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Ultradian oscillations of Stat, Smad and Hes1 expression in response to serum

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Running title: Serum-responsive ultradian oscillators

Summary

Serum response has been used as a model for studying signaling transduction for many biological events such as cell proliferation and survival. While expression of many genes is up- or down-regulated after serum stimulation, the Notch effector *Hes1* displays oscillatory response. However, the precise mechanism and biological significance of this oscillation remain to be determined. Here, we identified novel serum-induced ultradian oscillators, including molecules in Stat and Smad signaling. Stat and Smad oscillations involve activation of Stat3 and Smad1 and delayed negative feedback by their inhibitors Socs3 and Smad6, respectively. Moreover, Stat oscillations induce oscillatory expression of Hes1 by regulating its half-life, and loss of Hes1 oscillations leads to G1 phase retardation of the cell cycle. These results indicate that coupled Stat and Hes1 oscillations are important for efficient cell proliferation, and provide evidence that expression modes of signaling molecules affect downstream cellular events.

Introduction

Serum response has been used as a model for studying signaling transduction for many biological events such as cell cycle and growth, neuronal excitation, and immune response (1-3). It has been shown that expression of more than 500 genes is changed substantially in fibroblasts after serum stimulation: some are up-regulated while others are down-regulated and then gradually return to the original levels (1). It is generally thought that the duration and intensity of such changes in gene expression are important for downstream events.

We previously found that the Notch effector *Hes1*, a basic helix-loop-helix repressor gene, displays an oscillatory response with a 2-hr period to serum stimulation (4, 5). This oscillation depends on negative feedback and rapid degradation of the protein (4). While Hes1 is required for maintenance of neural progenitors (6-8), sustained Hes1 expression inhibits both proliferation and differentiation of these cells (6, 9), raising the possibility that oscillation is important for neural progenitors. The related gene *Hes7* and its target gene *lunatic fringe* (*Lfng*) also display cyclic expression and regulate periodic somite segmentation. Sustained *Hes7* or *Lfng* expression leads to fusion of somites, suggesting that oscillatory expression is required for this process (10-15). Importantly, in both cases, oscillatory versus sustained expression results in

different outcomes in biological events. However, although transcriptional response to serum stimulation has been analyzed for many genes, previous studies failed to detect oscillatory responses, because the temporal profiling is usually intensive only for the first one or two hours but not for longer periods.

Here, we analyzed temporal changes in gene expression more intensively for the first 4.5 hours after serum stimulation and identified novel ultradian oscillators, including molecules in Stat signaling. Loss of Stat oscillations leads to inhibition of Hes1 oscillations, which retards the cell cycle progression. These results provide a new insight into the significance of oscillatory expression in cell proliferation.

Results and Discussion

Identification of novel ultradian oscillators

In order to elucidate the dynamics of regulatory networks, we searched for ultradian oscillators by performing gene expression profiling of mouse fibroblasts (C3H10T1/2) after serum stimulation. Biotinylated cRNAs were prepared every 30 min from time = 0 to time = 4.5 hr (10 time points) and hybridized to high-density microarrays. For screening the data, we set the following three criteria. First, at least two peaks with >1.5-fold higher than the lowest signal value were required during the 4.5-hr period. Second, values of two peaks were flagged with "present" and higher than 100 to exclude genes with very weak expression levels. Third, the difference between the values of two peaks and the lowest was statistically significant. These criteria resulted in 7 candidate oscillators with a periodicity of about two hours out of 45,037 probe sets (Fig. 1A and Table S1 in the Supplemental Data). None of them had shorter periods, although oscillators with longer periods were not included in this study. Validation of this approach was the observation that the known ultradian oscillator *Hes1* was included in this list (Fig. 1A). These candidate genes were further examined, and four of them (*Socs3*, *Hes1*, *Gse1* and *Smad6*) were identified as oscillators by real-time PCR (Fig. 1B). *Socs3* and *Hes1* have the first peak at 1 hr, *Gse1* at 1.5 hr, and *Smad6* at 2 hr (Fig. 1A, B). Of particular interest are the genes for signal transduction *Socs3* and *Smad6*, downstream molecules of Jak-Stat and Smad signaling pathways, respectively (16-19), because these pathways are known to exhibit earliest responses to regulate many downstream genes. Jak-Stat signaling mediates the effects of various growth factors and cytokines while Smad signaling mediates the effects of TGF-β/BMP, and

both regulate cell proliferation and survival (16-19). Thus, we decided to further characterize these signaling pathways.

Stat-Socs oscillations

Socs3 is an inhibitor of Jak-Stat signaling (16, 17, 20): phosphorylated Stat3 (at Tyr705, p-Stat3) induces expression of Socs3, which in turn inhibits Jak-dependent phosphorylation of Stat3. Real-time PCR analysis with shorter intervals (10 min) indicated that *Socs3* mRNA expression oscillates with peaks at 50 min and 170 min after serum stimulation (Fig. 2A). Western blot analysis showed that Socs3 protein expression displays oscillation with peaks around 2 hr and 4 hr, indicating ~1-hr delay between mRNA and protein synthesis (Fig. 2C, control).

We next examined whether Stat3, an activator of Socs3, is also cyclic after serum stimulation. Stat3 mRNA and protein levels are almost constant after serum stimulation (Fig. S1A, B). However, p-Stat3 levels were found to be oscillatory with peaks around 1 hr, 3 hr and 5 hr (Fig. 2D, control), suggesting that cyclic activation of Stat3 regulates Socs3 oscillation. To show that Socs3 oscillation depends on cyclic phosphorylation of Stat3, we inhibited p-Stat3 formation. Treatment with AG490 inhibited Jak2-dependent phosphorylation of Stat3 (Fig. 2D, +AG490). Under this condition, expression of both Socs3 mRNA and protein are repressed, and their oscillations are abolished (Fig. 2B, C, +AG490), indicating that Jak2-dependent phosphorylation is essential for Socs3 oscillations. Similarly, in the presence of dominant-negative Stat3 (dnStat3), Socs3 oscillations are abolished (Fig. 2B, C, +dnStat3), indicating that Socs3 oscillations depend on periodic activation of Stat3.

We next examined whether periodic activation of p-Stat3 depends on Socs3 oscillations. When Socs3 is continuously expressed, periodic formation of p-Stat3 is inhibited (Fig. 2D, +Socs3), suggesting that Socs3 oscillations periodically inhibit formation of p-Stat3. To prove this suggestion, we next knocked down Socs3 expression by its specific siRNA. This siRNA successfully down-regulates both Socs3 mRNA and protein levels (Fig. 2B, C, +siSocs3, and Fig. S2F). Under this condition, p-Stat3 formation is persistently up-regulated, and its oscillation is abolished (Fig. 2D, +siSocs3). These results indicate that p-Stat3 and Socs3 oscillations depend on each other and are regulated by negative feedback.

Because Jak and Stat mediate IL-6 signaling, we next examined whether

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Socs3 expression oscillates after treatment with IL-6. *Socs3* mRNA was found to oscillate after IL-6 stimulation (Fig. S2A), just like serum stimulation, suggesting that Stat-Socs oscillations are induced by a single stimulator but not the results of mixed stimulators included in serum.

Smad oscillations

Smad6 is an inhibitor of Smad signaling (18, 19, 21): phosphorylated Smad1/5/8 (p-Smad1/5/8) induces expression of Smad6, which in turn inhibits phosphorylation of Smad1/5/8. Real-time PCR analysis with shorter intervals (10 min) indicated that *Smad6* mRNA expression oscillates with peaks at 120 min and 230 min after serum stimulation (Fig. 3A). Western analysis showed that Smad6 protein expression displays oscillation with peaks around 2.5 hr and 4.5 hr (Fig. 3C, control). mRNA and protein for Smad1, an activator of Smad6, do not oscillate after serum stimulation (Fig. S1C, D). However, p-Smad1/5/8 levels were found to be oscillatory with peaks around 1.5 hr, 3.5 hr and 5.5 hr (Fig. 3D, control), suggesting that periodic activation of Smad1/5/8 regulates Smad6 oscillation. To show that Smad6 oscillations depend on periodic phosphorylation of Smad1/5/8, we inhibited p-Smad1 activity. In the presence of dominant-negative Smad1 (dnSmad1), both Smad6 mRNA and protein oscillations are abolished (Fig. 3B, C, +dnSmad1), indicating that Smad6 oscillations depend on periodic activation of Smad1. We next asked whether periodic activation of Smad1 depends on Smad6 oscillations. When Smad6 is continuously expressed, periodic formation of p-Smad1 is inhibited (Fig. 3D, +Smad6), indicating that Smad6 oscillations periodically inhibit formation of p-Smad1. These results show that p-Smad1 and Smad6 oscillations depend on each other and are regulated by negative feedback.

Because Smads mediate BMP signaling, we next examined whether *Smad6* expression oscillates after treatment with BMP. *Smad6* mRNA was found to oscillate after BMP4 treatment (Fig. S3A), just like serum stimulation, suggesting that Smad oscillations are induced by a single stimulator but not the results of mixed stimulators included in serum.

Stat-Socs oscillations regulate Hes1 oscillations

The above results indicate that Stat-Socs and Smad oscillations are regulated by their own negative feedback loops, like Hes1 oscillation. We next examined whether

there are any cross-talks between these oscillators. When Stat-Socs oscillations are inhibited by AG490, dnStat3, or Socs3, both Smad6 and p-Smad1 oscillations are not affected (Figs. S3B-D, S4A-C). In addition, when Smad oscillations are inhibited, both Socs3 and p-Stat3 oscillations are not affected (Figs. S2B, C, S5A, B). Thus, there seems to be no clear cross-talk between Stat-Socs and Smad oscillations. Similarly, Hes1 oscillations are not affected in the absence of Smad oscillations (Fig. S6), while Smad oscillations are not affected in the absence of Hes1 oscillations (Figs. S3E, F, S4D, E), suggesting that there is no cross-talk between Smad and Hes1 oscillations.

