PAX6 P46 Binds Chromosomes in the Pericentromeric Region and Induces a Mitosis Defect When Overexpressed

Raffaella Zaccarini,1 Fabrice P. Cordelières,1 Patrick Martin,2 and Simon Saule1

Purpose. Haploinsufficiency and overexpression of the PAX6 gene are responsible for defective central nervous system development. The purpose of the current work was to identify and characterize a new potential role for the PAX6 transcription factor in cellular proliferation in addition to its role at the level of gene expression.

Methods. Expression vectors encoding tagged Pax6p46 protein were used to observe directly protein localization during the cell cycle in cell lines. Three dimensional (3-D) fluorescence microscopy imaging was used to observe in vivo mitotic progression and chromosome dynamics to define the mitotic step affected by p46DsRed as well as to validate endogenous p46 localization on chromosomes in quail retinal cells. Video imaging was used to identify the precise moment of onset of effects related to p46 overexpression in living cells. A pulldown assay in HEK cells was used to identify a specific partner of p46.

Results. Pax6p46 protein in transfected cells is localized on the chromosomes, predominantly in a pericentromeric area, and its localization changes as mitosis progresses. Overexpression of p46 protein induces incomplete chromatid separation, resulting in defective mitosis at the onset of the anaphase. A physical interaction between p46 and ESPL1 was identified.

Conclusions. The results suggest that Pax6 exerts an effect on mitosis through protein–protein interactions with proteins localized on chromosomes. Supported by the observation that p46 interacts with separase, an enzyme required for chromatid separation, the authors propose that this interaction is responsible for the mitosis defect observed in cells overexpressing Pax6. (Invest Ophthalmol Vis Sci. 2007;48:5408–5419) DOI: 10.1167/iovs.07-0413

Retinal precursor cells coexpress a set of transcription factors before the onset of cell differentiation and during the ensuing stages of retinogenesis. These factors have been implicated in a highly conserved genetic network that controls eye development, and forced expression of each of them in fish and frogs results in mutual cross-activation and promotes the formation of ectopic retina tissues.1-5 Pax6 is one of the highly conserved transcription factors that bear two DNA-binding domains, the paired and the homeodomain, and a transactivation domain.4,5 Pax6 encodes different proteins through alternative splicing and internal initiations.6-9 Three splice variants, Pax6p48, Pax6p46, and Pax6p43, of 48, 46, and 43 kDa, respectively, contain a paired domain, but this DNA-binding domain is absent in the two remaining isoforms of 33 and 32 kDa (Pax6p53 and Pax6p32, respectively). Pax6 is necessary for the proper development of the pancreas and the nervous system and is known to be critical in eye development.9 Two hundred eighty-six sequence variations in Pax6 have been reported to be associated with pathologic mutations that cause congenital eye malformation in humans (http://pax6.hgu.mrc.ac.uk).9 The most common of these malformations is aniridia, a congenital, hereditary, bilateral, extreme form of iris hypoplasia that may be associated with other ocular defects. It describes an extreme form of iris hypoplasia in which the iris appears absent but retains the iris root. Aniridia is not just an isolated defect in iris development but is a panocular disorder with macular and optic nerve hypoplasia, cataract, and corneal changes. Despite its early expression in the optic vesicle, Pax6 is not necessary for optic vesicle outgrowth or identity, as optic vesicles form in a homozygous Pax6 mutant.10 However, subsequent development of the optic vesicle is highly abnormal, with failure to form the optic cup, neural retina, pigmented retina, or optic stalk, and the associated epithelial lens placode fails to invaginate to form a lens. Surprisingly, neurons differentiate in Pax6 mutant optic vesicles5 suggesting that one function of Pax6 is to control the timing of retinal neurogenesis. Both the semidominant inheritance pattern of the Pax6 mutant phenotype6,12 and experimental manipulations using transgenes based on Pax6 locus6,15 indicate that achieving the correct level of Pax6 is important for development of a normal eye and that an overexpression of Pax6 is deleterious for retinal cell proliferation.13 Overexpression of Pax6 is involved in cellular transformation6 and lack of Pax6 is also associated with hypcellularity of the retina16 and in a more natural situation, widespread structural abnormalities including absence of the pineal gland and hypoplasia of the olfactory bulb are observed in brains of human heterozygotes for Pax6 mutations.17 During brain development of the Pax6 mouse mutant, the cell cycle is shortened early in corticogenesis but later proliferation is slowed with a large increase in the length of the S phase in the mutant cortex.18 During mitosis, the metaphase-to-anaphase transition is the final discrete event in duplication and separation of the genetic material of the cell. Centromeres of mitotic chromosomes specifically retain RAD21 subunits until anaphase, whereas most RAD21 dissociates from chromosome arms during the prophase.19 Sister chromatid cohesion at or near the centromere opposes the microtubule-dependent pulling forces exerted by the spindle before the onset of the anaphase and therefore helps to prevent premature dissociation of sister chromatids. The metaphase to anaphase transition is triggered by the timely degradation of the RAD21 cohesin subunit by a protease, ESPL1 (separase), which is also required for cytokinesis.20 To follow the proteins in living cells, we expressed each distinct Pax6 protein tagged with GFP or with DsRed. We observed p46 at discrete spots on the chromosomes and more...
specifically, as shown by the use of centromeric markers, on the pericentromeric area of the chromosomes. Most of the cells that expressed the p46-tagged protein were unable to exit mitosis, and video imaging of the transfected cells revealed that the mitotic defect resulted in persistent DNA bridges initiated after the metaphase step that occurred normally. These results suggest that the Pax6 transcription factor, in addition to its role at the gene expression level, may regulate cellular proliferation by interfering with sister chromatid cohesion.

