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ARTICLE

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miR-17-92 and miR-106b-25 clusters regulate beta cell mitotic checkpoint and insulin secretion in mice

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Abstract

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Aims/hypothesis Adult beta cells in the pancreas are the sole source of insulin in the body. Beta cell loss or increased demand for insulin impose metabolic challenges because adult beta cells are generally quiescent and infrequently re-enter the cell division cycle. The aim of this study is to test the hypothesis that a family of proto-oncogene microRNAs that includes miR-17-92 and miR-106b-25 clusters regulates beta cell proliferation or function in the adult endocrine pancreas.

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Methods To elucidate the role of miR-17-92 and miR-106b-25 clusters in beta cells, we used a conditional *miR-17-92/miR-106b-25* knockout mouse model. We employed metabolic assays *in vivo* and *ex vivo*, together with advanced microscopy of pancreatic sections, bioinformatics, mass spectrometry and next generation sequencing, to examine potential targets of miR-17-92/miR-106b-25, by which they might regulate beta cell proliferation and function.

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Results We demonstrate that miR-17-92/miR-106b-25 regulate the adult beta cell mitotic checkpoint and that miR-17-92/miR-106b-25 deficiency results in reduction in beta cell mass *in vivo*. Furthermore, we reveal a critical role for miR-17-92/miR-106b-25 in glucose homeostasis and in controlling insulin secretion. We identify protein kinase A as a new relevant molecular pathway downstream of miR-17-92/miR-106b-25 in control of adult beta cell division and glucose homeostasis.

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Conclusions/interpretation The study contributes to the understanding of proto-oncogene miRNAs in the normal, untransformed endocrine pancreas and illustrates new genetic means for regulation of beta cell mitosis and function by non-coding RNAs.

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Data availability Sequencing data that support the findings of this study have been deposited in GEO with the accession code GSE126516.

27

Keywords Beta cells · Cell cycle · Diabetes · Glucose-stimulated insulin secretion · GSIS · microRNA · PKA · Protein kinase A

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Abbreviations

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BrdU	Bromodeoxyuridine	MARK2	Microtubule affinity regulating kinase 2	43
FDR	False discovery rate	MEF	Mouse embryonic fibroblast	44
FRET	Fluorescence resonance energy transfer	miRNA	MicroRNA	46
GSIS	Glucose-stimulated insulin secretion	PHH3	Phosphorylated histone H3	49
KO	Knockout	PKA	Protein kinase A	50
		PRKAR1 α	Protein kinase cAMP-dependent type I regulatory subunit α	53
		qRT-PCR	Quantitative real-time RT-PCR	56
		smFISH	Single molecule fluorescence in situ hybridisation	58
				60

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Introduction

MicroRNAs (miRNAs) are small, non-coding RNAs that provide a broad post-transcriptional silencing mechanism [1], including in metabolism and diabetes [2–4]. miRNAs are

Research in context

What is already known about this subject?

- The microRNA clusters miR-17-92 and miR-106b-25 regulate the S (synthesis) checkpoint in many tumours
- miR-17-92/miR-106b-25 expression is reduced in juvenile rat beta cells through changes in nutrient supply during weaning
- Loss of a single *miR-17-92* allele out of the three in the mouse genome negatively affects insulin secretion and proliferation in mice

What is the key question?

- What is the contribution of the family of miR-17-92/miR-106b-25 miRNAs to adult beta cell cycle and beta cell function?

What are the new findings?

- We present a comprehensive mouse genetics study of *miR-17-92/miR-106b-25* loss of function alleles in adult beta cells
- miR-17-92/miR-106b-25 engage with the cell division cycle and play a regulatory role in the beta cell mitotic checkpoint
- The insulin secretion and proliferation phenotype is regulated by miR-17-92/miR-106b-25 in a pathway that involves modulation of protein kinase A (PKA) activity

How might this impact on clinical practice in the foreseeable future?

- This study ties miR-17-92/miR-106b-25 activity to incretin/PKA activity, and further investigation of this pathway may contribute to the development of new incretin-based therapies

65 essential for normal beta cell function and inactivation of
66 miRNA biogenesis in beta cells results in a diabetic phenotype
67 [5, 6].

68 Beta cell mass is a function of cell number and size, corre-
69 lates with body demand [7] and is controlled by beta cell
70 replication [8–10]. Cell division is a tightly regulated process,
71 with four main stages, and its three checkpoints (i.e. G1/S, G2/
72 M and mitotic checkpoint) guarantee the accomplishment of
73 necessary molecular activities before progression to the next
74 stage [11]. Aberrant cell cycle progression might result in cell
75 cycle failure, premature cell cycle exit and cell death. In beta
76 cells, impaired proliferation may result in low insulin levels
77 and hyperglycaemia/diabetes; therefore better understanding
78 of the molecular mechanisms controlling beta cell prolifera-
79 tion is valuable.

80 The miR-17-92 family contains 15 miRNAs that regulate
81 cell proliferation and apoptosis [12–14]. These miRNAs are
82 transcribed from three polycistronic clusters (*miR-17-92* on
83 mouse chromosome 13, *miR-106a-363* on chromosome X
84 and *miR-106b-25* on chromosome 5). The clusters share four
85 main ‘seed’ subtypes and hence joint downstream mRNA tar-
86 gets [15]. Genetic deletion of *miR-17-92* results in smaller
87 mouse embryos with severely hypoplastic lungs.
88 Furthermore, deletion of both *miR-17-92* and the homologous
89 *miR-106b-25* cluster is lethal for embryos [13].

90 Expression of miR-17-92 family members is regulated dur-
91 ing the cell cycle, at least in cultured cells, with the highest

92 levels measured at the G2/M transition [16]. This may allow
93 inhibition of target proteins involved in the transition between
94 the G1/S phases. Indeed, miR-17-92 family members are in-
95 terwoven into a regulatory network, wherein expression of
96 these miRNAs is induced by c-Myc and E2F and the
97 miRNAs repress the expression of E2F family members
98 through conserved binding sites at the 3’UTR of E2F1/2/3
99 [17–19].

100 The Regazzi laboratory demonstrated roles for the miR-17-
101 92 family in metabolic adaptation of beta cells in newborn rats
102 to changes in nutrient supply [20] and in regulating islet cir-
103 cadian gene expression [21]. We hypothesised that miR-17-
104 92/miR-106b-25 family members regulate adult beta cell di-
105 vision, given the interaction of this miRNA family with c-
106 Myc, a known driver of beta cell proliferation [22].

Methods

107
108 **Mouse strains** Female and male c57bl/6 mice were housed and
109 handled at the Weizmann Institute of Science and in accor-
110 dance with protocols approved by the Institutional Animal
111 Care and Use Committee of the Weizmann Institute of
112 Science. All mice we used were bred in-house. To generate
113 *Pdx1-Cre;miR-17-92^{LoxP/LoxP};miR-106-25^{-/-}* (*miR-17-92/*
114 *miR-106b-25-KO*) mice, we previously crossed *Pdx1-Cre*
115 transgenic mice [23] (a gift from D. Melton [Howard

116 Hughes Medical Institute, Harvard University, Boston, MA,
 117 USA) with *miR-17-92*^{LoxP/LoxP} and further with *miR-106b-*
 118 *25*^{-/-} mice [13] (both were gifts from T. Jacks [Howard
 119 Hughes Medical Institute, Massachusetts Institute of
 120 Technology, Boston, MA, USA] and A. Ventura [Memorial
 121 Sloan Kettering Cancer Center, New York, NY, USA]).

122 *ROSA-miR-17-92*^{conditional} overexpressing mice (were a
 123 gift from K. Rajewsky [Max Delbrück Center for Molecular
 124 Medicine, Berlin, Germany]) [24] and were crossed with
 125 *Pdx1-Cre* to achieve *Pdx1-Cre;ROSA-miR-17-92*^{conditional}
 126 mice. *CcnB1-GFP* transgenic mice were generated by Y.
 127 Dor (The Hebrew University of Jerusalem, Israel) [25, 26].

128 **Isolation of islets of Langerhans, flow cytometry and cell**
 129 **sorting** Islets were isolated using collagenase P (Roche,
 130 Switzerland) injected into the pancreatic duct, followed by
 131 Histopaque gradient (1119, 1083 and 1077; Sigma-Aldrich,
 132 Israel) as described in [27]. For miRNA profiling along the
 133 beta cell cycle, flow cytometry, islet dissociation and cell
 134 sorting were performed as described in [25].

