RESEARCH ARTICLE

An anterior limit of FGF/Erk signal activity marks the earliest future somite boundary in zebrafish

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ABSTRACT
Vertebrate segments called somites are generated by periodic segmentation of the anterior extremity of the presomatic mesoderm (PSM). During somite segmentation in zebrafish, mesp-b determines a future somite boundary at position B-2 within the PSM. Heat-shock experiments, however, suggest that an earlier future somite boundary exists at B-5, but the molecular signature of this boundary remains unidentified. Here, we characterized fibroblast growth factor (FGF) signal activity within the PSM, and demonstrated that an anterior limit of downstream Erk activity corresponds to the future B-5 somite boundary. Moreover, the segmentation clock is required for a stepwise posterior shift of the Erk activity boundary during each segmentation. Our results provide the first molecular evidence of the future somite boundary at B-5, and we propose that clock-dependent cyclic inhibition of the FGF/Erk signal is a key mechanism in the generation of perfect repetitive structures in zebrafish development.

KEY WORDS: FGF signalling, Clock, Segmentation, Somitogenesis

INTRODUCTION
Proper formation of a well-proportioned body in multicellular organisms requires spatiotemporal control of multiple biological processes during development. For instance, the regularity of repetitive structures in vertebrate bodies is derived from the vertebrate segments called somites, which are generated by periodic segmentation of the uniform presomatic mesoderm (PSM) (Pourquie, 2001). The periodicity of segmentation is regulated by oscillation of the segmentation clock genes, and the position of segmentation is determined by a gradient of fibroblast growth factor (FGF) (Dubrulle et al., 2001; Dubrulle and Pourquie, 2004; Holley, 2007; Pourquie, 2001; Sawada et al., 2001).

The first detectable sign of a future segment boundary, as visualized by the expression of mesoderm posterior homolog-b (mesp-b; mespba – Zebrafish Information Network), appears at a distance of two somite lengths posterior to B0 (B-2, between S-I and S-II; see Fig. 1D for nomenclature) in the anterior PSM in zebrafish (Sawada et al., 2000), although this may not be the earliest event in boundary determination. A brief exposure of zebrafish embryos to heat shock disrupts somite segmentation after four cycles of normal segmentation (Roy et al., 1999), suggesting that the boundaries of at least five somites (from B-1 to B-5, between S-IV and S-V) are predetermined somewhere in the uniform PSM. However, no molecular sign marking the position of the future somite boundary at B-5 has yet been identified. By precise measurement of the FGF active domain and transient manipulation of FGF signal activity in zebrafish embryos, we show in this study that the anterior limit of FGF/Erk activity corresponds to the future somite boundary at B-5.

RESULTS
The fgf8a gradient moves continuously towards the posterior during somitogenesis
In chick, an Fgf8 gradient, generated by restricted transcription and mRNA decay of Fgf8, is essential for setting up the position of the segmentation (Dubrulle and Pourquie, 2004). In zebrafish, segmentation defects can be seen in ace/ace embryos (fgf8a mutants) (supplementary material Fig. S1) (see also Reifers et al., 1998), suggesting that zebrafish fgf8a has a similar role as chick Fgf8 in the context of somitogenesis. However, it has been reported that at least three fgfs (fgf4, fgf8a and fgf24) are expressed in the tailbud region (data retrieved from the Zebrafish Information Network, http://zfin.org/, 4 April 2013), and that fgf8a and fgf24 are together required to generate posterior mesoderm (Draper et al., 2003). We thus observed their expression in the tailbud of zebrafish embryos at a particular somite stage and tested whether the fgf gradient is seen in zebrafish embryos. Although expression of fgf4 and fgf24 was detected in the midline structures of the tailbud, a clear gradient of neither fgf4 nor fgf24 was observed in the PSM (supplementary material Fig. S2). By contrast, fgf8a mRNA was most abundant in a region of the posterior PSM between Kupffer’s vesicle and the posterior end of the tailbud, at the 5-somite stage, and declined gradually towards the anterior (Fig. 1A). To determine how this fgf8a gradient moves during somite segmentation, we measured levels of fgf8a expression in each embryo and then compared the position of the anterior limit of the fgf8a gradient among several embryos, which are arranged in an order of time progression by both the somite number and PSM length as a time indicator (for details, see Materials and Methods). Although PSM length varied between embryos at the same 5-somite stage, the length of the fgf8a gradient between the anterior border and the posterior end remained constant (Fig. 2A,C; supplementary material Fig. S10). When a pair of new somites formed, meaning that the embryo had reached the 6-somite stage, the PSM shortened immediately by about one somite length, whereas the length of the fgf8a gradient did not change at the transition between the 5- and 6-somite stages or indeed throughout the 6-somite stage (Fig. 2A,C; supplementary material Fig. S10). These results suggest that the fgf8a gradient moves continuously towards the posterior as the tail elongates.

