Hindbrain rhombomere centers harbor an heterogenous population of dividing progenitors

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Final degree project

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Author: Ariadna Hernández Martin[1]

Co-directors: Cristina Pujades Corbi[1,2] and Lydvina Meister[1,2]

Academic tutor: Jose Francisco Aramburu Beltran[1,2]

[1]Facultat de Ciències de la Salut i de la Vida, Universitat Pompeu Fabra

[2]Departament de Medicina i Ciències de la Vida, Universitat Pompeu Fabra (MELIS-UPF)

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ABSTRACT:

During embryonic development, tissue growth and morphogenesis are interconnected processes and their regulation is essential to correctly develop an organism. This regulation involves the controlled production of different cell types and the precise allocation of stem cell capacities at the right time. The embryonic zebrafish hindbrain is a notable example of how the spatial and temporal acquisition of cell diversity is linked to tissue growth, as neurogenesis depends on the position of progenitor cells along the anteroposterior axis. However, the exact coordination mechanisms behind this process are not fully understood. Through a combination of cell lineage and *in vivo* imaging, we have discovered certain characteristics of the cell population located in the hindbrain rhombomeric centers. By performing clonal analysis, we have investigated the proliferative capacity of this region, providing new insights into the behavior of this cell population. This work shows the spatiotemporal molecular profile of rhombomeric center. Moreover, our findings suggest that rhombomere centers harbor a heterogeneous progenitor population where proliferative capacities get restricted around 48hpf and this territory gets arrested in G1 cell phase of the cell cycle. In conclusion, centers of rhombomeres present characteristics comparable to long lasting progenitors.

1. INTRODUCTION:

1.1 The Central Nervous System: an organ to study cell diversity:

Embryogenesis studies how we go from a single cell to an entire organism. To achieve this goal, it is essential to coordinate proliferation and differentiation in the different cell types. The Central Nervous System (CNS) is a suitable model to study the coordination between these two processes. Neural stem cells obtain their neuronal fate in a process called neurogenesis [1], occurring with the formation of the neural tube.

During early neurogenesis, neuroepithelial cells (NEC) undergo symmetric self-renewing division (PP) giving rise to progenitor cells, enabling to increase the stem cell pool [2]. These cells divide in the ventricular zone (VZ), a highly proliferative zone located along the surface of the neural tube [3]. As long as development proceeds, NEC become radial glia cells (RGC) [4] and change their division mode allowing neuronal differentiation: they undergo asymmetric divisions (PN), giving rise to an RGC and a neuron. These intermediate progenitor cells can also undergo symmetric neurogenic divisions (NN), generating two neurons that will further migrate to the basal domain called mantle zone (MZ) [2] [Fig1.B].

1.2 The embryonic hindbrain as a model:

During embryonic development, the neural tube acquires its final shape composed by three brain vesicles: the forebrain, the midbrain, and the hindbrain [5]. The hindbrain is the most posterior vesicle and will give rise to the pons, the cerebellum and the medulla oblongata in the adult brain [6] [Fig 1.A]. The cerebellum is in charge of the coordination of movement, while the pons and the medulla oblongata regulate breathing and circulation, between other essential physiological processes through the cranial efferent nerves [6].

Hindbrain development is a dynamic process, where proliferation and differentiation occur while it undergoes morphogenesis. The embryonic hindbrain is transiently formed by seven segments along the anteroposterior (AP) axis called rhombomeres [7]. These rhombomeres are units of gene expression, meaning that they have a specific molecular identity and a characteristic transcriptional signature [8]. The borders between them are called boundaries and become sharper as morphological bulges appear [9]. Actomyosin cables at hindbrain boundaries are generated to prevent cell intermingling between adjacent compartments [10]. At the same time that hindbrain get segmented, it undergoes morphogenesis involving the generation of the lumen [11], a cavity filled with embryonic cerebrospinal fluid [12].

1.3 The zebrafish as a model system to study the hindbrain:

The hindbrain is highly conserved among vertebrates due to its crucial role in controlling life-sustaining functions [13], thus, the zebrafish model can be an optimal choice to investigate tissue morphogenesis. It is well characterized by its transparency, genetic manipulability and rapid development, qualities that make it optimal to combine high-resolution *in vivo* imaging and genetic manipulation. Altogether, the zebrafish hindbrain is an interesting model as it is an accessible structure for visualizing processes of cell proliferation and differentiation.

1.4 The rhombomere centers cell population:

At early stages of hindbrain segmentation, neurogenesis is present in all regions of the rhombomeres, excluding hindbrain boundaries [14]. Later on, neurogenesis is restricted to the boundary flanking regions, thus, it is excluded from the hindbrain boundaries and the rhombomere centers. Indeed, during this temporal window, boundaries and centers of rhombomeres are acting as non-neurogenic territories since they are devoid of proneural expression. As development proceeds, boundaries engage in neurogenesis in a Notch3-dependent manner by asymmetric division [15]. In contrast, rhombomere centers are formed by a pool of progenitors that restrict their capacity to generate both oligodendrocyte progenitors and astroglia upon development [16]. These regions are regulated by the FGF signaling pathway [16], which contributes to maintain this cell population as non-neurogenic.

Despite these evidences, little is known on the neurogenic capacity, the cell division mode and the molecular signature of the center of rhombomere’s cell population. Finally, it has been recently shown that Notch pathway is also implicated in maintaining this population as progenitors for longer period [17]. Therefore, we aim to unveil the neurogenic capacity and the behavioral properties surrounding rhombomeres centers by combining cutting-edge technologies and high-resolution *in vivo* imaging [Fig. 1C].

2. BACKGROUND:

2.1 Rhombomere centers progenitor population expresses a specific set of genes:

In order to better characterize the molecular signature of rhombomeric centers, the team decided to undertake fluorescent *in situ* hybridization for six different genes and study their distribution in the hindbrain. For each profile, we show the dorsal view of the hindbrain zebrafish, where the anterior part is localized on the top and posterior part is on the bottom. We analyzed the expression of these genes at different timings in order to see the onset of expression.

