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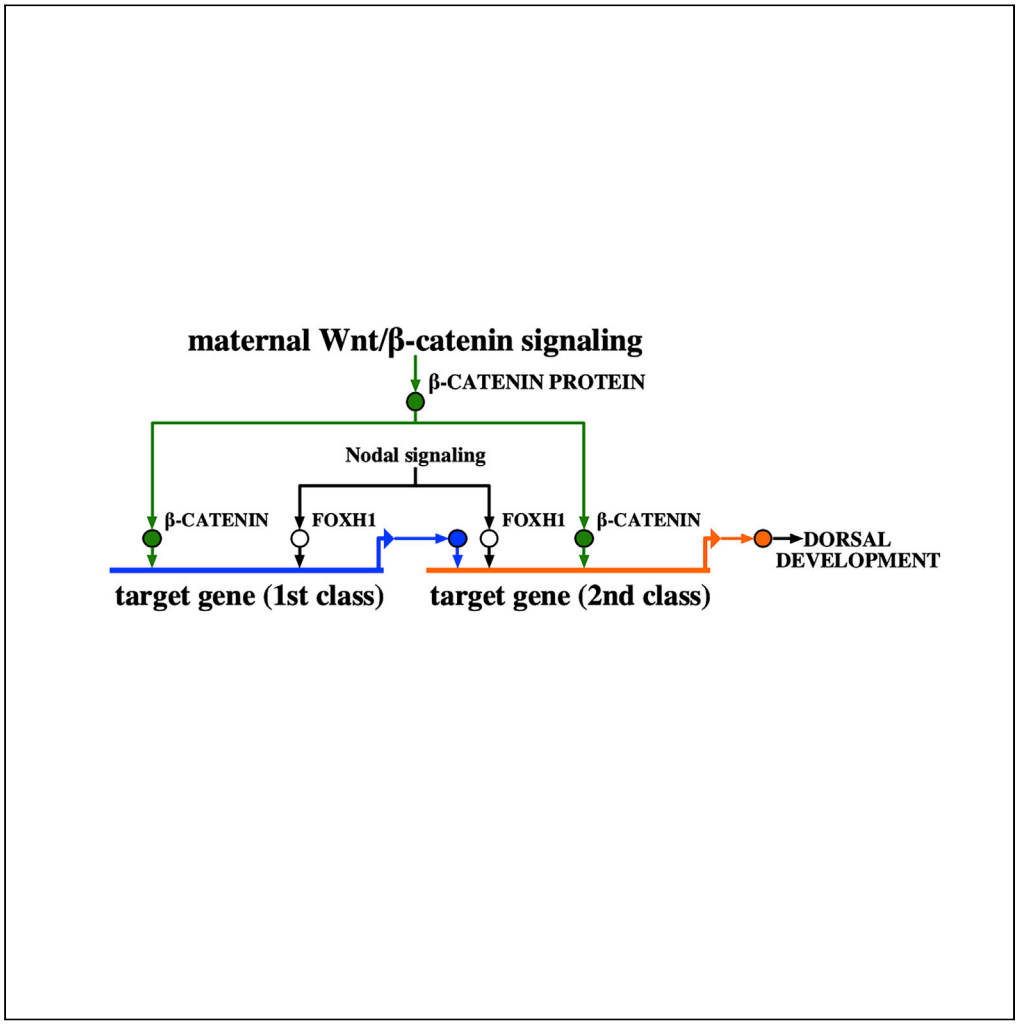

Boni A. Afouda, Yukio Nakamura, Sophie Shaw, ..., Ira L. Blitz, Ken W.Y. Cho, Stefan Hoppler

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HIGHLIGHTS

Combining RNA-seq and β-catenin ChIP-seq identifies direct Wnt target genes

Two distinct classes of direct maternal Wnt/β-catenin target genes can be discerned

We propose coherent feedforward regulation of gene expression of the second class

Maternal Wnt target gene expression of both classes requires Nodal/Foxh1 signaling

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Foxh1/Nodal Defines Context-Specific Direct Maternal Wnt/β-Catenin Target Gene Regulation in Early Development

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SUMMARY

Although Wnt/β-catenin signaling is generally conserved and well understood, the regulatory mechanisms controlling context-specific direct Wnt target gene expression in development and disease are still unclear. The onset of zygotic gene transcription in early embryogenesis represents an ideal, accessible experimental system to investigate context-specific direct Wnt target gene regulation. We combine transcriptomics using RNA-seq with genome-wide β-catenin association using ChIP-seq to identify stage-specific direct Wnt target genes. We propose coherent feedforward regulation involving two distinct classes of direct maternal Wnt target genes, which differ both in expression and persistence of β-catenin association. We discover that genomic β-catenin association overlaps with Foxh1-associated regulatory sequences and demonstrate that direct maternal Wnt target gene expression requires Foxh1 function and Nodal/Tgfβ signaling. Our results support a new paradigm for direct Wnt target gene co-regulation with context-specific mechanisms that will inform future studies of embryonic development and more widely stem cell-mediated homeostasis and human disease.

INTRODUCTION

The maternal-to-zygotic transition activates transcription of gene batteries under the control of transcription factors and signaling pathway components that are deposited in the egg by the maternal genome. Zygotic gene activation (ZGA) is initially controlled solely by these maternal factors, but maternal control is handed over to the zygotic genome following the synthesis of new gene products. How genes are differentially regulated by transcription factors to specify tissue-specific progenitor cells during this transition is an area of active investigation (reviewed by Nakamura and Hoppler, 2017). How transcription factors partner with one another to regulate expression of genes specifying different cell states is critical to this process. *Xenopus* has been used as an experimental model for the elucidation of germ layer specification (reviewed by Cao, 2015; Kiecker et al., 2016) and the maternal-to-zygotic transition (reviewed by Jukam et al., 2017).

Wnt signaling, mediated by the intracellular transducer β-catenin (Ctnnb1), plays drastically different roles before and after the maternal-to-zygotic transition (reviewed by Hikasa and Sokol, 2013; Zylkiewicz et al., 2014). Wnt/β-catenin functions in a regulatory switch mechanism to specify very different cell fates within a narrow window of developmental time. First, maternal Wnt signaling-regulated β-catenin protein controls subsequent expression of direct target genes (Blythe et al., 2010), including *siamois* (Brannon et al., 1997; Laurent et al., 1997) and *nodal3* (McKendry et al., 1997; Smith et al., 1995), by the midblastula stage. These genes are among the earliest zygotically expressed factors (Collart et al., 2014; Gentsch et al., 2019a, 2019b; Owens et al., 2016; Skirkanich et al., 2011; Tan et al., 2013; Yang et al., 2002) and function to establish dorsal embryonic cell fates (e.g., Ding et al., 2017; Kessler, 1997; Smith et al., 1995) together with subsequently expressed dorsal genes, such as *goosecoid* (*gsc*) and *noggin* (*nog*) (Ding et al., 2017; Wessely et al., 2001). Within an hour, zygotic Wnt8a signaling functions to regulate a radically different set of direct target genes (Christian et al., 1991; Ding et al., 2017; Hamilton et al., 2001; Hoppler et al., 1996; Nakamura et al., 2016), which then function to restrict dorsal and promote lateral and ventral cell fates (Christian and Moon, 1993; Hoppler et al., 1996). Context-specific direct Wnt/β-catenin

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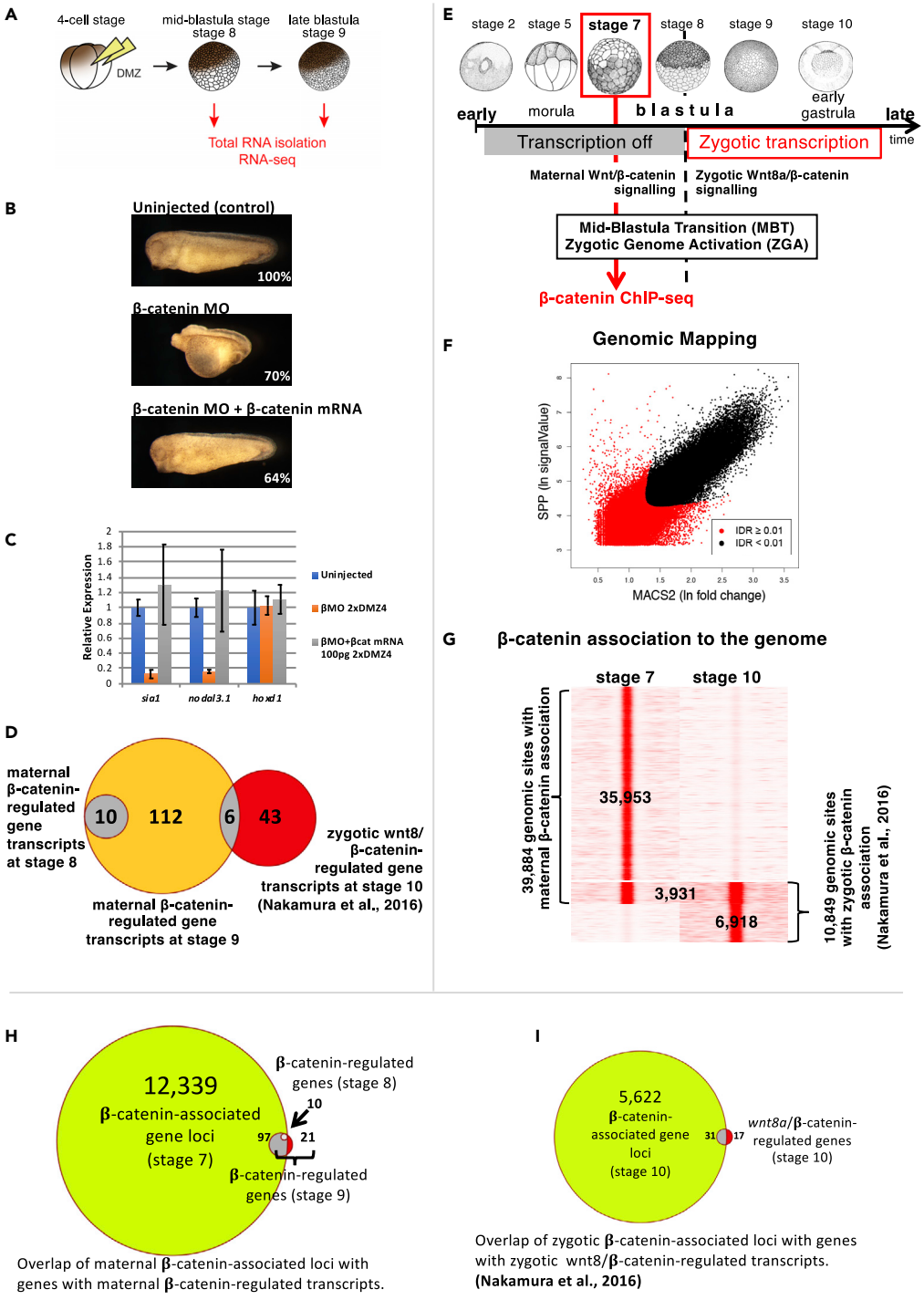

Figure 1. Identification of Maternal Wnt/β-Catenin Target Genes by Combining Transcriptomics (RNA-Seq Analysis) and β-Catenin-Association to Genomic Sequences (β-Catenin ChIP-Seq Analysis)

(A) Experimental design of transcriptomics analysis involved targeted injection into the prospective dorsal mesoderm (dorsal marginal zone) of four-cell-stage morula embryos with β-catenin Morpholino (MO, to knock down endogenous β-catenin protein expression) and (where indicated) with β-catenin mRNA (to experimentally rescue maternal Wnt/β-catenin signaling), with RNA expression subsequently sampled at the onset of ZGA (stage 8) and 1 h later (st. 9; with validated triplicate samples [see (C)] used for RNA-seq analysis).

