

**Supporting Information for
Post-transcriptional tuning of FGF signaling mediates neural crest induction**

Jacqueline Copeland and Marcos Simoes-Costa*

Department of Molecular Biology and Genetics, Cornell University, Ithaca, NY, USA. *Corresponding author:

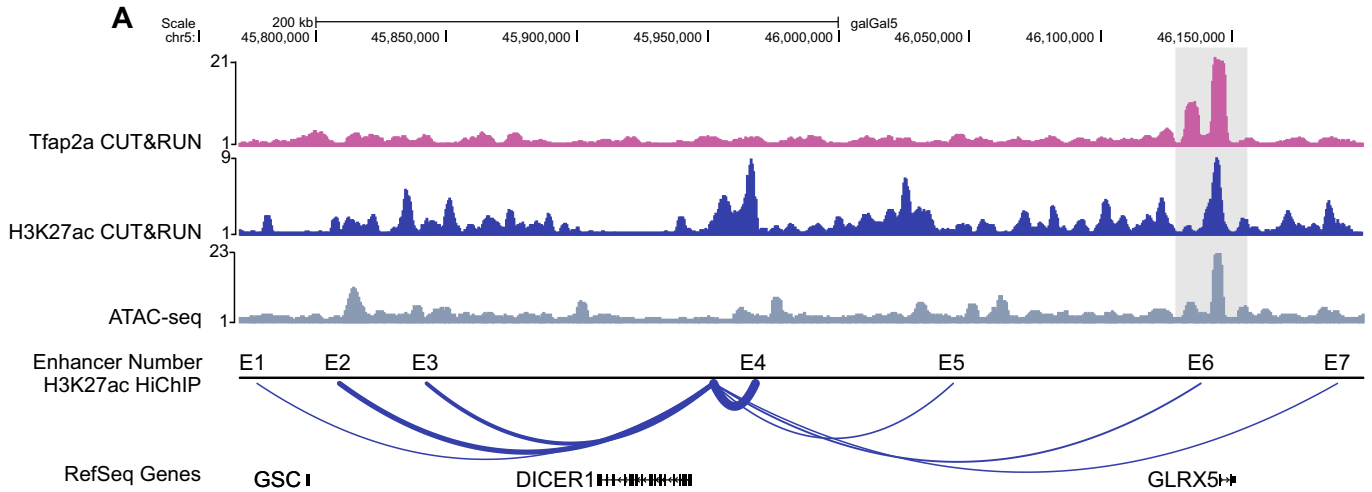
Marcos Simoes-Costa

Email: simoescosta@cornell.edu

This PDF file includes:

Figure S1-S5

Table S1-S3



B

Enhancer	Expression pattern
E1	No expression
E2	Neural
E3	Ectoderm
E4	Ectoderm
E5	No expression
E6	Neural Crest
E7	Neural/Neural Crest

Figure S1. (Related to Figure 1) Putative DICER enhancers exhibit a broad range of ectodermal tissue specificity

(A) TFAP2A CUT&RUN, H3k27Ac CUT&RUN, ATAC-seq profiles and H3k27Ac Hi-ChIP arc plot at the DICER locus. Region encompasses all enhancer-promoter loops identified in H3k27Ac Hi-ChIP. Putative enhancers were determined by presence of loop and are labeled in order of ascending genomic coordinates. **(B)** Description of expression patterns for all DICER enhancers tested via transient transgenesis in the chick embryo. Expression patterns were determined based on co-localization with the neural crest reporter Tfp2aE1:mCherry.

