

# Functional dissection of the paired domain of Pax6 reveals molecular mechanisms of coordinating neurogenesis and proliferation

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## SUMMARY

To achieve adequate organ development and size, cell proliferation and differentiation have to be tightly regulated and coordinated. The transcription factor Pax6 regulates patterning, neurogenesis and proliferation in forebrain development. The molecular basis of this regulation is not well understood. As the bipartite DNA-binding paired domain of Pax6 regulates forebrain development, we examined mice with point mutations in its individual DNA-binding subdomains PAI (Pax6<sup>Leca4</sup>, N50K) and RED (Pax6<sup>Leca2</sup>, R128C). This revealed distinct roles in regulating proliferation in the developing cerebral cortex, with the PAI and RED subdomain mutations reducing and increasing, respectively, the number of mitoses. Conversely, neurogenesis was affected only by the PAI subdomain mutation, phenocopying the neurogenic defects observed in full Pax6 mutants. Genome-wide expression profiling identified molecularly discrete signatures of Pax6<sup>Leca4</sup> and Pax6<sup>Leca2</sup> mutations. Comparison to Pax6 targets identified by chromatin immunoprecipitation led to the identification and functional characterization of distinct DNA motifs in the promoters of target genes dysregulated in the Pax6<sup>Leca2</sup> or Pax6<sup>Leca4</sup> mutants, further supporting the distinct regulatory functions of the DNA-binding subdomains. Thus, Pax6 achieves its key roles in the developing forebrain by utilizing particular subdomains to coordinate patterning, neurogenesis and proliferation simultaneously.

**KEY WORDS:** Transcriptional regulator, Radial glia, Cortex development, Mouse

## INTRODUCTION

During organ formation, cell proliferation and differentiation have to be tightly controlled and coordinated. This is of particular relevance in the CNS, as regulation of neuron numbers and subtypes profoundly affects functional properties of the nervous system. However, the molecular programs coordinating proliferation and neurogenesis are largely little understood. It is known that key transcriptional regulators influence both fate and patterning decisions as well as stem and progenitor cell proliferation (e.g. Zaret and Carroll, 2011), thereby acting as coordinators at the molecular level. The molecular programs by which these regulatory proteins exert their distinct functions remain poorly understood. The transcription factor Pax6 acts as a master regulator in various tissues, including the developing CNS and eye (Hanson and Van Heyningen, 1995; Dohrmann et al., 2000; Kozmik, 2008; Osumi et al., 2008). Pax6 has been established as a key regulator of patterning, fate and proliferation and is required to achieve appropriate CNS and eye development (Hanson and Van Heyningen, 1995; Stoykova et al., 1996; Stoykova et al., 1997; Götz

et al., 1998; Chapouton et al., 1999; Stoykova et al., 2000; Toresson et al., 2000; Yun et al., 2001; Estivill-Torrus et al., 2002; Heins et al., 2002; Haubst et al., 2004; Quinn et al., 2007; Sansom et al., 2009; Tuoc et al., 2009). In addition, Pax6 is sufficient to elicit eye formation (Altmann et al., 1997; Chow et al., 1999) and instruct neuron formation even from non-neurogenic glial cells (Heins et al., 2002; Berninger et al., 2007). Despite recent advances in identifying Pax6-regulated genes *in vivo* (Sansom et al., 2009; Wolf et al., 2009; Xie and Cvekl, 2011), how Pax6 instructs neurogenesis or how it coordinates various developmental aspects at the molecular level remains largely elusive.

Pax6 belongs to the class IV Pax transcription factors, which possess two complete domains, namely the paired domain (PD) and paired-type homeodomain (HD) for DNA binding followed by the C-terminal transactivation domain (Mansouri et al., 1996; Chi and Epstein, 2002). As the HD and PD recognize different Pax6 consensus binding sites (Chi and Epstein, 2002), they may regulate distinct functions by controlling particular targets either separately or in a cooperative manner (Jun and Desplan, 1996; Singh et al., 2000; Mikkola et al., 2001; Mishra et al., 2002; Xie and Cvekl, 2011). Indeed, Pax6 selectively utilizes the HD in the paired-less form of the protein to regulate the survival of mature dopaminergic neurons in the adult olfactory bulb via its  $\alpha$ -crystalline target (Ninkovic et al., 2010). The HD has also been implicated in regulating lens formation and retinal specification during eye development (Ashery-Padan et al., 2000; Ashery-Padan and Gruss, 2001; van Heyningen and Williamson, 2002), but has surprisingly little effect in forebrain development (Haubst et al., 2004; Ninkovic et al., 2010). These data imply that the multitude of effects that Pax6 exerts on patterning, neurogenesis and proliferation in the developing brain should be largely mediated by the PD.

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Interestingly, the PD itself is also structured in a modular, bipartite manner, with an N-terminal PAI subdomain and C-terminal RED subdomain, which can bind cooperatively or independently to their cognate sites (Epstein et al., 1994a; Yamaguchi et al., 1997). Alternative splicing of *Pax6* exon 5a regulates the insertion of 14 amino acids into the PAI subdomain, thereby abolishing PAI subdomain DNA binding while retaining RED subdomain activity (Epstein et al., 1994b; Kozmik et al., 1997; Anderson et al., 2002). Thus, it would be interesting to determine the function of these subdomains to see whether they regulate distinct programs in the developing CNS.

Recently, two mouse lines (*Pax6<sup>Leca4</sup>* and *Pax6<sup>Leca2</sup>*) were identified, each carrying a point mutation in one of the two bipartite DNA-binding subdomains of the PD (Thaung et al., 2002). These mutations (see supplementary material Fig. S1) result in the substitution of lysine for asparagine (N50K) in the PAI subdomain (*Pax6<sup>Leca4</sup>*) or of cysteine for arginine (R128C) in the RED subdomain (*Pax6<sup>Leca2</sup>*), a mutation that has also been observed in patients (Azuma et al., 1996). Both of these mutations would be expected to affect DNA binding of the respective subdomain (Thaung et al., 2002) (supplementary material Fig. S1) and the milder eye phenotype compared with full *Pax6* mutants (Thaung et al., 2002) would be consistent with a selective disruption of either subdomain. So far, only full mutants of *Pax6*, with either premature STOP codons that result in no protein or a truncated protein lacking the transactivation domain (Theiler et al., 1978; Hill et al., 1991; Matsuo et al., 1993; Lyon et al., 2000; Favor et al., 2001; Thaung et al., 2002; Graw et al., 2005) or the loss of a splice acceptor site resulting in the loss of exon 5a and 6 and a failure to translocate into the nucleus (Haubst et al., 2004; Graw et al., 2005; Dames et al., 2010), have been studied. Therefore, we set out to examine the extent to which the *Leca2* and *Leca4* mutations might affect the developing forebrain in a more distinct manner.

## MATERIALS AND METHODS

### Animals

All experimental procedures were performed in accordance with German and European Union guidelines. The *Pax6<sup>Leca2</sup>* and *Pax6<sup>Leca4</sup>* mouse lines (Thaung et al., 2002) were obtained from the GlaxoSmithKline Research & Development (UK) and maintained as previously published (Thaung et al., 2002). *Pax6<sup>Sey</sup>* mice (Hill et al., 1991) were maintained on a C57BL/6J×DBA/2J (B6D2F1) background.

### Immunohistochemistry and *in situ* hybridization

Whole heads and brains isolated from E12 to E17 embryos were fixed in 4% (w/v) paraformaldehyde in phosphate-buffered saline (PBS). Tissue preparation and immunostaining were performed as described (Haubst et al., 2004). Antibodies are described in supplementary material Table S1. Riboprobes for *Ngn2* (kindly provided by Francois Guillemot, NIMR, London, UK), *Rbp1* (Holm et al., 2007) and *Id2*, *Lgals1*, *Id4*, *Hjwp* and *Zic1* (templates generated by PCR cloning from mouse cDNA; see supplementary material Table S2 for primers) were synthesized with digoxigenin-labeled NTPs (Roche) according to the manufacturer's recommendation. *In situ* hybridization was performed on cryosections as described (Pinto et al., 2008).

### Chromatin immunoprecipitation-qPCR (qChIP) assay

The qChIP experiments were performed as previously described (Asami et al., 2011).

### Clonal analysis

Clonal analysis was performed as described (Haubst et al., 2004). For overexpression of *Pax6* with the *Leca* mutations, full-length cDNA of mouse *Pax6<sup>Leca4</sup>* and *Pax6<sup>Leca2</sup>* were cloned into a CAG-GFP retroviral

vector, with CAG-GFP vector as negative control and CAG-*Pax6*-IRES-GFP as positive control (Berninger et al., 2007; Blum et al., 2011).

### RNA isolation, microarray analysis and qRT-PCR

Total RNA was isolated from rostral cerebral cortex tissue of E14 embryos of *Pax6<sup>Leca4</sup>*, *Pax6<sup>Leca2</sup>*, *Pax6<sup>Sey</sup>* and respective wild-type littermates and the microarray analysis was performed as described (Pinto et al., 2008). Data sets were filtered for average expression exceeding 50 in at least one group (mutant or wild type), false discovery rate (FDR) below 10% and linear ratios exceeding 1.4× (mutant/wild type). Array data are available at GEO under accession number GSE35260.

For RT-PCR, extracted RNA was reverse transcribed to cDNA using the SuperScript II Kit (Invitrogen) according to the manufacturer's instructions. Real-time PCR was performed using gene-specific primers (supplementary material Table S2) as described (Pinto et al., 2008).

