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Photoreceptor-specific expression, light-dependent localization and transcriptional targets of the zinc-finger protein Yin Yang 1 in the chicken retina.

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ABSTRACT

The zinc finger transcription factor Yin Yang 1 (YY1) is a multifunctional protein that plays a critical role in embryonic development. Although it has been shown to play a role in eye development, its expression in the retina was not previously described. Here we investigated YY1 expression in chicken tissues and we identified the neural retina as one of the tissues with highest YY1 protein levels. Immunohistochemical detection of YY1 in the retina revealed a clear-cut photoreceptor-specificity and day/night differences in the cytoplasmic localization of the protein. YY1 was also present at high concentration in the nuclei of some photoreceptors. Gel-shift assays indicated YY1 bound to regulatory regions of several genes specifically expressed in photoreceptors. One of these genes, hydroxyindole-*O*-methyltransferase (HIOMT, EC 2.1.1.4), encodes the last-enzyme of the melatonin synthesis pathway. Although overexpression of chicken YY1 was not sufficient to activate the chicken HIOMT promoter in HEK293 cells, the YY1-binding site contained in this promoter was clearly required for full transcriptional activity in chicken embryonic retinal cells. These results suggest a role of YY1 in regulating the melatonergic function of retinal photoreceptors.

Keywords: Yin Yang 1, chicken, retina, photoreceptor, hydroxyindole-*O*-methyltransferase, melatonin.

Running title: photoreceptor-specific expression of YY1 in the chicken retina

INTRODUCTION

Yin Yang 1 (YY1) is an ubiquitous zinc-finger protein of the Polycomb group (PcG) that can act either as an activator or as a repressor of gene transcription, depending on promoter and cellular context (reviewed in: Shi *et al.*, 1997 ; Thomas and Seto, 1999 ; Gordon *et al.*, 2006). YY1 is multifunctional and plays critical roles in different aspects of cell biology, including proliferation, differentiation and apoptosis (Gordon *et al.*, 2006). As a regulator of cell proliferation, YY1 has been shown to act either through transcriptional activation of cell-cycle progression genes, such as *c-myc* (Riggs *et al.*, 1993), or through direct interaction with- and resulting inactivation of- the tumor suppressor gene product p53 (Sui *et al.*, 2004). The actions of YY1 on key regulators of cell proliferation and apoptosis have led to the notion that it may act as an initiator of tumorigenesis, and may therefore constitute an effective target for anti-cancer therapy (Gordon *et al.*, 2006). Beside its implication in tumorigenic proliferation, YY1 plays a fundamental role in physiological processes that are crucial for normal embryonic development. As a clear indication of this, targeted disruption of the YY1 gene in mice resulted in peri-implantation lethality (Donohoe *et al.*, 1999). Consistent with a role of YY1 in the control of cell division, analysis of heterozygote (YY1^{+/-}) mice revealed that a subset of these animals showed dramatic growth retardation. In addition, these mice displayed brain defects that suggested YY1 was required for normal progress of neurulation (Donohoe *et al.*, 1999). This was further supported by developmental studies in *Xenopus*, where overexpression of YY1 in one blastomere of two-cell stage embryos, induced the formation of an ectopic neural axis (Satijn *et al.*, 2001). During *Xenopus* development, YY1 was found to be predominantly expressed in the anterior neural tube, developing brain and optic vesicles (Kwon and Chung, 2003). Inhibition of YY1 synthesis by injection of antisense oligonucleotides in *Xenopus* embryos caused a slight developmental delay and a marked

reduction in the size of head structures and eyes (Kwon and Chung, 2003). Additional evidence involving YY1 in eye development came from a study showing that the abnormal anterior eye formation observed in Ring1-deficient mice was exacerbated in compound YY1^{+/-} Ring1^{-/-} mice (Lorente *et al.*, 2006). A similar phenotype was recently reported in Rybp (Ring1 and YY1 binding protein) heterozygous null mouse embryos (Pirity *et al.*, 2007). A result suggesting that Rybp, Ring1 and YY1 may interact to orchestrate normal vertebrate eye development. In addition, in *Xenopus* and mouse embryos YY1 has been shown to activate the expression of Otx2 (Kwon and Chung, 2003 ; Takasaki *et al.*, 2007), a homeobox transcription factor that plays a central role in retinal cell differentiation (Nishida *et al.*, 2003 ; Viczian *et al.*, 2003). The accumulating evidence relating YY1 to eye development prompted us to explore the hypothesis that YY1 may play a role in regulating retina-specific gene expression. In the present work, we examined the expression of YY1 in the chicken retina and we observed that this transcription factor is mainly expressed in photoreceptors. Functionally, we show that YY1 binds to regulatory regions of several genes specifically expressed in photoreceptors and we demonstrate that the YY1-binding site contained in the promoter of the gene encoding the last enzyme of the melatonin-synthesis pathway, hydroxyindole-*O*-methyltransferase (HIOMT, EC 2.1.1.4), is required for full transcriptional activation in chicken embryonic retinal cells.

MATERIALS AND METHODS

Animals

Fertilized chicken eggs (*Gallus domesticus*) were purchased from Rumulo C^{ie} (Quinçay, France) and were incubated at 37°C in an atmosphere of 70 % humidity. Embryos were staged according to Hamburger and Hamilton (1951).

Adult chickens (*Gallus domesticus*) were purchased at 8 weeks of age from Lecorne C^{ie} (Lencloître, France) and were kept for one week in a 12h light/12h darkness photoperiod (lights on zeitgeber time (ZT) 0-12), in a room at 27°C, with food and water *ad libitum*. They were anesthetized by an intraperitoneal injection of chloral hydrate and were sacrificed by decapitation, either at ZT23 (dark-adapted chickens, n = 3) or at ZT11 (light-adapted chickens, n = 3). Following dissection, tissues were either transferred in culture medium (for retinal cell culture) or frozen in liquid nitrogen (for western blot analysis) or fixed in Bouin's solution (for immunohistochemistry). Animal care was conducted in accordance with the standard ethical guidelines (European Community Guidelines on the Care and Use of Laboratory Animals; and French Agriculture and Forestry Ministry guidelines for handling animals, decree 87848, license E 86-040) and approved by the local ethical committee.

