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Direct regulation of *siamois* by VegT is required for axis formation in *Xenopus* embryo

HONG-YAN LI[1,2,3], WARIF EL YAKOUBI[1,2] and DE-LI SHI*[,1,2]

[1]Sorbonne Universités, UPMC Univ Paris 06, Institut de Biologie Paris-Seine (IBPS), UMR 7622, Laboratory of Developmental Biology, Paris, France, [2]CNRS, IBPS, UMR 7622, Laboratory of Developmental Biology, Paris, France and [3]Laboratory for Evolution and Development, Department of Marine Biology, Ocean University of China, Qingdao, China

ABSTRACT The homeobox gene *siamois* is one of the earliest genes expressed in the Spemann organizer and plays a critical role in the formation of the dorsoventral axis. It is directly regulated by maternal Wnt signalling and functions as an essential zygotic intermediary between maternal factors and the formation of the Spemann organizer. The maternal T domain transcription factor VegT interacts with Wnt signalling and is also involved in the formation of the Spemann organizer. However, the molecular mechanism of this functional interaction is not fully understood. Here we show that VegT is required for *siamois* expression through direct binding to the T-box binding sites in the *siamois* promoter. Mutational analysis of each of the five consensus T-box binding sites suggests that the proximal site close to the transcription start site is essential for activation of *siamois* promoter by VegT, while individual mutation of the four distal sites has no effect. VegT and Wnt signalling also functionally interact and are mutually required for *siamois* expression. In particular, VegT synergizes with Tcf1, but not Tcf3 and Tcf4, to induce *siamois* expression, and this is independent of Tcf/Lef-binding sites or the proximal T-box binding site in the *siamois* promoter. We further extend previous observations by showing that VegT cooperates with maternal Wnt signalling in the formation of the dorsoventral axis. These results demonstrate that maternal VegT directly regulates *siamois* gene transcription in the formation of the Spemann organizer, and provide further insight into the mechanism underlying the functional interaction between VegT and Wnt signalling during development.

KEY WORDS: *Dorsoventral axis, VegT, siamois, Wnt, Xenopus*

Introduction

During early amphibian development mesoderm is formed through inductive signals emanating from endoderm cells of the vegetal hemisphere and acting on the overlying equatorial cells. The molecular nature underlying this fundamental inductive event has been extensively studied and now partly elucidated. An essential pathway for mesoderm formation is triggered by VegT, a T domain family member of transcription factor, also known as Brat, Xombi and Antipodean (Lustig *et al.,* 1996; Stennard *et al.,* 1996; Zhang and King, 1996; Horb and Thomsen, 1997). VegT activates mesoderm formation by regulating the expression of TGF-β family genes, including *Xnr1*, *Xnr2*, *Xnr4* and derriere (Kofron *et al.,* 1999; Xanthos *et al.,* 2001), as well as a panel of other direct target genes (Tada *et al.,* 1998; Casey *et al.,* 1999; Taverner *et al.,* 2005; Sudou *et al.,* 2012). Maternal Wnt signalling also plays an essential role for the establishment of Spemann organizer in the dorsal region by activating the expression of specific target genes, in particular the homeobox gene *siamois*, which plays a key role in the formation of dorsoventral axis (Lemaire *et al.,* 1995; Fan and Sokol, 1997; Kessler, 1997). Thus, *siamois* is directly regulated by maternal Wnt signalling and functions as an essential zygotic intermediary between maternal factors and the formation of Spemann organizer (Carnac *et al.,* 1996; Brannon *et al.,* 1997; Fan *et al.,* 1998). It is therefore clear that the formation of dorsoventral axis involves a combinatory action of distinct signalling pathways and transcription factors.

VegT is supplied as a maternal mRNA localized in the vegetal

Abbreviations used in this paper: TCF/LEF, T-cell factor/lymphoid enhancer factor.

***Address correspondence to:** De-Li Shi. Sorbonne Universités, UPMC Univ Paris 06, Institut de Biologie Paris-Seine (IBPS), UMR 7622, Laboratory of Developmental Biology, F-75005, Paris, France. Tel: 33-1-4427-2772; Fax: 33-1-4427-3445. E-mail: de-li.shi@upmc.fr

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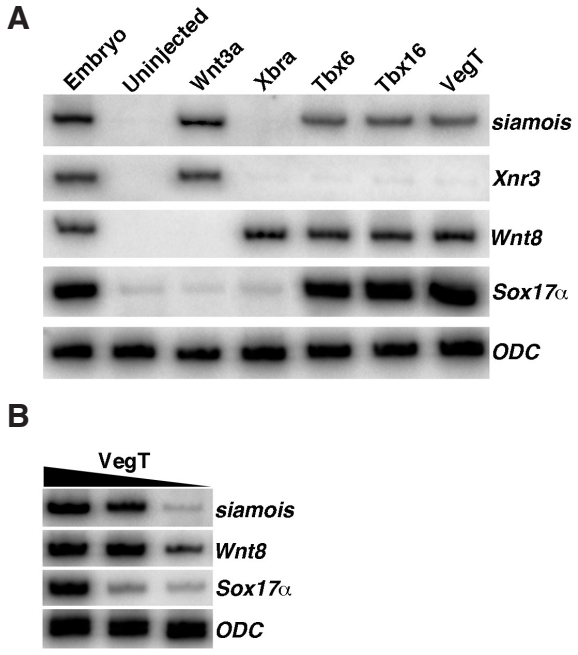

Fig. 1. T domain transcription factors induce ectopic *siamois* expression in ectoderm cells. (A) *Comparison of the* siamois *inducing activity between Wnt3a and different T domain transcription factors. Embryos at the 2-cell stage were injected with indicated synthetic mRNA in the animal pole region and ectoderm explants were dissected at the early gastrula stage for RT-PCR analysis of gene expression, as indicated. Wnt3a induces the expression of both* siamois *and* Xnr3*, but not other mesoderm and endoderm genes. Tbx6, Tbx16 and VegT similarly induce the expression of* siamois*,* Wnt8 *and* Sox17α *genes, but not* Xnr3*. Xbra only induces the expression of* Wnt8*.* **(B)** *VegT induces* siamois *expression in a dose-dependent manner. The expression of* siamois *is induced by high and intermediate doses of* VegT *mRNA, while low dose of* VegT *mRNA only induces* Wnt8 *expression. The endoderm gene* Sox17α *is induced by a high dose of* VegT *mRNA.* ODC *was used as a loading control for input RNAs.*

