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Supporting Information for

Left/right asymmetric collective migration of parapineal cells is mediated by focal FGF signalling activity in leading cells.

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SI. Materials and Methods

Embryos were raised and staged according to standard protocols (1).

Drug treatment

Embryos collected from *Tg(dusp6:d2EGFP)*^{pt6} outcrosses were dechorionated and treated from 25 hpf to 35 hpf with 10 µM SU5402 (Calbiochem) diluted from a 10 mM DMSO based stock solution in E3 medium or with an equal volume of DMSO diluted in E3 medium (controls).

Ectopic expression of CA-FgfR1 and Fgf8

Global misexpression of CA-FgfR1 or Fgf8 was induced in *Tg(hsp70:ca-FgfR1; cryaa:DsRed)*^{pd3} or *Tg(hsp70:Fgf8a)*^{x17} heterozygote embryos respectively, by performing heat shock before parapineal migration (25-26 hpf) (39°C, 45 minutes); a second short heat shock (15 min, 39°C) was carried out 3h later (28-29 hpf). For Fig. 3, in some cases, we performed an additional short heat shock at 32 hpf (39°C, 15 min).

Morpholino injection

Morpholino oligonucleotides (MO) targeting *no tail* (*ntl)* (2) *or southpaw* (*spaw*) (3) were solubilized at 1 mM in water and diluted to 0.5 mM working concentration; about 8 ng for *ntl* MO and 12 ng for *spaw* were injected into *Tg(dusp6:d2EGFP)* eggs at one cell stage. Embryos were subsequently fixed at 29-30 hpf and/or at 36-38 hpf and analyzed by confocal imaging after Topro-3 nuclear staining. For some 29-30 hpf embryos, we performed *pitx2 in situ* hybridization as a read-out of Nodal activity in the epithalamus to confirm that injection of *ntl* MO and *spaw* MO resulted, as previously described, in a majority of embryos with bilateral (4) or absent Nodal pathway (3) activation in the brain, respectively (**Table S2**).

In situ hybridization and immunohistochemical stainings

Embryos were fixed overnight at 4°C in BT-FIX (61), after which they were dehydrated through ethanol series and stored at −20°C until use. *In situ* hybridizations were performed using

antisense DIG labeled probes for *gfi1ab* (5), *sox1a* (6)*, dusp6* (7) and *ca-fgfr1* (8)*.* Hybridization step was performed at 65°C for *dusp6* and *ca-fgfr1* probes or at 60°C for *gfi1ab* and *sox1a* probes, in hybridization mix (formamide 50%, 4X SSC, yeast tRNA 1 mg/mL, heparin 0.05 mg/mL, Roche blocking reagent 2%, CHAPS 0.1%, EDTA 5mM, Tween 0.08%); details of the *in situ* hybridization protocol are available upon request. *In situ* hybridizations were completed using Fast Red (from Roche or Sigma Aldrich) as an alkaline phosphatase substrate. Immunohistochemical stainings were performed in PBS containing 0.5% triton using anti-GFP (1/1000, Torrey Pines Biolabs) and Alexa 488 or Alexa 555-conjugated goat anti-rabbit IgG (1/1000, Molecular Probes). For nuclear staining, embryos were incubated in Topro-3 (1/1000, Molecular Probes) as previously described (9).

Image acquisition

Bright field pictures were taken on a Nikon eclipse 80*i* microscope. Confocal images of fixed embryos were acquired on upright Leica SP5 or SP8 microscopes, using the resonant fast mode and oil x63 (aperture 1.4) or x20 (aperture 1.4) objectives. Live imaging was performed on an upright Leica SP8 microscope using a water x25 objective or an inverted Zeiss 710 with a 63x oil objective. Confocal stacks were analyzed using ImageJ software.

Quantification of the number and position of *Tg(dusp6:d2EGFP)* positive parapineal cells

The position and number of parapineal cells negative or positive for expression of the *Tg(dusp6:d2EGFP)* transgene were analyzed using ImageJ software (ROI Manager tool), the position of each cell being defined by the center of the cell nucleus detected with Topro-3 staining. The total number of parapineal cells was estimated by counting cell nuclei in the parapineal rosette using Topro-3 staining (as described in Fig. 1F, Fig. 4, Fig. 6 and Fig. S2) or by using *sox1a* expression as a specific marker of parapineal cell identity (Fig. S2). For each parapineal cell, we calculate its x and y position relative to the center of the parapineal (calculated as the mean of x and y positions of all parapineal cells).

To avoid possible bias in the counting procedure, when different genetic contexts were analyzed and compared (Fig. S2, Fig. 4 and Fig. 6), we quantified the mean intensity of the d2EGFP staining in an area corresponding to the cell nucleus by using the ROI Manager Tool (ImageJ). We then defined an intensity threshold above which the cell was considered to be *Tg(dusp6:d2EGFP)*+ and used the same intensity threshold to analyze the number of *Tg(dusp6:d2EGFP)*+ cells in each different context.

To create polar graphs, we plotted and quantified the numbers of total and *Tg(dusp6:d2EGFP)* positive parapineal cells in each semi-quadrant (1 to 8) of the parapineal. Semi-quadrants were defined relative to a line passing from anterior to posterior through the parapineal mean position (reference 0; centre of the polar graph) and progressing clockwise from the most anterior position: 0-45°C (1), 45-90°C (2) and so on. The number of cells per semi-quadrant was divided by the total number of embryos analyzed to obtain the mean number of total or *Tg(dusp6:d2EGFP)* positive parapineal cells per semi-quadrant and per embryo (left vertical scale).The polar graphs were created on R Studio.

Calculation of the Asymmetry Index (AI) of *Tg(dusp6:d2EGFP)* expression

Compared to control embryos, expression of *Tg(dusp6:d2EGFP)* and laterality of parapineal migration in *ntl* and *spaw* morphants was variable. Consequently, for each embryo, we calculated the parapineal mean position relative to the midline (using Topro-3 as a nuclear marker as described above) and an asymmetry index of *Tg(dusp6:d2EGFP)* expression using the following equation: [n(Rp) - n(Lp)] / [n(Rp) + n(Lp)], where n(Rp) is the number of *Tg(dusp6:d2EGFP)* positive cells in the right posterior quadrant and n(Lp), the number in the left posterior quadrant. This asymmetry index (AI) was used to define three groups of embryos, those with: expression of *Tg(dusp6:d2EGFP)* enriched on the left posterior side of the parapineal (AI ≤ -0.2); expression enriched on the right posterior side of the parapineal (AI ≥ +0.2); expression weakly or not lateralized (-0.2 ≤ AI ≥ +0.2, grey zone in Fig. 7J). Within each of these three groups and for each context (control, *ntl* MO, *spaw* MO), we averaged the

distribution and mean number of total or *Tg(dusp6:d2EGFP)* positive parapineal cells to create polar graphs in Fig. 6G-I".

