Supplementary Materials

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

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Figure S1

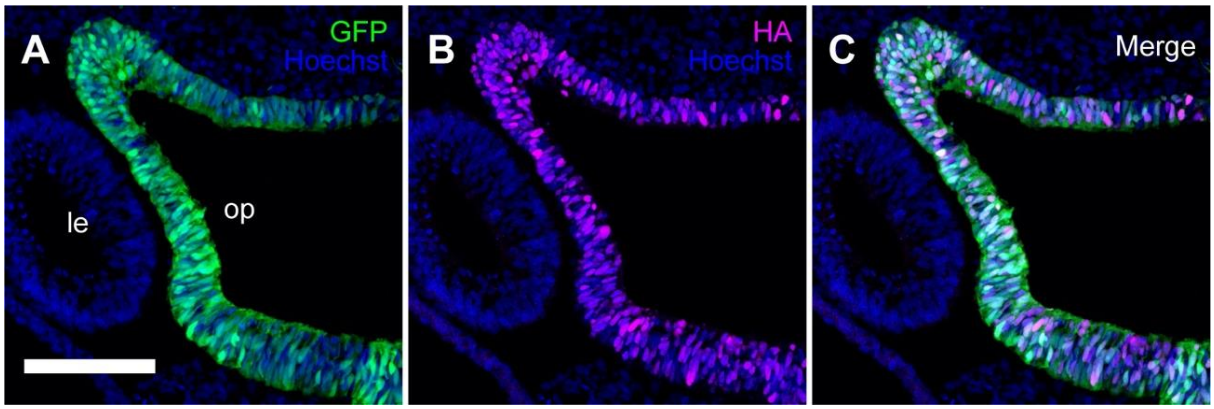

Figure S1 (data related to Figures 2 and 3): In the optic cup after *BHLHE40-HA* overexpression (from embryo #17, same as shown in Figure 3I-N), co-electroporated GFP fluorescence (**A**) is overlapped with BHLHE40-HA protein localization (**B**). Merged image of (**A**) and (**B**) is shown in (**C**). 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 S2

