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Overlapping requirements for Tet2 and Tet3 in normal development and hematopoietic stem cell emergence

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Summary

The Tet family of methylcytosine dioxygenases (Tet1, Tet2 and Tet3) convert 5-methylcytosine to 5-hydroxymethylcytosine. To date, functional overlap among Tet family members has not been examined systematically in the context of embryonic development. To clarify the potential for overlap among Tet enzymes during development, we mutated the zebrafish orthologs of *Tet1, Tet2* and *Tet3,* and examined single, double and triple mutant genotypes. Here, we identify Tet2 and Tet3 as the major 5-methylcytosine dioxygenases in the zebrafish embryo and uncover a combined requirement for Tet2 and Tet3 in hematopoietic stem cell (HSC) emergence. We demonstrate that Notch signaling in the hemogenic endothelium is regulated by Tet2/3 prior to HSC emergence and show that restoring expression of the downstream *gata2b/scl/runx1* transcriptional network can rescue HSCs in *tet2/3* double mutant larvae. Our results reveal essential, overlapping functions for *tet* genes during embryonic development and uncover a requirement for 5hmC in regulating HSC production.

Introduction

In vertebrate species, the epigenetically modified base 5-methylcytosine (5mC) is associated with transcriptional repression and is essential for normal development (Goll and Bestor, 2005). The mechanisms that establish and maintain 5mC are well defined; but less is known about how 5mC is removed (Wu and Zhang, 2011). The ten eleven translocation proteins

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(Tet1, Tet2 and Tet3) comprise a family of 2-oxoglutarate and Fe(II) dependent dioxygenases that convert 5mC to 5-hydroxymethylcytosine (5hmC) and its oxidative derivatives 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) (He et al., 2011; Tahiliani et al., 2009). Growing evidence suggests that conversion of 5mC to 5hmC and its derivatives can provide a first step toward DNA demethylation through active base excision or passive dilution of oxidized bases (Kohli and Zhang, 2013). Consistent with a role in regulating gene expression through DNA demethylation, 5hmC levels are most abundant in euchromatic regions including transcription start sites, enhancers and exons (Pastor et al., 2011; Stroud et al., 2011; Williams et al., 2011).

Individually, homozygous mutation of *Tet1, Tet2* or *Tet3* is compatible with mouse embryonic development, although *Tet3* mutant mice die perinatally (Kohli and Zhang, 2013). Conditional deletion of *Tet3* in oocytes results in delayed demethylation of the paternal genome and increased developmental failure, but viable pups can be recovered (Gu et al., 2011; Inoue et al., 2015). In contrast to single mutants, embryonic stem cells (ESCs) mutated for all three *Tet* genes contribute poorly to chimeras, suggesting that Tet family members have overlapping functions in promoting embryonic development (Dawlaty et al., 2014). *Tet1/2* double homozygous mutant mice can survive into adulthood; but other *Tet* mutant combinations have yet to be described (Dawlaty et al., 2013).

Tet regulation appears to be of particular importance in the hematopoietic lineage. *TET1* was first identified as a fusion partner of the mixed lineage leukemia (MLL) gene in acute myeloid leukemia and has essential oncogenic roles in MLL rearranged leukemias (Huang et al., 2013; Lorsbach et al., 2003). Moreover, *Tet1* mutations promote increased self-renewal of progenitor B cells and susceptibility to B cell lymphoma in mice (Cimmino et al., 2015). Mutations in *TET2* are common in human myeloid malignancies and *Tet2* mutation promotes myeloid transformation in mice and zebrafish (Gjini et al., 2014; Ko et al., 2011; Kunimoto et al., 2012; Li et al., 2011b; Moran-Crusio et al., 2011; Quivoron et al., 2011; Shide et al., 2012; Solary et al., 2014). Consistent with a role in promoting myeloid malignancy, *Tet2* mutation causes increased numbers of hematopoietic progenitor cells in the bone marrow and skewed differentiation toward the myelomonocytic lineage in mice (Ko et al., 2015). Similarly, human cord blood cells depleted for *TET2* and cells isolated from leukemia patients bearing *TET2* mutations exhibit an increase in myeloid-lineage differentiation at the expense of the erythroid-lineage (Madzo et al., 2014; Pronier et al., 2011). Recently, mutation of *Tet3* was shown to cause minor decreases in the absolute number of hematopoietic stem cells (HSCs) in the mouse bone marrow, while numbers of myeloid, erythroid and B lymphoid cells were unaffected (Ko et al., 2015).

While the importance of Tet regulation in the adult hematopoietic system is clear, less is known about requirements for *Tet* genes during early stages of hematopoietic development. Reports using antisense morpholino depletion in zebrafish and shRNA depletion in human ESCs have implicated Tet2 in the regulation of primitive hematopoiesis; but, these results are at odds with the normal primitive hematopoiesis observed in *Tet2* mutant mice and zebrafish (Ge et al., 2014; Gjini et al., 2014; Ko et al., 2015; Langlois et al., 2014). The *de novo* generation of HSCs during the definitive wave of hematopoiesis also appears normal in *Tet2* mutant mouse and zebrafish embryos; however, the potential for additional Tet

enzymes to contribute to HSC emergence during embryonic development has not been experimentally addressed in mutant animals (Gjini et al., 2014; Ko et al., 2015).

The zebrafish genome encodes single well-conserved orthologs of *Tet1, Tet2* and *Tet3* (Almeida et al., 2012). To define the requirements for these genes during development, we generated stable lines carrying mutations in each of the zebrafish *tet* orthologs and we derived single, double and triple homozygous mutant larvae representing all genetic combinations. In this study, we demonstrate that Tet2 and Tet3 are the major 5mC dioxygenases in the zebrafish embryo and that they have overlapping functions in promoting normal development. In addition, we describe a combined requirement for *tet2* and *tet3* in the *de novo* generation of HSCs in the embryo, and demonstrate the importance of *tet2/3* for Notch signaling and downstream expression of the *gata2b/scl/runx1* transcriptional program in the hemogenic endothelium. Our results provide a comprehensive analysis of *tet* mutants in the developing zebrafish embryo and identify requirements for Tet regulation in the early function of the hemogenic endothelium. These results underscore the importance of epigenetic regulation for the generation of HSCs and identify regulation of 5hmC as an additional variable to be considered in therapeutic applications such as the *in vitro* differentiation of HSCs from pluripotent precursors.

