

1 PAX4 loss of function alters human endocrine cell 2 development and influences diabetes risk

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45 **Abstract (220 words)**

46 Diabetes is a major chronic disease with an excessive healthcare burden on society¹. A
47 coding variant (p.Arg192His) in the transcription factor *PAX4* is uniquely and reproducibly
48 associated with an altered risk for type 2 diabetes (T2D) in East Asian populations²⁻⁷,
49 whilst rare *PAX4* alleles have been proposed to cause monogenic diabetes⁸. In mice,
50 *Pax4* is essential for beta cell formation but neither the role of diabetes-associated
51 variants in *PAX4* nor *PAX4* itself on human beta cell development and/or function are
52 known. Here, we demonstrate that non-diabetic carriers of either the *PAX4* p.Arg192His
53 or a newly identified p.Tyr186X allele exhibit decreased pancreatic beta cell function. In
54 the human beta cell model, EndoC-βH1, *PAX4* knockdown led to impaired insulin
55 secretion, reduced total insulin content, and altered hormone gene expression. Deletion
56 of *PAX4* in isogenic human induced pluripotent stem cell (hiPSC)-derived beta-like cells
57 resulted in derepression of alpha cell gene expression whilst *in vitro* differentiation of
58 hiPSCs from carriers of *PAX4* p.His192 and p.X186 alleles exhibited increased
59 polyhormonal endocrine cell formation and reduced insulin content. *In silico* and *in vitro*
60 studies showed that these *PAX4* alleles cause either reduced *PAX4* expression or
61 function. Correction of the diabetes-associated *PAX4* alleles reversed these phenotypic
62 changes. Together, we demonstrate the role of *PAX4* in human endocrine cell
63 development, beta cell function, and its contribution to T2D-risk.

64 **Keywords:** *PAX4*, human, endocrine cell, development, diabetes, human genetics,
65 induced pluripotent stem cell, beta cell, insulin

66 **Abbreviations**

67

68 2-DG: 2-deoxyglucose

69 AIRg: acute insulin response to glucose

70 AUC: area under the curve

71 BLC: beta-like cells

72 CABG: coronary artery bypass grafting

73 CHX: cycloheximide

74 CRF: chronic renal failure

75 DE: definitive endoderm

76 DI: disposition index

77 DM: diabetes mellitus

78 DR: diabetic retinopathy

79 ECAR: extracellular acidification rate

80 EP: endocrine progenitor

81 GDM: gestational diabetes mellitus

82 GO: gene ontology

83 GSIS: glucose-stimulated insulin secretion

84 HbA1c: hemoglobin A1c

85 HDR: homology directed repair

86 hiPSC: human induced pluripotent stem cells

87 HOMA-IR: homeostatic model assessment of insulin resistance

88 IGT: impaired glucose tolerance

89 IHD: ischemic heart disease

90 NAC: N-acetylcysteine

91 NMD: nonsense mediated decay

92 OCR: oxygen consumption rate

93 OGTT: oral glucose tolerance test

94 PBMC: peripheral blood mononuclear cell

95 PCA: principal component analysis

96 PE: pancreatic endoderm

97 PGT: primitive gut tube

98 PF: posterior foregut

99 PPM: permanent pace-maker implantation

100 PP1: pancreatic progenitor 1

101 PP2: pancreatic progenitor 2

102 PTV: protein truncating variant

103 qPCR: quantitative real-time PCR

104 RFU: relative fluorescence units

105 sgRNAs: single guide RNAs

106 Si: insulin sensitivity

107 SKAT: Sequence Kernel Association Test

108 SNP: single nucleotide polymorphism

109 T2D: type 2 diabetes

110 TPM: transcripts per million

111 UMAP: Uniform Manifold Approximation and Projection

112 WT: wildtype

113 **Introduction**

114 Diabetes is a chronic condition affecting more than 537 million people worldwide, giving
115 rise to devastating complications and healthcare burdens on society¹. In an effort to
116 identify novel disease-causing mechanisms and tractable targets for therapeutic
117 development, numerous genome-wide studies have been performed across different
118 ancestries to identify genetic variants that influence diabetes risk²⁻⁴. An Asian-enriched
119 *PAX4* p.Arg192His (rs2233580) coding variant has been reproducibly associated with
120 T2D risk [odds ratio of ~1.75]^{2,5}. Additional studies revealed that 21.4% of 2,886 people
121 with early-onset T2D carried at least one *PAX4* p.Arg192His allele⁶. Carriers of the T
122 allele (p.His192) have a dose-dependent earlier age of T2D-onset⁶ and have lower C-
123 peptide levels⁷, consistent with pancreatic beta cell dysfunction⁹. Earlier studies have
124 reported other rare coding allele(s) in *PAX4* as a cause of monogenic diabetes⁸. However,
125 the high frequency of the variants in the population and a lack of cosegregation with
126 diabetes has led to discussion over whether they are causal for diabetes and if *PAX4*
127 should be included in diagnostic testing panels for monogenic diabetes¹⁰.

128 *PAX4* is a paired-homeodomain transcription factor that has been shown to act as a
129 transcriptional repressor of insulin and glucagon promoters^{11,12}. In mice, *Pax4* is broadly
130 expressed throughout the developing pancreas^{12,13}. *Pax4* homozygous knockout mice
131 die three days postpartum from hyperglycemia, caused by a near complete absence of
132 insulin-producing beta cells¹³. Loss of *Pax4* in mice also leads to an increase in the
133 number of alpha cells and an upregulation of the alpha cell gene *Arx*^{13,14}. Conversely, *Arx*

134 *l⁻* mice upregulate *Pax4* and have more beta cells, leading to the model of mutual
135 repression of *Pax4* and *Arx* to direct the development of alpha and beta cell lineages,
136 respectively¹⁴. Similar mutual repression of *pax4* and *arx* has been detected in
137 zebrafish¹⁵; however, unlike in mice, *Pax4* is not required for beta cell development¹⁵. In
138 humans, it is currently unknown whether *PAX4* is required for endocrine cell formation.
139 As *PAX4* variants have been reported as a potential cause of monogenic diabetes^{8,16,17}
140 and are associated with altered T2D risk, investigating their role in human endocrine cell
141 formation may improve our understanding of the mechanism(s) underlying the genetic
142 association and clarify the potential role of *PAX4* variants as a cause of monogenic
143 diabetes.

144 Here we present detailed human *in vivo* and *in vitro* studies on two different *PAX4* coding
145 alleles, the East Asian population enriched p.Arg192His and a novel protein-truncating
146 variant (PTV) p.Tyr186X identified in a Singapore family with early onset diabetes. We
147 generated three independent human induced pluripotent stem cell (hiPSC) models: i)
148 *PAX4*-knockout and *PAX4* variant isogenic SB Ad3.1 cell lines using CRISPR-Cas9
149 genome editing; ii) donor-derived cells with p.Arg192Arg, p.Arg192His, p.His192His and
150 p.Tyr186X genotypes; and iii) genotype-corrected donor-derived cells, and differentiated
151 all lines into pancreatic beta-like cells (BLCs) using two different protocols. Consistently,
152 we found that *PAX4* deficiency and/or loss-of-function to result in derepression of alpha
153 cell genes, leading to the formation of polyhormonal endocrine cells *in vitro*, with reduced
154 total insulin content. This phenotype was confirmed independently in the human beta cell
155 line EndoC-βH1, and could be reversed in donor-derived hiPSC lines through correction

156 of diabetes-associated *PAX4* alleles. We conclude that, whilst *PAX4* is not essential for
157 *in vitro* stem cell differentiation to BLCs, both *PAX4* haploinsufficiency and loss-of-
158 function coding alleles increase the risk of developing diabetes by negatively impacting
159 human beta cell development and insulin secretion. Our observations are consistent with
160 rare *PAX4* alleles resulting in haploinsufficiency being insufficient to cause fully penetrant
161 monogenic diabetes but increasing the risk for T2D.

162 **Results**

163 **Carriers of the *PAX4* p.Arg192His T2D-risk allele exhibit decreased beta cell**
164 **function**

165 We recruited a total of 183 non-diabetic individuals and assessed their pancreatic beta
166 cell function by a frequently sampled intravenous glucose tolerance test. Carriers of the
167 T2D-risk allele (p.His192) had a decreased acute insulin response to glucose (AIRg,
168 $p=0.002$), which remained significant after adjusting for age, sex and BMI ($p_{adj}=0.04$)
169 (Fig. 1a). There were no differences in insulin sensitivity (Si , $p=0.105$) or disposition index
170 (DI, $p=0.203$) between the two groups (Fig. 1a). HOMA-B, a measurement of beta cell
171 function, was significantly reduced in p.His192 allele(s) carriers ($p=0.005$) but was no
172 longer significant after adjusting for age, gender and BMI ($p_{adj}=0.075$). A subset of the
173 recruited individuals [$n=57$] then underwent an oral glucose tolerance test (OGTT) which
174 revealed higher fasting and 2-hour glucose levels, as well as a lower ratio of area under
175 the curve (AUC) for insulin:glucose (Fig. 1b-d). HOMA-B was significantly poorer during
176 the OGTT in the p.His192 risk allele(s) carriers (132.5 ± 56.2) compared to controls
177 (190.6 ± 98.5) whether unadjusted ($p=0.008$) or adjusted for age, gender and BMI
178 ($p_{adj}=0.007$). There were no differences in fasting, 2-hour, or AUC glucagon (Extended
179 Data Fig. 1a-c). However, there was a significant decrease in the difference between
180 fasting and 2-hour glucagon (delta glucagon), suggesting carriers of p.His192 have less
181 glucagon suppression (Extended Data Fig. 1d). Consistent with the insulin sensitivity
182 measurement (Fig. 1a), there was no difference in HOMA-IR (Extended Data Fig. 1e). As

183 loss of *Pax4* impacts enteroendocrine cell formation in mice¹⁸, we also measured GLP-1
184 in p.His192 carriers and found no significant differences in GLP-1 level (Extended Data
185 Fig. 1f-h). Together, the clinical data are consistent with increased T2D-risk via defects in
186 pancreatic beta cell mass and/or function.

187 **Identification of a novel *PAX4* protein truncating variant p.Tyr186X in a family with
188 early onset diabetes**

189 A female proband (III-1) of Singapore Chinese ethnicity was diagnosed with early-onset
190 diabetes at the age of 10 years (random glucose 17 mmol/L) (Fig. 1e), verified to be GAD
191 antibody negative, and had detectable C-peptide (1.2 nmol/L). Upon diagnosis, she was
192 treated with a basal bolus insulin regimen and metformin for two weeks before being
193 switched to metformin-alone treatment. Following lifestyle modifications, she lost weight
194 (from 53.1 kg, BMI 25.3 kg/m² to 49.5 kg, BMI 23.6 kg/m²) and nine months post-diagnosis
195 her HbA1c was 7.1% (8.7 mmol/L). The early diabetes-onset, lack of evidence for type 1
196 diabetes, and persistence of diabetes despite weight loss prompted further assessment
197 in the family (Fig. 1e). II-11 was diagnosed with gestational diabetes (GDM) at the age of
198 29 years when she was pregnant with the proband. At age 40 years, while being
199 asymptomatic for diabetes, an OGTT confirmed a diagnosis of diabetes with a fasting
200 glucose of 5.6 mmol/L and a 2-hour glucose of 11.4 mmol/L.

201 Genetic testing for monogenic diabetes with a custom Illumina Nextera rapid capture
202 next-generation sequencing panel on an Illumina MiSeq sequencing platform (*HNF4A*,
203 *GCK*, *HNF1A*, *PDX1*, *HNF1B*, *NEUROD1*, *KLF11*, *CEL*, *PAX4*, *INS*, *ABCC8*, *KCNJ11*)

204 was performed on members of the family who were recruited for the clinical study. A novel
205 (not reported in gnomAD or ClinVar, date accessed Feb 2022), heterozygous *PAX4*
206 mutation (c.555_557dup) predicted to result in a truncated protein (p.Tyr186X) was
207 identified in the proband (III-1), the mother (II-11) and a female member of the family (II-
208 7) (Fig. 1e). No rare coding variants were detected in the other genes tested.

209 The other female heterozygous p.Tyr186X variant carrier (II-7; BMI 23.8 kg/m²) had a
210 history of GDM (age 26 years) and at the time of study (age 51 years) had impaired
211 glucose tolerance (IGT). Family members (II-4, II-5, II-8 and II-9) with diabetes and
212 another non-diabetic female family member (II-3, BMI 27.0 kg/m²) did not carry the
213 variant. Unfortunately, the proband's grandparents, both diagnosed with diabetes at the
214 age of 40 years, declined to take part in the study.

215 Given the high prevalence of diabetes in the family and both maternal grandparents
216 having diabetes, we evaluated measures of insulin resistance (HOMA-IR) and beta cell
217 function (DI) in family members with and without the *PAX4* p.Tyr186X variant. Family
218 member II-3, who does not have diabetes and does not carry the p.Tyr186X variant, has
219 the highest beta cell function as measured by the DI whilst those carrying the *PAX4*
220 variant (II-11 and II-7) have markedly reduced function (Fig. 1f). Of note, the family
221 members with diabetes who do not carry the *PAX4* variant all displayed evidence of
222 insulin resistance (HOMA-IR >2) and low DI, consistent with T2D (Fig. 1f). Taken
223 together, these findings are insufficient to provide support for the p.Tyr186X variant as

224 the cause of monogenic diabetes in this family but are consistent with the *PAX4* variant
225 being associated with decreased pancreatic beta cell function.

226 To further explore the role of rare coding variants in the *PAX4* gene on T2D risk, we
227 accessed aggregated gene-level exome-sequencing association data from 52K
228 individuals deposited in the Common Metabolic Disease Portal (<https://t2d.hugeamp.org>)
229 and in 281,852 individuals from UKBioBank (<https://www.ukbiobank.ac.uk/>). Burden and
230 Sequence Kernel Association Test (SKAT) analyses computed using a series of genotype
231 filters and masks provided nominal evidence for a gene level association that is
232 independent of the p.Arg192His variant (Supplementary Table 1a-b).

233 **Loss of *PAX4* alters hormone gene regulation, reduces insulin secretion function
234 and total insulin content in a human beta cell model**

235 To evaluate the consequence of *PAX4* loss on beta cell function, we first performed
236 siRNA- and shRNA-mediated knockdown of *PAX4* in human EndoC-βH1 cells (Fig. 2a).
237 Transient knockdown of *PAX4* using siRNAs significantly reduced *PAX4* transcript
238 expression (Fig. 2b) and glucose-stimulated insulin secretion (GSIS) in EndoC-βH1 cells
239 (Fig. 2c). To model a chronic loss of *PAX4* expression, we generated stable knockdown
240 *PAX4* EndoC-βH1 cells via lentiviral transduction of shRNA followed by antibiotic
241 selection (Fig. 2a). sh*PAX4* EndoC-βH1 cells had reduced *PAX4* transcript level
242 compared to shScramble control cells (Fig. 2d). Long-term knockdown of *PAX4*
243 completely abolished GSIS compared to shScramble control cells (Fig. 2e), accompanied
244 by reduced total insulin content in sh*PAX4* EndoC-βH1 cells (Fig. 2f). While there was no

245 difference in *INS* transcript (Fig. 2g), loss of *PAX4* increased *GCG* transcript by 6.2-fold
246 in sh*PAX4* EndoC-βH1 cells (Fig. 2h), consistent with *PAX4* being a repressor of *GCG*
247 expression^{12,19}.

248 ***PAX4* knockout in hiPSC-derived BLCs causes derepression of alpha cell gene
249 expression**

250 While *PAX4* transcript and protein can be detected in rat²⁰ and human islets²¹, its
251 expression is most abundant during embryonic development^{12,13}, suggesting that *PAX4*
252 variants may mediate disease risk early on during embryonic development. Homozygous
253 *Pax4* knockout mice die within three days of birth and have a near complete loss of
254 pancreatic beta cells¹³. Whether *PAX4* is similarly required for the formation of human
255 beta cells is unknown. We generated *PAX4* homozygous null isogenic hiPSC lines
256 (*PAX4*^{+/+}; *PAX4*^{-/-}) using CRISPR-Cas9 (Fig. 3a) and two single guide RNAs (sgRNAs)
257 designed for exons 2 and 5 (encoding the paired-domain and homeodomain) of the *PAX4*
258 gene (Extended Data Fig. 2a). Two independent cell lines had a homozygous deletion for
259 amino acids 64 through 200, whilst the other cell line was compound heterozygous for
260 two premature stop codons at amino acids 61 and 74, respectively (data not shown).
261 Three independent, unedited hiPSC lines generated during the CRISPR-Cas9 process
262 provided control *PAX4*^{+/+} lines (Fig. 3a). All six hiPSC lines were differentiated towards
263 BLCs using a seven-stage protocol²² (Fig. 3b; Protocol A). Flow cytometry analysis of
264 CXCR4+ and SOX17+ definitive endoderm (DE) cells determined that there was no defect
265 in the formation of DE (Extended Data Fig. 2b-c). As *PAX4* is a transcription factor, RNA-

266 seq analysis was used to determine the transcriptional consequence of *PAX4* knockout.
267 RNA-seq samples (n=8 per genotype) were collected at DE (before *PAX4* expression),
268 pancreatic endoderm (PE), endocrine progenitor (EP) (at the peak of *PAX4* expression)
269 and BLC (Fig. 3c and Supplementary Table 2). The *PAX4* transcript was significantly
270 reduced in *PAX4*^{-/-} PE (padj=4.25E-05), EP (padj=6.15E-05) and BLC (padj=1.27E-06)
271 (Fig. 3c), and the remaining transcripts were missing exons 2 through 5 (Extended Data
272 Fig. 2d).

273 Differential expression analysis using DESeq2 showed that the loss of *PAX4* resulted in
274 a de-repression of an alpha cell gene signature (*ARX*, *GCG*, *TTR*) and a repression of
275 the endocrine progenitor marker *FEV*^{23,24} in BLCs (Fig. 3d and Extended Data Fig. 2e-g).
276 To confirm our results, we differentiated the same *PAX4*^{+/+} and *PAX4*^{-/-} hiPSC lines into
277 BLCs using a second protocol (Fig. 3b; Protocol B)²⁵. Using Protocol B, we found a
278 significant reduction in *PAX4* transcript at the EP stage (Fig. 3e) and a larger number of
279 differentially expressed genes (Fig. 3f and Supplementary Table 3). Gene ontology (GO)
280 biological process analysis of the differentially expressed genes in *PAX4*^{-/-} BLCs revealed
281 a number of GO terms that included the alpha cell gene *ARX*.

282 Using a curated list of genes involved in beta and alpha cell lineages²⁶, we observed
283 directionally consistent, albeit nonsignificant, derepression of alpha cell genes (*ARX*,
284 *GCG*, *TTR*) (Fig. 3h). The expression of delta cell gene (*SST*), epsilon cell gene (*GHRL*)
285 and PP cell gene (*PPY*) was also derepressed in BLCs derived from *PAX4*^{-/-} lines (Fig.
286 3h). Some genes that are involved in beta cell maturation and hormone secretion (*MAFA*,

287 *ISL1, GRHL3, SLC17A6, PCSK2, EYA2*) were downregulated with the loss of *PAX4* (Fig.
288 3h). Importantly, *PAX4*^{+/+} and *PAX4*^{-/-} lines differentiating into BLCs repress pluripotency
289 genes and activate genes involved in endocrine cell fate in a similar manner (Extended
290 Data Fig. 3), suggesting that, unlike in mouse, *PAX4* is not required for human beta cell
291 differentiation *in vitro*. Rather, *PAX4* loss-of-function results in derepression of alpha cell
292 genes and a dysregulation of key endocrine maturation genes in hiPSC-derived BLCs.