Immunohistochemistry (IHC)

Retinas fixed in Bouin's solution were dehydrated in ethanol, then butanol, and embedded in paraffin. Sections (6 µm) were blocked in phosphate-buffered saline containing 0.05% Tween-20 (PBS-Tween), 1% BSA, 1% gelatin and were incubated overnight at 4°C with a rabbit polyclonal anti-YY1 antibody (Santa-Cruz, sc-281) diluted 1:1000 in PBS-Tween, 1% BSA, or with a rabbit polyclonal anti-LexA antibody (Affinity Bioreagents, PA1-4966) at the same dilution. The primary complexes were detected by incubation with biotinylated goat

anti-rabbit IgG (Vector laboratories) diluted 1/500 in PBS-Tween and with avidin-biotin-peroxidase complexes ("Vectastain" Kit, Vector Laboratories). Peroxidase activity was revealed with 0.05% diaminobenzidine, 38mM ammonium nickel sulfate and 0.003% H₂O₂ in 0.15M sodium acetate and 3.7mM acetic acid. Sections were observed on a light microscope (Zeiss Axioplan) equipped with a digital camera (Kappa).

Western blots

Protein extracts were prepared by sonicating the frozen tissues in phosphate buffer. After quantitation with Bradford dye (Bio-Rad), proteins were diluted in Laemmli buffer, fractionated by SDS-PAGE (8% gels) (Laemmli 1970) and electrotransferred on .nitrocellulose membrane. Non-specific sites were blocked in PBS-Tween with 1% BSA and 4% ProtoBlock™ (National Diagnostics) and the membranes were incubated overnight at 4°C with the anti-YY1 or anti-LexA antibodies used for IHC, or with a mouse monoclonal anti-beta-tubulin antibody (Sigma, T4026) diluted 1:400 in PBS-Tween, 1% BSA. For YY1 and LexA, antibody-antigen complexes were detected as described for IHC. For beta-tubulin, antibody-antigen complexes were detected with peroxidase-linked sheep F(ab')₂ fragments anti-mouse IgG (Amersham Biosciences) and peroxidase activity was detected as described for IHC.

Cloning of the full-length chicken YY1

The full-length chicken YY1 cDNA (GenBank accession number AJ719349) was obtained by RT-PCR from total RNA of chicken retina, with a sense primer containing the Kozak sequence and the first 14 bases of the YY1 coding sequence, with a KpnI site added at its 5'-end (5'-CCTGGTACCGCCATGGCCTCGGGAGA-3') and an antisense primer containing the last 21 bases of the YY1 coding sequence, with a BamHI site added at its 5'-end (5'-

TCCGGATCCTTTCACCTGGTTGTTTTGGCCTT-^{3'}). The PCR product obtained was cloned into pcDNA3 (InVitrogen) and sequenced with Big Dye reagent (ABI) (analysis on ABI Prism 310).

Electrophoretic mobility shift assay (EMSA)

Based on the consensus YY1-binding sequence published on the TESS software (Transcription Element Search Software on the www ; TRANSFAC matrix record M00059), eight putative YY1-binding sites were identified in different regulatory regions of genes specifically expressed in photoreceptors (Table 1). These sequences were analyzed by electrophoretic mobility shift assay (EMSA) as previously described (Bernard *et al.*, 2001 ; Dinet *et al.*, 2006). Proteins were extracted from chicken retinas, rat retinas and Y79 human retinoblastoma cells (ECACC). Recombinant chicken YY1 protein was synthesized *in vitro* with the transcription/translation-coupled reticulocyte lysate system (TNT[®], Promega), using the pcDNA3-YY1 plasmid described above as template. The double-stranded oligonucleotides used as DNA probes corresponded to the sequences described in Table 1 and were labelled with [γ -³²P]ATP (3000 Ci/mmol) using T4 polynucleotide kinase (Promega). The DNA-protein binding assay was carried out for 15 min at room temperature, with 5 fmoles of [³²P]-labelled DNA probe (~30, 000 cpm) and with either *in vitro*-translated YY1 (4 μ l of a TNT[®] reaction) or 20 μ g of protein extracts, in the absence or presence of a molar excess of non-radioactive DNA competitor (see figure legends for details). The final composition of the reaction was as previously described (Bernard *et al.*, 2001). In some reactions, the proteins were preincubated with 2 μ l of anti-YY1 antibody or 2 μ l of normal rabbit IgG for 45 min at room temperature before adding the radiolabelled probe. The DNA-protein complexes were analyzed on non-denaturing gels [4.5% acrylamide/bisacrylamide (30 : 1) in 40 mM Tris, 270 mM glycine and 2 mM EDTA]. The gels were dried and exposed

overnight to a PhosphorImager screen (Storm 820, Molecular Dynamics). The images were analyzed with the ImageQuant program (Molecular Dynamics).

Cell culture and transient transfections

HEK293 cells (a generous gift from the Laboratory of Prof. J. M. Müller, Poitiers) were maintained in Dulbecco's modified Eagle's medium supplemented with 100 U/mL penicillin/streptomycin and 10% fetal calf serum (Gibco BRL). Primary cultures of chicken embryonic retinal cells were prepared as previously described (Dinet *et al.*, 2006), with slight modifications. Embryonic day 8 (E8) neural retinas, free of pigment epithelial cells, were dissociated and cultured in polyornithine-coated plates, in M199 medium (Sigma) supplemented with 100 U/mL penicillin/streptomycin, 2 mM glutamine, 0.1% bovine serum albumin, and 10% newborn calf serum (Gibco BRL). HEK293 and retinal cells were incubated at 37°C in an atmosphere of 5% CO₂ in air. They were seeded in 6-well plates, one day before transfection, at a density of 2 x 10⁵ cells per well for HEK293 cells, or 3 x 10⁶ cells per well for retinal cells. Cells were transfected by the CaPO₄ method. Briefly, appropriate amounts of plasmid DNA (see Figure legends) were diluted in 75 µl of 0.25M CaCl₂ and were added dropwise to 75 µl of 2xHeBS (280 mM NaCl, 1.4 mM Na₂HPO₄, 50 mM HEPES, pH 7.1). Cells were incubated overnight with the precipitate (150 µl/well) and were cultured for 24 h in fresh medium before being assayed for luciferase activities. Transfections and assays were performed in triplicate for each experimental group.

