Zebrafish Kit ligands cooperate with erythropoietin to promote erythroid cell expansion

Jana Oltová[1], Ondřej Svoboda[1,2], Olga Machoňová[1], Petra Svatoňová[1], Michal Kolář[1], and Petr Bartůněk[1]

[1] *Institute of Molecular Genetics of the Czech Academy of Sciences, Prague, Czech Republic*
[2] *Department of Cellular and Molecular Medicine, University of California at San Diego, La Jolla, CA, USA.*

Correspondence should be addressed to P.B.
bartunek@img.cas.cz

Abstract

Kit ligand (Kitlg) is pleiotropic cytokine with a prominent role in vertebrate erythropoiesis. Although the role of Kitlg in this process has not yet been reported in *Danio rerio* (zebrafish), in the present study, we show that its function is evolutionary conserved. Since zebrafish possess two copies of Kitlg genes (Kitlga and Kitlgb) due to whole genome duplication, we tested both paralogues and we demonstrate that they contribute to the development of erythroid cells. To determine the role of each ligand in zebrafish, we performed a series of *ex vivo* and *in vivo* gain- and loss-of-function experiments. First, we generated recombinant versions of Kitlg proteins, and tested their biological activity in liquid culture on progenitor cells isolated from zebrafish whole kidney marrow. Using this assay, we demonstrate that Kitlga is necessary for expansion of erythroid progenitors *ex vivo*. To further address the role of *kitlga* and *kitlgb* in hematopoietic development *in vivo*, we performed gain-of-function experiments in zebrafish embryos, showing that both ligands cooperate with erythropoietin (Epo) to promote erythroid cell expansion. Finally, using the *kita* mutant ($kit^{b5/b5}$ or *sparse*), we show that Kita receptor is crucial for Kitlga/b cooperation with Epo in erythroid cells. In summary, using optimized liquid culture conditions with recombinant Kitlga, we are providing an indispensable tool to study normal and aberrant hematopoiesis in zebrafish. Furthermore, we conclude that although partial functional diversification of Kit ligands has been described in other processes, in erythroid development, both paralogs play a similar role and their function is evolutionary conserved.

Introduction

The most prominent cytokines that regulate proliferation and differentiation of erythroid cells in vertebrates are erythropoietin (EPO) and Kit ligand (KITLG or stem cell factor, SCF). Binding of EPO and KITLG to their cognate receptors ensures erythroid lineage commitment by triggering specific signaling events[1-3].

Besides its role in erythropoiesis, KITLG acts pleiotropically, affecting a wide range of tissues and cells, including hematopoietic (HSCs) and germ stem cells. It is an important regulator that plays a role in many processes, both during ontogenesis and in the adult organism[4,5]. In mammals, KIT signaling is associated with erythro-myelopoiesis[6], as well as neurogenesis and pigmentation[5,7-9]. Interestingly, there are two forms of KITLG occurring *in vivo* – transmembrane, important for the regulation of stem cells in their niches, and soluble, affecting more distant tissues[5,10]. Binding of KITLG to its receptor KIT, a member of receptor tyrosine kinase type-III family, leads to its autophosphorylation, further triggering various signaling cascades, e.g. PI-3K, MAPK, SRC and JAK kinase pathways[10,11].

In teleost species that include zebrafish, this scenario is more complex due to the fact that the whole ligand-receptor signalosome has been duplicated as a result of an extra round of whole genome duplication. Future studies are therefore dependent on the understanding of diversification of the functions of both ligands (Kitlga, Kitlgb) and receptors (Kita, Kitb) and their binding specificities. Similarly, there are also two copies of the epo gene present in the zebrafish genome - *epoa* and *epob*. The zebrafish *epoa* seems to play a similar role as its mammalian ortholog[12,13] but no role in hematopoiesis has been so far reported for *epob (OS, PB, unpublished).* For simplicity, we will use the designation Epo/*epo* for Epoa/*epoa* in this study.

Current data support the hypothesis that Kit receptor and Kit ligand paralogs have subspecialized during evolution. Kita is expressed in the neural crest, lateral line and notochord[14]. Overexpression of Kitlga results in a hyper-pigmentation phenotype[15] and *kita* receptor mutants (*kita*$^{b5/b5}$, *sparse*) have defective pigmentation[14]. On the other hand, the second zebrafish Kit paralog, Kitb, is expressed in neural tube and otic vesicles and likely does not play a role in melanogenesis[16,17]. Neither has a role in melanogenesis been reported for Kitlgb[17].

Although studied extensively, the role of Kit signaling in zebrafish hematopoiesis has remained unknown for a long time. So far, there are only two studies that suggest potential roles of Kitlg in hematopoiesis in *D. rerio*. The first reports a mild increase of HSCs upon overexpression of Kitlgb[18], the second reports a decrease in the number of HSCs upon downregulation of Kitb. The authors concluded that neither Kita nor

Kitlga are involved in hematopoiesis, but only in melanocyte formation, as suggested by previous studies [17]. Contradictory to the findings in other vertebrate models, and despite the fact that Kitlga is expressed in hematopoietic tissues, hematopoiesis is not affected in the *kita*$^{-/-}$ (*sparse*) mutants[14].

The objective of this study is to better understand the importance of Kit ligands during embryonic hematopoiesis in zebrafish, thus providing important insights for further research of hematopoietic development. Although *ex vivo* expansion of erythroid progenitors have been reported in different vertebrate organisms[19], a similar approach in zebrafish is not available. Here, we establish liquid culture conditions for the expansion of zebrafish erythroid progenitors and we investigate the role of the two zebrafish paralogs of Kit ligands (Kitlg) in zebrafish hematopoiesis using *in vivo* and *ex vivo* experimental approaches.

Methods

Animal stocks and embryos

Fish were mated, raised and staged according to "The zebrafish book. A guide for the laboratory use of zebrafish (*Danio rerio*)"[20] and the recommendations for zebrafish husbandry and housing[21]. For tracking the animals (age, number, health, genotype), we used a dedicated database solution called Zebrabase[22]. Fish were kept in ZebTEC aquatic system (Tecniplast). Transgenic reporter lines expressing fluorescent genes under the control of tissue specific promoters (*gata1*:*DsRed*[23] and *LCR:GFP*[24], as well as mutant lines (kita$^{-/-}$ mutant sparse)[14] and wild-type animals WT(AB) were used in this study. For ex vivo experiments, 6-month old fish were used to ensure optimal number and state of whole kidney marrow 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.

Ex vivo cultures

Zebrafish whole kidney marrow cells were isolated as previously described[25]. Cells were plated and cultivated in zfS13 medium (for composition see Supp. Table 3). Carp serum was obtained by the procedure described and published previously[25]. Specific cytokines and growth factors were added at final concentrations of (100 ng/ml), dexamethasone was added at final 1µM. 1/3 of the medium was exchanged every other day to ensure optimal growth of the cells.

