MODULATION OF EXCITATORY AND INHIBITORY NEURONAL BALANCE THROUGH REGULATION OF PTF1A BY FACTORS BINDING TO ZINC FINGER AND POU MOTIFS

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I dedicate this work to my family and friends
who have helped me on this journey and made
it possible for success in this endeavor.
I love you all.

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The proper function of the nervous system depends on a delicate balance between excitatory and inhibitory neurons. Transcription factors of the basic helix-loop-helix (bHLH) family have been shown to be particularly important in generating the correct numbers of these neurons during development. One of these, Ptf1a, is required in the specification of inhibitory neurons in multiple regions of the nervous system including the dorsal spinal cord. The absence of *Ptf1a* in null mice disrupts the balance of excitatory and inhibitory neurons, as Ptf1a is required for generating inhibitory neurons while suppressing the excitatory phenotype. Therefore, discovering the regulators of *Ptf1a* expression will identify mechanisms controlling the generation of a balanced neural network required for processing somatosensory information.

Using sequence conservation between divergent vertebrate species, a 1.2 kb enhancer that directs expression of a reporter gene to *Ptf1a* expressing domains in transgenic mice was identified approximately 11 kb 3' of the coding region. A series of mutations across the 1.2 kb enhancer were generated to identify sequences required for activity of this enhancer. The activity of the enhancer in directing expression specifically to the developing dorsal neural tube requires at least two distinct motifs: a putative POU motif required for activity, and a zinc finger which represses activity in non-*Ptf1a*-expressing populations

within the neural tube. The activities of these two motifs were tested by in chick and transgenic mice. Coupled with a bioinformatics approach, several candidates for the upstream transcription factors have been identified and were tested for their role in regulating the temporal and spatial specific-activity of the *Ptf1a*-enhancer. One factor, Zic1 was shown to repress expression of Ptf1a. Thus, a combination of transcriptional activators and repressors are required to control *Ptf1a* expression, which regulates the subsequent balanced generation of inhibitory and excitatory neurons in the dorsal spinal cord.

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Chapter One

Introduction

Gross development of the spinal cord

The spinal cord comprises a critical piece of the central nervous system that integrates sensory information and relays motor output in response to sensory input. The primary center of the sensory integration occurs in the dorsal horn of the spinal cord. In the dorsal horn, interneurons are the primary effectors for receiving and relaying sensory input to the brain; sensory input can include pain, proprioception, and temperature. Appropriate motor output is then relayed through the ventral horn of the spinal cord, the motor neuron center of the central nervous system. The super structure of the spinal cord is complex, and begins development during embryogenesis. The spinal cord arises from invagination and subsequent closure of the neural plate. This process forms the neural tube which is then subject to developmental signals to generate distinct populations of sensory and motor neurons essential for proper function of an organism's nervous system (Jessell 2000).

The overall structure of the neural tube is divided into two distinct regions, the mantle zone and the ventricular zone. Within the ventricular zone lie neural progenitors that give rise to glia and neurons present in the adult spinal cord. The mantle zone contains neurons that are undergoing specification, and eventually become mature neurons. Neurogenesis occurs primarily through embryonic day

9.5 (E9.5) and embryonic day 14.5 (E14.5), and is governed by neurogenic basic-helix-loop-helix (bHLH) transcription factors (Helms and Johnson 2003). These bHLH transcription factors are expressed in a spatially and temporally restricted fashion to generate distinct populations of neurons in the spinal cord. Spatial restriction of bHLH transcription factors is observed to follow along a dorsal-ventral axis in the neural tube (Helms and Johnson 2003). This axis is established, in part, by signaling cues from the roof plate and the floor plate. These two structures are specified by the surface ectoderm and the notochord, which are located immediately above and below the neural tube, respectively. The surface ectoderm and roof plate secrete Bone Morphogenic Proteins (BMPs), which creates a gradient from highest (dorsal) to lowest concentrations (ventral) in the neural tube (Helms and Johnson 2003). Family members of the BMP proteins implicated in this pathway include Bmp2, Bmp4, Bmp6 and Bmp7 (Le Dreau and Marti 2012). The opposite is true for Sonic Hedgehog (SHH), which is secreted from the floor plate and notochord, and creates a gradient that decreases in concentration toward the dorsal neural tube (Le Dreau and Marti 2012). Misexpression of either BMP or SHH in the neural tube dorsalizes or ventralizes neural tube neuronal populations, respectively (reviewed in Le Dreau and Marti 2012). The combinatorial effect of these opposing gradients generates a proper dorsal-ventral axis that contains molecular determinants, which lead to specific, spatial expression of bHLH transcription factors through genetic pathways that result in distinct populations of neurons in the spinal cord.

Regulation of gene expression

The central dogma of biology is that DNA is transcribed into RNA in the nucleus, which is then exported to the cytoplasm to be translated into a protein product. Each of these steps are tightly regulated and are responsive to external stimuli, and present in prokaryotes and eukaryotes. Regulation of gene expression is essential for processes such as cellular differentiation and morphogenesis. Methods used by cells to regulate expression of genes include: 1) modifications to the structure of chromatin and DNA; 2) RNA-mediated mechanisms that result in transcript degradation or translational processing; and 3) transcription factors, enhancers, and insulators that affect the transcription of a gene.

The overall structure chromatin can be classified as accessible (euchromatin) or inaccessible (heterochromatin) to *trans*-regulatory elements such as transcription factors. The accessibility of chromatin to transcription factors relies upon remodeling of the chromatin structure by post-translational modification of histones that form the nucleosome, the basic unit of packaging DNA in eukaryotes (Luger et al. 1997; Felsenfeld and Groudine 2003). Modifications of the histones facilitated by histone deacetylases (HDACs) or histone acetylases (HATs). These modifications by these enzymes render the DNA inaccessible (deacetylation of n-terminal lysine residues by HDACs) or accessible (acetylation of n-terminal lysine residues by HATs) (Grunstein 1997; Marmorstein and Roth 2001; Zupkovitz et al. 2006). Further elucidation of histone modifications, or marks, have revealed that certain modifications facilitate the

accessibility of chromatin such as trimethylation of histone H3 on lysine 4, lysine 36, or lysine 79 (H3K4me3, H3K36me3, H3K79me3 (Barski et al. 2007). Histone modifications are also correlated with levels of gene expression, modifications that affect a permissive state often correlate with a higher gene expression, and modifications that restrict chromatin accessibility correlate with a reduction in gene expression (Mikkelsen et al. 2007; Karlic et al. 2010). Methylation of CpG repeats and repressive histone marks has been linked to the recruitment of histone deacetylases that are known to render, generally, chromatin inaccessible (Boyes and Bird 1991; Jones et al. 1998; Billard et al. 2002; El-Osta et al. 2002; Rietveld et al. 2002). Furthermore, it has been resolved that distinct histone modifications such as monomethylation of H3K4 and acetylation of H3K27 may serve as predictors for *cis*-regulatory elements—e.g. gene enhancers and promoters (Barski et al. 2007; Heintzman et al. 2007).Recent work on histone modification has reinforced that distinct histone markers, specifically H3K79me3 and H3K27ac, and importantly, RNA polymerase II occupancy may accurately predict enhancer function in *Drosophila* embryos (Bonn et al. 2012).

The importance of modification to DNA is demonstrated by *Xenopus* and mouse models where methylation is disrupted by loss of the methyltranferases, Dnmt1, which results in embryonic lethality (Li et al. 1992; Lei et al. 1996; Panning and Jaenisch 1996; Stancheva et al. 2001). Other methyl transferases have been identified, such as Dnmt3a, Dnmt3b, and Dnmt3L, all of which are required for establishing methylation on DNA and are critical embryonic development (Lyko et al. 1999; Okano et al. 1999; Bourc'his et al. 2001; Hata et

al. 2002). Methylation of CpG repeats has been shown to block binding of transcription factors to their cognate sites (Tate and Bird 1993). Alternatively, methylation of some CpG repeats, may stimulate enhancer and promoter interactions that positively regulate gene expression by blocking binding of the insulator protein CTCF (Hark et al. 2000; Ohlsson et al. 2001). Mechanisms of modifying chromatin structure and DNA methylation will continue to be analyzed, and what may be appreciated is that the overall structure of the chromatin is a determining factor to how genes a regulated.

Another known mechanism of gene expression regulation is though RNA-mediated processes. One such RNA-mediated mechanism of gene expression regulation is dependent upon small RNA sequences, approximately 20 nucleotides in length, that are complementary to target transcripts (Elbashir et al. 2001). The two classes of small RNAs that mediate gene expression are small interfering RNAs (siRNAs) and microRNAs (miRNAs). Generally, endogenous transcripts that give rise to these interfering RNA molecules are 20-50 nucleotides in length, which fold back onto themselves to form double-stranded RNA (dsRNA) hairpins (Bartel 2004). These dsRNA hairpins are then processed in the nucleus by Drosha, a dnRNA-specific RNA-III-type endonuclease, in which the hairpin loops are removed resulting in a double stranded “base stems” (Lee et al. 2002; Lee et al. 2003). The resulting dsRNA products are then exported to the cytoplasm where they are further processed by another RNA endonuclease, Dicer, to yield approximately 21 base pair duplexes (Bernstein et al. 2001; Grishok et al. 2001; Hutvagner et al. 2001; Lee et al. 2004). A single strand,

typically the antisense due to a strand bias, is loaded into an RNA-induced silencing complex (RISC) which includes members of the Argonaut family; it is this complex that will mediate mRNA degradation or translational repression (Mourelatos et al. 2002; Bartel 2004; Okamura et al. 2004). RISC targets complementary or nearly complementary transcripts to the template mi- or siRNAs, and once targeted, the transcripts are degraded via a hydrolytic mechanism (Martinez et al. 2002; Martinez and Tuschl 2004; Schwarz et al. 2004). Translational repression by miRNAs has been shown by experiments which revealed association of miRNA to its cognate target site on an mRNA, and reduced levels of the target protein without a reduction in the levels of the target mRNA (Olsen and Ambros 1999; Seggerson et al. 2002).

Another mechanism of RNA-mediated regulation involves large intergenic non-coding RNAs (lincRNAs), or long non-coding RNAs (lncRNAs). Over 1000 lncRNAs have been found in the human genome and few have functions that have been defined (Mattick 2009). These RNA molecules are 200 nucleotides or greater in length and are transcribed from intergenic regions. The functions of lncRNAs are beginning to be revealed. Identification of lncRNAs centers around, but is not exclusive to, the presence of a short sequence of H3K3me3 marks and a longer stretch of H3K36me3 (Barski et al. 2007; Mikkelsen et al. 2007; Guttman et al. 2009; Khalil et al. 2009). lncRNA regulates gene expression through direct or indirect mechanisms that involve partners which modulate availability of *trans*-acting factors or modification of chromatin such that DNA is inaccessible to *trans*-acting factors. Two well-known lncRNAs, *HOTAIR* and *Xist*, have been shown to

recruit the methyltransferase Polycomb Repressive Complex 2 (PRC2) which in turn trimethylates H3K27—a marker for silent chromatin—on target loci (Bracken et al. 2006; Rinn et al. 2007; Ku et al. 2008; Zhao et al. 2008). Targeting of PRC2 to loci can be achieved directly, as in the case of *HOTAIR*, or through co-factors; *Xist* recruits YY1 to direct methylation of H3K27 to target loci (Rinn et al. 2007; Jeon and Lee 2011). In addition to lncRNAs associating with PRCs, they have been demonstrated to associate with other known repressor complexes including CoREST and REST which reveals that lncRNAs may be involved with lineage-specific repressive programs, e.g. repressing neural-specific expression in non-neural lineages, as lncRNAs are expressed in distinct patterns across various cellular lineages (Tsai et al. 2010). lncRNAs can also assist in activation of gene expression by recruiting specific gene-activating methyltransferases; *Mistral* is hypothesized to assist in the formation of chromatin loops between enhancers and promoters of target loci of the *HOXA* cluster by recruitment of MLL, a trimethyltransferase of the Trithorax family, which is known to be critical in activating expression of target genes (Bertani et al. 2011; Dean 2011; Schuettengruber et al. 2011; Wang et al. 2011). Other proteins that are recruited by lncRNAs include the Dnmt1 and Dnmt3a, which are recruited by *Kcnq1ot1* and *Tsix* respectively, which methylate CpG islands on target promoters (Sado et al. 2006; Sun et al. 2006; Mohammad et al. 2010). Additionally, a lncRNA may act as a “sink” that sequesters transcription factors from target genes. An example of this mechanism is *PANDA*, which competes for NF-YA binding and results in reduced expression of NF-YA target genes (Hung et al. 2011). While

long non-coding RNAs have been identified, and few have been functionally verified in contrast to their coding counterparts, it is becoming increasingly evident that this class of transcripts may play an important role in regulating gene expression through mechanisms which affect the structure of chromatin or interactions between *trans*- and *cis*-elements of gene expression.

Synergistic interactions between *cis*- and *trans*-regulatory elements dynamically regulate gene expression in addition to the mechanisms described previously. *Cis*-regulatory elements include promoters, enhancers, and insulators. *Trans*-regulatory elements are primarily transcription factors, which act upon *cis*-regulatory elements to affect levels of gene expression. A promoter may be sub-divided into a core promoter containing a transcriptional start site (TSS) that serves as a starting point for transcription of the gene being regulated, and a proximal promoter (upstream of the core promoter) that has recognition sites for transcription factors, and may be located near CpG islands. Enhancers are DNA sequences that are able to regulate transcription of a gene in a spatial and temporal manner, and independently of the distance or orientation (upstream or downstream) of a target gene. Enhancers have binding sites for multiple transcription factors, and these can be the same factors that bind to the proximal promoter (Maston et al. 2006). Another characteristic of enhancers is that they may have the ability to regulate more than one gene (Mohrs et al. 2001). Identification of developmentally critical enhancers has been aided by comparing sequences of vertebrate and mammalian species, and isolating sequences that have high degrees of conservation (Bejerano et al. 2004; Pennacchio et al.

2006). Testing highly conserved non-coding sequences has revealed that conservation is a valid method of identifying enhancers, as reports have shown as much as half of such sequences have shown enhancer-like activities (Pennacchio et al. 2006; Visel et al. 2008). Conservation is not a fail-safe method of enhancer identification as some sequences that exhibit high degrees of conservation do not have enhancer-like activities; conversely, there are sequences which are not highly conserved, but do exhibit enhancer-like activities (Blow et al. 2010; Schmidt et al. 2010). Chromatin marks, or modifications, have provided another avenue to identify putative enhancers as recent studies have shown that p300, H3K27ac, and H3K4me1 are shown to mark regions that have enhancer activity (Heintzman et al. 2007; Visel et al. 2009; Creyghton et al. 2010). The action through which enhancers regulate transcription is still being elucidated, but it appears that chromatin forms loops that serve to bring enhancers and promoters in close proximity of each other. Evidence for chromatin looping between enhancers and promoters is provided, in part, by chromosome conformation capture (3C) and Fluoresence *in situ* hybridization (FISH) which have shown physical interaction between distant regions of chromatin, and functional relevance for such looping interactions (Vakoc et al. 2005; Jiang and Peterlin 2008; Miele and Dekker 2008; Deng et al. 2012). Kagey *et al* demonstrated that Mediator, a transcriptional co-activator, and Cohesin, a protein involved in maintaining the separation of chromatin, co-occupy active genes, interact, and are predictive of looping interactions; furthermore, loss of either Mediator or Cohesin results in significant changes in expression levels of

genes where both are present, and a decreased frequency of interaction between core promoters and enhancers (Kagey et al. 2010). Genome-wide profiles have revealed that general transcription machinery (e.g. RNA pol II) are present at enhancers and can serve recruit factors necessary for the pre-initiation complex; other evidence of this is the presence and requirement of elongation factor, ELL3, for Pol II on enhancers (Koch et al. 2011; Lin et al. 2013). Enhancers serve important functions in mediating the expression of genes through interactions of transcription factors to specific DNA sequences that may act from considerable distances.

Insulators are DNA sequences that serve to block genes from transcriptional activity of neighboring genes and limit the activity of regulatory elements such as enhancers (Ghirlando et al. 2012; Herold et al. 2012; Kirkland et al. 2013). They may do so by blocking interactions between enhancers and promoters (enhancer blocking insulators) and preventing the proliferation of repressive chromatin marks (barrier insulators) (Sun and Elgin 1999; Recillas-Targa et al. 2002). Insulators are located in loci that contain a high number of genes and regulatory elements (Fourel et al. 2004). CCCTC-binding factor, or CTCF, is the primary vertebrate insulator protein that acts upon these insulator sequences (Ohlsson et al. 2001; Chaumeil and Skok 2012; Lee and Iyer 2012; Merkenschlager and Odom 2013). CTCF is expressed ubiquitously and is required for early development of the mouse embryo (Fedoriw et al. 2004; Wendt et al. 2008). CTCF is able to recruit transcriptional co-factors such as PRDM5 to chromatin (Galli et al. 2013). Interaction between CTCF and transcription

machinery prevents improper gene expression (Chopra et al. 2009; Erokhin et al. 2011). Accordingly, insulators block enhancer-promoter interactions and heterochromatin-mediated silence to check aberrant expression or silencing of genes.

Transcription factors are a large class of gene expression regulatory molecules that act upon discrete DNA sequences that occur with high regularity in gene regulatory elements (Bryne et al. 2008; Wunderlich and Mirny 2009). Transcription factors regulate gene expression in networks; their function can be redundant, and a particular factor's activity may be regulated by another transcription factor that is upstream or downstream in a given regulatory network (Thomas 1993; Laney and Biggin 1996; Davidson 2010; Young 2011). With the advent genome-wide sequencing, it has been repeatedly demonstrated that transcription factors bind at many thousands of sites throughout the euchromatic genome; this phenomenon has been observed across the commonly used genetic models (Johnson et al. 2007; Gerstein et al. 2010; Rey et al. 2011). Activities of certain transcription factors that are differentially expressed in a specific spatial or temporal pattern vary from factors that are ubiquitously expressed (Lupien et al. 2008; Gerstein et al. 2010; Roy et al. 2010). Furthermore, in addition to histone marks, occupancy of genomic regions by transcription factors may also be indicative of transcription control regions (Carr and Biggin 1999; Fullwood et al. 2009; MacArthur et al. 2009; Rey et al. 2011; Yu et al. 2011). Chromatin accessibility, as noted previously, is a significant factor in the binding patterns of transcription factors; transcription factors are able to bind

to “open” chromatin and not “closed” chromatin (Li et al. 2011). Transcription factors may interact with each other, other proteins, or transcriptional complexes such as general transcription factors to modulate specificity to gene targets (Laney and Biggin 1997; Ptashne and Gann 1997; Wagner 1997; Boyd et al. 1998). A critical component of gene expression regulation lies in the function of transcription factors that are able to recruit general transcription machinery to promoters and other *cis*-regulatory sequences to initiate or repress transcription of target sequences, this is regulated by co-factors that may enhance or suppress transcription factor activity in addition to chromatin structure. What follows is a description of the family of transcription factors that this work is concerned with, the basic-helix-loop-helix (bHLH) family of transcription factors, and their role in regulation of gene expression.

bHLH expression, function, and structure in neuronal differentiation

The neuronal populations in the neural tube are specified by neurogenic bHLH factors. These populations include (from dorsal-ventral): dI1-dI6, v0-v2, MN, and v3 (Lee and Pfaff 2001; Helms and Johnson 2003; Alaynick et al. 2011; Zhu et al. 2013). Each type of interneuron is determined by the bHLH factor that is expressed in a given progenitor cell. For example, dI1 is specified by progenitors expressing *Atoh1*; dI2 by *Ngn1/2*; dI3-5 by *Ascl1*; and dI4 by *Ptf1a* (Helms and Johnson 2003; Glasgow et al. 2005). These members of the bHLH family comprise a branch of the class II helix-loop-helix transcription factors, of which there are 7 classes, most of which have the ability to bind DNA and

regulate transcription of target genes (Massari and Murre 2000). All members of the helix-loop-helix (HLH) family are characterized by two alpha helices separated by a flexible loop region. Members of this family that bind directly to DNA do so through a hexameric sequence, CANNTG, or the E-box (Ephrussi et al. 1985). This hexameric sequence is readily observed throughout the genome including enhancers and promoters of developmentally critical genes in B-cell, pancreatic, and nervous system tissues (Massari and Murre 2000).

Class I HLH factors a widely expressed and are characterized by the ability to homodimerize with other class I HLHs, and heterodimerize with other bHLH classes (Murre et al. 1989). Two prominent factors within this group are E12 and E47, products of the *E2A* gene via alternative splicing events (Sun and Baltimore 1991). The crystal structure of an E47 homodimer bound to DNA revealed that Class I transcription factors interact with the major groove of DNA through a conserved basic region (Ellenberger et al. 1994). Moreover, both products of *E2A* mediate transcriptional events through two conserved activation domains, AD1 and AD2, located in the N-terminus of the respective proteins (Aronheim et al. 1993; Quong et al. 1993; Massari et al. 1996). It has been shown that E-proteins, through the HLH domain, have the ability to interact with chromatin modifying Histone Acetyltransferases (HATs) in order to potentiate transcription of target genes (Eckner et al. 1996; Ogryzko et al. 1996). Additional functions of the E-proteins have been ascribed to activation of B-cell lineage gene expression, as well as rudimentary tumor suppressor roles in induction of apoptosis of tumor tissue deficient in E12 or E47 (Murre et al. 1991; Bain et al.

1997; Engel and Murre 1999). E-proteins are widely expressed proteins that play a critical role in mediating binding of E-protein homodimers, as well as HLH heterodimers, to DNA and regulating transcriptional activities of developmentally critical genes.

Class II HLH transcription factors are characterized by restricted expression and an inability to homodimerize. In order for class II HLHs to effect transcription, a class II HLH preferentially forms a heterodimer with class I HLHs, such as the E-proteins mentioned above (Murre et al. 1989). Class II HLHs, similar to the E-proteins, have a conserved basic region that interacts directly with targeted E-boxes on DNA. Class II bHLH factors play an important role in tissue development. For example, Beta2/Neurod is expressed in the α and β cells, and is required for proper pancreatic development, as knockout models die several days after birth, partly due to severe diabetes (Naya et al. 1997). Beta2/Neurod is also expressed in the developing nervous system and is known to play a role in development of inner ear sensory neurons and granule cells of the cerebellum and hippocampus (Goebbels et al. 2005). The importance of class II bHLHs is further highlighted in the developing nervous system where these factors are critical for proper formation of the nervous system (Helms et al. 2005; Battiste et al. 2007; Galichet et al. 2008). The function of selected bHLH factors will be discussed below in detail below.

Class III HLHs contain an adjacent leucine zipper domain in adjacent to the distinctive bHLH (Zhao et al. 1993). Members of this family include Myc, and sterol-responsive element-binding proteins (Srebp1/2) (Le Dreau and Marti

2012). Myc is known to be involved with many developmental processes, in addition to its well-known oncogenic properties (Hurlin 2013). Myc and Srebp1/2 regulate transcription of target genes through recruitment of transcriptional complexes with HAT-activity that modify chromatin to enable activation of target genes (Grant et al. 1998; McMahon et al. 1998; Naar et al. 1998; Vassilev et al. 1998). Class IV HLHs, such as Max and Mad, have the ability to interact with class III HLHs and facilitate activation or repression of target genes, respectively (Blackwood and Eisenman 1991; Ayer et al. 1993; Zervos et al. 1994). The transcriptional activity of Max is mediated by HATs, and the repressive nature of Mad is due to its recruitment of a histone deacetylase complex, or HDAC that modifies DNA such that it is rendered inaccessible to transcription factors (Ayer et al. 1995; Schreiber-Agus et al. 1995; Alland et al. 1997; Heinzel et al. 1997).

Class V HLH members lack the basic region and do not bind DNA. Instead, they function as dominant-negative repressors; class V members sequester transcriptional activation of E-protein and other bHLH targets through formation of heterodimers, which are functionally inactive (Massari and Murre 2000). Notable class V members include the Id family. The Id family is comprised of four proteins Id1-4 and is known to bind to E-proteins (of class I) and class II bHLH factors (Langlands et al. 1997). Binding of any of the Id factors to class I or class II represses the ability of the binding member to effect transcription (Benezra et al. 1990). For example, Id3, which is expressed in the pancreas, has been shown to titrate away E47 (essential for Ptf1a activity) from binding to Ptf1a negatively affecting transcriptional activity of Ptf1a (Dufresne et al. 2011).

Class VI HLH factors contain a proline within their DNA-binding basic domain and may function as repressors. Important members of this class is Hairy and Enhancer of Split, which plays a role in regulation of Notch signaling (to be discussed below), represses gene transcription through recruitment of HDACs that modify the chromatin around target genes (Chen et al. 1999). Class VII factors are characterized by a Per-Arnt-Sim (PAS) domain in conjunction with the bHLH domain (bHLH-PAS). Aromatic hydrocarbon receptor (AHR) and AHR nuclear-translocator (Arnt) are two members that form a heterodimer and translocate into the nucleus in where they direct a genetic program that regulates detoxification of hydrocarbons (Ko et al. 1997; Crews 1998). Experiments have determined that AHR-activation mutants modify chromatin surrounding the target gene *CYP1A1* to allow for binding of other transcriptional activators (Ko et al. 1996).

Ascl1 and Ptf1a function in neurogenesis in the neural tube

In the developing spinal cord, neurogenesis occurs through two phases: an early phase, which occurs from E10 to E11, and a late phase that continues from E11.5 to E13.5 (Helms and Johnson 2003). During the early phase, Ptf1a-expressing progenitors constitute one of six distinct progenitor populations (dP1-6) that express neurogenic bHLH transcription factors, the dP4 population that will become GABAergic neurons (Glasgow et al. 2005). Ptf1a expression is transient and essential to specify progenitors towards the dI4 neuronal fate

(Glasgow et al. 2005). Ascl1, another bHLH transcription factor, expresses in dP3-5 populations, is critical for specification of glutamatergic neurons and is required for Ptf1a expression in the late phase of neurogenesis (Mizuguchi et al. 2006). In late phase neurogenesis, Ptf1a-expressing progenitors consist of the dP^A population, which differentiate into GABAergic dIL^A neurons (Glasgow et al. 2005).

Ptf1a-null mice die at birth and have a complete loss of dI4 and dIL^A GABAergic neurons in the dorsal spinal cord. In the absence of Ptf1a, the progenitor cells do not undergo apoptosis, but rather they switch to a dI5 and dIL^B glutamatergic (excitatory) fate (Glasgow et al. 2005). This demonstrates that Ptf1a executes two functions in the central nervous system: 1) to induce a GABAergic specification program and 2) suppress a glutamatergic fate in progenitors. This function for Ptf1a is not restricted to the spinal cord as loss of Ptf1a results in loss Purkinje cells and other inhibitory populations in the cerebellum with an increase in excitatory granule cells, and a loss of inhibitory amacrine and horizontal cells in the retina with in increase in retinal ganglia cells. Thus, understanding how Ptf1a expression is regulated becomes a critical question in unraveling how the balance of excitatory and inhibitory neurons in the CNS is controlled.

