Colony stimulating factor 1 signaling regulates myeloid fates in zebrafish via distinct action of its receptors and ligands

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- *csf1ra* and *csf1rb* are indispensable for macrophage differentiation and together with *csf1a* regulate embryonic macrophage fates in zebrafish
- *csf1rb* is important for granulocyte differentiation and migration and together with *il34* it regulates embryonic granulocytic fates in zebrafish

Abstract

Macrophage colony-stimulating factor receptor (M-CSFR/CSF1R) signaling is crucial for the differentiation, proliferation, and survival of myeloid cells. Therapeutic targeting of the CSF1R pathway is a promising strategy in many human diseases, including neurological disorders or cancer. Zebrafish are commonly used for human disease modeling and preclinical therapeutic screening. Therefore, it is necessary to understand the proper function of cytokine signaling in zebrafish to reliably model human-related diseases. Here, we investigate the roles of zebrafish Csf1rs and their ligands - Csf1a, Csf1b and Il34, in embryonic and adult myelopoiesis. The proliferative effect of exogenous Csf1a on embryonic macrophages is connected to both receptors as it is diminished in both *csf1ra*$^{\Delta 5bp}$ and *csf1rb*Δ^{4bp} mutants. There is no evident effect of Csf1b in zebrafish embryonic myelopoiesis. Further, we uncover an unknown role of Csf1rb in zebrafish granulopoiesis. Deregulation of Csf1rb signaling leads to failure in myeloid differentiation resulting in neutropenia throughout the whole lifespan. Surprisingly, Il34 signaling through Csf1rb seems to be of high importance as both *csf1rb*$^{\Delta 4bp}$ and *il34*$^{\Delta 5bp}$ deficient zebrafish larvae lack granulocytes. Our single-cell RNA sequencing analysis of adult whole kidney marrow (WKM) hematopoietic cells suggests that *csf1rb* is expressed mainly by blood and myeloid progenitors and that the expression of *csf1ra* and *csf1rb* is non-overlapping. We point out differentially expressed genes important in hematopoietic cell differentiation and immune response in selected WKM populations. Our findings could improve the understanding of myeloid cell function and lead to the further study of CSF1R pathway deregulation in disease, mostly in cancerogenesis.

Introduction

Hematopoiesis is a process of proliferation, differentiation, fate-commitment, and self-renewal of blood cells. It is primarily regulated by extrinsic signals, such as cytokines and growth factors that bind to cell receptors and activate internal signaling pathways.[1,2] One of the most prominent receptors that control the myeloid compartment is colony-stimulating factor 1 receptor (CSF1R, also known as macrophage-CSFR). In mammals, it is activated upon binding of two distinct cytokine ligands that have no obvious sequence homology – colony stimulating factor 1 (CSF1, M-CSF) and interleukin 34 (IL-34).[3-6] However, despite the fact that both of these cytokines bind to the same receptor and can equally support cell growth and survival, they achieve this by triggering different chemokine responses.[4,7] The CSF1R signaling pathway is in general critical for the proliferation, differentiation, survival, and activation of mononuclear phagocytic cells (MPCs) such as monocytes, macrophages, osteoclasts, or microglia in mammals,[8-10] birds,[11,12] and fish.[13] Deregulation of the CSF1R pathway was connected to disease phenotypes (reviewed in Hume et al.[14]) such as osteopetrosis,[9,10,15,16] brain disease,[17-20] or cancer.[21-24] Thus the CSF1R signaling is of high interest as a pathway for therapeutic targeting in neurological and infectious diseases and in tumorigenesis.[25,26] Particularly, myeloid cells, including both neutrophils and macrophages, can act negatively in carcinogenesis. Therefore, tumor associated macrophages (TAMs) are believed to be critical in tumor metastasis and are a good target in addition to conventional chemotherapy.[27-29] It has been shown in mouse that the number of TAMs can be efficiently reduced by the inhibition of CSF1R. Because of its low-throughput when testing compounds, other model organisms need to be utilized.[30] Zebrafish is a convenient model organism for human disease modeling[31-33] and the small size of zebrafish makes it advantageous for high-throughput pre-clinical drug screening.[32,34-38] Due to the genome re-duplication in teleost fish, many paralogs were generated that could possess redundant or novel biological functions.[39-41] This includes both, Csf1 and Csf1r, and therefore it is still needed to define the role and specificity of Csf1a, Csf1b and Il34 towards Csf1rs (Csf1ra/b) in zebrafish myelopoiesis. So far, it seems that the function of Csf1ra and Csf1rb is only partially redundant.[15] For instance, there are spatiotemporal differences in the importance of Csf1rs for microglia and HSC-derived myeloid cells development and seeding of the zebrafish brain.[42-44]

In this article, we focus on the roles of Csf1a, Csf1b, and Il34 cytokines in zebrafish embryonic myelopoiesis and adult hematopoiesis, shown by *ex vivo* tools and single cell RNA sequencing (scRNA-seq) of whole kidney marrows (WKM). We use a collection of zebrafish loss-of-function mutants to discern the effects of Csf1-receptor and -ligand functional defects. We show that Csf1a drives the expansion of embryonic macrophages, Csf1b has no evident role in embryonic myelopoiesis and Il34, acting through Csf1rb, is important for embryonic granulopoiesis. Finally, our observations suggest

evolutionarily interesting functions of CSF1R signaling in the myelopoiesis of non-mammalian vertebrates in addition to the conventional role of CSF1 in mammalian myelopoiesis[8,9,45] that should be taken into consideration when modelling human myeloid disorders in zebrafish.

Materials and methods

Animals

Zebrafish were bred, raised and kept in ZebTEC aquatic systems (Tecniplast) according to standard procedures,[46] tracked using Zebrabase.[47] Zebrafish *csf1* receptor mutant lines used in this study were *csf1ra*^{V614M} (Panther),[48] *csf1ra*^{t36ui}, (further *csf1ra*$^{\Delta 5bp}$), *csf1rb*^{re01}, (further *csf1rb*$^{\Delta 4bp}$)[42] and *csf1ra*^{V614M};*csf1rb*^{re01} double mutants.[42] The *csf1r* ligand mutants used were *csf1a*^{ins2bp}, *csf1b*$^{\Delta 2bp}$, and *il34*$^{\Delta 5bp}$.[49] Transgenic reporter zebrafish lines used were Tg(*mpeg1:EGFP*),[50] Tg(*fms:GAL4;UAS:mCherry*),[51] Tg(*mpx:EGFP*),[52] and Tg(*pax7:GFP*).[53] WT(AB) were used as controls. For *ex vivo* experiments, 6–12-month-old fish were used to get an optimal number of whole kidney marrow (WKM) cells. Animal care and experiments were approved by the Animal Care Committee of the Institute of Molecular Genetics, Czech Academy of Sciences (13/2016 and 96/2018) in compliance with national and institutional guidelines.

Multiplexed quantitative RNA fluorescence *in situ* hybridization

Hybridization chain reaction (HCR) v3.0 probe sets, amplifiers, and buffers, were used according to the manufacturer's protocols (Molecular Instruments).[54] Probes detecting zebrafish *csf1rb* (XM_009295703.3), *mpeg1* (NM_212737.1) and *mpx* (NM_001351837.1) were designed by the manufacturer. The Alexa 647, Alexa 546 and Alexa 488 amplifiers were used.