First, embryonic hindbrain is profiled with *sox9b* gene (Fig. 2A), a transcription factor necessary to maintain neural progenitors [17]. Rhombomere centers are enriched in *sox9b* expression at 36hpf. This gene is normally expressed by neural progenitors with RGC features rather than in NEC, so the team profiled the hindbrain with RGC progenitor markers such as *fabp7a* (as well known as *blbp*) and *slc1a2a* (glutamate transporter or as well called *glt-1*). We can observe at 48hpf that *slc1a2a2* (Fig. 2B) and *fabp7a* (Fig. 2C) expression is enriched in rhombomere centers, while it is not present in the flanking regions [17]. Next, the expression of *glula* (glutamine synthase), a later marker of RGC progenitors was analyzed. Although its expression was very faint, it is also localized in rhombomere centers (Fig. 2D) [17]. This suggests that rhombomere center cells are composed by neuronal progenitors expressing RGC features.

In order to investigate if some neural progenitors within rhombomeres centers undergo neurogenesis, the expression of the proneural gene *ascl1b* and the neuronal differentiation gene *neuroD4* was observed. It is well known that proneural genes promote neuronal differentiation and specify the different neuronal subtypes. The expression of *ascl1b* and *neuroD4* was compared between the boundary flanking regions and rhombomeres centers. At 48hpf, *ascl1b* (Fig. 2F) shows a high expression in flanking regions, however *ascl1b* is expressed in a very faint manner in rhombomeres centers. Then, the expression of *neuroD4* (Fig. 2E) was also analyzed. In the same manner, *neuroD4* is well expressed in rhombomeres except in the centers of the rhombomeres.

Altogether, previous data shows that rhombomere center cell population is enriched in *soxb9*, *slc1a2a*, *fabp7a* and *glula*, RGC progenitor markers, at different timings of development. However, at the same stage centers of rhombomeres show faint expression of the two proneurals genes *ascl1b* and *neuroD4,* in comparison with flanking regions.

3. RESULTS:

3.1 Rhombomere centers are devoid of *neurogenin1* expression:

To further investigate the neurogenic capacity within rhombomeric center cell population, we restored to perform a colocalization analysis of a proneural gene and an RGC marker specifically expressed in this population. Previous results from the team only showed the expression of RGC markers and proneural genes with simple fluorescent *in situ* hybridization (FISH) (Fig. 2). Thus, a double FISH of *fabp7a* and the proneural gene *neurogenin1* was performed. Double FISH could confirm co-expression or not within the progenitor domain of rhombomeres centers. We already know from previous results that *fabp7a* is enriched in rhombomere centers (Fig. 2C). *neurogenin1* is already known to be a proneural gene involved in neuronal differentiation and functions as a transcriptional activator [18].

We analyzed the expression of *fabp7a* and *neurogenin1* at 36 and 48hpf (Fig. 3). At 36hpf, transversal views of the centers of rhombomere 3 and 4 (Fig. 3-C1 and C2, respectively) were analyzed and compared with transversal views of the flanking regions (Fig. 3-F1 and F2, respectively). We can observe sparse co-expression of *fabp7* and *neurogenin1* in rhombomeric centers, while they clearly colocalize in flanking regions. *fabp7a* is also highly present in the lateral domains of the rhombencephalon, an expression that will be not detailed in this report. We can conclude from these results at 36hpf that few cells undergo neurogenesis in centers. Moreover, flanking regions show higher neurogenic capacity than centers at this stage.

At 48hpf, transversal views of the centers of rhombomeres were compared with the adjacent domains. We can notice that committed neurons in flanking regions are increased when compared to 36hpf. However, the expression in center remains the same. These results suggest that while flanking region get committed to neurons, cells in the rhombomeres centers essentially stay as progenitors, at least until 48hpf.

3.2 Rhombomere population harbor different proliferative capacity upon time:

To study whether rhombomere center progenitor cells display proliferative capacity, we analyzed the progression of this cell population through the cell cycle. The cell cycle consists to a series of steps undergoing in a cell in order to produce two cell derivates. It consists in 4 distinct phases: G1-phase, when the cell gets ready to synthesize the DNA; S-phase, when the cell duplicates its own DNA; G2-phase, when the cell prepares for dividing itself; and M-phase or mitosis, when the two daughter cells are created as a result of the whole process [19].

a. Rhombomere centers display proliferative capacity until 46hpf:

First, an analysis of EdU-incorporation was performed. EdU (5-etinil-2'-desoxiuridina) is a synthetic thymidine analogue that measures the S-phase synthesis of the cell cycle, allowing us to detect proliferative cells. The EdU treatment was performed every 2 hours between 42 and 48hpf, and embryos were fixed immediately after. After Click-It reaction in order to reveal EdU positive cells, the behavior of progenitor cells within centers of rhombomeres and flanking regions has been characterized. Following the EdU treatment, a fluorescence *in situ* hybridization (FISH) against *fabp7a* and *sgca* genes has been undergone in order to respectively label rhombomere centers and hindbrain boundaries. The center of rhombomere 4 (C) and the flanking region above it (F) (marked with a dashed line) (Fig. 4A), and transversal views of these levels have been analyzed at different stages of development (Fig. 4a-h).

First, we wanted to explore the proliferative capacity of flanking regions and rhombomeres centers upon time. We performed transverse sections at 42 (Fig. 4a and 4e), 44 (Fig. 4b and 4f), 46 (Fig. 4c and 4g), and 48hpf (Fig. 4d and 4h) of both regions. We can see EdU positive cells in the VZ (surface) of rhombomere centers between 42 and 46hpf. However, at 48hpf, few cells continue to divide. In contrast, flanking regions are actively dividing even after 48hpf. These results suggest that rhombomeres center progenitors show high proliferative activity until 46hpf, ceasing after that time. However, flanking region progenitor population display proliferative activity until at least 48hpf.

b. Rhombomeres centers and adjacent territories display different division modes in time:

Next, we wanted to explore the division mode of proliferative progenitors within rhombomeres centers. Thus, we performed an EdU pulse-chase strategy. Embryos were incubated with EdU every 2 hours (pulse) between 42 and 48hpf, and after being washed, they were incubated in embryo water for 6 hours (chase). Using this method, the positions of cell derivates can be detected. We used the same markers for the FISH. Transversal views of these embryos at the level of the center of rhombomere 4 and its flanking region have been analyzed between 48 and 54hpf (Fig. 4i-p).

