TETs Regulate Proepicardial Cell Migration through Extracellular Matrix Organization during Zebrafish Cardiogenesis

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SUMMARY

Ten-eleven translocation (Tet) enzymes (Tet1/2/3) mediate 5-methylcytosine (5mC) hydroxylation, which can facilitate DNA demethylation and thereby impact gene expression. Studied mostly for how mutant isoforms impact cancer, the normal roles for Tet enzymes during organogenesis are largely unknown. By analyzing compound mutant zebrafish, we discovered a requirement for Tet2/3 activity in the embryonic heart for recruitment of epicardial progenitors, associated with development of the atrial-ventricular canal (AVC). Through a combination of methylation, hydroxymethylation, and transcript profiling, the genes encoding the activin A subunit Inhbaa (in endocardium) and Sox9b (in myocardium) were implicated as demethylation targets of Tet2/3 and critical for organization of AVC-localized extracellular matrix (ECM), facilitating migration of epicardial progenitors onto the developing heart tube. This study elucidates essential DNA demethylation modifications that govern gene expression changes during cardiac development.

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AUTHOR CONTRIBUTIONS
Y.L., M.G.G., and T.E. conceived the study and designed experiments. Y.L. performed most of the experiments. H.P., B.D., and O.E. performed computational and bioinformatics analyses. C.L. provided essential reagents prior to publication. K.M.B. and J.S. performed experiments. Y.L. and T.E. wrote the manuscript. All authors edited and approved of the final draft.

SUPPLEMENTAL INFORMATION
Supplemental Information includes seven figures and two tables and can be found with this article online at https://doi.org/10.1016/j.celrep.2018.12.076.

DECLARATION OF INTERESTS
The authors declare no competing interests.
with striking temporal and lineage specificities, highlighting complex interactions in multiple cell populations during development of the vertebrate heart.

Graphical Abstract

In Brief

Lan et al. show that zebrafish larvae mutant for tet2 and tet3 fail to demethylate genes encoding Inhbaa (in endocardium) and Sox9b (in myocardium), leading to defects in ECM needed to form valves and to recruit epicardial progenitors onto the heart tube.

INTRODUCTION

Epigenetics refers to heritable changes in gene expression without DNA sequence alteration. Epigenetic modifications, including histone phosphorylation and methylation and DNA methylation and demethylation, can alter DNA accessibility and chromatin structure, thereby regulating gene expression (Loscalzo and Handy, 2014). In vertebrates, DNA methylation at the 5 position of cytosine (5mC) is often associated with transcriptional repression and is one of the key epigenetic mechanisms used during normal development (Goll and Bestor, 2005); alteration in DNA methylation patterns has been implicated in various disease states (Robertson, 2005). The mechanisms that establish and maintain 5mC are well defined, including de novo methylation through DNA methyltransferase-3 (Dnmt3) family proteins and maintenance methylation by Dnmt1 (Hu et al., 2012; Feng et al., 2010; Sen et al., 2010). Blocking the action of maintenance methylation leads to passive loss of 5mC through dilution of marks in replicating cells. However, there is good evidence that methyl marks can be actively removed, even in the absence of DNA replication (Wu and Zhang, 2017).

Recent studies identified the ten-eleven translocation (TET) proteins TET1, TET2, and TET3 as a family of 2-oxoglutarate-and Fe(II)-dependent dioxygenases that alter the methylation status of DNA by converting 5mC to 5-hydroxymethylcytosine (5hmC) and
then 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC), followed by replication-dependent dilution or thymine DNA glycosylase (TDG)-dependent base excision repair (He et al., 2011; Wu and Zhang, 2011; Pastor et al., 2013). Defects in this pathway are associated with multiple diseases, including cancer. Mutations in TET genes, most notably TET2, are associated with human hematopoietic malignancies (Ko et al., 2015; Madzo et al., 2014) and more recently are implicated in clonal hematopoiesis associated with risk for leukemia and cardiovascular disease (Solary et al., 2014; Turgeon et al., 2014). Animal and cell models have tested the impact of losing TET activity. Tet3 knockout mice die perinatally (Kohli and Zhang, 2013). Although Tet1 and Tet2 mutant mice are viable and fertile, half of Tet1−/−; Tet2−/− double null embryos exhibit midgestation abnormalities with perinatal lethality (Dawlaty et al., 2013). Both mouse and human embryonic stem cells (ESCs) carrying null mutations for all three Tet genes show impaired ability to differentiate and contribute poorly to teratomas or chimeras (Verma et al., 2018; Dawlaty et al., 2014). We (Li et al., 2015) and others (Seritrakul and Gross, 2017) reported overlapping requirements for tet2 and tet3 during zebrafish hematopoietic stem cell emergence and retinal neurogenesis, respectively. Less is known about specific requirements for TET genes during organogenesis and morphogenesis. DNA hydroxymethylation is associated with myocardial gene expression in maturation and hypertrophy (Kranzhöfer et al., 2016; Greco et al., 2016), suggesting that TET genes might be required during cardiogenesis.

The vertebrate heart forms from progenitor cells derived from multiple, distinct embryonic origins (Meilhac et al., 2004). The primitive heart tube forms from first-heart-field-derived mesoderm that generates myocardium associated with the underlying endocardium to form a beating heart tube. The atrial-ventricular canal (AVC) forms by repression of the muscle program to distinguish the primitive atrial and ventricular chambers and formation of cushions preceding valvulogenesis. Second heart field mesoderm adds to both the venous and arterial poles during formation of inflow and outflow tracts, respectively. Additional progenitors migrate to form an extracardiac rudiment called the proepicardium (PE) (comprising epicardial progenitors). Once the PE attaches to the heart, it undergoes morphogenesis to form an epithelial covering called epicardium, which is the source of cardiac pericytes and vascular smooth muscle cells and also acts as a sleeve, allowing ingrowth of the microvasculature (Chen et al., 2014; Dettman et al., 1998; Lindsey et al., 2014; Peralta et al., 2014; Poelmann et al., 1993; Ratajska et al., 2008; Red-Horse et al., 2010; Snarr et al., 2008). Here, we describe a combined requirement for Tet2 and Tet3 in facilitating zebrafish PE attachment. The results highlight exquisite spatial and temporal control of DNA methylation patterns underlying complex interactions of cell populations during cardiac morphogenesis.

