

RESEARCH ARTICLE

Tissue- and stage-specific Wnt target gene expression is controlled subsequent to β -catenin recruitment to cis-regulatory modules

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ABSTRACT

Key signalling pathways, such as canonical Wnt/ β -catenin signalling, operate repeatedly to regulate tissue- and stage-specific transcriptional responses during development. Although recruitment of nuclear β -catenin to target genomic loci serves as the hallmark of canonical Wnt signalling, mechanisms controlling stage- or tissue-specific transcriptional responses remain elusive. Here, a direct comparison of genome-wide occupancy of β -catenin with a stage-matched Wnt-regulated transcriptome reveals that only a subset of β -catenin-bound genomic loci are transcriptionally regulated by Wnt signalling. We demonstrate that Wnt signalling regulates β -catenin binding to Wnt target genes not only when they are transcriptionally regulated, but also in contexts in which their transcription remains unaffected. The transcriptional response to Wnt signalling depends on additional mechanisms, such as BMP or FGF signalling for the particular genes we investigated, which do not influence β -catenin recruitment. Our findings suggest a more general paradigm for Wnt-regulated transcriptional mechanisms, which is relevant for tissue-specific functions of Wnt/ β -catenin signalling in embryonic development but also for stem cell-mediated homeostasis and cancer. Chromatin association of β -catenin, even to functional Wnt-response elements, can no longer be considered a proxy for identifying transcriptionally Wnt-regulated genes. Context-dependent mechanisms are crucial for transcriptional activation of Wnt/ β -catenin target genes subsequent to β -catenin recruitment. Our conclusions therefore also imply that Wnt-regulated β -catenin binding in one context can mark Wnt-regulated transcriptional target genes for different contexts.

KEY WORDS: Wnt signalling, β -catenin, *Xenopus*, Gastrula, ChIP-seq, RNA-seq

INTRODUCTION

Key signalling mechanisms are deployed repeatedly during embryonic development to regulate differential gene expression, often in combination with each other and with other regulatory mechanisms. Wnt/ β -catenin signalling (hereafter referred to as Wnt signalling) is an important, evolutionarily conserved cell-to-cell signalling mechanism that regulates the transcription of specific

target genes (reviewed by Cadigan and Waterman, 2012; Hoppler and Nakamura, 2014). Wnt signalling operates repeatedly during embryogenesis, in stem cell-mediated homeostasis and in cancer (reviewed by Hoppler and Moon, 2014; Nusse et al., 2012). The textbook view asserts that activation of the canonical Wnt signalling pathway causes β -catenin stabilisation and nuclear localisation, where β -catenin associates with TCF/LEF transcription factors bound to so-called Wnt-response DNA regulatory elements (WREs) to activate the transcription of nearby Wnt target genes (reviewed by Nusse, 2012). Recruitment of nuclear β -catenin to target chromatin regions is therefore thought to be the critical step for Wnt-regulated target gene regulation. However, the developmental, cellular and transcriptional responses to Wnt signalling are often remarkably specific for particular stages, tissues and cell lineages, and the molecular mechanisms by which the specific Wnt/ β -catenin target genes are regulated in different cellular and developmental contexts are still largely unknown. Characterising these context-specific mechanisms is therefore important for understanding the specific functional roles of Wnt signalling in embryonic development and disease.

Early embryos represent ideal experimental models for studying the fundamental molecular mechanisms by which Wnt signalling regulates such context-specific responses, since there are rapid and fundamental changes in the cellular and developmental response to Wnt signalling (reviewed by Zylkiewicz et al., 2014). This is particularly prominent in the early *Xenopus* embryo (Fig. S1): maternally activated Wnt signalling before the general onset of zygotic transcription at the mid-blastula transition (MBT) (Newport and Kirschner, 1982) regulates specific genes that then function to establish dorsal development (e.g. Funayama et al., 1995; Heasman et al., 2000; McMahon and Moon, 1989); but, only shortly thereafter, early zygotic Wnt signalling promotes ventral development (Christian and Moon, 1993; Hoppler et al., 1996); yet, both are mediated by the β -catenin-dependent pathway (Hamilton et al., 2001). This radical change in the stage-specific response to Wnt signalling makes *Xenopus* embryos a unique model for dissecting the molecular mechanisms that determine context-specific responses to Wnt signalling. Direct target genes of maternally activated Wnt signalling have been described (e.g. Blythe et al., 2010; Brannon et al., 1997; Crease et al., 1998; Laurent et al., 1997); however, genes specifically regulated by early zygotic Wnt signalling are much less well understood. Identifying such direct Wnt target genes would not only be informative concerning the gene regulatory network that operates in the ventrolateral prospective mesoderm, but also more generally concerning the fundamental molecular mechanisms of context-specific Wnt target gene regulation.

Here, we report genome-wide identification of such stage-specific Wnt target genes through β -catenin chromatin immunoprecipitation followed by high-throughput sequencing (ChIP-seq) combined with RNA sequencing (RNA-seq) analysis of the relevant Wnt-regulated

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transcriptome. Although the early *Xenopus* embryo shows β -catenin occupancy at many genomic loci, our analysis reveals that transcriptional expression is Wnt regulated at only a subset of these loci. Thus, Wnt-regulated β -catenin recruitment to gene loci is required, but not sufficient, for Wnt target gene expression. We find instead that the tissue- and stage-specific context can regulate Wnt target gene expression subsequent to β -catenin recruitment to cis-regulatory modules at these loci.

RESULTS

Genome-wide mapping of β -catenin association in *Xenopus* early gastrulae

Nuclear localisation of β -catenin is the hallmark of canonical Wnt signalling (Schneider et al., 1996; Schöhl and Fagotto, 2002). In the nucleus, β -catenin regulates target gene expression in association with DNA-binding proteins, particularly those of the TCF/LEF family (reviewed by Cadigan and Waterman, 2012; Hoppler and Waterman, 2014). β -catenin ChIP-seq analysis had been used to identify direct transcriptional targets of Wnt signalling in cancer tissue and cultured cells (Bottomly et al., 2010; Park et al., 2012; Schuijers et al., 2014; Watanabe et al., 2014). We therefore reasoned that β -catenin ChIP-seq analysis in intact gastrula stage *Xenopus tropicalis* embryos would identify early gastrula-specific Wnt target genes.

We developed a reliable β -catenin ChIP protocol for analysis at the early gastrula stage (stage 10.25, Fig. 1A, Fig. S2) by optimising first chromatin shearing conditions for fragments of ~ 200 bp (Fig. S2A), then the immunoprecipitation of chromatin-associated β -catenin protein with two different β -catenin antibodies, as well as IgG as a negative control (see Materials and Methods). Specific binding of β -catenin by the antibodies was validated by western blotting and also by β -catenin ChIP-qPCR (Fig. S2B–D). In the ChIP-qPCR validation, we analysed known WREs in genes known to be Wnt regulated at this stage [*hoxd1* (Janssens et al., 2010) and *msgn1* (Wang et al., 2007)] as positive controls, and genomic regions not containing WREs (from *odc1* and *hoxd1*) as negative controls. ChIP DNA samples and input control DNA samples were each pooled from three validated ChIP experiments and sequenced.

Clear β -catenin ChIP-seq peaks (hereafter referred to as β -peaks) were found at known direct Wnt target loci in the *X. tropicalis* genome [e.g. the *hoxd1* locus (Janssens et al., 2010), Fig. 1B]. The β -catenin ChIP-seq also confirmed no β -catenin association at the negative control *odc1* locus (data not shown). Two independent peak-calling algorithms followed by stringent irreproducible discovery rate (IDR) analysis (Li et al., 2011) identified 10,638 reproducible β -peaks across the *X. tropicalis* genome (Fig. 1C),

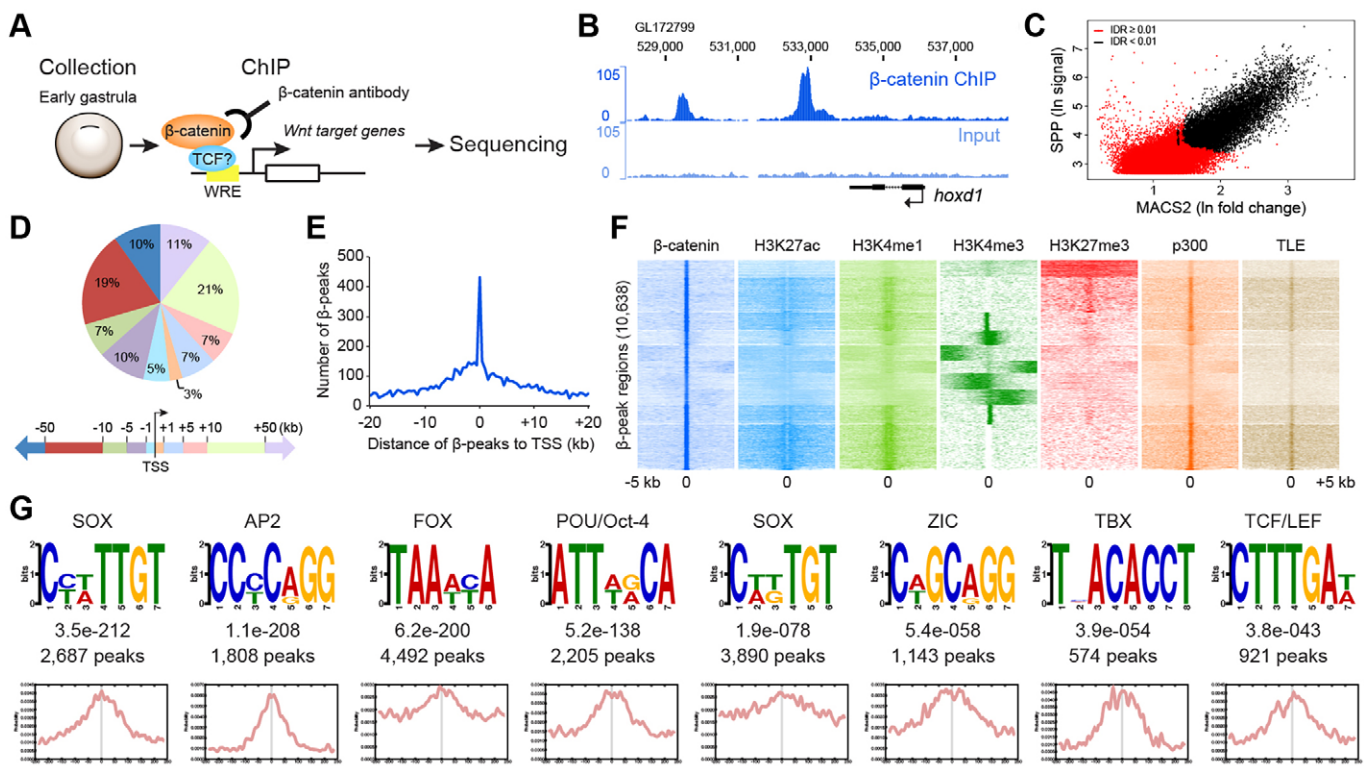


Fig. 1. β -catenin ChIP-seq analysis of *Xenopus* early gastrulae. (A) Experimental design of β -catenin ChIP-seq analysis. Early gastrulae were collected and fixed. Following chromatin shearing, β -catenin antibodies were used to selectively precipitate DNA fragments bound by β -catenin-containing protein complexes. Subsequently, the precipitated DNA fragments were sequenced. (B) Genome view of example β -catenin target gene *hoxd1*. Note the clear β -catenin ChIP-seq peaks (β -peaks) downstream (to the left) of the *hoxd1* locus. (C) Scatter plot combining peak calling analysis by SPP [considering signal strength, applying false discovery rate (FDR) ≤ 0.1] and MACS2 (considering fold change, applying $P \leq 0.01$) software, with black dots indicating 10,638 β -peaks reproducibly called [applying an irreproducible discovery rate (IDR) ≤ 0.01]. (D,E) β -peaks are associated with sequences throughout the genome (D) but enriched close to and just upstream (putative promoter) of the transcriptional start site (TSS) of nearby genes (E; analysed in 500 bp bins). Pie chart (D) shows the percentage of β -peaks according to their location relative to TSS (within 1 kb, 1–5 kb, 5–10 kb, 10–50 kb, over 50 kb upstream or downstream of TSS). (F) Heat map illustrating genome-wide association of β -peaks with histone modifications and transcription co-factor binding sites indicative of cis-regulatory modules (CRMs; such as promoters and enhancers) in patterns that can be clustered into ten groups. Each horizontal line represents the 5 kb downstream and upstream regions of ChIP-seq data around a β -peak. (G) Enriched motifs from *de novo* motif search of sequences under β -peaks. Note the identification of consensus TCF/LEF binding but also other known transcription factor binding motifs. Statistical significance (e-values) and the number of β -peaks are indicated below each motif logo. The analysis of motif distribution shows central enrichment of motifs within β -peak regions (500 bp window).

which can be assigned to 5193 genes (Table S1). β -peaks are widely distributed throughout the genome, close to and further away from the transcriptional start site (TSS) of annotated genes (Fig. 1D), but we find an enrichment close to and just 5' of the TSS of genes (Fig. 1E) and also a genome-wide correlation with putative cis-regulatory sequences, such as promoters, enhancers or silencers, which are collectively referred to here as cis-regulatory modules (CRMs) [Fig. 1F; data for H3K4me3 and H3K27me3 from Akkers et al. (2009), representing active promoters and inactive chromatin states, respectively; data for H3K27ac and H3K4me1 (both indicating active enhancers), for the transcriptional co-activator p300 and for the transcriptional co-repressor Transducin-like enhancer of split (TLE; also known as Groucho in *Drosophila*) from Yasuoka et al. (2014)]. For instance, correlation of β -catenin with p300-associated and with TLE-associated sites was 47.4% and 86.4%, respectively. We sought to detect enriched DNA sequences shared among the identified β -peaks by performing a *de novo* motif search on all β -peaks (Fig. 1G). As expected, consensus TCF/LEF core binding sequences were identified. Additionally, other known transcription factor binding motifs were found, some of which had also been identified in previous β -catenin ChIP-seq studies (Schuijers et al., 2014; Zhang et al., 2013) (see Discussion).

RNA-seq analysis of the *wnt8a*-regulated transcriptome

Independently, we performed transcriptome analysis using RNA-seq in order to identify Wnt-regulated transcripts at the early gastrula stage. Early zygotic Wnt signalling is activated in prospective ventral mesoderm by *wnt8a*, which is the predominant Wnt gene expressed during later blastula stages (Christian et al., 1991; Collart et al., 2014). We developed an experimental design that allowed us to identify genes regulated by Wnt8a signalling (*wnt8a*-regulated genes, Fig. 2A). We compared the mRNA expression profile a few hours after the onset of zygotic transcription at early gastrula (stage 10.25) in two control conditions with that in two experimental conditions: embryos in which endogenous *wnt8a* was knocked down with a previously validated antisense morpholino oligonucleotide (MO) (Rana et al., 2006); and the same *wnt8a* knockdown embryos but with experimentally reinstated expression with an MO-insensitive, Wnt8a-expressing DNA construct (Fig. S3A).

We initially optimised the experimental conditions so that the *wnt8a* knockdown not only consistently caused the well-established *wnt8a* loss-of-function morphological phenotype (Hoppler et al., 1996; Rana et al., 2006), but also is then substantially rescued to normal embryonic morphology by our experimentally targeted reinstatement of stage-specific Wnt8a expression (Fig. 2A) (Christian and Moon, 1993). We confirmed that the morphological changes caused by the knockdown and reinstatement of Wnt8a expression are accompanied by predicted changes in the expression of previously reported *wnt8a*-regulated genes (Fig. S3B,C). In addition, unaltered gene expression levels of the well-known maternal Wnt target gene *siamois* (*sia1*) (Fig. S3C) confirmed that our experimental manipulation at cleavage stages (MO and DNA microinjection) does not affect early gene regulatory and dorsal axis establishment processes controlled by maternal Wnt signalling (see below).

Statistical analysis of the RNA-seq results, applying a generalised linear model (GLM) (Anders and Huber, 2010), identified an initial longlist of 274 genes potentially positively regulated and 193 genes potentially negatively regulated by *wnt8a* (Fig. 2B, see Table S2). As expected, this list includes previously identified Wnt-regulated genes, such as *axin2/xarp* (Hufton et al., 2006), *hoxd1* (Janssens

et al., 2010), *sp5* (Weidinger et al., 2005) and *ventx1* (Hoppler and Moon, 1998). However, also included are genes with relatively subtle changes in gene expression, which might not be physiologically relevant for embryonic development. In order to create a more manageable shortlist for further detailed analysis we decided to focus on genes that were significantly affected by both knockdown and reinstatement of Wnt8a expression (Fig. 2B). This resulted in a shortlist of 14 high-confidence *wnt8a* positively regulated genes, which have reduced expression in *wnt8a* knockdown and are increased upon Wnt8a reinstatement. This included two uncharacterised genes (*ENSXETG00000010483* and *ENSXETG00000030701*), which showed strong sequence similarity to each other and resembled *Xenopus laevis* *marginal coil* (*xmc*, Fig. S4). We therefore named *ENSXETG00000010483* *xmc-like 1* (*xmcl1*) and *ENSXETG00000030701* *xmc-like 2* (*xmcl2*). Applying the same restrictive criteria for shortlisting suggested only one gene, *apt12a*, to be negatively regulated by Wnt8a signalling (Fig. 2B).

