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Supplemental Information

Mosaic Heterochrony in Neural Progenitors Sustains Accelerated Brain Growth and Neurogenesis in the Juvenile Killifish *N. furzeri*

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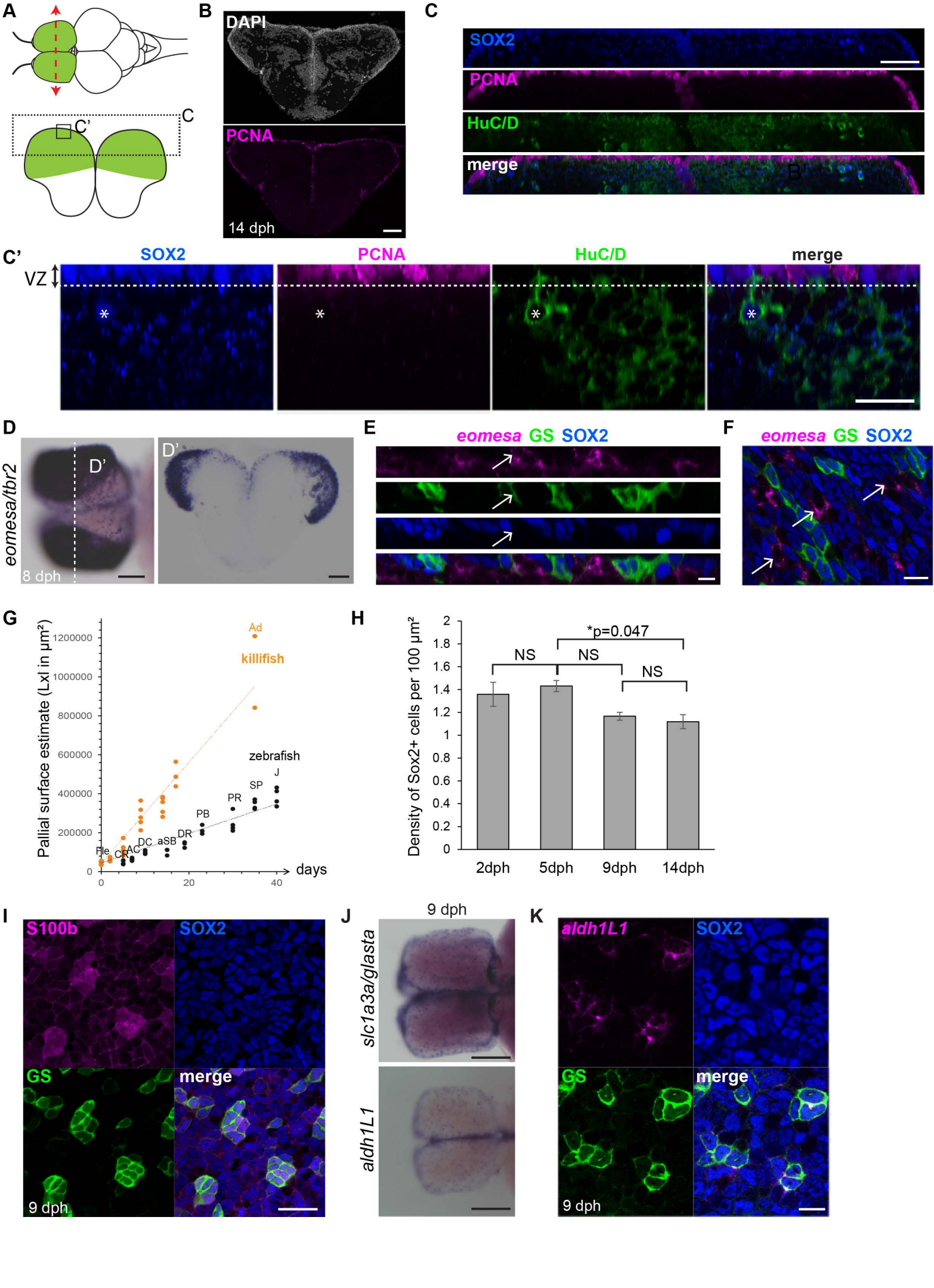

