

Identification of an Enhancer Critical for the *ephrin-A5* Gene Expression in the Posterior Region of the Mesencephalon

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Ephrin-A5 has been implicated in the regulation of brain morphogenesis and axon pathfinding. In this study, we used bacterial homologous recombination to express a LacZ reporter in various *ephrin-A5* BAC clones to identify elements that regulate *ephrin-A5* gene expression during mesencephalon development. We found that there is mesencephalon-specific enhancer activity localized to a specific +25.0 kb to +30.5 kb genomic region in the first intron of *ephrin-A5*. Further comparative genomic analysis indicated that two evolutionary conserved regions, ECR1 and ECR2, were present within this 5.5 kb region. Deletion of ECR1 from the enhancer resulted in disrupted mesencephalon-specific enhancer activity in transgenic embryos. We also found a consensus binding site for basic helix-loop-helix (bHLH) transcription factors (TFs) in a highly conserved region at the 3'-end of ECR1. We further demonstrated that specific deletion of the bHLH TF binding site abrogated the mesencephalon-specific enhancer activity in transgenic embryos. Finally, both electrophoretic mobility shift assay and luciferase-based transactivation assay revealed that the transcription factor *Ascl1* bound the bHLH consensus binding site in the mesencephalon-specific *ephrin-A5* enhancer *in vitro*. Together, these results suggest that the bHLH TF binding site in ECR1 is involved in the positive regulation of *ephrin-A5* gene expression during the development of the mesencephalon.

Keywords: bHLH transcription factor, EphA, ephrin-A5, mesencephalon

INTRODUCTION

Ephrin-A5 has a broad range of biological effects during embryonic brain development due to the fact it binds members of both the EphA and EphB receptor families (Kullander and Klein, 2002; Pasquale, 2005). *Ephrin-A5* is highly expressed during brain-wiring, particularly in the retina and superior colliculus. Consequently, it has been demonstrated that ephrin-A5 has a crucial role in forming the retinocollicular topographic map. For example, the retinal axons from the nasal region (containing high levels of ephrin-A5) are repelled by high levels of EphAs in the anterior region of the superior colliculus due to repulsive interactions. This leads to their termination zones mapping to the posterior part of the superior colliculus (containing low EphA expression) (Flanagan, 2006; Flanagan and Vanderhaeghen, 1998; O'Leary and Wilkinson, 1999). In addition, it was shown that neural tube closure defects are observed in *ephrin-A5*-null mutant embryos (approximately 30% penetrance) (Holmberg et al., 2000). It has been hypothesized that the regulation of cell adhesion may be impaired in the midline of the developing brain and this was a potential cause for the neural tube closure defects observed in *ephrin-A5* knockout embryos. Importantly, it was also shown that ectopic expression of ephrin-A5 in cortical progenitors expressing EphA7 resulted in increasing apoptosis, reducing cortical size (Depaepe et al., 2005). It was further demonstrated that brain region-specific apoptosis could occur in regions where EphAs cluster with

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neighboring ephrin-As through cell to cell contact (Noh et al., 2016; Park, 2013; Park et al., 2013). This suggests that Eph receptors and ephrin ligands have a role in the morphogenesis of the developing brain.

It appears that gradients in the expression of Eph receptors and ephrin ligands are key regulators of brain morphogenesis and wiring (Coulthard et al., 2002; Klein, 2012). However, understanding how these gradients of expression are regulated at the gene level during brain development has received little attention, except for the development of the neural retina. The opposing gradients of expression shown by ephrin-As and EphAs along the naso-temporal axis of the neural retina are regulated by chick brain factors 1 (CBF1, an orthologue of forkhead box G1 (FOXG1) in mice) and 2 (CBF2, an orthologue of FOXD1 in mice), respectively. CBF1 is a nasal-specific winged-helix transcription factor. Altered expression of CBF1 in the temporal retina was shown to repress the expression of CBF2 and EphA3, whereas it promoted the expression of ephrin-A2 and ephrin-A5 (Polleux et al., 2007; Takahashi et al., 2003). In contrast, CBF2/FOXD1 is expressed in the temporal retina and the knocking-out of FOXD1 was found to increase the expression of ephrin-A5 (Carreres et al., 2011). The opposing gradients of expression for ephrin-Bs and EphBs along the dorsal-ventral axis of the neural retina are regulated by T-box transcription factor 5 (TBX5) and ventral anterior homeobox 2 (VAX2), respectively. In the dorsal retina, TBX5 was shown to repress the expression of VAX2 and was sufficient to induce the expression of ephrin-B1 and ephrin-B2 (Koshiba-Takeuchi et al., 2000). Meanwhile, VAX2 was shown to regulate the expression of EphB2 and EphB3 in the ventral retina (Mui et al., 2002; Schulte et al., 1999). Overall, these findings suggest that the combination of various expression patterns of transcription factors along the naso-temporal and dorso-ventral axis of the retina determines the graded expression patterns of ephrin ligands and Eph receptors. These processes and patterns underlie embryo morphogenesis and brain wiring. However, many questions remain unaddressed. In particular, what are the transcriptional mechanisms that determine the gradients of expression for ephrin ligands and Eph receptors along the developing axis of the midbrain?

