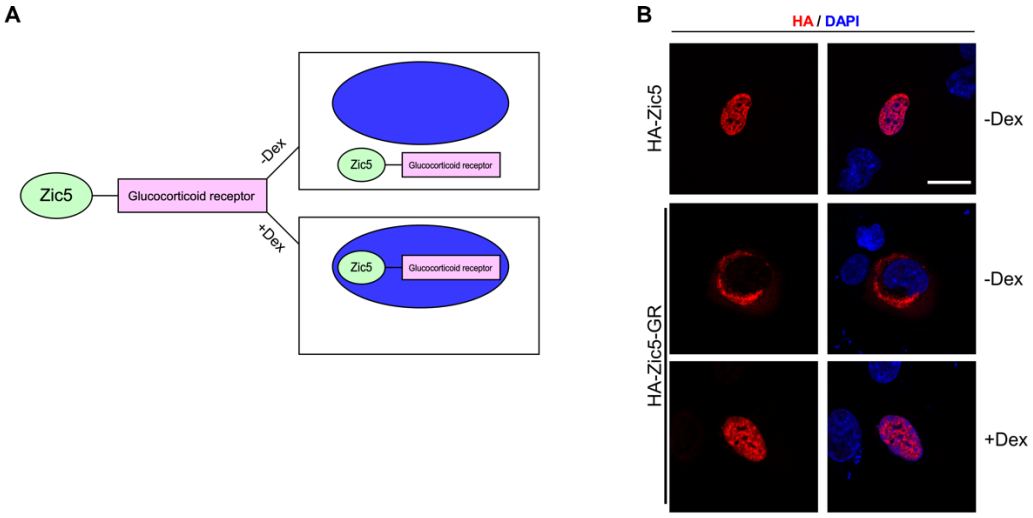

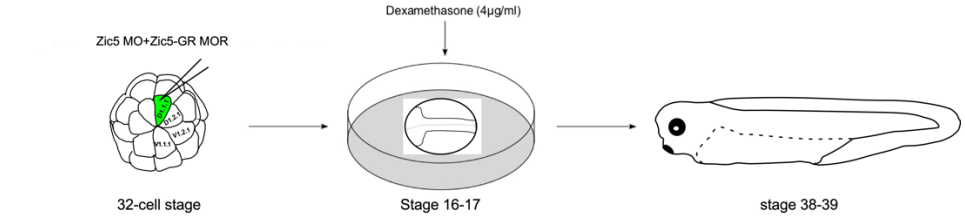

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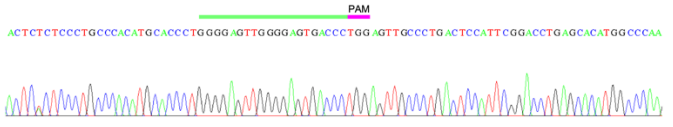
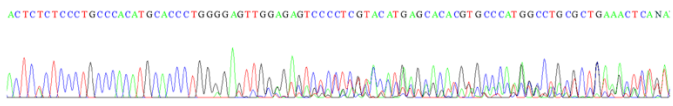
PAM



Cas9 alone



Cas9
+
Zic5 sgRNA

Figure S1. Related to Figure 1. Inducible Zic5-GR (glucocorticoid receptor) system and rescue experiment workflow.

(A) Scheme for working model of Zic5-GR fusion construct in cell.

(B) Validation of Zic5-GR construct in hRPE cells. HA-Zic5 localizes in the nuclear while HAZic5-GR shows cytoplasmic retention without dexamethasone treatment. This retention was released upon dexamethasone treatment for 30 min. Scale bar, 20 μm.

(C) Scheme for Zic5-GR rescue experiments.

(D) CRISPR/CAS9 targets the first exon of Zic5 gene. The direct sequencing of PCR amplicons shows the genomic DNA of Zic5 was edited by Cas9/Zic5 sgRNA co-injection.