The fgf8a gradient is converted into the Erk activity boundary
Fgf8 binds to and activates Fgf receptors (Fgfrs), which then transduces the FGF signal to the Ras/Erk pathway, leading to the transcriptional activation of target genes including positive and
In most developmental processes, a morphogen gradient is converted into a sharp response boundary within a uniform tissue to form proper patterns. We thus compared the $fgf8a$ and p-Erk gradients in different embryos and found that the p-Erk gradient was much steeper than the $fgf8a$ gradient (Fig. 1C). This result is further confirmed by comparisons of the $fgf8a$ and p-Erk gradients in the same embryos (supplementary material Fig. S4A-D). These results suggest that the gentle slope of $fgf8a$ is converted into the steep gradient of Erk activity, leading to generation of the Erk activity boundary in the uniform PSM. In agreement with this notion, we found that the anterior limit of Erk activity displayed stepwise changes within the PSM that correlated with the spatial periodicity of somite patterning (Fig. 2B,D; supplementary material Fig. S4E,F). Within a particular somite stage, the ON region of Erk activity elongated gradually owing to the tail elongation, whereas the length of the OFF region (distance between B-1 and the anterior limit of Erk activity; see also Fig. 1B) remained constant (Fig. 2B,D; supplementary material Fig. S4E,F). Interestingly, when a pair of new somites formed, the ON region shortened immediately by about one somite length so that the length of the OFF region was precisely maintained (Fig. 2B,D; supplementary material Fig. S4E,F). Furthermore, double staining of p-Erk and her1 expression confirmed that the stepwise changes in the anterior limit of Erk activity were consistent with both somite number and phase propagation of her1 expression (supplementary material Fig. S5). These results therefore suggest that the anterior limit of Erk activity displays stepwise movements that correlate with somite segmentation and corresponds to the future somite boundary in the uniform PSM.
The anterior limit of Erk activity represents the future somite boundary at B-5

Expression of mesp-b appears in the rostral parts of S-I and S-II in the anterior PSM, and is the molecular sign of the future somite boundary B-2 between S-I and S-II (Holley, 2007; Sawada et al., 2000). Double staining for p-Erk and mesp-b expression revealed that the anterior limit of Erk activity was not concomitant with the position at S-II where mesp-b is expressed (supplementary material Fig. S6). The anterior limit of Erk activity was located at a considerably more posterior site in the PSM (supplementary material Fig. S6), suggesting the possibility that the anterior limit of Erk activity marks a future somite boundary, such as B-5 between S-IV and S-V.

If this is the case, misplacement of the Erk activity boundary towards the anterior would result in an anterior shift of B-2 future somite boundary position, marked by mesp-b expression, leading to decreased size of the resultant somite. To test this possibility, we applied a strategy similar to that of the FGF signal perturbations reported by Sawada et al. (Sawada et al., 2001); we enhanced Erk activity for 4 minutes in embryos at the 2-somite stage using the dual-specificity phosphatase inhibitor BCI (Molina et al., 2009) (Fig. 3A). p-Erk signals were intensified immediately after BCI treatment (2-somite stage), resulting in an anterior shift (~14 μm) of the Erk activity boundary that is estimated by the difference of the OFF region length of p-Erk between embryos treated with DMSO (vehicle) and BCI (Fig. 3B,C; Table 1). However, the anterior limit of Erk activity returned to its normal position by the 3-somite stage (one round of segmentation after BCI treatment) (Fig. 3C). These results indicate that the Erk activity boundary transiently shifted towards the anterior immediately after BCI treatment.

