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Instability of Hes7 protein is crucial for the somite segmentation clock

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During somitogenesis, a pair of somites buds off from the presomitic mesoderm every 2 hours in mouse embryos, suggesting that somite segmentation is controlled by a biological clock with a 2-hour cycle[1–3]. Expression of the basic helix-loop-helix factor Hes7, an effector of Notch signaling, follows a 2-hour oscillatory cycle controlled by negative feedback; this is proposed to be the molecular basis for the somite segmentation clock[4–6]. If the proposal is correct, this clock should depend crucially on the short lifetime of Hes7. To address the biological importance of Hes7 instability, we generated mice expressing mutant Hes7 with a longer half-life (~30 min compared with ~22 min for wild-type Hes7) but normal repressor activity. In these mice, somite segmentation and oscillatory expression became severely disorganized after a few normal cycles of segmentation. We simulated this effect mathematically using a direct autorepression model. Thus, instability of Hes7 is essential for sustained oscillation and for its function as a segmentation clock.

Hes7 is expressed in a cyclical fashion, and each cycle of *Hes7* expression coincides with the generation of each pair of somites. Related basic helix-loop-helix genes and another Notch signaling component, lunatic fringe (*Lfng*), show the same dynamic expression in vertebrates[7–18]. We previously found that the cyclic expression of *Hes7* in the presomitic mesoderm (PSM) could be mimicked by *Hes1* in cultured cells[19]. Serum stimulation triggers an oscillation in *Hes1* mRNA

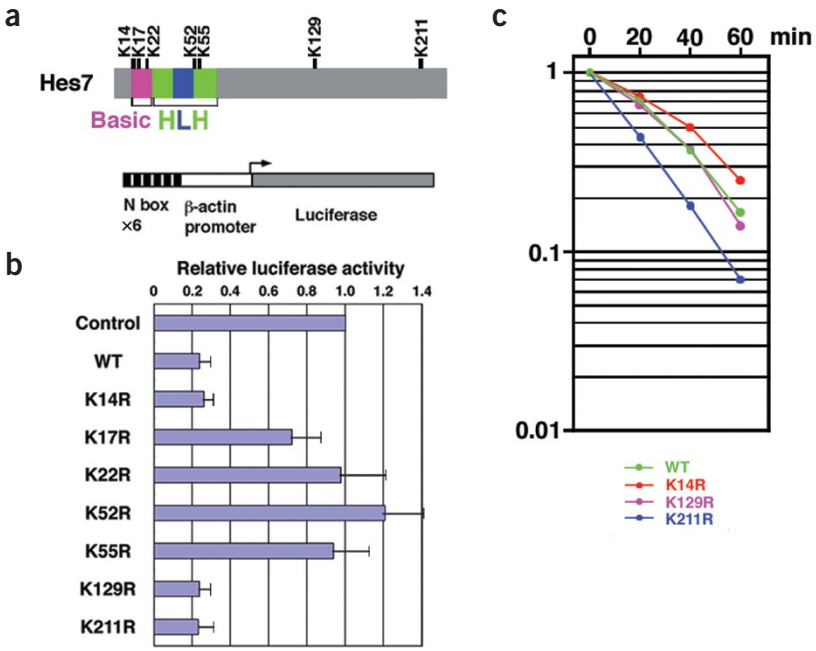

Figure 1 Repressor activities and half-lives of Hes7 mutants. (**a**) Structure of mouse Hes7 protein. Hes7 consists of 224 amino acid residues. Seven lysine residues are indicated. (**b**) C3H10T1/2 cells were cotransfected with the luciferase reporter gene under the control of the N box–containing promoter and wild-type (WT) or mutant Hes7 vectors. Relative luciferase activities are shown (mean ± s.d. for four independent experiments). Mutants K14R, K129R and K211R had normal repressor activities, whereas the others did not. (**c**) C3H10T1/2 cells were transfected with the HA-tagged wild-type (WT) or mutant Hes7 expression vectors. The next day, the cells were cultured in the presence of cycloheximide for 30 min and then collected at the indicated time points. Whole-cell extracts were probed with antibody to HA and the relative immunoreactive activities of the signals were measured. Wild-type Hes7 is degraded with a half-life of 22.3 min, whereas the half-lives of K14R, K129R and K211R mutant Hes7 are 30.3 min, 21.1 min and 15.6 min, respectively.

