Gene regulation by PAX6: Structural-functional correlations of missense mutants and transcriptional control of Trpm3/miR-204

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Purpose: Pax6 is a key regulatory gene for eye, brain, and pancreas development. It acts as a transcriptional activator and repressor. Loss-of-function of Pax6 results in down- and upregulation of a comparable number of genes, although many are secondary targets. Recently, we found a prototype of a Pax6-binding site that acts as a transcriptional repressor. We also identified the Trpm3 gene as a Pax6-direct target containing the miR-204 gene located in intron 6. Thus, there are multiple Pax6-dependent mechanisms of transcriptional repression in the cell. More than 50 Pax6 missense mutations have been identified in humans and mice. Two of these mutations, N50K (Leca4) and R128C (Leca2), were analyzed in depth resulting in different numbers of regulated genes and different ratios of down- and upregulated targets. Thus, additional studies of these mutants are warranted to better understand the molecular mechanisms of the mutants’ action.

Methods: Mutations in PAX6 and PAX6(5a), including G18W, R26G, N50K, G64V, R128C, and R242T, were generated with site-directed mutagenesis. A panel of ten luciferase reporters driven by six copies of Pax6-binding sites representing a spectrum of sites that act as repressors, moderate activators, and strong activators were used. Two additional reporters, including the Pax6-regulated enhancer from mouse Trpm3 and six copies of its individual Pax6-binding site, were also tested in P19 cells.

Results: Pax6 (N50K) acted either as a loss-of-function or neutral mutation. In contrast, Pax6 (R128C) and (R242T) acted as loss-, neutral, and gain-of-function mutations. With three distinct reporters, the Pax6 (N50K) mutation broke the pattern of effects produced by substitutions in the surrounding helices of the N-terminal region of the paired domain. All six mutations tested acted as loss-of-function using the Trmp3 Pax6-binding site.

Conclusions: These studies highlight the complexity of Pax6-dependent transcriptional activation and repression mechanisms, and identify the N50K and R128C substitutions as valuable tools for testing interactions between Pax6, Pax6(N50K), and Pax6 (R128C) with other regulatory proteins, including chromatin remodelers.

Pax6 functions as a key regulatory gene for vertebrate eye development [1-4]. Outside the eye, Pax6 regulates neurogenesis, as well as olfactory and pancreatic development [5-7]. To achieve these diverse roles, Pax6 proteins regulate common and distinct sets of genes in different cell types, and Pax6’s function clearly depends on the cell-type context [8-15]. Pax6 directly regulates genes encoding a wide range of regulatory, signaling, and structural proteins required for eye morphogenesis [16-21]. Comprehensive understanding of Pax6 function in individual cell types in conjunction with elucidation of molecular processes disrupted in the presence of disease-causing mutations in human PAX6, as well as developmental abnormalities linked to rodent Pax6 mutant alleles, are essential for understanding human organogenesis and tissue maintenance.

At the structural level, Pax6 contains two DNA-binding domains, a paired domain (PD) and an internal homeodomain (HD). The PD comprises two independent helix-turn-helix DNA-binding subdomains, called PAI and RED [22]. The amino acid sequences of the human and mouse Pax6/Pax6 gene are identical. Alternate splicing produces two main Pax6 proteins, Pax6/p46 and Pax6(5a)/p48 [23,24], which differ by an insertion of 14 amino acids into the PAI subdomain, encoded by exon 5a, that disrupts its DNA-binding activity [25]. DNA-binding studies identified consensus sequences recognized by isolated Pax6 and Pax6(5a) PDs [25,26] followed by identification of binding sites recognized by Pax6 PD/HD and PD(5a)/HD [21]. These studies lead to the identification of a continuum of eight Pax6-binding sequences that show high, moderate, and weak transcriptional activation. The ninth sequence variant (motif 1–2) functions as a transcriptional repressor of Pax6 and Pax6(5a) [21].