135 **Pancreas physiology assays** Blood glucose was determined
 136 using an Ascensia elite glucometer (Ascensia, Switzerland).
 137 Insulin levels in the pancreas and serum were determined
 138 using an ultrasensitive insulin ELISA kit (90,080; Crystal
 139 Chem, Elk Grove Village, IL, USA). GTTs and glucose-
 140 stimulated insulin secretion (GSIS) tests were performed by
 141 injecting glucose (2 mg/g) intraperitoneally after mice were
 142 fasted overnight (~18 h) at different time points (age 4 weeks
 143 to 12 months). ITT was performed by injecting insulin (0.8 U/
 144 g) intraperitoneally after 4- to 6-week-old mice were fasted for
 145 5 h. Blood for GTT and ITT was repeatedly sampled from the
 146 tail vein. Retro-orbital blood was sampled before the injection
 147 and 15 min post-injection for the in vivo GSIS test. Insulin
 148 secreted to the medium in the ex vivo GSIS was measured
 149 after 1 h of incubation with either 2.5 or 25 mmol/l glucose by
 150 fluorescence resonance energy transfer (FRET) (62IN2PEG;
 151 Cisbio, France). Protein kinase A (PKA) activity was quanti-
 152 fied using PKA Kinase Activity Assay Kit (ab139435;
 153 Abcam, UK). Islets for all in vitro assays were purified from
 154 4- to 6-week-old mice.

155 **Static and dynamic stimulation of insulin secretion** Insulin
 156 secretion studies were performed in KRB containing
 157 114.4 mmol/l NaCl, 5 mmol/l KCl, 24 mmol/l NaHCO₃,
 158 1 mmol/l MgCl₂, 2.2 mmol/l CaCl₂, 10 mmol/l HEPES and
 159 0.5% wt/vol. BSA, adjusted to pH 7.35. In static incubation
 160 experiments, 10–20 islets from 4- to 6-week-old mice were
 161 pre-incubated in basal KRB containing 2.5 mmol/l glucose for
 162 1 h. Islets were consecutively incubated at 2.5 and 25 mmol/l
 163 glucose for 1 h each. Medium was collected at the end of each
 164 incubation period. Insulin assays were performed in
 165 Eppendorf tubes at 37°C and 5% CO₂.

A perfusion system (Biorep, Miami Lakes, FL, USA) 166
 equipped with a peristaltic pump was used for dynamic 167
 assessment of insulin secretion. Forty size-matched islets were 168
 placed in columns and perfused at a flow rate of 100 µl/min 169
 with KRB (basal glucose concentration 2.8 mmol/l) at 37°C. 170
 After equilibration, high glucose (16.7 mmol/l) KRB was 171
 used. Insulin secreted to the medium was collected in 96- 172
 well plates, quantified by FRET (Cisbio) or ELISA (Crystal 173
 Chem) and normalised to total islet insulin content. 174

Pancreatic histology and immunohistochemistry Pancreases 175
 from 4- to 6-week-old mice were dissected and fixed in 4% 176
 vol./vol. paraformaldehyde for 24 h at 4°C and then processed 177
 into paraffin blocks. Sections (5 µm thick) were de-paraffinised, 178
 rehydrated and antigen retrieval was performed using a PickCell 179
 pressure cooker (PickCell, the Netherlands). The following pri- 180
 mary antibodies were used: guinea pig anti-insulin (1:200, 181
 A05641; Dako, Denmark); rabbit anti-activated caspase-3 182
 (1:50, c-96,615; Cell Signaling, Danvers, MA, USA); rabbit 183
 anti-Ki67 (1:200, SP6; Cell Marque, Rocklin, CA, USA), mouse 184
 anti-bromodeoxyuridine (BrdU) (1:200, RPN202; GE 185
 Healthcare, Chicago, IL, USA) and phosphorylated histone H3 186
 (PHH3) (1:200, c-9701; Cell Signaling). For TUNEL staining 187
 we used the ApopTag red in situ apoptosis detection kit (s7165; 188
 Merck, Germany). For DNA counter-stain, we used Hoechst 189
 33342 (1 µg/ml, H3570; Thermo Fisher, Waltham, MA, 190
 USA). Secondary antibodies conjugated to CY2, CY3 or CY5 191
 were all from Jackson Immunoresearch Laboratories Baltimore, 192
 MD, USA (1:200). All the antibodies were previously validated, 193
 and all immunostaining included a negative control (no primary 194
 antibody); a positive control was also used for the apoptosis 195
 staining. All primary and secondary antibodies were diluted in 196
 CAS-block (008120 Thermo Fisher). Fluorescence images were 197
 captured using a Zeiss LSM710/780/800 Laser Scanning/ 198
 confocal microscope system equipped with a Zeiss camera with 199
 ×40 / ×63 magnification (Thornwood, NY, USA). 200

Histomorphometry Digital images of consecutive paraffin- 201
 embedded pancreas sections (50 µm apart, spanning the entire 202
 pancreas, approximately 40 sections/pancreas) were obtained 203
 at a low magnification (×20) and stitched using NIS-Elements 204
 software (Nikon, Japan) and 3DHistech (Hungary) 205
 Panoramic Viewer. The fraction of insulin-positive surface 206
 was determined by insulin immunoreactivity and the whole 207
 pancreas area was determined by haematoxylin counter-stain. 208
 Beta cell mass was calculated as the product of pancreas 209
 weight and the fraction of tissue covered by beta cells. 210

RNA quantification Extraction of total RNA was carried out by 211
 the miRNeasy Mini Kit (Qiagen, Germany). mRNA cDNA 212
 was synthesised using an oligo d(T) primer (C1101; Promega, 213
 Madison, WI, USA) and SuperScript II reverse transcriptase 214
 (18064-014; Invitrogen, Carlsbad, CA, USA). Synthesis of 215

216 miRNA cDNA was created using Taqman MicroRNA qPCR
 217 Assays (Applied Biosystems, Foster City, CA, USA). mRNA
 218 quantitative real-time RT-PCR (qRT-PCR) analysis was per-
 219 formed on a LightCycler 480 System (Roche) using Kapa
 220 SYBR Green qPCR kit (Finnzymes, Finland). miRNA qRT-
 221 PCR was performed on ABI Step one (Thermo Fisher).
 222 miRNA and mRNA levels were normalised to the expression
 223 of small RNAs (sno234 and U6) or mRNA (*Gapdh* and *Hprt*),
 224 respectively.

225 **Mouse embryonic fibroblast isolation and adenovirus infec-**
 226 **tion** *miR-17-92/miR-106b*-knockout (KO) or control mouse
 227 embryonic fibroblasts (MEFs) were harvested as in [28], plated
 228 at 50–60% confluency and grown in monolayer cultures in
 229 DMEM supplemented with 20% vol./vol. FBS (Biological
 230 Industries, Israel), 1% vol./vol. penicillin–streptomycin, 1%
 231 vol./vol. L-glutamine, 1% vol./vol. sodium pyruvate and 1%
 232 vol./vol. MEM-non-essential amino acid (Biological Industries).
 233 Cells were infected the next day with Ad5CMVeGFP (*eGFP*-
 234 adenovirus) or Ad5CMVCRE-*eGFP* (CRE-adenovirus), 300 vi-
 235 ral particles/cell (Gene Transfer Vector Core, University of Iowa).
 236 Medium was added after 24 h, replaced after 48 h and cells were
 237 harvested 5 days post-infection.

238 **RNA sequencing** cDNAs were sequenced on Illumina 2500
 239 (Illumina, San Diego, CA USA) sequencing machine with
 240 50 bp single read protocol. Reads for each sample were
 241 mapped independently using TopHat2 version (<https://ccb.jhu.edu/software/tophat/index.shtml>) (v2.0.10) [29] against
 242 the mouse genome build mm9. Approximately, 85–90%
 243 mapping rate was observed. Only uniquely mapped reads
 244 were used to determine the number of reads falling into each
 245 gene with the HTSeq-count script (https://htseq.readthedocs.io/en/release_0.11.1/count.html) (0.6.1p1) [30]. Differentially
 246 expressed genes were calculated with the DESeq2 package
 247 (v1.4.5) [31]. Genes that were expressed on at least one
 248 sample were considered. Differentially expressed genes were
 249 determined by *p* value <0.05 and an absolute fold change >1.
 250 5. Benjamini–Hochberg correction was used to adjust *p* value
 251 with false discovery rate (FDR) <0.05. Hierarchical clustering
 252 using Pearson dissimilarity and complete linkage was per-
 253 formed in order to explore a pattern of gene expression.
 254 Clustering analysis was performed with Matlab software
 255 (<https://www.mathworks.com/products/matlab.html>) (8.0.0.
 256 783). Gene ontology (GO) term enrichment analysis was per-
 257 formed using DAVID (<https://david.ncifcrf.gov/>) [32, 33].

