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Oscillations of the *Snail* Genes in the Presomitic Mesoderm Coordinate Segmental Patterning and Morphogenesis in Vertebrate Somitogenesis

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Summary

The segmented body plan of vertebrate embryos arises through segmentation of the paraxial mesoderm to form somites. The tight temporal and spatial control underlying this process of somitogenesis is regulated by the segmentation clock and the FGF signaling wavefront. Here, we report the cyclic mRNA expression of *Snail1* and *Snail2* in the mouse and chick presomitic mesoderm (PSM), respectively. Whereas *Snail* genes' oscillations are independent of NOTCH signaling, we show that they require WNT and FGF signaling. Overexpressing *Snail2* in the chick embryo prevents cyclic *Lfng* and *Meso1* expression in the PSM and disrupts somite formation. Moreover, cells misexpressing *Snail2* fail to express *Paraxis*, remain mesenchymal, and are thereby inhibited from undergoing the epithelialization event that culminates in the formation of the epithelial somite. Thus, *Snail* genes define a class of cyclic genes that coordinate segmentation and PSM morphogenesis.

Introduction

The segmented body plan is a characteristic feature of the vertebrate embryo that becomes apparent very early in development. The first overt sign of segmentation is seen in the paraxial mesoderm as it progressively generates somites in an anteroposterior (AP) direction. Somitogenesis is under tight temporal control such that a new pair of somites forms according to a strict periodic schedule that is species specific. The periodicity of this process is under the control of the segmentation clock that drives oscillating mRNA expression of a number of "cyclic genes" in the unsegmented presomitic mesoderm (PSM) of vertebrates (Pourquie, 2003).

The majority of cyclic genes are downstream targets of the NOTCH signaling pathway and code for Hairy/Enhancer of split family members, the glycosyltransferase enzyme LUNATIC FRINGE (LFNG), and the NOTCH ligand DELTA. It is now a well accepted fact that this pathway plays a crucial role in somitogenesis since mutation of several of its components causes severe somitic defects in mice or zebrafish embryos (Rida et al., 2004). If the cyclic expression of genes of the NOTCH pathway such as *Lfng* or *Hes7* is perturbed by either gain or loss of function, somitogenesis is severely disrupted (Bessho et al., 2003; Dale et al., 2003; Serth et al., 2003). Nevertheless, even the most severe mouse and zebrafish NOTCH pathway mutants retain some degree of segmentation at the level of the most anterior somites, raising the possibility that there may be other pathways involved in the mechanism of the segmentation clock that can compensate in part for the loss of NOTCH (Rida et al., 2004).

Oscillations of *Axin2* and *Nkd1*, two members of the WNT signaling pathway in the mouse PSM, recently implicated this pathway in the segmentation clock (Aulehla et al., 2003; Ishikawa et al., 2004). Disruption of WNT signaling in the *Wnt3a* hypomorphic mutation *vestigial tail* (*vt*) leads to a loss of *Axin2* and of *Nkd1* expression. Interestingly, the dynamic expression of *Nkd1* is also dependent on NOTCH signaling, as it no longer oscillates in the absence of *Hes7* (Ishikawa et al., 2004). Taken together, these data suggest that the NOTCH and WNT pathways interact within the mechanism of the segmentation clock.

The FGF pathway is also known to be crucial to somitogenesis since it regulates the competence of PSM cells to undergo segmentation and thus controls the response of PSM cells to the segmentation clock (Dubrulle and Pourquie, 2004). FGF signaling establishes a traveling wavefront involved in the conversion of the pulsatile signal of the clock into the spatial periodic pattern of somites (Pourquie, 2003). In mouse, *Fgf8* expression is downregulated in the absence of *Wnt3a* (Aulehla et al., 2003), and in zebrafish, expression of the NOTCH target gene *her13.2*, required for cyclic gene oscillations, is regulated by FGF signaling (Kawamura et al., 2005). Thus, it appears that several levels of crosstalk exist between the NOTCH, the WNT, and the FGF pathways in somitogenesis.

The SNAIL superfamily of transcriptional repressors, most notably SNAIL1 (formerly SNAIL) and SNAIL2 (formerly SLUG), have been shown to play a critical role in vertebrate and invertebrate development and in cancer (Barrallo-Gimeno and Nieto, 2005). These proteins control major morphogenetic processes by controlling epithelium-to-mesenchyme transitions (EMTs) (Barrallo-Gimeno and Nieto, 2005). SNAIL proteins are able to repress genes coding for proteins associated with an epithelial phenotype such as E-CADHERIN or desmosomal proteins and can activate the expression of mesenchymal markers (Batlle et al., 2000; Cano et al., 2000; Savagner et al., 1997). In the developing vertebrate embryo, the first SNAIL-dependent EMT that occurs takes place in the primitive streak during gastrulation (Carver et al., 2001; Ciruna and Rossant, 2001). Driven by *Snail1* activity acting downstream of FGF signaling, epithelial primitive streak cells fated to become mesodermal progenitors downregulate E-CADHERIN and undergo

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EMTs. Consequently, they delaminate from the streak as individual mesenchymal cells and subsequently migrate to the various mesodermal locations of the developing embryo (Ciruna and Rossant, 2001). On exiting the primitive streak after an EMT, paraxial mesoderm progenitor cells populate the mesenchymal posterior PSM, where they are exposed to high levels of FGF signaling (Dubrulle et al., 2001) and maintain strong *Snail1* and *Snail2* expression in mouse and chick, respectively (Sefton et al., 1998). When paraxial mesoderm cells pass a threshold concentration of FGF signaling (termed the determination front) in the anterior PSM, they begin to acquire epithelial characteristics (Duband et al., 1987; Dubrulle et al., 2001; Nakaya et al., 2004). At this level, cells also become allocated to genetically defined segments that provide the templates upon which the epithelial somites will form at the anterior end of the PSM. How the morphogenetic program leading to epithelialization is coordinated to the segmentation process in the PSM is currently unknown.

The beginning of the epithelialization process in the PSM correlates with the downregulation of *Snail1* and *Snail2* (Sefton et al., 1998), thus raising the possibility that these genes are involved in the control of this morphological transition. We have closely investigated the expression profile of *Snail1* and *Snail2* within the PSM in mouse and chick embryos, respectively, and we found that their mRNA is rhythmically transcribed with a periodicity that matches the budding off of epithelial somites. *Snail1* oscillates largely in synchrony with the NOTCH cyclic genes, but its expression is independent of NOTCH signaling and relies upon WNT3A signaling. Misexpression of *Snail2* in the chick PSM blocks *Lfng* and *Meso1* expression. This disruption of the segmentation clock-driven oscillations suggests a role for *Snail* genes in the clock mechanism. Subsequently, cells overexpressing *Snail2* fail to epithelialize and form somites, remaining in a mesenchymal state. While this phenotype is reminiscent of that seen in embryos misexpressing FGF in the PSM, *Snail2* does not upregulate FGF targets like *Brachyury*, suggesting that it only mediates the morphogenetic aspect of the FGF response in the PSM. Together, these results indicate that *Snail* genes may identify a new class of cyclic genes and provide a link between the segmentation clock and the FGF signaling wavefront in vertebrate segmentation.