293 **Donor-derived hiPSCs from carriers of the *PAX4* p.Arg192His and p.Tyr186X alleles
294 have defects in endocrine cell differentiation *in vitro***

295 Having established the effect of *PAX4* loss during *in vitro* beta cell differentiation, we
296 next generated donor-derived hiPSCs from *PAX4* variant carriers and differentiated
297 them into BLCs. Skin biopsies and/or blood samples from recruited non-diabetic donors
298 were used to derive hiPSCs of the following genotypes: homozygous for the *PAX4*
299 p.Arg192 and p.Tyr186 alleles (wildtype), heterozygous for either the p.Arg192His or
300 p.Tyr186X alleles, and homozygous for the p.His192 allele (p.His192His) (Fig. 4a). To
301 account for possible line-to-line heterogeneity in hiPSC-based studies²⁷, three
302 independent hiPSC lines were generated from two donors for wildtype cells, four lines
303 from two donors for p.Arg192His, five lines from two donors for p.His192His, and three
304 lines from one donor (II-7; Fig. 1e) for the p.Tyr186X variant (Fig. 4a). All hiPSC lines
305 were characterized via pluripotency immunostaining, teratoma assay, and karyotyping
306 and genotypes were confirmed by Sanger sequencing (data not shown).

307 We simultaneously differentiated all 15 hiPSC lines into pancreatic BLCs using Protocol

308 B and performed qPCR, flow cytometry and immunostaining analyses. *PAX4* transcript
309 expression was unchanged in carriers of the *PAX4* p.His192 allele but elevated in EPs
310 derived from the p.Tyr186X carrier (Fig. 4b), consistent with transcriptional compensation
311 for the PTV. Heterozygous and homozygous carriers of the *PAX4* p.His192 allele had no
312 measurable differences in *INS*, *GCG*, or *SST* gene expression at the EP or BLC stages
313 (Fig. 4b-i). Similar to the *PAX4*^{-/-} knockout hiPSCs (Fig. 2h), the *PAX4* p.Tyr186X hiPSC
314 lines exhibited a derepression of the *GCG* gene in both EPs and BLCs (Fig. 4d and h),
315 suggesting that the PTV is loss-of-function. Immunostaining of endocrine hormones found
316 no significant differences in GCG+ or INS+ cells (Fig. 4j-l), but there was a significant
317 increase in the number of polyhormonal (C-PEP+/GCG+) BLCs from *PAX4* variant hiPSC
318 lines (Fig. 4j and m). BLCs from the *PAX4* p.Arg192His and p.Tyr186X hiPSCs had
319 lowered total insulin content (Fig. 4n), which is consistent with the *in vivo* clinical data
320 from *PAX4* variant carriers (Fig. 1a and f). Together, these data suggest that both *PAX4*
321 alleles result in a loss-of-function due to reduced *PAX4* gene dosage and/or altered *PAX4*
322 transcriptional activity, negatively affecting endocrine cell differentiation.

323 ***PAX4* p.Arg192His and p.Tyr186X alleles reduce the expression and/or function of
324 *PAX4* protein**

325 The *PAX4* protein consists of two functional domains, paired and homeodomain, that are
326 responsible for DNA binding, and two nuclear localization sequences (NLS) (Fig. 5a)^{28,29}.
327 Both p.Arg192His and p.Tyr186X variants are located within the functional homeodomain
328 of the *PAX4* protein (Fig. 5a). As the crystal structure of *PAX4* protein has not been

329 elucidated, we obtained the predicted three-dimensional molecular arrangement of PAX4
330 protein (AF-O43316-F1-model_v2) from the AlphaFold database^{30,31}. The p.Arg192
331 residue is located within a hydrophobic pocket (Extended Data Fig. 4a), suggesting that
332 substitution to an uncharged histidine may alter the DNA-binding function of the PAX4
333 protein. The *PAX4* p.Tyr186X (c.557-559 GTA duplication) variant causes a frameshift,
334 leading to the introduction of a premature stop codon at amino acid position 186 (Fig. 5a
335 and Extended Data Fig. 4a). Transcripts containing PTVs, such as *PAX4* p.Tyr186X, may
336 undergo nonsense mediated decay (NMD), resulting in haploinsufficiency³². To test
337 whether the *PAX4* variants undergo NMD, we performed allelic-specific qPCR following
338 treatment with the NMD inhibitor cycloheximide (CHX)^{33,34}. Treatment of hiPSC-derived
339 BLCs with CHX overnight stabilized the p.X186 allele (Fig. 5b) but had no effect on
340 p.His192 (Fig. 5c), confirming NMD of p.X186 and supporting *PAX4* haploinsufficiency as
341 the mechanism for the p.Tyr186X variant.

342 To understand the consequence of *PAX4* variants on protein function, we performed a
343 series of *in vitro* assays using overexpression of tagged WT and mutant (p.His192 and
344 p.X186) PAX4 protein (Extended Data Fig 4b). Western blot analyses demonstrated
345 successful PAX4 overexpression detected by PAX4 antibody³⁵ and V5 tag expression
346 (Extended Data Fig. 4c). Overexpression of the *PAX4* variants in AD293 cells confirmed
347 that the p.His192 allele does not prevent nuclear localization and that any p.X186 protein
348 that escaped NMD remained trapped in the cytoplasm due to the loss of downstream NLS
349 (Extended Data Fig. 4d). We observed fewer PAX4 antibody-positive cells in p.X186
350 transfected AD293 cells, despite no difference in overall transfection efficiency (% GFP+),

351 consistent with decreased stability of any truncated protein produced by the PTV
352 (Extended Data Fig. 4e). Treating AD293 cells overexpressing the *PAX4* constructs with
353 the proteasomal inhibitor MG132 revealed an accumulation of the p.X186 protein
354 compared to wildtype or p.His192, demonstrating that the overexpressed truncated
355 protein is subject to proteasomal degradation (Extended Data Fig. 4f-g).

356 It was previously reported that *PAX4* p.His192 results in defective transcriptional
357 repression of human *INS* and *GCG* gene promoters¹². In EndoC-βH1 cells,
358 overexpression of both WT and p.His192 *PAX4* proteins resulted in a significant
359 repression of *INS* promoter activity (Fig. 5d). Although the p.X186 variant most likely
360 results in NMD and haploinsufficiency, any translated protein was unable to repress *INS*
361 promoter activity (Fig. 5d). WT *PAX4* protein did not repress the *GCG* gene promoter in
362 EndoC-βH1 cells (Fig. 5e) but did so in the rodent alpha cell model αTC1.9 (Fig. 5f),
363 consistent with cell-type specific regulation of gene expression by *PAX4*. Both *PAX4*
364 p.His192 and p.X186 resulted in a derepression of the *GCG* promoter in beta cells (Fig.
365 5e) and a loss of repression activity in alpha cells (Fig. 5f). Luciferase assays for *INS*
366 gene promoter activity were unchanged in EndoC-βH1 cells following *PAX4* (sh*PAX4*)
367 knockdown (Fig. 5g). However, sh*PAX4* EndoC-βH1 cells had significantly increased
368 *GCG* promoter activity (Fig. 5h), consistent with the loss-of-repression of *GCG* promoter
369 activity observed in the presence of p.His192 or p.X186. Taken together, our studies
370 demonstrate that *PAX4* p.Arg192His and p.Tyr186X variant proteins have altered
371 expression and/or transcriptional activity.

372 **hiPSC-derived EPs have a distinct metabolic gene signature and exhibit a**
373 **bioenergetics switch from glycolysis to oxidative phosphorylation**

374 To evaluate the overall impact of diabetes-associated *PAX4* gene variants on the global
375 transcriptome of human pancreatic cells, RNA-seq was performed on *PAX4* variant
376 carrier donor-derived hiPSCs across four differentiation time points using Protocol B:
377 hiPSCs, PP2 cells, EPs and BLCs (Supplementary Table 4). Uniform Manifold
378 Approximation and Projection (UMAP) analyses of a total of 153 RNA samples
379 demonstrated that samples were clustered based on differentiation day (i.e., largest
380 source of variation is developmental time point) (Fig. 6a), indicating that the differentiation
381 protocol is robust in directing the hiPSCs toward BLCs. Volcano plots comparing the
382 *PAX4* p.Arg192His, p.His192His or p.Tyr186X against wildtype *PAX4* donor-derived
383 hiPSCs demonstrated that most differentially expressed genes were upregulated at the
384 EP stage (Fig. 6b), coinciding with the peak of *PAX4* expression (Fig. 3c and e). Principal
385 Component Analysis (PCA) revealed that EPs derived from *PAX4* variants clustered more
386 closely to each other than to wildtype *PAX4* (Fig. 6c), suggesting that the two *PAX4*
387 variants shared transcriptional similarity. Gene enrichment analyses of relevant biological
388 processes of the differentially expressed genes in the *PAX4* p.His192His and p.Tyr186X
389 EPs revealed an association with metabolic processes and cellular response to stress
390 (Fig. 6d). Between the *PAX4* p.His192His and p.Tyr186X genotypes, there were 2012
391 and 452 genes in common within the “metabolic processes” and “cellular response to
392 stress”, respectively, most of which were elevated in expression in the *PAX4* variant lines
393 (Extended Data Fig. 5a-b).

394 To further assess a potential defect in metabolism, we performed a Seahorse XFe96
395 Glycolysis Stress Test. The glycolysis stress test showed that EPs carrying one or two
396 p.His192 risk alleles had lower glycolytic function, including glycolytic capacity and
397 glycolytic reserve, and a modest downregulation of glycolysis (Fig. 6e-f). In addition, EPs
398 carrying the p.Tyr186X risk allele had decreased glycolytic reserve and non-glycolytic
399 acidification (Fig. 6f). Next, we hypothesized that EPs would seek alternative metabolic
400 processes to compensate for the reduction in energy production, such as oxidative
401 phosphorylation through mitochondrial respiration. Mitochondrial function was measured
402 via oxygen consumption in EPs using the Seahorse XFe96 analyzer. There was an
403 increase in oxidative phosphorylation activity in EPs harboring *PAX4* variants (Fig. 6g),
404 including basal respiration, non-mitochondrial O₂ consumption, ATP production, and H⁺
405 (proton) leak (Fig. 6h). Overall, EPs carrying *PAX4* diabetes risk alleles demonstrated a
406 bioenergetic switch from glycolysis to oxidative phosphorylation.

407 To investigate if the altered metabolic gene expression and bioenergetics profile
408 contributed to beta cell maturation from the EP stage, we treated differentiating cells with
409 the antioxidant N-acetylcysteine (NAC)³⁶ from EP (when *PAX4* expression peaks) to BLC
410 stage and extracted total insulin for assessment. *PAX4* p.His192His and p.Tyr186X
411 carrying BLCs revealed only a modest upregulation in total insulin content (Extended Data
412 Fig. 6a-b), suggesting that the alleviation of oxidative stress is insufficient to rescue the
413 total insulin content in BLCs. We postulate that the metabolic signature observed in our
414 donor-derived hiPSC model reflects the physiological status of the EPs rather than being
415 the immediate cause for the dysregulation of beta cell development and maturation.

416 **Correction of *PAX4* risk alleles in donor-derived hiPSCs with CRISPR-Cas9 rescues**
417 **dysregulated endocrine gene expression and metabolic phenotypes**

418 Next, we used CRISPR-Cas9 to correct the donor-derived hiPSC lines and to generate
419 *PAX4* variant isogenic hiPSC lines. We designed sgRNA#3 to target the donor-derived
420 homozygous p.His192His line and provided the homology-directed repair (HDR) template
421 to correct the rs2233580 T2D-risk allele (Fig. 7a). The II-11 donor-derived hiPSC line that
422 is heterozygous for a GTA duplication was corrected with sgRNA#4 and an HDR template
423 (Fig. 7b). From the CRISPR-Cas9 genome editing pipeline, we generated two corrected
424 p.Arg192Arg non-risk and two uncorrected p.His192His hiPSC lines (Fig. 7c). From the
425 II-11 donor-derived line, four corrected p.Tyr186Tyr and four uncorrected p.Tyr186X
426 hiPSC lines were derived (Fig. 7c). All the corrected and uncorrected lines were
427 differentiated towards BLCs using Protocol B, followed by RNA-seq analyses and
428 assessment of total insulin content (Fig. 7c).

429 Consistent with the BLCs derived from *PAX4*^{-/-} hiPSCs (Fig. 3), homozygous p.His192His
430 donor-derived BLCs had higher expression of non-beta cell genes *GCG*, *TTR*, *SST*, and
431 *PPY* (Fig. 7d and Supplementary Table 5). Correction of *PAX4* p.His192 variant to
432 p.Arg192 rescued the expression of several beta cell genes, such as *RFX6*³⁷, *ABCC8*³⁸
433 and *SLC30A8*³⁹ (Fig. 7d), which are involved in insulin content and secretion. Isogenic
434 hiPSC-derived BLCs homozygous for the p.His192 allele had directionally consistent
435 changes in gene expression (Fig. 7d), confirming that p.His192 is the cause of perturbed
436 beta cell gene expression. The differences between BLCs derived from corrected or

437 uncorrected *PAX4* p.Tyr186X hiPSCs and from isogenic BLCs homozygous for p.X186
438 or p.Tyr186X allele were smaller but directionally consistent (Fig. 7e and Supplementary
439 Table 6), supporting p.X186 (causing *PAX4* haploinsufficiency) resulted in dysregulated
440 gene expression. The glycolysis stress test revealed a rescue in glycolytic reserve only
441 in cells corrected for the p.His192 allele (Extended Data Fig. 7a-b) but not for cells
442 corrected for the p.Tyr186X allele (Extended Data Fig. 7c-d). Importantly, correcting the
443 *PAX4* p.His192His and p.Tyr186X mutations significantly increased and restored the total
444 insulin content of the BLCs (Fig. 7f-g), indicating that the *PAX4* variants were a direct
445 cause of reduced insulin content in the donor-derived BLCs.

446 Discussion

447 Rodent models have demonstrated that *Pax4* plays an important role in beta cell
448 specification during early pancreas development^{13,40}. However, differences between
449 rodent and human islets in architecture⁴¹ and gene expression⁴² make it challenging to
450 extrapolate data based on rodent studies directly to humans. For instance, heterozygous
451 *Pax4* knockout mice do not develop diabetes¹³ but *PAX4* variants causing altered
452 transcriptional activity are strongly associated with increased diabetes risk in
453 humans^{8,16,43,44}. These observations suggest that human beta cells could be more
454 sensitive to changes in *PAX4* gene dosage.

455 While *PAX4* p.Arg192His has been identified as one of the most reproducible variants
456 uniquely associated with East Asian T2D, the role of *PAX4* or its variant p.Arg192His in
457 human beta cell development has not been addressed. Our study capitalized on access
458 to East Asian carriers of T2D *PAX4* risk alleles to study their effect on human beta cell
459 function *in vivo*, and generate donor-derived hiPSCs as a versatile platform to interrogate
460 the role of *PAX4* during human pancreas development *in vitro*⁴⁵⁻⁴⁸. Our clinical
461 phenotyping of the *PAX4* p.Arg192His allele carriers demonstrated decreased pancreatic
462 beta cell function based on AIRg, HOMA-B, and lowered DI despite the donors being
463 insulin sensitive based on HOMA-IR measures. Whilst our investigation of the impact of
464 a novel variant predicted to result in a loss of *PAX4* function (p.Tyr186X) demonstrated
465 that *PAX4* haploinsufficiency is insufficient to cause monogenic diabetes, it was
466 consistent with a negative impact on beta cell function. This finding is further supported

467 by large sequencing studies that collectively show an association of rare alleles in *PAX4*
468 with an increased risk for diabetes or elevated HbA1c levels.

469 While donor hiPSC-derived beta cells can be used to study human pancreas development
470 *in vitro*, this experimental model suffers from the following challenges: 1) hiPSC line-to-
471 line variability, 2) the heterogenous nature of differentiated islet-like cells and 3)
472 incomplete functional maturity of differentiated beta-like cells⁴⁹. To circumvent these
473 challenges while leveraging the benefits of this model, we rigorously applied two
474 differentiation protocols to multiple donor-derived and isogenic genome-edited hiPSC
475 lines. Our three independent sets of RNA-seq data using two protocols^{22,25} in multiple
476 hiPSC models (*PAX4*^{-/-} knockout, donor-derived and isogenic hiPSCs carrying *PAX4*
477 variants, and donor-derived gene-corrected hiPSCs)²⁷ demonstrated that all
478 differentiating cells shared a similar trajectory towards pancreatic islet-like cells. Knockout
479 of *PAX4* did not result in the ablation of human beta cells, but rather, resulted in
480 compromised beta cells with elevated expression of multiple endocrine hormone markers
481 and lowered expression of genes associated with beta cell functional maturation. These
482 observations were similarly replicated across donor-derived hiPSCs of three independent
483 genotypes (p.Arg192His, p.His192His and p.Tyr186X), whereby BLCs carrying *PAX4*
484 alleles demonstrated increased polyhormonal gene expression and reduced total insulin
485 content. Our molecular assessments confirmed p.X186 to undergo NMD, while the
486 p.His192 resulted in altered transcriptional activity. Contrary to rodent models, our human
487 *in vivo* and *in vitro* findings indicate that differentiating human beta cells are sensitive to
488 the functional (haploinsufficiency; loss-of-function) *PAX4* gene dosage required to

489 maintain beta cell identity, insulin production and secretion. Our data are consistent with
490 a recent study on *HNF1A* deficiency⁵⁰ and support a model where *PAX4* T2D-risk alleles
491 mediate disease risk by biasing endocrine precursor cells towards an alpha cell fate.

492 Transcriptomic assessment of EPs identified altered metabolic signatures in carriers of
493 p.Arg192His or p.Tyr186X allele(s). Indeed, metabolic stress can compromise beta cell
494 identity and has been proposed to be one of the mechanisms underlying beta cell
495 exhaustion in T2D^{51,52}. Unfortunately, the use of NAC to alleviate oxidative stress was
496 insufficient to rescue the total insulin content in *PAX4* variant-expressing BLCs. BLCs
497 derived from gene-corrected hiPSCs demonstrated a rescue in total insulin content,
498 affirming the direct contribution of p.Arg192His or p.Tyr186X to decreased insulin content.
499 While we were unable to determine the direct cause of the altered metabolic signature
500 observed in EPs or whether the variants were a direct cause of the metabolic signature,
501 it is tempting to hypothesize that the inferior beta cells resulting from the *PAX4* variants,
502 compounded with cellular metabolic stress, hasten the eventual progression toward T2D
503 development.

504 As our hiPSC-derived beta cells were not functional *in vitro*, we included the study of
505 *PAX4* in the human beta cell line EndoC-βH1. Knockdown of *PAX4* led to a derepression
506 of the *GCG* gene promoter and elevated *GCG* gene expression in beta cells. *PAX4*
507 expression requires cooperative activation by several key transcription factors specific to
508 pancreatic beta cells¹² to specify *PAX4* exclusively in beta cells. In rodents, the
509 maintenance of pancreatic beta cell identity requires a continual repression of non-beta

510 cell gene expression^{51,52}. The expression of multiple hormonal markers, including GCG,
511 is one of the hallmarks of immature cells that could have diminished function in endocrine
512 hormone secretion. The reduced *PAX4* levels resulting in the loss-of-repression of non-
513 beta cell gene expression could possibly explain the co-expression of GCG in C-PEP-
514 expressing BLCs carrying the p.His192 or p.X186 allele(s). We have observed that
515 carriers of p.Arg192His or p.Tyr186X alleles secrete less insulin during GSIS (Fig. 1a and
516 f), and this was recapitulated in our si*PAX4* and sh*PAX4* EndoC-βH1 cells (Fig. 2). The
517 insulin content within pancreatic islet cells has a strong correlation with the amount of
518 insulin secreted during GSIS⁵³. The reduced total insulin content observed in our hiPSC
519 and EndoC-βH1 models and subsequent impaired GSIS in EndoC-βH1 cells collectively
520 suggest a role for *PAX4* in maintaining beta cell identity and regulating insulin secretion
521 function.

522 A limitation of our study is the description of a single family with a *PAX4* PTV, limiting the
523 confidence with which conclusions can be drawn from our observations of an effect of
524 *PAX4* haploinsufficiency in humans. We sought to strengthen our findings through
525 aggregation of exome-sequencing data from multiple publicly available datasets, which
526 both provided nominal evidence for a role of rare coding variation in elevated diabetes
527 risk and support mounting evidence that *PAX4* is not a monogenic diabetes gene¹⁰.

528 The loss of beta cell identity and the acquisition of polyhormonal cells have been reported
529 in the pancreatic islets of individuals with diabetes^{54,55}. The transdifferentiation of
530 metabolically stressed beta cells to express GCG has been proposed by several groups

531 as a mechanism underlying beta cell failure in T2D⁵⁵⁻⁵⁷. In the current study, we
532 demonstrate how coding gene variants in *PAX4* can influence pancreatic beta cell
533 development, identity, and function, thereby predisposing East Asian carriers to higher
534 risks of developing T2D.