Fluorescence imaging

Fluorescent images were acquired on Zeiss Axio Zoom.V16 with Axiocam 506 mono camera. Orthogonal projections were created in ZEN Blue 2.3 software. Images of HCR-stained embryos were acquired on, Dragonfly 503 microscope (Andor) using Zyla 4.2 PLUS sCMOS camera. All images were processed by Fiji and Adobe Photoshop CC 2021.[55]

Single cell RNA sequencing (scRNA-seq) and transcriptomics

WKM cells were isolated as described previously [56], fractionated with Biocoll (1.077 g/ml, Merck) and counted. Between 3,000 and 5,000 cells were used for preparation of Chromium 3' sequencing libraries using Chromium Single Cell 3' Chip kit v3.1 and sequenced with Illumina Nextseq 500. The Illumina FASTQ files were used to generate filtered matrices using CellRanger (10X Genomics) with

default parameters. To generate filtered matrices, data were loaded to Cellbender package[57] using the following parameters - expected-cells = 5000, total-droplets-included = 15000. Filtered matrices were then imported into R for exploration and statistical analysis using a Seurat V3 package.[58] Counts were normalized according to total expression, multiplied by a scale factor (10,000), and log-transformed. For cell cluster identification and visualization, gene expression values were also scaled according to highly variable genes after controlling for unwanted variation generated by sample identity. Cell clusters were identified based on UMAP of the first 20 principal components of PCA using Seurat's method, FindClusters, with an original Louvain algorithm and resolution parameter value 0.5. Following quality control and basic clustering of each sample, we subsetted individual datasets to contain 1,700 cells each and merged them together. To visualize marker gene expression, Seurat's method, Dot-Plot, was used. To merge individual datasets and to remove batch effects, Seurat V3 Integration and Label Transfer standard workflow were used.[58]

Other procedures and methods

The description of mRNA and protein microinjections, whole mount in situ hybridization (ISH) of zebrafish embryos using digoxigenin-labeled antisense riboprobes, cloning of constructs for recombinant protein expression, *ex vivo* WKM cell cultures, Sudan Black B (SBB) staining of embryos, fluorescence activated cell sorting (FACS) analysis, image processing, statistical analysis and data sharing statement of presented data are outlined in the supplemental Materials and Methods available in the online version of this article.

Data sharing statement

sc-RNAseq data are available in ArrayExpress under accession number E-MTAB-10360. Plasmids for cytokine expression are available via Addgene: 168103 (pAc-His-zfCsf1a), 168104 (pAc-His-zfCsf1b), 168105 (pAc-His-zfIl34) and for mRNA expression: 168110 (pCS2-zfCsf1a), 168111 (pCS2-zfCsf1b) and 168112 (pCS2-zfIl34).

Results

Zebrafish *csf1ra* and *csf1rb* are expressed from early embryonic development and have distinct expression patterns in adults

To determine whether the spatiotemporal expression pattern of zebrafish *csf1ra* and *csf1rb* overlap in embryonic development, we crossed *fms:GAL4;UAS:mCherry* (simplified as *csf1ra:mCherry*) and

mpeg1:EGFP reporter lines to generate triple transgenic animals (Figure 1Aa). At 72 hours post fertilization (hpf), almost all *mpeg1:EGFP+* macrophages are also *csf1ra:mCherry+* (Figure 1Ab, 1Ac) and single *csf1ra:mCherry+* cells in the skin are xanthophores,[48] zebrafish pigment cells.[53,59] On the contrary, by performing double fluorescent HCR using probes for *mpeg1 and csf1rb* at 72 hpf (Figure 1Ba), we showed that the majority of macrophages are *mpeg1* single-positive with only few *mpeg1* and *csf1rb* double-positive cells (Figure 1Bb-c).

To characterize the expression pattern of *csf1ra/b* during development and in adult tissues, we performed qRT-PCR. Here, we demonstrate that *csf1ra* starts to be expressed at 20 hpf, whereas *csf1rb* first appears at 36 hpf (Figure 1C). From this time point, the overall expression of both receptors during embryonic development gradually increases until 7 days post fertilization (dpf).

Similarly, qRT-PCR using selected adult zebrafish tissues (Supplemental Figure S1A) showed high expression of *csf1ra* in brain, moderate expression in spleen, muscles, eyes, kidneys and skin and weak expression in the remaining organs. The strongest expression of *csf1rb* was in adult kidney marrow and brain, whereas it was low in other organs. To summarize these results, we created representative schemas (Figure 1D).

To get a more detailed insight into the expression of *csf1ra/b* in adult hematopoietic tissues, we performed scRNA-seq (Figure 1E) and demonstrated that there is no overlap between *csf1ra and csf1rb* expression in adult WKM. Instead, the expression of *csf1ra* is restricted to a few cells within the population of monocytes-macrophages, whereas the *csf1rb*+ cells comprise blood and myeloid progenitors, monocytes-macrophages and granulocytes. This is in agreement with our HCR expression data (Figure 1Ba-c), where we show that only a subset of *csf1rb+* cells are macrophages.

csf1a drives the expansion and differentiation of zebrafish embryonic macrophages

To further characterize the effects of *csf1* ligands on hematopoietic cells, we *in vitro* transcribed and injected mRNA for *csf1a*, *csf1b* and *il34* ligands, into 1-cell stage zebrafish embryos and examined their caudal hematopoietic tissue (CHT) region at 72 hpf. We noticed that the overexpression of *csf1a* but not of *csf1b* or *il34* caused expansion of *csf1ra:mCherry+* (Figure 2A) and *mpeg1:EGFP+* macrophages (Figure 2B). These injected embryos had high expression of both *csf1rs* and macrophage specific markers, such as *mpeg1*, *mfap4* and *lcp1* (Supplemental Figure S2A). Increased expression of *lcp1* was also documented by ISH staining using *lcp1* probe (Supplemental Figure S2B). We also noticed that the overexpression of both *csf1a/b* highly increased the number of *csf1ra:mCherry+* cells across the whole fish. We saw the same expansion in the xanthophore-specific *pax7:EGFP* transgenic line (data not shown).

Embryonic macrophage fate is impaired with the loss of *csf1a* signaling in zebrafish

To study impaired macrophage development upon loss of *csf1ra* or *csf1rb*, we compared *lcp1* expression by ISH in CHT at 48 hpf between WT and other receptor mutants (*csf1ra*^{V614M}, *csf1ra*$^{\Delta 5bp}$, or *csf1rb*$^{\Delta 4bp}$). While there was no difference in the number of *lcp1* positive cells in the *csf1ra*^{V614M} (Figure 2C), *csf1rb*$^{\Delta 4bp}$ or in the *csf1ra*^{V614M}; *csf1rb*$^{\Delta 4bp}$ double mutant fish (Supplemental Figure S2C), it was significantly decreased in *csf1ra*$^{\Delta 5bp}$ mutant animals (Figure 2C).