All 14 *wnt8a* positively regulated genes were successfully validated (Fig. 2C). They were all shown to be expressed at the early gastrula stage when assayed by quantitative reverse transcription PCR (RT-qPCR) and, as expected, their expression was dependent on *wnt8a* function, although clearly to different degrees. However, the one gene that was apparently negatively regulated could not be validated. Therefore, consistent with the expected major role of Wnt signalling, we find that Wnt8a signalling mainly positively controls gene expression in early gastrula embryos and we proceeded to focus on *wnt8a* positively regulated genes. Expression of ten of the *wnt8a* positively regulated genes was detectable by whole-mount RNA *in situ* hybridisation in a pattern consistent with the expected signalling range of *wnt8a*-expressing cells mostly in the ventral and lateral prospective mesoderm, and, additionally, this expression was confirmed to be dependent on *wnt8a* function, but again clearly to varying degrees (Fig. 2D).

Identification of direct *wnt8a* target gene loci

By combining the β -catenin ChIP-seq and the *wnt8a*-regulated transcriptome datasets, we identified 13 from our shortlist of 14 and 179 from our longlist of 274 *wnt8a*-regulated genes among the 5193 genes associated with β -peaks (Fig. 3A; see also examples in Fig. S5 and Table S3). By definition, we considered these 13 and 179 genes as our shortlist and longlist of direct Wnt8a/ β -catenin target genes, respectively (Table S3).

We performed ChIP-qPCR analysis to examine whether Wnt8a signalling, as expected, controls β -catenin recruitment to the CRMs of Wnt8a/ β -catenin targets. Knockdown of endogenous *wnt8a* resulted in reduction of β -catenin binding compared with the control, confirming that β -catenin association with these 13 shortlisted *wnt8a* target gene loci was dependent on *wnt8a* function (Fig. 3B). To assess the transcriptional activity of the β -peaks, we selected five β -peak elements from proximal regions just upstream of the TSS and seven from more distant regions, and tested them in luciferase reporter assays (Fig. 3C). All β -peak sequences from proximal regions strongly induced expression of the luciferase reporter (greater than 10-fold compared with a control vector), and four out of the seven distant β -peak sequences activated a heterologous basal promoter driving luciferase expression with weaker activity. Taken together, these results support the conclusion that the identified β -peak genomic regions control β -catenin-mediated transcription in response to Wnt8a signalling.

Approximately one-third of apparently *wnt8a*-regulated genes were devoid of any identifiable associated β -peak. These 94 genes

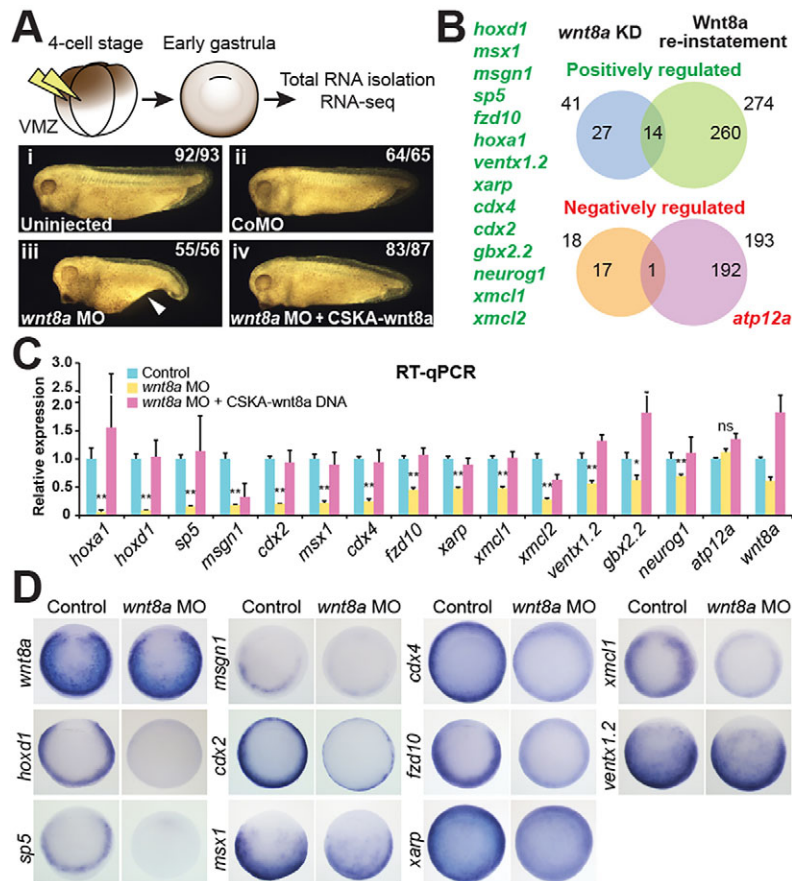


Fig. 2. RNA-seq analysis to identify *wnt8a*-regulated genes. (A) Experimental design to identify *wnt8a*-regulated genes. *wnt8a* MO and a standard control MO (CoMO) were microinjected into the ventral marginal zone (VMZ) of four-cell stage embryos (prospective endogenous *wnt8a*-expressing and ventral mesoderm tissue). For the reinstatement experiment, *wnt8a* MO was co-injected together with a DNA construct driving exogenous *Wnt8a* (CSKA-*wnt8a*) in the same tissue. Three biological replicates per experimental sample were sequenced. The experimental conditions were optimised by comparing the morphology of (i) uninjected embryos with the embryos injected with (ii) CoMO, (iii) *wnt8a* MO or (iv) *wnt8a* MO plus CSKA-*wnt8a* DNA, as well as expected changes in expression of candidate genes (Fig. S3). (B) Venn diagrams of genes that are positively (top) or negatively (bottom) regulated by *Wnt8a* signalling identified by generalised linear model (GLM) statistical analysis (FDR<0.1; see the supplementary Materials and Methods) of RNA-seq results. Forty-one genes were identified with reduced expression in the *wnt8a* knockdown (KD; blue, compared with uninjected and CoMO-injected controls) and 274 genes with increased expression when *Wnt8a* expression was reinstated (green, compared with *wnt8a* knockdown). A shortlist of 14 *wnt8a* positively regulated genes (listed on the left) was selected for further analysis by the overlap between these two groups of genes (see Table S2 for full gene lists). Eighteen genes with increased expression were identified in the *wnt8a* knockdown (amber) and 193 genes with reduced expression when *Wnt8a* expression was reinstated (purple), with one gene (*atp12a*) in the overlap and therefore apparently negatively regulated by *wnt8a*. (C) Validation of RNA-seq-discovered candidate genes by RT-qPCR. Transcripts collected from embryos microinjected into all four blastomeres with *wnt8a* MO, or with *wnt8a* MO co-injected with CSKA-*wnt8a* DNA, were compared with control (CoMO injected). All 14 positively *wnt8a*-regulated candidate genes of the shortlist were confirmed; but not *atp12a*, which had been suggested to be negatively regulated. Note the varying extent of dependence on *wnt8a* function for the different genes. * $P<0.1$, ** $P<0.05$; ns, not significant ($P\geq 0.1$); two-tailed Student's *t*-test. Error bars represent s.d. of two biological replicates. (D) Vegetal view of early gastrulae (with dorsal up) of control (uninjected) and *wnt8a* MO-injected embryos. Note the expression of *wnt8a*-regulated genes in a similar, but not always identical pattern, as *wnt8a*. Also note the reduced expression to varying extents in *wnt8a* MO-injected embryos.

were found in the *Wnt8a* reinstatement condition and might therefore be expressed due to *Wnt8a* overexpression. They might represent genes indirectly regulated by *Wnt*/ β -catenin signalling or by β -catenin-independent *Wnt* signalling mechanisms, but we did not analyse them further in the current study. Because *Wnt8a* signalling in the prospective ventral mesoderm is mediated by the β -catenin-dependent pathway (Hamilton et al., 2001), we instead investigated two classes of β -catenin-associated genes: the direct *Wnt8a*/ β -catenin target genes described above (i.e. the 13 and 179 genes of the shortlist and longlist, respectively) and 5009 β -catenin-bound but non-*wnt8a*-regulated genes (see also examples in Fig. S5E,F). We anticipated that comparing these two classes of genes would provide additional insight into how *Wnt*/ β -catenin target genes are regulated.

First, we performed gene ontology (GO) analysis to identify whether these different classes are predicted to function in different biological processes (Fig. 3D). Our analysis showed, however, that the different classes are enriched for similar developmental processes, such as mesoderm development, and also that they both mainly contain genes encoding transcription factors, such as homeobox genes. Despite these similarities, they show some differences (compare purple and amber with red bars in Fig. 3D); in particular, the non-*wnt8a*-regulated genes show an even higher association with metabolic and later developmental processes (e.g. muscle, neural and non-neural ectoderm development) (see Discussion).

Next, in order to identify context-specific *Wnt* signalling mechanisms, we characterised the genomic sequences under the β -peaks of *Wnt8a*/ β -catenin target genes when compared with

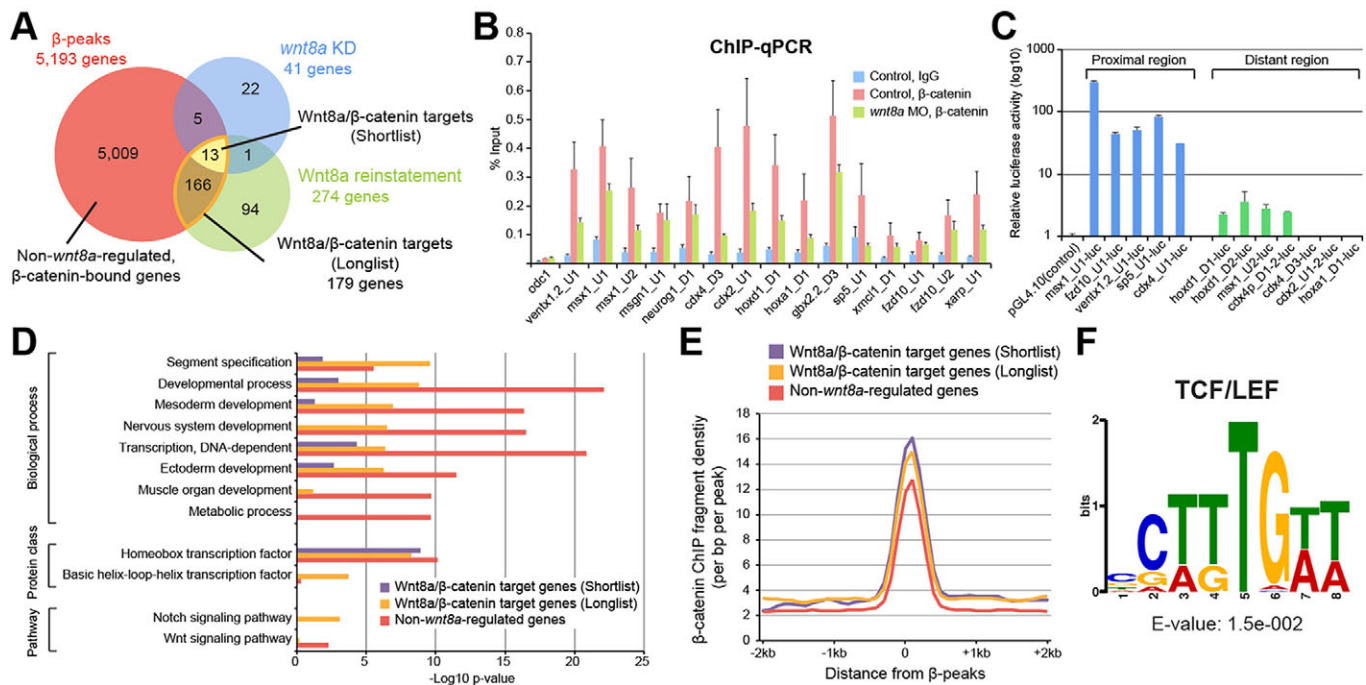


Fig. 3. Integrating β -catenin ChIP-seq and RNA-seq analysis to identify direct Wnt8a/ β -catenin target genes. (A) Venn diagram illustrating overlap between genes near β -peaks (red) and the *wnt8a* positively regulated genes (as in Fig. 2B). Note that from among the longlist of 274 potential *wnt8a*-regulated genes, 179 are associated with identified β -peaks (amber border around lens-shaped area), representing the longlist of probable direct Wnt8a/ β -catenin target genes. Also note that all but one (*xmcl2*) of the validated shortlist of *wnt8a* positively regulated genes are among these and therefore represent the shortlist of 13 direct Wnt8a/ β -catenin target genes (yellow). Also note that the majority of gene loci near β -peaks are not correlated with *wnt8a*-regulated genes and, conversely, that more than one-third of *wnt8a*-regulated genes in the longlist are not associated with identified β -peaks (most likely representing indirect *wnt8a* targets). (B) β -catenin ChIP-qPCR of identified β -peaks of our shortlist in chromatin extracted from control (uninjected) and *wnt8a* MO-injected embryos. Note that β -catenin association is reduced in the *wnt8a* loss-of-function experiment for most of the 15 β -peaks analysed. IgG antibodies were used as control. Error bars represent s.e.m. of three to five biological replicates. (C) Luciferase assays of reporter constructs containing sequences near identified β -peaks of *wnt8a*-regulated genes. Error bars represent s.d. of three biological replicates. (D) GO analysis suggests that β -peak-associated genes tend to encode transcription factors and also cell-to-cell signalling components, and to function in developmental processes, with different emphasis between *wnt8a*-regulated (purple) and non-regulated genes (red). (E) DNA occupancy level of β -catenin around the peak summit shows higher enrichment in direct Wnt8a/ β -catenin target gene loci [shortlist (purple) and longlist (amber)] compared with non-*wnt8a*-regulated genes (red). Read density was analysed using HOMER (bin size 100 bp). (F) TCF/LEF consensus motif is enriched under all 58 β -peaks associated with all 13 shortlisted Wnt8a/ β -catenin target genes.

β -catenin-bound but non-*wnt8a*-regulated genes. As shown above, all β -peaks were generally found to be enriched around TSSs (Fig. 1E); however, compared with non-*wnt8a*-regulated genes (10.1%), we found that *wnt8a*-regulated genes (30.7%) and particularly our shortlist (53.8%) exhibited an even higher enrichment of β -peaks within 1 kb regions upstream of the TSS. In addition, Wnt8a/ β -catenin target genes tend to have more clearly defined β -peaks than the non-*wnt8a*-regulated gene class (Fig. 3E; see examples in Fig. S5A,B,E,F). Therefore, these two classes of β -catenin-associated genes exhibit subtly different levels and relative genomic locations of β -catenin recruitment. However, *de novo* motif discovery among β -peaks associated with these *wnt8a*-regulated or non-regulated genes uncovered essentially the same motifs among their β -peaks (Fig. S6). Therefore, these other motifs in *wnt8a*-regulated and non-regulated genes appear to exist more generally in β -peaks, implying they are not involved in regulating context-specific *wnt8a* target gene expression. Interestingly, the TCF/LEF motif was the only shared sequence motif found among all 13 genes of the shortlist (Fig. 3F), suggesting that TCF/LEF motif-dependent actions might constitute the only shared mechanism regulating context-specific *wnt8a* target gene expression (see below).

Together, this analysis suggests subtle quantitative, but no obvious qualitative, differences between *wnt8a*-regulated and non-*wnt8a*-regulated β -catenin-associated loci.

β -catenin-chromatin association is not sufficient for transcriptional regulation of direct Wnt target genes

We had discovered β -catenin-associated loci in gastrula embryos that were not transcriptionally regulated by *wnt8a* function. We speculated whether these β -catenin-associated loci could represent Wnt target genes regulated in other tissues and at other stages. Conversely, we wondered whether our *wnt8a* target loci would bind β -catenin yet remain refractory to transcriptional regulation by Wnt signalling in a different developmental context. For that reason we investigated whether the identified *wnt8a* target genes have any potential to respond to earlier maternal Wnt signalling (see Fig. S1).