Results

Snail1 and *Snail2* mRNA Oscillate in the Mouse and Chick Embryo PSM, Respectively

We have examined the expression of *Snail1* mRNA in the PSM of E8.5–E10 mouse embryos by in situ hybridization. Large batches of embryos were collected and analyzed at each stage. We found that the expression pattern varied considerably among the batches of similar stage embryos. Thus, we found some embryos displaying a broad posterior pattern of expression plus a thin stripe in the anterior region of the PSM just caudal to the posterior limit of the next somite to be formed (Figures 1A and 1B); other embryos displayed only a wide posterior expression domain in the PSM (Figure 1C), while others displayed only a narrow band of expression in the anterior-most part of the PSM (Figure 1D). These

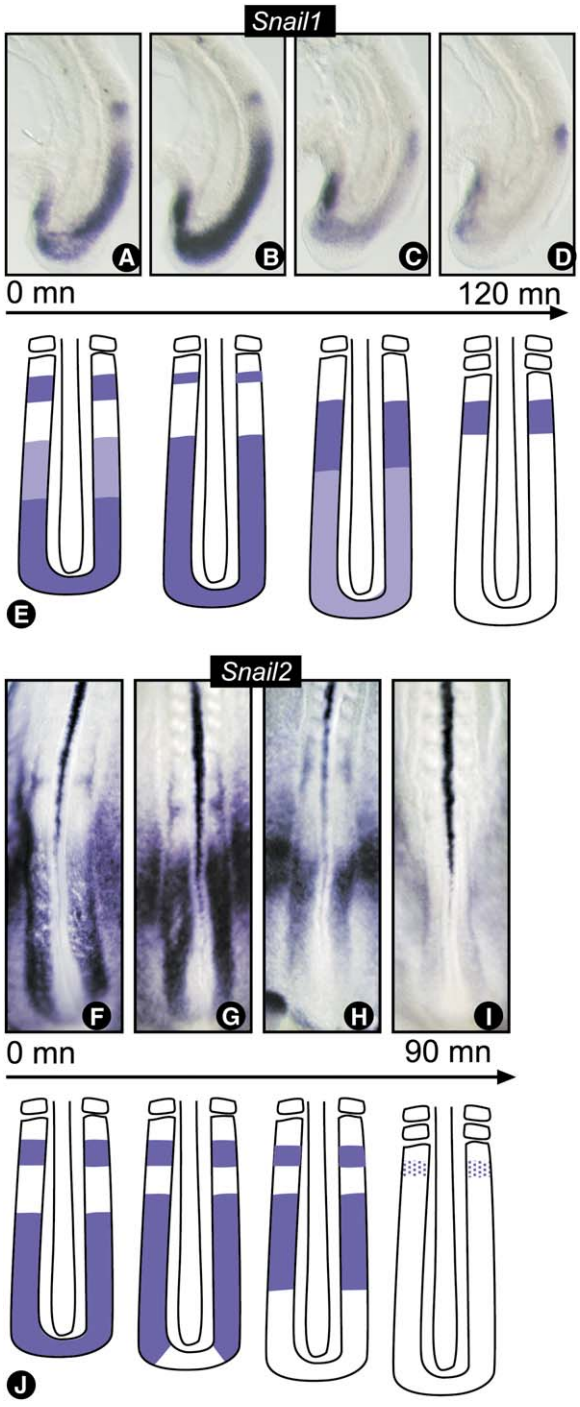

Figure 1. Oscillations of *Snail* Genes in Mouse and Chick PSM

(A–D) Posterior lateral view of E9.5 mouse embryos hybridized with the *Snail1* probe.

(E) Schematic representation of the dynamic expression of *Snail1* during the formation of one somite in the mouse embryo (120 min).

(F–I) Posterior dorsal view of 2-day-old chick embryos hybridized with the *Snail2* probe.

(J) Schematic representation of the dynamic expression of *Snail2* during the formation of one somite in the chick embryo (90 min).

different patterns were seen at each of the stages analyzed, strongly suggesting that *Snail1* may be oscillating in the PSM. To directly test if *Snail1* expression is oscillating, we bisected the posterior region of mice embryos

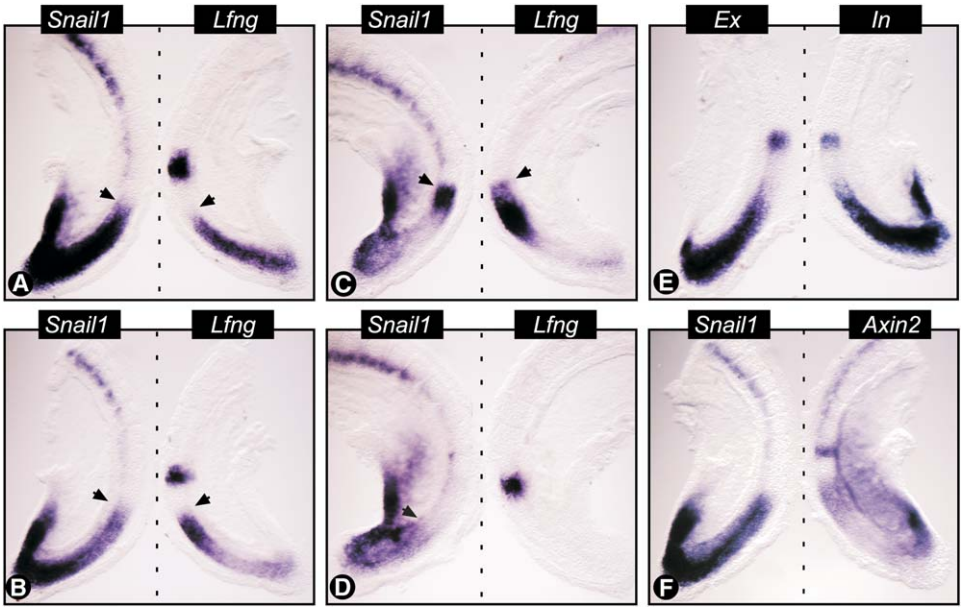

Figure 2. Comparison between *Snail1*, *Lfng*, and *Axin2* Expression in the Mouse Embryo PSM

(A–D) Comparison of *Snail1* and *Lfng* expression detected by whole-mount in situ hybridization in the two embryonic halves of the same E9.5 mouse embryos.

(E) Detection of *Snail1* by using an exonic (Ex) and an intronic (In) probe in the two embryonic halves of the same E9.5 embryos.

(F) Comparison of *Snail1* and *Axin2* expression detected by whole-mount in situ hybridization in the two embryonic halves of the same E9.5 mouse embryos.

Arrowheads mark the level of the anterior front of the posterior dynamic domain of the genes. Anterior is oriented toward the top.

down the midline and fixed one half while culturing the other half in vitro for a defined time period. The two halves were then analyzed for *Snail1* expression (Figure S1; see the Supplemental Data available with this article online). When the one half was cultured for 75, 90, or 105 min, we found that the expression of *Snail1* was different from that in the fixed half (Figure S1A, n = 6). However, when we cultured one half for 120 min, this explant formed an extra somite, and the expression domain was the same in the cultured and the fixed explants (Figure S1B, n = 5). These data demonstrate that *Snail1* mRNA is expressed according to a periodic wave in the mouse PSM (Figure 1E), and that the period of the oscillation is 120 min—the time it takes to form a new pair of somites from the mouse PSM.

There is a striking interchange in both the expression patterns and the functions of two members of the *Snail* superfamily, namely, *Snail1* and *Snail2*, in neural crest, nascent mesoderm, and paraxial mesoderm between chick and mice embryos (Locascio et al., 2002; Sefton et al., 1998). In the chick embryo, *Snail2*, not *Snail1*, is expressed in the PSM (Figures 1F–1I). We analyzed in detail the PSM expression of *Snail2* in a large series of stage-matched chick embryos and observed a clear dynamic expression pattern (Figures 1F–1J) similar to that seen in the mouse PSM for *Snail1*. Therefore, our data identify *Snail1* in mouse and *Snail2* in chick as cyclic genes regulated by the segmentation clock.

