# Supporting Information

# Insulin promoter-driven Gaussia luciferase-based insulin secretion biosensor assay for discovery of β-cell glucose-sensing pathways.

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## 1. Materials

### Assay buffer (adapted in part from Luft et al. [1] and US7718389 [2])

0.1% Disodium phosphate (1 mg/ml)
5% Glycerol
150 mM Sodium Bromide (Fisher # AC44680-1000) (enhances luciferase activity)
1 mM EDTA pH 8
25 mM Tris-HCl pH 8
300 mM Ascorbic acid (Sigma #A4034) (antioxidant to increase coelenterazine stability)
200 mM $Na_2SO_3$ (additive to lower background and improve signal/noise)

### Acidified Methanol (stabilizes coelenterazine in solution)

1.06% HCl in pure methanol

### *Gaussia* luciferase substrate

Coelenterazine (native) (Fisher #50-995-844)
Coelenterazine substrate stock solution:
1 mg/ml in acidified methanol
Aliquot 100 μl per tube and store at -80 °C

### GLuc assay working solution

Dilute *Gaussia* luciferase substrate 1:100 in Assay buffer immediately before use.

### Cell culture equipment

Standard flat bottom cell culture plates
Optiplate-96 (Perkin-Elmer #6005290 or Fisher #50-905-1575)
Nunc 384-well white cell culture dishes (Fisher 12-565-343)
Cell strainer, 40 μm (Falcon #352340)

## 2. Methods

### Plasmids

To generate the luciferase reporter, GeneArt Gene Synthesis (Life Technologies) was used to assemble human insulin with humanized *Gaussia* luciferase inserted into the C-peptide between Gly and Pro. The sequence for *Gaussia* luciferase was modified to remove the endogenous signal peptide as well as generate two mutations (M43I and M110I) which have been shown to increase glow-like properties.[3] pcDNA3.1+ was digested with MluI and NheI to excise the CMV promoter and replace with the 410bp rat Insulin1 promoter. This pcDNA3.1+(rIns) vector was digested with HindIII and XhoI to insert the Ins-GLuc sequence and form the final pcDNA3.1+(rInsp)-Ins-GLuc construct. The pLenti-(rInsp)-Ins-GLuc vector was generated by digestion of pLenti-EKAR2G2 (a gift from Olivier Pertz (Addgene plasmid # 40178))[4] with ClaI and XhoI to remove the CMV promoter and the EKAR2G2 insert. The rinsp-Ins-GLuc insert was amplified from the pcDNA3.1 vector by standard PCR to contain a 5' ClaI and 3'XhoI. The digested fragments were ligated to obtain the resulting lentiviral transfer vector.

### Cell Culture

MIN6 beta cells were cultured in Dulbecco's modified Eagle's medium (25 mM glucose) (Sigma D6429), supplemented with 15% fetal bovine serum, 100 units/ml penicillin, 100 µg/ml streptomycin, 292 µg/ml L-glutamine, and 50 µM β-mercaptoethanol as described previously. To prepare MIN6 cells for stimulation, they were washed twice with and incubated for 1 h in freshly prepared modified Krebs-Ringer bicarbonate buffer (MKRBB: 5 mM KCl, 120 mM NaCl, 15 mM HEPES, pH 7.4, 24 mM NaHCO3, 1 mM MgCl2, 2 mM CaCl2, and 1 mg/ml radioimmunoassay-grade BSA, with no glucose unless specified) followed by stimulations indicated in the figures. Cadaveric human islets were obtained via the Integrated Islet Distribution Program (IIDP). AlphaTC1-9 cells were cultured in DMEM with 16 mM glucose, supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 µg/ml streptomycin, and 1X non-essential amino acids (Cat# 091681049, MP Bio). Calcium influx measurements were completed by loading MIN6 cells with Fura-2-AM as indicated in the figure legend followed by monitoring excitation/emission using a Synergy2 H1 (BioTek).

### Transfection and Generation of a Stable Line

MIN6 cells were transfected with 4 μg of pcDNA3.1+(rIns)-hIns-eGluc2 or pcDNA3.1+(CMV)-hIns-eGluc2 in 35 mm dishes using a 2:1 ratio of Lipofectamine 2000/DNA. After 48 h cells were trypsinized and replated in medium without penicillin or streptomycin but including 750 μg/ml G418. After outgrowth for 2 weeks, cells were plated at 1e5 cells/well in 96-well culture dishes and screened for *Gaussia* luciferase activity. The rIns promoter-driven line was designated Ins-GLuc-MIN6 and the CMV-driven line was designated CMV-Ins-GLuc-MIN6. After confirmation of the line by assaying glucose-stimulated GLuc secretion, cells were maintained in normal MIN6 media with 250 μg/ml G418.

