
Involvement of a Basic Helix-Loop-Helix Gene *BHLHE40* in Specification of Chicken Retinal Pigment Epithelium

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Abstract: The first event of differentiation and morphogenesis in the optic vesicle (OV) is specification of the neural retina (NR) and retinal pigment epithelium (RPE), separating the inner and outer layers of the optic cup, respectively. Here, we focus on a basic helix-loop-helix gene, *BHLHE40*, which has been shown to be expressed by the developing RPE in mice and zebrafish. Firstly, we examined expression pattern of *BHLHE40* in developing chicken eye primordia by *in situ* hybridization. Secondly, *BHLHE40* overexpression was performed with *in ovo* electroporation and examined its effects on optic cup morphology and expression of NR

and RPE marker genes. Thirdly, we examined expression pattern of *BHLHE40* in *LHX1*-over-expressed optic cup. *BHLHE40* expression emerged in a subset of cells of the OV at Hamburger and Hamilton stage 14 and became confined to the outer layer of the OV and the ciliary marginal zone of the retina by stage 17. *BHLHE40* overexpression in the prospective NR resulted in ectopic induction of *OTX2* and repression of *VSX2*. Conversely, *BHLHE40* was repressed in the second NR after *LHX1* overexpression. These results suggest that emergence of *BHLHE40* expression in the OV is involved in initial RPE specification and that *BHLHE40* plays a role in separation of the early OV domains by maintaining *OTX2* expression and antagonizing a NR developmental program.

Keywords: Basic helix-loop-helix e40, *BHLHE40*, LIM homeobox 1, *LHX1*, chicken, optic vesicle, retinal pigment epithelium, RPE, neural retina

1. Introduction

The eye develops as bilateral optic vesicles from parts of the forebrain. Subsequently, the optic cup develops by invagination of the optic vesicle. The neural retina (NR) and the retinal pigment epithelium (RPE) differentiate from the inner and outer layers of the optic cup, respectively. The NR gives rise to various neuronal cells involved in photoreception (cone and rod cells) and processing of visual information (interneurons such as amacrine, horizontal and bipolar cells, projection neurons such as retinal ganglion cells, and Müller glia cells). The RPE is a single layered epithelium that prevents light scattering in the retina and sustains metabolism and maintenance of the photoreceptors. How these two retinal domains are initially specified and develop to mature cell layers consisting of various retinal cell types is a fundamental issue in developmental biology and regenerative medicine to alleviate congenital retinal disorders.

In chicken embryos, FGF1 secreted from the surface ectoderm abutting the optic vesicle can direct the neural retina (NR) domain in the optic vesicle [1]. NR cells are specified by stage 13 (19 pairs of somites), and before this stage, optic vesicle cells can exhibit a dorsal

telencephalic character when cultured alone. Then, the NR identity is established by BMP signals from the lens ectoderm [2]. However, another study shows that a high dose of BMP5 is able to reprogram NR into the RPE by inducing the expression of *MITF*, an RPE differentiation transcription factor, when a Wnt2b signal is present in the chicken optic cup [3]. This discrepancy is explained by that the optic cell behavior depends on the concentration of BMP5 as a low dose of BMP5 induces the expression of *VSX2*, an NR specification transcription factor, resulting in the conversion of the RPE to the NR [3]. Strikingly, embryonic stem cells from mammals can self-organize to form optic vesicles and develop the layered retina even without surface ectoderm [4,5]. Therefore, it seems likely that the NR and RPE compartments are initially determined by intrinsic factors, but their further development and sustained differentiation require extrinsic factors from the surrounding tissues.

Recent studies in zebrafish have shown that NR development is a default state and specification of RPE in the optic primordia separates the distinct NR and RPE domains in the optic cup [6]. However, differences between species are observed in the development of RPE: maturation of RPE is fulfilled by cell shape change in zebrafish but in amniotes cell proliferation occurs in that phase [7]. In this study, we focus on an RPE gene, *basic helix-loop-helix e40*, (*Bhlhe40*), shown to be expressed in the prospective and definitive RPE cells. In zebrafish, *bhlhe40* expression begins in the optic vesicle, becomes confined to its medial layer, the future RPE, and retains in the RPE throughout embryonic development [8]. Since zebrafish *bhlhe40* could mark the prospective RPE cells from as early as 12-somite stage [9], how the RPE progenitors expand over the optic cup has been visualized [7,9]. In the developing mouse eye, *Bhlhe40* is expressed in the prospective RPE by embryonic day 11.5 (E11.5) and its expression persists in the RPE at later stages [10]. *Bhlhe40*, also known as DEC1 [11], Stra13 [12], and SHARP2 [13], has many physiological and pathological functions such as in cellular differentiation, circadian rhythm, immunity, and cancer [14-16]. However, roles of *Bhlhe40* in RPE specification has been elusive. As a first step to know the conserved and species-specific systems in early optic vesicle morphogenesis, we examined the expression pattern of *BHLHE40* during chicken early eye development and the effects of its overexpression in the optic primordia.

2. Materials and Methods

2.1. Chicken Embryos

Fertilized White Leghorn chicken embryos were obtained from Goto Furanjyo (Gifu, Japan). Eggs were incubated on their sides in a humidified incubator at 38 °C until the desired embryonic stage. All embryos were staged according to Hamburger and Hamilton [17]. All experiments were performed in accordance with the Recombinant DNA Advisory Committee and the Animal Care and Use Committee, Okayama University, in agreement with relevant guidelines in Japan.

2.2. Isolation of Chicken cDNAs and Construction of Expression Vectors

Chicken embryonic heads were dissected from 10 embryos at Hamburger and Hamilton (HH) stages 16-17, 21 and 24 in ice-cold PBS, and RNA was extracted using an RNAqueous-Micro Total RNA Isolation Kit (Thermo Fisher Scientific). First strand complementary DNA (cDNA) was synthesized using RNA and SuperScript III Reverse Transcriptase (Thermo Fisher Scientific) with random primers. Partial cDNAs for chicken *BHLHE40* and other RPE marker genes were isolated using the cDNA and primers listed in Supplementary Table A1 by PCR. The amplified cDNAs were cloned into the pGEM-T Easy (Promega, Madison, WI) or pBluescript KS (+) (Stratagene) vector and used as templates for *in vitro* transcription. The synthesized RNAs were utilized for *in situ* hybridization probes. For *in ovo* electroporation, we obtained cDNA containing the entire coding sequence (cds) for chicken *BHLHE40*, which was further cloned into the expression vector pCAGGS [18].

2.3. Cryosectioning

Embryonic heads or whole embryos were fixed in 4%paraformaldehyde (PFA) in phosphate-buffered saline (PBS) overnight, washed with PBS, cryoprotected in 20% sucrose/PBS and embedded in OCT compound (Tissue-Tek; Sakura Finetek, Tokyo, Japan) and frozen at -80 °C. Sections were cut at 15 µm or 16 µm on a cryostat (Tissue-Tek Polar B, Sakura Finetek Japan or Leica 1860) and mounted on adhesive glass slides (Matsunami Glass, Kishiwada, Japan). The slides were air-dried for 1 hour and stored at -20 °C until further analysis.

2.4. In Situ Hybridization (ISH)

Whole-mount ISH (WISH) was performed according to Kawaue et al. [19] by the use of a semiautomatic ISH machine (HS-5100; Aloka, Tokyo, Japan). The ISH on sections was performed according to Sato et al. [20]. Each RNA probe was used at a concentration of 0.67 $\mu\text{g/mL}$ (WISH) or 0.17 $\mu\text{g/mL}$ (sections) in hybridization buffer. Fluorescence ISH using HNPP/Fast Red Fluorescent Detection set (Roche #11758888001) was performed basically according to the kit instructions, but without proteinase treatment for subsequent immunofluorescence (Figure 2B-D). ISH experiments were performed in at least in triplicate (Figure 1) or in duplicate (other data).

2.5. Western Blot Analysis

Chicken BHLHE40 (cBHLHE40) were tagged with the HA epitope (YPYDVPDYA) at the C-terminus and inserted into the mammalian expression vector pCAGGS. Expression constructs for Δ basic BHLHE40, and one with acidic extension were similarly tagged with HA. The nucleotide sequences were confirmed by Sanger sequencing. The expression of recombinant proteins was assessed by Western blot at least in duplicate as follows. The plasmid DNA was transfected into HEK293T cells (ATCC, Manassas, VA) using polyethyleneimine max (#24765-1; Polysciences, Warrington, PA). An empty pCAGGS vector was also transfected as a negative control. Two days after transfection, the cells were harvested and lysed with RIPA buffer (#08714-04; Nacalai Tesque, Kyoto, Japan) containing 1% SDS. The protein concentration in the lysate was quantified using a Bradford protein assay (5000006JA; Bio-Rad Laboratories, Hercules, CA) after diluting the SDS. The lysate was then mixed with an equal volume of SDS sample buffer (0.1 M Tris-HCl, 2% w/v SDS, 20% w/v glycerol, 0.02% w/v bromophenol blue, 5% w/v 2-mercaptoethanol, pH 6.8) and heated at 95 °C for 3 minutes. Ten micrograms of total protein in each sample was separated by SDS-PAGE (12.5% gel) and transferred onto a polyvinylidene difluoride (PVDF) membrane using a wet blotter. Membranes were blocked with Blocking One (#03953-95, Nacalai Tesque) at RT for 1 hour with gentle agitation. The PVDF membrane was then incubated overnight at 4 °C with primary antibodies against the HA epitope (anti-HA High Affinity from rat IgG₁, 1:2,000, #ROAHAHA Roche; Merck, Darmstadt, Germany) or alpha tubulin (alpha Tubulin Monoclonal Antibody DM1A, 1:600, #62204, Thermo Fisher Scientific). After washing four times for

10 min with Tris-buffered saline containing 0.1% (w/v) Tween 20 (TBST), the membrane was incubated for 90 minutes at RT with a peroxidase-conjugated anti-rat IgG (1:10,000, #SA00001-15; Proteintech, Rosemont, IL) or anti-mouse IgG (1:5,000, #7076; Cell Signaling Technology, Danvers, MA) secondary antibody. Primary and secondary antibodies were diluted in IMMUNO SHOT Reagent 1 and 2 (Cosmo Bio, Tokyo, Japan), respectively. After washing four times for 10 min with TBST, immunoreactive bands were visualized by ImmunoStar LD (Wako) for the HA epitope or ImmunoStar Zeta (Wako) for alpha-tubulin. Images of chemiluminescence were captured with a C-DiGit chemiluminescence Western blot scanner (LI-COR Biosciences, Lincoln, NE).

2.6. *In Ovo Electroporation*

In ovo electroporation was performed as described previously [21]. Briefly, chicken embryos were incubated for 36-39 hours until HH stage 10. Each expression plasmid in the pCAGGS vector [18] (1 µg/µL of *EGFP* and 5 µg/µL of *LHX1* or *BHLHE40*) with Fast Green FCF (0.1%) (FUJIFILM Wako Pure Chemical, Osaka, Japan) was first injected into the right forebrain vesicle using Nanoject II (Drummond Scientific, Broomall, PA) and the injection volume was 64.4 nL for each embryo. Immediately after DNA injection, the embryos were electroporated using a sharpened tungsten needle (CUY614T-200, and CUY615; Nepa Gene, Ichikawa, Japan) as a cathode and a platinum electrode (CUY611P3-1 and CUY580; Nepa Gene) as an anode. Twenty-four hours post electroporation, the embryos were observed under a fluorescence dissection microscope (Leica M165 FC, Leica Microsystems, Wetzlar, Germany) to evaluate their embryonic stages, location and extent of ectopic expression by co-electroporated GFP fluorescence, harvested, fixed in 4% PFA/PBS at 4 °C overnight, washed, and stored in 100% ethanol at -20 °C until further analysis.

2.7. *Immunofluorescence*

Immunofluorescence (IF) was essentially performed according to Kawaue et al.[19]. Briefly, after blocking with 1% Bovine Serum Albumin in PBS containing 0.1% Triton X-100 and anti-HA (1:200, rat IgG; Thermo Fisher Scientific) antibody was applied to the sections and incubated at 4 °C overnight. After washing, the sections were incubated with anti-rat IgG conjugated with Alexa Fluor 488 (1:1,000; A-11006; Thermo Fisher Scientific) or Alexa Fluor

568 (1:1,000; ab175708, Abcam) and Hoechst 33342 (1 μ g/mL) at RT for 60 min. Other antibodies used are listed in Table A2.