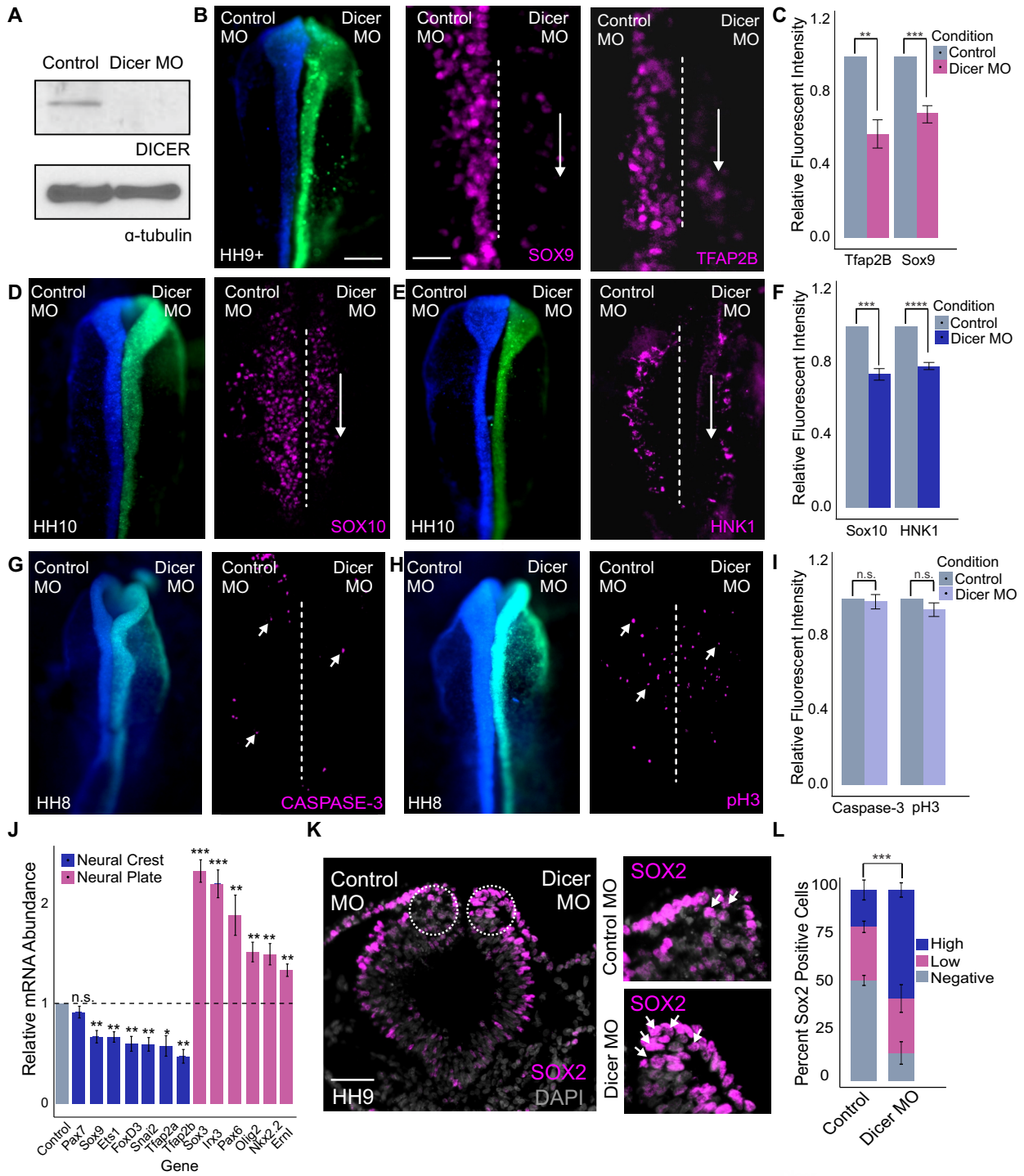


Figure S2. (Related to Figure 2) DICER knockdown controls and additional analysis of neural crest and neural plate markers upon DICER knockdown in avian embryos

(A) Western blot analysis revealed a decrease in DICER protein levels upon knockdown with translation inhibiting morpholino. **(B)** Dorsal whole mount view of HH9+ embryo with Control MO on the left and DICER MO on the right. Immunohistochemistry for neural crest markers TFAP2B and SOX9 upon DICER knockdown. Dotted line represents embryo midline. **(C)** Quantification of TFAP2B and SOX9-positive cells following DICER knockdown, normalized to the control side of the embryo (n=5). **(D)** Dorsal whole mount view of HH10 embryo with Control MO on the left and DICER MO on the right. Immunohistochemistry for

neural crest marker SOX10 upon DICER knockdown. Dotted line represents embryo midline. **(E)** Dorsal whole mount view of HH10 embryo with Control MO on the left and DICER MO on the right. Immunohistochemistry for neural crest marker HNK1 upon DICER knockdown. Dotted line represents embryo midline. **(F)** Quantification of SOX10 and HNK1-positive cells following DICER knockdown, normalized to the control side of the embryo (n=5). **(G)** Dorsal whole mount view of HH8 embryo with Control MO on the left and DICER MO on the right. Immunohistochemistry for cell death marker CASPASE-3 upon DICER knockdown. Dotted line represents embryo midline. **(H)** Dorsal whole mount view of HH8 embryo with Control MO on the left and DICER MO on the right. Immunohistochemistry for cell proliferation marker phospho-histone H3 upon DICER knockdown. Dotted line represents embryo midline. **(I)** Quantification of fluorescence revealed no change in cell death (Caspase-3) or proliferation (pH3) upon DICER knockdown (n=5). **(J)** Quantitative RT-PCR for select neural crest and neural plate genes in embryos electroporated with DICER morpholino. Phenotypes were assayed at stage HH8, comparing control to experimental sides of the embryo (n=6). **(K)** Immunohistochemistry (transverse section) for neural plate marker SOX2 at HH9 upon DICER knockdown. Dotted lines highlight differences in high intensity SOX2-positive cells (arrows) in control and DICER MO sides of the embryo. **(L)** Quantification of SOX2-positive (low and high intensity) and negative cells in the top $\frac{1}{4}$ of the dorsal neural tube upon DICER knockdown, as compared to the control. P-value represents students t-test comparing percentage of high intensity SOX2-positive cells between control and DICER MO sides of the embryo (n=5). Error bars in (C), (F), (I), (J), and (L) represent standard deviation. Arrowheads in (B), (D), (E), and (G) represent a decrease in marker expression upon knockdown. MO: morpholino, n.s.: not significant. Scale bars, 200uM in (B, D, E, G, and H) dorsal whole mount view of embryo with Control MO on the left and DICER MO on the right, 100uM in (B, D, E, G, and H) immunohistochemistry for neural crest markers, 50uM in (K).

