HHS Public Access

Author manuscript

Dev Dyn. Author manuscript; available in PMC 2016 June 01.

Published in final edited form as:

Dev Dyn. 2015 June ; 244(6): 797–807. doi:10.1002/dvdy.24275.

Wnt8a and *Wnt3a* Cooperate in the Axial Stem Cell Niche to Promote Mammalian Body Axis Extension

Thomas J. Cunningham[1], **Sandeep Kumar**[1], **Terry P. Yamaguchi**[2], and **Gregg Duester**[1,*]

[1]Development, Aging, and Regeneration Program, Sanford-Burnham Medical Research Institute, 10901 N. Torrey Pines Road, La Jolla, CA 92037, USA

[2]Cancer and Developmental Biology Laboratory, NCI-Frederick, National Institutes of Health, 1050 Boyles St., Frederick, MD 21702

Abstract

Background—Vertebrate body axis extension occurs in a head-to-tail direction from a caudal progenitor zone that responds to interacting signals. Wnt/β-catenin signaling is critical for generation of paraxial mesoderm, somite formation, and maintenance of the axial stem cell pool. Body axis extension requires *Wnt8a* in lower vertebrates, but in mammals *Wnt3a* is required, although the anterior trunk develops in the absence of *Wnt3a*.

Results—We examined mouse *Wnt8a*$^{-/-}$ and *Wnt3a*$^{-/-}$ single and double mutants to explore whether mammalian *Wnt8a* contributes to body axis extension and to determine whether a posterior growth function for *Wnt8a* is conserved throughout the vertebrate lineage. We find that caudal *Wnt8a* is expressed only during early somite stages and is required for normal development of the anterior trunk in the absence of *Wnt3a*. During this time, we show that *Wnt8a* and *Wnt3a* cooperate to maintain *Fgf8* expression and prevent premature *Sox2* upregulation in the axial stem cell niche, critical for posterior growth. Similar to *Fgf8*, *Wnt8a* requires retinoic acid (RA) signaling to establish its expression boundaries and possesses an upstream RA response element that binds RA receptors.

Conclusions—These findings provide new insight into interaction of caudal Wnt-FGF-RA signals required for body axis extension.

Keywords

Wnt8a; Wnt3a; body axis extension; somitogenesis; Fgf8; retinoic acid; mouse

INTRODUCTION

During late gastrulation in vertebrates, anterior-to-posterior body axis extension is driven by axial stem cells residing in the epiblast, lateral to the primitive streak, which give rise to both neural and paraxial mesodermal progeny (Tzouanacou et al., 2009; Wilson et al., 2009). Neural progeny derive from axial stem cells that remain in the epiblast epithelium,

*Correspondence to: Gregg Duester, Development and Aging Program, Sanford-Burnham Medical Research Institute, 10901 North Torrey Pines Road, La Jolla, California 9203. duester@sanfordburnham.org.

and subsequently differentiate to form the neural tube/spinal cord. Paraxial mesodermal progeny derive from axial stem cells that ingress ventrally through the primitive streak and undergo an epithelial-to-mesenchyme transition to form the presomitic mesoderm (PSM). As the primitive streak regresses, blocks of anterior PSM periodically and bilaterally segment via a mesenchyme-to-epithelial transition that is temporally and spatially regulated, thus forming pairs of somites on either side of the neural tube that later give rise to the axial skeleton, ribs, skeletal muscle, connective tissue, and dermis of the back (Pourquié, 2011). A cohort of signaling cues are needed for controlling gastrulation and the spatiotemporal dynamics of somite formation; among them is Wnt/β-catenin signaling, whereby secreted Wnt glycoproteins bind to a Frizzled cell membrane receptor, leading to stabilization and nuclear translocation of β-catenin that subsequently binds to Tcf/Lef transcription factors to modulate transcription (Logan and Nusse, 2004).

In the mouse, Wnt3a is the primary Wnt/β-catenin pathway ligand that is required during body axis extension. Genetic loss-of-function in *Wnt3a*$^{-/-}$ embryos leads to a major posterior truncation below the level of the forelimbs due to loss of *Fgf8* and *T (Brachyury)* expression required for maintenance of the axial stem cell pool and production of paraxial mesodermal progeny (Takada et al., 1994; Greco et al., 1996; Yoshikawa et al., 1997; Yamaguchi et al., 1999; Aulehla et al., 2003; Dunty et al., 2008; Dunty et al., 2014). Somite formation in *Wnt3a*$^{-/-}$ embryos is perturbed, with the first 7–9 somite pairs initially forming but successively becoming smaller and disappearing towards the truncated posterior region, while ectopic neural-like tissue expressing *Sox2* forms in place of paraxial mesoderm beyond the 7-somite stage (Takada et al., 1994; Yamaguchi et al., 1999; Nowotschin et al., 2012; Dunty et al., 2014). *Wnt3a*$^{vg/vg}$ hypomorphic embryos and conditional (*T-cre* driven) *β-catenin*$^{flox/flox}$ embryos demonstrate that caudal *Fgf8* expression is regulated downstream of Wnt/β-catenin signaling, which operates as a posterior to anterior gradient of nuclear β-catenin in parallel with FGF (Aulehla et al., 2003; Aulehla et al., 2008; Dunty et al., 2008). In addition, Wnt/β-catenin is promoted downstream of FGF signaling in the caudal progenitor zone demonstrating a mutually positive Wnt/β-catenin-FGF autoregulatory loop (Olivera-Martinez and Storey, 2007; Wahl et al., 2007; Naiche et al., 2011; Boulet and Capecchi, 2012).

Several lines of evidence suggest that Wnt/β-catenin signaling is also important for mesoderm patterning in lower vertebrates (frogs and fish), with *wnt8a* functioning as the primary ligand (see Table 1 for *Wnt8a* ortholog nomenclatures) (Christian et al., 1991; Hoppler et al., 1996; Hoppler and Moon, 1998; Lekven et al., 2001; Shimizu et al., 2005; Martin and Kimelman, 2009; Baker et al., 2010; Lu et al., 2011; Martin and Kimelman, 2012; Wylie et al., 2014). In *Xenopus*, overexpression of a dominant-negative form of *wnt8a* disrupts somite mesoderm specification and posterior development (Hoppler et al., 1996). Similarly, antisense morpholino interference or genetic knock-down of the bicistronic zebrafish *wnt8a* locus leads to a loss of posterior mesoderm expansion as well as defects in neural ectoderm posteriorization (Lekven et al., 2001; Shimizu et al., 2005; Baker et al., 2010; Wylie et al., 2014). Other experiments in zebrafish identified a positive Wnt/β-catenin—*Ntl/Bra* (the two zebrafish orthologs of *T*) autoregulatory loop required for specification of mesoderm from axial stem cells, while manipulations at the single-cell level using cell

autonomous Wnt inhibitors and activators demonstrated an additional role for specification of mesoderm into somites (Martin and Kimelman, 2008; Martin and Kimelman, 2010; Martin and Kimelman, 2012). A positive Wnt/β-catenin-FGF autoregulatory loop has also been found in zebrafish, needed for normal body axis extension, similar to mouse (Stulberg et al., 2012).

Thus, experiments across different model organisms are consistent with conserved multiplexed roles for canonical Wnt/β-catenin signaling in posterior body axis development and mesoderm specification during body axis extension. A clear discrepancy appears to be the specific Wnt ligands at play; *Wnt3a* in mouse versus *wnt8a* in *Xenopus* and zebrafish, despite the fact that all vertebrates possess both *Wnt3a* and *Wnt8a* orthologs (Garriock et al., 2007). Here, we investigated the function of mammalian *Wnt8a* during body axis extension using a genetic loss-of-function approach. We found that caudal *Wnt8a* is most strongly expressed during early somite stages, a time when the anterior trunk can form in the absence of *Wnt3a*. While we find that *Wnt8a*$^{-/-}$ embryos have no overt body axis defects, we discover that *Wnt3a*$^{-/-}$;*Wnt8a*$^{-/-}$ double knockout embryos in comparison to *Wnt3a*$^{-/-}$ embryos display a more severe posterior truncation, fewer somites, a more extensive loss of caudal *Fgf8* expression, and earlier manifestation of ectopic *Sox2* expression in the axial stem cell niche. These findings reveal that mouse *Wnt8a* cooperates with *Wnt3a* during early somite stages to maintain axial stem cell homeostasis required for normal body axis extension and somitogenesis. These findings thus demonstrate a wide conservation of *Wnt8a* function throughout vertebrates.