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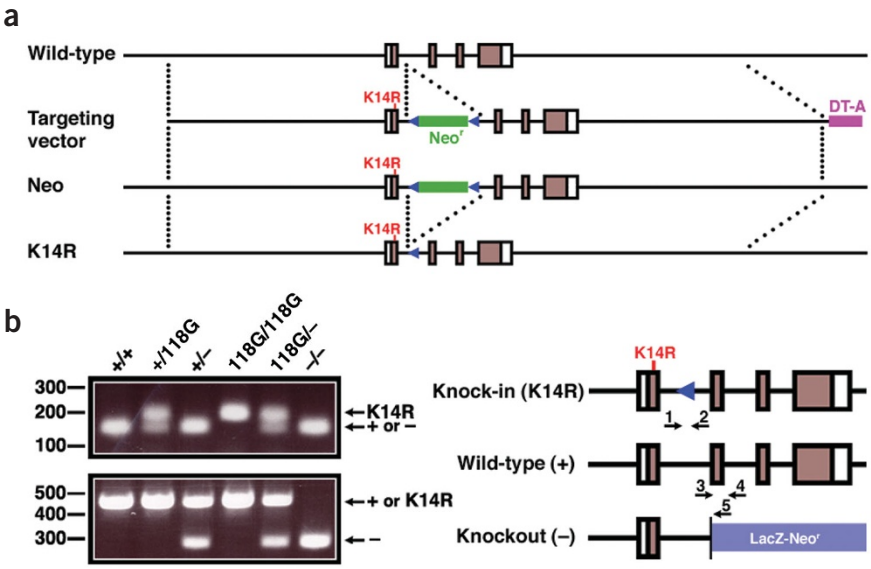

Figure 2 Generation of *Hes7*$^{118G/118G}$ knock-in mice for Hes7 K14R expression. (**a**) Targeting strategy. An A→G mutation was introduced at nucleotide residue 118 in exon 1 to code arginine instead of lysine, and the neomycin resistance gene cassette (Neo^r) was inserted into intron 1. Diphtheria toxin gene (DT-A) was used for negative selection. The blue arrowheads indicate the *lox*P sites. (**b**) Genotype analysis by PCR. The positions of PCR primers are shown in the right panel. The upper left panel shows that PCR with primers 1 and 2 generates a 191-bp band (*Hes7*^{118G}) and a 155-bp band (wild-type or knockout). The lower left panel shows that PCR with primers 3, 4 and 5 generates a 464-bp band (wild-type or *Hes7*^{118G}) and a 280-bp band (knockout).

and Hes1 protein levels governed by negative feedback: Hes1 protein represses *Hes1* transcription but is rapidly degraded by the proteasome system, thereby allowing the next bout of transcription. This negative feedback loop is the molecular basis for the 2-hour oscillation in Hes1 levels in cultured cells[19]. We recently found that the same mechanism works for Hes7 oscillation in the PSM[5]. The dynamic expression of Hes7 is thought to depend on its short lifespan: the half-life of Hes7 protein is only ~22 min, and this short half-life is expected to be required for genesis of the 2-hour periodicity[5,20–22]. To address the importance of the instability of Hes7, we manipulated its half-life.