**RESULTS**

**Overlapping Requirement for Tet2 and Tet3 in PE Morphogenesis**

Loss of any single tet1/2/3 gene is tolerated in zebrafish embryos and adults. By combining mutant alleles, we showed previously that Tet2 and Tet3 are the major 5mC dioxygenases in the zebra-fish embryo and that most hydroxymethylation is lost in the double mutant embryos, associated with developmental defects, including a failure to generate
hematopoietic stem cells, neural defects, and pericardial edema (Li et al., 2015), the latter of which indicates potential functions during cardiogenesis. Specification of cardiac progenitors and formation of a primitive heart tube was normal in tet2 and tet3 double homozygous mutant (tet2/3DM) larvae, assessed by expression patterns of gata4, nkx2.5, myh6, and myh7 using whole-mount in situ hybridization (WISH) (Figure S1A). RNA sequencing data at 28 h post-fertilization (hpf) indicated neural developmental defects in tet2/3DM larvae (Figure S1B). In contrast, an equivalent cardiac transcriptomic profile was found comparing tet2/3DM and wild-type or sibling larvae (Figure S1C), consistent with normal early heart development. Imaging of tet2/3DM larvae in the background of fluorescent reporter strains showed that endocardial marker kdr1 and myocardial marker myl7 were grossly normal during the first 2 days of development (Figures S2A-S2F). The mutant hearts beat and normal expression of klf2a (an immediate early responder to flow) suggest that blood flow is also grossly normal during the first 2 days of development (Li et al., 2015). This was confirmed by directly measuring heart rate, which did not differ significantly comparing sibling and mutant embryos (Figure S2G). Although expression of PE marker wt1 was also normal at 40 hpf, wt1 expression patterns were reduced and restricted at 54 hpf in tet2/3DM larvae compared to stage-matched sibling controls (Figure 1A), suggesting a defect in epicardial development.

To further evaluate epicardial development in tet2/3DM larvae, the mutant alleles were crossed onto the Tg(tcf21:NLS-EGFP) transgenic background, in which GFP is expressed in PE as well as epicardium (Figure 1B). Consistent with previous findings (Plavicki et al., 2014b), in sibling larvae, PE were observed near the heart at 46 hpf and found attaching to the heart around the atrioventricular (AV) junction at 52 hpf. By 72 hpf, in addition to the establishment of epicardial cells on the ventricle, a bridge can be seen between the AV junction and pericardium as a path for PE cells migrating onto the heart. Although PE could be found in the pericardial region at 46 hpf in tet2/3DM larvae, they failed to attach to or migrate onto the heart at 52 hpf or 72 hpf (Figure 1B), suggesting a PE morphogenesis defect in tet2/3DM larvae. To clarify whether this was a migration or proliferation defect, we quantified tcf21+ PE cell numbers and found a similar number of PE cells in sibling or tet2/3DM larvae at 48 hpf (Figure 1C). By 72 hpf, the total number of tcf21+ PE cells was not different comparing siblings and tet2/3DM larvae. However, in tet2/3DM larvae, the cells failed to migrate to the ventricle and accumulated in the pericardial region, indicated by increased numbers of tcf21+ cells on the yolk sac of tet2/3DM larvae (Figure 1C). Moreover, pH3 antibody staining suggested essentially no cell proliferation of tcf21+ PE cells at 48 hpf or 72 hpf (Figure S3). Therefore, in tet2/3DM larvae, there is normal specification and no impact on proliferation but rather a marked defect in migration during PE morphogenesis.

To validate that the phenotype was caused by loss of Tet function and specifically DNA demethylation, we attempted to rescue PE migration in tet2/3DM larvae by forced TET expression or by inhibiting DNA methylation. Embryos derived from tet2−/−tet3+/− intercrosses were injected with in vitro transcribed mRNA encoding human TET2 at the one-cell stage or cultured in the presence of the DNA methyltransferase inhibitor 5-aza-2′-deoxycytidin (5-aza) from 24 hpf. The number of tcf21+ epicardial cells associated with the developing ventricle was subsequently examined in tet2/3DM larvae by confocal imaging at 4 dpf. Either strategy partially rescued the PE migration and morphogenesis defect in
tet2/3<sup>DM</sup> larvae (Figure 1D). Moreover, injection of mRNA encoding a catalytically dead version of TET2 failed to rescue PE migration, directly implicating a critical function for tet2 and tet3 in regulating PE migration through DNA demethylation.

**Tet2 and Tet3 Regulate PE Migration by Modulating Myocardial Function**

An in vitro epicardial migration assay was performed to test whether the PE migration defect in tet2/3<sup>DM</sup> larvae is cell autonomous or non-cell autonomous for epicardium (Figure 2A). In the assay, donor hearts were isolated from embryos transgenic for the Tg(tcf21:DsRed) epicardial cell reporter at 72 hpf, and recipient hearts were isolated from embryos transgenic for the Tg(myl7:EGFP) myocardial reporter at 48 hpf, before epicardial cells had migrated onto the myocardium. After a week of co-culture, epicardial cells from wild-type donor hearts could be observed covering wild-type recipient hearts (Figure 2B). However, epicardial cells from wild-type donor hearts failed to migrate onto tet2/3<sup>DM</sup> recipient hearts (Figure 2C), demonstrating a non-cell autonomous role for Tet2/3 in epicardial cell migration. Because the recipient experiment could not be performed due to a lack of epicardium in tet2/3<sup>DM</sup> hearts, a cell-autonomous role for Tet2/3 could not be evaluated. Overall, our data suggest that myocardial Tet2/3 is required for promoting PE migration during cardiac development.

**Deficiency of Tet Activity Leads to Hypermethylation and Deregulation of Developmental Genes during Cardiogenesis**

Because tet2 and tet3 appear to regulate epicardium at least in part through a non-cell-autonomous fashion, we sought to identify potential factors responsible for these effects, utilizing both methylomic and transcriptomic analyses. The developing tet2/3<sup>DM</sup> larvae are largely depleted of 5hmC (Li et al., 2015), so it was important to first map the normal distribution of 5hmC sites. For this purpose, 5hMe-bead-integrated click-seq (5hMe-BIC-seq) was performed using genomic DNA from wild-type 48 hpf hearts. We identified 145,501 5hmC-occupied peaks and defined the distribution of these peaks in the zebrafish genome in four regions: promoters (2 kb upstream and downstream of transcription start sites); active enhancers (peaks overlapped with H3K4me1 and H3K27ac peaks, but not overlapped with promoters or exons); poised enhancers (peaks overlapped with H3K4me1 peaks only, but not overlapped with H3K27ac peaks, promoters, or exons); and other regions. The majority (over 60%) of 5hmC-associated regions were found in active enhancers, with another 5% mapping to transcriptional regulatory regions, including promoters and poised enhancers (Figure 3A), which is consistent with previous mouse and zebrafish data (Bogdanović et al., 2016; Hon et al., 2014).

To determine the impact of Tet enzyme loss and 5hmC depletion on DNA methylation, hearts from wild-type or tet2/3<sup>DM</sup> larvae were isolated at 48 hpf and ERRBS (enhanced reduced representation bisulfite sequencing) was performed to compare their 5mC profiles. In tet2/3<sup>DM</sup> heart tissue, a total of 10,494 differentially methylated regions were found with increased methylation (hyper-differentially methylated regions [DMRs]), and much fewer, only 829, showed decreased methylation (hypo-DMRs; Figure 3B), which is consistent with a function for Tet enzymes in DNA demethylation. Hyper-DMRs in the mutant hearts showed a distribution pattern similar to 5hmC in the wild-type embryos, specifically...
enriched in active enhancers and other regulatory regions (Figure 3A). Moreover, most hyper-DMRs in mutants co-localized with 5hmC peaks in wild-type (Figure 3C), demonstrating the close association of 5hmC during DNA demethylation. Gene pathway analysis of these hyper-DMR-associated genes showed a significant enrichment for developmental signaling pathways, including Notch, Wnt, transforming growth factor β (TGF-β), and bone morphogenetic protein (BMP) pathways (Figure 3D). Notably, these pathways are all highly associated with cardiac development (Azhar et al., 2003; Wang et al., 2013), suggesting that Tet2/3-mediated DNA demethylation regulates important signaling functions during cardiogenesis.