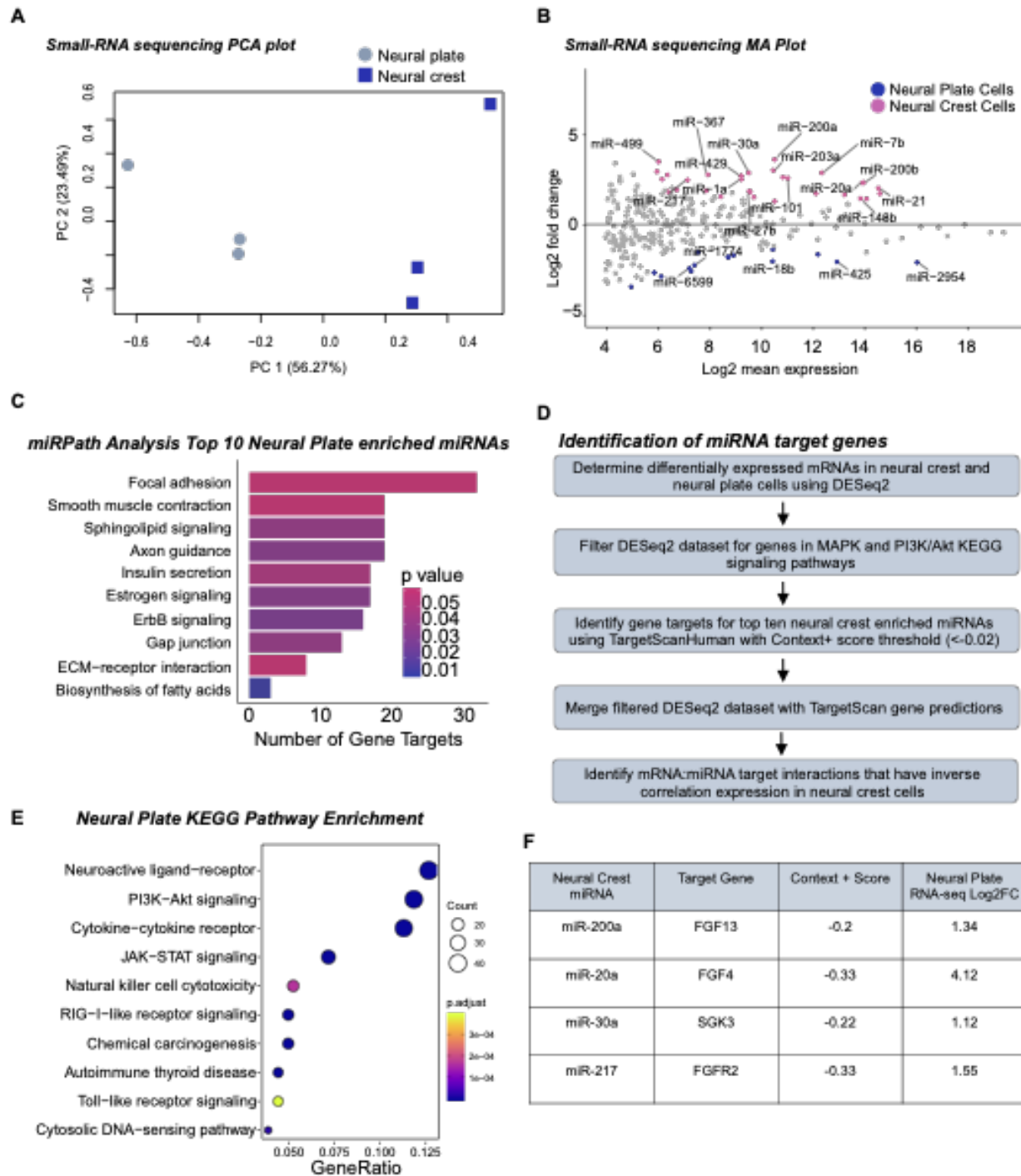


Figure S3. (Related to Figure 3) Additional analysis of small RNA-sequencing libraries and target identification. **(A)** Principal component analysis for neural crest and neural plate small RNA-sequencing libraries (n=3). **(B)** MA-plot from differential miRNA expression analysis between the neural crest and neural plate. Significant differentially expressed miRNAs were labeled based on FDR = 0.05, Log2 fold change > 1, padj < 0.05. **(C)** miRPath analysis for top ten neural plate enriched miRNAs. The plot shows the top ten KEGG pathways identified in this analysis. **(D)** Description of pipeline used to identify neural crest miRNA gene targets. **(E)** KEGG pathway enrichment analysis for neural plate enriched mRNAs shows upregulation of genes associated with FGF signaling cascades (PI3K-Akt and JAK-STAT). **(F)** Confidently predicted miRNA:mRNA target interactions based on analysis pipeline in Fig. S3D.

