

# Paired box 6 (PAX6) regulates glucose metabolism via proinsulin processing mediated by prohormone convertase 1/3 (PC1/3)

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## Abstract

**Aims/hypothesis** Human patients with aniridia caused by heterozygous *PAX6* mutations display abnormal glucose metabolism, but the underlying molecular mechanism is largely unknown. Disturbed islet architecture has been proposed as the reason why mice with complete inactivation of paired box 6 (PAX6) in the pancreas develop diabetes. This is not, however, the case in human aniridia patients with heterozygous *PAX6* deficiency and no apparent defects in pancreatic development. We investigated the molecular mechanism underlying the development of abnormal glucose metabolism in these patients.

**Methods** A human aniridia pedigree with a *PAX6* R240Stop mutation was examined for abnormal glucose metabolism using an OGTT. The underlying mechanism

was further investigated using *Pax6* R266Stop mutant small-eye mice, which also have abnormal glucose metabolism similar to that in *PAX6* R240Stop mutation human aniridia patients.

**Results** Paired box 6 (PAX6) deficiency, both in aniridia patients with a heterozygous *PAX6* R240Stop mutation and in mice with a heterozygous *Pax6* R266Stop mutation, causes defective proinsulin processing and abnormal glucose metabolism. PAX6 can bind to the promoter and directly upregulate production of prohormone convertase (PC)1/3, an enzyme essential for conversion of proinsulin to insulin. *Pax6* mutations lead to PC1/3 deficiency, resulting in defective proinsulin processing and abnormal glucose metabolism.

**Conclusions/interpretation** This study indicates a novel function for PAX6 in the regulation of proinsulin processing and glucose metabolism via modulation of PC1/3

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production. It also provides an insight into the abnormal glucose metabolism caused by heterozygous *PAX6* mutations in humans and mice.

**Keywords** Glucose metabolism · *PAX6* · PC1/3 · Proinsulin processing

### Abbreviations

ACTH	Adrenocorticotrophic hormone
ChIP	Chromatin immunoprecipitation
EIA	Electroimmunoassay
GLP-1	Glucagon-like peptide-1
IGT	Impaired glucose tolerance
IPGTT	Intraperitoneal glucose tolerance test
$\alpha$ -MSH	Alpha-melanocyte stimulating hormone
<i>PAX6</i>	Paired box 6
PC	Prohormone convertase
POMC	Pro-opiomelanocortin
qRT-PCR	Quantitative reverse transcription PCR
SSCP	Single-strand conformational polymorphism

### Introduction

*PAX6* encodes a transcription factor and belongs to the paired-box gene family. The protein contains a paired domain, a linker region, a homeodomain and a transactivation region [1, 2]. The paired domain together with the homeodomain binds to the promoter DNA, allowing the transactivation region to regulate the expression of its target gene [3, 4].

Paired box 6 (*PAX6*) is an evolutionarily conserved protein whose amino acid sequences are almost identical between humans and mice [5]. The *Pax6*-null mutant mouse has defects in the development of the eye, brain and pancreas, leading to neonatal lethality [6–10]. Mice with heterozygous *Pax6* mutations can survive but show a phenotype of small eyes [8]. This small-eye phenotype mirrors the condition seen in human aniridia patients, who are characterised by lack of irises caused by heterozygous *PAX6* gene mutations [9, 10].

In 2002, it was reported that four sporadic aniridia patients with *PAX6* mutations developed impaired glucose tolerance (IGT) [11], but the mechanism of how *PAX6* heterozygosity leads to abnormal glucose metabolism is still unclear. Another study showed that mice with a complete loss of *PAX6* in the pancreas developed diabetes [12]; these mutant mice exhibited disturbed islet architecture, suggesting that diabetes in *Pax6* mutant mice is due to abnormal pancreatic development [7, 12, 13]. This, however, is not the case in heterozygous *Pax6* mutant animals,

which do not show apparent defects in pancreatic development [14]. Therefore, it is still largely unknown why aniridia patients with *PAX6* mutations develop abnormal glucose metabolism.

To address these questions, we investigated an extended aniridia family and identified a pedigree carrying a heterozygous *PAX6* gene mutation. We also obtained mutant mice that had both the *Pax6* gene mutation and the small-eye phenotype. Using this heterozygous *Pax6* mutant mouse model, we further investigated how the *Pax6* gene mutation caused abnormal glucose metabolism in these mice and probably in the human aniridia patients, too.

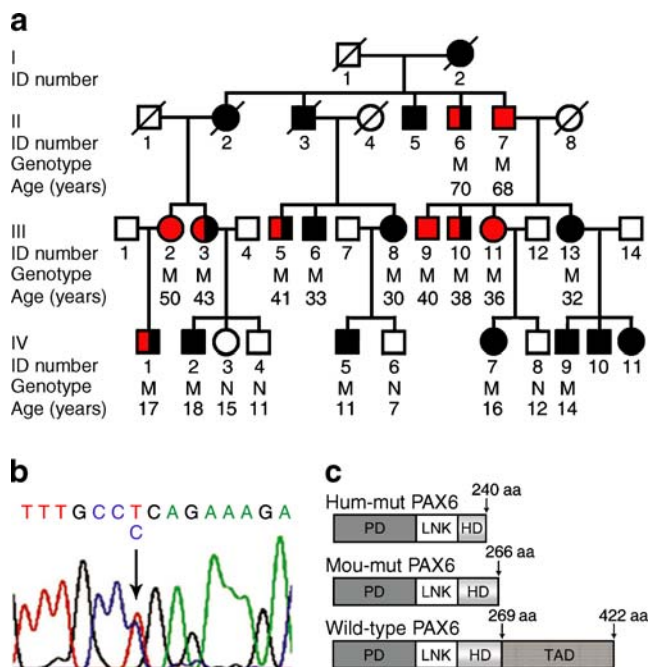
### Methods

*Participants: identification of a pedigree with the PAX6 gene mutation* The family with aniridia was identified at Peking University Third Hospital. This study was approved by the Ethics Committee of Peking University Health Science Center and carried out according to the guidelines of the World Medical Association Declaration of Helsinki ([www.wma.net/e/policy/b3.htm](http://www.wma.net/e/policy/b3.htm)). All participants (or their parents) were fully aware of the scope of this study and signed an informed consent form prior to enrolment.