To investigate the transcriptional consequences of these methylation changes, RNA sequencing (RNA-seq) was also performed using isolated wild-type and tet2/3 DM hearts at 48 hpf. In the tet2/3 DM hearts, 129 genes were downregulated and 160 genes were upregulated (Figure 3E). For a select subset of top differentially expressed genes, qPCR analysis confirmed cardiac transcriptional alterations in tet2/3 DM hearts, compared with sibling or wild-type hearts. Interestingly, these differences were not found in samples generated from whole embryos, demonstrating the requirement to isolate heart tissue to reveal cardiac-specific gene regulation changes (Figure S4A). Gene Ontology (GO) analysis suggested that downregulated genes in tet2/3 DM mutant hearts are involved in heart morphogenesis, vasculature development, cell motility and junction, and muscle differentiation (Figure S4B), further confirming the cardiac developmental defect in tet2/3 DM larvae. Gene pathway analysis also categorized pathways that were highly downregulated in tet2/3 DM hearts, including tumor necrosis factor alpha (TNF-α), TGF-β, Notch, and Wnt/beta-catenin signaling pathways (Figure 3F), which highly overlapped with hyper-DMR-related pathways. Taken together, these data link Tet-dependent demethylation with the proper activation of genes critical for cardiac development.

**AVC Development Is Disrupted in tet2/3 DM Larvae Temporally Associated with PE Migration Defect**

Both methylomic and transcriptomic analysis identified several pathways that were highly deregulated in 48 hpf tet2/3 DM hearts, including the Wnt/beta-catenin and TGF-β signaling pathways. Previous studies suggested tight regulation of the Wnt and TGF-β pathways is required for atrioventricular canal (AVC) development at this developmental stage (Azhar et al., 2003; Piven and Winata, 2017). Gene set enrichment analysis from RNA sequencing also showed disruption of epithelial-to-mesenchymal transition (EMT), extracellular matrix (ECM) organization, and collagen formation in tet2/3 DM hearts (Figure S4C), all of which are critical processes during AVC development (Moro et al., 2012; Todorovic et al., 2011; Wang et al., 2013). Proper patterning of the AVC is critical for several diverse processes within the developing heart, including cushion and AV valve formation and delay of the electrical impulse between the atria and ventricles (Peal et al., 2011; Wang et al., 2013). The AVC is also the first location where PE cells attach to the heart (Figure 1B), implying its potential function coordinating PE migration. Therefore, we evaluated AVC development in tet2/3 DM larvae.
During AVC development, endocardial-based Notch-Wnt signaling induces a myocardial Bmp-Tbx pathway, which then activates the expression of downstream ECM-related genes, such as has2, to initiate EMT as the first step toward heart valve formation (Shirai et al., 2009; Wang et al., 2013). Using transgenic biosensor reporter strains, we evaluated Notch and Wnt activity in tet2/3DM larvae and observed comparable Notch activity in both sibling and tet2/3DM endocardium (Figure S4D). We also observed specific Wnt activity in the AVC endocardium in 2 dpf sibling hearts, which is in agreement with previous work (Moro et al., 2012; Wang et al., 2013). However, this Wnt activity signal was strikingly lost in tet2/3DM hearts (Figure 4A). By WISH, transcript levels of Wnt downstream genes, including bmp4, tbx2b, and has2 (Ahuja et al., 2016; Camenisch et al., 2002; Singh et al., 2012; Verhoeven et al., 2011), were also reduced in the hearts of tet2/3DM larvae (Figures 4B and S4E), suggesting a quite early EMT defect in tet2/3DM larvae. As a result, the AV valve is absent in 4 dpf tet2/3DM larvae (Figure 4C). Moreover, conditional inhibition of the Wnt pathway using a small-molecule compound inhibitor of Wnt response-1 (IWR-1) led to AVC disruption and a PE morphogenesis defect, which phenocopied tet2/3DM larvae (Figure 4D), also suggesting a close association of proper AVC patterning and epicardial development.

Hypermethylation and Deregulation of inhbaa and sox9b Are Associated with AVC Development and PE Migration Defects in tet2/3DM Larvae

In order to identify candidate genes targeted by Tet2/3 to regulate AVC development, we filtered data from RNA-seq, 5hMe-BIC-seq, and ERRBS to identify genomic regions associated with genes that are hydroxymethylated in wild-type hearts but hypermethylated, and significantly downregulated, in tet2/3DM hearts (Table S1). This identified potential Tet target genes inhbaa and sox9b, both of which are associated in the literature with regulating AVC development. Inhbaa is the monomeric subunit of activin A, a secreted ligand that signals through a serine-threonine kinase complex consisting of type I receptor ActBIB (Alk4) and type II receptor ActRIIA or ActRIIB (DiMuccio et al., 2005; JaŹwińska et al., 2007; Shi and Massagué, 2003; Sun et al., 2006). In murine studies, transcripts for Inhba and its receptors were detected in AVC endocardium at embryonic day 9.5 (E9.5) and E10.5, induced by the Notch pathway leading to activation of the nitric oxide (NO) pathway to promote EMT (Chang et al., 2011). After EMT occurs in the AVC endocardium, mesenchymal cells invade and populate ECM to separate the endocardium and myocardium; these progenitors undergo further proliferation to eventually form heart valves (Person et al., 2005; de Vlaming et al., 2012). In mice, Sox9 is a BMP target required for mesenchymal cell expansion and ECM organization (Garside et al., 2015; Lincoln et al., 2007). In zebrafish studies, sox9b was detected in myocardium and required for epicardium as well as valve formation (Hofsteen et al., 2013; Plavicki et al., 2014a).

In tet2/3DM larval hearts, both WISH and qPCR showed a clear reduction of inhbaa transcripts, which can be rescued by injection of human TET2mRNA (Figures 5A and S5A). Because of widespread expression outside of the heart, this loss of inhbaa expression is not seen if whole embryos are analyzed; thus, the TET-dependent regulation is heart specific (Figures S5B and S5C). The ERRBS data, validated by gene-specific bisulfite sequencing, identified a hypermethylated region in the promoter of inhbaa in tet2/3DM hearts, within a broad peak of 5hmC marks that are present in wild-type hearts (Figure 5B).
Notably, compared with whole heart, the levels of methylation as measured by bisulfite sequencing is further reduced in samples derived from dissected AVC regions, yet this region remains highly methylated in similarly dissected samples derived from tet2/3DM hearts (Figure 5B). This AVC-specific methylation pattern is consistent with AVC-enriched transcript expression, suggesting that Tet-dependent DNA demethylation is restricted for this gene largely to AVC endocardium. Similarly, sox9b transcripts were also strikingly reduced in the heart, associated with promoter hypermethylation (Figures 5A and 5C), features that were not noted using whole-embryo samples, again indicating cardiac specificity (Figures S5B and S5C). Expression levels for sox9b could also be rescued by injection of human TET2 mRNA (Figure 5A).

