Differential Axial Requirements for Lunatic Fringe and Hes7 Transcription during Mouse Somitogenesis

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Abstract

Vertebrate segmentation is regulated by the “segmentation clock”, which drives cyclic expression of several genes in the caudal presomitic mesoderm (PSM). One such gene is Lunatic fringe (Lfng), which encodes a modifier of Notch signalling, and which is also expressed in a stripe at the cranial end of the PSM, adjacent to the newly forming somite border. We have investigated the functional requirements for these modes of Lfng expression during somitogenesis by generating mice in which Lfng is expressed in the cranial stripe but strongly reduced in the caudal PSM, and find that requirements for Lfng activity alter during axial growth. Formation of cervical, thoracic and lumbar somites/vertebrae, but not sacral and adjacent tail somites/vertebrae, depends on caudal, cyclic Lfng expression. Indeed, the sacral region segments normally in the complete absence of Lfng and shows a reduced requirement for another oscillating gene, Hes7, indicating that the architecture of the clock alters as segmentation progresses. We present evidence that Lfng controls the expression or activity of a long-range signal that regulates axial extension.

Introduction

Somites are repeated epithelial blocks of tissue that differentiate into the segmental units of the axial skeleton (vertebrae, intervertebral discs, ribs), attached skeletal and limb muscles, and additional mesodermal tissues. In the mouse, somites form between embryonic days E7.75 and E13.5 from the unsegmented, mesenchymal presomitic mesoderm (PSM), which lies towards the caudal end of the embryo. During this process, formation of new epithelial boundaries (every 2 h in the mouse) at the cranial end of the PSM generates a new, bilateral pair of somites. Cell migration and proliferation replenish the caudal PSM as the embryo grows [1,2, reviewed in 3,4].

The sequential process of somitogenesis is controlled by a molecular oscillator, the so-called “segmentation clock”, that is characterised by oscillatory transcription of genes in the PSM with the same periodicity as that of somitogenesis [5,reviewed in 6]. Expression of cycling genes is synchronised between neighbouring cells, but subject to phase delays along the length of the axis so that a wave of transcription appears to sweep cranially along the PSM, concomitant with the formation of a new pair of somites. The segmentation clock arises via delayed negative feedback, but details of the clock pacemaker circuitry remain unclear [5,7, reviewed in 6,8]. Amongst several oscillating genes that play essential roles in somitogenesis [reviewed in 9] is Lunatic fringe (Lfng), which encodes a β1,3-N-acetylglucosaminyl-transferase that acts in the Golgi to modify the extra-cellular domain of the Notch receptor before transport to the cell membrane [10,11,12]. Glycosylation by Lfng regulates the sensitivity of Notch receptors towards their ligands and, in the PSM, appears to repress Notch signalling. Thus, inactivating Lfng causes constant Notch1 activity (as measured by cleavage of its intracellular domain [13]), and ectopic Lfng expression depresses Notch signalling in the chick [14].

Lfng transcription in the mouse PSM comprises two dynamic domains, a cycling domain in the caudal PSM, and a cranial PSM stripe adjacent to the boundary that is about to form between somitomeres s–I and s0 (Figure 1B). Promoter analysis has revealed that oscillating Lfng transcription in the PSM is driven by an assembly of discrete cis-regulatory elements, and that a distinct 285 bp element (B-block [15]; block 3 [16]) drives stripe expression.

The relative significance of the two Lfng PSM domains is not clear. Lfng−/− embryos show irregular and incomplete segmentation of the PSM and perturbed cranial-caudal patterning of somites. The resulting vertebral column is truncated and irregular with incompletely formed and fused vertebrae; the thoracic cage is malformed and several ribs are fused [17,18]. Thus, the oscillating domain could be a component of the segmentation clock and contribute to periodic gene expression. Indeed, cyclic, spatially patterned Lfng transcription is essential for proper somitogenesis: continuous Lfng overexpression in the whole mouse PSM, both in wild-type and Lfng−/− embryos, leads to somite and vertebral column defects [19].
The cranial stripe could also be involved in boundary formation, either maintaining a preformed but morphologically invisible metameric pattern in the PSM, defining cranial-caudal compartments in the forming somite, or regulating the generation of intersomitic boundaries. *Drosophila* Fringe is essential for boundary formation in several imaginal tissues [reviewed in 20], and high levels of vertebrate *Lfng* in the cranial stripe may contribute to formation of the new somite boundary. Indeed, transplantation experiments in the chick support this view: apposition of *Lfng*-expressing cells adjacent to non-expressing cells in the middle of chick somitomere s-II induces formation of an extra somite boundary [21].

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**Figure 1. Expressing transgenic cLfng in the cranial PSM stripe.** (A) Constructs BB-cLfng and Lfng5kb-cLfng were used to generate transgenic lines BBL-1/-2 and 5kL-1/-2, respectively. cLfng ORF (orange) is under the control of either a duplication of the stripe-specific B-block enhancer and the β-globin basic promoter P (top), or 5 kb of the mouse *Lfng* promoter containing conserved cis-regulatory elements A-, B-, C-blocks (bottom; [15]). (B) The resulting transgenic expression (visualised by in situ hybridization with a cLfng probe on PSMs of hemizygous E10.5 embryos of lines BBL-1, BBL-2, 5kL-1 and 5kL-2) is confined to the cranial PSM stripe in BBL embryos or comprises stripe plus oscillatory domain in 5kL embryos. Position of PSM, somitomeres s-I and s0 and somites sI and sII are indicated on the left. Note the different expression levels, as reflected by the different times of colour development (in hours) given at the bottom of each panel. (C–F) Transgenic cLfng is expressed in the PSM (boxed in C–I) at all stages of segmentation (e.g. G–G': hemizygous BBL-1 embryos at embryonic day E8.5, E10.5 and E12.5) and in ectopic expression domains outside the PSM (e.g. H, I: 5kL-1 and 5kL-2; black arrows and arrowheads). Arrowed domains are seen in all transgenic lines, indicating that the *Lfng* promoter includes repressive regulatory elements that lie outside the proximal 5 kb region. doi:10.1371/journal.pone.0007996.g001
In this study, we dissect individual contributions of the two Lfng subdomains by generating transgenic mice that express Lfng predominantly in the cranial stripe. We show that oscillatory Lfng expression in the caudal PSM is required in the cranial body half, and that activity in the cranial Lfng stripe is required in the tail. Both modes of Lfng activity are dispensable in the intermediate sacral area. Analysis of a hypomorphic Hes7 allele indicates that requirements for oscillating Hes7 expression also vary during somitogenesis, suggesting that the gene network regulating Hes7 activity are dispensable in the intermediate sacral area. Analysis of a hypomorphic Hes7 allele indicates that requirements for oscillating Hes7 expression also vary during somitogenesis, suggesting that the gene network regulating Hes7 expression also vary during somitogenesis.