Figure 1. *Continued*

(B) Experimental conditions were initially optimized by monitoring expected morphological changes caused by β-catenin knockdown and maternal β-catenin rescue (shown phenotypes are representative of five independent experiments scoring a total of 157, 72, and 174 embryos, respectively, from top to bottom).

(C) Extracted RNA samples were validated by monitoring the expected reduced and recovered expression of known maternal Wnt/β-catenin target genes (*sia1*, *nodal3.1*; and a zygotic Wnt8/β-catenin target [*hoxd1*] as a negative control) by qPCR following knockdown and rescue, respectively (error bar represents standard deviation from two independent biological experiments with three technical replicates each), before three independent experiments were sequenced.

(D) Venn diagram illustrating the number of genes identified (false discovery rate [FDR] <0.05) to be transcriptionally regulated by maternal Wnt/β-catenin signaling at the onset of ZGA (st.8, Table S1A) and 1 h later (st. 9, Table S1B and Figure S1), compared with genes regulated by zygotic Wnt8/β-catenin signaling (st. 10, Table S1C, experimental data from Nakamura et al. [2016], Figure S2); for these two groups of maternal Wnt/β-catenin signaling-regulated genes, also see Figure 2 and Table S1D.

(E) Experimental design of β-catenin ChIP-seq analysis at early blastula stage (st.7; before the onset of ZGA) involved pooling of many embryos, since there are fewer cells at early embryonic stages, and therefore fewer nuclei and less DNA.

(F) Genomic mapping of β-catenin ChIP-seq experiment with two independent software tools (see Transparent Methods for detail) identifying 39,884 β-catenin-associated genomic locations, near to 12,436 annotated genes.

(G) Comparing β-catenin association to the genome before (st.7) and after the onset of ZGA (in the early gastrula, st.10, experimental data from Nakamura et al. [2016]) reveals 3,931 shared β-catenin-associated locations (i.e., same genomic location occupied at st. 7 by maternal β-catenin and at st. 10 by zygotic β-catenin), exclusively maternal β-catenin-associated (35,953), and exclusively zygotic β-catenin-associated locations (6,918).

(H) Identification of direct maternal wnt/β-catenin target genes from overlap between maternal β-catenin-associated loci (F and G) with genes with maternal β-catenin-regulated transcripts (D) at stage 8 (first surge of gene expression) and at stage 9 (second surge of gene expression) (Table S1E).

(I) As comparison, identification of zygotic Wnt8a/β-catenin targets from overlap between zygotic β-catenin-associated loci with genes with zygotic Wnt8/β-catenin-regulated transcripts (Table S1F, experimental data from Nakamura et al. [2016]).

target gene expression during these early gastrula stages is defined by co-regulation with Bmp and Fgf signaling (Nakamura et al., 2016); i.e., zygotically expressed Wnt8a regulates β-catenin recruitment to cis-regulatory sequences, whereas target gene transcription is determined by Bmp (Hoppler and Moon, 1998) or Fgf signaling (see also Kjolby et al., 2019). Remarkably, regulation by Bmp and Fgf signaling occurs independently of Wnt8a-regulated β-catenin recruitment to target loci (reviewed by Nakamura and Hoppler, 2017; and Ramakrishnan and Cadigan, 2017).

Here we investigate the regulation of direct gene targets of maternal Wnt/β-catenin signaling at the genome-wide level. Different from the later zygotic direct Wnt8a/β-catenin target genes, we find these direct maternal targets are co-regulated by Foxh1-mediated Nodal/Tgfβ signaling. Our results further define two distinct classes of direct maternal Wnt target genes, which differ both in persistence of β-catenin association and temporal expression, with early genes involved in controlling expression of later ones in an apparent feedforward regulatory loop.

RESULTS

Defining the Maternal Wnt/β-Catenin-Regulated Transcriptome

To identify genes regulated by maternal Wnt/β-catenin signaling, we used an experimental design involving not only knockdown of endogenous β-catenin expression (Ding et al., 2017; Gentsch et al., 2019a, 2019b) but also rescue with re-instated β-catenin expression (Figures 1A and 1B). We validated experimental samples using RT-qPCR by monitoring expected changes in expression of known marker genes (i.e., *sia1* and *nodal3*) at midblastula stage (Figure 1C).

Samples were then processed for RNA sequencing (RNA-seq) analysis. Remarkably, our knockdown and rescue experimental design identify transcripts of only ten genes significantly regulated by maternal Wnt signaling at the early onset of ZGA (midblastula). All turn out to encode paralogs of *siamois* or *nodal3*, *5*, *6* (Table S1A). Since other known Wnt-regulated, dorsally expressed genes (such as *gsc*, *nog*, *chrd*, and *fst1*, e.g., Wessely et al. [2001] and Ding et al. [2017]) were not among these genes, we analyzed from the same experiment samples collected later, 1 h after the initial onset of ZGA (late blastula, Table S1B). At this stage, we find transcripts of 128 genes significantly regulated by maternal Wnt signaling, among them the ten already identified at the initial onset of ZGA (Figure 1D).

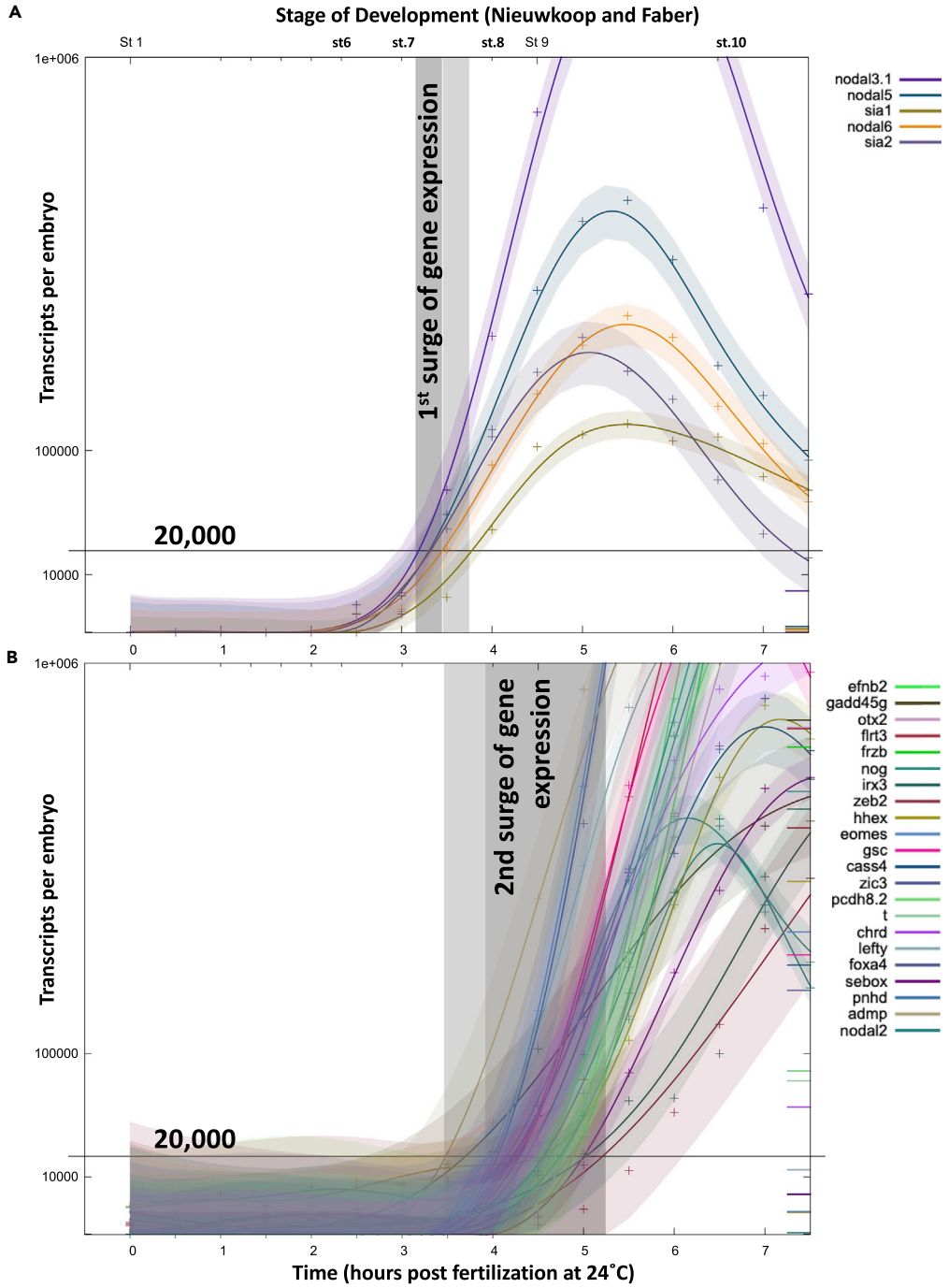

Figure 2. Two Surges of Maternal Wnt/β-Catenin Target Gene Expression

(A) First surge of maternal Wnt/β-catenin-regulated gene expression initiates between stage 7 and stage 7.5 (gray box), although sia1 is slightly delayed (lighter gray box) relative to the other genes in this class (sia2, nodal3.1, nodal 5, nodal 6).

(B) Second surge of maternal Wnt/β-catenin-regulated gene expression initiates between stage 8 and stage 9.5 (gray box), although admp and gadd45g are slightly earlier (lighter gray box) than the other genes in this class (e.g., eomes, gsc, chrd, frzb, noggin, nodal 2, and others as indicated). Data were mined from Owens et al. (2016) using the online tool http://genomics.crick.ac.uk/cgi-bin/profile-search.exe?dbe=http&dbs=INFO-PUBLIC&uid=guest&species=Xt&profiles=KBAP&src=search&tgt=main&menu=main_images&option=images&dataset=KBAP&project_key=0&version=0. The graphs shown are framed between zero and 1 million transcripts per embryo and between fertilization and stage 10.25. Of the ten maternal Wnt/β-catenin-regulated genes identified as a first surge of expression in our analysis (using version 9 of the *Xenopus tropicalis* genome

Figure 2. *Continued*
assembly, Figure 1D and Table S1A), the transcriptomics data from Owens et al. (2016, analyzed using version 7) contained information for five (see Table S1D), whereas of the 112 maternal Wnt/β-catenin-regulated genes expressed exclusively as part of the second surge of expression (118 minus the 10 genes already expressed from the first surge), 22 were used in this analysis both because transcriptomics data from Owens et al. (2016) were available and induction could be defined between low initial gene expression (less than 10k transcripts per embryo before st.6) and increased expression (more than 100k by stage 10, see Table S1D). The gray boxes indicate the first (in A) and second surge (in B) of expression, defined by 20,000 transcripts per embryo in the transcriptomics data from Owens et al. (2016).