If the Erk activity boundary represents the B-5 future somite boundary, the transient anterior shift of this boundary may alter mesp-b expression position towards the anterior only when three rounds of segmentation (5-somite stage) had occurred after BCI treatment. To test this possibility, we investigated the distribution of mesp-b expression in BCI-treated embryos. Although the anterior limit of Erk activity was shifted just after BCI treatment (Fig. 3C), mesp-b expression occurred normally at this time point (Fig. 3D). Only when three rounds of segmentation had occurred after BCI treatment (5-somite stage) was the width of mesp-b stripes shortened (~13 μm) (Fig. 3D,E; Table 1). Consistently, this manipulation eventually led to decreased size (~13 μm) of the resultant 7th somite (Fig. 3F,G; Table 1). Similar results could be obtained when 4- or 10-somite stage embryos were treated with BCI (Table 1). We also investigated whether mesp-b knockdown affected the distribution of the anterior limit of Erk activity. Although mesp-b knockdown resulted in mild disruption of the somite boundary, this manipulation did not affect the stepwise movement of the anterior limit of Erk activity seen in cycles of somite segmentation (supplementary material Fig. S7). These results therefore suggest that the anterior limit of Erk activity is the future somite boundary at B-5; this is an earlier sign of the future somite boundary than that of mesp-b expression at B-2.
Time-lapse imaging followed by p-Erk staining revealed that the position of the anterior limit of Erk activity was maintained in several cycles of somite segmentation (supplementary material Fig. S8A). We thus reasoned that targeted cell fluorescence labelling and time-lapse imaging could confirm whether this position represents the future somite boundary at B-5. To test this possibility, we photo-converted cells at the presumptive p-Erk boundary in Tg[her1:KikGR] embryos and traced the trajectory of the cells (supplementary material Fig. S8B and Movie 1). Although the alignment of the photo-converted cells (supplementary material Fig. S8B, red cells) changed during somitogenesis, a proportion of the cells finally resided at the rostral end of the somite when five rounds of segmentation had completed, meaning that the position adjacent to the photo-converted cells (supplementary material Fig. S8B, red arrowhead) corresponds to the future somite boundary at B-5 (see also Fig. 1D).

**Somite segmentation clock controls the stepwise movement of the Erk activity boundary during somitogenesis**

Because the repetitive structure of somites is generated from the temporal periodicity created by the somite segmentation clock (Oates et al., 2012; Pourquié, 2011), we hypothesized that proper functioning of the segmentation clock is required for the stepwise movement of the future somite boundary. To test this possibility, we disrupted the segmentation clock in zebrafish embryos by knocking down two segmentation clock genes, her1 and her7, and investigated the dynamics of the anterior limit of Erk activity in the manipulated embryos. As shown previously (Henry et al., 2002), double knockdown of her1 and her7 resulted in the loss of metameric structures of somites (supplementary material Fig. S9B). Erk activation occurred normally even in the manipulated embryos (supplementary material Fig. S9C,D). However, the anterior limit of Erk activity lost its stepwise pattern in each cycle of somite segmentation (Fig. 4A,B; supplementary material Fig. S13). These results therefore suggest that the segmentation clock is required for the stepwise movement of the anterior limit of Erk activity in the PSM (Fig. 4C).

**DISCUSSION**

In a clock and wavefront model, the clock controls when the somite boundaries form and the wavefront determines where they form (Cooke and Zeeman, 1976). It has been proposed that, in zebrafish, Her1 homodimer- and Her7:Hes6 heterodimer-based negative feedback loops constitute the clock, and FGF gradients constitute...
the wavefront (Holley, 2007; Pourquié, 2001; Schröter et al., 2012; Trofka et al., 2012). In this study, we provide additional information for the mechanism underlying zebrafish somitogenesis. Because the single peak \(fgf8a\) gradient moved continuously towards the posterior (Fig. 2A,C; supplementary material Fig. S4E,F), the \(fgf8a\) gradient acts as a steadily regressing wavefront. As the \(fgf8a\) gradient is converted into the Erk activity boundary (Fig. 1; supplementary material Figs S3, S4), which corresponds to the future B-5 somite boundary (Fig. 3; supplementary material Fig. S8B and Movie 1), and because the clock regulates the stepwise movements of the future somite boundary (Fig. 4; supplementary material Fig. S9), the anterior limit of Erk activity represents a ‘signal integration spot’ of the clock and the wavefront.

