Title: Signal transduction pathways controlling *Ins2* gene activity and β cell state transitions

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Abstract

Pancreatic β cells exist in low and high insulin gene activity states that are dynamic on a scale of hours to days. Cells with higher *Ins2* gene activity have a 'mature' β cell transcriptomic profile but are more fragile. Information remains unknown on the spatial relationship between these β cell states, their proteomic signatures, and the signaling mechanisms underlying state transitions. Here, we used live 3D imaging, mass spectrometry proteomics, and 48 targeted perturbations of β cell signaling pathways to comprehensively investigate *Ins2*(GFP)^{HIGH} and *Ins2*(GFP)^{LOW} β cell states. We found that the two *Ins2* gene activity states exist in intact isolated islets, and cells in the same state were more likely to be nearer to each other. We report the proteomes of pure β cells to a depth of 5555 proteins and show that β cells with high *Ins2* gene activity had increased transcriptional and mRNA processing factors, as well as increased translation rate. We identified activators of cAMP signaling (GLP1, IBMX) as powerful drivers of both GFP expression and transitions from *Ins2*(GFP)^{LOW} to the *Ins2*(GFP)^{HIGH} states. Okadaic acid and cyclosporine A had the opposite effects. This study provides new insight into the proteomic profiles and regulation of β cell states.

Article highlights

a. Why did we undertake this study?

- We sought to define the proteomic signatures of β cell maturity states and understand the mechanisms regulating *Ins2* gene activity and state transitions.

b. What are the specific questions we wanted to answer?

- What are the islet spatial distribution and proteomic profiles of β cell maturity states?
- What are the molecular mechanisms controlling *Ins2* gene activity and state transitions?

c. What did we find?

- In intact islets, β cells in the high *Ins2* state are more likely to be close to each other.
- IRF3, CLIP1, PAPSS2, YWHAZ, and AIFM1 are the most β cell-specific proteins in mouse islets.
- High *Ins2* activity coincides with upregulation of proteins involved in transcriptional regulation and processing.
- Agonists and drugs that augment cAMP signaling increase *Ins2* gene activity while calcineurin inhibitors cyclosporine A and okadaic acid reduced *Ins2* gene activity.

d. What are the implications of our findings?

- Cells with high *Ins2* gene activity have evidence of increased transcriptional capacity. cAMP and calcineurin signaling regulate *Ins2* gene activity and cell state transitions.

Introduction

Diabetes is characterized by dysfunctional glucose homeostasis which is caused by a relative (type 2 diabetes) or near-absolute (type 1 diabetes) deficiency in insulin, the body's main anabolic hormone (1). Insulin is produced by pancreatic islet β cells, whose dysfunction and/or death are prerequisites for diabetes progression (2-4). Not all β cells are the same (5-7). Several studies have shown that insulin content and mRNA can vary greatly between individual β cells (8; 9). Previously, live cell imaging using a dual *Ins1* and *Pdx1* fluorescent promoter reporter showed that mouse and human β cell lines could transition from less to more mature states (10; 11) and identified endogenous and chemical regulators of this process (12; 13). However, reporter constructs using abbreviated promoter fragments may not accurately report endogenous gene activity. Therefore, we used *Ins2*^{GFP} knock-in mice (14) to investigate endogenous *Ins2* gene activity and identified two distinct β cell states, one with high insulin gene activity, *Ins2*(GFP)^{HIGH}, and one with lower insulin gene activity, *Ins2*(GFP)^{LOW} (15). Live cell imaging revealed that β cells had the capacity to transition between the high and low GFP states in both directions. Single cell RNA sequencing (scRNAseq) demonstrated *Ins2*(GFP)^{HIGH} cells had a more mature β cell transcriptome profile, including upregulation of factors related to optimal insulin secretion. However, it has been shown that the transcriptome and proteome have weak correlation in islets (16), so it is critical to understand the proteomic profile of these states. It is also important to understand the molecular mechanisms that control β cell state transitions. ER stress and other cellular perturbations are also known to impact gene transcription (17; 18). For example, stresses associated with the high *Ins2* state lead to β cell fragility (15) and lower proliferation capacity (19), but the effects of stress itself on *Ins2* gene activity remains unclear. With robotic imaging technology and automated analysis, it is possible to study dozens of signaling pathways at once.

Live imaging of β cell states also presents the opportunity for fluorescence activated cell sorting (FACS) purification of selected cell populations, while advances in mass spectroscopy permit the identification and cataloguing of thousands of cell-selective proteins that could be effects markers or targets for drug delivery. Previous proteomic studies purified β cells using insulin immunofluorescence staining or FAD autofluorescence (20; 21), but these methods for enriching β cells have caveats such as the reliance on antibody specificity and that FAD autofluorescence may not identify all β cells. Thus, there is need to revisit this approach with updated and more sensitive proteomics equipment and analysis pipelines.

Here, we define the proteomic profiles of purified rodent β cells and of the two *Ins2* gene activity states using *Ins2*^{GFP} knock-in mice. We also systematically tested the effects of 48 drugs known to affect selected signaling pathways and cellular functions on *Ins2* gene activity and β cell state transitions. We present the first comprehensive comparison of the two *Ins2* expression states at the protein and functional level, and reveal multiple genes implicated in mechanisms related to *Ins2* gene activity and β cell state transitions.

Methods

Animal husbandry and breeding

Animals were housed in the Modified Barrier Facility at UBC, under protocols approved by the UBC Animal Care Committee in accordance with national and international guidelines. To maintain consistency with our prior study (15) and to keep the genotype as close to wild-type as possible, all mice in this study were *Ins2*$^{WT/GFP}$ heterozygous mice. Including the *Ins1* gene, these mice have 3 out of 4 wildtype insulin alleles. Mice used for islet isolations were between 60 and 65 weeks of age.

Islet isolation and dissociation

Pancreatic islets were isolated using collagenase when mice were aged 60 weeks, filtered, and hand-picked as previously described (22). Islets were cultured overnight (37°C, 5% CO2) in RPMI1640 medium (ThermoFisher, Waltham, United States) with 11 mM glucose (Sigma), 100 units/ml penicillin, 100 µg/ml streptomycin (ThermoFisher, Waltham, United States), and 10% vol/vol fetal bovine serum (FBS; ThermoFisher, Waltham, United States). For islet dissociation, islets were washed 4 times with minimum essential medium (Corning, Corning, United States) and immersed for 5 minutes in 0.05% trypsin at 37°C with pipetting. Cells were then resuspended in RPMI1640 medium, plated in appropriate reservoirs or plates, and rested for 24 hours (37°C, 5% CO2)**.** Cells were incubated for 2 hours in the presence of 0.05 µg/mL Hoechst 33342 (Sigma–Aldrich, St. Louis, United States) to mark nuclei and 0.5 µg/mL propidium iodide (Sigma–Aldrich, St. Louis, United States) to mark cell death. Drug/small molecules for the perturbation study were purchased from the sources indicated in Table 2 and applied immediately prior to imaging.

Fluorescence-activated cell sorting

Pancreatic islets were dispersed using 0.05% trypsin and resuspended in 1x PBS with 0.005% FBS. Dispersed islets were then filtered into 5 ml polypropylene tubes. Sorting was conducted on a Cytopeia Influx sorter (Becton Dickinson, Franklin Lakes, USA) at the Life Sciences Institute FLOW core facility. Cells were excited with a 488 nm laser (530/40 emission) and a 561 nm laser (610/20 emission).

Live cell imaging and analysis

For 3D live imaging, intact mouse islets were incubated overnight in culture media (RPMI1640 medium with 11 mM glucose, 100 units/ml penicillin, 100 µg/ml streptomycin, and 10% vol/vol FBS) with Hoechst 33342 (0.05 µg/mL) and propidium iodide (0.5 µg/mL). After 24 hours, cells were transferred into 2 well or 8 well µ-Slides (Ibidi) with fresh culture media. Images were then acquired using a LEICA THUNDER Imager Live Cell & 3D assay microscope at 25x water objective (numerical aperture 0.95). Analysis of 3D live cell imaging experiments was done using Imaris Microscopy Image Analysis software with the FIJI Labkit module and custom R scripts. Image processing, including smoothing and background subtraction, was performed using Imaris. Labkit facilitated image labeling and pixel classification for the identification of GFP fluorescence in individual β cells. Nearest neighbor analysis was done using Imaris Python XTensions. Distance-to-core analysis was conducted on Imaris, using a reference frame positioned at the islet's center to measure the distance of each cell from the origin reference frame. Plots were generated for visualization using Graphpad Prism v10 or custom R scripts.

To investigate the effects of small molecules on GFP fluorescence, we cultured dispersed islet cells from *Ins2*^{GFP} mice on 384-well glass bottom plates (Perkin Elmer, Waltham, United States) and performed live cell imaging using an ImageXpress^{Micro} environmentally controlled, robotic system (Molecular Devices, San Jose, United States) which employed a 300 W Xenon lamp as previously described (15). Cells were stained with Hoechst 33342 and propidium iodide as described above. Images were acquired at 10x air objective, numerical aperture 0.3, at 30-minute intervals up to 96 hours, with cells being exposed to 359 nm light for 110 ms, 491 nm light for 15 ms, 561 nm for 75 ms. Analysis of live cell imaging experiments on *Ins2*^{GFP} cells was done using MetaXpress analysis software and custom R scripts. Tracking of GFP fluorescence in individual cells was performed as previously described (15). The *Ins2*(GFP)^{HIGH} and *Ins2*(GFP)^{LOW} subpopulations were identified using model-based clustering (23).

Protein synthesis assay

One day after isolation, dispersed islets were seeded into an optical 96-well plate (Perkin Elmer, Waltham, United States) at a density of approximately 16,000 cells per well in culture media (RPMI1640 medium with 11 mM glucose, 100 units/ml penicillin, 100 µg/ml streptomycin, and 10% vol/vol FBS). Treatments were applied 3 hours after seeding. After 24 hours of incubation, fresh culture media was applied, then supplemented with 20 µM OPP (Invitrogen, Waltham, United States). The assay was performed according to instructions provided by the manufacturer. Cells were imaged at 10x, numerical aperture 0.3, with an ImageXpress^{Micro} high-content imager and analyzed with MetaXpress to quantify the integrated staining intensity of OPP-Alexa Fluor 594 in cells identified by NuclearMask Blue Stain.

Mass Spectrometry / Proteomics

For proteomics sample preparation, between 2,000 and 10,000 cells were diluted with ultrapure water, and then lysed by adding cold trifluoroethanol (TFE) (final 50% TFE) to the suspension. Samples were then chilled on ice for 10 min, vortexed for 1 min and further sonicated for 5-10 min in an ice bath. Tris (pH 8-8.5) was added to adjust the pH and concentration to final 100 mM Tris at pH 8-8.5. Total protein mass was estimated to be 0.3 ng/cell. Samples were reduced with tris(2-carboxyethyl)phosphine (1 µg TCEP: 50 µg protein) incubated for 20 min at room temperature, then alkalized with chloroacetamide (5 µg CAA:50 µg protein) incubated at 95 °C for 10 min. Then samples were diluted with 50mM Ammonium bicarbonate (final TFE < 10%). Proteins were digested with LysC/Trypsin (1 µg LysC/Trypsin: 50 µg protein) for 2 hours at 37 °C, followed by digestion with trypsin (1 µg trypsin: 50 µg protein) and incubated overnight at 37 °C. Samples were further digested with trypsin (1 µg trypsin: 125 µg protein) for 5 hours at 37 °C. Digestion was stopped by acidification. Samples were desalted with 6 mM C18; briefly, stage tips were conditioned with 100% methanol, equilibrated with 0.2% TFA, loaded with samples, then washed with 0.2% TFA, and finally eluted with 80 µL *2 of 40% ACN, 0.1% formic acid. Eluate was dried in vacuum then kept in -20°C. Before instrumental analysis, samples were reconstituted in in 0.5% acetonitrile, 0.1% formic acid and concentration was measured using NanoDrop One (ThermoFisher, Waltham, USA) with the A205 Scopes method (absorbance at 205 nm, baseline correction at 340 nm).

