Sumoylation activates the transcriptional activity of Pax-6, an important transcription factor for eye and brain development

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Pax-6 is an evolutionarily conserved transcription factor regulating brain and eye development. Four Pax-6 isoforms have been reported previously. Although the longer Pax-6 isoforms (p46 and p48) bear two DNA-binding domains, the paired domain (PD) and the homeodomain (HD), the shorter Pax-6 isoform p32 contains only the HD for DNA binding. Although a third domain, the proline-, serine- and threonine-enriched activation (PST) domain, in the C termini of all Pax-6 isoforms mediates their transcriptional modulation via phosphorylation, how p32 Pax-6 could regulate target genes remains to be elucidated. In the present study, we show that sumoylation at K91 is required for p32 Pax-6 to bind to a HD-specific site and regulate expression of target genes. First, in vitro-synthesized p32 Pax-6 alone cannot bind the P3 sequence, which contains the HD recognition site, unless it is preincubated with nuclear extracts precleared by anti–Pax-6 but not by anti-small ubiquitin-related modifier 1 (anti-SUMO1) antibody. Second, in vitro-synthesized p32 Pax-6 can be sumoylated by SUMO1, and the sumoylated p32 Pax-6 then can bind to the P3 sequence. Third, Pax-6 and SUMO1 are colocalized in the embryonic optic and lens vesicles and can be coimmunoprecipitated. Finally, SUMO1-conjugated p32 Pax-6 exists in both the nucleus and cytoplasm, and sumoylation significantly enhances the DNA-binding ability of p32 Pax-6 and positively regulates gene expression. Together, our results demonstrate that sumoylation activates p32 Pax-6 in both DNA-binding and transcriptional activities. In addition, our studies demonstrate that p32 and p46 Pax-6 possess differential DNA-binding and regulatory activities.

retina | lens | cataract | small eye mutation | pancreas

Pax-6 belongs to the Pax transcription factor family, which contains nine members that share a conserved paired domain (PD) and a homeodomain (HD) (1, 2). This evolutionarily conserved transcription factor acts high in the regulatory hierarchy, controlling eye and brain development in humans, mice, zebrafish, and *Drosophila* (1–6). The highly conserved amino acid sequence of Pax-6 proteins in different species suggests its critical function in regulating development of these organisms. Indeed, targeted expression of Pax-6 from a general promoter in *Drosophila* induces formation of ectopic compound eyes (7). Furthermore, haploinsufficiency or deletion of the Pax-6 gene causes many ocular diseases including aniridia, cataracts, and glaucoma. A homozygous mutation in Pax-6 is lethal at birth, with severe brain defects and absence of eyes and nose in humans and mice (4, 5).

At the molecular level, Pax-6 functions primarily to mediate the commitment of the ectoderm above the optic vesicle into the lens ectoderm and also to promote formation of the lens vesicle (6). Pax-6 controls transcriptional expression of genes encoding both transcription factors responsible for lens development, such as musculoaponeurotic fibrosarcoma (*L-maf*), SRY-box containing gene 1 (*Sox-1*), prospero homeobox 1 (*Prox-1*), and also lens structural proteins, such as α-, β-, and γ-crystallins (8–11). Previous studies have demonstrated the presence of at least four forms of Pax-6 in quail cellular extracts with molecular masses of 48, 46, 43, and 32 kDa, which were named "p48," "p46," "p43," and "p32," respectively (12). These isoforms vary from each other in their N-terminal structures. The common p46 isoform has a 128-amino acid PD at the N terminus and a 56-amino acid HD in the central region which bind specifically to different DNA sequences, P6CON and P3 (TAAT/ATTA), respectively (12–14). At the C terminus, a conserved domain rich in proline (P), serine (S), and threonine (T) residues exists in all Pax-6 isoforms and thus is named the "PST" domain (Fig. S1*A*). This domain mediates activation of Pax-6 through phosphorylation by p38 MAP kinase and homeodomain-interacting protein kinase 2 (15, 16). Several phosphorylation sites have been identified in the PST domain of human and zebrafish Pax-6. Our recent studies have shown that both p32 and p46 Pax-6 isoforms are subjected to negative regulation by protein serine/threonine phosphatase-1 (PP-1) in vitro and in vivo (17). However, the obvious absence of a PD domain in the N terminus might endow p32 Pax-6 with a DNA-binding specificity different from that of p46 Pax-6; hence, it may modulate different downstream genes. In this investigation, we have explored how p32 Pax-6 can bind to the HD-specific P3 sequence and exert its regulatory function.

The small ubiquitin-related modifier (SUMO) family of proteins is an important class of ubiquitin-like proteins that share a common ancestry, structural fold, and a core conjugation pathway but have distinct sequences and functional properties (18, 19). SUMO is structurally related to ubiquitin and also is ligated to lysine residues within its target proteins. In sumoylation, the target lysine generally falls within a recognizable consensus, namely ψ-Lys-X-Glu/Asp (where ψ is a large hydrophobic amino acid, most commonly isoleucine or valine, and X is any residue). The enzymatic machinery that adds and removes SUMO includes SUMO E1-activating enzyme AOS1/UBA2, SUMO E2 conjugation enzyme UBC9, and SUMO E3 ligase (18, 19). Sumoylation of target proteins is implicated in protein–protein interactions, subcellular localization, protein–DNA interactions, transcriptional activation and repression, and enzyme activity (18, 19). Three SUMO family members, SUMO1, -2, and -3, have been identified

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in mammals. Like ubiquitin, SUMO2 and SUMO3 can form polymeric chains. In contrast, SUMO1 is a 97-amino acid polypeptide of 11.5 kDa that is covalently monoconjugated to proteins at lysine residues, thereby playing a critical role in many cellular processes, as mentioned earlier, through rapidly reversible changes in sumoylation status (18, 19).

