

# Highly specific interactions between bHLH transcription factors and chromatin during retina development

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## Summary

**Basic helix-loop-helix (bHLH) transcription factors such as atonal homolog 5 (ATH5) and neurogenin 2 (NGN2) determine crucial events in retinogenesis. Using chromatin immunoprecipitation, we demonstrate that their interactions with target promoters undergo dynamic changes as development proceeds in the chick embryo. Chick ATH5 associates with its own promoter and with the promoter of the  $\beta$ 3 nicotinic receptor specifically in retinal ganglion cells and their precursors. NGN2 binds to the ATH5 promoter in retina but not in optic tectum, suggesting that interactions between bHLH factors and chromatin are highly tissue specific. The transcriptional**

**activations of both promoters correlate with dimethylation of lysine 4 on histone H3. Inactivation of the ATH5 promoter in differentiated neurons is accompanied by replication-independent chromatin de-methylation. This report is one of the first demonstrations of correlation between gene expression, binding of transcription factors and chromatin modification in a developing neural tissue.**

Supplemental data available online

Key words: Retinogenesis, Basic helix-loop-helix, Transcription, Chromatin modifications, Chick, CHRN3

## Introduction

Members of the basic helix-loop-helix (bHLH) superfamily of transcription factors have emerged as one of the major classes of positive and negative regulators of neural cell fate specification and differentiation (Anderson et al., 1997; Bertrand et al., 2002; Vetter and Brown, 2001). bHLH proteins share extensive homology within the basic and helix-loop-helix domains that mediate, respectively, binding to the DNA consensus sequence CANNTG, called an E-box, and dimerization. Both the heterogeneity of neural bHLH genes and their diversified expression patterns indicate that they mediate transcription of a large repertoire of genes within the developing and adult nervous systems. A pertinent question relates to the identity of the target genes that are directly regulated by these factors. Experiments performed in several laboratories have led to the identification and characterization of bHLH factors involved in retina genesis, including neurogenin 2 (NGN2) (Sommer et al., 1996), atonal homolog 5 (ATH5) (Kanekar et al., 1997; Matter-Sadzinski et al., 2001), NeuroM and NeuroD (Inoue et al., 2002; Marquardt et al., 2001), and others (Vetter and Brown, 2001). Among these, ATH5 has been shown to be involved in specification and differentiation of retinal ganglion cells (RGC). ATH5 is expressed almost exclusively in RGC precursors as well as in newborn RGCs and its targeted disruption in the mouse results in retinas that lack most RGCs and, consequently, in optic nerve agenesis (Brown et al., 2001; Wang et al., 2001).

The expression of ATH5 during chick retina development is

transient. The peak of activity of the ATH5 promoter coincides with the period when the majority of RGC precursors exit from the mitotic cycle and start to differentiate. In vitro and ex vivo approaches using chick embryos have suggested that ATH5 protein is involved in the regulation of its own expression exactly during this period, whereas NGN2 plays a role in the early activation of ATH5 (Matter-Sadzinski et al., 2001) (L. Matter-Sadzinski, M. Puzianowska-Kuznicka, J. Hernandez, M.B. and J.-M.M., unpublished). Additionally, the onset of expression of the  $\beta$ 3 subunit of a neuronal nicotinic acetylcholine receptor (nAChR; CHRN3 – Mouse Genome Informatics), a specific marker of RGC specification, appears to come under the control of ATH5 (Matter-Sadzinski et al., 2001).

We show that chromatin immunoprecipitation (ChIP) can be successfully adapted for use with developing central nervous system tissues, allowing the unequivocal identification of transcription factors that bind target promoters at different stages of development. Specifically, we use ChIP to monitor changes in the in vivo occupancy of target promoters by ATH5 and NGN2 during the course of retina development. We demonstrate that binding of the ATH5 protein to its own promoter as well as to the  $\beta$ 3 nAChR promoter coincides with the period of development when both genes are actively transcribed. We show that the differential occupancy of the ATH5 and  $\beta$ 3 promoters by NGN2 correlates well with its ability to activate these promoters. Moreover, we show a correlation between promoter activity and histone H3

hypermethylation on lysine 4 (K4), thus providing one of the first direct demonstrations that activation of neuron-specific promoters by bHLH transcription factors is associated with chromatin modifications known to reflect the transcriptional competence of target genes.

## Materials and methods

### Antibodies

Rabbit antibodies against chicken ATH5 (#AJ001178) and NGN2 (#AJ012659) were raised against bacterially expressed GST fusions of the N-terminal domains of each protein (ATH5: AA 1-39, NGN2 AA 1-79). Anti-THY1 antibody (French and Jeffrey, 1986) was kindly provided by Dr P. L. Jeffrey (Children's Medical Research Institute, Wentworthville, Australia). Anti-mouse FITC-conjugated antibody and anti dimethylated H3-K4 antibody were purchased, respectively, from Sigma (#F5897) and Upstate (#07-030).

