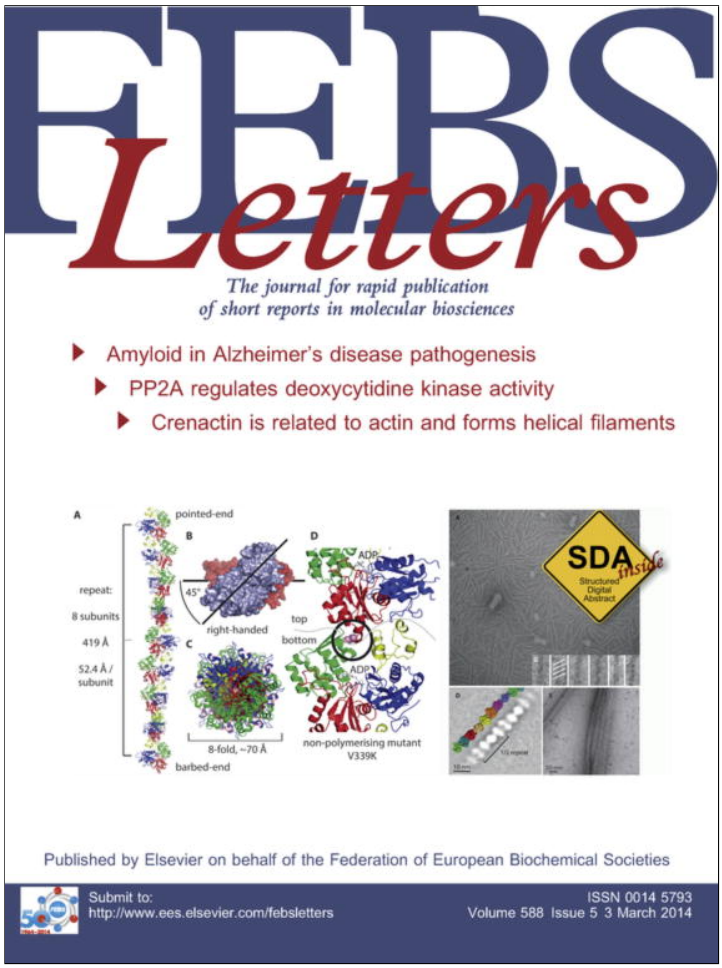

FEBS Letters 588 (2014) 770–775

FEBS Letters

journal homepage: www.FEBSLetters.org

# Transcription factor AP-2delta regulates the expression of polysialyltransferase ST8SIA2 in chick retina

Xiaodong Li, Amit R.L. Persad, Elizabeth A. Monckton, Roseline Godbout *

*Department of Oncology, University of Alberta, Cross Cancer Institute, 11560 University Avenue, Edmonton, Alberta T6G 1Z2, Canada*

ARTICLE INFO

*Article history:*
Received 12 December 2013
Revised 7 January 2014
Accepted 10 January 2014
Available online 23 January 2014

Edited by Ned Mantei

*Keywords:*
Retina
PSA-NCAM
Polysialyltransferase
ST8SIA2
Gel shift
Chromatin immunoprecipitation

ABSTRACT

**The AP-2δ transcription factor is restricted to a subset of retinal ganglion cells. Overexpression of AP-2δ in chick retina results in induction of polysialylated neural cell adhesion molecule (PSA-NCAM) accompanied by misrouting and bundling of ganglion cell axons. Two polysialyltransferases, ST8SIA2 and ST8SIA4, are responsible for polysialylation of NCAM. Here, we investigate the mechanism driving the increase in PSA-NCAM observed upon AP-2δ overexpression. We show that *ST8SIA2* is induced by AP-2δ overexpression in chick retina. We use chromatin immunoprecipitation and gel shift assays to demonstrate direct interaction between AP-2δ and the *ST8SIA2* promoter. We propose that up-regulation of *ST8SIA2* upon AP-2δ overexpression in retina increases ectopic polysialylation of NCAM which in turn causes premature bundling of axons and alters axonal response to guidance cues.**



## 1. Introduction

Activating protein 2 (AP-2) transcription factors have been implicated in various biological processes during development. Each member of the AP-2 family (AP-2α, β, γ, δ and ε) has been inactivated in mouse, with accompanying phenotypes indicating both redundant and non-redundant roles for the different members of the AP-2 family [1–8]. AP-2δ is the most divergent member of the family with only three of the eight amino acids in the transactivation domain considered to be critical for AP-2 function being conserved [9–10]. AP-2δ is expressed in both the inferior colliculus and the superior colliculus of mouse brain, as well as the dorsal thalamus and cortex. *AP-2δ*-/- mice are viable but lack the inferior colliculus [8].