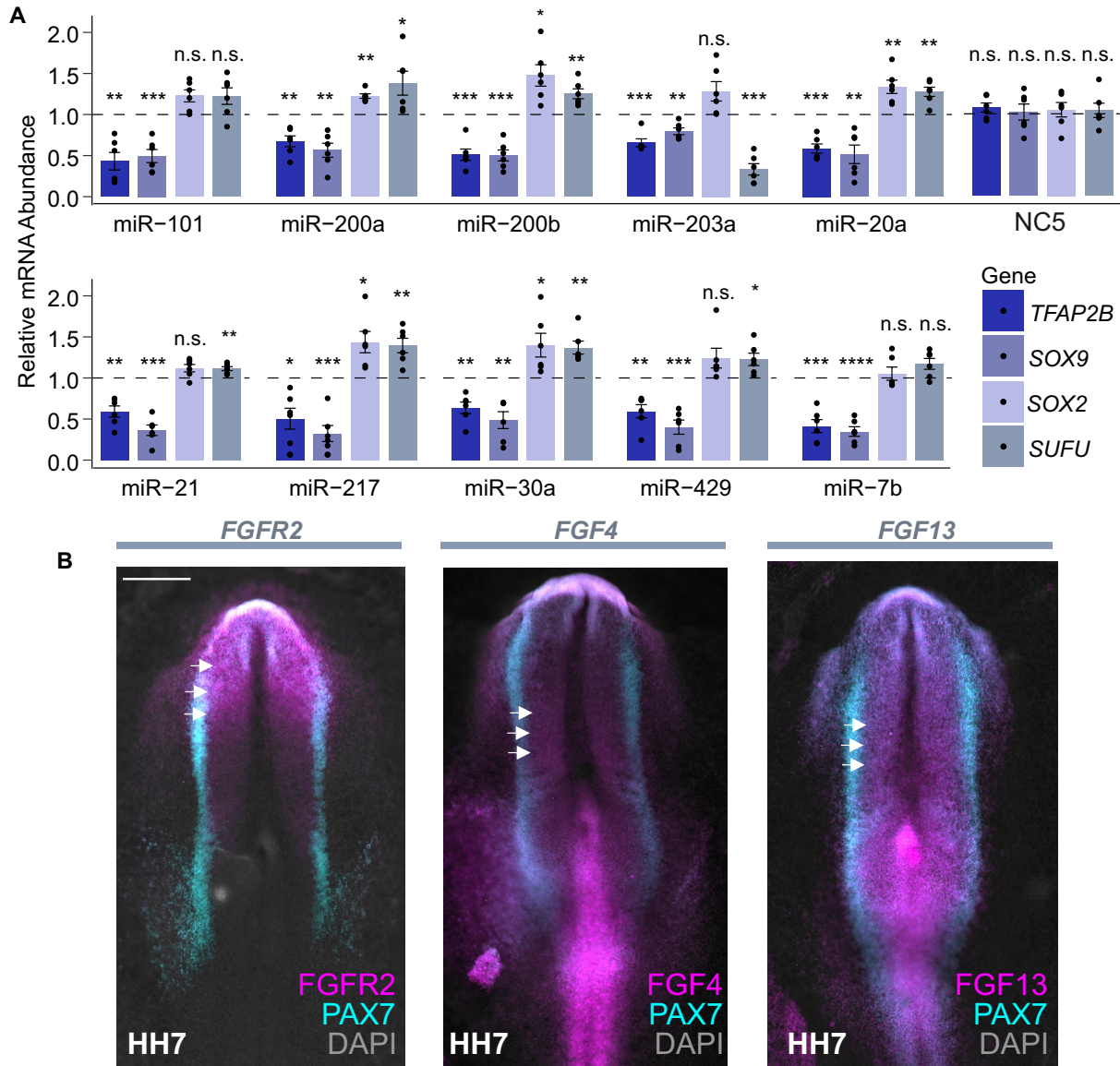


Figure S4. (Related to Figure 4) Additional neural crest miRNAs screened for DICER knockdown related phenotypes and candidate miRNA target gene expression patterns in avian embryos

(A) Quantitative RT-PCR for neural crest genes *TFAP2B* and *SOX9* and neural plate genes *SOX2* and *SUFU* upon miRNA loss-of-function for the top ten significantly enriched neural crest miRNAs (n=6). **(B)** Dorsal whole mount view of chicken embryos from double fluorescent in situ hybridization for each miRNA target gene (*FGFR2*, *FGF4*, or *FGF13*) and *PAX7* at developmental stage HH7. miRNA target genes are enriched in the neural plate, having low co-localization with the neural plate border marker *PAX7*. Error bars in (A) represent standard deviation. Arrowheads in (B) denote the neural plate border. Scale bars, 500 μ m in (B).