results

Generation of Transgenic Mice Expressing Lfng in the Cranial PSM Stripe

We examined the function of Lfng in the PSM by driving Lfng expression in the cranial PSM stripe via a tandem duplication of the B-block enhancer of the Lfng promoter which drives stripe expression more strongly than a single B-block (BB-Lfng; Figure 1A; data not shown; [15,16]). We expressed chick Lfng (referred to as cLfng for clarity) so that we could distinguish between transgenic and endogenous Lfng expression by in situ hybridisation. Although mouse and chick Lfng are 70% identical and 87% similar in aminoacid sequence, indicating that they should be functionally equivalent, the genes share less than 60% identity in DNA sequence, sufficient for a cLfng probe to be selective for the chick transcript under stringent hybridisation conditions (Methods). As a control, we used a 5 kb fragment of the mouse Lfng promoter to drive cLfng in both the stripe and the oscillating PSM domains (Lfng5kb–cLfng; Figure 1A; [15,16]).

We obtained 13 transgenic mouse lines with BB-cLfng and 8 lines with Lfng5kb–cLfng. All 8 Lfng5kb–cLfng lines express cLfng in the expected wildtype expression pattern: cyclically in the caudal PSM and stably in a stripe in the cranial compartment of somitomere s–I. 9 of the 13 BB-cLfng lines express cLfng similarly. Surprisingly, all 9 lines also weakly express cLfng dynamically in the caudal and mid-PSM. This staining indeed represents cLfng expression because no cross-reaction with endogenous Lfng is seen following equivalent staining of wildtype embryos (Figure 1C). Rather, it appears that the duplicated B-block can drive dynamic expression in the PSM, albeit at very low levels (see Discussion).

For further study, we selected two lines (referred to as BB-1 and BB-2) in which caudal expression is very weak and visible only after extended staining, and two control Lfng5kb–cLfng lines (referred to as 5KL-1 and 5KL-2) (Figure 1B, 1D–F). All four lines express the transgenes throughout somitogenesis (Figure 1G–I”, and not shown). The 5 kb fragment drives stronger stripe expression than the duplicated B-block (Figure 1B, 1G–I), indicating that the longer promoter includes additional elements that synergise with and enhance stripe expression from the B-block, and BB-1 expresses more highly than BB-2. The former is homozygous viable, allowing us to compare hemi- (BB-1/0) and homozygous (BB-1/BB-1) animals. Only hemizygous BB-2 animals were examined because the insertion is homozygous lethal (genotyping was performed following identification of the transgenic insertion site; Methods). Together, the transgene lines form an allelic series of cLfng stripe expression (5KL/0>BBL-1/ BB-1>BBL-1/0>BBL-2/0; Figure 1B).

cLfng Expression in the Cranial Stripe Rescues Embryonic Tail Growth but Not Adult Viability

The various transgenes were crossed with Lfng+/− mice and the patterning of Lfng−/+ × Lfng−/+ offspring (which we refer to by their transgene names) assayed as foetuses and adults. Lfng−/+ mice usually die before weaning, presumably because the deformed axial trunk skeleton reduces the thoracic space and impairs breathing [17,18]. In our experiments, only three non-transgenic Lfng−/+ animals survived to adulthood (equivalent to 9% of the expected number of homozygous offspring; Table 1), although a much higher proportion of Lfng−/+ foetuses survived till E18.5 (61%; Table 1).

Expressing cLfng in both the stripe and the oscillating PSM domains substantially restores adult viability (33% and 68% for 5KL-1 and 5KL-2, respectively; Table 1; see Methods S1 for a likely reason of the unexpected high lethality of 5KL mice), and also enhances foetal recovery (Table 1). Thus, chick Lfng is indeed functional in the mouse and can largely substitute for the endogenous protein. Rescue of adult viability when cLfng is expressed solely in the cranial stripe is less efficient, (9–24% in BB-1 and −2; Table 1).

Table 1. E18.5 and adult viabilities of transgenic animals in a Lfng−/+ background.

| Genotype of interest | 5KL-1 | 5KL-2 | BB-1 | BB-1/BB-1 | BB-1/0 | BB-2/0 | all lines |
|----------------------|-------|-------|-------|------------|--------|--------|----------|
| Total number of E18.5 (or adult) offspring | 87 (155) | 37 (116) | 23 (59) | 49 (49) | 18 (129) | 214 (308) |
| Number recovered | 18 (11) | 9 (11) | 7 (11) | 6 (2) | 2 (2) | 9 (3) |
| Number expected | 12.9 (33.2) | 7.5 (16.1) | 5.7 (14.7) | 9.2 (8.4) | 3.2 (23.1) | 14.9 (33.3) |
| Relative viability | 139% (33%) | 120% (68%) | 122% (20%) | 65% (24%) | 62% (9%) | 61% (9%) |

1Adult data are in brackets and italicised.
2For each line, 5KL-1, 5KL-2, BB-1, BB-2, we crossed parents of the genotype Lfng+/− × Lfng+/− or Lfng−/+ × Lfng+/− or Lfng+/− or Lfng−/+ × Lfng+/− (BB-1 only, shown in the 4 th column) and genotyped the offspring. We never obtained homozygous foetuses or adults of lines 5KL-1 and BB-2 (and we were unable to check for homozygosity of line 5KL-1). For sites of insertion and genotyping see Methods.
3Numbers for transgene-free Lfng−/+ in the 7 th column are pooled from all crosses of all lines.
4The expected numbers of offspring of the relevant genotype were calculated separately for each cross to take account of differing parental genotypes (cf. footnote 2), and then combined.

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confirming that transgenic cLfng oscillating thoracic and lumbar vertebrae are only slightly restored (e.g. [18]). Thus, the sacral area undergoes considerable segmentation in the mutant embryos (e.g. Figure 2B, 2C in [17], Figure 2C in [19]).

This phenotype is likely to be due to subtle alterations of Notch signalling in the PSM that lead to a delay in distal dorsal arches. At least three of the sacral vertebrae form the proximal dorsal arches. At least three of the sacral vertebrae form distinct ribs connecting the axial skeleton to the ilium of the pelvic girdle, whereas thoracic ribs and vertebrae undergo multiple fusions in the thorax (Figure 2C, 2G top). Relative normality of sacral structures is also evident in previous images of the mutant embryos (e.g. Figure 2B, 2C in [17], Figure 2C in [18]). Thus, the sacral area undergoes considerable segmentation independently of Lfng activity.

