*Fgf4* is critical for maintaining *Hes7* levels and Notch oscillations in the somite segmentation clock.

Matthew J. Anderson¹, Valentin Magidson², Ryoichiro Kageyama³, and Mark Lewandoski¹

¹ Genetics of Vertebrate Development Section, Cancer and Developmental Biology Lab, National Cancer Institute, National Institutes of Health, Frederick, MD 21702, USA

² Optical Microscopy and Analysis Laboratory, Frederick National Laboratory for Cancer Research, Frederick, MD 21702, USA

³ Institute for Frontier Life and Medical Sciences, Kyoto University, Kyoto 606-8507, Japan
During vertebrate development, the presomitic mesoderm (PSM) is periodically segmented into somites, which will form the segmented vertebral column and associated muscle, connective tissue, and dermis. The periodicity of somitogenesis is regulated by a segmentation clock of oscillating Notch activity. Here, we examined mouse mutants lacking only $Fgf4$ or $Fgf8$, which we previously demonstrated act redundantly to prevent PSM differentiation. $Fgf8$ is not required for somitogenesis, but $Fgf4$ mutants display a range of vertebral defects. We analyzed $Fgf4$ mutants by quantifying mRNAs fluorescently labeled by hybridization chain reaction within Imaris-based volumetric tissue subsets. These data indicate that FGF4 controls Notch pathway oscillations through the transcriptional repressor, HES7. This hypothesis is supported by demonstrating a genetic synergy between $Hes7$ and $Fgf4$, but not with $Fgf8$. Thus, $Fgf4$ is an essential Notch oscillation regulator and potentially important in a spectrum of human Segmentation Defects of the Vertebrae caused by defective Notch oscillations.
Introduction

A common developmental mode employed by many embryos is a segmentation clock that oscillates within a posterior growth zone; with each cycle, a segment forms. This stratagem has evolved independently within the three major bilaterian clades (annelids, arthropods, and chordates) as well as in plants. In chordates, a segmentation clock oscillates in the presomitic mesoderm (PSM); with each cycle, a pair of somites form flanking the neural tube. Somites differentiate into dermis, skeletal muscle, tendons, as well as the vertebral column, which retains the segmented attribute of the somites.

In vertebrates, genes with an oscillatory pattern within the PSM include those encoding components or targets of the FGF, WNT and Notch signaling pathways. However, which individual genes oscillate differs among species, with the exception of the Notch-responsive HES/HER transcription factors, suggesting that Notch signaling is at the core of the somitogenesis clock. Supporting this idea, genetic and pharmacological manipulations demonstrate that Notch pathway oscillation in the mouse embryo is essential for somitogenesis. In the mouse, oscillatory waves of the Notch1 receptor and Delta-like 1 (Dll1) ligand expression, as well as the activated Notch receptor (cleaved Notch intracellular domain, NICD) sweep from the posterior to anterior, where oscillations arrest. These oscillations are established through negative-feedback loops of Notch components such as the transcriptional repressor, HES7, and the glycosyltransferase, LFNG, both of which are encoded by oscillating genes under Notch pathway regulation. Notch oscillations arrest in the anterior PSM, where NICD cooperates with TBX6 to periodically activate Mesp2 expression. MESP2
then regulates formation of the nascent somite as well as its rostro-caudal patterning, which is essential for normal patterning of the subsequent vertebral column.\textsuperscript{17,18}

Mutations in nearly all of the aforementioned Notch pathway genes have been identified in human patients where defective somitogenesis is thought to be the cause of Segmentation Defects of the Vertebrae (SDV)\textsuperscript{19,20}. For example, frequently recessive mutations in DLL3, \textit{HES7}, \textit{MESP2} and \textit{LFNG} and a dominant mutation in \textit{TBX6}\textsuperscript{21,22} have been identified in patients with spondylocostal dysostosis (SCDO), which is characterized by severe vertebral malformations that include hemivertebrae, vertebral loss and fusion along the length of the axis.\textsuperscript{23} Whereas SCDO is relatively rare, congenital scoliosis (CS), defined as a lateral curvature of the spine exceeding 10\%, is much more common, with a frequency of 1:1000, which is suspected to be an underestimation because asymptomatic individuals do not seek medical care.\textsuperscript{24} Mutated alleles of \textit{HES7}, \textit{LFNG}, \textit{MESP2}, and \textit{TBX6} are all associated with CS.\textsuperscript{22-25}

The position in the anterior PSM where Notch oscillations arrest and \textit{Mesp2} expression establishes the future somite boundary is called the determination front.\textsuperscript{15,18,26} This region is also the anterior limit of the wavefront in the classical clock-and-wavefront, a theoretical model proposed over 40 years ago to explain the precipitous and periodic formation of segments in the PSM.\textsuperscript{27} In this model, wavefront activity prevents the PSM from responding to the segmentation clock; hence somites form only anteriorly, where the wavefront ends. In chick or zebrafish embryos, exogenously added FGF protein or pharmacological inhibition of FGF signaling will shift the determination front rostrally or caudally, respectively. In the mouse, Cre-mediated inactivation of \textit{Fgf4} and \textit{Fgf8} specifically in the PSM results in an initial expansion of \textit{Mesp2}
expression, followed by the premature expression of somite markers throughout the PSM\textsuperscript{28}. Canonical WNT signaling is also a wavefront candidate, mostly because ectopic activation of $\beta$-catenin in the PSM results in an expansion of this tissue and a rostral shift of Mesp2 expression\textsuperscript{29,30}. However, this expansion observed in $\beta$-catenin “gain-of-function” mutants requires FGF4 and FGF8 activity, suggesting that these FGF signals are synonymous with the wavefront\textsuperscript{28}.

In addition to its role in preventing somite differentiation, FGF signaling is also implicated in the regulation of key Notch components of the segmentation clock. Several studies have demonstrated that pharmacological inhibition of FGF disrupts Notch oscillations\textsuperscript{31,32}. In mouse embryos with PSM-specific loss of FGF receptor 1 or both Fgf4 and Fgf8, Notch pathway genes such as Hes7, are downregulated, although this analysis can be complicated due to the loss of PSM tissue in these mutants\textsuperscript{28,32}. Here, we focus on FGF-Notch interactions by analyzing mouse mutants that lack only one of the wavefront Fgf genes, Fgf8 or Fgf4. An indispensable technique in our analysis is whole mount \textit{in situ} hybridization chain reaction (HCR), which allows us to multiplex different gene expression domains and quantify mRNA levels within specific embryonic tissues\textsuperscript{33-35}. We demonstrate that, while Fgf8 is not required for somitogenesis, Fgf4 is required for normal Notch oscillations and patterning of the vertebral column.
Results

FGF4 activity is required in the presomitic mesoderm for normal segmentation of rostral somites.

To analyze the role of Fgf4 or Fgf8 expression in the primitive streak and PSM (Figure 1A - B), we inactivated each gene specifically within these tissues using TCre transgenic activity, thus generating “Fgf4 mutants” (TCre; Fgf4 flox/Δ; “Δ”= “deleted” or null) or “Fgf8 mutants” (TCre; Fgf8 flox/Δ; see Table 1). Whereas Fgf8 mutants do not survive much beyond birth due to kidney agenesis, Fgf4 mutants are viable and found at Mendelian ratios at weaning (n = 18 controls and n = 21 mutants). Skeleton preparations of Fgf8 mutant embryos at E18.5 (n = 22) revealed all vertebral bodies were present and normally patterned. Fgf8 mutants also presented with minor cervical and/or lumbar homeotic transformations, each with incomplete penetrance and expressivity: small ribs were sometimes present in the most posterior cervical vertebra (8/22, 4 bilateral and 4 unilateral) or on the most anterior lumbar vertebra (8/22, 4 bilateral and 4 unilateral). On the other hand, Fgf4 mutants display a variety of segmentation defects in the cervical and thoracic vertebrae with 100% penetrance (Compare Figure 1D, D’ with C). An average of 7.9 defects occurred per mutant and consisted of hemivertebrae, and misshapen and deleted vertebrae (Figure 1E).

We examined gross somite patterning in Fgf4 mutants by staining embryos at various stages for Uncx4.1 mRNA, which marks the posterior somite compartment. Analysis of 39 Fgf4 mutants revealed a frequency of irregular Uncx4.1 expression specifically in future cervical and thoracic somites with full penetrance (Figure 1F-J).
To determine if these malformed somites were due to an error in segmentation, we examined *Mesp2* expression, which occurs in the anterior presomitic mesoderm (PSM) and is required for normal somite segmentation and rostral-caudal somite identity. At the stages when irregular somites are emerging from *Fgf4* mutant PSM, we detected aberrant *Mesp2* expression, whether we analyzed gene expression by traditional wholemount *in situ* hybridization (WISH) staining (Figure 1K-L) or by fluorescent HCR analysis (Figure 2A, B). However, at later stages (22-24 somite stages), when properly segmented somites are emerging from *Fgf4* mutant PSM, *Mesp2* expression appears normal (Figure 1 - figure supplement 1). Therefore, we conclude that this indistinct pattern of *Mesp2* expression at early stages is likely responsible for the vertebral defects in *Fgf4* mutants.