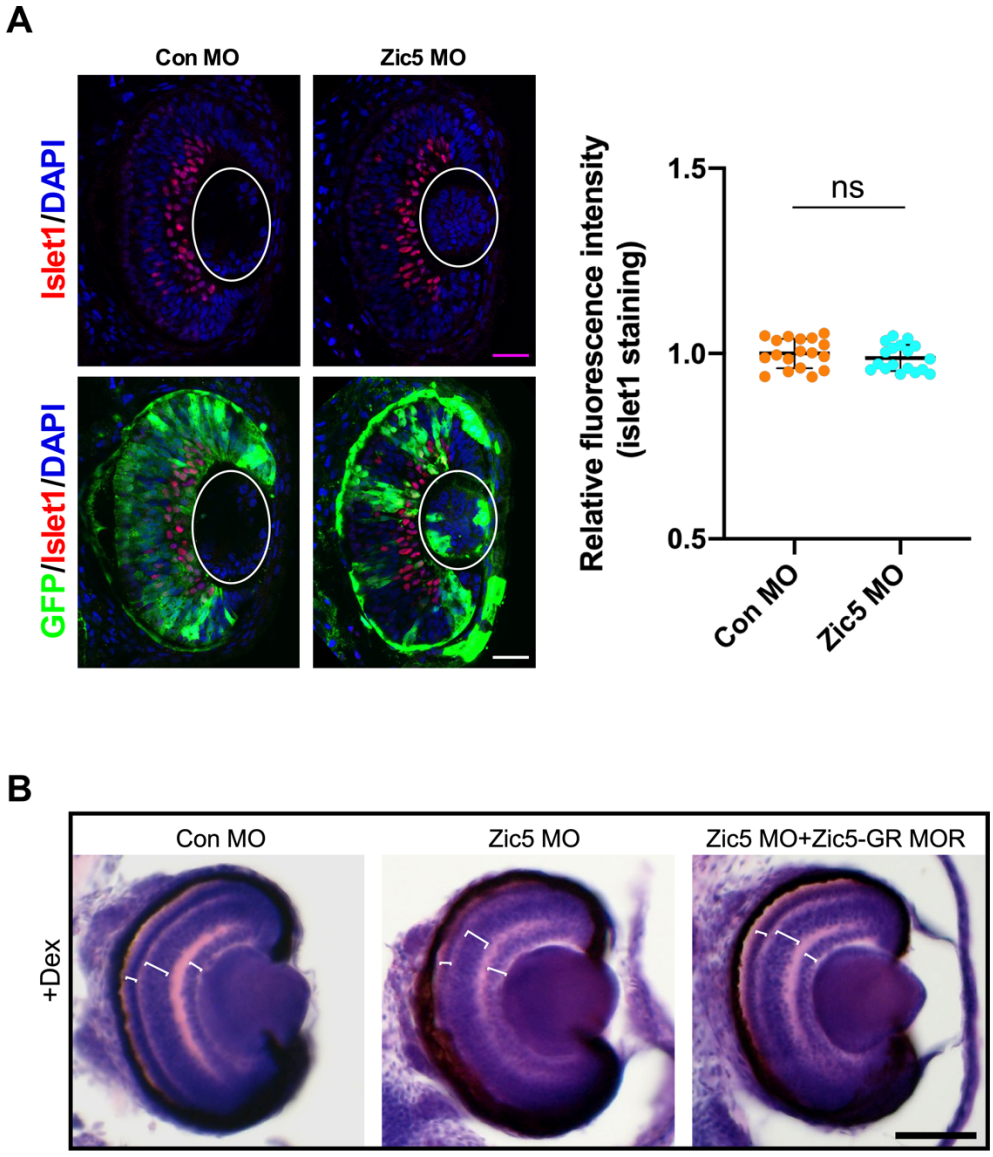


Figure S2. Related to Figure 2. Zic5 knockdown did not affect inner nuclear layer marker Islet1 expression as well as retina lamination.

(A) Con MO or Zic5 MO was injected with GFP RNA into the D1.1.1 blastomere at 32 -cell stage. Embryos were sectioned and immunostained with Islet1 antibody at stage 38. Quantification of relative Islet1 fluorescence intensity with unpaired t test, scatterplots represent means ± s.d from three biological repeats, ns: no statistical differences between the groups. Scale bar, 40 μm.

(B) Section H&E staining of stage 39 eyes injected with indicated morpholino or RNA. Scale bar, 150 μm.