**Lentivirus generation and human islet transduction**

Lentivirus was generated in HEK293T cells by transfection with pMD2.G, psPAX2, and pLenti-(rInsp)-Ins-GLuc. Virus-containing supernatant was concentrated and kept at -80°C until use. To transduce human islets, islets were first dissociated by 5 min digestion with Accutase, plated into wells of a 96 well dish pre-coated with Cultrex BME RGF Type 2 (Cat#: 50-591-050, Fisher) diluted 1:20 in CMRL complete medium (CMRL-1066, 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin). Lentivirus was added and plates were centrifuged at 30°C for 1h at 800g. The medium was replace with fresh CMRL complete medium and the cells were incubated overnight before fixation/permeabilization, blocking in normal donkey serum (NDS) blocking buffer for 1h at room temperature, and incubation for 1h at room temperature with goat anti-insulin (sc-7839, 1:300, Santa Cruz), mouse anti-glucagon (G2654, 1:1000, Sigma), rabbit anti-Gaussia (E8023, 1:100, NEB) antibodies, three 10 min washes followed by secondary fluorescent antibodies for 1h, one 5 min wash, a 5 min incubation with DAPI, and a 5 min wash. All washes and antibody solutions were made in NDS blocking buffer. The cells were imaged on an LSM 700 using a 20X objective. Images were processed using ImageJ.

***Gaussia* luciferase assays**

Ins-GLuc-MIN6 cells were plated in 96-well dishes at 1e5 cells/well and incubated for 2-3 days. Cells were then washed twice with KRBH and preincubated in 100 μl of KRBH (200μM diazoxide included where indicated in figure legends) for 1 h. The buffer was then removed and cells were washed one time with 100 μl of KRBH and then incubated in KRBH with or without the indicated glucose concentration (or 200μM diazoxide, 35mM KCl, 20mM Glucose where indicated) for 1 h. 50 μl of KRBH was collected from each well and pipetted into a white opaque 96-well plate (Optiplate-96, Perkin-Elmer #6005290). Fresh GLuc assay working solution was then prepared by adding coelenterizine stock solution 1:100 into assay buffer and the tube was wrapped in foil to protect from light. 10 μl of working solution was then rapidly added to the wells using a Matrix 125 μl electric multi-channel pipette. After adding reagent to the plate, luminescence was measured on a Synergy2 H1 (BioTek) plate reader within 10 min. The protocol was set to shake the plate orbitally for 3 seconds and then read the luminescence of each well with a 100 ms integration time.

**High-Throughput Screening**

For high-throughput screening of natural products, Ins-GLuc MIN6 cells were seeded at 6e6 cells per T75 flask until confluence and then trypsinized, resuspended in medium, passed through a 40 μm cell strainer and diluted to 4e5 cells/ml. Cells were dispensed at 50 μl per well in 384-well opaque white cell culture dishes using a liquid handler to yield 2e4 cells/well. The plates were centrifuged for 1 min at 500 x g prior to incubation at 37°C in a tissue culture incubator for 72 h. Next, 0.5 μl each of DMSO (negative control, 1%), thapsigargin (positive control, 1 μM), and natural product fractions (5 μg/ml) were added robotically to the plates and the cells were incubated 24 h. On the day of the assay, cells were washed twice with 50 μl of KRBH using a BioTek EL406 plate washer and then incubated in 30 μl of KRBH containing 200 μM diazoxide for 30 min. Next, using the BioTek plate washer 30 μl of 2X stimulation buffer (20 mM glucose, 35 mM KCl, 200 μM diazoxide) was added to yield a final concentration of 10 mM glucose, 15 mM KCl and 200 μM diazoxide in a total volume of 60 μl. The plates were incubated for 60 min at 37°C in a tissue culture incubator. Afterward, 10 μl of GLuc assay working solution was added to each well using a TiterTek Multidrop liquid handler. Within 10-20 min, the luminescence from each well of the plates was measured using a Perkin Elmer EnVision multi-mode plate reader with 0.1 sec integration time. The raw data were corrected for plate effects using a GeneData proprietary algorithm and then each value on a per plate basis was subjected to either single-point or two-point normalization, followed by calculation of the robust Z (RZ) score for each well. Natural products with an RZ score greater than 3 or less than -3 were selected as top hits.