In this study, we determined that a bHLH transcription factor binding site in the 800 bp mesencephalon-specific *ephrin-A5* enhancer plays a key role in regulating the graded expression pattern of *ephrin-A5* in the posterior region of the mesencephalon. This finding is an important step towards understanding the transcriptional mechanisms that underlie ephrinA-mediated midbrain development.

MATERIALS AND METHODS

Targeting vectors for BAC recombination

Homologous arms for modifying 4O15, 211L12, 375B7, and 23O22 BACs were amplified via PCR by using the following primer sets. For the A arm, forward 5'-GGTGATGTCTCCCGCAG-3' and reverse 5'-GGAGAGATCGGGGATCCAG-3' and for the B arm, forward 5'-TGCTCTTCTGGTGCTCT-3' and reverse 5'-CTTCCACTCACCCATCCCT-3'. For PCR am-

plification of the B arm to generate the 385B7del30.4 BAC, the primers were forward 5'-TAGCACTCTCATCTGTCTCTCGG-3' and reverse 5'-GGCACTTGTGGTCACAGAAATAG-3'. Homologous A and B arms were sub-cloned into a reporter vector containing a LacZ reporter gene and a SV40 poly (A) signal on a pGEM11Z vector backbone (Promega). Homologous A and B arms were cloned into the *SfiI/SacI* and *NotI/NsiI* site of the LacZ-SV40 poly (A)-FRT-Kana-FRT-pGEM11Z vector, respectively. Targeting vectors were digested with *SfiI/NsiI* and then recombined with the BAC using a bacterial homologous recombination method described previously (Kim et al., 2007).

C1, C2, and C3 fragments were synthesized via PCR by using the following primer sets. For C1 5'-TAAAGCACTGAAAAGTAGACCCAAG-3' and 5'-ATTTACTTCAGTGTGAAGCCA CTGT-3', for C2 5'-GTTTTAAGGCTGCAGTAATCACTGT-3' and 5'-TCTGAAAATTCTACAAAGTGCCCTA-3', and for C3 (5'-ATCCATATCCTCTTACCTCAGGA-3' and 5'-CCGAGA GACAGATGAGAGTGCTA-3'). Each amplifying fragment was cloned into the *SpeI/NotI* site of a pGEM11Z plasmid containing the ephrin-A5 basal promoter and LacZ.

For C3delECR1, a 240 bp fragment was amplified with primers 5'-GGTAACTCTTGCTTGGGCATGTG-3' and 5'-GCATTCTGCCTTCCAAGAGGCAACACACAGTGG-3', and a 320 bp fragment was amplified with primers 5'-CTGTGTGTTGCCTTGGGAAGGCAGAATGCTGA-3' and 5'-GCAGCAGC CAACAGAATTGCACCC-3'. The two partially complementary PCR fragments that were generated were annealed and used as the templates in a second PCR with primers 5'-GGTAACTCTTGCTTGGGCATGTG-3' and 5'-GCAGCAGCC AACAGAATTGCACCC-3'. The resulting 540 bp product was digested with *SpeI/NcoI* and sub-cloned into the corresponding region of the C3 vector. Similar procedures were used to generate other constructs. For C3delECR2, primers for the first PCR were 5'-ACTTAAAATGGCTGGGATGACCAGTTA-3' and 5'-CTAAAGCCTCAACGCCACATGGCCCTCACTACT-3', primers for second PCR were 5'-ACTTAAAATGGCTGGGAT GACCAGTTA-3' and 5'-CCGAGAGACCAGATGAGAGTGCTA-3'. For C3-bHLH, primers for first PCR were 5'-GGTAACTCTTGCTTGGGCATGTG-3' and 5'-CACTGAAAGATCACCG TTTATGAAGAAGGC-3', and primers for second PCR were 5'-GGTAACTCTTGCTTGGGCATGTG-3' and 5'-GCAGCAG CCAACAGAATTGCACCC-3'. For C3-hox, primers for first PCR were 5'-GGTAACTCTTGCTTGGGCATGTG-3' and 5'-CCCCTAAGGGACTGAATACCACTGAAAGAT-3', and primers for second PCR were 5'-GGTAACTCTTGCTTGGGCATGTG-3' and 5'-GCAGCAGCCAACAGAATTGCACCC-3'.

