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Wnt3a/β-catenin signaling controls posterior body development by coordinating mesoderm formation and segmentation

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Somitogenesis is thought to be controlled by a segmentation clock, which consists of molecular oscillators in the Wnt3a, Fgf8 and Notch pathways. Using conditional alleles of *Ctnnb1* (β-catenin), we show that the canonical Wnt3a/β-catenin pathway is necessary for molecular oscillations in all three signaling pathways but does not function as an integral component of the oscillator. Small, irregular somites persist in abnormally posterior locations in the absence of β-catenin and cycling clock gene expression. Conversely, Notch pathway genes continue to oscillate in the presence of stabilized β-catenin but boundary formation is delayed and anteriorized. Together, these results suggest that the Wnt3a/β-catenin pathway is permissive but not instructive for oscillating clock genes and that it controls the anterior-posterior positioning of boundary formation in the presomitic mesoderm (PSM). The Wnt3a/β-catenin pathway does so by regulating the activation of the segment boundary determination genes *Mesp2* and *Ripply2* in the PSM through the activation of the Notch ligand *Dll1* and the mesodermal transcription factors *T* and *Tbx6*. Spatial restriction of *Ripply2* to the anterior PSM is ensured by the Wnt3a/β-catenin-mediated repression of *Ripply2* in posterior PSM. Thus, Wnt3a regulates somitogenesis by activating a network of interacting target genes that promote mesodermal fates, activate the segmentation clock, and position boundary determination genes in the anterior PSM.

KEY WORDS: Wnt3a, β-catenin, Gastrulation, Mesoderm, Segmentation, Somitogenesis

INTRODUCTION

Gastrulation is a major early event in the development of the mammalian embryo. The primitive streak (PS) plays a crucial role in gastrulation because it is the site where mesoderm and endoderm progenitors are specified. The paraxial presomitic mesoderm (PSM) cells that will give rise to the musculoskeletal system of the trunk and tail, arise in the anterior PS (Tam and Beddington, 1987). The morphogenetic process of gastrulation continuously drives the movement of PSM progenitors from the streak to the posterior end of the PSM. Cells in the posterior PSM remain in an undetermined and immature state, and become anteriorly displaced as new cells are added to the posterior PSM. When PSM cells reach a prescribed position in the anterior PSM, they undergo a dramatic transition in gene expression, initiating a segmentation program that determines where and when a morphological segment boundary will form. The rhythmic formation of a new boundary in the anterior PSM, every 2 hours, leads to the formation of somites. Thus the coordinated addition of new mesodermal cells to the posterior PSM, coupled with the cleaving of new somites from the anterior PSM, is critical for the maintenance of the PSM and, ultimately, for the rapid growth and posterior extension of the body axis that occurs during vertebrate embryogenesis (Aulehla and Herrmann, 2004; Dubrulle and Pourquie, 2004; Pourquie, 2001; Saga and Takeda, 2001).

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The bHLH transcription factor Mesp2, under the control of the Notch signaling pathway, plays an important role in the segmentation program. *Mesp2* is expressed in a segmental prepattern in the anterior PSM prior to the formation of overt boundaries, and is required for segment polarity and boundary formation (Saga et al., 1997). The prevailing 'clock and wavefront', or 'clock and gradient' models postulate that segment boundaries are positioned along the anterior-posterior (AP) axis by gradients of fibroblast growth factor 8 (Fgf8) and/or Wnt3a and an opposing gradient of retinoic acid (RA), which together define a boundary determination front in the anterior PSM. The periodicity of boundary formation is thought to be controlled by an oscillating segmentation clock driven by the Wnt and Notch signaling pathways (Aulehla and Herrmann, 2004; Pourquie, 2003; Rida et al., 2004). The molecular mechanisms linking these signaling pathways to the clock and to boundary formation, are not well understood.

Feedback suppressor loops in the Wnt and Notch pathways are considered central molecular components of the segmentation clock. Notch activity oscillates in the PSM, driving periodic expression of its target genes lunatic fringe (*Lfng*) and *Hes7* (Bessho et al., 2001; Morimoto et al., 2005). The glycosyltransferase Lfng, and the transcriptional repressor Hes7, function as negative regulators of Notch signaling and are required for proper segmentation (Bessho et al., 2003; Evrard et al., 1998). Similarly, the Wnt target genes *Axin2* and *Nkd1* encode negative regulators of Wnt signaling, oscillate in the PSM, and are thought to function as integral components of the clock to periodically suppress Wnt signaling (Aulehla et al., 2003; Ishikawa et al., 2004). Oscillating genes in both the Wnt and Notch pathways depend upon *Wnt3a* (Aulehla et al., 2003; Nakaya et al., 2005), however the significance of Wnt-centered feedback loops for the clock remains unclear because mutations in *Axin2* or *Nkd1* do not lead to somite or mesodermal phenotypes (Li et al., 2005; Yu et al., 2005).

Wnt3a controls gene expression by stabilizing cytosolic levels of β-catenin, the central player in the canonical Wnt/β-catenin pathway. Stabilized β-catenin can then translocate to the nucleus, bind to Tcf/Lef transcription factors, and activate target genes (Stadeli et al., 2006; Willert and Jones, 2006). Embryos lacking *Wnt3a* display posterior axis truncations (Takada et al., 1994). This is due, at least in part, to changes in the expression of the target genes *T* (*Brachyury*), a T-box transcription factor gene necessary for mesoderm formation, and *Dll1*, which encodes a Notch ligand required for segmentation (Arnold et al., 2000; Aulehla et al., 2003; Galceran et al., 2001; Galceran et al., 2004; Hofmann et al., 2004; Nakaya et al., 2005; Yamaguchi et al., 1999). Although a few important target genes have been validated, the transcriptional network activated by Wnt3a in vivo remains largely unresolved. Since functional redundancy between Wnt ligands may confound a full understanding of the role of Wnts and their target genes in these processes, we have turned to conditional loss and gain of function alleles of β-catenin to determine the precise role that Wnt3a and β-catenin play in mesoderm formation and segmentation.

MATERIALS AND METHODS

Mice

Ctnnb1^{tm2Kem} were purchased from the Jackson Labs. The *Ctnnb1*$^{lox(ex3)}$ (Harada et al., 1999), *T-cre* (Perantoni et al., 2005), and *BATlacZ* mice (Nakaya et al., 2005), were described previously. Transgenic mice were generated in the Transgenic Core Facility, NCI-Frederick, by pronuclear injection following standard procedures. All animal experiments were performed in accordance with the guidelines established by the NCI-Frederick Animal Care and Use Committee.

Half-embryo explant cultures

PSM/somite explants were dissected from E9 outbred NIH Swiss embryos, and bisected down the midline as previously described (Correia and Conlon, 2000). One half was immediately fixed whereas the remaining half was cultured in 10% FBS in DMEM for 1 hour.

In situ hybridization

Single and double whole-mount in situ hybridization (WISH) was performed as previously described (Biris et al., 2007). Embryos were photographed on a Leica stereoscope or a Zeiss Axiophot compound microscope. Unless indicated otherwise, at least four mutant embryos were examined with each probe, and all yielded similar results.

Gene expression profiling

Total RNA was isolated with TRIzol reagent (Invitrogen) as previously described (Baugh et al., 2003) from microdissected node and primitive streak regions of E7.75-E8 wild-type and Wnt3a mutant embryos. Protocols for synthesis of cDNA and cRNA were performed using the Affymetrix Two-Cycle Target Labeling Kit (Affymetrix, Santa Clara, CA) according to the manufacturer's recommendations. Array hybridizations (GeneChip Mouse Genome 430 2.0, Affymetrix) were performed in triplicate per genotype. Subsequent washing, staining, and array scanning were carried out according to Affymetrix protocols. Statistical analysis was performed on probe-intensity level data using BRB ArrayTools (v3.2). Class comparison analysis was conducted using a random-variance F-test ($P \leq 0.001$). Hierarchical clustering was carried out for statistically significant genes using normalized $\log_2$ of the signal values.

