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Transcriptional activity of paired homeobox Pax6 is enhanced by histone acetyltransferase Tip60 during mouse retina development

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ABSTRACT

Pax6 is a member of the Pax family of transcription factors that contains a DNA binding paired-box and homeobox domain. In animals, including humans, Pax6 plays a key role in development, regulating organogenesis of the eye and brain. The current data show that histone acetyltransferase Tip60 physically interacts with Pax6 in developing post-natal day 4 (P4) mouse retinas. We also found that Tip60 binds with paired-domain of Pax6 using its chromo- and zinc-finger-containing regions, and that these protein interactions were needed for the effective full-transcriptional activation of Pax6. Furthermore, among the combinations of Pax6-target gene interactions using its two DNA binding domain, paired- and homeobox domain, Tip60 significantly enhanced the transcriptional activity of Pax6 on the paired-domain binding sequence (P6CON) containing reporter construct (pCON) than other homeo domain and chimera binding containing pP3 and pCON/P3 constructs. Taken together, these results suggest that Tip60 binds with Pax6 and that this physical interaction leads to the full-transcriptional activation of Pax6 during retina development.

1. Introduction

The *Pax6* gene encodes an evolutionarily conserved paired homeobox protein that is crucial for eye and retina development [1]. In vertebrates, ectopic expression of Pax6 induces ectopic eyes or lenses, indicating critical roles of Pax6 in eye-specific differentiation [2]. Genetic studies have demonstrated that the expression levels of Pax6 strongly influence the formation and growth of ocular tissues [3,4]. Pax6 protein is composed of several functional domains that include N-terminal paired domain, homeodomain, and C-terminal proline/serine/threonine (PST)-rich transactivation domain [5,6]. The paired domain and the homeodomain bind with their specific target DNA sequence independently, but can also been perform cooperatively to mediate the appropriate transcription [7].

Various nonsense or missense mutations of Pax6 are associated with eye diseases such as aniridia [8], familial foveal dysplasia [9], and keratitis [10]. The importance of cooperative DNA binding activity of Pax6 can be explained by the finding that many Pax6 mutations occur in the DNA binding domain [11–13]. Several truncation mutations in C-terminal region of Pax6 have also been documented with aniridia patients [13,14]. These truncations result in mutant proteins that have lost all or part of the transcriptional activity of Pax6, despite retaining the DNA binding domains. In this regard, both the DNA binding activity and the transcriptional activity of Pax6 appear to be critical for the proper functioning of Pax6 during eye development [14]. However, the underlying molecular mechanisms in the Pax6-mediated transcriptional regulation are not well identified.

Tip60 (HIV Tat-interacting protein, 60 kDa) was identified as a binding partner for the Tat protein from human immunodeficiency virus (HIV)-1 [15]. Tip60 contains the MYST (MOZ, Ybf2/Sas3, SAS2 and Tip60) domain as part of the MYST family of HAT proteins that are conserved from yeasts to humans [16]. Tip60 performs dual roles as a transcriptional regulator depending on the cellular context or specific promoter sites [17]. As a coactivator, Tip60 associates with transcriptional activators such as HIV-1 Tat [15], Type I nuclear hormone receptor [18], amyloid-β precursor protein (APP) [19], MyoD [20], and Neural retina leucine-zipper (Nrl) [21]. The coactivator function in these cases is mediated by the acetylation of histones within the target promoter region, whereas in other cases, Tip60 directly acetylates p53 and modulates its transcriptional activity [22]. In contrast, TIP60 has also been implicated in the negative regulation of gene expression by binding to CREB (cAMP response element-binding protein) [23], STAT3 [24], ZEB (zinc-finger E box-binding protein) [25], and p73 [26]. Moreover, *Tip60* mRNA is highly expressed in the early stages of neural retina development [27]. However, the role of Tip60-HAT activity in retinal development remains to be elucidated.

The present data verify that Tip60 specifically interacts with Pax6, in which a protein–protein interaction is important for

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regulation of the transcriptional activity of Pax6. The findings suggest that Tip60 is essential for retinal development by modulating Pax6-dependent retina-specific gene expressions.