RESULTS

Caudal *Wnt8a* Expression is Strongest During Early Somite Stages

From the headfold stage to the 1-somite pair stage (E7.5-E7.75), *Wnt8a* is expressed throughout the epiblast and primitive streak along with *Wnt3a* (Yamaguchi, 2008; Zhao and Duester, 2009); also *Wnt8a* is expressed in rhombomere 4 of the hindbrain (Niederreither et al., 2000). We further examined the expression pattern of *Wnt8a* in wild type mouse embryos during early and late somite stages to assess the potential for Wnt8a signaling to impact posterior growth (Fig. 1A). At the 5 and 7 somite pair stages, two distinct domains of *Wnt8a* expression were observed in the hindbrain and anterior two-thirds of the epiblast. We observed rapid downregulation of *Wnt8a* at the 10 somite pair stage, with only weak expression detected in the hindbrain. By the 12 somite pair stage, no detection of *Wnt8a* transcripts was observed. These observations suggest a potential role for *Wnt8a* in body axis extension during early somite stages.

Wnt8a and *Wnt3a* Function Redundantly During Early Somite Formation

To assess the function of *Wnt8a* during body axis extension, we generated a *Wnt8a*$^{-/-}$ knock out mouse line, which displayed no detectable expression of *Wnt8a* transcripts (Fig. 1B). Mendelian ratios for heterozygous [*Wnt8*$^{+/-}$ x *Wnt8a*$^{+/-}$] matings were normal with no indications for *Wnt8a*$^{-/-}$ embryonic morbidity or mortality. Both male and female *Wnt8a*$^{-/-}$ adult mice were healthy and fertile, with homozygous [*Wnt8a*$^{-/-}$ x *Wnt8a*$^{-/-}$] matings displaying normal litter sizes compared to previous heterozygous breeder pairs (data not

shown), consistent with a previously generated *Wnt8a*$^{-/-}$ mouse line (Vendrell et al., 2013). We did not observe any overt phenotype relating to body axis extension in *Wnt8a*$^{-/-}$ embryos (data not shown). To explore potential redundancy with *Wnt3a*, we mated *Wnt3a*$^{+/-}$ mice (Takada et al., 1994; Nakaya et al., 2005) with *Wnt8a*$^{+/-}$ mice and analyzed *Uncx4.1* expression marking somite formation at E8.75 and E9.5 (Fig. 2 and Table 2). *Wnt3a*$^{+/-}$;*Wnt8a*$^{-/-}$ embryos were indistinguishable from both *Wnt8a*$^{-/-}$ and wild-type embryos; each of these genotypes generated 9–14 somite pairs when collected at E8.75 (data not shown) and an average of 22–24 somite pairs when collected at E9.5 (Table 2). *Wnt3a*$^{-/-}$ embryos displayed a posterior truncation below the forelimbs, with only 7–9 somite pairs forming in total and with the posterior-most somites abnormally small in size, consistent with previous reports (Takada et al., 1994; Yoshikawa et al., 1997; Yamaguchi et al., 1999) (Fig. 2). *Wnt3a*$^{-/-}$;*Wnt8a*$^{+/-}$ embryos displayed a posterior truncation similar to that of *Wnt3a*$^{-/-}$ embryos (see Table 2). *Wnt3a*$^{-/-}$;*Wnt8a*$^{-/-}$ double knock-out embryos displayed the most severe phenotype, displaying a shorter axis than *Wnt3a*$^{-/-}$ embryos and forming on average 3 somite pairs (range 2–4); (Fig. 2). These observations demonstrate that *Wnt8a* functions together with *Wnt3a* to promote body axis extension at early somite stages.

Wnt8a and *Wnt3a* Function Redundantly to Maintain *Fgf8* Expression During Formation of Anterior Somites

A major role for Wnt/β-catenin signaling during body axis extension is maintenance of *Fgf8* expression in the caudal epiblast (and maintenance of the Wnt/β-catenin-FGF positive autoregulatory loop), required for self-renewal of the axial stem cell pool and control of PSM motility and maturation during somitogenesis (Dubrulle et al., 2001; Aulehla et al., 2003; Dubrulle and Pourquié, 2004; Dunty et al., 2008; Benazeraf et al., 2010; Naiche et al., 2011; Boulet and Capecchi, 2012). To assess whether *Wnt8a* functions as part of this process, we analyzed caudal *Fgf8* expression at E8.5, around the time when the posterior truncation becomes evident in *Wnt3a*$^{-/-}$ embryos. *Fgf8* expression was indistinguishable between *Wnt3a*$^{+/-}$;*Wnt8a*$^{-/-}$, *Wnt8a*$^{-/-}$, and wild-type embryos, with strong expression in the epiblast region (Fig. 3 and data not shown). *Wnt3a*$^{-/-}$ embryos displayed a modest, yet clear, narrowing of the epiblast region and corresponding smaller domain of caudal *Fgf8* expression, as expected, representing the early stages of posterior truncation. A similar expression pattern was observed in *Wnt3a*$^{-/-}$;*Wnt8a*$^{+/-}$ embryos (data not shown). However, at this same stage, *Wnt3a*$^{-/-}$;*Wnt8a*$^{-/-}$ double knock-out embryos displayed an extreme narrowing of the epiblast region and little to no caudal *Fgf8* expression, demonstrating that *Wnt8a* functions together with *Wnt3a* to maintain caudal *Fgf8* expression.

Wnt8a and Wnt3a Function Redundantly to Prevent Ectopic Sox2 Expression in the Axial Stem Cell Niche

Loss of *Wnt3a* is associated with ectopic neural-like tissue expressing *Sox2* in the paraxial mesoderm compartment after the 7-somite stage (Takada et al., 1994; Yamaguchi et al., 1999; Nowotschin et al., 2012; Dunty et al., 2014). We sought to investigate whether combined loss of *Wnt3a* and *Wnt8a* yielded a similar phenotype at an earlier stage than that observed for *Wnt3a* single mutants (i.e. prior to the 7-somite stage). All *Wnt3a*$^{-/-}$;*Wnt8a*$^{-/-}$ double knockout embryos examined displayed ectopic *Sox2*-positive neural-like tissue in the paraxial mesoderm compartment, including those examined at E8.25 with rostral

development matching wild-type embryos at the 5-somite stage (Fig. 4). In addition, whereas wild-type embryos normally exhibit low *Sox2* expression in the caudal epiblast, we observed a large increase in *Sox2* expression in *Wnt3a*$^{-/-}$;*Wnt8a*$^{-/-}$ caudal epiblast, similar to levels normally observed in wild-type neural plate. These observations indicate that *Wnt8a* functions together with *Wnt3a* to maintain the caudal epiblast in an undifferentiated "*Sox2*-low" state.

RA Repression of *Wnt8a* Is Associated With a Nearby Retinoic Acid Response Element

Raldh2$^{-/-}$ embryos, deficient in retinoic acid (RA) synthesis and signaling, display small somites and a shortened trunk due to loss of *Fgf8* repression, resulting in an anterior shift in the caudal expression boundary of *Fgf8* (Diez del Corral et al., 2003; Vermot et al., 2005; Sirbu and Duester, 2006; Kumar and Duester, 2014). *Raldh2*−/− embryos also display strong upregulation of caudal *Wnt8a* that expands into the developing trunk (Zhao and Duester, 2009). Here, we additionally analyzed *Rdh10*$^{-/-}$ embryos (Chatzi et al., 2013), also deficient in RA synthesis and signaling, and confirmed strong upregulation and expansion of *Wnt8a* at the 7 somite stage, finding that the caudal and hindbrain expression domains merge into one (Fig. 5A). Analysis of the *Wnt8a* genomic region revealed a putative RA response element (RARE) of the DR2 class (direct repeat separated by 2 bp) located 4.9 kb upstream of the transcription start site (Fig. 5B). We performed CHIP analysis using E8.5 mouse embryos, and found that all three RA receptors (RARα, RARβ, and RARγ) are recruited to this RARE in vivo (Fig. 5C). To further confirm RAR-RARE interaction, we performed electrophoretic mobility shift assays, using nuclear protein extracts from E8.5 mouse embryos, to determine whether the *Wnt8a* RARE was capable of binding RARs. The wild-type *Wnt8a* RARE, but not a mutant version, was shifted by the E8.5 nuclear extract, while super-shift assays using RAR antibodies verified that the wild-type RARE binds all three RAR isoforms (Fig. 5D). Together, these studies provide evidence that RA repression of *Wnt8a* may be mediated directly through this RARE.