The rescued mice also frequently exhibit homeotic transformations of single segments: Out of 32 5kL mice, 14 (44%) have an extra rib-bearing thoracic vertebra, 15 have an extra pair of “true ribs” (connected to the sternum), and 12 miss a lumbar vertebra. This phenotype is likely to be due to subtle alterations of Notch signalling in the PSM that lead to a delay in Hox gene expression in the PSM [22,23,24].

Finally, all rescued 5kL males (Lfng^{+/−}; cLfng) are sterile (7 5kL-1, 10 5kL-2), confirming the reduced fertility of Lfng^{+/−} males [25]. This finding excludes the possibility that skeletal malformations contribute to the reduced fertility, and indicates that a testis-specific Lfng enhancer lies outside the 5 kb promoter used in the 5kL lines.

By contrast with the 5kL transgenes, BBL transgenes only rescue segmentation in the tail areas. The sacral area of BBL mice is always regularly patterned, as in most Lfng_{5kL}^{−/−} mice. Cervical, thoracic and lumbar vertebrae are only slightly restored (e.g. dorsal arch quality; Figure 2D, 2E, 2G middle), indicating that oscillating Lfng expression is necessary for proper segmentation in these axial regions, and that this requirement cannot be met by the weak dynamic expression in the caudal PSM of these embryos.

However, the stripe domain is sufficient for proper segmentation of the tail (Figure 2D, 2E, 2G middle; compare tail to lumbar area [l] and to the short tail in Figure 2C; Table 2). The efficiency of tail rescue correlates with the strength of transgene expression (BBL-1>BBL-1/0>BBL-2/0, Figure 2F; Table 2). Even slight, respectively (Figure 2A; Table 2). Rescued BBL tails are often kinked, indicative of locally disrupted somitogenesis due to partially incomplete Lfng expression (Figure S1A).

Surprisingly, 15% (3/21) of the 5kL foetuses show no rescue of tail length (and of skeletal segmentation; not shown). Most likely, these represent individual embryos in which the transgene has been inactivated (see Methods S1), although we cannot exclude the possibility that sequence differences between mouse and chick Lfng also contribute to differences between the activity of the transgenic and endogenous genes.

**Table 2.** Comparison of E18.5 foetuses and adults of the four transgenic lines in the Lfng−/− background (data for adults are given in brackets and italics).

| Transgene | E18.5 | Adult |
|-----------|-------|-------|
| Lfng−/− | 10 | 10 |
| Lfng+/− | 1 | 1 |
| cLfng+/− | 2 | 2 |
| BBL-1 | 6 | 6 |
| BBL-1/0 | 7 | 7 |
| BBL-2 | 6 | 6 |
| BBL-2/0 | 1 | 1 |
| Lfng | 10 | 10 |

**Length of whole vertebral column (mm)**

| Transgene | E18.5 | Adult |
|-----------|-------|-------|
| Lfng−/− | 28.3 | 28.3 |
| Lfng+/− | 23.2 | 23.2 |
| cLfng+/− | 24.3 | 24.3 |
| BBL-1 | 24.3 | 24.3 |
| BBL-1/0 | 24.3 | 24.3 |
| BBL-2 | 24.3 | 24.3 |
| BBL-2/0 | 24.3 | 24.3 |
| Lfng | 28.3 | 28.3 |

**Length of cervical plus thoracic region (mm)**

| Transgene | E18.5 | Adult |
|-----------|-------|-------|
| Lfng−/− | 10.1 | 10.1 |
| Lfng+/− | 8.7 | 8.7 |
| cLfng+/− | 8.7 | 8.7 |
| BBL-1 | 8.7 | 8.7 |
| BBL-1/0 | 8.7 | 8.7 |
| BBL-2 | 8.7 | 8.7 |
| BBL-2/0 | 8.7 | 8.7 |
| Lfng | 10.1 | 10.1 |

**Length of sacral region (mm)**

| Transgene | E18.5 | Adult |
|-----------|-------|-------|
| Lfng−/− | 2.7 | 2.7 |
| Lfng+/− | 2.7 | 2.7 |
| cLfng+/− | 2.7 | 2.7 |
| BBL-1 | 2.7 | 2.7 |
| BBL-1/0 | 2.7 | 2.7 |
| BBL-2 | 2.7 | 2.7 |
| BBL-2/0 | 2.7 | 2.7 |
| Lfng | 2.7 | 2.7 |

**Length of tail (mm)**

| Transgene | E18.5 | Adult |
|-----------|-------|-------|
| Lfng−/− | 10.5 | 10.5 |
| Lfng+/− | 10.5 | 10.5 |
| cLfng+/− | 10.5 | 10.5 |
| BBL-1 | 10.5 | 10.5 |
| BBL-1/0 | 10.5 | 10.5 |
| BBL-2 | 10.5 | 10.5 |
| BBL-2/0 | 10.5 | 10.5 |
| Lfng | 10.5 | 10.5 |

**Number of regular sacral vertebrae**

| Transgene | E18.5 | Adult |
|-----------|-------|-------|
| Lfng−/− | 4 | 4 |
| Lfng+/− | 4 | 4 |
| cLfng+/− | 4 | 4 |
| BBL-1 | 4 | 4 |
| BBL-1/0 | 4 | 4 |
| BBL-2 | 4 | 4 |
| BBL-2/0 | 4 | 4 |
| Lfng | 4 | 4 |