Even though the number of *lcp1+* cells was unchanged in *csf1rb*$^{\Delta 4bp}$ mutants, positive cells aggregated more to the rostral part of the CHT as compared to the WT. In addition, we also examined *mpeg1* expression in *csf1ra*^{V614M} as well as in *csf1rb*$^{\Delta 4bp}$ mutants at 48hpf. As expected, based on published data[43,44] and *lcp1* expression data (Figure 2C), the number of *mpeg1*+ macrophages in *csf1ra*^{V614M} CHT did not differ from those in WT (Supplemental Figure S2D), however, it was significantly decreased in *csf1rb*$^{\Delta 4bp}$ fish (Supplemental Figure S2E). To reveal the Csf1a ligand-receptor specificity, we microinjected *csf1a* into both of these mutants and we demonstrate that ligand-overexpression induced macrophage expansion was defective in them. Neither the number of *mpeg1:EGFP*+ cells in *csf1ra*^{V614M} (Supplemental Figure S2F) nor of *mpeg1+* cells in *csf1rb*$^{\Delta 4bp}$ mutants (Supplemental Figure S2G) was changed as compared to the WT. Thus, Csf1a acts through both Csf1rs.

We examined *lcp1* expression in *csf1a* and *csf1b* ligand mutants, carrying frameshift mutations. ISH showed a significant decrease in the number of *lcp1* expressing cells in the *csf1a*^{ins2bp} but not in the *csf1b*$^{\Delta 2bp}$ mutants (Figure 2C). Likewise, this phenotype can be rescued at 36 hpf by injection of *csf1a* mRNA or Csf1a proteins (Supplemental Figure S3A, B) into *csf1a*^{ins2bp} mutant 1-cell stage embryos (Figure 2D).

Zebrafish *csf1rb* together with *il34* regulate embryonic granulocytic fates

To test whether *csf1r* signaling is involved in the generation of other myeloid cell types besides macrophages, we examined the granulocytic lineage in *csf1* ligand and receptor mutants. Mature granulocytes were visualized in zebrafish embryos and larvae by Sudan black B (SBB) staining and positive cells were counted in tails (Figure 3A).

Strikingly, the number of SBB+ granulocytes in the tail was lower comparing to the WT and failed to increase during the development in *csf1rb*$^{\Delta 4bp}$ as well as in *il34*$^{\Delta 5bp}$ mutants (Figure 3B), whereas it gradually increased in both *csf1ra* mutants at the same time (Supplemental Figure S4A). An intermediate phenotype was documented in the receptor double mutants, in which the size of the original granulocytic pool did not change significantly with time (Supplemental Figure S4A). In addition, the mutation in csf1a or csf1b ligands had no obvious effect on the number of granulocytes (data not

shown). Along with these findings, the expression of *mpx* was also significantly downregulated in the CHT region of *csf1rb*$^{\Delta 4bp}$ and *il34*$^{\Delta 5bp}$, but not in other receptor or ligand mutants at 4 dpf (Supplemental figure S4B and data not shown).

Further, we assessed the effects of *il34* injection on granulocytic expansion in WT as well as in *csf1rb*$^{\Delta 4bp}$ mutants. As a positive control, we injected *colony stimulating factor 3a* (*csf3a,* also known as *gcsfa*).[60] As expected, the injection of *csf3a* mRNA led to a significant increase of SBB positive granulocytes in either *csf1rb*$^{\Delta 4bp}$ mutant or WT fish at 4 dpf. Similarly, the injection of *il34* mRNA into WT fish also caused an increase, however in contrast to *csf3a*, this *il34* mediated phenotype was diminished in the *csf1rb*$^{\Delta 4bp}$ mutants (Figure 3C). The same effect was confirmed by *mpx* ISH of 4 dpf injected WT as well as *csf1rb*$^{\Delta 4bp}$ mutant embryos (Figure 3D). We also tested the other ligands, *csf1a* and *csf1b*, but *il34* was the only one to affect granulocytic expansion (Supplemental Figure S4C). Importantly, microinjection of *il34* induced granulocytic expansion in the *csf1ra*^{V614M} mutant (Supplemental Figure S4D). The co-expression of *mpx* with *csf1rs* in the CHT of 72 hpf embryos shows basically no overlap between *csf1ra* and *mpx* (Figure 3Ea, 3Eb). However, there is a small proportion of *csf1rb* and *mpx* double positive cells (Figure 3Fa, 3Fb). Taken together, these results indicate that Il34 regulates embryonic granulocyte development through Csf1rb.

Zebrafish *csf1rb* is indispensable for definitive granulopoiesis

To investigate the importance of *csf1rb* in adult granulopoiesis, we imaged and counted the number of *mpx*+ cells in tail fins of 6 months old *mpx:EGFP* transgenic WT and *csf1rb*$^{\Delta 4bp}$ mutant animals. There was a significantly reduced number of granulocytes in the periphery of *csf1rb*$^{\Delta 4bp}$ mutants (Figure 4A). Furthermore, we examined WKMs of *csf1ra*^{V614M}*, csf1ra*$^{\Delta 5bp}$*, csf1rb*$^{\Delta 4bp}$ *and il34*$^{\Delta 5bp}$ animals using FACS analysis (Figure 4B), noticing a significant decrease in the number of myeloid cells in *csf1rb*$^{\Delta 4bp}$ mutants only (Mean ± SD; WT: 39.7 ± 3.2%; *csf1rb*$^{\Delta 4bp}$: 16.9 ± 6.4%; *il34*$^{\Delta 5bp}$: 48.3 ± 1.7%). The other mutants were not affected (data not shown). Additionally, we prepared thin layer smears stained by May-Grünwald and Giemsa (MGG) from WKM cell suspensions (Supplemental Figure S5A). Most of the cells isolated from *csf1rb*$^{\Delta 4bp}$ mutants resembled immature undifferentiated cells. The morphology of *csf1rb*$^{\Delta 4bp}$ mutant granulocytes was abnormal with a significantly decreased frequency of lobulated mature cells that were much smaller in size compared to WT cells.

Csf1a, Csf1b and Il34 zebrafish proteins expand adult myeloid cells in *ex vivo* culture

To investigate cell autonomous effects of Csf1 and Il34 cytokines, we performed *in vitro* experiments using recombinant ligand proteins. Therefore, we isolated and seeded WKM cells from WT fish, as published previously,[56,60] with the addition of recombinant zebrafish Csf1a, Csf1b or Il34 proteins. After

3 days in suspension culture, we prepared histological smears of myeloid cells for enumeration. Specifically, we counted the number of monocytes, differentiated macrophages and granulocytes in proportion to other cells (mostly immature blood progenitors and lymphoid-like cells). In the presence of any of all three cytokines, suspension cells differentiated towards the myeloid lineage to mostly become mature macrophages (Figure 4C). Strikingly, mature multinucleated osteoclasts represented a major fraction of adherent cells. The addition of Csf1a or Il34 to the *ex vivo* culture, promoted the proliferation of osteoclast progenitors and their fusion. (Figure 4D).

