

Fig. S1. Sensor Assay to examine the efficiency of the knockdown of the designed hairpins

(A) GFP, in green, shows the transfection efficiency of the control, pRmiR-empty in HEK293T cells. (A') mCherry fluorescence, in red, shows the expression of the sensor vector containing the target region of the hairpins. (A'') Merged images of cells transfected with control, pRmiR-empty. (B) GFP, in green, shows the transfection efficiency of the test, *CNKSR2*-RNAi (1) in HEK293T cells. (B') mCherry fluorescence, in red shows the expression of the sensor vector upon transfection with *CNKSR2*-RNAi. (B'') Merged images of cells transfected with the test, *CNKSR2*-RNAi (1). (C) GFP, in green, showing the transfection efficiency of the test, *CNKSR2*-RNAi (2) in HEK293T cells. (C') mCherry fluorescence, in red shows the expression of the sensor vector upon transfection with *CNKSR2*-RNAi (2). (C'') Merged images of cells transfected with the test, *CNKSR2*-RNAi(2). (D) Quantification of the mean mCherry fluorescence by ImageJ followed by statistical analyses by Unpaired t-test (two-tailed) in GraphPad software between pRmiR-empty, *CNKSR2*-RNAi (1), and *CNKSR2*-RNAi (2), $p=0.0001$ (***) for pRmiR-empty and *CNKSR2*-RNAi (1), and $p=0.0002$ (***) for pRmiR-empty and *CNKSR2*-RNAi (2). Error bars indicate SEM. Scale bar indicates 100 μ m. $n=3$, where n represents one well and fluorescence intensity of three fields from one well for each condition were quantified, averaged, and values plotted as Mean \pm SEM.

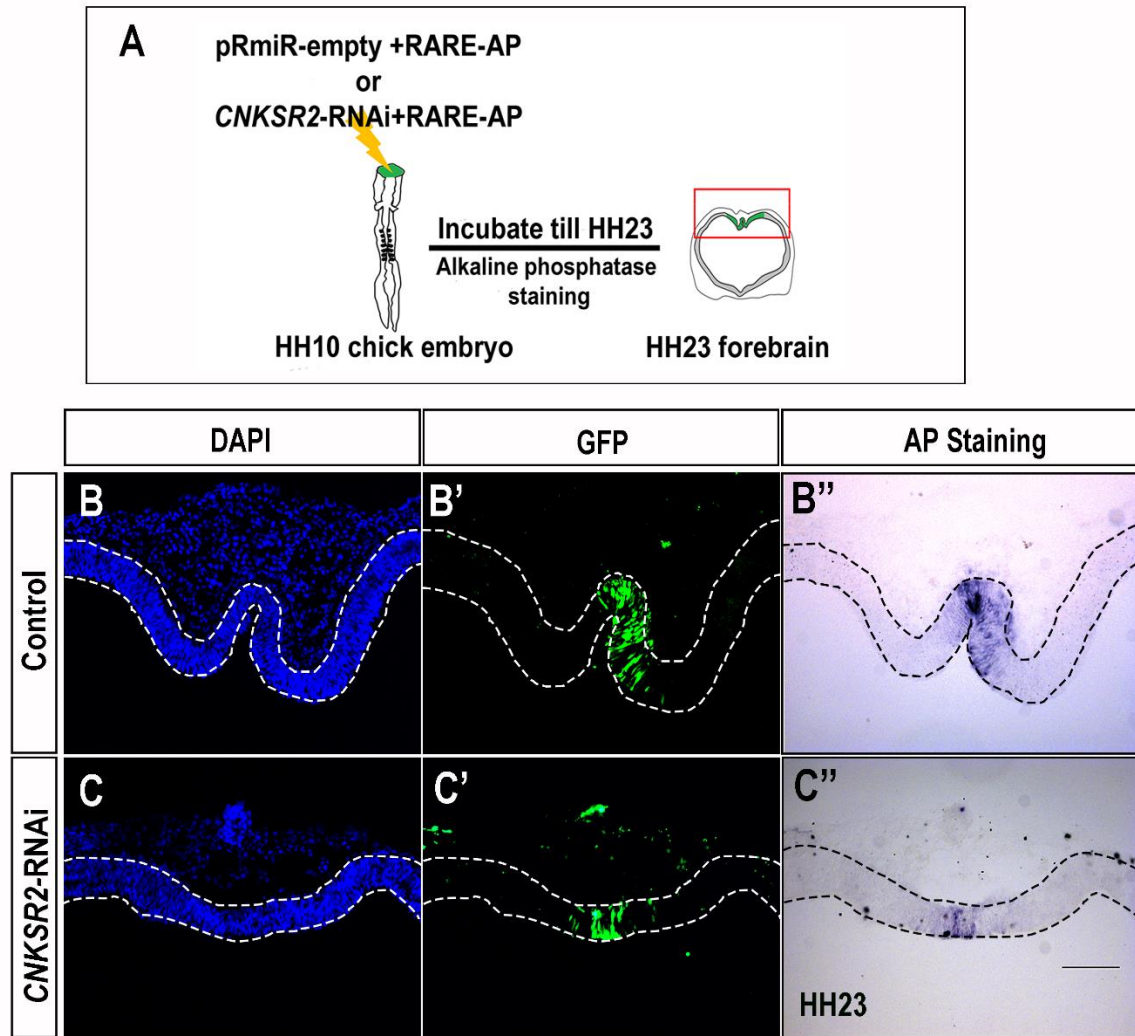


Fig S2. Knockdown of *CNKSR2* does not affect RA signaling in the invaginating forebrain roof plate

