**Supplementary Information:**

**A cross-species analysis in pancreatic neuroendocrine tumors reveals molecular subtypes with distinctive clinical, metastatic, developmental, and metabolic characteristics.**

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**Supplementary Information: Materials and Methods.**

**Processing of microarrays, data analysis and subtype identification.** The core clinical gene and miR expression datasets were from Missiaglia, *et al.* (GEO Omnibus accession number - **GSE73338)** (1) andRoldo, *et al*. (**GSE73350)** (2).

Affymetrix GeneChip® Human and Mouse Gene 1.0 ST arrays (Affymetrix, Santa Clara, CA, USA) were processed according to the manufacturer’s instructions. Patient (gene expression validation samples) RNA samples were quantitated, quality checked, amplified, hybridized to Affymetrix Human Gene 1.0 ST arrays and scanned by Lausanne Genomic Technologies Facility, Center for Integrative genomics (CIG), University of Lausanne, Lausanne, Switzerland. Mouse RNA samples were similarly processed using Affymetrix Mouse Gene 1.0 ST arrays by the UCSF Genomics Core Facilities, University of California San Francisco, San Francisco, USA.

The Robust Multi-array Analysis (RMA) of human Affymetrix microarrays were analyzed using a statistical framework R and Bioconductor (3) as described earlier (4, 5) and mouse microarrays using aroma.affymetrix (6). The normalized data for human samples are available at GEO Omnibus with accession number **GSE73339 and for mouse samples - GSE73514.**

Agilent Human miRNA Microarrays (based on miRBase release 16; Agilent Technologies) were processed according to the protocols provided by the manufacturer. 100 ng total RNA from each sample (miR validation samples) was labeled according to the Agilent miRNA Complete Labeling and Hybridization Kit. The labeled RNAs were hybridized to Agilent human miRNA microarrays for 20 hours at 55°C with rotation. After hybridization and washing the arrays were scanned with an Agilent microarray scanner using high dynamic range settings as specified by the manufacturer. Agilent Feature Extraction Software v 10.7.3.1 was used to extract the data. Normalization of the Agilent Human miRNA Microarrays profile data were performed as described (7) with the following modification. In order to avoid taking the logarithm (log) of zero and of values between zero and one, we added a small constant, 10, to the raw data values, such that all resulting values were greater than one (except those from the blank control). After this shift, the data were log2 transformed followed by normalization of the data to the same scale, using a quantile method that is available in the R based Bioconductor package “preprocessCore” (8). These procedures were performed by Lausanne Genomic Technologies Facility, Center for Integrative genomics (CIG), University of Lausanne, Switzerland. The normalized data for human samples are available at GEO Omnibus with accession number **GSE73367. Two different batches of Agilent arrays were batch corrected using ComBat** (9)**.**

For subtype identification, the variable genes (from core clinical gene expression dataset) with SD > 0.8 or miRs (from core clinical miR expression dataset) with SD > 0.5 from the normalized data were median-centered across samples for each gene and analyzed using the non-negative matrix factorization (NMF) algorithm (10) as described in our previous studies (4, 5). Significant Analysis of Microarrays (SAM) analysis (11) was performed to identify significant features (mRNA or miR) that are differentially expressed between subtypes, as described previously (4, 5). For (both gene and miR) validation datasets, SAM genes from the core clinical datasets were mapped followed by NMF analysis for subtype identification. Clusters were generated using Gene Cluster 3.0 (12) and heatmaps were generated using Genepattern-based Hierarchical Clustering Viewer (13).

**Merging of microarray data.** ComBat (9) and distance weighted discrimination (DWD) algorithms (14, 15) were used to correct the batch effects and merge human and mouse gene expression data. DWD was applied after sample or column minimal value-based centering and row or gene expression median-based centering. The data was column normalized to N(0,1) and row median centered after DWD analysis. These were performed as described in our previous publications (4, 5, 16). For merging the human and mouse data, we used official gene names from HUGO Gene Nomenclature Committee (HGNC) as references.

**Cross-comparison of gene expression and miR subtypes.** The hypergeometric test was performed using R based phyper library (17) for enrichment of samples between gene expression and miR subtypes, and the p values were plotted as a heatmap using the statistical framework R as described (18).

