

# Sonic hedgehog in temporal control of somite formation

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**Vertebrate embryo somite formation is temporally controlled by the cyclic expression of somitogenesis clock genes in the presomitic mesoderm (PSM). The somitogenesis clock is believed to be an intrinsic property of this tissue, operating independently of embryonic midline structures and the signaling molecules produced therein, namely Sonic hedgehog (Shh). This work revisits the notochord signaling contribution to temporal control of PSM segmentation by assessing the rate and number of somites formed and somitogenesis molecular clock gene expression oscillations upon notochord ablation. The absence of the notochord causes a delay in somite formation, accompanied by an increase in the period of molecular clock oscillations. Shh is the notochord-derived signal responsible for this effect, as these alterations are recapitulated by Shh signaling inhibitors and rescued by an external Shh supply. We have characterized chick *smoothed* expression pattern and have found that the PSM expresses both *patched1* and *smoothed* Shh signal transducers. Upon notochord ablation, *patched1*, *gli1*, and *fgf8* are down-regulated, whereas *gli2* and *gli3* are overexpressed. Strikingly, notochord-deprived PSM segmentation rate recovers over time, concomitant with *raldh2* overexpression. Accordingly, exogenous RA supplement rescues notochord ablation effects on somite formation. A model is presented in which Shh and RA pathways converge to inhibit PSM Gli activity, ensuring timely somite formation. Altogether, our data provide evidence that a balance between different pathways ensures the robustness of timely somite formation and that notochord-derived Shh is a component of the molecular network regulating the pace of the somitogenesis clock.**

somitogenesis | molecular clock | notochord

**V**ertebrate body segmented organization relies on the progressive formation of early embryonic vertebrae precursor structures—somites—flanking bilaterally the axial organs, notochord, and neural tube. Somitogenesis is a reiterated process occurring over time with strict periodicity. In the trunk of the chicken embryo, a pair of somites is formed every 90 min from the anterior tip of presomitic mesoderm (PSM). As development proceeds, ventral somitic cells migrate around the axial organs, giving rise to segmented structures such as vertebrae, intervertebral disks, and ribs, whereas more dorsal somitic cells give rise to the dermis of the back and all of the striated muscles of the adult body (1). Underlying somitogenesis periodicity is a molecular clock first evidenced by the cyclic expression of *hairyl* in chick PSM cells with 90-min periodicity, corresponding to the time of somite formation (2). Currently, multiple genes belonging to Notch, Wnt, and Fgf signaling pathways are known to have similar oscillatory behavior and to participate in the embryo segmentation clock (1, 3). Posterior PSM cells in an undetermined state are influenced by high FGF/Wnt levels, which fade away anteriorly, counteracted by an opposing gradient of RA (4–6). A determination front is established in the intersection of these opposite gradients at the level of

somites –III to –IV, and cells located anteriorly to this front are determined to form somites (6).

Packard et al. described somite formation to be independent of the neural tube and notochord (7, 8) and today this is widely accepted. The somitogenesis clock is also believed to operate independently of these axial structures (2). However, there is an intriguing report showing that when a quail PSM is grafted into a chicken embryo, the grafted tissue progressively adjusts somite formation to that of the host contralateral part (9). This is particularly evident in the somites formed by cells that were located posterior to the donor determination front when the graft was performed, strongly suggesting that undetermined PSM requires a signal coming from the embryo midline structures for subsequent somite formation. Notochord and neural tube floor plate produce Sonic hedgehog (Shh), a morphogen central to growth, patterning, and morphogenesis (10), implicated in somite sclerotome induction (11) and in survival of medial somitic cells (12, 13). These functions have been described to account for the lack of spinal column observed in Shh KO mice (14). However, it is commonly believed that Shh signaling does not play a role in PSM segmentation or in the somitogenesis clock.