Single-point normalization: (sampleData - medianOfCompounds)/medianOfCompounds x 100

Two-point normalization:

(sampleRawValues - medianOfDMSOcontrols)/( medianOfPositveControls-medianOfDMSOcontrols) x 100

Robust Z-score: (sampleData - Median Of Compounds) / STD Of Compounds

**Natural product library**

The natural product library used for this study was composed of extracts from 303 marine-derived bacterial strains and 20 marine invertebrates (19 sponges, 1 tunicate). The library of microbial natural product fractions was derived from marine-derived Actinomycetes (190), Firmicutes (60), and Proteobacteria (53). These bacteria were cultivated from marine sediment samples collected in the Gulf of Mexico (Texas and Louisiana), estuaries in South Carolina, and the Bahamas. Various techniques were used to isolate strains, including the use of small-molecule signaling compounds (N-acylhomoserine lactones, siderophores, and surfactins) that mimic the natural environment of the bacteria of interest. Nutrient-limited isolation media, such as those composed of only humic or fulvic acid, were used for isolation of actinomycetes and α-proteobacteria from mangrove and estuary sediments. Most of the bacterial isolates were characterized by 16S ribosomal RNA (rRNA) phylogenetic analysis following previously described methods (40). Universal 16S rRNA primers FC27 and RC 1492 were used. The sequences of 16S rRNA were compared to sequences in available databases, using the Basic Local Alignment Search Tool. Specific details of the strain isolation and phylogenetics for bacteria discussed in the text are included below. For bacterial isolates not characterized by 16S rRNA, we used morphology to ensure that strains in the collection were not duplicated. To generate the library, bacterial strains were fermented in 5 × 2.8–liter Fernbach flasks each containing 1 liter of a seawater-based medium (10 g starch, 4 g yeast extract, 2 g peptone, 1 g $CaCO_3$, 40 mg $Fe_2(SO_4)_3 \cdot 4H_2O$, 100 mg KBr) and shaken at 200 rpm for 7 days at 27°C. After 7 days of cultivation, XAD7-HP resin (20 g/liter) was added to adsorb the organic products, and the culture and resin were shaken at 200 rpm for 2 h. The resin was filtered through cheesecloth, washed with deionized water, and eluted with acetone to give a crude extract. Fractionation of the bacterial crude extracts was accomplished using an Isco medium-pressure automatic purification system with reversed-phase C18 chromatography (gradient from 90:10 $H_2O/CH_3CN$ to 0:100 $H_2O/CH_3CN$ over 25 min). Each bacterial strain gave rise to 20 fractions for further testing. All natural product fractions in the library were standardized to a concentration of 10 mg/ml in DMSO. The following generic code was used for the natural product fraction nomenclature, SN-X-###-##. SN-A designates strains for 2007 collection; SN-B designates strains for 2008 collection. As an example, SN-A-048 would be the 48th strain isolated in the 2007 collection. Further, numbers SN-F-009-# refer to the active fraction in each of the purification steps leading to the pure compound. For example, SN-A-009-7 would designate fraction number 7 from the first purification of the crude extract of SN-F-009 Identification of pure bioactive compounds requires regrowth of the bacterial culture followed by bioassay-guided fractionation. Intermediate purification fractions were labeled SN-X-###-#-#.

**Isolation of natural product fractions and bacterial strain information**

Natural product extracts from marine invertebrates were generated in the following way; ~ 1 g of freeze-dried tissue was dissected into small pieces and soaked in a 2:1 mixture of methanol/dichloromethane for 24 hours, filtered to remove insoluble material, and concentrated in vacuum to give a complex crude mixture. The crude extract was then sequentially extracted with *n*-hexane, ethyl acetate, *n*-butanol, and $H_2O$ to give four fractions for biological screening. The library of microbial and invertebrate natural product fractions was subjected to liquid chromatography-mass spectrometry (LC-MS) analysis using an Agilent Model 6130 single quadrupole mass spectrometer with a HP1200 HPLC. A photodiode array detector provided a chemical fingerprint of all fractions on the basis of molecular weight and UV profile. Fractions were dereplicated using various compound databases, including Antibase, Reaxys, and Scifinder Scholar.