Live imaging and time-lapse analysis

One cell stage eggs carrying the *Tg(dusp6:d2EGFP)* transgene were injected with 50-100 pg of mRNA encoding the nuclear red fluorescent protein H2B-RFP. Embryos were anesthetized with MS-222 at 24-25 hpf and mounted in drops of low melting agarose (0.6% in fish water) on a plastic petri dish (50 mm diameter; 4-8 embryos per plate) for imaging on an upright microscope or on a plastic petri dish (35 mm diameter) with a coverslip at the bottom (14 mm glass diameter) for imaging on an inverted microscope. Petri dishes were filled with fish water containing 0.5x MS-222 (0.08 mg/ml) and 0.5x PTU (0.0015%) to impede pigment formation. The embryos were imaged at 22-24°C. For four long duration (22h) movies, we quantified the number and position of all parapineal cells at each time points (26, 28, 30, 32, 36, 40, 44, 48 hpf) on a dorsal and a ventral section (+4.5 µm ventral relative to the dorsal section) containing parapineal cells. This allowed us to approximate all parapineal cells as most were included in these two sections. Parapineal cells could be identified without ambiguity from 30 hpf as their nuclei organize in a rosette-like structure and were backtracked to confirm their parapineal identity at 26 hpf or 28 hpf. The position of each parapineal cell was defined as the center of the cell nucleus detected by the H2B-RFP expression using ImageJ software (ROI Manager tool). For each cell counted, the mean intensity of d2EGFP staining was measured in a circular area positioned on the center of the cell nucleus (ROI Manager tool); this allowed us to define in an objective way the most intense d2EGFP-expressing cell in Fig. S3I and S3J. The x mean and y mean of all parapineal cells as well as the x,y position of each *Tg(dusp6:d2EGFP)*+ cells were normalized for each time point using the anterior epiphysis (y=0) and the midline of the epiphysis lumen (x=0) as references. The anterior limit and midline of the epiphysis was consistently defined for each time point on a similar z-section (8 µm ventral to the most dorsal section containing the epiphysis). From these data, polar graphs were created as described for fixed embryos. The orientation and distance of migration at each specific time point was

defined by an extrapolated line passing through the parapineal mean positions at T and at T+2h (for time point 26 hpf) or T+4h (for time points 28, 32, 36, 40, 44 hpf).

Quantification of size and number of cytoplasmic protrusions in migrating parapineal cells.

To visualize cytoplasmic protrusions from parapineal cells, we performed time lapse imaging of *Tg(dusp6:d2EGFP)*^{pt6}, *Tg(flhBAC:Kaede)*^{vu376} double transgenic embryos that express Kaede in all the pineal complex including the parapineal (6). Kaede was photoconverted from green to red fluorescence using UV light (on a Zeiss 710 confocal), so that we could visualize the cytoplasm of all or most parapineal cells in red. The size and number of cytoplasmic protrusions were quantified on z-sections from 5 embryos live imaged between 29 and 36 hpf, using the ROI manager tool (ImageJ). To avoid bias, quantification was performed using the Kaede red channel, blind for the d2EGFP channel, and we analyzed subsequently whether the counted cytoplasmic protrusions were positive or negative for *Tg(dusp6:d2EGFP)* expression.

Quantification of the number and position of *gfi1ab* positive parapineal cells

Parapineal migration was assessed by detecting the expression of the marker gene *gfi1ab* at 52 hpf (5). The position and number of *gfi1ab* positive parapineal cells were analyzed using the Multipoint tool on ImageJ software and determined as the center of the cell nucleus detected with Topro-3 staining. The position of each parapineal cell was measured relative to the brain midline (reference origin =0) as determined by a line passing through the center of the lumen of the epiphysis. For each embryo, we calculated the mean position of parapineal cells. The rare embryos for which we detected less than 4 *gfi1ab* positive parapineal cells (n=6/69 for Fig. 3) were excluded from the datasets.

IR-LEGO local heat shock experiment

Eggs collected from crosses between *fgf8*$^{+/-}$, *Tg(hsp70:ca-fgfr1)*$^{+/-}$ and *fgf8*$^{+/-}$ fish were injected with mRNA encoding the H2B-RFP red fluorescent protein. At 24 hpf, *fgf8*$^{-/-}$ embryos were

sorted based on their phenotype, anesthetized with MS-222 and embedded in a drop of Methyl-cellulose 2.5% on a petri dish (35 mm diameter) with a coverslip at the bottom (14 mm glass diameter). Embryos were imaged on a Nikon inverted spinning disk confocal microscope. H2B-RFP labeling of nuclei enabled visualization of the pineal complex given the stereotypical organization of cell nuclei around the lumen of the epiphysis and in the forming parapineal rosette. To irradiate target cells, we developed an optical system adapted from the IR-LEGO microscope described previously (10–12). An infrared laser (BrixX series narrow-bandwidth diode, Omicron) with nominal power output of 430 mW and a wavelength of 1480 nm was inserted into the beam path of the spinning disk microscope. We used a 60x (NA 1.3 immersion) objective in combination with a Zeiss immersion oil with an index of refraction n=1.3339, as it has a refractive index close to that of water while not exhibiting the strong absorption peak of water near 1480 nm. The laser was controlled with Omicron Laser Controller software, whereas for irradiation, the shutter was opened in a predetermined time-sequence that was triggered via an externally mounted Arduino Uno board controlled trigger. We tested different parameters of time and intensity and obtained the best activation of the *Tg(hsp70:ca-fgfr1)* transgene using time-sequences of 0.5 seconds and 80 mW intensity. On each embryo from 25 hpf to 29 hpf, we irradiated 2 to 3 cells located in the anterior part of the pineal complex. After focal heat shock, embryos recovered in fish water containing PTU 0.003% at 28°C. Some *fgf8*$^{-/-}$ embryos (n=25 in total) were fixed 1h to 3h after the heat shock to check the induction of the CA-FgfR1 transgene expression by *in situ* hybridisation; these embryos were genotyped for the presence of *Tg(hsp70:ca-fgfr1)* transgene (n=10/25). The remaining *fgf8*$^{-/-}$ embryos (n= 65 in total) were fixed at 50 hpf after *Tg(hsp70:ca-fgfr1)* positive embryos were sorted by expression of dsRED in the lens. In both groups of irradiated *fgf8*$^{-/-}$ mutants embryos (with or without CA-Fgfr1 transgene), we analyzed the mean position of the *gfi1ab* expressing parapineal cells relative to the midline as described in the previous section.

