Revisiting the involvement of signaling gradients in somitogenesis

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ABSTRACT

During embryonic development, formation of individual vertebrae requires that the paraxial mesoderm becomes divided into regular segmental units known as somites. Somites are sequentially formed at the anterior end of the presomitic mesoderm resulting from functional interactions between the oscillatory activity of signals promoting segmentation and a moving wavefront of tissue competence to those signals, eventually generating a constant flow of new somites at regular intervals. According to the current model for somitogenesis the wavefront results from the combined activity of two opposing functional gradients in the presomitic mesoderm involving the Fgf, Wnt and RA signaling pathways. Here, I use published data to evaluate the wavefront model. A critical analysis of those studies seems to support a role for Wnt signaling, but raise doubts regarding the extent to which Fgf and RA signaling contribute to this process.

MAIN TEXT

Formation of body segments is an integral part of animal embryonic development. In vertebrates, the best studied example of segmentation is the production of somites, an essential initial step in the generation of independent vertebral units from the paraxial mesoderm [1] (see Box 1). During embryonic development, somites are formed progressively in a head to tail sequence in close coordination with embryo extension at its posterior end [1]. Embryo growth is driven by the activity of axial progenitors, first in the epiblast and later in the tailbud, that add new tissue to the posterior end of the embryo, including the mesoderm required for the sequential formation of new somites [2]. Somites are, however, not directly derived from the tissue produced by the axial progenitors. Instead, the new mesoderm is incorporated to the posterior end of the so-called presomitic mesoderm (PSM), which represents the most posterior region of the paraxial mesoderm. The PSM, itself devoid of obvious signs of segmentation, serves as a kind of factory that produces a constant flow of new somites at its anterior end. The mechanisms of somitogenesis have fascinated embryologists and theoretical

biologists for decades as they lead not only to the steady production of somites during an extended developmental time but also manage to integrate this process with the growth of other embryonic structures to build a functional body.

The current model for somitogenesis

The current model for somitogenesis considers that formation of a new somite results from the combination of two basic elements, a molecular clock (also known as segmentation clock) that sets the pace of somite formation, and a wavefront of activity that defines the position of the posterior border of each new somite (Fig. 1)[1,3]. The molecular clock is provided by waves of signaling activity, moving from posterior to anterior along the PSM, one wave per somite. Notch, Fgf and Wnt signaling have been shown to oscillate in the PSM [4,5], although functional analyses indicate that Notch signaling is the key activator of the mechanisms producing intersomitic borders and, thus, liberating new somites from the anterior PSM. Waves of Notch signaling activity travel throughout the whole PSM but segmentation only becomes activated in a distinct region in the anterior PSM. This region is defined at the anterior margin of a wavefront of functional activity, thought to result from the combination of two opposite signaling gradients: a posterior to anterior gradient of Fgf and Wnt signaling and an anterior to posterior gradient of retinoic acid (RA) activity. Fgf and Wnt activities are thought to inhibit the segmentation process in a dose dependent fashion whereas RA is thought to counteract this activity in part by regulatory interactions with Fgf8 signaling [1,3]. At a particular position in the anterior PSM, the level of Wnt and Fgf signaling falls below their threshold of effective inhibitory activity, thus rendering the PSM competent to activate the segmentation process when exposed to the next incoming wave of Notch signaling. This region is often known as the determination front. The position of the determination front moves posteriorly following the growth of the embryo. This posterior displacement, together with the oscillation of the molecular clock results in the sequential production of somites as the embryo grows (Fig. 1).

One of the merits of this model is that it allows testable predictions. One of these is that under stable conditions, somite size will be determined by the extent of embryo growth between two waves of Notch activity reaching the determination front. Accordingly, if gradient size (see Box 1), embryo growth and clock oscillatory period are kept constant, the actual size of the gradient will have little effect on somite size. Under these stable conditions, what the gradient dimension determines is the position within the PSM where the new somite will be liberated. This will have direct impact on PSM size, which will be inversely proportional to the extension of the gradient (Fig. 2A,B). The dimension of the gradient is expected to have significant effects on somite size only if it changes between formation of consecutive somites, producing bigger somites if the gradient shrinks and smaller when it expands (Fig. 2A,B). The model also predicts that in the extreme case of the total inactivation of wavefront components, the PSM would be essentially non-existing and replaced by tissue with signs of somitic differentiation (Fig. 2C). Conversely, if activity levels of the wavefront molecules are kept over their functional threshold, the PSM would fail to produce somites, becoming bigger as the embryo grows posteriorly and keeping expression of markers for undifferentiated paraxial mesoderm (Fig. 2C). It should be noted, however, that these theoretical predictions are not always simple to test experimentally because the relevant signaling pathways involved in the wavefront model also play relevant roles in the production of mesodermal cells from the progenitors in the epiblast or tail bud [2], often complicating to separate what derives from signaling activity in the axial progenitors and what from their function in the PSM.