To confirm that deregulation of inhbaa impacts AVC development as well as PE migration, the small molecule SB431542 was used to block receptor function, or alternatively, 1-(2-[trifluoro-methyl]phenyl)imidazole (TRIM) was used to block the downstream nitric oxide synthase (NOS) inducer. Both treatments caused AVC defects (Figures S6A–S6D) as well as a PE migration defect (Figure S6G). Injection of inhbaa mRNA rescued expression of AVC-restricted markers has2 and bmp4 (Figure 5D) and partially restored PE migration (Figure 5E). Moreover, TRIM treatment also inhibited Wnt activity in AVC endocardium (Figures S6E and S6F), suggesting that inhbaa and NOS function upstream of the Wnt pathway during AVC development. Considering that Inhba is regulated by the Notch pathway and our analysis showed no defect for Notch signaling in endocardium of tet2/3DM larvae, the data implicate inhbaa as a mediator between Notch and Wnt pathways, which is critical for EMT during AVC development. The role of sox9b was next tested. Consistent with previous sox9b knockdown studies (Hofsteen et al., 2013; Lincoln et al., 2007), epicardial morphogenesis was inhibited in sox9b morphant larvae (Figure S6G). Injection of sox9b mRNA was sufficient to rescue has2 but failed to rescue bmp4 (Figure 5D), indicating that sox9b functions downstream of bmp4 but upstream of has2. Sox9b mRNA injection also partially restored epicardium in tet2/3DM larvae (Figure 5E). Notably, the extent of epicardial rescue in tet2/3DM larvae was modestly enhanced with combined injection of inhbaa and sox9b mRNA (Figure 5E), suggesting that Tet2/3 regulates targets that cooperate to facilitate PE recruitment and epicardial development.

**Tet2/3-Dependent AVC ECM Organization Is Critical for PE Attachment to the Heart**

In tet2/3DM larvae, both inhbaa and sox9b mRNA rescued expression of the ECM-related gene has2, which encodes the enzyme required to synthesize ECM constituent hyaluronic acid. Production of hyaluronan is important for the induction of epicardial cell differentiation and invasion in vitro (Craig et al., 2010). Studies in chick embryos also demonstrated that an ECM bridge guides PE cell migration to the myocardium (Nahirney et al., 2003), and we observed features consistent with this process in wild-type zebrafish larvae, lacking in tet2/3DM larvae (Figure 1B). These data suggest ECM may play important functions during PE cell migration. Another key AVC-associated ECM constituent protein gene, vcana (Hatano et al., 2012), was also strikingly deregulated in tet2/3DM larvae (Figure 6A), consistent with RNA sequencing data showing defects of ECM organization and collagen formation in tet2/3DM hearts (Figure S4C).
To test directly whether loss of ECM components could account for the PE migration defects observed in tet2/3<sup>DM</sup> larvae, has2 mRNA was reintroduced into tet2/3<sup>DM</sup> larvae. Injection of has2 mRNA into tet2/3<sup>DM</sup> embryos increased significantly the number of epicardial cells that migrate onto the heart of tet2/3<sup>DM</sup> larvae (Figures 6B and 6C). Although has2 transcript levels were strikingly decreased in tet2/3<sup>DM</sup> hearts, and there were robust 5hmC peaks in the gene body as well as promoter of the has2 gene in wild-type hearts, we observed no difference in methylation status at these regions comparing genomic DNA isolated from tet2/3<sup>DM</sup> and wild-type hearts (Figure S7). These observations indicate that has2 is regulated indirectly by Tet2/3 through ihhaa and sox9b as a downstream component of Tet-dependent PE morphogenesis.

Finally, we tested directly whether the ECM defect during AVC development is responsible for the PE migration defect by injecting the ECM protein collagen into the pericardial region of tet2/3<sup>DM</sup> larvae at 2 dpf. Strikingly, when tet2/3<sup>DM</sup> larvae were injected with 20 nL of a 0.5 mg/mL collagen solution, PE cells migrated onto the heart tube (Figures 6B and 6C), strongly supporting a non-cell-autonomous effect, specifically through TET-dependent ECM organization at the AVC. Siblings were not affected by this treatment. When a high concentration (8 mg/mL) of collagen solution was injected to the pericardial region, collagen aggregates were formed. Under these conditions, in sibling larvae, the PE cells migrated to the heart as well as attached to the collagen aggregate. In tet2/3<sup>DM</sup> larvae, PE cells were attracted to migrate to the collagen aggregate at the expense of attaching to the heart tube (Figure 6B). Taken together, these results uncover a requirement for Tet2/3 in AVC and epicardial development and identify ihhaa and sox9b as likely direct targets of Tet2/3 regulation during AVC-associated ECM organization, which then facilitates PE recruitment and attachment to the heart (Figure 6D).

**DISCUSSION**

During cardiogenesis, epigenetic mechanisms, including DNA methylation and demethylation, histone modification, and long-range chromatin organization, undergo dynamic changes to orchestrate lineage-and temporal-specific changes in gene expression (Backs and Olson, 2006; Hang et al., 2010; Kou et al., 2010; Vallaster et al., 2012), and epigenetic regulatory defects contribute to progression of cardiac diseases (Mano, 2008). However, specific functions and requirements for TET enzymes to regulate DNA demethylation in this context were not known. We found a primary defect in tet2/3<sup>DM</sup> embryos for the attachment to the heart, and subsequent migration to cover the heart, of epicardial progenitors, through defects in PE cell-extrinsic signaling pathways. The results are consistent with recent reports showing cell-non-autonomous functions for Tet enzymes in zebrafish retinal cell differentiation, mouse gastrulation, and for defining the relative balance of neuroectoderm and mesoderm derivatives (Dai et al., 2016; Li et al., 2016; Seritrakul and Gross, 2017). These studies suggest a general function for Tet enzymes during embryonic development to regulate important cell-extrinsic signaling pathways and coordinate cell interactions during organ development.

We found that failed recruitment of PE cells to the heart is associated with defective AVC development. Previous loss-of-function studies identified several pathways in the heart,
including BMP signaling emanating from the AVC myocardium, that are necessary for recruitment of PE cells (Hatcher et al., 2004; Ishii et al., 2010; Liu and Stainier, 2010; Yang et al., 1995). In our zebrafish model, Tet-dependent expression of activin-A is required to activate a downstream bmp4 pathway to coordinate the attachment of PE cells to an AVC-associated region of the heart. We also identified sox9b, a transcription factor regulating valve precursor cell proliferation and differentiation, as another Tet target gene. Both genes affect ECM scaffold formation. Injection of has2 mRNA at the one-cell stage or direct injection of collagen into the pericardial region at 2 dpf can efficiently rescue epicardial cell migration onto the heart in tet2/3DM larvae. Collagen injection experiments directly demonstrated the important function of ECM organization during PE recruitment to the heart. During cardiac development, ECM provides a bridge linking the AV canal and pericardial wall to help guide PE cell migration to the myocardium. Our data therefore indicate that AVC-specific, ECM-related genes, such as has2 and vcana, are not only critical for AV valve formation within the AV cushions but also appear required for ECM bridge generation in the pericardial cavity.