Generation of transgenic mice and genotyping

BAC DNA was purified using a Qiagen Large-Construct kit (Qiagen). Recombinant BACs for microinjection were diluted in injection buffer and then injected into 200 fertilized oocyte pronuclei from C57BL/6 mice, as described previously (Kim et al., 2007). Each BAC transgenic embryo was identified by PCR genotyping, using the primers 5'-GTTACAATAA AGCAATAGCATCACA-3' and 5'-GGTTGCTGCTGTCCAGT AGAC-3'. Transgenic embryos carrying plasmid vector were identified via PCR genotyping by using the following primer sets. For C1, 5'-GTTACAATAAAGCAATAGCATCACA-3' and

5'-AATACCTGGCCCATAGTAGACACTTTA-3', for C2, 5'-GTTACAATAAAGCAATAGCATCACA-3' and 5'-ATTTACTTCAGTGTGAAGCCACTGT-3', and for C3, 5'-GTTACAATAAAGCAATAGCATCACA-3' and 5'-ATTTTATTGTGTACTGAAGAGATGGG-3'.

Whole mount X-gal staining of transgenic embryos

For X-gal staining, embryos were dissected in phosphate buffered saline (PBS), fixed in 0.2% glutaraldehyde for 10 min, and finally washed and stained using procedures described previously (Park et al., 2013).

Electrophoretic mobility shift assay (EMSA)

Proteins for DNA binding assays were produced using a T7 TNT-coupled *in vitro* transcription-translation kit (Promega). For preparation of double-stranded oligonucleotides, oligonucleotides were mixed in a buffer containing 0.1 M KH₂PO₄ (pH 8.0) and 2 M KCl, incubated at >90°C for 10 min, and cooled. Double-stranded DNAs were ³²P-end labeled with Klenow enzyme and added to the DNA binding mixture (with 75 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 10 mM Tris-HCl (pH 7.5), 6% glycerol, 2 µg of bovine serum

albumin, 16 ng of poly(dI-dC), 0.1 µg of salmon sperm DNA, and the translation product). The DNA binding reaction mixtures were incubated at room temperature for 20 min. These were then separated on a 5% polyacrylamide gel in 0.25X Tris-borate-EDTA buffer. The gel was fixed and dried at room temperature overnight prior to exposure.

Luciferase assay

HEK293 cells were cultured in Dulbecco's modified Eagle's medium, supplemented with L-glutamine, 10% fetal bovine serum, and penicillin/streptomycin. A total of 0.9 µg of DNA containing 0.4 µg of luciferase reporter construct and 0.5 µg of expression construct (pcDNA-AscI1-tether-E47 was gift by Dr. Guillemot) was transfected using Lipofectamine (Invitrogen). A co-transfected Renilla luciferase reporter (18 ng per transfection) was used to normalize transfection efficiency. For generating eA5-E₆, eA5-Em₆, and Dll1-E₆ constructs, each concatemeric oligonucleotide containing six tandem repeats of E box (indicated in Fig. 4D) was sub-cloned into a pTAL-Luc vector (Promega). Cells were lysed 24 h after transfection, and the luciferase activity of each extract was assayed using a Dual Luciferase Assay Kit (Promega).