**RNA isolation and qRT-PCR analysis.** Total RNA was isolated using the miReasy kit (Qiagen, Hombrechtikon, Switzerland) or Ambion RiboPure kit (Life Technologies, Zug, Switzerland), and reverse transcription was performed using Superscript II (Life Technologies) as per the manufacturer’s instructions. qRT-PCR was performed using Qiagility (automated PCR setup, Qiagen), QuantiTect SYBR Green PCR kit (Qiagen), gene-specific primers (**Supplementary Table 4**) and a Rotor-Gene Q real time PCR machine (Qiagen). All qRT-PCR gene expression data were normalized to the levels of the housekeeping gene RPL13A or PPIA. The RNA integrity was assessed by NanoDrop 2000 (Thermo Scientific) and RNA Pico/Nano chip analysis using Agilent Bioanalyzer (Agilent Technologies).

**RIP1-Tag2 (RT2) transgenic mouse model of PanNET.** The breeding and generation of RT2 mice has been described previously (19). While majority of the mice in this study were on a C57Bl/6 background, which is typically used for this model, we also crossed C57BL/6J (B6) RT2 mice with A/J (B6AF1) or DBA/2J (B6D2F1) mice to produce F1 mice. All animal research was performed under the auspices of animal protocols approved by Experience sur animaux (EXPANIM) – Service de la consommation et des Affaires veterinaries (SCAV) in Switzerland.

**Isolation of RT2 tumor and pre-malignant lesions.** RT2 mouse PanNET tumors and liver metastases were dissected out of 14- to 21-wk-old RT2 mice and either snap-frozen in liquid nitrogen for DNA, RNA and protein isolation or formalin fixed and paraffin embedded for IHC as described previously (20, 21). Normal, hyperplastic, and angiogenic islets were isolated as described previously (20, 22) and snap-frozen in liquid nitrogen.

**RT2 mouse tumor cell lines:** βTC cell lines were previously derived from RT2 mouse model (23). These cell lines (passages between 20 and 30) were authenticated by growth, morphological characteristics and insulin gene and protein expression. These were also tested for mycoplasma using two different kits (MycoProbe™ Mycoplasma Detection Kit  (R&D Systems, Minneapolis, MN, USA) and PCR Mycoplasma test kit (Biological Industries, Kibbutz Beit-Haemek, Israel).

**Laser capture microdissection.** Snap frozen pancreatic sections (10µm) from 15-week old RT2 mice were mounted onto PET-membrane slides (MMI). Sections were incubated with ice-cold 70% ethanol and stained with cresyl violet. Individual tumors were microdissected using a PALM laser dissecting microscope (Zeiss, Feldbach, Switzerland). 100ng of total RNA was subjected to microarray experiments using Affymetrix GeneChip Mouse Gene 1.0 ST arrays.

**Human tissue microarray (TMA) analysis.** A human TMA containing pancreatic tumor samples were obtained from US Biomax (Rockville, MD, USA; #PA 2081a) and subjected to IHC analysis as described below.

**Immunohistochemistry (IHC).** IHC was performed as described in our previous studies (5, 24, 25) using the following primary antibodies: anti-Enpp2 (#10005375, Cayman Chemical, Ann Arbor, MI), 1:500; anti-CD31 (#DIA-310, Dianova GmbH, Hamburg, Germany), 1:40; anti-Ki67 (MIB1 antibody; DBA, Milan, Italy; for human samples; reference (1)); anti-Ki67 (#16667, Abcam, Cambridge, MA, USA), 1:100 (for mouse samples); anti-insulin (#A0564, DAKO), 1:250; anti-HK1 (#2024, Cell Signaling Technology, Danvers, MA, USA), 1:250; and anti-SV40-Tag polyclonal antibody (in house production), 1:1000. Slides were imaged using a Leica DM 5500 B microscope (Leica, St. Gallen, Switzerland). For Ki67 scoring of the human PanNET specimens, the area with the highest density of Ki67-positive cells was defined. Ki67 was then assessed by scoring at least 2,000 tumor cells in the selected areas, after which the percentage of positive cells was calculated as previously described (1). For mouse samples, ImageJ software was used to count the total number of Ki67 positive cells normalized to hematoxylin-stained cells. The number of CD31 positive blood vessels in random 20X magnification fields was determined independently by two investigators, and averaged.

**Immunoblotting.** 1g of total protein lysate extracted from laser capture microdissected tumor samples or 10g of cell lysate of TC cells was separated on a Mini-Protean TGX gel (Bio-Rad Laboratories AG, Fribourg, Switzerland) and subjected to immunoblot analysis using antibodies against HK1 (#2024, Cell Signaling Technology), insulin (#A0564, DAKO), PC (#ab126707, Abcam) and -actin (**#**4967, Cell Signaling Technology).