Because similar numbers of PE cells can be detected in the tet2/3DM larvae, and PE cells undergo little if any detectable proliferation at 48 or 72 hpf, Tet2/3 appears to be dispensable for early PE specification and proliferation. However, other cell-autonomous requirements in epicardial cells after they migrate to the heart cannot be ruled out. Because Tet2/3 function is required for EMT during cardiac AVC development and also for endothelial-to-hematopoietic transition (EHT) during hematopoietic stem cell emergence (Li et al., 2015), we speculate that Tet activity becomes progressively more important as specified PE cells become further differentiated and normally undergo EMT (Kalluri and Weinberg, 2009; Kovacic et al., 2012; Krainock et al., 2016). This may represent a common functional requirement for Tet activities in epithelial cells undergoing mesenchymal-like transitions and is likely relevant to the impact of deregulated Tet activity in cancer. Further studies using conditional epicardial lineage-specific knockout of tet2/3 are needed to clarify the function of Tet during later stages of epicardium-derived cell development.

Consistent with recent studies that identified distal regulatory elements as targets of 5mC remodeling in zebrafish embryogenesis and mouse stem cells (Bogdanović et al., 2016; Hon et al., 2014; Lee et al., 2015), depletion of Tet2/3 causes hypermethylation mainly at normally active enhancers. One study in murine ESCs found that knockdown of Tet1 results in loss of promoter oxidation, and depletion of Tet2 causes loss of 5hmC at gene bodies (Huang et al., 2014). We cannot rule out the possibility that biased enhancer hypermethylation is due to Tet1 activity in tet2/3DM larvae. However, in contrast to mouse stem cells, Tet1 expression is low during zebrafish embryogenesis (Ge et al., 2014) and hyper-DMRs are still enriched at enhancers in tet1/2/3knockdown larvae (Bogdanović et al., 2016). These data suggest that Tet1 plays a marginal role during zebrafish development. In promoters, CpG islands may be protected against ectopic DNA methylation by additional mechanisms, including exclusion of de novo DNMTs and polycomb-associated proteins (Boulard et al., 2015; Noh et al., 2015; Ooi et al., 2007; Rasmussen and Helin, 2016). In contrast, distal regulatory regions, such as enhancers, are more vulnerable to DNMT activity upon loss of Tet activity. Regardless, in the present study, we did identify functional hyper-DMRs in promoter regions (such as for inhbaa and sox9b). In summary, our study reveals
Tet2/3-mediated epigenetic modifications that regulate cardiogenesis and uncovers molecular pathways in AVC-dependent PE cell recruitment. It also highlights signaling interactions between distinct heart derivatives, including proepicardium, myocardium, and endocardium.

STAR★METHODS

CONTACT FOR REAGENTS AND RESOURCE SHARING

Further information and requests for reagents should be directed to and will be fulfilled by Todd Evans (tre2003@med.cornell.edu).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

All procedures were approved by the WCMC IACUC—Zebrafish: We used 1–4 days post fertilization larval zebrafish for this study, bred on AB background. Embryos were raised at 28.5°C and morphologically staged as described (Kimmel et al., 1995); No statistical methods were used to predetermine sample size, and animal selection was not randomized or blinded.

Previously described zebrafish lines were used as follows:: tet2^{mk17/mk17}, tet3^{mk18/mk18} double mutant (tet2/3^{DM}) (Li et al., 2015) for tet2/3 loss of function studies, Tg(myl7:EGFP) (Huang et al., 2003) for myocardium labeling, Tg(tcf21:NLS-EGFP) and Tg (tcf21:DsRed2)(Kikuchi et al., 2011) for epicardium labeling, Tg(7xTCF-Xla.Siam:GFP)\textsuperscript{ia4} (Moro et al., 2012) for Wnt pathway activity labeling, Tg(kdrl:EGFP-NLS) (Zygmunt et al., 2011) for endothelium labeling, Tg(p1:EGFP) (Parsons et al., 2009) for Notch pathway activity labeling. Embryos selected for experiments were typically less than 4dpf, a stage at which sex cannot be readily determined and is unlikely to influence the biological processes under study.

METHOD DETAILS

RNA Synthesis and Microinjection—The human TET2 vector used for mRNA production has been previously described (Li et al., 2015). Briefly, the human TET2 ORF corresponding to GenBank: NM_001127208 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). The inhbaa, sox9b and has2 ORF were PCR amplified from a 2 dpf zebrafish embryo cDNA library and cloned into the pCS2+ vector. Sequences of all clones were confirmed by conventional DNA sequencing. PCR primers used are listed in Table S1. Capped RNA was synthesized using mMESSAGE mMACHINE (Invitrogen) with Sp6 polymerase. For each experimental condition, mRNA was injected into at least 100 embryos derived from tet2^{mk17/mk17}, tet3^{mk18/+} intercrosses.

Whole-mount in situ hybridization (WISH)—Zebrafish embryos at the desired stages were fixed in 4% paraformaldehyde (PFA). Whole-mount RNA in situ hybridization (WISH)
was performed using standard methods (Jowett, 1999). For \textit{inhb}aa heart \textit{in situ}, embryonic hearts were micro-dissected at 2 dpf and processed with a standard WISH protocol. For \textit{inhb}aa and \textit{has}2 probe generation, the same vectors used for RNA synthesis were linearized by EcoRI (\textit{inhb}aa) or BamHI (\textit{has}2). The riboprobe was synthesized with T7 RNA polymerase. Additional probes were prepared as described previously: \textit{nkh}2.5, \textit{amhc, vmhc} (Reiter et al., 1999); \textit{gata}4 (Heicklen-Klein and Evans, 2004); \textit{wt}1 (Serluca, 2008); \textit{bmp}4 and \textit{tbx}2b (Chi et al., 2008); \textit{vcana} (Patra et al., 2011), \textit{sox}9b (Yan et al., 2005).

**Immunohistochemistry**—Whole-mount immunohistochemistry was performed using standard methods (Li et al., 2012). Anti-pH3 primary antibody (Santa Cruz) was used at 1:3000 dilution. Anti-GFP primary antibody (Invitrogen) was used at 1:1000 dilution. Alexa Fluor 555-conjugated anti-rabbit and Alexa Fluor 488-conjugated anti-mouse secondary antibodies (Invitrogen) were used at 1:2000 dilution.

**Morpholino and small molecule treatment of embryonic zebrafish**—Splice-blocking MO (5′ TGC AGT AAT TTA CCG GAG TGT TCT C 3′) for \textit{sox}9b was as described (Yan et al., 2005) and injected into one cell stage embryos. For chemical treatment, wild-type or \textit{tet}2/3 \textit{DM} embryos were manually dechorionated with forceps at 24 hpf and exposed to 5-aza (75 μM, Sigma), IWR-1(10 μM, Enzo Life Sciences), TRIM (2 μM, Sigma), or SB431542 (100 μM, Stemgent) from 24 hpf to identical stages for analysis in a solution of E3 medium (5.0 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl, 0.33 mM MgSO4).