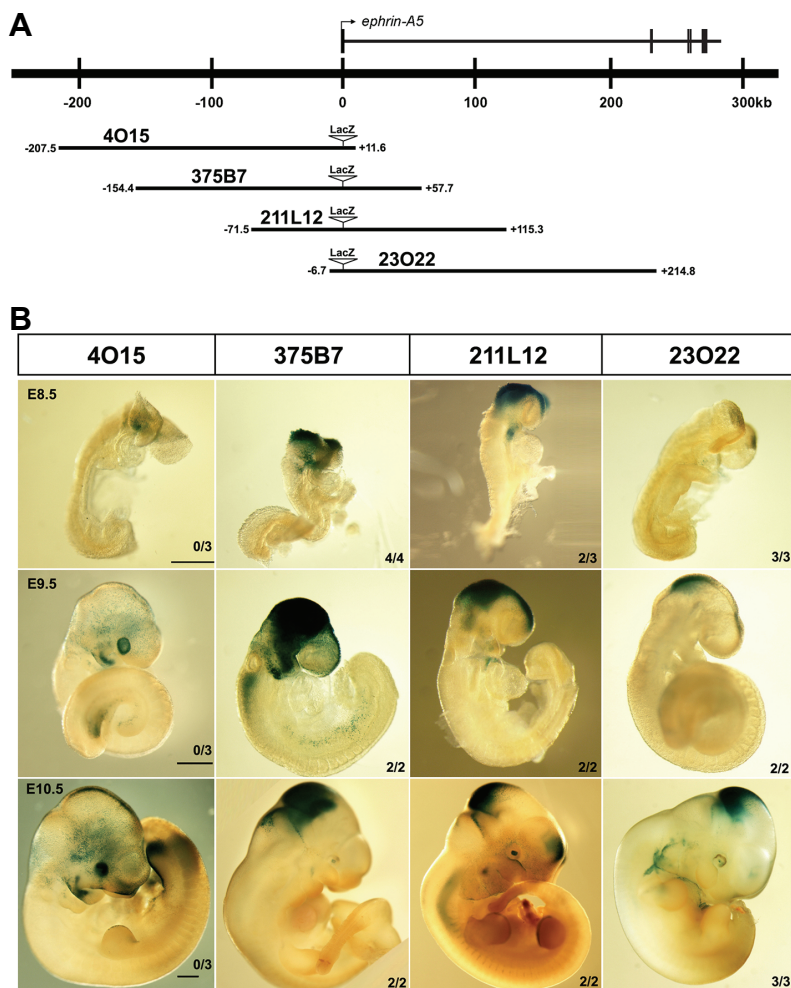


Fig. 1. Mapping the mesencephalon-specific ephrin-A5 enhancer using various recombinant BACs. (A) A physical map displaying *ephrin-A5* BAC clones. Overlapping BACs contain the first exon of *ephrin-A5*. BACs were targeted with the same targeting vector (LacZ) to generate recombinant BACs for transgenic lines. (B) Expression analysis of LacZ in representative BAC transgenic embryos, from E8.5 to E10.5. The number of embryos exhibiting the mesencephalon-specific reporter activity relative to the total number of transgenic embryos is indicated in each panel. The transgenic embryos carrying 375B7, 211L12, and 23022 BACs reveal LacZ expression in the mesencephalon. Scale bar, 500 µm.

RESULTS

The mesencephalon-specific enhancer of *ephrin-A5* maps to a 46.1 kb genomic region within the first intron

The mouse *ephrin-A5* gene spans approximately 277 kb and includes five exons. Homozygous *ephrin-A5* null mutant mice display several neural developmental defect phenotypes, including incomplete neural tube closure (Holmberg et al., 2000). Neural tube closure defects are most severe in the posterior region of the mesencephalon where *ephrin-A5* is abundantly expressed. To identify cis-acting elements responsible for *ephrin-A5* gene expression in the mesencephalon, we selected four different mouse BAC clones that spanned a genomic region -207.5 kb to +214.8 kb from the *ephrin-A5* transcription start site (TSS) (Fig. 1A). A common feature of these BACs was that they all contained the first exon of *ephrin-A5*. To efficiently modify these BAC clones, the same targeting vector was used to induce bacterial homologous recombination, as described previously. This vector contains the 0.4 kb *ephrin-A5* basal promoter (A arm), a LacZ reporter, a kanamycin selection marker, and 0.7 kb of the B arm. Bacterial homologous recombination was successfully performed by transforming the targeting vector into

each BAC clone. The resulting recombinant BACs were intensively examined via Southern analysis to confirm that the targeting vector was correctly inserted into the region encompassing the first exon of each BAC. Each recombinant BAC was injected into fertilized C57BL/6 zygotes. Embryos were then isolated from their surrogate mothers and genotyped by PCR. Whole mount X-gal staining of transgenic embryos at various developmental stages revealed that LacZ expression was detectable in the mesencephalon as early as E8.5, when the neural tube had not yet closed (Fig. 1B, top panels). In addition, LacZ expression in the mesencephalon was relatively high until E10.5 (Fig. 1B, second and third panels from the top). This developmental LacZ expression pattern was consistent with previous reports examining the developmental dynamics of *ephrin-A5*, suggesting that our BAC clones recapitulate the endogenous expression of *ephrin-A5*. Importantly, three BAC clones, including 375B7, 211L12, and 23O22, showed a LacZ temporal and spatial regulatory expression pattern that was consistent with the mesencephalon (Fig. 1B, second, third, and fourth panels from the left). However, the 4O15 BAC did not show any LacZ expression in the mesencephalon (Fig. 1B, first panel from the left). Taken together, our findings suggest that the