In the present study, we provide evidence showing that sumoylation acts as a posttranslational modification that activates p32 Pax-6 and that sumoylated p32 Pax-6 positively regulates expression of the target genes. Moreover, we demonstrate that sumoylation of p32 Pax-6 occurs in vivo mainly at embryonic day (ED) 9.5 and ED 11.5, possibly playing an important role in controlling eye and brain development of vertebrates. Finally, we show that the p32 and p46 Pax-6 isoforms display differential DNA-binding and regulatory activities and thus can regulate different downstream target genes.

Results

Various Pax-6 Isoforms Are Present in Mouse Eye Tissues and Human Ocular Cell Lines. Previous studies have shown that four different Pax-6 isoforms (p48, p46, p43, and p32) are present in quail (12) (Fig. S1*A*). To determine if these Pax-6 isoforms are also present in mammals, we examined their expression in developing and adult mouse eyes and human ocular cell lines with three different antibodies (Fig. S1*B*). The eye tissues of newborn mice contain all four Pax-6 isoforms (Fig. 1*A*). Although p46 and p32 Pax-6 are the major isoforms, a considerable amount of p48 was detected also. Among the four isoforms, p43 was the least abundant and was detectable only in the immunoprecipitated Pax-6 mixture (Fig. 1*A*). RT-PCR revealed that p43 in mice and humans was not derived from alternative splicing (Fig. S2). In eye tissues from adult mice, significant amounts of p46 and p32 Pax-6 were present in the retina and cornea (Fig. S1*C*). In contrast, a much reduced level of p43 was observed in these tissues. In the lens epithelium, p46 Pax-6 was relatively abundant, but p32 and p43 Pax-6 isoforms were barely detectable (Fig. S1*C*). A thorough analysis of Pax-6 in four human ocular cell lines (20, 21) revealed that p46, p43, and p32 were all present in these cells (Fig. S1*D*).

Among different eye tissues and cell lines, p32 and p46 Pax-6 are the two major Pax-6 isoforms. Therefore, we tested the transcriptional activities of these two isoforms on the αB-crystallin promoter (−427 to +44) containing four copies of Pax-6 binding sites (22). As shown in Fig. 1*B*, both p32 and p46 displayed significant amounts of transcriptional activity on the αB-crystallin gene promoter.

In Vitro-Translated Pax-6 Proteins Lack P3 Recognition Ability. Next, we studied the DNA-binding activities of p46 and p32 Pax-6. To do so, we synthesized wild-type p32 and p46 using a cell-free system (Fig. 1*C*). In addition, to examine whether phosphorylation at the five identified sites (Fig. S1*E*) modulates the DNA-binding ability of the two major Pax-6 isoforms, we also synthesized p32-5A and p46-5A Pax-6 (mimicking constant dephosphorylation) and p32-5D and p46-5D Pax-6 (mimicking constant phosphorylation) (Fig. 1*C*, *Upper*). Western blot analysis revealed that the wild-type and mutant proteins displayed some differences in their electrophoretic mobility (Fig. 1*C*, *Lower*). In addition, two bands (p46 and p32) were always generated when the cDNA encoding p46 Pax-6 was translated (Fig. 1*C*). Subsequently, we tested the DNA-binding activities of the two major Pax-6 isoforms, in either wild-type or mutated status, using EMSA. Nuclear extracts from fetal human lens epithelial (FHL-124) cells, telomerase-transfected human lens epithelial (T-HLE) cells, and human lens epithelial (HLE) cells were included for comparison. As shown in Fig. 1*D*, although the nuclear extracts bound the P3 sequence, presumably because of the presence of endogenous Pax-6, none of the in vitro-synthesized proteins displayed P3-binding activity. This result suggests that in vitro-synthesized Pax-6 needs to be modified further to recognize the P3 sequence. Because neither 5A nor 5D displayed P3-binding activity, the modification is unlikely to be a result of phosphorylation or dephosphorylation.

