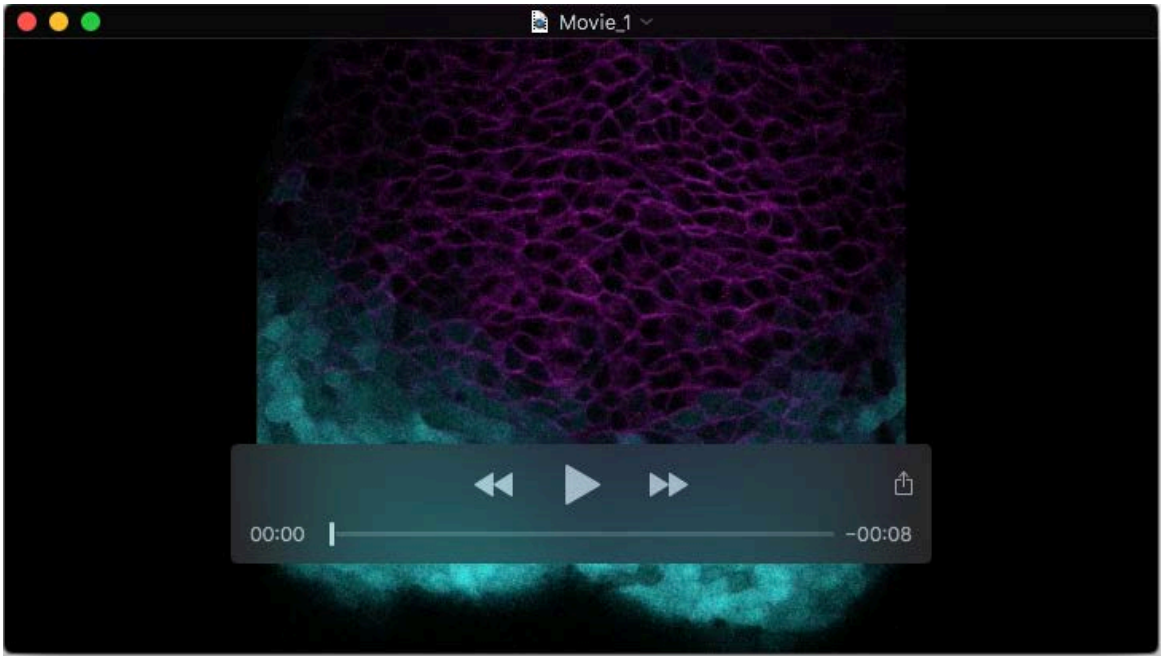
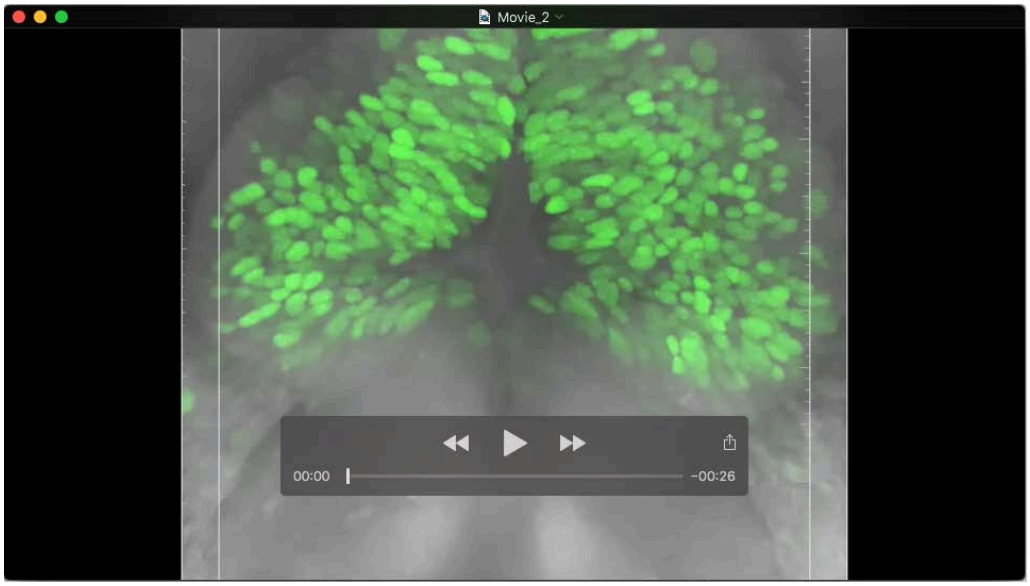
Supplementary data



