The role of the tumor suppressor Lkb1 in energy homeostatis

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Chapter 6

The tumor suppressor Lkb1 affects hematopoiesis during zebrafish development

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Abstract

Hematopoiesis is the differentiation of hematopoietic stem cells (HSCs) into functional blood cells. Research into the development and maintenance of HSCs is important in the understanding of cancer, ageing and blood-related disorders. HSC functioning and self-renewal is influenced by various factors, including systemic metabolism. An important gene that regulates metabolism and cell growth is *LKB1*. Recently it has been shown that HSC-specific knock-out of Lkb1 in mice results in severe pancytopenia as a result of loss of HSC quiescence. In this study, we show that deficiency in Lkb1 affects erythrocyte and neutrophil development in zebrafish larvae and that deregulation of whole-body metabolism impacts the maintenance of HSCs and their proper differentiation into downstream lineages. Our data establish *lkb1* zebrafish as a model to study the effects of Lkb1 and deregulated metabolism on hematopoiesis, and propose the use of this model as a screening platform to identify strategies that could ameliorate Lkb1- or metabolism-associated hematopoietic defects.

Introduction

Hematopoiesis is the generation of hematopoietic stem cells and differentiation of these cells in specific blood cell lineages with distinct functions. Hematopoietic stem cells have the potency to differentiate or to self-renew (Seita and Weissman, 2010). The balance between differentiation and self-renewal of HSCs is crucial for health of blood cells; differentiation into downstream lineages ensures replacement of cells, whilst a quiescent state prevents depletion of the HSC pool, securing a lifelong reservoir of cells (Zon, 2008). In addition to continuous replacement of aging blood cells, HSCs proliferate rapidly in response to external signals during disease and injury (Passegue et al., 2005). Disruption of the balance between self-renewal and differentiation can lead to hematological-related disorders. For instance, loss of quiescence can lead to pancytopenia (a shortage of all blood cells types) (Beerman et al., 2010); and aberrant differentiation of HSCs is a hallmark of leukemia (Zhao et al., 2010). These examples highlight the importance of a better understanding of the biology of HSCs and their relation to diseases.

Zebrafish are an extensively used research model in hematopoiesis due to their optical transparency, which facilitates the study of HSCs and other blood cell lineages *in vivo*. In zebrafish, all mature blood types, except platelets, are shared with mammals and most genes are conserved (Traver et al., 2003). Similar to other vertebrates, hematopoiesis is divided into two stages; the primitive- and definitive wave (de Jong and Zon, 2005). During the primitive wave, erythrocytes are created from the intermediate cell mass (ICM) and primitive macrophages from the anterior lateral mesoderm. The primitive hematopoiesis shifts to the posterior blood island after 24 hours post fertilization (hpf), where progenitors create multiple other lineages (Bertrand et al., 2008; de Jong and Zon, 2005). During the definitive wave, HSCs give rise to all blood cells needed (Figure 1A). The definitive wave starts at 30 hpf in the Aorta-Gonad-Mesonephros (AGM) region (Figure 1B). From this region, cells seed the caudal hematopoietic tissue (CHT), where early definitive hematopoiesis takes place. Finally, from the CHT, stem cells migrate to the kidney and thymus, the final locations of definitive hematopoiesis (Chen and Zon, 2009; de Jong and Zon, 2005). The innate immune system is already functional at one day post-fertilization (dpf) and consists of neutrophils and macrophages (Renshaw and Trede, 2012). Mature T-cells and B-cells (Seita and Weissman, 2010) (Page et al., 2013) of the adaptive immune system emerge after four to six weeks. Overall, conserved hematopoiesis combined with the many scientific advantages and sophisticated techniques such as *in vivo* lineage tracing and live imaging, enabled several insights into the detailed pathways of hematopoiesis and HSC biology (Gore et al., 2018).

Stem cell maintenance and function are greatly influenced by metabolic pathways. Many different stem cell types are regulated by bioenergetics signalling and dependent on anaerobic glycolysis (Ito and Suda, 2014). Metabolic state at cellular and organismal level has been shown to influence fate decisions in stem cells (Mans and Haramis, 2014; Rafalski and Brunet, 2011). A better understanding of the interactions between metabolism and HSCs and of how disruption of this interaction can lead to diseases such as cancer, ageing and blood disorders, is required to provide new insights and treatment strategies (Ito and Suda, 2014).

