THE Pax6b HOMEODEOMAIN IS DISPENSABLE FOR PANCREATIC ENDOCRINE CELL DIFFERENTIATION IN ZEBRAFISH

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Running head: Molecular dissection of Pax6b function in zebrafish pancreas
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Pax6 is a well conserved transcription factor that contains two DNA binding domains, a paired domain and a homeodomain, and plays a key role in the development of eye, brain and pancreas in vertebrates. The recent identification of the zebrafish sunrise mutant, harbouring a mutation in pax6b homeobox and presenting eye abnormalities but no obvious pancreatic defects, raised the question about the role of pax6b in zebrafish pancreas. We show here that pax6b does play an essential role in pancreatic endocrine cell differentiation as revealed by the phenotype of a novel zebrafish pax6b null mutant and of embryos injected with pax6b morpholinos. Pax6b-depleted embryos have almost no β cells, a strongly reduced number of δ cells, and a significant increase of ε cells. Through the use of various morpholinos targeting intron-exon junctions, pax6b RNA splicing was perturbed at several sites leading either to retention of intronic sequences or to deletion of exonic sequences in pax6b transcript. By this strategy, we show that deletion of Pax6b homeodomain in zebrafish embryos does not disturb pancreas development while lens formation is strongly affected. These data thus provide the explanation for the lack of pancreatic defects in the sunrise pax6b mutants. In addition, partial reduction of Pax6b function in zebrafish embryos performed by injection of small amounts of pax6b morpholinos caused a clear rise in α cell number and in glucagon expression, emphasizing the importance of the fine tuning of Pax6b level on its biological activity.

The transcription factor Pax6 is well conserved among metazoa and plays important roles in the development of several organs including the eye, brain, pituitary, and pancreas (1-5). The protein contains two DNA-binding domains, a paired domain (PD) and a paired-like homeodomain (HD), and the C-terminal proline-, serine-, and threonine-rich (PST) region which is acting as a transcriptional activation domain (TA). Many studies on the function of the murine Pax6 gene rely on the analysis of Pax6Sey (Small Eye) and Pax6Sey-Neu mutant mice, harbouring distinct point mutations in the Pax6 coding region. These often lead to C-terminal truncations in the Pax6 protein, disrupting its activity (6-8). These truncated Pax6 mutant mice exhibit a phenotype essentially identical to that of Pax6/LacZ knockout mice (9) and are thus considered as null mutants. While heterozygous Pax6 mutants display eye malformations (reduced lens size, iris and corneal anomalies) and some forebrain patterning defects (10,11), no obvious morphological abnormalities have been detected in the pancreas. Yet mice and human patients heterozygous for a Pax6 mutation often display impaired glucose tolerance at the adult stage (12,13). Rodents homozygous for a Pax6 null mutation lack eye and olfactory bulbs, have severe brain anomalies, and display severe defects in the differentiation of pancreatic endocrine cells (7,9,14-18). Also, overexpression of Pax6 within the eye, brain, or pancreas in transgenic mouse embryos perturbs the development of these organs, showing that an optimal level of PAX6 protein activity is crucial for proper organogenesis (19-21). In the murine pancreas, Pax6 is expressed in all endocrine cell types and its inactivation leads to significant
depletion of insulin- and somatostatin-expressing cells (β cells and δ cells, respectively), almost total loss of glucagon-expressing cells (α cells), and a strong increase in ghrelin-expressing cells (ε cells)(9,15,22,23).

Previous studies have shown that the PD is crucial for Pax6 function, since a mutation causing a deletion within this domain leads to a phenotype of the eyes and brain almost identical to that of Pax6 null mutants. On the other hand, the missense mutation Pax6\textsuperscript{Neu4} leading to an aminoacid substitution within the HD, preventing DNA binding through this domain, also affects eye development, but causes only subtle defects in telencephalon development (18). These data suggest that most Pax6 target genes in the brain are regulated through the PD, while in the eye the HD is also important. In vivo studies on the respective roles of the PD and HD in pancreatic cell differentiation have not yet been reported.

Because of a genome duplication early in the evolution of teleost fish, two pax6 genes exist in zebrafish. Both are active in ocular and brain structures but only one, pax6\textsuperscript{b}, is expressed in pancreatic endocrine cells because of sequence divergence within the pancreatic cis-regulatory regions (24,25). Recently, the sunrise zebrafish mutant was reported to contain a deleterious missense mutation in the homeobox of the pax6\textsuperscript{b} gene (24). While homozygous sunrise mutant embryos display a microphthalmia (small eye) phenotype with abnormal lens and corneal structures, they display normal number of α and β pancreatic cells and adult sunrise mutants are viable and fertile (24). This surprising finding was in sharp contrast with the severe pancreatic defects observed in the Pax6 mutant mice and prompted us to investigate further the role of pax6\textsuperscript{b} in zebrafish pancreas development. In the present study, we demonstrate the crucial role of Pax6\textsuperscript{b} for the differentiation of endocrine cells in the zebrafish pancreas and, by using morpholino disrupting pax6\textsuperscript{b} mRNA splicing, we show that the Pax6\textsuperscript{b} homeodomain is dispensable for pancreas development.