To study the clonogenic potential of the whole kidney marrow cells after addition of different cytokines, we performed clonal assays in semisolid media (methylcellulose), which prevents movement of the single cells plated in it. For the detailed protocol for these assays, please refer to Svoboda et al., 2016[25].

Cytokine cloning and expression

First, the amino acid sequence of zebrafish Kitlga/b, was subjected to protein structure prediction and hydrophobicity analysis using Phobius tool (http://phobius.sbc.su.se/). Two large hydrophobic regions (amino acid positions 1 to 24 and 206 to 224 for Kitlga, and amino acid positions 1 to 31 and 185 to 209 for Kitlgb) corresponding to the putative signal peptide (SP) and transmembrane (TM) domains, respectively, were identified (Figure S1A). To generate a Kitlga/b version devoid of both domains, two primers (Table S1) were used for PCR amplification of each Kitlg cDNA fragment corresponding to amino acid 25 to 182 (Kitlga) and 31 to 187 (Kitlgb) from adult zebrafish retina. We used the baculovirus expression system to produce soluble Kitlga/b in large quantities. We cloned the amplified fragment into pAc-His-GP67-B vector and generated the recombinant baculovirus by co-transfection of pAc-His-Kitlga/b and BaculoGold Bright Baculovirus DNA into sf21 insect cells. Virus-infected cells expressed GFP and secrete recombinant His-Kitlga/b extracellularly. Finally, we purified the secreted proteins on a Ni2+-NTA agarose column and used them for the following experiments (Figure S2B).

Similarly, to generate Kitlga/b CDS for mRNA injection experiments, the cDNA fragment was amplified using PCR to devoid the TM domain; however, the SP domain was maintained to support extracellular transport of mature protein during zebrafish development after mRNA injection. To generate this version of both coding sequences, primers corresponding to amino acid 1 to 182 (*kitlga*) and 1 to 187 (*kitlgb*) were used (Figure S1A).

Additional methods

For experimental details on the generation of recombinant cytokines, mRNA microinjection, benzidine staining, image analysis, qPCR, FACS-sorting, RNAseq and transcriptomics, please refer to supplemental methods.

Results

Cytokine cloning and expression

First, we cloned and expressed recombinant zebrafish Kitlga and Kitlgb. Using specific primers (Table S1), we amplified the mature form of zebrafish *kitlga* and *kitlgb* lacking the signal peptide-encoding region, intracellular and transmembrane region (Figure S1A) from adult retina. We cloned the amplified fragment into baculovirus expression vector and produced recombinant Kitlga and Kitlgb in sf21 insect cells (Figure S1B). His-tagged purified proteins were used in the following experiments.

Kit ligands promote erythroid and myeloid expansion of whole kidney marrow cells.

To test the biological activity of Kitlg proteins, we designed an experiment using whole kidney marrow cells isolated from adult, 6-month old zebrafish. To reveal differences that would suggest biological activity and potential cooperation of Kitlg proteins with other recombinant cytokines, we treated the cells with various cytokines, or their combination, and subsequently counted them at specific time points. After 3 days in culture, the potential enhancement in the myeloid lineage was assessed. We added both Kit ligands, either separately or combined with Gcsfa, which has been previously shown to support myeloid cell fate[26]. Interestingly, we observed an increase in the number of cells in all the conditions tested when compared to untreated control. In agreement with previously published studies, Gcsfa boosted significantly the growth of whole kidney marrow cells, and the effect was further potentiated by the addition of either of the Kit ligands. The Kit ligands alone exhibited reproducible mild activity that was however statistically not significant (Figure 1A).

Next, we tested the activity of Kit ligands during erythroid cell expansion. Cells were treated with both Kitlga and Kitlgb, either separately or combined with Epo. The increase in cell number was quantified after 7 days in culture. In agreement with previously published studies[12,27], Epo boosted cell growth significantly and again, there was a mild increase in the number of the cells when treated with any of the Kit ligands alone. Strikingly, when Epo was combined with Kitlga, we measured a significant increase in the number of cells at 7 days in culture compared to Epo alone (Figure 1B).

With the aim of maximum expansion of erythroid progenitors, we decided to improve the conditions for liquid culture of whole kidney marrow cells. Although there were no existing reports of liquid culture of zebrafish or hematopoietic progenitor cells, we supposed that similar conditions might be effective in liquid cultures as was shown in human and mouse [3,28]. As different steroids were reported as important players in the maintenance of self-renewal and proliferation of erythroid progenitors in chicken, mouse

and human [28-31], we included dexamethasone and tested different concentrations and combinations of all three factors.

We found that, in line with previous findings in other vertebrates, Kitlga, Epo and Dex act synergistically in *ex vivo* cultures, enabling erythroid expansion (Figure 1C, left). When compared to the cells treated with Epo and Dex, cells treated with Epo, Dex and Kitlga formed islets of round, highly *gata1:DsRed*-positive cells, corresponding to erythroid progenitors (Figure 1C, right). Additionally, we observed that Epo can cooperate with Kitlga to promote the growth of large erythroid colonies in colony forming assays in methylcellulose (Figure S2C). When Kitlga was combined with Epo in kidney marrow culture from *gata1:DsRed* transgenic fish, we observed significantly more, highly *gata1*:DsRed positive colonies that were also significantly larger (Figure S2A,B) However, no similar effect was observed after the addition of Kitlgb in either liquid or semisolid media (data not shown).

Kitlga, when combined with Epo and dexamethasone, leads to upregulation of erythroid-, translation- and cell cycle-related gene expression

To gain deeper insight into the mechanism of Kitlga action when combined with Epo and Dex in kidney marrow liquid cultures, we performed an RNAseq experiment, comparing samples from Epo, Dex, Kitlga and Epo, Dex-treated cells. Samples were collected after 8 days in culture. This was the first time-point where we could observe a significant boost in the proliferation of erythroid cells in the presence of Epo, Dex and Kitlga, as clearly shown in the growth curve (Figure 1C, left).

RNAseq analysis revealed that both culture conditions resulted in high expression of erythroid marker genes (data not shown). However, addition of Kitlga to the Epo, Dex combination caused an additional increase in expression of erythroid genes. Among the upregulated differentially expressed genes (see heatmap in Figure 1D and Supp. Table 4), we identified several erythroid (*g6fl*, *lias*) and cell cycle (*cdc27*) specific genes, confirming additional Kitlga-mediated erythroid cell expansion over Epo and Dex treatment alone. On the contrary, downregulated genes were mostly linked to the myeloid cell lineage (*pigrl2*.3, *blnk*, *nfkbiz*, *alox5a*, *inpp5d*), providing further evidence that Epo, Dex, Kitlga-induced expansion led to a more highly enriched erythroid cell population as compared to Epo, Dex culture alone.

