An isoxazole small molecule drives metabolic and gene expression changes to decrease proliferation and promote a neuroendocrine phenotype in islet cells.

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Supporting Information

Figure Legends

Figure S1. ISX induces acetylation of histones in multiple beta cell lines.

(A) ISX (20 μM) induces H3 and H4 acetylation in human beta cell line 1.1B4.

(B) ISX (20 μM) induces H3 acetylation in rat INS1 beta cells.

(C) GSEA of microarray data shows enrichment in genes for neuronal, beta cell, fatty acid, and cell cycle regulation. NES, normalized enrichment score.

(D) MIN6 cells were treated with PD0325901 (500nM), nifedipine (10 μM), BAPTA-AM (100 μM), cycloheximide (50 μg/ml), or compound C (10 μM) in the presence of absence of ISX (20 μM) for 6 hours and STMN2 expression was monitored by qPCR. Data are shown as the average and SEM of more than three independent experiments. *, $P < 0.05$.
Related to Figure 1.

(E) MIN6 cells treated with ISX (20 μM) for 0, 1, 6, or 24 hours. Total AMP kinase and phosphorylated Thr-174 AMP kinase were immunoblotted. Bar graph shows the average and SEM of three independent experiments.

Table S1. Human Islet Donor Information, Related to Figure 1.

Table S2. ISX microarray and beta cell-enriched/T2D-implicated genes. Related to Figure 1.

Table S3. Metabolomic analysis of ISX-treated MIN6 cells. Related to Figure 3.

Supporting Information

Methods

Metabolomic analysis

Three separate passages of MIN6 cells in 6-well dishes were incubated in either high (25 mM)

or low glucose (5 mM) complete MIN6 medium with either DMSO (0.1%) or ISX (10 μM) for two days. Cells were then washed with 1 ml ice cold saline and harvested in 1 ml of 80% methanol at -80°C. Cells were scraped and transferred to 1.5 ml tubes for three freeze thaw cycles in liquid nitrogen. Lysates were clarified at 14,000 rpm for 15 min at 4° C. Metabolite-containing supernatents were transferred to a new 1.5 ml tube and lyophilized using a SpeedVac. Breathable membranes were placed on top of the open tubes to prevent cross contamination and the SpeedVac was operated without heat for 4 h. Pellets were stored at -80° C prior to analysis by the Metabolomics Facility at Children's Medical Center Research Institute at UT Southwestern (Dallas, TX). Metabolites were reconstituted in 100 ml of 0.03% formic acid in analytical-grade water, vortex-mixed, and centrifuged to remove debris. Thereafter, the supernatant was transferred to a high-performance liquid chromatography (HPLC) vial for the metabolomics study. Targeted metabolite profiling was performed using a liquid chromatography-mass spectrometry/mass spectrometry (LC/MS/MS) approach. Separation was achieved on a Phenomenex Synergi Polar-RP HPLC column (150 × 2 mm, 4 μm, 80 Å) using a Nexera Ultra High Performance Liquid Chromatograph (UHPLC) system (Shimadzu Corporation). The mobile phases employed were 0.03% formic acid in water (A) and 0.03% formic acid in acetonitrile (B). The gradient program was as follows: 0-3 min, 100% A; 3-15 min, 100% - 0% A; 15-21 min, 0% A; 21-21.1 min, 0% - 100% A; 21.1-30 min, 100% A. The column was maintained at 35°C and the samples kept in the autosampler at 4°C. The flow rate was 0.5 ml/min, and injection volume 10 μl. The mass spectrometer was an AB QTRAP 5500 (Applied Biosystems SCIEX) with electrospray ionization (ESI) source in multiple reaction monitoring (MRM) mode. Sample analysis was performed in positive/negative switching mode. Declustering potential (DP) and collision energy (CE) were optimized for each metabolite by direct infusion of reference standards using a syringe pump prior to sample analysis. The MRM MS/MS detector conditions were set as follows: curtain gas 30 psi; ion spray voltages 5000 V

(positive) and -1500 V (negative); temperature 650°C; ion source gas 1 50 psi; ion source gas 2 50 psi; interface heater on; entrance potential 10 V. Dwell time for each transition was set at 3 msec. Samples were analyzed in a randomized order, and MRM data was acquired using Analyst 1.6.1 software (Applied Biosystems SCIEX).

