE components (red) followed by visualization by confocal microscopy. Scale bar; 5µm.

SUPPLEMENTAL TABLE LEGENDS

Table S1: List of all identified secretory proteins

A list of all identified secretory proteins with raw, normalized spectral counts and mean spectral counts are shown in first table and rest of the tables provide scoring information for peptides in each experimental replicates (six experimental replicates for control and insulin; three for proinsulin samples; IgG matched control runs for proinsulin and insulin).

Table S2: Mouse to human orthology mapping

Mouse proteins are mapped to their human orthologs using OrthoMCL. Mapping for each protein along with mean spectral count information is shown. Mapped human secretory proteins with Entrez ID, gene name, common name and mean spectral count for proinsulin and insulin is also shown in a separate sheet.

Table S3: Analysis of proteins involved in secretory pathway

List of proteins involved in the secretory pathway with mean spectral counts in insulin and proinsulin immunoprecipitate, Log_2 value for insulin: proinsulin binding, p value calculated for differential binding using Student's t-test as well as corrected for multiple testing are shown in the table. Comparison of 230 proteins in the secretory pathway with two other proteomic datasets (Schrimpe-Rutledge et al., 2012; Waanders et al., 2009) and two other gene expression data sets (Mahdi et al., 2012; Marselli et al., 2010) are shown in a separate sheet. Proteins that show significant change in response to glucose are indicated in red.

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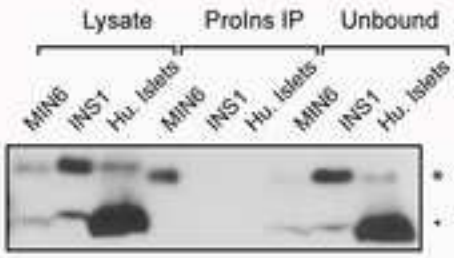
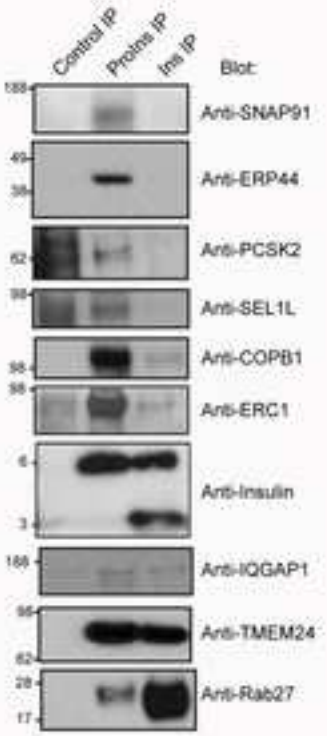
Supplemental Figure 1