Results

TALEN induced mutations reveal overlapping requirements for *tet2* and *tet3* in the zebrafish embryo

To systematically define requirements for 5hmC during development, we introduced mutations into the zebrafish orthologs of *Tet1, Tet2* and *Tet3* using TAL effector nucleases (TALENs) (Li et al., 2011a; Sander et al., 2011). RNAs encoding TALENs that targeted each gene were separately injected into one-cell stage embryos and we recovered individuals harboring germline transmissible mutations in each of the three genes. The recovered *tet2^{mk17}* allele deletes 4 base pairs in exon 8. This deletion results in a frame shift, causing early termination one amino acid 3' of an essential iron-binding residue (Figure 1A and 1B, Figure S1D-S1F). The *tet1^{mk16}* and *tet3^{mk18}* alleles harbor 4 and 14 base pair deletions, respectively, in the last coding exon. In addition to causing frame shift and premature termination, these deletions eliminate sequence encoding a C-terminal arginine residue that is required for 2-oxoglutarate binding (Hu et al., 2013) (Figure 1A and 1B, Figure S1A-C and S1G-I). Loss of specific residues involved in cofactor binding and catalysis is predicted to similarly compromise the dioxygenase activity of all three enzymes.

Modest (less than 3-fold) reductions in total 5hmC were observed in larvae that were homozygous mutant for *tet1, tet2,* or *tet3* (Figure 1C). More considerable reductions in 5hmC were observed in double mutants, with the *tet2$^{mk17/mk17}$*, *tet3$^{mk18/mk18}$* double mutant (*tet2/3^{DM}*) combination producing the most dramatic decrease (>30-fold) (Figure 1C). 5hmC levels were further reduced in *tet1/2/3* triple mutant (*tet1/2/3™*) larvae; indicating that all three mutated genes encode proteins that are compromised for catalytic activity (Figure 1D). The enhanced loss of 5hmC in *tet2/3^{DM}* larvae compared to other double mutant combinations argues that Tet2 and Tet3 are the predominant 5mC oxidases in the zebrafish

embryo and that they function redundantly to promote the formation of 5hmC during development.

Zebrafish that were homozygous for mutations in *tet1, tet2* or *tet3* were viable to adulthood, as were *tet1/2* and *tet1/3* double homozygous mutants. In contrast, combined mutation of *tet2* and *tet3* was not compatible with survival beyond the larval period. The *tet2/3^{DM}* larvae were morphologically indistinguishable from wild-type controls during the first 24 hours post fertilization (hpf), but subtle abnormalities in brain development emerged on the second day post fertilization (dpf). By 36 hpf, smaller eyes, abnormal brain morphology, altered pigmentation and a modest curvature of the trunk were apparent in 25% of larvae derived from intercrosses between *tet2$^{mk17/mk17}$*, *tet3$^{mk18/+}$* adults (Figure 1E). Genotyping (n=20) confirmed that the morphologically abnormal embryos all carried homozygous mutations in both *tet2* and *tet3,* demonstrating a combined requirement for the two genes in zebrafish development. Despite the additional reduction in 5hmC, *tet1/2/3^{TM}* larvae were morphologically indistinguishable from *tet2/3* double mutants at all stages examined (Fig 1F and data not shown).

Overlapping requirements for Tet2 and Tet3 in definitive but not primitive hematopoiesis

Given the combinatorial effects of *tet2* and *tet3* mutation on overall development, and the known roles of Tets in later stages of hematopoiesis, we tested whether *tet2* and *tet3* had overlapping roles in regulating hematopoiesis during embryonic development. Development of non-HSC derived primitive erythroid and myeloid cells was normal in *tet2/3^{DM}* larvae and *tet2$^{mk17/mk17}$* siblings as assessed by expression of *gata1, scl* and *pu.1* at 25 hpf by whole mount in situ hybridization (WISH) (Figure 2A-2I). In contrast, expression of HSC dependent definitive blood cell markers was dramatically reduced in *tet2/3^{DM}* larvae compared to stage matched wild-type controls. While mature *rag1* positive T-cells were readily observed in the thymus of wild-type larvae at 5 dpf, *rag1* positive cells were absent from the corresponding region of *tet2/3^{DM}* larvae (Figure 3A-3B). Similarly, the *Tg(lysC:GFP)* transgenic line, which labels definitive granulocytes (Hall et al., 2007), produced a clear fluorescent signal in blood from wild-type larvae at 6 dpf, but fluorescent cells were not detected in *tet2/3^{DM}* larvae at this stage (Figure 3C-3D).

The loss of differentiated definitive blood cells in *tet2/3^{DM}* larvae can be attributed to a defect in HSC development. During normal embryonic development, HSCs emerge from the hemogenic endothelium in the ventral wall of the dorsal aorta (DA). In *tet2/3^{DM}* larvae, we found that expression of the HSC-associated genes, *runx1* and c-*myb*, was reduced in the DA at 36 hpf, whereas *runx1* expression in *tet2* and *tet3* single homozygous mutant larvae was indistinguishable from wildtype (Figure 3E-3M). Moreover, in *tet2/3^{DM}* larvae, *c-myb* positive hematopoietic stem and progenitor cells (HSPCs) could not be detected in the caudal hematopoietic tissue (CHT) niche, a secondary, transient site for HSC amplification (Figure S2A-S2F). Importantly, *runx1* expression was rescued by injecting mRNA encoding either human TET2 or TET3 into one-cell stage embryos derived from *tet2$^{mk17/mk17}$*, *tet3$^{mk18/+}$* intercrosses (Figure 3N). Injection of mRNA encoding a catalytically dead version of TET2 failed to rescue wild-type levels of *runx1* expression, directly implicating the 5mC dioxygenase activity of TET2 in regulating HSC development (Figure 3N).

Normal vascular development, arterial specification, and blood flow-induced Nitric Oxide (NO) signaling are known prerequisites for HSC development (Adamo et al., 2009; Jagannathan-Bogdan and Zon, 2013; North et al., 2009). WISH for the vascular markers *kdrl* and *cdh5,* and the arterial marker *efnb2a* revealed similar expression in wildtype and *tet2/3^DM* larvae, demonstrating the presence of an overtly intact vasculature (Figure 4A-4F). Visual inspection of *tet2/3^DM* larvae by bright field microscopy showed that blood flow was grossly normal during the first two days of development, and *klf2a*, an immediate early responder to blood flow, was expressed at similar levels in wild-type and *tet2/3^DM* larvae (Figure 4G-4H). Moreover, while exposure to the NO agonist S-nitroso-N-acetyl-penicillamine (SNAP) was sufficient to rescue *runx1* expression in *silent heart* (*sih*) morpholino injected embryos lacking blood circulation, SNAP exposure was unable to rescue *runx1* expression in *tet2/3^DM* larvae (Figure S3A-S3E). Taken together, these results suggest that the HSC defects observed in *tet2/3^DM* larvae are not secondary to defects in vascular development or aberrant blood flow.