**Wavefront gene expression is normal in *Fgf4* mutants**

For the rest of our mRNA expression analysis we mostly relied on whole mount *in situ* HCR in conjunction with Ce3D++ tissue clearing. Because the fluorescent signal intensity corresponds proportionally to the number of mRNA molecules, HCR allows for quantitative and multiplexed mRNA detection. Accurate quantification of the HCR signal requires a method such as Ce3D++ tissue clearing (see Materials and Methods), which allows for imaging in deep tissues without signal loss.

We first addressed why a distinct and symmetrical band of *Mesp2* expression fails to form in *Fgf4* mutants. *Mesp2* expression is normally triggered by activated Notch (NICD) and TBX6 at the determination front in the anterior PSM, where cells fall below a critical threshold of wavefront FGF signaling. We previously showed that this FGF activity was encoded by *Fgf4*
or Fgf8; if we inactivated both Fgfs with TCre (Fgf4/Fgf8 double mutant), Mesp2 expression is transiently activated throughout the PSM, which then aberrantly expresses the paraxial mesoderm marker, Meox1. To determine if the wavefront position is altered in Fgf4 mutants we examined the anterior-posterior length and expression levels of genes responsive to this activity at early somite stages when aberrant Mesp2 expression occurs. Both Msgn1 and Tbx6 are required to specify the paraxial mesoderm and are silenced in the absence of wavefront activity in Fgf4/Fgf8 double mutants. Neither the length nor level of expression of Tbx6 and Msgn1 were significantly altered in Fgf4 mutants (Figure 2C, C”, D, D”, E, F). Consistent with this observation, the paraxial differentiation marker, Meox1, was not expanded into the PSM (Figure 2 C’ and D’), as occurs in Fgf4/Fgf8 double mutants.

Therefore, we conclude that wavefront activity is normal in Fgf4 mutants, an insight consistent with the observation that normal axis extension occurs in these mutants, with no loss of caudal vertebrae. We surmise that this normal wavefront position and signaling in Fgf4 mutants are likely maintained by Fgf8, which is expressed at normal levels (Figure 3A-C) and is sufficient for maintaining normal expression levels of the FGF target genes, Spry2, Etv4, and Spry4 (Figure 3D-F’’’); these FGF targets are silenced in Fgf4/Fgf8 double mutants. Therefore, neither a change in wavefront activity nor a change in determination front position explains the aberrant pattern of Mesp2 expression in Fgf4 mutants.

Oscillation of Notch family components is altered in the PSM of Fgf4 mutants. We then examined the pattern of activated Notch in Fgf4 mutants by immunostaining for Notch intracellular domain (NICD), which is an obligate factor in the transcriptional complex that
activates *Mesp2*\(^{17}\). Overall NICD levels were unchanged between *Fgf4* mutants and littermate controls at the embryonic stages when somitogenesis was abnormal (Figure 4A). At these stages, we always observed two to three distinct stripes of NICD along the anterior-posterior axis in both mutant and littermate control PSM (Figure 4B-C), demonstrating the oscillatory nature of Notch activation\(^{12,15,43}\). However, mutant oscillatory stripes of NICD were always less distinct, compared to controls. This was more evident when the relative intensities of NICD immunostaining signals were modeled using Imaris software. In controls, cells medial-lateral to each other had similar levels of activated NICD, whereas in mutants, this coordination was less distinct (Figure 4B’-C’). Importantly, this blurred pattern occurred in the anterior PSM, where NICD activates *Mesp2* expression at the determination front (Figure 4B’-C’, brackets).

A normal NICD pattern is achieved through a number of negative feedback loops including the transcriptional repressor, HES7\(^{14}\) and the glycosyltransferase, LFNG\(^{15}\); disruption of either gene encoding these factors causes uncoordinated Notch signaling\(^{14}\). *Hes7* and *Lfng* are themselves targets of activated Notch and therefore their expression oscillates\(^{14}\). Although oscillatory patterns of *Hes7* and *Lfng* are apparent in both *Fgf4* mutants and littermate controls (Figure 4D-E’’), the mutant patterns are clearly uncoordinated and indistinct, similar to the NICD immunostained mutant (Figure 4D-E’’). Together, these data suggest the aberrant *Mesp2* pattern in *Fgf4* mutants can be explained by imprecise oscillations of Notch signaling.

**Hes7 expression is reduced in the PSM of *Fgf4* mutants**

We proceeded to analyze the expression of *Hes7* in greater detail in *Fgf4* mutants. *Hes7* is within the *Hes/Her* class of transcriptional repressors that are the only oscillating clock genes conserved
amongst mouse, chicken, and zebrafish. In the mouse, mutations that accelerate HES7 production will accelerate the tempo of the segmentation clock. Hence, Hes7 is considered to be a fundamental pacemaker of the segmentation clock that controls somitogenesis.

Analysis of Hes7 mRNA in Fgf4 mutants at 5-6 somite stages, using conventional WISH, revealed a reduced and aberrant expression pattern in Fgf4 mutants (Figure 5- figure supplement 1). Analysis with HCR was much more informative, allowing unambiguous pattern analysis (Figure 4) and quantification (Figure 5, Figure 5-figure supplement 2, Figure 5- figure supplement 3). Characterization of an oscillatory gene expression pattern in the PSM is achieved by classifying static expression patterns into three phases. This approach has been used to characterize Hes7 expression at E9.5-E10.5, when one to two stripes of expression are observed. However, in the E8.5 PSM, when aberrant Notch signaling occurs in Fgf4 mutants, there are two to three Hes7 expression stripes in both controls and Fgf4 mutants, indicating a faster somitogenesis clock at this stage (Figure 4, 5). Therefore, we generated criteria for classification of phases at E8.5 as follows. Phase I: three stripes with the most posterior stripe limited to the posterior midline and the most anterior stripe having reached the anterior boundary of the PSM (Figure 5A). Phase II: two stripes with the posterior stripe having expanded laterally compared to phase I and the anterior stripe having not reached the anterior limit of the PSM (Figure 5B). Phase III: two distinct stripes and a third stripe initiating at the posterior midline (Figure 5C).

Control embryos are distributed nearly equally between each phase (Figure 5G) as is the case for Hes7 expression in older embryos. It is challenging to place Fgf4 mutants in phases, emphasizing the aberrant Hes7 expression pattern. However, in our assessment, we allocated
Fgf4 mutants within all three phases (Figure 5D-F), with most found in oscillation phase II (Figure 5G).

*Hes7* is expressed in both the PSM and neural ectoderm. To quantify expression only in the PSM, we used Imaris software to create a volumetric model of the PSM, based on *Tbx6* expression, which is limited to the PSM at these stages. By measuring the intensity of the *Hes7* HCR signal within this volume, we obtained a PSM-specific *Hes7* quantification for each embryo (see Figure 5 - figure supplement 3 and Material and Methods). PSM-specific *Hes7* expression is reduced in Fgf4 mutants to 51.8% of that expressed in litter mate controls (Figure 5H). A comparison between mutant and control *Hes7* expression levels within each phase reveals that the reduction in Fgf4 mutants is not due to the difference in phase allocation between Fgf4 mutants and littermate controls (Figure 5 - figure supplement 4). Moreover, after the 22 somite stage, when forming somites are normally segmented in mutants (Figure 1J), there is no significant difference in *Hes7* PSM expression levels between Fgf4 mutants and controls (Figure 5 - figure supplement 5). Therefore, we conclude that a reduction of *Hes7* expression may account for the defective segmentation during rostral somitogenesis in Fgf4 mutants.

To correlate *Hes7* expression levels with pattern, we created Imaris-generated spot models of the HCR signal, which correspond to a single mRNA molecule or clusters of mRNA molecules within a subcellular-volume (see Materials and Methods). We then colored-coded individual spots to reflect the intensity of the *Hes7* HCR signal, using a linear series of intensity-based cutoffs every 20% to generate five colors (quintiles) (Figure 6A-B). In controls, the lowest intensity quintile (blue) contains the most spots (40%) and each higher intensity group contains
10% fewer spots (Figure 6A, white bars in C). The distribution of spots in the Fgf4 mutant, compared to controls, is significantly skewed towards the lowest quintile at the cost of higher-level expression spots (Figure 6B, black bars in C). This modeling provides an effective illustration of the pattern of Hes7 expression.