**EXPERIMENTAL PROCEDURES**

**Transgenic lines and fish maintenance.** The AB strain, pax6\textsuperscript{b}:GFP / insulin:dsRed (25) and the ptf1a:GFP (26,27) transgenic lines, used for pax6\textsuperscript{b} morpholinos injections, were raised according to the Zebrafish Book. All the embryos were staged according to Kimmel et al. (28). The genotyping of pax6\textsuperscript{b}sa0686 and sunrise embryos was done on DNA extracted from tails of ISH-stained-embryos by performing nested PCR using primers as reported previously (24), and as recommended by the Zebrafish Mutation Resources, respectively.

**Whole-mount in situ hybridization.** Single whole-mount in situ hybridizations were performed as previously described by Hauptmann and Gerster (29). Anti-sense RNA probes were synthesized by transcription of cDNA clones with T7, T3 or SP6 RNA polymerase and using digoxigenin labelling mix (Roche). The following probes were used: glucagon (30), insulin (31), somatostatin\textsubscript{2} (32), ghrelin (NCBI: AL918922, digestion with Apa\textsubscript{I} and transcription with T3)(Verbruggen, unpublished results).

**Morpholino injections.** The anti-sense morpholino oligonucleotides of splicing (Gene Tools) used were: Mo1Pax6\textsuperscript{b} (5’-GGCTTCACAAGTCACCTGCAAAATC), Mo2Pax6\textsuperscript{b} (5’-TTGATTTGCACCTACGCTCGGTATG), Mo3Pax6\textsuperscript{b} (5’-AAAGTTGTGATCGTTCACCTTTCTC), Mo4Pax6\textsuperscript{b} (5’-GGCAACGTCTGAAAAATATAACA), Mo5Pax6\textsuperscript{b} (5’-TGCACACTGAATGGACAGCAATAT) and Mo1mutArx (5’-GCGTCTTTATTAGCTCGTCAACACA) as control morpholino. These morpholinos target exon – intron or intron – exon boundary. They were diluted in Danieau solution from which 1000pL were injected into the yolk of one-cell stage embryos. To check the injection efficiency, Rhodamine dextran was added at 0.5% in the injected solution.

**Control of the morpholinos efficiency.** Zebrafish embryos were injected at one cell stage with Mo1Pax6\textsuperscript{b} (6ng/embryo) or with Mo2Pax6\textsuperscript{b} (4.5ng/embryo) or Mo3Pax6\textsuperscript{b} (6ng/embryo), or Mo5Pax6\textsuperscript{b} (2ng/embryo) or with Mo2Pax6\textsuperscript{b} + Mo5Pax6\textsuperscript{b} (4.5ng+2ng/embryo) or with Mo3Pax6\textsuperscript{b} + Mo4Pax6\textsuperscript{b} (6ng+4ng/embryo) or with an arx mutated morpholino (1.7ng/embryo).
We performed a reverse transcription on 1µg of mRNA extracted from these morpholinos injected embryos or from non injected control embryos at 24hpf or 30hpf. The primers used for the PCR amplification on the obtained cDNA were: pax6b paired domain (upstream primer: 5’-GAACGGCAGACCGTTACCGGACTC; downstream primer: 5’-CTCTGCCGTTGAGCATTCTCAGC), pax6b homeodomain (upstream primer: 5’-GCAACAGATGGGTGCAGATGGC; downstream primer: 5’-CTGTATTCTTTGCTCGGAGGTC) and nkar6.2 (upstream primer: 5’-TATTCTGGCCGGGAATGATTCT; downstream primer: 5’-GCCTCTTTCGCCATTTAGTTCTT). cDNA were subjected to 30 cycles or 40 cycles of 30s at 94°C, 30s at 60°C, and 30s at 68°C, followed by a final 7min extension at 68°C.

