Using zebrafish to model erythroid lineage toxicity and regeneration

Anna Lenard,[1] Elisa Alghisi,[1] Hamina Daff,[2] Massimiliano Donzelli,[2] Claudia McGinnis[2] and Claudia Lengerke[1,3*]*
**equal contributors*

[1]Department of Biomedicine,, University Hospital Basel, Hebelstrasse 20; [2]Roche Pharmaceutical Research & Early Development (pRED), Pharmaceutical Sciences, Roche Innovation Center Basel, Grenzacherstrasse 124; and [3]Clinic of Hematology, University Hospital Basel, Switzerland

Correspondence: claudia.lengerke@unibas.ch
doi:10.3324/haematol.2016.142562

Supplementary Data

Supplementary Material and Methods

Zebrafish maintenance and stocks

Zebrafish of the *Tg(gata1:DsRed)*, *Tg(globin:EGFP)* lines were maintained at standard conditions [1]. Embryos were staged by hours post-fertilization (hpf) at 28.5 °C [2].

Compound exposure

Irinotecan (8 mM in DMSO stock solutions, ChemPacific Corporation) and topotecan (1.6 mM in DMSO stock solution, Sigma-Aldrich), were diluted in zebrafish E3 medium [1]. 10-12 embryos were incubated in 1.5 ml water with compound in a 24-well plate as indicated.

Phenylhydrazine (PHZ, Sigma-Aldrich, 1 mg/ml water stock solution) was diluted in the E3 medium to 1 µg/ml. Embryos were incubated in 24-well plates from 36 to 54 hpf and then washed 4 times with fresh E3 medium.

For irinotecan + PHZ experiments, embryos were incubated from 36 to 52 hpf in 1 µg/ml PHZ only, 1 µg/ml PHZ and 2 µM irinotecan, 2 µM irinotecan only or 0.1 % DMSO for control. At 52 hpf solutions were washed away and replaced with E3 or 2 µM irinotecan, respectively and analyzed at 4 dpf. For injection, irinotecan (32 µM), topotecan (32 µM) or DMSO (20%) as control respectively, were injected into the yolk sac of 24 hpf zebrafish embryos (drop size ~2nl). Embryos were kept in E3 medium and analyzed at 6 dpf.

Imaging

Embryos were anesthetized in 0.08 % tricaine and mounted in 0.7 % low melting point agarose (Sigma) in a glass bottom dish (MatTek glass bottom petri dish or Ibidi 4 chamber glass-bottom dish). For still pictures we used Leica TCS SP5 confocal microscope with 20x air objective or Zeiss LSM 710 confocal microscope with 20x air objective. For time-lapse experiments we used Leica TCS SP5 with 20x air objective. Images were taken with 0.8-1 µm step size and 40-50 z-slices. All images are maximum intensity z-projections, unless indicated otherwise. The images were analyzed with Imaris (Bitplane) and Fiji software.

Flow cytometry

Groups of 5-10 zebrafish embryos were incubated in 150 µl of Accumax Cell Dissociation Solution (Millipore) for 15 minutes prior to manual disintegration with a pestle in a 1.5 ml Eppendorf tube. Dissociated embryo cells were then diluted to 1000 µl with sterile 1x PBS and centrifuged for 10 min at 300 x g. After a washing step with PBS, cells were re-centrifuged and the pellet resuspended in 300 ul of PBS and filtered through a cell strainer (35 µm, Falcon). The sample volume was adjusted to be the same for all samples. 1:1000 DRAQ5 (Thermo Scientific) was added to label the nucleated cells. Cells were analyzed on the BD Accuri flow cytometer using the DRAQ5 positive population as “all fish cells” reference. Cell numbers were analyzed as percentage of all DRAQ5+ fish cells or as calculated cell number per embryo.

ImageStream (Amnis) imaging flow cytometer was used to acquire images of the cells within specific populations, with either 40x or 60x objective. The data were analyzed with Ideas software.

In situ hybridization and Immunofluorescence of whole mount zebrafish embryos

Double *in situ* hybridization was performed as describes in the manuscript. The *cmyb*-digoxigenin probe was detected with anti-digoxigenin antibody (Roche) conjugated to alkaline phosphatase, followed by use of the NBT/BCIP substrates (Promega), resulting in a blue precipitate. The *gata1*-fluorescein probe was detected with anti-fluorescein antibody (Roche) conjugated to alkaline phosphatase and followed by use of INT/BCIP substrate (Roche) resulting in a red precipitate.

The detection of the phosphohistone 3 (PH3) was performed using the rabbit polyclonal anti-PH3 (1:200, Cell Signalling), according to manufacturer´s datasheet. In brief 2 hpf and 3.5 dpf larvae were fixed in 4% PFA at room temperature for 2 hours and then rinsed, blocked, permeabilized with Acetone and incubated with the antibody overnight at 4°C. Subsequently Alexa Fluor 633 secondary antibody (1:500, Cell Signalling) was used to reveal PH3 positive cells. The expression of the transgenes, globin:GFP and gata:DsRed, was still visible at the microscope as green and red fluorescence respectively.

Bioanalytical compound uptake studies

Embryos were incubated in freshly prepared dilutions of irinotecan (1 and 5 μM) or topotecan (5 and 25 μM) from 36 hpf to 3 dpf. A sample of medium

was taken at the beginning and at the end of the experiment. The embryos were washed 3 times with filtered E3 medium, the excess of water was removed and the embryos were frozen for analysis.

Concentrations of topotecan and irinotecan were determined by reversed phase HPLC tandem mass spectrometry. 100 µL of incubation medium were mixed with 200 µL internal standard solution (Oxazepam 25 ng/mL in acetonitrile). Embryo samples (10 embryos) were mixed with 200 µL internal standard solution and homogenized in a Precellys® tube (2 cycles of 15 s at 5500 rpm).