HSC emergence is compromised in *tet2/3^DM* larvae

Beginning around 32 hpf, nascent HSCs emerge from the ventral aortic endothelium of the zebrafish embryo through a process termed the endothelial to hematopoietic transition (EHT) (Bertrand et al., 2010; Kissa and Herbomel, 2010). To test whether EHT was impacted by mutation of *tet2/3*, the *tet2* and *tet3* mutant alleles were introduced into a *Tg(kdrl:Ras-mCherry)^S896*, *Tg(kdrl:H2B-EGFP)^mu122* transgenic background (Chi et al., 2008; Kochhan et al., 2013). In this background, membrane mCherry and nuclear GFP are expressed in the vascular endothelium and emergent HSCs, allowing EHT events to be identified based on stereotypical changes in cell morphology (Bertrand et al., 2010; Kissa and Herbomel, 2010). Prior to the onset of EHT, *tet2/3^DM* larvae and siblings from *tet2^mk17/mk17*, *tet3^mk18/+* intercrosses exhibited similar fluorescent labeling with the two transgenes (Figure 5A-5B). A defined region of the DA was then monitored in *tet2/3^DM* larvae and sibling controls (n=3 each) between 30 and 46 hpf by time-lapse confocal microscopy. Analysis of image sets revealed a four-fold reduction in the number of EHT events detected in *tet2/3^DM* larvae compared to siblings, indicating that EHT is compromised in the double mutants (average of 3 vs 13 EHT events, p=0.001; Figure 5C and 5E, Movie S1).

In each time-lapse sequence from *tet2/3^DM* larvae, we also observed between one and three cells within the DA undergoing nuclear fragmentation (Figure 5D and 5F, Movie S2). In contrast, fragmentation was never detected in time-lapse sequences from siblings (Figure 5D). The nuclear fragmentation phenotype we observed in *tet2/3^DM* larvae is reminiscent of that described for zebrafish embryos depleted for *runx1* by morpholino injection, and suggests that a fraction of cells undergoing EHT also die via apoptosis in the absence of *tet2/3* (Kissa and Herbomel, 2010). Elevated levels of TUNEL positive cells were not observed in the DA or surrounding tissues of fixed *tet2/3^DM* larvae (n=10, data not shown). However, apoptotic events restricted to the few cells undergoing EHT would be difficult to capture in fixed embryos.

Tet2/3 are required for Notch signaling and expression of key hematopoietic transcription factors in the hemogenic endothelium

The reduction in EHT events in *tet2/3^{DM}* larvae suggested a requirement for Tet2/3 in the function or specification of the hemogenic endothelium, which gives rise to nascent HSCs. The hematopoietic transcription factors *runx1* and *scl* are both expressed in the hemogenic endothelium prior to the initiation of EHT and are required for this process (Kissa and Herbomel, 2010; Zhen et al., 2013). By WISH, we found that expression of both *runx1* and *scl* was reduced in the DA of *tet2/3^{DM}* larvae prior to HSC emergence, indicating that Tet2 and Tet3 are required to promote the hemogenic potential of the vascular endothelium (Figure 6A-6D). Expression of *scl* and *runx1* is controlled in part by the transcription factor Gata2 in mouse, and the zebrafish genome encodes for two Gata2 paralogs that have undergone subfunctionalization (Butko et al., 2015; Gao et al., 2013; Gottgens et al., 2002; Pimanda et al., 2007). Zebrafish *gata2a* is broadly expressed throughout the hematopoietic system, and is important for vascular morphogenesis (Zhu et al., 2011). In contrast, zebrafish *gata2b* is specifically detected in the hemogenic endothelium and is required for *runx1* expression within this tissue (Butko et al., 2015). Consistent with the HSC specific phenotypes observed in *tet2/3^{DM}* larvae, we found that *tet2/3* mutation compromised expression of *gata2b*, while *gata2a* expression was unaffected at similar stages (Figure 6E-6H). Collectively, these observations identify Tet2/3 as essential regulators of the *gata2b/scl/runx1* transcriptional network in the hemogenic endothelium.

To clarify whether disruption of *gata2b/scl/runx1* transcriptional program could account for the HSC defects observed in *tet2/3* double mutant larvae, we next tested whether reintroducing mRNA encoding *scl* or *gata2b* into *tet2/3^{DM}* embryos could rescue HSC production. *In vitro* transcribed mRNA encoding Scl or Gata2b was injected into one cell-stage embryos derived from *tet2$^{mk17/mk17}$*, *tet3$^{mk18/+}$* intercrosses and *runx1* expression was subsequently examined in the DA of *tet2/3* double mutants by WISH at 36 hpf. Injection of either mRNA was sufficient to rescue wild-type levels of *runx1* expression in the DA of *tet2/3^{DM}* larvae, identifying the *gata2b/scl/runx1* network as the primary hematopoietic program regulated by Tet2/3 during HSC emergence (*scl*: p=0.0004, *gata2b*: p=0.0008, Figure 6I-6M).

Analysis of genomic DNA from sorted vascular endothelial cells revealed that the promoters of *gata2b, runx1* and *scl* were similarly unmethylated in wildtype and *tet2/3^{DM}* larvae, suggesting that Tet2/3 do not directly regulate expression of these genes via promoter demethylation (Figure S4). This observation raised the possibility that Tet2/3 act upstream of *gata2b* in regulating HSC emergence. Notch signaling is required for the expression of *gata2b* in the zebrafish hemogenic endothelium and Gata2 is a direct target of Notch signaling in the mouse dorsal aorta, making this pathway a strong candidate for Tet2/3 regulation during HSC emergence (Butko et al., 2015; Robert-Moreno et al., 2005). To test whether mutation of *tet2/3* disrupted Notch signaling in the hemogenic endothelium, we introduced the *tet2* and *tet3* mutant alleles into a Notch reporter line, *Tp1:GFP*, which expresses GFP under the control of tandem Notch responsive elements (Parsons et al., 2009). At the whole embryo level, wildtype and *tet2/3^{DM}* larvae exhibited similar patterns of GFP expression from the *Tp1:GFP* transgene, suggesting that Notch signaling was not

globally compromised by mutation of *tet2/3* (Figure S5A-S5D). At higher resolution, morphologically wild-type embryos carrying the *Tp1:GFP* transgene exhibited the expected strong expression of *Tp1:GFP* along both the dorsal and ventral walls of the DA (Figure 7A). In *tet2/3^{DM}* larvae, *Tp1:GFP* expression on the dorsal side of the DA appeared similar to wild type, however, expression along the ventral wall appeared weaker and discontinuous (n=10/11, Figure 7B). Quantification of mean GFP fluorescent intensity in each region revealed a 3-fold reduction in the ratio of GFP fluorescence in the ventral DA compared to the dorsal DA in *tet2/3* double mutants (p=0.0002, Figure 7C). This specific reduction in GFP in the ventral DA reveals a requirement for Tet2/3 in regulating Notch signaling in the hemogenic endothelium and provides a mechanistic explanation for the down regulation of *gata2b* observed in *tet2/3^{DM}* larvae. Taken together these results uncover a requirement for Tet2/3 in the early function of the hemogenic endothelium and identify Notch signaling and the downstream expression of the *gata2b/scl/runx1* transcriptional network as key targets of Tet2/3 regulation during HSC emergence.