Human genetic studies on aniridia and related ocular syndromes have led to the identification of more than 400 PAX6 mutations, including more than 50 missense mutations. These include a mutation R128C in the RED subdomain that
caused foveal hypoplasia in two unrelated patients [27,28]. In contrast, a much smaller number of mouse missense mutations were isolated, including Pax6<sup>6<sub>eco2</sub></sup> (V256G), Pax6<sup>6<sub>eco2</sub></sup> (R128C), Pax6<sup>6<sub>eco2</sub></sup> (N50G) [29,30], and Pax6<sup>6<sub>neo</sub></sup> (S259P) [31]. Externally, the Leca4 allelic series are displayed as a lens-gonial adhesion: Leca2 displays central corneal and lens opacities, and Leca4 is characterized by cataract in the presence of a relatively normal iris [29]. Recent analysis of Leca2 and Leca4 homozygous mutations in the mouse forebrain using high-density oligonucleotide array hybridizations revealed 94 and 416 disregulated genes, respectively [32]. Interestingly, a higher proportion of downregulated genes were found in the Leca2 (59/94, 63%) compared to the Leca4 (179/416, 43%) model. The majority of these genes are unique suggesting that different types of Pax6-binding sites respond to these mutations.

The molecular mechanisms of transcriptional activation and repression by Pax6 remain to be elucidated [2]. Pax6 has been shown to bind the Brg1 and Brm catalytic subunits of the SWI/SNF complex [33-35]. In mouse lens, Brg1 and Pax6 regulate expression of a joint set of genes [36]. Pax6 also binds p300 histone acetyltransferase [37]. Use of different chromatin remodeling complexes recruited by Pax6 to specific genes is thought to play a pivotal role in Pax6's capacity to control critical steps in embryonic development [34]. However, it is not known if Pax6 missense mutations modulate these protein–protein interactions. Transcriptional repression by Pax6 is even less understood. Apart from the site-specific repression described above, two additional mechanisms address this Pax6 function. First, Pax6 activates expression of the Trpm3 gene [15], which harbors miR-204, a key regulator microRNA (miRNA) of mammalian eye development [38]. Thus, Pax6 indirectly controls expression of genes in the specific miR-204 pathway. Second, complex formation between Pax6 and pRb can also reduce gene expression by preventing Pax6 from binding DNA [39,40].

To gain additional insights into Pax6-mediated gene activation and repression, we examined the functional properties of a representative group of six missense PAX6 and Pax6<sup>(5a)</sup> mutants. In particular, Pax6 mutations included two mouse missense mutations, N50K (Leca4) and R128C (Leca2), as both were analyzed in depth resulting in different numbers of regulated genes and different ratios of down- and upregulated targets (see above). Notably, mouse Leca2 is identical to a human Pax6 mutation identified in multiple patients as described above. The R242T mutation represents one of the few mutations known within the Pax6 homeodomain [41]. The remaining PAX6 mutations, including G18W, R26G, and G64V, were examined elsewhere using P6CON/luciferase reporters [40,42,43]. Herein, we used a panel of 12 distinct luciferase reporters, with a primary focus on N50K and R128C, as genetic and molecular studies of these Pax6-mutated genes are now available for comparisons [32]. The reporter systems used here included a panel of ten Pax6-binding sites recognized by different combinations of the PAI, RED, and HD subdomains [21], coupled with two reporters based on a recently identified Pax6-dependent enhancer in the Trpm3/miR-204 locus [15]. We found that the N50K and R128C mutations in PAX6 have unexpected properties relative to other mutations that impair structure and function of either the PAI or RED subdomain that can be used for future mechanistic studies of protein–protein complexes recruited by Pax6 to chromatin DNA.

**METHODS**

**Reportor gene construction:** A 1.5 kb region (mm9: chr19:22524101–22525510) of the mouse Trpm3 gene that includes a previously identified Pax6-binding site [15] was synthesized by GenScript and cloned into a pGL3-Basic vector, which contains a firefly luciferase reporter gene. Six copies of Pax6-binding site 3.4 (5'-ACT TTA AAG GAT GAC AGA ATT-3'); Pax6-binding nucleotides underlined) and motifs P6CON, 1–1, 1–2, 1–3, 2–1, 2–2, 3–1, 3–2, 3–3, and 4–1 were also made as described above and cloned into the E4-TATA-firefly luciferase reporter as described earlier [21,42].