For liquid chromatography steps, 75 ng of peptides were injected and separated on-line using NanoElute UHPLC system (Bruker Daltonics Billerica, United States) with Aurora Series Gen2 (CSI) analytical column, (25 cm x 75 µm 1.6µm FSC C18, with Gen2 nanoZero and CSI fitting; Ion Opticks, Parkville, Australia) heated to 50 °C and coupled to timsTOF Pro (Bruker Daltonics, Billerica, USA). A standard 30 min gradient was run from 2% B to 12% B over 15 min, then to 33% B from 15 to 30 min, then to 95% B over 0.5 min, and held at 95% B for 7.72 min. Before each run, the analytical column was conditioned with 4 column volumes of buffer A. Where buffer A consisted of 0.1% aqueous formic acid and 0.5 % acetonitrile in water, and buffer B consisted of 0.1% formic acid in 99.4% acetonitrile. The NanoElute thermostat temperature was set at 7 °C. The analysis was performed at 0.3 µL/min flow rate.

For mass spectrometry steps, the Trapped Ion Mobility – Time of Flight Mass Spectrometer (TimsTOF Pro; Bruker Daltonics, Billerica, USA) was set to Parallel Accumulation-Serial Fragmentation (PASEF) scan mode for DIA acquisition scanning 100 – 1700 m/z. The capillary voltage was set to 1800V, drying gas to 3 L/min, and drying temperature to 180°C. The MS1 scan was followed by 17 consecutive PASEF ramps containing 22 non-overlapping 35 m/z isolation windows, covering the m/z range 319.5 – 1089.5 (more information in DIA windows). As for TIMS setting, ion mobility range (1/k0) was set to 0.70 – 1.35 V·s/cm^2, 100 ms ramp time and accumulation time (100% duty cycle), and ramp rate of 9.42 Hz; this resulted in 1.91s of total cycle time. The collision energy was ramped linearly as a function of mobility from 27 eV at 1/k0 = 0.7 V·s/cm^2 to 55

eV at 1/k0 = 1.35 V·s/cm^2. Mass accuracy: error of mass measurement is typically within 3 ppm and is not allowed to exceed 7 ppm. TimsTOF Pro was run with timsControl v. 4.1.12 (Bruker, Billerica, USA). LC and MS were controlled with HyStar 6.0 (6.2.1.13, Bruker, Billerica, United States). For the final search and quantification of the results, acquired diaPASEF data were then searched using DIA-NN (v. 1.8.1) to obtain DIA quantification, with the spectral library generated from the canonical proteome for *Mus Musculus* from UniProt. Quantification mode was set to "Any LC (high precision) and a two-pass search was completed. All other settings were left default. Mice with a GFP protein ratio of less than 3 when comparing their respective *Ins2*(GFP)^{HIGH} and *Ins2*(GFP)^{LOW} samples were excluded.

Statistics and data visualization

Statistics and data representation for nearest neighbor analyses, distance to core analyses, and OPP translation assays, were conducted using GraphPad Prism v10 (GraphPad Software, San Diego, USA). Statistics and data representation for live cell imaging experiments employed custom R scripts. Student's t-test and one-way ANOVAs were used for parametric data as indicated in figure legends. For all statistical analyses, differences were considered significant if the p value was less than 0.05. Error bars were presented as ± standard error of the mean (SEM).

For proteomics analyses, proteins that were present in less than 80% of samples were removed from analysis. Significantly differential proteins were defined with a threshold of p value < 0.05 and graphs were generated using custom R scripts. For STRING and Cytoscape analyses, significantly differentially abundant proteins were entered into the STRING website, where nodes and networks were generated. For STRING settings, only 'Experiments' and 'Databases' interactions were included, with medium confidence interaction scores. STRING networks and protein expression data were then imported into Cytoscape and reordered according to their cellular location. Background images were generated with Biorender.

For STRING and Cytoscape analyses in our small molecule screen, the primary binding proteins for small molecules were determined using online literature search. Proteins were then entered into STRING, where nodes and networks were rendered. For settings on STRING, all interactions were included, with medium confidence interaction scores. STRING networks, GFP fluorescence, cell death, and cell state transition data were then imported and finalized in Cytoscape. Background graphs were generated using Biorender.

Results

Live 3D imaging of intact islets from Ins2^{GFP} mice

Our previous analyses of *Ins2* gene activity in dispersed mouse islets revealed a bimodal distribution of GFP fluorescence from a reporter construct knocked into the endogenous *Ins2* gene locus (15) (Fig. 1A, B). In this study, we used live 3D imaging to define the prevalence and spatial relationship of cells in the two β cell insulin gene activity states in whole intact islets from *Ins2*^{GFP} knock-in mice (Fig. 1C). Consistent with dispersed cells, *Ins2* gene activity in intact islets also had a bimodal distribution (Fig. 1D). We also found that the range of GFP fluorescence was greater in larger islets compared to smaller ones (Fig. 1D). We did not find significant sex differences when comparing the ratios of *Ins2*(GFP)^{HIGH} and *Ins2*(GFP)^{LOW} cells in intact male and female islets (Fig. 1E), consistent with our previous work in dispersed β cells (22). Nearest neighbor analysis showed that cells in the same *Ins2* activity state were more likely to be found in proximity of each other (Fig. 1F; Supplemental Fig. 1B). We found no significant difference between the cell states and their distance to the core of the islet (Fig. 1G; Supplemental Fig. 1C). To summarize, live cell 3D imaging

analysis revealed the that the *Ins2*(GFP)^{HIGH} and *Ins2*(GFP)^{LOW} expression states exist in intact islets, with cells in the same expression states being more likely to be found next to each other.

Identification of β cell selective islet proteins

Proteins that are selective to β cells have potential as biomarkers and anchor targets for imaging and drug delivery (24). Previous studies have identified β cell-specific mRNAs using single cell RNA sequencing (25). However, mRNA and proteins in isolated islets only have a correlation coefficient of ~0.5 (16), so there is an unmet need for minimally biased, high-throughput characterization of β cell selective gene products at the protein level. Therefore, as a step towards identifying β cell specific proteins, we compared the proteomes of GFP positive cells to GFP negative cells from dispersed and sorted mouse islets to a depth of 5555 proteins (Fig. 2A; Supplemental Fig. 2A). As expected, GFP-negative, non-β cells were enriched for known markers of other islet cell types, such as GCG, PYY, and SST (Fig. 2A). To rank the proteins, we multiplied the p value by the fold change. The top novel non-β cell proteins were the cell surface protein tetraspanin 8 (TSPAN8), arginase 1 (ARG1), and clusterin (CLU) (Fig. 2A). The top 5 most significantly enriched β cell selective proteins were: 1) interferon regulatory factor (IRF3), a key transcription factor of type 1 interferon-dependent immunity; 2) CAP-Gly domain containing linker protein 1 (CLIP1), which links cytoplasmic vesicles to microtubules; 3) 3'-phosphoadenosine 5'-phosphosulfate synthase 2 (PAPSS2), which mediates two steps in the sulfate activation pathway; 4) tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (YWHAZ), also known as 14-3-3ζ, a scaffolding protein we have shown plays important survival and signaling roles in β cells (26); and 5) apoptosis inducing factor mitochondria associated 1 (AIFM1) (Fig. 2A). As expected, INS1 and IAPP proteins were also significantly enriched in pure β cells. The G protein-coupled receptor 158 (GPR158), a metabotropic glycine receptor (27) which may play a role in autocrine feedback (28), was highly enriched in β cells. Notably, proteins involved in ER homeostasis such as HSPA5 were increased in GFP positive β cells, suggesting higher ER processing, and possibly stress, in β cells compared to other islet cells (Fig. 2A).

We next undertook pathway analysis to identify themes in our proteomic data from non-β cells and pure β cells (Fig. 2C). Gene set enrichment analyses revealed that non-β cells are enriched for proteins that mediate metabolic and catabolic processes including 'cellular modified amino acid metabolic process' and 'carbohydrate derivative metabolic process'. In pure β cells, there was enrichment of pathways entitled 'skin development' and 'supramolecular fiber organization', which was driven by the relative abundance of multiple keratin family proteins, which are known to modulate β cell signalling and play roles in stress responses (29). Enrichment of 'regulation of cellular component biogenesis' and 'regulation of protein-containing complex assembly' pathways suggests β cells may exist in a more anabolic state than non-β cells. Pathways involving 'protein phosphorylation' and 'IκB/NFκB signalling' were also enriched in pure GFP-positive β cells (Fig. 2C). Interestingly, NFκB signalling has been implicated in a β cell subpopulation with lower PDX1 expression, which has similarities with our *Ins2*(GFP)^{LOW} cells (30). Overall, these results suggest that differences between *Ins2* expressing cells and other islet cells are primarily associated with protein production and signalling.

Proteomic profiles of FACS purified β cells in Ins2(GFP)^{HIGH} and *Ins2*(GFP)^{LOW} states

We next compared the proteomic profiles of the two distinct *Ins2* gene activity states in FACS purified β cells (Fig. 2B, Supplemental Fig. 2B). Using p value times fold change as a criteria again, the top 5 most enriched proteins in *Ins2*(GFP)^{LOW} cells were: 1) glucagon (GCG), a marker for alpha cells; 2) pancreatic polypeptide (PPY) a marker for PP cells; 3) Purkinje cell protein 4 (PCP4), a calmodulin regulator involved in calcium binding and signalling; 4) aldehyde dehydrogenase 1 family

member 3 (ALDH1L2), a mitochondrial enzyme implicated in β cell protection and secretory dysfunction (31); and 5) adenine phosphoribosyltransferase (APRT), an enzyme involved in the nucleotide salvage pathway (Fig. 2B). These results suggest that cells in the less mature *Ins2*(GFP)^{LOW} state was enriched in markers of 'multi-hormonal' cells, consistent with our previous scRNAseq analysis (15). We also found enrichment in proteins in endoplasmic reticulum processing, including HSPA5, HSP90B1, and ERO1L. This corroborates our prior scRNAseq analyses and cell death assays, which also revealed increased ER stress factors at the mRNA level and a marked resiliency to ER stress in the *Ins2*(GFP)^{LOW} state (17).The top 5 most enriched proteins (other than GFP) in *Ins2*(GFP)^{HIGH} cells were: 1) zinc finger AN1-type containing 2B (ZFAND2B), a protein associated with the proteasome for degradation of misfolded proteins; 2) zinc finger RANBP2-type containing 2 (ZRANB2), a protein involved in RNA splicing and mRNA processing 3) myocyte enhancer factor 2D (MEF2D), a calcium regulated transcription factor; 4) SWI/SNF related, matrix associated, actin dependent regulator Of chromatin subfamily A, member 2 (SMARCA2), a protein which regulates transcription via altering chromatin structure; and 5) phosphofurin acidic cluster sorting protein 2 (PACS2), a protein involved in ER calcium ion homeostasis. The protein poly(A) polymerase alpha (PAPOLA), a protein required for creation of the 3'-poly(A) tail of mRNAs, was also upregulated in *Ins2*(GFP)^{HIGH} cells (Fig. 2B; Supplemental Fig. 2B).