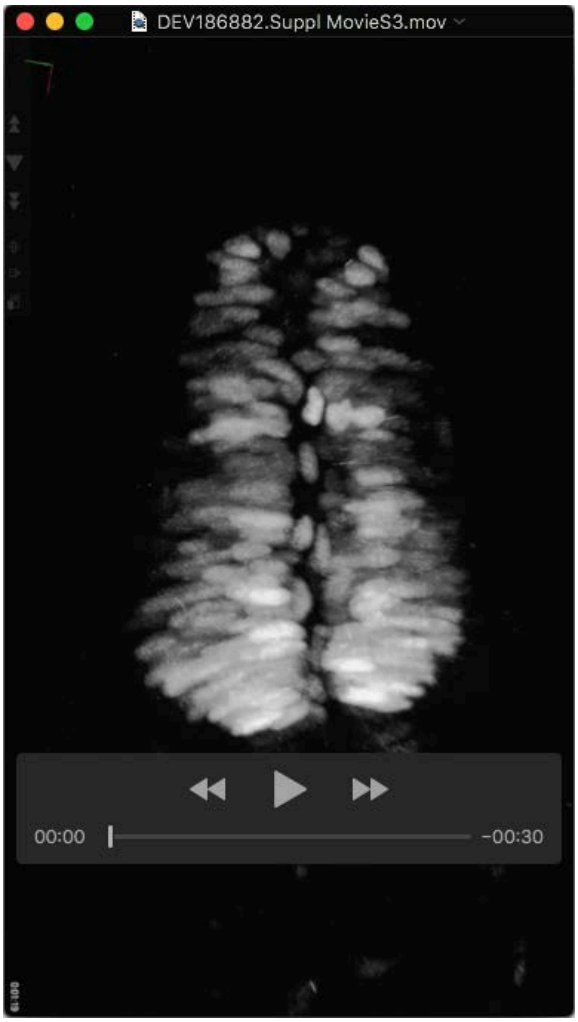
Movie 1: Initially overlapping domains of *otx2b-gbx1*

Time-lapse imaging of *otx2*+(magenta) and gbx1+(cyan) cells in *otx2b:HRAS-mKate2* and *gbx1:Venus* reporter lines. Embryos were dorsally mounted with the anterior end to the top and imaged from 10.5 hpf to 15 hpf. Tissue sections spanning about 20µm were chosen with a z interval of 1 µm. Images were acquired at 2:30 (min:sec) intervals. Maximum intensity projections (covering 3 µm) of the fluorescent images are shown. Overlapping expression domains of *otx2b* and *gbx1* could be observed from the neural plate to the neural rod stages, as evidenced by the presence of cells that were positive for both mKate2 and Venus (double-positive).