In contrast, Hes1 oscillations are significantly affected by inactivation of Stat-Socs oscillations. When p-Stat3 formation is constitutively suppressed by AG490, dnStat3, or Socs3, *Hes1* mRNA is transiently induced by serum treatment, but it remains suppressed at the basal level thereafter (Fig. 4A, compare with control). We also examined *Hes1* expression in individual cells by using the real-time imaging method, which used the ubiquitinated luciferase reporter gene under the control of *Hes1* promoter (5). *Hes1* expression oscillates after serum stimulation in individual cells (Fig. S7A), but this oscillation is suppressed at the single cell level in the presence of AG490 (Fig. S7B). In contrast, Hes1 protein is persistently up-regulated after serum treatment in the presence of AG490, dnStat3, or Socs3 (Fig. 4B, compare with control). These results suggest that Hes1 protein is stabilized in the absence of Stat signaling, which may lead to repression of Hes1 mRNA. In accordance with this suggestion, the half-life of Hes1 protein becomes prolonged from 22.4 ± 0.9 min to 40-50 min by AG490, Socs3 and dnStat3 (Fig. 4C, compare with control). Conversely, when p-Stat3 formation is constitutively up-regulated by knock-down of Socs3 with siSocs3, both Hes1 mRNA and protein oscillations are again abolished (Fig. 4D, E, compare with control). Under this condition, *Hes1* mRNA tends to be higher than the basal level, whereas the Hes1 protein is near the basal level (Fig. 4D, E). The half-life of Hes1 protein is shortened to 15.3 ± 1.6 min by constitutive activation of Stat3 (Fig. 4C, +siSocs3). Thus, both sustained activation and inactivation of Stat signaling abolish Hes1 oscillations, suggesting that Stat-Socs oscillations regulate Hes1 oscillations by controlling the half-life of Hes1 protein. We also examined whether Stat-Socs oscillations depend on Hes1 oscillations. However, Stat-Socs oscillations are not affected by overexpression of Hes1 or dnHes1, suggesting that they are independent of Hes1 oscillations (Figs. S2D, E, S5C, D). It was previously shown that Hes1 associates with both Jak2 and Stat3 and

thereby promotes Stat3 phosphorylation (22). Thus, Hes1 protein, but not Hes1 oscillation, is required for periodic Stat activation, and it is likely that coupled oscillations between Stat and Hes1 signaling pathways are occurring after serum stimulation.

Hes1 oscillations are important for efficient cell proliferation

All actively dividing cultured cells that we have examined express Hes1, but without serum stimulation, Hes1 expression levels seem to be stationary on Northern and western analyses. However, even under this condition, Hes1 expression was found to be oscillatory at the single cell level (5). Hes1 oscillation is just out of synchrony between cells without serum stimulation (5). Because Hes1 expression oscillates in proliferating cells, we next examined the effects of sustained and loss of Hes1 activity on proliferation of fibroblasts. Both cells that express Hes1 protein persistently and cells that lose Hes1 activity do not proliferate extensively, and their proliferation rates are reduced to half, compared to the control cells, where Hes1 expression oscillates (Fig. 5A). These results suggest that Hes1 oscillation is required for efficient cell proliferation.

We next examined effects of different Hes1 expression modes on several cell cycle regulators (23). Expression of the cyclin-dependent kinase (CDK) inhibitors p21 and p27, G1 phase markers, is up-regulated by persistent expression of Hes1 or dnHes1 (Fig. 5B, +Hes1 and +dnHes1), compared to the control, where Hes1 expression is oscillatory (Fig. 5B, control). Furthermore, expression of another G1-specific marker, cyclin D1, is also increased by persistent expression of Hes1 or dnHes1 (Fig. 5C, +Hes1 and +dnHes1), compared to the control (Fig. 5C, control). In contrast, expression of the S phase marker PCNA and the check point genes Wee1, Chk1 and Chk2 are not significantly affected by Hes1 or dnHes1 (Fig. 5B). Thus, both sustained Hes1 protein expression and knock-down of Hes1 protein activity increase expression of G1-specific markers, suggesting that persistent or down-regulated Hes1 expression leads to G1 phase retardation. It is likely that Stat-Socs oscillations promote cell proliferation by inducing Hes1 oscillation.

Mathematical simulation of Hes1 oscillations

Hes1 oscillation has been mathematically simulated using a negative

autorepression model (4, 24-26). Because a simple negative feedback loop is insufficient to maintain a stable oscillation, another factor or time delay has been postulated (4, 24-26). We found that p-Stat3 destabilizes Hes1 protein, suggesting that Hes1 protein half-life would be changing by p-Stat3 oscillation. With this assumption, Hes1 oscillation can be now readily simulated without postulating another factor or time delay (Figs. 6A and S8). To reproduce the experimental trend by numerical simulation, we adapted the framework of the negative feedback model with the change of Hes1 protein half-life. Let $m(t)$ and $p(t)$ be the numbers of *Hes1* mRNA and protein molecules, respectively, in a cell at time t. The rates of change of $m(t)$ and $p(t)$ are described as follows.

$$\frac{dp(t)}{dt} = am(t) - b(t)p(t) \tag{E1}$$

$$\frac{dm(t)}{dt} = \frac{k}{1 + \left(\frac{p(t)}{P_0}\right)^2} - cm(t) \tag{E2}$$

where the constant a *[h^{-1}]* is the rate of production of new proteins, c *[h^{-1}]* is the degradation rate (inverse half-life) of the mRNA molecules, and k *[h^{-1}]* and p_0 are the constants to represent the inhibitory process by the protein acting as a dimer. Throughout the simulations, we set $a = 270.0$, $c = 1.6$, $k = 1980.0$ and $P_0 = 40.0$ (24). To represent the change of Hes1 protein half-life by p-Stat3 oscillation, we introduced two types of time dependence to the degradation rate $b(t)$ *[h^{-1}]*, sinusoidal or flip-flop, as follows.

$$b(t) = 1.1\sin\left(\frac{2\pi}{T}t\right) + 1.9 \tag{E3}$$

$$b(t) = \begin{cases} 1.9 + 1.1(2.0 - \text{w})/\text{w} & \left(2(n-1)T \le t < 2(n-1)T + w\right) \\ 0.8 & \left(2(n-1)T + w \le t < 2nT\right) \end{cases} \quad (\frac{1}{2}T \le w < T) \tag{E4}$$

where T is the period of p-Stat3 oscillation, which is determined as 2.0 hours by experiment, w in (E4) is a parameter to characterize the flip-flop change of Hes1 protein half-life and n is an integer. From the experimental value of Hes1 protein half-life induced by p-Stat3 oscillation, we can estimate the average and the trough values of $b(t)$ as 1.9 and 0.8, respectively. With either of these $b(t)$ (E3, E4), Hes1 expression shows stable oscillation (Figs. 6A and S8). In contrast, when the Hes1

protein degradation rate is fixed at certain values, b(t) = 0.8, which represents the absence of p-Stat3 formation, or b(t) = 2.77, which represents persistent formation of p-Stat3, Hes1 oscillation becomes damped, well mimicking the effects of sustained inhibition and activation of Stat signaling (Fig. 6B, C).

Our study reveals an unexpected expression mode of signaling molecules in response to serum. We show that in addition to the Notch effector Hes1, Stat-Socs and Smad signaling molecules display oscillatory expression with the periodicity of about two hours. These oscillations are regulated by negative feedback, just like Hes1 oscillation (Fig. S9). Interestingly, Smad6 oscillation is delayed compared to Socs3 and Hes1 oscillations, although phosphorylation of Stat3 and Smad1 occurs simultaneously (Figs. 2D, 3D). Induction of *Socs3* mRNA also occurs simultaneously with phosphorylation of Stat3, whereas that of *Smad6* mRNA is delayed about one hour, suggesting that p-Stat3 functions faster than p-Smad1. Kinetics of these factors remains to be analyzed. Strikingly, Stat-Socs oscillations lead to Hes1 oscillations by regulating the stability of Hes1 protein. The mechanism by which Stat signaling regulates the Hes1 protein stability remains to be determined. Physical interaction with Jak and Stat (22) could lead to destabilization of Hes1 protein. Another possibility is that p-Stat3 induces expression of genes that regulate degradation of Hes1 protein. Further analysis on E3 ligase for Hes1 protein may be required to clarify this issue.

We also show that both sustained Hes1 expression and loss of Hes1 activity reduce cell proliferation by inducing G1 phase retardation, suggesting that Hes1 oscillation is important for efficient cell proliferation. It is likely that Hes1 is required at a certain point but should be down-regulated at another point of the cell cycle, although it remains to be determined at which points Hes1 promotes and inhibits the cell cycle progression. Another important question is whether the Hes1 oscillator links the cell cycle to the somite segmentation clock. However, no obvious defect in the segmentation and the cell cycle was observed in *Hes1*-null mice (7), probably due to compensation by its related genes such as *Hes7*. Analysis on compound mutations would be required to answer this question.

In our microarray analysis, none of the downstream genes in the Notch-Hes1, Stat-Socs and Smad pathways is found to display oscillatory expression. It is possible that oscillations in the Notch-Hes1, Jak-Stat-Socs and Smad pathways are important just to maintain expression of downstream genes within a critical range rather than

oscillatory. Alternatively, expression of downstream genes could easily become out of synchrony between cells, which might make it difficult to identify oscillatory expression. In that case, expression should be examined at the single cell level.

It has been reported that NF-κB and p53 signaling pathways also display oscillatory responses with the periods of about 100 min and 440 min, respectively (27-30). In these pathways, IκB and Mdm2 act as negative regulators for NF-κB and p53, respectively. In our present study, oscillations in the NF-κB signaling are not observed, probably because it is not activated by serum. We assume that other signaling pathways with negative feedback loops, which do not respond to serum, would also display oscillatory responses to different stimulations. Our results raise the possibility that many cellular activities are rhythmically controlled by combinations of different types of ultradian oscillators, and suggest that the number of pulses as well as the duration and intensity may be important for cells to make final decisions.