2.8. Image Capture and Processing

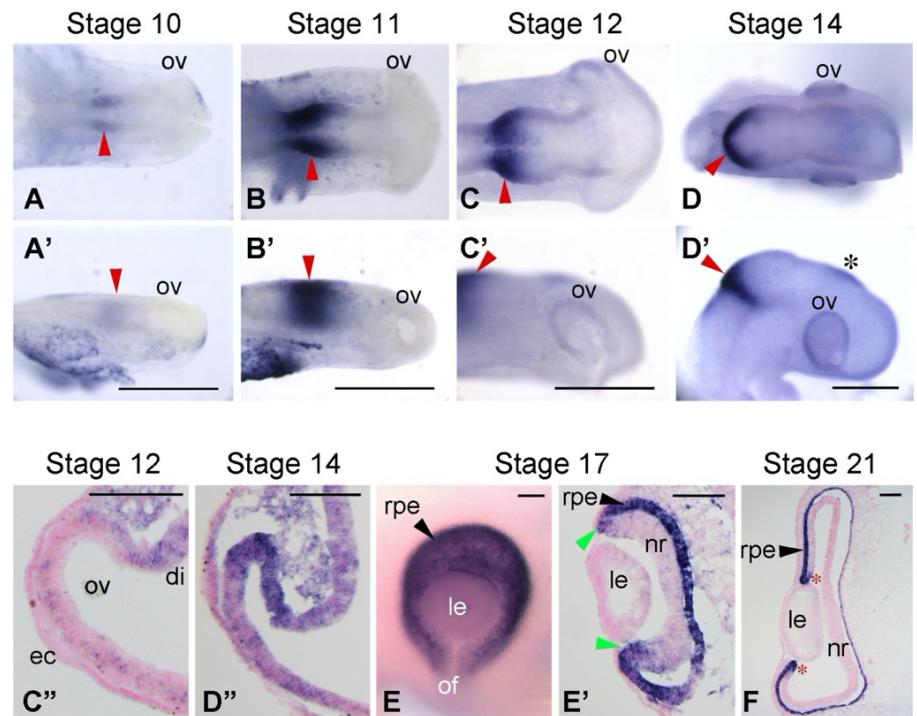
The colored embryos after WISH were photographed with a digital camera system (Leica DFC310). Tissue sections were observed with bright-field or differential interference contrast microscopy (Leica DM5000B) and photographed with a CCD camera system (Nikon DS-Fi1, Nikon, Japan). Brightfield images of *in situ* hybridization on tissue sections were also captured with an IDS UI-3290SE-C-HQ camera mounted on a Carl Zeiss Axioplan microscope. Fluorescent micrographs (Figures 3K, N; Figure A2K, N) were taken with a Leica upright DM5000B microscope equipped with a Nikon006 DS-Qi1 CCD camera system. Confocal fluorescence images were collected with a Carl Zeiss LSM 780 laser scanning confocal microscope system with 405, 488, 561 and 633-nm laser lines. Brightness and contrast adjustments were performed for some images, and image manipulation was performed using a ZEN 2012 SP1 black edition. Confocal z-stack images were acquired at 0.606- μ m intervals for a total depth of 6.66 μ m (Figure 2B-D), at 0.705- μ m intervals for a total depth of 9.87 μ m (Figure 3A), at 0.705- μ m intervals for a total depth of 11.99 μ m (Figure 3B-D), at 0.658- μ m intervals for a total depth of 12.51 μ m (Figure 3E) and at 0.634- μ m intervals for a total depth of 12.05 μ m (Figure 3F-H). Shown images are maximum intensity projections of these confocal z-series.

3. Results

3.1. Expression Patterns of *BHLHE40* in Chicken Early Eye Development

Bhlhe40 was reported to be expressed in the developing RPE of mice and zebrafish [8,12], but none of chicken was described. We therefore examined the expression pattern of *BHLHE40* in chicken embryos by *in situ* hybridization (ISH) (Figure 1). We found that

Figure 1. Expression pattern of *BHLHE40* in chicken early embryos. (A-D', E) Whole-mount *in situ* hybridization of chick embryos at Hamburger and Hamilton (HH) stages 10 to 14, and 17. Dorsal views (A-D) and lateral views (A'-D'; to the same scale of A-D, respectively) of embryonic heads or eye (E) are shown. Red arrowheads show the midbrain-hindbrain boundary. Asterisk in (D') shows the developing pineal gland. (C'', D'', E', F) Frontal sections of the



optic primordia at stages 12 to 21. Lateral is to the left. Dorsal is to the top. Light green arrow-heads in (E') and asterisks in (F) show the ciliary marginal zone. mRNA signals are in dark blue. For sections, cell nuclei were stained with nuclear fast red. di, diencephalon; ec, surface ectoderm; le, lens vesicle; nr, neural retina; of, optic fissure; ov, optic vesicle; rpe, developing RPE. Scale bars: 0.5 mm in (A-D'), 100 μ m (C'', D'', E, E', F).

BHLHE40 mRNA expression emerged in the dorsal portion of the optic vesicle (OV) toward the dorsal diencephalon at stage 12 (Figure 1C',C''). At stage 14, distinct signals of *BHLHE40* were observed in the dorsal region of the OV, the prospective RPE (Figure 1D', D'')[10]. At stage 17, *BHLHE40* was expressed in the outer layer of the optic cup, the nascent RPE and ciliary marginal zone (CMZ) (Figure 1E, E'). At stage 21, *BHLHE40* continued to be expressed in the early-stage RPE and CMZ (Figure 1F). As reported [8], *BHLHE40* was expressed in the midbrain-hindbrain boundary (Figure 1A-D, A'-D') and developing pineal gland (Figure 1D').

3.2. Overexpression of *BHLHE40* in the OV Drives Early RPE Development

Since *BHLHE40* was expressed in the RPE-lineage of chicken OV, we next examined whether overexpression of *BHLHE40* would drive early RPE development in chicken optic primordia. To discriminate endogenous transcripts for *BHLHE40* from ectopic ones, we constructed an expression plasmid for chicken *BHLHE40* with a human influenza virus hemagglutinin (HA)

tag (cBHLHE40-HA) at the C-terminus. We confirmed its expression and the protein size as approximately 45 kDa by Western blot analysis using HEK293T cells (Figure 2A), showing the constructed plasmid vector successfully produced full-length *BHLHE40* proteins.

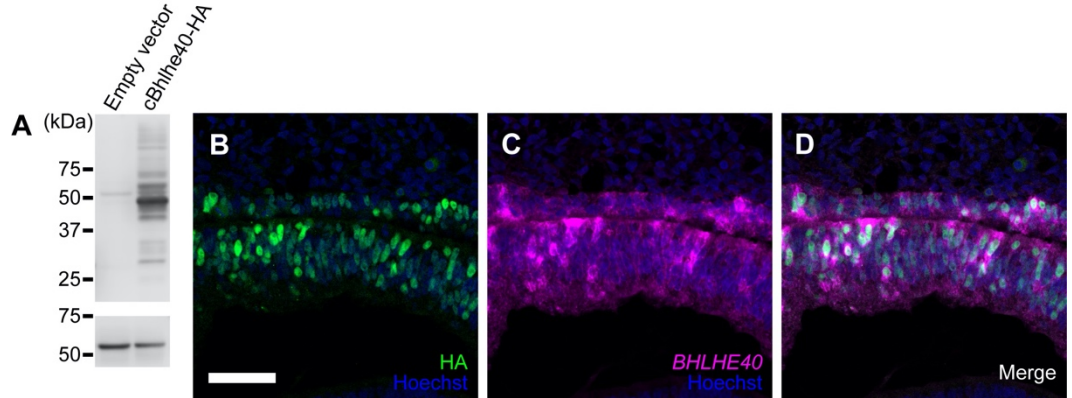


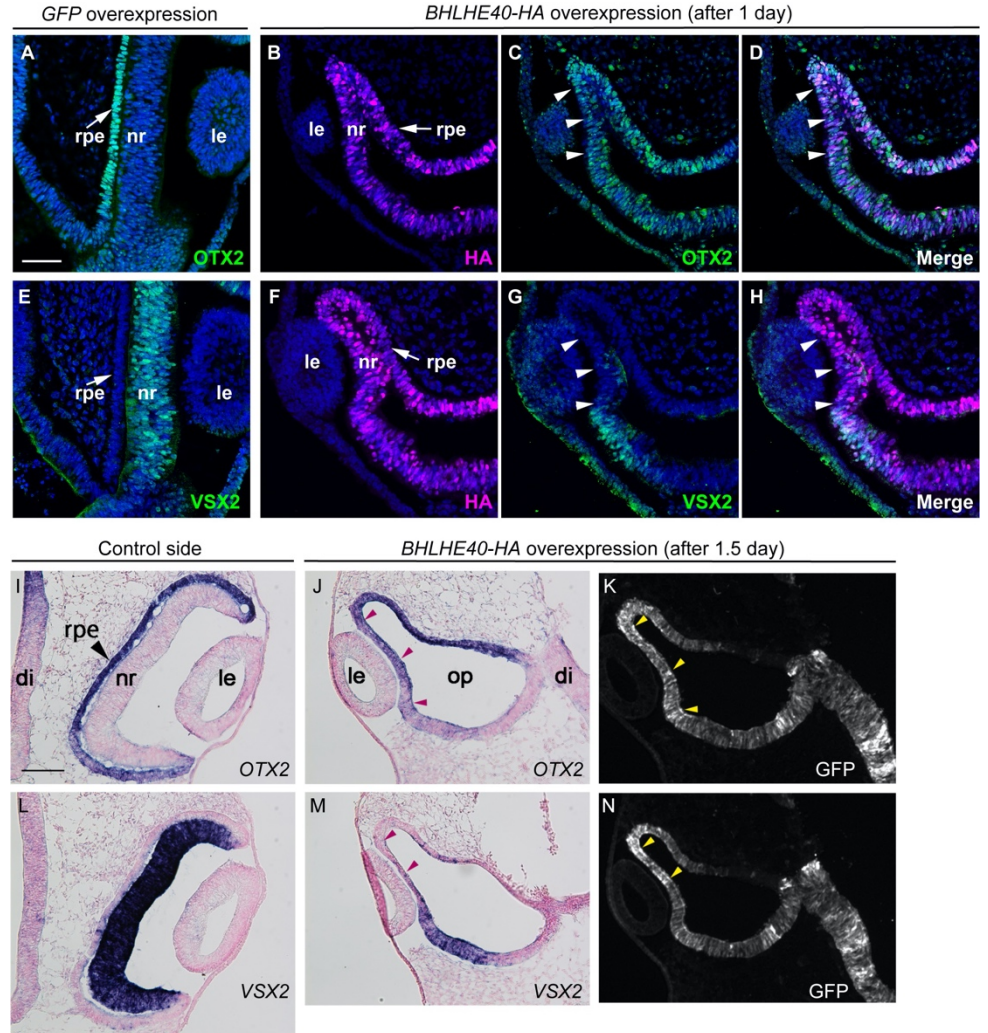
Figure 2. Validation of HA-tagged *BHLHE40* expression. (A) Whole cell extracts of HEK293T cells transfected with chicken *BHLHE40*-HA/pCAGGS, or empty pCAGGS vector were analyzed by Western blot. HA-tagged proteins were detected by anti-HA antibody. The predicted molecular mass of *BHLHE40* is 44,830 Da. Alpha-tubulin from the same samples was detected for loading reference. (B-D) Section of the developing RPE (upper) and NR (lower) layers at 1.5 days post electroporation (HA-tagged *BHLHE40* overexpression). (B) Immunofluorescence of HA, showing cell nuclei that express BHLHE40-HA proteins in both retinal layers. Secondary antibody to anti-HA was Alexa Fluor 488-conjugated anti-rat IgG. (C) *In situ* hybridization of *BHLHE40*, showing cells that express *BHLHE40* mRNA in both retinal layers. Color development was performed with HNPP/FastRed. (D) Merged image of (B) and (C). Cell nuclei were stained with Hoechst 33342. Scale bar: 50 μ m in (B) for (B-D).

By *in ovo* electroporation (EP), we overexpressed *cBHLHE40-HA* in the early optic vesicle. At 40 hours post EP, the ectopic expression of *BHLHE40*, HA-tagged BHLHE40 proteins, was verified by immunofluorescence with anti-HA antibody (Figure 2B). Along with a co-electroporated GFP plasmid (Supplementary Figure A1A), cells with ectopic BHLHE40 proteins were visualized in all over the OC (Figure A1B, C). We further verified that *BHLHE40* mRNA was overexpressed in the inner layer of the OC, the prospective NR, after electroporation of the *cBHLHE40-HA* plasmid (Figure 2C, D).

Under these experimental conditions, we examined whether the overexpression of *BHLHE40* could influence the morphology of the optic cup and the expression domains of an RPE specification gene, *OTX2* and a NR specification gene, *VSX2*. After overexpression of GFP alone, *OTX2* proteins were detected in the outer layer of the optic cup, the prospective RPE, while none of *OTX2* was detected in the inner layer, the prospective NR, abutting the lens vesicle (Figure 3A), similar to their normal expression patterns in the optic cup. After *BHLHE40* overexpression, however, *OTX2* was detected in the neuroepithelium abutting the lens vesicle (arrowheads in Figure 3C) and continued to localize to the dorsal epithelium of the OC. Merged view of HA (Figure 3B) and *OTX2* (Figure 3C) showed (Figure 3D) that *OTX2* protein in the inner layer of the optic cup abutting the lens vesicle was ectopically induced or its early expression in the optic vesicle was maintained under the influence of *BHLHE40* overexpression. Regarding *VSX2*, its expression was downregulated in that portion (arrowheads in Figure 3G, H), while it was observed in the ventral portion of the inner layer of OC (below the arrowheads).