In control embryos, each stripe of Hes7 expression contains a concentric gradient of signal intensity with highest expression at the center (white spots) and lowest expression at the outside (blue spots) (Figure 6A, D). Fgf4 mutants maintain some of this concentric organization, but the boundaries between groups is less clear (Figures 6B, E); in particular the trough between peaks of Hes7 expression is more shallow, and contains more higher-intensity spots (mostly second quintile, green) than littermate controls (Figures 6D’ and E’). To determine if this indicated a failure to repress Hes7 transcription in these regions, we performed HCR using a probe that hybridized to the Hes7 intronic sequences, and therefore specific to newly transcribed pre-mRNA. This analysis revealed that transcription of Hes7 is reduced in the posterior PSM and is more widespread, extending into the trough between Hes7 mRNA peaks in the Fgf4 mutant (white brackets in Figure 6 F’, G’). Therefore, we hypothesized that FGF4 is required to maintain Hes7 transcription in the PSM above a threshold required for normal Notch oscillation. The reduced level of HES7 in Fgf4 mutants is insufficient to fully autorepress its own expression, resulting in an aberrant pattern of expression.
A synergistic defect in Fgf4/Hes7 mutants reveals that Fgf4 is required to maintain Hes7 above a critical threshold

To test our hypothesis that a reduction of Hes7 expression causes the Fgf4 mutant vertebral defects, we asked if these defects worsen if we further reduce Hes7 expression by removing one gene copy. We compared such mutant to littermate controls that were simple Fgf4 mutants (with two wildtype Hes7 alleles) or Hes7 heterozygotes. These Hes7 heterozygotes also carried TCre and one floxed Fgf4 allele (see Table 1, and Figure 7) resulting in Fgf4 heterozygosity in the TCre expression domain. However, Fgf4 heterozygosity had no effect on the phenotype because the defects we observed were similar to the Hes7 heterozygous defects reported by the Dunwoodie lab. About 50% of our Hes7 heterozygotes had defective lower thoracic vertebrae with a frequency of 2.8 defects per animal. In littermate Fgf4 mutants, vertebral defects were completely penetrant, with an average of 7.7 defects per animal, a frequency similar to that of progeny in our original genetic cross (Figure 1). However, compound Fgf4/Hes7 mutants displayed a large set of defects with 25 defects per animal (Figure 7C-E), a frequency significantly greater than littermate Fgf4 mutants (3-fold greater, p < 0.005) or Hes7 heterozygotes (9-fold greater, p < 0.001). These data indicate a synergistic, as opposed to additive, effect of loss of one Hes7 allele in the Fgf4/Hes7 compound mutant.

We performed a similar genetic analysis where we determined if Hes7 heterozygosity likewise affects Fgf8 mutant defects. We examined littermates of the last cross described in Table 1 (Figure 7 – figure supplement 1). Fgf8 mutants in this cohort displayed the same homeotic transformations we previously observed with incomplete penetrance and expressivity: small ribs in the posterior cervical vertebra (3/5, 2 bilateral and 1 unilateral) or anterior lumbar vertebra...
(3/5, 1 bilateral and 2 unilateral). Hes7 heterozygotes (also heterozygous for a floxed Fgf8 allele) displayed a nearly identical frequency (2.7 defects per animal) as observed in the Fgf4/Hes7 experiment (Figure 7A). Vertebral defects in compound Fgf8/Hes7 mutants were not synergistic (4 defects per animal) but were clearly the Hes7 heterozygous defects added to the relatively mild Fgf8 defects (1.8 defects per animal, Figure 7 – figure supplement 1). Therefore, we demonstrate that the relationship between Hes7 and Fgf4 is unique in that there is no genetic interaction between Fgf8 and Hes7.

We then examined levels and spatial patterns of Hes7 mRNA expression in each genotype from the Fgf4/Hes7 mutant littermates, specifically within a volumetric model of the Tbx6 expression domain, as we had in Fgf4 mutants (Figure 5, 6). We observed a reduction in Hes7 expression that correlated with the severity of vertebral defects within each genotype (Figure 7E). Hes7 null heterozygotes had only a 19% reduction in expression, presumably because a loss of HES7 auto-repression results in enhanced transcription from the remaining wildtype allele. Such compensatory upregulation fails in compound Fgf4/Hes7 mutants because we observed an 80% Hes7 reduction to occur in these embryos (Figure 7E). Analysis of intensity-colored spot models of PSMs of these genotypes suggests a threshold effect of Hes7 levels on oscillatory gene expression (Figure 7F-I). Oscillations appear relatively normal if Hes7 levels are reduced 19% (in Hes7 heterozygotes, Figure 7G) and disordered with a 33% reduction (in Fgf4 mutants, Figure 7H). The synergistic 80% reduction of Hes7 that occurs in compound Fgf4/Hes7 mutants causes a severe dampening of oscillatory gene expression (Figure 7I). The resulting vertebral defects, though relatively severe (Figure 7A-D), are not as extreme as occurs in Hes7 null homozygotes ⁴⁷, indicating that this reduced level of Hes7 supports limited patterning during
segmentation. Together, our data support our hypothesis that FGF4 acts to maintain *Hes7* above a necessary threshold for normal somitogenesis. In *Fgf4* mutants, a reduction in *Hes7* levels, generates uncoordinated, asynchronous Notch oscillations. Disordered Notch oscillations initiate *Mesp2* unevenly in the anterior PSM leading to improperly shaped somites and subsequently, malformed vertebrae.
Discussion

Here we describe mutants with only $Fgf4$ or $Fgf8$ inactivated specifically in the PSM. We found that $Fgf4$ mutants display a range of cervical and thoracic vertebral defects that are caused by defective Notch oscillations during somitogenesis. In contrast, the vertebral columns of $Fgf8$ mutants are normally segmented, with about 30% displaying minor homeotic transformations; such alternations in vertebral identity are not likely due to defects in somitogenesis per se.

Previously, a role for FGF signaling in wavefront activity in chick and zebrafish embryos was supported by pharmacological manipulation $^{26,50}$ and in mouse mutants where both $Fgf4$ and $Fgf8$ are simultaneously inactivated using the same TCre activity that we use in this study $^{28}$. These double $Fgf4/Fgf8$ mutants as well as mutants with tissue-specific inactivation of $Fgfr1$ support a role for FGF signaling in clock oscillations, but these conclusions were complicated by a loss of PSM tissue due to potential wavefront defects $^{16,28,31,32}$. Compared to these Fgf pathway mutants, the phenotype of $Fgf4$ mutants is relatively subtle, with no wavefront defect, as indicated by no loss of caudal vertebrae $^{42}$ and no quantitative change in wavefront gene markers (Fig 2). This lack of any overt axis extension defect allows us to unambiguously identify $Fgf4$ as an Fgf ligand gene required for a normal segmentation clock. This insight is supported by recently published in vitro work on the human segmentation clock $^{51}$.

Our mutants model human Segmentation Defects of the Vertebrae (SDV); $Fgf4$ mutants resemble CS and the synergistic phenotype resulting from the additional removal of one $Hes7$ copy in these mutants resembles human SCDO. Both of these diseases are caused by mutations in Notch signaling components that we find are misexpressed in $Fgf4$ mutants, such as $HES7$, 

MESP2, and LFNG. With regard to the human FGF pathway and SDV, a straightforward gene-disorder relationship has not been uncovered, probably because of the pleiotropic phenotypes of such putative mutations would preclude embryonic survival. Sparrow et al. found that gestational hypoxia in mice results in an increase in the severity and penetrance of CS in Notch1, Mesp2, and Hes7 heterozygotes. Intriguingly, CS is more severe in children living at high altitudes, suggesting a similar environmental effect may affect human development. In mice, hypoxia was found to diminish FGF signaling components, but expression of Fgf8 was unchanged, and Fgf4 was unexamined. Our data suggest that FGF4 may be part of the system that is responsive to this environmental insult. However, if this is the case, reduced FGF4 activity cannot be the only response to reduced oxygen levels because gestational hypoxia reduces expression of the FGF target gene Spry4 in the PSM, whereas we found no change in expression in this or other such canonical FGF target genes in Fgf4 mutants.