### Chromatin immunoprecipitation

ChIP has been performed essentially as previously described (Takahashi et al., 2000) with some modifications. Dissected retinas and suspensions of immunopanned cells were incubated for 10 minutes at room temperature in 1% formaldehyde solution with douncing (J. Ripperger and U. Schibler, University of Geneva, unpublished). Crosslinking was stopped by the addition of glycine to a final concentration of 0.125 M. After washing with PBS, cells were rocked for 10 minutes at 4°C in a lysing solution containing 50 mM HEPES (pH 7.6), 140 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% NP-40, 0.25% Triton X-100 and protease inhibitors (10 µg/ml aprotinin, 1 µg/ml leupeptin). After centrifugation (5 minutes at 1800 g, 4°C), the pellet was resuspended in TE buffer containing 200 mM NaCl, 0.5 mM EGTA and protease inhibitors, and rocked for 10 minutes at room temperature. Nuclei were collected by centrifugation (for 5 minutes, at 1800 g, 4°C), resuspended in sonication buffer (1 mM EDTA, 0.5 mM EGTA, 10 mM Tris pH 8 and protease inhibitors) and sonicated on ice to an average DNA length of 700 bp. For immunoprecipitation, 10 µg (~3.5×10<sup>6</sup> cells from whole retina) or 5 µg (~2×10<sup>6</sup> immunopanned cells) of crosslinked chromatin were incubated in solutions [20 mM HEPES (pH 8), 200 mM NaCl, 2 mM EDTA, 0.1% NaDOC, 1% Triton X-100, 1 mg/ml BSA, 100 µg/ml salmon sperm DNA and protease inhibitors] containing either 20 µg of affinity-purified antibody (ATH5 or NGN2) or 2 µg of anti dimethylated K4 H3 antibody or the appropriate amount of rabbit preimmune serum as control. Immune complexes were captured for 2 hours at room temperature with protein A sepharose beads. Beads were washed seven times with modified RIPA buffer [50 mM HEPES (pH 7.6), 1 mM EDTA, 0.7% NaDOC, 1% NP-40, 0.5 M LiCl] and once with TE buffer. Immunoprecipitates were eluted from beads with 500 µl of 100 mM Tris pH 8, 1% SDS for 10 minutes at 65°C and digested with 100 µg of proteinase K in 200 mM NaCl for 2 hours at 42°C, and then overnight at 65°C to reverse crosslinks. DNAs were purified by phenol-chloroform extraction. DNA sequences present in the immunoprecipitates were quantified by real-time PCR using the primers listed below. Real-time PCR was performed using the iCycler iQ Real-Time PCR Detection System (BioRad) and a SYBR-Green based kit for quantitative PCR (iQ Supermix – BioRad). The amounts of immunoprecipitated DNA were calculated by comparison to a standard curve generated by serial dilutions of input DNA, subtracting values obtained with preimmune sera. The data were plotted as mean of at least two independent chromatin immunoprecipitation assays and three independent amplifications. Immunoprecipitation efficiency was calculated as the ratio of precipitated sequence over total sequence amount in input chromatin.

### Sequences and primers

The ATH5 and β3 genomic sequences are available, respectively, as

AJ630209 and X83740. The primers used for real-time amplifications (Fig. 1C) were as follows: ath5fwd, GCTGGGAAGGTACTGGGAT; ath5rev, CTTGACTGCCGTCGGAAGC; β3fwd, TTGCCTCACTTTGAATCCCAGAC; β3rev, GCTCCCTAAAGCACACTTCATTG; β3ORFfwd, GGCAGTATGGTGGACTTAATTC; β3ORFrev, CCTGTTGCCTTTCATACCTTTG; NeuroMfwd, TGCTGCTCCACCTGAGAGTTAATTG; NeuroMrev, CGGCGTGGATTAGGGTGT-TAATTAC; NeuroDfwd, AGCTGAACCCTGGCAGATG; NeuroDrev, AGCCTGGAGGTGCAATGTC.

### Immunopanning

Neuroretinas were dissected and cells dissociated as previously described (Matter-Sadzinski et al., 1992). Immunopanning was performed as described by Butowt et al. (Butowt et al., 2000), except that eight instead of five rounds of washing were applied after panning. Cells were detached from dishes by mild trypsinization (0.02%) and kept in suspension for crosslinking or seeded on plates covered with poly-L-ornithine for immunostaining.

### Immunostaining

Cells were fixed in 100% methanol for 4 minutes at –20°C. After 15 minutes of blocking with PBS containing 0.5% BSA and 0.1% Tween20, cells were incubated with anti-THY1 (0.25 mg/ml) antibody overnight at 4°C and revealed with FITC conjugated anti-mouse antibody (1:1000 dilution).

### In situ hybridization

In situ hybridizations on tissue sections and on dissociated cells were performed as previously described (Roztocil et al., 1997; Matter-Sadzinski et al., 2001).

## Results and discussion

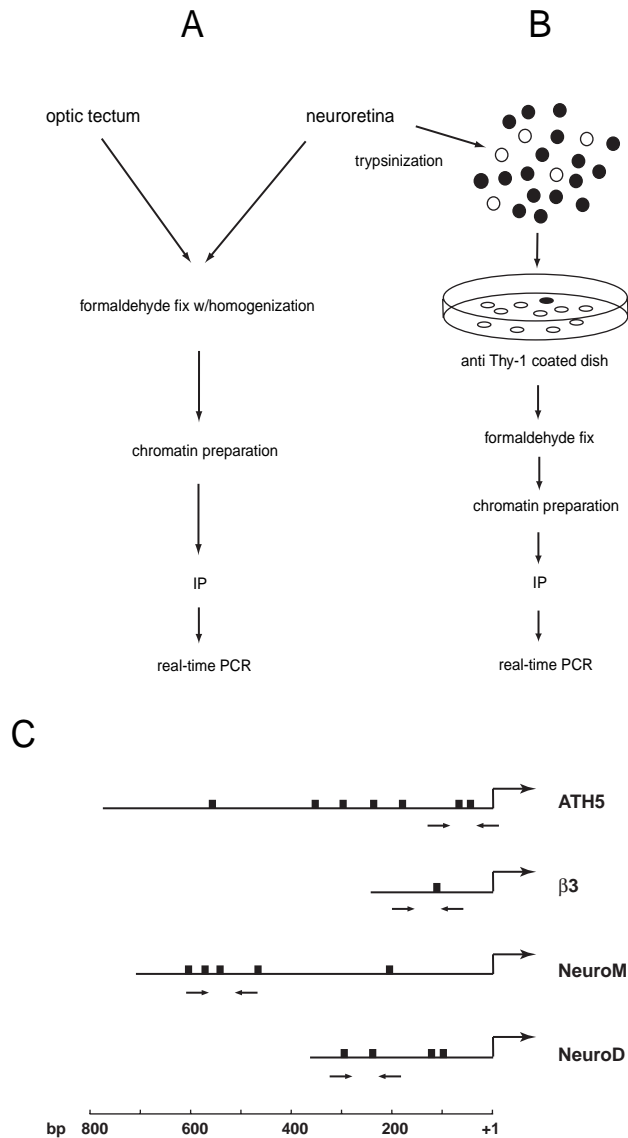
### The ATH5 and NGN2 bHLH proteins bind the ATH5 promoter during retina ontogenesis