The gradient of Wnt signaling activity

The existence of a gradient of Wnt activity in the PSM was first suggested in the mouse by the spatial distribution of *Axin2* expression, a downstream target of Wnt signaling [4], and has been later confirmed in zebrafish and mouse embryos by showing a graded posterior to anterior distribution of nuclear β-

catenin (a readout of canonical Wnt signaling activity) in this tissue [6,7]. A variety of genetic studies are consistent with a relevant role of this signaling in the wavefront. The cleanest example was provided by a recent study that uses an elegant genetic trick to modify the extension of the Wnt gradient in the PSM of zebrafish embryos without affecting mesoderm production, thus permitting functional evaluation of the Wnt gradient in somitogenesis [7]. With this system, it was observed that activation of the segmentation program, as determined by the expression of markers like *mespb* or the anterior stripes of *her7* expression, occurred at more posterior positions within the PSM when the Wnt gradient was reduced, thus resulting in a shorter PSM. Interestingly, the somites formed during the time when the Wnt gradient was changing were bigger than those in normal embryos but somites formed after the stabilization of the shorter gradient were normal sized. As the pace of the molecular clock was not influenced by the Wnt gradient size, these observations are consistent with a relevant role for Wnt activity as a functional component of the wavefront system. Quite surprisingly, the Fgf gradient seemed to be largely not affected in embryos with shorter PSM, questioning the involvement of the Fgf signaling in wavefront activity [7].

A variety of experiments performed in mouse embryos also support a role of Wnt signaling in establishing the position of the new intersomitic border. The earliest set of experiments involved the analysis of *Wnt3a* hypomorphs, which had aberrant somitogenesis mostly restricted to the posterior somites [4]. These studies were later further extended by modulating Wnt signaling through the production of targeted alterations in β-catenin activity. When β-catenin was removed from mesodermal cells (I will refer to them as $Ctnnb1^{Tcre}$) expression of markers for differentiated paraxial mesoderm expanded almost until the posterior end of the embryo, and expression of markers for undifferentiated PSM was essentially not detectable [8]. This pattern is consistent with a posterior expansion of the somitic mesoderm and the extreme reduction of PSM predicted for a complete absence wavefront components the PSM. However, the observation that these embryos became truncated very early in development

adds uncertainties to the interpretation of this phenotype. In particular, while this truncation might result from the failure of the PSM to undergo proper development, it could also have resulted from the exhaustion of the progenitor pool, which also requires *Wnt3a* for its production [2], thus strongly compromising the production of new mesoderm.

In the complementary experiment, Wnt signaling was kept high in the mesoderm of mouse embryos through the conditional expression of a constitutively active form of β-catenin in the paraxial mesoderm [6,8]. As predicted by the wavefront model these embryos showed strong expansion of their PSM together with all associated molecular characteristics. Interestingly, maintenance of high Wnt activity levels seemed to have very little effect on the segmentation clock, which kept oscillating along most of the PSM length but failed to trigger formation of a new somitic border [6,8].

Together, genetic data strongly support that a gradient of Wnt signaling activity might interact with the segmentation clock to determine the position of the posterior border of the new somite, in agreement with Wnt being an essential molecular component of the wavefront. It has been proposed that the drop in Wnt signaling Notch signaling leaves the oscillatory activity and activates the segmentation process [3]. It should be noted, however, that the mechanistic relationship between Wnt signaling and the determination front might include additional components because, at least in zebrafish, Wnt activity reaches undetectable levels midway through the PSM and not at the determination front as would be expected according to the current model for segmentation [7]. It has been proposed that the drop in Wnt signaling activity results in the loading of positional information into posterior PSM cells that is later transported by cell flow to the anterior PSM, where it is interpreted to activate the segmentation program [7]. Whether this is indeed the case and understanding the molecular basis of this alternative model will require properly designed experiments.