hemisphere of oocytes and early embryos, it is also transcribed zygotically within the entire marginal zone of the early gastrula (Heasman, 1997; White and Heasman, 2008). Deleption of maternal *VegT* transcripts prevents the formation of mesendoderm (Zhang *et al.,* 1998), while its mis-expression in ectoderm suppresses head formation (Zhang and King, 1996). This suggests that VegT may operate sequentially in several developmental pathways during embryogenesis, including dorsoventral and posterior patterning. Thus, in addition to initiate general mesoderm induction, VegT is also involved in the formation of Spemann organizer and in the patterning of embryonic axis, and its interaction with maternal Wnt signalling in these processes is well documented (Agius *et al.,* 2000; Xanthos *et al.,* 2002). In particular, both VegT and β-catenin are required for the activation of a panel of mesendoderm genes at the late blastula and early gastrula stages (Agius *et al.,* 2000; Xanthos *et al.,* 2002). Thus, VegT represents an important pathway for the formation and function of Spemann organizer. However, the molecular mechanism underlying this functional interaction is not fully understood.

Here we show that VegT is directly involved in the expression of the Spemann organizer gene *siamois* through two related mechanisms. First, we demonstrate that it directly activates *siamois* expression by binding to the T-box binding sites present in the *siamois* promoter. This induction occurs independently of Wnt signalling. Second, maternal VegT and Wnt signalling functionally interact and are mutually required for *siamois* expression. In particular, VegT strongly synergizes with Tcf1 to induce *siamois* expression, even in the absence of Tcf/Lef-binding sites. These results unravel a novel mode of *siamois* regulation in the formation of Spemann organizer in *Xenopus*.

Results

Induction of siamois *ectopic expression by a subset of T domain proteins in ectoderm cells*

We have previously used a hormone-inducible form of Tbx6VP16, Tbx6VP16-GR, in which Tbx6VP16 was fused with the ligand-binding domain of the human glucocorticoid receptor (Fig. S1A), to identify Tbx6 target genes during *Xenopus* early development (Li *et al.,* 2006). Synthetic *Tbx6VP16-GR* mRNA (200 pg) was injected in the animal pole region at the 2-cell stage and ectoderm explants were dissected at early gastrula stage and incubated in 10 μM dexamethasone (DEX) for 1.5 hours. RT-PCR was performed to analyze the expression of a panel of early mesoderm and endoderm markers. Among different genes whose expression was induced in this condition, we have made an unexpected observation that the expression of *siamois*, a specific target gene of maternal Wnt signalling (Carnac *et al.,* 1996; Brannon *et al.,* 1997; Fan *et al.,* 1998), was also strongly induced, while no expression of *Xnr3*, another specific Wnt target gene (McKendry *et al.,* 1997), was detected (Fig. S1B). This indicates that Tbx6VP16 may directly induce *siamois* expression.

To see if other T domain proteins were also able to induce *siamois* expression in ectoderm explants, we injected synthetic mRNA corresponding to *Xbra* (500 pg), *Tbx6* (500 pg), zebrafish *Tbx16*

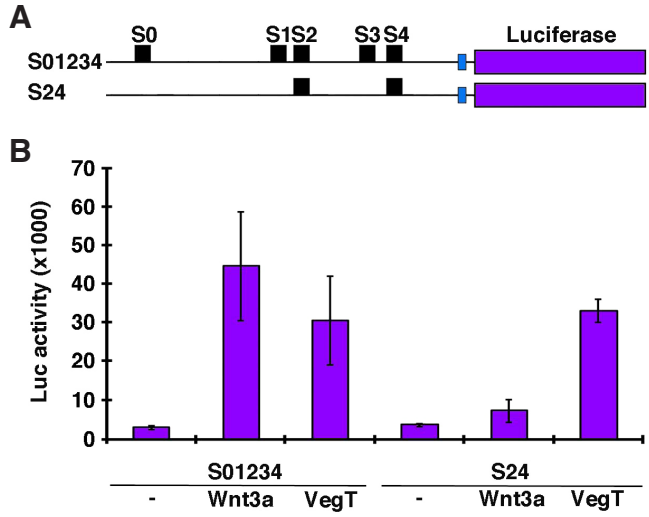

Fig. 2. VegT activates *siamois* promoter reporter independently of Tcf/Lef-binding sites. (A) *Schematic representation of wild-type (S01234) and mutated (S24)* siamois *promoter luciferase reporters, with the location of Tcf/Lef sites indicated (not in scale).* **(B)** *Both Wnt3a and VegT activate the S01234* siamois *promoter with 3 sites (S0, S1, and S3) that conform to the Tcf/Lef- consensus binding sites and 2 sites (S2 and S4) that diverge at the 3′-most base. VegT, but not Wnt3a, also activates the S24* siamois *promoter mutated at the S0, S1 and S3Tcf/Lef-binding sites. Data represent the mean of triplicate experiments (error bars indicate s.d.).*

