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4 **Heterogeneity of Proliferative Markers in Pancreatic β -Cells of Patients with Severe Hypoglycemia**
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7 **Following Roux-en-Y Gastric Bypass**

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5 **ABSTRACT**
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7 **Aims:** Severe postprandial hypoglycemia with neuroglycopenia is an increasingly recognized,
8 debilitating complication of Roux-en-Y gastric bypass (RYGB) surgery. Increased secretion of insulin
9 and incretin hormones are implicated in its pathogenesis. Histopathologic examination of pancreas has
10 demonstrated increased islet size and/or nuclear diameter in post-RYGB patients who underwent
11 pancreatectomy for severe refractory hypoglycemia with neuroglycopenia (RYGB+NG). We aimed to
12 determine whether β -cell proliferation or apoptosis are altered in RYGB+NG.
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21 **Methods:** We performed an observational study to analyze markers of proliferation, apoptosis, and cell
22 cycle, and transcription factor expression in pancreatic tissue from affected RYGB+NG patients (n=12),
23 normoglycemic patients undergoing pancreatic surgery for benign lesions (controls, n=6, and individuals
24 with hypoglycemia due to insulinoma (n=52).
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31 **Results:** Proliferative cell nuclear antigen (PCNA) expression was increased in insulin-positive cells in
32 RYGB+NG patients (4.5-fold increase, $p < 0.001$ vs. controls) and correlated with β -cell mass. Ki-67
33 immunoreactivity was low in both RYGB+NG and controls, but did not differ between groups. Phospho-
34 histone H3 levels did not differ between RYGB+NG and controls. PCNA and Ki-67 were both
35 significantly lower in both controls and RYGB+NG than insulinomas. Markers of apoptosis and cell
36 cycle (M30, p27, and p21) did not differ between groups. PDX1 and menin exhibited similar expression
37 patterns, while FOXO1 appeared to be more cytosolic in RYGB+NG.
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47 **Conclusions:** Markers of proliferation are heterogeneous in patients with severe post-RYGB
48 hypoglycemia. Increased β -cell proliferation in some individuals may contribute to increased β -cell mass
49 observed in severely affected patients.
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54 **Keywords:** islet, hypoglycemia, human, gastro-entero pancreatic factors
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56 **Abbreviations:** roux-en-Y gastric bypass (RYGB), neuroglycopenia (NG)
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6 **Introduction**
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9 Bariatric surgery is increasingly performed for treatment of both obesity and type 2 diabetes (T2D).
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11 Randomized clinical trials comparing bariatric surgery with medical management have demonstrated that
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13 surgery achieves not only weight loss, but also improved glycemic control while reducing the need for
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15 medications [1-3]. One rare but increasingly recognized complication of Roux-en-Y gastric bypass
16
17 (RYGB) surgery is severe hypoglycemia with neuroglycopenia [4, 5]. While severe hypoglycemia may
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19 occur in fewer than 1% of patients [6-8], mild, often unrecognized, hypoglycemia is more frequent [9, 10]
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21 and may contribute to increased appetite and weight regain after surgery [11]. More severely affected
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23 patients can develop profound neuroglycopenia, with falls, motor vehicle accidents, loss of consciousness,
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25 and seizures refractory to dietary management. Neuroglycopenic symptoms typically appear two or more
26
27 years after RYGB surgery, well after weight loss has stabilized. Hypoglycemia usually occurs in the
28
29 postprandial period and is accompanied by inappropriately high plasma insulin [12]. Higher levels of
30
31 glucagon-like peptide-1 (GLP1) and other incretins may promote excessive insulin secretion [13, 14].
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33 Increased insulin-independent glucose uptake may also contribute to hypoglycemia [15]. Some patients
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35 initially improve with medical nutritional therapy but may require progressive pharmacologic strategies,
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37 including acarbose to reduce carbohydrate absorption, and somatostatin analogs, diazoxide, and/or
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39 calcium channel blockade to reduce insulin secretion [16]. Surgical procedures which increase gastric
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41 restriction [17, 18] or tube feeding into the gastric remnant [19] have been successful for some patients.
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47 Reversal of gastric bypass may improve hypoglycemia, but is not uniformly successful [4, 20].
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50 Partial pancreatectomy was previously performed in a small number of patients with severe
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52 neuroglycopenia refractory to medical management, in an attempt to reduce the frequency and severity of
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54 hypoglycemia [4, 5]. While some patients experience initial improvements following partial
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56 pancreatectomy, hypoglycemia often recurs [4, 21], suggesting progressive β -cell dysregulation and
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58 inappropriate insulin secretion. Pathologic examination of pancreatic tissue from severely affected
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4 patients who underwent pancreatectomy reveals large and small islets, often of irregular shape and
5 clustered near ducts, with nuclear atypia, similar to findings seen with nesidioblastosis unrelated to gastric
6 surgery [4, 5, 22]. Insulinoma has also been reported rarely in post-bypass patients [23]; whether this is
7 linked to stimulation of proliferation and clonal expansion or to increased diagnostic attention in post-
8 RYGB patients remains uncertain. β -cell mass is quantitatively increased in some [24] but not all series
9 [25], potentially due to small cohort sizes or heterogeneity among case or control samples.

10
11 To determine whether cell proliferation or apoptosis are altered in pancreatic β -cells from patients with
12 post-RYGB hypoglycemia with neuroglycopenia (RYGB+NG), we examined pancreatic samples
13 obtained from patients who had undergone partial pancreatectomy for refractory hypoglycemia. Results
14 were compared with those obtained in pancreatic samples from patients with normoglycemia undergoing
15 pancreatic resection for benign mass lesions or other diseases, and from patients with hypoglycemia due
16 to insulinoma.

17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 **Research Design and Methods:**

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37 *Patients:* Consecutive patients presenting to either Joslin Diabetes Center (n=8) or University of Texas
38 Health Science Center at San Antonio (n=4) for surgical management of post-RYGB hypoglycemia
39 refractory to medical management were included in this cross-sectional observational study. Diagnosis of
40 post-RYGB+NG was made on the basis of documented venous hypoglycemia with neuroglycopenia,
41 inappropriately high insulin levels at the time of spontaneous hypoglycemia, and normal glucose and
42 appropriately suppressed plasma insulin after an overnight fast or prolonged inpatient fast. Sample size
43 was determined by the availability of specimens from patients with post-RYGB+NG who underwent
44 palliative partial pancreatectomy in an attempt to control severe hypoglycemia; this number is limited, as
45 pancreatic surgery is no longer performed in our institution for this condition due to incomplete efficacy.
46 Control pancreatic tissues were obtained from patients without diabetes who underwent pancreatic
47 surgery for benign pancreatic mass lesions (n=6) at Brigham and Women's Hospital or Beth Israel
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4 Deaconess Medical Center. Patients requiring surgery for malignancy were excluded. Fifty-two sporadic
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6 insulinomas in patients with severe hypoglycemia treated at Haukeland University Hospital (n=7) or at
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8 Ospedale di Circolo (n=45) were also analyzed.
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11 The study was approved by the Institutional Review Board or Regional Ethics Committee at each
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13 institution, and performed according to the Helsinki Declaration.
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16 Surgical collection of samples: Partial pancreatectomy was performed for patients with severe post-
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18 RYGB hypoglycemia after extensive hormonal evaluation, noninvasive imaging, and arteriography with
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20 selective calcium-stimulated insulin secretion testing to rule out insulinoma and define the anatomic site
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22 of dominant insulin secretion. Routine diagnostic evaluation of pancreatic specimens was performed by
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24 pathologists at each institution, and detailed gross and microscopic analysis to rule out insulinoma or
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26 other neuroendocrine tumors was performed in all patients. Some cases included in our study were
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28 previously described in clinical case series on post-RYGB hypoglycemia [4], analysis of GLP1R
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30 expression [24], or prior studies of insulinoma [26, 27]. Patient gender and age are provided in **Table 1**;
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32 for one control and two post-RYGB patients, only de-identified information was available. For
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34 insulinomas, tumors and adjoining pancreatic parenchyma were resected at laparotomy. Large or
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36 infiltrating tumors were resected using standard surgical procedures (Whipple resection, middle or distal
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38 pancreatectomy), whereas small (≤ 2 cm) well-defined, encapsulated and superficially localized
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40 insulinomas were enucleated. Insulinomas were classified according to the 2010 WHO classification of
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42 tumors of the digestive system [28].
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48 Immunostaining protocols: Pancreas tissues were fixed in 10% buffered formalin at 4°C overnight, and
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50 embedded in paraffin. Serial sections (5 μ m thick) were used for all immunostainings. After being
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52 microwaved for 20 minutes in 10 mM sodium citrate, sections were blocked with donkey serum (5%) and
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54 incubated with primary antibodies against: a) PCNA (Dako, mouse monoclonal, clone PC10, 1:400), b)
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56 phosphohistone H3 (Millipore, 06570), c) Ki-67 (Dako, mouse monoclonal, clone B56 or MIB1, 1:100
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58 dilution), d) P27 (BD, mouse monoclonal, clone 57, 1:25), e) P21 (Dako, mouse monoclonal, clone
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4 SX118, 1:20), anti-caspase cleavage product of cytokeratin 18 (M30 CytoDeath, Hoffmann-LaRoche
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6 mouse monoclonal, clone M30, 1:100), f) PDX1 (Cell Signaling, rabbit polyclonal, 1:400), g) FoxO1
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8 (Cell Signaling, rabbit polyclonal, 1:50), h) Menin (Abcam, rabbit polyclonal, 1:1600), and i) insulin
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10 (Abcam, Guinea pig polyclonal, 1:200, or Cell Signaling, rabbit polyclonal, 1:400 or BioGenex, mouse
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12 monoclonal, clone AE9D6, 1:100). Specific signals were detected using fluorescence-conjugated
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14 secondary antibodies (Jackson Immunoresearch, Alexa 488 or Alexa 594, 1:400). P27, P21 and PCNA
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16 were detected by polymer-HRP/AP-conjugated secondary antibody (Dako), followed by color
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18 development with 0.03% 3,3'-diaminobenzidine tetrahydrochloride (DAB) or permanent red substrate.
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20 For quantification of Ki-67 or PCNA positivity, sections were co-stained with PCNA or Ki-67 and
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22 insulin, and then counterstained with DAPI.
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27 Slides were examined under AXIO Imager A2 Fluorescence/Bright Field microscope (Zeiss) and images
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29 were captured with the same background setting by a single observer masked to group. Cell counting was
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31 conducted using Image J software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda,
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33 Maryland, USA, <http://rsb.info.nih.gov/ij/>, 1997-2009). For each sample, 1000-2000 β -cells (insulin
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35 positive cells) were counted; the number also positive for PCNA, pHH3, or Ki-67 was counted and the
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37 percentage of insulin-positive cells was calculated [28]. The number of patients analyzed for each
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39 antibody is indicated in figure legends. M30 immunostainings were performed as described previously
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41 [29].
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45 *β -cell area and nuclear area:* Immunofluorescent staining for insulin was carried out by using guinea pig
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47 anti-insulin primary antibody (Abcam) and Alexa-conjugated secondary antibody. Images were captured
48
49 using Olympus BX60 fluorescence microscope under both 4x and 20x magnification for the whole
50
51 pancreas and all of the individual islets. Nuclear area was measured in 500 randomly selected β -cells
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53 from each section. Images were analyzed using Image J software. β -cell area was expressed as a fraction
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55 of the pancreas area (multiplied by 100).
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4 Statistics: Student's t-tests (2-tailed, unequal variance) or χ^2 tests were used to assess between-group
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6 comparisons. Linear regression was used to assess the relationship between proliferative markers and
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8 islet mass. Results were considered significant if $P < 0.05$.
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10 11 12 13 14 **Results:**