An important challenge in the study of the regulation of transcription during neural development is the identification of the targets that are bound under physiological conditions by transcription factors known to be key regulators of cell fate specification and differentiation. We have focused our study on the *ATH5* gene, which offers several advantages in addressing this issue: (1) it is specifically required for the differentiation of RGCs, (2) the transient expression of the *ATH5* gene is regulated at the transcriptional level and (3) the functional properties of the *ATH5* promoter have been characterized. The onset of *ATH5* expression in the chick retina is detected at day 2 of embryonic development (E2), but expression remains low until E5. *ATH5* mRNA levels rapidly increase around E5, peak at E6 and then decrease rather abruptly to low values by E9 and beyond. We have isolated and characterized ~800 bp of the promoter of *ATH5* gene. The region encompasses seven E-boxes, four of which are essential for proper promoter activity (Fig. 1C; J. Hernandez, L. Matter-Sadzinski, D.S.-K., J.-M.M. and M.B., unpublished). Functional analyses of the *ATH5* promoter indicate that both the ATH5 and NGN2 proteins are involved in the regulation of *ATH5* transcription (Matter-Sadzinski et al., 2001) (L. Matter-Sadzinski, M. Puzianowska-Kuznicka, J. Hernandez, M.B. and J.-M.M., unpublished; Fig. 1C; J. Hernandez, L. Matter-Sadzinski, D.S.-K., J.-M.M. and M.B., unpublished). To determine if these two factors bind the *ATH5* promoter during retina development, we prepared chromatin from retina at E3 to E12 and performed ChIP using antibodies directed against the N-terminal domains of ATH5

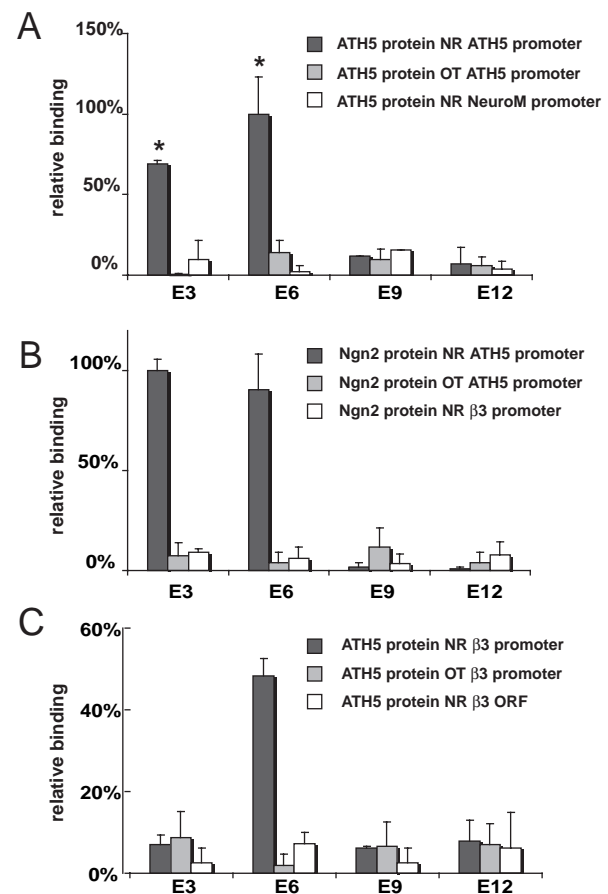
and NGN2 (Fig. 1A). Importantly, these antibodies do not crossreact with other bHLH transcription factors (see Fig. S1 at <http://dev.biologists.org/cgi/content/full/131/18/4447/DC1>). The enrichment of the target DNA sequences in the immunoprecipitates was quantified by real-time PCR. It should be noted that the resolution of the technique (average size of DNA fragments ~700 bp) allows overall detection of promoter occupancy but does not allow the determination of which out

of several closely spaced E-boxes is bound by the targeted transcription factor.

In retina, ATH5 is bound to its own promoter both at E3 and at E6, but not at E9 or at E12 (Fig. 2A). In situ hybridization on tissue sections and on dissociated cells has shown that similar proportions of retinal cells express ATH5 at E2.5 and E6 (Fig. 3). The mildly enhanced binding detected at E6 may thus reflect an increased involvement of ATH5 in its own transcription at this stage. This pattern of association coincides with the period of development when ATH5 promoter activity is highest and is consistent with functional data suggesting that ATH5 protein stimulates the activity of its own promoter more efficiently in E6 than in E3 retina. At E9, the ATH5 promoter is downregulated, while at E12 the promoter is turned off (Matter-Sadzinski et al., 2001) (L. Matter-Sadzinski, M. Puzianowska-Kuznicka, J. Hernandez, M.B. and J.-M.M., unpublished). The specificity of the ATH5 immunoprecipitation reaction was confirmed by performing



**Fig. 1.** Schematic representation of chromatin immunoprecipitation experiments. (A) Outline of ChIP experiment in retina and optic tectum. Dissected tissues were homogenized in the presence of formaldehyde. Crosslinked chromatin was prepared using a standard procedure, sonicated and incubated with appropriate antibodies. Specific DNA fragments in immunoprecipitates were quantified by real-time PCR. (B) Retinal ganglion cells (RGC) isolated using panning with anti-THY1 antibody. Dissociated retinal cells expressing THY1 were retained on dishes coated with anti-THY1 antibody and fixed with formaldehyde. Fixed cells were processed for ChIP as in A. (C) Schematic representation of upstream regions of the analyzed genes. The black squares are E-boxes and arrows indicate the primers used for amplification.



**Fig. 2.** In vivo occupancy of neuronal-specific promoters by ATH5 and NGN2 as a function of developmental stage. Antibodies directed against ATH5 (A,C) and NGN2 (B) were used to immunoprecipitate crosslinked chromatin fragments prepared from E3 to E12 neuroretinas and optic tecta. Immunoprecipitates were analyzed for the abundance of ATH5 (A,B),  $\beta 3$  (B,C), NeuroM (A) regulatory sequences and  $\beta 3$  ORF (C) by real-time PCR. Data are normalized relative to ATH5 promoter occupancy by ATH5 protein (A,C) in E6 retina (IP efficiency: 0.08%), and for NGN2 protein (B) in E3 retina (IP efficiency: 0.08%). NR, neuroretina; OT, optic tectum. \* $P=0.02$ , Student's *t*-test.