**Number of regular tail vertebrae**

| Transgene | E18.5 | Adult |
|-----------|-------|-------|
| Lfng−/− | 30.4 | 30.4 |
| Lfng+/− | 26.1 | 26.1 |
| cLfng+/− | 26.1 | 26.1 |
| BBL-1 | 26.1 | 26.1 |
| BBL-1/0 | 26.1 | 26.1 |
| BBL-2 | 26.1 | 26.1 |
| BBL-2/0 | 26.1 | 26.1 |
| Lfng | 30.4 | 30.4 |
Figure 2. Regional and dose-dependent rescue of the Lfng phenotype by transgenic expression in the stripe domain. (A) Dosage-dependent rescue of the truncated tail phenotype of Lfng-/-;cLfng BBL E18.5 foetuses. Homozygous BBL-1 tails resemble those of wildtype/5kL, and weak hemizygous BBL-2 resemble Lfng-/- . White arrowheads point at the tip of the tails; scale bar, 5 mm; asterisk, midgut that failed to return to the reduced peritoneal cavity (found in 5/42 Lfng-/-;cLfng E18.5 foetuses). (B, C) E18.5 skeletons of 5kL embryos show regular vertebrae all along the length axis like wildtype [B, lateral (upper panel) and dorsal (lower panel) view] whereas Lfng-/- skeletons (C) exhibit regular vertebrae only in the sacral area. (D, E) BBL skeletons show a regional rescue in the tail area adjacent to the sacral area. The cervical, c; thoracic, t; lumbar, l; sacral, s; and tail regions of the vertebral columns are indicated, as are: ar, vertebral arch; bo, vertebral body; il, ilium. Regular sacral vertebrae in the bottom panels of (C) and (E), magnifications of the middle panels, are labelled with white asterisks. Note the cranial shift of the left side of sacral vertebrae in the Lfng-/- foetus (C bottom), indicative of homeotic transformation. Scale bar (bottom of D), 5 mm. (F) A schematic quantitative comparison of lengths of cervical/thoracic (c+t, green boxes), lumbar (l, yellow boxes), sacral (s, blue boxes), and tail (orange boxes) sections of vertebral columns of various genotypes indicates a dose-dependent rescue of the tail length in BBL foetuses, but not of the length of the cervical-thoracic area. Boxes for each region are aligned to the left (cranial) end. (G) Skeleton preparations of adult mice (dorsal views) show the truncated tail but normal Lfng-/- sacral area (top), a partial tail rescue in hemizygous BBL-1 mice (middle) and an overall rescue in 5kL mice (bottom); scale bar, 10 mm.

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the weakest BBL-2-rescued foetus shows three regular tail vertebrae. These results suggest that stripe expression of Lfng in the cranial PSM helps maintain somitogenesis in the tail (see below).

**Lfng Stripe Expression Restores Somite Organisation in the Tail**

The rescue of tail vertebrae by Lfng stripe expression could be due to restoration of cranial-caudal compartmentalisation of somites. We tested this idea by visualising expression of Uncx4.1, a marker of caudal somite halves [26].

Segmentation and cranial-caudal differentiation are both greatly disrupted in Lfng−/− embryos, in which Uncx4.1 is expressed in irregularly spaced and sized stripes and patches [17,18]. Nevertheless, from about somite 28 onwards, Lfng−/− embryos form 17±2 (n=5) somites that are regular in the sacral and adjacent tail area but become increasingly irregular towards the end of the truncated tail (Figure 3A–3C). The Uncx4.1 stripes in these somites look slightly less distinct than in wildtype, but are much more regular than in somites 1–27 of the same Lfng−/− embryos (n=9; Figure 3A, B).

The cranial somite marker Tbx18 [27] also reveals regularly relative segments in the sacral and tail area of Lfng−/− embryos (Figure S2A–D), while the myogenic marker MyoD [28] suggests that the regionalisation is less distinct for the myotomal than for the sclerotomal compartment (Figure S2E–H).

Regular segmentation of the sacrum and adjacent tail in Lfng−/− embryos is not due to compensatory expression of another fringe parologue. Neither Manic fringe (Mfng) or Radical fringe (Rfng) is expressed in wildtype PSMs between E8.0 and E11.5 [29, our own observation], and Mfng and Rfng mRNA levels are not increased in Lfng−/− embryos compared to wildtype embryos as determined by quantitative RT-PCR on dissected E10.25 PSMs (data not shown).

These relatively well-patterned somites are incorporated into both regular and the severely disorganised Lfng−/− sacral/tail regions (Figure 2C, 2G top). The latter observation shows that cranial-caudal compartmentalisation is not sufficient for proper development of the sclerotome, which derives from a ventral portion of the somite [reviewed in 3].

Scl attenuates repair almost normally for Uncx4.1 (n=7; Figure 3E, 3F). Lfng stripe expression in either BBL line also restores orderly Uncx4.1 expression in the tail (n=10; Figure 3G–3L), albeit less regularly in BBL-2 tails (Figure 3I), but not in more cranial regions (Figure 3G). These results argue that Lfng stripe expression is necessary for tail segmentation.

**Lfng Regulates Dorsal-Ventral Compartmentalisation in Tail Somites**

As Lfng−/− tail vertebrae were more irregular than expected from their regular cranial-caudal compartmentalisation (compare Figure 3B and 3C with 2G), we examined sclerotome development in Lfng−/− embryos using the marker Paxl [30,31]. We found that lack of Lfng affects dorsal-ventral compartmentalisation during tail development.

In wildtype embryos, sclerotones are regular and clearly separated all along the length axis (n=17; Figure 3J, 3K). In Lfng−/− embryos, sclerotones cranial to the hindlimb buds are fused, while the more caudal ones appear distinctly separated (n=10; Figure 3L, 3M). Sclerotome borders in the mutant never become as clearly visible as in wildtype because Paxl expression is less concentrated at the borders, but a relative reduction of Paxl in the cranial area is discernible in some sacral sclerotones (Figure 3L). The Paxl domain in the truncated Lfng−/− E11.5 tails is much thicker than in wildtype tails (compare Figures 3K and 3M and corresponding transverse sections in Figures 3N and 3O), indicating that Lfng limits formation of sclerotome. Presumably, over-production of Paxl-expressing cells leads to blurring of the borders between sclerotomes in Lfng−/− embryos (Figure 3L, 3M). This morphological difference between sacral and tail sclerotomes (which is not prefigured by a difference in cranial-caudal compartmentalisation as visualised by Uncx4.4; compare Figure 3B, 3C to 3L, 3M) is likely to cause the difference between normal sacral vertebra and the deformed/fused tail vertebrae in Lfng−/− mice.

**Lfng and Axial Extension of the Tail**

Lfng plays a role in maintaining axial extension, a process whereby axial progenitor cells which lie at the chordoneural hinge of the tailbud generate caudal growth of the embryo and elongation of the body axis [32,33,34]. In Lfng−/− embryos, axial extension ceases around E10.5, resulting in truncated tails. This phenotype can be rescued by increased Lfng expression in the cranial PSM (cf. tail-lengths in hemi- and homozygous BBL-1 and in BBL-2 embryos; Figure 2A, 2F; Table 2), indicating that Lfng expression in the cranial stripe domain is sufficient for sustained segmentation of the tail. Such a dose-dependent rescue of the length of the vertebral column is not observed in the cranial body half, which is similar in BBL-1/BBL-1, BBL-1/0, BBL-2/0 and Lfng−/− (Figure 2F).

How might cranial Lfng expression sustain progenitors in the tailbud? Lfng regulates Notch signalling cell-autonomously [reviewed in 35], suggesting that Lfng activity acts indirectly to influence the behaviour of axial progenitor cells. For example, Lfng activity might be needed to maintain the Fgf8 and Wnt signalling that is required for axial extension.