During retinogenesis, six major types of neurons (ganglion, amacrine, bipolar, horizontal, cone photoreceptors and rod photoreceptors) and one type of glial cell (Müller glia) are generated from multipotent retinal progenitor cells. Retinal ganglion cells are the only projection neurons of the retina. These cells convey visual information to the brain via the optic nerve. Polysialic acid (PSA) is a linear carbohydrate homopolymer of alpha-2,8-linked sialic acid residues that is predominantly found on the neural cell adhesion molecule (NCAM). Four major NCAM isoforms have been identified, including three isoforms (NCAM-120, -140, -180) that transduce extracellular signals to the cytosol and one soluble isoform [11]. Polysialylation of NCAM increases cell motility and promotes axon growth, guidance and fasciculation by interfering with NCAM-protein interactions and reducing contact-dependent interactions between cells [12–14]. In retina, PSA-NCAM becomes increasingly restricted to the ganglion cell axons at later stages of development [15–17].

ST8 α-N-acetyl-neuraminide α-2.8-sialyltransferase (ST8SIA) is a family of 6 sialyltransferases (ST8SIA 1–6) that catalyses sialic acid addition through α-2.8 linkages [18]. Two ST8SIAs, ST8SIA2 (STX) and ST8SIA4 (PST), are responsible for the synthesis of PSA [14,19–21]. ST8SIA2 and ST8SIA4 have distinct tissue-specific and cell-specific expression profiles, with both ST8SIA2 and ST8SIA4 expressed in the central nervous system [19]. *ST8SIA2* transcript levels peak during brain development, whereas *ST8SIA4* transcript levels remain high throughout development and in adult brain [22]. These data suggest a preferential role for ST8SIA2 in the developing brain.

At least four of the five AP-2 family members are expressed in developing retina. AP-2α is primarily expressed in amacrine cells, although it has also been detected in horizontal cells prior to their full maturation [23–26]. AP-2β is detected in both amacrine and horizontal cells [23–25], whereas AP-2γ is restricted to amacrine cells with a later onset compared to AP-2α and AP-2β [24]. The only AP-2 documented to be expressed in ganglion cells is AP-2δ.

* Corresponding author. Fax: +1 7804328892.
*E-mail address:* rgodbout@ualberta.ca (R. Godbout).

0014-5793/$36.00 
http://dx.doi.org/10.1016/j.febslet.2014.01.024

Approximately one-third of ganglion cells in developing chick retina express AP-2δ [10]. It is not known whether inactivation of the *AP-2δ* gene affects retinal structure or function as only the midbrain of *AP-2δ* knock-out mice has been examined to date [8]. Ectopic expression of AP-2δ in chick retina results in increased PSA levels, accompanied by abnormal axonal routing and bundling [17]. Here, we show that overexpression of AP-2δ in the developing chick retina up-regulates the polysialyltransferase *ST8SIA2*. Our results indicate that *ST8SIA2* is a direct target of AP-2δ, suggesting that the axonal defects and increased PSA levels observed upon AP-2δ overexpression is a direct consequence of *ST8SIA2* transcriptional activation.

## 2. Materials and methods

### 2.1. Western blot analysis

Retinal tissue from E5, E7, E10 and E15 chick embryos was homogenized in RIPA buffer (50 mM Tris–HCl pH 7.5, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 0.5 M NaCl, 10 mM $MgCl_2$ and Roche Complete protease inhibitors). Proteins were separated in an 8% SDS–polyacrylamide gel, transferred to a nitrocellulose membrane and NCAM detected with mouse anti-NCAM antibody (1:1000 dilution) (5e; Developmental Studies Hybridoma Bank) and actin with mouse anti-actin antibody (1:100,000 dilution; Sigma).