2. Materials and methods

2.1. Animals

Post-natal day 4 (P4) ICR strain mice (SAM IBRS#301) were purchased from Samtaco (Osan, Korea). The retinas were then excised rapidly by removing the lenses on an ice plate, after which they were stored at −70 °C. The Chung-Ang University Institutional Review Board (IRB) approved (Approval No. 40) all procedures involving mice used in this study.

2.2. Cell culture and transfection

HEK 293 cells obtained from the American Type Culture Collection (Manassas, VA, USA) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, USA) and penicillin–streptomycin (50 U/ml). Transient transfection was conducted using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

2.3. Plasmids

The human Tip60 full-length coding region was amplified from hTip60 cDNA in the human brain library (Clontech, Mountain View, CA, USA) using polymerase chain reaction (PCR) and was introduced to pCRII-TOPO vector (Invitrogen); the product was designated pCRII-TOPO-Tip60. The Tip60 clone was verified by DNA sequencing. Tip60 full-length (FL) was amplified from pCRII-TOPO-Tip60 using PCR reaction: forward 5′-gaa ttc ATG GCG GAG GTG GGG-3′, reverse 5′-tct aga TCA CCA CTT CCC CCT-3′. Tip60-chromo, -Zn, and -HAT truncated mutants were PCR-amplified from Tip60-FL; for Tip60-chromo: forward 5′-gaa ttc ATG GCG GAG GTG GGG-3′, reverse 5′-tct aga GAG GAC AGG CAA TGT-3′; for Tip60-Zn: forward 5′-gaa ttc ACC CCC ACT AAG AAC-3′, reverse 5′-tct aga ATT GTA GTC TTC CGT-3′; for Tip60-HAT: forward 5′-gaa ttc CTA CGA CAT CCT CCA-3′, reverse 5′-tct aga TCA CCA CTT CCC CCT-3′. These PCR products were introduced into the pCRII-TOPO vector (Invitrogen) and subcloned into the pEGFPC2 vector between *Eco*RI and *Xba*I and verified by DNA sequencing. The mouse Pax6 full-length coding region was amplified from P4 mouse retina cDNA generated by reverse transcriptase (Intron Biotechnology, South Korea). Amplified Pax6 was introduced into the RBC T&A cloning vector (Real Biotech Corp.), and the Pax6 clone was verified by DNA sequencing. Pax6 full-length (FL) was amplified from RBC-T&A-Pax6 using PCR: forward 5′-aag ctt c ATG CAG AAC AGT CAC AGC-3′, reverse 5′-gtc gac TTA CTG TAA TCG AGG CCA-3′. Pax6-PD, -HD, and -TAD truncated mutants were PCR-amplified from Pax6-FL; for Pax6-PD: forward 5′-aga tct ATG CAG AAC AGT CAC AGC-3′, reverse 5′-gtc gac TGT GCC CCA GCT TCC-3′; for Pax6-HD: forward 5′-gaa ttc CGC CCT GGT TGG TAT-3′, reverse 5′-gtc gac GGC CTG TCT TCT CTG-3′; for Pax6-TAD: forward 5′-gaa ttc AGG AAC CAG AGA AGA-3′, reverse 5′-gtc gac TTA CTG TAA TCG AGG CCA-3′. These PCR products were introduced into the RBC T&A cloning vector and subcloned into the pEGFPC1 or pEGFPC2 vector and verified by DNA sequencing.

2.4. Luciferase assay

HEK 293 cells were cultured in 60-mm-diameter dishes and transfected using Lipofectamine 2000 (Invitrogen) with the luciferase reporter constructs (0.1 μg), pCMV-β-galactosidase, and Pax6 encoding plasmids. The cells were lysed in reporter lysis buffer 48 h after transfection (E3971; Promega, Madison, WI, USA). Cell extracts were analyzed with the luciferase reporter assay system using a glomax luminometer (Promega). Luciferase activities were normalized based on the β-galactosidase activity of the co-transfected vector. All transfection experiments were repeated independently at least three times.