A
B
DAPI
PCNA
14 dph
C
C'
C
SOX2
PCNA
HuC/D
merge
B'
C'
VZ
SOX2
PCNA
HuC/D
merge
D
eomesa/tbr2
D'
D'
8 dph
E
eomesa GS SOX2
F
eomesa GS SOX2
G
Pallial surface estimate (Lxl in µm²)
1200000
1000000
800000
600000
400000
200000
0
0
20
40
days
Ad
killifish
zebrafish
J
SP
PR
PB
DR
aSB
DC
AC
CR
Ple
H
Density of Sox2+ cells per 100 µm²
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*p=0.047
NS
NS
NS
2dph
5dph
9dph
14dph
I
S100b
SOX2
GS
merge
9 dph
J
9 dph
slc1a3a/glasta
aldh1L1
K
aldh1L1
SOX2
GS
merge
9 dph

Figure S1. Apical progenitors at the surface of the rapidly growing killifish pallium, related to Figure 1

A. Scheme illustrating the location and morphology of the killifish pallium (green). The bottom panel illustrates a transverse section, and highlights the areas displayed in C and C'. **B**. Maximum intensity projection of a 50 µm-vibratome transverse section through the killifish pallium at 14 dph, showing immunostaining for PCNA (magenta) and nuclear DAPI counterstaining (grey). Note that PCNA-positive cells are only found along the T-shaped ventricle. Scale bar, 50 µm. **C**. 5 µm transverse section through a 3D-reconstruction showing immunostaining of a 7 dph killifish pallium for the markers Sox2 (blue), PCNA (magenta) and HuC/D (green). HuC/D (encoded by *elavl3/4* genes) is a marker of differentiated neurons. **C'**: higher magnification. Note that dividing neural progenitors (Sox2+PCNA+) are restricted to the ventricular zone (VZ), right above differentiated neurons. A few Sox2+/HuC+ neurons are found deep in the parenchyme (asterisk); these cells were excluded from our quantifications. Scale bar, 20 µm. **D-F**. Expression of the intermediate progenitor marker *eomesa/tbr2* in the killifish pallium. **D-D'**. Whole-mount ISH (blue) in a dorsal view (D) and vibratome transverse section (D'). Scale bar, 100 µm. **E-F**. ISH for *eomesa/tbr2* (magenta) together with immunostaining for the markers Sox2 (blue) and GS (green). High magnification of the ventricular zone in a 2 µm transverse section through a 3D-reconstruction (E) High-magnification of the pallial surface on a single optical z-plane (F). Arrows point to few *eomesa*+ cells located at the ventricle. These cells are devoid of Sox2 expression. Scale bar, 10 µm. **G**. Comparison of pallial growth between the killifish *N. furzeri* and zebrafish. Pallial surface was estimated by measuring pallial maximal length (L, along the antero-posterior axis) and width (l, along the left-right axis). Measurements were performed on samples from pre-hatching diapause III stage (t0) to sexually adult stage (35 dph) in *N. furzeri* and from Fle stage (~5 d post-fertilization, t0) to juvenile (~45 d post-fertilization) stage in zebrafish. Zebrafish larvae were staged according to [S1] and plotted using the corresponding average developmental time in AB zebrafish. (Fle: early flexion; CR: caudal fin ray appearance; AC: anal fin condensation; DC: dorsal fin condensation; aSB: anterior swim bladder appearance; DR: dorsal fin ray appearance; PB: pelvic bud appearance; PR: pelvin fin ray; SP: squamation onset posterior; J: Juvenile; Ad: Adult). **H**. Density of Sox2-positive cells at the pallial surface of the killifish. To estimate density, a surface was created on one pallial hemisphere using the Imaris surface function and the total number of Sox2-positive cells was quantified. (*) corrected p-value. Data were analysed with a one-way ANOVA, followed by pairwise comparisons using Holm's procedure. Proportions were rank transformed prior to analysis. Data are represented as mean ± SEM; n=6, 5, 5 and 3 for 2, 5, 9 and 14 dph respectively. **I**. Triple immunostaining for S100b, Sox2 and GS at 9dph. Images show high-magnifications of the pallial surface on a single optical z-plane. **J**. Dorsal views of the killifish pallium at 9 dph with a whole-mount ISH for *slc1a3a/glasta* and *aldh1l1* (blue) Note the salt-and-pepper distribution of the signal, matching the sparsed distribution of RG. Scale bar, 200 µm. **K**. ISH for *aldh1l1* (magenta) combined with immunostaining for GS (green) and Sox2 (blue) at 9 dph. Images are high magnifications of the pallial surface on a single optical z-plane. Note the co-localization of the ISH signal and GS. Scale bar, 10 µm.