Standard procedures for 16*S* rRNA analysis were used for phylogenetic characterization of bacterial strains. For example, *Streptomyces* sp. strain SN-F-009 was isolated from a sample collected from Gulf of Mexico. The sediment was desiccated and stamped onto agar plates using gauze 1 acidic media (20 g starch, 1 g $NaNO_3$, 0.5 g $K_2HPO_4$, 0.5 g $MgSO_4$, 0.5 g NaCl, 0.01 g $FeSO_4$, 10 μM cadaverine, 10 μM spermidine, 750 mL seawater, 15 g agar, pH adjusted to 5.3 with phosphate buffer). Bacterial colonies were selected and streaked for purity using the same agar media. Analysis of the strain by 16S rRNA revealed 100% identity to *Streptomyces parvulus*.

**Cultivation and Extraction of SN-F-009.** Bacterium SNF-009 was cultured in 5 × 2.8 L Fernbach flasks each containing 1 L of a seawater-based medium (10 g starch, 4 g yeast extract, 2 g peptone, 1 g $CaCO_3$, 40 mg $Fe_2(SO_4)_3 \cdot 4H_2O$, 100 mg KBr) and shaken at 200 rpm at 27°C. After 7 days of cultivation, sterilized XAD-7-HP resin (20 g/L) was added to adsorb organic chemicals produced by the bacterium, and the culture and resin were shaken at 200 rpm for 2 h. The resin was extracted with acetone and filtered through cheesecloth. The acetone-soluble portion was dried *in vacuo* to yield 2.0 g of extract.

**Purification of SN-F-009** The extract (300 mg out of 2.0 g) was subjected to reversed-phase medium-pressure liquid chromatography (MPLC) with 5% $CH_3OH/H_2O$ for 1 min, followed by a linear gradient of $CH_3OH/H_2O$ from 5% to 100% over 15 min, 4 min of column washing with pure $CH_3OH$, and substitution of the column with 5% $CH_3OH/H_2O$ for 2 min to provide 20 library fractions (SNF-009-1 to -20). The active fraction SNF-009-8 was divided into ten subfractions (SNF009-8-1 to -7 and SNF009-catchall-1 to -3) by reversed-phase HPLC using 10%

$CH_3CN/H_2O$ (0.1% formic acid) for 5 min, followed by a linear gradient of $CH_3CN/H_2O$ from 10% to 100% over 20 min, and 5 min of column washing with pure $CH_3CN$.

**SUPPORTING REFERENCES**


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4. Fritz, R. D.; Letzelter, M.; Reimann, A.; Martin, K.; Fusco, L.; Ritsma, L.; Ponsioen, B.; Fluri, E.; Schulte-Merker, S.; van Rheenen, J.; Pertz, O., A versatile toolkit to produce sensitive FRET biosensors to visualize signaling in time and space. *Sci Signal* **2013,** *6* (285), rs12.