As previously mentioned in the introduction, neuronal progenitors within the hindbrain can undergo three different modes of division: progenitors can divide symmetrically (PP) [2], can undergo asymmetric division (PN) giving rise to a progenitor and a differentiated neuron or can undertake neurogenic symmetric division (NN) generating two neurons. Regarding this information and the position of the cell derivative, we can hypothesize on the mode of division in the hindbrain.

EdU positive cells visible in the VZ can be either arrested in G2 phase stage after having incorporated the EdU and have not yet divided, can be undergoing a PP division, or a PN division and the migration to the MZ has not started yet. On the other part, EdU positive cells visible in the MZ can be committed neurons derived from a PN or NN divisions. In conclusion, cells within the VZ were considered as progenitors and cells within the MZ as differentiated neurons.

From these experiments, we explored the mode of division in both centers and flanking regions. We can see at 48 (Fig. 4i and 4m), 50 (Fig. 4j and 4n) and 52hpf (Fig. 4k and 4o) (respectively EdU pulsed at 40-42hpf, 42-44hpf and 44-46hpf) some EdU positive cells in the VZ and others in MZ (more in the VZ) both in the centers and the flanking regions. However, at 54hpf (Fig. 4l and 4p) (pulse at 46-48hpf) EdU positive cells are not any more visible in the VZ but some are still detectable in the MZ.

Overall, these results suggest that rhombomere centers cells undergo mainly PP or PN division between 40 and 42hpf. Between 42 and 44hpf, the progenitor pool in rhombomeres center almost ceases doing PP division and still undergo PN division. Between 46hpf and 48hpf, this progenitor pool dividing between 40 and 42hpf is almost visible in the MZ, suggesting PN or NN division. In contrast, progenitor cells in flanking regions mainly undergo PN and PP divisions between 40 and 44hpf. After this time window, the progenitor cells in flanking regions decrease their division rate and probably undertake mainly PN and NN division modes.

c. *Rhombomere progenitor cells divide more actively in flanking regions when compared to centers:*

To study this further, we wanted to compare the proliferative capacity between centers of rhombomeres and adjacent domains. Between 40 and 42hpf, flanking regions and rhombomeres centers seems to divide equivalently. However, at 44hpf, we can clearly detect more Edu positive cells in the flanking regions. At 52hpf, in the rhombomeres centers, we can’t see almost no cells in the ventricular and the mantle zone. In comparison, few cells are visible in the flanking regions.

Regarding these results, we can conclude that rhombomere centers and flanking region cells display the same proliferative capacity at 42hpf. At 44 and 46hpf, progenitor cells divide more actively in flanking regions when compared to centers. Finally, at 48hpf flanking regions are still proliferatively active while rhombomere centers completely stop their division.

3.3 Rhombomere centers display less proliferative capacity than flanking regions at 48hpf:

In order to decipher whether rhombomere centers have a different proliferative capacity than flanking regions, zebrafish embryos were injected with the mRNA of Proliferating cell nuclear antigen (PCNA) coupled with GFP. This gene acts as a reporter of cell division since plays critical roles in many aspects of DNA replication. Depending on the nuclei distribution of PCNA-GFP, cell cycle phases can be identified (Fig. 5A). G1 and G2 exhibit homogeneous nuclear distribution, however S-phase is characterized by small dots covering the nucleus and conferring a granular aspect. Finally, M phase shows a diffuse cellular distribution since the nuclear envelope is broken. For this experiment, the Tg[Mü4127] transgenic line was used as a positional landmark along the anterior-posterior axis since it labels rhombomeres 3 and 5.

On the Fig. 5B, we delimitated an area of the center and the flanking region of rhombomere 3, 4 and 5 in several embryos. We then analyzed the ratio of the number of nuclei in S-phase per total number of nuclei labelled by PCNA-GFP at two different timings: 42 and 48hpf. Finally, the percentage of S-phase cells in both flanking regions and centers was calculated in order to compare the number of proliferative cells located in these regions upon time.

The percentage of S-phase cells is similar between flanking regions and rhombomere centers at 42hpf (Fig. 5C). However, we can clearly observe that the percentage of S-phase cells is significantly higher in flanking regions than in rhombomere centers at 48hpf (Fig. 5D). Thus, we can conclude that rhombomere centers exhibit the same proliferative capacity than flanking regions until 42hpf. However, at 48hpf, flanking regions have a significant higher rate of proliferation than rhombomere centers coinciding with previous results using the EdU technology.

3.4 Rhombomere centers are cell cycle arrested in G1:

In order to study the cell cycle state of rhombomere center cell population, a fluorescence ubiquitin cell cycle indicator (FUCCI) based system was used. It distinguishes cell cycle phases by labeling G1 phase of the cell cycle in mCherry (Fig. 6A). A dorsal view of Tg[ubi:zFucci] embryo at 48hpf labeling G1 cells in red can be observed (Fig. 6B). The centers of rhombomere 3 and 4 centers are highlighted. We can clearly observe that centers and boundaries show an accumulation of G1 cells. In conclusion, this experiment revealed that rhombomeric centers are enriched in G1 cells at 48hpf, indicating that this cell population could be arrested in the G1 phase.

4. DISCUSSION:

a. *Rhombomeric centers display a specific molecular signature:*

In this project we have explored how progenitor and neurogenic capacities are allocated within the hindbrain.