Statistical analysis

The mean position and number of parapineal cells were compared between datasets using R Studio software. For each dataset, we tested the assumption of normality with the Shapiro-Wilks test and variances homogeneity with the Bartlett test for multiple comparison or F test for two-sample comparision. When datasets were normal, we compared them with a two-sample T test or a Welsh T test when variances differed. When datasets did not distribute normally, we compared them using the Wilcoxon rank sum non-parametric test. For Fig. 3, we compared the four *fgf8*$^{-/-}$ datasets (*fgf8*$^{-/-}$ mutants carrying or not *Tg(hsp70:ca-fgfr1)* transgene and heat-shocked or not) two by two in a pairwise Wilcoxon test (p-value adjusted with Holm method) (Table S1). Unless otherwise mentioned in Figure legends, data are representative of at least three independent experiments. Numbers of parapineal cells are reported as mean ± standard deviation. Statistical significance is indicated on boxplots with one star (p-value<0.05) or two stars (p-value<0.01).

Data availability

The data that support the findings of this study are available from the corresponding author upon request.

Supplementary Figures S1 to S9

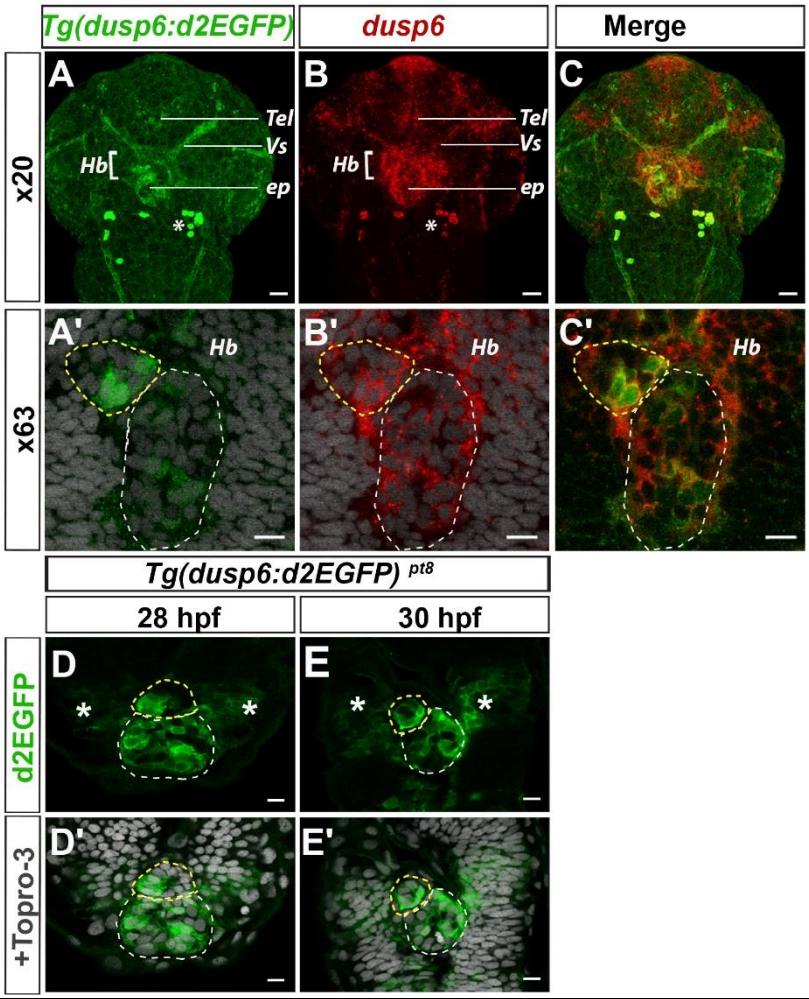
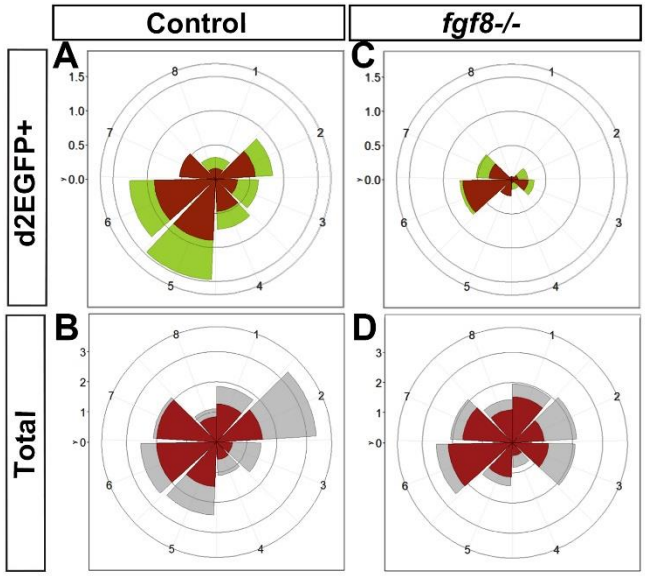


Figure S1. Endogenous *dusp6* and *Tg(dusp6:d2EGFP)*^{pt8} transgene are focally expressed in few parapineal cells as observed for the *Tg(dusp6:d2EGFP)*^{pt6} allele.

(A-C') *Tg(dusp6:d2EGFP)*^{pt6} expression recapitulates endogenous *dusp6* expression in the epithalamus. Confocal maximum projection (100 µm, stepsize 2.5 µm; scale bar: 25 µm) (A-C) or high magnification confocal sections (A'-C'; scale bar: 10 µm) showing the expression of *Tg(dusp6 :d2EGFP)*^{pt6} after immunostaining against GFP (green; A, A') and *dusp6* gene (red; B, B') detected by *in situ* hybridization at 32 hpf; merges are shown in C and C'; pictures in A' and B' are merged with cell nuclear staining (Topro-3, grey). As for the *Tg(dusp6:d2EGFP)* transgene, the endogenous *dusp6* gene is expressed in both the epiphysis (ep, white circle) and the parapineal (yellow circle), in the head vessels (Vs), in the telencephalon (Tel), in the presumptive habenular domain (Hb) and in a group of neurons in the Tectum (*).

