Histone H4 Lys 20 monomethylation by histone methylase SET8 mediates Wnt target gene activation

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Histone methylation has an important role in transcriptional regulation. However, unlike H3K4 and H3K9 methylation, the role of H4K20 monomethylation (H4K20me-1) in transcriptional regulation remains unclear. Here, we show that Wnt3a specifically stimulates H4K20 monomethylation at the T cell factor (TCF)-binding element through the histone methylase SET8. Additionally, SET8 is crucial for activation of the Wnt reporter gene and target genes in both mammalian cells and zebrafish. Furthermore, SET8 interacts with lymphoid enhancing factor-1 (LEF1)/TCF4 directly, and this interaction is regulated by Wnt3a. Therefore, we conclude that SET8 is a Wnt signaling mediator and is recruited by LEF1/TCF4 to regulate the transcription of Wnt-activated genes, possibly through H4K20 monomethylation at the target gene promoters. Our findings also indicate that H4K20me-1 is a marker for gene transcription activation, at least in canonical Wnt signaling.

epigenetic regulation | zebrafish embryonic development

Covalent modifications on histone tails act sequentially or in combination to create docking sites for effectors and thus, change the chromatin packing status (1, 2). These modifications are implicated in gene transcription, DNA replication, and DNA repair to orchestrate DNA-based biological processes (3–5). Among these modifications, histone methylations are more stable and believed to have important effects on epigenetic information inheritance between cell divisions. Also, histone methylation has attracted considerable attention because of its diversity and complexity (6). Both lysine and arginine residues can be methylated. For lysine at histone N tails, it can be mono-, di-, or trimethylated (me-1, me-2, and me-3, respectively). The effects of methylation on DNA metabolism rely on the specific site and the number of the methylation sites (1, 6).

Great progress has been made in characterizing histone methylation function. H3K4me-3 is well-known as a gene activation marker, whereas H3K9me-3 is associated with repression. Although modifications on H3 attract more attention, the N tail of histone H4 is essential for chromatin structure packing (7), and only K20 among the five lysine residues in histone H4 could be methylated in mammalian cells. However, the relationship between H4K20me-1 and gene transcription remains controversial. Since the discovery of related methylase SET8 (also known as PR-Set7), H4K20me-1 was identified as a transcription repression marker (8), and the H4K20me-1 related reader/effector L3MBT1 was identified (9). However, accumulating evidence shows that H4K20me-1 could function as a transcription activator. H4K20me-1 was reported to be associated with Pol II (10) and up-regulated in the promoter and coding regions of the active genes (10, 11). A genomic screen found that H4K20me-1 was enriched in the coding regions of active genes, hinting at a positive role of H4K20me-1 in gene transcription (12). More recent evidence has shown that SET8 and H4K20me-1 play a positive role in peroxisome proliferator-activated receptor (PPAR)γ expression and adipogenesis (13).

Wnt signaling plays an important role in a wide range of biological and pathophysiological processes (14–16). The canonical Wnt/β-catenin signaling leads to the stabilization of cytosolic β-catenin by destruction of the Axin/Adenomatous Polyposis Coli (APC)/glycogen synthase kinase 3β (GSK3β) complex. β-catenin translocates to the cell nucleus and forms a transcription complex with LEF1/TCFs. Concomitantly, a number of coactivators are recruited to this transcription complex at target gene promoters, such as Pygopus, legless, c-Jun, Dvl, and others (17–19). Other than these coactivators, some histone modification enzymes are recruited into the β-catenin/TCF4 complex to facilitate gene transcription by alter chromatin status, such as CBP/P300 and SET1 (20, 21). However, the role of the epigenetic modifications, particularly histone methylations, in Wnt signal transduction has not yet been thoroughly characterized.

To investigate histone methylation changes during Wnt signaling, in this work, we performed a ChIP screen and found that H4K20me-1 increased robustly with Wnt3a stimulation. The oscillation of H4K20me-1 at TCF-binding element (TBE) is coordinated with β-catenin, indicating its positive role in gene regulation. We provide evidence that the increased H4K20me-1 on Wnt stimulation is catalyzed by SET8 and that SET8 could participate in Wnt signaling in both mammalian cells and zebrafish. Furthermore, we have discovered that TCF4 directly interacts with SET8, and β-catenin enhances this complex formation by expelling transcription repressor Groucho from TCF4.

Results

Wnt Stimulates H4K20me-1 Enrichment at Target Gene Promoters. To explore the epigenetic mechanism involved in Wnt target gene transcription regulation, we performed a ChIP screen to detect alterations in histone methylations at the TBE of the Wnt target gene *AXIN2* (Fig. 1*A*). HEK293 cells were treated with control or Wnt3a-conditioned medium (CM) for 1 h, and the antibodies specific to various histone methylations were used for ChIP assay. As controls, we also examined the interactions of TCF4 and β-catenin with the *AXIN2* TBE. Consistent with a previous report (21), Wnt3a treatment did not alter the level of TCF4 but markedly increased that of β-catenin at the TBE (Fig. 1*A*). Although most of the histone methylations showed no or marginal changes, there was, surprisingly, an approximately fourfold increase in H4K20me-1 (Fig. 1*A*). However, the H4K20me-1 en-

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See Commentary on page 3097.