535 **Methods**

536 **Clinical studies**

537 We recruited 183 non-diabetic individuals by genotype (62 carriers of p.Arg192His allele
538 and 121 controls) from existing research programs in Singapore. The inclusion criteria
539 were Chinese ethnicity, age between 21 and 80 years, non-smoker or no use of nicotine
540 or nicotine-containing products for at least 6 months. Subjects with a known history of
541 diabetes mellitus, screening HbA1c greater than 6.5% or fasting plasma glucose greater
542 than 7.0 mmol/L were excluded. Subjects with weight loss greater than 5% of body weight
543 in the preceding six months, major surgery in the last three months, a history of
544 malignancy, estimated creatinine clearance based on the MDRD formula less than 60
545 mL/min, current corticosteroid use, or any clinically significant endocrine, gastrointestinal,
546 cardiovascular, hematological, hepatic, renal, respiratory disease, or pregnancy were
547 also excluded. The study was approved by the National Healthcare Group Domain
548 Specific Review Board (2013/00937), and informed consent was obtained from all
549 participants. Data on demographics and medical history were obtained through an
550 interviewer-administered questionnaire. Height and weight were measured. All subjects
551 underwent confirmation of genotype by polymerase chain reaction-restriction fragment-
552 length polymorphism analysis.

553

554

555 **Frequently sampled intravenous glucose tolerance test**

556 A 3-hour intravenous glucose tolerance test was performed after an overnight 10- to 12-
557 hour fast. Subjects were required to abstain from strenuous physical activity, alcohol and
558 caffeinated beverages 24 hours before the procedure. A bolus of intravenous 50%
559 glucose (0.3 g/kg body weight) was given within 60 seconds into the antecubital vein.
560 Regular insulin (Actrapid; NovoNordisk, Copenhagen, Denmark) was administered as a
561 bolus injection at 20 min at a dose of 0.03 units/kg body weight. Blood was sampled from
562 the contralateral antecubital vein at -15, -10, -5, 0, 2, 3, 4, 5, 6, 8, 10, 14, 19, 22, 25, 30,
563 40, 50, 70, 100, 140 and 180 min for assessment of plasma glucose (YSI 2300
564 STATPLUS; YSI Incorporated, Life Sciences, Yellow Springs, OH, USA) and insulin
565 (Advia Centaur; Siemens Health-care Diagnostics, Hamburg, Germany). AIRg (acute
566 insulin response to glucose) and Si (insulin sensitivity) were estimated using
567 mathematical modeling methods (MINMOD Millennium, ver. 6.02) Disposition index (DI)
568 was calculated as AIRg x Si.

569 **Oral glucose tolerance test (OGTT)**

570 Fifty-seven subjects (29 heterozygous p.Arg192His carriers and 28 p.Arg192Arg controls)
571 were invited to return for a 3-hour oral glucose tolerance test. The test was performed
572 after an overnight 10 to 12-hour fast. A 75-gram glucose drink in 200 mLs of water was
573 administered orally over 5 min. Blood samples were collected via an intravenous cannula
574 at -10, 0, 10, 20, 30, 45, 60, 75, 90, 120, 150 and 180 min for glucose (YSI 2300
575 STATPLUS; YSI Incorporated, Life Sciences, Yellow Springs, OH, USA), insulin (Advia

576 Centaur; Siemens Health-care Diagnostics, Hamburg, Germany), glucagon (Human
577 Glucagon ELISA; BioVendor R&D, Shizuoka, Japan) and GLP-1 (Glucagon-like peptide-
578 1 total ELISA; IBL International, Hamburg, Germany). HOMA-B was calculated using the
579 formula: $20 \times \text{fasting insulin } (\mu\text{IU/mL})/\text{fasting glucose } (\text{mmol/mL}) - 3.5$. HOMA-IR was
580 computed using the formula: $\text{fasting insulin } (\mu\text{IU/ml}) \times \text{fasting glucose } (\text{mmol/mL})/ 22.5$.

581 **Statistical analysis of clinical data**

582 Analyses were carried out using SPSS software version 18.0 (SPSS Inc., Chicago, IL,
583 USA). Independent t-test and Chi-square tests were used to compare continuous and
584 categorical variables between carriers and controls, respectively. A multiple linear
585 regression model was used with adjustment by age, sex and BMI. Data are shown as the
586 means (SD), and a p-value of <0.05 was considered statistically significant.

587 **Cell culture**

588 The use of human cells is covered by A*STAR IRB 2020-096. All mammalian cells were
589 routinely tested to be mycoplasma free using a MycoAlertTM PLUS mycoplasma detection
590 kit (Lonza Bioscience, LT07-710). All mammalian cells were cultured in a 5% CO₂
591 humidified incubator at 37°C. Unless otherwise stated, cells were passaged using 0.25%
592 trypsin. Mouse insulinoma 6 (MIN6) cells were cultured in high glucose DMEM (HyClone,
593 SH30021.01) supplemented with 15% FBS (HyClone, SV30160.03), 1% sodium pyruvate
594 (ThermoFisher Scientific, 11360070) and 55 μM beta mercaptoethanol (Gibco, 21985-
595 023). Alpha TC clone 9 mouse pancreatic adenoma cells (αTC1.9) (ATCC, CRL-2350TM)

596 were cultured in low glucose DMEM (1.0 g/L) supplemented with an additional 1.0 g/L
597 glucose (final glucose concentration to be 2.0 g/L), 10% FBS, 15 mM HEPES
598 (ThermoFisher Scientific, 15630080), 1% NEAA (Gibco, 11140-50), 0.02% Bovine serum
599 albumin (BSA) (Sigma-Aldrich, A9418) and 1.5 g/L sodium bicarbonate (ThermoFisher
600 Scientific, 25080094). AD293 (Agilent, 240085) and 293FT (Invitrogen, R70007) human
601 embryonic kidney cell lines were cultured in high glucose DMEM supplemented with 10%
602 FBS and 1% NEAA. EndoC- β H1 cells⁵⁸ (Human Cell Design) were cultured according to
603 the manufacturer's recommendations. Briefly, tissue culture plates were precoated with
604 high glucose DMEM supplemented with 2 μ g/mL fibronectin (Sigma-Aldrich, F1141) and
605 1% ECM (Sigma-Aldrich, E1270) at least 30 min prior to cell plating. Low glucose DMEM
606 (Gibco, 11885084) supplemented with 2% BSA, 10 mM nicotinamide (Sigma-Aldrich,
607 N0636 or N3376), 2 mM GlutaMAXTM (Gibco, 35050061), 50 μ M beta mercaptoethanol,
608 5.5 μ g/mL transferrin (Sigma-Aldrich, T8158) and 6.6 ng/mL sodium selenite (Sigma-
609 Aldrich, 214485). Cells were passaged weekly with 0.05% or 0.25% Trypsin and
610 neutralized with 20% FBS in DPBS and plated at a density of 70,000 cells/cm². The
611 isogenic SB Ad3.1 hiPSC line derived from human skin fibroblasts from a Caucasian
612 donor with no reported diabetes (Lonza CC-2511, tissue acquisition number 23447) was
613 obtained from the Human Biomaterials Resource Centre, University of Birmingham. The
614 SB line and donor-derived hiPSC lines generated herein were cultured in TeSRTM-E8TM
615 or mTeSR-1TM medium (StemCell Technologies, 05990 or 85850) with daily media
616 changes. hiPSCs were passaged twice weekly using ReLeSRTM (StemCell Technologies,
617 05872) or Accutase (Gibco, A1110501) according to the manufacturer's instructions.

618 Culture plates were precoated with 0.1% gelatin in cell culture grade water for at least 10
619 min and then with MEF media for at least 48 hours prior to plating or with Corning Matrigel
620 hESC-Qualified Matrix (VWR International, BD354277) for at least an hour prior to plating.

621 **Generating donor-derived hiPSC lines**

622 Skin punch biopsies were obtained from the upper forearm of recruited subjects and
623 cultured in low glucose DMEM supplemented with 10% heat-inactivated FBS and 1%
624 MEM non-essential amino acids (Gibco, 11140-50) to obtain fibroblasts. A Human Dermal
625 Fibroblast Nucleofector™ Kit (Lonza Bioscience, VDP-1001) was used for episomal
626 reprogramming of fibroblasts. Cells were trypsinized and washed with DPBS and 500,000
627 cells were resuspended in Nucleofector™ Solution according to manufacturer's
628 instructions. The following Yamanaka factors from Addgene were added at 1 µg to the
629 cell suspension: pCXLE-hOCT3/4-shp53-F (plasmid #27077), pCXLE-hSK (plasmid
630 #27078), and pCXLE-hUL (plasmid #27080). Nucleofection program P22 was used to
631 transfet cells. At the end of the nucleofection, cells were plated onto mitotically-
632 inactivated CF1-MEF (plated one day in advance) (Lonza Bioscience, GSC-6201G) and
633 cultured in DMEM/F12 (Gibco, 10565018) media supplemented with 20% KnockOut™
634 serum replacement (Gibco, 10828010), 1% NEAA, and 10 ng/mL FGF-2 (Miltenyi Biotec,
635 130-093-842). Media were replaced daily until hiPSC colonies emerged.

636 Peripheral blood mononuclear cells (PBMCs) were extracted from donor blood using a
637 BD Vacutainer® CPT™ Mononuclear Cell Preparation Tube (BD Biosciences, 362753).
638 The white buffy coat layer containing PBMCs was collected, washed twice with DPBS,

639 and centrifuged at 300xg for 10 min to pellet the cells. One to two million cells were
640 seeded and cultured in expansion media: IMDM media (Gibco, 12440053) supplemented
641 with 10% FBS, 50 µg/mL of L-ascorbic acid (Sigma-Aldrich, A8960), 50 ng/mL of Stem
642 Cell Factor (RnD Systems, 255-SC-010), 10 ng/mL IL-3 (StemCell Technologies, 78040),
643 2 U/mL Erythropoietin, 40 ng/mL IGF-1 (BioVision, 4119), 1 µM dexamethasone (Sigma-
644 Aldrich, D8893) and 0.2% Primocin (Invivogen, ant-pm-1). PBMCs were reprogrammed
645 following manufacturer's instructions using CytoTune™-iPS 2.0 Sendai Reprogramming
646 Kit (Invitrogen™, A16517) to obtain hiPSCs. For reprogramming, 200,000 PBMCs were
647 plated in 12-well plates, and Sendai viruses [hKOS (MOI5), hc-Myc (MOI5), and hKlf4
648 (MOI3)] were added to culture media supplemented with 8 µg/mL of Polybrene (Sigma-
649 Aldrich, TR-1003). Media was replaced the next day. Cells were collected and plated onto
650 mitotically-inactivated CF1-MEFs (pre-seeded one day in advance) and cultured in
651 DMEM/F12 media supplemented with 20% KOSR (Gibco, 10828010), 1% NEAA (Gibco,
652 11140-50) and 10 ng/ml FGF-2 (Miltenyi Biotec, 130-093-842), supplemented with 50
653 µg/mL of L-ascorbic acid, 50 ng/mL of Stem Cell Factor, 10 ng/mL IL-3, 2 U/mL
654 Erythropoietin, 40 ng/mL IGF-1, and 1 µM dexamethasone for the first two days. The
655 reprogrammed PBMCs were then maintained in basal media without growth factor and
656 small molecule supplementation until hiPSC colony formation. hiPSC colonies were
657 handpicked and cultured with a TeSR™-E8™ Kit. Each colony was designated to be one
658 hiPSC line and expanded for cryopreservation, with two to three independent lines per
659 donor.

660 Immunofluorescence staining was performed on all hiPSC lines used in this study to
661 confirm the expression of pluripotency markers OCT3/4, SOX2, NANOG, SSEA-4 and
662 TRA1-60. One representative donor-derived hiPSC line from each donor was submitted
663 for karyotyping (Cytogenetic laboratories, Singapore General Hospital) and for teratoma
664 assay (A*STAR Biological Resource Centre (BRC) Animal Facility). Teratomas were then
665 sent to Advanced Molecular Pathology Laboratory (AMPL, A*STAR) for paraffin block
666 processing, sectioning and H&E staining. Derivation of all three germ layers (definitive
667 endoderm, mesoderm and ectoderm) was confirmed using light microscopy.

668 **CRISPR-Cas9 genome editing of hiPSCs**

669 To generate *PAX4*^{-/-} isogenic SB Ad3.1 hiPSC lines, a strategy was designed to mirror
670 the well-studied *Pax4*^{-/-} mice where almost all of the functional domains were replaced
671 with a beta galactosidase-neomycin resistance cassette¹³. To delete the majority of the
672 paired and homeodomains, sgRNAs were designed targeting exon 2 (sgRNA#1:
673 CTAGGGCGTTACTACCGCAC) and exon 5 (sgRNA#2: TATCCTGATTCAAGTGGCCCG)
674 of *PAX4* gene (ENST00000341640.6). To generate the p.Arg192His and p.Tyr186X
675 variants in the SB Ad3.1 hiPSC line, sgRNA#2 was electroporated with HDR template
676 with either rs2233580 (G>A) mutation or GTA duplication, respectively. To correct the
677 donor-derived p.His192His and p.Tyr186X hiPSCs, sgRNA#3
678 (GGCAGTAGCCAGCTTCCAT) or sgRNA#4 (ATCTCCGCAGAGTTCCAGCG) were
679 electroporated with an HDR repair template. sgRNAs were synthesized following
680 manufacturer's instructions using the EnGen sgRNA Synthesis Kit, *S. Pyogenes* (NEB,

681 E3322), followed by DNase treatment and RNA purification using the RNA Clean &
682 Concentrator Kit (Zymo Research, R1017). Ribonucleoprotein (RNP) complexes were
683 formed by combining 20 μ M (681 ng) sgRNA, 20 μ M Cas9 (NEB, M0646T) and Buffer R
684 (ThermoFisher, MPK109R) in a total volume of 6 μ L and incubating at room temperature
685 for 15 min. The RNP complex was then combined with 250,000 hiPSCs in 15 μ L of Buffer
686 R and incubated on ice for 5 min. Ten microliters of the RNP+cell mixture was
687 electroporated in two separate electroporations using the NeonTM Transfection System
688 10 μ L Kit (ThermoFisher, MPK1025). Electroporated cells were seeded into Matrigel-
689 coated plates with mTeSR media and 10 μ M Y-27632 (StemCell Technologies, 72302).
690 Forty-eight hours after electroporation, hiPSCs were plated at low-density (5,000 cells/60
691 mm dish) on Matrigel-coated plates with mTeSR and 10 μ M Y-27632. The resulting
692 colonies were handpicked and expanded for further genotyping and quality control
693 measures.

694 **hiPSC differentiation to into BLCs**

695 For differentiation experiments using Protocol A, hiPSCs were cultured in mTeSRTM1 with
696 daily media changes and passaged using Accutase. Cells were plated at 10^6 cells/well in
697 Growth Factor Reduced Matrigel (Corning, 356230)-coated CellBind 12-well tissue
698 culture plates (Corning, 356230 and 3336) in mTeSR1 (StemCell Technologies, 85850)
699 supplemented with 10 μ M of Y-27632 dihydrochloride (AbCam, ab120129). The following
700 morning, the medium was changed to mTeSRTM1, and differentiation was started 24
701 hours after plating. Directed differentiation protocol was adapted from Rezania et al. and

702 basal differentiation media (using MCDB-131) was formulated accordingly²². Media was
703 changed daily to basal media supplemented with growth factors and small molecules
704 (Table 1) with the following modifications: Activin A and CHIR 99021 were used for Stage
705 1; all stages were performed in planar culture; and stages 6 and 7 were both 6 days in
706 length.

707 For differentiation experiments using Protocol B, hiPSCs were plated and maintained in
708 10 cm plates until 80-90% confluence. Following, hiPSCs were washed with DPBS and
709 dissociated into single cells using TrypLE™ Express (Gibco, 12605-010). Cells were
710 seeded at a density of 1 million cells per mL of mTeSR™1 kit supplemented with 10 µM
711 of Y-27632 (StemCell Technologies, 72303) on non-treated 6 well plate. Cells were then
712 incubated in tissue culture incubator on an orbital shaker with a shaking speed set at 80
713 rpm over a duration of 24 – 48 hours before the start of differentiation by changing to
714 differentiation media supplemented with growth factors and/or small molecules. Directed
715 differentiation protocol was adapted from Pagliuca et al. and basal differentiation media
716 (S1, S2, S3, S5 and S6) were formulated accordingly²⁵. Fresh differentiation media
717 supplemented with growth factors and small molecules were added at stipulated
718 timepoints for directed differentiation over a duration of 35 days. The details of the culture
719 medium and key reagents used for Protocol B can be found in Table 1.

720 **Cloning**

721 *PAX4* plasmid (pLenti6.2/V5-DEST-PAX4, HsCD00329734) was purchased from DNASU
722 plasmid repository. Full-length *PAX4* sequence was confirmed via Sanger sequencing

723 before subcloning into an engineered lentiviral vector pCDH-MCS-EF1-GFP to include a
724 5' Flag tag and a 3' V5 tag within the multiple cloning site (MCS). The full-length *PAX4*
725 sequence was subcloned into the pCDH-MCS-EF1-GFP vector for protein expression.
726 Refer to Table 2 for the list of primers used for cloning in this study. Cloning primers
727 h*Pax4*FLXba1F (forward) and h*Pax4*FLV5Xho1R (reverse) were used to amplify full-
728 length *PAX4* sequence. Polymerase chain reaction (PCR) was performed using
729 Phusion™ High-Fidelity DNA Polymerase (ThermoScientific, F530) for sequence
730 amplification. Thermal cycling conditions were set according to the manufacturer's
731 manual. Restriction enzyme digestion was performed on the pCDH-MCS-EF1-GFP and
732 amplified *PAX4* sequence independently using Xba1 (New England Biolabs, R0145) and
733 Xhol (New England Biolabs, R0146) according to the manufacturer's manual. Digested
734 products were resolved using gel electrophoresis and gel extraction was performed using
735 Purelink™ Quick Gel Extraction Kit (Invitrogen, K210012). Ligation was performed using
736 Quick Ligation Kit (New England Biolabs, M2200S) according to the manufacturer's
737 manual. The ligated plasmids were transformed into home-made competent cells
738 propagated from Stbl3™ competent cells (Invitrogen, C7373-03) and sequentially
739 amplified in LB broth for plasmid extraction using PureLink™ HiPure Plasmid Filter
740 Maxiprep Kit (Invitrogen, K210017). To introduce R192H and Y186X mutations into the
741 pCDH-*PAX4* plasmid, site-directed mutagenesis (SDM) primers were designed (refer to
742 Table 2) and SDM was performed according to the procedures described.
743 For gene promoter cloning, human genomic DNA extracted from AD293 cells was used
744 as template. Basic luciferase vector, pGL4.10 (Promega) was used as cloning vector for

745 gene promoters. Briefly, primers targeting the promoter region (-1 nucleotide from ATG
746 translational start site) were designed (refer to Table 2). Targeted promoter regions were
747 amplified, digested with restriction enzyme, ligated and transformed into competent cells
748 similarly as described in the previous section. With the exception of the insulin gene
749 promoter, all other gene promoters used in this study were amplified from human gDNA
750 and subcloned into pGL4.10 at the multiple cloning site. The pGL4.10 *INS* promoter
751 plasmid was synthesized by IDT (gBlocks™ Gene Fragments). Length of the various
752 gene promoters used is as follows: *PAX4* – 1384 bp, *INS* – 1499 bp, *GCG* – 1068 bp,
753 *SST* – 718 bp.

754 The design of shRNA sequence to knockdown *PAX4* gene was referenced to Genetic
755 Perturbation Platform (Broad Institute), Clone ID TRCN0000015989. The shRNA targets
756 the coding sequence CGGATCCTTAAGGTATCTAAT within *PAX4* gene (Table 2). The
757 shRNA was ligated into pLKO.1 vector using Quick Ligation Kit (New England Biolabs,
758 M2200S) according to the manufacturer's instructions. Successfully ligated sh*PAX4*
759 plasmid was amplified for subsequent experiments.