ChIP experiments with chromatin isolated from optic tectum, a tissue in which *ATH5* is not expressed (Fig. 2A). In addition, as there is no evidence suggesting that *NeuroM* can be directly regulated by *ATH5*, we have monitored the enrichment in *ATH5* immunoprecipitates of the *NeuroM* upstream region, which contains several E-boxes (Fig. 1C; J. Hernandez and M.B., unpublished). The absence of *ATH5* on the *NeuroM* promoter (Fig. 2A) is further evidence for the specificity of the procedure.

*NGN2* plays an important role in inducing expression of the *ATH5* gene. It has been shown that both the *NGN2* and *ATH5* genes are expressed in the same subset of retinal cells. Moreover, electroporation and transfection experiments have demonstrated that *NGN2* is able to activate transcription from the *ATH5* promoter when ectopically expressed in E3 to E6 retinal cells (Matter-Sadzinski et al., 2001) (L. Matter-Sadzinski, M. Puzianowska-Kuznicka, J. Hernandez, M.B. and J.-M.M., unpublished). These studies, however, have not excluded the possibility that *NGN2* may control *ATH5* promoter activity indirectly rather than by a direct interaction. To determine if *NGN2* binds directly to the *ATH5* promoter, we have performed ChIP experiments with chromatin isolated from E3-E12 retina. The results indicate that *NGN2* binds efficiently to the *ATH5* promoter at E3 and E6 but not at E9 and E12 (Fig. 2B), which is in good agreement with our previous *NGN2* overexpression experiments. Interestingly, no binding of *NGN2* to the *ATH5* promoter was detected in chromatin isolated from optic tectum (Fig. 2B), although *NGN2* is expressed in this tissue, thus demonstrating a correlation between binding by *NGN2* and activation of the *ATH5* promoter. In addition, this result provides evidence

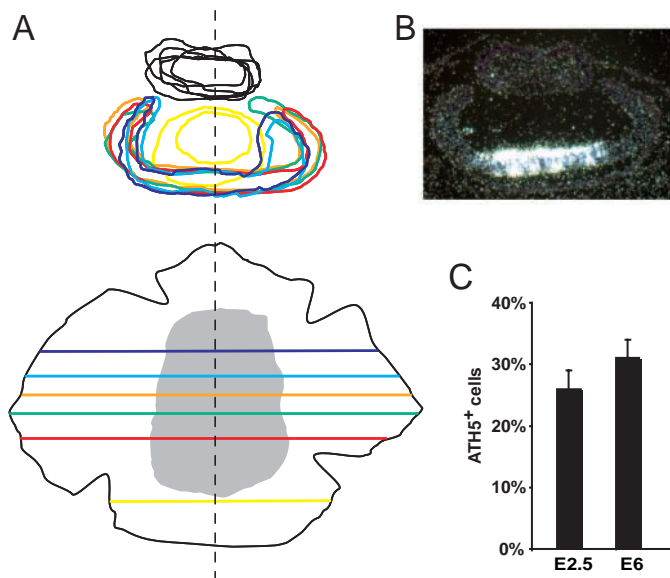
suggesting that *NGN2* is recruited to the *ATH5* promoter by neuroretina-specific factors.

Although essential regulatory elements (e.g. E-boxes) are very well conserved in the chicken (GenBank AJ630209) and mouse (Brown et al., 2002) *ATH5* regulatory regions, suggesting that distant vertebrate species may use quite similar strategies to regulate *ATH5*, there are reported differences in the *ATH5* and *NGN2* expression patterns of birds and mammals. In the developing mouse retina, the studies by Brown et al. (Brown et al., 2001) and Wang et al. (Wang et al., 2001) show that autoregulation is not required for mouse *ATH5* expression. Using a *lacZ* reporter assay, Yang et al. (Yang et al., 2003) argue that mouse *ATH5* expression is restricted to post-mitotic cells, in contradiction with the work of Brown et al. (Brown et al., 1998) and Marquardt et al. (Marquardt et al., 2001) who find that mouse *NGN2* and *ATH5* are expressed in retinal progenitor cells. At this point, it is possible to argue that the regulation of *ATH5* differs in birds and mammals, based on the 48 hours delay separating the onset of *ATH5* and *NGN2* expression in the mouse (Brown et al., 1998) and the quasi-simultaneous onsets in the chick (Matter-Sadzinski et al., 2001).

### The $\beta 3$ nAChR promoter is transiently and selectively bound by *ATH5*

The  $\beta 3$  subunit of the neuronal nicotinic receptor is selectively expressed in the RGCs and expression of the  $\beta 3$  gene starts as soon as retinal precursor cells are committed to the RGC fate (Matter et al., 1995). Functional analysis of the  $\beta 3$  promoter indicates that *ATH5* regulates the  $\beta 3$  gene in the developing retina (Matter-Sadzinski et al., 2001). To examine whether *ATH5* binds to the  $\beta 3$  promoter, we performed ChIP experiments with chromatin isolated from E3-E12 retinas. Remarkably, although binding of *ATH5* to the  $\beta 3$  promoter was undetectable in chromatin isolated from retinas at E3, E9 and E12, it was readily detected at E6 (Fig. 2C). A single E-box is implicated in the regulation of the  $\beta 3$  promoter (Fig. 1C) (Roztocil et al., 1998) and *ATH5* was bound exclusively to sequences encompassing this region, but not to coding sequences located downstream of the transcription start site (ORF) (Fig. 2C). Control ChIP experiments indicated that *ATH5* binding was nearly undetectable in chromatin derived from the optic tectum (Fig. 2C). The transient binding of *ATH5* on the  $\beta 3$  promoter in E6 retina is in agreement with previous studies showing that *ATH5* has the capacity to transactivate the  $\beta 3$  promoter during the rather narrow period of development when RGCs are generated, but not at later stages of development (Matter-Sadzinski et al., 2001). Taken together, these data suggest that *ATH5*-regulated transcription of the  $\beta 3$  gene in RGC precursors and in newborn RGCs correlates well with binding of *ATH5* protein to the  $\beta 3$  promoter. Furthermore, as *ATH5* is not expressed in fully differentiated RGCs, transcription of the  $\beta 3$  gene in these neurons must be directed by another, uncharacterized factor.