The pathways significantly enriched in the *Ins2*(GFP)^{LOW} state relative to the *Ins2*(GFP)^{HIGH} state included: 'organic acid metabolic process', 'cellular respiration', 'generation of precursor metabolites and energy', and 'oxidative phosphorylation', suggesting the less mature cells may have more energic capacity. They are also enriched in proteins involved in 'fatty acid metabolic processes' and 'lipid metabolic processes', suggesting the possibility of metabolic substrate preference and switching between these cell states (Fig. 2D). A subpopulation of human β cells identified by Xin et al in 2018 with low *INS* mRNA and high UPR related factors also had upregulation in oxidative phosphorylation pathways, and it was suggested that upregulation of this pathway may be a form of protection against oxidative stress (8). The *Ins2*(GFP)^{LOW} state was enriched in 'hormone metabolic process' which was also enriched in non-β cells relative to β cells, including GCG, PYY, and SST. Gene set enrichment analysis revealed that the pathways most enriched in the *Ins2*(GFP)^{HIGH} state were related to transcriptional upregulation and mRNA processing (Fig. 2D). The upregulation of 'DNA-templated transcription' and 'RNA biosynthetic process' pathways may suggest that the *Ins2*(GFP)^{HIGH} state is more transcriptionally active. The *Ins2*(GFP)^{HIGH} state also enriched for 'RNA processing', a pathway which involves mRNA capping and addition of the 3'poly(A) tail. Regulation and processing of the 5' and 3' UTRs of insulin mRNA is known for their importance in maintaining mRNA stability, and our prior scRNAseq and qPCR data indeed showed that the *Ins2*(GFP)^{HIGH} state had more *Ins2* mRNA (15). 'Chromosome organization' and 'cell cycle' pathway upregulation is also indicative of regulation of the transcriptional landscape. Overall, this data suggests that differences between the *Ins2*(GFP)^{HIGH} and *Ins2*(GFP)^{LOW} states were related to transcription mechanisms and processes.

We next analysed the relationship between our differentially abundant proteins using STRING and mapped them to a cell diagram in Cytoscape to visualize protein-protein networks in their most common cellular locations (Fig. 3A, B). Protein-protein interaction networks enriched in GFP negative cells, beyond the known markers from other islet cell types (GCG, PYY, PPY, SST), included protein networks related to lipid metabolism (GPX3, HEXB) and factors related to amino acid synthesis and transport (SLC7A8, SLC3A2, ARG1) (Fig. 3A). Protein-protein interaction networks enriched in GFP positive cells, compared with non-GFP, included those involved in GPCR and cAMP signalling, glycolysis, and the secretory pathway (ER, Golgi, granules, cytoskeleton). Specifically, pure β cells showed enrichment for multiple cytoskeletal factors, including CLASP2, a protein which associates with LL5β, which in turn is needed for clustering of insulin secretory machinery; VIL1, a member of

calcium regulated actin binding proteins and is known plays a role in insulin exocytosis regulation; and CLIP1, a protein associated with granule trafficking (32-34). These data provide data on β cell specific protein networks at unprecedented detail.

Next, we examined network differences between the *Ins2*(GFP)^{HIGH} state and the *Ins2*(GFP)^{LOW} state. The *Ins2*(GFP)^{LOW} state was enriched for networks related to mitochondria function and cell respiration, including members of the pyrroline-5-carboxylate reductase family (PYCR1, PYCR2), and subunits of the NADH dehydrogenase (ubiquinone) complex (NDUFA6, NDUFB6, NDUFB10) (Fig. 4A). We also noted a number of factors with links to proteasome function (PSMA3, PSMB3, PSMB8), ER stress factors (HSPA5, HSP90B1, PDIA4, PDIA6), and translation factors (EIF3B, EIF2S1, EEF2) (Fig. 4A). This suggests that cells with low *Ins2* gene activity may be recovering from various sources of stress and may also be in the process of regulating translation machinery. Interestingly, RHOA, an F-actin regulator which reduces insulin secretion when activated, was upregulated in the *Ins2*(GFP)^{LOW} state (35). Another actin protein, ARPC2, which was downregulated in human islets when treated with insulin, was also enriched in *Ins2*(GFP)^{LOW} cells (36). On the other hand, many of the enriched proteins in the *Ins2*(GFP)^{HIGH} state were located in the nucleus and were linked to transcriptional regulation, including prominent transcription factors TOP1, CDK9, KAT7 and INTS1 (Fig. 4A). Several nuclear proteins were associated with mRNA processing, such as ZRANB2, SRPK1, and PAPOLA (37-39) (Fig. 4B). We also noted a number of factors in the Golgi, including: HOOK2, a component of the FTS/Hook/FHIP (FHF) complex which promotes vesicle trafficking; STRIP1, a protein involved in the localization of the Golgi; and VCPIP1, a protein involved in Golgi reassembly. There were also a fair number of proteins in the ER, including the aforementioned ZFAND2B and PACS2; DNAJA1, a protein which stimulates ATP hydrolysis; and NGLY1, an enzyme involved in the degradation of misfolded proteins. Proteins related to the cell cytoskeleton and protein trafficking were also upregulated, including CLIP1, CLASP2, and protein transport regulator KIF3A, suggesting upregulation in protein export in the *Ins2*(GFP)^{HIGH} state. Taken together, the *Ins2*(GFP)^{HIGH} state is primarily associated with the regulation of transcription, mRNA processing, and protein trafficking, while the *Ins2*(GFP)^{LOW} state may be in the process of recovering from stress associated with high *Ins2* gene activity and a shift in protein translation machinery.

Our proteomics data revealed enrichment of 'regulation of cellular component biogenesis' and 'regulation of protein-containing complex assembly' pathways in the *Ins2*(GFP)^{HIGH} state, so we tested whether β cell translation capacity was different compared to other islet cell types using the O-propargyl-puromycin (OPP) assay, which incorporates a fluorescent marker into nascent proteins (Fig. 5A). Using GFP as a marker for β cells, we found that β cells had higher translational capacity compared to other islet cells (Fig. 5B). The upregulation of DNA template transcriptional regulation and RNA processing pathways in *Ins2*(GFP)^{HIGH} cells relative to *Ins2*(GFP)^{LOW} cells is suggestive of increased downstream translational capacity. We found that cells in the *Ins2*(GFP)^{HIGH} state had significantly higher translational capacity compared to cells in the *Ins2*(GFP)^{LOW} state (Fig. 5C, D). We did not note any significant differences when comparing the translational capacity of the two *Ins2* expression states in the context of sex differences (Supplemental Fig. 4A). Overall, these data reveal the differences between the *Ins2*(GFP)^{HIGH} and *Ins2*(GFP)^{LOW} expression states at the protein level, and show how some of these differences can translate to differences in β cell function.

Mechanistic interrogation of Ins2 gene activity regulation

The molecular mechanisms regulating *Ins2* gene activity are not clear. We chose 48 drugs known to perturb key β cell signaling pathways to study their regulation of β cell states (Table 2)(Fig. 6A). Concentrations for the various compounds were decided based on documented doses used in *in vitro* assays. Dispersed islet cells from *Ins2*^{GFP} mice were treated with compounds and imaged in a high-throughput format (Fig. 6B-L). Although all of the experiments were conducted in parallel using

a robotic imaging system, we visualized the results from each family of compounds separately. Compounds which activate cAMP signalling, namely the PDE inhibitor 3-isobutyl-1-methylxanthine (IBMX) and the cAMP inducer GLP-1, significantly increased *Ins2* gene activity when tracked over time (Fig. 6B). The rate of increase for GFP fluorescence was considerably greater in *Ins2*(GFP)^{HIGH} cells in response to cAMP pathway activators compared to *Ins2*(GFP)^{LOW} cells (Fig. 7B, C). Compared to DMSO control, the calcineurin pathway inhibitors cyclosporine A, okadaic acid, and tacrolimus reduced *Ins2* gene activity (Fig. 6C). Inhibitors of the endonuclease IRE1A caused a steep drop in *Ins2* gene activity after prolonged treatment, with APY29 inducing a strong increase prior to the drop (Fig. 6D). Effectors of ER pumps and channels had relatively few effects on *Ins2* gene activity, with ATP2A2 inhibitor thapsigargin slightly increasing *Ins2* activity (Fig. 6E). Compounds which impacted insulin signalling also had some interesting effects, in particular the protein kinase inhibitor staurosporine, which significantly increased *Ins2* gene activity, and PI3K inhibitor LY294002, which caused a spike at 48 hours followed by a steep decline (Fig. 6F). The glucocorticoid agonist dexamethasone reduced *Ins2* activity, while PPARG agonist rosiglitazone slightly increased it (Fig. 6G). The three nutrients we tested all increased *Ins2* gene activity, with arginine and sodium butyrate having a more significant increase over time compared to palmitic acid (Fig. 6H). Compounds which affected cell membrane excitability also had interesting effects, with K_{ATP} channel (ABCC8 subunit) activator diazoxide and CACNA1C inhibitor verapamil decreasing *Ins2* gene activity, while K_{ATP} channel (ABCC8 subunit) inhibitor tolbutamide modestly increased it near the end of our experiment (Fig. 6I). Perturbing protein degradation factors also yielded effects, in particular ERAD inhibitor eeyarestatin I, which decreased *Ins2* gene activity rapidly after treatment (Fig. 6J). As expected, many compounds which affected transcriptional machinery also impacted *Ins2* gene activity (Fig. 6K). TFIIH inhibitor Triptolide and RNA polymerase II inhibitor alpha amanitin resulted in an increase in *Ins2* activity, peaking at around 48 hours and rapidly declining afterwards (Fig.6K). CDK9 inhibitor 5, 6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) also increased *Ins2* gene activity (Fig. 6K). TOP1 inhibitor actinomycin D caused a steep decline in *Ins2* gene activity 24 hours after treatment (Fig. 6K). HDAC inhibitors MGCD0103 and trichostatin A both reduced *Ins2* gene activity, but not as abruptly as triptolide and alpha amanitin (Fig. 6K). Compounds which affected translation that had an effect on *Ins2* gene activity include EEF2 inhibitor cyclohexamide, which increased *Ins2* activity followed by a steep decline after 48 hours, PKR inhibitor, which increased *Ins2* gene activity, and PERK activator CCT020312 and EIF2S1 inhibitor sal003, which reduced *Ins2* gene activity (Fig. 6L).

We performed hierarchical clustering using various cell behavior traits in the context of comparing cells from male and female mice (Supplemental Fig. 3A,B). Cell activity traits we used included mean GFP (average GFP fluorescence over time), max GFP (peak GFP fluorescence), and mean cell death (average PI intensity). Overall, the strongest effectors of GFP fluorescence did not show any significant sex differences (Supplemental Fig. 3A).

Rapid drops in GFP fluorescence are indicative of cell death, so we explored the relationship between GFP expression and cell viability by plotting fluorescence in the green wavelength (GFP) against the red wavelength (PI) (Fig. 6M) (15). We note that the cAMP agonists IBMX and GLP-1 amide had the highest GFP fluorescence with relatively low cell death (Fig. 6M). We found that okadaic acid and trichostatin A had the greatest decrease in GFP fluorescence without significant cell death (Fig. 6M). The subset of drugs which had increasing GFP fluorescence followed by a rapid drop (APY 29, triptolide, alpha amanitin, cyclohexamide, DRB, LY294002) had significant cell death, indicating the drugs were toxic after prolonged treatment (Fig. 6M). This is supported by an OPP assay done in the context of several small molecules of interest (GLP-1 amide, IBMX, APY 29, triptolide), in which a significant drop in translational capacity can be observed after 24 hours of treatment, well before the drop in GFP fluorescence and noticeable cell death (Supplemental Fig.