The gradient of Fgf signaling activity

The involvement of Fgf signaling in the wavefront is not so clear cut. The existence of an *Fgf8* gradient in the PSM has been convincingly documented [9,10]. Also, initial experimental evidence from chicken and zebrafish embryos using bead implantation approaches to produce local alterations of Fgf8 levels seemed consistent with a role for Fgf8 as a component of the wavefront [9,11]. However, a variety of different experimental results seem to be at odds with such a role for Fgf8. The first piece of data difficult to fit in the model is the nature of the receptor receiving the Fgf signal. FgfR1 seems to be the only known member of the Fgf receptor family expressed in the PSM and is thus the candidate for such a role [10,12]. However, the spatial distribution of *FgfR1* transcripts in the PSM does not match the expected pattern in any vertebrate species where it was analyzed, as it can be detected at high levels in the anterior PSM but it is almost undetectable in the posterior PSM [10,12]. It could be still argued that the posterior PSM keeps FgfR1 proteins produced at the progenitor stage and that these are the receptor molecules channeling the Fgf8 signal. This hypothesis, however, requires direct experimental evaluation. Also against a role of *Fgf8* in the establishment of the wavefront is the finding that inactivation of this gene in the paraxial mesoderm of mouse embryos had no negative effects on somitogenesis [13]. This result could be explained if Fgf8 activity is compensated by other Fgf signals. Consistent with this, *Fgf3* and *Fgf17* are also expressed in the PSM following a posterior to anterior gradient [12]. Redundancy among Fgf signals was thus tested in mouse embryos by inactivating *FgfR1* in mesodermal tissues (*FgfR1*$^{T\text{-}cre}$ embryos), hence removing all Fgf activity from the PSM [12]. These mutant mice survived to term but developed strong malformations in their axial skeleton at thoracic and more posterior levels, derived from abnormal somitogenesis posterior to the forelimb bud. However, markers of the determination front, like *Mesp2* or the anterior stripe of *Lfng*, still mapped to positions roughly similar to those observed in wild type embryos. Similarly, expression of markers for differentiated paraxial mesoderm did not extend to more posterior positions than in wild type embryos. Therefore, in *FgfR1*$^{T\text{-}cre}$

embryos, the PSM largely conserved its normal anterior-posterior dimensions, thus failing to follow the predictions of the wavefront model. Interestingly, Notch signaling did not oscillate in the PSM of *FgfR1*$^{T\text{-}cre}$ embryos [12], which could by itself provide a suitable explanation for the absence of somites. Indeed, the phenotypic characteristics of *FgfR1*$^{T\text{-}cre}$ mutants are remarkably similar to those described in mutant mice for the oscillating gene *Hes7* [14], thus adding some support to this hypothesis. Also, it should not be ignored that *FgfR1* expression is strong at the anterior PSM [10,12], opening the unexplored possibility of a potential requirement of Fgf signaling for the segmentation process itself.

More recent genetic evidence, however, was consistent with the involvement of *Fgf8*, redundantly with *Fgf4*, in the establishment of the determination front. In particular, simultaneous removal of both genes in mesodermal tissues following a strategy similar to that used to inactivate *FgfR1* (*Fgf8/4*$^{T\text{-}cre}$ embryos) produced an embryonic phenotype [15] closely resembling that observed in *Ctnnb1*$^{T\text{-}cre}$ mutants [8], which follows the patterns predicted for a total absence of wavefront activity. The apparent redundant role of *Fgf4* and *Fgf8* in the PSM is however surprising because bead implantation experiments indicated that Fgf8 and Fgf4 produced very different effects when placed on the PSM of chicken embryos [9]. In addition, the possible involvement of Fgf8 and Fgf4 as components of the wavefront is at odds with the phenotype of *FgfR1*$^{T\text{-}cre}$ embryos, which are expected to have no Fgf activity in the paraxial mesoderm [12]. Also against this interpretation is the finding that the *Fgf8/4*$^{T\text{-}cre}$ phenotype was not reproduced in embryos lacking mesodermal activity of the same two Fgfs but generated using a slightly different conditional strategy (*Fgf8/4*$^{b1\text{-}cre}$ embryos) [16]. In these latter embryos, somitogenesis was fairly well conserved during early developmental stages and only later in development could negative effects on development of the paraxial mesoderm be scored. A variety of molecular and cellular studies indicated that the *Fgf8/4*$^{b1\text{-}cre}$ phenotype mostly derived from reduced expansion of axial progenitors [16]. The negative impact of the absence of *Fgf4* and Fgf8 on the expansion of axial progenitors fits better with the