**Cell cultures, transfections, and reporter assays:** The transient transfections were conducted in embryonic carcinoma P19 cells that lack endogenous Pax6 expression as described earlier [21]. PAX6 and Pax6<sup>(5a)</sup> cDNAs were generated in CMV-containing vector pKW10 as described earlier [32,42]. Briefly, the cells were cultured on 10 cm dishes in DMEM (Life Technologies, Carlsbad, CA, Cat # 11965) medium supplemented with 10% fetal bovine serum (FBS). Sixteen hours before transfection, 5 × 10<sup>4</sup> cells were seeded into each well of the 24-well microplate in the medium without antibiotics. The cells were transfected with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, Cat # 11668027) with 0.6 µg of firefly luciferase reporter plasmids, and 50 ng of empty vector (pKW10) or expression plasmids encoding PAX6, Pax6<sup>(5a)</sup>, and their mutants. As an internal control, 20 ng of Renilla-TK luciferase plasmids was also included in the transfection mixtures. After 4 h, fresh medium was added, and the cells were incubated for another 26–30 h. The dual luciferase reporter assay system (Promega, Fitchburg, WI, Cat# E1960) was used to measure promoter activity. The experiments were performed in triplicate with two independent repeats. For statistical evaluation of the results, p values were calculated.
from paired Student t tests between reactions with cDNA vector and wild-type Pax6/Pax6(5a) cDNA for each motif. Paired Student t tests were also performed between wild-type Pax6/Pax6(5a) and each Pax6 mutant.

Homology model of human PAX6 bound to DNA: PAI and RED domains in complex with cognate DNA were obtained from the Protein Data Bank (PDB) coordinate file of 6PAX. Separately, a homology model of the human HD domain was generated using the Drosophila melanogaster HD domain template structure (PDB entry 3A01). A set of seven protein–nucleotide atom–atom distances were extracted from this complex. The combined complex structure containing PAI, RED, and HD domains with the cognate DNA was constructed using these structures, models, and distance restraints. The crystal structure of the human TBP domain in complex with DNA (PDB entry ICDW) and the structure of the mouse bZIP bound to DNA (PDB entry 4AUW) were also included in the Pax6 model. The homology model was produced using Modeller 9v2. The figure was prepared with Pymol 1.1r1 (PyMOL Molecular Graphics System; Schrödinger, LLC, Portland, OR).

RESULTS AND DISCUSSION

The PAX6 mutations studied here are shown in Figure 1A. In the first set of experiments, we used a panel of ten reporter genes driven by Pax6-binding sites of different structure. They represent a continuum of sequences that provide high and moderate levels of Pax6 activation as well as transcriptional repression [21]. The 6xP6CON reporter is derived from the Pax6 consensus binding site identified by Epstein et al. [26] (see Figure 1B,C). This reporter was activated by wild-type Pax6 approximately ten times in the P19 cells. Five Pax6 mutants, including G18W, R26G, N50K, G64V, and R242T, resulted in reduced activation. In contrast, the R128C mutation elicited nearly 2.5-fold activation of the P6CON. As expected, no activation of P6CON was found with Pax6(5a) as this splice variant does not bind P6CON [25,44]. These results are similar to our earlier studies of G18W, R26G, G64V, and R128C in P19 cells [43] and the N50K mutation in HEK293 cells [32]. The R242T mutation was evaluated for the first time and showed reduced activation of P6CON in P19 cells.

A group of three Pax6-binding site variants, motifs 1–1, 1–2, and 1–3, are related to the P6CON sequence (Figure 2A); however, motifs 1–1 and 1–3 activate the reporter gene (range of activation two- to four-fold) while motif 1–2 acts as a transcriptional repressor (Figure 2B-D). The Pax6 N50K showed the lowest activation on the motif 1–1 reporter, while the other mutants mostly showed no change (Figure 2B). Pax6 N50K and R128C showed no significant repression on the motif 1–2 reporter. Both these activities were similar to the wild type Pax6 (Figure 2C). In contrast, the R26G and G64V Pax6 proteins were more potent repressors compared to the wild-type Pax6 (Figure 2C). Motif 1–3 was not activated by Pax6 carrying mutations G18W, R26G, N50K, and G64V (Figure 2D). The R128C mutation apparently did not abrogate Pax6 function on motif 1–3 (Figure 2D). Although Pax6(5a) can bind motifs 1–1, 1–2, and 1–3 [21], none of the mutants studied here showed any significant difference compared to the wild-type Pax6(5a); Figure 2B-D).