DISCUSSION

Evolutionary Conservation of Caudal *Wnt8a* Expression Suggests a Role in Mammalian Body Axis Extension

The Wnt/β-catenin signaling pathway is utilized throughout the animal kingdom and is found in even the most basic multicellular animals (placozoa), with roots potentially dating back to the Metazoa–Protozoa divergence around 700 million years ago (Croce and McClay, 2008; Srivastava et al., 2008; Holstein, 2012). The complexity of the *Wnt* gene family is similarly ancient and very highly conserved; of the 13 definitive *Wnt* gene subfamilies (*Wnt1-11*, *Wnt16*, and *WntA*), 11 are conserved between humans and the sea anemone *Nematostella vectensis* (Cnidaria) that diverged over 550 million years ago (Kusserow et al., 2005). Vertebrates possess 12 *Wnt* subfamilies (*Wnt1-11* and *Wnt16*), with less ancient gene duplications accounting for variation among different vertebrate classes; the *WntA* subfamily was lost in the chordate lineage (Croce and McClay, 2008; Holstein, 2012). Human and mouse genomes have 7 duplicated *Wnt* subfamily genes, with 19 *Wnt* genes in total; chick has 8 duplications and 20 *Wnt* genes; *Xenopus* has 12 duplications and 24 *Wnt* genes; and zebrafish has 15 duplications and 27 *Wnt* genes (Garriock et al., 2007).

Conservation of Wnt function appears to be coupled to the ancient conservation of Wnt complexity, with a key role in maintenance of a posterior growth zone common among bilateria (Martin and Kimelman, 2009; Petersen and Reddien, 2009). Wnt8 orthologs appear to be the most widely conserved Wnt ligands for posterior growth; loss of function analyses (using dominant negative mRNAs, RNAi, morpholino interference, and genetic knockout) have identified a requirement from frogs and fish (*Wnt8a*) to the house spider *Achaearanea tepidariorum* (*at-wnt8*) (Hoppler et al., 1996; Lekven et al., 2001; Shimizu et al., 2005; McGregor et al., 2008; Baker et al., 2010; Wylie et al., 2014).

In this study, we set out to examine the function of *Wnt8a* in late gastrulation mouse embryos to determine whether it plays a role during early mesoderm formation and body axis extension as previously reported in lower vertebrates. We found that *Wnt8a* expression is strongest during the early somite stages when anterior somites form, but that it is downregulated during posterior somite development, positioning *Wnt8a* at the right time and location for a role in body axis extension during development of the anterior trunk.

Wnt8a and *Wnt3a* Cooperate to Form Anterior Somites, Maintain Caudal *Fgf8* Expression, and Prevent Ectopic *Sox2* Expression

Our analyses of $Wnt8a^{-/-}$ embryos revealed no body axis extension deficiency, leading us to explore the possibility that *Wnt3a* and *Wnt8a* might function redundantly. The anterior-most 7–9 somite pairs still form in both $Wnt3a^{-/-}$ embryos and conditional (*T-cre* driven) $\beta\text{-}catenin^{flox/flox}$ embryos (although they later degrade in the latter), suggesting that anterior somites and the anterior trunk may form independently of Wnt/β-catenin signaling or are under the control of another Wnt gene (Takada et al., 1994; Yoshikawa et al., 1997; Yamaguchi et al., 1999; Aulehla et al., 2003; Dunty et al., 2008; Nowotschin et al., 2012).

We found that $Wnt3a^{-/-}$;$Wnt8a^{-/-}$ double knockout embryos exhibit a more severe posterior truncation versus $Wnt3a^{-/-}$ single knockouts, revealing that *Wnt8a* is required for formation of somite pairs ~3–9 in the absence of *Wnt3a*. The first 2–3 somite pairs could be Wnt/β-catenin independent; however, it seems more likely that an additional Wnt ligand(s) plays a role at this very early stage. A prime candidate is the *Wnt3a* paralog *Wnt3*, which is strongly expressed prior to somite development and is required for primitive streak formation and initiation of mesoderm formation; *Wnt3* mRNA disappears after E7.5 prior to onset of somitogenesis (Liu et al., 1999), but sufficient protein may be present up to E8.0 to function during formation of the first 2–3 somite pairs. Interestingly in zebrafish, morpholino interference of *wnt3a* yields little to no phenotype during body axis extension; however, combined *wnt8a/wnt3a* morpholino interference yields severe posterior truncations, including significant reductions in somite number that are more severe than single *wnt8a* morphants (Shimizu et al., 2005). Thus, *Wnt8a* and *Wnt3a* function during body axis extension is conserved from fish to mammals, with *Wnt8a* being dominant in lower vertebrates and *Wnt3a* being dominant in higher vertebrates.

Another critical signal for body axis extension and somite formation is FGF, with $Fgfr1^{-/-}$ embryos failing to form somites due to a loss of FGF4 and FGF8 signaling (Yamaguchi et al., 1994; Naiche et al., 2011; Boulet and Capecchi, 2012). We discovered that $Wnt3a^{-/-}$;$Wnt8a^{-/-}$ double knockouts had an absent or severely diminished caudal *Fgf8*

expression domain at E8.5 (10-somite stage), versus a modest reduction of caudal *Fgf8* expression in *Wnt3a*$^{-/-}$ single knockouts, thus accounting for the reduced number of somites and more severe posterior truncation in the double knockout. A positive caudal Wnt/β-catenin—FGF autoregulatory loop exists to maintain both of these critical signals (Aulehla et al., 2003; Olivera-Martinez and Storey, 2007; Wahl et al., 2007; Dunty et al., 2008; Naiche et al., 2011; Boulet and Capecchi, 2012; Stulberg et al., 2012) and *Wnt8a* evidently plays its part in this pathway during anterior somite formation. A second positive autoregulatory loop exists between *Wnt3a* and its target *T*, required for mesoderm formation at gastrulation (Martin and Kimelman, 2008). *T*$^{-/-}$ mouse embryos display a similar phenotype to *Wnt3a*$^{-/-}$ embryos and only form the first ~7 somite pairs (Herrmann et al., 1990; Yamaguchi et al., 1999; Martin and Kimelman, 2008). This observation suggests that the anterior-most somites can form independently of the Wnt3a/β-catenin—T autoregulatory loop and that *Wnt8a* does not participate in the T autoregulatory loop, consistent with the fact that *Wnt8a* expression is positioned a little more anterior to *T* and *Wnt3a* (which overlap in the caudal-most tip of the embryo). Thus, we hypothesize a scenario whereby a Wnt3/Wnt3a/Wnt8a/β-catenin—FGF positive autoregulatory loop is established early to promote anterior trunk development and to initiate formation of the anterior somites, followed later by Wnt3a/β-catenin—FGF and Wnt3a/β-catenin—T positive autoregulatory loops, with the former functioning to maintain somitogenesis and form posterior somites and the latter functioning to maintain prolonged mesoderm production during later stages (Martin and Kimelman, 2009) (Fig. 6).

Previously, it has been reported that *Sox2*-positive neural-like tissue forms in place of paraxial mesoderm in *Wnt3a*$^{-/-}$ embryos (Takada et al., 1994; Yamaguchi et al., 1999; Nowotschin et al., 2012; Dunty et al., 2014), suggesting a role for Wnt3a/β-catenin signaling in maintaining mesoderm specification potential. Here, we demonstrate that *Wnt3a* and *Wnt8a* cooperate in this role during early somite stages. We further report here that mutant embryos also exhibit high levels of *Sox2* expression in the caudal epiblast, compared to wild-type embryos that maintain low levels, and only upregulate *Sox2* to high levels when cells undergo neural differentiation in the neural plate. This suggests that Wnt/β-catenin signaling acts to restrict *Sox2* expression to a low level in the caudal epiblast to prevent precocious neural differentiation and thus maintain an undifferentiated pool of axial stem cells. Wnt has previously been proposed to activate the *Sox2* caudal enhancer N1 in conjunction with FGF signals (Takemoto et al., 2006; Takemoto et al., 2011). In contrast, we observe ectopic *Sox2* expression despite a loss of *Wnt3a*, *Wnt8a*, and *Fgf8*. Thus, exactly how Wnt and FGF signals interact to control the axial stem cell niche is yet to be fully understood.

RA Control of Caudal Wnt and FGF Signaling

RA, generated in the somites, signals to antagonize both *Fgf8* and Wnt caudally (Diez del Corral et al., 2003; Vermot et al., 2005; Sirbu and Duester, 2006; Olivera-Martinez and Storey, 2007; Zhao and Duester, 2009; Kumar and Duester, 2014), thus adding an additional layer of control to body axis extension (Fig. 6). Here, we demonstrate that *Wnt8a* is likely a direct target of RA signaling in mouse embryos, through a RARE located 4.9 kb upstream of the transcription start site of *Wnt8a* that is only partially conserved in humans, perhaps due

to the redundant nature of Wnt8a signaling with other Wnt ligands. This RARE is a DR2 type element (2 bp spacing between hexameric repeats), comparable to the DR2 element seen upstream of *Fgf8*, required for RA-mediated caudal *Fgf8* repression (Kumar and Duester, 2014). Furthermore, it would be interesting to discover precisely what repressive RAREs have in common that determine their repressive modality, compared to the majority of in vivo-verified RAREs near other target genes (discovered to date) that elicit transcriptional activation (Cunningham and Duester, 2015).