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16 Islet morphology and proliferation were evaluated in 12 patients with RYGB+NG and 6 normoglycemic
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18 controls without malignancy (**Table 1**). For comparison, 52 patients with sporadic insulinoma were also
19
20 analyzed (**Supplementary Table 1**).
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24 Morphology: Fractional β -cell area was increased in pancreas samples from RYGB+NG patients versus
25
26 controls (5.13 ± 0.42 vs. $1.74 \pm 0.46\%$, $p < 0.001$) (**Figure 1A**). Since nuclear area has been suggested to
27
28 reflect β -cell activity [25], we measured nuclear area in 500 randomly selected β -cells from each section.
29
30 There was no difference in nuclear diameter between RYGB+NG and controls (**Figure 1B** and
31
32 **Supplementary Figure 1**).
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35 Proliferation of Insulin-Positive Cells: To determine whether increased β -cell mass in this population is
36
37 accompanied by increased proliferation, pancreas sections were co-immunostained with insulin and either
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39 PCNA and Ki-67. The percentage of cells co-immunostaining for PCNA and insulin was 4.5 fold higher
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41 in pancreatic tissue from RYGB+NG patients with neuroglycopenia as compared with control pancreas
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43 ($36.5 \pm 4.1\%$ versus $8.1 \pm 1.8\%$, $p < 0.0001$, **Figure 2A**). The percentage of PCNA-positive insulin-positive
44
45 cells correlated with β -cell mass ($r = 0.60$, $p = 0.02$, **Supplementary Figure 2**). Similarly, the percentage of
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47 insulin-positive cells also positive for the mitosis marker phospho-histone H3 (Serine 10) was
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49 numerically higher in a subset of 6 patients with RYGB \pm NG (0.68 ± 0.18) as compared with 5 controls
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51 (0.29 ± 0.15 , $p = 0.13$, **Supplementary Figure 3A/B**).
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4 Ki-67-positive β -cells were less abundant (range 0-0.05%, **Figure 2B**) than PCNA-positive β -cells in all
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6 samples. Four of 12 samples from patients with RYGB+NG had detectable Ki-67 immunoreactivity,
7
8 compared to only one of 6 control samples ($p=0.44$, Fisher's exact test).
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10
11 PCNA and Ki-67 data were compared to values in patients with insulinoma, since both insulinoma and
12
13 RYGB+NG are characterized by hyperinsulinemia, and increased levels of insulin or signaling through
14
15 the insulin/IGF-1 receptors could potentially contribute to proliferation in non-neoplastic pancreas [30].
16
17 As predicted, tumor samples had a higher percentage of positivity for the proliferation markers PCNA
18
19 ($92.2\pm 1.4\%$) and Ki-67 ($0.8\pm 0.3\%$) as compared with non-neoplastic islets in the same individuals and
20
21 with both RYGB+NG and controls (**Figure 2A/2B** and **Supplementary Figure 4**). Similarly higher
22
23 proliferation (Ki-67) was observed in a larger independent series of insulinoma, with a mean Ki-67 index
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25 of $2.4\pm 0.4\%$ ($n=45$). In islets outside of, but adjacent to, the insulinoma (at least $25\ \mu\text{m}$ away from the
26
27 tumor), the percentage of PCNA-positive insulin-positive cells was $35.0\pm 9.0\%$, and the percentage of Ki-
28
29 67-positive insulin-positive cells was $0.06\pm 0.06\%$ (**Figure 2A/2B**, right column, and **Supplementary**
30
31 **Figure 4**). These values were lower than for the adjacent tumor, as expected, but higher than control
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33 samples and quantitatively similar in magnitude to RYGB+NG islets. Percent positivity for PCNA
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35 correlated with percent positivity for Ki-67 across the cohort of both insulinoma and post-RYGB patients
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37 ($r=0.34$, $p=0.03$).
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43 Apoptosis: Reduced apoptosis could also contribute to increased β -cell mass. To investigate this
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45 possibility, consecutive pancreatic sections were immunostained with the apoptosis marker M30
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47 antibody. No significant M30 positivity was detectable in either RYGB+NG patients or controls
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49 (representative sections, **Figure 3A**). Similarly, there were no differences in overall immunoreactivity or
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51 subcellular distribution for the additional apoptosis markers p27 or p21 (**Figure 3B/C** and
52
53 **Supplementary Figure 5**). In insulinoma samples, there were no detectable differences in p27
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55 immunoreactivity in comparison with surrounding non-neoplastic islets (**Supplementary Figure 5A**),
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57 p21 was 7-fold higher in tumor ($p<0.01$, **Supplementary Figure 5B/C**).
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4 *Transcription Factors:* Pancreatic sections were immunostained for key transcription factors that regulate
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6 β -cell development and differentiation, including pancreatic and duodenal homeobox 1 (PDX1), forkhead
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8 box O1 (FOXO1), and menin. PDX-1 expression in β -cells from RYGB+NG patients was both nuclear
9
10 and cytoplasmic in distribution, whereas it was localized in the nucleus of control β -cells (**Figure 4, top**
11
12 **row**). Likewise, FoxO1 was largely excluded from the nucleus in RYGB+NG subjects; by contrast, in
13
14 control samples the majority of insulin-positive β -cells exhibited nuclear FoxO1 expression with minimal
15
16 cytoplasmic immunoreactivity (**Figure 4, middle row**). Menin, implicated in β -cell replication and
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18 MEN1-linked tumorigenesis [31], was similarly expressed in the nucleus of all β -cells and non-islet cells
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20 in both RYGB+NG and control patients (**Figure 4, lower row**).
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25 In pancreas sections from patients with insulinoma, the transcription factors PDX1, FOXO1, and menin
26
27 were detected in both tumor and peri-tumor islets. Both nuclear and cytoplasmic expression of all factors
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29 was observed in tumor cells (**Supplementary Figure 6A-C, upper panels**), while expression was
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31 predominantly nuclear in peri-tumor islets (**Supplementary Figure 6A-C, lower panels**).
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7 **Discussion**
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9 Hypoglycemia following RYGB performed for severe obesity is a challenging and sometimes serious
10 clinical syndrome. Increased postprandial insulin secretion, together with increased insulin-dependent
11 and insulin-independent glucose disposal, may contribute to postprandial hypoglycemia [13, 14].
12
13 Unfortunately, severely affected patients do not fully respond to currently available therapies aimed at
14 reducing insulin and incretin secretion, underscoring the need to better understand the pathophysiology of
15 this syndrome. Interestingly, hypoglycemia can also be observed following sleeve gastrectomy but rarely
16 after gastric banding [32].
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19 Partial pancreatectomy has previously been utilized in severely affected patients with post-bariatric
20 hypoglycemia in an attempt to reduce hypoglycemia and improve patient safety. This approach is no
21 longer routinely employed due to high morbidity and poor long-term efficacy [21]. However, some
22 severely affected patients have achieved reductions in hypoglycemia frequency and severity with partial
23 pancreatectomy, raising the possibility that increased islet mass could contribute to the pathophysiology
24 of this syndrome. Using surgical samples from these patients, we aimed to determine whether β -cell
25 mass, proliferation, and apoptosis are altered, as compared with normal pancreas tissue from surgical
26 resections for benign pancreatic lesions, and compared to pathology with hypoglycemia due to
27 insulinoma.
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29
30 Histologic analysis of pancreatic samples from this patient cohort revealed significant increases in
31 insulin-positive cells, as compared with surgical controls. Increased islet mass has been detected in some
32 [4, 5, 24] but not all studies [25]. Whether this reflects the heterogeneity of pancreatic pathology and β -
33 cell mass in this syndrome, or the wide variation in β -cell mass even in healthy individuals across a
34 spectrum of obesity [33], remains uncertain. We cannot exclude a role for prior severe obesity as driver
35 of increased β -cell mass in individuals with post-RYGB hypoglycemia.
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4 Increases in β -cell mass in severely affected post-RYGB patients could reflect alterations in proliferation
5 or apoptosis. Markers of apoptosis, including M30, p27, and p21, were low in all patient groups, and did
6 not differ between controls and post-RYGB patients with neuroglycopenia. By contrast, PCNA was 4.5
7 fold increased in insulin-positive cells from patients with RYGB+NG, as compared with normoglycemic
8 patients with benign pancreatic lesions. Similarly, phospho-histone H3 and Ki-67 expression were
9 numerically higher in insulin-positive cells in neuroglycopenic patients; these values were within a range
10 similar to those reported by us previously and others [34, 35], suggesting variable extent of increased
11 mitoses and proliferation in this population. Additionally, expression levels of Ki-67 were low in all non-
12 insulinoma samples (range 0-0.095% for Ki-67). While reasons for differences between the magnitude of
13 PCNA elevation and those for the mitotic markers PHH3 and Ki-67 remain uncertain, Ki-67 has recently
14 been demonstrated to be particularly susceptible to loss of immunoreactivity during processing [36].
15 Alternatively, increases in PCNA may reflect not only S phase entry into the cell cycle, but also DNA
16 repair synthesis after damage [37] or growth factor-stimulated protein expression [38]. Despite these
17 mechanistic possibilities and differences in magnitude, PCNA and Ki-67 markers correlated across all
18 samples, consistent with prior studies showing overall concordance of these markers in human pancreatic
19 tissue [39]. Moreover, PCNA positivity correlated positively with β -cell area in patients with post-RYGB
20 neuroglycopenia (**Supplementary Figure 2**).