### Luciferase assay

The pGL3-based promoter plasmids containing six copies of each DNA-binding motif, the parental expression plasmid pKW10, and pKW10 expressing wild-type *Pax6* have been described previously (Xie and Cvekl, 2011). The pKW10 expression vectors containing the mutant *Leca2* and *Leca4* forms of *Pax6* were generated using the QuikChange mutagenesis system (Stratagene). The luciferase assay was performed as previously described (Asami et al., 2011).

### Quantification and statistical analysis

All quantifications were performed on images from the dorsal telencephalon in rostral regions using level-matched sections of at least three stage-matched embryos of each genotype from at least three different litters. Statistical analysis was performed using the one-way ANOVA test.

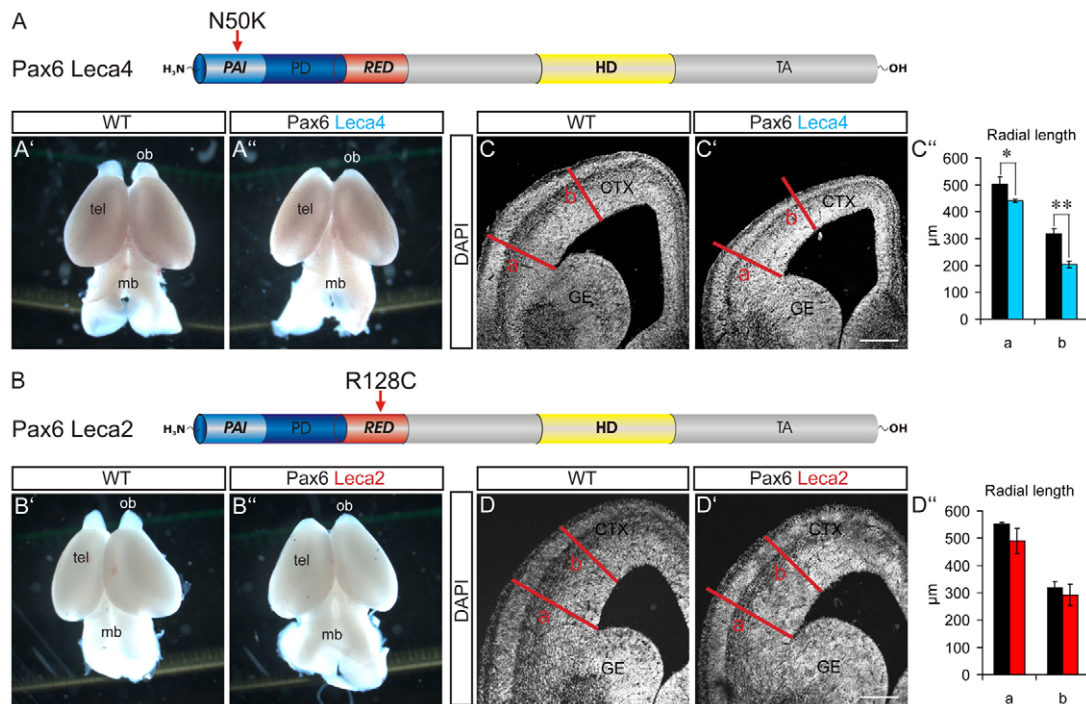
## RESULTS

### Telencephalon development in *Pax6<sup>Leca4</sup>* (PAI subdomain) and *Pax6<sup>Leca2</sup>* (RED subdomain) mutant mice

The first gross morphological analysis of homozygous *Pax6<sup>Leca4</sup>* and *Pax6<sup>Leca2</sup>* mutant mouse embryos showed similar eye and craniofacial defects (supplementary material Fig. S2) to the functional null allele (*Pax6<sup>Sey</sup>*; supplementary material Fig. S2) (Hill et al., 1991; Tzoulaki et al., 2005). However, in contrast to *Pax6<sup>Sey</sup>* and *Pax6<sup>Leca4</sup>* mutants, which both die at birth, *Pax6<sup>Leca2</sup>* mutants developed to adulthood in an almost Mendelian ratio (21% instead of 25%,  $n=64/311$ ; supplementary material Fig. S2). Notably, craniofacial abnormalities were no longer grossly evident in adult *Pax6<sup>Leca2</sup>* mutants.

In contrast to *Pax6<sup>Sey</sup>*, both subdomain mutants (Fig. 1A,B) had a detectable olfactory bulb (OB), although of reduced size (Fig. 1A',A'',B',B''; supplementary material Fig. S2, note the incomplete penetrance in *Pax6<sup>Leca2</sup>* in S2B,C'''). The absence of the OB in *Pax6<sup>Sey</sup>* is due to misspecification of the olfactory placode, which normally expresses *Pax6*, and the aberrant assembly of OB neurons within the ventral telencephalon forming the olfactory bulb-like structure (OBLS) (Jiménez et al., 2000; Nomura and Osumi, 2004). However, staining for reelin to detect mitral cells revealed no such aberrant OBLS in *Pax6<sup>Leca4</sup>* or *Pax6<sup>Leca2</sup>* brains (supplementary material Fig. S3A-C), suggesting an initially normal formation of the OB anlage, which then fails to further extend in these mice.

Next we examined the thickness of the cerebral cortex by comparing anatomically matched, DAPI-stained coronal sections of the embryonic day (E) 14 telencephalon (Fig. 1C,C',D,D'). In *Pax6<sup>Leca4</sup>* homozygotes, radial cortical thickness was significantly reduced in the rostral cortex (Fig. 1C-C'), where *Pax6* is expressed at highest levels (Bishop et al., 2000). This phenotype resembles that of *Pax6<sup>Sey</sup>* cortices. By contrast, no reduction in radial length



**Fig. 1. A point mutation in the PAI or RED subdomain of the Pax6 DNA-binding domain impairs telencephalon development.** (A,B) The domain structure of Pax6. Arrows indicate the position of the point mutations in the PAI (Pax6<sup>Leca4</sup>, A) and RED (Pax6<sup>Leca2</sup>, B) subdomains. (A',A'',B',B'') E14 brains of Pax6<sup>Leca4</sup> (A'), Pax6<sup>Leca2</sup> (B'') mice and their WT littermates (A',B'). (C,C',D,D') Micrographs of representative sections of the E14 telencephalon of Pax6<sup>Leca4</sup> (C'), Pax6<sup>Leca2</sup> (D') and their WT littermates (C,D). (C',D') Radial length of the cerebral cortex at the positions (a, b) indicated by red lines in the sections. Black bars, WT; blue or red bars, Leca4 or Leca2 mutant. Data are mean  $\pm$  s.e.m.;  $n \geq 3$  (embryos analyzed); \* $P < 0.05$ , \*\* $P < 0.01$ . ob, olfactory bulb; tel, telencephalon; mb, midbrain; CTX, cortex; GE, ganglionic eminence. Scale bars: 150  $\mu$ m.

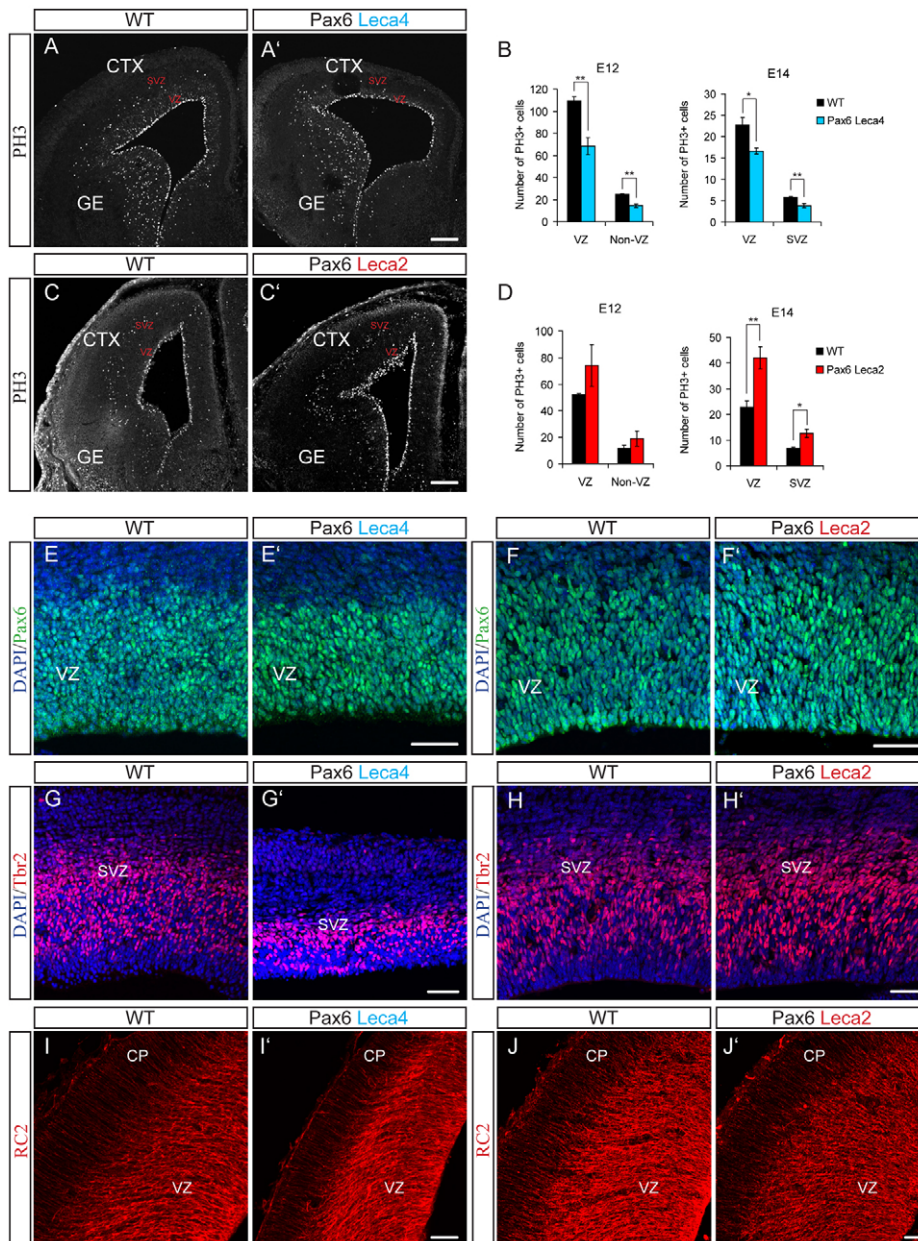
was detectable at comparable positions in the Pax6<sup>Leca2</sup> mutant (Fig. 1D').