Myelopoiesis is partially blocked in the *csf1rb*$^{\Delta 4bp}$ mutants

Our results thus far have shown that embryonic granulopoiesis is altered in *csf1rb*$^{\Delta 4bp}$ mutants. With the noted differences in composition of individual hematopoietic population between WT and mutant animals, we were interested in characterizing these changes at the single cell level. We thus utilized scRNA-seq to profile WKM cells isolated from 12 months old WT, *csf1ra*$^{\Delta 5bp}$ and *csf1rb*$^{\Delta 4bp}$ mutants.

Via unsupervised clustering of single cell transcriptomes and based upon known lineage marker genes, we named each cluster based on likely cell type origins (Figure 5A). The percentage of cells in selected clusters of blood progenitors (BP), myeloid progenitors (MP), monocytes/macrophages (M/M) and granulocytes (G) is shown in a table (Figure 5B). We saw increased numbers of cells in BP and MP populations for *csf1ra*$^{\Delta 5bp}$ and surprisingly G for *csf1rb*$^{\Delta 4bp}$ (Figure 5B, Supplemental Figure S6A), whereas the number of M/M was decreased in *csf1rb*$^{\Delta 4bp}$ as expected.

Further, we picked out representative markers of hematopoiesis that characterize BP (green), MP (blue), M/M (orange), and G (pink) populations and created dot plot expression schemes showing their relative expression and the percent of cells expressing them (Figure 5C). We observed deregulation in most of these markers in both mutants. However, for *csf1ra*$^{\Delta 5bp}$ mutant the differences were more prominent in progenitors (BP and MP) and for *csf1rb*$^{\Delta 4b}$ they were more prominent in differentiated myeloid cells (M/M and G). Additionally, we observed that the *csf1rb* gene was upregulated in *csf1ra*$^{\Delta 5bp}$ mutant and *vice versa*. After a closer look at the discrepancy to the decreased number of embryonic granulocytes and the peripheral neutropenia observed in adult *csf1rb*$^{\Delta 4bp}$ fish, we noted that a high proportion of granulocyte-like cells accumulate in WKM (Supplemental Figure S6A) and there are aberrantly expressed progenitor (Supplemental Figure S6B), migration as well as inflammation markers (Figure 5C, Supplemental Figure S6C).

Discussion

Differentiation, survival and maturation of myeloid cells is tightly controlled by extrinsic factors, such as cytokines. To the most prominent ones belong CSF1, CSF2 (also known as granulocyte-macrophage-

CSF, GM-CSF), and CSF3. The critical role of CSF1 signaling on proper macrophage cell differentiation and survival was shown in mice mutants that lack *CSF1R* or *CSF1*.[9,61,62] Interestingly, *CSF1*, *CSF2* and *CSF3* triple mutant mice are not completely devoid of macrophages or granulocytes.[62] This indicates that the role of these factors can be replaced by other cytokines, such as IL-6 or IL-34.[4,63,64] Although CSF signaling has been historically studied mainly in mammalian and bird animal models and many transgenic and mutant lines are available, zebrafish has recently become a popular alternative model organism for modeling hematopoietic human diseases.

Here, we studied the *in vivo* function of zebrafish Csf1 receptor paralogs (*csf1ra*, *csf1rb*) and their ligands (Csf1a, Csf1b, Il34) to describe their involvement in fish myelopoiesis and to characterize their diversification. Our findings demonstrate that Csf1a and Csf1b sub-functionalized during embryonic myelopoiesis. Specifically, we show that only the Csf1a ligand is important in the development of embryonic macrophages, while both, Csf1a and Csf1b, are involved in pigmentation. This is consistent with previous studies demonstrating the role of Csf1a/b in adult pigment patterning.[15,65]

To get detailed insight into the involvement of Csf1 signaling in embryonic and adult macrophage development in zebrafish, we decided to study Csf1 ligand and receptor mutants. Zebrafish *csf1ra, csf1rb* and *il34* mutants have been previously described for the lack of microglia.[42,49] It was shown that macrophages develop, migrate or proliferate aberrantly in *il34*$^{\Delta 5bp}$ mutants,[43,49] and in *csf1ra*^{V614M};*csf1rb*$^{\Delta 4bp}$ double mutant fish,[66] whereas they develop normally in *csf1ra*^{V614M} (known as Panther)[42,43] and *csf1rb*^{sa1503} mutant[44] embryos. The status of macrophage development in *csf1rb*$^{\Delta 4bp}$ single mutant animals has remained unnoticed.

In contrast to these studies, we demonstrate that the number of embryonic macrophages in the CHT of *csf1ra*$^{\Delta 5bp}$ and *csf1rb*$^{\Delta 4bp}$ mutant embryos is decreased. We suppose that the discrepancy in the number of embryonic macrophages between *csf1ra*^{V614M} and *csf1ra*$^{\Delta 5bp}$ or *csf1rb*^{sa1503} and *csf1rb*$^{\Delta 4bp}$ could be explained by the fact that *csf1ra*$^{\Delta 5bp}$ and *csf1rb*$^{\Delta 4bp}$ mutants have stronger phenotypes because they carry frameshift mutations with a premature stop codon. This has been also shown in another Panther mutant with a C-terminal deletion mutation (*csf1ra*^{j4blue}).[48,67,68] Surprisingly, macrophage expansion is diminished in both *csf1ra*^{V614M} and *csf1rb*$^{\Delta 4bp}$ mutants after *csf1a* microinjection. Therefore, we hypothesize that Csf1a drives macrophage development via either of both, Csf1ra or Csf1rb, receptors.

Besides the role of Csf1 signaling in macrophage differentiation, we noticed that it might also be involved in zebrafish granulocyte differentiation. Despite decades of CSF1R research, only sparse evidence exists linking CSF1R signaling to granulocyte differentiation in mammals.[69] Strikingly, we show that *csf1rb*$^{\Delta 4bp}$ and also *il34*$^{\Delta 5bp}$ mutant zebrafish embryos have major defects in granulopoiesis

and that the overexpression of *il34* in WT animals leads to granulocyte expansion. Further, we show that the pool of embryonic granulocytes cannot be expanded in the *csf1rb*$^{\Delta 4bp}$ mutants by *il34*. This implies that Il34 plays a previously unknown role in the regulation of embryonic granulopoiesis and that it can act through Csf1rb, providing a novel Csf3r alternative pathway that is important for granulocyte differentiation.

As a next step, we decided to examine adult macrophages in receptor mutant animals. In contrast to defects in embryonic macrophage myelopoiesis, we did not see any evident myeloid defects in WKM of adult *csf1ra*$^{\Delta 5bp}$ mutants when examined histologically and by FACS. Nevertheless, the scRNA-seq analysis of *csf1ra*$^{\Delta 5bp}$ mutants revealed that there is a slight downregulation of macrophage-specific markers, such as *mfap4*, *mhc2dab*, or *mrc1b* and an upregulation of progenitor specific markers, such as *myb*, *cebpa* and *gata2b* and the overall number of blood and myeloid progenitors is elevated as well, however, the other populations are mostly unchanged. Despite these less severe phenotypes in adult WKM cells, we hypothesize that besides Csf1ra role in development of embryonic macrophages, it also plays a minor role during adult hematopoiesis as previously not shown. The decreased number of monocytes/macrophages was also observed in *csf1rb*$^{\Delta 4bp}$ mutant using scRNA-seq. In addition, markers specific to macrophage host defense/phagosome and antigen presentation are decreased (*mrc1b, nccrp1, mhc2dab*) in this mutant. These observations led us to the conclusion that Csf1rb is equally important during embryonic as well as adult macrophage development.