Pax6 antibody, immunofluorescence and western blotting. Pax6 antibody was purified from rabbit antiserum after several injections of purified Pax6b C-terminal domain expressed in E. Coli (last 88 amino-acid of Pax6b). This antibody reacts against both zebrafish Pax6a and Pax6b proteins. Immunofluorescence was performed on 22-24 hpf zebrafish embryos fixed in 2 % PFA using anti-Pax6 Ab (1/200) and Alexa-488 anti-rabbit (1/500, Molecular Probe) as secondary antibody. Briefly, washes were performed in PBS/Triton 0.3% and antibody incubations in PBS/Triton 0.3%/4% BSA. For Western blotting, crude extracts were prepared from 40 zebrafish embryos injected with various morpholinos. One fifth of extracts were loaded on a acrylamide gel, analyzed with the anti-Pax6 (1/200) or an anti-tubulin (1/1000, Abcam) as primary antibodies, then with the second HRP-coupled antibody (1/2000, Cell singaling) and revealed with the ECL chemoilluminescence kit (Pierce).

Imaging. Microscope pictures were performed with an Olympus DP70 photocamera fixed on BX60 Olympus microscope. Pax6 immunostaining were captured with a Leica TCS SP2 laser confocal microscope. All the pictures were processed using Adobe Photoshop and Adobe Illustrator softwares.

RNA extraction, reverse transcription and Q-PCR. Total RNA was extracted with Quiazol (Qiagen) from 2 different batches of 25 larvae injected with Mo2 or control morpholinos. cDNA was synthesized from 1µg total RNA by reverse transcription with the RT Transcriptor First Strand cDNA Synthesis Kit (Roche Clinical Laboratories, Indianapolis, IN) according to the manufacturer’s instructions. One twentieth of the resulting cDNA was used for real-time PCR with the one-step 2X Mastermix (Diagenode, Liège, Belgium) containing SYBR green. Thermal cycling was performed on an Applied Biosystems 7000 detection system (Applied Biosystems, Foster City, CA). For all reactions, negative controls were run with no template present, and random RNA preparations were also subjected to sham qRT-PCR (no reverse transcription) to verify lack of genomic DNA amplification. The relative transcript levels for the genes of interest (glucagon, insulin, somatostatin transcripts) were obtained by the relative standard curve method and normalized with respect to the mean of two reference genes EF1α and Rpl13a. Primers, the sequences of which are available upon request, were designed with the Primer Express 2.0 software (Apply Biosystem, Forster City, USA) and selected so as to span exon–exon junctions, to avoid detection of genomic DNA.

RESULTS

Pax6b plays a key role in the differentiation of pancreatic endocrine cells in zebrafish embryos.

To determine the function of Pax6b in the zebrafish pancreas, we first injected fertilized zebrafish eggs with an antisense morpholino (Mo2) annealing to the exon6-intron6 junction of the pax6b gene. This morpholino disrupts RNA splicing at that junction causing the removal of exon 6 coding sequences in pax6b transcripts, leading to a frame-shift and a premature stop codon in the paired domain (see Fig. 4 and below for further detailed descriptions). Almost no full-length Pax6b protein could be detected in the pancreas of injected embryos by immunostaining while expression of the paralog Pax6a protein was still easily detected in the neural tube by the same anti C-terminal Pax6 antibody (Fig. 1 A,B). Formation of the different pancreatic endocrine cells was then analysed in the Mo2 morphants at 3 days post-fertilization (dpf) and compared with
those of uninjected embryos or of embryos injected with an unrelated-sequence morpholino used as a control. Almost no insulin-expressing \( \beta \)-cells were detected in the Mo2 morphants, as opposed to an average of 25 to 35 insulin-expressing cells in control embryos (Co) (Fig. 1C,D). The number of somatostatin-expressing cells was also drastically decreased in about 90% of the morphants (Fig. 1 E,F). Conversely, ghrelin-expressing cells were barely detectable in wild-type embryos but numerous and easily observed in all the Mo2 morphants (Fig. 1 G,H). The number of glucagon-expressing cells was not significantly affected in the morphants injected with 4.5 ng of Mo2 (Fig 1 I,J). However, injection of 9 ng of Mo2 morpholino resulted in a reproducible decrease of glucagon-cells (data not shown). To determine whether pax6b knockdown affected development of the pancreatic exocrine tissue, Mo2 morpholino was injected in the ptf1a:GFP transgenic zebrafish line. No obvious change of the exocrine tissue could be detected in the morphants (Fig.1 K, L), consistent with the lack of pax6b expression in these cells. Very similar endocrine pancreatic defects were observed when embryos were analysed 30 hours post-fertilization (hpf) (Supplemental Data S1).