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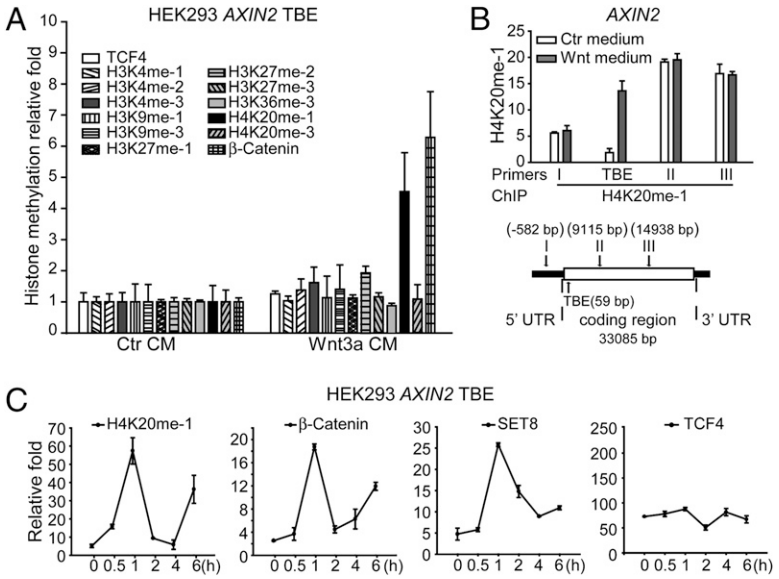

Fig. 1. H4K20me-1 is a transcriptional activation marker at the promoter of the Wnt target gene. (*A*) ChIP screen for histone methylation variation on *AXIN2* TBE. HEK293 cells were treated with control or Wnt3a CM for 1 h before ChIP with different antibodies. All of the precipitated DNA samples were quantified by real-time PCR and normalized by input DNA. Quantitative PCR results of control CM-treated cells were set as 1. (*B*) H4K20me-1 status at control regions in *AXIN2* after Wnt stimulation. HEK293 cells were treated with control or Wnt3a CM for 1 h before ChIP with H4K20me-1 antibody. Regions targeted by different primer pairs are as indicated. (*C*) Kinetic ChIP for H4K20me-1, β-catenin, SET8, and TCF4 on promoter of *AXIN2* in HEK293. Quantitative PCR results of IgG were set as 1. All graphs show average with SD.

richment was not observed at the *AXIN2* coding region or promoter region containing no TBEs (Fig. 1*B*).

We then performed a kinetic ChIP to detect the time-course variation of H4K20me-1. Dynamics of H4K20me-1 at the TBEs of both *AXIN2* and *c-MYC* over a time course of 6 h under Wnt3a treatment were examined. The analyses revealed that the H4K20me-1 level peaked at approximately 1 h after Wnt stimulation, dropped back to the basal level between 2 and 4 h, and picked up again at 6 h in both cases (Fig. 1*C* and Fig. S1*A*). This Wnt-induced H4K20me-1 enrichment was not observed at the *GAPDH* or *IκB* promoter (Fig. S1*B*) or the control region in promoter (Fig. S1*C*). Furthermore, the Wnt stimulation induced H4K20me-1 enrichment at TBEs of other target genes, such as *LMO2*, *NKD1*, and *DKK1* (Fig. S1*D*). We also investigated the binding of β-catenin and TCF4 at TBEs of *AXIN2* and *c-MYC*. The recruitment of β-catenin to TBE showed similar kinetics to H4K20me-1 at the TBEs, whereas TCF4 maintained a steady state with marginal variation (Fig. 1*C* and Fig. S1*A*), implying a correlation between β-catenin and H4K20me-1. The early onset of H4K20me-1 also indicates that this modification should be a transcription activation marker instead of a feedback repression marker. The concomitant occurrence of β-catenin recruitment and H4K20me-1 suggests that H4K20me-1 may play a positive role in regulating Wnt target gene expression.

H4K20me-1 Methylase SET8 Is Involved in Wnt Signaling in Mammalian Cells. H4K20me-1 modification is catalyzed by SET8, the only known enzyme in vertebrate so far (8, 22, 23). Thus, we tested whether SET8 was involved in Wnt signaling. Knockdown of SET8 using specific shRNAs abolished the Wnt3a-induced elevation of H4K20me-1 at the TBEs of *AXIN2* and *c-MYC* (Fig. 2*A* and Fig. S2*A*) in HEK293 cells, suggesting that the increase in H4K20me-1 during Wnt signaling depends on SET8. Additionally, when SET8 was knocked down, the whole genomic H4K20me-1 level reduced significantly (Fig. S2*B*). ChIP analyses also revealed that the binding of SET8 to the TBE showed an oscillation pattern similar to H4K20me-1 and β-catenin (Fig. 1*C* and Fig. S1*A*). These results together indicate that SET8 participates in Wnt signaling.

The Wnt reporter gene LEF1-luc, which is sensitive to Wnt stimulation, contains multiple TBE sites and forms a chromatin-like structure (24). To investigate the function of SET8 in canonical Wnt/β-catenin signaling, we overexpressed SET8 with the Wnt reporter gene in National Institutes of Health 3T3 cells and found that, although SET8 overexpression did not change the basal reporter gene activity, it potentiated the Wnt3a-induced activity (Fig. S2*C*). We also tested some other histone methyltransferase, SET9, Suv39h1, G9a, and Dot1L. The results showed that these methyltransferases had no effect on Wnt reporter activity (Fig. S2*C*). In addition, SET8 overexpression did not affect TNFα-stimulated NF-κB reporter gene activity (Fig. S2*D*), suggesting that the effect of SET8 on transcriptional activation may not be universal. We further tested SET8 function in Wnt signaling at the endogenous target gene level in NIH 3T3 cells. Previous studies showed that deletion of the N-terminal 113 aa of SET8 (named SET8-114, containing residues 114–352) led to elevated methylation activity, whereas mutation of His-299 and Asp-338 to Ala impaired its methylase activity and histone binding capacity, respectively (22, 25). In the target gene assay, SET8 overexpression increased *Axin2* mRNA abundance and the SET8-114 truncation mutant exhibited a higher activity, whereas the SET8-114 truncation mutants, carrying a H299A mutation (114H299A) or D338A mutation (114D338A), failed to potentiate Wnt stimulation (Fig. 2*B*). These results show the importance of the methyltransferase activity and histone binding capacity for the function of SET8 in Wnt signaling. Consistently, knockdown of mouse SET8 (mSET8) inhibited the Wnt3a-stimulated *Axin2* upregulation, and this inhibitory effect could be reverted by human SET8 expression in a rescue experiment (Fig. 2*C*).