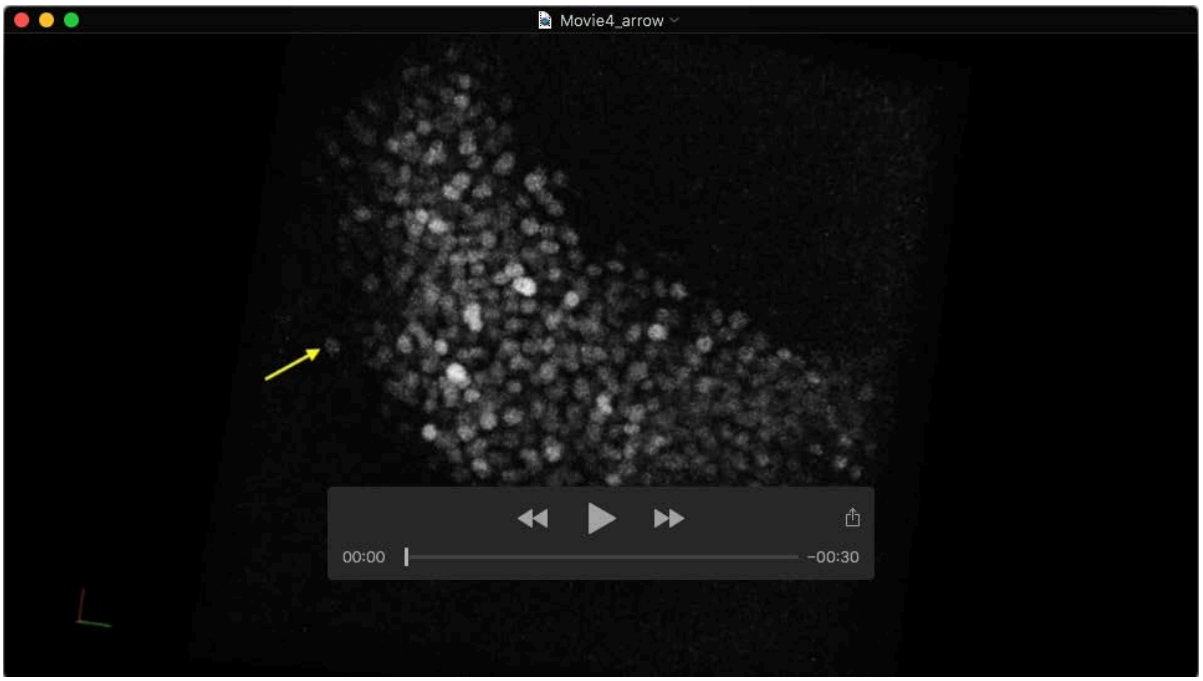


Movie 2: Short-term lineage (temporary)tracing of *Otx2b*-derived cells

Time-lapse imaging of *otx2*-positive cells in the *otx2b:Venus-NLS* reporter line. Embryos were dorsally mounted with the anterior end to the top and imaged from 26 hpf to 32hpf. Tissue sections covering about 40µm were chosen with a z interval of 1 µm. Images were acquired at 2:30 (min:sec) intervals. Maximum intensity projections of the fluorescent images merged with transmitted light images are shown. Perdurance of the Venus fluorescent protein in the hindbrain domain (marked with a yellow circle) can be seen. An image file highlighting the region of interest (circle) was inserted within the movie subsequently using iMovie (10.1.13).

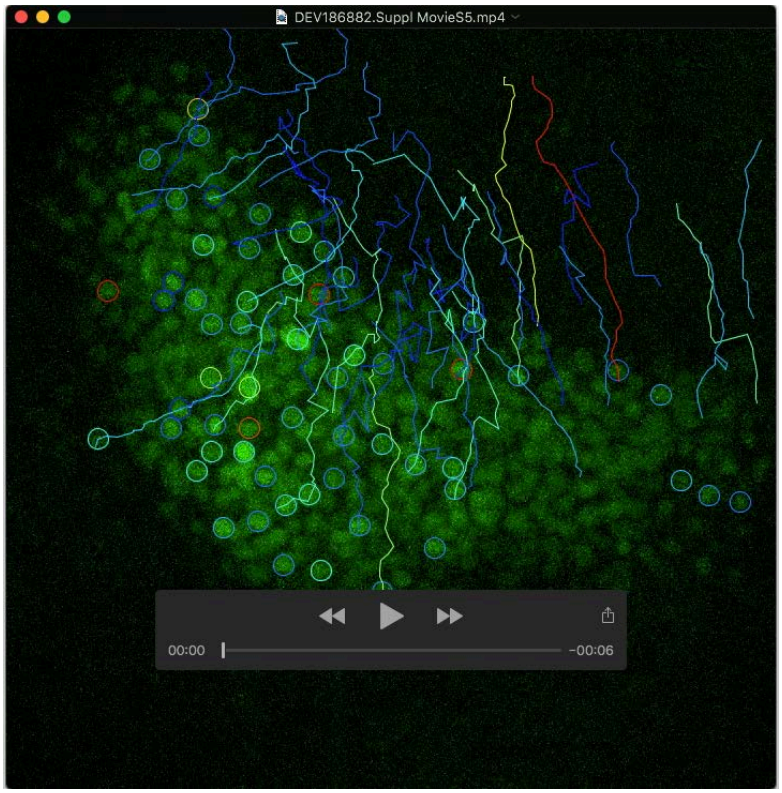


Movie 3: Short-term lineage tracing of *wnt1*-derived cells Time-lapse imaging of *wnt1*+cells (green) in the *wnt1:Venus-NLS* reporter line. Embryos were dorsally mounted with the anterior end to the top and imaged from 14 hpf to 26 hpf. Tissue sections spanning about 40µm were chosen with a z interval of 1 µm. Images were acquired at 2:30 (min:sec) intervals. Maximum intensity projections of the fluorescent images were merged with the transmitted light images and are shown. Perdurance of Venus fluorescent protein in the hindbrain domain (marked with arrow) can be observed. An image file highlighting the region of interest (arrow) was inserted at the beginning of the movie frame using iMovie (10.1.13).

