Supplementary Materials and Methods

Patient sample characteristics

Detailed data on insulinoma patient’s age, sex, metastatic disease, disease stage and tumor grade and size are provided as supplementary table 1. The study was approved by the responsible ethics committee of Bern (KEK-BE 105-2015). Swiss patient samples were collected before 2014 and used according to Art. 34 of the Swiss legislation (Human research act); as a consequence samples of patients having vetoed were excluded. German patients have been registered since 2004 with the German NET-register under the guidance of the German Society for Endocrinology and have given their written informed consent. Dutch patient material was collected before 2002 and was used according to the Code for Proper Secondary Use of Human Tissue in The Netherlands (https://www.federa.org/, update 2011).

All 108 insulinoma patients had hyperinsulinism followed by a hypoglycaemia syndrome. The initial treatment consisted of surgical removal of the primary tumor, and if present liver and/or lymph node metastases. Follow-up treatment for patients with metastatic disease included surgery, Transarterial Embolization or Transarterial Chemoembolization. The tumors were all sporadic, not associated with MEN1 syndrome and classified according to the World Health Organization 2010 staging and grading system.

Of 48 insulinomas snap frozen tissue was available for mRNA analysis, and from 26 cases also formalin fixed paraffin-embedded material for immunohistochemistry. 37 patients had localized and 11 metastatic disease. Patient’s age at surgery ranged from 20-82 years (mean 50, median 44) in the group with localized disease and 36-69 years (mean 58, median 60) in the group with metastatic disease. The insulinomas were retrieved from the archives of the Insulinoma and GEP-Tumor Centre, Department of Internal Medicine, Heinrich Heine University, Düsseldorf, Germany, the archives of the departments of
Pathology of the University Medical Centers of Rotterdam, Nijmegen and Utrecht, The Netherlands and the University of Bern, Switzerland.

Furthermore, two paraffin-embedded tissue micro arrays (TMAs) were available for immunohistochemical analysis, containing 49 insulinomas (TMA1) and a second TMA with 11 additional insulinomas (TMA2) (2 cores per tumor). TMA1 furthermore contained 92 additional PanNETs (12 gastrinomas, 11 glucagonomas, 10 vipomas and 59 non-functioning PanNETs), of which data can be found in Supplementary Table 6.

In total 178 PanNETs could be analyzed with immunohistochemistry, including 86 insulinomas (52 from patients with localized disease mean age at surgery 50 years, range 20-82, 18 from patients with metastatic disease, mean age at surgery 57 years, range 26-73 and 16 unknown), and 92 other PanNETs. These included 33 other functioning PanNETs (mean age at surgery 57 years, range 12-79) and 59 non-functioning PanNETs (mean age at surgery 57 years, range 14-85). Follow-up time of the insulinoma patients after surgery ranged from 1 to 314 months (median 86). Follow-up time for the other functioning PanNETs ranged from 8-161 months (median 86) and for the non-functioning PanNETs from 2-247 months (median 73). Patient material was used according to the Code for Proper Secondary Use of Human Tissue in The Netherlands (https://www.federa.org/, update 2011) and according to the cantonal ethics committee of Bern (KEK-BE 105-2015).

**RNA isolation**

Total RNA was isolated from snap frozen insulinoma tissue using the RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany). Briefly, 5-10 freshly cut 20 µm tissue sections were picked up with teasing needles and transferred to sterile Costar micro centrifuge tubes (Corning Inc., NY, USA) containing 350 µl of lysis buffer. After addition of approximately 25 glass beads (1.0 mm, BioSpec Products, Biolab, Barendrecht, The Netherlands), the tissue was disrupted by vortexing vigorously for 1 min. Following centrifugation of the lysate for 3 min in a micro-
centrifuge (Eppendorf AG, Hamburg, Germany) at 12,000 RPM, RNA was extracted from the supernatant according to the manufacturer’s instructions, including an on-column RNAse free DNase digestion step. RNA concentrations were determined using a Nanodrop ND-1000 Spectrophotometer (NanoDrop Technologies, USA). RNA integrity was analyzed (2100 Bioanalyzer, Agilent Technologies, USA) and RNA with a RIN value higher than 6.5 was included in the study. Adjacent 5 µm slides were stained with hematoxylin and eosin (H&E) and assessed by a pathologist to make sure that ≥ 70% or more of the section comprised tumor tissue.

Control MPV™ Total RNA from normal human pancreas, liver, lung and adrenal gland was purchased from Stratagene (Agilent Technologies Company, Santa Clara, CA, USA). Total RNA from normal, single donor human pancreatic islets was a gift of Dr E. de Koning, Department of Endocrinology, Leiden University Medical Center, Leiden, The Netherlands.

Quantitative Real-Time PCR

Total RNA (500 ng) was converted to cDNA using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer’s instructions. mRNA levels for the genes CGA, SYP, INS, EGFR, AKT, ERK1, ERK2, EIF4EBP1 (coding for 4EBP1 protein) and the housekeeping genes HPRT, GUSB, ACTB and CYP2 were analyzed by quantitative Real-Time PCR (qRT-PCR) using an iQ5 and CFX 96 Real-Time PCR Detection System (Bio-Rad Laboratories).