After one and half day post electroporation, as shown by the normal optic cup on the contralateral control side, the outer layer of the OC remains a single layer of *OTX2*-expression cells (Figure 3I), while the neuroepithelial cells in the inner layer of the OC proliferates to become a thickened NR expressing *VSX2* by this stage (Figure 3L). After *BHLHE40* overexpression, however, the dorsal portion of the neuroepithelium abutting the lens vesicle remained thin (arrowheads in Figure 3J, M), where *BHLHE40* was overexpressed judging from the localization of co-electroporated GFP (Figure 3K, N). At this stage, *OTX2* mRNA was detected in the neuroepithelium abutting the lens vesicle (arrowheads in Figure 3J), while *VSX2* expression was downregulated in that portion (Arrowheads in Figure 3M).

In another embryo (#22), the OV development was disrupted and no lens vesicle formation was observed (Figure A2I-N). Although *OTX2* is normally expressed in the dorsal portion of the OV, the prospective RPE, it was expressed in the distal portion of the deformed OV



(Figure A2J) where *BHLHE40* was overexpressed, judging from the localization of co-electroporated GFP (Figure A2K). Regarding *VSX2*, its expression was

Figure 3. Expression pattern of *OTX2* and *VSX2* in the optic cup after GFP and *BHLHE40* overexpression. Frontal sections of the optic cup from three embryos (*GFP*#2, *BHLHE40*#14, *BHLHE40*#17) are shown. Dorsal is to the up. (A-H) Optic cups at 1 day post electroporation (for GFP alone and HA-tagged *BHLHE40* overexpression). (A, E) Immunofluorescence of *OTX2* (A) and *VSX2* (E) in *GFP* overexpression control (#2). (B-D) Immunofluorescence of HA (B) and *OTX2* (C) in HA-tagged *BHLHE40* overexpression (#14). Secondary antibodies to anti-HA and anti-*OTX2* were Alexa Fluor 568-conjugated anti-rat IgG and Alexa Fluor 647-

conjugated anti-rabbit IgG, respectively. **(D)** Merged image of **(B)** and **(C)**. **(F-H)** Immunofluorescence of HA **(F)** and VSX2 **(G)** in HA-tagged *BHLHE40* overexpression. Secondary antibodies to anti-HA and anti-VSX2 were Alexa Fluor 568-conjugated anti-rat IgG and Alexa Fluor 488-conjugated anti-sheep IgG, respectively. **(H)** Merged image of **(F)** and **(G)**. Cell nuclei were stained with Hoechst 33342 **(A-H)**. Similar data from another embryo (#11) are shown in Supplementary Figure A2A-H. **(I-N)** Optic cups at 1.5 day post electroporation (HA-tagged *BHLHE40* overexpression, #17). **(I, L)** Optic cups on the control side are shown. *OTX2* is expressed in the outer layer of the optic cup **(I)**, while *VSX2* is expressed in the inner layer of the optic cup **(L)**. In **(K, N)**, co-electroporated GFP, showing the domain of *BHLHE40* overexpression. Images for GFP were taken before ISH. **(J, M)** In embryo #17, the inner layer of the optic cup abutting the lens, where *BHLHE40* is ectopically expressed (arrowheads in **J**), remains thin and expresses *OTX2* **(M)**. In the same domain, *VSX2* expression is downregulated (arrowheads in **M**). Cell nuclei were stained with nuclear fast red **(I-N)**. di, diencephalon; le, lens vesicle; nr, neural retina; op, optic cup or deformed optic vesicle in **(G)**; rpe, developing RPE. Scale bar: 50 μ m in **(A)** for **(A-H)**, 100 μ m in **(I)** for **(I-N)**.

reduced but present partly (Figure A2M). We further examined whether *BHLHE40-HA* overexpression might induce cell death by immunostaining with anti-cleaved caspase 3 antibody. At one day post electroporation, we did not find any positive cells for cleaved caspase 3 in the HA-immunoreactive cells (Figure A3). These data suggest that *BHLHE40* could maintain *OTX2* expression in the optic vesicle, drive early RPE development and repress the developmental program for NR.

3.3 Effects of LHX1 Overexpression on *BHLHE40* Expression

BHLHE40 lacking a basic region (Δ basic *BHLHE40*) is known to act as a dominant negative form of *BHLHE40* [14]. Therefore, we next examined whether overexpression of Δ basic *BHLHE40* could alter the morphology of the optic cup, such as lack of RPE development or formation of double NR. We constructed an expression plasmid for Δ basic *BHLHE40* with an HA tag, whose calculated molecular mass was 42,877 Da and confirmed its expression by Western blot analysis (Figure A4A). We overexpressed Δ basic *BHLHE40* in the early optic

vesicle. However, the eye morphology was not altered (Figure A4B and not shown). Additionally, an extension of 12 acidic amino acids in place of the basic region of bHLH transcription factors is known to stabilize heterodimerization between the dominant negative forms and endogenous bHLH proteins [22]. Therefore, we further constructed an acidic extension form of Δ basic *BHLHE40* and expressed it in the optic vesicle. However, despite confirmed protein expression for the acidic extension form (39,998 Da with HA) (Figure A4C), overexpression of this construct did not alter the eye morphology, either (Figure A4D and not shown).

We therefore sought to utilize the *LHX1* overexpression. Previously, we found that *LHX1* overexpression in the early chicken OV induced the second NR formation, verified by ectopic expression of NR marker genes, *RX*, *SIX3*, *SOX2*, *SIX6*, and *VSX2*, and called double NR [19]. Recent studies in zebrafish show that the default state of neuroepithelium of the OV is the NR and expression of RPE specification genes drive the development of RPE and separates the NR and RPE domains [6]. In chicken embryo, however, deducing from overlapping expression domains of the above NR genes and RPE genes such as *OTX2* and *MITF* suggests bipotential retinal progenitor cells in the nascent OV. Furthermore, *LHX1* expression in the posterior portion of the chicken OV corresponds to the future central NR domain [23], suggesting its ability to drive NR formation by antagonizing RPE differentiation. Thus, we examined whether *BHLHE40* expression was downregulated in the *LHX1*-overexpressed optic cup. We analyzed it in chicken embryos at 24 hours post electroporation, of approximately stages 15 to 16, by ISH (Figure 4). After *LHX1* overexpression,

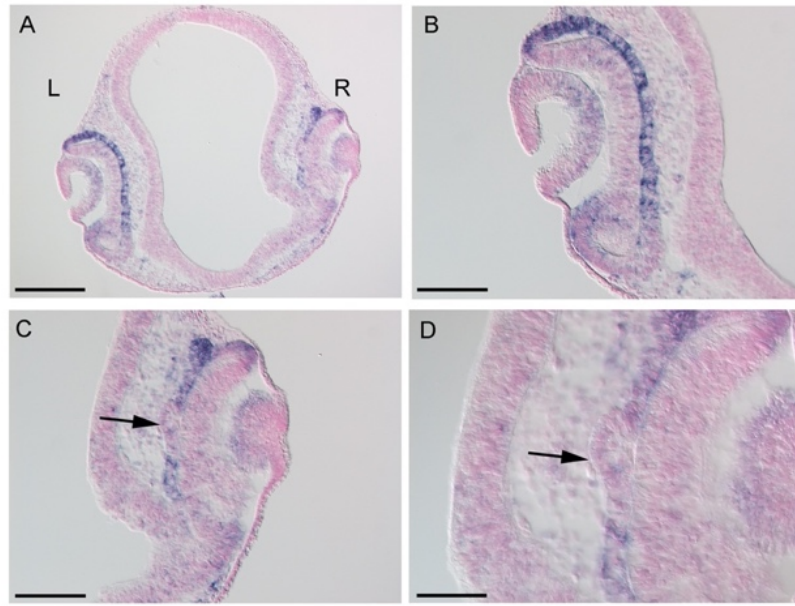


Figure 4. *BHLHE40* expression after *LHX1* overexpression. (A) *In situ* hybridization (ISH) on the frontal section of the chicken embryonic head after 1 day post electroporation (embryo #5). Enlarged view of the left (L) control optic cup (OC) is shown in (B), and that of the right (R) *Lhx1*-overexpressed one is shown in (C, D). *BHLHE40* expression is downregulated in a portion of the outer layer of the OC (arrows in C, D). Cell nuclei were stained with nuclear fast red. Scale bars: 200 μ m (A), 100 μ m (B, C), 50 μ m (D). Similar data from other embryos are shown in Supplementary Figure A5.

protruding regions were observed from the outer layer of the optic cup, where *OTX2* and *MITF*, the RPE genes are repressed [19]. Sectioning of the *LHX1*-overexpressed embryonic heads and ISH on sections revealed that *BHLHE40* expression was downregulated in the portion transforming to NR from the nascent RPE in the outer layer of the OC (arrows in Figure 4C, D and Figure A5C, D, G). Thus, *BHLHE40* expression is negatively regulated by a molecular force of NR formation, *LHX1*.

4. Discussion

A bHLH transcription factor gene *Bhlhe40* was reported to be expressed in the developing RPE as well as somites and other organ primordia of mice and zebrafish [8,12]. Recently, this gene has been used to visualize the cell behavior during early RPE development [7,9]. Here

we firstly described the early onset of *BHLHE40* expression in the dorsal region of the optic vesicle, the prospective RPE of the chicken [10]. While we focused on the role in RPE development, it has been known that BHLHE40 is involved in circadian rhythm and tumor progression [24]. There is a paralogous gene of *BHLHE40*, *BHLHE41*, which is also expressed in the developing RPE of zebrafish [6], chicken (this study, Figures A6 and A7), and human iPS cell-derived RPE [25]. In human tumor cells, there are functional differences in their roles: BHLHE40 induces apoptosis and epithelial-mesenchymal transition (EMT), whereas BHLHE41 inhibits them [26]. This is considered to be due to their structural differences, in which both of BHLHE40/41 have bHLH and Orange domains, but BHLHE41 has an additional alanine/glycine-rich region in the C-terminus [26] (Figure A8). Regarding this, although there are similar upstream regulators for the two paralogs, their downstream effectors and influences are rather opposite. For example, BHLHE40 negatively regulates E-cadherin and Bcl-2, while BHLHE41 negatively regulates Slug (Snail2), Twist1, and caspase-8 [26]. In addition to suppression of these target genes by binding to E-boxes, BHLHE40/41 regulate each other by binding to their E-boxes. Although we have yet to examine the effect of *BHLHE41* overexpression, the involvement of BHLHE41 might be correlated to the result of no phenotypes when dominant negative (DN) forms of *BHLHE40* were overexpressed, as overexpression of the DN forms might induce some compensation by the paralog.

Not a few downstream targets of BHLHE40 have been identified in other contexts [16]. Along with EMT-related genes, BHLHE40 represses Cyclin D1 and STAT1, and upregulates beta-catenin by cooperating with other co-transcription factors. When acting as a repressor, BHLHE40 is known to recruit histone deacetylase, HDAC1. These known target genes represent its multi-functionality and studies on the candidate target genes and transcriptome analysis of *BHLHE40*-overexpressed embryonic eyes will further illuminate more detailed molecular aspects of its functions in the chicken eye development.

As we mentioned in Introduction, *Bhlhe40* was originally isolated as a retinoic acid-inducible gene, *Stral3* [12] and they described that P19 cell clones expressing higher amount of STRA13 died after the passage in culture. In our electroporation method, we did not observe any cell death in *BHLHE40-HA* expressing chicken optic cup cells as shown in Figure A3.

Boudjelal et al.[12] also said that the low expressing cells did not undergo efficient neuronal differentiation, and in this study we could not determine the expression level of *BHLHE40* that robustly altered the morphology and expression of *OTX2* and *VSX2*, which would be our next challenge.

One of the upstream factors of *BHLHE40/41* is hypoxia, which is often molecularly exemplified by *HIF1a* [26]. RPE cell-specific knockout mice of this pathway genes show that regulation of *Hif1a* in the RPE is required for normal RPE and related ocular development [27]. Therefore, during chicken RPE development, it would be intriguing to know the effect of *HIF1A*-hypoxia pathway genes on the expression *BHLHE40*, which will be studied in the next study. Another important upstream regulator of *BHLHE40/41* in mouse tumor cells is TGF beta [26,27]. It has been reported that TGF beta member activin from the extraocular mesenchyme surrounding the optic primordia patterns the optic vesicle in the chicken [28]. It is therefore conceivable that the expression of *BHLHE40* would be induced by TGF beta family signals from the extraocular mesenchyme at the early phase of optic cup development.