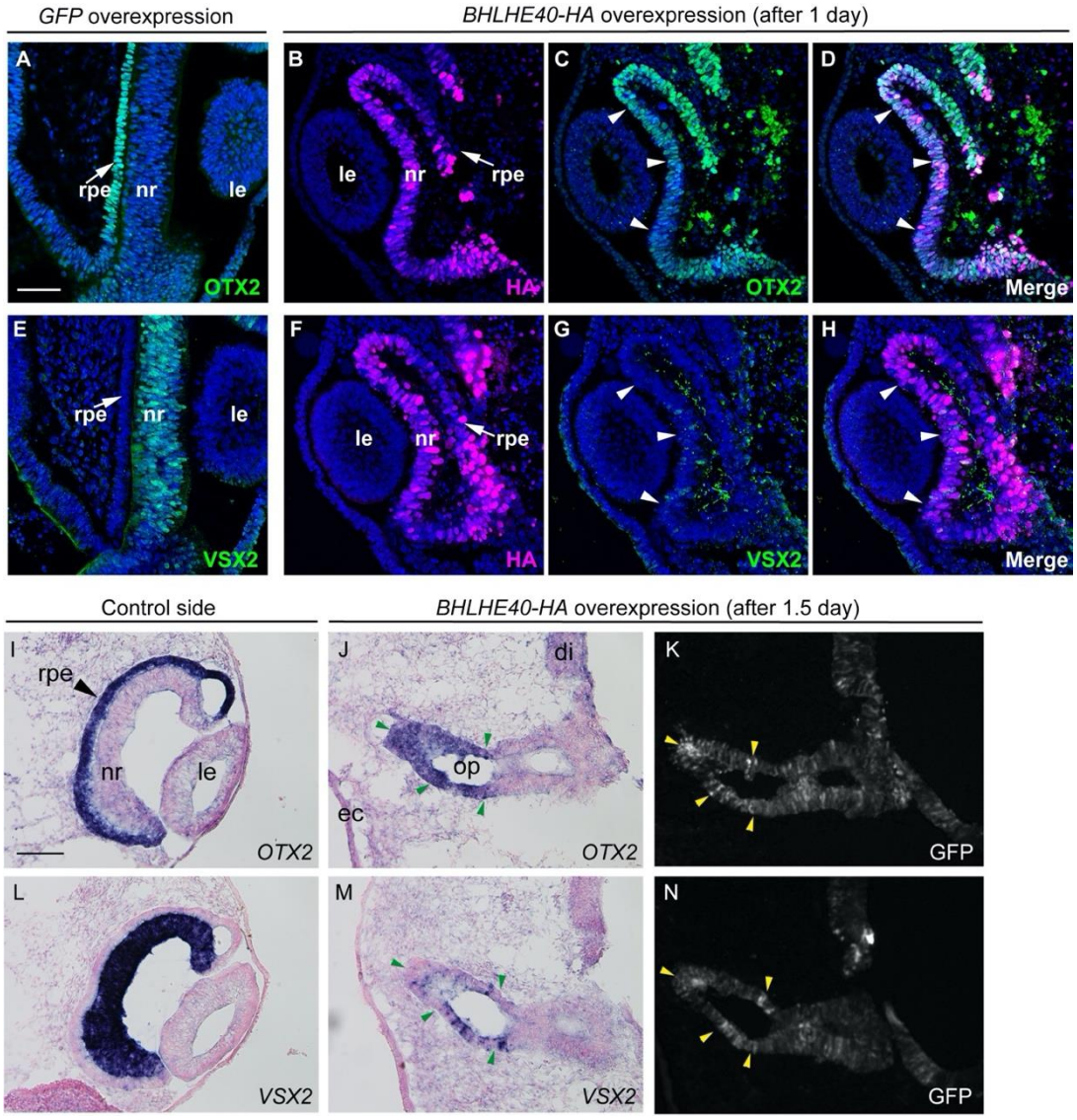

Figure S2 (data related to Figure 3): Similar data of Figure 3 from other embryos, except for those after GFP expression (**A, E**), which are identical images as shown in Figure 3A and E. Frontal sections of the optic cup from three embryos (*GFP*#2, *BHLHE40*#11, *BHLHE40*#22) are shown. Dorsal is 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 to 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 S3

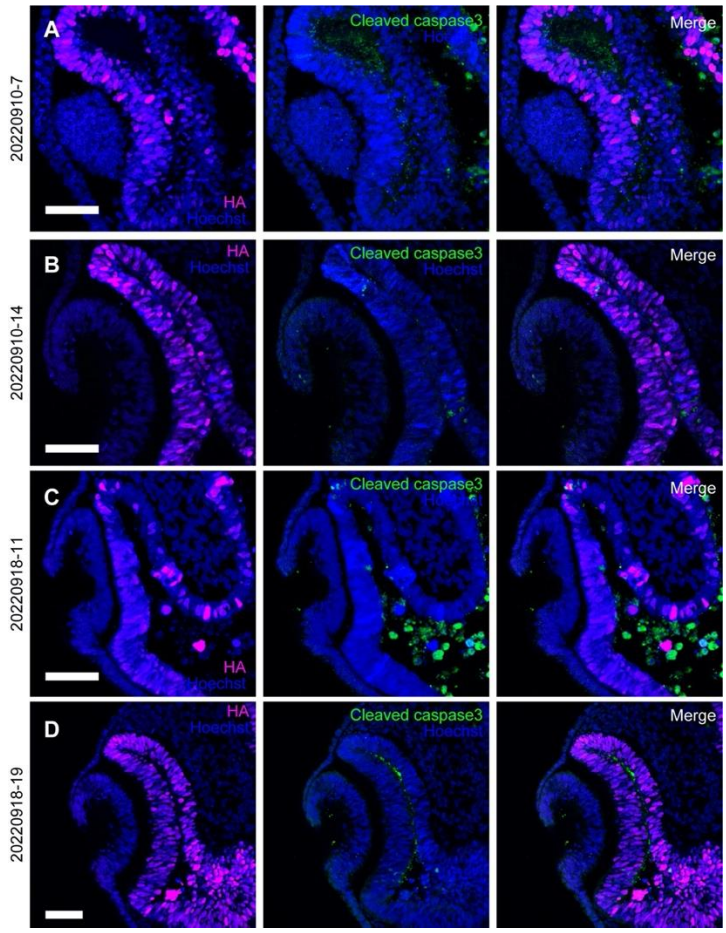

Figure S3: *BHLHE40-HA* expressing cells do not show cell death as revealed by immunofluorescence using anti-cleaved caspase 3. In four embryos examined (**A-D**) (embryo identification numbers are shown on the leftmost), none of the HA-immunoreactive cells are positive for cleaved caspase 3. Cell nuclei were stained with Hoechst 33342. Shown images are maximum intensity projections of confocal z-series. Scale bar: 50 μm in (**A-D**) for all images.