2.5. Immunohistochemistry

P4 mouse eyes were fixed in 4% paraformaldehyde (#15710; Electron Microscopy Sciences, Hatfield, PA, USA) in phosphate buffered saline (PBS) for 1 h and incubated in 20% sucrose–PBS for 1 day followed by freezing in OCT compound (Sakura Finetek, Torrance, CA, USA). Sections were incubated for 1 h in a blocking solution that contained 5% normal donkey serum in PBS with 0.1% Triton X-100. Mouse monoclonal antibody against Pax6 (1:100, sc-81649) and mouse monoclonal antibody against Tip60 (1:100, sc-5725) were incubated for 2 h at room temperature. Fluorescence images were obtained with a LSM-510 META laser scanning microscope (Carl Zeiss, Oberkochen, Germany) after staining with fluorescein isothiocyanate (FITC)-conjugated donkey anti-goat antibody and Rhodamine-conjugated donkey anti-mouse antibody for 1 h at room temperature.

2.6. In vivo binding assay and Western blotting

HEK 293 cells were seeded in 100-mm-diameter plates at an initial density of 2×10^6 cells and allowed to grow for 12 h. The cells were transfected with the respective plasmids, further incubated for 24 h, and lysed in a buffer containing 1% Triton X-100, 150 mM NaCl, 50 mM Tris–HCl, pH 7.5, 0.1% sodium dodecyl sulfate (SDS), 1% Nonidet P-40, and 1 mM phenylmethanesulfonylfluoride, and centrifuged at 12,000 rpm at 4 °C for 20 min. For immunoprecipitation assays, the supernatants were pre-cleaned with 20 μl of protein A/G agarose bead (50% slurry) and then incubated at 4 °C overnight with 50 μl of protein A/G beads in the presence of appropriate antibodies. The beads were washed three times in PBS, resuspended in SDS sample buffer, and boiled for 10 min. The protein samples were resolved by 10% SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to a PORTRAN nitrocellulose membrane (Whatman, Buckinghamshire, UK). The membrane was blocked with 5% skim milk in a solution of 20 mM Tris–HCl (pH 7.6), 137 mM NaCl, and 0.1% Tween 20 and incubated with appropriate dilutions of the primary antibody at room temperature for 3–5 h. Samples were analyzed by Western blotting using the appropriate antibody to detect protein expression. Polyclonal antibodies against Tip60 (sc-5725) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Monoclonal antibodies against green fluorescence protein (GFP-1814 460) and FLAG-M2 (F3165) were purchased from Roche Diagnostics (Indianapolis, IN, USA) and Sigma–Aldrich (St. Louis, MO, USA), respectively.

2.7. Statistical analysis

Statistical analysis of variances between two different experimental groups was conducted with Tukey's *post hoc* comparison test using SPSS version K12 (SPSS, Chicago, IL, USA). All experiments were repeated at least three times. The levels were considered significant at $p < 0.05$ (∗) and very significant at $p < 0.01$ (∗∗), obviously significant at $p < 0.001$ (∗∗∗), or not significant (*n.s.*).