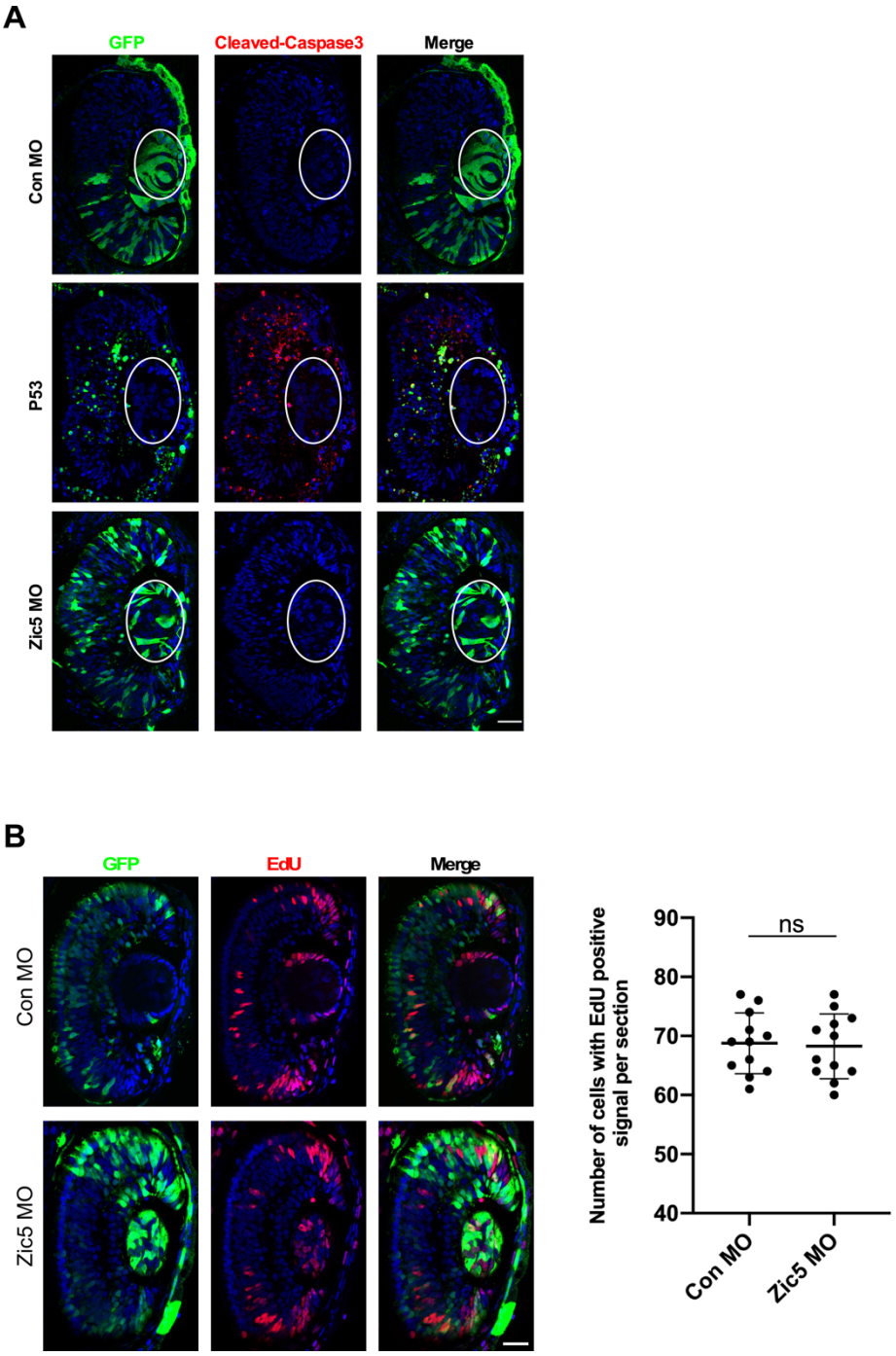

A
GFP
Cleaved-Caspase3
Merge
Con MO
P53
Zic5 MO
B
GFP
EdU
Merge
Con MO
Zic5 MO
Number of cells with EdU positive signal per section
ns
90
80
70
60
50
40
Con MO
Zic5 MO

Figure S3. Related to Figure 2. Knockdown of Zic5 did not induce cell apoptosis or EdU incorporation in the eye.

(A) Con MO or Zic5 MO was injected with GFP RNA into the D1.1.1 blastomere at 32 -cell stage. Embryos were sectioned and immunostained with cleaved-Caspase3 antibody at stage 37. P53 RNA injection was used as a positive control. Scale bar, 40 μm.