Previous studies have highlighted the role of LKB1 in hematopoietic stem cell maintenance (Gurumurthy et al., 2010; Krock et al., 2011; Nakada et al., 2010a). LKB1 is a serine threonine kinase that regulates many cellular processes, such as cell growth and metabolic pathways (Shackelford and Shaw, 2009). HSC-specific *Lkb1* mutation in mice resulted in a decrease in bone marrow-derived myeloid and red and white blood cells, as well as an increase in apoptosis in HSCs and progenitor cells. The rapid loss of HSCs and progenitor cells was preceded by an increase in proliferation and number of HSCs, which suggests that LKB1 has a cell autonomous role in HSC quiescence (Gurumurthy et al., 2010; Nakada et al., 2010a). Besides the regulation of cellular processes, LKB1 also regulates energy homeostasis on the organismal level. It has been shown that organismal energy state during embryogenesis influences HSC development and function as well (Suda et al., 2011). Experimentally manipulating the metabolic state in zebrafish is relatively straightforward and was shown to affect hematopoiesis, since treatment with glucose led to an increase in the number of HSCs, (Harris et al., 2013) and knock-down of the metabolism-related gene *fgf21* resulted in defective hematopoiesis (Yamauchi et al., 2006).

The *lkb1* mutant zebrafish model is characterized by deregulated metabolism upon yolk depletion (Mans et al., 2017; van der Velden et al., 2011). In this study, we used this model to study the effect(s) of whole-body LKB1 depletion on the physiology of HSCs and downstream lineages, and assess the impact of deregulated systemic metabolism on hematopoiesis. We examined HSC presence and differentiation into downstream lineages in wild type, heterozygous and *lkb1* mutant larvae. We found no dramatic differences in HSC (*cmyb*$^{+}$) cells in *lkb1* larvae compared to wild type or heterozygous larvae during embryonic development. However, *lkb1* larvae showed depletion of neutrophils after 5 dpf and dramatically low levels of *gata1*$^{+}$ erythrocytes throughout development. Therefore, our results provide new evidence that whole-body metabolism has an important role in HSC maintenance and support a cell-specific role for Lkb1 in the differentiation of HSCs into erythrocytes.

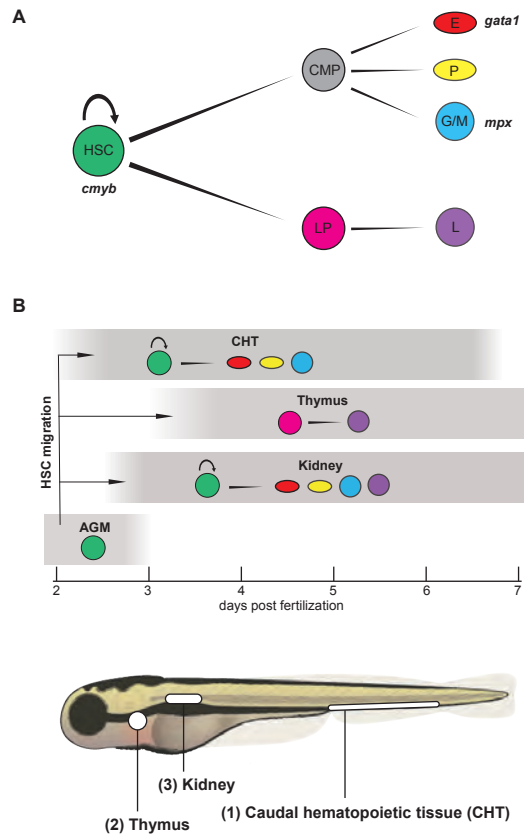

Figure 1. Definitive hematopoiesis in zebrafish larvae. (A) Hematopoietic stem cells (HSCs), characterized by expression of *cmyb*, are progenitor cells that have the potential to self-renew and to give rise all downstream lineages via differentiation. (CMP, common myeloid progenitor; E, erythrocyte expressing *gata1*; P, platelet; G/M, granulocyte/monocyte with neutrophils cells expressing *mpx*; LP, lymphoid progenitor; L, lymphocyte). **(B)** Location and timing of progenitor cell migration during larval development (adapted from (de Jong and Zon, 2005)). Definitive hematopoiesis starts in the aorta-gonad mesonephros (AGM). After 2 dpf, progenitor cells migrate from the AGM to colonize three regions: (1) the CHT, which is the intermediate location of blood development, (2) the thymus, which is a site of lymphocyte maturation, and (3) the kidney, which is the larval and adult location for production of all blood cell types.

