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 PSM (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).

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).

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Wnt3a controls gene expression by stabilizing cytosolic levels of \( \beta \)-catenin, the central player in the canonical Wnt/\( \beta \)-catenin pathway. Stabilized \( \beta \)-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 mesendoderm 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 \( \beta \)-catenin to determine the precise role that Wnt3a and \( \beta \)-catenin play in mesendoderm formation and segmentation.

**MATERIALS AND METHODS**

**Mice**

Ctnnb1tm2Kem were purchased from the Jackson Labs. The Ctnnb1\textsuperscript{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 (\( P<0.001 \)). 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; SacI-Xhol) with and without the Ripply2 promoter (1.1 kb; Xhol-Neol) into pGL4.10[luc2] or pGL4.23[luc2 miniP] 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 \( \times \) 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-\( \beta \)-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.

**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; Huelskens et al., 2000), we conditionally inactivated Ctnnb1tm2Kem (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 \( T^{-}\text{Cre;Ctnnb1}^{\text{flLOF}} \), gastrulated normally but displayed posterior truncations (Fig. 1B) that were similar but more severe than that observed in Wnt3a\textsuperscript{−/−} mutants (Takada et al., 1994). Head structures were normal but mutants lacked a PS, posterior mesoderm and obvious somites. Loss of \( \beta \)-catenin activity was confirmed by monitoring the expression of the BATlacZ transgene, an in vivo reporter of \( \beta \)-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 \( \beta \)-catenin [\( \text{Ctnnb1}^{\text{lox(ex3)}} \) (Harada et al., 1999), hereafter referred to as \( \text{Ctnnb1}^{\text{GOF}} \)] in streak-derived mesoderm through the T-Cre-mediated deletion of the phosphorylation/degradation domain of \( \beta \)-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 \( T^{-}\text{Cre;Ctnnb1}^{\text{GOF}} \) mutants, confirming the enhanced activity of \( \beta \)-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 \( T^{-}\text{Cre;Ctnnb1}^{\text{GOF}} \) 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.
were absent from the T-Cre;Ctnnb1flLOF/1990; Hrabe de Angelis et al., 1997; Sun et al., 1999). All four genes necessary for posterior mesoderm formation, migration or segmentation (Chapman and Papaioannou, 1998; Herrmann et al., addition to the somite defects, kinked neural tubes, and an enlarged allantois 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) BATlacZ expression in -5 ss wild-type (D-F), T-Cre;Ctnnb1flLOF/-gal (I-K), and T-Cre;Ctnnb1flGOF/-gal (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 -gal activity in the T-Cre;Ctnnb1flLOF/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), 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;Ctnnb1flLOF/ (L,M), and T-Cre;Ctnnb1flGOF/-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 Q, 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.

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;Ctnnb1flLOF/-embryos, 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;Ctnnb1flLOF/-PSM. Similarly, the expression of the oscillating Notch target genes Lfng and Hes7 were not observed in the T-

Fig. 1. Conditional loss and gain of β-catenin function causes mesoderm and segmentation phenotypes. (A-C) Wnt/β-catenin (BATlacZ) reporter activity in E9 wild-type (A), T-Cre;Ctnnb1flLOF/ (B) and T-Cre;Ctnnb1flGOF/ (C) embryos. β-galactosidase (-gal) activity was reduced posteriorly in the truncated T-Cre;Ctnnb1flGOF/ mutants, and highly upregulated in the grossly enlarged PSM of the T-Cre;Ctnnb1flGOF/-embryo. In addition to the somite defects, kinked neural tubes, and an enlarged allantois 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) BATlacZ expression in -5 ss wild-type (D-F), T-Cre;Ctnnb1flLOF/-gal (I-K), and T-Cre;Ctnnb1flGOF/-gal (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 -gal activity in the T-Cre;Ctnnb1flLOF/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;Ctnnb1flLOF/ (L,M), and T-Cre;Ctnnb1flGOF/-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 Q, 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.
marker (A), is expressed in the E8.5 mesoderm and PS markers.

and Tam, 2001), was anteriorized in the expression, along with the first segment border B0 (black bar) (Pourquie LFng

PSM (C). Expression of the PSM marker T-Cre;Ctnnb1flGOF/+ Cre;Ctnnb1flGOF/+ genes in conditional

Fig. 2. Examination of mesoderm markers and oscillating clock

DEVELOPMENT

条纹在延长的Hes7 mRNA中仍可检测到（Fig. S1 in the supplement 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;Ctnnb1flGOF/+ 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;Ctnnb1flGOF/+ 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;Ctnnb1flGOF/+), 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;Ctnnb1flLOF/+ embryos, uncx4.1+ expression in embryos that only express the stabilized form of β-catenin (i.e. T-Cre;Ctnnb1flGOF/+), 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.

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;Ctnnb1flGOF/+ mutants (Fig. 3I). Nevertheless, interspersed, fuzzy stripes of Tbx18 and Uncx4.1
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;Ctnnb1flLOF/+ 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;Ctnnb1flLOF/+ 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–/–; T-Cre;Ctnnb1flLOF/+ mutants by stabilized 10 ss (somite-stage) littermate (cf. Fig. 3L,P with Fig. 3K,O). Notably, Wnt3a–/–; T-Cre;Ctnnb1flGOF/+ mutants (not shown), although it was easily detected in the wild-type 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–/–; T-Cre;Ctnnb1flGOF/+ embryos, abutting the posterior-most end of the node (Fig. 3T).

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;Ctnnb1flGOF/+ 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 coactivator Groucho and repressing the zebrafish Mesp2 homolog, mesp-b.
target genes such as Hes7 (Fig. 4F,G), and were undetectable by 3-6 ss (Hes7, the Notch pathway, including overlapping with intense anterior PSM expression of components of PSM in one or two stripes in prospective somites S0 and S-I, demonstrated that Ripply2 mRNA was expressed 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 reporter (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 (Rip2PΔTbx6lac) 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.

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;Ctnnb1flLOF/Δ 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;Ctnnb1flLOF/Δ embryos (not shown). However, in contrast to the multiple stripes of Mesp2 expression observed in T-Cre;Ctnnb1flLOF/Δ embryos (Fig. 5V), 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.

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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;Ctnnb1flLOF/Δ 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;Ctnnb1flLOF/Δ embryos (not shown). However, in contrast to the multiple stripes of Mesp2 expression observed in T-Cre;Ctnnb1flLOF/Δ embryos (Fig. 5V), 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.
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
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<sup>ΔLOF</sup> 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<sup>ΔGOF</sup> 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<sup>ΔLOF</sup> 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<sup>ΔGOF</sup> 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<sup>Δ</sup> 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/β-catenin and segment boundaries

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 (Galeran 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 developmental 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
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