Methods

Cell culture and RNA and protein purification

C3H10T1/2 mouse fibroblast cells were treated with serum at time = 0, as previously described (4), and RNA was prepared every 10 min or 30 min. Proteins were prepared at t = 45 min (0.75 h) and every 30 min thereafter. Total RNA was purified using TRIzol reagents (Invitrogen) from cells in 100mm dishes or 6 well plates. Protein was purified from cells in 100mm dishes by Cell Lysis Buffer (50mM Tris-Cl pH 8.0, 5mM EDTA, 150mM NaCl, 1% NP-40) with Protease Inhibitor Cocktail (Nacalai Tesque) and phosphatase inhibitors (1mM NaF, 1mM Na_3VO_4, 1mM β-glycerophosphate, 1mM sodium pyrophosphate). For treatment with AG490, the final concentration of 50μM was added to the medium with serum stimulation. For treatment with BMP4 or IL-6, medium containing 100 ng/ml each without serum was added at time = 0.

Microarray analysis

Total RNA was prepared from cultured cells at indicated time points (serum stimulation at time = 0). Microarray analysis using Gene Chip Mouse Genome 430 2.0 Array (Affymetrix) was done, according to the manufacturer's protocols. Data were analyzed using GCOS (Affymetrix) and Gene Spring (Agilent Technologies). In brief, per chip normalization was done using the value of median, and then, per gene normalization

was done using the value of time = 0. To explore the candidates for new oscillators, the following three criteria were set. First, at least two peaks with >1.5-fold higher than the lowest signal value were required during the 4.5-hr period. This result should be obtained in at least two independent experiments among the three. Second, the signal intensities of prospective peak points should be flagged with "Present" and higher than 100. Third, the difference between the peak signal intensities and the lowest signal intensities should be statistically significant ($p<0.05$ at unpaired one-tail t-test). The microarray data will be deposited in the Genome Network Platform < http://genomenetwork.nig.ac.jp/index_e.html>.

Real time PCR

Total RNA was reverse-transcribed using ReverTra Ace (TOYOBO) and Random Primer (TOYOBO). Real time PCR was done using Applied Biosystems 7500 Real Time PCR System (Applied Biosystems) and SYBR Premix EX Taq (TAKARA), according to the manufacturer's protocols. PCR primers are described in Supplemental Data. GAPDH was used as a control, and data were normalized to the value of time = 0.

Western blotting

Antibodies used for western blotting are as follows: anti-Hes1 (gift from Dr. Sudo), anti-Socs3 (Fusion Antibodies, FA1017), anti-Smad6 (IMGENEX, IMG-555), anti-phospho-Y705-Stat3 (Cell Signaling Technology, #9131), anti-Smad1 (Cell Signaling Technology, #9512), anti-phospho-Smad1/5/8 (Cell Signaling Technology, #9511), anti-Stat3 (BD Transduction Laboratories, 610189), anti-Cyclin D1 (Santa Cruz Biotechnology, sc-718), anti-Actin (SIGMA, A2066), anti-rabbit IgG (Amersham Biosciences, NA9340V) and anti-mouse Ig antibodies (Amersham Biosciences, NA9310). Signal was detected by SuperSignal West Dura Extended Duration Substrate (Pierce) and Hyperfilm ECL (Amersham Biosciences) or LAS-3000 mini (Fujifilm). Means with S.E. were calculated from three independent experiments.

Expression vectors

For mis-expression of Hes1, Socs3 and Smad6, the coding region of each cDNA was cloned into pCI expression vector (Promega). For dnHes1, mouse Hes1 cDNA with mutations of E43A, K44A and R47A was cloned into pCI expression vector. For

dnStat3, mouse Stat3 cDNA with mutation of Y705F was cloned into pCI expression vector. For dnSmad1, mouse Smad1 with mutation of A422stop was cloned into pCI expression vector. For knockdown of Socs3 by the siRNA method, the following fragments were cloned into psiRNA-h7SKneo vector, according to the manufacturer's protocol (InvivoGen): siSocs3 sense strand, acctcGCATCTTTGTCGGAAGACTGTtcaagagACAGTCTTCCGACAAAGATGCtt; siSocs3 antisense strand, caaaaaGCATCTTTGTCGGAAGACTGTctcttgaACAGTCTTCCGACAAAGATGCg; siGFP sense strand, acctcGCAAGCTGACCCTGAAGTTCAccaccTGAACTTCAGGGTCAGCTTGCtt; and siGFP antisense strand, caaaaaGCAAGCTGACCCTGAAGTTCAggtggTGAACTTCAGGGTCAGCTTGCg. siGFP was used as a control.

Transfection of expression vectors

Cells were transfected with expression vectors with pCI-neo using Lipofectamin reagent and Plus reagent (Invitrogen), and transfected cells were selected by 1mg/ml G418 (GIBCO).

Measurement of Hes1 protein half-life

Cells were treated with 100μM cycloheximide and harvested at indicated time points (cycloheximide treatment at time = -20 min), and cell extracts were subjected to western blotting using anti-Hes1 antibody.

Real-time imaging

Cells containing the reporter Hes1-Ub2-Luc were cultured in DMEM/10% FBS/1 mM luciferin (Nacalai Tesque), and bioluminescence was measured, as previously described (5).

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Figure Legends

Figure 1. Identification of serum-induced ultradian oscillators by microarray analysis. (A) Microarray analysis of serum-induced ultradian oscillators. Seven candidate genes are found. (B) Time course of *Socs3*, *Hes1*, *Gse1* and *Smad6* expression in real-time PCR analysis. Means with S.E. of three independent experiments are shown. Patterns of microarray and real-time PCR experiments are very similar to each other.

Figure 2. Oscillations in Stat signaling. Cells were treated with serum at time = 0 in the absence (control) or presence of AG490, dnStat3, Socs3 or siSocs3. (A) Expression profiles of *Socs3* mRNA were analyzed with 10 min intervals by real-time PCR. The peaks of *Socs3* mRNA appear at 50 min and 170 min after serum stimulation. (B) Expression profiles of *Socs3* mRNA were analyzed by real-time PCR. *Socs3* mRNA oscillation is abolished in the presence of AG490, dnStat3 or siSocs3. (C) Expression profiles of Socs3 protein in the absence (control) or presence of AG490, dnStat3 or

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siSocs3 were analyzed by western blotting. Socs3 protein oscillation is abolished in the presence of AG490, dnStat3 or siSocs3. (D) Expression profiles of p-Stat3 in the absence (control) or presence of AG490, Socs3 or siSocs3 were analyzed by western blotting. p-Stat3 oscillation is abolished in the presence of AG490, Socs3 or siSocs3. Means with S.E. of three independent experiments are shown in all graphs.

Figure 3. Oscillations in Smad signaling. Cells were treated with serum at time = 0 in the absence (control) or presence of dnSmad1 or Smad6, and mRNA and protein levels were quantified by real-time PCR and western blots, respectively. (A) Expression profiles of *Smad6* mRNA were analyzed with 10 min intervals. The peaks of *Smad6* mRNA appear at 120 min and 230 min after serum stimulation. (B) Expression profiles of *Smad6* mRNA in the absence (control) or presence of dnSmad1 were analyzed. *Smad6* mRNA oscillation is abolished by dnSmad1. (C) Expression profiles of Smad6 protein in the absence (control) or presence of dnSmad1 were analyzed. Smad6 protein oscillation is abolished by dnSmad1. (D) Expression profiles of p-Smad1/5/8 in the absence (control) or presence of Smad6 were analyzed. p-Smad1/5/8 oscillation is abolished by Smad6. Means with S.E. of three independent experiments are shown in all graphs.

Figure 4. Regulation of Hes1 oscillation by Stat signaling. (A) Expression of *Hes1* mRNA oscillates after serum stimulation (time = 0), but this oscillation is abolished by AG490, dnStat3, or Socs3. (B) Expression of Hes1 protein oscillates after serum stimulation (time = 0) (control), but this oscillation is abolished by AG490, dnStat3, or Socs3. (C) Measurement of Hes1 protein half-life. Hes1 protein levels were measured in the presence of cycloheximide (100μM), which blocks new protein synthesis. Hes1 protein is degraded with the half-life of 22.4 ± 0.9 min (n = 3). This half-life is elongated by AG490, dnStat3, or Socs3 and shortened by siSocs3. Thus, Stat signaling regulates Hes1 protein stability. (D) Hes1 mRNA oscillation is abolished by siSocs3. (E) Hes1 protein oscillation is abolished by siSocs3. All values are the average of three independent experiments with S.E.