Materials and Methods

Plasmids

Red (DsRed) and green fluorescent proteins (GFP) were expressed from the pVNC7-DsRed and pVNC3-EGFP vectors, respectively. Quail cDNA encoding the BBG p46Pax6 proteins was digested by XhoI and NotI, and the 1269-bp fragment was inserted in XhoI-BspBI-digested pVNC3MiRed21 vector to obtain pVNC3p46DsRed. The same fragment inserted into pVNC7HA digested by the same enzymes yielded pVNC7p46HA. pVNC7p48DsRed and pVNC7p43DsRed were created by inserting the 692-bp DsRed BamHI-NotI fragment from pDsRed-N3 into the pVNC7p48 or p43 clones digested by the same enzymes. pVNC7p30DsRed was constructed from pVNC7p48 digested by XhoI-NdeI, Klenow-treated, and ligated. This p30-encoding vector was then digested with BamHI-NotI and the 692-bp DsRed BamHI-NotI fragment from pDsRed-N3 inserted in the plasmid digested with the same enzymes. The p46del170-342 mutant results from a KpnI fragment deletion on pSG5p46. EGFP and DsRed tags were inserted in the C-terminal part of the proteins. pVNC3p46EGFP and GST-p46 paired domain and homeodomain have been described.21 HP1α-encoding vectors were kindly provided by Jean-Claude Courvalin. V5-tagged ESPL1 encoding vector was kindly provided by David S. Pellman.22

Cell Cultures and Transfection Assays

HeLa cells were grown in DMEM without l-glutamine (Invitrogen-Gibco, Cergy-Pontoise, France) supplemented with 10% FCS, 1% MEM nonessential amino acids (100×; Invitrogen-Gibco) and 1% L-glutamine (200 mM; Invitrogen-Gibco). HeLa and HEK cell transfections were performed with PEI (exgen 500; Euromedex, Souffelweyersheim, France) reagent according to the manufacturer’s protocol. MC29-transformed Pax6-expressing cells have been described.23 HeLa histone H2B-EGFP was kindly provided by Kevin F. Sullivan.

Immunofluorescence

HeLa, 293T human epithelial kidney (HEK) and CenpB-GFP-expressing human osteosarcoma U2OS24 cell lines were cultured on glass coverslips for 48 hours after transfection. For the colocalization experiment, HeLa cells were lysed for 2 minutes in 0.5% Triton X-100 and fixed in 4% paraformaldehyde at room temperature for 10 minutes, washed three times in PBS and further permeabilized for 25 minutes in 0.1% Triton X-100 in PBS. The cells were treated with 50 mM NH4Cl in PBS for 10 minutes, washed three times for 5

![Figure 1](https://iovs.arvojournals.org/)