In zebrafish, RPE progenitors expands around the NR in two phases; firstly RPE progenitors are specified with an increase in cell number, and then the RPE stretches around the NR with an elongation of RPE progenitor cells [9]. In amniotes (chicken and human), however, in the second phase, the RPE still expands by cell proliferation with a less pronounced cell flattening [7]. Correspondingly, a reported transcriptome analysis has revealed two waves of transcription factor genes regulating the identity of the RPE [6]:Early peaking genes include *tead3b*, *tfap2a*, and *tfap2c*, and representative late peaking genes are *klf5a* and *klf2b* as well as *otx2* and *bhlhe40*. According to this, we cloned the cDNAs and firstly examined the expression patterns of *TEAD3*, *TFAP2A*, *TFAP2C*, *KLF2*, and *KLF5* in chicken embryos, but none of the genes except *TEAD3* were expressed in the developing RPE (Figures A9-A11 and Table A1). Only *TEAD3* was found to be expressed in the developing chicken RPE but its expression domains were not restricted to RPE progenitors as seen for *BHLHE40*. Thus, we suggest that the molecular network regulating RPE specification may not be identical between zebrafish and chicken, except for established RPE genes such as *OTX2* and *BHLHE40*.

This study showed *BHLHE40* overexpression upregulated *OTX2* expression and down-regulated *VSX2* expression, suggesting *BHLHE40* would drive RPE specification in bipotential retinal progenitor cells of the chicken. Since it was reported that *OTX2* alone could not induce the expression of *MITF*, a melanogenic and differentiation factor for RPE in the chicken embryo [29], *BHLHE40* alone might not proceed with later RPE development in the chicken. In this regard, *MITF* was found to be negatively regulated by *VSX2* in the developing chicken eye [30]. Furthermore, direct repression of *Mitf* by *Vsx2* have been reported in mouse [31]. As *VSX2* was markedly repressed in the optic primordia where *BHLHE40* was ectopically expressed, it is conceivable to think that *BHLHE40* positively regulates *MITF* at least through repression of *VSX2*. Also, RNA levels of *Cyclin D1* are significantly decreased in the *Vsx2*-deficient *or^J* mouse [32]. Since Cyclin D1 is known to promote cell proliferation [33], repression of *Cyclin D1* would have disrupted expansion of the prospective NR layer after *BHLHE40* overexpression, also via downregulation of *VSX2*. In cancer cell lines, *BHLHE40* (DEC1) interacts with Cyclin E and stabilize it, leading to prolong the S phase and suppress cell proliferation [34]. Thus, a direct negative effect of *BHLHE40* on the cell cycle of nascent OV cells would be conceivable as well.

We did not succeed in the functional inhibition of *BHLHE40* in the developing chicken eye, to see if the presumptive RPE could transdifferentiate into the NR in the stage of bipotential retinal progenitors. Instead, we utilized *LHX1* overexpression and found that *BHLHE40* is repressed in the transdifferentiating outer layer of the optic cup. Previously, we reported that the second NR formation by *LHX1* overexpression was not mediated by ectopic induction of *FGF8* expression in the early phase [19]. A recent work using fate mapping in chicken embryos has shown that the central NR is defined within the posterior optic vesicle (OV), while the peripheral retina, including future CMZ actually comes from the distal OV [23]. They also showed *LHX1* expression in the posterior portion of the OV corresponds to the future central NR domain. Accordingly, *LHX1* would antagonistically regulate *BHLHE40* in the very early stage of optic vesicle development and determine the central NR domain.

In conclusion, a bHLH gene, *BHLHE40* is expressed in the prospective and definitive RPE cells as well as CMZ of the developing chicken eye. *BHLHE40* can influence the

expression of *OTX2* and *VX2* and its expression can be downregulated by a NR-inducing factor LHX1, suggesting its definite role in the NR/RPE developmental programs of the optic vesicle. Conserved expression of *BHLHE40* in the RPE lineage across vertebrates, and its effects on the RPE/NR specification genes verify that this RPE gene belongs to the transcriptional node group among diversified RPE developmental processes.

Author Contributions: Conceptualization, T.K., K.S., T.N. and H.O.; methodology, T.K., K.S., T.B., T.M., S.M., T.N. and H.O.; validation, T.K., K.S., T.B., T.N. and H.O.; formal analysis, T.K., K.S., T.B., T.N. and H.O.; investigation, T.K., K.S., T.B. and H.O.; resources, K.S., T.B., T.M. and H.O.; data curation, T.K., K.S. and H.O.; writing—original draft preparation, T.K., K.S. and H.O.; writing—review and editing, T.K., K.S., T.N. and H.O.; visualization, T.K., K.S., T.N. and H.O.; supervision, T.N. and H.O.; project administration, H.O.; funding acquisition, K.S. and H.O. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: All experiments were performed in accordance with the Recombinant DNA Advisory Committee (protocol codes 13044, 18066 on 24 September 2013, 21 June 2018) and the Animal Care and Use Committee, Okayama University, in agreement with relevant guidelines in Japan.

Data Availability Statement: Not applicable.

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Appendix

Supplementary Figures (Figures A1-A11) and Tables (Table A1 and A2)

Figure A1 (data related to Figure 2): In the optic cup after *BHLHE40-HA* overexpression (from embryo #17, same as shown in Figure 3I-N), co-electroporated GFP fluorescence (**A**) is overlapped with BHLHE40-HA protein localization (**B**). Merged view of (**A**) and (**B**) is shown in (**C**). Cell nuclei were stained with Hoechst 33342. Shown images are maximum intensity projections of confocal z-series. le, lens vesicle; op, optic cup. Scale bar: 100 µm in (**A**) for (**A-C**).

Figure A2 (data related to Figure 3): Similar data of Figure 3 from other embryos, except for those after GFP expression (**A, E**), which are identical images as shown in Figure 3A and E. Frontal sections of the optic cup from three embryos (*GFP*#2, *BHLHE40*#11, *BHLHE40*#22) are shown. Dorsal is to the up. (**A-H**) Optic cups at 1 day post electroporation (for GFP alone and HA-tagged *BHLHE40* overexpression). In embryo #11 after BHLHE40 overexpression, OTX2 protein is localized in the inner layer of the OC (arrowheads in **C, D**), where VSX2 protein is not detected (arrowheads in **G, H**). Cell nuclei were stained with Hoechst 33342. Shown images are maximum intensity projections of confocal z-series. (**I-N**) Optic cups at one and half day post electroporation (HA-tagged *BHLHE40* overexpression). (**I, L**) Optic cups on the control side are shown. In (**K, N**), co-electroporated GFP, showing the domain of *BHLHE40* overexpression. (**J, M**) In embryo #22, optic cup development is disrupted and there is no lens formation after *BHLHE40* overexpression. *OTX2* is expressed in the distal portion of the optic cup (**J**), where *BHLHE40* is ectopically expressed (arrowheads in **J, K**). In the same domain, *VSX2* expression is downregulated but remains in some cells (arrowheads in **M, N**). Cell nuclei were stained with nuclear fast red. di, diencephalon; ec, surface ectoderm; le, lens vesicle; nr, neural retina; op, optic cup or deformed optic vesicle in (**J-N**); rpe, developing RPE. Scale bar: 50 µm in (**A**) for (**A-H**), 100 µm in (**I**) for (**I-N**).

Figure A3: *BHLHE40-HA* expressing cells do not show cell death as revealed by immunofluorescence using anti-cleaved caspase 3. In four embryos examined (**A-D**) (embryo identification numbers are shown on the leftmost), none of the HA-immunoreactive cells are positive for cleaved caspase 3. Cell nuclei were stained with Hoechst 33342. Shown images are maximum intensity projections of confocal z-series. Scale bar: 50 μ m in (**A-D**) for all images.

Figure A4: Validation of expression of HA-tagged presumed dominant negative forms of *BHLHE40*. (**A, C**) Western blot analysis of HEK293T cells transfected with chicken Δ basic *BHLHE40*-HA/pCAGGS (**A**) or Δ basic with acidic extension *BHLHE40*-HA (**C**) vectors. Alpha-tubulin from the same samples was detected for loading reference. (**B, D**) Confocal microscopic images show that HA-tagged presumed dominant negative forms of *BHLHE40* are detected in all over the optic cup by immunofluorescence using the anti-HA antibody. Secondary antibody to anti-HA was Alexa Fluor 568-conjugated anti-rat IgG. Co-electroporated GFP images and merged view are shown in the middle and right panels. Cell nuclei were stained with Hoechst 33342. Shown confocal images are maximum intensity projections of z-series. Scale bar: 50 μ m in (**B**) for all confocal images.

Figure A5 (data related to Figure 4): *In situ* hybridization (ISH) on the frontal section of the chicken embryonic head after 1 day post electroporation (embryo #7 for **A-D** and #4 for **E-H**). Enlarged view of the optic cup (OC) in (**A**) on the left (L) control side is shown in (**B**), and that of the right (R) *Lhx1*-overexpressed one is shown in (**C, D**). *BHLHE40* expression is downregulated in a portion of the outer layer of the OC (arrows in **C, D**). In embryo #4, the head sections were cut oblique and the left control OC is not seen in (**A**). Enlarged view of the right OC is shown in (**G**) and that of the left control OC is shown in (**H**). *BHLHE40* expression is downregulated in two portions of the outer layer of the OC (arrows in **G**). Cell nuclei were stained with nuclear fast red. Scale bars: 200 μ m (**A, E, F**), 100 μ m (**B, C, H**), 70 μ m (**G**), 50 μ m (**D**).

Figure A6: Expression patterns of *BHLHE40* (**A,A'**) and *BHLHE40* (**B,B'**) in the developing chicken eye at stage 20. *BHLHE41* is expressed by developing retinal ganglion cells at the

central retina in addition to the developing RPE and ciliary marginal zone. le, lens vesicle; nr, neural retina; rpe, developing RPE. Scale bars: 100 μ m.

Figure A7: Alignment of coding DNA sequences for chicken BHLHE40 and BHLHE41. The nucleotide identity is 63%. Identical nucleotides are highlighted in yellow.

Figure A8: Alignment of BHLHE40 and BHLHE41 proteins of chicken, human, mouse and zebrafish. bHLH and orange domains, and alanine and glycine-rich region in the C-terminus are shown. Gene IDs for referenced amino acid sequences are as follows: CHICK BHLHE40 (416108), HUMAN BHLHE40 (8553), MOUSE BHLHE40 (20893), DANRE BHLHE40 (324413), CHICK BHLHE41 (101750689), HUMAN BHLHE41 (79365), MOUSE BHLHE41 (79362), DANRE BHLHE41 (563771).

Figure A9: Expression patterns of *TEAD3* and *TFAP2A* in the developing chicken eye. (A-D) *TEAD3* is expressed in the surface ectoderm (SE) and developing eye at stages 16 (A,B) and 20 (C,C',D). (C') Enlarged view of the developing retina is shown. *TEAD3* mRNA signals are observed in the RPE as well as the basal vitread surface of the neural retina. (E-H) *TFAP2A* is expressed in the SE and lens vesicle. Results using sense control probes are shown in (B,D,F,H). il, inner layer of the optic cup; le, lens vesicle; nr, neural retina; ol, outer layer of the optic cup; rpe, developing RPE. Scale bars: 50 μ m (A,B,E,F), 100 μ m (C,D,G,H), 25 μ m (C').

Figure A10: Expression patterns of *TFAP2C* and *KLF2* in the developing chicken eye. (A-D) *TFAP2C* is expressed in the surface ectoderm (SE), lens vesicle, and by a subset of cells in the ocular mesenchyme (C). (E-H) *KLF2* is expressed by a subset of cells in the ocular mesenchyme just beneath the RPE (G). il, inner layer of the optic cup; le, lens vesicle; nr, neural retina; ol, outer layer of the optic cup; rpe, developing RPE. Scale bars: 100 μ m.

Figure A11: Expression pattern of *KLF5* in the developing chicken eye. *KLF5* is expressed in the surface ectoderm of the developing eye (**A,C**) and outside the eye (**E**) as well as the endoderm of developing trachea, bronchus and esophagus. br, bronchus; es, esophagus; il, inner layer of the optic cup; le, lens vesicle; nr, neural retina; ol, outer layer of the optic cup; rpe, developing RPE; tr, trachea. Scale bars: 100 μ m.

Table A1: PCR primer sequences used in this study.

Table A2: Antibodies used in this study.