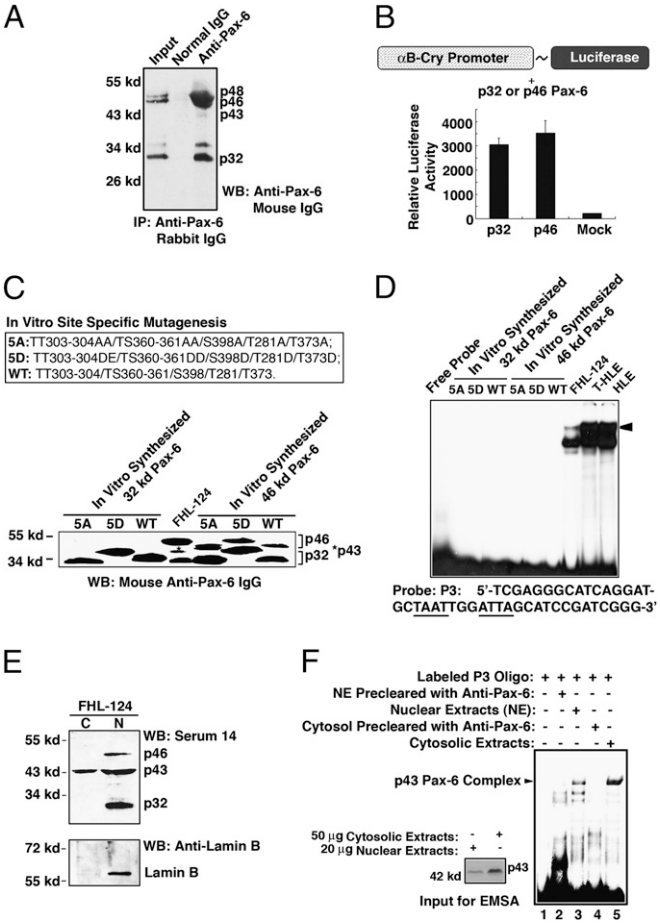

Fig. 1. Detection of four Pax-6 isoforms and exploration of DNA-binding and transcriptional activities of p32 and p46 Pax-6. (*A*) Detection of different Pax-6 isoforms in newborn mouse eyes. Note that p46 and p32 are present in relatively high levels. In contrast, p43 is much reduced. (*B*) Both p46 and p32 Pax-6 isoforms positively regulate the expression of αB-crystallin as tested in FHL-124 cells. (*C*) (*Upper*) In vitro site-specific mutagenesis-generated mutants mimicking constant dephosphorylation (5A) or phosphorylation (5D) in human Pax-6 (WT). (*Lower*) Western blot analysis of in vitro-generated mutant or wild-type p32 and p46 Pax-6 proteins; note their differential electrophoretic mobilities. In addition, the mutant and wild-type 46-kDa Pax-6 plasmids generate both p46 and p32 because of the activation of an internal ATG initiator. (*D*) In vitro-synthesized p32 and p46 Pax-6 cannot bind to the P3 sequence. In contrast, nuclear extracts from HLE cells and the P3 sequence form three DNA–protein interacting complexes. The arrowhead designates the complex derived from Pax-6 and the P3 sequence (see legend of Fig. 2 for explanation). (*E*) Western blot analysis of different Pax-6 isoforms in cytosolic (C) and nuclear (N) extracts of FHL-124 cells. (*F*) EMSA demonstrating that p43 Pax-6 from FHL-124 cytosol and the P3 sequence form a strong interacting complex. The relative amounts of nuclear and cytosolic p43 Pax-6 are shown in the Western blot.

The Cellular 43-kDa Pax-6 Isoform Can Bind to the P3 Sequence. To understand the binding patterns exhibited by the nuclear extracts of HLE cells and to explore what kind of modification is necessary for binding to the P3 sequence, we further examined the Pax-6 expression profile in the nucleus and cytoplasm of FHL-124 cells. As shown in Fig. 1*E*, although FHL-124 nuclear extracts contained three Pax-6 isoforms, p46, p43, and p32, the cytoplasm contained only a single isoform, p43. When we conducted EMSA with nuclear extracts of FHL-124 cells and the P3 sequence (Fig. S3*A*), three DNA–protein complexes were detected (Fig. S3*B*, lane 2). Furthermore, when mouse Pax-6 antibody was used to remove the endogenous Pax-6 from FHL-124 nuclear extracts, the only band depleted was the one with the slowest mobility and weakest P3

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binding (Fig. S3*B*, lane 3). These experiments suggest that the other two complexes were derived from non–Pax-6 proteins.

To identify which Pax-6 isoform was responsible for binding to the P3 sequence, we conducted EMSA using cytosolic extracts of FHL-124 cells. As shown in lane 5 of Fig. 1*F*, the p43 Pax-6 in the FHL-124 cytosol exhibited strong binding to the P3 sequence. This complex displayed similar mobility to that of the top complex formed by the nuclear extracts and the P3 sequence (compare lane 3 and lane 5, Fig. 1*F*), which was inhibited by preclearing with the mouse anti–Pax-6 antibody (Fig. 1*F*, lane 2). Together, these experiments strongly suggest that p43 Pax-6 is responsible for binding to the P3 sequence.

In Vitro-Translated p32 Pax-6 Binds to P3 After Preincubation with Pax-6–Depleted Nuclear Extracts. To further explore what kind of modification is necessary for the in vitro-synthesized Pax-6 to bind to the P3 sequence, we combined in vitro-translated wild-type p32 Pax-6 protein with Pax-6–depleted FHL-124 nuclear extracts. After incubation for 1 h at 30 °C, the mixture was analyzed by EMSA. As shown in lane 5 of Fig. 2*A*, the mixture exhibited a strong interaction with the P3 sequence, forming a DNA–protein complex with the same migration position as the complex formed by the FHL-124 nuclear extracts and the P3 sequence (Fig. 2*A*, lane 2). Thus, a modification of the in vitro-synthesized p32 Pax-6 by the Pax-6–depleted FHL124 nuclear extracts most likely conferred the ability to bind to the P3 sequence.

We then examined whether phosphorylation/dephosphorylation would modulate the binding of p32 Pax-6 to the P3 sequence using synthesized p32 mutant proteins mimicking constant dephosphorylation (5A) or constant phosphorylation (5D) (Figs. 1*C* and 2*B*) to conduct EMSA. As shown in Fig. 2*C*, after incubation with the Pax-6–depleted nuclear extracts, the 5A mutant demonstrated a reduced (27% less) P3-binding ability compared with wild-type (WT) p32 Pax-6. In contrast, the 5D mutant exhibited enhanced (63% more) P3-binding ability. As mentioned earlier (Fig. 1*D*), none of the in vitro-translated proteins could bind to the P3 sequence on their own. Thus, phosphorylation/dephosphorylation may further modulate binding to the P3 sequence after the proteins are modified and enabled to bind P3 by the nuclear extracts.