In the present work, we evaluated the notochord and Shh signaling contribution to PSM segmentation preceding somite formation. We show that notochord ablation delays somite formation and molecular clock oscillations, and that Shh is the notochord-derived signal regulating these events. Furthermore, our results show that the delay in somite formation in notochord-deprived PSMs is overcome in time, mediated by RA signaling. Altogether, our data identify embryo midline-derived Shh as a component of the molecular network regulating the pace of the somitogenesis clock and reveal a cross-talk with the RA pathway to ensure the robustness of somite formation.

## Results

**Timely Somite Segmentation of Undetermined PSM Tissue Requires Presence of Notochord.** To evaluate the role of the notochord in PSM segmentation, the posterior part of chicken embryos was cut along the embryo midline, generating two embryo explants (Fig. 1A): a control explant containing half of the neural tube, the floor plate, and the notochord (No<sup>+</sup>), and an experimental explant with half of the neural tube but without the floor plate and notochord (No<sup>-</sup>). Explant pairs were cultured separately for different time periods (4.5, 7.5, 9, and 12 h), and the number of somites formed in culture was assessed (Fig. 1B and C). The

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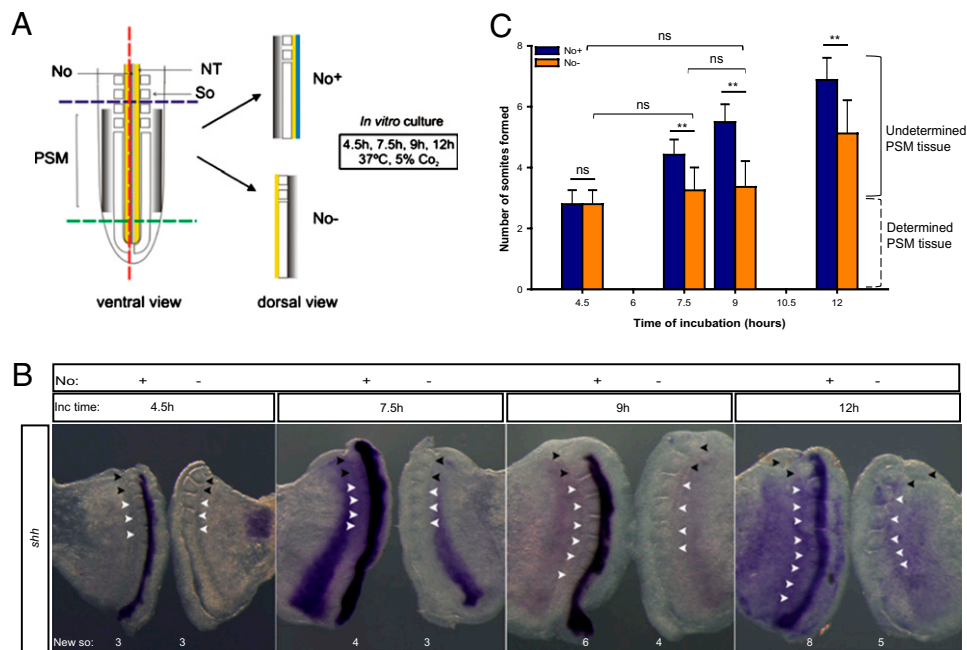
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**Fig. 1.** Notochord removal impairs timely somite segmentation of the undetermined PSM in vitro. (A) Schematic representation of explant delimitation and excision. No, notochord; NT, neural tube; So, somite; PSM, presomitic mesoderm. (B) Evidence for the presence (No<sup>+</sup>)/absence (No<sup>-</sup>) of the notochord in control and experimental explants, by in situ hybridization for *shh*. Black arrowheads indicate somites formed before culture; white arrowheads indicate somites formed during incubation (New so). (C) Graphic representation of mean number of somites formed de novo upon 4.5, 7.5, 9, or 12 h of incubation. Data are mean  $\pm$  SD. \*\* $P < 0.05$ . ns, not statistically significant.

presence or absence of notochord on the control and experimental explants was randomly confirmed by in situ hybridization using a *sonic hedgehog* (*shh*) antisense RNA probe (Fig. 1B).