expression profiles of these two Fgf molecules as *Fgf4* expression is not detected in the PSM, being restricted to the progenitor-containing compartment [12], and *Fgf8* is also expressed and functionally relevant in the progenitor-containing compartment [10,17]. Removal of *Fgf4* and *Fgf8* activity from the axial progenitor compartment would actually also provide a suitable explanation for the *Fgf8/4*$^{T\text{-}cre}$ phenotype.

Interestingly, although at E8.5 somitogenesis appeared largely unaffected in *Fgf8/4*$^{b1\text{-}cre}$ mutants, clear somite malformations were observed at E9.5, together with a reduction of the PSM size [16], thus compatible with shorter wavefront-producing gradients. While this could derive from reduced Fgf signaling in the PSM, the finding that *Wnt3a* expression levels in the caudal end of these embryos were fairly normal at E8.5 but strongly reduced at E9.5, suggests that the PSM phenotype of *Fgf8/4*$^{b1\text{-}cre}$ might be the consequence of the effects that Fgf signaling have on *Wnt3a* expression. Consistent with this hypothesis, *Wnt3a* expression was preserved in *FgfR1*$^{T\text{-}cre}$ embryos [12] but absent from *Fgf8/4*$^{T\text{-}cre}$ mutants [15], thus correlating with the PSM size of those embryos. It is therefore not clear to which extent formation of the wavefront requires direct Fgf activity in the PSM. It could be argued that the total loss of wavefront activity (as it could have happened in *Ctnnb1*$^{T\text{-}cre}$ and *Fgf8/4*$^{T\text{-}cre}$ embryos) requires complete and simultaneous Fgf and Wnt signaling inactivation, thus suggesting functional redundancy between the two signaling pathways. Consistent with this, *Fgf8* was down regulated in *Ctnnb1*$^{T\text{-}cre}$ embryos [15]. However, the finding that reducing the size of the Wnt gradient was enough to affect the position of the segmental plate even in the presence of fairly normal distribution of Fgf signaling [7], seems to suggest that if Fgf signaling plays a role in this process, it might weight less than that of Wnt signaling.

The involvement of Retinoic Acid in the wavefront of activity

The involvement of the third component of the wavefront system, the RA gradient, is also questionable on the basis of existing experimental data.

According to the current paradigm, the anterior to posterior RA gradient in the PSM results from a production/sink mechanism, whereby RA is synthesized by Raldh2 in newly formed somites and cleared at more posterior areas of the PSM by Cyp26a1 produced at the caudal embryonic end [1]. Whether this RA gradient in the PSM is indeed produced is not clear because reporter experiments often show that the posterior end of RA activity in the paraxial mesoderm is abrupt rather than progressive [18]. Also, it has been described that in embryos showing a complete down regulation of *Cyp26a1* expression RA signaling activity did not extend farther from the RA-producing tissue into the PSM [12,16], thus questioning the role of Cyp26a1 in the production of a hypothetical RA gradient in this tissue.

RA is thought to participate in the production of the wavefront by modulating the extension of the *Fgf8* gradient in the PSM. This was first proposed on the basis of the observation that *Fgf8* expression expanded anteriorly in the PSM of chicken embryos deficient in RA synthesis [19]. A similar anterior expansion of Fgf8 expression was observed in mouse and zebrafish embryos lacking RA activity [20,21], thus confirming that RA signaling limits *Fgf8* expression in the PSM. However, it is highly unlikely that the restriction of *Fgf8* expression in the PSM derives from a dose-dependent effect provided by graded RA activity in the PSM. In particular, it has been shown that the *Fgf8* gradient does not result from differential transcriptional rates along the PSM. Instead, *Fgf8* transcription occurs in the axial progenitors or nascent mesodermal tissue, becoming silent when cells enter the posterior end of the PSM [10]. The gradient then results from the progressive decay of the initial *Fgf8* transcript load as cells occupy more anterior positions within the PSM [10]. Therefore, the characteristics of the *Fgf8* mRNA gradient to a large extent depend on the initial load and transcript stability. RA controls gene expression at the transcriptional level with no described effects on mRNA stability [22]. Therefore, the expanded *Fgf8* expression observed in the PSM in the absence of RA signaling can be better explained by RA impacting the initial level of *Fgf8* mRNA loaded into cells