Regulation of Ptf1a in the central nervous system

A Ptf1a-dependent regulatory mechanism operates though a 2.3 kb enhancer located 13.4 kb 5’ of the Ptf1a coding region. The 5’ enhancer contains

two consensus-binding sites for Ptf1a that are required for enhancer activity (Meredith et al. 2009). Furthermore, the enhancer is not active in *Ptf1a* mutants. Taken together, the 5' *Ptf1a* enhancer requires Ptf1a for its activity.

Another enhancer, 12.4 kb in length, was identified downstream of the *Ptf1a* coding region that directed expression of a reporter transgene to the dorsal neural tube in mice and chicken. In contrast to the auto-regulatory enhancer described above, this enhancer directed expression to only some of the Ptf1a expression domains (Meredith et al. 2009). Furthermore, although the enhancer directed restricted expression to the dorsal neural tube, expression was not restricted to Ptf1a lineage cells and was maintained to later stages. Nevertheless, the 12.4 kb enhancer contains information for initiating expression restricted to the dorsal neural tube. In this study, we show that a 1.2 kb sequence is necessary and sufficient to recapitulate the activity of the 12.4 kb enhancer in the dorsal neural tube. In addition, integration of both activator and repressor activities through this enhancer are required for the dorsal restricted expression. These activities require the POU motif for activation, and a zinc finger transcription factor-binding motif for restricting expression to the dorsal domain.

Thesis rationale and goals

This work seeks to address the question of how Ptf1a, a key molecular determinant of a properly specified central nervous system, is regulated. A study by Meredith et al uncovered an enhancer sequence 12.4 kb in length that directs

reporter activity to Ptf1a-expressing regions in the neural tube, retina and hindbrain. Moreover, this enhancer acts independent of Ptf1a expression suggesting that the enhancer sequence contains information necessary for function of Ptf1a. In knowing how Ptf1a expression is initiated, an important regulator of proper nervous system specification will be uncovered. To this end, Chapter 2 will seek to demonstrate that within the 12.4 kb enhancer lies a 1.2 kb sequence that is sufficient for enhancer activity. Furthermore, an ultra-conserved 132 bp sequence is necessary for activity of the 1.2 kb *Ptf1a* enhancer. Chapter 3 will show that there lies both activator and repressor elements in the ultra-conserved sequence and that the activator element is due to a predicted POU motif whose activity is also required for enhancer function in mammals. Also, amongst multiple candidates tested that have predicted motifs within the 1.2 kb *Ptf1a* enhancer, only Zic1 functioned when overexpressed in the chick neural tube to repress Ptf1a expression and repress Ptf1a enhancer activity.

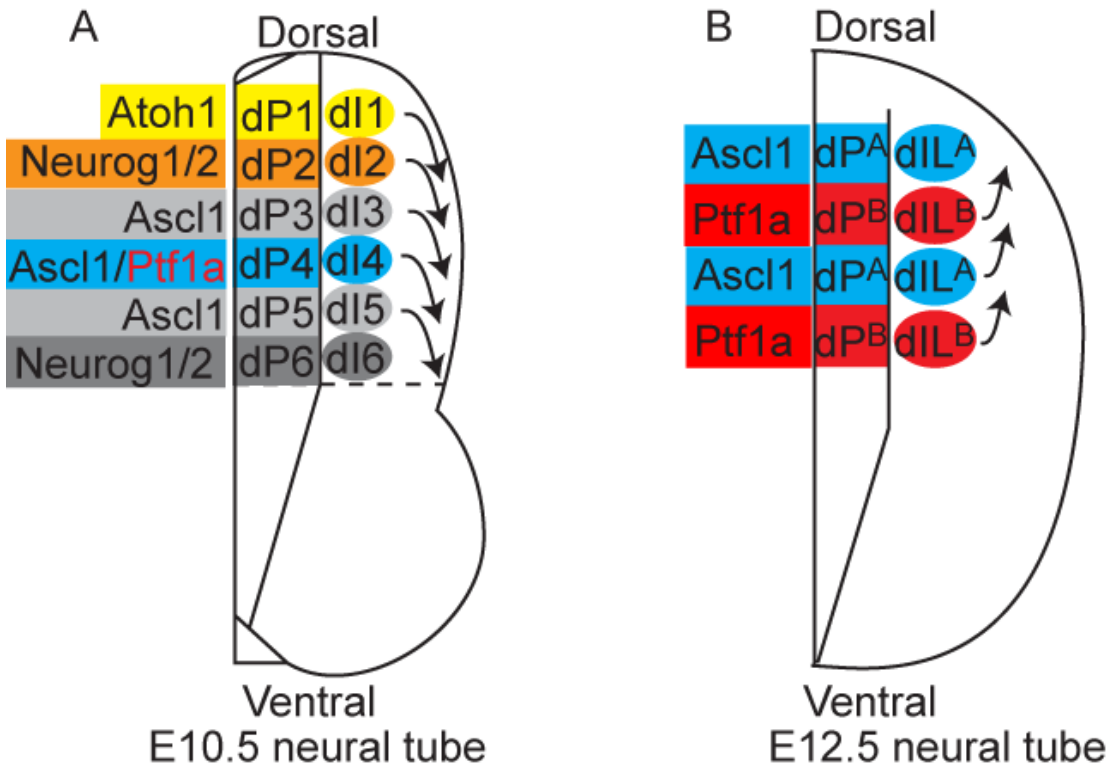

Figure 1.1. Expression of bHLH transcription factors in spinal cord development. The bHLH family plays a critical role in cell-fate decisions of progenitor populations in the spinal cord during early and late phases of neurogenesis. **(A)** During the early phase of neurogenesis, members of the bHLH family are expressed in distinct domains that specify particular lineages of interneurons. Upon transient expression of these factors, progenitors begin to differentiate and migrate out of the ventricular zone and into the mantle zone. **(B)** During late phases of neurogenesis, the two interneuron populations specified by Ascl1 and Ptf1a are dIL^A and dIL^B, respectively. These two factors are not restricted to distinct domains as in the early phase. Instead, progenitors that express Ascl1 or Ptf1a are dispersed in a salt-and-pepper pattern.

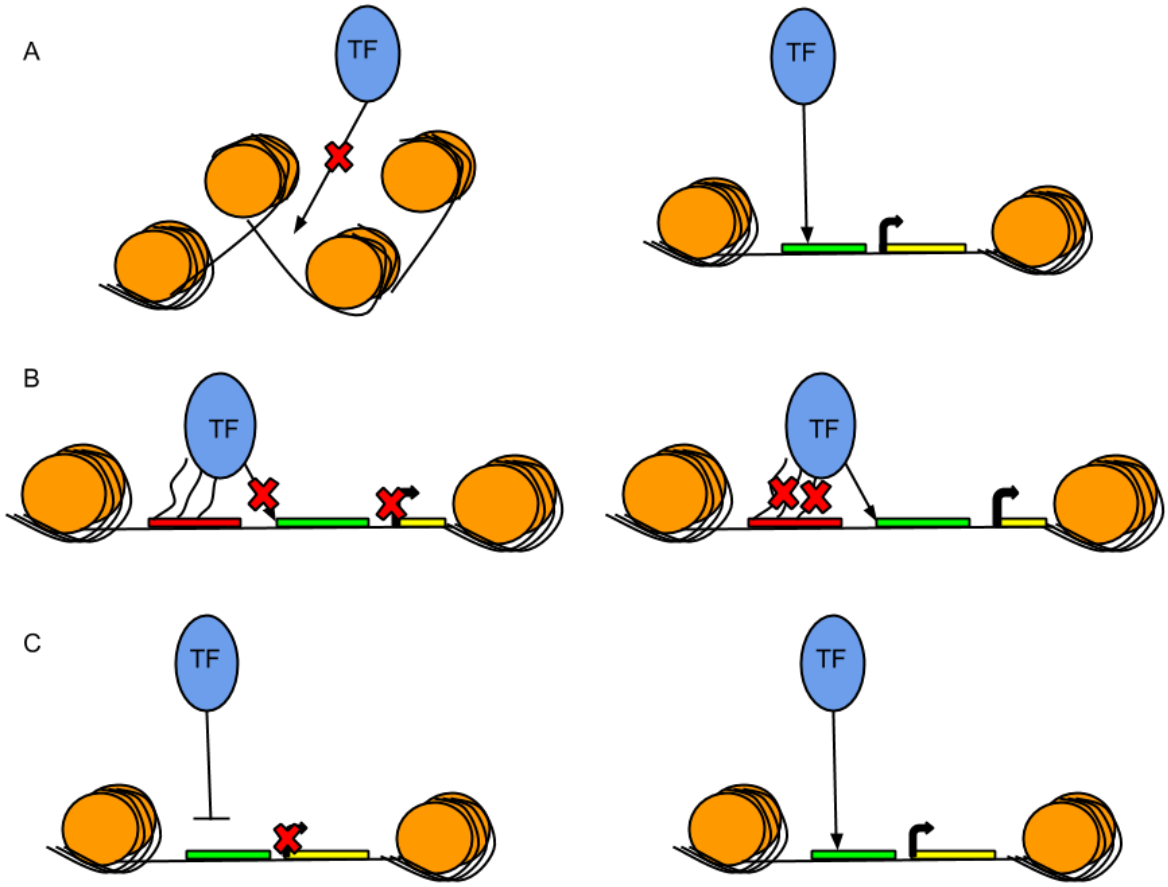

Figure 1.2. Major gene regulatory mechanisms. Gene regulation can be accomplished by three main mechanisms: **(A)** when chromatin is wound tightly around histones, it is inaccessible to transcription factors and genes cannot be transcribed. When histones become acetylated, they "loosen" the chromatin which allows for transcription factors to interact with targets on cis-regulatory elements (green box) to allow for transcription of genes (yellow box). **(B)** Long non-coding RNAs (lncRNAs) can act as "sinks" for transcription factors such that they are unavailable to transcribe targets. When lncRNAs are not transcribed, transcription factors can interact with their targets to effect transcription of target genes. **(C)** Trans-regulatory elements can directly regulate gene expression by interacting with cis-regulatory elements in such a way that can promote or repress gene expression. This is contingent upon the trans-regulatory element and any co-factors that may interact with them. (Histones, orange; enhancer, green box; gene, yellow box; transcribed intergenic region, red box).

Chapter Two

Regulation of *Ptf1a* enhancer activity lies in a 1.2 kb sequence

Introduction

A balance of excitatory and inhibitory neurons is necessary for a properly functioning nervous system; perturbation of this balance may result in a variety of neurological disorders such as schizophrenia and chronic pain (Fields et al. 1991; McCormick and Contreras 2001; Berry et al. 2003). Originally identified in rat pancreatic cells, Ptf1a, Pancreas Transcription Factor 1a, is a basic-helix-loop-helix (bHLH) transcription factor that interacts with two binding partners: an E-protein, and RBP-J (Cockell et al. 1989; Obata et al. 2001; Beres et al. 2006). Loss of Ptf1a function results in agenesis of the Pancreas and is observed to function in exocrine cell specification (Krapp et al. 1998; Kawaguchi et al. 2002; Sellick et al. 2004).

Ptf1a also plays a critical role in proper specification of the nervous system. Ptf1a is expressed in the cerebellum, developing spinal cord and retina (Obata et al. 2001; Glasgow et al. 2005; Hoshino et al. 2005; Fujitani et al. 2006; Dullin et al. 2007; Pascual et al. 2007). In the cerebellum, Ptf1a specifies inhibitory neurons; for example, Purkinje Cells and GABAergic interneuron populations including basket, golgi, and stellate cells (Hoshino et al. 2005; Pascual et al. 2007). Expression commences and is abundant at E12 and

persists in the cerebellar ventricular zone through E14, after which it is detected in single cells until E16 (Pascual et al. 2007). Ptf1a function in the cerebellum was uncovered as a part of the *cerebelless* phenotype upon which cerebellar dysplasia and a loss of GABAergic, inhibitory neurons is seen in *cerebelless* mouse mutants. This mouse contains a 313 kb deletion of the chromosomal region approximately 60 kb downstream of *Ptf1a* (Hoshino et al. 2005). Experiments using *Ptf1a*$^{Cre/+}$; *R26R* models revealed Ptf1a^{+} lineages in the cerebellum express markers for inhibitory neurons (Hoshino et al. 2005; Pascual et al. 2007). Furthermore, in *Ptf1a*$^{Cre/cbl}$, a transheterozygote that has a *Ptf1a* null allele in a *cerebelless* background, revealed that loss of *Ptf1a* was the cause of the *cerebelless* phenotype (Hoshino et al. 2005). Human mutations in *PTF1A* result in truncation of the C-terminal 32 amino acids, causing cerebellar and pancreatic agenesis culminating in permanent neonatal diabetes mellitus (Sellick et al. 2004). Lineage tracing of Ptf1a-derived cells indicates that these populations settle in the Purkinje cell layer (PCL) and the cerebellar parenchyma, but not in the external granule cell layer (EGL), and co-localize with markers for Purkinje cells (PC) and interneurons, but not with granule cell markers (Pascual et al. 2007). Ptf1a is required for generation of PCs and interneurons as loss of Ptf1a results in the agenesis of these populations. Ptf1a also suppresses granule cell fate; loss of Ptf1a results in cells, normally fated to become PCs or interneurons, to become granule cells and abnormally localize to the EGL.

Neurogenesis in the retina occurs in a stepwise fashion, as distinct populations of neurons are generated at distinct timepoints throughout retinal development. The retina is composed of seven cellular populations, six neuronal types and one glial: rod and cone photoreceptors, amacrine, horizontal, bipolar, ganglion, and Müller glia. Genesis of these cellular populations begins around E11 and proceeds through post-natal day (P)11. Each population is specified temporally during retinogenesis with cone photoreceptors and horizontal cells generated from E11-E16; followed by ganglion and amacrine cells (E11-P1); Rod cells (E14-P10); Bipolar cells (E16-P10); and Müller glia (E17-P11). Structurally, the retina is organized into three layers, 1) the outer nuclear layer (ONL) containing photoreceptors, 2) the inner nuclear layer (INL) containing amacrine, bipolar, horizontal, and Müller glial cells, and 3) the ganglion cell layer containing ganglion cells. Retinal expression of Ptf1a serves to specify horizontal and a subset of amacrine cells. *Ptf1a*$^{Cre/+}$;*R26R* mice reveal that as in other organ systems, Ptf1a is expressed in post-mitotic cells in the outer neuroblastic layer (Fujitani et al. 2006). Specifically, Ptf1a-derived cells express markers for horizontal cells and amacrine cells, both populations of GABAergic neurons in the retina (Fujitani et al. 2006). Loss of Ptf1a results in a complete loss of horizontal cells, partial loss of amacrine cells. This suggests that amacrine cell specification is governed by two genetic pathways, one that is Ptf1a-dependent and another that is independent of Ptf1a expression (Fujitani et al. 2006; Dullin et al. 2007). These observations indicate that Ptf1a is required to specify

populations of GABAergic neurons and suppression of a ganglionic fate in the retina.

Ptf1a is expressed in the dorsal neural tube during early and late neurogenesis (Obata et al. 2001; Glasgow et al. 2005). During both stages of neurogenesis in the neural tube, Ptf1a expresses in post-mitotic neural progenitors in discrete domains: the dP4 domain during early neurogenesis, and dPLA during late neurogenesis (Glasgow et al. 2005). These Ptf1a-derived lineages give rise to dorsal populations of inhibitory GABAergic neurons as loss of Ptf1a results in a loss of dI4 and dILA neurons (Glasgow et al. 2005). Furthermore, Ptf1a is required to suppress dI5 and dILB lineages, in early and late neurogenesis respectively, as loss of Ptf1a results in a dorsal expansion of excitatory, glutamatergic dI5 and dILB neurons (Glasgow et al. 2005). Ptf1a function in the neural tube is similar to that in the cerebellum and the retina; Ptf1a specifies populations of inhibitory neurons and suppresses excitatory neuronal specification.

Two regulatory regions for Ptf1a have been identified: a 2.3 kb sequence upstream of the Ptf1a coding region that directs reporter expression to all Ptf1a domains and a 12.4 kb sequence downstream of Ptf1a that directs expression to the hypothalamus, neural tube and the retina. Activity of the 2.3 kb Ptf1a enhancer is observed in $Ptf1a^{+}$ progenitors in the pancreas, cerebellum, neural tube, developing hypothalamus, and hindbrain (Meredith et al. 2009). Two PTF1 motifs, an E-box and adjoining TC-box, were identified and binding of the PTF1-J transcriptional complex was demonstrated to be required for activity of the 2.3 kb

enhancer (Meredith et al. 2009). These data revealed that through this enhancer, Ptf1a is auto-regulated by a feed forward loop, but is not responsible for initiating Ptf1a expression due to the enhancer's inactivity in a Ptf1a mutant background. The 12.4 kb enhancer is neural specific, as reporter expression is observed in most neural Ptf1a domains, except the cerebellum. There are no conserved PTF1 sites within the enhancer and activity is independent of Ptf1a. In the neural tube, enhancer activity is observed in the dP4 progenitor pools during early neurogenesis; and during late neurogenesis, reporter expression is observed in both dILA and dILB neurons. These results demonstrate that this enhancer of Ptf1a is sufficient to initiate expression of *Ptf1a* in the developing nervous system (with the exception of the cerebellum), though activity is not solely restricted to progenitors that will express Ptf1a.

The importance of Ptf1a function in neuronal specification has been highlighted above and understanding how Ptf1a is regulated will provide insight as to how the correct balance of excitatory and inhibitory neurons, and thusly a balanced nervous system, are established.

Materials and Methods

Identification of conserved regions in the 12.4 kb Ptf1a enhancer

Identification of the 12.4 kb enhancer is described by Meredith et al. Further assessment of the 12.4 kb sequence was performed using UCSC

Genome browser (http://genome.ucsc.edu/cgi-bin/hgGateway) and mammalian conservation was used to identify seven distinct, conserved regions. The same 12.4 kb sequence was then analyzed in the ECR Browser (http://ecrbrowser.dcode.org) against mouse, chicken, and *Fugu* genomes for conserved regions.

Description of reporter constructs

Genomic region for the 12.4 kb *Ptf1a* enhancer is chr2: 19447313-19459713. R1: chr2: 19448442-19449514; R2: chr2: 19450395-19452622; R3: chr2: 19453616-19454733; R4/5: chr2: 19454751-19456577; R6: chr2: 19457563-19458031; R7: chr2: 19458547- 19459713; 132 bp ECR: chr2: 19458902-19459007. Chromosome locations were obtained from the mm10 mouse genomic assembly. Each region was cloned into a nuclear mCherry or GFP reporter vector using SalI-HF (NEB, Cat. #R3138S) and BamHI-HF (NEB, Cat. #R3136S). The reporter vectors used are described in Meredith et al. 2009, BGn-EGFP and BGn-mCherry (see map in Appendix G). Regions 1-7 of the 12.4 kb *Ptf1a* enhancer that were cloned into the reporter vectors, described above, were then referred to as *R1-GFP*, *R3-GFP*, *R6-GFP*, *R7-GFP*, *R2-mCherry*, and *R4/5-mCherry*. A PCR mutagenesis strategy was used to generate the *R7Δ132-GFP* mutation.

Transgenic mice

Each transgene was isolated from the recombinant plasmid described

above using SalI-HF (NEB, Cat. #R3138S), XhoI (NEB, Cat. #R0146S), and ScaI-HF (NEB, Cat. #R3122S), run on a standard 0.8% agarose gel, and isolated using gel purification and resuspended in 10mM Tris/25mM EDTA. Transgenic mice were generated by standard procedures (Brinster et al. 1985) using fertilized eggs from B6SJLF1 (C57BL/6JxSJL) crosses. The Transgenic Core Facility of UT Southwestern, Dallas microinjected each transgene at 1-3 ng/μl into pronuclei of fertilized eggs. Transgenic embryos were identified using DNA isolated from embryonic yolk sacs and using PCR to detect *GFP*. For transient transgenic analysis, embryos were harvested at E10.5, E12.5, and E14.5.

In ovo chick electroporation

Fertilized White Leghorn eggs were obtained from the Texas A&M Poultry Department and incubated for 2 days at 38^{o}C. Plasmid DNA for the constructs described above was diluted (1-4 mg/mL) and were injected into the neural tube of chick embryos at stages HH13-15 (Nakada et al. 2004). A Myc-tagged inactive mutant of Ascl1 was electroporated as a control (Ascl1^{AQ}) (Nakada et al. 2004; Hori et al. 2008). Injected embryos were then pulsed 5 times (at 25 mV, 50 msec intervals). Embryos were harvested 48 hours later at stages HH22-23, then fixed with 4% paraformaldehyde for 1 hour, washed 3 times in PBS sunk in 30% sucrose overnight. Embryos were cryosectioned at 30 μm and processed for immunofluorescence using antibodies described below.

Tissue processing and immunofluorescence

Mouse embryos at E10.5 and E12.5 were dissected in ice-cold PBS, fixed in 4% paraformaldehyde for 2 hours at 4^{o}C, and washed three times in PBS. E14.5 embryos were fixed in 4% paraformaldehyde overnight at 4^{o}C and washed with PBS overnight. Embryos were then sunk in 30% sucrose, embedded in OCT, and cryosectioned at 30 µm. Immunofluorescence was performed on sections incubated using the antibodies described below at the indicated dilutions in 1% goat serum/01% NP-40/1xPBS followed by incubation with the appropriate secondary antibodies conjugated with either Alexa Fluor 488, 567, or 647 (Invitrogen). The primary antibodies used include guinea pig anti-Ascl1 (1:10,000 (Battiste et al. 2007)); guinea pig anti-Ptf1a (1:5,000 (Hori et al. 2008)); mouse anti-c-myc (1:1000; Santa Cruz Biotechnology, Cat. #sc-789, A-14); rabbit anti-Pax2 (1:500; Invitrogen, Cat. #716000); and guinea pig anti-Tlx3 (1:20,000 (Muller et al. 2005)). Secondary antibodies used include goat anti-guinea pig (1:500, Invitrogen, Cat. #A11075); goat anti-mouse (1:500, Invitrogen, Cat. #A11004); goat anti-rabbit (1:500, Invitrogen, Cat. #A11011); goat anti-guinea pig (1:250, Invitrogen, Cat. #A21450); and goat anti-rabbit (1:250, Invitrogen, Cat. #A21244).

Results

A conserved 1.2 kb element is an enhancer of Ptf1a

Previous work had identified a 12.4 kb enhancer of Ptf1a which directs reporter expression to Ptf1a expressing populations in the nervous system independent of Ptf1a expression (Meredith et al. 2009). This enhancer contains several distinct regions that are highly conserved between multiple mammalian species (Fig. 2.1A). To assess which of these conserved regions, if any, are sufficient to drive Ptf1a-specific expression, each region was tested in chick using a heterologous reporter system. In this assay, reporter constructs were generated with each homology region (R1-R7) cloned upstream of a reporter cassette containing a basal promoter driving either GFP or mCherry (Fig. 2.1A). These reporter constructs were electroporated into the neural tube of HH12-13 chick embryos and embryos were analyzed for reporter expression 48 hours later at HH24-25. To demonstrate electroporation efficiency along the whole dorsal/ventral extent of the neural tube, an activity dead Ascl1 expression vector was added that was detected with an Ascl1 antibody (Fig. 2.1C'-J'). This Ascl1 antibody also detects the endogenous chick Ascl1 allowing the boundaries of progenitor domains for dI3-dI5 to be delineated (Fig. 2.1C'-I'). Similar to the robust dorsal neural tube restricted expression seen with the 12.4 kb enhancer (Fig. 2.1C), one homology region, R7, also directed expression of the reporter to the dorsal neural tube (Fig. 2.1I; n=4). In contrast, none of the other six regions had this activity (Fig. 2.1D-H; n>4, exact "n" is shown in Appendix B). Note the

ventral expression detected with these constructs is intrinsic to the reporter cassette (Fig. 2.1B, I, asterisk). These results demonstrate that the 1.2 kb R7 sequence is the sequence within the 12.4 kb *Ptf1a* enhancer sufficient to direct reporter expression to the dorsal neural tube.

Activity of the Ptf1a enhancer is conserved in mouse

The R7 sequence of the 12.4 kb enhancer is sufficient to drive reporter expression to the dP4/dI4 domain in chick. To ascertain whether activity of the Ptf1a enhancer is preserved in mammals, transgenic mice were generated using the same construct. Embryos were harvested and analyzed at E10.5 (n=6), E12.5 (n=4) and E14.5 (n=1); stages at which Ptf1a expression is present in the central nervous system. In addition to identifying whether Ptf1a enhancer activity was present within the Ascl1^{+} and Ptf1a^{+} populations, of interest was determining whether enhancer activity was specific to these lineages during specification. Specification in the neural tube is driven by defined factors, which can be used to mark distinct populations: Ascl1^{+}/Ptf1a^{-} lineages express LIM-Homeodomain (LIM-HD) specification markers Tlx1/3, and Ptf1a^{+} lineages express Lhx1/5 and Pax2 (Fig. 2.2C).

At E10.5, the 1.2 kb enhancer has robust expression in the dorsal neural tube in the *R7-GFP* transgenic mice (Fig. 2.2D). Expression in the neural tube is also detected in the ventral neural tube due to inherent ectopic expression of the vector backbone that was also observed in chick. To resolve in which populations enhancer activity is observed, immunohistochemistry for Ascl1 and Ptf1a was

performed (Fig. 2.2D', E', F', G'). Activity of the 1.2 kb enhancer is detected in dP3/dP4, but not dP5 populations as the GFP reporter co-localizes with Ascl1 in the dP3 and dP4 domains in the ventricular zone, but not the dP5 domain (Fig. 2.2E"). Furthermore, staining with Ptf1a (Fig. 2.2 G') confirms that the lowest limit of enhancer activity in the dorsal neural tube is the dP4 population (Fig. 2.2G"). Using the LIM-HD factors Lhx1/5 and Tlx3 to mark dI2/dI4 and dI3/dI5 populations, respectively, revealed that dI2 through dI4 populations co-localize with reporter expression (Fig. 2.2H', H", H"', arrows). These results indicate that during early neurogenesis in the neural tube, the 1.2 kb enhancer of Ptf1a maintains activity from the Ptf1a-expressing dP4 and other dorsal populations, but not in the Ascl1^{+}/Ptf1a^{-} dI5 domain.