**Tissue explant culture**—Hearts from \textit{myl}7:EGFP and \textit{tcf}21:DsRed2 larvae were isolated at 48 hpf and 72 hpf, respectively, as previously described (Burns and MacRae, 2006). Briefly, approximately 100 embryos were anesthetized, washed three times with embryo disruption medium (EDM) [Leibovitz’s L-15 Medium (Fisher) containing 10% fetal bovine serum (Sigma), ice-cold at every step] and resuspended in 1.25 mL EDM. The microfuge tube, a 19-gauge needle and a 6-mL syringe were secured to a ring stand. Approximately 1 mL EDM containing embryos was drawn into the needle and syringe and immediately expelled back into the microfuge tube 30 times at a rate of 1 s per syringe motion. Isolated hearts were isolated under a fluorescence microscope. Hearts were mixed in a 1:1 ratio and placed in culture plates coated with low melting agarose. Heart cultures were incubated at 28°C in tissue culture media containing Leibovitz’s L-15 (Fisher) with 10% fetal bovine serum (Sigma) and 4x penicillin/streptomycin (Invitrogen). Cultures were monitored daily and media was refreshed every other day. On day 7, heart clusters were removed and imaged by confocal microscopy.

**Collagen injection**—For low concentration (0.5mg/ml) collagen injection, Corning Collagen I, High Concentration, Rat Tail (Fisher, 8mg/ml) was diluted in tissue culture medium. 20nl of diluted collagen was microinjected into the pericardial region of 2 dpf larvae. For high concentration collagen injection, 10nl of 8mg/ml collagen was directly microinjected into the pericardial region of 2 dpf larvae to generate a collagen aggregate.

**Image acquisition and analysis**—WISH preparations were mounted in glycerol and imaged using a Nikon SMZ1500 microscope with an Insight Firewire 2 digital camera and...
SPOT advanced imaging software. Transgenic embryos, embryonic hearts and cultured heart clusters were mounted in low melting agarose and imaged using the Zeiss LSM510 or LSM800 confocal microscope with Zen software. Images were analyzed in ImageJ and Adobe Photoshop. Epicardial cell number was counted manually at 4 dpf using the Zeiss LSM510 confocal microscope. 4% tricaine (Sigma) was added immediately before counting to stop heart beating. At least 10 embryos were counted for each biological replicate.

**RNA isolation and quantitative RT-PCR**—Whole embryo and dissected heart total RNA was isolated with the RNeasy Mini kit (QIAGEN) or RNeasy Micro kit (QIAGEN). At least 5 fish or 40 dissected hearts were used for each biological replicate. RNA was reverse transcribed with the Superscript III First-Strand Synthesis System (Invitrogen). The qPCR analysis was performed on a LightCycler 480 II (Roche) using LightCycler 480 Sybr Green master mix (Roche). Primer sequences are provided in Table S1. Relative gene expression was determined as described (Holtzinger et al., 2010).

**RNA sequencing**—For RNA sequencing, whole embryo (n = 3) or dissected heart (n = 2) total RNA from wild-type, sibling or tet2/3DM embryos were isolated as described above. For dissected heart, 1 ng total RNA was used to prepare amplified double-stranded cDNA using the Ovation RNA-Seq System V2 (Nugen). Amplified cDNA was purified using a QIAquick PCR purification kit (QIAGEN) and 200 ng of amplified cDNA was fragmented in a final volume of 50 ul using S220 Focused-ultrasonicator (Covaris) to obtain 150 bp DNA fragment size (peak incident power: 175W, duty factor: 10%, cycles per burst: 200, time: 280 s). Fragmented DNA samples and whole embryo RNA samples were used to prepare libraries with the TruSeq RNA Library Prep Kit v2 (Illumina) and submitted to WCMC Genomics Resources Core Facility for sequencing. RNA-seq data were aligned to the GRCz10 reference genome. RNA seq alignment, differential gene expression analysis, orthology to human genes and GSEA were performed as described (Anelli et al., 2017). Differentially expressed genes were defined by log2 fold change greater than 2 or less than −2 and an adjusted p value < 0.05.

**ERRBS**

For ERRBS, genomic DNA was isolated from wild-type and tet2/3DM dissected hearts using DNeasy Blood & Tissue Kits (QIAGEN). At least 40 hearts were collected for each biological group. Genomic DNA was submitted to the Weill Cornell Medical College Epigenomics core for ERRBS. The WCMC Computational Genomics core facility supported alignment and methylation extraction for ERRBS data as described (Akalin et al., 2012). DMRs were defined as regions containing at least five differentially methylated CpGs (DMCs; false discovery rate = 20%; chi-square test) and whole methylation was more than 10%. DMR calling was performed with RRBSseqer with default parameters (Pan et al., 2015). Peak annotation was performed with R-3.3.2. Histone datasets were obtained from GSE32483 (Bogdanovic et al., 2012).

**5hMe BIC-seq**—Genomic DNA was isolated from wild-type (n = 2) dissected hearts using DNeasy Blood & Tissue Kits (QIAGEN). At least 40 hearts were collected for each
biological group. Genomic DNA was submitted to the Weill Cornell Medical College Epigenomics core for 5hMe-Bead-Integrated Click-seq (5hMe BIC-seq) as described below:

250 ng of genomic DNA were sonicated in a Covaris S220 instrument (Covaris, Woburn, MA) to obtain mean fragment sizes of 250bp (Peak Incident Power 175, Duty Factor 10%, Cycles per Burst 200, 150secs) and DNA was end-repaired and A-tailed using New England Biolabs enzymes (Ipswich, MA). 10ng of this DNA was used as Input control by adding TruSeq barcoded adapters. The rest of the sample was used to selectively label 5-hydroxymethylcytosine with azido-modified-glucose (UDP-6-N3_Glucose, P19-11019, Proactive Molecular Research, Alachua, FL) in the presence of β-glucosyltransferase (New England Biolabs, Ipswich, MA) by a 16 hr incubation at 37C. Biotin was added through Click chemistry with Dibenzocyclooctyne-PEG4-biotin (BP-22295, Broad-Pharm, San Diego, CA), and the biotinylated-gluco-modified DNA was isolated by affinity capture with streptavidin magnetic beads (Dynabeads ® MyOneStreptavidin, ThermoFisher/ LifeTechnologies, Waltham, MA). After ligation of TruSeq barcode adapters the 5hmC-enriched sample was PCR amplified using 12 cycles with Turbo Pfu Cx Polymerase (Agilent, Santa Clara, CA). To control the labeling and enrichment of 5hmC-containing DNA, a parallel reaction containing sheared E. coli DNA spiked in with the 5-hydroxymethylcytosine APC-spike control (cat # 55008) obtained from Active Motif (Carlsbad, CA) was performed. Input and 5hmC-enriched libraries were clustered on a paired end read flow cell and sequenced for 50 cycles on an Illumina HiSeq 2500 to obtain about 30M reads per libraries. Primary processing of sequencing images was done using Illumina Real Time Analysis software (RTA) as suggested by Illumina. CASAVA 1.8.2 software was used to perform image capture, base calling and demultiplexing. Reads passing Illumina’s purity filter were adaptor trimmed and aligned to the genome using the BWA aligner.

ChIP-seq data were aligned to the danRer7 reference genomes using bwa-0.7.12 with default parameters by the WCMC Computational Genomics core facility (Li and Durbin, 2009). Peak calling and analysis of read density in peak regions were performed by macs14 1.4.2 with default parameters (Zhang et al., 2008).

