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

Zhenfei Li1, Fen Nie1, Sheng Wang, and Lin Li2

State Key Laboratory of Molecular Biology, Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200031, China

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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 the interaction between 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 L3MBTL1 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/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, legsad, 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 altering 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. L4). 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. L4). Although most of the histone methylations showed no or marginal changes, there was, surprisingly, an approximately fourfold increase in H4K20me-1 (Fig. L4). However, the H4K20me-1 en-
richment was not observed at the AXIN2 coding region or promoter region containing no TBEs (Fig. 1B).

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. 1C and Fig. S1A). This Wnt-induced H4K20me-1 enrichment was not observed at the GAPDH or IκB promoter (Fig. S1B) or the control region in promoter (Fig. S1C). Furthermore, the Wnt stimulation induced H4K20me-1 enrichment at TBEs of other target genes, such as LMO2, NKD1, and DKK1 (Fig. S1D). We also investigated the binding of β-catenin to TBE showed similar kinetics to H4K20me-1 at the TBEs, whereas TCF4 maintained a steady state with marginal variation (Fig. 1C and Fig. S1A), 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. 2A and Fig. S2A) 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. 2B). ChIP analyses also revealed that the binding of SET8 to the TBE showed an oscillation pattern similar to H4K20me-1 and β-catenin (Fig. 1C and Fig. S1A). 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. S2C). 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. S2C). In addition, SET8 overexpression did not affect TNFα-stimulated NF-κB reporter gene activity (Fig. S2D), 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 114–299) 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. 2B). These results show the importance of the methyltransferase activity and histone binding capacity for the SET8 function in Wnt signaling. Consistently, knockdown of mouse SET8 (mSET8) inhibited the Wnt3a-stimulated Axin2 up-regulation, and this inhibitory effect could be reverted by human SET8 expression in a rescue experiment (Fig. 2C).

Consistently, Wnt target genes, such as AXIN2, c-MYC, NKD1, and LEF1, were regulated by SET8 in human cell lines (Fig. 2D). 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),
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. 2E 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 \( \text{NKD1} \), \( \text{ELP4} \), and \( \text{MSX2} \). Wnt stimulation led to H4K20me-1 enrichment at these TBEs but not \( \text{CAMK2B} \) TBE, a gene regulated by Wnt signaling but not SET8 (Fig. 2F). 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. 2G). 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. S3A). When set8a was knocked down by morpholino oligonucleotides (MOs), the H4K20me-1 abundance at \( \text{tbx6} \) promoter was reduced (Fig. 3A). Consistently, knockdown of \( \text{wnt8} \) also led to reduction of H4K20me-1 (Fig. 3A). In situ hybridization analysis showed that set8a was broadly expressed by 24 h postfertilization (hpf) (Fig. S3B). 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 \( \text{wnt8} \) morphants (26, 27) (Fig. 3B). These phenotypes became more severe with increased dosages of the set8a MO (Fig. 3C). Importantly, these phenotypes were rescued by set8a mRNA or human SET8-114 mRNA injection (Fig. 3C and Fig. S3C). Consistently, the in situ hybridization experiment revealed that the set8a morphants at 10 somite stage exhibited a shortened trunk marked by \( \text{myod} \) (adaxial cells and somites) and a slight expansion of \( \text{opl} \) (a telencephalon marker) (26, 27) in a dose-dependent manner, similar to \( \text{wnt8} \) morphants (Fig. 3D). 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 \( \text{wnt8} \) MOs (28), and these morphants at 26 hpf displayed an enlarged telencephalon coupled with a shortened trunk and tail, phenocopying the potent \( \text{wnt8} \) knockout effects.
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, 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 neuroectodermal 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.