Previous functional studies have shown that the  $\beta 3$  promoter is efficiently activated by *ATH5*, but not by *NGN2* (Matter-Sadzinski et al., 2001). To elucidate the mechanism of this discrimination, we have examined the  $\beta 3$  promoter by ChIP using an antibody raised against *NGN2*. Although *NGN2* is expressed in these cells, there was no enrichment of the  $\beta 3$  promoter region in the *NGN2* immunoprecipitates (Fig. 2B),



**Fig. 3.** *ATH5*-expressing cells in the developing chick retina. (A,B) Serial transverse sections at stage E2.5 were hybridized with an *ATH5*-specific antisense riboprobe. *ATH5* is expressed in the central retina (gray) in a domain corresponding to ~30% of the surface of the retina. (C) Cell counting reveals that ~88% of the cells in the central domain express *ATH5*, representing ~26% of the total retinal cell population. The dissociated cells of E6 retinas were hybridized with an *ATH5*-specific antisense riboprobe and the ration of labeled to unlabeled cells was determined.

demonstrating that NGN2 protein is not bound to the  $\beta 3$  promoter in developing retina and further documenting the remarkable specificity with which key bHLH transcription factors interact with target genes in neuronal cells.

### Histone methylation correlates positively with activity of the ATH5 and $\beta 3$ promoters

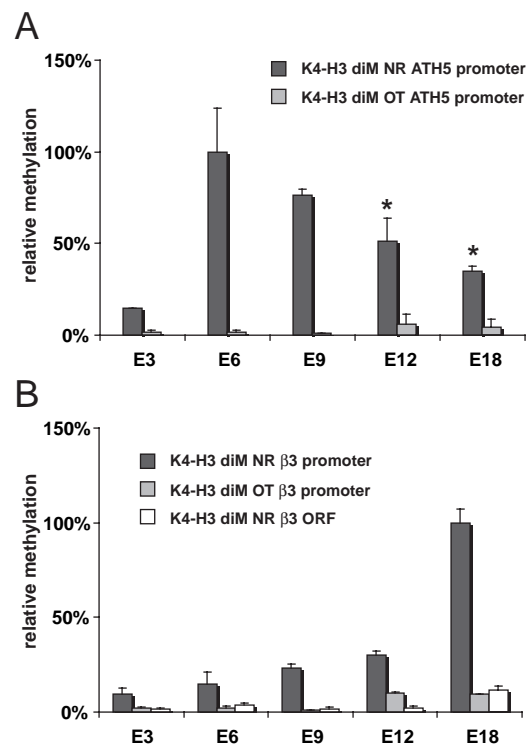
Even though there are numerous studies on gene expression in the nervous system, very few have addressed the issue of chromatin structure modification in relation to transcriptional competence [see Guan et al. (Guan et al., 2002), for pioneering work in the context of memory storage]. Essentially nothing is known about histone modification within the promoter regions of genes involved in CNS development. We decided to monitor lysine 4 (K4) dimethylation of histone H3, a modification known to reflect the transcriptional competence of several genes involved in differentiation (Kouzarides, 2002) and development (Litt et al., 2001). We performed immunoprecipitation of chromatin isolated from retinas and optic tecta using an anti-dimethylated K4 H3 antibody (H3-K4). As depicted in Fig. 4A, we observed a selective enrichment of ATH5 promoter sequences in the immunoprecipitates at all stages of retina development (E3–E18). Strikingly, enrichment peaks at E6 and then decreases at a steady rate to low levels by E18, in exact register with the reported kinetics of ATH5 promoter activity (Matter-Sadzinski et al., 2001). In the developing optic tectum, where the ATH5 gene is not expressed, H3-K4 dimethylation was not detected on the ATH5 promoter (Fig. 4A). Methylation, however, decreases in retina at a much slower rate than promoter activity. Histone demethylation is known to proceed at a slow rate and to be dependent on mechanisms that can be either replication dependent or replication independent (Bannister et al., 2002). All RGCs are born before E12 (Prada et al., 1991), strongly suggesting that the operating mechanism of de-methylation in developing retinal cells is replication independent.

Another positive correlation between methylation and transcriptional activity was detected on the  $\beta 3$  promoter (Fig. 4B), where methylation reaches its highest level in E18 retina and is lowest in E3–E6 retinas. This correlates very well with accumulation of  $\beta 3$  mRNA in the developing retina during development and with its continued presence in the mature retina (Hernandez et al., 1995). By contrast, the transient stimulation of  $\beta 3$  expression regulated by ATH5 at E6 does not correlate with histone methylation of the  $\beta 3$  promoter region. We surmise that the proportion of retinal cells that express  $\beta 3$  at this stage of development might be too low for detection by our assay. When it occurred, methylation was detected only on the promoter region but not downstream of the transcription start site (Fig. 4B), suggesting its relevance for transcriptional regulation. The same ChIP analysis performed in parallel on chromatin isolated from optic tecta, where the  $\beta 3$  gene is not expressed, did not show any enrichment of the  $\beta 3$  regulatory sequences in H3-K4 immunoprecipitates (Fig. 4B).

### Histone H3 methylation correlates with transcriptional competence

To determine whether the correlation between promoter activity and histone H3-K4 dimethylation is a general phenomenon, it was of interest to analyze the methylation patterns of genes expressed both in the retina and in the optic

tectum. NeuroM and NeuroD are good candidates for this study as they are dynamically expressed in both tissues. In the optic tectum, the transient expression of NeuroM peaks at E6, at a time when the various cell classes exit from the mitotic cycle. In the retina, NeuroM expression obeys the same principle as in the optic tectum; however, expression does not stop at the end of neurogenesis but persists in mature bipolar and horizontal cells (Roztocil et al., 1997). In the optic tectum and retina, NeuroD has a later onset than NeuroM. In early (E4–E6) retina, expression of NeuroD is detected in precursor cells (Roztocil et al., 1997) and may correlate at later stages with the differentiation of photoreceptors and amacrine cells (Morrow et al., 1999). In the optic tectum, NeuroD expression is detected at around E6 and increases slowly during development of the tissue (Roztocil et al., 1997). We performed immunoprecipitation of chromatin from retina and optic tectum using an anti dimethylated H3-K4 antibody and observed correlations in both tissues between histone methylation and the known expression patterns of NeuroM and NeuroD (Fig. 5). In retina, methylation of the NeuroM promoter is detected at E3 and reaches its highest level at E9 (Fig. 5A). It remains high in the developed retina, in accordance with the sustained NeuroM mRNA expression seen in this tissue (Roztocil et al., 1997). In the optic tectum, the transient expression of this gene is at a much lower level than in the retina (Roztocil et al., 1997) and no significant