4B). Overall, our small molecule screen shows how various drugs and small molecules may impact *Ins2* gene activity and how it may correlate to β cell health.

Cell state transitions in the context of media conditions and perturbations

Various culture conditions can impact cell state transitions, with higher glucose concentrations and the addition of FBS inducing a greater rate of *Ins2*(GFP)^{LOW} to *Ins2*(GFP)^{HIGH} transitions (Fig. 7A). We next analyzed the rate of transitions for cells from *Ins2*^{GFP} knock-in mice in the context of our small molecule screen (Fig. 7D). In general, most small molecules had higher *Ins2*(GFP)^{HIGH} to *Ins2*(GFP)^{LOW} transitions rates compared to *Ins2*(GFP)^{LOW} to *Ins2*(GFP)^{HIGH}, with our DMSO control at possessing a ~3:1 ratio (9.1% *Ins2*(GFP)^{HIGH} to *Ins2*(GFP)^{LOW}, 3.2% *Ins2*(GFP)^{LOW} to *Ins2*(GFP)^{HIGH}). Compared to our DMSO control, we found that cAMP agonists GLP-1 amide and IBMX had the greatest increase in *Ins2*(GFP)^{LOW} to *Ins2*(GFP)^{HIGH} transitions (19% for GLP-1 amide, 14% for IBMX), with relatively low levels of *Ins2*(GFP)^{HIGH} to *Ins2*(GFP)^{LOW} transitions (Fig. 7B-D). Okadaic acid and cycplosporin A induced the highest rate of *Ins2*(GFP)^{HIGH} to *Ins2*(GFP)^{LOW} transitions (Fig. 7D). Interestingly, we found that several drugs with high GFP, including triptolide, APY 29, and alpha amanitin, had very few transitions of either type (Fig. 7D). This shows that the increase in GFP fluorescence observed in these treatments was primarily driven by increasing GFP fluorescence in the *Ins2*(GFP)^{HIGH} cells, and not in the *Ins2*(GFP)^{LOW} cells.

STRING/Cytoscape analyses helped contextualize the major binding partners of our small molecule screen (Fig. 8A). Several of the strongest effectors of GFP fluorescence were found to impact genes located within the nucleus, including CDK9, TOP1, POL2A, ERCC3, HDAC1, HDAC2, SP1, and PAK1. Many of these proteins have known roles in transcriptional mechanisms (40-45). On the membrane, GLP1R and NOS1 were receptors which had effects on GFP fluorescence. In the ER, perturbing ERN1, a major factor in UPR response, impacted GFP fluorescence and cell health (46). In the cytosol, perturbation of VCP, a major component of ERAD, resulted in heavy cell death, while PIK3CA, PDE3A, EEF2 influenced GFP fluorescence. In the context of cell state transitions, perturbing PDE3A and GLP1R induced the highest rate of *Ins2*(GFP)^{LOW} to *Ins2*(GFP)^{HIGH} transitions, while perturbing PPP3CC and PPP2CA resulted in the highest rate of *Ins2*(GFP)^{HIGH} to *Ins2*(GFP)^{LOW} transitions. Overall, these results show how perturbing various β cell signalling pathways may impact *Ins2* gene activity and cell state transitions.

Discussion

This study aimed to further our understanding of dynamic *Ins2* gene activity states, their proteomic profiles, and their regulation by key cellular processes. Our 3D imaging experiments showed how the *Ins2*(GFP)^{HIGH} and *Ins2*(GFP)^{LOW} expression states existed in live intact islets. The two *Ins2* gene expression states were present in intact islets. Cells in the same expression states were more likely to be next to each other. We did not find any correlation for either cell population to the islet periphery or core. This is similar to what was observed by Johnston *et al*, in which hub cells, a β cell sub-set with similarities to *Ins2*(GFP)^{LOW} cells, did not show any clear preference for the islet center or periphery (47). A caveat of our 3D imaging was the lack of penetrance into the center of larger islets. Future studies could employ two-photon microscopy to resolve this issue (48).

Our study characterized the *Ins2*(GFP)^{HIGH} and *Ins2*(GFP)^{LOW} expression states at the proteome level and revealed mechanisms governing *Ins2* gene activity and cell state transitions. Minimally biased interrogation of proteins and protein networks selectively present in β cells and β cell states builds on past efforts to map the β cell proteome. Previously established FACS purifying mouse β cell populations based on insulin immunoreactivity yielded 6955 proteins across 36 islet samples with

various diets and islet cell types (20), and led to the identification of a subpopulation of β cells with low CD81 expression which had enrichment for factors in spliceosome and RNA processing pathways, but reduced oxidative phosphorylation factors, similar to our *Ins2*(GFP)^{HIGH} cells (20). Two β cell subpopulations identified in a study on human β cell scRNAseq data had low *INS* expression, and while one high UPR marker expression, the other had enrichment in pathways related to oxidative stress (8). These two β cell subpopulations possess similarities with our *Ins2*(GFP)^{LOW} cells, and it was proposed that these low *INS* expressing cells were in a state of recovery from the various stresses of high insulin production (8). Overall, our study identifies novel and previously established β cell markers at the protein level and presented results showing how the cells in a high *Ins2* gene expression state had enrichment for factors related to RNA processing and transcription, with reduced oxidative phosphorylation and ER stress markers.

Our targeted perturbation analysis revealed pathways which impact endogenous *Ins2* gene activity and cell state transitions. The calcineurin inhibitors tacrolimus, cyclosporine A, and okadaic acid reduced *Ins2* gene activity (Fig. 6C), consistent with known roles for calcineurin on insulin mRNA in transgenic mice with a human insulin promoter (49). Okadaic acid and cyclosporine A are the two top inducers of *Ins2*(GFP)^{HIGH} to *Ins2*(GFP)^{LOW} transitions. NFAT, a calcineurin target, has been reported to be required for cAMP-mediated upregulation of insulin transcription in INS-1E cells (50). Interestingly, IRS2, a reported target of calcineurin for regulation of human β cell survival, is enriched in *Ins2*(GFP)^{HIGH} cells, further supporting a role for calcineurin in cell state regulation (51).

A key finding of our study was that GLP-1 amide and IBMX robustly increased *Ins2* activity over time, peaking at around 25 hours for GLP-1 amide and 55 hours for IBMX (Fig. 6B). This is consistent with previous data demonstrating that cAMP signaling plays a critical role in insulin production and secretion (52). Interestingly, GLP-1 and IBMX, also had the highest rates of *Ins2*(GFP)^{LOW} to *Ins2*(GFP)^{HIGH} transitions (Fig. 7D). Our proteomics analyses revealed that *Ins2*(GFP)^{HIGH} cells had upregulation in two phosphodiesterase (PDE) isoforms, PDE1C and PDE8A, both of which are known to play major roles in regulating cAMP and GSIS in rodent β cells (53; 54). Okadaic acid, the strongest inducer of *Ins2*(GFP)^{HIGH} to *Ins2*(GFP)^{LOW} transitions, is known to increase PDE expression in rat adipocytes (55). It is also worth noting that despite strongly upregulating GFP expression, many treatments including APY29, triptolide, alpha amanitin, staurosporine, sodium butyrate, and arginine, did not induce nearly as many cell state transitions as IBMX and GLP-1 amide. Taken together, these results may suggest that the dynamics between calcineurin, cAMP, and PDE concentrations play a major role in regulating *Ins2* gene activity and cell state transitions.

Some small molecules had effects on GFP fluorescence that were strongly correlated to cell health. For example, actinomycin D and eeyarestatin I killed cells rapidly upon treatment, resulting in a rapid collapse of GFP fluorescence. Interestingly, a subset of drugs including APY29 (IRE-1A inhibitor), triptolide (TFIIH inhibitor), alpha amanitin (RNA polymerase II inhibitor), cyclohexamide (EEF2 inhibitor), DRB (CDK9 inhibitor), and LY294002 (PI3K inhibitor) induced a strong increase in GFP fluorescence followed by rapid cell death after prolonged treatment. The endonuclease IRE-1A is an important factor in β cell stress regulation (46). Inhibition of general transcriptional machinery such as TFIIH and RNA polymerase II is highly detrimental to cell health after prolonged periods of time (56; 57). Inhibition of translation and cell cycle factors are also known to be toxic to cells (58; 59). The PI3K signalling pathway is linked to cell survival and resistance mechanisms (60)., The toxicity of these drugs may result in a buildup of stress cumulating in eventual cell death. It is interesting to note that the increase in GFP fluorescence in these drugs only occurs in *Ins2*(GFP)^{HIGH} cells, but not *Ins2*(GFP)^{LOW} cells. This may be due to the inherent fragility in *Ins2*(GFP)^{HIGH} cells, which may make them more vulnerable to the effects of stress (15). It is also worth noting that the two small molecules which induced the greatest increase in GFP fluorescence, GLP-1 amide and IBMX, are also well known to have protective effects on β cells (61; 62). The OPP assay done in the context of several

small molecules of interest also supports this, with cells treated to 24 hours of triptolide and APY 29 displaying reduced translational capacity prior to cell death and reduction in GFP fluorescence. These findings may suggest links between cell health and *Ins2* gene activity.

In summary, we provide data which further reveals the differences between the *Ins2*(GFP)^{HIGH} and *Ins2*(GFP)^{LOW} expression states beyond our previous study. Our live cell 3D imaging captures the two cell states in intact living islets, and our proteomic and translational assays characterizes them at the protein and functional level. The small molecule screen reveals pathways which may impact *Ins2* gene activity and cell state transitions, including prominent effects by effectors of cAMP signalling. Our study highlights the importance of cell expression states and cell state transitions in the islet, and how targeting factors which influence cell state transitions may help manage β cell function in diabetes.