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43 Rapid changes in systemic metabolism, growth factors, improved insulin sensitivity, or paracrine factors
44 could contribute to increased PCNA and/or proliferation of insulin-positive cells after RYGB. For
45 example, activation of intracellular insulin signaling has been shown to increase β -cell PCNA [40], while
46 infusion of transforming growth factor (TGF) or epidermal growth factor (EGF) in healthy rats increases
47 PCNA expression in both pancreatic islets and ducts independent of ^3H -thymidine incorporation [38].
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4 GLP1 receptor density, independent of weight loss [41]. Likewise, both duodenal-jejunal bypass and
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6 vertical sleeve gastrectomy in rodents result in increased β -cell mass 3 months postoperatively, assessed
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8 by positron emission tomography [42]. One potential contributor to both increased PCNA, proliferation,
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10 and insulin secretion in post-RYGB patients could be the incretin hormone GLP1. While GLP1 is
11
12 associated with β -cell proliferation in rodents, it remains unknown whether the increased endogenous
13
14 secretion of GLP1 observed after RYGB could also contribute to β -cell proliferative responses in humans
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16 [30]. Indeed, levels of GLP1 are markedly increased, up to 10-fold higher after meals in post-RYGB
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18 patients as compared with nonsurgical controls, and are even higher in patients with hypoglycemia [12].
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22 While GLP1 receptors are expressed in human islets, expression is not increased in patients with post-
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24 bariatric hypoglycemia as compared with controls [24]. Nevertheless, it remains possible that
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26 exceedingly high levels of GLP1 in post-bypass patients could contribute to GLP1-dependent responses.
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28 We acknowledge that alterations in β -cell mass are not consistently observed in patients with post-RYGB
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30 hypoglycemia [25], indicating that increased proliferation and/or β -cell mass may not be absolutely
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32 essential for functional dysregulation of insulin secretion observed in this syndrome.
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36 We find localization of FOXO1 is more cytosolic in those with post-RYGB neuroglycopenia compared to
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38 nonsurgical controls. Potential mediators of this pattern include increases in levels or action of growth
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40 factors, such as insulin or hepatocyte growth factor [43-45]. Since these localization patterns of FOXO1
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42 differed between insulinoma and post-RYGB hypoglycemia patients, additional factors beyond high
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44 circulating insulin concentrations are likely to contribute in the post-RYGB setting. For example,
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46 increased expression of insulin-like growth factor-2 (IGF2) and insulin-like growth factor-1 receptor- α
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48 (IGF1R α) have been reported in post-RYGB patients [22] and could also contribute to both altered
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50 FOXO1 regulation and islet function.
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54 A single case report indicated increased PDX1 expression [46] in a post-RYGB patient, as seen in rodent
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56 models [47]; however, PDX1 expression patterns did not differ in our series of post-RYGB hypoglycemic
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58 patients. Menin was localized to the nucleus in both β -cells and ductal cells, implicating a potential
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4 functional role in both cell types [31]. However, immunostaining for menin did not differ in post-bypass
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6 hypoglycemia patients compared to control, indicating menin is not likely to be a major contributor to the
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8 post-RYGB hypoglycemia syndrome.
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11 Parallel analysis of samples from patients with insulinoma, who have hypoglycemia due to excessive and
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13 autonomous insulin secretion by tumor cells, revealed the expected increases in both Ki-67 and PCNA,
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15 thus serving as a positive control. Levels of Ki-67 were not as high in adjacent normal islets, consistent
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17 with prior data [48]. However, PCNA was also increased in adjacent normal islets, with magnitude
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19 similar to that of RYGB+NG islets. These findings raise the possibility that endocrine or paracrine
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21 effects of excessive insulin secretion by either tumor or non-tumor mechanisms could contribute to PCNA
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23 positivity in β -cells.
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27 Both insulinoma tumor and adjacent cells showed presence of nuclear PDX1, FOXO1, and menin. As
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29 noted, these patterns are distinct from those in post-RYGB neuroglycopenia, despite common increases in
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31 insulin levels. Cellular phenotypes exclusive to insulinoma could be linked to transformation or
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33 transdifferentiation; the effects of sustained increases in insulin/IGF-1 signaling via phosphoinositide 3-
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35 kinase (PI3K) or Akt-dependent pathways [4], the autocrine effects of local growth factors, alteration in
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37 regulation of cell cycle regulatory proteins [49] or additional mechanisms exclusive to tumor cells (e.g.
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39 increases in GLP1 receptor density) [24] will require additional investigation.
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43 In summary, we find the proliferation marker PCNA is increased in parallel with increased β -cell area in
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45 patients with post-RYGB neuroglycopenia. Whether such alterations in pancreatic pathology are linked
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47 to dysregulation of postprandial insulin secretion, potentially via common upstream mechanisms, or are
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49 independent of each other, remains uncertain and cannot be addressed by examination of tissue pathology.
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51 We recognize our study is limited by sample size, heterogeneity between patients, and the inability to
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53 compare pre- and post-RYGB pancreatic samples from the same patient. Moreover, these data may not
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55 be generalizable to the entire population of less severely affected patients with post-RYGB hypoglycemia,
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4 or to those post-RYGB patients without clinically recognized hypoglycemia. Future studies will be
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6 required to analyze potential transcriptional contributors to increased insulin secretion.
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9 More broadly, understanding processes that may lead to improved β -cell function is an area of significant
10 interest given the need to find novel ways to counter progression of T2D. Multiple studies provide
11 convincing evidence that rodent β -cells can be stimulated to proliferate, leading to increased functional β -
12 cell mass. A series of emerging reports support this possibility in human β -cells [34, 35, 50-52].
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14 Whether the ability to proliferate can be harnessed to modulate the overall functional β -cell mass for
15 therapeutic purposes warrants further investigation and should continue to spur efforts to identify both
16 systemic and paracrine mediators of islet mass and functional capacity.
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CONFLICT OF INTEREST: The authors declare that they have no conflict of interest.

STATEMENT OF HUMAN AND ANIMAL RIGHTS: All procedures performed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008. This article does not contain any studies with animal subjects.

INFORMED CONSENT: Exemption from informed consent was obtained from the institutional review board as these studies were performed on discarded clinically obtained tissues.

AUTHOR CONTRIBUTIONS: MEP, ABG, and RNK wrote the manuscript. JH, DH, and SL performed immunohistochemical analysis. RNK, AM, and SL performed data analysis. JG and WHS contributed to sample procurement and discussion. AM, SL, and FF contributed to discussion and reviewed/edited manuscript.