**Figure 1.** Pax6p46DsRed localization on chromosomes during mitosis in HeLa nuclei. HeLa cells were transiently transfected with a plasmid driving expression of Pax6p46 protein fused to DsRed (A, D, G, J). DNA was labeled with DAPI (B, E, H, K). Only in the early phases of mitosis, until the metaphase (D), did Pax6p46DsRed localize in discrete double-spotted areas, whereas from the anaphase (G) until segregation of the chromosomes and the beginning of DNA decondensation (J), Pax6p46 appears to be spread along the chromosome arms. (C, F, I, L) Merged images. *Blue:* DAPI; *red:* Pax6. Scale bar, 5 μm.
minutes each in PBS, blocked in PBS-BSA (PBS containing 0.1% BSA), and labeled with antibody for 1 hour at 37°C. Anti-paired–specific rabbit serum has been previously described. Anti H3S10ph (to follow the nomenclature of histone tail modifications) was from Upstate Laboratories (Lake Placid, NY), CENP-F (anti-mitosin) was from BD Transduction Laboratories (Lexington, KY). After they were washed in PBS-BSA, the cells were incubated with secondary antibodies for 45 minutes.

3-D Microscopy

Pictures of fixed cells were collected using a 3-D deconvolution imaging system which consisted of a Leica DM RXA microscope, equipped with a piezoelectric translator (PIFOC; PI, Lederhose, Germany) placed at the base of a 100× NA 1.4 objective (PlanApo, Carl Zeiss Meditec, GmbH, Oberkochen, Germany), and a 5-MHz interline CCD camera (Micromax 1300Y; Roper Instruments, Evry, France). Stacks containing fluorescence images were collected automatically at 0.2-μm intervals (Metamorph software; Universal Imaging, West Chester, PA). Wavelength selection was achieved by switching to the corresponding motorized selective filter block (Leica, Wetzlar, Germany) before each stack acquisition. Automated batch deconvolution of each z-series was computed using a measured point spread function and constrained iterative deconvolution.

Pulldown Assays

HEK cells (5 × 10^5) transfected with 2 μg of plasmid pIND/ESPL1-HIS using PEI reagent. After 24 hours, cells were washed twice with PBS 1× and after centrifugation were resuspended in 150 μL of lysis buffer (20 mM HEPES [pH 7.4], 150 mM NaCl, 1% Triton X-100, 0.2 mg/mL BSA, 2 mM dithiothreitol [DTT], 2 mM EDTA, protease inhibitor cocktail (Complete Mini; Roche Diagnostics, Mannheim, Germany) on ice for 30 minutes and then centrifuged at 15,000g for 20 minutes at 4°C. To analyze the interaction between Pax6 and ESPL1, cell lysates were incubated at 4°C for 1.5 hours under gentle agitation with GST-fusion proteins or GST alone immobilized on glutathione beads in the presence of incubation buffer (same composition as lysis buffer). The beads were washed three times with NETN buffer (100 mM NaCl, 20 mM Tris-HCl [pH 8], 1 mM EDTA, and 0.5% Triton X-100) and boiled in Laemmli sample buffer. Proteins were separated by SDS/PAGE on an 8% polyacrylamide gel and transferred to a PVDF membrane. Human ESPL1 was detected by Western blot analysis with anti-v5 monoclonal antibody, as previously described.

**FIGURE 2.** Analysis of Pax6 localization on chromosomes. (A-D) To study Pax6p46 localization on chromosomes. (A) HeLa cells were transfected with Pax6p46 and visualized by 3-D microscopy using DsRed fluorescence. Pax6 localization was then compared with that of the set of centromeric proteins recognized by the CREST antiserum, visualized with a secondary FITC-conjugated antibody (green) (B). (D) Pax6 protein was very close to the set of proteins recognized by CREST but did not occupy the same region. (E-K) Pax6 (E) localized in a region next to that identified by the kinetochore marker (CREST serum; F) and also to the region recognized by an outsider marker such as DCTN1 (G). Merged images: (I) Pax6p46 protein (green) localized in a region next to CREST (red); (J) CREST (red) signal was correctly localized, as previously described, next to the DCTN1 (green) stain; (K) Pax6p46 fusion protein (red) did not colocalize with the region marked by the DCTN1 (green). (F–K) enlargements of the corresponding panels. (L–O) Pax6 (L) localized in a region next to that identified by the kinetochore marker (M) DCTN1 in proliferating quail retinal cells. (N) Merged imaging showing Pax6 protein (green) localized in a region next pDCTN1 (red). DNA (O) was labeled with DAPI. Scale bar, 5 μm.
Video Microscopy