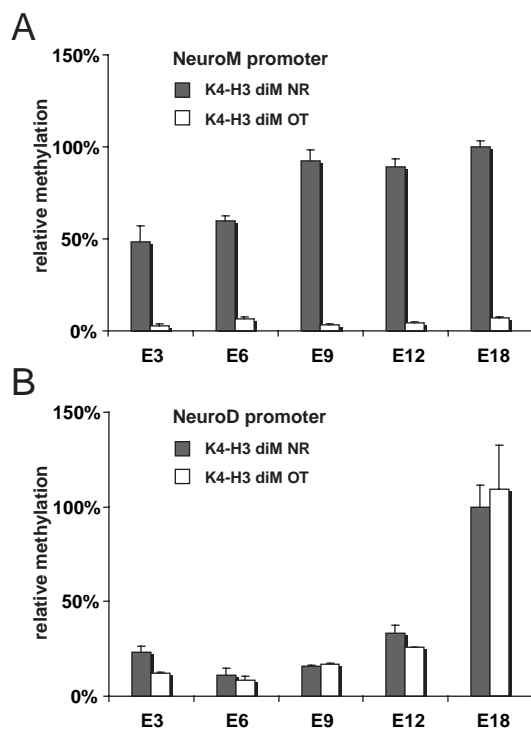


**Fig. 4.** Histone H3 K4 methylation on the ATH5 and  $\beta 3$  promoters during retina and optic tectum development. Chromatin from E3–E18 retinas and optic tecta were immunoprecipitated with an antibody specific for the dimethylated K4 of histone H3. ATH5 and  $\beta 3$  promoters sequences in the precipitates were quantified by real-time PCR. Methylation levels are shown relative to E6 retina in A for the ATH5 promoter (IP efficiency, 0.7%) and to E18 retina in B for the  $\beta 3$  promoter (IP efficiency, 1.1%). \* $P=0.02$ , Student's *t*-test.

enhancement of methylation was detected (Fig. 5A). This could reflect the fact that the fraction of tectal cells that express NeuroM is too small to be detected in our assay, or it may suggest different histone modification requirements for brief versus continuous expression of the gene. The level of methylation of NeuroD promoter sequences remained very low during retina and optic tectum development, but was strongly enhanced in the developed retina and optic tectum (Fig. 5B). This delayed methylation of the NeuroD promoter is congruent with the late onset of NeuroD expression in both tissues. Incidentally, our ability to detect H3 methylation at the NeuroD promoter in both retina and optic tectum demonstrates that the paucity in optic tectum methylation we observe for other promoters is physiologically relevant and not due to a tissue-specific bias in chromatin quality.

### Histone methylation positively correlates with gene activity in RGCs

In the developing retina, both the ATH5 and the  $\beta 3$  promoters are expressed in RGCs and their precursors (Matter et al., 1995; Matter-Sadzinski et al., 2001). We were thus concerned that histone modifications observed in chromatin isolated from whole retina may not necessarily reflect changes that occur on promoters in the RGC lineage. At E9, a significant fraction of the RGCs that have already reached the ganglion cell layer are still expressing ATH5, whereas expression is suppressed in the vast majority of RGCs at E12 (Matter-Sadzinski et al., 2001).

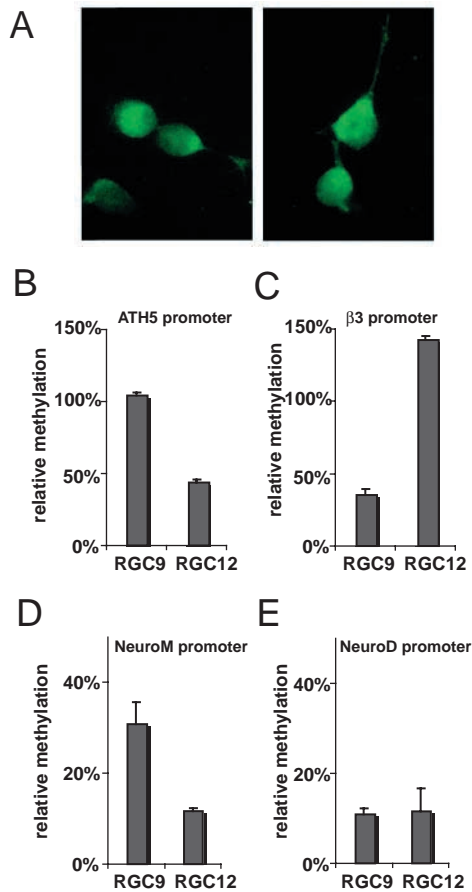


**Fig. 5.** Methylation of histone H3 at NeuroM and NeuroD promoters as a function of retina and optic tectum developmental stage. Quantitative ChIP experiments were performed with an antibody specific for the dimethylated K4 of histone H3. Results are normalized relative to the value observed for E18 retina [NeuroM promoter (A), IP efficiency 1.2%; NeuroD promoter (B), IP efficiency 0.2%].

$\beta 3$  mRNA is first detected by northern blot and in situ hybridization at E6 and steadily accumulates in RGCs to peak at E12, when the majority of RGCs have completed their differentiation (Hernandez et al., 1995; Matter et al., 1995). We dissociated E9 and E12 retinal cells and used plates coated with an anti-THY1 antibody (Butow et al., 2000) (see Materials and methods) to select a population much enriched in RGCs (Fig. 1B). THY1 expression commences in RGCs that are completing differentiation (Sheppard et al., 1988) and the proportion of THY1-expressing RGCs was expected to be high enough at these stages to allow retrieval of sufficient amounts of chromatin. We have calculated an eightfold enrichment in RGCs after immunopanning (~80% purity) at E9 and E12 (Fig. 6A). Next, we conducted a ChIP analysis on the chromatin of the immunopanned cells, monitoring (as before) the increased levels of the ATH5 and  $\beta 3$  promoters in dimethylated H3 immunoprecipitates. We found that the methylation changes in RGCs (Fig. 6B,C) accurately reflected those previously seen in chromatin from whole retina (Fig. 4), thereby strengthening the conclusions drawn using whole retina chromatin. However, we noted that the differences between E9 and E12 levels of methylation of the ATH5 and  $\beta 3$  promoters were much higher when the chromatin was isolated from RGCs. We believe that these differences reflect the experimental procedure that we used to enrich in RGCs. The onset of THY1 expression takes place when RGCs are completing differentiation and coincides with the period of development when expression of ATH5 is downregulated, so that only a small fraction of RGCs should co-express THY1 and ATH5. Because the majority of THY1-expressing RGCs complete their differentiation between E9 and E12, it is likely that the fraction of cells that express ATH5 would be selectively diminished in the enriched population of THY1-expressing cells relative to the total population of retinal cells. Moreover, THY1-expressing cells selected at E9 and E12 are newborn RGCs. The significant de-methylation detected between these two stages thus occurs in cells that have exited the mitotic cycle (Fig. 6B). Taken together, these results suggest that de-methylation of the ATH5 promoter is replication independent and correlates very well with the decreased promoter activity seen in RGCs. Likewise, the strong increase in the methylation of the  $\beta 3$  promoter that we detect between E9 and E12 in immunopanned cells when compared with whole retina most probably reflects the strong enrichment in differentiated RGCs, the cell type expressing  $\beta 3$  (Fig. 6C).