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Gender	Age (years)	BMI at RYGB	BMI at Pancreatic Surgery	Pathology
CONTROLS (no RYGB)				
F	28	-	18	Microcystic serous cystadenoma
F	38	-	25.1	Leiomyoma, normal pancreas
n/a	n/a	-	n/a	Localized pancreatic tumor
M	69	-	n/a	Serous cystadenoma
M	65	-	n/a	Intraductal papillary mucinous adenoma
F	43	-	n/a	Ampullary adenoma, normal pancreas
POST-RYGB NEUROGLYCOPENIC HYPOGLYCEMIA				
M	46	40.6	23.1	Islet hyperplasia
F	42	n/a	30	Islet hyperplasia

TABLE 1

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F	35	42.3	37.5	Islet hyperplasia
F	37	n/a	n/a	Islet hyperplasia
F	29	46.8	26.3	Islet hyperplasia
F	33	40	25.8	Islet hyperplasia
F	49	43.5	29.7	Islet hyperplasia
F	69	n/a	n/a	Islet hyperplasia
n/a	n/a	n/a	n/a	Islet hyperplasia
F	54	n/a	n/a	Islet hyperplasia
F	36	n/a	n/a	Islet hyperplasia
n/a	n/a	<u>n/a</u>	<u>n/a</u>	Islet hyperplasia

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7 **TABLE AND FIGURE LEGENDS**
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10 **Table 1.** Characteristics of patients from whom pancreatic samples were obtained at surgery. n/a, data
11 not available.
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14 **Figure 1.** A. Area (% of total pancreas area) and B. nuclear area (μm) in insulin-positive cells in controls
15 (n=6) and patients with post-RYGB hypoglycemia (RYGB+NG, n=9). Nuclear size was assessed in 500
16 cells per patient.
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21 **Figure 2.** Representative images (left) and quantification (right) for (A) PCNA and (B) Ki-67 staining,
22 expressed as percentage of insulin-positive cells in samples from controls (n=6), patients with
23 RYGB+NG (n=11 for PCNA, 12 for Ki-67). These data are compared with neoplastic cells from patients
24 with insulinoma (n=7), and with surrounding non-neoplastic islets. 1000-2000 β -cells were counted for
25 each patient. *** indicates $p < 0.0001$.
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33 **Figure 3.** Representative images for the apoptosis markers and related proteins (A) M30, (B) p27 and (C)
34 p21. 1000-2000 β -cells were counted.
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39 **Figure 4.** Representative images of immunostaining for the transcription factors PDX1, FOXO1, and
40 menin (green), and insulin (red).
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7 **Supplementary Table and Figure Legends**
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10 **Supplementary Table 1.** Characteristics of patients with insulinoma.
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15 **Supplementary Figure 1.** Representative images of β -cells stained with DAPI (blue) and for insulin
16
17 (red).
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20 **Supplementary Figure 2.** Correlation between % PCNA-positive insulin-positive cells and β -cell mass
21
22 (% of area). Red symbols denote RYGB+NG, while black symbols indicate controls. Spearman $r=0.68$,
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24 $p<0.01$.
25

26
27 **Supplementary Figure 3.** A. Representative images of proliferation marker phospho-histone H3 in
28
29 pancreas sections, expressed as percentage of insulin-positive cells in samples from controls (n=5) and
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31 patients with RYGB+NG (n=6). Arrows indicate cells selected for display in higher-magnification inset.
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33 B. Quantification of phosph-histone H3 positive, insulin-positive cells.
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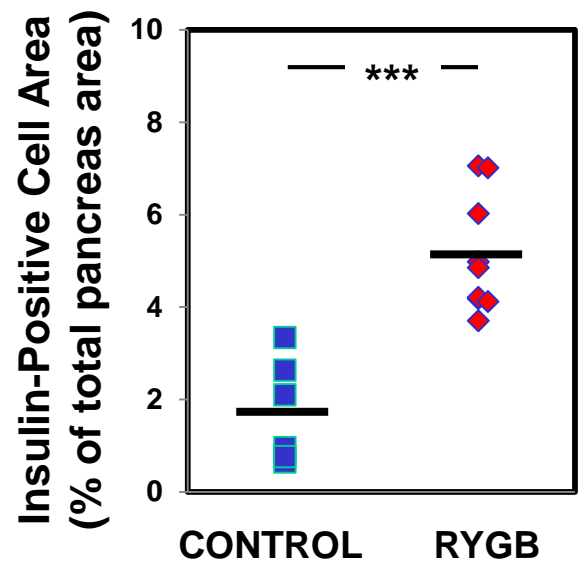
36
37 **Supplementary Figure 4.** Representative images of proliferation markers (A) PCNA and (B) Ki-67 in
38
39 pancreas sections from 7 patients with insulinoma (INS1-7). Upper panels depict normal pancreas
40
41 (adjacent to tumor) within the surgical specimen, while lower panels shows insulinoma tumor tissue.
42
43 Arrows indicate cells selected for display in higher-magnification inset.
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45

46
47 **Supplementary Figure 5.** Representative images of apoptosis markers (A) p27 and (B) p21 in pancreas
48
49 sections from 7 patients with insulinoma (INS1-7). Upper panels depict normal pancreas (adjacent to
50
51 tumor) within the surgical specimen, while lower panels shows insulinoma tumor tissue. Arrows indicate
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53 cells selected for display in higher-magnification inset. (C) Quantification of percentage of p21+ cells in
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55 insulinoma and adjacent non-neoplastic insulin-positive cells. *indicates $p=0.004$.
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4 **Supplementary Figure 6.** Representative immunostaining images for the transcription factors (A) PDX1,
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6 (B) FOXO1, and (C) menin (green) and insulin (red) in normal and tumor tissue from patients with
7
8 insulinoma. Arrows indicate cells selected for display in higher-magnification inset.
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Figure 1.

A.



B.

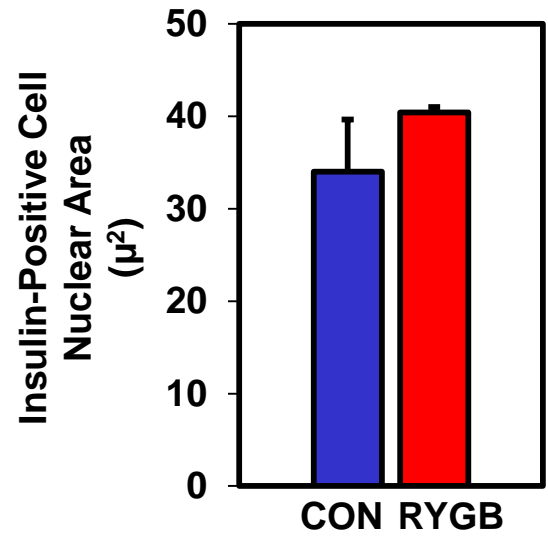
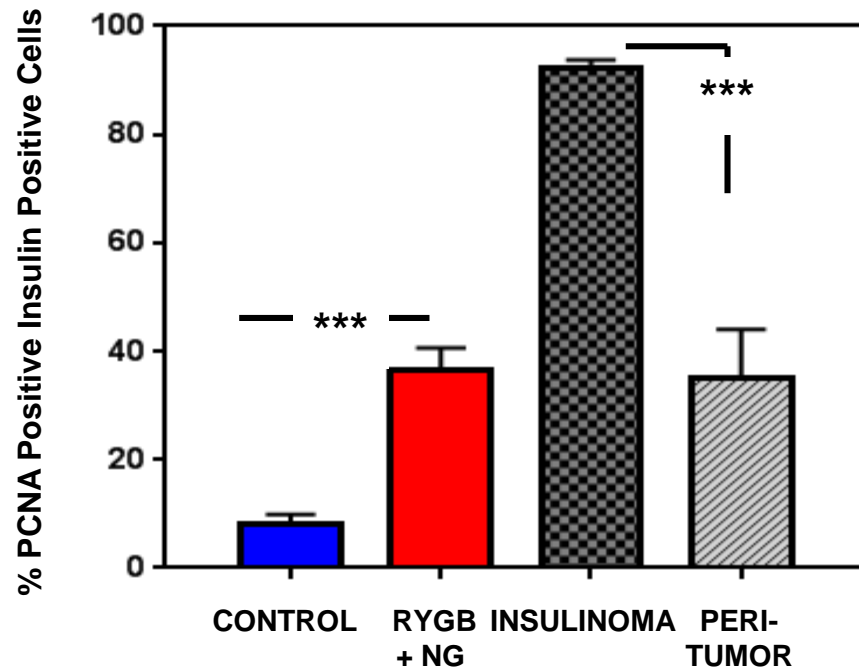
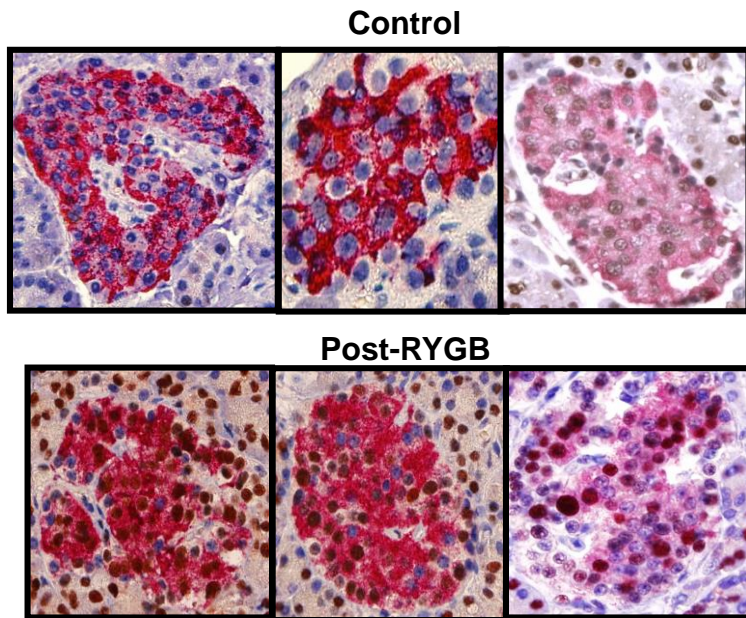