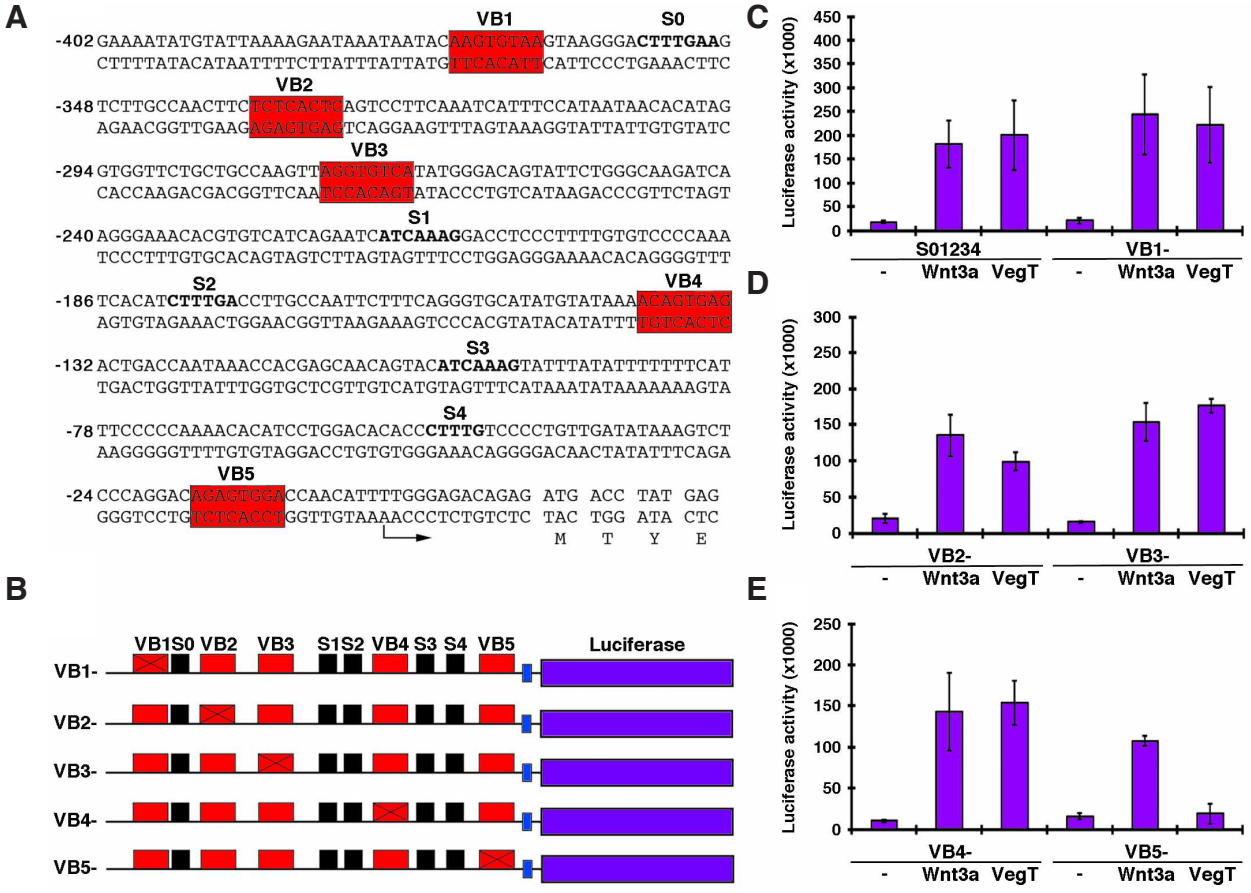

Fig. 3. VegT activates *siamois* promoter through T-box binding sites. (A) *Nucleotide sequence of the 0.4 kb* siamois *promoter fragment. The first 12 nucleotides of the* siamois *coding sequence with its deduced amino acids and 402 nucleotides of* siamois *promoter region 5′ to the transcription start site (arrow) are shown. The Tcf/Lef-binding sites are indicated as S0, S1, S2, S3 and S4, as previously reported (Brannon* et al., *1997). The putative T-box binding sites are boxed and identified above the sequence, as VB1, VB2, VB3, VB4 and VB5. All these sites conform to the T-box binding consensus.* **(B)** *Schematic representation of* siamois *reporter constructs mutated at the VegT-binding sites.* **(C-E)** *Luciferase reporter assay using ectoderm explants previously injected with wild-type or mutant* siamois *promoter reporter DNAs and synthetic mRNAs, as indicated. Mutation of VB1, VB2, VB3 or VB4 does not affect the response of* siamois *promoter to Wnt3a or VegT. Mutation of VB5 abolishes the activation of* siamois *promoter induced by VegT, but not by Wnt3a. Data represent the mean of triplicate experiments (error bars indicate s.d.).*

(500 pg) and *VegT* (200 pg) in the animal pole region and analyzed ectopic *siamois* expression in the ectoderm explants. *Wnt3a* mRNA (2 pg) was also injected to compare its activity with these T domain proteins. RT-PCR analysis performed on ectoderm explants at the early gastrula stage clearly showed that Tbx6, Tbx16 and VegT, but not Xbra, similarly induced ectopic *siamois* expression, although this induction was less efficient than did Wnt3a (Fig. 1A). However, Wnt3a also induced *Xnr3* expression as expected, but not Tbx6, Tbx16 and VegT. As previously reported, these T domain proteins also induced the expression of the mesoderm gene *Wnt8* and the endoderm gene *Sox17*α, with the exception of Xbra, which only induced *Wnt8* expression at the dose injected (Fig. 1A). This result indicates that different T domain proteins have distinct activity to induce *siamois* ectopic expression in ectoderm cells.

Among these T domain proteins, only VegT is maternally expressed and plays an important role in the formation of Spemann organizer (Zhang *et al.,* 1998; Kofron *et al.,* 1999), it is thus likely that Tbx6 and zebrafish Tbx16 only mimicked the activity of VegT. We then performed a dose-response analysis to see how VegT induces the expression of *siamois* and mesoderm and endoderm genes in the ectoderm explants. Injection of different amounts of *VegT* mRNA (1000 pg, 400 pg and 100 pg) indicated a dose-dependent manner of *siamois* induction. VegT induced *siamois* expression at high and intermediate doses, but not at low dose, while it induced the expression of the endoderm gene *Sox17*α only at high dose. The expression of the ventral mesoderm gene *Wnt8* was induced both at high and low doses (Fig. 1B). These observations demonstrate that some T domain proteins, in particular VegT, are able to activate *siamois* expression.

Tcf/Lef site-independent activation of siamois *promoter reporter by VegT*

To further examine how VegT activates *siamois* expression, we first tested if and how it activates the *siamois* promoter reporter. A previously characterized 0.8-kb fragment of the *siamois* promoter was shown to contain three typical consensus Tcf/Lef-binding sites, CTTTG(A/T)(A/T) (S0, S1 and S3), and two additional sites (S2 and S4) differing from the Tcf/Lef core consensus sequence at the 3′-most base (Brannon *et al.,* 1997; Fig. 2A; see also Fig. 3A). In ectoderm explants, the wild-type promoter reporter (S01234) was strongly

activated by Wnt signalling through injection of *Wnt3a* mRNA (Fig. 2B). Consistent with its ability to induce *siamois* expression in ectoderm explants, coinjection of *VegT* mRNA (400 pg) with S01234 promoter reporter DNA strongly increased the luciferase activity (Fig. 2B). We then tested the activity of VegT on the S24 promoter reporter, which was mutated at the three typical consensus Tcf/Lef-binding sites (S0: CTTTGAA to ATTCCAA, S1: ATCAAAG to ATGAAAC, S3: ATCAAAG to ATGAATTC) and did not respond to Wnt signalling (Brannon *et al.,* 1997). Indeed, coinjection of *Wnt3a* mRNA (2 pg) with S24 promoter reporter DNA did not significantly increase the luciferase activity (Fig. 2B), however, coinjection of *VegT* mRNA (400 pg) with S24 promoter reporter DNA increased the luciferase activity similarly as its coinjection with the wild-type S01234 promoter reporter (Fig. 2B). This result clearly indicates that VegT could activate *siamois* promoter independently of Tcf/Lef-binding sites.

