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Kaiso is a bimodal modulator for Wnt/β-catenin signaling

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ABSTRACT

The Wnt family of secreted ligands plays critical roles during embryonic development and tumorigenesis. Here we show that Kaiso, a dual specific DNA-binding protein, functions as a bimodal regulator of canonical Wnt signaling. Loss-of-function analysis of Kaiso abrogated Wnt-mediated reporter activity and axis duplication, whereas gain-of-function analysis of Kaiso dose-dependently resulted in synergistic and suppressive effects. Our analyses further suggest Kaiso can regulate TCF/LEF1-activity for these effects via modulating HDAC1 and β-catenin-complex formation. Our studies together provide insights into why Kaiso null mice display resistance to intestinal tumors when crossed onto an $Apc^{Min/+}$ background.

Stuctured summary:
MINT-6823807: *HDAC1* (uniprotkb:Q13547) *physically interacts* (MI:0218) with *beta catenin* (uniprotkb:P35222) by *anti tag coimmunoprecipitation* (MI:0007)
MINT-6823820: *axin* (uniprotkb:O15169) *physically interacts* (MI:0218) with *beta catenin* (uniprotkb:P35222) by *anti tag coimmunoprecipitation* (MI:0007)

1. Introduction

DNA methylation on CG repeats is important for epigenetic regulation leading to gene-silencing events. Dysregulation of DNA methylation is causative for cancers as well as embryonic defects [1,2]. Kaiso is a methylated DNA binding protein that binds to methylated diCpG nucleotides through its first two zinc finger domains, unlike other methylated DNA binding proteins, such as MBD1, MBD2, MBD4 and MeCP2 [3], which use a so-called methyl CpG binding domain (MBD). In addition, Kaiso can recognize the non-methylated DNA sequence (TNGCAGGA) [4,5]. Kaiso was originally identified by a yeast-two hybrid screen using an adhesion protein p120ctn as a bait [6] and the Kaiso protein consists of a POZ/BTB domain and three zinc finger domains. Kaiso also interacts with the insulator protein CTCF and the N-CoR (nuclear receptor corepressor 1) protein through its POZ/BTB domain [7,8]. Kaiso is thought to repress target gene expression by recruiting a repressor complex [8]. However, the expression of rapsyn is positively regulated by Kaiso with δ-catenin, suggesting that Kaiso may possess bimodal activity with regards to its regulation of gene expression [9].

Interestingly, Kaiso has been shown to function in the Wnt signaling pathway. Wnt signaling plays key roles in early development and its dysregulation causes numerous pathologies including cancer formation and osteoporosis [10]. Wnt/β-catenin signaling, which is also termed canonical Wnt signaling, controls cell differentiation and cell proliferation [11], whereas the non-canonical Wnt pathway (Wnt/PCP and Wnt/Ca^{2+}) regulates cell polarity and cell movement [12,13]. In the canonical Wnt pathway, the level of β-catenin protein is tightly regulated by a degradation complex. In the absence of Wnt, β-catenin is phosphorylated by CKIα and GSK3 on the scaffolding protein Axin, leading to the ubiquitination and degradation of β-catenin via the proteasome pathway. The tumor suppressor gene adenomatous polyposis coli (APC) can complex with Axin to promote β-catenin phosphorylation, thereby enhancing β-catenin degradation. In the presence of Wnt, Frizzled and co-receptor LRP5/6 form a ternary complex, recruiting the cytoplasmic factors Dishevelled and Axin to the plasma membrane. Thus β-catenin is no longer efficiently phosphorylated, resulting in the cytoplasmic stabilization of β-catenin. β-catenin subsequently translocates into the nucleus and mediates activation of Wnt-responsive genes mainly by binding to members of the transcription factor TCF/LEF family.

