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Proper Activity of Histone H3 Lysine 4 (H3K4) Methyltransferase Is Required for Morphogenesis during Zebrafish Cardiogenesis

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While increasing evidence indicates the important function of histone methylation during development, how this process influences cardiac development in vertebrates has not been explored. Here, we elucidate the functions of two histone H3 lysine 4 (H3K4) methylation enzymes, SMYD3 and SETD7, during zebrafish heart morphogenesis using gene expression profiling by whole mount *in situ* hybridization and antisense morpholino oligonucleotide (MO)-based gene knockdown. We find both *smyd3* and *setd7* are highly expressed within developing zebrafish heart and knock-down of these genes led to severe defects in cardiac morphogenesis without altering the expressions pattern of heart markers, including *cmlc2*, *vmhc*, and *amhc*. Furthermore, double knock-down by coinjection of *smyd3* and *setd7* MOs caused the synergistic defects in heart development. As similar to knock-down effect, overexpression of these genes also caused the heart morphogenesis defect in zebrafish. These results indicate that histone modifying enzymes, SMYD3 and SETD7, appear to function synergistically during heart development and their proper functioning is essential for normal heart morphogenesis during development.

INTRODUCTION

During vertebrate development, heart is formed by a highly stereotypic and organized process, and the expression of genes in these processes need be tightly regulated in a spatiotemporal manner (Barnett et al., 2012; Wang, 2012). Recent reports suggest that epigenetic regulation modulates genetic program during differentiation and development of various organs including heart and somitic lineages (Wamstad et al., 2012). For instance, modifications of histone substantially alter the accessibility and structure of chromatin, therefore modifying transcriptional activity of specific gene clusters. Histones are known to undergo specific modification such as methylation and acetylation: while methylation and demethylation of histone are coordinated by histone methyltransferases (HMTs) and demethylase (HDMs), acetylation of histone is modulated by acetyltransferases (HAT) and deacetylases (HDAC) (Bhaumik et al., 2007; Helin and Dhanak, 2013). Due to their significance in regulating genetic program during development and diseases, recent efforts were directed to identify specific factors that mediate this process (Arrowsmith et al., 2012; Cayuso Mas et al., 2011; Kim et al., 2012a; 2012b). In case of methylation, histone H3 lysine 4 residue (H3K4) is sequentially modified through mono-, di-, and tri-methylation status, each of which step is mediated by specific enzymes. MLLs (Mll1-5), SETDB1, and SETDB2 have broad roles for H3K4 methylation, and can methylate all type of H3K4, such as non-, mono-, di-methylated H3K4 (Black et al., 2012; Sims and Reinberg, 2004). In contrast, SMYD3 methylate mono- and di-methylated H3K4, and SETD7 can only catalase the non-methylated H3K4 to transit to mono-methylated H3K4 (Hamamoto et al., 2004; Wang et al., 2001).

SET and MYND domain-containing proteins (SMYDs), involving SMYD1-5, are high conserved protein family across plant, fungi, and animal as well as some protozoa, and have two functional protein domains, SET and MYND domains (Del Rizzo and Trievel, 2011; Dillon et al., 2005). Especially, SET domain is important for histone lysine methylation activity and MYND domain can mediate the protein-protein interaction and bind to DNA motifs. SMYD3 was originally reported as histone lysine methyltransferase (HMT), which methlylates the mono-(H3K4me1) and di-methylated lysine 4 residue (H3K4me2) to generate H3K4me3, of histone H3, but SMYD3 also can bind to 5′-CCCTCC-3′ motif on promoter region of Nkx2.8 gene to induce its expression in cancer cells (Hamamoto et al., 2004). Recent researches showed the global decrease of histone H4 lysine 5 (H4K5) methylation in SMYD3 knock-down condition and structural preference for histone H4 lysine 20 (H4K20) of

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SMYD3, suggesting the substrate diversity of SMYD3 (Foreman et al., 2011; Van Aller et al., 2012). It is known that SMYD3 is highly expressed in a various cancers, including liver, prostate, rectal, and breast carcinomas, and stimulates the oncogenic activities, such as cell proliferation, adhesion and migration, of tumor cells (Frank et al., 2006; Hamamoto et al., 2006; Silva et al., 2008). In addition, SMYD3 is essential for myogenic differentiation through MyoD regulation (Proserpio et al., 2013). Actually, the deficient zebrafish embryos displayed the developmental cardiac and somite abnormalities, illuminating its functions in cardiac and somite muscle formation (Fujii et al., 2011).