(A) Experimental strategy for detecting active RA signaling upon knockdown of *CNKSR2* in the invaginating forebrain roof plate. (B) DAPI staining (blue) on control, pRmiR empty, and pRARE-AP electroporated forebrains. (B') GFP (green) on control, pRmiR empty, and pRARE-AP electroporated forebrains. (B'') Alkaline phosphatase staining at HH23 showing active RA signaling in the invaginating forebrain roof plate in control electroporated forebrains. (C) DAPI staining (blue) on the test, *CNKSR2*-RNAi, and pRARE-AP electroporated forebrains. (C') GFP (green) on the test, *CNKSR2*-RNAi, and pRARE-AP electroporated forebrains. (C'') Alkaline phosphatase staining at HH23 showing active RA signaling in the invaginating forebrain roof plate in test electroporated forebrains, showing no change in RA signaling upon knockdown of *CNKSR2* in the invaginating roof plate, n=3 each for control and test. Scale bar indicates 100µm.

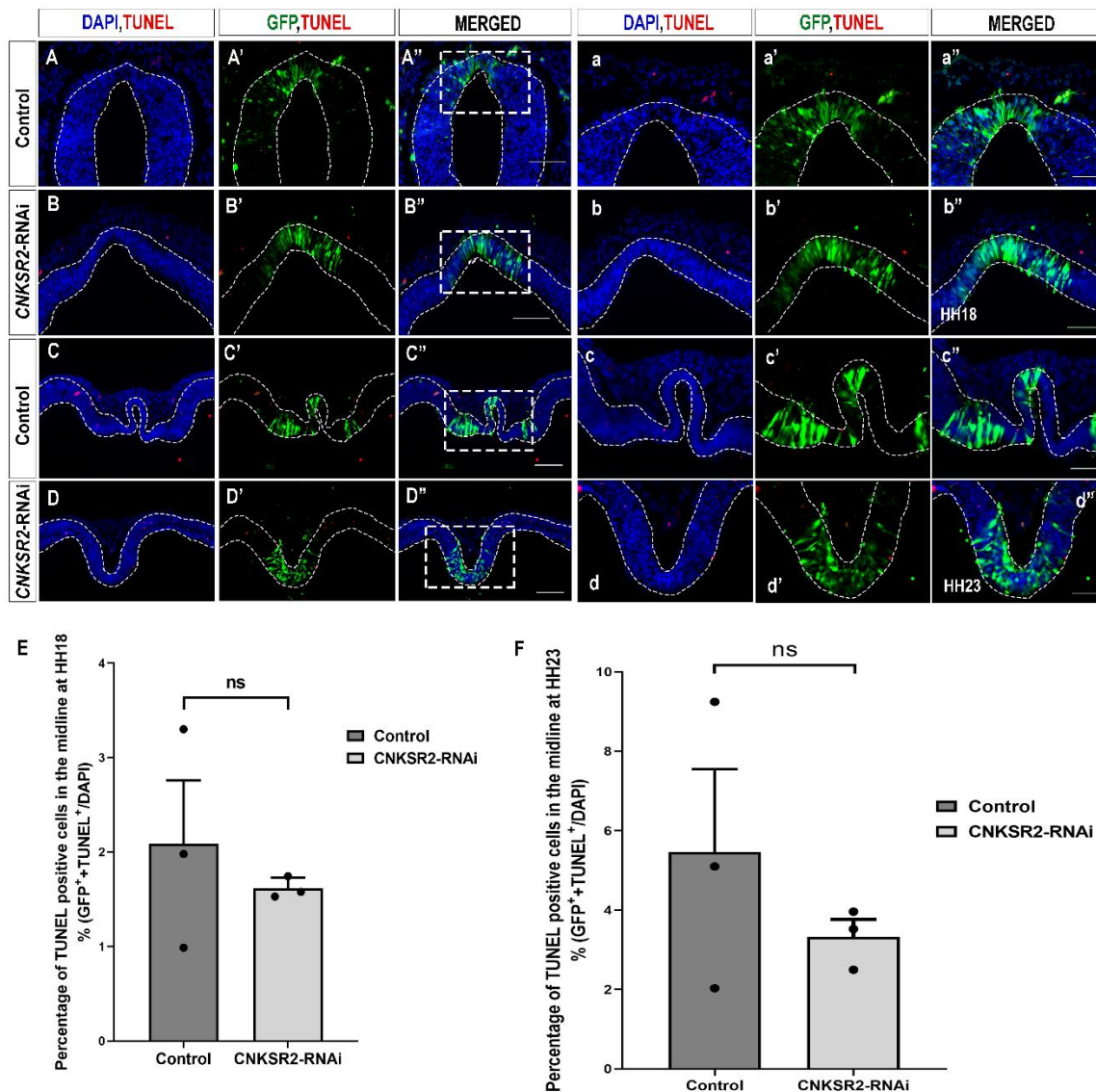


Fig. S3. Knockdown of *CNKSR2* does not affect apoptosis in the invaginating forebrain roof plate