At E12.5, Ascl1 and Ptf1a are expressed in progenitors in the ventricular zone in a "salt and pepper" pattern during neuronal development. Ptf1a enhancer activity in the spinal cord during late neurogenesis largely mirrors what is observed at E10.5. Enhancer activity is observed in the neural tube on whole-mount E12.5 embryos (Fig. 2.3A, dot). Activity of the 1.2 kb enhancer is highly enriched in the dorsal neural tube (Fig. 2.3B). Ventral neural tube reporter expression is present, likely as a consequence of intrinsic expression from the transgene similar to what is seen in the chick assays (Fig. 2.3B, asterisk). Enhancer activity is present in both Ascl1^{+} and Ptf1a^{+} progenitors in the ventricular zone of the neural tube (Fig. 2.3D", 3E"). Furthermore, reporter expression is present in both mature populations of dILA and dILB interneurons marked by Lhx1/5 and Tlx3 (Fig. 2.3F"'). Therefore, this enhancer directs

expression to the dorsal neural tube during both early and late stage neurogenesis in the appropriate pool of progenitors, as well as other dorsal populations, suggesting that there may be a sequence that lies outside of the full length 12.4 kb sequence which may be responsible in suppressing initiation of Ptf1a expression.

A highly conserved 132 base pair sequence is required for Ptf1a enhancer activity

While regions 1 through 7 of the 12.4 kb Ptf1a enhancer are well-conserved in mammals, it was of interest to determine if there were regions of the 1.2 kb Ptf1a enhancer (R7) that were conserved in other vertebrates. Using evolutionary conserved regions (ECRs) between human and puffer fish sequences, important regulatory regions were elucidated in *DACH* (Nobrega MA et al, 2003). This approach was applied to the 1.2 kb Ptf1a enhancer, which revealed that a highly conserved 132 base pair sequence was largely conserved between human, mouse, chicken, and zebrafish (Fig. 2.4). Deletion of this highly conserved sequence in the *Ptf1a* enhancer resulted in a dramatic reduction of reporter expression in the dorsal neural tube in contrast to the wildtype enhancer (Fig. 2.5A-C; n=7). In order to test whether the requirement of this sequence for activity was preserved in mammals, transgenic mice carrying the mutant Ptf1a enhancer were generated. In these models, harvested at E10.5, reporter expression was lost in the dorsal neural tube in whole mount embryos, while ventral expression remained (Fig. 2.5E, dorsal expression demarcated by white

dot; n=3). Sections revealed nearly complete loss of reporter expression in the dorsal neural tube with few GFP^+ cells at the very dorsal-lateral domain. As observed in whole mount embryos, ventral expression of the transgene was not affected (Fig. 2.5E', ventral expression marked by asterisk). To assess whether the GFP^+ cells at the dorsal-lateral edge of the neural tube were derived from dorsal neural progenitor lineages, immunohistochemistry using Lhx1/5, Pax2, and Tlx3 was performed on R7 and R7Δ132 E10.5 transgenic mouse sections. In R7 Tg GFP^+ cells, reporter expression did co-localize with markers of dorsal interneurons, in contrast, R7Δ132 Tg GFP^+ cells at the periphery of the neural tube did not co-localize with any dI markers (Fig 2.5F-F''', 5G-G'''). These results reveal that within the 1.2 kb Ptf1a enhancer is a 132 bp sequence that is highly conserved and is critical for enhancer activity in vertebrates.

Discussion

Ptf1a is a critical factor necessary for the proper development of the pancreas and nervous system. Loss of Ptf1a results in agenesis or malformation in these organ systems (Krapp et al. 1996; Krapp et al. 1998; Sellick et al. 2004; Dullin et al. 2007; Pascual et al. 2007). Therefore, knowing how Ptf1a expression is initiated and regulated is of prime importance. In this chapter, I described the identification of an enhancer of Ptf1a, which is sufficient for driving expression of a heterologous reporter to the dorsal neural tube overlapping the Ptf1a

expression domain. A highly conserved sequence within this enhancer was identified that is necessary for activity of the Ptf1a enhancer.

A 1.2 kb conserved sequence is sufficient for Ptf1a enhancer activity

A 12.4 kb enhancer for Ptf1a was identified downstream of the Ptf1a coding region and was demonstrated to direct reporter expression to appropriate domains in the developing nervous system, except the cerebellum (Meredith et al. 2009). This sequence was demonstrated to be sufficient to initiate Ptf1a expression in appropriate domains during neurogenesis in the spinal cord, as well as in the retina, hindbrain, and hypothalamus. I tested sequences within this enhancer that were conserved in mammals and found that one 1.2 kb sequence was sufficient to drive reporter expression in the neural tube. Activity of the enhancer was observed more widely than that of the full-length enhancer, with activity being observed in dP2-dP4 domains, but not in the dP5 population. These results suggest that the dP2-dP4 populations may express the factor(s) necessary for initiation of *Ptf1a* expression, while the dP5 population does not. Another possibility is that all dorsal populations may express the factor(s) necessary for initiation of *Ptf1a* expression. This reveals that while the 1.2 kb enhancer is sufficient for enhancer activity, this activity is not restricted to the *Ptf1a* lineage in the dorsal spinal cord mirroring the pattern of the full-length 12.4 kb *Ptf1a* enhancer. Therefore another sequence(s) outside of the 12.4 kb enhancer acts to repress initiation of Ptf1a expression in inappropriate domains.

A highly conserved element in the 1.2 kb Ptf1a enhancer is required for activity

Using a bioinformatics approach, I determined that there was an evolutionary conserved region (ECR) of 132 base pairs in length, which was highly conserved. Prior studies using ECRs that have the same degree of conservation have uncovered important regulatory elements (Nobrega et al, 2003). These 132 base pairs are required for Ptf1a enhancer activity in both chick and mouse, revealing a conserved function in regulating of *Ptf1a* expression in the spinal cord. The factor(s) that are responsible for initiating expression of *Ptf1a* must bind through this sequence.

Concluding remarks

The function of Ptf1a is critical to the proper specification of cellular populations in the pancreas and developing nervous system. Particularly, in the nervous system, it is critical for promotion of an inhibitory neural fate and suppression of excitatory neural specification. While the 1.2 kb sequence significantly narrows down the critical component regulating initiation of Ptf1a expression, it is still to be determined what transcription factor(s) are acting through the enhancer to do so. Elucidation of such a mechanism will provide insight as to how spatially distinct regions in the developing nervous system specify the precise balance of excitatory and inhibitory neurons.

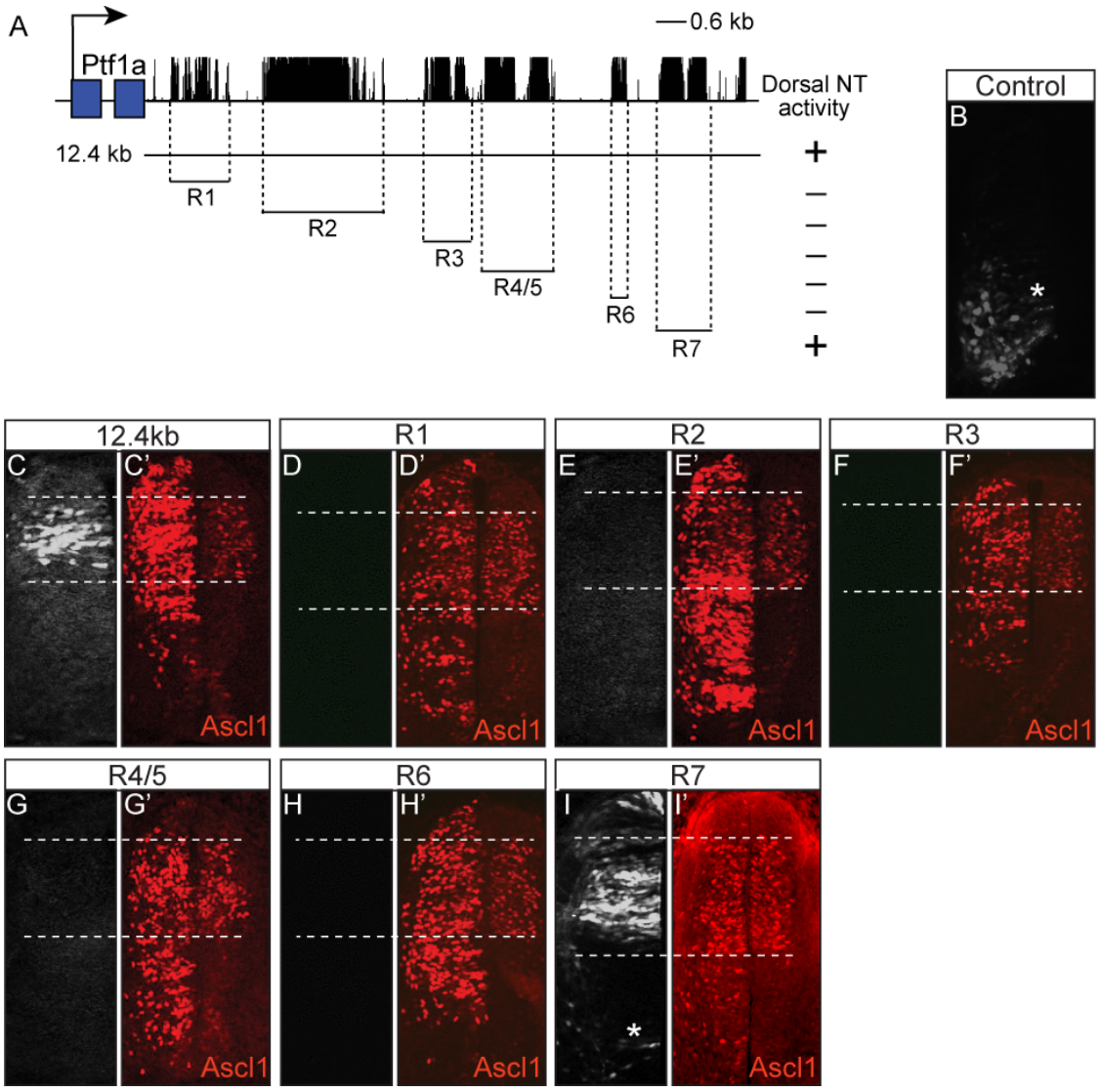

Figure 2.1. Activity of a 1.2 kb sequence is sufficient for 12.4 kb Ptf1a enhancer activity. (A) Mammalian conservation analysis of the 12.4 kb Ptf1a enhancer yields seven distinct conserved sequences. **(C-I)** Each region was tested in chicken embryos to determine if any were sufficient to drive dorsal expression of a fluorescent reporter. **(C'-J')** Using a control vector, which expressed an inactive form of Ascl1, staining for Ascl1 allowed for both electroporation efficiency and demarcation of dP3-dP5 domains (region between dotted lines). **(C)** The activity of the 12.4 kb Ptf1a enhancer lies in the dP4 domain between the Ascl1^{+} dP3 and dP5 domains (compare reporter expression in C to endogenous Ascl1 expression in C'). **(D-H)** Regions 1-6 are not sufficient direct reporter expression to the neural tube. **(I)** Region 7, a 1.2 kb sequence, is sufficient to drive reporter expression. Ectopic expression in the ventral neural tube of embryos electroporated with R7 **(I, asterisk)** is likely due to endogenous activity of the expression vector as the same ventral expression is observed in the empty vector control **(B)**. (Corresponding embryos shown for each region: 12.4 kb, 080412.4#6; R1, 0503R1#2; R2, 0804R2#2; R3, 0410R3gfp#1; R4/5, 0804R45#2; R6, 0607R6b; R7, 0806R7mycemb1;n>4 for all expression constructs).

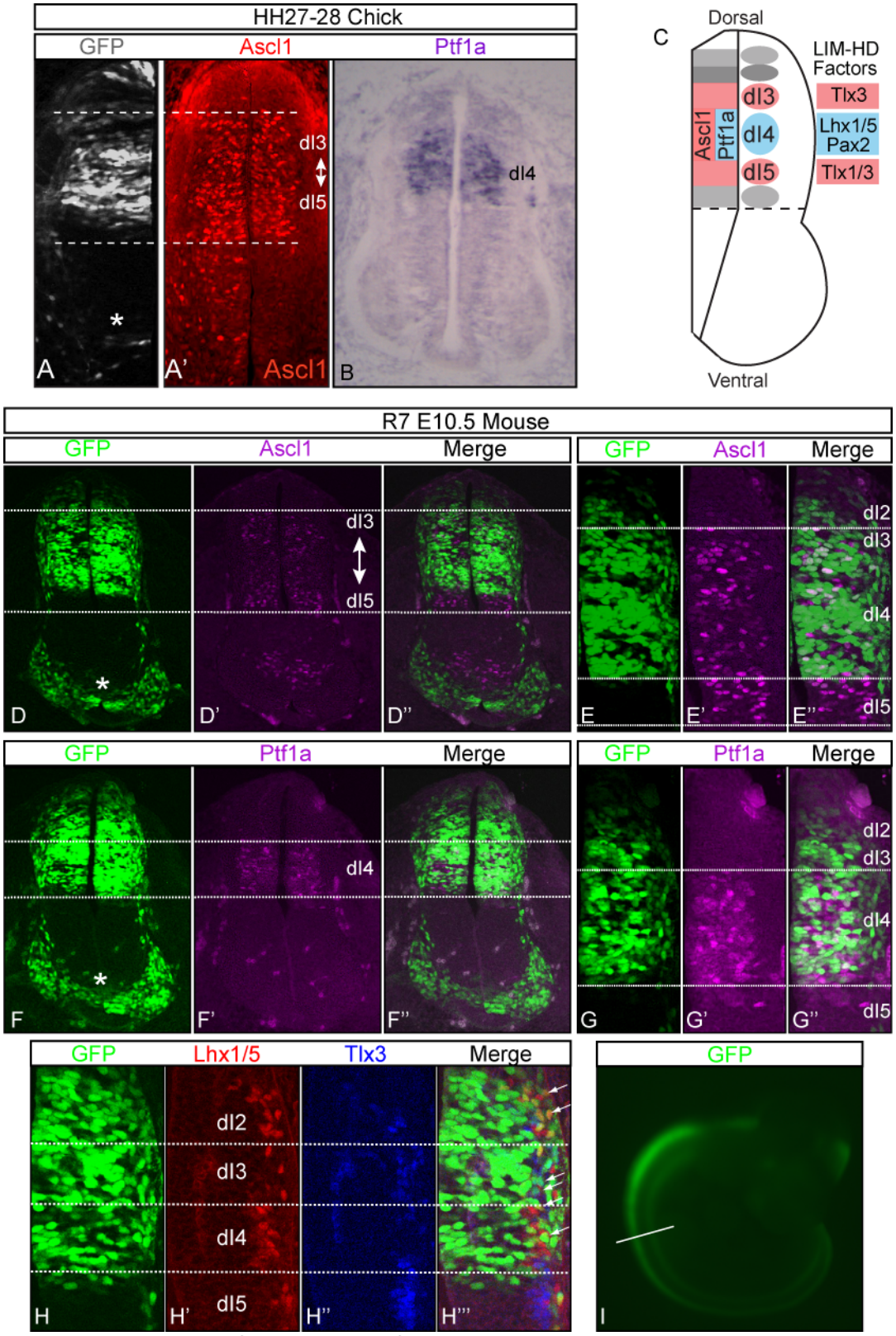

Figure 2.2. Activity of the 1.2 kb Ptf1a enhancer during early neurogenesis in the neural tube. (A, A') The 1.2 kb Ptf1a enhancer directs expression to the dorsal neural

tube in chick and expression is enriched in the dP4 domain. **(B, C)** Ptf1a is expressed in ventricular zone of the dorsal spinal cord, and Ptf1a-derived neurons undergo specification through expression of LIM-HD factors Lhx1/5 and Pax2, where as Ascl1-derived neurons express Tlx3 or Tlx1/3 in dI3 or dI5 populations, respectively. **(D)** At E10.5 in transgenic mouse embryos, the GFP reporter strongly labels the dorsal neural tube with ectopic expression in the ventral neural tube mirroring patterns observed in chick **(D, asterisk)**. **(D', D")** R7 activity overlaps with Ascl1 expression in the dorsal neural tube; however, expression of the reporter does not precisely mirror Ascl1 expression, as the reporter appears to extend past the dP3 domain and not express in the presumptive dP5 population. **(E, E', E")** Indeed, upon closer inspection, reporter expression indicates that the activity of the 1.2 kb Ptf1a enhancer is not restricted to the $Ascl1^{+}$ populations, but extends to the dP2 domain. Furthermore, activity of the enhancer is restricted to within the $Ascl1^{+}$ population to the dP3/dP4 domains, as reporter expression does not overlap with Ascl1 expression in the dP5 domain. **(G, G', G")** The lower limit of 1.2 kb Ptf1a enhancer activity is the dP4 domain as confirmed by co-localization of Ptf1a, but not lower than dP4. **(H, H', H'', H''')** *R7-GFP* expression co-localizes with Lhx1/5 and Tlx3, marking dI2/dI4 and dI3/dI5 neural populations respectively. Notably, transgene expression is observed dI4 neurons while also co-localizing with dI2 and dI3, but not dI5 neurons (arrows). **(I)** In transgenic mice at E10.5, expression of *R7-GFP* is observed in the dorsal and ventral neural tube from the hindbrain to the tail. (n=6; embryo reference #: 2378-3).

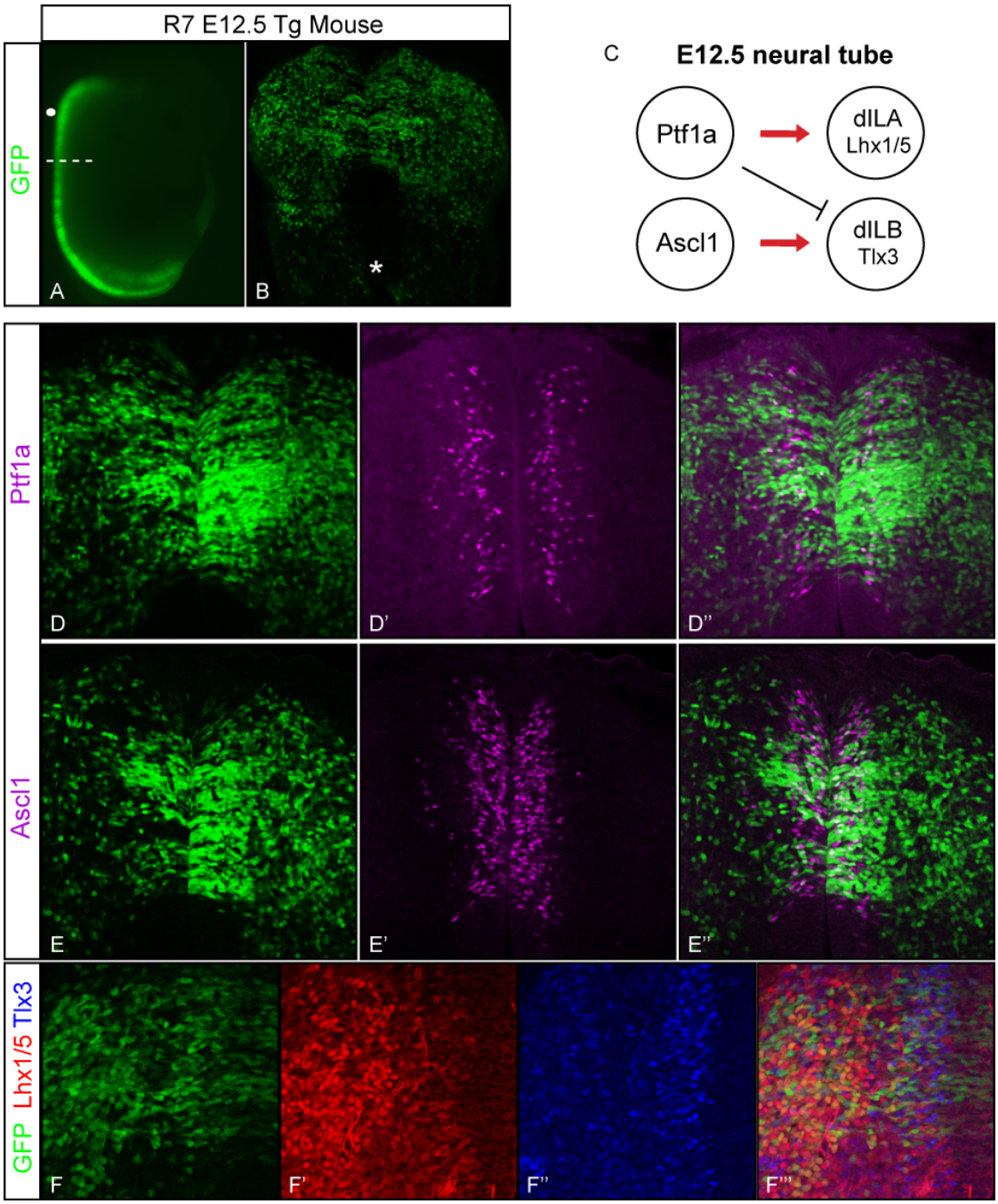

Figure 2.3. Activity of the 1.2 kb Ptf1a enhancer is present in dPLA and dPLB neural progenitors. (A) *R7-GFP* expression in E12.5 mouse embryos reflects patterns in E10.5 embryos, with strong dorsal expression in the neural tube from the hindbrain to the tail (white dot). **(B)** A representative cross-section of the neural tube reveals strong reporter expression dorsally, which extends beyond the ventricular zone. Ectopic expression is observed in the ventral neural tube, as in E10.5 embryos (asterisk). **(C)** dPLA progentors expressing Ptf1a give rise to inhibitory dILA neurons which express Lhx1/5 and Pax2. Ptf1a is required to suppress the excitatory dILB fate, marked by Tlx3, which are derived from dPLB population that expresses Ascl1. **(D, D', D")** Expression of Ptf1a during late neurogenesis is restricted to the ventricular zone of the dorsal spinal