### 2.2. In ovo electroporation of chick embryos

*In ovo* electroporation of RCASBP(B)-GFP and RCASBP(B)-GFP-AP-2δ DNA in the eyes of chick embryos at embryonic day 2 was carried out as previously described [17]. Embryos were harvested at embryonic day 8 (E8) and screened for GFP expression by epifluorescence. Fertilized eggs from White Leghorn chickens were obtained from the University of Alberta Farm poultry unit. Chicken embryo research was carried out with institutional approval following the Canadian Council on Animal Care guidelines.

### 2.3. RT-PCR

One microgram of total RNA from at least two different batches of pooled E5, E7, E10 and E15 chick retina, as well as RNA from E8 retinal tissue *in ovo* electroporated with GFP and GFP-AP-2δ retroviral vectors, were reverse transcribed with Superscript II reverse transcriptase (Invitrogen). Single-strand cDNAs were PCR-amplified using the following primers for *ST8SIA2*: 5′-GAGGC AGAGGTACAATCAGA-3′ (top strand) and 5′-CACCTGATGACAA AGCTGTG-3′ (bottom strand); and for *ST8SIA4*: 5′-TTCTGGCA TCCTTCTGGACA-3′ (top strand) and 5′-GCGTGTACATGAGGA-GACC-3′ (bottom strand).

### 2.4. In situ hybridization

A 614 bp *ST8SIA2* cDNA fragment (bases 107–720) was generated by PCR amplification and cloned into the pGEM-T Easy vector. Antisense riboprobe labeled with digoxigenin (DIG) was synthesized by *in vitro* transcription of the linearized plasmid. Retinal tissue was fixed in 4% paraformaldehyde, cryoprotected in sucrose and embedded in OCT along the dorsal–ventral axis. Frozen tissue sections were prehybridized, hybridized, washed and the signal detected as previously described [10]. Coverslips were mounted with Faramount aqueous mounting medium (Dako). Image acquisitions were at room temperature using a Zeiss Axioskop 2 plus microscope with a NA 0.75 Zeiss FluAR lens and Zeiss Axiocam camera. Images were acquired with AxioVs40V4.7.1.0 software.

### 2.5. Chromatin immunoprecipitation

E10 chick retinal tissue was dissociated with trypsin and cross-linked with 1% formaldehyde for 10 min at room temperature. Cells were homogenized in lysis buffer (0.5 mM PIPES pH 8.5, 85 mM KCl, 0.5% NP-40 and Roche Complete protease inhibitors) and sonicated at 50% output (1/8 inch microprobe; Biologics, Inc.). After sonication, the lysates were precleared by incubation with protein A-Sepharose beads. The precleared lysates were immunoprecipitated with anti-AP-2δ antibody [10]. Rabbit IgG was used as a control for immunoprecipitations. Protein-DNA complexes were eluted from the beads. Cross-links were reversed and protein digested with proteinase K. The DNA was purified by phenol/chloroform extraction followed by precipitation in ethanol. PCR-amplification was done using primers flanking the potential AP-2 binding sites in the *ST8SIA2* promoter region (listed in Fig. 3B).

### 2.6. Electrophoretic mobility shift assay

Complementary oligonucleotides flanking potential AP-2 binding sites in *ST8SIA2*, sites #4 (upstream, 5′-CACGCCGG GCCCTGGGGATGCTG-3′; downstream, 5′-TGGCACAGCATCCCCAG GGCCCG-3′) and #5 (upstream, 5′-TCACGAGGCCCCAATGGCAC CTG-3′; downstream, 5′-CCTTGCAGGTGCCATTGGGGCCT-3′) were annealed and radiolabeled with $^{32}P$. Nuclear extracts from E7 chick retina were prepared as described [27]. One μg of nuclear extracts was incubated with AP-2 oligonucleotides. Supershifts were carried out with the following antibodies: AP-2α (3B5; Developmental Studies Hybridoma Bank), AP-2β (H87; Santa Cruz Biotechnology), AP-2δ (both affinity-purified and antiserum) [10] and NFI (antiserum) [28]. DNA–protein complexes were resolved in a 6% polyacrylamide gel in 0.5X Tris–borate EDTA buffer.

