Supplemental Figure 1. pINSGFP^{+} caps form spontaneously and independently of maturation media

A, imBC clusters were cultured for 10 days in the presence of endocrine differentiation media (ENDO), maturation media (containing ALK5i and thyroid hormone (T3)) (MAT) and minimal maturation media (lacking ALK5i and T3 (MIN)), schematic representation. **B,** pINSGFP images of clusters in different media at day 20 and day 30 (scale bars represent 200 μm). **C**, quantitative PCR analysis of insulin gene expression in clusters at day 30 of differentiation. Error bars are representative of the mean +/- the standard deviation, n = 2 independent differentiation experiments. **D**, flow cytometric quantification of hormone expression (ins= insulin, gluc= glucagon, sst= somatostatin) in iPSC derived imBC and seBC clusters (n = 3 independent differentiation experiments). **E**, representative immunofluorescence analysis of sections from iPSC derived seBC clusters for enterochromaffin marker SLC18A, C-peptide and ENTPD3.

Supplemental Figure 2. Ca2^{+} analysis of imBC and seBC clusters

A fraction of area within intact cluster showing elevations in Ca^{2+} activity of individual imBC and seBC clusters at 2 mM and 11 mM glucose (n = 3 independent differentiation experiments with > 10 clusters measured per condition). **B**, fraction of area within intact cluster exhibiting coordinated Ca^{2+} activity (n = 3 independent differentiation experiments with > 10 clusters measured per condition). *p<0.05 **p<0.01 ***p<0.001 error bars are representative of the mean +/- SEM.

Supplemental Figure 3. Analysis of alternative beta cell differentiation trajectories identified by trajectory inference. A, Branchpoint Expression Analysis Modeling (BEAM) demonstrating top 200 genes differentially expressed across branches indicated by arrows. egfp shown in cluster 2 corresponds to the expression of the pINSGFP transgene. **B**, Same as panel A, but performed for the branch indicated by arrows.

Supplemental Figure 4. Single cell RNA-seq profiling of eBC differentiation.

A, schematic representation of eBC differentiation and sorting. **B**, tSNE projection of 4,178 eBC labeled by inferred cell types. **C,** heatmap showing scaled abundance of the top ten marker genes for each cell type identified by single cell RNA-seq. **D,** tSNE projection with RNA velocity vector estimates overlayed. **E & F**, differentiation start-point (**E**) and end-points (**F**) modeled using a markov diffusion process on RNA velocity transmission probabilities. Start and end points were sampled from a uniform 100 x 100 grid, then imputed for all cells using K = 10 K-nearest neighbor pooling. Values range from 0 (yellow) to 1 (dark blue). **G**, RNA velocity endpoints (left) and INS-eGFP transgene expression (right) overlayed on tSNE projection. **H,** tSNE embedding of both seBC and eBC single cell RNA-seq datasets colored by respective dataset. Datasets were aligned using Seurat v2 integration methods. **I**, tSNE projections colored by the expression (log-normalized) of key genes related to beta-cell differentiation.

Supplemental Figure 5. ENTPD3 sorting strategy in pINSGFP reporter cell line

A, seBC were dissociated and sequentially incubated with anti-ENTPD3 (mouse) antibody and anti-mouse 555 secondary antibody then sorted first based on pINSGFP

expression and second based on +/- ENTPD3-555. **B**. Cells were plotted on FSC vs SSC linear axes and gated to remove cell debris. Remaining cells were plotted by FSC area vs FSC height and gated to excluded non-single cells. Single cells were then plotted against DAPI stain and gated to remove dead cells. Live cells were plotted against pINSGFP reporter and those positive were then plotted for ENTPD3-555 in unstained, secondary antibody only and ENTPD3 conditions.