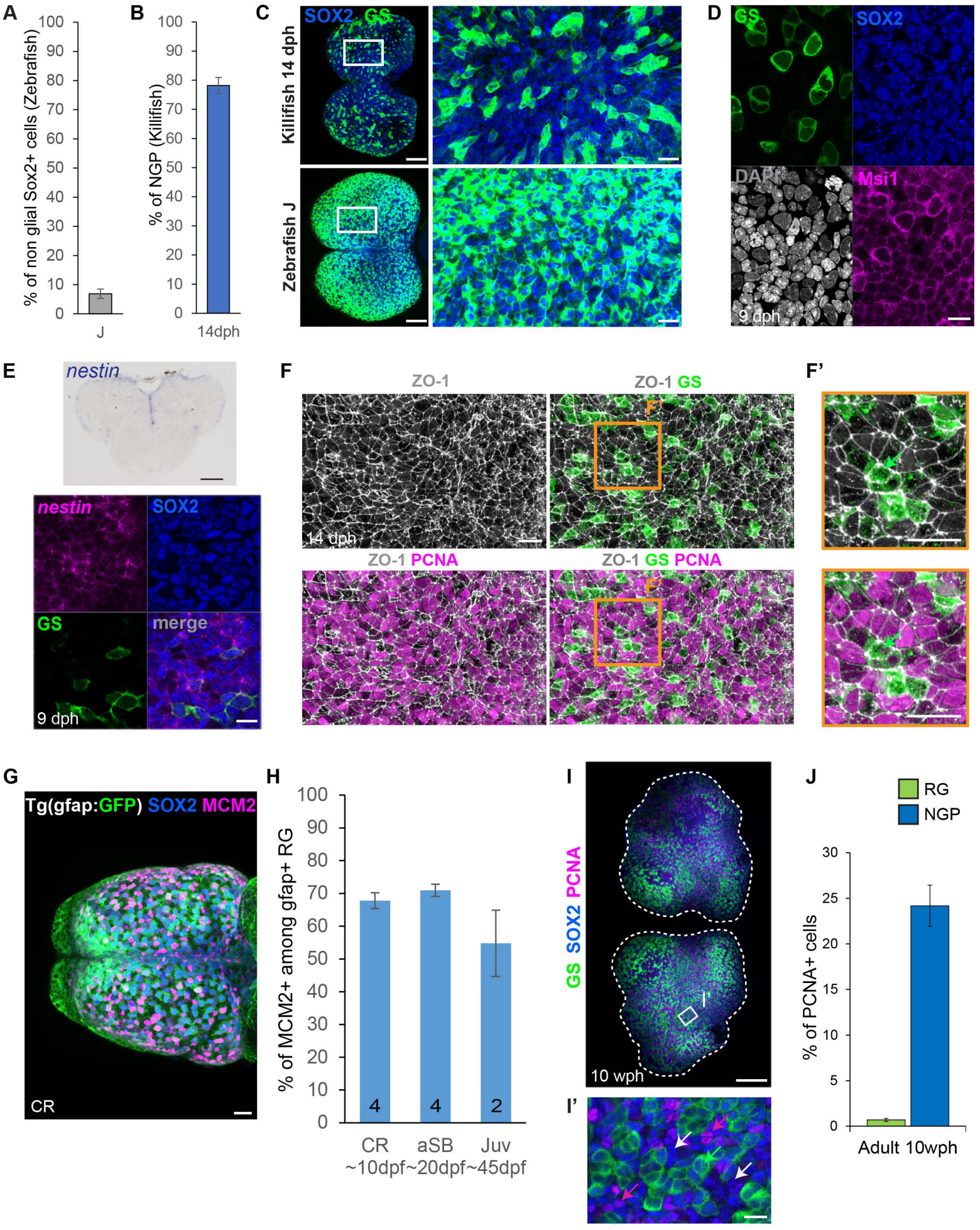