760 **Lentiviral-mediated sh*PAX4* stable line generation**

761 3rd generation lentivirus system was used for this study. Lentivirus plasmids used for virus
762 production: pRC/CMV-Rev (Rev), pHDM-HIVgpm (Gag/Pol) and pHDM-G (Vsv-g). Non-
763 targeting shScramble and shRNA targeting human *PAX4* (sh*PAX4*) gene were subcloned
764 into pLKO.1 vector for lentiviral packaging in 293FT cells. For the generation of stable
765 lines, EndoC-βH1 cells were plated onto 10 cm plates. The cells were then transduced

766 with pLKO.1 shScramble or shPAX4 lentiviruses in the presence of 8 µg/mL polybrene.
767 After 72 hours, the transduced cells were cultured in EndoC media supplemented with
768 500 µg/ml of G418 antibiotic (Invivogen, ant-gn-1). In parallel, one plate of untreated
769 EndoC-βH1 cells (plated at the same density) was cultured in the same antibiotic
770 supplemented media as a control. Media were replenished routinely during this selection
771 process. Thereafter, the surviving EndoC-βH1 cells were expanded to obtain stable lines.

772 **EndoC-βH1 gene silencing using siRNAs**

773 Knockdown studies in EndoC-βH1 cells were performed using Lipofectamine RNAiMAX®
774 transfection protocol and 15 nM SMART pool ON-TARGETplus siRNAs (Horizon
775 Discovery Biosciences, siNT: D-001810-10-05, siPAX4: L-012240-00-0005) diluted in
776 Opti-MEM reduced serum-free medium (ThermoFisher Scientific, 31985062) and 0.4%
777 RNAiMAX® (ThermoFisher Scientific, 13778150). Silencing efficiency was determined by
778 qPCR from samples collected during GSIS, five days post-transfection.

779 **Glucose-stimulated insulin secretion (GSIS) assay**

780 siNT and siPAX4 EndoC-βH1 cells were seeded in 48-well plates at a density of 180,000
781 cells six days prior to GSIS. Cells were gently washed three times with pre-warmed
782 secretion assay buffer (114 mM sodium chloride, 4.7 mM potassium chloride, 1.2 mM
783 calcium chloride, 1.2 mM potassium phosphate, 1.16 mM magnesium sulphate, 25 mM
784 sodium bicarbonate, 0.2% fatty acid-free BSA (Proliant, 68700), 20 mM HEPES, adjusted
785 to pH 7.3). Cells were then incubated in secretion assay buffer for 1 hour before being

786 stimulated with 2.8 mM or 16.7 mM glucose for 40 min. Supernatant was collected at the
787 end of 40 min for insulin secretion measurements and insulin content was collected using
788 RIPA buffer. AlphaLISA human insulin research kit (Perkin Elmer, AL204C) was used to
789 measure insulin secretion and content. Total protein measurements were determined
790 using a Pierce BCA Protein Assay Kit (Life Technologies, PI23227). Stimulation index
791 was calculated by normalizing to total protein and relative to 2.8 mM glucose.

792 shPAX4 and shScramble EndoC- β H1 cells were seeded in 12-well plate prior to GSIS
793 assay. Before GSIS assay, cells were gently washed three times with warm Krebs Ringer
794 bicarbonate (KRB) buffer (125 mM sodium chloride, 4.74 mM potassium chloride, 1 mM
795 calcium chloride, 1.2 mM potassium phosphate, 1.2 mM magnesium sulfate, 5 mM
796 sodium bicarbonate, 0.1% fatty acid-free BSA, 25 mM HEPES, adjusted to pH 7.5 (± 0.2)
797 with 1 M sodium hydroxide). After that, cells were subjected to normalization at 2.8 mM
798 glucose for 1 hour before being stimulated at 2.8 mM and 16.7 mM glucose for 30 min
799 each sequentially. At the end of each stimulation step, KRB buffer was collected for
800 human insulin ELISA (Mercodia, 10-1113-10). Stimulation index was computed by insulin
801 secreted at 16.7 mM divided by insulin secreted at 2.8 mM glucose. Total insulin was
802 extracted from each sample after the whole process of GSIS was completed.

803 **Total insulin content extraction**

804 At the end of the 35-day directed differentiation using Protocol B, BLCs from each hiPSC
805 line were handpicked, and 400 μ l of acid/ethanol solution was added. Cells were
806 vigorously vortexed and subjected to repeated pipetting to break up cell clumps. BLCs

807 were then incubated at 4°C overnight before total insulin extraction. For EndoC-βH1 cells,
808 500,000 cells were seeded onto each well of a 12-well plate. At the end of the experiment,
809 cells were washed thrice with DPBS before adding 500 µl of acid/ethanol solution to each
810 well and incubated at 4°C overnight prior to insulin extraction. After overnight incubation,
811 the insulin extracts were centrifuged at 1000 rpm for 5 min. The top aqueous layer
812 containing insulin was collected and subjected to human insulin ELISA assay (Mercodia,
813 10-1113-10) while the bottom layer (containing cell pellet) was boiled to dryness at 80°C
814 on a heat block. The dried cell pellet was resuspended in water for total DNA
815 quantification. All data involving total insulin content quantification were normalized to
816 total DNA. For donor hiPSC-derived BLCs, the average total insulin content extracted
817 from various cell lines from each donor in one experiment is represented as a single data
818 point on the graph. For CRISPR-edited cells, each data point represents the average of
819 total insulin extracted from one cell line in one experiment. For EndoC-βH1 cells, each
820 data point represents the average of total insulin extracted in independently sampled
821 triplicates in one experiment.

822 **Gene expression analysis**

823 Total RNA was extracted using MN NucleoSpin RNA Kit (Macherey-Nagel). RNA was
824 quantified and reverse transcribed to cDNA using High-capacity cDNA reverse
825 transcription kit (Applied Biosystems, 4368813). QPCR was performed using iTaq™
826 Universal SYBR ® Green Supermix (Bio-rad, 172-5124). Thermal cycling was performed
827 using CFX384 Touch Real-Time PCR System (Bio-rad). Relative quantification of each

828 gene expression was normalized to *ACTIN*, calculated by the 2^{-ddCt} method. The qPCR
829 primers used in this study are summarized in Table 3.

830 **Taqman allelic discrimination assay**

831 Custom Taqman® Assay Design Tool (ThermoScientific) was used to design probes
832 specific for either the wildtype (p.Arg192 or p.Tyr186) or *PAX4* variant transcripts
833 (p.His192, ANT2HTM or p.X186, ANU7DDJ). A template sequence of approximately 600
834 bp around the SNP of interest was used as a reference to design custom assay probe.
835 For Taqman assays qPCR, cDNA from EPs was used as templates. A 5 μ l assay with
836 TaqMan® SNP Genotyping MasterMix (Applied Biosystems, 4351384) was prepared
837 according to the manufacturer's manual. Relative fluorescence units (RFUs) from the
838 HEX probe (wildtype allele) and the FAM probe (either Arg192 or X186 allele) were
839 analyzed using the CFX384 Touch Real-Time PCR System (Bio-rad).

840 **Immunofluorescence staining**

841 In preparation for pluripotency IHC, each donor-derived hiPSC line was seeded onto a
842 few wells of precoated 12-well plate. AD293 and EndoC- β H1 cells were seeded onto
843 uncoated and coated coverslips in 12-well plates, respectively. For overexpression
844 studies, transfection was performed on seeded cells using Lipofectamine™ 2000
845 transfection reagent (Invitrogen, 11668-019) or FuGENE® 6 transfection reagent
846 (Promega, E2691) according to the manufacturer's instructions. hiPSC-derived EPs and
847 BLCs were collected and sent to Advanced Molecular Pathology Laboratory (AMPL,

848 A*STAR) for cryo-embedding, cryo-block processing and sectioning. For IHC,
849 cryosections were thawed and dried at room temperature before staining. Cells were
850 washed thrice with DPBS and fixed with 4% paraformaldehyde (WAKO, 163-20145) for
851 20 min. Blocking and cell membrane permeabilization were performed using DPBS
852 supplemented with 5% Donkey serum (Merck Millipore, S-30) and 0.1% Triton-X-100
853 (Merck Millipore, 9410) for 1 hour at 4°C. Cells were incubated with primary antibodies
854 overnight at 4°C. Cryosections were then incubated with corresponding secondary
855 antibodies for 1 hour at room temperature. For nuclear staining, cryosections were
856 incubated with DAPI (1:5000) (Sigma-Aldrich, D9542) in DPBS for 20 min before
857 mounting onto glass slides for imaging with Olympus Fluoview Inverted Confocal
858 microscope. Refer to Table 4 for the list of antibodies used and their respective dilution
859 factors for IHC.

860 **SDS-PAGE and Western blot**

861 Cells were washed with DPBS and lysed in M-PER™ (Mammalian protein extraction
862 reagent) (ThermoScientific, 78501) in the presence of protease inhibitor cocktail (Sigma-
863 Aldrich, P8340), phosphatase-2 inhibitor (Sigma-Aldrich, P5726), and phosphatase-3
864 inhibitor (Sigma-Aldrich, P0044). Protein was quantified using Pierce™ BCA protein
865 assay kit (ThermoScientific, 23227) according to the manufacturer's instructions before
866 being separated with SDS-PAGE and transferred to PVDF membrane. Protein blots were
867 first blocked with 5% milk in TBST (1X Tris-Buffered Saline, 0.1% Tween 20) for 1 hour
868 before incubating with primary antibody for either 2 hours at room temperature or

869 overnight at 4°C. Blots were washed and then incubated with the respective HRP-
870 conjugated secondary antibody for 1 hour. Chemiluminescence signals were visualized
871 after incubation with Super Signal™ West Dura Extended Duration Substrate
872 (ThermoScientific, 34076). Refer to Table 4 for the list of antibodies used and their
873 respective dilution factors for western blotting.

874 **Flow cytometry**

875 DE cells generated with Protocol A were collected at the end of Stage 1 using Accutase.
876 For extracellular staining, cells were washed twice with 1X Flow Cytometry Staining Buffer
877 (RnD, FC001). Cells were blocked in Flow Cytometry Staining Buffer with FC block for 5
878 min before adding human anti-CXCR4 antibody for 45 min. Cells were then washed twice
879 with Flow Cytometry Staining Buffer. For intracellular staining, cells were fixed using BD
880 CytoFix Buffer (BD Biosciences, 554655) for 20 min on ice before washing twice with
881 PBS. Using BD Perm/Wash Buffer (BD Biosciences, 554723), fixed cells were
882 permeabilized for 30 min on ice, washed three times, and human anti-SOX17 antibody
883 was incubated for 1 hour at 4 °C before a final wash step in PBS. Stained cells were
884 acquired on SH800 Cell Sorter (Sony) and data analysis was performed using FlowJo™
885 10.6.0.

886 EPs and BLCs were collected on D20 and D35, respectively, following differentiation with
887 Protocol B before being dissociated into single cells using TrypLE™ Express (Gibco,
888 12605-010). Cells were passed through a 40 µm cell strainer and single cells were fixed
889 with 4% PFA for 20 min on ice. Antigen blocking and cell permeabilization were performed

890 using DPBS supplemented with 5% FBS (HyClone, SV30160.03) and 0.1% Triton-X-100
891 for 30 min on ice. Cells were stained with primary antibodies for 1 hour at room
892 temperature. The cells were then washed three times with DPBS and incubated with
893 corresponding secondary antibodies for 1 hour at room temperature. Flow cytometry
894 analyses were performed with BD® LSR II Flow Cytometer (BD Biosciences) and data
895 was analyzed using FlowJo™ software (BD Biosciences). Refer to Table 4 for the list of
896 antibodies used and their respective dilution factors for flow cytometry.

897 **Luciferase assays**

898 Cells were plated in triplicate one day prior to co-transfection with 0.5 µg of pCDH-
899 overexpression constructs encoding *PAX4* or its variants, 0.4 µg of pGL4.10 luciferase
900 vector and 10 ng of TK Renilla vector. Transfection was performed using either
901 Lipofectamine 2000 (Invitrogen, 11668-019) or FuGENE® 6 transfection reagent
902 (Promega, E2691). Cells were lysed with lysis buffer at the end of transfection (24 hours
903 for AD293, 48 hours for MIN6/αTC1.9 and 72 hours for EndoC-βH1 cells). Luciferase
904 assay was performed using Dual-Glo® Luciferase Reporter Assay Kit (Promega, E2920)
905 following the manufacturer's instructions. The luciferase firefly activity was normalized
906 against the Renilla readings within each well to account for variation in transfection
907 efficiency across replicate wells. Each triplicate was normalized to the mean of the pCDH-
908 MCS-EF1-GFP-empty control.

909

910 **Seahorse metabolic assays**

911 EPs on D19 of directed differentiation using Protocol B were dissociated into single cells
912 using TrypLE™ Express (Gibco, 12605-010) before passing through 40 μ m cell strainer.
913 80,000 or 120,000 cells were plated with S5 differentiation medium²⁵ supplemented with
914 10 μ M of Y-27632 onto pre-coated Seahorse microplate one day prior to analysis. The
915 same number of cells were seeded across all cell lines within each experiment. On the
916 day of glycolysis stress test (Agilent Seahorse XF Glycolysis Stress Test Kit), cells were
917 washed with unbuffered serum-free assay medium (DMEM 5030, Sigma-Aldrich;
918 supplemented with 2 mM L-glutamine). Following, the cells were incubated in assay
919 medium in a non-CO₂ incubator at 37°C for 1 – 2 hours before measurements were taken.
920 Extracellular acidification rates (ECAR) were measured using Seahorse XFe96 analyzer
921 (Seahorse Bioscience) at pre-set timings prior to and following sequential injections of 10
922 mM glucose, 1.5 μ M oligomycin and 50 mM 2-deoxy-glucose (2-DG). The same number
923 of cells was seeded across the various genotypes for individual experiments. Four to eight
924 technical replicates were seeded for each cell line. Each data point on graph represents
925 the average of all replicates from one cell line. For the Mito Stress Test (Agilent Seahorse
926 XF Cell Mito Stress Kit), cells were washed with unbuffered serum-free assay medium
927 supplemented with 20 mM glucose (keeping the glucose level consistent with S5
928 differentiation medium), 2 mM pyruvate and 2 mM L-glutamine prior to incubation in assay
929 medium in a non-CO₂ incubator at 37°C for 1 – 2 hours before analysis. Oxygen
930 consumption rates (OCR) were measured using Seahorse XFe96 analyzer prior to and
931 following sequential injections of 1.5 μ M oligomycin, 1 μ M FCCP and 0.5 μ M

932 rotenone/antimycin-A. Similarly, the same number of cells was seeded across the various
933 genotypes for individual experiments. Four to eight technical replicates were seeded for
934 each cell line. One data point on the graph represents the average of all replicates from
935 one cell line.

936 **RNA sequencing and analysis**

937 Total RNA was extracted from samples generated using differentiation Protocol A at the
938 end of Stage 1 (DE), 4 (PE), 5 (EP), and 7 (BLC) using RNeasy Mini Kit (Qiagen, 74104)
939 following manufacturer's instructions. Polyadenylated transcripts were isolated using
940 NEBNext PolyA mRNA Magnetic Isolation Module (New England Biolabs, E7490).
941 Sequencing libraries were prepared using the NEBNext Ultra Directional RNA Library Kit
942 with 12 cycles of PCR and custom 8 bp indexes (New England Biolabs, E7420). Libraries
943 were multiplexed and sequenced on the Illumina NovaSeq 6000 as 150-nucleotide
944 paired-end reads. Reads were mapped to human genome build hg19 (GRCh37) using
945 STAR v.2.5⁵⁹, with GENCODE v19
946 (https://www.gencodegenes.org/human/release_19.html) as the transcriptomic
947 reference. featureCounts from the Subread package v1.5
948 (<http://subread.sourceforge.net/>) was used to perform gene-level quantification.
949 Differential expression analysis was performed per stage using DESeq2⁶⁰ comparing
950 *PAX4*^{+/+} and *PAX4*^{-/-} cell lines. First, the model was fit using a likelihood ratio test with
951 genotype as a factor of interest and experiment as a covariate. From this, genes not within
952 the top 5000 most significant genes were used as an empirical control (affected only by

953 unwanted experimental variation) and the estimated factor of unwanted variation (k=1)
954 was calculated using the RUVg function from RUVSeq⁶¹. To identify differentially
955 expressed genes, DESeq2 was performed using a likelihood ratio test and including the
956 factor from RUV as a covariate along with the technical replicate (experiment).
957 Significance was determined by padj <0.05. Sashimi, TPM, and volcano plots were
958 generated using ggplot2.

959 Total RNA was extracted from samples generated using differentiation Protocol B on day
960 0 (hiPSC), day 13 (PP2), day 20 (EP) and day 35 (BLC). Poly-A mRNA (10 – 100 ng) was
961 used to construct multiplexed strand-specific RNA-seq libraries (NEXTflexTM Rapid
962 Directional RNA-SEQ Kit, dUTP-Based, v2). The quality of individual libraries was
963 assessed and quantified using Agilent 2100 Bioanalyzer and Qubit 2.0 fluorometer before
964 pooling for sequencing using a HiSeq 2000 (1x101 bp read). Prior to cluster formation,
965 pooled libraries were quantified using the KAPA quantification kit (KAPA Biosystems).
966 The processing of raw RNA sequencing data was performed in collaboration with
967 Molecular Engineering Laboratory (A*STAR) to remove low quality sequence reads.
968 Filtered read sequences were mapped onto human genome (hg19). Fragments per
969 kilobase million (FPKM) was used to calculate differential expression between patient
970 lines using DESeq2. Using UMAP (Uniform Manifold Approximation and Projection) and
971 PCA (Principal Component Analysis) dimension reduction clustering for all four time-
972 points, 11 out of 164 samples were classified as outliers and excluded from clustering
973 analyses.

974 For the generation of PCA plot for EPs differentiated using protocol B, the TPM read
975 counts for each gene within the transcriptome were first normalized to calculate a
976 standardized score. To derive a standardized score, we applied the following formula:
977 $\log_{10}(((\text{GOI's TPM counts for sample of interest} + 1) / (\text{average TPM counts for GOI across})) + 1)$, where GOI represents a gene of interest within the whole transcriptome.
978 Using this standardized score, PCA analysis was performed in R via the prcomp()
979 function. The ggplot2 package was used to plot the final PCA biplot based upon the PC1
980 and PC2 loadings obtained from prcomp(). Finally, the stat_ellipse() function was used to
981 cluster the transcriptome of cells with the various *PAX4* genotypes based upon a
982 confidence interval of 90%.

984 **Data availability**

985 Protocol A *PAX4*^{-/-} RNA-seq data: EGAS00001006036
986 Protocol B RNA-seq data 1: Submission into GEO in progress under NCBI #22948635
987 Protocol B RNA-seq data 2: GSE202206 (secure token: epufowcsnjcvvgv)

988

989 **Statistical analysis**

990 Statistical analyses were performed using GraphPad Prism version 9. Data are presented
991 as the standard error of the mean (SEM). Unless otherwise specified, unpaired Student's
992 t tests were performed to compare the means of two groups, and one-way ANOVA was
993 performed to compare the means among three or more groups. A p-value of less than
994 0.05 indicates statistical significance.

