Wilms Tumor 1b Expression Defines a Pro-regenerative Macrophage Subtype and Is Required for Organ Regeneration in the Zebrafish

Highlights

- Wt1b+ macrophages reveal a pro-regenerative gene expression signature
- Wt1b controls migration behavior of macrophages during fin and heart regeneration
- Wt1b regulates differentiation of macrophages in the kidney marrow
- wt1b mutants reveal impaired fin and heart regeneration

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In Brief

Sanz-Morejón et al. identify Wilms tumor 1b (Wt1b)+ macrophages with a pro-regenerative gene signature in injured fins and hearts in the zebrafish. They show that Wt1b controls macrophage migration and differentiation. Regeneration is impaired in wt1b mutants, supporting a role for this gene, likely within macrophages, in organ regeneration.
Wilms Tumor 1b Expression Defines a Pro-regenerative Macrophage Subtype and Is Required for Organ Regeneration in the Zebrafish

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SUMMARY

Organ regeneration is preceded by the recruitment of innate immune cells, which play an active role during repair and regrowth. Here, we studied macrophage subtypes during organ regeneration in the zebrafish, an animal model with a high regenerative capacity. We identified a macrophage subpopulation expressing Wilms tumor 1b (wt1b), which accumulates within regenerating tissues. This wt1b+ macrophage population exhibited an overall pro-regenerative gene expression profile and different migratory behavior compared to the remainder of the macrophages. Functional studies showed that wt1b regulates macrophage migration and retention at the injury area. Furthermore, wt1b-null mutant zebrafish presented signs of impaired macrophage differentiation, delayed fin growth upon caudal fin amputation, and reduced cardiomyocyte proliferation following cardiac injury that correlated with altered macrophage recruitment to the regenerating areas. We describe a pro-regenerative macrophage subtype in the zebrafish and a role for wt1b in organ regeneration.

INTRODUCTION

Proper control of the inflammatory response, including its duration and the specific immune cell types recruited to the injury site, can determine whether a damaged tissue undergoes fibrotic healing or proceeds to regeneration (Godwin et al., 2017b). In recent years, macrophages have emerged as key players in organ regeneration (Mescher, 2017; Wynn and Vannella, 2016). Macrophage depletion leads to the blockage of several regenerative processes such as limb regeneration in the axolotl (Godwin et al., 2013) and heart regeneration in the zebrafish, axolotl, and neonatal mice (Aurora et al., 2014; Godwin et al., 2017a; Lai et al., 2017).

Macrophages are classically considered to be recruited to the site of damage in two phases (Amici et al., 2017). In the first phase, monocytes are polarized to a pro-inflammatory phenotype by a specific set of cytokines, including tumor necrosis factor (TNF). In the second reparative-regenerative phase, macrophages with different gene signatures are detected at the site of injury (Amici et al., 2017). Whether pro-inflammatory and pro-regenerative phases represent a phenotypic switch within individual macrophages in response to environmental cues or whether newly arriving pro-regenerative macrophage waves displace the original pro-inflammatory population remains unclear. Accumulating evidence challenges the classical M1 and M2 classification and suggests a much more complex spectrum of macrophage polarization states (Murray et al., 2014).

In the zebrafish, a model organism with a high regenerative capacity, macrophages are necessary for the regeneration of the lateral line (Carrillo et al., 2016), fins (Li et al., 2012; Petrie et al., 2014), spinal cord, and the heart (Lai et al., 2017; Tsarouchas et al., 2018). As a first response, macrophages with a pro-inflammatory gene signature are attracted to the site of injury. Similar to that in mammals, the expression of tnf alpha (tnfa) is a hallmark of zebrafish pro-inflammatory macrophages (Nguyen-Chi et al., 2015). During wound resolution, tnf alpha expression in macrophages is downregulated, suggesting a transition from a pro- to an anti-inflammatory population or polarization state (Nguyen-Chi et al., 2015). Nevertheless, the precise dynamics and changes in the gene expression profile of macrophages during zebrafish organ regeneration remain poorly understood.
In a recent transcriptomics study in mouse hearts, the gene encoding the zinc finger transcription factor Wilms tumor suppressor 1 (WT1) was found to be expressed in cardiac leukocytes in the neonatal mouse but not in adults, coinciding with the time window during which heart regeneration occurs in mice (Quaife-Ryan et al., 2017). Aberrant WT1 expression has been identified in some myeloid leukemias in mammals (Rossi et al., 2016), but WT1 has not previously been reported in healthy differentiated myeloid cells. Regarding the heart, WT1 has mostly been studied during the formation of the epicardium, the outer layer covering the myocardium (Martínez-Estrada et al., 2010; Moore et al., 1999). Whereas WT1 is downregulated in the adult epicardium in the mouse, its expression is reactivated upon myocardial infarction (Zhou et al., 2011). The zebrafish has two WT1 orthologs, wt1a and wt1b, which are expressed in the kidney and mesothelium of several organs, including the heart. Similar to the mouse, wt1b is upregulated in the epicardium in response to cardiac damage (González-Rosa et al., 2011; Schnabel et al., 2011) and in sheath cells during notochord repair (Lopez-Baez et al., 2018). Hence, wt1b may represent a marker gene for the early phase of regeneration, although its roles during regenerative processes remain unknown.

RESULTS

wt1b is Expressed in a Subtype of Macrophages

Upon cardiac insult, immune cells are recruited to the injury area and actively participate in debris clearance and inflammatory response control, among other roles. The epicardium, the outer layer covering the myocardium, plays an active role in the recruitment of immune cells to the injured heart (Huang et al., 2012). To characterize macrophage recruitment dynamics following cardiac injury, we crossed the transgenic zebrafish Tg(wt1b:eGFP), which labels epicardium-derived cells (EPDCs) that infiltrate the damaged tissue (Sim dés and Riley, 2018), with Tg(mpeg1: mCherry), a line widely used to label macrophages in the zebrafish (Ellett et al., 2011).

In double transgenic animals, we performed immunofluorescence staining on heart sections of the cardiac ventricle at different stages post-cryoinjury or sham operated (Figures 1A–1G) and co-immunostained with the pan-leukocyte marker L-plastin (Feng et al., 2010). A subset of the mpeg1: mCherry;L-plastin* population was also positive for wt1b:eGFP expression. Flow cytometry analysis further confirmed the presence of wt1b:eGFP*:mpeg1:mCherry* cells (Figures 1H and 1I). The percentage of wt1b:eGFP*:mpeg1:mCherry* versus mpeg1:mCherry* cells increased at 4 dpi (from 50% ± 16% to 69% ± 11%); the total number of mCherry* cells was sham 761 ± 347, n = 11; 4 dpi 2,567 ± 1,405, n = 19). At late stages post-injury, 21 dpi, the percentage of wt1b:eGFP:expressing macrophages declined (44% ± 10%; total number of mCherry* cells 1,526 ± 1,342, n = 12).

Histological staining of isolated wt1b:eGFP*:mpeg1:mCherry* and mpeg1:mCherry* cells from cryoinjured hearts revealed a typical myeloid cell shape with large cytoplasmic areas and an irregular nuclear shape. Double-positive cells were slightly larger than mpeg1:mCherry* cells (Figures 1J and 1K), suggesting that wt1b:eGFP expression distinguished a specific macrophage population in the cryoinjured zebrafish heart. qRT-PCR analysis confirmed that the wt1b:eGFP*:mpeg1:mCherry* population expressed higher levels of wt1b mRNA than mpeg1:mCherry* cells (Figure 1L), providing evidence that the Tg(wt1b:eGFP) reporter line does indeed recapitulate the endogenous wt1b expression pattern.

To further test whether wt1b:eGFP*:mpeg1:mCherry* cells represent a macrophage population, we confirmed the phagocytic activity of wt1b:eGFP* cells by injecting fluorescently labeled inactivated Escherichia coli into the trunk of Tg(wt1b:eGFP) larvae followed by in vivo imaging (Video S1; observed in 5 of 6 larvae).