**Proliferation assay.** Cells were plated (5 x 103) into 96-well plates in DMEM Glutmax with 10% FBS media (Life Technologies) at day 0. The following day (day 1), glucose-dropout media (Life Technologies) was added, and the cells were assayed by CellTiter-Glo on day 3, as described in our previous studies (4, 5).

**Metabolic profiling.** To characterize central carbon metabolism, targeted LC-MS/MS was performed (26). Briefly, βTC cells were grown in DMEM (25mM glucose, 2mM glutamine, without pyruvate) to 80% confluency, and metabolites were extracted using 80% methanol at dry ice temperatures. Metabolite fractions were normalized to protein concentration from a biological replicate processed in parallel. For 13C-labeling experiments, cells were grown in DMEM (without pyruvate, glucose and glutamine) in the presence of 10% dialyzed FBS, supplemented with 13C-glucose (25mM; Cambridge Isotope Laboratories, Tewksbury, MA; #CLM-1396) for 20hrs.

**Next-Generation Sequencing.** Human DNA samples were analyzed with a panel targeting all coding sequences of *MEN1*, *ATRX*, *DAXX*, *PTEN*, *TSC2* and *ATM*, and mouse (tumor and normal) samples were similarly analyzed with a panel to detect mutations in the *Atrx* and *Daxx* genes.DNA was prepared from human tumor tissue after neoplastic cell enrichment to about 70%, by manually microdissecting 10 consecutive 4-µm FFPE sections. The mouse tumors were microdissected for enrichment of neoplastic cells, as described above. Next-generation targeted sequencing was performed as previously described (27) using an Ion AmpliSeq custom panel (Life Technologies). Twenty nanograms of DNA were used for each multiplex PCR amplification. Sequencing was performed on an Ion Torrent Personal Genome Machine (PGM, Life Technologies) loaded with 318 chips. Data analysis was done using the Torrent Suite Software v.3.6 (Life Technologies). Those filtered mutations/variants were annotated using the SnpEff software v.3.1 (28). Alignments of the DNA sequence were visually verified with the Integrative Genomics Viewer; IGV v.2.2 (29, 30).

**Proteomic analyses by mass spectrometry.** Proteins from laser capture microdissected tumor sections were extracted using 0.1 % RapiGest SF Surfactant (Waters, Baden-Dättwil, Switzerland) reagent. Protein extracts were in-solution digested as previously described (31). The reaction was stopped and RapiGest was cleaved by adding pure trifluoroacetic acid (TFA) during a final one hour incubation at 37°C. Peptides were desalted using stageTips (32) and dried using a vacuum concentrator. For LC-MS/MS analysis, resuspended peptides were separated by reverse phase chromatography on a Dionex Ultimate 3000 RSLC nano UPLC system connected in-line with an Orbitrap Fusion (Thermo Fisher Scientific, Waltham,USA). Database searches were performed using Mascot (Matrix Science, Boston, USA), MS-Amanda (33) and SEQUEST in Proteome Discoverer v.1.4. against a mouse UniProt protein database. Data was further processed and inspected in ScaffoldTM 4.4.1.1 (Proteome Software, Portland, USA).

**Supplementary Figure Legends.**

**Supplementary Figure 1. NMF analysis of PanNET miR expression and its validation. (A.)** NMF analysis of PanNET core clinical miR expression data set (n=40). (**B.)** NMF analysis of the independent validation dataset of human PanNET miR expression (n=50). (**C.)** Heatmap showing validation of three PanNET miR expression subtypes using the independent validation data set (n=50). In the rainbow bar beneath the heatmaps, red indicates elevated expression, blue decreased, and white no change. PanNETassigner-miR signatures associated with this dataset are listed in the same order in **Supplementary Table 1b**.

**Supplementary Figure 2. NMF analysis of PanNET gene expression and its validation. (A.)** NMF analysis of the PanNET core (‘training’) clinical gene expression dataset (n=86). (**B.)** NMF analysis of the validation dataset of human PanNET gene expression data (n=29). (**C.)** Heatmap confirming the existence of three PanNET gene expression subtypes using the independent validation data set (n=29). In the rainbow bar beneath the heatmaps, red indicates elevated expression, blue decreased, and white no change. PanNETassigner-mRNA signatures associated with the subtypes in the heatmap are listed in the same order in **Supplementary Table 1e.**

**Supplementary Figure 3. Cross-species analysis of gene expression subtypes in human and mouse PanNETs.** Heatmap showing cross-species analysis and association of human (IT, MLP and intermediate; from core clinical gene expression dataset; n=72) and mouse RT2 PanNET (IT and MLP; n=10) subtypes using DWD-merged gene expression profiles. In all the heatmaps, the rows indicate differentially expressed genes amongst the profiled transcriptomes. In the rainbow bar beneath the heatmaps, red indicates elevated expression, blue decreased, and white no change. Genes and samples in the same order as that of the heatmap are shown in **Supplementary Table 1i**.

