Notch Is a Critical Component of the Mouse Somitogenesis Oscillator and Is Essential for the Formation of the Somites

Zoltan Ferjentsik1, Shinichi Hayashi2, J. Kim Dale1,9, Yasumasa Bessho7, An Herreman3,4, Bart De Strooper3,4, Gonzalo del Monte5, Jose Luis de la Pompa5, Miguel Maroto1*

1 Division of Cell and Developmental Biology, College of Life Sciences, University of Dundee, Dundee, Scotland, United Kingdom, 2 Graduate School of Biological Sciences, Nara Institute of Science and Technology, Ikoma, Nara, Japan, 3 Department of Molecular and Developmental Genetics, Vlaams Instituut voor Biotechnologie, Leuven, Belgium, 4 Center for Human Genetics, KULeuven, Leuven, Belgium, 5 Cardiovascular Developmental Biology Department, Centro Nacional de Investigaciones Cardiovasculares, Madrid, Spain

Abstract

Segmentation of the vertebrate body axis is initiated through somitogenesis, whereby epithelial somites bud off in pairs periodically from the rostral end of the unsegmented presomitic mesoderm (PSM). The periodicity of somitogenesis is governed by a molecular oscillator that drives periodic waves of clock gene expression caudo-rostrally through the PSM with a periodicity that matches somite formation. To date the clock genes comprise components of the Notch, Wnt, and FGF pathways. The literature contains controversial reports as to the absolute role(s) of Notch signalling during the process of somite formation. Recent data in the zebrafish have suggested that the only role of Notch signalling is to synchronise clock gene oscillations across the PSM and that somite formation can continue in the absence of Notch activity. However, it is not clear in the mouse if an FGF/Wnt-based oscillator is sufficient to generate segmented structures, such as the somites, in the absence of all Notch activity. We have investigated the requirement for Notch signalling in the mouse somitogenesis clock by analysing embryos carrying a mutation in different components of the Notch pathway, such as Lunatic fringe (Lflng), Hes7, Rbpj, and presenilin1/presenilin2 (Psen1/Psen2), and by pharmacological blocking of the Notch pathway. In contrast to the fish studies, we show that mouse embryos lacking all Notch activity do not show oscillatory activity, as evidenced by the absence of waves of clock gene expression across the PSM, and they do not develop somites. We propose that, at least in the mouse embryo, Notch activity is absolutely essential for the formation of a segmented body axis.

Introduction

Segmentation is a key feature of the body plan of all vertebrates, including humans, that initiates very early in embryonic development. The first sign of metamersism or segmentation is seen when vertebrate embryos develop somites, the precursors of several segmented organs such as the axial skeleton, body skeletal muscles and part of the dermis. Somites are formed in a highly regulated process called somitogenesis from the unsegmented presomitic mesoderm (PSM) [1–3]. During the formation of somites the most mature PSM cells located at the rostral end of the PSM bud off as an epithelial sphere of cells to form the somite. Somite formation occurs simultaneously with the recruitment of newly generated mesenchymal cells from the primitive streak/tail bud into the caudal region of the PSM [2–4].

Critical molecular and embryological experimental data obtained in the last ten years has shown that somitogenesis is governed by a molecular oscillator [5] that drives cyclic expression of genes in the PSM and which is coupled to the formation of the somites [2,3,6–8]. Expression of these cyclic genes is coordinated such that a wave of expression travels caudo-rostrally throughout the PSM during the formation of one somite. All cyclic genes identified to date encode either (a) components or modulators of the Notch pathway (b) components of the Wnt pathway or (c) components of the FGF pathway [2,3,6–8].

There are discrepancies in the literature regarding the role(s) of Notch signalling during the process of somite formation. At least in the zebrafish embryo it seems clear that Notch signalling has a predominant function in the synchronization of clock gene oscillations, where inhibition of Notch is not sufficient to interrupt the generation of a segmented body plan [7–11]. This view of Notch as a clock synchronizer has also been proposed to operate during mouse somitogenesis [12,13]. On the other hand, data generated in chick, mouse and zebrafish is consistent with Notch...
Author Summary

Vertebrate animals generate their segmented body plan during embryogenesis. These embryonic segments, or somites, form one after another from tissue at the tail end of the embryo in a highly regulated process controlled by a molecular oscillator. This oscillator drives the expression of a group of genes in this tissue and determines the periodicity of somite formation. To date the genes regulated by this molecular clock comprise components of the Notch, Wnt, and FGF pathways. Recent data in the zebrafish embryo have suggested that the only role of Notch signalling in this process is to synchronise gene oscillations between neighbouring cells and that somite formation can continue in the absence of Notch activity. However, we show that mouse embryos lacking all Notch activity do not show oscillatory activity and do not develop somites. We propose that, at least in the mouse embryo, Notch activity is absolutely essential for building a segmented body axis.