Discussion

Our systematic analysis of *tet* mutant phenotypes revealed Tet2 and Tet3 to be the major 5mC dioxygenases in the zebrafish embryo. To date, a number of studies in mouse have focused on *Tet1* and *Tet2*, likely due to the fact that these are the only *Tet* orthologs expressed in ESCs (Wu and Zhang, 2011). Nonetheless, *Tet3* is up regulated upon differentiation of ESCs and is highly expressed in many differentiated primary tissues (Dawlaty et al., 2013; Li et al., 2015). Our analysis of zebrafish *tet* mutants, combined with the survival of *Tet1/2* mutant mice and the poor differentiation capacity of *Tet1/2/3* mutant ESCs, suggest that *Tet2* and *Tet3* may also have important overlapping requirements in promoting mammalian development (Dawlaty et al., 2014; Dawlaty et al., 2013). Intriguingly, while at least *Tet3* is maternally deposited in mouse, few, if any, *tet* transcripts are detected in RNA-seq data from 2-cell stage zebrafish embryos and 5hmC is not detected in the zebrafish embryo by immunofluorescence until the bud stage (Almeida et al., 2012; Gu et al., 2011). These observations imply that mRNAs encoding the Tet enzymes are not maternally deposited in zebrafish and suggest that, in contrast to mammals, the zebrafish genome does not contain significant amounts of 5hmC prior to segmentation. Zebrafish do not undergo the same Tet dependent erasure and reestablishment of global 5mC patterns observed during mammalian preimplantation development, providing one potential explanation for this distinction (Jiang et al., 2013; Potok et al., 2013). The lack of maternal deposition and limited dependency on Tet enzymes during the first 24 hours post fertilization make zebrafish a powerful system for examining Tet requirements in later developmental processes, including those associated with tissue specific development and differentiation.

In the current study, we examine requirements for Tet2/3 during embryonic stages of hematopoietic development. Defects in primitive hematopoiesis following antisense morpholino depletion of *tet2* in zebrafish and impaired differentiation of primitive embryonic/yolk sac progenitors following shRNA depletion of *TET2* in human ESCs have been reported (Ge et al., 2014; Langlois et al., 2014). However, these results are difficult to reconcile with the normal primitive hematopoiesis observed in published mouse and

zebrafish models of *Tet2* mutation (Gjini et al., 2014; Ko et al., 2011; Kunimoto et al., 2012; Li et al., 2011b; Moran-Crusio et al., 2011; Quivoron et al., 2011; Shide et al., 2012; Solary et al., 2014). While it is difficult to definitively address the discrepancies between these studies, both shRNA and antisense morpholino technologies can be susceptible to off target effects (Kok et al., 2015; Scherer and Rossi, 2003). Similar to other published studies of *Tet2* mutants, we find that primitive hematopoiesis proceeds normally in the *Tet2* homozygous mutant zebrafish generated by our laboratory, and we demonstrate that combinatorial elimination of Tet2 and Tet3 catalytic functions does not further impact primitive hematopoiesis. It is important to note that because the truncation mutations used in this study leave N-terminal coding sequence intact, we cannot rule out the possibility that Tet2/3 have combinatorial dioxygenase independent functions in regulating primitive hematopoiesis.

The earliest stages of definitive hematopoiesis also appear unaffected in *Tet2* single mutant mice and zebrafish, although diminished expression of *c-myb,* but not *runx1,* was observed in the DA of *tet2* morpholino injected zebrafish embryos (Ge et al., 2014; Gjini et al., 2014; Ko et al., 2011; Kunimoto et al., 2012; Li et al., 2011b; Moran-Crusio et al., 2011; Quivoron et al., 2011; Shide et al., 2012; Solary et al., 2014). In genetic models, loss of *Tet2* eventually leads to an expansion of hematopoietic progenitor cells in the bone marrow and skewed myeloid differentiation (Gjini et al., 2014; Ko et al., 2011; Li et al., 2011b; Moran-Crusio et al., 2011). However, the long latency that precedes these phenotypes suggests secondary somatic mutations may be a contributing factor. In contrast to these later hematopoietic phenotypes, we find that combined mutation of *tet2/3* causes an early loss of definitive blood cells, resulting from compromised HSC production. Importantly, abnormalities in HSC development occurred in embryos that were morphologically quite normal, and had normal expression of vascular markers. The relatively normal development of *tet2/3^{DM}* larvae supports a specific role for *tet2* and *tet3* in regulating transcription of the embryonic HSC developmental program rather than a more generalized role in regulating global transcription.

Compared to *tet1*, both *tet2* and *tet3* transcripts are relatively enriched in the DA of the developing zebrafish embryo (Ge et al., 2014). This expression pattern provides a potential explanation for the specific overlapping Tet2/3 requirements in HSC production. Analysis of double mutant larvae revealed a combined requirement for *tet2/3* in regulating Notch signaling in the hemogenic endothelium, suggesting a role for 5hmC in the specification or early function of this tissue. Enrichment of 5hmC has been reported at Notch receptor and ligand genes in other tissues, but the functional significance of these changes has not been determined (Terragni et al., 2014). Intriguingly, we find Notch signaling to be relatively intact in other tissues of *tet2/3^{DM}* larvae, indicating that Tet2/3 are likely be involved in fine-tuning the activation of this pathway in select cell types. Notch signaling is essential for HSC development in vertebrates, and has been implicated in both specification of the dorsal aorta and downstream HSC production (Jagannathan-Bogdan and Zon, 2013; Robert-Moreno et al., 2005). Tet2/3 appear to be dispensable for Notch regulation of arterial specification, as we observe normal expression of the arterial marker *ephrinb2* in *tet2/3^{DM}* larvae. Instead, disruption in Notch signaling in the hemogenic endothelium favors a select

requirement for Tet2/3 in the regulation of HSC specification. This model is consistent with the downstream disruption of the *gata2b/scl/runx1* transcriptional network observed in *tet2/3^{DM}* larvae and our observation that reintroducing *scl* or *gata2b* mRNA can rescue HSC production in double mutants. Notably, Notch regulation is dispensable for *scl* expression during the primitive wave of hematopoiesis, which is consistent with the normal *scl* expression observed in *tet2/3^{DM}* primitive erythrocytes (Burns et al., 2005; Kim et al., 2013).

Collectively, these results uncover a requirement for Tet regulation of 5hmC in the early function of the zebrafish hemogenic endothelium. A deeper understanding of how HSC generation is regulated *in vivo*, is expected to facilitate the *in vitro* production of HSCs for therapeutic purposes. Importantly, our results identify regulation of 5hmC as an additional variable to be considered in the optimization of protocols for HSC differentiation from pluripotent progenitors. This observation may be of particular relevance given recent data highlighting the impact of cell culture conditions on global 5hmC levels (Blaschke et al., 2013; Nestor et al., 2015; Yin et al., 2013).

Experimental Procedures

Zebrafish husbandry

Zebrafish maintenance and breeding were conducted under full animal use and care guidelines with approval by the institutional animal care and use committee. Zebrafish were raised under standard conditions at 28° C.