Figure 5. Hes1 oscillation in cell cycles. (A) Comparison of growth curves. Cells that express Hes1 protein persistently and cells that lose Hes1 activity do not proliferate
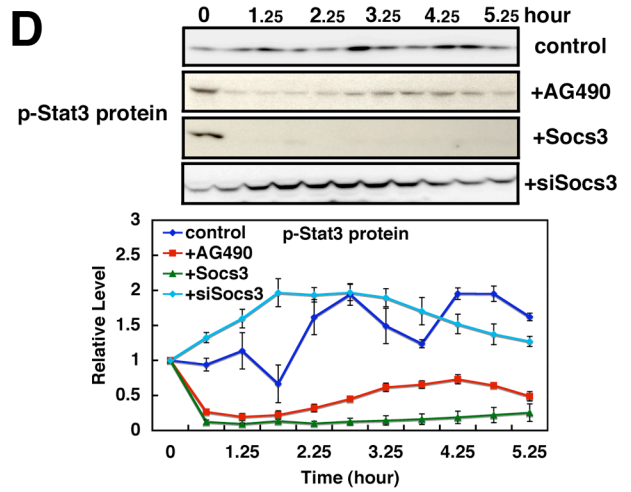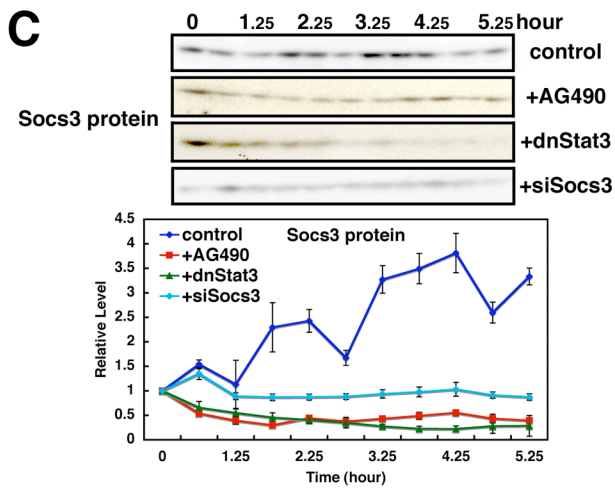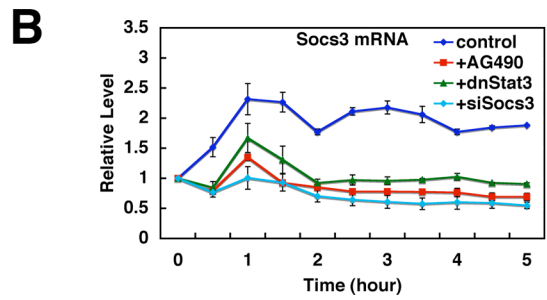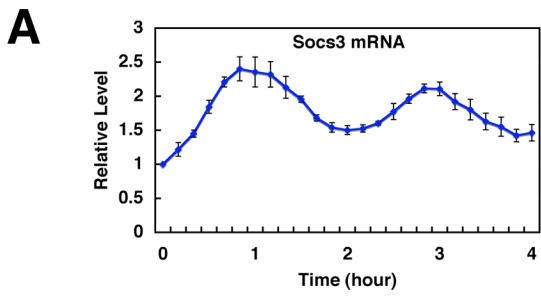
extensively, compared to the control, where Hes1 expression oscillates. (B) Regulation of cell cycle regulators by Hes1. Expression was examined by real-time PCR. Both sustained Hes1 expression (+Hes1) and knock-down of Hes1 activities (+dnHes1) increase expression of p21 and p27, G1 phase genes, compared to oscillatory Hes1 expression (control). Transfected cells with the control vector and non-transfected cells gave the same results (data not shown). (C) Expression of cyclin D1, a G1-specific marker. Both Hes1 and dnHes1 increase cyclin D1 expression, compared to control. All values are the average of three independent experiments with S.E.
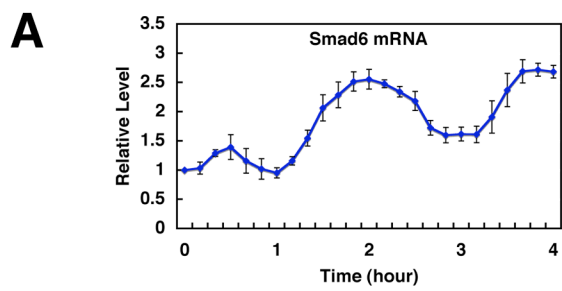
Figure 6. Mathematical simulation of Hes1 oscillations. Hes1 oscillations are mathematically simulated using a negative autorepression model. When Hes1 protein half-life is changing, Hes1 expression shows stable oscillation (A). In contrast, when the Hes1 protein degradation rate is fixed at the values $b(t) = 0.8$, which represents the absence of p-Stat3 formation (B), and $b(t) = 2.77$, which represents persistent formation of p-Stat3 (C), Hes1 oscillation becomes damped. This simulation well mimics Stat3-dependent Hes1 oscillations.
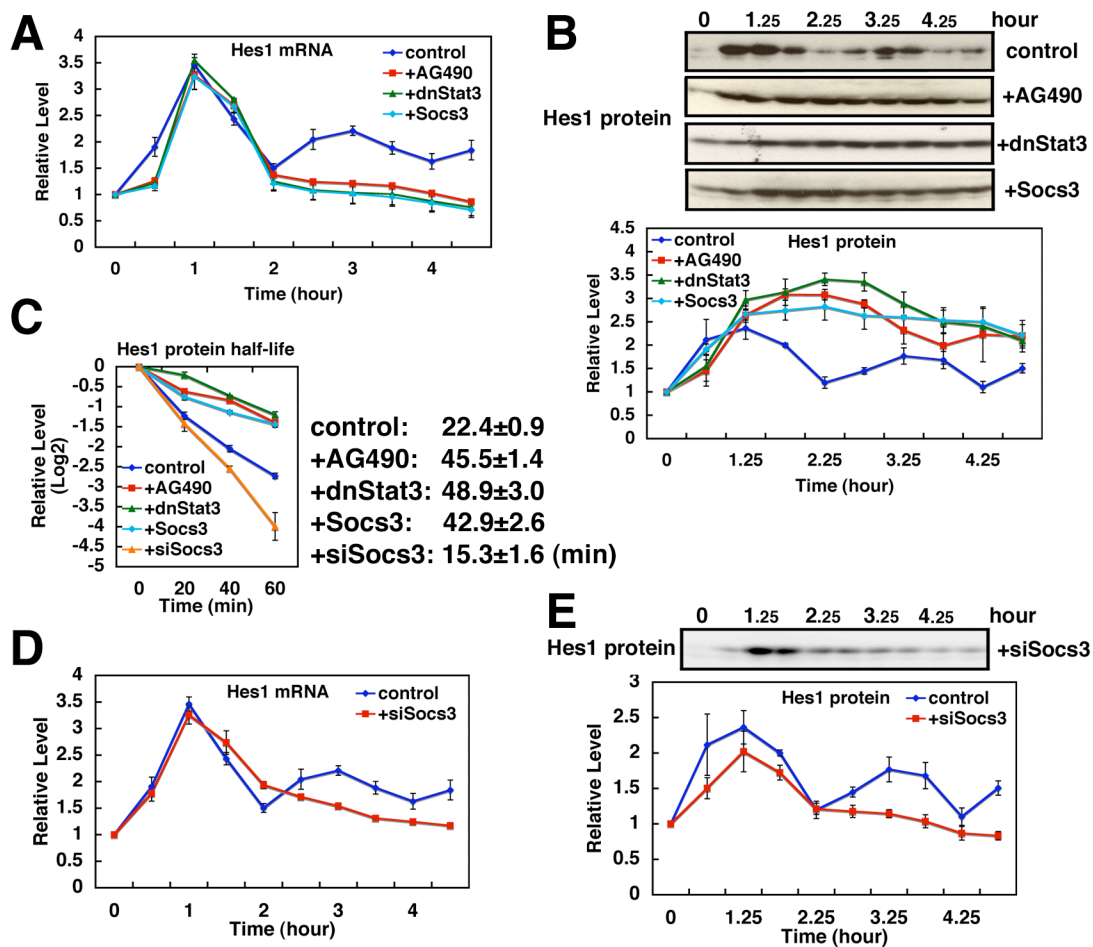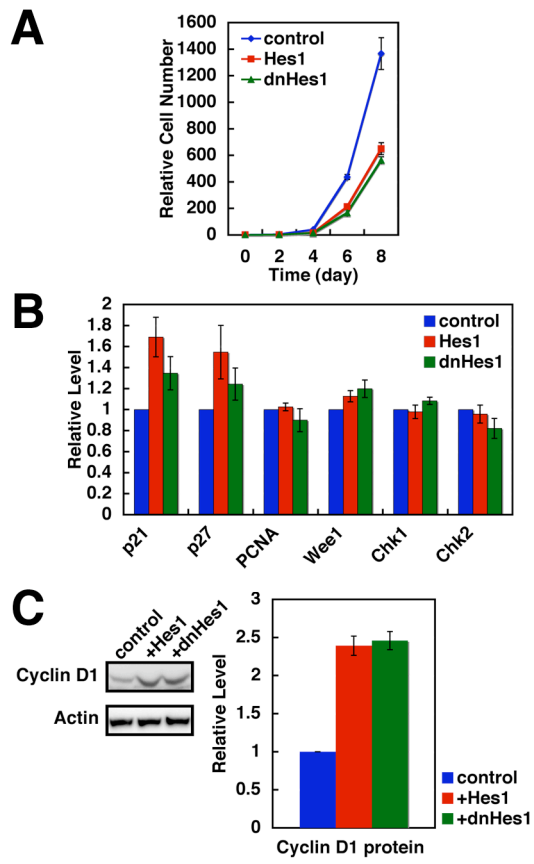
A

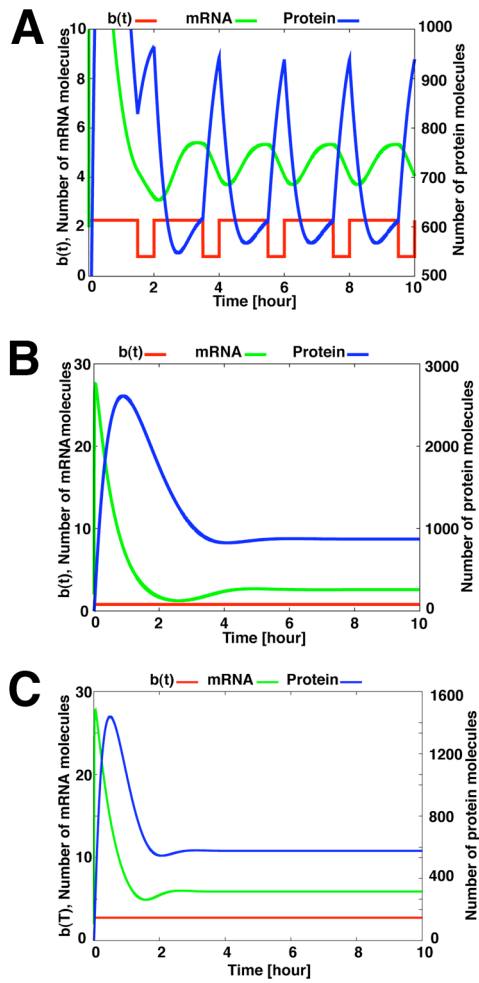| 0	1	2	3	4	hour
					Socs3
					Hes1
					Gse1
					Smad6
					Lasp1
					Wdr57
					Pelo