995 **Reagent Table 1: directed differentiation protocols A and B.**

Reagent	Source, Catalog number
MCDB 131 media	Corning, 15-100-CV; Gibco, 10372-019
CMRL-1064 supplemented	Mediatech, 99-663-CV
Bovine serum albumin	Roche, 10775835001; Proliant, 68700
Insulin-Transferrin-Selenium-Ethanolamine	Gibco, 51500-056
L-Ascorbic acid	Sigma-Aldrich, A4544 or A8960
Heparin	Sigma-Aldrich, H3149
Human recombinant Activin A	Peprotech, 120-14; StemCell Technologies, 78001.2
CHIR 99021	Axon Medchem, 1386; Tocris, 4423
KGF/FGF-7	Peprotech, 100-19; StemCell Technologies, 78046.2
Retinoic acid	Sigma-Aldrich, R2625; WAKO, 186-01114
SANT1	Sigma-Aldrich, S4572; Santa Cruz, sc-203253
Phorbol 12,13-dibutyrate	Tocris, 4153
LDN-193189	Stemgent, 04-0074; Sigma-Aldrich, SML0559
Compound E, γ -secretase inhibitor XXI	EMD Millipore, 565789; Cayman, 15579-20
Alk5 inhibitor II	ENZO, ALX-270-445-M001
L-3,3',5-triiodothyronine	Sigma-Aldrich, T6397; Merck Millipore, 642511
Human betacellulin	Cell Signalling, 5235SF
Alpha-Amyloid Precursor Protein Modulator	EMD Millipore, 565740
N-acetyl cysteine	Sigma-Aldrich, A9165
R428	SelleckChem, S2841
Trolox	EMD Millipore, 648471
Zinc Sulfate	Sigma-Aldrich, Z0251

996

997 **Reagent Table 2: primers for cloning.**

Cloning primers	Primer sequence (5' – 3')	Purpose
hPax4FLXbal1F	GCTCTAGAATGAACCAGCTTGGGG GGCT	To clone full-length <i>PAX4</i> sequence
hPax4FLV5Xho1R	CCGCTCGAGTTCCAAGCCATACAG TAGTGGG	
SDM primers	Primer sequence (5' – 3')	Purpose
Y186XSDM-F	GTGGGCAGTAGTCCTGATTCA	To create Y186X
Y186XSDM-R	TGAATCAGGACTACTGCCAC	
R192HSDM-F	CAGTGGCCCATGGAAAGCTG	To create R192H
R192HSDM-R	CAGCTTCCATGGGCCACTG	
Promoter cloning primers	Primer sequence (5' – 3')	Purpose
hINSP-1.5KpnIF	GGGGTACCGCCTGGCTCT	<i>INS</i> gene promoter
hINSP-1EcoRVR	CCCGATATCGGCAGAAGGACA	
hGcgP-1068NheIF	CTAGCTAGCCACAGCTGGTCAATA ACAGCAA	<i>GCG</i> gene promoter
hGcgP-1 HindIIIR	CCCAAGCTTTCTGCTGTCTTCTG GTAGTGT	
hARXPKpn-1084F	GGGGTACCAAGGTGAACAGCCTCA GGGTGAAG	<i>ARX</i> gene promoter
hARXPEcoRV-7R	GGGGATATCGGCTTTCCCAGGG CGCAGA	
hSSTP-724FEcoRV	GGGGATATAGGGAGGGTGAGCCA GAGGT	<i>SST</i> gene promoter
hSSTP-6RHindIII	TTTAAGCTTCGCCCGCAAAGCCGA GC	
shRNA cloning primers	Primer sequence (5' – 3')	
shPAX4nt315F	CGGC GGATCCTTAAGGTATCTAATCTCGAGATTAGATACC TTAAGGATCCGTTTG	
shPAX4nt315R	ATTCAAAAACGGATCCTTAAGGTATCTAATCTCGAGATTA GATACCTTAAGGATCCG	

998

999 **Reagent Table 3: qPCR primers.**

Targeting gene	Primer	Primer sequence (5' – 3')
<i>ACT/N</i>	hActinF	TTGCCGATCCGCCGCCGTC
	hActinR	CCCATGCCACCATCACGCCCTGG
<i>ARX</i>	hArxF	GGACGTCTTCACCAGGGAGGAAC
	hArxR	TCTCCCGCTTGCGCCACTT
<i>GCG</i>	hGcgF	ACAGCACACTACCAGAAGACAGCA
	hGcgR	TGTGCCCTGTGAATGGCGCT
<i>INS</i>	hIns1-3F2	CCTGCAGGTGGGGCAGGTGGAGC
	hIns1-3R2	CGGGTGTGGGGCTGCCTGCG
<i>NKX6.1</i>	hNkx6.1F	ACGCACGCCTGGCCTGTACCCC
	hNkx6.1R	CCCTCTGGGCCCGCCAAGTA
<i>PAX4</i>	hPax4F5	AGGACACGGTGAGGGTCTGGT
	hPax4R5	CAGTGGTTCCAGGGCAGGCA
<i>PDX1</i>	hPdx1F	CCTTCCCGGAGGGAGCCGAGCC
	hPdx1R	GTAGGCCGTGCGCGTCCGCT
<i>SST</i>	hSstF	GCTGCGCTGTCCATCGTCCT
	hSstR	TTGGCCAGTTCCCTGCTTCCCC

1000

1001

Reagent Table 4: antibodies.

Antibody	Dilution factor	Cat#	RRID
Primary antibody			
Anti-beta Actin (Mouse monoclonal)	WB 1:10000	Sigma-Aldrich, A5441	AB_476744
Anti-C-PEPTIDE (Rat monoclonal)	IF 1:100	DSHB, GN-ID4	AB_2255626
Anti-FLAG M2 (Mouse monoclonal)	WB 1:1000; IF 1:100	Sigma-Aldrich, F1804	AB_262044
Anti-Glucagon Antibody (N-17) (Goat polyclonal)	IF 1:100	Santa cruz, sc-7780	AB_641025
Anti-Insulin antibody (Guinea pig polyclonal)	IF 1:100; FC 1:100	Abcam, ab7842	AB_306130
Anti-PAX4 (Goat polyclonal) *Not validated to stain endogenous protein expression	IF 1:100; WB 1:1000	RnD, AF2614	AB_2159529
Anti-SOX2 antibody (Rabbit polyclonal)	IF 1:200	Abcam, ab97959	AB_2341193
Anti-Human SSEA-4 Antibody, Clone MC-813-70 (Mouse monoclonal)	IF 1:100	StemCell Technologies, 60062AD	AB_528477
Somatostatin Antibody (D-20) (Goat polyclonal)	IF 1:100	Santa cruz, sc-7819	AB_2302603
Anti-Human TRA-1-60 Antibody, Clone TRA-1-60R (Mouse monoclonal)	IF 1:100	StemCell Technologies, 60064AD	AB_2686905
Anti-V5 tag antibody (SV5-Pk1) (Mouse monoclonal)	IF 1:100; WB 1:1000	Abcam, ab27671	AB_471093
Anti-CXCR4 PE-conjugated (Mouse monoclonal IgG ^{2B} Clone #44717)	FC 1:10	RnD, FAB173P	AB_357083
FC block or anti-mouse CD16/CD32 (Rat monoclonal)	FC 1:500	BD, 553141	AB_394656
Alex Fluor® 488 anti-Human Sox17 (Mouse monoclonal Clone #P7-969)	FC 1:20	BD, 562205	AB_10893402
Fluorophore-conjugated secondary antibody			
Alexa Fluor® 488 anti-goat	IF 1:500; FC 1:2000	Invitrogen, A11055	AB_2534102
Alexa Fluor® 488 anti-mouse	IF 1:500; FC 1:2000	Invitrogen, A21202	AB_141607

Alexa Fluor® 488 anti-rabbit	IF 1:500; FC 1:2000	Invitrogen, A21206	AB_2535792
Alexa Fluor® 488 anti-rat	IF 1:500; FC 1:2000	Invitrogen, A21470	AB_10561519
Alexa Fluor® 594 anti-goat	IF 1:500; FC 1:2000	Invitrogen, A11058	AB_2534105
Alexa Fluor® 594 anti-Guinea pig	IF 1:500; FC 1:2000	Invitrogen, A11076	AB_141930
Alexa Fluor® 594 anti-mouse	IF 1:500; FC 1:2000	Invitrogen, A21203	AB_141633
Alexa Fluor® 594 anti-rabbit	IF 1:500; FC 1:2000	Invitrogen, A21207	AB_141637
Alexa Fluor® 647 anti-Guinea pig	IF 1:500; FC 1:2000	Jackson, 706-605-148	AB_2340476
Alexa Fluor® 647 anti-mouse	IF 1:500; FC 1:2000	Jackson, A31571	AB_162542
Alexa Fluor® 647 anti-rabbit	IF 1:500; FC 1:2000	Invitrogen, A31573	AB_2536183
Alexa Fluor® 488 Isotype	IF 1:500; FC 1:2000	BD Pharmingen, 565572	AB_2869685
HRP-conjugated secondary antibody			
Donkey anti-goat IgG-HRP	WB 1:10000	Santa cruz, sc-2020	AB_631728
Mouse anti-goat IgG-HRP	WB 1:5000	Santa cruz, sc-2354	AB_628490
Goat anti rabbit IgG HRP	WB 1:10000	Santa cruz, sc-2004	AB_631746
Goat anti-mouse IgG-HRP	WB 1:10000	Santa cruz, sc-2005	AB_631736
Goat anti-mouse IgG-HRP	WB 1:10000	Santa cruz, sc-2055	AB_631738
m-IgGκ BP-HRP	WB 1:5000	Santa cruz, sc-516102	AB_2687626
Mouse anti-rabbit IgG-HRP	WB 1:5000	Santa cruz, sc-2357	AB_628497

1002 WB: Western blot; IF: Immunofluorescence; FC: Flow cytometry.

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1179

1180 **Acknowledgments**

1181
1182 The authors thank A/P Yee Joo Tan for her support with antibodies and Daniela Moralli
1183 (University of Oxford) for her support with hiPSC karyotyping. We thank the Oxford
1184 Genomics Centre at the Wellcome Centre for Human Genetics (funded by Wellcome
1185 Trust grant reference 203141/Z/16/Z for the generation and initial processing of the
1186 sequencing data. H.H.L. is supported by the Institute of Molecular and Cell Biology
1187 (IMCB) Scientific Staff Development Award (SSDA) for her part-time Ph.D. N.A.J. K. is
1188 supported by the Stanford Maternal and Child Health Research Institute Postdoctoral
1189 Fellowship. A.L.G. is a Wellcome Senior Fellow in Basic Biomedical Science. A.L.G. is
1190 funded by the Wellcome (200837) and National Institute of Diabetes and Digestive and
1191 Kidney Diseases (NIDDK) (U01-DK105535, U01-DK085545, UM1DK126185,
1192 U01DK123743, U24DK098085) and the Stanford Diabetes Research Center (NIDDK
1193 award P30DK116074). A.K.K.T. is supported by IMCB, A*STAR, Lee Foundation Grant
1194 SHTX/LFG/002/2018, FY2019 SingHealth Duke-NUS Surgery Academic Clinical
1195 Programme Research Support Programme Grant, Precision Medicine and Personalised
1196 Therapeutics Joint Research Grant 2019, the 2nd A*STAR-AMED Joint Grant Call
1197 192B9002, HLTRP/2022/NUS-IMCB-02, Paris-NUS 2021-06-R/UP-NUS (ANR-18-IDEX-
1198 0001), OFIRG21jun-0097, CSASI21jun-0006 and MTCIRG21-0071.
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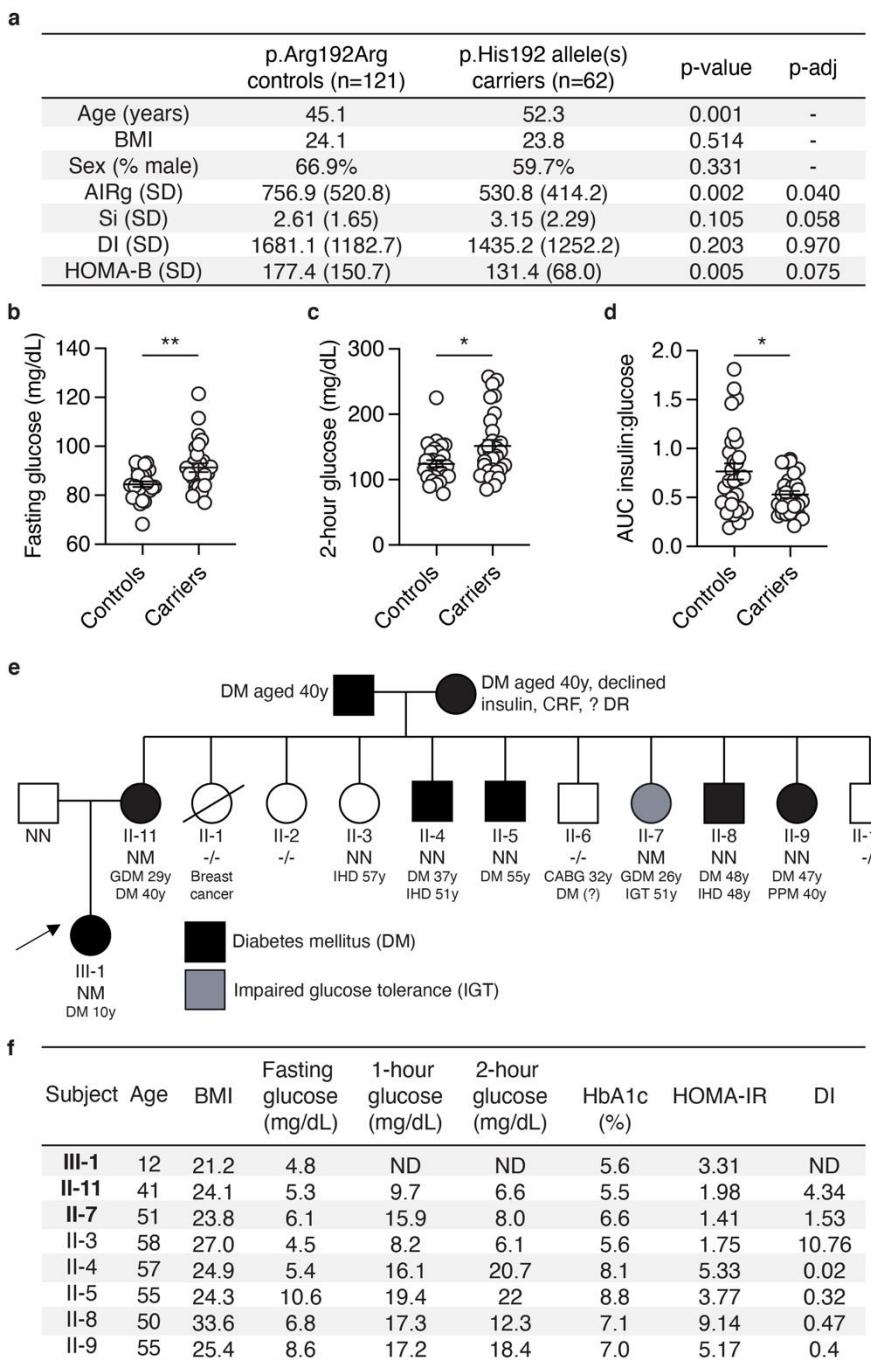
1200 **Author contributions**

1201
1202 Conceptualization: E.S.T., A.L.G., A.K.K.T.
1203 Data curation: M.P.A., H.S., A.J.
1204 Formal Analysis: H.H.L., N.A.J.K., M.P.A., H.S., A.J., A.L.G., A.K.K.T.
1205 Funding acquisition: E.S.T., A.L.G., A.K.K.T.
1206 Investigation: H.H.L., N.A.J.K., F.A., J.W.C., J.A., S.G., B.C., S.H., A.N.S.T., D.G., S.L.K.,
1207 A.L.G., A.K.K.T.
1208 Methodology: H.H.L., N.A.J.K., F.A., M.P.A., J.A., A.L.G., A.K.K.T.
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1213 Validation: H.H.L., N.A.J.K.
1214 Visualization: H.H.L., N.A.J.K.
1215 Writing – original draft: H.H.L., N.A.J.K., A.L.G., A.K.K.T.
1216 Writing – review & editing: All authors approved
1217

1218 **Competing interest statement**

1219
1220 A.L.G.'s spouse is an employee of Genentech and holds stock options in Roche. A.K.K.T.
1221 is a co-founder of BetaLife Pte Ltd.

1222 **Figures**

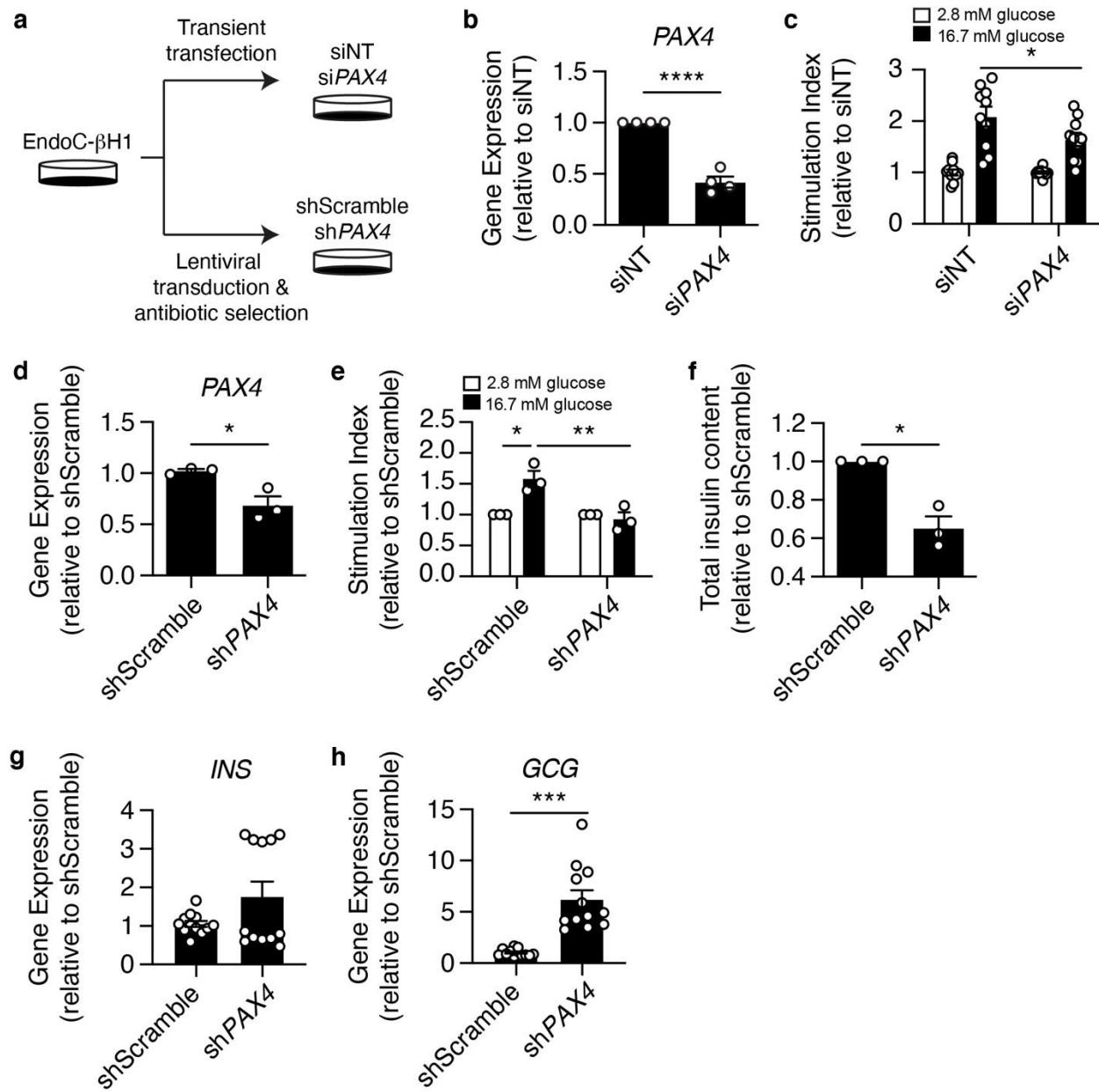


1223

1224 **Figure 1. Reduced pancreatic beta cell function in carriers of diabetes-associated**
 1225 **PAX4 variants.**

1226 **(a)** Mean age and BMI of heterozygous and homozygous carriers of p.His192 PAX4 allele
 1227 (n=62) and homozygous p.Arg192Arg controls (n=121) who underwent frequently