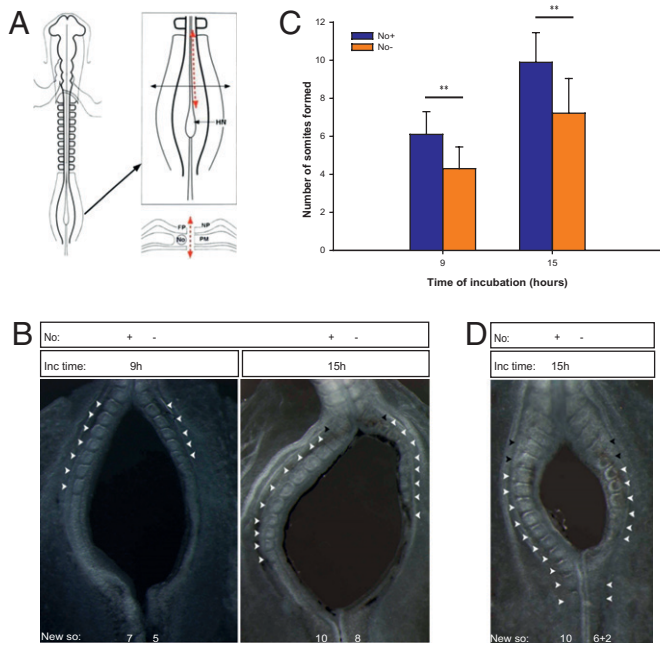
Explants incubated for 4.5 h formed three somites at the expected rate of one somite/90 min, even in the absence of the notochord ( $n = 68$ ) (Fig. 1B and C). Surprisingly, for greater incubation periods of 7.5 h ( $n = 33$ ) and 9 h ( $n = 100$ ), the PSM tissue did not segment to the same extent in the absence of notochord as in control explants (Fig. 1B and C). In fact, somite formation considerably slowed down from 4.5 h to 9 h of incubation. Both PSM cell apoptosis and proliferation levels were assessed, and showed that the results obtained were not caused by alterations in either of these cellular processes (Fig. S1). Strikingly, from 9 h to 12 h ( $n = 25$ ), an average of two new somites was formed even in the absence of the notochord, suggesting a recovery of the system (Fig. 1B and C). Our experimental approach was reproduced in ovo; upon 9 h ( $n = 28$ ) or 15 h ( $n = 24$ ) of reincubation, the results obtained recapitulate those observed in explant cultures: the side without notochord consistently formed less somites than the control part (Fig. 2 and Movie S1). Moreover, somite formation rate is recovered over time and, accordingly, a difference of two somites in control and experimental sides is maintained after 9, 12, or 15 h of incubation (Figs. 1C and 2C). In some embryos the size of the slit allowed to observe a delay in somite segmentation in the PSM facing the incision, whereas timely bilateral somite formation occurred caudal to the slit, leaving a rostral gap of unsegmented tissue with approximately two somites length (Fig. 2D). Because somite formation periodicity in the absence of the notochord is already reestablished at 15 h (Figs. 1C and 2C), No<sup>-</sup> tissue will undergo further segmentation in a timely fashion, concomitantly with the generation of new somites caudal to the slit. Accordingly, after a 24 h incubation, both control and experimental PSMs are completely segmented indicating that notochord deprivation does not impair, but transiently delays somite formation (Fig. S2A and B).

Altogether, we show that the undetermined PSM tissue depends on the presence of the notochord for timely somite segmentation. Moreover, as long as the PSM cells retain contact with the notochord, they preserve autonomous correct temporal information.

PSM rostral to the determination front no longer requires the notochord to segment at normal rate.

