SPATIO-TEMPORAL REGULATION OF THE *ATONAL HOMOLOG 1* GENE

APPROVED BY SUPERVISORY COMMITTEE

Jane Johnson, PhD, Mentor
Professor, Neuroscience

Jenny Hsieh, PhD
Assistant Professor, Molecular Biology

Rolf Joho, PhD
Associate Professor, Neuroscience

SPATIO-TEMPORAL REGULATION OF THE *ATONAL HOMOLOG 1* GENE

by

CHRIS ARIES ADAMS

THESIS

Presented to the Faculty of the Graduate School of Biomedical Sciences

The University of Texas Southwestern Medical Center at Dallas

In Partial Fulfillment of the Requirements

For the Degree of

MASTER OF SCIENCE

The University of Texas Southwestern Medical Center at Dallas

Dallas, Texas

August, 2007

SPATIO-TEMPORAL REGULATION OF THE *ATONAL HOMOLOG 1* GENE

Chris Aries Adams, M.S.

The University of Texas Southwestern Medical Center at Dallas, 2007

Supervising Professor: **Jane Johnson, Ph.D.**

Controlled spatio-temporal expression of atonal homolog 1 is necessary for the correct development of the cerebellar granule cells, the dorsal interneuron 1 population, gut goblet cells and the cochlear and vestibular hair cells of mice. The purpose of these studies was to determine how atonal homolog 1 is regulated by analyzing the activity of atonal homolog 1 regulatory regions. This was accomplished by deleting different conserved regions in a bacterial artificial chromosome (318GFPBAC) containing ~180 kb of sequence 5' and 3' of atonal homolog 1 and assaying for green fluorescent protein expression in transgenic mice. Transgenic embryos were analyzed at embryonic day 10.5 in the neural tube, metencephalon and rhombencephalon and at embryonic day 16.5 in merkel cells, inner ear cells, vestibular cells and the cerebellum all tissues where endogenous atonal homolog 1 is expressed. Auto-regulation from an enhancer region 3' of the atonal homolog 1 gene (enhancer AB) was previously shown to be sufficient for

atonal homolog 1-specific expression. In this paper, I show that enhancer AB is required for expression from the 318GFPBAC indicating that there are no other auto-regulatory elements for atonal homolog 1 expression in the 200 kb tested. Further, I have located an evolutionarily conserved region that has not previously been tested 12 kb 3' of the atonal homolog 1 coding region (enhancer C). When enhancer C is deleted from the 318GFPBAC, green fluorescent protein expression is not affected at embryonic day 10.5 or embryonic day 16.5. Also, enhancer C cannot drive green fluorescent protein expression by itself at embryonic day 10.5 or embryonic day 16.5. Thus no role for this conserved sequence around atonal homolog 1 was detected in these studies. To date, the AB enhancer is the only sequence shown *in vivo* to function in atonal homolog 1 regulation being both necessary and sufficient to direct expression in an atonal homolog 1 pattern.

CONTENTS

PRIOR PUBLICATIONS

Matsumoto, S., Banine, F., Struve, J., Xing, R., **Adams, C**., Liu, Y., Metzger, D., Chambon, P., Rao, M. S., and Sherman, L. S. (2006). Brg1 is required for murine neural stem cell maintenance and gliogenesis. Dev Biol *289*, 372-383.

FIGURES

TABLES

Table Page

APPENDICES

ABBREVIATIONS

Atoh1- Atonal homolog 1

BAC- Bacterial artificial chromosome

bHLH- basic helix-loop-helix

Chlor- Chloramphenicol

dI- dorsal interneuron

GFP- Green fluorescent protein

Kan- Kanamycin

LB- Luria Broth

Neo- Neomycin

Strep- Streptomycin

CHAPTER ONE

Introduction

Atonal Homolog 1

Atonal homolog 1 (Atoh1) is a basic helix-loop-helix (bHLH) transcription factor. The basic helix-loop-helix motif contains a DNA-binding domain and a protein interaction domain. In order for Atoh1 to bind DNA, it must dimerize with an E-protein such as Tcfe2a, Tcf4 or Tcf12. *Atonal* was first discovered in *Drosophila* as necessary for the formation of the chordotonal organs, which are stretch receptors for proprioception in the peripheral nervous system (Jarman et al., 1993). When the mammalian homolog of *atonal*, *Atoh1*, is knocked out, the cerebellum does not develop properly, cochlear hair cells do not differentiate, dI1 interneurons do not form and goblet cells do not form in the gut (Bermingham et al., 1999; Bermingham et al., 2001; Gowan et al., 2001; Yang et al., 2001). Lack of *Atoh1* regulation can lead to inappropriate specification; for instance, when *Otx2* is knocked out in neurons with a nestin-Cre promoter, Atoh1 protein is ectopically expressed in the dorsal midbrain leading to an ectopic cerebellar-like structure (Vernay et al., 2005). Also, over-expression of Atoh1 results in ectopic protein expression of NEUROD and DCX in the cerebellum in inappropriate layers (Helms et al., 2001).

Spatio-temporal Expression of Atoh1

To understand the regulatory mechanism for *Atoh1* expression, one must first determine its spatio-temporal expression. *Atoh1* expression has been found in various regions of the nervous system from embryonic day (e) 9.5 to adult. Its expression begins at e9.5 in the dorsal neural tube and its expression here increases until around e11.5 (Ben-Arie et al., 2000; Helms et al., 2000). Later, *Atoh1* is expressed in diverse places ranging from hairy skin to the cerebellum. A summary of Atoh1 expression is in **Table 1.1** and **Table 1.2**.

Age	Cerebellum/ metencephalon	Hindbrain/ Rhomb-encephalon	Dorsal spinal cord	Cerebellar peduncles	Pons + Medulla
E10.5	X[4]	X[3]	X[3,4]		
E12.5	X[2]		Weak[3]		
E15.5	X[3]		Weak[3]	X[3]	X[3]
P0 and adult	X[3,4]				

Table 1.1 *Atoh1* Expression in the Brain and Spinal Cord[1]

[1]Gensat also showed Atoh1 expression in the globus pallidus, olfactory bulb, caudate putamen, midbrain, telencephalon and hypothalamus at e15.5, but this is not confirmed with other studies.
[2](Akazawa et al., 1995)
[3]Gensat
[4](Lumpkin et al., 2003)

Age	Hairy Skin (Merkel cells)	Vibrissae (Merkel cells)	Ear (Hair cells)	Intestine (goblet)	mandibular condylar cartilage
E15.5 – e16.5	X[1,2]	X[2]	X[2]	X[3]	
P0-Adult					X[4]

Table 1.2 *Atoh1* Expression in Hairy Skin, Vibrissae, Hair Cells, Goblet Cells and Manidbular Cells.

[1]Gensat
[2](Helms et al., 2000)
[3](Mutoh et al., 2006)
[4](Shimizu et al., 2005)

Spatio-temporal regulation at the Atoh1 enhancer

In order to study the spatio-temporal regulation of *Atoh1*, one must determine the regulatory domains of *Atoh1* that are required for precise expression. In *Drosophila*, *atonal* is regulated by a 3' and a 5' enhancer (Sun et al., 1998). The 3' enhancer is thought to initiate expression in the eye. The 5' enhancer, which is auto-regulated by *atonal*, is thought to enhance and maintain expression (Sun et al., 1998). Over-expression of mouse Atoh1 induces *Xath1* expression, indicating it also auto-regulates itself (Kim et al., 1997). So both *Drosophila* and *Xenopus atonal* homologs show auto-regulation.

To determine whether there is a similar regulatory mechanism in mammals, enhancer regions have been identified and deletions used to find the critical sequences. Helms et al. tested sequences around the *Atoh1* coding region with a reporter construct expressing lacZ (Helms et al., 2000). They found a 1.4 kb region 3 kb 3' of the *Atoh1* coding sequence that directed *Atoh1* expression to all expression domains (Helms et al., 2000). They compared this sequence across species finding two regions of homology within this 1.4 kb fragment (>80%), which they termed enhancers A and B (Helms et al., 2000).

To determine the functions of these enhancer regions in mammals, transgenic mice were made using different regions of the enhancers placed in front of lacZ with a β-globin promoter and expression was analyzed (Helms et al., 2000). They found that enhancer A without enhancer B drives expression in the hindbrain (e10.5), ear (e14.5), whisker (e14.5) and EGL (e14.5) but not the spinal cord (Helms et al., 2000). On the other hand, they found that enhancer B without enhancer A drives expression in all of the Atoh1 protein expression domains including the spinal cord (Helms et al., 2000).

E-boxes were identified within enhancers A and B, which may explain why they can drive expression in similar domains without other apparent sequence conservation (Helms et al., 2000). The enhancer B e-box is required for enhancer B function (Helms et al., 2000). Gel shift assays showed that Atoh1 can bind this region (Helms et al., 2000). Further, *Atoh1* enhancer-driven lacZ expression was lost in *Atoh1*-/- mice expressing a transgene including enhancers A and B (Helms et al., 2000). Likewise, electroporation of an Atoh1 expressing plasmid in chick neural tube causes up-regulation of *Cath1* (the chicken ortholog of Atoh1) (Gowan et al., 2001). So, similar to *Drosophila* and *Xenopus* Atoh1, mammalian *atonal* appears to auto-regulate itself.

Three genes have been found to regulate *Atoh1* in either enhancer A or B. Zic1, a zinc finger transcription factor, was found to inhibit *Atoh1* auto-regulation in the neural tube (Ebert et al., 2003) (**Fig 1.1**). PC3 (pheochromacytoma cell-3), a BGT family

protein, induces lacZ expression in a construct containing enhancers A and B in PC12 cells and cerebellar precursor cells (Canzoniere et al., 2004) (**Fig 1.1**). Finally, CDX2, a homeodomain transcription factor, induces luciferase expression from a reporter construct containing enhancers A and B in the intestinal cell line IEC-6 (Mutoh et al., 2006) (**Fig 1.1**).

Regions outside enhancers A and B may be necessary for *Atoh1* expression. When lacZ is knocked into the *Atoh1* locus to produce *Atoh1*-/- animals, there is still lacZ expression in spinal cord at e11.5 (Bermingham et al., 2001). As mentioned above, enhancers A and B cannot drive lacZ expression in the *Atoh1* mutant. Because lacZ is expressed when lacZ is knocked in to the *Atoh1* locus in *Atoh1-/-* animals, regions outside enhancer A and B are probably necessary for regulating *Atoh1* expression. Also, there must be other mechanisms for *Atoh1* expression than auto-regulation since something must initiate expression before autoregulation can function. Altogether, these findings indicate that sequences outside enhancers A and B may be necessary for the initiation and maintenance of *Atoh1* expression.

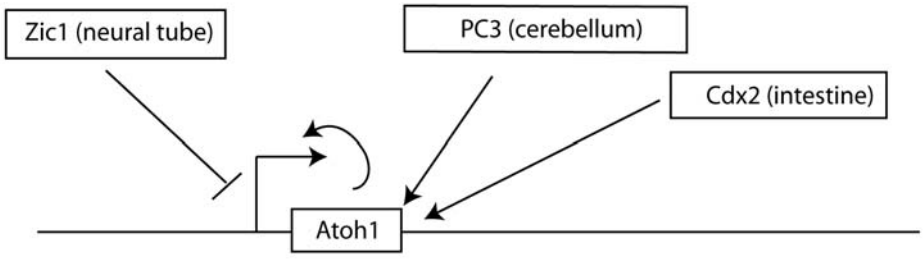


Figure 1.1 Atoh1 regulation in the cerebellum, inner ear and neural tube.