Finally, we were interested specifically in changes in expression of erythroid-specific genes. The analysis of RNAseq data reveled that, compared to cells treated with Epo and Dex, cells treated with Epo, Dex and Kitlga display higher expression of many of the important erythroid genes – namely globins (*hbae1.1*, *hbaa1*), genes involved in heme and iron metabolism (e.g. *urod*, *slc11a2*, *steap3*), erythroid cytoskeleton

(e.g. *rhd*, *sptb*, *eph41b*) and signaling (*gata1a*, *jak3*) (Figure 1E, Supp. Table 5). Based on GO prediction, we revealed an enrichment in specific categories of biological processes – namely in translation (GO:0006412, GO:0002181, GO:0000028, GO:0000027), erythropoiesis (GO:0042541, GO:0030218, GO:0048821) and cell cycle regulation (GO:0051726). For a full list of enriched GO terms in all three standard GO term categories, refer to Table S6.

Both Kit ligands cooperate with Epo to promote erythroid expansion in zebrafish embryos

To test the function of both Kit ligands *in vivo*, we microinjected cytokine mRNA(s) into 1-cell stage embryos. When combined with *epo*, both *kitlga* and *kitlgb* showed an enhancement in *gata1*:*DsRed* expression (Figure 2A) and hemoglobinization (Figure 2B) at 72 hpf compared to *epo* alone. Quantification of benzidine staining in the tails of 72 hpf embryos confirmed the observed trend. Quantitative PCR (qPCR) analysis from whole injected embryos also showed an increase in the expression of erythroid markers at 72 hpf (Figure 2D) when *epo* was combined with either of the ligands, however, these changes were rather subtle.

The expression of both kit receptors is gradually increasing during embryonic development

To better understand the mechanism of cooperation of Epo with Kit ligands, we first analyzed the expression of both kit receptors in tissues and during the development by qPCR. Among all the tissues analyzed, the highest *kita* expression was observed in kidney (Figure S3A) while the highest kitb expression was observed in the retina (Figure S3B). During zebrafish development, the highest relative expression was observed at 72 hpf for both *kita* and *kitb* (Figure 3A). As we saw a significant expansion of erythroid, *gata1:DsRed*+ cells in *ex vivo* cultures, we wanted to analyze the dynamics of the receptor expression in erythroid cells during development. Therefore, we collected samples from 24 hpf, 48 hpf and 72 hpf *gata1:DsRed* and *LCR:GFP* embryos and FACS-sorted *gata1*:*DsRed*-positive cells (Figure 3B) or *LCR*:*GFP*-positive cells (Figure 3C), respectively. In both cases, we found that there is a gradually increasing expression of both receptors in erythroid cells during the course of development (Figure 3D).

Kita mediates Kitlg-dependent erythroid expansion in vivo

To understand the molecular basis of the effect we observed on erythroid cells, we used the *sparse* mutant (*kita*^{b5}) zebrafish, lacking the functional Kita receptor. Our data show that in *sparse* mutants, the effect of cooperation between any of the *kitlg* and *epo* is lost; however, we observed the effect of *epo* alone at comparable levels to the wild-type animals (Figure 4A). These findings are also supported by qPCR data

showing that the expression of β-globin (*hbbe1*) and *gata1a* is not increased after the injection of *epo* with either of the kit ligands, as is the case in wild-type embryos (Figure 4B).

To summarize, we have cloned and expressed both Kit ligand zebrafish paralogs. Using the purified recombinant proteins, we tested various cytokine combinations in liquid culture of whole kidney marrow cells and defined an optimal factor combination, composed of Epo, Kitlga and dexamethasone, which enables efficient expansion of erythroid progenitors. Also, we tested gain of function effect of both *kitlga and kitlgb* in embryonic hematopoiesis and observed an increase in the number of erythroid cells in *epo, kitlgb* and *epo, kitlgb* mRNA-injected fish compared to *epo* alone. Finally, by employing the *kita*$^{-/-}$ (*sparse*) mutants, we showed that Kita is mediating Kitlg-dependent erythroid expansion *in vivo*.

Discussion

In this study, we explored the role of zebrafish Kit ligands in hematopoiesis. In zebrafish, multiple paralogs of many important genes resulted from an extra round of whole genome duplication 250-350 million years ago[32,33], including hematopoietic cytokines. After such an event, the process of pseudogenization often leads to a loss of function of one of the paralogs[34-36]. Alternatively, paralogs of an ancestral gene can either remain functionally redundant, split the original function between the two (subfunctionalization), or acquire completely new functions (neofunctionalization)[37]. Here, we studied the hematopoietic function of two retained zebrafish paralogs of a crucial hematopoietic cytokine, the Kit ligand (KITL or Stem Cell Factor – SCF), designated as Kitlga and Kitlgb.

So far, KIT signaling has not been associated with erythropoiesis in zebrafish [14,15]. This is in contrast with previous studies in human[2], mouse[38] and chicken[6,39,40] model organisms, where it has been shown that KITL is critical for proper erythroid development[4,41,42]. By focusing on Kit function during erythropoiesis in zebrafish, we were able to prove for the first time, using *ex vivo* and *in vivo* gain- and loss-of-function experiments, that Kit signaling does indeed play a role in zebrafish erythroid differentiation. This happens in a similar manner as in other vertebrate species, via induction of cell proliferation coupled with differentiation, promoting cell cycle progression and enhancing protein translation in differentiating cells.

To examine the role of Kit ligands *ex vivo*, we used clonal ex vivo assays and liquid culture experiments[13,26,27,43]. Since the reported cross-reactivity of mammalian hematopoietic cytokines in zebrafish is very limited, it was essential to use bona fide zebrafish Kit ligands as recombinant proteins. For both ligands, we cloned the extracellular portion of the corresponding cDNAs, lacking the N-terminal

signal peptide (SP), transmembrane domain (TM) and cytosolic part (C). This approach allowed us to generate soluble form of Kit ligands to stimulate kidney marrow progenitor cells *ex vivo*[2,5].

As a result, treatment of hematopoietic progenitor cells isolated from kidney marrow with Kitlga, but not Kitlgb, in combination with the other master regulator of erythroid lineage commitment, Epo[12,13], enabled erythroid expansion of cells in liquid cultures and clonal assays. Next, we also tested the activity of dexamethasone (Dex) in addition to Epo and Kitlga. Dex is a glucocorticoid receptor agonist that has been reported to support self-renewal and differentiation of erythroid progenitors[3,28-30]. Indeed, addition of Dex to Kitlga and Epo led to synergistic erythroid expansion in liquid cultures.