Consistently, Wnt target genes, such as *AXIN2*, *c-MYC*, *NKD1*, and *LEF1*, were regulated by SET8 in human cell lines (Fig. 2*D*). We further carried out a gene expression microarray to investigate the role of SET8 in Wnt signaling. HEK293 cells transfected with control or SET8 shRNA were treated with control or Wnt3a CM for 6 h followed by expression microarray analysis. The microarray results showed that 55 genes were regulated after Wnt3a CM stimulation (1.5-fold change, $P < 0.05$),

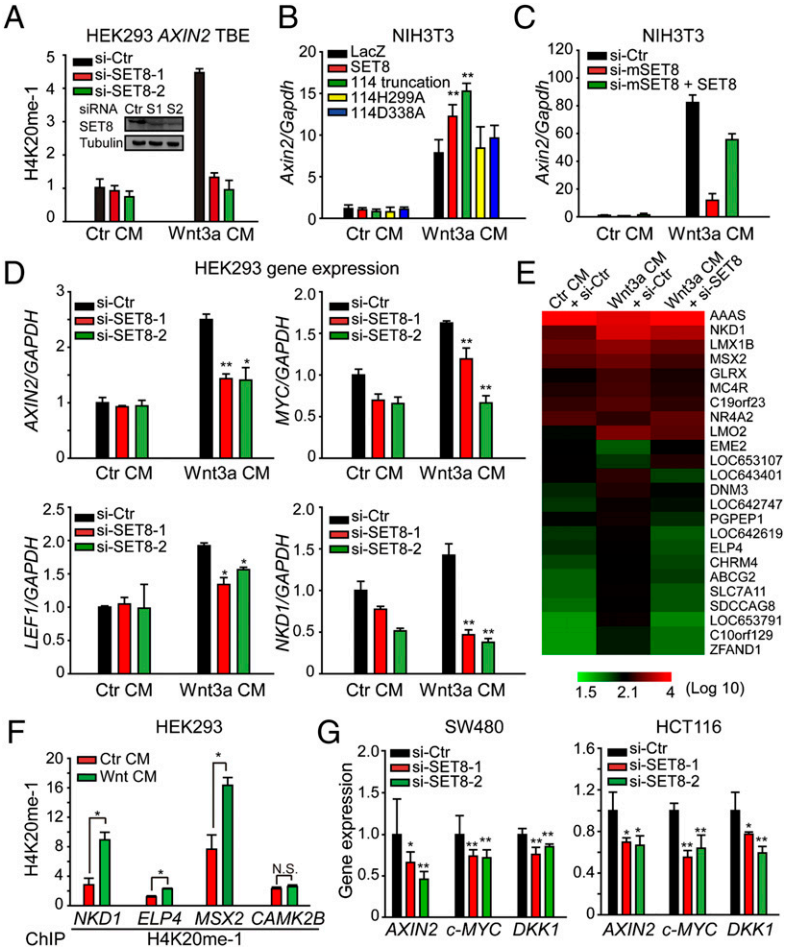

Fig. 2. H4K20me-1 related histone methylase SET8 is critically involved in Wnt signaling activation. (*A*) SET8 knockdown inhibited H4K20me-1 enrichment at TBE of *AXIN2*. (*B*) SET8 overexpression enhanced Wnt target gene activity in NIH 3T3. Quantitative PCR results of *Axin2* were normalized by *Gapdh*. *P* values were calculated between LacZ and SET8 truncations overexpression in Wnt3a CM treatment. (*C*) Expression of *Axin2* was abrogated by mSET8 knockdown and restored by human SET8 overexpression. (*D*) SET8 knockdown-abrogated Wnt target genes *AXIN2*, *c-MYC*, *NKD1*, and *LEF1* activation in HEK293 cells. The expression of Wnt target genes was detected by quantitative real-time PCR and normalized by *GAPDH* gene expression. *P* values were calculated between control shRNA and SET8 shRNA in Wnt3a CM treatment. (*E*) Expression pattern of 24 genes regulated by both Wnt and SET8 selected against overlapping probes with 1.5-fold change. (*F*) Wnt stimulation induced H4K20me-1 enrichment at promoters of target genes regulated by both Wnt signaling and SET8. (*G*) SET8 knockdown inhibited Wnt target gene expression in colon cancer cell lines. All graphs show average with SD. Student *t* test was applied to determine statistical significance. $^{**}P < 0.01$; $^{*}P < 0.05$.

including the well-known Wnt target genes. Among these genes, about 24 genes were regulated by SET8 RNAi (1.5-fold change, $P < 0.05$). These results showed that SET8 plays an important role in Wnt target gene regulation (Fig. 2*E* and Table S1). We also tested the H4K20me-1 status at TBEs of genes that are regulated by both Wnt signaling and SET8 (listed in Table S1 as the microarray results), such as *NKD1*, *ELP4*, and *MSX2*. Wnt stimulation led to H4K20me-1 enrichment at these TBEs but not *CAMK2B* TBE, a gene regulated by Wnt signaling but not SET8 (Fig. 2*F*). These results indicate the correlation between H4K20me-1 and Wnt-induced gene expression. Moreover, SET8 was shown to regulate Wnt signaling in SW480 and HCT116, two colon cancer cell lines (Fig. 2*G*). These results, taken together, show that SET8 has a positive role in regulating Wnt signaling and that this role depends on its methyltransferase and histone binding activities.