In Vitro-Translated p46 Pax-6 Cannot Bind to the P3 Sequence even After Mixing with the Pax-6–Depleted FHL124 Nuclear Extract. To test whether in vitro-generated p46 Pax-6 also can gain the ability to bind P3 after mixing with Pax-6–depleted FHL-124 nuclear extracts, we combined in vitro-translated p46 and p32 Pax-6 proteins with the Pax-6–depleted FHL-124 nuclear extracts. After incubation for 1 h at 30 °C, the mixture was analyzed by EMSA with the P3 sequence. As shown in lanes 2–4 of Fig. S3*C*, the mixture of p46 and p32 Pax-6 yielded a strong P3-binding complex after incubation with the Pax-6–depleted nuclear extracts. This complex migrated to a position similar to that of the top complex generated by the FHL-124 nuclear extracts and the P3 sequence (Fig. S3*C*, lane 6). This binding activity was inhibited by immunoprecipitation of the in vitro-translated protein with serum 14 but not serum 11 (Fig. 2*D*). As shown in Fig. 2*E*, serum 11 precipitated only p46, whereas serum 14 precipitation depleted both p46 and p32. Thus, only p32 can bind to the P3 sequence after incubation with the Pax-6–depleted nuclear extracts.

Sumoylation Converts p32 Pax-6 into p43 Pax-6 and Endows It with P3-Binding Ability. The fact that p43 Pax-6 from either the cytosol or the nucleus of FHL-124 cells can bind to P3 oligo, whereas p32

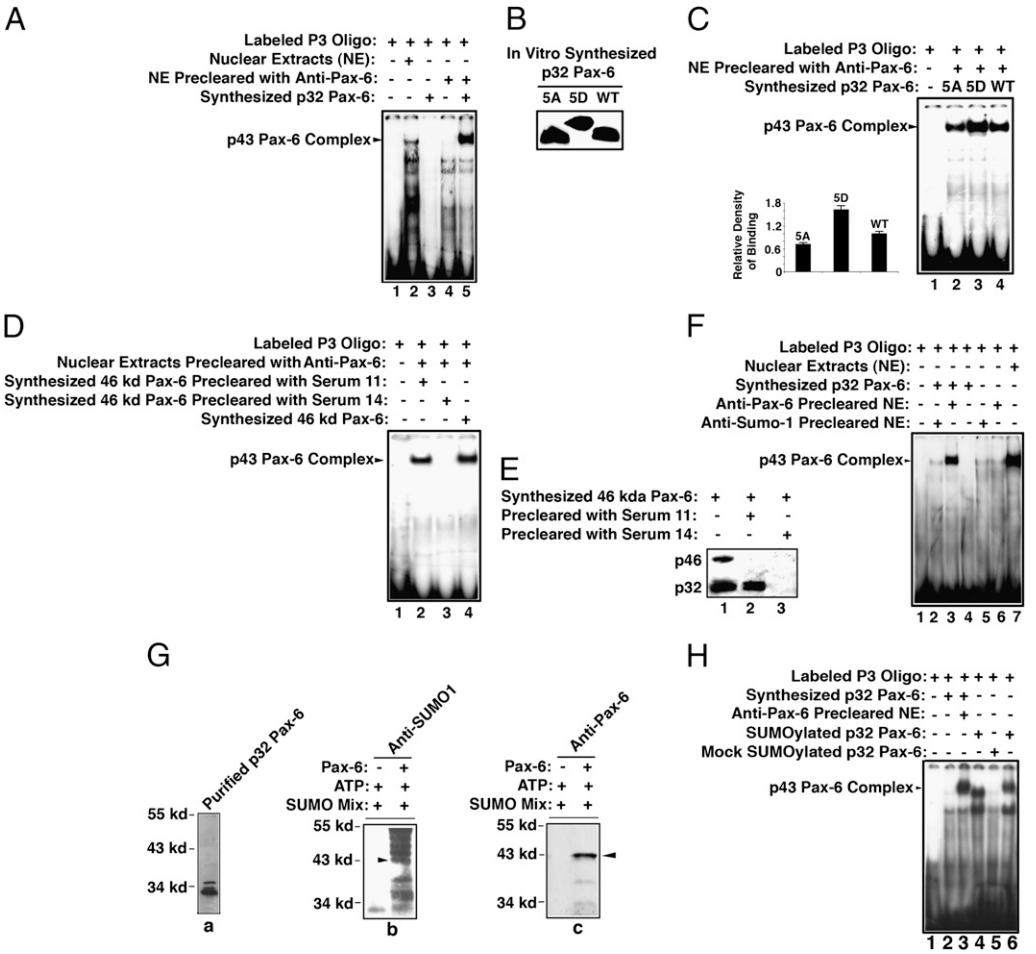

Fig. 2. Demonstration that sumoylation of the p32 Pax-6 activates its DNA-binding activity. (*A*) Gel mobility-shifting assay showing that in vitro-translated p32 Pax-6 can bind to the P3 sequence only after preincubation with Pax-6–depleted FHL-124 nuclear extract. (*B*) Western blot analysis indicating the amounts of in vitro-synthesized mutant and wild-type p32 Pax-6 used for EMSA described in *C*. (*C*) EMSA demonstrating that phosphorylation and dephosphorylation modulate DNA binding of p32 Pax-6 to the P3 sequence. (*D*) EMSA demonstrating that p32 but not p46 Pax-6 binds to the P3 sequence. (*E*) Western blot analysis of in vitro-synthesized p46 and p32 proteins from Pax-6 full-length cDNA without (lane 1) or with preclearance by serum 11 (lane 2) or by serum 14 (lane 3). (*F*) EMSA demonstrating that sumoylation is necessary for p32 Pax-6 to bind to the P3 sequence. (*G*) Demonstration that p32 Pax-6 can be sumoylated by SUMO1 in vitro. (*a*) The in vitro-generated GST–p32 Pax-6 fusion protein was purified by thrombin cleavage and GST column. (*b* and *c*) Purified p32 Pax-6 was subjected to sumoylation with a kit from Biomol (UW8955). After sumoylation, the reaction products were identified by anti-SUMO1 (*b*) or anti–Pax-6 antibodies (*c*). Arrowheads indicate p43 Pax-6. (*H*) EMSA demonstrating that sumoylation of p32 Pax-6 in vitro activates its DNA binding to the P3 sequence.