Overall, these findings establish the presence of wt1b* macrophages in the heart upon cardiac injury.

wt1b* Macrophages Present a Pro-regenerative Gene Signature

We next sought to further analyze whether wt1b expression reflects the presence of a specific macrophage subtype. To do this, we performed RNA sequencing (RNA-seq) analysis of sorted wt1b:eGFP*:mpeg1:mCherry* cells and compared their transcriptome to the rest of mpeg1:mCherry* cells sorted from adult zebrafish hearts at 4 dpi (Figures 2A and S1A–S1C). In total, 278 genes, including wt1b itself, were upregulated in the wt1b:eGFP*:mpeg1:mCherry* subset, whereas 314 genes were upregulated in mpeg1:mCherry* cells (Figure 2B; Table S1).

The leucocyte marker gene lcp1, the myeloid marker genes spi1a and mpeg1.1, tlr7, tlr9, arg2, and rln1 (Table S2). In contrast, the wt1b* macrophages presented an upregulation of genes related to extracellular matrix remodeling and tissue homeostasis restoration such as tmp2b, mmp14a, vcanb, mafb, mafb, c1qa, and c1qb genes (Figures 2B, 2C, and S1D; Table S2). In this population, we also detected the upregulation of il1b, a cytokine that plays a pivotal role in the modulation of spinal cord regeneration in the zebrafish (Tsarouchas et al., 2018). We also investigated whether direct targets of WT1, which can act as a transcriptional activator or repressor (Chau and Hastie, 2012), could be found among the differentially expressed genes. myca, which is repressed by WT1, is downregulated, while mafb and mafb genes, which are positively regulated by WT1, are upregulated in wt1b* macrophages (Dong et al., 2015; Hewitt et al., 1995). This suggests that wt1b itself may be involved in the regulation of the pro-regenerative gene signature observed in this population (Figures 2B, 2C, and S1D).

Some of the target and upregulated genes were validated by RNAscope in situ hybridization. In this manner, we confirmed the expression of wt1b, mafb, and mmp14a transcripts in wt1b:eGFP*:mpeg1:mCherry* macrophages (Figures S1E–S1J).

At the same time, Gene Ontology (GO) biological processes analysis revealed enrichment in blood vessel development, leukocyte migration, and regulation of the inflammatory response, among others, in the wt1b* macrophage population (Figure 2D;
wt1b+ macrophages present a more pro-regenerative gene signature.

wt1b+ Macrophages Preferentially Persist in the Injured Tissue during the Regenerative Phase

The transcriptome analysis suggests different cell behaviors, including migration in wt1b+ macrophages. Accordingly, we next examined their migratory capacity and localization at the injury site using a tissue regeneration system that allows in vivo monitoring of cell migration—caudal fin resection in zebrafish larvae (Roehl, 2018) (Figure 3A).

In uninjured larvae, mpeg1:mCherry+ macrophages within the caudal tail were mostly negative for wt1b:eGFP expression (Figures 3B–3C’’), whereas wt1b:eGFP+; mpeg1:mCherry+ cells were present in larval fins post-amputation (Figures 3D–3M’’). We analyzed wt1b:eGFP expression dynamics during macrophage infiltration and their localization during the regenerative process. During the first 12 h post-amputation (hpa), mpeg1:mCherry+ and a few wt1b:eGFP+; mpeg1:mCherry+ cells reached the site of resection (Figures 3D–3G’’; Video S2). This time frame corresponds to a previously described early wave of pro-inflammatory macrophages homing to the site of injury (Nguyen-Chi et al., 2015). Some mpeg1:mCherry+ cells that homed to the injury area showed wt1b:eGFP upregulation near the amputation site (Video S2), providing evidence for wt1b+ upregulation in a subset of macrophages in response to injury. At later stages of fin regeneration (between 24 and 72 hpa), when the presence of pro-inflammatory macrophages has been reported to decline (Nguyen-Chi et al., 2015), we observed that wt1b:eGFP+; mpeg1:mCherry+ cells preferentially accumulated at the regenerating area, whereas wt1b+ mpeg1:mCherry+ cells were found to be more scattered throughout the larval trunk (Figures 3H–3M’’). In vivo imaging during 34 hpa revealed that the accumulation of wt1b+ macrophages at the regeneration front is mainly a result of the upregulation of wt1b:eGFP expression in macrophages during migration rather than the arrival of a
second wave of wt1b:eGFP* (Video S3; Figure S2). This result suggests a shift of macrophage populations or a polarization state in the regenerating tissue coinciding with the transition from the inflammatory to the regenerative phase, being wt1b-expressing macrophages enriched during the latter (Figure 3N).

**Wt1b Influences the Migratory Behavior of Macrophages in Response to Injury**

We next compared the migration speed of wt1b:eGFP*;mpeg1:mCherry* and mpeg1:mCherry* macrophages in the larval caudal fin amputation model. Cell tracking during the first 12 hpa revealed that double-positive cells migrated at a lower speed than mpeg1:mCherry* cells (4.7 ± 2.1 versus 8 ± 0.9 μm/5 min) (Figure 3O).

To investigate the possible role of Wt1b in modulating macrophage migratory behavior upon injury, we generated a Tg(eGFP-UAS-RFP;mpeg1:Gal4) line to inhibit Wt1b function by overexpressing a dominant-negative isoform of wt1b (hereafter called wt1bDN) in macrophages when crossed with the Tg(mpeg1:Gal4) line (Ellett et al., 2011) (Figures S3A–S3D). The wt1bDN truncated protein lacks the four DNA binding zinc finger motifs and has previously been shown to interfere with endogenous WT1 function (Englert et al., 1995; Holmes et al., 1997). As a control, we used a Tg(eGFP-UAS-RFP;mpeg1:Gal4) line. In vivo imaging of eGFP* cells was performed in both transgenic lines after amputation of the caudal fin in zebrafish larvae (Figure 3P). We tracked wt1bDN-expressing macrophages for 12 hpa of the caudal fin, and mean cell migration speed was compared with that from the control RFP-expressing line (Figure 3Q; Video S4). Results showed that the mean migration speed of macrophages overexpressing wt1bDN was on average 30% faster than the RFP-expressing controls (15 ± 2 versus 11 ± 5 μm/10 min). Consistent with a higher degree of motility, we observed that the number of wt1bDN-expressing macrophages persisting in the regenerating caudal fin was reduced compared to the control group at late stages of regeneration (Figures 3R–3T).

Overall, these results show that the endogenous expression of wt1b defines a population of macrophages that accumulates after the initial pro-inflammatory response occurring during the early stage of fin regeneration. The finding that wt1b+ macrophages remain at the site of injury for a longer period suggests...
that Wt1b plays a role in the retention of macrophages at the regenerating tissue. We demonstrate that Wt1b itself influences macrophage migration dynamics.

**Hematopoietic Niche-Derived Cells Can Contribute to the Cardiac wt1b+ Macrophage Population**

Upon myocardial infarction in the mouse, monocytes derived from the bone marrow and spleen home to the heart and differentiate into macrophages (Honold and Nahrendorf, 2018). The source of macrophages contributing to regeneration in zebrafish remains, nevertheless, unknown.

The kidney marrow is the equivalent of the mammalian bone marrow in the zebrafish. To characterize wt1b expression within the hematopoietic niche, we studied the whole kidney marrow (WKM) composition of adult Tg(wt1b:eGFP;mpeg1:mCherry) zebrafish by flow cytometry (Figures 4A and 4B). wt1b:eGFP+ cells clustered mainly into the hematopoietic progenitor pool (gate 2) (Moore et al., 2016; Traver et al., 2003) (Figures 4B and 4B’). However, as observed in the heart, a subset of mpeg1:mCherry+ cells was also wt1b:eGFP+ (6.49% ± 4.55%) (Figures 4C and 4D). Histochemical staining of WKM-sorted wt1b:eGFP+; mpeg1:mCherry+ revealed a typical macrophage morphology and larger cell area than the remainder mpeg1:mCherry+ cells (Traver et al., 2003) (Figures 4E and 4F), which is consistent with our observations in the heart (Figures 1J and 1K).

To study whether the wt1b+ macrophage subpopulation in the regenerating heart derived from the hematopoietic niche, we transplanted the WKM from adult Tg(wt1b:eGFP;mpeg1:mCherry) into irradiated wild types (Figure 4G). After reconstitution of the hematopoietic stem cell niche, the recipients’ hearts were cryoinjured and fixed at 4 dpf to assess the presence of wt1b-expressing macrophages (Figures 4H–4I”). wt1b:eGFP+;mpeg1:mCherry+ and mpeg1:mCherry+ cells were identified in 5 of 7 animals, suggesting that hematopoietic niche-derived wt1b+ macrophages can home to the injured heart.