Conclusions

In this study, we reveal that cooperative Wnt3a/Wnt8a signaling maintains the axial stem cell niche needed for posterior growth during early somite stages, thus demonstrating that Wnt/β-catenin signaling is required during anterior as well as posterior body axis extension in mammals. By assigning a function for *Wnt8a* during somite formation in mouse, which primarily utilizes *Wnt3a* for this purpose, we demonstrate a tighter conservation of the Wnt ligands at play during body axis extension between mammals and lower vertebrates, which primarily utilize *Wnt8a*. In the only other loss-of-function study conducted to date on both of these ligands, *Wnt3a* was found to be a non-essential but redundant partner with *Wnt8a* in zebrafish (Shimizu et al., 2005), reciprocal to the situation in mouse. Thus, cooperative Wnt3a/Wnt8a signaling is conserved from fish to mammals, but genetic drift has seemingly switched the dominant partner during evolution of the mammalian lineage. Our studies also provide new insight into how Wnt-FGF-RA signals interact during body axis extension.

EXPERIMENTAL PROCEDURES

Generation of *Wnt8a*, *Wnt3a*, and *Rdh10* Knockout Mice

Heterozygous *Wnt8a*+/− mutant frozen embryos were implanted in pseudopregnant female mice to generate *Wnt8a*+/− mice purchased from the Knockout Mouse Project (KOMP, University of California, Davis); KOMP *Wnt8a*+/− embryos carry a deletion that encompasses all exons of *Wnt8a*. Matings of *Wnt8a*+/− mice generated a homozygous *Wnt8a*−/− mouse line that was then maintained by interbreeding *Wnt8a*−/− mice. Double mutant mice were generated by mating *Wnt8a*−/− mice with *Wnt3a*+/− mice (Nakaya et al., 2005) to generate *Wnt8a*+/−;*Wnt3a*+/− adult mice that were mated to produce double mutant embryos. *Rdh10*−/− embryos were generated as described previously (Chatzi et al., 2013).

For timed matings, noon on the day of vaginal plug detection was considered embryonic day 0.5 (E0.5). Embryos derived from timed matings were genotyped by PCR analysis of yolk sac DNA. *Wnt8a* mutants were genotyped with mutant primers (5′-GGT AGG AGA CCT GCT TCA GC-3′ and 5′-GTC TGT CCT AGC TTC CTC ACT G-3′ that generate an 81 bp PCR product) and wild-type primers (5′-GCT TCC GTC ATC TTC TTA GCA C-3′ and 5′-GGG CAC TCC TAA CCC TGT C-3′ that generate a 361 bp PCR product). *Wnt3a* mutants were genotyped as previously described (Nakaya et al., 2005) as were *Rdh10* mutants (Chatzi et al., 2013). All mouse studies conformed to the regulatory standards adopted by the Animal Research Committee at the Sanford-Burnham Medical Research Institute.

Whole-mount in situ Hybridization

Whole-mount in situ hybridization was performed using digoxigenin-labeled antisense RNA probes as previously described (Wilkinson, 1992); probes for *Wnt8a*, *Uncx4.1*, and *Fgf8* were previously reported (Zhao and Duester, 2009; Cunningham et al., 2013); the *Sox2* probe was described previously (Zappone et al., 2000). Wild-type and mutant embryos were stained for the same length of time.

Chromatin Immunoprecipitation (ChIP) Assay

ChIP was performed according to the manufacturer's protocol (Active Motif) as described (Frank et al., 2001). To perform whole-embryo ChIP, nuclear extracts were prepared from pooled wild-type E8.25 mouse embryos as previously described (Kumar and Duester, 2014); antibodies included anti-RARα or anti-RARγ (Santa Cruz Biotechnology) or anti-RARβ (Affinity Bioreagents). ChIP samples were subjected to PCR using primers flanking the mouse *Wnt8a* RARE near −4.9 kb (5′-ATC TTG GGT TGA GGC AGA GTC TC-3′ and 5′-CGC TGA GCC ACC TCT ACA ATC TT-3′ that generate a 280bp PCR product) or primers flanking a non-specific region located at −9.7 kb (5′-TCA GAC ATG GCC TCC ACT AGA AC-3′ and 5′-CCC CGT TGA GGT ATT TCT TTT GGC-3′ that generate a 219bp PCR product).

Electrophoretic Gel Mobility Shift Assay (EMSA)

Nuclear extracts were prepared from whole wild-type E8.25 embryos as described (Dignam et al., 1983). Biotin-labeled double-stranded oligonucleotide probes containing the wild-type *Wnt8a* RARE sequence or a mutant *Wnt8a* RARE sequence (see Fig. 4D for sequences) were bound to nuclear extracts. Binding reactions were executed using the LightShift Chemiluminescent EMSA Kit according to the manufacturer's instructions (Pierce, Thermo Scientific). After incubation of reaction mixtures for 20 min at room temperature, samples were mixed with 5x loading buffer and subjected to electrophoresis on a 6% non-denaturing polyacrylamide gel using 0.5 X Tris-Borate-EDTA buffer for 90min at 100V on ice, then transferred onto Biodyne nylon membrane (Thermo Scientific) at 380mA for 1 hour also in 0.5 X Tris-Borate-EDTA buffer on ice. The membrane was optimally UV-light cross-linked and detection was performed using a LightShift Chemiluminescent EMSA Kit according to the manufacturer's instructions (Pierce, Thermo Scientific). For supershift analysis, nuclear extracts were first incubated on ice for 20 min with 3 μg of anti-RARα (sc-551, Santa Cruz Biotechnology), anti-RARβ (PA1-811, Pierce, Thermo Scientific), or anti-RARγ antibodies (sc-550, Santa Cruz Biotechnology) before mixing with the biotin-labeled oligonucleotide probes.

Acknowledgments

Grant sponsors: National Institutes of Health GM062848 (G.D.).

We thank the Sanford-Burnham Animal Resources Core Facility for performing timed-matings to generate embryos. G.D. is funded by the National Institutes of Health.

References

Aulehla A, Wehrle C, Brand-Saberi B, Kemler R, Gossler A, Kanzler B, Herrmann BG. Wnt3a plays a major role in the segmentation clock controlling somitogenesis. Dev Cell. 2003; 4:395–406. [PubMed: 12636920]

Aulehla A, Wiegraebe W, Baubet V, Wahl MB, Deng C, Taketo M, Lewandoski M, Pourquie O. A beta-catenin gradient links the clock and wavefront systems in mouse embryo segmentation. Nature Cell Biol. 2008; 10:186–193. [PubMed: 18157121]

Baker KD, Ramel MC, Lekven AC. A direct role for Wnt8 in ventrolateral mesoderm patterning. Dev Dyn. 2010; 239:2828–2836. [PubMed: 20845427]

Benazeraf B, Francois P, Baker RE, Denans N, Little CD, Pourquie O. A random cell motility gradient downstream of FGF controls elongation of an amniote embryo. Nature. 2010; 466:248–252. [PubMed: 20613841]

Boulet AM, Capecchi MR. Signaling by FGF4 and FGF8 is required for axial elongation of the mouse embryo. Dev Biol. 2012; 371:235–245. [PubMed: 22954964]

Chatzi C, Cunningham TJ, Duester G. Investigation of retinoic acid function during embryonic brain development using retinaldehyde-rescued Rdh10 knockout mice. Dev Dyn. 2013; 242:1056–1065. [PubMed: 23765990]

Christian JL, McMahon JA, McMahon AP, Moon RT. Xwnt-8, a Xenopus Wnt-1/int-1-related gene responsive to mesoderm-inducing growth factors, may play a role in ventral mesodermal patterning during embryogenesis. Development. 1991; 111:1045–1055. [PubMed: 1879349]

Croce JC, McClay DR. Evolution of the Wnt pathways. Methods Mol Biol. 2008; 469:3–18. [PubMed: 19109698]

Cunningham TJ, Duester G. Mechanisms of retinoic acid signalling and its roles in organ and limb development. Nature Rev Mol Cell Bio. 2015; 16:110–123. [PubMed: 25560970]

Cunningham TJ, Zhao X, Duester G. Uncoupling of retinoic acid signaling from tailbud development before termination of body axis extension. Genesis. 2011; 49:776–783. [PubMed: 21538808]

Cunningham TJ, Zhao X, Sandell LL, Evans SM, Trainor PA, Duester G. Antagonism between retinoic acid and fibroblast growth factor signaling during limb development. Cell Reports. 2013; 3:1503–1511. [PubMed: 23623500]

Diez del Corral R, Olivera-Martinez I, Goriely A, Gale E, Maden M, Storey K. Opposing FGF and retinoid pathways control ventral neural pattern, neuronal differentiation, and segmentation during body axis extension. Neuron. 2003; 40:65–79. [PubMed: 14527434]

Dignam JD, Lebovitz RM, Roeder RG. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res. 1983; 11:1475–1489. [PubMed: 6828386]