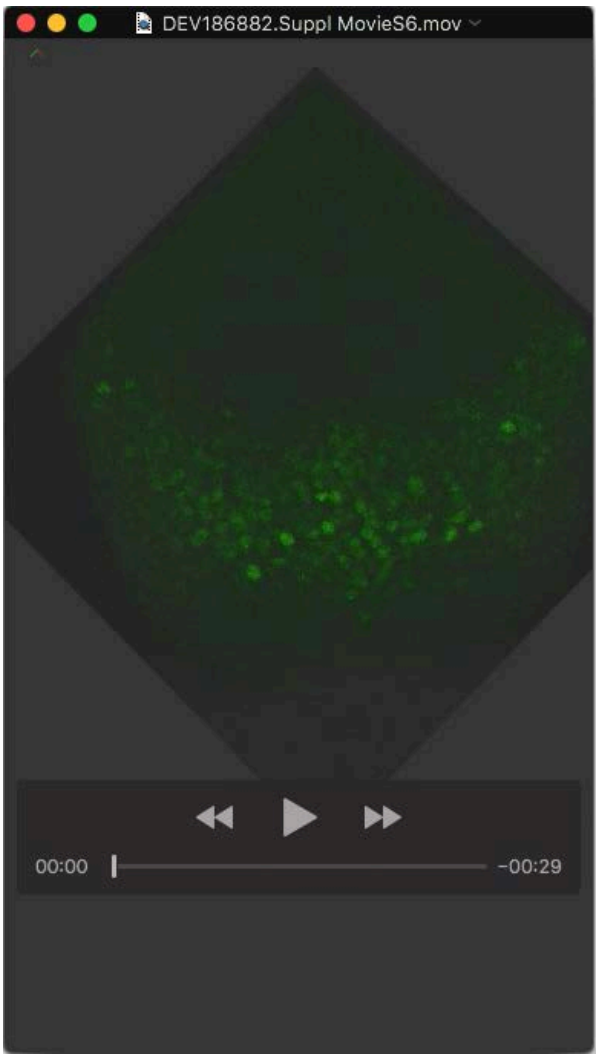


Movie 4: Cell sorting at the MHB

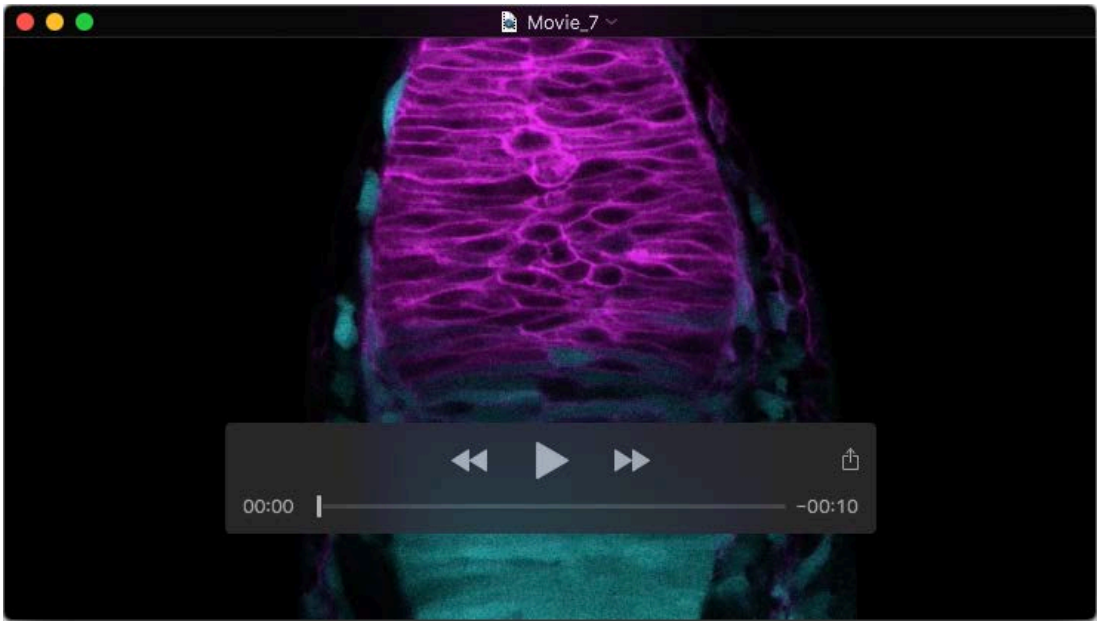
Time-lapse imaging of *wnt1*+cells in the *wnt1:Venus-NLS* reporter line (supporting movie file for images shown in Fig.5A). Embryos were dorsally mounted with the anterior end to the top and imaged from 10.5 hpf to 12 hpf. Tissue sections covering about 30µm were chosen with a z interval of 1 µm. Images were acquired at 2:30 (min:sec) intervals. Maximum intensity projection of images are shown. The movement of a straggling wnt1-cell (arrow) and its active migration towards the group of other boundary cells is shown. An image file highlighting the region of interest (arrow) was inserted at the beginning of the movie frame using iMovie (10.1.13).