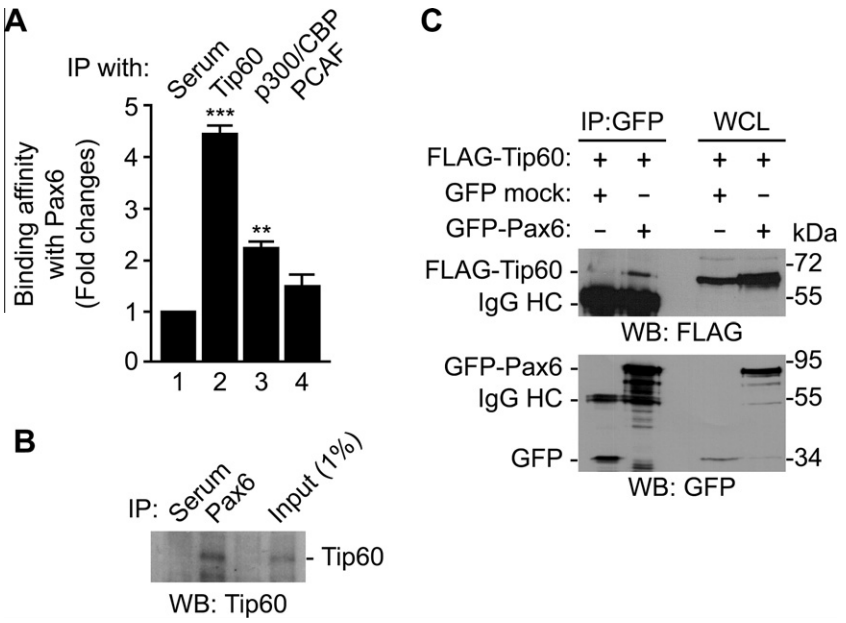

Fig. 1. Tip60 physically interacts with Pax6. (A) *In vivo* interaction screening of endogenous Pax6 with Tip60, p300/CBP, and PCAF proteins was determined in P4 mouse retina. Cell lysates were immunoprecipitated with anti-Tip60, p300/CBP, and PCAF antibodies. Co-precipitated proteins were eluted and then detected by enzyme-linked immunosorbent assay using Pax6 antibody. (B) *In vivo* interaction of endogenous Tip60 with Pax6 proteins determined in the P4 mouse retina. Cell lysates were immunoprecipitated with anti-Pax6 antibody and co-precipitated proteins were detected by Western blotting using Tip60 antibody. (C) HEK 293 cells were transfected with plasmids expressing FLAG-Tip60 (1 μg) together with either GFP empty (1 μg) or GFP-Pax6 (1 μg). Twenty-four hours after transfection, cells were harvested and lysates were immunoprecipitated with anti-GFP antibody. By Western blotting, proteins were detected by using appropriate antibodies as indicated.

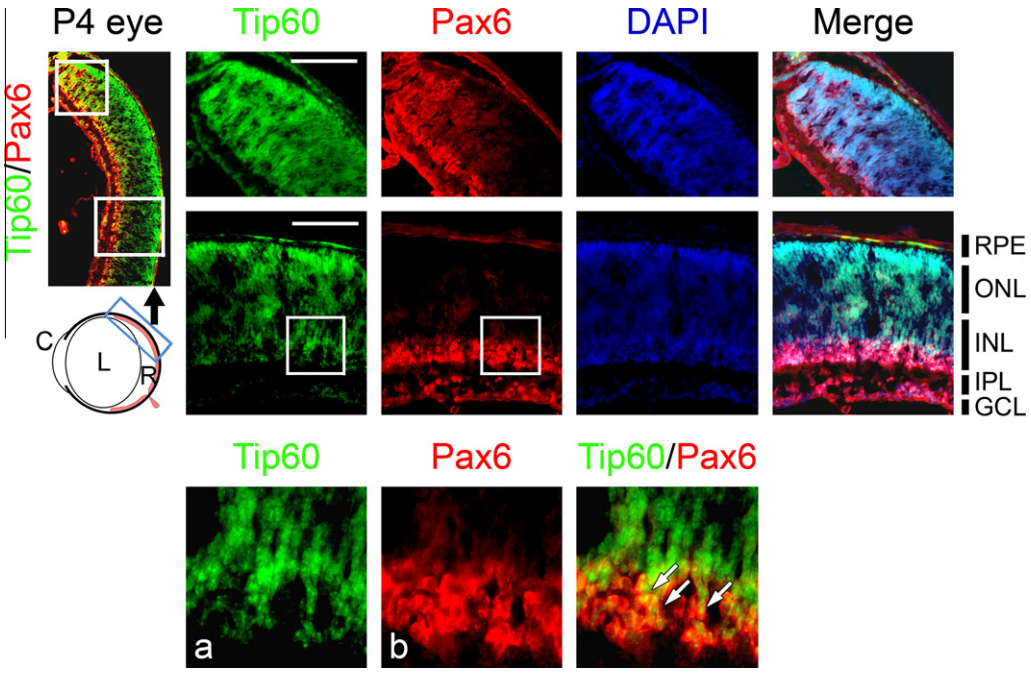

Fig. 2. Tip60 interacts with Pax6 in P4 mouse retina. P4 mouse retinas were immunolabeled with anti-Tip60 (green) and anti-Pax6 (red) antibodies. The nucleus was counterstained by 4′,6-diamidino-2-phenylindole (DAPI, blue). *Abbreviations:* PRE, retinal pigment epithelium; ONL, outer nuclear layer; INL, inner-nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Bars denote 50 μm.