One of the putative direct target genes of Kaiso is *Xenopus* Wnt11 [14], which regulates dorsal axis formation [15] and convergent extension in the frog embryo [16]. In addition, it has been demonstrated that Kaiso negatively regulates the Wnt/β-catenin target genes siamois and matrilysin/MMP7 [17,18]. An additional connection between Kaiso and the Wnt/β-catenin pathway was uncovered when the Kaiso binding protein p120ctn was shown

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to associate with Frodo [19] which interacts with the cytoplasmic protein Dishevelled and transcription factor TCF3 [20,21]. In the *Xenopus* embryo, Kaiso is expressed maternally and present throughout early development [22]. Kaiso knockdown via morpholino antisense oligonucleotides (MO) caused gastrulation defects [14], as well as an apoptotic phenotype when injected into animal hemisphere at the 1-cell stage [23]. Somewhat surprisingly however, Kaiso null mice showed no obvious phenotype [24]. However, when crossed with tumor-susceptible $Apc^{Min/+}$ mice, which have a mutation in APC and thus have up-regulated β-catenin signaling, $Kaiso^{-/y}$ $Apc^{Min/+}$ mice showed less tumors, implying that Kaiso possess oncogenic activity [24]. Surprisingly, the expression levels of putative target genes were not significantly changed in Kaiso null mice. The reason for the considerable differences in observed phenotypes between Kaiso knocked-down *Xenopus* embryos and the targeted null mouse phenotype may be that *Xenopus* appears to lack a zBTB4 homolog which is also a methylated DNA binding protein whose specificity partly overlapped with Kaiso in the mouse.

Here we examine the effect of Kaiso in Wnt signaling and show that Kaiso has bimodal activity in Wnt signaling in *Xenopus* embryos. Kaiso knockdown by MO resulted in suppression of Wnt/β-catenin activity. While mild ectopic expression of Kaiso enhanced Wnt/β-catenin signaling, moderate and higher expression of Kaiso inhibited Wnt/β-catenin signaling. Kaiso can decrease complex formation between β-catenin and HDAC1, implying that Kaiso may alleviate the negative effects of HDAC1 from the β-catenin/TCF complex. Furthermore, the positive role of Kaiso in Wnt/β-catenin signaling can partly explain why Kaiso null mice develop less tumors on a cancer susceptible background.

2. Materials and methods

2.1. Embryo manipulation, RT-PCR and explant assays

RNA was *in vitro* transcribed using SP6 mMESSAGEmachine (Ambion). Eggs were obtained from female *Xenopus laevis*, and embryos were raised in 0.1× MMR buffer (100 mM NaCl, 2 mM KCl, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 5 mM HEPES, 1 mM EDTA, pH 7.8). Embryos were staged according to Nieuwkoop and Faber [25]. Embryos were microinjected with 5 nl per blastomere of *in vitro* synthesized RNA synthesized (mMessage mMachine; Applied Biosystems) and/or DNA in 3% Ficoll/0.5× MMR buffer. For RT-PCR, total RNA was isolated from *Xenopus* animal caps or whole embryo by TRIzol (Invitrogen) according to the manufacturer's protocol. PCR primers for Xnr3, siamois and EF-1α were as previously described [26].

2.2. Luciferase assay

Twenty picograms of firefly luciferase reporter constructs in combination with 2 pg of renilla luciferase (renilla-TK: Promega) per embryo were injected into two blastomeres of VMZ or DMZ at the 4-cell stage, or animal pole at the 2-cell stage with MO and/or mRNAs. Luciferase levels were measured from lysates of three whole embryos in duplicate or triplicate using the Dual Luciferase Assay Kit (Promega).

2.3. Plasmids and oligonucleotides

Xenopus Kaiso cDNA was obtained from Open Biosystems and human Kaiso cDNA was obtained from OriGene. Both cDNAs were subcloned into pCS2 using EcoRI and XhoI sites. All morpholino antisense oligonucleotides were purchased from Gene Tools. *Xenopus* Kaiso Morpholino oligonucleotide (xKaiso MO) sequence was according to Kim et al. [14]. A standard Control Morpholino oligonucleotide was used as a negative control. To generate xKaiso MO-resistant construct, we introduced five mismatched silent mutations into coding region of xKaiso, substituting ATGGAGACAAAAAAGCTG with ATGGAAACTAAGAAACTT by QuickChange (Stratagene).