Three categories of plasmids were used for transient transfection experiments. HEK293 cells and retinal cells were transfected with the pGL3-HIOMT promoter-reporter construct, containing ~3 kb of the chicken HIOMT promoter upstream of the *firefly* luciferase reporter gene (described as pGL3A in Bernard *et al.*, 2001). The pGL3-HIOMT(cH1mut) corresponded to pGL3-HIOMT with a mutation in the YY1-binding site (CCCATTT →

CCCCTTT) and was obtained by site-directed mutagenesis (Quikchange[®], Stratagene). The mutated plasmids were controlled by direct sequencing. The pcDNA3-YY1 plasmid described above was used to overexpress YY1 in transfected HEK293 cells. A plasmid expressing the rat Crx transcription factor (Bernard *et al.*, 2001) was used as a positive control and the empty pcDNA3 vector was used as a negative control. Corrections for variations in transfection efficiencies were performed with pRL-SV40 (Promega), a plasmid expressing the *Renilla* luciferase under control of the SV40 promoter end enhancer.

Firefly and *Renilla* luciferase activities, were assayed using the Dual-Glo[™] luciferase assay system (Promega). Light emission was measured on a Beckman LS 6500 counter, on the luminometer program (Beckman Instruments). Transfection efficiency was normalized to the firefly/*Renilla* luciferase activity ratio. The statistical significance of the results were verified by the Student's *t*-test.

RESULTS

Tissue specificity of YY1 expression

Western blot analysis of a series of chicken tissues indicated that YY1 is expressed predominantly in the neural retina, retinal pigment epithelium (RPE), pineal gland and duodenum (Fig. 1A). Lower expression levels could be detected in telencephalon, cerebellum, liver and heart, while YY1 remained undetectable in skeletal muscle (Fig.1A). In agreement with previous reports in human and rat (Shi *et al.*, 1997 ; Ellis *et al.*, 2002), YY1 was detected as a ~63 kDa protein on western blots. Depending on tissues, some amount of non-specific reaction could be observed, as determined with an irrelevant antibody (Fig. 1A). The neural retina stood out as the tissue where 63 kDa YY1 was detected with the highest specificity (Fig.1A). As illustrated in figure 1B, the YY1 protein was readily detectable in the retina of 6-day embryos (E6). The signal gradually decreased until day 1 post-hatch (P1), then stabilized between P1 and P15 (Fig. 1B). The use of tubulin as an internal standard confirmed the decrease in YY1 intensity was real and did not reflect differences in the total amount of protein loaded on the gel (compare E6 and P15 in Fig.1B).

Cellular localization of YY1 in dark-adapted retina

Immunohistochemical detection of YY1 in the retina of dark-adapted chickens was highly specific of photoreceptors (Fig. 2A, B). In the central retina, YY1 labelling was mainly concentrated in inner segments above the outer limiting membrane (Fig. 2A, C), while in more peripheral zones it gradually extended to the outer nuclear layer and to the basal pedicles of photoreceptors (Fig. 2B, D-I). Different morphological subtypes of photoreceptors were observed. The strongest labelling was found in accessory cones of the abundant double-cones, particularly in the myoid region and around the large paraboloid and ellipsoid regions

(Fig. 2C-H). Some accessory cones displayed strong reactions in the basal pedicle and nucleus (Fig. 2D, E, F, H) and some were also intensely labelled at the apex of the inner segment, just proximal to the lipid droplet (Fig. 2F-H). Principal cones of double-cones showed a more diffuse and weaker labelling than accessory cones, but some had a distinct nuclear labelling (Fig. 2C, G). YY1 labelling could also be identified in rod nuclei, typically localized at the vitreal side of the outer nuclear layer (Fig. 2I, J). The number of labelled photoreceptor nuclei and the intensity of the nuclear labelling showed large individual and zonal variations. YY1 was not detected in outer segments, but was found in the calycal process of some photoreceptors (Fig. 2C, E, I).

Upon extended development of the chromogen, a low intensity labelling could be observed in some nuclei of the inner nuclear layer and in the cytoplasmic extensions of Müller glial cells (Fig. 2K). This low intensity labelling was not background noise, because it was not observed with an irrelevant antibody (Fig. 2L).

Effect of light on the subcellular localization of YY1

When YY1 immunohistochemical labelling was compared in light-adapted and dark-adapted retinas, striking differences could be observed in the subcellular localization of the protein (Fig. 3, compare A and B ; also compare to dark-adapted retina in Fig. 2). Light-adapted retinas displayed a spatially more restricted YY1 labelling, mostly overlying the outer limiting membrane (Fig. 3A). In accessory cones of double-cones, YY1 was still present in the myoid region of the inner segment, but the intense labelling around the paraboloid/ellipsoid region and in the basal pedicle was no longer observed (Fig. 3C). Principle cones and single cones displayed a more diffuse labelling in the inner segment. Labelled photoreceptor nuclei were still observed in light-adapted retina (Fig. 3C). Our attempts to quantify light-induced differences in the number of YY1-immunoreactive

photoreceptor nuclei remained inconclusive, due to large individual variation. As in dark-adapted retinas, a low-level labelling could be observed in the inner nuclear layer of light-adapted retinas upon extended chromogen development (data not shown).

Western blot analysis of dark-adapted *versus* light-adapted retinas revealed no significant differences in the amount or size of the YY1 protein (Fig. 3D).

Interaction of YY1 with regulatory regions of genes expressed in photoreceptors

Strong YY1 immunochemical labelling in the nuclei of some rods and cones prompted us to examine YY1 binding to promoter regions of genes specifically expressed in photoreceptors. Based on the consensus sequence for YY1 binding elements (see Materials and Methods for details), putative binding sites could be identified in the chicken rhodopsin promoter, as well as in the promoters of two genes of the melatonin pathway, arylalkylamine N-acetyltransferase (AANAT, EC 2.3.1.87) and hydroxyindole-*O*-methyltransferase (HIOMT) (Table 1). Using EMSA analysis, only one element of the chicken HIOMT promoter (cH1) showed saturable, sequence-specific interaction with a protein from chicken retina (Fig. 4A and B). In contrast, the other putative sites found in HIOMT, AANAT and rhodopsin promoters gave non-specific shifted bands of weak intensity, that were not displaced by the unlabelled homologous probes (Fig. 4A). YY1 binding to the cH1 element was further characterized. The retinal protein that bound to cH1 could be identified as YY1, because its binding to DNA was specifically inhibited by an anti-YY1 antiserum, raised against the C-terminal zinc-finger domain of YY1 (Fig. 4C). Moreover, the cH1 element clearly bound the full-length *in vitro*-expressed chicken YY1 protein (Fig. 4C).