Rather, as is the case for hypoxia-treated embryos, Hes7 expression is reduced in Fgf4 mutants and we propose that this leads to aberrant segmentation. An indispensable tool to this insight was the use of multiplex HCR imaging. The simultaneous fluorescent imaging of multiple mRNA domains with this technique is particularly useful in a complex embryonic process, such as somitogenesis, where many complex and dynamic gene expression patterns need to be analyzed. HCR is a relatively new tool for mutant embryo analysis, just beginning to be used to analyze mutant embryos. We used HCR to examine the expression of multiple genes in a single tissue by combining it with Imaris image analysis software. This combination of HCR and Imaris modeling is broadly applicable for in situ quantification of gene expression within any complex embryonic and clinical sample. With distinct molecular markers, one can use this
approach to quantify gene expression in any cell population with a precision that heretofore was not possible and still retain the intact whole mount embryo or tissue. Here, we generated and analyzed Imaris-based volumetric models of the PSM, based on Tbx6 expression, and determined that Hes7 levels are reduced by about 40-50% specifically in the PSM of Fgf4 mutants during early somite stages when the progenitors of aberrant vertebrae are segmenting.

We propose that this reduction in Hes7 expression is the primary defect in Fgf4 mutants, and subsequently causes an irregular activated Notch (NICD) pattern, misexpression of Mesp2, and ultimately leads to vertebral defects. Given the molecular feedbacks during genetic oscillations in the PSM, we considered other possible models, but they do not fit our data. For example, we observed aberrant expression of Lfng, which encodes a glycosyltransferase that modulates Notch signaling. Although embryos completely lacking Lfng display aberrant Hes7 oscillations, overall Hes7 mRNA levels are not reduced, unlike the case in our Fgf4 mutants. Another factor required for Hes7 transcription is encoded by Tbx6, but we found no significant difference in expression of this gene. We support our proposal that the observed 50% reduction in Hes7 is the cause of the Fgf4 mutant with the synergistic worsening of the vertebral segmentation defect that occurs when we further reduce Hes7 levels by removal of one wildtype allele. From another perspective, the mild CS caused by loss of one Hes7 allele in a wildtype background is worsened 9-fold by removing Fgf4 (Figure 7). Therefore, Fgf4 is a robustness-conferring gene that buffers somitogenesis against a perturbation in Hes7 gene dosage.

We observed that in Fgf4 mutants Hes7 mRNA levels are reduced during rostral somitogenesis at E8.5 (5-6 somite stages), but are unaffected at about E9.5 (24-26 somite stages) when the
embryo is beginning to generate correctly patterned somites that will differentiate into normally patterned vertebrae. Tam showed that between these two stages of mouse development, somites more than double in size. Such an increase can be due to a slower segmentation clock and/or faster regression of the determination front. Gomez et al. found that the caudal movement of the wavefront does not significantly change at these stages; therefore the clock must slow between E8.5 and E9.5. Consistent with a slowing clock, we observed at least 2 and frequently 3 bands of Hes7 expression at the Fgf4-sensitive stages (5-6 somite stages; Figure 4, 5), but only 1 to 2 bands when Fgf4 loss has no effect on Hes7 expression (24-26 somite stages; Figure 5 - figure supplement 5). Thus, it appears that FGF4 activity is required to maintain Hes7 mRNA levels above a certain threshold when the segmentation clock is faster; when Notch oscillations slow, they may become FGF-independent, or other FGFs may be at play. If this is the case in all vertebrates, we might expect embryos with faster segmentation clocks, such as snakes, to have a longer window of FGF4-dependence for normal somitogenesis.

This study and past work indicate that FGF4 and FGF8 have both shared and unique roles in the vertebrate embryo’s posterior growth zone. They redundantly encode wavefront activities, preventing PSM differentiation. Here, we show that FGF4 uniquely regulates Notch oscillations that control segmentation. Future efforts will explore why Fgf8 loss-of-function causes the incompletely penetrant homeotic transformations that we observed - possibly FGF8 regulates Hox gene expression. These insights are intriguing in the context of the evolution of the vertebrate body plan. Vertebrates, together with Cephalochordates and Urochordates make up the phylum Chordata. Somitogenesis predates vertebrate evolution as it occurs in Cephalochordata, the most basal living chordates. In both Cephalochordates and Urochordates,
the FGF essential for embryonic axis extension is an *Fgf8* ortholog\(^{69,70}\), suggesting that this *Fgf* ("*Fgf8/17/18*") may be the ancestral gene in this process. However, *Fgf8/17/18* activity in these invertebrate chordates does not control gene oscillations as these embryos apparently lack a segmentation clock\(^{69,71}\). Therefore, we speculate that the recruitment of an *Fgf4* role during the evolution of vertebrate axis extension may have coincided with the development of gene oscillations that control somitogenesis.

**Materials and Methods**

**Alleles, Breeding and Genotyping**

All mice were kept on a mixed background. All genetic crosses are shown in Table 1, with the female genotype shown first and the male genotype shown second. PCR-genotyping for each allele was performed using the following primer combinations, *Fgf4flo*\(^{72}\) (5'-CAGACTGAGGCTGGACTTGAGG and 5'-CCTCTTGGGATCTCGATGCTGG), *Fgf4Δ*\(^{72}\) (5'-CTCAGGAACCTCTGAGGTAGATGGGG and 5'-ATCGGATTCACCTGCAGGTGC), *Fgf8flo*\(^{73}\) (5'-GGTCTTTCTTAGGCTATCCAC and 5'-GACAAGAGGGCCAGGTGGCAGGTG), *Fgf8Δ*\(^{73}\) (5'-CCAGAGGTGGAGTCTCAGGT and 5'-ATCGGATTCACCTGCAGGTGC), *Hes7Δ*\(^{47}\) (5'-AGAAGGGCATTTTTTCTCTCT and 5'-TTGGCTGCAGCCCGGGATCCCC), TCre\(^{36}\) (5'-GGACCCATTTTCTCTTCT and 5'-CCATGAGTTACACCGACCTGG)
HCR

Hybridization chain reaction fluorescent in situ was carried out as described with the modification of using 60 pmol of each hairpin per 0.5 ml of amplification buffer. Hairpins were left 12-14 hours at room temperature for saturation of amplification to achieve highest levels of signal to noise. Stained embryos were soaked in DAPI solution (0.5 ug/mL DAPI in 5x SSC with 0.1% TritonX-100, 1% Tween20) overnight at room temperature. Split initiator probes (V3.0) were designed by Molecular Instruments, Inc.

Whole Mount Immunohistochemistry, Colorimetric WISH, and Skeletal Staining

Notch Intracellular Domain staining (NICD) was performed following a significantly modified protocol; briefly, embryos were dissected in cold PBS, briefly fixed for 5 minutes in 4% PFA, then fixed for 35 minutes in equal parts DMSO:Methanol:30% Hydrogen Peroxide at room temperature. They were then rinsed 3 x 5 minutes in 50mM Ammonium Chloride at room temperature, blocked in TS-PBS (PBS, 1% Triton X-100, 10% Fetal Calf Serum) for 30 minutes at room temperature, then incubated overnight in 1:100 anti-NICD (CST #4147) in TS-PBS at 4°C rocking. The next day, embryos were washed 4 x 10 minutes in TS-PBS, then incubated overnight in anti-rabbit-alexa647 (ThermoFisher A32733) 1:100 in TS-PBS at 4°C rocking. Embryos were then washed 4x 10 minutes in TS-PBS then soaked overnight in DAPI, as described above in the HCR section, and embedded and cleared as described below. WISH and skeletal staining were performed as previously described.
Embedding and Clearing

Embedding: Stained embryos were mounted in coverslip bottomed dishes suspended in ultra-low gelling temperature agarose (Sigma, A5030) that had been cooled to room temperature. Once correct positioning of embryos was achieved the dishes were moved to ice to complete gelling.

Clearing: For tissue clearing we utilized Ce3D+, a modified version of Ce3D (Gerner et al., 2017), in which the concentration of iohexol (Nycodenz, AN1002424, Accurate Chemical & Scientific Corp) is increased to match the refractive index (RI) of the mounting solution to that of standard microscopy oils (nD = 1.515; not utilized in this study) and 1-thioglycerol is omitted to reduce toxic compounds within the solution (thereby making the solution safer to handle) and increase shelf life. To account for dilution by water from a sample (including agarose volume) we used Ce3D++, in which the iohexol concentration is further increased. Ce3D++ was designed to produce desired RI after two incubations - detailed protocol is available upon request. The protocol for preparing these solutions is as follows: in a 50 mL tube, add iohexol powder (20 g for Ce3D or 20.83 g for Ce3D++), then 10.5 g of 40% v/v solution of N-Methylacetamide (M26305, Sigma) in 1X PBS, and then 22.5 mg Triton-X-100 (T8787, Sigma) for a final concentration of 0.1%. The solution is mixed overnight at 37 C on an orbital rocker with intermittent vortexing then stored at room temperature. Embedded embryos were cleared using 2 changes of Ce3D++ solution while rocking at room temperature for a twenty-four-hour period.