Expression constructs, transfections, cell culture and luciferase reporter assays

Luciferase reporter constructs were generated by cloning the *Ripply2* enhancer fragment (2.1 kb; *Sac*I-*Xho*I) with and without the *Ripply2* promoter (1.1 kb; *Xho*I-*Nco*I) into pGL4.10[*luc*2] or pGL4.23[*luc*2/minP] vectors (Promega), respectively. Two deletion constructs were generated, one consisting of the 1.1 kb *Ripply2* promoter lacking the proximal putative Tbx6 binding site and E-box, and a second containing three repeats of this 44 bp region upstream of a minimal promoter. HEK293 cells were seeded at 0.55×10^5 cells per well in 24-well plates and grown to 70% confluency. A total of 400 ng DNA containing the reporter plasmid (200 ng) and empty vector were co-transfected with or without expression vectors, pCS2-3X FLAG Tbx6 (10 ng), p3X FLAG-CMV Mesp2 (50 ng), and pCDNA3 ΔN-β-catenin-myc (150 ng) using Fugene 6 (Roche). Cells were lysed 48 hours after transfection and luciferase activity was measured using the Dual Luciferase Assay Kit (Promega) as per manufacturer's recommendation. For each condition,10 ng pGL4.74[hRluc/TK] Vector (Promega) was used as an internal control to normalize for transfection efficiency. Fold change was calculated as a ratio of the luciferase vector containing *Ripply2* regulatory elements relative to empty luciferase vector for identical experimental conditions, normalized to a control condition minus expression vectors. The reported values consist of one experiment but are representative of at least three independent experiments.

Electrophoretic mobility shift assay

Double-stranded DNA oligonucleotides were end labeled and protein-DNA complexes were analyzed using the DIG Gel Shift Assay Kit (Roche) according to the manufacturer's instructions. A 3× Flag-Tbx6 protein was made using the TNT Reticulocyte Lysate System (Promega) in vitro. Binding reactions were incubated at room temperature for 15 minutes and the protein-DNA complexes were analyzed on 6% DNA retardation gels (Invitrogen). Oligonucleotide sequences used in the EMSA assays are as follows: 5′-CGTT**CACAC**CCGCGCGCGGCCCGCGGCGCC-3′ (wt Tbx6 BS); 5′-CGTT**GATAT**CCGCGCGCGGCCCGCGGCGCC-3′ (mut Tbx6 BS).

RESULTS

Mesoderm formation and segmentation defects in conditional *Ctnnb1* mutants

To bypass the pre-gastrulation lethality observed in embryos null for *Ctnnb1* (Haegel et al., 1995; Huelsken et al., 2000), we conditionally inactivated *Ctnnb1*^{tm2Kem} (Brault et al., 2001) in the PS on E7.5 (one day after gastrulation begins) using the *T* promoter to express Cre recombinase (Perantoni et al., 2005). Conditional *Ctnnb1* loss of function (LOF) mutants, hereafter referred to as *T-Cre;Ctnnb1*$^{flLOF/\Delta}$, gastrulated normally but displayed posterior truncations (Fig. 1B) that were similar but more severe than that observed in *Wnt3a*$^{-/-}$ mutants (Takada et al., 1994). Head structures were normal but mutants lacked a PS, posterior mesoderm and obvious somites. Loss of β-catenin activity was confirmed by monitoring the expression of the *BATlacZ* transgene, an in vivo reporter of β-catenin/Tcf function (Nakaya et al., 2005). Reporter activity was down-regulated specifically in the posterior embryo, with residual activity only detectable in surface ectoderm (Fig. 1B). Conversely, conditional stabilization of β-catenin [*Ctnnb1*$^{lox(ex3)}$ (Harada et al., 1999), hereafter referred to as *Ctnnb1*$^{flGOF/+}$] in streak-derived mesoderm through the T-Cre-mediated deletion of the phosphorylation/degradation domain of β-catenin, led to a grossly enlarged PSM, at the apparent expense of somites, and impaired embryo turning (Fig. 1C). *BATlacZ* expression appeared upregulated and anteriorized in the *T-Cre;Ctnnb1*$^{flGOF/+}$ mutants, confirming the enhanced activity of β-catenin.

Mutants were examined at earlier developmental stages (E8.2-8.5) to assess segmentation phenotypes. Somites were not observed in the anterior paraxial mesoderm of *T-Cre;Ctnnb1*$^{flLOF/\Delta}$ embryos (Fig. 1I), although they were clearly distinguishable in controls (Fig. 1D). Histological analyses confirmed that the mutant anterior paraxial mesoderm was unsegmented, thickened and disorganized, but small, incompletely epithelialized somites were observed in posterior regions where the PSM would normally lie (cf. Fig. 1L,M with Fig. 1G,H). The *BATlacZ* reporter was specifically downregulated in the mesoderm (Fig. 1J) and posterior streak (Fig.