Four additional consensus sites were found in photoreceptor-expressed genes of other species (Table 1). EMSA analysis with anti-YY1 antibody interference positively identified two of these sequences as YY1-binding sites: one in the first intron of the rat AANAT gene, the

other one in the 5'-flanking region of the human cone alpha-transducin gene (Fig. 4D). A result suggesting that YY1 may target photoreceptor-expressed genes in different species.

To keep our focus on the chicken retina, where photoreceptor-specificity and light-dark regulation of YY1 were clearly demonstrated, we moved on to examine the functional impact of YY1 on the chicken HIOMT promoter. HEK293 cells were cotransfected with a promoter-reporter plasmid containing a 3kb version of the chicken HIOMT promoter and with an expression plasmid encoding chicken YY1. Although transfection of HEK293 cells led to overexpression of the YY1 protein, as verified by western blot (data not shown), the HIOMT promoter remained inactive in these cells (Fig. 4E, left panel). In contrast, we verified that the rat Crx transcription factor was able to activate the HIOMT promoter in HEK293 cells (Fig. 4E, left panel), as previously shown in COS7 cells (Bernard *et al.*, 2001). Although YY1 was apparently not sufficient to directly activate the HIOMT promoter, a role of the YY1-binding site contained in this promoter could be clearly established upon transfection of the promoter-reporter plasmid into chicken embryonic retinal cells. Indeed, a single-nucleotide mutation that abolishes YY1-binding in EMSA (Fig. 4B), also reduced by 70% the HIOMT promoter activity (Fig. 4E, right panel).

DISCUSSION

The present study provides the first evidence of a photoreceptor-specific expression of YY1 in the chicken retina and further shows that light causes a subcellular translocation of YY1 in photoreceptors. In addition, we demonstrate that YY1 binds to regulatory regions of genes specifically expressed in photoreceptors and we show that its binding-site in the promoter of one of these genes -HIOMT- is required for full transcriptional activity in embryonic chicken photoreceptors.

The first analyses of YY1 expression by whole-mount *in situ* hybridization in mouse embryos have led to the description of YY1 as an ubiquitous protein (Donohoe *et al.*, 1999). This notion was somewhat revised in *Xenopus* embryos, where YY1 expression was found to be ubiquitous in early embryos, but progressively restricted to anterior neural structures during later embryonic stages (Kwon and Chung, 2003). In keeping with these results, our western blot analysis indicates a relatively widespread tissue distribution of YY1 in adult chicken, with neural retina being one of the tissues with highest YY1 expression level. More surprisingly, our immunohistochemical examination of the retinal neuroepithelium revealed that YY1 is not present in all chicken retinal cells, but is predominantly expressed in photoreceptors. Although an implication of YY1 in normal eye development has been suggested in different studies (Kwon and Chung, 2003 ; Lorente *et al.*, 2006 ; Purity *et al.*, 2007), the cellular and molecular basis of this action still remain unknown. The data presented herein suggest that this action of YY1 may include a specific role on photoreceptor differentiation and/or homeostasis. The contrast observed here between ubiquitous tissue-distribution and high cellular-specificity in the retina warrants further immunohistochemical studies in different tissues, aimed at identifying the YY1-expressing cell types. Because

partial inactivation of YY1 causes strong defects in neurulation and/or eye development in mouse and *Xenopus* (Donohoe *et al.*, 1999 ; Kwon and Chung, 2003), the immunohistochemical identification of YY1-expressing cells in the brain and eyes of these animals would be worthwhile, as it should help specify the mechanisms involved in these defects.

Another intriguing aspect of our findings is the effect of light on the subcellular localization of YY1 in photoreceptors. Consistent with previous observations in other cell types (Ficzyc *et al.*, 2001 ; Palko *et al.*, 2004) we found that YY1 was not exclusively nuclear. In most photoreceptors indeed, the protein was very abundant in the inner segment and in the basal pedicle. However, we observed that the spatial distribution of the protein was strikingly narrowed following light-adaptation. These changes in YY1 localization are apparently not related to degradation of the protein or to major post-translational modifications, because the size of the immunoreactive YY1 is the same in dark-adapted and light-adapted retinas. The light-induced redistribution of the protein is most visible in accessory cones of double-cones, and is mainly characterized by a concentration of the protein within the myoid region of the voluminous inner segment. This cellular compartment has previously been shown to contain actin filaments that are involved in the day/night photomechanical movements of rods and cones (O'Connor and Burnside, 1982). In addition, the myoid cytoskeletal proteins contribute to the subcellular trafficking of proteins in photoreceptors. In mouse, rat, chicken and *Xenopus*, several phototransduction proteins have been shown to shuttle between the inner and outer segments, on a day-night basis. Transducin and MEKA are translocated from the inner to the outer segment in response to darkness, while arrestin is translocated to the outer segment in response to light (Brann and Cohen, 1987 ; Kuo and Miki, 1989 ; McGinnis *et al.*, 2002 ; Zhu *et al.*, 2002). In *Xenopus* retina, these movements were clearly shown to be

controlled by the cytoskeletal network, because they were affected by pharmacological agents that disrupt microfilaments and microtubules (Peterson *et al.*, 2005). Together, these data suggest that the subcellular trafficking of YY1 may rely on a similar interaction with myoid cytoskeletal proteins. Further studies utilizing cytoskeleton depolymerizing agents will be needed to elucidate this point and to determine the possible consequences of YY1 trafficking in the cytoplasm of photoreceptors. Previous studies in smooth muscle cells have indicated that an interaction of YY1 with polymerized γ -smooth muscle actin could sequester YY1 in the cytoplasm and thereby inhibit its nuclear translocation (Favot *et al.*, 2005). We did not find any evidence of a similar regulation in our study, because the number of YY1-containing nuclei was not significantly different between light-adapted and dark-adapted photoreceptors. However, this lack of significance should be re-evaluated in dedicated studies utilizing a larger number of animals and a more quantitative method for immunodetection.