(A) DAPI staining (blue) and TUNEL staining (red) on control, pRmiR empty electroporated forebrains. (A') GFP (green) and TUNEL staining (red) on control electroporated forebrains at HH18. (A'') Merged images of A and A'. (a) Magnified images of DAPI and TUNEL staining of boxed region A''. (a') Magnified images of GFP and TUNEL staining of boxed region A''. (a'') Magnified images of boxed region in A''. (B) DAPI staining (blue) and TUNEL staining (red) on test, *CNKSR2*-RNAi electroporated forebrains. (B') GFP (green) and TUNEL staining (red) on test electroporated forebrains at HH18. (B'') Merged images of B and B' at HH18. (b) Magnified images of DAPI and TUNEL staining of boxed region B''. (b') Magnified images of GFP and TUNEL staining of boxed region B''. (b'') Magnified images of boxed region in B''. (C) DAPI

staining (blue) and TUNEL staining (red) on control, pRmiR empty electroporated forebrains at HH23. (C') GFP (green) and TUNEL staining (red) on control electroporated forebrains at HH23. (C'') Merged images of C and C' at HH23. (c) Magnified images of DAPI and TUNEL staining of boxed region C''. (c') Magnified images of GFP and TUNEL staining of boxed region C''. (c'') Magnified images of boxed region in C''. (D) DAPI staining (blue) and TUNEL staining (red) on test, *CNKS2*-RNAi electroporated forebrains at HH23. (D') GFP (green) and TUNEL staining (red) on test electroporated forebrains at HH23. (D'') Merged images of D and D' at HH23. (d) Magnified images of DAPI and TUNEL staining of boxed region D''. (d') Magnified images of GFP and TUNEL staining of boxed region D''. (d'') Magnified images of boxed region in D''. (E and F) Quantification of the percentage of TUNEL positive cells in the midline between test and control at HH18 and HH23 respectively. Statistical analysis was performed by Unpaired t-test (two-tailed) using GraphPad software and values plotted as Mean \pm SEM. Error bars represent SEM. $p = 0.5520$ (ns) at HH18 and $p = 0.3748$ (ns) at HH23. $n = 3$ each for test and control for both stages. Scalebar for panels A-D'' 100 μ m and a-d'' 50 μ m.