Imaging

All images were obtained on an Olympus FV1000 confocal microscope, with an image size of 1620 x 1200 pixels and with a Kalman averaging of 3 frames. To capture the entire tissue a 10x
UPlanApo objective (NA= 0.4) was used achieving a pixel size of 0.9um x 0.9um. Tissues were oriented in the same way with the anterior-posterior axis of the tissue oriented from left-right within the center of the field. Microscope settings were kept consistent between imaging. Intensity calibration and shading correction was performed as previously described, however fluorophore dye concentrations used for shading correction were 0.05mg/L for fluorescein (Sigma 46960), 1mg/L for acid blue 9 (TCI CI42090), and 2mg/L for rose bengal (Sigma 198250). Dyes were placed in coverslip bottomed dishes. The Shading Correction plugin within Fiji was then used with the median flat field images acquired using the dye solutions.

**Image processing**

Images within figures were processed using Fiji and represent max projections of z-stacks. Compared images are presented with identical intensity ranges for each channel. Orthogonal projections were made using Imaris software (Imaris V9.2.1, Bitplane Inc).

**Statistical analysis**

For all analysis at least 3 embryos were used unless otherwise stated in the text or caption. Significance was determined using a Students two-tailed t-test.

**Imaris Fluorescence Quantification and Modeling**

**HCR data:** Flat-fielded image stacks were imported from Fiji into Imaris (Imaris V9.2.1, Bitplane Inc). A baseline subtraction was then performed for each probe, using a cutoff value specific for each probe and fluorophore combination. For all probes, except Hes7, the baseline cutoff was adjusted until signal from a tissue known not to express the gene was no longer
detectable (e.g. neural epithelium for Tbx6 \textsuperscript{40,49}. For Hes7, this cutoff was determined by using embryos that were homozygous for a Hes7 null allele \textsuperscript{47} that lacked sequences complementary to the HCR probes; the cutoff was chosen that resulted in no signal in these mutants (Figure 5 - figure supplement 5 ).

The Surface model tool was used to build surfaces for each expression domain to be quantified except for Fgf8 and Hes7, which were quantified using a surface derived from the Tbx6 expression domain. Quantification of Spry2, Spry4 and Etv4 were determined by measurement of fluorescence intensity per cubic micrometer within the volume the respective gene expression domain. Surface models were generated using a surface detail value of 3uM, absolute intensity setting, and an absolute intensity threshold cutoff that was set to exclude background signal in tissues known not to express the gene being modeled. Once established for each probe, the same intensity threshold cutoff values were used for generating surfaces for all embryos. For generating Tbx6 surfaces, a range of absolute intensity threshold cutoffs were used to achieve a final surface volume between 9.0e6 and 1.1e7 um\(^3\). Values for mRNA expression represent the intensity sum within the volumetric model divided by the volume of the model.

**Immunostaining data:** In images where NICD was immunostained, the surface creation tool was used. The mesoderm was selected, eliminating the ectodermal and endodermal tissue, on alternating z-planes through the entirety of the z-stack. The resulting pattern was interpolated for intervening planes. Values for NICD expression represent the intensity sum within the volumetric model divided by the volume of the model.

**Spot modeling:** Spot diameter was set to 4uM with a point spread function value of 8uM, local background subtraction was used, and sum intensity of the channel being modeled was used to threshold. Threshold cutoff values for spot models were set at 1,500 for all figures except Figure 23.
6. In Figure 6 the thresholds values (a.u.) for the sum intensity are: low (blue), 1,000-6,000; low-medium (green), 6,000-11,000; medium (yellow), 11,000-16,000; medium-high (red), 16,000-21,000; high (white), 21,000 or greater. “Heat maps” in Figures 4 and 7 were generated within the Imaris software using a linear color scale; the scale in Figure 4 ranged from intensity values of 7,000 (blue) to 20,000 (red), the scale in Figure 7 ranged from 1,000 (blue) to 15,000 (red).

Acknowledgements We thank E. Kamiya and W. Heinz of the NCI Optical Microscopy and Image Analysis Lab, for their assistance with clearing using Ce3D+ and Imaris, respectively. We are grateful for technical assistance from T. Kuruppu, C. Elder, E. Truffer, and M. Boylan. We thank M. Kaltcheva, P. Abete-Luzi and A. Perantoni for critical reading of the manuscript.

Competing Interests The authors declare that no competing interests exist.
References

1. Clark, E., Peel, A.D. & Akam, M. Arthropod segmentation. Development 146 (2019).
2. Richmond, D.L. & Oates, A.C. The segmentation clock: inherited trait or universal design principle? Curr Opin Genet Dev 22, 600-6 (2012).
3. Chipman, A.D. Parallel evolution of segmentation by co-option of ancestral gene regulatory networks. Bioessays 32, 60-70 (2010).
4. Ten Tusscher, K. Of mice and plants: Comparative developmental systems biology. Dev Biol (2018).
5. Balavoine, G. Segment formation in Annelids: patterns, processes and evolution. Int J Dev Biol 58, 469-83 (2014).
6. Pourquie, O. Vertebrate segmentation: from cyclic gene networks to scoliosis. Cell 145, 650-63 (2011).
7. Hubaud, A. & Pourquie, O. Signalling dynamics in vertebrate segmentation. Nat Rev Mol Cell Biol 15, 709-21 (2014).
8. Christ, B., Huang, R. & Scaal, M. Amniote somite derivatives. Dev Dyn 236, 2382-96 (2007).
9. Krol, A.J. et al. Evolutionary plasticity of segmentation clock networks. Development 138, 2783-92 (2011).
10. Dequeant, M.L. et al. A complex oscillating network of signaling genes underlies the mouse segmentation clock. Science 314, 1595-8 (2006).
11. Ferjentsik, Z. et al. Notch is a critical component of the mouse somitogenesis oscillator and is essential for the formation of the somites. PLoS Genet 5, e1000662 (2009).
12. Bone, R.A. et al. Spatiotemporal oscillations of Notch1, Dll1 and NICD are coordinated across the mouse PSM. Development 141, 4806-16 (2014).
13. Shimojo, H. et al. Oscillatory control of Delta-like1 in cell interactions regulates dynamic gene expression and tissue morphogenesis. Genes Dev 30, 102-16 (2016).
14. Niwa, Y. et al. Different types of oscillations in Notch and Fgf signaling regulate the spatiotemporal periodicity of somitogenesis. Genes Dev 25, 1115-20 (2011).
15. Morimoto, M., Takahashi, Y., Endo, M. & Saga, Y. The Mesp2 transcription factor establishes segmental borders by suppressing Notch activity. Nature 435, 354-9 (2005).
16. Oginuma, M., Niwa, Y., Chapman, D.L. & Saga, Y. Mesp2 and Tbx6 cooperatively create periodic patterns coupled with the clock machinery during mouse somitogenesis. Development 135, 2555-62 (2008).
17. Takahashi, Y. et al. Mesp2 initiates somite segmentation through the Notch signalling pathway. Nat Genet 25, 390-6 (2000).
18. Saga, Y., Hata, N., Koseki, H. & Taketo, M.M. Mesp2: a novel mouse gene expressed in the presegmented mesoderm and essential for segmentation initiation. Genes Dev 11, 1827-39 (1997).
19. Eckalbar, W.L., Fisher, R.E., Rawls, A. & Kusumi, K. Scoliosis and segmentation defects of the vertebrae. Wiley Interdiscip Rev Dev Biol 1, 401-23 (2012).
20. Giampietro, P.F. et al. Progress in the understanding of the genetic etiology of vertebral segmentation disorders in humans. Ann N Y Acad Sci 1151, 38-67 (2009).
21. Sparrow, D.B. et al. Autosomal dominant spondylocostal dysostosis is caused by mutation in TBX6. Hum Mol Genet 22, 1625-31 (2013).
22. Lefebvre, M. et al. Autosomal recessive variations of TBX6, from congenital scoliosis to spondylocecostal dysostosis. *Clin Genet* **91**, 908-912 (2017).
23. Takeda, K. et al. Screening of known disease genes in congenital scoliosis. *Mol Genet Genomic Med* **6**, 966-974 (2018).
24. Giampietro, P.F. et al. Clinical, genetic and environmental factors associated with congenital vertebral malformations. *Mol Syndromol* **4**, 94-105 (2013).
25. Sparrow, D.B. et al. A mechanism for gene-environment interaction in the etiology of congenital scoliosis. *Cell* **149**, 295-306 (2012).
26. Dubrulle, J., McGrew, M.J. & Pourquie, O. FGF signaling controls somite boundary position and regulates segmentation clock control of spatiotemporal Hox gene activation. *Cell* **106**, 219-32 (2001).
27. Cooke, J. & Zeeman, E.C. A clock and wavefront model for control of the number of repeated structures during animal morphogenesis. *J Theor Biol* **58**, 455-76 (1976).
28. Naiche, L.A., Holder, N. & Lewandoski, M. FGFR4 and FGFR8 comprise the wavefront activity that controls somitogenesis. *Proc Natl Acad Sci U S A* **108**, 4018-23 (2011).
29. Aulehla, A. et al. A beta-catenin gradient links the clock and wavefront systems in mouse embryo segmentation. *Nat Cell Biol* **10**, 186-93 (2008).
30. Dunty, W.C., Jr. et al. Wnt3a/beta-catenin signaling controls posterior body development by coordinating mesoderm formation and segmentation. *Development* **135**, 85-94 (2008).
31. Wahl, M.B., Deng, C., Lewandoski, M. & Pourquie, O. FGF signaling acts upstream of the NOTCH and WNT signaling pathways to control segmentation clock oscillations in mouse somitogenesis. *Development* **134**, 4033-41 (2007).
32. Niwa, Y. et al. The initiation and propagation of Hes7 oscillation are cooperatively regulated by Fgf and notch signaling in the somite segmentation clock. *Dev Cell* **13**, 298-304 (2007).
33. Choi, H.M., Beck, V.A. & Pierce, N.A. Next-generation in situ hybridization chain reaction: higher gain, lower cost, greater durability. *ACS Nano* **8**, 4284-94 (2014).
34. Choi, H.M.T. *et al.* Third-generation in situ hybridization chain reaction: multiplexed, quantitative, sensitive, versatile, robust. *Development* **145**(2018).
35. Trivedi, V., Choi, H.M.T., Fraser, S.E. & Pierce, N.A. Multidimensional quantitative analysis of mRNA expression within intact vertebrate embryos. *Development* **145**(2018).
36. Perantoni, A.O. *et al.* Inactivation of FGFR8 in early mesoderm reveals an essential role in kidney development. *Development* **132**, 3859-71 (2005).
37. Mansouri, A. *et al.* Paired-related murine homeobox gene expressed in the developing sclerotome, kidney, and nervous system. *Dev Dyn* **210**, 53-65 (1997).
38. Neidhardt, L.M., Kispert, A. & Herrmann, B.G. A mouse gene of the paired-related homeobox class expressed in the caudal somite compartment and in the developing vertebral column, kidney and nervous system. *Dev Genes Evol* **207**, 330-339 (1997).
39. Chapman, D.L. & Papaioannou, V.E. Three neural tubes in mouse embryos with mutations in the T-box gene Tbx6. *Nature* **391**, 695-7 (1998).
40. Nowotschin, S., Ferrer-Vaquer, A., Concepcion, D., Papaioannou, V.E. & Hadjantonakis, A.K. Interaction of Wnt3a, Msgn1 and Tbx6 in neural versus paraxial mesoderm lineage commitment and paraxial mesoderm differentiation in the mouse embryo. *Dev Biol* **367**, 1-14 (2012).
41. Chalamalasetty, R.B. et al. The Wnt3a/beta-catenin target gene Mesogenin1 controls the segmentation clock by activating a Notch signalling program. *Nat Commun* **2**, 390 (2011).