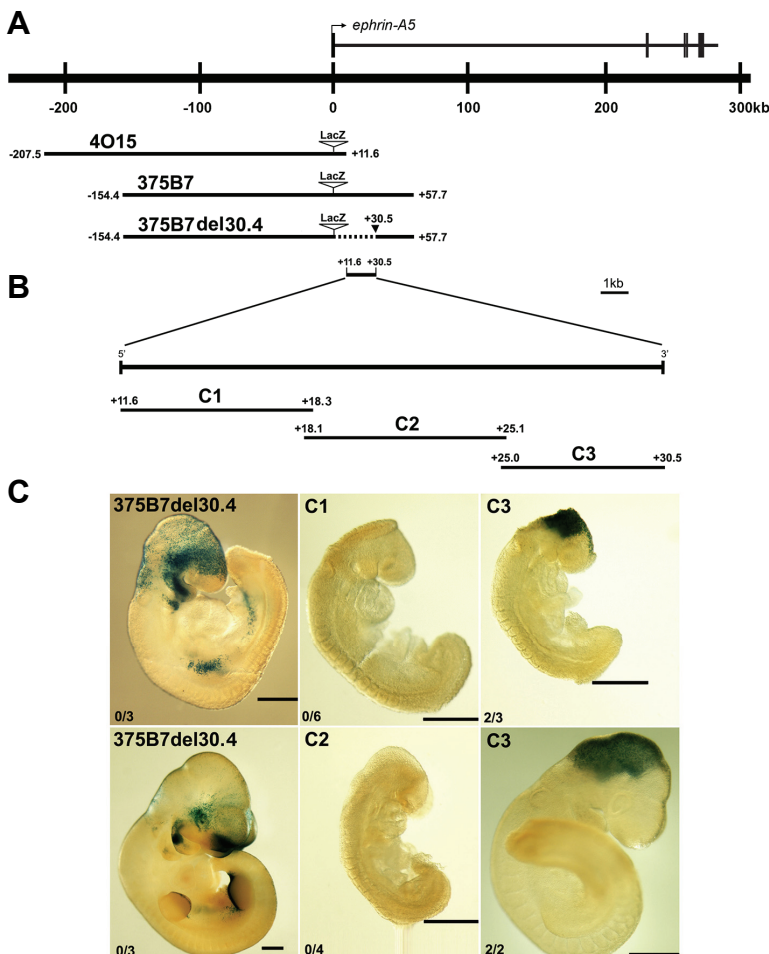


Fig. 2. A 5.5-kb enhancer region directs the expression of LacZ in the mesencephalon. (A) Schematic of BAC clones 4O15, 375B7, and 375B7del30.4. The dashed line in 375B7del30.4 BAC represents a genomic portion deleted by BAC recombination. (B) Schematic of C1 (from +11.6 kb to 18.3 kb), C2 (from +18.1 kb to 25.1 kb), and C3 (from +25 kb to 30.5 kb) fragments amplified via PCR. (C) Analysis of LacZ expression using transgenic embryos. Transgenic embryos carrying 375B7del30.4, C1, and C2 do not show robust LacZ expression in the posterior part of the mesencephalon (first panel, E9.5; second panel, E8.5; fourth panel, E10.5; fifth panel, E8.5). Only C3 transgenic embryos show LacZ expression in the mesencephalon (third panel, E8.5; sixth panel, E9.5). Scale bar, 500 μ m.

mesencephalon-specific enhancer of *ephrin-A5* resided in a genomic region that is +11.6 kb to +57.7 kb from the TSS and is within the first intron (Fig. 1A).

Identification of a 5.5 kb intronic DNA region as a potential enhancer for mesencephalon-specific *ephrin-A5* expression

To further restrict the possible locations of the mesencephalon-specific enhancer within the first intron, the 375B7 BAC was modified using a different targeting vector. This vector was identical to the targeting vector described for Fig. 1, except the 1.1 kb B arm consisted of the region +30.5 kb to +31.6 kb from the TSS. The resulting BAC (375B7del30.4) was verified based on the deletion of the genomic region spanning +0.1 kb to +30.5 kb (Fig. 2A). Importantly, transgenic embryos carrying this recombinant BAC did not show significant LacZ expression in the mesencephalon (Fig. 2C, first panel from the left). This suggested that the deleted 30.4 kb region included the mesencephalon-specific enhancer of *ephrin-A5*. As the 4O15 BAC did not show enhancer activity in transgenic embryos, it was concluded that

the enhancer most likely resides in a smaller 18.9 kb region spanning +11.6 kb to +30.5 kb from the TSS (Fig. 2A). This 18.9 kb genomic region was further divided into three regions, designated C1, C2, and C3 (Fig. 2B). Each genomic fragment was amplified by PCR, sub-cloned, and then intensively analyzed by DNA sequencing. Validated genomic DNA was selected and inserted into a site immediately upstream of a 0.4 kb *ephrin-A5* basal promoter, followed by LacZ in a plasmid vector. Transgenic embryos carrying the C1 and C2 genomic DNA did not show LacZ expression in the mesencephalon (Fig. 2C, second panel from the left). However, C3 transgenic embryos had a consistent expression pattern of LacZ in the mesencephalon (Fig. 2C, third panel from the left). This suggests that the potential enhancer for *ephrin-A5* expression in the mesencephalon was in a 5.5 kb genomic region from +25 kb to +30.5 kb of the TSS.

A binding site for bHLH transcription factors is critical for the mesencephalon-specific enhancer activity of *ephrin-A5*

To search for the mesencephalon-specific *ephrin-A5* enhancer within the 5.5 kb genomic region, we used a comparative