3. Results

3.1. Tip60 physically interacts with Pax6 in mouse retina

Although the key transcription factor, paired homeobox Pax6 protein, is expressed in the early developmental stages, photoreceptor development is mostly completed post-natally [28]. This indicates that various combinations of additional regulatory factors are required to precisely control the retina-specific gene expressions. To identify the physically interacting partners that might bind with Pax6 to regulate the transcriptional activity of Pax6 via histone modification, P4 mouse retina was isolated and its cell lysates were immunoprecipitated with anti-Tip60, anti-p300/CBP, and anti-PCAF antibodies. Co-precipitates were eluted

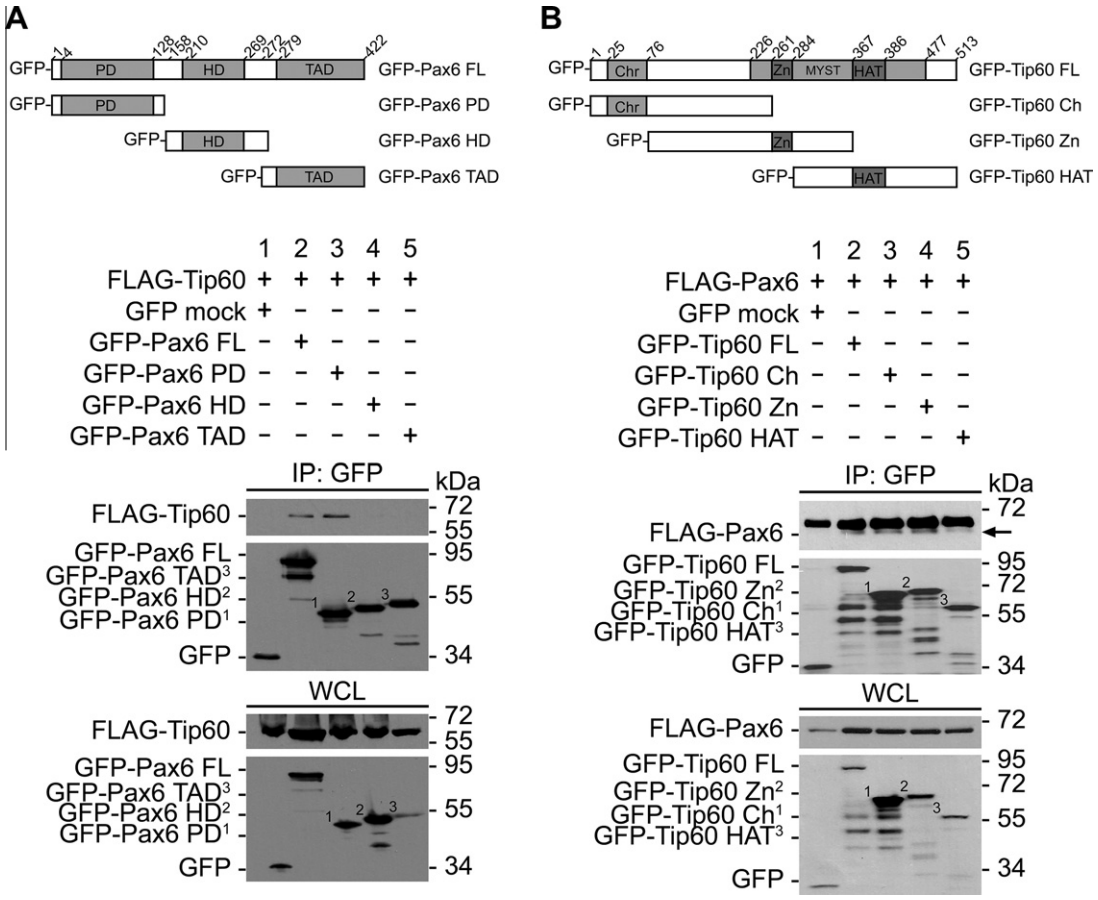

Fig. 3. Paired domain of Pax6 interacts with chromo-domain and zinc-finger domain of Tip60. (A) Schematic representation of Pax6 protein domains (upper panel). HEK 293 cells were co-transfected with expression plasmids of FLAG-Tip60 and GFP-pax6 full-length or each domains of Pax6. After the whole cell lysates were immunoprecipitated with anti-GFP antibody, the expression of GFP-pax6 and FLAG-Tip60 proteins was determined by Western blot analysis using anti-GFP and anti-FLAG antibodies, respectively. Abbreviations: PD; paired domain, HD; homeobox domain, TAD; transactivation domain. (B) Schematic representation of Tip60 protein domains (upper panel). HEK 293 cells were co-transfected with expression plasmids of FLAG-Pax6 and GFP-Tip60 full-length or each domains of Tip60. After the whole cell lysates were immunoprecipitated with anti-GFP antibody, the expression of GFP-Tip60 and FLAG-Pax6 proteins was determined by Western blot analysis using anti-GFP and anti-FLAG antibodies, respectively. *Abbreviations:* Ch; chromo domain, Zn; zinc-finger domain, HAT; histone acetyltransferase domain.

