Retinal Patterning by Pax6-Dependent Cell Adhesion Molecules

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ABSTRACT: Long-standing evidence gained from *Pax6* mutant embryos pointed to an involvement of Pax6-dependent cell adhesion molecules in patterning the central nervous system and, in particular, the retina. However, direct evidence for such pathways remained elusive. We here present direct evidence that knockdown of Pax6 expression by morpholino antisense molecules in *Xenopus* embryos and knockdown of maternal N-cadherin (mNcad), N-cadherin (Ncad) and neural cell adhesion molecule (NCAM) produce similar phenotypes. Eye formation is reduced and retinal lamination is heavily disorganized. In Pax6 knockdown embryos, the levels of mRNAs coding for these cell adhesion molecules are markedly reduced. Overexpression of Pax6 efficiently

rescues the phenotype of Pax6 knockdown embryos and restores expression of these putative target genes. Rescue of Pax6-deficiency by the putative target gene mNcad moderately rescues eye formation. The promoters of the genes coding for cell adhesion molecules contain several putative Pax6 binding sites, as determined by computer analysis. Chromatin immunoprecipitation shows that, in embryonic heads, Pax6 binds to promoter regions containing such predicted binding sites. Thus, several cell adhesion molecules are direct target genes of Pax6 and cooperate in retinal patterning.

Keywords: cadherins; NCAM; duplication; rosette; RPE

INTRODUCTION

The pivotal role of the paired box and homeobox-containing transcription factor Pax6 in eye formation was discovered by analyzing the spontaneous “small eye” mutations in vertebrates, namely murine *Sey* (Hill et al., 1991; Walther and Gruss, 1991) and human *Aniridia* (Ton et al., 1992). Eye development starts with the induction of the eye field in the anterior region of the neural plate by Rx1a controlling Pax6 (Mathers et al., 1997) and several other interact-

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ing factors. However, Pax6 is also expressed at later stages of organogenesis in different compartments of the developing eye, brain, spinal chord, and many other organs (reviewed by Jean et al., 1998; Ashery-Padan and Gruss, 2001; Simpson and Price, 2002; Job and Tan, 2003; Kozmik, 2005; Nakamura and Watanabe, 2005).

In the context of this study, it is important to note that numerous defects linked to Pax6 deficiency point to a disturbed cell adhesion. In the absence of functional Pax6 protein, cytoarchitectonic alterations, failure of cell migration and altered cell adhesion, occur in the developing cortex and ganglia (Stoykova et al., 1997; Chapouton et al., 1999; Kawano et al., 1999; Jimenez et al., 2002; Tyas et al., 2003). Pax6 deficiency may be accompanied by disturbed expression of extracellular matrix components such as reelin (Stoykova et al., 2003), axonal guidance molecules such as semaphorins and netrin (Jones et al., 2002) and cell adhesion molecules (CAM) such as L1-CAM (Kawano et al., 1999) and R-cadherin (Stoykova et al., 1997). Electroporation of R-cadherin into Pax6-deficient embryos rescued correct outgrowth of pioneer axons during early brain patterning in the mouse (Andrews and Mastick, 2003). Overexpression of Pax6 in transfected HeLa cells induces changes in cellular shape and migration, prompts massive expression of neuronal α tubulin, and establishes a postmitotic phenotype (Cartier et al., 2006). Moreover, it was shown that Pax6 regulates the transcription of cadherin 4 (Liu et al., 2001), L1-CAM (Chalepakakis et al., 1994; Meech et al., 1999), NCAM (Holst et al., 1997) and α 4integrin (Zaniolo et al., 2004). Pax6 target genes in mouse lens have been identified by microarray screening and include JAM-1, L1-CAM, NCAM-140, and neogenin (Chauhan et al., 2002). Pax6 may indirectly control N-cadherin through optomedin (Grinchuk et al., 2005; Lee and Tomarev, 2007). In spite of many observations regarding Pax6 binding to promoters of putative target genes, or disturbed expression of cell adhesion molecules in Pax6-deficient mutants (reviewed by Simpson and Price, 2002), direct evidence for Pax6-dependent disturbances linked to abnormal cell adhesion has not been provided.

Several Pax6 cDNAs have been cloned for *Xenopus laevis* (Hirsch and Harris, 1997; Li et al., 1997; Jaworski et al., 1997; Hollemann et al., 1998). The expression pattern of Pax6 mRNA, mapped by *in situ* hybridization (Hirsch and Harris, 1997; Li et al., 1997; Hollemann et al., 1998; Zuber et al., 2003), is similar to the pattern in the mouse embryo. Ectopic Pax6 expression induced heterotopic eyes containing all major cell types in a rather correct spatial arrangement whereas

coexpression of a dominant negative form of Pax6 suppressed ectopic as well as endogenous eye formation (Chow et al., 1999). Similarly, the inhibition of Pax6 function by overexpressing a dominant negative transcription factor, Xtll (*Xenopus* homologue of *tailless*) resulted in down-regulation of Pax6 and blocked the evagination of the eye vesicle (Hollemann et al., 1998). However, little is known regarding specific Pax6 functions in this genus during retinal development.

Using morpholino antisense molecules (Summerton and Weller, 1997) against Pax6, we were able to inhibit Pax6 expression until advanced stages of *Xenopus* development. Some of the effects on eye development, namely reduced eye size and folding of retinal layers, are closely similar to the defects observed in the *Sey*-mouse and in chimeric (Quinn et al., 1996; Collinson et al., 2000) or Cre/loxP mice (Ashery-Padan et al., 2000; Marquardt et al., 2001). However, duplications of inner retinal layers, defects in RPE formation, and rosette formation by photoreceptors observed in this study, were not described so far in conjunction with Pax6 deficiency. Duplications and rosette formation have been observed in the zebrafish mutants *parachute* (Erdmann et al., 2003; Masai et al., 2003) and *glass onion* (Malicki et al., 2003) coding for defective N-cadherin and in R-cadherin mutants (Babb et al., 2005). A potential impact of Pax6 on the transcriptional regulation of these genes by Pax6 was not yet investigated.

We show here that, direct antisense inhibition of several cell adhesion molecules, namely XmN-cadherin (mNcad), a maternally expressed *X. laevis* homolog of R-cadherin or cdh4 (Tashiro et al., 1996), N-cadherin or cdh2 (Ncad; Ginsberg et al., 1991), and the neural cell adhesion molecule (NCAM; Kudo et al., 1998) produces strikingly similar disorders in ocular development and retinal lamination, as does Pax6 inhibition. The mRNA levels of these genes are markedly reduced in Pax6 knockdown embryos. As revealed by chromatin immunoprecipitation (ChIP), Pax6 binds to promoter regions of these genes containing several Pax6-binding motifs. Thus, several Pax6-dependent genes coding for cell adhesion molecules cooperate in establishing retinal patterning.

MATERIALS AND METHODS

Knockdown

Morpholino oligomers were synthesized by Gene Tools, Corvallis, Oregon. Sequences were chosen following the recommendations by the manufacturer: www.gene-tools.com. The antisense morpholino molecules are 25 bases long and span the AUG initiation region (CAT):

Pax6 (U7753226; Li et al., 1997): 5' GCTGTGACTGT TCTGCATGTGCGAG 3'; mNcad (S82457; Tashiro et al., 1996): 5' CAGTCATACTGCTCCCGGTCTCGGT 3'; Ncad (X57675; Ginsberg et al., 1991): 5' GAAGGGCTCTTTCCG GCACATGGTG 3'; NCAM (AB008162; Kudo et al., 1998): 5' AGATGAGATCCTTAATGTGCAGCAT 3'.