Our transcriptomics analysis therefore reveals two groups of maternal Wnt-regulated genes: the first surge of expression includes what has previously been described as "pre-MBT" transcription (Yang et al., 2002), and then the second surge of gene expression concurs with more general onset of ZGA, including dorsally expressed genes, including *gsc* and *nog*, which had previously been shown to be Wnt regulated (Ding et al., 2017; Wessely et al., 2001). These two distinct surges of gene expression related to these two groups can also be seen in the staged transcriptomics data by Owens et al. (2016) (Figures 2A and 2B).

β-Catenin Protein Associates with Genomic Loci Prior to ZGA

In order to identify direct Wnt/β-catenin target genes among maternal Wnt-regulated genes, we embarked on β-catenin chromatin immunoprecipitation sequencing (ChIP-seq) analysis. β-Catenin indirectly associates with genomic DNA sequences by binding sequence-specific DNA binding transcription factors, principally of the LEF/TCF protein family (e.g., Nakamura et al., 2016). We performed β-catenin ChIP-seq analysis in the early blastula, revealing β-catenin association with 39,884 specific genomic sites (Figures 1E–1G), which can be bioinformatically assigned to 12,339 annotated genes (Figure 1H).

We then compared this genome association of maternal Wnt-regulated β-catenin before with the genome association of zygotic Wnt-regulated β-catenin well after ZGA (early gastrula, Nakamura et al., 2016, reanalyzed the same way as the new data). This comparison revealed 35,953 exclusively maternal β-catenin bindings sites (peaks) distinct from 6,918 exclusively zygotic binding sites, with 3,931 overlapping (i.e., loci associated with β-catenin before and after ZGA, Figure 1G).

When comparing our transcriptomics with our genome association results, we find that all ten maternal Wnt/β-catenin-regulated genes in the first group, i.e., with an early surge of expression, have nearby maternal β-catenin association, indicating that, as expected, they are all direct target genes (cf. Blythe et al., 2010), as are 82% of the second group of maternal Wnt/β-catenin-regulated genes with a later surge of gene expression (Figure 1H, compare with direct Wnt8a/β-catenin target genes at gastrulation, Figure 1I). All maternal Wnt-regulated genes expressed in the first group show β-catenin binding in the early blastula stage ChIP-seq data, but significantly, not at early gastrula stage (Figures 1G, S3A, and S3B). In contrast, maternal Wnt-regulated genes expressed in the second surge show β-catenin binding both before and after ZGA (Figures 1G, S3C, and S3D; data from Nakamura et al. [2016]).

Both transcriptomics and β-catenin ChIP-seq analyses therefore independently identify the same two distinct classes of direct maternal Wnt/β-catenin target genes in the early embryo.

Coherent Feedforward Regulation of Direct Maternal Wnt/β-Catenin Target Genes of the Second Class by Gene Products of the First

What could account for the temporal difference in timing of expression between these two classes of direct maternal Wnt/β-catenin target genes? We wondered whether products of direct maternal Wnt target genes expressed as part of the first surge might be required for regulation of direct maternal Wnt targets in the second surge, since some of the genes in this second class of direct Wnt target genes had previously been shown to be regulated by *siamois* (e.g., Bae et al., 2011; Carnac et al., 1996). MO knockdown of *sia1* and *sia2* indeed results in reduced expression of some direct maternal Wnt target genes of the second class (Figure 3A, as also previously shown in Bae et al. [2011]), which is reinstated by rescuing Sia activity (Figure 3B), whereas the earlier surge of expression of the first class of direct target genes (in midblastula), such as *sia1* itself and *nodal3*, is not affected (Figure 3C).

These results support our hypothesis that maternal Wnt/β-catenin regulates these two classes differently, with second-class genes additionally requiring products of the first class. This suggests that a coherent

feedforward regulatory mechanism (Figure 3D) promotes expression of direct Wnt target genes of the second class.

Foxh1/Nodal Signaling Is Required for Context-Specific Regulation of Direct Maternal Wnt/β-Catenin Target Genes in the Early Embryo

Previously we had found that β-catenin association with cis-regulatory sequences is insufficient for transcriptional regulation of Wnt8a/β-catenin target genes in specification of ventral tissue in the early gastrula (Nakamura et al., 2016; Nakamura and Hoppler, 2017). Bmp or Fgf signaling was identified as critical for the context-specific expression of these zygotic Wnt8a/β-catenin target genes (Hoppler and Moon, 1998; Kjolby et al., 2019; Nakamura et al., 2016). Here, we aimed to determine what context-determining mechanism is involved in regulating direct maternal Wnt/β-catenin target genes, which are expressed earlier in the dorsal marginal zone of blastula-stage embryos.

We used *de novo* motif analysis to identify shared cis-regulatory sequences suggesting transcription factor binding sites among direct maternal Wnt/β-catenin target genes. As expected, these genes share sequences for LEF/TCF-binding sites (also known as WRE, reviewed by Ramakrishnan and Cadigan, 2017). Importantly, in addition, they also harbor motifs matching the consensus binding site for Foxh1 (Table 1). Motif analysis of zygotic Wnt8a/β-catenin target sequences had not identified Foxh1 consensus binding sequences (Nakamura et al., 2016). This difference suggests that Foxh1 plays a context-determining role in selecting which of the many maternal β-catenin-bound genes are transcriptionally regulated by maternal Wnt/β-catenin.

We used Foxh1 ChIP-seq data (as in Charney et al., 2017; Chiu et al., 2014) to explore this hypothesis. Comparing β-catenin-bound with Foxh1-bound regions reveals a substantial (54%) correlation before ZGA (Figure 4A), but not thereafter (9%) (Figure 4B). A similar finding of enrichment for Foxh1 was recently reported by Gentsch et al. (2019b). There is also a strong correlation between these genomic loci that share both β-catenin and Foxh1 association with the maternal Wnt/β-catenin targets that we had identified above (Figures 4C and 4D, 80% and 70%, respectively; but less so [25%] with zygotic Wnt8a/β-catenin targets Figure 4E). We also compared Wnt/β-catenin-regulated genes with altered gene expression in an MO-mediated knockdown of *foxh1* function (Figures 4F–4H, see Transparent Methods). There is higher correlation in late blastula (Figure 4G, i.e., 23% of maternal Wnt/β-catenin-regulated transcriptome) than later in early gastrula (Figure 4H, i.e., 5% of zygotic Wnt8a/β-catenin-regulated transcriptome). This analysis correlates context-specific regulation of maternal Wnt/β-catenin target genes with a requirement for Foxh1 function.

Since Foxh1 function in the early embryo mediates embryonic Nodal/Tgfβ signaling (Chen et al., 1996; Chiu et al., 2014; Hill, 2018), we compared the maternal Wnt/β-catenin-regulated transcriptome directly with transcripts reduced after treatment with a pharmacological Nodal/Tgfβ signaling inhibitor, SB431542 (Figures 4F–4H). We expected to find such a correlation, since cooperative regulation by Nodal/Tgfβ and Wnt/β-catenin-signaling had been demonstrated for some of the genes identified here as direct maternal Wnt/β-catenin target genes of class 1 (*sia*) and class 2 (*gsc*, *chrd*) (Crease et al., 1998; Nishita et al., 2000). As with Foxh1 above, there is indeed correlation between Nodal//Tgfβ- and maternal Wnt/β-catenin-regulated genes (Figure 4G, 23%), which, however, is only slightly higher than with zygotic Wnt8a/β-catenin-regulated genes (Figure 4H, 18%), which may reflect a mostly Foxh1-independent role for Nodal/Tgfβ signaling in control of zygotic Wnt8a/β-catenin-regulated genes (e.g., Charney et al., 2017; Coda et al., 2017; Germain et al., 2000; Kunwar et al., 2003). In conclusion, maternal Wnt/β-catenin signaling target genes could be co-regulated by Foxh1/Tgfβ.

β-Catenin Association with Target Genes Is Independent of Foxh1/Nodal Signaling

We directly validated the requirement of Foxh1 function and Nodal/Tgfβ signaling activity for regulation of direct maternal Wnt/β-catenin target genes using a *foxh1* knockdown and a pharmacological Nodal/Tgfβ signaling inhibitor (Chiu et al., 2014). *foxh1* knockdown (Figure 4I) and inhibition of Nodal/Tgfβ signaling (Figure 4K) caused reduced expression of direct maternal Wnt/β-catenin target genes at the late blastula stage, both those of the first class (*sia1*, *nodal3*) and of the second class (*gsc*, *nog*).

Since we had previously shown that loss of context-defining Bmp or Fgf signaling had no effect on β-catenin recruitment to zygotic Wnt8a/β-catenin target loci (Nakamura et al., 2016), we tested here whether Foxh1/Tgfβ signaling could influence β-catenin recruitment to relevant WREs in blastula stage embryo, using

A: *De Novo* Motif Analysis First Surge Genes					B: *De Novo* Motif Analysis Second Surge Genes				
Rank	Discovered Motif	Best Match	p Value	%	Rank	Discovered Motif	Best Match	p Value	%
1		TCF7L2	110^{-20}	26	1		FOXH1	1×10^{-99}	49
2		FOXH1	1×10^{-19}	32	2		Tbx21-like T-box	1×10^{-41}	14
3		LEF1	1×10^{-18}	35	3		Helix-turnhelix (homeobox?)	1×10^{-28}	5
4		Pan/dTCF	1×10^{-18}	23	4		TCF7L2	1×10^{-28}	18
5		Pan/dTCF	1×10^{-18}	16	5		C2H2 zinc finger	1×10^{-26}	4
6		C4 zinc finger (GATA?)	1×10^{-16}	16	6		ROX1-like HMGbox	1×10^{-25}	7
7		Zinc finger	1×10^{-15}	23	7		NFkB-like	1×10^{-25}	42
8		SOX	1×10^{-14}	13	8		TDA9-like zinc finger	1×10^{-24}	6
9		Meis1	1×10^{-13}	42	9		MBP1-like helix-turnhelix	1×10^{-22}	15
10		TOD6-like	1×10^{-13}	26	10		NHP10-like HMG box	1×10^{-14}	7

Table 1. *De Novo* Motif Analysis of β-Catenin-Associated Cis-regulatory Sequences

De novo motif analysis of sequences surrounding β-catenin-associated locations (at early blastula st.7) in maternal Wnt/β-catenin-regulated genes at the onset of zygotic transcription (ZGA) (Table 1A, mid-blastula st.8, note Tcf/Lef and Foxh1 consensus motifs) and approximately 1 h later (Table 1B, late blastula st. 9, note Foxh1 and Tcf/Lef consensus binding motifs).