The lost of \( \beta \) cells in the Mo2 morphants sharply contrasts with the apparent normal number of \( \alpha \) cells reported in the zebrafish pax6b sunrise mutant at 5 dpf (24). Thus, we decided to further analyse the pancreatic phenotype of the sunrise mutants at 30 hpf and 3 dpf. No difference could be detected between sunrise homozygous embryos and their wild-type siblings in the number of either \( \alpha \), \( \beta \), \( \delta \) or \( \epsilon \) cell types (Supplemental Data S2). To confirm that the endocrine pancreatic defects in Mo2 morphants are due to the knockdown of pax6b and not of Mo2 off-targets, we analysed the pancreas of a novel zebrafish pax6b mutant recently identified at the Zebrafish Mutant Resource. This pax6b mutant allele (sa0086) harbors a C to A substitution changing codon 109 (Tyr) to a premature Stop codon. Thus, this allele encodes a Pax6b protein possessing a truncated paired domain and lacking the whole homeodomain plus the transactivation C-terminal domain (Fig. 2). Based on the pax6 allelic mutant series described in mammals and in Drosophila, the pax6b\(^{sa0086}\) allele corresponds to a null mutation. Crosses between pax6b\(^{sa0086}\) carrier zebrafish generated homozygous mutant embryos all displaying severe lens defects (Fig. 2 A,B). However, the expressivity was variable with a reduction of the lens size in some mutants to a complete lack of lens in others. The pancreatic phenotype of the homozygous pax6b\(^{sa0086}\) mutants was similar to the Mo2 morphants with an almost lack of \( \beta \) cells and a strong increase of \( \epsilon \) cells in all homozygous mutants (Fig2 C-F). A marked diminution of somatostatin expressing cells was also detected in mutants, as in Mo2 morphants, but the level of reduction varied between mutant embryos (n=14), one mutant displaying a complete lack of \( \delta \) cells, 9 mutants containing 1 to 5 \( \delta \) cells and 3 possessing 6 to 10 \( \delta \) cells (Fig2 G-I). With regard to glucagon-expressing \( \alpha \) cells, some mutants were presenting almost normal or a slight decrease of \( \alpha \) cells (n=7), while other mutants displayed a severe reduction (n=5)(Fig. 2 J-L). Thus, the analysis of the pax6b\(^{sa0086}\) mutant strengthens the knockdown data, confirming the essential role of Pax6b in the differentiation of endocrine cells in the zebrafish pancreas.

Requirement of similar Pax6b levels for lens and pancreatic-\( \beta \)-cell development. Given the important role of Pax6b in the endocrine pancreas, the apparent normal pancreas of the sunrise mutant is quite surprising. One explanation might be that the sunrise missense mutation occurring in the pax6b homeobox is a hypomorphic mutation causing only partial loss of Pax6b activity, sufficient to produce morphological abnormalities in the eye but not sufficient to affect cell differentiation in the endocrine pancreas. To test this hypothesis, we injected zebrafish eggs with different amounts of pax6b Mo2 morpholino and we examined the eyes and counted the number of insulin-expressing cells in each morphant (Fig. 3). We used a doubly transgenic zebrafish line harbouring the Insulin:dsred transgene, for monitoring the number of \( \beta \) cells, and the Pax6b:GFP transgene, for monitoring the entire endocrine pancreatic cell lineage and the retinal neurons. Expression of the endogenous insulin gene was also checked by whole-mount in situ hybridization (inlets in the lower panels of Fig 3A). As the amount of injected Mo2 morpholino increased, the lens appeared increasingly smaller, almost disappearing at 4.5 ng (Fig. 3, upper panels). Precise measurement of the lens surface for each experimental condition revealed a dose-
Thus, the phenotypic difference between the

control embryos was subjected to RT-PCR
design to amplify a pax6b cDNA region    leads to a frame shift in the coding sequence,

Control of β, δ, and ε cell differentiation
does not require the Pax6b homeodomain.

Another hypothesis that might explain the
different thresholds of Pax6 expression for development of the eyes and

We also determined the splicing defects
caused by other morpholinos (Mo1, Mo2, and
Mo5) designed to affect coding regions within the

Mo2 very efficiently perturbed splicing at the exon
5a-intron5 junction, blocked splicing at this site