Snail1 May Define a New Class of Cyclic Genes

The genes of the NOTCH pathway *Lfng*, *Hes1*, and *Hes7* cycle in synchrony in the mouse PSM; whereas the WNT pathway inhibitor *Axin2* oscillates out of phase with them (Aulehla et al., 2003). In order to determine whether *Snail1* cycles in synchrony with any of these genes, we bisected the posterior region of mice embryos down the midline and hybridized one half for *Lfng* expression and the other half for *Snail1* expression. We found that, for the main part of the cycle, the anterior progression domain of the expression front progresses in synchrony for the two genes (arrowheads, Figures 2A–2C). However, there are two differences in the expression profiles of these genes in the PSM. First, at the onset of a new wave, *Snail1* expression is initiated prior to that of *Lfng* in the posterior PSM (Figure 2D), and, subsequently, the *Lfng* expression front catches up with that of *Snail1* (Figure 2A). Second, unlike *Snail1*, the posterior domain of the expression of *Lfng* is rapidly degraded as the expression front moves anteriorly up the PSM (Figures 2B–2D). This may be due to different rates of mRNA stability/degradation for the two genes.

We tested this possibility indirectly by comparing the expression of *Snail1* detected with either an intronic probe or an exonic probe in bisected explants as described above (Figure 2E). In all cases, the pattern of expression in each half was the same for the two probes (Figure 2E, n = 11), demonstrating that the domains of expression of the exonic probe are sites of active transcription. Thus, *Snail1* continues to be transcribed in domains of the PSM where *Lfng* transcription has already been shut off. Due to these two differences, the ratio of embryos that do or do not display the broad posterior domain of expression is very different for the two genes. Thus, only 20% of the embryos analyzed display only the stripe of *Snail1* expression in the anterior-most PSM, which may account for why the authors who originally

described the expression of *Snail1* in the PSM did not report the dynamic expression in this tissue (Locascio et al., 2002; Nieto et al., 1992; Sefton et al., 1998; Smith et al., 1992). The differences in the expression profiles of the NOTCH pathway-related cyclic genes as compared to *Snail1* in the mouse PSM suggest that *Snail1* expression is not regulated in the same way as *Lfng*, *Hes1*, and *Hes7*.

Axin2 was shown to oscillate in opposite phase to the NOTCH pathway-related cyclic genes in the mouse PSM (Aulehla et al., 2003), and, as expected based on the expression described above, which was largely in synchrony with *Lfng* when we directly compared *Snail1* and *Axin2* in half-embryos, they were also observed to be out of synchrony (Figure 2F, n = 18).

Periodic Expression of *Snail1* Is Independent of NOTCH, but Is Downstream of the WNT Pathway

In order to test whether *Snail1* is regulated by NOTCH or by WNT signaling, we analyzed the PSM expression of *Snail1* in mutant mice embryos in which these pathways were altered. We found that at E9–E9.5, when mutant embryos already exhibit strong segmentation defects and expression of *Lfng* and *Hes7* is severely disturbed, *Snail1* continues to be expressed strongly in the PSM of *Notch1*$^{-/-}$ (n = 12), *Hes7*$^{-/-}$ (n = 17), and *Lfng*$^{-/-}$ (n = 3) homozygous null embryos at levels equivalent to these seen in heterozygous and wild-type littermates (Figures 3A and 3C–3F). We also analyzed the expression of *Snail1* at E8.5 in *Rbpjk*$^{-/-}$ homozygous null embryos that display the most severe segmentation phenotype of the NOTCH mutants (Oka et al., 1995). Whereas *Lfng* expression is absent from the PSM of these mutants (data not shown) (Morales et al., 2002), we found that *Snail1* is still expressed (Figure 3B, n = 13/14). Therefore, NOTCH signaling is not required for *Snail1* expression in the PSM.

We next investigated if NOTCH signaling was required for the oscillations of *Snail1* in the mouse PSM. To this end, we examined *Snail1* expression in *Hes7* null mouse mutants in which oscillations of the NOTCH pathway-related cyclic genes are lost (Bessho et al., 2001). While the anterior stripe of *Snail1* expression was absent in the PSM of *Hes7* mutants, we did observe different patterns of expression when comparing a large number of embryos, implying that a dynamic expression of *Snail1* is maintained in these mutants (Figures 3E and 3F). These data strongly suggest that *Snail1* dynamic expression in the PSM is not dependent on NOTCH activity.

To complement this approach, we conducted a 2 hr in vitro culture of bisected E9.5 posterior mouse embryo explants in the presence or absence of 10 μM DAPT (Dovey et al., 2001), an inhibitor of γ-secretase-mediated NOTCH cleavage. Under these conditions, while cyclic *Lfng* transcription is abolished in the PSM (Figure 3G, n = 3), *Snail1* continues to be expressed (Figure 3H, n = 6). Moreover, the expression domains of *Snail1* are the same in the treated and untreated explant halves from one embryo, but they differ from those of other embryos, suggesting, again, that oscillating *Snail1* expression in the PSM is independent of NOTCH signaling.

To test if the regulation of *Snail2* in the chick embryo PSM is similarly independent of NOTCH signaling, we

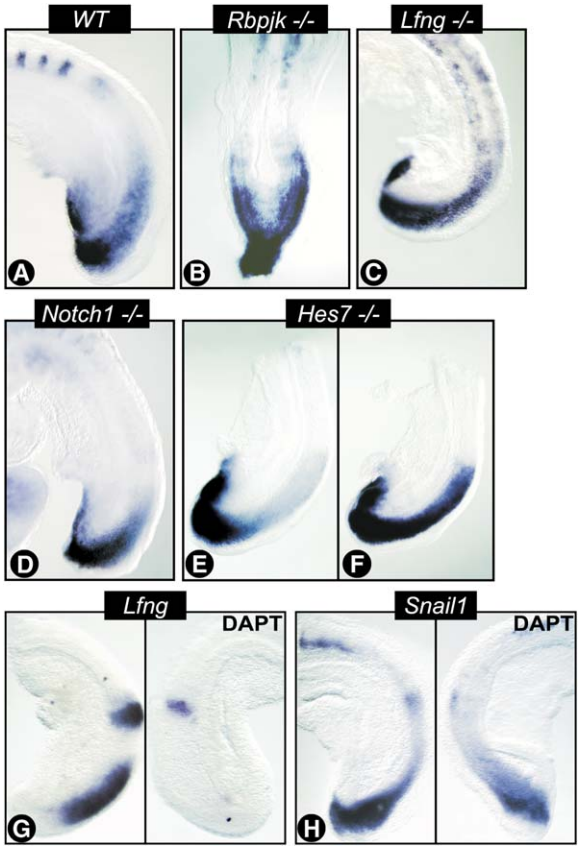

Figure 3. *Snail1* Oscillations Are Not Regulated by NOTCH Signaling in the Mouse Embryo

(A–F) Expression of *Snail1* in the posterior PSM in a (A) wild-type (WT) embryo and in homozygous null mutants for (B) *Rbpjk*, (C) *Lfng*, (D) *Notch1*, and (E and F) *Hes7*. Embryos were colabeled with *Uncx4.1*, which labels the posterior part of the newly formed somites.

(G and H) Comparison of embryonic halves from E9.5 CD1 WT mouse embryos cultured in control DMSO (left) and DAPT-containing medium (right) hybridized in whole mount for (G) *Lfng* and (H) *Snail1* expression.

overexpressed by in ovo electroporation the activated form of the NOTCH receptor, or a LUNATIC FRINGE expression construct that was shown to inhibit NOTCH signaling in the PSM (Dale et al., 2003). In both cases, no effect on *Snail2* expression could be observed in the PSM (data not shown). Together, these data suggest that, in mouse and chick, the dynamic expression of *Snail* genes in the PSM is not regulated by NOTCH signaling.