We experimentally induced ectopic and enhanced activation of maternal Wnt signalling and examined the expression of several *wnt8a* target genes by RT-qPCR in blastula embryos at the MBT (Fig. 4A), as well as the known maternal Wnt targets *sial* (Brannon et al., 1997) and *nodal3.1* (also known as *Xnr3*; McKendry et al., 1997) as controls. Enhanced activation of maternal Wnt signalling significantly increased expression of the maternal targets, as expected, but did not change expression of *wnt8a* target genes (Fig. 4A). This is consistent with the established idea that the *wnt8a* target genes represent ventral mesoderm-specific zygotic Wnt targets. However, β -catenin ChIP analysis revealed that, remarkably, β -catenin is associated with both maternal Wnt and *wnt8a* target gene loci in pre-MBT embryos (100-cell stage), when β -catenin is

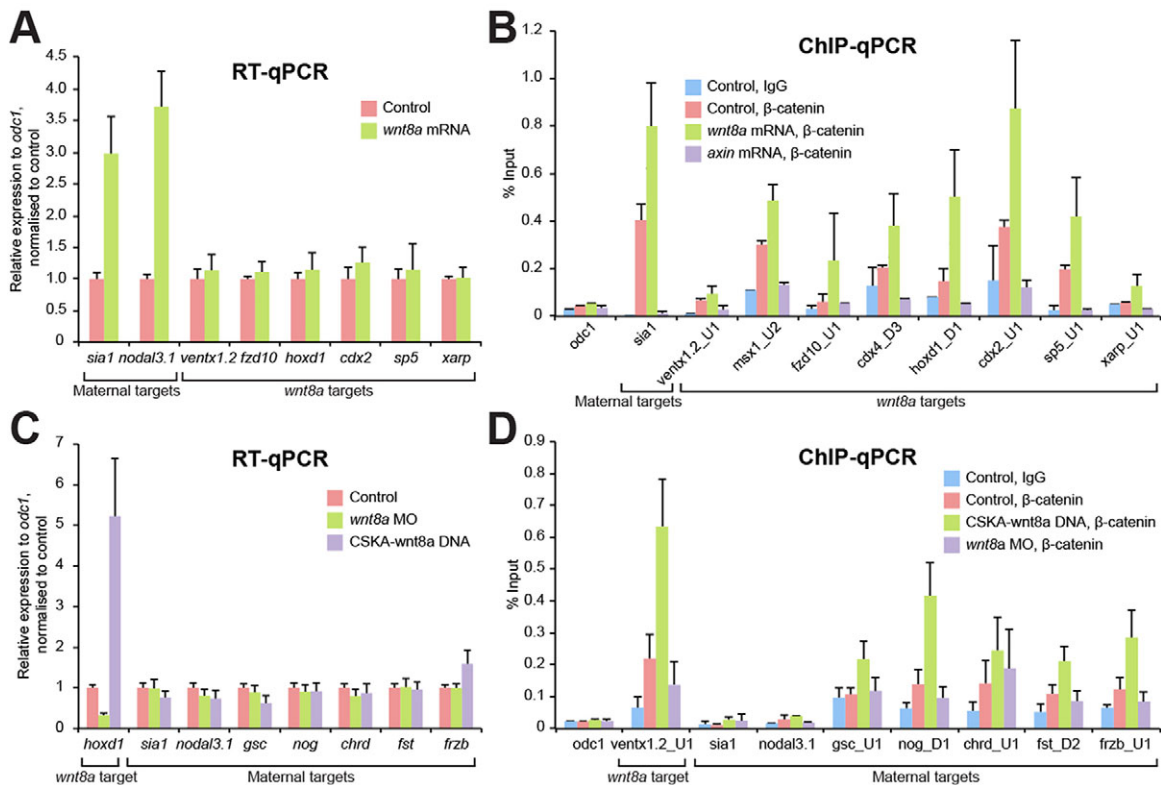


Fig. 4. β-catenin recruitment is not sufficient for transcriptional regulation. (A,B) Maternally activated Wnt/β-catenin signalling regulates transcription of only context-specific maternal Wnt/β-catenin target genes. Experimental enhancement of maternal Wnt signalling, by injection of *wnt8a* mRNA at the two- to four-cell stage, increases expression of the maternal Wnt targets *sia1* and *nodal3.1* when analysed at the MBT, compared with the uninjected control (A). By contrast, expression levels of *wnt8a* target genes remain unchanged. However, β-catenin binding increases at both maternal Wnt target and zygotic *wnt8a*-regulated target loci at the 1000-cell stage (B). Note that the reduction of β-catenin binding following injection of *axin* mRNA indicates that maternally regulated endogenous β-catenin associates with not only maternal Wnt target genes but also zygotic *wnt8a* target genes. (C,D) Zygotically activated β-catenin controls the expression of only zygotic *wnt8a* targets. *wnt8a* MO or CSKA-*wnt8a* DNA were injected at the two- to four-cell stage and gene expression and β-catenin binding were analysed at the early gastrula stage. Knockdown of *wnt8a* reduces, and zygotic activation of Wnt8a signalling increases, expression of the *wnt8a* target *hoxd1*, as a control. Whereas *wnt8a* knockdown or overexpression does not affect the expression of maternal Wnt-regulated genes (C), overactivation of Wnt8a signalling increases β-catenin binding to some maternal Wnt-regulated loci (D) but not to the well-characterised direct maternal Wnt target genes *sia1* and *nodal3.1*. Error bars indicate s.d. and s.e.m. of three biological replicates for RT-qPCR and ChIP-qPCR, respectively.

regulated by maternal Wnt signal and well before the onset of zygotic Wnt8a signalling (Fig. 4B, pink). Furthermore, the β-catenin occupancy increased with enhanced maternal Wnt activity (Fig. 4B, green). This observation was confirmed by pharmacological activation of maternal Wnt signalling activity with BIO (Fig. S7). β-catenin binding was reduced following experimental inhibition of endogenous maternal Wnt signalling (Fig. 4B, purple). This result clearly shows that maternal Wnt signalling controls β-catenin recruitment before the MBT not only to maternal Wnt target genes but also to *wnt8a* target loci. Thus, there appears no obvious qualitative difference in β-catenin recruitment between maternal Wnt target genes and *wnt8a* target genes.

Conversely, as expected, the transcription of genes known to be regulated by maternal Wnt signalling (Brannon et al., 1997; Crease et al., 1998; Wessely et al., 2001) remained unaffected by either *wnt8a* knockdown or experimentally enhanced Wnt signalling activity in gastrula embryos (Fig. 4C). However, β-catenin ChIP analysis in the same experiment revealed differences among maternal Wnt-regulated gene loci; for some (*gsc*, *nog*, *chrd*, *fst* and *frzb*), levels of β-catenin binding were increased by experimentally enhanced Wnt8a activity in gastrula embryos (Fig. 4D, green), similar to *wnt8a* targets (e.g. *ventx1.2*); whereas others (*sia1* and *nodal3.1*) were neither associated with endogenous β-catenin nor with experimentally activated β-catenin in gastrula

embryos (Fig. 4D; see Table S4 for β-peaks of maternal Wnt-regulated gene loci). Together, these results demonstrate in two different developmental contexts that Wnt-regulated β-catenin association is not sufficient for transcriptional activation.

Context-specific expression of *wnt8a* target genes is regulated by BMP and FGF signalling subsequent to β-catenin recruitment

Beyond the expected TCF/LEF motifs, *de novo* motif discovery among *wnt8a* target genes failed to identify further shared enriched motifs. We therefore sought to test earlier proposed hypotheses that combinatorial signalling underlies the context-specific expression of *wnt8a*-regulated genes. It has previously been suggested that, among our *wnt8a*-regulated genes, *ventx1.2* is co-regulated by BMP signalling (e.g. Hoppler and Moon, 1998). To investigate whether co-regulation by Wnt and BMP signalling represents a shared mechanism for regulating context-specific expression of *wnt8a* targets (reviewed by Itasaki and Hoppler, 2010), we examined the requirement of BMP signalling for *wnt8a* target gene regulation by blocking the BMP pathway while maintaining constant levels of Wnt8a signalling. We found that the expression of another four genes, in addition to *ventx1.2*, is dependent on BMP signalling (Fig. 5A), but, importantly, not that of all 13 genes in the shortlist. Thus, although decisive for context-specific expression of some

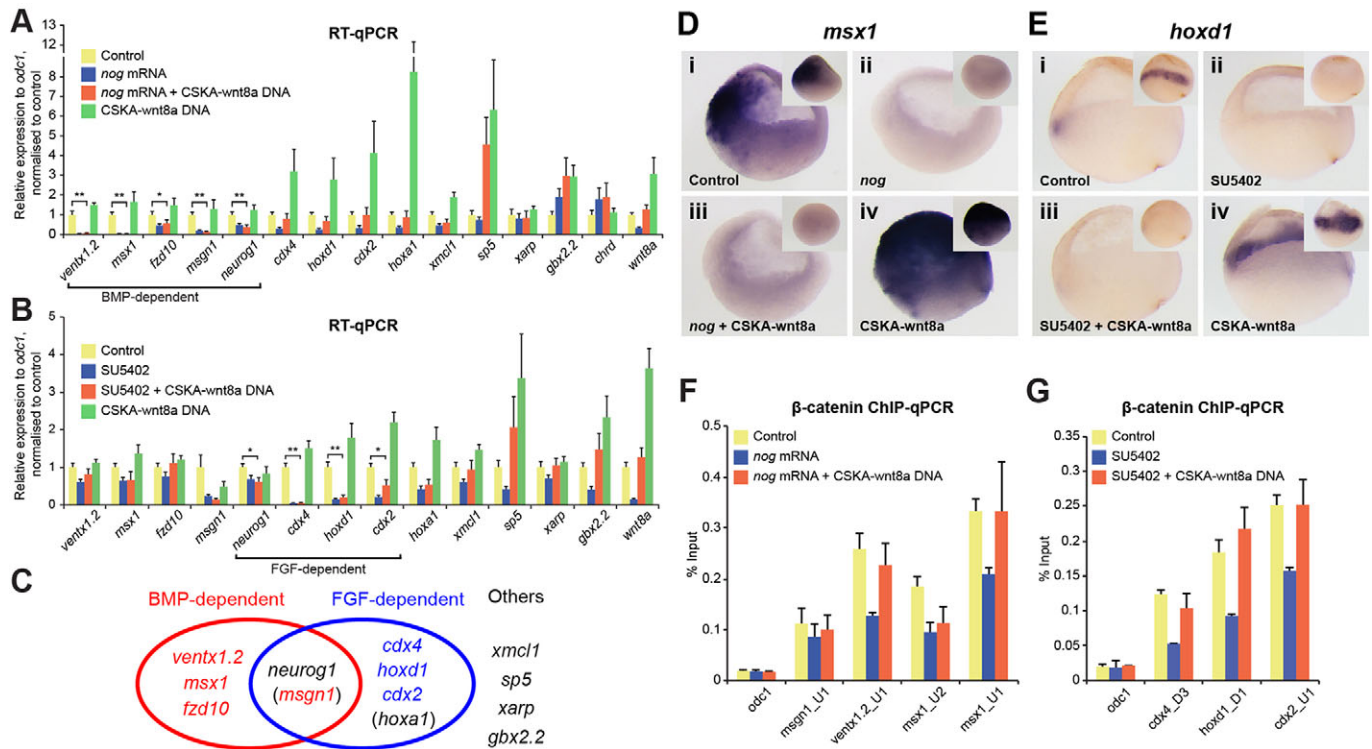


Fig. 5. BMP or FGF signalling is required for *wnt8a* target gene expression but not for β -catenin recruitment. (A) BMP signalling is required for context-specific transcriptional regulation by *Wnt8a* signalling, but only of some *wnt8a* target genes. Two- to four-cell stage embryos were injected with BMP antagonist *noggin* (*nog*) mRNA. CSKA-*wnt8a* DNA was injected additionally to reinstate *Wnt8a* expression (as endogenous *wnt8a* expression is itself regulated by BMP signalling). Expression was analysed by RT-qPCR at the early gastrula stage. When BMP signalling is blocked, expression of BMP-dependent genes remains reduced even when *Wnt8a* expression is reinstated. (B) FGF signalling is required for context-specific transcriptional regulation by *Wnt8a* signalling, but only of some *wnt8a* target genes. Embryos were treated with the FGFR inhibitor SU5402 from the 1000/2000-cell stage through the early gastrula and injected where indicated with CSKA-*wnt8a* DNA at the two- to four-cell stages (to reinstate *Wnt8a* expression, as endogenous *wnt8a* expression is itself regulated by FGF signalling). When FGF signalling is inhibited, expression of FGF-dependent genes remains reduced, even when *Wnt8a* expression is reinstated. (C) *wnt8a* targets can therefore be classified into BMP-dependent or FGF-dependent genes. Note that some genes belong to both groups and others are neither BMP nor FGF dependent. (D,E) *In situ* hybridisation shows expression of *msx1* (D) and *hoxd1* (E) in sagittal sections and lateral views (insets) of control uninjected and experimentally manipulated embryos as indicated (dorsal to the right). (F) BMP signalling is not required for *wnt8a*-regulated β -catenin recruitment to BMP-dependent *wnt8a* target gene loci. Embryos were treated as in A and analysed by β -catenin ChIP-qPCR at the early gastrula stage. (G) FGF signalling is not essential for *wnt8a*-regulated β -catenin recruitment to FGF-dependent *wnt8a* target gene loci. Embryos were treated as in B and analysed by β -catenin ChIP-qPCR at the early gastrula stage. Uninjected, untreated embryos were used as controls in A,B,D-G. * $P < 0.1$, ** $P < 0.05$; two-tailed Student's *t*-test. Error bars represent s.d. of four biological replicates (A,B) or s.e.m. of three biological replicates (F,G). Note that *wnt8a* gene expression itself was decreased by BMP or FGF pathway inhibition (*wnt8a* blue bars in A,B) but restored by co-injection of CSKA-*wnt8a* DNA (*wnt8a* orange bars in A,B) compared with controls (*wnt8a* yellow bars in A,B), and that higher *wnt8a* expression levels with CSKA-*wnt8a* DNA (*wnt8a* green bars in A,B) reflect both expression of endogenous *wnt8a* and expression from CSKA-*wnt8a* DNA, resulting in upregulation of several *wnt8a* target genes (in A,B).

genes in this tissue, BMP signalling is not an indispensable element of any general mechanism for context-specific regulation of *wnt8a* target genes.

Among our other *wnt8a* targets, *cdx4* had been shown to be co-regulated by FGF signalling (Haremak et al., 2003). To examine whether other *wnt8a* targets are similarly co-regulated by FGF signalling, we analysed *wnt8a* target gene expression while inhibiting the FGF pathway under constant levels of *Wnt8a* signalling. Interestingly, compared with the BMP experiments, a largely distinct subset of *wnt8a* target genes was found to be FGF dependent (Fig. 5B). These results suggest that *wnt8a*-regulated genes can be categorised into at least two different groups based on co-regulation by different signalling pathways (Fig. 5C), and that there is therefore no collectively shared context-specific *Wnt8a* signalling mechanism that prevails in the ventral prospective mesoderm of gastrulae.

Since the BMP and FGF pathways are activated in different regions of early gastrulae (Fig. S8A) (Schohl and Fagotto, 2002), we examined whether contexts in which *wnt8a* target genes are

regulated by these two pathways are spatially restricted. We performed whole-mount *in situ* hybridisation of several BMP-dependent or FGF-dependent *wnt8a* targets. Expression of the BMP-dependent *wnt8a* target gene *msx1* was detected in the prospective ectoderm and mesoderm and, as expected, it was significantly reduced in both tissues when BMP signalling was inhibited (Fig. 5D). Experimentally enhanced *Wnt8a* activity increased the expression in both tissues only when endogenous BMP signalling was active (similar results are shown for *fzd10* in Fig. S8B). On the other hand, the FGF-dependent *wnt8a* targets *hoxd1* (Fig. 5E) and *cdx2* (Fig. S8C) are expressed more exclusively in prospective mesoderm (the marginal zone). Blocking FGF signalling decreased their expression in prospective mesodermal cells. Activation of *Wnt8a* signalling did not reinstate their expression when FGF signalling was blocked, but did cause strongly induced expression of both genes when endogenous FGF signalling was active, specifically in the marginal zone. These results suggest that the BMP and FGF pathways provide different, spatially restricted contexts where *wnt8a* target genes can be

activated in response to Wnt8a signalling; however, their respective spatially restricted contexts overlap in the prospective mesoderm.

We uncovered one shared aspect of gene regulation of these context-specific *wnt8a* targets. Since BMP and FGF signalling are required for normal *wnt8a* target gene regulation, we wondered whether these signalling mechanisms would regulate β -catenin recruitment to these *wnt8a* target loci. We observed, however, that β -catenin is still able to bind to *wnt8a* target loci at comparable levels to controls even when BMP or FGF signalling is inhibited, provided constant levels of Wnt8a signalling are maintained (Fig. 5F,G). This demonstrates that neither BMP nor FGF signalling restricts β -catenin recruitment to WREs. Rather, our results suggest that context-specific transcriptional regulation of *wnt8a* targets by the BMP or FGF pathway takes place in addition, and subsequent, to Wnt-regulated β -catenin binding to cis-regulatory sequences associated with these genes.

DISCUSSION

β -catenin is required but not sufficient for Wnt-regulated transcriptional activation

The interaction of nuclear β -catenin with target genomic loci has been shown to be sufficient to activate target gene transcription for many specific examples studied in a variety of tissues and experimental systems (recently reviewed by Zhang and Cadigan, 2014). Our results, however, demonstrate that chromatin association of β -catenin does not necessarily imply transcriptional activation. This is also consistent with data from a cell culture model of colorectal cancer demonstrating chromatin association of β -catenin near to many genes that are not regulated by β -catenin function (Watanabe et al., 2014). Our study is the first to investigate this phenomenon and to provide evidence that β -catenin binding to target loci can be Wnt regulated even in embryonic contexts, in which these genes are not transcriptionally Wnt responsive (Fig. 4). Furthermore, we uncover that molecular mechanisms (e.g. BMP or FGF pathways) required for context-specific transcriptional regulation of direct target genes do not influence the Wnt-regulated chromatin association of β -catenin (Fig. 5).