The high level of YY1 expression in the chicken retina and its marked photoreceptor-specificity raise one major question: what role might this transcription factor play in retinal physiology? Considering its well-established role on cell cycle control, the first hypothesis that comes to the mind is that of a contribution of YY1 to retinoblast proliferation. The studies performed in *Xenopus* embryos favoured this assumption, because they showed that inactivation of YY1 in the embryo generated microphthalmia (Kwon and Chung, 2003). Based on this result, we may surmise that YY1 could play a similar role in the chicken retina. However, here we detected YY1 in the nuclei of mature photoreceptors, a result indicating that the role of YY1 may not be restricted to the maintenance of retinal precursors in a proliferative status. Rather, the presence of YY1 in the nuclei of fully differentiated photoreceptors would suggest a role of this transcription factor in photoreceptor-specific gene expression. The data presented here support this hypothesis, by showing that YY1 can bind to

the regulatory regions of genes specifically expressed in photoreceptors. Moreover, we demonstrate that the promoter of one of these genes (HIOMT) requires an intact YY1 binding-site in order to be fully activated in embryonic photoreceptors. Since HIOMT is the enzyme that catalyzes the last step of melatonin synthesis, we suggest that one contribution of YY1 to retinal function might be to favour the production of melatonin by photoreceptors. However, it seems likely that YY1 would act in conjunction with other transcription factors, because we found that YY1 alone was not sufficient to activate the HIOMT promoter in HEK293 cells. Homeodomain-containing factors of the Crx/Otx family might be involved in this process, because they are known to play a major role in the differentiation and maintenance of the photoreceptor phenotype (Furukawa *et al.*, 1997 ; Furukawa *et al.*, 1999 ; Nishida *et al.*, 2003 ; Viczian *et al.*, 2003), and we showed in previous studies that they could directly activate the chicken HIOMT promoter (Bernard *et al.*, 2001 ; Dinet *et al.*, 2006). Of course, additional factors expressed in chicken photoreceptors could also be implicated, for instance Otx2 (Toy *et al.*, 1998), NeuroD (Yan and Wang, 1998 ; Yan and Wang, 2004), Ngn2 (Yan *et al.*, 2001) and RaxL (Ohuchi *et al.*, 1999 ; Chen and Cepko, 2002). Even though HIOMT is the only gene identified here as a potential YY1 target in the chicken retina, we cannot exclude an involvement of YY1 in the transcription of other genes that define photoreceptor-specific functions, particularly those encoding phototransduction proteins. The possibility of an indirect influence of YY1 on photoreceptor-specific transcription should also be hypothesized, because YY1 has been shown to be an essential activator of Otx2 expression in anterior neuroectoderm (Kwon and Chung, 2003 ; Takasaki *et al.*, 2007), and Otx2 has the ability to directly activate the promoter of genes specifically expressed in photoreceptors: interphotoreceptor retinoid-binding protein (Bobola *et al.*, 1998) ; Crx (Nishida *et al.*, 2003) ; rhodopsin and M-cone opsin (Peng and Chen, 2005), HIOMT (Dinet *et al.*, 2006) and rhodopsin kinase (Young *et al.*, 2007).

In the light of recent data suggesting an involvement of YY1 in eye development (Kwon and Chung, 2003 ; Lorente *et al.*, 2006 ; Pirity *et al.*, 2007) and based on the results of our study demonstrating the strong YY1 expression in retinal photoreceptors, it might now be interesting to re-examine the retinas of YY1^{+/-} heterozygote mice and of YY1-deficient *Xenopus* eyes. This would help determine whether decreased YY1 levels in the retina affect photoreceptor-specific gene expression and, as a consequence, photoreceptor differentiation.

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Table 1. Sequences of putative YY1-binding elements in photoreceptor-expressed genes.

Based on the consensus YY1-binding sequence defined in the TESS software, eight putative YY1-regulatory elements were identified in the 5'-upstream regions and intronic regions of genes specifically expressed in photoreceptors. The GenBank accession numbers of these regulatory regions are: AF116454 (chicken HIOMT promoter) ; AF193072 (chicken AANAT promoter) ; D00702 (chicken rhodopsin promoter) ; U77455 (rat AANAT promoter and intron 1) ; contig AC091784 and Zhu *et al.* (2002) (mouse cone arrestin promoter) ; U66698 (human cone alpha-transducin promoter) ; U16824 (human rhodopsin promoter).

Gene	Sequence name	Sequence
chicken HIOMT (promoter region)	cH1	5' TGTGAGTGCCCATTTTTAAAGATG 3'
chicken HIOMT (promoter region with mutation)	cH1mut	5' TGTGAGTGCCCC <u>C</u> TTTTTAAAGATG 3'
chicken HIOMT (promoter region)	cH2	5' CCTCTGGTCCCATTTCATTGGGCT 3'
chicken AANAT (promoter region)	cNAT	5' GGATAATCCCCATTTGGTTCCACT 3'
chicken rhodopsin (promoter region)	cRho	5' TAACGTGTCCCATTTCATATCC 3'
rat AANAT (intron 1)	rNATi	5' CGCCATCGCCCATTTAAAAAGATT 3'
mouse cone arrestin (promoter region)	mArr	5' CGGTTCTTCCCATTTTGGCTACAT 3'
human cone alpha-transducin (promoter region)	hTrans	5' ATGATCTGCCCATTTTGGCCTCCC 3'
human rhodopsin (promoter region)	hRho	5' TTATCATTCCCATTTTACACGAGT 3'

FIGURE LEGENDS

Figure 1. Western blot immunodetection of YY1 in chicken tissues and in the developing chicken retina. A. Proteins from different adult chicken tissues were fractionated by SDS-PAGE (8 % acrylamide gels ; 10 µg protein/lane) and the western blot was immunostained with an anti-YY1 antibody. The primary complexes were revealed with a biotinylated secondary antibody and with avidin-biotin-peroxidase complexes. RPE: retinal pigmented epithelium. The arrow points to YY1. Asterisks indicate non-specific bands (ns) that were also detected with an irrelevant anti-LexA antibody (unillustrated). B. Proteins from neural retinas of developing chickens were fractionated by SDS-PAGE (12.5 % acrylamide gels ; 20 µg protein/lane). The western blot was incubated successively with an anti-YY1 antibody and with an anti-tubulin antibody. The primary complexes were revealed either as indicated for panel A (for YY1) or with a peroxidase-labelled secondary antibody (for tubulin). E: embryonic days ; P: post-hatch days ; Tub: tubulin.