**The Composition of the Hematopoietic Populations Changes in wt1b Mutants**

Having identified a link between wt1b and the immune system, we next investigated its role in the zebrafish adult hematopoietic niche. Given that our wt1bDN UAS-driven line is silenced in adults, we used the CRISPR-Cas9 platform to generate a stable mutant wt1b line, lacking 5 nucleotides in exon 2 (named wt1bD5), which is predicted to induce a premature stop codon (Figures S3E and S3F). The loss of wt1b expression was confirmed by anti-Wt1 immunostaining in cryoinjured hearts. We failed to detect Wt1 protein in macrophages in wt1bD5/D5 mutants, but readily observed staining in wt1b:eGFP+;mpeg1:mCherry+ cells in wild-type hearts (Figures S3G–S3K). In addition, qPCR analysis of embryos revealed a decrease in wt1b mRNA expression levels without a concomitant compensatory increase in wt1a expression levels (Figure S3L).

We then compared the composition of immune cells in the WKM of wt1b+/+, wt1bD5/D5, and wt1bD5/D5 lines (Figures 4J–4J”). Although we observed no changes in lymphoid cells (population 1) or cells from the precursor pool (population 2), in wt1bD5/D5 animals there was a significant decrease in the numbers of the larger, more granular cells corresponding to myeloid cells, including differentiated macrophages (population 3) (Figure 4J”). Thus, the loss of function of wt1b decreases the number of myeloid cells in the adult hematopoietic niche.

Since the genetic background used in this experiment was Tg(wt1b:eGFP;mpeg1:mCherry), we further characterized the eGFP+, mCherry+, and double-positive cells, as well as the non-fluorescent cells in wt1b+/+, wt1bD5/D5, and wt1bD5/D5 animals (Figure S4A). We observed no differences in single wt1b:eGFP+; mpeg1:mCherry+ or double-positive WKM-derived populations (Figure S4B). However, in the non-fluorescent cell population, we detected a significant decrease in the number of myeloid cells (population 3) in wt1bD5/D5 animals when compared with wt1b+/+ (Figure S4B). These findings suggest that in the adult kidney

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**Figure 3. wt1b:eGFP+ Macrophages Home to the Site of Injury during Caudal Fin Regeneration, and Wt1b Regulates Their Migratory Behavior**

(A) Caudal fins from Tg(wt1b:eGFP;mpeg1:mCherry) zebrafish larvae were amputated at 3.5 dpf and either fixed at different time points and processed for IF or embedded for in vivo imaging.

(B–M”) Whole-mount IF on caudal fins. Merged and single eGFP and mCherry channels of the magnified views from boxed areas are shown on the right panels. The yellow arrowheads point to double-positive cells, and the blue arrowheads to single mCherry+ cells. The white and yellow dotted lines outline the remote and regenerating areas, respectively. The regenerating area is defined as 100 μm distal from the amputation plane until the fin tip. Maximum intensity projections are shown. Also shown are representative images from 6.hpa (n = 7), 12 hpa (n = 6), 24 hpa (n = 8), 36 hpa (n = 4), and 72 hpa (n = 5) samples from 2 experimental replicates.

(N) Accumulation index of eGFP+; mCherry+ or mCherry+ macrophages at the regenerating area of animals from (D)–(M”). Calculated as cell density at the regenerating area/cell density at the remote area. Two-way ANOVA, followed by Sidak’s post hoc test.

(O) Quantification of the migration speed of eGFP+; mCherry+ versus mCherry+ macrophages. The dots indicate mean values for macrophages counted in n = 7 larvae from 3 experimental replicates. Means ± SDs are shown; two-tailed unpaired t test.

(P) Analysis of migratory behavior of macrophages during the first 12 hpa upon Wt1b inhibition by expressing a dominant-negative isoform in macrophages using larvae from 3 experimental replicates. Means ± SDs are shown. Individual points represent the average migration of all macrophages per embryo from 3 experimental replicates. Two-tailed unpaired Student’s t test.

(R–S) Whole-mount IF on caudal fins from Tg(eGFP:UAS:RFP) (R) and Tg(eGFP:UAS:wt1bDN) (S) lines, both in Tg(mpeg1:Gal4) background. (R’) and (S’) are magnified views of boxed areas in (R) and (S), respectively; arrowheads point to eGFP+ macrophages. The white and yellow dotted lines outline the remote and regenerating areas, respectively. The regenerating area is defined as 100 μm distal from the amputation plane until the fin tip. The maximum intensity projections are shown.

(T) Accumulation index of eGFP:UAS:RFP or eGFP:UAS:wt1bDN macrophages at the regenerating area of animals from (R)–(S’) at 12, 24, 48, and 72 hpa, calculated as in (N). An average of n = 12 embryos was analyzed per time point; two-tailed unpaired t test. Scale bars, 50 μm (B, D, F, H, J, L, R, and S) and 20 μm (magnified views). dpf, days-post-fertilization; hpa, hours-post-amputation.

See also Figures S2 and S3.
marrow, Wt1b function is required within a precursor cell population for the maintenance of the myeloid cell pool.

Heart and Fin Regeneration Are Impaired in wt1b Mutants

Our findings reveal that wt1b expression defines a subset of pro-regenerative macrophages. We next aimed to dissect wt1b function during regeneration. To that purpose, we analyzed the regenerative capacity of wt1bD5/D5 using fin resection and cardiac ventricle cryoinjury. We performed adult caudal fin amputation to wt1bD5/D5 mutants and wt1b+/+ wild-type adults, and fin regrowth was quantified periodically until complete regeneration at 18 days post-amputation. We observed a significant delay in fin regrowth from 2 to 5 dpi, showing that fin regeneration is affected in wt1bD5/D5 mutants (Figures 5 A and 5B).

We next studied whether the cardiac regenerative capacity would also be affected in wt1b mutants. During cryoinjury, fibrotic tissue deposition is followed by cardiomyocyte proliferation and fibrosis regression (González-Rosa et al., 2011). Ventricular cryoinjury was performed on wt1bD5/D5 and wt1b+/+, and cardiomyocyte proliferation was assessed by 5-bromo-2'-deoxyuridine (BrdU) incorporation at 7 dpi (Figures 5 C–5G’’). Results showed that cardiomyocyte proliferation in wt1bD5/D5 mutants was significantly lower than in controls, with a 60% decrease in the number of BrdU+ cardiomyocytes (Figure 5 H).

yellow arrowheads indicate eGFP+;mCherry+ cells. Representative examples from 3 biological replicates from 2 technical replicates are shown. 

(J–J”) Composition of immune cells in the WKM of wt1b+/+, wt1bD5/D5, and wt1bD5/D5 lines. Boxplots of normalized cell numbers of cell populations in gates 1 (J, lymphoid), 2 (J’, precursors), or 3 (J”, myeloid) in wt1b+/+(n = 15), wt1bD5/D5(n = 15), and wt1bD5/D5(n = 14) WKM are shown. Data from 3 experimental replicates. Normalized cell numbers relate to cell numbers per 106 events of living single cells. Myeloid cell numbers (J”) are significantly lower in wt1bD5/D5 than in wt1b+/+ by one-way ANOVA, followed by Tukey’s post hoc test. Scale bars, 20 μm (E, E’, and H) and 100 μm (J–J”). See also Figures S3 and S4.
However, neither fibrotic tissue deposition nor fibrosis regression was impaired in wt1b<sup>a5</sup>/Δ5 animals (Figures S4C–S4I).