To understand the mechanism triggering this substantial cell expansion, we performed an RNAseq experiment. We decided to compare the effect of Epo and Dex versus Epo, Dex, and Kitlga treatment on adult kidney marrow cells. Transcriptional profiling showed expression of erythroid specific genes in both conditions, as expected due to Epo-mediated signaling. However, we observed relatively subtle changes in expression between the Epo and Dex and the Epo, Dex, and Kitlga cultures. The trends of expression changes showed a distinct erythroid fingerprint in cells treated with Epo, Dex and Kitlga, compared to a more mixed/myeloid one in cells treated with Epo and Dex alone, as suggested by the increased expression of myeloid-specific genes. This corresponds to the fact that isolation of kidney marrow cells yields, in addition to progenitor cells, also a fraction of myeloid cells with the ability to survive in the culture. However, this effect becomes negligible during erythroid expansion of cells treated with Epo, Dex, and Kitlga, when myeloid cells are overgrown by expanding erythroid progenitors. Importantly, gene ontology analysis revealed an enrichment especially in translation and expression of erythroid-specific genes in Epo, Dex and Kitlga cultures. Therefore, we hypothesize that Kitlga might enhance erythroid cell development and cell proliferation potentially through upregulation of genes related to protein translation and cell cycle progression.

To confirm these findings, we decided to test whether Kitlg signaling is involved also in zebrafish erythropoiesis *in vivo*. Surprisingly, and in contrast to *ex vivo* cultures, we found that in addition to kitlga, also kitlgb was potent in expansion of erythroid cells at 72 hpf when injected together with *epo* mRNA (Figure 2D). We hypothesize that the discrepancy between the *ex vivo* and *in vivo* results in respect to Kitlgb activity, could be partially explained by its ability to act on HSPCs *in vivo* as previously described[18]. Erythroid progenitor cells may not be responsive to Kitlgb in the cell-autonomous manner required in *ex vivo* cultures.

Finally, we investigated, if Kita receptor is dispensable in Kitlg-mediated erythroid cell expansion *in vivo*. As a result, we did not observe the cooperation of *epo* with either of the *kit* ligands in erythroid differentiation in *kita*$^{-/-}$ (*sparse*) mutant fish. These data suggest that Kita is responsible for Kit signal transduction in erythroid cells. Although Kitb might also be involved in this process, it couldn't compensate for missing Kita, and Kita seems to be required for Kit signaling in zebrafish erythroid cells.

To summarize, in this study we have addressed the question of the role of Kitlg in hematopoiesis in zebrafish using both *in vivo* and *ex vivo* approaches. Importantly, we established optimized liquid culture conditions for erythroid expansion of kidney marrow progenitor cells that require the addition of Kitlga. Together with colony forming assays, liquid cultures might serve as indispensable tools in disease modelling, allowing analysis of normal and aberrant hematopoiesis in zebrafish blood mutants (vlade, moonshine, mindbomb, etc.)[44]. This will enable further biochemical, genomic and proteomic analysis of *ex vivo* cultured and expanded cells.

Interestingly, zebrafish Kitlga and Kitlgb seem to possess only partial redundancy in its function: Kitlga retains the role in melanogenesis and pigmentation, which is lost in the case of Kitlgb, but both ligands contribute to the overall process of erythroid commitment and development in zebrafish *in vivo*. Moreover, based on the differences in *ex vivo* and *in vivo* activity of Kiltgb, we hypothesize that although finally contributing to the same process of erythroid progenitor expansion, both ligands might be targeting different subsets of hematopoietic progenitors. In *ex vivo* cultures, Kitlga might be targeting relatively more abundant erythroid-committed progenitors present in kidney marrow by cell autonomous manner, whereas Kitlgb might rather affect relatively rare upstream HSPCs, in agreement with previous studies[17,18]. However, the exact mechanism of such functional diversification has yet to be investigated. Altogether, we demonstrate that role of Kit signaling is evolutionarily conserved as previously unnoticed[14], and our results further support the use of zebrafish as a model to study vertebrate hematopoiesis.

Acknowledgements

We thank Nikol Pavlu and Tereza Hojerova for animal care, Tereza Mikulasova for graphical work and cDNA samples, Trevor Epp for editing the manuscript and Leonard Zon for providing *gata1:DsRed* and *LCR:GFP* reporter fish lines. Supported by the Czech Science Foundation (16-21024S), Ministry of Health (NV19-07-00412), Ministry of Education, Youth and Sports - Program NPU I (LO1419) to PB. OS was partially funded by American Heart Association (19POST34380328).

Authorship contribution

J.O., O.M., O.S. performed the experiments, J.O, O.S., P.S., M.K. and P.B. designed the experiments, analyzed the data and wrote the manuscript.

References

1 Krantz, S. B. Erythropoietin. Blood 77, 419-434 (1991).

2 Broudy, V. C. Stem cell factor and hematopoiesis. Blood 90, 1345-1364 (1997).

3 Panzenbock, B., Bartunek, P., Mapara, M. Y. & Zenke, M. Growth and differentiation of human stem cell factor/erythropoietin-dependent erythroid progenitor cells in vitro. Blood 92, 3658-3668 (1998).

4 Ding, L., Saunders, T. L., Enikolopov, G. & Morrison, S. J. Endothelial and perivascular cells maintain haematopoietic stem cells. Nature 481, 457-462, doi:10.1038/nature10783 (2012).

5 Lennartsson, J. & Ronnstrand, L. Stem cell factor receptor/c-Kit: from basic science to clinical implications. Physiol Rev 92, 1619-1649, doi:10.1152/physrev.00046.2011 (2012).

6 Hayman, M. J., Meyer, S., Martin, F., Steinlein, P. & Beug, H. Self-renewal and differentiation of normal avian erythroid progenitor cells: regulatory roles of the TGF alpha/c-ErbB and SCF/c-kit receptors. Cell 74, 157-169 (1993).

7 Grabbe, J., Welker, P., Dippel, E. & Czarnetzki, B. M. Stem cell factor, a novel cutaneous growth factor for mast cells and melanocytes. Arch Dermatol Res 287, 78-84, doi:10.1007/bf00370723 (1994).

8 Alexeev, V. & Yoon, K. Distinctive role of the cKit receptor tyrosine kinase signaling in mammalian melanocytes. J Invest Dermatol 126, 1102-1110, doi:10.1038/sj.jid.5700125 (2006).

9 Gong, Y. et al. SCF/SCFR signaling plays an important role in the early morphogenesis and neurogenesis of human embryonic neural retina. Development 146, doi:10.1242/dev.174409 (2019).

10 Ashman, L. K. The biology of stem cell factor and its receptor C-kit. The international journal of biochemistry & cell biology 31, 1037-1051, doi:10.1016/s1357-2725(99)00076-x (1999).

11 Abbaspour Babaei, M., Kamalidehghan, B., Saleem, M., Huri, H. Z. & Ahmadipour, F. Receptor tyrosine kinase (c-Kit) inhibitors: a potential therapeutic target in cancer cells. Drug Des Devel Ther 10, 2443-2459, doi:10.2147/DDDT.S89114 (2016).

12 Paffett-Lugassy, N. et al. Functional conservation of erythropoietin signaling in zebrafish. Blood 110, 2718-2726, doi:10.1182/blood-2006-04-016535 (2007).