Figure 2. Immunohistochemical detection of YY1 in the retina of dark-adapted chickens. Retinas from dark-adapted 9-week-old chickens (n = 3) were immunostained with anti-YY1 (A-K) or anti-LexA (L) immunoglobulins. The primary complexes were revealed with a biotinylated secondary antibody and with avidin-biotin-peroxidase complexes. A and B are low magnification views of central (A) or peripheral (B) retina. Abbreviations: RPE, retinal pigmented epithelium ; OS, photoreceptor outer segments ; IS, photoreceptor inner segments ; ONL, outer nuclear layer ; INL, inner nuclear layer ; IPL, inner plexiform layer ; GCL, ganglion cell layer. The labelling reveals the presence of YY1 only in inner segments (IS) and nuclei (ONL) of photoreceptors. C, D, E, F, G, H, I and J are high magnification views of the labelled photoreceptor layer. Abbreviations: e, ellipsoid ; p, paraboloid ; my,

myoid ; nu, nucleus ; cp, calycal process ; ld, lipid droplet. In K, an extended chromogen development reveals a faint labelling of YY1 in the inner nuclear layer (INL), in addition to the strong labelling observed in the photoreceptor layer (PL). In L, no labelling is detected with an irrelevant (anti-LexA) antibody. Scale bar= 25µm.

Figure 3: Immunohistochemical detection of YY1 in the retina of light-adapted chickens.

In A to C, retinas from light-adapted (A, C) or dark-adapted (B) 9-week-old chickens (n = 3 in each experimental group) were immunostained with anti-YY1 immunoglobulins. A and B are observations at low magnification, while C is a high magnification view of the labelled photoreceptor layer. In light-adapted retinas, the YY1 labelling is concentrated in the myoid region of photoreceptors. Abbreviations: RPE, retinal pigmented epithelium ; OS, photoreceptor outer segments ; IS, photoreceptor inner segments ; ONL, outer nuclear layer ; INL, inner nuclear layer ; IPL, inner plexiform layer ; GCL, ganglion cell layer ; NFL, ganglion cells nerve fibers layer ; e, ellipsoid ; p, paraboloid ; my, myoid ; nu, nucleus ; cp, calycal process. Scale bar= 25µm. D shows a western blot analysis of YY1 (20µg protein per lane ; SDS-PAGE on 12.5% gels) in retinas from light- or dark-adapted chickens (n = 3 in each experimental group). The arrow shows the position of YY1. The additional band labelled with an asterisk is non-specific (ns).

Figure 4. Analysis of putative YY1-regulatory elements, by EMSA and transient

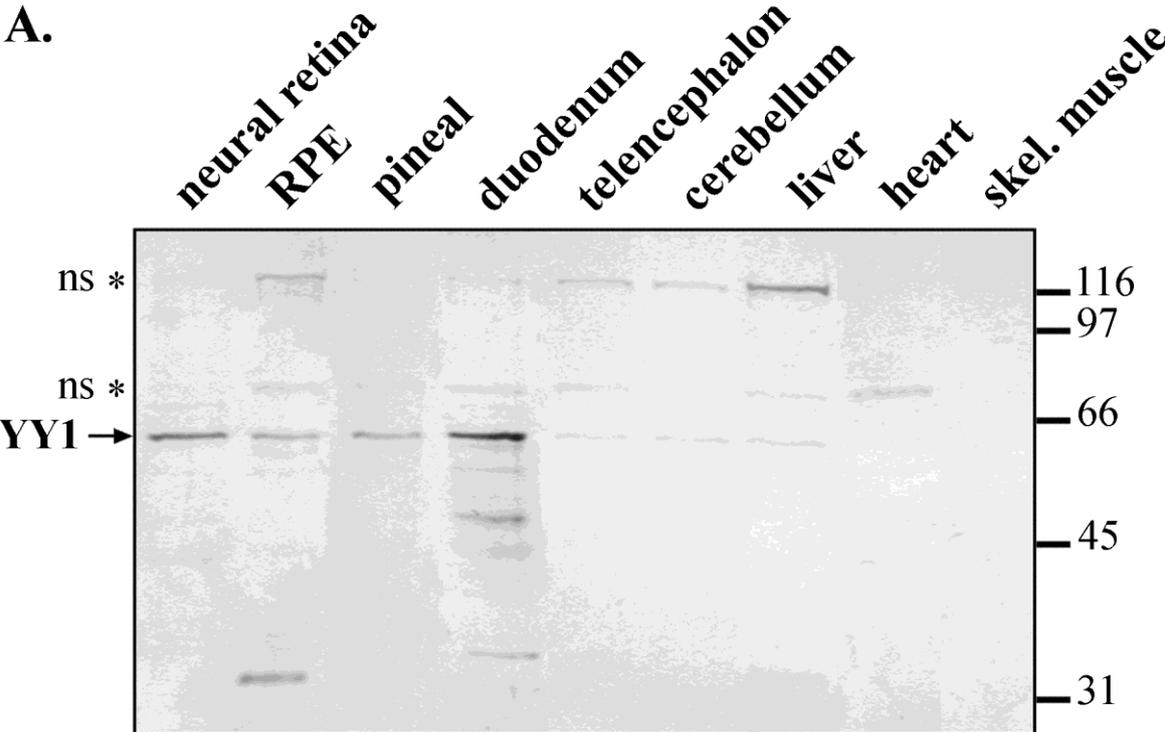
transfections. In A-D, ³²P-labelled double-stranded oligonucleotides corresponding to putative YY1-binding elements (see Table 1) were analyzed by EMSA. In A, 20 µg of proteins extracted from chicken retina were incubated with putative YY1-binding sites identified in the chicken HIOMT promoter (cH1 and cH2), in the chicken rhodopsin promoter (cRho) and in the chicken AANAT promoter (cNAT). The reactions were carried out in the