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Table 1. Key resources

Reagent/resource	Designation	Source/reference	RRID
Ins2^{GFP} knock-in mouse model	Mouse model	Wakae-takada et al., 2013(14)	N/A
Chemical compound	Collagenase P	Sigma, catalog number C7657	N/A
Reagent	RPMI 1640	Invitrogen, catalog number 11875	N/A
Reagent	Fetal bovine serum	Invitrogen, catalog number 12483020	N/A
Software	GraphPad Prism	GraphPad Software	N/A
Software	ImageXpress	Molecular Devices	N/A
Software	R	R Foundation for Statistical Computing	N/A

Table 2. Reagents used in the small molecule screen

Drug	Concentration	Vendor	Catalogue #	Reference PMID
Cyclohexamide	355 μM	Sigma Aldrich	C7698	32844746
Sal003	5 μM	Sigma Aldrich	S4451	28009255
CCT020312	0.2 μM	Calbiochem	324879	28148553
DHBDC	20 μM	Calbiochem	5315510001	23784735
ISRIB	0.2 μM	Sigma Aldrich	SML0843	25858979
GSK2606414 / Perk inhibitor 1	0.03 μM	Sigma Aldrich	516535	22827572
PKRi/C16	2 μM	Sigma Aldrich	I9785	31723021
Thapsigargin	1 μM	Millipore	586005	36690328
Tunicamycin	0.1 μM	Sigma Aldrich	T7765	32268491
Eeyarestatin I	10 μM	Sigma Aldrich	E1286	21124757
APY 29	20 μM	Sigma Aldrich	SML2381	25018104
Sunitinib	5 μM	Sigma Aldrich	PZ0012	28449948
Azoramide	20 μM	Sigma Aldrich	SML1560	26084805
Tacrolimus / FK506	0.03 μM	Selleck Chem	S5003	12761338
Cylcosporin A	5 μM	Selleck Chem	S2286	12761338
LY 294002	50 μM	Sigma Aldrich	L9908	18202127
Wortmannin	0.1 μM	Sigma Aldrich	W1628	8666133
Sodium butyrate	1000 μM	Sigma Aldrich	B5887	2843409
Trichostatin A (TSA)	0.625 μM	Sigma Aldrich	T1952	25305500

MGCD0103/Mocetinostat	25 μM	Selleck Chem	S1122	18413790
Somatostatin (SST)	1 μM	Sigma Aldrich	S9129	7907998
Epinephrine	50 μM	Sigma Aldrich	E4250	7907998
GLP-1 amide	0.01 μM	R&D systems	2082	10909973
Rosiglitazone	10 μM	Sigma Aldrich	R2408	19338578
Staurosporine	0.1 μM	Sigma Aldrich	S4400	14510500
Okadaic	0.1 μM	Sigma Aldrich	O9381	8183910
Palmitate	0.5 μM	Sigma Aldrich	P9767	15944145
Metformin	0.5 μM	Sigma Aldrich	PHR1084	27662780
Dexamethasone (DEX)	0.1 μM	Sigma Aldrich	D4902	28406939
Isoxazole 9 (ISX-9)	40 μM	Selleck Chem	S7914	22143803
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Diazoxide	100 μM	Sigma Aldrich	D9035	17229937
IBMX	100 μM	Sigma Aldrich	I5879	6201163
Arginine	10000 μM	Sigma Aldrich	A8094	25205820
Verapamil	2.7 μM	Sigma Aldrich	V4629	1095438
Ryanodine	10 μM	Millipore	559276	15033925
5-amino-imidazole carboxamide riboside (AICAR)	300 μM	Sigma Aldrich	A9978	15823553
Pramlintide	1 μM	Sigma Aldrich	SML2523	1311671
Tolbutamide	0.37 μM	Sigma Aldrich	T0891	6988275
HNMPA	100 μM	Abcam	ab141566	18202127
S961	0.1 μM	Phoenix Peptide	051-86	26740601
Bortezomib	0.004 μM	Sigma Aldrich	5043140001	19190324
MG132	0.4 μM	Sigma Aldrich	M8699	19190324
xestospongin C	2 μM	Sigma Aldrich	X2628	23302634
Actinomycin D	2 μM	Sigma Aldrich	A9415	21922053
α-amanitin	2 μM	Sigma Aldrich	A2263	24213132
Triptolide	0.1 μM	Sigma Aldrich	T3652	24213132
5,6-Dichloro-1-beta-Ribo-furanosyl Benzimidazole (DRB)	50 μM	Sigma Aldrich	D1916	24213132

Figure Legends

Figure 1. Live 3D imaging of intact *Ins2*^{GFP} mouse islets. (A) Schematic of the *Ins2*^{GFP} knock-in mouse model, in which the second exon of the wildtype *Ins2* gene has been partially replaced with GFP. **(B)** FACS purification of *Ins2*(GFP)^{LOW} and *Ins2*(GFP)^{HIGH} populations of β cells, with β cells isolated from a WT mouse for comparison. **(C)** Example images of 3D live cell imaging of intact islets from *Ins2*^{GFP} knock-in mice. Hoechst is a cell nuclei dye. Scale bar is 30 µm. **(D)** Density plot of cells from *Ins2*^{GFP} islets. Each distribution represents an islet. Blue distributions are small islets with less than 150 counted GFP positive cells, while red islets are large islets with over 150 counted GFP positive cells (n = 40 islets). **(E)** Comparison of ratios of high GFP cells to low GFP cells in male and female mice. Each dot represents an islet (n = 20 male and 20 female islets from 3 male and 3 female mice). **(F)** Nearest neighbor analysis of cells from *Ins2*^{GFP} knock-in mice (n = 40 islets). One-way ANOVA. **(G)** Distance to core of cells from *Ins2*^{GFP} knock-in mice. Student's T-test. Density: probability density function, represents the probability a data is located within the defined range (i.e. ratio of the total). * $p < 0.05$

Figure 2. Proteomic analysis of FACS purified *Ins2*^{GFP} β cells. (A) Volcano plot of differentially expressed proteins comparing FACS purified GFP positive and GFP negative cells. **(B)** Volcano plot of differentially expressed proteins comparing FACS purified *Ins2*(GFP)^{HIGH} and *Ins2*(GFP)^{LOW} cells. **(C)** Gene set enrichment analysis of pathways enriched in GFP positive and GFP negative cells. **(D)** Gene set enrichment analysis of pathways enriched in *Ins2*(GFP)^{HIGH} and *Ins2*(GFP)^{LOW} cells.

Figure 3. Integration of STRING networks and protein expression data in Cytoscape reveals differences in protein production and export between GFP positive and GFP negative cells. (A) Integration of STRING networks and protein expression data of differentially abundant proteins (top 100 with p value lower than 0.05) in GFP negative cells in Cytoscape. Deeper color of the nodes represents the fold change (GFP negative/GFP negative), while the thickness of the line around the node represents p value. **(B)** Integration of STRING networks and protein expression data of differentially abundant proteins (top 100 with p value lower than 0.05) in GFP positive cells in Cytoscape. Deeper color of the nodes represents the fold change (GFP positive/GFP negative), while the thickness of the line around the node represents p value.

Figure 4. Integration of STRING networks and protein expression data in Cytoscape reveals differences in transcription and mRNA processing between GFP expression states. (A) Integration of STRING networks and protein expression data of differentially abundant proteins (p value lower than 0.05) in *Ins2*(GFP)^{HIGH} cells in Cytoscape. Deeper color of the nodes represents the fold change (*Ins2*(GFP)^{LOW}/*Ins2*(GFP)^{HIGH}), while the thickness of the line around the node represents p value **(B)** Integration of STRING networks and protein expression data of differentially abundant proteins (p value lower than 0.05) in *Ins2*(GFP)^{LOW} cells in Cytoscape. Deeper color of the nodes represents the fold change (*Ins2*(GFP)^{HIGH}/*Ins2*(GFP)^{LOW}), while the thickness of the line around the node represents p value.

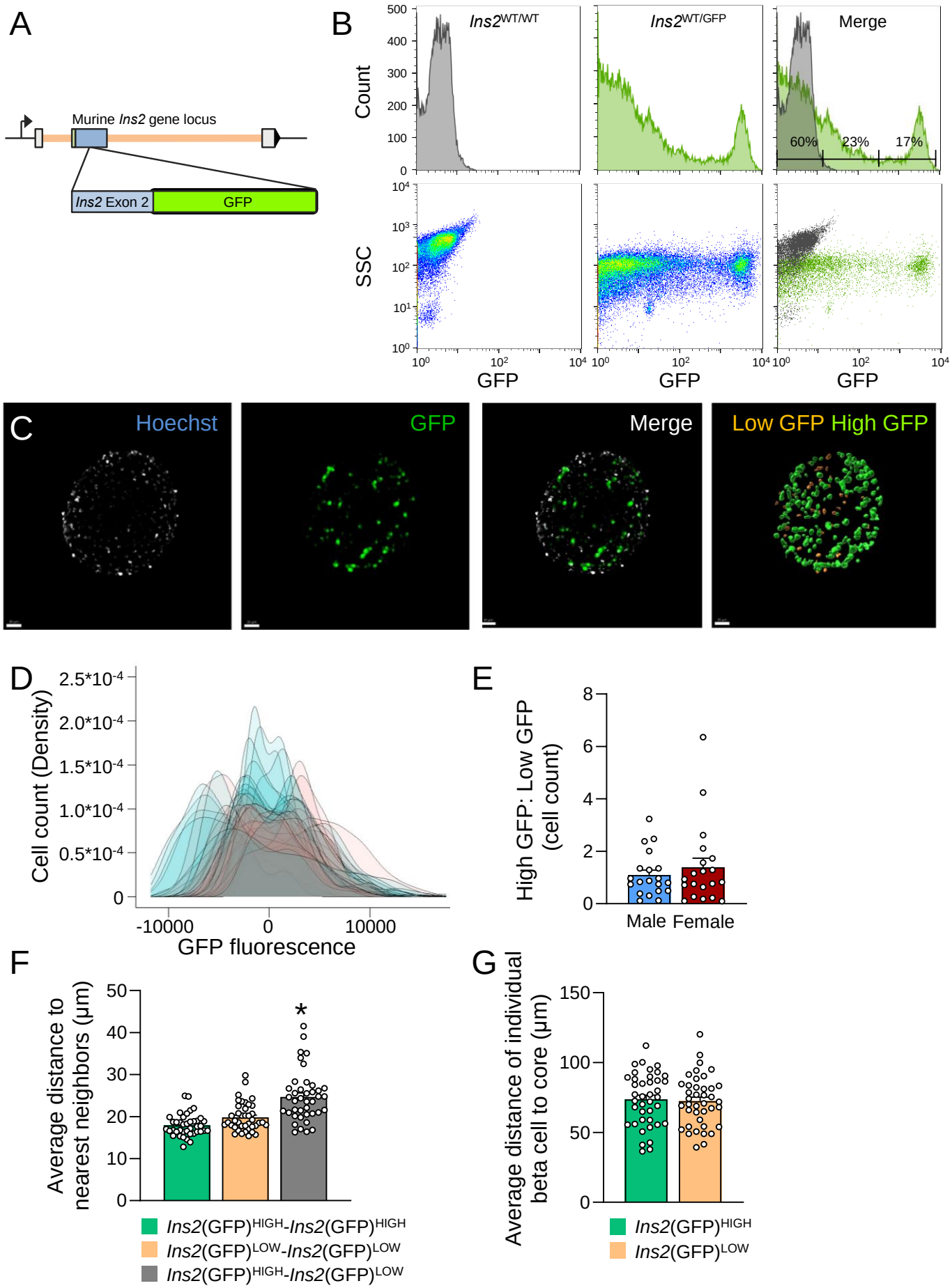
Figure 5. OPP protein synthesis assay of *Ins2*(GFP)^{LOW} and *Ins2*(GFP)^{HIGH} cells. (A) Representative images of OPP protein synthesis assay on *Ins2*$^{WT/GFP}$ mice. The blue wavelength is the Nuclearmask cell nuclei stain, the green wavelength is GFP, and the red wavelength is the OPP marker for nascent peptide formation. Scale bar is 40 µm. **(B)** Comparison of translational capacity of GFP positive β cells and GFP negative cells (n = 6 mice). Student's t-test. **(C)** Comparison of

translational capacity of *Ins2*(GFP)^{HIGH} and *Ins2*(GFP)^{LOW} cells (n = 6 mice). Student's t-test. **(D)** GFP fluorescence of *Ins2*(GFP)^{LOW} β cells and *Ins2*(GFP)^{HIGH} β cells. Student's t-test (n=6 mice).