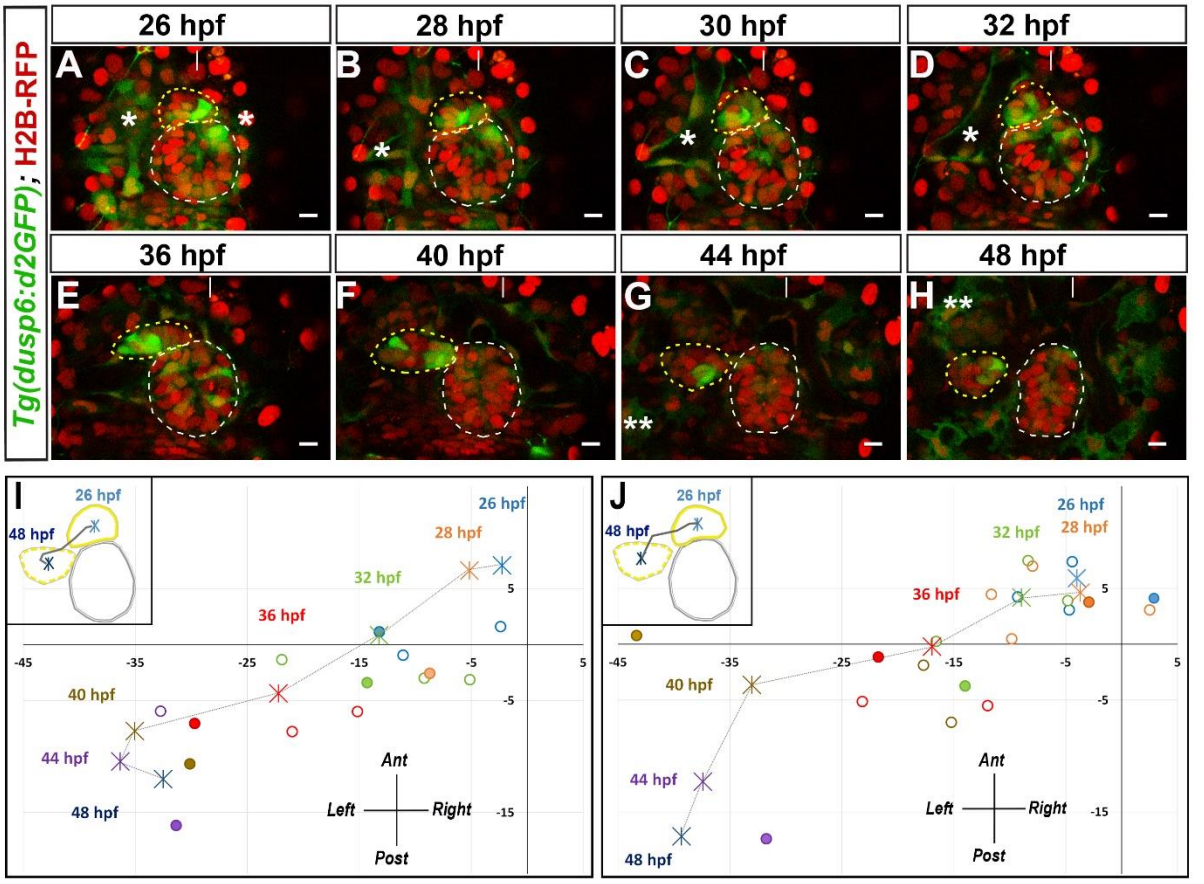
(D-E') d2EGFP shows localised parapineal expression in the pt8 *Tg(dusp6:d2EGFP)* allele as in the pt6 allele. Confocal sections showing the expression of *Tg(dusp6 :d2EGFP)*^{pt8} transgene

(Green) at 28 hpf (D, D') and 30 hpf (E, E'), alone (D, E) or merged with nuclear staining (Topro-3, grey) (D', E'); scale bar: 10 µm. *Tg(dusp6 :d2EGFP)*^{pt8} transgene is expressed in both in pineal (white circle) and in the parapineal (yellow circle); weak staining in also detected in the presumptive habenular domain (*). *Tg(dusp6 :d2EGFP)*^{pt8} expression in the parapineal is mosaic both at 28 hpf (n=10) and 30 hpf (n=7) and usually enriched at the leading edge as observed for *Tg(dusp6:d2EGFP)*^{pt6} allele (Figure 1). Embryos are viewed dorsally with anterior up. *In situ* hybridizations are representative of two independent experiments.



<u>Figure S2.</u> Focal expression of *Tg(dusp6:d2EGFP)* FGF pathway reporter in the parapineal depends on Fgf8.

Polar graph showing the distribution and mean number of total (B,D) and *Tg(dusp6:d2EGFP)* positive parapineal cells (A,C) in each of the eight 45°C semi-quadrants (1 to 8) relative to the parapineal mean position for control embryos (A,B; n=19) or *fgf8*$^{-/-}$ mutants (C,D; n=21) at 32 hpf. The radial axis (vertical scale on the left side) represents the mean number of cells per semi-quadrant per embryo. The total number of parapineal cells was estimated by counting cell nuclei (grey area in B,D) or by using *sox1a* marker (red area in B,D); as some cells can be part of the parapineal rosette but do not express *sox1a*, the total number of *sox1a* positive parapineal cells (about 10 cells) is lower than the number that we estimated by counting cell nuclei in the parapineal rosette (about 16 cells) (B,D; red versus grey polar bars). Graph A and C show the distribution and mean number of *Tg(dusp6:d2EGFP)* expressing parapineal cells among the *sox1a* positive cells (red area in A,C) or among the total counted cell nuclei (green area in A,C); the distribution of *Tg(dusp6:d2EGFP)* positive cells, enriched in the left posterior quadrants 5 and 6, was similar in both cases (A, red versus green polar bars), validating the use of nuclear staining to define parapineal cells. The mean number of *Tg(dusp6:d2EGFP)* positive cells is strongly reduced in *fgf8*$^{-/-}$ parapineals (C) while the total number of parapineal cells is not significantly reduced (D).



<u>Figure S3.</u> Focal activation of *Tg(dusp6:d2EGFP)* FGF reporter is enriched at the leading edge of the migrating parapineal.

(A-H) Comparable analysis to Figures 2A-2H for a second embryo: embryo n°2 shown in Movie S2.

(I) Position of *Tg(dusp6:d2EGFP)* positive cells (circle) relative to the mean position of all parapineal cells (star) analyzed on a representative confocal section of embryo n°1 (shown in Figures 2A-2H and Movie S1), at different stages of parapineal migration: 26 hpf (light blue), 28 hpf (orange), 30 hpf (yellow), 32 hpf (green), 36 hpf (red), 40 hpf (brown), 44 hpf (purple), 48 hpf (dark blue). The y line (x=0) represents the brain midline and the x line (y=0) represents the anterior limit of the epiphysis. For each time points, the brightest d2EGFP expressing cell is shown as a color filled mark (filled circle). Black dotted line represents the extrapolated displacement of the parapineal mean position from T to T+2h or T+4h. Left corner: schematic showing how the parapineal (yellow circle) migrates relative to epiphysis (grey circle) between 26 and 48 hpf.

(J) Comparable analysis to (I) for embryo n°2, shown in Figures S3A-S3H and in Movie S2.