(B) Con MO or Zic5 MO was injected with GFP RNA into the D1.1.1 blastomere at 32 -cell stage. Embryos were then injected intra-abdominally EdU (5-ethynyl-2′-deoxyuridine; 1 mM) at stage37. Three hours after injection, embryos were harvested. Quantification of EdU positive cells in retina with unpaired t test, Scatterplots represent means ± s.d from three biological repeats, ns: no statistical differences between the groups. Scale bar, 40 μm.

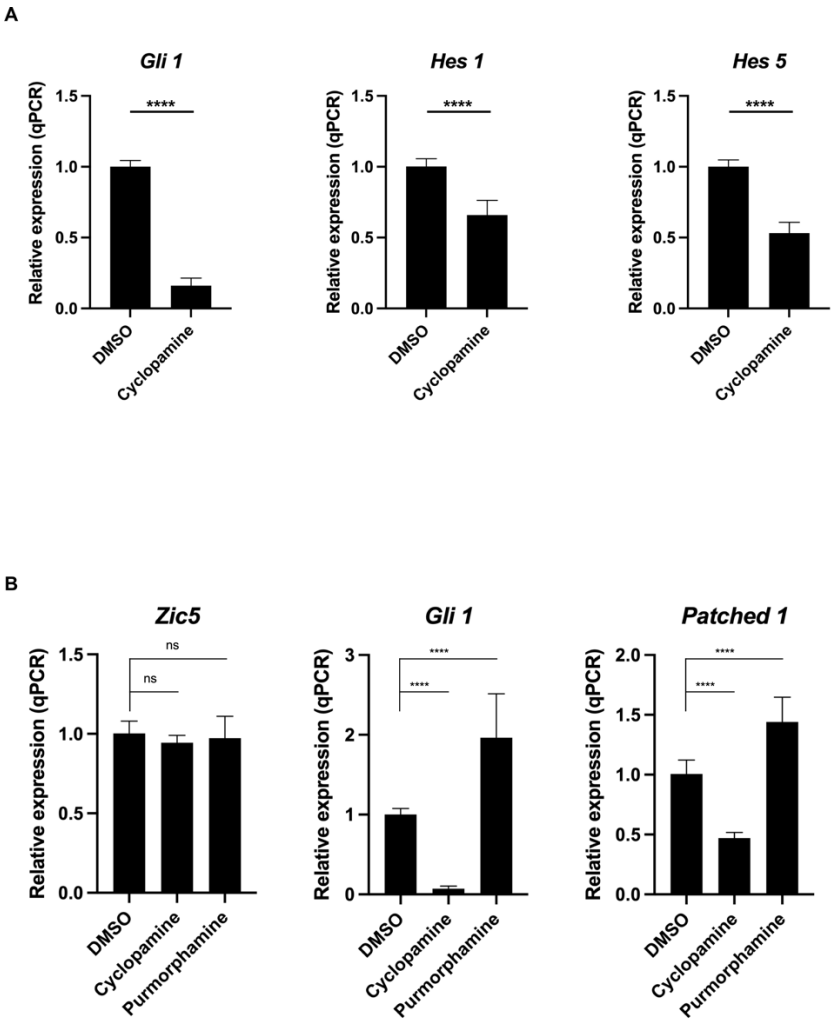


Figure S4. Related to Figure 3. The expression of *Hes1* and *Hes5* but not *Zic5* was regulated by Hh signal in the developing eyes.

(A) Embryos were treated with DMSO or cyclopamine from stage 18-19. 40 eyes per group were then dissected at stage 38 for quantitative PCR analysis. Quantification of normalized fold expression of indicated genes with unpaired t test, ****, $P < 0.0001$. Histograms represent means ± s.d.

(B) Embryos were treated with DMSO or cyclopamine or purmorphamine from stage 18-19. 40 eyes per group were then dissected at stage 38 for quantitative PCR analysis. Quantification of normalized fold expression of indicated genes with one-way ANOVA (Dunnett's multiple comparisons test), ****, $P < 0.0001$. Histograms represent means ± s.d. ns: no statistical differences between the groups.