SET domain containing protein 7 (SETD7), also termed as SET7/9, is another type of histone lysine methyltransferase (HMT) and only have SET domain for methyltransferase activity, but not MYND domain (Wang et al., 2001). It was initially discovered as a specific methyltransferase for only non-methylated histone H3 lysine 4 (H3K4) that converted to mono-methylated histone H3 lysine 4 (H3K4me1) (Wang et al., 2001). Subsequently, recent accumulating evidence suggests that non-histone proteins, such as Yap, DNMT1, E2F1, and STAT3, are also the substrates for SETD7 (Estève et al., 2009; Oudhoff et al., 2013; Pradhan et al., 2009; Yang et al., 2010). The mouse knock out allele of SETD7 is a viable and did not show any developmental and gross defect (Campaner et al., 2011; Lehnertz et al., 2011). In zebrafish developmental model, knock-down of *setd7* showed the defects in skeletal muscle formation and myofibril structures (Tao et al., 2011).

Here, we examined the function of *smyd3* and *setd7* in heart development in zebrafish. Collectively, our results demonstrate that SMYD3 and SETD7 function synergistically for skeletal muscle development and non-redundantly for heart morphogenesis.

METHODS AND MATERIALS

Zebrafish husbandry

Zebrafish AB strain as wild-type control for all experiments, expect the experiments using *Tg(cmlc2:EGFP)* line. Zebrafish strains were maintained as previously described (Westerfield, 1993). To visualize the heart morphology in live embryos, *Tg(cmlc2:EGFP)* embryos were subjected to several experiments (Huang et al., 2003).

Injection of anti-sense morpholino oligonucleotides (MOs)

Control, *smyd3*, and *setd7* MOs were designed by author and ordered to Genetools Company. MOs were injected with 0.1% phenol-red solution into zebrafish embryos at 1-2 cell stages as desired concentrations. The sequences of MOs used are Control MO, 5′-CCTCTTACCTCAGTTACAATTTATA-3′; *smyd3* MO, 5′-AAGAGCTCTTACCTGACACTGAACG-3′; and *setd7* MO, 5′-AGCTTCTCGTACCTTCCACCACTTC-3′. Embryos injected MOs were observed or collected at desired developmental stages for further experiment.

Whole mount *in situ* hybridization

To make digoxigenin-labelled antisense ribo-probe, zebrafish *smyd1a (NM_205540.1)*, *smyd2* (NM_001013550.1), *smyd3* (NM_001037400.1) and *setd7* (NM_001002456.1) full ORFs were cloned into pGEM-T vector (Promega) after amplification of 24 hpf cDNA. Cloned genes were validated by sequencing. Following primers were used for PCR reaction: *smyd1a* forward, 5′-AGCATGACCGTGGAGAAGAC-3′; *smyd1a* reverse, 5′-TGTTCATGCTTTGATCTGCAC-3′; *smyd2* forward, 5′-AGCA GCAGGTCAATAGCGATG-3′; *smyd2* reverse, 5′-TTCCTCC-CATTATGTCTGCTC-3′; *smyd3* forward, 5′-ATGGACGGTCA-CTG CAGATGA-3′; *smyd3* reverse, 5′-CTAGAGCGTGTTGA-GCTC AGC-3′; *setd7* forward, 5′-AACATGGACAGCGATGA-TGAC-3′, and *setd7* reverse, 5′-CAGGTGTTCAGAGGAGTC-3′. The reaction producing ribo-probes were carried out according to manufacture protocol (Roche). Whole mount *in situ* hybridization were routinely performed as previously described (Thisse and Thisse, 2008). As brief, zebrfish embryos were fixed by 4% paraformaldehyde (PFA) and stored in methanol. To experiment, fixed embryos washed and digested with proteinase K solution as desired concentration and times. Hybridization reaction was carried out at 65°C, and the detection of hybridized RNA probe was achieved by anti-digoxigenin Fab fragment conjugated to alkaline phosphatase and BCIP/NBT substrate (Roche). Staining were stopped according to desired detection status and imaged in 75% glycerol solution for proper orientation.