The aniridia pedigree consisted of 19 living patients (Fig. 1a) and four unaffected consanguineous members (Fig. 1a). Of the 19 patients, 16 (all patients except for II-5, IV-10 and IV-11; Fig. 1a) were available for this study, together with all four unaffected consanguineous members (IV-3, IV-4, IV-6 and IV-8; Fig. 1a). To screen for the *PAX6* mutation, each exon of the *PAX6* gene was amplified from genomic DNA as others have reported [9, 15]. The mutation site was determined by analysing PCR products from each exon by single-strand conformational polymorphism (SSCP) [16]. The PCR products found to have an abnormal pattern were sequenced to confirm the mutation.

*Collection of human and mouse blood samples* Venous blood samples were collected from all available members in the family at fasting and at 30, 60 and 120 min after glucose loading. The amount of glucose ingested for adults was 75 g per person. For children this was adjusted according to the formula 1.75 g/kg body weight, with total intake held to less than 75 g. Genomic DNA was prepared from venous blood samples.

For the intraperitoneal glucose tolerance test (IPGTT) in mice, the blood samples were collected at fasting and at 30, 60 and 120 min after injection of glucose. Blood samples were collected from the tail vein into tubes containing anticoagulant (about 1,000 U of heparin per millilitre of blood). Immediately after centrifugation (2,500 g) at 4°C, plasma was separated and stored at –70°C for further analysis.



**Fig. 1** *PAX6* gene mutation leads to IGT and diabetes. **a** Illustration of a single human pedigree with the heterozygous *PAX6* mutation, in which nine of 16 available patients developed IGT or diabetes. White symbols, family members without *PAX6* mutation; black symbols, participants with the *PAX6* mutation but without abnormal glucose metabolism; red symbols, patients with diabetes; black and red symbols, patients with IGT. N, normal allele; M, mutant allele. Genotype and age (years) of each participant are indicated under the ID number. **b** Sequence of the PCR product of exon 9 in *PAX6*. Arrow indicates the mutation site. A heterozygous C to T mutation converts an arginine codon (CGA) to a stop codon (TGA). **c** Schematic mapping of the wild-type *PAX6*, human mutant *PAX6* (Hum-mut *PAX6*) and mouse mutant *PAX6* (Mou-mut *PAX6*). The *PAX6* polypeptide consists of paired domain (PD), linker region (LNK), homeodomain (HD) and transactivation domain (TAD). aa, amino acid

**Measurement of plasma total insulin, proinsulin, adrenocorticotrophic hormone and  $\alpha$ -melanocyte stimulating hormone** Human insulin and proinsulin levels were measured with RIA kits (Linco Research, St Charles, MO, USA). Specific electroimmunoassay (EIA) kits from Phoenix Pharmaceuticals (Mountain View, CA, USA) were used to detect adrenocorticotrophic hormone (ACTH) and alpha-melanocyte stimulating hormone ( $\alpha$ -MSH) levels in humans. The American Diabetes Association criteria were used for diagnosis of IGT and diabetes [17, 18].

Mouse insulin was measured with an ELISA kit from Shibayagi Company (Ishihara Shibukawa-shi, Gumma, Japan). Mouse proinsulin was measured using an ELISA kit for rat proinsulin (Merckodia, Uppsala, Sweden) according to the manufacturer's instructions. Since this kit is for rat proinsulin, the calculated ratios of proinsulin over total insulin in mice are likely to be not precise, but are acceptable for the present purposes of comparison. Specific

EIA kits and protocols (Phoenix Pharmaceuticals, Mountain View, CA, USA) were used to measure levels of ACTH and  $\alpha$ -MSH in tissue extracts of mice.

**Animals, *N*-ethyl-*N*-nitrosourea mutagenesis, mapping and genotyping** C57BL/6J and C3H/HeJ mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). Injection of the chemical mutagen *N*-ethyl-*N*-nitrosourea into male mice often induces sporadic A–T transition in the germ cell genome of the animals. After breeding with normal mice, the mutation can be transmitted to newborns. Based on the small-eye phenotype, mice with the *Pax6* gene mutation can be selected. The mutant mice were crossed to C3H/HeJ to generate F1 mice. The F1 mice with the small-eye phenotype were back-crossed to C57BL/6J to generate F2. Tail DNA samples of F2 mice were extracted and PCR-amplified with D2Mit249 micro-satellite marker of the Whitehead/MIT database (<http://www-genome.wi.mit.edu>). PCR products were separated by electrophoresis with 4% (wt/vol.) agarose II gel (BioBasic, Toronto, ON, Canada). Mice were genotyped by PCR analysis with primers specific for *Pax6*: forward primer 5'-GATGAGATGGGAGTTGGGTG-3', reverse primer: 5'-AAGTTACCAGGTGTGGTGGG-3'.