Notch is essential to the mouse segmentation clock

In principle, the observed upregulation of Notch downstream targets in the Lfng⁻/⁻ embryos could culminate in an accumulation of the mRNA for these genes along the PSM. To test this, we decided to measure the amount of Hes7 mRNA present in the rostral half of the PSM of wild type or Lfng⁻/⁻ embryos. To that end we isolated the total RNA from pooled rostral half PSM samples of several wild type and mutant embryos of unknown cyclic phases and then performed quantitative RT-PCR. We observed that the relative expression level of Hes7 revealed no statistically significant difference between wild type (n = 12) and Lfng⁻/⁻ (n = 10) PSM samples (Figure 2A; t-test, df = 20, P = 0.130). One possible explanation for this lack of accumulation of Hes7 mRNA in the Lfng⁻/⁻ embryos might be that the transcripts are in fact produced and degraded as in the wild type embryo, albeit not quite as efficiently. We decided to re-examine Hes7 mRNA expression in these Lfng⁻/⁻ embryos in more detail. When we re-analysed Lfng⁻/⁻ embryos with the Hes7 probe carefully monitoring the intensity of the revelation step we observed different patterns or phases of expression (n = 13, Figure 2B and 2C; [22]). Longer staining of the same mutant embryos could culminate in an accumulation of Hes7 mRNA in these Lfng⁻/⁻ embryos. To that end we isolated the total RNA from pooled rostral half PSM samples of several wild type and mutant embryos of unknown cyclic phases and then performed quantitative RT-PCR. We observed that the relative expression level of Hes7 revealed no statistically significant difference between wild type (n = 12) and Lfng⁻/⁻ (n = 10) PSM samples (Figure 2A; t-test, df = 20, P = 0.130). One possible explanation for this lack of accumulation of Hes7 mRNA in the Lfng⁻/⁻ embryos might be that the transcripts are in fact produced and degraded as in the wild type embryo, albeit not quite as efficiently. We decided to re-examine Hes7 mRNA expression in these Lfng⁻/⁻ embryos in more detail. When we re-analysed Lfng⁻/⁻ embryos with the Hes7 probe carefully monitoring the intensity of the revelation step we observed different patterns or phases of expression (n = 13, Figure 2B and 2C; [22]). Longer staining of the same mutant embryos led to the general upregulation of Hes7 described above (Figure 2B and 2C, Figure 1I). Under similar conditions of longer staining this general upregulation is not observed using wild type embryos (n = 25, data not shown). To further corroborate these data we analysed Lfng⁻/⁻ embryos using a Hes7 intronic probe in order to detect nascent pre-spliced mRNA and thereby to show the location of active transcription [23]. The Lfng⁻/⁻ embryos (n = 6) presented patterns of Hes7 expression similar to those observed in wild type embryos (n = 6, Figure 2D and 2E, data not shown). To confirm that these different patterns corresponded indeed to a dynamic activity we performed a half embryo analysis, in which the tail of an embryo is split longitudinally in two halves, then one half is immediately fixed and the other is cultured for 60 minutes before fixation [24,25]. In situ hybridisation with an intronic Hes7 probe on samples prepared using this type of analysis showed that the two halves displayed different patterns of expression (n = 5, Figure 2F), which clearly indicates that in the absence of Lfng activity the expression of the Notch-related cyclic gene Hes7 is still dynamic. Similarly, when we analysed the expression of a second Notch-related cyclic gene, Narp, we also
found different patterns of expression similar to those observed in wild type embryos (n = 8 and n = 12 respectively, Figure 2G and 2H, Figure 3C and 3D; [26]). In addition, we analysed the expression of Hes7 protein in tails of E10.5 Lfng−/− embryos (n = 8) using a specific anti-Hes7 antibody [19] and found that Hes7 protein also displayed different phases of expression, consistent with it being cyclic, although the boundaries of expression were not as sharp as in the wild type (n = 8 and n = 10 respectively, Figure 2I and 2J; data not shown). Finally, we tested the expression of the cleaved intracellular portion of Notch (NICD), which is the active fragment of Notch and is generated when Notch receptor, after its interaction with the ligand, is processed by the γ-secretase complex. Once NICD is produced it translocates to the nucleus where it binds to RBPj and the complex becomes a transcriptional activator of downstream targets [21]. NICD has previously been reported to display a dynamic expression profile in the PSM of wild type mice embryos [27,28]. Using an antibody specific for NICD we stained tail sections of E10.5 Lfng−/− embryos and observed different patterns of NICD expression (n = 9, Figure 2K and 2L), consistent with Notch activity still being dynamic. These results clearly indicate that in the absence of the glycosyltransferase Lfng it is possible to detect dynamic NICD and dynamic expression of Notch-related cyclic genes, although not with sharp boundaries of expression, which suggests that Lfng could be an important but not a critical component of the mouse oscillator.

Oscillations of Notch cyclic genes are absent in the PSM of Hes7−/− embryos but not those of Wnt and FGF cyclic genes

In order to examine more closely whether the Hes7−/− embryos also retained some cyclic Notch activity, we first analysed these embryos by in situ hybridisation with an intronic probe against the Notch-regulated cyclic gene Lfng. Strikingly, we found a different situation in the Hes7−/− embryos than we had observed in the Lfng−/− embryos and we were not able to detect the existence of different phases of Lfng expression. During the course of a controlled staining all Hes7−/− embryos (n = 10) displayed the same profile of a broad rostro-caudal gradient of expression (Figure 3B). Longer staining of the same mutant embryos led to the general upregulation of Lfng described above (Figure 3B', Figure 3A). Similarly, the expression pattern of the Notch-regulated cyclic gene Nrarp was identical in all Hes7−/− embryos (n = 7, Figure 3E). These non-dynamic patterns are
consistent with the fact that in these Hes72/2 embryos we did not see different patterns of NICD expression either, rather it was detected in a rostro-caudal gradient of expression across the PSM (n = 6, Figure 3F). Expression of the non-cyclic Notch target gene Mesp2 [29,30] was retained in the rostral region of the PSM of Hes72/2 embryos, although the expression domain was not as sharp as in wild type embryos (Figure 3G and 3H; [16]). It has been reported that Mesp2 expression is severely compromised in Dll12/2 and Rbpj2/2 mutant embryos [31]. Thus, the Mesp2 band of expression observed in the Hes72/2 embryos is probably due to the presence of non-dynamic Notch signalling activity in the PSM. In summary, there is a non-dynamic expression of Notch-based cyclic genes in the PSM of Hes72/2 embryos, which mirrors the non-dynamic Notch activity in this tissue.

Since in the Hes72/2 embryos, unlike the Lfng2/2 embryos, there does not appear to be cyclic activity of Notch-regulated cyclic genes we investigated whether any of the FGF or Wnt regulated cyclic genes were oscillating in the PSM of these Hes72/2 embryos. Based on the patterns of expression observed in Dll12/2 embryos it has been proposed that the expression of Axin2 is independent of Notch activity [32]. We first examined the expression of the Wnt-related cyclic gene Axin2 in the PSM of Hes72/2 embryos (n = 30) and observed different patterns of expression (Figure 4D and 4E; [20]). In addition, a fix and culture assay using E10.5 caudal explants of Lfng2/2 embryo confirms oscillatory Hes7 expression. Arrowheads demarcate distinct domains of expression in the PSM.