Over-expression of *smyd3* and *setd7* by the injection of synthetic mRNA

To generate the synthetic *smyd3* and *setd7* mRNAs, we first cloned the full open reading frames (ORF) of *smyd3* and *setd7* into pCS2+ expression vector, which have SP6 promoter sequence and SV40 poly-A tail sequence for *in vitro* transcription. These plasmids were cut by NotI digestion to linearize, and capping mRNA were produced using m7G(5′)PPP(5′)G (Roche, Germany) and SP6 RNA polymerase (Roche, Germany). Synthetic mRNA was diluted as 100 μg/ml and phenol red was added prior to injection as 0.1% solution. Injection was achieved at 1-2 cell stages as optimal concentrations (about 100-200 pg/embryo).

RESULTS

To delineate functions of HMTs, such as *smyd1a*, *smyd2*, *smyd3*, and *setd7*, during heart development, we have first analyzed the expression pattern of these genes by whole mount *in situ* hybridization analyses and conventional reverse-transcriptase (RT) PCR. Transcripts of all HMTs examined appear to be maternally deposited, and widely detected at early stages. At later stages, the expression becomes gradually restricted to several organs, including somite, brain and eye (Figs. 1 and 2). While *smyd1a* and *smyd2* showed higher expressions in developing somite region from 10 somite stage (ss) than *smyd3* and *setd7,* hinting their roles in somitogenesis (Figs. 1 and 2) (Sesé et al., 2013), the developmental expression regions of *smyd3* and *setd7* were highly expressed within the heart forming region at 36 h post-fertilization (hpf) and 48 hpf, respectively (red arrow heads in Fig. 2). These gene expression profiling data suggest functions of zygotic SMYD3 and SETD7 in heart development.

To further delineate the roles of HMTs in heart development, we carried out knock-down experiments of SMYD3 and SETD7. First, to determine the functional requirement of SMYD3 during zebrafish heart development, we knocked SMYD3 down with a morpholino (MO)-based gene targeting system. For this purpose, splicing blocking MO that binds to the junction of exon2 and intron2 of *smyd3* pre-matured mRNA was designed (Fig. 3A). To validate the MO efficacy, PCR primers were designed to detect normal and aberrant *smyd3* transcripts in wild-type embryos and *smyd3* morphants, and carried out conventional RT-PCR (Fig. 3B). In *smyd3* morphant embryo, mature transcript that amplified by F+R2 primer was not presented, while aberrant transcript that produced by F+R1 primer was dis-

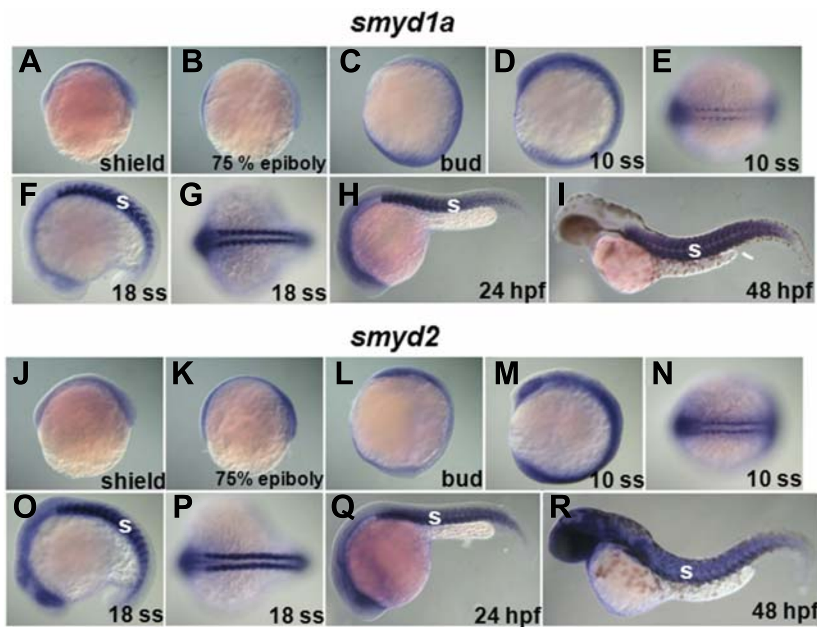

Fig. 1. The expressions of *smyd1a* and *smyd2* are ubiquitous in early developmental stages and restricted into somite during late development in zebrafish. (A-I) Images by whole mount *in situ* hybridization experiment using *smyd1*a ribo-probe. (J-R) Developmental expressions of *smyd2*. Lateral views (A-D, F, H-M, O, Q, and R) and dorsal views (E, G, N, and, P). Anterior is left (E-F and N-R). S; somite.