42. Anderson, M.J., Southon, E., Tessarollo, L. & Lewandoski, M. Fgf3-Fgf4-cis: A new mouse line for studying Fgf functions during mouse development. *Genesis* **54**, 91-8 (2016).

43. Huppert, S.S., Ilagan, M.X., De Strooper, B. & Kopan, R. Analysis of Notch function in presomitic mesoderm suggests a gamma-secretase-independent role for presenilins in somite differentiation. *Dev Cell* **8**, 677-88 (2005).

44. Harima, Y., Takashima, Y., Ueda, Y., Ohtsuka, T. & Kageyama, R. Accelerating the tempo of the segmentation clock by reducing the number of introns in the Hes7 gene. *Cell Rep* **3**, 1-7 (2013).

45. Kageyama, R., Niwa, Y., Isomura, A., Gonzalez, A. & Harima, Y. Oscillatory gene expression and somitogenesis. *Wiley Interdiscip Rev Dev Biol* **1**, 629-41 (2012).

46. Pourquie, O. & Tam, P.P. A nomenclature for prospective somites and phases of cyclic gene expression in the presomitic mesoderm. *Dev Cell* **1**, 619-20 (2001).

47. Bessho, Y. *et al.* Dynamic expression and essential functions of Hes7 in somite segmentation. *Genes Dev* **15**, 2642-7 (2001).

48. Bessho, Y., Hirata, H., Masamizu, Y. & Kageyama, R. Periodic repression by the bHLH factor Hes7 is an essential mechanism for the somite segmentation clock. *Genes Dev* **17**, 1451-6 (2003).

49. Chapman, D.L., Agulnik, I., Hancock, S., Silver, L.M. & Papaioannou, V.E. Tbx6, a mouse T-Box gene implicated in paraxial mesoderm formation at gastrulation. *Dev Biol* **180**, 534-42 (1996).

50. Sawada, A. *et al.* Fgf/MAPK signalling is a crucial positional cue in somite boundary formation. *Development* **128**, 4873-80 (2001).

51. Diaz-Cuadros, M. *et al.* In vitro characterization of the human segmentation clock. *Nature* (2020).

52. Hou, D., Kang, N., Yin, P. & Hai, Y. Abnormalities associated with congenital scoliosis in high-altitude geographic regions. *Int Orthop* **42**, 575-581 (2018).

53. Lignell, A., Kerosuo, L., Streichan, S.J., Cai, L. & Bronner, M.E. Identification of a neural crest stem cell niche by Spatial Genomic Analysis. *Nat Commun* **8**, 1830 (2017).

54. Kim, H.S., Neugebauer, J., McKnite, A., Tilak, A. & Christian, J.L. BMP7 functions predominantly as a heterodimer with BMP2 or BMP4 during mammalian embryogenesis. *Elife* **8**(2019).

55. Attardi, A. *et al.* Neuromesodermal progenitors are a conserved source of spinal cord with divergent growth dynamics. *Development* **145**(2018).

56. McKinney, M.C., McLennan, R. & Kulesa, P.M. Angiopoietin 2 signaling plays a critical role in neural crest cell migration. *BMC Biol* **14**, 111 (2016).

57. Kopan, R. & Ilagan, M.X. The canonical Notch signaling pathway: unfolding the activation mechanism. *Cell* **137**, 216-33 (2009).

58. Stauber, M., Sachidanandan, C., Morgenstern, C. & Ish-Horowicz, D. Differential axial requirements for lunatic fringe and Hes7 transcription during mouse somitogenesis. *PLoS One* **4**, e7996 (2009).
59. Hayashi, S., Nakahata, Y., Kohno, K., Matsui, T. & Bessho, Y. Presomitic mesoderm-specific expression of the transcriptional repressor Hes7 is controlled by E-box, T-box, and Notch signaling pathways. *J Biol Chem* **293**, 12167-12176 (2018).

60. Gonzalez, A., Manosalva, I., Liu, T. & Kageyama, R. Control of Hes7 expression by Tbx6, the Wnt pathway and the chemical Gsk3 inhibitor LiCl in the mouse segmentation clock. *PLoS One* **8**, e53323 (2013).

61. Felix, M.A. & Barkoulas, M. Pervasive robustness in biological systems. *Nat Rev Genet* **16**, 483-96 (2015).

62. Tam, P.P. The control of somitogenesis in mouse embryos. *J Embryol Exp Morphol* **65 Suppl**, 103-28 (1981).

63. Oates, A.C., Morelli, L.G. & Ares, S. Patterning embryos with oscillations: structure, function and dynamics of the vertebrate segmentation clock. *Development* **139**, 625-39 (2012).

64. Gomez, C. et al. Control of segment number in vertebrate embryos. *Nature* **454**, 335-9 (2008).

65. Boulet, A.M. & Capecchi, M.R. Signaling by FGF4 and FGF8 is required for axial elongation of the mouse embryo. *Dev Biol* **371**, 235-45 (2012).

66. Carapuco, M., Novoa, A., Bobola, N. & Mallo, M. Hox genes specify vertebral types in the presomitic mesoderm. *Genes Dev* **19**, 2116-21 (2005).

67. Putnam, N.H. et al. The amphioxus genome and the evolution of the chordate karyotype. *Nature* **453**, 1064-71 (2008).