### Proliferation is differently affected in the cerebral cortex of Pax6<sup>Leca4</sup> and Pax6<sup>Leca2</sup> mice

The reduction in the radial thickness of the Pax6<sup>Leca4</sup> neocortex might be the consequence of impaired proliferation, impaired neurogenesis and/or increased cell death, as Pax6 has been implicated in all these functions (Schmahl et al., 1993; Götz et al., 1998; Chapouton et al., 1999; Stoykova et al., 2000; Toresson et al., 2000; Yun et al., 2001; Estivill-Torrus et al., 2002; Heins et al., 2002; Nikolettou et al., 2007; Ninkovic et al., 2010). We first examined the number of proliferating cells by immunostaining for the phosphorylated form of histone H3 (PH3) present in the G2/M phase of the cell cycle (Fig. 2). This allows the discrimination of apical progenitors, which undergo cell divisions at the ventricular surface, and basal progenitors, which undergo cell division distant from the ventricle (Fig. 2A,C). The numbers of both apical and basal PH3<sup>+</sup> cells were significantly decreased at E12 and E14 in the rostral cerebral cortex of Pax6<sup>Leca4</sup> mutants (Fig. 2B), whereas they were almost doubled ( $P < 0.05$ ) at E14 in the Pax6<sup>Leca2</sup> cerebral cortex (Fig. 2D). Apical and basal progenitors were Pax6<sup>+</sup> and Tbr2<sup>+</sup> (Eomes – Mouse Genome Informatics), respectively, in both mutants (Fig. 2E-H'), and also the morphology of Pax6<sup>+</sup> radial glia stained with RC2 (see supplementary material Table S1) appeared grossly normal (Fig. 2I,J'). Thus, only the number of cells in mitosis, but not the identity of these progenitors, is distinctly affected in the Pax6<sup>Leca4</sup> and Pax6<sup>Leca2</sup> mutants.

### Dorsoventral patterning is largely normal in the telencephalon of Pax6<sup>Leca4</sup> and Pax6<sup>Leca2</sup> mice

Intriguingly, the proliferation phenotypes of both the Pax6<sup>Leca4</sup> and Pax6<sup>Leca2</sup> cerebral cortex differ from the phenotype of full Pax6 mutants, such as Pax6<sup>Sey</sup> or conditional Pax6 deletion, which have normal numbers of apical, but increased numbers of basal, progenitors (Haubst et al., 2004; Tuoc et al., 2009). As a particularly high number of basal progenitors is characteristic of the ventral telencephalon, and because the transcription factors that are normally restricted there, such as Gsx1/2, Dlx1/2 and Olig2, spread into the dorsal telencephalon, i.e. the pallium, in full Pax6 mutants (Stoykova et al., 2000; Toresson et al., 2000; Yun et al., 2001), this ventralization of the cortex contributes to the aberrations in proliferation and can be partially rescued in Pax6<sup>Sey</sup>;Gsx2 double-mutant mice (Toresson et al., 2000). In contrast to Pax6<sup>Sey</sup>, ventral telencephalic genes were not expressed in most of the dorsal telencephalon, the dorsal and medial pallium in either Leca mutant (Fig. 3A-C). However, at the pallial-subpallial (dorsal telencephalon-ventral telencephalon) boundary (PSB), scattered Gsx2<sup>+</sup> and Olig2<sup>+</sup> cells spread into the ventral pallium (the region ventral to the sulcus but dorsal to the boundary; see Fig. 3M) in the Pax6<sup>Leca4</sup>, but not Pax6<sup>Leca2</sup>, mutant (Fig. 3A',B',D',E'). Note that Olig2<sup>+</sup> cells had spread within the Pax6<sup>Leca4</sup> ventricular zone and hence are not oligodendrocyte progenitors migrating normally into the dorsal telencephalon at subventricular zone positions (arrowheads in Fig. 3D-F). Taken together, these data suggest that patterning of most of the dorsal telencephalon is normal in both Pax6<sup>Leca4</sup> and Pax6<sup>Leca2</sup> mutants, whereas the ventral pallium close to the PSB is ventralized in Pax6<sup>Leca4</sup> homozygotes. Notably, the



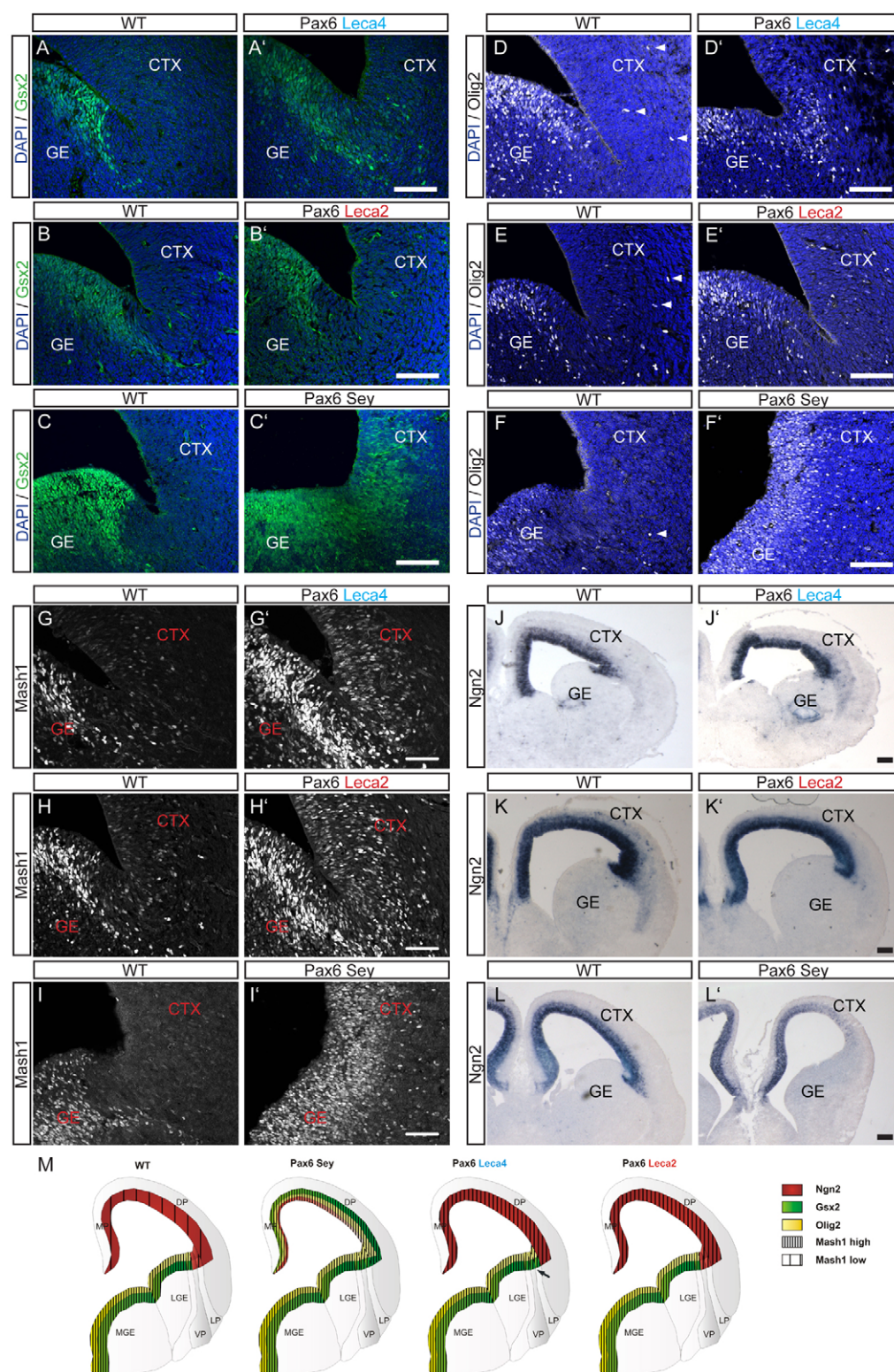
**Fig. 2. Mutations in the PAI and RED subdomains of Pax6 affect the number of apical and basal mitoses in an opposing manner.** (A,A',C,C') Micrographs of E14 telencephalon sections showing cells immunoreactive for phosphohistone H3 (PH3) in Pax6<sup>Leca4</sup> and Pax6<sup>Leca2</sup> mice and their WT littermates. (B,D) The number of PH3<sup>+</sup> progenitors in Pax6<sup>Leca4</sup>, Pax6<sup>Leca2</sup> and their WT littermates at E12 and E14. Data are mean  $\pm$  s.e.m.,  $n \geq 3$  (embryos analyzed); \* $P < 0.05$ , \*\* $P < 0.01$ . Note the opposite effects of the Pax6<sup>Leca4</sup> and Pax6<sup>Leca2</sup> mutations on cortical progenitors. (E-J') Micrographs showing immunoreactivity for Pax6 (E-F'), Tbr2 (G-H') and RC2 (I-J') on E14 dorsal telencephalon of Pax6<sup>Leca4</sup>, Pax6<sup>Leca2</sup> and WT littermates. CTX, cortex; GE, ganglionic eminence; VZ, ventricular zone; SVZ, subventricular zone. Scale bars: 100  $\mu$ m in A,A',C,C'; 50  $\mu$ m in E-J'.

sulcus, which constitutes the morphological dorsal border of this region, is still maintained in both Leca mutant cortices, whereas it is virtually absent in the Pax6<sup>Sey</sup> cerebral cortex (Fig. 3C',F',I',L').