Figure 2

A. PCNA



B. Ki67

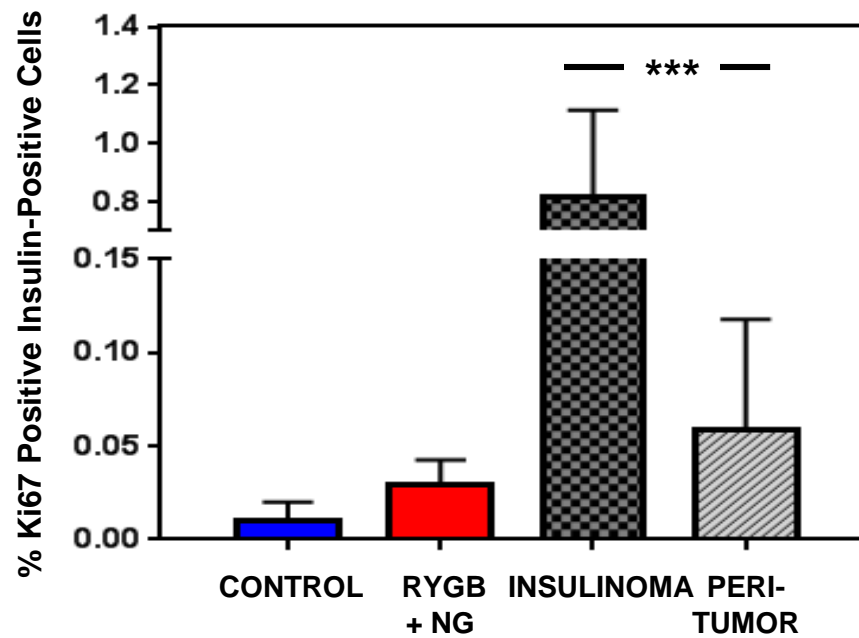
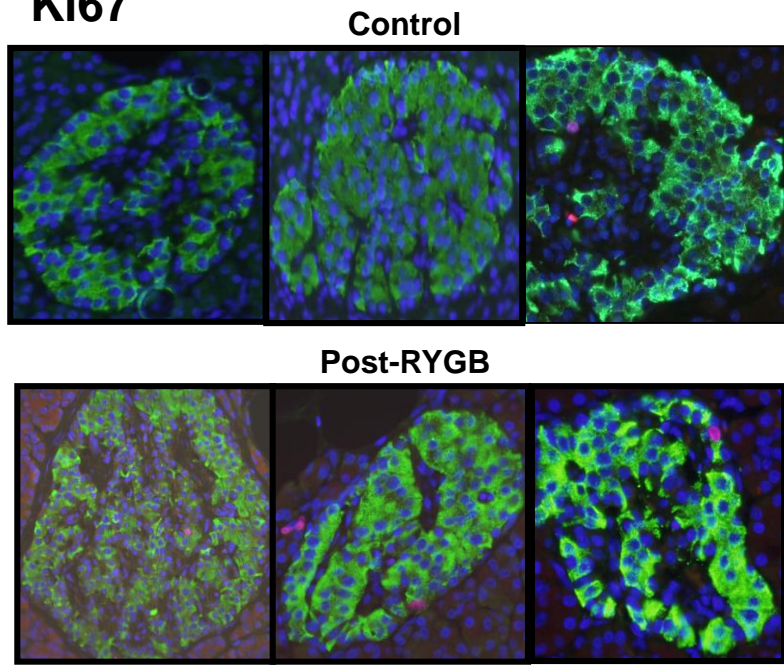
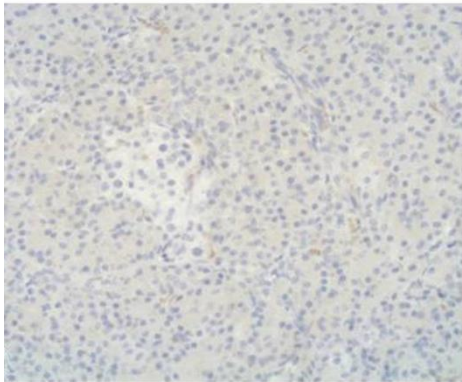


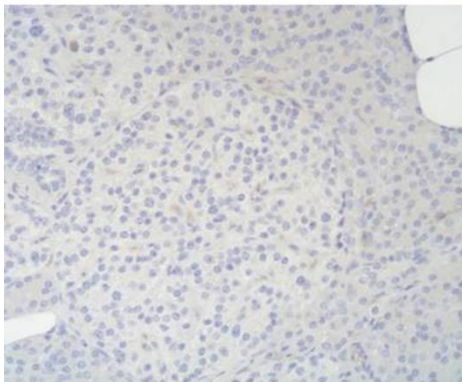
Figure 3.
Figure 3.

A. M30

Control

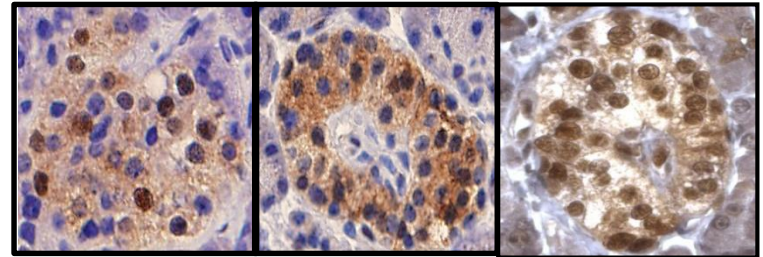


Post-RYGB

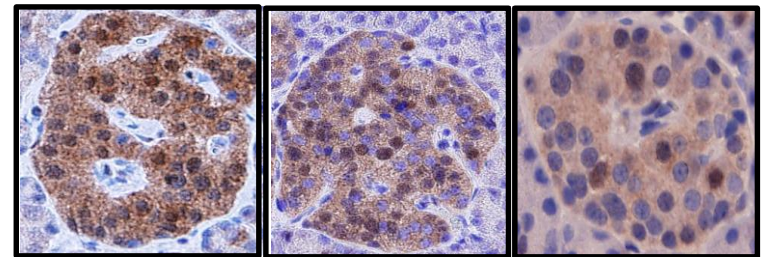


B. p27

Control

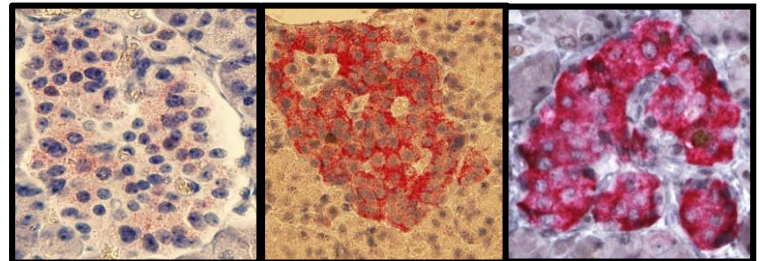


Post-RYGB



C. p21

Control



Post-RYGB

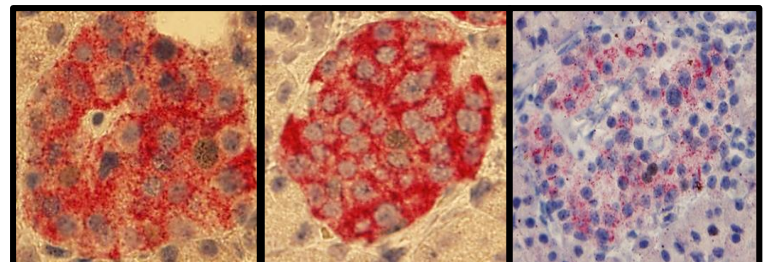
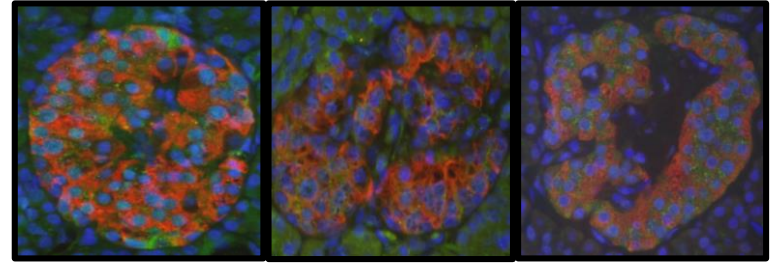
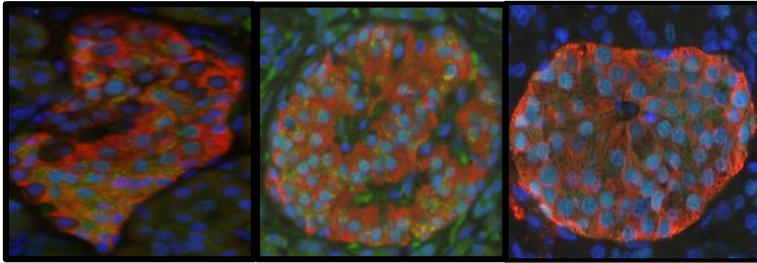


Figure 4.