**Notochord Ablation Alters Clock and Determination Front Gene Expression.** Because notochord ablation affected temporal control of morphological somite formation, we evaluated the expression of genes involved in the somitogenesis clock and determination front establishment. Explants such as those previously described (No<sup>+</sup> and No<sup>-</sup>) were incubated for 4.5 or 9 h and hybridized with probes for the clock genes *hairy2* and *lunatic fringe* (*lfng*), and determination front genes *fgf8* and *raldh2* (Fig. 3). Molecular clock gene expression was significantly altered in the absence of the notochord both after 4.5 h (*lfng*,  $n = 4/4$ ; *hairy2*,  $n = 8/11$ ) and 9 h (*lfng*,  $n = 19/22$ ; *hairy2*,  $n = 15/18$ ) (Fig. 3). The same could be observed upon in ovo microsurgery (Fig. S2C). Furthermore, in notochord-deprived explants the expression domains of both *fgf8* (Fig. 3) and its downstream target *sprouty1* (Fig. S3) were shifted caudally. However, this was not the primary cause for somite formation delay, because FGF8 supplementation did not rescue somite formation in No<sup>-</sup> explants and inhibition of FGF signaling in No<sup>+</sup> explants did not mimic Shh deprivation (Fig. S4). *Raldh2* expression was unaltered by notochord ablation upon 4.5 h of incubation ( $n = 9/10$ ) but was highly overexpressed after 9 h ( $n = 4/5$ ) (Fig. 3). The expression of *meis2*, a RA downstream target, further confirms these results and was already slightly up-regulated after 4.5 h (Fig. S3). As a control, two equivalent No<sup>+</sup> explants were generated by splitting the notochord in half, and no alterations in segmentation rate or gene expression were observed (Fig. S5). In conclusion, somite formation delay in the absence of the notochord is accompanied by alterations in both molecular clock and determination front gene expression.

**Ectopic Shh Replaces Notochord in Temporal Control of Gene Expression and Somite Formation.** *Shh* is one of the most well-studied signaling molecules produced by the notochord. Shh-producing (QT6-Shh) or control (QT6-ctrl) QT6 cells were juxtaposed to No<sup>-</sup> explants which were cultured for 9 h in parallel to their No<sup>+</sup> counterpart (Fig. 4A). No<sup>-</sup>/QT6-ctrl ( $n = 43$ ) failed to form the expected number of somites and presented gene expression perturbations, similarly to what had been previously observed for the No<sup>-</sup>



**Fig. 2.** Notochord ablation in ovo delays somite segmentation of the undetermined PSM. (A) Schematic representation of embryo microsurgical operation. Only one of the embryo's PSMs (PM) was left in contact with the notochord (No). HN, Hensen's node; FP, floor plate; NP, neural plate. (B) Representative results obtained in operated embryos after a reincubation period of 9 or 15 h. (C) Graphic representation of the mean number of somites formed after 9 or 15 h. Data are mean  $\pm$  SD.  $**P < 0.001$ . (D) Operated embryo reincubated for 15 h where a gap in somite formation is observed, resulting from the absence of notochord (No<sup>-</sup>) in the PSM tissue juxtaposed to the surgical slit, whereas reestablished contact of the posterior PSM with the notochord reinstates timely somite formation. Presence (No<sup>+</sup>) or absence (No<sup>-</sup>) of notochord is indicated. Black arrowheads indicate somites formed before the surgical intervention; white arrowheads indicate somites formed during reincubation (New so).

explants (Fig. 4 B and C). Notably, No<sup>-</sup>/QT6-Shh explants and No<sup>+</sup> controls presented the same number of somites and comparable expression patterns for the molecular clock genes *hairy2* ( $n = 9/14$ ) and *lfng* ( $n = 9/12$ ) and for the determination front genes *fgf8* ( $n = 4/6$ ) and *raldh2* ( $n = 3/3$ ) (Fig. 4 B and C). Altogether, the results indicate that Shh is able to replace the notochord in control of timely somite segmentation and underlying gene expression dynamics.