In contrast to Gsx2 or Olig2, however, Mash1 (Ascl1 – Mouse Genome Informatics) was upregulated in a rather widespread manner in both Pax6<sup>Leca2</sup> and Pax6<sup>Leca4</sup> cerebral cortices (Fig. 3G,H'), although in fewer cells than in Pax6<sup>Sey</sup> (Fig. 3I,I'). As the bHLH transcription factors Neurog1/2 and Mash1 negatively regulate each other (Fode et al., 2000; Britz et al., 2006) and Neurog1/2 were downregulated in the Pax6<sup>Sey</sup> cerebral cortex (Haubst et al., 2004), we examined Neurog2 as a possible cause for the increase in Mash1 expression. In pronounced contrast to the Pax6<sup>Sey</sup> cerebral cortex, a strong Neurog2 mRNA signal was present throughout the cerebral cortex of both Pax6<sup>Leca4</sup> and Pax6<sup>Leca2</sup> mutants (Fig. 3J-L'). Thus, the function of both PD subdomains is required to restrict Mash1 expression in the cerebral cortex, whereas mutation of either subdomain is dispensable for grossly normal expression of Neurog2, Gsx2 and Olig2.

### Neurogenesis is selectively impaired in the cerebral cortex of Pax6<sup>Leca4</sup> but not Pax6<sup>Leca2</sup> mice

The change in the number of progenitors in Leca mutants, but the normal expression of progenitor markers such as Neurog2 and Tbr2, prompted us to analyze neurogenesis in Leca mutants by immunostaining for Map2, which labels all differentiating neurons in the cortical plate (CP), and for Tbr1, which is enriched in layer VI neurons (Fig. 4). Whereas the CP was notably thinner in the Pax6<sup>Leca4</sup> cerebral cortex at E14 and E17, in Pax6<sup>Leca2</sup> it was comparable to that of wild-type (WT) littermates (Fig. 4; supplementary material Fig. S4). Furthermore, neurons in the deep layers of the cerebral cortex that were immunopositive for Ctip2 (Bcl11b – Mouse Genome Informatics) and Foxp2 (supplementary material Fig. S4A,B) and Cux1<sup>+</sup> upper layer neurons (supplementary material Fig. S4A,B) were reduced in number in the Pax6<sup>Leca4</sup> cerebral cortex, but grossly normal in number in the Pax6<sup>Leca2</sup> cerebral cortex at E17 (supplementary material Fig. S4A,B) and adult (supplementary material Fig. S4C). Thus, neurons



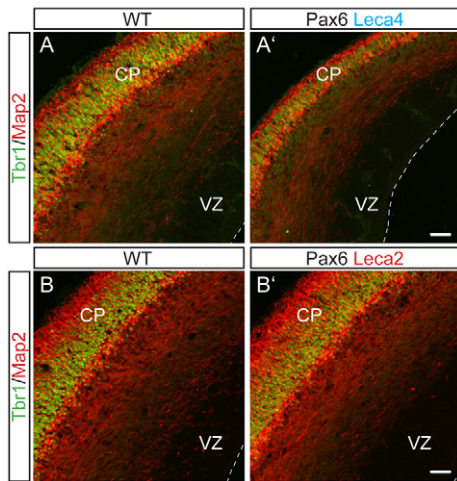
**Fig. 3. PAI and RED subdomains are largely redundant in controlling dorsoventral telencephalic patterning.**

(A-I') Micrographs showing the expression of typical ventral markers in the E14 telencephalon of Pax6<sup>Leca4</sup>, Pax6<sup>Leca2</sup>, Pax6<sup>Sey</sup> mice and their WT littermates. Note that only a few scattered Gsx2<sup>+</sup> or Olig2<sup>+</sup> cells are present in the Pax6<sup>Leca2</sup> and Pax6<sup>Leca4</sup> cerebral cortices in contrast to the overall extension in Pax6<sup>Sey</sup>. White arrowheads (D-F) indicate migrating Olig2<sup>+</sup> oligodendrocyte progenitors. (J-L') Micrographs showing Ngn2 (marking dorsal telencephalon) expression in E14 dorsal telencephalon of Pax6<sup>Leca4</sup>, Pax6<sup>Leca2</sup>, Pax6<sup>Sey</sup> and WT littermates. (M) Summary of the patterning in each mutant. The arrow indicates the ectopic expression in the ventral and lateral pallium in the Pax6<sup>Leca4</sup> mutant. CTX, cortex; GE, ganglionic eminence (L and M indicate lateral and medial); P, pallium (M, D, V and L indicate medial, dorsal, ventral and lateral). Scale bars: 100  $\mu$ m.

in the CP are reduced in the PAI subdomain mutant Pax6<sup>Leca4</sup>, but unaffected in the RED subdomain mutant Pax6<sup>Leca2</sup>.

The latter finding is intriguing given the increase in mitotic cells in the Pax6<sup>Leca2</sup> cerebral cortex, which might result in delayed neuronal differentiation or even an increase in neuron numbers if the increased progenitors all generate neurons. As Pax6 has also been implicated in the regulation of neuron survival

(Nikolopoulos et al., 2007), we investigated a possible alteration in cell death rate by quantifying cells immunoreactive for activated caspase 3 in the rostral cerebral cortex of each mutant as compared with their respective WT littermates (supplementary material Fig. S5). No change in the number of activated caspase 3<sup>+</sup> cells was detected in Pax6<sup>Leca4</sup>, but in the Pax6<sup>Leca2</sup> cerebral cortex almost double the number of activated caspase 3<sup>+</sup> cells was observed



**Fig. 4. Neurogenesis is impaired by the PAI subdomain mutation (Pax6<sup>Leca4</sup>) but not the RED mutation (Pax6<sup>Leca2</sup>).** (A–B') Micrographs showing cells immunoreactive for Tbr1 marking layer VI neurons and Map2 marking cortical plate in coronal sections of E14 dorsal telencephalon of (A') Pax6<sup>Leca4</sup>, (B') Pax6<sup>Leca2</sup> and (A,B) WT littermates. Note the cortical plate reduction in the Pax6<sup>Leca4</sup> mice. CP, cortical plate; VZ, ventricular zone. Scale bars: 100  $\mu$ m.

(supplementary material Fig. S5). Interestingly, apoptotic cells were selectively increased in the progenitor layers of Pax6<sup>Leca2</sup> cerebral cortex (supplementary material Fig. S5), i.e. in the region of increased PH3<sup>+</sup> cells.

#### **In vitro clonal analysis demonstrates effects of Pax6<sup>Leca4</sup>, but not Pax6<sup>Leca2</sup>, mutation on neurogenesis and proliferation**

To directly investigate the neurogenic capacity of Pax6 harboring the Leca2 and Leca4 mutations we first examined the progeny of single progenitor cells after overexpression of the Leca mutant forms in dissociated primary cultures of WT E14 cerebral cortex. Replication incompetent MLV-based retroviral vectors encoding Pax6<sup>Leca2</sup>, Pax6<sup>Leca4</sup>, WT Pax6 or controls expressing an eGFP reporter (Fig. 5B) were used to infect fewer than 50 cells per coverslip, and distinct clusters of infected cells were considered as clones and analyzed after 1 week *in vitro* (Heins et al., 2002; Haubst et al., 2004). Clones were classified as pure neuronal (NeuN<sup>+</sup> cells), mixed (NeuN<sup>+</sup> and NeuN<sup>-</sup> cells) or pure non-neuronal clones (NeuN<sup>-</sup> cells) (NeuN is also known as Rbfox3 – Mouse Genome Informatics) (Fig. 5A).

Forced expression of WT Pax6 increased pure neuronal clones (Fig. 5C), consistent with our previous data (Heins et al., 2002; Haubst et al., 2004). Conversely, clone composition did not differ from that with the GFP-only control vector after overexpression of the PAI mutant Pax6<sup>Leca4</sup>, suggesting that the Leca4 mutant has lost its neurogenic capacity (Fig. 5C). In pronounced contrast, Pax6<sup>Leca2</sup> transduction significantly increased the number of neuronal clones, thus indicating maintenance of its neurogenic capacity (Fig. 5C). These data were further corroborated by analysis of clones infected with the GFP control virus in cultures isolated from the Pax6<sup>Leca4</sup> cerebral cortex at E14, which generated significantly fewer pure neuronal clones than those from their WT littermates (Fig. 5D), suggesting a defect in neuronal progenitor cells. Conversely, Pax6<sup>Leca2</sup> cells showed no change in clone composition (Fig. 5E) consistent with their apparently normal neurogenesis. Taken together, these data suggest a selective role of the N50 residue

within the PAI subdomain for neurogenic fate instruction in cortical progenitors, whereas the R128C mutation within the RED domain does not impair this function.