Mo1, encompassing the exon5-intron5 junction, blocked splicing at this site

To determine the splicing defects generated by morpholino injection, RNA
extracted from morphants and from uninjected control embryos was subjected to RT-PCR
designed to amplify a pax6b cDNA region corresponding to the HD. To that end, one pair of primers was used (see blue arrows in the upper panel of Fig.4) and the amplicons were analysed directly by sequencing and gel electrophoresis (see lower panels in Fig.4). The injection of Mo3 alone, encompassing the exon8-intron8 junction, efficiently disrupted splicing at this site, as revealed by the slightly larger amplified cDNA fragment. Direct sequencing of this amplicon revealed the insertion of the first 22 bp of intron 8 in the majority of pax6b transcripts. This insertion

controlled decrease (Fig 2B). In parallel, a similar progressive reduction was also detected in the expression of both the insulin:dsred transgene and the endogenous insulin gene. In contrast, the total number of pancreatic endocrine cells expressing the Pax6b-GFP transgene was not affected (Fig. 3, middle panels and data not shown). Quantitative analyses showed that the morpholino dose required to reduce the lens size was the same as the dose required to reduce the number of β cells. Thus, the phenotypic difference between the pax6bα0086 mutants and the sunrise mutants cannot be explained by different thresholds of Pax6 activity required for development of the eyes and of the pancreas.

We also determined the splicing defects
caused by other morpholinos (Mo1, Mo2, and
Mo5) designed to affect coding regions within the

The pax6b mRNA was analysed by RT-PCR followed by direct sequencing and gel electrophoresis (fig. 4). Mo1, encompassing the exon5-intron5 junction, blocked splicing at this site causing retention of intron 5. This insertion introduces 14 amino acids and a stop codon just after exon 5. Splicing was not totally blocked at this site, however, since correctly spliced pax6b mRNAs were still detected in the Mo1 morphants. Mo2 very efficiently perturbed splicing at the exon 6-intron 6 site for almost all pax6b transcripts causing a 29-bp deletion in exon 6 and leading to a premature stop codon at the level of the paired domain. In the Mo5 morphants, most pax6b transcripts were found to contain exon 5a and intron 5a introducing a stop codon just after it. Co-injection of Mo2 and Mo5 caused specific deletion of exon 6, but only in a minority of pax6b

leads to a frame shift in the coding sequence, generating 9 added codons followed by a stop codon 4 bp downstream from the insertion. Thus, we can infer that Mo3 morphants express a Pax6b protein containing the paired domain and the C-terminal transactivation domain and a functional homeodomain (see schematic representations of Pax6b protein in Fig. 4). In zebrafish embryos injected with a mixture of the Mo3 and Mo4 morpholinos, pax6b transcripts displayed only a deletion of exon 8, as shown by gel electrophoresis and direct sequencing. As this deletion does not cause a frame shift, translation of these transcripts results in synthesis of a Pax6b protein containing the paired domain and the C-terminal transactivation domain but lacking the C-terminal part of the linker region and the N-terminal part of the homeodomain. Western blotting using a Pax6 antibody raised against the C-terminal domain confirmed the expression of this truncated Pax6b protein in Mo3+Mo4 morphants (Supplemental figure S3). When eye morphology and the number of pancreatic β cells were examined in these morphants (Fig. 5), the lens appeared significantly reduced in size in both Mo3 and Mo3+Mo4 morphants, but β cell formation was strongly reduced only in the former. This demonstrates that the Pax6b homeodomain is not essential for β cell differentiation.
transcripts. The bulk of these transcripts possessed the combined modifications caused by Mo2 and Mo5. Thus, we can infer that the Mo2+Mo5 morphants expressed mainly C-terminal truncated Pax6b proteins with low levels of Pax6b lacking a functional paired domain. RT-PCR analysis also demonstrated that all the pax6b morpholinos affected splicing only at their respective target sites, while splicing at the other exon-intron junctions within pax6b transcripts or of another mRNA remained unaffected (Supplemental data S4). We next analysed eye morphology and pancreatic cell types in these morphants possessing pax6b transcripts disrupted at the level of the PD coding sequence. All of them displayed either an absent or a strongly reduced lens (Fig 5 and data not shown). The Mo2 and Mo2+Mo5 morphants were found to have no or few β cells (Fig 5). These data indicate that the whole C-terminal part of the Pax6b protein is crucial for eye and pancreas development.

We also analysed the other pancreatic endocrine cell types in all the different pax6b morphants (Fig 6). All the morphants expected to produce a Pax6b protein deleted in the C-terminal region (Mo1, Mo2, and Mo3) or in the paired domain (Mo2+Mo5) displayed not only a drastic reduction of β cells but also a significantly decreased number of somatostatin-expressing cells and an increased number of ghrelin-expressing cells. In contrast, the Mo3+Mo4 morphants expressing a Pax6b protein with a truncated homeodomain did not display any change of β, δ, and ε cell numbers compared to the control embryos. Thus, we can conclude that the Pax6b homeodomain is not required for the differentiation of all pancreatic cell types. Thus, these findings explain the phenotypic difference between the sunrise mutants and the null pax6b mutants/morphants.