doi:10.1371/journal.pgen.1000662.g002

Figure 2. Notch-based cyclic gene expression is still dynamic in the PSM of Lfng2/2 embryos. (A) Bar chart showing that the relative amount of Hes7 mRNA in the PSM of wild type and Lfng2/2 embryos is not statistically different as judged by qRTPCR (P = 0.130). Error bars represent standard deviation. (B–L) Lateral views of the caudal region of E9.5–10.5 Lfng2/2 embryos analysed by in situ hybridisation or immunocytochemistry using (B,B’,C,C’) Hes7, (D–F) intronic Hes7, (G,H) and Nrarp probes, (I,J) anti-Hes7 or (K,L) anti-NICD antibodies. (F) Fix and culture assay using E10.5 caudal explants of Lfng2/2 embryo confirms oscillatory Hes7 expression. Arrowheads demarcate distinct domains of expression in the PSM.
We tested the expression of Dusp6 \( (n = 30) \) and Sprouty2 \( (n = 32) \) in Hes7\(^{2/2}\) embryos and observed different patterns of expression, consistent with dynamic FGF activity in the PSM of these embryos (Figure 4O, 4P, 4S, and 4T). In summary, we conclude that embryos lacking Hes7 retain dynamic activity of the Wnt-regulated genes and FGF-regulated genes of the somitogenesis oscillator, which is likely to underlie the generation of periodicity and the formation of irregular somites in these embryos.

Somitogenesis is lost in the Psen1/Psen2 double mutant embryos

Our data indicate that in Lfng\(^{-/-}\) embryos the cyclic gene oscillations comprise elements of the Notch pathway (Figure 2). This is in contrast to the situation in the Hes7\(^{-/-}\) embryos because the oscillatory mechanism appears to be based only on Wnt and FGF-dependent genes. It is not clear, however, if this Wnt/FGF-based oscillator is sufficient to generate segmented structures, such as the somites, in the complete absence of all Notch activity, since in these Hes7\(^{-/-}\) embryos there remains clear evidence of non-dynamic Notch activity, which could be a critical requirement for the proper function of an Wnt/FGF-based segmentation oscillator and/or the formation of the somites. To more definitively test the relevance of Notch activity during the process of somitogenesis we decided to re-analyse homozygous null embryos from two other mutant lines widely accepted to develop in the complete absence of Notch activity, Rbpj\(^{-/-}\) and Psen1\(^{-/-}\);Psen2\(^{-/-}\) [39–41]. We first evaluated the situation in Rbpj\(^{-/-}\) embryos. RBPj is the transcriptional repressor to which NICD binds in the nucleus in order to activate expression of downstream target genes [21,42,43]. Rbpj\(^{-/-}\) embryos die at approximately E9.5 after forming a variable number (zero to five) of disorganized and irregular somite-like structures [39]. As expected, we observed that at stage E8.5–9.0 the expression of the Notch-regulated cyclic gene Lfng \( (n = 6) \) was lost in the PSM (Figure 5B). Barrantes and colleagues have previously reported that in a few cases they were able to detect a single faint stripe of Lfng in the rostral PSM [31]. Surprisingly, however, we found that the Notch-related cyclic gene Hes7 was still present along the PSM of these Rbpj\(^{2/2}\) embryos \( (n = 5, \) Figure 5D and 5E; [34]). In fact, Hes7 expression can be detected with different patterns of expression in a broad caudal domain and in restricted bands in the rostral region, similar to the expression observed in wild type embryos (Figure 5C), suggesting that its dynamic character may still be functional in these mutant embryos. Similarly we also found that the Wnt/FGF-based cyclic gene Snail1 displayed different patterns of expression, including both a caudal domain and a rostral band of expression \( (n = 14, \) Figure 5I and 5J; [33]). The Wnt-related cyclic gene Axin2 \( (n = 4) \) and the FGF-related cyclic genes Dusp6 \( (n = 4) \) and Sprouty2 \( (n = 5) \) were also found expressed along the PSM with patterns of expression similar to those found in wild type embryos (Figure 5G, 5L, and 5N). Of critical importance to this study is the fact that in Rbpj\(^{-/-}\) embryos two Notch-dependent cyclic genes, Lfng and Hes7, respond differently to the absence of RBPj activity and at least Hes7 displays patterns of expression in the PSM similar to those observed in the wild type, which may be dynamic. These data raise the question of whether these mutant embryos do in fact develop in the complete absence of
Notch activity or whether there remains some residual RBPj-independent Notch activity similar to what has been described in Drosophila [44,45]. Feller and colleagues have shown that the expression of these two Notch targets, Lfng and Hes7, is also differentially affected following perturbation of Notch activity in the PSM of embryos expressing constitutive-activate Notch (T-NICD) [13]. Thus, it would appear that they are not equally sensitive to the levels of NICD as an input to their expression. It is formally possible that the absence of RBPj results in a severe decrease of Notch signalling leading to a loss of Lfng but that a certain level of RBPj-independent NICD activity remains, which could act to maintain the expression of Hes7. To further investigate this possibility we decided to explore if NICD is expressed in the PSM of the Rbpj2/2 embryos. Our immunostaining on sections clearly indicate that indeed it is possible to detect weak NICD expression in the PSM of these mutant embryos (Figure 5P; [46]). From these two results, the existence of different patterns of expression of Hes7 in the PSM and the expression of NICD, we conclude that the Rbpj2/2 mutant line is not appropriate to definitively test the significance of a complete lack of Notch activity during the process of somitogenesis.

We next examined embryos from the double knockout line Psen1/Psen2, which generate mutant embryos lacking all presenilin activity [40,41]. Presenilin is the catalytic component of the γ-secretase complex responsible for the cleavage of Notch receptor and the generation of NICD. In principle, this mutant mouse line should lack all Notch activity. Psen1+/−;Psen2−/− embryos retaining one allele of presenilin1 did not display a somitic phenotype (n = 20, Figure 6A) and showed normal patterns of Hes7 expression along the PSM (Figure 6C and 6D). In contrast, Psen1−/−;Psen2−/− embryos lacking the two presenilins failed to form any somites (n = 22, Figure 6B; [40]) and Hes7 expression was absent in the medial and rostral PSM, consistent with this expression domain being entirely Notch dependent. Nevertheless, it was possible to detect weak Hes7 expression in the tail bud region (n = 4, Figure 6E), which may be indicative of FGF-induced activation in this domain since FGF is reported to be responsible for the initiation of Hes7 expression in the caudal PSM [34,35]. Consistent with the idea that these double mutant embryos develop in the complete absence of Notch activity is the fact that NICD was not detected by
imunostaining on sections prepared from these double mutant embryos (Figure 6J). To confirm this negative result we also performed western blot analysis using protein samples prepared with embryonic fibroblasts from the \( Psen1^{-/-};Psen2^{-/-} \) embryos [47] and observed that NICD is not produced (Figure 6K; [48]). These data indicate that, in contrast to our observations in the \( Rbpj^{-/-} \) embryos, the \( Psen1^{-/-};Psen2^{-/-} \) embryos develop in the complete absence of Notch activity, as judged by the absence of NICD production and the absence of cyclic expression of Notch-dependent \( Hes7 \). Interestingly, we also found that in these double mutant embryos the expression of \( Axin2 \) (n = 4), \( Snail1 \) (n = 4), \( Dusp6 \) (n = 4) and \( Sprouty2 \) (n = 4) is lost along the PSM and when they are detected the remaining staining is restricted to the neural tube or caudal tail bud (Figure 6F–6I). Together these results indicate that the Notch activity appears to be critical both to maintain any form of dynamic clock gene expression in the PSM and to generate somites.