Figure S4

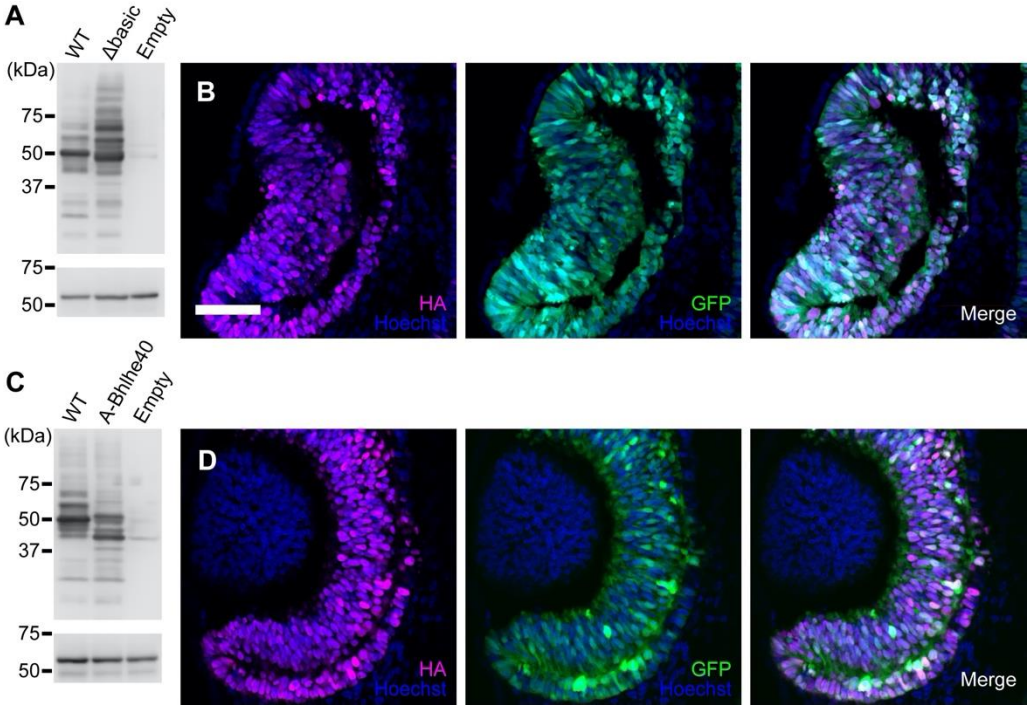

Figure S4: Validation of expression of HA-tagged presumed dominant negative forms of BHLHE40. (**A, C**) Western bot analysis of HEK293T cells transfected with chicken Δbasic *BHLHE40*-HA/pCAGGS (**A**) or Δbasic with acidic extension *BHLHE40*-HA (**C**) vectors. Alpha tubulin from the same samples was detected for loading reference. (**B, D**) Confocal microscopic images show that HA-tagged presumed dominant negative forms of BHLHE40 are detected 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 and merged images are shown in the middle and right panels, respectively. Cell nuclei were stained with Hoechst 33342. Shown confocal images are maximum intensity projections of z-series. Scale bar: 50 μm in (**B**) for all confocal images.

Figure S5

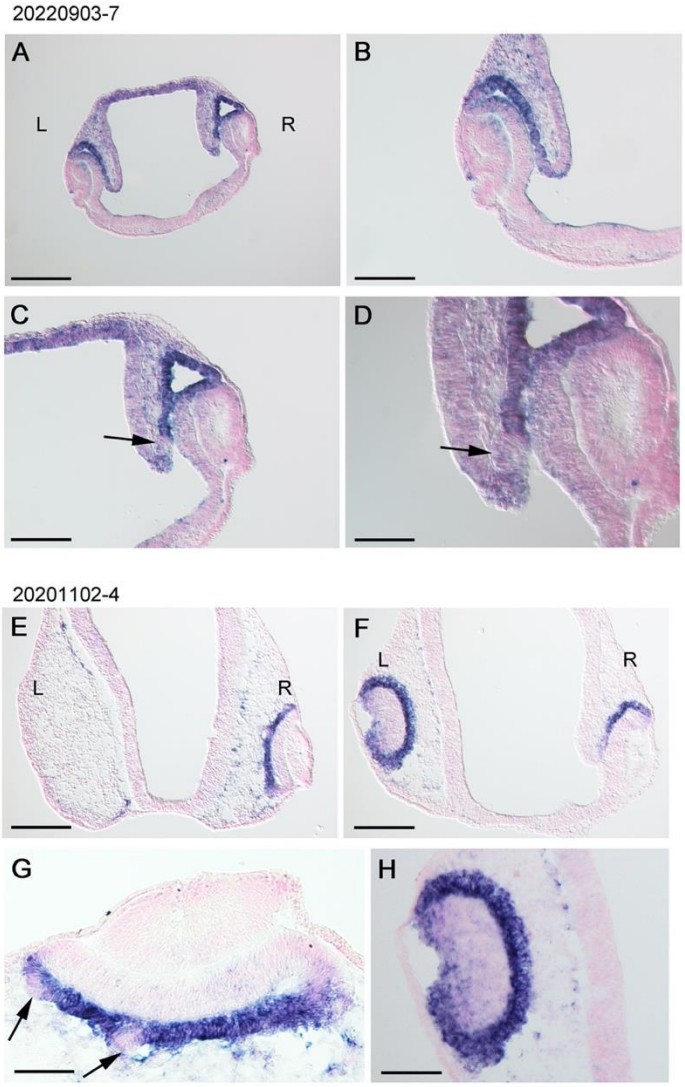

Figure S5 (data related to Figure 4): In situ hybridization (ISH) on the frontal section of the chicken embryonic head after 1 day post electroporation (embryo #7 for **A-D** and #4 for **E-H**). Enlarged view of the optic cup (OC) in (**A**) on the left (L) control side is shown in (**B**), and that 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 μm (**A, E, F**), 100 μm (**B, C, H)**, 70 μm (**G**), 50 μm (**D**).