Dubrulle J, McGrew MJ, Pourquié O. FGF signaling controls somite boundary position and regulates segmentation clock control of spatiotemporal *Hox* gene activation. Cell. 2001; 106:219–232. [PubMed: 11511349]

Dubrulle J, Pourquié O. fgf*8* mRNA decay establishes a gradient that couples axial elongation to patterning in the vertebrate embryo. Nature. 2004; 427:419–422. [PubMed: 14749824]

Dunty WC Jr, Biris KK, Chalamalasetty RB, Taketo MM, Lewandoski M, Yamaguchi TP. Wnt3a/beta-catenin signaling controls posterior body development by coordinating mesoderm formation and segmentation. Development. 2008; 135:85–94. [PubMed: 18045842]

Dunty WC Jr, Kennedy MW, Chalamalasetty RB, Campbell K, Yamaguchi TP. Transcriptional Profiling of Wnt3a Mutants Identifies Sp Transcription Factors as Essential Effectors of the Wnt/beta-catenin Pathway in Neuromesodermal Stem Cells. PLoS ONE. 2014; 9:e87018. [PubMed: 24475213]

Frank SR, Schroeder M, Fernandez P, Taubert S, Amati B. Binding of c-Myc to chromatin mediates mitogen-induced acetylation of histone H4 and gene activation. Genes Dev. 2001; 15:2069–2082. [PubMed: 11511539]

Garriock RJ, Warkman AS, Meadows SM, D'Agostino S, Krieg PA. Census of vertebrate Wnt genes: isolation and developmental expression of Xenopus Wnt2, Wnt3, Wnt9a, Wnt9b, Wnt10a, and Wnt16. Dev Dyn. 2007; 236:1249–1258. [PubMed: 17436276]

Greco TL, Takada S, Newhouse MM, McMahon JA, McMahon AP, Camper SA. Analysis of the vestigial tail mutation demonstrates that Wnt-3a gene dosage regulates mouse axial development. Genes Dev. 1996; 10:313–324. [PubMed: 8595882]

Herrmann BG, Labeit S, Poustka A, King TR, Lehrach H. Cloning of the T gene required in mesoderm formation in the mouse. Nature. 1990; 343:617–622. [PubMed: 2154694]

Holstein TW. The evolution of the Wnt pathway. Cold Spring Harbor Perspectives in Biology. 2012; 4:a007922. [PubMed: 22751150]

Hoppler S, Brown JD, Moon RT. Expression of a dominant-negative Wnt blocks induction of MyoD in Xenopus embryos. Genes Dev. 1996; 10:2805–2817. [PubMed: 8946920]

Hoppler S, Moon RT. BMP-2/-4 and Wnt-8 cooperatively pattern the Xenopus mesoderm. Mech Dev. 1998; 71:119–129. [PubMed: 9507084]

Kumar S, Duester G. Retinoic acid controls body axis extension by directly repressing *Fgf8* transcription. Development. 2014; 141:2972–2977. [PubMed: 25053430]

Kusserow A, Pang K, Sturm C, Hrouda M, Lentfer J, Schmidt HA, Technau U, von Haeseler A, Hobmayer B, Martindale MQ, Holstein TW. Unexpected complexity of the Wnt gene family in a sea anemone. Nature. 2005; 433:156–160. [PubMed: 15650739]

Lekven AC, Thorpe CJ, Waxman JS, Moon RT. Zebrafish wnt8 encodes two wnt8 proteins on a bicistronic transcript and is required for mesoderm and neurectoderm patterning. Dev Cell. 2001; 1:103–114. [PubMed: 11703928]

Liu P, Wakamiya M, Shea MJ, Albrecht U, Behringer RR, Bradley A. Requirement for Wnt3 in vertebrate axis formation. Nature Genet. 1999; 22:361–365. [PubMed: 10431240]

Logan CY, Nusse R. The Wnt signaling pathway in development and disease. Annu Rev Cell Dev Biol. 2004; 20:781–810. [PubMed: 15473860]

Lu FI, Thisse C, Thisse B. Identification and mechanism of regulation of the zebrafish dorsal determinant. Proc Natl Acad Sci USA. 2011; 108:15876–15880. [PubMed: 21911385]

Martin BL, Kimelman D. Regulation of canonical Wnt signaling by Brachyury is essential for posterior mesoderm formation. Dev Cell. 2008; 15:121–133. [PubMed: 18606146]

Martin BL, Kimelman D. Wnt signaling and the evolution of embryonic posterior development. Curr Biol. 2009; 19:R215–219. [PubMed: 19278640]

Martin BL, Kimelman D. Brachyury establishes the embryonic mesodermal progenitor niche. Genes Dev. 2010; 24:2778–2783. [PubMed: 21159819]

Martin BL, Kimelman D. Canonical Wnt Signaling Dynamically Controls Multiple Stem Cell Fate Decisions during Vertebrate Body Formation. Dev Cell. 2012; 22:223–232. [PubMed: 22264734]

McGregor AP, Pechmann M, Schwager EE, Feitosa NM, Kruck S, Aranda M, Damen WG. Wnt8 is required for growth-zone establishment and development of opisthosomal segments in a spider. Curr Biol. 2008; 18:1619–1623. [PubMed: 18926703]

Naiche LA, Holder N, Lewandoski M. FGF4 and FGF8 comprise the wavefront activity that controls somitogenesis. Proc Natl Acad Sci USA. 2011; 108:4018–4023. [PubMed: 21368122]

Nakaya MA, Biris K, Tsukiyama T, Jaime S, Rawls JA, Yamaguchi TP. Wnt3a links left-right determination with segmentation and anteroposterior axis elongation. Development. 2005; 132:5425–5436. [PubMed: 16291790]

Niederreither K, Vermot J, Schuhbaur B, Chambon P, Dollé P. Retinoic acid synthesis and hindbrain patterning in the mouse embryo. Development. 2000; 127:75–85. [PubMed: 10654602]

Nowotschin S, Ferrer-Vaquer A, Concepcion D, Papaioannou VE, Hadjantonakis AK. Interaction of Wnt3a, Msgn1 and Tbx6 in neural versus paraxial mesoderm lineage commitment and paraxial mesoderm differentiation in the mouse embryo. Dev Biol. 2012; 367:1–14. [PubMed: 22546692]

Olivera-Martinez I, Storey KG. Wnt signals provide a timing mechanism for the FGF-retinoid differentiation switch during vertebrate body axis extension. Development. 2007; 134:2125–2135. [PubMed: 17507413]

Petersen CP, Reddien PW. Wnt signaling and the polarity of the primary body axis. Cell. 2009; 139:1056–1068. [PubMed: 20005801]

Pourquié O. Vertebrate segmentation: from cyclic gene networks to scoliosis. Cell. 2011; 145:650–663. [PubMed: 21620133]

Shimizu T, Bae YK, Muraoka O, Hibi M. Interaction of Wnt and *caudal*-related genes in zebrafish posterior body formation. Dev Biol. 2005; 279:125–141. [PubMed: 15708563]

Sirbu IO, Duester G. Retinoic acid signaling in node ectoderm and posterior neural plate directs left-right patterning of somitic mesoderm. Nature Cell Biol. 2006; 8:271–277. [PubMed: 16489341]

Srivastava M, Begovic E, Chapman J, Putnam NH, Hellsten U, Kawashima T, Kuo A, Mitros T, Salamov A, Carpenter ML, Signorovitch AY, Moreno MA, Kamm K, Grimwood J, Schmutz J, Shapiro H, Grigoriev IV, Buss LW, Schierwater B, Dellaporta SL, Rokhsar DS. The Trichoplax genome and the nature of placozoans. Nature. 2008; 454:955–960. [PubMed: 18719581]

Stulberg MJ, Lin A, Zhao H, Holley SA. Crosstalk between Fgf and Wnt signaling in the zebrafish tailbud. Dev Biol. 2012; 369:298–307. [PubMed: 22796649]

Takada S, Stark KL, Shea MJ, Vassileva G, McMahon JA, McMahon AP. Wnt-3a regulates somite and tailbud formation in the mouse embryo. Genes Dev. 1994; 8:174–189. [PubMed: 8299937]

Takemoto T, Uchikawa M, Kamachi Y, Kondoh H. Convergence of Wnt and FGF signals in the genesis of posterior neural plate through activation of the Sox2 enhancer N-1. Development. 2006; 133:297–306. [PubMed: 16354715]

Takemoto T, Uchikawa M, Yoshida M, Bell DM, Lovell-Badge R, Papaioannou VE, Kondoh H. Tbx6-dependent *Sox2* regulation determines neural or mesodermal fate in axial stem cells. Nature. 2011; 470:394–398. [PubMed: 21331042]

Tzouanacou E, Wegener A, Wymeersch FJ, Wilson V, Nicolas JF. Redefining the progression of lineage segregations during mammalian embryogenesis by clonal analysis. Dev Cell. 2009; 17:365–376. [PubMed: 19758561]

Vendrell V, Vazquez-Echeverria C, Lopez-Hernandez I, Alonso BD, Martinez S, Pujades C, Schimmang T. Roles of Wnt8a during formation and patterning of the mouse inner ear. Mech Dev. 2013; 130:160–168. [PubMed: 23041177]

Vermot J, Llamas JG, Fraulob V, Niederreither K, Chambon P, Dollé P. Retinoic acid controls the bilateral symmetry of somite formation in the mouse embryo. Science. 2005; 308:563–566. [PubMed: 15731404]

Wahl MB, Deng C, Lewandoski M, Pourquie O. FGF signaling acts upstream of the NOTCH and WNT signaling pathways to control segmentation clock oscillations in mouse somitogenesis. Development. 2007; 134:4033–4041. [PubMed: 17965051]

Wilkinson, DG. Whole mount *in situ* hybridization of vertebrate embryos. In: Wilkinson, DG., editor. *In Situ* Hybridization: A Practical Approach. Oxford: IRL Press; 1992. p. 75-83.