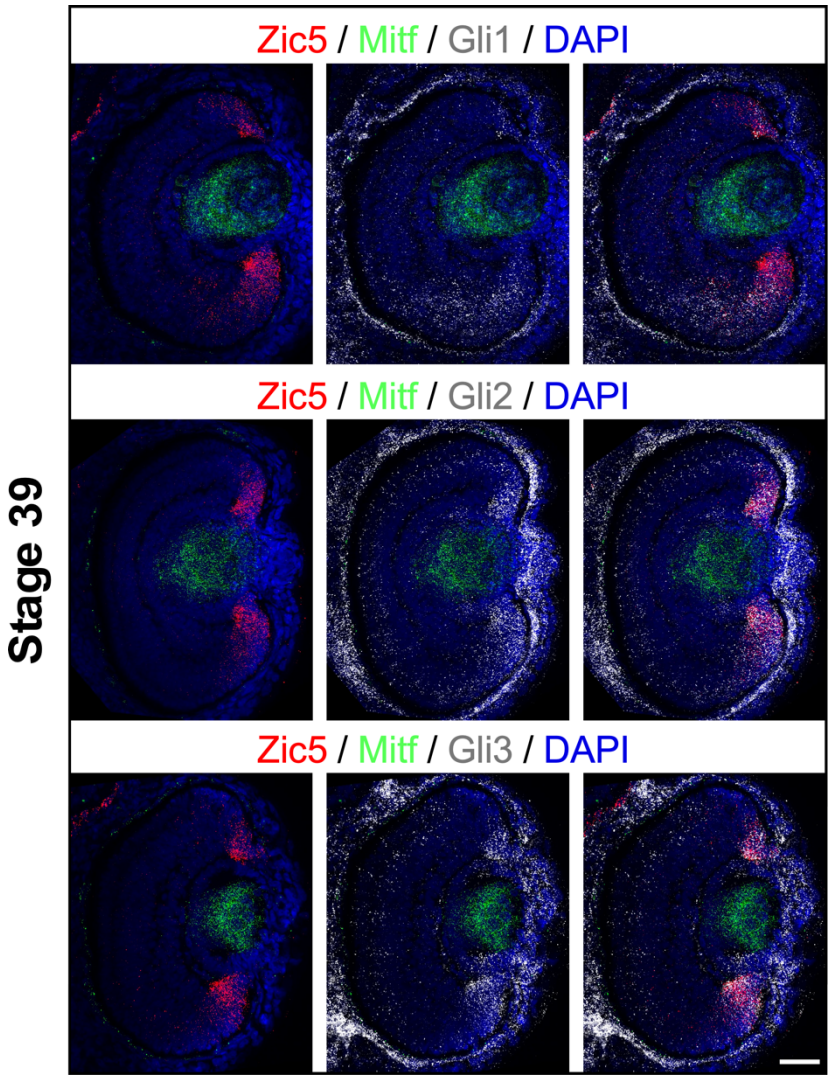


Figure S5. Related to Figure 4. Expression pattern of Zic5 and Glis in eyes at stage 39.
Section HCR analysis was performed using embryos from stage 39 with indicated probe sets. Mitf probes were used as a RPE marker. Scale bars, 40 μm.