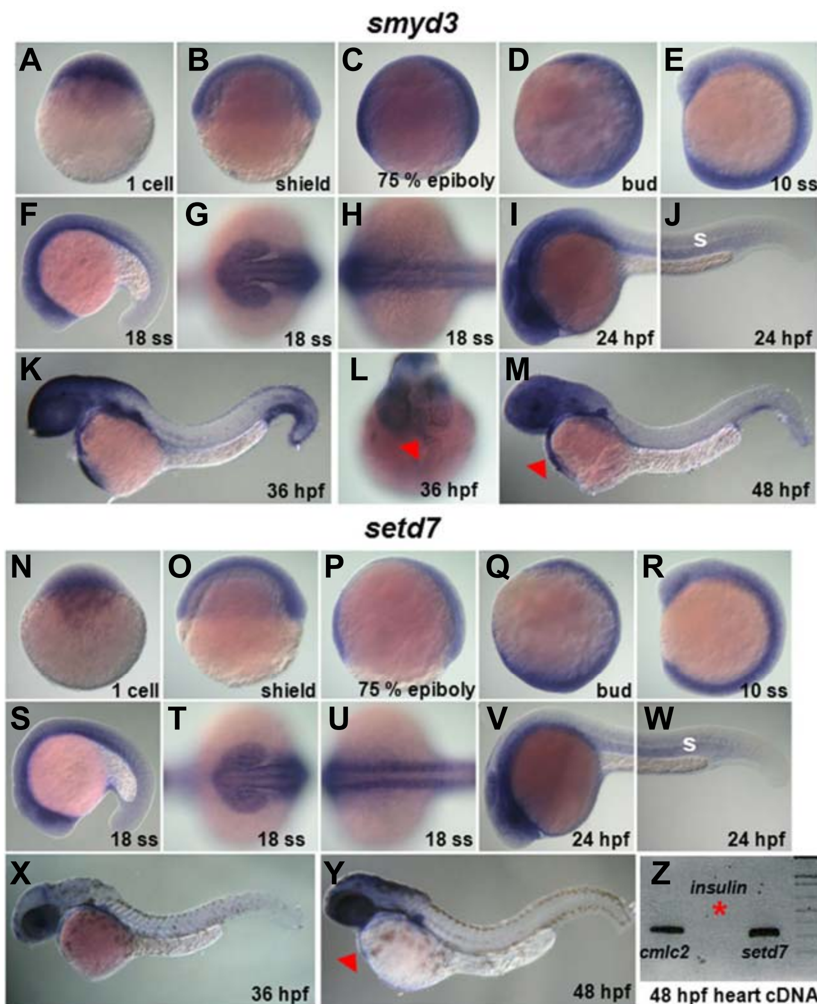

Fig. 2. *smyd3* and *setd7* express in developing heart in zebrafish. (A-M) Developmental expressions of *smyd3* by whole mount *in situ* hybridization experiment. (N-Y) The detection of *setd7* expressions by whole mount *in situ* hybridization. Lateral views (A-F, I-K, M-S, and V-Y), anterior views (G, L, and T), and dorsal views (H, U). Anterior is left (F-K, M, and S-Y). Red arrowheads point to heart regions. S; somite. (Z) *setd7* expression in heart was determined by conventional reverse-transcriptase (RT) PCR method using 48 hpf zebrafish cDNA. *insulin* (red asterisk), which not expressed in heart sample prepared here, was used as control.