Additionally, *csf1rb*$^{\Delta 4bp}$ adult fish have lower number of myeloid cells in WKM, and also fewer *mpx*+ cells in the periphery. Since the majority of myeloid cells in WKM are neutrophils[70,71] we attribute the reduced number of myeloid cells to the loss of mature, physiologically normal granulocytes. However, surprisingly our scRNA-seq data indicate that *csf1rb*$^{\Delta 4bp}$ mutants have more granulocytes in WKM. We explain this discrepancy by the fact that *csf1rb*$^{\Delta 4bp}$ granulocyte progenitors cannot fully differentiate and migrate, and progenitor-like early and also late granulocytes accumulate in WKM. This is proven by scRNA-seq marker gene expression profiling and also by FACS and histological staining of WKM cells, demonstrating that *csf1rb*$^{\Delta 4bp}$ fish have a high proportion of small cells with abnormal morphology and low granularity. Using scRNA-seq analysis, the predicted granulocyte population of *csf1rb*$^{\Delta 4bp}$ WKMs showed an overexpression of *myb, cbfb, spi1b* and *cebp* markers, known to play essential roles in progenitor hemostasis.[72-76] Further we observed deregulated expression of chemokine and inflammation markers (*il34*, *itgb2*, *mmp13a*, *il6r*, *ifngr1*), and markers connected to granulocyte migration (*cxcr4b*, *ifngr1, cxcl8b*).[77,78] Abnormal activation of the zebrafish *ifngr1* signaling has been previously shown in neutropenic zebrafish.[79]

Next, we have shown that *csf1r* paralogues have functionally diverged in the course of teleost evolution. Consistently with previous findings,[44] we suggest that the function and expression of *csf1* receptor paralogues is mostly non-overlapping. At the single cell level, using scRNA-seq analysis of adult WKM cells, we showed that there is no expression overlap between *csf1ra* and *csf1rb* and that *csf1rb* is highly expressed in blood and myeloid progenitors as well as in a small subset of monocytes/macrophages and granulocytes. In contrast, the *csf1ra* expression is restricted only to a small subset of monocytes/macrophages. In correlation with these expression data, we also demonstrated that mutation in *csf1ra* primarily affects embryonic macrophage development, while the mutation in *csf1rb* is equally important during embryonic as well as adult development of both macrophages and granulocytes. Regarding ligand-receptor signaling specificities, we found that Csf1a acts via both, Csf1ra and Csf1rb and that the granulocyte related function of Il34 is executed via Csf1rb. In contrast, Csf1b has no function during embryonic myelopoiesis and it expands xanthophores instead, together with Csf1a. Importantly, based on the fact that there is low expression of *csf1rb* in the skin, we assume that the Csf1 dependent xanthophore expansion is mediated via Csf1ra.

In addition, our *ex vivo* experiments suggest that recombinant Csf1a/b and Il34 cytokines are functional in *ex vivo* cultures and drive WKM-derived myeloid cell differentiation towards monocyte-macrophage and osteoclast fates. However, terminal granulocytic differentiation is not affected by Csf1r ligands.

In summary, we demonstrate that zebrafish continues to provide new biological insights relevant to disease. Using a wide range of convenient *in vivo* and *ex vivo* tools, it is possible to characterize new exciting roles of cytokines under steady-state as well as non-steady-state conditions. Myeloid cells, including neutrophils and macrophages are critical actors in cancerogenesis[80-82] and the CSF1 pathway is a promising target for clinical treatments.[24,83] Here we performed detailed characterization of Csf1 signaling in zebrafish making it suitable for preclinical disease modeling in high-throughput discovery of new therapeutics.

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Authorship

Contribution: M.H., T.M., O.M., A.P., U.I. performed research. O.S. and P.B. designed the research. T.J.v.H. and C.N.-V. provided critical reagents for the work. M.H., T.M., P.B. and O.S. wrote the manuscript.

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Figure 1: *csf1ra* and *csf1rb* have distinct expression patterns in zebrafish.

(A) Co-expression of *csf1ra* (red) and *mpeg1* (green) visualized in 72 hpf Tg(*fms:GAL4;UAS:mCherry*);Tg(*mpeg1:EGFP*) double transgenic embryos: (Aa) whole embryo, (Ab) head, (Ac) caudal hematopoietic tissue (CHT) region. (B) HCR WISH of 72 hpf embryos for *csf1rb* (red) and *mpeg1* (green). **(**Ba) whole embryo, (Bb) head, (Bc) CHT region. Fluorescence images were taken on Dragonfly 503 microscope (Andor) using Zyla 4.2 PLUS sCMOS camera, with 10x magnification and processed with the Fusion software, FIJI and Adobe Photoshop. (C) qRT-PCR analysis of pooled zebrafish embryos showing the expression dynamics of *csf1ra* and *csf1rb* in zebrafish development. Pool of 15-20 embryos/sample in 2-6 biological replicates. The expression was normalized to *mob4* gene and to the time point with the highest expression (14 dpf for *csf1ra* and 7 dpf for *csfrb*). (D) qRT-PCR analysis of adult zebrafish tissues. Pool of 3-5 fish organs/sample in 3-5 biological replicates. The expression was normalized to *ef1α* gene. (E) scRNA-seq data showing the expression of *csf1ra* and *csf1rb* in whole kidney marrow (WKM) cell populations. E - erythroid cells; EP - erythroid progenitors; M/M - monocytes & macrophages; KSC - kidney support cells; BP - blood progenitors; TC - T-cells; BC - B-cells; NKC - NK cells; MP - myeloid progenitors; EC - endothelial cells; HSPCs - hematopoietic stem and progenitor cells; G – granulocytes.

Figure 2: *csf1a,* but not *csf1b* and *il34* drive the expansion of embryonic macrophages *in vivo.*

(A-B) *csf1a, csf1b* and *il34* ligands were overexpressed by mRNA microinjection in 1-cell stage transgenic embryos. Control embryos were injected with PBS. Fluorescence images were acquired at 72 hpf and the area of fluorescent cells was calculated in the caudal hematopoietic tissue (CHT, area inside of the red box) by FIJI. Results were normalized to injected controls. Scatter plots on the right represent quantification of fluorescent cells in CHT. Each dot in the scatter plot represents one larva. (A) Tg(*fms:GAL4;UAS:mCherry*) (B) Tg(*mpeg1:EGFP*) (C) WISH of 48 hpf embryos showing the expression of *lcp1* in WT, two *csf1ra* mutants: *csf1ra*^{V614M} (panther) and *csf1ra*$^{\Delta 5bp}$, and in *csf1a*^{ins2bp}, *csf1b*$^{\Delta 2bp}$ ligand mutants. (D) *lcp1* WISH of 36 hpf WT embryos injected with PBS (control), and *csf1a*^{ins2bp} mutant embryos injected with PBS (control), *csf1a* mRNA or recombinant zebrafish Csf1a protein. (C-D) Violin plot graphs show the level of *lcp1* expression in the CHT region of individual embryos (L = low, M = medium, H = high) with median represented by a black line. (A-D) The level of statistical significance was determined by unpaired two-tailed t-test. *P < 0.04, **P < 0.006, ***P < 0.0001. All fluorescent images were acquired on Zeiss Axio Zoom.V16 with Zeiss Axiocam 506 mono camera and ZEN Blue software. Bright field images of WISH were acquired on Zeiss Axio Zoom.V16 with Zeiss Axiocam 105 color camera and processed using the Extended Depth of Focus module in the ZEN Blue software. FIJI and Adobe Photoshop were used for image processing.