absence (-) or in the presence (+) of a 200-fold molar excess of unlabelled DNA competitor homologous to the labelled probe. Only cH1 shows a retarded band that is specifically displaced by the unlabelled competitor (arrow). In B, the cH1 labelled probe was incubated with 20 μ g of proteins from chicken retina, in the absence (-) or in the presence of increasing amounts (50-, 100-, 200- or 500-fold molar excesses) of unlabelled DNA competitor homologous to the labelled probe (cH1) or carrying a point mutation (cH1mut: CCATTT \rightarrow CCCTTT). The cH1mut DNA was also used as a 32 P-labelled probe, in the absence or presence of the homologous unlabelled competitor (200-fold molar excess). The retinal protein that binds cH1 can be saturated by unlabelled cH1 DNA, but does not bind unlabelled or labelled cH1mut. In C, retinal proteins were incubated with 32 P-labelled cH1, in the presence of the anti-YY1 antibody (α -YY1) or in the presence of normal rabbit IgG (ctrl-Ig). The binding of the retinal protein to cH1 is inhibited by the anti-YY1 antibody, but not by control immunoglobulins. A retarded band is also observed when the cH1 probe is incubated with *in vitro*-expressed chicken YY1 (arrow). This band is absent when the cH1 probe is incubated with the reticulocyte lysate alone (RL). In D, 32 P-labelled probes corresponding to putative YY1-binding sites identified in intron1 of the rat AANAT gene (rNATi) and in the human transducin promoter (hTrans) were tested by EMSA. When rNATi was incubated with proteins from rat retina and when hTrans was incubated with proteins from human Y79 retinoblastoma cells, retarded bands that were recognized by the anti-YY1 antibody but not by control immunoglobulins were observed. The bands pointed out by the long arrow have the same electrophoretic mobility as the one observed with cH1. A second band of higher mobility that is also recognized by the anti-YY1 antibody is observed with the rNATi probe (short arrow). This band might correspond to a truncated form of YY1 still containing the C-terminal domain. In E, left panel, HEK293 cells were cotransfected with 50 ng of a promoter-reporter vector containing 3kb of chicken HIOMT promoter (pGL3-HIOMT) and 2 μ g of

pcDNA3 plasmids expressing either chicken YY1 (pcDNA3-YY1) or rat Crx (pcDNA3-Crx). As a negative control, a group of HEK293 cells was cotransfected with pGL3-HIOMT and an empty pcDNA3 plasmid (not shown). In each experimental group, the cells were cotransfected with 1 ng of pRL-SV40, to correct for variations in transfection efficiency. In E, right panel, chicken embryonic retinal cells were transfected with 2 μ g of the wild-type promoter-reporter vector pGL3-HIOMT or with 2 μ g of pGL3-HIOMT(cH1mut), a pGL3-HIOMT vector carrying a mutation of the cH1 sequence that abolishes YY1-binding (see panel B). The retinal cells were cotransfected with 200 ng of pRL-SV40, to correct for variations in transfection efficiency. In HEK293 and retinal cells, *firefly* and *Renilla* luciferase activities were assayed 24h post-transfection. The ratios of *firefly*/*Renilla* luciferase activities were calculated and were expressed relative to (pGL3-HIOMT+ empty pcDNA3) in HEK293, and to pGL3-HIOMT in retinal cells. Histograms represent the mean \pm S.E.M. (n=3 in HEK cells ; n=12 in retinal cells). Overexpression of YY1 did not activate the HIOMT promoter in HEK293 cells, while overexpression of Crx did ($p < 0.05$ at the *Student's t*-test). In retinal cells the mutation of the YY1-binding site decreased the HIOMT promoter activity by 70% ($p < 0.005$ at the *Student's t*-test).

Figure 1

A.



B.