13 Stachura, D. L. et al. Clonal analysis of hematopoietic progenitor cells in the zebrafish. Blood 118, 1274-1282, doi:10.1182/blood-2011-01-331199 (2011).

14 Parichy, D. M., Rawls, J. F., Pratt, S. J., Whitfield, T. T. & Johnson, S. L. Zebrafish sparse corresponds to an orthologue of c-kit and is required for the morphogenesis of a subpopulation of melanocytes, but is not essential for hematopoiesis or primordial germ cell development. Development 126, 3425-3436 (1999).

15 Hultman, K. A., Bahary, N., Zon, L. I. & Johnson, S. L. Gene Duplication of the zebrafish kit ligand and partitioning of melanocyte development functions to kit ligand a. PLoS genetics 3, e17, doi:10.1371/journal.pgen.0030017 (2007).

16 Mellgren, E. M. & Johnson, S. L. kitb, a second zebrafish ortholog of mouse Kit. Development Genes and Evolution 215, 470-477, doi:10.1007/s00427-005-0001-3 (2005).

17 Mahony, C. B., Pasche, C. & Bertrand, J. Y. Oncostatin M and Kit-Ligand Control Hematopoietic Stem Cell Fate during Zebrafish Embryogenesis. Stem cell reports 10, 1920-1934, doi:10.1016/j.stemcr.2018.04.016 (2018).

18 Mahony, C. B., Fish, R. J., Pasche, C. & Bertrand, J. Y. tfec controls the hematopoietic stem cell vascular niche during zebrafish embryogenesis. Blood 128, 1336-1345, doi:10.1182/blood-2016-04-710137 (2016).

19 von Lindern, M. et al. The glucocorticoid receptor cooperates with the erythropoietin receptor and c-Kit to enhance and sustain proliferation of erythroid progenitors in vitro. Blood 94, 550-559 (1999).

20 Westerfield, M. A guide for the laboratory use of zebrafish (Danio rerio). 4th ed., (Univ. of Oregon Press, Eugene, 2000).

21 Alestrom, P. et al. Zebrafish: Housing and husbandry recommendations. Lab Anim, 23677219869037, doi:10.1177/0023677219869037 (2019).

22 Oltova, J. et al. Zebrabase: An Intuitive Tracking Solution for Aquatic Model Organisms. Zebrafish 15, 642-647, doi:10.1089/zeb.2018.1609 (2018).

23 Traver, D. et al. Transplantation and in vivo imaging of multilineage engraftment in zebrafish bloodless mutants. Nature immunology 4, 1238-1246, doi:10.1038/ni1007 (2003).

24 Ganis, J. J. et al. Zebrafish globin switching occurs in two developmental stages and is controlled by the LCR. Developmental biology 366, 185-194, doi:10.1016/j.ydbio.2012.03.021 (2012).

25 Svoboda, O. et al. Ex vivo tools for the clonal analysis of zebrafish hematopoiesis. Nature protocols 11, 1007-1020, doi:10.1038/nprot.2016.053 (2016).

26 Stachura, D. L. et al. The zebrafish granulocyte colony-stimulating factors (Gcsfs): 2 paralogous cytokines and their roles in hematopoietic development and maintenance. Blood 122, 3918-3928, doi:10.1182/blood-2012-12-475392 (2013).

27 Stachura, D. L. et al. Zebrafish kidney stromal cell lines support multilineage hematopoiesis. Blood 114, 279-289, doi:10.1182/blood-2009-02-203638 (2009).

28 Kolbus, A. et al. Cooperative signaling between cytokine receptors and the glucocorticoid receptor in the expansion of erythroid progenitors: molecular analysis by expression profiling. Blood 102, 3136-3146, doi:10.1182/blood-2003-03-0923 (2003).

29 England, S. J., McGrath, K. E., Frame, J. M. & Palis, J. Immature erythroblasts with extensive ex vivo self-renewal capacity emerge from the early mammalian fetus. Blood 117, 2708-2717, doi:10.1182/blood-2010-07-299743 (2011).

30 Leberbauer, C. et al. Different steroids co-regulate long-term expansion versus terminal differentiation in primary human erythroid progenitors. Blood 105, 85-94, doi:10.1182/blood-2004-03-1002 (2005).

31 Schroeder, C., Gibson, L., Nordstrom, C. & Beug, H. The estrogen receptor cooperates with the TGF alpha receptor (c-erbB) in regulation of chicken erythroid progenitor self-renewal. EMBO J 12, 951-960 (1993).

32 Hoegg, S., Brinkmann, H., Taylor, J. S. & Meyer, A. Phylogenetic timing of the fish-specific genome duplication correlates with the diversification of teleost fish. J Mol Evol 59, 190-203, doi:10.1007/s00239-004-2613-z (2004).

33 Amores, A., Catchen, J., Ferrara, A., Fontenot, Q. & Postlethwait, J. H. Genome evolution and meiotic maps by massively parallel DNA sequencing: spotted gar, an outgroup for the teleost genome duplication. Genetics 188, 799-808, doi:10.1534/genetics.111.127324 (2011).

34 Nei, M. & Roychoudhury, A. K. Probability of fixation and mean fixation time of an overdominant mutation. Genetics 74, 371-380 (1973).

35 Takahata, N. & Maruyama, T. Polymorphism and loss of duplicate gene expression: a theoretical study with application of tetraploid fish. Proceedings of the National Academy of Sciences of the United States of America 76, 4521-4525 (1979).

36 Watterson, G. A. On the time for gene silencing at duplicate Loci. Genetics 105, 745-766 (1983).

37 Force, A. et al. Preservation of duplicate genes by complementary, degenerative mutations. Genetics 151, 1531-1545 (1999).

38 Antonchuk, J., Hyland, C. D., Hilton, D. J. & Alexander, W. S. Synergistic effects on erythropoiesis, thrombopoiesis, and stem cell competitiveness in mice deficient in thrombopoietin and steel factor receptors. Blood 104, 1306-1313, doi:10.1182/blood-2004-04-1522 (2004).

39 Bartunek, P. et al. Avian stem cell factor (SCF): production and characterization of the recombinant His-tagged SCF of chicken and its neutralizing antibody. Cytokine 8, 14-20, doi:10.1006/cyto.1996.0003 (1996).

40 Dolznig, H., Bartunek, P., Nasmyth, K., Mullner, E. W. & Beug, H. Terminal differentiation of normal chicken erythroid progenitors: shortening of G1 correlates with loss of D-cyclin/cdk4 expression and altered cell size control. Cell Growth Differ 6, 1341-1352 (1995).

41 Chabot, B., Stephenson, D. A., Chapman, V. M., Besmer, P. & Bernstein, A. The proto-oncogene c-kit encoding a transmembrane tyrosine kinase receptor maps to the mouse W locus. Nature 335, 88-89, doi:10.1038/335088a0 (1988).

42 Huang, E. et al. The hematopoietic growth factor KL is encoded by the Sl locus and is the ligand of the c-kit receptor, the gene product of the W locus. Cell 63, 225-233, doi:10.1016/0092-8674(90)90303-v (1990).