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Supplementary Data for

Involvement of a basic helix-loop-helix gene *BHLHE40* in specification of chicken retinal pigment epithelium

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Figure A1

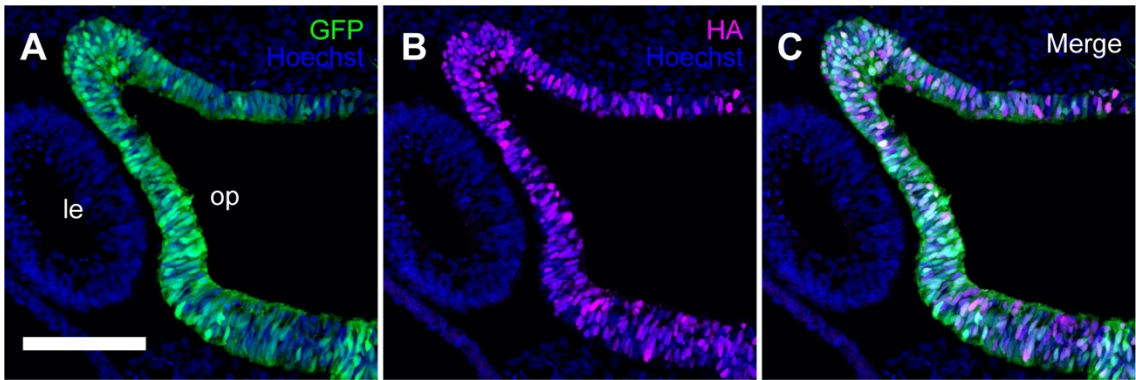


Figure A2

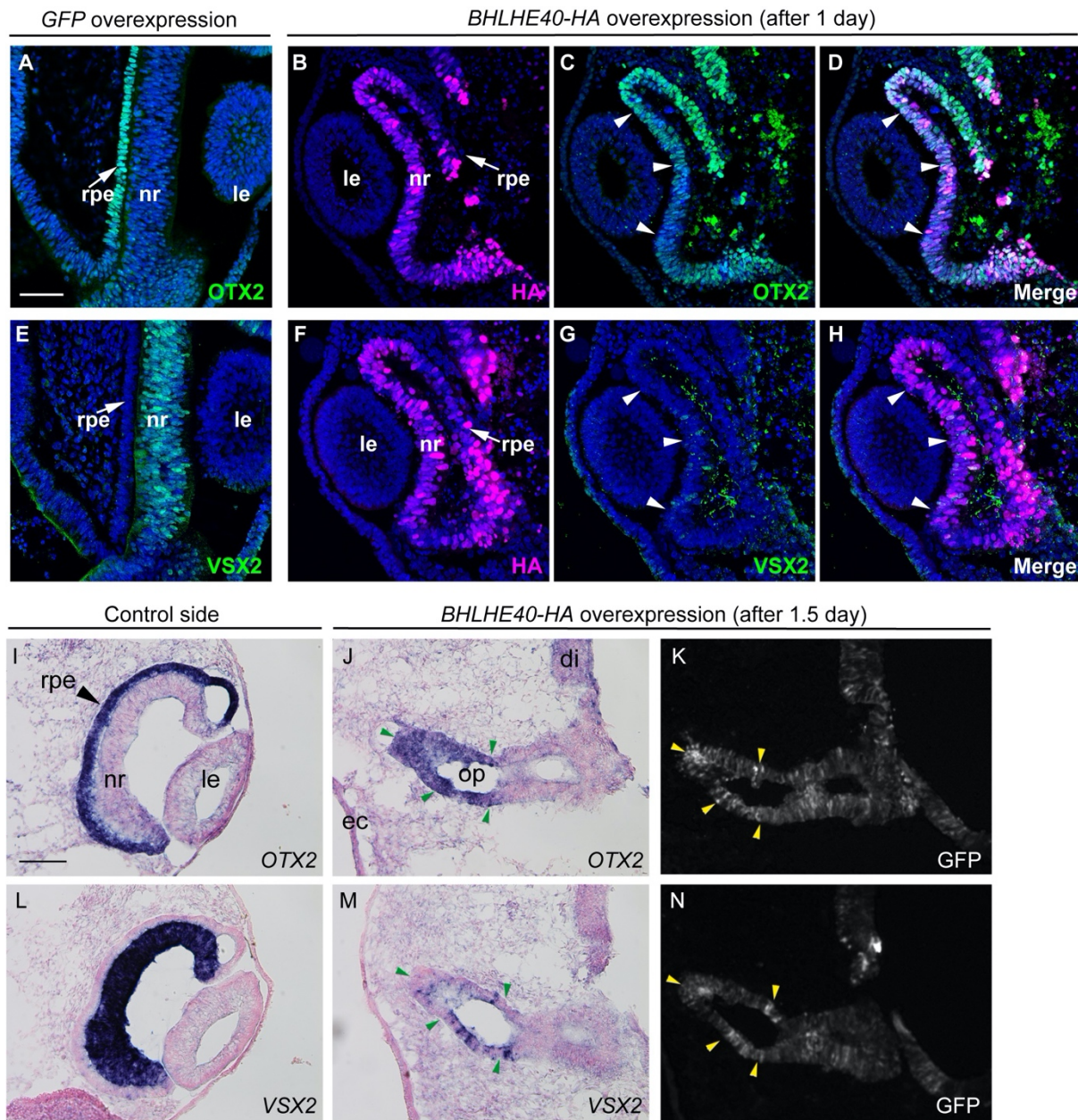


Figure A3

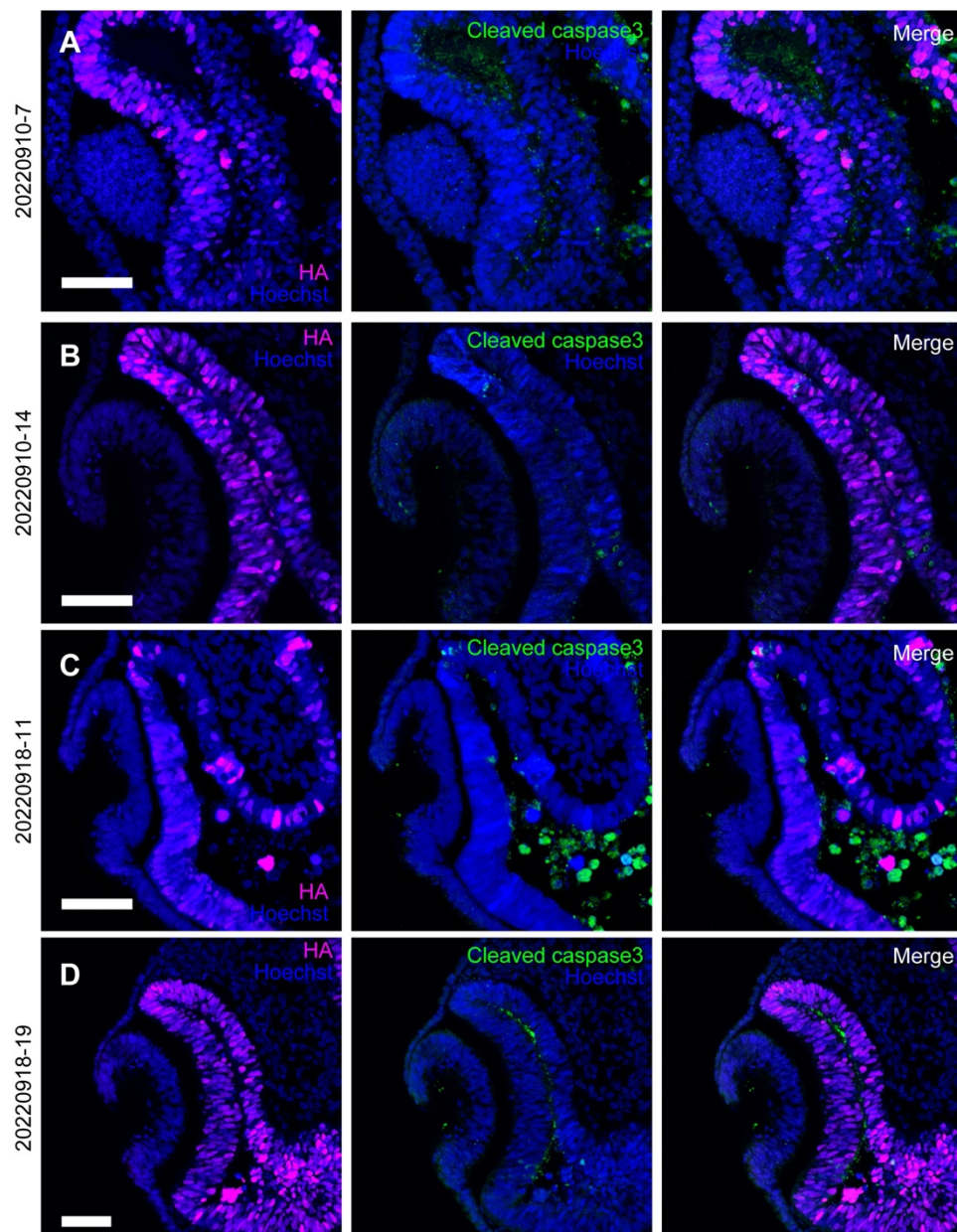


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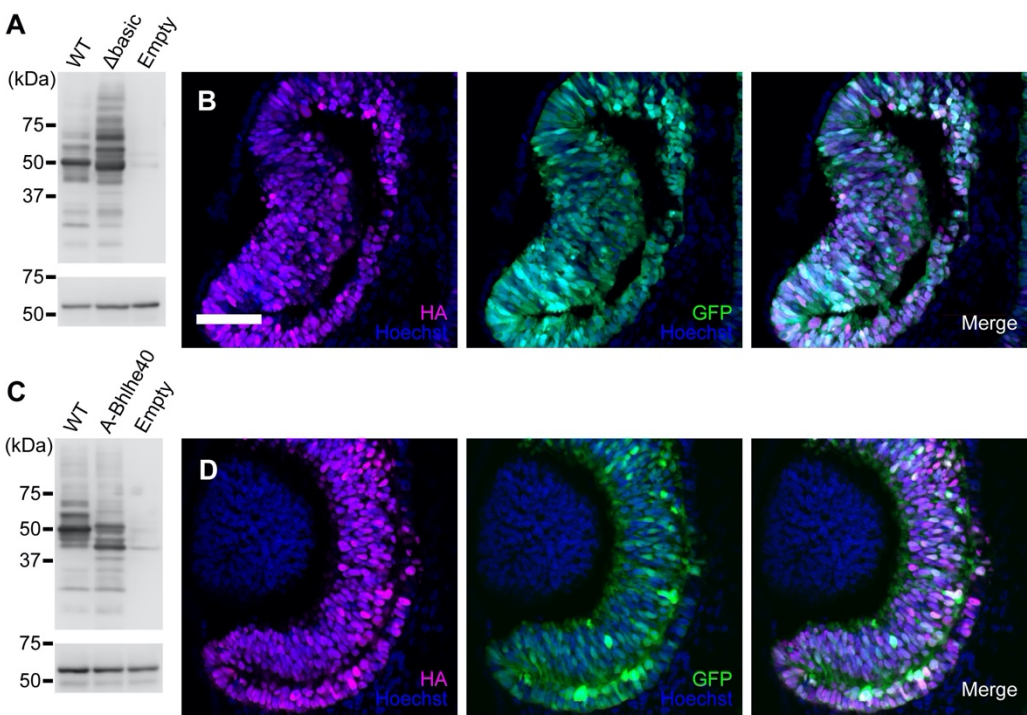
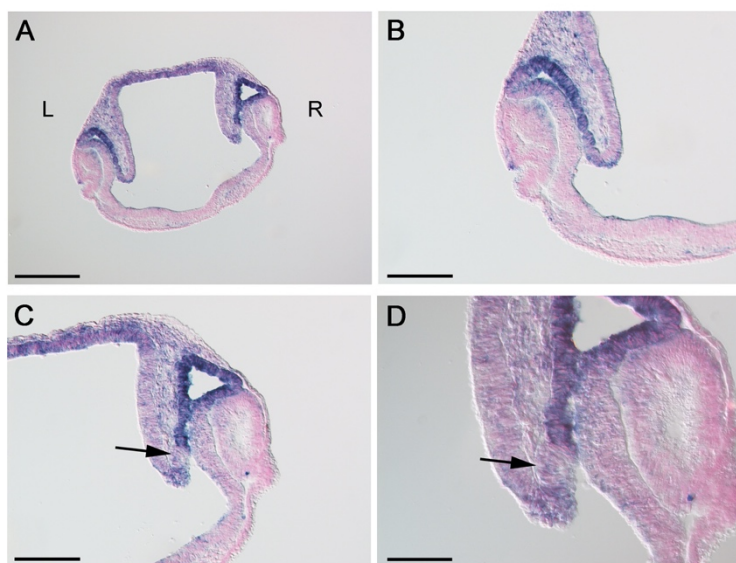


Figure A5

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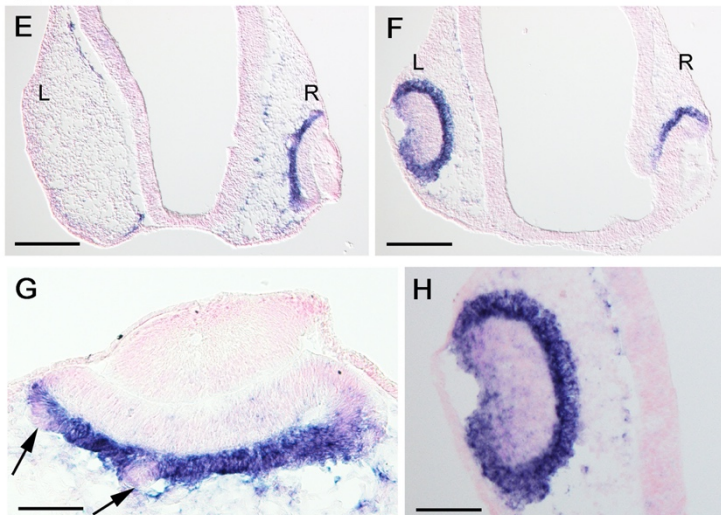