Video microscopy experiments were performed 2 days after transfection with Pax6p46 protein. Live videomicroscopy was performed with an imaging system. Cells were grown on a glass coverslip and then mounted in a Ludin chamber. Both the microscope and the chamber were maintained at 37°C. Stacks of 12 images with a z-step of 0.3 μm were acquired with a 100× NA 1.4 oil-immersion objective coupled to a piezo device (PI). Images were collected in stream mode with a camera (Micromax; Roper Scientific) set at 2 × 2 binning with an exposure time of 50 to 100 ms (frequency 6/min). All stacks were treated by automatic batch deconvolution using the PSF (point spread function) of the optical system. Projections, animations, and analyses were generated on computer (Metamorph software; Universal Imaging Corp.).

RESULTS

Pax6 Localization on Chromosomes during Mitosis

Transfection of HeLa cells with expression vectors encoding tagged p46 protein allowed us to follow p46 protein localization during the cell cycle directly, using 3-D fluorescence microscopy imaging. We observed (Figs. 1A, 1D, 1G, 1J) that p46 remained on the condensed chromosomes during the different mitosis phases. Pax6p46DsRed staining displayed an accumulation in very bright small spots identified as double-dotted in prometaphase and metaphase, and during these phases the Pax6p46DsRed signal was not identified in any other region of the chromosome structures. Conversely, in the late anaphase, when the two halves of each chromosome are pulled apart by the mitotic spindle apparatus, and in the telophase, the Pax6p46DsRed stain, previously visible as bright spots, was significantly reduced (although a low signal was still detected). To verify that this surprising protein localization is not the consequence of the tag fusion with p46, we performed additional experiments with GFP (Fig. 2E and data not shown) as well as HA p46-tagged proteins. An identically punctate localization of p46 was observed with all the chimeras used (data not shown).

Pax6 Binding to Pericentromeric Region of Chromosomes

The punctate pattern suggest that the protein is located within a particular chromosomal area. Since the localization of p46 frequently appeared as doublet dots on the chromosomes from metaphase cells (Fig. 1D), we performed indirect immunofluorescence staining with antibodies against centromeric and kinetochore proteins (CREST and DCTN1, also known as p150Glued, the largest polypeptide of the dynactin complex.

![Image of colocalization](https://example.com/figure3.png)

**Figure 3.** Colocalization of HP1α with Pax6p46. Exponentially growing HeLa cells were transiently transfected with plasmid expressing HP1α (B, F, J, N) and, individually, all Pax6 DsRed proteins (A, E, I, M). The two different proteins were individually visualized by 3-D microscopy with the two different immunofluorescent tags fused to the proteins (EGFP for HP1α and DsRed for Pax6 proteins). DNA was counterstained with DAPI, which highlighted the A/T-rich repeat sequences present in heterochromatic foci (bright blue patches) (C, G, K, O). (D, arrow) Colocalization between the bright spots can be identified only in the merged image between Pax6p46DsRed and HP1α. Scale bar, 5 μm.
which binds directly to microtubules and to cytoplasmic dynein) within HeLa cells expressing p46DsRed and in HeLa cells expressing p46GFP protein (Fig. 2); CREST labels the inner kinetochore, and DCTN1 labels the outer kinetochore. Codetection experiments presented in Figures 2E to 2K demonstrate that CREST staining correctly localized next to the region stained by the DCTN1 antibody (Fig. 2J). The Pax6p46 fusion protein signal was in very close proximity to the signals from both CREST and DCTN1 but did not colocalize with either (Fig. 2I-K). Therefore, the region recognized by the