Chromatogram review and peak area integration were performed using MultiQuant software version 2.1 (Applied Biosystems SCIEX). Although the numbers of cells were very similar and each sample was processed identically and randomly, the peak area for each detected metabolite was normalized against the protein content of that sample to correct any variations introduced from sample handling through instrument analysis. The normalized area values were used as variables for the multivariate and univariate statistical data analysis. The chromatographically co-eluted metabolites with shared MRM transitions were shown in a grouped format, i.e., leucine/isoleucine. Principle component analysis (PCA) was completed using SIMCA-P software (version 13.0.1, Umetrics).

ISX time course and mRNA stability in MIN6

MIN6 cells were grown in 12 well culture dishes to ~80% confluence in normal media. For ISX treatments, media was changed to either ISX- or DMSO-containing low glucose MIN6 media (Dulbecco's modified Eagle's medium (5.5 mM glucose), supplemented with 15% fetal bovine serum, 100 units ml-1 penicillin, 100 g ml-1 streptomycin, 292 µg ml-1 L glutamine, and 50 µM β-mercaptoethanol) for the indicated times prior to harvest.

Data visualization and analysis

Line and bar graphs were generated using Graphpad Prism 6. Microarray plot shown in Figure 1A was generated using MatLab. Clustering and heatmap generation for metabolomic data along with the volcano plots shown in Figure 3 were completed using MatLab. Code for analysis

is available upon request. Gene set enrichment analyses (GSEA) were run using software freely obtained from the Broad Institute. For metabolomics data, all values are normalized to the mean of the DMSO low glucose control. Hierarchical clustering was performed on the normalized matrix using MATLAB function, clustergram (Figure 3A). For each pair of treatments (Figures 3B-E), Student's t-test was used to determine a p-value for each metabolite, and subsequently was adjusted using Benjamini and Yekutieli's FDR controlling procedure [1]. The fold change cut-off for all comparisons was chosen at 1.3-fold due to a narrow dynamic range of ISX treatment compared to DMSO under high glucose treatment (Figure 3D).

Supporting References

1. Yekutieli, D. and Benjamini, Y. (1999) Resampling-based false discovery rate controlling multiple test procedures for multiple testing procedures., *J. Stat. Plan. Inf. 82*, 171-196.
2. Williams, M.J., Almen, M.S., Fredriksson, R., and Schioth, H.B. (2012) What model organisms and interactomics can reveal about the genetics of human obesity, *Cell Mol Life Sci 69*, 3819-3834.

Table of qPCR primers used:

Target Gene	Species	Sequence 5'-3'
ACTB	Human/Mouse	AGGTCATCACTATTGGCAACGA
		CACTTCATGATGGAATTGAATGTAGTT
INS (exon2)	Human	TGTCCTTCTGCCATGGCCCT
		TTCACAAAGGCTGCGGCTGG
NeuroD1	Human	ATGACCAAATCGTACAGCGAG
		GTTCATGGCTTCGAGGTCGT
Glucagon	Human	CTGAAGGGACCTTTACCAGTGA
		CCTGGCGGCAAGATTATCAAG
MafA	Human/Mouse	CTTCAGCAAGGAGGAGGTCATC
		CTCGTATTTCTCCTTGTACAGGTCC
Glucokinase (variant 1, beta cell)	Human	TGTGACTGAACCTCAAACCCC