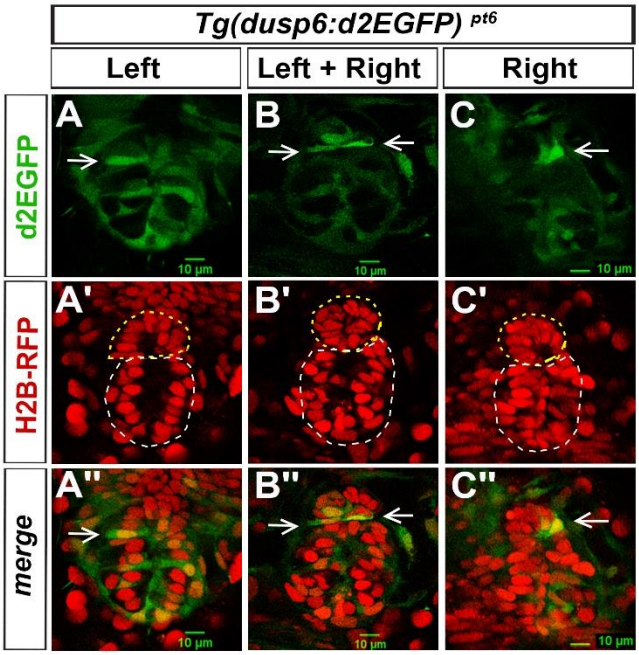
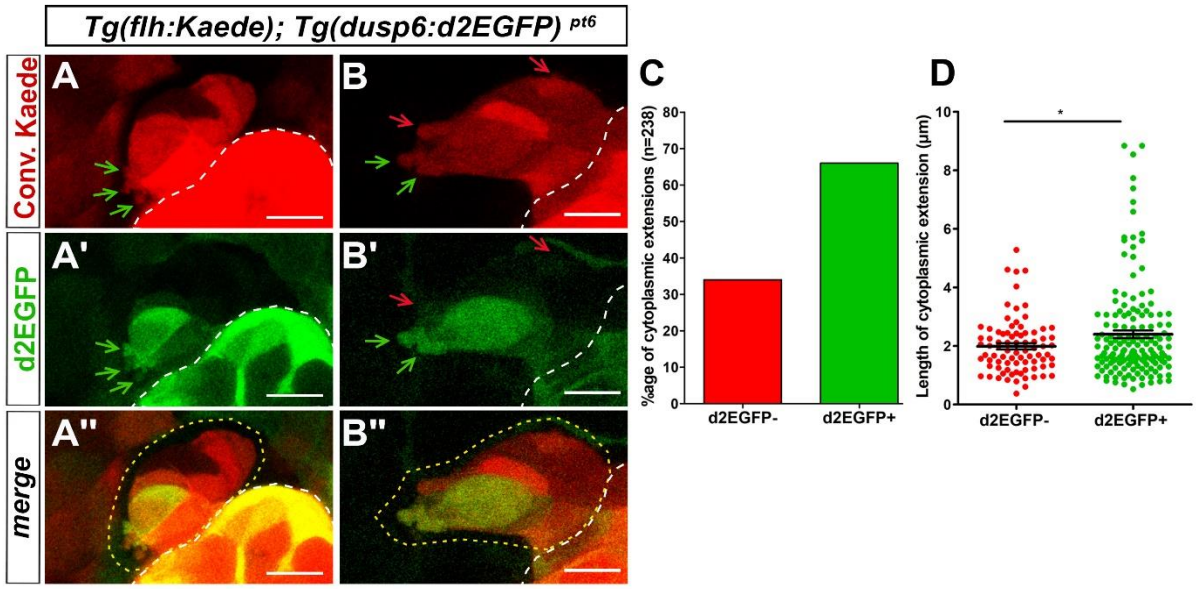


Figure S4. *Tg(dusp6:d2EGFP)* expression is initiated at the interface between the epiphysis and nascent parapineal.

Confocal section of live imaged *Tg(dusp6:d2EGFP)*^{pt6} embryos (green) expressing H2B-RFP protein (red) in cell nuclei between 26 and 28 hpf (A-C''); scale bar: 10 µm. The epiphysis and the parapineal are shown as a white or yellow dotted circle in (A'-C'). When first detected in the parapineal, d2EGFP expression is usually found in one or two parapineal cells on the left posterior side (A-A''; n=26/41). In some cases, *Tg(dusp6:d2EGFP)* can be expressed on both left and right sided parapineal cells (B-B'' ; n=10/41) or rarely on right sided cells (C-C'' ; n=5/41). Embryo view is dorsal, anterior is up; white arrows show *Tg(dusp6:d2EGFP)* expressing cells in the parapineal.



<u>Figure S5.</u> Cytoplasmic protrusions from parapineal cells are enriched in *Tg(dusp6:d2EGFP)* expressing cells and are longer.

(A-B'') Zoom-in illustrative confocal sections of 2 live imaged embryos showing the expression of *Tg(flh:Kaede)* transgene after photoconvertion of Kaede (Red; A, B) and of *Tg(dusp6 :d2EGFP)* FGF reporter (Green; A', B') at 32 hpf (A-A'') or 35 hpf (B-B''); merge are shown in A'' and B''; scale bar: 10 µm. The epiphysis and the parapineal are outlined with white or yellow dots (A-B''). Green arrows show cytoplasmic protrusions from parapineal cells expressing both d2EGFP and photoconverted Kaede while red arrows show cytoplasmic protrusions from parapineal cells expressing Kaede only.

(C) Graph showing the percentage of Kaede positive cytoplasmic protrusions observed from parapineal cells that were negative (red, d2EGFP-, 34%) or positive (green, d2EGFP+, 66%) for d2EGFP expression in *Tg(flh:Kaede); Tg(dusp6:d2EGFP)* transgenic embryos; a total of 238 protrusions were counted on 5 live imaged embryos.

(D) Dot plot showing the length of cytoplasmic protrusions that express photoconverted Kaede only (red, d2EGFP-, n=82) or Kaede plus *Tg(dusp6:d2EGFP)* (green, d2EGFP+, n=156). The average length of cytoplasmic protrusions is significantly higher in parapineal cells that express d2EGFP (Mean± SEM = 2.39 ± 0.13 µm) than in those that are d2EGFP negative (Mean± SEM=1.99 ± 0.10 µm); p=0.015 in a Welch's T Test (* indicates statistical significance).