Figure S1

A



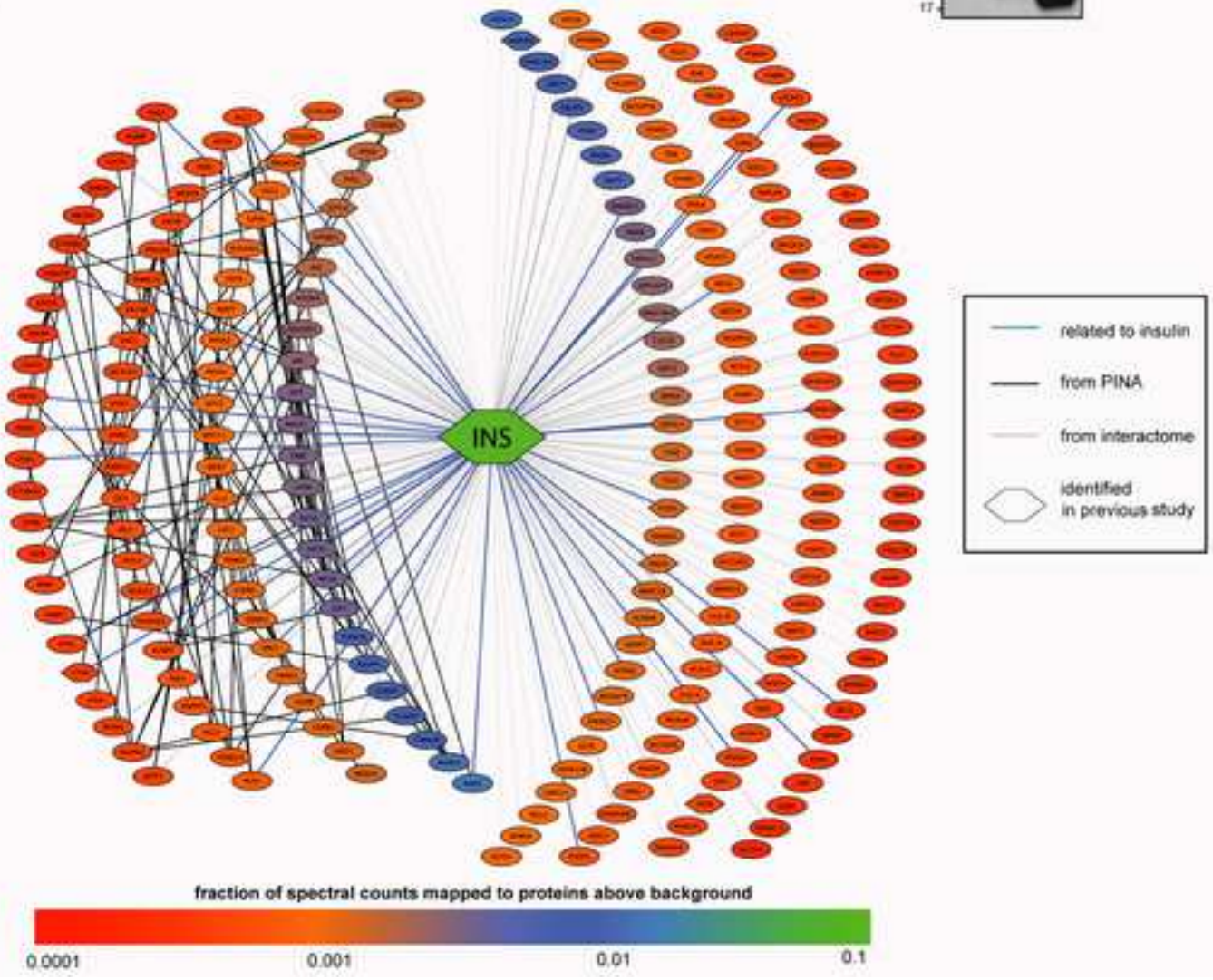
C



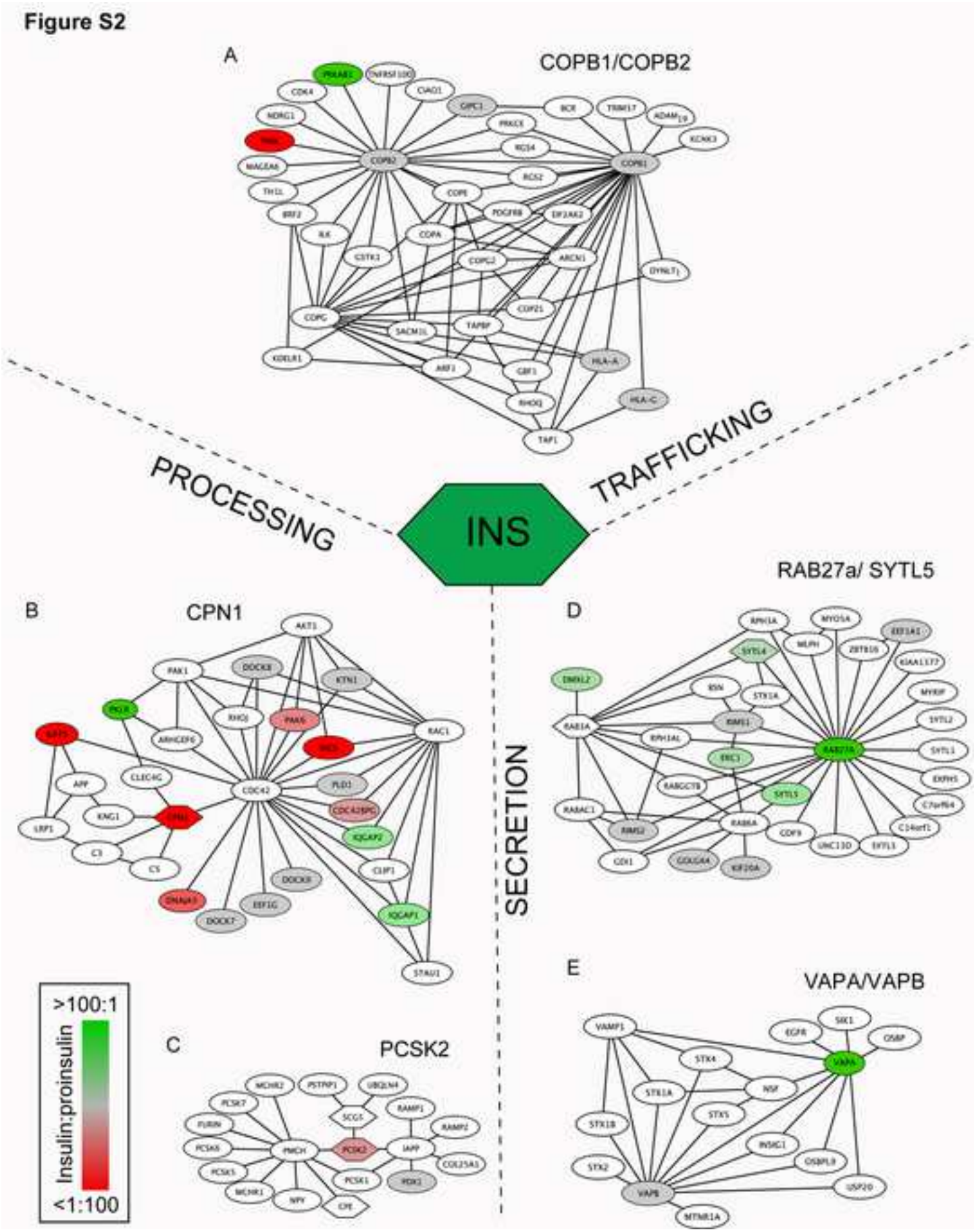
B

	Other Proteomic Studies			Gene Expression Analysis		
	Schrimpe et al (2012)	Waanders et al (2009)	Schrimpe et al & Waanders et al	Mahdi et al (2012)	Marselli et al (2010)	Mahdi et al & Marselli et al
Pottekat et al	156 (67.8%)	168 (73%)	191 (83%)	225 (99.5%)	224 (97.3%)	230 (100%)