To test whether the effect on heart regeneration could be a consequence of altered macrophage distribution in wt1b<sup>a5</sup>/Δ5 mutants, we analyzed the composition of wt1b:eGFP<sup>+</sup>;mpeg1:mCherry<sup>+</sup> and mpeg1:mCherry<sup>+</sup> cells in hearts from wt1b<sup>a5</sup>/+ and wt1b<sup>a5</sup>/Δ5 animals at 7 dpi (Figures S5I–S5L'). We found that the percentage of wt1b:eGFP<sup>+</sup>;mpeg1:mCherry<sup>+</sup> cells in the heart was reduced in wt1b<sup>a5</sup>/Δ5 compared to wt1b<sup>a5</sup>/+ (Figure 5M). Furthermore, the number of single- and double-positive cells was also reduced in wt1b<sup>a5</sup>/Δ5 compared to wt1b<sup>a5</sup>/+ siblings (Figure 5N). Within the 100 μm of myocardium adjacent to the injury area, only the number of wt1b:eGFP<sup>+</sup>;mpeg1:mCherry<sup>+</sup> cells was reduced in wt1b<sup>a5</sup>/Δ5, while that of mpeg1:mCherry<sup>+</sup> cells was unaffected (Figure 5O). Thus, the reduction of wt1b<sup>a5</sup> macrophages in the regenerating myocardium correlates with the reduced proliferation of cardiomyocytes.

These data support a role for wt1b function during adult caudal fin regeneration and cardiomyocyte proliferation upon cardiac injury.
DISCUSSION

Studies exploring the wt1b expression pattern during organ regeneration have previously described its upregulation in the epicardium during heart regeneration and in sheath cells during notochord regeneration (González-Rosa et al., 2011; Lopez-Baez et al., 2018; Schnabel et al., 2011). However, a direct role for Wt1 during the regeneration of these structures has not been reported. Our present results demonstrate that wt1b upregulation correlates with an injury response in the zebrafish and that Wt1b has a dedicated function during organ regeneration.

We identified a macrophage subtype defined by wt1b expression whose genetic profile suggests an overall pro-regenerative behavior, characterized by an anti-inflammatory phenotype and the expression of extracellular matrix remodeling proteins that promote tissue regeneration. The temporal dynamics of wt1b+ macrophage accumulation during fin regeneration inversely correlate with the dynamics previously observed for tnfα macrophages (Nguyen-Chi et al., 2015, 2017). We found that tnfα expression levels at the early stages of the injury response were indistinguishable between wt1b+ and wt1b− cells in the heart. These findings suggest that pro-inflammatory tnfα cells may adopt a reparative character during the later phases of regeneration. The differential expression of some known WT1 targets in wt1b+ macrophages supports a role for Wt1b as a transcription factor in the definition of this specific macrophage subtype.

The enrichment of wt1b+ macrophages in the adult heart and larval caudal fin beyond the early phase of the injury response, during which pro-inflammatory macrophages have been described to be predominant, further suggests that wt1b expression identifies a population of pro-regenerative macrophages. Following larval caudal fin amputation, we observed both the homing of wt1b+ macrophages to the site of injury and the upregulation of wt1b upon their arrival. This suggests that the homing of different macrophage populations to the damaged tissue coexists with a local change in polarization. We provide evidence that wt1b expression defines a subset of macrophages and that Wt1b modulates their migratory behavior.

We also investigated the origin of cardiac wt1b+ macrophages. Upon myocardial infarction, tissue resident and circulating monocyte-derived macrophages home to the damaged tissue (Chen and Frangogiannis, 2017). Results from transplantation assays suggest that kidney marrow-derived wt1b+ macrophages can infiltrate into the injured heart. However, the contribution from cardiac resident macrophages in homeostatic conditions cannot be discarded, since circulating macrophages may be reconstituting niches of irradiation-depleted pre-existent cells (Guilliams and Scott, 2017). Furthermore, we show that wt1b is required in a cell non-autonomous manner within the hematopoietic niche in the adult zebrafish to maintain the myeloid pool, shedding light on a long-standing debate on the role of Wt1 in mouse hematopoiesis (Chau and Hastie, 2012); wt1b mutants showed decreased maturation of the hematopoietic myeloid lineage, which could influence the number and polarization state of kidney marrow-derived macrophages that home to injured tissues, thus influencing the regenerative process.

Recent studies have highlighted the importance of the immune response and particularly the role of macrophages during regenerative processes (Wynn and Vannella, 2016), including in the fin and the heart (Aurora et al., 2014; Nguyen-Chi et al., 2017). We found delayed adult fin regeneration and impaired cardiomyocyte proliferation upon injury in wt1b mutants. Regarding the heart, wt1b is not expressed in adult cardiomyocytes. Therefore, the cardiomyocyte proliferation impairment upon injury observed in the wt1b mutants must occur in a non-cell-autonomous manner. Impaired cardiomyocyte proliferation correlated with a decrease in wt1b+ macrophages in the hearts of wt1b mutants. Besides the wt1b+ macrophage subpopulation, wt1b is also expressed in EPDCs (González-Rosa et al., 2012). Thus, it cannot be excluded that wt1b also plays a role within the epicardium to support heart regeneration (Wang et al., 2015). Cardiac fibroblasts are derived from EPDCs (Kikuchi et al., 2011) and are the main source of fibrosis in response to cryoinjury (Sánchez-Iranzo et al., 2018). The fact that the fibrotic response is not affected may indicate that Wt1b does not act on regeneration primarily through its role in EPDCs. Our results indicate that Wt1 may play a role in regeneration not only through its function in a specific macrophage population but also through the regulation of myeloid cell differentiation in WKM. Furthermore, the observation that wt1b mutants also show delayed fin regeneration upon caudal fin amputation further supports a role for wt1b in macrophages during the modulation of the regenerative response.

In conclusion, this study represents the description of a role for Wt1 during organ regeneration and characterizes a wt1b+ pro-regenerative macrophage subtype that accumulates within regenerating tissues in the zebrafish. These findings can help to open horizons for the study of macrophage etiology, heterogeneity, and function in a model organism with high regenerative capacity, and they will enrich our understanding of organ regeneration, possibly paving the way for future diagnostic and therapeutic interventions.

STAR METHODS

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  - FACS and Flow Cytometry
  - Cytology
  - RT-qPCR on Heart Samples
  - RT-qPCR for wt1a and wt1b in Zebrafish Larvae

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o RNaseq Analysis
o GO Biological Processes enrichment analysis
o Macrophage Migration Assay
o Macrophage Intensity Analysis
o Whole Kidney Marrow Transplantation Assays
o Adult Caudal Fin Regeneration upon Amputation
o BrdU Pulse-Chase Experiments during Heart Regeneration
o Macrophage Localization Assessment in Regenerating Hearts
o Quantification of Fibrotic Tissue in Regenerating Hearts

QUANTIFICATION AND STATISTICAL ANALYSIS
DATA AND CODE AVAILABILITY

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.cebrep.2019.06.091.

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AUTHOR CONTRIBUTIONS

A.S.-M. designed and performed most of the experiments. A.B.G.-R. performed most of the preliminary experiments leading to the conclusions of this manuscript. I.J.M. and J.M.G.-R. generated the Tg(mpeg1:mCherry) line. A.G. and S.M. generated the wt1β5/5 line. H.R. and T.B. performed some experiments with the wt1β5/5 line. M.B. performed the bioinformatics analysis. I.J.M., M.G.-C., I.P., A.E., J.M.G.-R., and X.L. assisted with the experiments. N.M., C.E., M.R.-O., A.M.B., and M.S. provided funding. A.S.-M. and N.M. wrote the manuscript. A.B.G.-R., I.J.M., H.R., T.B., and C.E. contributed to the interpretation of the results and to writing the manuscript. T.B., H.R., and C.E. conceived the WKF FACS experiments. N.M. conceived the project, designed the experiments, and interpreted the results.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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### STAR★METHODS

#### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Chicken polyclonal anti-GFP | Aves Labs | Cat#GFP-1010; RRID:AB_2307313 |
| Mouse living colors polyclonal anti-DsRed | Takara Bio | Cat#632392; RRID:AB_2801258 |
| Rat monoclonal anti-mCherry | Thermo Fisher Scientific | Cat#M11217, RRID:AB_2536611 |
| Mouse monoclonal anti-mouse (for paraffin sections) | Developmental Studies Hybridoma Bank | Cat#MF 20, RRID:AB_2147781 |
| Mouse monoclonal anti-mouse (for gelatin sections) | Developmental Studies Hybridoma Bank | Cat#f59, RRID:AB_528373 |
| Mouse monoclonal anti-BrdU | BD Biosciences | Cat#563445, RRID:AB_2738210 |
| Rabbit anti-L-Plastin | Paul Martin lab | N/A |
| Goat anti-Chicken IgY (H+L) Secondary Antibody, Alexa Fluor 488 | Jackson ImmunoResearch Labs | Cat#111-066-003, RRID:AB_2337966 |
| Goat anti-Rabbit IgG (H+L) Secondary Antibody, Alexa Fluor 647 | Jackson ImmunoResearch Labs | Cat#112-065-167, RRID:AB_2338179 |
| Goat anti-Mouse IgG1 Cross-Adsorbed Secondary Antibody, Alexa Fluor 647 | Jackson ImmunoResearch Labs | Cat#SA1010 |
| Streptavidin Cy3 conjugate | Thermo Fisher Scientific | Cat#SA1011 |
| 16% Paraformaldehyde (formaldehyde) aqueous solution | Electron Microscopy Sciences | Cat#15710 |
| N-Phenylthiourea (PTU) | Sigma-Aldrich | Cat#P7629 |
| 5-Bromo-2`-deoxyuridine (BrdU) | Sigma-Aldrich | Cat#B5002 |
| Phosphate buffered saline (PBS) | Sigma-Aldrich | Cat#P4417 |
| Fetal Bovine Serum (FBS) | Sigma-Aldrich | Cat#F2442 |
| Ethyl 3-aminobenzoate methanesulfonate (Tricaine) | Sigma-Aldrich | Cat#E10521 |
| Trypsin / EDTA | Thermo Fisher Scientific | Cat#15400054 |
| Tri Reagent | Sigma-Aldrich | Cat#T9424 |
| 4`, 6-diamidino-2-phenylindole dihydrochloride (DAPI) | Merck | Cat#124653 |
| Q5 High-Fidelity DNA Polymerase | New England Biolabs | Cat#M0491 |
| Gibson Assembly Master Mix | New England Biolabs | Cat#E2611 |
| Zymoclean Gel DNA Recovery Kit | New England Biolabs | Cat#D4007 |
| DNA Clean & Concentrator | New England Biolabs | Cat#D0413 |
| DreamTag Green PCR Master Mix | Thermo Fisher Scientific | Cat#K1081 |
| Quick-Step Ligase | Bioline | Cat#BIO-27027 |
| High-Capacity cDNA Reverse Transcription Kit | Thermo Fisher Scientific | Cat#4368813 |
| QuantiTect Rev. Transcription Kit | QIAGEN | Cat#205310 |
| PicoPure RNA Isolation Kit | Thermo Fisher Scientific | Cat#KIT0204 |
| PowerUp SYBR Green Master Mix | Thermo Fisher Scientific | Cat#A25742 |
| SYBR GreenER | Thermo Fisher Scientific | Cat#11762100 |
| mMESSAGE mMACHINE T7 Transcription Kit | Thermo Fisher Scientific | Cat#AM1344 |
| iScript cDNA synthesis kit | Bio-Rad | Cat#1708890 |
| SYTOX Blue Dead Cell Stain | Thermo Fisher Scientific | Cat#S34857 |

(Continued on next page)
LEAD CONTACT AND MATERIALS AVAILABILITY

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Nadia Mercader (nadia.mercader@ana.unibe.ch).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Zebrafish

Experiments were conducted with zebrafish embryos and adults aged 4–15 months, raised at maximal 5 fish/l and maintained under the same conditions: 28 °C, 650–700 μs/cm, pH 7.5, the lighting conditions were 14:10 hours (light: dark) and 10% of water exchange a day. Feeding schedule was: three times per day, once artemia (Ocean Nutrition) and twice dry food (ZM-000, Gemma Micron 150 and 300 for larvae, juveniles and adults stages, respectively). Approximately equal sex ratios were used for experiments. As controls, siblings or same-staged animals were used. Experiments were approved by the Community of Madrid “Dirección General de Medio Ambiente” in Spain, the Landesamt für Verbraucherschutz Thüringen, Germany and the “Amt für Landwirtschaft und Natur” from the Canton of Bern, Switzerland. All animal procedures conformed to EU Directive 86/609/EEC and Recommendation 2007/526/EC.

Continued

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Raw bulk RNA-seq wt1b:eGFP+;mpeg1:mCherry+ versus wt1b:eGFP−;mpeg1:mCherry− cells at 4 days post ventricular cryoinjury | This paper | GEO: GSE115381 |

Oligonucleotides

- sgRNA used to generate wt1bD5/D5 mutants: 5′-GTCGACGGAATTCCCAGTTACGG-3′

Please see Table S3 for primer sequences used in this study

Recombinant DNA

- p5E-mpeg1.1 promoter
  - Eliett et al., 2011
  - N/A
- pME-mCherry
  - Gift from Nathan Lawson lab
  - N/A
- p3E-polyA
  - Villefranc et al., 2007
  - N/A
- βGI-eGFP:E1b-5xUAS-E1b:RFP-βGI
  - Gift from the Reinhard W. Köster lab
  - N/A
- pDestTol2pA2AC
  - Kwan et al., 2007
  - N/A

Software and Algorithms

- Fiji/ImageJ
  - NIH
  - RRID:SCR_002285
- GraphPad Prism 7
  - GraphPad Software
  - N/A
- Imaris 8.4.1
  - Bitplane
  - N/A
- FlowJo-X
  - FlowJo
  - N/A
- REST
  - Pfaffl, 2001; Pfaffl et al., 2002
  - N/A
- Zen
  - Zeiss
  - N/A

Other

- Dako Fluorescence Mounting Medium
  - Dako
  - Cat#S3023
- 35 mm Dish, No. 0 Coverslip, 20 mm Glass Diameter, Uncoated
  - MatTek
  - Cat#P35G-0-20-C
- Avidin/Biotin Blocking Kit
  - Vector Laboratories
  - Cat#SP-2001
- Certified Low Melt Agarose
  - Bio-Rad
  - Cat#9012-36-6
regarding the protection of animals used for experimental and other scientific purposes, enforced in Spanish law under Real Decreto 1201/2005. Experiments in Switzerland were conducted under the license BE95/15 and BE64/18. Published strains used in this study include: wild-type AB, Tg(wt1b:eGFP)1119 (Pemer et al., 2007), Tg(mpeg1:Gal4)1116 (Bar et al., 2011), the newly generated Tg(mpeg1:mCherry;cryaa:mCherry)1116, Tg(GFP:5xUAS-wt1bDN;cryaa:eCFP)1114, Tg(GFP:5xUAS-RFP;cryaa:eCFP)1115 and wt1b5/5. Transgene sequences are available upon request.

METHOD DETAILS

Construction of mpeg1:mCherry Zebrafish

To generate the mpeg1:mCherry transgenic line, the following DNA fragments were assembled using Gateway cloning (Kwan et al., 2007): p5E mpeg1 promoter element containing a 1.8 kb region upstream of the ATG of the mpeg1 gene (Bar et al., 2011), pME- mCherry and the p3E-polyA fragment from Villefranc et al. (2007). The entire construct was flanked with Tol2 sites to facilitate transgenesis. The plasmid was injected into one-cell-stage zebrafish embryos and those with strong and broad mCherry expression were injected with a solution containing the single guide sgRNA (15 ng/mg). Selected embryos were incubated for 48 hours at 28°C. The generation of wt1b Mutant (wt1b5/5) Zebrafish

To generate wt1b5/5 mutant animals, a unique undigested 480 bp DNA band can be identified. Primers used for genotyping are described in Table S3.

Zebrafish Cardiac Cryoinjuries

Cardiac ventricular cryoinjury experiments were conducted using adult zebrafish as described (González-Rosa and Mercader, 2012). Briefly, adult fish were anesthetized and their pericardial cavity opened to expose the heart. A copper filament cooled in liquid nitrogen was placed on the ventricular surface of the heart until thawing. After surgery, animals were revived by gently directing water to their gills using a plastic Pasteur pipette.