Figure S6

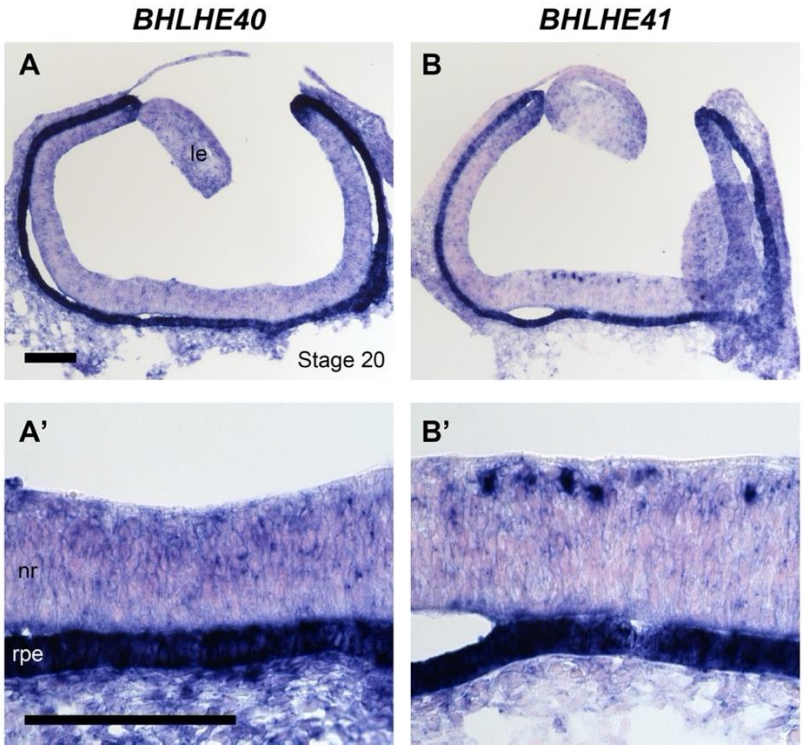

Figure S6: Expression patterns of *BHLHE40* (A,A') and *BHLHE41* (B,B') in the developing chicken eye at stage 20. *BHLHE41* is expressed by developing retinal ganglion cells at the central retina in addition to the developing RPE and ciliary marginal zone. le, lens vesicle; nr, neural retina; rpe, developing RPE. Scale bars: 100 μm.

Figure S7

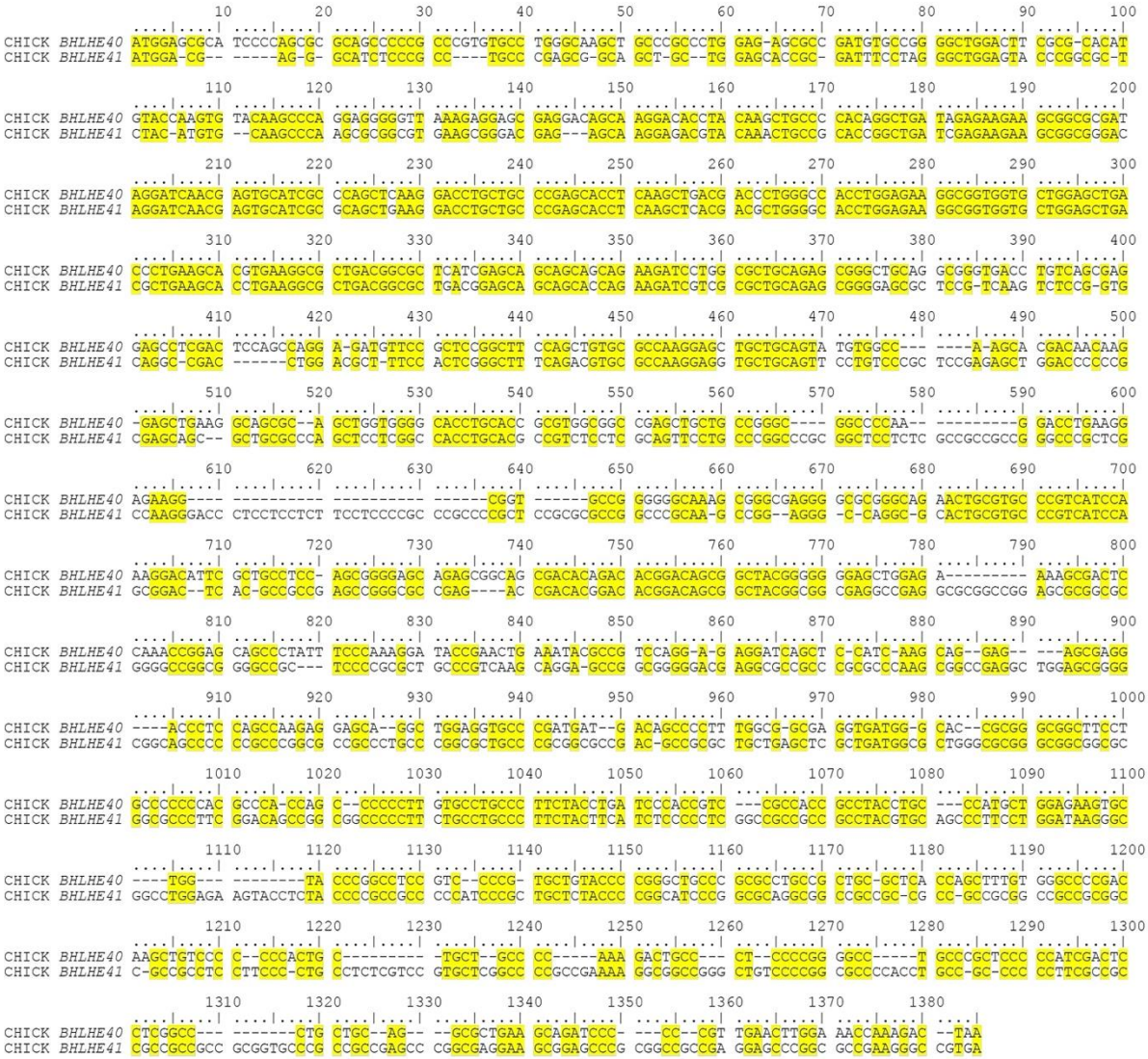

Figure S7: Alignment of coding DNA sequences for chicken BHLHE40 and BHLHE41. The nucleotide identity is 63%. Identical nucleotides are highlighted in yellow.