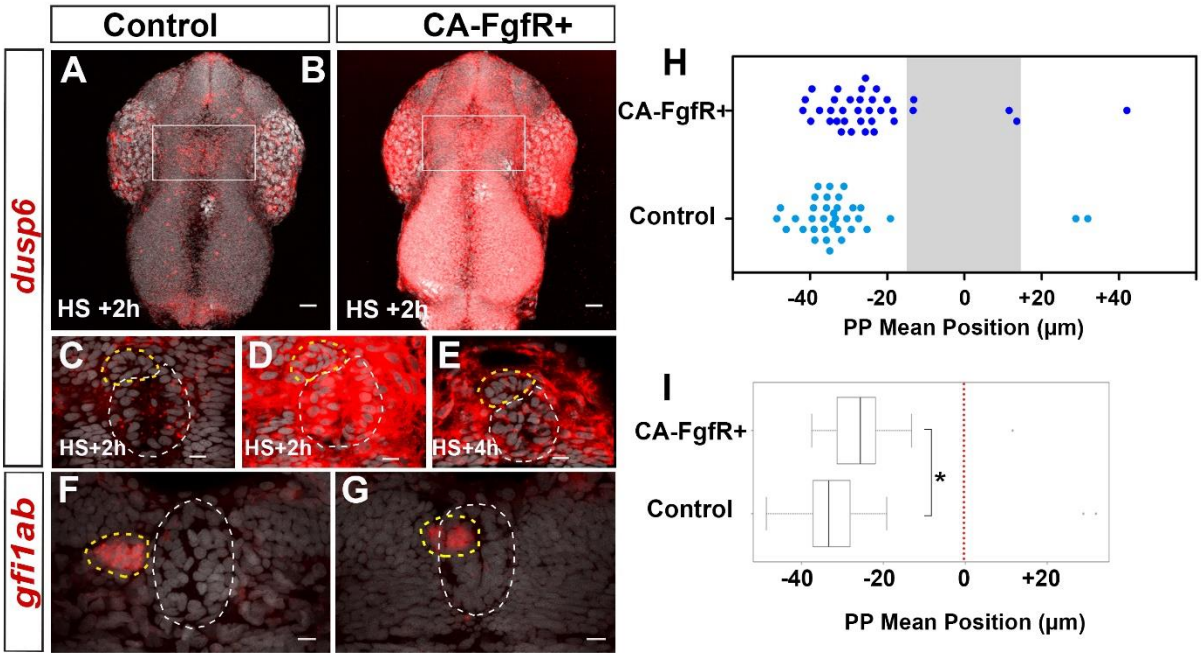


Fig. S6: Ectopic expression of a constitutively activated version of the FgfR1 receptor compromises parapineal migration in wild-type embryos.

(A-E) Confocal maximum projection (A, B) (100 µm projection with a 5 µm step size; scale bar: 25 µm) or confocal sections (C-E; scale bar: 10 µm) showing the expression of the endogenous *dusp6* gene (red) and cell nuclei (grey) in embryos that carry the *Tg(hsp70:ca-fgfr1)* transgene (B, D, E) or not (A, C). Zoom out embryos were heat-shocked at 26 and 29 hpf and fixed at 31 hpf, 2h after the last heat shock (A, n=16; B, n=9). Zoom in embryos were heat-shocked at 28 hpf (C, n=33; D, n=21) or 26 hpf (E, n=12) and fixed at 30 hpf respectively, 2h or 4h after heat shock.

(F, G) Confocal maximum projections (5 µm) showing *gfi1ab* expression (red) and cell nuclei (grey) in the forebrain of 52 hpf embryos that carry the *Tg(hsp70:ca-fgfr1)* transgene (F) or not (G) after being heat-shocked at 26 hpf and 29 hpf. *gfi1ab* expression labels the parapineal nucleus (yellow outline); epiphysis (white outline). In A-G, embryo view is dorsal, anterior is up.

(H) Dot plot showing, for each embryo, the mean parapineal position in µm distant to the brain midline (x=0) in embryos expressing (dark blue) or not (light blue) CA-FgfR1 after two heat-shocks at 26 hpf and 29 hpf or three heat-shock at 26, 29 and 32 hpf. Grey shaded zone between -15 µm and +15 µm defines the 'no migration' domain as corresponding to the average width of the epiphysis; grey dotted lines show -25 µm and +25 µm.

(I) Boxplot showing the distribution of parapineal mean position relative to the brain midline (reference 0, red dotted line) in embryos expressing CA-FgfR1 or in controls. Parapineal

migration is compromised in the embryos that overexpress CA-FgfR1 relative to control embryos; p-value=0.023 in a Wilcoxon test (* indicates statistical significance).

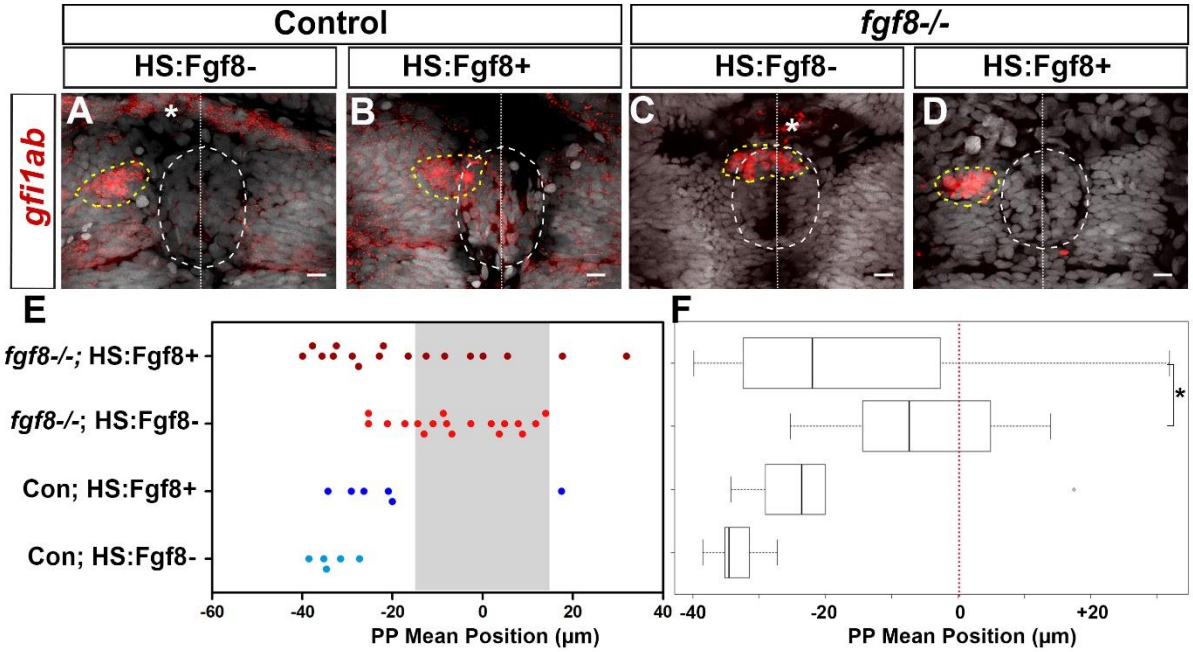
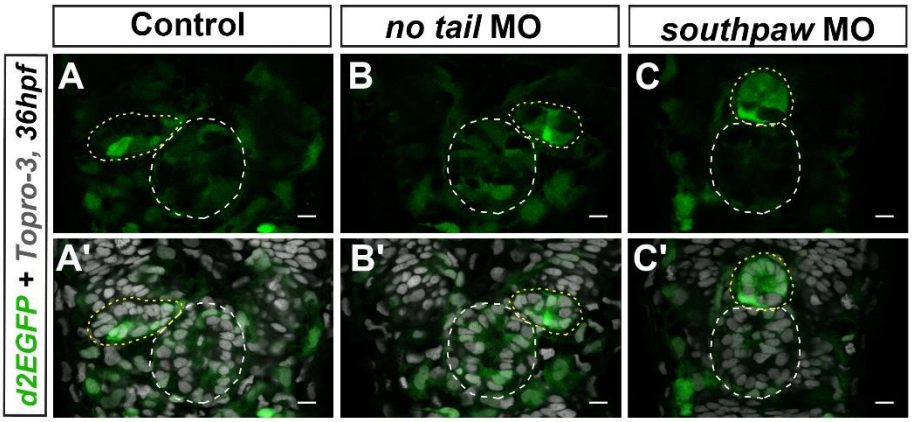