Results

Decreased levels of *cmyb*⁺ cells in CHT, thymus and kidney at 7 dpf in a subset of *lkb1* larvae.

To investigate the effect of deregulated metabolism or Lkb1 depletion on the HSC population, we assessed expression of the HSC-specific marker *cmyb* by whole-mount *in situ* hybridization (WISH) (de Jong and Zon, 2005). Expression of *cmyb* marks the onset of definitive hematopoiesis in HSCs from 2 dpf onwards (Bertrand et al., 2008; de Jong and Zon, 2005) (Figure 1). We examined wild-type (wt), heterozygous (het) and *lkb1* larvae from 2 until 7 dpf, comparing the intensity of *cmyb* staining in the three most important hematopoietic regions, the caudal hematopoietic tissue (CHT), the kidney and the thymus.

cmyb⁺ cells were present in the CHT from 2 dpf onwards in all genotypes (Figure 2A) to varying extent. Seeding of the thymus occurs at around 3 dpf (Figure 2B) and seeding of the kidney at around 4 to 5 dpf (Figure 2C). We did not observe dramatic differences in *cmyb* staining between the different genotypes. In the thymus however, we observed a subset of *lkb1* larvae that lack staining over the whole course of development. At 7 dpf we found that approximately 20% of *lkb1* larvae showed reduced staining in the kidney and thymus region compared to wt and heterozygous larvae. Moreover, this lack of *cmyb* staining in the kidney or thymus was always accompanied by absence of *cmyb* staining in the CHT. These results indicate that there is variation in the effects of whole body depletion of Lkb1 depletion on *cmyb*⁺ cells, with a subset of *lkb1* larvae presenting with depletion of *cmyb*⁺ cells throughout the organism after 7 dpf.

Failure to maintain the neutrophil population in *lkb1* larvae at 7 dpf

We next examined whether the reduction in the number of *cmyb*⁺ cells in the thymus affected the development of other hematopoietic subpopulations in the *lkb1* larvae. To assess neutrophil differentiation, we used the TSA staining for myeloperoxidase (Mpx) activity (Benard et al., 2015; Mathias et al., 2006). The neutrophils develop from the HSCs as early as 2 dpf together with macrophages. These cell types are part of the innate immune system, important in the defence against pathogens until the adaptive immune system matures at four to six weeks post-fertilization (Renshaw and Trede, 2012). We found that large numbers of Mpx-positive neutrophils were present in the tail region of wt and heterozygous larvae at 5 dpf, which we used as a reference point. We did not observe a difference in the number of neutrophils between wt, het and *lkb1* mutant larvae at this time-point. This finding indicates that the HSCs present in the larvae are properly dividing into