Expression

Min   Max

B

Socs3 mRNA

Hes1 mRNA

Smad6 mRNA

Gse1 mRNA
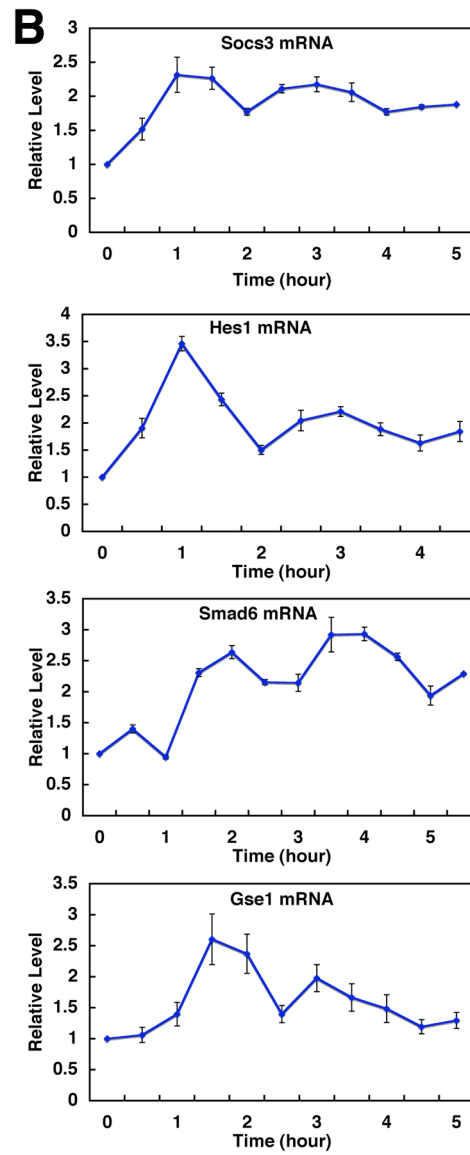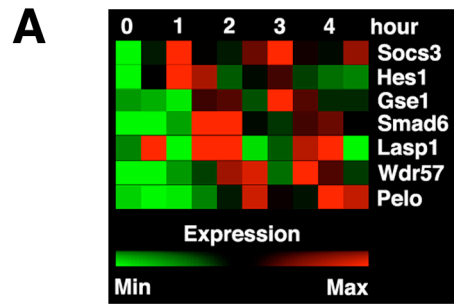
Fig.1

Fig.2

Fig.3

Fig.4

A

B

C

Cyclin D1

Actin
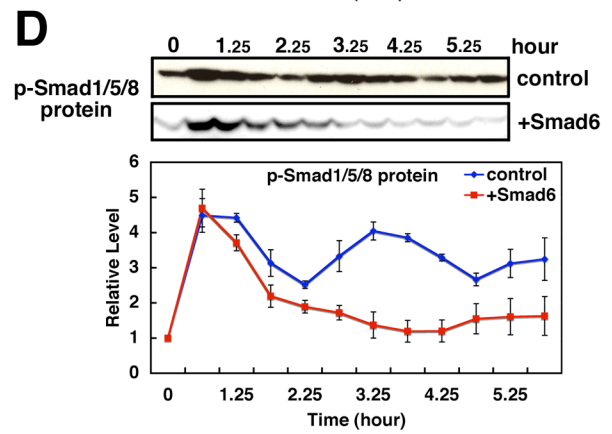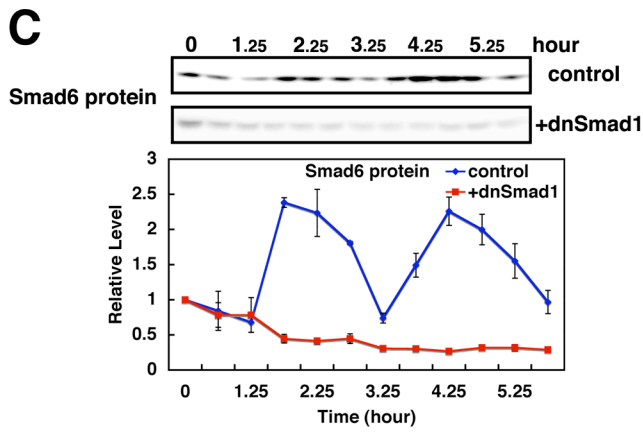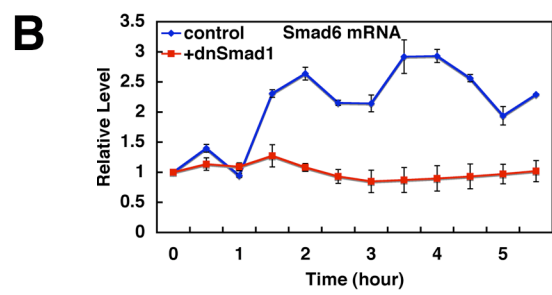
**Fig.5**

Fig.6

Supplemental Data

Methods

The following primers were used for real-time PCR: Hes1 forward, tgaaggattccaaaaataaaattctctggg; Hes1 reverse, cgcctcttctccatgataggctttgatgac; Socs3 forward, ccgctggtactgagccgacctctctcctcc; Socs3 reverse, tggagcatcatactgatccaggaactcccg; Smad6 forward, tctcggctgtctcctcctgaccagtacaag; Smad6 reverse, ccacgctgcaccagtggctcggcttggtgg; Stat3 forward, tacagcgacagcttccccatggagctgcgg; Stat3 reverse, cacccaagagattatgaaacaccaacgtgg; Smad1 forward, aaagacctgtggcttccgtct; Smad1 reverse, ttatcgtggctccttcgtcag; Gse1 forward, accaaccatagctctccagctagcacgccc; Ges1 reverse, agccagagtcctggcggctctcgcttctcc; p21 forward: aggagcaaagtgtgccgttgt, p21 reverse: tcaaagttccaccgttctcgg, p27 forward: tccgcctgcagaaatctcttc, p27 reverse: cgaaattccacttgcgctga, Wee1 forward: tcggatcccacaagtgcttt, Wee1 reverse: tgaatgcttcaccagctccat, Chk1 forward: tctgctcctctggctttgctt, Chk1 reverse: tggcctctttgctcctctgtt, Chk2 forward: ggaacaagcgcctgaaagaag, Chk2 reverse: ccggctttaagtcccgatgta, PCNA forward: aaggtgctggaggctctcaaa, PCNA reverse: tatgtgtcgaagccttcggag, GAPDH forward, atcttcttgtgcagtgccagcctcgtcccg; GAPDH reverse, agttgaggtcaatgaaggggtcgttgatgg.

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| Gene ID	Genbank No.	Common Name	Time=0	Time=0.5	Time=1	Time=1.5	Time=2	Time=2.5	Time=3	Time=3.5	Time=4	Time=4.5
1455899_x_at	BB241535	Socs3	1	1.514	2.625	1.776	1.533	2.006	2.762	1.81	1.568	2.144
			1	1.229	1.502	0.962	0.801	0.99	1.365	1.029	1.029	0.856
			1	1.282	2.464	1.414	1.352	1.725	1.577	1.663	1.236	0.817
1418102_at	BC018375	Hes1	1	3.188	7.274	5.004	2.133	2.985	4.065	2.318	1.959	1.822
			1	3.13	9.698	8.421	3.516	3.741	5.143	4.175	3.138	2.955
			1	2.639	6.269	3.804	2.555	2.599	3.091	2.312	1.426	0.967
1429653_at	BE863648	Gse1	1	0.922	0.735	2.046	2.163	1.257	3.027	2.148	1.415	1.44
			1	1.111	1.497	3.859	3.135	2.85	5.004	4.125	2.252	1.523
			1	3.028	4.481	5.875	6.01	5.487	12.35	8.537	4.936	3.39
1422771_at	AF010133	Smad6	1	1.103	1.052	3.006	3.192	1.911	1.961	1.735	2.624	2.568
			1	0.933	1.272	3.606	3.71	1.976	1.764	2.587	2.736	2.224
			1	1.203	1.548	3.559	4.491	2.353	2.493	2.988	3.217	2.278
1439264_x_at	BB433798	Lasp1	1	1.502	0.814	1.52	1.522	0.921	1.029	1.399	1.99	0.556
			1	1.151	1.178	1.784	2.437	3.045	1.495	2.591	1.968	1.751
			1	0.843	1.193	1.332	2.027	2.354	1.92	1.565	2.423	2.845
1452713_a_at	AK002371	Wdr57	1	1.196	0.714	1.222	1.334	1.282	0.672	2.089	1.947	1.239
			1	1.006	1.156	1.343	2.008	2.172	1.25	2.388	1.814	1.374
			1	1.31	1.262	1.385	1.798	2.677	2.23	1.688	3.028	2.964
1434071_a_at	AV288264	Pelo	1	0.822	0.941	1.249	1.627	2.75	2	1.67	3.135	2.666
			1	1.102	0.715	0.866	1.578	1.688	1.25	1.125	2.013	1.885
			1	1.491	0.87	1.317	1.74	1.999	1.511	2.045	2.33	2.571