D



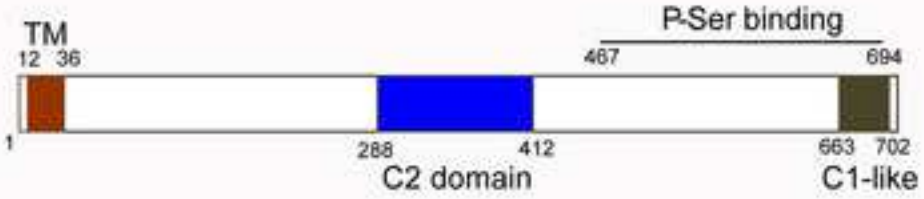
Supplemental Figure 2



Supplemental Figure 3

Figure S3

A



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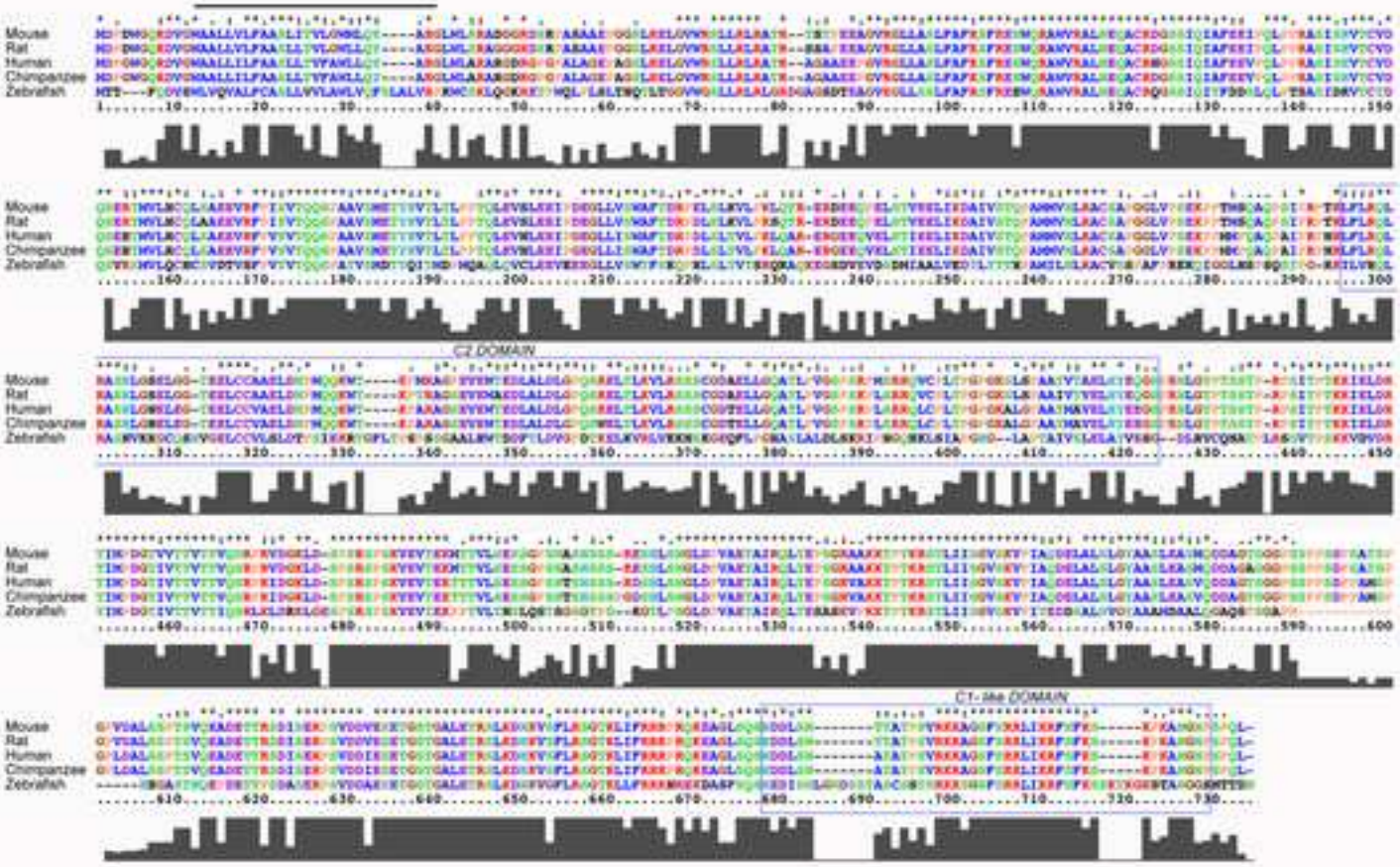