During the first wave of neurogenesis happening between 24 and 36hpf [20], the entire hindbrain mainly undergoes differentiation except hindbrain boundaries [14]. At 36hpf, while flanking regions and boundaries start to massively differentiate contributing to the neural pool, the centers of rhombomeres get devoid of proneural genes. Our results support coherently previous studies from the team, showing that rhombomeres centers do not express the proneural gene *neurogenin1.*

At the same time, this cell population start to get a specific molecular signature. The center of rhombomere progenitor population start to express the transcription factor *sox9b* from 30hpf. This cell population also get enriched in RGC markers such as *fabp7a* and *slc1a2a* from 36hpf. Finally, at later stages, *glula*, a gene for glutamine synthase get specific to this cell population. The acquisition of these different genes appears in a sequential manner.

Moreover, between 42 and 48hpf, the rhombomeres centers progenitor pools start to decrease the proliferative rate and get arrested at the G1 phase stage of the cell cycle. RGC at the centers of rhombomeres seem to be maintained at a slow proliferative state, and specifically with a longer G1 phase.

b. *Progenitors within the hindbrain display different division modes:*

In order to understand cell proliferation during hindbrain growth, we explored the committed cells within rhombomeres emerging from the neural stem cells after division. Through an Edu Pulse-chase experiment, we could follow the lineage of each proliferative cell. Clonal analysis studies in the hindbrain suggested that the majority of neurons are born from symmetric neurogenic divisions. During the first phase of differentiation and growth of the tissue: rhombomeres centers and its adjacent domains mainly undergo symmetric self-renewing division in order to expand the stem cell niche and symmetric neurogenic divisions or terminal differentiation in order to allow tissue growth. From 44hpf onwards, there is a drastic change of division mode in rhombomeres centers probably with final PN division or NN division. These changes are shift in flanking regions by 46hpf. Further clonal analysis may be done in order to follow individual cells at different timing in order to determine the special mode of division in each domain.

We can conclude from our research that rhombomere centers harbor a heterogeneous progenitor population that divide symmetrically and asymmetrically in different proportions, self-renewing and contributing to the neuronal differentiated domain. At 48hpf, centers stop dividing when they start expressing RGC progenitor markers and being cell cycle arrested, potentially becoming quiescent neural progenitors or long-lasting progenitors [21].

5. ACKNOWLEDGMENTS:

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6. MATERIALS AND METHODS:

Ethics declarations and approval for animal experiments:

All procedures were approved by the institutional animal care and use ethic committee (Comitè Etica en Experimentació Animal, PRBB) and the Generalitat of Catalonia (Departament de Territori i Sostenibilitat), and implemented according to National and European regulations. Government and University veterinary-inspectors examine the animal facilities and procedures to endure that animal regulations are correctly followed. The PRBB animal house holds the AAALAC International approval B9900073. All the members entering the animal house have to hold the international FELASA accreditation. The Project License covering the proposed work (Ref 10642, GC) pays particular attention to the 3Rs.

Zebrafish strains:

Embryos were obtained by mating of adult fish using standard methods. All zebrafish strains were maintained individually as inbred lines. Tg[Mü4127] (Distel et al., 2009) transgenic line was used as landmarks of rhombomeres 3 and 5. Tg[ubi:zFucci] line labels the G1 (with mCherry) cell phase of the cell cycle in all tissues (Bouldin & Kimelman, 2014).

Confocal imaging of whole mount embryos:

Anesthetized live embryos expressing genetically encoded fluorescence or stained fixed samples were mounted in 0,8% low melting point (LMP) agarose with the hindbrain positioned towards the glass-bottom of Petri dishes (Mattek) to achieve dorsal views of the hindbrain. Imaging was performed under an LSM980 AiryScan or a SP8 Leica confocal microscope.

Whole mount *in situ* hybridization:

Embryo whole mount *in situ* hybridization was adapted from (Thisse and Thisse, 2008). The following riboprobes were generated by *in vitro* transcription from cloned cDNAs: *neurog1* (Itoh and Chitnis, 2001). The other probes were generated by PCR amplification adding the T7 or Sp6 promoter sequence in the Rv primers: *fabp7a* Fw: 5' –GAC TGA ACT CAG CGA CTG TAC– 3' and Rv: 5' –AGG CCT CAA TAA TAC ACT CCC–T7 3'. For fluorescent *in situ* hybridization, FLUO- and DIG-labeled probes were detected with TSA Fluorescein and Cy3, respectively.

***In toto* embryo immunostainings:**

Embryos were blocked in 10% neutralized goat serum and 2% Bovine Serum Albumin (BSA) in in PBS-Tween20 (PBST) during 2h at room temperature (RT), except after *in situ* hybridization that they were blocked in 5% neutralized goat serum in PBST during 1h. They were then incubated O/N at 4°C with the corresponding primary antibody: mouse anti-GFP ([1:100]; ThermoFisher). After extensive washings with PBST, embryos were incubated with secondary antibodies conjugated with Alexa Fluor®488 to label cell nuclei.

Cell proliferation:

EdU-incorporation experiments:

Cells in S-phase were detected by EdU-incorporation using the Click-It™ EdU Alexa Fluor™ 647 Imaging Kit (C10340, Thermo Fisher Scientific) following the supplier instructions, with the following modifications: Embryos were dechorionated, incubated in 500µM EdU diluted in 7% DMSO and placed on ice during the first hour for better EdU-incorporation. Afterwards, they were either washed three times with embryo medium to wash out the EdU or fixed in 4%PFA during 4 hours at RT, and dehydrated in MetOH. After progressive rehydration, embryos were permeabilized with 10 mg/ml Proteinase K (Invitrogen) for 35min, post fixed 40min in 4%PFA, and washed in PBT. Embryos were then incubated 1h in 1%DMSO/1% Triton X-100/PBS. The Click-iT reaction was carried out according to the manufacturer's instructions for 60 min at RT, washed into PBT and used for *in situ* hybridization with *fabp7a*. In order to precisely determine the position of boundaries and rhombomeres centers, we used Tg[BCP:H2B-GFP] line and revealed the GFP by immunostaining. Imaging was undertaken with the AiryScan2 LSM980 using a 20× dry objective.