**Figure 5.** Notch-based cyclic gene \( Hes7 \) displays different patterns of expression in the PSM of the \( Rbpj^{-/-} \) embryos. (A–J) Dorsal and (K–N) lateral views of caudal region of E8.5–9.5 (A,C,F,H,K,M) wild type or (B,D,E,G,I,J,L,N) \( Rbpj^{-/-} \) embryos analysed by in situ hybridisation using (A,B) \( Lfng \) and \( Uncx4.1 \), (C–E) \( Hes7 \), (F,G) \( Axin2 \) and \( Uncx4.1 \), (H–J) \( Snail1 \) and \( Uncx4.1 \), (K,L) \( Dusp6 \) and (M,N) \( Sprouty2 \) probes. (O,P) Immunostaining prepared using the anti-NICD antibody on cross-sections of E8.5 (O) wild type and (P) \( Rbpj^{-/-} \) embryos showing expression in the PSM. Red arrowheads demarcate a distinct, discrete rostral domain of expression of \( Hes7 \) in the PSM of the mutant embryo. White arrowhead indicates the weak NICD expression detected in the PSM of the mutant embryo.

doi:10.1371/journal.pgen.1000662.g005
We decided to confirm these observations by blocking all Notch activity in the PSM using a pharmacological treatment and then analysed the effect of this treatment on the expression of oscillatory genes and somite formation, similar to the approach used in recent studies focusing on the role of Notch during somitogenesis in the zebrafish embryo [6,10,11,49]. We first performed the treatment using the half embryo assay in the presence or absence of either 100 μM DAPT or 100 nM LY411575, two reagents that block Notch by inhibiting the γ-secretase cleavage of the Notch receptor [50–54]. We confirmed by western blot that after a 3 hour exposure to the Notch-blocking drugs the expression of NICD was abrogated (Figure 6K), as previously reported [48,55]. Furthermore the expression of Ltg and Niap was completely abolished from the PSM, as expected (n = 8, Figure 7A; [24,25]; Wright et al., submitted). In addition, the drug-treated samples showed no expression of Hes7 in the rostral PSM, consistent with this expression domain being entirely Notch dependent. In the rest of the PSM these treated explants also showed either no expression of Hes7 at all or only a restricted weak caudal domain in the tail bud (n = 14, Figure 7D), which is reminiscent of the Hes7 pattern of expression observed in the Psen1−/−;Psen2−/− embryos (Figure 6E). As mentioned above, Hes7 expression in the caudal PSM is initiated by FGF activity [34,35]. Therefore, if Notch and

**Figure 6. Cyclic gene expression is lost or restricted to the caudal PSM in Psen1/Psen2 double mutant embryos.** (A–E) Lateral and (F–I) dorsal views of caudal regions of E9.5 (A,C,D) Psen1+/−;Psen2−/− or (B,E,F–I) Psen1−/−;Psen2−/− double mutant embryos analysed by in situ hybridisation using (C–E) Hes7, (F) Axin2, (G) Snail1, (H) Dusp6 and (I) Sprouty2 probes. (J) Immunostaining using the anti-NICD antibody on cross-sections of an E9.5 Psen1−/−;Psen2−/− embryo showing no expression in the PSM. (K) Western blot analysis with control embryonic extract, embryonic extract after treatment with DAPT/LY411575 and embryonic fibroblast prepared from Psen1−/−;Psen2−/− embryos analysed with anti-α-tubulin and anti-NICD antibodies. Black arrowhead demarcates a distinct, discrete caudal domain of expression of Hes7 in the PSM. doi:10.1371/journal.pgen.1000662.g006
FGF activities are blocked simultaneously. Hes7 expression should disappear completely from the PSM. Consistent with this idea, when half embryo samples were cultured in the presence of both DAPT and SU5402, a drug that blocks FGF signalling, the expression of Hes7 was completely abolished in the caudal PSM (n = 10, Figure 8G). Drug treatment with DAPT/LY411575 also drastically reduced Axin2 expression throughout the PSM as compared to untreated controls, and when detected it was restricted to a weak caudal domain (n = 14, Figure 7G). These data indicate that drug treatment blocked the rostral progression of cyclic expression of both the Notch-based gene Hes7, as well as that of the Wnt-regulated gene Axin2 across the PSM. To test the effect of DAPT/LY411575 treatment on somite formation we cultured E8.0–8.5 wild type mouse embryos for 18–20 hours using an in vitro roller culture system in the presence or absence of the drug. During this period of time control embryos formed 9–10 extra somites (n = 16, Figure 7B, 7E, 7H, 7K, 7N, and 7Q), however embryos treated with Notch-blocking drugs formed a very limited number of somites, varying from 0 to 2 (n = 65, Figure 7C, 7F, 7I, 7L, 7O, and 7R). As expected, drug-treated embryos did not show Lfng expression (Figure 6C). In addition, none of the treated embryos expressed Hes7 in the rostral or medial PSM and in those that did show expression it was very weak and restricted to the caudal end of the tail bud (n = 9). Figure 7F). Likewise, none of these treated embryos expressed Axin2 in the rostral PSM but a proportion showed some weak expression in the caudal domain (n = 8, Figure 7I). To test if FGF-dependent cyclic genes are also affected under these conditions we evaluated the expression of Snail1, Dusp6 and Sprouty2 and found that their expression seems to be not affected following a 3 hour exposure to Notch-blocking drugs (n = 10, n = 8 and n = 9 respectively, Figure 7J, 7M, and 7P), but they were severely downregulated in the PSM after overnight incubation with the drugs (n = 8, n = 8 and n = 11 respectively, Figure 7L, 7O, and 7R). Thus, these results are consistent with those obtained by analysis of the Psen1/Psen2 double mutant embryos and show that, at least in the mouse, Notch activity is critical for both the maintenance and rostral progression of oscillations of the segmentation clock along the PSM and for somite formation.