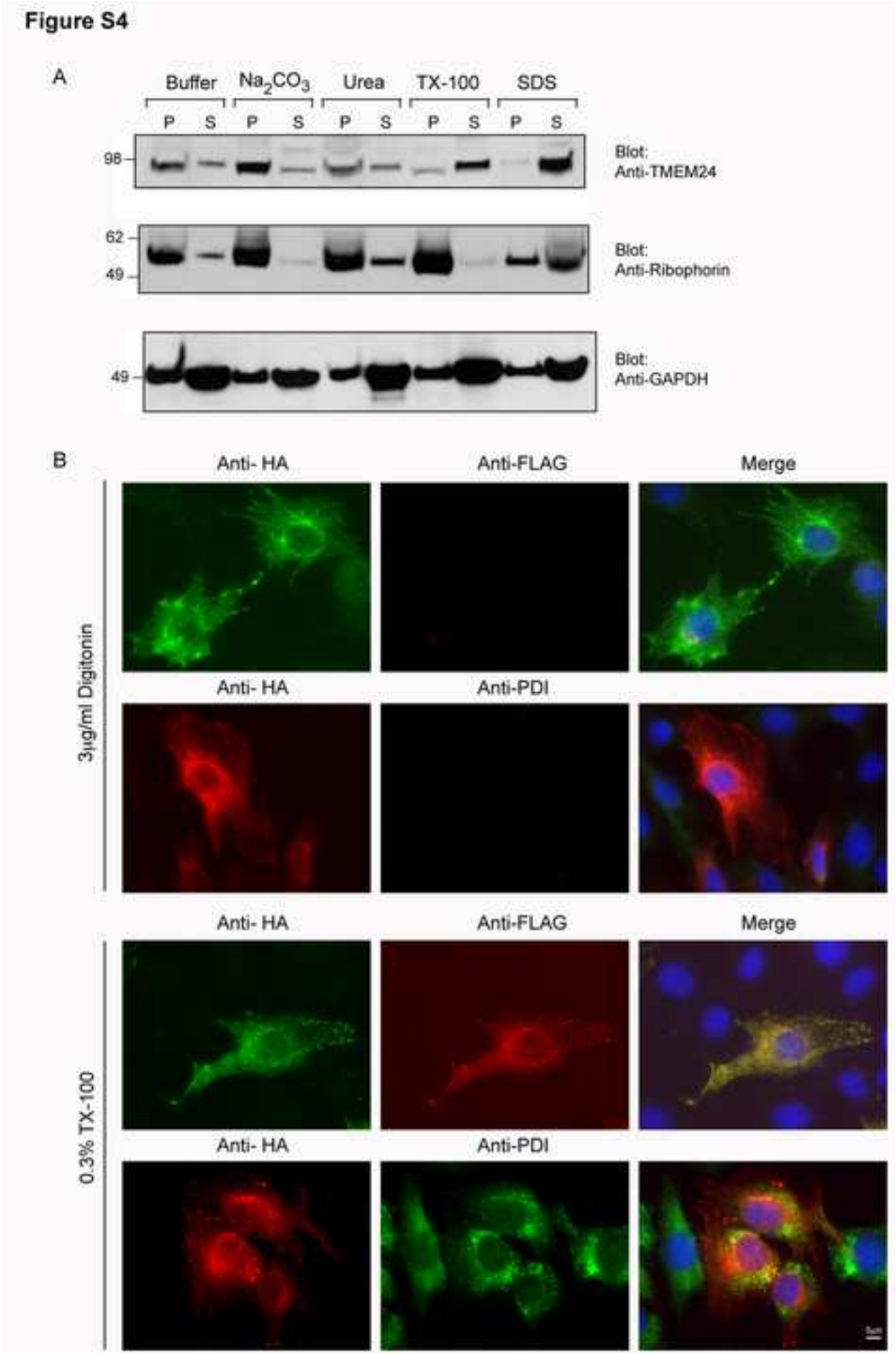


C

	Human	Chimpanzee	Mouse	Rat
Human	100%	99%	90%	91%
Chimpanzee	99%	100%	90%	91%
Mouse	90%	90%	100%	97%
Zebrafish	49%	49%	48%	48%

Supplemental Figure 4



Supplemental Figure 5