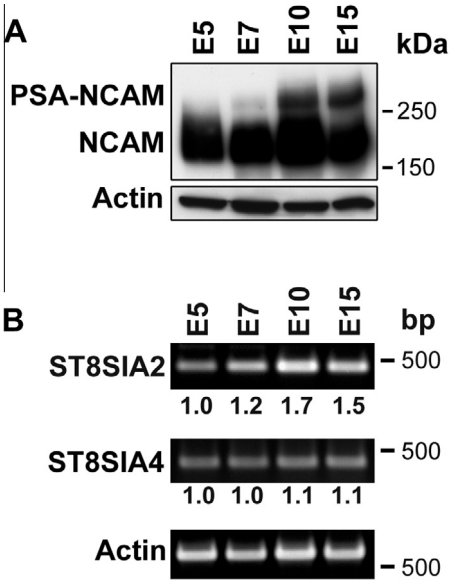


**Fig. 1.** Expression pattern of PSA-NCAM and polysialyltransferases in developing retina. (A) Protein lysates from retinal tissue at different developmental stages as indicated were separated in an 8% SDS–polyacrylamide gel and transferred to a nitrocellulose membrane. NCAM was detected with mouse anti-NCAM antibody. (B) RT-PCR of *ST8SIA2* and *ST8SIA4* using cDNAs prepared from E5, E7, E10 and E15 retina tissue. Actin served as the loading control. DNA signal density was quantified by densitometric analysis using Adobe Photoshop. Values under each lane indicate the average of 5 experiments with average intensity of signal depicted as the ratio of ST8SIA2/actin or ST8SIA4/actin, relative to d5 retina, with a ratio of 1 arbitrarily set for E5 retina.