We then examined whether *Snail1* expression is regulated by WNT signaling. Both *Wnt5a* and *Wnt3a* are expressed in the mouse PSM, and their mutation leads to segmentation defects (Takada et al., 1994; Yamaguchi et al., 1999). We therefore analyzed *Snail1* expression in the PSM of both *Wnt5A* homozygous null embryos and in the hypomorphic *Wnt3a* mutant *vt* (Greco et al., 1996). In *Wnt5a* null embryos, expression of *Snail1* continues to oscillate in the tail bud (Figures 4A and 4B, n = 7). However, in *vt/vt* embryos, *Snail1* transcription is severely downregulated in the middle PSM and tailbud regions (compare Figures 4C and 4E to Figures 4D and

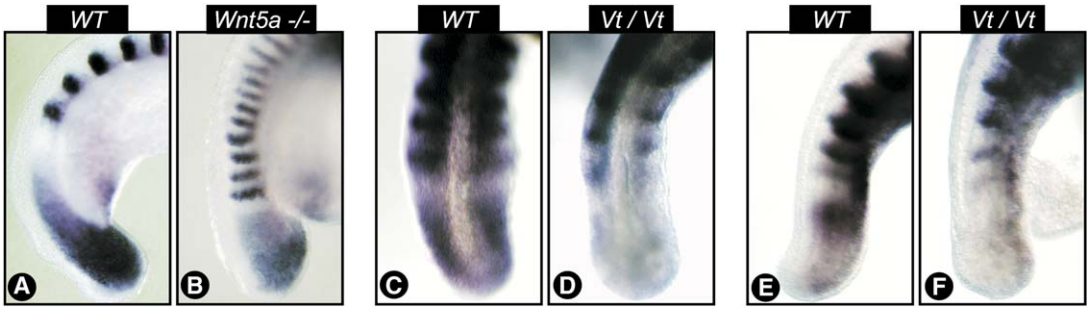

Figure 4. *Snail1* Expression Is Downstream of *Wnt3a* Signaling

(A and B) Comparison of *Snail1* expression between (A) wild-type (WT) and (B) *Wnt5a* homozygous null mutant E9.5 mouse embryos.
(C–F) Comparison of *Snail1* expression between (C and E) WT and (D and F) *vt* mutant E10.5 mouse embryos.
Embryos were colabeled with *Uncx4.1*, which labels the posterior part of the newly formed somites. (A, B, E, and F) lateral views and (C and D) dorsal views of the PSM region.

4F, n = 29). Thus, like the other cyclic genes characterized thus far (Aulehla et al., 2003), *Snail1* oscillations appear to be dependent on WNT3A signaling.

Snail2 Overexpression in the Chick Embryo Blocks Segmentation

To analyze the role of *Snail* genes in segmentation, we misexpressed *Snail2* in the developing paraxial mesoderm in the chick embryo by in ovo electroporation and then analyzed the resulting effects on somitogenesis. In the segmented region of embryos strongly expressing *Snail2*, green fluorescent protein (GFP)-positive cells formed large aggregates extending along the neural tube and lateral plate in which no morphological segmentation could be observed (Figures 5A, 5C, 5E, and 5G; see also Figures 6A and 7C and 7E). *Uncx4.1*, normally segmentally expressed in the posterior part of somites, was absent from these unsegmented domains showing strong levels of GFP (asterisks, Figure 5B; n = 6). Because of their preferential localization between the somites and the neural tube, we examined if the cells that strongly overexpressed *Snail2* could correspond to neural crest rather than paraxial mesoderm by staining with the neural crest markers *Sox10* (Figures 5C and 5D; n = 7) (Southard-Smith et al., 1998) or HNK1 (data not shown) (Le Douarin and Dupin, 1993). No expression of these markers was observed in cells overexpressing *Snail2*, suggesting that they do not belong to the neural crest. In many cases, *Snail2*-overexpressing cells also localized

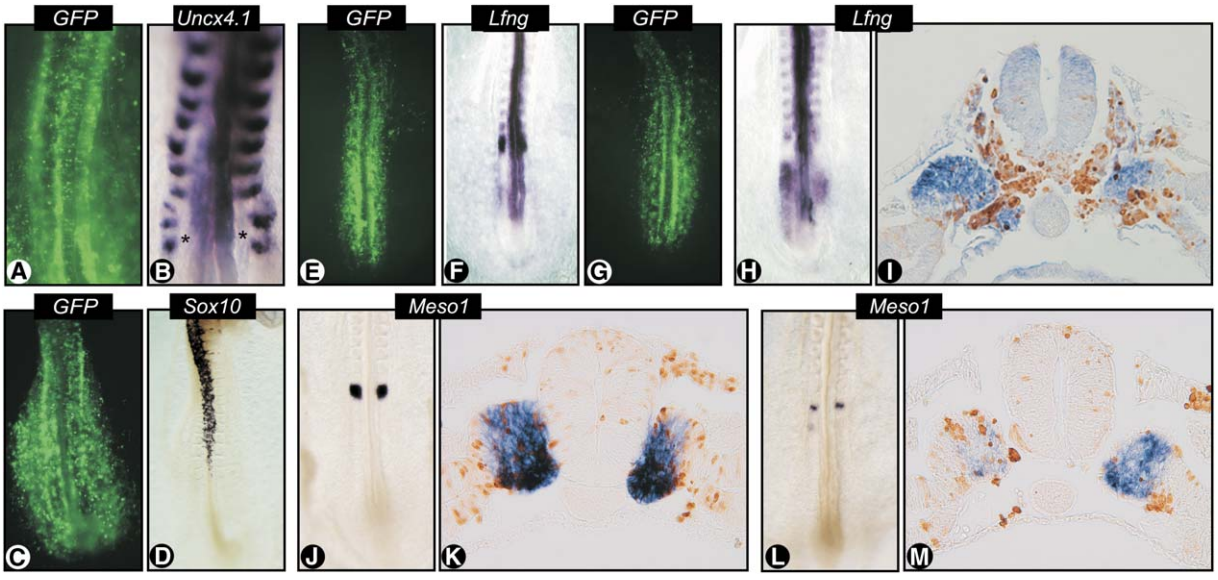

Figure 5. Overexpression of *Snail2* in the Chick Embryo PSM Disrupts Segmentation

(A–H) (A, C, E, and G) Dorsal views showing GFP expression in chick embryos electroporated with the *Snail2-IRES-GFP* construct. (B, D, F, and H) The corresponding right panels show the same embryo after in situ hybridization with (B) *Uncx4.1*, (D) *Sox10*, (F and H) *Lfng* antisense probes.
(I) Transverse section of an embryo electroporated with the *Snail2-IRES-GFP* construct, hybridized with the *Lfng* probe (blue), and labeled with an antibody against GFP (brown).
(J–M) Embryos overexpressing (J and K) a control PCIG-GFP vector or (L and M) the *Snail2-IRES-GFP* construct and hybridized with *Meso1* (in blue) shown in (J and L) dorsal view and in (K and M) transverse section after staining with an anti-GFP antibody (brown label).