SET8 Regulates Zebrafish Embryonic Development. The importance of SET8 in Wnt signaling was also investigated using the zebrafish model in which Wnt signaling regulates its embryonic ventral and posterior cell fates (26). Sequence analysis revealed that the catalytic SET domain was evolutionally conserved between human and zebrafish. Zebrafish Set8 (Set8a) could also potentiate Wnt activity in NIH 3T3 cells (Fig. S3*A*). When *set8a* was knocked down by morpholino oligonucleotides (MOs), the H4K20me-1 abundance at *tbx6* promoter was reduced (Fig. 3*A*). Consistently, knockdown of *wnt8* also led to reduction of H4K20me-1 (Fig. 3*A*). In situ hybridization analysis showed that *set8a* was broadly expressed by 24 h postfertilization (hpf) (Fig. S3*B*). When *set8a* was knocked down by an MO against the 5′-UTR region of *set8a* (*set8a* MO), the morphants exhibited shortened trunk and tail phenotypes, which were similar to the typical phenotypes of *wnt8* morphants (26, 27) (Fig. 3*B*). These phenotypes became more severe with increased dosages of the *set8a* MO (Fig. 3*C*). Importantly, these phenotypes were rescued by *set8a* mRNA or human *SET8-114* mRNA injection (Fig. 3*C* and Fig. S3*C*). Consistently, the in situ hybridization experiment revealed that the *set8a* morphants at 10 somite stage exhibited a shortened trunk marked by *myod* (adaxial cells and somites) and a slight expansion of *opl* (a telencephalon marker) (26, 27) in a dose-dependent manner, similar to *wnt8* morphants (Fig. 3*D*). These results suggest that Set8a is involved in zebrafish embryonic development.

To explore the role of Set8a in Wnt signaling, we coinjected *set8a* MO with a suboptimal dose of *wnt8* MOs (28), and these morphants at 26 hpf displayed an enlarged telencephalon coupled with a shortened trunk and tail, phenocopying the potent *wnt8*

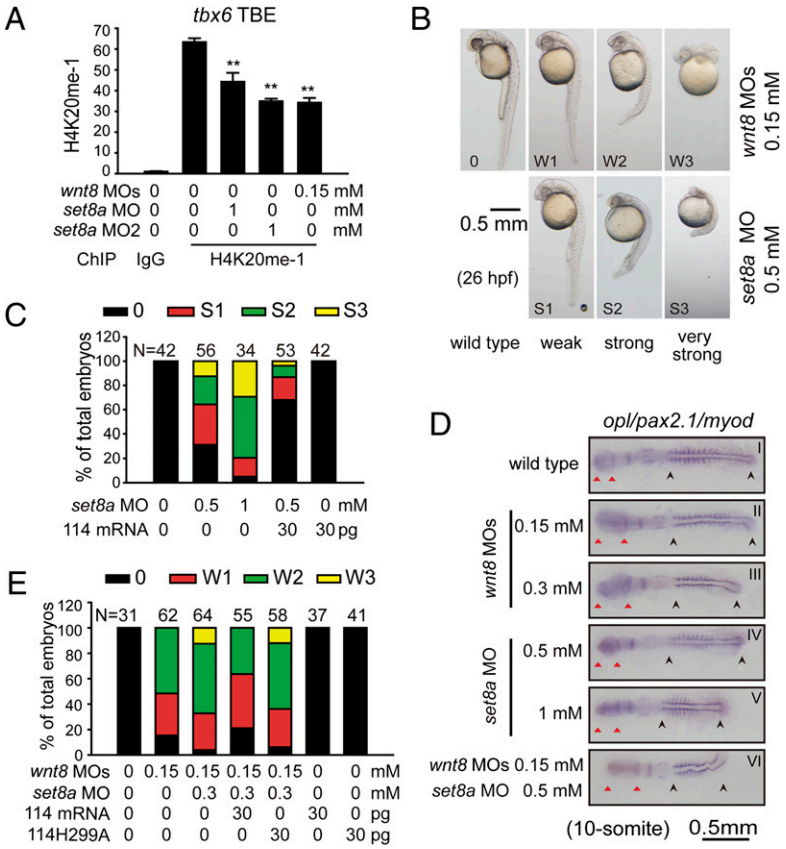

Fig. 3. Set8a participates in Wnt signaling to regulate zebrafish embryonic development. (*A*) *set8a* MO led to H4K20me-1 reduction at *tbx6* TBE. Zebrafish embryos were injected with distinct *set8a* or *wnt8* MOs at one-cell stage and collected at shield stage for ChIP experiment. Quantitative PCR results of IgG were set as 1. *P* values were calculated between control MO and *set8a* or *wnt8* MO injection. $^{**}P < 0.01$. (*B*) Morphology of *set8a* (0, S1, S2, and S3) and *wnt8* (0, W1, W2, and W3) morphants at 26 hpf. The *wnt8* morphants were classified into four categories, with 0 being WT and W3 being the most severe phenotype. The *set8a* morphants were classified into four categories, with S1 displaying relatively small head, S2 displaying small head and shortened trunk and tail, and S3 displaying small head and severely shortened trunk and tail. (*C*) *SET8-114* mRNA restored the *set8a* MO phenotype. The number of embryos scored (*N*) is shown on top of each bar, and the concentration of MO is indicated. The morphant phenotypes were classified into four categories: 0, S1, S2, and S3. (*D*) *set8a* MO enhanced the neuroecodermal and mesodermal defect of *wnt8* MOs. Embryos were examined at the 10-somite stage. In situ markers used were *opl* (telencephalon), *pax2.1* (midbrain/hindbrain boundary), and *myod* (adaxial cells and somites). Red arrowheads, *opl* expression; black arrowheads, notocord. (*E*) *SET8-114* mRNA but not *114H299A* rescued the synergistic effect of *set8a* MO and *wnt8* MOs. The morphants phenotypes were classified into four categories: 0, W1, W2, and W3.