Cell cycle phase dynamics:

In order to follow the cell cycle phase dynamics, Tg[Mu4127] embryos were injected between 16-cell stage and 32-cell stage with PCNA-GFP mRNA and *in vivo* imaged under the *AiryScan2* LSM980 microscope. Finally, the distribution of PCNA-GFP in the cell nuclei was assessed.

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8. FIGURES:

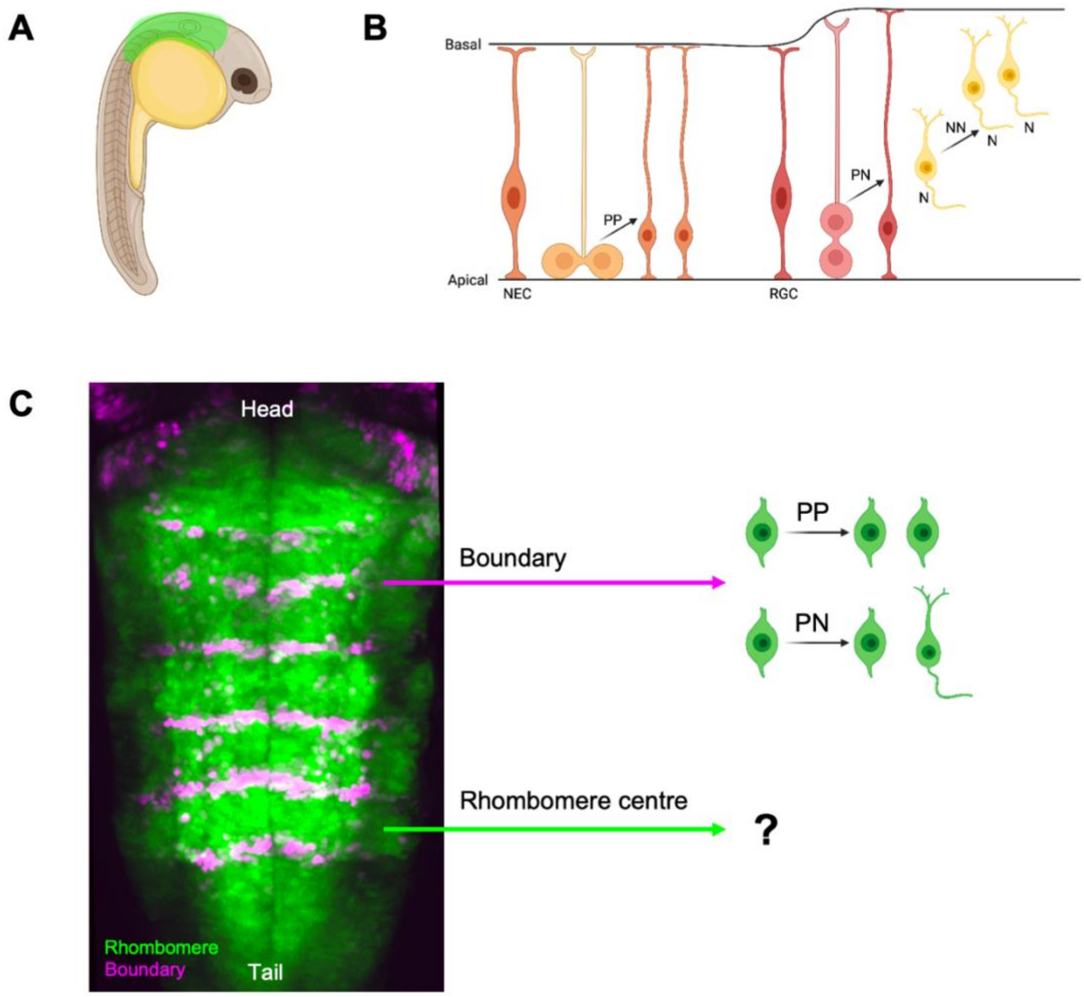

Figure 1. The zebrafish hindbrain and neural stem cells division modes. (A) Lateral view representation of a 24hpf zebrafish embryo. The hindbrain has been highlighted in green. **(B)** Representation of the mode of division in the ventricular zone of the hindbrain. Firstly, neuroepithelial cells (NEC) undergo symmetric proliferative divisions (PP). Later, NEC become radial glial cells (RGC) that undergo asymmetric divisions, giving rise to an RGC and a neuron (N). Neurons can undergo symmetric neurogenic divisions (NN), generating two neurons that migrate to the basal domain. **(C)** Dorsal view of a zebrafish hindbrain where rhombomeres are visualized in green and boundaries in magenta. Hindbrain boundaries display symmetric self-renewing division (PP) and asymmetric division (PN). Nevertheless, behavioral properties of rhombomeres centers are still unknown.