**Animal care** Mice were maintained in a pathogen-free facility under a 12 h light–dark cycle and were given free access to food and water. The studies were approved by the Peking University animal ethics committee. Animal experiments were carried out in accordance with institutional guidelines and complied with The 1996 Guide for the Care and Use of Laboratory Animals [19].

**IPGTT and insulin tolerance test in mice** After fasting for about 15 h, baseline blood glucose levels were measured. The glucose solution (20%, wt/vol.) was then injected intraperitoneally at 10  $\mu$ l/g body weight and blood glucose levels were measured at 30, 60 and 120 min after the injection. For the insulin tolerance test, mice were fasted for 6 h and then injected with human insulin (Novo-Nordisk, Bagsværd, Denmark) at 0.75 U/kg body weight. Blood glucose levels were measured at fasting and at 15, 30, 45 and 60 min following insulin injections. Blood glucose concentrations were measured with a One Touch Ultra meter (Lifescan, Milpitas, CA, USA).

**Isolation and purification of mouse islets** The islets of mice were isolated and characterised following the method of Hori [20]. Briefly, the pancreas was digested by type V collagenase (Sigma-Aldrich, St Louis, MO, USA) and then purified by centrifugation (2,700 *g*) on Histopaque-1077 gradients (Sigma-Aldrich). Individual islets, free of attached acinar, vascular and ductal tissues, were handpicked under a dissecting microscope.

**Immunofluorescence** The mouse pancreas was fixed for 10 h in 4% (wt/vol.) paraformaldehyde/PBS at 4°C before cryosection. The cryosections were immunostained with normal rabbit or mouse IgG, rabbit antibody to glucagon or mouse antibody to insulin (Zhongshan Goldenbridge Biotechnology, Beijing, China) and incubated overnight at 4°C with anti-rabbit IgG or anti-mouse IgG, and then with mounting medium (DAPI; Vector Laboratories, Burlingame, CA, USA).

**Cell culture** NIT-1 mouse insulinoma cells were cultured in RPMI 1640 with 10% (vol./vol.) FBS. NIH3T3 cells were cultured in DMEM/High Glucose with 10% (vol./vol.) FBS. All cell culture media were obtained from Gibco BRL (Gaithersburg, MD, USA) and the FBS from Hyclone (Thermo Fisher Scientific, Waltham, MA, USA).

**Quantitative RT-PCR** RNA of islets was extracted with RNeasy Micro Kit (Qiagen, Valencia, CA, USA) and reversed to cDNA. The quantitative RT-PCR (qRT-PCR) was performed with primers specific for *Pc1/3* (also known as *Pcsk1*): forward primer 5'-ATGGGCGGCGGAGATC-3', reverse primer 5'-CCAATCTGACCCAAAAGGTCATAC-3'. *GAPDH* was used as a control: forward primer 5'-GCACCACCAACTGCTTAGC-3', reverse primer 5'-TCTTCTGGGTGGCAGTGATG-3'.

**Western blotting analysis** The proteins of islets were separated by 10% (wt/vol.) SDS-PAGE and probed with different antibodies including rabbit PAX6-specific antibody (Chemicon, Temecula, CA, USA), rabbit prohormone convertase (PC)1/3-specific antibody (Abcam, Cambridge, MA, USA) and mouse  $\beta$ -actin-specific monoclonal antibody (Sigma-Aldrich).

**Luciferase reporter assay** A 4,194-bp mouse *Pc1/3* promoter sequence was cloned from mouse genomic DNA and inserted into pGL<sub>3</sub>-basic luciferase reporter vectors. The primers were as follows: forward primer 5'-CGGGGTACCGGGCTGGGAAGGAATAAAATCTGGAGTATAAA-3', reverse primer 5'-CCGCTCGAGTCTTGACGTCAGATCTACCTGGACTGAGAACTGGAT-3'. *Pax6* was cloned and inserted into pcDNA3.1 (-) vectors. The luciferase reporter assay was performed in NIH3T3 cells. The cells were cultivated in 24 well plates for 24 h and transfected by transfection reagent (Lipofectamine 2000; Invitrogen, Carlsbad, CA, USA) with the mouse *Pc1/3* promoter along with either intact or mutated *Pax6* cDNA at several doses. Cells were collected 48 h after transfection and assayed in triplicate with the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA).