68. Holland, L.Z. & Onai, T. Early development of cephalochordates (amphioxus). *Wiley Interdiscip Rev Dev Biol* **1**, 167-83 (2012).

69. Bertrand, S. et al. Evolution of the Role of RA and FGF Signals in the Control of Somitogenesis in Chordates. *PLoS One* **10**, e0136587 (2015).

70. Pasini, A., Manenti, R., Rothbacher, U. & Lemaire, P. Antagonizing retinoic acid and FGF/MAPK pathways control posterior body patterning in the invertebrate chordate Ciona intestinalis. *PLoS One* **7**, e46193 (2012).

71. Onai, T., Aramaki, T., Inomata, H., Hirai, T. & Kuratani, S. On the origin of vertebrate somites. *Zoological Lett* **1**, 33 (2015).

72. Sun, X. et al. Conditional inactivation of Fgf4 reveals complexity of signalling during limb bud development. *Nat Genet* **25**, 83-6 (2000).

73. Meyers, E.N., Lewandoski, M. & Martin, G.R. An Fgf8 mutant allelic series generated by Cre- and Flp-mediated recombination. *Nat Genet* **18**, 136-41 (1998).

74. Geffers, I. et al. Divergent functions and distinct localization of the Notch ligands DLL1 and DLL3 in vivo. *J Cell Biol* **178**, 465-76 (2007).

75. Anderson, M.J., Schimmang, T. & Lewandoski, M. An FGF3-BMP Signaling Axis Regulates Caudal Neural Tube Closure, Neural Crest Specification and Anterior-Posterior Axis Extension. *PLoS Genet* **12**, e1006018 (2016).

76. Li, W., Germain, R.N. & Gerner, M.Y. Multiplex, quantitative cellular analysis in large tissue volumes with clearing-enhanced 3D microscopy (Ce3D). *Proc Natl Acad Sci USA* **114**, E7321-E7330 (2017).

77. Model, M.A. Intensity calibration and shading correction for fluorescence microscopes. *Curr Protoc Cytom Chapter 10*, Unit10 14 (2006).

78. Schindelin, J. et al. Fiji: an open-source platform for biological-image analysis. *Nat Methods* **9**, 676-82 (2012).
| Experimental Cross | Experimental Genotype (Freq) | Control Genotype (Freq) |
|--------------------|-----------------------------|-------------------------|
| *Fgf4*^flox/fox*   | *Tcre*^{TG/0}; *Fgf4*^flox/fox* | *Tcre*^{TG/0}; *Fgf4*^flox/fox* (1/2) |
| *Fgf8*^flox/flox*  | *Tcre*^{TG/0}; *Fgf8*^flox/flox* | *Tcre*^{TG/0}; *Fgf8*^flox/flox* (1/2) |
| *Hes7*^fl/ft*      | *Hes7*^fl/ft* | *Hes7*^fl/ft* (1/4) |
| *Fgf4*^flox/fox*; *Hes7*^fl/ft* | *Tcre*^{TG/0}; *Fgf4*^flox/fox*; *Hes7*^fl/ft* (1/4) | |
| *Fgf8*^flox/flox*; *Hes7*^fl/ft* | *Tcre*^{TG/0}; *Fgf8*^flox/flox*; *Hes7*^fl/ft* (1/4) | |

*Experimental cross is always written “female x male”*

Δ = “deletion” or null
Figure 1. Fgf4 mutants have defects in vertebral and somite patterning.

A, A') Max intensity projection (MIP) of HCR staining of Fgf4 and Fgf8 mRNA expression (A) or Fgf4 only (A') in the PSM of a 5 somite stage wildtype embryo (dorsal view). PS: primitive streak, PSM: presomitic mesoderm, S: somite. B) MIP of 4 parasagittal z-sections (approximately 5μm each) of HCR staining of Fgf4 and Fgf8 mRNA expression, of a 10 somite stage wildtype embryo (lateral view, mediolateral to the midline). C, D) Skeletal preparations (ventral view after removing ribcage) of E18.5 control and Fgf4 mutants. D') Tracing of the Fgf4 mutant vertebral pattern in D. E) Histograms representing the vertebral column and associated ribs (C = cervical, T = thoracic, L = lumbar), showing variety and location of vertebral defects in E18.5 Fgf4 mutants (n = 12). Each block in the histogram represents a single defect as indicated in the key, bottom right. F-I) Wholemount in situ hybridization (WISH) detection of Uncx4.1 mRNA expression in control and Fgf4 mutants at E10.5 (F, G lateral views, anterior top; G' dorsal view of embryo in G) and at 9-10 somite stage (H, I dorsal view, anterior left); dotted line in F and G marks the anterior boundary of the forelimb (FL). J) Graph depicting the percentage of abnormal somites scored with a mispatterned Uncx4.1 mRNA WISH pattern (F-I) in Fgf4 mutants at the somite position listed on the x-axis. Somites 1-6 were scored in somite stage 7 Fgf4 mutants (n=20) and somites 7-26 were scored in 26-30 somite stage Fgf4 mutants (n=19). Note that posterior to somite 21 the Uncx4.1 pattern is normal. K, L) Dorsal view (anterior left) of 5-6 somite stage control (K) and Fgf4 mutant (L) embryos WISH-stained for Mesp2 mRNA expression. In all control embryos (n=7) the Mesp2 pattern was normal; in 5/8 mutant embryos, the pattern was abnormal.

Linked to Figure 1 is following figure supplement:

Figure 1 - figure supplement 1. Mesp2 pattern is normal at 24-26 somite stage in Fgf4 mutants.
Figure 1 - figure supplement 1. *Mesp2* pattern is normal at 24-26 somite stage in *Fgf4* mutants. 

A-B’) HCR staining showing no difference in the patterns of *Mesp2* and *Tbx6* expression between control and *Fgf4* mutant 24-26 somite stage embryos. A and B: MIP, lateral view with anterior to the left. A’ and B’: dorsal projections of A and B, anterior to the left. (control, n = 10; mutant, n = 11).
Figure 2. Fgf4 mutants maintain a normal wavefront. A, B) HCR staining of 5-6 somite stage control (A, n = 8) and Fgf4 mutant embryos (B, n = 11). In mutants, Msgn1 expression was similar between mutants and controls, but Mesp2 expression was abnormal (6/11). C-D’’) HCR staining of 5-6 somite stage control and Fgf4 mutant embryos showing normal expression of Meox1 (control, n = 4; mutant, n = 4), Msgn1 (control, n = 9; mutant, n = 8), and Tbx6 (control, n = 10; mutant, n = 8). E) There is no significant difference in the anterior-posterior length of Tbx6 and Msgn1 domains in control (n = 9) and mutant embryos (n = 8; green and red bars in C and C’). F) Quantification of Tbx6 and Msgn1 mRNA expression, determined by measurement of fluorescence intensity per cubic micrometer within the volume of the Msgn1 or Tbx6 domain of expression, respectively. There is no significant difference between control and mutant embryos. In E and F, data are mean±s.e.m, significance determined by a Student’s t-test. Msgn1: control n=7; mutant n = 8. Tbx6: control, n = 9; mutant, n = 8. All images: MIP, dorsal view, anterior left. Same embryo shown in C-C’’ and D-D’’.
Figure 3. Fgf8 is sufficient for maintaining canonical FGF-responsive gene expression in Fgf4 mutants. A, B) HCR staining of 5-6 somite stage control (n=6) and Fgf4 mutant (n=3) embryos showing comparable expression of Fgf8, quantified in C). C) Quantification of Fgf8 mRNA expression, determined by measurement of fluorescent intensity per cubic micrometer within the volume the mesoderm-specific Tbx6 expression domain. Note, there is no significant difference between control and mutant embryos. D) Quantification of HCR analysis of Spry2, Etv4, and Spry4 expression in 5-6 somite stage control (n =4) and Fgf4 mutant (n =4) embryos imaged in E-F””. Data in C and D are mean±s.e.m, significance determined by a Student’s t-test. All images: MIP, dorsal view, left. Same embryo shown in E- E’”” and F- F’””. 
Figure 4. Pattern of Notch signaling is abnormal in Fgf4 mutants. A) Quantification of immunostained NICD fluorescent signal specifically in the mesoderm of 5-6 somite stage embryos. Note, there is no significant difference between Fgf4 mutants (n=5) and littermate controls (n=5). Data are mean±s.e.m, significance determined by a Student’s t-test. B, C) 5-6 somite stage control (B) or Fgf4 mutant (C) embryos immunostained for NICD. B’ C’) Same images as in (B,C) but visualized using the Imaris spot modeling function where each fluorescent signal is represented as a colored sphere according to pixel intensity (lowest value is purple and highest value is red, as indicated). Note that the pattern in the Fgf4 mutant (C’) is less distinct and blurred compared to littermate control (B’); brackets indicate anterior PSM. D-E’’) HCR staining of representative 5-6 somite stage control (n = 10) and Fgf4 mutant (n = 8) embryos for the indicated genes. Note Fgf4 mutant expression of Hes7 and Lfng in mutants is abnormal. All images: MIPs, dorsal view, anterior left. Same embryo shown in B, B’; C, C’; D-D’’; E-E’’.
Figure 5. *Hes7* expression is abnormally patterned and reduced in *Fgf4* mutants.