loss of function (Fig. 3*E* and Fig. S3*D*). In addition, injection of *SET8-114* mRNA, but not *SET8-114H299A*, partially reverted these phenotypes (Fig. 3*E*). Furthermore, a suboptimal dose of *set8a* and *wnt8* MOs coinjection gave rise to morphants with an expansion of *opl* (a telencephalon marker) and reduction of the posterior, such as the trunk was shortened and somites in the tail missing (marked by *myod*) (26, 27), which was similar to the phenotypes of morphants that were injected with high-dose *wnt8* MOs (Fig. 3*D*). We also tested another *set8a* ATG-MO, *set8a* MO2. This MO gave the same result (Fig. S4 *A*–*C*). These data taken together revealed the synergistic effect of Set8a with Wnt8 and indicated the importance of Set8a in zebrafish development.

SET8 Regulates Wnt Target Genes in Zebrafish. Besides the phenotype observed, we went on carrying out in situ hybridization analysis to determine the effect of the *set8a* MO on Wnt target gene expression. Embryos injected with *wnt8* MOs diminished the expression of *tbx6* and *cdx4* (ventrolateral mesodermal marker and Wnt target genes) and expanded *goosecoid* expression (*gsc*, a dorsalateral mesodermal marker, and a Wnt-responsive gene). Meanwhile, the *no tail* (*ntl*) gene, as a negative control (29), was not affected (Fig. 4 *A* and *B*). Injection of *set8a* MO alone led to similar results, although milder than *wnt8* MOs, and this effect could be rescued by human *SET8-114* mRNA injection (Fig. 4 *A* and *B*). Additionally, we coinjected *set8a* MO with *wnt8* MOs and found that *set8a* MO could also cooperate with *wnt8* MOs to affect the expression of Wnt-responsive genes (Fig. 4*C*). Quantitative real-time PCR analysis was performed to confirm the Set8a effect on Wnt target genes expression. The results showed that the mRNA levels of *cdx4* and *tbx6* were attenuated by injection of *set8a* MO and *wnt8* MOs, respectively (Fig. 4*D*). In addition, injection of *SET8-114* mRNA rescued the effect of both the *set8a* MO and the *wnt8* MOs (Fig. 4*D*). Additionally, coinjection of *set8a* MO and *wnt8* MOs led to more severe reduction of *cdx4* and *tbx6* expression (Fig. 4*E*). Together, these results revealed the important role of Set8a in Wnt signaling in zebrafish. Furthermore, another *set8a* ATG-MO, *set8a* MO2, also gave a similar result (Fig. S4*D*). In summary, these results collectively indicate the conserved role of Set8a in Wnt signaling in the early development of zebrafish.

SET8 Interacts with TCF4. Next, we wanted to understand the mechanism by which SET8 participates into Wnt signaling. Because SET8 was recruited into the TBE to which LEF1/TCF4 also binds, we hypothesized that SET8 may interact with LEF1/TCF4. A coimmunoprecipitation (co-IP) assay showed that both TCF4 and LEF1 interacted with SET8 in HEK293T cells and vice versa (Fig. 5 *A* and *B*). Consistently, endogenous SET8 also formed a complex with TCF4 under Wnt3a stimulation in vivo (Fig. 5*C*). We further mapped the interaction sites between SET8