A group of two Pax6-binding site variants (2–1 and 2–2) is characterized by a presumptive HD-binding site, 5’-ATTA-3’ (Figure 3A). This sequence is localized upstream from the target sequence recognized by the PAI, b-turn, and linker regions and is similar to P6CON (Figure 3A). The 2–1 and 2–2 motifs were activated approximately 15-fold by Pax6 (Figure 3B,C). For motif 2–1, the majority of missense mutants showed approximately 10% of the wild-type Pax6 activity (Figure 3B), and the overall pattern of their properties was similar to motif 1–3 (compare Figure 2D and Figure 3B). On motif 2–2, this pattern was “broken” by mutant N50K, which had about one third of the wild-type Pax6 activity (Figure 3C). Although motifs 2–1 and 2–2 differed only at the G residue in the linker region, motif 2–1 (Figure 3B), but not motif 2–2 (Figure 3C), was nearly three-fold activated by Pax6(5a). No change in reporter gene activity on motif 2–1 was found for Pax6(5a) carrying mutations G18W, R26G, N50K, and G64V (Figure 3B). In contrast, Pax6(5a) R128C (R242T) showed moderate loss (gain) of activity, respectively (Figure 3B). Interestingly, no change in reporter gene activity was found on motif 2–2 (Figure 3C).

The third group of Pax6-binding sites, including motifs 3–1, 3–2 and 3–3, were characterized by nucleotides recognized by the linker region and RED subdomain (Figure 4A). Sites 3–1 and 3–2 preferred Pax6(5a) over Pax6, while site 3–3 was bound and activated only by Pax6 [21]. On motif 3–1, five Pax6 mutants, including G18W, R26G, G64V, R128C, and R242T, showed at least 50% loss of their activation potential (Figure 4B). In contrast, Pax6 N50K retained more than 80% of the wild-type Pax6 activity (Figure 4B). On motif 3–2, wild-type Pax6 acted as a weak activator, and the G18W mutation produced a moderate approximate 1.6-fold activation (Figure 4C). Three mutants, including G18W, R26G, and G64V, evaluated with motif 3–3 lost their activation potential (Figure 4D). The replacement of the T to A nucleotide in the PAI-RED linker binding region (ATT to AAT) had only a marginal effect in Pax6 R128C (Figure 4D). The most notable finding in the Pax6(5a) series (Figure...
Figure 1. Transcriptional regulation by PAX6 and PAX6(5a) on P6CON luciferase reporters. A: A schematic diagram of PAX6 and PAX6(5a) proteins including six missense mutations G18W, R26G, N50K, G64V, R128C, and R242T. Paired domain, PD; homeodomain, HD; proline/serine/threonine-rich domain, PST; β-turn, β; linker between the PAI and RED subdomains, L. B: Distribution of nucleotides in the P6CON Pax6-binding site. The DNA sequence for gene synthesis is aligned with the P6CON motif. The binding sites and flanking sequences are in upper and lower case, respectively. C: A schematic representation of the 6x Pax6 DNA-binding motif/E4TATA-luciferase reporter. D: Evaluation of PAX6 (black bars) and PAX6(5a) (gray bars) series in cotransfected P19 cells. The data are expressed as relative fold changes elicited in the presence of PAX6 or PAX6(5a) compared with the changes found with the empty vector, pKW10. The sample size n=6 from two independent triplicates. The error bars represent the standard deviation. The significant fold-changes are indicated by the asterisks. The range of the p values are: *** <0.001, ** 0.001~0.01, * 0.01~0.05. The p values between empty vector and wild-type Pax6/Pax6(5a) are calculated with paired Student t tests. The p value for each mutant is calculated by comparing it with the wild-type.
4B–D) was strongly reduced activation by PAX6(5a) N50K using the motif 3–1 reporters (Figure 4B).