cord and co-labels with expression of *R7-GFP*. **(E, E', E")** Ascl1 is also expressed in the ventricular zone in a greater number of progenitors than Ptf1a (compare D' to E'). Moreover, *R7-GFP* expression also, overlaps and co-labels with Ascl1. **(F-F"')** Using Lhx1/5 and Tlx3 to mark dILA and dILB neurons, respectively, *R7-GFP* co-localizes with both markers. Thusly, the 1.2 kb enhancer is active in progenitor pools of both dILA and dILB lineages; this is reflective of the broad activity that is also present during early neurogenesis in the neural tube of the E10.5 embryo. (n=4; embryo reference #: 2380-8).

```
Human       TGGGAGGGGCAACATGACATTT CACTTGGCAGTTCTAAACAGGT
Mouse       TGGGAGGGGCAACATGACATTT CACTTGGCAGTTCTAAACAGGT
Chicken     TGGGAGGGGCGACATGACATTT CCCTCGGCAGTTCTAAACAGGT
Zebrafish   TGGGAGGGGCAACATGACATTT CCCTGGCCGATTCAAAACAGGT
            ********** ********** **** * *  *** ********

Human       CACAGACCTTTCACAGACCCTA AGGCAGTGGGAGGGAGCTCTTT
Mouse       CACAGACCTTTCACAGACCCTA AGGCAGTGGGAGGGAGCTCTTT
Chicken     CACAGACCTTTCACAGACCCTA AGGCAGTGGGAGGGAGCTCTTT
Zebrafish   CACGGACCCTCCATAGACACCA AGGCGGTGGGAGGGAGAACTTA
            *** **** * ** **** * * **** ***********  ***

Human       ACTAAACCGTGTAACCAAATCA AACCAATGGTTACCCCTTTTCA
Mouse       ACTAAACCGTGTAACCAAATCA AACCAATGGTTACCCCTTTTCA
Chicken     ACGAAACCGTGTAACCAAATCA AACCAATGGTTACCCCTTTTCA
Zebrafish   ACCACACCGTGTAACCGAATCA AACCAGTGGTTACCCCTTTTCA
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Figure 2.4. **A 132 base pair sequence is conserved to fish.** The 1.2 kb Ptf1a enhancer is conserved to mammals. Using the ECR Browser (http://ecrbrowser.dcode.org/), and whole genome analysis of human, mouse, chicken, and zebrafish, a 132 bp sequence is conserved throughout all four species. Evolutionary Conserved Regions (ECRs) have been shown to correlate to critical regulatory elements of genes. This 132 bp ECR may be integral to activity of the 1.2 kb Ptf1a enhancer.

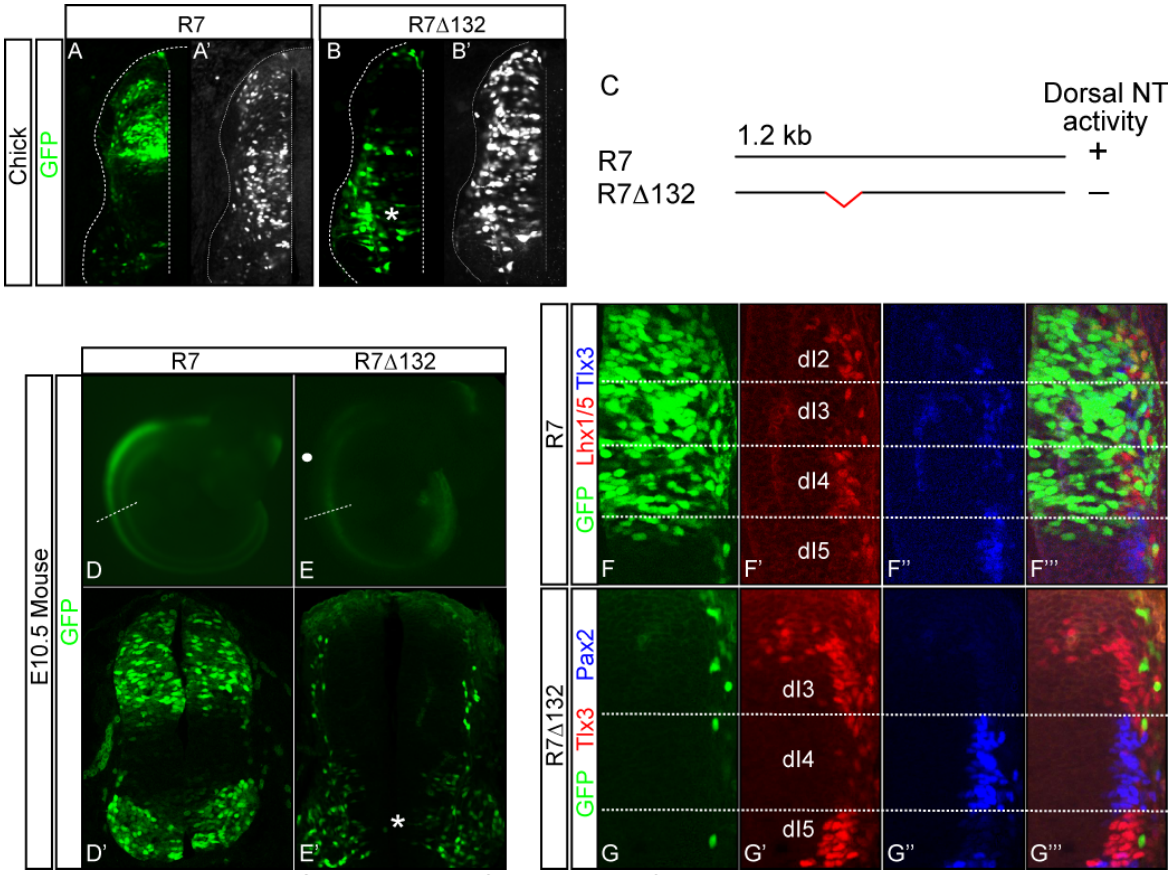

Figure 2.5 A 132 bp ECR is critical for 1.2 kb Ptf1a enhancer activity in the dorsal neural tube. (A) The activity of the 1.2 kb Ptf1a enhancer, R7, is enriched dorsally in the chick spinal cord. **(B)** Deletion of the 132 bp ECR results in severe abrogation of dorsal enhancer activity highlighting the ECRs function in dorsal Ptf1a enhancer activity. **(A', B')** Electroporation efficiency was determined by myc-staining of a control expression vector. **(C)** The relative position of the 132 bp ECR in R7 is shown, deletion results in abrogation of dorsal neural tube activity. **(D, D')** Activity of R7, in *R7-GFP* transgenic mice, is enriched in the neural tube, specifically in the dorsal and ventral regions. **(E, E')** In E10.5 *R7Δ132-GFP* embryos, deletion of the 132 bp ECR results in abrogation of dorsal enhancer activity (E, white dot). Sections reveal a dramatic loss of GFP expression in the dorsal neural tube with ectopic ventral expression, seen in R7, not affected (E', asterisk). **(F-F''')** *R7-GFP* expression co-localizes with markers for dI2-dI4 populations. **(G-G''')** *R7Δ132-GFP* expression in cells of the dorsal-lateral neural tube do not co-localize with any markers of dI2-dI5 neurons and therefore are not derived from any progenitors from dP2-dP5 cellular populations. (n=7 for chick R7Δ132; embryo reference #: R7Δ132-GFPchick, n=3 for mouse *R7Δ132-GFP*; embryo reference #: 1430-1).

Chapter Three

Two motifs differentially regulate activity of the *Ptf1a* enhancer

Introduction

Spatial and temporal regulation of transcription is dependent upon transcription factors, regulatory co-factors, and other cooperative interactions with DNA. Transcription factor binding to a defined region of regulatory DNA are referred to as *cis*-regulatory modules (CRMs); and these CRMs have the ability to direct tissue-specific expression of genes during development (Makeev et al. 2003; Howard and Davidson 2004). Enhancers also contain densely clustered transcription factor binding sites and exert their activity regardless of position relative to the transcription start site (Banerji et al. 1981; Woolfe et al. 2005). Enhancers are critical for gene expression as illustrated when expression of immunoglobulin heavy chain and CD4 were lost when the respective enhancers for each gene was deleted (Grosschedl and Marx 1988; Chong et al. 2010). Enhancers can be located in proximity to or several kilobases away from a promoter. The mechanism through which enhancers may effect gene expression is through the formation of chromatin looping which brings the CRMs in close proximity with transcription machinery at the promoter (Murrell et al. 2004; Horike et al. 2005).

ECRs may function as enhancers and recapitulate expression patterns of local genes (Gu and Spitzer 1995; Nobrega et al. 2003; Bejerano et al. 2004; Visel et al. 2009). The previous chapter described a 1.2 kb enhancer of Ptf1a and the requirement of a 132 bp ECR in its activity in the developing nervous system. Considering its importance in activity of the 1.2 kb Ptf1a enhancer, it may contain binding sites for transcription factors which serve to initiate expression of Ptf1a and are therefore critical for generating the fine balance of excitatory and inhibitory neurons. To this end, I analyzed the 132 bp sequence for transcription factor motifs which were cross-referenced to expression data from Ptf1a^{+} and Ptf1a^{-} lineages. Together, these data were used to generate a list of candidates that may regulate enhancer and Ptf1a activity. Below I will discuss a select few candidates that have effects on neurogenesis and neurotransmitter specification.

During spinal cord development, spontaneous calcium currents are characteristic of embryonic neurons and have functional consequences on specifying neurotransmitter phenotypes. During embryonic development, neurons become excitable even prior to synapse formation (Gu and Spitzer 1995). These neurons are characterized by sustained action potentials with large influxes of calcium current and small, repolarizing potassium currents, which induce brief elevations in intracellular calcium levels (Spitzer and Lamborghini 1976; O'Dowd et al. 1988). Furthermore, the calcium spikes that are a feature of the developing spinal cord specify neuronal neurotransmitter type. Altering the frequency of the calcium spikes *in vitro* or *in vivo* perturbs the phenotypes of developing neurons. Suppression of calcium spike activity results in an

expansion of excitatory cholinergic and glutamatergic neurons, while decreasing the numbers of inhibitory GABAergic and glycinergic neurons (Borodinsky et al. 2004). However, enhancement of calcium spike activity generates an expansion of GABAergic and glycinergic neurons, and a concomitant decrease in the number of excitatory cholinergic and glutamatergic neurons (Borodinsky et al. 2004). These changes in neurotransmitter specification in response to calcium spiking were demonstrated to be homeostatic and functional (Borodinsky et al. 2004). Moreover, the window of competence for re-specification of neurons is limited to five hours within the initiation of calcium spiking (Borodinsky et al. 2004). In *Xenopus*, cJun was identified as a factor mediating calcium spiking mediated specification of neurotransmitter phenotype. Calcium spiking causes phosphorylation of cJun, which translocates to the nucleus and binds to the promoter of *tlx3* and suppresses its activity (Marek et al. 2010). *Tlx3* is critical for specification of excitatory neurons in the spinal cord as loss of Tlx3 function results in ectopic inhibitory neurons (Cheng et al. 2005). The consensus motif for cJUN is TGA(C/G)TCA and can occur in variants of this motif, as the cJun motif in *tlx3* acts through a variant (Marek et al. 2010). cJun is also implicated in recruiting additional factors such as NF-kB, and histone acetyltransferases to mediate transcription (Wolter et al. 2008; Tiwari et al. 2012). Also, cJUN interacts with GLI to enhance transcription of targets of each factor where both motifs are present (Laner-Plamberger et al. 2009). Also, this interaction between cJUN and GLI is not necessarily direct and may be a part of a larger transcriptional complex (Laner-Plamberger et al. 2009). This demonstrates genetic integration of calcium

signaling through pathways, which have been implicated in specification of neurons.

The Zic family of transcriptional repressors is composed of five members (Zic1-5), which play an important role in the developing nervous system. Mutation of Zic1 and Zic4 results in Dandy-Walker malformation, which involves malformation of the cerebellum to the most severe phenotype, cerebellar agenesis (Grinberg et al. 2004; Blank et al. 2011). Zic2 also cooperates in cerebellar development; disruption of both Zic1 and Zic2 leads to malformed cerebella and disruption of neurogenesis (Aruga et al. 2002b). Additionally, disruption of Zic factors cause neural tube defects (Grinberg and Millen 2005). Zic factors may bind to GC-boxes and repress target genes (Aruga 2004). Zic function in the spinal cord serves to repress neurogenesis and promote expansion of neural progenitor cells (Aruga et al. 2002b). Zic1 is known to bind to a GC-box in the *Atoh1* enhancer, a known neurogenic bHLH, and repress *Atoh1* expression (Ebert et al. 2003).

NF-kB is a transcriptional regulator that acts as a dimer and in mammals is composed of five family members: p65 (Rel-A), p50, p52, C-Rel, and RelB. Homo- or heterodimerization between any of these five family members is bound by IkB in the cytoplasm and is maintained in a sequestered state, rendering NF-kB inactive. In response to environmental cues, IkB dissociates from NF-kB dimers, which then translocate to the nucleus and regulates gene expression (Lipton 1997; Mattson et al. 2000). The importance of NF-kB in neurogenesis is underscored by its requirement in early differentiation of neurons; loss of NF-kB

results in decreased neurogenesis as well as a decreased progenitor pool indicating a requirement in asymmetric cell division (Zhang et al. 2012). Furthermore, NF-kB induces expression of the proneural bHLH, *scute*, in *Drosophila* (Ayyar et al. 2007). The mechanism through which NF-kB effects neurogenesis may be through recruitment of widely-expressed transcriptional regulators such as the Sp family of transcription factors, as presence of both NF-kB and Sp motifs (GC-boxes) of the *NR1* promoter are required for activity (Liu et al. 2004).

In this chapter, I describe two motifs that regulate activity of the 1.2 kb Ptf1a enhancer and a candidate screen which resulted in a known repressor with the ability to regulate enhancer activity and Ptf1a expression in chick.

Materials and Methods

Identification of putative binding sites

Putative binding sites in the 132 bp ultra-conserved sequence and the surrounding region were identified using Transcription Element Search System, or TESS, (http://www.cbil.upenn.edu/tess) and JASPAR (http://jaspar.genereg.net/) by inputting the relevant regions. Results given by the databases varied depending upon the stringency of the search, and putative binding sites relied upon for further analyses were primarily from TESS.

Description of mutant zinc finger and POU motif reporter constructs

Region 7 of the 12.4 kb *Ptf1a* enhancer with the mutations in the putative zinc finger and POU motifs were cloned into a nuclear BGnEGFP reporter vector (previously described in Chapter 2 and Meredith et al., 2009) to generate *R7mZF-GFP* and *R7mPOU-GFP*. These mutations were introduced using PCR mutagenesis strategy. *R7mZF-GFP* contained the mutation TGGAAGAGAC from the wild-type putative zinc finger motif, TGGGAGGGGC; *R7mPOU-GFP* contained the mutation GCCCAAGC from the wild-type putative POU motif AACCAAAT. Sources for zinc finger and POU motif mutations may be found at (Luo et al. 2009) and (Park et al. 2009), respectively.

Transgenic mice

Each transgene was isolated from the recombinant plasmid described above using SalI-HF (NEB, Cat. #R3138S), XhoI (NEB, Cat. #R0146S), and ScaI-HF (NEB, Cat. #R3122S), run on a standard 0.8% agarose gel, and isolated using gel purification and resuspended in 10mM Tris/25mM EDTA. Transgenic mice were generated by standard procedures (Brinster et al. 1985) using fertilized eggs from B6SJLF1 (C57BL/6JxSJL) crosses. The Transgenic Core Facility of UT Southwestern, Dallas microinjected each transgene at 1-3 ng/μl into pronuclei of fertilized eggs. Transgenic embryos were identified using DNA isolated from embryonic yolk sacs and using PCR to detect *GFP*. For transient transgenic analysis, embryos were harvested at E10.5, E12.5, and E14.5.

Zic1;Zic4 mutant mice

Mouse mutants for *Zic1;Zic4* were gifted by Kathleen J. Millen (Seattle Children's Hospital). These mice were generated by targeted deletion of the first exon of both *Zic1* and *Zic4*. Mice null for both results in the deletion of the first exon of *Zic1* and *Zic4* in addition to the 17.6 kb intergenic region between both genes (Grinberg et al. 2004). Embryos, at E10.5 and E12.5 from this line of mice were shipped from Seattle from the Millen laboratory who also provided the genotypes for each.

In ovo chick electroporation

Fertilized White Leghorn eggs were obtained from the Texas A&M Poultry Department and incubated for 2 days at 38^{o}C. Plasmid DNA for the constructs described above was diluted (1-4 mg/mL) and were injected into the neural tube of chick embryos at stages HH13-15 (Nakada et al. 2004). A Myc-tagged inactive mutant of Ascl1 was electroporated as a control (Ascl1^{AQ}) (Nakada et al. 2004; Hori et al. 2008). Injected embryos were then pulsed 5 times (at 25 mV, 50 msec intervals). Embryos were harvested 48 hours later at stages HH22-23, then fixed with 4% paraformaldehyde for 1 hour, washed 3 times in PBS sunk in 30% sucrose overnight. Embryos were cryosectioned at 30 μm and processed for immunofluorescence using antibodies described below.

Constructs for over-expression