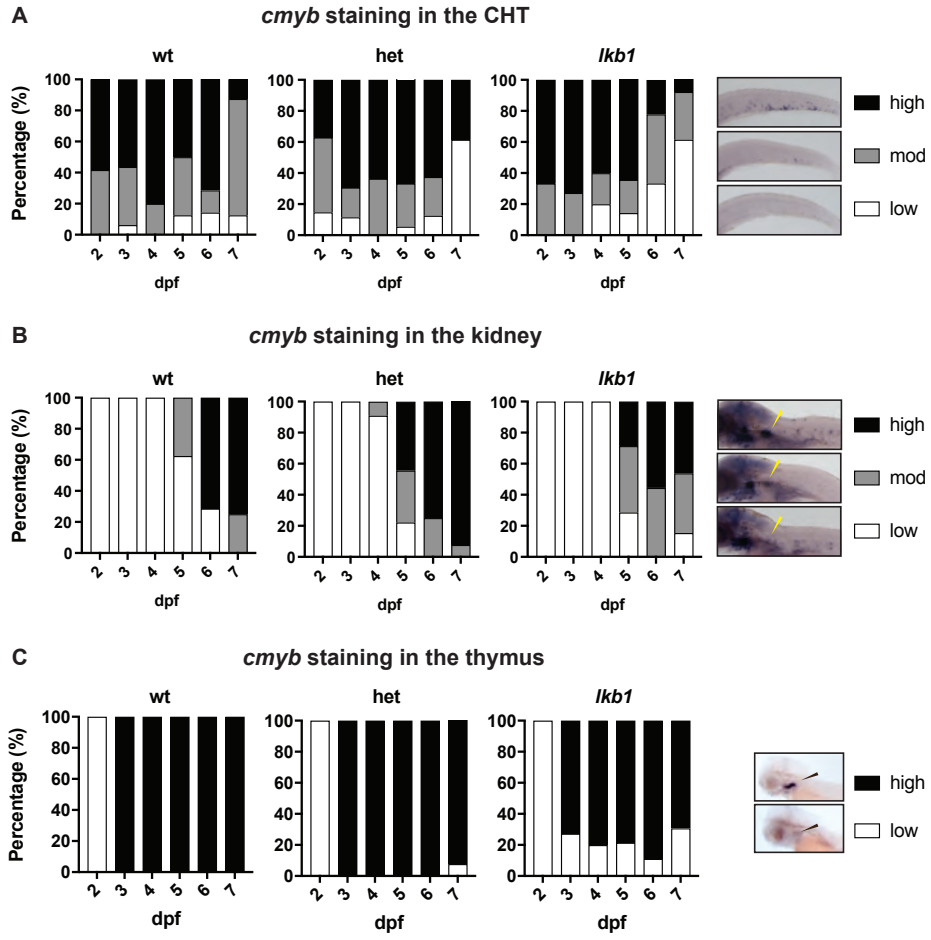

Figure 2. Reduced number of *cmyb+* cells in a subset of *lkb1* larvae. Whole-mount *in situ* hybridization for cmyb expression to visualize HSCs, in wt, heterozygous and *lkb1* larvae between 2-7 dpf in three hematopoietic regions. **(A)** Analysis of *cmyb+* cells in the CHT revealed varying numbers in all genotypes throughout development. **(B)** In the kidney (yellow arrow), *cmyb+* cells were observed from 4 dpf onwards in all genotypes. At 7 dpf we observed a subpopulation of *lkb1* larvae without *cmyb+* cells. **(C)** In the thymus (black arrow), seeding of *cmyb+* cells was observed from 2 dpf in all genotypes. In *lkb1* larvae a subpopulation showed no staining in the thymus throughout development. Staining was scored as high, medium, and low with representative images indicating the region of interest. The graph presents the percentage of the total population of larvae (8 > n < 19, from three independent experiments).

downstream lineages. We next compared the population of neutrophils between wt, heterozygous and *lkb1* larvae at 7 dpf, the developmental time point when the starvation phenotype of *lkb1* larvae becomes apparent. Surprisingly, we observed near complete absence of Mpx activity in the CHT of the mutant larvae compared to the wt (Figure 3). These results suggest that while at 5 dpf, neutrophil populations are similar between wt and *lkb1* larvae, the population is not maintained in *lkb1* larvae after 7 dpf.

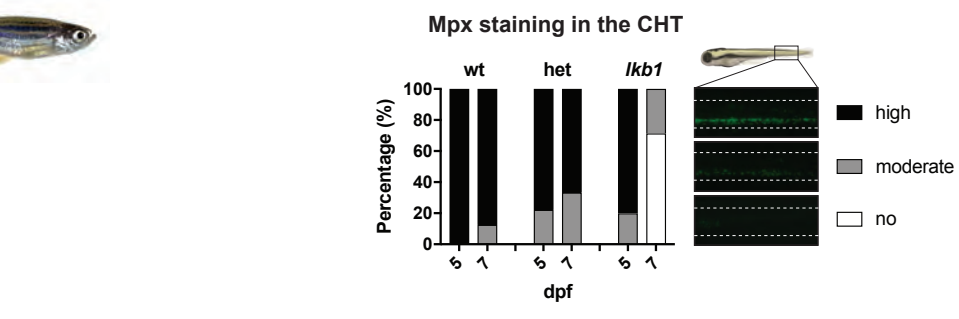

Figure 3. Low levels of Mpx in the CHT of *lkb1 larvae at 7 dpf.* Enzymatic staining for Mpx to visualize neutrophil cells, in wt, heterozygous and *lkb1* larvae at 5 and 7 dpf in the CHT. Staining was scored as high, medium, and low with representative images indicating the region of interest. At 5 dpf all genotypes showed high or medium Mpx staining in the CHT. At 7 dpf, wt and heterozygous larvae show similar staining as at 5 dpf, but *lkb1* larvae showed dramatically reduced levels of Mpx. The graph presents the percentage of the total population of larvae ($4 > n < 9$, from two independent experiments).

