

## Oscillatory links of Fgf signaling and Hes7 in the segmentation clock

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## Summary

Somitogenesis is controlled by the segmentation clock, where the oscillatory expression of cyclic genes such as *Hes7* leads to the periodic expression of *Mesp2*, a master gene for somite formation. Fgf signaling induces the oscillatory expression of *Hes7* while *Hes7* drives coupled oscillations in Fgf and Notch signaling, which inhibits and activates *Mesp2* expression, respectively. Because of different oscillatory dynamics, oscillation in Fgf signaling dissociates from oscillation in Notch signaling in S-1, a prospective somite region, where Notch signaling induces *Mesp2* expression when Fgf signaling becomes off. Thus, oscillation in Fgf signaling regulates the timing of *Mesp2* expression and the pace of somitogenesis. In addition, Fgf signaling was found to be a primary target for hypoxia, which causes phenotypic variations of heterozygous mutations in *Hes7* or *Mesp2*, suggesting gene-environment interaction through this signaling.

## Introduction

Somites, metameric structures that later give rise to vertebrae, ribs, skeletal muscles, and subcutaneous tissues, are formed by periodic segmentation of the anterior parts of the presomitic mesoderm (PSM). This periodic event is regulated by a biological clock called the segmentation clock, which involves the oscillatory expression of cyclic genes such as the basic helix-loop-helix (bHLH) gene *Hes7* (Figure 1) [1-5]. Genes in the Notch, Fgf, and Wnt pathways are expressed in an oscillatory manner in the mouse PSM [6-9]. One major outcome of such oscillatory expression is periodic activation of the bHLH gene *Mesp2*, a master gene for somite formation, in the prospective somite region termed S-1 (note that S0 is the next forming somite while S-1 is the PSM region that will form a somite after S0) (see Figure 3) [10,11]. So, the question is how cyclic genes periodically induce *Mesp2* expression in S-1.

Oscillatory expression is halted in the anterior region of the PSM, where overt somite differentiation begins, and the interface between this anterior region and the posterior oscillatory region is called the wavefront. It was previously shown that the wavefront is established by morphogens such as *Fgf8* [12,13]. The *Fgf8* gene is transcribed only in the posterior end of the PSM, but due to slow degradation, the amount of *Fgf8* mRNA gradually decreases in PSM cells, which move anteriorly as the PSM grows posteriorly, forming the posterior to anterior *Fgf8* gradient [14]. The

anterior border of the Fgf gradient corresponds to the wavefront, and after passing this line, PSM cells express *Mesp2* because *Mesp2* expression is repressed by Fgf8 [11]. Fgf signaling seems to sweep back at a steady speed as the PSM grows, suggesting that the wavefront might regress steadily. However, *Mesp2* expression initiates synchronously in the whole S-1 region, and this synchronous expression seems to be important for the subsequent somitogenic processes [11]. These results raise the alternative possibility that the wavefront periodically jumps. Recent studies revealed that oscillators in the Fgf and Notch pathways are essential for periodic induction of *Mesp2* expression in S-1.

### **Fgf signaling induces traveling waves**

The Notch intracellular domain (NICD), an active form of Notch, activates the *Hes7* promoter both in in-vitro culture and in-vivo transgenic mouse studies, indicating that *Hes7* expression is controlled by Notch signaling [8,15-17]. In agreement with this notion, *Hes7* expression is severely down-regulated in the absence of *Rbpj*, an essential mediator of Notch signaling [8,17]. However, a low level of *Hes7* expression still oscillates in the PSM of *Rbpj*-null mice, suggesting that another signaling may be responsible for oscillatory expression of *Hes7* [8,17]. Indeed, Fgf signaling is required for *Hes7* expression because *Hes7* expression totally disappears in the presence of Fgf inhibitors or in the absence of *Fgfr1*, an Fgf receptor gene essential for somitogenesis [8,18,19]. Thus, *Hes7* expression is cooperatively regulated by both Fgf and Notch signaling. In zebrafish, it was previously proposed that non-synchronous oscillatory expression remains in embryos mutant for Notch signaling [20], and it was recently shown that expression of *her1*, a zebrafish *Hes7* homologue, still oscillates non-synchronously in such mutants [21•]. In contrast, treatment with an inhibitor of Fgf signaling abolished *her1* expression, whereas transplanted Fgf8-soaked beads induced ectopic traveling waves of *her1* expression in zebrafish embryos [22••]. These results indicate that Fgf signaling is essential for *Hes7/her1* oscillations in the PSM.