The following coding sequences were cloned into a N-terminus 5x myc-tagged expression vector, pMIWIII (Hori, K et al 2008) using NcoI-HF (NEB, Cat. #3193S) and XbaI (NEB, Cat. #0145S): Brn2 (*Mus musculus*, NM_008899); c-Jun (*Mus musculus*, NM_010591); Sox2 (*Mus musculus*, NM_011443). The Zic1 (*Mus musculus*, NM_009573) expression vector used was previously published by (Ebert et al. 2003). C/EBP expression vector was a gift from Beverly Rothemel (University of Texas—Southwestern Medical Center). Pbx1b was a gift from Raymond MacDonald (University of Texas—Southwestern Medical Center).

Tissue processing and immunofluorescence

Mouse embryos at E10.5 and E12.5 were dissected in ice-cold PBS, fixed in 4% paraformaldehyde for 2 hours at 4^{o}C, and washed three times in PBS. E14.5 embryos were fixed in 4% paraformaldehyde overnight at 4^{o}C and washed with PBS overnight. Embryos were then sunk in 30% sucrose, embedded in OCT, and cryosectioned at 30 µm. Immunofluorescence was performed on sections incubated using the antibodies described below at the indicated dilutions in 1% goat serum/01% NP-40/1xPBS followed by incubation with the appropriate secondary antibodies conjugated with either Alexa Fluor 488, 567, or 647 (Invitrogen). The primary antibodies used include guinea pig anti-Ascl1 (1:10,000 (Battiste et al. 2007)); guinea pig anti-Ptf1a (1:5,000 (Hori et al. 2008)); mouse anti-c-myc (1:1000; Santa Cruz Biotechnology, Cat. #sc-789, A-14); rabbit anti-Pax2 (1:500; Invitrogen, Cat. #716000); guinea pig anti-Tlx3 (1:20,000 (Muller et

al. 2005)); and rabbit anti-Zic (1:400, a gift from Rosalind Segal, Dana Farber Institute, Harvard University). Secondary antibodies used include goat anti-guinea pig (1:500, Invitrogen, Cat. #A11075); goat anti-mouse (1:500, Invitrogen, Cat. #A11004); goat anti-rabbit (1:500, Invitrogen, Cat. #A11011); goat anti-guinea pig (1:250, Invitrogen, Cat. #A21450); and goat anti-rabbit (1:250, Invitrogen, Cat. #A21244).

Electrophoretic mobility shift assay and in situ hybridization

EMSA was performed as previously described in (Henke et al. 2009) using the Zic1 expression vector described previously and the following probes:
Atoh1 ZF: TCCAGGCCGCTCCCCGGGGAGCTGAGCGGC;
R7-ZF1: TCCCATTTGGGAGGGGCAACATGAC;
R7mZF: TCCCATTTGGAAGAGACAACATGAC (red letters indicate mutant nucleotides). Zic1 was synthesized using SP6 and T7 TNT Quick Coupled lysate systems (Promega). Lysates were then incubated in binding buffer (10 mM HEPES, pH 7.9, 4 mM Tris-HCl, pH 8.0, 80 mM NaCl, 0.5 mM EDTA, pH 8.0, 12% glycerol, 5 mM DTT, 1.6 µg Poly dI/dC) with ($^{\gamma\text{-}32}$P) end-labeled oligo probes (50,000 cpm) in a total volume of 20 µL at 30°C for 30 minutes. Reactions were transferred to ice for 15 minutes before loading and running on a 4.5% polyacrylamide matrix gel in 1x TGE at 4°C. For competition assay Non-radiolabeled probes were added in excess from 10x and 100x molar concentrations of radiolabeled probes during the reaction. Probe sequence of *Atoh1* ZF is derived from Ebert et al. 2003.

In situ hybridization was performed as previously described (Birren et al. 1993). Modifications to the Birren et al. 1993 protocol include the use of Triton-X 100 in wash buffers after exposure to alkaline phosphatase. The c*Ptf1a* probe used was synthesized using an approximately 400 bp sequence at the 3' sequence of the c*Ptf1a* mRNA. This probe was labeled with Digoxygenin (DIG) to generate DIG-labeled antisense RNA probes used at 1-5 mg/ml. These probes were then hybridized to chick sections overnight at 65°C, incubated with anti-DIG AP antibody (Roche), followed by incubation with NBT/BCIP (Roche).

Results

A zinc finger motif and POU motif regulate activity of the 1.2 kb Ptf1a enhancer in chick

Analysis of the 132 ECR identified motifs for important neurogenic transcription factors. These include motifs for: zinc fingers, cJun, POU, SOX, and NF-kB (Fig. 3.1). Mutations to the first zinc finger motif and a POU motif affected activity of the 1.2 kb Ptf1a enhancer. Mutation of the zinc finger motif (ZF1) resulted in a ventral expansion of reporter expression when compared to the wildtype enhancer (Fig. 3.2A, C; n=6). This reveals that the zinc finger motif is critical for repression of enhancer activity in the ventral domains. Also, there is a subtle increase in reporter expression in the dorsal most region of the neural tube. In contrast, mutation of the POU motif severely affects enhancer activity as

there is significant abrogation of reporter activity when compared to the wildtype enhancer, and reflects to a degree the phenotype of loss of the 132 bp ECR (Fig. 3.2A, B, D; n=5). Electroporation efficiency was analyzed using a tagged control vector (Fig. 3.2A-D, inset)

The POU motif in the 132 bp ECR is essential for 1.2 kb Ptf1a enhancer activity in the mouse spinal cord

In order to test whether the effects of the zinc finger and POU motifs in the 132 bp ECR are conserved in mammals, I generated transgenic mice that harbor each of these mutations and analyzed expression of a GFP reporter. As previously described, the POU motif is essential for robust activity of the Ptf1a enhancer, as mutation of the motif resulted in severe abrogation of enhancer activity (Fig. 3.3A, B). Electroporation efficiency was determined using a control vector expressing an inactive mutant form of Ascl1, which immunostaining for Ascl1 allowed for detection of endogenous Ascl1 on the unelectroporated hemisphere of the neural tube (Fig. 3.3A', B'). The POU motif is also necessary for Ptf1a enhancer activity in transgenic mice as mutation of the motif results in abrogation of dorsal reporter expression evident in both the whole-mount and sectioned neural tube (Fig. 3.3E, white dot; n=3). This closely mimics loss of the 132 bp ECR in transgenic mice as well (compare Fig. 3.3D, D' to E, E'). During late neurogenesis in the spinal cord, activity of the Ptf1a enhancer is also severely impaired as reporter expression in E12.5 mouse neural tubes is significantly reduced as observed in whole-mount embryos (Fig. 3.4A, B; n=3).

Noticeably, reporter expression in R7mPOU embryos is mosaic, which may be a consequence of generating transgenic mice in founder lines due to the site of transgene integration in the donor embryo (Wilkie et al. 1986; Burdon and Wall 1992). Spinal cord sections at this stage confirm the importance of the POU motif for enhancer activity at this stage; reporter expression is impaired when compared to activity of the wildtype enhancer (Fig. 3.4A', B'). These data demonstrate that the POU domain is necessary for robust 1.2 kb Ptf1a enhancer activity, and is likely the primary motif through which the transcription factor(s) responsible for initiation of Ptf1a activity acts.

The zinc finger motif is dispensable for regulation of 1.2 Ptf1a enhancer activity in the mouse spinal cord

In chick, the first zinc finger motif is critical for repression of Ptf1a enhancer activity in inappropriate domains. To test if this activity is also observed in mammals, I generated transgenic mice that expressed GFP under control of the mutant Ptf1a enhancer *R7mZF*. GFP expression at E10.5 was similar to control (Fig. 3.5A, B; n=3). Spinal cord sections of *R7mZF-GFP* transgenics indicated no patterning abnormalities with respect to controls (Fig. 3.5C, D). Furthermore, staining with Ptf1a (Fig. 3.5C', D') reveals that *R7mZF* expression overlaps with the dP4/dI4 domain, which reflects the patterning of the wildtype Ptf1a enhancer, R7 (Fig. 3.5 C", D"). During late neurogenesis in the spinal cord, mutation of the zinc finger also had no appreciable affect on the pattern of

enhancer activity. Strong dorsal expression of GFP is observed in whole-mount transgenic embryos containing mutant or wildtype enhancers (Fig. 3.6A, B; n=3) and is reflected in sections of the spinal cord in both lines (Fig. 3.6A',B'). The intensity of reporter expression in the dorsal spinal cord does appear to be decreased in *R7-mZF* transgenic embryos. Thusly, during both early and late neurogenesis in the spinal cord, the repressive activity of the zinc finger motif observed in chick is not conserved in mouse.

A zinc finger motif is dispensable, while a POU motif is necessary for activity of the Ptf1a enhancer in the retina and cerebellum

Meredith *et al* described the activity of the 12.4 kb Ptf1a enhancer in the hindbrain and retina, noting that its activity was observed in both regions at E14.5. The 1.2 kb Ptf1a enhancer has activity in the retina, in the presumptive neuroblastic (NBL) and ganglionic cell layers (GCL) (Fig. 3.7A; n=1). Mutation of the zinc finger motif does not affect patterning of enhancer activity as expression in both NBL and GCL is still observed, and co-labeling of cells expressing GFP and Ptf1a indicates that the mutation does not affect expression in the Ptf1a lineage (Fig. 3.7B, arrows; n=2). Reporter expression is restricted to the Ptf1a^{+} lineage as no co-localization is observed with Ascl1 (data not shown). Furthermore, *R7mZF* activity in the hindbrain, but not the cerebellum (Fig. 3.7B', hindbrain indicated by arrowhead, Cb designates cerebellum; n=2) phenocopies activity of the 12.4 kb Ptf1a enhancer (Meredith et al. 2009). Reflecting the

importance of the POU motif in the Ptf1a enhancer activity, reporter expression is lost in both the retina and the hindbrain of *R7mPOU* transgenic mice demonstrating that the POU motif is integral, not only to enhancer activity in the spinal cord but to other domains of the nervous system where Ptf1a is expressed (Fig. 3.7C, C'; n=2). Further replicates are required for these results to significant, but the strong phenotype shown at this late stage reinforces the importance of the putative POU motif.

A candidate screen reveals a zinc finger transcription factor as a regulator of the 1.2 kb Ptf1a enhancer

Candidate transcription factors that might function to regulate Ptf1a expression through the 1.2 kb enhancer were identified. These candidates were chosen because of a known involvement in neurogenesis and expression in the dorsal neural tube overlapping Ptf1a. These candidates were tested by over-expression assays in chick neural tubes. The initial screen included members from families described earlier in the chapter. In order to evaluate whether the candidates affected expression of Ptf1a, an *in situ* probe against cPtf1a mRNA was used, as the Ptf1a antibody available does not recognize cPtf1a. The sole candidate that was able to regulate Ptf1a expression was Zic1, which repressed Ptf1a upon over-expression (Table 3.1).

Zic1 represses expression of Ptf1a and activity of 1.2 kb Ptf1a enhancer in chick, but not in mouse

Zic1 was identified as a candidate to regulate expression of Ptf1a enhancer activity. As previously described, it represses expression of the neurogenic bHLH *Atoh1* by interactions with the *Atoh1* enhancer, which made it an ideal candidate for regulation of Ptf1a (Ebert et al. 2003). To this end, I over-expressed Zic1 with the 1.2 kb Ptf1a enhancer (R7) in chick spinal cords, and when compared to over-expression of a control expression vector, or compared to the non-electroporated side, Zic1 repressed Ptf1a as observed by *in situ* using cPtf1a-specific probes (Fig. 3.8A, B; n=4). Furthermore, R7 activity was repressed as GFP expression was abrogated in spinal cords where Zic1 was over-expressed (Fig. 3.8A', B', electroporation efficiency, inset). This revealed that Zic1 is capable of repressing Ptf1a expression, and it was likely that this mechanism of repression operated through the 1.2 kb Ptf1a enhancer.

Zic family members, *Zic1-Zic3*, are expressed in the developing spinal cord in chick during HH18-19 and in mouse at E10.5 (Ebert et al. 2003; McMahon and Merzdorf 2010). In mouse models, *Zic1* mutants do not show a significant morphological change; when two *Zic* loci are mutated a dramatic morphological change is observed (Aruga et al. 2002a; Grinberg et al. 2004). I obtained *Zic1*$^{-/-}$;*Zic4*$^{-/-}$ mutants from Kathleen Millen (Grinberg et al. 2004) in order to further test the model that Zic1, and Zic4 by association, was able to repress expression of Ptf1a. Embryonic spinal cords at E10.5 were analyzed for

loss of Zic1 and Zic4 by use of a pan-Zic antibody. The Zic mutant embryos expressed Zic in significantly fewer cells than did wild-type embryos, which notably, Zic expression was lost in the ventral-most regions, but is still present dorsally (Fig. 3.8C,D; n=2). Also, Expression of Ptf1a did not change between *Zic1;Zic4* controls or *Zic1*$^{-/-}$*;Zic4*$^{-/-}$ mutants (Fig. 3.8C',D'). Co-localization of Ptf1a^{+} and Zic^{+} cells did occur in controls, but did not occur as frequently in mutant embryos (Fig. 3.8C",D"). These data had few replicates, two or less, and expression levels were not quantitated. These data indicate that in mice deficient in Zic1 and Zic4, Ptf1a expression is not affected.

Zic1 does not bind to the 1.2 kb Ptf1a enhancer

To determine whether Zic1 binds to the putative zinc finger motif 1 in the 1.2 kb *Ptf1a* enhancer, I used an electrophoretic mobility shift assay with probes corresponding to a 20 base pair sequence encompassing the first zinc finger motif. This experiment would reveal whether repression of Ptf1a expression and enhancer activity, observed when Zic1 is overexpressed, is achieved through directly binding the zinc finger motif. If Zic1 binds to the zinc finger motif this would be suggestive of Zic1 directly repressing 1.2 kb Ptf1a enhancer activity. If Zic1 does not bind the motif, then its repressive activity is likely due to an indirect effect. An issue with the experiment was revealed when one of the controls, the rabbit reticulocyte lysate alone which did not have any protein expressed, had a band which indicated a non-specific reactive species in the lysate bound the