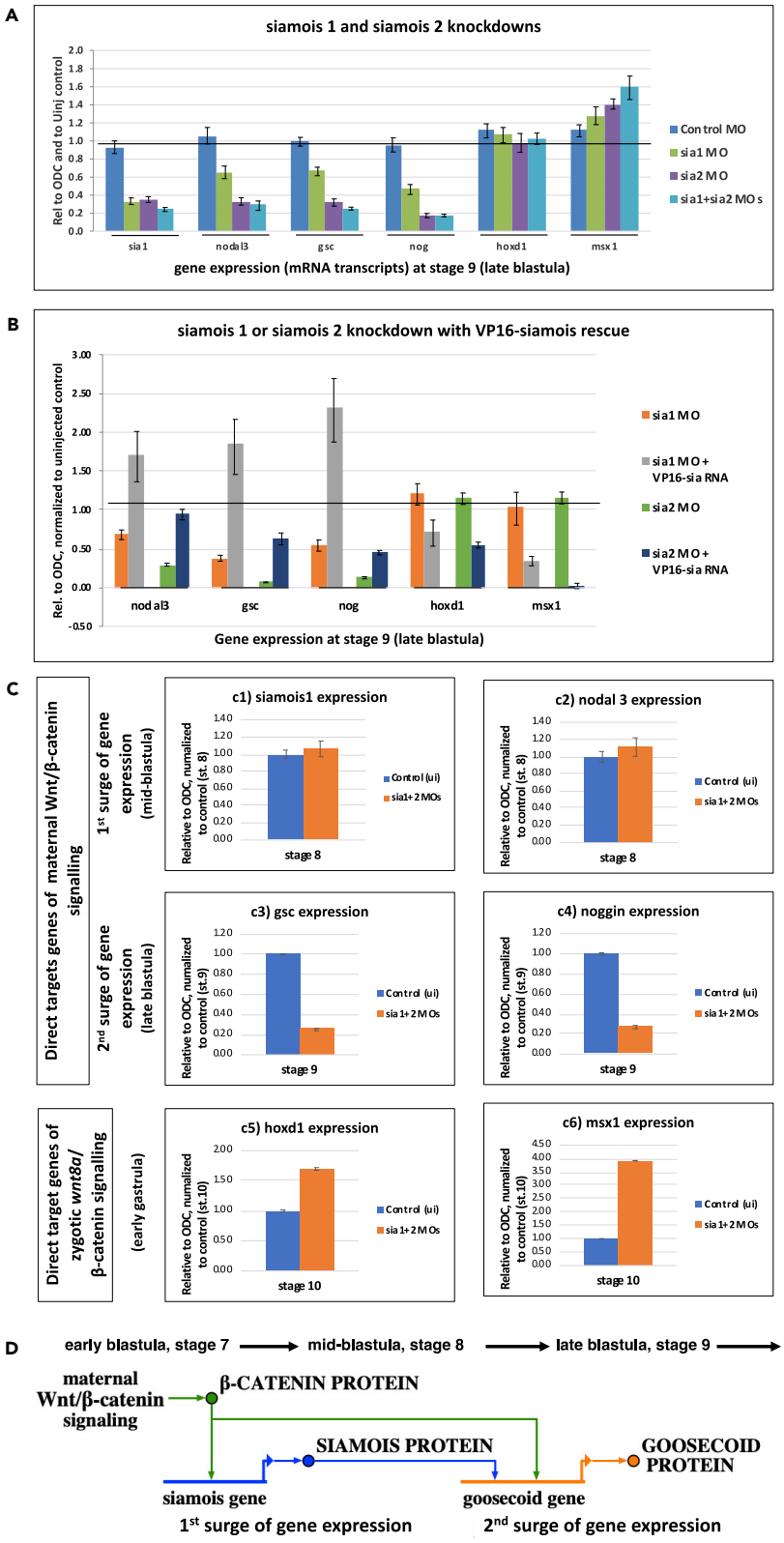
A
siamois 1 and siamois 2 knockdowns
Rel to ODC and to Uinj control
Control MO
sia1 MO
sia2 MO
sia1+sia2 MOs
sia1
nodal3
gsc
nog
hoxd1
msx1
gene expression (mRNA transcripts) at stage 9 (late blastula)
B
siamois 1 or siamois 2 knockdown with VP16-siamois rescue
Rel. to ODC, normalized to uninjected control
sia1 MO
sia1 MO + VP16-sia RNA
sia2 MO
sia2 MO + VP16-sia RNA
nodal3
gsc
nog
hoxd1
msx1
Gene expression at stage 9 (late blastula)
C
Direct targets genes of maternal Wnt/β-catenin signalling
1st surge of gene expression (mid-blastula)
2nd surge of gene expression (late blastula)
c1) siamois1 expression
c2) nodal 3 expression
c3) gsc expression
c4) noggin expression
c5) hoxd1 expression
c6) msx1 expression
Relative to ODC, numalized to control (st. 8)
Relative to ODC, numalized to control (st.9)
Relative to ODC, numalized to control (st.10)
Control (ui)
sia1+2MOs
stage 8
stage 9
stage 10
Direct target genes of zygotic wnt8a/ β-catenin signalling
(early gastrula)
D
early blastula, stage 7
mid-blastula, stage 8
late blastula, stage 9
maternal Wnt/β-catenin signaling
β-CATENIN PROTEIN
SIAMOIS PROTEIN
GOOSECOID PROTEIN
siamois gene
goosecoid gene
1st surge of gene expression
2nd surge of gene expression

Figure 3. Coherent Feedforward Regulation of Maternal Wnt/β-Catenin Target Genes in Late Blastula

(A) *siamois1*, *siamois2* (also known as *twin*) and double Morpholino (MO) knockdown causes reduced expression of maternal Wnt/β-catenin target genes (at late blastula stage 9) (see also Figures S4A–S4F).

(B) Rescue of *siamois1* and *siamois2* knockdown with constitutively activating siamois construct (which is not targeted by either MO) re-instates expression of maternal Wnt/β-catenin target genes (*nodal3*, *goosecoid*, *noggin*).

(C) Stage-specific sampling of gene expression reveals that direct maternal Wnt/β-catenin target genes of the first class (*siamois1* and *nodal3*) remain unaffected (st. 8), whereas expression of direct maternal Wnt/β-catenin target genes of the second class (*goosecoid*, *noggin*) is reduced (st.9). Also note that expression of zygotic Wnt8/β-catenin target genes (*hoxd1*, *msx1*) is not reduced but may be increased presumably owing to indirect mechanisms.

(D) Coherent feedforward regulation of some direct maternal Wnt/β-catenin target genes of the second class (e.g., *goosecoid*) involves *siamois* genes, which are among direct maternal Wnt/β-catenin target genes of the first class. Control Morpholino (control MO-injected embryos); Uninjected Control (uninjected embryos); sia1 MO, sia2 MO (embryos injected with Morpholino targeting *siamois1* or *siamois2* [also known as twin], respectively; VP16-sia RNA (*Xenopus tropicalis* embryos injected with *Xenopus laevis* constitutively active siamois mRNA [Kessler, 1997]). Data are from one representative of three independent experiments; error bars represent mean ± SEM of three technical replicates with $p \leq 0.05$.

β-catenin ChIP-qPCR. No reduction of β-catenin association at maternal Wnt/β-catenin target loci is detected when Foxh1 function (Figure 4J) or Nodal/Tgfβ signaling is inhibited (Figure 4L). These results demonstrate that Foxh1 function and Nodal/Tgfβ signaling are required independently of Wnt-regulated β-catenin association at maternal Wnt/β-catenin target loci for their context-specific transcriptional expression.

DISCUSSION

Initially two kinds of direct Wnt/β-catenin target genes were expected in the early embryo (reviewed by Zylkiewicz et al. [2014], Nakamura and Hoppler [2017] and Esmaeili et al. [2020]): direct maternal Wnt/β-catenin target genes, such as *sia1* and *nodal3* (involved in dorsal specification), and direct zygotic Wnt8a/β-catenin target genes, such as *hoxd1* and *ventx1* (involved in ventral/lateral specification). Our previous analysis of direct zygotic Wnt8a/β-catenin target genes had revealed at least two contexts (Bmp-regulated and Fgf-regulated contexts, Nakamura et al., 2016). Here, we describe a much greater developmental complexity of direct maternal Wnt/β-catenin target genes, implicating an additionally dorsally expressed class of genes, expression of some of which were known to be influenced by Wnt signaling (e.g., Ding et al., 2017; Wessely et al., 2001). These two classes of direct maternal Wnt/β-catenin target genes can be defined both by their timing of gene expression and by their dynamics of β-catenin-association with respective genomic loci.

Yet our analysis discovers a shared Foxh1- and Nodal/Tgfβ signaling-dependent context-defining mechanism for both the first and second class of direct maternal Wnt/β-catenin target genes. Cooperative regulation of early dorsal embryonic development by Nodal/Tgfβ and Wnt/β-catenin signaling is deeply conserved among vertebrates and even with closely related invertebrate chordates (Kozmikova and Kozmik, 2020). Thus, maternal Wnt/β-catenin regulation of direct transcriptional targets occurs in a different co-regulatory context (i.e., Foxh1 and Nodal/Tgfβ) than for direct zygotic Wnt8a/β-catenin targets (i.e., Bmp or Fgf). Importantly, Wnt signaling regulates β-catenin association with direct Wnt/β-catenin target loci in all these different contexts independently of any of those various context-defining co-regulatory mechanisms, which in turn only regulate the expression of, not β-catenin-association with, these Wnt target genes.

However, the first class of direct maternal Wnt/β-catenin target genes lose β-catenin association by gastrulation, precisely when chromatin accessibility at such loci is found to be restricted (Esmaeili et al., 2020). Developmental competence of direct target genes to respond to Wnt/β-catenin signaling in a context-specific way is therefore likely to be regulated not only by combinatorial signaling as highlighted here but also by developmentally regulated chromatin modification, which we have not further explored (see also Hontelez et al., 2015).

Wnt-activated nuclear β-catenin associates widely with chromatin across the genome, including to many loci that are not expressed at the stages analyzed (see also Nakamura et al., 2016). It is likely that such extra binding, which is not regulating stage-specific transcription nearby, may function as a buffering mechanism to fine-tune the response and prevent inadvertent promotion of transcription (as initially proposed for transcription factors by Lin and Riggs [1975] and discussed in the context of Wnt/β-catenin signaling in Nakamura and Hoppler [2017]).