Movie 5: Cell tracking shows cell mixing within compartments This is the supporting movie file for images shown in Fig.5B. An analysis of cellular movement (shown in Movie 4) was performed using Trackmate-ImageJ (FIJI), which showed zig-zag movements and crossovers among many *wnt1*+ cells (green).

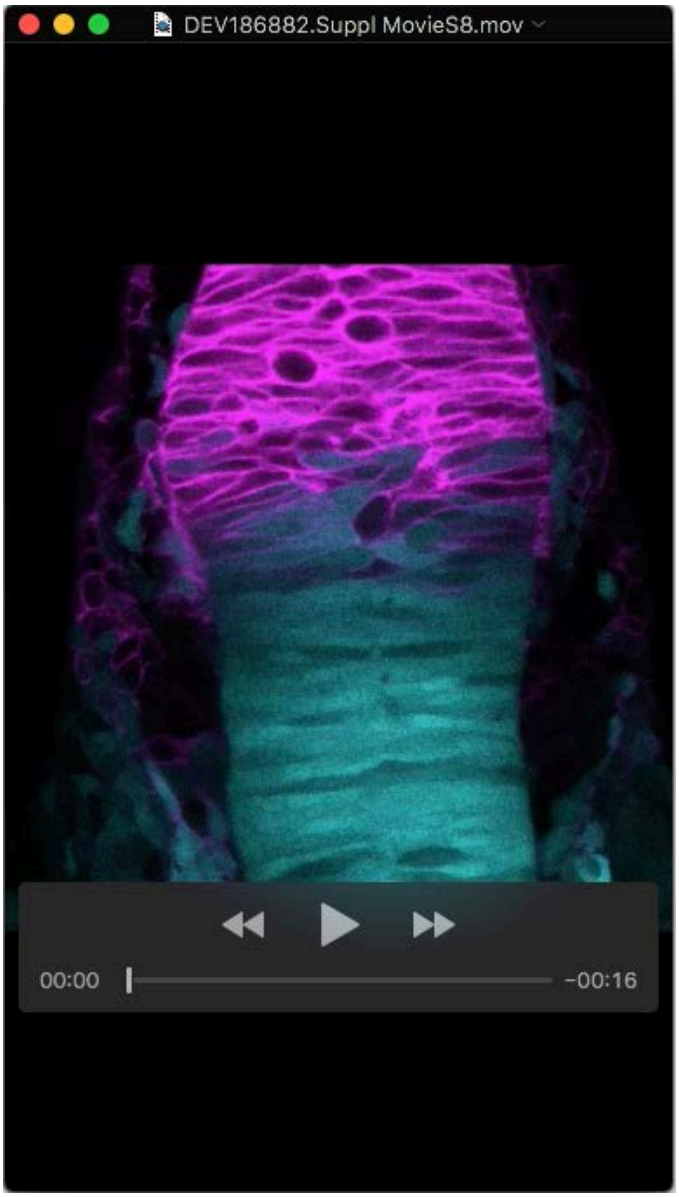


Movie 6: Time-lapse imaging of *wnt1*+(green) cells in the *wnt1:Venus-NLS* reporter line. This is an additional representative time-lapse movie depicting cell sorting behavior (arrow) at the MHB. An image file highlighting the region of interest (arrow) was inserted within the movie frame using iMovie (10.1.13).



Movie 7:

A sequence of optical sections (z-sections, z =1 µm interval, total 40 µm), acquired on the dorsal-ventral axis of the double transgenic fish, *otx2b:HRAS-mKate2* and *gbx1:Venus*, is shown. These are un-injected control fish at 14hpf. An overlapping *otx2* (magenta*)-gbx1* (cyan) domain, spanning a few cell layers, can be observed.



Movie 8: *otx2:HRAS-mKate2* and *gbx1:venus* (sol-efnb2a)

A sequence of optical sections (z-sections, z =1 µm interval, total 40 µm), acquired on the dorsal-ventral axis of the double transgenic fish, *otx2b:HRAS-mKate2* (magenta)- and *gbx1:Venus* (green), is shown. These embryos expressed sol-efnb2a and were imaged at 14hpf. The overlapping *otx2b-gbx1* domain is irregular and larger than that seen in control fishes, and many *otx2b*-positive (single-positive) cells could be observed far from the Otx-Gbx overlapping domain.