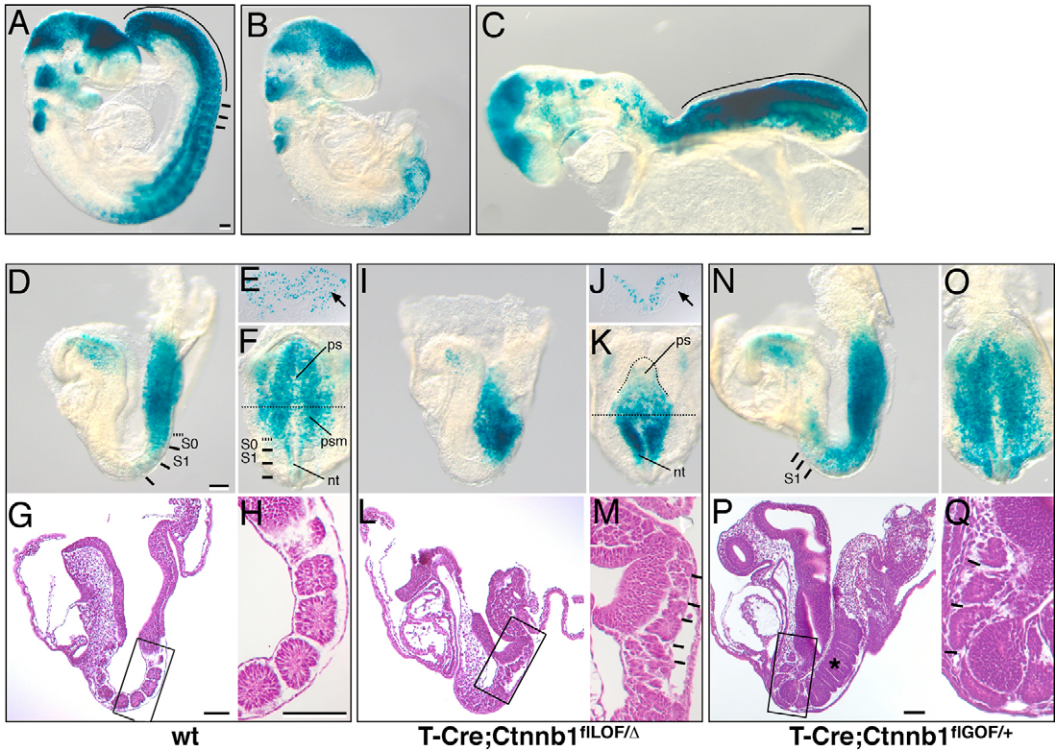

Fig. 1. Conditional loss and gain of β-catenin function causes mesoderm and segmentation phenotypes. (**A-C**) Wnt/β-catenin (BAT*lac*Z) reporter activity in E9 wild-type (A), *T-Cre;Ctnnb1*$^{flLOF/\Delta}$ (B) and *T-Cre;Ctnnb1*$^{flGOF/+}$ (C) embryos. β-galactosidase (β-gal) activity was reduced posteriorly in the truncated *T-Cre;Ctnnb1*$^{flLOF/\Delta}$ mutants, and highly upregulated in the grossly enlarged PSM of the *T-Cre;Ctnnb1*$^{flGOF/+}$ embryo. In addition to the somite defects, kinked neural tubes, and an enlarged alantois were observed in the GOF mutants, whereas enlarged pericardia, and heart-looping defects were found in both LOF and GOF mutants. Bars, segment borders; curved line, the extent of the PSM. (**D-Q**) BAT*lac*Z expression in ~5 ss wild-type (D-F), *T-Cre;Ctnnb1*$^{flLOF/\Delta}$ (I-K), and *T-Cre;Ctnnb1*$^{flGOF/+}$ (N,O) embryos. (E,J) Cross-sections through the PS and PSM (level indicated by dotted lines in F and K) illustrate that the remaining β-gal activity in the *T-Cre;Ctnnb1*$^{flLOF/\Delta}$ PS (I) was found only in the ectoderm and not in the mesoderm (arrow in J). (G,H,L,M,P,Q) Hematoxylin and Eosin stained sections of E8.5 wild-type (G,H), *T-Cre;Ctnnb1*$^{flLOF/\Delta}$ (L,M), and *T-Cre;Ctnnb1*$^{flGOF/+}$ (P,Q) embryos. High-power magnifications illustrated in H, M and Q are taken from boxed regions in G, L and P. The asterisk in P indicates the kinked neural tube. All embryo images are lateral views, with the exception of F, K, and O, which offer a ventral-posterior perspective. S0, forming somite; SI, first somite; ps, primitive streak; psm, presomitic mesoderm; nt, neural tube. Scale bars: 100 μm.

1I,K), but remained easily detectable in the streak ectoderm and posterior neural tube. Similar small disorganized somites were also found in *T-Cre;Ctnnb1*$^{flGOF/+}$ mutants at early somite stages, but were only detectable anteriorly, rostral to the enlarged PSM (Fig. 1N-Q). As expected, the β-catenin reporter was upregulated in the streak and anteriorized in the elongated PSM (Fig. 1N,O). At these stages, the somites generally formed anterior to the *BATlacZ* expression domain in both the wild-type and *T-Cre;Ctnnb1*$^{flGOF/+}$ embryos (Fig. 1D,F,N).

The PS and/or early mesoderm markers, *T/Brachyury* (Fig. 2A-C), *Tbx6* (Fig. 2D-F), *Fgf8* (Fig. 2G-I), and *Dll1* (not shown) are necessary for posterior mesoderm formation, migration or segmentation (Chapman and Papaioannou, 1998; Herrmann et al., 1990; Hrabe de Angelis et al., 1997; Sun et al., 1999). All four genes were absent from the *T-Cre;Ctnnb1*$^{flLOF/\Delta}$ PS and PSM, but were upregulated and anteriorly expanded in *T-Cre;Ctnnb1*$^{flGOF/+}$ embryos. These results suggest that β-catenin is necessary and sufficient for the specification of posterior PSM fates and for the maintenance of the PS. Furthermore, they demonstrate that *Fgf8*, which is thought to be important for the determination of segment boundary position, lies downstream of β-catenin. Anterior paraxial mesoderm was present in both LOF and GOF mutants, as indicated by the continued expression of the somite markers *Mox1* (Fig. 2A-C) and *Paraxis* (not shown). However, neither *Mox1* nor *Paraxis* (also known as *Tcf15* – MGI) were expressed in a segmental fashion in the anterior paraxial mesoderm of *T-Cre;Ctnnb1*$^{flLOF/\Delta}$ embryos. Coupled with the histological observations that small somites were only found posteriorly, the data indicate that segmentation is delayed but ultimately proceeds in the absence of β-catenin.

Oscillating gene expression in conditional *Ctnnb1* mutants

To determine whether the somitogenesis defects were due to abnormal cycling of the segmentation clock, we examined embryos for the expression of oscillating target genes of the Wnt, Fgf and Notch signaling pathways. As expected from analyses of *Wnt3a* mutants (Aulehla et al., 2003; Nakaya et al., 2005), the Wnt3a target gene *Axin2* was not expressed in the *T-Cre;Ctnnb1*$^{flLOF/\Delta}$ streak and PSM, although easily detected in the head (see Fig. S1 in the supplementary material). Analysis of oscillating genes associated with the Fgf signaling pathway (Dequeant et al., 2006) such as *Dusp6/Mkp3* (Fig. 2J,K), which encodes an extracellular signal-related kinase (ERK) phosphatase, and the FGF pathway inhibitor *Spry2* (not shown), also revealed little to no expression in the *T-Cre;Ctnnb1*$^{flLOF/\Delta}$ PSM. Similarly, the expression of the oscillating Notch target genes *Lfng* and *Hes7* were not observed in the *T-*