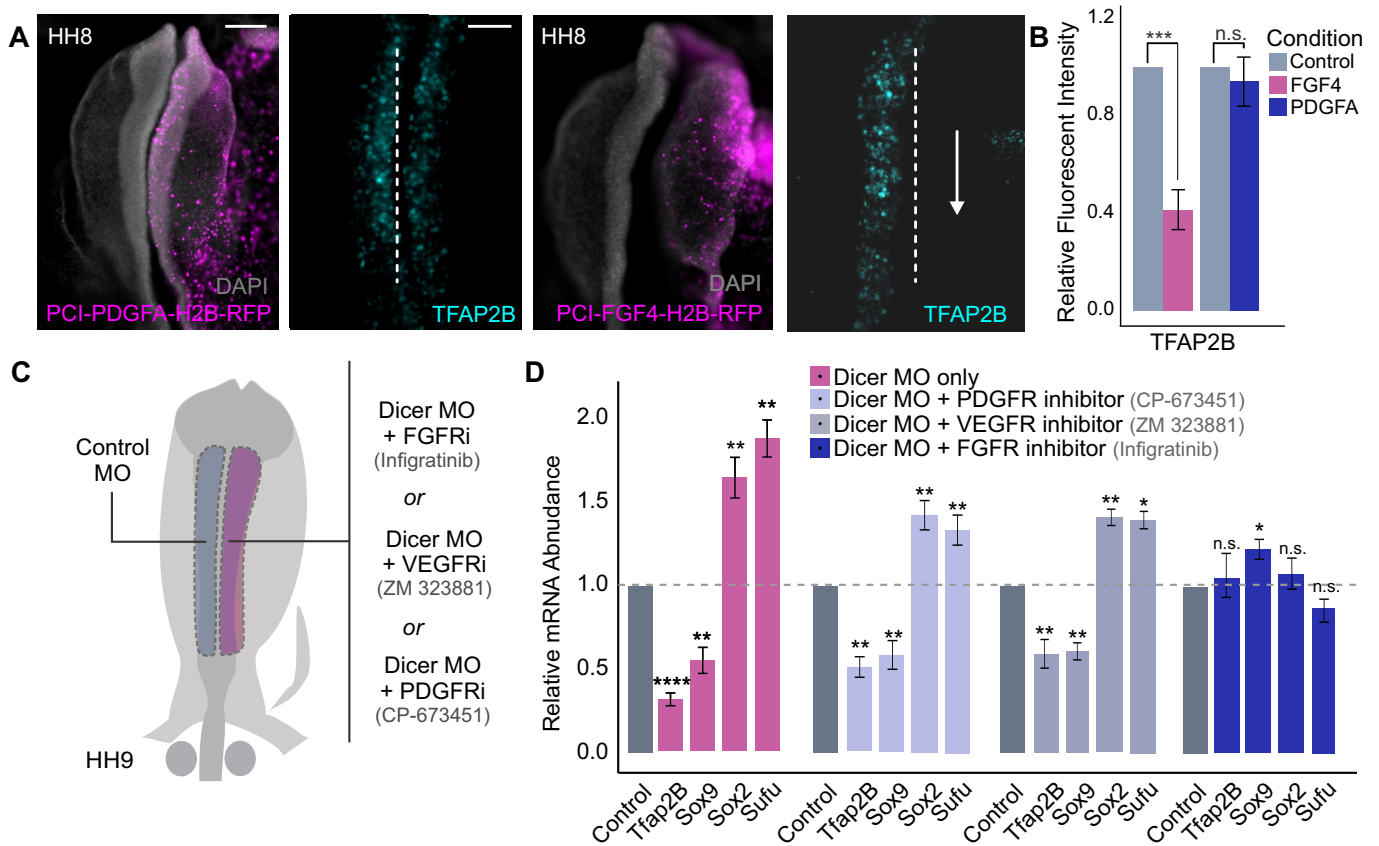


Figure S5. (Related to Figure 5) Manipulation of FGF signaling alters DICER knockdown phenotype as compared to manipulation of other RTK signaling pathways

(A) Dorsal whole mount view of HH8 embryos unilaterally transfected with PCI H2B RFP FGF4 or PCI H2B RFP PDGFA overexpression constructs. Immunohistochemistry for neural crest marker TFAP2B upon overexpression. Dotted line represents embryo midline. **(B)** Quantification of TFAP2B positive cells following overexpression of FGF4 or PDGFA, normalized to the control side of the embryo (n=6). **(C)** Electroporation scheme for DICER MO rescue experiments. **(D)** Quantitative RT-PCR for neural crest specification genes *TFAP2B* and *SOX9* and neural plate genes *SOX2* and *SUFU* in embryos electroporated with DICER MO alone or rescued by incubation with either FGFR1/2/3 inhibitor (Infigratinib), VEGFR2 inhibitor (ZM 323881), or PDGFR α/β inhibitor (CP-673451). Each experimental condition was normalized to the control side of bilaterally electroporated embryos for that given condition (n=6). Error bars in (B) and (D) represent the standard deviation. Arrowheads in (A) represent a decrease in marker expression upon perturbation. HH: Hamburger Hamilton, n.s.: not significant, MO: morpholino * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$. Scale bars, 200uM in (A) dorsal whole mount view of embryo with control on the left and experimental condition on the right, 100uM in (A) immunohistochemistry for TFAP2B.

Table S1. H3K27Ac Hi-ChIP enhancer-promoter contact locations in the DICER locus.