2.4. Cell culture, conditioned medium (CM) and transfection

293T cells were cultured in DMEM containing 10% FBS at 37 °C in 5% CO_2. Conditioned media (CM) was prepared by following "Protocol for the Purification of Wnt Proteins" at The Wnt Homepage (http://www.stanford.edu/~rnusse/assays/W3aPurif.htm#medium). Cells in a 12-well plate were transfected with Lipofectamine (Invitrogen) according to manufacturers' instructions.

2.5. Antibodies and immunoprecipitation

Monoclonal antibody for Flag (M2) was from Sigma and Myc (9E10) was from Millipore. Whole cell lysate was prepared 24 h post-transfection with lysis buffer (25 mM Tris–HCl [pH 7.4], 137 mM NaCl, 2.68 mM KCl, 0.6% Triton X-100) including protease inhibitor cocktail (Complete Mini, Roche). Lysates were precipitated with anti-Flag antibody, resolved by 10% SDS–PAGE and blotted with anti-Myc, or anti-Flag antibody.

3. Results

3.1. Kaiso regulation in siamois promoter

The siamois gene is a marker for organizer formation and is required for dorso-ventral patterning. Its promoter contains three TCF/LEF1 binding sites within −350-bp and one Kaiso consensus sequence (KCS) centered around −300-bp (Fig. 1a). First, in order to examine the role of Kaiso in dorso-ventral pattern formation using siamois as a target-gene read-out, we performed a luciferase assay harvesting embryos at st. 10.5 after injection of the reporter gene with ~900 bp of the siamois promoter region [27] together with *Xenopus* Kaiso morpholino (xKaiso MO) or control MO into animal poles, dorsal marginal zone (DMZ) or ventral marginal zone (VMZ). Unexpectedly, we observed that xKaiso MO significantly reduced siamois promoter activity at the DMZ (Fig. 1b). In addition, Kaiso knockdown caused inhibition of reporter activity even with G to T substitution in KCS injected into DMZ (Fig. 1b), indicating that Kaiso can act as a positive regulator in Wnt/β-catenin signaling independent of its KCS.

3.2. Kaiso in β-catenin signaling

To test whether Kaiso regulates Wnt/β-catenin signaling through TCF/LEF1 activity, we used the Super8XTOPFlash luciferase reporter as a read-out [28]. We injected xKaiso MO or control MO into the animal pole, VMZ or DMZ, and then harvested embryos at st. 11–12. Kaiso knockdown caused severe reduction of β-catenin activity at the DMZ (Fig. 1c) consistent with the siamois promoter results. The endogenous β-catenin pathway is activated within the ventral mesoderm after midblastula transition [29] when robust zygotic transcription starts. We observed inhibition of β-catenin signaling by xKaiso MO at the VMZ, which represents zygotic β-catenin activity. Since these results contradicted previously published data [17], we re-evaluated the position of Kaiso in Wnt/β-catenin signaling. We co-injected xKaiso MO with β-catenin pathway activators. Indeed, not only was Wnt8 or LRP6 activity reduced, but LEF1 activity was also reduced by xKaiso MO (Fig. 2a). xKaiso knockdown also repressed the activity of dom-