These unexpected mechanistic findings suggest a more general paradigm for Wnt-regulated transcriptional mechanisms. Thus, chromatin association of β -catenin, even to functional WREs, is only productive for Wnt signalling-regulated transcriptional activation in the appropriate developmental context (Fig. 6). This new insight helps explain why we identify chromatin association of β -catenin near many genes that are not overtly transcriptionally regulated by the Wnt signalling mechanism operating at this stage (Fig. 3). Taken at face value, this would suggest that β -catenin ChIP-seq analysis is not of sufficient use on its own for detecting direct transcriptionally Wnt-regulated target genes, and it raises questions about the biological significance of apparently widespread β -catenin binding across the genome.

Molecular mechanisms regulating context-specific Wnt/ β -catenin target gene expression

Identifying direct Wnt8a/ β -catenin targets was motivated by our ambition to uncover a unifying mechanism for context-specific Wnt/ β -catenin target gene regulation in the ventrolateral prospective mesoderm. We wondered whether it would be possible to predict Wnt-regulated target genes from many β -catenin-bound loci. We found that *wnt8a* targets tend to show stronger and clearer β -catenin binding than non-*wnt8a*-regulated loci (Fig. 3E). While β -peaks generally appear to be enriched close to TSSs (as previously observed by Watanabe et al., 2014), this enrichment is even higher

in confirmed *wnt8a* targets. These observations are consistent with the notion that transcriptionally regulated direct target genes exhibit high levels of transcription factor occupancy at nearby binding sites (Biggin, 2011). Although we anticipated that *wnt8a*-regulated genes would share specific DNA sequences under their β -catenin-bound regions, which we hoped would reveal a shared tissue-specific molecular regulatory mechanism, our sequence motif analysis suggested that only TCF/LEF-mediated mechanisms are shared (Fig. 3F). Individual β -catenin-associated genomic sequences contain consensus binding sequences for other transcription factors; however, such sequences are found both in the Wnt8a/ β -catenin target genes that we identified and in non-*wnt8a*-regulated loci, and as such are not informative in the context of a shared tissue-specific molecular regulatory mechanism for Wnt/ β -catenin target genes in the ventrolateral mesoderm. The presence of regulatory sequences for other transcription factors in some *wnt8a* target loci could indicate additional regulation of these genes, particularly by T-box transcription factors driving mesoderm induction and development in this tissue and at this stage (Gentsch et al., 2013). Overall, these trends do not add up to reliable criteria for predicting *wnt8a*-regulated genes from among all β -catenin-bound genomic loci, let alone Wnt transcriptionally regulated genes more generally.

Since direct target genes of maternal Wnt/ β -catenin signalling were shown to be regulated by combinatorial Wnt and Smad2 (Activin/Nodal/TGF β) signalling (Crease et al., 1998; Laurent et al., 1997), we hypothesized that context-specific *wnt8a* target genes shared an analogous common regulatory mechanism, possibly involving combinatorial signalling with another signalling mechanism. Indeed, we find that combinatorial signalling is important; however, more gene-specific mechanisms are unearthed: some *wnt8a* target genes are co-regulated by BMP, some by FGF signalling. The discovery of several classes of *wnt8a* target genes confirmed that there is no single, collectively shared tissue-specific mechanism for restricting Wnt/ β -catenin target gene regulation in the context that we have investigated, and therefore studying these molecular mechanisms would not reveal shared ventral mesoderm-specific processes. This explains our inability to identify any shared motifs beyond potential TCF/LEF binding sequences. However, as expected, all *wnt8a* target genes co-regulated by BMP signalling contain potential Smad1 and Smad4 binding sequences, and all *wnt8a* targets co-regulated by FGF signalling contain potential ETS binding motifs.

β -catenin binding to Wnt target genes in alternative contexts

Our β -catenin ChIP-seq analysis at the early gastrula stage found β -peaks at gene loci known to be transcriptionally regulated by maternal Wnt signalling at an earlier stage. However, when zygotic Wnt8a signalling is experimentally activated, β -catenin occupancy increases at these gene loci, but not gene expression. Conversely, we can detect β -catenin binding to *wnt8a* target loci even before the onset of endogenous *wnt8a* expression (Fig. 4). This precocious β -catenin binding to *wnt8a* target loci is regulated by maternal Wnt signalling, but this binding does not cause increased transcriptional expression. These results support our conclusion that chromatin association of β -catenin does not imply Wnt-regulated transcriptional activation and are therefore also consistent with context-specific regulatory mechanisms acting subsequent to Wnt-regulated β -catenin binding, as discussed above.

Widespread distribution of β -catenin binding

Widespread binding to the genome is common for some DNA-binding transcription factors and is thought to be mediated via

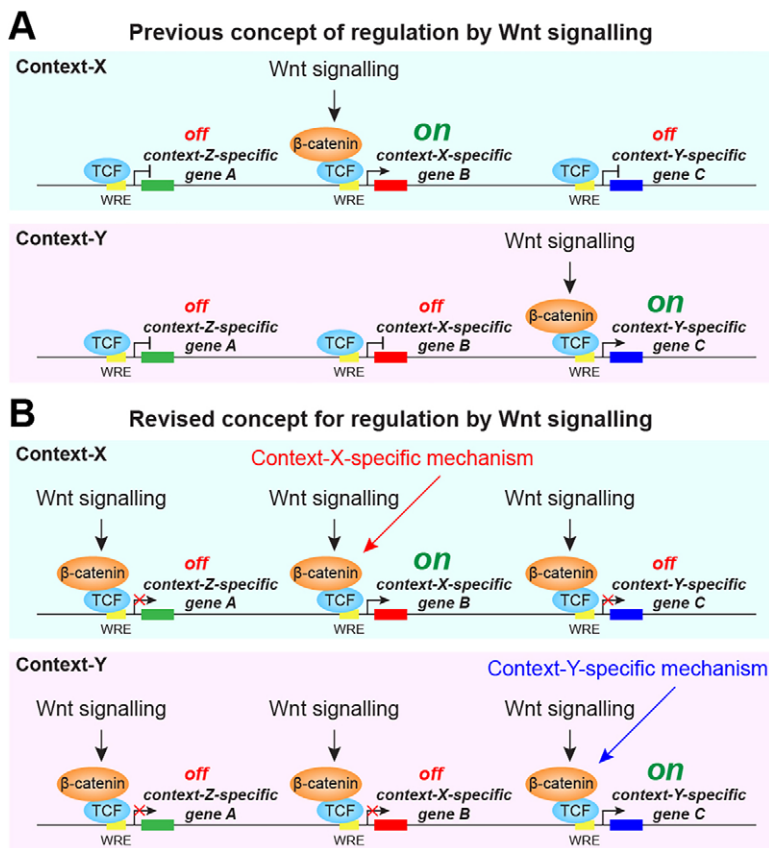


Fig. 6. Model for context-specific Wnt/ β -catenin target gene regulation. (A) In the previous concept established from studies of individual genes, Wnt signalling specifically controls β -catenin recruitment to the Wnt-response element (WRE) of context-specific target genes and leads to their transcription (e.g. *gene B* in the context-X). (B) In the revised concept from our studies, Wnt-regulated β -catenin recruitment takes place at numerous loci. Transcriptional activation at those loci is conditional on context-specific mechanisms (e.g. a context-X-specific mechanism for *gene B* in context-X).

low-affinity sites (Biggin, 2011; MacQuarrie et al., 2011). However, β -catenin association does not, on the whole, result from indiscriminate binding across the genome but rather β -catenin tends to be recruited to putative CRMs (promoter and enhancer sequences). In particular, we find a significant level of overlap between our β -peaks and TLE ChIP-seq peaks, which have recently been found to be indicators of tissue-specific CRMs (Yasuoka et al., 2014).

β -catenin is reported to be predominantly associated with TCF/LEF motif-containing chromatin, both in cancer cells with activated Wnt signalling (Schuijers et al., 2014; Watanabe et al., 2014) and Wnt-induced embryonic stem cells (Zhang et al., 2013). Our analysis also identified TCF/LEF as the only shared sequence motif among the validated 13 direct *wnt8a* target genes, suggesting that positive gene regulation by Wnt/ β -catenin signalling is mediated by TCF/LEF-dependent mechanisms. However, our *de novo* motif search revealed that non-*wnt8a*-regulated, yet β -catenin-bound, loci also contain consensus binding sequences for transcription factors other than TCF/LEF, suggesting that some β -catenin protein may interact with those transcription factors when associated with genomic sequences of non-*wnt8a*-regulated genes. In fact, such interactions have previously been reported for OCT4 (POU5F1) (Kelly et al., 2011), TBX5 (Rosenbluh et al., 2012), SOX proteins (Kormish et al., 2010) and FOX proteins (Zhang et al., 2011); and, among them, OCT4 (Abu-Remaileh et al., 2010) and SOX proteins (Kormish et al., 2010) are known to negatively regulate β -catenin-dependent transcriptional regulation. Thus, the β -catenin-bound yet non-*wnt8a*-regulated gene loci identified in our analysis might be deliberately repressed by these transcription factors. Alternatively, chromatin association of β -catenin via these transcription factors might act as part of a buffering system to fine-tune the availability of β -catenin for transcriptional regulation at Wnt/ β -catenin target

genes, similar to that previously suggested for fine-tuning the availability of functional DNA-binding transcription factors (MacQuarrie et al., 2011). In particular, our analysis of motifs enriched in β -peaks close to non-*wnt8a*-regulated loci identifies the same combined SOX and OCT4 motif that has previously been reported in embryonic stem cell studies (Zhang et al., 2013). Although technical bias cannot currently be excluded, the β -catenin chromatin association observed in our *Xenopus* embryos seem more similar to that of embryonic stem cells than cancer cells (Schuijers et al., 2014; Watanabe et al., 2014). Future analysis might confirm that β -catenin association with chromatin containing the combined SOX and OCT4 motif in particular is specifically prevalent in embryonic cells.

Wnt/ β -catenin target genes in the genome

Our results do not allow us to rule out the possibility that low levels of nuclear β -catenin associate with chromatin to mediate other, as yet undiscovered functions for β -catenin in the genome or to be part of a buffering system to fine-tune the availability of β -catenin for transcriptional regulation, as mentioned above. β -catenin-bound, yet non-*wnt8a*-regulated, gene loci in our analysis could more generally represent real Wnt target genes, but those that are regulated by Wnt signalling in other tissues and at other stages. Consistent with this idea, the GO analysis suggests that such genes are more associated with functions at later stages of development, after the stage of our analysis in early gastrulation, such as neural development, and also with metabolism (Fig. 3D). As a particular example, *sall4*, which is among our β -catenin-bound but not our *wnt8a*-regulated genes, has recently been identified as a direct *wnt3a* target gene during neural development (Young et al., 2014). In addition, our *wnt8a* target *msx1* showed a β -peak (*msx1_U2*) that is located at a conserved limb bud-specific enhancer (Miller et al.,

2007), consistent with our hypothesis that β -catenin recruitment already occurs during early embryonic stages to cis-regulatory elements responsible for Wnt-mediated regulation in other tissues at later stages. Furthermore, ~60% of orthologues of the Wnt target genes listed at the curated Wnt homepage (<http://web.stanford.edu/group/nusselab/cgi-bin/wnt/>) are represented in our list of β -catenin-bound genes. Likewise, our list contains 70% of homologues of direct β -catenin-regulated target genes identified in a colorectal cancer cell line (Watanabe et al., 2014). Therefore, many potential direct Wnt targets in the genome could be associated with β -catenin, even if their expression is not Wnt regulated in the tissue analysed.

Conclusions

Our investigation challenges the fundamental concept that β -catenin recruitment to individual Wnt target genes predictably drives transcriptional expression (Fig. 6); instead, it introduces a more general paradigm for Wnt-regulated transcriptional mechanisms, which is more relevant for the repeated and tissue-specific functions of Wnt/ β -catenin signalling in embryonic development, stem cell-mediated homeostasis and cancer. We discovered that chromatin association of β -catenin, even to functional WREs, does not imply transcriptional activation. Wnt signalling regulates β -catenin binding to target loci even in embryonic contexts, in which these gene loci are not transcriptionally Wnt responsive. Chromatin association of β -catenin is only productive for Wnt signalling-regulated transcriptional activation in the appropriate developmental context. Mechanisms regulating this developmental context therefore do not necessarily influence the Wnt-regulated association of β -catenin with chromatin. Our findings will also be relevant beyond early embryogenesis, with implications for cancer research and other Wnt-related diseases, where an abnormal subtle change in cellular context may induce the anomalous expression of genes, with deleterious consequences.

MATERIALS AND METHODS

Embryo experiments

Xenopus tropicalis (Gray, 1864) embryos were obtained by natural mating of adult males and females or by *in vitro* fertilisation as described by del Viso and Khokha (2012) and staged according to Nieuwkoop and Faber (1967). The fertilised embryos were injected with MOs and mRNAs, and treated with chemical inhibitors as indicated, and then cultured in 0.1× Marc's Modified Ringer (MMR) at 28°C. MOs (Gene Tools) were: CoMO, 5'-CCTCTTAC-CTCAGTTACAATTATA-3' (Heasman et al., 2000); *wnt8a* MO, 5'-GGAGACTGCTATCCAGGGTAATGCT-3' (Rana et al., 2006). pCSKA-wnt8a was created as a *wnt8a* MO-insensitive *wnt8a* gene by introducing nucleotide substitutions (Fig. S3A). Capped mRNA was synthesized using the mMESSAGE mMACHINE Kit (Ambion) and the following linearised DNA templates were used: pCS2+ Xwnt-8, Axin/CS2mt and pCS2+ noggin. SU5402 (SML0443) was purchased from Sigma. See the supplementary Materials and Methods for further details of *Xenopus* embryos and treatment with MOs, mRNAs and chemical inhibitors.

Whole-mount RNA *in situ* hybridisation

Digoxigenin-labelled antisense RNA probes were synthesized from linearised template plasmids (see the supplementary Materials and Methods) using the MEGAscript Transcription Kit (Life Technologies) for use in whole-mount RNA *in situ* hybridisation as described by Lavery and Hoppler (2008).

RT-PCR

Total RNA was isolated from whole embryos as described by Lee-Liu et al. (2012). cDNA was synthesized using the QuantiTech Reverse Transcription Kit (Qiagen).

qPCR

qPCR was performed using a LightCycler 480 and SYBR Green I Master reagents (Roche). For RT-qPCR, relative expression levels of each gene to *odc1* were calculated and then normalised to the control. For primer sequences for RT-qPCR and ChIP-qPCR, see the supplementary Materials and Methods.

ChIP

ChIP analysis was carried out as described (Akkers et al., 2012; Blythe et al., 2010; Janssens et al., 2010) with slight modifications: after homogenisation, embryos were sonicated with a Bioruptor Plus (Diagenode). Two β -catenin antibodies, namely anti-*Xenopus* β -catenin antibody (1:28; Blythe et al., 2009) and anti- β -catenin antibody [1:28 (2 µg); H-102; sc-7199, Santa Cruz Biotechnology], and normal rabbit IgG [1:56 (2 µg); sc-2027, Santa Cruz Biotechnology] were used for immunoprecipitation. For optimised conditions of the β -catenin ChIP experiment, see Fig. S2 and the supplementary Materials and Methods.

ChIP-seq

β -catenin ChIP was performed using anti- β -catenin antibody (H-102) as described above. Two Illumina TrueSeq ChIP libraries were constructed from ChIP and input control DNA and sequenced using Illumina HiSeq 2500 at The Genome Analysis Centre (TGAC, Norwich, UK). Sequenced reads were mapped to the *X. tropicalis* genome assembly JGI 4.2. Briefly, MACS2 (Zhang et al., 2008) and SPP (Kharchenko et al., 2008) were used for peak calling. Reproducible peaks were identified using the IDR method (Li et al., 2011). Peaks were assigned to closest genes using the distanceToNearest function [rtracklayer (Lawrence et al., 2009) and GenomicRanges (Lawrence et al., 2013)]. Heat maps were created using HOMER, Cluster 3.0, and Java Treeview. Histograms were visualised using HOMER and Excel. *De novo* motif discovery was performed using MEME-ChIP. ChIP-seq and RNA-seq data were visualised on the UCSC genome browser. GO analysis was performed using the PANTHER classification system (Mi et al., 2013). We carried out statistical over-representation tests using PANTHER GO annotations (PANTHER GO-Slim Biological Process, PANTHER Protein Class, PANTHER Pathways). See the supplementary Materials and Methods for details. The ChIP-seq data sets are available in GEO under the accession number GSE72657.

RNA-seq

Total RNA was extracted as described by Lee-Liu et al. (2012). Illumina TruSeq RNA libraries were constructed and sequenced using Illumina HiSeq 2000 at TGAC. Sequenced reads were aligned to the *X. tropicalis* genome JGI 4.2 with gsnap. Aligned reads were counted using HTSeq (Anders et al., 2015) and further differential gene expression analysis was carried out using DESeq2. See the supplementary Materials and Methods for details. The RNA-seq data sets are available in GEO under the accession number GSE72657.

Reporter constructs and luciferase assay

Genomic fragments of β -peaks were amplified by PCR and subcloned into the pGL4.10 vector (Promega) or a derivative vector p β -actin-luc carrying a heterologous basal promoter. Embryos were injected with 40 pg reporter plasmid DNA together with 40 pg pRL-CMV (Promega) at the two- to four-cell stage, collected at the early gastrula stage, and assayed for luciferase activity. For cloning into luciferase reporter constructs, see the supplementary Materials and Methods.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Y.N. designed research, carried out all experiments and data analysis, and wrote the paper. E.d.P.A. carried out most post-sequencing data analysis. G.J.C.V. participated in the experimental design and processing of sequencing data, and contributed to data analysis. S.H. designed research, helped with microinjection experiments, analysed data and wrote the paper.