The stripes of both *Hes7* and *her1* become narrower as they move anteriorly in the PSM, and this narrowing is owing to slowing oscillations (longer periods) [23,24]. The *Fgf8* gradient could be involved in the slowing oscillations because the Fgf level affects the length (or narrowing) of *her1* stripes [22••]. However, pERK, an effector of Fgf signaling [13,25], is expressed in an oscillatory manner and does not form any apparent gradients in the mouse PSM (see below) [8,26••], indicating that the Fgf8

gradient is not translated into pERK levels. It was shown that Notch signaling-mediated intercellular coupling regulates the pace of the segmentation clock [27]. Fgf signaling could cross-talk with Notch signaling and thereby control the period of oscillatory expression. Further study will be required to understand the mechanism of how *Hes7/her1* oscillations slow in the anterior PSM and whether Fgf signaling is involved in this slowing.

### **Coupled and dissociating oscillations of Notch and Fgf signaling**

In the mouse PSM, expression of the bHLH repressor gene *Hes7* oscillates by negative feedback [15]. *Hes7* oscillation drives the oscillatory expression of the Notch modulator *Lunatic fringe (Lfng)*, which inhibits Notch signaling [5,17,28-30]. When Notch signaling is activated by its ligands such as Delta-like1, the transmembrane protein Notch is processed, releasing NICD. *Lfng* oscillation periodically inhibits Notch signaling, resulting in cyclic formation of NICD in the PSM (Figure 2) [26,31,32]. It has been shown that NICD activates *Mesp2* expression in collaboration with Tbx6, a T-box protein required for PSM differentiation and segmentation [11,33]. *Hes7* also drives the oscillatory expression of *Dusp4*, a phosphatase of phosphorylated ERK (pERK), an effector of Fgf signaling. Owing to *Dusp4* oscillation, pERK is periodically dephosphorylated, and the amount of pERK also oscillates in the PSM (Figure 2) [8,26]. It was shown that *Hes7* induces oscillatory expression of another Fgf inhibitor gene, *Sprouty4*, in the mouse PSM, which could also contribute to pERK oscillation [34]. Thus, *Hes7* induces oscillations of both Notch and Fgf signaling in the PSM. Conversely, Notch and Fgf signaling cooperatively up-regulate *Hes7* expression (Figure 2) [7]. These results indicate that Notch and Fgf signaling molecules and *Hes7* form the oscillatory gene network. The next question is how the oscillatory expression of NICD, an activator of *Mesp2*, and pERK, a repressor of *Mesp2*, induces *Mesp2* expression in S-1.

Detailed expression analysis showed that the dynamics is different between NICD and pERK oscillations: NICD expression travels like a wave, narrowing down to a near somite size, while pERK exhibits an On-Off pattern (Figure 3). Narrowing of the NICD expression domain is due to slower oscillation in the anterior PSM compared to the posterior PSM, whereas the pERK oscillation does not slow. When NICD is expressed in the posterior to middle PSM, pERK is also present, inhibiting NICD from

inducing *Mesp2* expression (phases II and III, Figure 3). However, when the NICD expression domain moves and narrows down to the region around S-1, pERK suddenly disappears, which allows NICD to induce *Mesp2* expression synchronously in the whole S-1 region (Figure 3, panel furthest to the right) [26••]. Thus, two types of oscillations in Notch and Fgf signaling, which are coupled by *Hes7* in the posterior PSM, dissociate in the anterior PSM, and this dissociation allows a group of cells (NICD<sup>+</sup>pERK<sup>-</sup>) to express *Mesp2* simultaneously in the whole S-1 region (Figure 3).