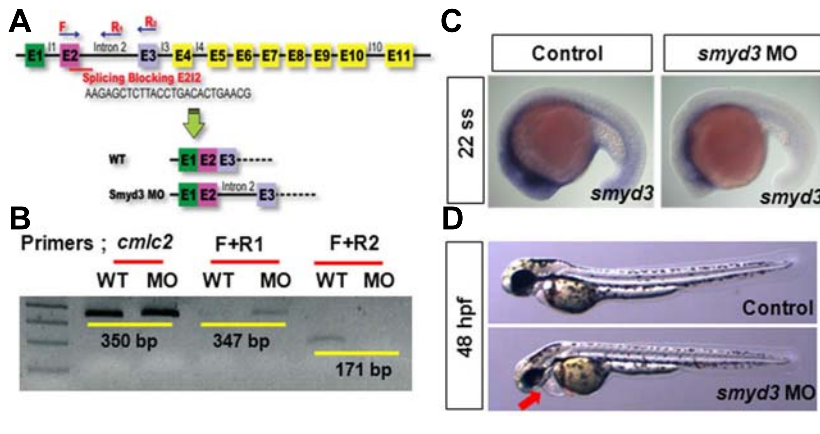

Fig. 3. The knock-down of *smyd3* causes the developmental cardiac edema. (A) Schematic showing the design of splicing blocking morpholino oligonucleotide (MO) targeting to *smyd3*. (B) Efficacy validation of *smyd3* MO by conventional RT-PCR using 24 hpf cDNA. Normal PCR product by F and R2 primers was largely decreased, while aberrant PCR product was presented by F and R1 primers in *smyd3* morphant embryos. (C) Images by whole mount *in situ* hybridization experiment at 22 somite stage (ss). Endogenous *smyd3* expression was reduced by *smyd*3 MO injection. (D) Phenotypes of embryos injected control and *smyd3* MO. Red arrow points to cardiac edema at 48 hpf. Anterior is left.

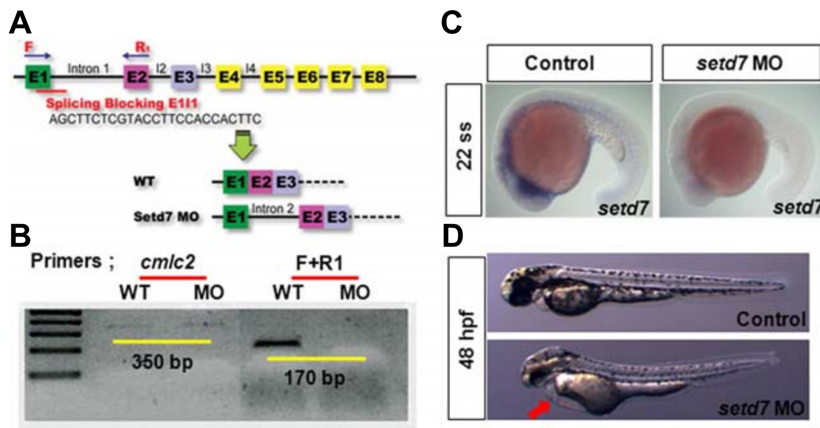

Fig. 4. SETD7-deficent embryo shows the developmental heart edema. (A) Design of the splicing blocking morpholino oligo (MO) targeting to *setd7*. (B) Validation of *setd7* MO efficacy. Normal transcript of *setd7* presenting RT- PCR by F and R1 primers was largely reduced. (C) The endogenous expression of *setd7* was determined by whole mount *in situ* hybridization experiment in control and *setd7* knock-down embryos. (D) Phenotypes of embryos injected control and *setd7* MO. Red arrow points to cardiac edema at 48 hpf. Anterior is left.

played (Fig. 3B), validating the efficacy of *smyd3* MO. In addition, *smyd3* expression was drastically reduced in *smyd3* morphants at 22 ss, suggesting non-sense mediated decay of aberrant transcript caused by MO injection (Fig. 3C). At optimal concentration (approximately 3.0 ng/embryo), gross morphology of *smyd3* morphants was comparable to control embryos (Fig. 3D and data not shown). However, pronounced heart edema (red arrow) was apparent in *smyd3* morphants at 48 hpf, suggesting that SMYD3 function may be essential for cardiac development (Fig. 3D).

Similarly, we examined the function of SETD7 during development. MOs targeting *setd7* was designed in a way to target the junction of exon 1 and intron 1 of *setd7* pre-mature mRNA (Fig. 4A). The efficacy of the MO was validated by conventional RT-PCR (Fig. 4B). In the *setd7* MO-injected embryos, normal transcript was largely abolished, detecting by PCR primer F+R1 (Fig. 4B). In addition, expression of *setd7* was noticeably decreased by MO injection at 22 ss, suggest that *setd7* MO is a functional to block the normal transcription of *setd7* gene (Fig. 4C). Similar to SMYD3 deficiency, the knock-down embryos of *setd7* at optimal concentration (1.0 ng/embryo) displayed cardiac edema (red arrow) without causing any obvious morphological defects (Fig. 4D and data not shown). Collectively, our results illustrate that the proper activities of H3K4 methyltransferases, specifically SMYD3 and SETD7, are important for zebrafish heart formation.