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Data availability

The ChIP-seq and RNA-seq data sets are available at Gene Expression Omnibus under accession number GSE72657.

Supplementary information

Supplementary information available online at <http://dev.biologists.org/lookup/doi/10.1242/dev.131664.supplemental>

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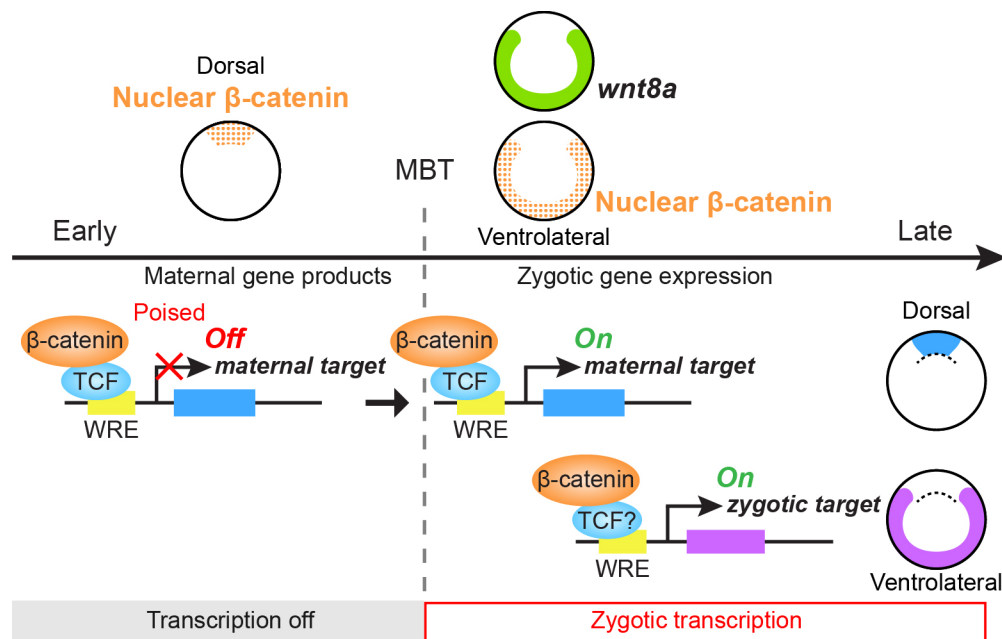


Fig. S1. Wnt/β-catenin target genes change during early development.

Embryonic development from early (left) to late (right) is illustrated. Maternal gene products (mRNA and protein) sustain the earliest embryonic development until Mid-Blastula Transition (MBT), when zygotic transcription is initiated to regulate further development. Maternal Wnt/β-catenin signalling localises β-catenin protein to nuclei of prospective dorsal cells (orange) (Schohl and Fagotto, 2002). After the MBT, *wnt8a* is expressed in ventral and lateral prospective mesoderm (green) (Christian et al., 1991) causing nuclear β-catenin localisation in ventrolateral cells (Schohl and Fagotto, 2002). Maternally regulated nuclear β-catenin initiates poised transcription in specific maternal Wnt/β-catenin target genes (blue), which are transcribed after MBT (Blythe et al., 2010) in dorsal mesoderm. *wnt8a*-regulated nuclear β-catenin is expected to initiate transcription of different specific zygotic Wnt/β-catenin target genes (violet) in ventrolateral embryonic cells.

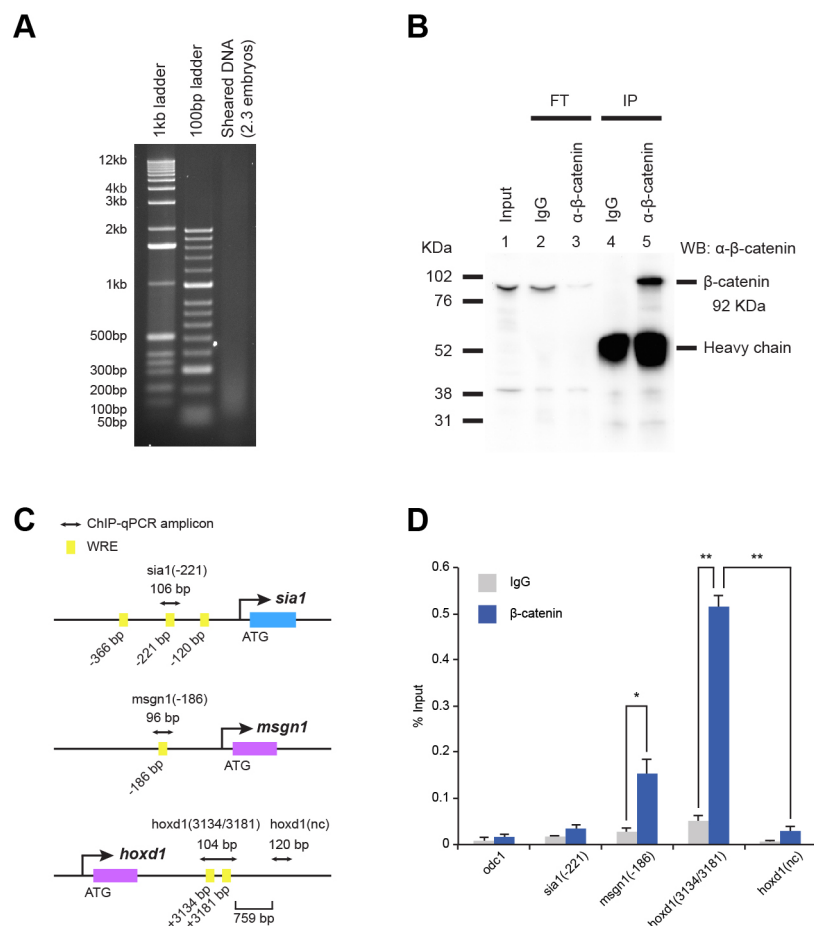


Fig. S2. β-catenin ChIP experimental condition.

(A) Chromatin shearing with optimised condition. Chromatin was extracted from 50 early gastrula embryos that were fixed for 30 minutes, and were sheared during 2.5 rounds of 10 cycles of 30 seconds ON/30 seconds OFF at high power setting using the Bioruptor Plus Instrument. After de-crosslinking and purification, the sheared DNA from 2.3 embryos was analysed by agarose gel electrophoresis. The sheared chromatin was enriched around 200 bp. (B) Immunoprecipitation for chromatin-associated β-catenin protein with optimised condition. Chromatin extract from approximately 42 embryos was incubated with 10 μg of either IgG (negative control) or β-catenin antibodies overnight at 4°C and subsequently precipitated with 50 μl of Dynabeads Protein G for 1 hour, followed by washing and de-crosslinking. Input chromatin extracts before antibody incubation (Input), flow-through supernatant after antibody incubation (FT), and immunoprecipitated samples following immunoprecipitation and elution (IP) were analysed for β-catenin protein by western blotting with β-catenin antibody. β-catenin protein was efficiently immunoprecipitated with β-catenin antibody (see lanes 3 and 5), while significant amount of β-catenin protein remained in supernatant after incubation with IgG (lane 2). Heavy chain indicates denatured IgG and β-catenin antibodies after heat incubation during elution. (C) Schematic diagrams of the *sia1*, *msgn1*, and *hoxd1* genomic loci. Known Wnt-response-elements (WREs, yellow boxes) are shown with the positions relative to the translation start site (ATG). The locations of amplicons (double-headed arrows) analysed by ChIP-qPCR are shown above the corresponding WREs. (D) Validation of β-catenin ChIP by qPCR. Co-immunoprecipitation of β-catenin protein with predicted genomic regions containing target WREs was

analysed by qPCR. Genomic regions of known WREs at the *msgn1* and *hoxd1* loci were detected at greater levels in β -catenin ChIP sample than in the IgG ChIP control sample. Negative control sites [*odc1* and *hoxd1(nc)*] and a WRE site of the maternal Wnt target *sial* were not efficiently co-immunoprecipitated with β -catenin protein (at gastrula stage). Note that a significant difference in β -catenin ChIP recovery between *hoxd1* (3134/3181) and a genomic region only 759 bp downstream of it [*hoxd1(nc)*] demonstrates a high resolution of chromatin shearing. $*p < 0.01$; $**p < 0.00001$ (two-tailed Student's *t*-test). The error bars represent s.d. of three technical replicates.

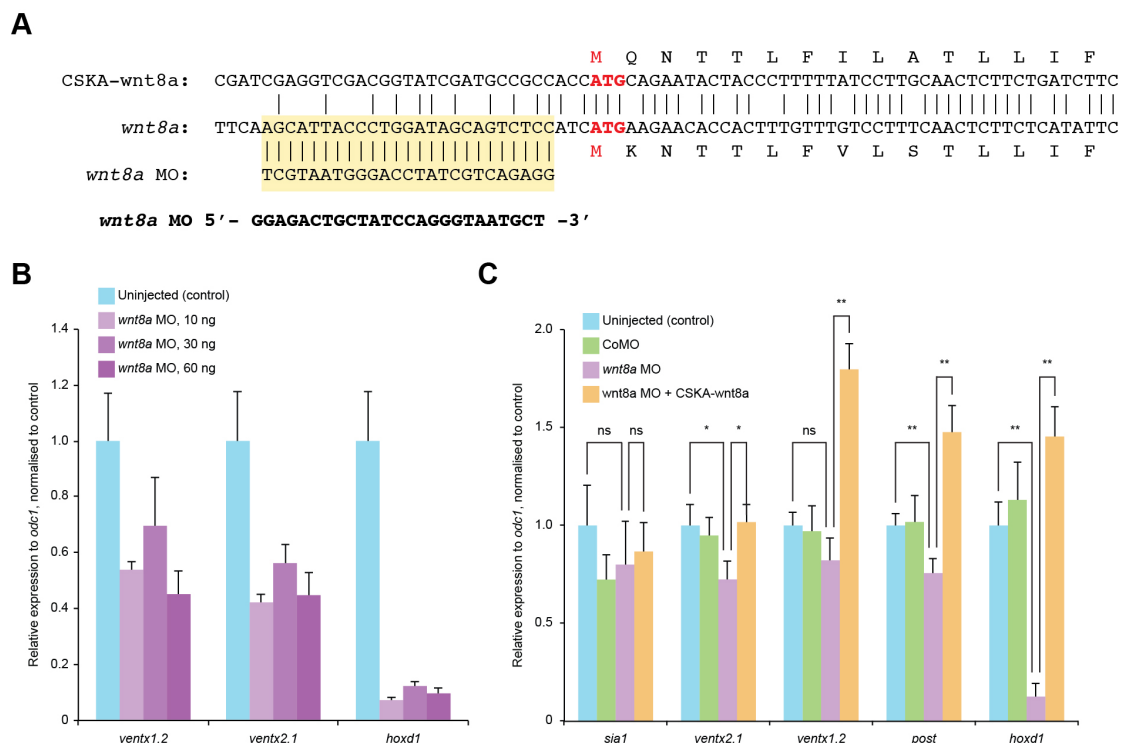


Fig. S3. RNA-seq experiment for identifying *wnt8a*-regulated genes.

(A) *wnt8a* MO and CSKA-*wnt8a* DNA construct. CSKA-*wnt8a* was created as a *wnt8a* MO-insensitive rescue DNA construct (see Supplementary Materials and Methods for details). Note that CSKA-*wnt8a* lacks nucleotide sequences targeted by previously validated *wnt8a* MO (Rana et al., 2006). A yellow box shows sequence homology between *wnt8a* MO and its target sequence of *wnt8a*. The start codons are shown in red. (B) Optimisation of *wnt8a* knockdown. Different amounts of *wnt8a* MO were injected into embryos at the two- to four-cell stage, and expression levels of known *wnt8a*-regulated genes were compared. Note that 10 ng of *wnt8a* MO is adequate to knockdown *wnt8a* activity resulting in downregulation of the known *wnt8a* target genes. Error bars represent s.d. of three technical replicates. (C) Validation of positive control *wnt8a*-regulated gene expression in samples for subsequent RNA-seq analysis. Embryos at the four-cell stage were injected at the marginal zone of both ventral blastomeres with either control MO (CoMO, 2.5 ng per blastomere), *wnt8a* MO (2.5 ng per blastomere), or a combination of *wnt8a* MO (2.5 ng per blastomere) and CSKA-*wnt8a* DNA (6.25 pg per blastomere). mRNA was extracted at the early gastrula stage and analysed by RT-qPCR. Expression levels were normalised to *odc1* and to uninjected control. Expression of known *wnt8a*-regulated genes such as *ventx2.1*, *ventx1.2*, *post*, and *hoxd1* decreased with *wnt8a* MO, and increased with *wnt8a* MO and CSKA-*wnt8a* DNA. In contrast, the known maternal Wnt target *sia1* did not show significant difference in gene expression in these experiments. * $p < 0.1$; ** $p < 0.05$; ns, not significant ($p \geq 0.1$); two-tailed Student's *t*-test. Error bars represent s.e.m. of three biological replicates.