Medium and embryos probes were mixed for 30 s, centrifuged (6400g, 10min, 10 ºC) and 10 µL of the supernatant was injected into the LC-MS/MS system for analysis.

Chromatographic separation was performed on a Shimadzu UHPLC system (Shimadzu AG) coupled to a triple quadrupole tandem mass spectrometer (API6500, AB Sciex) operating in positive electrospray ionization mode.

Samples were eluted on an UPLC BEH C18 column (50*2.1 mm, 1.7µm, Waters) at 50 ºC. The total run time was 2.0 min. The following gradient was applied: from 0.02 to 0.3 min. 90% solvent A (0.1 % formic acid in water), from 0.3 to 1.0 min the proportion of solvent B (0.1 % formic acid in methanol) linearly increased from 10 to 90 %, at 1.51 min, the percentage of B was maintained for 0.5 min. At 2 min, the initial conditions were re-established.

A calibration curve was constructed plotting analyte concentration vs. peak area ratios of the analyte/ internal standard measured in samples.

The assay was linear between 1 and 1000 ng/mL and the lower limit of quantification was 1ng/mL for both analytes.

Statistical analyses

Statistical analyses were performed with GraphPad Prism 6 software. Multiple samples were compared using Ordinary One-way ANOVA test. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$

Supplementary Figures legends

Figure S1. Confocal images of transgenic embryos *Tg(gata1:DsRed;globin:GFP)* over the first 5 days of development.

Confocal images representing 5 consecutive days of zebrafish embryo development with (left) and without (right) the bright field channel. Gata1:DsRed expressing cells are red, globin:GFP expressing cells are green.

Figure S2. Gating strategy for flow cytometry analyses of cells from whole zebrafish embryos.

(A) Forward and side scatter analyses of whole zebrafish embryo cell sample. (B) Nucleated cells labeled with DRAQ5 DNA dye constitute the main population of zebrafish cells (blue frame). (C) DRAQ5 positive cell population back-gated on a forward vs side scatter graph (blue). (D) Flow cytometry analyses representing 5 consecutive days of zebrafish embryo development, for double transgenic embryos *Tg(gata1:DsRed;globin:GFP)*, labeling two erythrocyte populations: double fluorescent (gata1+/globin+) mature erythrocytes (green frame) and red-only (gata1+/globin-) erythroid progenitors (red frame). (E) Mature erythrocyte (green) and erythroid progenitor (red) populations represented as percentage of all embryo cells over the first 5 days of development. Each result is an average of 5 flow cytometry experiments. (F) Mature erythrocyte and erythroid progenitor populations represented as cell number per embryo, over the first 5 days of development. Each result is an average of 5 flow cytometry experiments.

Figure S3. Irinotecan and topotecan toxicity to erythroid progenitors

Graphs representing flow cytometry results of irinotecan (IRI) and topotecan (TOP) exposure experiments on double transgenic zebrafish embryos *Tg(gata1:DsRed;globin:GFP),* summarized in Figure 2. Each result is an average of 3 biological replicates with average and standard deviation, presented as cell numbers per embryo. Compound concentrations are indicated on the graph in µM (e.g. IRI4 indicates 4 µM irinotecan in E3 medium) Different incubation schemes were used to assess the treatment effect on mature and progenitor erythrocytes: (A-B) embryos treated from 24 hpf continuously and analyzed at 3 dpf (A) or 6 dpf (B); (C-D) embryos treated from 24 hpf until 32 hpf, then washed and analyzed at 3 dpf (C) or 6 dpf (D); (E-F) embryos treated from 32 hpf continuously and analyzed at 3 dpf (E) or 6 dpf (F); (G) simplified summary of all drug treatment results. Decreased cell number is indicated with a downward arrow. Equality sign represents not affected cell number. (H) Embryos injected with ~2 nl of 32 µM irinotecan or topotecan.

Figure S4. Compounds bioavailability

(A-B) LC/MS analysis of irinotecan and topotecan concentrations in embryo compared to medium. Embryos (10 embroys per condition, in triplicates) were treated from 32 hpf to 3 dpf with 2 different concentrations of each compound, then sampled, washed, homogenized and analyzed. The corresponding incubation media were also sampled. Increase in the medium concentration caused a proportional increase in the compound absorbed by the embryo. The efficiency of drug absorbance, presented as the percentage of the

embryo concentration to the medium concentration, is significantly higher for irinotecan than topotecan.

Figure S5. Phagocytosis of hemolytic erythrocytes in the CHT

Still from a time lapse video (video 3) showing phagocytosis of damaged erythrocytes in a PHZ treated embryo at ~2.5 dpf in a transgenic embryo *Tg(gata1:DsRed;globin:GFP)*. First, a green cell (green arrow) is phagocytosed by a red cell of irregular shape (white arrow), most likely a macrophage (A). Second, the same macrophage phagocytoses a larger, red cell (B, red arrow). See also video 3.

Figure S6. Recovery from hemolytic anemia

(A) Confocal images of double transgenic embryos *Tg(gata1:DsRed;globin:GFP)* at 3dpf for control (A) and PHZ treated fish (A'). In the PHZ treated embryo (A') the number of circulating erythrocytes increases slightly when compared to images at 4 dpf (Figure 2). (B) At later stage is possible to visualize the increase of gata1 positive progenitors in treated fish (B') compared to controls (B). (C-D) Flow cytometry analyses of erythrocyte populations in double transgenic embryos *Tg(gata1:DsRed;globin:GFP)* over 5 consecutive days after the PHZ exposure, presented as percentage of all embryo cells. Mature erythrocytes (C) almost completely gone at 3 dpf, gradually increase in number over the following days. Erythroid progenitors (D) almost double in number over 3-5 dpf. (E) Immunohistochemistry analyses of Phospho-Histone H3 (white) in the CHT of double transgenic embryos *Tg(gata1:DsRed;globin:GFP)* at 3.5 dpf

(red-gata1, green-globin). Much more cells are labeled in the PHZ treated embryos (E') comparing to the control (E).