Because of the oscillatory nature of cyclic gene expression in the caudal PSM of normal non-treated embryos it is possible that the remaining expression of these genes observed in the tail bud region after treatment with the drugs is indicative of the existence of an FGF/Wnt based pacemaker operational and dynamic in the absence of Notch activity. Under this paradigm the oscillations might be generated in the primitive streak/tail bud by a Notch-independent mechanism and Notch would then act to propagate these oscillations along the PSM. To test the possible existence of this Notch-independent pacemaker we used the half embryo assay and treated both halves with DAPT or LY411575 for 3 hours, then fixed one half and cultured the second half for 1 additional hour before fixation. When we performed this analysis we found that the expression of Lfng was completely absent in both halves, as expected (n = 3, Figure 8A). Under these conditions, if the expression of cyclic genes, such as Hes7, continues to oscillate in the caudal PSM after drug treatment we should find pairs of samples in which the expression domain is different in the two sides such that the cyclic gene is present in one half and absent in the other. When we analysed Hes7 we found that the expression was weak in the two halves as
compared with the intensity observed in control non-treated samples, as described above. By extending the colour revelation step we observed that \textit{Hes7} expression was present and restricted to the caudal region of the PSM of drug-treated samples (n = 8, Figure 8B). Similarly, \textit{Axin2} displayed a weak expression profile restricted to the caudal end of the PSM (n = 9, Figure 8C). In addition, we observed that \textit{Snail1}, \textit{Dusp6} and \textit{Sprouty2} displayed similar patterns of expression in both halves (n = 12, n = 11 and n = 14 respectively, Figure 8D–8F), indicating that their expression was also not dynamic. In summary, all these results taken together demonstrate that, in the mouse embryo, in the absence of Notch signalling the expression of cyclic genes is lost in the rostral and medial PSM and is dramatically reduced in the caudal PSM where it loses its dynamism, and this is not consistent or compatible with the existence of a Notch-independent pacemaker.

Figure 8. Cyclic gene expression in the tail bud is lost or stops being dynamic after treatment with Notch-blocking drugs. (A–F) Half embryo explants from E9.5–10.5 wild type embryos cultured in vitro in the presence of 100 \textmu M of DAPT or 100 nM LY411575 for 3 hours (left) or 4 hours (right) and then analysed by in situ hybridisation for the expression of (A) \textit{Lfng}, (B) \textit{Hes7}, (C) \textit{Axin2}, (D) \textit{Snail1}, (E) \textit{Dusp6} and (F) \textit{Sprouty2}, showing that after drug treatment the cyclic gene expression is lost in the medial and rostral PSM, and that remaining expression still present in the tail bud is not dynamic. (G) Half embryo explants from E9.5 wild type embryos cultured in vitro in the absence (left half) or presence (right half) of 100 \textmu M of DAPT plus 50 \textmu M SU5402, a FGF signalling inhibitor, and then analysed by in situ hybridisation for the expression of \textit{Hes7}. (H) Schematic representation of the data from the \textit{Psen1}^{−/−};\textit{Psen2}^{−/−} embryos and the pharmacological treatment with Notch-blocking drugs showing that the oscillations of all cyclic genes along the PSM detected in wild type or untreated embryos are lost in the absence of Notch signalling. The expression of cyclic genes is completely lost or is severely down regulated and restricted to the caudal region of the PSM where it is non dynamic. In addition, in \textit{Psen1}^{−/−};\textit{Psen2}^{−/−} embryos or in wild type embryos treated with Notch-blocking drugs the formation of somites is lost.
Discussion

In this study we have investigated the implication of Notch signalling in the mechanism of the mouse somitogenesis oscillator. We found that in the absence of Lfng the mouse embryo is still able to display dynamic expression of all cyclic genes analysed. In the absence of Hes7, however, only the expression of FGF and Wnt-regulated cyclic genes is still dynamic in a PSM that displays non-cyclic Notch activity along its extension. Surprisingly, in the absence of RBPj there is still some RBPj-independent Notch activity, as evidenced by the expression profile of Hes7. Our data show that the double mutant embryos Psen1−/−;Psen2−/− develop in the complete absence of Notch activity and they do not form somites or display oscillatory gene expression, as evidenced by the lack of expression of cyclic genes along the PSM. Similar defects are produced in wild type embryos cultured in the presence of Notch-blocking drugs. We propose that, contrary to what by the lack of expression of cyclic genes along the PSM. Similar defects are produced in wild type embryos cultured in the presence of Notch-blocking drugs. We propose that, contrary to what happens during zebrafish development, in the mouse embryo Notch activity, cyclic or non-cyclic, is critically required both for the maintenance of the somitogenesis oscillator and for the formation of the somites (Figure 8H).