xmc ENSXETG00000010483 ENSXETG00000030701	CGGTGGAAAGATTGTGACCCAGAGCTGCACTGAAGCCCTGGAGGAAGTGTGGAACCTAGGACGGTGTCTGGAAATCTGTACTGTGAACCTAAAGGGTTGGTAAGAATACTGTT 120 -----
xmc ENSXETG00000010483 ENSXETG00000030701	TTTTTTTTTATTATATATAAATTTATTTTAAAGTTAGCACTCTTTATATATACATAGCTGGCTTACTCACTGTAATAGCACTAAGGAGAAATGTCAGACCTGCTGCTTACTATATT 240 -----ATGGCTTTAGTCACTGTAATAGCACTAAGGAGAAATGTCAGACCTGCTGCTTACTATATT -----ATGGCTTTAGTCACTGTAATAGCACTAAGGAGAAATGTCAGACCTGCTGCTTACTATATT 66 *****
xmc ENSXETG00000010483 ENSXETG00000030701	AAAAATCATGGGGACCATATGAATCCCTCAAGAAATTAATAAATTCATGGGATTTAATGAAAGATTTTATAGCTGACAGTGTTTTTAATAAGAACTAAGGAAGCAAAACGCAAAAGGA 360 AAAAATCATGGGGACCATATGAATCCCTCAAGAAATTAATAAATTCATGGGATTTAATGAAAGATTTTATAGCTGACAGTGTTTTTAATAAGAACTAAGGAAGCAAAACGCAAAAGGA 186 AAAAATCATGGGGACCATATGAATCCCTCAAGAAATTAATAAATTCATGGGATTTAATGAAAGATTTTATAGCTGACAGTGTTTTTAATAAGAACTAAGGAAGCAAAACGCAAAAGGA 186 *****
xmc ENSXETG00000010483 ENSXETG00000030701	CTTATCATACAGGGATTGGTCTCTGCTGTTTAAAAATGGCAGAAATTTAAACAAAAGGAGACTGATATCAGAGAACAGGCAGATAAGTATACGACTTTTACTGAGAGAGTCAAAATGAA 480 CTTATCATACAGGGATTGGTCTCTGCTGTTTAAAAATGGCAGAAATTTAAACAAAAGGAGACTGATATCAGAGAACAGGCAGATAAGTATACGACTTTTACTGAGAGAGTCAAAATGAA 306 CTTATCATACAGGGATTGGTCTCTGCTGTTTAAAAATGGCAGAAATTTAAACAAAAGGAGACTGATATCAGAGAACAGGCAGATAAGTATACGACTTTTACTGAGAGAGTCAAAATGAA 306 *****
xmc ENSXETG00000010483 ENSXETG00000030701	AAGATCTCAGAGTTAAGGTTACATGCCATAAACCACTGGGGAGGCATTGCTTGAGAGGCCAATCTATGTGACCACTTAACCAAGTGTAAATGACGAATTTGTCATAAATTTACAGGATGTA 600 AAGATCTCAGAGTTAAGGTTACATGCCATAAACCACTGGGGAGGCATTGCTTGAGAGGCCAATCTATGTGACCACTTAACCAAGTGTAAATGACGAATTTGTCATAAATTTACAGGATGTA 426 AAGATCTCAGAGTTAAGGTTACATGCCATAAACCACTGGGGAGGCATTGCTTGAGAGGCCAATCTATGTGACCACTTAACCAAGTGTAAATGACGAATTTGTCATAAATTTACAGGATGTA 426 *****
xmc ENSXETG00000010483 ENSXETG00000030701	GAAAAGGAGCTGGAGGACATAAACATGCAATAAATATTGCTTTTGATAAGGTTGGGAATAATTCTGACCAACTTTGTGAAAACAATATTTCTAAATTTAAACACAGAAATTAAGGACAT 720 GAAAAGGAGCTGGAGGACATAAACATGCAATAAATATTGCTTTTGATAAGGTTGGGAATAATTCTGACCAACTTTGTGAAAACAATATTTCTAAATTTAAACACAGAAATTAAGGACAT 546 GAAAAGGAGCTGGAGGACATAAACATGCAATAAATATTGCTTTTGATAAGGTTGGGAATAATTCTGACCAACTTTGTGAAAACAATATTTCTAAATTTAAACACAGAAATTAAGGACAT 546 *****
xmc ENSXETG00000010483 ENSXETG00000030701	AAACAATGTACCCCTTGAATGAATTAGATCAAGCATGTAGTTTCCAGCAGCCCTGTGTCACTACAATGTATTAATACTGACATACAGGGAGGTACAGTCATCTCAAAACAA 840 AAACAATGTACCCCTTGAATGAATTAGATCAAGCATGTAGTTTCCAGCAGCCCTGTGTCACTACAATGTATTAATACTGACATACAGGGAGGTACAGTCATCTCAAAACAA 666 AAACAATGTACCCCTTGAATGAATTAGATCAAGCATGTAGTTTCCAGCAGCCCTGTGTCACTACAATGTATTAATACTGACATACAGGGAGGTACAGTCATCTCAAAACAA 666 *****
xmc ENSXETG00000010483 ENSXETG00000030701	TTAAAGCCTCAAGAGATGGATGCAATAGTTAGAGAGATTGGGCAAGTACCAAGATATGATATCAACAGATTCAATGAAGTGGTCTGTGAGTTGCAAGGGTCAGAGAGAGCTAATAATTG 960 TTAAAGCCTCAAGAGATGGATGCAATAGTTAGAGAGATTGGGCAAGTACCAAGATATGATATCAACAGATTCAATGAAGTGGTCTGTGAGTTGCAAGGGTCAGAGAGAGCTAATAATTG 786 TTAAAGCCTCAAGAGATGGATGCAATAGTTAGAGAGATTGGGCAAGTACCAAGATATGATATCAACAGATTCAATGAAGTGGTCTGTGAGTTGCAAGGGTCAGAGAGAGCTAATAATTG 786 *****
xmc ENSXETG00000010483 ENSXETG00000030701	AATCCTGAGGATGTAGATAGAGTGTACAGAGAGTGTGAAATGTTTATGGTAAAGGATTGTACAGAACTGTGACAGCAGCCAGGACAAAGGATGTGTTAAAGGAAATCTTAGA 1080 AATCCTGAGGATGTAGATAGAGTGTGACAGAGTGTGAAATGTTTATGGTAAAGGATTGTACAGAACTGTGACAGCAGCCAGGACAAAGGATGTGTTAAAGGAAATCTTAGA 903 AATCCTGAGGATGTAGATAGAGTGTGACAGAGTGTGAAATGTTTATGGTAAAGGATTGTACAGAACTGTGACAGCAGCCAGGACAAAGGATGTGTTAAAGGAAATCTTAGA 906 *****
xmc ENSXETG00000010483 ENSXETG00000030701	GCATTGTATGATATCACTCAAAATGTGCTTTTGTCCGTTAAATTAAGCAAAATGAAGCAGGAATGTCTTATGAATTTATCAGATAGATAGCCACAGTAATGGAACAAATTTCAATTTGAT 1200 GCATTGTATGATATCACTCAAAATGTGCTTTTGTCCGTTAAATTAAGCAAAATGAAGCAGGAATGTCTTATGAATTTATCAGATAGATAGCCACAGTAATGGAACAAATTTCAATTTGAT 1023 GCATTGTATGATATCACTCAAAATGTGCTTTTGTCCGTTAAATTAAGCAAAATGAAGCAGGAATGTCTTATGAATTTATCAGATAGATAGCCACAGTAATGGAACAAATTTCAATTTGAT 1026 *****
xmc ENSXETG00000010483 ENSXETG00000030701	AATCCAGGATTTGAACATGGCGGTTTAAATTCATAGGTCATGTTGTTAGAGCTTTAGAGGAGGATGTCAAGGAAGGAATATTATCTATCACTCCTGATCCTACGGATCTAAAAGACATT 1320 AATCCAGGATTTGAACATGGCGGTTTAAATTCATAGGTCATGTTGTTAGAGCTTTAGAGGAGGATGTCAAGGAAGGAATATTATCTATCACTCCTGATCCTACGGATCTAAAAGACATT 1143 AATCCAGGATTTGAACATGGCGGTTTAAATTCATAGGTCATGTTGTTAGAGCTTTAGAGGAGGATGTCAAGGAAGGAATATTATCTATCACTCCTGATCCTACGGATCTAAAAGACATT 1146 *****
xmc ENSXETG00000010483 ENSXETG00000030701	CTGTTGAGGGCAGATAATTTGTGCGAAGGAAGAAATGAAGGAGAAATTTTGTAGTGATGCTGCTAGAAATTTTGAAGTAGAGGGACAGCAGAGAAAGGATGTGTTGATTTTAAAGGG 1440 CTGTTGAGGGCAGATAATTTGTGCGAAGGAAGAAATGAAGGAGAAATTTTGTAGTGATGCTGCTAGAAATTTTGAAGTAGAGGGTCAACAAAGGAAGGATGTGTTGATTTTAAATGG 1263 CTGTTGAGGGCAGATAATTTGTGCGAAGGAAGAAATGAAGGAGAAATTTTGTAGTGATGCTGCTAGAAATTTTGAAGTAGAGGGACAGCAGAGAAAGGATGTGTTGATTTTAAAGGG 1266 *****
xmc ENSXETG00000010483 ENSXETG00000030701	CCTAGGCAGGGAAATATTCTCAATTCAGAAAGCCAGAAAGAAATTAAGATTCAGATGGCAGAAATAGACAAACAAATCAAGAGATTGCAAAATCTAGAACTTGGATACCCCTTTCC 1560 CCTCAAGAGGAAGGGGTTTAAATTCAGAGCCAGAAAGAAATTAAGATTCAGATGGCAGAAATAGACAAACAAATCAAGATTTCCATTAATCTAGAACTTGGATACCCCTTTCC 1383 CCTAGGCAGGGAAATATTCTCAATTCAGAAAGCCAGAAAGAAATTAAGATTCAGATGGCAGAAATAGACAAACAAATCAAGAGATTGCAAAATCTAGAACTTGGATACCCCTTTCC 1386 ***
xmc ENSXETG00000010483 ENSXETG00000030701	CAACTAAAAGGGAACATGAAAACCTTAAATTTGAATATGATAGATCAGGGCTGAAAGGGATCTTTTAAACAAACAGTTCTTTACTGGAACAGAACTTTTCAAAATTAAGGGGTGCC 1680 CAACTAAAAGGGAACATGAAAACCTTAAATTTGAATATGATAGATCAGGGCTGAAAGGGATCTTTTAAACAAACAGTTCTTTACTGGAACAGAACTTTTCAAAATTAAGGGGTGCC 1503 CAACTAAAAGGGAACATGAAAACCTTAAATTTGAATATGATAGATCAGGGCTGAAAGGGATCTTTTAAACAAACAGTTCTTTACTGGAACAGAACTTTTCAAAATTAAGGGGTGCC 1506 *****
xmc ENSXETG00000010483 ENSXETG00000030701	CCTCTTACACATTTCTGTTTAAAGACCATGGGGAGGAACTTTAATAGAATTTTAAACCCATACATGCCACTTAAATGTTCAATTGCACTTTCCTTTAATTTATCTCGAGCAATGTAA 1800 CCTCTTACACATTTCTGTTTAAAGACCATGGGGAGGAACTTTAATAGAATTTTAAACCCATACATGCCACTTAAATGTTCAATTGCACTTTCCTTTAATTTATCTCGAGCAATGTAA 1560 CCTCTTACACATTTCTGTTTAAAGACCATGGGGAGGAACTTTAATAGAATTTTAAACCCATACATGCCACTTAAATGTTCAATTGCACTTTCCTTTAATTTATCTCGAGCAATGTAA 1563 *****
xmc ENSXETG00000010483 ENSXETG00000030701	TTTATGTAGATATATGAATTAATATTTTCTCTAAGTTTATCTTCCAGGAAAGATCTCAAGCAGAAAGGATAAATGTGCTGAATTTTGTGGTCAATGTTAACTGTTTGTGTTT 1920 -----
xmc ENSXETG00000010483 ENSXETG00000030701	TTTTTCTTCTATCTCACACTTAAGCAGCTTTTCAAAATTTTAAATGACAGTAAACATTGGTAGATGAGACCTTAAAGTTTCCAAAGTGGGGGTGTGAAGGAATTTGATGACACAGAGG 2040 -----
xmc ENSXETG00000010483 ENSXETG00000030701	AGAGGTGTTGGTGTGACCCACAGAGTTTTTACTGGATGCAATGTGCAATGGGCTGGAT 2105 -----

Fig. S4. Sequence similarity of *xmc* genes.

ClustalW2-based multiple sequence alignment of DNA sequences of the *Xenopus tropicalis* homologue of *Xenopus laevis* *marginal coil* (*xmc*) [GenBank: XM_002944874.1] and of two *xmc*-like genes (*ENSXETG00000010483* and *ENSXETG00000030701*). *ENSXETG00000010483* and *ENSXETG00000030701* show 97% and 98% identities with the *Xenopus tropicalis* *xmc* gene, respectively.

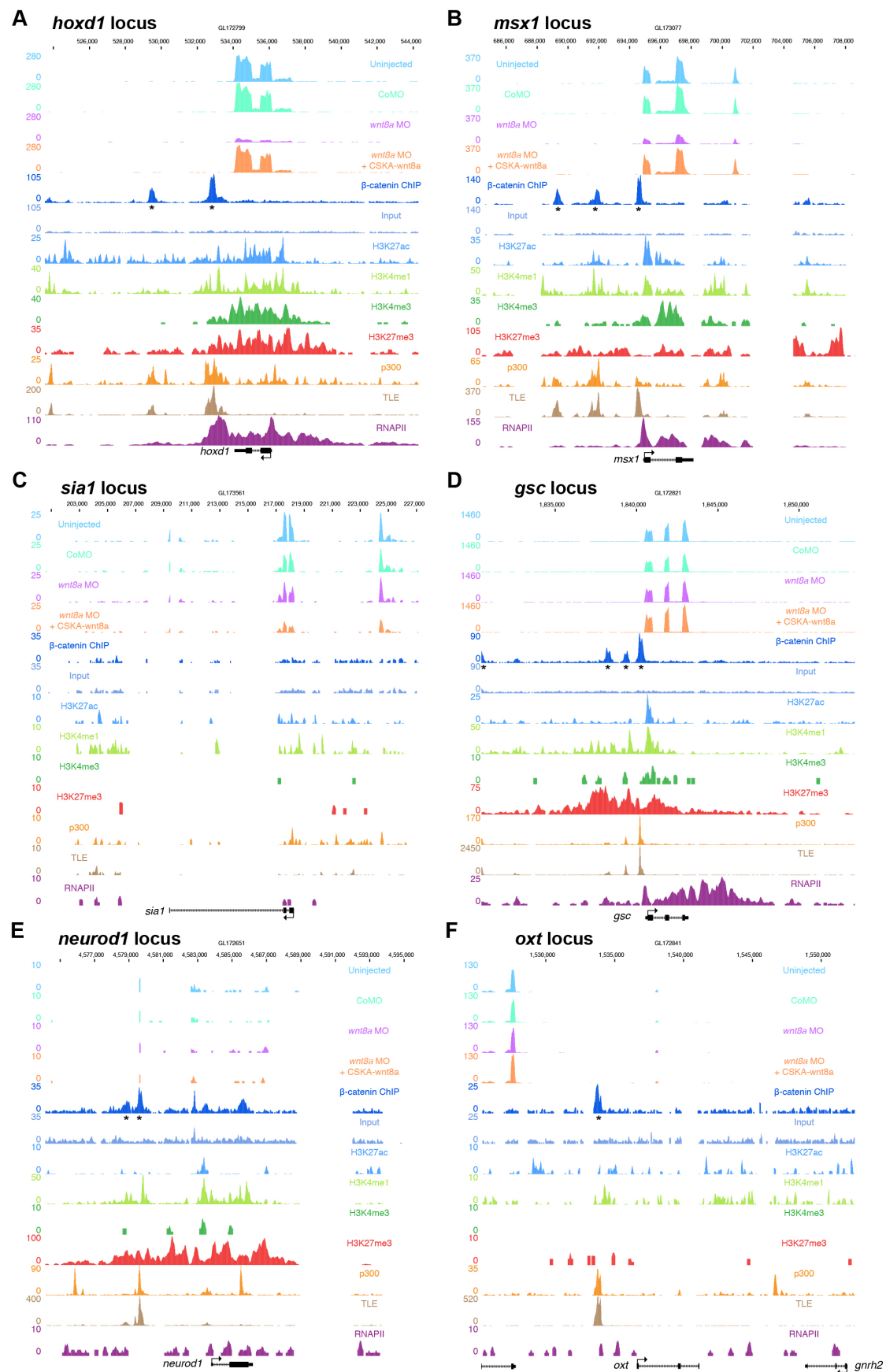


Fig. S5. Genome browser representation of ChIP-seq and RNA-seq data in exemplary genes.

Genomic loci of *wnt8a*-regulated target genes *hoxd1* (A), *msx1* (B), maternal Wnt/ β -catenin target *sial* (C), maternal Wnt regulated gene *gsc* (D), non-*wnt8a*-regulated gene *neurod1* (E) and transcriptionally silent gene *oxt* (F) are visualised as indicated with RNA transcript profiles of four experimental samples from RNA-seq analysis (Uninjected, CoMO-, *wnt8a* MO-, and *wnt8a* MO + CSKA-*wnt8a* DNA-injected), β -catenin ChIP-seq data (β -catenin ChIP and Input), ChIP-seq data of histone marks (H3K27ac, H3K4me1, H3K4me3, and H3K27me3) and RNA polymerase II (RNAPII), and of transcriptional co-factors (p300 and TLE). *denotes β -peak position identified by peak calling with the IDR method. Note β -peaks correlate with the histone mark and transcriptional co-factor peaks. *hoxd1* and *msx1* show RNA transcript profiles that correlate with RNA-seq experimental conditions where they are expressed at low levels in *wnt8a* MO but expressed in the re-instatement *wnt8a* MO + CSKA-*wnt8a* at comparable levels to the Uninjected and CoMO controls. There is no β -peak in the proximal promoter of the *sial* locus, which has been shown to contain functional TCF/LEF-binding sequences mediating response to maternal Wnt/ β -catenin signalling. The *gsc* locus contains multiple β -peaks that correlate with p300 and TLE peaks. The *neurod1* and *oxt* loci associate with two β -peaks (-4 kb and -5kb regions) and one β -peak (-3 kb region), respectively, but mRNA expression levels are low and remain unaffected by change in Wnt8a activity.

A *de novo* motif discovery with 10,009 β -peaks from 5,009 non-*wnt8a*-regulated genes

MEME				DREME			
Motif logo	E-value	Sites	TF	Motif logo	E-value	Sites	TF
	1.8e-1845	2,614	AP2		2.1e-205	2,525	SOX
	2.7e-470	784	ZNF		1.9e-186	5,220	FOX
	2.9e-577	3,835	SOX		3.3e-206	1,487	AP2
	5.5e-445	1,115	ZIC		4.8e-099	1,902	POU/Oct-4
	1.5e-210	554	TBX		3.6e-079	3,650	SOX
	3.9e-169	2,409	POU/Oct-4		1.0e-061	463	TBX
	2.7e-147	696	FOX		1.2e-053	1,073	ZIC
	1.5e-071	211	Eve		6.8e-036	1,152	TCF/LEF
	6.1e-098	387	TCF/LEF		1.2e-026	515	ZNF
	1.0e-039	73	NR4A2		7.4e-026	377	AP2
	3.4e-005	105	Homeobox		1.1e-025	782	TCF/LEF

B *de novo* motif discovery with 624 β -peaks from 179 *Wnt8a*/ β -catenin target genes

MEME				DREME			
Motif logo	E-value	Sites	TF	Motif logo	E-value	Sites	TF
	1.9e-066	143	AP2		5.4e-014	166	POU/Oct-4
	3.3e-033	43	ZNF		4.2e-011	158	SOX
	2.2e-029	80	TBX		1.2e-006	82	TBX
	2.3e-026	170	SOX		1.6e-007	93	AP2
	6.4e-010	63	FOX		6.0e-005	178	FOX
	2.5e-007	65	No hit		1.1e-002	57	Homeobox
	1.3e-003	77	SOX/FOX		1.8e-002	39	Homeobox
					3.8e-002	31	TCF/LEF

Fig. S6. *De novo* motif discovery on β -peaks in comparing non-*wn8a*- and *wnt8a*-regulated genes.

De novo motif search was performed on β -peaks of 5,009 non-*wnt8a* regulated genes (A) and 179 *wnt8a*-regulated genes (B), using the MEME-ChIP software with MEME and DREME search functions. *De novo* motif logo, e-value, the number of sites containing the motif, and transcription factor names having similar target motif are indicated. Top eleven motifs are shown for non-*wnt8a*-regulated genes.