**Chromatin immunoprecipitation assays** Chromatin immunoprecipitation (ChIP) assays were performed in a mouse

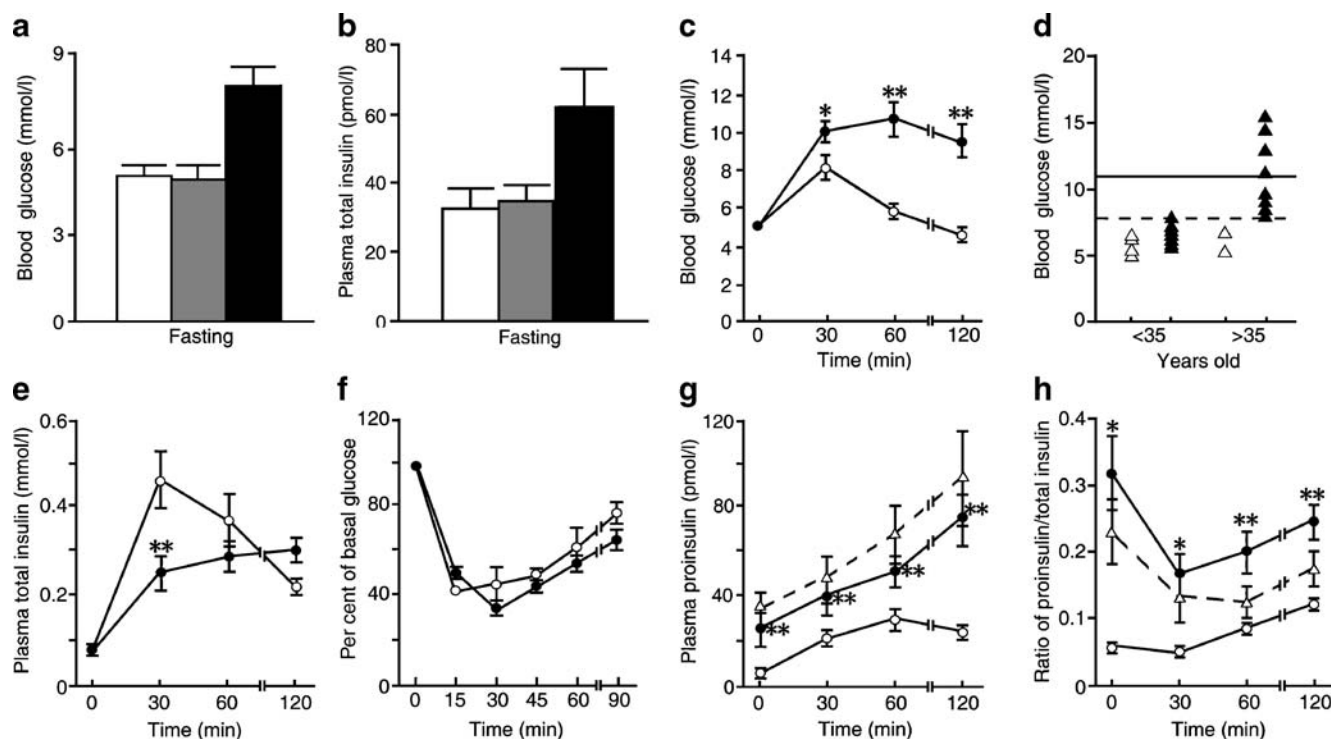
beta cell line, NIT-1 cells from the American Type Culture Collection (ATCC, Manassas, VA, USA). Briefly, the chemical-linked complexes of genomic DNA and PAX6 protein were immunoprecipitated with antibodies specific to PAX6 (Chemicon). The DNA was amplified after ChIP using primers of *Pc1/3* promoter (forward primer 5'-TCATCAAGTCTTGCCTCGTT-3', reverse primer 5'-ATTATCGCTGTCCACACAT-3').

**Statistical analysis** Statistical analysis was performed using an independent samples *t* test. Differences were considered statistically significance at  $p < 0.05$ .

## Results

*A heterozygous PAX6 R240Stop mutation was identified in the aniridia pedigree; affected patients exhibited abnormal proinsulin processing and impaired glucose tolerance* To determine whether there was any mutation in the *PAX6* gene in the identified pedigree, we first narrowed down the region bearing a potential mutation to exon 9 of the *PAX6* gene by SSCP (not shown). Within exon 9 we identified a C-to-T mutation at nucleotide 1080 that converted an Arg codon (CGA) to a stop codon (TGA; Fig. 1b). This mutation resulted in a truncated polypeptide that prematurely terminated at residue 240 (*PAX6* R240Stop mutation; hereafter referred to as the *PAX6* mutation; Fig. 1c), whereas the full length *PAX6* consists of 422 amino acids (Fig. 1c). The R240Stop truncated polypeptide lacked the entire transactivation domain and a small portion of the homeodomain (Fig. 1c). The mutation was not found in the four unaffected consanguineous family members or in the sample of 100 unaffected people.

To investigate whether the stop mutation in *PAX6* might lead to abnormal glucose metabolism in humans, we measured fasting glucose and insulin levels in the blood of each participant and then performed OGTT. Four non-mutation family members and 12 healthy adults were included as normal controls, as well as eight patients with type 2 diabetes (but without the *PAX6* mutation, obesity or diabetic complications). While the patients with type 2 diabetes had significantly higher fasting glucose (Fig. 2a) and insulin levels (Fig. 2b) than the other two groups, there was no difference between the patients with the heterozygous *PAX6* mutation and the normal controls (Fig. 2a,b). In OGTT, on the other hand, the post-load blood glucose levels in the patients with the *PAX6* mutation were significantly higher than those in the controls (Fig. 2c). Interestingly, among the 19 available patients bearing the *PAX6* mutation, seven aged 11 to 33 years (IV-5, IV-9, IV-7, IV-2, III-8, III-13, III-6) appeared to have normal glucose



**Fig. 2** Patients with the heterozygous *PAX6* mutation exhibit abnormal glucose metabolism. Fasting blood glucose levels (**a**) and plasma total insulin levels (**b**) in normal controls (white bars), patients with the *PAX6* mutation (grey bars) and patients with diabetes (black bars). **c** Blood glucose levels in normal controls (white circles) and patients with the heterozygous *PAX6* mutation (black circles) as measured in OGTT. **d** The development of IGT (dashed line) and diabetes (continuous line) in patients with the heterozygous *PAX6* mutation correlated with the ages of the patients. White triangles, normal controls; black triangles, patients with the *PAX6* mutation. **e** Plasma total insulin levels in normal controls (white circles) and

patients with the *PAX6* mutation (black circles) during OGTT. **f** Insulin tolerance test. The percentage of blood glucose levels over basal levels at different time points after injection of insulin in normal controls (white circles) and in patients with the *PAX6* mutation (black circles). **g** Plasma proinsulin levels in normal controls (white circles), patients with the *PAX6* mutation (black circles) and in patients with diabetes (white triangles). **h** The plasma proinsulin/total insulin ratio in normal controls (white circles), patients with the *PAX6* mutation (black circles) and patients with diabetes (white triangles). Each data point represents the mean  $\pm$  SE. \* $p < 0.05$ , \*\* $p < 0.01$

metabolism despite having aniridia (Fig. 1a), but five aged 17 to 70 years (IV-1, III-10, III-5, III-3, II-6) had IGT (Fig. 1a) and four from ages 36 to 68 years (III-11, III-9, III-2, II-7) even developed diabetes (Fig. 1a). It appears that the phenotypes vary according to the ages of the patients bearing the *PAX6* mutation (Fig. 2d). Neither non-mutation family members nor the 12 healthy adults exhibited abnormal glucose metabolism. The clinical variables are shown in Electronic supplementary material (ESM) Table 1.