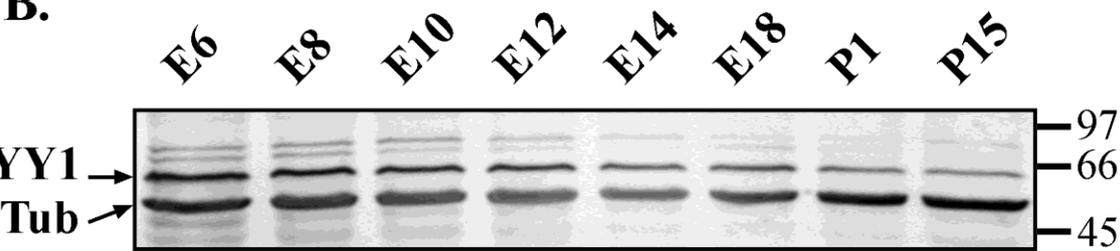


Figure 2

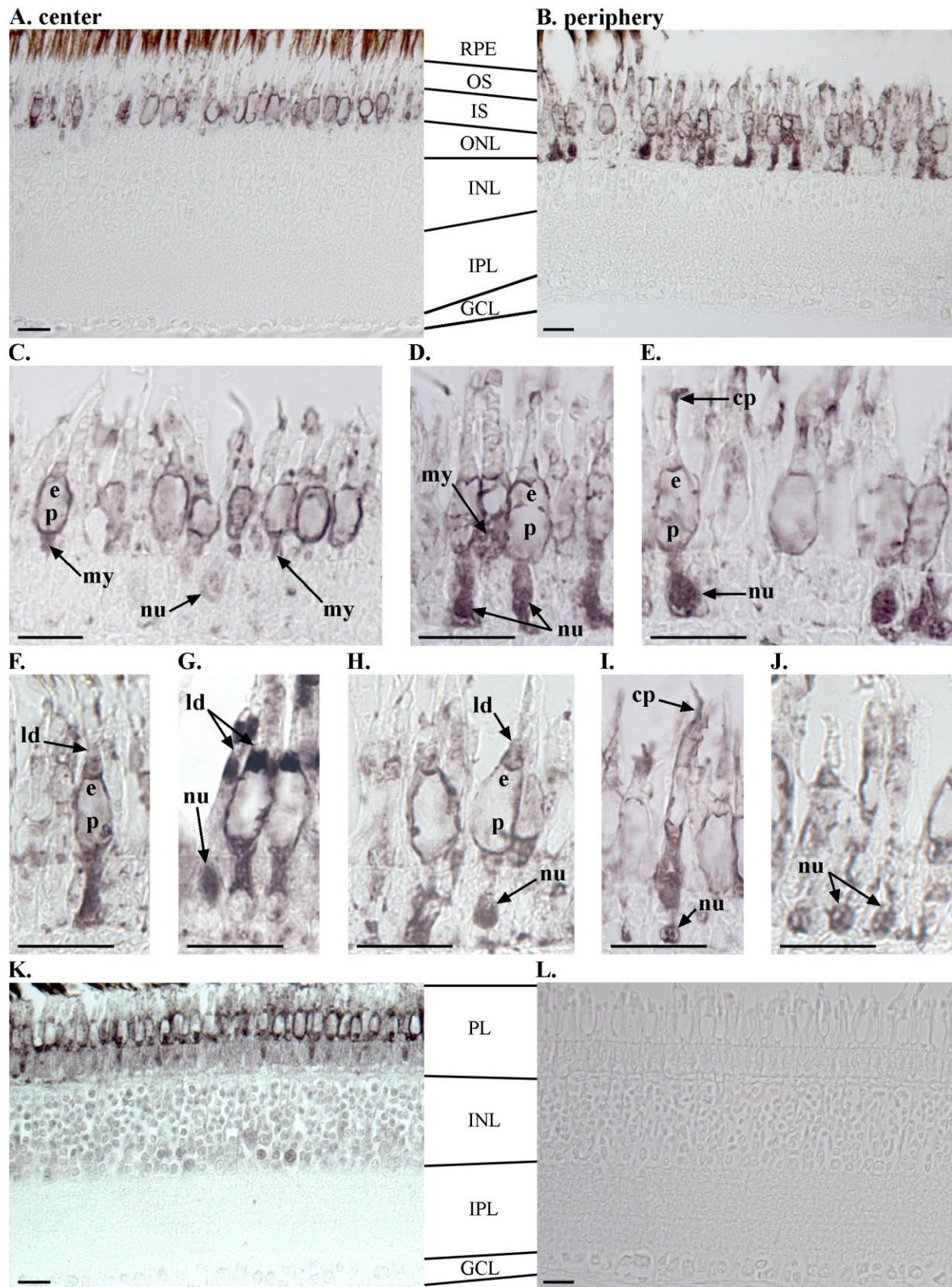
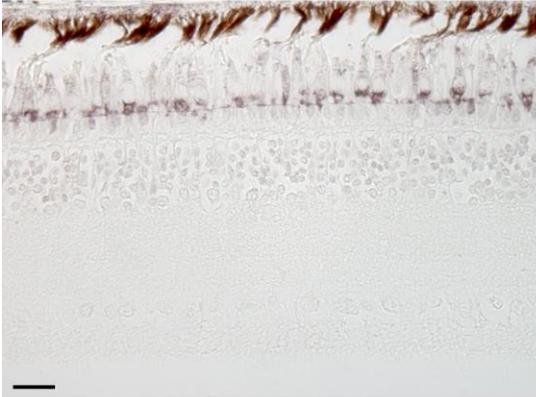


Figure 3

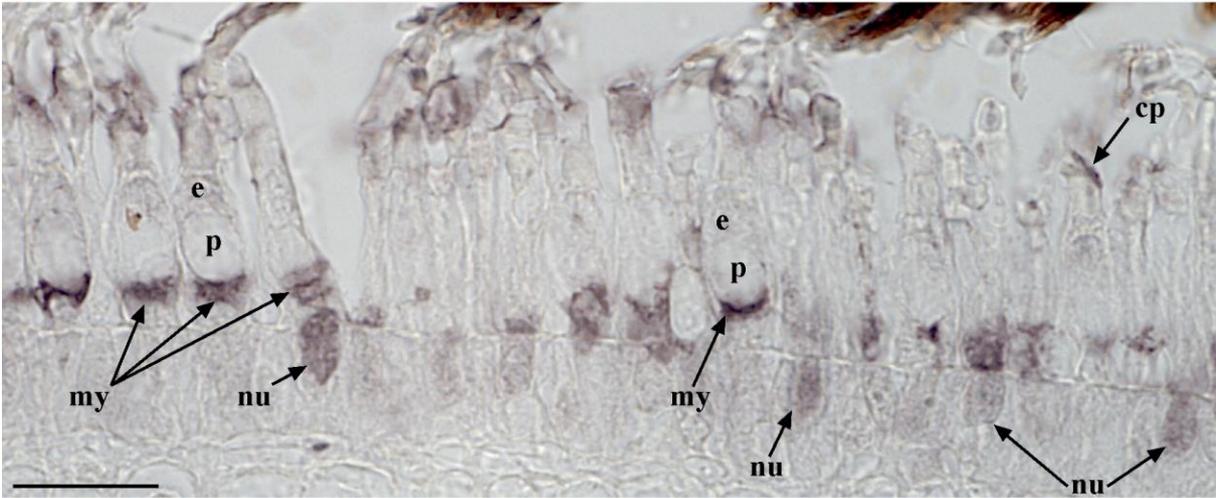
A. light-adapted



B. dark-adapted



C.



D. western blot

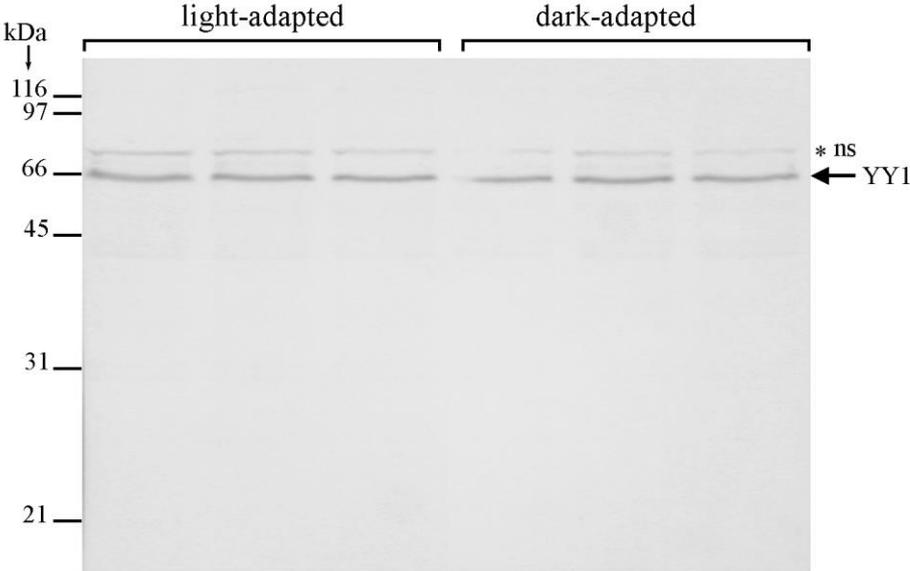


Figure 4