Figure A6

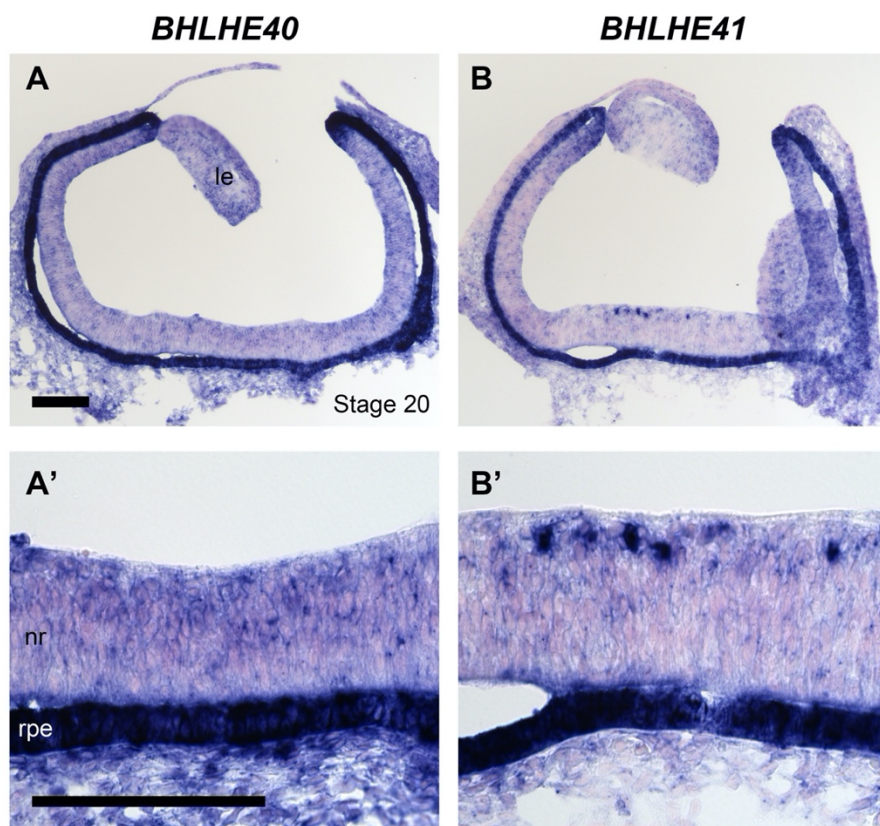


Figure A7

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CHICK BHLHE40 ATGGAGCGCA TCCCCAGCGC GCAGGCCCGC CCGTGTGGC TGGGCAAGGT GCCCGCCCTG GAG-AGCGGC GATGTCCCG GGTGGACIT GCGG-CACAT
CHICK BHLHE41 ATGGA-CG-- ----AG-G- GCATCTCCCG CC----TGCC CGAGCG-GCA GCT-GC--TG GAGCACCGG- GATTTCCTAG GGTGGAGTA CCGGGGCG-T

      110     120     130     140     150     160     170     180     190     200
CHICK BHLHE40 GTACCAAGTG TACAAGCCCA GGAGGGGGTT AAAGAGGAGC GAGGACAGCA AGGACACCTA CAAAGCTGCC CACAGGCTGA TAGAGAAGAA GCGGCGCGAT
CHICK BHLHE41 CTAC-ATGTG --CAAGCCCA AGCGCGGCGT GAAGCGGGAG GAG---AGCA AGGAGACTA CAAACTGCCG CACCGGCTGA TCGAGAAGAA GCGGCGGGAC

      210     220     230     240     250     260     270     280     290     300
CHICK BHLHE40 AGGATCAACG AGTGCATCGC CCAGCTCAAG GACCTGTGTC CCGAGCACCT CAAGCTGACG ACCCTGGGCG ACCTGGAGAA GGCGGTGGTG CTGGAGCTGA
CHICK BHLHE41 AGGATCAACG AGTGCATCGC GCAGCTGAAG GACCTGTGTC CCGAGCACCT CAAGCTCAGC ACGCTGGGCG ACCTGGAGAA GGCGGTGGTG CTGGAGCTGA

      310     320     330     340     350     360     370     380     390     400
CHICK BHLHE40 CCTGAAGCA CTTGAAGGGC CTGACGGGCG TCATCGAGCA GCAGCAGCAG AAGATCCTGG CGCTGCAGAG CCGGCTGCG GCGGTGACC TGTGACCGAG
CHICK BHLHE41 CCTGAAGCA CTTGAAGGGC CTGACGGGCG TCATCGAGCA GCAGCAGCAG AAGATCCTGG CGCTGCAGAG CCGGAGGCGC TCCG-TCAG TCTCCG-GTG

      410     420     430     440     450     460     470     480     490     500
CHICK BHLHE40 GAGCCTCGAC TCCAGCCAGG A-GATCTTCC GCTCCGGCTT CCAGCTGTGC GCCAAGGAGC TGGTGCAGTA TGTGGCC-- --A-AGGA CGACAACAAG
CHICK BHLHE41 CAGGC-CGAC ----CTGG ACGCT-TTCC ACTCCGGCTT TCAGACGTGC GCCAAGGAGG TGGTGCAGTT CCTGTCCCGC TCCGAGAGGT GGACCCCGCG

      510     520     530     540     550     560     570     580     590     600
CHICK BHLHE40 -GAGCTGAAG GCAGCGCG-A GCTGGTGGGG CACCTGCACC GCGTGGCGGC CGAGCTGCTG CCGGGC-- GGCCTCAA-- GACCTGAAGG
CHICK BHLHE41 CGAGCAG-- GCTGCGGCCA GCTCCTCGGC CACCTGCACC CCGTCTCTCT CGAGTCTCTG CCAGGGCCGC GGTCTCTCTC GCCGCGCGCG GCGGCGCTCG

      610     620     630     640     650     660     670     680     690     700
CHICK BHLHE40 AGAAGG-- ----CGGT ----GCGG GGGGGCAAAG CCGGCGAGGG GCGCGGGCAG AACTGCGTGC CCGTCATCCA
CHICK BHLHE41 CCAAGGACC CTCCTCTCTC TCCTCCCGCG CCGGCCCGT CCGCGCGCGG GCGCGCAA-G CCGG--AGGG -C-CAGGG-G CACTGCGTGC CCGTCATCCA

      710     720     730     740     750     760     770     780     790     800
CHICK BHLHE40 AAGGACATTC GGTGCCTCC- AGGCGGGAGC AGAGCGGCAG CGACACAGAC ACGGACAGCG GCTACGGGGG GGAGCTGGAG A----- AAAGCGACTC
CHICK BHLHE41 GCGGAC--TC AC-GCCCGCG AGCGGGGCGC CGAG---AC GCACACAGAC ACGGACAGCG GCTACGGCGG CGAGGCCGAG GCGCGGCCGG AGCGCGGGCG

      810     820     830     840     850     860     870     880     890     900
CHICK BHLHE40 CAACCCGGAG CAGCCCTATT TCCCAAGGA TACCGAACTG AAATACGGCG TCAGG-A-G AGGATCAGCT C-CATC-AAG CAG--GAG-- --AGCGAGG
CHICK BHLHE41 GGGGCCGGCG GGGCGC-- TCCCGCGCT GCCCTCAAG CAGGA-GCGG GCGGGGACG AGGCGCCGCG CCGGCCAAG CCGCGGAGGC TGGAGCGGGG

      910     920     930     940     950     960     970     980     990     1000
CHICK BHLHE40 ---ACCTTC CAGCCAAGAG GAGCA--GGC TGGAGGTGCC CGATGAT--G ACAGCCCTT TGGCG-GCGA GGTGATGG-G CAC--CGCGG GCGGCTTCT
CHICK BHLHE41 CGGCAGCCCG CCGCCCGCGC CGCCCTGCC CGCGCTGCC CGCGCGCGCG AC-GCCGCG AC-GCCGCG GCTGATGGC GCTGATGGC TTGGGCGCGG GCGGCGGGCG

      1010    1020    1030    1040    1050    1060    1070    1080    1090    1100
CHICK BHLHE40 GCGCCCCAC GCGCA-CCAG C--CCCCCT GTGCTGCCG TTCTAGCTGA TCCACCGTC --CGCCACC GCCTACCTGC ---CCATGCT GGAGAAGTGC
CHICK BHLHE41 GCGCCCCCT GCGCAGCCGG GGGCCCCCT GTGCTGCCG TTCTAGCTGA TCTCCGCTG GCGCGCGCG GCCTAGTGC AGCCTTCTT GGATTAAGGGC

      1110    1120    1130    1140    1150    1160    1170    1180    1190    1200
CHICK BHLHE40 ---TGG--- ----TA CCGGCCCTCC GTC--CCCG- TGCTGTACCC CCGGCTGCC GCGGCTGCC CTGC-GCTCA CCAGCTTTGT GGGCCCCGAC
CHICK BHLHE41 GGCCTGGAGA AGTACCTCTA CCGCGCGCGC CCGATCCCG CCCTTACCC TGCTGTACCC CCGCATCCG GCGGAGCGG CCGCGC-CG CG-GCGCGG CCGCGCGGGC

      1210    1220    1230    1240    1250    1260    1270    1280    1290    1300
CHICK BHLHE40 AAGCTGTGCC C-GCCACTG C---GCTT- TCGT-CCG CC---AAA GACTGCC- CT--CCCGG GGGG---T GGGCGCTGCC GATTCGAATC
CHICK BHLHE41 C-GCGCGTC CTTCCG-CTG GCTCTCGTCC GTGCTCGGCG CCGCCGAAA GGGGGCGGG CTGTCCCCGG CGCCCACTT GCC-GC-CCG CCTTCGCGCG