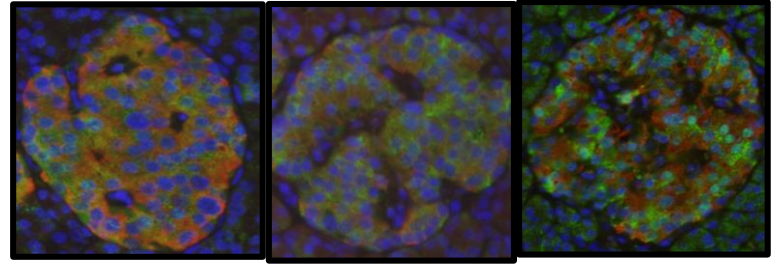
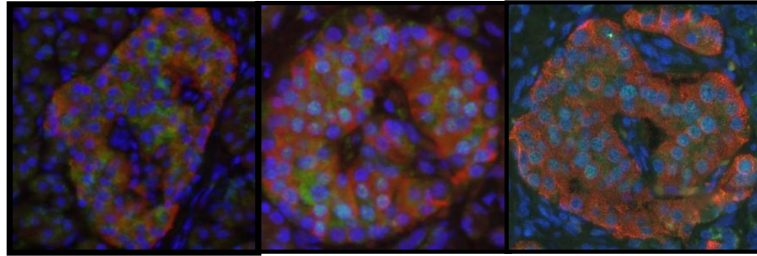
Control

Post-RYGB

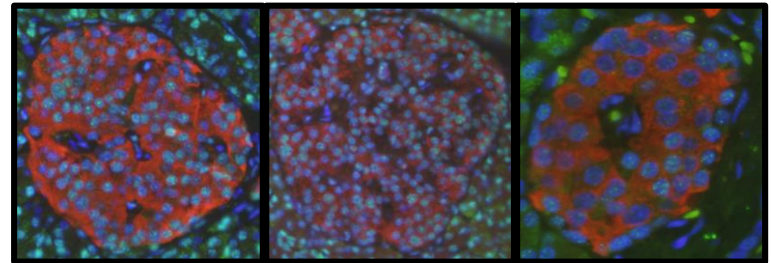
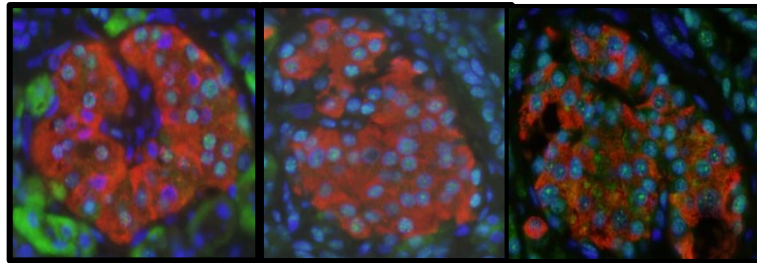
PDX1
Insulin



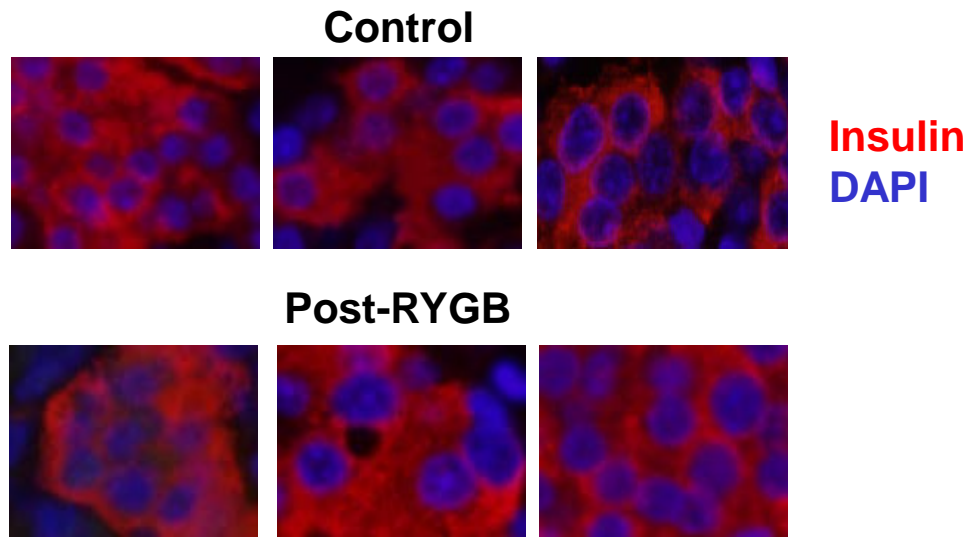
FoxO1
Insulin



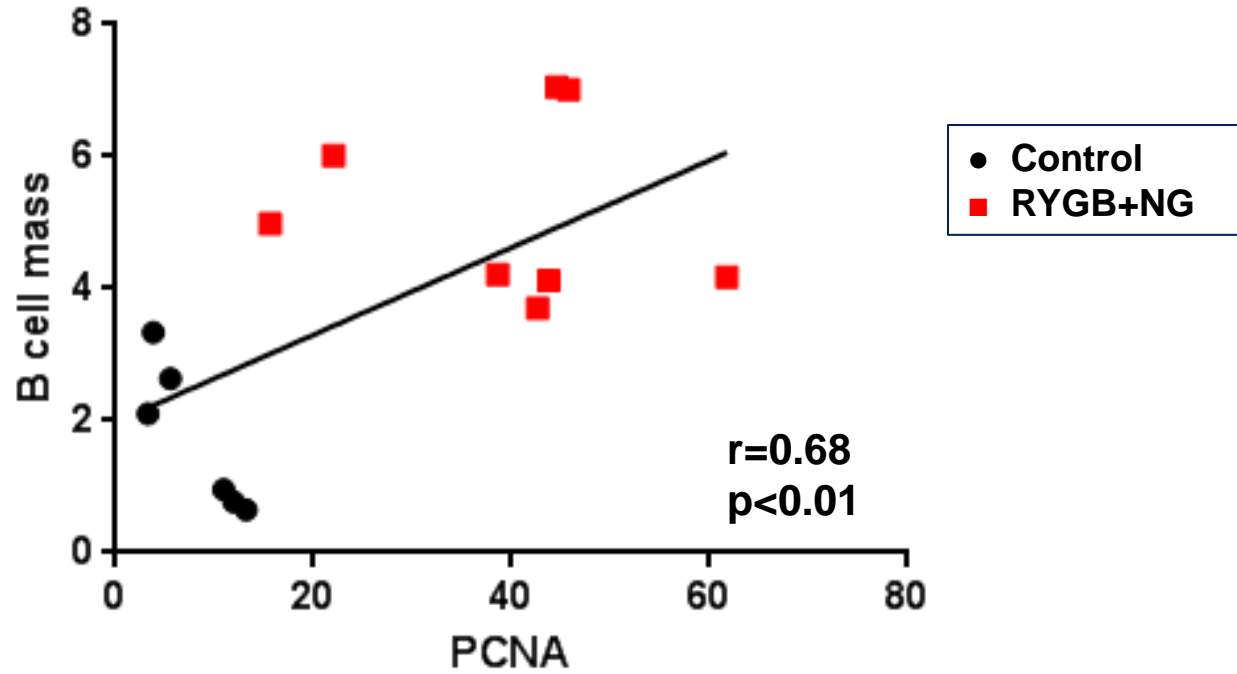
Menin
Insulin



Supplementary Figure 1.



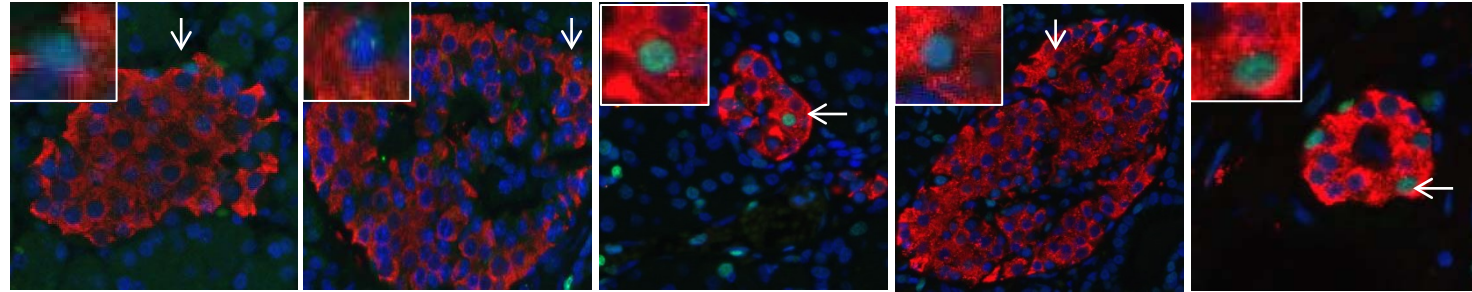
Supplementary Figure 2.



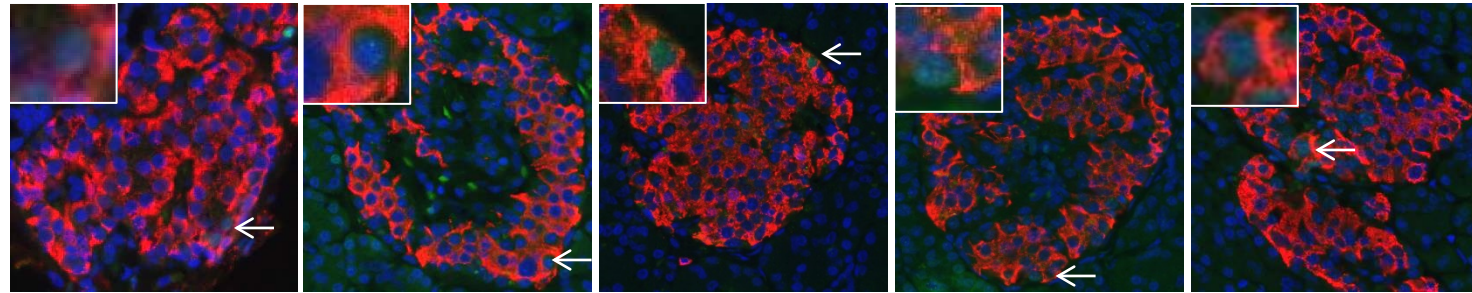
Supplementary Figure 3.