Partial loss of Pax6b activity leads to increased glucagon expression. When glucagon expression was analysed in detail in the pax6b morphants, a reproducible increase of α cells was detected specially in Mo1 morphant: the number of α cells was at least 150% higher on average in Mo1 morphant compared to control embryos (see Fig. 6). As the Mo1 morpholino did not disrupt pax6b RNA splicing as efficiently as the other morpholinos (see Fig. 4), we wondered if the number of α cells might depend on the levels of endogenous Pax6b protein. This prompted us to analyse the various pancreatic cell types in embryos producing different amounts of functional Pax6b protein. For this, we injected various quantities of the Mo2 morpholino (Fig 7A). As the amount of injected Mo2 increased, the number of insulin+ and somatostatin+ cells gradually decreased. In contrast, the number of glucagon+ cells was found first to increase with a maximum level at 1.5-ng injected Mo2 morpholino, and then to gradually decrease at higher morpholino doses. The number of ghrelin+ cells was found to rise, appearing maximal at around 3 ng. In order to verify more precisely these effects, quantitative RT-PCR were performed on RNA extracted from 3-dpf morphants (Fig 7B). The results clearly show that, while the somatostatin and insulin transcripts decreased progressively with the dose of injected Mo2 to reach respectively about 20% (sst) and 4% (ins) compared to control embryos, the glucagon transcripts rised to 160% at 1.5 ng of Mo2 and then were down-regulated to about 50% at 4.5 ng of Mo2. In order to quantify more precisely the increase of α cell number, we counted the glucagon positive cells at 30-hpf as stained cells are more easily discerned at this earlier stage compared to 3-dpf. This analysis showed an increase of about 1.5 fold in the number of α cells after injection of 1.5 ng Mo2 compared to control embryos (Fig7C). Thus, these findings reveal the importance of the fine-tuning of Pax6 expression level for its biological activity.

**DISCUSSION**

We have previously shown that only one of the zebrafish duplicated pax6 genes, namely pax6b, is expressed in all endocrine cell types of the pancreas (25). We demonstrate here by morpholino knock-down approach and analysis of the sunrise and pax6b<sup>⁰⁰⁰⁰⁰⁵</sup> zebrafish mutants that pax6b is required for proper endocrine cell differentiation but its homeodomain is not essential for this function. Both the paired domain and the homeodomain are the most conserved parts of Pax6 from invertebrates to vertebrates (5) indicating strong evolutionary pressure to maintain the function of these 2 DNA binding domains. However, our data show that a truncation in Pax6b homeodomain does not disturb pancreas
development while this deletion affects the eyes. It is possible that subtle pancreatic defects may have not been detected after deletion of Pax6b homeodomain, but the fact that sunrise homozygous mutants survive to adulthood is a strong argument for a nonessential role of Pax6b homeodomain in the pancreas. Thus, we can infer that Pax6b regulates expression of target genes in pancreatic cells mostly, if not exclusively, through DNA-binding of its paired domain. By analysing Pax6\textsuperscript{Avy}\textsuperscript{18} and Pax6\textsuperscript{Avay} mutant mice, possessing a mutated paired domain and a mutated homeodomain respectively, Haubst and collaborators have previously drawn similar conclusions for the action of PAX6 in the murine brain cortex (18). All together, these data indicate that the Pax6 homeodomain is important for eye development in mammals and fish. In contrast, in drosophila, eye development can be triggered by a Pax6 protein devoid of the entire homeodomain (33) suggesting that the role of the homeodomain in the eye has been acquired during vertebrate evolution.