		TCCATCCTGGCTCTGTCGTC
SLC27A2	Human	TACTCTTGCCTTGCGGACTAA
		CCGAAGCAGTTCACCGATATAC
ENPP5	Human	TAAAGTTCCAACGCCCCATTT
		GCAAAGAGGCCAGTTACCAAAG
STMN2	Human	GCTCTTGCTTTTACCCGGAAC
		AGGCACGTTTGTTGATTTGCT
VAT1L	Human	AAGATCCGCGTCAAAGCCTG
		GAGCTTCAACAATCCCAGAACA
GPR160	Human	CCAGCCATCTACCAAAGCCTG
		GCCAGTAACTCTGAATGCTGACA
INA	Human	CGCCACCTTCGGGAATACC
		GTGCTAAAACGTGTCTCCTCG
FAM105A	Human	GTGACCAAGTTCACTCCTGGA
		CCAACATCACAAAGCCTTTTGC
RGS9	Human	CCCTGGATCACCGATGACAC
		CGTTCCACTCGCATCTTGGTT
SLC39A8	Human	TTCCCCACGAGTTAGGAGACT
		TTGTTGCCCACCAAAATGCC
PCSK1	Human	TCGCGCCTCCTAGCTCTTCGCA
		GCAGACTCCAGGCTCTTCGCTC
CLU	Human	CCAATCAGGGAAGTAAGTACGTC
		CTTGCGCTCTTCGTTTGTTTT
NEGR1	Human	GCTTGTTGCTCGAACCAGTG
		CCCCTTTTCTGACCATCATGTT
SLC6A6	Human	GGAGAAGTGGTCTAGCAAGATCG
		AGAAACGCACCTCCACCATTC
NTRK2	Human	GCAATCCATTTACATGCTCCTGT
		CATATTAGGAACCGGATCACCTG
FGF19	Human	CGGAGGAAGACTGTGCTTTCG
		CTCGGATCGGTACACATTGTAG
SCD5	Human	TGCGACGCCAAGGAAGAAAT
		CCTCCAGACGATGTTCTGCC
MAFB	Human	GACGCAGCTCATTCAGCAG
		CTCGCACTTGACCTTGTAGGC
PDX1	Human	ATAAGAGGACCCGTACAGCTT
		CTCCGTCAAGTTGAGCATCAC
ENTPD3	Human	GCTGCGTGAGACTGAGAGTT
		CTTGAGGCCTGCTTGCTCAC
CDK1	Human	GGATGTGCTTATGCAGGATTCC
		CATGTACTGACCAGGAGGGATAG

CCL11	Human	CCCCTTCAGCGACTAGAGAG
		TCTTGGGGTCGGCACAGAT
TOP2A	Human	TGGCTGTGGTATTGTAGAAAGC
		TTGGCATCATCGAGTTTGGGA
ARHGAP11A	Human	CTGCATCCTGCCTCAGAGAG
		TTTTACCCCCTATTTCCGTGGC
KIF11	Human	AGCAAGCTGCTTAACACAGTT
		CCTTCTTACGATCCAGTTTGGAA
PLK4	Human	TTCTCGATACCTTCGTAGAGCTT
		CTGAGTGACATCGTTCCATTGT
CDC25C	Human	TCTACGGAACTCTTCTCATCCAC
		TCCAGGAGCAGGTTTAACATTTT
INSI/II	Mouse	TGAAGTGGAGGACCCACAAGT
		AGATGCTGGTGCAGCACTGAT
TOP2A	Mouse	CAACTGGAACATATACTGCTCCG
		GGGTCCCTTTGTTTGTTATCAGC
MYC	Mouse	ATGCCCCTCAACGTGAACTTC
		GTCGCAGATGAAATAGGGCTG
DUSP1	Mouse	GTTGTTGGATTGTCGCTCCTT
		TTGGGCACGATATGCTCCAG
STMN2	Mouse	CAGAGGAGCGAAGAAAGTCTCA
		CTAGATTAGCCTCACGGTTTTCC