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, 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 neuroectodermal 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. 3E and Fig. S3D). In addition, injection of SET8-114 mRNA, but not SET8-114H299A, partially reverted these phenotypes (Fig. 3E). 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. 3D). We also tested another set8a ATG-MO, set8a MO2. This MO gave the same result (Fig. S4A–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 dorsolateral mesodermal marker, and a Wnt-responsive gene). Meanwhile, the no tail (ntl) gene, as a negative control (29), was not affected (Fig. 4A 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. 4A 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. 4C). 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. 4D). In addition, injection of SET8-114 mRNA rescued the effect of both the set8a MO and the wnt8 MOs (Fig. 4D). Additionally, coinjection of set8a MO and wnt8 MOs led to more severe reduction of cdx4 and tbx6 expression (Fig. 4E). 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. S4D). 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 coinmunoprecipitation (co-IP) assay showed that both TCF4 and LEF1 interacted with SET8 in HEK293T cells and vice versa (Fig. 5A and B). Consistently, endogenous SET8 also formed a complex with TCF4 under Wnt3a stimulation in vivo (Fig. 5C). We further mapped the interaction sites between SET8 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 dorsolateral mesodermal marker, and a Wnt-responsive gene). Meanwhile, the no tail (ntl) gene, as a negative control (29), was not affected (Fig. 4A 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. 4A 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. 4C). 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. 4D). In addition, injection of SET8-114 mRNA rescued the effect of both the set8a MO and the wnt8 MOs (Fig. 4D). Additionally, coinjection of set8a MO and wnt8 MOs led to more severe reduction of cdx4 and tbx6 expression (Fig. 4E). 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. S4D). In summary, these results collectively indicate the conserved role of Set8a in Wnt signaling in the early development of zebrafish.
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 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/TF4 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).
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. 2B) and rescue the phenotype of set8a morpholino (Fig. 3E and Fig. S4C). 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. 2B) indicates that SET8 potentiates 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-xB pathway (Fig. S2D). 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. 6D, 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 (14C)-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′-AGTCAAAGATCTATGCTA3′. 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 instruction. 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 × 106) 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 tx6, human AXIN2, and human c-MYC were described previously in refs. 19, 40, and 21, respectively. Detailed ChIP procedure and other primer pairs used...
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'GAGCAGCGAGAGGCGTCCACCTCCTCCT-3', and set8a MO2: 5'TATTCTTACCGCGACAAAAATGTC-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 instructions. Whole-mount in situ hybridizations were performed by 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 2 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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Supporting Information

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SI 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 (14β-catenin; BD Biosciences), TCF4 (6H5; Millipore), H4K20me1 (#05-735; Upstate), and SET8 (ab3744 and ab3798; Abcam). Wnt3a-conditioned medium (CM) and the control were described previously (1). shRNAs targeting human SET8 mRNA were described previously (2, 3), and the mouse SET8 mRNA sequence was 5′-AGTCTCAAGAG-TCTATGCCTA-3′. The β-catenin shRNA was described previously (1).

Cell Culture and Transfection. HEK293, NIH 3T3, HCT116, and SW480 were maintained in DMEM (Invitrogen) supplemented with 10% FBS (Gibco). Cells were transfected with plasmids and shRNA by Lipofectamine Plus or lipofectamine 2000 (Invitrogen), respectively, according to the manufacturer’s protocols.

Reporter Gene Assay. Cells were transfected with DNA using Lipofectamine Plus or with shRNA using Lipofectamine 2000 (Invitrogen), according to the manufacturer’s instructions. LEP1-luc reporter and GFP were transfected together with the indicated plasmids or shRNA. Cells were treated with control or Wnt CM for ~6–8 h before luciferase activity was measured. The luciferase activities presented were normalized against the GFP expression level.