and the bound proteins were analyzed by enzyme-linked immunosorbent assay using anti-Pax6 antibody. As shown in Fig. 1A, Pax6 was significantly co-precipitated with Tip60 in P4 mouse retina. To examine the physical interactions between Pax6 and Tip60 *in vivo*, endogenous Pax6 was immunoprecipitated from P4 mouse retina with either anti-Pax6 polyclonal antibody or the corresponding control rabbit serum, then Western blotting was performed with anti-Tip60 antibody. Endogenous Pax6 was associated with Tip60 in the developing P4 mouse retina *in vivo*. To further test the physical interactions between Pax6 and Tip60, HEK 293 cells were transiently transfected with FLAG-Tip60 and GFP-mock or GFP-Pax6 expression plasmids. Whole cell lysates were immunoprecipitated with anti-GFP antibody and verified via Western blotting using anti-FLAG and anti-GFP antibodies. Consistent with the *in vivo* interactions from P4 mouse retina, FLAG-Tip60 protein was co-immunoprecipitated with GFP-Pax6, but not with only GFP (Fig. 1C, lane 2). To evaluate the co-localization of Pax6 with Tip60 *in vivo*, P4 mouse retina were cryosectioned and co-immunostained with anti-Pax6 and anti-Tip60 (Fig. 2). Although Tip60 was strongly expressed in the outer and inner-nuclear layers, Pax6 was exuberantly expressed in the inner-nuclear layer and ganglion cell layer (Figs. 2a and b, merged panel). Taken together, these results support the idea that Tip60 physically interacts with Pax6 in the inner-nuclear layer of the developing mouse retina *in vivo*.

3.2. Paired-domain of Pax6 interacts with the chromo-domain and Zn-domain of Tip60

To determine the binding region of Pax6 required for interaction with Tip60, HEK 293 cells were co-transfected with the Pax6 encoding GFP-fused to the Pax6 full-length, paired- (Pax6-PD), homeobox- (Pax6-HD), and transactivation domain (Pax6-TAD) containing region together with full-length of FLAG-Tip60 expression plasmid (Fig. 3A, upper panel). Whole cell lysates were immunoprecipitated with anti-GFP antibodies and then Western blotting was performed with anti-FLAG and anti-GFP antibodies. As shown in the lower panel of Fig. 3A, Tip60 strongly interacted with the paired domain of Pax6, but not with the homeobox- and transactivity domain-containing regions. To define the region of Tip60 required for interaction with Pax6, HEK 293 cells were co-transfected with Tip60 encoding GFP-fused to full-length Tip60, the Tip60-chromo domain (Tip60-Ch), the Tip60 zinc-finger domain (Tip60-Zn) or the Tip60 histone acetyltransferase domain (Tip60-HAT) together with the FLAG-Pax6 expression plasmid (Fig. 3B, upper panel). After whole cell lysates were immunoprecipitated with anti-GFP antibodies, Western blotting was performed with anti-FLAG and anti-GFP antibodies. Although the HAT domain-containing region (amino acids 285–513) of Tip60 did not appear to interact with Pax6, chromo (amino acids 1–260) and zinc-finger (amino acids 77–366) domain-containing regions