Our study also highlights the usefulness of morpholinos targeting exon-intron junctions allowing to generate various truncations in a studied protein within zebrafish embryos, thereby revealing the role of specific parts of a protein in vivo. When a single morpholino was injected in zebrafish eggs, the splicing process was disturbed specifically at the targeted junction site, either leading to the presence of the intron sequence within the final transcript or leading to the use of nearby cryptic donor/acceptor splicing sites. Importantly, this work reveals for the first time that the combined injection of two morpholinos targeting the two splicing sites flanking the same exon can induce the specific removal of this exon from the spliced transcript. The efficiency of such deletion seems to vary among the splicing sites as, on the two tested pax6b exons, one (exon8) was deleted with a high efficiency while the other (exon6) was removed only in a minority of pax6b transcripts. The fact that the pancreatic endocrine defects caused by the injection of morpholino Mo3 were rescued by the combined injection of morpholino Mo4 clearly demonstrates that i) the homeodomain is not essential in pancreas and ii) the endocrine defects obtained by the Mo3 injection are due to pax6b knockdown and not to other unspecific Mo3 off targets. In addition, the fact that the same pancreatic perturbations were observed with three different pax6b morpholinos and were also detected in the pax6b\textsuperscript{sa0086} mutant further consolidates the validity of our morpholino knock-down data. The only difference noted between the pax6b\textsuperscript{sa0086} mutants and the pax6b morphants is a reduction of glucagon expressing α-cells which was observed in 40% of mutants while α-cells were not significantly affected after injection of 4.5 ng of Mo2 morpholino. This slight divergence may be due to a small residual expression of Pax6b in morphants which would be sufficient to drive α cell differentiation. On the other hand, the fact that α-cell differentiation is not strongly affected in about 60% of pax6b\textsuperscript{sa0086} mutants indicate that a Pax6b-independent pathway may also be used to generate glucagon cells. The variation in α-cell number, as well as in δ-cells and in lens size, observed among pax6b\textsuperscript{sa0086} mutants could be due to different genetic background in each mutant embryos. Indeed, the pax6b\textsuperscript{sa0086} fish have been recently identified following a ENU large scale mutagenesis and it is possible that other loci influence the phenotype severity of pax6b\textsuperscript{sa0086} homozygotes. Several outcrosses of the pax6b\textsuperscript{sa0086} mutant will be necessary to verify this hypothesis.

Another unexpected and important finding of this study is the increase in glucagon expression after partial pax6b knockdown. This increase of α cells was initially noticed after injection of the Mo1 pax6b morpholino. As this morpholino was much less efficient for disrupting pax6b splicing compared to the other pax6b morpholinos (see Fig. 4 and Fig. 6), this prompted us to inject various amounts of the more efficient Mo2 morpholino. This dose-response analysis revealed that the increase of α cells and of glucagon transcripts was maximal with 1.5ng of injected Mo2 corresponding to a mild reduction of pax6b activity. Previous studies have shown that a precise range of Pax6 protein level is important for the proper development of the eyes, brain and pancreas as an increase of Pax6 expression in transgenic mice leads to abnormalities in these organs (19,20,34). Moreover, heterozygous Pax6 individuals display anomalies in eye morphology and brain patterning (10). However, no obvious morphological abnormality has been reported yet at the level of pancreas in Pax6 heterozygous mice.
and we did not detect any obvious anomalies in pax6b<sup>−/−</sup> heterozygous larvae (data not shown). Recently, pancreatic expression of the prohormone convertases PC1/3 has been reported to be downregulated in Pax6 heterozygous mice, thereby contributing to the abnormal glucose metabolism (12). This example indicates that a 50% reduction of Pax6 levels is sufficient to perturb expression of some pancreatic target genes. However, our observation of the increase of α cells after partial knockdown of pax6b is quite peculiar as this effect is not occurring after full knockdown of pax6b. A similar phenomenon has been recently shown for the pancreatic transcription factor Ptf1a. Indeed, a partial reduction of Ptf1a levels in zebrafish embryos strongly promotes endocrine cell fate while such effect is not observed when Ptf1a is completely repressed (27). The precise molecular mechanisms determining the formation distinct endocrine cell types from pancreatic progenitor cells are still largely unknown. Our data indicate that the precise levels of Pax6 protein may be one of the clues for pancreatic cell fate choice. This also highlights the importance of carefully monitoring transcription factor levels when rescue or gain-of-function experiments are performed.

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FIGURE LEGENDS

Fig. 1: Pancreatic function of the zebrafish pax6b gene. A,B Pax6 Immunostaining of whole-mount 1-dpf zebrafish embryos injected with a control morpholino (Co)(A) or with the pax6b Mo2 morpholino (4.5ng/embryo)(B); pax6b protein is not detected in pancreatic cells (P) while pax6a expression is not affected in the neural tube (NT) in the morphants. C-L Analysis of pancreatic markers in

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control embryos (left column) and in pax6b morphants (right column) by in situ hybridization. Ventral views of the pancreatic area of 3-dpf embryos with the anterior side to the left. ins: insulin, gcg: glucagon, sst: somatostatin, ghr: ghrelin, Ptf1a gene. The number of insulin- and somatostatin-expressing cells decreases in the pax6b morphants while ghrelin expression increases. Scale bar A-J: 50 µm, K,L: 200 µm.

**Fig. 2**: Pancreatic cell endocrine differentiation is perturbed in sa0086 pax6b−/− zebrafish mutants. Upper panels: Schematic drawing of Pax6 structures in wild-type and sa0086 mutant. A-L Phenotype comparison of wild-type (left column) and sa0086 mutant (right column) 3.5-dpf larvae; A,B Eye and lens morphology; C-L Expression of pancreatic hormones by in situ hybridization. Ventral views of the pancreatic area with the anterior side to the left. ins: insulin, gcg: glucagon, sst: somatostatin, ghr: ghrelin. The number of insulin and somatostatin cells are significantly reduced in the mutants while ghrelin cells increase dramatically. A high variability is observed for glucagon expression among mutants.