In the neural tube, atoh1 is negatively regulated by ZIC1 (Ebert et al., 2003). In the cerebellum, *Atoh1* is positively regulated by PC3 (Canzoniere et al., 2004). In the intestine, *Atoh1* expression is positively regulated by cdx2 (Mutoh et al., 2006). All of these transcription factors regulate expression through directly binding the *Atoh1* enhancer AB.

To determine whether regions outside of enhancers A and B regulate *Atoh1* expression, a BAC spanning ~80 kb 5' and ~100 kb 3' of *Atoh1* coding sequence has

been created where the *Atoh1* coding region is replaced with green fluorescent protein (GFP). Expression from the 318GFPBAC is restricted to Atoh1 expression domains (**Fig 1.2**). This is in contrast to the J2X-GFP mouse, which contains about 1.2 kb of enhancer sequence and contains enhancers A and B, where GFP is ectopically expressed in the cortex, hippocampus and ventral spinal cord (Lumpkin et al., 2003). Other than the ectopic expression, the J2X-GFP mouse expresses GFP in an Atoh1-specific pattern. To determine whether the expression driven by the 200 kb 318GFPBAC requires Atoh1, the 318GFPBAC transgenic mouse line was crossed with *Atoh1*-/+ mice. 318GFP+; *Atoh1-/-* embryos express GFP in the dorsal neural tube at e10.5 indicating that the Atoh1-independent regulatory region is present (**Fig 1.2**). Note that although GFP expression is detected, the pattern is disrupted reflecting the phenotype in *Atoh1-/-* neural tubes (Bermingham et al., 2001; Gowan et al., 2001). For my thesis research, I set out to determine 1) whether enhancers A and B were required for detectable expression from the 318GFPBAC and 2) whether regulatory regions outside of enhancers A and B could be identified.

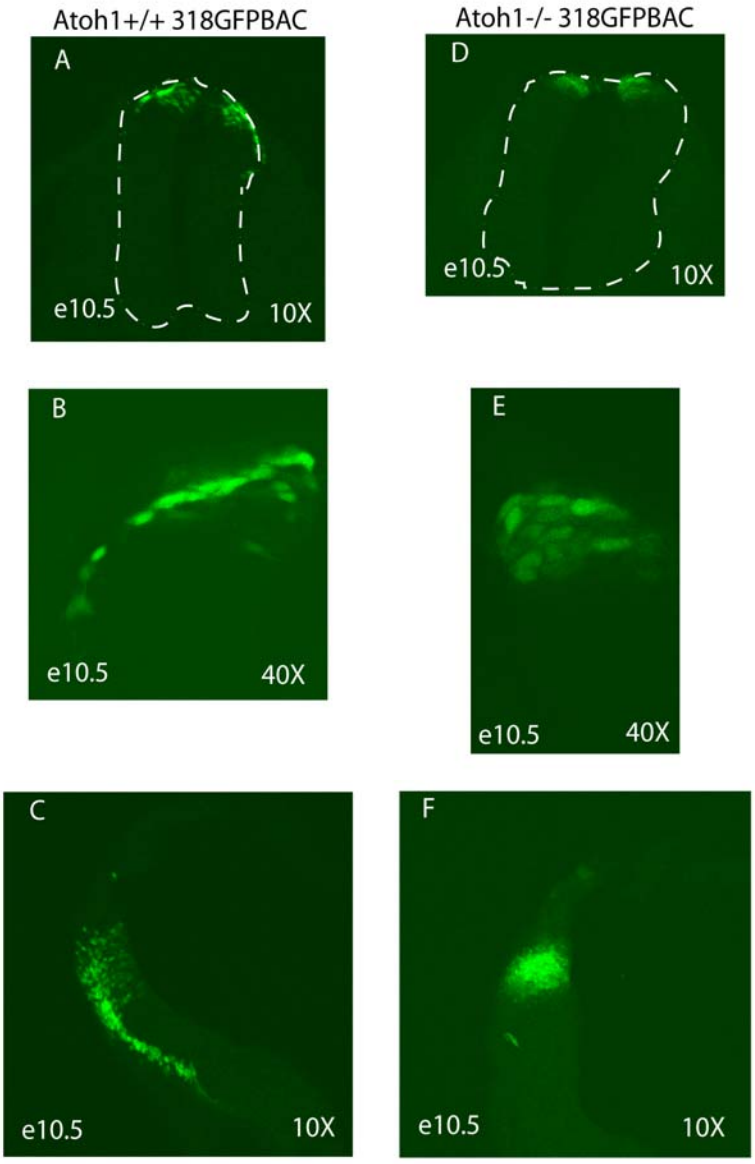


Figure 1.2 GFP Expression from the 318GFPBAC in Wild Type and *Atoh1*-/- Mice.

At e10.5, *Atoh1*+/+ 318GFPBAC and *Atoh1*-/- 318GFPBAC transgenic mice express GFP in the dorsal neural tube (A, B, D and E) and the hindbrain (C and F). The 318GFPBAC expresses GFP in *Atoh1*-/- mice indicating that *Atoh1* is not necessary for the maintenance of its own expression. These images were taken by Trisha Savage.

CHAPTER TWO

Materials and Methods

The Copeland method was used to modify the 318GFPBAC (Liu et al., 2003).

Bacterial Strains and Plasmids

EL250 cells, which contain the arabinose-inducible FLP recombinase gene, were used in this study (Liu et al., 2003). The PL451 plasmid, which contains a neo cassette flanked by two FRT sites, was used to create the targeting vectors.

Construction of the Enhancer AB Targeting Vector to Delete Enhancers A and B from the 318GFPBAC

For the 5' homology region (PCR product 1), the following PCR primers were used. For-5'EnhA/Bdel-XhoI: AAAActcgagAGATTGCCACCAAAGATGCT (for); Rev-5'EnhA/Bdel-EcoRI: AAAAaagcttGGCAGAAAACAATGATGCTG (rev). The 5' homology arm was 175 bp. For the 3' homology region (PCR product 2), the following PCR primers were used. For-3'EnhA/Bdel-BamHI: AAAAggatccAGACAGGATTCCCTCGGTCT (for); Rev-3'EnhA/Bdel-NotI: AAAAgcggccgcTTGTTTGGGACCAAAAGAGG (rev). The 3' homology arm was 219 bp. Forty nanograms of PCR product 1 (XhoI/EcoRI) was ligated with 120 ng of PL451 (XhoI/EcoRI) (Fig 3). Then, 40 ng of PCR product 2 (BamHI/NotI) was ligated with 100 ng PL451-5'EnhAB (BamHI/NotI) (Fig 3). For each reaction (20 µl), 2 µl of 10X ligation buffer and 1 µl of T4 DNA ligase was added. The reactions were incubated at 16˚C overnight. Then, 0.5 µl of the reaction was transformed into DH10B cells (Invitrogen). The construct was designed to delete a 1889 bp region around enhancer AB. The J2X region is 1,384 bp so 505 bp around the J2X region was deleted in addition to the J2X region in the 318GFPBACΔEnhAB. The deletion starts 2.8 kb from the Atoh1 stop codon. Construction of the enhancer C targeting vector to delete enhancer C from the 318GFPBAC

Construction of the enhancer C targeting vector to delete enhancer C from the 318GFPBAC

For the 5' homology region (PCR product 4), the following PCR primers were used. For-5'EnhCdel-XhoI: AAAActcgagAGATTGCCACCAAAGATGCT (for); Rev-5'EnhCdel-HindIII***:*** AAAAaagcttGGCAGAAAACAATGATGCTG (rev). The 5' homology arm was 209 bp. For the 3' homology region (PCR product 3), the following PCR primers were used. For-3'EnhCdel-BamHI: AAAAggatccGGAAATTTGCCTGAAAAGCA; Rev-3'EnhCdel-NotI: AAAAgcggccgcGCCTGAGAAAGGACATCCAA. The 3' homology arm was 270 bp. Forty nanograms of PCR product 3 (XhoI/HindIII) was ligated with 80 ng of PL451 (XhoI/HindIII) (**Fig 2.1**). Then, 40 ng of PCR product 4 (BamHI/NotI) was ligated with 80 ng of PL451-5'EnhC (BamHI/NotI) (**Fig 2.1**). For each reaction (20 µl), 2 µl of 10 ligation buffer and 1 µl of T4 DNA ligase was added. The reactions were incubated at 16˚C overnight. Then, 0.5 µl of the reaction was transformed into DH10B cells (Invitrogen). This construct was designed to delete a 1,361 bp sequence covering the enhancer C homology. The deletion starts about 11.8 kb from the Atoh1 stop codon.

PCR amplification steps for enhancer C and enhancer AB deletion constructs

The amplification steps were as follows: 95 degrees for 2 minutes, 95 degrees for 30 seconds, 60 degrees for 20 seconds and 72 degrees for 30 seconds. This was followed by 33 cycles of 95 degrees for 30 seconds, 60 degrees for 20 seconds and 72 degrees for 30 seconds. There was a final extension step of 10 minutes at 72 degrees.

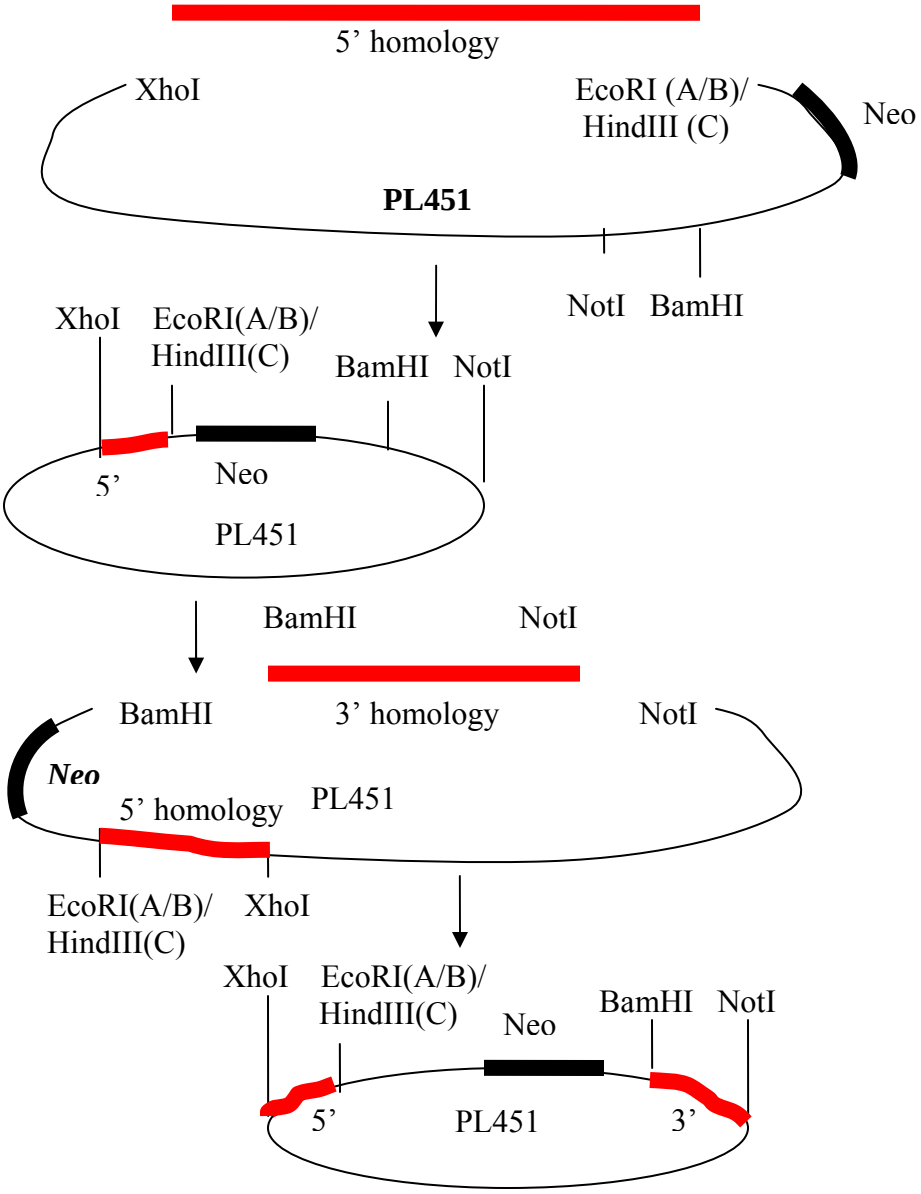


Figure 2.1 Protocol for cloning the 5’ and 3’ homology regions into the PL451 vector.