**Figure 4.** Pax6p48 (A–L) and Pax6p30 (M–R) localization during mitosis in HeLa cells. (A–L) HeLa cells were transfected with plasmid overexpressing Pax6p48 splice variant fused to DsRed (A, D, G, J). Chromosomes were labeled with DAPI (B, E, H, K). Pax6p48 localized on the whole chromosome area in metaphase as well as up to the end of mitosis (DNA starts to decondense (J). (C, F, I, L) Merged images of DAPI (blue) staining and immunofluorescence from Pax6 (red). (M–R) HeLa cells were transfected with plasmids expressing Pax6p30 isoform fused to DsRed (M, P). DNA was labeled with DAPI (N, Q). Pax6p30 localized on the whole chromosome area from the early phases of mitosis (prometaphase; M) to the latest phases (P). Scale bar, 5 μm.
Pax6 product has to be in the pericentromeric area of the chromosomes, next to the regions identified by CREST and DCTN1. To demonstrate that this particular localization was not the result of Pax6p46 fusion with either GFP or DsRed, or of overexpression, we performed indirect immunofluorescence staining with antibodies against kinetochore proteins (DCTN1) and Pax6 within mitotic quail retinal cells. The Pax6 protein signal was in very close proximity to the signal from DCTN1 (Fig. 2N). As mitosis progressed, the p46 signal diffused along the arms of the chromosomes and at interphase appeared as discrete spots within the nucleus (Fig. 3A).

We also examined the localization of the other Pax6 splice variants during mitosis. In contrast to the p46DsRed protein signal, p48DsRed staining resulted in widely distributed, abundant, and diffuse signals along the entire chromosomes without any discrete, specific, marked areas during all the mitotic phases as shown in Figures 4A to 4L. Although, the p30DsRed isoform stain was also diffusely associated with the chromosomes (Figs. 4M–R), it appeared to be more organized in small, abundant dots spread on the chromosome arms. We next studied whether the other Pax6 splice variants bind to the pericentromeric region of chromosomes. We used a Cenp-B-GFP-expressing human cell line U2OS transfected with Pax6DsRed proteins. As shown in Figure 5, only p46 exhibited a significant close proximity with the Cenp-B signal as evidenced by cross-correlation function (CCF). Only a marginal signal was visualized with p48DsRed and had no correlation at all with p30DsRed.

**Colocalization of Pax6 with HP1α in Transfected Cells**

In vertebrates, centromeric regions occur within the context of constitutive heterochromatin. As such, they are organized by nucleosomes that are hypoacetylated and hypermethylated, accounting for the presence of chromodomain-containing heterochromatin proteins, such as HP1, which bind H3K9me3.30 In human cells, of the three related HP1 proteins, only HP1α is associated with centromeres in metaphase. To study a possible colocalization between each Pax6 proteins and HP1α, HeLa cells were cotransfected with a vector encoding EGFP-tagged HP1α and with one of four plasmids from a set containing each of the Pax6 splice variants fused with DsRed, and the cells were examined by 3-D microscopy. Signals, captured sequentially from the same cells, permitted distinction between Pax6 proteins and HP1α protein. We found, as shown in Figure 3A, that p46DsRed formed large spots that precisely overlaid a subset of HP1α dots (Fig. 5D), whereas there was no obvious overlap between HP1α fusion protein and any of the other Pax6 proteins (Figs. 3H, 3L, 3P). Confocal experiment using anti-HP1α antibodies also demonstrated colocalization between p46 and endogenous HP1α in transfected cells (data not shown).

**Pax6-Induced Mitosis Exit Defect in Transfected Cells**

Examination of p46 transfected HeLa cells revealed that Pax6 expression induces a mitotic failure (Fig. 6). DNA staining with 4′,6-diamino-2-phenylindole (DAPI) and the red fluorescence analysis of Pax6 protein in transfected HeLa cells revealed that adjacent, well-separated cells were connected by a structure (a bridge) containing not only Pax6 protein but also DNA (Figs. 6A–C). This particular mitosis defect was also observed for the p46GFP or p46 wild-type proteins (data not shown). To confirm that the lagging DNA was chromatinized, we overexpressed p46DsRed construct in HeLa cells stably expressing GFP-histone 2B fusion protein to visualize chromosomal DNA. Again, with these particular cells, we frequently observed lagging chromatinized DNA in the bridge (Figs. 6D–F). The same DNA bridges were found when p46 was overexpressed in retinal cells (Figs. 6G–I). Chromosomal condensation is depen-
p46 Paired-Domain Induction of the Mitosis Exit Defect in Transfected Cells