A. Phospho-Histone H3 and Insulin Co-Immunostaining

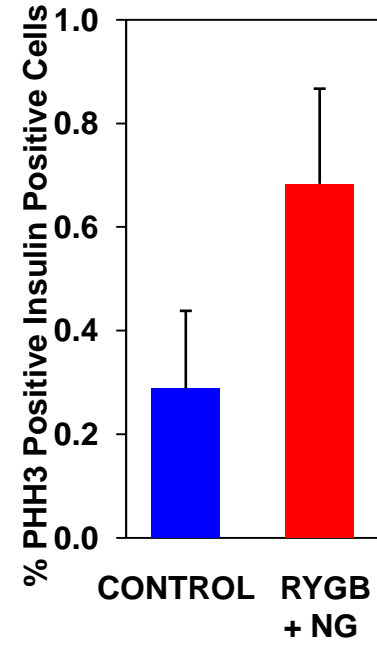
Control



Post-RYGB

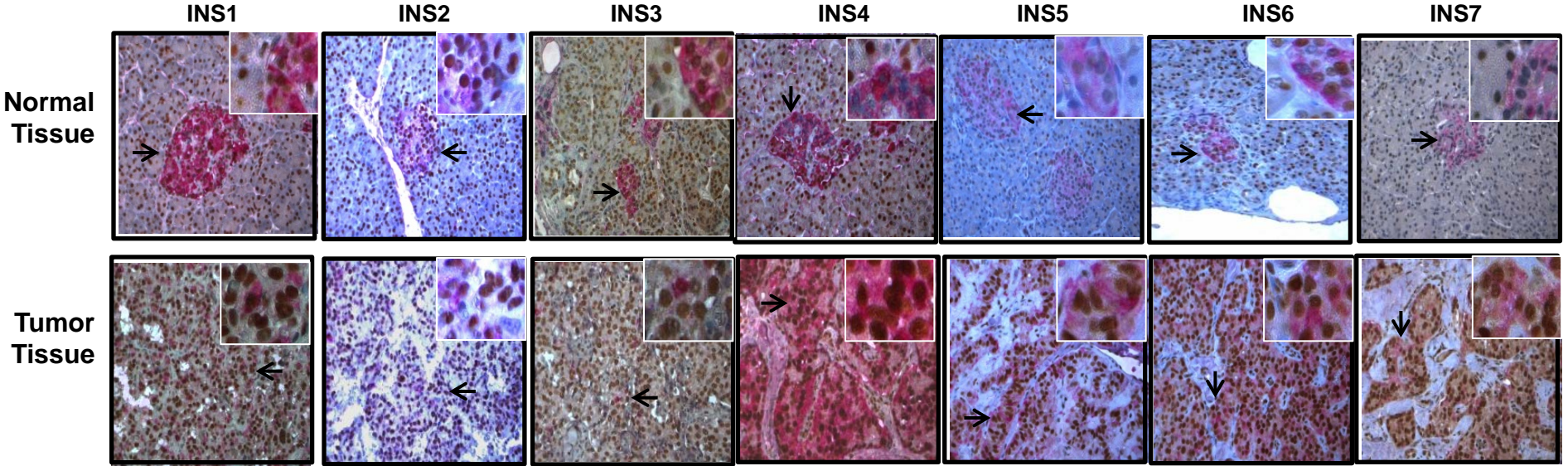


B. Quantification

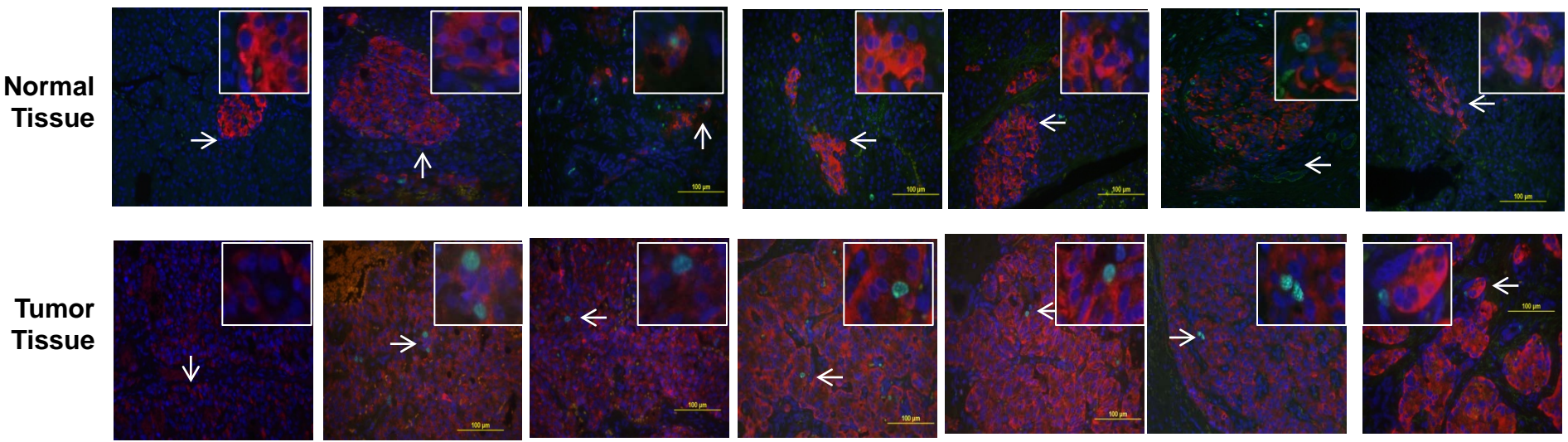


Supplementary Figure 4.

A. PCNA



B. Ki67



Supplementary Figure 5.