In order to clone the enhancer AB or enhancer C homology regions into the PL451 vector, first the 5’ homology region (XhoI and EcoRI for enhAB or HindIII for enhC) was cloned into the vector. Second, the 3’ homology region (BamHI and NotI) was cloned into the vector.

Electroporating the 318GFPBAC into EL250 bacterial cells

The 318GFPBAC was electroporated into EL250 bacteria since these bacteria contain heat-inducible RecA. The RecA allows homologous recombination so that enhancer regions can be deleted from the BAC. The bacteria also contain an arabinose-inducible FLP recombinase so the selection marker, Neomycin (Neo), can be removed from the cells once recombinants are selected. A single colony of EL250 bacteria was picked from an LB-Streptomycin (Strep) (10 μg/ml) and inoculated into a 5 ml LB-Strep (10 μg/ml) culture at 32°C O/N (16-20 hrs.) to an OD600 = 0.5 – 0.7. Then, the cells were collected by centrifuging them at 5000 rpm (0°C) for 6 min using a 15 ml Falcon® tube. The supernatant was poured off and the cell pellet was resuspended with 888 μl of ice-cold water. The cells were then transferred to a 1.5 ml eppendorf tube on ice and centrifuged using a bench-top centrifuge at room temperature for 20 sec at 14,000 rpm. This process is repeated a total of three times. Finally, the cell pellet was resuspended in 50 μl of ice-cold water and 75 μl of the cell suspension transferred to a disposable electroporation cuvette (0.1 cm gap). Two microliters of 318GFPBAC DNA (100ng) was added. The cells were electroporated using the Biorad GenePulser electroporator under the following conditions: 1.75kV, 25uF with the pulse controller set at 200 ohms. The time constant was around 4.0. After electroporating the cells and adding 1.0 ml SOC, the cells were transferred to a 15 ml Falcon® tube and incubated in a shaker at 32°C for 1 hour. The cells were plated at 1/10 and 9/10 onto separate LB-Chlor plates (12.5 μg/ml) for 24-48 hrs at 32°C. To verify BAC integrity, ten colonies were picked and the BAC DNA was purified using the modified BAC miniPrep procedure. To ensure no bacteria lacking the BAC was contaminating the colony, EL250 containing the BAC were inoculated in 5 ml of LB-Strep (10 μg/ml) / Chlor (12.5 μg/ml) overnight. Finally, glycerol stocks were prepared and stored at -80C.

Generation of enhancer AB and C deletion BAC recombinants

A midiprep was made for each PL451 construct. Each construct was digested with XhoI and NotI and gel isolated. The resulting linear fragment is named 5'-FRT-Neo-FRT-3'. A 5 ml LB-Strep (10 μg/ml) / Chlor (12.5 μg/ml) culture was inoculated

with a single colony of the EL250-BAC bacteria picked from an LB-Strep (10 μg/ml) / Chlor (12.5 μg/ml). The bacteria were incubated at 32°C shaking O/N (16-20 hrs) to an OD600 = 0.5 – 0.7. One milliliter of O/N culture was added to 20 ml LB in a 50 mL conical tube and incubate at 32°C shaking for 2-3 hrs or OD600=0.5. Ten milliliters of cells were transferred to a new 50 mL conical and shaken in a 42°C water bath for 15 min. Immediately after the 42°C induction, the tube was placed into wet ice and shaken on an orbital shaker to make sure the temperature dropped as fast as possible. Cells were prepared for electroporation as above. Then, the pellet was resuspended in 50 μl of ice-cold water and 75 μl were transferred to a disposable electroporation cuvette (0.1 cm gap) on ice. Two microliters of the 5'-FRT-Neo-FRT-3' (50 ng) was then added. The cells were electroporated with the Biorad GenePulser electroporator as described above. Then 1.0 ml of SOC was added, the cells were transferred to a falcon cap tube and incubated at 32°C for 1 hour. The cells were plated at 1/10 and 9/10 onto separate LB-Chlor (12.5 μg/ml) / Kan (25 μg/ml) for 24-48 hrs at 32°C. A single colony was picked, inoculated into 5 ml LB and incubated at 32°C O/N in a shaker. To induce the FLP recombinase, 1.0 ml of the O/N culture was inoculated into 10ml of LB at 32°C for 2-3 hrs or OD600=0.5. Then, 100 μl of 10% L (+) arabinose (sigma A-3256) was added to the culture (0.1% final concentration) for one hour. Dilutions of 10^{-4} and 10^{-6} were plated onto separated LB-Chlor (12.5 μg/ml) plates for 24 hours at 32°C. Ten single colonies were picked and inoculated into 5 mL LB-Chlor in the absence of Kan at 32°C shaking O/N. The cultures were streaked onto LB-Chlor (12.5 μg/ml) AND an LB-Kan (25 μg/ml) plates and incubated O/N at 32°C. Colonies that grew on the LB-Chlor but not the LB-Kan plates contained the mutated BAC. These colonies were picked. To screen the colonies, PCR primers across the neo cassette were used. To confirm that the neo cassette was flipped out, colonies with the expected PCR products were sequenced.

Generation of enhancer AB+C deletion BAC recombinant

318GFPBACΔEnhAB made previously was used as starting material for deleting the enhancer C region. The enhancer C region was deleted as discussed above. However, the procedure had to be modified to only flip out the neo cassette. Since the

318GFPBACΔEnhAB still contains a FRT site, inducing FLP recombinase in the bacteria led to recombination of the enhancer AB FRT site with the FRT site 3' of the Neo cassette in the enhancer C deletion region. To get a deletion construct that lacked enhancer C but contained the regions between enhancer C and enhancers A and B, FLP recombination was induced for 5, 10, 20, 30 and 50 minutes to flip out the neo cassette. The colonies were then plated at 10^{-4} and 10^{-6}. The next day, 5 colonies from each plate were tested for whether they contained the region between enhancer AB and C while lacking the Neo cassette that replaced enhancer C. Only the 5 minute induction contained colonies that lacked the neo cassette that replaced enhancer C while maintaining the region between enhancer AB and C. However, sequence from colonies induced for 5 minute lacked the region between enhancer AB and C. Then, 96 colonies were picked from the 5 minute induction plate and resuspended in a 96-well plate. The colonies were then stamped onto either Chlor or Kan plates and incubated at 32˚C overnight. The colonies were picked, resuspended in LB, spun down, resuspended in water and boiled. After boiling, 1 µl from each clone was used for PCR. Colonies that grew on the Chlor but not the Kan plates were screened using PCR to verify the correct deletion event. The correct deletion event occurred in one of the 96 picked colonies.

Preparation of 318GFPBAC deletion constructs for injection

318GFPBAC deletion constructs were prepared for injection using the UT Southwestern transgenic facility BAC DNA prep protocol. To determine the quality of the DNA, the 260/280 ratio was measured a spectrophotometer. Aliquots of digested and undigested DNA were then run out on a gel to determine whether there was degradation. The digested DNA banding pattern was compared to the original BAC to determine whether there were any chromosomal rearrangements. Finally, 318GFPBACΔEnhAB was injected into stage 2 mouse oocytes at 120 ng/µl and 318GFPBACΔEnhC was injected at 750 ng/µl by the UTSW Transgenic Core.

BGnEGFP-enhancer C subcloning

The purpose of creating this construct was to determine whether enhancer C is sufficient for *Atoh1* expression. Enhancer C was amplified (PCR product 5) from the 318GFPBAC using the following primers. For-EnhC-BgnEGFP-SalI (for): AAAAgtcgacCAGGAGAGGCTTCAAATGGT; Rev-EnhC-BgnEGFP-NotI (rev): AAAAgcggccgcGGCATAGGAGCAAAC ACACA. The product is about 1.8 kb. Sixty-six ng of PCR product 5 (SalI/NotI) was ligated with 40 ng of BGnEGFP (SalI/NotI). BGnEGFP contains a β-globin promoter to drive EGFP expression (Lumpkin et al., 2003). For each reaction (20 µl), 2 µl of 10 ligation buffer and 1 µl of T4 DNA ligase was added. The reactions were incubated at 16˚C overnight. Then, 0.5 µl of the reaction was transformed into DH10B cells (Invitrogen). After isolating the BGnEGFP-EnhC fragment from plasmid using SalI and NotI, this transgene was prepared for injection using the EluTip method and was injected at a concentration of 75 ng/µl. To determine the quality of the DNA, the 260/280 ratio was measured with a spectrophotometer. A small quantity was also run on a gel to determine whether there was degradation. Only constructs with a 260/280 greater than 1.7 were used for injection. Finally, EnhC-BGnEGFP was injected into stage two mouse oocytes at 100 ng/µl by the UTSW Transgenic Core.

PCR amplification steps for BGnEGFP-enhancer C construct

The amplification steps were as follows: 95 degrees for 2 minutes, 95 degrees for 30 seconds, 60 degrees for 20 seconds and 72 degrees for 30 seconds. This was followed by 35 cycles of 95 degrees for 30 seconds, 60 degrees for 20 seconds and 72 degrees for 30 seconds. There was a final extension step of 10 minutes at 72 degrees.

Tissue preparation and sectioning

Embryonic day 10.5 embryos were fixed for 2 hours at 4˚C. They were then washed 3 times for 10 minutes. Then, the head was cut just above the upper limbs. The tissue was sunk in 30% sucrose overnight at 4˚C. The head and body were embedded in

OCT and then frozen in liquid nitrogen. Horizontal sections were taken from the head and transverse sections were taken from the body.

First, the heads of e16.5 embryos were then cut from the bodies. Second, they were fixed overnight at 4˚C. They were then washed 3 times for 10 minutes. The heads and bodies were then sunk in 30% sucrose overnight at 4˚C. The next day, the heads and bodies were embedded in OCT and frozen on dry ice. Sagittal sections were taken from the head and transverse sections were taken from the body.