It has been previously shown that Lfng and Hes7 are two important components of the Notch pathway and interfering with their functions seems to affect the somitogenesis oscillator [19,20,24], an idea supported by the phenotype displayed by Lfng−/− and Hes7−/− embryos [17–19]. Homozygous mutant embryos have clear somitic abnormalities that later in development result in skeletal malformations of vertebrae and ribs. However, the fact that these mutant embryos make somites at all indicates that the somitogenesis oscillator may still be producing oscillations and generating periodicity in the absence of these two proteins. A first analysis suggested that the Notch pathway was upregulated along the entire PSM of Lfng−/− embryos, as judged by the expression of Notch-dependent cyclic genes, although a more careful analysis revealed dynamic Hes7 expression. The existence of this dynamism in the PSM of the Lfng−/− embryos is corroborated by the results obtained by immunostaining with the anti-Hes7 and anti-NICD antibodies that demonstrated there is periodic production of Hes7 mRNAs and periodic production of NICD. Based on the patterns of expression described in the literature the induction of all Notch-based cyclic genes, including Hes7, is unaffected [13]. Thus, in both Hes7−/− and Psen1−/−;Psen2−/− embryos we found that they do not display any kind of activity of the segmentation clock, as suggested by the lack of different patterns of expression of different Notch-based, Wnt-based and FGF-based cyclic genes, and because they do not form somites. We also found that NICD is not produced in these embryos, a clear indication that these double mutant embryos really develop in the absence of Notch signalling. We can not rule out the formal possibility that there is a Notch-independent γ-secretase activity implicated in segmentation [27]. However, we think this is unlikely because amongst the list of type I transmembrane proteins known to be substrates for γ-secretase [60,61] only the Notch components have been described to be implicated in the oscillator involved in somitogenesis. In addition, when we treated samples from wild type mouse embryos in vitro with drugs that block Notch cleavage we also inhibited the dynamic expression of all cyclic genes and the generation of somites beyond those already determined or in the process of being formed in the rostral PSM at the time of treatment (1 or 2). Thus, the generation of temporal and spatial periodicity in the mouse embryo absolutely requires Notch activity. In the absence of all Notch activity no oscillations occur and no somites are formed.

Feler et al. recently reported that NICD is not detected in the PSM of Pifia1−/− embryos, a mutant mouse line carrying a mutation in another relevant component of the Notch pathway, although these embryos are nevertheless still able to generate a significant number of irregular somites [13]. The authors concluded that these Pifia1−/− mutant embryos develop and generate somites in the absence of Notch signalling. A further explanation for the difference in their interpretation compared to ours is the detection limit of our respective assays for NICD. Indeed the expression of NICD we detect in Rbpj−/− embryos, which also develop a limited number of somites, is very weak and could easily be overlooked. As yet an analysis of the expression profile of different Notch-based cyclic genes, including Lfng and/or Hes7, has not been performed with the Pifia1−/− embryos. Further investigation will be required to clarify these discrepancies.

The only Hes7 expression domain remaining in the T-NICD transgenic embryos [13] Wnt-based genes continue to oscillate in a background of non-cyclic Notch activity. It remains unclear whether the presence of somites and the existence of cyclic gene expression in these two genetic backgrounds, Hes7−/− and T-NICD, are due to the combination of non-cyclic Notch together with cyclic Wnt signalling or whether cyclic Wnt alone is sufficient to account for this. The analysis of Rbpj−/− embryos, thought to develop in the absence of Notch activity, failed to resolve this issue since, surprisingly, the results show that at least one Notch-related cyclic gene, Hes7, is still expressed with different patterns of expression along the PSM suggesting its expression is still dynamic. While it remains a formal possibility that this residual Hes7 expression is Notch-independent the fact that we report NICD is present at low levels in the PSM of the Rbpj−/− embryos strongly support that the Hes7 expression is a consequence of a poorly defined RBPj-independent Notch activity in this tissue [21].
the expression of *Hes7* and *Axin2* in the caudal PSM, which indicates that at least in the mouse embryo there does not appear to be a Notch-independent pacemaker. It will be of great interest to study the mechanism by which Notch activity modifies the regulation of non-cyclic *Hes7* and *Axin2* in the caudal PSM such that it becomes oscillatory.

As mentioned above, it is widely accepted that in the zebrafish embryo the main function of Notch is to synchronise the oscillations of her cyclic genes and that Notch inhibition does not interrupt the generation of oscillations and the resulting segmented body plan [7–11,62]. The present study does not provide evidence for a role for Notch in synchronizing the mouse somitogenesis oscillator, but it also does not seem to preclude such a role. Nevertheless our data clearly indicate that in mouse this signalling pathway plays a critical central role in the mechanism of the segmentation clock, which is not the case in zebrafish. This crucial difference in the role for Notch during mouse and zebrafish somitogenesis could be due to species-differences in the complexity of the core mechanism of the segmentation clock; an idea supported by the fact that in mouse the oscillator mechanism drives periodic expression of cyclic genes from three signalling pathways whereas in zebrafish the mechanism seems solely based on the oscillations of *her* genes which are both Notch and FGF dependent [2,36]. So far no Wnt-based cyclic genes have been reported in the zebrafish.

In summary, our data show that in the mouse embryo Notch signalling is absolutely required to generate periodicity by the somitogenesis oscillator, as evidenced by the expression of cyclic genes and the formation of somites. The different signalling pathways implicated in this oscillator mechanism all appear to be interconnected via Notch signalling. A better knowledge of these reciprocal interactions will be of great relevance to gain a deeper understanding of the fundamental workings of this oscillatory mechanism.

**Material and Methods**

**Explant culture and mouse embryo culture**

Wild type CD1 *Mus musculus* embryos were obtained from timed mated pregnant females between 8.0 and 10.5 days postcoitum (dpc). *Lfg−/−*, *Hes7−/−*, *Rbpj−/−* and *Psen1−/−;Psen2−/−* embryos were obtained and genotyped by PCR analysis of the yolk sacs as described [18,19,39,41]. For half embryos analysis E9.5–10.5 mouse embryos were isolated and the caudal portion was divided into two halves by bisecting the tissue along the midline. Explants were cultured on medium composed of DMEM/F12 supplemented with 10% fetal bovine serum, 10 ng/ml bFGF, and 50 U/ml penicillin/streptomycin. At the end of the culture period the explants were transferred into 4% paraformaldehyde fixative solution and then analysed by *in situ* hybridisation for gene expression. Four different series of experiments were performed: (A) One half explant was fixed and the other half was cultured for 60 minutes. (B) The two halves were cultured for 3 hours in medium in the absence or presence of either 100 μM DAPT (Calbiochem) or 100 nM LY411575 to inhibit the Notch activity, or the equivalent volume of DMSO as control. (C) The two halves were cultured for 3 hours in medium in the presence or absence of a mix of 100 μM DAPT plus 50 μM SU5402 (Calbiochem). (D) The two halves were cultured for 3 hours in medium in the presence of 100 μM DAPT or 100 nM LY411575, then one half was fixed and the second half was cultured for 1 additional hour before fixation. Whole embryo mouse culture was performed as previously described [63,64] using wild type embryos. In short, E8.0–8.5 mouse embryos with their membranes intact were cultured for about 18–20 hours in standard whole embryo roller culture conditions: 50% rat serum in F12 medium plus 1 mM sodium pyruvate, 2 mM glutamine and non-essential amino acids at 37°C with 5% CO2. Media was supplemented with either 100 μM DAPT or 100 nM LY411575 or the appropriate amount of DMSO as control. All animals were handled in strict accordance with good animal practice as defined by the relevant national and/or local animal welfare bodies, and all animal work were approved by the ethical committees for experiments with animals of the University of Dundee (UK), Nara Institute of Science and Technology (Japan), University of Leuven (Belgium) and Centro Nacional de Investigaciones Cardiovasculares (Spain).