All primers were purchased from Biolegio (Nijmegen, The Netherlands). The primer sequences are listed in Supplementary Table 1. qRT-PCR reactions were performed using two commercially available SYBR green mixes. ACTB and CYP2 were analyzed using the iQ™ SYBR® Green Supermix (BioRad, Veenendaal, The Netherlands) and the remaining genes were analyzed using the SensiMix™ SYBR & Fluorescein Kit (BioLine, London, England). The PCR reaction mix included the following components: 7.5 or 12.5 µl of either SensiMix™
SYBR & Fluorescein or iQ™ SYBR® Green Supermix, 300 nM of each primer and 12.5 ng cDNA template in a total volume of 15 µl or 25 µl, respectively. PCR programs and respective PCR reaction mix are listed in Supplementary Table 1. Data-analysis was performed using the Bio-Rad iQ5 software (version 2.0.148.60623) or the Bio-Rad CFX Manager 2.0 software (BioRad, version 2.0.885.0923), respectively. A cDNA pool was made from RNA isolated from the endocrine cell lines H720, H727, H295, SW13, Bon, QGP and CM. From this cDNA pool a standard curve was prepared by 10-fold serial dilutions. From the slope of the resulting regression line the primer efficiency could be determined. Primers were accepted when the efficiency was 100±10%. For more accurate and reliable normalization of mRNA expression the most stable housekeeping gene from a panel of 4 was selected, using the geNorm algorithm (Vandesompele et al. 2002). qRT-PCR analysis of the IGF pathway genes IGF1, IGF1R, IGF2, IGF2R, IGFBP1, IGFBP2, IGFBP3, IGFBP6, INSR-A, INSR-B and the mTOR pathway genes mTOR and RPS6KB1 (coding for PS6K protein) were carried out as described by De Martino (De Martino et al. 2012), using an ABI-PRISM-7900 sequence detection system.

Immunohistochemistry

Freshly cut 4 µm thick formalin fixed, paraffin embedded tissue sections were deparaffinized and rehydrated. Antigen retrieval was performed as summarised in Supplementary Table 2. Endogenous peroxidase activity was quenched by treatment with 0.3% H$_2$O$_2$ in methanol for 30 min at RT. The slides were subsequently blocked for 60 min at RT with either 3% bovine serum albumin (BSA, fraction V, Roche Diagnostics GMBH, Mannheim, Germany) in phosphate buffered saline (PBS) or 5% normal goat serum (NGS) in Tris-buffered saline (TBS) /0.1% Tween-20 (Acros Organics, New Jersey, USA). Details of primary antibodies directed against the proteins EGFR, IGF2, p-AKT, p-ERK, IGF1R, INSR, p-S6K and p-4EBP1, appropriate secondary antibodies and enhancement protocols are summarized in
Supplementary Table 2. After overnight incubation at 4°C or 1 hr at RT the slides were washed and incubated with the appropriate secondary antibodies (Supplementary Table 2) for the indicated times. After washing, staining was visualized by incubation for 5 min with diaminobenzidine (DAB; Dako REAL™ EnVision Detection System, Peroxidase/DAB+, Dako Denmark A/S). The sections were counterstained with haematoxylin (Merck KGaA, Darmstadt, Germany), dehydrated and mounted in Entellan (Merck). Control tissues with proven positivity for the proteins of interest were included (Supplementary Table 2). In negative controls the primary antibody was omitted. Normal human pancreas tissue sections were stained for comparison in evaluation.

Immunohistochemical staining was scored as: 0, absent; 1, weakly positive in ≥10% of cells; 2, moderately positive in ≥10% of cells, 3, strongly positive in ≥10% of cells. For statistical analysis 2 groups were defined, score 0 and 1 taken together versus score 2 and 3 taken together.

Statistical analysis

Statistical analysis was performed using SPSS (version 20, IBM® SPSS®, IBM Corporation Software Group, Somers, NY, USA). Mean relative gene expression levels between groups were compared with the F-test and Student t-test. Associations between relative gene expression levels and immunostaining levels were determined using Pearson’s correlation. Results were considered statistically significant if p-values were ≤ 0.05 in two-sided tests. Survival curves were created using the Kaplan–Meier method and the log-rank test was used to test for differences between subgroups. Overall 10 years survival or 10 years disease free survival rates were calculated from the date of primary surgery until patient’s death or the last date the patient was known to be alive. In order to determine whether the mRNA expression level correlated with survival, the relative expression levels of all genes were subdivided into quartiles, using the 25th, 50th or 75th percentile as the arbitrary cut-off
points. In order to correlate overall 10 years and 10 years disease free survival rates with protein expression, the protein expression data were subdivided into low (negative and weakly positive immunostaining) and high (moderately and strongly positive immunostaining) expression levels. Kaplan-Meier survival curves were created as described for mRNA expression. Cox-regression was used for multivariate analysis.

References to the Supplementary Materials and Methods