260 Sequencing data that support the findings of this study have
 261 been deposited in GEO with the accession codes GSE126516
 262 [34].

263 **Mass spectrometry** The samples were subjected to in-solution
 264 tryptic digestion followed by a desalting step. The resulting
 265 peptides were analysed using nanoflow liquid

266 chromatography (nanoAcquity, Milford, MA, USA) coupled 266
 267 to high-resolution, high-mass-accuracy MS (Q Exactive Plus, 267
 268 Thermo Fisher). Samples were separately analysed in random 268
 269 order. Data were normalised to the sample total ion current 269
 270 and searched against the mouse protein database, to which a 270
 271 list of common laboratory contaminants was added (Mascot 271
 272 algorithm). Quantitative analysis was performed using 272
 273 Genedata Expressionist (UK). Only proteins identified by 273
 274 more than two peptides and more than nine amino acids/ 274
 275 peptides were considered. *p* values were corrected for multiple 275
 276 hypothesis using Benjamini–Hochberg procedure with FDR 276
 277 <0.05. The MS proteomics data have been deposited to the 277
 278 ProteomeXchange Consortium via the PRIDE [35] partner 278
 279 repository with the dataset identifier PXD012610. 279

280 ***Xist* single molecule fluorescence in situ hybridisation and**
 281 **image analysis** Single molecule in situ hybridisation
 282 (smFISH) was as in [36]. TransQuant (<https://ars.els-cdn.com/content/image/1-s2.0-S1046202315301559-mmcl.zip>;
 283 accessed 13 Dec 2018) used for *Xist* smFISH signal
 284 segmentation and analysis [37]. Ilastik (<https://www.ilastik.org/>) (1.3.1) was used for cell cycle image segmentation [38]. 285
 286

287 **Statistical analysis**

288 Data are expressed as means (SEM) and a two-sided Student’s
 289 *t* test was used for statistical comparisons. 289

290 **Results**

291 **miR-17-92 expression in developing and adult mouse**
 292 **endocrine pancreas**

293 miR-17 and miR-20a are expressed only from the *miR-17-92* 293
 294 cluster, miR-363 from *miR-106a-363* and miR-25 from *miR-*
 295 *106b-25* cluster (Fig. 1a), enabling the discrimination of 295
 296 expressed clusters by qRT-PCR. miR-106a-363 expression 296
 297 was undetected, consistent with its reported limited expression 297
 298 pattern [13], whereas miR-17-92 and miR106b-25 clusters 298
 299 were expressed in mouse embryonic pancreas (Fig. 1b). 299

300 Expression of miR-17, miR-20a and miR-25 significantly
 301 increased at embryonic day 15 (E15.5) relative to their expres-
 302 sion earlier in pancreas development (Fig. 1c), consistent with
 303 their reported role in cell proliferation and tissue growth. To
 304 evaluate the expression levels of the *miR-17-92* clusters in
 305 adult replicating beta cells, we obtained sorted beta cells from
 306 *CcnB1-GFP* transgenic mice, which express *eGFP* in repli-
 307 cating beta cells [25, 26]. mRNA levels of *Ki67* (also known
 308 as *Mki67*) and the gene encoding DNA topoisomerase II α
 309 (*Top2a*) were upregulated in the sorted cells, confirming that
 310 this population is indeed in the cell division cycle (Fig. 1d). 310

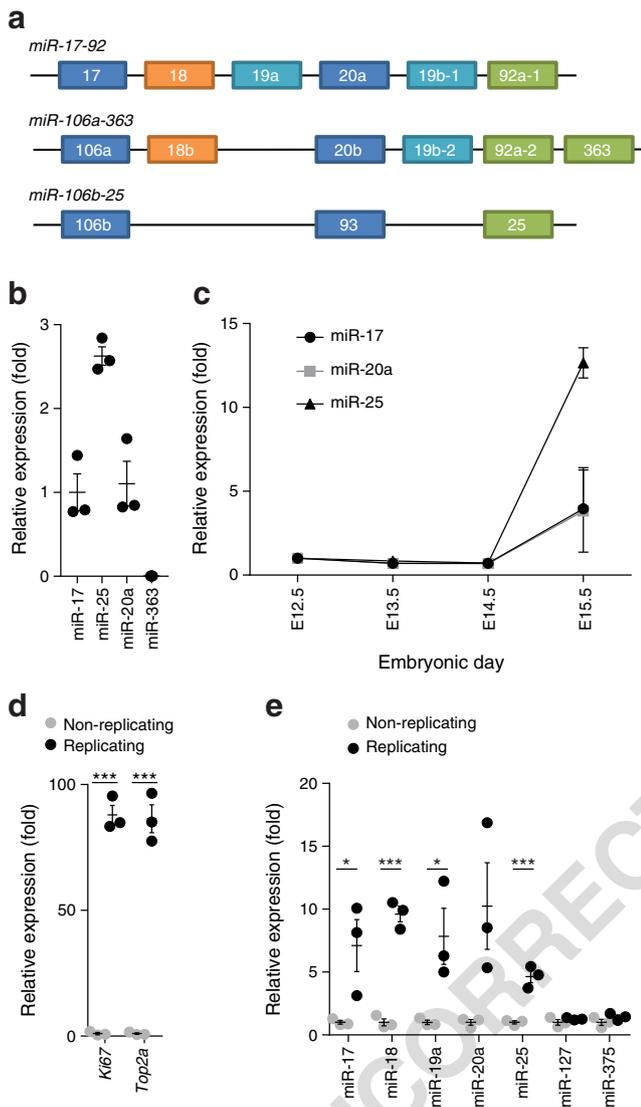


Fig. 1 Dynamics of miRNA expression from the three paralogous genomic clusters encoding the miR-17-92 family members in the developing pancreas of the mouse. **(a)** Diagram of the genomic organisation of the *miR-17-92* and *miR-106b-25* clusters. miRNAs are colour coded according to seed sequences that define target specificity. **(b)** Relative expression levels of miR-17, miR-25 (*miR-106b-25* cluster, chromosome 5), miR-20a (*miR-17-92* cluster, chromosome 13) and miR-363 (*miR-106a-363* cluster, chromosome X) in pancreatic buds, isolated from mouse embryos at embryonic day E14.5. Expression relative to miR-17 is shown and is normalised to the expression of the non-coding RNA U6. It is noteworthy that miR-363 is undetected. $n=3$. **(c)** miR-17, miR-20a and miR-25 expression at multiple time points in the pancreas during embryonic development. miRNA expression is normalised to U6 and to expression at E12.5. RNA for the embryonic measurements was extracted from three embryonic pancreatic buds (three embryos) for each sample. **(d, e)** mRNA levels of *Ki67* and *Top2a*, two cell cycle markers **(d)**, and of specific miRNAs **(e)**, in sorted replicating vs non-replicating beta cells. Data are shown as mean \pm SEM and expression is relative to that in non-replicating beta cells. * $p<0.05$ and *** $p<0.001$ (two-sided Student's *t* test)

311 The expression of *miR-17-92* and *miR-106b-25* clusters was
312 upregulated in proliferating cells, while the expression of

other miRNAs (miR-375, miR-127) was unchanged (Fig. 313
1e). Therefore, miR-17-92/miR-106b-25 are induced in divid- 314
ing beta cells. 315

miR-17-92/miR-106b-25 involvement in endocrine function 316

To study miR-17-92/miR-106b-25 family function in the 317
mouse pancreas, we crossed mice carrying the *miR-17-92* 318
conditional allele with *Pdx1-Cre* transgenic mice and further 319
mated the pedigree to mice carrying the miR-106b-25 whole- 320
body KO allele [13] (Fig. 2a). The *Pdx1-Cre; miR-17-92^{LoxP/}* 321
LoxP; miR-106b-25^{-/-} cross resulted in significant downregula- 322
tion of miR-17, miR-25 and miR-20a in islets, relative to 323
control mice (harbouring *miR-17-92^{LoxP/LoxP}; miR-106b-25^{+/-}* 324
alleles; Fig. 2b). 325