Loop Position	Enhancer side of loop (bp)	Promoter side of loop (bp)	Enhancer Designation	Loop logFC
Chromosome 5	46166839	46213256	DicerE1	1.33
Chromosome 5	45772137	45782937	DicerE2	1.0
Chromosome 5	45825755	45858813	DicerE3	0.94
Chromosome 5	45793675	45824322	DicerE4	0.91
Chromosome 5	45961494	45974179	DicerE5	0.72
Chromosome 5	46127097	46148620	DicerE6	0.68
Chromosome 5	46034137	46052549	DicerE7	0.63

Table S2. Number of biological replicates and p-values for quantitative experiments.

Experiment	No. of replicates/embryos	p-value	Figure
miRNA biogenesis enzymes qPCR	5 replicates/gene/stage 1 replicate=1,000 GFP+/- sorted cells		
Dicer HH8		0.03	Fig 1D
Dicer HH10		0.139	Fig 1D
Drosha HH8		0.04439	Fig 1D
Drosha HH10		0.1116	Fig 1D
Ago2 HH8		0.1416	Fig 1D
Ago2 HH10		0.1116	Fig 1D
Dicer single cell suspension immunohistochemistry	10 embryo heads/sample	p < 1.00e-04	Fig 1E
Dicer enhancer loss-of-function assay qPCR	3 independent replicates 1 replicate=100 GFP+/- sorted cells		
Dicer		0.0285	Fig 1I
Dicer knockdown immunohistochemistry quantification	5 embryos/immunostaining		
Ap2B HH8		9.86E-05	Fig 2A-B
Sox9 HH8		0.00381	Fig 2A-B

Ap2B HH9		0.002767	Fig S2B-C
Sox9 HH9		0.001163	Fig S2B-C
Sox10 HH10		0.0003209	FigS2D, F
HNK1 HH10		7.883e-05	FigS2E, F
Caspase-3		0.9822	Fig S2G, I
pH3		0.3621	Fig S2H, I
Sox2 HH8		Control High vs Dicer MO High= 0.0001352	Fig 2G-H
Sox2 HH8		Control Low vs Dicer MO Low = 0.000437	Fig 2G-H
Sox2 HH8		Control Neg vs Dicer MO Neg = 0.01677	Fig 2G-H
Sox2 HH9		Control High vs Dicer MO High= 0.0006557	Fig S2K-L
Sox2 HH9		Control Low vs Dicer MO Low = 0.0319	Fig S2K-L
Sox2 HH9		Control Neg vs Dicer MO Neg = 0.1047	Fig S2K-L
Dicer knockdown qPCR for neural crest and neural plate genes	5 replicates/condition/gene 1 replicate = 1 bilaterally transfected embryo		
Pax7		0.2118	FigS2J
Sox9		0.005788	FigS2J
Ets1		0.002922	FigS2J
FoxD3		0.005529	FigS2J
Snai2		0.00384	FigS2J
Tfap2a		0.01421	FigS2J
Tfap2B		0.001493	FigS2J
Sox3		0.0002941	FigS2J
Irx3		0.0009952	FigS2J

Pax6		0.01184	FigS2J
Olig2		0.006345	FigS2J
Nkx2.2		0.009916	FigS2J
Ernl		0.005921	FigS2J
Dicer MUT/WT rescue qPCR	5 replicates/condition/gene 1 replicate = 1 bilaterally transfected embryo		
Ap2B-mutant		0.0298	Fig 2C
Ap2B-WT		0.03027	Fig 2C
FoxD3-mutant		0.005175	Fig 2C
FoxD3-WT		0.05286	Fig 2C
Sox9-mutant		0.001102	Fig 2C
Sox9-WT		0.05744	Fig 2C
miRNA qPCR	5 replicates/miRNA 1 replicate= 3,000 GFP-/+ sorted cells		
miR-200a		0.01714	Fig 3D
miR-200b		0.001592	Fig 3D
miR-203a		0.004788	Fig 3D
miR-30a		0.002435	Fig 3D
miR-429		0.005544	Fig 3D
miR-7b		0.005832	Fig 3D
miR-217		0.03295	Fig 3D
miR-21		0.003784	Fig 3D
miR-20a		0.01237	Fig 3D
miR-101		0.1845	Fig 3D
miR-18b		0.0001192	Fig 3D
qPCR upon miRNA gain-and-loss-of-function	6 replicates/condition/gene 1 replicate = 1 bilaterally transfected embryo		
Ap2B	miR-200a GOF	0.2519	Fig 4C
Sox9		0.06174	Fig 4C
Sox2		0.01486	Fig 4C
Sufu		9.22E-03	Fig 4C
Ap2B	miR-200a LOF	4.25E-03	Fig 4B
Sox9		0.003771	Fig 4B
Sox2		0.000435	Fig 4B
Sufu		0.04702	Fig 4B