Wilson V, Olivera-Martinez I, Storey KG. Stem cells, signals and vertebrate body axis extension. Development. 2009; 136:1591–1604. [PubMed: 19395637]

Wylie AD, Fleming JA, Whitener AE, Lekven AC. Post-transcriptional regulation of wnt8a is essential to zebrafish axis development. Dev Biol. 2014; 386:53–63. [PubMed: 24333179]

Yamaguchi TP. Genetics of Wnt signaling during early mammalian development. Methods Mol Biol. 2008; 468:287–305. [PubMed: 19099264]

Yamaguchi TP, Harpal K, Henkemeyer M, Rossant J. *fgfr-1* is required for embryonic growth and mesodermal patterning during mouse gastrulation. Genes Dev. 1994; 8:3032–3044. [PubMed: 8001822]

Yamaguchi TP, Takada S, Yoshikawa Y, Wu N, McMahon AP. T (Brachyury) is a direct target of Wnt3a during paraxial mesoderm specification. Genes Dev. 1999; 13:3185–3190. [PubMed: 10617567]

Yoshikawa Y, Fujimori T, McMahon AP, Takada S. Evidence that absence of Wnt-3a signaling promotes neuralization instead of paraxial mesoderm development in the mouse. Dev Biol. 1997; 183:234–242. [PubMed: 9126297]

Zappone MV, Galli R, Catena R, Meani N, De Biasi S, Mattei E, Tiveron C, Vescovi AL, Lovell-Badge R, Ottolenghi S, Nicolis SK. Sox2 regulatory sequences direct expression of a (beta)-geo transgene to telencephalic neural stem cells and precursors of the mouse embryo, revealing regionalization of gene expression in CNS stem cells. Development. 2000; 127:2367–2382. [PubMed: 10804179]

Zhao X, Duester G. Effect of retinoic acid signaling on Wnt/beta-catenin and FGF signaling during body axis extension. Gene Expr Patterns. 2009; 9:430–435. [PubMed: 19539783]

Key findings

- *Wnt8a* and *Wnt3a* exhibit overlapping functions during the early stage of body axis extension.
- *Wnt8a*−/− embryos do not exhibit body axis extension defects.
- *Wnt8a*/*Wnt3a* double knockouts cease body axis extension earlier than *Wnt3a* mutants correlating with early termination of caudal *Fgf8* expression and ectopic *Sox2* expression.
- Embryo ChIP identified an RA response element associated with RA repression of caudal *Wnt8a* expression.
- Our findings provide new insight into how Wnt-FGF-RA signals interact during body axis extension.

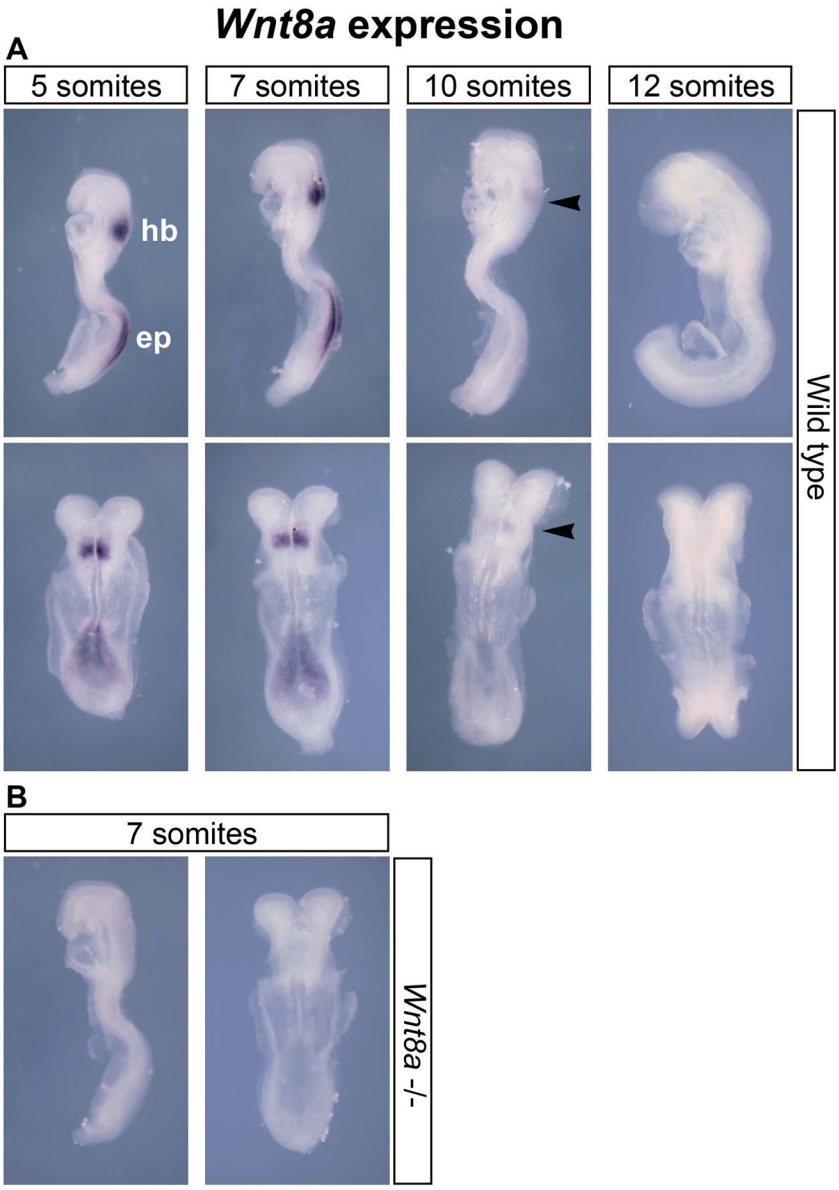

Fig. 1.

Wnt8a expression analysis. **A:** *Wnt8a* expression during somite pair stages 5, 7, 10, and 12 in wild type embryos; lateral views (top) and dorsal views (bottom) are shown. **B:** *Wnt8a* expression during somite pair stage 7 in *Wnt8a*$^{-/-}$ embryos; shown is a lateral view (left) and dorsal views (right). hb, hindbrain, ep, epiblast. Arrowheads indicate weak staining in the hindbrain.