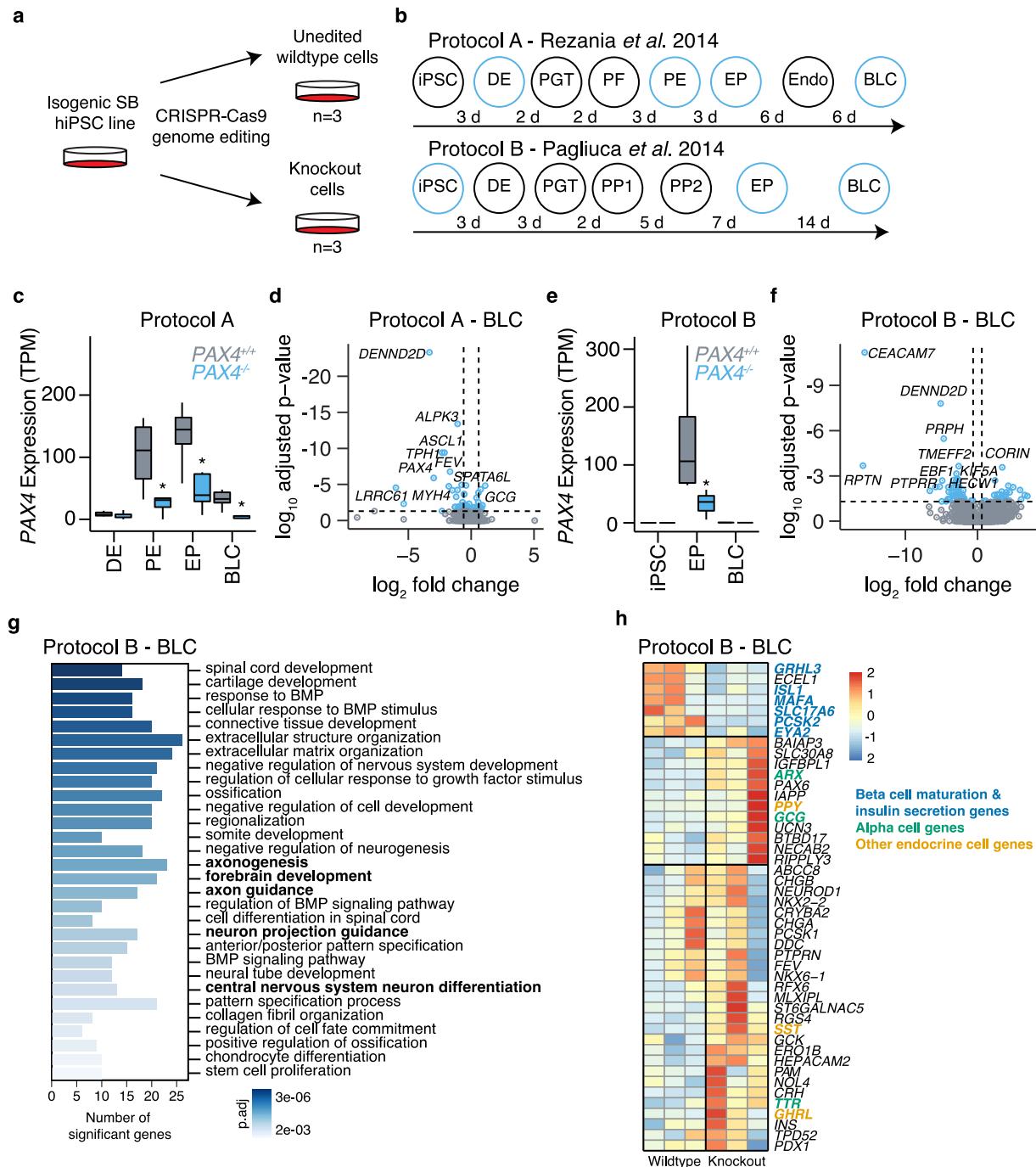
1228 sampled intravenous glucose tolerance tests to measure Acute Insulin Response to
1229 glucose (AIRg), insulin sensitivity (Si), disposition index (DI), and HOMA-B. Unadjusted
1230 p-value and adjusted p-value (adjusted for age, sex and BMI) are indicated in the table
1231 for AIRg, Si, DI and HOMA-B. **(b-c)** Plasma glucose level (mg/dL) at the **(b)** fasting and
1232 **(c)** 2-hour time points during the oral glucose tolerance test (OGTT) of heterozygous or
1233 homozygous p.Arg192His carriers (n=29) and p.Arg192Arg controls (n=28). **(d)** Ratio of
1234 area under the curve (AUC) insulin to glucose during the 2-hour oral glucose tolerance
1235 test. **(e)** Family pedigree of a Singaporean family with a novel p.Tyr186X *PAX4* variant.
1236 NN, wild-type; NM, heterozygotes; -, genotype not accessible. An arrow indicates the
1237 proband (III-1). Age of diagnosis for diabetes mellitus (DM), gestational diabetes mellitus
1238 (GDM), ischemic heart disease (IHD), coronary artery bypass grafting (CABG), impaired
1239 glucose tolerance (IGT), permanent pace-maker implantation (PPM), chronic renal failure
1240 (CRF), and diabetic retinopathy (DR). **(f)** Summary of measures of beta cell function
1241 between family members in **(e)** during a 2-hour 75 g glucose oral glucose tolerance test.
1242 Carriers of the p.X186 allele are in bold. HbA1c, hemoglobin A1c; HOMA-IR, homeostatic
1243 model assessment of insulin resistance (value >2 indicates insulin resistance); DI,
1244 disposition index (Matsuda); ND, not done. Data are presented as mean \pm SEM in Fig. 1b-
1245 d. Statistical analyses were performed using unpaired t-test. *p<0.05, **p<0.01.



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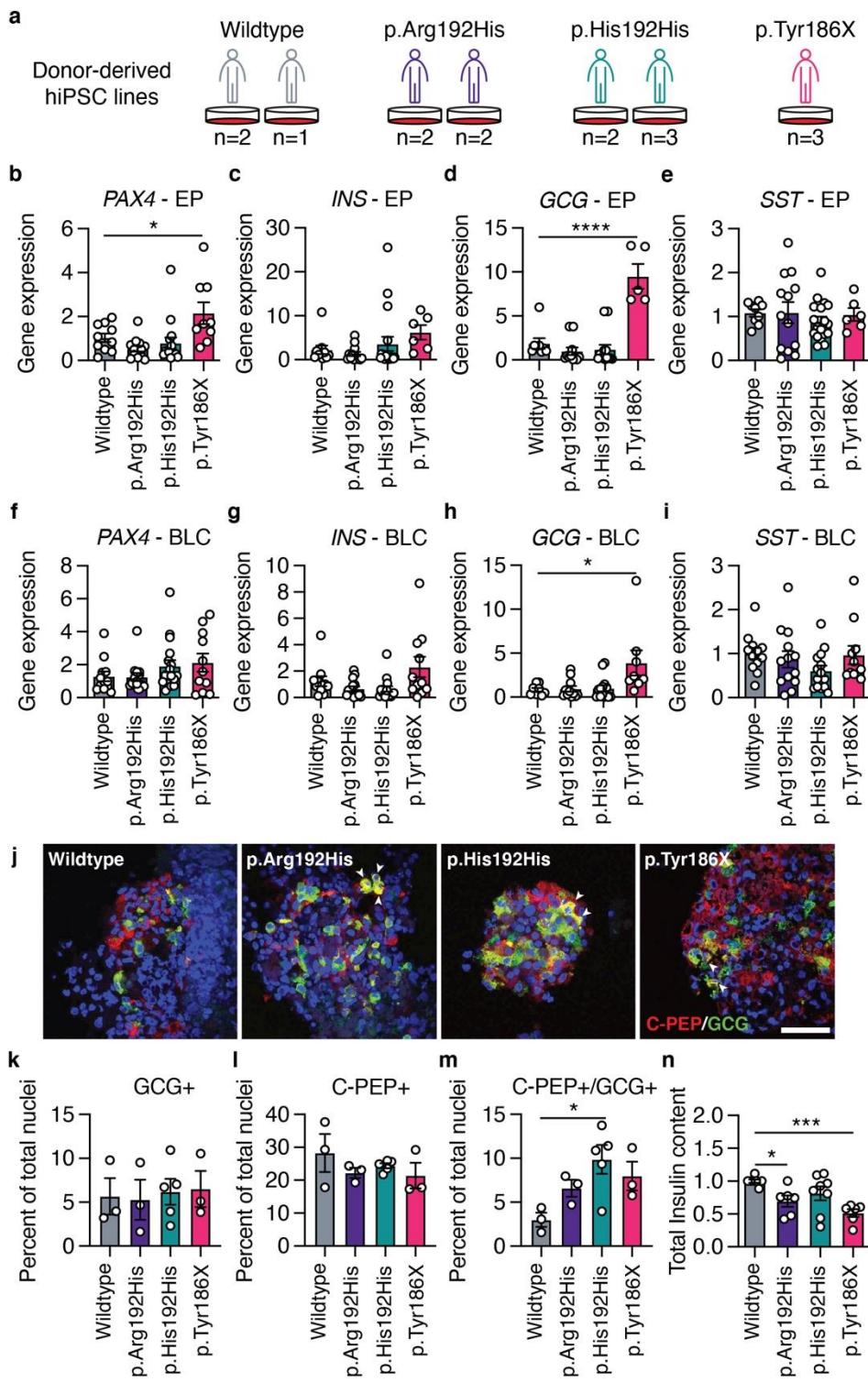
1247 **Figure 2. PAX4 knockdown and knockout impairs glucose-stimulated insulin-
1248 secretion and reduces insulin content in human EndoC- β H1 cells.** (a) Experimental
1249 design for PAX4 knockdown approaches using siRNA and shRNA in EndoC- β H1 cells.
1250 (b) PAX4 gene expression following transient transfection of siPAX4 and non-targeting
1251 (siNT) control in EndoC- β H1 cells. (c) Glucose-stimulated insulin secretion of 2.8 mM and
1252 16.7 mM glucose in siNT and siPAX4 EndoC- β H1 cells, normalized to total protein then
1253 to 2.8 mM glucose. (d) PAX4 gene expression in PAX4-knockdown (shPAX4) and control
1254 (shScramble) EndoC- β H1 cells following six passages of antibiotic selection. (e) Glucose-
1255 stimulated insulin secretion assay comparing shPAX4 and shScramble EndoC- β H1 cells,
1256 normalized to total DNA and then to 2.8 mM glucose. (f) Relative fold change in total

1257 insulin content in shPAX4 and shScramble EndoC-βH1 cells, normalized to total DNA
1258 content. **(g)** *INS* transcript expression in shScramble and shPAX4 EndoC-βH1 cells. **(h)**
1259 *GCG* transcript expression in shScramble and shPAX4 EndoC-βH1 cells. Data are
1260 presented as mean±SEM. Statistical analysis of two samples was performed by paired t
1261 test or a two-way ANOVA for comparison of multiple groups. *p<0.05, ***p<0.001,
1262 ****p<0.0001. n=3-12.



1264 **Figure 3. Isogenic *PAX4* knockout human induced pluripotent stem cell lines have**
1265 **defects in endocrine cell formation during *in vitro* differentiation.** (a) CRISPR-Cas9
1266 genome editing was used to generate three independent isogenic hiPSC *PAX4*
1267 homozygous knockout cell lines and three unedited wildtype control cell lines. (b)
1268 Schematic outline of seven-stage protocol A and six-stage protocol B from human
1269 induced pluripotent stem cells (hiPSC), definitive endoderm (DE), primitive gut tube
1270 (PGT), posterior foregut (PF) or pancreatic progenitor 1 (PP1), pancreatic endoderm (PE)
1271 or pancreatic progenitor 2 (PP2), endocrine progenitor (EP), endocrine (Endo), towards
1272 beta-like cells (BLC). RNA-seq samples were collected at the end of stages highlighted
1273 in blue. (c) Expression of *PAX4* in transcripts per million (TPM) in *PAX4*^{+/+} and *PAX4*^{-/-}
1274 cells at DE, PE, EP and BLC derived from Protocol A. (d) Volcano plot of differentially
1275 expressed genes in *PAX4*^{-/-} versus *PAX4*^{+/+} BLCs derived from Protocol A. The top ten
1276 differentially expressed genes are highlighted. (e) Expression of *PAX4* in TPM in *PAX4*^{+/+}
1277 and *PAX4*^{-/-} cells at hiPSCs, EPs and BLCs derived from Protocol B. (f) Volcano plot of
1278 differentially expressed genes in *PAX4*^{-/-} versus *PAX4*^{+/+} BLCs derived from Protocol B.
1279 The top ten differentially expressed genes are highlighted. (g) Gene Ontology (GO)
1280 analysis of differentially expressed genes in *PAX4*^{-/-} compared to *PAX4*^{+/+} BLCs from
1281 Protocol B. (h) Heatmap of relative gene expression for pancreatic endocrine genes in
1282 BLCs derived from Protocol B.

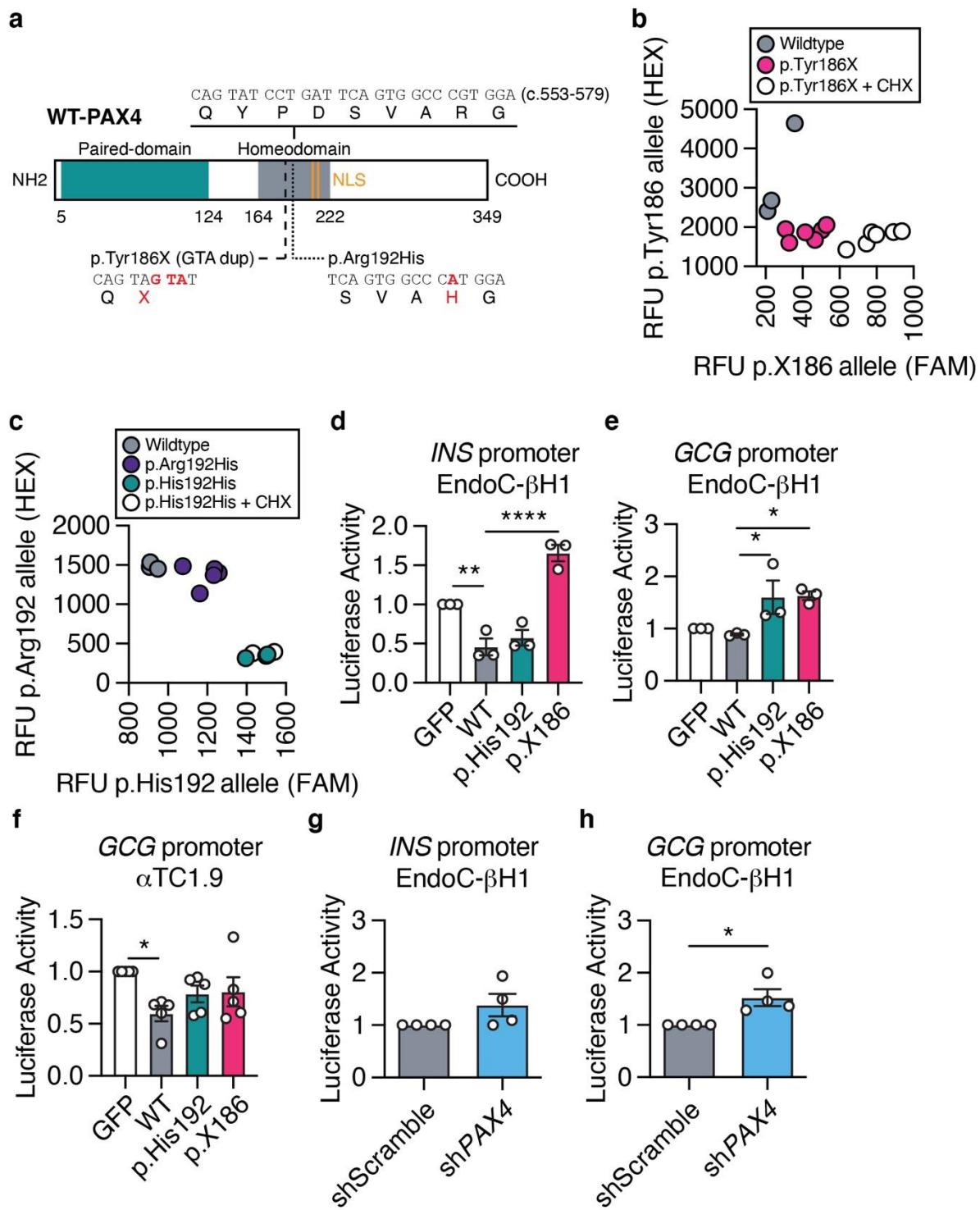
1283



1284

1285 **Figure 4. *PAX4* p.Arg192His and p.Tyr186X donor-derived hiPSCs have**
 1286 **perturbations in differentiation towards BLCs. (a)** Donor-derived hiPSC lines were
 1287 generated from the following genotypes: three lines from two wildtype donors; four lines

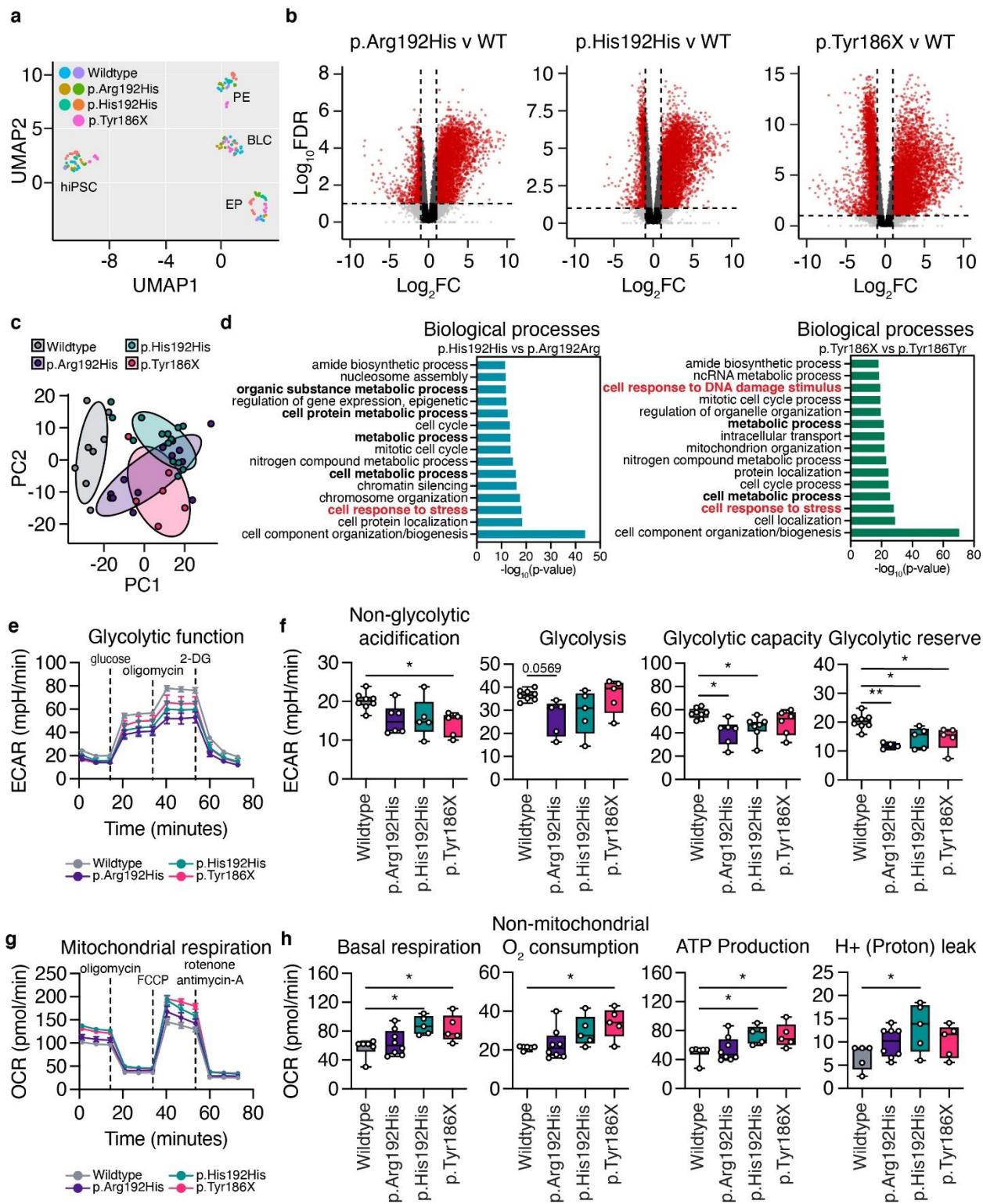
1288 from two p.Arg192His donors; five lines from two p.His192His donors; and three lines
1289 from one p.Tyr186X donor. **(b-e)** Transcript expression of **(b)** *PAX4*, **(c)** *INS*, **(d)** *GCG*
1290 and **(e)** *SST* in hiPSC-derived endocrine progenitor (EP) cells using differentiation
1291 Protocol B. **(f-i)** Transcript expression of **(f)** *PAX4*, **(g)** *INS*, **(h)** *GCG* and **(i)** *SST* in hiPSC-
1292 derived beta-like cells (BLCs) using differentiation Protocol B. **(j)** Representative
1293 immunofluorescence images of hiPSC-derived beta-like cells with C-peptide in red,
1294 glucagon in green, and nuclei in blue. Arrows indicate C-PEP+/GCG+ double-positive
1295 cells. Scale bar: 50 μ m. **(k-m)** Quantification of immunofluorescence images for the
1296 percentage of cells expressing **(k)** *GCG* (monohormonal), **(l)** C-PEP (monohormonal) or
1297 **(m)** C-PEP+/GCG+ (polyhormonal). **(n)** Total insulin content normalized to total DNA from
1298 hiPSC-derived BLCs. Data are presented as mean \pm SEM. n>3. Statistical analyses were
1299 performed using one-way ANOVA. *p<0.05, ***p<0.001, and ****p<0.0001.