A. P27

INS1

INS2

INS3

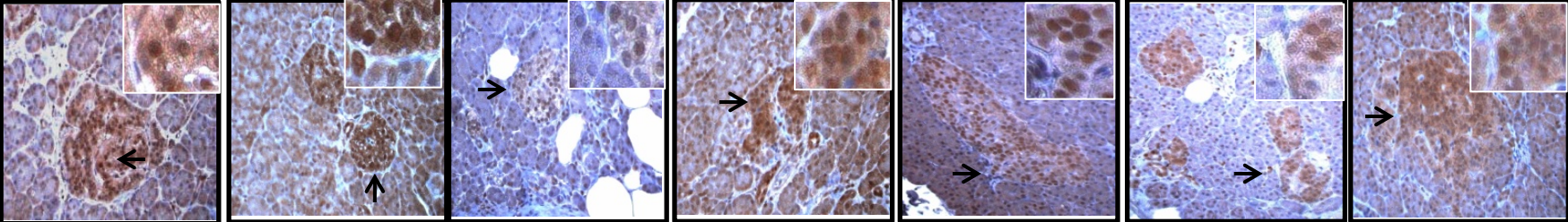
INS4

INS5

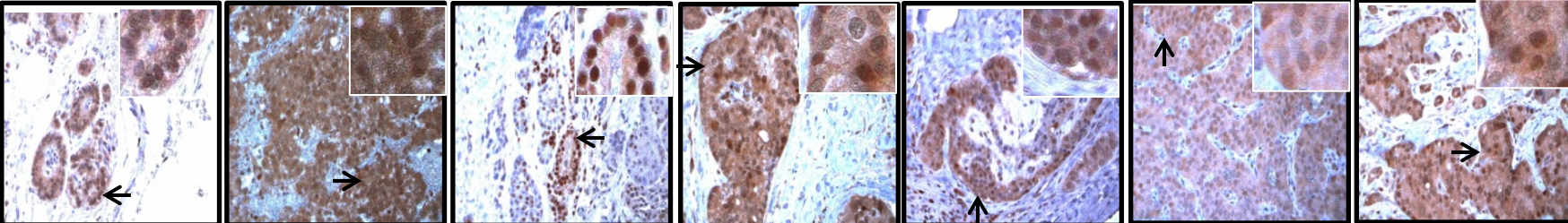
INS6

INS7

Normal Tissue

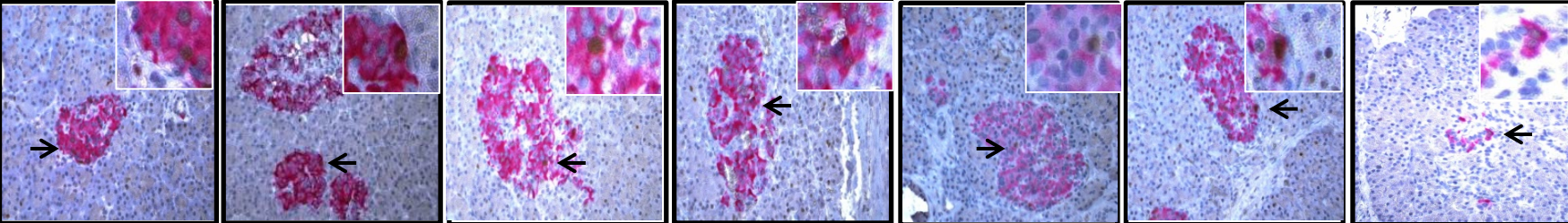


Tumor Tissue

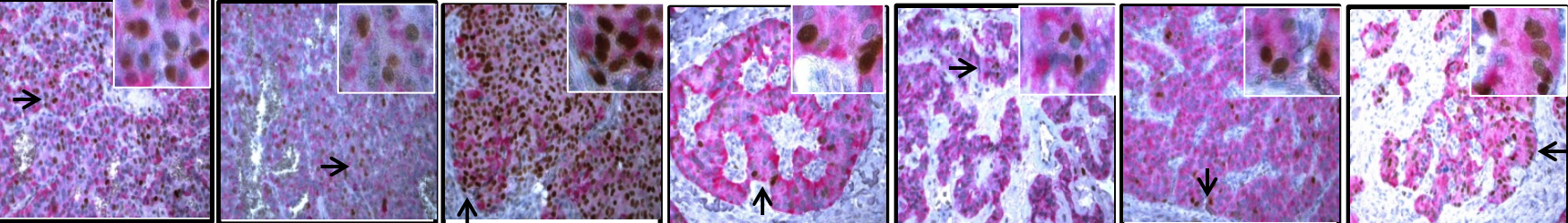


B. P21

Normal Tissue

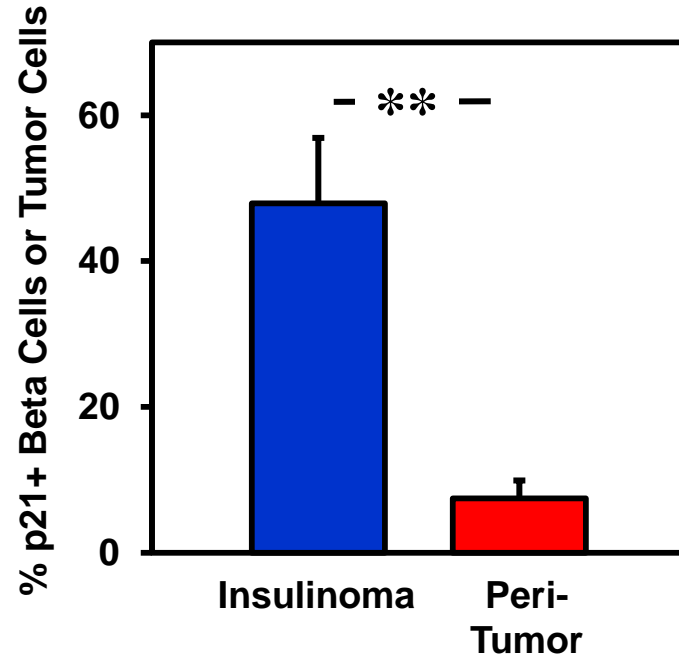


Tumor Tissue

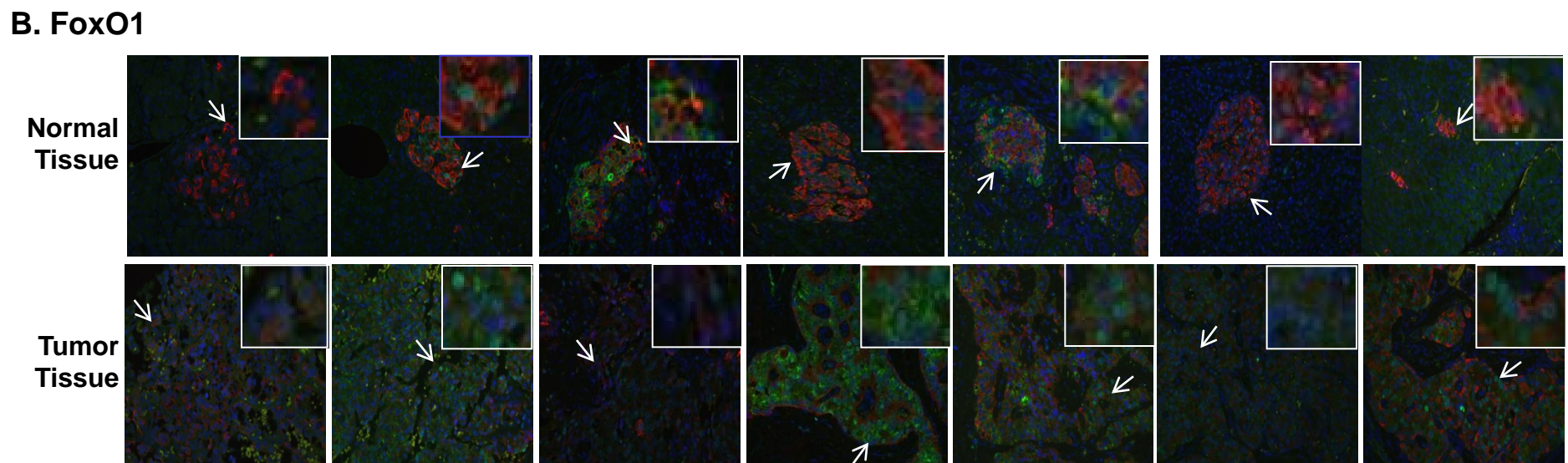
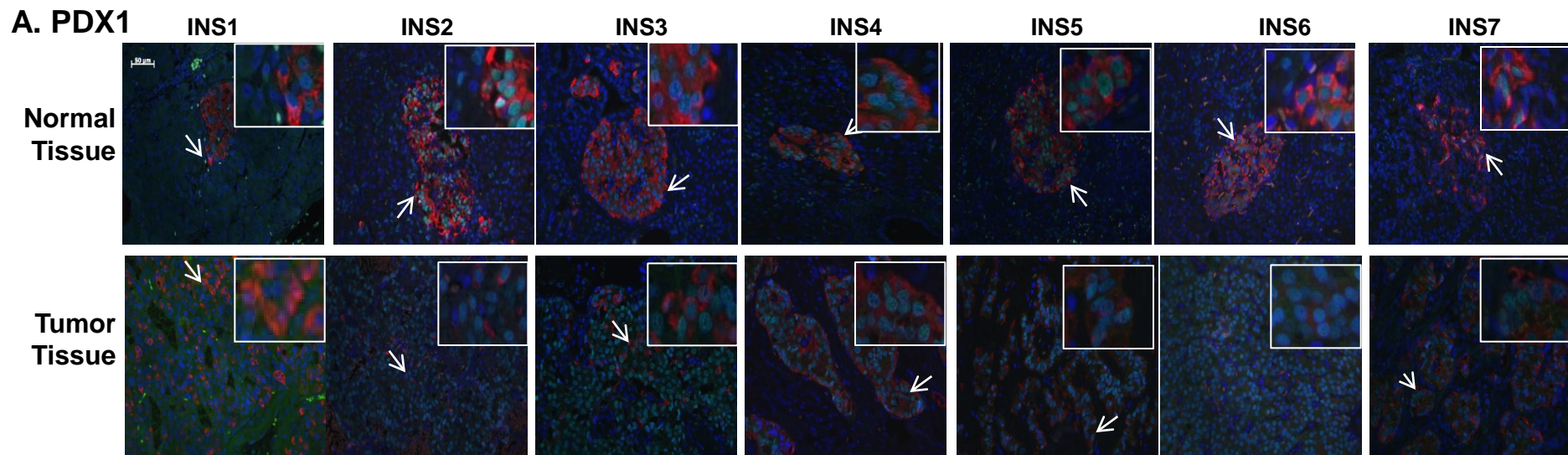


Supplementary Figure 5.

C.

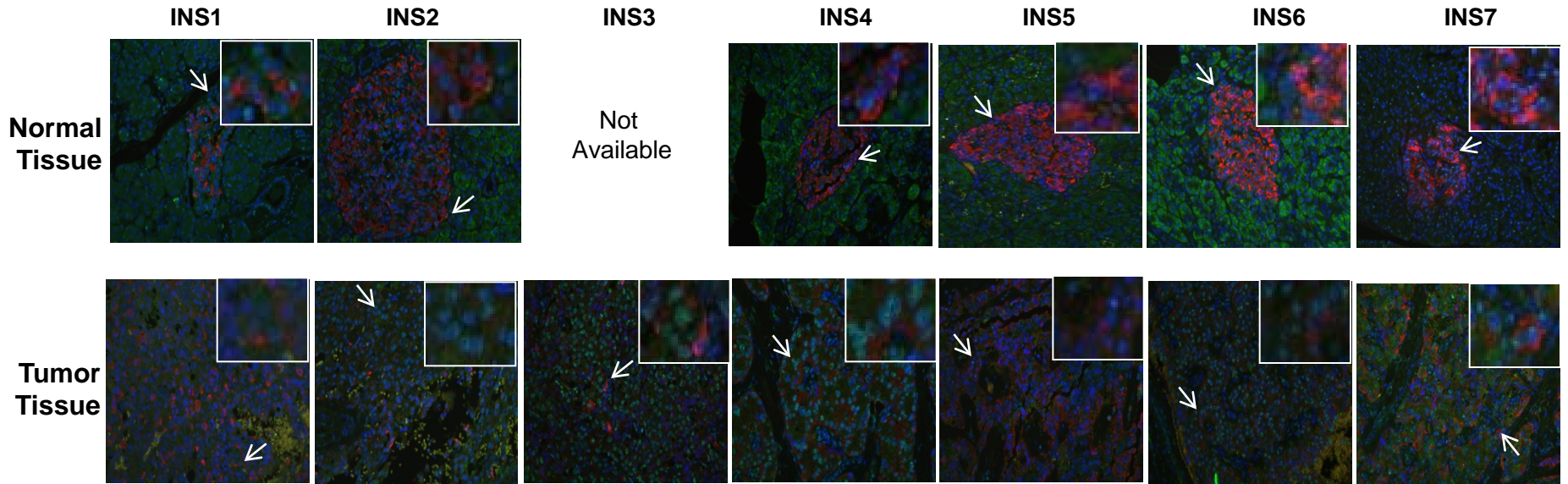


Supplementary Figure 6.



Supplementary Figure 6 (continued).

C. Menin



Supplementary Table 1

INSULINOMA SAMPLES		
Sex	Age	Ki-67 (%)
Series 1 (Norway)		
3M , 4F	60.3 \pm 6.3	0.82 + 0.29%
Series 2 (Italy)		
23 M, 22 F	47.9 \pm 2.6	2.44 \pm 0.42%