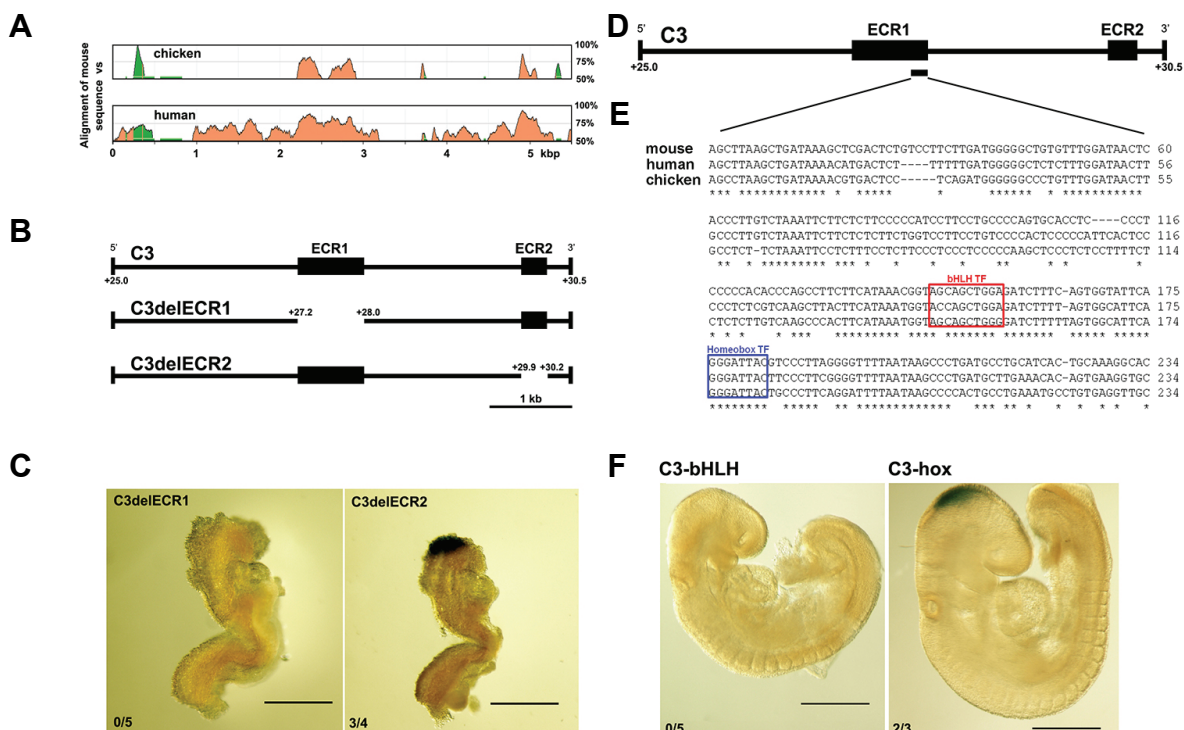


Fig. 3. A bHLH transcription factor binding site is critical for the *ephrin-A5* enhancer activity. (A) Pair-wise alignment of mouse *ephrin-A5* sequence with chicken and human orthologues using the ECR browser (<http://ecrbrowser.dcode.org/>). (B) Schematic of the C3, C3delECR1, and C3delECR2 constructs. ECR1 and 2 (highlighted) were defined as genomic regions with >40% similarity over a minimum distance of 100 bp. (C) Analysis of LacZ expression for transgenic embryos carrying the indicated C3 ECR1- or ECR2-deleted constructs. Transgenic embryos carrying C3delECR1 did not show any LacZ expression in the mesencephalon at E8.5. (D, E) The 3'-234 bp sequence of the mouse *ephrin-A5* ECR1 aligned with the corresponding human and chicken *ephrin-A5* genomic DNA sequences using a multiple sequence alignment tool (Clustal Omega: <http://www.ebi.ac.uk/Tools/msa/clustalo/>). bHLH and homeobox transcription factor binding sites are indicated using red and blue boxes, respectively. (F) Analysis of LacZ expression for transgenic embryos carrying the indicated C3 constructs lacking either the bHLH or homeobox transcription factor binding site. Transgenic embryos carrying C3-bHLH show no LacZ expression in the mesencephalon at E9.0. Scale bar, 500 μ m.

genomic analysis between mice, chick, and humans to identify two evolutionary conserved regions (ECR1 and ECR2) (>40% identity over 100 bp, ECR browser: <http://ecrbrowser.dcode.org/>). These regions are highly conserved in both chick and human lineages, despite these being distantly related organisms (Fig. 3A). However, these ECRs were not found in teleosts. To determine whether these ECRs contain the *ephrin-A5* enhancer, the C3 genomic DNA template was modified with PCR to delete either ECR1 or ECR2 (Fig. 3B). We found that transgenic embryos carrying the ECR1-deletion construct (C3delECR1) did not exhibit any LacZ expression in the mesencephalon. However, transgenic embryos with the ECR2 deletion (C3delECR2) still showed prominent expression of LacZ in the mesencephalon (Fig. 3C). This suggests that the 0.8 kb ECR1 contains an enhancer sequence that is critical for the regulation of *ephrin-A5* in the mesencephalon. We further focused on a 234 bp region

located in a 3' region of ECR1 that contained a consensus sequence for binding basic helix-loop-helix (bHLH) (CANNTG) or homeobox (TAAT) transcription factors (TFs) (Fig. 3D and 3E). C3 genomic DNA was further modified to delete the bHLH binding site (C3-bHLH) or homeobox TF binding sites (C3-hox). Transgenic embryos carrying the C3-hox DNA still had high LacZ expression in the mesencephalon (Fig. 3F, right panel). However, transgenic embryos carrying the C3-bHLH DNA with the bHLH consensus deletion did not show any LacZ expression in the mesencephalon (Fig. 3F, left panel). In addition, we found that the activity of a luciferase reporter in HEK293 cells co-transfected with ECR1 and Ascl1 was increased by approximately three-fold (data not shown). This strongly suggests that the bHLH TF binding site is a critical part of the mesencephalon-specific *ephrin-A5* enhancer.