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Pax-6 can bind to the P3 sequence only if it is preincubated with the Pax-6–depleted nuclear extract, suggests that p32 Pax-6 may be converted to p43 Pax-6 by the extract. Among different protein modifications, sumoylation could add a peptide of about 11 kDa to p32 Pax-6 and generate a 43-kDa Pax-6. To explore this possibility, we searched the putative SUMO acceptor sites in p32 Pax-6 and identified K91 as a putative site (Fig. S4*A*). Western blot analysis of the total FHL-124 proteins by anti-SUMO1 or anti–Pax-6 antibodies identified the same 43-kDa Pax-6 band (Fig. S4*B*). Moreover, the strong binding complex generated by in vitro-synthesized p32 and the P3 sequence in the presence of the Pax-6–depleted FHL124 nuclear extracts was diminished when the mixtures were precleared with a SUMO1 antibody (Fig. 2*F*, lane 2). Together, these results suggest that sumoylation converts p32 Pax-6 into p43 Pax-6 and endows p32 Pax-6 with P3-binding ability.

In Vitro-Synthesized p32 Pax-6 Can Be Sumoylated into p43 Pax-6. To confirm that p32 indeed can be sumoylated into p43 Pax-6, we conducted an in vitro sumoylation reaction with purified p32 Pax-6 (Fig. 2*Ga*). As shown in Fig. 2*G b* and *c*, the sumoylated mixture contained a 43-kDa protein, which was detected by both anti-SUMO1 (except for p43, other bands detected in Fig. 2*Gb* seem to be derived from oligomerization of SUMO1 itself; see ref. 23) and anti–Pax-6 antibodies, suggesting that sumoylation indeed converts p32 Pax-6 into p43 Pax-6. Next, we confirmed that sumoylated p32 can bind to the P3 sequence. As shown in lanes 4 and 6 of Fig. 2*H*, the in vitro-sumoylated p32 Pax-6 exhibited strong P3-binding activity, but the mock sumoylation without ATP did not yield any P3 binding (Fig. 2*H*, lane 5).

Sumoylation Enhances p32 Pax-6 DNA-Binding and Transcription Activities. To examine the importance of sumoylation of p32 Pax-6 by SUMO1, we conducted several lines of experiments. First, we tested if a mutation in the sumoylation site (K91R) would change its P3-binding ability. As shown in lanes 3 and 4 of Fig. 3*A*, compared with wild-type p32 Pax-6, the K91R mutation significantly reduced binding to the P3 sequence in the presence of the Pax-6–depleted nuclear extracts. The presence of some DNA binding in the K91R mutant p32 suggests the possible existence of other sumoylation sites. However, mutation of the only other putative sumoylation site, K110, had no effect on p32 Pax-6 DNA binding (Fig. 3*B*, lane 8). EMSA results with K91R p32 Pax-6 and P3 oligo alone also ruled out the possibility that the K91R mutation itself may activate its DNA binding (Fig. S5). Thus, the small amount of DNA binding by K91R could be from background, because it is not always present (Fig. S5), or from sumoylation in an unknown nonconsensus site. In addition, we found that the N terminus of p32 Pax-6 is necessary for its DNA binding (Fig. 3*B* and Fig. S5). Second, we explored sumoylated p32 regulation on the exogenous gene. Transfection of the wild-type p32 significantly increased the reporter gene activity driven by a minipromoter containing three copies of the P3 sequence (Fig. 3*C*). Cotransfection of SUMO1 further enhanced the reporter gene activity (Fig. 3*C*). In contrast, transfection of the K91R p32 without or with cotransfection of