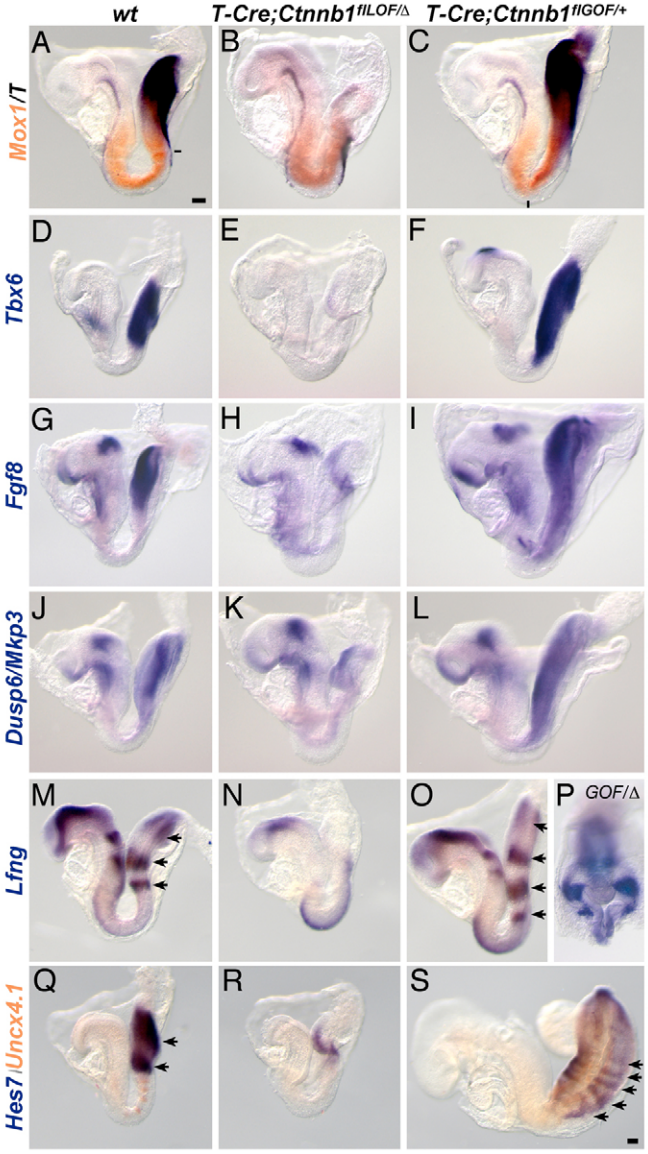

Fig. 2. Examination of mesoderm markers and oscillating clock genes in conditional β-catenin mutants. (**A-I**) Expression analysis of mesoderm and PS markers. *Mox1* (orange), a somite and anterior PSM marker (A), is expressed in the E8.5 *T-Cre;Ctnnb1*$^{flLOF/\Delta}$ (B) and *T-Cre;Ctnnb1*$^{flGOF/+}$ (C) paraxial mesoderm despite the relative lack of segments. *T* (purple) was expressed in the node and notochord of the *T-Cre;Ctnnb1*$^{flLOF/\Delta}$ mutant but was absent from the PS and PSM (B). *T* expression, along with the first segment border B0 (black bar) (Pourquie and Tam, 2001), was anteriorized in the *T-Cre;Ctnnb1*$^{flGOF/+}$ streak and PSM (C). Expression of the PSM marker *Tbx6* (D), and the PS marker *Fgf8* (G), were similarly dependent upon β-catenin, being absent from the *T-Cre;Ctnnb1*$^{flLOF/\Delta}$ PSM (E,H) but upregulated and anteriorized in the *T-Cre;Ctnnb1*$^{flGOF/+}$ mutants (F,I). (**J-S**) Expression analysis of oscillating genes in the Fgf and Notch pathways. *Dusp6/Mkp3*, an oscillating component of the Fgf signaling pathway, is not expressed in the PSM in the absence of β-catenin (K), but is expressed in an anteriorized fashion when β-catenin is stabilized (L). Striped domains of cycling *Lfng* and *Hes7* expression in the PSM (arrows in M,Q) were not apparent in *T-Cre;Ctnnb1*$^{flLOF/\Delta}$ embryos (N,R). By contrast, ectopic *Lfng* and *Hes7* stripes were observed in the elongated *T-Cre;Ctnnb1*$^{flGOF/+}$ PSM (O,S). *Lfng* mRNA stripes were also easily detected in the *T-Cre;Ctnnb1*$^{flGOF/\Delta}$ mutant PSM (P). All views are lateral, with the exception of the ventral view in (P) which afforded a clear view of the *Lfng* stripes. Scale bars: 100 μm.

Cre;Ctnnb1$^{flLOF/\Delta}$ PSM (Fig. 2M,N,Q,R). These results demonstrate that β-catenin is upstream of all signaling pathways known to oscillate in the PSM.

We reasoned that if β-catenin is an integral component of the segmentation clock [predicted by a Wnt3a-β-catenin-*Axin2* negative feedback loop (Aulehla et al., 2003)], then stabilization of β-catenin should disrupt the molecular oscillations associated with the clock. Examination of *Axin2* expression revealed that it was indeed upregulated and anteriorized in the paraxial mesoderm of *T-Cre;Ctnnb1*$^{flGOF/+}$ embryos (see Fig. S1 in the supplementary material), consistent with it being a Wnt3a/β-catenin target gene. The Fgf pathway genes *Dusp6/Mkp3* (Fig. 2L) and *Spry2* (not shown) were also similarly upregulated and anteriorized in *T-Cre;Ctnnb1*$^{flGOF/+}$ embryos. Remarkably, stripes of *Lfng* and *Hes7* mRNA were still observed in the enlarged PSM (Fig. 2O,S), demonstrating that the oscillations of the Notch pathway persist despite the highly stabilized, non-cycling expression of β-catenin and *Axin2*. Although no more than two or three domains of *Lfng* or *Hes7* expression were observed in the wild-type PS and PSM (Fig. 2M,Q), four to seven stripes were observed in the elongated *T-Cre;Ctnnb1*$^{flGOF/+}$ PSM, depending upon embryo age and PSM size (Fig. 2O,S, and data not shown). It is formally possible that the continued periodicity observed in these embryos is attributable to the presence of the wild-type *Ctnnb1* allele. However, examination of *Lfng* expression in embryos that only express the stabilized form of β-catenin (i.e. *T-Cre;Ctnnb1*$^{flGOF/\Delta}$), revealed persistent pairs of *Lfng* stripes in the anterior PSM (Fig. 2P). These results show that the oscillating Notch pathway is surprisingly robust in the presence of stabilized β-catenin, and suggest that Wnt/β-catenin signaling defines a cellular state that is permissive, but not instructive, for the segmentation clock.

Segmental gene expression occurs in *Wnt3a* and *Ctnnb1* mutants

The abnormal somites observed in conditional *Ctnnb1* mutants prompted us to examine mutants for segment polarity defects because the establishment of segment polarity correlates with proper boundary formation (Pourquie, 2001). Expression of the paired-type homeobox gene *Uncx4.1* is restricted to the posterior halves of segmented somites (Mansouri et al., 1997). Initial analyses indicated that *Uncx4.1* was absent in *Ctnnb1* mutants compared with wild-type embryos (Fig. 2Q-S); however, a detailed examination using higher contrast chromogens revealed surprising results. Despite our demonstration that periodic expression of Fgf, Notch and Wnt target genes was arrested in *T-Cre;Ctnnb1*$^{flLOF/\Delta}$ mutants, *Uncx4.1* was still expressed in a segmented pattern in the mutant somites at E8.5 (Fig. 3C,D). *Uncx4.1* stripes were notably absent from the anterior-most paraxial mesoderm, and were compressed and posteriorly shifted, demonstrating that segmentation proceeded but was delayed. These stripes of *Uncx4.1* mRNA were no longer detectable by E9-9.5 (not shown) suggesting that β-catenin is required for the maintenance of the epithelial segment boundary. Interestingly, stabilization of β-catenin in *T-Cre;Ctnnb1*$^{flGOF/+}$ embryos resulted in an anterior shift of the *Uncx4.1* spatial domain (Fig. 3E,F,J). *Uncx4.1* was expressed as two distinct stripes anteriorly, but was fused and disorganized posteriorly.

To compare segment polarity in *Wnt3a* and *Ctnnb1* mutants, we assessed the expression of *Uncx4.1* together with *Tbx18*, a marker of the anterior half-somite (Kraus et al., 2001). Segment polarity was well preserved in the anterior *Wnt3a*$^{-/-}$ somites (Fig. 3H), but was aberrant and less well defined in *T-Cre;Ctnnb1*$^{flLOF/\Delta}$ mutants (Fig. 3I). Nevertheless, interspersed, fuzzy stripes of *Tbx18* and *Uncx4.1*