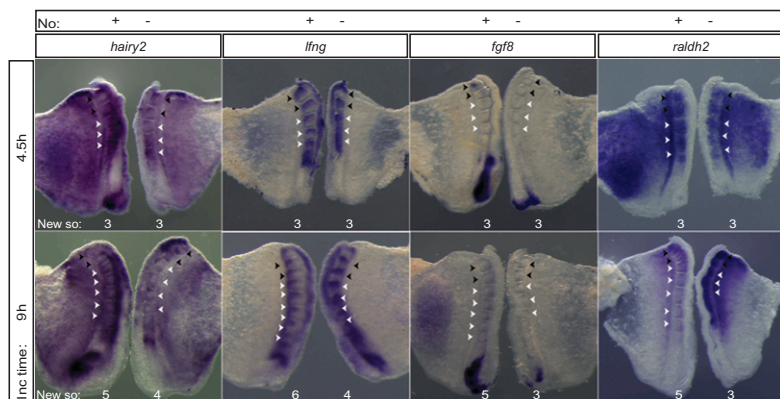
**Shh Signaling Controls Clock Pace and Somite Formation.** Shh signaling is transduced by a receptor complex that includes two transmembrane proteins, Patched (Ptc) and Smoothed (Smo). Shh association with Ptc allows Smo activity, whereas in the

absence of Shh, Ptc binds and inhibits Smo. Among the many target genes regulated by Shh signaling are *ptch* and *gli* signal transducers (15). To investigate the Shh pathway components operating in somite segmentation, we characterized the expression patterns of *smo* and *ptch1/2* (Fig. S6). We found that notochord-adjacent PSM cells express both *smo* and *ptch1/2*, enabling them to respond to notochord-derived Shh.

We performed chemical inhibition of Shh pathway in No<sup>+</sup> explants and the results obtained mimicked the absence of notochord, leading to a similar delay in somite formation (Fig. 5, A–C). Furthermore, the expression patterns of molecular clock genes are different in control and Shh-inhibited explants (Fig. 5B). Shh inhibition, however, does not halt molecular clock oscillations: cyclopamine-treated explants incubated for time periods differing by 30 min (6 h and 6.5 h) presented dissimilar phases of molecular clock gene expression (Fig. 5D). In accordance to the observed delay in somite formation, Shh-inhibited explants incubated for time periods differing by 90 min (6 h and 7.5 h) differed in the phases of molecular clock gene expression (Fig. 5E). Taken together, these results indicate that, in the absence of Shh signaling, the period of the clock is no longer 90 min.

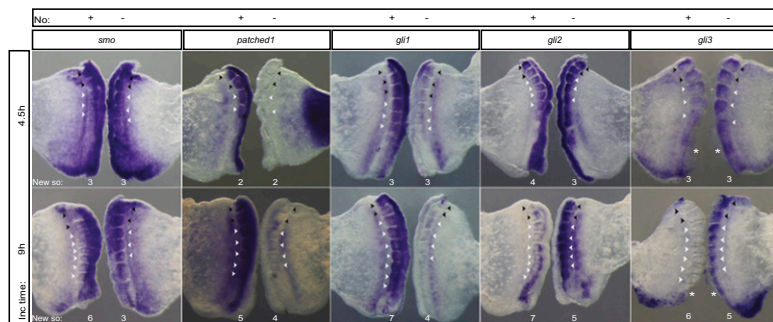
To evaluate the delay in the molecular clock, we cultured pairs of Shh-inhibited explants for time periods differing by 1 h 10 min, 2 h, 2 h 15 min, or 2.5 h. All explant pairs presented different phases of expression of the clock and the same number of somites. When explants were incubated with a time difference of 2 h 45 min (Fig. 5F), 27% of the cases presented similar clock gene expression, but no extra somite formed. Finally, when differing by 3 h, 85% of the explants had formed one extra somite but already presented different molecular clock patterns. These results indicate that clock oscillations are severely delayed in the absence of Shh, with a new time period between 2 h 45 min and 3 h. The imprecision in the determination of the new oscillation period could be due to differing levels of perturbation of the system imposed by Shh deprivation, which can be dependent on the phase of the molecular clock at the moment of notochord ablation. In conclusion, Shh ensures timely somite segmentation by setting the pace of the somitogenesis molecular clock.