We performed GTTs on four intermediate genotypes (ESM 326
Fig. 1a), revealing an additive role for *miR-17-92* and *miR-* 327
106b-25 clusters in glucose homeostasis. Complete nullifica- 328
tion resulted in the most severe impairment in glucose toler- 329
ance. We therefore investigated mutant mice lacking miR-17- 330
92 and miR-106b-25 (*Pdx1-Cre; miR-17-92^{LoxP/LoxP}; miR-* 331
106b-25^{-/-}, referred to as *miR-17-92/miR-106b-25-KO*) vs lit- 332
termate controls (*miR-17-92^{LoxP/LoxP}; miR-106b-25^{+/-}*). 333
Impaired glucose tolerance was evident at 3 months of age 334
and progressively deteriorated at 6 and 12 months (Fig. 2c,d 335
and ESM Fig. 1b), comparable with the results of Chen et al 336
[39]. An ITT demonstrated normal response to insulin in mu- 337
tant and control mice (ESM Fig. 1c), indicating that whole- 338
body *miR-106b-25-KO* does not cause insulin resistance in 339
peripheral tissues under these experimental conditions. 340

Morphometric analysis (Fig. 2e) revealed reduced beta cell 341
mass in *miR-17-92/miR-106b-25-KO* vs control mouse 342
pancreases (Fig. 2f). Moreover, there was a 50% decrease in 343
total pancreatic insulin content in *miR-17-92/miR-106b-25-* 344
KO vs control mice (Fig. 2g). 345

Immediately after i.p. injection of glucose, serum insulin 346
levels were significantly diminished in *miR-17-92/miR-106b-* 347
25-KO vs control mice (Fig. 2h). To distinguish between an 348
intrinsic insulin secretion defect and a secondary effect due to 349
reduced beta cell mass, we performed ex vivo GSIS tests on 350
islets isolated from *miR-17-92/miR-106b-25-KO* mice or lit- 351
termate control mice. Insulin secretion from isolated *miR-17-* 352
92/miR-106b-25-KO mouse islets was diminished, even when 353
normalised to insulin content (Fig. 2i). We therefore conclude 354
that, in addition to controlling beta cell mass, miR-17-92/miR- 355
106b-25 are required autonomously in beta cells for normal 356
GSIS. 357

To characterise insulin secretion further, we isolated islets 358
and performed GSIS in a perfusion apparatus. Perfused islets 359
from *miR-17-92/miR-106b-25-KO* mice secreted insulin in a 360
manner comparable with control islets in low glucose but 361
failed to display enhanced insulin secretion in response to high 362
glucose (Fig. 3a). Of note, the temporal secretion pattern was 363
normal and insulin was released in early (first) and delayed 364

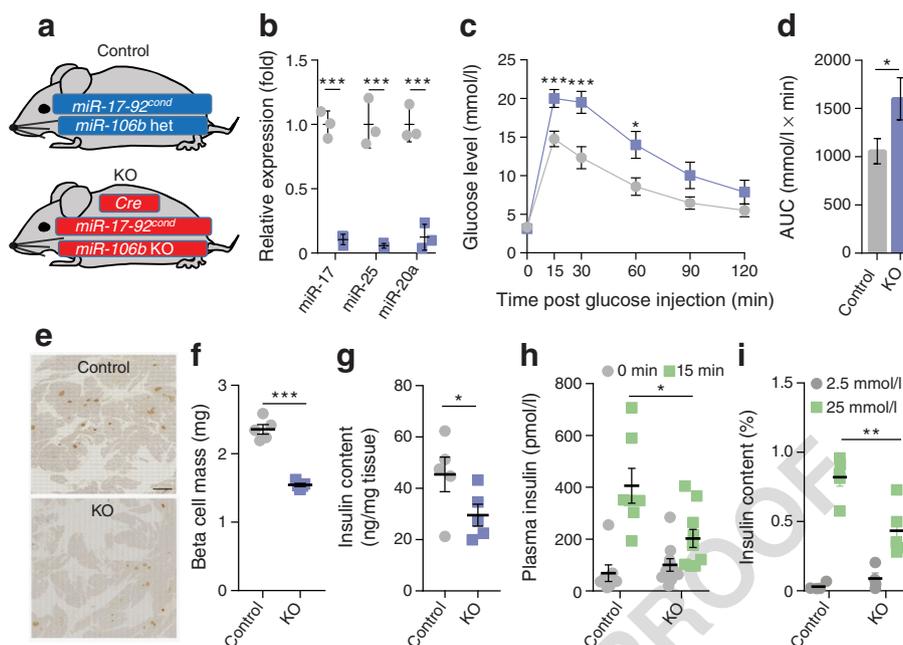


Fig. 2 Pancreas-specific loss of miR-17-92/miR-106b-25 impairs endocrine function. **(a)** Diagram of mouse genetics: *miR-17-92*^{LoxP/LoxP}; *miR-106b-25*^{+/-} (control) vs *Pdx1-Cre*; *miR-17-92*^{LoxP/LoxP}; *miR-106b-25*^{-/-} (*miR-17-92/miR-106b-25-KO*). **(b)** Relative expression levels of miR-17, miR-20a and miR-25 in 6-week-old KO and control mouse islets. Expression is normalised to the expression level in control tissue and to the expression of the non-coding RNA U6. *n*=3 mice. **(c)** GTT at multiple time points after glucose i.p. bolus (2 mg/g) in 6-month-old mice. *miR-17-92/miR-106b-25-KO* mice develop glucose intolerance, relative to control littermates. *n*=15 mice. **(d)** Area under the GTT curve shows an increase in the integrated sum of the blood glucose levels in the *miR-17-92/miR-106b-25-KO* vs control mice. **(e)** Representative micrograph of insulin immunohistochemistry in control and *miR-17-92/miR-106b-25-KO* mouse pancreases counter-stained with haematoxylin. Scale bar, 1000 μ m. **(f)** Beta cell mass calculated as the fraction of insulin-positive area in consecutive sections spanning the entire pancreas (approximately

35 sections/pancreas) and multiplied by the pancreas mass in 4-month-old mice. *n*=5 mice. **(g)** ELISA measurement of insulin after ethanol extraction from whole pancreases of 4-month-old mice. *n*=5 mice. **(h)** Plasma insulin levels in peripheral blood before (0) or 15 min after i.p. injection of glucose (2 mg/kg) revealed reduced insulin secretion in 4- to 6-week-old KO mice relative to control mice. *n*=7 (control) or 10 (KO) mice. **(i)** Insulin secretion in isolated cultured islets of Langerhans, 60 min after low (2.5 mmol/l) or high (20 mmol/l) glucose stimulation, relative to total islet insulin. KO islets exhibit impaired response to glucose stimulus. *n*=5 mice. Data are presented as mean \pm SEM. Except for **(h)** and **(i)**, grey circles and bars, control mice; purple squares and bars, *miR-17-92/miR-106b-25-KO* mice. Significance was assessed with two-sided Student's *t* test. **p*<0.05, ***p*<0.01 and ****p*<0.001. For **(c)** significance was calculated for the comparison between KO and control mice at different time points. *miR-17-92*^{cond}, *miR-17-92*^{conditional}; *miR-106b* het, miR-106-25 heterozygous; *miR-106b* KO, *miR-106b-25* KO

365 (second) phases (Fig. 3a,b). We noted a 50% reduction in
 366 insulin secretion in the *miR-17-92/miR-106b-25-KO* vs control
 367 mouse islets (Fig. 3a). However, forced-depolarisation of
 368 *miR-17-92/miR-106b-25-KO* mouse beta cells with the non-
 369 nutrient secretagogue KCl produced a response comparable
 370 with that of control islets (Fig. 3a,b). These results demon-
 371 strate that miR-17-92/miR-106b-25 impact islet insulin secre-
 372 tion via a mechanism acting upstream of the plasma mem-
 373 brane depolarisation. Finally, we tested insulin secretion in
 374 the presence of cytochalasin B, a cell-permeable c-mycotoxin,
 375 which inhibits actin polymerisation and thus increases GSIS
 376 [40]. Cytochalasin B normalised insulin secretion in *miR-17-92/*
 377 *miR-106b-25-KO* mouse islets (Fig. 3c,d), suggesting that
 378 the capacity to synthesise insulin and assemble it into secre-
 379 tory granules is maintained in *miR-17-92/miR-106b-25-KO*
 380 mouse beta cells, while the regulated secretion pathway is
 381 impaired at a position upstream of potassium-dependent cell
 382 membrane depolarisation.

miR-17-92/miR-106b-25 KO do not affect beta cell apoptosis 383
 384 Reduced beta cell mass could result from either a defect in
 385 proliferation or from beta cell apoptosis, consistent with the
 386 impact of miR-17-92/miR-106b-25 on proliferation and apo-
 387 ptosis in other tissues [22]. miR-17-92/miR-106b-25 family
 388 members are suppressors of the proapoptotic genes *BIM* (also
 389 known as *BCL2L1*) and *PTEN* [41], suggesting that an in-
 390 crease in apoptosis may occur, when miR-17-92/miR-106b-
 391 25 genes are deleted. Apoptosis was neither detected with
 392 activated caspase 3 nor with TUNEL staining of pancreas
 393 sections from mice aged 4 weeks or 12 months (ESM Fig.
 394 2), in accordance with similar data from [39]. Therefore, it is
 395 likely that miR-17-92/miR-106b-25 family members regulate
 396 beta cell mass via proliferation rather than beta cell apoptosis.