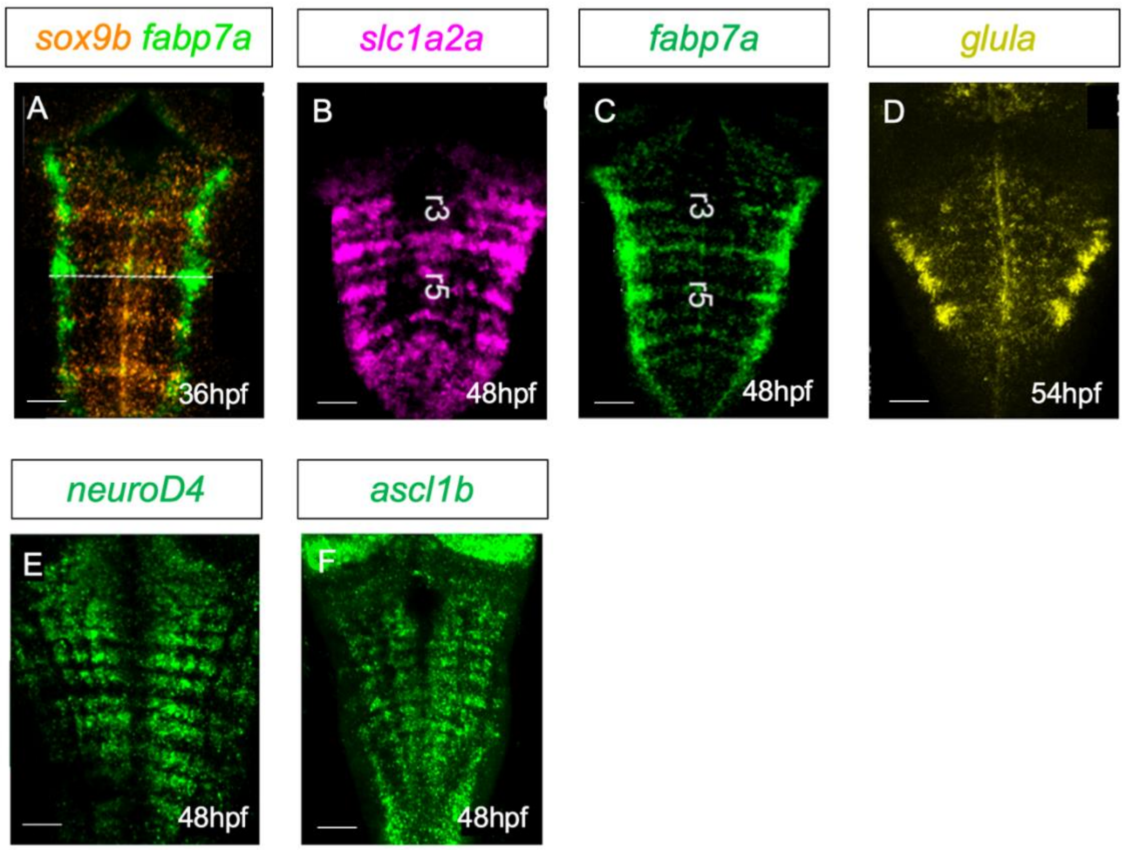

Figure 2. Rhombomeric centers display long-lasting progenitor potential. Dorsal view of embryos *in situ* hybridized with *sox9b* and *fabp7a* (A) at 36hpf, *fabp7a* (A) and *slc1a2a* (B) at 48hpf, *glula* (D) at 54hpf, *neuroD4* (E) and *ascl1b* (F) at 48hpf. Distribution of *sox9b*, *fabp7a, slc1a2a and glula* expression is enriched in rhombomeric centers while *ascl1b* and *neuroD4* are not expressed in this localization, indicating that centers are devoid of neural differentiation at this stage and are maintained as non-committed progenitors. Scale bars correspond to 50µm.

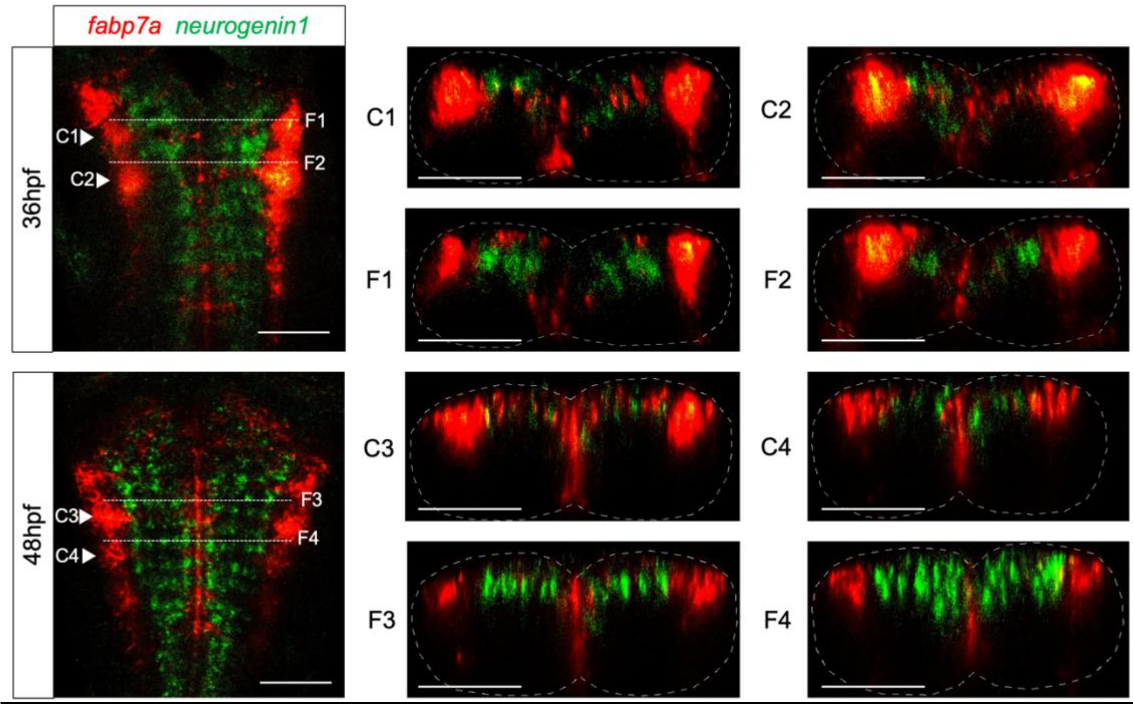

Figure 3. Rhombomere centers are non-commited progenitors compared to flanking regions. Whole-mount fluorescent *in situ* hybridizations of *fabp7a* (red) and *neurogenin1* (green) in embryos at 36 and 48hpf (dorsal views). C1 and C2 are transversal views of rhombomere 3 and 4, respectively, at the level of the arrowheads. F1 and F2 are transversal views of the flanking regions above rhombomere 3 and 4, respectively, at the level of the dashed lines. C3 and C4 are transversal views of rhombomere 4 and 5, respectively, at the level of the arrowheads. F1 and F2 are transversal views of the flanking regions above rhombomere 4 and 5, respectively, at the level of the dashed lines. Scale bars correspond to 50μm.