TALEN mutagenesis

TALEN sequences were selected using Targeter 2.0 software (Doyle et al., 2012). TAL repeat assembly was achieved using the Golden Gate assembly method and assembled repeats were integrated into the GoldyTALEN scaffold (Bedell et al., 2012; Cermak et al., 2011). Assembled vectors served as templates for *in vitro* mRNA transcription using the T3 mMessage mMachine kit (Ambion) according to manufacturer's instructions. 50-100 pg of mRNA was injected into wild-type embryos at the one-cell stage. Details of mutation recovery and genotyping can be found in Supplemental Experimental Procedures.

5hmC dot blot

Genomic DNA was isolated from larvae at 5 dpf by phenol-chloroform extraction and ethanol precipitation. Following RNAse treatment and denaturation, serially diluted DNA was spotted onto nitrocellulose membranes. Cross-linked membranes were incubated with 0.02% Methylene Blue to validate uniform DNA loading. Membranes were blocked with 5% bovine serum albumin and incubated with anti-5hmC antibody (1:10,000; Active Motif) followed by a horseradish peroxidase-conjugated antibody (1:15,000; Active Motif). Signal was detected using the ECL Prime Detection Kit (GE).

WISH

WISH was performed as described (Thisse and Thisse, 2008). For all probes except *gata2b*, 10% dextran sulfate was added to the hybridization buffer.

RNA synthesis and microinjection

The human *TET3* vector used for mRNA production has been previously described (Ko et al., 2013). The human *TET2* ORF corresponding to Genebank: NM_001127208 was was amplified from cDNA made from SH-SY5Y neuroblastoma cells. Following sub-cloning, the *TET2* ORF was introduced into the pEF1/V5-His vector (Invitrogen) to allow for *in vitro* transcription. Mutant TET2 (H1382Y, D1384A) was generated using the QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent). pExpress-1-gata2b was purchased from Transomic Technologies. For *scl-β*, RT-PCR amplified *scl-β* cDNA with sequence corresponding to Genbank: EF488003 was cloned into pCS2+. Sequences of all clones were confirmed by conventional DNA sequencing. In all cases, capped RNA was synthesized using mMessage mMachine (Ambion) with Sp6 or T7 polymerase as appropriate to the vector. For each experimental condition, mRNA was injected into at least fifty embryos derived from *tet2^{mk17/mk17}*, *tet3^{mk18/+}* intercrosses.

Time-lapse confocal microscopy

Embryos were anesthetized with 0.02% tricaine and embedded in 0.5% low-melt agarose. Embryos were scanned using a SP8 confocal microscope (Leicia) at 28.5°C. Confocal z-stacks were acquired every 7 to 10 minutes between 30 and 46 hpf. Approximately 25 planes were collected per time point at a spacing of 3 μm. Data was analyzed using Imaris software and exported in QuickTime. All confocal planes were sequentially analyzed to identify changes in cell morphology consistent with EHT and to identify nuclear fragmentation events.

Tp1:GFP imaging and quantification

Mounted samples were scanned using an SP8 confocal microscope (Leica) using 40X water immersion objective at 26 hpf. For each image, approximately 60 planes were captured at a spacing of 0.38 μm. Data processed using Imaris software and quantified using ImageJ.

Statistical analysis

The Student unpaired 2-tailed t-test was used for statistical analysis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Tet2 and Tet3 are the major 5-methylcytosine dioxygenases in the zebrafish embryo

- Tet2 and Tet3 have overlapping requirements in hematopoietic stem cell emergence

- Loss of Tet2/3 compromises expression of the *gata2b/scl/runx1* hematopoietic program

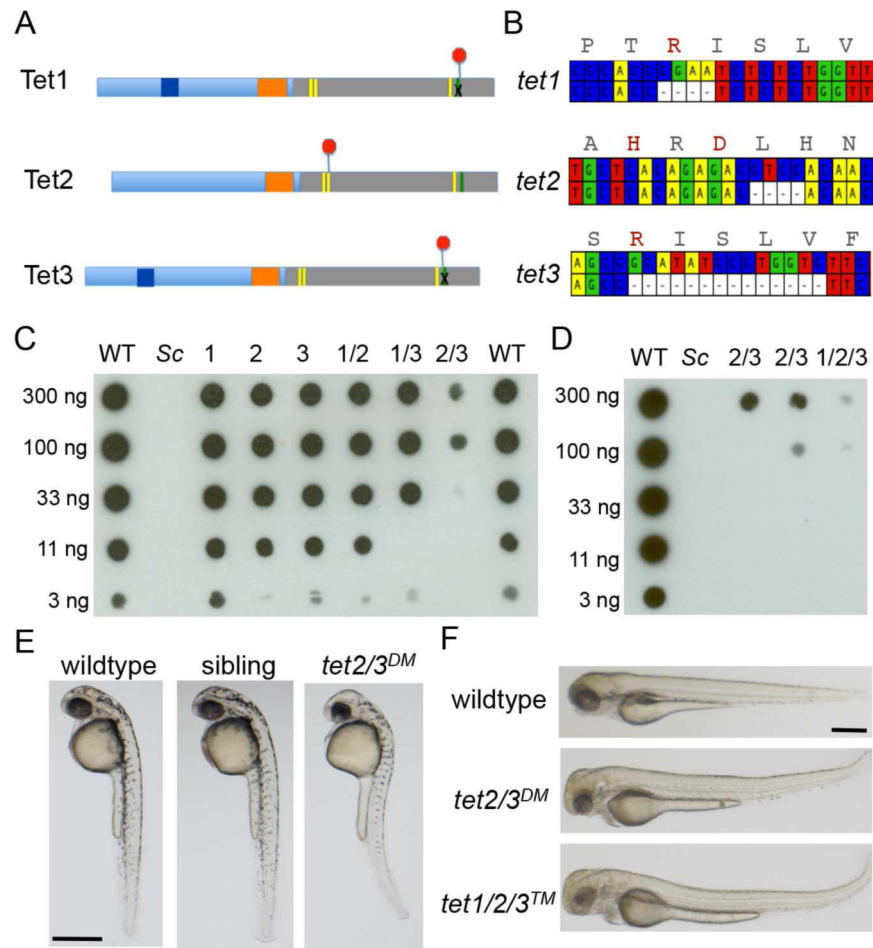- Notch signaling in the hemogenic endothelium is dependent on Tet2/3

Figure 1. Mutation of zebrafish *tet1, tet2* and *tet3*

(A) Schematic illustrating early termination caused by TALEN mutations in zebrafish *tet1, tet2* and *tet3*. Red octagons indicate the position of early termination signals. Yellow bars indicate conserved iron binding residues, the green bar indicates the arginine required for 2-oxoglutarate binding.

(B) Schematic depicting deleted bases in zebrafish *tet1, tet2* and *tet3*. Corresponding amino acid sequences for the wild-type allele are included, with residues required for cofactor binding or catalysis indicated in red.