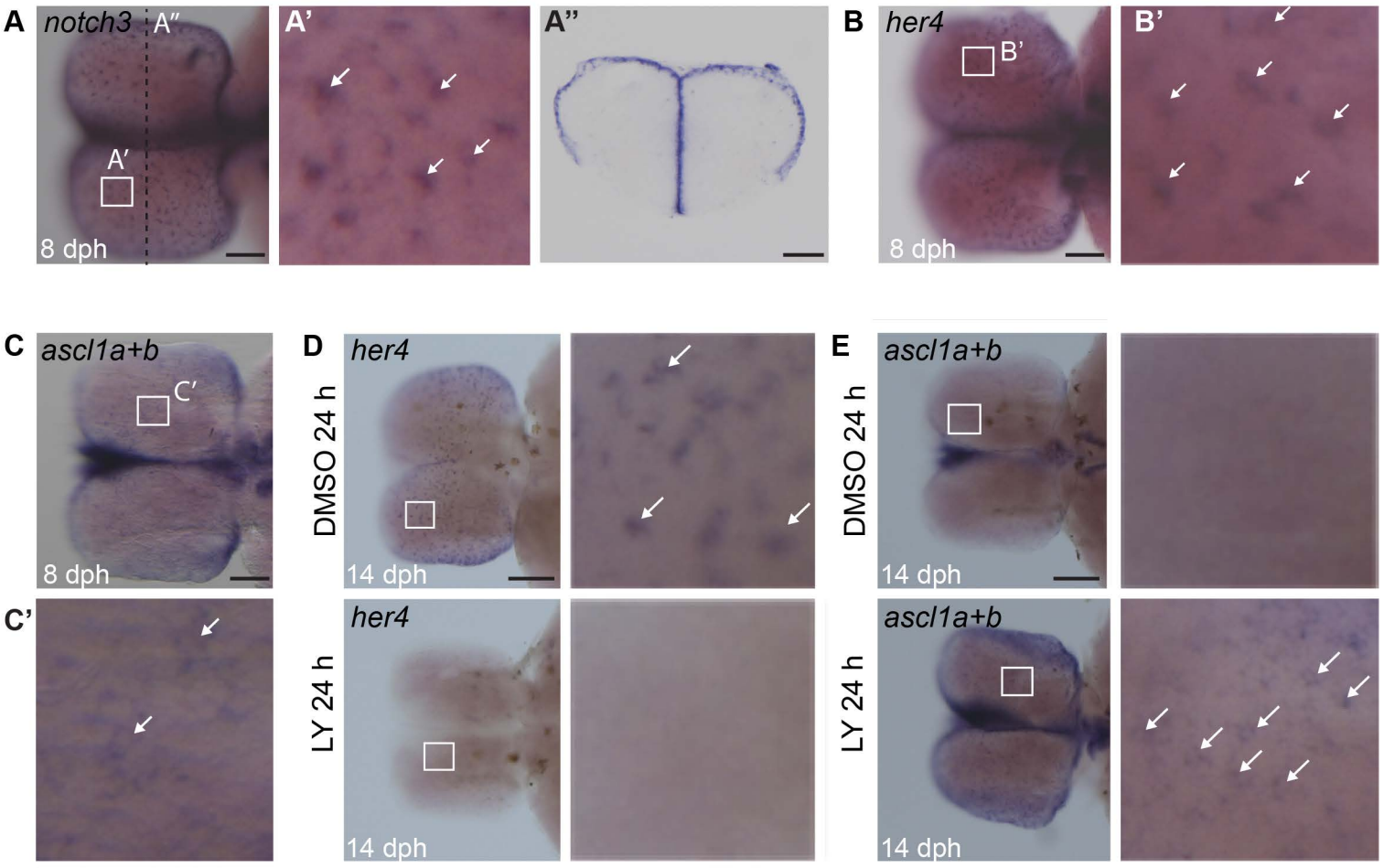