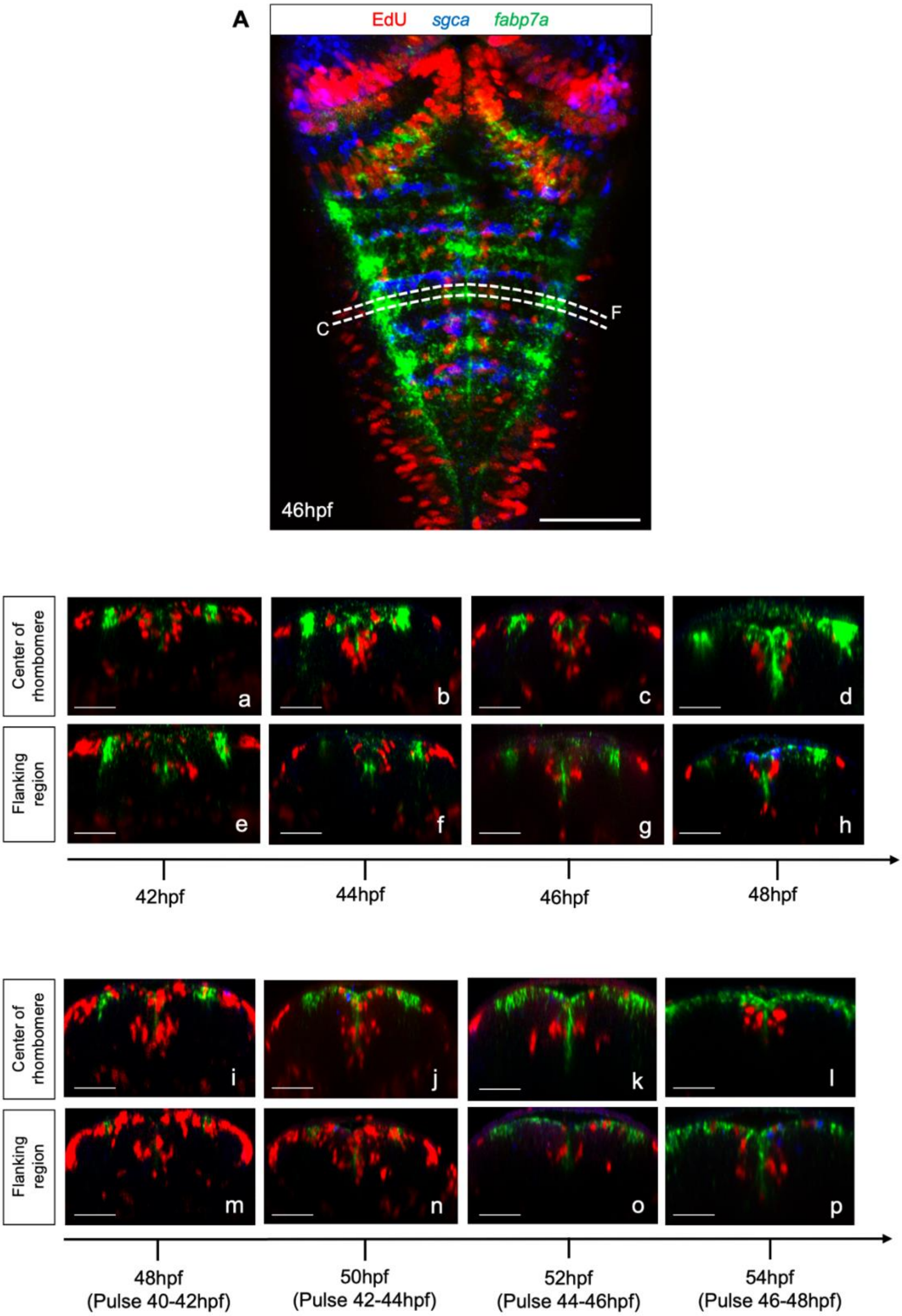

Figure 4. Rhombomere centers display proliferative activity. (A) Dorsal view of a 46hpf embryo with EdU positive cells labelled in red (cells in S-phase), *fabp7a* (gene expressed in rhombomere centers) labelled in green, and *sgca* (gene expressed in hindbrain boundaries) marked in blue. Transversal view of rhombomere center (C) at the

level of the dashed line in rhombomere 4 in embryos being fixed just after EdU treatment at 42hpf (a), 44hpf (b), 46hpf (c) and 48hpf (d), as well as its flanking regions at 42hpf (e), 44hpf (f), 46hpf (g) and 48hpf (h). Transversal view of rhombomere center at the level of the dashed line in rhombomere 4 in embryos after a pulse-chase EdU treatment at 48hpf (i), 50hpf (j), 52hpf (k) and 54hpf (l), as well as its flanking regions at 48hpf (m), 50hpf (n), 52hpf (o) and 54hpf (p). Scale bars correspond to 50μm.