CHAPTER THREE

Results

Enhancer AB is required to direct detectable expression from the 318GFPBAC in transgenic mice

Enhancer AB has been shown to be sufficient for expression in transgenic animals. This enhancer responds to auto-regulation and requires Atoh1 since enhancer AB is not active in *Atoh1*-/- mice (Helms et al., 2000). However, when lacZ is knocked into the *Atoh1* locus to create an Atoh-/- mouse, lacZ is still expressed (Bermingham et al., 2001). Together, these results suggest that regions outside of enhancers A and B are required for *Atoh1* expression, particularly for initiation of expression, which should be Atoh1 independent.

To determine whether enhancers A and B are required for *Atoh1*-like expression from the 318GFPBAC and whether sequence outside A and B are sufficient to detect initiation of expression at the locus, they were deleted from the BAC that contains 100 kb 3' and 100 kb 5' of the *Atoh1* gene (**Fig 3.1**). In this BAC, the coding sequence for *Atoh1* is replaced with GFP. The modified BAC was tested for GFP expression in transgenic mice containing the 318GFPBAC lacking enhancers A and B (318GFPBACdelEnhAB).

In order to assay 318GFPBACdelEnhAB expression, embryos were taken at e10.5 and e16.5. At e10.5, expression was analyzed in the hindbrain and dorsal neural tube. At e16.5, expression was analyzed in the cerebellum, vibrissae, cochlear hair cells and vestibular hair cells. GFP was expressed in 0 out of 10 e10.5 embryos and 0 out of 1 e16.5 embryos (**Fig 3.2**). Based on PCR analysis, the GFP gene appears to be intact ruling out disruption of GFP expression as the cause for no expression.

Thus, enhancer AB is required for all detectable expression of GFP from the 318GFPBAC. The 318GFPBAC does not contain regulatory regions outside enhancer AB that are sufficient to direct detectable expression.

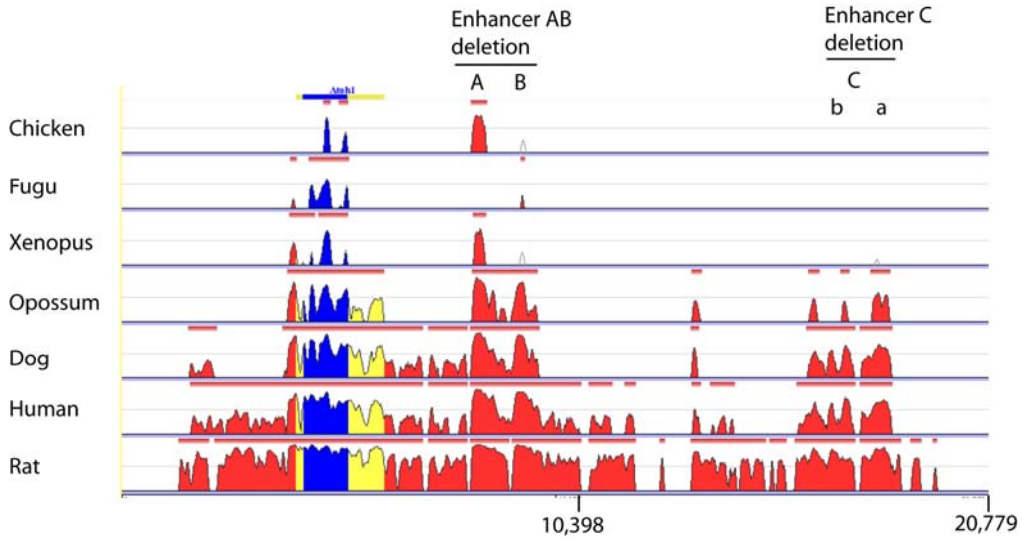


Figure 3.1 Alignment of Possible Mouse *Atoh1* Enhancer Regions with Possible Chicken, *Fugu*, *Xenopus*, Opossum, Dog, Human and Rat Enhancer Regions.

Enhancers A, B and C are designated A, B and C. Bars under the enhancer AB deletion and the enhancer C deletion indicate the size of the deletions. Enhancer C is divided into regions a and b and sequence is shown in Appendix. The enhancer alignment was created using RVista (http://rvista.dcode.org/). Ebert et al. found homology for enhancer B in the chicken genome (Ebert et al., 2003). RVista appears to have incomplete sequence for this region in the chicken genome. Based on Vista, there is homology between mouse and *Xenopus* enhancer B and C regions (http://genome.lbl.gov/vista/index.shtml).

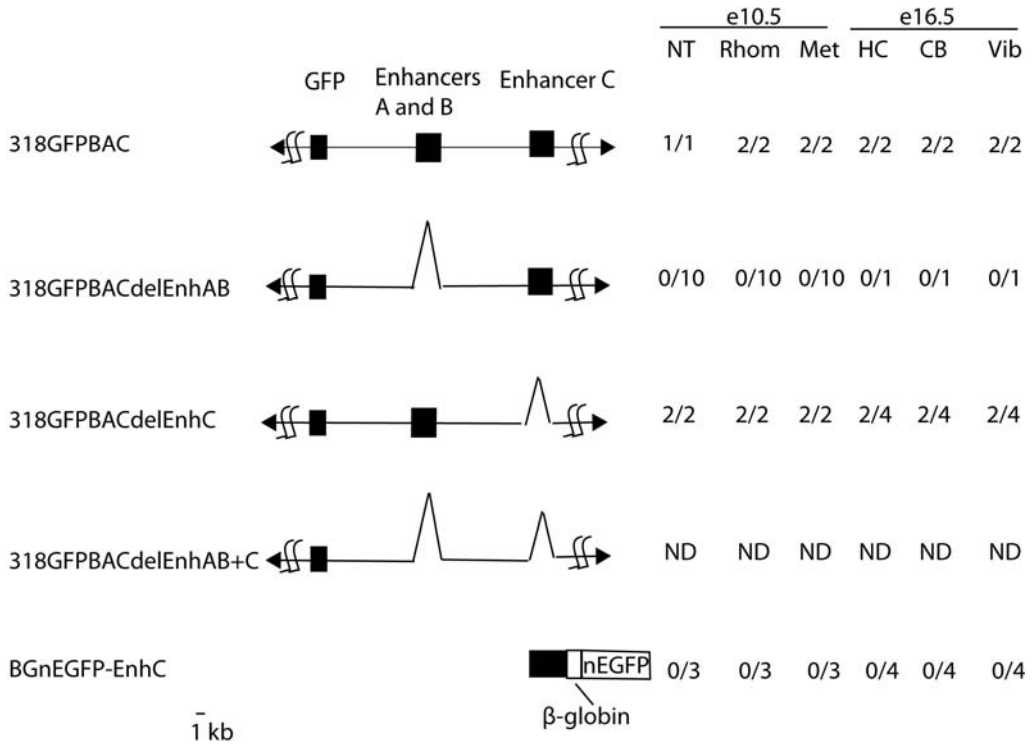


Figure 3.2 Summary of Results for Transgenic Constructs.

318GFPBAC transgenic mouse embryos expressed GFP in the neural tube (NT) and Rhombencephalon (Rhom) at e10.5 and in hair cells (HC), the cerebellum (CB) and vibrissae at e16.5. Neither BGnEGFP-EnhC nor 318GFPBACΔEnhAB transgenic mice drove GFP expression at e10.5 or e16.5. Also, 318GFPBACΔEnhC transgenic mice expressed GFP at e10.5 and e16.5. The *Atoh1* coding sequence was replaced with GFP as shown above. The 318GFPBAC contains sequence from positions 64,583,516 to 64,764,919 on chromosome 6.

Enhancer C is not required for expression from the 318GFPBAC at e10.5 or e16.5

Upon analysis of the *Atoh1* locus using RVista to compare genomic sequence from multiple species, I identified a region further 3' of *Atoh1* than the previously tested AB enhancer (**Fig 3.1**) **(**Helms et al., 2000). To determine whether this highly conserved region 3' of *Atoh1* (enhancer C) was necessary for appropriate 318GFPBAC expression, transgenic mice were created that contain the 318GFPBAC lacking enhancer C (318GFPBACdelEnhC) (**Fig 3.1** and **Appendix**).

The rationale for studying enhancer C was that it showed high conservation in mammals and some regions showed conservation to *Xeonpus* so enhancer C could contain important regulatory information (**Fig 3.1** and **Appendix**). Conservation of the enhancer C region between the dog, human, opossum, mouse, monkey, rat and *Xenopus* is shown in the **Appendix**. The sequence shown in enhancer C was deleted from the 318GFPBAC (**Appendix**).

In order to assay 318GFPBACdelEnhC expression, transgenic embryos were taken at e10.5 and e16.5. At e10.5, 318GFPBACdelEnhC expression was analyzed in the hindbrain and dorsal neural tube. At e16.5, 318GFPBACdelEnhC expression was analyzed in the cerebellum, vibrissae, cochlear hair cells and vestibular hair cells. Expression was maintained in 2 out of 2 e10.5 embryos in a pattern that matched that of transgenic embryos expressing the 318GFPBAC (**Fig 3.2** and **Fig 3.3**). Expression was also maintained in 2 of 4 e16.5 embryos in a patterned that matched that of transgenic embryos expressing the 318GFPBAC (**Fig 3.2** and **Fig 3.4**).

These results demonstrate that the sequence conserved across multiple species in enhancer C is not required for *Atoh1* expression at e10.5 or e16.5.