      1310    1320    1330    1340    1350    1360    1370    1380
CHICK BHLHE40 CTGGGCGC ---CTG CTGC--AG-- --GCGCTGAA GCAGATCCG- ---CG--CGT TGAACCTGGA AACCAAGAC --TAA
CHICK BHLHE41 CCGCGCGGCC GCGGTGCCG CCGCCGAGCC CGCGGAGAA GCGGAGCCCG CGCGCGCGA GAGGCCCGG GCGGAAGGC CGTGA
```

Figure A8

		10	20	30	40	50	60	70	80	90	100
CHICK	BHLHE40	M-ERIPSAQ	PPVCILGKLP	LESADVPGLD	FAHMYQVYK	RRGLKRSRSD	KD TYKLPHRL	TEKKRRDRIN	ECIAQLKDLL	PEHLKLITLG	H--LEKAVVL
HUMAN	BHLHE40	M-ERIPSAQ	PPACILPKAP	LEHGDLPGMY	PAHMYQVYKS	RRGIKRSRSD	KET TYKLPHRL	TEKKRRDRIN	ECIAQLKDLL	PEHLKLITLG	H--LEKAVVL
MOUSE	BHLHE40	M-ERIPSAQ	PPPTCLKFAP	LEHGDLSGMD	FAHMYQVYKS	RRGIKRSRSD	KET TYKLPHRL	TEKKRRDRIN	ECIAQLKDLL	PEHLKLITLG	H--LEKAVVL
DANRE	BHLHE40	M-ERISASQ	PP--CMSKFPG	LDISDMGMD	FP--MY-VYKP	RRMKRSRSD	KD TYKLPHRL	TEKKRRDRIN	ECIAQLKDLL	PEHLKLITLG	H--LEKAVVL
CHICK	BHLHE41	MDEGISRLPE	RQ-----L	LEHRDFLGL	YPALY-MCKP	KRGVKR-DES	KET TYKLPHRL	TEKKRRDRIN	ECIAQLKDLL	PEHLKLITLG	H--LEKAVVL
HUMAN	BHLHE41	MDEGIPLHQE	RQ-----L	LEHRDFLGL	YSSLY-MCKP	KRSMKR-DDT	KD TYKLPHRL	TEKKRRDRIN	ECIAQLKDLL	PEHLKLITLG	H--LEKAVVL
MOUSE	BHLHE41	MDEGIPLHQE	RQ-----L	LEHRDFLGL	YSSLY-MCKP	KRSLKR-DDT	KD TYKLPHRL	TEKKRRDRIN	ECIAQLKDLL	PEHLKLITLG	H--LEKAVVL
DANRE	BHLHE41	MEERISRMQN	RQ-----F	LDHADFLGVE	YSSLY-MCKS	KRGMKR-EEG	KD TYKLPHRL	TEKKRRDRIN	ECIGQLKDLL	PEHLKLITLG	H--LEKAVVL
basic helix-loop-helix (bHLH) domain											
		110	120	130	140	150	160	170	180	190	200
CHICK	BHLHE40	ELTLKHVKAL	TALIEQQQQK	IALAQSGLQA	GDLARSRLSD	QGM F RSFGQ	LC AKEL LQYV	AKHD--N-KE	LKAQQLVGH	HRVAEELLQG	G-----
HUMAN	BHLHE40	ELTLKHVKAL	TALIEQQQQK	IALAQSGLQA	GELSGRNVET	SQEM F CSGFQ	TCAREVLQYL	AKHE--NTRD	LKSSQLVTHL	HRVSELLPQG	G-----T
MOUSE	BHLHE40	ELTLKHVKAL	TNLIDQQQQK	ITIALQSGLQA	GDLSGRNLEA	QGM F CSGFQ	TCAREVLQYL	AKHE--NTRD	LKSSQLVTHL	HRVSELLPQG	G-----A
DANRE	BHLHE40	ELTLKHVKAL	NNLLEQQQQK	ITSLQNGSLQ	GKGNGPNSEN	SEEM F RSFGH	LC AKEL LQFL	ANQE--TMRD	LTTAHIIEHL	QKVASELIQS	G-----P
CHICK	BHLHE41	ELTLKHKHAL	QALTEQQHQK	IVALQSGHGS	VK----SPVQA	DLDA F HSFGQ	TC AKEL VQYL	SRFESWTTPRE	QRCAQLLGH	HAVVSQFLPG	PRLLSPPPGP
HUMAN	BHLHE41	ELTLKHKHAL	TALTEQQHQK	IALQNGERS	LK----SPIQS	DLDA F HSFGQ	TC AKEL VQYL	SRFESWTTPRE	PRCVQLINHL	HAVATQFLPT	PQLLTQQV-P
MOUSE	BHLHE41	ELTLKHKHAL	TALTEQQHQK	IALQNGERS	LK----SPVQA	DLDA F HSFGQ	TC AKEL VQYL	ARFESWTTPRE	PRCAQLVSHL	HAVATQLL-T	PQV-----P
DANRE	BHLHE41	ELTLKHLNAL	TAVTEQQHQK	ITIALQNGERS	LK----SSLQA	DLDA F HSFGQ	AC AKEL VQYL	NKVENWTARE	QRCTRILINHL	HKVSAQFPQG	TGILQQ----
Orange domain											
		210	220	230	240	250	260	270	280	290	300
CHICK	BHLHE40	-----PK	DLKEKAVPG-	--GKAGEGRG	QNCVPVIQRT	FAASSGEQSG	SDTDTDSGYG	GELEKSDSKP	EQP--YFPKD	TEBKVAVQER	TSIIKIQE--S
HUMAN	BHLHE40	SRKPSDPAPK	VMDFKKEKPS	P-AKSGSEGPG	KNCVPVIQRT	FAHSSGEQSG	SDTDTDSGYG	GESEKGDLSR	EQP--CFKSD	HGRRFTMGER	IGAIIKQE--S
MOUSE	BHLHE40	SRKPLDSAPK	AVDLKEKPSF	L-AKSGSEGPG	KNCVPVIQRT	FAPSGGEQSG	SDTDTDSGYG	GELEKGDLSR	EQP--YFKSD	HGRRFAVGER	VSTIKIQE--S
DANRE	BHLHE40	SPRLDEPASF	AQESREKPSG	LQPKAAEGHA	KNCVPVIQRT	YPHSS-EQSG	SDTDTDSGYG	GEYEKRDQAS	QRPDCCYVKS	GALKYS----	--SIIKEE--Q
CHICK	BHLHE41	LAKG--PS	SSSSPPAP-L	RAPAKPEGQ	AHCVPVIQRT	H-AAE-PGAE	TDTDSDSGYG	GAEAAERPRE	AGP-----	----AGPLP	ALPVKIQEPP
CHICK	BHLHE41	LSKGTG-APS	AAGSAAAPCL	ERAGQKEPL	AYCVPVIQRT	QPSAE-LAAE	NDTDTDSGYG	GAEAAERPRE	KGK-----	----GAGLS	RVTIKQEPAG
MOUSE	BHLHE41	SGRGSGRAPC	SAGAAAAA--	-----GPERV	ARCVPVIQRT	QPTE--PE	HTDSDSGYG	GAEAQG----	-----GAGAS	-----	RAAVKQEPGP
DANRE	BHLHE41	-----FV	AGDDAPER--	-----DTQRDPQ	ANCVPVIQRT	Q--NLEINE	NDTDTDSGYG	GAEAKGDGKS	EKG-----	-----CDTAK	GVKIQE--FG
		310	320	330	340	350	360	370	380	390	400
CHICK	BHLHE40	EDPPAKRSRL	EVPDDDD--	-----	-----	-----	-----SPFG	GEVMGTAGGF	LPPHAHQFPL	CLPFYLIPPS	AT-AYL-PM-
HUMAN	BHLHE40	EEPTTKKNRM	QLSDDE---	-----	-----	-----	-----GHFTS	SDLIS--SPF	LGPHPHQPFP	CLPFYLIPPS	AT-AYL-PM-
MOUSE	BHLHE40	EEPTTKKNRM	QLSEEE---	-----	-----	-----	-----GHFAG	SDLMG--SPF	LGPHPHQPFP	CLPFYLIPPS	AT-AYL-PM-
DANRE	BHLHE40	DEPPSKRPRS	DSSEDE---	-----	-----	-----	-----SLSG	LDVGVGHSYP	VSFSPQP-PL	CMFFYLFPFG	AAAAYL-PM-
CHICK	BHLHE41	DEAPPAPKRP	RLEGGG---	-----	-----SPPPG	AALPGAA RGA	DAALLSSILMA	LGAGGGAAPF	GQP--AAAF	CLPFYFISIPS	AAAAYVQFPL
HUMAN	BHLHE41	EDSP-APKRM	KLDSRGGGSG	GGPGGGAAAA	AAALLGPDPA	AA--AAL RP	DAALLSSLIVA	FGGGGG-APF	PQPAAAAAPF	CLPFCFLSPS	AAAAYVQFPL
MOUSE	BHLHE41	DSSP-APKRP	KLDEARG--	-----	-----	-----ALIGP	EPALLGSLIVA	LGGG--APF	AQP--AAAPF	CLPFYLLSPS	AA--AYVQFPL
DANRE	BHLHE41	DERVTKKAMR	NWSANG--	-----	-----GSDST	ST----- RP	DVALMNSIMG	MTGVGG----	-----QQTPE	CMFFYFINPS	AAASYM-PLF
		410	420	430	440	450	460	470	480	490	500
CHICK	BHLHE40	---LEKWCY	PAS-----	--VPVLYPGLP	-----AP	AAALTSFVGP	DKLSPPLLLP	QRLSPSPGPA	-----	---RSPIDSS	ALLQALKQTP
HUMAN	BHLHE40	---LEKWCY	PTS-----	--VPVLYPGLN	-----AS	AAALSSFMMNP	DKISAPLLMP	QRLSPSLPA-	-----	---HPSVDSS	VLLQALKQTP
MOUSE	BHLHE40	---LEKWCY	PTS-----	--VPVLYPGLN	-----TS	AAALSSFMMNP	DKIPTPLLLP	QRLPSLPA-	-----	---HSSLDSS	ALLQALKQTP
DANRE	BHLHE40	---LEKWCY	PGA-----	--MPVLYPGLG	-----SS	PASLS--P	EKLPSMVMMS	SRVSGPVST-	-----	---TSMDSM	ALLQALKQTP
CHICK	BHLHE41	DKGGLEK YLY	PAA-----	FIPLLYPGIP	---AQAAAAA	AAAAAAASF	CLSSVLGPA-	EKAAGLSAP	HLP-----	---PFPAAAA	AAVPAEPGE