We performed an analysis to identify the statistical significance of DNA bridge formation once cells were transiently transfected with p46DsRed splice variants. This experiment was performed on an HEK cell population, in which transfection efficiency is higher than in HeLa cells, but similar observations were also made in HeLa cells (Fig. 8). Chromatin bridges were only occasionally observed in control cells. Differences between control and p46-transfected cells were found to be significant by Student’s t-test \((P < 0.001)\), strongly suggesting a role for p46 in mitosis exit failure. Since Pax6 encodes proteins that differ in their DNA-binding domains through alternative splicing and internal initiations, we tested whether all splice variants would induce a similar mitosis failure. As shown in Figure 8, the percentage of cells exhibiting DNA bridges was slightly higher than in the control cells, but was significantly lower for all the splice variants when compared to p46. p48DsRed, exhibiting an insertion of 14 amino acids in the paired domain, was inefficient in the induction of the mitosis failure, suggesting that an intact p46 paired domain is needed to perturb mitosis efficiently and not the homeodomain or the transactivation domain of the protein. To verify this point, we used the \(p46del170-342\) mutant lacking the homeodomain and most of the transactivation domain fused, as for p46, to the DsRed protein. The effect of transfecting HeLa and HEK with a vector encoding the \(p46del170-342\) transcription-dead protein was similar to that of transfecting with p46DsRed vector, resulting in the same mitosis failure (Fig. 9H). \(p46del170-342\) was found indistinguishable from p46 (Figs. 9A–C) with respect to localization in the pericentromeric area of metaphase chromosomes; the \(p46del170-342\) signal was found adjacent to CENP-F kinetochore \((H)\) immunolabeling (Figs. 9D–G). Therefore, we observed a positive correlation between mitosis exit failure and overexpressed Pax6 protein on the pericentromeric chromosomal area through its paired domain.

**Effect of Overexpression of Pax6 on Sister Chromatid Separation**

We performed experiments to follow in vivo mitotic progression and chromosome dynamics in live cells after transfection of HEK cells with the p46DsRed splice variants, to define the mitosis step affected by p46DsRed. Most of the cells overexpressing Pax6 did not divide during the observation time (at least 10 hours). One particular mitosis exit defect is presented in Figure 10A. All the chromosomes correctly aligned at the metaphase plate before anaphase onset (see 2 hours and 26 minutes in Fig. 10A). Separation of the chromatids was not complete, and none of the lagging chromosomes we observed were incorporated in the daughter nuclei during nuclear envelope reformation, but instead migrated to just one of the daughter nuclei (Fig. 10A). In Figure 10B, the chromosomes were correctly aligned and metaphase plate formation had occurred (Fig. 10B; 28 minutes). At the anaphase step, the chromatids failed to separate, and a strong enrichment of p46 protein was visible as bright spots on both sides of the chromosomes. These observations suggest that the DNA bridges derive from the inability of the chromatids to separate after correct alignment on the metaphase plate. This event eventually leads to aneuplody in the daughter cells.

Finally, we performed an experiment to test the possibility that p46 directly targets ESPL1 itself. As shown in Figure 11, a direct interaction between the p46 paired domain and human ESPL1 was demonstrated by a GST pulldown experiment. This experiment was performed using lysates from...
V5-tagged ESPL1-transfected cells incubated with GST-fusion proteins bound to glutathione-agarose beads. ESPL1 recovered after extensive washing was revealed with V5 antibodies.

**DISCUSSION**

Indirect immunofluorescence and GFP- or DsRed-labeled Pax6 proteins showed a substantial proportion of p46 to be associated with pericentromeric heterochromatin, and overexpression of p46 resulted in a mitosis defect. This effect was specific to the p46 protein, since overexpression of other Pax6 splice variants did not significantly hamper mitosis. Similar patterns of protein localization in the pericentromeric chromosome area have been reported for several proteins that play multiple roles in modifying chromosomal structure and gene expression. GAGA factor, a product of the Trithorax-like locus, is associated with pericentromeric heterochromatin in diploid...
cells and plays a role in chromosome condensation and segregation.\textsuperscript{37} ATRX, a member of the helicase/ATPase SNF2 family, bears a plant homeodomain zinc finger in its N-terminal region that is essential for its association with pericentromeric heterochromatin and with HP1\textsubscript{α}/H9251.\textsuperscript{38} Tal1/SCL, a lineage-specific bHLH transcription factor, binds to pericentromeric DNA and increases H3K9me3 and HP1\textsubscript{α}/H9251 association and transcription repression.\textsuperscript{39} YY1 a transcription factor belonging to a polycomb group of proteins involved in the repression of homeotic genes, binds to pericentromeric heterochromatin and colocalizes with HP1\textsubscript{α}.\textsuperscript{40}