Table S1. List of candidate oscillator genes in microarray analysis. Signal intensity at each time point was normalized to that of time = 0. Data from three independent experiments are shown.
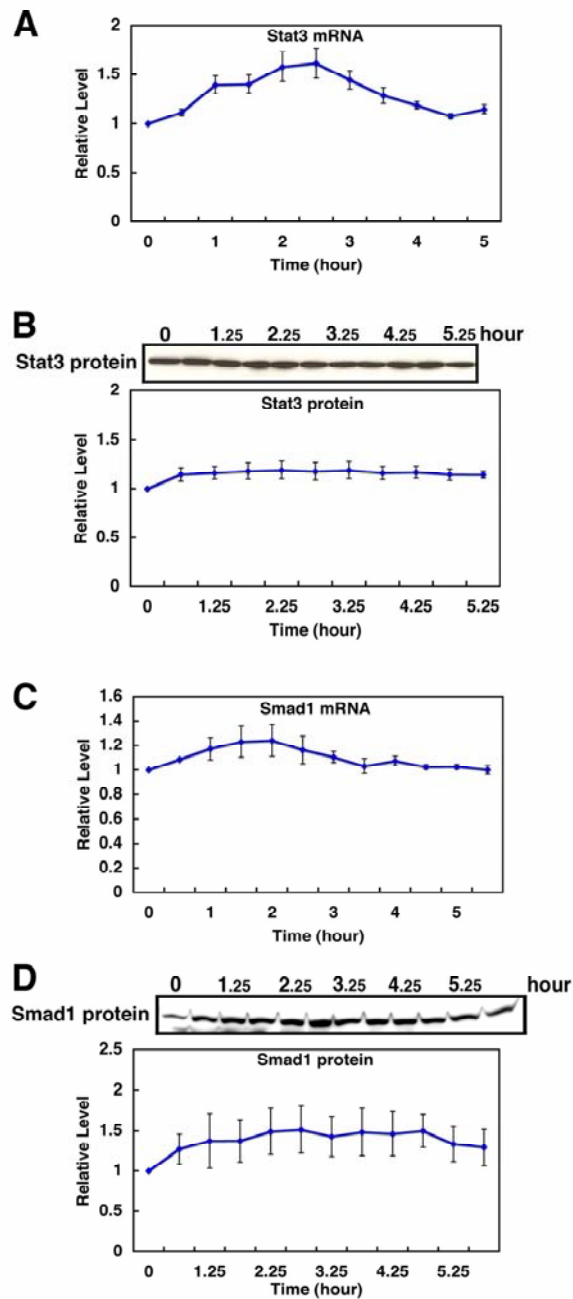
Figure S1. Expression of Stat3 mRNA, Stat3 protein, Smad1 mRNA and Smad1 protein after serum stimulation. Total RNA (A, C) and protein (B, D) were prepared from fibroblasts after serum stimulation at indicated time points (serum stimulation at time = 0) and subjected to real time PCR analysis and western blotting, respectively. Values in graphs are means ± S.E. of three independent experiments. Expression of Stat3 mRNA (A), Stat3 protein (B), Smad1 mRNA (C) and Smad1 protein (D) does not oscillate after serum stimulation.
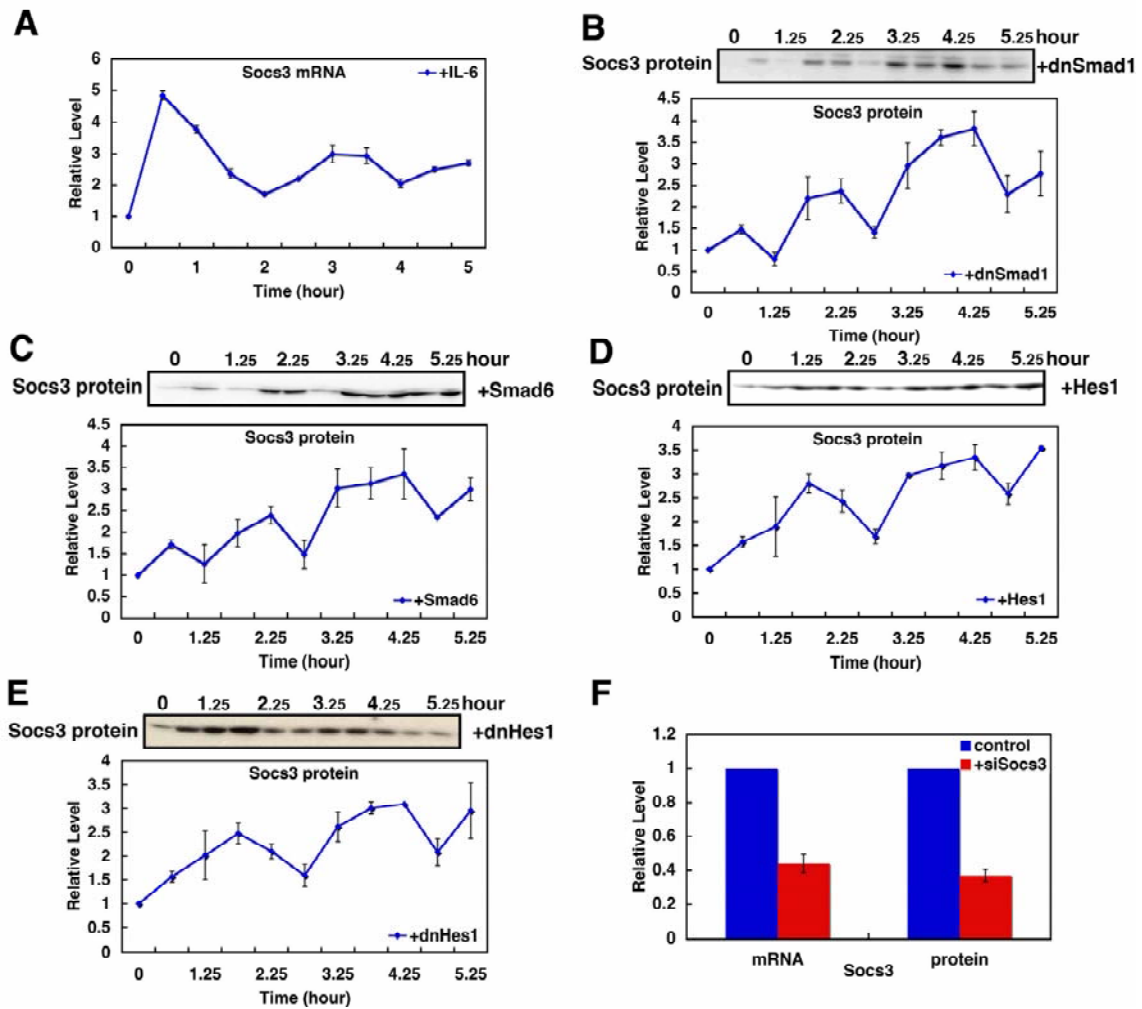
Figure S2. Regulation of Socs3 oscillation. (A) *Socs3* mRNA levels were examined by real-time PCR. *Socs3* mRNA oscillation is induced by IL-6 (100 ng/ml). (B-E) Protein was prepared from fibroblasts after serum stimulation (time = 0) at indicated time points and subjected to western blotting. Socs3 protein oscillation is not affected by dnSmad1 (B), Smad6 (C), Hes1 (D) or dnHes1 (E). (F) Socs3 mRNA and protein expression is suppressed by a specific siRNA for Socs3 but not by a control (siGFP). Values are means ± S.E. of three independent experiments.
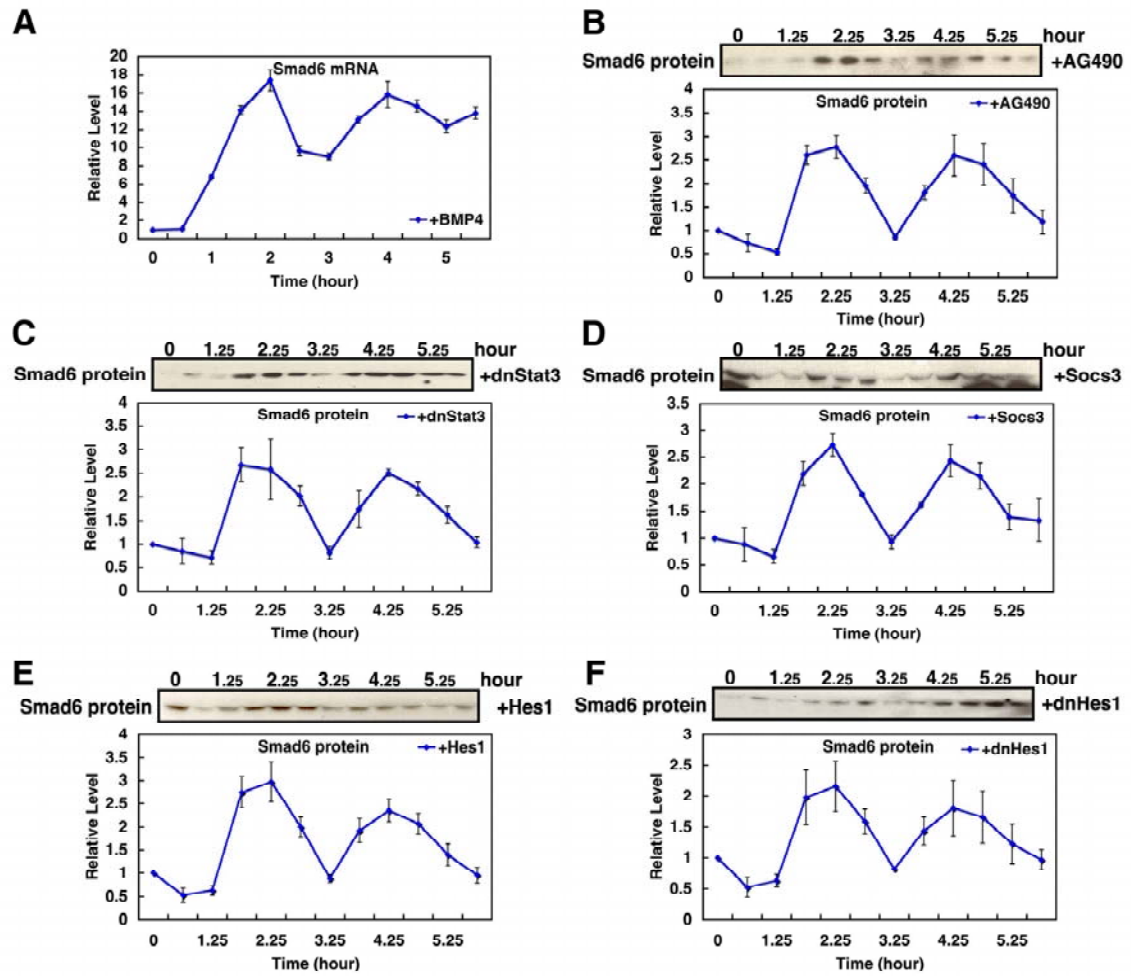