Figure S1

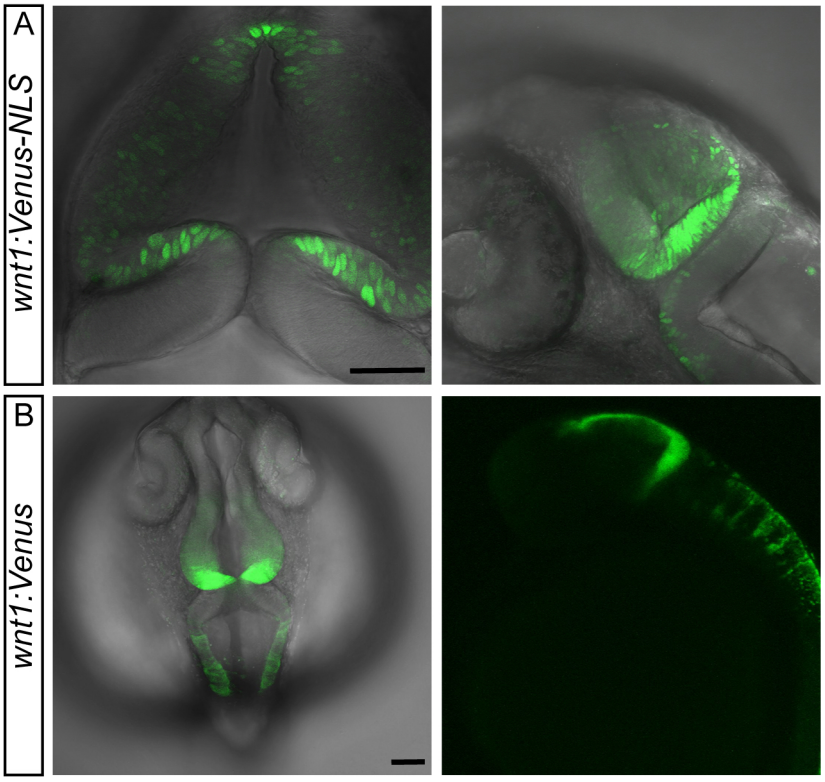


Fig S1: Identical reporter expression in *wnt1:Venus-NLS and wnt1:Venus* transgenic line

(A and B) Live imaging show identical expression patterns of venus fluorescent protein (nuclear (*venus_NLS*) and cytoplasmic (*venus*)) in two independent transgenic line. Both dorsal and lateral views are provided for better clarity. Scale bar 20µm.