During histone H3 lysine 4 (H3K4) methylation, SETD7 can only methylate non-methylated H3K4 to produce mono-methylated H3K4 (H3K4me1), which then is further methylated by SMYD3 to become H3K4me2 and H3K4me4 (Hamamoto et al., 2004; Wang et al., 2001). Individual roles of SMYD3 and SETD7 during cardiac and skeletal muscle development in zebrafish have been reported (Fujii et al., 2011; Tao et al., 2011). Given that SMYD3 and SETD7 successively methylate H3K4, we can assume that these two enzymes function synergistically during zebrafish development. To test this possibility, we examined whether co-injection of *smyd3* and *setd7* MOs at a suboptimal concentration can give rise a synergistic effect for induction of developmental defects (Fig. 5). The *smyd3* and *setd7* MOs, by themselves at optimal concentrations (3.0 ng/embryo for *smyd3* and 1.0 ng/embryo for *setd7*), produced no discernible gross morphological defects except cardiac edema at 48 hpf (Figs. 3 and 4). However, co-injection of these MOs at optimal concentrations caused severe somitogenesis defects as indicated by the phenotype of shortened and curled body morphology (Fig. 5A). In addition, the severity of the abnormalities was sharpened by co-injection of increased concentrations of these MOs (Fig. 5B). As expected, these results suggest that SMYD3 and SETD7 have a synergistic function in zebrafish development.

Next, we delineated the synergistic roles of SMYD3 and SETD7 in zebrafish heart development. For this purpose, we used the heart-specific transgenic fish *Tg(cmlc2:EGFP)* line to allow easy visualization of the heart morphology in live embryos

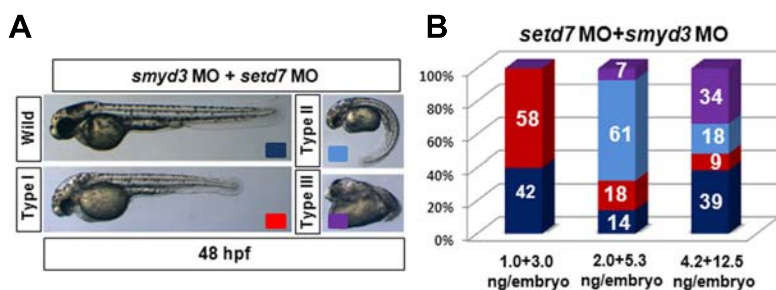

Fig. 5. Synergistic defect of body formation by double knock-down of *smyd3* and *setd7*. (A) Developmental phenotypes of embryos injected both *smyd3* and *setd7* MOs. Embryos were injected both MOs at 1-2 cell stages and imaged at 48 hpf. The embryos in type I group showed shortened body axis. Type II group was sorted by appearance of curved body formation. Monster phenotypes were grouped into type III. Anterior is left. (B) Phenotypic contributions were determined by titrated-MO injections. Ratios were indicated in graph (N > 100).

(Fig. 6A). The injections of control, *smyd3*, *setd7*, and *smyd3* + *setd7* MOs were carried out at the optimal concentrations (3.0 ng/embryo for *smyd*3, 1.0 ng/embryo for *setd7*). Morphants without body morphological defects were selected for further analyses of heart development. At 48 hpf, heart tubes in the *smyd3* and *setd7* morphants displayed defects in the looping process and remained linear in shape (Fig. 6A). Single knock-down of these genes caused a defect in heart morphogenesis, suggesting that each gene is indispensable for heart morphogenesis. Interestingly, double knock-down of SMYD3 and SETD7 presented a more severe defect than single knock-down of either of those genes (Fig. 6A). However, the global expression level of *clmc2* detected by whole mount *in situ* hybridization (Kim et al., 2014) was not altered in these *smyd3*, *setd7*, and *smyd3* + *setd7* morphant embryos at 48 hpf (data not shown), suggesting that specification and/or maintenance of cardiomyocytes were not compromised in those embryos. Collectively, our results show that SMYD3 and SETD7 likely function synergistically in zebrafish development. Moreover, our data suggest that heart development is more sensitive than somitogenesis to the decrease of SMYD3 and SETD7 levels.

To test whether the developing heart defects in the SMYD3- and SETD7-deficient embryos are caused by the expressional changes of early cardiac muscle genes, we determined the early expressions of *cmlc2*, *vmhc*, and *amhc* (Kim et al., 2014) by whole mount *in situ* hybridization experiment. The expression level of these genes was not altered by the knock-down of SMYD3 and/or SETD7 and the gathered shape of cardiac precursor cells in these morphant embryos was also comparable to wild-type embryos (Fig. 6B). This finding suggests that SMYD3 and SETD7 are dispensable for proper expression of early cardiac muscle genes and differentiation of cardiac precursor cells.