To test this idea, we analysed PSMs of Lfng−/− embryos. Lacking known markers for axial progenitor cells, we analysed the expression of Fgfr, a target of Wnt signalling [36], which forms a caudal-cranial concentration gradient and reflects the maturation of the PSM and of the general PSM marker Tbx6 [37,38]. Fgfr expression in Lfng−/− embryos is indistinguishable from that in control Lfng+/− embryos (Figure 3P–U) indicating that despite of cessation of progenitor proliferation the Lfng−/− PSM is still largely intact when segmentation terminates. Thus, Lfng is not required to maintain longrange signals of Fgf8, Wnt and retinoic acid in the caudal PSM (see Discussion). We conclude that continued tail segmentation promoted by the cranial Lfng stripe in BBL mice does not depend on known long-range signals in the PSM.

Further evidence that loss of Fgfr expression does not cause the short-tail phenotype comes from our finding that Fgfr becomes downregulated between E10.5 and E11.5 in Lfng−/− embryos (n=21; Figure 3Q), i.e. about two days before the end of wildtype tail segmentation. This result indicates that other factors control progenitor maintenance from about E11.

**Regular Formation of the Sacral Axial Skeleton Does Not Require Hes7 Oscillation**

The dispensability of Lfng for the formation of sacral somites and vertebrae might be due to changing requirements for Notch signalling during axial elongation. To test this idea, we analysed mutants of Hes7, a cycling Notch target gene required for somitogenesis that encodes a Notch effector [39,40,41]. Hes7 oscillates in the caudal PSM in phase with Lfng, but is not expressed in the cranial PSM [39,42]. Lfng and Hes7 oscillations...
Figure 3. Influence of Lfng PSM domains on segmentation and extension of the tail. (A–I) Transgenic Lfng stripe expression improves somite compartmentalisation only in the tail. (A–C) Cranial-caudal somite compartmentalisation in Lfng<sup>−/−</sup> embryos (visualised by in situ hybridization with Uncx4.1) is irregular in the cranial body half but regular in the sacral (red box) and first tail somites. Lfng<sup>−/−</sup> E11.5 tails are shorter than wildtype-like Lfng<sup>+/−</sup> tails (compare C and D). The lack of Uncx4.1 stripes close to the PSM suggests that segmentation has ceased in the mutant (red bar in C). (E, F) Transgenic cLfng expression in 5kL lines (5kL−1/0) almost fully rescues compartmentalisation in trunk and tail (arrows in F indicate occasional irregular stripes). (G–I) Transgenic cLfng expression in BBL lines (G, BBL−1/0; H, BBL−1/0; I, BBL−2/0) rescues cranial-caudal compartmentalisation mainly in the tail (arrows in I indicate irregular stripes). (J–O) Lfng is important for clear separation of sclerotomes (ventral compartments of somites) except for those in the sacrum, and influences dorsal-ventral patterning of the paraxial mesoderm of the tail. Sclerotomes (stained with Pax-1) are regular and segmented in wildtype mice (J, K) but enlarged and undefined in Lfng<sup>−/−</sup> embryos except in the sacral area (L, M). 10 μm transverse sections of embryos in K and M at tail region indicated by a dash (N, O; same magnification) counterstained with nuclear fast red (Vector Labs) show an increase in sclerotome size in the mutant (Pax1-positive area encircled with a dashed line on right side of sections). (P–U) Expression of PSM markers Fgf8 (P–R; red arrows point at PSM expression in the tail; expression in Q is weak and restricted, and undetectable in slightly older embryos, not shown) and Tbx6 (S–U) is indistinguishable between Lfng<sup>+/−</sup> and Lfng<sup>−/−</sup> embryos. The PSM is boxed in blue. Forelimb buds (fl) align to somites 8–14, hindlimb buds (hl) to somites 25–30; nt, neural tube.
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Differential Requirements for Hes7 Activity During Segmentation in the Sacral Area

As threshold requirements for Lfng activity show axial variations, we hypothesized that the same might be true for Hes7 activity. Thus, we examined embryos expressing a partial loss-of-function Hes7 allele in which a 14-aminoacid biotin acceptor peptide (BAP) was knocked into the fourth exon of Hes7. 17 aminoacids upstream of the C-terminus. Although the tagged protein (Hes7<sup>BAP</sup>) retains wildtype-like repressor activity in cultured cells (Figure 5A), it shows greatly impaired activity in vivo. Only 22% of the expected number of homozygous Hes7<sup>BAP/BAP</sup> (BAP/BAP) mice survive till weaning (14/254), and the survivors' tails are truncated although not as severely as in Hes7<sup>−/−</sup> mice (Figure 5B and Figure S1B, Table 3; [39]).

Hes7<sup>BAP</sup> transcript levels do not oscillate in the BAP/BAP knock-in embryos (Figures 5C and S3D,E; n = 27 covering all stages between E9.5 and E11.5). Hes7 protein is expressed uniformly in the whole PSM (Figure 5D, n = 7~E10.25). Likewise, Lfng is expressed continuously throughout the BAP/BAP PSM (Figure 5E). A likely explanation for these findings is that BAP-tagging Hes7 has disrupted its repressive activity so that it is unable to maintain an auto-inhibitory feedback loop in vivo. This hypothesis is supported by analysis of heterozygous Hes7<sup>BAP/BAP</sup> (BAP/+), embryonos, in which Hes7 and Lfng transcript levels are normal and oscillating (Figure 5C, 5E), and by our observation that Hes7 protein, the repressor Hes7, is upregulated in BAP/BAP embryos (Figure 5C,D).

BAP/BAP mice display strongly regionalised segmental phenotypes, indicating that Hes7 activity, like that of Lfng, is required differentially during the course of segmentation. The mutant mice show severe segmentation defects in the cervical, thoracic and
Figure 5. Differential requirement for hypomorphic Hes7\textsuperscript{BAP} during segmentation. (A, B) BAP-tagged Hes7 (Hes7\textsuperscript{BAP}) shows full activity in luciferase assays of cultured mouse fibroblast cells (A, relative luciferase activities are shown as mean±s.d. for three experiments) but causes a hypomorphic Hes7 phenotype in vivo (B; note tail lengths of E18.5 foetuses, arrowheads point at the tip of the tails). (C–E) Hes7 (C, in situ hybridisation with an intron probe; D, staining with a Hes7 antibody) and Lfng (E, in situ hybridisation with a cDNA probe) are oscillating in heterozygous (BAP/+; n = 8 Hes7, n = 9 Lfng) but not in homozygous (BAP/BAP; n = 9 Hes7, n = 4 Lfng) E10.5 embryos. (F–H) Skeletons of heterozygous BAP/+ foetuses (F) and adults (H left) are regular and resemble wildtype whereas homozygous (BAP/BAP) foetuses (G) and adults (H right) are regular only in the sacrum and adjacent tail, similar to BBL mice. Foetal skeletons are shown in lateral and dorsal view, adult skeletons in dorsal view; magnification of sacral and adjacent areas of the middle panel in (F,G) are shown in the bottom panel of (F,G); for annotation see Figure 2; arrowheads in (H) point at kinks in tails.