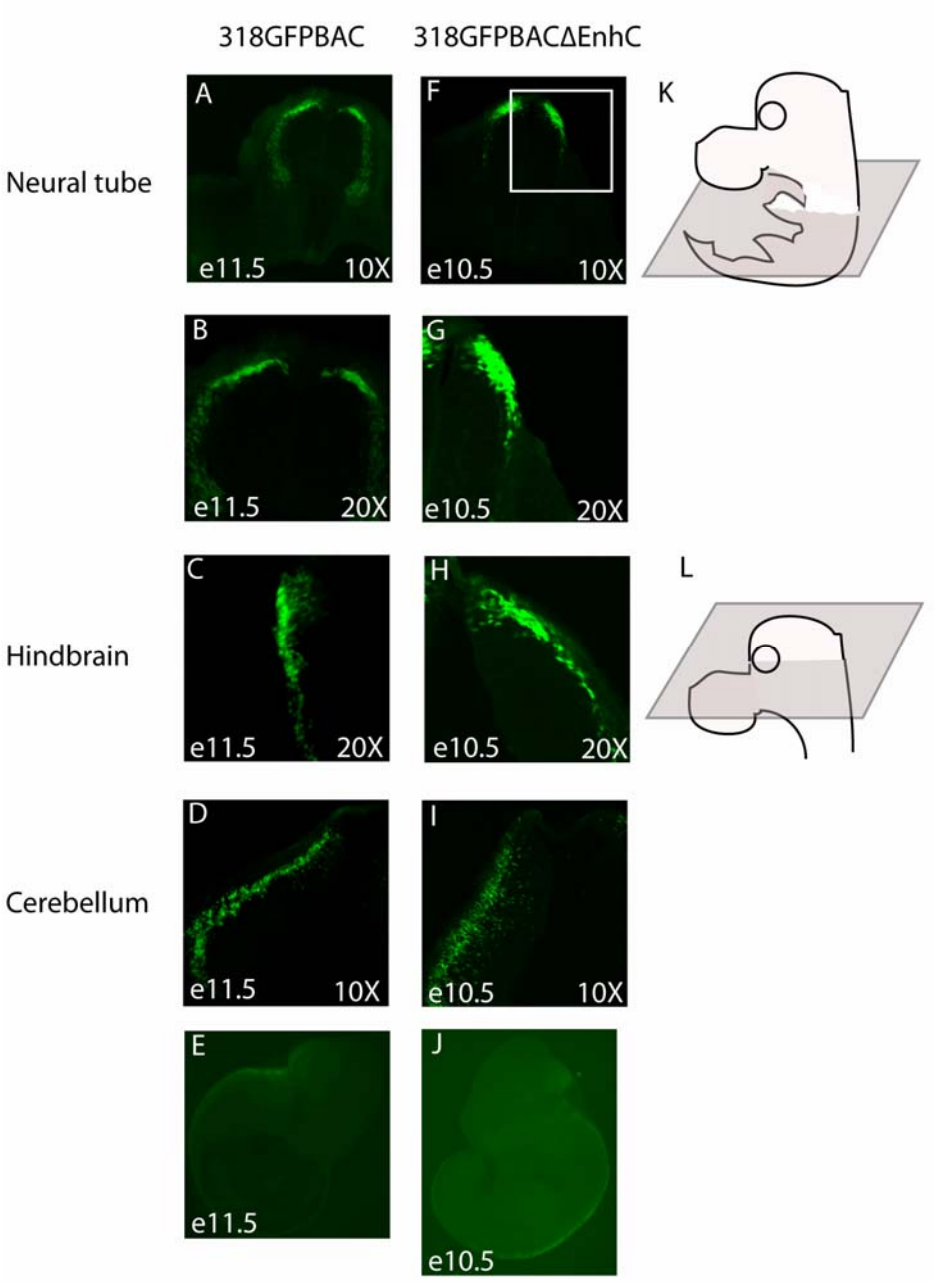


Figure 3.3 GFP Expression from 318GFPBAC and 318GFPBACΔEnhC at e10.5 and e11.5.

Both 318GFPBACΔEnhC and 318GFPBAC express GFP in the neural tube (A, B, E and J) hindbrain (C, H, E and J) and cerebellum (D, I, E and J). Sectioning planes are shown (K and L).

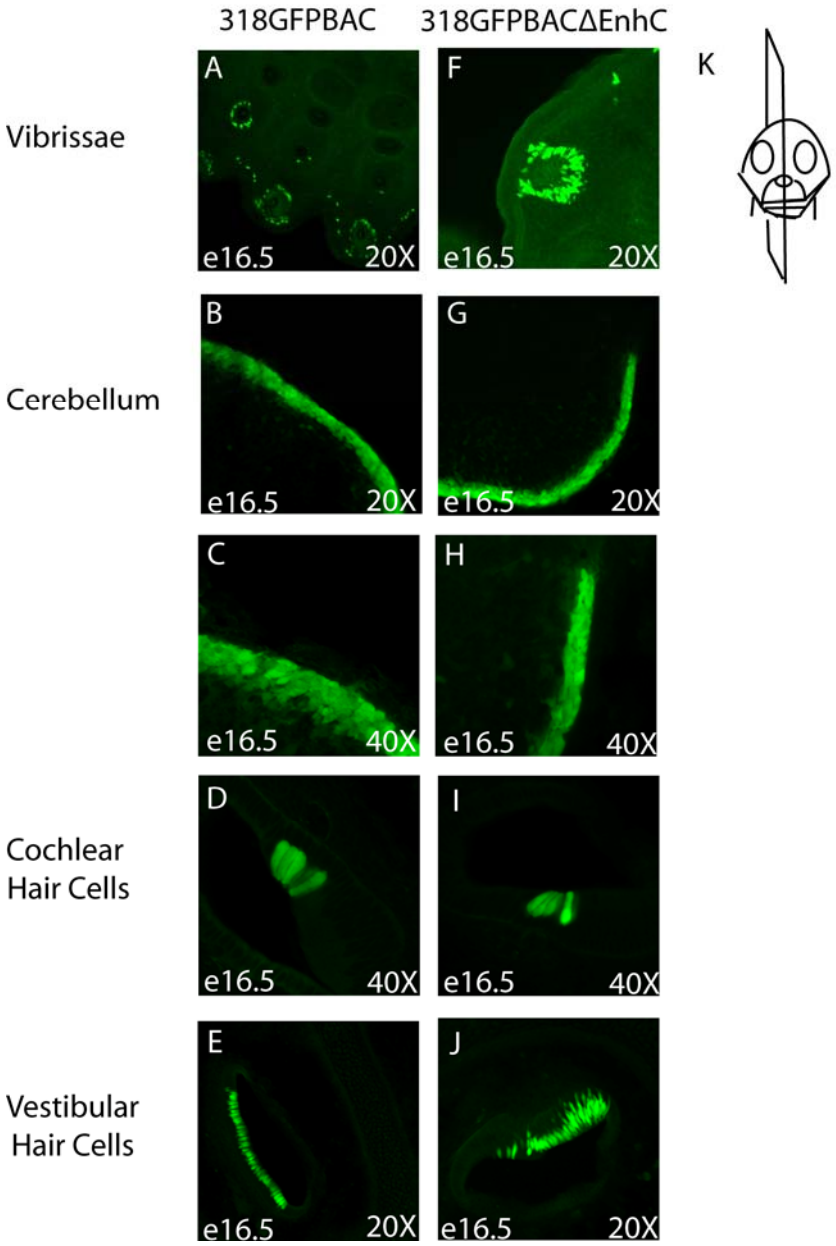


Figure 3.4 Expression from 318GFPBAC and 318GFPBACΔEnhC at e16.5.

In both the 318GFPBACΔEnhC and the 318GFPBAC drive GFP expression in merkel cells of the vibrissae (A and F), cerebellum (B, C, G and H), cochlear hair cells (D and I) and vestibular hair cells (E and J). The plane of sectioning is diagramed in K.

Enhancer C is not sufficient for Atoh1 expression

Though enhancer C is not necessary for expression from the 318GFPBAC, there could be redundant elements in the 318GFPBAC that obscure its function in this assay. It is possible that enhancer C is sufficient for *Atoh1* expression but the auto-regulation through enhancer AB that remains in the 318GFPBAC may mask this. To determine whether enhancer C is sufficient for *Atoh1* expression, transgenic mice expressing GFP driven by a basal β-globin promoter and enhancer C were created.

In order to assay BGnEGFP-EnhC expression, transgenic embryos were taken at e10.5 and e16.5. At e10.5, GFP expression was analyzed in the hindbrain and dorsal neural tube. At e16.5, GFP expression was analyzed in the cerebellum, vibrissae, cochlear hair cells and vestibular hair cells. Expression was detected in 0 out of 3 e10.5 embryos and 0 out of 4 e16.5 embryos (**Fig 3.2**). This compares with 7 out of 8 embryos expressing in an Atoh1 pattern seen with a similar strategy testing the AB enhancer (Helms et al., 2000).

In summary, enhancer C is neither necessary nor sufficient for driving GFP expression in an Atoh1 pattern. Further, enhancer AB is to date, the only sequence shown *in vivo* to function in *Atoh1* regulation being both necessary and sufficient to direct expression in an Atoh1 pattern.

CHAPTER FOUR

Discussion

Enhancer AB is necessary for expression in an Atoh1 pattern from the 318GFPBAC

Previous research has shown that enhancer AB contains regulatory information to drive GFP expression in an Atoh1-specific pattern (Helms et al., 2000). The enhancer AB region cannot drive expression in the *Atoh1-/-* mouse indicating that there is an auto-regulatory site within enhancer AB (Helms et al., 2000). Since 318GFPBACEnhAB does not express GFP, there are no other detectable Atoh1 auto-regulatory domains other than within enhancer AB. Also, there is also no Atoh1-independent expression as seen with the 318GFPBAC; *Atoh1*-/-. So enhancer AB contains regulatory information responsible for Atoh1-independent expression as well as Atoh1-dependent expression.

There are different possibilities to explain why the 318GFPBACΔEnhAB does not express GFP. One possibility is that the BAC contains chromatin information not contained in the BGnEGFP-EnhAB construct so the 318GFPBAC can express in the *Atoh1-/-* mouse while BGnEGFP-EnhAB cannot (Helms et al., 2000). A second possibility is that, in addition to providing auto-regulation, enhancer AB is necessary to coordinate the binding of transcription factors associated with other enhancer regions. A third possibility is that *Atoh1*expression is regulated at a region outside of enhancer AB and that Atoh1 negatively regulates its own expression so that, without enhancers A and B to maintain expression, Atoh1 expression is turned off. There is evidence that Atoh1 negatively regulates itself in cerebellar cells by non-cell-autonomously inducing *Hes1* expression (Gazit et al., 2004).

There are various caveats to using the 318GFPBAC to assay *Atoh1* regulatory domains. For instance, re-arrangements can occur in BACs after homologous recombination is induced and if the re-arrangement is subtle we would not detect it in our gel system. For instance, the 318GFPBACΔEnhAB may contain unwanted rearrangements that disrupt important enhancer regions outside of enhancers A and B. To increase confidence in the results provided here, and to eliminate this caveat, a second, independently generated, enhancer AB deletion BAC should be tested.

Enhancer C is neither necessary nor sufficient for expression in an Atoh1 pattern from the 318GFPBAC

Enhancer C shows high sequence homology between different species and one region appears to be conserved to *Xenopus*. However, this region does not seem to be necessary for *Atoh1* expression at e10.5 or e16.5 since the 318GFPBACΔEnhC shows neither loss of GFP expression nor ectopic GFP expression at e10.5 or e16.5. If there were positive-regulatory domains, then BGnEGFP-EnhC transgenic mice would express GFP. If enhancer C contained inhibitory domains, then 318GFPBACΔEnhC transgenic mice would express GFP ectopically.

Though 318GFPBACΔEnhC expressed GFP comparable to the 318GFPBAC at e10.5, expression driven by auto-regulation at enhancer AB may be too great to detect more subtle regulation at enhancer C.