Figure 6. A small molecule screen for effectors of *Ins2* gene activity. (A) Experiment pipeline. Islets were isolated and dispersed from *Ins2*^{GFP} knock-in mice. Cells were then stained with Hoechst cell nuclei dye and propidium iodide (PI) cell death marker for two hours, then subjected to treatments. Live cell imaging was then performed, and fluorescence for all three wavelengths was tracked across 96 hours of imaging. **(B-L)** Average GFP fluorescence of cells, across all treatments, over the period of 96 hours. Treatments were categorized based on their primary binding partners. Deceased cells were removed by thresholding for cells with high PI fluorescence. Dotted black lines indicate DMSO controls, dashed lines indicate inhibitors of their respective categories, and solid lines indicate activators of their respective categories (n = 6 mice). **(M)** Analysis of GFP fluorescence against cell death. GFP fluorescence is scaled GFP fluorescence relative to background after 48 hours of treatment. Cell death is scaled PI fluorescence relative to background after 72 hours of treatment. Size of dots represents p value (adjusted). Colors of dots represents categories, which were decided based on their primary binding partners (n = 6 mice). Two-way ANOVA, adjusted p value indicated by size of dots.

Figure 7. Cell state transitions in the context of various media conditions and perturbations. (A) Transition rates between the *Ins2(*GFP)^{HIGH} and *Ins2(*GFP)^{LOW} cell states in the context of various media conditions. % of cells transitioning: percent of subpopulation transitioning to other cell state after 48 hours of culture. **(B)** Example images of increasing GFP fluorescence in response to IBMX. **(C)** Example traces of GFP upregulation and cell state transitions in response to IBMX and GLP-1 amide. **(D)** Comparison of *Ins2(*GFP)^{HIGH} to *Ins2(*GFP)^{LOW} and *Ins2(*GFP)^{LOW} to *Ins2(*GFP)^{HIGH} transition rates in the context of the small molecule screen. Data shown is average transition rate by the end of 48 hours of treatment (n = 6 mice). Size of the dots denotes p value (adjusted). Color of the dots denotes scaled GFP fluorescence. Two-way ANOVA.