In mouse, the clock (Notch oscillators such as Hes7 and Lfng) controls oscillation of Erk activity, and Erk oscillation periodically allows Notch signals to induce \(Mesp2\) expression at S-I, suggesting that Erk oscillation represents a rippled wavefront (Niwa et al., 2011). These findings suggest that, although the detailed mechanisms are different, there is a common logic of somitogenesis in zebrafish and mouse: Notch oscillators periodically modulate the FGF/Erk wavefront at the signal integration spot to regulate spatiotemporal periodicity. In chick, although Erk activity declines gradually towards the anterior (Delfini et al., 2005), whether the gradient of Erk activity corresponds to a future somite boundary and whether the gradient shows an oscillatory pattern remain unknown. Therefore, it would be of great interest to understand a common and/or specific logic of somitogenesis in vertebrates by comparing regulatory mechanisms of somite formation in such different species.

**Conclusion**

Pattern formation is one of the most important processes of vertebrate morphogenesis. In the case of somites, pre-patterning over a distance of about five somites is thought to be determined within apparently uniform tissue composed of somite progenitor cells. Using somitogenesis as a model to investigate how patterns are generated during development, we reveal that a morphogen gradient is converted into a sharp response boundary of intracellular signalling, providing positional information as a future segment boundary, and that clock-dependent stepwise movement of this sharp response boundary is required to generate the perfect metameric pattern of the vertebrate body during development.

**MATERIALS AND METHODS**

**Zebrafish**

Wild-type and \(ace^{n252a}\) (\(fgf8a\) mutant) zebrafish were used in this study. As described previously (Matsui et al., 2011), substitution from G to A in the \(ace\) allele was detected by sequence analyses of the DNA fragment obtained from each embryo after *in situ* hybridization or immunohistochemistry.

**Whole-mount in situ hybridization and immunohistochemistry**

Whole-mount *in situ* hybridization and immunohistochemistry were performed as described previously (Matsui et al., 2005; Matsui et al., 2011). cDNA fragments of \(fgf8a\), \(mesp-b\) and \(her1\) were used as templates for antisense probes.

**Morpholinos (MOs) and injection**

The following antisense MO oligonucleotides against \(her1\), \(her7\) and \(mesp-b\) and a control MO were obtained from Gene Tools: control-MO: 5'-CCTTTACCTCGTCAATTTATA-3'; \(her1\)-MO: 5'-TTCGACTTCGTTTTTGGAGTAAC-3' (Henry et al., 2002); \(her7\)-MO: 5'-CAGTCTGCGAGGAGGCTAAGC-3'; \(mesp-b\)-MO: 5'-TCGTTTCTGCTTGTGGATG-3'; \(her1\)-MO plus \(her7\)-MO (6.25 ng each), \(mesp-b\)-MO (5 ng) or control-MO (12.5 ng or 5 ng) were injected into the yolk of one-cell-stage zebrafish embryos.
as described previously (Matsui et al., 2011). Injected embryos were collected and fixed at the indicated time points, and then used for experiments including in situ hybridization and immunohistochemistry.

**Transient treatment of BCI**

Embryos were obtained by mating wild-type fish and were cultured normally until 10.5-11 hours post-fertilization (hp). Embryos at the 2-somite stage were treated with 0.1 mg/ml BCI (Dual Specificity Protein Phosphatase 1/6 Inhibitor, Calbiochem) for 4 minutes, and then washed extensively with embryo medium. Embryos were fixed just after treatment (2-somite stage), or after one (3-somite stage), three (5-somite stage) or eight (10-somite stage) rounds of segmentation following BCI treatment.

**Graphical analyses**

The embryos were flat-mounted in Vectashield mounting medium, which contains propidium iodide (PI) (Vector Laboratories). Immunofluorescence signals were visualized and photographed using an LSM510-META or LSM710 confocal microscopic (Zeiss). Sequential confocal images were stacked. The position of the somite boundary and posterior end of the PSM was estimated by visual observation of PI staining in combination with bright-field microscopy. Embryos were grouped according to somite number, and were arranged in order of PSM length, because the PSM gradually elongates along with tail elongation. Signal intensity was measured by using ImageJ software (National Institutes of Health), and background was subtracted to remove any tissue autofluorescence from the signal. The position of the anterior limit of fgf expression and the Erk activity was determined by the comparison between visual observation and signal intensity plot.