Total insulin levels (including proinsulin and true insulin) in the blood of patients with the *PAX6* mutation were significantly lower than those in the controls post glucose loading (Fig. 2e). At 2 h after the glucose challenge, total insulin production in the patients with the *PAX6* mutation finally caught up with the levels in the controls (Fig. 2e); nonetheless, blood glucose levels in the same patients with the *PAX6* mutation remained high (Fig. 2c). This suggests that these patients might have defects in insulin production and insulin signalling. To exclude the latter possibility, an insulin tolerance test was

performed. The participants received an insulin injection and blood glucose levels were measured at different time points. As shown in Fig. 2f, patients with the *PAX6* mutation and controls showed no difference in terms of insulin sensitivity. Interestingly, the blood proinsulin levels (Fig. 2g) and the proinsulin/C-peptide ratios (ESM Fig. 1) were much higher in both diabetic patients and in the patients with the *PAX6* mutation than in the controls.

Although blood proinsulin levels in the patients with the *PAX6* mutation were lower than in the patients with type 2 diabetes (Fig. 2g), the proinsulin/total insulin ratio in the former was significantly higher than in the latter (Fig. 2h). Similar results were also observed when the patients with the *PAX6* mutation were subdivided into groups with (1) normal glucose metabolism and (2) abnormal glucose metabolism, i.e. either IGT or diabetes (ESM Fig. 2). Therefore, unlike in the type 2 diabetes patients whose high proinsulin levels may be due to overstimulation by hyperglycaemia [21, 22], the high proinsulin levels in the patients with the *PAX6* mutation most probably resulted from a

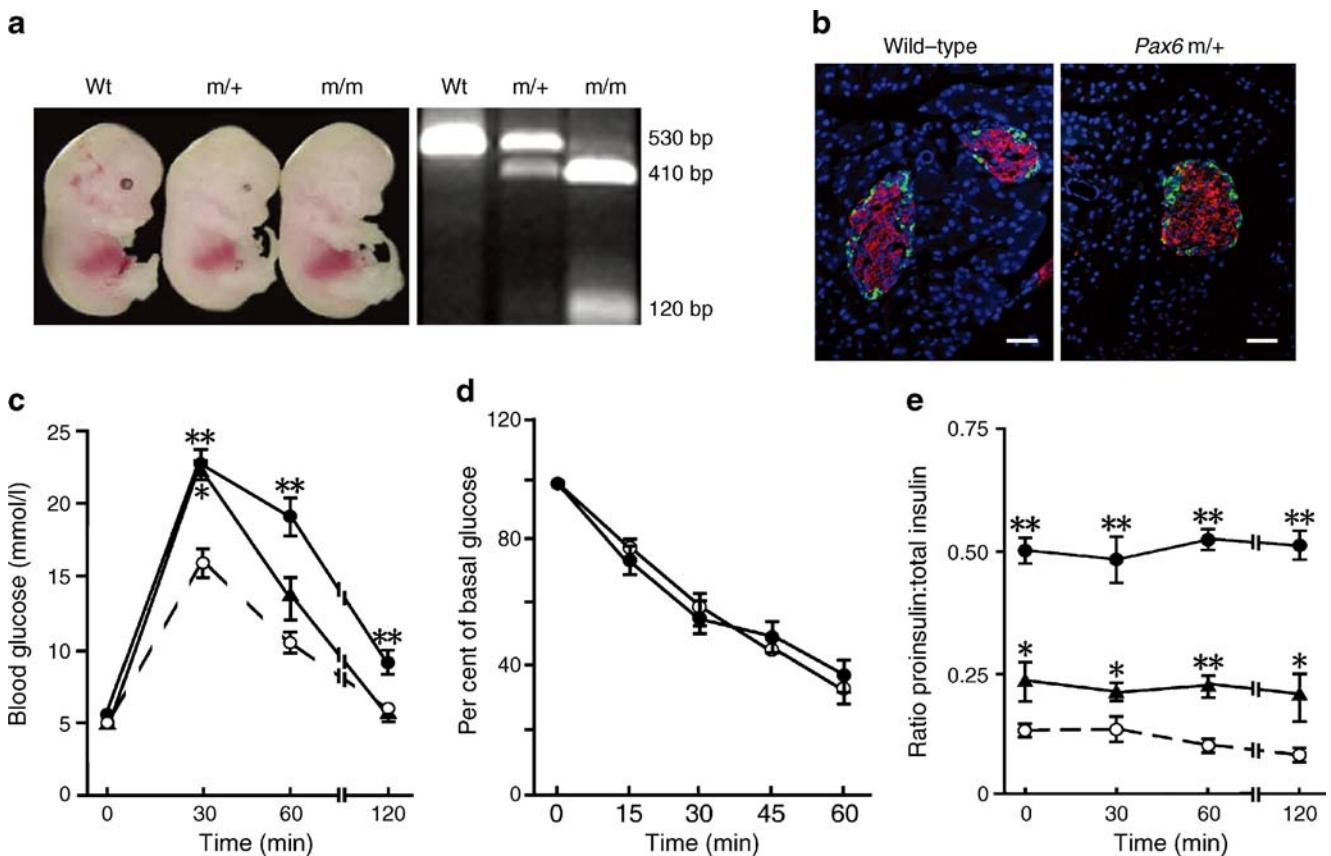
defect in proinsulin processing. In other words, defective proinsulin processing may be the primary cause for development of abnormal glucose metabolism in patients with the *PAX6* mutation.