Figure 8. Integration of STRING protein networks and a small molecule screen on Cytoscape. (A) Primary binding partners for small molecules used in the screen localized in Cytoscape. Binding partners (bold) are chosen based on their affinity to the small molecule. Color of the nodes represents GFP fluorescence (scaled) after 48 hours of treatment. Borders of the nodes represents cell death (scaled PI fluorescence) after 72 hours of treatment. Height of green triangles represents *Ins2(*GFP)^{LOW} to *Ins2(*GFP)^{HIGH} transition rates. Height of orange triangles represents *Ins2(*GFP)^{HIGH} to *Ins2(*GFP)^{LOW} transition rates. Blue lines represent STRING interactions. Pointed arrows with grey lines represents upregulation effect. Inhibitor arrows with grey lines represents downregulation effect.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Hoechst
GFP
Merge
Low GFP High GFP
D
Cell count (Density)
GFP fluorescence
E
High GFP: Low GFP (cell count)
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Female
F
Average distance to nearest neighbors (µm)
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Ins2(GFP)LOW-Ins2(GFP)LOW
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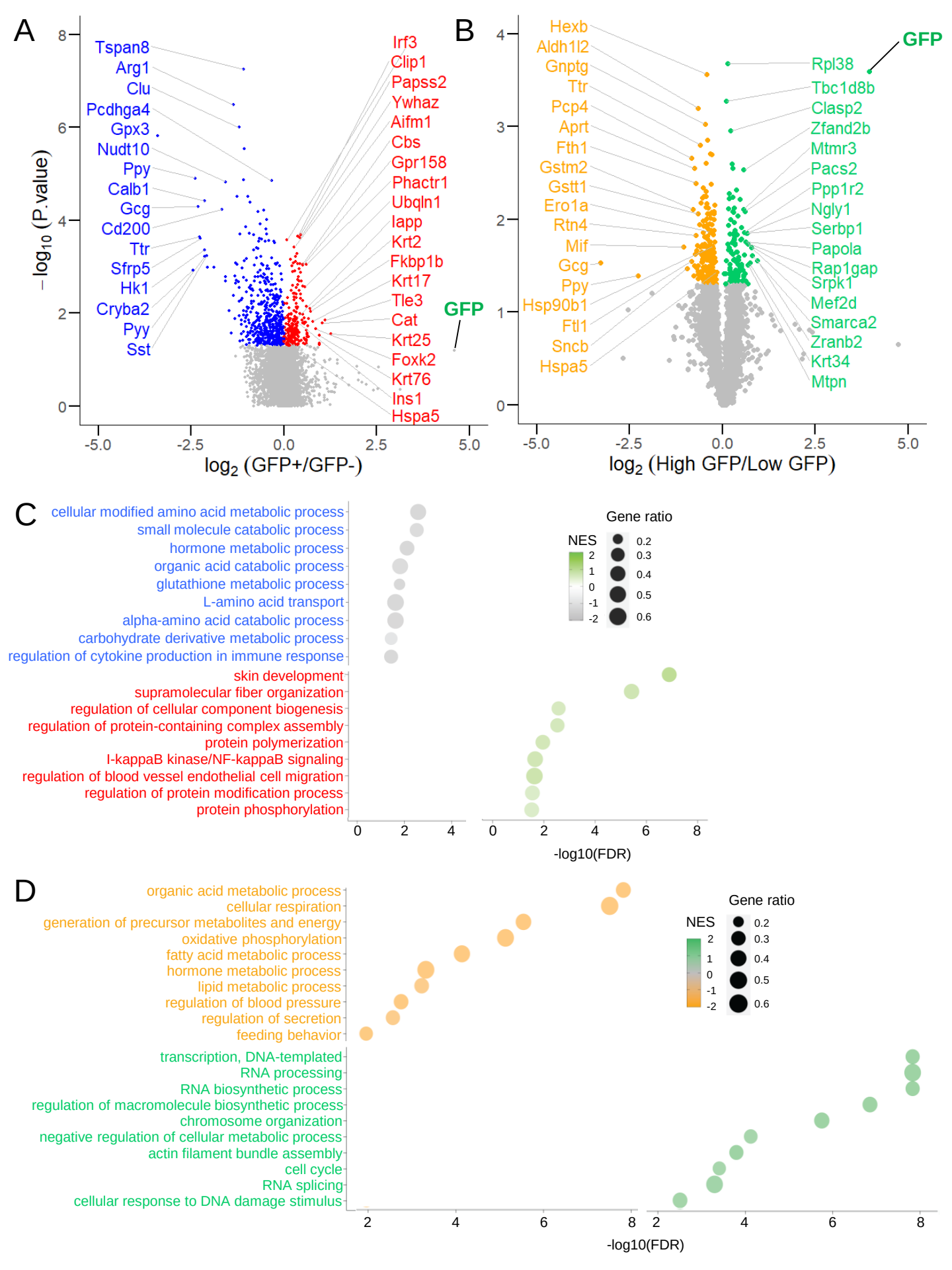
Average distance of individual beta cell to core (µm)
Ins2(GFP)HIGH
Ins2(GFP)LOW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hormone metabolic process
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skin development
supramolecular fiber organization
regulation of cellular component biogenesis
regulation of protein-containing complex assembly
protein polymerization
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regulation of blood vessel endothelial cell migration
regulation of protein modification process
protein phosphorylation
Gene ratio
NES
-log10(FDR)
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lipid metabolic process
regulation of blood pressure
regulation of secretion
feeding behavior
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RNA processing
RNA biosynthetic process
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Gene ratio
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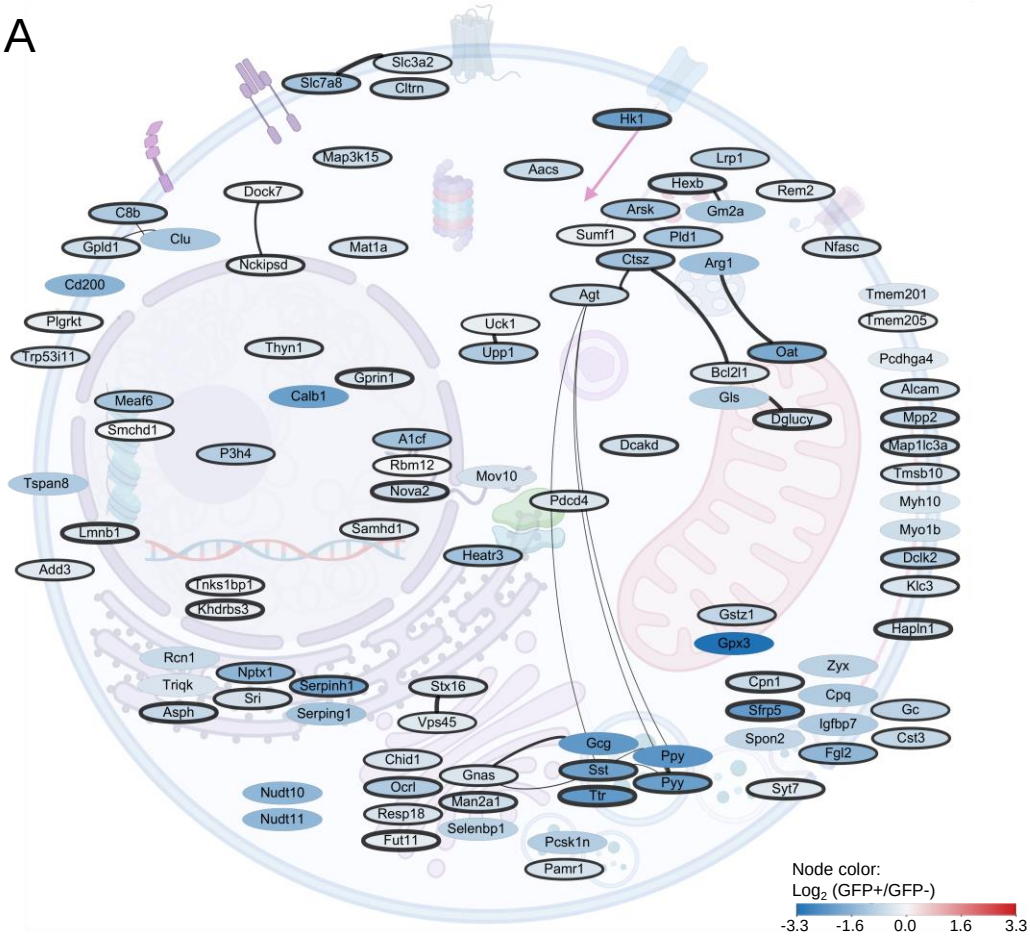
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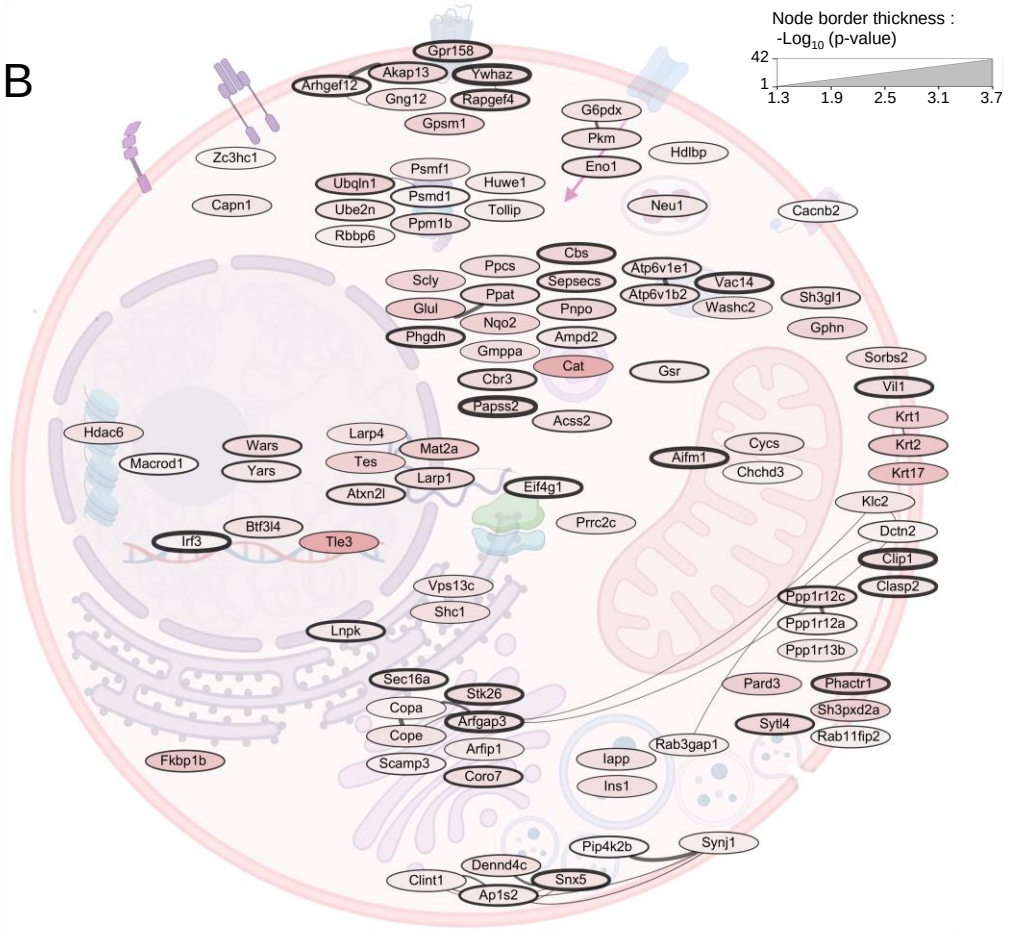
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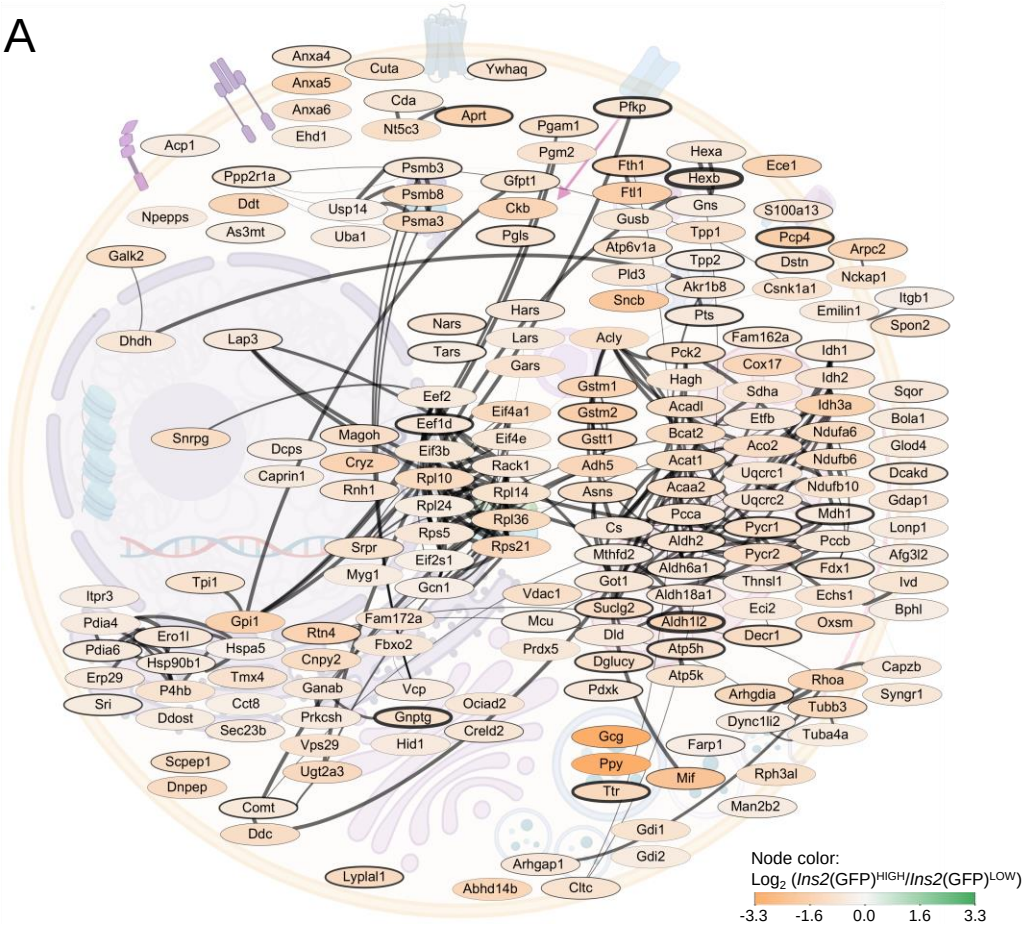
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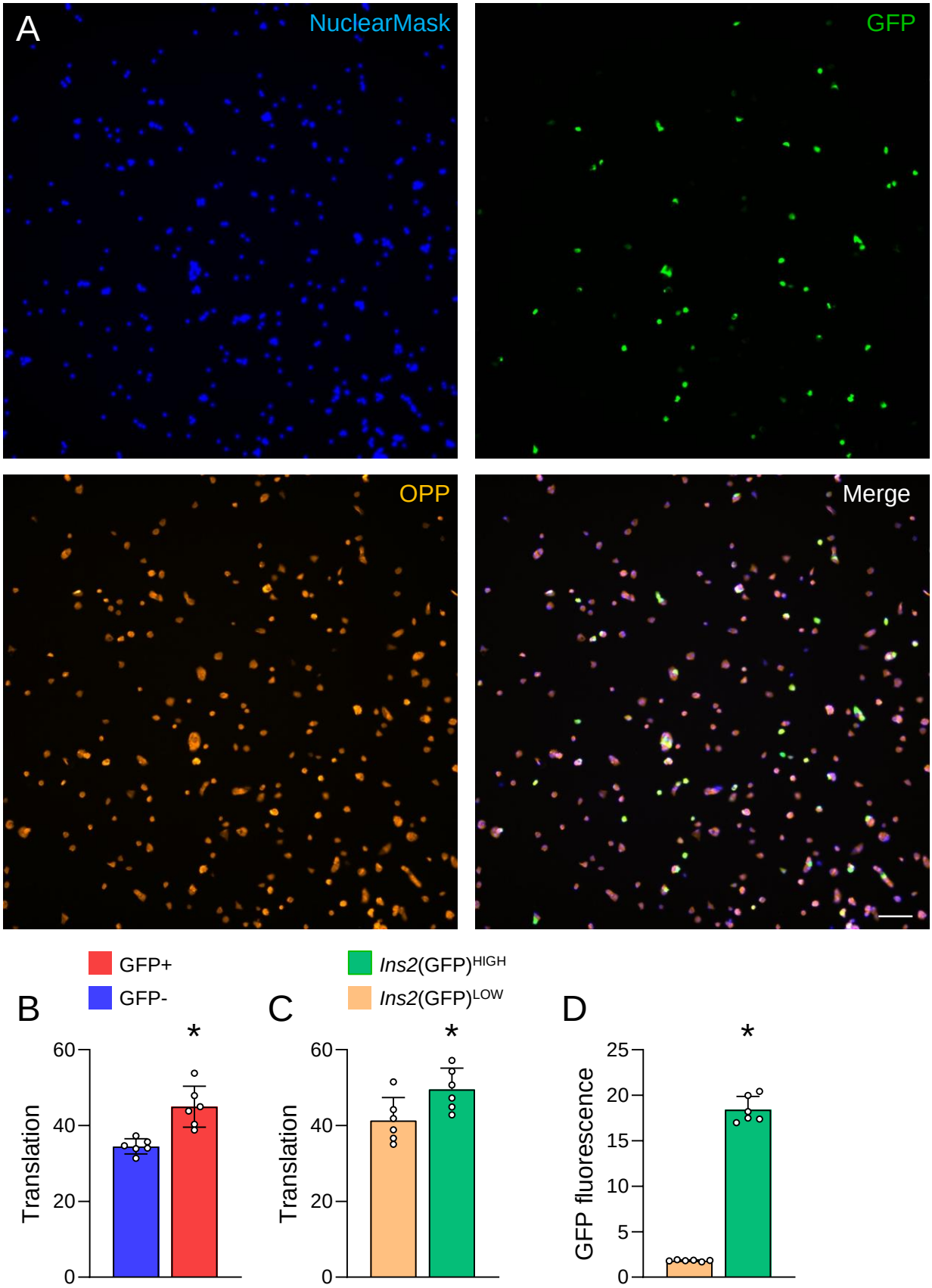
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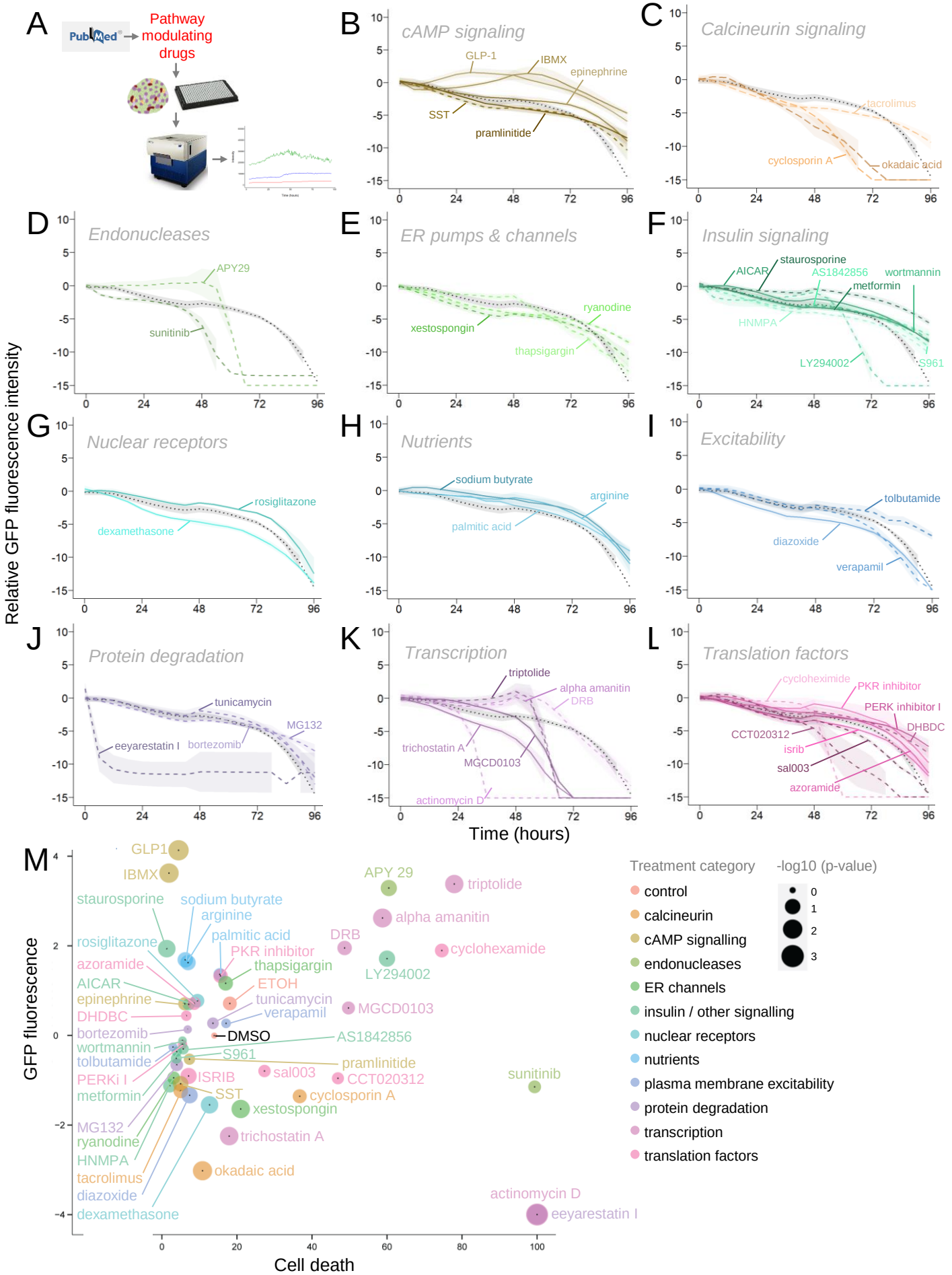
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arginine
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PKR inhibitor
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thapsigargin
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ETOH
epinephrine
tunicamycin
DHDBC
verapamil
bortezomib
DMSO
wortmannin
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tolbutamide
S961
pramlinitide
PERKi I
ISRIB
sal003
CCT020312
metformin
SST
cyclosporin A
xestospongin
MG132
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DRB
cyclohexamide
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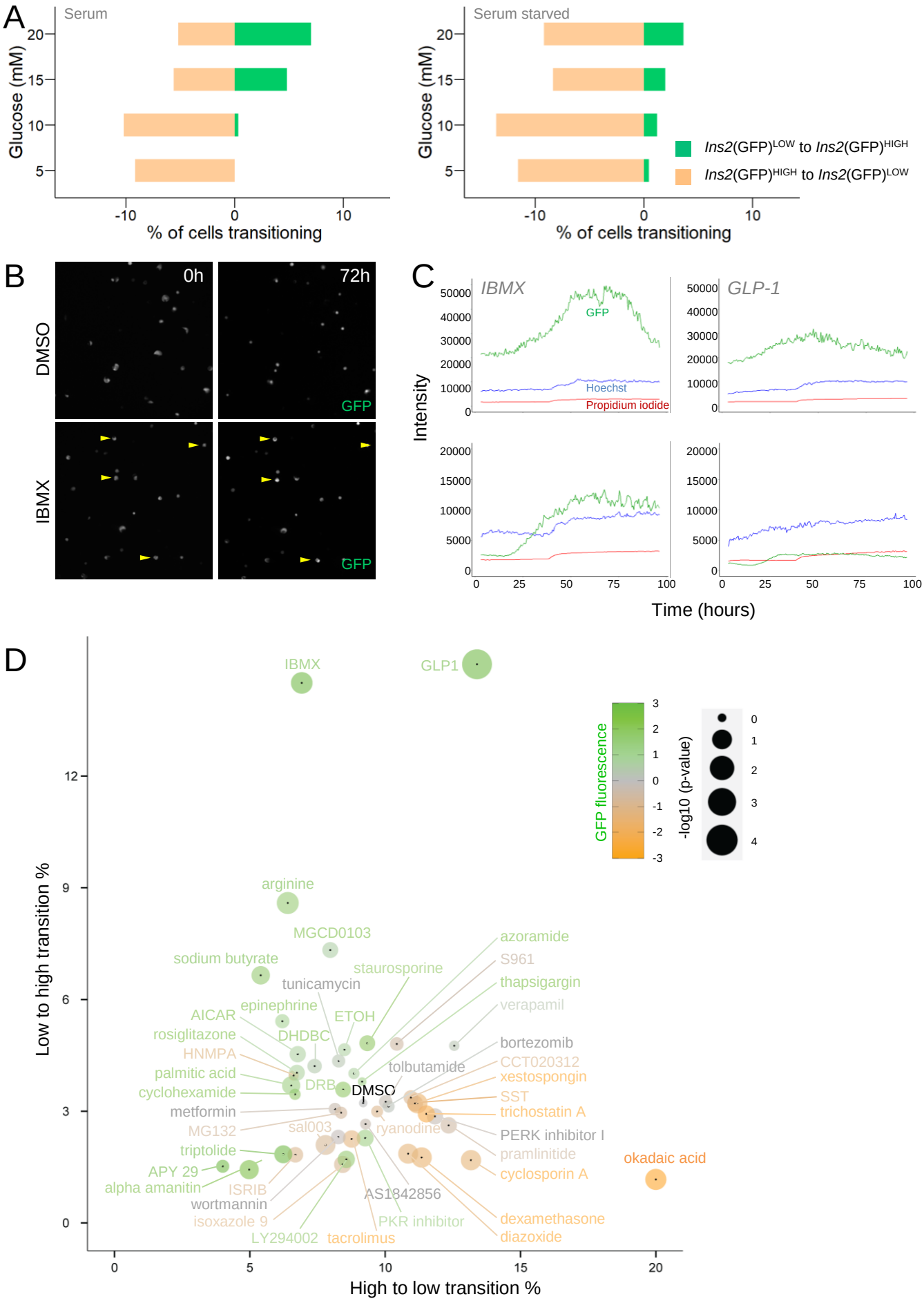
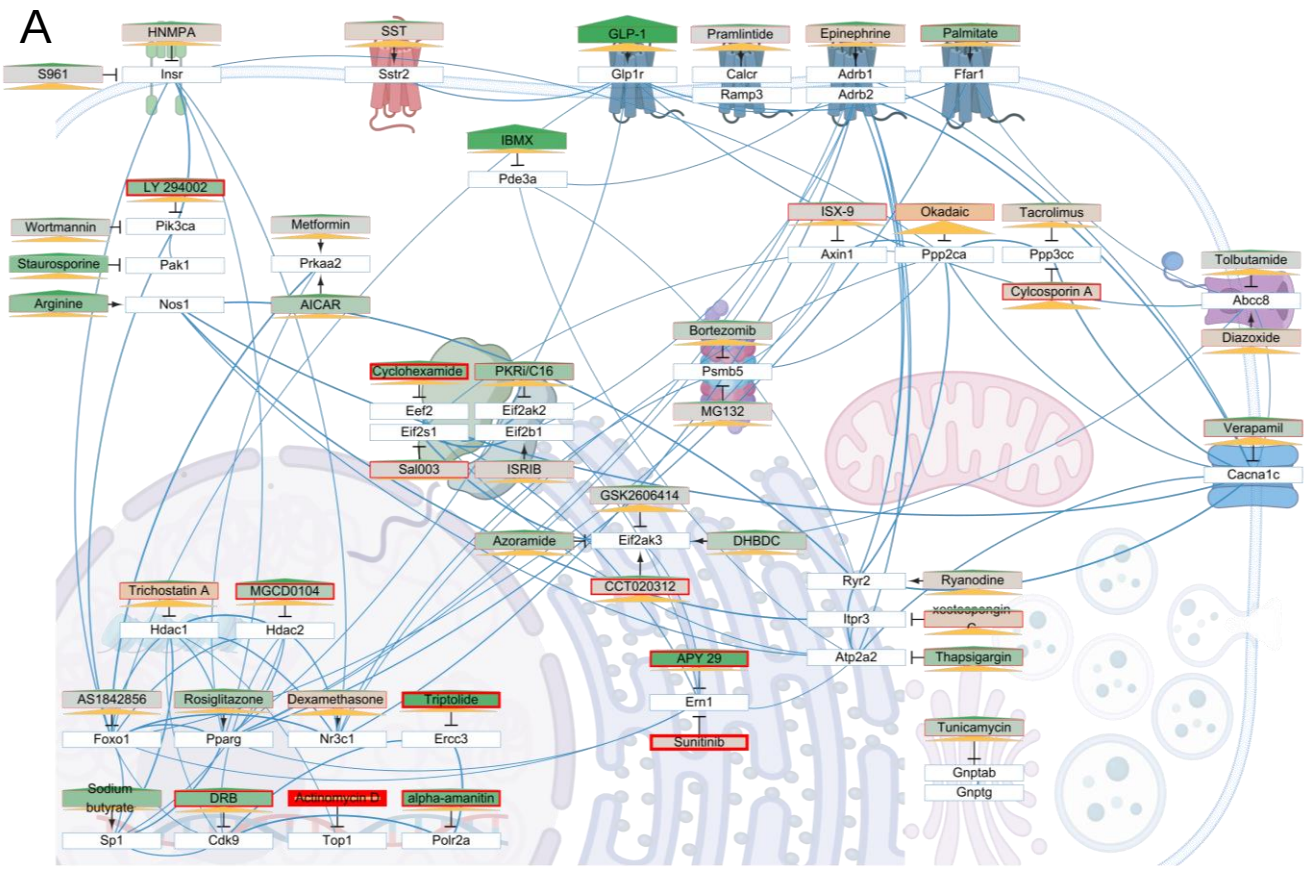
control
calcineurin
cAMP signalling
endonucleases
ER channels
insulin / other signalling
nuclear receptors
nutrients
plasma membrane excitability
protein degradation
transcription
translation factors
-log10 (p-value)
0
1
2
3