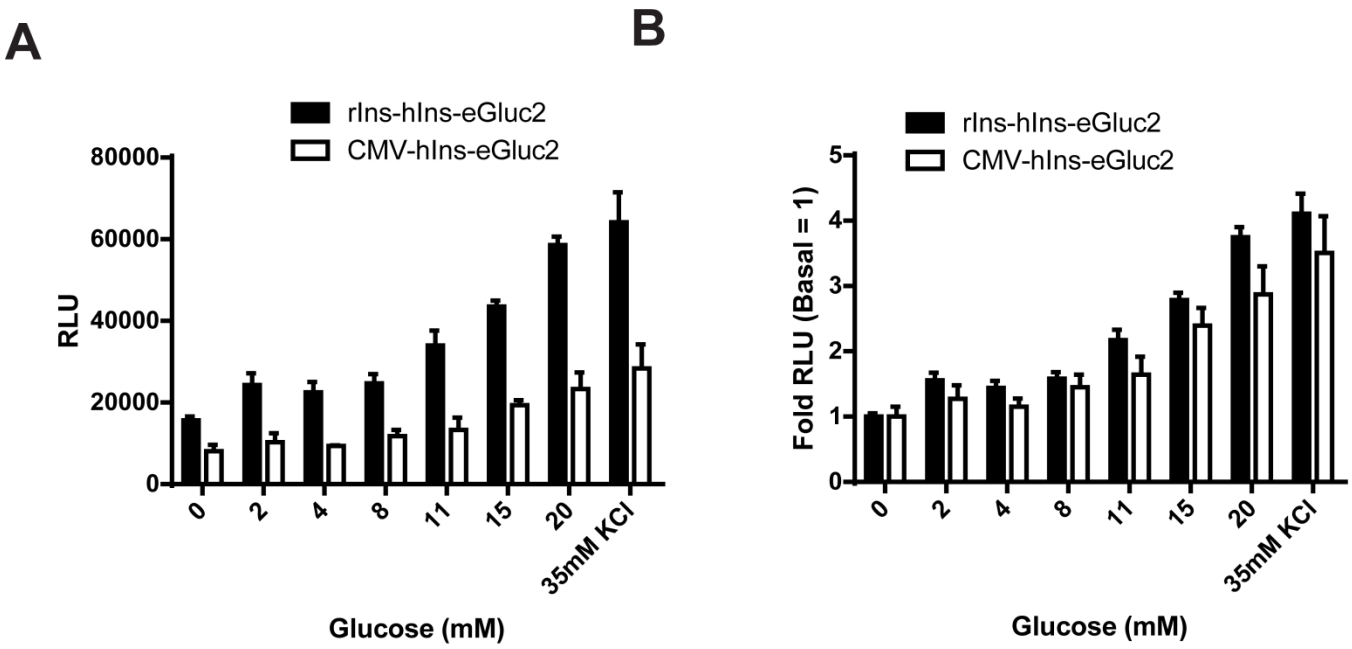


**Figure S1. Glucose dose-response curves in Ins-GLuc MIN6 cells. A)** Either rIns or CMV promoter-driven reporter lines were tested in a glucose dose-response and the secreted GLuc activity was measured. **B)** Data from (**A**) replotted as fold of unstimulated (0mM glucose).

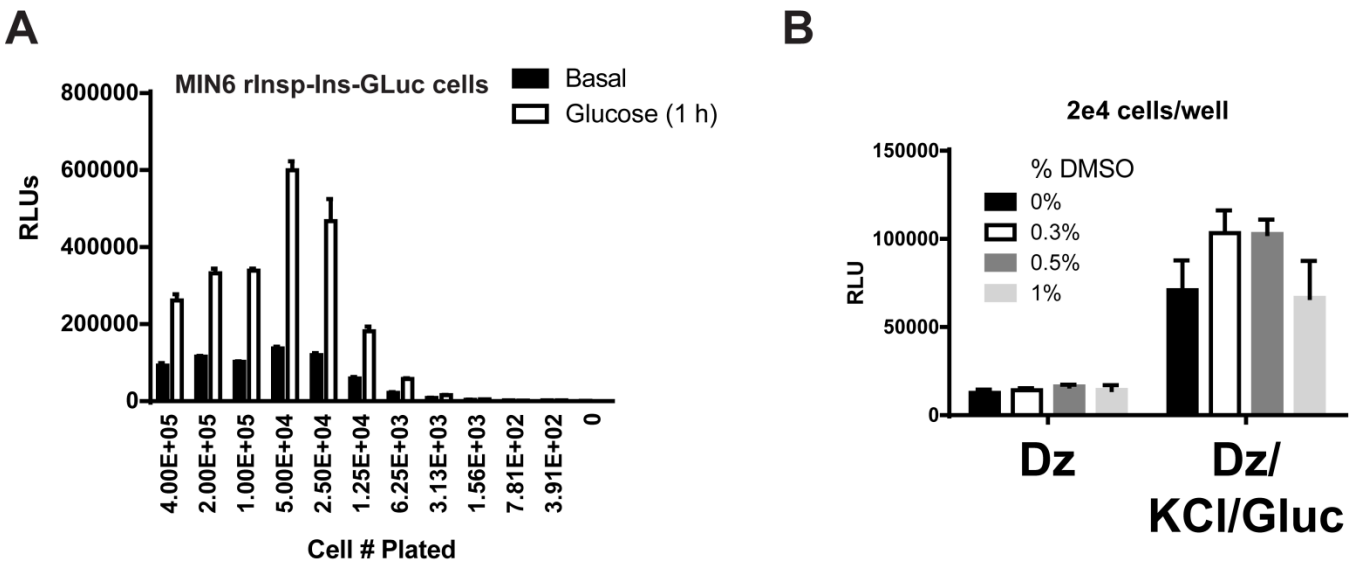


**Figure S2. Optimization of Ins-GLuc MIN6 cells for high-throughput screening A)** Between 2e4 and 5e4 cells per well are optimal for stimulation of Ins-GLuc secretion. **B)** Incubation with 0-1% DMSO during preincubation and stimulation stages had no major impact on luciferase secretion.