As a control, we used the unspecific standard control oligo, recommended by the manufacturer: 5' CCTCTTACC TCAGTTACAATTTATA 3'. These molecules were fluorescein-labeled, allowing to follow their distribution and persistence in *Xenopus* embryos. Moreover, we used a "5-base mismatch" of the anti-mNcad morpholino: 5' CACT CATGGTGCTCGCGGTCTCCGT 3' as a specific control.

In vitro fertilization, physiological solutions, calibration of needles and the microinjection procedure are described elsewhere (Giovannini and Rungger, 2002). Animal care and experimental conditions were in agreement with Swiss laws on animal protection and were authorized by the Veterinary office of the State of Geneva.

The morpholino molecules were dissolved in 0.5x injection buffer (1xIB; 88 mM NaCl, 1 mM KCl, 15 mM Hepes, pH 7) to a concentration of 8.5 mg/mL (1 mM), and aliquots frozen at -80°C . Immediately before use, they were further diluted to 1, 3, or 5 mg/mL, respectively. After preliminary verification, we used 30 ng of morpholino per fertilized egg except for anti-mNcad. Because of high lethality, its dose was reduced to 15 ng/embryo.

Rescue

Pax6 expression vectors were produced by inserting the SacI-ScaI fragment of *Xenopus laevis* Pax6 cDNA (U77532, Li et al., 1997) into pDS Red1-N1 (Clontech, BD Biosciences, Allschwil, Switzerland). The resulting pCMV-Pax6-Red was used to visualize ectopic expression by monitoring red fluorescence. For functional rescue, pCMV-Pax6 without the Red-coding segment was produced by cutting pCMV-Pax6-Red with BamHI and NotI, blunting, and religating the large fragment.

CMV-mNcad was produced by amplifying a 2936 bp segment comprising the entire coding sequence (99 bp before ATG to position 2979) of XmNcad (Tashiro et al. 1996, kind gift of Dr. K. Tashiro, Tokyo). The primers were FP: CACTCGAGCGGGACACTAAGGGAAGCTA with an extension carrying a XhoI recognition sequence (italics) and RP: CTGCGGCCGCAAAGAATGCAGC CAGGAAGA with a NotI extension. The amplicon was cut with XhoI and NotI and inserted into the corresponding sites of pDSRed1-N1 (Clontech). All constructs were verified by sequencing.

Real Time PCR

RNA from pools of five embryos, or from dissected head regions and remaining body, was isolated on RNeasy columns (Qiagen, Basel, Switzerland) and reverse transcribed with Superscript II polymerase (Invitrogen, GIBCO, Basel, Switzerland) in the presence of random hexamer primers (Roche, Dübendorf, Switzerland).

Amplification was done by the Taqman assays on an Applisystem 770 or 790 apparatus (Applied Biosystems, Rotkreuz, Switzerland) at a reassociation temperature of 60°C . 18S rRNA, served as an internal standard (18S rRNA TaqMan[®] Ribosomal RNA control, VIC). Relative mRNA levels were calculated: $2^{-\text{CTmRNA}/2^{-\text{CTrRNA}}}$. For rRNA measurements, each sample was diluted 1000 $\times$. The relative amounts of mRNA indicated in the figures thus roughly correspond to % of rRNA. Statistical evaluation was done by Student's *t*-tests.

The forward primers (FP) and reverse primers (RP) and internal probes (IP; FAM) for the genes of interest were determined by the Primer3 program (www.genome.wi.mit.edu/cgi-bin/primer/):

Pax6: FP 79 AGGAAGGAGCAAGGAGGAAG; RP 299 TCCTGGGAAGGAGACAGAGA; IP 108 ATTGCTG ACAGGGGTCAGTC. mNcad: FP: 534 CCAGAGCCAGG AGTCAGAAC; RP: 705 GTCAGAGCGAATCAGCAC AA; IP 632 TAATACCGCCCGTTAACGTC. Ncad: FP: 248 AAGGGCAACCGCTTCTAAAT; RP 403 AAAATTC TGCCTGCTCTGGA; IP 279 TGA CTGCGGTGCTGATA GAC. NCAM: FP 52 GTGGCTTTGGAAGTGACCAT; RP 214 CGTTTTTCACCACGGAGATT; IP 124 CAAGTAA GCGGAGAAGCCAC.

Western Blotting

Antero-dorsal portions were cut from control or morpholino-treated embryos, lysed in 10 mM Hepes pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, and 5 mM NaF, supplemented with Complete Mini protease inhibitor cocktail (Roche Diagnostics, Rotkreuz, Switzerland). The lysate was centrifuged at 10,000g for 15 min (0°C). The supernatant was precipitated with ice-cold trichloro acetic acid at a final concentration of 10%. The precipitates were washed three times in ice-cold acetone, dried, and solubilized in SDS-sample buffer.

Proteins (equivalent to one embryo per slot) were run on a 5 to 15% SDS-polyacrylamide gradient gel and blotted onto nitrocellulose. Blocking was done by TBS (10 mM Tris-HCl, pH 8.0, 150 mM NaCl) containing 0.05% Tween 20 and 5% fat-free milk powder. Blots were incubated with rabbit anti-Pax6 antibody, directed against the C-terminal peptide of murine Pax6 (Covance, Eurogentec, Herstal, Belgium), diluted 1:300, or with murine monoclonal anti-actin C4 antibody, diluted 1:10,000 (Chemicon, VWR International AG—Life Sciences, Dietikon, Switzerland) overnight at 4°C . Second antibodies were donkey anti-rabbit F(ab)₂ fragments coupled to peroxidase (1:3000, Jackson Immunoresearch, Milan Analytica, La Roche, Switzerland). Reaction was revealed by an ECLTM chemiluminescence kit (Amersham Pharmacia Biotech, Dübendorf, Switzerland).

ChIP Experiments

Sequence information to design ChIP probes were available only for *Xenopus tropicalis*. Accession numbers for the

Table 1 Gene Names, Accession Numbers, and Scaffolds

	<i>X. laevis</i>	<i>X. tropicalis</i>
mNcad	XmNcad	Rcad, retinal, cdh4
cDNA	S82457	NM_001123479.1
Gene ID		100144729
5' & exons 1–2		Scaffold 1028 (–) +1: 200,099
Exons 3–12 (13 missing)		Scaffold 531 (+) +303: 254,910
Ncad	EPcad, Ncad	N2cad, cdh2
cDNA	X57675	NM_001127418.1
Gene ID		10015172
5' & coding		Scaffold 84 (+) +1: 15,522,001
NCAM	NCAM	NCAM
cDNA	AB008162	NM_001097400.1
Gene ID		100038291
5' & start		Scaffold 436 (+) +1: 950,595
Exon 1		Scaffold 318 (–) 1,278,474–1,333,359
Exons 2–13		

(–) = minus strand; (+) = plus strand.

X. laevis and corresponding *X. tropicalis* genes are given in Table 1. Target Explorer (Sosinsky et al., 2003) was used to search for putative Pax6-binding sites.