Centromeres are fundamental to eukaryotic biology. Centromeric DNA attaches to spindle microtubules through the proteinaceous kinetochore. The outer kinetochore assembles when the kinetochore condenses and microtubules attach to it for mitosis, and it disassembles after chromosomes have segregated. The inner kinetochore remains with the DNA throughout the cell cycle and consists of pericentromeric chromatin and associated proteins. Centromeric DNA comprises long stretches of short tandem repeat satellite DNA sequences that are found only there and in surrounding pericentric heterochromatin, but without conserved sequence across species.\textsuperscript{41} Genomic integrity relies on the cell cycle-dependent deposition of protein complexes that mediate association, precise alignment, and efficient packaging of sister chromatids after replication, the most important being the evolutionarily conserved RAD21 and condensin complexes.\textsuperscript{12} Numerous observations suggest that an intimate relationship exists between the formation and function of the centromere-kinetochore and the establishment and maintenance of sister chromatid cohesion. We observed an interaction between Pax6 and ESPL1. It is therefore possible that p46 inhibits the function of ESPL1 in a

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure9.png}
\caption{Chromosomal localization and statistical analysis showed p46del170-342 localization in HeLa nuclei to be similar to that of Pax6p46 protein, suggesting that Pax6 localization in the pericentromeric area is strictly dependent on the paired domain of the protein. (A–C) Analysis of chromosomes derived from transfected HeLa cells with plasmid expressing p46del170-342 protein shows that the localization of the mutant form is organized in doubled-spotted areas, as is that described for Pax6p46 protein fused to DsRed. Paired containing protein is detected with specific anti-paired serum 11\textsuperscript{15} (A). DNA was labeled with DAPI (B). Arrows: p46del170-342 localization in greater detail. (D–G) p46del170-342 protein detected (D), as the Pax6p46DsRed form, localizes in an area close to the kinetochore region as detected by the use of the CENP-F (E) antibody. (H) Counting of adjacent cells determined that the mutant form p46del170-342 is as competent as Pax6p46 in causing the formation of DNA bridges. Scale bar, 5 μm.}
\end{figure}
similar manner to the action of securin (PTTG1). p46DsRed are more efficient in the induction of mitosis defect than the EGFP-tagged or wild-type p46. Clearly, the other Pax6 proteins fused with DsRed do not induce significant mitosis defect. Thus, DsRed only is not responsible for the observed defect.

Since tetramerization through DsRed is possible, we favor the hypothesis that, with this p46 chimera, the amount of protein at the pericentromeric area of the chromosome is increased with respect to the amount of p46EGFP or wild-type p46 found, and therefore, that the function of ESPL1 is more efficiently inhibited. Both paired and homeodomain were found to interact with ESPL1, suggesting that the high rate of mitosis defect induced by p46 is most probably due to the greater accumulation of p46 in the pericentromeric area than for the other proteins. Of interest, in ESPL1-deficient mouse embryonic fibroblasts abnormal mitosis reminiscent of the defects observed in p46-expressing cells was also observed.43

Centromeric regions are organized by hypoacetylated nucleosomes, hypermethylated on lysine 9 of histone H3 (H3K9me3).44 The latter feature accounts for the presence of chromo-domain–containing heterochromatin proteins that bind H3K9me3 at the centromere,30,31,45 and these proteins have been implicated in the recruitment and/or stabilization of centromere-proximal RAD21.46,47 We observed that p46 localizes at the pericentromeric region in the condensed chromosomes and that most of the p46-expressing cells were unable to separate their DNA at the end of mitosis. A colocalization between Pax6p46 and HP1 suggests that p46 accumulated in the pericentromeric area may perturb the function of HP1 and lead to the observed mitosis defect. Indeed, this phenotype is reminiscent of a loss-of-function phenotype of Drosophila HP1.48 Since the p46 paired domain efficiently perturbs mitosis, this effect may depend either on the DNA-binding properties of this domain or alternatively on the paired protein–protein interaction interface. Indeed, a mutant of p46 devoid of homeodomain and most of the transactivation domain still exhibits a biological