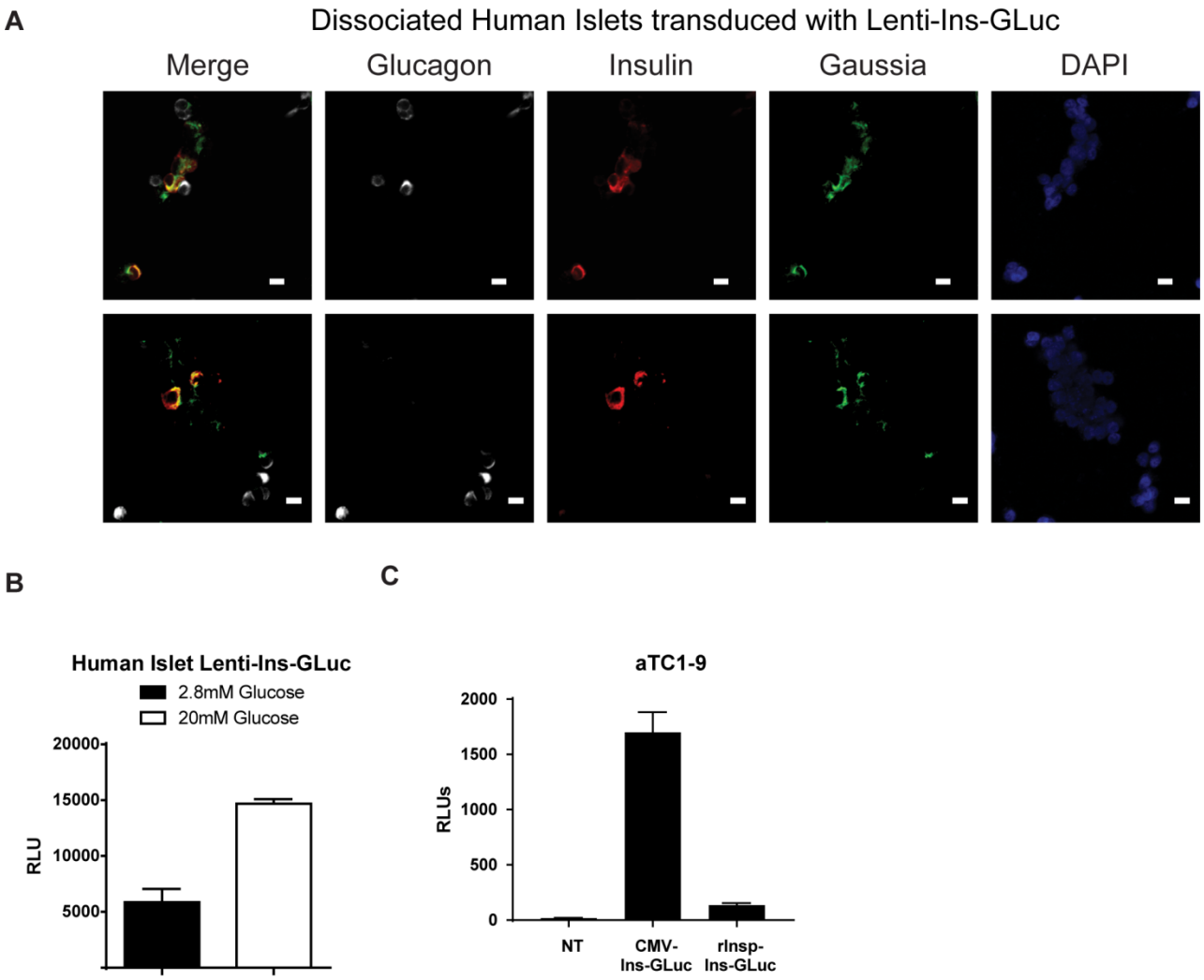


**Figure S3. rIns promoter drives expression in beta cells from dissociated human islets and is secreted in response to glucose stimulation. A)** Human islets were dissociated and infected with lentivirus expressing the rinsp-Ins-GLuc biosensor. The merge channel highlights in yellow where red (insulin+) beta cells overlap with green Gaussia+ cells. The white glucagon+ alpha cells are largely unlabeled. Scale bar, 10 μm. **B)** In similar experiments, dissociated human islets infected with lentivirus expressing the rInsp-Ins-GLuc biosensor were subjected to static glucose-induced insulin secretion assays. Cells exposed to 20 mM glucose secreted Gaussia luciferase as detected by luminescence assay. **C)** aTC1-9 cells were transfected with CMV- or rInsp-driven biosensor and conditioned media was tested for Gaussia luciferase activity.