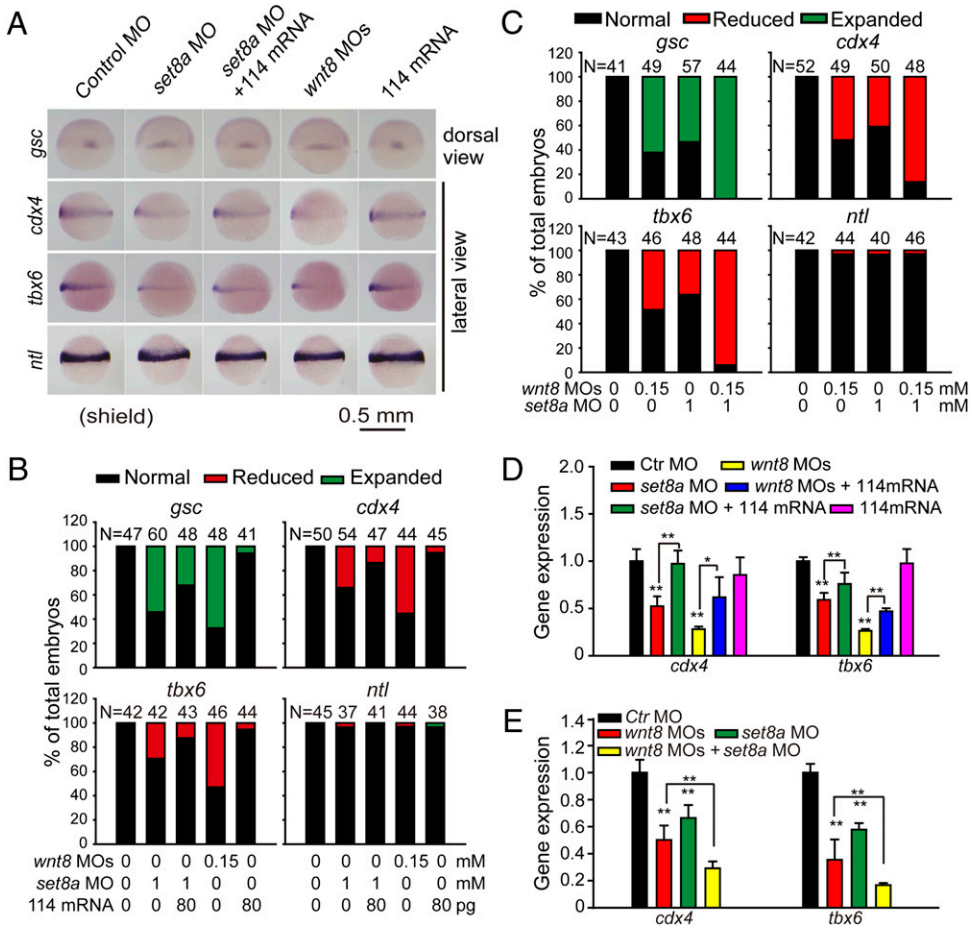

Fig. 4. SET8 regulates Wnt target genes in zebrafish. (*A* and *B*) Knockdown of *set8a* resulted in dorsal mesodermal expansion. Embryos injected with the indicated MOs were fixed at the shield stage and stained for *tbx6*, *cdx4*, *gsc*, and *ntl*. The graph shows the statistical data for each gene, respectively. (*C*) *set8a* MO showed synergistic effect with *wnt8* MOs on Wnt responsive genes. The graph shows the statistical data of the in situ experiment for each gene, respectively. (*D*) Set8a regulated *cdx4* and *tbx6* expression. The embryos injected MOs, as indicated, were collected at the shield stage. The *cdx4* and *tbx6* expression was detected by quantitative real-time PCR and normalized by *ntl* gene expression. (*E*) *set8a* MO and *wnt8* MOs reduced the transcription level of *cdx4* and *tbx6*, both alone and synergistically. $^{**}P < 0.01$; $^{*}P < 0.05$.

and LEF1. The high-mobility group (HMG) domain (residues 244–397) of LEF1 and the SET domain (residues 191–352) of SET8 are responsible for this interaction (Fig. 5*D* and Fig. S5 *A* and *B*). In vitro pull-down assay using the purified recombinant proteins expressed in *Escherichia coli* revealed that SET8 bound to LEF1 and TCF4 directly (Fig. 5*E*). These data taken together show that LEF1/TCF4 bridges SET8 into Wnt signaling.

β-Catenin Facilitates SET8/TCF4 Complex Formation. We noticed that SET8 and TCF4 could form a complex only in Wnt stimulation in vivo. This Wnt-regulated interaction prompted us to investigate the effect of β-catenin on the TCF4–SET8 interaction. We generated a stable HEK293 cell line expressing a low level of Flag-tagged SET8. In this cell line, endogenous TCF4 was coprecipitated with Flag-SET8 under stimulation with Wnt3a CM (Fig. S5*C*). In addition, β-catenin RNAi reduced the interaction of TCF4 with SET8 (Fig. S5*C*). The basal interaction between SET8 and TCF4 in this cell line possibly is because of the increase of SET8 abundance. Consistently, overexpression of a truncated form of β-catenin (ΔN-β-catenin) with constitutive activity in HEK293 increased the binding of SET8 to the *AXIN2* TBE without affecting the binding of TCF4 (Fig. 6*A*). In contrast, knockdown of β-catenin using shRNA diminished the occupation of SET8 at the TBE on Wnt stimulation (Fig. 6*B*), and this situation was not observed at the control region (Fig. S6*A*). These results indicate that β-catenin was involved in the TCF4–SET8 complex formation. However, β-catenin exhibited little affinity to SET8, and purified β-catenin did not facilitate the interaction between LEF1 and SET8 in vitro (Fig. S6*B*). Groucho, a transcription repressor, is known to bind to TCFs in the absence of Wnt. On Wnt stimulation, β-catenin removes Groucho from TCFs as part of the transcription activation process (30). Groucho binds to the HMG domain of TCF4 (31) and thus, might prevent SET8 from binding to TCF4. We hypothesized that β-catenin might promote the binding of SET8 to TCF4 by removing Groucho. To test this hypothesis, we overexpressed SET8, Groucho, TCF4, and ΔN-β-catenin in HEK293T cells and found that Groucho inhibited the interaction of SET8 with TCF4 (Fig. 6*C* and Fig. S6*C*). However, expression of ΔN-β-catenin alleviated this inhibition by expelling Groucho from TCF4 (Fig. 6*C* and Fig. S6*C*). These data suggest that Wnt regulates the interaction of SET8 with TCF4 family transcription factors through the β-catenin–mediated removal of Groucho from TCFs.

Discussion

In this study, we show that, under Wnt stimulation, SET8 is recruited into the β-catenin/TCF4 complex and functions as a coactivator, possibly by catalyzing H4K20me-1 as a transcription activation marker. Knockdown of SET8 abolished H4K20me-1 enrichment at Wnt target gene promoters (Fig. 2*A* and Fig. S2*A*)