To investigate whether enforced expression of *smyd3* and *setd7* causes the cardiac defect in zebrafish, synthetic mRNA encoding SMYD3 or SETD7 was injected (Fig. 7A). The co-injection of *smyd3* and *setd7* mRNA as optimal dose (each 100-200 pg/embryo) caused similar developmental defects as in embryos lack either *smyd3* or *setd7*, such as defective body axis formation. Moreover, these embryos showed pronounced cardiac edema, indicating the defects in heart formation (red arrow in Fig. 7B). To better examine the defects caused by overexpression of these *smyd3* and *setd7*, we analyzed the expression of known cardiac markers, including *cmlc2*, *vmhc*, and *amhc*. Despite the obvious cardiac edema, the expression of these genes was unaltered in embryos overexpressing *smyd3* and *setd7* (Fig. 7C). Therefore, it appears that excessive H3K4 methyltransferase activity can lead to defects in heart and skeletal muscle development in zebrafish.

Taken together, our results indicate that methylation can provide essential regulatory input during organ development in zebrafish. Since similar developmental defects were observed in embryos with either excessive or reduced level of SMDY3 and/or SETD7 activity, it appears that appropriate level of histone methylation is critical to ensure the formation of cardiac and skeletal muscle in zebrafish.

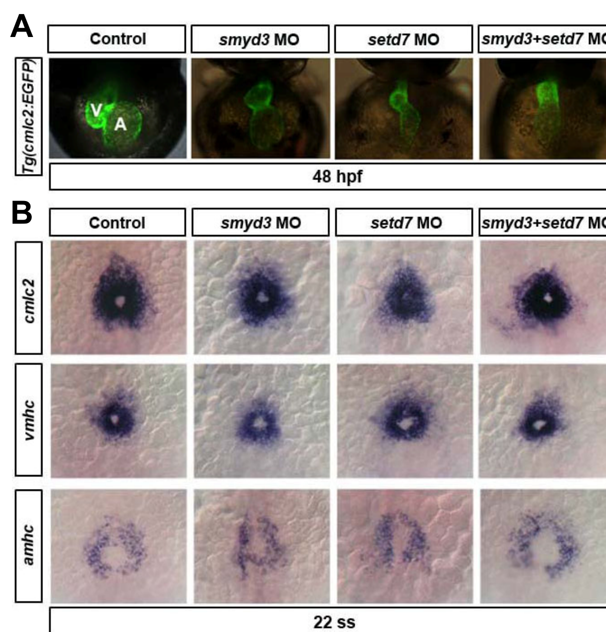

Fig. 6. Heart morphogenesis was affected by *smyd3* and/or *setd7* knock-down in *Tg(cmlc2:EGFP)* fish, but not the expressions of early heart markers. (A) Developmental heart morphology showing the GFP expression by *cmlc2* promoter in *Tg(cmlc2:EGFP)* transgenic fish. MOs were injected at 1-2 cell stages and imaged by 48 hpf. *smyd3* and *setd7* knock-down cause the looping defect in zebraifsh developing heart. (B) Early heart marker expressions by whole mount *in situ* hybridization. *cmlc2, vmhc,* and *amhc* expressions were not changed in *smyd3* and *setd7* knock-down embryos. MOs were injected at 1-2 cell stage and embryos were collected at 22 somite stage (ss). Dorsal views and anterior is up.

DISCUSSION

Heart morphogenesis in vertebrate development is a highly complicated event that achieved by correct cell specification, migration, proliferation, apoptosis, and transition as well as controlled beating of heart myocytes (Barnett et al., 2012). In