VegT activates siamois *expression through T-box binding sites*

The above observations led us to examine whether consensus sequence for T-box binding site, TNNCAC(C/T)(T/C), as determined by previous works (Kispert and Hermann, 1993; Conlon *et al.,* 2001), was present in the *siamois* promoter sequence. We identified 5 potential consensus sites within the 0.4 kb *siamois* promoter region upstream of the transcription start site, which we designated as VB1, VB2, VB3, VB4 and VB5 for VegT-binding sites (Fig. 3A). VB1 is located 8 nucleotides upstream of the S0 Tcf/Lef site, while VB2 is located 14 nucleotides downstream. VB3 is located 51 nucleotides upstream of the S1 Tcf/Lef site, while VB4 is located 29 nucleotides upstream of the S3 Tcf/Lef site. VB5 is located 8 nucleotides upstream of the transcription start site (Fig. 3A). To analyze the contribution of each of these putative VegT-binding sites in the activation of *siamois* promoter by VegT, we have individually mutated the core consensus VegT-binding sequence in the wild-type S01234 promoter (Fig. 3B). The resulting mutant promoter reporters were designated as VB1- (TTACACTT to TTAATCTT), VB2- (TCTCACTC to TCTAGATC), VB3- (TGACACCT to TGAATTCT), VB4- (TGTCACTC to TGTAGATC) and VB5- (TCTCACCT to TCTCGAGT). We then used ectoderm explants to test the activation of these mutant reporters in response to VegT, in comparison with Wnt3a. Our results showed that the *siamois* promoter mutants in the VB1, VB2, VB3 or VB4 site exhibited similar background activation in the ectoderm as the wild-type S01234 promoter, and that they all responded similarly to the induction by Wnt3a and VegT (Fig. 3 C-E). However, mutation of the VB5 site did not reduce the background activation but completely abolished the activation by VegT, but not by Wnt3a (Fig. 3E). These observations indicate that the VB5 site, which is in close proximity of the transcription start site, is indispensable for the activation of *siamois* promoter by VegT, while individual mutation of VB1 to VB4 sites has no effect. However, we cannot exclude the possibility that a combination of these sites may be required to respond to VegT.

VegT binds to siamois *promoter*

To provide further evidence that VegT directly regulates *siamois* expression, we performed ChIP experiments to examine if VegT indeed binds to the *siamois* promoter. One-cell stage embryos were injected in the animal pole region with Flag-tagged *VegT* mRNA (500 pg) and cultured to the early gastrula stage. Chromatin from both uninjected and injected embryos was immunoprecipitated

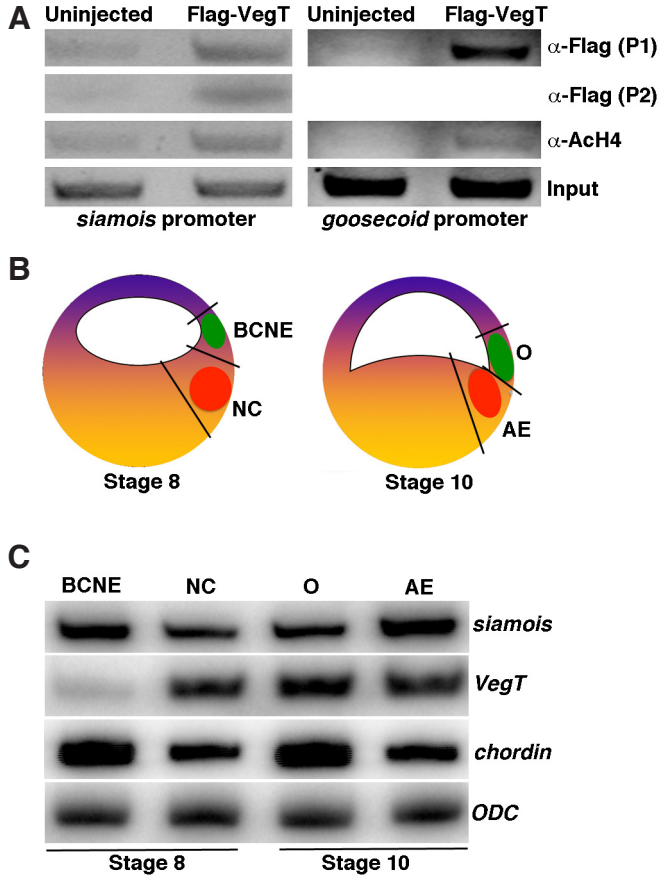

Fig. 4. Binding of VegT to the *siamois* promoter and overlapping expression domains between *VegT* and *siamois* at blastula and gastrula stages. (A) *ChIP assays were performed using early gastrula stage embryos previously injected at the 1-cell stage with Flag-tagged* VegT *mRNA. A representative semi-quantitative PCR result is shown with* siamois *and* goosecoid *promoters. P1 and P2 refer to* siamois *primer pair 1 and 2. PCR amplifications were also performed on chromatin isolated before immunoprecipitation to control the input level.* **(B)** *Schematic representation of the regions dissected at blastula and gastrula stages.* **(C)** *RT-PCR analysis of the expression of* siamois, VegT *and* chordin *in blastula* chordin- *and* noggin-*expressing (BCNE) center and Nieuwkoop center (NC) at the blastula stage, and in the Spemann organizer (O) and anterior endoderm (AE) at the early gastrula stage.* ODC *was a loading control.*

using anti-Flag M2 antibody and analyzed by semi-quantitative PCR. The results showed that, in the presence of VegT, a -500/-168 (relative to transcription start site as +1) *siamois* promoter region containing VB1, VB2 and VB3 sites was amplified using primer pair 1 from chromatin precipitated by anti-Flag M2 antibody (Fig. 4A). A -174/+26 *siamois* promoter region containing VB4 and VB5 sites was also amplified using primer pair 2 (Fig. 4A). As a positive control, we have examined the *goosecoid* promoter, which was shown to be a direct target of VegT (Sudou *et al.,* 2012), to validate the ChIP experiment. Consistent with previous observations, a -390/-167 *goosecoid* promoter region was amplified (Fig. 4A). This indicates that VegT binds to the *siamois* promoter, a region with also an increased acetylated histone H4 mark (Fig. 4A), reflecting an opened state of chromatin in this location (Shogren-Knaak *et al.,* 2006). This result further demonstrates that *siamois* is a direct target of VegT.