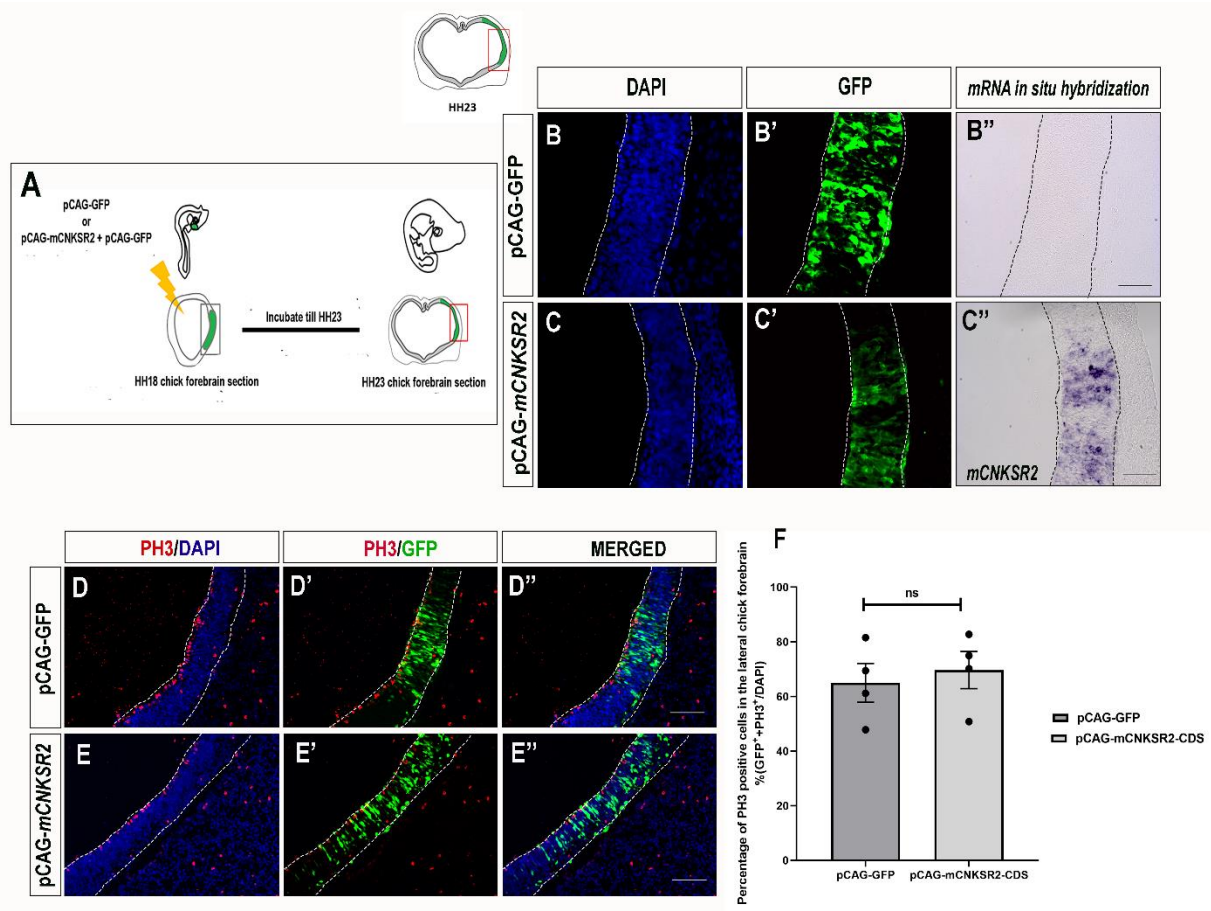


Fig. S4. Misexpression of *mCNKSR2* does not affect the number of PH3 positive cells in the lateral chick forebrain.

(A) Schematic illustration of the experimental strategy for the misexpression of *mCNKSR2* in the lateral chick forebrain. (B) DAPI staining (blue) of control, pCAG-GFP electroporated chick forebrains. (B') GFP (green), showing the extent of electroporation of control, pCAG-GFP in the lateral chick forebrain. (B'') Expression of *mCNKSR2* transcript on pCAG-GFP electroporated samples. (C) DAPI staining (blue) of control, pCAG-*mCNKSR2* electroporated chick forebrains. (C') GFP (green), showing the extent of electroporation of control, pCAG-*mCNKSR2* in the lateral chick forebrain. (C'') Expression of *mCNKSR2* on pCAG-*mCNKSR2* electroporated samples in the lateral chick forebrain. (D) DAPI staining (blue) and PH3 immunostaining (red) on control, pCAG-GFP electroporated chick forebrains. (D') GFP (green), showing the extent of electroporation of control, pCAG-GFP, and PH3 immunostaining (red) in the lateral chick forebrains. (D'') Merged images of D and D'. (E) DAPI staining (blue) and PH3 immunostaining (red) on test pCAG-*mCNKSR2* electroporated chick forebrains. (E') GFP (green), showing the extent of electroporation of test, pCAG-*mCNKSR2*, and PH3 immunostaining in the lateral chick forebrains. (E'') Merged images of E and E'. (F) Quantification of the percentage of PH3 positive cells and statistical analysis performed by Unpaired t-test (two-tailed) in GraphPad software between control and test in the lateral forebrain and values plotted as Mean \pm SEM. $p=0.6500$ (ns), $n=3$ each for control and test. Error bars represent SEM. Scale bar indicates 100 μ m.

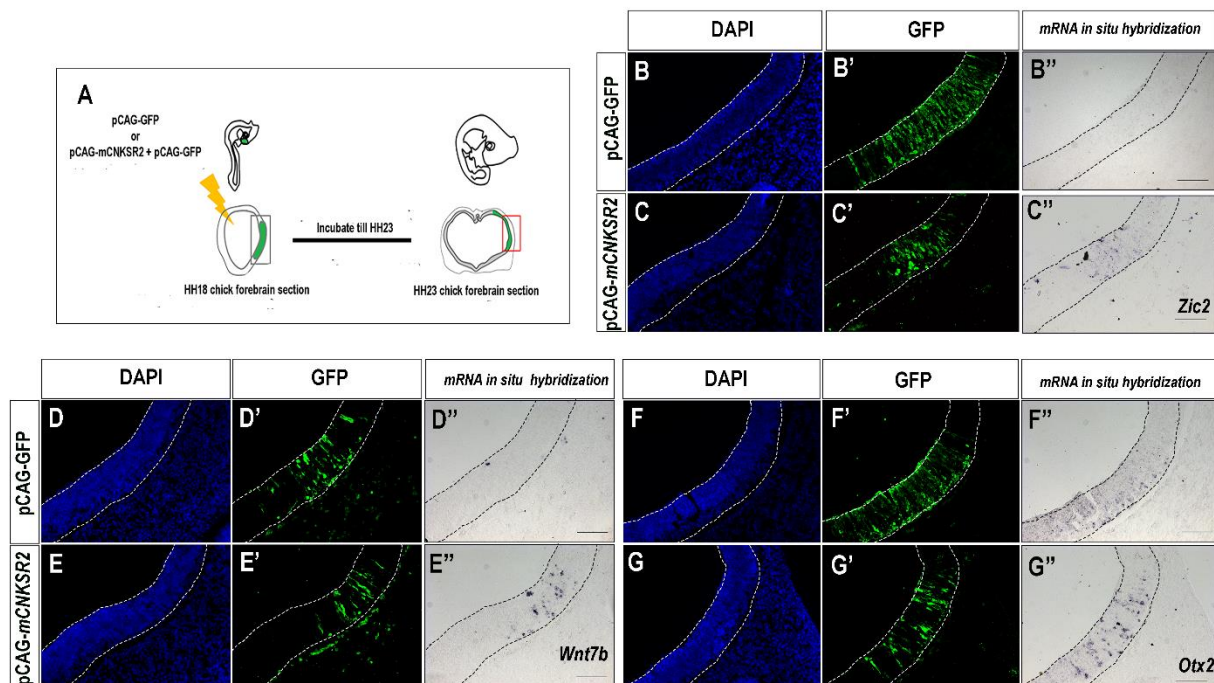


Fig. S5. Misexpression of *mCNKSR2* is sufficient to induce roof plate markers, *Zic2*, *Wnt7b*, and *Otx2*.