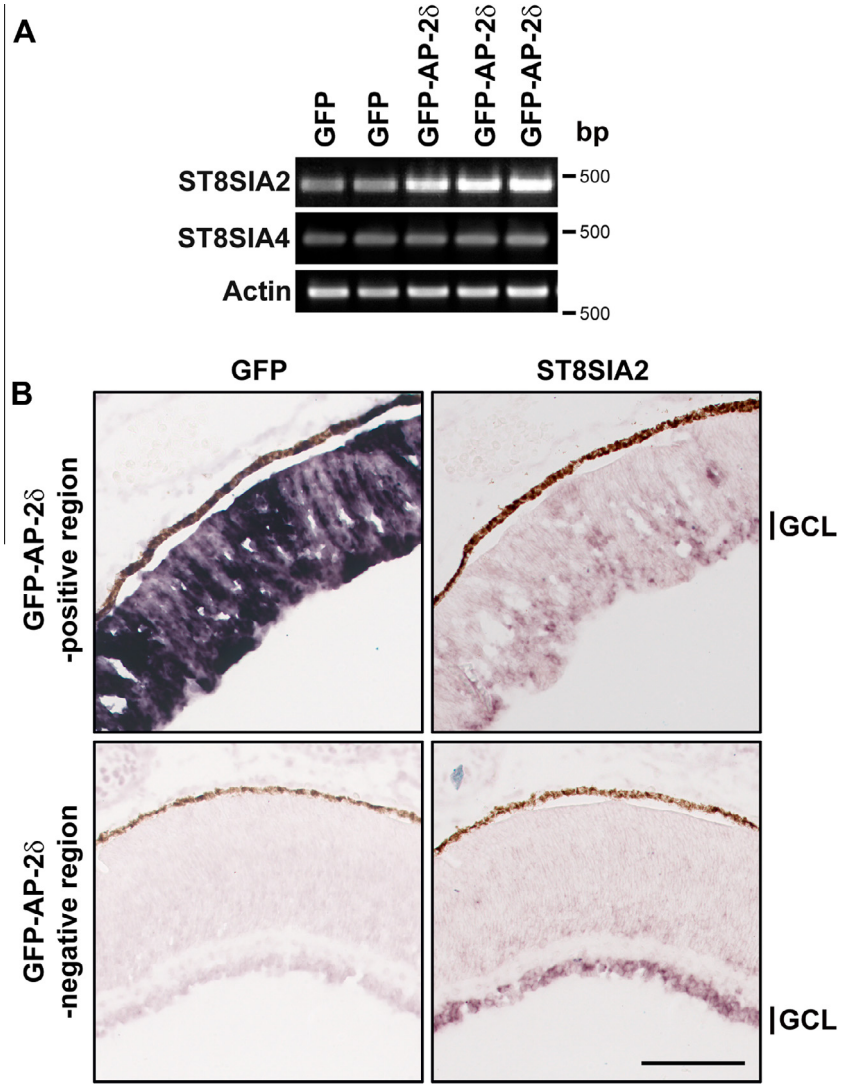


**Fig. 2.** *ST8SIA2* is a putative target of AP-2δ. (A) RT-PCR of *ST8SIA2* and *ST8SIA4* using cDNAs prepared from E8 embryos *in ovo* electroporated with GFP (two different retinas) or GFP-AP-2δ (three different retinas) RCAS expression constructs. Actin served as the loading control. (B) Retinal tissue sections from E8 embryos *in ovo* electroporated with a GFP-AP-2δ RCAS expression construct were hybridized with DIG-labeled *GFP* or *ST8SIA2* antisense RNA probes. The signal was detected using alkaline phosphatase-coupled DIG antibody (purple color). GFP-negative and GFP-positive regions from the same *in ovo* electroporated retina are shown here. GCL, ganglion cell layer. Photographs were taken with a 20× lens using a Zeiss Axioskop 2 plus microscope. Scale bar = 100 μm.

## 3. Results

PSA-NCAM has previously been shown to play a key role in promoting and directing the growth of retinal ganglion cell axons [29–30]. We first examined the expression pattern of PSA-NCAM in the developing chick retina by western blotting using an antibody that recognizes the extracellular domain of NCAM molecules. Similar levels of NCAM-180 were detected at all developmental stages examined (Fig. 1A). There was clear induction of a higher molecular weight form of NCAM representing PSA-NCAM, between embryonic day 7 (E7) and 10 when axonal growth is at its peak [31]. We next examined the expression patterns of *ST8SIA2* and *ST8SIA4* in the developing chick retina by semi-quantitative RT-PCR. A gradual increase in *ST8SIA2* mRNA levels was observed from E5 to E10, with a reduction in *ST8SIA2* RNA levels observed at E15. In contrast, *ST8SIA4* mRNA levels remained relatively constant during development. Thus, the temporal expression pattern of *ST8SIA2* coincides most closely with that of PSA-NCAM in the developing chick retina. *In ovo* electroporation of RCAS/GFP-AP-2δ in the eyes of chick embryos results in induction of PSA-NCAM and axonal misrouting [17]. A similar phenotype is observed upon *in ovo* electroporation of ST8SIA4 but not ST8SIA2 in embryonic chick eyes [16].

To address the possibility that AP-2δ overexpression can directly affect polysialyltransferase expression, we examined the mRNA levels of both *ST8SIA2* and *STASIA4* in GFP control and GFP-AP-2δ-positive retinas by RT-PCR. *ST8SIA2* mRNA was clearly induced in GFP-AP-2δ-positive retinas whereas no change was observed in *ST8SIA4* mRNA levels (Fig. 2A). Next, *ST8SIA2* RNA levels in GFP-positive versus GFP-negative regions of E8 GFP-AP-2δ-electroporated retinas were compared by *in situ* hybridization. In agreement with our RT-PCR results, increased levels of *ST8SIA2* were observed in GFP-AP-2δ-positive regions of *in ovo* electroporated retinas (Fig. 2B). Of note, our *in situ* hybridization data show that *ST8SIA2* is normally expressed in ganglion cells (Fig. 2B). As AP-2δ is also expressed in ganglion cells, these combined results support the idea that *ST8SIA2* is a target of AP-2δ.