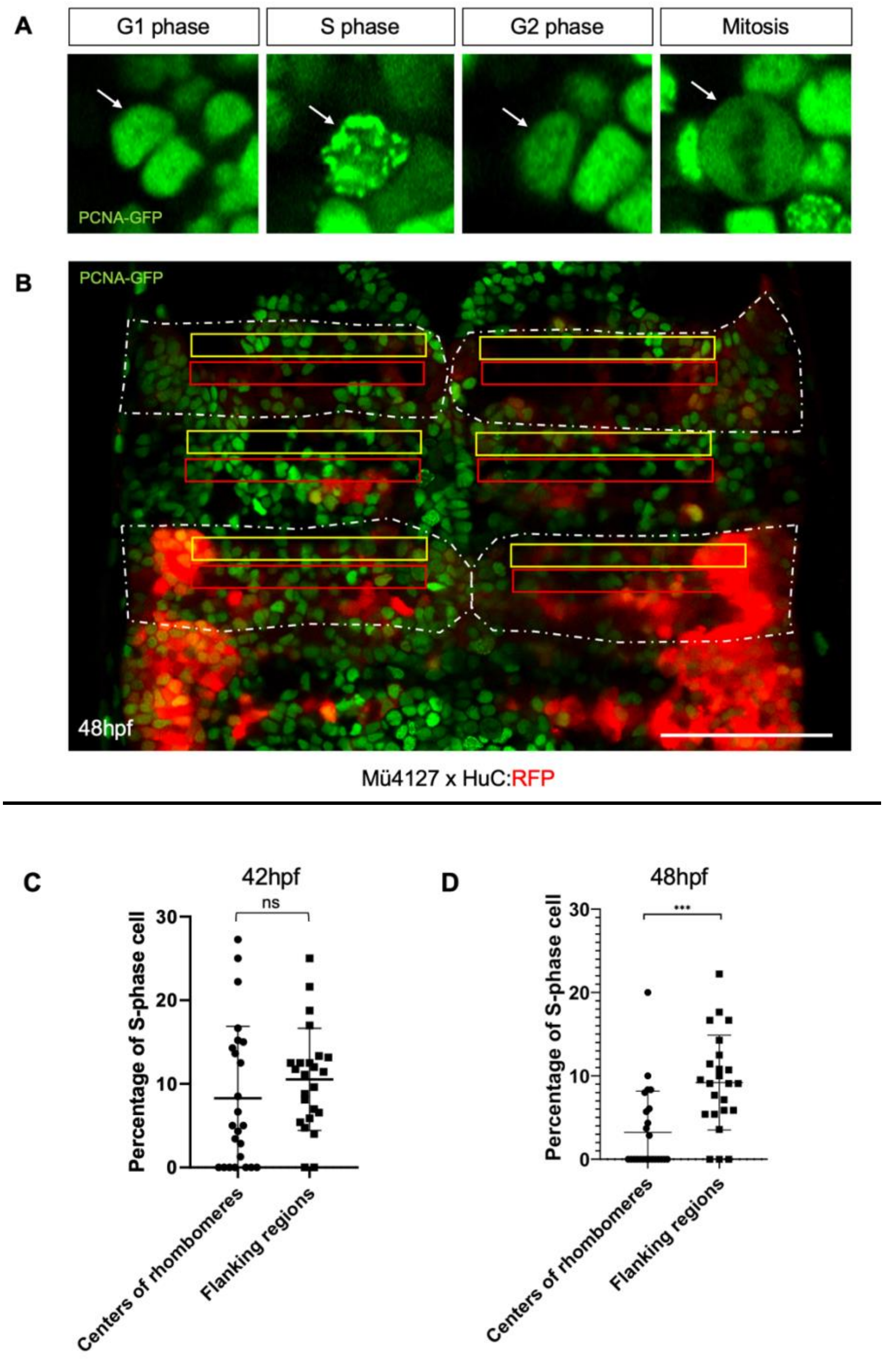

Figure 5. Rhombomere centers display less proliferative activity when compared to flanking regions. (A) Images of zebrafish hindbrain nuclei through the cell cycle. Phases can be identified by the differential distribution of PCNA-GFP (white arrows). **(B)** Dorsal view of a 48hpf embryo where HuC is marked in red (differentiated neurons), PCNA is seen in green (reporter of cell division) and rhombomere 3 and 5 are highlight with a white dashed line (Tg[Mü4127]). The red straight line is a landmark of rhombomere centers while yellow straight line marks out the area of flanking regions. **(C)** Plot containing a comparison of the percentage of S-phase cells at 42hpf in rhombomere

centers and flanking regions. **(D)** Plot containing a comparison of the percentage of S-phase cells at 48hpf in rhombomere centers and flanking regions. For both plots, the top and bottom of each box indicate upper and lower quartiles, respectively; the horizontal line represents the median and Wilcoxon test was performed. ******* $p < 0.0001$. 000Scale bars correspond to 50μm.

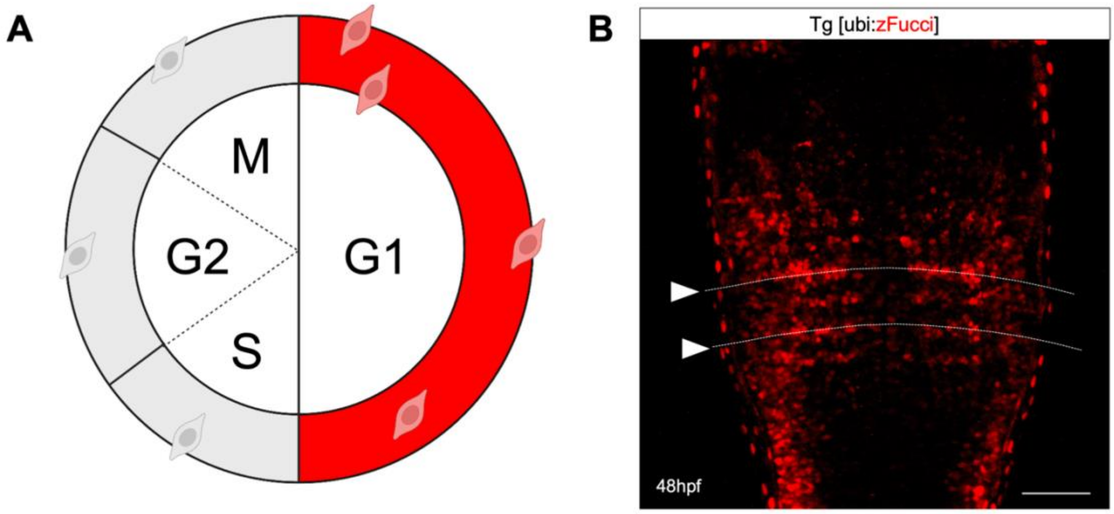

Figure 6. Rhombomere center population is enriched in G1-phase. (A) Scheme of the phases of the cell cycle. The G1 phase is depicted in red. **(B)** Tg[ubi:zFucci] embryo at 48hpf. White arrowheads indicate the localization of rhombomere 3 and 4 centers, respectively. Scale bars correspond to 50µm.