(A) Experimental strategy for analyzing induction roof plate markers upon misexpression of *mCNKSR2* in the lateral chick forebrain. (B) DAPI staining (blue) of control, pCAG-GFP electroporated lateral chick forebrain. (B') GFP (green) shows the extent of electroporation of control, pCAG-GFP in lateral chick forebrain. (B'') Expression of *Zic2* on control, pCAG-GFP electroporated lateral chick forebrains. (C) DAPI staining (blue) of the test, pCAG-mCNKSR2 electroporated lateral chick forebrain. (C') GFP (green) shows the extent of electroporation of the test, pCAG-mCNKSR2 in lateral chick forebrain. (C'') Expression of *Zic2* transcript on the test pCAG-mCNKSR2 in lateral chick forebrain. (D) DAPI staining (blue) of control, pCAG-GFP electroporated lateral chick forebrain. (D') GFP (green) shows the extent of electroporation of control in lateral chick forebrain. (D'') Expression of *Wnt7b* transcript on control electroporated lateral chick forebrains. (E) DAPI staining (blue) of the test, pCAG-mCNKSR2 electroporated lateral chick forebrain. (E') GFP (green) shows the extent of electroporation of the test in lateral chick forebrain. (E'') Expression of *Wnt7b* on the test electroporated lateral chick forebrain. (F) DAPI staining (blue) of control, pCAG-GFP electroporated lateral chick forebrain. (F') GFP (green) shows the extent of electroporation of control, pCAG-GFP in lateral chick forebrain. (F'') Expression of *Otx2* on control electroporated lateral chick forebrains. (G) DAPI staining (blue) of the test, pCAG-mCNKSR2 in lateral chick forebrain. (G') GFP (green) shows the extent of electroporation of the test in lateral chick forebrain. (G'') Expression of *Otx2* transcript on the test electroporated lateral chick forebrain. Scale bar indicates 100µm. n=3 for all panels in this figure.

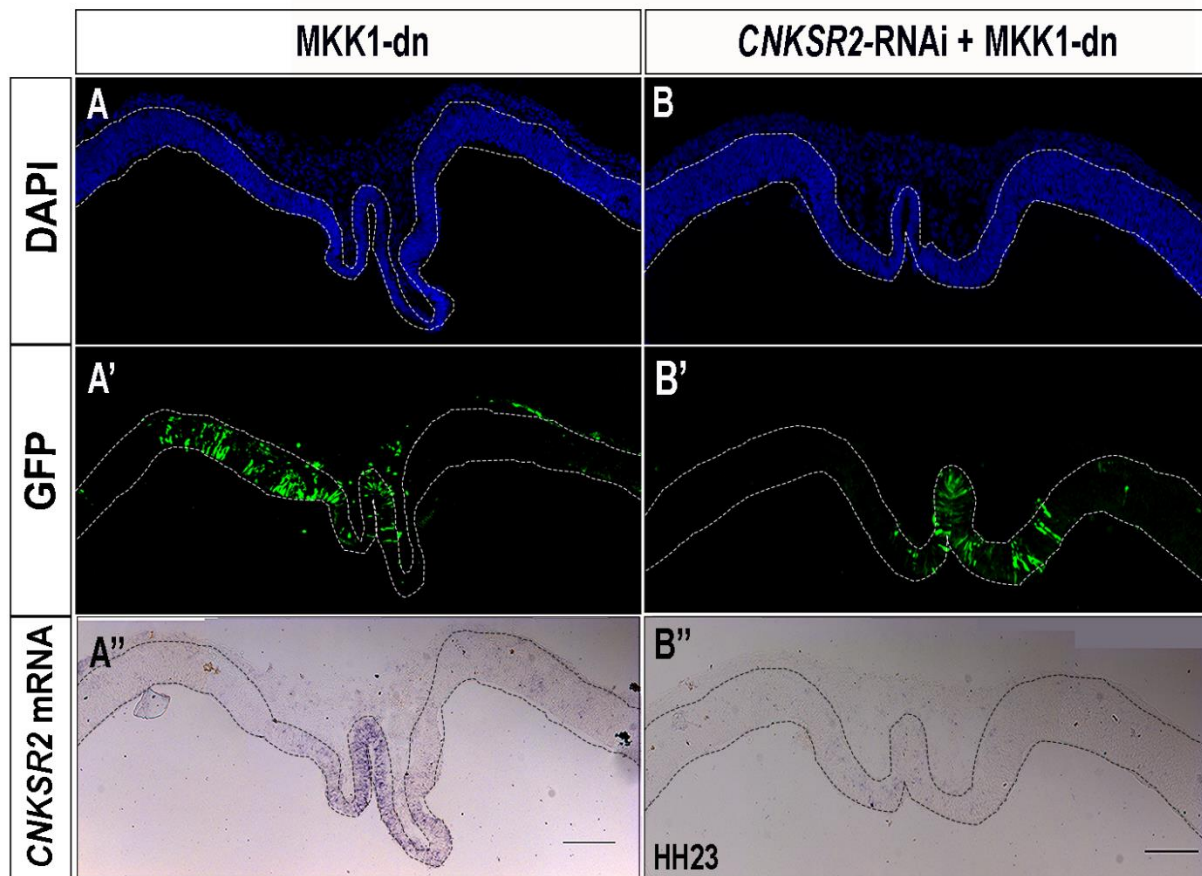


Fig. S6. Knockdown of *CNKSR2* on MKK1-dn and *CNKSR2*-RNAi+ MKK1-dn electroporated samples.