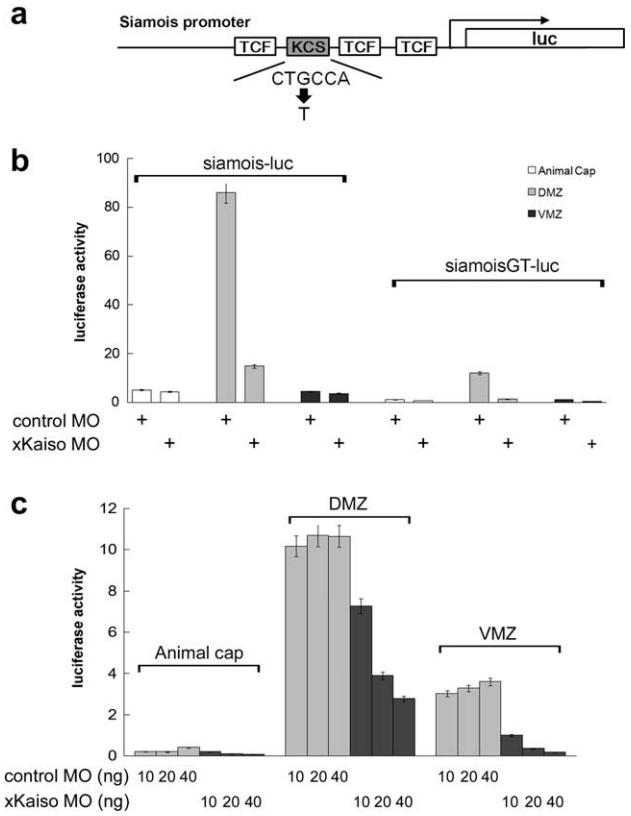

Fig. 1. (a) A schematic figure of siamois-luc. The promoter region of siamois-luc contains three TCF binding sites and one Kaiso consensus sequence (KCS). siamoisGT-luc has a point mutation at KCS. (b) Kaiso knockdown by specific MO (xKaiso MO) injection reduced siamois promoter activity. xKaiso MO had similar effect with siamoisGT-luc. Embryos were harvested at st10.5. (c) xKaiso MO injected into DMZ and VMZ inhibited β-catenin/TCF activity in a dose dependent manner. Embryos were harvested at st. 11–12.

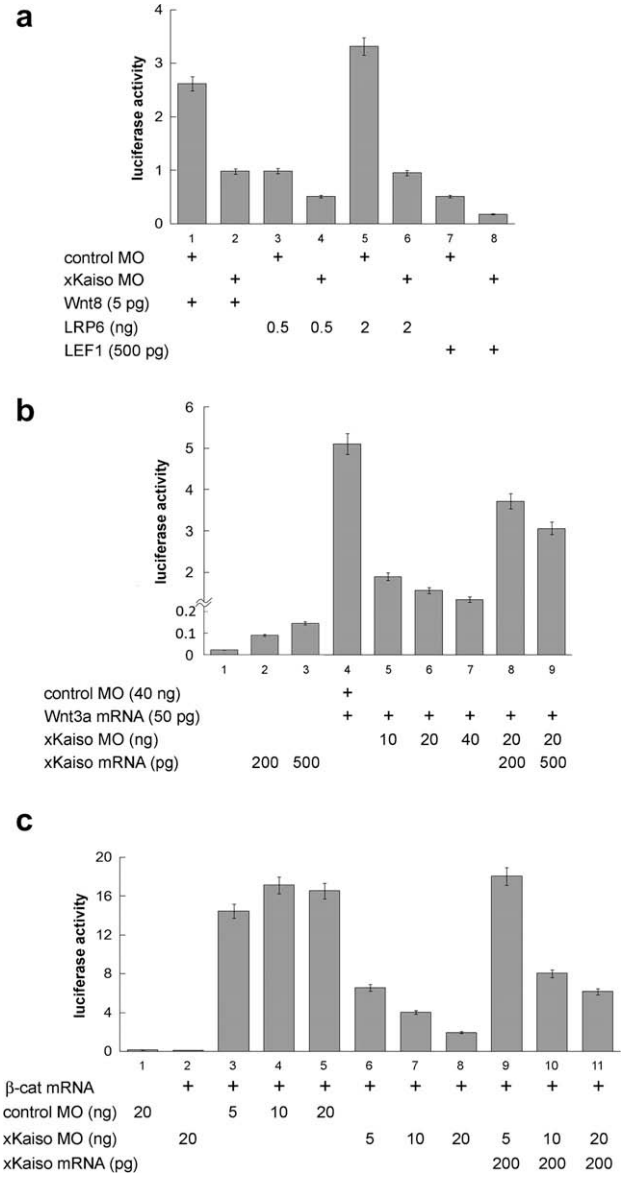

Fig. 2. (a) Twenty nanograms of xKaiso MO antagonized Wnt8, LRP6 and LEF1 activity when injected into animal pole at both blastomeres at 2-cell stage. (b) xKaiso mRNA by itself mildly activated Super8XTOPFlash (lanes 2 and 3). MO-resistant xKaiso mRNA rescued xKaiso MO partially (lanes 8 and 9) Injection was done at 2-cell stage into both blastomeres at animal pole. (c) β-Catenin into VMZ was antagonized by xKaiso MO, which was also rescued by MO-resistant xKaiso mRNA. Eggs were harvested at st. 10.5.