**Retinoic Acid Is Able to Recover Somite Formation Rate in the Absence of Notochord.** Notochord-deprived PSMs present delayed somite formation from 4.5 h of incubation onward, accompanied by an overexpression of *gli2* ( $n = 4/4$ ) and *gli3* ( $n = 11/11$ ), a slight up-regulation of *smo* ( $n = 5/5$ ) and down-regulation of both *ptch1* ( $n = 5/5$ ) and *gli1* ( $n = 3/3$ ) (Fig. 6). Timely somite formation is resumed after 9 h and this is not due to a re-establishment of Shh pathway components throughout this time period, as their levels of expression do not revert to basal state (Fig. 6). At 9 h, an intriguing overexpression of *raldh2* is observed that is not present at 4.5 h (Fig. 3). To test whether increased amounts of retinoic acid (RA) could mediate the recovery of somite formation rate, No<sup>-</sup> explants were incubated in RA-conditioned medium, and the number of formed somites after 9 h was assessed (Fig. 7 A and B). Noticeably, a greater number of somites is formed in No<sup>-</sup> explants when the medium is supplemented with RA. Moreover, the total number of somites formed in these conditions is not significantly



**Fig. 3.** Notochord ablation alters somitogenesis clock and determination front gene expression. Expression patterns of *hairy2*, *lfng*, *fgf8*, and *raldh2*, evaluated by in situ hybridization in explants cultured for 4.5 or 9 h. Presence (No<sup>+</sup>) or absence (No<sup>-</sup>) of the notochord is indicated. Black arrowheads indicate somites formed before culture; white arrowheads indicate somites formed during incubation (New so).





**Fig. 6.** Expression of Shh pathway components in the PSM is altered by notochord ablation. Expression patterns of *smo*, *patched1*, *gli1*, *gli2*, and *gli3* genes evaluated by in situ hybridization in explants cultured for 4.5 or 9 h. \*Neural tube removal from explants for better visualization of PSM *gli3* expression. Presence (No<sup>+</sup>) or absence (No<sup>-</sup>) of the notochord is indicated. Black arrowheads indicate somites formed before culture; white arrowheads indicate somites formed during incubation (New so).

formation observed in the absence of Shh? When PSM tissue is deprived of Shh signaling, either by notochord ablation or chemical inhibition, *hairy2/lfng* expression patterns are significantly different from those obtained in control conditions. Moreover, an exogenous Shh supply to notochord-ablated explants recovered molecular clock expression patterns. Hence, we could detect a clear correlation between the presence of Shh and both timely somite formation and molecular clock gene oscillations. Our group has previously shown that only the medial-most PSM (M-PSM) cells possess intrinsic information for both somite formation and molecular clock gene expression (21). The study suggested that a signal supplied by M-PSM travels along the medial-lateral PSM axis, ensuring both timely segmentation and cyclic clock expression. However, the nature of this signal was not identified (21). The results obtained throughout the present work suggest that M-PSM cells are exposed to notochord-derived Shh signaling, as evidenced by *ptch1/2* restricted expression, providing them crucial information for timely clock oscillations and somite formation. M-PSM cells are then capable of instructing and recruiting their lateral neighbors for segmentation. This hypothesis is supported by the observation that somites formed in the absence of Shh are smaller along the medial-lateral (M-L) axis (Fig. S2B). How this is achieved in vivo is subject of ongoing studies in the laboratory.