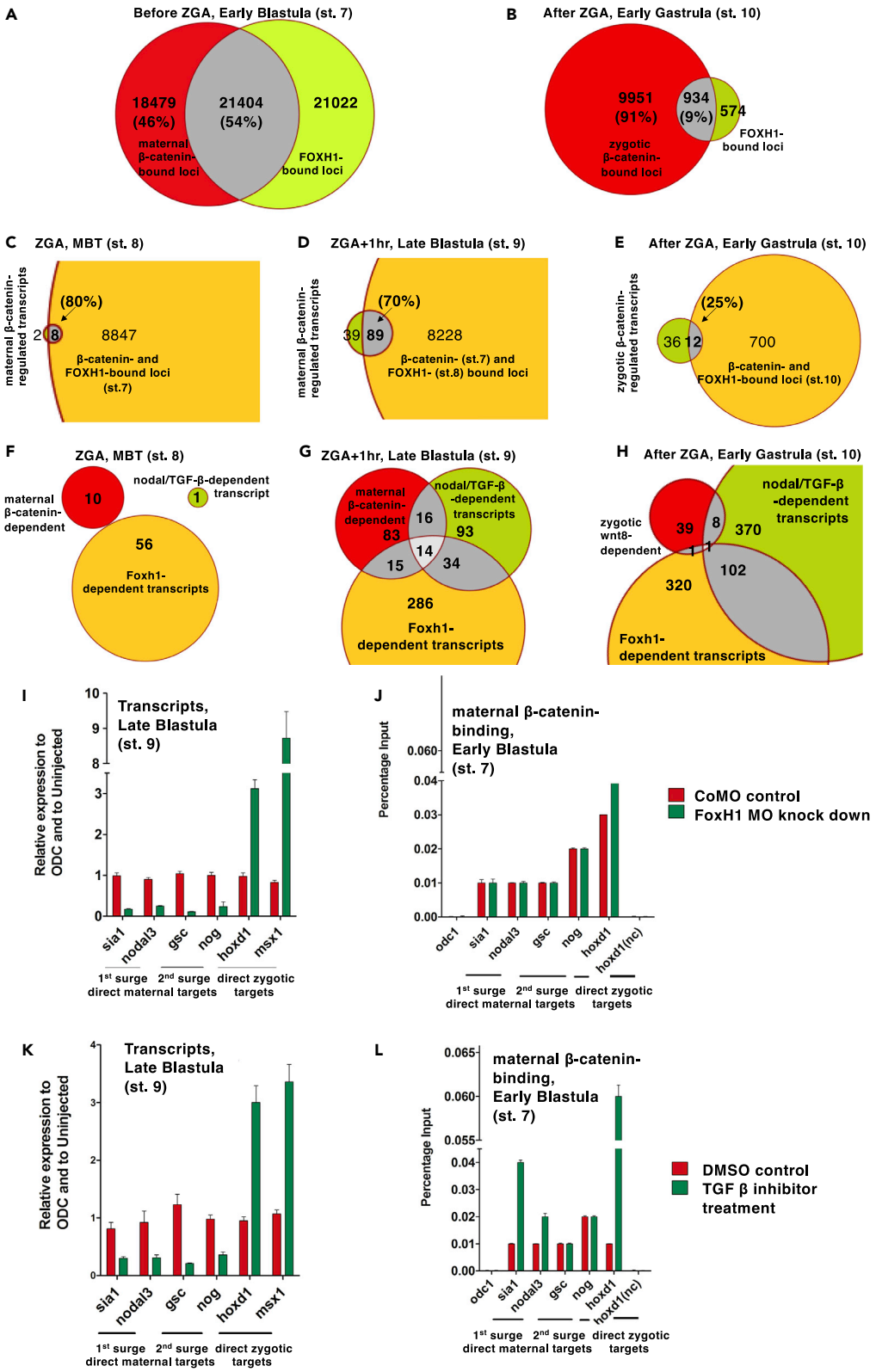
A
Before ZGA, Early Blastula (st. 7)
18479 (46%)
21404 (54%)
21022
maternal β-catenin-bound loci
FOXH1-bound loci
B
After ZGA, Early Gastrula (st. 10)
9951 (91%)
934 (9%)
574
zygotic β-catenin-bound loci
FOXH1-bound loci
C
ZGA, MBT (st. 8)
maternal β-catenin-regulated transcripts
(80%)
2
8
8847
β-catenin- and FOXH1-bound loci (st.7)
D
ZGA+1hr, Late Blastula (st. 9)
maternal β-catenin-regulated transcripts
(70%)
39
89
8228
β-catenin- (st.7) and FOXH1- (st.8) bound loci
E
After ZGA, Early Gastrula (st. 10)
zygotic β-catenin-regulated transcripts
(25%)
36
12
700
β-catenin- and FOXH1-bound loci (st.10)
F
ZGA, MBT (st. 8)
maternal β-catenin-dependent
10
nodal/TGF-β-dependent transcript
1
56
Foxh1-dependent transcripts
G
ZGA+1hr, Late Blastula (st. 9)
maternal β-catenin-dependent
83
16
nodal/TGF-β-dependent transcripts
93
14
15
34
286
Foxh1-dependent transcripts
H
After ZGA, Early Gastrula (st. 10)
zygotic wnt8-dependent
39
8
nodal/TGF-β-dependent transcripts
370
1
1
320
102
Foxh1-dependent transcripts
I
Transcripts, Late Blastula (st. 9)
Relative expression to ODC and to Uninjected
sia1
nodal3
gsc
nog
hoxd1
msx1
1st surge direct maternal targets
2nd surge
direct zygotic targets
J
maternal β-catenin-binding, Early Blastula (st. 7)
Percentage Input
odc1
sia1
nodal3
gsc
nog
hoxd1
hoxd1(nc)
1st surge direct maternal targets
2nd surge
direct zygotic targets
CoMO control
FoxH1 MO knock down
K
Transcripts, Late Blastula (st. 9)
Relative expression to ODC and to Uninjected
sia1
nodal3
gsc
nog
hoxd1
msx1
1st surge direct maternal targets
2nd surge
direct zygotic targets
L
maternal β-catenin-binding, Early Blastula (st. 7)
Percentage Input
odc1
sia1
nodal3
gsc
nog
hoxd1
hoxd1(nc)
1st surge direct maternal targets
2nd surge
direct zygotic targets
DMSO control
TGF β inhibitor treatment

Figure 4. Foxh1/Nodal Signaling Is Required for Expression of Maternal Wnt Target Genes but Not for β-Catenin Recruitment

(A and B) Bioinformatics analysis of overlap between genomic loci with maternal β-catenin and Foxh1 association before the onset of zygotic transcription (Zygotic Gene Activation, ZGA) (A, st.7, Early Blastula); and after (B, st.10, Early Gastrula). Note considerable overlap before the onset of ZGA (A, compared with B; also see Tables S2A and S2B).

(C–E) Overlap between Wnt/β-catenin-regulated gene loci and genomic loci sharing β-catenin and Foxh1 association; comparing (C) maternal Wnt/β-catenin-regulated gene loci at the onset of ZGA (st. 8, midblastula) with shared β-catenin/Foxh1-associated loci just before the onset of ZGA (st. 7, early blastula); (D) maternal Wnt/β-catenin-regulated gene loci after the onset of ZGA (st. 9, late blastula) with shared β-catenin/Foxh1 loci at the onset of ZGA (comparing β-catenin-associated loci at st.7 with Foxh1-associated loci at st. 8, midblastula); and (E) Wnt8a/β-catenin-regulated gene loci with β-catenin/Foxh1 loci at early gastrulation (st. 10, early gastrula). Note correlation between maternal Wnt/β-catenin-regulated gene loci and corresponding β-catenin/Foxh1 loci (80% and 70%, respectively) of maternal Wnt/β-catenin signaling-regulated gene loci expressed in the first surge (C) and in the second surge of gene expression (D), compared with less than 25% of zygotic Wnt8a/β-catenin signaling-regulated gene loci in (E) (see also Tables S2C–S2E).

(F–L) Overlap between Wnt/β-catenin-regulated genes and transcripts reduced in a zygotic Foxh1 morpholino knockdown, and following pharmacological inhibition of Nodal/Tgfβ signaling (with SB431542, SB), at the onset of ZGA (F, st.8, midblastula), 1 h later (G, st. 9, late blastula), and during early gastrulation (H, st. 10, early gastrula) (see also Tables S2F–S2H). Note correlation between maternal Wnt/β-catenin-regulated genes and those reduced in Foxh1 knockdown and with inhibited Nodal signaling (G), compared with zygotic Wnt8a/β-catenin-regulated genes at st.10 in (H). Absence of overlap when analyzed at midblastula stage (st.8 in [F]) is likely due to maternal rescue, i.e., maternal FOXH1 protein not affected by Foxh1 morpholino knockdown. Foxh1 Morpholino knockdown (I and J) and pharmacological inhibition of Nodal signaling with SB431542 (K and L) cause reduced gene expression of representative maternal Wnt/β-catenin target genes (I and K; analyzed with qRT-PCR, see also Figures S4G, S4H, S4J, and S4K) but does not cause reduced β-catenin association at these loci (J and L; analyzed with β-catenin ChIP-qPCR, see also Figures S4I and S4L). Data are from one representative of three independent experiments; error bars represent propagation error of three technical replicates with $p \leq 0.05$.

The two classes of direct maternal Wnt/β-catenin target genes differ in that the specific context-defining mechanism controlling gene expression of the second class includes a coherent feedforward mechanism involving an additional input from products of genes of the first class of direct maternal Wnt/β-catenin targets (Figure 3D). Such a coherent feedforward regulatory network motif was shown to serve as a persistence detector (a so-called sign-sensitive delay element, e.g., Mangan and Alon, 2003), suggesting here that only persistent maternal Wnt/β-catenin signaling will promote second class target gene expression and subsequent dorsal axis development. Additional gene regulatory mechanisms are not ruled out, particularly since additional consensus transcription factor binding motifs were discovered in relevant β-catenin-associated genomic DNA sequences (Table 1, e.g., Sox3, see also Doumpas et al., 2019; Gentsch et al., 2019b; Kormish et al., 2010; Zhang et al., 2003).

The concepts we uncover about regulation of direct Wnt/β-catenin target genes in the early *Xenopus* embryo provides a general novel paradigm for the role of context in Wnt target gene regulation in other developmental settings and in human disease, such as cancer (e.g., Koval and Katanaev, 2018; Madan et al., 2018).

Limitations of the Study

The concept of feedforward regulation emphasized here implies redundancy in gene regulation, which may have evolved for improved robustness. This redundancy, by definition, makes it difficult to disentangle direct from indirect inputs and demonstrate that both are required independently for gene activation.

Resource Availability

Lead Contact

Stefan Hoppler (s.p.hoppler@abdn.ac.uk).

Materials Availability

Requests for materials and reagents should be directed to the Lead Contact.

Data Availability

Raw sequencing data generated for this study have been deposited in the ArrayExpress database at EMBL-EBI under the accession number E-MTAB-8555 (http://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-8555).

Previously published datasets used in this study are available from Gene Expression Omnibus at NCBI under the accession numbers GSE53654, GSE72657, and GSE85273.

METHODS

All methods can be found in the accompanying Transparent Methods supplemental file.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.isci.2020.101314.