inant negative GSK3 and the stabilized form of β-catenin (data not shown). These data indicate that Kaiso positively modulates β-catenin signaling at the transcriptional level. Moreover, the effect of the xKaiso MO was effectively rescued by the co-injection of xKaiso mRNA which contains five silent mismatch mutations with regards to the morpholino-recognition sequence (Fig. 2b), demonstrating the specificity of the xKaiso MO. When injected into VMZ, xKaiso MO resulted in inhibition of β-catenin activity and again this inhibition was rescued by the MO-resistant mRNA (Fig. 2c).

3.3. Kaiso potentiates Wnt/β-catenin signaling

To examine whether ectopic expression of Kaiso can activate Wnt/β-catenin signaling, we injected xKaiso mRNA with Wnt3a or Lef1 into animal caps. Reporter activity was increased by co-injection of xKaiso with Wnt3a or Lef1 (Fig. 3). Moreover, xKaiso by itself upregulated Super8XTOPFlash activity (Fig. 2b), which is reciprocal to the knockdown studies suggesting that Kaiso has positive role in Wnt/β-catenin pathway. The xKaiso mRNA activity was blocked by ΔN-TCF3 (data not shown) which lacks a β-catenin binding domain and acts as a dominant negative [30], implying that Kaiso cannot substitute for the absence of β-catenin within the TCF complex. Taken together, these results support a model in which Kaiso can enhance Wnt/β-catenin signaling. However, xKaiso mRNA overexpression ventrally was unable to induce axis duplication (data not shown), suggesting that Kaiso functions as a modulator in Wnt/β-catenin signaling.

3.4. Kaiso positively and negatively regulates β-catenin signalin

Next, we tested whether Kaiso modulates expression of endogenous Wnt-target genes. Previously it has been shown that siamois expression was regulated by TCF and Kaiso [17]. We found that xKaiso mRNA injection can synergize with Wnt3a for siamois induction using animal cap assays (Fig 4a, lanes 4–6) as observed with our Super8XTOPFlash experiments (Fig. 3a). Injection of xKaiso MO with a minimal amount of Wnt3a mRNA caused up-regulation of siamois (Fig 4a, lanes 8 and 9) as reported previously [17]. These results indicate that both the loss-of-function and gain-of-function of Kaiso can modulate siamois expression *in vivo*. To monitor effects within the embryo, we injected xKaiso MO or mRNA with β-catenin mRNA into the VMZ of the two blastomeres at 4-cell stage. We observed xKaiso MO reduced axis duplication

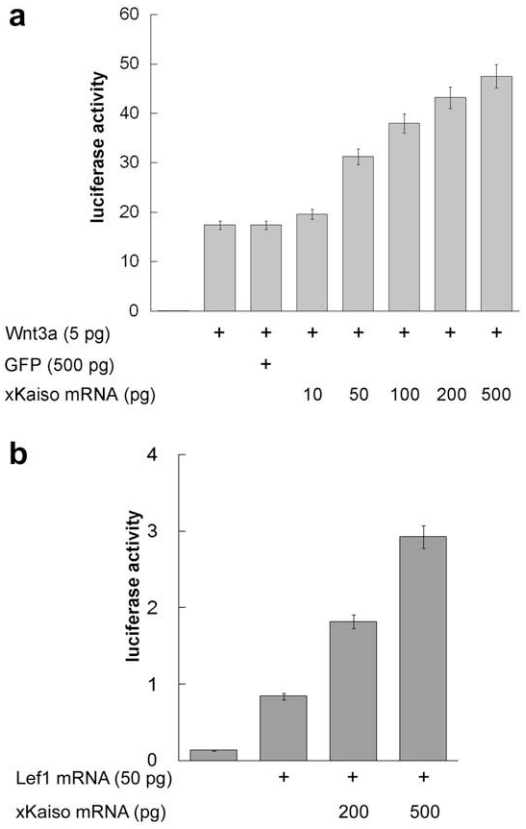

Fig. 3. xKaiso mRNA synergized with Wnt3a (a) and Lef1 (b) in Super8XTOPFlash luciferase reporter assay. mRNAs were injected into animal pole at both blastomere of 2-cell stage embryos.