**Statistical analyses**

The coefficient of variation (C.V.) was calculated by the ratio of the s.d. to signal. The position of the anterior limit of fgf expression and the Erk activity was determined by the comparison between visual observation and signal intensity plot.

**Time-lapse imaging**

In order to label PSM cells with a photo-convertible fluorescent protein named KikGR, we generated a transgenic line Tg[her1:KikGR] using the Tol2 system (Kawakami et al., 2004; Usasaki et al., 2006). Tg[her1:KikGR] carries the zebrafish her1 promoter, KikGR full length cDNA (MBL), nuclear localization signal sequence and SV40 poly A sequence. A 405 nm laser was used in photo-conversion of KikGR. Time-lapse image acquisition was performed by using Olympus FV-1000-D confocal microscope and FLUOVIEW software.

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**Supplementary material**

Supplementary material available online at http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.098905/-/DC1

**References**


Figure S1. *ace/ace* embryos (*fgf8a* mutants) shows segmentation defects. (A-C) Representative images of p-Erk signals (A), *mesp-b* (B) or *uncx4.1* expression (C) in wild-type (left panel) or *ace/ace* (right panel) embryos at 10- to 12-somite stages. Although Erk activity was reduced in the small and narrow PSM of all *ace/ace* embryos (n = 10), the Erk activity boundary could be seen (A). Consistent with the change of Erk activity at mid-somitogenesis stages, *mesp-b* expression at S-I and S-II was partially fused and patchy in *ace/ace* embryos, and the angle of *mesp-b* stripes became steeper than that of wild-type (B; 70%, n = 51). In addition, expression of *uncx4.1* became abnormal in 79% *ace/ace* embryos (C; n = 29).
Figure S2. Neither *fgf4* nor *fgf24* mRNA shows graded distribution in the PSM. (A, B) Representative images of *fgf4* (A) or *fgf24* (B) expression. Signal intensities (upper images; AU, arbitrary unit) were measured by ImageJ software from the original pictures (lower). Dorsal view of tailbud regions in flat-mounted embryos, anterior to the left. The horizontal white lines in the lower images mark the paths along which the signal intensities shown in the upper panel were recorded. Scale bar: 100 µm.
Figure S3. p-Erk distribution in the PSM. (A) Left: flat-mounted wild-type embryo at the 6-somite stage immunostained with p-Erk antibody (green). Nuclei are stained with PI (red). Right: p-Erk signal intensity was measured along the white line shown in the left image. (B-D) PSM cells viewed at a higher magnification, p-Erk signals in the nuclei are a sign of Erk activation. In the anterior PSM, nuclear p-Erk signals were not detected (B), whereas nuclear p-Erk signals were detected in all the cells in the posterior PSM (D). A boundary of nuclear p-Erk signals could be seen at the middle of the PSM (white dotted line in panel C). Scale bar: 40 µm.
Figure S4. Double staining of fgf8a and p-Erk in the same embryo. (A) fgf8a expression. (B) p-Erk distribution. (C) Merged image of fgf8a (green) and p-Erk (red). Signal intensities (upper images; AU, arbitrary unit) were measured by ImageJ software from the original pictures (lower panels). Anterior and posterior ends of the PSM are marked by white lines. Scale bar: 100 µm. The position of the anterior extremity (dotted lines) was estimated by comparison of signal intensity (upper panel) and visual observation (lower panel). Dorsal view of tailbud region in the flat-mounted embryo, anterior to the left. The horizontal white lines in the lower images mark the paths along which the signal intensities shown in the upper panel were recorded. (D) Comparison of slopes between fgf8a expression and p-Erk distribution. Approximation formulae of fgf8a and p-Erk were calculated from average values of signal intensities, respectively (n = 7). Dots indicate averages and green/red bars indicate standard deviations. (E) Representative images of fgf8a expression (green) and p-Erk distribution (red) in double stained embryos at the 4- to 5-somite stages. Dorsal view of tailbud regions, anterior to the top. Scale bar: 100 µm. (F) Quantitative data of fgf8a expression (left) or p-Erk distribution (right) in double stained embryos (n = 13). Statistical significance of variation (2P < 0.05) could be seen in fgf8a expression region (C.V. = 0.028) versus fgf8a non-expression region (C.V. = 0.100) and in the OFF region (C.V. = 0.028) versus the ON region of Erk activity (C.V. = 0.046).