For ChIP analysis, the heads and tails of 300 *X. tropicalis* tadpoles (Stage 42) were cut and processed in groups of 50 fragments. Each batch was fixed in 1 mL of PBS with 1% formaldehyde for 10 min, quenched by the addition of 1 mL of 250 mM glycine, and washed once with 0.5 mL of 250 mM glycine. At this point, the corresponding batches were pooled and resuspended in 0.5 mL of 165 mM NaCl, 22 mM Tris pH 7.5, 2.2 mM EDTA and homogenized by 50 strokes with a loose pestle, followed by 50 strokes with a tight pestle in a Dounce glass homogenizer. After centrifugation at 500 rpm for 5 min, the supernatant was recovered. The presence of intact nuclei was verified by phase contrast microscopy. The prepared chromatin was adjusted to 1% SDS, 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 2 mM EDTA, and sonicated 10 times 15 sec using a microtip for a VT300 sonicator (BioLogics, Manassas, VA). The chromatin was then adjusted to 0.1% SDS, 1% Triton X-100, 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 2 mM EDTA. From here on the procedure followed the described protocol (Ripperger and Schibler, 2006). Five microliters of each sample were directly used in TaqMan real-time PCR reactions of 15 μ l final volumes on a RotorGene 6200 machine (Corbett Life Science, Sydney, Australia) with 1 $\times$ Sybr-Ex TaqTM PCR master mix (TaKaRa Bio Inc., Shiga, Japan) and the specific PCR probes. Antibodies used in these experiments have been described (Ripperger and Schibler, 2006). Anti-

Histone H3 and anti-Pax6 antibody were from Abcam (Cambridge, UK).

The primers used were: mNcad-PRO FP: 5'-AGCCCAT AAGACCCCTTGGCAGGA-3' and mNcad-PRO RP: 5'-AG CTCTCCCAGGCAGCAGT-3'; mNcad-CO FP: 5'-ACTG GGCTGTATTGGGAGTG-3' and mNcad-CO RP: 5'-GCC CAATGGGAGATACTCAG-3'; Ncad-PRO FP: 5'-GGGA GATGGCCTTATCGTGAGC-3' and Ncad-PRO RP: 5'-AGTGCAGCGACATGCAACCA-3'; NCAM-PRO FP: 5'-CACCAGCATTTTCAGGCTGTGAGT-3' and NCAM-PRO RP: 5'-TGCAGTAAGTCACAAGTGAAGACGA-3'.

Morphology

The external phenotypes were documented on living *Xenopus* embryos and tadpoles using a Leica MZ FLIII stereomicroscope equipped with a Nikon Coolpix 990 digital camera. For histological analysis, embryos were fixed in Smith's fixative (Peng, 1991), abundantly rinsed in tap water, transferred into 0.1 M cacodylate buffer (pH 7.4), dehydrated through ethanol, and embedded in Epon. Semithin transversal sections were stained with methylene blue and viewed by Nomarski optics on a Zeiss Axiophot. Pictures were captured with a ProgRes 3008 digital camera and introduced into Adobe Photoshop.

Immunofluorescence Microscopy

Embryos (Stage 33–48) were fixed in either ice-cold methanol/dimethyl sulfoxide (4:1) or MEMFA (Peng, 1991) and stored in methanol (–20°C) or cryoprotected in 30% sucrose. Cryostat sections (7 μ m) were stained with polyclonal rabbit anti-Pax6 antibodies (dilution 1:300, Covance), murine monoclonal anti-syntaxin antibody (dilution 1:200, Sigma, Buchs, Switzerland), murine monoclonal anti-*Xenopus* vimentin antibody (clone XI-VIM-14-13, dilution 1:8; antibodies-online GmbH, Aachen, Germany), murine anti-human CD57/HNK (dilution 1:500, Pharmingen, Basel, Switzerland) or rabbit serum against *Xenopus* rod outer and inner segments (Rungger-Brändle et al., 1987), diluted 1:75. Antibody dilutions and washing steps were done in TBS containing 0.05% Triton X-100. Incubations were performed overnight (4°C). Secondary antibodies were coupled to FITC or Texas Red (1:150, Jackson ImmunoResearch). Sections were viewed with a 20 $\times$, 40 $\times$, or 63 $\times$ objective of a Zeiss LSM 410 confocal microscope and optical sections taken at intervals of 0.5 or 1 μ m, respectively. Stacks of 10–12 images were processed using Imaris software (Bitplane, Zürich, Switzerland).

RESULTS

Antisense Inhibition of Pax6

To document antisense inhibition which acts on translation, Pax6 protein levels were measured by Western blotting on lysates from total, or isolated heads of

control and Pax6-inhibited *Xenopus* embryos. The polyclonal antibody against murine Pax6 reacts with a cluster of bands between M_r 48–54 kDa. At all developmental stages tested (20, 32, 38) the smallest and largest bands were strongly reduced by antisense treatment, whereas an intermediate band was hardly affected [Fig. 1(A)]. Immunocytochemistry reveals a more or less drastically decreased number of nuclei that stained for Pax6 in the two inner retinal layers formed by ganglion and amacrine cells (Hirsch and Harris, 1997) and a corresponding deficiency in the size and differentiation of the lens. The stringency of Pax6 knockdown is variable and the resulting disturbances in the retina range from near normal size with prominent folding and duplications to rudimentary structures comprising a few clusters of cells exhibiting normal staining intensity for Pax6.

Similar Phenotypes of Knockdown Embryos Deficient in Pax6 or in Cell Adhesion Molecules

As compared to uninjected siblings, embryos carrying nonspecific standard control morpholino developed somewhat more slowly but, in their large majority, exhibited entirely normal phenotypes [Fig. 2(A)]. Antisense treatment against Pax6 (at the optimal dose of 30 ng per egg) induced specific abnormalities in the external phenotype. Micro- and acephalic, or else deformed embryos occurred with a similar frequency [Fig. 2(C)] as in embryos carrying the control morpholino and thus represent nonspecific defects due to experimental manipulation.

The most characteristic features of Pax6 deficiency were reduced eye size and disturbances in the intestinal differentiation. The stringency of antisense inhibition was variable and the severity of the external phenotype more or less pronounced. The primary, specific defect, observed in over 80% of the embryos [Fig. 2(C)], resided in reduced eye size resulting, in severe cases, in the complete absence of externally visible eye structures [Fig. 2(A)]. Moreover, the nasal placode was defective or missing and, the differentiation of the digestive tract was markedly delayed and abnormal, eventually causing the intestine to prolapse [Fig. 2(A), arrow]. A number of the treated tadpoles were immobile, seemingly caused by abortive outgrowth of motoneurons. Indeed, staining for acetylated tubulin to label outgrowing neurons revealed heavy deficiencies in ventral somitic innervation (Supporting Information Fig. S1). Over 90% of the Pax6 antisense-treated tadpoles, including individuals presenting a normal external phenotype, died between Stage 46 and 49.

Figure 1 Reduction of Pax6 protein by morpholino treatment. A: Western blot using polyclonal Pax6 antibodies on proteins from whole embryos carrying control morpholino (MO CO) or Pax6 antisense-morpholino (MO Pax6), or from isolated heads at developmental Stages 20, 32, and 38. Immunoreactive bands migrate between M_r 48 and 54 kDa. Aliquots of the same extracts were analyzed for β actin (lower panel). B: Immunofluorescent staining for Pax6 on eyes from control (CO MO) and Pax6-inhibited (Pax6 MO) Stage 42 tadpoles. Small-eyed phenotypes show retinal folding (top right, bottom left) and duplications (bottom middle). Rudimentary eye anlagen contain a reduced number of Pax6 expressing cells (bottom right). Bar, 50 μ m. [Color figure can be viewed in the online issue, which is available at [wileyonlinelibrary.com](http://www.interscience.wiley.com).]