Figure S7. Generation of globin+ erythrocytes from gata1+ erythroid progenitors in the CHT during post-anemia recovery

(A) Stills from a time lapse video (Video 5) showing new erythrocyte formation after PHZ induced hemolytic anemia in a transgenic embryo *Tg(gata1:DsRed;globin:GFP).* A small gata1+/globin- cell in the CHT acquires globin:GFP expression and turns green. The cell divides and generates two bright green new eryhtrocytes. (B) Higher magnification of the images from picture (A).

Figure S8. Cell proliferation analyses in the CHT.

(A) Immunohistochemistry analyses of Phospho-Histone H3 (white) in the CHT of double transgenic embryos *Tg(gata1:DsRed;globin:GFP)* at 2.5 dpf (red-gata1, green-globin). Much more cells are labeled in the PHZ treated embryos (A') comparing to the control (A). (B) *In situ* hybridization analyses of stem cell markers (cmyb and runx) expression in zebrafish embryos at 2.5 dpf. Embryos treated with PHZ (B') show increase of cmyb/runx staining in comparison to control (B). (C) *In situ* hybridization analyses of stem cell marker cmyb (blue) and erythroid progenitor marker gata1 (orange) at 3.5 dpf. Full images used for zoomed-in pictures in Figure 2D.

Figure S9. Phenotypic analyses of erythroid cell populations in normal and anemic zebrafish embryos using ImageStream imaging flow cytometer

(A) The graph represents flow cytometry plot of all zebrafish cells (DRAQ5 positive) from transgenic embryos *Tg(gata1:DsRed;globin:GFP)* analyzed for red and green fluorescence. (B) The gata+/globin+ cells (green gate from A) were further separated in regards to "aspect ratio" and "cell area" parameters, based on bright field images. Several populations were marked to distinguish small round, small oval, and big round cells. (C) The graph represents contribution of cells of defined shapes to the gata+/globin+ population for control and PHZ treated embryos. The values are absolute cell numbers counted within 10 000 of all fish cells. The table shows the percentage of gata+/globin+ and gata+/globin- cells in embryos treated with PHZ and control (two first rows), and the percentage of different cell shapes within the gata+/globin+ population, corresponding to the graph. (D-G) Representative images of blood cells from the gates in A and B, acquired with the ImageStream, showing bright field, gata1:DsRed and globin:GFP signals. "Small oval" and "big round" cell populations are comparable in PHZ-treated and control embryos (D and E), and represent mature erythrocytes. Among "small round" and "small rounder" cells, in the PHZ treatment there are more cells resembling erythroid progenitors, whereas in the control, more cells resemble mature erythrocytes (F-G').

Supplementary Videos Legend

Video S1. Time lapse imaging of transgenic zebrafish embryo *Tg(gata1:DsRed;globin:GFP)* after hemolytic anemia induction

Control (upper panel) and PHZ (bottom panel) treated embryos were imaged in the tail region behind the yolk extension, between 2.5 and 3 dpf. In the control, multiple gata1+/globin+ erythrocytes are circulating within the blood vessels. A few gata1+/globin- erythroid progenitors are visible in the CHT, between dorsal aorta and posterior cardinal vein. In the PHZ treated embryo (bottom), the circulating erythrocytes are significantly reduced and the CHT is filled up with damaged erythrocytes.

Video S2. Phagocytosis of hemolytic erythrocytes in the CHT

Close up of a time-lapse video of a PHZ treated embryo, showing phagocytosis of damaged erythrocytes in the CHT. Large, red phagocytosing cells are marked with white arrows.

Video S3. Close up of erythrocyte phagocytosis from Video 2

A single cell in the middle of the screen (white arrow) is phagocytosing multiple erythrocytes.

Video S4. Time lapse imaging of transgenic zebrafish embryo *Tg(gata1:DsRed;globin:GFP)* during recovery from hemolytic anemia