**Supplementary Figure 4.** ENPP2 and INS mRNA expression in human PanNET subtypes as assessed using human PanNET core clinical gene expression microarray dataset.

**Supplementary Figure 5. Comparison of mouse PanNET subtypes to microdissected invasive IT vs. non-invasive IC2 of mouse PanNET tumors: Relationship to PanNET histotypes and progenitor gene signatures.** **(A.)** A heatmap of hierarchical clustering of DWD merged gene expression profiles from RT2 progression stages (including IT, MLP and met samples) and microdissected highly invasive IC2 and non-invasive IT of RT2 tumors (from published data (21)). **(B.)** A heatmap showing expression of genes that distinguish progenitor (including metabolic and EMT) from EMT and mature  cell genes in microdissected non-invasive (from IT) and invasive (IR; from IC2) regions of RT2 tumors (samples and microarray data from Chun et al., (21)).

**Supplementary Figure 6. Metabolic profiles of IT and MLP cell lines. (A-B.)** Heatmap showing hierarchical clustering of samples with metabolites (with SD>0.2) from LC-MS/MS analyses that are differentially abundant between (**A.**) MLP (TC1b) and IT (TC4), and (**B.**) MLP (TC1e) and IT (TC3) cell lines. The experiments in (**A.**) and (**B.**) were done as different batches and hence, were analyzed separately. The analyses were performed in triplicate for each sample.

**Supplementary Table Legends.**

**Supplementary Table 1. Underlying data from microRNA and mRNA transcriptome profiling of human and mouse PanNET samples associated with the main and supplementary figures. (a-b.)** microRNA profiles and PanNETassigner-miR signatures from (**a.)** human PanNET core (‘training’) clinical miR dataset (n=40) in the same order top to bottom as in **Figure 1A**, and (**b.)** from the human miRNA validation dataset (n=50), in the same order top to bottom as in **Supplementary Figure 1C. (c.)** Gene expression profiles and PanNETassigner-mRNA signatures from human PanNET core clinical gene expression dataset (n=86) in the same order from top to bottom as in **Figure 2A**. **(d.)** Human PanNET core clinical gene expression dataset (n=72), with combined MLP-1 plus MLP-2 subtypes as one class and IT and intermediate as other class and their associated genes are in the same order as in **Figure 2B**. (**e.)** Gene expression profiles and PanNETassigner-mRNA signatures from the human gene expression validation (n=29) dataset, in the same order top to bottom as in **Supplementary Figure 2C. (f.)** mRNA profiles and genes associated with different progression stages of RT2 mouse model, in the same order top to bottom as in **Figure 2E**. (**g.)** mRNA profiles and signatures (m-PanNETassigner-mRNA) from mouse (RT2) PanNET subtypes (IT and MLP) in the same order top to bottom as in **Figure 2F**. **(h-i.)** Merged gene expression profiles of human (only tumor samples) and mouse (only IT and MLP) using (**h.**) ComBat (in the same order top to bottom as in **Figure 2G)** and (**i.**) DWD (in the same order top to bottom as in **Supplementary** **Figure 3)**.

**Supplementary Table 2. Patient and tumor characteristics of the core clinical samples used in the miR and mRNA profiling.** The dataset includes clinical syndrome, mutations, gender, age at diagnosis, sporadic or familial cancer, site of tumor, percent Ki67-positive cells and NET grades.

**Supplementary Table 3.**

a) Evaluation of Insulin and Enpp2 IHC staining of human PanNET TMA. ‘-‘ indicates no staining, ‘+’ weak staining, ‘++’ intermediate staining, ‘+++’ strong staining.

b) Protein expression levels determined by global proteomic analysis of micro dissected IT and Ins-lo MLP tumor samples from RT2 mice. The number of unique peptides, the normalized total spectrum and the fold change comparing IT to Ins-lo MLP are depicted.

**Supplementray Table 4.** List of primer sequences used in this study.

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