Although NeuroM is transiently expressed in newborn RGCs, its expression persists in other retinal cell types until adulthood (Roztocil et al., 1997). To compare the pattern of methylation of the NeuroM promoter in RGCs and in other retinal cells, we quantified the NeuroM regulatory sequence in dimethyl K4-H3 immunoprecipitates from RGC chromatin. Methylation at both E9 and E12 (Fig. 6D) was much lower than in experiments using whole retina (Fig. 5A), suggesting that methylation of the NeuroM promoter in RGCs represents only a small fraction of the methylation observed in whole retinas. Whereas the level of methylation decreased in RGCs between E9 and E12, it remained stable in whole retina during the same period. These results are consistent with the differential expression patterns of NeuroM in RGCs and in other retinal cell types at these stages.

We have also monitored methylation of the NeuroD promoter region in enriched populations of RGCs. The low and



**Fig. 6.** Histone H3 methylation monitored in isolated RGCs. (A) THY1 immunostaining of ganglion cells purified with anti-THY1 antibody. (B-E) Chromatin from purified RGCs was immunoprecipitated with an antibody directed against the dimethylated K4 of histone H3. Enrichments of ATH5 (B),  $\beta 3$  (C), NeuroM (D) and NeuroD (E) promoter sequences are normalized relative to the value observed for E6 (B) or E18 (C-E) in the corresponding whole retina experiment.

constant methylation that we detected at E9 and E12 (Fig. 6E) may reflect the weak expression of NeuroD in newborn RGCs (Roztocil et al., 1997). We suggest that the significant increase in methylation detected in whole retina between E9 and E12 (Fig. 5B) mostly reflects NeuroD expression in photoreceptors (Roztocil et al., 1997; Yan and Wang, 1998).

## Conclusions

In this report, we show that ChIP can indeed be used to address basic questions regarding functional interactions between transcription factors and their target genes within the developing central nervous system. The method is capable of identifying highly specific interactions between bHLH proteins and neuron-type specific promoters in a native chromatin environment. Moreover, we show that ChIP can be applied to study chromatin modifications in the promoter regions of genes expressed in selected subclasses of retinal neurons. Specifically, we demonstrate that, *in vivo*, the ATH5 factor directly interacts with the regulatory sequences of the  $\beta 3$  and *ATH5* genes. Our results further indicate that stable interactions

between bHLH proteins and specific regulatory elements occur only when these factors are engaged in regulating transcriptional activity. The demonstration that ATH5 binds the  $\beta 3$  promoter, whereas NGN2 does not, suggests that the remarkable property of the  $\beta 3$  promoter to discriminate between bHLH proteins is most probably regulated, *in vivo*, at the level of DNA binding. The specific binding of NGN2 to the *ATH5* promoter in neuroretina suggests that recruitment of NGN2 to this regulatory element involves tissue-specific factors. The fact that both NGN2 and ATH5 bind the *ATH5* promoter is congruent with our mutational and functional analysis showing that both proneural proteins require the same E-boxes to mediate their effect upon the promoter (Matter-Sadzinski et al., 2001) (Fig. 1C; J. Hernandez, L. Matter-Sadzinski, D.S.-K., J.-M.M. and M.B., unpublished). We surmise that they are acting in dynamic equilibrium and that the preponderance of one protein over the other may change in the course of development. Increased binding of ATH5 at E6 may reflect the fact that autostimulation is prevalent in driving promoter activity at this stage. We have also noted a remarkable coincidence between the expression patterns of the *ATH5*, *NeuroM*, *NeuroD* and  $\beta 3$  genes, and the histone methylation of their promoters during development. These congruent events are one of the first demonstrations that gene expression in the developing nervous system is associated with major changes in histone modification. Furthermore, we note a striking correlation between diminished levels of dimethylated K4 on histone H3 and diminished expression, suggesting that an uncharacterized, replication independent mechanism may operate in neurons to remove histone modification and dampen gene expression.

The correlation that we found between gene expression, binding of transcription factors and chromatin modification indicates that ChIP is a powerful and robust tool with which to investigate neural gene regulation. The approach should be easily extended to the developing and adult nervous tissues of other vertebrate species. Moreover, ChIP and DNA arrays may be combined into a unique tool to screen for new target of transcription factors known to play key roles in the neural development and maintenance of the differentiated state.