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AUTHOR CONTRIBUTIONS

S.H., Y.N., K.W.Y.C., I.L.B., and R.M.C. conceived this project; Y.N., B.A.A., I.L.B., and R.M.C. performed experiments and molecular analysis. S.S., Y.N., and K.D.P. performed bioinformatics analysis. S.H. wrote the manuscript with help from co-authors.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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iScience, Volume *23*

Supplemental Information

Foxh1/Nodal Defines Context-Specific Direct Maternal Wnt/β-Catenin Target Gene Regulation in Early Development

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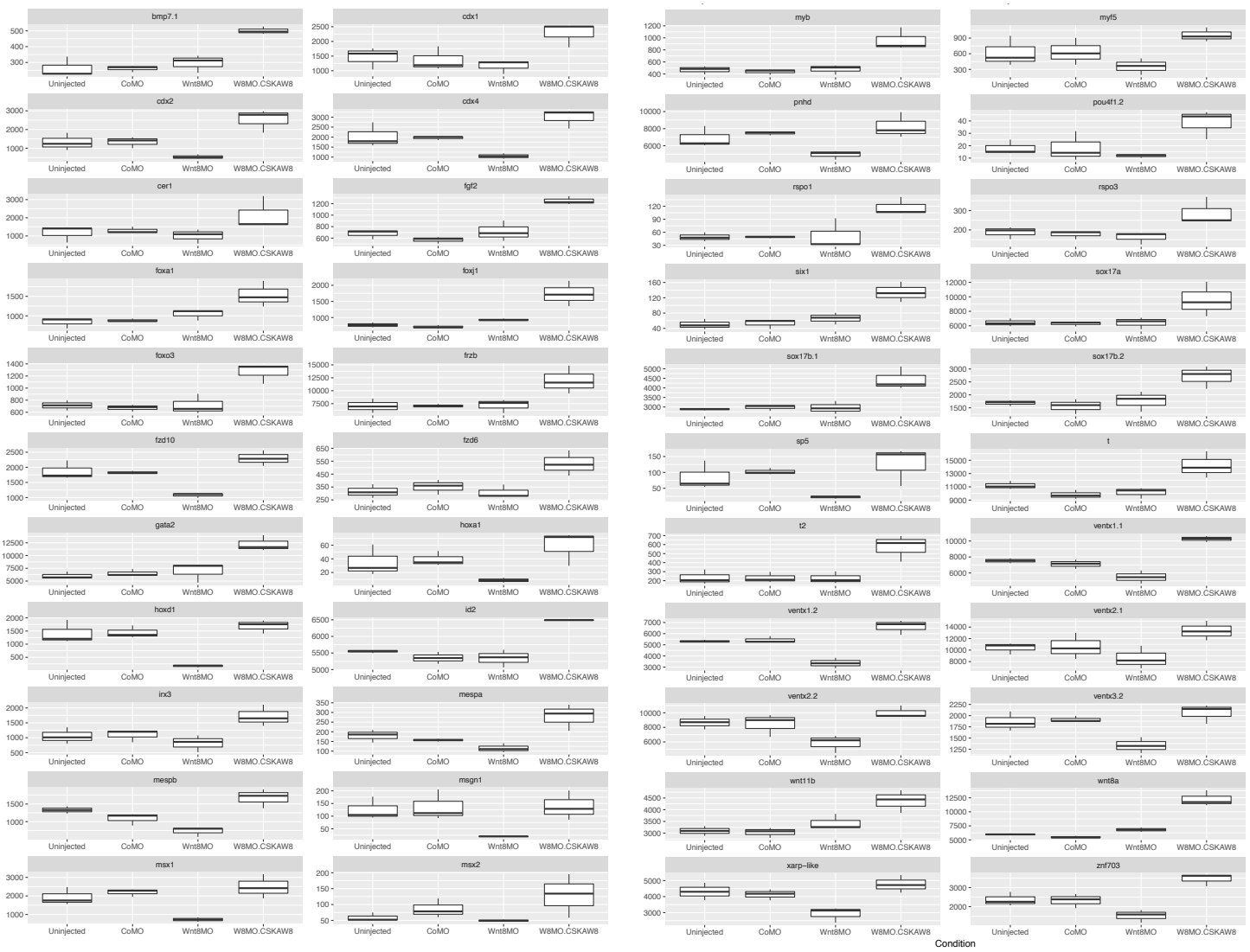
SUPPLEMENTAL FIGURES


Count
cdh2
cdh22
cdh7
cdx2
cdx4
chrd
ctnnb1
drosha
eomes
fgf20
fgfr4
foxa4
foxb1
frzb
fst
fzd10
fzd8
gbx2.2
gsc
hes3−like
hhex
hoxa1
hoxa2
hoxd1
id3
irx3
lef1
lefty
msx2
myb
nodal3.2
nodal5.2
nodal6
nog1
nrg3
otx2
pitx2
pnhd
pou3f2
rdh5
sia1
sp5
syngap1
t
tbx2
ventx1.2
ventx2.1
wnt8a
xarp−like
Uninjected
bCatMO
bMObmRNA

Suppl. Fig.1

Suppl. Figure 1: Transcriptomics of maternal Wnt/β-catenin signaling-regulated genes at stage 9 (late blastula), Related to Figure 1D

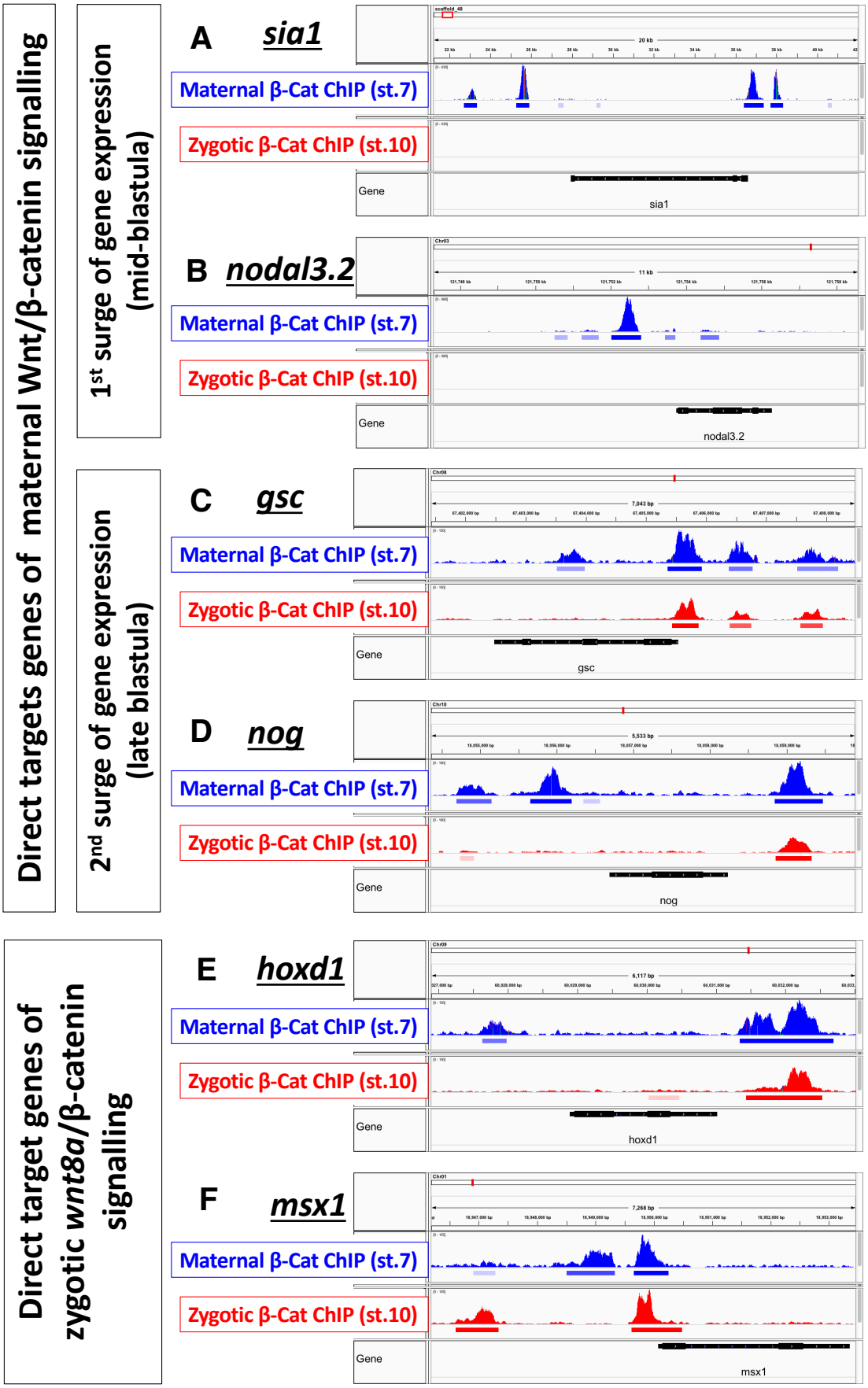
Sequence counts for individual genes (in alphabetical order) in the individual samples of the experiment involving control, knock-down and rescue of maternal β-catenin signaling, as indicated.


bmp7.1
cdx1
cdx2
cdx4
cer1
fgf2
foxa1
foxj1
foxo3
frzb
fzd10
fzd6
gata2
hoxa1
hoxd1
id2
irx3
mespa
mespb
msgn1
msx1
msx2
myb
myf5
pnhd
pou4f1.2
rspo1
rspo3
six1
sox17a
sox17b.1
sox17b.2
sp5
t
t2
ventx1.1
ventx1.2
ventx2.1
ventx2.2
ventx3.2
wnt11b
wnt8a
xarp−like
znf703
Uninjected
CoMO
Wnt8MO
W8MO.CSKAW8
Condition

Suppl. Fig.2

Suppl. Figure 2: Transcriptomics of zygotic Wnt8a/β-catenin signaling-regulated genes at stage 10 (early gastrula), Related to Figure 1D

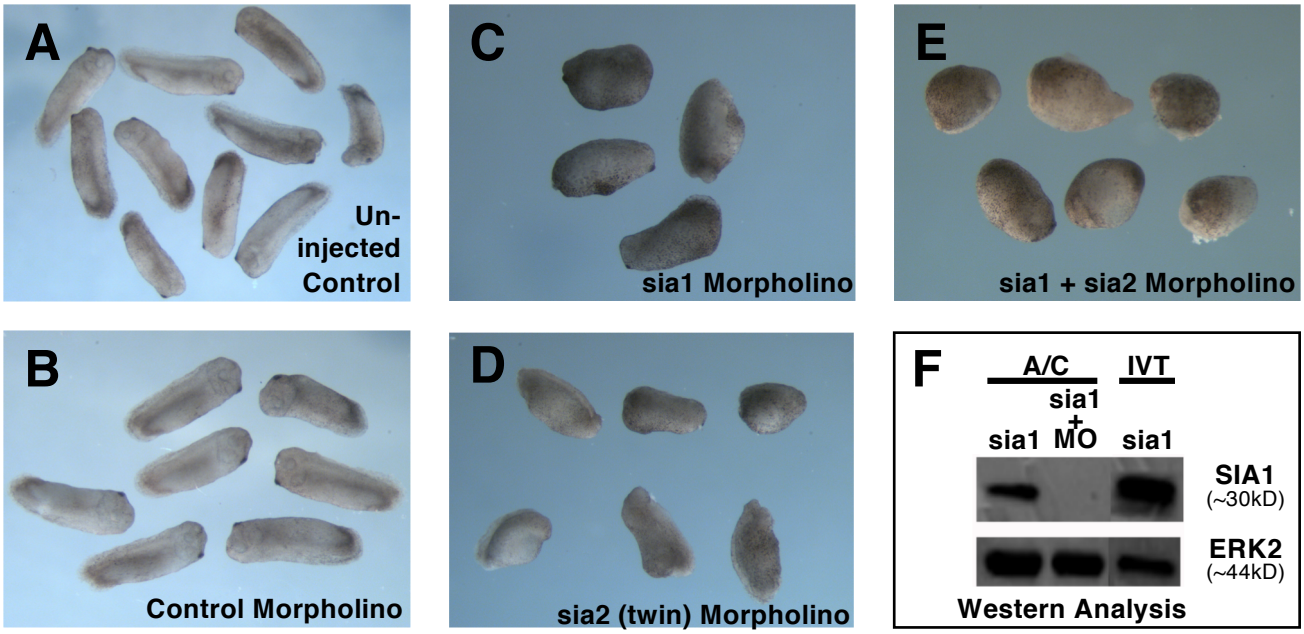
Sequence counts for individual genes (in alphabetical order) in the individual samples of the experiment involving controls, knock-down and rescue of zygotic Wnt8a signaling, as indicated. Data from Nakamura et al. (2016) reanalyzed.