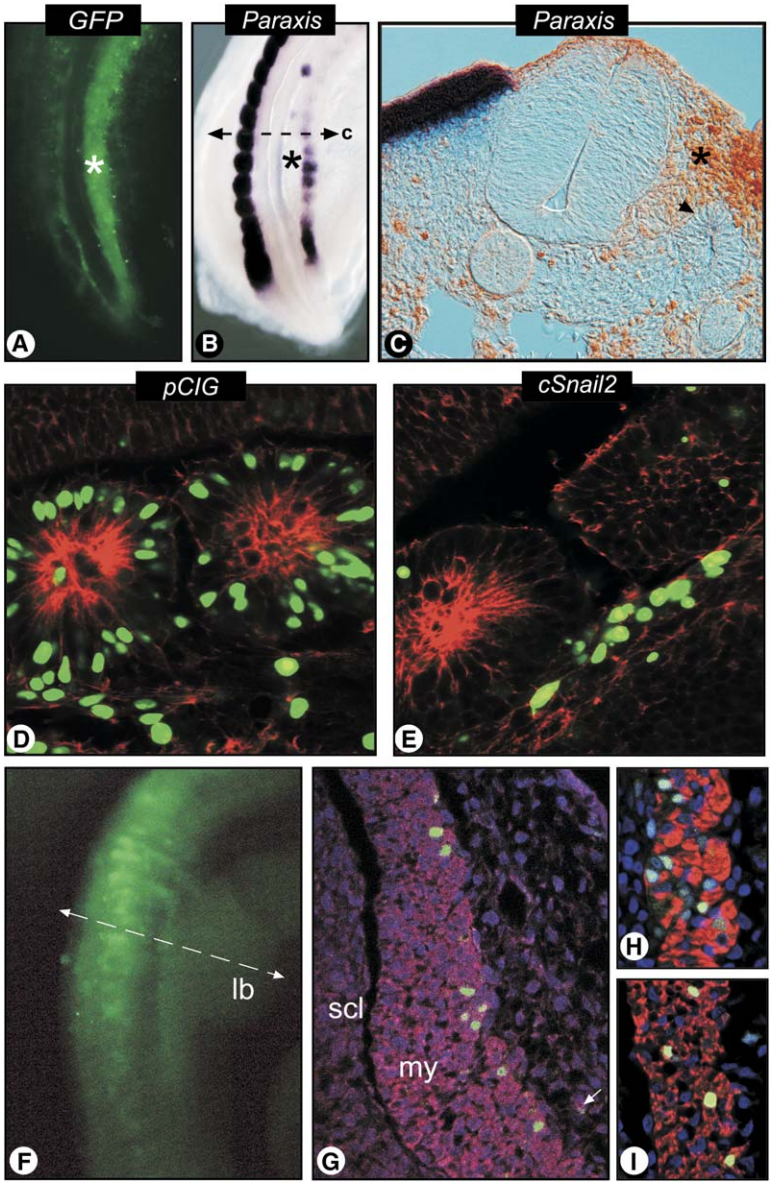

Figure 6. Overexpression of *Snail2* in the Chick Embryo PSM Prevents Epithelialization

(A) GFP expression in chick embryos electroporated with the *Snail2-IRES-GFP* construct.

(B) The corresponding right panel shows the same embryo after in situ hybridization with *Paraxis*. The hatched line shows the level of the section in (C).

(C) Transverse section of the embryo shown in (A) and (B) labeled with an antibody against GFP (in brown). The arrowhead points to the *Paraxis*-negative small dermomyotome.

(D and E) Confocal sections of embryos electroporated with (D) control *pCIG-GFP* or with the (E) *Snail2-IRES-GFP* constructs and phalloidin-AF546, which highlights the actin-rich apical side of somitic cells.

(F) GFP expression in the paraxial mesoderm of an E4 chick embryo electroporated with a *Snail2-IRES-GFP* construct. The hatched line marks the level of the section shown in (G).

(G) Confocal image of a transverse section of an E4 chick embryo electroporated with *Snail2-IRES-GFP* and labeled with an antibody against MF20 (in red) and with DAPI (purple) to evidence the nuclear localization of the GFP. The white arrowhead indicates an isolated positive cell in the dermis.

(H and I) Confocal section showing a higher magnification highlighting the nuclei of GFP-positive cells (green) in the MF20-positive myotome (red) in (H) a *Snail2*-overexpressing E4 embryo and (I) a control E4 embryo.

Lb, limb bud; Scl, sclerotome; My, myotome. Dorsal views; anterior is oriented toward the top in (A), (B), and (F).

between the paraxial mesoderm and lateral plate. However, these cells were found to be negative for expression of *BMP4* and *Pax2*, which, respectively, label the lateral plate or the intermediate mesoderm (data not shown). Thus, overexpressing *Snail2* appears to block segmentation and somite formation in a cell-autonomous manner.

To test whether these segmentation defects are linked to an alteration of the segmentation clock oscillations, we analyzed the effect of *Snail2* electroporation on *Lfng* expression. Following in situ hybridization, embryos that strongly expressed GFP showed the different phases of *Lfng* expression in the PSM (Figures 5E–5H). In a fraction of the cases, the *Lfng* expression domains were offset between the two sides (Figure 5H), but such desynchronizations were also frequently observed in control embryos, suggesting that they rather result from a nonspecific effect of the electroporation on the segmentation clock oscillations. Because electroporation results in mosaic expression of the overexpressed constructs, embryos were sectioned and stained with an anti-GFP antibody to examine the effect of *Snail2* at the cellular level. This revealed that cells expressing *Snail2* cell-autonomously downregulate *Lfng* (Figure 5I; n = 5). Thus, our observations suggest that misexpression of *Snail2* alters the normal expression of *Lfng*.

In the mouse embryo, *Mesp2* was recently shown to play a critical role in the anterior PSM to maintain *Lfng* expression at the level of the future somite boundary (Morimoto et al., 2005). We examined the expression of the chick homolog of *Mesp2*, *Meso1* (Buchberger et al., 1998), in embryos overexpressing *Snail2* in the PSM. In most embryos showing strong GFP expression in the PSM (n = 10), *Meso1* expression was altered, resulting in a smaller and irregular expression domain (compare Figure 5J to Figure 5L). In sections of these embryos, *Meso1* and GFP showed a complementary expression pattern, suggesting that *Snail2* can repress *Meso1* expression (Figures 5K–5M). These results indicate that *Snail2* can block *Lfng* and *Meso1* expression,

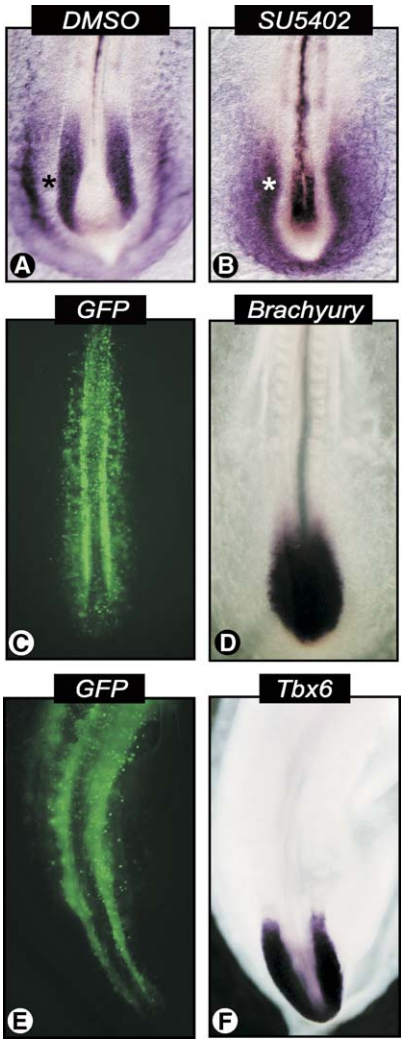

Figure 7. *Snail2* Expression Is Regulated by FGF Signaling

(A and B) Chick embryos treated with (A) DMSO and (B) SU5402 and hybridized with a *Snail2* probe. Asterisks mark the lateral plate.