Conclusion and future research

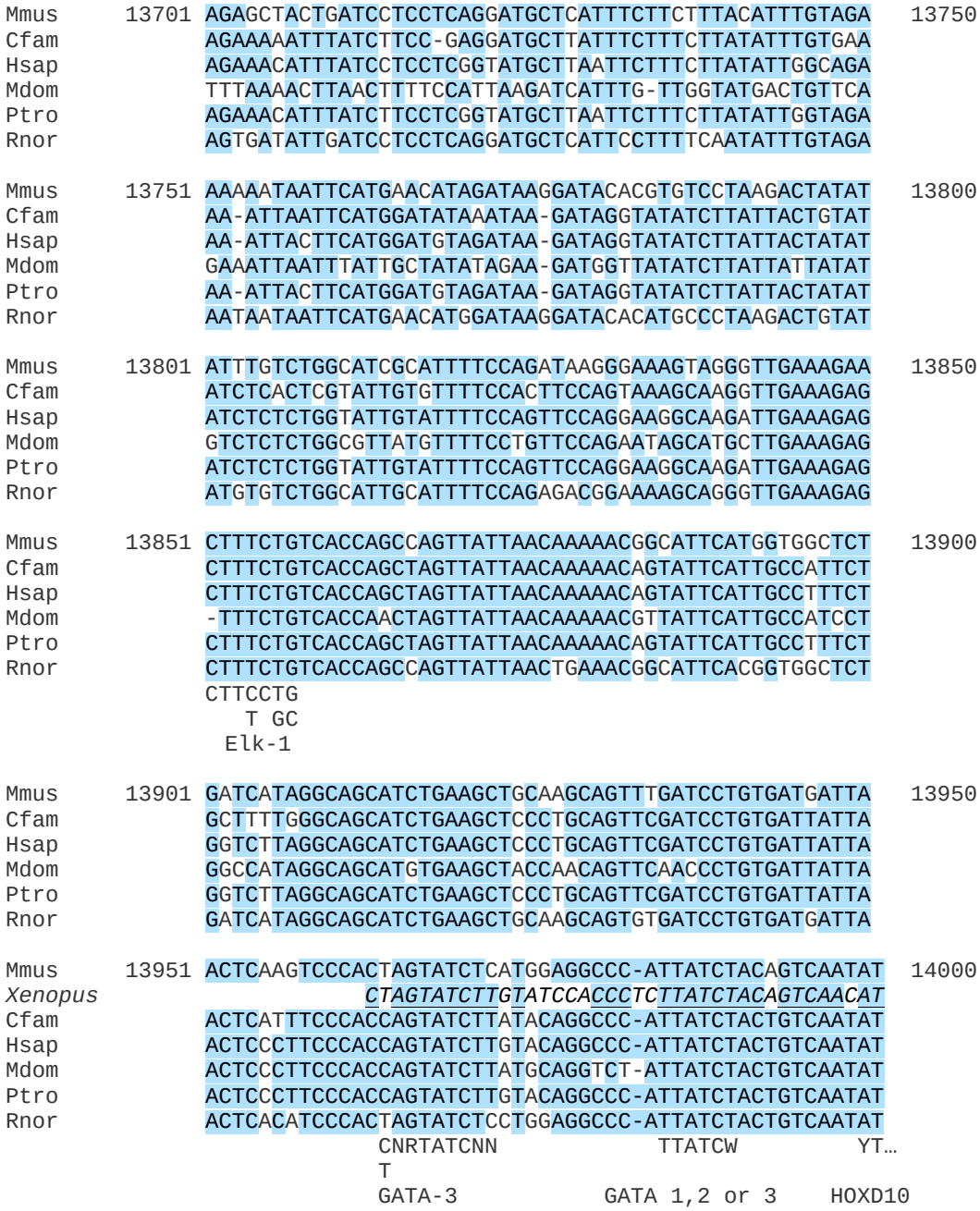
In conclusion, this study in combination with previous studies shows that enhancer AB is the only known regulatory region that is both necessary and sufficient to direct expression in an Atoh1-specific pattern. Since enhancer AB is necessary for expression from the 318GFPBAC, it may contain regions outside of the auto-regulatory domain that can drive expression in different tissues. Future research could be conducted to determine whether there are other regulatory domains in the enhancer AB region. To do this, the enhancer AB auto-regulatory regions, the ebox sequences, could be deleted from the 318GFPBAC and expression could be analyzed. If the finding of this future research are that the auto-regulatory domains are not necessary for expression, then it can be concluded that enhancer AB directs expression of GFP from the 318GFPBAC by other means than auto-regulation.