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During segmentation and tail extension, and the significance of the allele. We discuss the individual functions of each subdomain roles in mouse segmentation. The oscillating, caudal segmentation. Nevertheless, both domains play crucial and distinct stripe domain may fulfil a more fundamental role during novelty of higher vertebrates, and the more widely conserved skeleton. Expression of cLfng appears essential for the formation of the cranial half of the axial embryos express cranial PSM but does not oscillate [48,49,50,51]. Corn snake and medaka, where it is expressed in one or two stripes in the also acts in somitogenesis of lower vertebrates such as zebrafish esis is indicated by its expression pattern, and by the effects of Regional Requirements for Different Modes of Lfng Expression An important role for Lfng during mouse and chick somitogenesis is indicated by its expression pattern, and by the effects of manipulating its activity [14,17,18,45,46,47]. It is less clear if Lfng also acts in somitogenesis of lower vertebrates such as zebrafish and medaka, where it is expressed in one or two stripes in the cranial PSM but does not oscillate [48,49,50,51]. Snail embryos express Lfng in up to nine stripes of variable size and spacing in the mid- and cranial PSM, but they too lack a causal oscillating domain [52]. Thus, the oscillating, caudal domain may be an evolutionary novelty of higher vertebrates, and the more widely conserved stripe domain may fulfil a more fundamental role during segmentation. Nevertheless, both domains play crucial and distinct roles in mouse segmentation. The oscillating, caudal Lfng domain appears essential for the formation of the cranial half of the axial skeleton. Expression of cLfng in the cranial stripe fails to rescue somite formation and cranial-caudal patterning of somites 1 to ~30, and few BBL pups, in which this is the only domain of Lfng expression, survive to weaning (Figures 2A, 2D–G, 3G–I; Table 1). By contrast, the stripe domain appears to be sufficient and, in the absence of the oscillating domain, necessary for caudal somite formation and patterning, from somite ~34 onwards (Figures 2A, 2D–G, 3G–I).

Alternatively, the regional phenotypes may reflect different threshold requirements for striped Lfng in different body regions. Transgenic Lfng expression levels are below wildtype levels, and might be too low for regular segmentation cranial to the sacral somites, but sufficiently high for tail somites. In this scenario, high Lfng stripe expression is needed to form somites 1 to ~30, while weak Lfng stripe expression is sufficient for the formation of tail somites from somite ~35 on. However, requirement of oscillatory Lfng for normal segmentation of the cranial body has been demonstrated previously [14,19].

Shifley et al. [53] recently reached similar conclusions to our former model by analysing mice in which Lfng PSM-expression is restricted to the stripe domain. They studied mice (Lfng\textsuperscript{FCE1} in which FCE1/A block, the major enhancer element driving caudal oscillating expression, has been deleted from the endogenous Lfng locus. They too found that Lfng stripe expression rescues caudal but not cranial segmentation, indicating that Lfng requirements differ between trunk and tail formation.

Although a single B-block drives expression only in a cranial stripe, our transgenic BBL lines (with tandem B-blocks) also express very low levels of dynamic–presumably oscillating cLfng in the PSM. Multiple transgenic lines show such expression, indicating that it is indeed due to the duplicated B-block, not the effects of chromosomal flanking sequences on the transgene, i.e. the B-block includes a cryptic oscillator element whose ability to express is enhanced by the tandem duplication. This result is consistent with previous evidence that the Lfng promoter includes multiple cyclic enhancers. In particular, Lfng reporter transgenes lacking the A block still cycle in the caudal PSM, albeit weakly (mlf\textsuperscript{1.8}; [15]). Such expression is visible only after extended staining, perhaps explaining why such cyclic expression was not observed in the AFCE1 embryos [53].

This weak cyclic expression is unlikely to contribute to the rescue of tail segmentation in BBL and AFCE1 mice. For this to be the case, segmentation of the trunk would have to require more oscillatory Lfng than tail segmentation, although the intermediate sacral region does not require oscillatory Lfng at all. The oscillatory

Table 3. Comparison of Hes7\textsuperscript{BAP/+, BAP/BAP, and Hes7\textsuperscript{KO/KO}} E18.5 foetuses and adults (data for adults are given in brackets and italics).

|                      | BAP/+ | BAP/BAP | KO/KO |
|----------------------|-------|---------|-------|
| Number of E18.5 (adult) skeletons analysed | 3 (4) | 4 (5) | 4 (0) |
| Length of whole vertebral column (mm) | 30.6±2.3 (147.5±2.8) | 20.1±1.9 (74.4±12.5) | 16.3±0.5 |
| Length of cervical plus thoracic region (mm) | 9.3±0.6 | 5.5±0.6 | 5.6±0.8 |
| Length of lumbar region (mm) | 3.8±0.3 | 3.6±0.8 | 4.0±0.4 |
| Length of sacral region (mm) | 1.96±0.05 | 1.6±0.2 | 1.6±0.3 |
| Length of tail (mm) | 11.5±0.5 (87.25±4.8) | 6.5±2.4 (32.4±10) | 4.9±0.6 |
| Number of regular lumbar vertebrae | 6±0 (6±0) | 2.3±0.9 (1±1) | 0 |
| Number of regular sacral vertebrae | 4±0 (4±0) | 3.8±0.5 (3.2±1) | 0 |
| Number of regular tail vertebrae | 28±1 (27±1.4) | 6.5±4.4 (4±3.4) | 0 |
| Number of ribs, left and right counted separately | 13±0 (13±0) | 9±0 (9.8±0.8) | 7.3±0.7 |

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caudal expression in BBL embryos is insufficient to rescue segmentation in the trunk (Figures 1D, 1E, 2C–G) and to maintain Hes7 oscillation (Figure S3C). Most likely, restoration of the tail is indeed due to the strong, cranial stripe domain of Lfng expression.

Unexpectedly, the sacral region segments almost normally in Lfng<sup>−/−</sup> embryos: somites ~31 to ~34 are regular and cranial-caudal compartmentalised, and form normal vertebrae (Figures 2C, 2G, 3A–C). The ability of this region to segment independently of Lfng activity has not been noted previously, and is further evidence that the segmentation circuitry varies along the cranial-caudal body axis. Although Hes7 activity is needed for somites to form normally in vivo, even though it retains repressive activity in cultured cells. The homozygous Hes7<sup>BAP/BAP</sup> phenotype is almost as strong as that of Hes7<sup>−/−</sup> embryos, and is fully recessive. Hes7 is overexpressed in homozygous but not heterozygous BAP embryos, indicative of a recessive failure of repression.