(C and E) GFP expression in chick embryos electroporated with the *Snail2-IRES-GFP* construct.

(D and F) The corresponding right panel shows the same embryo after in situ hybridization with (D) *Brachyury* and (F) *Tbx6* antisense probes. Dorsal views; anterior is oriented toward the top.

thus preventing overexpressing cells from forming somites.

Snail2 Regulates the Mesenchyme-to-Epithelium Transformation Leading to Somite Formation

The posterior expression domain of mouse *Snail1* and chick *Snail2* correlates with the mesenchymal domain in the PSM, suggesting that these genes might be involved in the control of the mesenchymal state of these cells. To test whether *Snail2* is involved in the control of this transition, we examined the effect of overexpressing *Snail2* on PSM epithelialization. To that end, we analyzed the effect on the expression of *Paraxis*, a transcription factor whose expression is associated with the onset of epithelialization in the anterior PSM and is subsequently expressed in the epithelial somites and dermomyotomes (Burgess et al., 1995). In chick embryos strongly overexpressing *Snail2*, *Paraxis* was found to be absent from the GFP-positive regions of the anterior PSM and somites (Figures 6A and 6B; n = 9). The strongly expressing unsegmented domains lying between the neural tube and somites were often flanked by a row of smaller somites composed of GFP-negative cells (Figures 6A and 6B). In sections, expression of *Snail2* was clearly associated with a loss of the epithelial structures in the somite or the dermomyotome (asterisk, Figure 6C), and *Paraxis* was found to be downregulated in a cell-autonomous fashion in these domains (Figure 6C and data not shown). In some cases, *Paraxis* was also found to be downregulated in the remaining small dermomyotome, which, nonetheless, does not express *Snail2* (arrowhead, Figure 6C). This non-cell-autonomous downregulation is probably caused by the physical separation of the dermomyotome from the overlying ectoderm by the *Snail2*-overexpressing cells, since it has been reported that *Paraxis* expression in the dermomyotome depends upon close contact with the ectoderm (Sosic et al., 1997). Furthermore, confocal analysis of electroporated embryos clearly showed that GFP-positive cells overexpressing *Snail2* were largely excluded from epithelial somites (Figures 6D and 6E). We counted the GFP-positive cells in the somitic region of the paraxial mesoderm in control embryos; 135 GFP-positive cells were observed in 5 somites in 2 control embryos, whereas no cell was seen outside these somites in the paraxial mesoderm region. In 2 embryos overexpressing *Snail2*, 27 GFP-positive cells were counted in 5 somites, while 128 cells were found to lie adjacent to these somites in the paraxial mesoderm region. Therefore, ectopic activation of *Snail2* can block the incorporation of paraxial mesoderm cells in epithelial somites.

We then examined the fate of the *Snail2*-overexpressing cells compared to control GFP-overexpressing cells in E4 embryos (Figure 6F; n = 4 experimentals and n = 4 controls). A difficulty with this experiment is that the plasmids electroporated become diluted in tissues that are actively dividing, like the sclerotome or the dermis. In contrast, because the muscle fibers are postmitotic, a bright GFP signal is retained in the nuclei even at late stages like E4 (Figure 6G). Therefore, we have compared the number of GFP-positive cells per myotome in sections of E4 embryos overexpressing *Snail2* (Figure 6H) and overexpressing the control GFP construct (Figure 6I). We found an average of 11.9 GFP-positive cells/myotome (143 cells on 12 sections from 3 control embryos) in control embryos and 12.2 GFP-positive cells/myotome (183 cells on 15 sections from 4 experimental embryos) in the *Snail2*-overexpressing embryos. Therefore, a similar contribution to the myotome is observed for control and *Snail2*-overexpressing embryos, indicating that *Snail2*-overexpressing cells can differentiate into paraxial mesoderm derivatives.

FGF Signaling Is Necessary, but Not Sufficient, for *Snail2* Expression in the PSM

In the PSM, the EMT is controlled by the level of FGF signaling, and the epithelialization process begins once the cells enter the anterior region characterized by lower FGF signaling (Delfini et al., 2005; Dubrulle et al., 2001). Our data suggest that this transition could be controlled

by *Snail* genes acting downstream of FGF. Consistently, *Snail1* expression is lost in the PSM of mouse mutants for FGFR1 (Ciruna and Rossant, 2001). To test whether *Snail2* is regulated in a similar way in the chick embryo, we treated chick embryo explants for 4 hr in culture with 100 μm SU5402, which blocks the activity of FGFR1 (Mohammadi et al., 1997). In treated embryos, *Snail2* was found to be downregulated in the PSM, whereas it was upregulated in the neural tube and the lateral plate (Figures 7A and 7B; n = 10). Therefore, FGF signaling is required for *Snail2* expression in the PSM, whereas this pathway represses its expression in the neural tube and lateral plate.

The cell-autonomous blockade of segmentation observed when overexpressing *Snail2* is reminiscent of that seen when overactivating the ERK pathway that acts downstream of FGF signaling in the PSM (Delfini et al., 2005). In these experiments, electroporation of a constitutively active form of the MEKK1 kinase, ca-MEKK1, results in an ectopic activation of *Brachyury* in the overexpressing cells of the anterior PSM and somites. Since *Snail* genes act downstream of FGF signaling in the PSM, we investigated whether *Snail2* in the chick may be acting as the transcriptional effector of the FGF response. We therefore examined the effect of misexpressing *Snail2* on the expression of *Brachyury*, whose expression is associated with posterior PSM identity (Ciruna and Rossant, 2001; Delfini et al., 2005; Dubrulle et al., 2001). Unlike in FGF8 or activated MEKK1-overexpressing embryos, no ectopic anterior expression of *Brachyury* was observed in *Snail2*-overexpressing embryos (Figures 7C and 7D; n = 8). Therefore, *Snail2* and *Brachyury* appear to be regulated independently by FGF signaling. To further investigate if this reflects an impairment of maturation of the paraxial mesoderm cells, we also examined the expression of *Tbx6*, a T box factor expressed specifically in the PSM (Knezevic et al., 1997). No expression of *Tbx6* could be detected in cells overexpressing *Snail2* in the somitic region (Figures 7E and 7F; n = 6). These results indicate that whereas PSM cells overexpressing *Snail2* fail to epithelialize and segment, they do not maintain a PSM identity.

To test whether ectopic FGF signaling in the PSM results in *Snail2* expression, we overexpressed FGF8 by electroporation. Whereas somite formation was blocked, as previously reported (Dubrulle et al., 2001), no ectopic expression of *Snail2* was observed in the paraxial mesoderm of FGF8-overexpressing embryos (data not shown). Together, these experiments suggest that FGF signaling is necessary, but not sufficient, for *Snail2* expression in the chick PSM.

Discussion

Here, we show that *Snail1* and *Snail2* may identify a novel class of cyclic genes showing periodic expression in the mouse and chick PSM, respectively. We observe that while *Snail1* expression dynamics show some similarities with those of the NOTCH pathway-related cyclic genes like *Lfng*, they also exhibit clear differences, particularly at the tail bud level. Thus, *Snail1* transcription is initiated prior to that of the NOTCH cyclic genes at the onset of a new wave of expression in the posterior PSM. Interfering with the NOTCH pathway does not alter the dynamic expression profile of the *Snail* genes, suggesting that their regulation is independent of NOTCH signaling. In contrast, *Snail1* expression is lost in the *Wnt3a* hypomorph mutant *vt*, thus confirming the important role of this pathway upstream of the segmentation clock mechanism. In the chick embryo PSM, overexpressing the *Snail2* gene downregulates the expression of *Lfng* and *Meso1* and results in a block of segmentation. Such gain of function ultimately prevents targeted cells from activating *Paraxis*, thereby blocking them in a mesenchymal state. We show that FGF signaling is required for maintenance of *Snail2* expression in the PSM. While the *Snail2* overexpression phenotype in the chick embryo strongly resembles that observed after the ectopic activation of the FGF pathway in the PSM, no ectopic expression of genes associated with the posterior PSM identity is seen upon *Snail2* misexpression. Together, these data suggest that *Snail* genes link the segmentation clock to PSM morphogenesis downstream of the FGF pathway.