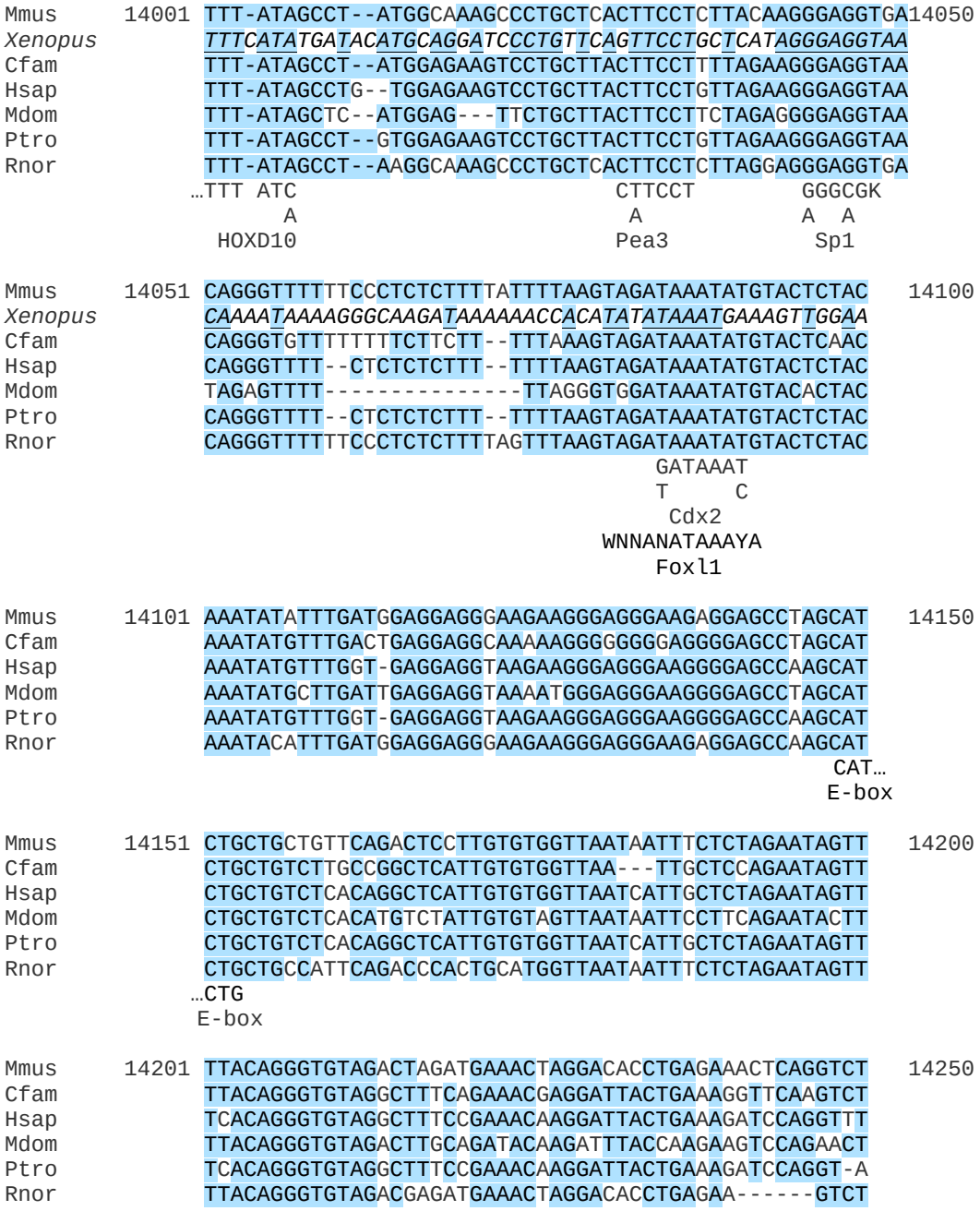
APPENDIX

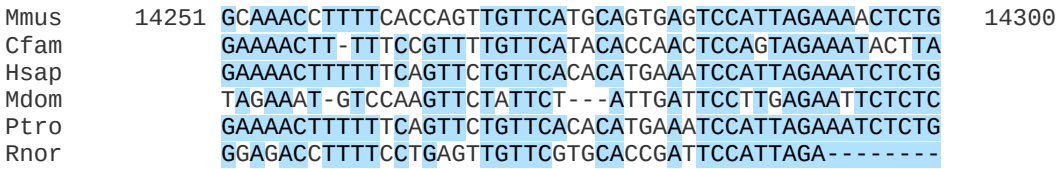
Enhancer C alignment

```
Region A
Mmus     12951 GGAGAGGCTTCAAATGGTCTCATCACTTGCTCTGAAAGGTAAATCGTGGC   13000
Cfam           GCAGAAGTTTCAAATCATTTCAACATTTATCCCATAAATTAAACCATGAA
Hsap           GTAGATGCTTTAAATGATTTCAACATTTACCCCATAAGTTAAAT------
Mdom           GAGAACGCTTCAAGTGATTTCTTAATTTCCCAAGTAACTGAAAT------
Ptro           GTAGATGCTTTAAATGATTTCAACATTTACCCCATAAGTTAAAT------
Rnor           GTAGAGGCTTCTAATGGTCTCATCACTTCCTCTGAAAGGTGAACCATGGA

Mmus     13001 AGAGTAAGGAATTCAGTTAAAGTTTGGAGAAAGGCAATGATTTCTGCAGA   13050
Cfam           TCAACAGGACACCTAGTTAGAATACAAAGAGGGGGAGAGATTGCTAGGGA
Hsap           -GAATAAGACATTTGGTTAAAATACAGAGAAAGAGAAAGATTGCTAGTGA
Mdom           -AAATCAATCATTCAGTAAAATTCCAC--AAGG----------------A
Ptro           -GAATAAGACATTTGGTTAAAATACAGAGAAAGAGAAAGATTGCTAGTGA
Rnor           AGAGTAAGGAATTCGGTTAAAGTTTGGAAAAGGGCAATTGTTGCTACAGA

Mmus     13051 GGAGAGAGAGAGCTGTGCCTGGGTAATTTCCTAGCTTCTCAATGTGTGCT   13100
Cfam           ----GAAGGGATTTGCACCTGTAGGACTTG-TGGTTACCTAATGT-----
Hsap           ----CGAGGGATTTGCACCTGTACAACTTG-TGGTTACTTAATGTCTCCT
Mdom           ----GAATGACTTTGCACCCGAACAATTTG-TA-CTACTTAGTGTCTCTG
Ptro           ----CGAGGGATTTGCACCTGTACAACTTG-TGGTTACTTAATGTCTGCT
Rnor           GAGGAGAGAGAGCTGCACCTGGGCAAGTTCCTAGCTTCCCAATGTGTGCT

Mmus     13101 CAAGTCTAGAGGTATTTGCCAGGACAGCTGGCAAATAGGGGGTTAATCAT   13150
Cfam           GAAGTCTAGAGGTGTTCACTGAGACAGCTGGCAAATAGGGGGTTAATCAT
Hsap           GAAATCTAGAAGTACTCACCAAGACAGCTGGCAAATAGGGGGTTAATCAT
Mdom           CAGATCTAAAGGCATTCATCATAACAGCTGGCAAAGAGAGGGTTAATCAT
Ptro           GAAATCTAGAAGTACTCACCAAGACAGCTGGCAAATAGGGGGTTAATCAT
Rnor           CAAGTCAGGAGGTATTCACGCGGACAGCTGGCAAATAGAGGGTTAATCAT
                                       CAGCTGGC
                                      HEB (e-box)

Mmus     13151 TACAGAAAGTAGTGCATCTTAGCTTTCAGTTTGGTATAGACTGAATGGCA   13200
Cfam           TACAGAAGATCATGCATCCTATCTTTCAGTTTGGTTTATACAGAAGGGCA
Hsap           TACAGAAAATGATGCATCATATCTTTCAGTTTGGTTTATACTGAAGGGCA
Mdom           TACTGAAAATGATGTTTCATATCTTTCAGTCTTGTTTATTCA-TAGGACA
Ptro           TACAGAAAATGATGCATCATATCTTTCAGTTTGGTTTATACTGAAGGGCA
Rnor           TACAGAAAATGCTGTCTCATATCTTTCAGTTTGGTTTATACCGAAAGGCA

Mmus     13201 GTAAAGATTTATGCTTCAGGACAGGGTAGAACTGTTATTTTCAGAGTGTG   13250
Cfam           GTAAAGATTTATGCTTTGGGAAAGGATAAAACTATTATTTTCAGAGTGTA
Hsap           GTAAAGATTTATGCTTCAGG--AGGATAAAACTGTTATTTTCAGAGTGTG
Mdom           GTAAAGAGCTGTGT--GGGG-AAAGGGAGTTTTTTTTTTTCAATGGGTG
Ptro           GTAAAGATTTATGCTTCAGG--AGGATAAAACTGTTATTTTCAGAGTGTG
Rnor           GGAAAGATTTATGCTTCAGAGGAGGATAGAACTGCTATGTGCAGAGTGTG

Mmus     13251 GCTGGCTCCATTGATTAATTTCTGAGGGTATGTAGATACATCACAAAGCA   13300
Cfam           GTTGATTCCTTTGCTTAATTTCTAAGGAAATGTAGATATATTATAAAACA
Hsap           GTTGTCTCCTTTGATTATTTTCTGAGGCAATGTAGATATATTATAGAACA
Mdom           GATGGCTTTTTTGATTACTA----CAGTGATATCAA-AAACCACAAAGGA
Ptro           GTTGTCTCCTTTGATTATTTTCTGAGGCAATGTAGATATATTATAGAACA
Rnor           GTTGGCTCCACTGATTAATTTCGGAGGCCCTGGAGACACATCACAAAGCA
```

```
Mmus     13301 TCCTTGTCTCCCACTCCAAAAGAGCTGGATTTAGAAATTGTTATTTTGTC   13350
Cfam           CCTT-AGCTAT--TCGTAAAAGAACTCT----GGTGCTGGTACTTTAATC
Hsap           TCCT-AGTGCT--CCCTAAAAGAACTCTC--------TGGTACTTTAATC
Mdom           TCCCTGTTTTC--CCTTAAAAGAA--------------------------
Ptro           TCCT-AGTGCT--CCCTAAAAGAACTCTC--------TGGTACTTTAATC
Rnor           TCCT-ATCTCCCACTCCAAAAGAGCTGAATTGGGGAATTGTGTTCTTGTC

Mmus     13351 ACAAGGACCCTCTTAGGCTAGATGGAAACTTCTTGGCCTTCTAAAAAATA   13400
Cfam           ACCTTGATCTTCTTAGACTATATGCAAACTTCTTAGGTTTCTGAA-----
Hsap           ATCTGGAT-GTATTAGGCTATATGAAAACGTCTTGGGTTTCTGAA-----
Mdom           --------------------------------------------------
Ptro           ATCTGGAT-GTATTAGGCTATATGAAAACGTCTTGGGTTTCTGAA-----
Rnor           ACAGGGACCATCTTATGCTATATGAAAATGTGGTGACCTTCTAAAAGATA

Mmus     13401 CTAATATCAAGCCATGTGTGGCATCTAACGTCCGTAATCCCAGCATAAGG   13450
Cfam           --------------------------------------------------
Hsap           --------------------------------------------------
Mdom           --------------------------------------------------
Ptro           --------------------------------------------------
Rnor           CTAATATTAAGCCCTGTGTGGTATCTAACATTTGTAATCCCAGCATAAGG

Mmus     13451 AGGCTGGTGCAGGTGATCTCTAGTTTCAGACAGCCTTGGTTACATAGCAA   13500
Cfam           --------------------------------------------------
Hsap           --------------------------------------------------
Mdom           --------------------------------------------------
Ptro           --------------------------------------------------
Rnor           AGACTGAGGCAGGCGCTCTCCAGTTTCAGACACACTTGGCTACACAGCAA

Region b
Mmus     13501 GATATCTCAAAGAACTCAAACAAAAGTTGTAACATCTGAGGCTTAAAAGA   13550
Cfam           ----------------------AAGTTCTGACACCTAAGGCTCATACGA
Hsap           ----------------------ATGTTCTAACACCTAAGGCTTATAAGA
Mdom           ----TCTCCAGGAAATGGAAGATAAGTCCC-AAATATGAGACCAAAGAAA
Ptro           ----------------------ATGTTCTAACACCTAAGGCTTATGAGA
Rnor           GCTGTCTCAGAGAAGTCAAGCAAAGGTTGTAACATCTGAGGCAGATAAGA

Mmus     13551 ATATGCGAGTATAGCAATATTTTTTAAAAGAGAAAATTTCTTCTTAAGTT   13600
Cfam           ATATTGAACTTCAGTAACATT-TTTAAAGGAGGAAATTCTCTTTTAAATT
Hsap           ATATTGAGCTATAGTAACATT-TTAAAAACAGAAGATTCCTTATTAAATT
Mdom           ATGTGCCAGCATAGAAAAATCTTTTAAAAAAGAAAATCCATATTTCAGCT
Ptro           ATATTGAGCTATAGTAACATT-TTAAAAACAGAAGATTCCTTATTAAATT
Rnor           ATATTAGATTATAGAAATGTTTTTCAAAAGAGAAAATTTCTTAGTAAGTT

Mmus     13601 AGGTAACATTATCTGGCTAAATGAGTGTCTCCTTTCTTACAATGTTTTTC   13650
Cfam           AGATAATACTGTTTGGCTGAGTTAGTT--TACTTTCTTGCAGCATTTTTA
Hsap           AGGTAATACTGTTTGTCTACATCAGTTGCCACTTTCTTGCAGTATTTTTC
Mdom           GGCTAACTTTAT-GGCCTTAATGGGCTTCCCTGAAATTCCAGTCTTATTT
Ptro           AGGTAATGCTGTTTGTCTACATCAGTTGCCACTTTCTTGCAGTATTTTTC
Rnor           AGGTAACATTCACTGGCTAAATGAGTGTCTCCTTTCTTATAATGCTTTGC

Mmus     13651 AAAGTAGGGTTTTCTTTTCCAGTATTTCTTCTTACTGCTAATCACACTGC   13700
Cfam           AAA-GGGAGTTTTATCCTCCAATACTTCTAATTACTGCTAATCACATTTT
Hsap           AAAAGATAGTTTTATCCTCCAATATTTTCAATTGCTGCTAACTGCATTTT
Mdom           AAAAGAGGGAAA--------AATTCCTGAAGATACTGCTAAT---ATCTT
Ptro           AAAAGATAGTTTTATCCTCCAATATTTTCAATTACTGCTAACTGCATTTT
Rnor           AAAGTCGGGTTTCCTCTGCCAGTATTTCTTCTTACTGCCAATCCCATTGC
```


Mmus 13701 AGAGCTACTGATCCTCCTCAGGATGCTCATTTCTTCTTTACATTTGTAGA 13750
Cfam AGAAAAATTTATCTTCC-GAGGATGCTTATTTCTTTCTTATATTTGTGAA
Hsap AGAAACATTTATCCTCCTCGGTATGCTTAATTCTTTCTTATATTGGCAGA
Mdom TTTAAAACTTAACTTTTCCATTAAGATCATTTG-TTGGTATGACTGTTCA
Ptro AGAAACATTTATCTTCCTCGGTATGCTTAATTCTTTCTTATATTGGTAGA
Rnor AGTGATATTGATCCTCCTCAGGATGCTCATTCCTTTTCAATATTTGTAGA
Mmus 13751 AAAAATAATTCATGAACATAGATAAGGATACACGTGTCCTAAGACTATAT 13800
Cfam AA-ATTAATTCATGGATATAAATAA-GATAGGTATATCTTATTACTGTAT
Hsap AA-ATTACTTCATGGATGTAGATAA-GATAGGTATATCTTATTACTATAT
Mdom GAAATTAATTTATTGCTATATAGAA-GATGGTTATATCTTATTATTATAT
Ptro AA-ATTACTTCATGGATGTAGATAA-GATAGGTATATCTTATTACTATAT
Rnor AATAATAATTCATGAACATGGATAAGGATACACATGCCCTAAGACTGTAT
Mmus 13801 ATTTGTCTGGCATCGCATTTTCCAGATAAGGGAAAGTAGGGTTGAAAGAA 13850
Cfam ATCTCACTCGTATTGTGTTTTCCACTTCCAGTAAAGCAAGGTTGAAAGAG
Hsap ATCTCTCTGGTATTGTATTTTCCAGTTCCAGGAAGGCAAGATTGAAAGAG
Mdom GTCTCTCTGGCGTTATGTTTTCCTGTTCCAGAATAGCATGCTTGAAAGAG
Ptro ATCTCTCTGGTATTGTATTTTCCAGTTCCAGGAAGGCAAGATTGAAAGAG
Rnor ATGTGTCTGGCATTGCATTTTCCAGAGACGGAAAGCAGGGTTGAAAGAG
Mmus 13851 CTTTCTGTCACCAGCCAGTTATTAACAAAAACGGCATTCATGGTGGCTCT 13900
Cfam CTTTCTGTCACCAGCTAGTTATTAACAAAAACAGTATTCATTGCCATTCT
Hsap CTTTCTGTCACCAGCTAGTTATTAACAAAAACAGTATTCATTGCCTTTCT
Mdom -TTTCTGTCACCAACTAGTTATTAACAAAAACGTTATTCATTGCCATCCT
Ptro CTTTCTGTCACCAGCTAGTTATTAACAAAAACAGTATTCATTGCCTTTCT
Rnor CTTTCTGTCACCAGCCAGTTATTAACTGAAACGGCATTCACGGTGGCTCT
CTTCCTG
T GC
Elk-1
Mmus 13901 GATCATAGGCAGCATCTGAAGCTGCAAGCAGTTTGATCCTGTGATGATTA 13950
Cfam GCTTTTGGGCAGCATCTGAAGCTCCCTGCAGTTCGATCCTGTGATTATTA
Hsap GGTCTTAGGCAGCATCTGAAGCTCCCTGCAGTTCGATCCTGTGATTATTA
Mdom GGCCATAGGCAGCATGTGAAGCTACCAACAGTTCAACCCTGTGATTATTA
Ptro GGTCTTAGGCAGCATCTGAAGCTCCCTGCAGTTCGATCCTGTGATTATTA
Rnor GATCATAGGCAGCATCTGAAGCTGCAAGCAGTGTGATCCTGTGATGATTA
Mmus 13951 ACTCAAGTCCCACTAGTATCTCATGGAGGCCC-ATTATCTACAGTCAATAT 14000
Xenopus CTAGTATCTTGTATCCACCCTCTTATCTACAGTCAACAT
Cfam ACTCATTTCCCACCAGTATCTTATACAGGCCC-ATTATCTACTGTCAATAT
Hsap ACTCCCTTCCCACCAGTATCTTGTACAGGCCC-ATTATCTACTGTCAATAT
Mdom ACTCCCTTCCCACCAGTATCTTATGCAGGTCT-ATTATCTACTGTCAATAT
Ptro ACTCCCTTCCCACCAGTATCTTGTACAGGCCC-ATTATCTACTGTCAATAT
Rnor ACTCACATCCCACTAGTATCTCCTGGAGGCCC-ATTATCTACTGTCAATAT
CNRTATCNN TTATCW YT…
T
GATA-3 GATA 1,2 or 3 HOXD10