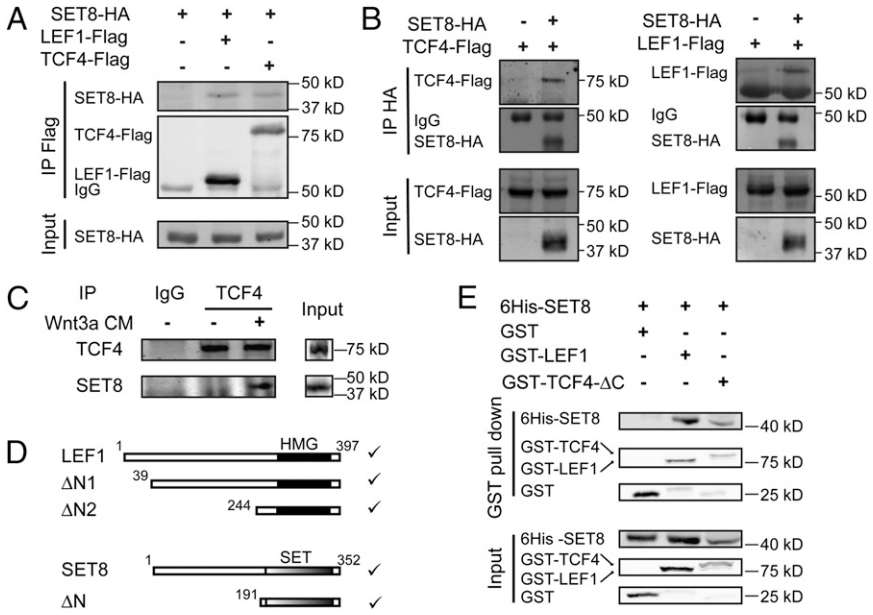

Fig. 5. SET8 interacts with the TCF4 family. (*A* and *B*) Coimmunoprecipitation of SET8 and TCF4/LEF1 in HEK293T cells. (*C*) Endogenous SET8 and TCF4 could form a complex under Wnt3a stimulation. HEK293 cells were treated with control or Wnt3a CM for 1 h. (*D*) Schematic representation of interaction domain between SET8 and LEF1. (*E*) SET8 directly interacted with LEF1 and TCF4. GST-TCF4-ΔC (1–499 aa, containing HMG domain), GST-LEF1, and GST were purified and mixed with full-length SET8 separately for 3 h with GST beads. DNase was added to prevent the interference of DNA.

and inhibited expression of target genes in mammalian cells and zebrafish (Figs. 2 and 4). The intrinsic methylase ability of SET8 is necessary for its activator role, because only WT, instead of the methylase defect mutant, could activate the Wnt target gene (Fig. 2*B*) and rescue the phenotype of *set8a* morpholino (Fig. 3*E* and Fig. S4*C*). More substrates of SET8 have been found other than histone H4, such as the p53 protein (32). It is not clear whether SET8 modifies other proteins to affect Wnt signaling, because neither β-catenin nor TCF4 contains a substrate motif (25). However, the fact that the D338A mutant of SET8, which is unable to bind to histone, exhibited marginal activation on Wnt signaling (Fig. 2*B*) indicates that SET8 potentializes Wnt signaling by at least catalyzing H4 methylation.

We observe that SET8 responds to Wnt signaling and then is recruited by TCF4, establishing H4K20me-1 modification around TBE. This H4K20me-1 modification might attract additional transcriptional factors to modulate chromatin structure. However, it is also possible that the function of H4K20me-1 is site-specific or context-related, because its activation function does not extend to the GAL4 reporter (22) or NF-κB pathway (Fig. S2*D*). It is also possible that different readers of H4K20me-1 lead to diverse biological effects of H4K20me-1. Although the mechanism by which H4K20me-1 activates transcription under Wnt stimulation is unclear, we provide clear evidence that H4K20me-1 under Wnt stimulation is linked to transcription activation. Interestingly, SET8 is well-known for cell cycle control, and the abundance of SET8 and H4K20me-1 reaches the highest level at the G2/M cell cycle phase when LRP6 phosphorylation and Wnt signaling are also enhanced (33, 34).

In summary, on the basis of the observations made by ourselves and others, we propose a model, depicted in Fig. 6*D*, that shows the involvement of the H4K20 methyltransferase SET8 in the canonical Wnt signaling pathway. In this model, we suggest that SET8 is recruited to the TBE of Wnt target genes and replaces Groucho with assistance from β-catenin, directly binding to LEF1/TCF4 on Wnt stimulation/β-catenin accumulation, and that SET8-mediated H4K20me-1 promotes transcription of the Wnt target genes.

Accumulating evidence has shown that histone methyltransferases is crucial for gene transcription regulation (1) and can serve as a potential target in cancer therapy (35). It is possible that SET8 is a key regulator in tumor formation. It has been reported that SET8 negatively regulates the function of p53 (32). Additionally, SET8 is involved in promoting the cell cycle and DNA replication (36–38), indicating its importance in cell proliferation. Wnt signaling is also well-investigated in cancer development. Our finding that SET8 participates in the Wnt signaling pathway suggests an interesting link between SET8 and carcinogenesis. Furthermore, as a histone methyltransferase identified to act on LEF1/TCF4, SET8 has potential as a target interface for drug development and cancer therapy.

Materials and Methods

Plasmids and Reagents. cDNA-encoding human SET8 was amplified from total RNA of HEK293 cells by RT-PCR. The antibodies used were β-catenin (14/β-catenin; BD Biosciences), TCF4 (6H5-3; Millipore), H4K20me-1 (#05–735; Upstate), and SET8 (ab3744 and ab3798; Abcam). Wnt3a CM and the control were described previously (19). shRNAs targeting human SET8 mRNA were described previously (32, 39), and the mouse SET8 shRNA sequence was 5′-AGTCAAAGATCTATGCCTA-3′. The β-catenin shRNA was described previously (19).

Cell Culture, Transfection, and Reporter Gene Assay. Cell culture, transfection, and reporter gene assay details are listed in *SI Materials and Methods*.

Target Gene Assay. Total RNA was extracted from cultured cells or zebrafish embryos at shield stage with TRIzol. Additionally, the reverse transcription of purified RNA was performed using oligo(dT) priming and the superscript III first-strand synthesis system (Invitrogen) according to the manufacturer's instructions. The quantification of gene transcripts was measured by quantitative real-time PCR. Gene expression was normalized by *GAPDH*. More details, information, and primer pairs used for target genes are listed in *SI Materials and Methods*.