43 Svoboda, O. et al. Dissection of vertebrate hematopoiesis using zebrafish thrombopoietin. Blood 124, 220-228, doi:10.1182/blood-2014-03-564682 (2014).

44 Ransom, D. G. et al. Characterization of zebrafish mutants with defects in embryonic hematopoiesis. Development 123, 311-319 (1996).

Figure legends

Figure 1. The effect of zebrafish Kitlga and Kitlgb on self-renewal and proliferation of hematopoietic cells *ex vivo*.

(A, B) Quantification of the number of whole kidney marrow cells after treatment with PBS (control) or specific cytokine combinations for 3 days (A) and 7 days (B). Statistical significance was assessed using standard one-way ANOVA.

(C) Growth curve for *ex vivo* culture of whole kidney marrow cells with different cytokine combinations. Biological duplicates for every condition and timepoint are shown. Representative photomicrograph (right) of whole kidney marrow cells isolated from transgenic *gata1:DsRed* fish treated with PBS (control) or a specific cytokine combinations and cultivated for 8 days. Scale bar represents 100 um.

(D, E) An expression heatmap of Epo, Dex and Epo, Dex, Kitlga treated cells showing the top predicted differentially expressed genes (D) and representative markers of erythroid differentiation (E). Color coding by z-score for heatmaps in E and F.

Figure 2. *In vivo* effects of *kitlga* and *kitlgb* at 72 hpf.

(A) Representative micrographs of *gata1*:*DsRed* transgenic reporter larvae at 72 hpf after injection corresponding mRNAs. Control represents uninjected larvae. Images were acquired on Zeiss Axio Zoom.V16.

(B) Representative micrographs of benzidine stained embryonic tails at 72 hpf after injection of corresponding mRNAs. Control represents uninjected embryos. Images were acquired on Zeiss Axio Zoom.V16 as z-stacks and processed using Extended Depth of Focus module in the ZEN blue software.

(C) Quantification of the benzidine stained embryonic tails in (B). Values were plotted using the mean of *epo* as the relative expression fold 1.

(D) qPCR expression analysis of *gata1a* and *hbbe1* from whole injected embryos at 72 hpf. Data have been normalized using *mob4* as the housekeeping gene and using uninjected control as the relative expression fold 1. Bars represent a mean of three triplicates with SD.

Figure 3. Expression of *kit* receptors in the development

(A) qPCR expression analysis of *kita* (left) and *kitb* (right) at different developmental stages. Data was normalized using *ef1a* as the housekeeping gene and using the 24 hpf sample as relative expression fold 1.

(B) Sorting strategy used for FACS isolation of *gata1:DsRed* positive cells (top left) from transgenic zebrafish at 24 hpf stage. qPCR expression analysis of *gata1* (top right), *kita* (bottom left) and *kitb* (bottom right) in *gata1:DsRed* negative and positive cells. Data was normalized using *ef1a* as the housekeeping gene and using the *gata1:DsRed* positive 24 hpf sample as relative expression fold 1.

(C) Sorting strategy used for FACS isolation of *LCR:GFP* positive cells (top left) from transgenic zebrafish at 24 hpf stage. qRT-PCR expression analysis of *gata1* (top right), *kita* (bottom left) and *kitb* (bottom right) in *LCR:GFP* negative and positive cells. Data was normalized using *ef1a* as the housekeeping gene and using the *LCR:GFP* positive 24 hpf sample as relative expression fold 1.

Figure 4. The effect of *kitlga* and *kitlgb* in *sparse* mutants.

(A) Quantification of the benzidine staining in control (uninjected) or *epo*, *kitlga* and *kitlgb* mRNA injected WT or *kita*$^{-/-}$ (*sparse*) embryos. Values were plotted relatively to the mean of *epo* (=1). NOTE that Figure 4A left is re-used from Figure 2C for demonstrative purposes.

(B) qPCR expression analysis of *gata1a* and *hbbe1* from whole injected *kita*$^{-/-}$ (*sparse*) embryos at 72 hpf. Data was normalized using *mob4* as the housekeeping gene and uninjected control as the relative expression fold 1. Bars represent mean of three triplicates with SD.

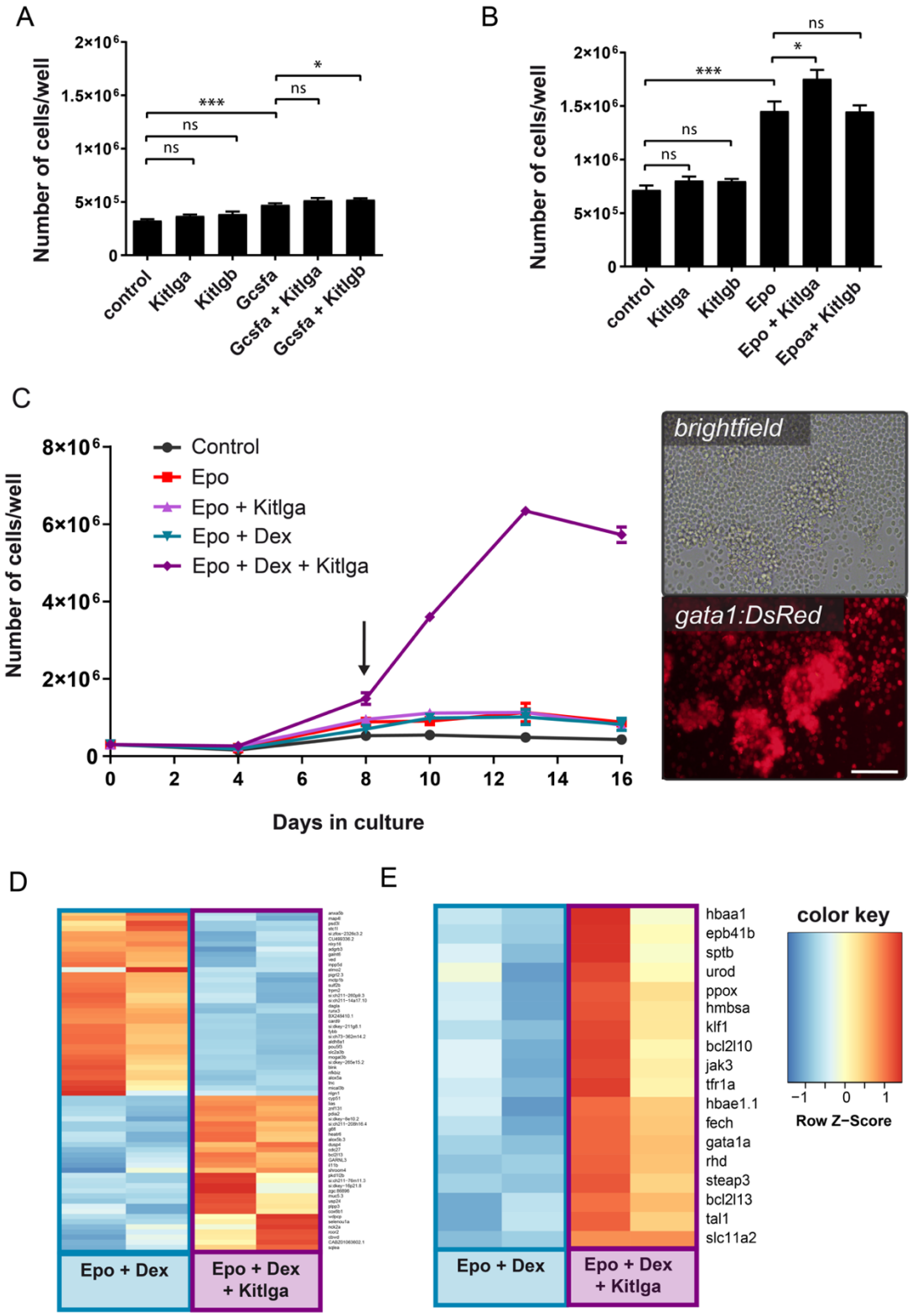
A
Number of cells/well
2×10⁶
1.5×10⁶
1×10⁶
5×10⁵
0
***
ns
ns
*
ns
control
Kitlga
Kitlgb
Gcsfa
Gcsfa + Kitlga
Gcsfa + Kitlgb
B
Number of cells/well
2×10⁶
1.5×10⁶
1×10⁶
5×10⁵
0
***
ns
ns
ns
*
control
Kitlga
Kitlgb
Epo
Epo + Kitlga
Epoa+ Kitlgb
C
Number of cells/well
8×10⁶
6×10⁶
4×10⁶
2×10⁶
0
Control
Epo
Epo + Kitlga
Epo + Dex
Epo + Dex + Kitlga
0
2
4
6
8
10
12
14
16
Days in culture
brightfield
gata1:DsRed
D
Epo + Dex
Epo + Dex + Kitlga
E
hbaa1
epb41b
sptb
urod
ppox
hmbsa
klf1
bcl2l10
jak3
tfr1a
hbae1.1
fech
gata1a
rhd
steap3
bcl2l13
tal1
slc11a2
Epo + Dex
Epo + Dex + Kitlga
color key
−1
0
1
Row Z−Score