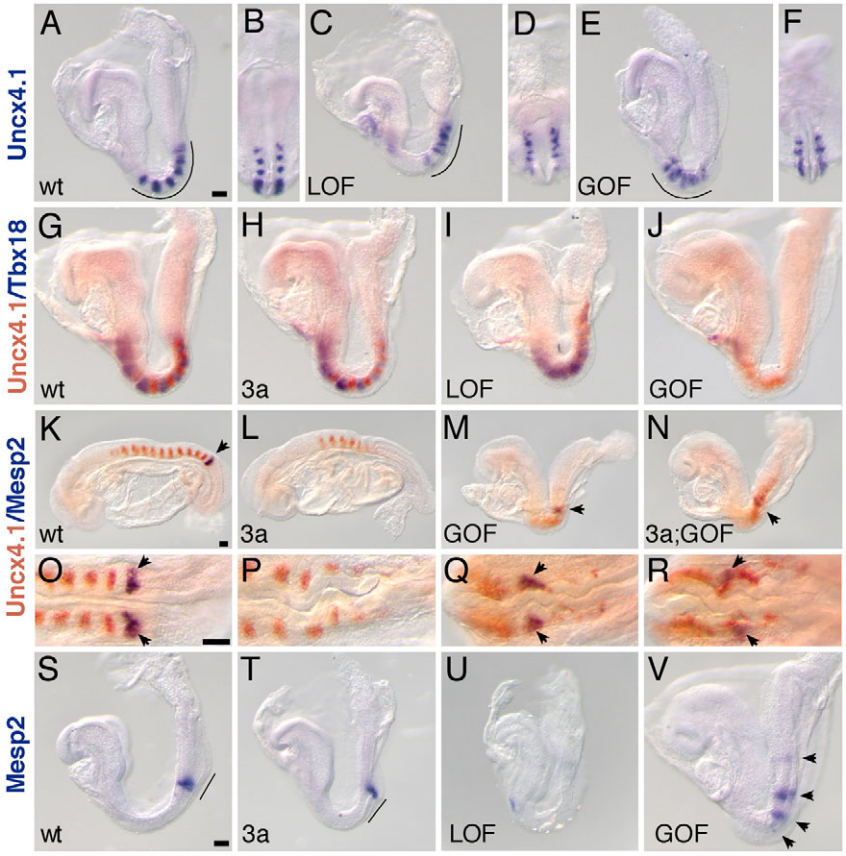

Fig. 3. Wnt3a and β-catenin regulate segment polarity and boundary formation by positioning the expression of segment determination genes. (**A-F**) *Uncx4.1* expression in wild-type (A,B), *T-Cre;Ctnnb1*$^{flLOF/\Delta}$ (C,D), and *T-Cre;Ctnnb1*$^{flGOF/+}$ (E,F) 4-6 ss embryos. Note that B and D are ventral-posterior views, whereas F is an anteroventral view. The curved line depicts the extent of the expression domain along the AP axis. (**G-J**) Two-color WISH depicting *Uncx4.1* (orange) and *Tbx18* (purple) expression in wild-type (G), *Wnt3a*$^{-/-}$ (H), *T-Cre;Ctnnb1*$^{flLOF/\Delta}$ (I), and *T-Cre;Ctnnb1*$^{flGOF/+}$ (J) 5-7 ss embryos. (**K-R**) Two-color WISH depicting *Uncx4.1* (orange) and *Mesp2* (purple, arrows) expression in wild-type (K,O), *Wnt3a*$^{-/-}$ (L,P), *T-Cre;Ctnnb1*$^{flGOF/+}$ (M,Q), and *Wnt3a*$^{-/-}$*; T-Cre;Ctnnb1*$^{flGOF/+}$ (N,R) 10 ss embryos. O-R are high-power magnifications of dorsal (O,P) and ventral (Q,R) views. (**S-V**) *Mesp2* expression in S-I (S) is posteriorized, relative to the node (line), in *Wnt3a*$^{-/-}$ embryos (T), and nearly absent from *T-Cre;Ctnnb1*$^{flLOF/\Delta}$ 2-5 ss embryos (U). *Mesp2* is anteriorized and expressed in multiple stripes (arrows) in *T-Cre;Ctnnb1*$^{flGOF/+}$ embryos (V). 3a, *Wnt3a*$^{-/-}$; LOF, loss of function; GOF, gain of function. Scale bars: 100 μm.

were still detected posteriorly, indicating that rudimentary polarized gene expression and segment borders can form in the absence of β-catenin. By contrast, the caudal limit of the *Uncx4.1* expression domain was shifted anteriorly in *T-Cre;Ctnnb1*$^{flGOF/+}$ embryos, whereas the somitic expression of *Tbx18* was almost completely repressed (Fig. 3J).

Wnt/β-catenin signaling positions the site of segment boundary determination

Our demonstration that reduced β-catenin activity led to a posterior shifting of somites and somite-specific gene expression, whereas elevated β-catenin activity had the opposite effect suggests that the level of Wnt/β-catenin activity controls the AP position of segment boundary formation in the PSM. Examination of mutants for *Mesp2* expression, which is required for proper boundary formation, revealed its absence in *Wnt3a*$^{-/-}$ (n=6) embryos and *T-Cre;Ctnnb1*$^{flLOF/\Delta}$ mutants (not shown), although it was easily detected in the wild-type 10 ss (somite-stage) littermate (cf. Fig. 3L,P with Fig. 3K,O). Notably, *Mesp2* expression and PS and PSM cells, were rescued in *Wnt3a*$^{-/-}$ mutants by stabilized β-catenin (n=4) (Fig. 3N,R). The *Wnt3a*$^{-/-}$*; T-Cre;Ctnnb1*$^{flGOF/+}$ double mutant phenocopies the *T-Cre;Ctnnb1*$^{flGOF/+}$ mutant (Fig. 3M,Q), demonstrating that *Ctnnb1* is epistatic to *Wnt3a*, and that *Mesp2* lies downstream of both genes. In addition, the data provides formal genetic proof that β-catenin mediates the mesoderm-inducing activity of Wnt3a.

Because the loss of *Mesp2* expression in E8.5 mutants may simply be due to the absence of posterior mesoderm, we examined the position of *Mesp2* transcripts in younger *Wnt3a*$^{-/-}$ embryos (0-4 ss), which still possess posterior mesoderm. Although the *Mesp2* stripes always lay adjacent to the anterior end of the wild-type node (Fig. 3S), *Mesp2* expression was posteriorized in *Wnt3a*$^{-/-}$ mutants, abutting the posterior-most end of the node (Fig. 3T). Examination of *T-Cre;Ctnnb1*$^{flLOF/\Delta}$ embryos revealed that *Mesp2* was generally absent (5/6 embryos) (Fig. 3U). In the remaining E8.2 embryo, the *Mesp2* stripe was undefined and further posteriorized than in *Wnt3a*$^{-/-}$ embryos (not shown). Remarkably, multiple anteriorized stripes of *Mesp2* expression, reminiscent of the multiple *Lfng* and *Hes7* stripes, were observed in the expanded PSM of *T-Cre;Ctnnb1*$^{flGOF/+}$ embryos (Fig. 3V). Wnt3a and β-catenin presumably control the spatiotemporal activation of *Mesp2* indirectly, since Notch and Tbx6 are known to directly activate *Mesp2* (Yasuhiko et al., 2006).

Identification of *Ripply2*, a putative boundary determination gene

Mesp2 is thought to function in the anterior PSM to arrest the segmentation clock by activating *Lfng*, and thereby suppressing Notch activity (Morimoto et al., 2005). The ectopic stripes of *Lfng*, *Hes7*, and *Mesp2* mRNA observed in the *T-Cre;Ctnnb1*$^{flGOF/+}$ PSM indicates that the cycling Notch clock failed to arrest despite the expression of *Mesp2* and *Lfng*. These observations suggest that genes in addition to *Mesp2* may be required for segment boundary determination. In a genome-wide microarray screen designed to identify the in vivo target genes of Wnt3a during gastrulation and somitogenesis (unpublished), we identified a RIKEN EST (C030002E08) that was differentially expressed (P<0.001) in *Wnt3a* null mutants compared with the wild type (Fig. 4A). This cDNA represents a putative isoform of the recently described Zebrafish *Ripply2* (Kawamura et al., 2005). The closely related family member *Ripply1* functions in fish to regulate somitogenesis by binding to the transcriptional corepressor Groucho and repressing the zebrafish *Mesp2* homolog, *mesp-b*.