Figure S8

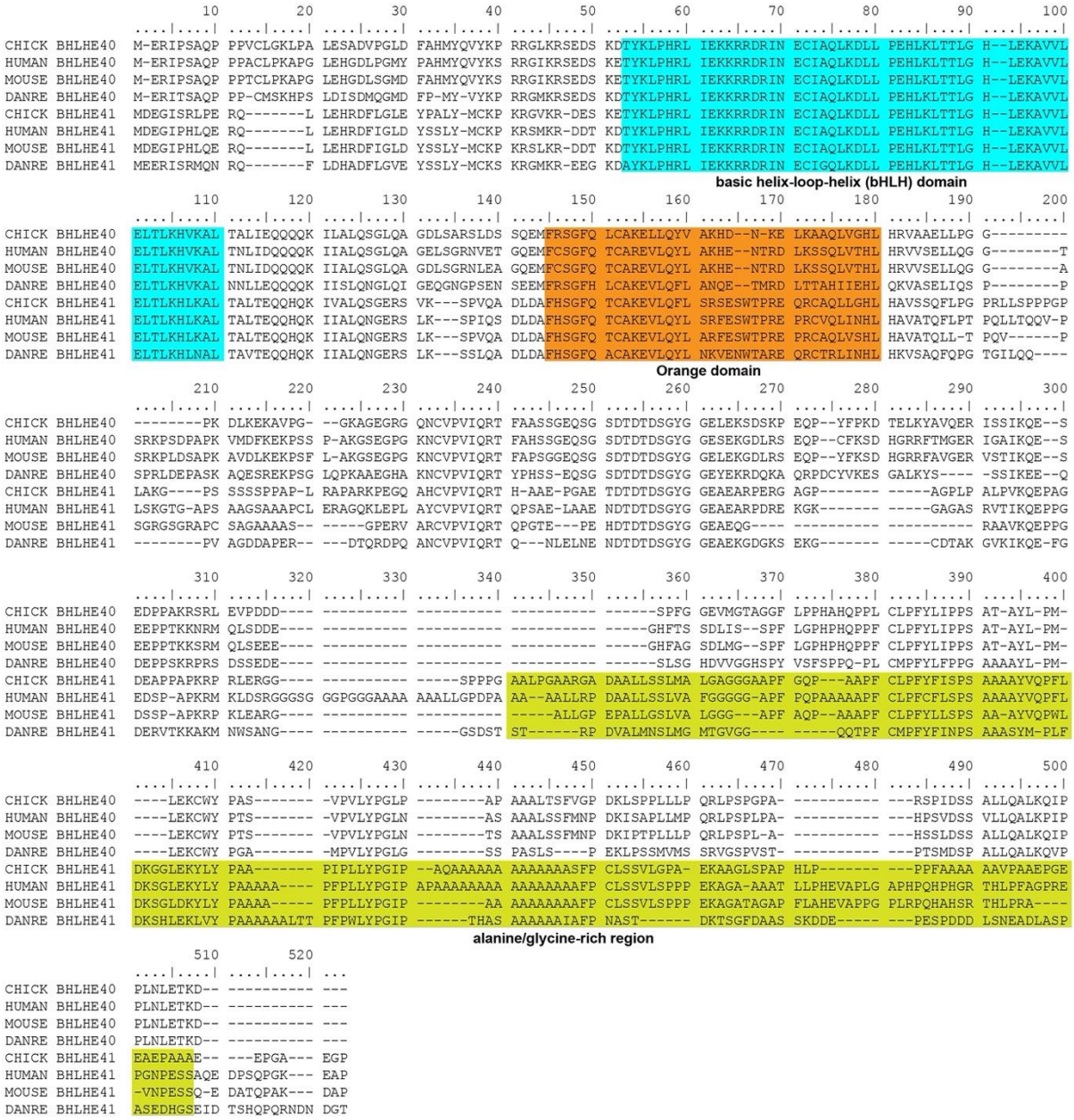

Figure S8: Alignment of BHLHE40 and BHLHE41 proteins of chicken, human, mouse and zebrafish. bHLH and orange domains, and alanine and glycine-rich region in the C-terminus are shown. Gene IDs for referenced amino acid sequences are as follows: CHICK BHLHE40 (416108), HUMAN BHLHE40 (8553), MOUSE BHLHE40 (20893), DANRE BHLHE40 (324413), CHICK BHLHE41 (101750689), HUMAN BHLHE41 (79365), MOUSE BHLHE41 (79362), DANRE BHLHE41 (563771).