**Whole-mount in situ hybridisation**

Mouse intronic and exonic *Lfg*, intronic and exonic *Hes7*, *Axin2*, *Mesp2*, *Snail1*, *Unctx1*, *Dusp6* and *Sprouty2* probes were prepared as described [16,20,23,30,32,37,65,66]. Whole-mount *in situ* hybridisation was done basically as described [67]. The following modifications to this protocol were used for intronic probe *in situ* hybridisation. Samples were hybridised with probe for 40 hours in a low stringency hybridisation mix (50% formamide, 5 × SSC, 5 mM EDTA, 50 μg/ml tRNA, 0.2% Tween-20, 0.1% SDS, 100 μg/ml heparin) and post-hybridisation washes were performed in post-hybridisation buffer (50% formamide, 0.1% Tween-20, 1 × SSC). Samples were processed either by hand or using the InsituPro VS Robot (Intavis AG). All images were captured on a Leica MZ16 APO microscope using a Jenopik camera. Images were recorded using Openlab software version 4.0.3.

**Quantitative real-time RT–PCR**

The reaction was accomplished in the presence of SYBR Green Supermix (BioRad) and the reactions were measured in a Mastercycler ep realplex (Eppendorf) using the following cycling conditions: 95°C for 5 min, 40 cycles at 95°C for 15 sec and 53°C for 60 sec. Primers to quantify *Hes7* mRNA levels were designed using Primer3. The two primers used were 5'-GAAGCGGTTGGTGAGAAG-3' and 5'-GGCTTCGCTCCCTCAAGTAG-3'. Normalization was performed against β-actin amplified using the primers 5'-GGCT- GTATTTCCCTCCATCG-3' and 5'-CCAGTTGGTAAACATGC- CCATGT-3'.

**Alcian Blue and Alizarin Red staining**

E18.5 mouse embryos were fixed in 95% ethanol overnight at room temperature and stained with 150 mg/ml Alcian Blue in 1:4 mixture of acetic acid and 95% ethanol at RT for 24–48 h. After washing with 95% ethanol for 1 h, the embryos were treated with 1% KOH for 24 h with several changes. Embryos were subsequently stained with 75 mg/ml Alizarin Red S in 1% KOH solution for 12–24 h and cleared in a solution of 20% glycerol and 1% KOH for a week with daily changes. Samples were transferred to 50% glycerol, 50% ethanol for photography and storage.

**Immunohistochemistry**

Whole-mount immunohistochemistry with anti-Hes7 and anti-NICD antibodies was performed as described previously [20,27,46]. Briefly, for analysis with the anti-Hes7, embryos were fixed with 4% paraformaldehyde in PBS at 4°C for 3 h and treated with 0.1% H2O2 overnight. Then the embryos were incubated with anti-Hes7 antibody (1:100 diluted) at 4°C for 3–5 days and next with HRP-conjugated anti-guinea pig IgG (Chemicon) overnight at 4°C. The peroxidase deposits were
visualized by 4-chloro-1-naphthol. For analysis with the anti-NICD on transversal sections 5-μm paraffin-embedded sections were immersed in 10 mM sodium citrate pH 6.0 and boiled 10 min to enable antigen retrieval. Immunostaining was performed with cleaved Notch1 antibody (Val1744, 1:100 diluted, Cell Signaling Technology) overnight at 4°C, followed by biotinylated anti-rabbit IgG antibody (1:100 diluted, Vector Laboratories) for 60 min at RT. Finally, the signal was amplified in two steps; first with avidin/biotin-HRP (ABC kit, Vector Labs) and then with peroxidase solution (Pierce.) Followed by visualization with DAB and counterstaining with hematoxylin.

Western blot analysis
Samples were prepared with caudal fragments of E9.5–10.5 mouse embryo control or treated with drugs and with mouse embryonic fibroblast derived from Pem1−/−;Pem2−/−; embryos [47]. 20 μg of protein samples were used to perform electrophoresis in MOPS running buffer. Gels were then blotted and the resulting membrane was incubated with the anti-NICD antibody (Val1744, 1:1000 dilution, Cell Signaling Technology) overnight at 4°C followed by anti-rabbit-HRP antibody (1:10000) for 60 min at RT and then standard ECL revelation (Pierce). α-Tubulin staining was preformed with a 1:20000 dilution (Abcam, ab7291) followed by a 1:2000 dilution of anti-mouse-HRP antibody.

References
1. Bryson-Richardson RJ, Currie PD (2000) The genetics of vertebrate myogenesis. Nat Rev Genet 1: 375–386.
2. Dequcne M, Pourque O (2008) Segmental patterning of the vertebrate embryonic axis. Nat Rev Genet 9: 370–382.
3. Holley SA (2007) The genetics and embryology of zebrafish metamorphosis. Dev Dyn 236: 1422–1449.
4. Shook DR, Keller R (2008) Epithelial type, ingestion, blastopore architecture and the evolution of chordate mesoderm morphogenesis. J Exp Zool B Mol Dev Evol 310: 85–110.
5. Cooke J, Zeeman EC (1976) A clock and wavefront model for control of the number of repeated structures during animal morphogenesis. J Theor Biol 58: 455–476.
6. Cinquin O (2007) Understanding the somitogenesis clock: what’s missing? Mech Dev 124: 501–517.
7. Jiang YJ, Aerne BL, Smithers L, Haddon C, Ish-Horowicz D, et al. (2000) Notch regulates MyoD and Myf-5 expression in the periodic segmentation of somites. Genes Dev 14: 455–476.
8. Saga Y (2007) Segmental border is defined by the key transcription factor Mesp2, by means of the suppression of Notch activity. Development 135: 899–908.
9. Saga Y, Hata N, Koseki H, Taketo MM (1997) Mesp2: a novel mouse gene expressed in the presegmented mesoderm and essential for segmentation initiation. Genes Dev 11: 1827–1839.
10. Saga Y, Hata N, Koseki H, Taketo MM (2003) Wnt3a expressed in the presegmented mesoderm and essential for segmentation initiation. Genes Dev 17: 2148–2152.
11. Saga Y, Hata N, Koseki H, Taketo MM (2006) Oscillator Responsive to Notch Signaling. Dev Cell 3: 63–74.
12. Dequcne M-L, Lynn E, Gaudenz K, Wahl M, Chen J, et al. (2006) A complex oscillating network of signaling genes underlies the mouse segmentation clock. Science 314: 1593–1598.