To assess the contribution of putative Pax6 target genes coding for cell adhesion molecules to phenotypic traits of Pax6 deficiency, the expression of mNcad, Ncad, and NCAM was directly inhibited by selective antisense morpholino. Injection of 30 ng of morpholino against mNcad caused arrest of development during or after gastrulation in 86% of the treated embryos that exhibited weak cell adhesion and eventually dissociated, corroborating earlier findings (Hojyo et al., 1998). Similarly, injecting 15 ng of morpholino into one cell of the two-cell stage resulted in embryos with one body half composed of loosely adherent cells [Fig. 2(B)]. To avoid such heavy loss, we reduced in the following experiments the dose of anti-mNcad morpholino to 15 ng per egg.

Under this condition, about 25% of the embryos dissociated. Among the survivors, 67% were small-eyed, corresponding to 50% of the total [Fig. 2(C)]. Reduction in eye size and abnormalities in the digestive tract were less severe than in Pax6-deficient embryos [Fig. 2(A)]. The control morpholino and an antisense mNcad morpholino carrying five mismatches, produced no specific phenotype (data not shown).

In contrast to mNcad, Ncad is not maternally expressed (Ginsberg et al., 1991). Accordingly, its direct antisense inhibition did not affect gastrulation. At later stages, eye size was as frequently, yet less severely reduced than by Pax6 inhibition [Fig. 2(A,B)]. On the other hand, defects in the digestive tract and sometimes of the entire body were more severe than in the mNcad or Pax6 knockdown embryos. About 20% of the Ncad-deficient embryos

not only had small eyes but remained tiny, differentiated poorly, and eventually disintegrated.

Inhibition of NCAM had little effect on the external phenotype. Only 23% of the embryos were moderately small-eyed and, rarely, the development of the digestive tract was slightly delayed [Fig. 2(A,C)]. NCAM seems to be only marginally involved in determining eye size. However, as shown below, patterning of the retina is similarly affected as in mNcad and Ncad-deficient embryos.

Ocular Disorders

The overall ocular defects induced by inhibition of either Pax6 or genes coding for cell adhesion molecules exhibit striking similarities also on the histological level. As compared with the normal eye [Fig. 3(A)], the “small-eyed” phenotype caused by knocking down Pax6 [Fig. 3(B,C)], mNcad [Fig. 3(I)], or Ncad [Fig. 3(H)] often displayed a distortion of the ocular axis and a reduced and poorly differentiated lens [Fig. 3(C)]. Even in the “eyeless” phenotype [cf. Fig. 2(A)], in which the position of the eye anlagen was externally marked by an epidermal fold, a differentiating lens was present but reduced to a small cell cluster [cf. Fig. 1(B)]. Knockdown of NCAM had lit-

Figure 2 External phenotypes of Pax6, mNcad, Ncad, and NCAM knockdown embryos. A: Representative tadpoles at Stage 45 carrying control morpholino (CO MO) or antisense morpholino against Pax6 (Pax6 MO) or individual Pax6-dependent genes coding for the cell adhesion molecules (mNcad MO, Ncad MO, or NCAM MO). Pax6 MO, two small-eyed Pax6-deficient tadpoles and one “eyeless” individual. Note the delay in differentiation of the intestinal tract, the most severe case presenting prolapse (arrow). mNcad MO, two small-eyed individuals with delayed differentiation of the intestinal tract. Ncad MO, two small-eyed individuals with delayed differentiation including the intestinal tract. NCAM MO, tadpole with slightly decreased eye size and normal differentiation of the body. B: Normal neurula (CO MO) and two arrested gastrulae (mNcad MO) with dissociating cells in one body half, resulting from injection of 15 ng of morpholino directed against mNcad into one cell of the two-cell stage. C: Frequency of external phenotypes, scored at Stage 42. Averages of 3–6 experimental series with >100 injected embryos. Embryos were injected with no (none), control (CO) or with antisense morpholino (MO) against Pax6, mNcad, Ncad, or NCAM. OK, normal embryos; eye, reduced or rudimentary eyes; def, various deformities (microcephaly, axial disturbance, bent tail) considered to be non-specific; diss, entirely dissociated embryos, most often, accompanied by disturbances in the digestive tract; dead, lethal before Stage 42. [Color figure can be viewed in the online issue, which is available at [wileyonlinelibrary.com](http://www.interscience.wiley.com).]

Figure 3 Retinal disturbances in tadpoles deficient for Pax6 (B,C,E), mNcad (F,G,I) or Ncad (H). Representative semithin sections through eyes between Stages 38–48. A: Control Stage 42 with correct retinal lamination. ON, optic nerve. B: Eye of Pax6-deficient Stage 48 tadpole with small photoreceptor rosettes (arrowheads) and reduced number of ganglion cells (arrows). C: Eye of a Pax6-deficient, albino tadpole (Stage 48) displaying distorted eye axis, a poorly differentiated lens, and severely disturbed lamination with rosette formation and a displaced cluster of RPE cells (arrowhead). Note retarded neuronal differentiation. D: RPE-neural retinal interface of a control retina (Stage 40) with correct apposition of photoreceptors onto RPE. E: Age-matched Pax6-deficient retina with a poorly defined interface between RPE and neural retina (arrows). F,G: Disturbed interface (arrows) with rosettes and displaced RPE cells (arrowheads) in an mNcad-deficient retina (Stage 38). H: Small-eyed phenotype caused by Ncad deficiency (Stage 42). I: Multiple rosettes (arrowheads) and a duplication of inner retina (arrow) in an mNcad-deficient Stage 46 tadpole. Bars, 50 μ m (A), (D–G), (B,C,H,I).

the impact on eye size and did not affect the ocular axis (not shown).

Disturbed laminar organization of the neural retina was observed in Pax6 as well as mNcad, Ncad, and NCAM knockdown embryos. Photoreceptor cells formed rosettes around clusters of RPE cells that were displaced into the inner retina [Figs. 3(B,C,E–G,I) and Fig. 4]. Even eyes with an else apparently correct laminar organization could contain minute rosettes consisting of a few cells only [Fig. 3(B)]. Massive rosette formation occurred in severely disorganized retinas [Fig. 3(C,E–G,I)]. Rosettes were also detected in ventral regions of the diencephalon (not shown). In Pax6-deficient eyes, pigmentation in the RPE was often reduced [Fig. 3(C,E–G)] as compared to controls [Fig. 3(A,D)]. The extraocular mesenchymal tissue apposing to the RPE was poorly defined with enlarged intercellular spaces and pigment bear-

Figure 4 Photoreceptor rosettes in retinas deficient for Pax6 (A), mNcad (B), Ncad (C), and NCAM (D). Cryostat sections and confocal microscopy of Stage 42 tadpoles using antiserum to photoreceptors (red) and monoclonal antibody to vimentin (green) to reveal radial glia. A: Müller cell endfeet (arrowheads) directed toward the RPE layer (arrows), indicating the presence of a second retina in mirror orientation. B: Rosette (arrowhead) and a corresponding gap (arrows) in the photoreceptor layer suggesting displacement of outer into inner retina. C: Heavy disruption of photoreceptor layer (arrows) and rosette formation (arrowheads). D: Numerous rosettes (arrowheads) and Müller cell endfeet (arrow) in the outer retina. Bar, 50 μ m.

ing cells resembling phagocytes [Fig. 3(C,E)]. Similar cells were observed within the neural tube.