The apparent difference between the ex and in vivo activity of Hes7<sup>BAP</sup> could be due to the considerable overexpression in the former context that might allow a greatly weakened Hes7<sup>BAP</sup> still to repress target genes. Alternatively, the insertion in Hes7<sup>BAP</sup> prevents binding of an accessory factor that is only required in the in vivo environment. Clearly, because a protein is active in a cultured cell assay, it cannot be assumed to retain activity in vivo.

**Temporal Changes of the Segmentation Machinery**

Axial changes in the segmentation circuitry were first suggested by the phenotype of Notch signalling mutants. The first 7–9 somites form normally in Notch1/Notch2 double knock-out mutants and in mice lacking the common, downstream transcription factor RBP-Jk/CBF1 [54,55]. Notch pathway mutations in zebrafish also retain the first ~7 somites [56,57]. However, this apparent regional phenotype might be due to gradual desynchronisation of neighbouring oscillators during axial elongation, not to changes in regulatory circuitry [58]. An example of differential regulation of mesoderm development along the length axis is the changing hierarchy of the T-box genes tobal, spadetail and thanx in trunk versus tail in zebrafish embryos [59]. Several other genes exert regionalised effects on somite formation by unclear mechanisms, e.g. z-fra169 [60].

Our results define at least three phases of segmentation during the 5 days of mouse somitogenesis (Figure S4). Phases A (somites 1–30; E7.75–E10) and C (somites ~35–65; E10.5–E13.5) rely mainly on oscillating Lfng and the cranial PSM stripe, respectively, and have also been described by Shilley et al [53]. However, we also see clear evidence of a transition phase B (somites ~31–34) that is associated with a drop in the requirement for Lfng activity such that, even in the complete absence of Lfng, mice frequently develop normal sacral vertebrae. This sacral region is also less sensitive to the reduced levels of Hes7 activity in homozygous Hes7<sup>BAP/BAP</sup> animals (Figure 5G, 5H right). Hes7 requirements may be more stringent for vertebrae in the tail; 43% of adult Hes7<sup>−/−</sup> animals have a kinked tail [59].

As our BBL mice express some cyclic Lfng in addition to the cranial PSM stripe that is probably below wildtype levels, we cannot formally exclude the alternative but unlikely model (see discussion above) that stronger, wildtype-like stripe expression would alleviate the Lfng phenotype in the trunk, or that the weak oscillating caudal domain in BBL embryos contributes to the tail rescue. A transgenic line expressing only the cranial PSM stripe at wildtype levels without a caudal PSM domain would allow to unambiguously assign individual expression domains (oscillatory vs. stripe) to phases A and C. However, such a desirable transgenic line cannot be made because the Lfng promoter is more complex than previously thought: the "stripe-specific" enhancer (B-block) also drives a faint caudal domain, and other promoters are unlikely to precisely match timing, position, extent and expression level of the wildtype Lfng stripe.

Requirements for Wnt signalling, which is needed for mouse Lfng to oscillate [61,62], might also show regional variation. Mice with reduced canonical Wnt signalling due to an in-frame insertion of lacZ in the Left1 gene form skeletons with severely disorganised vertebrae. These mice exhibit a regularly patterned sacral and cranial tail region similar to that found in BBL animals (Figure 1B in [63]).

It is unclear what positions and triggers the different phases. In wildtype mice, phase B is inconspicuous, and not revealed by any obvious variability in oscillatory modes of expression. The beginning of phase B coincides with the time when mesodermal cells are recruited from the tailbud and no longer by involution and streaming through the primitive streak as for phase A (~30-somite stage; [64]). The phase B-mode could lead to a partial loss of synchronisation of PSM cells due to changed cell movements, and the segmentation clock circuitry might be modified to take this into account.

The onset of phase B is slightly variable: the site of the first regular vertebra in Lfng<sup>−/−</sup> mice varies between the last two lumbar vertebrae and the first two sacral vertebrae. Thus, the lumbosacral boundary, which is positioned by the activity of Hox9 and Hox10 paralogous genes in the PSM [22,24], does not directly determine the onset of phase B. Nevertheless, the number of vertebrae covered by phase B is relatively invariant (~12–15 somite stage; [64]). The phase B-mode could lead to a partial loss of synchronisation of PSM cells due to changed cell movements, and the segmentation clock circuitry might be modified to take this into account.

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Lfng Is Important for the Maintenance of Somitogenesis

Premature termination of axial extension in Lfng<sup>−/−</sup> embryos indicates that progenitor cells become depleted in these embryos at around E10.5. The low variability of Lfng<sup>−/−</sup> tail lengths hints at a clear-cut temporal change in the requirement of Lfng for maintenance of axial extension in trunk vs. tail when the segmentation machinery shifts from phase B to C.

Our transgenic BBL lines show an unexpected Lfng expression domain in the caudal PSM. Although this caudal domain could contribute to continued axial extension of the tail by short-range signalling, its expression is very weak and unable to promote segmentation (discussed above). Rather, the rescue of the Lfng<sup>−/−</sup> short-tail phenotype by stripe expression of Lfng in BBL mice suggests that Lfng expression in the cranial PSM regulates expression of a component that signals caudally to regulate axial extension.

Our results suggest that this signal is not mediated by retinoic acid, a long-range signal made in newly formed somites; retinoic acid regulates expression of Fgf8 in the caudal PSM and tailbud [65,66], yet Fgf8 expression is normal in Lfng<sup>−/−</sup> E10.5 embryos (Figure 3P, 3R). Either retinoic acid signalling is unaffected in the
mutant embryos, or Lfng modifies retinoic acid expression or activity in a manner that does not affect Fgfβ transcription.

Indeed, Fgfβ transcription may not be required to sustain proliferation of axial progenitor cells at late stages of somitogenesis. Fgfβ transcripts are no longer detectable in wildtype PSMs from stage E11.5 (Figure 3Q), yet somitogenesis and Tbx6 and Lfng transcription persists for a further two days (Figure 3T, and not shown). This lack of linkage between Fgf and Lfng expression suggests that other signals from the cranial PSM or somites are involved in maintaining axial elongation.