Figure S9

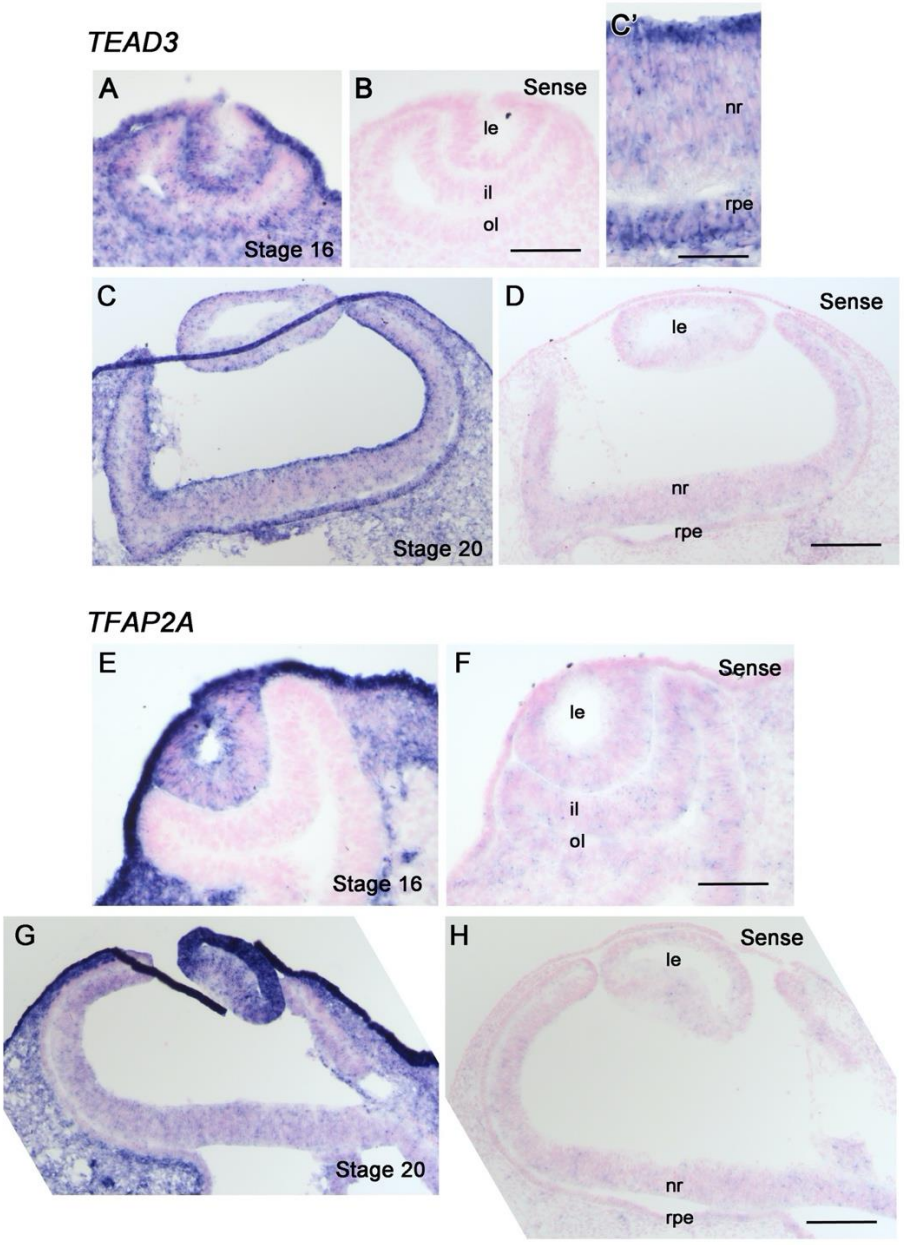

Figure S9: **Expression patterns of *TEAD3* and *TFAP2A* in the developing chicken eye.** (**A-D**) *TEAD3* is expressed in the surface ectoderm (SE) and developing eye at stages 16 (**A,B**) and 20 (**C,C',D**). (**C'**) Enlarged view of the developing retina is shown. *TEAD3* mRNA signals are observed 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 μm (**A,B,E,F**), 100 μm (**C,D,G,H**), 25μm (**C'**).