Target Gene Assay. Cells were transfected with plasmids for ~24 h or with shRNA for ~48 h, and they were treated with control or Wnt3a CM for ~7 h. 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 using the Quantitative SYBR green PCR kit (TaKaRa). Gene expression was normalized by GAPDH. The primer pairs used for target genes are as follows: zebrafish adk4: 5′-CTCCGGAGCAGGATCTCTAT-3′ and 5′-CTCCTGCTTCTGGTGTTCC-3′; tubα: 5′-CAAGCTTGATTAGGCTGGAC-3′ and 5′-GGGGTTTGTTGAAAGGCTGATA-3′; nilf: 5′-GAAAAGTCTGGGATTTGAGCAGA-3′ and 5′-TGGTGGAGGCTCTCCCTGT-3′; human AXIN2: 5′-AGTGGTGAGATTCCACG-GAAG-3′ and 5′-CTTCACACTGCGATGCATTT-3′; human LEF1: 5′-AACATGGTGAAAGAAGC-3′ and 5′-GGGTAGTTGAGCTGAGTC-3′; human c-MYC: 5′-TGTCGCGGCCCGCTCCACTCTC-3′ and 5′-AATGGTGTGAGCCAGCGA-3′; human GAPDH was described previously (1); mouse Axin2: 5′-CTCCCACCTGGTAAAGAGC-3′ and 5′-ACTGGTCTGCTTCTGGAA-3′; mouse Gpap4: 5′-GGCTGCTTCACCACCTCTA-3′ and 5′-CAAGGTCTCATCCATGCAACTC-3′.

ChIP Assay. HEK293 (2 × 10⁶) 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 the one-cell stage with indicated morpholino oligonucleotides (MOs) and harvested at the shield stage. The primer pairs used for zebrafish tubα, human AXIN2, and human c-MYC were described previously in refs. 1, 4, and 5, respectively. Primer pairs: AXIN2 promoter control region (1): 5′-GTGGGTGTGGGAAAGA-3′ and 5′-GCGGATTGGCCCTGAGTC-3′; AXIN2 coding region (2): 5′-CCAGCCCTGACTCTGTTGC-TC-3′ and 5′-GGCTGTTGAAATACCCCAACTT-3′; AXIN2 coding region (3): 5′-TGTTGGAGACCAGCTTGATC-3′ and 5′-CCACAGGATGAAAGCAG-3′; AXIN2 transcriptional region (4): 5′-TCCGATCGTGTTGAAAGG-3′ and 5′-GGCAAGATGAGAGGAGC-3′; AXIN2 transcriptional region (5): 5′-TCGAATTCTGGGATTTGAGC-3′ and 5′-GAGCTGCTGCTTCTCCTCTGTA-3′; Mouse E2B: 5′-TCCGATCGTGTTGAAAGG-3′ and 5′-GGCAAGATGAGAGGAGC-3′; AXIN2 transcriptional region (6): 5′-TCCGATCGTGTTGAAAGG-3′ and 5′-GGCAAGATGAGAGGAGC-3′; Camk2B 2R: 5′-TCCGATCGTGTTGAAAGG-3′ and 5′-GGCAAGATGAGAGGAGC-3′.

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 and the RNaseq 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 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 RNA 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 the 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 theTuebingen strain. Anti sense MO and a standard control MO were obtained from Gene Tools. The MO sequences used were setba MO: 5′-GGAGCGCAGAGA-GCCCTGACTCTGTTGC-3′ and setbka MO: 5′-TATCTCATTCAG-GGCAGGAAACTC-3′; wntβ MOs (wntβ-ORF1 MO + wntβ-ORF2 MO) have been described previously (6). 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 instructions. Whole-mount in situ hybridizations were performed following the standard methods (7) with minor modifications. The complete ORF of zset8a (DQ343297) was inserted into the pCS2+ vector for a template of the zset8a probe, and other probe sequences were described previously (8). The embryos were photographed as described (9). 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 Escherichia 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.


Fig. S2. SET8 is involved in Wnt/β-catenin signaling. (A) SET8 knockdown inhibited H4K20me-1 enrichment at the TBE of c-MYC. HEK293 cells were transfected with SET8 or control shRNA for more than 48 h and treated with control or Wnt3a CM for 1 h before the ChIP experiment was carried out. (B) H4K20me-1 reduction after SET8 RNAi. HEK293 cells were transfected with control or SET8 RNAi for 48 h and collected directly with SDS loading buffer for Western blot. (C) Effect of diverse histone methylation enzymes on the LEF1-luc in NIH 3T3. (D) SET8 had little effect on the NF-κB reporter gene in HEK293 cells.