miR-17-92/miR-106b-25 regulate beta cell proliferation 397
 398 To test directly the contribution made by miR-17-92/miR-106b-
 399 25 to the proliferation of beta cells, we examined the

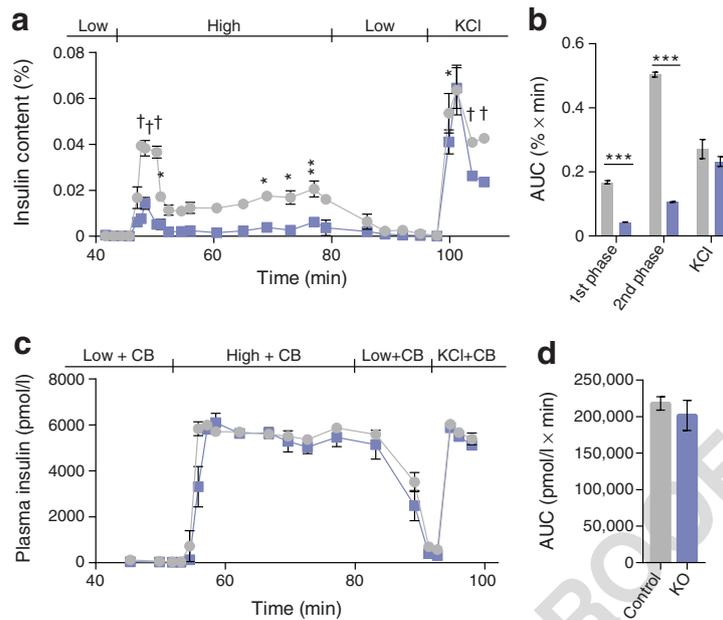


Fig. 3 miR-17-92/miR-106b-25 are necessary for continuous GSIS. (a) GSIS of control vs miR-17-92/miR-106b-25-KO islets in a perfusion apparatus under conditions of low (2.8 mmol/l) or high (16.7 mmol/l) glucose concentration, or when depolarised with KCl (30 mmol/l). (b) Quantification of secreted insulin integral as AUC for the first and second phases of secretion (45–52 min and 52.3–95 min, respectively) and in response to KCl. (c) Continuous GSIS with cytochalasin B (CB, 10 mmol/l) under conditions as in (a). (d) Comparable insulin secretion by control and miR-17-92/miR-106b-25-KO islets in response to cytochalasin B. AUC of secreted insulin for the whole perfusion period (45–95 min). Islets from 3 mice, 4- to 6-week-old per experimental condition. Statistical significance was calculated in (a) and (c) for the comparison between KO and control mice at different time points with two-way ANOVA followed by Sidak's multiple comparison test. In (b) and (d), data are presented as mean \pm SEM. Significance was assessed with two-sided Student's *t* test. **p*<0.05, ***p*<0.01 and ****p*<0.001; †*p*<0.001 in (a). Grey circles and bars, control mice; purple squares and bars, miR-17-92/ miR-106b-25-KO mice.

400 expression levels of cell-cycle-related genes in mutant mice.
 401 Both nuclear markers of proliferation Ki-67 and DNA topo-
 402 isomerase II α (encoded by *Top2a*), serve as sensitive prolif-
 403 eration markers in the endocrine pancreas [42, 43]. *Ki67* and
 404 *Top2a* mRNA levels were downregulated in *miR-17-92/miR-*
 405 *106b-25-KO* vs control mouse islets (Fig. 4a) and the percent-
 406 age of Ki-67-positive beta cells was also reduced (Fig. 4b).

Therefore, miR-17-92/miR-106b-25 activity is important for
 beta cell proliferation.

Next, we hypothesised that miR-17-92 family members af-
 fect the ability of beta cells to enter or to successfully complete
 the cell division cycle. We quantified the percentage of beta
 cells engaged in DNA synthesis by BrdU labelling and deter-
 mined the percentage of BrdU-positive beta cells out of total

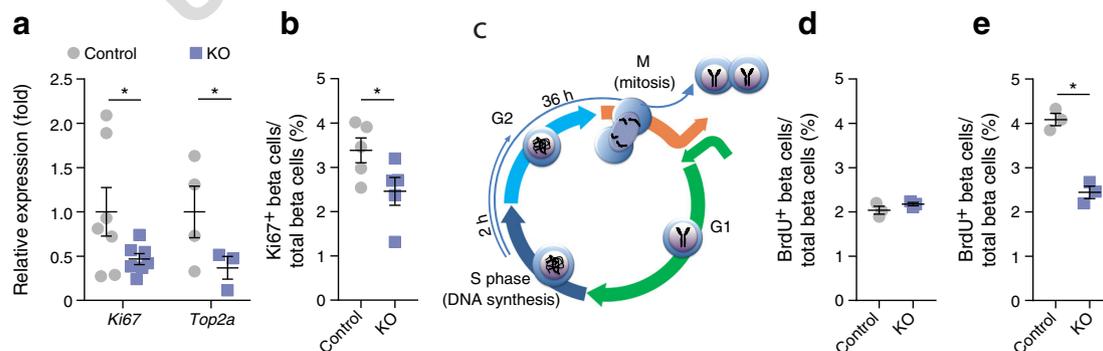


Fig. 4 Impaired beta cell proliferation in *miR-17-92/miR-106b-25-KO* mice. (a) *Ki67* and *Top2a* mRNA expression levels in islets isolated from *miR-17-92/miR-106b-25-KO* mice or control littermates. *n*=7 (*Ki67*) or *n*=4 (*Top2a*). (b) Quantification of beta cells, which are positive for insulin and Ki67 immunostaining, in sections of *miR-17-92/miR-106b-25-KO* and control mouse pancreases (*n*=5 mice per group, >60 islets per mouse). (c) Diagram of the experimental design, depicting BrdU pulse

and a chase of either 2 h or 36 h. (d) The percentage of BrdU-positive beta cells is comparable in *miR-17-92/miR-106b-25-KO* and control mouse islets 2 h post BrdU injection. (e) The percentage of BrdU-positive beta cells is reduced at 36 h post injection in *miR-17-92/miR-106b-25-KO* vs control mouse islets. *n*=3 mice per condition, >60 islets per mouse, >5000 beta cells counted per mouse. Data are shown as individual values and as mean \pm SEM, **p*<0.05 (two-sided Student's *t* test)

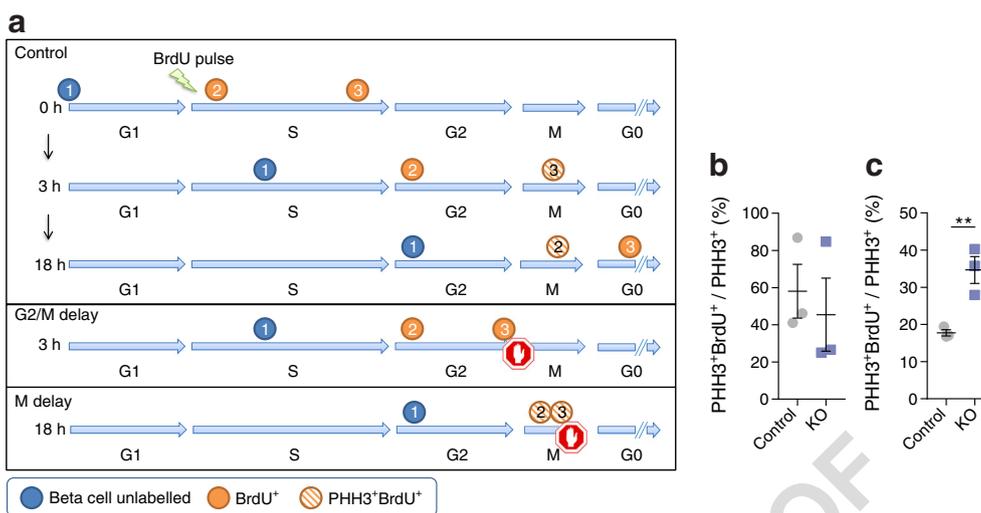


Fig. 5 *miR-17-92/miR-106b-25-KO* mouse beta cells are delayed in mitosis. **(a)** Diagram of cells progressing through the cell cycle. When BrdU is injected, cells (2) and (3) are at the DNA synthesis stage and incorporate BrdU, while most cells in the tissue (1) are unlabelled. Later, some cells exit S and retain BrdU (2), progress to mitosis and are co-labelled with PHH3 (3). A delay in transition through the G2/M checkpoint may be observed as relative depletion of PHH3⁺BrdU⁺ cells at 3 h, whereas a delay in M checkpoint will result in accumulation of PHH3⁺BrdU⁺ cells

at 18 h. **(b)** The percentage of PHH3⁺BrdU⁺ cells (expressed as a percentage of the total PHH3⁺ cells) 3 h after BrdU injection was comparable in *miR-17-92/miR-106b-25-KO* and littermate control mouse beta cells. **(c)** The percentage of PHH3⁺BrdU⁺ cells 18 h after BrdU injection was doubled in *miR-17-92/miR-106b-25-KO* vs control mouse beta cells, suggesting a delay at the M checkpoint. Mice aged 4–6 weeks were used. Data are shown as individual values and as mean ± SEM. *n*=3. ***p*<0.01 (two-sided Student's *t* test)