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## References

- Anderson, D. J., Groves, A., Lo, L., Ma, Q., Rao, M., Shah, N. M. and Sommer, L. (1997). Cell lineage determination and the control of neuronal identity in the neural crest. *Cold Spring Harb. Symp. Quant. Biol.* **62**, 493-504.
- Bannister, A. J., Schneider, R. and Kouzarides, T. (2002). Histone methylation: dynamic or static? *Cell* **109**, 801-806.
- Bertrand, N., Castro, D. S. and Guillemot, F. (2002). Proneural genes and the specification of neural cell types. *Nat. Rev. Neurosci.* **3**, 517-530.
- Brown, N. L., Kanekar, S., Vetter, M. L., Tucker, P. K., Gemza, D. L. and Glaser, T. (1998). *Math5* encodes a murine basic helix-loop-helix

- transcription factor expressed during early stages of retinal neurogenesis. *Development* **125**, 4821-4833.
- Brown, N. L., Patel, S., Brzezinski, J. and Glaser, T.** (2001). Math5 is required for retinal ganglion cell and optic nerve formation. *Development* **128**, 2497-2508.
- Brown, N. L., Dagenais, S. L., Chen, C. M. and Glaser, T.** (2002). Molecular characterization and mapping of ATOH7, a human atonal homolog with a predicted role in retinal ganglion cell development. *Mamm. Genome* **13**, 95-101.
- Butowt, R., Jeffrey, P. L. and von Bartheld, C. S.** (2000). Purification of chick retinal ganglion cells for molecular analysis: combining retrograde labeling and immunopanning yields 100% purity. *J. Neurosci. Methods* **95**, 29-38.
- French, P. W. and Jeffrey, P. L.** (1986). Partial characterization of chicken Thy-1 glycoprotein by monoclonal antibodies. *J. Neurosci. Res.* **16**, 479-489.
- Guan, Z., Giustetto, M., Lomvardas, S., Kim, J. H., Miniaci, M. C., Schwartz, J. H., Thanos, D. and Kandel, E. R.** (2002). Integration of long-term-memory-related synaptic plasticity involves bidirectional regulation of gene expression and chromatin structure. *Cell* **111**, 483-493.
- Hernandez, M. C., Erkman, L., Matter-Sadzinski, L., Roztocil, T., Ballivet, M. and Matter, J. M.** (1995). Characterization of the nicotinic acetylcholine receptor beta 3 gene. Its regulation within the avian nervous system is effected by a promoter 143 base pairs in length. *J. Biol. Chem.* **270**, 3224-3233.
- Inoue, T., Hojo, M., Bessho, Y., Tano, Y., Lee, J. E. and Kageyama, R.** (2002). Math3 and NeuroD regulate amacrine cell fate specification in the retina. *Development* **129**, 831-842.
- Kanekar, S., Perron, M., Dorsky, R., Harris, W. A., Jan, L. Y., Jan, Y. N. and Vetter, M. L.** (1997). Xath5 participates in a network of bHLH genes in the developing *Xenopus* retina. *Neuron* **19**, 981-994.
- Kouzarides, T.** (2002). Histone methylation in transcriptional control. *Curr. Opin. Genet. Dev.* **12**, 198-209.
- Litt, M. D., Simpson, M., Gaszner, M., Allis, C. D. and Felsenfeld, G.** (2001). Correlation between histone lysine methylation and developmental changes at the chicken beta-globin locus. *Science* **293**, 2453-2455.
- Marquardt, T., Ashery-Padan, R., Andrejewski, N., Scardigli, R., Guillemot, F. and Gruss, P.** (2001). Pax6 is required for the multipotent state of retinal progenitor cells. *Cell* **105**, 43-55.
- Matter, J. M., Matter-Sadzinski, L. and Ballivet, M.** (1995). Activity of the beta 3 nicotinic receptor promoter is a marker of neuron fate determination during retina development. *J. Neurosci.* **15**, 5919-5928.
- Matter-Sadzinski, L., Hernandez, M. C., Roztocil, T., Ballivet, M. and Matter, J. M.** (1992). Neuronal specificity of the alpha 7 nicotinic acetylcholine receptor promoter develops during morphogenesis of the central nervous system. *EMBO J.* **11**, 4529-4538.
- Matter-Sadzinski, L., Matter, J. M., Ong, M. T., Hernandez, J. and Ballivet, M.** (2001). Specification of neurotransmitter receptor identity in developing retina: the chick ATH5 promoter integrates the positive and negative effects of several bHLH proteins. *Development* **128**, 217-231.
- Morrow, E. M., Furukawa, T., Lee, J. E. and Cepko, C. L.** (1999). NeuroD regulates multiple functions in the developing neural retina in rodent. *Development* **126**, 23-36.
- Prada, C., Puga, J., Perez-Mendez, L., Lopez, R. and Ramirez, G.** (1991). Spatial and temporal patterns of neurogenesis in the chick retina. *Eur. J. Neurosci.* **3**, 559-569.
- Roztocil, T., Matter-Sadzinski, L., Alliod, C., Ballivet, M. and Matter, J. M.** (1997). NeuroM, a neural helix-loop-helix transcription factor, defines a new transition stage in neurogenesis. *Development* **124**, 3263-3272.
- Roztocil, T., Matter-Sadzinski, L., Gomez, M., Ballivet, M. and Matter, J. M.** (1998). Functional properties of the neuronal nicotinic acetylcholine receptor beta3 promoter in the developing central nervous system. *J. Biol. Chem.* **273**, 15131-15137.
- Sheppard, A. M., Konopka, M. and Jeffrey, P. L.** (1988). Thy-1 expression in the retinotectal system of the chick. *Brain Res.* **471**, 49-60.
- Sommer, L., Ma, Q. and Anderson, D. J.** (1996). neurogenins, a novel family of atonal-related bHLH transcription factors, are putative mammalian neuronal determination genes that reveal progenitor cell heterogeneity in the developing CNS and PNS. *Mol. Cell. Neurosci.* **8**, 221-241.
- Takahashi, Y., Rayman, J. B. and Dynlacht, B. D.** (2000). Analysis of promoter binding by the E2F and pRB families in vivo: distinct E2F proteins mediate activation and repression. *Genes Dev.* **14**, 804-816.
- Vetter, M. L. and Brown, N. L.** (2001). The role of basic helix-loop-helix genes in vertebrate retinogenesis. *Semin. Cell Dev. Biol.* **12**, 491-498.
- Wang, S. W., Kim, B. S., Ding, K., Wang, H., Sun, D., Johnson, R. L., Klein, W. H. and Gan, L.** (2001). Requirement for math5 in the development of retinal ganglion cells. *Genes Dev.* **15**, 24-29.
- Yan, R. T. and Wang, S. Z.** (1998). neuroD induces photoreceptor cell overproduction in vivo and de novo generation in vitro. *J. Neurobiol.* **36**, 485-496.
- Yang, Z., Ding, K., Pan, L., Deng, M. and Gan, L.** (2003). Math5 determines the competence state of retinal ganglion cell progenitors. *Dev. Biol.* **264**, 240-254.