entering the PSM rather than by modulation of *Fgf8* transcript levels within the PSM by the RA gradient. Accordingly, it has been shown that RA controls expression *Fgf8* levels in the PSM by direct interaction with *Fgf8* promoter elements [20]. Also, in *Rdh10*^{trex} mutants that lack RA activity in the paraxial mesoderm [23], *Fgf8* expression in the PSM is similar to that observed in wild type embryos [24].

An additional consideration is related to the role that RA might play in somitogenesis. It has been described that chicken embryos lacking RA signaling have slightly longer PSM, thus compatible with the proposed role for RA in the wavefront system, although this increase in PSM length is much smaller than what would have been expected from the strong expansion of the *Fgf8* gradient observed in these embryos [19]. The picture is less clear in mouse embryos. *Raldh2* mutants, which lack most endogenous RA, can form 10-13 somite pairs before their development is arrested between E8.5 and E9.0 [18]. These somites are smaller and formed at different paces in the left and right embryonic sides [18]. Asymmetric somite production has also been described in zebrafish embryos treated with anti-*Raldh2* morpholinos [21], thus indicating a conserved role of RA in bilateral synchronization of paraxial mesoderm differentiation. To which extent this is mediated by effects on the *Fgf8* gradient is unclear because *Fgf8* seems to be symmetrically expressed in the PSM of RA-deficient mouse embryos [18] but asymmetrically in *Raldh2* morphant zebrafish embryos [21]. Importantly, however, somite malformations in RA-deficient embryos are only seen on somites formed before these embryos become developmentally arrested. *Raldh2* mutants resume fairly normal embryonic development if exposed to an acute external dose of RA that somehow overcomes the cause of the developmental arrest [18]. In these rescued embryos, somitogenesis follows totally normal patterns even in the absence of any sign of RA activity [18]. Therefore, if RA plays a direct role in somitogenesis, its functional weight is different at different levels of the embryonic axis. Alternatively, RA might not play a role in the somitogenesis process itself but its activity be essential for other

early developmental processes, whose alteration have indirect impact on somite formation. Indeed, the embryonic arrest of RA-deficient embryos might result from interference with such processes. The observation that *Rdh10*^{trex} mutant embryos develop without any obvious negative effect on somite formation in the absence of detectable RA activity in the paraxial mesoderm [23,24] is consistent with the latter possibility.

In conclusion, functional data are consistent with the involvement of Wnt signaling in the wavefront of functional competence that sets the position of the somite borders. However, a considerable amount of data seems to question the role that Fgf and RA signaling play in this process. Solving these uncertainties will be necessary to move forward in our understanding of the molecular mechanisms of somitogenesis.

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FIGURE LEGENDS

Figure 1. Schematic representation of somitogenesis. The drawings in B, D and F represent a dorsal view of the posterior end of the embryo with the paraxial mesoderm at both sides of the neural tube. The paraxial mesoderm is composed of somites (squares) being liberated at the anterior border of the presomitic mesoderm (PSM). The images in C, E and G show diagrams representing the interactions between the gradient (left side) and segmentation clock (right side) in the PSMs represented in B, D and F, respectively. Two opposite gradients, a posterior to anterior gradient of Wnt and Fgf signaling (red triangle in A) and an anterior to posterior gradient of retinoic acid (RA, green triangle in A) generate a gradient of activity (red in the left side of the schemes in B to G) that blocks tissue competence to respond to differentiation signals provided by Notch signaling activity (in blue in the right side of the diagrams). In the anterior part of the PSM, the activity of the gradient falls below a threshold level of inhibitory activity, producing a window of tissue competence (commonly known as the determination front, represented as a blue bracket), where PSM cells are able to activate the segmentation program. B,C. When a wave of Notch activity (in blue on the right side of the schemes) reaches the determination front, the segmentation program becomes activated (yellow ray) and produces a new intersomitic border. D-G. The position of the determination front moves posteriorly following axial embryo growth but a new intersomitic border is only formed when the next wave of Notch activity reaches this position (in the scheme it occurs in F,G but not in D,E panels). The size of each new somite will result from the extent of embryo growth (double headed arrow) between two waves of the segmentation clock reaching the determination front.