Figure 3: *il34* binds to *csf1rb* and regulates the embryonic granulocytic fate:

(A) Sudan black B (SBB) staining of a 4 dpf larva. The analyzed area is marked with a red rectangle. (B-C) SBB positive cells were manually counted and the level of statistical significance was determined by unpaired two-tailed t-test. **P < 0.006, ***P < 0.0001. (B) SBB in WT, *csf1rb*$^{\Delta 4bp}$ and *il34*$^{\Delta 5bp}$ at 4 dpf. The graph on the right shows the number of SBB positive cells during zebrafish embryonal and larval development, 2 – 7 dpf. (C) *il34* and *csf3a* ligands were overexpressed by mRNA microinjection in 1-cell stage WT or *csf1rb*$^{\Delta 4bp}$ mutant embryos. Control embryos were injected with PBS. SBB staining was performed at 4 dpf. The graph on the right shows the number of SBB positive cells. (D) WISH of 4 dpf larvae showing the expression of *mpx* in WT or mutant *csf1rb*$^{\Delta 4bp}$ embryos with overexpressed *il34* or *csf3a* ligands. Violin plots show the level of *mpx* expression in individual embryos (L = low, M = medium, H = high) with median represented by a black line. ***P < 0.001. (E) Co-expression of *csf1ra* (red) and *mpx* (green) visualized in 72 hpf double transgenic embryos Tg(*fms:GAL4;UAS:mCherry*);Tg(*mpx:EGFP*): (Ea) whole embryo, (Eb) caudal hematopoietic tissue (CHT) region. (F) HCR WISH of 72 hpf embryos for *csf1rb* (red) and *mpx* (green). (Fa) whole embryo, (Fb) CHT region. All SBB staining and WISH bright field images were acquired on Zeiss Axio Zoom.V16 with Zeiss Axiocam 105 color camera and processed using the Extended Depth of Focus module in the ZEN Blue software. FIJI and Adobe Photoshop were used for image processing. Fluorescence images were taken on Dragonfly 503 microscope (Andor) using Zyla 4.2 PLUS sCMOS camera, with 10x magnification and processed with the Fusion software, FIJI and Adobe Photoshop.

Figure 4: *csf1rb* is indispensable for definitive granulopoiesis.

(A) Number of neutrophils in adult Tg(*mpx:EGFP*) = WT and Tg(*mpx:EGFP*);*csf1rb*$^{\Delta 4bp}$ = *csf1rb*$^{\Delta 4bp}$ fish tails. Neutrophils were manually counted in the area of the yellow square. The level of statistical significance was determined by unpaired two-tailed t-test. *P < 0,04.

(B) FACS analysis of whole kidney marrow (WKM) cell suspension from WT, *csf1rb*$^{\Delta 4bp}$ and *il34*$^{\Delta 5bp}$ adult zebrafish. The numbers in FSC/SSC plots represent the mean percentage with SD in the gates of myeloid cells (pink gate), progenitors (blue gate) and lymphoid and small progenitor cells (green gate). The percentage of WKM cells in the myeloid gate is also shown in the bar graph on the right. (C-D) *Ex vivo* culture of WKM cells treated with Csf1a, Csf1b or Il34 proteins. (C) After 3 days in culture, smears of suspension cells were stained on microscopic glass slides with May-Grünwald and Giemsa (MGG) and the number of differentiated cells (monocytes, macrophages and granulocytes) was counted. The graph on the bottom shows the mean percentage of cells with SD. *P < 0,04. The scale bar on the

microscopic image is 20 µm. (D) After 3 days in culture, adherent cells on the dish were washed with PBS, stained with MGG and the number of small, medium and large osteoclasts was counted in 20 fields of view with a 20x magnification objective. The scale bar on the microscopic image is 50 µm. Fluorescence images were acquired on Zeiss Axio Zoom.V16 with Zeiss Axiocam 506 mono camera and ZEN Blue software. ImageJ and Adobe Photoshop were used for image processing. Bright field images of *ex vivo* cultures were acquired on Leica DM 2000 microscope with Zeiss Axiocam 105 color camera.

Figure 5: Single-cell RNA sequencing (scRNA-seq) of adult WT, *csf1ra*$^{\Delta 5bp}$ and *csf1rb*$^{\Delta 4bp}$ WKM cells shows differentially expressed hematopoietic genes.

(A) Clusters in Dim plots represent individual selected populations of WKM hematopoietic cells. The identity of each cluster is based on likely cell origin according to lineage marker gene expression. Green – blood progenitors (BP), blue – myeloid progenitors (MP), orange – monocytes/macrophages (M/M), pink – granulocytes (G), gray – other cells (OC). (B) Table representing the percentage of cells in each population for WT and respective mutants. (C) Dot plot visualization of scRNA-seq gene expression in individual populations of WKM hematopoietic cells of WT and respective mutants. The color of each dot represents the level of expression (also depicted in the histogram) and the size of the dot is showing the percentage of cells expressing each individual gene. Green – BP, blue – MP, orange – M/M, pink – G.