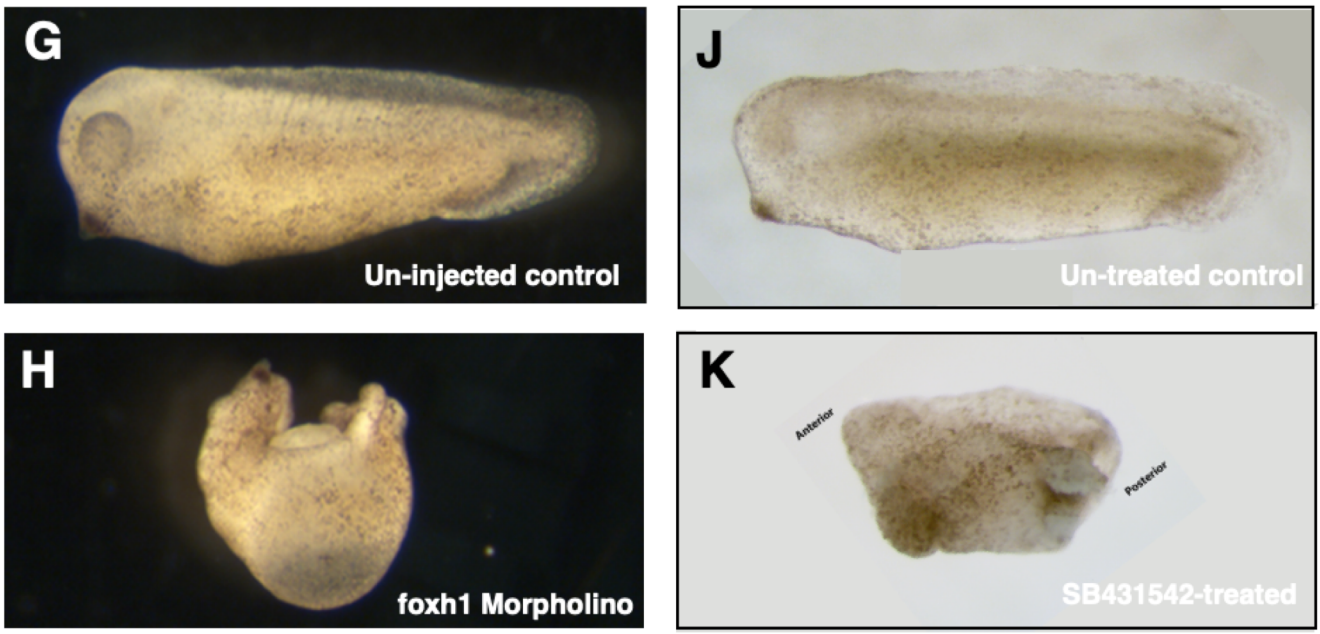

Direct targets genes of maternal Wnt/β-catenin signalling
1st surge of gene expression (mid-blastula)
2nd surge of gene expression (late blastula)
A sia1
Maternal β-Cat ChIP (st.7)
Zygotic β-Cat ChIP (st.10)
Gene
sia1
B nodal3.2
Maternal β-Cat ChIP (st.7)
Zygotic β-Cat ChIP (st.10)
Gene
nodal3.2
C gsc
Maternal β-Cat ChIP (st.7)
Zygotic β-Cat ChIP (st.10)
Gene
gsc
D nog
Maternal β-Cat ChIP (st.7)
Zygotic β-Cat ChIP (st.10)
Gene
nog
Direct target genes of zygotic wnt8a/β-catenin signalling
E hoxd1
Maternal β-Cat ChIP (st.7)
Zygotic β-Cat ChIP (st.10)
Gene
hoxd1
F msx1
Maternal β-Cat ChIP (st.7)
Zygotic β-Cat ChIP (st.10)
Gene
msx1

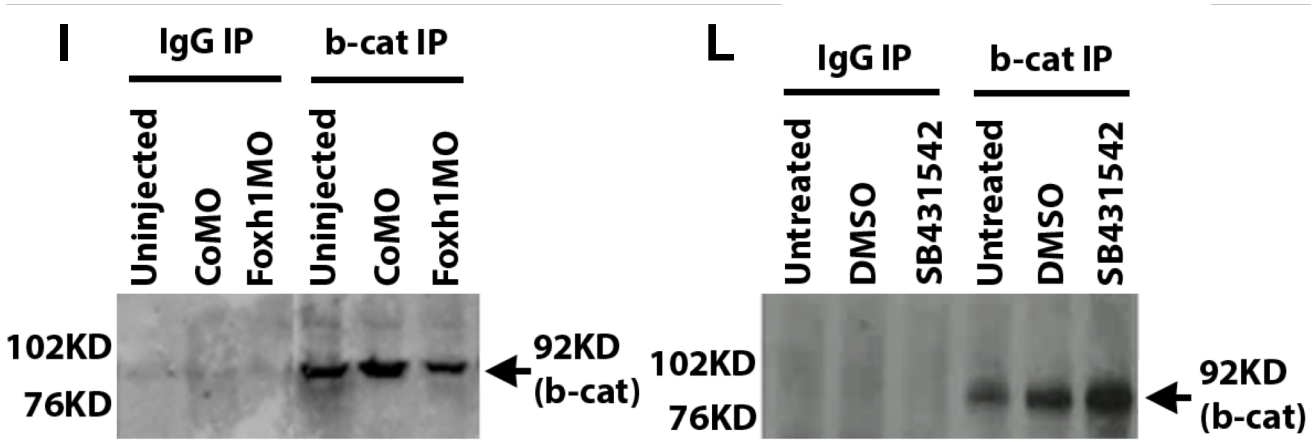
Suppl. Fig. 3

Suppl. Figure 3: Comparing maternal and zygotic β-catenin ChIP-seq signals at example gene loci, Related to Figure 1G

Genome alignment of β-catenin ChIP-seq signals at example gene loci at stage 7 (in blue, to illustrate genome association by β-catenin regulated by maternal Wnt/β-catenin signaling) and stage 10 (in red, to illustrate genome association by β-catenin regulated by zygotic *wnt8*/β-catenin signaling; data from Nakamura et al. (2016). A) *siamois1* (*sia1* gene locus), B) *nodal3.2* gene locus C) *gsc* gene locus D) *noggin* (*nog* gene locus) E) *hoxd1* gene locus and F) *msx1* gene locus.


A
Un-injected Control
C
sia1 Morpholino
E
sia1 + sia2 Morpholino
B
Control Morpholino
D
sia2 (twin) Morpholino
F
A/C
IVT
sia1
+
MO
sia1
sia1
SIA1
(~30kD)
ERK2
(~44kD)
Western Analysis


G
Un-injected control
J
Un-treated control
H
foxh1 Morpholino
K
Anterior
Posterior
SB431542-treated


I
IgG IP
b-cat IP
Uninjected
CoMO
Foxh1MO
Uninjected
CoMO
Foxh1MO
102KD
76KD
92KD
(b-cat)
L
IgG IP
b-cat IP
Untreated
DMSO
SB431542
Untreated
DMSO
SB431542
102KD
76KD
92KD
(b-cat)

Suppl. Fig. 4

Suppl. Figure 4: Specificity of siamois Morpholino, FoxH1 Morpholino and SB431542 TGFβ inhibitor, Related to Figures 3 and 4

A-E) *Xenopus tropicalis* embryos were injected into the two-dorsal blastomeres of the four-cell embryo with 10ng of either control Morpholino MO (B; N=45), sia1 MO (C; N=27) or sia2 (twn) MO (D; N=32); or both sia1 and sia2 MO (F; N=22)(A is un-injected control, see Transparent Methods for MO sequences). Note lack of A-P and D-V patterning in the single-injected morphants (C, D) and more severe effects in the double-injected morphants (E). (F) *Xenopus laevis* embryos were injected at one-cell stage into the animal pole with 10pg of mRNA encoding *Xenopus tropicalis* sia1 (Haramoto et al., 2017) or combined with *Xenopus tropicalis* sia1 MO, as indicated. Animal caps explants (A/C) were excised at stage 8 and explants cultured until stage 12 to monitor protein expression using an anti-rabbit Sia antibody (Sudou et al., 2012). Note that MO efficiently blocks Sia protein production. IVT: In Vitro Translation. (G-I) *Xenopus tropicalis* Foxh1 MO (Chiu et al., 2014) was injected into *Xenopus tropicalis* embryos (see Transparent Methods) and embryos collected when control un-injected embryos (G) reached stage 32. (H) Foxh1 morphant display severe A-P and D-V defects. (I) ChIP qPCR analysis; i.e. immunoprecipitation for chromatin-associated β-catenin protein (see Transparent Methods). (J-L) Embryos were treated with TGFβ inhibitor SB431542 (see Transparent Methods) until stage 32 when control untreated embryos reached stage 32 (J). (K) SB431542-treated embryos lack distinctive A-P patterning and (L) ChIP analysis performed using conditions and reagents as in (I).

TRANSPARENT METHODS

Embryo manipulations:

Xenopus tropicalis embryos were obtained by *in vitro* fertilization (del Viso and Khokha, 2012) and staged according to Nieuwkoop and Faber (1967). The fertilized embryos were either injected with morpholinos (MOs) and/or mRNA or treated with chemical inhibitors (see below), as indicated. The injected embryos were cultured in 0.1x Marc's Modified Ringer (MMR) at 28°C. Sequences of MOs obtained from Gene Tools (Philomath, Oregon, United States) were as follow:

- CoMO: 5'-CCTCTTACCTCAGTTACAATTTATA-3' (Khokha et al., 2002);
- *Xenopus tropicalis* (Xt) β-catenin (ctnnb1) MO (Khokha et al., 2002): 5'-TTTCAACAGTTTCCAAAGAACCAGG-3';
- Xt foxh1 MO (Chiu et al., 2014): 5'-TCATCCTGAGGCTCCGCCCTCTCTA-3';
- Xt sia1-1 MO: 5' GCTCCATTTCAGCCTCACAGGTCAT 3' (X. tropicalis equivalent to X.laevis sia1 MO from Bae et al., 2011, shown in Fig. 3 and Suppl. Fig. 4);
- Xt sia1-2 MO: 5' TTCGCCTCACAGGTCATGTCTGTC 3' (X. tropicalis equivalent to X. laevis sia1 MO from Ishibashi et al., 2008, used as additional control, not shown);
- Xt sia2-1 MO: 5' GCTCAAGCTCAGAGTCACAAGTCAT 3' (X. tropicalis equivalent of X. laevis twn MO from Bae et al., 2011, used as additional control, not shown);
- Xt sia2-2 MO: 5' CTCAGAGTCACAAGTCATCCTTGAA 3' (X. tropicalis equivalent of X. laevis twn MO from Ishibashi et al., 2008, shown in Fig.3 and Suppl. Fig. 4).