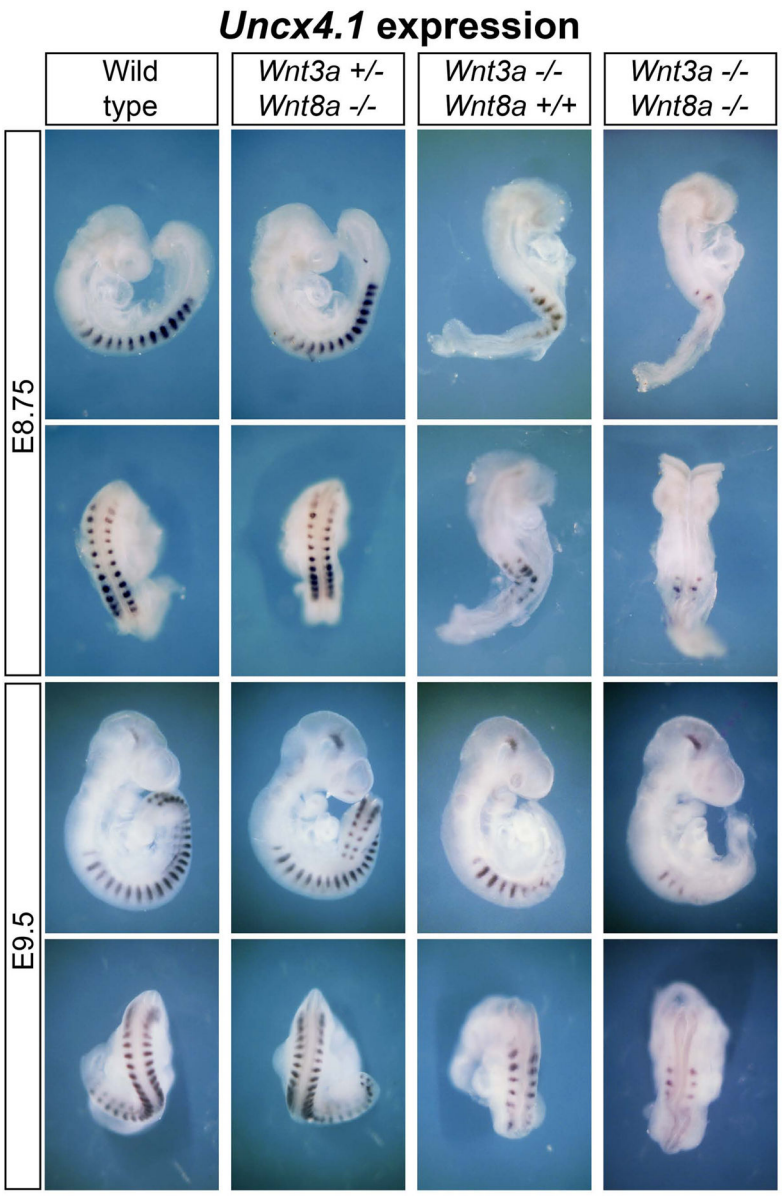

Fig. 2.

Somite formation analysis in *Wnt8a* and *Wnt3a* mutants. Expression of *Uncx4.1* marking the somites is shown in wild type, $Wnt3a^{+/-};Wnt8a^{-/-}$, $Wnt3a^{-/-};Wnt8a^{+/+}$, and $Wnt3a^{-/-};Wnt8a^{-/-}$ embryos at E8.75 and E9.5. For each stage, lateral views (top) and dorsal views (bottom) are shown.

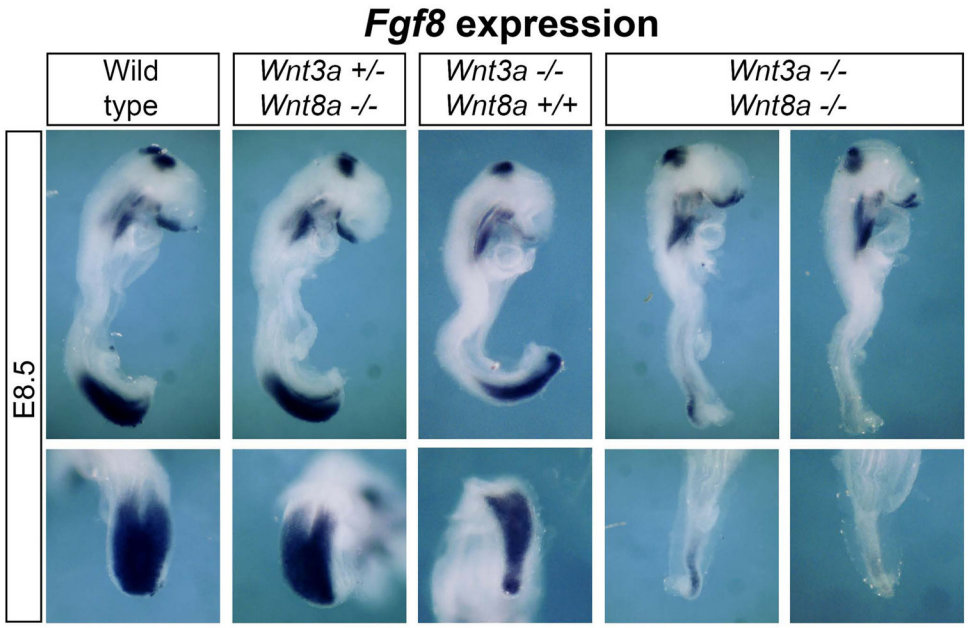

Fig. 3.

Caudal *Fgf8* expression analysis in *Wnt8a* and *Wnt3a* mutants. Expression of *Fgf8* is shown in wild-type, $Wnt3a^{+/-};Wnt8a^{-/-}$, $Wnt3a^{-/-};Wnt8a^{+/+}$, and $Wnt3a^{-/-};Wnt8a^{-/-}$ embryos at E8.5. Two different $Wnt3a^{-/-};Wnt8a^{-/-}$ embryos are shown. Lateral views (top) and dorsal views (bottom) of the caudal *Fgf8* expression domain are shown.

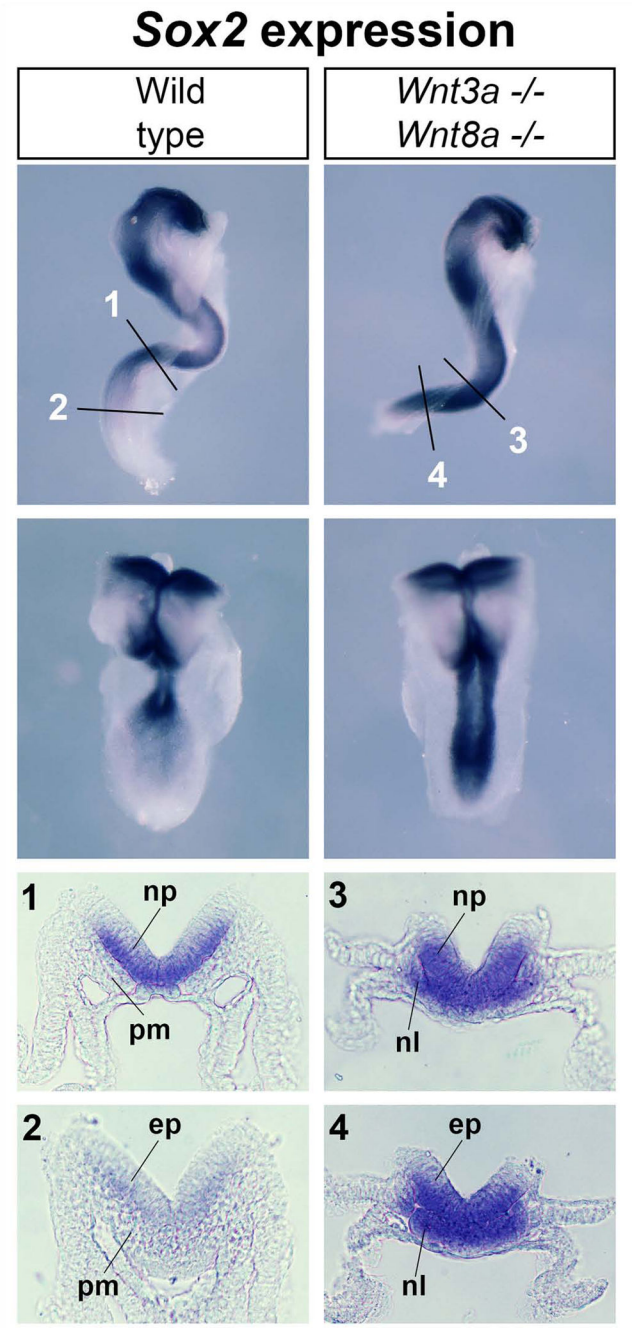

Fig. 4.

Sox2 expression analysis in *Wnt8a/Wnt3a* double mutants. Expression of *Sox2* is shown in wild-type and *Wnt3a*$^{-/-}$;*Wnt8a*$^{-/-}$ embryos at E8.25. The wild-type embryo has 5 somite pairs, while the mutant has 3 somite pairs but is at an equivalent stage based on similar rostral development. Lateral and dorsal views are shown. Lower panels are sections from planes indicated by numbers. ep, caudal epiblast; nl, neural-like tissue; np, neural plate; pm, paraxial mesoderm.