Figure 1

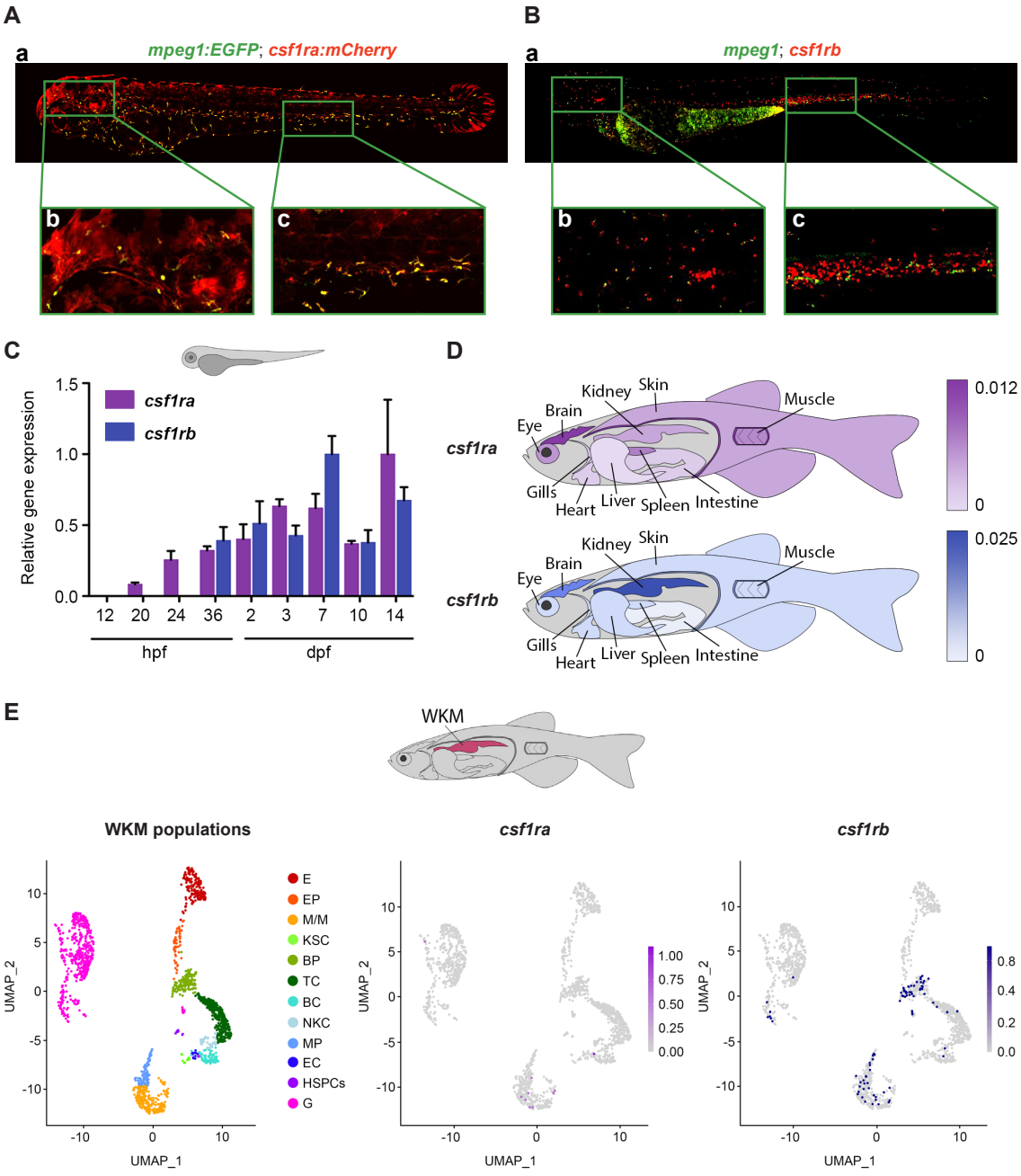
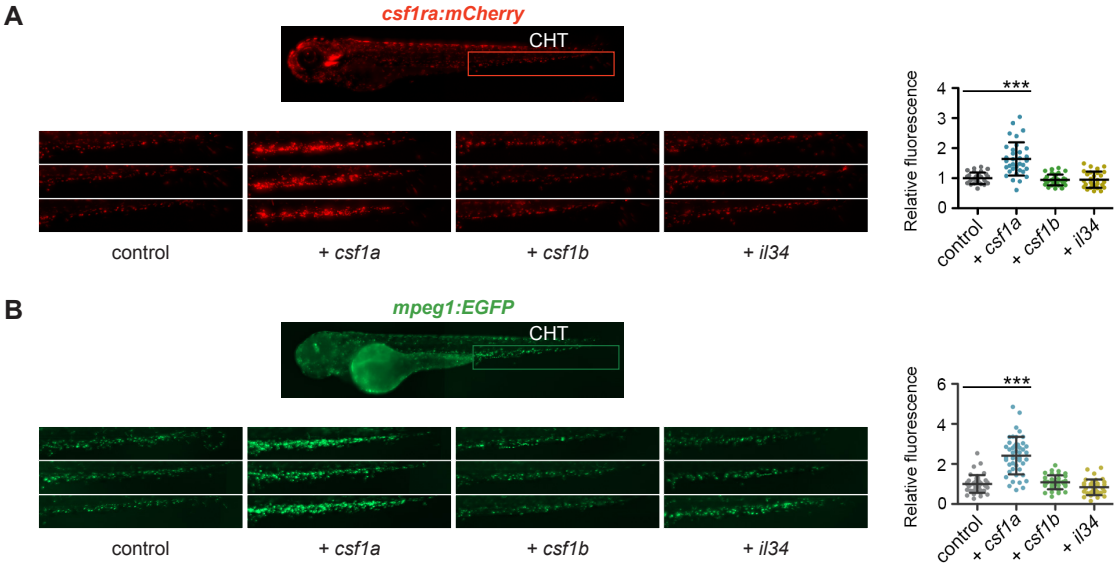