Immunostaining of the small-eyed phenotype by an antibody recognizing photoreceptor outer and inner segments ascertained the participation of these cells in rosette formation in Pax6 [Fig. 4(A)], mNcad [Fig. 4(B)], Ncad [Fig. 4(C)], and NCAM-deficient [Fig. 4(D)] embryos.

Staining for vimentin revealed endfeet-like differentiation in mirror orientation in the outer retina close to the RPE [Fig. 4(A,D)], hinting to the presence of glial cell structures normally found in the inner retina. Indeed, duplication of inner layers could be detected by double staining for Pax6 and syntaxin, a membrane marker for amacrine cells and the inner plexiform layer (Barnstable et al., 1985). Figure 5 shows such duplications in Pax6 (A, B), mNcad (C), Ncad (D), and NCAM (E)—deficient embryos. The formation of an additional retina seems not to be linked to an excess of neurons, since it occurred in near-normal-sized [Fig. 5(A,E)] as well as rudimentary eyes with a reduced complement of neurons and a small cluster of lenticular cells [Fig. 5(B–D)]. In addition to disturbed laminar organization of the retina, knockdown of Ncad also caused false orientation of single neurons [Supporting Information Fig. S2(A,B)]. Moreover, defective axonal bundling [Supporting Information Fig. S2(C,D)] was observed in all knockdown embryos deficient in mNcad, Ncad, or NCAM [Supporting Information Fig. S2(D)].

Conclusively, in spite of the knockdown of individual cell adhesion molecules, differentiation of the major retinal cell types takes place even in rudimentary eye anlagen. Prominent disturbances of the laminar organization are reflected by folding and duplication of inner retinal layers and, by rosette formation of photoreceptors associated with displaced RPE cells. The striking similarity between knockdown embryos deficient in Pax6, mNcad, Ncad and, on the level of retinal organization also NCAM, suggests that these cell adhesion molecules are part of the Pax6 pathway.

Pax6-Dependent Expression of Cell Adhesion Molecules

During development, transcription of the putative Pax6 target genes Ncad and NCAM started shortly after the onset of Pax6 expression at Stage 18, peaked at Stages 24 and 28, respectively, and somewhat decreased until Stage 38. No maternal mRNA stocks for either Ncad or NCAM could be detected [Fig. 6(A)]. By contrast, consistent maternal stocks of mNcad mRNA were present in the zygote and steadily diminished to disappear by Stages 18 to 24. Zy-

Figure 5 Duplication of inner retinal layers in Pax6 (A,B), mNcad (C), Ncad (D), and (E) tadpoles (Stage 42). Cryostat sections and confocal microscopy using polyclonal antibodies to Pax6 (red) and monoclonal antibody to syntaxin (green). Duplications are marked by an arrowhead. B: Note duplication in the rudimentary eye, in spite of reduced number of neurons. D: Cluster of Pax6-positive nuclei in outer retina in the absence of a differentiated inner plexiform layer (arrowhead). D,E: Staining of the lens is not specific. Bars, 50 μ M (A–D), (E).

gotic expression of this gene increased after Stage 24 (see also Tashiro et al., 1996).

Lowering Pax6 protein expression by antisense treatment led to a significant reduction of the mRNA levels of the three genes examined. At embryonic Stage 32, the ratio of Pax6 mRNA levels in antisense-treated over control embryos remained close to

Figure 6 Normal and reduced expression of mRNAs coding for Pax6 and the cell adhesion molecules mNcad, Ncad or NCAM. A: Normal expression during development (Stage 1–38). Relative mRNA levels are normalized to 18S rRNA. B: Average mRNA levels of Pax6 ($n = 7$ batches of 5 embryos each), mNcad ($n = 12$), Ncad ($n = 6$) and NCAM ($n = 9$) in Pax6 knockdown embryos at Stage 32. The respective mRNA levels are given as ratio over that of sibling control embryos. For statistical significance see text. C: Expression of mNcad mRNA in control (MO CO) and Pax6 knockdown (MO Pax6) embryos during development. Average of 5 independent experimental series. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

one, those of the target gene mRNAs were significantly reduced in the Pax6 knockdown larvae. On the average, the mNcad mRNA level was lowered by 70%, those of both Ncad and NCAM by about 40% [Fig. 6(B)]. Whereas the average ratio of unaffected Pax6 mRNA levels ($n = 7$ batches of 5 embryos each) is close to one, those of the putative target genes mNcad ($n = 12$, $p < 2 \times 10^{-9}$), Ncad ($n = 6$, $p < 4 \times 10^{-4}$) and N-CAM ($n = 9$, $p < 3 \times 10^{-5}$) are significantly reduced.

The maternal and zygotic levels of mNcad mRNA during development were scored in five independent series of control and Pax6 knockdown embryos. Figure 6(C) gives the average levels present in the zygote and up to Stage 38. As expected, the maternal mNcad mRNA pool was not affected by Pax6 deficiency, whereas zygotic expression was strongly reduced.

Since Pax6 is predominantly expressed in the head region, we measured the mRNA levels of Pax6 and of the target genes also in isolated heads and trunks of Stage 31 embryos (data not shown). For Pax6, the head:body ratio was 67:1. This regional difference was less pronounced for NCAM (13:1), Ncad (3:1)

and mNcad (2:1). Following antisense treatment against Pax6, the mNcad mRNA level was reduced to about the same degree (60%) in both head and trunk, whereas Ncad and NCAM mRNAs were reduced in the head (40%) but not in the trunk. Pax6 dependence of these target genes may thus be tissue-specific.

Rescue of Pax6 Inhibition

To verify Pax6-dependence of the putative target genes coding for cell adhesion molecules, we carried out rescue experiments. Overexpression of Pax6 was first tested by injection of CMV-Pax6-Red, producing a red fluorescent fusion protein. CMV-promoter driven expression from episomal plasmids was pronounced all over ectodermal and mesodermal derivatives but somewhat patchy (not shown).

For the rescue experiments, zygotes were injected with either a CMV-Red control plasmid, or a CMV-Pax6 expression vector. Control (MO-CO/CMV-Red), Pax6 overexpressing (MO-CO/CMV-Pax6), knockdown (MO-Pax6/CMV-Red) and rescued (MO-Pax6/CMV-Pax6) individuals ($n = 100$ for each mixture) carried all the same amounts of DNA (100 ng) and morpholino (15 ng). Of note that the dose of control and anti-Pax6 morpholino was half of that applied in the above experiments and the resulting mRNA levels may thus differ from the values given in Figure 6.

The mRNA levels of Pax6, mNcad, Ncad, and NCAM in such embryos were determined by real-time PCR on batches of 5 Stage 30 embryos [Fig. 7(A)]. Overexpression of Pax6 (MO-CO/CMV-Pax6) more than doubled the level of Pax6 mRNA yet did not increase mNcad mRNA over normal (MO-CO/CMV-Red). However, it somewhat increased Ncad, and markedly NCAM expression. Knockdown of Pax6 (MO-Pax6/CMV-Red), strongly reduced mNcad and, to a lesser extent, Ncad and NCAM mRNA levels. Overexpression of Pax6 in Pax6 knockdown embryos (MO-Pax6/CMV-Pax6) dramatically increased mNcad, Ncad and NCAM expression compared to MO-Pax6/CMV-Red.