Figure 1

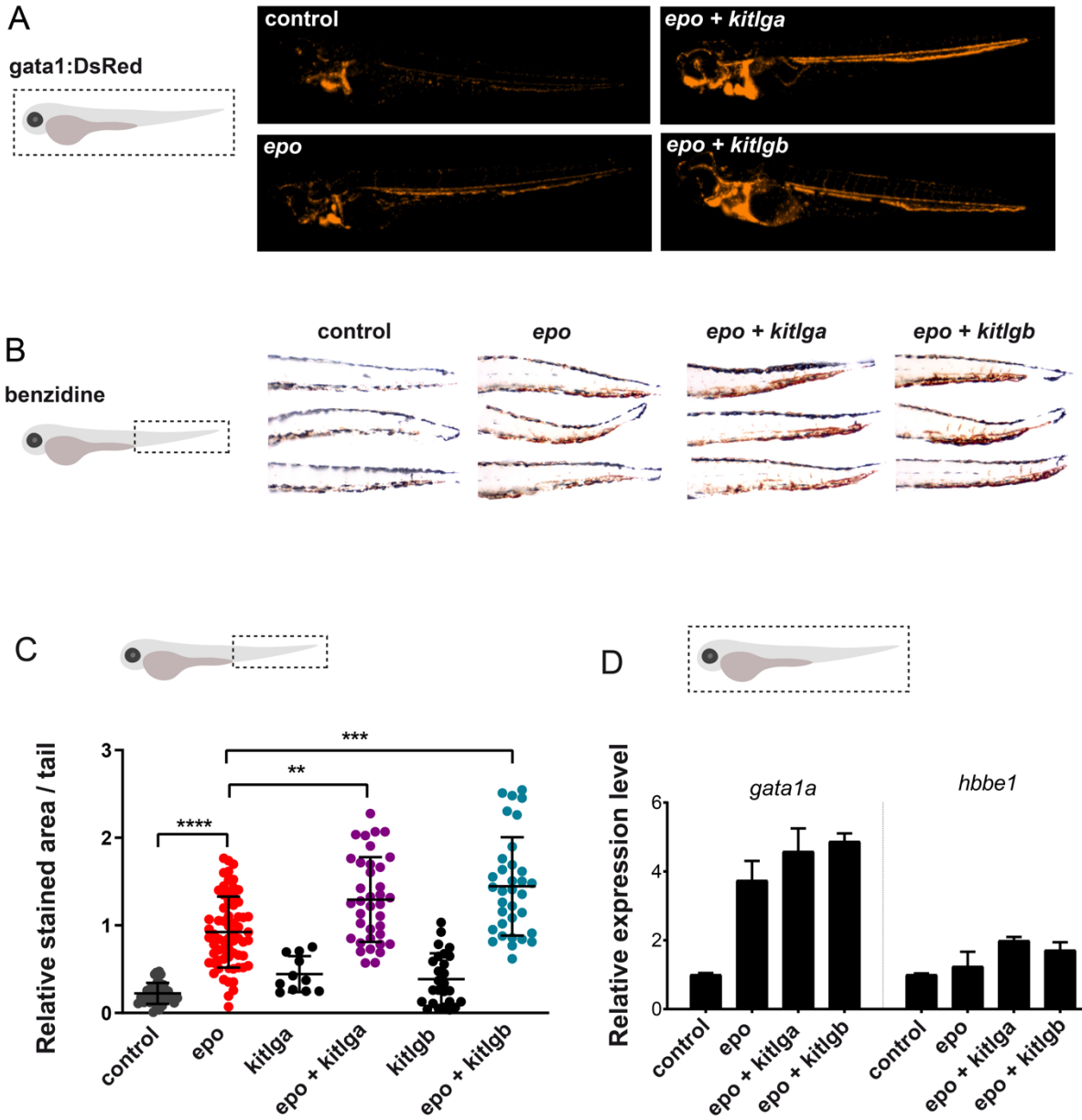
A
gata1:DsRed
control
epo + kitlga
epo
epo + kitlgb
B
benzidine
control
epo
epo + kitlga
epo + kitlgb
C
Relative stained area / tail
****
**
***
control
epo
kitlga
epo + kitlga
kitlgb
epo + kitlgb
D
Relative expression level
gata1a
hbbe1
control
epo
epo + kitlga
epo + kitlgb