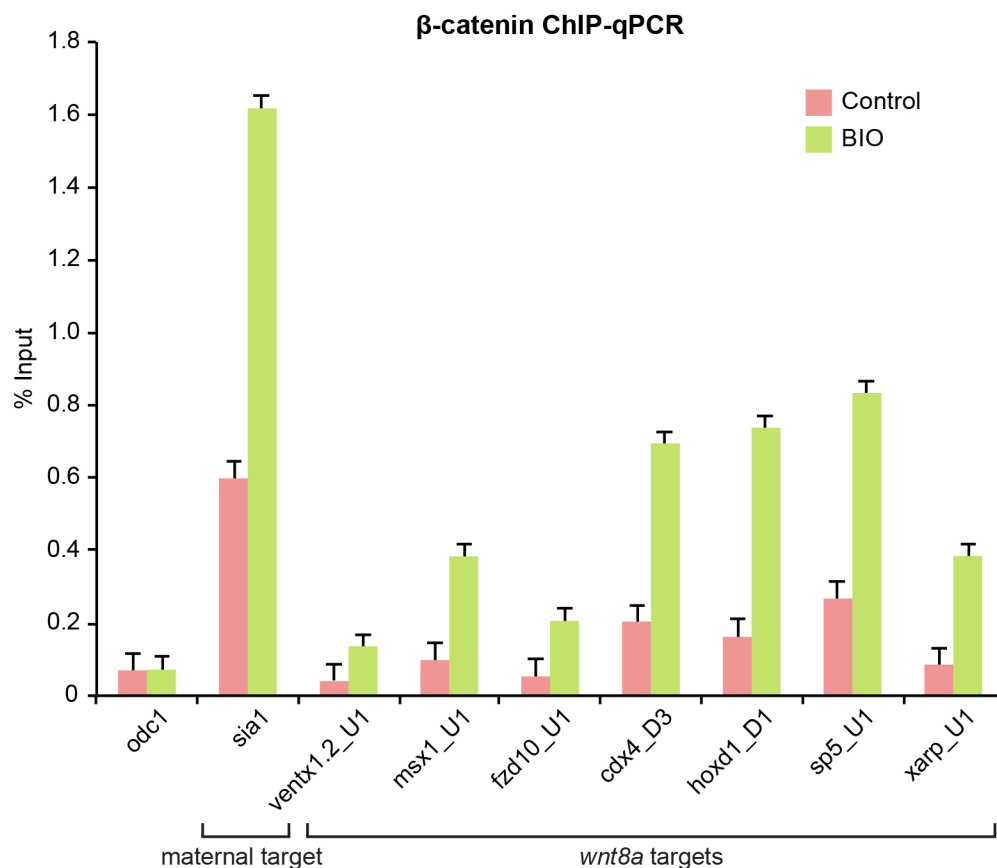


Fig. S7. Maternal Wnt signalling regulates β -catenin recruitment to both maternal Wnt target and zygotic *wnt8a* target loci.

In order to experimentally activate maternal Wnt/ β -catenin signalling, embryos were treated with the glycogen synthase kinase-3 inhibitor BIO at the four-cell stage until the 1000-cell stage, when the embryos were collected for β -catenin ChIP analysis to compare β -catenin occupancy levels with untreated control embryos. Enhanced activity of maternal Wnt signalling with BIO increased β -catenin binding levels at both maternal Wnt target *sia1* and zygotic *wnt8a* target gene loci. Error bars represent s.d. of two biological replicates.

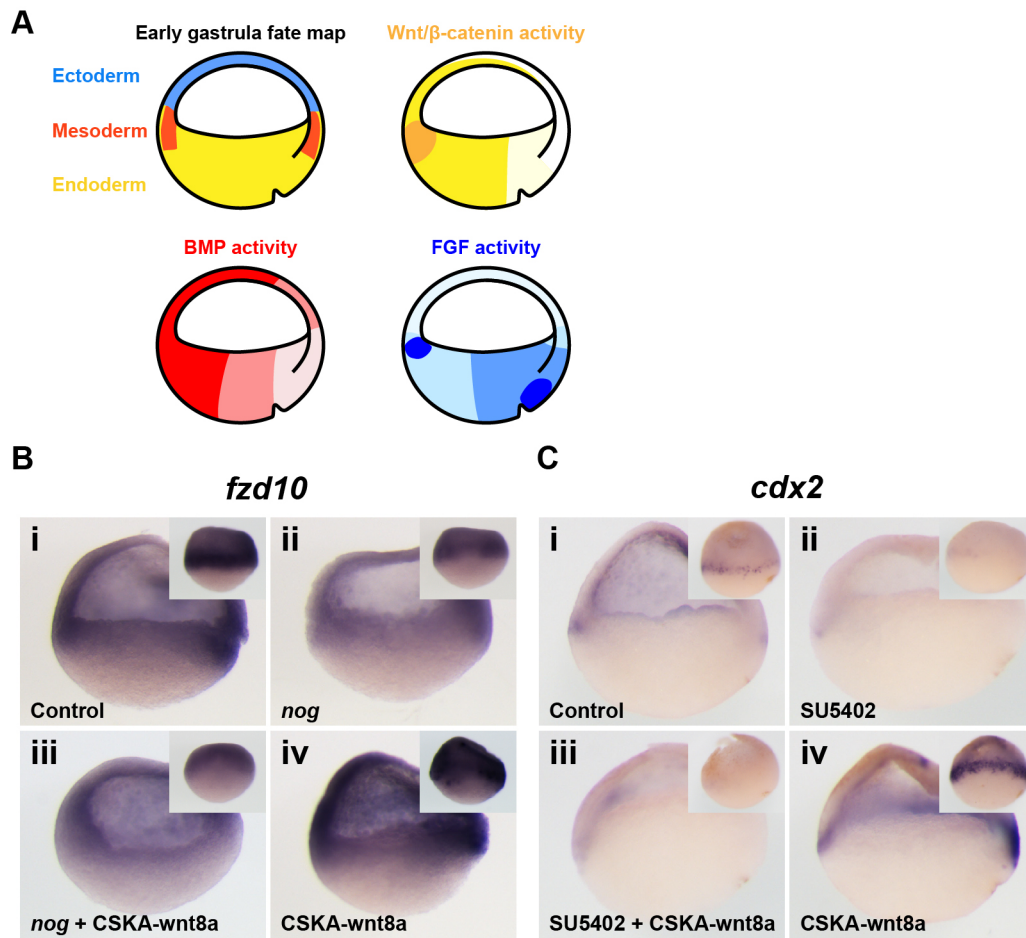


Fig. S8. BMP or FGF signalling is required for context-specific regulation of *wnt8a* target gene expression.

(A) Schematic diagrams of fate map in early gastrula and of Wnt/ β -catenin, BMP, and FGF signalling pathway activities (Schohl and Fagotto, 2002). Regions with higher pathway activities are shown in darker colours. (B,C) *In situ* hybridisation shows expression of *fzd10* (B) and *cdx2* (C) in sagittal sections and lateral views (insets) of control uninjected, untreated embryos and experimentally manipulated embryos as indicated, with dorsal right.

Table S1. β -peaks identified in β -catenin ChIP-seq data.

Sheet 1: 68,045 peaks called by MACS2 (version 2.0.10)(p-value cutoff of 0.01) using β -catenin ChIP-seq data. “fold-change” was used for IDR analysis. Sheet 2: 60,888 peaks called by SPP (version 1.10.1)(FDR cutoff of 0.1) using β -catenin ChIP-seq data. “signalValue” was used for IDR analysis. Sheet 3: 10,638 β -catenin ChIP-seq peaks (β -peaks) identified by the irreproducible discovery rate (IDR) method (IDR threshold of 0.01) using the MACS2 and SPP peaks. The position of each β -peak (“peakname”) is indicated by “scaffold”, “matchstart” and “matchend”, with “summit” of the peak. The nearest genes of β -peaks were annotated using distanceToNearest function (rtracklayer version 1.2.26 and GenomicRanges version 1.12.5). The nearest genes are indicated by “gene” and “gene name” with their start (“genestart”) and end (“geneend”) positions, and direction of gene-encoding (“genestrans”) on the scaffold. “genedistance” indicates the distance of a β -peak from the transcriptional start site of the nearest gene. “IDR” shows IDR of each β -peak.

[Click here to Download Table S1](#)

Table S2. Identification of *wnt8a*-regulated genes from RNA-seq analysis data.

Sheet 1: 14 *wnt8a*-regulated target genes (shortlisted). The identified *wnt8a* targets are indicated with their “Gene ID”, “Gene Name”, and “Gene Function” using the Xenbase annotation. “Fold change” and “FDR” indicate those values of “*wnt8a* knockdown” experiments (compared CoMO control) or of “Wnt8a re-instatement” experiments (compared *wnt8a* MO) obtained from GLM analysis. Sheet 2: gene list from GLM analysis of *wnt8a* knockdown, including Uninjected, CoMO, and *wnt8a* MO samples (FDR < 0.1). “Down” and “Up” indicate decreased and increased expression of corresponding gene in *wnt8a* MO against CoMO condition, respectively. Sheet 3: gene list from GLM analysis of Wnt8a re-instatement, including Uninjected, CoMO, *wnt8a* MO, and *wnt8a* MO + CSKA-*wnt8a* samples (FDR < 0.1). “Down” and “Up” indicate negative and positive regulation of expression of corresponding gene in *wnt8a* MO + CSKA-*wnt8a* against *wnt8a* MO condition, respectively. Sheet 4: pair-wise analysis of Uninjected and CoMO (FDR < 0.1). “Down” and “Up” indicate downregulation and upregulation of corresponding gene in CoMO against Uninjected condition, respectively. These genes affected by CoMO injection were excluded from analysis identifying *wnt8a*-regulated genes.

[Click here to Download Table S2](#)

Table S3. β -peaks of *wnt8a*-regulated genes.

Sheet 1: a list of 179 genes that are positively regulated by Wnt8a signalling and that are associated with β -peaks. The 179 *wnt8a*-positively regulated genes contain 624 β -peaks (see also Table S1 for details). Sheet 2: a list of β -peaks associated with 13 shortlisted Wnt8a/ β -catenin target genes. The β -peaks were named as U (upstream) or D (downstream) plus a number corresponding to the position of the peak relative to the transcriptional start site.

[Click here to Download Table S3](#)

Table S4. β -peaks of maternal Wnt-regulated genes.

A list of β -peaks associated with genes that are transcriptionally regulated by maternal Wnt signalling (see text for detail). The β -peaks were named as U (upstream) or D (downstream) plus a number corresponding to the position of the peak relative to the transcriptional start site.

[Click here to Download Table S4](#)

Supplementary Materials and Methods:

Embryo experiments

Xenopus tropicalis (Gray) were purchased from Nasco (<http://www.enasco.com/xenopus/>) and the European Xenopus Resource Centre (EXRC)(<http://www.port.ac.uk/research/exrc/>). Adult female and male *X. tropicalis* were primed with 10 units of human chorionic gonadotropin (HCG) 12 hours before boosting. 5 hours before embryo collection, female and male frogs were boosted with 200 units and 100 units of HCG, respectively. For natural mating, single pairs of female and male frogs were placed together, and embryos were harvested approximately every 1 hour after the onset of egg laying. For *in vitro* fertilisation, testes were dissected from euthanized male frogs and kept in 1x Modified Barth's Saline (MBS) + 0.1% BSA on ice. The testes were subsequently minced and used to fertilise eggs. Fertilised embryos were dejellied in 3% cysteine/0.1x Marc's Modified Ringer (MMR), washed with 0.1x MMR, and kept at 18°C until they developed to desired stages. Injection experiments were performed by microinjecting the following reagents (MOs, mRNA, and DNA) into the marginal zone of two ventral blastomeres of four-cell-stage embryos for the RNA-seq, and into the marginal zone of each blastomere of embryos at the two- to four-cell stage for other experiments:

- CoMO and *wnt8a* MO, 2.5 ng per blastomere: 5 ng per embryo for the RNA-seq analysis (Fig. 2A; Fig. S3C) and 10 ng per embryo for the other experiments;
- CSKA-wnt8a DNA, 6.25 pg per blastomere: 12.5 pg per embryo for the RNA-seq analysis (Fig. 2A; Fig. S3C) and 25 pg per embryo for the re-instatement experiments (Fig. 2C) and 100 pg for the overexpression experiments (Figs. 4C,D, 5;
- *wnt8a* mRNA, 40 pg per embryo;
- *axin* mRNA, 2 ng per embryo;
- *nog* mRNA, 500 pg per embryo.

SU5402

SU5402 (Sigma, SML0443) stock was made in DMSO and freshly diluted to 50 μ M in 0.1x MMR before use.

BIO

BIO (Tocris Bioscience, 3194) stock was made in DMSO and freshly diluted to 20 μ M in 0.1x MMR before use.

pCSKA-wnt8a construct

pCSKA-wnt8a plasmid DNA construct was created by introducing nucleotide substitutions and Kozak consensus sequence into the *Xenopus laevis* (Daudin) *wnt8a* (*Xwnt8*) gene. A *Xwnt8* DNA fragment was PCR-amplified from pCSKA-Xwnt-8 (Christian and Moon, 1993) as a template, using the following forward and reverse primers (restriction enzyme sites are underlined and Kozak consensus sequence is *italic* and *bold*):
 5'-CCATCGATGCCGCCACCATGCAGAATACTACCCTTTTTATCCTTGCAAC
 TCTTCTG-3' and 5'-GGAATTCTCATCTCCGGTGGCCTC-3'. The resulting PCR product was digested with ClaI and EcoRI and subcloned into pCSKA digested with

ClaI and EcoRI, which resulted in the rescue construct carrying *wnt8a* coding sequence that is not recognised by the *wnt8a* MO.

ChIP

Embryos were harvested at the developmental stage of interest and fixed at room temperature with 1% formaldehyde in phosphate-buffered saline (PBS) for 45 minutes (1000-cell stage embryos) or for 30 minutes (early gastrulae). Immediately after fixation, the embryos were incubated with 125 mM glycine/PBS for 10 minutes and washed three times with ice-cold PBS for 5 minutes. Batches of 50 embryos were snap-frozen in liquid nitrogen and stored at -80°C for future use. For the following method, all solutions and samples were kept on ice. RIPA buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% IGEPAL CA-630, 0.25% Sodium deoxycholate, 0.1% SDS, 0.5 mM DTT) supplemented with Protease Inhibitor Cocktail (Sigma, P8340) was added to frozen embryos. Embryos were thawed on ice for 10-15 minutes, homogenised, and then kept on ice for 10 minutes. After re-homogenisation, the embryo extracts were transferred to TPX microtubes (Diagenode) and sonicated during 2.5 rounds of 10 cycles with 30 seconds ON/30 seconds OFF at high power setting using the Bioruptor Plus Instrument (Diagenode). The sonicated samples were centrifuged at 14,000 rpm for 10 minutes at 4°C, and the supernatant was transferred to a 1.5ml tube for subsequent use for ChIP and input samples. A small aliquot of the supernatant was used for checking chromatin shearing. The input samples were stored at -20°C for later usage. The supernatant for ChIP were incubated for 1 hour at 4°C with Dynabeads Protein G (Life technologies) that had been blocked with 5% BSA/PBS for 1 hour at 4°C. After snap-spin, the supernatant was transferred to a 1.5ml safe-lock tube and incubated with antibodies (2 ug) overnight at 4°C. On the following day, chromatin was precipitated with 5% BSA/PBS-blocked Dynabeads Protein G for 1 hour at 4°C and then the beads were successively washed with ChIP buffer 1 (20 mM Tris pH 8.0, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.1% SDS), ChIP buffer 2 (20 mM Tris pH 8.0, 500 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.1% SDS), ChIP buffer 3 (10 mM Tris pH 8.0, 250 mM LiCl, 1 mM EDTA, 1% IGEPAL CA-630, 1% Sodium deoxycholate), ChIP buffer 4 (10 mM Tris pH 8.0, 1 mM EDTA) for 5 minutes each. Chromatin was eluted from the beads with elution buffer (50 mM Tris pH 8.0, 10 mM EDTA, 1% SDS) for 20 minutes in a thermoshaker (65°C, 900 rpm). At this stage, the frozen input samples were supplemented with elution buffer. ChIP and input samples were incubated with RNase A at 37°C for 30 minutes. The samples were then added with NaCl and incubated for over 16 hours in a thermoshaker (65°C, 900 rpm). The samples were further treated with proteinase K for 2 hours in a thermoshaker (65°C, 900 rpm). Eventually, the de-crosslinked DNA fragments were purified with phenol:chloroform:isoamylalcohol and precipitated in ethanol for qPCR or using MinElute Reaction Cleanup Kit (QIAGEN) for sequencing.