Figure S7: Ectopic and global expression of Fgf8 partially restores parapineal migration in *fgf8*$^{-/-}$ mutants.

(A-D) Confocal (8 µm) maximum projection showing expression of *gfi1ab* (red) with cell nuclei (Topro-3, grey) in the heads of representative control embryos (A-B) and *fgf8*$^{-/-}$ mutants (C-D) that carry (B,D) or don't carry (A,C) the *Tg(hsp70:fgf8a)* transgene; scale bar: 10 µm. Control embryos are siblings of *fgf8*$^{-/-}$ mutants thus corresponding to both wild-type and *fgf8*$^{+/-}$ heterozygotes. All embryos were heat-shocked at 26 hpf and 29 hpf. The expression of *gfi1ab* labels the parapineal nucleus (yellow outline) while global nuclear staining was used to visualize the epiphysis (white outline) and to define the brain midline (reference 0; dotted white line in the center of the epiphysis lumen). Non-specific fluorescent staining is shown as (*).

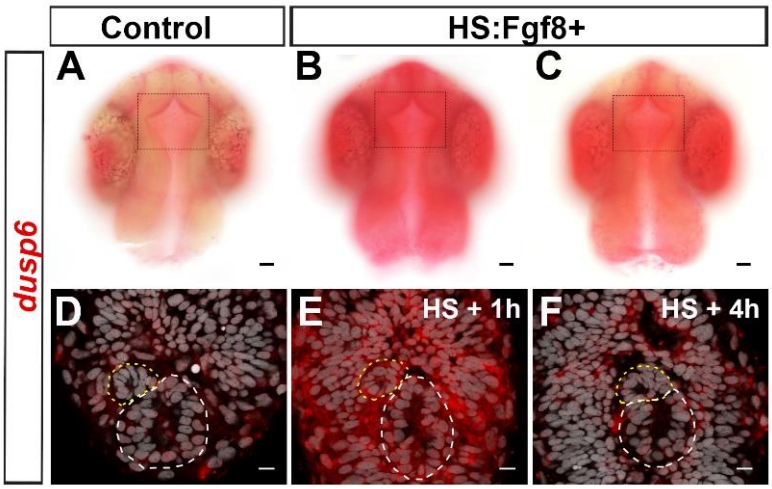
(E) Dot plot showing, for each embryo, the mean parapineal position in µm distant to the brain midline (x=0), at 52 hpf, in *fgf8*$^{-/-}$ mutant embryos that express or not the *Tg(hsp70:fgf8a)* transgene after heat-shock at 25 hpf and 29 hpf. Grey shaded zone (-15 µm and +15 µm) define the 'no migration' domain as corresponding to the average width of the epiphysis. Parapineal migration is compromised in control embryos that express *Tg(hsp70:fgf8a)* transgene (B and E, light blue dots; n=5) compared to controls that do not (A and E, dark blue; n=6); p-value=0,03 in a Wilcoxon test. In *fgf8*$^{-/-}$ mutants that do not express the *Tg(hsp70:fgf8a)* transgene, the parapineal either stays at the midline or migrates partially (C and E, light red; n=18) while the migration is partially rescued in mutants that over-express Fgf8 (D and E, dark red; n=17); p-value=0,049 in a Wilcoxon test.

(F) Boxplot showing the distribution of parapineal mean position relative to the brain midline (reference 0) in the same set of embryos; p-value=0,049 (* indicates statistical significance).



<u>Figure S8</u>: Parapineal migration is delayed in absence of Nodal signaling.

(A-C') Confocal sections showing the expression of *Tg(dusp6:d2EGFP)* (green) at 36 hpf in parapineals (yellow circles) of a control embryo (A, A'; n=17) and in illustrative *ntl* (B, B'; n=26) and *spaw* morphants (C, C'; n=32); images A'-C' show images A-C superimposed on nuclear staining (grey) allowing visualization of the epiphysis (white outline) and parapineal (yellow outline). At 36 hpf, the parapineal has migrated in all controls and *ntl* morphants but is still found at the midline in 25% of *spaw* morphants (numbers of embryo with a left, right or no migrated parapineal are shown in Table S5). Embryo view is dorsal, anterior is up; scale bars: 10 µm.



<u>Figure S9</u>. Global ectopic expression of Fgf8 induces *dusp6* expression.

(A-C) Bright field pictures (scale bar: 25 µm) showing the expression of the endogenous *dusp6* gene (red) and confocal sections (D-F; scale bar: 10 µm)) showing the expression of the endogenous *dusp6* gene (red) and cell nuclei (grey) in representative embryos that carry the *Tg(hsp70:fgf8)* transgene (B-C, E-F) or not (A, D). Embryos were heat-shocked at 29 hpf (A-B, D-E; n=20 and n=27) or 26 hpf (C, F; n=19) and fixed at 30 hpf respectively 1h or 4h after heat shock. *In situ* hybridizations are representative of two independent experiments.

Supplementary Tables S1 to S5

Table S1. Pairwise comparisons of parapineal mean position between the four *fgf8*$^{-/-}$ mutant embryo contexts presented Figure 4.