Figure 2

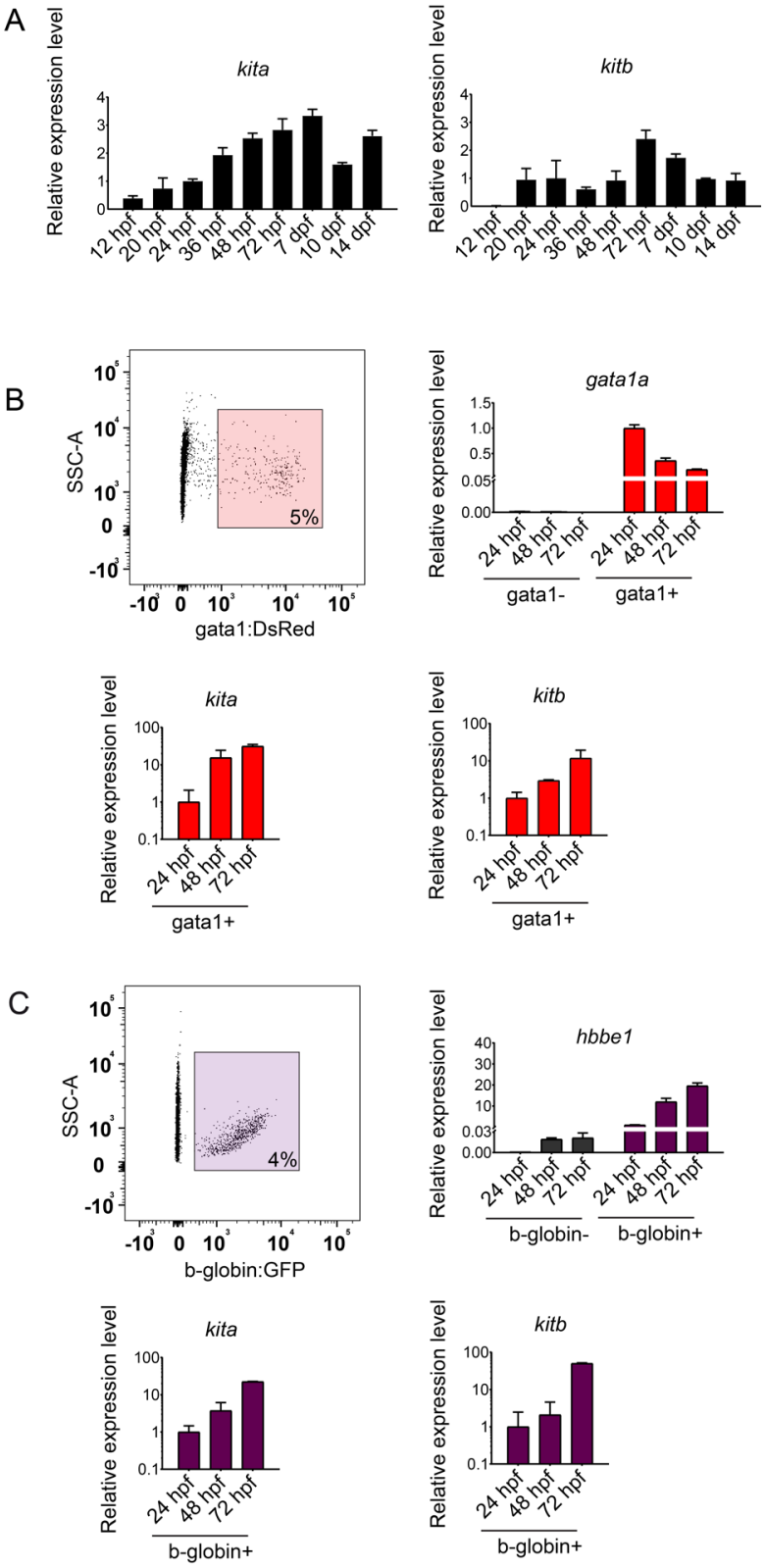
A
kita
kitb
Relative expression level
12 hpf
20 hpf
24 hpf
36 hpf
48 hpf
72 hpf
7 dpf
10 dpf
14 dpf
B
SSC-A
gata1:DsRed
5%
gata1a
gata1-
gata1+
kita
kitb
C
b-globin:GFP
4%
hbbe1
b-globin-
b-globin+

Figure 3

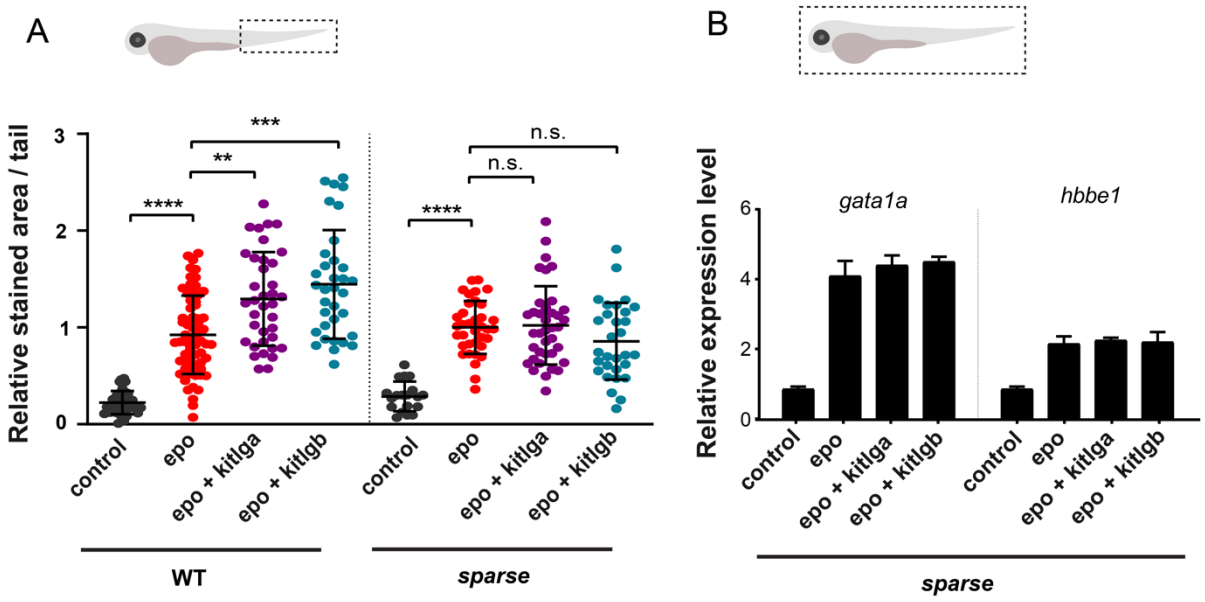
A
B
Relative stained area / tail
3
2
1
0
****
**
***
****
n.s.
n.s.
control
epo
epo + kitlga
epo + kitlgb
control
epo
epo + kitlga
epo + kitlgb
WT
sparse
Relative expression level
6
4
2
0
gata1a
hbbe1
control
epo
epo + kitlga
epo + kitlgb
control
epo
epo + kitlga
epo + kitlgb
sparse

Figure 4