probe containing the zinc finger motif, R7-ZF (Fig. 3.9, Lane 1). Expressing Zic1 in this system resulted in no bands of significant intensity indicating that Zic1 does not interact with the zinc finger motif (Fig. 3.9, Lane 2). Non-radioactive competitor probes which contained the wild-type motif or a mutated motif, R7-mZF, were used in order to determine if Zic1 binding, should it have occurred, was specific to the motif (Fig 3.9, Lanes 3-6). Since Zic1 did not interact with the motif, the competition experiment was not informative. As a positive control, Zic1 lysate did bind to the GC-box identified in the *Atoh1* enhancer identified by Ebert *et al*. indicating that Zic1 used in the experiment does recognize a zinc finger motif (Fig. 3.9, Lanes 9 and 10, arrow). Thus, in this in vitro assay, it can be concluded that Zic1 does not directly bind the zinc finger motif present in the 1.2 *Ptf1a* enhancer under the conditions tested. Inherent limitations to this experiment are the presence of non-specific binding to the R7-ZF and R7-mZF probes used; that it does not preclude the possibility that other family members of the Zic family, which may exert a repressive effect on Ptf1a expression; Zic1 may require a binding partner(s) to bind the R7 motif.

Discussion

Evolutionary conserved regions can be bound by transcriptional regulators, and can act as enhancers for gene expression(Makeev et al. 2003; Howard and Davidson 2004). Binding of transcription factors, co-factors and other components to an enhancer represents a *cis*-regulatory module (Makeev et

al. 2003; Howard and Davidson 2004). Within these CRMs lie motifs through which transcriptional regulators bind and regulate enhancer activity (Makeev et al. 2003; Howard and Davidson 2004). Identification and motif analysis of a 132 base pair ECR in the 1.2 kb Ptf1a enhancer revealed a few binding sites which correlate with candidate factors that could potentially regulate enhancer activity, and expression of Ptf1a. Through mutational analysis, I determined that two motifs were critical for regulating enhancer activity as assessed by electroporation in the chick neural tube but only one retained its activity when tested in transgenic mice. A predicted zinc finger motif was critical for repression of Ptf1a enhancer activity in ventral domains of the chick spinal cord. This activity was not observed in transgenic mouse as reporter expression mimicked that of transgenics expressing a wild-type enhancer construct. This suggests that there is another region in the 1.2 kb Ptf1a enhancer that is critical for repression of activity in domains where Ptf1a is not expressed. It is a possibility that the second zinc finger motif within the 132 ECR may offer an alternative binding site for the presumptive transcriptional repressor to bind. A transgenic mouse with both zinc fingers mutated would resolve this possibility.

A predicted POU motif is necessary for activity of the Ptf1a enhancer as mutation of the motif results in abrogation of reporter expression in both chick and mouse. This reveals that the mechanism of Ptf1a activation in chick and mouse is shared between the two species and places primary importance to the transcriptional activator that binds to the motif. An HMG/Sox motif is also predicted in close proximity to the POU motif. POU and Sox transcription factors

are known to interact and synergistically regulate expression of targets. It would be of prime importance to test whether known POU and Sox factors are able to regulate initiation of Ptf1a expression. However, testing Sox2 and Brn2 alone or in combination did not induce Ptf1a expression or induce Ptf1a enhancer activity (data not shown).

Zic family members are zinc finger-containing transcriptional repressors, and Zic1 is able to repress expression of *Atoh1* through a GC-box located in the enhancer of *Atoh1*. I have demonstrated that Zic1 is also able to repress activity of the Ptf1a enhancer as well as Ptf1a expression, but this effect is not through the zinc finger motif located in the 132 base pair ECR. Furthermore, in *Zic1;Zic4* mutant mice, Ptf1a expression is not altered. This maybe due to compensatory repressive action by Zic2 and Zic3 as all Zic factors can bind the same motifs.

Concluding Remarks

While I have determined that a POU motif in the 132 bp ECR is necessary for Ptf1a enhancer activity, it still remains an open question as to which transcription factor is regulating initiation of Ptf1a expression. Intriguingly, over-expression of cJun is not sufficient to affect expression of Ptf1a or enhancer activity suggesting that the specification properties of calcium waves in the developing spinal cord are genetically interpreted by a mechanism that requires additional factors. Also, repressing Ptf1a expression in inappropriate domains is another critical issue that needs to be addressed. What is the role of Zic factors

in repression of Ptf1a expression? Does the second zinc finger motif in the 132 bp ECR play a role in repression of Ptf1a enhancer activity? Resolving these questions will contribute to further understanding how a balanced nervous system develops.

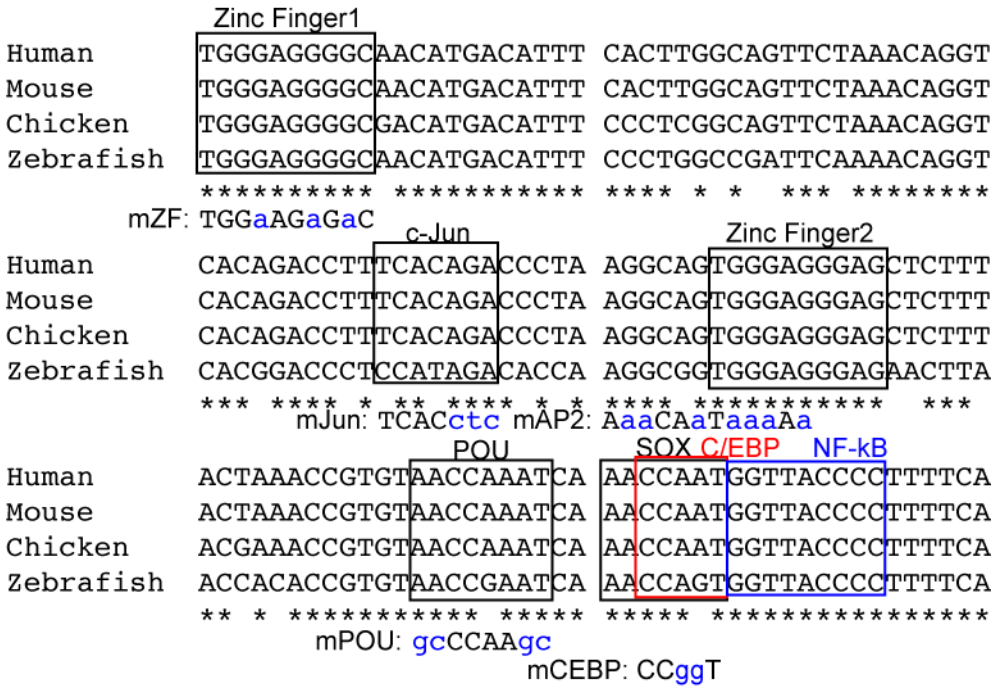

Figure 3.1. Predicted motifs in the 132 bp ECR. Analysis of the 132 bp ECR with the Transcription Element Search System (http://www.cbil.upenn.edu/tess) and JASPAR (http://129.177.120.189/cgi-bin/jaspar2010/jaspar_db.pl) transcription factor databases, which predicted motifs for two zinc fingers, cJun, POU, Sox, and NF-kB. Mutations to selected motifs were generated and mutated nucleotides are shown in blue, lowercase letters.

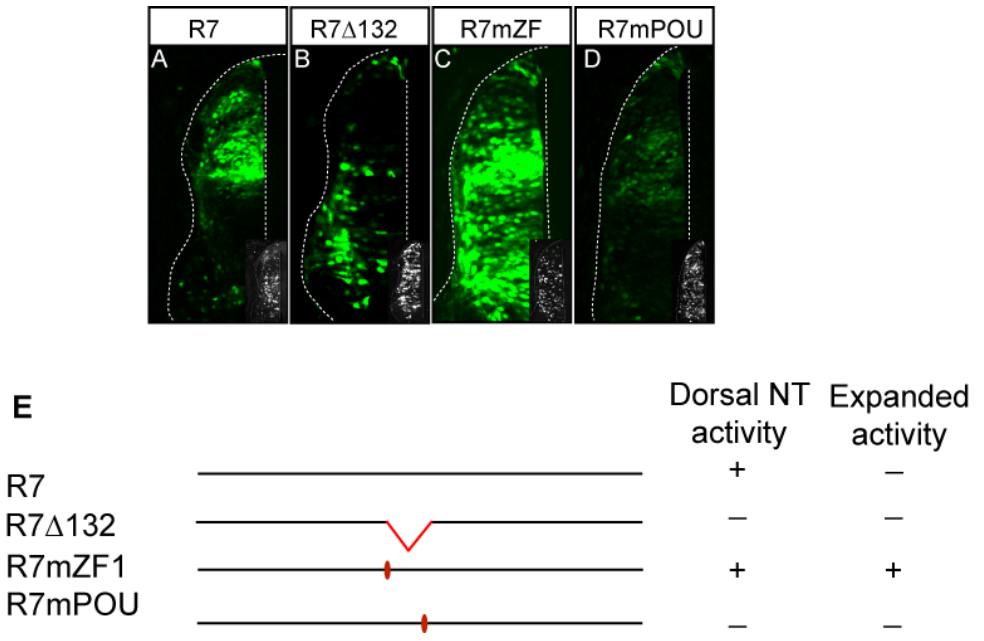

Figure 3.2. A zinc finger and POU motif differentially regulate *Ptf1a* enhancer activity. Mutations mZF and mPOU from Fig. 3.1 were tested in chick revealing that these motifs are essential to proper activity of the 1.2 kb Ptf1a enhancer. **(A, B)** As previously described, a 132 bp ECR is necessary for dorsal activity of the Ptf1a enhancer. **(C)** Mutation of the first zinc finger motif, ZF1, results in a dramatic increase of reporter expression along the dorsal-ventral axis of the spinal cord. This demonstrates that the zinc finger motif is critical for repression of enhancer activity in some domains of the spinal cord. **(D)** The POU domain is essential for robust enhancer activity in the spinal cord, as mutation of the motif results in abrogation of reporter expression. Taken together, the 132 ECR contains motifs that have differential regulatory effects on Ptf1a enhancer activity. (n>4 for each construct tested in chick; embryo reference #s: R7mZF, 1009 mOct1b emb2; R7mPOU, mMzf emb 3).

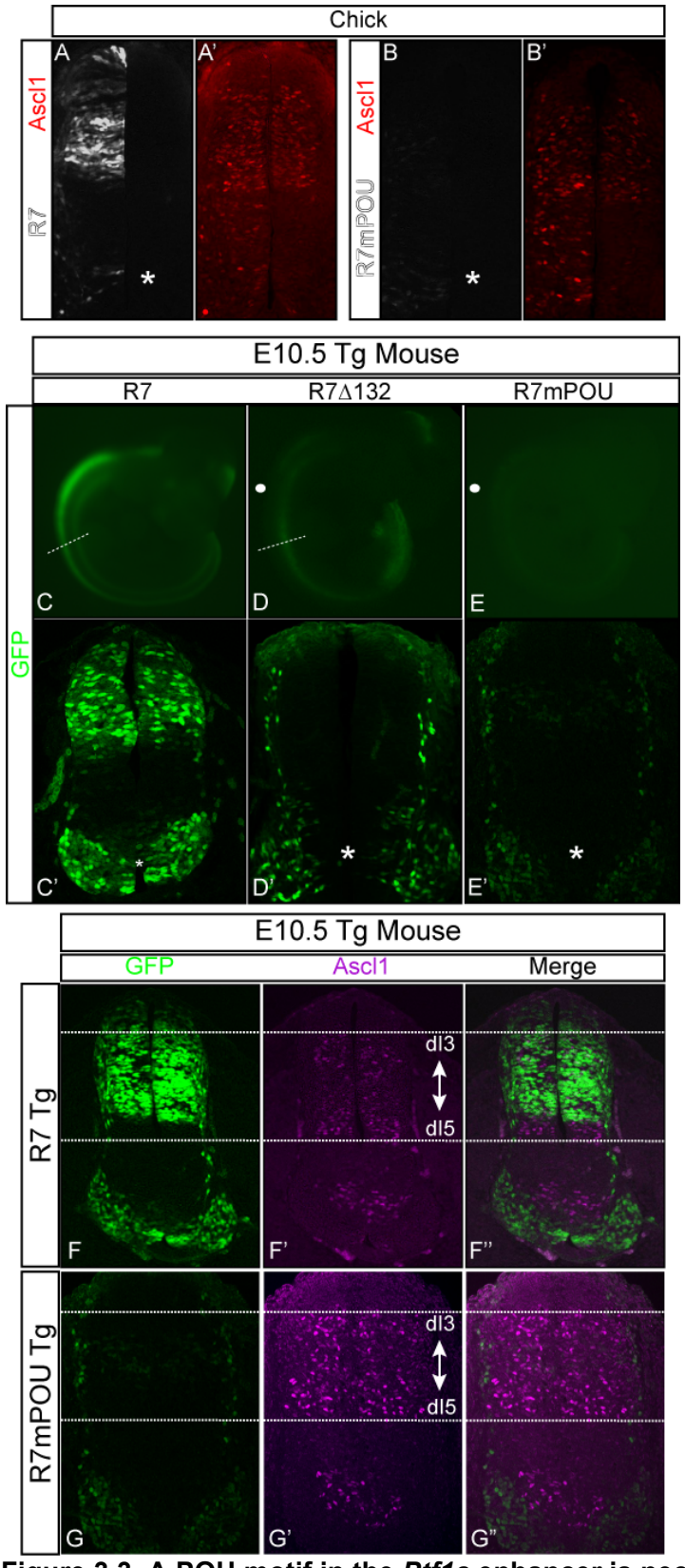

Figure 3.3. A POU motif in the *Ptf1a* enhancer is necessary for activity in the spinal cord at E10.5. (A-B') As previously described, a POU domain is necessary for

Enhancer activity in the chick neural tube and **(C, D, E)** this activity is phenocopied in the mouse at E10.5, as dorsal activity is strikingly lost in whole-mount *R7mPOU-GFP* transgenic mouse embryos. **(C', D', E')** Furthermore, sections reveal, that mutation of the POU domain is necessary for robust enhancer activity and almost completely phenocopies the deletion of the 132 bp ECR, as reporter expression in *R7mPOU-GFP* transgenic mice is severely abrogated. Note that ectopic ventral expression is not affected by mutations (asterisk). **(F-F'', G-G'')** Staining of mutant embryos reveals that the few cells expressing low levels of reporter lay within the Ascl1 domain. These data confirm that the POU motif has a conserved function, between chick and mouse, in being critical for Ptf1a enhancer activity. (n=3; embryo reference #: 2489-6).

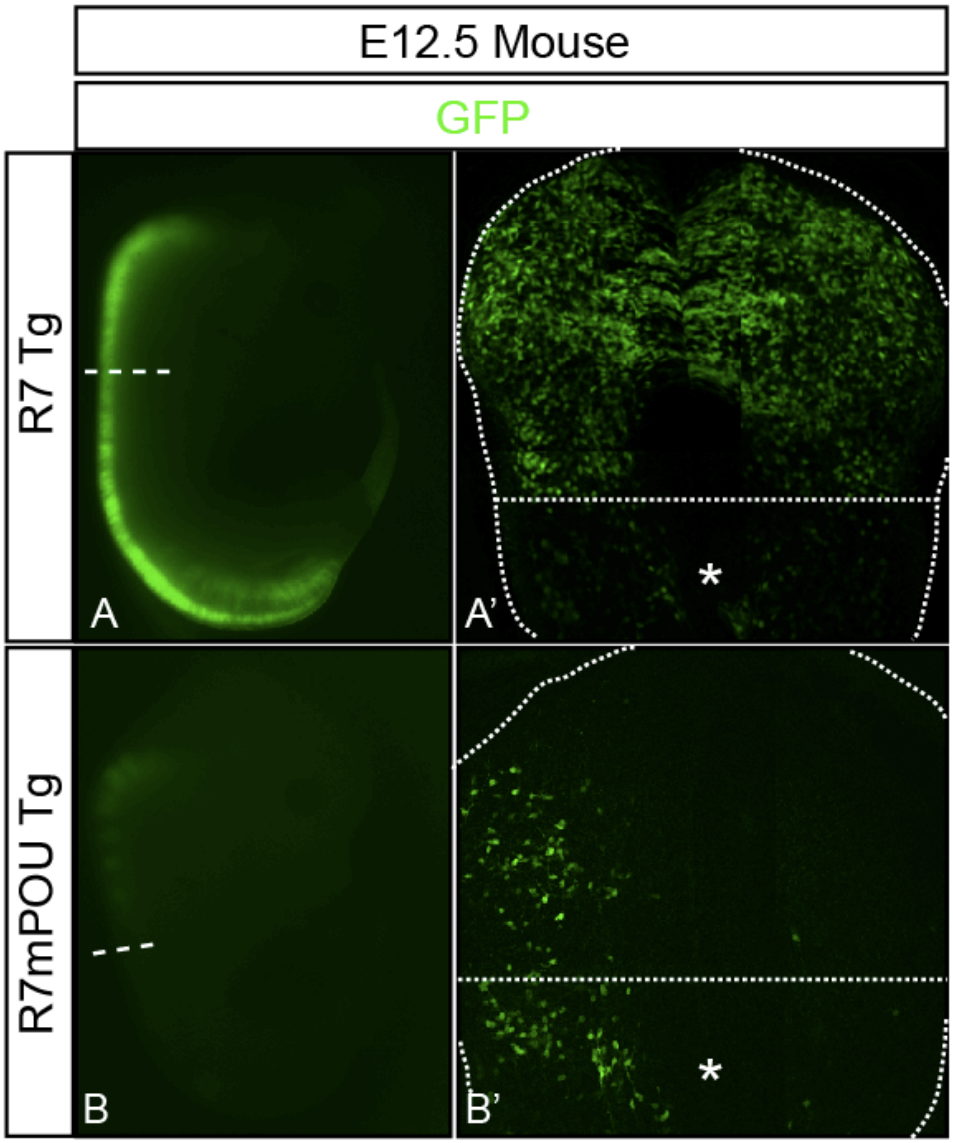

Figure 3.4. The POU motif is necessary for proper activity of the *Ptf1a* enhancer at E12.5. (A, B) Whole-mount imaging of *R7mPOU-GFP* transgenic mice at E12.5 have a similar phenotype to E10.5 embryos in that reporter expression in the dorsal spinal cord is severely impaired. **(A', B')** Cross-sections of *R7mPOU-GFP* transgenic spinal cords reveal that reporter expression is significantly reduced. Specifically, reporter expression in the dorsal spinal cord (area above the dashed line) is severely ablated in spinal cords expressing the transgene the mutated POU motif. Note the R7mPOU Tg embryos had mosaic expression as seen in the wholemount image B and in the asymmetry in expression in the neural tube section in B'. (Asterisk indicates region of ectopic reporter expression). (n=3; embryo reference #: 2482-9).

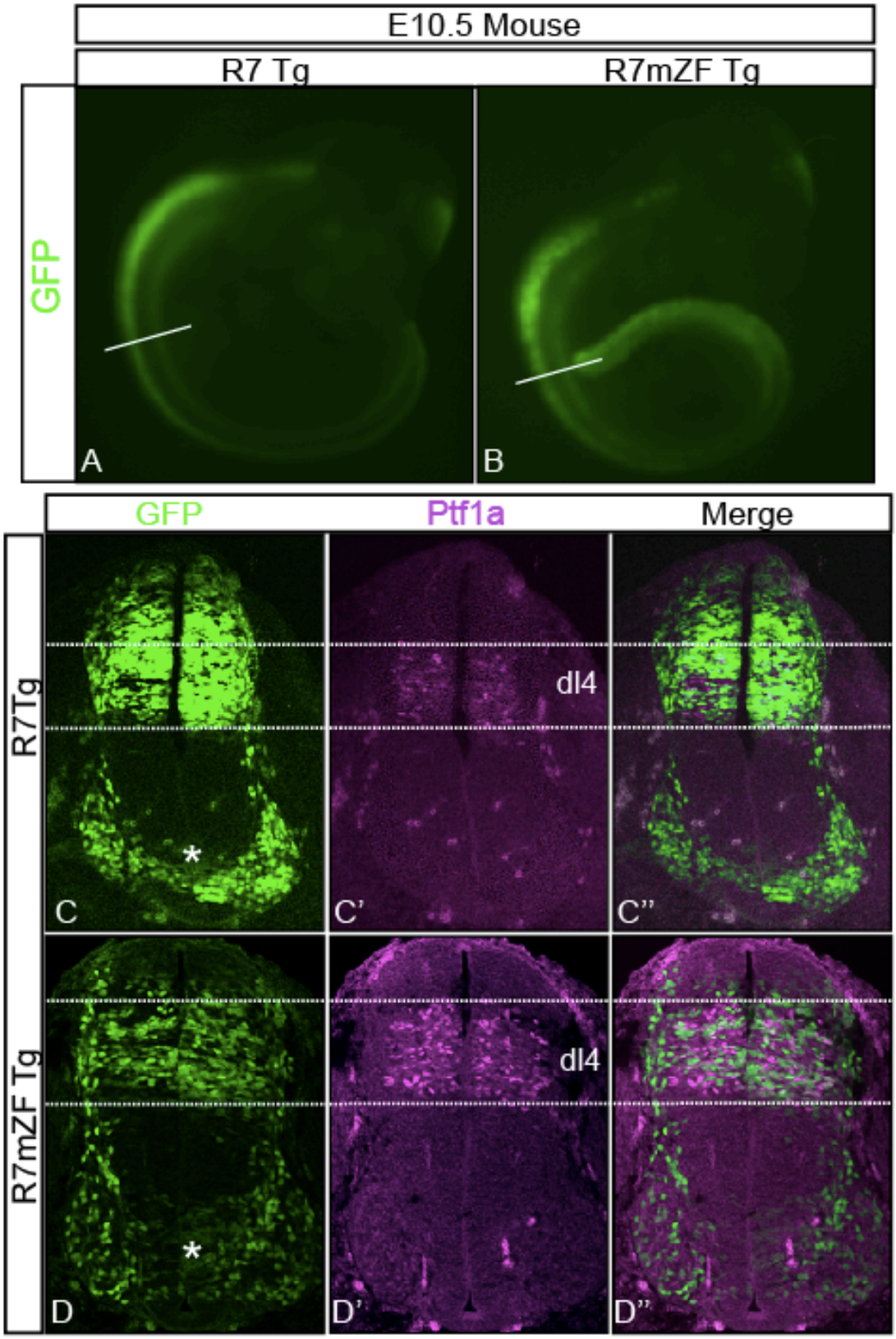

Figure 3.5. A zinc finger motif is not integral for repression of *Ptf1a* enhancer activity in transgenic mice. (A, B) At E10.5, reporter expression in *R7mZF-GFP*

transgenic embryos mirrors GFP expression in *R7-GFP* transgenic mice. **(C-C", D-D")** Patterning of enhancer activity is not altered in *R7mZF-GFP* in the E10.5 spinal cord. These data indicate that mutation of the predicted zinc finger motif is dispensable for regulation of Ptf1a enhancer activity. (Asterisks indicate ectopic reporter expression). (n=3; embryo reference #: 2449-1).

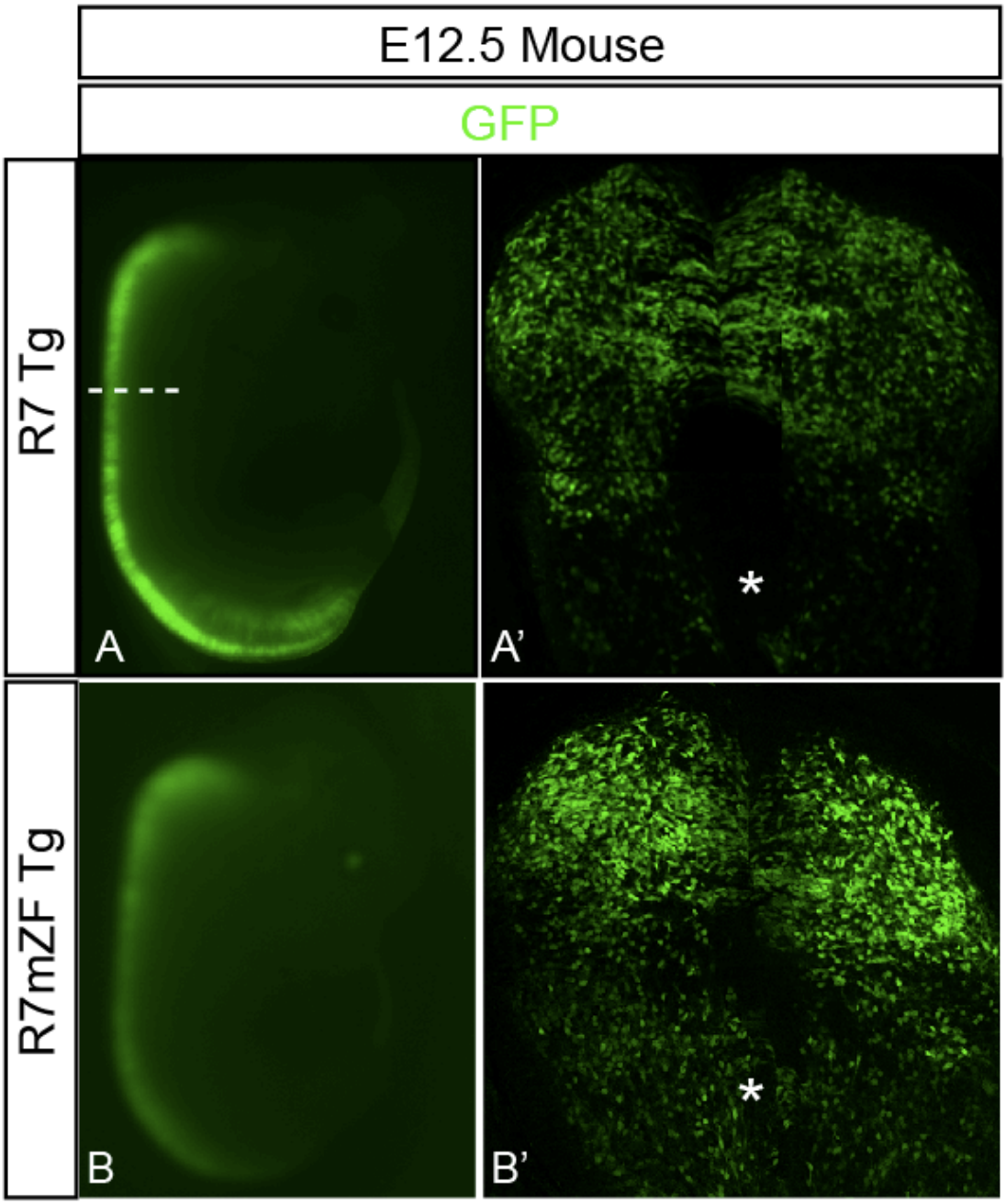

Figure 3.6. Reporter expression of *R7mZF-GFP* in the spinal cord of transgenic mouse embryos is not affected by mutation of the zinc finger motif at E12.5. (A, B) Strong reporter expression is observed in the spinal cord at E12.5 in whole-mount embryos of both *R7-GFP* and *R7mZF-GFP*. **(A', B')** Furthermore, reporter expression is indistinguishable between *R7-GFP* and *R7mZF-GFP* in spinal cord sections. Therefore, mutation of the predicted zinc finger motif exerts no effect on activity of the *Ptf1a* enhancer. (Asterisk indicates region of ectopic reporter expression). (n=3; embryo reference #: 2437-8)

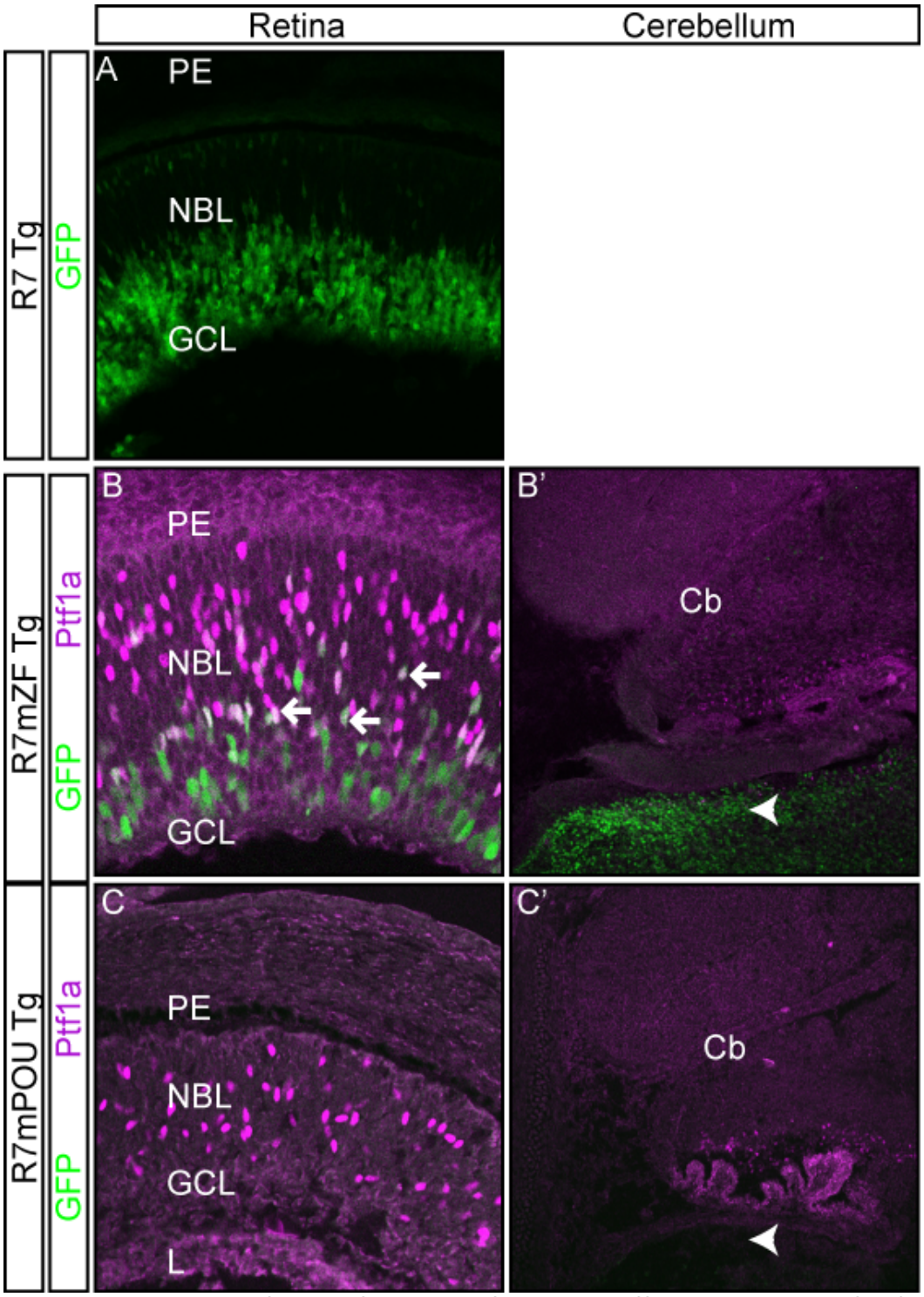

Figure 3.7. Mutation of a zinc finger motif does not affect expression of *Ptf1a* enhancer activity, and a POU motif is necessary for activity of the enhancer in E14.5 retina and hindbrain. (A) Expression of *R7-GFP* is observed in the retina in both the neuroblastic layer (NBL) and the ganglionic cell layer (GCL). Hindbrain at E14.5 was not analyzed. **(B)** In *R7mZF-GFP* transgenic mouse retinas, reporter expression is observed in the NBL and GCL, and co-localizes with Ptf1a expression (arrows). **(B')** Expression of *R7mZF-GFP* is observed in the hindbrain, but not in the cerebellum

(arrowhead). Meredith *et al* demonstrated that the full-length 12.4 kb *Ptf1a* enhancer does not express in the cerebellum. Therefore, mutation of the zinc finger motif does not change activity of the *Ptf1a* enhancer. **(C)** Mutation of the predicted POU domain results in abrogation of *Ptf1a* enhancer activity of in the retina when compared to *R7-GFP* and *R7mZF-GFP*. **(C')** Reporter expression is significantly reduced in the hindbrain of *R7mPOU-GFP* transgenics (arrowhead). As in other stages, the zinc finger motif does not affect expression of *Ptf1a* enhancer activity in domains where *Ptf1a* enhancer is active. The POU domain is necessary for enhancer activity as reporter expression is lost in the retina and cerebellum. (n=2 for *R7mZF* and *R7mPOU*; n=1 for *R7-GFP*)

Candidate TF	Ptf1a(+) Expression (RPKM)	Motif Present in enhancer	Phenotype of motif mut	*In situ* (Ptf1a)
Brn2 (Pou3f2)	117.74	Yes	LoA	No change
Jun	42.76	Yes	No change	No change
Sox2	196.62	Yes	No change	No change
Zic1**	172.19	Yes	LoR	Repression
C/EBP	0.19	Yes	No change	No change

Table 3.1. List for candidates for *Ptf1a* enhancer-regulating factors. Analysis of mRNA-seq data from Ptf1a(+) populations, sorted from *12.4 kb-mCherry* mouse neural tubes at E12.5, yielded a selection of candidates. Selected candidates were tested if motifs for the candidates were present in the 132 bp ultraconserved element. These motifs were mutated and analyzed for activity that was binned into loss of activity (LoA) or loss of repression (LoR) if a change in the activity of the R7 enhancer was observed. A predicted motif for Brn2 is required for robust activity of the R7 as mutation of this motif significantly reduced reporter expression in the chick spinal cord. Mutation of a predicted zinc finger motif resulted in an expansion of reporter expression suggesting that this predicted site is a silencer element repressing activity of R7 in certain domains of the spinal cord. Mutation of a predicted C/EBP motif, which overlaps with a predicted Sox site, does not have an effect on enhancer activity. The only candidate, when tested by overexpression, which was able to affect expression of Ptf1a was Zic1. Zic1 repressed Ptf1a expression.

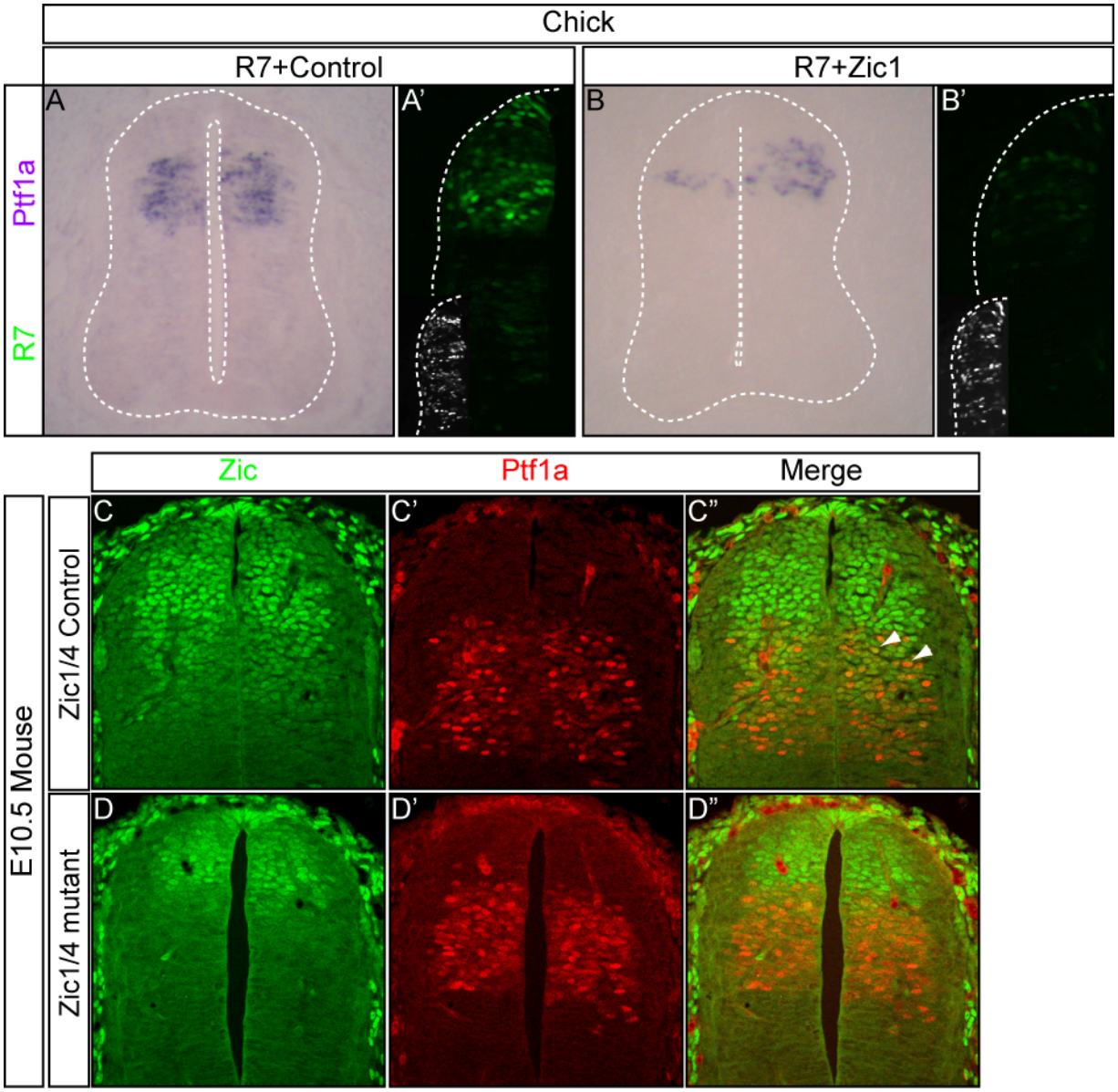

Figure 3.8. Zic1 represses Ptf1a expression and enhancer activity in chick, but not in mouse. (A, B) In chick, over-expression of Zic1 represses expression of Ptf1a when compared to over-expression of a control vector. **(A', B')** Zic1 also represses activity of the *Ptf1a* enhancer (inset, electroporation efficiency. **(C-C")** In wild-type mice at E10.5, Zic expression is restricted to the dorsal neural tube. Moreover, Zic co-labels with $Ptf1a^{+}$ cells. **(D-D")** At E10.5, $Zic1^{-/-};Zic4^{-/-}$ mutant mice have considerably fewer Zic^{+} cells, but expression of Ptf1a was not altered. n=2

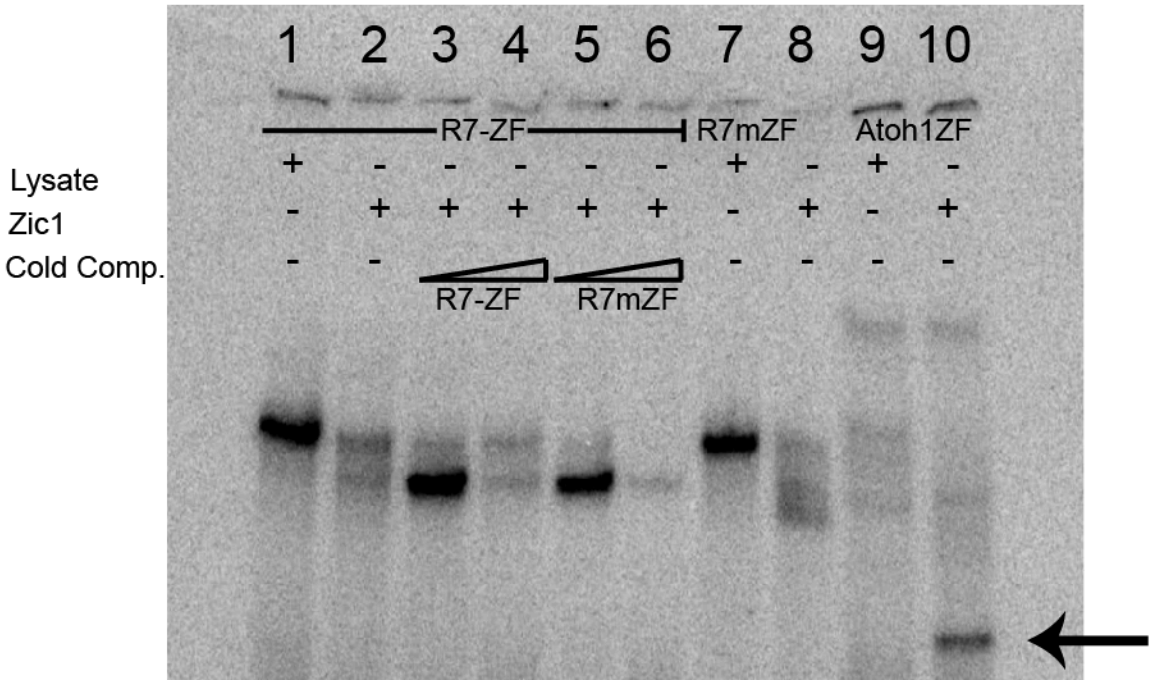

Figure 3.9. Zic1 does not bind to the zinc finger motif in the 132 bp ECR. A non-specific reactive species in the lysate bound the probe containing the zinc finger motif, R7-ZF **(Lane 1)**. Expression of Zic1 in this system resulted in no bands of significant intensity which indicates that Zic1 does not interact with the zinc finger motif **(Lane 2)**. Non-radioactive competitor probes which contained the wild-type motif, R7-ZF, or a mutated motif, R7-mZF, were used in order to determine if Zic1 binding, should it have occurred, was specific to the motif; there was no observable interaction **(Lanes 3-6)**. Zic1 did not interact with the motif, and the competition assay was not informative. A GC-box identified in the *Atoh1* enhancer, identified by Ebert *et al*., was used as a positive control to determine if Zic1 being expressed in the experiment was able to bind to a known target. A strong band in reaction with the known Zic1 motif indicates that Zic1 used in the experiment can bind a known motif **(Lanes 9 and 10, arrow)**.

Chapter Four

Conclusion and Future Directions