**FIGURE 10.** (A, B) Video microscopy has been used to follow the formation of DNA bridges in HEK cells transfected with plasmid over-expressing Pax6p46. (A) Images obtained at different times demonstrate that DNA bridges are identified in cells that later divide by entering metaphase within the period of live observation (2–26 hours). If all lagging chromosomes appeared in cells in which all the chromosomes successfully aligned at the metaphase plate before anaphase onset (see 2 hours and 26 minutes), none of the lagging chromosomes observed were correctly split in the two daughter nuclei, but instead migrated in just one of the daughter nuclei causing the production of one anuclear cell.

Video microscopy (B) shows that although the chromosomes are correctly aligned and metaphase plate formation has occurred (28 minutes) at the anaphase step, the chromatids fail to separate, and a strong enrichment of p46 protein is observed as bright spots on both sides of chromosomes that fail to separate (see enlargements, 42 and 44 minutes), causing the production of aneuploid daughter cells.

**FIGURE 11.** Detection of interaction between Pax6p46 and human ESPL1. HEK cells were transfected with pIND/ESPL1 V5-HIS. After 24 hours, cells were lysed, and the cell lysate was incubated with individual GST fusion proteins bound to glutathione beads, as indicated at the top of the figure. The proteins recovered after extensive washing were then analyzed by Western blot with anti-v5 monoclonal antibody. As previously shown, wild type ESPL1 after 12 to 48 hours of expression appears as two polypeptide bands: a full protein (220 kDa) and a 65-kDa fragment.
activity in vivo, suggesting that protein–protein interactions are still operative with this disabled molecule. We observed that p46DsRed was able to bind in the pericentromeric area of quail retinal cells and was able to induce a mitosis defect similar to that found in the human cell lines HeLa or HEK. Since the DNA sequence between human and quail species was not expected to be conserved in this chromosome area, we favor the hypothesis that protein–protein interactions are involved, most probably including ESPL1 and HP1α. Expression of p46 in CenpB-GFP U2OS cells resulted in larger accumulation of CenpB (a centromeric satellite DNA-binding protein) than in nontransfected or p30-expressing cells. This finding suggests that p46 binding to the pericentromeric area modifies CenpB recruitment to the chromosome. The direct interaction between p46 and Cenp-B remains to be investigated. We have observed that endogenous Pax6 is present on the chromosomes of proliferating retina cells, and similarly located in transiently transfected HeLa cells, as judged from DCTN1 co-labeling. Clearly, the function of Pax6 is not to perturb mitosis during neural cell division. The deleterious effect observed on p46 overexpression may reflect a checkpoint to control accidental overexpression of this transcription factor and associated proteins deleterious for normal development. This may explain why overexpression of Pax6 results in abnormal eye development as the aneuploid cells obtained after mitosis suffered from a proliferation-inducing mutation, the accumu-

Clonal analysis on oligodendrocyte precursor cells suggests that an intrinsic clock operates within each cell to help control when it stops dividing and differentiates. It is probable that a similar internal clock, whose mechanism may be influenced by extracellular signals, acts to determine the rate of division and differentiation of neural progenitors. The results we present suggest that Pax6 may be an essential component through its control of mitosis. Regulating the rate of progression through the cell cycle may be a primary function of Pax6 in early neuronal progenitors, since in the absence of Pax6, the cell cycle is shortened. Because of its long (12-hour) half-life and particular chromosomal location, Pax6 may be an internal clock measuring the number of mitoses. As soon as a critical threshold of p46 is reached on the chromosomes, mitosis is no longer possible, and differentiation may occur. If such a ne-

Acknowledgments

The authors thank Jim Dompiere and Frederic Coquelle for helpful discussions; Oceane Anzano for technical help; Olivier Albarghi, who first pointed out and evidenced the colocalization between overexpressed pax6(p46) and centromeric heterochromatin; and Edward Tate for suggestions on the manuscript.