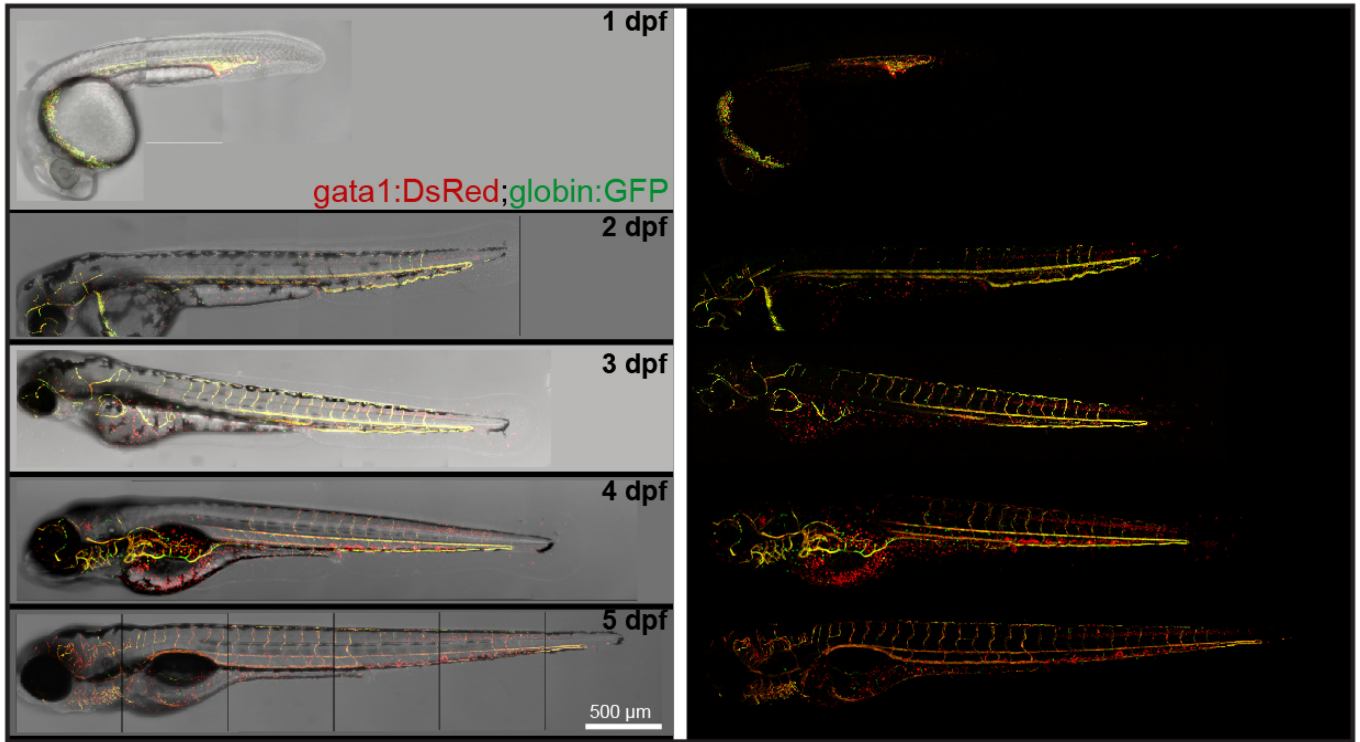
Control (upper panel) and PHZ (bottom panel) treated embryos were imaged in the tail region behind the yolk extension, between 3.5 and 4 dpf. In the control, multiple gata1+/globin+ erythrocytes are circulating within the blood

vessels (yellow stream). A few gata1+/globin- erythroid progenitors are visible in the CHT. In the PHZ treated embryo, singe cells are circulating and the CHT is full of gata1+/globin- erythroid progenitors, many of which are dividing. Several of them are turning green, meaning the globin promoter is activated, indicating generation of new erythrocytes.

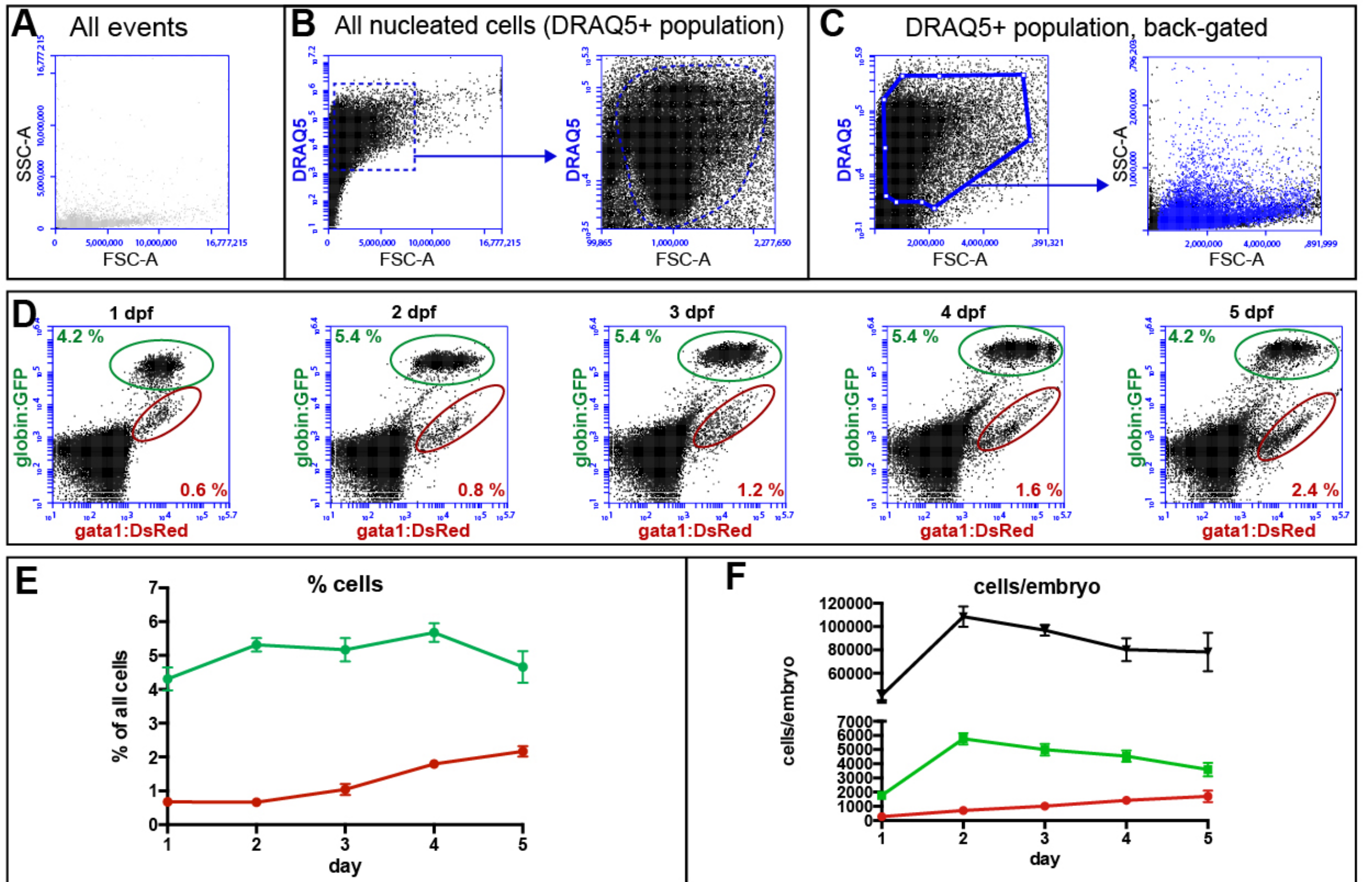
Video S5. Generation of globin+ erythrocytes from gata1+ erythroid progenitors in the CHT during post-anemia recovery

A close up of an erythroid progenitor from Video 4. The cell gradually acquires globin:GFP expression, divides and generates two new erythrocytes.