Figure S10

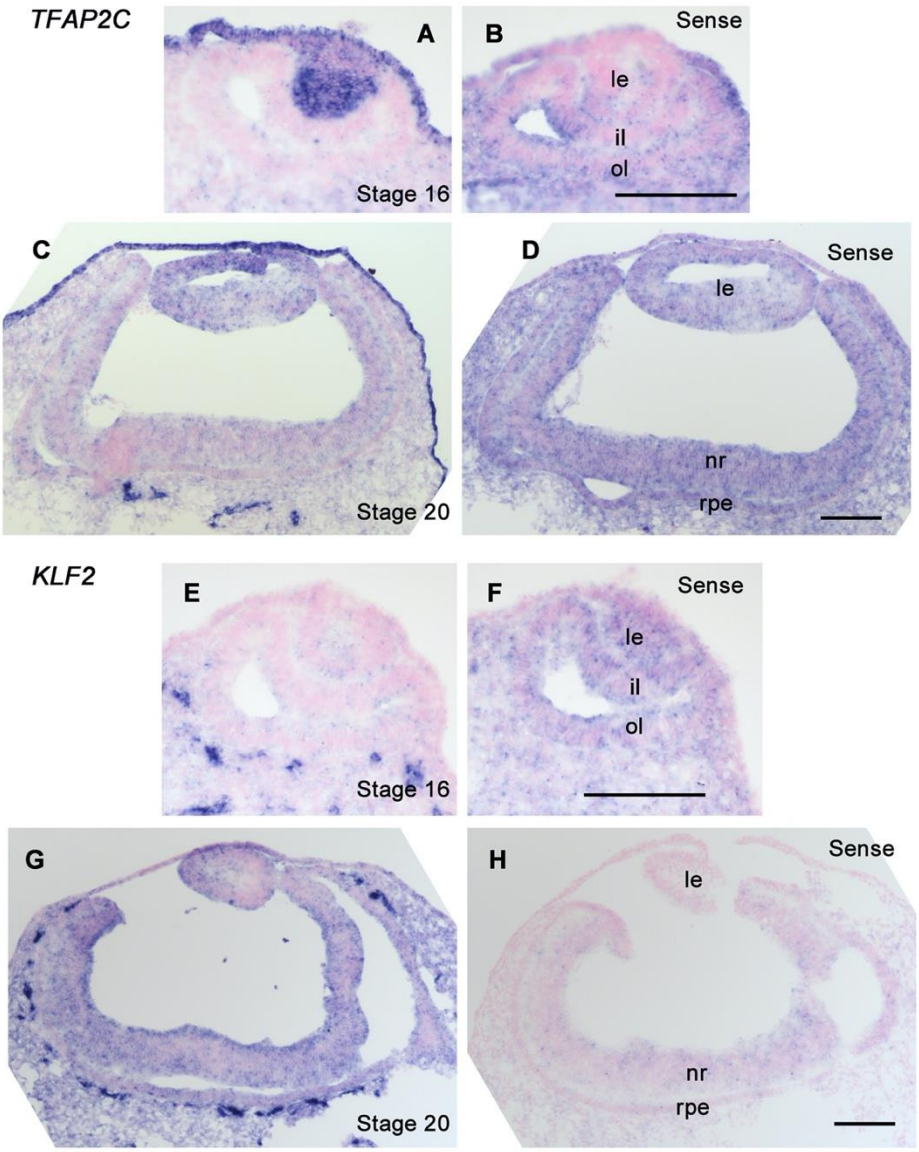

Figure S10: Expression patterns of *TFAP2C* and *KLF2* in the developing chicken eye. (**A-D**) *TFAP2C* is expressed in the surface ectoderm (SE), lens vesicle, and by a subset of cells in the periocular mesenchyme (**C**). (**E-H**) *KLF2* is expressed by a subset of cells in the periocular mesenchyme 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 μm.

