Title	Deubiquitinating enzymes regulate Hes1 stability and neuronal differentiation.
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Citation	The FEBS journal (2015), 282(13): 2411-2423
Issue Date	2015-04-23
URL	http://hdl.handle.net/2433/201615
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Type	Journal Article
Textversion	publisher

Deubiquitinating enzymes regulate Hes1 stability and neuronal differentiation

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Hairy and enhancer of split 1 (Hes1), a basic helix-loop-helix transcriptional repressor protein, regulates the maintenance of neural stem/progenitor cells by repressing proneural gene expression via Notch signaling. Previous studies showed that Hes1 expression oscillates in both mouse embryonic stem cells and neural stem cells, and that the oscillation contributes to their potency and differentiation fates. This oscillatory expression depends on the stability of Hes1, which is rapidly degraded by the ubiquitin/proteasome pathway. However, the detailed molecular mechanisms governing Hes1 stability remain unknown. We analyzed Hes1-interacting deubiquitinases purified from mouse embryonic stem cells using an Hes1-specific antibody, and identified the ubiquitin-specific protease 27x (Usp27x) as a new regulator of Hes1. We found that Hes1 was deubiquitinated and stabilized by Usp27x and its homologs ubiquitin-specific protease 22 (Usp22) and ubiquitin-specific protease 51 (Usp51). Knockdown of Usp22 shortened the half-life of Hes1, delayed its oscillation, and enhanced neuronal differentiation in mouse developing brain, whereas mis-expression of Usp27x reduced neuronal differentiation. These results suggest that these deubiquitinases modulate Hes1 protein dynamics by removing ubiquitin molecules, and thereby regulate neuronal differentiation of stem cells.

Introduction

Hairy and enhancer of split 1 (Hes1) is a member of the basic helix-loop-helix family of transcriptional repressor proteins, and is a downstream effecter of canonical Notch signaling. Hes1 regulates many developmental events by repressing the expression of target genes, including proneural genes that encode transcriptional activators that promote neuronal differentiation [1,2]. Hes1 expression oscillates with a period of 2–3 h in cultured fibroblasts [3] and neural stem/progenitor cells [4]. The expression dynamics of the Hes1 gene

(*Hes1*) play crucial roles in proper developmental timings and fate determination steps in both embryonic stem (ES) cells and neural stem cells [5–12]. Hes1 oscillation dynamically changes the fate preference of ES cells by controlling Notch signaling activity, and contributes to heterogeneous responses of ES cells [5]. In embryonic neural stem cells, Hes1 oscillation drives the oscillatory expression of proneural factors. During neural development, Hes1 expression disappears, while proneural gene expression becomes sustained in differentiating neurons but continues to oscillate in neural stem cells [4]. Sustained Hes1 expression represses the expression of genes involved in cell-cycle progression, suggesting that Hes1 oscillation is important for the proliferation of neural stem cells [4,9].

Hes1 oscillation is regulated by an auto-negative feedback loop [3]. After activation of *Hes1* transcription via Notch signaling or serum stimulation, translated Hes1 represses the expression of its own gene by directly binding to N-box sequences in its promoter. The Hes1 gene products, both mRNA and protein, are very unstable and degraded with a short half-life of approximately 20 min, which enables *Hes1* expression to be restarted in a few hours. Previous reports demonstrated that the instability of Hes1 gene products is critical for its oscillation [3,13], but the detailed mechanism of regulation of Hes1 stability remains to be determined.

Ubiquitination and deubiquitination are well-regulated processes that modulate protein stability and are driven by specific ubiquitin ligases (E3 ligases) and deubiquitinases (DUBs), respectively [14–18]. DUBs are proteases that cleave ubiquitin moieties from ubiquitinated substrates and regulate various cellular responses by controlling substrate abundance and activity [19,20]. Hes1 was shown to be rapidly degraded by the ubiquitin/proteasome system [3], but neither the specific E3 ligases nor the DUBs have been identified. Here, we identified DUBs regulating Hes1 stability by affinity purification of Hes1 from ES cell lysates, followed by LC/MS/MS, and analyzed the functional significance of Hes1 protein stability with respect to its oscillation and neuronal differentiation.