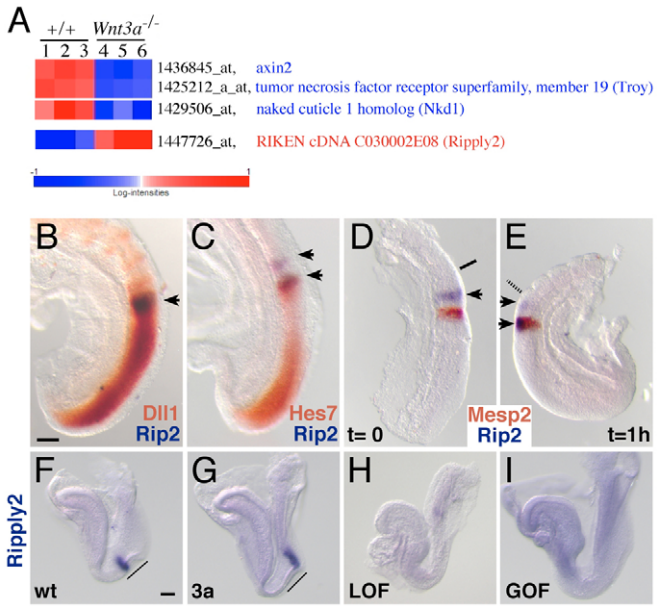

Fig. 4. Transcriptional profiling of 0-2 ss *Wnt3a*$^{-/-}$ embryos identified the putative segment boundary determination gene *Ripply2*. (**A**) Hierarchical clustering of select differentially expressed genes revealed that several previously identified direct Wnt/β-catenin target genes such as *Axin2, Tnfrsf9(Troy)* and *Nkd1* were downregulated (blue) in the *Wnt3a*$^{-/-}$ mutants, as expected. The Riken EST C030002E08 (Ripply2) was identified in a class of genes that were upregulated (red) in embryos lacking Wnt3a. (**B-E**) Two-color WISH illustrating striped *Ripply2* (purple) expression in the anterior PSM (arrows), overlapping with the Notch pathway components (orange) *Dll1* (B) and *Hes7* (C). A representative half-embryo culture experiment illustrates that at time 0, *Ripply2* (purple) and *Mesp2* (orange) are expressed as mutually exclusive domains in adjacent presumptive somites (S-I and S-II, respectively) in one half of the sagitally bisected embryo (D). The solid line represents segment border B0, which is out of the focal plane. After culturing the complementary half-embryo for 1 hour (E), *Ripply2* was strongly co-expressed with *Mesp2* in S-I, while disappearing in S0. The forming segment border B0 is represented by a dashed line. (**F-I**) *Ripply2* expression in S-I (F) is posteriorized relative to the node (line) in *Wnt3a*$^{-/-}$ embryos (G), and nearly absent from T-Cre;Ctnnb1$^{flLOF/\Delta}$ embryos (H). *Ripply2* is not expressed in T-Cre;Ctnnb1$^{flGOF/+}$ embryos (I). Scale bars: 100 μm.

Two-color whole-mount in situ hybridization (WISH) demonstrated that *Ripply2* mRNA was expressed in the anterior PSM in one or two stripes in prospective somites S0 and S-I, overlapping with intense anterior PSM expression of components of the Notch pathway, including *Dll1* (Fig. 4B), *Lfng* (not shown), *Hes7* (Fig. 4C) and *Mesp2* in S-I (see below). To examine the temporal aspects of *Ripply2* and *Mesp2* expression in the anterior PSM, half-embryo culture experiments were performed. These experiments revealed that *Ripply2* expression was dynamic and was periodically activated in S-I after *Mesp2* was activated there (Fig. 4D,E). Similarly, *Ripply2* overlapped, but was expressed out of phase with, *Hes7* (not shown).

Investigation of the role of the Wnt3a/β-catenin pathway in *Ripply2* expression in vivo showed that Wnt signaling indeed regulated *Ripply2*. Like *Mesp2*, *Ripply2* stripes were posteriorized in 0-2 ss *Wnt3a*$^{-/-}$ embryos (n=3), compared with the wild type (Fig. 4F,G), and were undetectable by 3-6 ss (n=3, not shown). Expression was weak to undetectable in similarly staged E8.2 *T-Cre;Ctnnb1*$^{flLOF/\Delta}$ embryos (Fig. 4H), indicating that Wnt3a and β-catenin are required for the maintenance of *Ripply2* expression. Again, similarly to *Mesp2* expression, weak, posteriorized expression was observed in one (n=3) E8.2 *T-Cre;Ctnnb1*$^{flLOF/\Delta}$ embryos (not shown). However, in contrast to the multiple stripes of *Mesp2* expression observed in *T-Cre;Ctnnb1*$^{flGOF/+}$ embryos (Fig. 3V), *Ripply2* was strikingly absent (n=4, Fig. 4I). These results are consistent with the microarray data and together, suggests that *Ripply2* is exquisitely sensitive to the levels of Wnt3a/β-catenin signaling.

Tbx6, Mesp2 and the Wnt pathway regulate *Ripply2*

To investigate the molecular mechanisms underlying the control of *Ripply2* expression, we compared the human and mouse *Ripply2* loci to identify potential regulatory elements. Two conserved regions were found, a putative promoter adjacent to the initiator codon, and a putative enhancer 6 kb upstream (Fig. 5A). Both fragments were tested for their ability to drive expression of a *lacZ* reporter in vivo. Analysis of transgenic founder embryos revealed that the combined enhancer and promoter fragments (Rip2EP) drove expression in the anterior PSM and the anterior halves of the newly formed somites (Fig. 5A1). The distal enhancer element (Rip2E) directed reporter expression in the PSM and throughout posterior somites (Fig. 5A2), whereas expression from the promoter element (Rip2P) was largely restricted to a stripe in the anterior PSM and the anterior halves of posterior somites (Fig. 5A3). We conclude that these fragments contain *cis*-acting regulatory elements sufficient to drive appropriate *Ripply2* expression in the anterior PSM in vivo.

Since *Ripply2* expression overlapped with the expression of several components of the Notch pathway, we hypothesized that transient *Ripply2* expression in S-I is activated by Notch signaling. To address this, we asked whether the activated Notch intracellular domain (NICD), or transcription factors such as Tbx6 or Mesp2 that function downstream or in parallel with Notch (Yasuhiko et al., 2006), could activate the *Ripply2* regulatory elements in luciferase reporter assays in vitro. Although NICD (not shown) or Mesp2 had minimal activity on *Ripply2* regulatory elements, Tbx6 activated the promoter (Fig. 5B2), and strongly stimulated the combined enhancer and promoter elements 26-fold (Fig. 5B1). Expression of T had similar activity to Tbx6 (not shown). Coexpression of NICD and Tbx6 resulted in a modest increase in transcriptional activation of the *Ripply2* promoter (not shown); however, strong synergistic activation was observed when Tbx6 and Mesp2 were coexpressed (Fig. 5B1,2). Since the same qualitative effect was observed with the *Ripply2* promoter or the combined promoter/enhancer, subsequent experiments focused on the promoter.

An E-box, capable of binding Mesp2 (Nakajima et al., 2006), and a near-consensus putative Tbx6 binding site (BS) (White and Chapman, 2005), are conserved in the proximal mouse and human *Ripply2* promoters (Fig. 5C). Electrophoretic mobility shift assays (EMSA) confirmed that the putative Tbx6 BS specifically bound FLAG-Tbx6 (Fig. 5D). A 44 bp deletion that removed the E-box and Tbx6 BS (Rip2PΔTbx6*luc*) strongly diminished the ability of Tbx6, or Tbx6 and Mesp2 together, to activate the *Ripply2* promoter (Fig. 5B2,3). Multimerizing the 44 bp region was sufficient to restore the synergistic activation of *Ripply2* by Tbx6 and Mesp2 to levels observed in the full promoter construct (Fig. 5B4). Since *Tbx6* and *Mesp2* expression overlaps only in S-I (Yasuhiko et al., 2006), we suggest that they function together to control the periodic activation of *Ripply2* in S-I.