The corresponding phenotypes were scored on the surviving sibling embryos ($n = 27$ –41) at Stage 36 [Fig. 7(B)]. Overexpression of Pax6 (MO-CO/CMV-Pax6) had no major impact on eye size or overall phenotype. Morpholino inhibition of Pax6 (MO-Pax6/CMV-Red), even at the reduced dose used, produced 70% of small eyes. CMV-driven overexpression of Pax6 rescued eye size in the morpholino-treated embryos (MO-Pax6/CMV-Pax6), reducing the frequency of small-eyed individuals to 25%.

Figure 7 Rescue of target gene expression by Pax6. A: Steady state Pax6, mNcad, Ncad, and NCAM mRNA levels in embryos (Stage 30) carrying either control (MO-CO) or anti-Pax6 morpholino (MO-Pax6), mixed with a CMV-driven expression vector coding for either red fluorescent protein (CMV-Red) or Pax6 (CMV-Pax6). B: Frequency of phenotypes (Stage 36) in sibling embryos (treated as in A). OK, normal; eye, small or defective eye; def, other malformations. C: Western blots for mNcad and β actin in knock-down and rescued embryos (as in A, Stage 30). The smaller reactive band is a degradation product. D: mNcad mRNA levels in embryos (Stage 30) carrying anti-Pax6 (MO-Pax6) or anti-mNcad morpholino (MO-mNcad) and CMV-Red or CMV-mNcad vector. E: Frequency of phenotypes in sibling embryos (Stage 36; treated as in D, phenotypes as in B). [Color figure can be viewed in the online issue, which is available at [wileyonlinelibrary.com](http://www.wileyonlinelibrary.com).]

For mNcad, Pax6-dependent expression was demonstrated on the protein level using an antibody raised against a synthetic mNcad peptide [Fig. 7(C)]. mNcad protein was strongly reduced in Stage 30 embryos carrying anti-Pax6 morpholinos and CMV-Red DNA and was rescued in individuals that had been injected with antisense morpholino and CMV-Pax6 expression vector.

Rescue of Pax6 knockdown was also attempted by overexpressing the putative Pax6 target mNcad. This analysis was carried out in parallel to the Pax6 rescue, under the same conditions. PCR analysis on Stage 30 embryos [Fig. 7(D)] shows that MO-Pax6/CMV-mNcad embryos expressed levels of mNcad mRNA five times higher than that of Pax6 knockdown embryos (MO-Pax6/CMV-Red). Direct knockdown of mNcad (MO-mNcad/CMV-Red) did not affect the normal level of mNcad mRNA. The CMV-mNcad vector produced elevated levels of mRNA also in the presence of mNcad antisense molecules (MO-mNcad/CMV-mNcad). On the level of the phenotypes [Fig. 7(E)], mNcad overexpression (MO-Pax6/CMV-mNcad) rescued eye size in only a few Pax6-deficient embryos compared with MO-Pax6/CMV-Red. Direct rescue of mNcad knockdown (MO-mNcad/CMV-Red) by mNcad overexpression (MO-mNcad/CMV-mNcad) was more efficient.

Pax6 Binding to Genes Coding for Cell Adhesion Molecules

To verify, whether Pax6 directly modulates transcription of the target genes coding for cell adhesion molecules, we searched for putative Pax6-binding sites in their promoters. For lack of sequencing data in *X. laevis*, this study was done on *X. tropicalis*. The first exon, 5' end and upstream sequences of mNcad were found on scaffold 1028, whereas the main coding segment was situated on scaffold 531. The Ncad gene is contiguous (scaffold 84) whereas the NCAM promoter and gene were again attributed to different contigs (scaffolds 318 and 436, respectively; for overview see Table 1). Screening with Target Explorer (Sosinsky et al., 2003) was done using a consensus sequence integrating different sequences binding to Pax6 *in vitro* (Epstein et al., 1994). Numerous, partly clustered, putative Pax6 binding motifs with scores between 2 and 8 were found in the promoters of all three genes examined [Fig. 8(A), Table 2].

For ChIP experiments, we chose PCR primers in the vicinity of some high-scoring putative Pax6 targets [Fig. 8(A)]. The cluster of binding sites in the proximal promoter of the mNcad gene could not be analyzed because it is not completely sequenced. Moreover, this region is highly repetitive, resulting in extremely high PCR amplification even in the controls. We thus limited our analysis to the upstream cluster for this gene plus a control region located in the first intron of the mNcad gene, far from any calculated Pax6-binding motif. For Ncad the most upstream putative binding site was analyzed, whereas the NCAM probe covered the entire cluster of Pax6 motifs.

Figure 8 Chromatin immunoprecipitation of Pax6-binding motifs in genes coding for cell adhesion molecules. A: Sketch of promoter regions of the genes coding for mNcad, Ncad, and NCAM. Hypothetical Pax6-binding sites, revealed by computer search, are indicated by circles of varying diameter, reflecting their respective scores. Amplicons (boxes) of promoter regions (PRO) or an intragenic region (CO) may originate from DNA fragments extending 250–500 bp (lozenge) on either side. Repetitive elements (stippled line) and so far not sequenced segments (N) preclude analysis of the proximal promoter region of mNcad. B: ChIP analysis of the four gene regions mNcad PRO and CO, Ncad PRO and NCAM PRO, with histone H3 antibodies yields near-equal values in both head and tail chromatin, expressed as percent of the input material precipitated. Average from two experiments $\pm$ SD. C: ChIP analysis with anti-Clock and anti-Pax6 antibodies on the same gene regions as in B. Note that about twice as many fragments of the promoter regions of the three genes coding for cell adhesion proteins were precipitated from chromatin preparations of heads by Pax6 antibody as compared to the nonspecific anti-Clock antibody. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

X. tropicalis embryos were grown to Stage 42. Chromatin from head or tail was submitted to immunoprecipitation with various antibodies. Comparable PCR signals were obtained with all primers for the different genomic regions after precipitation of head and tail chromatin with anti-histone 3 antibodies used as a positive control [Fig. 8(B)]. To determine the level of background binding, we used an antibody directed against the unrelated Clock protein (Ripperger and Schibler, 2006). Head and tail chromatin precipitated with antibodies directed against this factor yielded virtually identical background signals. Precipitation of tail chromatin with anti-Pax6 antibodies and amplification with primers for the promoter region of all three genes and for the mNcad control region, also gave near identical background signals. By contrast, precipitation of the chromatin prepared from embryonic heads with antibody directed against Pax6 yielded clearly elevated signals for the promoter region of all three genes analyzed, whereas the intragenic control region of the mNcad gene gave low signals with chromatin from both head and tail nuclei [Fig. 8(C)]. Accordingly, Pax6 binds *in vivo* to the chromatin regions with our predicted Pax6-binding motifs within the promoters of mNcad, Ncad, and NCAM.