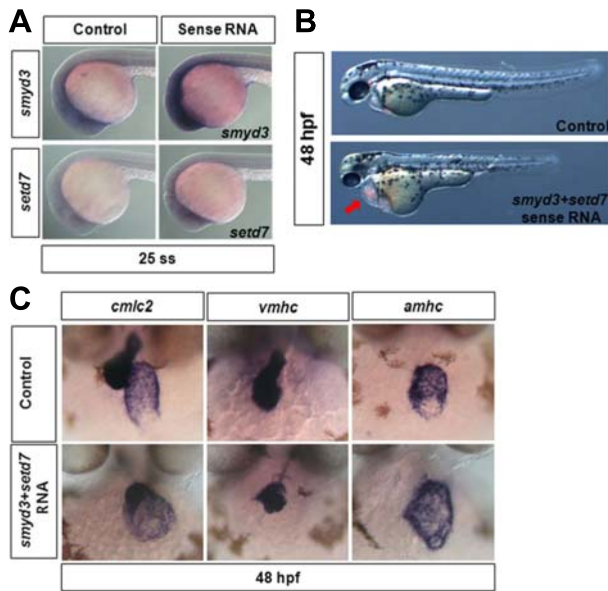

Fig. 7. Augmented activities of SMYD3 and SETD7 cause the defect in heart morphogenesis. (A) Endogenous expressions of *smyd3* and *setd7* were determined by whole mount *in situ* hybridization experiment in control and synthetic *smyd3*- or *setd7* mRNA-injected embryos. Higher expression was shown in embryos injected sense RNA compared with control at 25 ss. Developmental phenotypes of control and both *smyd3* and *setd7* sense RNA-injected embryos at 48 hpf. Embryos injected sense RNA of these genes displayed the heart edema (red arrow). (C) Heart morphology was addressed by expression shapes of heart marker genes, such as *cmlc2*, *vmhc,* and *amhc,* in control and sense RNA injected embryos at 48 hpf. Expression levels of heart marker genes were not changed, but expression shapes were largely altered in embryos injected sense RNA. MOs were injected at 1-2 cell stage and embryos were collected at 48 hpf. Frontal views and anterior is up.

this process, proper gene expressions for structure of cardiac chamber at early developmental stages are critical for late heart forming event. Our data presented here indicates that proper level of histone methylation, which is mediated by a series of enzymes commonly known as HMTs, including *smyd3*, and *setd7* appears to be essential for heart development.

During zebrafish development, the expression pattern of HMTs indicates that transcripts encoding these proteins may be maternally deposited. In addition, considering their ubiquitous expression, these proteins may provide essential functions in development by regulating histone methylation for all types of cells (Figs. 1 and 2). However, knock-down of *smyd3* and *setd7* in zebrafish embryos did not cause any obvious defects in early development, such as in gastrulation movement, germ layer formation, cell proliferation, or cell death. Therefore, these genes may redundantly function to regulate early vertebrate development (Figs. 1 and 2). In later developmental stages, the expressions of all HMTs tested in this paper were gradually restricted and often increased in specific organs, including somite, brain, eye primordial cells, fin primordial region, and heart. For instance, *smyd2* becomes highly expressed within eye primodium, while *smyd3* and *setd7* are enriched in developing heart, suggest that each HMTs might have a non-redundant and organ-specific roles during late vertebrate developmental periods (Figs. 1 and 2).

It was report that knocked-down *smyd3* and *setd7* by MO led to developmental defects in heart and/or skeletal muscle (Fujii et al., 2011; Tao et al., 2011). Consistent with previous reports, we also find that MO injections as high concentrations (over 10 ng/embryo for *smyd3* MO and 4.2 ng/embryo for *setd7* MO) cause the similar somite defects. However, the optimal injection conditions (concentrations under 3.0 ng/embryo for *smyd3* and 1.0 ng/embryo for *setd7* MO) morphant embryos showed only developmental heart abnormality without any somite defects, indicating that each organ may have different sensitivity to the fluctuation in Smyd3 and Setd7 level during organogenesis.

Interestingly, in skeletal muscle development, SETD7 directly interacts with MyoD to induce myogenin and MEF2 expression to generate differentiated myocytes (Tao et al., 2011). Therefore, it is tempting to speculate that SETD7 manipulation in zebrafish may lead to the alteration in expression of certain structural genes. However, we did not find any discernible change in the expression of *cmlc2*, *vmhc*, and *amhc* in our SMYD3 and SETD7 double-deficient or overexpressed embryos, suggesting the presence of distinct mechanisms underlying SETD7-mediated differentiation of the cardiac muscle and skeletal muscle cells. Moreover, SMYD3 and SETD7 appear to be dispensable for the specification of cardiomyocytes, since the lack of SMYD3 or SETD7 did not abrogate the expression of cardiac markers (Figs. 6 and 7).

Here, we have shown the knock-down and overexpression phenotypes of SMYD3 and SETD7 in zebrafish development. The results suggest that H3K4 methyltransferases are crucial genetic regulators for normal heart development. These findings extend our current knowledge of the roles of HMTs and increase our understanding of the functional mechanism of HMTs in heart development.

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