We also observed intriguing differences in clone size. Forced expression of WT Pax6 resulted in reduced clone size, as previously described (GFP control,  $5.9 \pm 1.4$  cells per clone; Pax6-GFP,  $3.6 \pm 0.8$ ) (see Haubst et al., 2004). Whereas overexpression of the PAI mutant Pax6<sup>Leca4</sup> was still able to reduce clone size (Leca4-GFP,  $3.5 \pm 0.7$ ), clone size resembled that of the GFP control upon forced expression of the RED mutant Pax6<sup>Leca2</sup> (Leca2-GFP,  $5.9 \pm 1.5$ ). These data further support defects in the anti-proliferative role of Pax6 by the R128C mutation within the RED domain, whereas the PAI domain mutation does not affect this function.

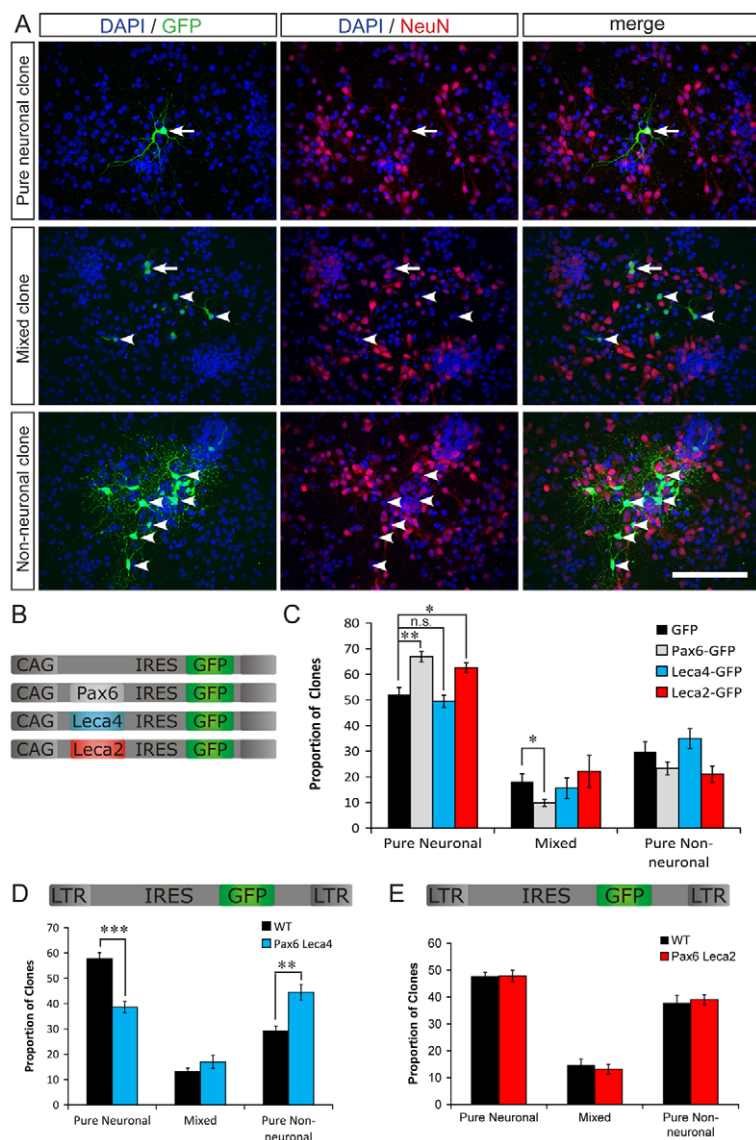
#### **Distinct changes in genome-wide expression in the Pax6<sup>Leca4</sup> versus Pax6<sup>Leca2</sup> cerebral cortex**

Given the notably different phenotypes in the cerebral cortex of individual PAI and RED subdomain missense mutants, we set out to determine the transcriptional alterations caused by these mutations. We analyzed genome-wide expression in rostral regions of E14 WT and mutant cerebral cortices by Affymetrix MOE430 2.0 arrays. Differences between WT and mutant littermates were determined by a stringent filter consisting of statistical significance (FDR < 10%), an average expression level exceeding 50 and at least 1.4-fold difference in expression. This revealed 416 probe sets altered in the PAI domain mutant Pax6<sup>Leca4</sup>, of which 179 (43%; supplementary material Table S3) were reduced in expression level (Fig. 6A), whereas only 94 probe sets differed from WT in the RED subdomain mutant Pax6<sup>Leca2</sup> (63% downregulated; supplementary material Table S4) (Fig. 6C). The reliability of this transcriptome analysis was confirmed by qRT-PCR of independent samples for 26 genes (Fig. 6B,B',D,D'). Importantly, only 17 probe sets were altered in both mutants (Fig. 6E; supplementary material Table S6), revealing largely discrete sets of gene expression differences in the PAI and RED domain mutant mice, consistent with the distinct phenotypes of these mutants.

Genes dysregulated in the Pax6<sup>Leca2</sup> cerebral cortex encode transcription factors promoting proliferation, such as Zic1 (Fig. 6G; Fig. 7A,B) (Pourelbrahim et al., 2007; Brill et al., 2010; Watabe et al., 2011) and Zic3 (Inoue et al., 2007), or regulating the progression through the cell cycle, such as Id4 (Fig. 6G) (Yun et al., 2004). In addition, pro-apoptotic genes such as *Hrk* and *Bcl2l1* (Putchala et al., 2001; Ghosh et al., 2011) were upregulated in agreement with increased cell death (supplementary material Fig. S5).

Conversely, in the Pax6<sup>Leca4</sup> cerebral cortex, we observed significant downregulation of pro-proliferative factors such as Id2 (Fig. 6G) (Uribe and Gross, 2010) and a series of neurogenic transcription factors including Dmrt1, Meis1 (Fig. 7A,B), Tcfap2d (Tcfap2d; AP-2 $\delta$ ), Tcfap2c (Tcfap2c; AP-2 $\gamma$ ), Pou3f4, Sall3 and Cux2 (Shimazaki et al., 1999; Cubelos et al., 2008; Pinto et al., 2009). In addition, other genes encoding factors involved in neurogenesis, such as members of the prokineticin family (Prkcb, Prkg2), components of the retinoic acid signaling pathway (Rbp1, Rbp1, Pbx3) (Fig. 6G; Fig. 7A,B) and Wnt signaling pathway (downregulation of Wnt7a) (Hirabayashi et al., 2004) were reduced in expression in the Pax6<sup>Leca4</sup> cerebral cortex, and negative regulators of Wnt signaling such as Lgals1 (Fig. 6G) (Satelli and Rao, 2011) were increased. Interestingly, these genes involved in neurogenesis were also dysregulated in Pax6<sup>Sey</sup>, but not in Pax6<sup>Leca2</sup> (Fig. 6F).

In order to identify direct targets among the differentially regulated genes, we compared those affected in the Leca mutants



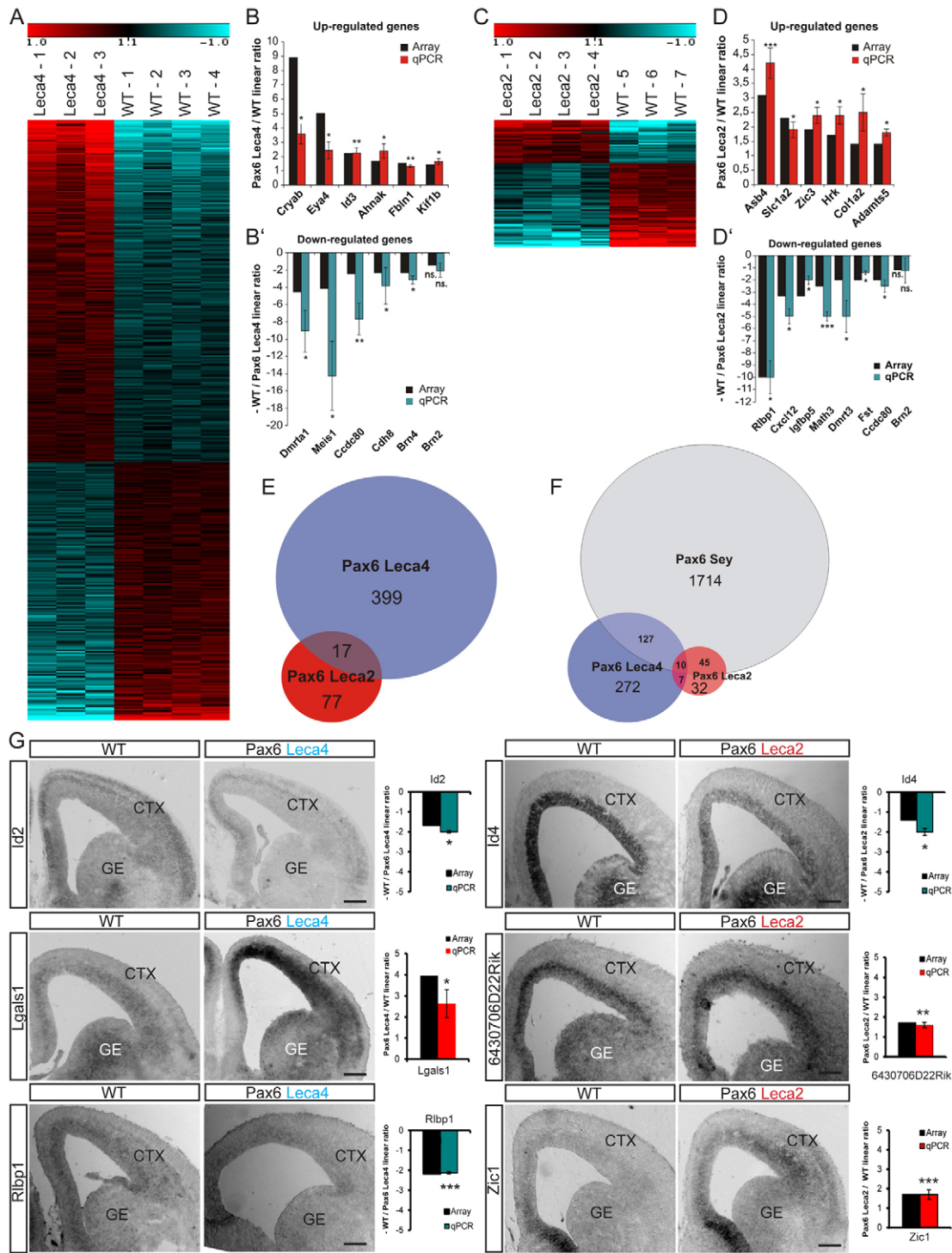
**Fig. 5. The PAI subdomain mutation interferes with neuronal fate commitment.** (A) Representative images of neuronal, non-neuronal and mixed clones. Arrows indicate neuronal cells and arrowheads non-neuronal cells. Scale bar: 50  $\mu$ m. (B) Summary of the retroviral constructs employed in C. (C) The clone types generated after overexpression of Pax6 or its mutated forms Pax6<sup>Leca4</sup> and Pax6<sup>Leca2</sup> in E14 WT mouse cortical progenitors. Note the significant increase in pure neuronal clones after overexpression of Pax6 (gray) and Pax6<sup>Leca2</sup> (red) but not Pax6<sup>Leca4</sup> (blue) compared with the control GFP-expressing virus. (D,E) The clone types generated from the E14 cortical progenitors isolated from the Pax6<sup>Leca4</sup> mutant (D), Pax6<sup>Leca2</sup> mutant (E) or WT littermates. Data are mean  $\pm$  s.e.m.;  $n \geq 6$  (coverslips analyzed); \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ; n.s., not significant.