Figure 2



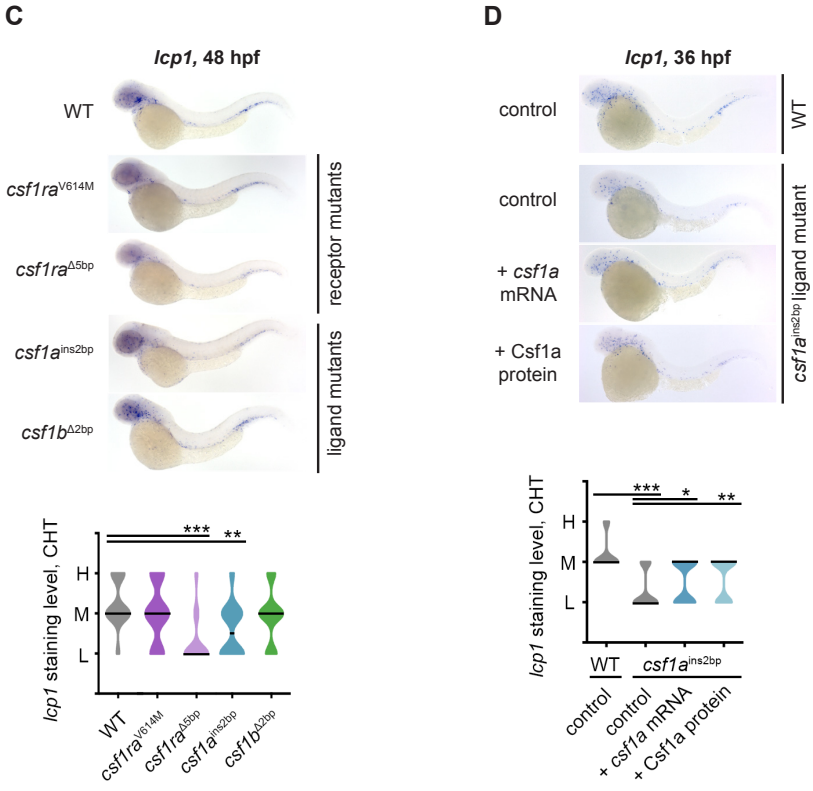


Figure 3

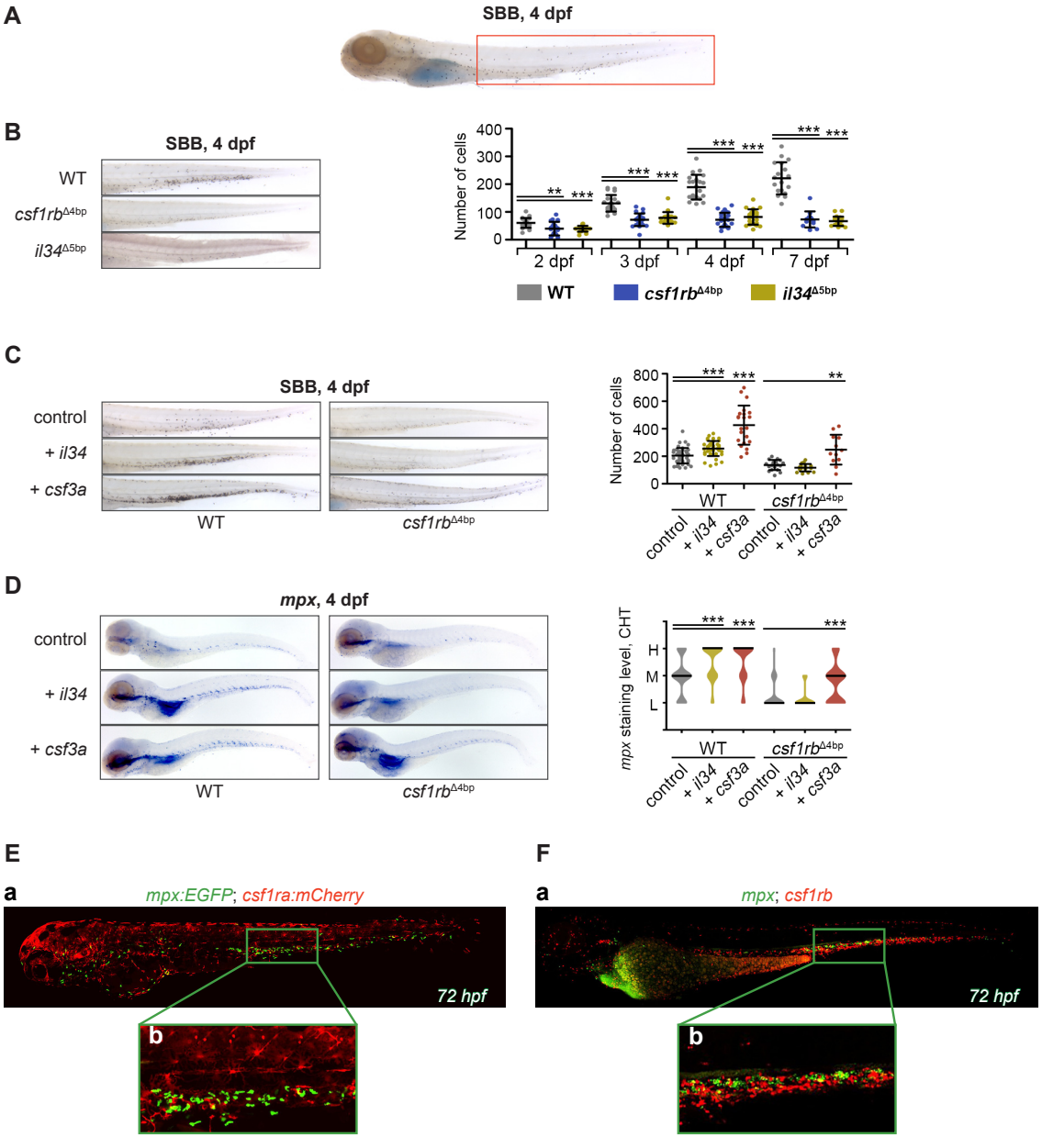


Figure 4

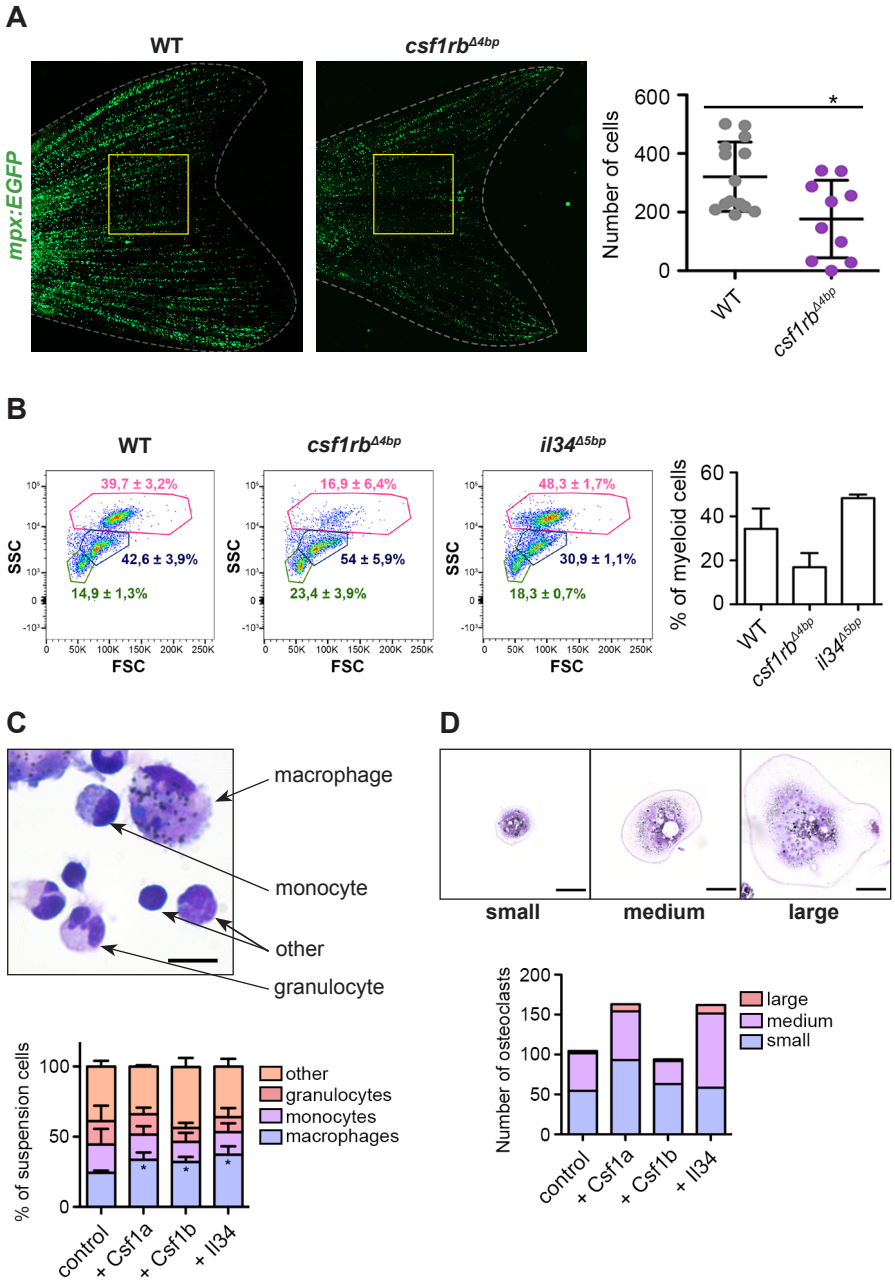


Figure 5