We first screened for Hes7 mutants with long half-lives and normal repressor activity. Because lysine residues are the typical ubiquitination targets, we focused on seven lysine residues in Hes7 (**Fig. 1a**). We introduced a lysine-to-arginine point mutation at each lysine residue and created seven mutants (Hes7 K14R, Hes7 K17R, Hes7 K22R, Hes7 K52R, Hes7 K55R, Hes7 K129R and Hes7 K211R). To analyze the transcriptional activity of these Hes7 mutants, we carried out a transient transfection assay with the luciferase reporter gene under the control of the N box–containing promoter. Wild-type Hes7 repressed luciferase gene expression (**Fig. 1b**). Hes7 K14R, Hes7 K129R and Hes7 K211R also repressed transcription, whereas the other mutants, which have a mutation in the basic helix-loop-helix domain (**Supplementary Fig. 1** online), did not repress transcription and were not studied further. We next determined the half-lives of the three Hes7 mutants with normal repressor activity. The protein half-life of wild-type Hes7 was as short as 22.3 min, whereas those of Hes7 K14R, Hes7 K129R and Hes7 K211R were 30.3 min, 21.1 min and 15.6 min, respectively (**Fig. 1c**). Because of its longer half-life (30.3 min) and normal repressor activity, we chose to study the effect of mutant Hes7 K14R on the mouse segmentation clock.

To examine the role of Hes7 half-life in somitogenesis, we generated knock-in mice expressing Hes7 K14R instead of wild-type Hes7. This was done by introducing an A→G point mutation at nucleotide residue 118 of *Hes7* in embryonic stem (ES) cells (**Fig. 2a**). The