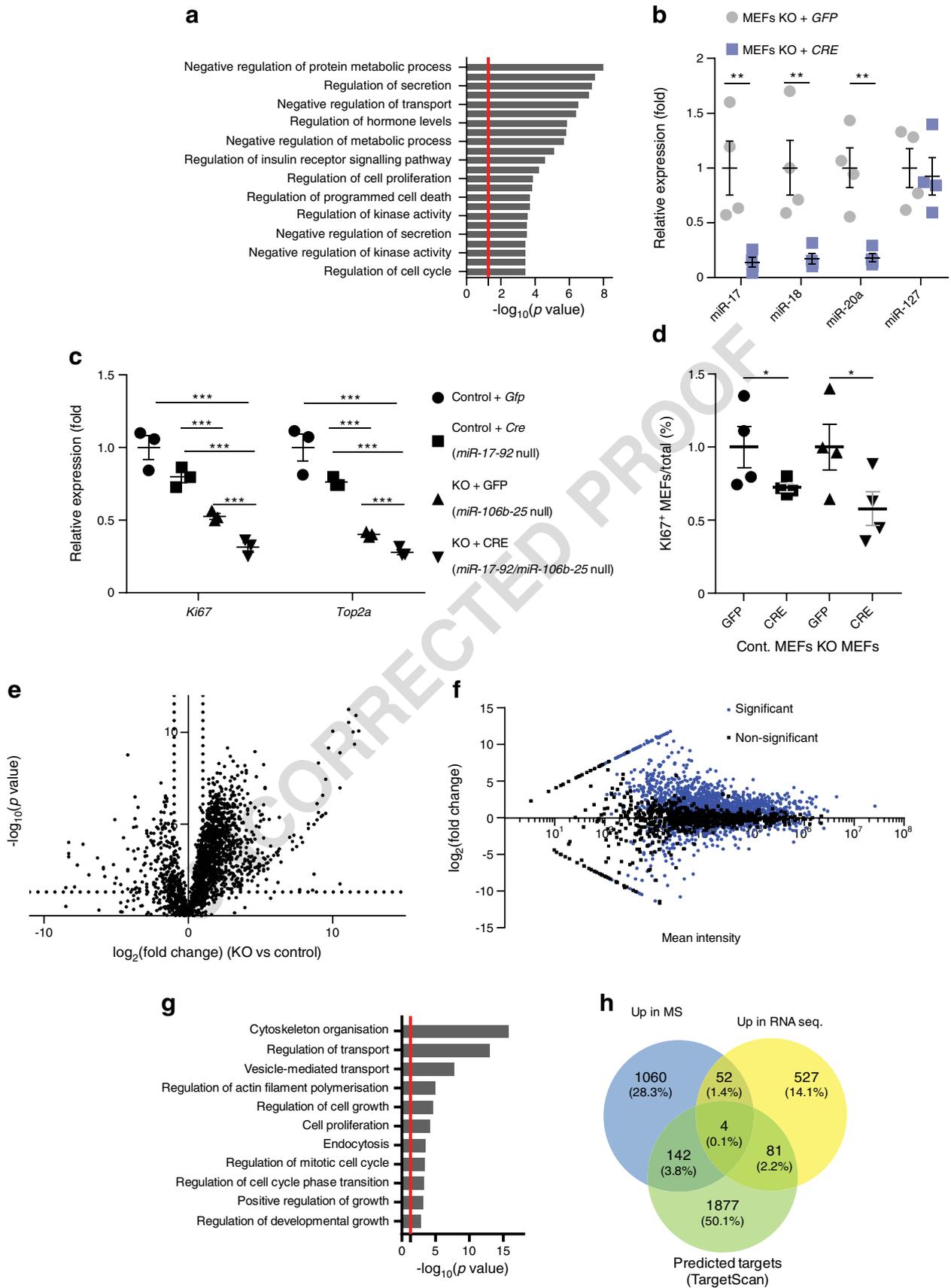
414 insulin-positive cells ([44], diagram in Fig. 4c). BrdU incorporation at 2 h post-injection represents the fraction of beta cells
 415 engaged in the S phase (DNA synthesis) at the time of the study
 416 (Fig. 4d). Unexpectedly, we observed a similar proportion of
 417 BrdU-positive beta cells in control and *miR-17-92/miR-106b-*
 418 *25-KO* mouse pancreases, suggesting that miR-17-92 family
 419 members are not essential for beta cell DNA synthesis.
 420

421 To discover whether *miR-17-92/miR-106b-25-KO* mouse
 422 beta cells fail to accomplish the cell division cycle, we extend-
 423 ed the study to 36 h after BrdU incorporation (i.e. longer than
 424 the full beta cell cycle [45]). After accomplishment of cyto-
 425 kinesis, the fraction of BrdU-positive wild-type beta cells dou-
 426 bled (from ~2% at 2 h to ~4% at 36 h, Fig. 4d,e), consistent
 427 with the expected doubling of the cell population. However,
 428 the fraction of BrdU-positive beta cells in *miR-17-92/miR-*
 429 *106b-25-KO* mouse islets remained unchanged (~2% at 2 h
 430 and 36 h), suggesting a severe defect in the cell division cycle.
 431 The defect was observed in islets of all sizes (ESM Fig. 3).
 432 Therefore, miR-17-92 is required for beta cells to effectively
 433 proceed through the cell cycle, at a point that is later than
 434 DNA synthesis, in contrast to miR-17-92 family activity de-
 435 scribed in other contexts. [13].

436 ***miR-17-92/miR-106b-25-KO* mouse beta cells are delayed in**
 437 **the mitotic checkpoint** Because it appears that G1/S transition
 438 is not the main target of miR-17-92/miR-106b-25 in beta cells,
 439 we tested whether later checkpoints, G2/M and M, are regu-
 440 lated. We performed dual labelling of PHH3, a marker of M
 441 phase [45], along with BrdU. The transition through cell cycle

checkpoints is disclosed by calculating the fraction of the total
 PHH3-positive beta cells that are positive for both BrdU and
 PHH3 (Fig. 5a). The BrdU⁺PHH3⁺ / PHH3⁺ ratio post-BrdU
 pulse revealed that the fraction of cells undergoing mitosis
 (M) and engaged in DNA synthesis (S) at the 3 h time window
 of the experimental chase was comparable in *miR-17-92/miR-*
 447

Fig. 6 Integrated transcriptome and proteome analyses. **(a)** Biological pathway over-representation analysis of the genes with significantly altered expression in the *miR-17-92/miR-106b-25-KO* islets, depicting pathways involved in regulation of secretion, cell cycle and kinase activity. The red line indicates a *p* value of 0.05. **(b)** The expression of miR-17-92/miR-106b-25 family members in MEFs is reduced upon transduction with the CRE-adenovirus compared with control GFP-adenovirus. *n*=4. **(c)** Expression of cell cycle markers is downregulated in MEFs upon loss of miR-17-92/miR-106b-25. *n*=3. **(d)** The percentage of MEFs engaged in the cell cycle is reduced in MEFs infected with CRE- compared with control GFP-adenovirus. *n*=4. **(e)** Volcano plot of the proteins downstream of miR-17-92/miR-106b-25 KO. **(f)** MA-plot for differential analysis of the mass spectrometry samples. **(g)** PANTHER analysis of the proteins that were significantly different in the *miR-17-92/miR-106b-25-KO* MEFs compared with control MEFs reveals enrichment in biological pathways related to cytoskeleton organisation, regulation of mitosis and cell cycle phase transitions. The red line indicates a *p* value of 0.05. **(h)** 664 mRNA species were upregulated (Up) in miR-17-92/miR-106b-25-KO mouse islets, relative to control. 1258 proteins were upregulated in *miR-17-92/miR-106b-25-KO* MEFs (determined by MS). Of these, only four genes were upregulated with a corresponding effect at the protein level and are also predicted miR-17-92/miR-106b-25 targets from TargetScan. *n*=6 MEFs for each condition, corrected *p* value <0.05. Data are presented as individual values and as mean ± SEM. **p*<0.05, ***p*<0.01 and ****p*<0.001 (two-sided Student's *t* test)



448 *106b-25-KO* and control beta cells. This suggests that miR-
449 17-92/miR-106b-25 do not contribute to beta cell G2/M
450 checkpoint dynamics (Fig. 5b).