Figure S2

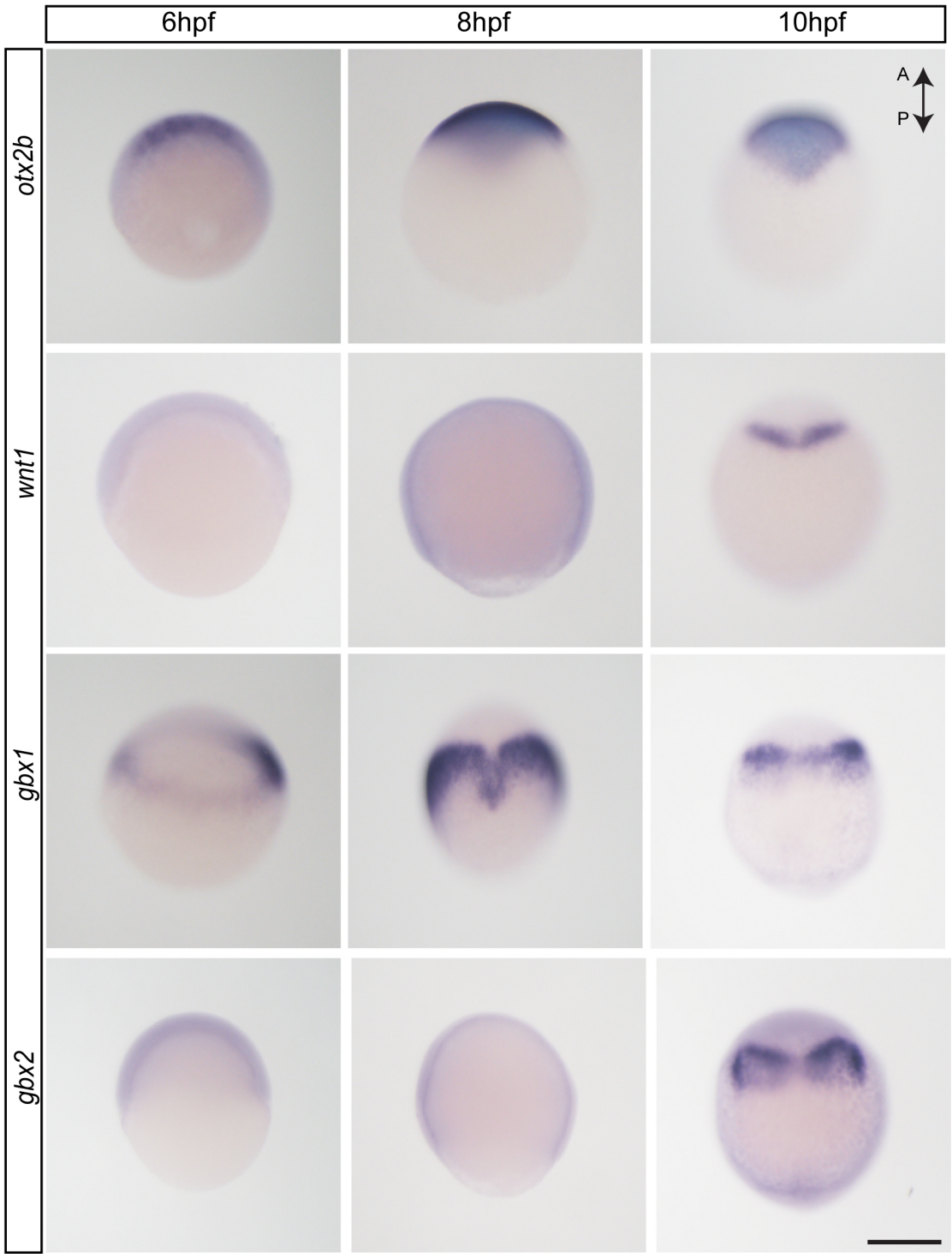


Fig S2: *in situ* hybridization showing onset of gene expression.

Whole mount *in situ* hybridization in embryos at 6, 8, and 10hpf for midbrain markers, namely *otx2b* and *wnt1*, and hindbrain markers *gbx1* and *gbx2*, show that both otx2 and gbx1 show an early onset (6hpf) compared to *wnt1* and *gbx2*, which show late onset (10hpf) of gene expression. The anterior-posterior axis of the embryo is marked with A-P and an arrow. Scale bar 200µm.