Figure S3. Regulation of Smad6 oscillation. (A) *Smad6* mRNA levels were examined by real-time PCR. *Smad6* mRNA oscillation is induced by BMP4 (100 ng/ml). (B-F) Protein was prepared from fibroblasts after serum stimulation (time = 0) at indicated time points and subjected to western blotting. Smad6 protein oscillation is not affected by AG490 (B), dnStat3 (C), Socs3 (D), Hes1 (E) or dnHes1 (F). Values are means ± S.E. of three independent experiments.
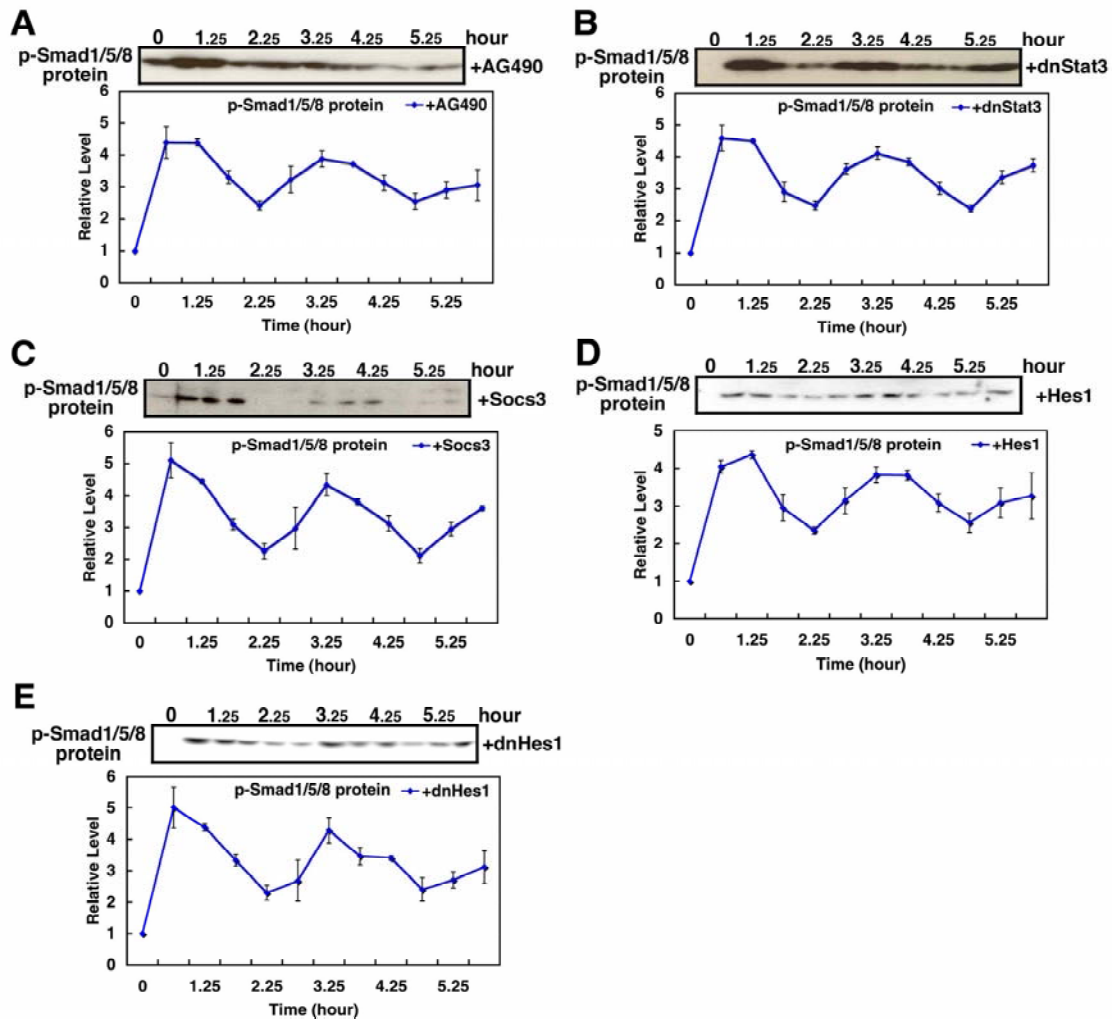
Figure S4. Regulation of p-Smad1/5/8 oscillation. Protein was prepared from fibroblasts after serum stimulation (time = 0) at indicated time points and subjected to western blotting. p-Smad1/5/8 oscillation is not affected by AG490 (A), dnStat3 (B), Socs3 (C), Hes1 (D) or dnHes1 (E). Values are means ± S.E. of three independent experiments.
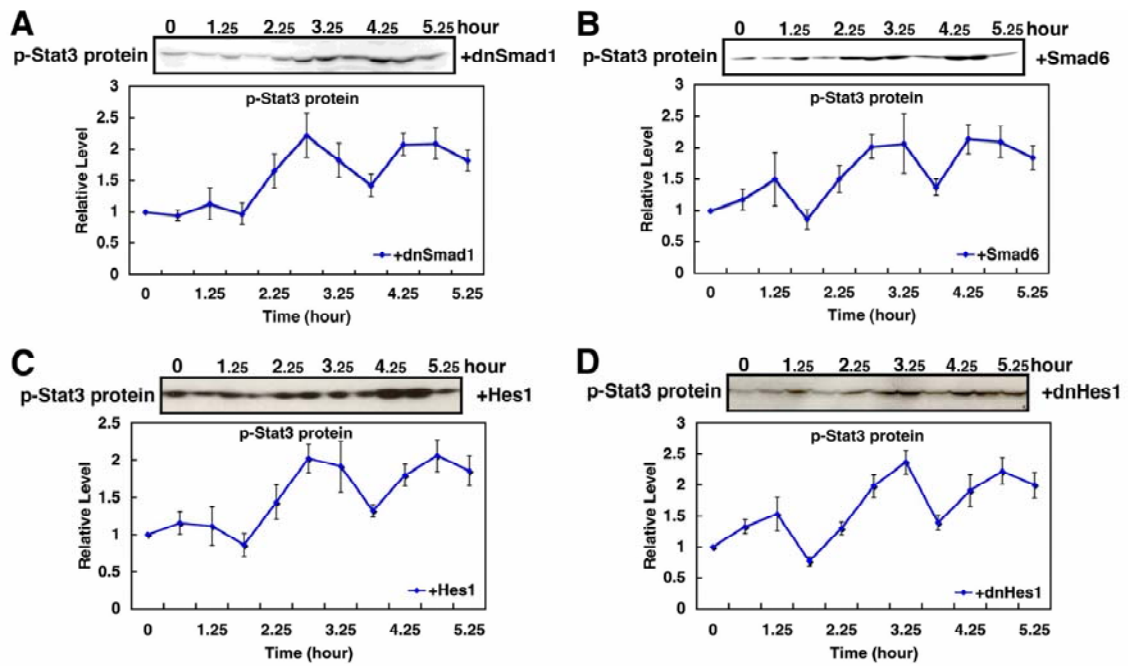
Figure S5. Regulation of p-Stat3 oscillation. Protein was prepared from fibroblasts after serum stimulation (time = 0) at indicated time points and subjected to western blotting. p-Stat3 oscillation is not affected by dnSmad1 (A), Smad6 (B), Hes1 (C) or dnHes1 (D). Values are means ± S.E. of three independent experiments.
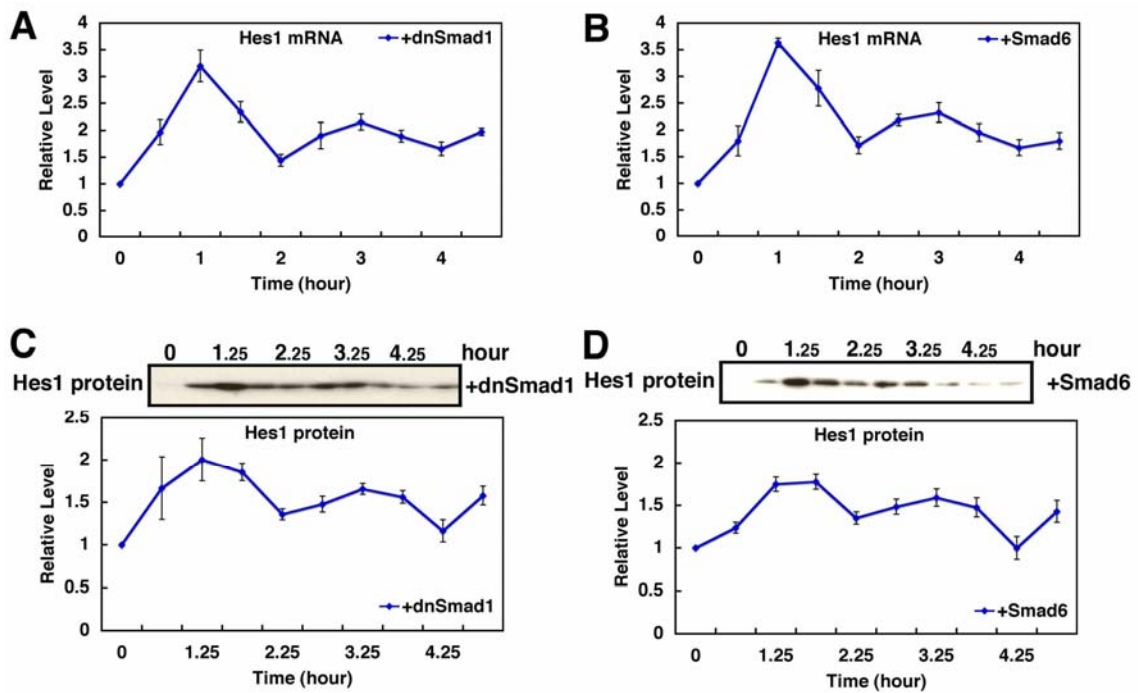
Figure S6. Regulation of Hes1 oscillation. mRNA and protein were prepared at indicated time points from fibroblasts after serum stimulation (time = 0) and subjected to real time PCR and western blotting, respectively. Hes1 mRNA (A, B) and protein oscillations (C, D) are not affected by dnSmad1 (A, C) or Smad6 (B, D). Values are means ± S.E. of three independent experiments.