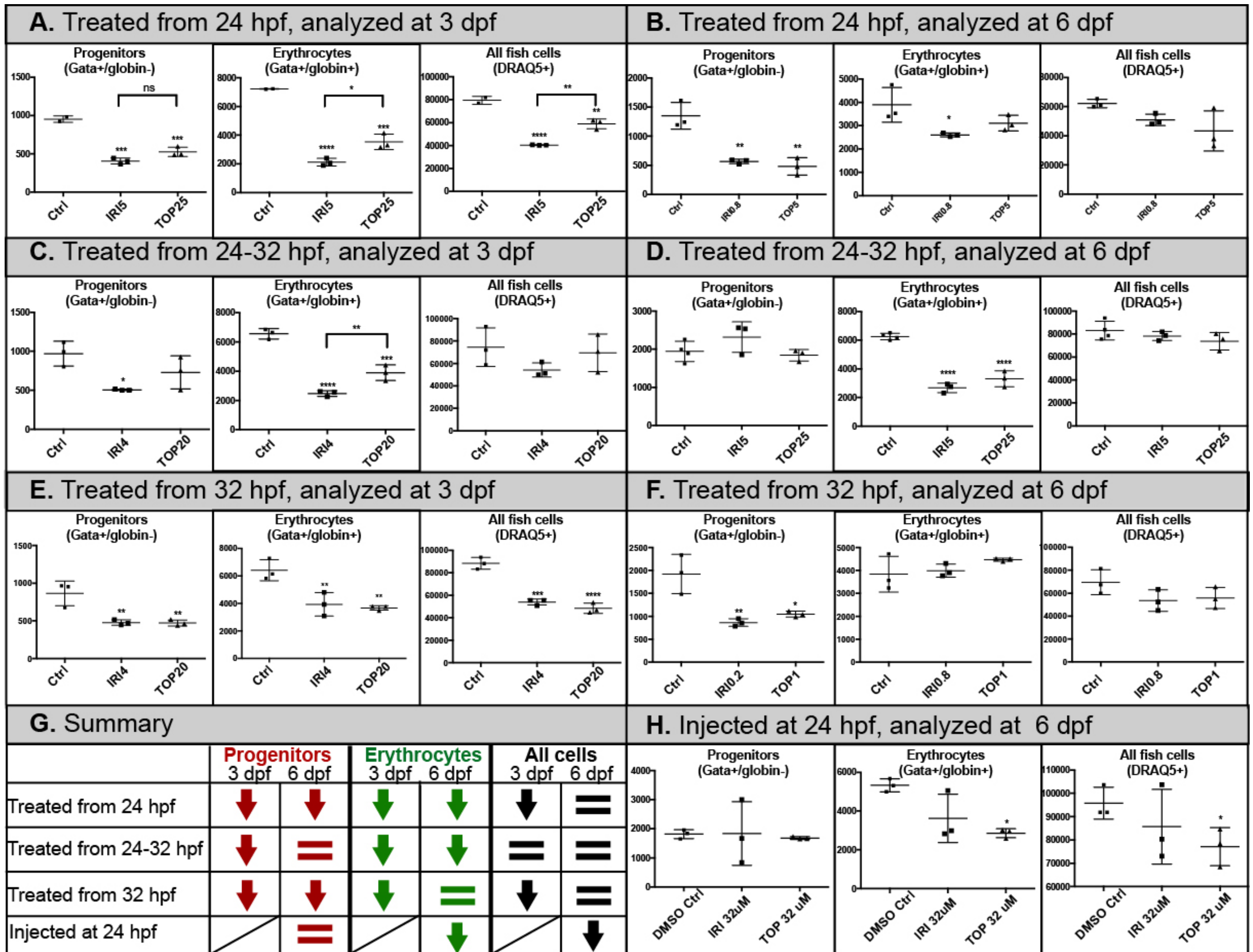
Supplementary figure 1



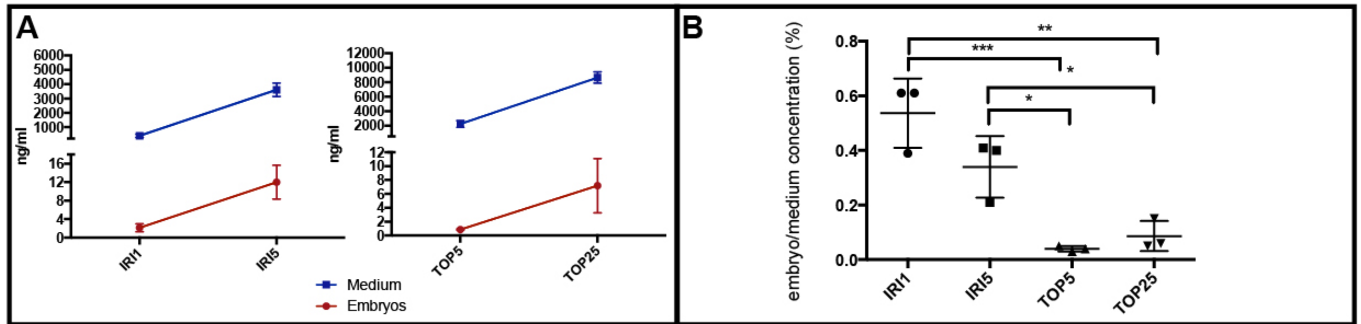
Supplementary figure 2



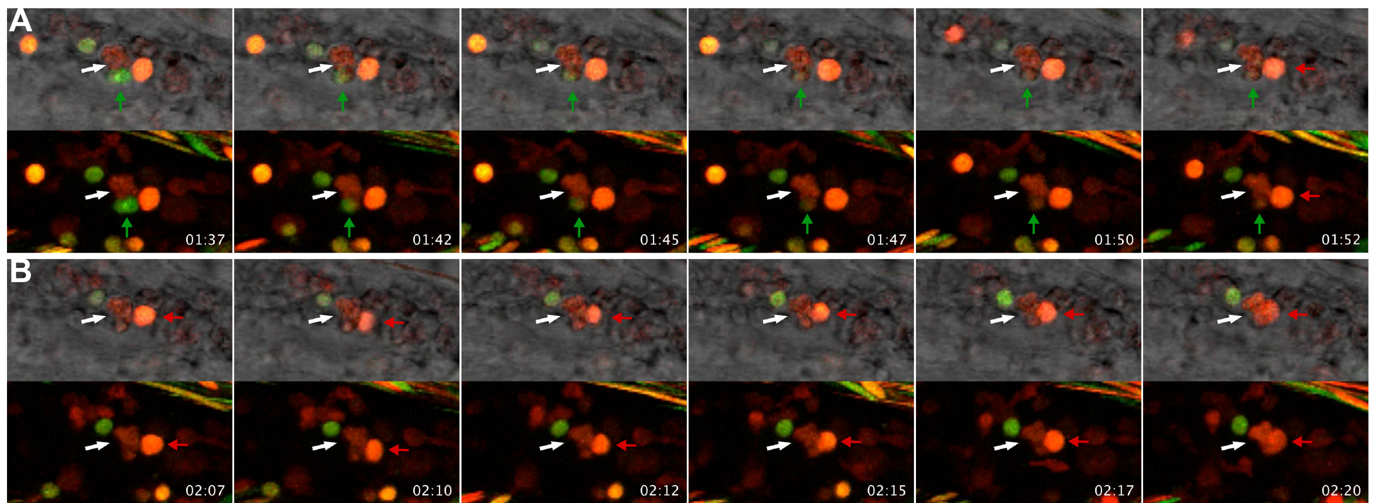
Supplementary figure 3



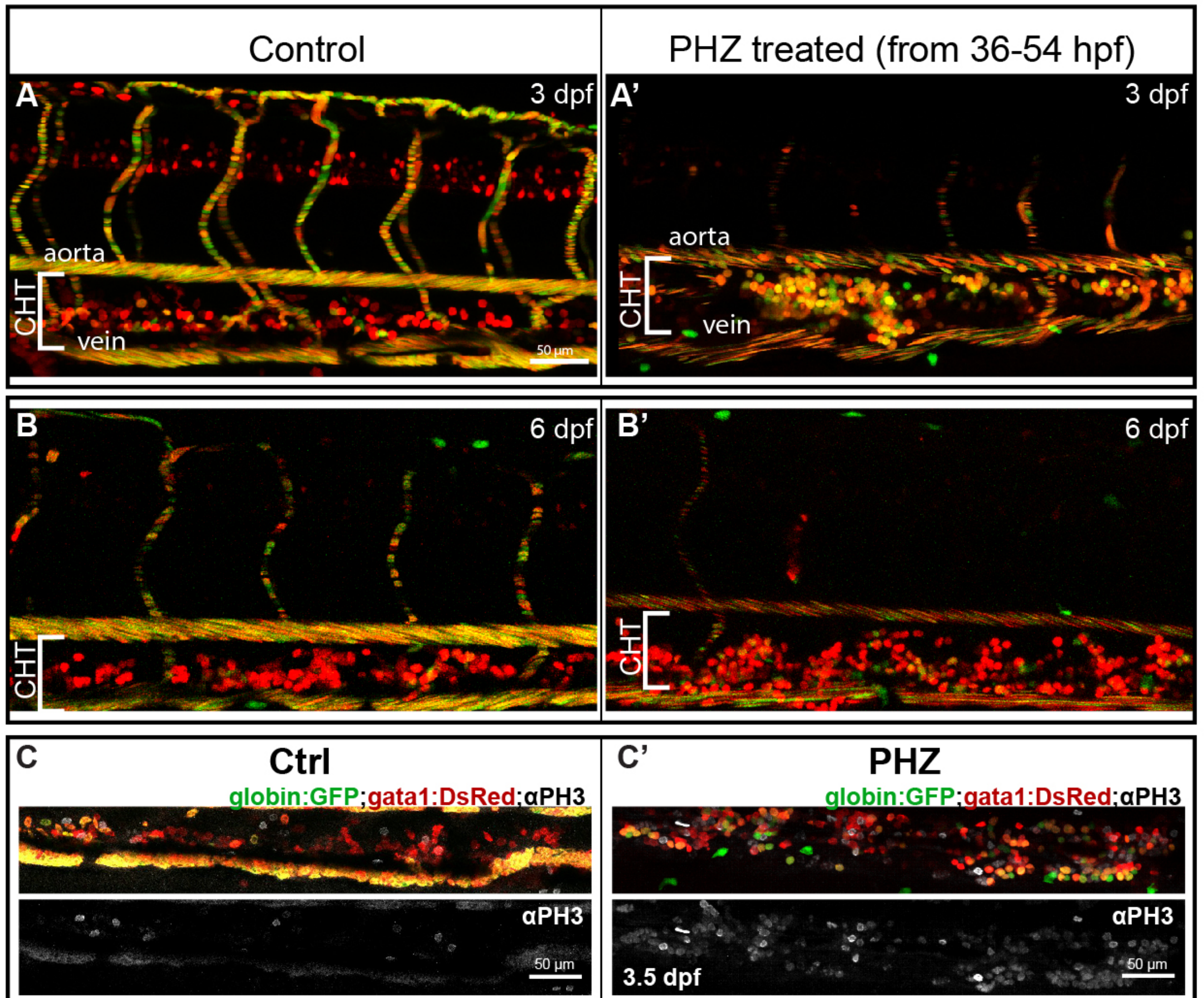
Supplementary figure 4