Figure S3

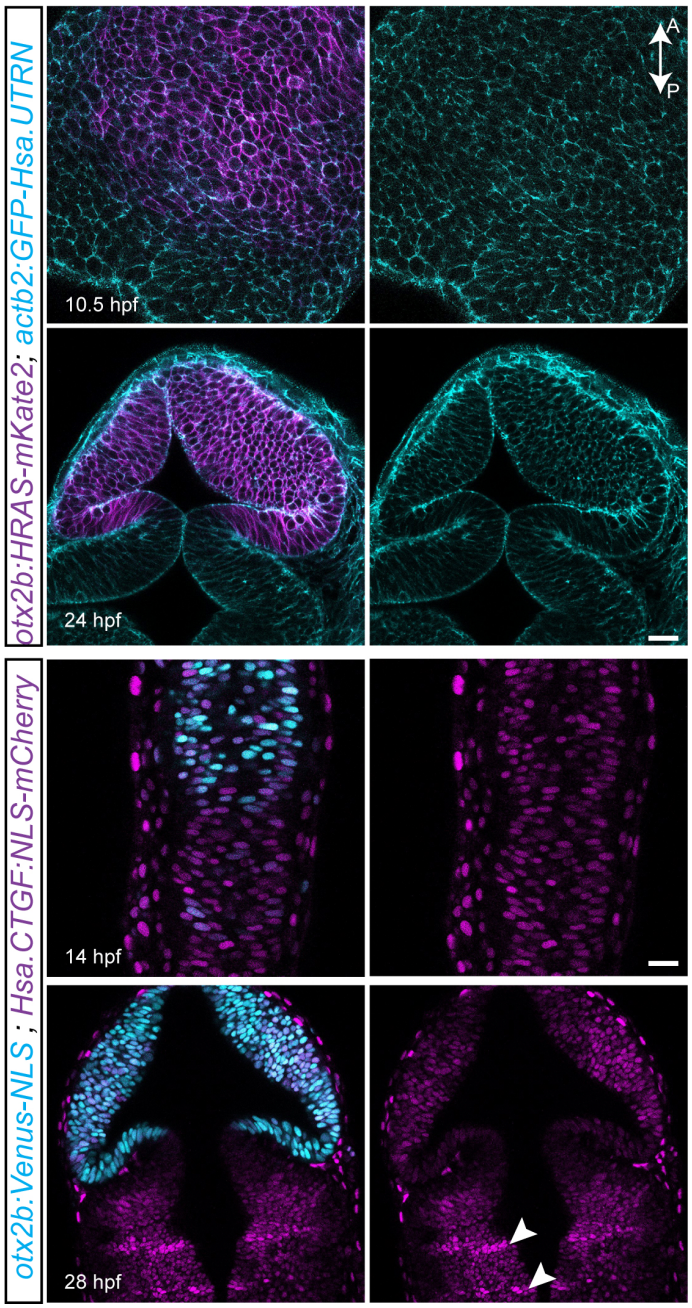


Fig S3: F-actin and Yap signaling atMHB

The F-actin transgenic reporter fish were combined with *Otx2b* reporter (*otx2b:HRAS-mKate2; actb2GFP_Hsa.UTRN).* Double transgenic embryos were dorsally mounted and imaged at 10.5 hpf and 24 hpf. No obvious actin belt structures could be observed at the *otx2b* boundary. Similarly, active Yap signaling reporter *Has.CTGF:NLS-mCherry*, in combination with *otx2b*, did not show any significant mCherry signal at the MHB. In contrast, boundary cells in the rhombomeres showed a strong mCherry signal (arrowheads) as expected. The anterior-posterior axis of the embryo is marked with A-P and an arrow. Scale bar 20µm.

Table S1. List of CRISPR/Cas9 target site and primer sequences for generating bait plasmids

Gene	Target site with PAM (underlined)	Primers used for bait generation
otx2	GGAACCCGGCTAATTGTCTCAGG	F: GGGTGACGCTGAACTTATGTTCACC R: TTTACCCCCCACAACCATCTTTAGC
gbx1	GGTTATCCTGGCGCTGCTGTAGG	F: TTAATTCTCCCCTATTTTATAAGC R: GGTGAACTGAGCGCGGTCTGGTC
wnt1	GGAGGGAGGAAAAAAACAGAGGG	F: ACACGAGGAATCTCTGGACG R: GCACACACTGTCAGATATAGCC
gbx2	GGCGCGGCCAGAGCTCATGGTGG	F: TGCAAACACTCTGACCATACT R: GTCCTGAAGTCTGGGAGAAGC
fgf8a	GGACAGCTCGGGATTTCCTCGGG	F: AGCCTTGCACAATAGCCTCG R: TCAGGTTTTCCAGCTCAAATGT
N-cad(cdh2)	AACGATGTACCGTTCCGGAGG CTCGTTCTCGGTGAAACCAGG	

Table S2. List of CRISPR/Cas9-mediated knock-in lines and transgenic zebrafish lines used in this study

Strain	Source	ZFIN Identifier
Tg(otx2b:Venus)	Kesavan et al., 2017	*tud40Tg(AB)*
Tg(otx2b:Venus-NLS)	This study; Träber et al., 2019	N/A
Tg(otx2b:mKate2)	This study	N/A
Tg(gbx1:Venus)	This study	N/A
Tg(wnt1:Venus-NLS)	This study	N/A
Tg(wnt1:Venus)	This study	N/A
Tg(gbx2:Venus-NLS)	This study	N/A
Tg(dusp6:d2egfp)	Molina et al., 2007	*Pt10Tg*
Tg(otx2b:CreERT2)	Kesavan et al., 2018	*tud44Tg(AB)*
Tg(fgf8a:CreERT2)	This study	N/A
Tg(hsp70l:loxP-DsRed- loxP-EGFPNLS)	Knopf et al., 2011	*tud9Tg*
Tg(ubb:lox2272-loxp-RFP-lox2272-CFP- loxp-YFP)	Pan et al., 2013	*a131Tg*
Tg(actb2:GFP-Hsa.UTRN)	Behrndt et al., 2012	*e116Tg*
Tg(Hsa.CTGF:NLS-mCherry	Astone et al., 2018	*Ia49Tg*

Table S3. Other reagents

Reagent	Source	Identifier
pCS2:HRAS-mkate2	Weber et al., 2014	N/A
pBut2:sol-efnb2a	Cavodeassi et al., 2013	N/A
Hsp70l:loxp_nonFP_loxp_Cas9eGFP	This study	N/A
N-cad *(cdh2)* (morpholino)	TCTGTATAAAGAAACCGATAGAGTT	ZDB-MRPHLNO-060815-1(ZFIN)