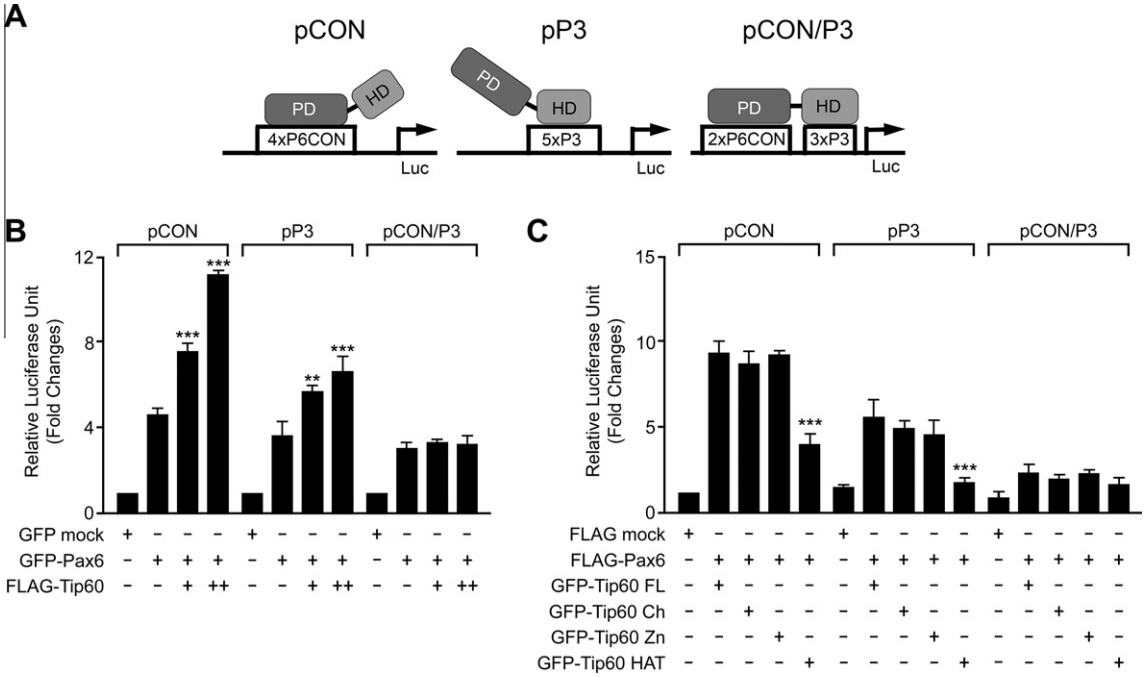

Fig. 4. Tip60 enhances transcriptional activity of Pax6 through the protein–protein interactions. (A) Schematic representation of Pax6-dependent reporter genes. (B) HEK 293 cells were co-transfected with combined expression plasmids of GFP-Pax6 and FLAG-Tip60, together with a luciferase reporter plasmid pCON, pP3, and pCON/P3 constructs. The data were normalized to β-galactosidase activity and are expressed in relative fold increase of luciferase units (RLU). Statistical significance is represented by Tukey's *post hoc* test (***$p < 0.001$, **$p < 0.01$). (C) HEK 293 cells were co-transfected with combined expression plasmids of FLAG-Pax6 and GFP-fused Tip60 truncation mutants together with a luciferase reporter plasmid pCON, pP3 and pCON/P3 constructs. The data were normalized to β-galactosidase activity and are expressed in RLU. Statistical significance is represented by Tukey's *post hoc* test (***$p < 0.001$).

strongly interacted with FLAG-Pax6 (Fig. 3B, lower panel), indicating the interaction of Pax6 with chromo- and zinc-finger containing domains of Tip60. Together, these results were consistent with the suggestion that the chromo-domain and zinc-finger domain of Tip60 interacts with the paired-domain-containing region of Pax6 in HEK 293 cells.