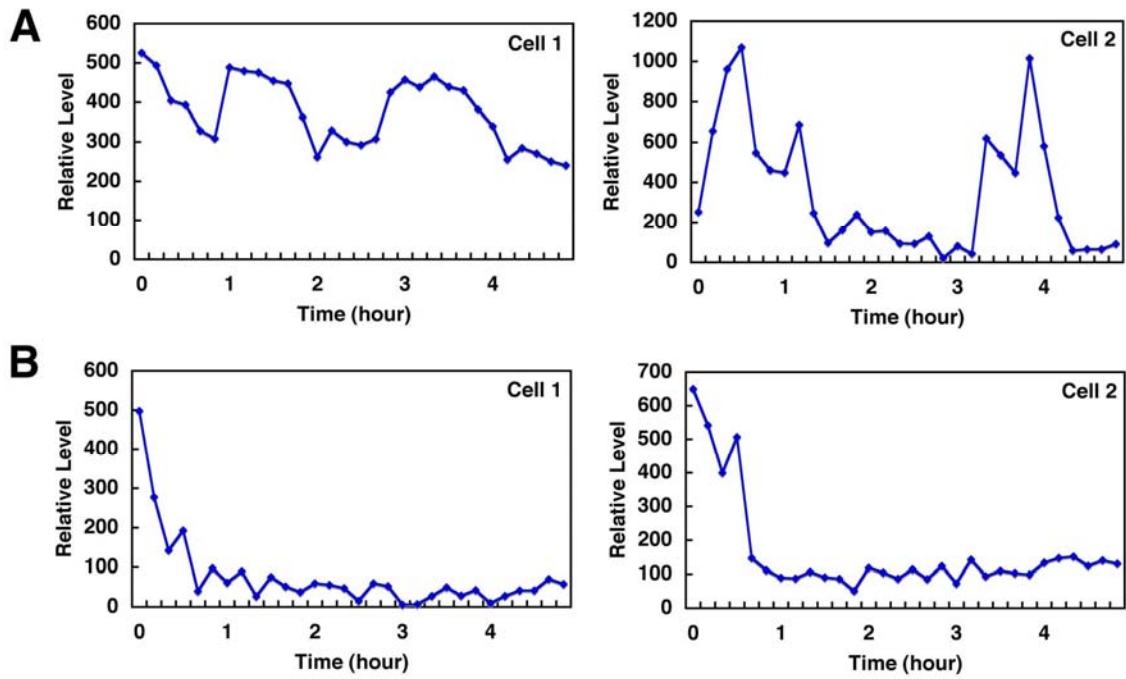
Figure S7. Real-time monitoring of *Hes1* expression at the single cell level. Bioluminescence of fibroblasts carrying the reporter Hes1-Ub2-Luc was quantified in the absence (A, control) or presence of AG490 (B). Two representative cells (among 10 cells analyzed for each condition) are shown for each condition. (A) *Hes1* expression is oscillatory in both cells. (B) *Hes1* oscillation is suppressed by AG490 in each individual cell.
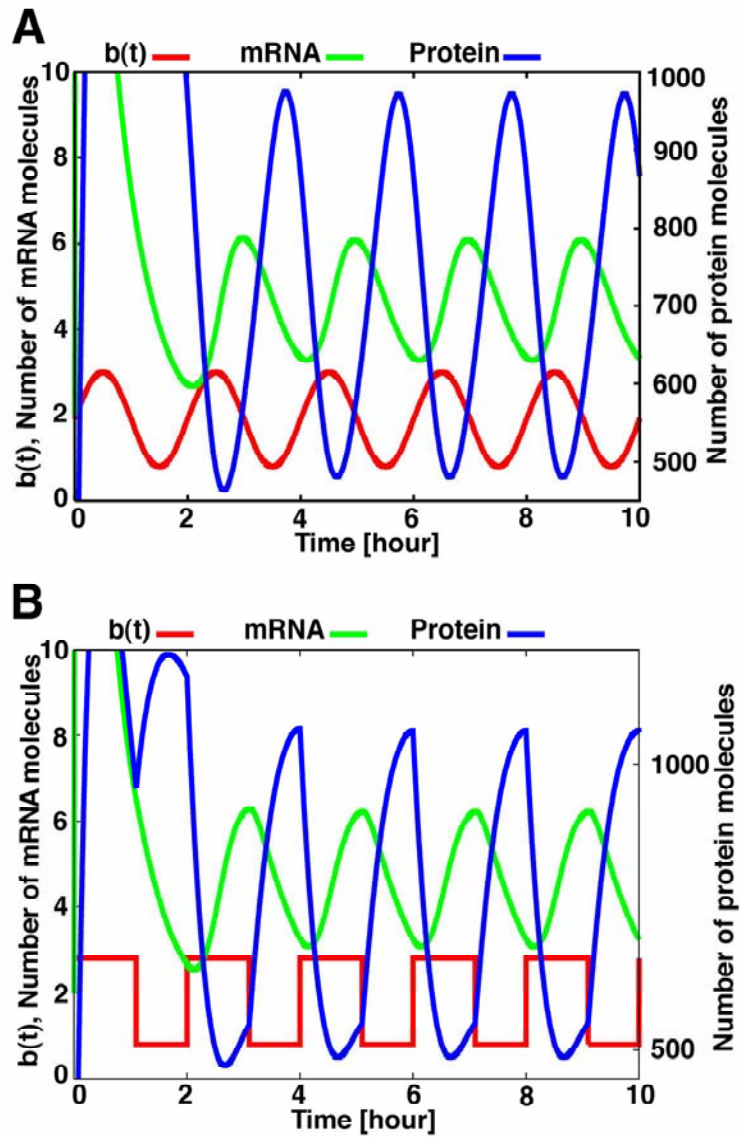
Figure S8. Mathematical modeling of Hes1 oscillation. The green and blue lines show the time courses of the numbers of *Hes1* mRNA [$m(t)$] and Hes1 protein molecules [$p(t)$], respectively, while the red line represents the change of Hes1 protein half-life, $b(t)$, induced by p-Stat3 oscillation. Simulation was performed, according to negative feedback model with sinusoidal change of $b(t)$ in (E3) (A) and with the flip-flop change of $b(t)$ with $w = 1.1$ in (E4) (B).
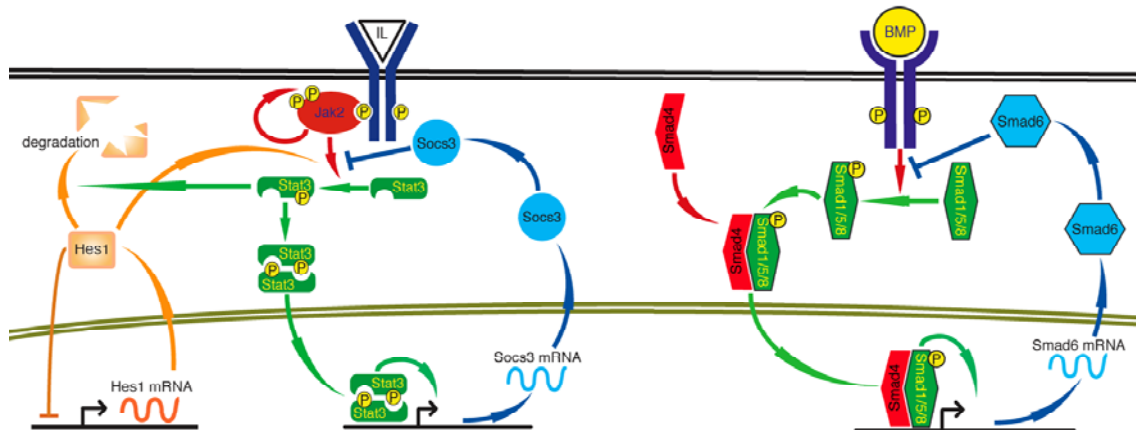
Figure S9. Schematic representation of a model of Jak-Stat-Socs, Smad and Hes1 oscillations. p-Stat3 and Socs3 oscillations depend on each other and are regulated by negative feedback. Similarly, p-Smad1 and Smad6 oscillations depend on each other and are regulated by negative feedback. p-Stat3-Socs3 oscillations control Hes1 oscillation by regulating the Hes1 protein stability, while Hes1 promotes Jak2-dependent phosphorylation of Stat3.