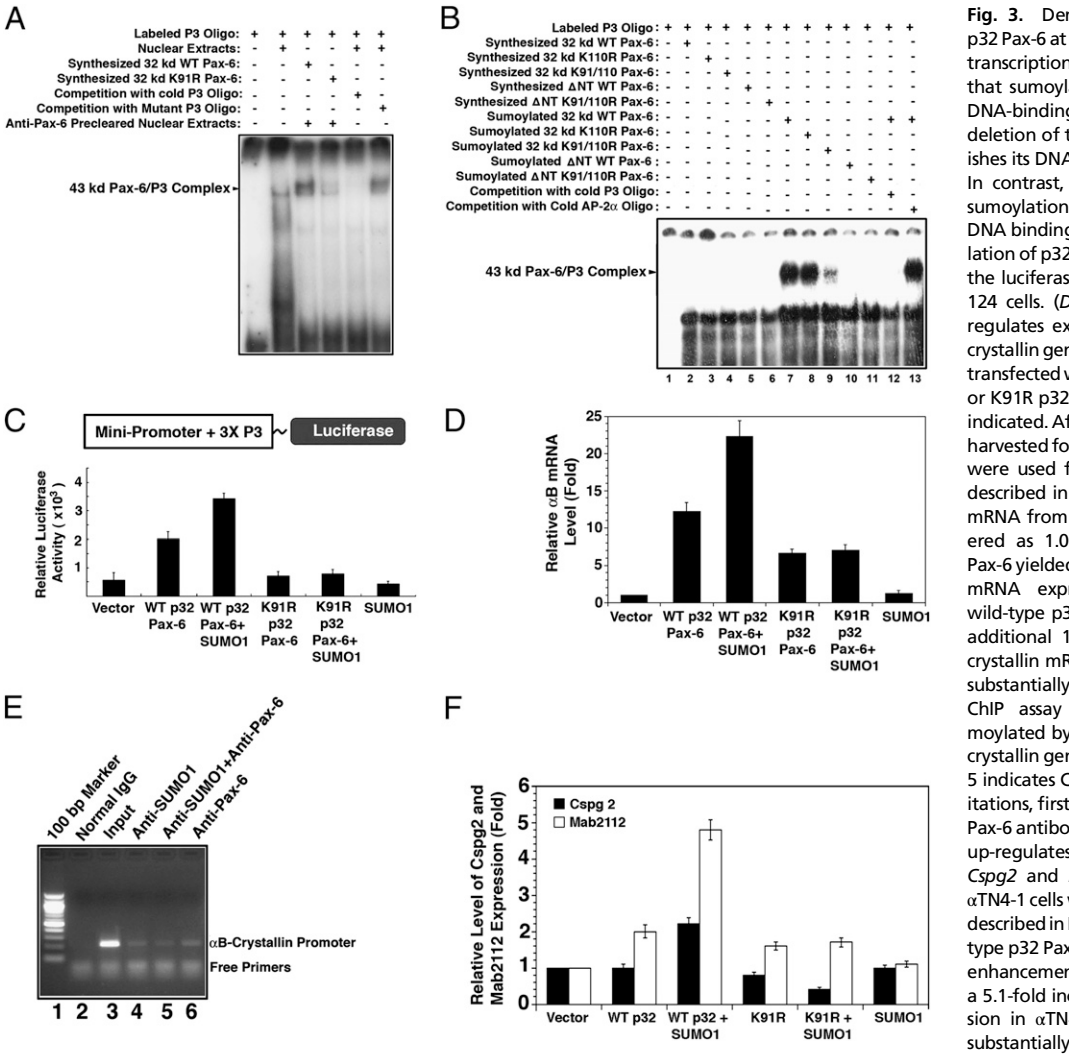

Fig. 3. Demonstration that sumoylation of p32 Pax-6 at K91 activates its DNA-binding and transcriptional activities. (*A*) EMSA showing that sumoylation at K91 activates p32 Pax-6 DNA-binding activity. (*B*) EMSA showing that deletion of the N terminus of p32 Pax-6 abolishes its DNA-binding activity (lanes 5 and 10). In contrast, mutation in the other putative sumoylation site, K110, has no effect on the DNA binding of p32 Pax-6 (lane 8). (*C*) Sumoylation of p32 Pax-6 activates its transactivity on the luciferase reporter gene as tested in FHL-124 cells. (*D*) Sumoylation of p32 Pax-6 up-regulates expression of the endogenous αB-crystallin gene in ARPE cells. ARPE-19 cells were transfected with vector alone or with wild-type or K91R p32 Pax-6 with or without SUMO1 as indicated. After 36 h, the transfected cells were harvested for preparation of total RNAs, which were used for quantitative real-time PCR as described in *Methods*. Note that αB-crystallin mRNA from vector-transfected cells is considered as 1.0. Transfection of wild-type p32 Pax-6 yielded a 12-fold increase in αB-crystallin mRNA expression. Cotransfection of the wild-type p32 Pax-6 with SUMO1 leads to an additional 11-fold enhancement of the αB-crystallin mRNA expression. The K91R mutant substantially decreased its transactivity. (*E*) ChIP assay demonstrating that Pax-6 sumoylated by SUMO1 binds directly to the αB-crystallin gene promoter in ARPE-19 cells. Lane 5 indicates ChIP result from sequential precipitations, first by anti-SUMO1 and then by anti–Pax-6 antibodies. (*F*) Sumoylation of p32 Pax-6 up-regulates expression of the endogenous *Cspg2* and *Mab2112* genes in αTN4-1 cells. αTN4-1 cells were transfected and processed as described in Fig. 3*D*. Cotransfection of the wild-type p32 Pax-6 with SUMO1 leads to a 2.2-fold enhancement of *Cspg2* mRNA expression and a 5.1-fold increase of *Mab2112* mRNA expression in αTN4-1 cells. The p32 K91R mutant substantially decreased its transactivity.

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SUMO1 yielded only a weak increase in the reporter gene activity (Fig. 3*C*). Next, we cotransfected wild-type or K91R p32 with or without SUMO1 into human retinal pigment epithelial (ARPE-19) cells and measured the mRNA expression level of the endogenous αB-crystallin gene in the transfected cells using quantitative real-time PCR. As shown in Fig. 3*D*, transfection of the wild-type p32 Pax-6 into ARPE cells significantly increased the mRNA expression level of the αB-crystallin gene. Furthermore, cotransfection of SUMO1 cDNA with wild-type p32 Pax-6 cDNA led to further up-regulation of αB-crystallin mRNA. In contrast, mutation at K91 caused a substantial decrease in p32 Pax-6 transactivity. A ChIP assay further confirmed that sumoylated Pax-6 binds directly to the αB-crystallin gene promoter in ARPE-19 cells (Fig. 3*E*). Finally, we cotransfected wild-type or K91R p32 with or without SUMO1 into mouse lens αTN4-1 cells and measured the mRNA expression levels of two additional Pax-6 target genes, *Cspg2* and *Mab21l2*. Sumoylation of p32 Pax-6 significantly increased the mRNA expression levels of both genes. This up-regulation was largely inhibited in the cotransfection of K91R p32 with SUMO1 (Fig. 3*F*). Thus, sumoylation of K91 by SUMO1 within the DNA-binding domain (HD) significantly enhances both DNA-binding activity and transactivity of the p32 Pax-6.