The expression of *siamois* is first detected in the dorsal animal region of the blastula *chordin*- and *noggin*-expressing (BCNE) center at the blastula stage, and then in the anterior endoderm (AE) at the early gastrula stage (Kuroda *et al.,* 2004; Rankin *et al.,* 2011; Sudou *et al.,* 2012). To examine if the expression domains of *siamois* and *VegT* overlaps at blastula and gastrula stages, we dissected the regions corresponding to BCNE and Nieuwkoop center (NC) at stage 8, and the Spemann organizer and anterior endoderm at stage 10 (Fig. 4B). The expression of *siamois* and *VegT* was detected in all these regions although *VegT* was only weakly detected in the BCNE at stage 8 (Fig. 4C). This result further supports the possibility that VegT is involved in the regulation of *siamois* expression.

Functional interaction between VegT and Wnt signalling in the activation of siamois *expression*

We have established that *siamois* is a direct target gene of VegT, the question remains how VegT and Wnt signalling interact to regulate *siamois* expression. We thus used the wild-type and different mutant *siamois* promoter reporters to examine this issue. First, low dose of *VegT* mRNA (100 pg) was coinjected with low dose of *Wnt3a* mRNA (0.2 pg). We found that VegT strongly synergized with Wnt3a to activate the wild-type S01234 *siamois* promoter (Fig. 5A). We then tested if VegT also cooperates with different Tcf factors (Tcf1, Tcf3 and Tcf4) to activate *siamois* promoter reporters. Injection of high amount of *Tcf1* mRNA (200 pg) potently activated *siamois* promoter reporter, and injection of *Tcf4* mRNA (200 pg) moderately activated *siamois* promoter reporter, while injection of *Tcf3* mRNA (200 pg) had no effect (Fig. S2). This is consistent with a previous study showing that high dose of Tcf1 increases the expression of Wnt target genes in the ventral region of early gastrula (Standley *et al.,* 2005). When low dose of *Tcf1*, *Tcf3* or *Tcf4* mRNA (50 pg) was coinjected with low dose of *VegT* mRNA (100 pg), we found that VegT cooperated with Tcf1, but not Tcf3 or Tcf4, to activate the wild-type *siamois* promoter reporter (Fig. 5A; Fig. S2). Unexpectedly, VegT also cooperated with Wnt3a or Tcf1 to activate the mutated S24 *siamois* promoter reporter lacking all consensus Tcf/Lef binding sites, which does not respond to Wnt signalling (Fig. 5B). In addition, we also observed a strong synergy between VegT and Wnt3a or Tcf1 in the activation of the VB5- *siamois* promoter reporter, which does not respond to VegT alone (Fig. 5C). This result indicates that VegT functionally interacts with Wnt signalling to activate *siamois* expression, and that the presence of either Tcf/Lef-binding sites or VegT-binding sites may be sufficient for the interaction. We further tested this possibility by deleting the VB2 to VB4 sites, and mutating the VB5 site in the S24 construct (Fig. S3A). In this case, no cooperation between VegT and Tcf1 could be observed (Fig. S3B), indicating that the presence of VB1 site alone is not sufficient to respond to VegT.

To see if the activity of VegT is required for the activation of the *siamois* promoter reporter (S01234) induced by Wnt signalling, we injected at the 2-cell stage in the animal pole region synthetic mRNA (200 pg) corresponding to the dominant negative VegT mutant (VegT-ENR), in which the DNA binding domain of VegT (amino acids 1 to 284) is fused the *Drosophila* Engrailed repressor domain (amino acids 2 to 298), with *Wnt3a* mRNA (2 pg) or *Tcf1* mRNA (200 pg), and assayed luciferase activity in ectoderm explants at the early gastrula stage. The result showed that blocking the activity of VegT strongly inhibited the activation of the *siamois* promoter induced by Wnt3a or Tcf1 (Fig. 5D). This result further suggests that VegT cooperates with Wnt signalling and is required for *siamois* expression. We then performed RT-PCR and *in situ* hybridization analyses to confirm the functional interaction between VegT and Wnt signalling. As observed in *siamois* promoter reporter assays, coinjection of low amounts of *VegT* and *Wnt3a* mRNAs was able to strongly induce *siamois* expression in ectoderm explants (Fig. 6A), indicating a synergy of the two pathways. In addition, the ectopic *siamois* expression induced by Wnt3a or Tcf1 in ectoderm explants was strongly inhibited by the presence of VegT-ENR (Fig. 6A). Conversely, inhibition of Wnt signalling by the dominant negative Tcf3 (dnTcf3), with the N-terminal 87 amino acids of the β-catenin-binding domain deleted, also potently inhibited *siamois* ectopic expression induced by VegT (Fig. S4). This result clearly suggests that VegT and Wnt signalling are mutually required for *siamois* expression.