ChIP-seq

Three independent β -catenin ChIP experiments were performed as described above, and sheared chromatin was collected from approximately 750 early gastrula embryos (stage 10.25) in total. Each ChIP DNA and input control DNA was purified using MinElute Reaction Cleanup Kit (QIAGEN) and pooled to one sample. The purified DNA was quantified using Qubit dsDNA HS Assay Kits (Life technologies) by Qubit 2.0 Fluorometer (Life technologies). Two Illumina TrueSeq ChIP libraries were constructed from the ChIP DNA and the input control DNA samples and sequenced

using 50 bp single-end reads by Illumina HiSeq 2500 at The Genome Analysis Centre (TGAC, Norwich, UK). After quality control using FasQC, sequenced reads were mapped to the *X. tropicalis* genome assembly JGI 4.2/xenTro3 using bwa (version 0.7.5a) (Li and Durbin, 2009). Multi-mapped reads were filtered with samtools version 1.1.19 (Li et al., 2009). In order to focus our analysis on a set of highly reliable peaks we performed peak calling using two different methods [SPP (version 1.10.1)(Kharchenko et al., 2008) and MACS2 (version 2.0.10.20120913)(Zhang et al., 2008)] and then used IDR (Li et al., 2011) to identify the peaks, which were reproducible using the two methods. This approach has an advantage over using the overlap of the peaks obtained using the two methods since IDR quantitatively assesses when the findings are no longer consistent across replicates. The ranked list of peaks for both methods was generated with a low confidence threshold, which is necessary for IDR to separate signal from noise. We used $p \leq 0.01$ for MACS2 and $FDR \leq 0.1$ for SPP resulting in 68,045 and 60,088 peaks respectively, which include both high and low quality peaks. These were used as input for IDR with threshold of 0.01 and resulted in 10,638 peaks. Peaks were assigned to closest genes using distanceToNearest function (rtracklayer version 1.2.26 and GenomicRanges v 1.12.5). For visualising heat maps, in addition to β -catenin ChIP-seq data, ChIP-seq data were used of H3K27ac, H3K4me1, p300 and TLE (all of these from *X. tropicalis* early gastrula whole embryos at stage 10.5) from Yasuoka et al. (2014) and of H3K4me3 and H3K27me3 (each of these from *X. tropicalis* gastrula whole embryos at stage 11-12) from Akkers et al. (2012). The coverage for each base was divided by the total number of all bases of ChIP-seq data, and then normalised by multiplying a million. Using the normalised ChIP-seq data, a heatmap data matrix files were generated using HOMER (Heinz et al., 2010), clustered using Cluster 3.0 with *k*-means clustering ($k=10$)(de Hoon et al., 2004), and visualised using Java Treeview (version 1.1.6r4)(Saldanha, 2004). Histograms of β -catenin occupancy level around the peak summit were generated using HOMER with bin size in 100 bp and visualised in Excel. For *de novo* motif discovery, DNA sequences of 100 bp regions centred on the peak summit were analysed using MEME-ChIP with MEME and DREME algorithms coupled with CentriMo and Tomtom algorithms to find the motif width from 5 to 10 nucleotides (Ma et al., 2014). Genome browser representation files were generated by converting ChIP-seq data to bigWig format. This was done using genomeCoverageBed from bedtools v 2.17.0 to generate a bed file then UCSC bedGraphToBigWig to convert the bed to bigWig format.

RNA-seq

X. tropicalis embryos were injected into both ventral blastomeres at the four-cell stage. Total RNA was extracted as described in Lee-Liu et al. (2012) from stage 10.25 uninjected embryos and embryos that had been injected with MOs (2.5 ng per blastomere) or together with pCSKA-wnt8a (6.25 pg per blastomere). The quality of total RNA was assessed using Agilent 2100 Bioanalyzer whether the RNA integrity number was 7 or higher. Illumina TruSeq RNA libraries were constructed from 12 total RNA samples (biological triplicates of each experimental conditions: uninjected, CoMO-injected, *wnt8a*MO-injected, *wnt8a*MO and pCSKA-wnt8a-coinjected). The libraries were sequenced using 100 bp paired-end reads on Illumina HiSeq 2000 at TGAC. Sequenced reads were checked for base qualities, trimmed where 20% of the bases were below quality score 20, and filtered to exclude sequences that were shorter than 20 bp using Fastx (Version 0.0.13). Sequences were aligned to the *X. tropicalis* genome JGI 4.2/xenTro3 with gsnap (Wu and Watanabe, 2005) with parameters -B 4

-E 100 -N 1. Aligned reads were counted using HTSeq (version 0.5.4p2) with parameters -m intersection-strict -s no -a 20 and further differential gene expression analysis was carried out using DESeq2 (version 1.0.19)(Love et al., 2014) with defaults. To identify genes that are differentially expressed in *wnt8a* knockdown or Wnt8a re-instatement, we used generalised linear model (GLM) analysis as follows. Differentially expressed genes for *wnt8a* knockdown were obtained using a GLM with explanatory variables “CoMO control”, “*wnt8a* knockdown” and tested against the null hypotheses where the “*wnt8a* knockdown” variable was excluded. Differentially expressed genes for Wnt8a re-instatement were obtained using a GLM with explanatory variables “CoMO control”, “*wnt8a* knockdown”, “Wnt8a re-instatement” and tested against the null hypotheses where the “Wnt8a re-instatement” variable was excluded. DESeq2 was used to normalise the counts by size factor, estimate dispersions and perform Wald test on a negative binomial model. The p-values were adjusted for multiple testing with the Benjamini-Hochberg procedure using DESeq2’s default setting. RNA-seq profiles was displayed on the UCSC genome browser as described above for ChIP-seq data.

RT-qPCR primers

<i>odc1</i> :	F:	TTTGGTGCCACCCTTAAAAC
	R:	CCCATGTCAAAGACACATCG
<i>hoxa1</i> :	F:	ACCAACTTCACCACCAAACAGC
	R:	AGAGCAGCAGCAATTTCTACCC
<i>hoxd1</i> :	F:	CAAGTATCTCACCAGGGCAAG
	R:	GAGTTTTTACGCAGATACTGGATG
<i>sp5</i> :	F:	ACTCAGATTGCTGCACTACTGC
	R:	ACCACTGGAAGTTTGGCAGTTG
<i>msgn1</i> :	F:	AACCTTCCATGACAGTCCAACG
	R:	AAATTGTTGCGCAGGGTGTG
<i>cdx2</i> :	F:	AATCTGGGGCTTTCGGAGAGAC
	R:	ATTTTGGCCAGTCTGAGTCTGC
<i>msx1</i> :	F:	TTTAGGTTTGGGGAGCTTGGC
	R:	AACGCAAACAGACAGTGCTG
<i>cdx4</i> :	F:	TTTGTCTCACACAGCTGCCAAC
	R:	AAGTCGATTGCACGGTTTTCCC
<i>fzd10</i> :	F:	ATTTAGCAGCCTGGGCAATTCC
	R:	ATTGACATCCATGCTGCCAACG
<i>xarp</i> :	F:	ACGCTTTTCCGCATGTACTTGG
	R:	TTGATGTCTTGGGTTCACACGG
<i>xmcl1</i> :	F:	ACAATTCAGACCGCCAGAAAGG

	R:	TGGGGAAAGGGTATCCAAGTTC
<i>xmcl2:</i>	F:	ACTTGGATACCCTTTCCCCAAC
	R:	AAGAGGGGCACGCCTTAATTTG
<i>ventx1.2:</i>	F:	GGATTCTCTATTGACCTCATTC
	R:	CTTTCTCCTTGGTATCTCCTTG
<i>gbx2.2:</i>	F:	TGCATTCTGTTCCAGCCTTGC
	R:	TTTCAAAGGCCCCATGCAGAC
<i>neurog1:</i>	F:	AGTAAAGAATGACGCCGTGCTG
	R:	TTGTGCATTCGGTTCCTTTCCC
<i>atpl2a:</i>	F:	AAGCAATTGCACGGTGTGTAGG
	R:	TTCACCACACAAGCACATGC
<i>sial:</i>	F:	TTGACCCCCTAGTCAACAGC
	R:	ACCAGCGGCCTCTTACATT
<i>nodal3.1:</i>	F:	AGGAAGGTGGACATGTTTGTGG
	R:	GCATCGTCCGTCTCATTCAAGTGG
<i>gsc:</i>	F:	GTTGCACGTACAGACGCCTA
	R:	TAAGGGAGCATTTGGTGAGG
<i>chrd:</i>	F:	TGAAGCAGTGGGATTCTAGAGG
	R:	GGCAGGATTTAGAGTTGCTTC
<i>nog:</i>	F:	AGGTTTTGGCCTCGCTATGTG
	R:	TGGCAGCTTTGCAAACCATG
<i>fst:</i>	F:	AAGAAGAACAAGCCGAGGTGTG
	R:	TTTGCCATCTATTCCGCACACG
<i>frzb:</i>	F:	AACGCTCACTGTGCTTCATGTG
	R:	AATGGCATTGGCTTGAGTGC
<i>wnt8a:</i>	F:	CTGCAGTGATAATGCAGAATTTG
	R:	TGCAAGTCTTCCTGCTTCATTG
<i>ventx2.1:</i>	F:	GGCTTCTGAGAGACGGAAAC
	R:	TTGTATTTTCATCCTGCGGTTC
<i>post:</i>	F:	AAGCAAGGTGGGATACAGTGAG
	R:	CTGATTGGGGGCTAAAGAGAG

ChIP-qPCR primers

odc1	F: GTGCACGCCTGAATTCTTTCT R: GGCTCAGCAATGATGGTCACT
ventx1.2_U1	F: GCCCATTCTGATAGCTATTATCCA R: AGTTGTGTGTACACAAAGCCTATG
msx1_U1	F: CGCTCCTATTAAACCGGCTTAGC R: GCTCTTGTTGTTGACTCGCTTC
msx1_U2	F: TGCGAGTTAACCTCCTCAATGG R: GCGCCTGCATTGCTAATTGC
mshn1_U1 (-186)	F: GACCAGTCCATTTTCCATGTTGA R: GGCCCTTTTATACAGACCTGCTAA
neurog1_D1	F: GAAGCTGAAACAAGCAAGCC R: TTACGGGCAGCCAATCACAG
cdx4_D3	F: GCTCATTGTCTTCTCCTAGCTCAG R: TCCATCTCCCTTTGATCCTTCC
cdx2_U1	F: AGGGGGTCTTTGTTCTTCCTTG R: AGGGGCAGATGTATAGGCACTG
hoxd1_D1 (3134/3181)	F: TGTTGTAGATGCTGATGCTTATCG R: AACAGAAAATCAAAGGCTTGCA
hoxa1_D1	F: TCTAAAGAAACACGGCGGAGTC R: TAAGCCGTGCCACCATTAC
gbx2.2_D3	F: TCCTCTCCAGGCAACAATTAGG R: ACAACCTCTTCCTGCACTGTTC
sp5_U1	F: AAGTTTGCCGCTGCCCAATC R: CACTCCATGAGGGCTTTGTACATTC
xmcl1_D1	F: CATCAAACAGTATCCAGCCCATTG R: AGAGGGAGAGGTGTTGGATGTTG
fzd10_U1	F: AGTGCCACAATCCCACACTTTC R: ACAGTGAGCAATAACGGCCAAG
fzd10_U2	F: AACCAAACAGACCCAACGTG R: AAAAAGAGCTCAGGGGTCCATC
xarp_U1	F: CCCCCTCTGGTTAAGAAAAAGAG R: GGGATGAAAGGAATAGCTGCTG
sial (-221)	F: AAGATCAAGGGAACCAGGTG R: TTGCACCCTACAAACATGGG

nodal3.1	F: ATAGCTTCAATGTACCACAGTGCA R: AGAGTCTGGCAGGTCCCTG
gsc_U1	F: ACCATTTCTTACCCAGAGAAACG R: TCCTTGCTCTCAATCCCAATCC
nog_D1	F: TTGGCAATCTCTCCTCTGATGTCC R: AGGGGCCATTCAAAAGGTGTC
chrd_U1	F: TGGGACTAGCGCAGGATTTATAGG R: ACTCATCAACTCCCAGAGTGAGTG
fst_D2	F: ACAGGACCAGTGTAGGTAAACG R: AAATTGGCCGACCCTTTCACAC
frzb_U1	F: AAATCCACAGGAGGGACGTTTC R: AGCCCAGAGATACAAGAGTGTAC
hoxd1(nc)	F: GTACCACATAGCAACCAATCAG R: GGCTGCATGCATGGCAAATC

cDNA plasmid clone for *in situ* hybridisation

cDNA was amplified by PCR using primers described below, from first-strand cDNA synthesized from mRNA extracted from *X. tropicalis* gastrula embryos. The amplified DNA fragments were subcloned into pGEM-T vector (Promega). For making digoxigenin-labelled antisense RNA probes, the following restriction enzymes (RE) and RNA polymerases (RNA pol) were used.

Gene	Plasmid	Primer	RE	RNA pol
<i>wnt8a</i>	pYNX22	CCATCGATTGGCTGAGGATACTGTTCAAGCATTAC CCATCGATGTCTCCGGTGGTGGCCTCTGTTCTTC	ApaI	SP6
<i>hoxd1</i>	pYNX27	CACGTGACCGCCACTCTATATTAGG CTAGCTGTGAGTCTTTATACTTAAACGTCC	NcoI	SP6
<i>sp5</i>	pYNX36	AGGGGAGGCTACCTCACTAACTG AGTATGAAAACAAGGTATCCTCTCCAAG	NotI	T7
<i>msgn1</i>	pYNX35	GAAGCTCCTGGTTGGAACCATTTAG ATATACACAAACCATGGGGTATTTACAG	NotI	T7
<i>cdx2</i>	pYNX39	ACAGGATTATGCAGCTAGCTGGCAC ATTGCCGACCCGAACAATGTGCAC	SphI	SP6
<i>msx1</i>	pYNX44	TTCCCAGCTCGGATATCTCTGTATG CATACAATCCCTTCCAAAGGGATTATTG	NcoI	SP6
<i>cdx4</i>	pYNX56	GTAGCATCAAGGCACCGCCTAAC GCAGTGCCCCAGACATAAGGATTTAC	NotI	T7
<i>fzd10</i>	pYNX43	CTGGAGCAAGGATGACAAGAAGTTTG ACCTTAGCATGCAGTCTCTGGTTTG	NotI	T7
<i>xarp</i>	HAR-199	(obtained from EXRC)	EcoRI	T7
<i>xmcl1</i>	pYNX34	ATGGCTTTAGTCAGTGGTAATAGCAC GGGCACGCCTTAATTTGGAAAGTTC	NcoI	SP6
<i>ventx1.2</i>	HAR-56	(obtained from EXRC)	EcoRI	T7

Luciferase reporter constructs and assay

Genomic fragments of β -peaks were amplified from *X. tropicalis* genomic DNA by PCR using primers described below and cloned into pGEM-T (Promega). Luciferase reporter constructs for β -peaks in the proximal regions were created by introducing a genomic fragment of the β -peak region into the pGL4.10 vector (Promega). For β -peaks in distant regions, corresponding genomic fragments were subcloned into p β -actin-luc, which contains a chicken β -actin basal promoter in front of the luciferase reporter gene. p β -actin-luc was generated by subcloning a chicken β -actin basal promoter as a SmaI-HindIII fragment from pBSSK2+ β EGFP (Ogino et al., 2008) into the EcoRV and HindIII sites of pGL4.10. Reporter plasmid DNA (40 pg per embryo) was injected together with pRL-CMV (40 pg per embryo, Promega) into the marginal zone of both dorsal and ventral blastomeres at the two- to four-cell stage. Embryos were collected at the early gastrula stage (stage 10.25) and assayed for luciferase activity. Primers used for cloning are as follows (restriction enzyme sites are underlined with their names on the right side):

msx1-U1-luc	<u>GGAAGATCTAGCAGATTTATTTATATGGATAACAGG</u>	BglII
	<u>CCCAAGCTTACAGAGATATCCGAGCTGGGAA</u>	HindIII
fzd10-U1-luc	<u>CCGCTCGAGACACAAAATACACAACAGTGAGC</u>	XhoI
	<u>CCCAAGCTTGCCCGCAGCCCAACTCG</u>	HindIII
ventx1.2-U1-luc	<u>CGGGATCCATGGGATTCACTGCCGCGCCAATG</u>	BamHI
	<u>CCCAAGCTTCTGAAGGGAAACCTGCTCTGG</u>	HindIII
sp5-U1-luc	<u>GGAAGATCTTACAGTGTGTGGCCACCTTAG</u>	BglII
	<u>CCCAAGCTTAGTCCAGCTCCTACAGGTGC</u>	HindIII
cdx4-U1-luc	<u>GGAAGATCTGGTTGGGTAGTTGTTAGTGGATG</u>	BglII
	<u>CCCAAGCTTTCCTAGGCGAGATCCTTGGTG</u>	HindIII
hoxd1_D1-luc	<u>CTAGCTAGCGGCAATTGAATGAAGGA</u>	NheI
	<u>CCGCTCGAGACAAAATGTCACTGATAGGA</u>	XhoI
hoxd1_D2-luc	<u>CTAGCTAGCGGCTAATCAGAGCTCACTTGAAC</u>	NheI
	<u>CCGCTCGAGTTACAGACACGTTAATGCAATTATC</u>	XhoI
msx1-U2-luc	<u>CTAGCTAGCGGTTGGAAAGCAGCAAAGCTTTG</u>	NheI
	<u>CCGCTCGAGAAAGTGGAGAGTGGTGCATGAAG</u>	XhoI
cdx4p_D1-2-luc	<u>CTAGCTAGCTATGCCTGCATTTTGTCAATCAATG</u>	NheI
	<u>CCGCTCGAGTGCCACTCTTATTACCATACCTG</u>	XhoI
cdx4p_D3-luc	<u>CTAGCTAGCGAGGACAGTAATTATGCCTTATAC</u>	NheI
	<u>CCGCTCGAGTTAAACATGACTGAGCATTTGTATG</u>	XhoI
cdx2p_U1-2-luc	<u>CTAGCTAGCTGACTCCATTAGGGCATATTCTG</u>	NheI
	<u>CCGCTCGAGTTTGCTAAATACAAGTGCTATACAG</u>	XhoI
hoxa1_D1-luc	<u>CTAGCTAGCTGCGCCAACGTTTCGTTTTTATTC</u>	NheI
	<u>GCGTCGACATTTTTGTGATACAGTATGGAAGT</u>	Sall

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