DISCUSSION

Pax6-Dependent Cell Adhesion Molecules

Many of the abnormalities observed earlier in Pax6 mutants of several species suggested that cell adhesion is disturbed (see Introduction). Knocking down Pax6 by morpholino antisense molecules in *Xenopus* embryos, we show here that the expression of the cell adhesion molecules, mNcad, Ncad, and NCAM depends, at least in part, on normal Pax6 levels. Moreover, direct knockdown of these genes produces phenotypic traits similar to those found in Pax6-deficient larvae. A computer analysis revealed clusters of putative Pax6-binding sites in the promoter region of the target genes and, ChIP experiments showed that, in head tissues, Pax6 does bind to these potential regulatory regions.

The levels of Pax6 mRNA are not reduced by the addition of morpholinos directed against Pax6. Antisense morpholino molecules thus inhibit translation but do not induce degradation of the target mRNA as this is the case with antisense RNA (Nichols et al., 1995). As far as the putative Pax6 target genes are concerned, the maternal stocks of mNcad mRNA nor-

Table 2 Putative Pax6-Binding Sites

Gene	Position ^a	Orientation ^b	Sequence ^c	Score
mNcad (cdh4)	−2018	F	<u>CAAATAATGCTTCACTGC</u>	7.11
	−873	F	<u>TATTTAATGGTTAAATGA</u>	2.14
	−761	F	<u>TATTTTCATGGTTAAATGA</u>	4.14
	−649	F	<u>TATTTAATGGTTAAATGA</u>	2.14
	−562	F	<u>TATTTTCATGGTTAAATGA</u>	4.14
	−424	F	<u>TATTTAATGGTTAAATGA</u>	2.14
	−1	R	<u>GAGATCGGGCGGACAGTG</u>	3.61
Ncad (cdh2)	−3661	F	<u>AGTTGCATTCTTCAATTG</u>	6.64
	−2897	F	<u>CATTCTCTCGTATGACCTG</u>	7.78
	−1200	R	<u>GCAGCCATGCATGTGTGT</u>	6.17
NCAM	−704	F	<u>ATTTGCTCGCCCCATTGC</u>	4.92
	−620	R	<u>TCAGTCATAAGTAAATCA</u>	6.09
	−533	F	<u>GACTTACTGCATTATTTA</u>	3.41
	−522	R	<u>TATCCAATGCATAATGTT</u>	2.92
	−296	F	<u>AGCCTCACTCATTTCTGA</u>	3.56
	−79	F	<u>AGTCTCTTGGCTGGGCGC</u>	2.29

^aRelative to mRNA start (+1).

^bF, forward; R, reverse.

^cComparison with consensus sequence ANNTTCACGC(A/T)T(G/C)ANT(G/T)(A/C) from Table II (Epstein et al., 1994).

mally persist until Stage 18 and are not altered. Zygotic expression, however, is significantly lowered by over 60%, that of Ncad and NCAM by 40%. As knockdown of Pax6 is probably not complete, it remains an open question whether, in some tissues and at specific developmental stages, Pax6 is absolutely required for the expression of these target genes or whether it more or less stringently modulates their transcription.

In this context, it is important to note that, during early development, the expression levels of Pax6 and its target genes considered here are much higher in the head region than in the remaining body. The low levels of mRNA for Ncad and NCAM in the body were not measurably affected in Pax6-deficient Stage 32 embryos. In these tissues, Ncad and NCAM expression may thus not, or only weakly be regulated by Pax6. The expression of mNcad, however, was equally dependent on Pax6 in both head and trunk. At later stages of development, all target mRNA levels slowly normalized. This recovery may be due to early lethality of strongly affected individuals. Alternatively, during development, these cell adhesion molecules may become expressed in numerous tissues where transcription is independent from Pax6 regulation.

Pax6-dependence of expression of the genes coding for cell adhesion molecules considered here, is underscored by the rescue experiments. Coinjecting a CMV-Pax6 expression vector together with the Pax6 antisense morpholino normalized the phenotypes and restored the mRNA levels transcribed from the target

genes. The Pax6 antisense morpholino used is complementary to all known *Xenopus* Pax6 mRNAs (cDNA clones), including the one (Li et al., 1997) used for overexpressing Pax6 in our rescue experiments. The rescue effect may thus at least in part reside on titrating away antisense morpholino by the supplementary mRNA formed. Whatsoever, reducing the severity of Pax6 inhibition clearly favored normal eye development and enhanced the expression of the putative Pax6 target genes.

Compared with rescue of Pax6 deficiency by overexpressed Pax6, rescue of Pax6 by mNcad was modest. This is not too surprising. Pax6 is known to control eye formation through several signaling pathways not related to cell adhesion (citations above). In addition, according to the data presented here, several cell adhesion molecules are dependent on Pax6. Rescuing a single component of this complex network may well be futile. Rescue of mNcad deficiency by mNcad was somewhat more efficient but still rather limited, maybe because expression of mNcad under control of the CMV promoter was neither correctly timed, nor dosed, nor tissue specific. Nonetheless, the partial rescue of Pax6-deficiency by mNcad argues in favor of mNcad being part of the Pax6 pathway. In turn, its limited success underscores the idea that several Pax6-dependent cell adhesion molecules cooperate in eye formation.

Computer screening for putative Pax6-binding sites with Target Explorer (Sosinsky et al., 2003) needs several input sequences to define a reliable weight matrix. We used 23 sequences containing the

entire consensus region, out of the array of sequences that bound to Pax6 *in vitro* (Epstein et al., 1994). This analysis revealed several positively scoring, putative binding sites within the promoter region of the *Xenopus tropicalis* genes coding for cell adhesion molecules (map in Fig. 8, scores in Table 2).

To evaluate whether these results are meaningful, we used the same weight matrix to determine the scores of several known Pax6-binding sites in the promoters of the L1 (Chalepakis et al., 1994; Meech et al., 1999), NCAM (Holst et al., 1997), the cortical transcription factor Er81 (Tuoc and Stoykova, 2008) and various other genes (Czerny et al., 1993; Czerny and Busslinger, 1995; Wolf et al., 2009). With the exception of a strong false positive, namely 5.5 for sequence TGFb210 (Wolf et al., 2009), two nonbinding mutants scored 1.6 and 1.8 but the other 10 scored negatively. Eleven sequences binding Pax6 *in vitro* scored positive in our computer analysis (between 2.6 and 15.4), similar to the binding sites pointed out by our search (details in Supporting Information Table S1). We thus chose the regions to be analyzed by ChIP close to these putative binding motifs.

In our ChIP experiments, consistent amounts of amplicons were only obtained with chromatin from embryonic heads precipitated with anti-Pax6 antibodies and amplified with primers close to putative binding sites in the mNcad, Ncad, as well as NCAM promoters. All controls, including tail chromatin, irrelevant antibody, or intragenic primers, yielded similar background levels. The limitation of the positive signal to twofold over baseline, is probably due to the fact that only a small subset of cells in the head express Pax6 and, the anti-Pax6 antibody used was risen against murine Pax6, and its crossreactivity in *Xenopus* may be weak. Nevertheless, these data strongly suggest that Pax6 binds *in vivo* to the motifs present in the promoters of genes coding for cell adhesion molecules.

Phenotypes

Most of the defects arising in *Xenopus* embryos have also been observed in the murine *Sey* mutant (reviewed by Ashery-Padan and Gruss, 2001). Contrary to the heterozygous *Sey* mouse, and in spite of the variable stringency of antisense inhibition, all Pax6-deficient *Xenopus* embryos died after developmental Stage 46, including tadpoles with normal external phenotypes and formerly small-eyed individuals having recovered a near-normal eye size. Deficient peripheral innervation and disturbed differentiation of endocrine organs might cause this general lethality. In the mouse, Pax6 has been shown to be

involved in development, differentiation, and function of pancreas and gastrointestinal endocrine cells (Schönhoff et al., 2004).