In the whole embryo, to examine the requirement of both VegT

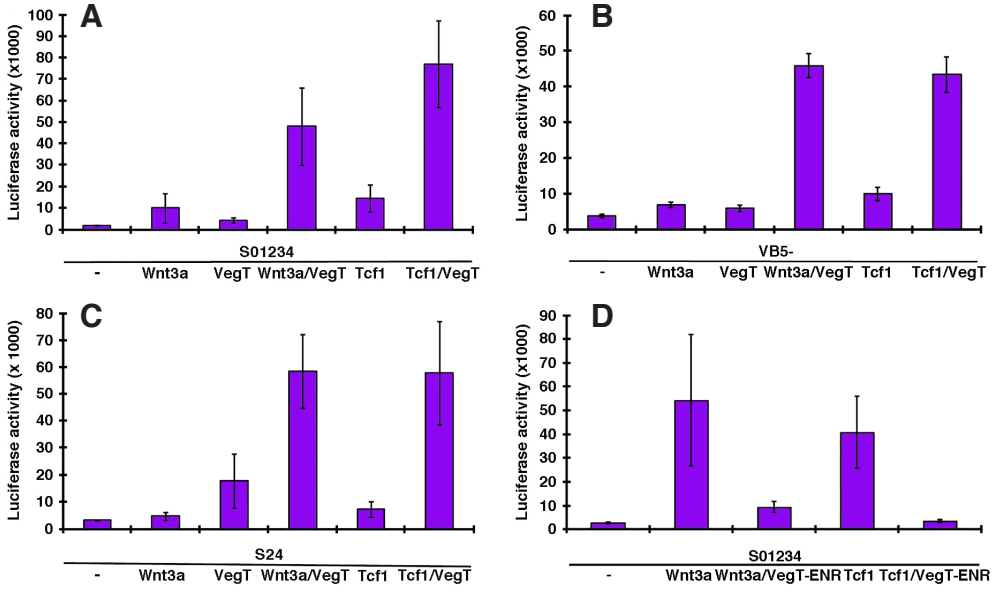

Fig. 5. VegT synergizes with Wnt signalling to activate *siamois* promoter reporter. *Two-cell stage embryos were injected with synthetic mRNA along with wild-type or different mutant* siamois *promoter DNAs, as indicated, in the animal pole region and ectoderm explants were dissected at the early gastrula stage for luciferase assay.* **(A)** *At low dose, VegT synergizes with Wnt3a or Tcf1 to activate luciferase activity driven by the S01234 wild-type* siamois *promoter.* **(B)** *A similar synergy can be observed using the S24 mutant promoter lacking the three canonical Tcf/Lef-binding sites.* **(C)** *VegT cooperates with Wnt3a or Tcf1 to activate the VB5-*siamois *promoter reporter mutated at VB5 site.* **(D)** *The dominant negative VegT mutant blocks activation of the S01234 wild-type* siamois *promoter reporter induced by injection of high dose of* Wnt3a *or* Tcf1 *mRNA. Data represent the mean of triplicate experiments (error bars indicate s.d.).*

and Wnt signalling in the formation of Spemann organizer, we injected *VegT-ENR* mRNA (50 pg), *dnTcf3* mRNA (50 pg) or the same amounts of both *VegT-ENR* and *dnTcf3* mRNAs in the dorsal equatorial region at the 4-cell stage, *in situ* hybridization was first performed to analyze the expression of *chordin* at the early gastrula stage. Single injection of *dnTcf3* or *VegT-ENR* inhibited *chordin* expression in the dorsal region (Fig. 6 B-D). Coinjection of *dnTcf3* and *VegT-ENR* nearly blocked *chordin* expression (Fig. 6E). This result was further confirmed by RT-PCR analysis, which also showed an increase in the expression level of the ventral mesoderm gene *BMP4* in singly or doubly injected early gastrula (Fig. S5). Consistent with these molecular analyses, examination of the phenotypes at the late tail-bud stage indicates that single injection of *VegT-ENR* or *dnTcf3* resulted in head deficiency and shortened trunk and tail regions (Fig. 6 F-H,J). Coinjection of *VegT-ENR* and *dnTcf3* resulted in the absence of head and severely affected trunk and posterior regions (Fig. 6 I,J). These observations suggest that maternal Wnt signalling and VegT cooperate to regulate the formation of Spemann organizer and anterior development by activating the expression of *siamois*. In addition, when RT-PCR analysis was performed at the start of zygotic transcription (stage 8) on whole embryos injected with *VegT-ENR* or *dnTcf3*, or coinjected with *VegT-ENR* and *dnTcf3*, the result indicates that blockade of maternal Wnt signalling and VegT function inhibited *siamois* expression (Fig. S6), indicating that thay are required for initiation of *siamois* expression.

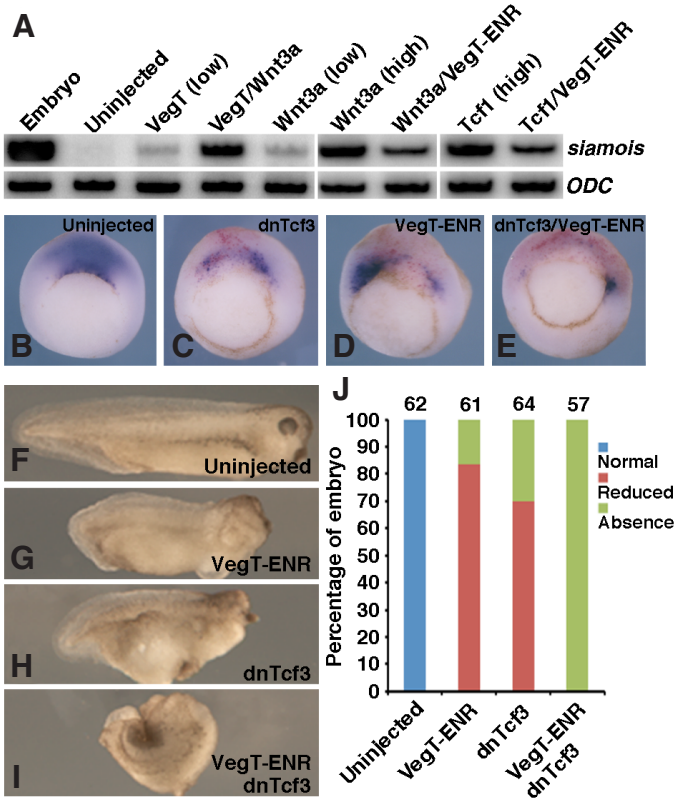

Fig. 6. Functional interaction between VegT and Wnt signalling in regulating Spemann organizer formation. (A) *RT-PCR analysis of* siamois *ectopic expression in ectoderm explants injected with indicated mRNAs. VegT synergizes with Wnt signalling to induce* siamois *expression. It is also required for* siamois *expression activated by Wnt signalling.* ODC *was a loading control.* **(B-E)** In situ *hybridization analysis of* chordin *expression in control* **(B)** *and stage 10.5 early gastrula previously injected with* dnTcf3 **(C)** *or* VegT-ENR **(D)** *mRNA, or coinjected with* dnTcf3 *and* VegT-ENR *mRNAs* **(E)**. β-*gal staining was used to localise the injected region. Coinjection of* dnTcf3 *and* VegT-ENR *more efficiently blocks* chordin *expression than single injection of* dnTcf3 *or* VegT-ENR. **(F-I)** *Phenotypic analysis of control* **(F)** *and embryos injected with* VegT-ENR **(G)**, dnTcf3 **(H)** *or* dnTcf3 *and* VegT-ENR **(I)**, *at the late tail-bud stage.* **(J)** *Statistical analysis of the phenotypes. Numbers on the top of each stacked column indicate total embryos scored from two independent experiments.*

Discussion

In this study, we showed that VegT binds to the *siamois* promoter and directly activates *siamois* expression through putative VegT-binding sites. In addition, it also cooperates with Wnt signalling to regulate *siamois* expression and the formation of Spemann organizer in the *Xenopus* embryo. Our finding suggests that the presence of either Tcf/Lef-binding sites or VegT-binding sites is sufficient for a synergistic interaction between VegT and Wnt signalling.