Materials and Methods

Mouse Lines

The Lfng null line was obtained from Randy Johnson [17] and the Hes7 null line from Ryoihiro Kageyama [39]. Transgene constructs were injected into F1 x F1 (CBA x C57Bl/6 SJ) mouse embryos. Transgenic mice were kept in the C57Bl/6J background. C57Bl/6J also served as wildtype control. All animals were handled in strict accordance with good animal practice as defined by the Animals (Scientific Procedures) Act 1986 and in accordance with the Codes of Practice issued for the use of animals in scientific procedures issued by the UK Home Office. All animal work was approved by the ethical committees for experiments with animals of the London Research Institute.

Preparation of cLfng Transgene Vectors and Hes7BAP Targeting Construct

The construction of transgene vectors BB-cLfng and Lfng5kb-cLfng (Figure 1A) and of the targeting vector used to generate Hes7BAP mice is described in Methods S1.

Thermal Asymmetric Interlaced (TAIL) PCR

We mapped the integration sites in the transgenic lines by amplifying the flanking genomic regions using TAIL-PCR with a combination of degenerate and nested primers ([67]; for primers see Table S1).

In 5kL-1 mice, the transgene is inserted into chromosome 19, position 31,973,131 (Build 36), i.e. into the second intron of the APOBEC-1 complementation factor (ACF) locus. We never obtained homozygous transgenics from various hemizygous 5kL-1 parents (100 offspring genotyped for homozygosity), consistent with the pre-implantation lethality of homozygous ACF+/− embryos [68].

The BBL-1 transgene is inserted into chromosome 8, position 44,990,545. Homozygous BBL-1 transgenics are viable and fertile and appear normal.

In BBL-2 we found three integration sites located within a distance of 500 kb on chromosome 4, all in introns of the “unclassifiable” cDNA AK139518 (positions 23,085,307/23,086,135 resulted in a rearrangement of the genomic locus. No homozygous transgenic mice of BBL-2 were identified among the offspring of several hemizygous parents (46 offspring genotyped for homozygosity), indicating that homozygous insertion is embryonic lethal.

We were unable to identify the genomic integration site of the 5kL-2 line, but found tandem insertions of the transgene, as were also present in the other three lines.

In Situ Hybridisation and Immunochemistry

Whole-mount in situ hybridisation was performed by a modification of the method of Henrique et al. [69]; described in the Supporting Information). RNA probes were made from 2.1 kb cLfng cDNA [70], 0.7 kb Fgfβ cDNA [71], 0.7 kb Hes7 ORF [72], 1.2 kb mouse Lfng cDNA (IMAGE clone 408467), 1.9 kb MyoD cDNA [28], 0.9 kb Pax1 cDNA [30], 0.7 kb Tbx6 cDNA [38], 1.4 kb Tbx18 (PCR-amplified from exon 8) [27], and 0.7 kb Unx4.1 cDNA [73]. The Hes7 intron probe was synthesised from a 1 kb PCR product of the first intron cloned into pCR-TOPO vector (Invitrogen) and used as for the cDNA probes except that hybridisation was at 65°C instead of 70°C. Hes7 antibody staining was performed as described [40].

Skeleton Preparation

Adult and E18.5 skeletons were prepared and stained with alcian blue/alizarin red S following standard procedures. All photos were taken with a Leica DC500 digital camera and Leica Firecam version 1.7.1 software. Several photos were assembled for adult skeletons in Figure 2G and 5H.

Luciferase Reporter Assay

C3H10T1/2 cells were plated at a density of 8×10^4 per well of a 24-well tissue culture plate 24 hours before transfection, 100 ng of the firefly luciferase reporter under the control of six N-boxes and the β-actin promoter [74] was cotransfected with 200 ng pCI (Promega); pCI-Hes7 or pCI-Hes7-BAP using GeneJuice (Novagen) transfection reagent at a ratio of 1:3; DNA:GeneJuice. 4 ng of the Renilla luciferase vector pRL-TK (Promega) was used in each sample as reference reading. After incubation for 24 hours at 37°C and 5% CO₂, the assay was analysed using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer’s guidelines. Firefly and Renilla activities were read using the EnVision Multilabel Reader. The values of the reporter readings were normalised to the values of the Renilla reading. The resulting luciferase activity alone was taken 100%. Each experiment was done in triplicates and repeated at least three times.

Supporting Information

Figure S1 Adult tail phenotypes of 5 kL and BBL transgenes and Hes7BAP knock in mice. Transgenic mice of the 5 kL lines (without endogenous Lfng) resemble wildtype and Lfng+/−/mice; BBL lines (without endogenous Lfng) show a tail rescue of variable degree (A, arrowheads point at kinks). Hes7BAP/+ mice resemble wildtype; Hes7BAP/BAP mice have truncated, kinky tails (B).

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Figure S2 Expression of Tbx18 and MyoD in Lfng−/−/ E11.5 embryos. Expression of Tbx18 (A–D) and MyoD (E–H) in Lfng−/−/ mice as compared to wildtype as captured by MyoD and Tbx18 staining, indicating that MyoD expression is less obvious than in Uncx4.1 or Tbx18 stainings, i.e. (E, F). Regionalisation of the Lfng−/−/ domains can be observed in any region of the length axis by MyoD is less obvious than in Uncx4.1 or Tbx18 stainings, i.e. (E, F). Regionalisation of the Lfng−/−/ null phenotype visualised by MyoD is less obvious than in Uncx4.1 or Tbx18 stainings, i.e. fused stripes can be observed in any region of the length axis including the sacrum, indicating that the observed requirements for Lfng activity mainly apply to the sacral, and not to the myotomal compartment.

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Figure S3 Non-oscillatory Hes7 expression in E11.5 Lfng−/−/ mice, BBL-1, and BAP/BAP embryos. Transcription of Hes7 visualised
by in situ hybridisation of Lfng+/− (A), Lfng−/− (B, n = 5), BBL-1 (C, n = 4), BAP/+ (D) and BAP/BAP (E). Black arrows point at regular somite borders in an embryo with uniform Hes7 expression throughout the PSM in (C).

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**Figure S4** Three phases of mouse segmentation. Segmentation phase A, which comprises the cervical, thoracic and lumbar region, requires oscillatory Lfng and oscillatory Hes7+. During phase B, the segmentation of the sacrum, Lfng expression in both PSM domains is dispensable; Hes7 does not need to have full activity (as in the hypomorphic allele Hes7/BAP) and does not need to oscillate. Phase C, segmentation of the tail, requires the cranial Lfng stripe expression and oscillatory Hes7+, but the oscillatory Lfng domain is less important.

Found at: doi:10.1371/journal.pone.0007996.s004 (0.45 MB EPS)

**Table S1** List of primers.

Found at: doi:10.1371/journal.pone.0007996.s005 (0.08 MB DOC)

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