Cleavage of proinsulin mainly depends on prohormone convertases. Both PC1/3 and PC2 are members of a family of serine proteases encoded on human chromosome 5q14-q21 and 20p11.2, respectively [23, 24]. While pancreatic alpha cells mainly produce PC2, pancreatic beta cells produce PC1/3 and PC2 [25–27]. Based on the pedigree study, we hypothesised that *PAX6* may regulate the production of the PCs and that the *PAX6* gene mutation causes PC deficiency, leading to defective proinsulin processing and, in turn, abnormal glucose metabolism.

*Pax6* heterozygous R266Stop mutant mice exhibited abnormal glucose metabolism similar to that in the human patients with the *PAX6* mutation To identify a *Pax6* mutant

mouse model that displays a mutation similar to the aniridia patients with the *PAX6* mutation, we screened *N*-ethyl-*N*-nitrosourea mutagen-induced mutagenic mice for *Pax6* gene mutations based on the typical small-eye phenotype of *Pax6* mutants [8, 28] and identified a strain of mice with a truncation at residue 266 of the mouse *PAX6* protein (*Pax6* heterozygous R266Stop mutant mice; hereafter referred to as *Pax6* mutant mice) (Figs 1c and 3a).

These mutant mice did not show obvious structural abnormalities in the pancreas (Fig. 3b) as revealed by immunofluorescent staining of insulin and glucagon. Glucose metabolism as measured by IPGTT appeared to be nearly normal in these mutant mice when younger than 4 months (Fig. 3c). However, when older than 6 months, most of these *Pax6* mutant mice, like patients with the *PAX6* mutation above 35 years of age in the pedigree (Fig. 2d), exhibited IGT detected by IPGTT (Fig. 3c), but responded normally to exogenous insulin (Fig. 3d). Simi-



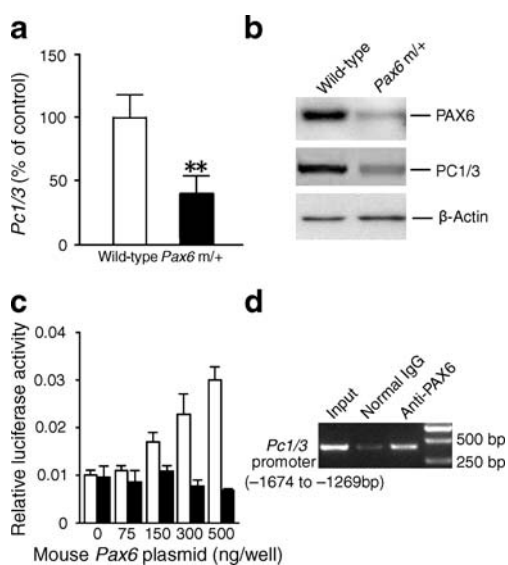
**Fig. 3** *Pax6* mutant mice (*Pax6* m/+) exhibited abnormal glucose metabolism similar to that observed in human patients with the heterozygous *PAX6* mutation. **a** Photo showing phenotypes and genotypes for wild-type (Wt), *Pax6* m/+ and *Pax6* m/m mice. **b** Immunofluorescent staining for total insulin and glucagon in the pancreas of these mice. Red, insulin; green, glucagon; blue, DAPI. Scale bar, 50  $\mu$ m. **c** Blood glucose levels during IPGTT in wild-type mice ( $n=18$ ) (white circles) and in 4-month-old ( $n=7$ ) (black triangles) and 6-month-old (black circles) *Pax6* m/+ mice ( $n=13$ ).

**d** Insulin tolerance test. The percentage of blood glucose levels over the basal levels at different time points after injection of insulin in wild-type mice ( $n=6$ ; white circles) or in 6-month-old *Pax6* m/+ mice ( $n=6$ ; black circles). **e** The plasma proinsulin/total insulin ratios in *Pax6* mutant mice at ages 4 months ( $n=7$ ) (black triangles) or 6 months ( $n=6$ ; black circles) were significantly higher than those in wild-type mice ( $n=7$ ; white circles). Each value represents the mean  $\pm$  SE. \* $p<0.05$ , \*\* $p<0.01$

larily to the patients with the *PAX6* mutation, the proinsulin/total insulin ratios in the *Pax6* mutant mice were significantly higher than in wild-type controls (Fig. 3e). Actually, a high proinsulin/total insulin ratio was also observed in mutant mice at age 4 months that had not shown abnormalities at 120 min in the IPGTT (Fig. 3c,e). These observations indicate that the abnormal proinsulin/total insulin ratio was present before the development of abnormal glucose metabolism in the *Pax6* mutant mice.

In analysing glucose metabolism in these mutants, we documented that the *Pax6* mutant mice share a similarity with the human aniridia patients with the *PAX6* mutation with regard to abnormal glucose metabolism. Using this animal model, we then investigated whether PCs are deficient and if so, how the *Pax6* mutation leads to such a deficiency.