Zebrafish Caudal Fin Amputations

Larval and adult caudal fins amputations were performed using a scalpel. For larvae, caudal fin amputations were performed at 3.5 or 4.5 dpf including the very distal part of the notochord. For adults, amputations were performed only to half of the caudal fin.
(Invitrogen, 1:250), mouse anti-DsRed (Takara, 1:250), rabbit anti-L-plastin (a kind gift from Paul Martin; 1:500), anti-Myosin Heavy Chain (DSHB Iowa Hybridoma Bank (MF20 for paraffin sections or F59 for gelatin sections, 1:20), mouse anti-BrdU (BD PharMingen; 1:250), rabbit anti-WT1 (a kind gift from Elizabeth Patton; 1:1000) (Lopez-Baez et al., 2018). Secondary antibodies were Alexa Fluor 488, 568, 647 (Life Technologies, 1:250) and biotin anti-rabbit (Jackson Immuno Research, 1:250) or biotin anti-rat (Jackson Immuno Research, 1:250) followed by incubation with Cy3 or Cy5 streptavidin conjugate (Molecular Probes, 1:250). Nuclei were counterstained with DAPI and slides were mounted in Dako Fluorescence Mounting Medium.

To detect wt1b, mafbb, mmp14a and mCherry transcripts, RNAscope (Advanced Cell Diagnostics) was performed following the manufacturer’s instructions for PFA-fixed paraffin-embedded samples with standard tissue pretreatment and Fluorescent Multiplex Assay detection kit. Following transcript detection, an anti-GFP immunofluorescence was performed on the same slides.

A Zeiss LSM 880 or Leica SP8 confocal microscopes were used to image immunostained sections.

Acid fuchsin-orange G (AFOG) stain was used to detect fibrotic tissue. Muscle, fibrin/cell debris and collagen were stained brown-orange, red and blue, respectively.

**FACS and Flow Cytometry**
Hearts collected in ice-cold PBS were digested at room temperature in 0.5% trypsin and repeatedly passed through a micropipette tip to obtain a single cell suspension. Digestion was stopped by adding ice-cold PBS, 10% fetal bovine serum (FBS), and cells were pelleted by centrifugation (200 x g, 10 min, 4°C) and resuspended in 10% FBS in PBS. Dead cells were excluded by staining with 1 μg/ml DAPI (Sigma). Cells were analyzed for forward scatter, side scatter and eGFP and mCherry fluorescence on an Aria Canto 3L HTS FAC (Beckton Dickinson). Percentages of mCherry+ and mCherry;eGFP+ cells were determined by analyzing 100,000 cells per sample.

WKM was isolated following dissection of adult kidney tissue, which was pressed through a 40 μm cell strainer. Cells were pelleted by centrifugation (300 g, 5mins, 4°C) and resuspended in PBS and analyzed on a BD FACS Aria Illu device (Beckton Dickinson). Gating for transgenic cells was controlled prior to analysis with non-transgenic control samples. For all samples, debris, doublets and dead cells (SYTOX Blue+, ThermoFisher Scientific) were removed from analysis.

WKM experiments were performed with a sequential application of two interim analyses prior to final statistical calculations (Neumann et al., 2017). Significance levels were applied with interim α-values of 0.001 to avoid type I errors and a final α-value of 0.05. Three replicates with total n numbers wt1b/+/ = 15, wt1b+/ΔΔ = 15, wt1bΔΔ/ΔΔ = 14 were performed. WKM experiments were analyzed using FlowJo-X and plots were generated using R (ggplot2 package).

**Cytology**
Kidney- and cardiac-derived wt1b:eGFP*:mpeg1:mCherry+ and mpeg1:mCherry+ cells were isolated as described in “FACS and Flow Cytometry” section, separated by FACS and concentrated by cytocentrifugation at 250 x g for 5 min onto glass slides using a Shandon Cytospin 4 (Thermo Fisher Scientific). Slides were fixed and stained with May-Grünwald Giemsa protocol as described in Stachura et al. (2009). Briefly, samples were fixed in methanol for 15 minutes, May-Grunwald for 20 minutes, washed with ddH2O followed by Giemsa staining for 10 minutes, washed with ddH2O, briefly dried and mounted with DPX.

**RT-qPCR on Heart Samples**
Hearts from Tg(wt1b:eGFP; mpeg1:mCherry) zebrafish were cryoinjured and at 4 dpi mCherry+ and eGFP+;mCherry+ cells were FACs purified. RNA was extracted and a total of 1 μg of RNA was reverse-transcribed into cDNA with random hexamers. Quantitative PCR (qRT-PCR) was performed on a 7500 Fast ABI System (Invitrogen Life Technologies). PCR cycles proceeded as follows: initial denaturation for 30 s at 95°C, followed by 40 cycles at 95°C for 5 s and 60°C for 30 s. Melting curve analysis was performed in SYBR green reactions to show PCR product specificity. To calculate the relative index of gene expression, we employed the 2^ΔΔCt method, where ef1α gene served as the internal control. Primers used are described in Table S3.

**RT-qPCR for wt1a and wt1b in Zebrafish Larvae**
Total RNA was isolated from larvae at 5 dpf using trizol according to the manufacturer’s protocol. 10 samples containing 10 wt1bΔΔ/ΔΔ or wt1bΔΔ/ΔΔ larvae each were collected per genotype. RNA was reverse transcribed with iScript cDNA synthesis kit (Bio-Rad) and qRT-PCR was carried out in triplicates for each sample using SYBR GreenER (Thermo Fisher Scientific) on the CFX384 Real-Time System (Bio-Rad). PCR efficiencies and relative expression were calculated, and significances determined by using pairwise reallocation randomization test (using REST) as previously described (Pfaffl, 2001; Pfaffl et al., 2002). For normalization we used ef1α. Primers used are described in Table S3.

**RNaseq Analysis**
Hearts from Tg(wt1b:eGFP; mpeg1:mCherry) zebrafish were cryoinjured and eGFP+;mCherry+ and eGFP+;mCherry+ cells were FACs purified at 4 dpi. RNA was extracted from 4 pools of eGFP+/mCherry+ and of eGFP+;mCherry+. One ng of RNA was used to generate barcoded RNA-seq libraries using the Ovation Single Cell RNA-Seq System (NuGEN) with two rounds of library amplification. The size of the libraries was calculated using the Agilent 2100 Bioanalyzer. Library concentration was determined using the Qubit fluorometer (ThermoFisher Scientific). Libraries were sequenced on a HiSeq2500 (Illumina) to generate 60 base single reads. FastQ files for each
sample were obtained using CASAVA v1.8 software (Illumina). Four biological replicates consisting of five pooled hearts were used per sample.

Sequencing adaptor contaminations were removed from reads using cutadapt 1.7.1 software (Martin, 2011) and the resulting reads were mapped and quantified on the zebrafish genome (Zv11, release 94) using RSEM v1.2.20 (Li and Dewey, 2011). Only genes with at least 1 count per million in at least 4 samples were considered for statistical analysis. Data were then normalized and differential expression tested using the Bioconductor package limma (Robinson et al., 2010). We considered as differentially expressed those genes with a Benjamini-Hochberg adjusted pvalue < 0.05 and abs(Log2FC > 1). Raw data has been deposited in the GEO Database with the reference GEO: GSE115381.

GO Biological Processes enrichment analysis
We used the R language Clusterprofile package (Yu et al., 2012). Differentially expressed genes (Benjamini-Hochberg adjusted pvalue < 0.05) were used with enrichDavid function with the parameters (pvalueCutoff = 0.01 annotation = ’GOTERM_BP_ALL’). Further zscore = (up - down / \sqrt{genes}) column was included using R for circular plotting with GEnrichPlot package and GOChord function (Walter et al., 2015). The workflow can be visualized using Docker and has been deposited in https://doi.org/10.17632/v2fxyb8nyj.1.

Macrophage Migration Assay
Larvae were transferred to E3 medium containing 0.2 mg/ml tricaine and 0.0033% phenyl-thiourea and immobilized in 1% low melting agarose in a 35 mm Petri dish with a glass cover. The caudal fin was transected with a sterile scalpel at 3.5 dpf or 4.5 dpf in Tg(wt1b:eGFP;mpeg1:mCherry) or the UAS-driven lines, respectively. Amputated larval caudal fins were imaged from 0.5 to 12 hpa using a Zeiss LSM 880 inverted confocal microscope with a 20x air objective. A z stack of 100 \upmu m was acquired every 5 min for Tg(wt1b:eGFP;mpeg1:mCherry) line and every 10 min for the Tg(mpeg1:Gal4;eGFP-UAS-RFP) and Tg(mpeg1:Gal4:eGFP-UAS-wt1bDN) lines. The 4D files generated from time-lapse acquisitions were processed using Zen software and compressed into maximum intensity projections. Brightness, contrast, and color levels were adjusted for maximal visibility and drift correction was applied. Migration speed of macrophages was quantified using the points and statistic function in Imaris. The average of macrophage subpopulation mean migration speed was calculated for each embryo.