Figure S11

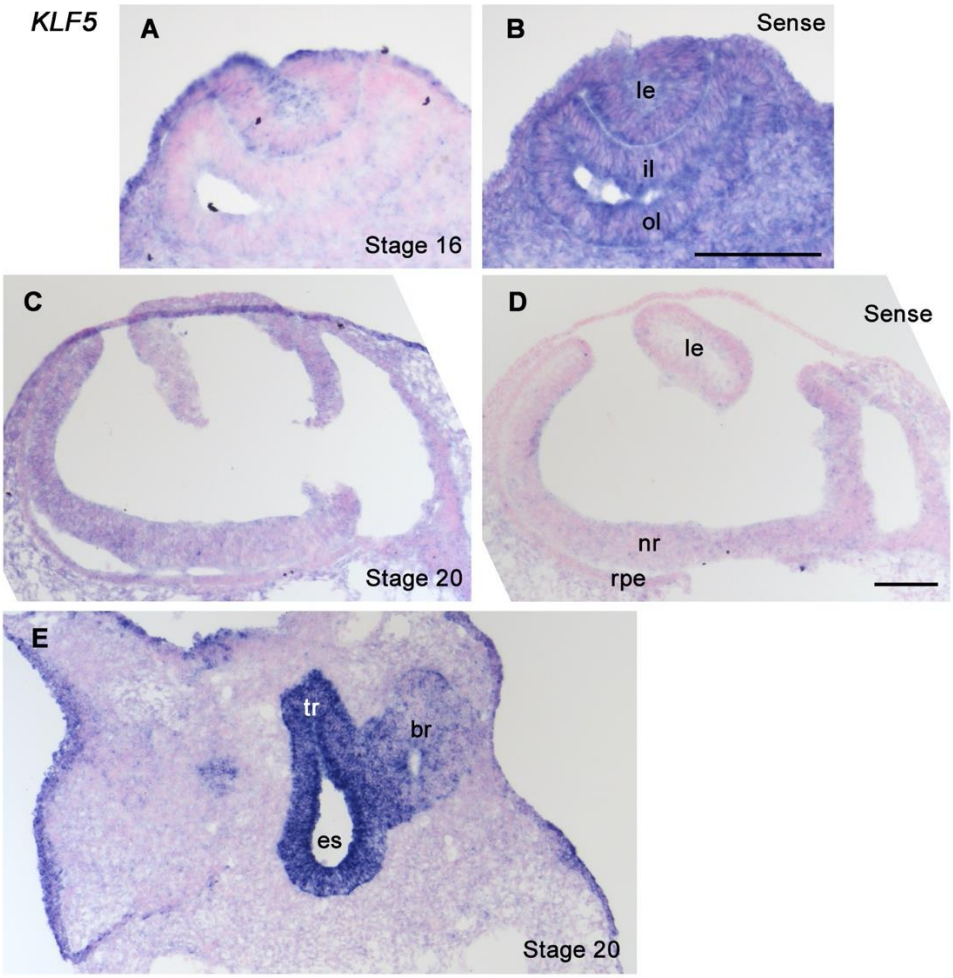

Figure S11: Expression pattern of *KLF5* in the developing chicken eye. *KLF5* is expressed in the surface ectoderm of the developing eye (**A,C**) and outside the eye (**E**) as well as the endoderm of developing trachea, bronchus and esophagus. br, bronchus; es, esophagus; il, inner layer of the optic cup; le, lens vesicle; nr, neural retina; ol, outer layer of the optic cup; rpe, developing RPE; tr, trachea. Scale bars: 100 μm.

Table S1: PCR primer sequences used in this study.

Gene name	Amplicon size (bp)	Primer name	Primer/DNA sequences (5'->3')	Purpose
chicken BHLHE40 (cBHLHE40)	1193	cBhlhe40 forward	CTGACCCTGAAGCACGTGAA	cloning a template cDNA to synthesize riboprobes
		cBhlhe40 reverse	GACACATGGCAGCTGACTGA	
cBHLHE40	1206	cBhlhe40 full forward	ATGGAGCGCATCCCCAGC	cloning a full-coding sequence for *cBHLHE40*
		cBhlhe40 full reverse	TTAGTCTTTGGTTTCCAAGTTCAAC	
cBHLHE40	1252	Bhlhe40_Fw	TAGCGTTTAAACTTAAGCTTGCCACCATGGAGCGCATCCCCAGC	insertion of *cBHLHE40* into pCAGGS vector
		Bhlhe40_Rv	ACGGGCCCTCTAGACTCGAGTTAGTCTTTGGTTTCCAAGTTCAAC	
cBHLHE40	1279	Bhlhe40_pCAG_Fw	AGCGTTTAAACTTAAGCTTGGCCACCATGGAGCGCATC	insertion of *cBHLHE40* into pCAGGS vector with HA-tag
		Bhlhe40_HA_pCAG_Rv	CGGGCCCTCTAGACTCGAGCTTACGCATAATCCGGCACATCATACGGATAGTCTTTGGTTTCCAAGTT	
delta_basic cBHLHE40	194	ggaBhlhe40_delbasic_Fw	AGGAGCGAGGACAGCAAGGATAGGATCAACGAGTGC	amplification of N terminal portion of HA-tagged cBHLHE40 before basic region
		ggaBhlhe40_delbasic _Rv	GCACTCGTTGATCCTATCCTTGCTGTCCTCGCTCCT	
delta_basic cBHLHE40	1076	pCAG_fw_HindIII	AGCGTTTAAACTTAAGCTTG	amplification of C terminal portion of HA-tagged cBHLHE40 after basic region
		pCAG_Rv_XhoI	CGGGCCCTCTAGACTCGAGC	
acidic extension cBhlhe40	106	a-Bhlhe40_ins_Fw_pCAG	AGCGTTTAAACTTAAGCTTGGCCACCATGGCATCAATGACTGGA	acidic extension sequence for replacement of N terminal portion of cBHLHE40 including basic region
		a-Bhlhe40_ins_Rv	AACTCTTCCAGCTCTTCTTCATCATCCTCCTCCTCATCGGGGTCCCTCCCCATCTGCTGACCTCCAGTCATTGATGCCAT	
acidic extension cBhlhe40	1068	a-Bhlhe40_Fw	AGAAGAGCTGGAAGAGTTGGAAGAGTGCATCGCCCAGCTC	amplification of C terminal portion of HA-tagged cBHLHE40 after basic region
		pCAG_Rv_XhoI	CGGGCCCTCTAGACTCGAGC	
chicken BHLHE41	1776	gga_bhlhe41_Fw	CCCGCGGATCCCCGAAGC	cloning template cDNAs to synthesize riboprobes
		gga_bhlhe41_Rv	GCACTGCGGCCGCCCATAAA	
chicken TEAD3	1299	gga_tead3_Fw	ATGGACAAGAGCCTGGACAATGACGCCGAG	
		gga_tead3_Rv	CTAGTCCTTCACCAACTTGTAGACATGATG	
chicken TFAP2A	1640	gga_tfap2a_Fw	GGGATCGGACCCTCTCCCGC	
		gga_tfap2a_Rv	CTGGCTCCGTCCCCCTGTTT	
chicken TFAP2C	1347	gga_tfap2c_Fw	ATGTTGTGGAAACTGGCAGATAACGTCAAG	
		gga_tfap2c_Rv	TTACTTCCTGTGCTTGTCCATTTTATCGAT	
chicken KLF2	1536	gga_klf2_Fw	CCATGGCGCTGAGCGATACCA	
		gga_klf2_Rv	AACACCGAGCTACAGCAACGCTC	
chicken KLF5	1504	gga_klf5_Fw	GAGGCATTGACGACGCCCGA	
		gga_klf5_Rv	GCCTCGGCAACAACTCTGGCT	

Table S2: Antibodies used in this study.

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