A
% of non glial Sox2+ cells (Zebrafish)
J
B
% of NGP (Killifish)
14dph
C
SOX2 GS
Killifish 14 dph
Zebrafish J
D
GS
SOX2
DAPI
Msi1
9 dph
E
nestin
nestin
SOX2
GS
merge
9 dph
F
ZO-1
ZO-1 GS
F'
14 dph
ZO-1 PCNA
ZO-1 GS PCNA
F'
F'
G
Tg(gfap:GFP) SOX2 MCM2
CR
H
% of MCM2+ among gfap+ RG
4
4
2
CR
aSB
Juv
~10dpf~20dpf~45dpf
I
GS SOX2 PCNA
I'
10 wph
I'
J
RG
NGP
% of PCNA+ cells
Adult 10wph

Figure S2. NGPs express primary progenitors markers and proliferate up to adulthood in killifish, related to Figure 2

A. Proportion of non-glial Sox2-positive cells at the ventricular surface of the zebrafish pallium at the Juvenile (J) stage. Sox2-positive cells were quantified in the transgenic line Tg(*gfap:GFP*)^{mi2001}, and cells without GFP signal were scored as non-glial progenitors. Data are represented as mean ± SEM; n=2. **B.** Proportion of NGP at the ventricular surface of the killifish pallium at 14 dph. Quantifications were performed on brains immunostained for GS, Sox2 and PCNA (see Figure 1A-B) and the Sox2-positive GS-negative cells are scored as NGP. Data are represented as mean ± SEM; n=3 **C.** Direct comparison of the distribution of RG cells at the surface of the pallium in zebrafish at the juvenile stage (left panel) and killifish at 14 dph (right panels) at the same magnification, using a double immunostaining for GS (green) and Sox2 (blue). A dorsal view of a 3D reconstruction is shown. Scale bar, 100µm for the global view and 20µm for high magnification views. **D**. Triple immunostaining for Msi1 (magenta), GS (green) and Sox2 (blue) with a DAPI counterstaining (grey) at 9 dph, showing that both RG and NGP express Msi1. Images are high magnifications of the pallial surface on a single optical z-plane. Scale bar, 10 µm **E**. ISH for *nestin* in the killifish pallium at 9 dph. Top panel: 50 µm-vibratome transverse section with ISH for *nestin* in blue. Note the localization of the signal to the ventricular surface. Scale bar, 100 µm. Bottom panel: ISH for *nestin* (magenta) combined with immunostaining for GS (green) and Sox2 (blue). Images are high magnifications of the pallial surface on a single optical z-plane and show that both RG and NGP express *nestin*. Scale bar, 10 µm. **F-F'**. Triple immunostaining for ZO-1 (grey), GS (green) and PCNA (magenta) at 14 dph. Images are high-magnifications of the pallial surface from a 3D reconstruction. Green arrows point to GS+ RG cells. Scale bar, 20 µm. **G**. Dorsal views of 3-D reconstruction of the zebrafish pallium at stage CR (~10 dpf). Neural progenitors are labelled with Sox2 antibody (blue), RG are labelled by a GFP reporter of the Tg(*gfap*:GFP)^{mi2001} line and proliferating cells are labelled with a MCM2 antibody (magenta). Scale bar, 70µm. **H.** Proportion of MCM2-positive proliferating cells among the RG population (GFP-positive in the Tg(*gfap*:GFP)^{mi2001} line). RG cells maintain a quite stable proliferation rate across stages. Data are represented as mean ± SEM. The number of samples used for each stage is indicated on the graph. Comparisons between these stages show no statistically significant differences. Data were analysed with a one-way ANOVA, followed by pairwise comparisons using Holm's procedure. Proportions were rank transformed prior to analysis. **I-I'** Dorsal 3D-views of killifish pallium (anterior left) at 10 weeks post-hatching (wph) with a whole-mount immunostaining for Sox2 (blue), PCNA (magenta) and GS (green). A dotted line contours the two pallial hemispheres. I' is a higher magnification of the pallial surface. Green arrows point to RG and white arrows to NGPs. Scale bars, 50 µm (I) and 10 µm (I'). **J**. Proportion of PCNA-positive cells among RG (green bars) and NGPs (blue bars) at 10 wph. Data are represented as mean ± SEM; n=3.


A
notch3
A''
A'
8 dph
A'
A''
B
her4
B'
8 dph
B'
C
ascl1a+b
C'
8 dph
C'
D
her4
DMSO 24 h
14 dph
LY 24 h
14 dph
E
ascl1a+b
DMSO 24 h
14 dph
LY 24 h
14 dph

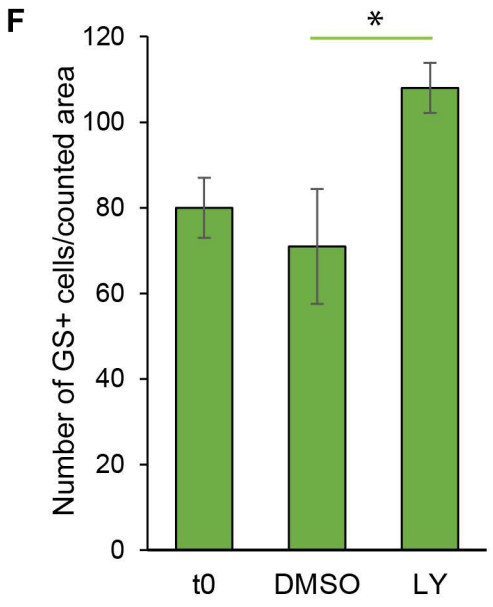

F
Number of GS+ cells/counted area
120
100
80
60
40
20
0
*
t0
DMSO
LY

Figure S3. Expression of Notch pathway genes involved in the quiescence/activation cycle of RG, related to Figure 3