Figure S5. Posterior shift of p-Erk signal coincides temporally with the transition from phase III to I of cyclic her1 expression. p-Erk (green) and her1 (red) were stained in the same embryos. Embryos are displayed in order of phase propagation of her1 oscillation (from phase I to III). Scale bar: 100 µm. Length of p-Erk ON and OFF regions was measured and listed in the bottom table.
Figure S6. Spatial distribution of p-Erk and *mesp-b* mRNA in the PSM. Double staining of p-Erk (A, green) and *mesp-b* (B, red). Scale bar: 100 µm. The anterior limit of Erk activity is located about three somite lengths posterior to B-2.
Figure S7. Knockdown of *mesp-b* does not affect the stepwise pattern of p-Erk. (A, B) Somite morphology in control-MO-injected (A) or *mesp-b*-MO- injected (B) embryos. Nuclei are stained by PI (red). Somites are outlined by dotted lines. Scale bar: 100 µm. (C, D) p-Erk signals in control (C) or *mesp-b* (D) morphants at the 5- to 6-somite stages. (E, F) Quantitative data for p-Erk signals in control (E, n = 21) or *mesp-b* (F, n = 17) morphants. Even when *mesp-b* was knocked down, the stepwise movement of p-Erk occurred normally.
Figure S8. The anterior limit of Erk activity represents the future somite boundary at B-5. (A) Time-lapse imaging followed by p-Erk staining. Each embryo was immediately fixed and stained for p-Erk after somite formation was observed for 30, 60 or 90 min by time-lapse. Lateral view, anterior to the top. To measure the length of the p-Erk OFF region, the embryo was flat-mounted (right panel). Mean ± s.d. of the OFF region length is shown. (B) Specific fluorescent labeling and time-lapse imaging. Photo-conversion of KikGR revealed that the position at about 200 µm away from B-1 (red arrowhead) represents the future somite boundary at B-5. White brackets mark newly formed somites during time-lapse imaging. Images are frames of Movie 1.
Figure S9. Spatial distribution of p-Erk in her1 and her7 double-knockdown embryos. (A, B) Somite morphology in wild-type embryos (A) or embryos co-injected with her1-MO and her7-MO (B). Nuclei are stained by PI (red). Somites are outlined by dotted lines. Scale bar: 100 µm. Double knockdown of her1 and her7 disrupted the proper repetitive structures of somites. (C) Representative images of p-Erk distribution (green) showing the bimodal pattern in double-knockdown embryos of her1 and her7. Signal intensity (upper image; AU, arbitrary units) was measured from the original picture (lower). Dorsal view of tailbud regions in flat-mounted embryos, anterior to the left. (D) Intensity plot of the upward slope region of the gradient represented by the yellow line in (C). Mean ± s.d. of intensity (n = 7) is shown. The equation describing the slope obtained from these her1 and her7 double-knockdown embryos is almost the same as that of wild-type (Figure 1C), suggesting that the somite segmentation clock does not affect the steepness of the p-Erk gradient.
Figure S10. Expression of fgf8a in wildtype embryos (n = 29).
Figure S11. Distribution of p-Erk signals in wildtype embryos (n = 39).
Figure S12. Expression of *fgf8a* (green) and distribution of p-Erk signals (red) in double stained embryos (n = 13).
Figure S13. Distribution of p-Erk signals in *her1* and *her7* double knockdown embryos (n = 35).
Movie 1. Time-lapse imaging of the presumptive p-Erk boundary. This movie shows the dorsal view of a Tg[her1:KikGR] embryo (shown in Fig. S8B). Movements of photoconverted cells (red) located around the Erk activity boundary were traced in 5 rounds of segmentation.