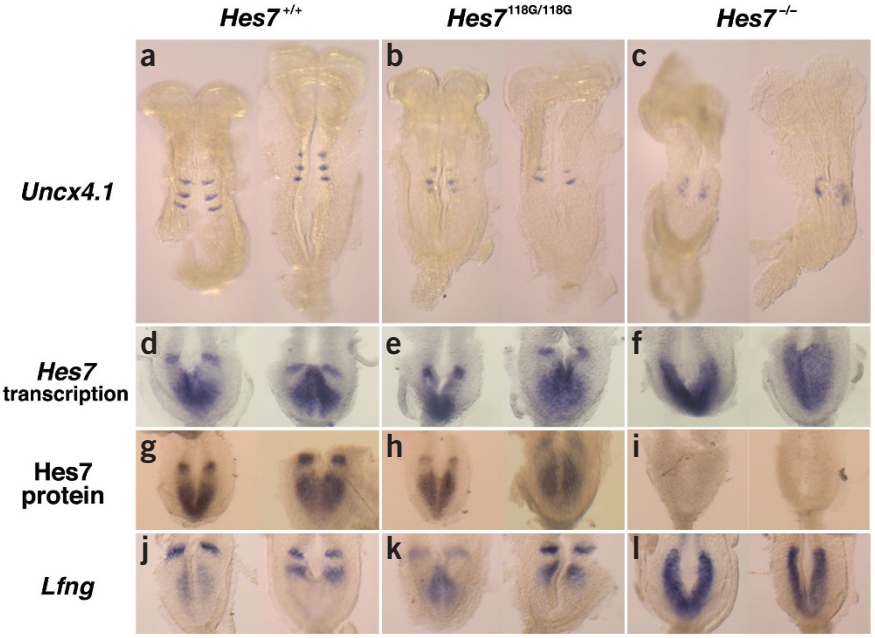

Figure 3 Dynamic expression of Hes7 K14R and *Lfng* at the two- or three-somite stage. (**a–c**) *In situ* hybridization with *Uncx4.1* probe (marker for posterior half of each somite) indicates that the somite segmentation occurs normally in *Hes7*$^{118G/118G}$ and *Hes7*$^{+/+}$ embryos but not in *Hes7*$^{-/-}$ embryos. (**d–f**) *In situ* hybridization with *Hes7* intron probe detects the expression of nascent transcripts of all alleles (+, –, 118G). The expression is dynamic in *Hes7*$^{118G/118G}$ and *Hes7*$^{+/+}$ embryos. In contrast, the nascent transcript is constitutively expressed throughout the PSM in *Hes7*$^{-/-}$ embryos. (**g–i**) Immunostaining with antibody to Hes7 shows the dynamic patterns of Hes7 protein expression in *Hes7*$^{118G/118G}$ and *Hes7*$^{+/+}$ embryos, whereas Hes7 protein is not expressed in *Hes7*$^{-/-}$ embryos. (**j–l**) *Lfng* mRNA expression is also dynamic in *Hes7*$^{118G/118G}$ and *Hes7*$^{+/+}$ embryos but not in *Hes7*$^{-/-}$ embryos. Thus, the PSM in *Hes7*$^{118G/118G}$ embryos shows periodic expression for *Hes7* and *Lfng* oscillators.

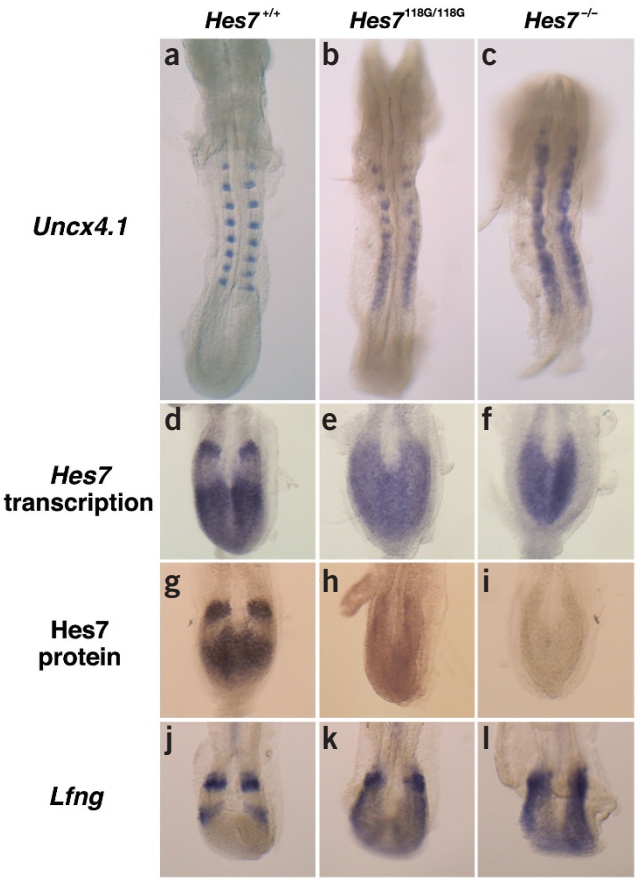

Figure 4 Damped oscillations of Hes7 K14R and *Lfng* expression at the eight-somite stage. (**a–c**) *In situ* hybridization with *Uncx4.1* probe indicates that, in contrast to *Hes7*$^{+/+}$ embryos, somite segmentation is disturbed after the three- to four-somite stage in *Hes7*$^{118G/118G}$ embryos and is disturbed from the first somite stage in *Hes7*$^{-/-}$ embryos. (**d–f**) *Hes7* nascent transcript is constitutively expressed throughout the PSM in *Hes7*$^{118G/118G}$ and *Hes7*$^{-/-}$ embryos, whereas it is expressed in a dynamic fashion in *Hes7*$^{+/+}$ embryos. The expression pattern in *Hes7*$^{118G/118G}$ and *Hes7*$^{-/-}$ embryos is not scattered, suggesting that the oscillation is not out of synchrony but lost. (**g–i**) Hes7 protein is expressed in a dynamic manner in the PSM of *Hes7*$^{+/+}$ embryos. In contrast, Hes7 protein is constitutively expressed at a low level throughout the PSM of *Hes7*$^{118G/118G}$ embryos and is not expressed at all in *Hes7*$^{-/-}$ embryos. (**j–l**) *Lfng* mRNA expression is also steady in *Hes7*$^{118G/118G}$ and *Hes7*$^{-/-}$ embryos but periodic in *Hes7*$^{+/+}$ embryos. *Hes7*$^{118G/118G}$ and *Hes7*$^{-/-}$ embryos show steady expression of both *Hes7* intron and *Lfng* mRNA, but their Hes7 protein expression patterns are different.