The spinal cord serves as a conduit that receives and sends sensory input from the environment to the brain, and relays motor output to the limbs and body. The proper operation of the spinal cord is dependent upon a precise balance in the composition of neurons that populate it; excitatory and inhibitory neurons are both required for the correctly functioning nervous system. This balance is established during the neurogenic period of embryonic development, between embryonic days 9.5 and 14.5 in mouse, where discrete populations of neural precursors acquire a distinct identity based upon the expression of specific factors that regulate their developmental fates (Jessell 2000).

Transcription factors such as Ascl1 and Ptf1a are part of a regulatory program, which specifies a particular expression pattern of neurogenic genes. The expression of these genes is tightly regulated by variety of mechanisms. One such mechanism is modification of chromatin into “accessible” or “inaccessible” forms. This is mediated primarily through modifications of chromatin structural elements, histones. Acetylation or deacetylation on particular lysine residues on histones will result in chromatin being accessible to *trans*-regulatory factors, or being condensed rendering DNA inaccessible (Barski et al. 2007; Mikkelsen et al. 2007; Karlic et al. 2010). Recent work has established that several histone marks are predictive of key *cis*-regulatory elements, enhancers

and promoters (Barski et al. 2007; Heintzman et al. 2007). Using these marks, functional enhancers have been isolated and shown to be active in specific tissues at defined time points. While histone markers are useful in *cis*-regulatory element predictions, it is not completely accurate as there are many genomic region which contain histone markers associated with *cis*-regulatory elements, but do not have *cis*-regulatory activity (Visel et al. 2009). Accordingly, the presence of select histone marks is not a guarantee of a functional enhancer. It would be important to determine what histone markers are present along the auto-regulatory 2.3 kb, 1.2 kb, the full-length 12.4 kb *Ptf1a* enhancers at the early and late stages of neurogenesis. This would reveal whether there are any dynamic changes in the chromatin structure of the *cis*-regulatory elements of *Ptf1a*. Any dynamic changes in histone marks that might be observed would indicate that histone modification plays a potential role in the regulation *Ptf1a*.

A second mechanism of directing gene expression is through RNA-mediated processes. Small RNAs such as siRNAs and miRNAs can repress gene expression though direct binding of target mRNA transcripts and inducing destruction or translational pausing of those target transcripts (Olsen and Ambros 1999; Martinez et al. 2002; Seggerson et al. 2002; Martinez and Tuschl 2004; Schwarz et al. 2004). This mechanism is currently in use as a tool to study function of genes as it can be easily manipulated in cellular- and animal-based systems. Another RNA-mediated gene regulation mechanism is long non-coding RNAs, which are transcripts that are generated from non-coding stretches of the genome. These transcripts are believed to regulate gene expression by acting as

scaffolds that integrate chromatin remodeling proteins and transcriptional machinery at gene transcription start sites (Bracken et al. 2006; Rinn et al. 2007; Ku et al. 2008; Zhao et al. 2008; Tsai et al. 2010; Bertani et al. 2011; Dean 2011; Schuettengruber et al. 2011; Wang et al. 2011). Another mode of regulation by lncRNAs is through siphoning off factors required for transcription of target genes leading to repression through a deficit in transcriptional machinery necessary for transcript synthesis (Hung et al. 2011). lncRNAs are a burgeoning field and discoveries will prove to be interesting in the potential impact to gene expression, especially taking into consideration the large number of non-coding transcripts that are generated in vertebrates. Thusly, it would be helpful to determine if there are regions in the 3' *Ptf1a* enhancer that are transcribed in early and late phases of neurogenesis. If there are regions within the enhancer that are transcribed, these transcripts may play a critical role in regulating enhancer activity and Ptf1a expression.

Regulatory elements that occur in *cis*- or *trans*- are the most recognizable and classically understood in the field of gene expression. *Cis*-regulatory elements of the highest notoriety are the promoter, enhancer, insulator, and silencer. Transcriptional machinery is known to interact with *cis*-regulatory elements, and this interaction is necessary for transcription initiation. It has been an open question as to how distant *cis*-regulatory elements interact with a promoter that could be kilobases away (Maston et al. 2006). Evidence now points to chromatin looping as a potential model: chromatin can form loops with itself, forming interactions between distant genomic regions (Vakoc et al. 2005; Jiang

and Peterlin 2008; Miele and Dekker 2008; Dean 2011; Deng et al. 2012). Two key proteins, cohesin and mediator, facilitate such looping interactions. Genome-wide analysis of cohesin and mediator reveal binding patterns that mirror genes that are being expressed to a significant degree (Kagey et al. 2010). Furthermore, interaction of general transcription factors with cohesin and mediator suggest that recruitment of the transcriptional machinery to the promoter may be effected, in part, by such looping interactions (Kagey et al. 2010). It is possible that transcription factors required for Ptf1a expression are recruited to the 1.2 kb enhancer which then loops and interacts with the promoter of *Ptf1a* and initiates expression. To interrogate this possibility the use of chromatin conformation capture at stages immediately prior to Ptf1a expression and into late neurogenesis would address the following questions: 1) does the 1.2 kb enhancer form a loop with the *Ptf1a* promoter and 2) if a loop does form, is it a transient interaction. The answer to these two questions can elucidate a potential mechanism through which the 1.2 kb enhancer can initiate Ptf1a expression.

Regulation of this key determinant is achieved through two enhancers. The first is an auto-regulatory element that contains two PTF1 functional binding sites, and is highly conserved in mammals. This enhancer element is 2.3 kb in length and lies 13.4 kb upstream of the Ptf1a coding region. Mutation of these sites results in the loss of enhancer activity. Furthermore, enhancer activity *in vivo* is lost in mice that do not have functional Ptf1a (Meredith et al. 2009). Another enhancer of Ptf1a lies immediately downstream of the Ptf1a coding

region and is 12.4 kb in length (Meredith et al. 2009). This enhancer has activity in the appropriate Ptf1a domains in the spinal cord and retina, but not the cerebellum. Furthermore, the activity of the 12.4 kb enhancer is maintained in the absence of Ptf1a, supporting the hypothesis that this enhancer contains information that is necessary for the initiation of Ptf1a expression. The key regulatory sites for the 2.3 kb enhancer lay in the PTF1 sites, but the regulatory sites in the 12.4 kb enhancer still has yet to be uncovered.

To this end, the work contained in this volume elucidated the following: 1) out of the seven regions of conserved sequence contained in the 12.4 kb enhancer, one region could recapitulate the activity of the 12.4 kb enhancer, R7; 2) a central 132 bp ultraconserved region is required in the context of R7 for enhancer activity; 3) a predicted zinc finger motif, though necessary for restricting activity of the mouse R7 enhancer in chick assays, does not have a significant role in regulating enhancer activity when tested in transgenic mice; 3) a putative POU motif is required for robust enhancer activity, and this activity is conserved to mouse.

Using conservation analysis, which has been used to predict enhancers in the field, I identified seven conserved regions. Testing each region individually (save for two that were combined into one) in chick revealed that only one, region seven (R7) was sufficient to drive expression to appropriate Ptf1a-expressing domains in the dorsal spinal cord. Within R7 lies a highly conserved region that is approximately 132 bp, which when deleted ablates dorsal activity in the spinal cord. Therefore, the site through which the activity of this Ptf1a enhancer is

initiated in must lie in the highly conserved 132 bp region. I analyzed the region for potential consensus sequences for known transcription factor binding sites and cross-referenced the sites with transcription factors that were known to play a role in nervous system development in addition to transcription factors that were known to be present in the spinal cord at the developmental time points I was investigating. This led me to mutate sites that I hypothesized to be important in the activity of the R7 Ptf1a enhancer. A predicted zinc finger and POU motif resulted in being necessary for repression and activity of the enhancer, respectively, in chick. In mouse, the predicted zinc finger motif was dispensable as it did not affect activity of R7, but the predicted POU motif revealed a stronger phenotype than in the chick as nearly complete loss of activity was observed in areas where the wild-type enhancer was normally active. Contrasting this approach, I also tested several candidate transcription factors of which only one, Zic1, was able to repress activity of R7.

Taken together these results do not completely address the central question that was posed, which is, how is Ptf1a expression regulated? Specifically, how is Ptf1a expression initiated? However, the evidence presented in this work has significantly narrowed down the sequence, which carries information sufficient for *Ptf1a* enhancer activity, a putative motif that can be presumably bound by a family of transcription factors critical in nervous system development, the POU family. It is important to note that this may not be the sole sequence responsible for regulating *Ptf1a* activity. The next significant step is to identify what factor(s) bind to this motif. In order to do so, two methods can be

employed. The first is an unbiased approach, which would involve the use of a yeast one-hybrid assay where the putative POU motif would be used as bait for a cDNA library. This method will provide a relatively non-biased approach to identifying a potential transcription factor(s) that interacts with the bait sequence. Some issues with this methodology can miss a DNA-transcription factor interaction may be a part of an obligate heterodimer or require post-translational modifications in order to bind DNA (Walhout 2011). With these caveats of the yeast one-hybrid system, this assay can provide a transcription factor(s) that interact with the putative POU motif. POU transcription factors bind DNA as homodimers, and if there is an interaction between a POU family member and the putative POU motif this assay will be able to detect it (Scholer 1991). The second approach is to continue to search for candidates that bind to the putative POU motif using expression data from neural tube populations from *2.3kb-GFP* and *12.4kb-mCherry* transgenic mice (data not published). These data sets formed the basis for the candidate selection described in the previous chapter, but this candidate list did not exhaustively identify transcription factors that bound a POU motif. Therefore, efforts must be concentrated to look for such factors. Presumptive factors that come out of these two methods must then be tested in a similar fashion to what has been established in this work. First and foremost is over-expression of the candidate factor and analyzing whether Ptf1a expression is affected, in addition to any effect on *1.2 kb Ptf1a* enhancer activity. This can be readily performed using chick *in ovo* electroporation. More stringent testing can

be accomplished through mutant mice in any candidate which affects Ptf1a expression and *1.2 kb Ptf1a* enhancer activity should such mutants be available. The repression of Ptf1a expression coupled with the repression of *1.2 kb Ptf1a* enhancer activity by Zic1 reveals that this transcription factor may play a role in determining the balance of excitatory and inhibitory neurons. The balance of excitatory and inhibitory interneurons in the spinal cord should be assayed in the same manner as Glasgow et al. using markers for the relevant populations in later stages of spinal cord development of Zic mutant mice. The lack of change in Ptf1a expression in Zic mutant mice would suggest that there might be no appreciable change in the balance of excitatory or inhibitory neurons, but a quantitative assessment would be helpful in concluding the effect Zic mutations. Furthermore, since the repressive effect of Zic1 on Ptf1a is readily observed in chick, it will be of important to determine if Ptf1a repression leads to a disrupted balance of excitatory and inhibitory neurons in this system. In order to determine if the repression of Ptf1a by Zic1 is direct, I propose that EMSA assays be confirmed with the probe containing the wild-type putative zinc finger motif. A future EMSA would have to eliminate the ectopic bands there were observed in the lysate samples used. This will serve to confirm whether or not Zic1 is binding directly to the putative zinc finger motif. If the repression of Ptf1a is indirect, this will provide an opportunity to find which factor(s) are directly repressing Ptf1a expression; the yeast one-hybrid assay mentioned previously could give a list of candidates effecting Ptf1a enhancer activity if the bait is the putative zinc finger motif.