A-F) HCR staining for *Hes7* expression in 5-6 somite stage control and *Fgf4* mutant embryos sorted by phase of oscillation, showing abnormal expression of *Hes7* in mutants. G) Distribution of phases in control and *Fgf4* mutants shown in A-F; controls: phase I, n = 9; phase II, n = 10; phase III, n = 11. *Fgf4* mutants: phase I, n = 6; phase II, n = 9; phase III, n = 2. H) Quantification of *Hes7* expression specifically within the PSM (all phases combined) in 5-6 somite stage control (n=11) and *Fgf4* mutant (n=8) embryos shows significantly decreased expression in the *Fgf4* mutant. Quantification of *Hes7* mRNA expression, determined by measurement of fluorescence intensity per cubic micrometer within the volume the mesoderm-specific *Tbx6* expression domain. Data in H are mean±s.e.m, significance determined by a Student’s t-test. All images are MIPs, dorsal view, anterior to left.

Linked to Figure 5 are the following figure supplements:

Figure 5 - figure supplement 1. *Hes7* expression is abnormal in *Fgf4* mutants.

Figure 5 - figure supplement 2. Using *Hes7* null homozygotes to adjust Imaris baseline cutoff.

Figure 5 - figure supplement 3. Imaris-modeling HCR Expression.

Figure 5 - figure supplement 4. *Hes7* expression in *Fgf4* mutants is reduced, irrespective of oscillation phase.

Figure 5 - figure supplement 5. *Hes7* expression is normal at 24-26 somite stage in *Fgf4* mutants.
**Figure 5 - figure supplement 1.** *Hes7* expression is abnormal in *Fgf4* mutants.  
A-D) Chromogenic whole mount in situ hybridization of *Hes7* mRNA in 5-6 somite stage control and *Fgf4* mutant embryos. Development of chromogenic signal was either not saturated (developed for ~1hr in A, B) or saturated (developed for ~8hr in C, D). Note decreased expression in *Fgf4* mutants (B) and abnormal pattern of oscillations (D). All images are dorsal views, anterior left and representative: A, n = 4; B, n = 4; C, n = 4; D, n = 6.
Figure 5 - figure supplement 2. Using Hes7 null homozygotes to adjust Imaris baseline cutoff. 

A, B) HCR staining of 5-6 somite stage control (n=3) and Hes7 mutant (n=3) embryos using probes complimentary to the deleted region of Hes7. Note that the probe does not detect any product generated by the Hes7 null allele. 

A’, B’) Composite image with Tbx6 and DAPI staining in combination with Hes7 of the same embryos as in A and B. All images are representative MIPs, dorsal view, anterior left. The baseline cutoff for Imaris-modeling of control and Fgf4 mutant embryos was adjusted until a signal was absent from such Hes7 null homozygotes. Same embryo shown in A, A’ and B, B’.
Figure 5 - figure supplement 3. Imaris-modeling HCR Expression.

A) HCR staining of Hes7 and Tbx6 in a 5-6 somite stage wildtype embryo; dorsal view, anterior left. B) Imaris-generated surface model of Tbx6 expression domain from A. C) Imaris-generated spot modeling of Hes7 expression domain from A. D) Anterior view of transverse projection of spot model in C. E) Spot model in D with Hes7 spots outside of the Tbx6 surface model colored yellow. F) Spot Hes7 model from C with spots outside the Tbx6 domain removed, thus only mesodermal spots remain. A similar Tbx6 surface model was used in the quantification of Hes7 expression levels in Figures 5H and Figure supplement 1 and 4. Spot modeling in F demonstrates how the spot models were generated in Figures 6 and 7.
Figure 5 - figure supplement 4. Hes7 expression in Fgf4 mutants is reduced, irrespective of oscillation phase.
Quantification of Hes7 expression within the PSM of control and Fgf4 mutants at 5-6 somite stage. Data and embryos are identical to Figure 5H, except that fluorescence intensity quantification values are segregated by phase.
Figure 5 – figure supplement 5. *Hes7* expression is normal at 24-26 somite stage in *Fgf4* mutants.

A-F) HCR staining of representative 24-26 somite stage control and *Fgf4* mutant embryos showing normal patterns of *Hes7* expression for each phase of oscillation. MIP, lateral view with anterior to the left. G) Quantification of *Hes7* expression within the PSM, reveals no difference between controls and *Fgf4* mutants. Measurement of *Hes7* fluorescence intensity per cubic micrometer within the volume the mesoderm-specific *Tbx6* expression domain; data are mean ± s.e.m, significance determined by a Student’s t-test. Controls: phase I, n = 3; phase II, n = 3; phase III, n = 4. Mutants: phase I, n = 4; phase II, n = 4; phase III, n = 3.
Figure 6. Reduced Hes7 expression with less distinct peaks and troughs in the Fgf4 mutant PSM.

A, B) Spot models based on HCR analysis of Hes7 expression, in 5-6 somite stage control and Fgf4 mutant embryos within the PSM, as defined by the Tbx6 expression domain. Localized Hes7 expression is colored by level of expression from low (blue) to high expression (white), as indicated. Note there are less high expressors (yellow, red and white) and more low expressors (blue). This insight is quantified in C), where the percentage of spots found in each expression-level group is graphed. Data are mean±s.e.m, significance determined by a Student’s t-test; control, n = 6; mutant, n = 5. D-E’) Composite of spot models from A and B. Note that the Hes7 expression trough (boxed region, expanded in D’ and E’) between anterior and posterior oscillatory peaks is less distinct in mutant (E’) with more higher expressors (green) than in control embryos (D’). F-G’) MIP of HCR staining of 5-6 somite stage control and Fgf4 mutant embryos using probes against Tbx6, Hes7, and Hes7intron which specifically labels active Hes7 transcription. Note the trough of Hes7intron signal between peaks (brackets) in mutants is less pronounced than controls, indicating a failure of transcriptional repression of Hes7 in this region. Representative embryos are shown; control, n = 6; mutant, n = 5. All images are dorsal views, anterior left. Same embryo shown in F, F’; G, G’.
Figure 7. Removal of one Hes7 allele exacerbates vertebral defects in Fgf4 mutants.

A-C) Histograms representing the vertebral column and associated ribs (C = cervical, T = thoracic, L = lumbar), showing variety and location of vertebral defects at E18.5 in the genotypes indicated (A and B, n = 7; C, n = 6). Each block in the histogram represents a single defect as indicated in the key in A. D) Skeletal preparation of a representative E18.5 compound Fgf4 mutant- Hes7 heterozygote (ventral view). E) Quantification of Hes7 expression within the PSM in 5-6 somite stage embryos of the indicated genotype: control (n=10), Fgf4-Hes7 double heterozygotes (n = 6), Fgf4 mutants (n = 10), and compound Fgf4 mutant- Hes7 heterozygotes (n = 7). Quantification of Hes7 mRNA expression, determined by measurement of fluorescent intensity per cubic micrometer within the volume of the mesoderm-specific Tbx6 expression domain. Data in E are mean±s.e.m, significance determined by a Student’s t-test. F-I) Spot models based on HCR analysis of Hes7 expression, in embryos of the indicated genotype, within the PSM, as defined by the Tbx6 expression domain. Localized Hes7 expression is colored by level of expression from low (purple) to high expression (red), as indicated. Images F-I are dorsal views, anterior left.

Linked to Figure 7 is following figure supplement:

Figure 7 - figure supplement 1. Removal of one Hes7 allele has no effect on vertebral defects in Fgf8 mutants.
Figure 7 - figure supplement 1. Removal of one *Hes7* allele has no effect on vertebral defects in *Fgf8* mutants.

Histograms representing the vertebral column and associated ribs (C = cervical, L = thoracic, L = lumbar), showing variety and location of vertebral defects at E18.5 in the genotypes indicated (A, n = 6; B, n = 5; C, n = 8). Each block in the histogram represents a single defect as indicated in the key in A. Note that the vertebral defects in the *Fgf8/Hes7* mutants are additive, not synergistic.