We then examined the 5′ flanking sequence of the chicken *ST8SIA2* gene for putative AP-2 binding sites. We found 5 putative AP-2 binding sites within a 1.6 kb region upstream of the *ST8SIA2*

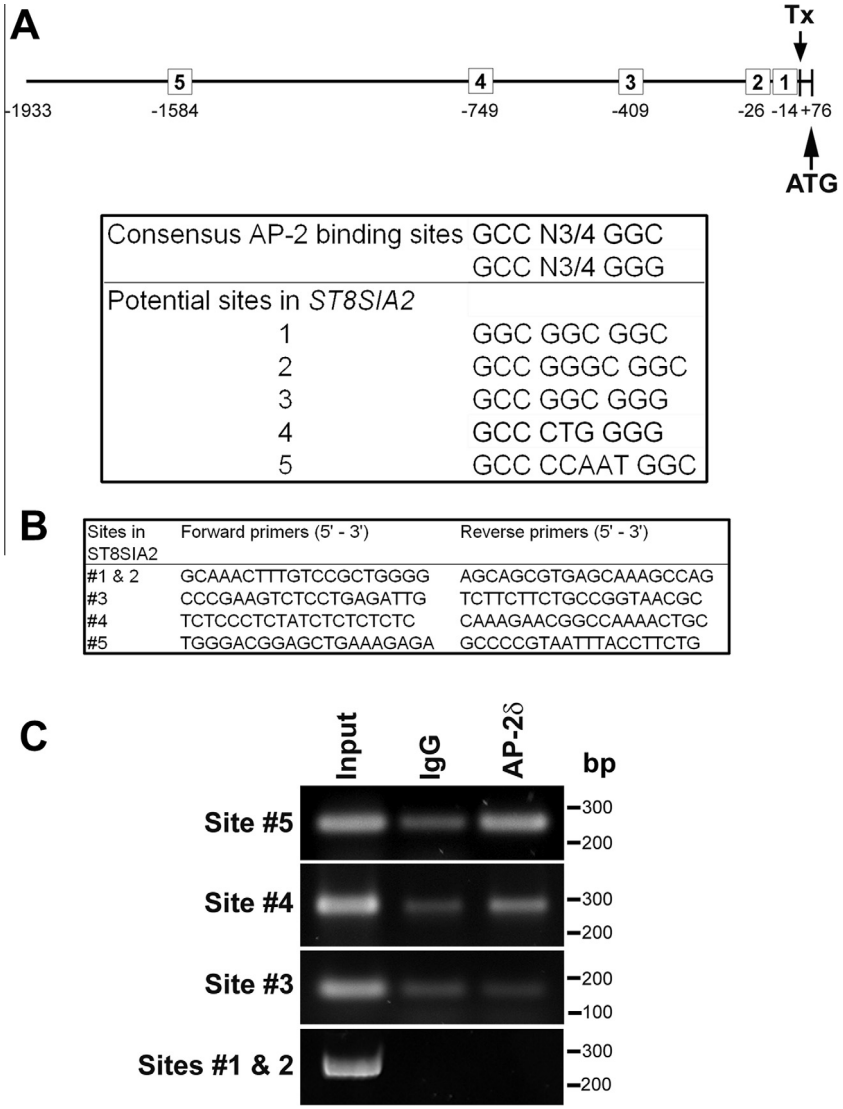


**Fig. 3.** ChIP analysis demonstrating that AP-2δ occupies the *ST8SIA2* promoter region. (A) Five potential AP-2 binding sites (denoted as box 1, 2, 3, 4, 5) are located within 2 kb of the transcription start site of *ST8SIA2*. Numbers under the boxes indicate base pairs relative to the transcription start site. Tx, predicted transcription start site, based on EST sequences; ATG, start codon. (B) Sequences of primers flanking potential AP-2 binding sites for ChIP analysis. (C) Retinal tissue at E10 was cross-linked and genomic DNA-AP-2δ complexes immunoprecipitated with anti-AP-2δ antibody. DNA purified from cross-linked complexes was PCR-amplified using primer pairs flanking each of the potential AP-2 binding sites located within 1.6 kb of the *ST8SIA2* transcription start site. Normal rabbit IgG served as the negative control for these experiments. Input is total genomic DNA.

transcription start site (Fig. 3A). We next tested whether *ST8SIA2* might be a target of AP-2δ by chromatin immunoprecipitation (ChIP) experiments using E10 chick retina tissue and an antibody that specifically recognizes AP-2δ [10]. Normal rabbit IgG was used as a negative control. DNA cross-linked to AP-2δ was PCR-amplified using primer pairs flanking each of the potential AP-2 binding sites (Fig. 3B). The DNA spanning AP-2 binding sites #4 and #5 in the promoter region of the *ST8SIA2* gene was preferentially amplified in AP-2δ-immunoprecipitated DNA compared to control lanes, suggesting that *ST8SIA2* is a direct target of AP-2δ (Fig. 3C).