Retinoic acid (RA) signaling forms an opposite gradient to and antagonizes the Fgf8 signaling [35], and it has been proposed that the bistability between antagonistic Fgf and RA gradients leads to jumps of the wavefront [36]. However, this notion remains to be experimentally analyzed. A recent study with the monolayer PSM culture system shows that the phase of *Lfng* oscillation is delayed in the anterior PSM compared to the posterior PSM [37•]. Interestingly, the phase difference between anterior and posterior cells (phase gradient) was found to be inversely proportional to the sizes of formed segments, suggesting that the segment size or the wavefront jump is predetermined by the phase gradient [37•]. Strikingly, the phase gradient in monolayer PSM culture tissues is maintained even in the absence of an anterior opposing gradient [37•], suggesting that the segment size is controlled independently of an anterior opposing gradient.

### **Oscillation in Fgf signaling is important for the timing of somite formation**

It was previously thought that Notch signaling function as a pacemaker of the segmentation, while the distance that the wavefront (the anterior boarder of Fgf signaling) travels during one oscillation cycle defines the somite size [1]. However, recent data on the oscillator networks needs revision of this view. Cyclic down-regulation of pERK seems to be important for the S-1 cells to express *Mesp2* periodically, suggesting that pERK oscillation in Fgf signaling regulates the pace of segmentation (Figures 3 and 4A) [26••]. In contrast, the NICD expression domain narrows in the anterior PSM, and this narrowing is important for the size of somites, because the NICD<sup>+</sup> region that co-expresses *Tbx6* induces *Mesp2* expression after being freed from Fgf-pERK regulation (Figures 3 and 4A) [26••,31]. Thus, NICD oscillation plays an important role in the spatial regulation of somitogenesis. It was previously shown that increased Fgf activity reduces the somite size without affecting the pace of

segmentation [12]. Fgf was shown to caudalize the PSM and thereby shift the wavefront anteriorly [12]. In addition, as discussed above, Fgf signaling could be involved in narrowing of *Hes7* stripes, which would lead to narrowing of NICD stripes via Lfng. These effects could secondarily affect the somite size. Further analysis will be required to determine the role of Fgf signaling in somite formation to differentiate between direct and indirect activities.

In *Hes7* KO mice, although segmentation is severely defective, *Mesp2* is expressed in the S-1 region, as observed in wild-type mice [5,26••]. In the absence of *Hes7*, Lfng and Dusp4 expression becomes non-oscillatory, and therefore NICD and pERK expression become steady (Figure 4B) [26••]. In *Hes7* KO mice, NICD expression continues longer than pERK, forming the NICD<sup>+</sup>pERK<sup>-</sup> region around S-1 where *Mesp2* expression is induced. However, time-lapse imaging analysis showed that this *Mesp2* expression domain moves steadily as the PSM grows (Figure 4B). As a result, in the absence of *Hes7*, the onset of *Mesp2* expression does not occur simultaneously in the whole S-1 region but gradually proceeds from the anterior to posterior even in the same S-1 region (Figure 4B), which may cause the segmentation defects. Thus, although a snapshot of *Mesp2* expression is not significantly different between the wild-type and *Hes7* KO embryos (green signals in the left panels of Figure 4A,B), the spatiotemporal profiles of *Mesp2* are totally different (green signals in the middle panels of Figure 4A,B) [26••].

The notion that *Hes7*-induced NICD and pERK oscillations regulate periodic expression of *Mesp2* raises the possibility that *Hes7* is a key pacemaker of the segmentation clock. It has been suggested that the negative feedback with a delayed timing is essential for sustained oscillation with an appropriate period [38-42], and that negative feedback with shorter delays accelerates the tempo and dampens or abolishes the oscillation. It was recently shown that intronic delays, which include the time required for transcription and splicing of intron sequences, constitute an important part of such proper delays. Due to rapid transcription, splicing events rather than the intron length may be more important for the intronic delay [43]. The *Hes7* gene has three introns, and deletion of all three introns reduces the delay by 19 min and completely abolishes oscillatory expression, leading to steady *Hes7* expression and fusion of all somites [44]. In contrast, deletion of two introns of the *Hes7* gene reduces the delay by 5 min and accelerates the pace of the segmentation clock, although the oscillation is

eventually halted [45•]. Interestingly, these mutant mice have nine cervical vertebrae whereas the wild-type mice have seven, indicating that two more pulses of *Hes7* oscillation occur during formation of cervical vertebrae [45•]. It is likely that oscillations in both Notch and Fgf signaling are also accelerated in these mutant mice, suggesting that *Hes7* is a fundamental pacemaker of the segmentation clock.