(A) DAPI staining (blue) of MKK1-dn-IRES-GFP electroporated forebrains. (A') GFP (green) shows the extent of electroporation of MKK1-dn-IRES-GFP. (A'') Expression of *CNKSR2* on control electroporated samples. (B) DAPI staining (blue) of *CNKSR2*-RNAi and MKK1-dn electroporated forebrains. (B') GFP (green) shows the extent of electroporation of *CNKSR2*-RNAi and MKK1-dn. (B'') Expression of *CNKSR2* on test electroporated samples. Scale bar indicates 100 μ m. n=3 for control and test for all panels in this figure.

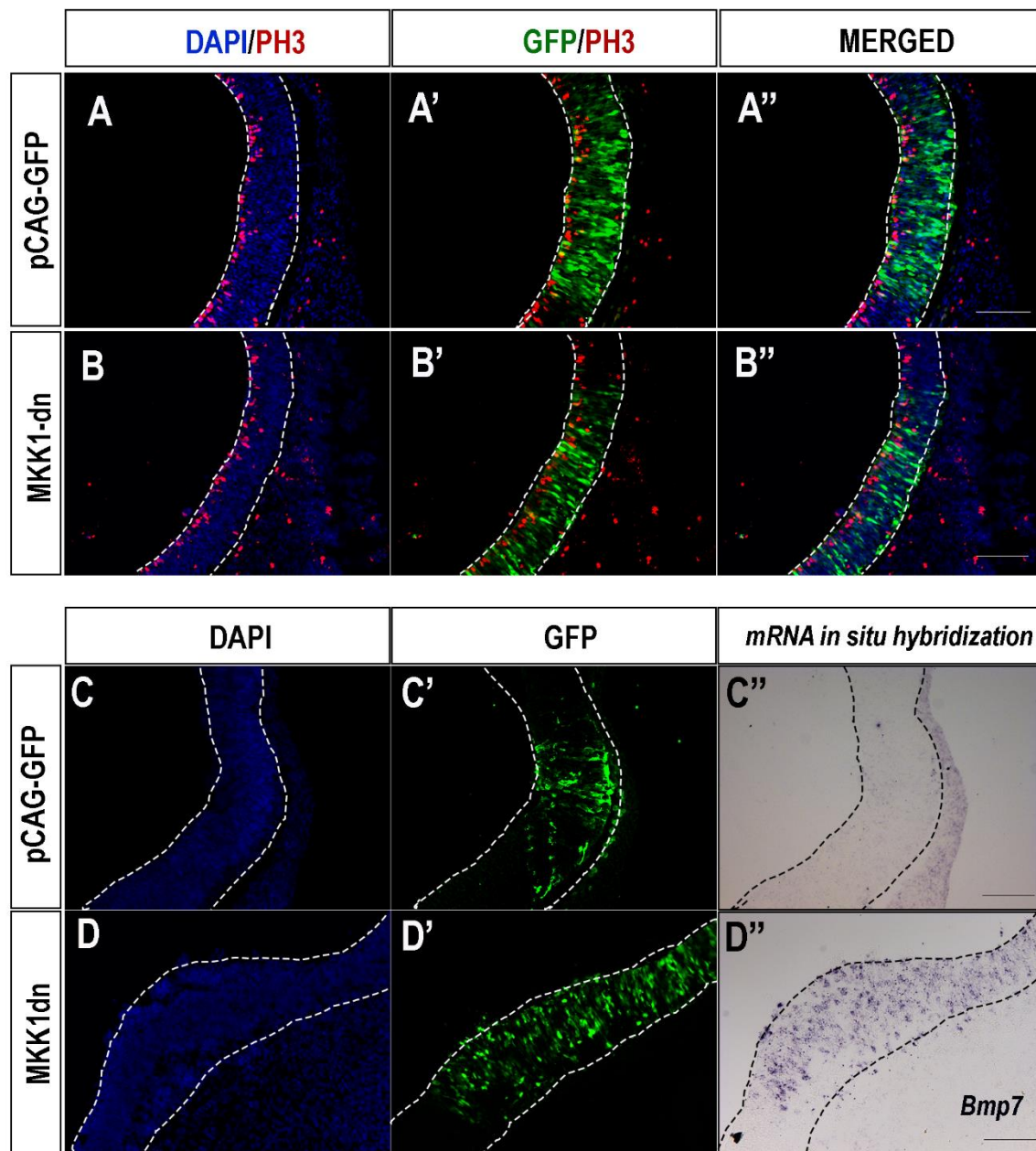


Fig. S7. Ectopic downregulation of Ras/Raf/MEK is sufficient to induce the expression of *Bmp7* but not regulation of cell proliferation.