Results

Usp27x and its homologs deubiquitinate Hes1

We analyzed Hes1-interacting DUBs purified from mouse ES cell lysates using Hes1-specific antibody, and identified Usp19 and Usp27x, both of which are ubiquitin-specific cysteine proteases (USPs) [17], by LC/MS/MS. We examined their interactions with Hes1 in HEK293T cells. FLAG-tagged Usp19 and Usp27x, together with hemagglutinin (HA)-tagged Hes1, were expressed and immunoprecipitated using FLAG antibody. Both Usp19 and Usp27x interacted with Hes1 (Fig. 1A, lanes 8 and 9). To determine whether these DUBs remove ubiquitin from Hes1, we co-transfected His_6-tagged Hes1 with both HA-tagged ubiquitin and the corresponding Usp genes in C3H10T1/2 cells, and then purified His_6-tagged Hes1 after treatment with the proteasome inhibitor MG132. Usp27x reduced the amount of ubiquitinated Hes1 (Fig. 1C, lane 4), but Usp19 and the catalytically inactive point mutants of Usp27x, C87A (mt1) and H380A (mt2), did not

Fig. 1. Usp27x and its homologs interact with and deubiquitinate Hes1. (A) Interaction of USPs with Hes1. Each of the FLAG-tagged Usp vectors was co-transfected with HA-tagged Hes1 into HEK293T cells. Cell lysates were immunoprecipitated using FLAG antibody. The whole-cell extracts (input) and immunoprecipitates (IP) were blotted using FLAG antibody (FLAG) and HA antibody (Hes1). Asterisks indicate degradation products of Usp proteins. (B) Interaction between endogenous Hes1 and Usp22. C3H10T1/2 cells were subjected to immunoprecipitation with control rabbit IgG (IgG) or Hes1 antibody (Hes1). The whole-cell extracts (input) and immunoprecipitates (IP) were blotted with Usp22 and Hes1 antibodies. (C) Usp27x and its homologs deubiquitinate Hes1. His_6-tagged Hes1 and HA-tagged ubiquitin were co-transfected with FLAG-tagged USPs into C3H10T1/2 cells. The transfection efficiencies were approximately 20% in all samples. Cells were cultured in the presence of MG132 for 2 h, and lysed in denaturing buffer for purification. His-tagged Hes1 was purified with Ni-NTA agarose beads and detected by western blotting using HA and Hes1 antibodies. (D) Lack of deubiquitination of the Hes1 homolog Hes7. His_6/FLAG-tagged Hes7 was co-transfected with both HA-tagged ubiquitin and each of the USPs into