(C) Dot blot for 5hmC on genomic DNA isolated from larvae at 5 dpf. Numbers indicate the mutated *tet* gene(s) in each sample. *Sc* indicates DNA isolated from *Saccharomyces cerevisiae*. Horizontal rows depict 3-fold serial dilutions of DNA.

(D) Dot blot for 5hmC on genomic DNA isolated from larvae at 5 dpf including DNA isolated from *tet1/2/3*™ larvae.

(E) Lateral views of a representative wild-type larva, a *tet2/3^DM* larva and a sibling larva derived from a *tet2^mk17/mk17*, *tet3^mk18/+* intercross at 36 hpf.

(F) Lateral views of a representative wild-type larva, a *tet2/3^DM* larva and *tet1/2/3*™ larva at 3 dpf. All scale bars indicate 500 μM. See also Figure S1.
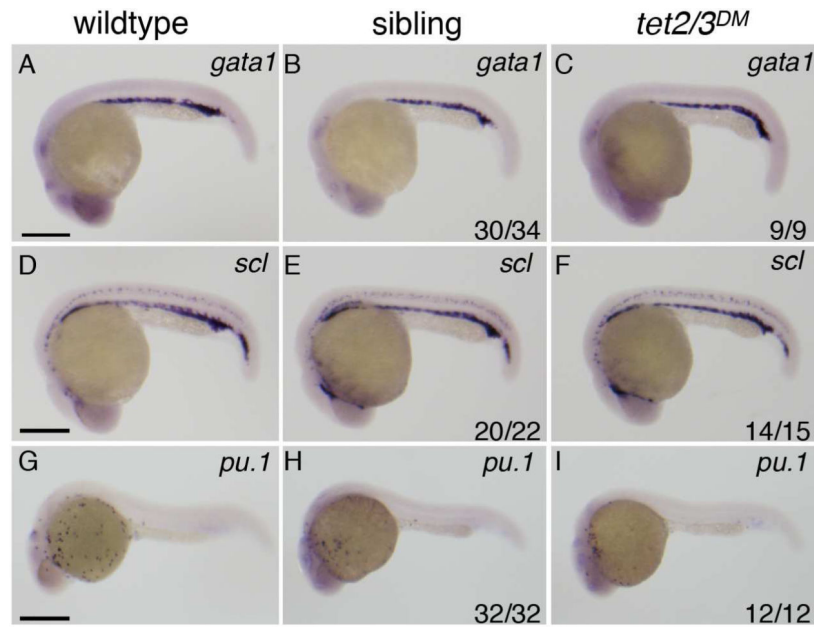
Figure 2. Markers of primitive hematopoiesis are similarly expressed in wildtype, *tet2^{mk17/mk17}* and *tet2/3^{DM}* larvae

(A-I) Representative lateral views of wild-type larvae compared to *tet2/3^{DM}* and *tet2^{mk17/mk17}* sibling larvae derived from *tet2^{mk17/mk17}*, *tet3^{mk18/+}* intercrosses.

(A-C) WISH for the primitive erythroid marker *gata1* at 25 hpf.

(D-F) WISH for the primitive erythroid marker *scl* at 25 hpf.

(G-I) WISH for the primitive myeloid marker *pu.1* at 25 hpf.

Numbers in the lower right hand corner indicate the fraction of embryos exhibiting WISH labeling similar to the representative image. Scale bars indicate 500 μM.
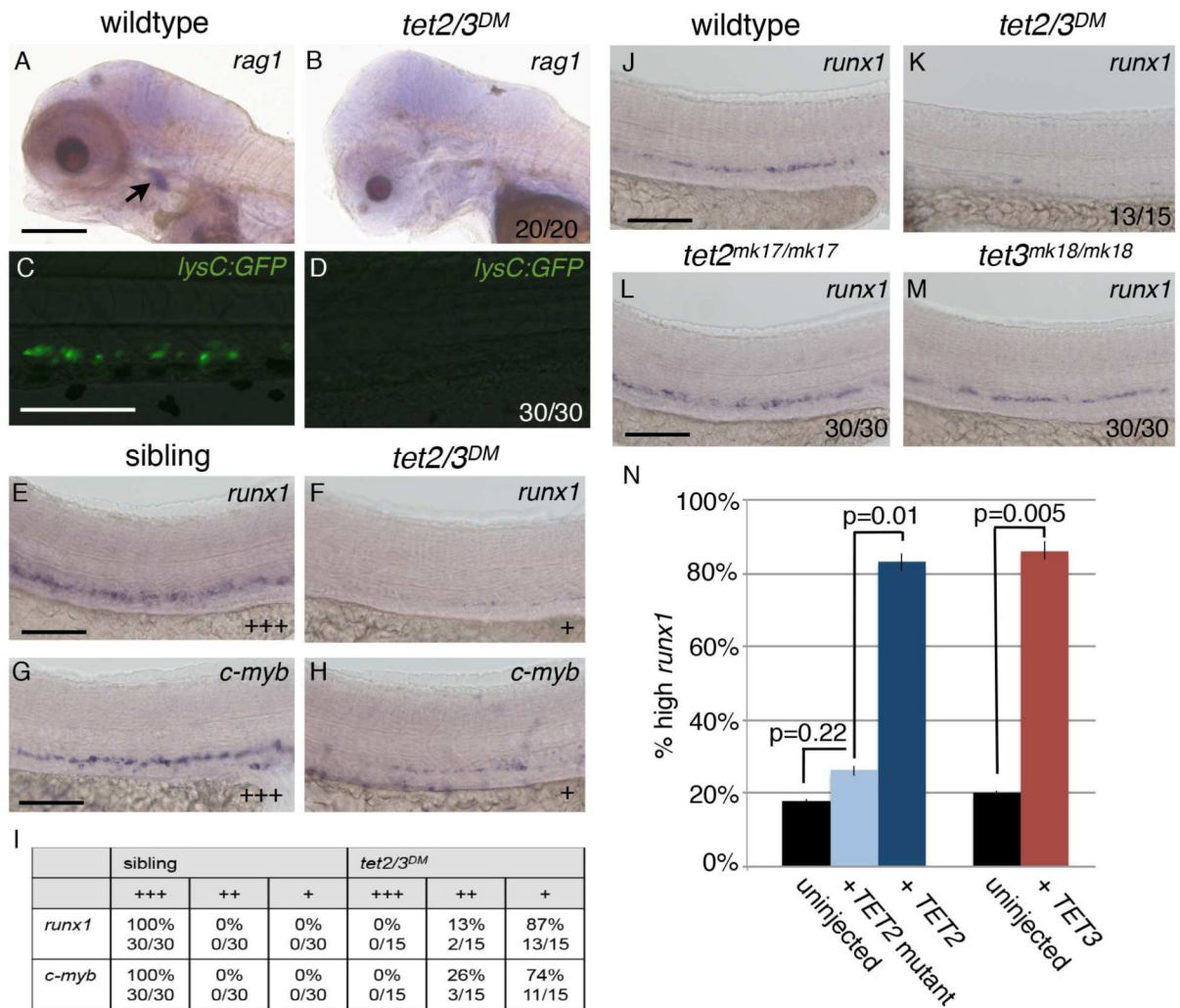
Figure 3. *tet2* and *tet3* have overlapping functions in HSC development

(A and B) WISH for *rag1* at 5 dpf. Arrow indicates thymic T-cells in wild-type larvae.