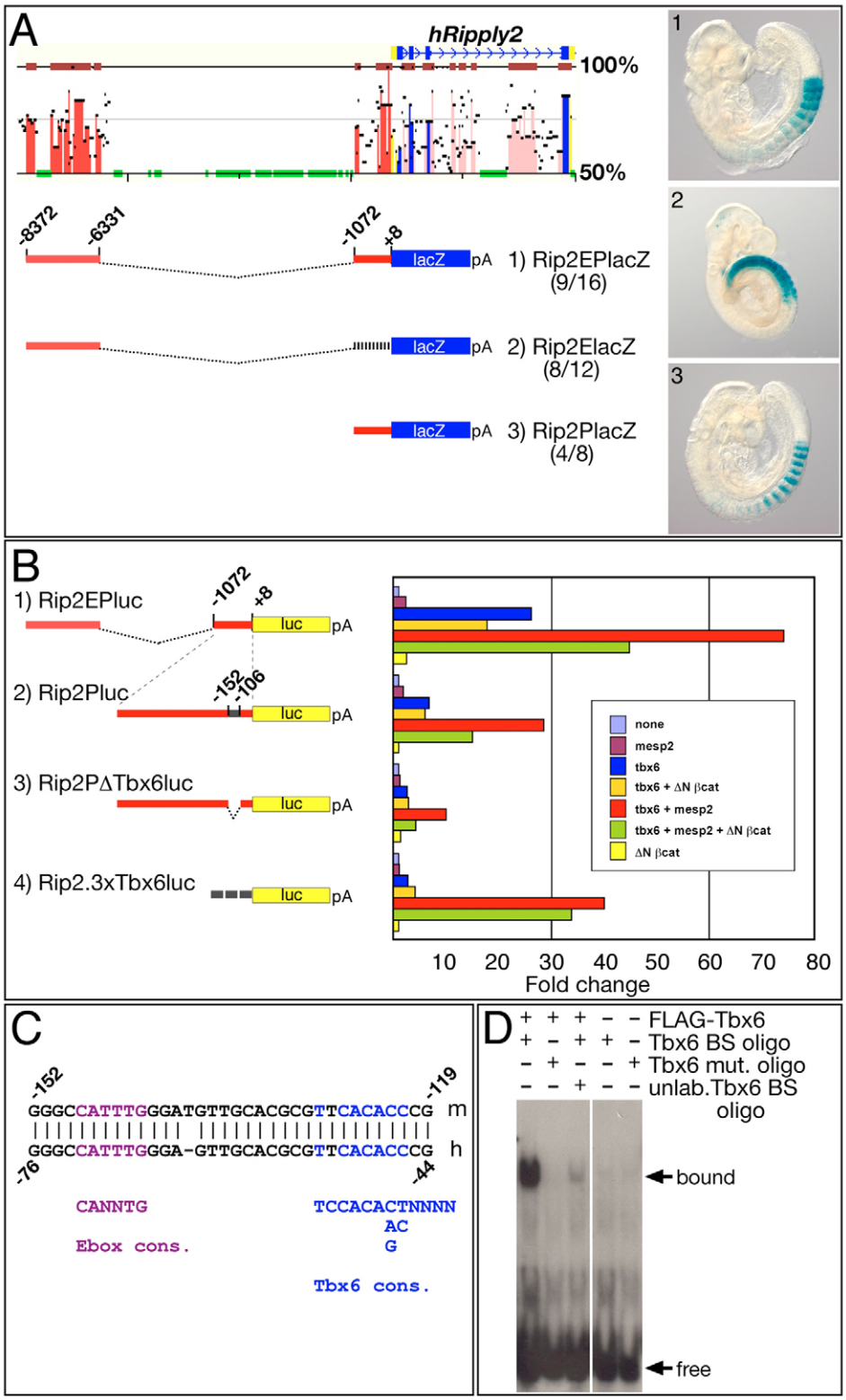

Fig. 5. *Ripply2* expression is regulated by the Notch and Wnt pathways. (**A**) Comparative Vista plots of the mouse and human *Ripply2* loci identify highly conserved putative regulatory regions (red) and coding exons (blue). Sequence identity (%) is indicated on the right. *lacZ* reporter constructs driven by these *Ripply2* regulatory elements are depicted below the Vista plot. Representative transgenic founder embryos expressing the 'enhancer/promoter' reporter (1), the 'enhancer' reporter (2), or the 'promoter' reporter (3) are shown on the right. The number of β-gal-positive transgenic embryos/total number of transgenic founders is shown in brackets. The gradually diminishing reporter expression in anterior somites is probably due to the perdurance of the stable β-gal protein. (**B**) Luciferase reporter constructs are depicted on the left. The enhancer and promoter elements are the same as in A. Graph displaying activity of *Ripply2* luciferase constructs in HEK293 cells is shown on the right. Note that stabilized β-catenin expression had no effect on Mesp2 or Tbx6 protein levels as assessed by western blot (not shown). (**C**) Highly conserved region (33/34 bp identical) in the mouse (m) and human (h) *Ripply2* promoters contains an Ebox and near-consensus Tbx6 binding site (BS). (**D**) An oligo containing the putative Tbx6 BS bound specifically to FLAG-Tbx6 in EMSA assays. Binding of Flag-Tbx6 protein to the wild-type oligo is specifically competed by adding 125× excess unlabeled oligo to the binding reaction.

Our genetic analyses suggest that elevated Wnt signaling represses *Ripply2*. To test whether Wnt signaling can suppress the ability of Tbx6 and Mesp2 to activate the *Ripply2* promoter, we cotransfected stabilized β-catenin with Tbx6 and Mesp2 and assessed luciferase activity. Consistent with the in vivo data, expression of activated β-catenin reduced Mesp2/Tbx6-mediated activation of the enhancer/promoter and the promoter construct (Fig. 5B).

DISCUSSION

We have investigated the role of the Wnt3a/β-catenin signaling pathway in mesoderm formation and segmentation during mammalian embryogenesis. We show that β-catenin plays a central role in somitogenesis, functioning to link mesodermal determinants with genes that control segment boundary formation. Previous studies demonstrating oscillatory expression of the Wnt target gene *Axin2*, a

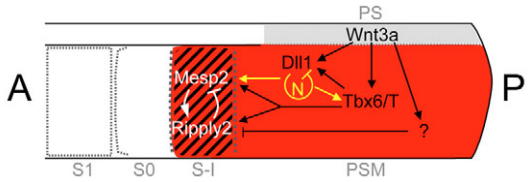

Fig. 6. Wnt3a-dependent transcriptional networks coordinate mesoderm formation with segment boundary determination. Schematic of selected target genes and pathways activated in the PSM by Wnt3a, see text for details. The red color represents the expression domain of the Wnt3a and β-catenin target genes (*Dll1, T, Tbx6*) in the PSM. *T* and *Tbx6* are grouped together for the sake of simplicity. Yellow indicates a representative oscillating negative-feedback loop in the Notch (N) pathway in the segmentation clock. Expression of the segment boundary determination genes *Ripply2* and *Mesp2* (black hatching) in S-I, overlap with the anterior domain of *Tbx6* expression. The proposed Wnt3a/β-catenin target repressor (?) is expressed in the posterior PSM where it ensures that boundary determination gene expression is repressed. A, anterior; P, posterior.

negative regulator of Wnt signaling, led to the proposal that negative feedback loops in the Wnt3a/β-catenin pathway are important components of the oscillating segmentation clock (Aulehla et al., 2003). This is supported by recent studies identifying additional cyclic Wnt target genes (Dequeant et al., 2006). Although our genetic studies demonstrate that Wnt3a and β-catenin are necessary in the PSM for oscillating gene expression in the Wnt, Fgf and Notch pathways, the continued periodic expression of Notch target genes in the presence of a stabilized form of β-catenin that is refractory to proteolytic degradation does not support a role for β-catenin as a core component of the oscillator. Moreover, we can find little evidence for striped expression of Wnt/β-catenin reporters in wild-type PSM, either at the protein or RNA level (Nakaya et al., 2005) (not shown), suggesting that β-catenin activity itself does not oscillate. Since only a small subset of Wnt/β-catenin target genes oscillate, out of a much larger number of target genes (Dequeant et al., 2006; Lickert et al., 2005; Morkel et al., 2003) (our unpublished data), it appears that additional regulatory inputs are required for the oscillation of select Wnt target genes. The functional significance of oscillating Wnt target gene expression is not currently well understood. We conclude that the Wnt3a/β-catenin pathway plays a permissive, and not instructive, role in the regulation of oscillating clock genes.