Figure 2. Representation of the theoretical consequences of reduced or expanded gradients in the PSM. A. The case of a reduced gradient (effective within a shorter anterior posterior distance). b, c, d show the effect on somite formation while the gradient is undergoing reduction but keeping a constant rate of posterior growth. This reduction results in a progressively more posterior position of the determination front between two adjacent somites, thus resulting in the activation of segmentation at a more posterior position with the creation of a longer somite. Panel e shows the condition after gradient stabilization. The position of the determination front will move at the same rate as PSM posterior growth, resulting in somites of normal size. The PSM size (D') will, however, be shorter than in the presence of a normal sized gradient (D). B. The case of an increased gradient (effective within a longer anterior posterior distance). b, c, d show the effect on somite formation during gradient expansion but keeping a constant rate of posterior growth. This expansion results in a more anterior position of the determination front in two adjacent somites, thus resulting in the activation of segmentation at a more anterior position and the creation of a shorter somite. Panel e shows the condition after gradient stabilization. The position of the determination front will move at the same rate as PSM growth,

thus producing normal-sized somites. The PSM size (D') will however be longer than in the presence of a normal-sized gradient (D). C. Representation of extreme cases: a. the total absence of gradient-forming molecules the PSM would be strongly reduced and signs of differentiation (somitogenesis) would be identified throughout the paraxial mesoderm. b. If the concentration of gradient forming molecules is high and rather uniform throughout the paraxial mesoderm the PSM would be strongly extended and signs of differentiation virtually absent.

Box 1. Definition of a few essential concepts

- The **paraxial mesoderm** is the part of the mesoderm responsible for the formation of the axial skeleton and all our body muscles. It is located at both sides of the developing neural tube, extending along the whole main body axis. For most of its length, it is divided in discrete segments called **somites**. The existence of somites is the first sign anticipating the segmental nature of our skeleton. The posterior end of the paraxial mesoderm is not segmented. It is known as **presomitic mesoderm (PSM)**. The PSM acts as the factory where new somites are made through the process of **somitogenesis**. During development the PSM grows at the caudal end by addition of new tissue and progressively liberates new somites at its anterior end.

- Vertebrate embryos are made in a head to tail sequence by progressive addition of new cells at the posterior embryonic end. This caudal growth results from the activity of a group of cells generally known as **axial progenitors**. The activity of these cells must be finely balanced so that it can both produce the new cells that extend mesodermal, endodermal and neural structures, and self-renew to guarantee that further embryonic growth.

- Gradient size refers to the length of PSM where the activity of the molecules producing the gradient can be sensed. I will essentially refer to the graded posterior to anterior activity that keeps silent the Notch-dependent segmentation program. Gradient size depends on several variables, the most important being the initial level of activity at the posterior PSM and the rate at which this activity decays as cells occupy more anterior positions in the PSM