(C and D) GFP labeled macrophages and neutrophils in 6 dpf larvae carrying the *Tg(lysC:GFP)* transgene.

(E and F) WISH for the HSC marker *runx1* in the DA at 36 hpf.

(G and H) WISH for the HSC marker *c-myb* in the DA at 36 hpf.

(I) Number of sibling and *tet2/3^DM^* embryos with wild type (+++), reduced (++), or nearly absent (+) *runx1* labeling in the DA. Numbers are representative of three independent crosses.

(J-M) WISH for *runx1* in the DA at 32 hpf.

(N) Graph indicating the percent of *tet2/3^DM^* larvae exhibiting high *runx1* staining in the DA in uninjected controls or following injection with 100 pg of mRNA encoding TET2, TET2 H1382Y.D1384A (TET2 mutant) or TET3. Numerical data is presented as the mean ± standard error of the mean (SEM).

Numbers in the lower right corner of images indicate the fraction of larvae with WISH labeling similar to the representative image. All scale bars indicate 100 μM. See also Figure S2.
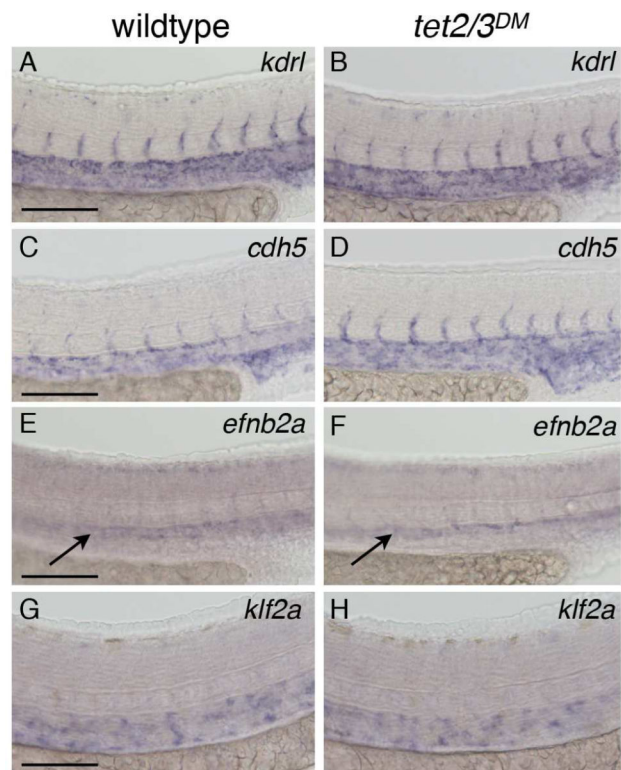
Figure 4. Normal vasculature and blood flow in *tet2/3^DM* larvae

(A and B) WISH for the vascular marker *kdrl* at 31 hpf.

(C and D) WISH for vascular marker *cdh5* at 31 hpf.

(E and F) WISH for the arterial marker *efnb2a* at 31 hpf.

(G and H) WISH for the blood flow dependent marker *klf2a* at 36 hpf.
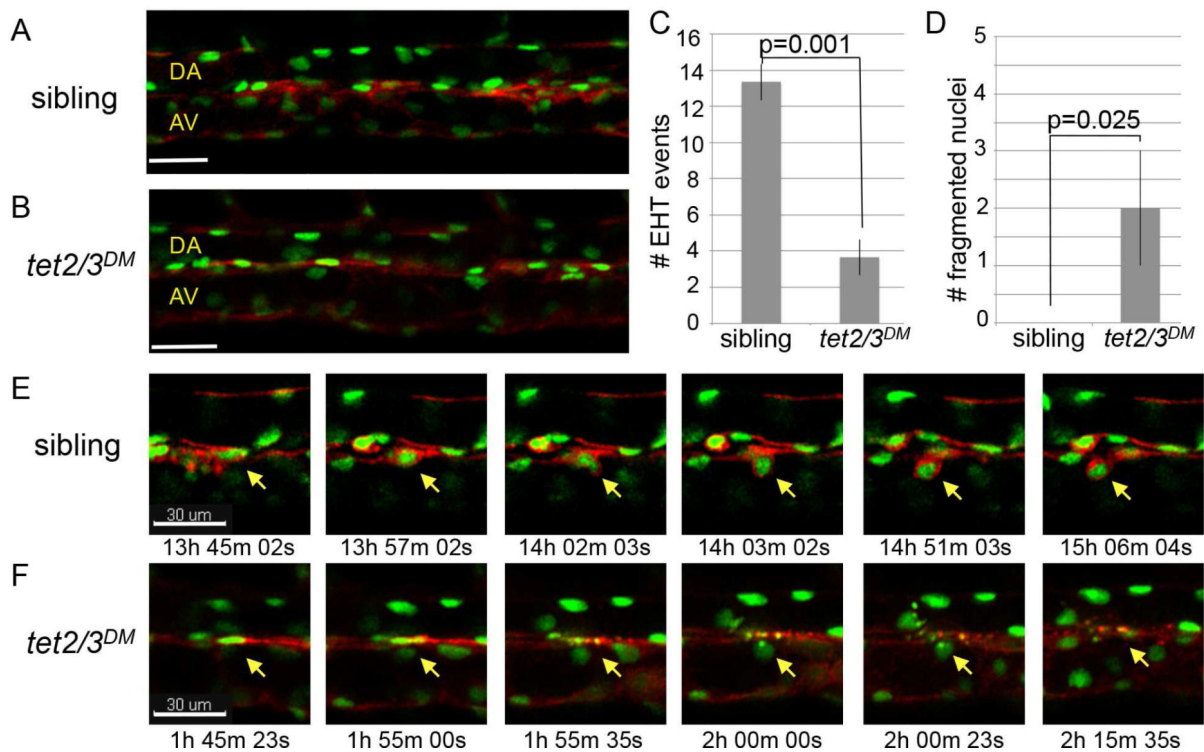
Scale bars indicate 100 µM. See also Figure S3.

Figure 5. Tet2/3 are required for HSC emergence through the endothelial to hematopoietic transition

(A and B) Merged images depicting GFP and mCherry labeling of the vasculature in *Tg(kdrl:Ras-mCherry), Tg(kdrl:H2B-EGFP)* transgenic larvae at 30 hpf.

(C) Number of EHT events detected between 30 and 46 hpf in *tet2/3^{DM}* larvae and siblings. Numerical data is presented as the mean ± SEM.