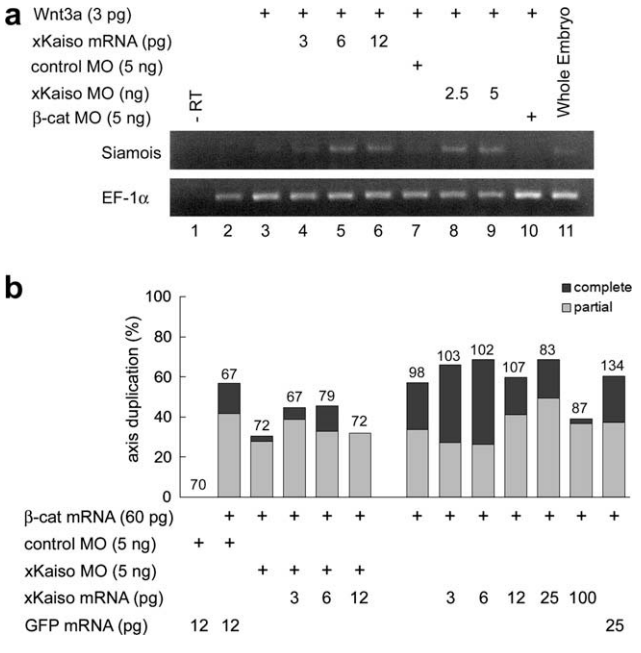

Fig. 4. Kaiso has bimodal effects on endogenous Wnt/β-catenin pathway. (a) xKaiso synergized with Wnt3a. Three picograms of Wnt3a mRNA injection barely induced siamois expression. When xKaiso mRNA was co-injected with Wnt3a, siamois expression was upregulated (lanes 4, 5 and 6). Kaiso MO co-injection with Wnt3a also increased siamois expression in animal caps (compare lane 7 with lanes 8 and 9). b) Kaiso knockdown reduced axis duplication induced by β-catenin. The xKaiso MO was rescued by 3 or 6 pg of MO-resistant mRNA. Co-injection of Kaiso (3 or 6 pg per embryo) and β-catenin increased double axis formation, but mild overexpression of Kaiso inhibits β-catenin activity.