with Pax6 ChIP data obtained with E15 WT cerebral cortex, lens and pancreas cells (Xie and Cvekl, 2011; Xie et al., 2013). Interestingly, Pax6 was bound to the promoters of ~20% of the genes regulated in either Pax6<sup>Leca4</sup> or Pax6<sup>Leca2</sup> (supplementary material Tables S3, S4 and S7). To gain further insights into direct Pax6 targets, we also compared gene expression changes with the full Pax6<sup>Sey</sup> mutant, isolating tissue at the same stage and from the same region. A much larger number of genes were differentially regulated in this mutant (Fig. 6F), which might result from a partially redundant function of the PAI and RED subdomains, as observed for patterning. Indeed, patterning is profoundly altered in the full Pax6 mutant, with *Olig2*, *Gsx1/2*, *Mash1* and *Dlx* transcription factors ectopically increased in the Pax6<sup>Sey</sup> cerebral cortex, which will in turn further affect gene expression, whereas only *Mash1* was increased in both Leca mutants, and *Olig2* mRNA (but not the protein; see Fig. 3) was elevated in Pax6<sup>Leca2</sup> (supplementary material Tables S3–S5). Moreover, comparing Pax6 ChIP data with the genes regulated in the respective Leca mutants and the full Pax6 mutant revealed an overlap of 23% (supplementary material Tables S7–S9), and 93% of these were regulated in the same manner (i.e. up- or downregulated), suggesting that they are direct targets and not indirectly affected by patterning.

Taken together, our data revealed largely distinct batteries of genes with disrupted expression in both Pax6 missense mutants versus the Pax6<sup>Sey</sup> nonsense mutant, suggesting their differential regulation by the PAI or the RED subdomain.

### The Leca4 and Leca2 mutations of Pax6 selectively affect transactivation of PAI- or RED domain-containing motifs

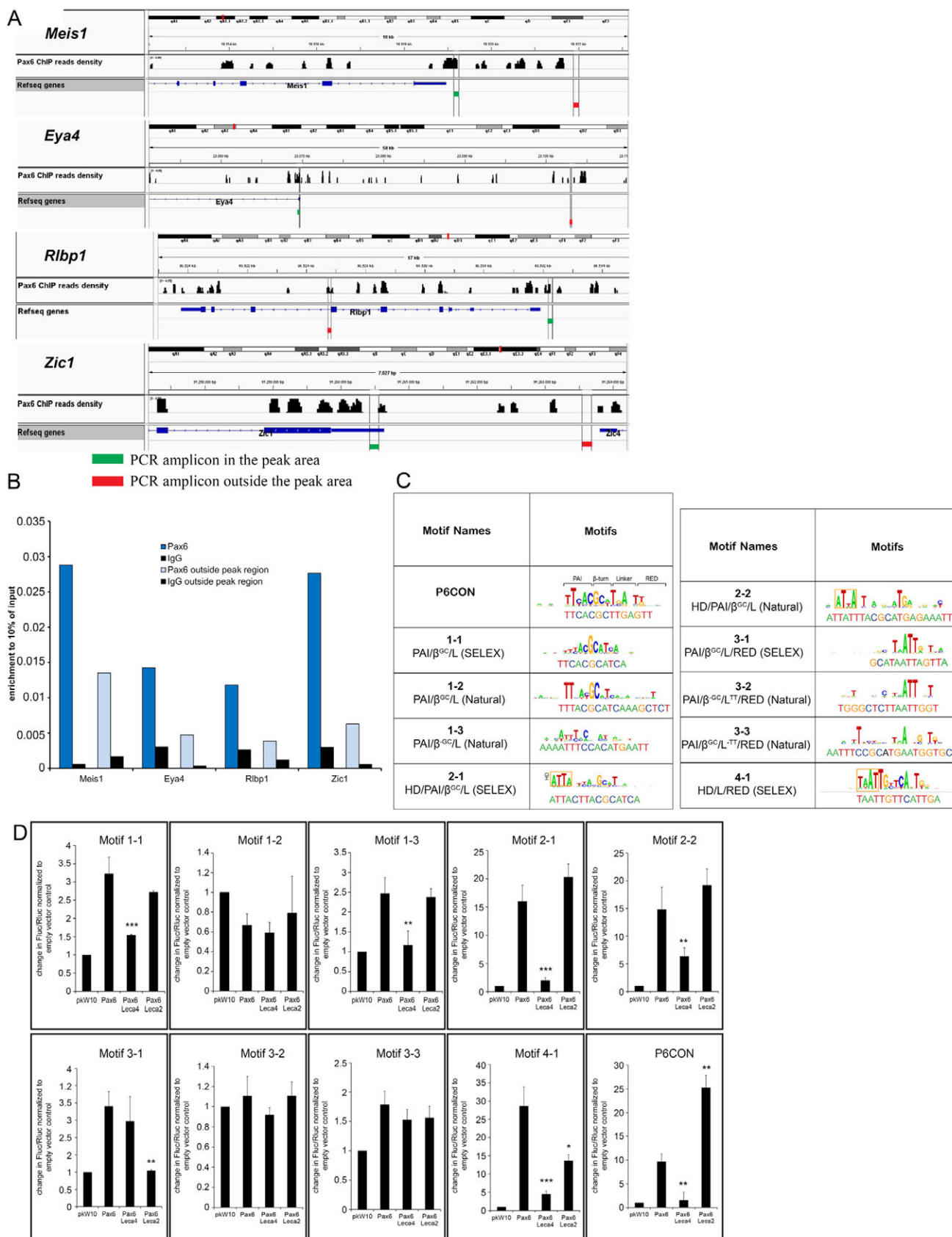
The largely discrete alterations in gene expression in the Pax6<sup>Leca4</sup> and Pax6<sup>Leca2</sup> cerebral cortices suggest distinct effects of the respective mutations on gene expression. Indeed, bioinformatic and crystallographic analyses also predicted a selective effect of the N50 amino acid that is mutated in the Pax6<sup>Leca4</sup> line on DNA binding of the PAI subdomain. N50 is the first residue in the DNA-contacting helix ( $\alpha 3$ ) of the PAI subdomain and part of a cluster of seven amino acid residues that render Pax6 unique DNA binding specificity compared with other Pax proteins (Czerny and Busslinger, 1995). Crystallographic data established direct contact between N50 and an invariant T residue found in many Pax6 binding site motifs (Xu et al., 1999; Xie and Cvekl, 2011). Therefore, the N50K mutation is predicted to disrupt a crucial interaction between the PAI subdomain of Pax6 and DNA (supplementary material Fig. S1B) (Thaung et



**Fig. 6. PAI and RED subdomain mutations affect different gene sets.** (A,C) Heat map representing the expression of dysregulated genes in the Pax6<sup>Leca4</sup> (A) or Pax6<sup>Leca2</sup> (C) E14 mouse cortex compared with WT littermates. Red/blue indicate higher/lower expression values. (B,B',D,D') Gene expression changes in Pax6<sup>Leca4</sup> and Pax6<sup>Leca2</sup> compared with WT littermates as measured by Affymetrix array analysis or qRT-PCR. (E,F) Venn diagrams depicting the overlap between the dysregulated probe sets in the Pax6<sup>Leca4</sup> and Pax6<sup>Leca2</sup> cortices (E) and in these versus the Pax6<sup>Sey</sup> cortex (F). (G) Micrographs showing *in situ* hybridization on sections of E14 telencephalon of Pax6<sup>Leca4</sup>, Pax6<sup>Leca2</sup> and WT littermates for *Id2*, *Lgals1*, *Rbp1*, *Id4*, *Hjurp* and *Zic1*. To the right is shown the expression changes detected in the microarray and by qPCR. Data are mean  $\pm$  s.e.m., \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ; ns, not significant. CTX, cortex; GE, ganglionic eminence. Scale bars: 200  $\mu$ m.

al., 2002). The R128 amino acid mutated in the Pax6<sup>Leca2</sup> line is located within the sixth helix ( $\alpha 6$ ) of the PD domain and has a perfect DNA binding distance of 2.1 Å, suggesting that the R128C

mutation disrupts the DNA binding capacity of the RED subdomain (supplementary material Fig. S1C). This is in agreement with decreased or absent DNA binding as previously reported



**Fig. 7. Leca4 and Leca2 mutations in Pax6 affect its transactivation properties on Pax6 DNA-binding motifs.** (A) Pax6 binding regions in the promoters of target genes and position of primers used for ChIP-qPCR. (B) Enrichment of Pax6 binding to target loci in the area containing the newly identified motifs (binding within the peak regions) and in the adjacent area (binding outside of the peak regions). (C) DNA-binding site variants regulated by Pax6. (D) The transactivation properties of WT Pax6, Pax6<sup>Leca2</sup> and Pax6<sup>Leca4</sup>. Data are shown as mean  $\pm$  s.e.m.; \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

(Yamaguchi et al., 1997; Chauhan et al., 2004). Thus, *in silico* analysis predicts selective disruption of the DNA binding activity of the respective subdomains in the mutant proteins (supplementary material Fig. S1B,C).