A. Whole-mount ISH for *notch3* (blue) at 8 dph. A: dorsal view, A': 50 µm-vibratome transverse section A'' higher magnification. Note the salt-and-pepper distribution of the signal, matching the sparse distribution of RG (White arrows). **B**. Whole-mount ISH for *her4.2* (blue) at 8 dph. B: dorsal view, B' higher magnification. **C**. Whole-mount ISH for *ascl1a* and *ascl1b* (blue) at 8 dph. C: dorsal view, C' higher magnification. Only a few cells express *ascl1* orthologs in the pallium (White arrows). **D**. Effect of Notch inhibition on the expression of the canonical Notch target *her4.2*. Whole-mount ISH of the pallium are shown (blue) as well as a higher magnification in the right panels. *her4.2* signal totally disappears after 24 h of LY treatment. **E**. Effect of Notch inhibition on the expression of *ascl1* orthologs. Whole-mount ISH of the pallium are shown (blue) as well as a higher magnification in the right panels. Barely any signal for *ascl1a+b* is detected in control brains at 14 dph, in keeping with the low activation rate of RG at this stage. In contrast, mosaic expression of *ascl1a+b* in some cells of the ventricular surface is detected after LY treatment (white arrows). **F**. Number of RG cells (Sox2+GS+) per counted area at t0 and after 3 days in DMSO or LY. (*) corrected p-value < 0.05; one-way ANOVA, followed by pairwise comparisons using Holm's procedure. Data were rank transformed prior to analysis. Data are represented as mean ± SEM; n=3 for each treatment condition. Scale bars, 100 µm (A-C) and 200 µm (D-E).