SUMO1 and Pax-6 Are Colocalized in Embryonic Mouse Eyes. To confirm further that Pax-6 sumoylation takes place in vivo, we examined the expression of SUMO1 and Pax-6 in the embryonic eye from ED 9.5 to ED 19.5. As shown in Figs. S6 and S7, both SUMO1 and Pax-6 were expressed at ED 9.5, and their expression became much stronger at ED 11.5. At these two stages, colocalization of SUMO1 and Pax-6 was detected in neural tube (Fig. S7) and optic and lens vesicles (Fig. 4 *A* and *B*). Expression of both SUMO1 and Pax-6 was down-regulated by ED 12.5 (Figs. S6 and S7) and became significantly decreased from ED 14.5 to ED 18.5. At birth (ED 19.5), the SUMO1 signal was diminished (Figs. S6 and S7).

The observed colocalization reflects the specific interaction between p32 Pax-6 and SUMO1, because p43 Pax-6 was detected only in the cotransfected cells by wild-type p32 and SUMO1 but not with K91R p32 and SUMO1 (Fig. S8). This specific interaction also exists in vivo, as revealed by coimmunoprecipitation-linked Western blot analysis (Fig. 4 *C* and *D*). Together, our results demonstrated that during the early stages of mouse eye development, sumoylation activation of p32 Pax-6 occurs in vivo.

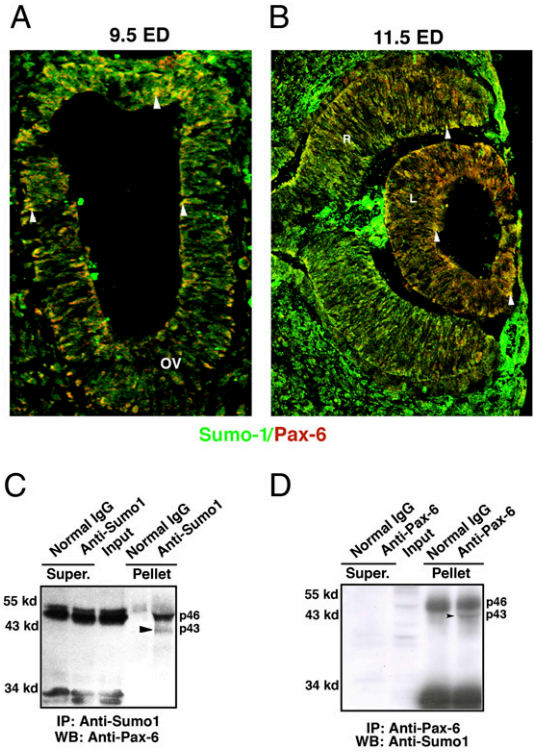

Fig. 4. Presence of p32 Pax-6 sumoylation in the developing mouse embryonic eye. (*A* and *B*) Immunohistochemistry analysis of Pax-6 and SUMO1 in the optic vesicle and lens vesicle of a mouse embryonic eye. Note that both Pax-6 and SUMO1 are clearly detected in the optical vesicle at ED 9.5 (*A*) and in the lens vesicle and retina tissues at ED 11.5 (*B*). In most cases, Pax-6 and SUMO1 overlap each other to generate the yellow fluorescence (arrowheads) (*C* and *D*) Total proteins were extracted from ED 11.5 embryonic eyes and were used for immunoprecipitation-linked Western blot analysis. Note that p43 Pax-6 (arrowhead in *C*) immunoprecipitated by anti-SUMO1 can be detected by anti–Pax-6 antibody. Similarly, p43 Pax-6 (arrowhead in *D*) immunoprecipitated by anti–Pax-6 can be detected by anti-SUMO1 antibody. L, lens; OV, optic vesicle; R, retina.

Discussion

Various Pax-6 Isoforms Are Generated Through Different Mechanisms of Gene Expression. The early results that quail cells contain four Pax-6 isoforms (12) are confirmed here in mice (Fig. 1*A* and Fig. S1*C*) and humans (Fig. S1*D*). The presence of various Pax-6 isoforms in different vertebrate species suggests the existence of common regulatory mechanisms for Pax-6 expression, which are essential to govern brain and eye development in these organisms.

Among the four different isoforms of Pax-6, p48 is derived from alternative splicing (24) with an additional 14 amino acids inserted into the PD. This insertion interrupts the N-subdomain (PAI) of the PD but leaves the C-subdomain (RED) of the PD intact (25). Thus, compared with p46 Pax-6, p48 Pax-6 is restricted in both DNA binding and regulation of downstream genes (26). Overexpression of p48 Pax-6 driven by a lens-specific promoter changes the gene-expression profile and leads to lens pathology (11). The p32 Pax-6 is derived from alternative translation of an internal ATG codon in full-length Pax-6 mRNA (12). Proteins generated through usage of the internal ATG codon have been observed in other genes, and such proteins sometimes act as repressors of the activities of full-length proteins (26). In the present study, we observed that p32 Pax-6 generated from an internal ATG codon of the full-length Pax-6 mRNA may or may not repress the full-length p46 Pax-6 function, depending on the target genes. Both p32 and p46 Pax-6 isoforms show positive regulation on the αB-crystallin gene (Fig. 1*B*). However, the two Pax-6 isoforms display differential controls on *Cspg2* and *Mab21l2*. In the Pax6$^{+/-}$ lens, the expression levels of *Cspg2* and *Mab21l2* are increased, suggesting that p46 Pax-6 represses these genes (27). In contrast, cotransfection of p32 Pax-6 and SUMO1 positively regulates their expression (Fig. 3*F*). Thus, p32 and p46 Pax-6 isoforms display differential regulations on the same target genes.