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1301 **Figure 5. Molecular function of PAX4 variants in human EndoC-βH1 cells. (a)**
1302 Illustration of human full-length wildtype (WT) PAX4 protein. Functional domains are
1303 depicted in green (paired-domain) and grey (homeodomain). Nuclear localization

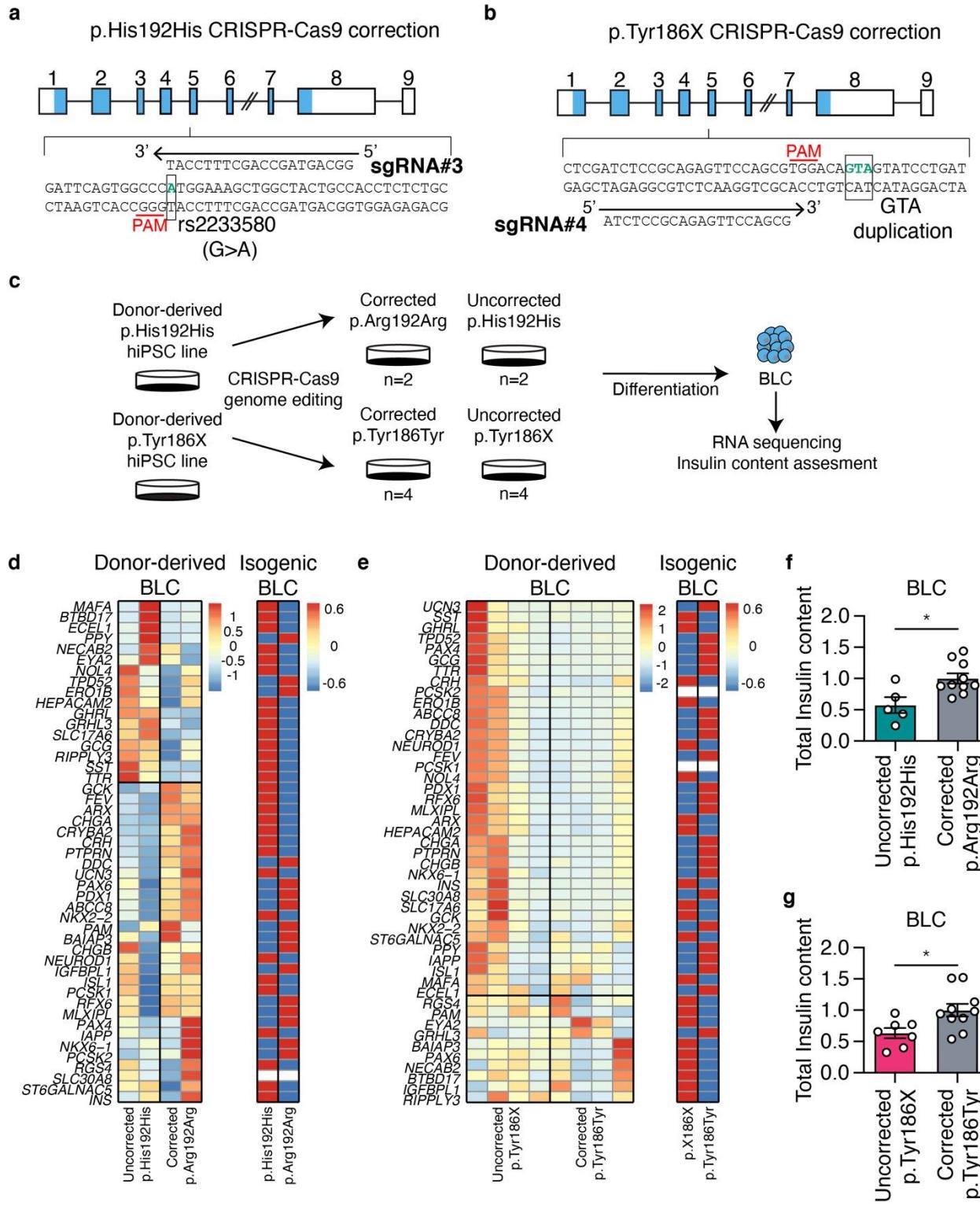
1304 sequences (NLS; orange) are located at amino acids 206-210 and 212-216. Part of the
1305 homeodomain sequence that contains the *PAX4* variants (c.553-579) and the amino acid
1306 changes of p.Tyr186X and p.Arg192His are highlighted. **(b)** Allele-specific qPCR of *PAX4*
1307 transcript for p.Tyr186 and p.X186 alleles following cycloheximide (CHX) treatment. **(c)**
1308 Allele-specific qPCR of *PAX4* transcript for p.Arg192 and p.His192 alleles following CHX
1309 treatment. **(d-e)** Luciferase activity of **(d)** *INS* and **(e)** *GCG* gene promoters in EndoC-
1310 β H1 cells overexpressing WT-*PAX4*, p.His192 and p.X186. **(f)** Luciferase activity of the
1311 *GCG* gene promoter in α TC1.9 cells overexpressing WT-*PAX4*, p.His192 and p.X186. **(g-
1312 h)** Luciferase activity of **(g)** *INS* and **(h)** *GCG* gene promoters in shScramble and sh*PAX4*
1313 EndoC- β H1 cells. Data are presented as mean \pm SEM. Statistical analyses were
1314 performed by t-test or two-way ANOVA. n>3. *p<0.05, **p<0.01, ****p<0.0001.
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Figure 6. Metabolic seahorse assays revealed alterations in glycolysis function and oxidative phosphorylation in the presence of the *PAX4* p.Arg192His or p.Tyr186X

1320 **variants.** **(a)** Uniform Manifold Approximation and Projection (UMAP) of 153 RNA
1321 samples at the hiPSC, PE, EP and BLC stages of *in vitro* differentiation using Protocol B.
1322 **(b)** Volcano plots [$\text{Log}_{10}\text{FDR}$ and $\log_2(\text{FC})$] demonstrating pairwise comparisons of
1323 p.Arg192His, p.His192His, and p.Tyr186X against wildtype, respectively. Red circles
1324 represent transcripts with $\log_2\text{FC} < -2$ or > 2 and $p < 0.05$. **(c)** Principal component analysis
1325 (PCA) of RNA-seq data for *PAX4* donor hiPSC-derived EP cells. PC1: 35%; PC2: 11%.
1326 **(d)** Gene ontology (GO) analysis of differentially expressed genes in EP comparing
1327 p.His192His against p.Arg192Arg (*PAX4*^{+/+}) or p.Tyr186X against p.Tyr186Tyr (*PAX4*^{+/+}),
1328 $\text{FC} < 0.67$ or $\text{FC} > 1.5$. The bars denote $-\log_{10}(\text{p-value})$ with $\text{FDR} < 0.05$. **(e)** Extracellular
1329 acidification rate (ECAR) measurements of wildtype, p.Arg192His, p.His192His, and
1330 p.Tyr186X EP cells following a sequential addition of glucose, oligomycin, and 2-
1331 deoxyglucose (2-DG). **(f)** Non-glycolytic acidification, glycolysis, glycolytic capacity, and
1332 glycolytic reserve measurements during the ECAR of wildtype, p.Arg192His,
1333 p.His192His, and p.Tyr186X EP cells. **(g)** Oxygen consumption rate (OCR)
1334 measurements of wildtype, p.Arg192His, p.His192His, and p.Tyr186X EP cells following
1335 a sequential addition of oligomycin, FCCP, rotenone and antimycin-A. **(h)** Basal
1336 respiration, non-mitochondrial O_2 respiration, ATP production, and H^+ (proton) leak of
1337 wildtype, p.Arg192His, p.His192His, and p.Tyr186X EP cells. Data are presented as
1338 mean \pm SEM. $n > 3$. Statistical analyses were performed by one-way ANOVA. * $p < 0.05$,
1339 ** $p < 0.01$.



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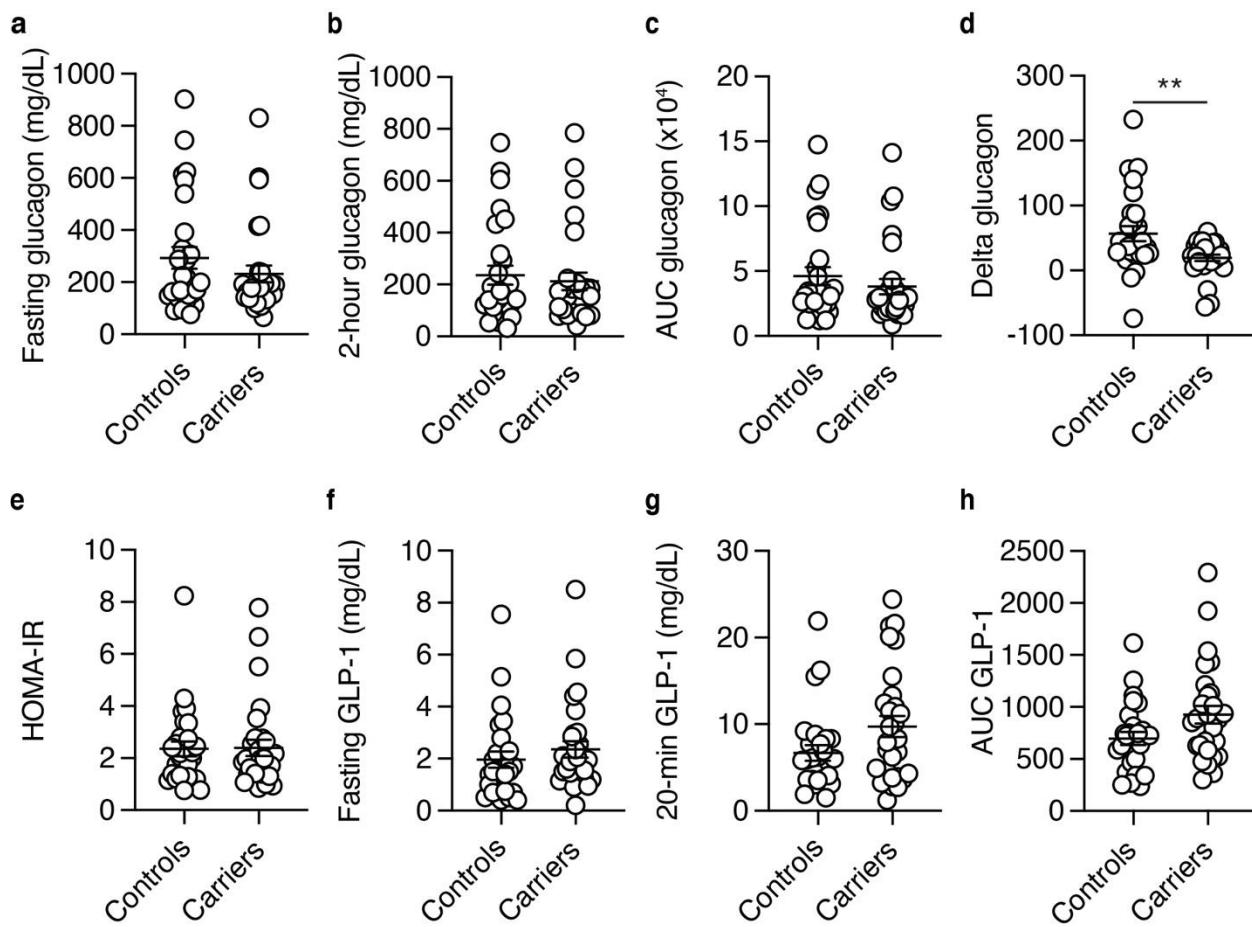
Figure 7. CRISPR-correction of p.Arg192His or p.Tyr186X allele demonstrated rescue in beta cell identity and total insulin content. (a) CRISPR-Cas9 gene correction strategy for p.His192His genotype. (b) CRISPR-Cas9 gene correction strategy

1344 for p.Tyr186X genotype. **(c)** Experimental design for CRISPR-Cas9 mediated gene
1345 correction and differentiation strategies. **(d-e)** Targeted heatmap of selected beta and
1346 alpha specific genes comparing uncorrected and corrected donor-derived BLCs and
1347 isogenic BLCs for **(d)** p.His192 and **(e)** p.X186 alleles. **(f-g)** Total insulin content of BLCs
1348 derived from **(f)** uncorrected p.His192His and corrected p.Arg192Arg and **(g)** uncorrected
1349 p.Tyr186X and corrected p.Tyr186Tyr. n>3. Data are presented as mean±SEM.
1350 Statistical analyses were performed by Student's t-test, *p<0.05.

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Extended Data Figures

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Extended Data Fig. 1 | Clinical assessment of glucagon, HOMA-IR, and GLP-1 in carriers of p.Arg192His PAX4 variant.

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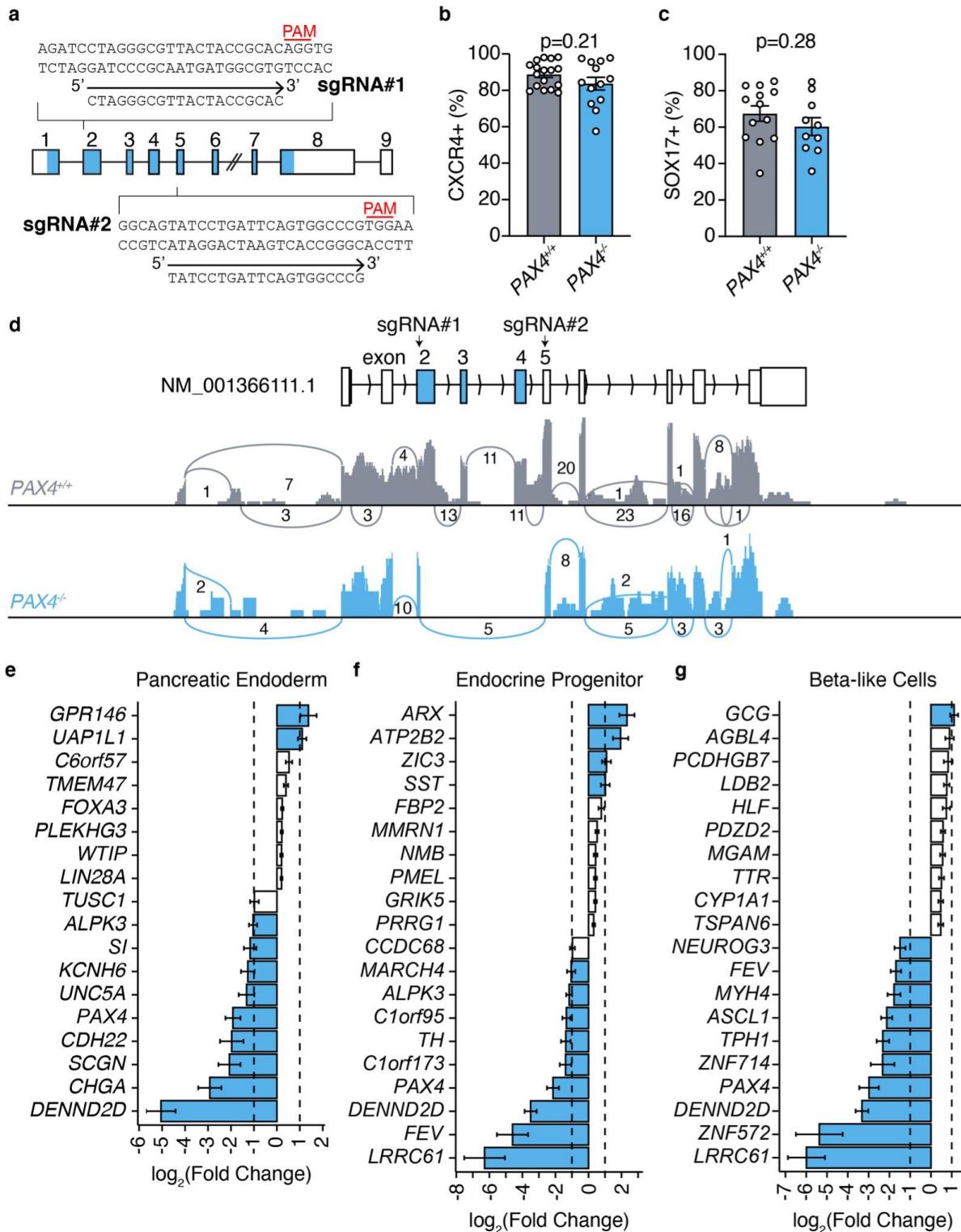
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(a-d) Plasma glucagon level (mg/dL) at (a) fasting, (b) 2-hour time point, (c) area under the curve (AUC), and (d) delta glucagon during oral glucose tolerance test of p.His192 allele carriers (n=29) and p.Arg192Arg controls (n=28). (e) HOMA-IR measurement of p.Arg192Arg controls and p.His192 carriers during the 2-hour oral glucose tolerance test. (f) Fasting, (g) 20-min, and (h) AUC GLP-1 measurements during oral glucose tolerance test. Data are presented as mean \pm SEM. Statistical analyses were performed using unpaired t-test. *p<0.05, **p<0.01.