**Fig. 3**: Lens size and β-cell number are controlled by similar Pax6b activity levels. A Injection of increasing doses of pax6b Mo2 (from 0.75 to 4.5 ng/embryo) leads to progressive reduction of the lens size (black lines, upper panels) and of β-cell number (lower panels), while the total number of endocrine pancreatic cells expressing the pax6b:GFP transgene is not significantly altered (middle panels). Insulin expression was determined by expression of the Insulin:ds-Red transgene and by in situ hybridization (inlets in the lower panels). Anterior part of 3-dpf embryos, with the anterior side to the left. Scale bars: 200 µm. B Lens surface was measured in the lateral view and insulin-expressing cells were counted in injected embryos for each condition. Means for control embryos were taken as 100% and relative percentages were calculated for the injected embryos. Error bars: standard deviation.

**Fig. 4**: pax6b RNA splicing defects generated by injection of morpholinos. Schematic representation of pax6b gene structure between the 5th exon (e5) and the 10th exon (e10), with regions coding for the paired domain (red boxes) and the homeodomain (blue boxes) highlighted. Mo1, Mo5, and Mo2 target splicing junctions within regions coding for the paired domain, and Mo3 and Mo4 target junctions within the homeobox (green lines). Embryos were injected with Mo1 (6 ng/embryo), Mo2 (4.5 ng/embryo), Mo3 (6 ng/embryo), Mo5 (2 ng/embryo), Mo2 + Mo5 (respectively 4.5 + 2 ng/embryo), and Mo3 + Mo4 (respectively 6 + 4 ng/embryo). The RNA splicing defects caused by the different morpholinos were determined by amplifying the cDNAs corresponding to the paired domain and the homeodomain through RT-PCR with two distinct pairs of primers (red and blue arrows respectively) and analysed by gel electrophoresis and sequencing. The combination of Mo3 and Mo4 leads to deletion of the 8th exon. The predicted Pax6b proteins produced in each morphants are represented at the bottom of the corresponding gel pictures. M: marker (from top to bottom, 1 kb, 800 bp, 600 bp, 400 bp, and 200 bp), Co: control, MO: morpholino-injected embryos. Purple italic characters: stop codon. Green characters: predicted amino acid sequences with the corresponding non-wild-type amino acids in bold. PD: paired domain, HD: homeodomain, TA: C-terminal transactivation domain

**Fig. 5**: The Pax6b homeodomain is not required for the differentiation of insulin-expressing cells. First column: Pax6b protein prediction resulting from altered splicing following morpholino injection. PD: paired domain (red), HD: homeodomain (green), TA: C-terminal transactivation domain (blue). Second column: dorsal views of the anterior part of injected embryos at 3-dpf and lateral views of the eyes in the inlets, with the size of the lens highlighted by a red line. Scale bars: 200 µm. Third column: insulin expression determined by in situ hybridization. Scale bars: 50 µm.

**Fig. 6**: Effects of the different pax6b morpholinos on the pancreatic endocrine cell types. Expression of pancreatic hormone genes as detected by in situ hybridization in control (Co) and morpholino-injected embryos (Mo). Ventral views of the pancreatic area of 3-dpf embryos, with the
anterior side to the left. Scale bars: 50 µm. ins: insulin transcripts, sst: somatostatin transcripts; ghr, ghrelin transcripts; gcg: glucagon transcripts.

**Fig. 7:** Partial knockdown of pax6b leads to an increased level of glucagon gene expression. A Hormone-gene expression pattern determined by in situ hybridization following injection of increasing doses of morpholino Mo2. Ventral views of the pancreatic area of 3-dpf embryos, with the anterior side to the left. Scale bars: 25 µm. ins: insulin transcripts; sst: somatostatin transcripts; ghr: ghrelin transcripts; gcg: glucagon transcripts. B Quantitative RT-PCR of glucagon, insulin and somatostatin transcripts in two independent mRNA preparations from zebrafish embryos injected with various doses of Mo2 morpholinos. C Number of glucagon-positive cells in 30hpf-embryos injected with 1.5 of control morpholino or of Mo2 morpholino. Each point in the graph represents the α-cell number of one embryo. Cell counting was performed directly under the microscope at high magnification on whole-mount embryos by focusing successively on each layer of stained cells.
The Pax6b homeodomain is dispensable for pancreatic endocrine cell differentiation in zebrafish

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