Our results also detected the presence of p43 Pax-6 in both newborn and adult mouse eyes. Where does p43 Pax-6 come from? In quail cells, one source for p43 Pax-6 is the alternative splicing (12). In mice and humans, however, we could not detect the alternative splicing products (Fig. S2). Thus, the origin of p43 Pax-6 must be different. In our study, we noticed that in eye tissue from both newborn and adult mice, p43 Pax-6 is present at a very low level (about 5% compared with the total p32), suggesting that its presence may be transitory. Indeed, our study reveals that p43 Pax-6 in mice and humans is derived from SUMO1-conjugated sumoylation of p32 Pax-6 (see discussion below). Thus, four different Pax-6 isoforms reflect differential expression mechanisms of the Pax-6 gene at transcriptional (p46), splicing (p48), translational (p32), and posttranslational (p43) levels.

Sumoylation of p32 Pax-6 Activates Its DNA-Binding and Transcriptional Activities. In the present study, we demonstrated that in vitro-synthesized p32 Pax-6, although containing the HD for DNA binding, cannot bind directly to the P3 sequence. This result is surprising, because the P3 sequence contains the conserved HD recognition site. Knowing that p43 Pax-6 is present at a very low level in newborn and adult mouse eye tissues and that the cytosolic p43 Pax-6 can bind to the P3 sequence, we predicted that in vitro-

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synthesized p32 Pax-6 needs to be modified to bind to the P3 sequence. Indeed, when in vitro-synthesized p32 Pax-6 is mixed with Pax-6–depleted FHL-124 nuclear extracts, it displays strong binding affinity to the P3 sequence. However, when FHL-124 nuclear extracts are precleared with anti-SUMO1 antibody, in vitro-synthesized p32 Pax-6 loses the ability to bind to the P3 sequence. These results led us to conclude that p32 Pax-6 is sumoylated by SUMO1 before it can bind to the P3 sequence. Additional experiments further confirmed our conclusion. First, purified synthetic p32 from a cell-free system can be sumoylated in vitro with SUMO1, yielding a 43-kDa protein that can be recognized by anti-SUMO1 and anti–Pax-6 antibodies. Second, bioinformatics and in vitro mutagenesis identify K91 as a SUMO1 acceptor site, an analysis that is confirmed by EMSA and ex vivo functional assays. Finally, in the developing mouse embryo at ED 9.5 and ED 11.5, immunocytochemistry demonstrates the colocalization of endogenous SUMO1 and Pax-6. This result is confirmed further by the in vitro cotransfection study and in vivo immunoprecipitation-linked Western blot analysis, which identify the same p43 Pax-6 by anti-SUMO1 and mouse anti–Pax-6 antibody. Together, our data show that p32 Pax-6 is converted to p43 Pax-6 through sumoylation and elucidate the in vivo source of p43 Pax-6 in mice and humans.

Protein sumoylation, in addition to regulating many cellular processes (18, 19), seems to be especially important for the regulation of gene expression (28). Although most studies have revealed that sumoylation of various transcriptional factors leads to repression of gene expression (28), considerably less is known about sumoylation promoting transcription activation (29). In the present study, our results demonstrate that sumoylation of p32 Pax-6 at K91 activates its ability to bind to the P3 sequence, which contains the HD recognition site. How could sumoylation activate the DNA-binding activity of p32 Pax-6? It is possible that sumoylation may remove the intramolecular inhibition on the HD domain. However, our finding that deletion of the N terminus abolishes its DNA binding (Fig. 3*B* and Fig. S5) rules out this possibility. Alternatively, sumoylation may stabilize p32 Pax-6 in a configuration favoring DNA binding. In any case, the N terminus of the p32 Pax-6 is essential for sumoylation-activated DNA binding.

Our results also show that sumoylation of p32 Pax-6 enhances its transactivity to regulate the expression of either the exogenous or the endogenous genes. ChIP assay reveals that the sumoylated p32 Pax-6 can bind directly to the target gene promoter. Our results are consistent with several recent studies that found that sumoylation activates other transcription factors (29, 30). Because chondroitin sulfate proteoglycan 2 (*Cspg2*), Mab-21-like-2 (*Mab2112*), and αB-crystallin are actively involved in cell proliferation or differentiation, regulation of these genes by the sumoylated p32 Pax-6 likely produces profound effects on early brain and eye development. These effects are consistent with our observations that the interactions between SUMO1 and Pax-6 occur in mouse embryonic neural tube, optic vesicles, and lens vesicles (Fig. 4 and Figs. S6 and S7).

p46 Pax-6 contains the same homeodomain as p32 Pax-6. However, our results show that p46 Pax-6 cannot bind to the P3 sequence even after incubation with Pax-6–depleted FHL-124 nuclear extract. This result suggests several possibilities. First, the tertiary structure of p46 Pax-6 protein may prevent its homeodomain from being sumoylated. Alternatively, sumoylation of p46 Pax-6 may occur, but the presence of the PD in p46 Pax-6 may prevent its binding to the P3 sequence. In any case, our results reveal that p32 and p46 Pax-6 isoforms also display differential DNA-binding activities, and thus probably regulate different target genes.

Methods

Animals. Mice used in this study were handled in compliance with the *Guide for the Care and Use of Laboratory Animals* (National Academy Press, Washington, DC). Embryonic mice of different stages (ED 9.5 to ED 19.5), neonatal mice, and 4-wk-old adult mice were obtained from the University of Nebraska Medical Center breeding facility.

Other analytical methods used in this study are detailed in *SI Methods*. Oligo primers used in the generation of 5A and 5D mutants are listed in Table S1.

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