(A) DAPI staining (blue), and PH3 immunostaining (red) of control, pCAG-GFP in lateral chick forebrain. (A') GFP (green) shows the extent of electroporation and PH3 immunostaining (red) of control in lateral chick forebrain. (A'') Merged images of A and A'. (B) DAPI staining (blue), and

PH3 immunostaining (red) of the test, pCIG-MKK1-dn-IRES-GFP in lateral chick forebrain. (B') GFP (green) shows the extent of electroporation and PH3 immunostaining on the test, pCIG-MKK1-dn in lateral chick forebrain. (B'') Merged images of B and B'. (C) DAPI staining (blue) of control, pCAG-GFP in lateral chick forebrain. (C') GFP (green) shows the extent of electroporation of control, pCAG-GFP in lateral chick forebrain. (C'') mRNA *in situ* hybridization of *Bmp7* on control, pCAG-GFP in lateral chick forebrains. (D) DAPI staining (blue) of the test, pCIG-MKK1-dn in lateral chick forebrain. (D') GFP (green) shows the extent of electroporation of the test, pCIG-MKK1-dn in lateral chick forebrain. (D'') mRNA *in situ* hybridization of *Bmp7* on the test, pCIG-MKK1-dn in lateral chick forebrains. Scale bar indicates 100µm. n=3 for control and test for all panels in this figure.

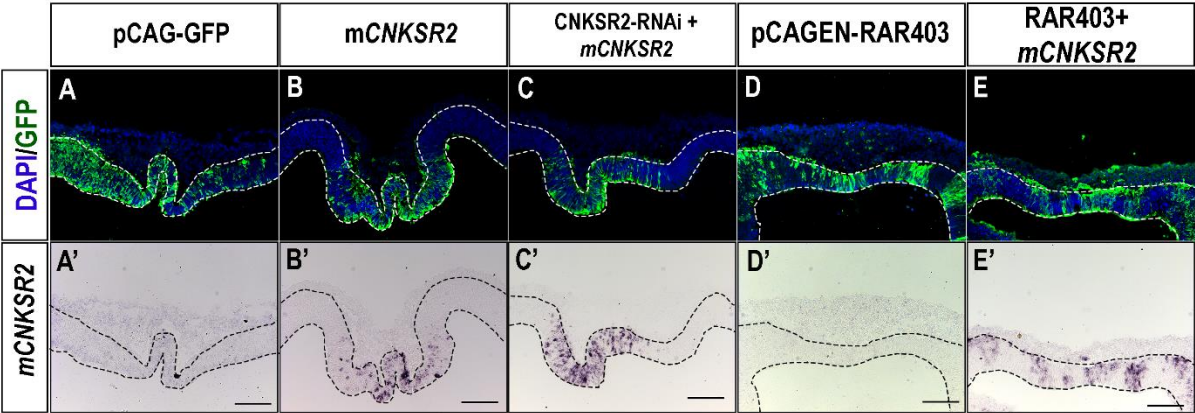


Fig. S8. Expression of mouse *CNKSR2* on pCAG-GFP, m*CNKSR2*, *CNKSR2*-RNAi + m*CNKSR2*, RAR403, and RAR403+m*CNKSR2* electroporated forebrains.

(A) DAPI staining(blue) and GFP (green) of pCAG-GFP electroporated forebrain roof plate midline. (A') m*CNKSR2* *in situ* hybridization on pCAG-GFP electroporated forebrains. (B) DAPI staining (blue) and GFP (green) of m*CNKSR2*+GFP. (B') m*CNKSR2* *in situ* hybridization on m*CNKSR2*+GFP electroporated forebrains. (C) DAPI staining (blue) and GFP (green) of *CNKSR2*-RNAi+m*CNKSR2* electroporated forebrains. (C') m*CNKSR2* *in situ* hybridization on *CNKSR2*-RNAi+m*CNKSR2* electroporated forebrains, showing partial rescue in invagination. (D) DAPI staining (blue) and GFP(green) of pCAGEN-RAR403+GFP. (D') m*CNKSR2* *in situ* hybridization on pCAGEN-RAR403+GFP electroporated forebrains. (E) DAPI staining (blue) and GFP(green) of pCAGEN-RAR403+GFP+ m*CNKSR2*. (D') m*CNKSR2* *in situ* hybridization on pCAGEN-RAR403+GFP+ m*CNKSR2* electroporated forebrains, showing invagination defects. Scale bar indicates 100µm. n=3 for control and test for all panels in this figure.