The two sia1 and the two sia2 MOs were tested in pilot experiments and confirmed to induce the expected phenotype (Bae et al., 2011). Since they induced these phenotypes at lower injection amounts or in a higher percentage of embryos when injected at the same amount (2.5ng per blastomere, 5ng per embryo), we continued our experiments with sia1-1 and sia2-2 MOs. Capped mRNA was synthesized using mMESSAGE mMACHINE Kit (Ambion) according

to manufacturer instructions. β-catenin plasmid (Yost et al., 1996) and constitutively active siamois VP16-sia plasmid (Kessler, 1997) were linearized with NotI and *in vitro* transcribed with SP6. The VP16-siamois fusion construct was used as an additional rescue control for the sia1 and sia2 Morpholino knockdown (e.g. Fig.3B), since its sequence was not targeted by any of the four MO used to knockdown *sia1* and *sia2* gene expression. The Nodal/TGFβ-signaling inhibitor SB431542 (Tocris Bioscience) was reconstituted to 10mM and diluted to 100μM in the culture medium. Four-cell stage embryos were immersed in 100μM SB431542 in 1/9xMMR and cultured at 25°C until mock (solvent)-treated siblings reached desired stage (see also Chiu et al., 2014).

Relevant regulatory standards

Experiments conducted at the University of Aberdeen were initially assessed and approved and in 2017 reviewed and renewed each time first by the University of Aberdeen Ethical Review Committee and then by the United Kingdom Home Office Inspector. All animal experiments were subsequently carried out under license from the United Kingdom Home Office: PPL 60-04376 (until 19 September 2017) and PPL PA66BEC8D (since 20 September 2017). For experiments conducted at the University of California Irvine, animals were raised and maintained in accordance with the University of California, Irvine Institutional Animal Care Use Committee (IACUC) and guided by husbandry methods developed by the National Xenopus Resource (Marine Biological Laboratory, Woods Hole, MA).

RNA extraction and RNA expression analysis with qPCR and RNA-seq

Total RNA was isolated from whole embryos using the RNeasy Mini Kit, according to manufacturer's instructions (QIAGEN) for processing of animal tissues (see also Lee-Liu et al., 2012; Nakamura et al., 2016). The abundance of RNAs was determined using a LightCycler 480 and SYBR Green I Master Reagents (Roche). Relative expression levels of genes was determined using ΔΔC(t) or Livak method (Taneyhill and Adams, 2008). For the RNA-seq analysis

of the functional β-catenin experiments Illumina TruSeq RNA libraries were constructed and sequenced using Illumina HiSeq 2000 at the Earlham Institute, Norwich, UK. For the RNA-seq analysis of the foxh1 MO knockdown and the Nodal/TGFβ signaling inhibition experiments total RNA was extracted from ~25-30 control and experimental early gastrula embryos using the acid guanidinium thiocyanate phenol chloroform method (Chomczynski and Sacchi, 1987) followed by selective precipitation of RNA using 2.5M LiCl. The quality of the RNA was examined using an Agilent BioAnalyzer 2100 instrument. 1µg of total RNA was subjected to oligo(dT) selection to extract polyadenylated RNA, which was then chemically fragmented and libraries were generated for single-end sequencing according to Illumina's RNA-seq sample preparation kit (see also Chiu et al., 2014).

β-catenin ChIP and β-catenin ChIP-seq

β-catenin ChIP qPCR and ChIP-seq experiments were conducted using anti-β-Catenin (Ctnnb1) antibody (H-102). *Xenopus tropicalis* embryos were harvested at stage 7 and fixed at room temperature with 1% formaldehyde in phosphate-buffered saline (PBS) for 45 minutes. 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 procedures, 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, homogenized, and then kept on ice for 10 minutes. After re-homogenization, the embryo extracts were transferred to TPX microtubes (Diagenode) and sonicated during 25 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). The de-crosslinked DNA fragments were purified with phenol:chloroform:isoamylalcohol and precipitated in ethanol using 50 embryos for qPCR (e.g. Fig.4J,K). For sequencing, sheared chromatin was collected from approximately 25,000 stage 7 embryos. 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). 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 Earlham Institute, Norwich, UK (see also Akkers et al., 2012; Nakamura et al., 2016).

Foxh1 ChIP-seq experiment

Foxh1 ChIP-seq experiments were carried out with a custom anti-Foxh1 antibody (Chiu et al., 2014) using 4 µg of antibody per 100-embryo-equivalents of chromatin for ChIP. Embryos were cultured in 1/9X MMR at 25°C until the indicated stage and fixed in 1% formaldehyde at room temperature for 45 minutes with gentle rocking. Crosslinking reactions were neutralized by the removal of the formaldehyde solution and incubation with 1ml 0.125M glycine solution for 10 minutes on ice. Embryos were then washed with cold RIPA buffer (50 mM Tris-HCl pH7.4, 150mM NaCl, 1mM EDTA, 0.25% sodium deoxycholate, 1%NP40, 0.1% SDS, 0.5mMDTT, and Roche cOmplete protease inhibitor cocktail), flash frozen, and stored at -80°C. The fixed embryos were homogenized in RIPA buffer and incubated on ice for 10 minutes. Samples were then microfuged at 14,000 rpm for 15 minutes at 4°C. Pellets were resuspended in RIPA buffer and sonicated on ice using a Branson Digital Sonifier 450 resulting in an average fragment size between 200-500bp. The samples were microfuged at 14,000 rpm for 20 minutes at 4°C to remove insoluble cellular debris. The chromatin was then ‘‘pre-cleared’’ by incubating with Protein A-coated Dynabeads (Invitrogen) for 2 hour at 4°C with rotation. Antibodies were pre-bound to blocked Protein A Dynabeads by incubating at 4°C for 30 min. A sample of sheared chromatin was frozen for use as an input control. Pre-cleared chromatin was added to antibody-bound Dynabeads, and incubated overnight at 4°C on an end-over-end rotator. The next day, the beads were washed for 20 minutes each with ice-cold ChIP wash solution I (50mM HEPES-KOH pH7.5, 2mM EDTA, 150mM NaCl, 0.1% sodium deoxycholate, 1% Triton X-100, 1mM DTT, and 0.4mM PMSF), ChIP wash solution II (50mM HEPES-KOH pH7.5, 2mM EDTA, 500mM NaCl, 0.1% sodium deoxycholate, 1% Triton X-100, 1mM DTT, and 0.4mM PMSF), ChIP wash solution III (0.25 M LiCl, 1 mM EDTA, 10 mM Tris-HCl pH 8.0, 0.5% NP-40, 0.5% sodium deoxycholate, 1 mM DTT, and 0.4 mM PMSF), and TE (10mM Tris, 1mM EDTA, 1 mM DTT, and 0.4 mM PMSF). The DNA was then eluted with TE buffer containing 1% SDS, and reverse-crosslinked at 65°C overnight. The sonicated input control was diluted 3-fold with elution buffer, and also incubated at 65°C. All samples were treated with

RNAse A, Proteinase K, phenol/chloroform extracted, and ethanol precipitated overnight. DNA pellets were resuspended in Qiagen EB solution. 10-30ng of total ChIP DNA was used for library construction using the NEXTflex ChIP-seq kit (Bioo Scientific). Sequencing was performed using the Illumina HiSeq 2500 and 50bp single-end reads were obtained (see also Charney et al., 2017; Chiu et al., 2014).

Bioinformatics:

RNA-seq differential expression analysis

Maternal Wnt/β-catenin-regulated transcriptome RNA sequencing data was quality control checked using FastQC (Andrews, 2015, version 0.11.3), aligned to the *Xenopus tropicalis* V9 reference genome (James-Zorn et al., 2015; Karpinka et al., 2015) using STAR (Dobin et al., 2013, version 2.4.0), converted to bam format and sorted using SAMtools (Li et al., 2009, version 1.2), and quantified at gene regions using HTSeq (Anders and Huber, 2010, version 0.6.1).

RNA sequencing data from Foxh1 MO and TGFβ inhibitor (SB431542) experiments were quality control checked using FastQC (Andrews, 2015, version 0.11.3) and TrimGalore! (Krueger, 2015, version 0.4.0), aligned to the *Xenopus tropicalis* V9 reference genome (James-Zorn et al., 2015; Karpinka et al., 2015) using HISAT2 (Kim et al., 2015, version 2.1.0), converted to bam format and sorted using SAMtools (Li et al., 2009, version 1.2), and quantified at gene regions using featureCounts (Liao et al., 2014, from the Subread package version 5.0-p1) with the parameter enabled to split multi-mapped reads as a fraction across hits.

In all cases, differential expression analysis was carried out with DESeq2 (Love et al., 2014, version 1.14.1) using generalized linear models with the LRT function. For Foxh1 and SB data sets, significance was identified by an FDR < 0.1. For the β-catenin samples, two models were used, incorporating the data from the rescue strain, genes with an FDR < 0.1 were selected, and an intersect of these genes between the two models was used to identify significance.

ChIP Sequencing analysis

ChIP sequencing peak data for zygotic β-catenin (st.10) was obtained from Nakamura et al. (2016). Previously published raw ChIP sequencing data for Foxh1 from Chiu et al. (2014) and Charney et al. (2017) was downloaded from the Gene Expression Omnibus using SRA toolkit (Alnasir and Shanahan, 2015, www.ncbi.nlm.nih.gov/sra, version 2.8.2). To ensure consistency in the analysis, previously published and new Foxh1 sequencing data (stage 7) was analyzed in an identical manner to Nakamura et al. (2016). In short, this encompassed quality filtering with FastQC (Andrews, 2015, version 0.11.3) and TrimGalore! (Krueger, 2015, version 0.4.0), alignment to the *Xenopus tropicalis* v9 reference genome (James-Zorn et al., 2015; Karpinka et al., 2015) using BWA aln (Li, 2013, version 0.7.12), conversion to sam format with BWA samse (Li, 2013, version 0.7.12), conversion to bam format with SAMtools (Li et al., 2009, version 1.2) incorporating removal of unmapped reads and non-primary alignments, peak calling independently with MASC2 (Zhang et al., 2008, version 2.1.20160309) and SPP (Kharchenko et al., 2008, version 1.14), before consensus peak calling with IDR (Li et al., 2011, version 2.0.2), and finally identifying common peaks between replicates, and between Foxh1 and β-catenin ChIP samples, using BEDTools (Quinlan, 2014; Quinlan and Hall, 2010, version 2.26.0). Peak selection was based on any genomic region that had overlapping regions of any length between two peak files. Closest adjacent genes were identified using BEDTools (Quinlan, 2014; Quinlan and Hall, 2010, version 2.26.0). Heatmaps of peak regions were created using Homer (Heinz et al., 2010, version 4.8.3). De-novo motif analysis was performed using Homer (Heinz et al., 2010, version 4.8.3) and MEME-ChIP (Machanick and Bailey, 2011, version 4.11.2).

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