### **Hypoxia and Fgf signaling**

Congenital scoliosis, which is caused by vertebral defects, occurs about 1 in 1,000 human live births, and it has been shown that heterozygous mutations in *HES7* or *MESP2* cause this disease [46••]. Interestingly, some of those who have the same mutations do not have any abnormal vertebrae, indicating partial penetrance. Partial penetrance of vertebral defects is also observed in mice carrying heterozygous mutations in *Hes7* or *Mesp2* [46••]. Notably, short-term hypoxia induces significantly higher rates and severity of vertebral defects in *Hes7* or *Mesp2* heterozygous mutant mice, suggesting that the phenotypes of genetic disorders are affected by environmental conditions such as hypoxia [46••,47]. Hypoxia does not alter *Fgf8* and *Fgfr1* expression in the PSM, but pERK expression becomes absent or significantly reduced (Figure 2). Furthermore, *Hes7* expression becomes absent or significantly reduced, which is similar to the phenotype caused by treatment with Fgf signaling inhibitors [46••]. These results suggest that hypoxia primarily affects Fgf signaling, although it may also affect other pathways like Wnt and Notch signaling (Figure 2). The mechanism of how hypoxia affects pERK formation remains to be determined.

### **Perspectives**

In the mouse PSM, NICD makes a traveling wave, while pERK makes an On-Off pattern, and the mechanism by which the dynamics of NICD and pERK oscillations are different remains to be determined. The kinetics of the cell-cell communication may be different between Notch and Fgf signaling. One possibility is that Notch signaling travels relatively slowly because the ligand activates Notch only in adjacent cells, while Fgf is secreted and may rapidly reach distant cells, making the whole population respond simultaneously. Further analysis will be required to determine the parameter values of each signaling communication speed.

Another important aspect of Fgf signaling is its role in elongation of the

embryonic axis [48-50]. Fgf signaling activates random motility of PSM cells, particularly in the posterior region, but not the proliferation of these cells, and this graded motility (high in the posterior and low in the anterior) seems to contribute to the axis elongation [48]. It remains to be determined how Fgf signaling coordinates the axis elongation with the pace of segmentation.

Recent studies showed that Fgf signaling induces the oscillatory formation of pERK in non-PSM cells such as fibroblasts, and pERK pulses are shown to affect cell cycle progression [51,52]. Thus, the oscillation in Fgf signaling is not specific to the PSM but is involved in other biological events of many cell types. Further analyses will be required to understand the significance and mechanism of oscillation in Fgf signaling activity not only in the segmentation clock but also in other biological events.

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## **Figure legends**

Figure 1. Oscillatory expression of Hes7 in the PSM during somite segmentation. Spatiotemporal profiles of Hes7 expression are shown in the lower middle. The x axis represents time, while the y axis represents space. Anterior is top, and posterior is bottom. The spatial patterns of Hes7 expression at different time points are shown at both sides and on top. Note that the posterior end of the PSM grows posteriorly (downward).

Figure 2. Hes7-mediated coupled oscillations in Fgf and Notch signaling in the segmentation clock. Hes7 drives oscillatory expression of Dusp4, leading to pERK oscillation. Hes7 also drives oscillatory expression of Lfng, leading to oscillatory formation of NICD. Thus, pERK-Dusp4 and Lfng-NICD oscillations are coupled by Hes7 oscillations. Conversely, Hes7 oscillations are cooperatively regulated by Fgf and Notch signaling in the PSM.

Figure 3. Dynamic expression of NICD, pERK and Mesp2 (adapted from [26••]). The

posterior NICD domain moves anteriorly and narrows, while the pERK domain expands anteriorly, covering the NICD domain (middle two panels). After segmentation, pERK expression is down-regulated, and NICD now induces *Mesp2* expression in the S-1 region (panel furthest to the right). Thus, oscillations in Notch signaling periodically segregate a group of synchronized cells, and oscillations in Fgf signaling release these synchronized cells for somitogenesis at the same time.