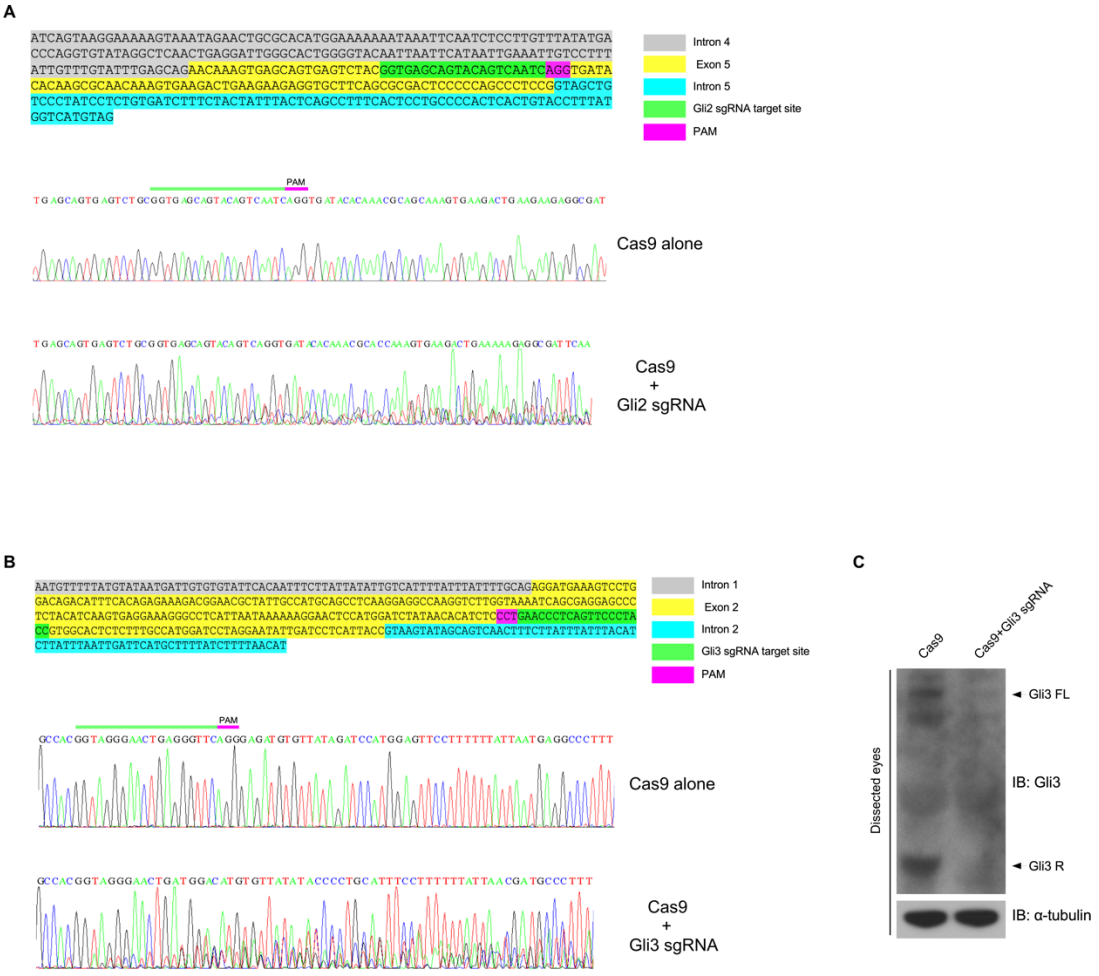


Figure S6. Related to Figure 4. Gli2 and Gli3 knockout by Crispr/Cas9

(A) Gli2 sgRNA targets the 5th exon of Gli2 gene. The direct sequencing of PCR amplicons shows the genomic DNA of Gli2 was edited by Cas9/Gli2 sgRNA co-injection.

(B) Gli3 sgRNA targets the 2nd exon of Gli3 gene. The direct sequencing of PCR amplicons shows the genomic DNA of Gli3 was edited by Cas9/Gli3 sgRNA co-injection.

(C) Verification of Gli3 knockout by western blot of endogenous Gli3 protein level.