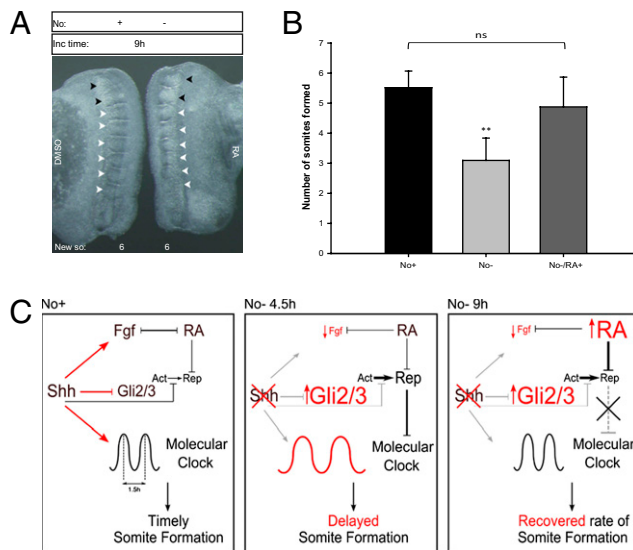
Inhibition of Wnt signaling has also been described to perturb the molecular clock (22). We found that *axin2* expression is unaltered in 60% of the notochord-ablated explants (Fig. S8) and the other 40% are only slightly down-regulated after 4.5 h. Because clock expression is perturbed in a much higher percentage of cases (80%), somite formation delay in the absence of Shh should not be due primarily to an impairment of Wnt signaling. Moreover, Wnt does not seem to be involved in the recovery of timely segmentation, as *axin2* is clearly down-regulated after 9 h (Fig. S8).

Somitogenesis clock gene expression perturbation in the absence of Shh signaling was not due to a halt in gene oscillations, but to an alteration of the clock period. In fact, the period of *hairy2* expression cycles is considerably enlarged in the absence of Shh signaling, from 1.5 h to a time point between 2 h 45 min and 3 h. Significantly, somite formation in these conditions is also greatly delayed, reinforcing the dependence of timely somite formation on the pace of clock oscillations. Interestingly, somite formation delay observed upon Shh inhibition was dose dependent, as fewer somites were formed with increased inhibitor concentrations (Fig. S9). In situ hybridization on sections suggests the existence of a *ptch1* expression gradient, with higher levels in the determined PSM (Fig. S6D). It is tempting to postulate that an anterior-posterior gradient of Shh activity could account for the different segmentation rates observed for rostral (early) and caudal (late) somites. Shh knock-out mice present a striking absence of the entire spinal column and most of the ribs (14). Our data suggest that a perturbation in somite segmentation could be contributing to this phenotype.

**Shh and RA Pathways Interplay with the Molecular Clock to Ensure Timely Somite Formation.** This work shows that Shh produced by the notochord/floor plate is required to maintain timely operation of the molecular clock and the correct pace of somite formation (Fig. 7C, Left). An important consequence of PSM isolation from its source of Shh is the up-regulation of *gli2* and *gli3* (Fig. 7C, Middle), which corroborates previous reports (19). This strongly suggests

that low *gli* levels in the PSM are not due to the lack of Shh signaling readout, as is commonly believed, but rather are actively restricted by notochord-derived Shh. Another striking molecular consequence of notochord ablation is the down-regulation of *fgf8* expression in the PSM, showing that Shh signaling is required to preserve the *fgf8* gradient underlying the determination front. These molecular changes, observed in the PSM after 4.5 h of incubation in the absence of Shh, most probably account for the subsequent delay in somite formation rate and molecular clock oscillations.

The PSM *fgf8* gradient counteracts an opposing RA gradient (5). Hence, the reduction of *fgf8* levels in the absence of Shh could allow the observed up-regulation of *Raldh2* after 9 h of incubation (Fig. 7C, Right). Under these molecular conditions, there is a recovery of somite formation rate. Accordingly, we show that an external RA supply is able to rescue timely somite formation in the absence of Shh (Fig. 7A and B). Although RA has been shown to