Fig. S3. Set8a acts synergistically with Wnt signaling in regulating zebrafish embryo development. (A) Set8a boosted the LEF1-luc in NIH 3T3. (B) In situ hybridization for expression of zebrafish set8a. set8a is broadly expressed at the 16-cell stage, sphere stage, and 60% epiboly. By 24 h postfertilization (hpf), set8a is more highly expressed in the brain region and blood island. Embryos hybridized with set8a sense probe were the negative control. (C) Injection of set8a mRNA rescued the set8a morphant phenotype. The morphants were classified into four categories: 0, S1, S2, and S3. (D) The set8a MO acted synergistically with the wnt8 MOs. The morphants were classified into four categories: 0, W1, W2, and W3.
**Fig. S4.** The ATG-MO set8a MO2 displays similar results as the set8a UTR-MO. (A) Injection of SET8-114 mRNA rescued the set8a morphant phenotype. The morphants were classified into four categories: 0, S1, S2, and S3. (B) The set8a MO2 acted synergistically with the wnt8 MOs. The morphants were classified into four categories: 0, W1, W2, and W3. (C) Injection of SET8-114 mRNA but not the 114H299A mutant rescued the synergistic effect of set8a MO2 and wnt8 MOs. (D) set8a MO2 injection led to dorsal mesoderm expansion, which could be rescued by SET8-114 mRNA. Embryos injected with the indicated morpholinos were fixed at the shield stage and stained for tbx6, cdx4, gsc, and ntl. The graph shows the statistical data for each gene, respectively.

**Li et al.** www.pnas.org/cgi/content/short/1009353108

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**Fig. S5.** SET8 interacts with LEF1 and TCF4. (A and B) Mapping of the essential domain for SET8 and LEF1 interaction. Different truncations of LEF1 were cotransfected with SET8 in HEK293T, and coimmunoprecipitations were performed. ΔN1- (39–397 aa) and ΔN2-LEF1 (244–397 aa) containing the high-mobility group (HMG) domain could bind to SET8. 6His-ΔN-SET8 (191–352 aa) containing SET domain could interact with LEF1 directly as the pull-down assay showed. (C) Interaction between SET8 and TCF4 was regulated by Wnt stimulation and β-catenin accumulation. Stable cells containing Flag-SET8 were transfected with control or β-catenin shRNA for more than 48 h and then treated with control or Wnt CM for 1 h. Nuclear extractions were immunoprecipitated with IgG or Flag beads overnight. The effect of Wnt stimulation and β-catenin RNAi on cytosolic β-catenin level was indicated.

Li et al. www.pnas.org/cgi/content/short/1009353108
**Fig. S6.** Wnt/β-catenin regulates SET8 and TCF4 interaction. (A) Wnt stimulation and β-catenin RNAi did not alter SET8 at the control region specifically. HEK293 cells were transfected with β-catenin or control shRNA for more than 48 h and then treated with control or Wnt3a CM for 1 h before the subsequent ChIP experiment. (B) β-catenin showed no effect on the interaction between LEF1 and SET8 in vitro. 6His-SET8, 6His-β-catenin, and GST-LEF1 or GST were purified separately and mixed for 3 h with GST beads. DNase was added to prevent the interference of DNA. (C) SET8 competed with Groucho in binding to TCF4. TCF4-HA, SET8-Flag, Groucho-Myc, and ΔN-β-catenin-HA were transfected into HEK293T cells as indicated. Cell lysates were immunoprecipitated with HA antibody.
<table>
<thead>
<tr>
<th>Table S1. List of genes regulated by Wnt3a and SET8</th>
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<tr>
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1–2, pairs of si-control + control CM; 3–4, pairs of si-control + Wnt3a CM; 5–6, pairs of si-SET8 + Wnt3a CM. Genes are regulated by Wnt3a and SET8 (fold > 1.5, P < 0.05; 24 unique genes).