Direct antisense inhibition of mNcad indeed caused defects in the digestive tract similar to those observed upon inhibition of Pax6. As mNcad expression in the trunk is Pax6-dependent, its role in differentiation of the digestive tract thus seems to be part of the Pax6 pathway. In the mouse, R-cadherin was shown to be expressed during organogenesis of the pancreas and gastrointestinal tract (Sjödén et al., 1995). In *Xenopus*, its homologue mNcad but also Ncad appear to be involved in similar differentiation steps. Ncad deficiency caused even more severe disturbances than Pax6 inhibition. As Ncad mRNA levels in the trunk were not measurably diminished by Pax6 knockdown, Ncad function in trunk tissues seems not to be controlled by Pax6. As far as externally visible features are concerned, NCAM appears not to play a major role during early development of the body as only a slight delay in differentiation of the digestive tract was noted but this by no means excludes a more subtle involvement of NCAM later during organogenesis.

Ocular Defects

Reduction in eye size is the predominant phenotype of Pax6-deficient *Xenopus* embryos with over 80% of the treated embryos being small-eyed or, by external inspection, eyeless. Variability in the stringency of antisense inhibition thus produces a wide range of phenotypes which may correspond to homozygous and heterozygous mammalian *Sey*⁻ mutants (reviewed by Cvekl and Tamm, 2004). A less pronounced reduction in eye size was also observed in mNcad- and Ncad-inhibited embryos and only few of the NCAM-deficient individuals exhibited a slightly reduced eye.

Clearly, reduced eye size reflects the well-known pivotal role of Pax6 in inducing ocular development and specifying the size of the primordia of both lens and retina, also in *Xenopus* (Altmann et al., 1997; Li et al., 1997; Zygarr et al., 1998; Chow et al., 1999; Hirsch and Grainger, 2000). Our observation that, even the most severe “eyeless” phenotypes formed a rudimentary eye with a few elongated lens fibers and a small complement of differentiated retinal cells, indicates that Pax6 inhibition is stringent but not complete. The conspicuous presence of a thickened epidermal fold in the “eyeless” tadpoles, and the presence of only few lenticular cells [Figs. 1(B) and 5(B)] suggest that Pax6 levels in the surface ectoderm were not sufficient to promote induction of a consist-

ent lens placode. This corroborates the conclusion that the surface ectoderm needs expression of Pax6 to become a lens placode (Ashery-Padan et al., 2000, Hirsch and Grainger, 2000). Direct inhibition of the Pax6-dependent cell adhesion molecules mNcad and Ncad readily produced small-eyed embryos. Ncad is expressed in a Pax6-dependent manner in the murine lens placode (van Raamsdonk and Tilghman, 2000) but only its very stringent inhibition seems sufficient to entirely block lens formation in *Xenopus* embryos.

Inefficient transmission of Pax6-dependent inductive signals from a defective lens may account not only for the reduced size of the posterior segment but also for the conspicuous folding of the neural retina observed in knockdown embryos. Our observations corroborate earlier findings in the *Sey* mouse where, in absence of a lens or after its ablation, the posterior globe remains small and presents retinal convolutions (Quinn et al., 1996; Ashery-Padan et al., 2000). The influence of mNcad, Ncad, and NCAM on the size of the posterior globe appears to be minor, as their direct knockdown did not produce obvious convolutions.

In all Pax6 knockdown embryos, including the “eyeless” phenotype, differentiation of ganglion, amacrine, and Müller glial cells took place at least to a state expressing the respective markers used. Rod and cone photoreceptors were also formed. As judged by Pax6 immunoreactivity, folded retinas exclusively comprised layers of the inner retina formed by ganglion and amacrine cells. This observation is in line with those made in conditional knockout mice, where neural progenitor cells lacking Pax6 preferentially entered amacrine differentiation (Marquardt et al., 2001).

Defective lamination of the neural retina, as reflected by duplication of inner retinal layers, not only occurred in Pax6-deficient embryos but also after knockdown of mNcad, Ncad, and NCAM. We infer that these retinal dysmorphologies are due to missing clues from a poorly differentiated RPE. Instability of the RPE may entail transdifferentiation into neural retina and elicit its inverse orientation (Coulombre and Coulombre, 1965; Stroeve and Mitashov, 1983). This process can be promoted by mutations in the *Mitf* gene (Quinn et al., 1996; Planque et al., 2001; Bäumer et al., 2003) or by its disturbed interaction with Pax6 (Martinez-Morales et al., 2004). Another reason for poor differentiation of the RPE may be disturbed migration of cells forming the extraocular mesenchyme in Pax6-deficient embryos and a resulting poor induction emitted by this tissue. Our data add evidence for a direct involvement of the Pax6-dependent cell adhesion molecules mNcad, Ncad, and NCAM in correct RPE differentiation. All three adhesion molecules have been shown to be synthesized by RPE cells in a

spatially and timely defined manner (Liu et al., 1997; Marmorstein et al., 1998; Bailey et al., 2004). In our study, deficiency of one of these components was sufficient to cause failure of normal RPE lining and, displacement of cell clusters into the neural retina.

Poorly defined adhesion in the RPE of Pax6, mNcad, Ncad, or NCAM knockdown embryos may thus be at the origin of photoreceptor rosettes that formed around single, or small clusters of displaced RPE cells. So far, rosettes were not reported in association with Pax6 deficiency but they are a common feature of certain retinal pathologies such as retinitis pigmentosa (Tulvatana et al., 1999). They may also form after transplantation of retinal cell sheets (Sharma et al., 1997) or because of a defective glia limitans (Willbold et al., 2000; Sakata-Haga et al., 2001). Rosettes also arise in conjunction with a mutation in *Mitf* (Bumsted and Barnstable, 2000) again demonstrating the importance of RPE integrity for photoreceptor support. In tune with our observations, massive rosette formation has been described for the zebrafish mutants *parachute* (Erdmann et al., 2003; Masai et al., 2003) and *glass onion* (Malicki et al., 2003), coding for defective Ncad, as well as for mutants lacking R-cadherin *Cdh4* (Babb et al., 2005).

Conclusively, morpholino antisense inhibition of Pax6 provides long-lasting effects and produces the phenotypes expected from the known principal functional roles of this transcription factor. The variable stringency of inhibition disclosed novel features, as yet not linked to Pax6 deficiency, such as instability of a poorly differentiated RPE, rosette formation, and duplication of inner retinal layers. The phenotypic similarities between Pax6, mNcad, Ncad, and NCAM knockdown embryos suggest that many of the disturbances are linked to impaired expression of Pax6-dependent cell adhesion molecules. Indeed, at least in the head region and during early development, transcription of these target genes depends on normal Pax6 levels being strongly reduced in Pax6-deficient embryos but fully rescued by Pax6 overexpression. The genes coding for the cell adhesion molecules considered in this study contain numerous hypothetical Pax6 binding sites, clustered in their promoter regions. As shown by ChIP experiments, the chromatin regions containing such theoretical binding sites indeed do bind Pax6 *in vivo*. Normal eye formation and, in particular, correct retinal lamination thus appear to depend, besides the already established pathways, on the cooperative function of several cell adhesion molecules under the control of Pax6.

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