HUMAN	BHLHE41	DKSGLEK YLY	PAAAAA---	FPPLLYPGIP	APAAAAAAA	AAAAAAAFP	CLSSVLSPPP	EKAGA-AAAT	LLPHEVAPL	APHPQHPHR	THLPFAGRP
MOUSE	BHLHE41	DKSGDK YLY	PAAAAA---	FPPLLYPGIP	-----AA	AAAAAAAFP	CLSSVLSPPP	EKAGATAGP	FLAHEVAPPG	PLRPQHAHSR	THLPRA----
DANRE	BHLHE41	DKSHLEK LYY	PAAAAAALT	FPFWLYPGIP	-----THAS	AAAAAAIAP	NAST-----	DKTSGFDAAS	SKDE-----	---PESDDDD	LSNEADLASP
alanine/glycine-rich region											
		510	520								
CHICK	BHLHE40	PLNLETKD--	-----	---							
HUMAN	BHLHE40	PLNLETKD--	-----	---							
MOUSE	BHLHE40	PLNLETKD--	-----	---							
DANRE	BHLHE40	PLNLETKD--	-----	---							
CHICK	BHLHE41	EAEPA AAE	---EPGA---	EGP							
HUMAN	BHLHE41	PGNP ESSAQE	DPSQPGK--	EAP							
MOUSE	BHLHE41	VN PESSQ--E	DATQPAK--	DAP							
DANRE	BHLHE41	ASED HGSEID	TSHQQRNDN	DGT							

Figure A9

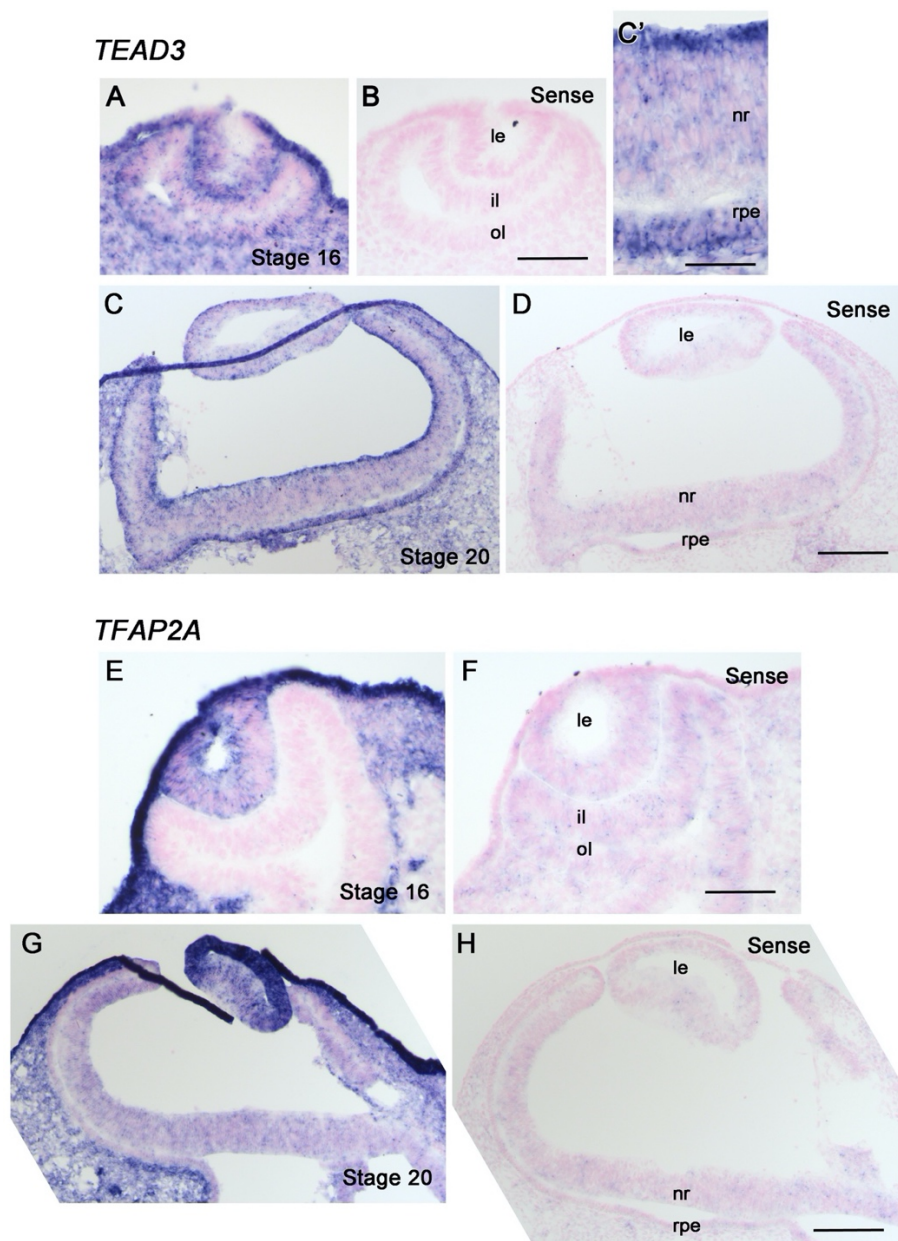


Figure A10

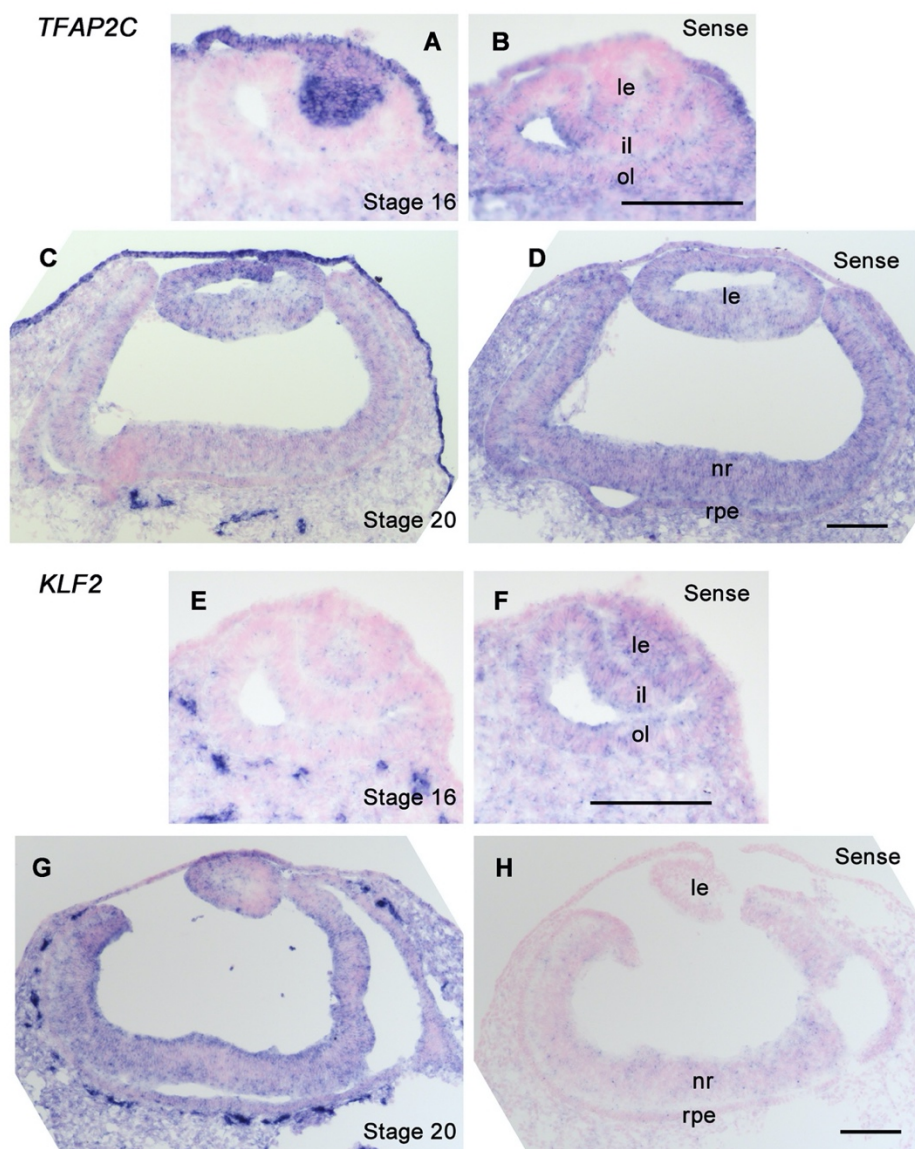


Figure A11

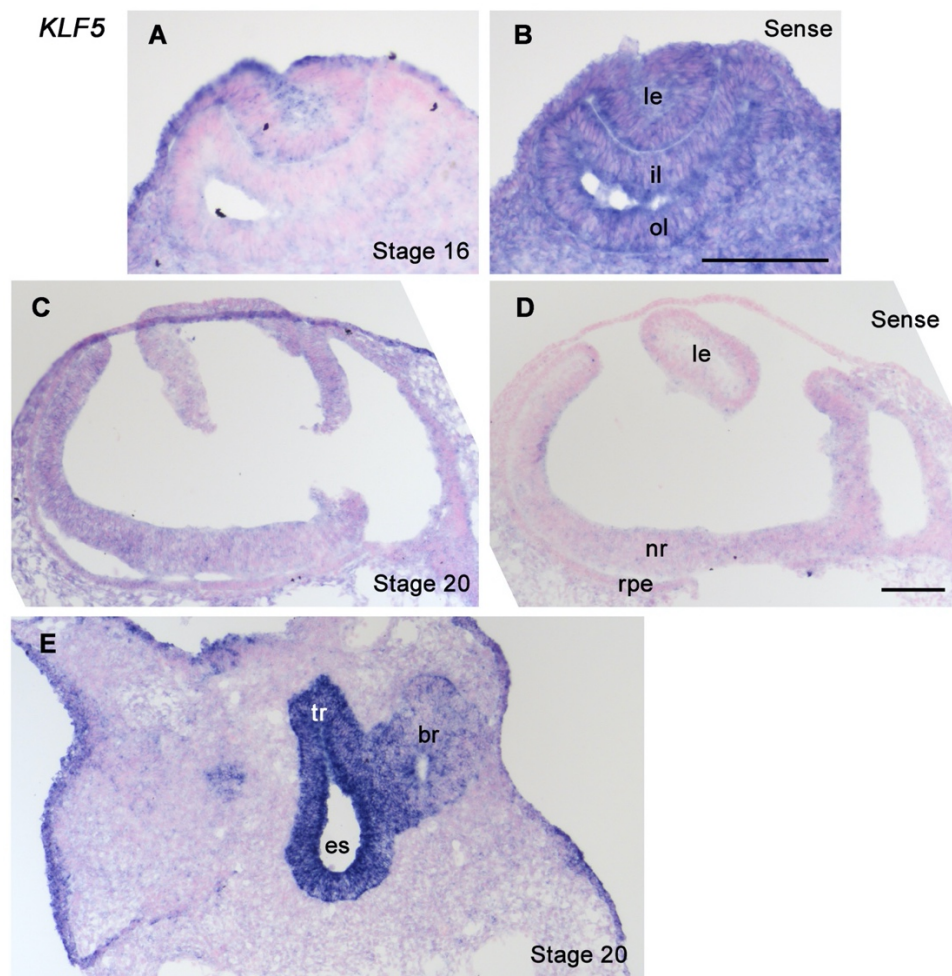


Table A1

Gene name	Amplion size (bp)	Primer name	Primer/DNA sequences (5'-3')	Purpose
<i>chicken</i> <i>BHLHE40</i> <i>cBHLHE40</i>	1193	<i>cbhlhe40</i> forward	CTGACCTGAAGCACGTCAA	doning a template cDNA to synthesize riboprobes
		<i>cbhlhe40</i> reverse	GACACATGCCAGCTGACTGA	
		<i>cbhlhe40</i> full forward	ATGGAGCGCATCCCCAGC	
<i>cBHLHE40</i>	1206	<i>cbhlhe40</i> full reverse	TTAGTCTTTGGTTTCCAAGTTCAAC	doning a full-coding sequence for <i>cBHLHE40</i>
<i>cBHLHE40</i>	1252	<i>Bhlhe40_Fw</i>	TAGCGTTAAACTTAAGCTTGGCAGCATGGCGATCCCCAGC	insertion of <i>cBHLHE40</i> into pCAGGS vector
		<i>Bhlhe40_Rv</i>	ACGGCCCTCTAGACTCGAGTGTAGTCTTTGGTTTCCAAGTTCAAC	
<i>cBHLHE40</i>	1279	<i>Bhlhe40_pCAG_Fw</i>	AGGGTTTAAACTTAAGCTTGGCAGCATGGCGAGCGCAIC	insertion of <i>cBHLHE40</i> into pCAGGS vector with HA-tag
		<i>Bhlhe40_HA_pCAG_Rv</i>	CGGGCCCTCTAGACTCGAGTGTAGCATAATCCGCACATCATACGGATAGTCTTTGGTTTCCCAAGTT	
<i>delta_basic</i> <i>cBHLHE40</i>	194	<i>ggcBhlhe40_delbasic_Fw</i>	AGGAGCGGAGACAGCAAGGATAGGATCAACGAGTGC	amplification of N terminal portion of HA-tagged <i>cBHLHE40</i> before basic region
		<i>ggcBhlhe40_delbasic_Rv</i>	GCACTCGTGTATCTATCTTGTGTCTCGCTCCT	
<i>delta_basic</i> <i>cBHLHE40</i>	1076	<i>pCAG_fw_HindIII</i>	AGCGTTTAAACTTAAGCTTG	amplification of C terminal portion of HA-tagged <i>cBHLHE40</i> after basic region
		<i>pCAG_Rv_XhoI</i>	CGGGCCCTCTAGACTCCGAGC	
<i>acidic</i> <i>extension</i> <i>cBhlhe40</i>	106	<i>a-Bhlhe40_ins_Fw_pCAG</i>	AGCGTTTAAACTTAAGCTTGGCCACATGGCATCAATGACTGGA	acidic extension sequence for replacement of N terminal portion of <i>cBHLHE40</i> including basic region
		<i>a-Bhlhe40_ins_Rv</i>	AACCTTCCAGCTCTCTCTTCATCATCTCTCTCTCATCGGGTCCCTCCCACTGCTGACCTCCAGTCAATGATGCCAT	
<i>acidic</i> <i>extension</i> <i>cBhlhe40</i>	1068	<i>a-Bhlhe40_Fw</i>	AGAAGAGCTGGAAAGAGTTGGAAGAGTGCATCGCCAGCTC	amplification of C terminal portion of HA-tagged <i>cBHLHE40</i> after basic region
		<i>pCAG_Rv_XhoI</i>	CGGGCCCTCTAGACTCCGAGC	
<i>chicken</i> <i>BHLHE41</i>	1776	<i>ggc_bhlhe41_Fw</i>	CCCGCGGATCCCGAAAGC	
		<i>ggc_bhlhe41_Rv</i>	GCACTGCGCGCGGCCATATAA	
<i>chicken</i> <i>TEAD3</i>	1299	<i>ggc_lead3_Fw</i>	ATGGACAAGAGCTCTGGACAATGACGCCGAG	
		<i>ggc_lead3_Rv</i>	CTAGTCTCTCAACAACTGTAGACATCATG	
<i>chicken</i> <i>TEAP2A</i>	1640	<i>ggc_tfap2a_Fw</i>	GGGATCGGACCCCTCTCCCGC	doning template cDNAs to synthesize riboprobes
		<i>ggc_tfap2a_Rv</i>	CTGGCTCCGTCGCCCTGTTT	
<i>chicken</i> <i>TEAP2C</i>	1347	<i>ggc_tfap2c_Fw</i>	ATGTTGTGGAAACTGGCAGATAACGTCAAG	
		<i>ggc_tfap2c_Rv</i>	TTACTTCTGCTGCTTGCTCATTTATCGAT	
<i>chicken</i> <i>KLF2</i>	1536	<i>ggc_klf2_Fw</i>	CCATGGCGCTGAGCGGATACCA	
		<i>ggc_klf2_Rv</i>	AACACCGAGCTAGAGCAACGCTC	
<i>chicken</i> <i>KLF5</i>	1504	<i>ggc_klf5_Fw</i>	GAGGCATTGAGGAGCGCGA	
		<i>ggc_klf5_Rv</i>	GCCTCGGCAACAACCTCTGGCT	

Table A2

Antibody		Vender	Catalog#	Dilution	Final concentration	Diluted in	Antigen retrieval	Antibody concentration
Anti-OTX2	Rabbit polyclonal	abcam	ab21990	1000	1 ug/mL	IMMUNO SHOT immunostaining, Fine	None	1 mg/mL
Anti-VSX2	Sheep polyclonal	Ex Alpha	X1180p	400	0.25 ug/mL	IMMUNO SHOT immunostaining, Mild	in TE (pH 8.0) at 98°C, 45 min	1 mg/mL
Anti-Cleaved caspase 3	Rabbit monoclonal	R & D systems	MAB835	1000	0.5 ug/mL	IMMUNO SHOT immunostaining, Fine	None	0.5 mg/mL
Anti-HA	Rat monoclonal	Roche	ROAHAHA	200 or 400	0.5 or 0.25 ug/mL	IMMUNO SHOT immunostaining, Fine or Mild	None	0.1 mg/mL
Anti-OTX2 + Anti-HA				1000(OTX2), 200 (HA)		IMMUNO SHOT immunostaining, Fine	None	
Anti-VSX2 + Anti-HA				400(VSX2), 400 (HA)		IMMUNO SHOT immunostaining, Mild	in TE (pH 8.0) at 98°C, 45 min	
Anti-sheep IgG Alexa Fluor 488	Donkey polyclonal	Thermo Fisher Scientific	A-11015	1000	2 ug/mL	1% BSA, 0.1% Triton-X100 in PBS	Not applicable	2 mg/mL
Anti-rat IgG Alexa Fluor 488	Goat polyclonal	Thermo Fisher Scientific	A-11006	1000	2 ug/mL	1% BSA, 0.1% Triton-X100 in PBS		2 mg/mL
Anti-rat IgG Alexa Fluor 568	Donkey polyclonal	abcam	ab175708	1000	2 ug/mL	1% BSA, 0.1% Triton-X100 in PBS	Not applicable	2 mg/mL
Anti-rabbit IgG Alexa Fluor 647	Goat polyclonal	abcam	ab150087	1000	2 ug/mL	1% BSA, 0.1% Triton-X100 in PBS	Not applicable	2 mg/mL