To confirm that AP-2δ binds directly to site #4 and/or site #5 in the promoter region of the *ST8SIA2* gene, we carried out gel shift assays. Nuclear extracts from E7 retina were used since relatively high levels of AP-2δ are present at this developmental stage [10]. Strong binding was observed when nuclear extracts prepared from E7 retina were incubated with $^{32}$P-labeled double-stranded oligonucleotides corresponding to site #4 (Fig. 4 – lane 2) but not site #5, suggesting that only site #4 contains a *bona fide* AP-2δ binding element. The shifted band disappeared in the presence of 100X excess cold oligonucleotide as competitor (Fig. 4 – lane 3). As sites #4 and #5 are only 800 bp apart, we postulate that the ChIP DNA immunoprecipitated with anti-AP-2δ antibody contained both sites #4 and #5, even though only site #4 was occupied by AP-2δ.

Next, we tested whether the band observed in the presence of oligonucleotide #4 could be supershifted with anti-AP-2 antibodies. Supershifted bands were observed with all three anti-AP-2 antibodies tested, with stronger signals obtained with anti-AP-2α and AP-2β antibodies compared to AP-2δ antibody (Fig. 4 – lanes 4–6). These results indicate that AP-2 binding sites located upstream of *ST8SIA2* can be bound by the other members of the AP-2 family *in vitro*. Weak supershift with anti-AP-2δ antibody is not surprising given that only ~3% of cells in the retina express AP-2δ. Supershifts with anti-AP-2δ antiserum compared to anti-NFI antiserum demonstrate the specificity of the interaction with AP-2 antibodies. As AP-2α and AP-2β are expressed in amacrine and horizontal cells, and AP-2δ and ST8SIA2 are expressed in ganglion cells, these combined data suggest that the *ST8SIA2* promoter is occupied by AP-2δ in ganglion cells.

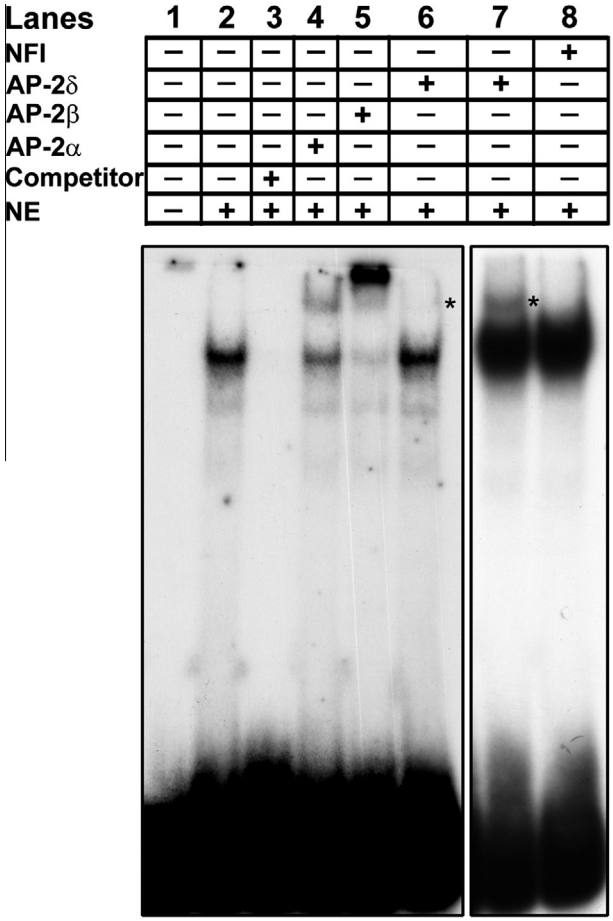


**Fig. 4.** AP-2 binds to the *ST8SIA2* promoter. Gel shifts were carried out with $^{32}$P-labeled *ST8SIA2* (site #4) double-stranded oligonucleotides and nuclear extracts from E7 chick retina. Unbound DNA and DNA–protein complexes were separated in a 6% native polyacrylamide gel. A 100-fold excess of unlabeled site #4 oligonucleotide was used as a competitor in lane 3. Supershifts were carried out with the indicated antibodies (lanes 4–6) or antisera (lanes 7 and 8). The DNA-AP-2δ supershifted complex is indicated by the asterisks.