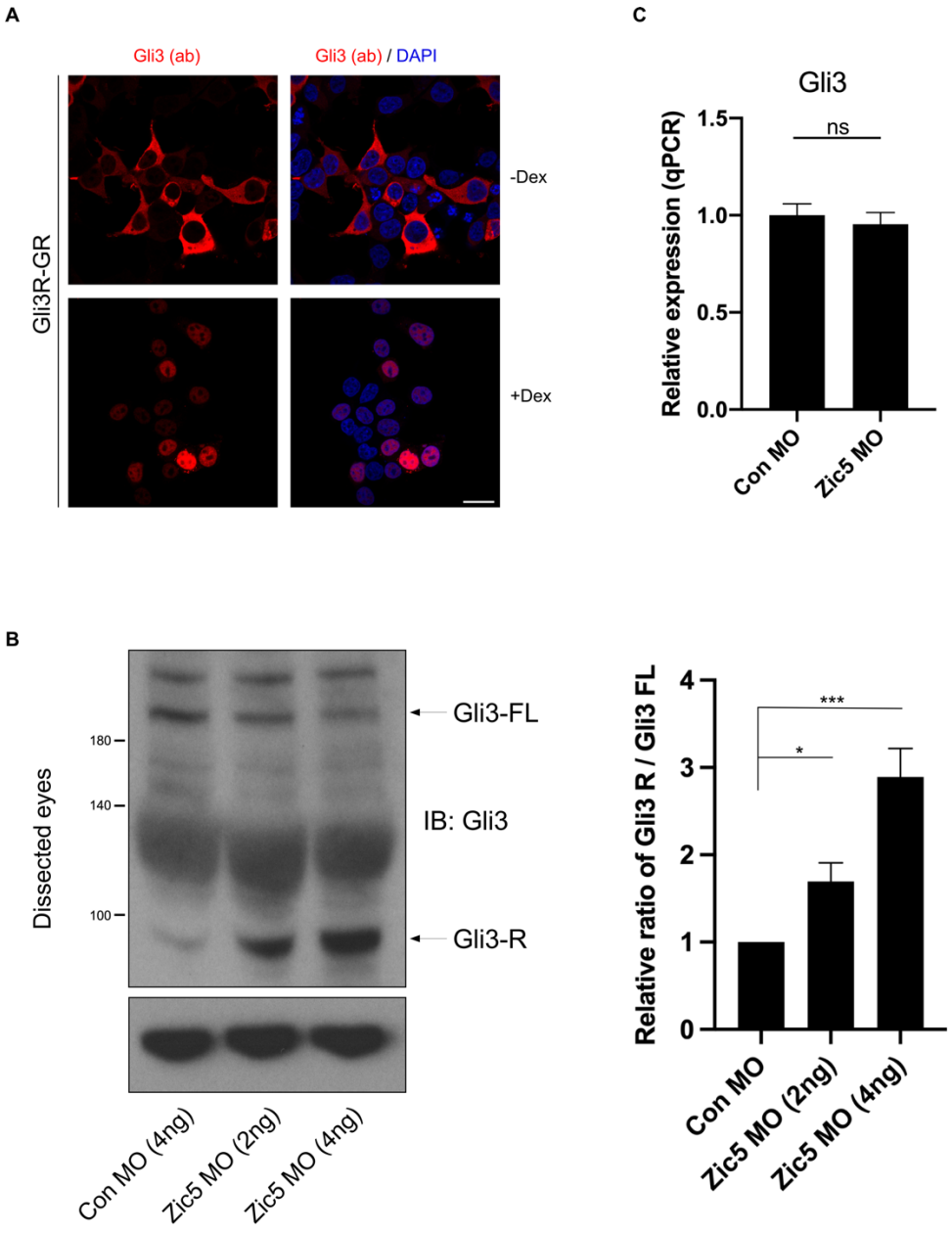


Figure S7. Related to Figure 4. Zic5 knockdown increases ratio Gli3R/Gli3 FL without affecting Gli3 mRNA level in the eye.

(A) Validation of Gli3R-GR construct in HEK 293T cells.

(B) Different doses of Zic5 MO were injected into D1.1.1 blastomere at 32-cell stage. Approximately, 46 Eyes were dissected at stage 35, lysed and immunoblotted with anti-Gli3 N-terminal antibody. Quantification of relative ratio of Gli3R/Gli3FL of western bolt with one-way

ANOVA (Dunnett's multiple comparisons test), *, $P < 0.05$, ***, $P < 0.001$. Histograms represent means ± s.d from three biological repeats.

(C) Con MO or Zic5 MO was injected into the D1.1.1 blastomere at 32 -cell stage. 40 eyes per group were then dissected at stage 38 for quantitative PCR analysis. Quantification of normalized fold expression of Gli3 with unpaired t test. Histograms represent means ± s.d. ns: no statistical differences between the groups.

Table S1. Real-time RT-PCR primer sequences. Related to STAR Methods.

Gene	Sequences
ODC	F: 5'-GCTTCTGGAGCGGGCAAAGGA-3' R: 5'-CCAAGCTCAGCCCCCATGTCA-3'
Gli1	F: 5'-GCACAAGCCAGCGATCATTT-3' R: 5'-ATCAATGCTGGCATCCGACA-3'
Gli3	F: 5'-CCCAGCAAAGGGGCTATCAA-3' R: 5'-ATTGCCCCTGCCTAACTGAC-3'
Hes1	F: 5'-GGCATGAACTACCCTACCCAG-3' R: 5'-CACTTTGGCAGCTTCGACTG-3'
Hes5.2	F: 5'-CATTTCCATTCAAATGCGGCAG-3' R: 5'-CTACCATGGCCTCCAAAGACT-3'
Patched 1	F: 5'-CAGCTGCCCAGCCGAGGGTA-3' R: 5'-GGGCGAAATTGGCATCGCAGTA-3'
Cyclin D1	F: 5'-CATCCGCAAACACGCCCAGA-3' R: 5'-GACACTGCCAGCGGCGATCA-3'