neomycin resistance gene was excised by transient expression of Cre recombinase. From these ES cells, we generated *Hes7*$^{118G/118G}$ knock-in mice, which express Hes7 K14R (**Fig. 2b**). To examine cyclic gene expression in the PSM, we carried out *in situ* hybridization with probes for *Uncx4.1*, *Hes7* intron and *Lfng* and immunostaining with antiserum to Hes7. At the two- or three-somite stage, expression of the homeobox gene *Uncx4.1* showed the normal striped pattern in both *Hes7*$^{118G/118G}$ and *Hes7*$^{+/+}$ embryos but was disorganized in *Hes7*$^{-/-}$ embryos (**Fig. 3a–c**). Because intron regions are present only in nascent transcripts, *in situ* hybridization with intron probes identifies only the regions where the gene is actively transcribed[5,23]. *In situ* hybridization at the two- or three-somite stage with a *Hes7* intron probe showed that *Hes7* was dynamically transcribed in *Hes7*$^{118G/118G}$ and *Hes7*$^{+/+}$ embryos, whereas *Hes7* transcription was constantly upregulated in *Hes7*$^{-/-}$ embryos (**Fig. 3d–f**). In agreement with this observation, we noted oscillatory expression of Hes7 protein in *Hes7*$^{118G/118G}$ and *Hes7*$^{+/+}$ embryos but not in *Hes7*$^{-/-}$ embryos (**Fig. 3g–i**). Similarly, we observed cyclic expression of *Lfng* in *Hes7*$^{118G/118G}$ and *Hes7*$^{+/+}$ embryos, whereas *Lfng* mRNA was constantly upregulated in *Hes7*$^{-/-}$ embryos (**Fig. 3j–l**). Thus, periodic gene expression in *Hes7*$^{118G/118G}$ embryos occurs normally, indicating that the longer half-life of Hes7 K14R does not disrupt early somite segmentation.

To examine somite segmentation at later stages, we carried out *in situ* hybridization and immunostaining with embryos at the eight-somite stage. *In situ* hybridization with the *Uncx4.1* probe showed that, in contrast to *Hes7*$^{+/+}$ embryos, somite segmentation in *Hes7*$^{118G/118G}$ embryos was disrupted after three to four somites had been generated, whereas in *Hes7*$^{-/-}$ embryos, we observed fused somites throughout the entire anterior-posterior axis (**Fig. 4a–c**). *Hes7* transcription was not periodic in either *Hes7*$^{118G/118G}$ or *Hes7*$^{-/-}$ embryos, whereas *Hes7* was transcribed dynamically in *Hes7*$^{+/+}$ embryos (**Fig. 4d–f**). At the protein level, Hes7 expression was cyclical in *Hes7*$^{+/+}$ embryos, continuous at a low level in *Hes7*^{118GA118G} embryos and absent in *Hes7*$^{-/-}$ embryos (**Fig. 4g–i**). In addition, *Lfng* was expressed at a steady level in both *Hes7*$^{118G/118G}$ and *Hes7*$^{-/-}$ embryos but was expressed periodically in *Hes7*$^{+/+}$ embryos (**Fig. 4j–l**). In *Hes7*$^{118G/118G}$ embryos, the peaks and troughs of expression became ill-defined at the four- to five-somite stage (data not shown).