451 We next addressed the hypothesis that miR-17-92/miR-
452 106b-25 regulate the M phase checkpoint by a longer (18 h)
453 chase period after BrdU labelling, as in [45]. At this point,
454 normal beta cells, which were initially at the S phase, have
455 already accomplished cytokinesis and accordingly downregu-
456 lated PHH3. Unexpectedly, we observed an increase in the
457 percentage of double-stained *miR-17-92/miR-106b-25-KO* vs
458 control mouse beta cells (Fig. 5c). We interpret this as evi-
459 dence in support of miR-17-92/miR-106b-25 activity at the
460 mitotic checkpoint. To test the hypothesis that *miR-17-92/*
461 *miR-106b-25-KO* mouse beta cells fail the mitotic checkpoint
462 and accumulate in a tetraploid state, we quantified X-inactive
463 specific transcript, a long non-coding RNA that coats one X-
464 chromosome in female cells. The smFISH study of duplicated
465 Xist signal, used to detect duplicated X-chromosomes in tet-
466 raploid cells, did not reveal an increase in the abundance of
467 tetraploid cells in *miR-17-92/miR-106b-25-KO* vs control
468 mouse beta cells (ESM Fig. 4a). Therefore, we could not sup-
469 port the hypothesis that *miR-17-92/miR-106b-25-KO* cells un-
470 dergo polyploidisation.

471 **Transcriptome and proteome analysis of *miR-17-92/miR-***
472 ***106b-25-KO* cells** To discover relevant miR-17-92/miR-
473 106b-25 targets, we profiled islet mRNA by next generation
474 sequencing (NGS), differential mRNA expression and gene
475 ontology analysis [46]. This depicted terms related to hor-
476 mone (insulin) secretion and to cell cycle regulation among
477 the over-represented biological pathways (Fig. 6a).

478 Bulk analysis in primary beta cells is limiting because only
479 a small fraction of the beta cells are engaged with the cell
480 division cycle. Therefore, we studied miR-17-92/miR-106b-
481 25 activity in MEFs (*miR-17-92^{LoxP/LoxP};miR-106-25^{-/-}*
482 MEFs) derived from MEFs from the same mouse allele and transduced
483 with either *GFP*-adenovirus (control) or *CRE*-adenovirus.
484 Comparing miRNA activity in endocrine pancreas and fibro-
485 blasts is biased towards detection of proteins that are
486 expressed in both cell types and thus overlooks cell-type-
487 specific expression. However, *Ki67* and *Top2a* were downregu-
488 lated in miRNA-deficient MEFs, reminiscent of *miR-17-92/*
489 *miR-106b-25-KO* beta cells (Fig. 6b,c). Furthermore, the per-
490 centage of Ki-67-positive cells in MEFs depleted of miRNA
491 was reduced relative to control MEFs (Fig. 6d).

492 MS analysis was performed on *miR-17-92^{LoxP/LoxP};miR-*
493 *106-25^{-/-}* MEF lysate, without or with *CRE*-Adenovirus.
494 *miR-17-92^{LoxP/LoxP};miR-106-25^{+/+}* MEFs with *GFP*-adenovi-
495 rus served as a control. Comparable results were gained when
496 *miR-17-92^{LoxP/LoxP};miR-106-25^{-/-}* MEFs with *GFP*-adenovi-
497 rus were used as controls. MS in six experimental repeats
498 depicted 16,005 unique peptides, corresponding to 2715 dif-
499 ferent proteins. The expression level of 64% of the proteins

was significantly changed by knocking out *miR-17-92/miR-* 500
106b-25. The majority of significantly changed proteins were 501
upregulated (84.6%, Fig. 6e,f). Intriguingly, mitosis and cell 502
cycle regulation were among the significantly enriched GO 503
terms along with cytoskeleton organisation and actin filament 504
polymerisation regulation (Fig. 6g). Out of the 1258 upregu- 505
lated proteins, 146 were predicted targets of at least one 506
miRNA from the miR-17-92/miR-106b-25 family (Fig. 6h). 507
Fifty-six gene products were significantly upregulated at both 508
the mRNA (in islets) and protein (in fibroblasts) levels. Four 509
out of these 56 were predicted direct targets of at least one 510
member of the miR-17-92/miR-106b-25 family, making a 511
short list of highly relevant targets across tissues. These targets 512
include *Mark2* encoding microtubule affinity regulating kin- 513
ase 2 (MARK2), *Jpt1/Hn1* encoding Jupiter microtubule as- 514
sociated homolog 1, *Sqstm1* encoding sequestosome 1 and 515
Prkar1a encoding protein kinase cAMP-dependent type I regu- 516
latory subunit α (PRKAR1 α). 517

518 Both MARK2 and PRKAR1 α are part of the protein kinase 518
A (PKA) pathway, the cellular sensor of cAMP, which regu- 519
lates cell division cycle and insulin secretion. [47–49]. 520
Prkar1a levels were validated by qRT-PCR (Fig. 7a). 521
Prkar1a and *Mark2* upregulation suggests a potential role 522
for the PKA pathway downstream of miR-17-92. We there- 523
fore studied the hypothesis that PKA activity is reduced in the 524
islets isolated from *miR-17-92/miR-106b-25-KO* mice. PKA 525
activity was downregulated in *miR-17-92/miR-106b-25-KO* 526
vs control littermate mouse islets. Furthermore, islets 527
harbouring the miR-17-92 overexpression transgene *Pdx-* 528
Cre;ROSA-miR-17-92^{conditional} displayed elevated PKA ac- 529
tivity (Fig. 7b and ESM Fig. 4b). Taken together, PKA ac- 530
tivity is sensitive bidirectionally to miR-17-92/miR-106b-25 531
levels in beta cells. Control of PKA by miR-17-92/miR-106b-25 is a 532
new convergence point for seemingly disparate processes of 533
proliferation and insulin secretion. 534

535 Discussion

536 Using mouse genetics we discovered that miR-17-92/miR- 536
106b-25 alleles regulate islet function via control of beta cell 537
mass and insulin secretion. The miR-17-92/miR-106b-25 538
family is important for normal endocrine function and, accord- 539
ingly, loss of the miRNAs results in endocrine failure. 540

541 *miR-17-92/miR-106b-25-KO* mice exhibited normal insu- 541
lin tolerance and miR-106b-25 deficiency in insulin- 542
responsive tissues (muscle, liver and adipose) did not modify 543
target organ insulin sensitivity, in vivo. Therefore, endocrine 544
pancreas failure manifesting as a reduction in GSIS is a plau- 545
sible cause of glucose intolerance. 546

547 Our study suggests a more pronounced role for miR-17-92 547
in GSIS than was previously reported [39], in part, since nul- 548
lification of both *miR-17-92* and *miR-106b-25* clusters in our 549