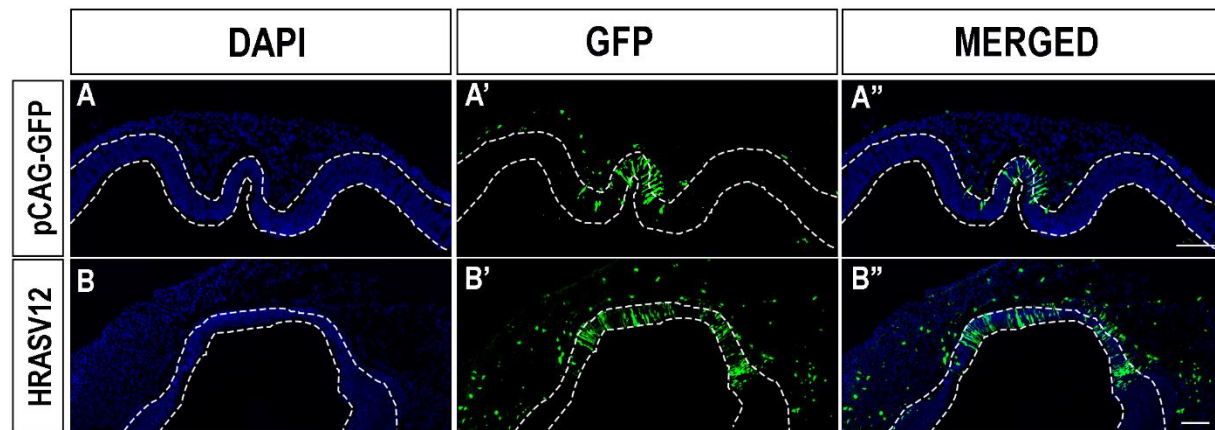


Fig. S9. Invagination defects seen upon constitutive activation of the Ras/Raf/MEK signaling in the roof plate.

(A) DAPI staining (blue) of control, pCAG-GFP in forebrain roof plate midline. (A') GFP (green) shows the extent of electroporation of pCAG-GFP. (A'') Merged images of A and A'. (B) DAPI staining (blue), of the test, pCDNA-HRASV12 electroporated forebrain roof plate midline. (B') GFP (green) shows the extent of electroporation of the test, pCDNA-HRASV12. (B'') Merged images of B and B' showing invagination defects, Scale bar indicates 100 μ m. n=3 for control and test for all panels in this figure.

***Gallus gallus* CNKSR2- 2kb upstream sequence**

accatttggtaaggacactttggctgagacacatttagttaagcagggtcagtatgaaaagtcattcagctctctttggacttctaccctagaagaagagaacagtaataaaatcaggcgagcaataaatttagagcaaattatattaaaaaaatacttctatgtagcacatgtcctgactatggaatagtaatagggtcatcaaaataaatactatgactggataattttatcatcactcgtactgagccatggctaagccaaccgaaataagaatatttggccaagtaaaagatgaatttaccagtagttataagactgctatgctcaatgagaagaacaaaatgtaattagagctctgggtcagtagacagcttctattattgaagtgggggagtaagctcccttttatagttccgtaatttgcagaaaccactacaaaggggctttaaagtgataggtactgcttactgatacagcagtgggtcagatgctatagtgagaacctgactctagctccaaattactctagcaagctataggaaatcatagtgacattctgtaacaaggaaatcatcagggcgttggtacatatgttctgagaacttacttccccctctcaccataaagaacttctcaacacaatgagggtcacacaatggtgttataaatcatatgccaggttcagttctgatcttgggtattcatttccaaacctttcaaaataccagtttctaattaaaattatgacacgttctttcaacaacaataaccagtatgcatggaactgtgtacctttcaaatctttcataaggcaaagagtcagccttaatttgaagtaaaagttattgttccggtggcttttaggtaatgagaactccttagaaacagactgccatgtcaggaatgtgacacaagacacatggctaggggccaaattgaagggaagtccagtgtcaaacccccccccccccccccccccctttcagtagaaaaagacttcactttcaactataaatgcaattccagcctcaccttgagtgtctctaggaacggggcatccatcacctctctggacaacctgggtctccaggggagaggctatggatcccttgagtgccttcttggcacttactgacagaatttcagtcccccataatgaacaagagagctgggtgactgcccagctatgcaaagggatgcctttatcattgcttctcggggctgagggaatcctcttttttcatcatgtacagacgttaaatgacagatgcagcagctggcattctgcagtcctcagagggaactccttgatggtcattgcctcagccttggcaggagaaagcagggggagatgcaaatgtaggcaaaaaatctgcactggccaactcaaaagcaggaaaacaccttcagtgggtatttatgcttggcttggagctctgggagtagacagaggctactagaaaaaggtgatggagaagcactcacgaccatccttaggaggaatcccttgcactgcgagggattcctacagccagaagcagatgtcacaaaggtatgtgggaaatgtccctttggggcagcattagccgagctttgctccttcaggatgaagtgggtgtgggggacagccaacacacctcagtggtgaaactcgggtggccagcacgacctcctgcctgagacagcctaattcctacccgagcctgccgtggctcctaagccgctttgccccatcacctctccctcggggagaagcagtgaagtaatgccctctcctctcggggctctttggaagcatccgccatctaccaccagcatttaacagcctggcttattctcagagagaggggaagaaaaaaaaaaaaaaaaaagaggagaggaaggaaggaaaaaaaccaaccttaccaccacgcagatgctttttaaagggaagaagaaaaaaaaaacatagaaaacccaacggaacagcagcgcatccttctggctgcgtc

Fig. S10. 2kb upstream sequence of the *Gallus gallus* CNKSR2 gene. Highlighted in yellow are the half-sites of RARE (Retinoic Acid Response Element) (PuG(G/T)TCA)- AGGTCA, GGGTCA