Mutations in components of the Notch signaling pathway lead to somite phenotypes in both fish and mice, clearly demonstrating a conserved role for Notch signaling in segmentation (Rida et al., 2004). However, normal segmentation of the anterior-most 7-10 somites is a feature common to virtually all Notch pathway mutants (Rida et al., 2004), including *Mesp2* mutants (Saga et al., 1997). These observations are difficult to explain if Notch signaling drives the segmentation clock. Studies of zebrafish segmentation have led to the alternative proposal that Notch functions to synchronize oscillations between neighboring PSM cells (Horikawa et al., 2006; Jiang et al., 2000). This hypothesis nicely accounts for the normal formation of the anterior somites in embryos lacking Notch pathway activity because it does not require Notch to drive the oscillator. Our observation that segmental *Uncx4.1* expression becomes progressively indistinct and fused posteriorly in both LOF and GOF *Ctnnb1* mutants, is consistent with Notch functioning in the coupling of oscillators. The fact that partial segment boundaries and polarized segmental gene expression occur in the *T-Cre;Ctnnb1*$^{flLOF/\Delta}$ mutants, despite the absence of oscillating gene expression in the Wnt or Fgf/Notch pathways, suggests that boundary formation in the anterior PSM can proceed independently of oscillating gene expression in the posterior PSM. Alternatively, it remains possible that an additional component(s) of an oscillating clock mechanism remains to be discovered, although this seems unlikely given the recent comprehensive, genome-wide survey of oscillating gene expression in the PSM (Dequeant et al., 2006).

Examination of *Mesp2* and *Ripply2* expression in the *Wnt3a* and *Ctnnb1* mutants demonstrates that Wnt3a/β-catenin signaling regulates segment boundary determination. Mesp2 is known to participate in this process (Morimoto et al., 2005; Nomura-Kitabayashi et al., 2002; Saga et al., 1997; Takahashi et al., 2003; Takahashi et al., 2000). The elongated PSM, segmentation defects, ectopic *Mesp2* expression and complete absence of *Ripply2* transcripts in the *T-Cre;Ctnnb1*$^{flGOF/+}$ embryos are consistent with the segmentation phenotype and ectopic *mesp-b* expression observed in zebrafish *Ripply1* knockdowns (Kawamura et al., 2005). Interestingly, *Ripply1* mRNA overexpression also caused segmentation defects, and downregulated *mesp-b* expression in a Groucho-dependent manner, suggesting that Ripply1 represses *mesp-b* transcription. Similar results have been obtained with *Xenopus* Ripply orthologs (Chan et al., 2006; Kondow et al., 2006). Our demonstration that *Ripply2* expression follows *Mesp2* expression in S-I is consistent with a role for Mesp2 in the activation of *Ripply2*, as well as a reciprocal role for Ripply2 in the repression of *Mesp2*. The synergistic activation of the *Ripply2* promoter by Mesp2 and Tbx6 indicates a direct role for Mesp2 in *Ripply2* activation. This is supported by recent complementary studies, which demonstrated that Mesp2 alone can directly bind and activate the *Ripply2* enhancer (Morimoto et al., 2007). Moreover, analyses of *Ripply2* null mutants are consistent with Ripply2 negatively regulating *Mesp2*. Together, the data strongly suggest that segment boundary determination is regulated by an Mesp2-centered negative feedback loop in which Mesp2 and Tbx6 activate *Ripply2*, and Ripply2, in turn, represses *Mesp2*.

Although segment boundaries form in conditional *T-Cre;Ctnnb1* mutants, the small somites appear incompletely epithelialized. Wnt/β-catenin signaling has been implicated in the control of somite epithelialization in chick through the activation of *Paraxis* (Linker et al., 2005). *Paraxis* expression is unaffected by the *T-Cre;Ctnnb1*$^{flLOF/\Delta}$ mutation (not shown), indicating that the epithelial defects are not dependent upon *Paraxis*. In addition to the regulation of Wnt target gene transcription, β-catenin has a well-characterized role in cell adhesion (Nelson and Nusse, 2004). Future studies will address the potential role that β-catenin, localized to adherens junctions, may play in somite epithelialization.

Previous studies have implicated graded Fgf8 signals in the positioning of the boundary determination front (Dubrulle et al., 2001), and have suggested that Fgf8 functions downstream of Wnt3a (Aulehla et al., 2003). Our data are consistent with these suggestions, because the *Fgf8* gradient is modestly reduced in *Wnt3a* mutants (not shown), absent in the *Ctnnb1* LOF embryos and greatly expanded in the *Ctnnb1* GOF mutants. Interestingly, *Fgf8* mRNA is still expressed as a gradient in the *T-Cre;Ctnnb1*$^{flGOF/+}$ PSM (Fig. 2I). Since the stabilization of β-catenin in these mutants anteriorly extended the *Fgf8* gradient but had little to no effect upon the gradient itself, we conclude that Wnt/β-catenin signaling is not instructive for establishment of the *Fgf8* gradient and therefore indirectly controls *Fgf8* expression in the streak. Although conditional *T-Cre; Fgf8*$^{fl/\Delta}$ mutants do not display segmentation phenotypes (Perantoni et al., 2005), the expression of other *Fgfs* presumably compensate. Experiments designed to test Fgf redundancy during PSM specification and somitogenesis are ongoing.

Wnt3a-dependent transcriptional networks coordinate mesoderm formation and segmentation

Our data suggest that Wnt3a controls posterior development by stimulating the canonical β-catenin/Tcf pathway in multipotent PS stem cells, initiating a cascade of gene expression that links mesoderm fate specification to the oscillatory segmentation clock and segment boundary formation (Fig. 6). The Wnt3a/β-catenin target genes *Dll1*, *T/Tbx6*, and additional unidentified target genes are critical for understanding how the Wnt/β-catenin signaling pathway regulates the spatiotemporal expression of segment boundary determination genes. Wnt3a activates the Tbox transcription factor gene *T* in the PS (Yamaguchi et al., 1999). *T*, together with the Notch pathway (White et al., 2005), in turn activates *Tbx6* in the PS and PSM (Hofmann et al., 2004) to specify mesoderm fates. Wnt3a and Tbx6 then synergistically activate the Notch ligand *Dll1* in the PS and PSM (Galceran et al., 2004; Hofmann et al., 2004), to define a PSM domain that is permissive for oscillating gene expression in the Notch pathway. Tbx6 functioning together with the activated Notch pathway activates the segment boundary determination gene *Mesp2* in the anterior PSM (Yasuhiko et al., 2006). We show here that Tbx6 and Mesp2 subsequently activate *Ripply2* in the anterior PSM. Ripply2 then functions as a feedback suppressor, bound to the transcriptional repressor Groucho, to repress *Mesp2* (Kawamura et al., 2005; Morimoto et al., 2007). Thus Tbx6 emerges as a major regulator of posterior development downstream of Wnt3a, functioning to integrate the Wnt and Notch pathways during boundary formation.

Additionally, we have shown that stabilized Wnt/β-catenin signaling anteriorly extends the *Tbx6* and *Dll1*-positive PSM, allowing additional cycles of the segmentation clock and ectopic activation of *Mesp2*. Despite the elevated Tbx6 and Mesp2 expression, stabilized β-catenin signaling repressed *Ripply2*. Since Wnt/β-catenin signaling activates target gene transcription, we suggest that Wnt signaling is indirectly repressing the Mesp2/Tbx6-mediated activation of *Ripply2* by activating a currently unknown transcriptional repressor. Repression of *Ripply2* in posterior PSM presumably ensures that the segmentation program initiates only in the anterior PSM. Future studies will address the nature of this unknown Wnt target gene(s). Given the demonstrated links between Wnt3a and mesoderm formation, segmentation, left-right and AP patterning, and axial elongation, we suggest that Wnt3a is the principal organizer of mammalian trunk and tail development.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/135/1/85/DC1

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