(D) Number of fragmented nuclei observed in the DA of *tet2/3^{DM}* larvae and their siblings between 30 and 46 hpf. Numerical data is presented as the mean ± SEM.

(E) Sequences from Supplemental Movie S1 documenting the stepwise emergence of an HSC from the the DA of a sibling larva. For each time point, merged GFP and mCherry images are shown. The yellow arrow indicates the cell undergoing EHT.

(F) Sequences from Supplemental Movie S2 documenting a cell undergoing nuclear fragmentation in the DA of a *tet2/3^{DM}* larva. For each time point, merged GFP and mCherry images are shown. The yellow arrow indicates the cell with nuclear fragmentation.
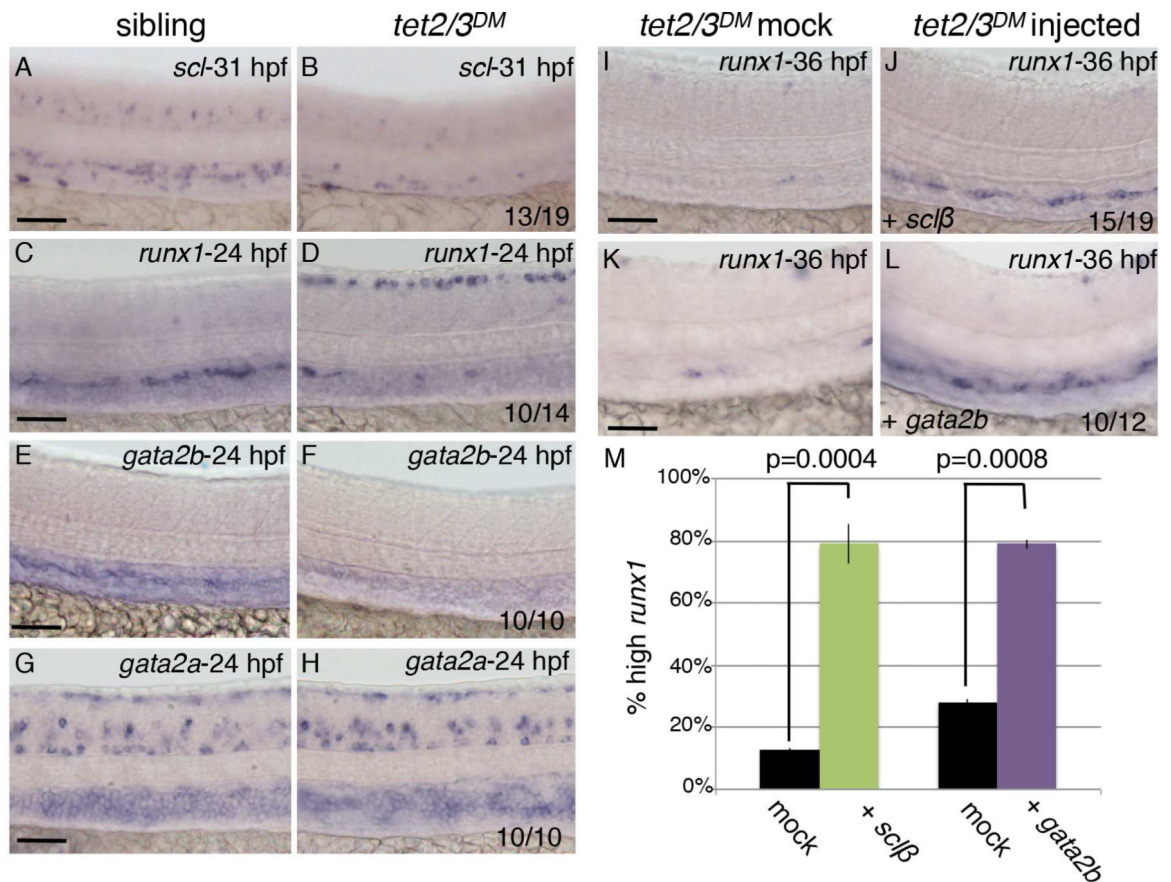
All scale bars indicate 30 μM. See also Movies S1-S2.

Figure 6. Tet2/3 regulate expression of the *gata2b/scl/runx1* transcriptional network in the hemogenic endothelium

(A-B) WISH for *scl* at 31 hpf.

(C-D) WISH for *runx1* at 24 hpf.

(E-F) WISH for *gata2b* at 24 hpf.

(G-H) WISH for *gata2a* at 24 hpf.

(I-J) WISH for *runx1* in the DA of mock injected and *sclβ* mRNA-injected *tet2/3^DM* embryos at 36 hpf.

(K-L) WISH for *runx1* in the DA of mock injected and *gata2b* mRNA-injected sibling embryos at 36 hpf.

(M) Percent of *tet2/3^DM* embryos with high *runx1* expression following mock injection, injection with mRNA encoding Sclβ (50pg) or Gata2b (200pg). Numerical data is presented as the mean ± SEM.

Numbers in the lower right corner of images indicate the fraction of larvae with WISH labeling similar to the representative image. All scale bars indicate 50 μM. See also Figure S4.
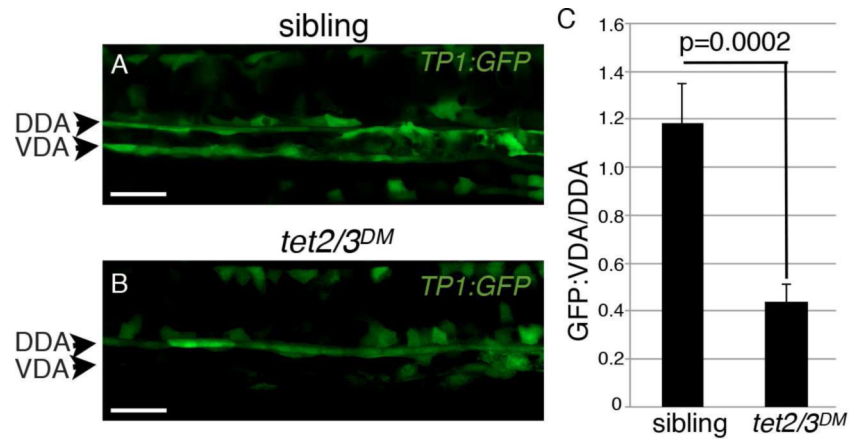
Figure 7. Tet2/3 are required for Notch signaling in the hemogenic endothelium
(A-B) Confocal images of *Tp1:GFP* expression in the dorsal aorta of sibling and *tet2/3^{DM}* larvae at 28 hpf. DDA indicates the dorsal wall of the dorsal aorta. VDA indicates the ventral wall of the dorsal aorta.

(C) Ratio of GFP fluorescence intensity in the VDA/DAA in sibling and *tet2/3^{DM}* larvae (n=11 per genotype). Numerical data is presented as the mean ± SEM. Scale bars indicate 100 μM.