**Fig. 7.** Shh and RA signaling pathways interplay in the PSM to ensure timely somite formation. (A and B) Retinoic acid mediates the recovery of timely somite formation in absence of notochord. (A) Explants with (No<sup>+</sup>) or without (No<sup>-</sup>) notochord were cultured for 9 h with DMSO (control explant) or with 15  $\mu$ M of retinoic acid (RA) (experimental explant). Black arrowheads indicate somites formed before culture; white arrowheads indicate somites formed during incubation period (New so). (B) Graphic representation of mean number of somites formed by explants with notochord (No<sup>+</sup>), without notochord (No<sup>-</sup>) or without notochord but with RA (No<sup>-</sup>RA<sup>+</sup>). \*\* $P < 0.05$  vs. No<sup>+</sup> or No<sup>-</sup>RA<sup>+</sup>. ns, Not statistically significant. Data are mean  $\pm$  SD. (C) Proposed model for molecular events underlying temporal control of somite formation in the presence (No<sup>+</sup>) or absence (No<sup>-</sup>) of notochord over time. In the presence of notochord-derived Shh, *gli2/3* expression in the PSM is repressed, and timely molecular clock oscillations and somite formation occur. When Shh signaling is disrupted, *gli2/3* are overexpressed, and there is a delay in both the clock period and PSM morphological segmentation. The normal rate of these events is reestablished over time due to an up-regulation of RA that counteracts Gli activity (detailed in text).

inhibit *gli2* expression (23), this does not occur within our experimental time frame. Because RA also modulates Gli activity (24), our results suggest that *raldh2* overexpression after 9 h of notochord ablation rescues somite formation through an inhibition of Gli2/3 activity in the PSM (Fig. 7C, Right). It is plausible that somite formation recovery may further involve the inhibition of *gli2* expression levels later in time. Our work strongly suggests that Shh and RA pathways interplay in the PSM to ensure timely somite formation and positions Gli2/3 at the intersection of these two pathways. Gli2 was recently reported to exert direct transcriptional regulation on the murine *hes* gene in retinal explants (25). In our experimental setup, *gli2* overexpression was concomitant with a deregulation in the tempo of *hairly2* transcriptional bursts, suggesting that in the PSM the same transcriptional regulation could take place. Hence, Shh-mediated restriction of Gli activity in the PSM seems to be essential to ensure the pace of both molecular clock oscillations and somite formation. Interestingly, Shh has been recently described to be essential for proper timing of neural progenitor differentiation, and RA was proposed to compensate for Shh deficiency in this developmental system (26).

Somitogenesis is a robust developmental mechanism. This can be achieved only by the integration of multiple signaling pathways. In fact, Notch, Wnt, Fgf, and RA signaling pathways operate in the PSM molecular clock and/or determination front (3, 4, 6). In the present work, we provide conclusive evidence that Shh signaling is a component of the intricate molecular machinery responsible for temporal control of somite formation.

Multiple congenital vertebral malformations have been associated with mutations in somitogenesis clock genes (1, 3). Furthermore, there are reports associating the Shh signaling pathway with congenital vertebral anomalies (27, 28). We report a previously uncharacterized association between these two molecular sys-

tems operating in the formation of early embryonic vertebrae precursor structures. A deeper understanding of the multiple molecular interactions occurring during the somitogenesis process is crucial for an adequate scientific contribution to the medical community, striving to better comprehend and treat such human malformations.

## Materials and Methods

Detailed materials and methods can be found in *SI Materials and Methods*. Chick embryos were staged according to Hamburger and Hamilton (HH) (29), and somites were considered to be formed when a definite cleft was observed (30). Antisense digoxigenin-labeled RNA probes were produced as described: *shh* (31), *hairly2* (17), *lunatic fringe* (32), *fgf8* (33), *raldh2* (34), *patched-1* (20), *gli1* and *gli2* (35), and *gli3* (19). A *smoothed-specific* probe was generated. For explant cultures, ventrally positioned embryos were divided into two halves by cutting across the three germ layers at the midline level, either parallel to the embryonic axis or bisecting the axial structures. In Shh-graft experiments, clumps of either QT6-ctrl or QT6-Shh cells (36) were juxtaposed to the No<sup>-</sup> experimental explants. For chemical treatments, 15 μM of RA (Sigma), 10 μg/mL cyclopamine (Calbiochem), 25 μg/mL forskolin (Sigma), or DMSO was added to the culture medium. In situ hybridization was performed as previously described (37).

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