Supplemental Figure 6. Human islet sorting strategy

A, representative image of immunofluorescence staining of intact human islet sections with ENTPD3, c-PEP and NKX6.1. **B**, representative gating strategy for ENTPD3$^{+/-}$ cells. **C**, quantification of immunofluorescence analysis of pancreatic hormone markers to verify presort and sorted populations (ENTPD3$^{+/-)}$ by single cell cytospin and counting using Image J analysis. **D**, insulin content per 1,000 pINSGFP^{+}ENTPD3^{+} sorted cells (n = 3 separate human islet preps, with 3 x 1,000 cell analyzed per prep). **E**, Proinsulin to insulin content molar ratio (n = 3 separate human islet preps, with 3 x 1,000 cell analyzed per prep). **F**, quantitative PCR analysis of mtDNA normalized to gDNA in pINSGFP^{+}ENTPD3^{+} sorted cells (n = 3 separate human islet preps, with 3 x 500 cell analyzed per prep). **G,** global levels of 5-hydoxmethylcytosine in pINSGFP^{+}ENTPD3^{+} sorted cells (n = 3 separate human islet preps, with 1 x 500 cell analyzed per prep). Error bars are representative of the mean +/- the standard deviation.

Supplemental Figure 7. ENTPD3^{+} caps form continuously after removal of already formed pINSGFP^{+}ENTPD3^{+} cells

A, seBC sorted for ENTPD3$^{+/-}$ and the ENTPD3^{+} cells discarded, the remaining pINSGFP^{+} and pINSGFP^{-} cells reaggregated for 7 days in maturation media. **B**, immunofluorescence staining of reaggregated intact clusters collected after 1 and 7 days of culture (scale bar represents 20 μm).

Supplemental Figure 8. T1D-iPSC established from patient-specific PBMC

A, schematic of type-1 diabetic induced pluripotent stem cell (T1D-iPSC) generation from patient-derived peripheral blood mononuclear cells (PBMC). PBMC were isolated from a blood drawn from a T1D patient and reprogrammed using episomal OKITA factor nucleofection to generate patient specific hiPSC. **B**, micrograph images of isolated T1D-PBMC and T1D-iPSC colony generated after reprograming (scale bar representation of 200 μm). **C**, representative karyotype of established T1D-iPSC line. **D**, quantitative PCR analysis for episomal vector expression in T1D-iPSC after 4 passages (positive control, PBMC 3 days after electroporation with episomal vector). **E**, quantitative PCR for key pluripotency factors in T1D-iPSC (expression normalized to GAPDH). **F**, immunofluorescence staining for key pluripotency transcription factors in T1D-iPSC (scale bar is representative of 50 μm).

Supplemental Figure 9. Generation of CTRL-iPSC line using Sendai viruses

A, schematic of the generation of control (CTRL) induced pluripotent stem cell. **B**, micrograph images of isolated CTRL-iPSC colony generated after reprograming. **C**, flow-based analysis of CTRL-iPSC for pluripotency markers. **D**, flow-based analysis after for

markers of the three germ layers after differentiation of CTRL-iPSCs. **E**, representative karyotype of established CTRL-iPSC line.

Supplemental Figure 10. The beta cell surface marker ITGA1 displays wide-spread expression across all maturity levels of pINSGFP^{+} seBC

A, tSNE projection of insulin transgene (pINSGFP), ENTPD3 and ITGA1 expression in 4,143 seBC. **B**, relative ITGA1 gene expression in pINSGFP^{+} imBC and seBC, (bulk RNA-seq experiment described in Fig. 1). **C**, relative ITGA1 gene expression in pINSGFP^{+}ENTPD3^{-} and pINSGFP^{+}ENTPD3^{+} cells (bulk RNA-seq experiment described in Fig. 4). Error bars are representative of the mean +/- the standard deviation. **D**, Flow based quantification of the percentage of ITGA1+ or ENTPD3+ cells present in iPSC derived seBC clusters. Paired students t-test, *<0.05. **E**, Flow based quantification of the percentage of INS and GCG expressing cells within ITGA1+ or ENTPD3+ cell populations. Paired one-way anova, *<0.05. **D** and **E**, n= 3 independent differentiation experiments.

Supplementary Figure 11: Quality analysis of single cell sequencing data.

A-D, the cutoffs chosen to exclude low-quality cells were based on examining the distribution of the # of UMIs (**A**), # of genes detected (**B**), and proportion of mitochondrial UMIs (**C** and **D**).