	***fgf8*$^{-/-}$**	***fgf8*$^{-/-}$_CAFgfR$^{+/-}$**	**No HS_f*gf8*$^{-/-}$**
***fgf8*$^{-/-}$_CAFgfR$^{+/-}$**	**0.03974**	-	-
No HS_*fgf8*$^{-/-}$	1.00000	**0.00252**	-
No HS_*fgf8*$^{-/-}$_CAFgfR$^{+/-}$	1.00000	**0.00021**	1.00000

The datasets were compared on R Studio using the function 'pairwise.wilcox.test' with the p-value adjustment method 'holm'; 'No HS_': non heat shocked embryos.

Table S2. Proportions of embryos showing laterality of *pitx2* expression at 29 hpf.

***pitx2* expression**	**Control (n=28)**	***ntl* MO (n=31)**	***spaw* MO (n=25)**
Left	**75 %**	0 %	4 %
Absent	0 %	3 %	**84 %**
Bilateral	** 25 %	**97 %**	8 %
Right	0 %	0 %	4 %

** Among the embryos annotated with a bilateral expression of *pitx2* (n=7/28, 25%), 5 embryos (18% of total embryos) clearly showed a stronger expression on the left side while only 2 embryos (7%) displayed a non-biased bilateral expression. Summary of 3 experiments.

Table S3. Distribution of embryos according to their asymmetry index (AI) in the number of *Tg(dusp6:d2EGFP)* positive cells in the left versus right posterior quadrant of the parapineal at 30 hpf.

AI in *Tg(dusp6:d2EGFP)*	**Control (n=36)**	***ntl* MO (n=37)**	***spaw* MO (n=38)**
Left (AI < or = -0,2)	78 %	38 %	34 %
Non Lateralized (-0,2< AI >0,2)	11 %	30 %	34 %
Right (AI > or = +0,2)	11 %	32 %	32 %

Table S4. Distribution of embryos according to their parapineal mean position (µm) relative to the brain midline (x=0) at 30 hpf.

Parapineal mean position (µm) at 30 hpf	**Control (n=36)**	***ntl* MO (n=37)**	***spaw* MO (n=38)**
Left (< -5 µm)	94 %	30 %	11 %
Midline (-5 µm < > +5 µm)	**6 %**	**35 %**	**76 %**
Right (> +5 µm)	0 %	35 %	13 %

Table S5. Distribution of embryos showing parapineal lateralization at 36-38hpf *.

Parapineal laterality at 36-38 hpf	**Control (n=17)**	***ntl* MO (n=26)**	***spaw* MO (n=32)**
Left	100 %	54 %	38 %
Midline	**0 %**	**0 %**	**25 %**
Right	0 %	46 %	38 %

* Summary of two experiments where embryos were fixed at 36 hpf or 38 hpf.

Captions for Movies S1 to S5

Movie S1. Dynamic expression of the *Tg(dusp6:d2EGFP)* FGF reporter transgene in the parapineal during its migration (embryo n°1).

Time series from 26 hpf to 48 hpf (1 frame /15 min) of thin confocal maximum projection (4.5 µm) of the dorsal brain of a live *Tg(dusp6 :d2EGFP)*^{pt6} embryo (green) expressing H2B-RFP protein (red) in cell nuclei. Embryo view is dorsal, anterior is up; the epiphysis and the parapineal are shown as a white or yellow circle respectively every 4h and the brain midline as the antero-posterior white line. *Tg(dusp6:d2EGFP)* expression is enriched in parapineal cells at the leading edge of the migration.

Movie S2. Dynamic expression of the *Tg(dusp6:d2EGFP)* FGF reporter in the parapineal during its migration (embryo n°2).

Time series from 26 hpf to 48 hpf (1 frame /15 min) of thin confocal maximum projections (4.5 µm) of the dorsal brain of a live *Tg(dusp6 :d2EGFP)*^{pt6} embryo (green) expressing H2B-RFP protein (red) in cell nuclei. Embryo view is dorsal, anterior is up; the epiphysis and the parapineal are shown as a white or yellow circle respectively every 4h and the brain midline as the anterior to posterior white line. The initial expression of *Tg(dusp6:d2EGFP)* on the right side correlates with a delay in the migration that only initiates at 30-32 hpf when d2EGFP expressing cells are eventually relocated to the left side.

Movie S3. Ectopic expression of the *Tg(dusp6:d2EGFP)* FGF reporter in the parapineal correlates with a delay in parapineal migration (embryo n°3).

Time series from 26 hpf to 48 hpf (1 frame /15 min) of thin confocal maximum projections (4.5 µm) of the dorsal brain of a live *Tg(dusp6:d2EGFP)*^{pt6} embryo (green) expressing H2B-RFP protein (red) in cell nuclei. Embryo view is dorsal, anterior is up; the epiphysis and the

parapineal are shown as white and yellow circles respectively every 4h and the brain midline as an anterior to posterior white line. Expression of the *Tg(dusp6:d2EGFP)* FGF reporter fails to be restricted to leading cells in the early migration phases; this correlates with a strong delay in parapineal migration that only starts around 38 hpf.

Movie S4. *Tg(dusp6:d2EGFP)* positive cells can exchange leading position (embryo n°4)

Time series from 30 hpf to 42 hpf (1 frame /15 min) of thin confocal maximum projection (4,5 µm) of the dorsal brain of a live *Tg(dusp6:d2EGFP)*^{pt6} (green) embryo expressing H2B-RFP protein (red) in cell nuclei. Embryo view is dorsal, anterior is up; the epiphysis and the parapineal are shown as white and yellow circles respectively every 2h and brain midline as a white anterior to posterior line. *Tg*(*dusp6:d2EGFP)* positive cells are identified as n°1 to 3. Cell n°1, initially located at the border between the parapineal and the epiphysis, strongly expresses *Tg(dusp6:d2EGFP)* and remains at the leading front from 30 hpf to 36 hpf. As d2EGFP expression decreases in cell n°1, other parapineal cells (cell n°2 at 34-36 hpf and n°3 at 38 hpf) start expressing *Tg(dusp6:d2EGFP)* and behave as new leading cells.

Movie S5. Cytoplasmic protrusions from parapineal cells are enriched in *Tg(dusp6:d2EGFP)* expressing cells.

Time series from 29 hpf to 34 hpf (1 frame /15 min) of confocal sections of the head of a live embryo expressing *Tg(dusp6:d2EGFP)*^{pt6} (green) and *Tg(flh:Kaede)* transgene after photoconvertion of Kaede H2B-RFP protein (red); scale bar: 10 µm. The parapineal is shown as a yellow dotted circle on the first frame. Green arrows show cytoplasmic protrusions from parapineal cells expressing both d2EGFP and photoconverted Kaede while red arrows show cytoplasmic protrusions from parapineal cells expressing Kaede only.

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