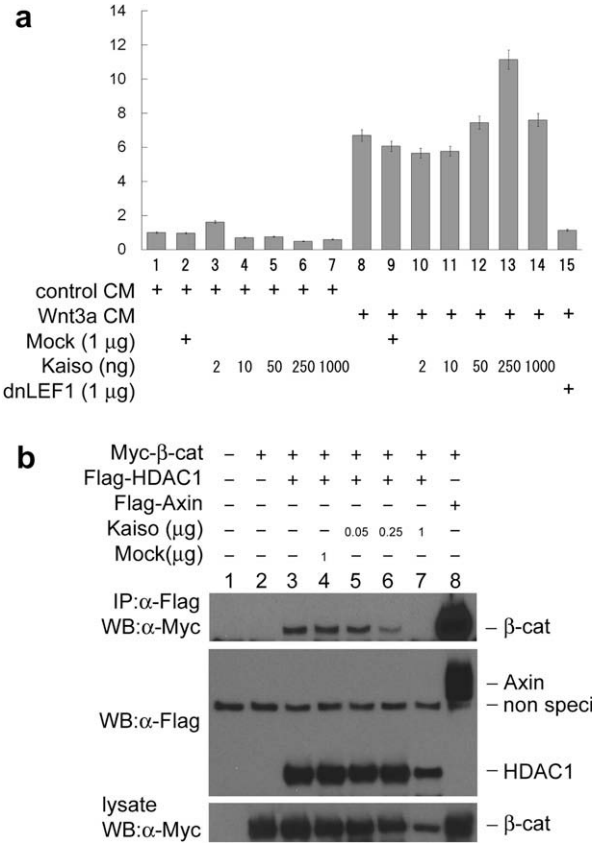

Fig. 5. (a) Kaiso functions bimodally for Wnt/β-catenin signaling in 293T cells. Super8XTOPFlash (50 ng) and RL-TK (5 ng) were cotransfected together with Kaiso constructions (0.002, 0.01, 0.05, 0.25, 1.0 μg). Dominant-negative form of LEF1 (dnLEF1) was used as a negative control. Conditioned media were added in a 1:1 ratio to growth medium 12 h post-transfection. Luciferase activity was measured at 8 h after CM-exposure. (b) Kaiso blocks the interaction of β-catenin with HDAC1. Plasmids encoding 6×Myc-β-catenin SA (0.5 μg), HDAC1-FLAG (0.1 μg), and Kaiso constructions (0.05, 0.25, 1.0 μg) were cotransfected for coimmunoprecipitation assays. Axin-FLAG (0.25 μg) was used as a positive control.

efficiency induced by β-catenin, suggesting that Kaiso can positively regulate the β-catenin pathway activity *in vivo*. This effect was rescued by 3 or 6 pg of MO-resistant Kaiso mRNA injection (Fig. 4b). However, an injection of 12 pg or higher of xKaiso mRNA decreased the induction of the secondary axes. xKaiso mRNA injection at 3 and 6 pg per embryo had an additive effect with β-catenin. Again, 12 pg or more xKaiso mRNA injection caused mild inhibition of β-catenin signaling in axis duplication assay. These *in vivo* data therefore suggest that Kaiso has bimodal activity for β-catenin signaling. We further examined Kaiso function in mammalian 293T cell using Super8XTOPFlash assays. While reporter activity was repressed by high amount of Kaiso cDNA transfection (Fig. 5a, lane 14), mild overexpression of Kaiso with Wnt3a CM treatment had additive effects on reporter activity (Fig. 5a, lanes 12 and 13). This data indicates that bimodal activity of Kaiso in Wnt/β-catenin signaling is not limited to *Xenopus* embryos.

3.5. Kaiso inhibits the interaction of β-catenin with histone deacetylase HDAC1

In the absence of Wnt stimulation, repressor Groucho/TLE binds to TCF/LEF1 [11], whose inhibitory effect was mediated by the interaction of HDAC1 [31]. Indeed it has been shown that HDAC1 is an inhibitory factor of Wnt/β-catenin signaling and forms a complex with LEF1 and β-catenin [32]. To delineate a molecular

mechanism how Kaiso positively modulate Wnt/β-catenin signaling, we performed coimmunoprecipitation assay of HDAC1 and LEF1 or β-catenin with dose dependent Kaiso expression. When cotransfected a stabilized form of β-catenin and HDAC1 with Kaiso in 293T cells, less β-catenin protein was co-immunoprecipitated with HDAC1 in a Kaiso-dependent manner, suggesting that Kaiso blocked the association between β-catenin and HDAC1 (Fig. 5b). We also observed that Kaiso overexpression reduced protein levels of β-catenin and HDAC1 (Fig. 5b, lanes 5 and 6) likely caused by the general repressor activity of Kaiso. These results imply Kaiso may release β-catenin from the HDAC1-complex, resulting in more β-catenin that is available for TCF/LEF1-complexes.

4. Discussion

In this study, we demonstrate that Kaiso has bimodal activity in Wnt/β-catenin signaling. Using a loss-of-function approach with an xKaiso morpholino, β-catenin signaling was dramatically reduced as demonstrated by the reporter activity read-out. We observed that mild overexpression of Kaiso enhanced the signaling, whereas higher levels of Kaiso expression inhibited β-catenin signaling *in vivo*. Our co-injection results of xKaiso MO plus Lef1 mRNA or xKaiso mRNA with ΔN-TCF3 further suggesting that Kaiso functions at the level of the transcription factor TCF/LEF1. In addition, we showed that complex formation between β-catenin and HDAC1 was blocked by Kaiso overexpression, implying a shift in the balance towards greater availability of β-catenin for TCF/LEF1.