Direct regulation of siamois *by VegT*

VegT is a maternal mRNA localized vegetally in the early embryos, it is also transcribed zygotically within the equatorial region of the early gastrula (Zhang and King, 1996; Lustig *et al.,* 1996; Stennard *et al.,* 1996; Horb and Thomsen, 1997). Maternal *VegT* has been shown to play an essential role in embryonic axis formation (Zhang *et al.,* 1998; Xanthos *et al.,* 2002). The homeobox gene *siamois* is zygotically expressed in the dorsal region including the Spemann organizer and the prospective anterior endoderm (Lemaire *et al.,* 1995; Kuroda *et al.,* 2004; Rankin *et al.,* 2011; Sudou *et al.,* 2012), and is required for the formation of dorsal axis (Kessler, 1997; Fan and Sokol, 1997). In addition, we showed that the expression domains of *VegT* and *siamois* overlap at the blastula and early gastrula stages. This supports the possibility that VegT may be involved in the regulation of *siamois* expression. Indeed, we have demonstrated that a subset of T domain transcription factors could induce *siamois* ectopic expression in ectoderm cells. In addition, VegT could also activate the *siamois* promoter independently of Tcf/Lef-binding sites.

The consensus sequence TNNCAC(C/T)(T/C) has been shown to be the potential binding site for VegT (Kispert and Hermann, 1993; Conlon *et al.,* 2001), according to this criterion, we have identified 5 putative VegT-binding sites within the 0.4 kb *siamois* promoter region. Individual mutation of any one of the four distal sites (VB1

to VB4) did not affect the response of the *siamois* promoter reporter to VegT. By contrast, mutation of the proximal VB5 site located at close proximity of the transcription start site indicates that it is indispensable for the activation of *siamois* promoter by VegT, but not by Wnt signalling. However, this result does not allow us to conclude whether VB5 site is sufficient for VegT-induced *siamois* transcription, or whether a combination of other sites may be also necessary for binding of VegT to the *siamois* promoter. It remains possible that the presence of VB5 site alone may not be sufficient and that the presence of multiple sites may be required for VegT to activate *siamois* transcription. Consistent with this proposal, our ChIP assay showed that VegT was able to bind to the regions including VB1, VB2 and VB3 sites, as well as VB4 and VB5 sites.

We have also observed that different T domain proteins exhibit distinct activity to activate *siamois* expression although they were shown to bind essentially to the same core consensus sequence (Kispert and Hermann, 1993; Conlon *et al.,* 2001). In particular, VegT, but not Xbra, activates *siamois* ectopic expression in ectoderm cells. There are at least two possibilities, which could explain this difference. First, Xbra usually binds to palindromic sequence, with two core sequences and without intervening nucleotides, which is not the case in the *siamois* promoter. However, VegT can interact with a single core sequence (Conlon *et al.,* 2001). Second, the functional difference between Xbra and VegT may depend on their T domains. There is evidence showing that the lysine residue (K) at position 149 of Xbra and the asparagine residue (N) at the corresponding position of VegT may play a differential role in the inductive activity for Xbra and VegT (Conlon *et al.,* 2001). In addition, the specificity of T domain proteins could be conferred by the N-terminal region, which interacts with different protein partners to elicit a specific response (Messenger *et al.,* 2005). Thus, these differences may largely account for their differential activity to induce *siamois* expression.

Interaction between VegT and Wnt signalling

Another finding made in this work concerns the mode of interaction between Wnt signalling and VegT in the activation of the *siamois* promoter. Since both Tcf/Lef-binding sites and VegT-binding sites are present in the *siamois* promoter, it can be expected that the two pathways functionally interact to regulate the transcription of *siamois*. We found that, in reporter assays, they indeed functionally interact and act synergistically to activate *siamois* promoter reporter. However, it is surprising that VegT and Wnt signalling also synergize to activate mutant promoters lacking either all consensus Tcf/Lef-binding sites or the VB5 site, which is required for VegT-induced *siamois* transcription in the absence of Wnt signalling. Promoter deletion analysis indicated that the absence of all the consensus Tcf/Lef-binding sites and the four distal VegT-binding sites completely abolished the activation of *siamois* promoter reporter in the dorsal region of the early gastrula (Brannon *et al.,* 1997). Consistent with this observation, our *in vitro* analysis indicated that removal of the all the consensus Tcf/Lef-binding sites and deletion or mutation of VB2 to VB5 sites abolished the interaction between VegT and Tcf1 to activate *siamois* promoter in ectoderm cells. Further analysis through simultaneous mutation of all VegT-binding sites should help to clarify their implication in VegT response of *siamois* promoter.

The functional interaction between VegT and Wnt signalling raises the possibility that the two pathways may physically interact, and this is supported by a previous study showing that VegT binds directly to Tcf3 (Cao *et al.,* 2007). A physical interaction between VegT and T cell factors could explain why dominant negative VegT mutant blocks *siamois* expression induced by Wnt signalling, and vice versa. Nevertheless, our reporter assay using the wild-type *siamois* promoter did not reveal a functional interaction between VegT and Tcf3 or Tcf4. We also failed to detect a direct interaction between VegT and Tcf1 by coimmunoprecipitation (data not shown). Thus, it is still unclear how VegT and Tcf1 synergize to activate *siamois* expression. It remains possible that there is weak and/or indirect interaction between VegT and Tcf1, this could be sufficient for their functional cooperation on the *siamois* regulatory region.

The implication of VegT in the formation of Spemann organizer has been well documented. Depletion of maternal VegT strongly reduces the transcription of a panel genes expressed in the Spemann organizer, including *siamois*. Simultaneous depletion of VegT and β-catenin further reduces *siamois* expression before and during gastrulation (Xanthos *et al.,* 2002). This suggests that VegT has the potential to regulate *siamois* expression. Our present study thus provides a molecular mechanism underlying the regulation of *siamois* by VegT and Wnt signalling in the formation of Spemann organizer. It also helps to understand the mode of interaction between T domain proteins and Wnt signalling.