It has been reported that Ascl1, a bHLH TF, is expressed in the mesencephalon. Additionally, mice with an *Ascl1* null

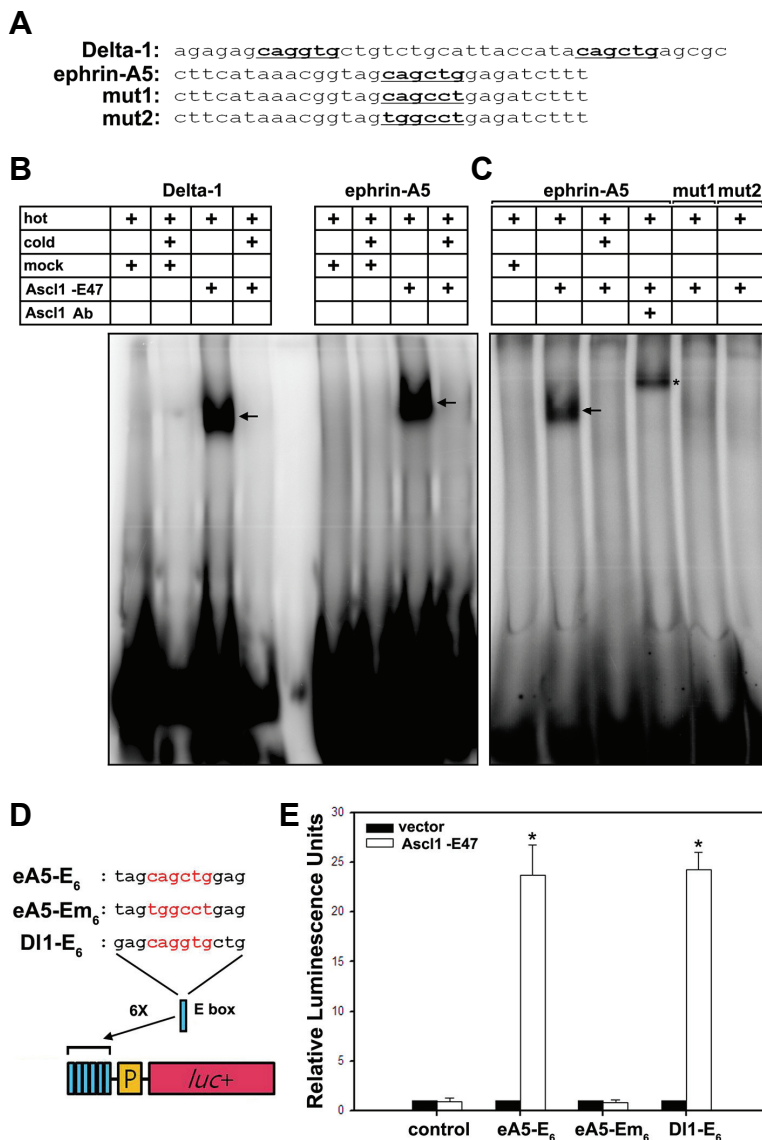


Fig. 4. The bHLH transcription factor Ascl1 directly binds to the mesencephalon-specific ephrin-A5 enhancer. (A) DNA sequences of the oligonucleotides used for EMSA. bHLH transcription factor binding sites are underlined in bold. (B and C) Electrophoretic mobility shift assay demonstrating the binding of Ascl1-E47 to the *ephrin-A5* enhancer containing a CAGCTG motif. The binding specificities of proteins were tested using cold probes, specific antibodies, or mutated versions of the *ephrin-A5* enhancer as indicated (+) above each lane. Representative results presented in the figure are from at least three independent experiments. (D) Schematic showing the luciferase reporter constructs containing a concatemer of six E box tandem repeats. eA5-E₆ represents a long concatemer consisting of tandem repeats of the indicated 12 bp sequence (top line). eA5, *ephrin-A5*; DI1, *Delta1*. (E) Luciferase activity assay showing that Ascl1-E47 fusion protein induces transcriptional activation through the *ephrin-A5* E-box DNA. Data represent means ± S.E. *P < 0.001, Student's *t*-test.