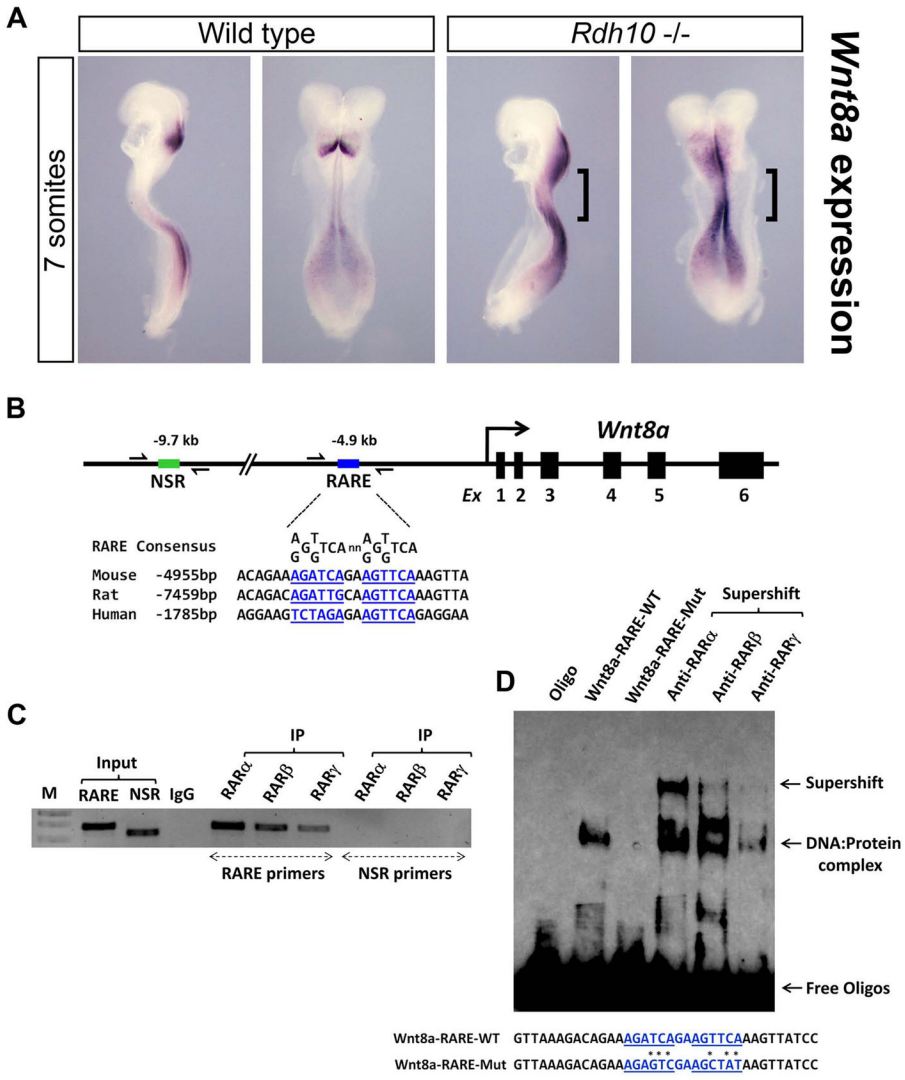

Fig. 5.

Evidence for RA regulation of *Wnt8a*. **A:** Expression analysis of *Wnt8a* in wild-type versus *Rdh10*$^{-/-}$ embryos at the 7 somite stage; lateral view (left) and dorsal view (right). **B:** Schematic view of the mouse *Wnt8a* locus highlighting a RA response element (RARE) located 4.9 kb upstream of the transcription start site and conservation with other species; the RARE consensus sequence is shown for a DR2 RARE which has 2 bp separating the direct repeats; also shown is a non-specific region (NSR) as well as arrows indicating locations of ChIP primers. **C:** ChIP analysis from pooled E8.25 mouse embryos, primers amplifying the *Wnt8a* RARE region or non-specific region (NSR) primers, and antibodies against RAR isoforms or IgG. **D:** Electrophoretic mobility supershift assay, using nuclear protein extracts from E8.25 mouse embryos, a biotin-labeled double-stranded oligonucleotide with wild-type or mutated *Wnt8a* RARE sequences, and antibodies against RAR isoforms.

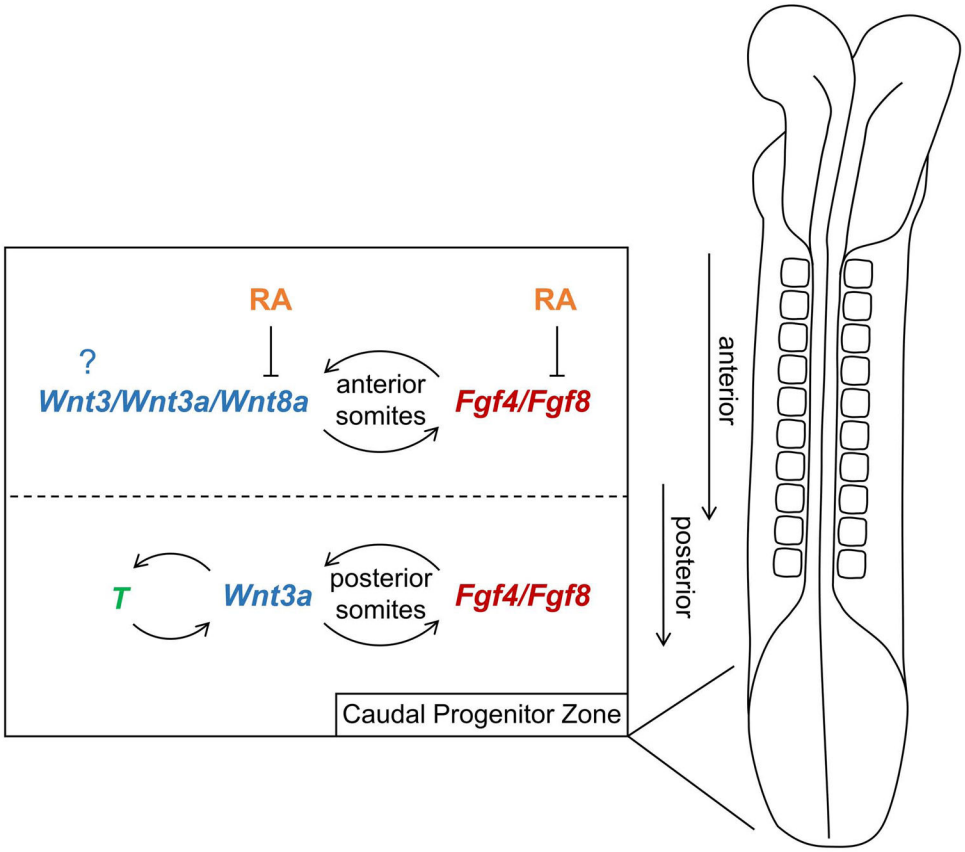

Fig. 6.

Schematic representation of Wnt–FGF-RA and Wnt–T regulatory loops during formation of anterior and posterior somites. During anterior somitogenesis in which the first 7–9 somite pairs form, at least two Wnt ligands (Wnt3a and Wn8a) cooperate to form a positive autoregulatory loop with FGFs (Wnt3 may also function to generate the first 3 anterior somite pairs); at the same time, RA generated in the somites directly antagonizes both *Wnt8a* and *Fgf8* expression. During posterior somitogenesis, only Wnt3a is critical, forming positive autoregulatory loops with both T and FGFs. At later stages (beyond E8.5) when posterior somites form, RA is no longer required to antagonize caudal *Fgf8* expression (Cunningham et al., 2011) and RA repression of *Wnt8a* is moot since it is no longer expressed.

Table 1

Wnt8a ortholog aliases among vertebrate model organisms (older names in parenthesis). Zebrafish has a bicistronic *wnt8a* gene with distinct wnt8a.1 and wnt8a.2 proteins that arose via a teleost-specific duplication event, whereas other species shown have a single *Wnt8a* gene.

Zebrafish	*Xenopus*	Chicken	Mouse	Human
wnt8a.1 (wnt8.1; wnt8) wnt8a.2 (wnt8.2)	*wnt8a (wnt-8; Xwnt-8)*	*Wnt8a (Wnt8c)*	*Wnt8a*	*Wnt8a*

Table 2

Somite pair counts in *Wnt8a* and *Wnt3a* mutants at E9.5.

	Wild Type (n=4)	***Wnt3a* +/− *Wnt8a* −/− (n=6)[1]**	***Wnt3a* −/− *Wnt8a* +/+ (n=7)[2]**	***Wnt3a* −/− *Wnt8a* +/− (n=6)[3]**	***Wnt3a* −/− *Wnt8a* −/− (n=6)[4]**
Somite pairs observed in individual embryos	19	19	8	7	3
	22	18	8	8	3
	28	28	8	7	3 (left), 4 (right)
	20	30	9	7	2 (left), 3 (right)
		27	8	7	3
		25	8	6	3
			7		
Average	**22.5**	**24.5**	**8**	**7**	**3**

Significance values are as follows (Student's t-test):

[1] not significant, p>0.05 (wild-type vs. $Wnt3a^{+/-};Wnt8a^{-/-}$),

[2] p<0.0001 (wild-type vs. $Wnt3a^{-/-};Wnt8a^{+/+}$),

[3] p<0.05 ($Wnt3a^{-/-};Wnt8a^{+/+}$ vs. $Wnt3a^{-/-};Wnt8a^{+/-}$),

[4] p<0.0001 ($Wnt3a^{-/-};Wnt8a^{+/+}$ vs. $Wnt3a^{-/-};Wnt8a^{-/-}$) and ($Wnt3a^{-/-};Wnt8a^{+/-}$ vs. $Wnt3a^{-/-};Wnt8a^{-/-}$).