Lkb1 depletion prevents development of (*gata1*$^+$) erythrocytes

To investigate the effect of Lkb1 depletion on the differentiation of erythrocytes, we compared wt, het and *lkb1 l*arvae from 2 until 7 dpf. We stained for *gata1*, a transcription factor that is a well-established, widely used marker for erythrocytes (de Jong and Zon, 2005). In wt and het larvae, *gata1*$^+$ cells were found in the CHT region from 2 dpf onwards, and in the kidney from 4 dpf. Both genotypes showed very robust staining in the CHT and kidney once the regions were seeded. In contrast, *lkb1* larvae showed no staining in these tissues, at any time-point during development (Figure 4). This implies that Lkb1 depletion affects (terminal) differentiation of *gata1*$^+$ erythrocytes.

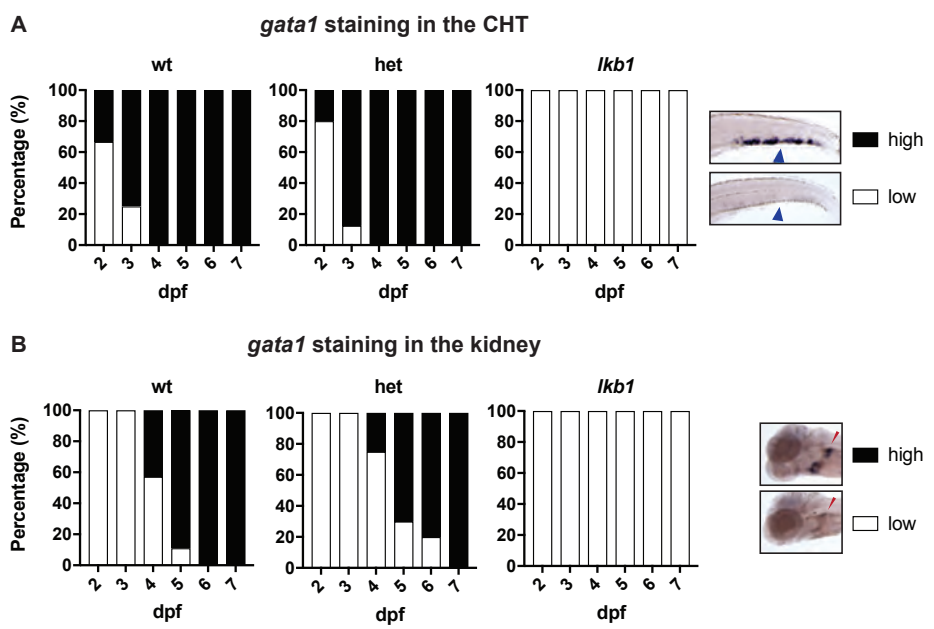

Figure 4. *gata1* expression is absent in the kidney and CHT of *lkb1* larvae throughout development. Whole-mount in situ hybridization for *gata1* expression to visualize erythrocytes, in wt, heterozygous and *lkb1* larvae between 2-7 dpf in two hematopoietic regions. **(A)** Analysis of *gata1*$^+$ cells in the CHT (blue arrow) revealed presence of this population in the CHT from 2 dpf onwards in wt and heterozygous larvae. In *lkb1* larvae, *gata1* was undetectable at any day of development. **(B)** In the kidney (red arrow), *gata1*$^+$ cells were observed from 4 dpf onwards in wt and heterozygous larvae. In *lkb1* larvae, *gata1* was undetectable at any day of development. Staining was scored at high, medium and low with representative images indicating the region of interest. The graph presents the percentage of the total population of larvae ($7 > n < 15$, from three independent experiments).

Discussion

In this study, we used the *lkb1* mutant zebrafish as an *in vivo* model to show that depletion of Lkb1 affects hematopoiesis. We found that *lkb1* mutation resulted in a decreased number of HSCs (*cmyb*$^{+}$ cells) in the thymus of a subset of *lkb1* larvae. The most dramatic loss of *cmyb*$^{+}$ cells was visible in a subset of *lkb1* larvae at 7 dpf at which time-point we observed low *cmyb* staining in all the hematopoietic regions (CHT, kidney and thymus). The variations in phenotype between *lkb1* larvae, and especially the almost normal number of *cmyb*$^{+}$ cells in some *lkb1* larvae were surprising, since HSC-specific depletion of LKB1 in mice was shown to be detrimental for HSC maintenance (Gan et al., 2010; Gurumurthy et al., 2010; Nakada et al., 2010a). Our results suggest that systemic inactivation of Lkb1 affects HSC maintenance. However, the metabolic state of the larvae also plays an important role, since loss of HSCs in all the hematopoietic regions was observed only at 7 dpf. The differences in *cmyb* staining between *lkb1* larvae at 7 dpf could be attributed to the varying onset of the starvation phenotype.