mutation have a decreased mesencephalon (Casarosa et al., 1999). We therefore investigated whether *Ascl1* is able to bind to the putative bHLH TF binding site we identified in ECR1. For this experiment, *Ascl1*-E47 fusion protein was *in vitro*-translated and then mixed with double strand (DS) oligonucleotides that were end-labeled with ³²P. These were then examined using an EMSA. As *Delta1* is one of the target genes for *Ascl1* (Castro et al., 2006), a 40-mer *Delta1* ds DNA oligonucleotide containing two *Ascl1* binding sites was used as a positive control for the EMSA (Fig. 4A, top line). In addition, 30-mer *ephrin-A5* ds DNA oligonucleotides containing the putative bHLH TF binding site (Fig. 4A, second line) and two mutant *ephrin-A5* oligonucleotides (mut1 and mut2) (Fig. 4A, third and fourth lines) were also examined by EMSA to test whether the core sequence is indeed critical for *Ascl1* binding. The mixtures containing protein/DNA complexes were analyzed via EMSA on a non-denaturing polyacrylamide gel (Fig. 4B). As expected, incubation with *Ascl1*-E47 gave rise to a slower migrating product with both *Delta1* and *ephrin-A5* DS DNA oligonucleotides (Fig. 4B, lanes 3 and 7). This was eliminated by cold-competitor oligonucleotides (Fig. 4B, lanes 4 and 8). In addition, when *Ascl1* antibody was mixed with pre-formed *Ascl1*-E47/DNA complexes, a super-shifted band with much slower mobility was observed (Fig. 4C, lanes 2 and 4). However, both mut1 and mut2 *ephrin-A5* DNAs failed to produce slow migrating products (lanes 5 and 6), demonstrating that *Ascl1*-E47 protein was bound specifically to the bHLH TF consensus sequence we identified in the *ephrin-A5* DNA.

To further assess whether *Ascl1*-E47 fusion protein directly induces transcriptional activation via interaction with *ephrin-A5* DNA, we examined the activity of a luciferase reporter in transiently transfected HEK293 cells. For this assay, 12-mer *ephrin-A5* or *Delta1* DNA oligonucleotides were linked to form long concatemers containing six repeats. These were then sub-cloned into β -globin basal promoter-driven luciferase plasmid vector (Fig. 4D). As expected, co-transfection of *Ascl1*-E47 with *ephrin-A5* or *Delta1* DNA increased luciferase activity by approximately 25-fold (Fig. 4E, lanes 4 and 8). However, this enhanced activity was not observed when *ephrin-A5* DNA oligonucleotides containing mutations in the bHLH TF core sequence were transfected (Fig. 4E, lane 6). Taken as a whole, our results suggest that *Ascl1* can be considered as a candidate regulator of the transcriptional activation of *ephrin-A5* in the mesencephalon.

DISCUSSION

In this study, we have presented *in vivo* evidence that a bHLH TF binding site in an evolutionary conserved region is involved in the positive regulation of *ephrin-A5* gene expression in the posterior part of the mesencephalon. The identification of transcription factors that directly regulate *ephrin-A5* gene expression may lead to uncovering the molecular and genetic pathways that lead to development of the mesencephalon. An important issue that arises from our study is understanding the identity of the bHLH TF that is physiologically involved in the direct regulation of *ephrin-A5* gene expression during mesencephalon development. Previous

studies have shown that the brains from *Ascl1* null mutants have distinct morphological defects in the medial ganglionic eminence (MGE) and the mesencephalon (Casarosa et al., 1999). In addition, a genome-wide association study suggested that *Ascl1* is required for both expansion of neural progenitors and for neurogenesis, acting as a molecular link between the two distinct developmental phases (Castro et al., 2011). Based on these studies, it was hypothesized that *Ascl1* was potentially one of the bHLH TFs involved in the *ephrin-A5* enhancer activity. Our *in vitro* experiments, using both EMSA and a luciferase-based transactivation assay, showed that *Ascl1* was directly bound to the bHLH consensus binding site in the mesencephalon-specific *ephrin-A5* enhancer. However, we failed to demonstrate direct binding of *Ascl1* to the *ephrin-A5* enhancer using a chromatin immunoprecipitation (ChIP) assay. We do not rule out the possibility that the low quality of the *Ascl1* antibody may have prevented us from demonstrating a direct interaction between *Ascl1* and the *ephrin-A5* enhancer *in vivo*. It is also conceivable that the *Ascl1* protein interacts with other transcription factors, such as homeobox TFs, to positively regulate *ephrin-A5* gene expression. These may factors play a synergistic role in regulating the division of progenitors and neurogenesis during the development of the mesencephalon. It will be particularly interesting to further investigate whether homozygous deletion of the bHLH TF binding site in the *ephrin-A5* enhancer using CRISPR-Cas9 results in a neural tube closure defect of the mesencephalon, similar to that shown in *ephrin-A5* null mutant brains (Fulco et al., 2016; Gilbert et al., 2013; Lopes et al., 2016).

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