Ap2B	miR-20a GOF	0.04489	Fig 4C
Sox9		0.01736	Fig 4C
Sox2		0.002046	Fig 4C
Sufu		0.0229	Fig 4C
Ap2B	miR-20a LOF	0.0006973	Fig 4B
Sox9		0.007732	Fig 4B
Sox2		0.008913	Fig 4B
Sufu		0.004327	Fig 4B
Ap2B	miR-217 GOF	0.02456	Fig 4C
Sox9		0.09155	Fig 4C
Sox2		0.00139	Fig 4C
Sufu		0.0004143	Fig 4C
Ap2B	miR-217 LOF	0.01147	Fig 4B
Sox9		0.000957	Fig 4B
Sox2		0.02016	Fig 4B
Sufu		0.006467	Fig 4B
qPCR upon miRNA loss-of-function	5 replicates/condition/gene 1 replicate = 1 bilaterally transfected embryo		
Ap2B	miR-101 LOF	0.003116	Fig S4A
Sox9		0.001433	Fig S4A
Sox2		0.02689	Fig S4A
Sufu		0.0767	Fig S4A
Ap2B	miR-200a LOF	0.004248	Fig S4A
Sox9		0.003771	Fig S4A
Sox2		0.000435	Fig S4A
Sufu		0.04702	Fig S4A
Ap2B	miR-200bLOF	0.0008324	Fig S4A
Sox9		0.0006817	Fig S4A
Sox2		0.01475	Fig S4A
Sufu		0.008529	Fig S4A
Ap2B	miR-203a LOF	0.0008299	Fig S4A
Sox9		0.00418	Fig S4A
Sox2		0.06262	Fig S4A
Sufu		0.0002114	Fig S4A
Ap2B	miR-20a LOF	0.0006973	Fig S4A
Sox9		0.007732	Fig S4A
Sox2		0.008913	Fig S4A
Sufu		0.004327	Fig S4A
Ap2B	miR-21 LOF	0.001869	Fig S4A

Sox9		0.0001751	Fig S4A
Sox2		0.05532	Fig S4A
Sufu		0.005038	Fig S4A
Ap2B	miR-217 LOF	0.01147	Fig S4A
Sox9		0.000957	Fig S4A
Sox2		0.02016	Fig S4A
Sufu		0.006467	Fig S4A
Ap2B	miR-30a LOF	0.003399	Fig S4A
Sox9		0.004122	Fig S4A
Sox2		0.03991	Fig S4A
Sufu		0.005429	Fig S4A
Ap2B	miR-429 LOF	0.003815	Fig S4A
Sox9		0.0009412	Fig S4A
Sox2		0.1006	Fig S4A
Sufu		0.04939	Fig S4A
Ap2B	miR-7b LOF	0.0007051	Fig S4A
Sox9		9.99E-05	Fig S4A
Sox2		0.5422	Fig S4A
Sufu		0.05865	Fig S4A
Ap2B	NC5 neg control	0.2304	Fig S4A
Sox9		0.8308	Fig S4A
Sox2		0.5966	Fig S4A
Sufu		0.6556	Fig S4A
qPCR for target genes upon miRNA loss-and-gain-of-function	5 replicates/condition/gene 1 replicate = 1 bilaterally transfected embryo		
SGK3	miR-30a LOF	0.111	Fig 4E
AKT3	miR-30a LOF	0.9715	Fig 4E
FGF4	miR-20a LOF	0.0008305	Fig 4E
FGF13	miR-200a LOF	0.0009129	Fig 4E
FGFR2	miR-217 LOF	0.04482	Fig 4E
FGF4	miR-20a GOF	0.005547	Fig 4F
FGF13	miR-200a GOF	0.003863	Fig 4F
FGFR2	miR-217 GOF	0.00175	Fig 4F
FGF4	NC5 control	0.3179	Fig 4F
FGF13	NC5 control	0.3205	Fig 4F
FGFR2	NC5 control	0.2064	Fig 4F
Dual luciferase 3'UTR reporter assays	3 individual replicates/condition (3 technical replicates each)		

FGF4	miR-20a mimic/control	0.01479	Fig 4H
FGF13	miR-200a mimic/control	0.02796	Fig 4H
FGFR2	miR-217 mimic/control	0.008378	Fig 4H
FGF4	miR-20a WT/Mutant	0.002929	Fig 4I
FGF13	miR-200a WT/Mutant	0.003736	Fig 4I
FGFR2	miR-217 WT/Mutant	0.01391	Fig 4I
Western blot quantification upon Dicer knockdown	3 individual replicates		
dpERK1/2		0.01013	Fig 5B-C
pAkt1/2/3		0.9612	Fig 5D-E
N2:eGFP enhancer fluorescent intensity quantification			
miRNA inhibitor		0.01053	Fig 5G-H
miRNA mimic		0.003401	Fig 5G-H
FGF4/PDGFA overexpression qPCR analysis			
Tfap2B	pCI-H2B-RFP-FGF4	1.363e-05	Fig 5I
Sox9	pCI-H2B-RFP-FGF4	0.0001364	Fig 5I
Sox2	pCI-H2B-RFP-FGF4	0.01028	Fig 5I
Sufu	pCI-H2B-RFP-FGF4	0.003127	Fig 5I
Tfap2B	pCI-H2B-RFP-PDGFA	0.8684	Fig 5I
Sox9	pCI-H2B-RFP-PDGFA	0.3875	Fig 5I
Sox2	pCI-H2B-RFP-PDGFA	0.3675	Fig 5I
Sufu	pCI-H2B-RFP-PDGFA	0.1133	Fig 5I
FGF4/PDGFA overexpression immunohistochemistry analysis			
Tfap2B	pCI-H2B-RFP-FGF4	0.0008537	Fig S5A-B
Tfap2B	pCI-H2B-RFP-PDGFA	0.602	Fig S5A-B
Dicer knockdown rescue experiments	6 replicates/condition/gene 1 replicate = 1 bilaterally transfected embryo		
Ap2B	Dicer MO only	5.58E-05	Fig 5K
Sox9		0.001966	Fig 5K
Sox2		0.006462	Fig 5K
Sufu		0.001437	Fig 5K
Ap2B	Dicer MO + SU5402 inhibitor	0.08349	Fig 5K
Sox9		0.5	Fig 5K
Sox2		0.8691	Fig 5K
Sufu		0.4728	Fig 5K