A
Serum
Serum starved
Glucose (mM)
% of cells transitioning
Ins2(GFP)LOW to Ins2(GFP)HIGH
Ins2(GFP)HIGH to Ins2(GFP)LOW
B
0h
72h
DMSO
IBMX
GFP
C
IBMX
GLP-1
GFP
Hoechst
Propidium iodide
Intensity
Time (hours)
D
IBMX
GLP1
GFP fluorescence
-log10 (p-value)
arginine
MGCD0103
sodium butyrate
staurosporine
tunicamycin
epinephrine
AICAR
ETOH
rosiglitazone
DHDBC
HNMPA
palmitic acid
DRB
cyclohexamide
DMSO
metformin
MG132
sal003
ryanodine
triptolide
APY 29
alpha amanitin
ISRIB
wortmannin
isoxazole 9
LY294002
tacrolimus
AS1842856
PKR inhibitor
tolbutamide
azoramide
S961
thapsigargin
verapamil
bortezomib
CCT020312
xestospongin
SST
trichostatin A
PERK inhibitor I
pramlinitide
cyclosporin A
okadaic acid
dexamethasone
diazoxide
Low to high transition %
High to low transition %


A
HNMPA
SST
GLP-1
Pramlintide
Epinephrine
Palmitate
S961
Insr
Sstr2
Glp1r
Calcr
Ramp3
Adrb1
Adrb2
Ffar1
IBMX
Pde3a
LY 294002
Wortmannin
Pik3ca
Metformin
Staurosporine
Pak1
Prkaa2
Arginine
Nos1
AICAR
ISX-9
Okadaic
Tacrolimus
Axin1
Ppp2ca
Ppp3cc
Cylcosporin A
Tolbutamide
Abcc8
Diazoxide
Bortezomib
Psmb5
MG132
Cyclohexamide
PKRi/C16
Eef2
Eif2ak2
Eif2s1
Eif2b1
Sal003
ISRIB
Verapamil
Cacna1c
GSK2606414
Azoramide
Eif2ak3
DHBDC
CCT020312
Ryr2
Ryanodine
Itpr3
xestospongin c
Atp2a2
Thapsigargin
Trichostatin A
MGCD0104
Hdac1
Hdac2
APY 29
Ern1
Sunitinib
AS1842856
Rosiglitazone
Dexamethasone
Triptolide
Foxo1
Pparg
Nr3c1
Ercc3
Tunicamycin
Gnptab
Gnptg
Sodium butyrate
DRB
Actinomycin D
alpha-amanitin
Sp1
Cdk9
Top1
Polr2a