ChIP Assay. HEK293 (2×10^6) was plated for ~48 h and treated with control or Wnt3a CM for 1 h before formaldehyde cross-linking. ChIP in zebrafish was performed with ~200 embryos injected at one-cell stage with indicated MOs and harvested at shield stage. The primer pairs used for zebrafish *tbx6*, human *AXIN2*, and human *c-MYC* were described previously in refs. 19, 40, and 21, respectively. Detailed ChIP procedure and other primer pairs used

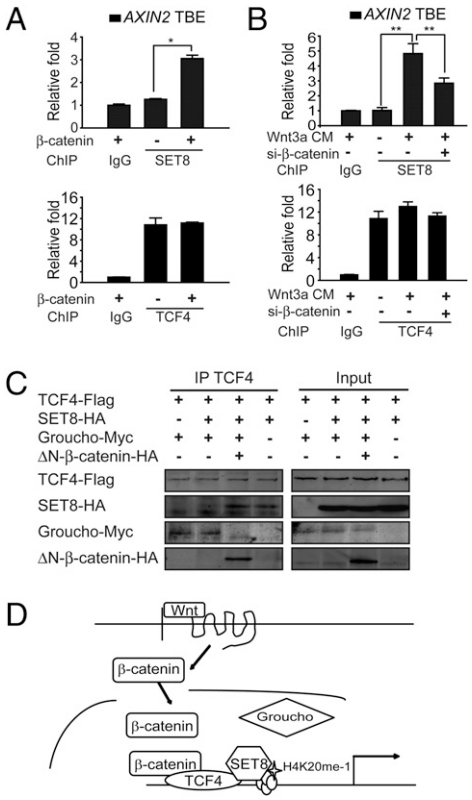

Fig. 6. Wnt/β-catenin facilitates SET8/TCF4 complex formation. (*A*) Overexpression β-catenin facilitated endogenous SET8 augmentation on the *AXIN2* promoter. HEK293 cells were transfected with ΔN-β-catenin or LacZ, and ChIP experiments were performed with SET8 or TCF4 antibodies. Quantitative PCR results of IgG were set as 1. (*B*) RNAi β-catenin inhibited Wnt-induced SET8 augmentation. HEK293 was transfected with β-catenin or control shRNA for more than 48 h and then treated with control or Wnt CM for 1 h before the subsequent ChIP experiment. All graphs show the average with SD. $^{**}P < 0.01$. (*C*) β-catenin expelled Groucho from TCF4 to facilitate SET8 binding. TCF4-Flag, SET8-HA, Groucho-Myc, and ΔN-β-catenin-HA were transfected in HEK293T as indicated. Cell lysates were immunoprecipitated with TCF4 antibody. (*D*) Schematic representation of a model for the role of SET8 and H4K20me-1 in the Wnt signaling cascade. When Wnt signaling is activated, β-catenin translocates into the nucleus and binds to TCF4, releasing repressors such as Groucho. Then, SET8 is recruited to TCF4/LEF1 and establishes H4K20me-1 as a gene transcription active marker to facilitate target gene expression.

are listed in *SI Materials and Methods*. All of the precipitated DNA samples were quantified by real-time PCR and normalized by input DNA. The value of IgG sample was always set as 1, and the ChIP results are presented as the relative fold of IgG. All ChIP experiments were performed three or more times on independent chromatin preparations. *P* value was calculated using a two-tailed Student *t* test.

Microarray Analysis. Total RNA was extracted from HEK293 cells after shRNA transfection (control or SET8 shRNA) and induced with control or Wnt3a CM for 7 h using TRIzol (Invitrogen) and the RNeasy kit (Qiagen). Three pairs of samples were used: si-control + control CM, si-control + Wnt3a CM 7 h, and si-SET8 + Wnt3a CM. Samples were amplified and labeled using a NimbleGen One-Color DNA Labeling Kit, and mRNA of SET8 and AXIN2 was detected as a positive control before hybridization in the NimbleGen Hybridization System. After hybridization, the processed slides were scanned with the Axon GenePix 4000B microarray scanner. Raw data were extracted as pair files by NimbleScan software (version 2.5). NimbleScan software's implementation of robust multichip average offers quantile normalization and background correction. The six gene summary files were imported into Agilent GeneSpring Software (version 11.0) for further analysis. Genes that have values greater than or equal to lower cutoff of 50.0 in all samples were chosen for data analysis. Differentially expressed genes were identified through fold-change screening. *P* value was calculated. Array data are available in the Gene Expression Omnibus database under accession number GSE24708.

Zebrafish Experiment Microinjection. Embryos were produced by pair-mating of fish raised under standard conditions. The WT embryos were derived from the Tuebingen strain. Antisense MO and a standard control MO were obtained from Gene Tools. The MO sequences used were *set8a* MO: 5-GAGCAGCGAGAAGCCTCCACTTCCT-3, and *set8a* MO2: 5-TATCTCATTCACGGCAGGAAACATG-3. *wnt8* MOs (*wnt8*-ORF1 MO + *wnt8*-ORF2 MO) have been described previously (8). For sense RNA injections, capped mRNA was synthesized using the mMessage mMachine kit (Ambion); 2 nL MOs or mRNA were injected into the yolk of the embryo at the one-cell stage.

Whole-Mount in Situ Hybridization. Linearized plasmids were used as templates. Digoxigenin-UTP–labeled antisense RNA probes were generated by in vitro transcription using the DIG RNA Labeling kit (Roche) according to the manufacturer's instruction. Whole-mount in situ hybridizations were performed following the standard methods (41) with minor modifications. The embryos were photographed as described (27). Embryos at the shield stage are shown in a dorsal view with the animal pole to the top or a lateral view with dorsal to the right. Embryos at the 10-somite stage were flat-mounted with the anterior to the left.

In Vitro Binding Assay. Recombinant proteins (GST or 6His-tagged) were expressed in *E. coli*. Proteins were purified and then mixed in GST buffer for 3 h at 4 °C along with GST beads. DNase was added to prevent the interference of DNA. GST beads were washed three times and resuspended in SDS loading buffer.

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