**5mC and 5hmC quantification**—Bisulfite sequencing was performed using the EZ DNA Methylation-Direct kit (Zymo Research). Embryonic hearts at 48 hpf were dissected (n = 4 per condition). Converted DNA was amplified using Taq DNA Polymerase (NEB) and bisulfite-specific primer pairs (listed in Table S1). PCR amplicons were sub-cloned using the TOPO TA Cloning kit (Invitrogen) for sequencing. At least 8 clones were sequenced for each condition. Sequencing traces were analyzed using Lasergene (DNASTAR).

**QUANTIFICATION AND STATISTICAL ANALYSIS**

The Student unpaired 2-tailed t test was used for statistical analysis. We performed Shapiro-Wilk normality tests and all samples passed the test; therefore, we could justify using parametric tests (t test). Data are presented as mean ± SD derived from at least three independent biological replicates. Statistical analysis was performed using Excel and Prism 7. The significance is indicated as *p < 0.05, **p < 0.01, ***p < 0.001, ns indicates not significant.
DATA AND SOFTWARE AVAILABILITY

The accession number for all the sequencing data reported in this paper is NCBI GEO: GSE121991.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

The authors thank the WCMC Optical Microscopy Core for imaging support; WCMC Epigenomics Core and Genomics Resources Core for performing ERRBS, ShmC ChIP-seq, and RNA sequencing; Hual-Jen Tsai for providing the Tg(my17:EGFP) transgenic line; Kenneth D. Poss for providing Tg(tc21::NLG-EGFP) and Tg(tc21::DsRed2) transgenic lines; James F. Amatruda for providing the Tg(7fTCF-Xla.Siam:GFP)transgenic line; and Jesus Torres Vazquez for providing the Tg(kdrl:EGFP-NLS) transgenic line. These studies were supported by a grant from the NHLBI (R35 HL135778 to T.E.) and from the Tri-Institutional Stem Cell Initiative (2014-010 to M.G.G. and T.E.). Y.L. is supported by a Tri-Institutional Starr Stem Cell Scholars Fellowship.

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Highlights

• Zebrafish larvae lacking Tet2 and Tet3 fail in AVC and epicardium development

• Inhbaa (in endocardium) and Sox9b (in myocardium) are targets of Tet2/3 demethylation

• These genes help coordinate ECM needed by epicardial progenitors
Figure 1. tet2 and tet3 Have Overlapping Functions in PE Recruitment to the Heart
(A) WISH for PE marker *wt1* at 40 hpf and 54 hpf. Arrows indicate PE cells with *wt1* transcripts.
(B) Lateral view of hearts showing GFP-labeled PE and epicardium in 46-hpf, 52-hpf, and 72-hpf larvae carrying the Tg(tcf21:NLS-EGFP) transgene. White arrow indicates the extracellular matrix bridge between AVC and the pericardial wall.
(C) Ventral view of hearts showing GFP-labeled PE and epicardium in 48-hpf and 72-hpf sibling or tet2/3DM larvae carrying the Tg(tcf21:NLS-EGFP) transgene. White arrows indicate *tcf21*+ PE and epicardial cells located on the heart. Yellow arrows indicate *tcf21*+ PE and epicardial cells located on the yolk sac. Graph indicates the total number of PE and...
epicardial cells in 48-hpf or 72-hpf sibling and tet2/3^{DM} larvae and the number of PE and epicardial cells located on heart or yolk sac at 72-hpf sibling and tet2/3^{DM} larvae.

(D) The PE migration defect is partially rescued by TET2 mRNA injection or 5-aza treatment. GFP-labeled PE and epicardium in 4-dpf sibling, tet2/3^{DM}, and tet2/3^{DM} injected with wild-type hTET2 mRNA, mutant hTET2 mRNA, or tet2/3^{DM} exposed to 75 μM 5-aza larvae carrying the Tg(tcf21:NLS-EGFP) transgene. Graph indicating the number of epicardial cells located on the heart at 4 dpf is shown. Scale bars: (A) 50 μm; (B–D) 100 μm. **p < 0.01; ***p < 0.001; ns indicates not significant. Data are presented as mean ± SD derived from at least three independent biological replicates.
Figure 2. Epicardial Cells from Donor Hearts Do Not Migrate onto tet2/3<sup>DM</sup> Recipient Hearts

(A) Schematic of epicardial cell migration assay. Wild-type tcf21:DsRed donor hearts (isolated at 72 hpf) were co-cultured with either wild-type or tet2/3<sup>DM</sup> myl7:GFP recipient hearts (isolated at 48 hpf) for one week and then confocal images taken.

(B) Confocal images showed epicardial cells from wild-type donor heart can migrate onto wild-type recipient hearts. In 30 pairs of co-cultured hearts, 6 of recipient hearts were observed having epicardial cells migrated from donor hearts.

(C) Confocal images showed epicardial cells from wild-type donor heart failed to migrate onto tet2/3<sup>DM</sup> recipient hearts. In 60 pairs of co-cultured hearts, none of the recipient hearts were observed having epicardial cells from donor hearts.

Scale bar: 50 μm.
Figure 3. Hypermethylation and Deregulation of Cardiac Developmental Genes in tet2/3DM Larvae

(A) Enrichment of various regulatory regions in 5hmC peak and hyper-DMR by 5hmC chromatin immunoprecipitation (ChIP)-sequencing and ERRBS.

(B) Total number of hyper-and hypo-DMR (tet2/3DM heart versus wild-type heart) by ERRBS.

(C) Overlapping locations between hyper-DMRs and 5hmC peaks.

(D) Pathway enrichment analysis for genes associated with hyper-DMR.

(E) Scatterplot of RNA sequencing data illustrating transcriptional changes in 48-hpf tet2/3DM heart as compared to wild-type heart.

(F) Pathway enrichment analysis for downregulated genes in 48-hpf tet2/3DM heart as compared to wild-type heart by RNA sequencing.
Figure 4. AVC Development Shows Disruption in tet2/3DM Larvae

(A) GFP-labeled AVC endocardium represents Wnt activity in sibling heart, but not tet2/3DM heart. Hearts were dissected from 48-hpf larvae carrying the Tg(7xTCF-Xla.Siam:GFP) transgene. White arrows indicate AVC endocardial cells with Wnt activity.

(B) WISH for AVC markers bmp4 and has2 at 48 hpf.

(C) GFP-labeled endocardium represents AV valve formation in 4-dpf sibling, but not tet2/3DM larvae carrying the Tg(kdrl:EGFP) transgene. White arrows indicate the AV valve. Bottom left images show higher magnification views of AV valve regions.

(D) WISH for AVC marker bmp4 in 48 hpf and confocal imaging for GFP-labeled PE and epicardium in 72-hpf control and IWR-1-treated larvae. Scale bars: 50 μm.
Figure 5. Tet2/3-Dependent Aberrant Promoter Hypermethylation and Deregulation of *inhbaa* and *sox9b* Leads to AVC and PE Migration Defects

(A) RT-PCR analysis of *inhbaa* and *sox9b* transcripts in 48-hpf embryonic heart.