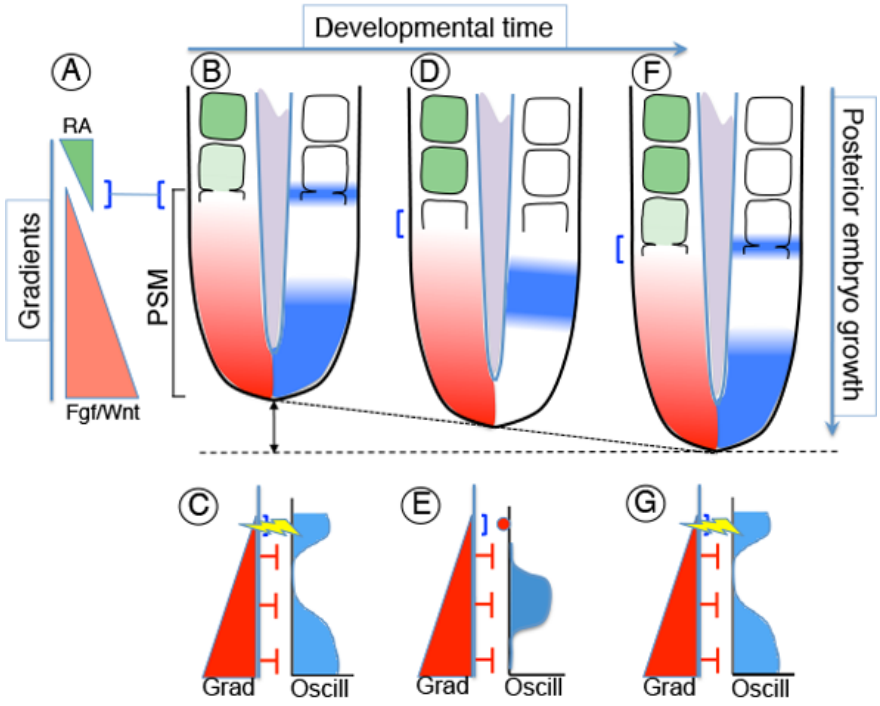
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Figure 1

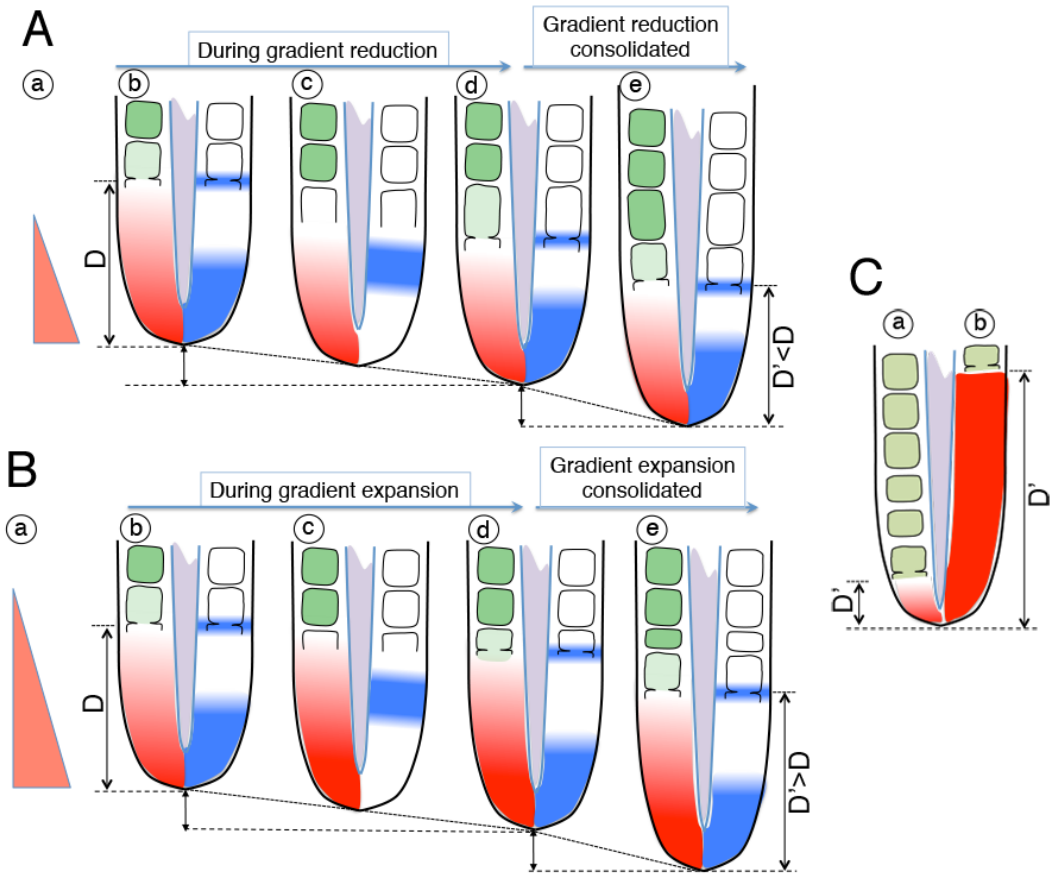
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Figure 2