Acknowledgments
We are grateful to the JKD and MM laboratories, to A. Muller for critical reading of the manuscript, and to M. Milan for his insights. We thank the groups of B. Herrmann, O. Pourrique, R. Kageyama, D. Ish-Horowicz, A. Gossler, A. Nieto, and Y. Saga for providing the plasmids to prepare probes for Aum2, Sprouty2, intronic and exonic Hes7, intronic and exonic Engrailed, Uncx4.1, Daches, Snail1, and Mesp2, respectively. We also thank N. Shiroy for the synthesis of the LY411575 compound, K. Craessaerts for her technical support with the Pem1−/−;Pem2−/−; mutant line, S. Huppert and S. Gibb for their help with the western analysis, and M. Pryde and M. Reilly for their administrative support.

Author Contributions
Conceived and designed the experiments: JKD YB MM. Performed the experiments: ZF SH JKD AH GdM MM. Analyzed the data: ZF SH JKD YB AH BDS GdM JLdP MM. Contributed reagents/materials/analysis tools: ZF SH JKD YB AH BDS GdM JLdP MM. Wrote the paper: JKD MM.

Notch is Essential to the Mouse Segmentation Clock
38. Hayashi S, Shimoda T, Nakajima M, Tsukada Y, Sakamura Y, et al. (2009) Spromly, an FGF inhibitor, displays cyclic gene expression under the control of the notch segmentation clock in the mouse PSN. PLoS One 4: e5603. doi:10.1371/journal.pone.0005603.

39. Oka C, Nakano T, Wakeham A, de la Pompa JL, Mori C, et al. (1995) Disruption of the mouse RBPJ kappa gene results in early embryonic death. Development 121: 3291–3301.

40. Donoviel DB, Hadjantonakis AK, Ikeda M, Zheng H, Hyslop PS, et al. (1999) Mice lacking both presenilin genes exhibit early embryonic patterning defects. Genes Dev 13: 2801–2810.

41. Herreman A, Hartmann D, Annaert W, Saftig P, Craessaerts K, et al. (1999) Presenilin 2 deficiency causes a mild pulmonary phenotype and no changes in amyloid precursor protein processing but enhances the embryonic lethal phenotype of presenilin 1 deficiency. Proc Natl Acad Sci U S A 96: 11072–11077.

42. Kato H, Taniguchi Y, Kurooka H, Minoguchi S, Sakai T, et al. (1997) Functional gamma-secretase inhibitors reduce beta-amyloid peptide levels in mouse brain. J Neurochem 76: 173–181.

43. Lewis J (2003) Autoinhibition with transcriptional delay: a simple mechanism for the notch segmentation clock in the mouse PSM. PLoS One 4: e5603. doi:10.1371/journal.pone.0005603.

44. Kato H, Taniguchi Y, Kurooka H, Minoguchi S, Sakai T, et al. (1997) Monitoring Notch1 activity in development: evidence for a feedback regulatory loop. Dev Dyn 216: 253–264.

45. Herreman A, Van Gassen G, Bentahir M, Nyabi O, Craessaerts K, et al. (1999) Gamma-Secretase activity requires the presenilin-dependent trafficking of nicastrin through the Golgi apparatus but not its complex glycosylation. J Cell Sci 112: 1127–1136.

46. Saxena MT, Schroeter EH, Munn JS, Kopan R (2001) Murine notch homologs (N1-4) undergo presenilin-dependent proteolysis. J Biol Chem 276: 40268–40273.

47. Hayashi S, Shimoda T, Nakajima M, Tsukada Y, Sakamura Y, et al. (2009) Spromly, an FGF inhibitor, displays cyclic gene expression under the control of the notch segmentation clock in the mouse PSN. PLoS One 4: e5603. doi:10.1371/journal.pone.0005603.

48. Lewis HD, Perez Revuelta BI, Linam A, Neduvelil JG, Harrison T, et al. (2003) Catalytic site-directed gamma-secretase complex inhibitors do not discriminate pharmacologically between Notch S1 and beta-APP cleavages. Biochemistry 42: 7580–7586.

49. Fujimaki R, Taya Y, Hozumi N, Tezuka K (2006) Involvement of Notch signaling in initiation of prion-hydrogen condensation and nodular formation in limb bud micromas cultures. J Bone Miner Metab 24: 191–196.

50. Bettenhausen B, Hrabe de Angelis M, Simon D, Greten JL, Gossler A (1995) Transient and restricted expression during mouse embryogenesis of Dil1, a murine gene closely related to Drosophila Delta. Development 121: 2407–2418.

51. Dunwoodie SL, Henriques D, Harrison SM, Beddington RS (1997) Mice Dil1: a novel divergent Delta gene which may complement the function of other Delta homologues during early pattern formation in the mouse embryo. Development 124: 3063–3076.

52. Lanz TA, Hosley JD, Adams WJ, Merchant KM (2004) Studies of Abeta pharmacodynamics in the brain, cerebrospinal fluid, and plasma in young (paule-free) Tg2576 mice using the gamma-secretase inhibitor N2-[2576-2/3,5-dimethoxyphenyl-2-hydroxyethanoyl]-N1-[78]-5-methyl-6-oxo-6,7-dihydro-3H-dibenzo[b,d]azepin-7-yl-L-alaninamide (LY-411575). J Pharmacol Exp Ther 309: 49–55.