Mmus 14001 TTT-ATAGCCT--ATGGCAAAGCCCTGCTCACTTCCTCTTACAAGGGAGGTGA 14050
Xenopus TTTCATATGATACATGCAGGATCCCTGTTCAGTTCCTGCTCATAGGGAGGTAA
Cfam TTT-ATAGCCT--ATGGAGAAGTCCTGCTTACTTCCTTTTAGAAGGGAGGTAA
Hsap TTT-ATAGCCTG--TGGAGAAGTCCTGCTTACTTCCTGTTAGAAGGGAGGTAA
Mdom TTT-ATAGCTC--ATGGAG---TTCTGCTTACTTCCTTCTAGAGGGGAGGTAA
Ptro TTT-ATAGCCT--GTGGAGAAGTCCTGCTTACTTCCTGTTAGAAGGGAGGTAA
Rnor TTT-ATAGCCT--AAGGCAAAGCCCTGCTCACTTCCTCTTAGGAGGGAGGTGA
…TTT ATC CTTCCT GGGCGK
A A A A
HOXD10 Pea3 Sp1
Mmus 14051 CAGGGTTTTTTCCCTCTCTTTTATTTTAAGTAGATAAATATGTACTCTAC 14100
Xenopus CAAAATAAAAGGGCAAGATAAAAAACCACATATATAAATGAAAGTTGGAA
Cfam CAGGGTGTTTTTTTTCTTCTT--TTTAAAGTAGATAAATATGTACTCAAC
Hsap CAGGGTTTT--CTCTCTCTTT--TTTTAAGTAGATAAATATGTACTCTAC
Mdom TAGAGTTTT---------------TTAGGGTGGATAAATATGTACACTAC
Ptro CAGGGTTTT--CTCTCTCTTT--TTTTAAGTAGATAAATATGTACTCTAC
Rnor CAGGGTTTTTTCCCTCTCTTTTAGTTTAAGTAGATAAATATGTACTCTAC
GATAAAT
T C
Cdx2
WNNANATAAAYA
Foxl1
Mmus 14101 AAATATATTTGATGGAGGAGGGAAGAAGGGAGGGAAGAGGAGCCTAGCAT 14150
Cfam AAATATGTTTGACTGAGGAGGCAAAAAGGGGGGGGAGGGGAGCCTAGCAT
Hsap AAATATGTTTGGT-GAGGAGGTAAGAAGGGAGGGAAGGGGAGCCAAGCAT
Mdom AAATATGCTTGATTGAGGAGGTAAAATGGGAGGGAAGGGGAGCCTAGCAT
Ptro AAATATGTTTGGT-GAGGAGGTAAGAAGGGAGGGAAGGGGAGCCAAGCAT
Rnor AAATACATTTGATGGAGGAGGGAAGAAGGGAGGGAAGAGGAGCCAAGCAT
CAT…
E-box
Mmus 14151 CTGCTGCTGTTCAGACTCCTTGTGTGGTTAATAATTTCTCTAGAATAGTT 14200
Cfam CTGCTGTCTTGCCGGCTCATTGTGTGGTTAA---TTGCTCCAGAATAGTT
Hsap CTGCTGTCTCACAGGCTCATTGTGTGGTTAATCATTGCTCTAGAATAGTT
Mdom CTGCTGTCTCACATGTCTATTGTGTAGTTAATAATTCCTTCAGAATACTT
Ptro CTGCTGTCTCACAGGCTCATTGTGTGGTTAATCATTGCTCTAGAATAGTT
Rnor CTGCTGCCATTCAGACCCACTGCATGGTTAATAATTTCTCTAGAATAGTT
…CTG
E-box
Mmus 14201 TTACAGGGTGTAGACTAGATGAAACTAGGACACCTGAGAAACTCAGGTCT 14250
Cfam TTACAGGGTGTAGGCTTTCAGAAACGAGGATTACTGAAAGGTTCAAGTCT
Hsap TCACAGGGTGTAGGCTTTCCGAAACAAGGATTACTGAAAGATCCAGGTTT
Mdom TTACAGGGTGTAGACTTGCAGATACAAGATTTACCAAGAAGTCCAGAACT
Ptro TCACAGGGTGTAGGCTTTCCGAAACAAGGATTACTGAAAGATCCAGGT-A
Rnor TTACAGGGTGTAGACGAGATGAAACTAGGACACCTGAGAA------GTCT



Enhancer C Homology among Dog (Cfam), Human (Hsap), Opossum (Mdom), Mouse (Mmus), Monkey (Ptro) and Rat (Rnor). The alignment is from the Ensemble Genome Browser. All binding sites except for CDX2 are from TRANSFAC. The CDX2 binding site is from Suh et al. (Suh et al., 1994). The numbers indicate the nucleotide position relative to the Atoh1 start site.

BIBLIOGRAPHY

Akazawa, C., Ishibashi, M., Shimizu, C., Nakanishi, S., and Kageyama, R. (1995). A mammalian helix-loop-helix factor structurally related to the product of Drosophila proneural gene atonal is a positive transcriptional regulator expressed in the developing nervous system. J Biol Chem *270*, 8730-8738.

Ben-Arie, N., Hassan, B. A., Bermingham, N. A., Malicki, D. M., Armstrong, D., Matzuk, M., Bellen, H. J., and Zoghbi, H. Y. (2000). Functional conservation of atonal and Math1 in the CNS and PNS. Development *127*, 1039-1048.

Bermingham, N. A., Hassan, B. A., Price, S. D., Vollrath, M. A., Ben-Arie, N., Eatock, R. A., Bellen, H. J., Lysakowski, A., and Zoghbi, H. Y. (1999). Math1: an essential gene for the generation of inner ear hair cells. Science *284*, 1837-1841.

Bermingham, N. A., Hassan, B. A., Wang, V. Y., Fernandez, M., Banfi, S., Bellen, H. J., Fritzsch, B., and Zoghbi, H. Y. (2001). Proprioceptor pathway development is dependent on Math1. Neuron *30*, 411-422.

Canzoniere, D., Farioli-Vecchioli, S., Conti, F., Ciotti, M. T., Tata, A. M., Augusti-Tocco, G., Mattei, E., Lakshmana, M. K., Krizhanovsky, V., and Reeves, S. A.*, et al.* (2004). Dual control of neurogenesis by PC3 through cell cycle inhibition and induction of Math1. J Neurosci *24*, 3355-3369.

Ebert, P. J., Timmer, J. R., Nakada, Y., Helms, A. W., Parab, P. B., Liu, Y., Hunsaker, T. L., and Johnson, J. E. (2003). Zic1 represses Math1 expression via interactions with the Math1 enhancer and modulation of Math1 autoregulation. Development *130*, 1949-1959.

Gazit, R., Krizhanovsky, V., and Ben-Arie, N. (2004). Math1 controls cerebellar granule cell differentiation by regulating multiple components of the Notch signaling pathway. Development *131*, 903-913.

Gowan, K., Helms, A. W., Hunsaker, T. L., Collisson, T., Ebert, P. J., Odom, R., and Johnson, J. E. (2001). Crossinhibitory activities of Ngn1 and Math1 allow specification of distinct dorsal interneurons. Neuron *31*, 219-232.

Helms, A. W., Abney, A. L., Ben-Arie, N., Zoghbi, H. Y., and Johnson, J. E. (2000). Autoregulation and multiple enhancers control Math1 expression in the developing nervous system. Development *127*, 1185-1196.

Helms, A. W., Gowan, K., Abney, A., Savage, T., and Johnson, J. E. (2001). Overexpression of MATH1 disrupts the coordination of neural differentiation in cerebellum development. Mol Cell Neurosci *17*, 671-682.

Jarman, A. P., Grau, Y., Jan, L. Y., and Jan, Y. N. (1993). atonal is a proneural gene that directs chordotonal organ formation in the Drosophila peripheral nervous system. Cell *73*, 1307-1321.

Kim, P., Helms, A. W., Johnson, J. E., and Zimmerman, K. (1997). XATH-1, a vertebrate homolog of Drosophila atonal, induces a neuronal differentiation within ectodermal progenitors. Dev Biol *187*, 1-12.

Liu, P., Jenkins, N. A., and Copeland, N. G. (2003). A highly efficient recombineering-based method for generating conditional knockout mutations. Genome Res *13*, 476-484.

Lumpkin, E. A., Collisson, T., Parab, P., Omer-Abdalla, A., Haeberle, H., Chen, P., Doetzlhofer, A., White, P., Groves, A., Segil, N., and Johnson, J. E. (2003). Math1-driven GFP expression in the developing nervous system of transgenic mice. Gene Expr Patterns *3*, 389-395.

Mutoh, H., Sakamoto, H., Hayakawa, H., Arao, Y., Satoh, K., Nokubi, M., and Sugano, K. (2006). The intestine-specific homeobox gene Cdx2 induces expression of the basic helix-loop-helix transcription factor Math1. Differentiation *74*, 313-321.

Shimizu, T., Tsujigiwa, H., Nagatsuka, H., Okafuji, N., Kurihara, S., Nagai, N., and Kawakami, T. (2005). Expression of Notch1 and Math1 in mandibular condyle cartilage in neonatal mice. Angle Orthod *75*, 993-995.

Suh, E., Chen, L., Taylor, J., and Traber, P. G. (1994). A homeodomain protein related to caudal regulates intestine-specific gene transcription. Mol Cell Biol *14*, 7340-7351.

Sun, Y., Jan, L. Y., and Jan, Y. N. (1998). Transcriptional regulation of atonal during development of the Drosophila peripheral nervous system. Development *125*, 3731-3740.

Vernay, B., Koch, M., Vaccarino, F., Briscoe, J., Simeone, A., Kageyama, R., and Ang, S.-L. (2005). Otx2 regulates subtype specification and neurogenesis in the midbrain. J Neurosci *25*, 4856-4867.

Yang, Q., Bermingham, N. A., Finegold, M. J., and Zoghbi, H. Y. (2001). Requirement of Math1 for secretory cell lineage commitment in the mouse intestine. Science *294*, 2155-2158

VITAE

Chris Aries Adams was born in Delaware, Ohio, on July 4, 1981, the son of Kathleen Marie Angona and Ronald Jerry Adams. After completing his work at Lake Oswego High School, Lake Oswego, Oregon in 2000, he entered Reed College in Portland, Oregon. Work from his undergraduate thesis contributed to the article, "Brg1 is required for murine neural stem cell maintenance and gliogenesis", which was published in the journal Developmental Biology. He worked with researchers at the Oregon Health and Science University to complete his undergraduate thesis. During the summers of 2001and 2004 he attended the University of Texas at Dallas. He worked as a fellow during the summers of 2002 and 2003 at the University of Texas Southwestern Medical Center at Dallas. He received the degree of Bachelor of the Arts with a major in Biology from Reed College in December, 2004. In September, 2005 he entered the Graduate School of Biomedical Sciences at the University of Texas Southwestern Medical Center at Dallas.

Permanent Address: 2921 Beverly Dr.
Plano, TX 75235