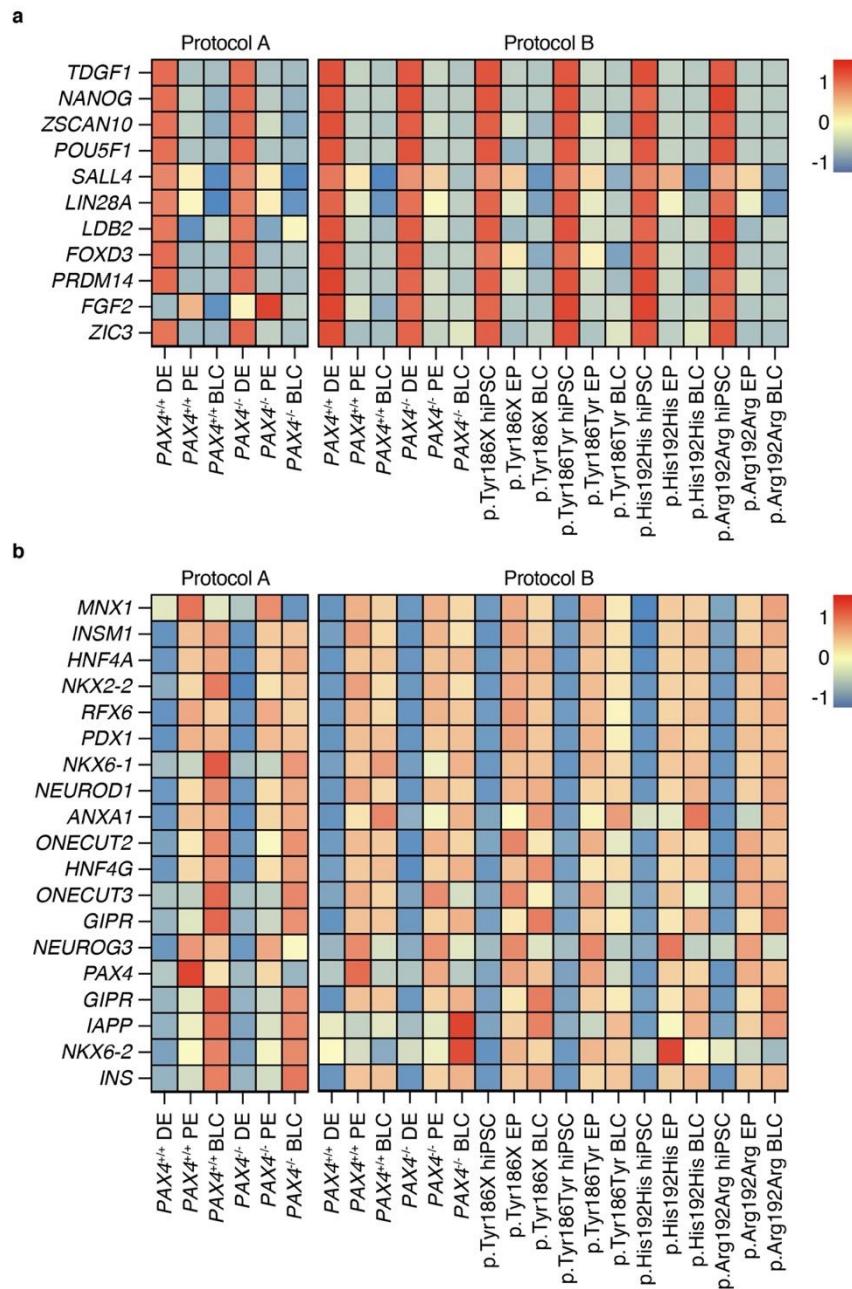
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1366 **Extended Data Fig. 2 | Validation of *PAX4*^{-/-} human induced pluripotent stem cells.**

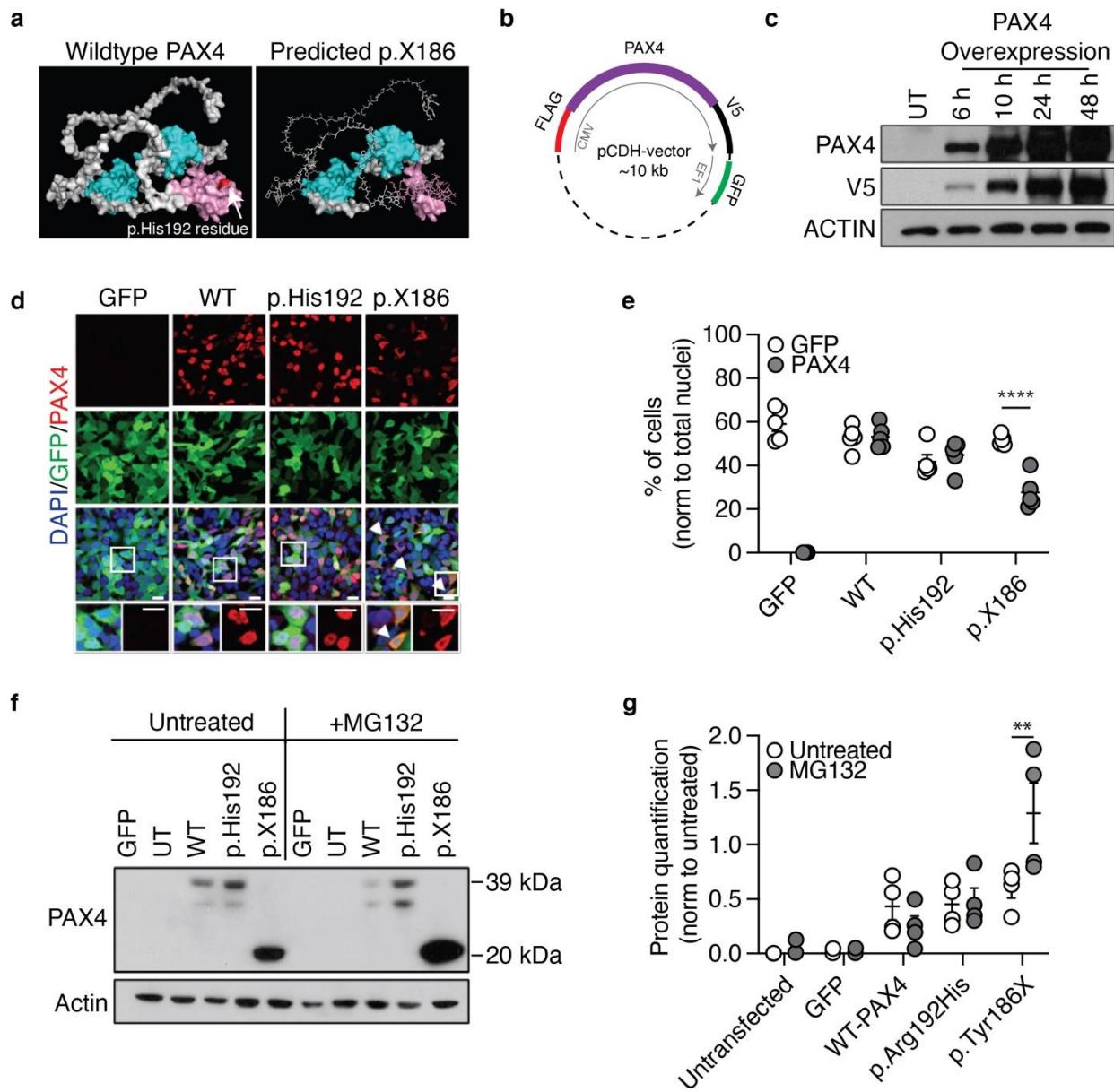
1367 **(a)** CRISPR-Cas9 genome editing strategy to generate *PAX4*^{-/-} hiPSC isogenic line. Two

1368 sgRNAs were designed to target exon 2 (sgRNA#1) and exon 5 (sgRNA#2). PAM
1369 genomic sequence is highlighted in red. **(b-c)** Flow cytometry assessment of definitive
1370 endoderm markers **(b)** CXCR4 and **(c)** SOX17 of wildtype (*PAX4^{+/+}*) and *PAX4*-knockout
1371 (*PAX4^{-/-}*) DE cells. **(d)** Sashimi plot of *PAX4* transcript from Protocol A confirmed the loss
1372 of exons 2 through 5 in *PAX4^{-/-}* lines. **(e-g)** Log₂(Fold Change) expression of top
1373 differentially expressed genes that are expressed in **(e)** pancreatic endoderm, **(f)**
1374 endocrine progenitor, and **(g)** beta-like cell stages. Blue bars represent genes with a
1375 log₂(Fold Change) >1 or <-1.



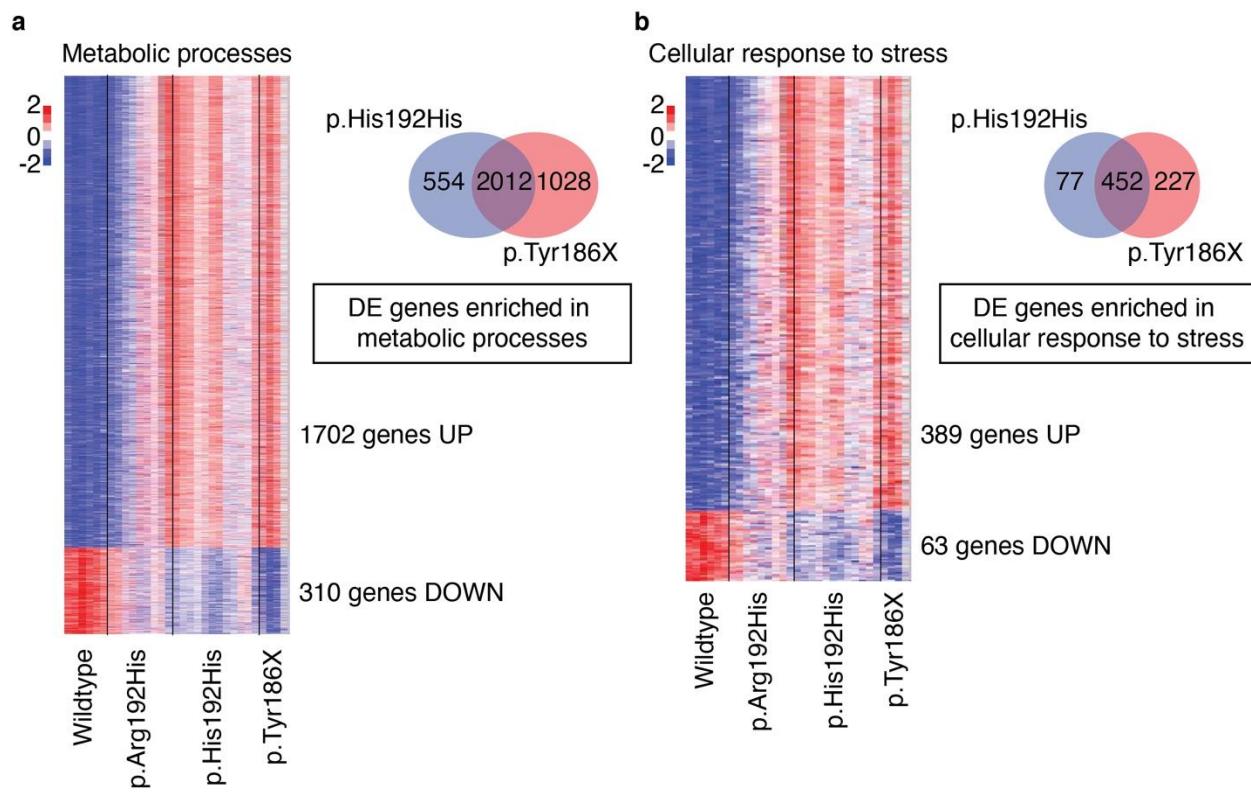
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1378 **Extended Data Fig. 3 | *PAX4*^{-/-} and variant lines have similar repression of**
1379 **pluripotency and activation of endocrine genes as wildtype and corrected lines. (a)**
1380 **Key pluripotency and (b) endocrine progenitor gene expression in hiPSCs, DE cells, EPs,**
1381 **and BLCs differentiated using Protocols A and B of *PAX4* wildtype (*PAX4*^{+/+}), knockout**
1382 **(*PAX4*^{-/-}), *PAX4* variants (p.His192His and p.Tyr186X), and corrected (p.Arg192Arg and**
1383 **p.Tyr186Tyr) donor-derived hiPSC lines.**



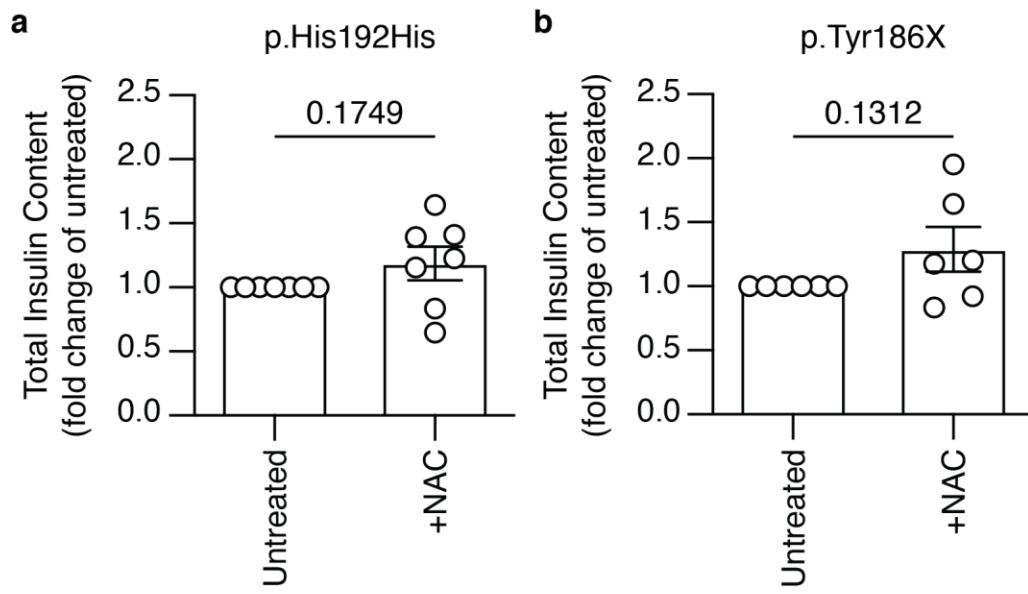
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1386 **Extended Data Fig. 4 | Characterization of PAX4 and its variant proteins.**
1387 (a) Predicted PAX4 protein structure obtained from AlphaFold (AF-O43316-F1-
1388 model_v2). PyMOL was used for molecular visualization. Using wildtype PAX4 as
1389 template, p.X186 protein was extrapolated to demonstrate protein truncation. (b) (c)
1390 Construct design for PAX4 overexpression studies. (c) Western blot assessment of PAX4
1391 protein, V5 tag (~37 kD) and ACTIN loading control in AD293 cells transfected with pCDH-
1392 WT-PAX4 plasmid for 6, 10, 24 and 48 hours compared to untransfected (UT) control. (d)
1393 Representative immunofluorescent images of PAX4 (red), GFP (green), and nuclei
1394 (DAPI; blue) in AD293 cells following transfection of WT PAX4, p.His192, or p.X186
1395 expressing plasmids. Scale bar = 10 μ m. (e) Quantification of GFP- and PAX4-expressing

1396 cells from immunofluorescence in (d). Percentage of cells expressing PAX4 or GFP was
1397 normalized to the total number of nuclei (DAPI). Statistical analyses were performed using
1398 two-way ANOVA and Sidak's multiple comparisons test, ***p<0.0001. (f) Representative
1399 image of western blot assessment and (g) densitometry quantification for WT PAX4,
1400 p.His192 and p.X186 was overexpressed in AD293 cells and normalized to ACTIN
1401 loading control. Cells were treated with or without 10 μ M of MG132 for 24 hours
1402 posttransfection. Molecular weights of 37 kD and 20 kD correspond to WT PAX4 and
1403 p.X186 truncated protein, respectively. n = 4. Statistical analyses were performed using
1404 two-way ANOVA and Sidak's multiple comparisons test, **p<0.01.

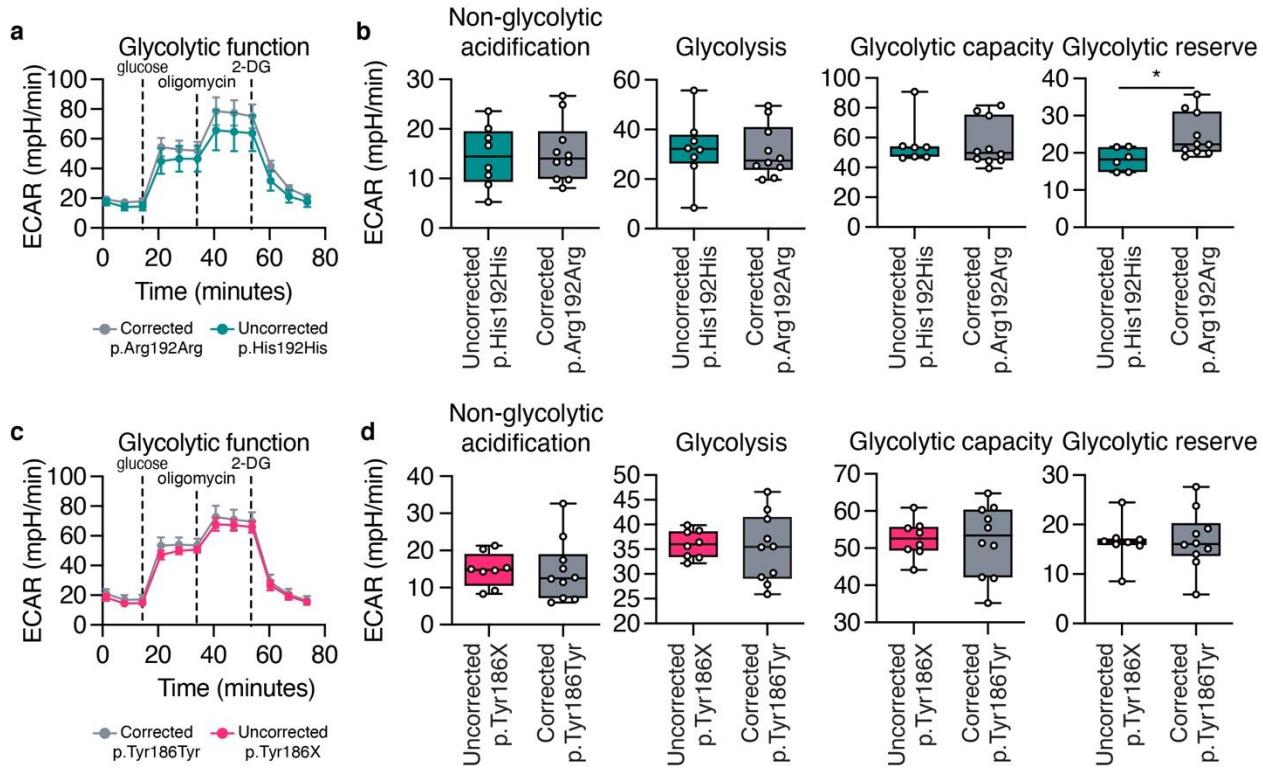


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Extended Data Fig. 5 | RNA-seq revealed elevated metabolic stress in endocrine progenitors derived from donor-derived hiPSCs carrying *PAX4* variants. Targeted heatmap of differentially expressed genes in endocrine progenitors that are involved in (a) metabolic processes (total gene count: 2012; upregulated: 1702; downregulated: 310) and (b) cellular response to stress (total gene count: 452; upregulated: 389; downregulated: 63). Venn diagram illustrating differentially expressed (DE) genes enriched in GO terms (a) metabolic processes or (b) biological processes when comparing p.His192His or p.Tyr186X against *PAX4*^{+/+} with FC < 0.5 or FC > 2.



1418 **Extended Data Fig. 6 | Antioxidant treatment does not rescue the total insulin**
1419 **content in compromised BLCs.** Total insulin content of BLCs treated with 10 μ M
1420 antioxidant NAC from EP to BLC stage carrying (a) p.His192His or (b) p.Tyr186X. Each
1421 dot represents an average of technical replicates of one hiPSC line from one experiment.
1422 n>3. Data are presented as mean \pm SEM. Statistical analyses were performed by Student's
1423 t-test, *p<0.05.



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1425 **Extended Data Fig. 7 | Metabolic stress is not the main causative factor for**
 1426 **compromised BLCs.** Glycolysis stress test on hiPSC-derived EP cells generated using
 1427 protocol B. Extracellular acidification rate (ECAR) profiles of EP cells of (a-b) p.Arg192Arg
 1428 (corrected) against p.His192His (uncorrected) and (c-d) p.Tyr186Tyr (corrected) against
 1429 p.Tyr186X (uncorrected). Each data point represents the average measurement rate of
 1430 technical replicates from one cell line. n>3. Data are presented as mean±SEM. Statistical
 1431 analyses were performed by Student's t-test, *p<0.05.

1432 **Supplementary Tables**

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1434 **Table S1: Aggregated gene-level exome-sequencing association data from**
1435 **Common Metabolic Disease Portal and UKBioBank.**

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1437 **Table S2: Differential Expression Analysis of *PAX4*^{+/+} and *PAX4*^{-/-} cells**
1438 **differentiated using Protocol A, Related to Figure 3.**

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1440 **Table S3: Differential Expression Analysis of *PAX4*^{+/+} and *PAX4*^{-/-} cells**
1441 **differentiated using Protocol B, Related to Figure 3.**

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1443 **Table S4: Differential Expression Analysis of donor-derived hiPSC lines (*PAX4*^{+/+},**
1444 **p.Arg192His, p.His192His, p.Tyr186X) differentiated using Protocol B, Related to**
1445 **Figure 4.**

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1447 **Table S5: Differential Expression Analysis of donor-derived hiPSC lines**
1448 **(uncorrected p.His192His and CRISPR-Cas9 corrected p.Arg192Arg) differentiated**
1449 **using Protocol B, Related to Figure 7.**

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1451 **Table S6: Differential Expression Analysis of donor-derived hiPSC lines**
1452 **(uncorrected p.Tyr186X and CRISPR-Cas9 corrected p.Tyr186Tyr) differentiated**
1453 **using Protocol B, Related to Figure 7.**