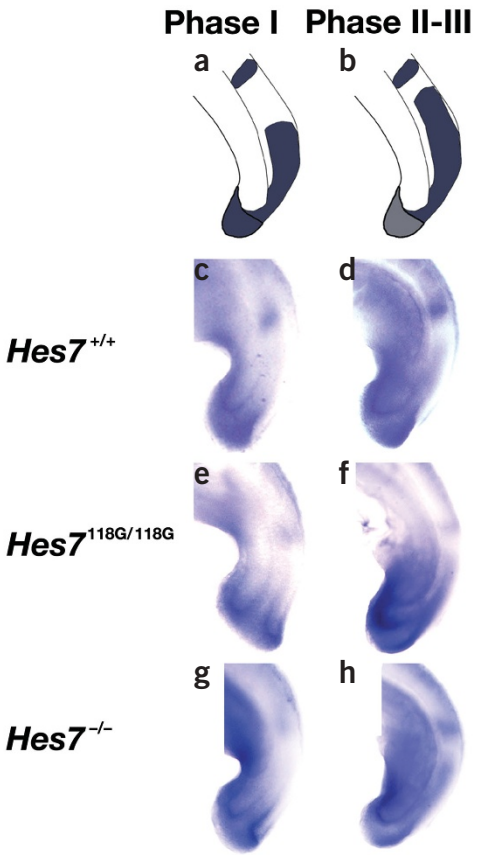

Figure 5 Dynamic expression of *Axin2* in *Hes7* mutant mice. (**a**,**b**) Illustration of dynamic expression of *Axin2* in the PSM. (**c–h**) *In situ* hybridization of *Axin2* in wild-type (**c**,**d**), *Hes7*$^{118G/118G}$ (**e**,**f**) and *Hes7*-null mice (**g**,**h**). *Axin2* expression is still dynamic in *Hes7* mutant mice (**e–h**), although the patterns are somewhat affected.

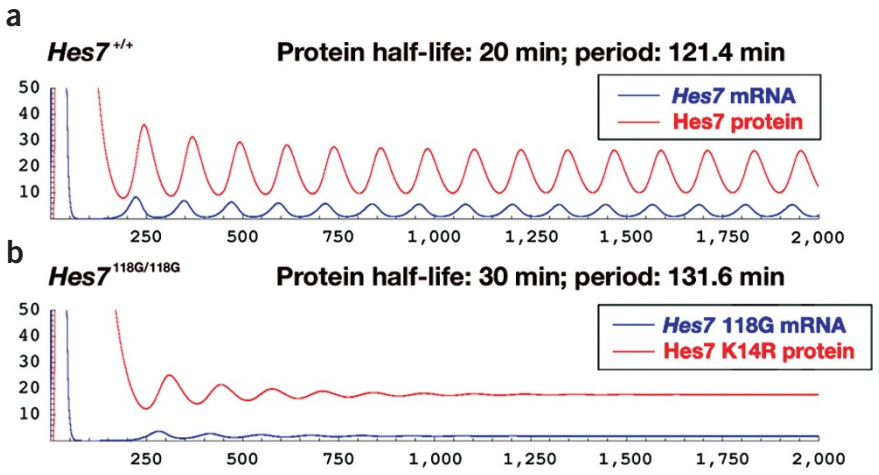

Figure 6 Mathematical simulation of Hes7 mRNA and protein oscillations. (**a**) For a protein half-life of 20 min, we obtained sustained oscillations with a period of 121.4 min. (**b**) For a protein half-life of 30 min, as measured for Hes7 K14R, the dynamic expression of protein and mRNA is attenuated after three to four cycles of oscillations. These values are based on data from eukaryotic cells and could be different from those of the mouse PSM. According to the mathematical theory, however, the crucial half-life, beyond which sustained oscillations become impossible (23–24 min, in this case), is largely determined simply by the period of the cycle and depends only weakly on the other parameters, whose values therefore are not essential to our conclusion. Blue and red lines indicate the relative levels of Hes7 mRNA and protein, respectively.