Motif 4–1 was comprised from a presumptive HD-binding sequence 5′-TAAT-3′ followed by nucleotides preferentially recognized by PAX6(5a) (Figure 5A) though PAX6 activation was higher compared to PAX6(5a) (Figure 5B). All PAX6 mutants evaluated lost their activation potential with the highest effect found for PAX6 N50K (Figure 5B). In the PAX6(5a) series, the highest decrease in promoter activity was found for R128C. In contrast, the activity of mutant PAX6(5a) R242T nearly doubled compared to wild-type PAX6(5a). To visualize similar and distinct properties of individual PAX6 mutations examined here, Figure 6 provides a color-coded summary of data from Figure 1–Figure 5.

The Trpm3/miR-204 model is a novel Pax6-regulated system [15]. We first tested a 1.5 kb genomic fragment from intron 6 of the mouse Trmp3 locus. We found that this fragment was activated by PAX6 by a factor of 1.8 in cotransfected P19 cells (Figure 7A,B). This result was nearly identical to the activity of the zebrafish homologous genomic fragment tested in HeLa cells [15]. We next generated a reporter plasmid driven by six copies of the Pax6-binding site (Figure 7C) identified by a combination of electrophoretic mobility shift assays (EMSAs) and chromatin immunoprecipitation (ChIP) assays [15]. With PAX6, this reporter yielded approximately six-fold activation that was reduced in the presence of all PAX6 mutants (Figure 7D). PAX6(5a) did not activate this promoter although the R242T mutant showed almost two-fold activation (Figure 7D).
Previous systematic studies of PAX6 missense mutants primarily focused on the correlation between their transactivation and DNA-binding in in vitro assays [43,45,46]. For mutations G18W, R26G, G64V, and R128C, we did not find any correlation between their DNA-binding and reporter gene activity [43]. Similarly, no correlation between in vitro DNA binding and transactivation/repression was found using the current panel of reporters used here [21]. Although no data are available to explain this apparent discrepancy, it is obvious that a typical missense mutation does not work as a DNA-binding but rather as a functional loss-of-function mutant in the context of the execution of the transcription process. More recently, extensive analyses of 50 PAX6 missense mutations predicted that the majority (77%) of these mutations influence intrinsic protein stability while the minority (30%) are thought to impair DNA binding [45]. The HD mutation R242T gave higher levels of activation using the P3 HD-dimeric reporter in HeLa cells [41]. Reduced sensitivity to trypsin digestion was found for the mutated protein concomitant with increased amounts of the nuclear proteins raising the possibility that the increased activation of the reporter gene is a protein dosage effect. In the current studies, we found that PAX6 R242T mutants had either decreased or unchanged activation (Figure 1, Figure 2, Figure 3, Figure 4, Figure 5, Figure 7). Only PAX6(5a) R242T produced more activity using motifs 3–1 and 4–1 and Trpm3.4 (Figure 4, Figure 5, Figure 7). Thus, multiple mechanisms can be used to explain the pleiotropic effects of this HD mutation.

To put in a structural context of the studied mutations, a PAX6 protein–DNA model was built (Figure 8). In this model, we used a DNA-binding site recognized by all five DNA-binding subdomains (β-turn, PAI, linker, RED and HD; e.g., motifs 2–1 and 2–2) with the HD-binding region upstream of the paired domain. The PAX6 PD–DNA interaction is based on a crystallographic model determined with...
P6CON [47]. The PAX6 HD interaction with DNA was based on similar data [48]. The structure of the approximate 80 amino acid region that connects the PD and HD is not known, and, thus, was not included in Figure 8. The present data show that the N50K and R128C mutations differ from each other (see Figure 6) and that they represent “outliers” from the panel of mutations investigated here and those tested in our earlier studies, including A33P, S43P, I87R, and V126D [43]. Both mutations are at the interface of protein–DNA interaction. The N50 residue is located in the beginning of the third α-helix comprising the PAI subdomain [47]. A substitution of polar uncharged asparagine for the charged lysine residue established a new type of interaction with the DNA, though not experimentally examined by Alibes et al. [45]. The outlier property of this substitution is documented on motifs 2–2 and 3–3 [31,41] activated by PAX6 [PAX6(5a)], respectively. The R128 residue is located in the central part of the third α-helix comprising the RED subdomain [47] (Figure 8). In addition to the change from a positively charged residue to a neutral one, the nonsynonymous arginine to cysteine substitution is known to disrupt helical conformation in many proteins [49]. The odd property of this substitution is documented on motifs P6CON (hyperactivation) and a larger group, including 1–1, 1–3, 2–1, 2–2, 3–2, and 3–3 (no change), by PAX6. It was argued earlier that the R128C mutation does not cause any significant conformational change in the mutated PAX6.
Figure 5. Transcriptional regulation by PAX6 and PAX6(5a) on motif 4–1 luciferase reporters. 