Ap2B	Dicer MO + miRNA mimic mix	0.05534	Fig 5K
Sox9		0.9401	Fig 5K
Sox2		0.2759	Fig 5K
Sufu		0.8077	Fig 5K
Ap2B	Dicer MO + FGFR inhibitor (Infigratinib)	0.7211	FigS5C-D
Sox9		0.02476	FigS5C-D
Sox2		0.5115	FigS5C-D
Sufu		0.07605	FigS5C-D
Ap2B	Dicer MO + VEGFR inhibitor (ZM 323881)	0.007863	FigS5C-D
Sox9		0.001399	FigS5C-D
Sox2		0.001343	FigS5C-D
Sufu		0.001786	FigS5C-D
Ap2B	Dicer MO + PDGFR inhibitor (CP-673451)	0.00118	FigS5C-D
Sox9		0.007359	FigS5C-D
Sox2		0.00944	FigS5C-D
Sufu		0.02037	FigS5C-D

Table S3. Custom reagents utilized in this study

Resource	Designation	Source or reference	Sequence	Additional information
Sequence based reagent	Dicer Morpholino	Genetools (this paper)	5'TGCAAAGCAGGGCTT3'	
Sequence based reagent	gga-miR-200a-3p inhibitor	IDT (this paper)	5'UAACACUGUCUGGUAACGAUGU'3	Detailed in Methods section
Sequence based reagent	gga-miR-20a-5p inhibitor	IDT (this paper)	5'UAAAGUGCUUUAUGUGCAGGUAG'3	Detailed in Methods section
Sequence based reagent	gga-miR-217-5p inhibitor	IDT (this paper)	5'UACUGCAUCAGGAACUGAUUGGAU'3	Detailed in Methods section
Sequence based reagent	gga-miR-101-3p inhibitor	IDT (this paper)	5'GUACAGUACUGUGUAACUGAA'3	Detailed in Methods section
Sequence based reagent	gga-miR-200b-3p inhibitor	IDT (this paper)	5' UAAUACUGCCUGGUAUUGAUGAU'3	Detailed in Methods section
Sequence based reagent	gga-miR-203a inhibitor	IDT (this paper)	5' GUGAAAUGUUUAGGACCACUUG'3	Detailed in Methods section
Sequence based reagent	gga-miR-21-3p inhibitor	IDT (this paper)	5' CAACAACAGUCGGUAGGCUGUC'3	Detailed in Methods section
Sequence based reagent	gga-miR-30a-5p inhibitor	IDT (this paper)	5' UGUAAACAUCCUCGACUGGAAG'3	Detailed in Methods section
Sequence based reagent	gga-miR-429-3p inhibitor	IDT (this paper)	5' UAAUACUGUCUGGUAUUGCCGU'3	Detailed in Methods section
Sequence based reagent	gga-miR-7b inhibitor	IDT (this paper)	5' UGGAAGACUAGUGAUUUUUGUU'3	Detailed in Methods section
Sequence based reagent	NC5 control inhibitor	IDT (this paper)	5'ACCAUAUUGCGCGUAUAGUCGC'3	Detailed in Methods section
Sequence based reagent	gga-miR-200a mimic	Qiagen (this paper)	5'UAACACUGUCUGGUAACGAUGU'3	Detailed in Methods section
Sequence based reagent	gga-miR-20a mimic	Qiagen (this paper)	5'UAAAGUGCUUUAUGUGCAGGUAG'3	Detailed in Methods section
Sequence based reagent	gga-miR-217 mimic	Qiagen (this paper)	5'UACUGCAUCAGGAACUGAUUGGAU'3	Detailed in Methods section

Sequence based reagent	AllStars Negative Control siRNA	Qiagen (this paper)		Predesigned/validated by Affymetrix GeneChip Array and cell-based assays
Chemical	SU5402	Sigma		SU5402 is a potent multi-targeted receptor tyrosine kinase inhibitor for VEGFR2, FGFR1, and PDGF-R β Cat # SML0443
Chemical	Infigratinib (BGJ398)	SelleckChem		Potent and selective FGFR 1/2/3 inhibitor Cat # S2183
Chemical	CP-673451	SelleckChem		CP-673451 is a selective inhibitor of PDGFR α / β Cat # S1536
Chemical	ZM 323881	SelleckChem		ZM 323881 is a potent and selective VEGFR2 inhibitor Cat # S2896