Macrophage Intensity Analysis
Tg(wt1b:eGFP;mpeg1:mCherry) 3.5 dpf larvae were subjected to caudal fin amputation and imaged from 0 to 34 hpa using a Zeiss LSM 880 inverted confocal microscope with a 20x air objective. A z stack of 100 \upmu m was acquired every 5 min using 2x1 tile scan, which were then stitched together using Zen software. In FIJI, a maximum intensity projection followed by a 2 pixel (Px) mean filter was applied to the time-lapse data to increase the homogeneity within individual cells. The ImageJ MtrackJ tool was used to analyze the migration of macrophages. For cell tracking the intensity signal was measured by applying the local cursor snapping function to detect the Px with the maximal fluorescence intensity within a cell. Starting from the last time point, double positive wt1b:eGFP‘;mpeg1:mCherry’ cells were tracked back until their first appearance. Subsequently, the tracked XY coordinates and the GFP fluorescence intensity over time were exported. To correct for growth and obtain a reference location the amputation site was tracked. The analysis was performed in MATLAB R2017a.

Each cell was categorized whether it is migrating positive for wt1b:eGFP or is upregulating wt1b:eGFP expression while migrating toward the injury. Two criteria to categorize for migration or activation were defined: First criterion for migration, once during its first five time-points (25 min), after appearance, the cell has to pass 33% of its own maximal GFP intensity. Second criterion, the cell has to be in the 25% most anterior tracked distance to the injury site during one of the first five time-points after appearance. The percentage of migration versus activation in each embryo was calculated and from these the mean percentage was obtained.

Each measured intensity was divided by the maximal measured GFP intensity of the individual embryo to increase comparability. The time-course was divided in 20 intervals (binning of 20 \times 5 min). The mean intensities per embryo and mean intensities of all embryos per interval were calculated.

Whole Kidney Marrow Transplantation Assays
A previously published protocol was followed with minor modifications (Li et al., 2015). Briefly, wild-type AB zebrafish were irradiated (23 Gy, 7 min). WKM cells from donor Tg(wt1b:GFP;mpeg1:mCherry) adult fish were isolated in ice-cold PBS and repeatedly passed through a micropipette tip to obtain a single cell suspension. Then, cells were pelleted by centrifugation (200 g, 10 min, 4 °C) and re-suspended in PBS. Cells were transplanted into irradiated fish by retro-orbital injection and, after two weeks, to allow reconstitution of the hematopoietic stem cell niche, hearts were cryoinjured and collected at 4 dpi.

Adult Caudal Fin Regeneration upon Amputation
Caudal fin amputation was performed to wild-type wt1b+/+ or mutant wt1bA5Δδ adult zebrafish. The 3 most regrown rays were measured per animal and time-point and normalized by the initial amputated fin length.
BrdU Pulse-Chase Experiments during Heart Regeneration

Ventricular cryoinjury was performed to wild-type wt1b+/+ or mutant wt1bΔ5/Δ5 adult zebrafish. At 6 dpi, animals were injected intraperitoneally with 20 μL of 2.5 mg/ml BrdU in phosphate buffered saline (PBS) and hearts were collected at 7 dpi. To calculate BrdU cardiomyocyte labeling indices, ventricular sections were immunostained with anti-MHC and anti-BrdU, antibodies and nuclei counterstained with 4’6-diamidino-2-phenylindole (DAPI). 3 ventricular sections containing the largest injury areas were imaged per heart. BrdU+/MHC+ cardiomyocytes were counted manually using ImageJ software in the whole ventricle and then normalized by total ventricular MHC area. The cardiomyocyte proliferation index from individual sections was averaged to establish a proliferation index for each animal.

Macrophage Localization Assessment in Regenerating Hearts

Ventricular cryoinjury was performed to Tg(wt1b:eGFP;mpeg1:mCherry) either wt1b+/+ or wt1bΔ5/Δ5 adult zebrafish and hearts were collected at 7 dpi. mpeg1:mCherry+ and wt1b:eGFP+;mpeg1:mCherry+ macrophages localized in the whole ventricle and those localized in the 100 μm of myocardium surrounding the injury area were manually counted and classified according to their relative position to the cryoinjured area.

Quantification of Fibrotic Tissue in Regenerating Hearts

To quantify the fibrotic area in regenerating hearts at 7 and 28 dpi, images of evenly-spaced AFOG-stained serial sections of the whole heart were scanned. Masks of every ventricular section per slide were manually generated and quantified using ImageJ Threshold Color based on differential staining: muscle (brown/orange); fibrotic area (fibrin (red) and collagen (blue)). To calculate the percentage of ventricular injured area, the total fibrotic area was normalized to the total ventricular area for each heart.

QUANTIFICATION AND STATISTICAL ANALYSIS

Sample sizes were chosen based on previous publications and are indicated in each figure legend. The experiments were not randomized, and the investigators were not blinded to allocation during experiments and outcome assessment. All statistical values are displayed as mean ± standard deviation. Sample sizes, statistical test and P values are indicated in the figures or figure legends. Data normality was determined before using parametric or non-parametric statistical test. All statistical tests were performed using GraphPad Prism 7 software.

DATA AND CODE AVAILABILITY

Raw data has been deposited at Mendeley under the link https://doi.org/10.17632/v2fyxb8ryj.1. RNA-seq raw data has been deposited in the GEO Database with the reference GEO: GSE115381. Zebrafish line information has been deposited at ZFIN.
Supplemental Information

Wilms Tumor 1b Expression Defines a Pro-regenerative Macrophage Subtype and Is Required for Organ Regeneration in the Zebrafish

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Figure S1. Differential gene expression of wt1b-positive macrophages. Related to Figure 2 and Table S1. A, wt1b:eGFP;mpeg1:mCherry and mpeg1:mCherry cells were FAC-sorted from cryoinjured adult zebrafish hearts at 4 dpi and their transcriptomes analyzed using RNA-seq. B, Principal component analysis of
wt1b:eGFP;mpeg1:mCherry\(^+\) and mpeg1:mCherry\(^+\) cells. C, Heatmap indicating all significant differentially expressed genes between both populations. D, Heatmap of genes from (C) whose function has been described in macrophages. Classification of gene function in macrophages according to literature: grey, genes involved in macrophage regulation including differentiation, phagocytosis and apoptosis; light green, genes involved in homeostasis restoration; dark green, genes involved in disease progression. E-J\(''''\), Validation of RNA-seq target genes by RNAscope in situ hybridization followed by anti-GFP immunostaining on cryoinjured wt1b:eGFP;mpeg1:mCherry heart sections at 4 dpi. Signal from wt1b, mafbb and mmp14a antisense riboprobes co-localizes with mCherry mRNA and eGFP signal (arrowheads). Note that large dots for mCherry channels represent background staining, and small dots correspond to signal. dpi, days post injury; FACS, fluorescence-activated cell sorting; PC, principal component; Mφ, macrophage. Scale bars, 50 µm (E, G, I) and 5 µm (F-F\(''''\), H-H\(''''\) and J-J\(''''\)).
Figure S2. Activation of *wt1b:eGFP* during migration towards the amputation plane. Related to Figure 3.