However, cooperative effects have also been observed between these and other subdomains of Pax6, raising the question of the extent to which these mutant forms of Pax6 disrupt binding to various Pax6 binding sites. To clarify this, we examined the transactivation properties of the mutant proteins in luciferase assays using PAI and RED binding sites (Fig. 7C) derived from *in vivo* Pax6 binding sites (Xie and Cvekl, 2011). Strikingly, 80% (four of five) of the motifs containing ‘dominant’ PAI over RED binding motifs were impaired in their regulation by the Leca4 mutant (Fig. 7D). By contrast, no defects in activation of these constructs were observed with the Leca2 mutant Pax6, supporting the contention that these mutations exclusively affect one subdomain (Fig. 7D). From these studies, we conclude that the N50C residue of Pax6 is important for Pax6 function on sites 1-1, 1-2, 1-3, 2-1, 2-2, 4-1 and P6CON, but not for motifs 3-1, 3-2 and 3-3. By contrast, the Leca2 mutant form of Pax6 (R128C) showed loss of activity with two of the four novel domains containing a RED motif (Fig. 7D), one of which (3-1) was not affected in transactivation by the Leca4 mutation in the PAI subdomain of Pax6 (Fig. 7D). One motif was affected by either Leca4 or Leca2 mutation (4-1), whereas others (1-2, 3-2 and 3-3) were not affected in regulation by any of these mutations. Thus, the Leca4 and Leca2 mutations exert largely selective effects on motifs containing PAI or RED motifs, respectively, consistent with the distinct battery of genes regulated in these mutants *in vivo*. Indeed, the observation that one motif was affected by both mutations is reminiscent of a minority of genes being dysregulated in both Pax6<sup>Leca2</sup> and Pax6<sup>Leca4</sup> mutants, whereas others were affected by none of the mutations, reminiscent of the absence of a severe patterning phenotype *in vivo*.

Although *in vivo* regulation is too complex to claim that these elements were one to one responsible for the altered gene expression in the mutants, it is noteworthy that 80% of the genes with Pax6 binding (ChIP<sup>+</sup>; see supplementary material Table S9) and significant dysregulation in the Pax6<sup>Leca2</sup> cerebral cortex contained one or more of the motifs that the Leca2 mutant failed to activate normally in the reporter assay (Table 1). By contrast, only 35.5% (using the Genomatix platform) of the promoters of randomly selected cortical genes (Pinto et al., 2008) contain at least one of these binding motifs. Taken together, phenotypic and genome-wide expression analyses, ChIP and *in vitro* transactivation assays all support the distinct behaviors of these mutant proteins, with selective effects *in vitro* and *in vivo*.

DISCUSSION

Here we unravelled a molecular logic of how a single transcriptional regulator coordinates neurogenesis, proliferation and patterning. We show that Pax6 utilizes distinct subdomains to control proliferation or to exert selective effects on neurogenesis, whereas these subdomains act largely in a redundant manner for patterning. Our data underline the concept of a modular organization of Pax6 function, not only performing distinct tasks via the PD and HD, but also by assigning distinct roles to the PAI and RED subdomains.

The PAI and RED subdomains regulate dorsoventral patterning

Whereas the multitude of phenotypes observed after deletion of the entire Pax6 protein (Schmahl et al., 1993; Quinn et al., 2007; Tuoc et al., 2009) makes it difficult to dissect individual roles of Pax6, analysis of the Leca mutants not only elucidates the role of the distinct DNA-binding domains, but also helps to discriminate direct effects on neurogenesis and proliferation from indirect effects due to alterations in patterning of the cerebral cortex. In

Table 1. Dysregulated genes in Pax6<sup>Leca2</sup> cortex and in Pax6 ChIP analysis, including Pax6 DNA-binding motifs identified for the individual genes

Probe set	Gene symbol or ID	Leca2 significant probe sets (20/94)	Pax6 ChIP binding sites*	Leca4 significant probe sets (3)	Sey significant probe sets (13)	Motifs
1418310_a_at	Rlbp1 <sup>4</sup>	0.12	Lens	0.46	0.02	P1-2 P1-3 P4-1 P1-2
1418054_at	Neurod4	0.28	Cortex		0.10	P1-1 P1-2
1438551_at	Neurog1	0.38	Cortex	2.53	0.16	P2-1 P2-1 P3-1
1424186_at	Ccdc80	0.51	Cortex	0.41	0.55	P1-1
1423260_at	Id4	0.58	Cortex			P1-1 P2-1 P1-3 P2-1
1420459_at	Ripply3	0.59	Lens		0.69	P3-1 P3-2 P4-1
1421365_at	Fst	0.66	Cortex			P3-1 P3-2
1433782_at	Cldn12	0.67	Cortex, pancreas			P1-3 P3-1
1417872_at	Fhl1	0.67	Cortex		0.6	P1-1 P1-1 P4-1 P3-1
1442312_at	Tbl1xr1	0.69	Cortex, lens			P3-3 P1-1 P1-3 P4-1
1418172_at	Hebp1	0.70	Lens			P4-1
1430798_x_at	Mrpl15	1.46	Pancreas			P2-2 P3-1 P1-1 P2-2 P3-1 P4-1 P4-1 P4-1
1447628_x_at	Mrps5	1.50	Lens			P2-1 P3-3
1456005_a_at	Bcl2l11	1.67	Lens		1.95	P1-3 P2-1 P2-1 P2-1 P3-1 P4-1
1439854_at	Hrk	1.80	Cortex		2.00	P2-1 P3-1 P3-1 P3-2 P4-1
1452526_a_at	Pax6	1.81	Lens, pancreas		1.52	P2-2 P3-1 P3-3 P1-2 P2-1 P4-1 P1-3 P2-1 P2-1 P3-2 P3-3 P4-1 P3-3 P3-1 P3-2 P3-2
1439627_at	Zic1	1.87	Pancreas		2.73	P1-3 P2-2 P4-1 P1-1 P1-2 P2-1 P2-1 P2-1 P2-1 P2-2 P3-3 P4-1 P4-1 P4-1 P4-1 P1-1 P3-1
1438737_at	Zic3	1.90	Lens		2.83	P1-3
1438194_at	Slc1a2	2.66	Cortex		1.61	P1-3 P1-1 P4-1 P4-1 P3-3 P4-1 P4-1 P4-1
1433919_at	Asb4	3.54	Cortex		12.29	P1-3 P3-1 P4-1 P4-1 P2-1 P3-3

Important binding domains are highlighted. P1-1, PAI/βGC/L (Selex); P1-2, PAI/βGC/L (Natural); P1-3, PAI/β-GC/L (Natural); P2-1, HD/PAI/βGC/L (Selex); P2-2, HD/PAI/βGC/L (Natural); P3-1 (light gray shading), PAI/βGC/L/RED (Selex); P3-2, PAI/β-GC/L/TT/RED (Natural); P3-3, PAI/βGC/L-TT/RED (Natural); P4-1 (dark gray shading), HD/LRED (Selex).

\* (Xie et al., 2013).

<sup>4</sup>Identified by ChIP-seq in cortex chromatin (see Fig. 7A,B).

all Pax6 mutants, including conditional deletions in a cortex-specific manner (Tuoc et al., 2009), mouse chimera (Quinn et al., 2007) or even upon acute deletion of *Pax6* by Cre electroporation (Asami et al., 2011), the ectopic misexpression of genes characteristic of the ventral telencephalon, such as *Gsx2*, *Olig2*, *Mash1* or *Dlx* transcription factors, occurred, changing neuronal fate from glutamatergic to GABAergic (Stoykova et al., 2000; Toresson et al., 2000; Heins et al., 2002; Kroll and O'Leary, 2005). Given that proliferation differs somewhat between the ventral and dorsal telencephalon, with a faster cell cycle and a much higher number of subventricular zone progenitors in the former, it has thus far not been possible to dissect direct effects of Pax6 on proliferation and neurogenesis from indirect effects due to altered regionalization.