C3H10T1/2 cells, purified with Ni-NTA beads, and blotted with FLAG and HA antibodies. (E) Deubiquitination of endogenous Hes1 by Usp27x. Plasmid encoding Usp27x or shRNA was co-transfected with HA-tagged ubiquitin into C3H10T1/2 cells. Cells were cultured in the presence of 10 μM MG132 (proteasome inhibitor) for 2 h and lysed. Hes1 was immunoprecipitated using Protein A beads cross-linked with Hes1 antibody, and analyzed by western blotting. Rabbit IgG was used as a negative control. (F) Deubiquitination of endogenous Hes1 in *Usp22* knockdown cells. C3H10T1/2 cells were transfected with control or *Usp22*-specific siRNAs. Endogenous Hes1 was immunoprecipitated using Hes1 antibody and analyzed by western blotting. The amount of polyubiquitinated Hes1 was quantified using an LAS3000mini luminescent image analyzer, in which the HA signals were in the linear range, and normalized against that of precipitated Hes1 (bottom). nd, not detected. (G) Deubiquitination of endogenous Hes1 by Usp22. Usp22 plasmid or control vector was co-transfected with HA-tagged ubiquitin into C3H10T1/2 cells, and endogenous Hes1 was analyzed by immunoprecipitation. The molecular masses of Hes1, HA-tagged Hes1, His_6-tagged Hes1 and His_6/FLAG-tagged Hes7 are 29.7, 30.8, 30.7 and 26.9 kDa, respectively.
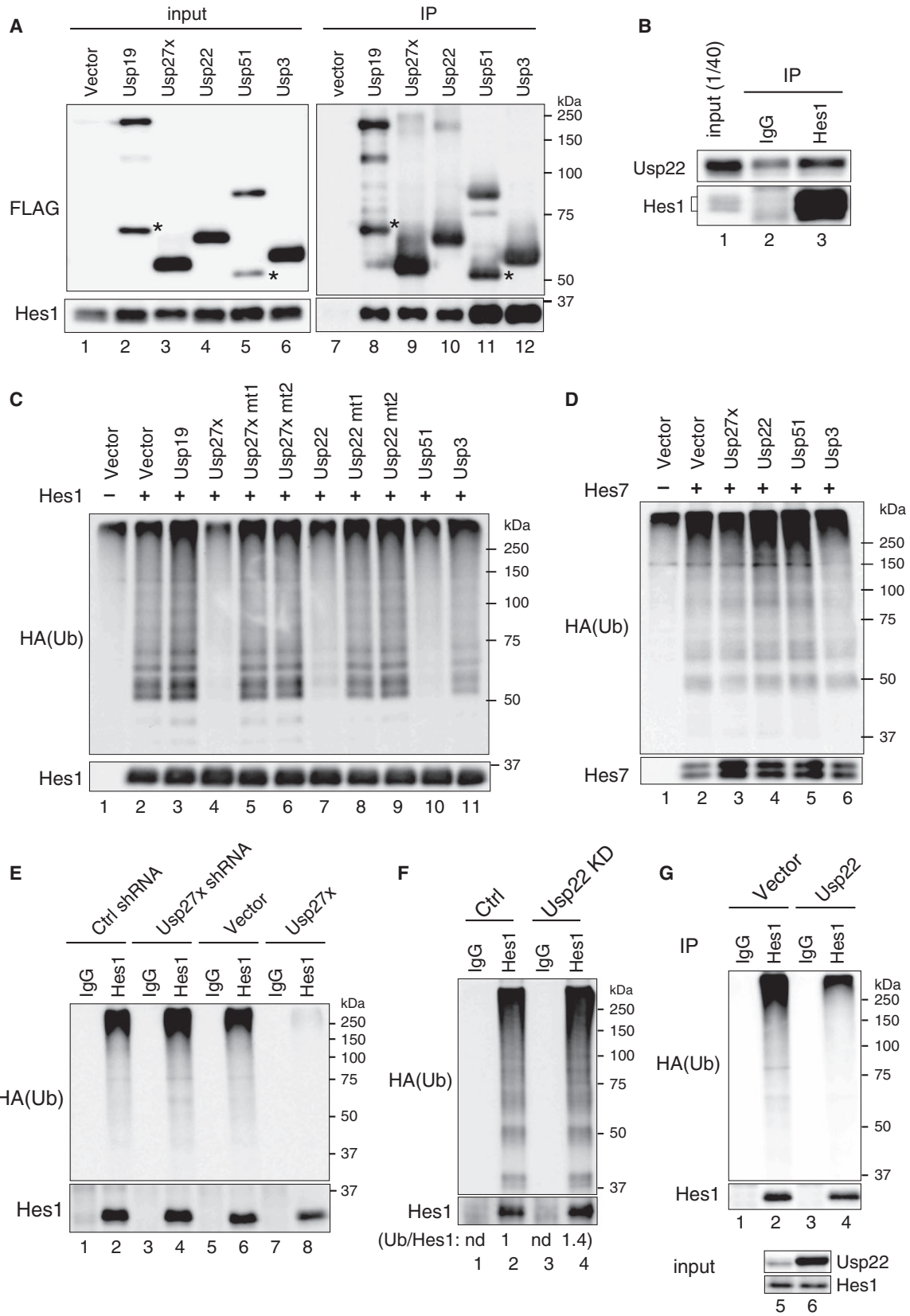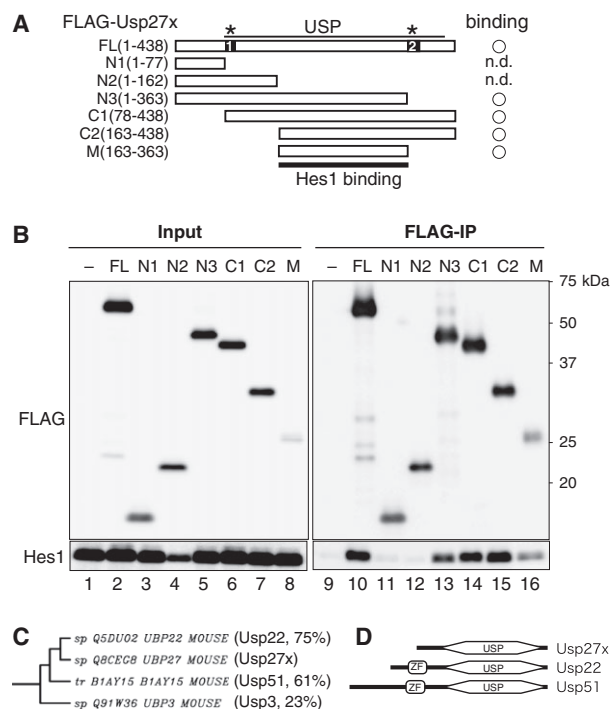
Fig. 2. Interactions of Usp27x and its deletion mutants with Hes1. (A) Schematic representation of Usp27x and its mutants, showing the region to which Hes1 binds. Usp27x has a catalytic USP domain. Hes1 binds to the region between signatures 1 and 2 (asterisks) in the USP domain. n.d., not detected. (B) Co-immunoprecipitation of Hes1 with Usp27x deletion mutants. HA-tagged Hes1 was co-transfected with FLAG-tagged Usp27x and its deletion mutants into HEK293T cells; their interactions were then analyzed by immunoprecipitation with FLAG antibody and western blotting using FLAG and HA antibodies. (C) Sequence alignment of Usp27x homologs. Part of the phylogenetic tree generated by ClustalW [39] from all deubiquitinases in mouse, with their identity scores relative to Usp27x in parentheses (%). (D) Schematic representation of the domains in Usp27x, Usp22 and Usp51. ZF, zinc finger.