A. Caudal fins from Tg(*wt1b:eGFP;mpeg1:mCherry*) zebrafish larvae were amputated at 3.5 dpf and *in vivo* imaging was performed from 0.5 to 34 hpa. B, Overview of merged eGFP and mCherry channels at 0.5 hpa. C–C′′, Merged and single eGFP and mCherry channels of the zoomed views from boxed areas in panel B are shown. D, Overview of merged eGFP and mCherry channels at 33 hpa. E–E′′, Merged and single eGFP and mCherry channels of the zoomed views from boxed areas in panel B are shown. F, Scheme of macrophage tracking and fluorescence intensity measurement. *wt1b:eGFP* macrophages were tracked during the entire movie and the maximal GFP intensity within the macrophage was detected simultaneously. G, For each of 41 tracked macrophages in 4 embryos an intensity- and localization-based decision (25% most anterior location and at least 33% of the macrophage’s maximal intensity) defines whether they upregulate *wt1b:eGFP* during migration or are eGFP+ from the initial time of tracking. The graph shows the overall mean percentage of upregulation (activation) and migration as bars plus the percentage for each of 4 embryos. H, Overall mean GFP intensity of the 41 tracked macrophages at different time points (grey bars). Colored dots indicate mean GFP intensity ± SD of macrophages in each of the 4 larvae. eGFP levels over time were normalized by maximal measured intensity per larvae. Time-intervals of 1.7 h (20 x 5 min) were binned. Paired t-test was performed. Scale bars: overview images 100 µm (B and D), zoomed views 10 µm (C-C′′ and E-E′′).
Figure S3. Validation of genetic lines to assess wt1b function. Related to Figures 3, 4 and 5.

**A-D.** Validation of transgenic lines for macrophage-specific inhibition of wt1b function. A. Caudal fins of Tg(mpeg1:Gal4; GFP:UAS:RFP) larvae were amputated at 4.5 dpf and eGFP and RFP positive cells imaged at 12 hpa. B–B'''. Images of the caudal fin showing RFP and eGFP positive cells. B' and B'' are single channels for eGFP and RFP. Orange arrowheads, double positive cells; magenta arrowheads, RFP'' cells; and green arrowheads, eGFP'' cells. C. Quantification of the percentage of eGFP'', RFP'' and double-positive cells. Dots indicate individual fish, shown are means ± SD. Note that over 70% of macrophages are double-positive. D. Confirmation of wt1bDN overexpression using the Tg(eGFP:UAS:wt1bDN) line. Gal4FF mRNA was injected into 1-cell stage wildtype, embryos from the transgenic control line Tg(eGFP:UAS:RFP) or Tg(eGFP:UAS:wt1bDN). qRT-PCR was performed on cDNA obtained from these larvae. Shown are mean values ± SD (n= 4 biological replicates per
condition). Statistical analysis by Mann-Whitney test. **E-L.** Generation of the \textit{wt1b}^{Δ5} mutant line. Related to Figures 4 and 5. **E.** Schematic drawing of the \textit{wt1b} gene locus and workflow for \textit{wt1b} mutant generation. A fish line that lacks 5 nucleotides in exon 2 of the \textit{wt1b} gene (\textit{wt1b}^{Δ5}) was established. Blue arrows indicate the forward and reverse primer positions and the \textit{BsrI} restriction site, used to detect indels, is highlighted in red. The sgRNA target sequence is shown in bold. **F.** The 5 nucleotides deletion is predicted to result in a frame shift (blue) and a premature termination codon that leads to a truncation of the protein. **G-J***. Immunostaining with anti-WT1, anti-GFP and anti-mCherry on cryoinjured heart sections at 4 days postinjury from \textit{wt1b}^{+/+}; \textit{wt1b}\textit{eGFP};\textit{mpeg1}\textit{mCherry} and \textit{wt1b}^{Δ5,Δ5}; \textit{wt1b}\textit{eGFP};\textit{mpeg1}\textit{mCherry} zebrafish. Cell nuclei are counterstained with DAPI. Colocalization (arrowheads) of WT1 with GFP and mCherry is detected in \textit{wt1b}^{+/+} but absent in \textit{wt1b}^{Δ5,Δ5}. **K.** Quantification of the relative mean WT1 nuclear fluorescence in images of \textit{wt1b}\textit{eGFP};\textit{mpeg1}\textit{mCherry} cells normalized by background signal. Two-tailed unpaired t test. **L.** Quantitative RT-qPCR analysis of \textit{wt1a} and \textit{wt1b} expression in \textit{wt1b}^{Δ5,Δ5} vs. wildtype larvae. Error bars represent standard error. p-values are calculated by pairwise fixed reallocation randomization with REST (n=10). dpf, days post fertilization; hpa, hours post amputation. Scale bars 50 µm (G and I), 40 µm (B-B'') and 5 µm (H-H'*** and J-J'***).
Figure S4. Phenotypic characterization of \textit{wt1b}^{Δ5} mutants. Related to Figure 4 and Figure 5. Composition of the whole kidney marrow cell populations in \textit{wt1b}^{Δ5} mutants. \textbf{A}, Whole kidney marrow (WKM) cells of \textit{wt1b}^{+/+}, heterozygous \textit{wt1b}^{+/Δ5} or homozygous \textit{wt1b}^{Δ5/Δ5} crossed into a Tg(\textit{wt1b}:eGFP; mpeg1:mCherry) background fish were isolated. This is the same experiment as described in Figure 4, but here, eGFP\textsuperscript{+}, mCherry\textsuperscript{+}, double positive populations and the non-fluorescent fraction were analyzed by flow cytometry separately. \textbf{B}, Shown are boxplots of normalized cell numbers of cell populations in gate 1 (lymphoid), 2 (precursors) or 3 (myeloid). Normalized cell numbers relate to cell numbers per 10\textsuperscript{6} events of living single cells. Negative (non-fluorescent) cell numbers in gate 3 are significantly lower in \textit{wt1b}^{Δ5/Δ5} than in \textit{wt1b}^{+/+} by one-way ANOVA followed by a Tukey’s post-hoc test. \textbf{C-I}, Fibrotic tissue deposition and regeneration in \textit{wt1b}^{Δ5} mutants. \textbf{C}, Ventricular cryoinjury was performed to \textit{wt1b}^{+/+} and \textit{wt1b}^{Δ5/Δ5} adult fish and fibrosis assessed at 7 and 28 dpi on sectioned hearts using AFOG histological staining to detect collagen. \textbf{D, E}, Representative sagittal section of a \textit{wt1b}^{Δ5/Δ5} and \textit{wt1b}^{+/+} heart stained with AFOG at 7dpi. \textbf{F}, Quantification of injured area versus total ventricular area. Data from two independent experiments. Two-tailed unpaired \textit{t} test. \textbf{G-I}, Representative images and quantification of injured cardiac ventricular area at 28 dpi, as shown in D-F. Two-tailed unpaired \textit{t} test. dpi, days postinjury. Scale bars, 100 µm.
| Primers to genotype wt1bΔ5/Δ5 mutants: | For: 5'-GTGAACTCTTGAATGTCACTACAAGC-3’ |
|----------------------------------------|-----------------------------------------|
|                                        | Rev: 5'-ACCGCTGTGAATAAAGGGGACTAAAC-3’ |
| qPCR primers, wt1b (heart RT-qPCR):    | Fw: 5'-GGCCTGGAATCTCCTAGTC-3’          |
|                                        | Rv: 5'-CAGAGGAGGTGCCCTGTGAAG-3’        |
| qPCR primers, wt1b (larvae RT-qPCR):   | Fw: 5'-TGCTGTCCCTCCTTCTAGCC-3’         |
|                                        | Rv: 5'-GAACGGAGGGATTGTGCCGTGC-3’       |
| qPCR primers, wt1a (larvae RT-qPCR):   | Fw: 5'-GGGAACCAAGAGATGTTTAG-3’         |
|                                        | Rv: 5'-GGGCTGTTTGAAGGAGTGG-3’          |
| qPCR primer, ef1α (larvae RT-qPCR):    | Fw: 5'-AGGGAACCACCAGAGTCTTGT-3’        |
|                                        | Rv: 5'-CTGCTGTTTGAAGGAGTGG-3’          |
| qPCR primers, wt1bDN (larvae RT-qPCR): | Fw: 5'-TATTTGCCAGGTACGTCATGGA-3’       |
|                                        | Rv: 5'-TCGGGTCCCTCCTGTGGATAAG-3’       |
| qPCR primers, ef1α (larvae RT-qPCR):   | Fw: 5'-CAGCTGATCGGTGGAGTCAA-3’         |
|                                        | Rv: 5'-TGTATGCGTACTGCTCCTTGAAGC-3’     |

Table S2. List of primers used to genotype wt1bΔ5/Δ5 mutants and to perform RT-qPCRs. Related to STAR Methods and the Key Resources Table.