To investigate the effect of Lkb1 on the differentiation of HSCs into downstream lineages, we used markers for neutrophil cells (Mpx) and erythrocytes (*gata1*). Mpx staining revealed comparable numbers of neutrophil cells in wt and *lkb1* larvae at 5 dpf, but dramatically low numbers in *lkb1* larvae at 7 dpf. This could suggest an essential role for Lkb1 in myelopoiesis during definitive hematopoiesis; the neutrophils we observed at 5 dpf may be remnants from primitive hematopoiesis (Le Guyader et al., 2008), which have been shown to be present in the circulation as late as 6 dpf (Chen and Zon, 2009) and express *mpx* (Lieschke et al., 2001). Another possible explanation is that the energy status of *lkb1* larvae is the cause for the depletion of neutrophils at 7 dpf. Decreased numbers of T-cells in response to starvation have been described in mice (Wing et al., 1988), and acute starvation was shown to reduce numbers and proliferation capacity of lymphocytes in cats (Freitag et al., 2000).

Furthermore, we observed an unambiguous depletion of *gata1*$^{+}$ erythrocytes in *lkb1* larvae throughout development; we did not detect any erythrocytes in *lkb1* larvae at any time-point. These results could indicate a cell autonomous role of Lkb1 during differentiation of *gata1*$^{+}$ erythrocytes.

Overall, our data confirm that systemic Lkb1 loss of function affects hematopoiesis. We propose that the interaction between Lkb1 depletion and the metabolic state of the larvae following yolk depletion affects the abundance of HSCs. and their differentiation to downstream lineages. Furthermore, we show that Lkb1 depletion results in lack of seeding of HSCs to the thymus in a subset of *lkb1* larvae, and disruption of erythrocytes differentiation in all *lkb1* larvae throughout development. Thus, the zebrafish *lkb1* mutant is a valuable model to study the interaction between systemic metabolic state and genetic inactivation of Lkb1 and represents a promising platform for screening compounds that positively affect hematopoiesis.

Materials & Methods

Zebrafish strains and Screening Methods

Zebrafish were handled in compliance with the local animal welfare regulations and were maintained according to standard protocols (zfin.org). Their culture was approved by the local animal welfare committee (DEC) of the University of Leiden and all protocols adhered to the international guidelines specified by the EU Animal Protection Directive 2010/63/EU. Genotype analysis for *lkb1*-mutant embryos was performed with SNP-specific PCR analysis. From 24 hpf larvae were grown in eggwater containing 0.003% PTU to prevent pigmentation (Karlsson et al., 2001).

Whole-mount *in situ* hybridization

The plasmids encoding *gata1* and *cmyb* were a gift from the Schulte-Merker lab at the Hubrecht Institute. Generation of RNA probes for whole-mount *in situ* hybridization (WISH) was done by transcribing the plasmids containing specific cDNA with an SP6 or T7 polymerase. After completion, the probes were stored at -20°C.

In order to detect specific gene activity, standardized WISH was preformed (Thisse and Thisse, 2008). Larvae were fixed at 5 and 7 dpf in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) overnight before being dehydrated and stored in 100% methanol at -20°C until use. After rehydration, larvae were permeabilized for 90 minutes in 10µg/ml Proteinase K and post-fixed in 4 % PFA for 20 minutes. Next, they were pre-hybridized for 2 hours after which the larvae were hybridized overnight at 68°C in 400-500 ng/500 µg of gene-specific probe. Anti-DIG antibody (1:5000) was added after the probe was removed and incubated overnight at 4°C. Finally, the larvae were washed several times before being stained with BM purple at room temperature (RT).

Enzymatic staining for neutrophils

A Green fluorescent enzymatic staining was performed to label neutrophils in 5 and 7 dpf larvae. The larvae were fixed overnight in 4% PFA and washed with PBSTx (PBS with 0.05% Triton × 100; Sigma-Aldrich). The neutrophil-specific Mpx

was labeled according to previous determined protocols (Benard et al., 2015) with the TSA Plus Fluorescein System Kit (Perkin-Elmer).

Imaging

Stained larvae were visualized by the use of a Leica MZ16FA stereo fluorescence microscope. Pictures were taken with the Leica DC420C camera mounted on the microscope. This setup was used for both the fluorescence and non-fluorescence images. Leica Application Suite version 3.8.0 [build: 878] was used to operate the microscope and provided both separate and overlayed pictures for the different fluorescence channels.

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