These results show that in *Hes7*$^{118G/118G}$ embryos, periodic gene expression was maintained in early somitogenesis (one to four somites) but not at later stages. This suggests that the increase of just 8 min in Hes7 half-life disabled the oscillator, although it is also possible that the K14R mutation is hypomorphic and thereby leads to damped oscillation. *Hes7*$^{+/118G}$ mice had a normal trunk but a kinked tail, indicating that somite segmentation was damped in these embryos, but less rapidly than in *Hes7*$^{118G/118G}$ embryos (data not shown).

We next examined the relationship between *Hes7* and *Axin2*, a Wnt-driven oscillator[24] (**Fig. 5a–d**). We found that *Axin2* expression remained dynamic in *Hes7* mutant embryos (**Fig. 5e–h**). On the other hand, somite segmentation seemed to occur normally in *Axin2*-null mice (W. Birchmeier, personal communication). These results suggest that *Axin2* oscillation and Hes7 oscillation are each capable of occurring independently, but that the Hes7 oscillation is essential for segmentation. Alternatively, these oscillations could be interdependent and the damped oscillation in *Hes7*$^{118G/118G}$ embryos could be due to uncoupling of Notch- and Wnt-driven oscillators.

To see whether an increase in Hes7 half-life would be expected to disrupt the segmentation clock in the way we observed, we simulated the Hes7 oscillation. The simulation is based on a recent model proposed for the zebrafish somitogenesis oscillator[20]. In this model, oscillations are generated by direct autorepression by its own protein product, and the transcriptional (T_m) and translational (T_p) delays (the times from initiation to completion of synthesis of an individual mRNA or protein molecule) have crucial roles. For sustained oscillation, the half-lives τ_m and τ_p of the mRNA and the protein, respectively, must be short compared with the sum of these delays, $T_m + T_p$. The period of oscillation is then $2(T_m + T_p + (\tau_m/\ln 2) + (\tau_p/\ln 2))$, approximately $2(T_m + T_p + 1.4\tau_m + 1.4\tau_p)$. These conditions impose quite narrow constraints on the delays and lifetimes required to give sustained oscillations with the observed 120-min period. If either of the half-lives is longer than a certain threshold value of somewhat less than $T_m + T_p$, sustained oscillation becomes impossible, but the system will show damped oscillations if it is started far from its steady state. Assuming that the protein half-life $\tau_P = 20$ min, we obtained sustained oscillations with a period of ~120 min (**Fig. 6a**). To model the lengthening of protein half-life from 20 min to 30 min, we repeated the computation substituting $\tau_P = 30$ min but without changing other parameters. This, in essence, simulates the effect of the K14R mutation. Oscillations were no longer sustained but were damped and became severely attenuated after three to four cycles (**Fig. 6b**). The damped oscillations of the model thus mimic well the experimental phenomena that result from slight stabilization of Hes7 protein. Mathematical simulation with the mixed half-lives of 20 min and 30 min for *Hes7*$^{+/118G}$ also showed damped oscillation but less rapidly than for *Hes7*$^{118G/118G}$, mimicking our results in heterozygous embryos (data not shown). Taken together, these simulation results and the Hes7 K14R observations suggest that the half-life of Hes7 protein is crucial for sustained oscillation as a vertebrate segmentation clock.

METHODS

Generation of Hes7 mutants. We created the Hes7 mutants by introducing point mutations in hemagglutinin (HA)-tagged *Hes7* cDNA to change the lysine residue (AAA or AAG) to the arginine residue (AGA or AGG, respectively) by the Quick Change method (Stratagene). We subcloned HA-tagged wild-type or mutated *Hes7* cDNAs into the eukaryotic expression vector containing the cytomegalovirus promoter, pCI-vector (Promega).

Luciferase assay. We cotransfected C3H10T1/2 cells with the luciferase reporter (0.2 g) under the control of β-actin promoter with six repeats of N boxes with or without the expression vector for HA-tagged wild-type or mutant Hes7 (1 g) and plated them in 6-multiwell plates. We cotransfected them with the vector for *Renilla* luciferase under the control of the SV40 promoter (5 ng, Promega) as an internal standard to normalize the transfection efficiency. After 24–36 h, we collected the cells and measured luciferase activities.