Other questions arise out of this work. The activity of the *12.4 kb Ptf1a* enhancer is not restricted to the *Ptf1a* lineage in the dorsal neural tube and therefore another sequence outside of the enhancer must serve as a silencing *cis*-regulatory element. What is the sequence and where is it located? Also, is the *1.2 kb* sequence required for the activity of the *12.4 kb* enhancer? As the *1.2 kb* enhancer is the only conserved element that has activity, this result is likely, but as some studies have pointed to before, conservation is not the only predictor of enhancer function. It is plausible that another sequence within the *12.4 kb* enhancer, that is not conserved, has an effect on the activity of the *12.4 kb Ptf1a* enhancer. Enhancers have spatial- and temporal-specific regulatory activities; the enhancer of *Ptf1a* that I have identified has activity in the retina and the neural tube, but not in the cerebellar *Ptf1a* lineage. Consequently, It remains an open question as to what is the *cis*-regulatory element that initiates cerebellar *Ptf1a* expression. In the *cerebelless* mutant, *Ptf1a* was pinpointed as the gene responsible for the mutant phenotype. *Ptf1a* is located 60 kb from one end of the region deleted in *cerebelless* mice. It is a possibility that the *cis*-regulatory element that initiates expression of Ptf1a in the cerebellum lies within this genomic area. Therefore, it should be a point to test conserved regions in this remaining area to determine if information required to drive Ptf1a expression is present.

Within the framework of gene expression regulation, this work has supported that a repressor and activator element can be located relatively close

to each other, and enhancers are regulated by complex interactions. Moreover, this work potentially informs that a large transcription factor family (POU), known to be critical in neuronal differentiation, may play a role in the generation of inhibitory neurons and generating a balanced neural network. It is a distinct possibility that while the sequence is identified as a POU domain, other families of transcription factors may recognize, bind to, and regulate expression of *Ptf1a* through it. Therefore, a more rigorous analysis of which transcription factors may potentially act through the predicted motif is of the utmost value. The importance of a detailed genetic pathway determining the formation of a neural network cannot be stressed enough, as a properly functioning nervous system allows the individual to interact successfully with the environment.

Appendix A

Sequences for *Ptf1a* enhancer regions tested, R1-R7. As described in chapter 2, below are the sequences for each of the conserved regions of the *12.4 kb Ptf1a* enhancer that were tested in chick.

Sequence for *Ptf1a* enhancer, region 1:

CCTCCTACCCCCACCCCCTCTCCACCCCGGCCTGGCCGGAGGACAACGTACAGACAGCAT

GCTAATCGCTTTAACCTAGTTTCAGACCCACCGAGGGGTTCCCCCGCGCGCGCTGCGGGT

GCCATGGACTTCATCCAAGGGTAATTAAAGCAAGGCTAGGAGCTGGACCGACCCCGCAGA

GCGGCCTGGCCAGACACGCTGAGATTCTGGGTCGCGACGCTCCCAGGAGGCCTTGAGCCC

TGGATCCTCTCACTTCTCCAGCTTTCTCCTCTTCTCCTGTCTCCTCTCCCTCTAAAAGTT

AGTTTTACACTGGGGATGTTTGAGAAGGAGAAGAAAAATAATTCGATTTTGTCTTGTATT

ATGGCCTCATTAAACACGAAAGTTGCTTTTGTACAAATGCTACCATCAGGCATGTAATCT

CATTACACTCATTAGAAAGTCAAATGTTAGACAGACTTCAACTTCATTATAAGTTATGGA

AGTGTATCGTTTTTTTCTCCCTCCTGTGTAGACCTGCCTTTCGATTGGATGAGGCAGTGT

GGCACTTCGCAGTTCAAACACTACAATCATTGTGGTGTGGCACCTTTTATTTCGAATGCC

ACGTTTTTTTGTGTGTGATTCAATGCATCAACACATCCTTTAGTGCTGTGCAGATCAAGC

GGAAGAGCCCAGCTAAGCGTTCAAAGCACCAAGGTCTCTATCTTGTCACTCTTGTGTCCT

GACTTGGCAAGTCCCTTTAAGCACAATCTTCAAAGAGACTGACAGTTTCTTTTTATTTAC

TCATAATCTATTCATTGCAGTGTAGCAATTCTTTTTCTTAAAAAAAAAATACAGCAGAAA

AAGCAGAACAAACGTATCATTTCAGAGCCTCCTTTGGCATCAGCGAGGCCAGGCCTGAAC

TGTGCTGGACACAGGGGAGCCAGGCTGGAGAGGCTCTGGCCTGCAGCATCTCTGGCCCAT

AGATACAGCTTGGCACTGGGAAGACCTTGAGGGGACAACTTGAGTGATGGGTATGCAGCA

GTCCTAAGTCAATA

Sequence for *Ptf1a* enhancer, region 2:

TCTCACAGGGCTGAGAGAGTAGGTGGGAAAGAGTGAGTGTCCCACTTCAGGCTGGGGTCA

GAGGGCATAAAGAACCTTGTTGGAGCTCCTGTGAAACCGGCTGCCAGCCATTCCCAGGAC

TGCTCATCTGCCAGTACCAGGATTAACGCTGCTCCATGGAGAGGGTGTGAAGCCTTTTAA

ATCCACAAACTCATCCTATTTCCATTAATGGGCCTCTGCGGTAGCACCGAGTACCAGCTG

CAAGGTTCAACAGACACCAGTTGAGTACTCCCTGGCGAATCTCTCTGAGAAGAGCCCTTT

CCCCTCCCTCTCGCACGTCCCCTCCCCTCCCACTGCCTTTCCAAAGCCTTCTGCAGCCTG

TGGGAATGGTCCTAAATAATTCCACAACCAATTGCATTTTTGAGATCTGGAACCACTGGT

AGGAAAAGATTCCCACGACAGGATGTGAGGTCTGGCCAACAGGTGTTCGGATTTGGCCCA

GTGAGATGGAGAAACCCAGGGAGGGCGAGAGACAGGCCTGCCAGAAAGCAGACTGCTCAG

GGCCTCCCGGCTTTACAGGCCGTGGAAGCCGCAGACAATCGAAATGATATCTTTATTGCT

AGGTTCATGTCCTGGGCTATAACATATCAATCCCTGTCTTGGTTCTCTGGGATGCACCTG

GTATTTCAAACTTGTTCTTTTTGTGCTTCTGGGTCCTGGACTGCAGCTGCCTAAAACAAG

CAAAGAGAGAGGCCCTTACAATGTCTCCAAGACGTGTTGGCATGGCCCGCCTGTCTTTAA

TGTACCATTAACCATTGTCTTTACACAATATGGAGACTGTAAAGCATGACATGTGTTATA

ATAAAACACATTTTCAATGATACACTTGGACTTGGGGCTGGGGTGTAGCAAACAAACAGG

CCCGAATCCTGTTTTGTCTCCCTTGCTAAGCCCACTGGCAATTAAACTTAAGACCAGTGT

TTCCCCCGCTCCTCATCAGCCAATTAAGTTTTATGTCTCCTAATTTTTCACACAGAAAAA

AAGTGTCAGTGTTGGTCCCTAAAGACATTGATTTTCTTGCTATCACCTGGCGCTCAGATC

TTAGTCCCATTTATCAAGAAGGGAACGTCAGGCGTTACATAAAGTGACTCTGTTACCATA

AGGCTCTCACCATCCTGGCCCATTGTCTTCCCTTGTTAATAACTCATTACCAGGATTTAA

CAAATCAACCATTACATATGTTTTGTGTCTCCATATTTTGATCCGTACGATTAAGCAGAT

GTTACGATTGAAAATTGAAAAGTCCAAGGCTACTCCAGTCTGAAAGAAAGAGGGCAGCCT

CAAACAACGAGCTAACAATGAGATTCAGAAGATCTTCAGAAAAACATTTACCACCAATAA

ACAGACTCCAACCTGCTCCATTTCAACCATTGTGTGCTTTGGGAGAATAATTAAGTTTAA

TAGGAATAGGTCTGGAGGGTTTTCAATGCCAAGACCACAGGTGCGGGGTGGCATGATGAA

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AAATATTGTCATTTCTTTTAATTCACAGCATTACTTTCAAACCGAACAAAGGCCGGAGCC

GGGGGGGAGGCGGGGGAACGGCCCGGCTGTACTGAAGCGCTTCGGAGGCGGAGGTGCA

GC

CCAAGAGGCTGAGGCGGCAACGGAGCGCGCTGCTGCGTTTGTGCGCAAGTGACACTCCTG

TGCCACACACAAAAGGCCTTCTTCTCCCCACCTTCTGCTGCTTTTCAGCCCTCGGGCCCC

TGCCAAACCGGCTCATAGGACGCCCGGGGGCTCCTGGCCCCCTATCCAGGGCAACTCAGC

CTTTGGGACATCCATCGCTGCCAAGGCAAACCTCTCCAAACTTTCCCCAAACTCGGACGG

CTGGGGAGGGACCCTGGCTGGAGGCGCGCATGCGCCCCGTCTGCTGCTGCCAGTTCCCAG

GGTTCCGGAGAGCCGGGGTGGAGTTGGAGGTGTAGCCCTAGAGGCCTGCCTAACCCCTAC

TATACCAAAAGTCGGGGTTCGGGTGCGGTCTGTCCGCCCAGGAGGCAGGAAGCAGCTGCC

CGGCACCAGCTCTCCACCATTTTTCTACCTTTGCGCATGGAAATCTCCAAAAAGGGATCC

AAGAAGCGGCGAGTTGAATTGGGGGTCATCGGCCTGGAAGAAGCCGGG

Sequence for *Ptf1a* enhancer, Region 3:

CTAGGAGAGGTTTCTAGGAGTTTATTATGAAATCCAGTTGAACGAGCTTTGACATTAAAA

AAAAAAACTGGGCATGCTTGCACTGGATGAATTCAGAAAAAATTCATGTCCCACTAACTG

ATCTGAAACAAGAATAGAAAATTTTAAAATGAGCCACTTCCAAATGAAGGGCGCTTGGCT

GCAACCTCACAAAGACCACAAGTGCATACAATTAAATATTAGGGCTCAGCAATGAGCATT

TGTGGCAATAGCAAATGTGTGACTCCTCGCAGCAATTACTTGCAAGTTTTTCCGTACTTT

TGAGAACATACTTGACGCCTCATTTCTTTGATGACTGCTCAATAGACACCCTGACACAGT

AGTGGAGCTGCCCCTGCCCCAGCTCCCCTCTAGCTCACAGGGCTTTCATCCAGGGCTTGA

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AAAGTTCTGGAAATCGGTATATTTTGCAGGTGTAGAATGTGCCTAATAGCCATGGCTTGT

GCGTGCTCCCCGATGGTGAAGGTCTGACAAGGACAGCTGGCTAGTTTTCAGTGTGAAAAC

ACCCCCTCCGTGGACCAAACACAACGACACTGACCTTTCTGCAGGGGAACCAGAGATGTG

CCTTTGACTGAATTAGCCACAAAGACGTCTTGCTATGACTTTTGTTCACCACGAAAGCAA

AGAAATATTGTGTCTAATATGAAAAATACTGTTCACAGCTTTTCTGTGCCCCTAAAGAAG

TATTACAGTGCTGATTTACTGGCCCCGTTCTCTAGAAATGGCTGGACACAAGGCGGGGTG

GAGGGGTGGGATGTGGGAATGGGACAAGGAGGAGGAGTTGGTAAGTTAAATTGCCTGCTG

CTGTTTCCAAGTTCTCTCTGTGCAGCAGCAAATTTATCAATCTTTTCACCTGACAATACT

TTGAAATATATCATTGTCATCCCAAGTTGTTTGATAGATTAACTGTAGGAACACAGCCTT

TTCCAAGGTCAAGTTGAAGATTCTTGTTTGCTGTTTAACTAAGTTGTTTAAAAACTCAGA

ACAATGCCTGCCTGTTAAATTTCCACTTGTTCTCATGGGATATTGGAAAGCAAAGTTTCC

TGTTGACTGAGTGTGTGGACACGTCATCTTTTTGAGTTAAATTCAGCAAACGGGATTAGC

AATGAGATCAACCCCTTGCAGACACAGAGGAAATTAAAGCCCTCTCTTATCCTTTCAATC

TGTGTACTAATGTTTATGTGTGTCAGGGGCTGGTTACAGCCACACATATCACGTTCAGCT

TTCTAATTTTACCCCAGTGGGAAGGTACAAATGTGCTCTTCTAAAGCAGGAAGGCACAGA

AATTGTGGCCACTCCTGTGGGGTTTTG

Sequence for *Ptf1a* enhancer, regions 4/5:

CAGAGCATAACCTGTTGCCTTAAAGCTTCAGTAAAAATAAAGCCAATTCAAAACCACCAT

GTTTGTGGACATCCTTATTTCTGTTTTTAACCCCACAATTGGCTTCACAATTTCCTCTAT

AACTGCAATGATTGATTTTTTTTTTATTCAACTAACTTTTATTTACCTTGCAATAATGTTC

TGGACCAATGTGATAAATTACAGATATGCAAATTTCTTTGAATGGTGTTGTAAGTGTGTC

TAGCTGGAGCTGAATGACTCCTTGCAAGTCAGTCTGTGCCGGCTCAGGACCCCAGGGAGC

TGATCAAAGCACCCATTCTCTTTCATCCCCAGTATTCTCCTCCAAACTATTTCGATACAA

TATGTTTCCATGATGCACTTAATGTGCTAGGGACATAGAACTCATTACAACCTAGCTAGT

TCTGCCGACCTCTTTGTTCCCCTGCAGAAAGTCAAAGAAAAAAGGAAAAAACCCTGCCAG

TTGCCAGCTTTCTGAAATGCTAAAGCATGCGTCATTTTTCACCAGGTCTGGTCTTGATAT

CCTCAAAAGACAAACTATGAAACCGGCCTCAGCCCTTTCTGGCATTAATTACACTGCTGT

CCAGCCGATCTGTTTTTCCTATTATATGCATTTCTCAGCAGGGATTTTTTTTTTTTTTTT

TGCAAGACTAATGCAGCTGCAAGTGAAACTATGAATGCATTTTTATCACTATGGAAAAAA

ATGAGTGAAAGGACTTCGAAGCATACAGCAAAAACTTTTGAAAGCATTCTGGTGGGTAAT

AGATTTAGGGCAGGCTGGGGGTTGGGGGGATGCAAGGTCTCTAAATCCAAACTTGACTGA

GAGTCTCTGAAGTTTCCAATGCGCTCAGTGTCTGTTGGTGACACATATTGTGCCATTTGA

AATTTCCCTTTACTACCCAGATAAATCTTAAAGGTTAAAGAGTCCCAGCAAAATTTTGAA

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GTGGAAATGGCTGGCAGCCAGTTCTGGGAAAGATTCCAGACCTGACTCCGATTAACCCTC

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TAAGCCAATTCATTTCTAAAGGAGAAAAATTCCTTTCTTTAGCCAAACTGATGGGAGGAA

ATTTGAAAGAAGCGCCAAACTGTGTAACTGTAATTCAGCCAAGGACTGCTAAAACAAGGT
GTTGATATATAGCAGCAGATTTAAAACAAGTTTCTAGGGCAACACTCCTTTGGAGGCGGT
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GTACTAGCCTTCAAAGTAACAGGTTTCTCTCCAATTAAATCTTGTGCCTTTTACGCACAA
ATAAATCGCCTCTCCAGCTTTCTTTTGGACCAGTCTATGCCAGCTTTATGTGACAGCTAC
TTCAAATATATAGCTTGCCACTTAGTTAATAGCCTTAGAATTTTGTTAACCAGATTCTAG
GATCAGGTACAGAGGCAGGGAGCCTTGAT

Sequence for *Ptf1a* enhancer, region 6:

GAAAGTTAGGAATGGGGAGGAAGGAAAAGACAAGAAGGGCCACTGAGACAGAGCAACTGG
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GACATTTCAGCTTTGAAGGGGAACAAAGGCCTGGAACTAAACTCAGAGAAAGCCAGGAGC
ATGGAGAAGCTAACAAAAGACTTTGAGCCTGTGGCCACTGGTAGGTGTCAATGTGGACAC
ATACAGACTGGGCATTAGGAGAGGGGAACACAGAGCCAGGCTGTCACTCAAGGCTTAACA
GGAACGAAGATAAAAACGTGACAGCCTCCACTTTCAATAGTGGTGAGACGCAAAGTGGAG
TGCTCCAGAGTCTGTGTGGGTGAAGTCTTGGGAAACGGGGCCTCCCCCT

Sequence for *Ptf1a* enhancer, region 7:

CAGACACATTCCCCCTATAAATTCTAGTAGTAAGACAAAATGCAATTTTTAAAAAAACAT
AATATTTCAAAATTCTGATTGTCTTTAATCAATGCAGGTCTCTCAAATAATTTTAAAAAG
GGGATGTAAAGATCCCTAAAAGACTAATGACATTTCACAGGTAATGCAATTTAGAAAGAG
ACACTTCCATGTTTTCTTTTCCAACAATGTGCATTTTTGTAAACTGCTATAAATTGCAAT
TCAAAAGCAGTGATCTCACCATCCCCAATCATTTTTTATACTGATCAAATGAATGCAGAT
ACTCCCATTTGGGAGGGGCAACATGACATTTCACTTGGCAGTTCTAAACAGGTCACAGAC
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ACCAATGGTTACCCCTTTTCAAAACCATTGGAATGGTTTGCACAATGCTATTCACATAGC
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CATATATGGTCCACATGCATTTTTGTTGGCACTGCATAGCATAGCTGTAACTGTCCATTT

GTATGCATATATAAGCACATGTATGTCTATGTATGTCTATTTGTTGGAAATGTTTCTTTG

TTTGAAATCATAGGCACAAAGACACAACATGGCTGCCCTGGAGATGACCTATTGCTTTCA

AGTTGCTTGAACACATCAGAATTTCTATTGTTAAGGTTGATTAATTGAAACTGATAAGTT

ACCATGGATAAAGATGGTTTCTCTGTTTAATGTGGTGGGCCTGATTTGTAAATACCTCAT

TCATGTAGCTTTGCAGGGCAGCATATGGGCAAGAGTCATTCAGTATCACAAACATACACA

GTTTATTATGCTCGTATGGGCACGACCAAAAGGCCTTTCTGTTTTAATGACAAAATTTCA

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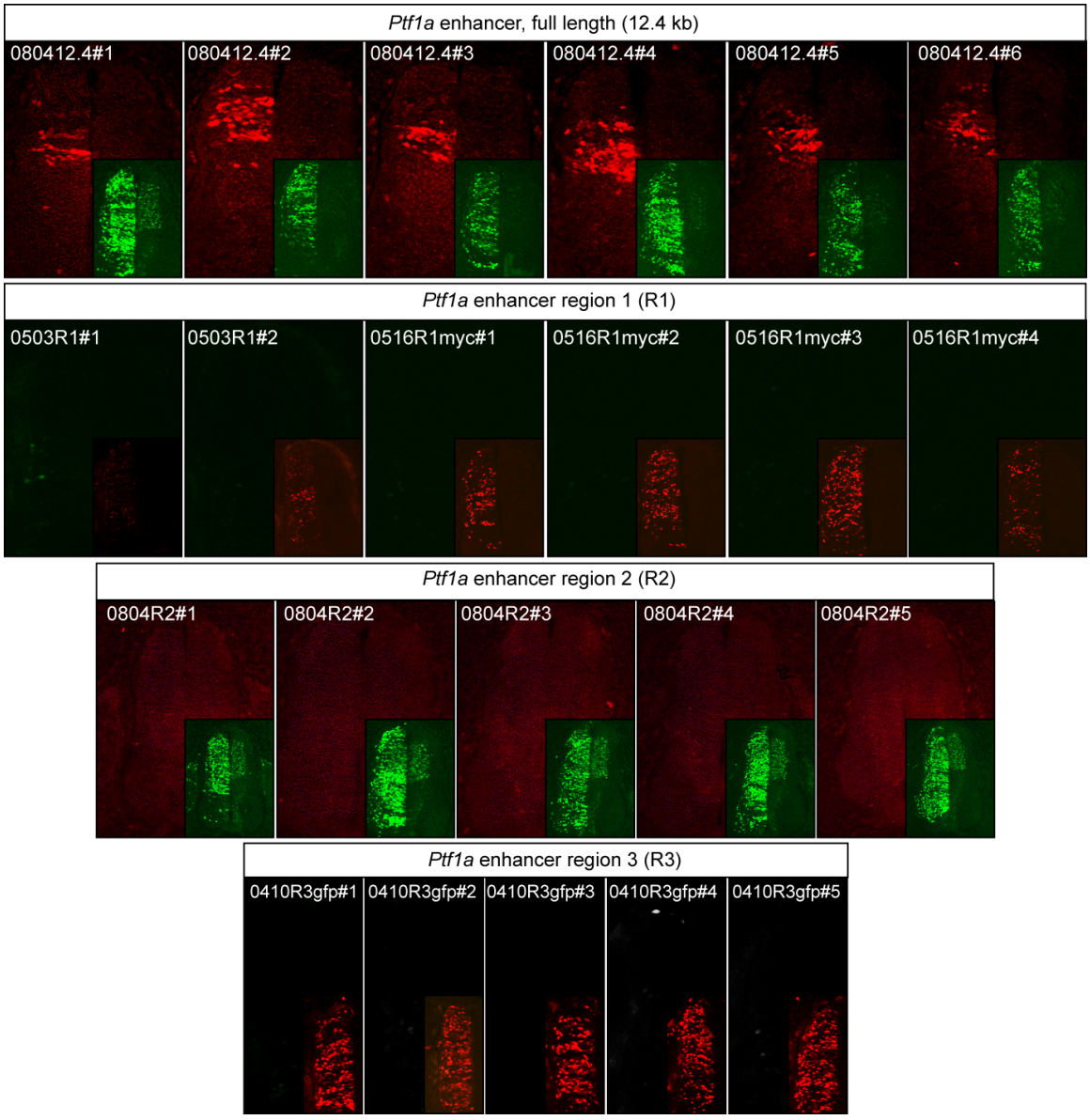
Appendix B


Ptf1a enhancer, full length (12.4 kb)
080412.4#1
080412.4#2
080412.4#3
080412.4#4
080412.4#5
080412.4#6
Ptf1a enhancer region 1 (R1)
0503R1#1
0503R1#2
0516R1myc#1
0516R1myc#2
0516R1myc#3
0516R1myc#4
Ptf1a enhancer region 2 (R2)
0804R2#1
0804R2#2
0804R2#3
0804R2#4
0804R2#5
Ptf1a enhancer region 3 (R3)
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0410R3gfp#3
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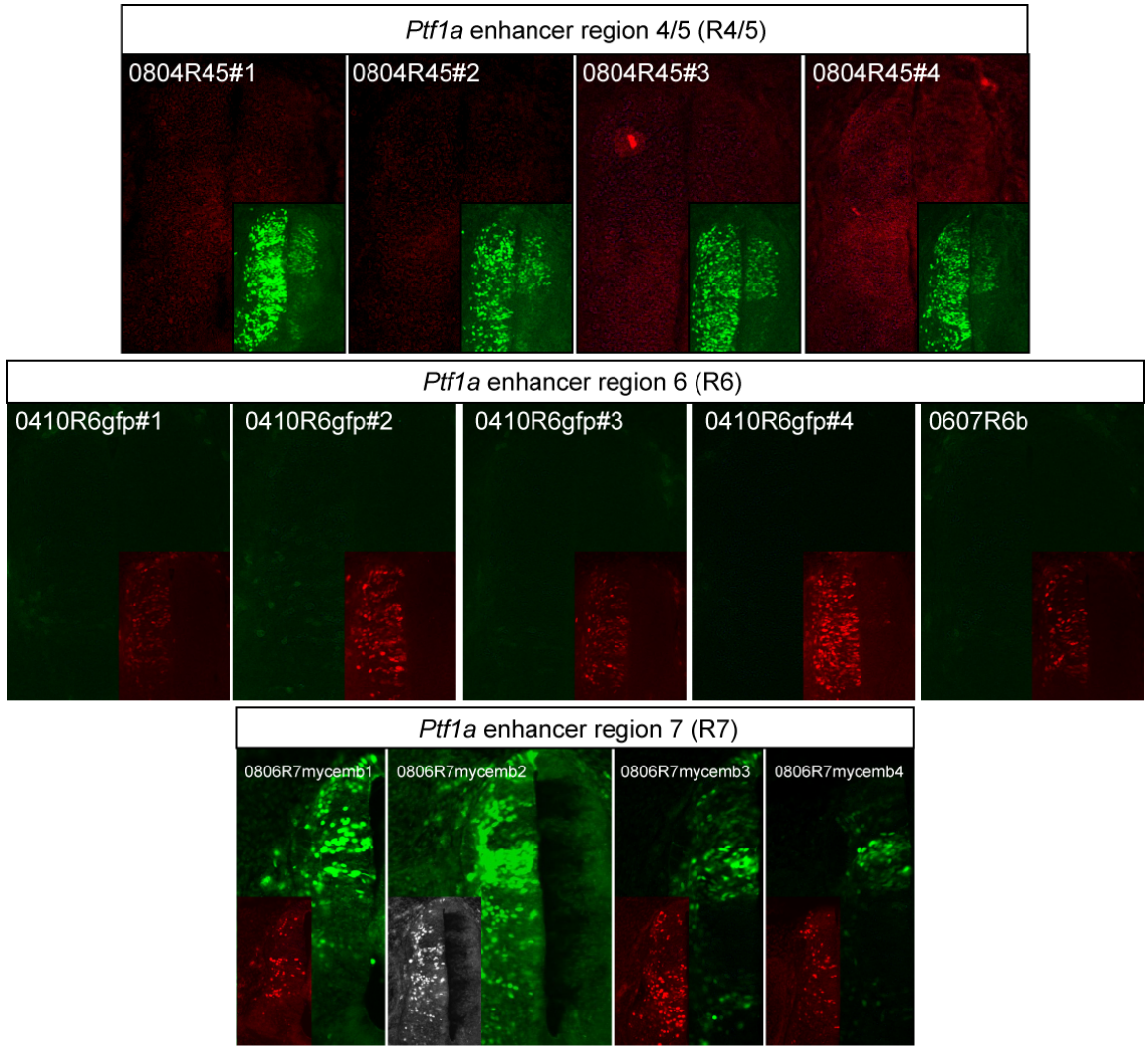
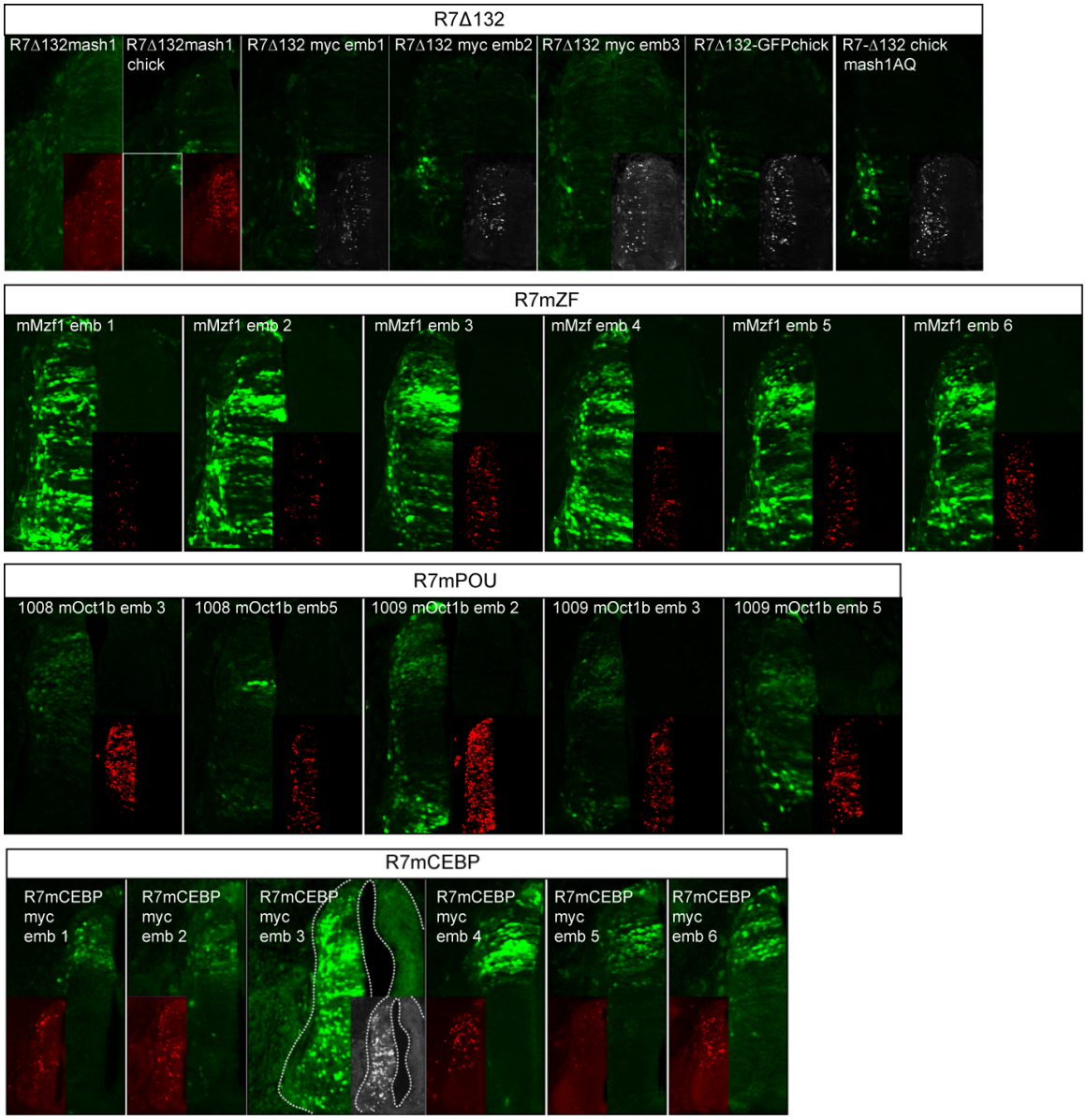


Composite of *Ptf1a* enhancer regions tested. Conserved regions of the 12.4 kb *Ptf1a* enhancer were cloned into *Bgn-mCherry* or *Bgn-GFP* expression vectors and tested in chick. Only one region, R7, drove expression of the reporter expression in the dorsal neural tube. Insets are electroporation controls, with which were electroporation efficiency determined using an inert myc-tagged expression vector.

Appendix C

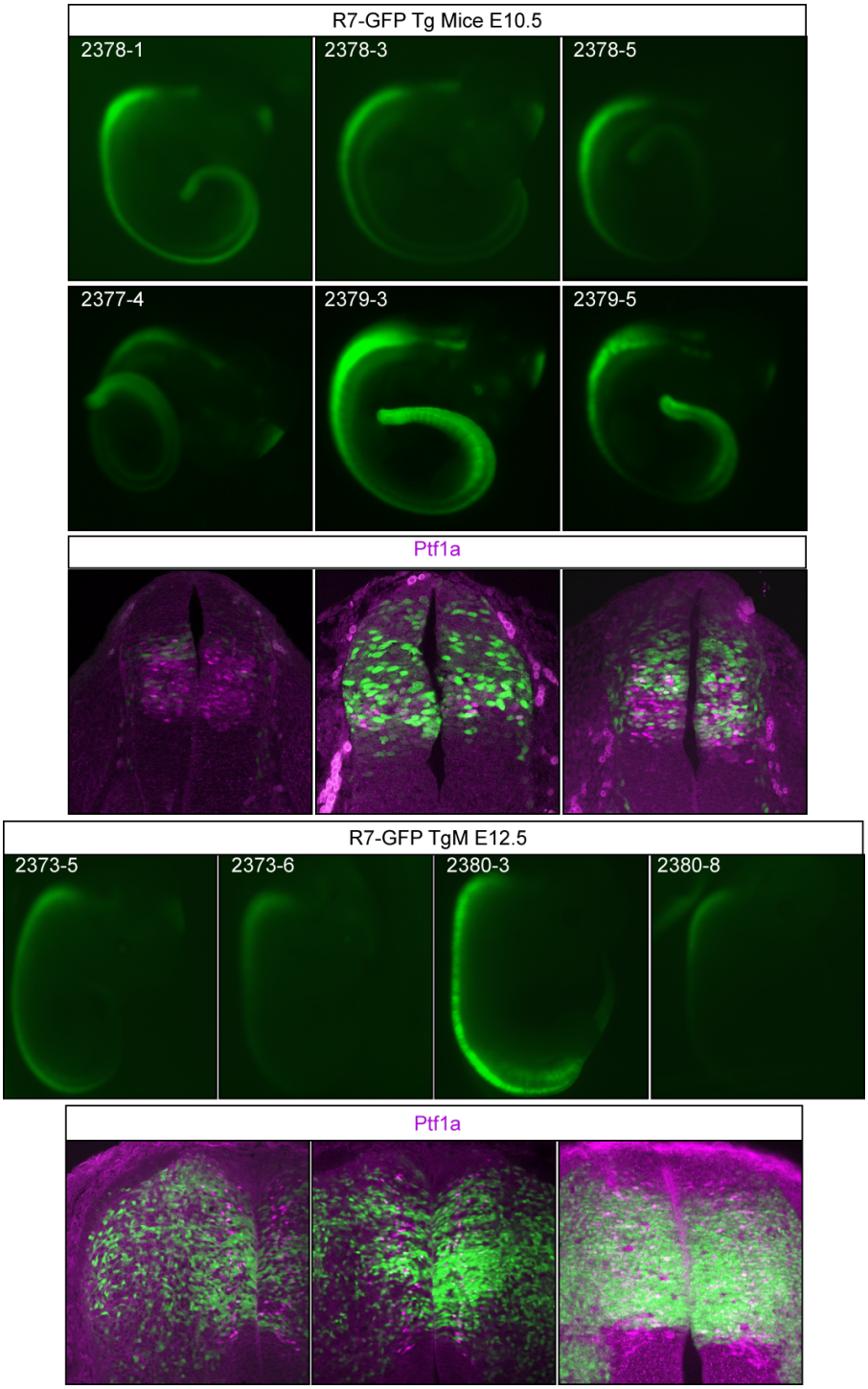
Ptf1a enhancer, region seven mutations composites



Composite images of the mutations to the predicted binding sites in the 132 bp ultraconserved sequence in R7 from Figure 3.1. Mutation of the predicted zinc finger motif (zinc finger motif 1) resulted in an expansion of R7 enhancer activity, whereas mutation of the putative POU motif revealed that the motif was required for robust enhancer activity. Mutation of a predicted overlapping CEBP/Sox motif did not greatly affect enhancer activity. Insets are electroporation controls, with which were electroporation efficiency determined using a control expression vector.

Appendix D

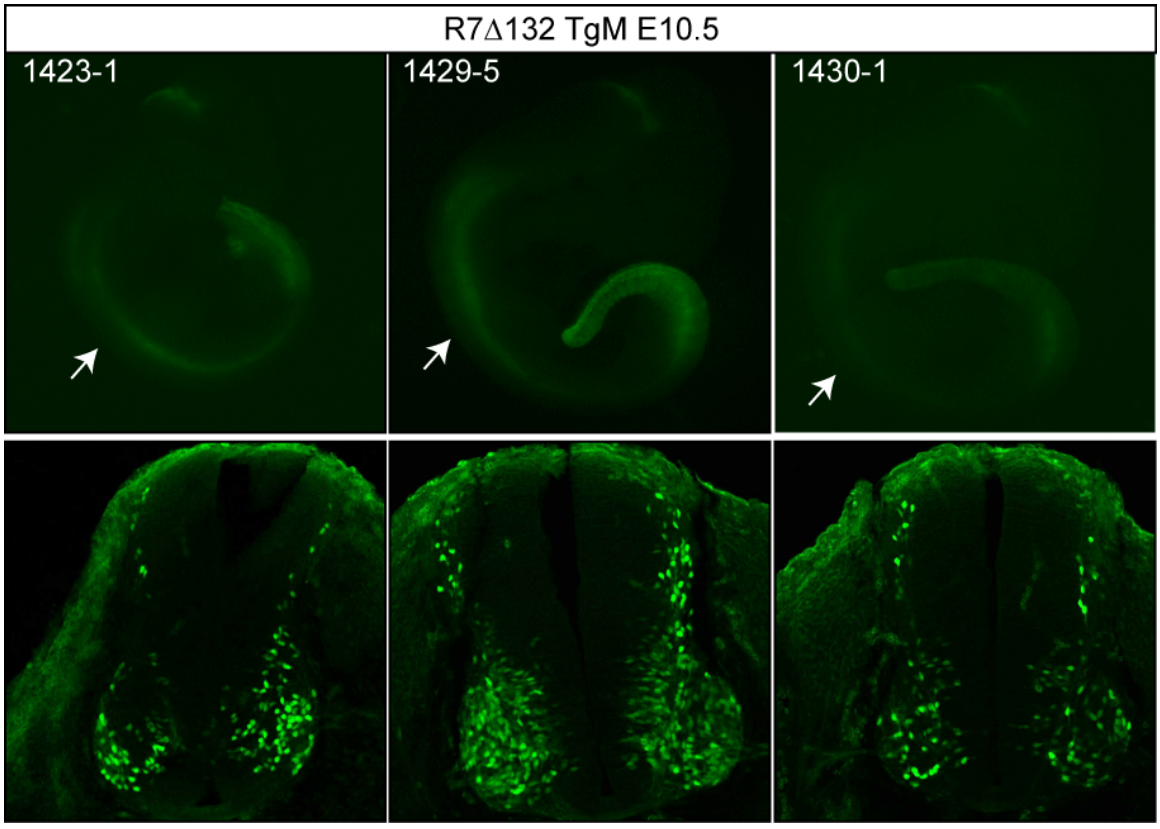
R7-GFP TgM composites



Composite images of *R7-GFP* mouse transgenic embryos analyzed from E10.5 (n=6) and E12.5 (n=4)

Appendix E

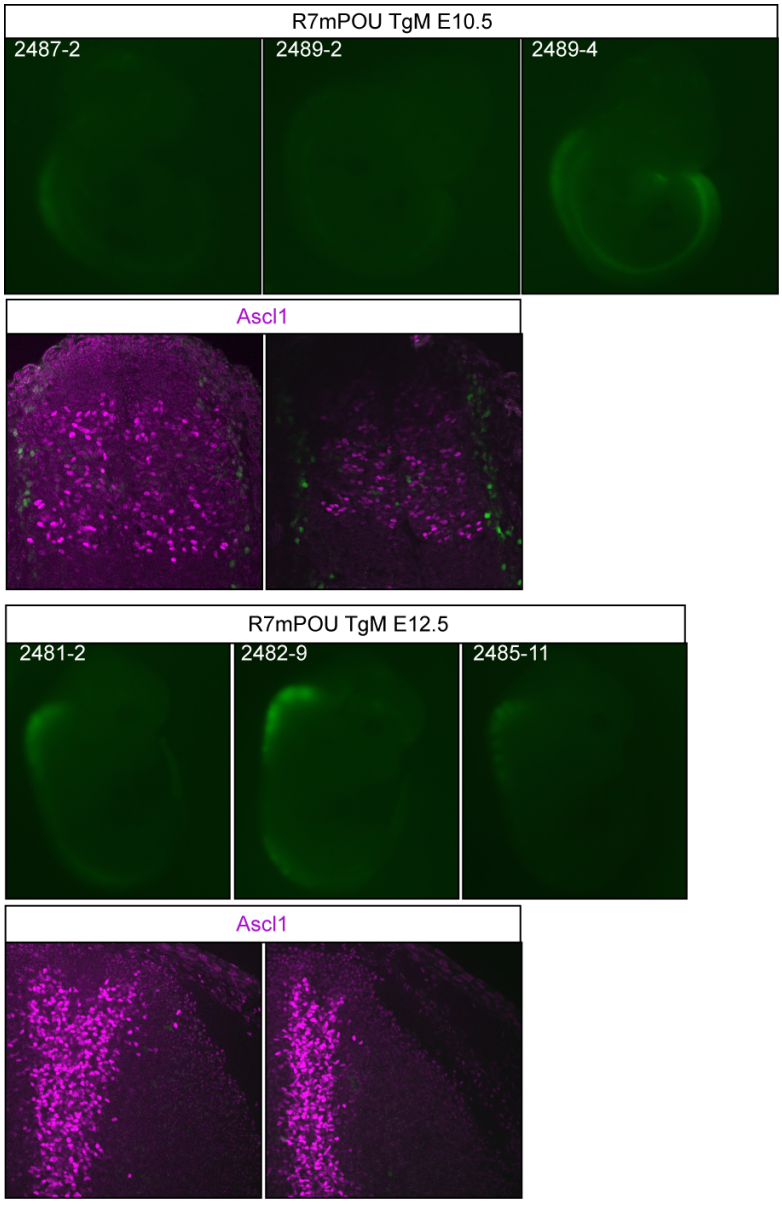
R7Δ132-GFP TgM composites



Composite images of *R7Δ132-GFP* mouse transgenic embryos at E10.5. Complete loss of reporter expression in the ventricular zone of the dorsal neural tube reveals that the ultraconserved 132 bp sequence is required for activity of R7 in the dorsal neural tube. n=3

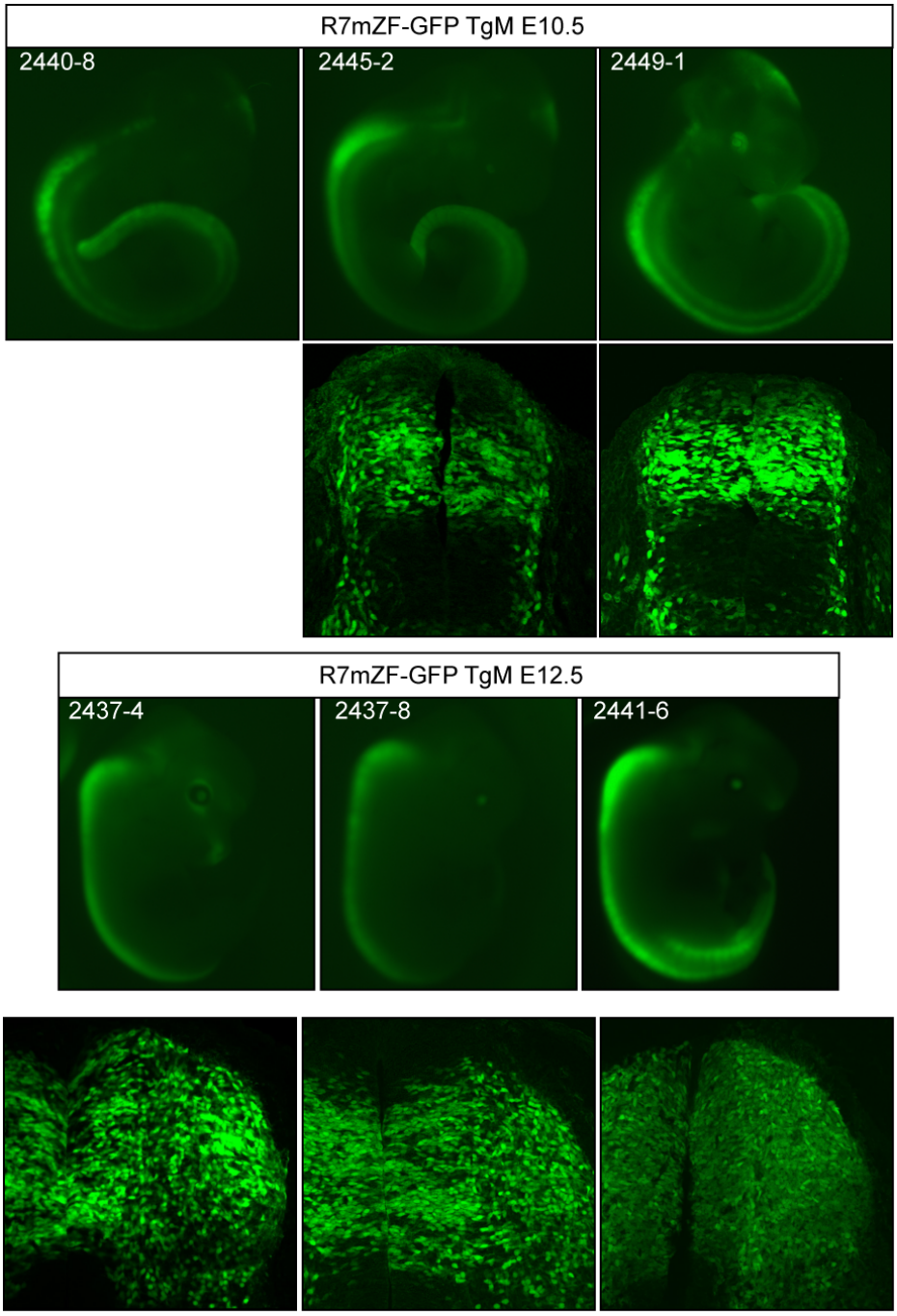
Appendix F

R7mPOU-GFP TgM composites



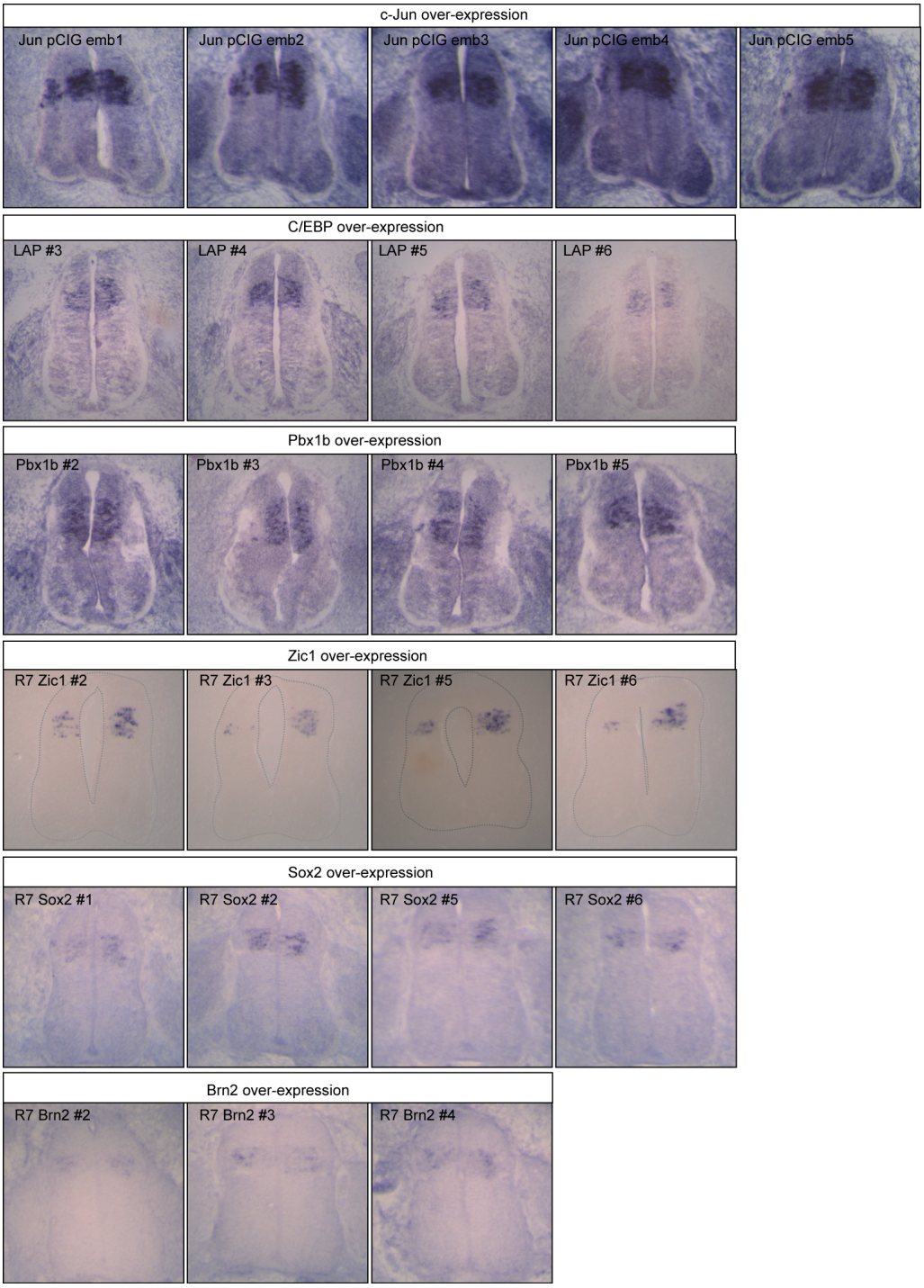
Composite images *R7mPOU-GFP* mouse transgenic embryos taken at E10.5 (n=3) and E12.5 (n=3) with selected sections immunostained with Anti-Ascl1 (1:5000-1:10000). Note that mutation of the putative POU motif results in severe reduction of enhancer activity.

Appendix G



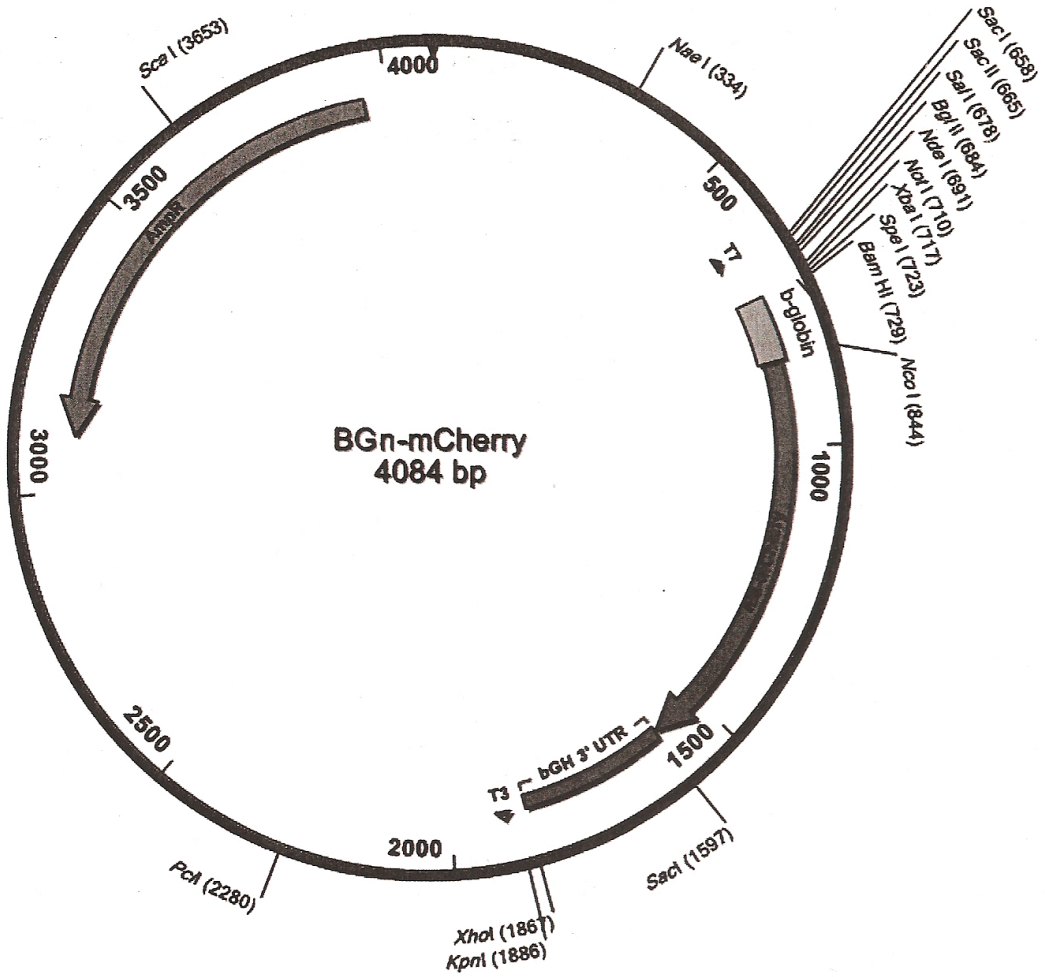
Composite images of *R7mZF-GFP* mouse transgenic embryos at E10.5 (n=3) and E12.5 (n=3) with sections of the neural tube showing native fluorescence. Patterning of R7 enhancer activity is not affected compared to *R7-GFP* embryos.

Appendix H



Composite images of candidate transcription factor overexpression. Electroporated hemisphere of the chick neural tube lies on the left, un-electroporated hemisphere lies on the right. These images show that only one candidate, Zic1, was able to modify expression of Ptf1a (compare left and right hemispheres of the neural tube). All other candidates did not cause a change in Ptf1a expression.

Appendix I



Description:

- Created by cutting N1-13 out of 1N-64 with SmaI/EcoRI (EcoRI end blunted)
- Unique sites for common enzymes shown
- Used for subcloning and chick injections

N.B. – Gives ventral expression pattern in chick.

Map of expression vector used in chick and mouse. All enhancer constructs made used this vector (or a variation where the mCherry reporter has been replaced with GFP, but all other regions are carried over). SalI and BamHI restriction cut sites were used to clone enhancer sequences into the expression vector.

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