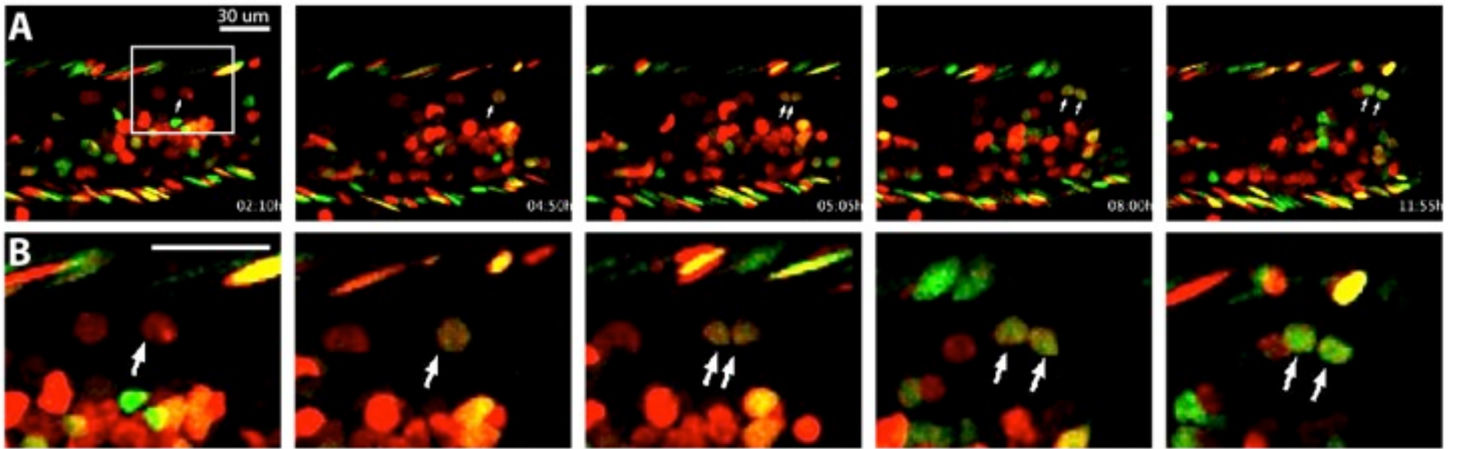
Supplementary figure 5



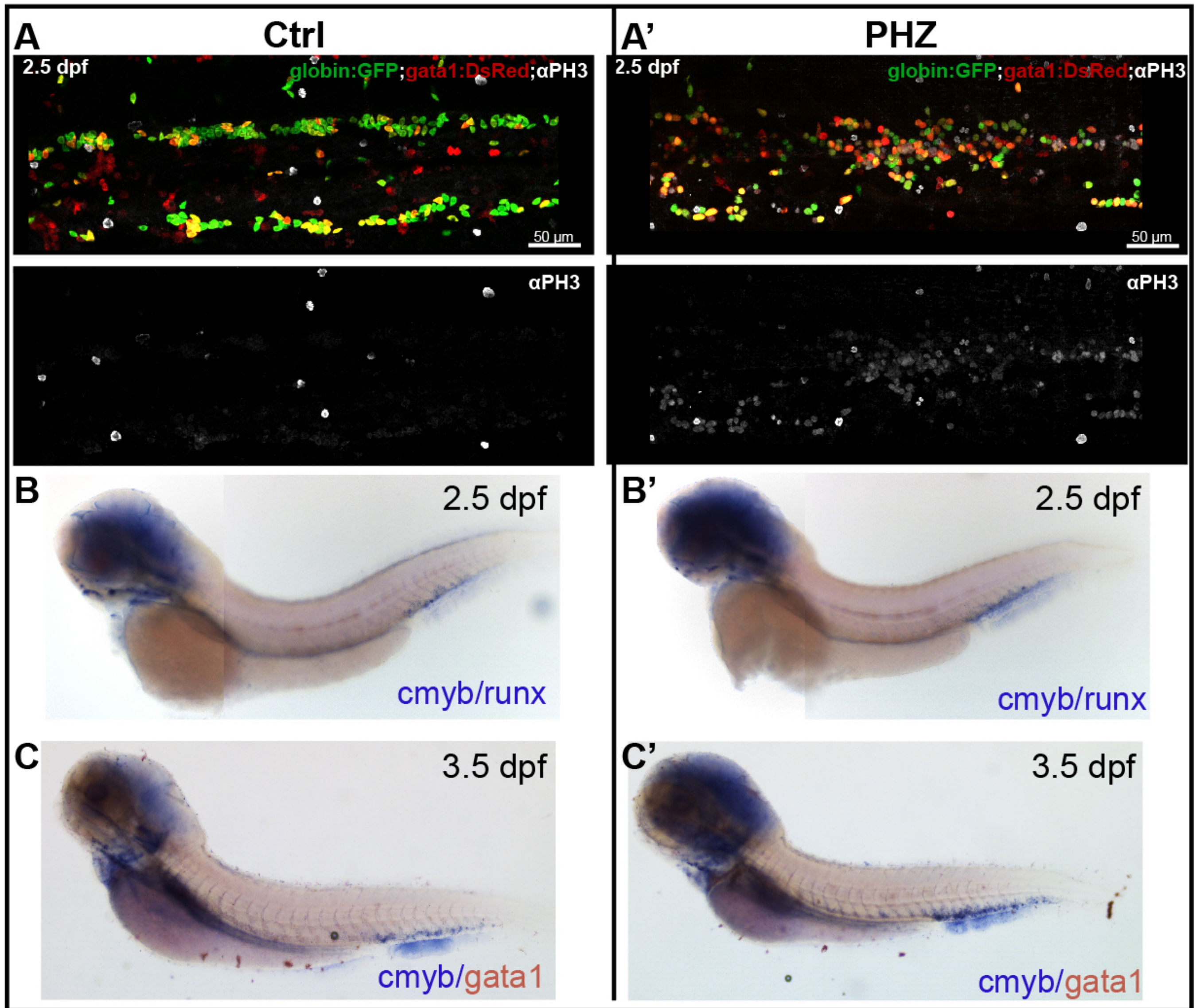
Supplementary figure 6



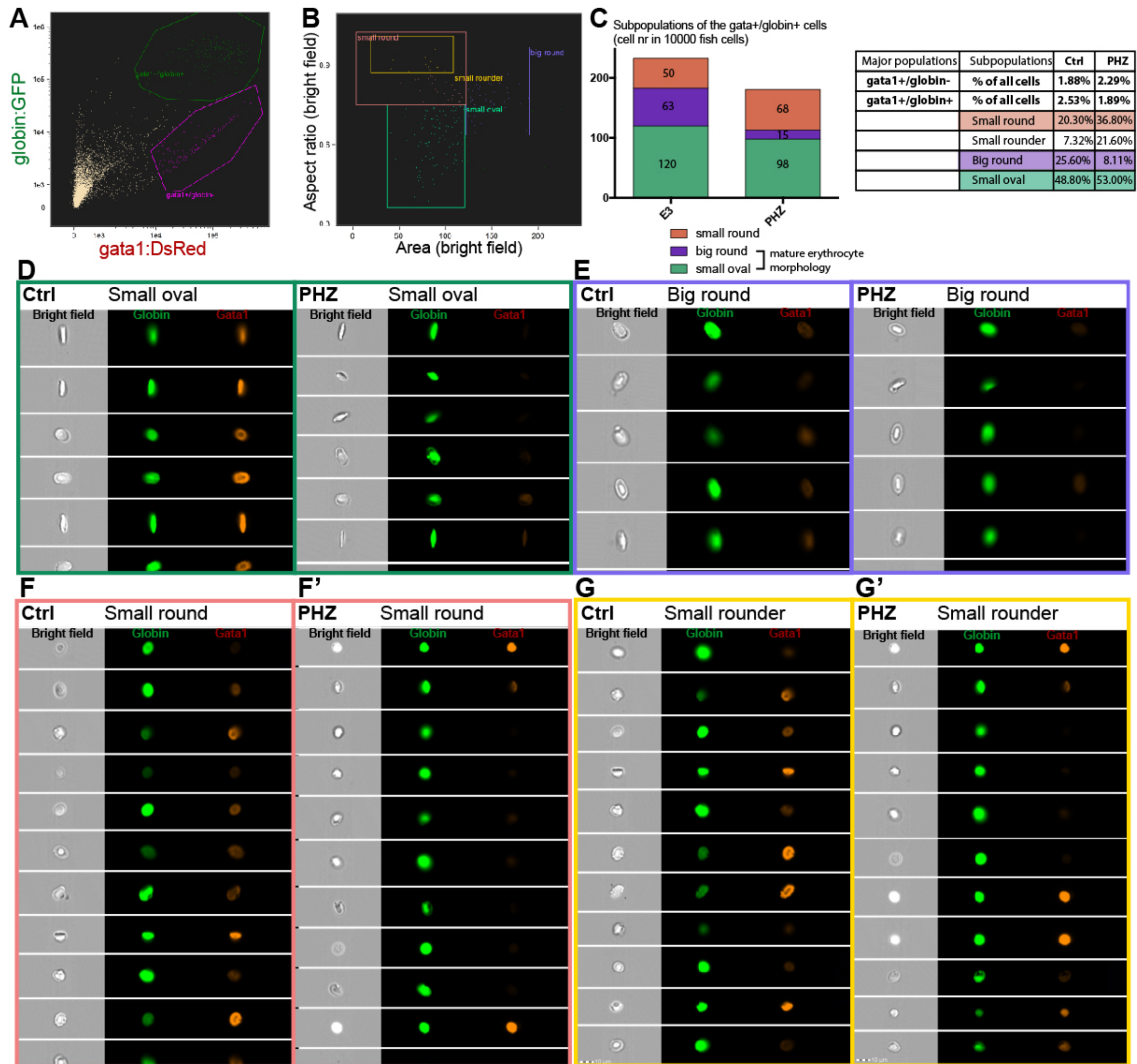
Supplementary figure 7



Supplementary figure 8



Supplementary figure 9



Major populations	Subpopulations	Ctrl	PHZ
gata1+/globin-	% of all cells	1.88%	2.29%
gata1+/globin+	% of all cells	2.53%	1.89%
	Small round	20.30%	36.80%
	Small rounder	7.32%	21.60%
	Big round	25.60%	8.11%
	Small oval	48.80%	53.00%

Supplementary references

1. Westerfield M. The zebrafish book. A guide for the laboratory use of zebrafish Danio rerio. 4 ed: Univ. of Oregon Press, Eugene, 2000.
2. Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF. Stages of embryonic development of the zebrafish. Developmental dynamics : an official publication of the American Association of Anatomists. 1995;203(3):253-310.