*PAX6* directly regulates *PC1/3* levels and the *Pax6* mutation leads to *PC1/3* deficiency We first analysed the expression of PC genes in the *Pax6* mutant mouse islets by qRT-PCR and found that, between *Pc1/3* and *Pc2*, it was *Pc1/3* that was significantly decreased in the islets of the *Pax6* mutant mice (Fig. 4a). This decrease in *Pc1/3* expression was then confirmed at the protein level by western blotting analysis (Fig. 4b, *PC1/3*).



**Fig. 4** *PAX6* directly regulates *Pc1/3* expression and *Pax6* mutation leads to *PC1/3* deficiency. Expression of *Pc1/3* in isolated islets as measured by qRT-PCR (**a**) or by western blotting analysis (**b**) in wild-type versus *Pax6* mutant mice. **c** *PAX6* (white bars), but not the truncated *PAX6* (black bars), upregulated *Pc1/3* expression as analysed using a luciferase report system. **d** *PAX6* binds directly to the *Pc1/3* promoter in NIT-1 cells as analysed by ChIP assay. Each bar represents the mean  $\pm$  SE. \*\* $p < 0.01$

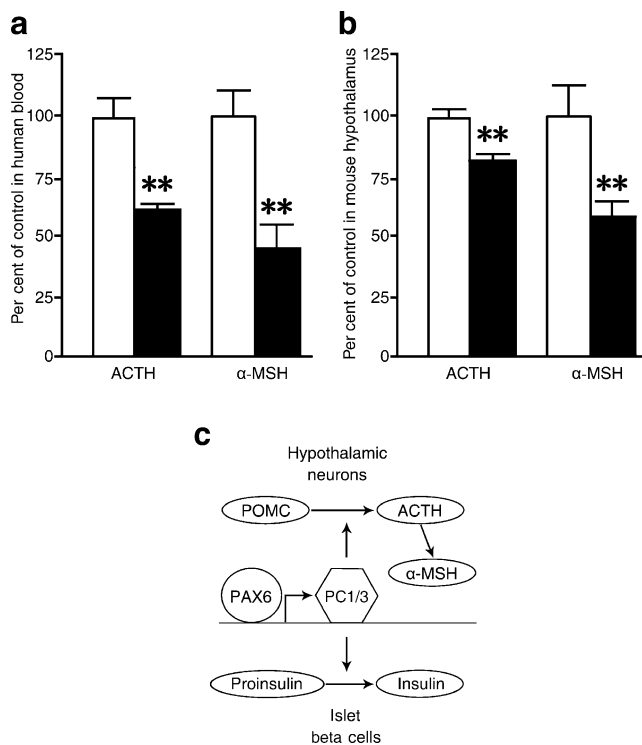
To investigate how the *Pax6* mutation leads to *PC1/3* deficiency, we performed a luciferase reporter assay using *Pc1/3* promoter activity as the variable. *PAX6*, but not the truncated moiety, was found to promote the expression of *Pc1/3* in a dose-dependent manner (Fig. 4c). In agreement with these data, we detected the binding of *PAX6* to the *Pc1/3* promoter by ChIP assay (Fig. 4d) in NIT-1 cells [29, 30]. We therefore concluded that the *Pax6* mutation caused *PC1/3* deficiency, leading to defective proinsulin processing. Since the truncated *PAX6* polypeptide did not show any effect on *Pc1/3* expression (Fig. 4c), we concluded that the *Pax6* mutation leading to *PC1/3* deficiency is a dose-dependent phenomenon rather than a dominant negative effect.

*ACTH* and  $\alpha$ -*MSH* were decreased in the blood of the aniridia patients with the *PAX6* mutation as well as in the brain of *Pax6* mutant mice In addition to the pancreas, *PC1/3* is also present in the hypothalamus, where it converts pro-opiomelanocortin (POMC) into *ACTH*. *ACTH* is further converted into  $\alpha$ -*MSH*, which plays a role in regulation of food intake in mice and humans [31]. If the *PAX6* gene mutation does indeed decrease *PC1/3* production, the production of *ACTH* as well as  $\alpha$ -*MSH* should be reduced in the brain. This was indeed the case. Both *ACTH* and  $\alpha$ -*MSH* were significantly decreased in the peripheral blood of the patients with the *PAX6* mutation (Fig. 5a) as well as in the hypothalamus of the *Pax6* mutant mice (Fig. 5b).

## Discussion

Based on our investigation of aniridia patients with the heterozygous *PAX6* mutation and of small-eye mice with the *Pax6* mutation, we demonstrated that the *PAX6* mutation causes a decrease in *PC1/3*. The resulting *PC1/3* deficiency leads to defective proinsulin processing and subsequent abnormal glucose metabolism. This study thus indicates a novel function for *PAX6* in the regulation of proinsulin processing and glucose metabolism via modulation of *PC1/3* production.

Our finding is consistent with studies by Yasuda et al. [11] and Ashery-Padan et al. [12] in that *PAX6* deficiency leads to abnormal glucose metabolism in mice and in humans. Moreover, we provide evidence that *PAX6* mutations cause *PC1/3* deficiency, resulting in defective proinsulin processing and subsequent abnormal glucose metabolism. This conclusion is partially supported by the fact that *PC1/3* deficiency causes abnormal glucose metabolism in humans and mice [32–35]. However, the phenotype and the primary mechanism may be different.