A: Distribution of the nucleotides in Pax6-binding motif 4–1. The DNA sequences for gene synthesis are aligned with the corresponding motifs. The binding sites and flanking sequences are in upper and lower case, respectively. 

B: Evaluation of PAX6 (black bars) and PAX6(5a; gray bars) series with 4–1 motif luciferase reporters in cotransfected P19 cells. The sample size n=6 from two independent triplicates. The error bars represent the standard deviation. The significant fold-changes are indicated by the asterisks. The range of the p values are: *** <0.001, ** 0.001~0.01, * 0.01~0.05.
proteins bound to the Pax6-binding site evaluated with FoldX [45]. However, the most likely explanation is that the major impact is on PAX6(5a) type-binding sites (i.e., motifs 3–1 and 4–1), recognized by PAX6 and PAX6(5a) proteins, an idea that remains to be experimentally tested with FoldX and potentially via direct crystallographic studies taking advantage of choosing from the DNA-binding motifs evaluated here. We conclude that the N50K and R128C PAX6 missense mutations represent useful systems for further dissecting molecular mechanisms of Pax6 in gene activation and repression.

Indeed, recent functional studies of the Leca4 (N50K) and Leca2 (R128C) Pax6 alleles revealed similarities and differences in cellular responses controlled by Pax6 [32]. Pax6 target genes, disrupted by the N50K mutation, include genes that control forebrain patterning, neurogenesis, and decreasing cell proliferation. In contrast, Pax6-dependent genes, affected by the R128C mutation, include genes that
increase cell proliferation, without any apparent impact on neurogenesis. An attractive hypothesis is that these mutations impair interactions with chromatin remodeling enzymes, including Brg1 and Brm [33-35]. The availability of specific antibodies for ChIP-seq studies in conjunction with mouse genetic studies of Brg1, Brm, BAF155, and BAF170 alleles crossed with the Pax6Leca2/Leca4 alleles allows direct testing of this model.

To further illustrate how PAX6 mutations can disrupt physical interactions with other proteins bound, we included a model of bZIP factor-bound to DNA (Figure 8, left) and TATA-box binding protein (TBP, Figure 8, right). It has been shown earlier that c-Maf, a bZIP transcription factor, binds to Pax6 [50], and local configurations of Pax6- and Maf-binding sites support different levels of transcriptional synergism [42]. Pax6 and c-Maf are known to bind p300 histone acetyltransferase [51]. Pax6 and CBP/p300 regulate multiple common genes during the lens placode formation [52] and crystallins in differentiating lens fibers [53]. Binding of TBP to the TATA box induces large DNA bending (Figure 8). The Pax6/TBP interaction has been established elsewhere [39]. Taken together, the present models show that major and minor conformational changes in mutated PAX6 proteins can have large impacts on the protein–protein interactions with other regulatory proteins, including c-Maf, TBP, and their associated chromatin remodeling complexes.

Some PAX6 missense mutations display pleiotropic activity in different cell types examined, such P19, 293T, CHO-K1, and N/N1003A [43]. The P19 cells were used here as the panel of ten luciferase reporters displayed a wide range of transcriptional activation and repression [21] and mutations N50K and R128C gave similar results in P19 and HEK293 cells [32]. In conclusion, the present studies emphasize the complexity of Pax6-dependent transcriptional activation and repression mechanisms, and identify the N50K and R128C substitutions as valuable tools for testing interactions between Pax6, Pax6N50K, and Pax6R128C with other regulatory proteins, including chromatin remodelers and their impact on eye and brain development.

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