Figure 4. Spatiotemporal profiles of NICD, pERK and *Mesp2* (adapted from [26••]). (A) Spatiotemporal patterns of NICD, pERK and *Mesp2* expression in the wild type. *Mesp2* expression is induced periodically by NICD in the whole S-1 region after pERK expression disappears. (B) Spatiotemporal patterns of NICD, pERK and *Mesp2* expression in *Hes7* KO mice. In *Hes7* KO mice, pERK expression steadily regresses, and *Mesp2* expression also steadily regresses in the anterior PSM after Fgf/ERK signaling is turned off. Thus, *Mesp2* expression occurs at different time between the anterior and posterior cells even in the same prospective somites.

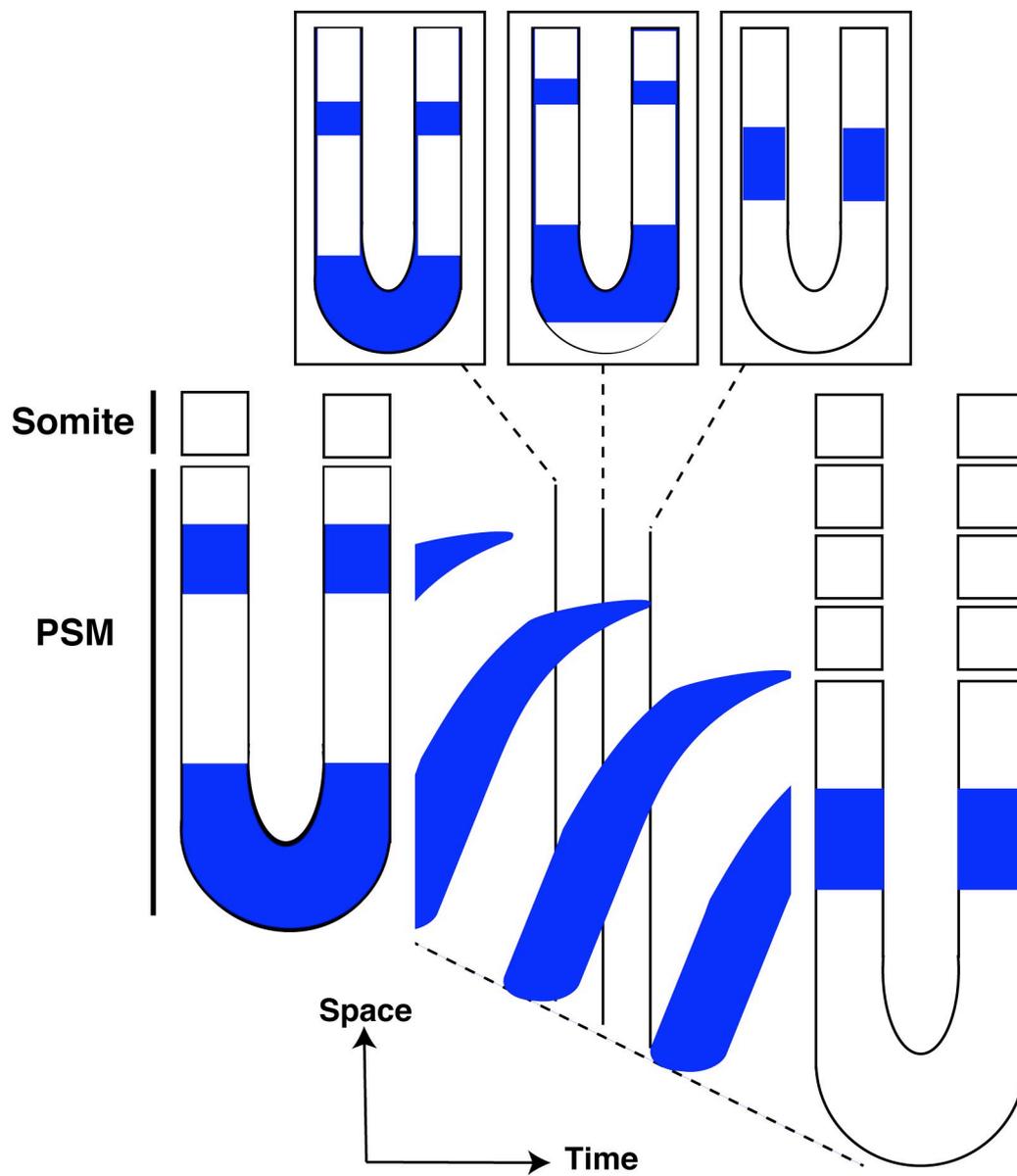


Figure 1

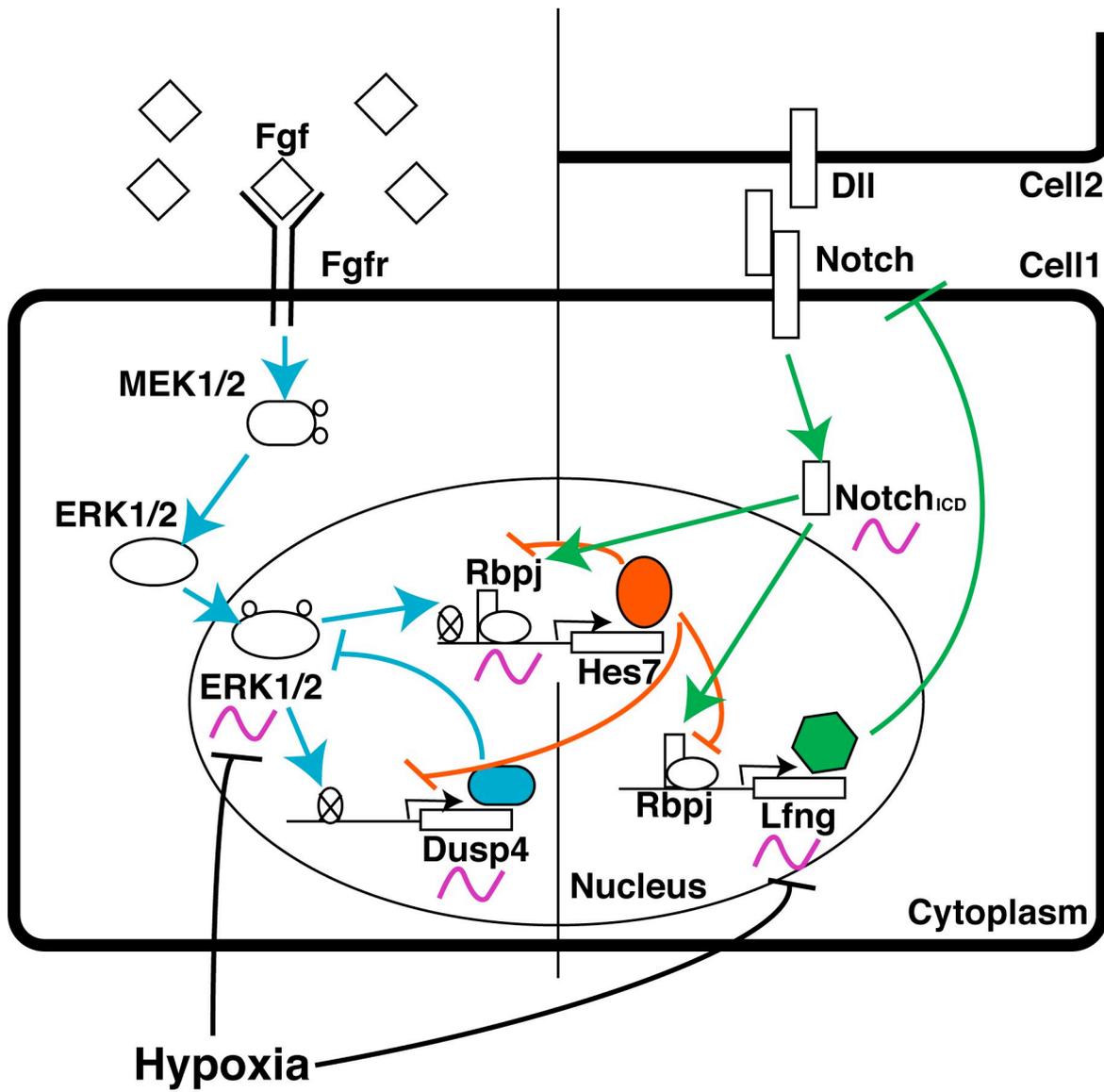


Figure 2

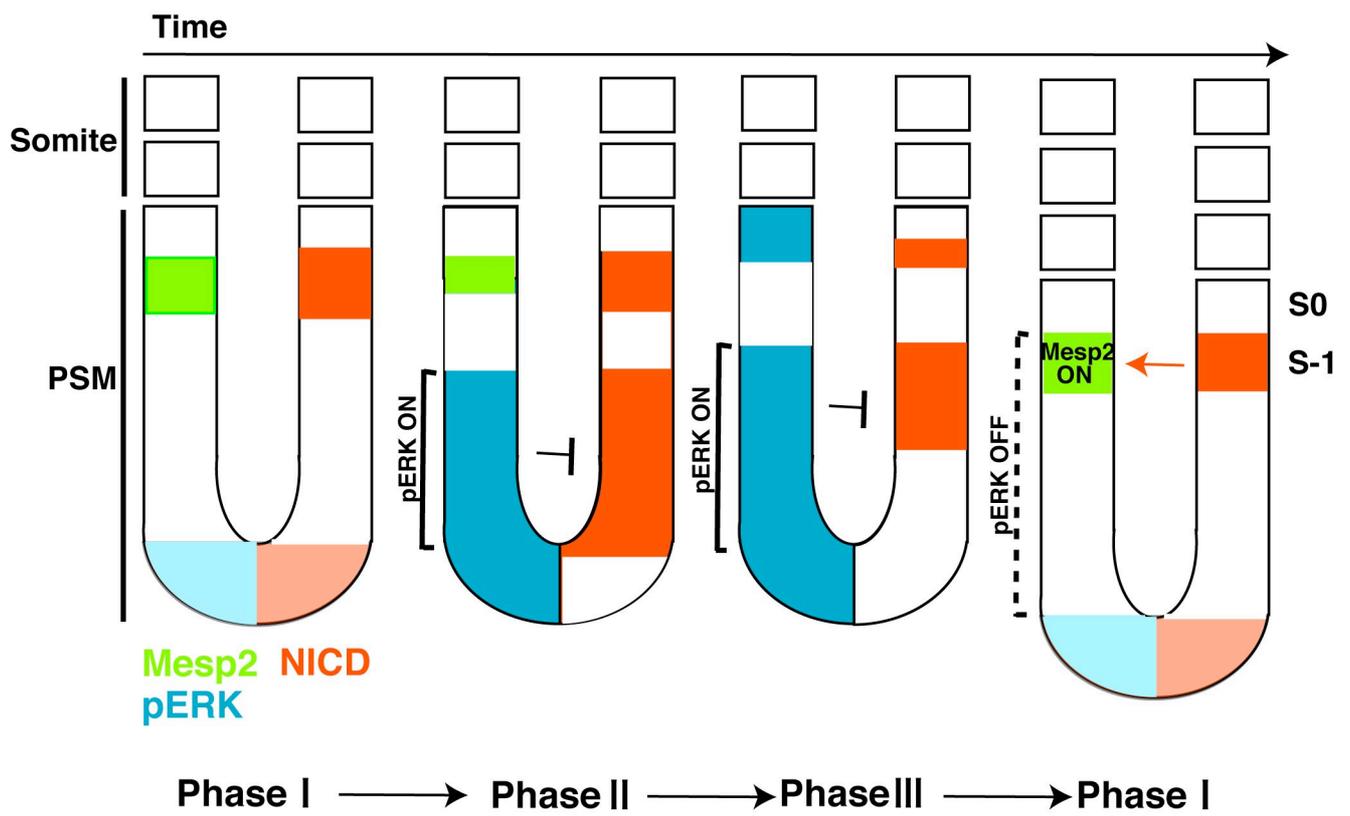


Figure 3

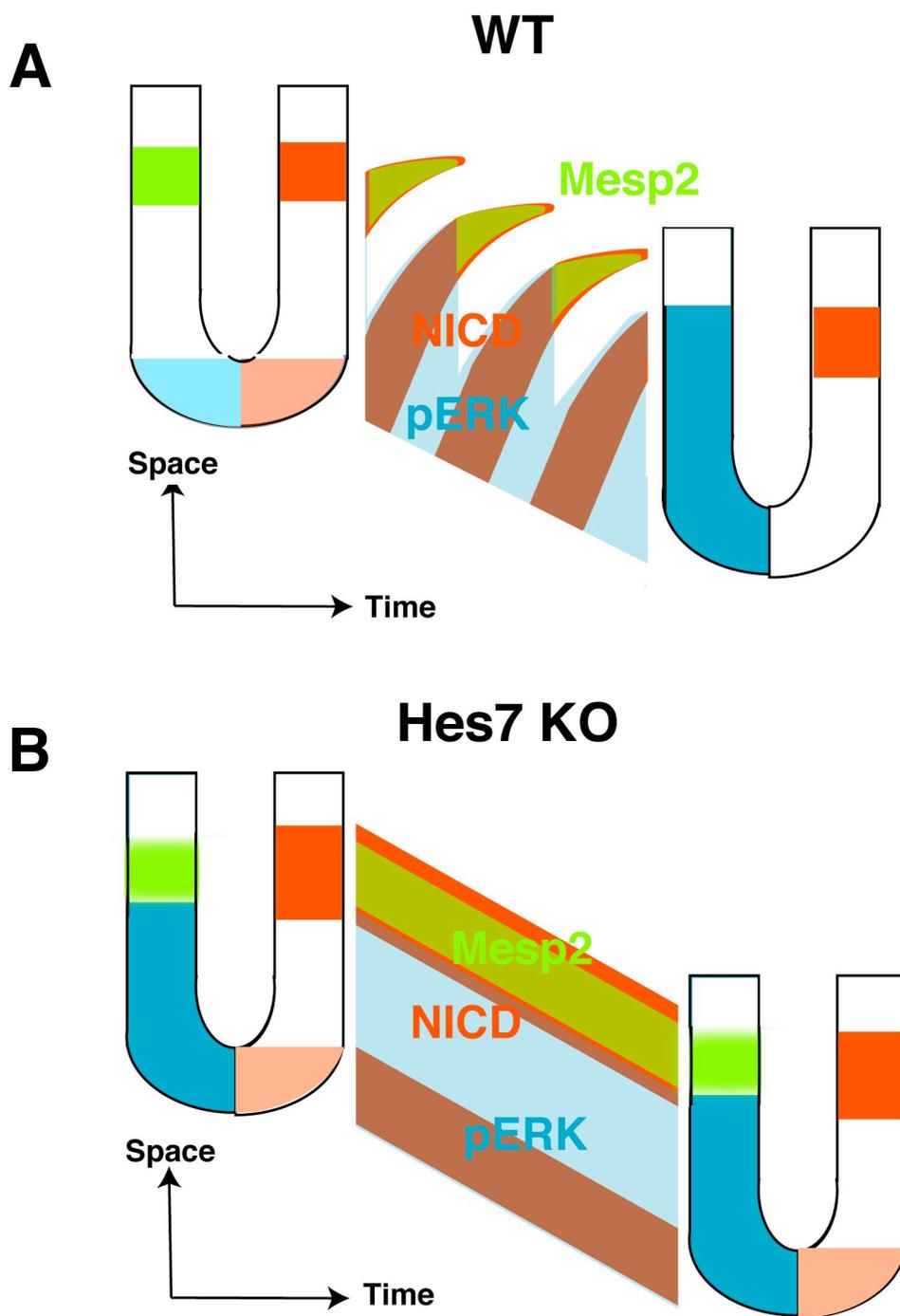


Figure 4