**Fig. 5** ACTH and  $\alpha$ -MSH levels were decreased in *PAX6* haploinsufficiency mice and patients with heterozygous *PAX6* mutations. **a** ACTH and  $\alpha$ -MSH peripheral blood contents was measured in humans by RIA. The plasma from patients with *PAX6* mutations (black bars) and normal controls (white bars) was collected 120 min after oral glucose. Levels are shown as percentage of control levels (mean $\pm$ SE).  $n=6$  for *Pax6* mutant mice;  $n=9$  for patients with *PAX6* mutations. **b** ACTH and  $\alpha$ -MSH contents in hypothalamus tissue of mice were measured by RIA. Hypothalamus tissue of wild-type (white bars) and *Pax6* mutant mice (black bars) was extracted 120 min after glucose ingestion to detect the concentration of ACTH and  $\alpha$ -MSH. **c** Proposed model for *PAX6* in regulation of proinsulin processing in the islets and of POMC processing in the hypothalamus. Each bar represents the mean $\pm$ SE. \*\* $p<0.01$

For instance, human patients with *PC1/3* mutation or mice with *Pc1/3* N222D/N222D mutation exhibited significant obesity [33, 35]. In our study, we saw no obesity either in the *Pax6* mutant mice or in the patients with the *PAX6* mutation, as evidenced by their normal BMI (ESM Table 1), even though  $\alpha$ -MSH levels were decreased in the human and mice mutant groups. This observation is in relative agreement with what is seen in *Pc1*-null mice, which showed  $\alpha$ -MSH deficiency but did not develop obesity [34]. It appears that an obese phenotype such as this depends on the dosage of *PC1/3*, since mice with *PC1/3* hemizygous deficiency indeed exhibited mild obesity [34].

It has been reported that *Pax6*<sup>sey/sey</sup> mice with homozygous *Pax6* mutation exhibited decreased levels of glucagon-like peptide-1 (GLP-1) in the small and large intestine [36]. However, we did not detect significant differences in GLP-1 production in the patients with the mutation versus normal controls (not shown). One possible explanation is

that, unlike in mouse intestine, *PAX6* may not be present in significant amounts in adult human intestinal cells, as suggested by a published study [37]. Compared with its production in human pancreas, *PAX6* expression was barely detected in human intestine [37]. Moreover, we found that all IVGTT measurements were very similar to those from the OGTT (ESM Fig. 3). These data indicate that the abnormal glucose metabolism we observed in this study cannot be due to changes in the intestinal GLP-1 level.

Since *PAX6* controls many downstream events, the primary cause of abnormal glucose metabolism in the *PAX6*-deficient participants may differ from that in the *PC1/3*-deficient animals. Some of the downstream factors regulated by *PAX6* may play a role opposed to that of  $\alpha$ -MSH in the regulation of food intake; even though both *PC1/3* and  $\alpha$ -MSH were reduced in our participants, we did not observe obesity in those with the *PAX6* mutation. The definitive explanation of these discrepancies awaits further investigation.

Using *PAX6*-deficient mouse models, several previous studies have proposed that the disturbed architecture and the resulting deficiency in insulin production in pancreas is the reason why mutant mice develop diabetes [7, 12, 13]. In heterozygous *Pax6* mutant mice, however, we did not see any obviously abnormal pancreatic architecture (Fig. 3b) [14]. Instead, we found that the proinsulin/total insulin ratio both in the patients with the *PAX6* mutation (Fig. 2g,h) and in the *Pax6* mutant mice was significantly higher than in wild-type controls (Fig. 3e). Furthermore, we found that, even though type 2 diabetes patients often have high proinsulin levels due to overstimulation by hyperglycaemia [21, 22], the proinsulin/total insulin ratio in the patients with the heterozygous *PAX6* mutation was significantly higher than in the type 2 diabetes patients (Fig. 2h), suggesting, again, that defective proinsulin processing may be the primary cause of diabetes in the patients with the *PAX6* mutation. This observation of the effect of the *PAX6* mutation is partially supported by a report that a point mutation in the human insulin gene gives rise to abnormal glucose metabolism and hyperproinsulinaemia [38].

It is important to note that the majority of the *Pax6* mutant mice and the patients with the *PAX6* mutation did not develop IGT until advanced ages. For example, most patients with the *PAX6* mutation exhibited abnormal glucose metabolism at ages above 35 years (Fig. 2d), which was similar to the comparative age (>6 months) at which abnormal glucose metabolism appeared in *Pax6* mutant mice (Fig. 3c), suggesting that other age-dependent factors may possibly exist and work in coordination with *PAX6* in the regulation of glucose metabolism. This also raises an interesting question regarding proinsulin levels in the participants with the heterozygous mutation in *PAX6* who did not yet show abnormal glucose metabolism.



Before age 4 months, the *Pax6* mutant mice did not show significant differences from wild-type mice in blood glucose levels during the IPGTT (Fig. 3c), but did exhibit higher proinsulin/total insulin ratio than wild-type animals (Fig. 3e). Therefore the high ratio of proinsulin/total insulin in the *Pax6* mutant mice occurs prior to onset of abnormal glucose metabolism, suggesting, again, defective proinsulin processing as the cause rather than an effect of abnormal glucose metabolism in the animals with the heterozygous *Pax6* mutation. The presence of such additional factors that contribute to the development of abnormal glucose metabolism in the animals with the *Pax6* mutation is currently under further investigation.

Glucose metabolism is an extraordinarily complex process that is regulated by multiple mechanisms. Our conclusion that defective proinsulin processing causes abnormal glucose metabolism in the participants with *PAX6* mutations does not exclude the possibility that other mechanisms, such as the  $\alpha$ -MSH regulatory pathway, may also contribute to the disorder. Nonetheless, our study provides a novel function of *PAX6* in the regulation of proinsulin processing and glucose metabolism via modulation of *PC1/3* production. It also provides an insight into how *PAX6* mutations lead to abnormal glucose metabolism in mice and humans.

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