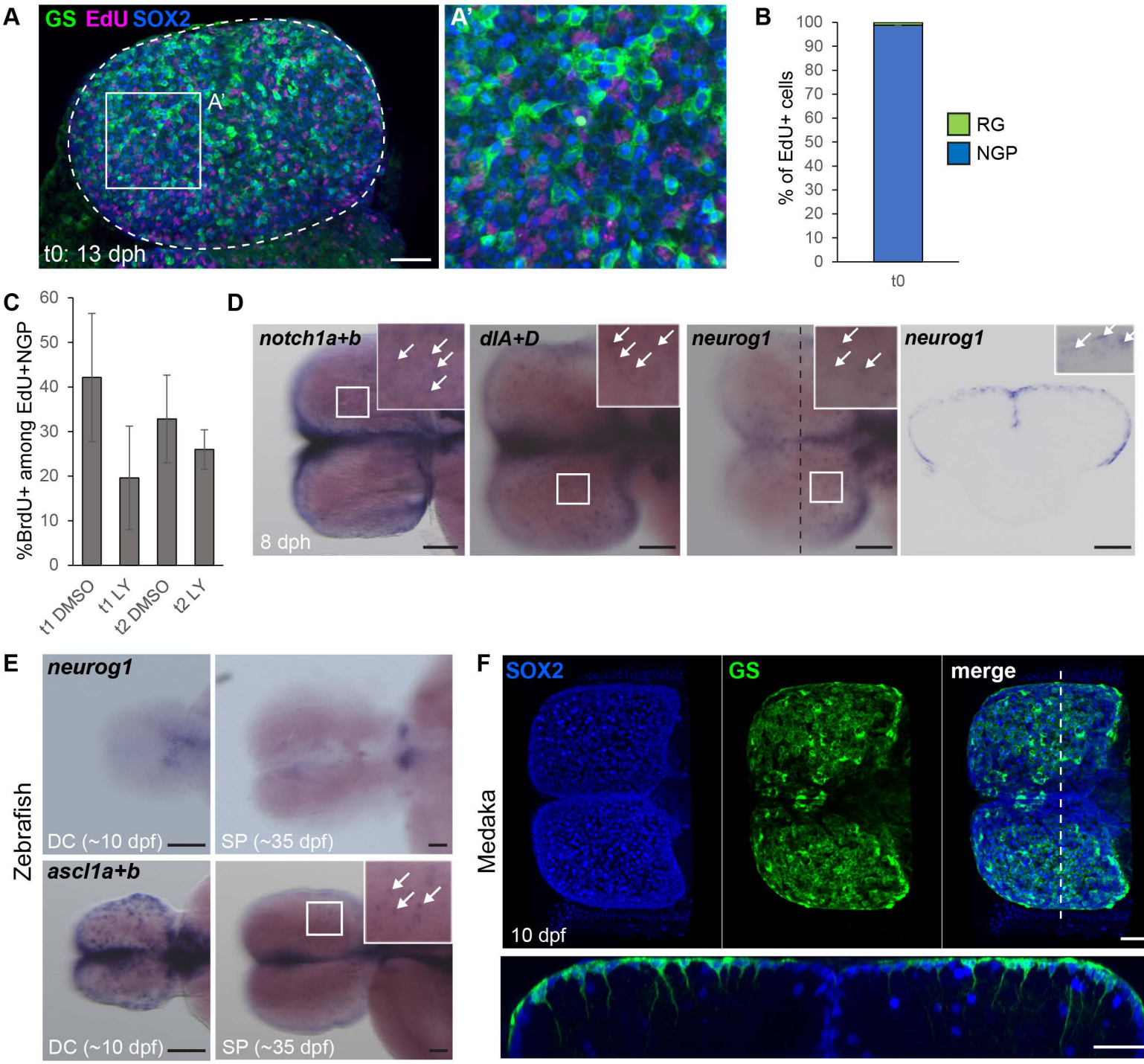

A
GS EdU SOX2
A'
t0: 13 dph
A'
B
% of EdU+ cells
100
90
80
70
60
50
40
30
20
10
0
t0
RG
NGP
C
%BrdU+ among EdU+NGP
60
50
40
30
20
10
0
t1 DMSO
t1 LY
t2 DMSO
t2 LY
D
notch1a+b
8 dph
dlA+D
neurog1
neurog1
E
Zebrafish
neurog1
DC (~10 dpf)
SP (~35 dpf)
ascl1a+b
DC (~10 dpf)
SP (~35 dpf)
F
Medaka
SOX2
GS
merge
10 dpf

Figure S4. Self-renewing and neurogenic NGPs maintain embryonic-like marker expression, related to figure 4

A-A'. Dorsal view of a killifish pallium at 13 dph with an immunostaining for Sox2, GS and detection of EdU. A' is a higher magnification of the boxed area in A. Most EdU-labeled cells are NGP. Scale bar, 50 µm **B**. Proportion of EdU-labeled cells that are NGP (blue, Sox2+GS-) or RG (green, Sox2+GS+) immediately following the EdU pulse (t0). Data are represented as mean ± SEM; n=2. **C**. Proportion of EdU-labeled NGP cells that have incorporated BrdU immediately following the BrdU pulse (t1) or after 3 days of chase. Data are represented as mean ± SEM; n=2 (t1) or n=3(t2) for each treatment condition. Statistical analysis does not reveal any statistical difference (2-way ANOVA approach followed by pairwise comparisons, data were ranked transformed prior to analysis). **D**. *notch1* orthologs, *Dll1* orthologs and *neurog1* expression in the killifish pallium at 8 dph. Whole-mount ISH of the pallium are shown as well as higher magnification in the top right corner. Some signal-positive cells are indicated by white arrows. A 50 µm-vibratome section is also shown for *neurog1*, highlighting the salt-and-pepper expression in the ventricular layer. Scale bar, 100 µm. **E**. ISH for *neurog1* and *ascl1a* at the DC and SP stages in zebrafish showing that neural progenitors express the proneural *ascl1a* but not *neurog1*. Scale bar, 100 µm **F**. Dorsal view (top panel) and cross section (bottom panel) of a 10 dpf medaka pallium immunostained for GS (green) and Sox2 (blue), showing that the ventricular surface is paved with RG like in zebrafish. Scale bar, 50 µm