3.3. Tip60 significantly enhances the transcriptional activity of Pax6 through their protein–protein interactions

To demonstrate whether Tip60 could affect Pax6-dependent transcriptional activation, HEK 293 cells were co-transfected with Tip60 together with the FLAG-Pax6 expression plasmid plus a luciferase reporter gene pCON and pP3, which contains the P6CON and P3 consensus binding sequences for Pax6-PD and HD domains, respectively, or the pCON/P3 chimeric construct, which contains fragments of both P6CON and P3 (Fig. 4A) [29]. As shown in Fig. 4B, consistent with previous reports [30], Pax6 alone strongly transactivated pCON, pP3, and pCON/P3 reporter constructs (lanes 2, 6, and 10, respectively). The presence of Tip60 significantly enhanced the transcriptional activity of Pax6 on pCON and pP3 reporter construct (Fig. 4A; lanes 3, 4, and 7, 8). The transcriptional activity of Pax6 on pCON was approximately 2-fold stronger than the pP3 reporter gene. To further demonstrate whether Tip60 could affect Pax6-dependent transcriptional activation through a protein–protein interaction, HEK 293 cells were co-transfected with Tip60 truncated mutants (described in Fig. 3B, upper panel) together with the FLAG-Pax6 expression plasmid plus luciferase reporter constructs of pCON, pP3, and pCON/P3. The transcriptional activity of Pax6 was markedly enhanced by the full-length Tip60 and the chromo-domain and zinc-finger domain-containing region of Tip60 (Fig. 4C, lanes 2–4), but not by the HAT domains of Tip60 (lane 5), indicating that a physical association with the chromo-domain and zinc-finger domain of Tip60 is necessary for enhancement of Pax6 transactivation. Taken together, these findings indicate that Tip60 enhances the transcriptional activity of Pax6, which is the critical intrinsic factor involved in retinogenesis via the up-regulation of P6CON containing Pax6-target genes.

4. Discussion

In the present study, we demonstrate that Tip60 enhances the transcriptional activity of Pax6 through their protein–protein interaction (Fig. 1). Pax6 and Tip60 co-localized at inner-nuclear layer of P4 mouse retina and their interactions were detected at endogenous protein level (Figs. 1 and 2). Over-expression of Tip60 specifically enhanced Pax6-mediated transcriptional activity on the paired-domain binding promoter containing reporter gene (Fig. 4A). Pax6 interacted with Tip60 using its paired-domain-containing region (Fig. 3A), and Pax6 bound with the chromo-domain and Zn-finger containing region of Tip60 (Fig. 3B). Also, these protein interactions were required for the effective transcriptional activation of Pax6 (Fig. 4B). Furthermore, Tip60 specifically activated the Pax6 target genes containing paired-domain binding sequences (Fig. 4).

While much effort has been devoted to characterizing *Pax6* expression patterns and genetic regulatory networks, the regulatory mechanisms of Pax6-mediated transcription in the developing retina have not been defined. Homeodomain-interacting protein kinase phosphorylates Pax6 at the transactivation domain, and this modification leads to the recruitment of p300/CBP on the Pax6 target promoter regions during retinogenesis [31]. Because Pax6 transcriptionally activates its target genes in exact time and space, we searched for a histone acetyltransferase capable of modulating the transcriptional activity of Pax6 target gene expression. Tip60 strongly interacted with Pax6 in P4 mouse retina, more so than the p300/CBP interaction (Fig. 1A). Previous report showed that

Tip60 mRNA is highly expressed in the early stages of neural retina development [27]. Furthermore, we have reported that the histone acetyltransferase activity of Tip60 is required for full-transcriptional activation of Nrl (Neural retina leucine-zipper) which is important for rod/cone decision [21]. Thus, we suggest that Tip60 regulates the transcriptional activity of various transcription factors important in retina development.

Pax6 encodes a protein containing a paired domain, homeo domain, and a C-terminal transactivation domain [5,6]. The Pax6 paired domain is 128 amino acids, and was first described in the *Drosophila* segmentation genes paired, gooseberry, and gooseberry neuro [32]. The Pax6 paired domain binds to two half sites in the adjacent major grooves in the target promoter DNA as a monomer [33]. Also, the Pax6 homeodomain binds to a palindromic its target sequences surrounding a conserved central motif [34]. Furthermore, it was reported that the paired and homeodomains could interact directly by performing of co-immunoprecipitation assays with other homeodomain including proteins [35]. These results suggested that the Pax6 DNA binding regions also have the potential of protein–protein interaction both intramolecular and intermolecular manner. Presently, Tip60 preferentially interacted with the paired domain of Pax6 (Fig. 4B). This protein–protein interaction was required for full-transcriptional activation of Pax6 (Fig. 4C). This raises the possibility that Tip60 specifically modulates only the paired-domain binding region containing target genes, and that this could be decisive in retinogenesis through the Pax6-dependent target gene expressions.

Despite recent intensive studies on Pax6-mediated retinal development, the transcriptional regulation of Pax6 is not fully understood. In this regard, it is important to note that our findings contribute to a functional linkage between Tip60 and Pax6 in the biological network of retina, demonstrating that Tip60 associates with and regulates Pax6 via its paired-domain bound specific expression during retinogenesis.

Acknowledgments

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