(B) DNA methylation status of *inhbaa* in 48-hpf isolated heart or isolated AVC. Diagram indicates *inhbaa* locus and the associated regulatory regions. Gray box represents 5hmC peak. Black box represents the coding sequence. White box represents hyper-DMR identified by ERRBS. Profiles of 5mC + 5hmC in hyper-DMR region were validated by bisulfite sequencing. n = 4 per condition.

(C) DNA methylation status of *sox9b* in 48 hpf isolated heart. Diagram indicates *sox9b* locus and the associated regulatory regions. Gray box represents 5hmC peak. Black box represents the coding sequence. White box represents hyper-DMR. Profiles of 5mC + 5hmC in hyper-DMR region were validated by bisulfite sequencing. n = 4 per condition.

(D) WISH for AVC markers *bmp4* and *has2* at 48-hpf sibling, tet2/3^DM_, and tet2/3^DM_ injected with *inhbaa* mRNA or *sox9b* mRNA larvae. Scale bar: 50 μm.

(E) Number of epicardial cells on the heart of 4-dpf sibling, tet2/3^DM_, and tet2/3^DM_ injected with *inhbaa* mRNA, *sox9b* mRNA, or *sox9b* combined with *inhbaa* mRNA larvae carrying...
the Tg(tcf21::NLS-EGFP) transgene. Data are presented as the mean ± SD. The significance is indicated as *p < 0.05; **p < 0.01; ***p < 0.001; ns indicates not significant.
Figure 6. Tet2/3 Regulate PE Migration through Extracellular Matrix Organization

(A) WISH for ECM constituent gene *vcana* at 48 hpf. Black arrows indicate AVC-specific expression of *vcana* in sibling, but not *tet2/3* DM heart.

(B) GFP-labeled PE and epicardium in 4-dpf larvae carrying the Tg(tcf21:NLS-EGFP) transgene. Sibling, *tet2/3DM*, and *tet2/3DM* injected with has2 mRNA and *tet2/3DM* injected with low concentration (0.5 mg/mL) collagen larvae were shown in lateral views. Sibling and *tet2/3DM* injected with high concentration (8 mg/mL) collagen larvae were shown in ventral views to represent collagen aggregate and heart clearly. The heart is outlined with white dashed line. The collagen aggregate is outlined with red dashed line.

(C) Number of epicardial cells on the heart of 4-dpf sibling, *tet2/3DM*, and *tet2/3DM* injected with sox9b mRNA or *tet2/3DM* injected with low concentration (0.5 mg/mL) collagen larvae carrying the Tg(tcf21:NLS-EGFP) transgene. Numbers data are presented as the mean ± SD derived from 3 independent biological replicates.

(D) Working model shows Tet2/3-dependent demethylation regulates the expression of *inhbaa* and *sox9b*, which subsequently regulate AVC ECM organization and PE migration. Scale bars: 50 μm. The significance is indicated as *p < 0.05; **p < 0.01; ***p < 0.001.
### KEY RESOURCES TABLE

| REAGENT or RESOURCE                        | SOURCE               | IDENTIFIER            |
|--------------------------------------------|----------------------|-----------------------|
| Antibodies                                 |                      |                       |
| p-Histone H3 Antibody (Ser 10)             | Santa Cruz           | Cat# sc-8656-R; RRID: AB_653256 |
| GFP Monoclonal Antibody (3E6)              | Invitrogen           | Cat# A-11120; RRID: AB_221568 |
| Bacterial and Virus Strains                |                      |                       |
| One Shot TOP10 Chemically Competent E. coli| Invitrogen           | Cat# C404006          |
| Chemicals, Peptides, and Recombinant Proteins |                    |                       |
| 5-Aza-2-deoxycytidine                      | Sigma                | A3656-5MG             |
| IWR-1                                      | Enzo Life Sciences   | BML-WN103-0005        |
| TRIM(1-[2-(Trifluoromethyl)phenyl]imidazole)| Sigma                | T7313-100MG           |
| SB431542                                   | Stemgent             | 04-0010-10            |
| Coming Collagen I                          | Fisher               | 354249                |
| Critical Commercial Assays                 |                      |                       |
| mMESSAGE mMACHINE                          | Invitrogen           | AM1340                |
| RNasey Mini kit                            | QIAGEN               | 74106                 |
| RNasey Micro kit                           | QIAGEN               | 74004                 |
| LightCycler 480 Sybr Green master mix      | Roche                | 04-887-352-001        |
| Ovation RNA-Seq System V2                  | Nugen                | N/A                   |
| TruSeq RNA Library Prep Kit v2             | Illumina             | RS-122-2001           |
| DNeasy Blood & Tissue Kits                 | QIAGEN               | 69506                 |
| EZ DNA Methylation-Direct kit              | Zymo Research        | D5021                 |
| Deposited Data                             | This paper           | GSE121991             |
| Histone datasets                           | Bogdanovic et al.,  2012 | GSE32483          |
| Zebrafish GRCz10 reference genome          | Genome Reference Consortium | https://www.ncbi.nlm.nih.gov/assembly/GCF_000002035.5/ |
| Zebrafish danRer7 reference genomes        | Wellcome Trust Sanger Institute | https://www.ncbi.nlm.nih.gov/assembly/GCF_000002035.4/ |
| Experimental Models: zebrafish Strains     |                      |                       |
| tet<sup>2α/l7αl17, tet<sup>α4/18/α4l18 double-mutant te2/pDM</sup> | Li et al., 2015      | N/A                   |
| Tg(α17:EGFP)                               | Huang et al., 2003   | N/A                   |
| Tg(tcf21:NLs-EGFP)                         | Kikuchi et al., 2011 | N/A                   |
| Tg(tcf21:DsRed2)                           | Kikuchi et al., 2011 | N/A                   |
| Tg(7αTCF-Xla.Siam:GFP)<sup>mut</sup>       | Moro et al., 2012    | N/A                   |
| Tg(kdrl:EGFP-NLS)                          | Zygmun et al., 2011  | N/A                   |
| Tg(tp1:EGFP)                               | Parsons et al., 2009 | N/A                   |
| Oligonucleotides                           |                      |                       |
| Splice-blocking MO (5’ TGC AGT AAT TTA CCG GAG TGT TCT C 3’) for sox9b | Yan et al., 2005 | N/A                   |
| PCR primer sequences, see Table S1         | This paper           | N/A                   |
| Recombinant DNA                            |                      |                       |
| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Plasmid: pEF1/V5-His-hTET2 | Li et al., 2015 | N/A |
| Plasmid: pEF1/V5-His-mutant hTET2 | Li et al., 2015 | N/A |
| Plasmid: pCS2+-inhbaa | This paper | N/A |
| Plasmid: pCS2+-sox9b | This paper | N/A |
| Plasmid: pCS2+-has2 | This paper | N/A |
| Software and Algorithms | | |
| SPOT advanced imaging software | SPOT Imaging | N/A |
| Zen software | ZEISS | N/A |
| macs14 1.4.2 | Shirley Liu lab | http://liulab.dfci.harvard.edu/MACS/00README.html |
| Lasergene | DNASTAR | N/A |
| Prism 7 | GraDhDad | N/A |