## 4. Discussion

Of the five AP-2 family members, AP-2δ is the only one known to be expressed in ganglion cells. AP-2δ is expressed in one-third of ganglion cells in the developing chick retina from E7 to E10, peak phases of axonal growth in the chick eye [10]. Overexpression of AP-2δ in chick retina causes axonal misrouting and bundling, defects accompanied by induction of PSA-NCAM [17]. Here, we show that the polysialyltransferase *STASIA2* is a direct target of AP-2δ and postulate that induction of *ST8SIA2* is involved in the axonal phenotype observed in AP-2δ-overexpressing chick retina.

PSA-NCAM has been reported to play critical roles in neuronal differentiation and maturation, including axon growth/guidance/fasciculation, synapse formation and synaptic plasticity [14,21,32]. PSA is a negatively-charged moiety that reduces NCAM adhesion thereby promoting axonal growth [12–13]. NCAM polysialylation is dependent on two alpha 2,8-polysialyltransferases, ST8SIA2 and ST8SIA4, whose role is to synthesize PSA. While knock-out of *NCAM* in mice affects the number of retinal ganglion cells, these cells can still project to their correct targets in the brain [33]. Notably, germ-line disruption of either *ST8SIA2* or *ST8SIA4* shows only a partial loss of PSA with mild but distinct phenotypes [34–35]. ST8SIA2 deficiency causes a reduction in the level of PSA during the perinatal stage, whereas ST8SIA4 deficiency results in a decrease of PSA in the adult brain. However, when both *ST8SIA2* and *ST8SIA4* are disrupted, there is no detectable PSA in brain [36]. These double knock-out mice, which die shortly after birth, show massive axonal tract defects including complete absence of the anterior commissure connecting the olfactory nuclei and temporal parts of the cortex, underlining the importance of PSA-NCAM in brain maturation [36–37].

Interestingly, overexpression of ST8SIA4, but not ST8SIA2, in developing chick retina results in disruption of retinal layers and increased PSA levels [16]. The absence of a 'ST8SIA2' phenotype in chick retina has been attributed to the observation that ST8SIA4 adds more sialic acid residues to NCAM than ST8SIA2 [38–40], thereby magnifying the consequence of ST8SIA4 overexpression compared to ST8SIA2 overexpression. However, it should be noted that examination of the PSA chain length in *ST8SIA2-/-* versus *ST8SIA4-/-* mice suggests that loss of ST8SIA2 has a stronger effect on PSA chain length than loss of ST8SIA4, at least at early stages of brain development [37,41]. Regardless of exact mechanism, our results indicate that the effect of AP-2δ overexpression on axonal misrouting and bundling is at least in part mediated through *ST8SIA2*. It is likely that other AP-2δ target genes also play a role in the phenotype observed in our *in ovo* electroporated retinas. Previously identified AP-2δ target genes that may play a role in axonal routing include *FGFR3*, associated with neurite outgrowth [42–43] and *Pou4f3 (Brn3c)* [8], expressed in retinal ganglion cells and associated with axonal routing [44].

In conclusion, we have identified *ST8SIA2* as a new target of the AP-2δ transcription factor based on induction of *ST8SIA2* RNA levels upon AP-2δ overexpression, chromatin immunoprecipitation, gel shifts and supershift assays. We propose that induction of *ST8SIA2* is responsible for the induction of PSA-NCAM observed in AP-2δ overexpressing retinas and is in part responsible for the axonal misrouting and bundling abnormalities observed in our *in ovo* electroporated retinas.

## Acknowledgements

We are grateful to Dr. Cairine Logan for teaching us the *in ovo* electroporation technique, and to Drs. Stephen Hughes, Bruce Morgan and Donna Fekete for sharing the pSLAX12 and RCAS constructs. This work was supported by the Canadian Institutes of Health Research – Funding Reference No. 114993.

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