Snail Genes May Define a New Class of Cyclic Genes

Here, we show that the mouse *Snail1* gene (formerly *Snail*) and the chick *Snail2* gene (formerly *Slug*) oscillate in the PSM with very similar expression kinetics in the two species (Figure 1). Such an inversion of expression and function between *Snail1* and *Snail2* in mouse and chick is not unexpected since it has been observed in most embryonic tissues, including neural crest and mesodermal derivatives (Locascio et al., 2002; Sefton et al., 1998). In heart formation, *Snail1* was shown to act downstream of NOTCH to control the EMT leading to the development of cardiac valvular primordia (Timmerman et al., 2004). In contrast, in mouse and chick PSM, our data suggest that expression of *Snail* genes is not regulated by the NOTCH pathway. This is in sharp contrast to the NOTCH pathway-related cyclic genes, such as *Lfng*, which are regulated by NOTCH signaling in the PSM (Dale et al., 2003). Also, expression of the *Snail* genes was found to be downstream of FGF signaling in the PSM (Figures 7A and 7B) (Ciruna and Rossant, 2001); however, expression of the NOTCH pathway-related genes, like *Lfng* or *Hairy/Hes*, was not affected by SU5402 treatment (Delfini et al., 2005; Dubrulle et al., 2001). *Snail1* has been shown to control EMTs in the mouse paraxial mesoderm (Ciruna and Rossant, 2001), and our overexpression data in the chick support the notion that *Snail2* plays a similar role in the chick PSM. Together, this data argue that *Snail1* and *Snail2* genes are functional homologs in the PSM of mouse and chick embryos, respectively.

The mouse *Snail1* homozygous null mutant does not form paraxial mesoderm, thus precluding analysis of the role of this gene in the segmentation clock function (Carver et al., 2001). We show that, in the chick PSM, overexpressing *Snail2* can repress *Lfng* expression, consistent with the well-known repressor function of *Snail* family genes (Nieto, 2002). SNAIL1 was shown to bind E boxes in the E-CADHERIN promoter (Batlle et al., 2000), and several such sites are found in the *Lfng* promoter, in agreement with the idea that *Snail* genes might directly regulate *Lfng* (Cole et al., 2002; Morales et al., 2002). Strikingly, however, *Snail1* and *Lfng*

are coexpressed for a portion of the oscillation cycle, although *Snail1* transcription continues in the posterior PSM in embryos that have downregulated *Lfng* (Figures 2C and 2D). This could suggest that accumulation of SNAIL1 protein during one oscillation cycle is required for repressing *Lfng* expression in the posterior PSM, or that *Lfng* repression by *Snail1* is indirect. Therefore, despite their coexpression for a significant portion of the oscillation cycle, our data indicate that *Snail* genes are regulated differently from the NOTCH pathway-related cyclic genes.

The link between WNT and NOTCH signaling remains one of the central unsolved mechanisms underlying both the generation and regulation of the segmentation clock. Here, we show that *Snail1* transcription is downstream of WNT3A, but not of NOTCH, signaling. Interestingly, however, *Snail1* oscillations were out of phase when compared to *Axin2*, suggesting that whereas both genes are regulated by WNT signaling, the modality of this regulation might be different. *Snail1* transcription was recently shown to be inhibited by GSK3β activation (Bachelder et al., 2005). The WNT pathway oscillations reported in the PSM (Aulehla et al., 2003) are expected to result in a periodic inactivation of GSK3β that would, in turn, lift the inhibition of *Snail1* transcription and, together with FGF signaling, would lead to periodic *Snail1* activation in the PSM. The periodic production of the SNAIL1 protein downstream of the WNT oscillations could trigger the periodic repression of NOTCH targets. The SNAIL1 protein is highly unstable and is targeted for nuclear export and proteasome-mediated degradation by means of GSK3β-mediated phosphorylation (Zhou et al., 2004). Thus, its effect on NOTCH signaling should be transient and periodic. *Snail1* oscillations might therefore provide a link between the WNT and NOTCH signaling pathways in the segmentation clock.

Snail Genes Coordinate PSM Morphogenesis and Segmentation

In the mouse embryo, cells of the primitive streak that receive high FGF signaling activate *Snail1* expression, which, in turn, represses E-CADHERIN transcription, thus allowing the EMT leading to the formation of mesodermal tissues such as the posterior PSM (Batlle et al., 2000; Ciruna and Rossant, 2001). The downregulation of E-CADHERIN expression was proposed to release a pool of β-CATENIN from the cell membrane, thus allowing these cells to respond to WNT signaling (Ciruna and Rossant, 2001). The most prominent *Snail1* and *Snail2* expression domains in the mouse and chick PSM extend from the primitive streak/tail bud to the level of the determination front and therefore nicely correlate with the mesenchymal area of the PSM (Figure 1) (Sefton et al., 1998). In chick and mouse embryos, E-CADHERIN is specifically excluded from the PSM, where the *Snail* genes are predominantly expressed (Cano et al., 2000; Thiery et al., 1984). The *Snail* genes' expression domain within the posterior PSM also correlates with the region where the oscillations of the WNT cyclic gene *Axin2* occur (Aulehla et al., 2003). Inhibition of cadherin levels, mediated by *Snail* genes and associated with the maintenance of the mesenchymal state of the PSM, might be required to ensure that a sufficient β-CATENIN pool is available for the WNT-driven oscillations underlying the segmentation clock. At the determination front, the downregulation of FGF and Wnt signaling (Aulehla et al., 2003) would result in the arrest of oscillating *Snail* expression. Thus, the repression of the epithelial phenotype is relieved at this level, and cadherin expression levels begin to increase with the progressive epithelialization of the anterior PSM (Duband et al., 1987; Linask et al., 1998). The arrest of clock gene oscillations at the determination front might therefore be linked to the reexpression of cadherins and the consequential reduced availability of β-CATENIN due to its sequestration at the membrane by cadherins. Such crosstalk between cadherins, WNT, and FGF signaling, resulting in the control of β-CATENIN levels, has been reported in several systems (Nelson and Nusse, 2004). The *Snail* genes would thus act as competence factors for the segmentation clock. Because they act downstream of FGF and Wnt signaling, these genes would provide a means of temporally integrating the clock mechanism with progression of the wavefront.

The anterior limit of the posterior expression domain of *Snail* genes in the PSM roughly corresponds to the position of the determination front. This is the level at which periodic NOTCH signaling controlled by the segmentation clock triggers the segmental activation of downstream targets such as the *Mesp* family genes (Jen et al., 1999; Morimoto et al., 2005; Takahashi et al., 2000). Here, we show that overexpression of *Snail2* in the chick PSM, prevents expression of the *Mesp2* homolog, *Meso1* (Figures 5J–5M) (Buchberger et al., 1998). This suggests that *Snail* genes might repress expression of *Mesp2*/*Meso1* in most of the PSM, thus restricting expression of these genes to the narrow *Snail*-negative stripe in the anterior PSM. Such a role in positioning the NOTCH response has been previously reported in the fly, where *Snail* was proposed to restrict NOTCH signaling at the future midline by a combination of cell-autonomous and non-cell-autonomous repressing and stimulating activities (Cowden and Levine, 2002; Morel et al., 2003).