Our Kaiso knockdown results using the reporter assays are somewhat contradictory to previous studies that showed Kaiso can negatively regulate siamois expression by repressing TCF/LEF1 activity [17,19], even though we employed the identical MO sequence and reporter construct for the siamois promoter. We believe this discrepancy may be due to where and how much of the reporter constructs were injected. While Park et al. injected 0.5 ng of reporter constructs per embryo into one- or two-cell stage embryos, we specifically injected only 20 pg of reporter constructs into DMZ or VMZ together with xKaiso MO, where the endogenous Wnt/β-catenin signaling is high and detectable. Alternatively, we injected reporter constructs into the animal pole region with positive regulators of the Wnt/β-catenin pathway where the endogenous Wnt/β-catenin signaling is very low. Though we observed kinked embryos and apoptotic phenotypes in xKaiso morphants as previously shown [22,23], we did not observe defects of dorsal axis formation when xKaiso MO was injected into DMZ (data not shown). As the Kaiso protein is present maternally [22,23] and a MO only inhibits protein translation, it is possible that the levels of maternal Kaiso protein may mask the effects of the xKaiso MO.

It is curious that Kaiso knockdown caused an opposite effect on the luciferase reporter assay and endogenous expression level of siamois, while small doses of ectopic expression of Kaiso in animal caps increased siamois expression when co-injected with Wnt3a, complementing the reporter assay data. It is plausible that a chromatin-based structure might lessen the requirement of Kaiso for siamois transcriptional activity. Alternatively, Kaiso might regulate siamois expression negatively through a more upstream or downstream region than that used in the siamois-luc construct. As shown in Fig. 4, a minor overexpression of Kaiso mRNA (3 or 6 pg per embryo) has a positive role in induction of siamois expression and axis duplication assay, whereas even very mild overexpression of Kaiso mRNA (12 pg or higher per embryo) can perturb Wnt/β-catenin signaling. This result implies that the endogenous levels of Kaiso must be tightly regulated.

Our studies demonstrating Kaiso's positive role in Wnt/β-catenin pathway can partly explain the phenotype of the Kaiso null mice on the $Apc^{Min/+}$ background. $Kaiso^{-/y}$ $Apc^{Min/+}$ mice show less tumor formation than $Kaiso^{+/y}$ $Apc^{Min/+}$. It is possible that Kaiso-target genes may only partially overlap with TCF regulated genes and Wnt/β-catenin responsive gene in the intestine might be less activated when Kaiso is targeted, thus $Kaiso^{-/y}$ $Apc^{Min/+}$ demonstrate less intestinal cancer.

Although it was shown that Kaiso and δ-catenin positively regulate rapsyn expression [9] which suggests that Kaiso has dual activity in transcriptional regulation, it remains unclear how Kaiso functions as a positive modulator in Wnt/β-catenin pathway. It is plausible that Kaiso may inhibit the expression of repressors that interact with TCF/LEF such as CtBP or Groucho. Alternatively, Kaiso or Kaiso-interacting proteins may affect complex formation with TCF/LEF1. Indeed, we observed that Kaiso reduced the interaction of β-catenin with HDAC1 (Fig. 5b). When β-catenin, HDAC1 and LEF1 were co-expressed, β-catenin preferably associated with HDAC1 to Lef1 [32]. Our binding studies further suggest that Kaiso inhibited HDAC1 association with β-catenin thereby increasing the availability of β-catenin for TCF/LEF1-interaction. How Kaiso may affect HDAC1 or possibly HDAC3 activity remains a central question to be addressed, since it has been recently showed that N-CoR and SMRT directly binds to β-catenin as well as TCF4 for attenuation of Wnt/β-catenin signaling [33]. Interestingly, this complex was observed to contain HDAC3 instead of HDAC1 [34]. Another recent study showed the methylated-DNA binding proteins, MeCP2, functioned not only as transcriptional a repressor but also as an activator by recruiting by CREB1 [35], suggesting such a bimodal function may not be limited to Kaiso. Thus, it is of interest to ascertain whether Kaiso is also capable of associating with co-activator complexes.

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