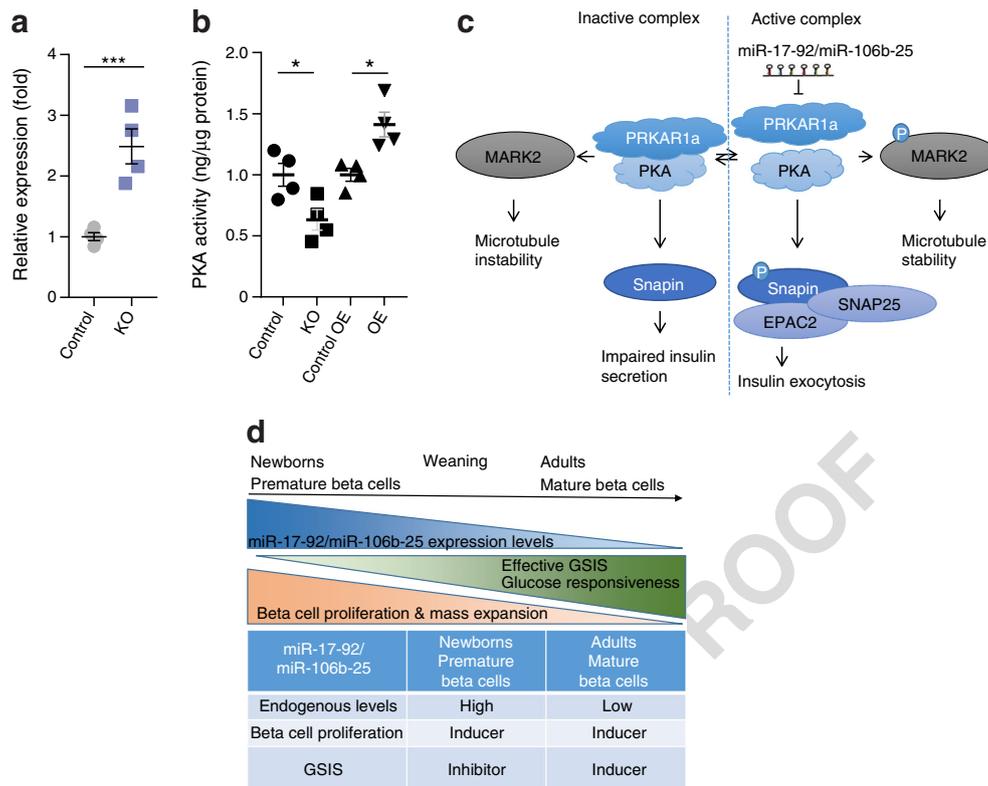


Fig. 7 PKA activity and suggested model downstream of miR-17-92/miR-106b-25. **(a)** *Prkar1a* expression is elevated in isolated *miR-17-92/miR-106b-25-KO* mouse islets relative to control littermates. *n*=4. **(b)** PKA activity is reduced in *miR-17-92/miR-106b-25-KO* mouse islets, while it is elevated in islets harbouring the *miR-17-92* overexpression transgene (*Pdx-Cre;ROSA-miR-17-92^{conditional}* [OE] and *ROSA-miR-17-92^{conditional}* [Control OE]). Data are shown as individual values and mean ± SEM. *n*=4. **p*<0.05 and ****p*<0.001 (two-sided Student's *t* test). **(c)** Suggested model for the regulation of the mitotic checkpoint and insulin secretion downstream of miR-17-92/miR-106b-25. PRKAR1α, the most abundant regulatory subunit of the PKA holoenzyme complex, keeps the complex in an inactive form. Upon release, the PKA catalytic subunit can phosphorylate its downstream targets, thus contributing to

microtubule stabilisation while preventing accumulation of cells in the G2 and M stages of the cell cycle. Moreover, different phosphorylated downstream targets promote insulin exocytosis. **(d)** miR-17-92/miR-106b-25 expression is downregulated when beta cells mature, correlating with a reduction in beta cell proliferation. Insulin exocytosis is inhibited by miR-17-92 in immature beta cells [20], whereas we demonstrated that miR-17-92 and miR-106b-25 induce GSIS in mature beta cells. Changes in miRNA levels, or changes in the functions of specific mRNA targets that are differentially expressed in immature vs mature beta cells, might contribute to explaining this discrepancy. EPAC2, exchange protein directly activated by cAMP2; SNAP25, synaptosome associated protein 25; Snapin, SNAP associated protein

550 model reduces the overall miRNA levels from these clusters,
 551 relative to the reduction achieved by KO of just the *miR-17-92*
 552 cluster. Additional GSIS study in a perfusion apparatus re-
 553 vealed that miR-17-92/miR-106b-25 are involved in insulin
 554 secretion at a stage preceding membrane depolarisation.
 555 Accordingly, non-nutrient secretagogue KCl normalised insu-
 556 lin exocytosis, suggesting that voltage-dependent calcium
 557 channels and successive events, such as granule docking and
 558 fusion, are insensitive to miR-17-92/miR-106b-25. Likewise,
 559 a study with cytochalasin B revealed that insulin synthesis,
 560 granule assembly, docking and fusion are unaffected by
 561 miR-17-92/miR-106b-25. Taken together, these analyses de-
 562 lineate miR-17-92/miR-106b-25 activity at steps earlier than
 563 membrane depolarisation.

564 Several studies proposed that miR-17-92/miR-106b-25 up-
 565 regulation is important for S phase entry in transformed cell
 566 lines and tissues [16, 50], whereas our work uncovers a new

567 role for miR-17-92/miR-106b-25 in regulation of the M
 568 checkpoint. The involvement in the M checkpoint might have
 569 been overlooked until now or alternatively could reflect dif-
 570 ferent miR-17-92/miR-106b-25 functions in genetically stable
 571 tissues. Interestingly, miR-17-92 expression is highest during
 572 G2/M and lowest in S phase even in transformed cells [16].

573 The final consequences of miR-17-92/miR-106b-25 defi-
 574 ciency in adult replicating beta cells are unclear, since we
 575 observed neither increased apoptosis nor accumulation of
 576 polyploid beta cells in young or old *miR-17-92/miR-106b-*
 577 *25-KO* mouse islets. Thus, the most likely explanation is that
 578 dividing adult *miR-17-92/miR-106b-25-KO* mouse beta cells
 579 undergo non-apoptotic cell death and rapid tissue clearance at
 580 a rate higher than our assay sensitivity.

581 *c-Myc* (also known as *Myc*) overexpression in beta cells
 582 induces cell cycle and reduces insulin expression and apopto-
 583 sis [22]. miR-17-92/miR-106b-25 might act as *c-Myc*

584 effectors in mediating at least some of its functions, as occurs
 585 in some cancers. In addition, *c-Myc* resides in a positive feed-
 586 back loop with PKA [51, 52]. Therefore, miR-17-92/miR-
 587 106b-25 family, *c-Myc* and PKA activities may be
 588 interwoven.

589 High cAMP levels induce insulin secretion via PKA- and
 590 *Epac2* (also known as *Rapgef4*)-dependent recruitment of insulin
 591 granules and/or granule fusion to the plasma membrane [53, 54].
 592 PRKAR1 α inhibits insulin secretion, whereas cAMP
 593 antagonises PRKAR1 α and releases PKA from PRKAR1 α sub-
 594 unit. Thus, cAMP facilitates PKA-dependent induction of
 595 *Snapin-Snap25-Epac2* pathway activity, resulting in increased
 596 insulin exocytosis [55, 56]. In silencing *Prkar1a*, miR-17-92/
 597 miR-106b-25 induce PKA activity and insulin exocytosis.
 598 Furthermore, PKA regulates microtubule stability and potentially
 599 the M checkpoint by phosphorylating *Mark2* on S409 [57–59].

600 Therefore, PKA is a convergence point that contributes both
 601 to insulin exocytosis and to mitotic checkpoint, connecting two
 602 seemingly disparate properties, namely beta cell division and
 603 insulin secretion, downstream of miR-17-92/miR-106b-25
 604 family (Fig. 7c). However, our current study does not provide
 605 direct experimental evidence that connects microtubule stability
 606 to PKA activity in *miR-17-92/miR-106b-25-KO* mice.

607 More broadly, miR-17-92/miR-106b-25 activity may be a
 608 new regulatory element, contributing to activity of incretin-
 609 stimulated pathways via PKA and to some of the therapeutic
 610 actions of glucagon-like peptide-1 (GLP-1) on insulin exocy-
 611 tosis and beta cell proliferation.

612 We suggest that in adult mice miR-17-92/miR-106b-25 ex-
 613 pression is upregulated significantly but transiently in dividing
 614 beta cells, contributing to beta cell proliferation via a mecha-
 615 nism similar to that reported in early postnatal maturation [20].
 616 Mature beta cells express only low miR-17-92/miR-106b-25
 617 levels, which primarily affect insulin secretion (Fig. 7d).

618 Overall, our study deciphers the involvement of miR-17-
 619 92/miR-106b-25 family in adult beta cell replication and in-
 620 sulin secretion, suggesting an important role for proto-
 621 oncogene miRNAs in regulating glucose homeostasis in the
 622 normal, untransformed endocrine pancreas. In mice, miR-17-
 623 92/miR-106b-25 appear to regulate many facets of the adult
 624 beta cell life, connecting mitosis and insulin secretion by a
 625 single post-transcriptional pathway, encouraging similar stud-
 626 ies in human beta cells.

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Data availability Sequencing data that support the findings of this study
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