Snail2 overexpression in the chick results in a cell-autonomous blockade of the somitogenesis process accompanied by a downregulation of *Paraxis* and *Uncx4.1* expression in the anterior PSM and somites. This phenotype is very similar to that observed after either constitutive activation of the ERK pathway via misexpression of a constitutively active form of the MEKK1 kinase in the PSM (Delfini et al., 2005) or overexpression of FGF8 in the PSM (Dubrulle et al., 2001). However, whereas FGF8 and caMEKK1 also ectopically activate expression of posterior PSM markers like *Brachyury* in the anterior PSM, this phenotype was not observed when overexpressing *Snail2*. This result is consistent with the homozygous null mutation of *Snail1* in the mouse in which *Brachyury*-positive mesoderm forms but retains a polarized epithelial character expressing E-CADHERIN (Carver et al., 2001). Overexpressing FGF8 in the PSM also blocks the subsequent differentiation of paraxial mesoderm cells (Dubrulle et al., 2001). In contrast, *Snail2*-overexpressing cells differentiate into muscle, dermis, and sclerotome, suggesting that while patterning is disrupted, paraxial mesoderm differentiation is not affected. These results are consistent with the phenotype of the *Paraxis* null mutant in which the paraxial

mesoderm does not form epithelial somites, but differentiates into normal myotomes (Burgess et al., 1996). Therefore, this suggests that *Snail* does not control the maturation of PSM cells, but rather controls the morphogenesis along this tissue. Together, our results suggest that *Snail* genes play a dual role in the segmentation process: first, they play a role at the level of the segmentation clock by integrating the NOTCH, WNT, and FGF pathways, and, second, they play a role by controlling the morphogenetic process associated with epithelial somite formation.

Experimental Procedures

Embryos

Fertilized chick eggs were obtained from Ozark Hatcheries (Neosho, MO) and incubated at 38ºC in a humidified incubator. Embryos were staged according to the developmental table of Hamburger and Hamilton (HH) (Hamburger and Hamilton, 1992) and by counting somite pairs.

Wild-type CD1 mice embryos were harvested from timed mated pregnant females between 8.5 and 10 days postcoitum (dpc). *Notch1*$^{-/-}$, *Lfng*$^{-/-}$, *Rbpjk*$^{-/-}$, *Hes7*$^{-/-}$, *Wnt5a*$^{-/-}$, and *vt/vt* embryos were obtained and genotyped by PCR analysis of the yolk sacs as described (Aulehla et al., 2003; Bessho et al., 2003; Conlon et al., 1995; Oka et al., 1995; Yamaguchi et al., 1999; Zhang and Gridley, 1998).

Mouse and Chick Embryo Explant Culture

E9.5 CD1 mice embryos were harvested, and their posterior part was divided into two halves by cutting along the neural tube. The explants were cultured in hanging drops of culture medium composed of DMEM/F12 supplemented with 10% fetal calf serum, 10 ng/ml bFGF, and 50 U/ml penicillin/streptomycin. Experiments were performed as described by Palmeirim et al. (1997). Half of the explant was immediately fixed, and the other half was cultured for 75–120 min prior to fixation. The two explants were then analyzed for expression of *Snail1* mRNA by in situ hybridization. Alternatively, the two halves of the explant were fixed immediately and were used to compare the expression domains of *Snail1* and *Lfng* or *Snail1* and *Axin2* or exonic *Snail1* and intronic *Snail1* by in situ hybridization. N-(N-(3,5-difluorophenacetyl)-L-alanyl)-S-phenylglycine t-butyl ester, DAPT (Calbiochem), was diluted into culture medium from stock solutions in dimethylsulphoxide (DMSO) to maintain a final DMSO concentration of 1%. Half-embryo assays were performed as described above. One half was cultured in medium containing DAPT (10 μM), whereas the control side was cultured in normal medium. Both sides were cultured for 2 hr and then analyzed for expression of *Lfng* mRNA or *Snail1* mRNA by in situ hybridization. Chick embryo explants were cultured in vitro as described in Delfini et al. (2005). Posterior half-embryo explants were cultured for 4 hr in a chick culture medium (5% chick serum, 2.5% FCS, and 1% bicarbonate in DMEM or L-15, GIBCO-BRL) containing 100 μM of the FGFR inhibitor SU5402 (Pfizer) dissolved in DMSO. Explants were then fixed and processed for in situ hybridization.

Plasmids and In Ovo Electroporation

In ovo electroporations were performed as described (Dubrulle et al., 2001). Eggs were windowed, and the DNA solution was injected between the vitelline membrane and the epiblast in stage-4 to stage-5 HH embryos. The primitive streak was coated with the DNA solution from the node until mid-streak. Two platinum electrodes tied together were used: one was placed directly on the streak posterior to the node, and the second was inserted into the yolk. A series of electric pulses (4 pulses, 30 volts, 50 ms) was directed with a square wave electroporator (BTX). For electroporations, constructs were cloned into the *pCIG* (Megason and McMahon, 2002) or *pCAGGS* (Niwa et al., 1991) expression vectors, which were purified with an endotoxin-free maxi kit (Qiagen) and used at a final concentration of 2 mg/ml or 1 mg/ml, respectively, in a PBS solution containing 1 mM MgCl2 and 0.4 mg/ml fast green FCF (Sigma). *pCIG* is a bicistronic vector that drives the expression of a nuclear GFP reporter in addition to the gene of interest (Megason and McMahon, 2002). Full-length *Snail2* was cloned into the *pCIG* vector. The FGF8, activated NOTCH, and LFNG expression constructs have been previously described (Dale et al., 2003; Dubrulle et al., 2001). Control embryos were either electroporated with empty *pCIG* or coelectroporated with *pCAAGS-GFP* and empty *pCAAGS*. After electroporation, eggs were reincubated for 24, 40, 48, or 96 (for E4 embryos) hr and were assayed for GFP expression. Embryos were then fixed and processed for in situ hybridization or incubated overnight with phalloidin AF546 (Molecular Probe, 1:100) and analyzed by confocal microscopy.

Whole-Mount In Situ Hybridization and Immunohistochemistry on Section

Whole-mount in situ hybridizations were performed as described (Henrique et al., 1995). The chick *Snail2*, *Brachyury*, *Tbx6*, *Lfng*, *Uncx4.1*, *Paraxis*, and *Sox10* (Dale et al., 2003; Knezevic et al., 1997; Sefton et al., 1998; Sosic et al., 1997; Southard-Smith et al., 1998) and the mouse *Lfng* and *Axin2* (Aulehla et al., 2003) probes have been described. The intronic *Snail1* probe was produced by polymerase chain reaction (PCR) amplification of a fragment corresponding to nucleotides 1004–1553 of the mouse *Snail1* genomic sequence (GenBank accession code NT 039210). Some of the labeled embryos were embedded for cryosection in gelatin-sucrose and cut at 14 μm. For some embryos, 10 μm paraffin sections were also prepared. GFP was detected on section with an anti-GFP mAb (Roche # 1814460, 1:200) by diaminobenzidine (DAB) or fluorescence staining. Differentiated muscles were evidenced in section by using the anti-Myosin Heavy Chain MF20 mAb detected by fluorescence. Sections were counterstained with DAPI staining to visualize nuclei.

Supplemental Data

Supplemental Data including Figure S1 are available at http://www.developmentalcell.com/cgi/content/full/10/3/355/DC1/.

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