Measurement of protein half-lives. We transfected C3H10T1/2 cells with expression vectors for HA-tagged wild-type or mutant Hes7. The next day, we exchanged the culture medium for one containing cycloheximide (20 M, Nacalai Tesque). Thirty minutes after the medium change (time = 0) and every 20 min after that, we collected the cells and subjected them to western blotting with rat monoclonal antibody to HA-peroxidase (3F10, Roche; diluted 1:5,000). We visualized the immunoreactive bands with ECL Plus system (Amersham) and measured their intensities by Typhoon9410 (Amersham).

Generation of *Hes7*$^{118G/118G}$ knock-in mice. All animals used in this study were maintained and handled according to protocols approved by Kyoto University. We constructed the targeting vector by replacing exon 1 with mutated exon 1 and by inserting *lox*P-*neo*-*lox*P into intron 1. We identified ES cell lines with the *neo* allele by PCR and Southern-blot analysis. We then transiently expressed the Cre expression vector by electroporation and identified ES cell lines with the *Hes7*^{118G} allele. We confirmed the 200-bp promoter and all the exons and introns of *the Hes7*^{118G} allele in ES cells by sequencing. We generated chimeric mice and bred them with ICR mice. Primer sequences used for PCR are available on request. After 35 cycles of the reaction (94 °C for 30 s, 68 °C for 30 s and 72 °C for 1 min), we separated the products by electrophoresis on 1.5% agarose gels.

Whole-mount immunostaining and whole-mount *in situ* hybridization. We carried out whole-mount immunostaining as described previously[25]. We fixed embryos at embryonic day 8.0 or 8.5 with 4% paraformaldehyde in phosphate-buffered saline at 4 °C for 3 h and treated them with 0.1% H_2O_2 overnight. We then incubated the embryos with antibody to Hes7 (diluted 1:100) for 5 d at 4 °C and then with peroxidase-conjugated antibody to guinea pig IgG (Chemicon) overnight at 4 °C. We visualized the peroxidase deposits with 4-chloro-1-naphthol. We carried out whole-mount *in situ* hybridization as described previously[4]. We detected *Hes7* nascent transcripts with the *Hes7* intron probe that corresponds to the entire first intron (1.0 kb).

Mathematical simulation. We computed the behavior of the Hes7 oscillator by numerically solving the following equations:

$$\frac{dp(t)}{dt} = am(t - T_p) - bp(t)$$

$$\frac{dm(t)}{dt} = f(p(t - T_m)) - cm(t)$$

in which $p(t)$ and $m(t)$ are the quantities of functional Hes7 protein and mRNA, respectively, per cell at time t, and $f(p)$ is the rate of initiation of transcription, which depends on the amount of the protein, p, present at the time of initiation. a is the rate constant for translation, and b and c are the degradation rate constants for protein and mRNA, respectively, which are simply related to the half-lives of the molecules:

$$b = \frac{\ln 2}{\tau_p}, \quad c = \frac{\ln 2}{\tau_m}$$

To represent the case where transcription is inhibited by Hes7 protein, which acts as a dimer, we assume that

$$f(p) = \frac{k}{1 + (\frac{p}{p_{crit}})^2}$$

where k is the number of molecules of *Hes7* mRNA synthesized per unit time in the absence of inhibition and p_{crit} is the amount of protein that gives half-maximal inhibition. As in the zebrafish model[20], we set $a = 4.5$ protein molecules per mRNA molecule per min, $p_{crit} = 40$ molecules per cell, $k = 33$ mRNA molecules per cell per min, $\tau_m = 3$ min. We assume that $T_m + T_P = 37$ min and that the protein half-life $\tau_p = 20$ min for wild-type Hes7 and 30 min for Hes7 K14R.

Note: Supplementary information is available on the Nature Genetics website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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