In pronounced contrast, none of the ventral transcription factors *Dlx1/2/5/6*, *Gsx1/2* or *Olig1/2* was misexpressed in a widespread manner in the dorsal cerebral cortex of *Leca2* or *Leca4* mice, with the sole exception of *Mash1*. Interestingly, despite the expanded *Mash1* expression, *Neurog2* (*Ngn2*), which is normally regulated in an opposing manner to *Mash1* in the forebrain (Fode et al., 2000; Parras et al., 2002), and *Tbr2* were both still expressed normally in the cerebral cortex of both *Leca* mutants. Both *Neurog2* and *Tbr2* are virtually absent in the Pax6<sup>Sey</sup> cerebral cortex at this stage (Stoykova et al., 2000; Muzio et al., 2002; Scardigli et al., 2003). As they are involved in glutamatergic neurogenesis (Schuurmans et al., 2004; Sessa et al., 2008), this further contributes to the conversion of glutamatergic to GABAergic neurogenesis in the full Pax6 mutants (Kroll and O'Leary, 2005; Quinn et al., 2007; Tuoc et al., 2009). Accordingly, glutamatergic neurogenesis is maintained in the *Leca* mutants and GABAergic neurons are neither increased nor accumulate ectopically in the white matter as observed in Pax6<sup>Sey</sup> mutants (supplementary material Fig. S4; data not shown). These phenotypes reveal that the remaining Pax6 subdomain function (PAI or RED) is still sufficient to achieve relatively normal dorsoventral patterning and therefore to allow the role of Pax6 in neurogenesis and progenitor proliferation to be determined in the absence of mispatterning.

Interestingly, the subdomain of the dorsal telencephalon closest to the boundary to the ventral telencephalon, the ventral pallium, appears most susceptible to mispatterning and ectopically expressed *Gsx2* and *Olig2* in the PAI, but not in the RED, subdomain mutant of Pax6. Thus, the PAI subdomain is crucial in the previously described antagonistic role of *Gsx2* and Pax6 (Toresson et al., 2000) and the RED subdomain on its own is not sufficient to fully restrict *Gsx2* to its normal region of expression. Moreover, a profound upregulation of the ventral pallium-specific transcription factor *Dbx1* is observed in the genome-wide microarray expression analysis of the Pax6<sup>Leca4</sup> cerebral cortex to an even higher extent than in the Pax6<sup>Sey</sup> mutant, whereas no change in *Dbx1* was observed in the Pax6<sup>Leca2</sup> cerebral cortex. This further supports abnormalities in the ventral pallium upon N50K mutation in the PAI domain, whereas the R128C mutation in the RED domain does not affect patterning.

### PAI domain-specific function in neurogenesis

Despite the absence of mispatterning, the gross morphology of the cerebral cortex of Pax6<sup>Leca4</sup> mice resembled the morphology observed in full Pax6 mutant mice, with a smaller, shorter cerebral cortex (Fig. 1) (Asami et al., 2011) and a thinner CP with reduced numbers of neurons (Schmahl et al., 1993; Heins et al., 2002). By contrast, this phenotype was not visible in mice carrying the R128C mutation in the RED domain, consistent with normal neurogenesis

in these mice. These selective effects on neurogenesis were further corroborated by clonal analysis *in vitro*. Thus, the PAI domain is essential for neuronal fate instruction, whereas the R128C mutation in the RED domain does not affect this function of Pax6.

### Distinct effects of the PAI and RED subdomain mutations on cell proliferation

The numbers of mitotic cells are clearly increased by E14 in the cerebral cortex of the RED domain Pax6<sup>Leca2</sup> mutants, whereas their number is decreased in the Pax6<sup>Leca4</sup> cerebral cortex, supposedly contributing to the smaller size of the Pax6<sup>Leca4</sup> cerebral cortex. Notably, the number of apical mitoses is unaffected in the full Pax6 mutant cortex, suggesting that the effects of both subdomains largely outcompete each other in these progenitors and that the selective increase in progenitors dividing at non-apical positions in the full Pax6 mutant (Götz et al., 1998; Haubst et al., 2004; Tuoc et al., 2009) might largely result from ventralization of the cortex. However, the *Leca* mutations affect proliferation of both apical and basal progenitors in the dorsal pallium in the absence of patterning defects, suggesting a different, indirect mechanism of how Pax6 might regulate basal progenitor divisions, as it is not expressed in basal progenitors, including in the *Leca* mutants.

The novel concept that the same transcription factor affects proliferation in opposing manners via its DNA-binding subdomains also has implications with regard to the diverse effects of Pax6 on region- and cell type-specific proliferation (Marquardt et al., 2001; Haubst et al., 2004; Sakurai and Osumi, 2008; Sansom et al., 2009). Loss of Pax6 can lead to a decrease in proliferation, such as in the developing diencephalon (Warren and Price, 1997), retina (Marquardt et al., 2001) and postnatal glial progenitors (Sakurai and Osumi, 2008), or to increased proliferation in the telencephalon as described above. Interestingly, even in the same cell type, i.e. the progenitors of the cerebral cortex, Pax6 has been observed to both promote and inhibit proliferation (Holm et al., 2007; Osumi et al., 2008; Sansom et al., 2009). Thus, this analysis of the specific mutations in the paired subdomains sheds new light on how Pax6 affects proliferation and cell division at multiple levels (see Asami et al., 2011).

Consistent with the distinct effects of the *Leca2* and *Leca4* mutations, genes promoting cell cycle exit such as *Gadd45b* are upregulated in the Pax6<sup>Leca4</sup> cerebral cortex, whereas the pro-proliferative factors *Id2* (Uribe and Gross, 2010) and *tenascin C* (von Holst et al., 2007) are decreased in expression. Conversely, transcription factors that promote proliferation, such as *Zic1* and *Zic3* (Inoue et al., 2007; Pourebrahim et al., 2007; Brill et al., 2010; Watabe et al., 2011), were upregulated in the Pax6<sup>Leca2</sup> cerebral cortex. Interestingly, genes encoding centrosome-associated proteins that were prominently affected in expression in the Pax6<sup>Sey</sup> cerebral cortex or after acute Pax6 deletion (Asami et al., 2011), such as the direct Pax6 target *Spag5*, which regulates the orientation of cell division, are not affected in either of the *Leca* mutants. Instead, increased apoptosis occurs in the progenitor zone of the Pax6<sup>Leca2</sup> cerebral cortex in agreement with the increased expression of pro-apoptotic genes such as *Bcl2l1* and *Hrk*. The extent to which this is due to aberrant events in mitosis or proliferation or reflects direct regulation of these genes by Pax6 remains to be determined. Importantly, our analysis addresses the roles of the PAI and RED subdomains within the canonical Pax6 isoform, as the alternatively spliced Pax6(5a) isoform (Epstein et al., 1994a) is less abundant in the developing cortex at the stages we analyzed (Haubst et al., 2004) and genomic deletion of exon 5a does not impair proliferation, neurogenesis or patterning of the cerebral cortex (Haubst et al., 2004).

## Pax6<sup>Leca4</sup> (PAI subdomain) versus Pax6<sup>Leca2</sup> (RED subdomain) mutations affect Pax6 function through a series of distinct sites *in vitro* and *in vivo*

The distinct phenotypes in Pax6<sup>Leca4</sup> and Pax6<sup>Leca2</sup> mutants also imply that the respective point mutations do not have a deleterious effect on the Pax6 protein, as this would result in a phenocopy of the complete null mutation. As predicted by structural analysis and confirmed by gene expression reporter assays, these mutations affect DNA binding of each subdomain selectively. The Leca4 mutation in the PAI subdomain interfered only with transcriptional activation mediated by the PAI, but not RED, binding site motifs. Similarly, the Leca2 mutation in the RED subdomain largely spares activation via the PAI site motifs, even though a few motifs were affected in regulation by both of the Leca mutant forms (4-1) and the Leca2 mutation also resulted in superactivation of the P6CON motif. Cooperation between PAI and RED subdomains has been observed previously (Yamaguchi et al., 1997; Chauhan et al., 2004) and Leca mutations might affect the interaction of Pax6 with other proteins (Cvekl et al., 1999; Kamachi et al., 2001; Planque et al., 2001; Sivak et al., 2004; Tuoc and Stoykova, 2008) and thereby alter transcription. Indeed, the effects of Pax6 point mutations are complex and it is important to note that the two mutations analyzed here might also differ in their respective severities (Alibés et al., 2010).

Notwithstanding these considerations, both luciferase experiments and genome-wide expression analyses support the rather discrete and modular effects of these subdomain mutations, rather than one being a subset of the other. Moreover, comparing the ChIP data (Xie et al., 2013) with the transcriptomes of the Pax6<sup>Sey</sup> and Leca mutant mice revealed an overlap of 23% (supplementary material Tables S7-S9), with almost all of these genes regulated in the same direction as in the mutants, suggesting that they are direct targets of Pax6 and not, for example, indirectly affected by mispatterning in the Pax6<sup>Sey</sup> cerebral cortex. Thus, our analysis provides candidates for novel direct Pax6 targets that are regulated preferentially by distinct subdomains *in vivo*.

Beyond these individual targets, however, the concept of modular Pax6 function is of broader relevance. Pax6 not only utilizes its modular structure to perform rather distinct functions via its HD and PD (Haubst et al., 2004; Ninkovic et al., 2010), but also even individual helix-turn-helix PD subdomains seemingly exert distinct and even partially opposing functions. Our data thereby provide a molecular framework of how the same transcription factor can affect proliferation in opposing manners and regulate progenitor numbers rather precisely depending on subdomain activity, Pax6 levels and the cellular context. Thus, the co-activation of both pro- and anti-proliferative genes in the same cell population, as is the case for Pax6 (Sansom et al., 2009), might allow particularly fine-tuned regulation of proliferation, implementing the complex differences in the cell cycle progression of self-renewing or committed progenitors, a crucial aspect in the ontogeny and phylogeny of the cerebral cortex.

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### Competing interests statement

The authors declare no competing financial interests.

### Supplementary material

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