Gene name	Genbank identifier	Zebrafish ortholog	Mouse ortholog	Primer FWD (5'-3')	Primer REV (5'-3')	insert length (bp)
Nf_aldh1l1	GAIB01094203	*aldh1l1*	*Aldh1L1*	ATCTACCACCCCTCTCTGCTC	GTTCATCTTGCCCCACTCTC	1134
Nf_ascl1a	GAIB01132435	*ascl1a*	*Ascl1*	CCTGGACACTGGGTGACTTT	TCCGTGGTTCTGTGAGGA	692
Nf_ascl1b	GAIB01133487	*ascl1b*	*Ascl1*	AAACTGCAACCATCACCACC	AGGGGCGGAAATGTACATCA	995
Nf_dlA	GAIB01004767	*dlA*	*Dll1*	ATCTAATATGGGGCGCGTCA	CTCTCTCAGGGTTGTCAGCA	1078
Nf_dlD	GAIB01092115	*dlD*	*Dll1*	GCATTCACAGTGACAGCCAA	CCATCCAAGTCATTCCTGCG	1063
Nf_eomesa	GAIB01100423	*eomesa*	*Eomes/Tbr2*	GTGAGGCAGAGGGGGACT	GGGAAGGTGAAGGTTTGG	904
Nf_her4.2	GAIB01137651	*her4.2*	*Hes5*	ATGGCTCCTACAATCACTGC	CCGTAGATTCAGTCGTGCAA	643
Nf_nestin	GAIB01121120	*nestin*	*nestin*	AGGGAAGCTCATCACAACGA	TGAGGCACCATGGATCTGTT	935
Nf_neurog1	GAIB01038862	*neurog1*	*Neurog1*	GAGCAAAGAAACGCATAGAGG	CCATCAGGAGGAGAAGTGGA	975
Nf_notch1a	GAIB01090429	*notch1a*	*Notch1*	ACGGCTTGGCTTCCTTCA	GGGTTGCTCTCACATTCATT	1114
Nf_notch1b	GAIB01101509	*notch1b*	*Notch1*	ACTGTGAGAGCCGTTACCTTCC	TACTTGTTTGGTCCGTCTGTG	1008
Nf_notch3	GAIB01090895	*notch3*	*Notch3*	TTCCTAATCCCTGCCTAAATG	CCACCCACTCTATCCACACA	1018
Nf_slc1a2a	GAIB01110701	*slc1a2a*	*slc1a2/Glt-1*	CCAATCCATCCAGATATTGTCAT	CTTGCTACAACCTCCAAGTCCT	707
Nf_slc1a3a	GAIB01014210	*slc1a3a*	*Glast*	TACACCACAACAACTGTCATCG	GGCAGAACTAGAAGAGGTTCCA	699

Table S1: List of primers used in this study to clone killifish genes, related to STAR Methods

Secondary Antibody Name	Target species	Host species	Coupled to	Source	Identifier
DyLight 405-AffiniPure Goat Anti-Mouse IgG2a	Mouse IgG2a	Goat	DyLight405	Jackson ImmunoResearch Labs	Cat#115-475-206, RRID:AB_2338800
Goat anti-Mouse IgG2a-488	Mouse IgG2a	Goat	Alexa Fluor 488	Thermo Fisher Scientific	Cat#A-21131, RRID:AB_2535771
Goat anti-Mouse IgG2a-546	Mouse IgG2a	Goat	Alexa Fluor 546	Thermo Fisher Scientific	Cat#A-21133, RRID:AB_2535772
Goat anti-Mouse IgG2a-633	Mouse IgG2a	Goat	Alexa Fluor 633	Thermo Fisher Scientific	Cat#A-21136, RRID:AB_2535775
Goat anti-Mouse IgG2b-633	Mouse IgG2b	Goat	Alexa Fluor 633	Thermo Fisher Scientific	Cat#A-21146, RRID:AB_2535782
DyLight™ 405 Goat anti-Mouse IgG1	Mouse IgG1	Goat	DyLight405	Biolegend	Cat#409109, RRID:AB_10642830
Goat anti-Mouse IgG1-546	Mouse IgG1	Goat	Alexa Fluor 546	Thermo Fisher Scientific	Cat#A-21123, RRID:AB_2535765
Goat anti-Mouse IgG1-647	Mouse IgG1	Goat	Alexa Fluor 647	Thermo Fisher Scientific	Cat#A-21240, RRID:AB_2535809
Goat anti-Rabbit-546	Rabbit IgG (H+L)	Goat	Alexa Fluor 546	Thermo Fisher Scientific	Cat#A-11010, RRID:AB_2534077
Goat anti-Rabbit-633	Rabbit IgG (H+L)	Goat	Alexa Fluor 633	Thermo Fisher Scientific	Cat#A-21071, RRID:AB_2535732
Goat Anti-Chicken IgG-488	Chicken IgY	Goat	Alexa Fluor 488	Thermo Fisher Scientific	Cat#A-11039, RRID:AB_142924
Goat anti-Rat IgG-488	Rat IgG (H+L)	Goat	Alexa Fluor 488	Thermo Fisher Scientific	Cat#A-11006, RRID:AB_2534074

Table S2: List of secondary antibodies used in this study, related to STAR Methods

Supplemental Reference

S1. Parichy, D.M., Elizondo, M.R., Mills, M.G., Gordon, T.N., and Engeszer, R.E. (2009). Normal table of postembryonic zebrafish development: staging by externally visible anatomy of the living fish. Dev. Dyn. Off. Publ. Am. Assoc. Anat. *238*, 2975–3015.