Materials and Methods

Embryos and ectoderm explants

Xenopus eggs were obtained from females injected with 500 IU of human chorionic gonadotropin (Sigma) and artificially fertilized. Eggs were dejellied with 2% cysteine hydrochloride (pH 7.8) and kept in 0.1x modified Barth solution (MBS) to appropriate stages. Microinjections of embryos were done in 0.1x MBS containing 3% Ficoll-400. Dissection of ectoderm explants was performed in 1x MBS and cultured in this solution until appropriate stages. The identification of Tbx6 target genes using hormone-inducible version of Tbx6VP16 was performed as previous described (Li *et al.,* 2006). *LacZ* mRNA (500 pg) was injected as a cell lineage tracer and β-galactosidase (β-gal) staining was performed using red-gal as a substrate (Li *et al.,* 2010).

Plasmid constructs and site-directed mutagenesis of the siamois *promoter*

The *siamois* reporter constructs (S01234 and S24), *Xenopus Wnt3a*, *Xbra*, *Tbx6*, *Tbx6VP16-GR*, Flag-tagged *VegT* (*Flag-VegT*), dominant negative *VegT* (*VegT-ENR*), dominant negative *Tcf3* (*dnTcf3*) and Cerberus-short (*cer-S*) were described previously (Brannon *et al.,* 1997; Piccolo *et al.,* 1999; Shi *et al.,* 2002; Li *et al.,* 2006). The full-length zebrafish *Tbx16* coding sequence was PCR-amplified from embryonic mRNA according to the sequence in the database (accession number NM_131058) and cloned into pCS2 vector. Myc-tagged *Xenopus Tcf1*, *Tcf3* and *Tcf4* coding sequences were cloned into pCS2MT vector inframe with the six myc epitopes. Mutations of the core consensus VegT-binding (VB) sites in the *siamois* promoter (S01234) were performed using QickChange XL site-directed mutagenesis kit (Stratagene) according to manufacture's instructions. The mismatched oligonucleotides used to mutate each VB site are as follow: VB1, 5'-GAATAAATAATACAAGCTTAAGTAAGGGCTTTG-3'; VB2, 5'- CTTGCCAACTTCTCTAGATCAGTCCTTCA-3'; VB3, 5'-GCTGCCAAGTTAGAATTCATATGGGACAG-3'; VB4, 5'- GCATATGTATAAAACATCTAGACTGACCAATAACC-3'; VB5, 5'- CTCCCAGGACAGAGCTCACCAACATTTTGG-3'. Mutated nucleotides in the core consensus VB sites are underlined. The S4VB1 *siamois* reporter construct was obtained by deletion in the S24 construct of an internal *Eco* RI fragment comprising the 3 consensus Tcf/Lef-binding sites and VB2 to VB4 sites, followed by mutation of the VB5 site. All constructs were sequenced before use.

Luciferase assay

The reporter plasmids (200 pg) were either injected alone or coinjected with different synthetic mRNAs in the animal pole region at the 2-cell stage. Cell lysates were prepared from 10 ectoderm explants dissected at the early gastrula stage and luciferase assay was performed using the luciferase assay system (Promega). The luciferase activity was measured using a Lumat LB 9507 luminometer (Berthold). The experiments were carried out in triplicate using different batches of embryos and the data were analyzed using Student's *t*-test.

RT-PCR and* in situ *hybridization

Extraction of total RNA from whole embryos or ectoderm explants was performed using guanidine isothiocyanate/phenol followed by LiCl precipitation to remove genomic DNA and polysaccharides. RNA samples were further treated with RNAse-free DNAse I (Roche) and were reverse-transcribed using 200 units M-MLV reverse transcriptase (Invitrogen). PCR primers for *siamois, goosecoid*, *chordin*, *BMP4*, *Wnt8, Sox17*α *Xnr1*, *Xnr2*, *Xnr3*, *Xnr4*, *Xnr6* and *ODC (ornithine decarboxylase*) were as described previously (Umbhauer *et al.,* 2000; Shi *et al.,* 2002; Li *et al.,* 2006, 2013). One-twentieth of the reverse-transcribed cDNA was used for PCR amplification in a reaction mixture containing 1 μCi of [α-^{32}P]dCTP (GE Healthcare). PCR products were resolved on a 5% non-denaturing polyacrylamide gel and visualized by a Phospho-Imager (BioRad). For some experiments, unlabelled PCR products were resolved on a 1.5% agarose gel. Whole-mount *in situ* hybridization was performed according to standard protocol (Harland, 1991). The probe was labelled using digoxigenin-11-UTP and appropriate RNA polymerase (Roche).

Chromatin immunoprecipitation (ChIP)

ChIP assay was performed as described (Li *et al.,* 2013), with minor modifications. One-cell stage embryos were injected in the animal pole region with 500 pg Flag-tagged *VegT* mRNA, 20 uninjected or injected embryos at the early gastrula stage were homogenized in 1 ml lysis buffer (2.2 M sucrose, 3 mM $CaCl_2$, 10 mM Tris-HCl, pH 7.5, 0.5% Triton X-100, 1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml pepstatin) and centrifuged at 53,000 rpm for 3 hours at 4°C using a TL-100 ultracentrifuge with a fixed-angle rotor (Beckman). The nuclei pellet was resuspended in 360 μl of nuclear isolation buffer (0.25 M sucrose, 10 mM Tris-HCl, pH 7.5, 3 mM $CaCl_2$, 1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml pepstatin). Proteins were crosslinked to DNA by adding formaldehyde (37%) directly to the nuclear suspension to a final concentration of 1% and incubated for 10 minutes on ice and then 20 minutes at room temperature. The lysate was sonicated 16 times by using a sonicator set to 20% low amplitude to reduce DNA length between 200 to 800 bp. Sonicated chromatin (25 μg) was incubated with 10 μg of anti-Flag M2 antibody (Sigma) or 8 μg of anti-acetylated histone H4 (AcH4) antibody (Millipore). ChIP products were analyzed by PCR using specific primers for *siamois* and *goosecoid* promoters (*siamois* primer pair 1: 5′-CATCCTTGGCCTGTTCTAAG-3′ and 5′-GGCAAGGTCAAAGATGT-GAT-3′; *siamois* primer pair 2: 5′-CCTTGCCAATTCTTTCAGGG-3′ and 5′-CCTCATAGGTCATCTCTGTC-3′; *goosecoid*: 5′-CCAGAGAAACAAAA-CAGTCATTCC-3′ and 5′-GCAGACTCTCCCTGTAGTTATTCAC-3′). The experiments were performed in triplicate.

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