(Fig. 1C, lanes 3, 5 and 6). We also examined the activities of the Usp27x homologs Usp22, Usp51 and Usp3, which have homologous USP domains (Fig. 2C, D). Immunoprecipitation analyses showed that all of these homologs interacted with Hes1 (Fig. 1A, lanes 10–12). Furthermore, Usp22 and Usp51 reduced the amount of ubiquitinated Hes1 (Fig. 1C, lanes 7 and 10). However, a high level of ubiquitinated Hes1 remained in Usp3-expressing cells (Fig. 1C, lane 11). The catalytically inactive point mutants of Usp22, C185A (mt1) and H479A (mt2), were also unable to remove ubiquitin from Hes1 (Fig. 1C, lanes 8 and 9). These results indicate that Usp27x, Usp22 and Usp51, but not Usp19 or Usp3, act as Hes1 DUBs, and that their catalytic activities are required to remove poly-ubiquitin from Hes1.

We next examined whether Usp27x, Usp22 and Usp51 have DUB activity specific to Hes1 or also act on other Hes family proteins. FLAG-tagged Hes7 and HA-tagged ubiquitin were co-expressed with or without the various DUBs. Neither Usp27x nor its homologs reduced the large amount of ubiquitinated Hes7, a homolog of Hes1 (Fig. 1D, lanes 3–6), compared with the control (lane 2). We further analyzed ubiquitination of endogenous Hes1 by immunoprecipitation from C3H10T1/2 cells transfected with HA-tagged ubiquitin. Over-expression of Usp27x reduced the level of ubiquitinated endogenous Hes1 (Fig. 1E, lane 8). However, knockdown of Usp27x by shRNA (79.5% efficiency of knockdown as determined by quantitative PCR) did little to alter the amount of ubiquitinated Hes1 (lane 4), suggesting that Usp27x activity is compensated for by its homologs. These results indicate that Usp27x, Usp22 and Usp51 are able to specifically deubiquitinate Hes1, and suggest that these Usp27x homologs compensate for the functions of one another.

We next sought to determine the region in Usp27x that is required for the interaction with Hes1. Co-immunoprecipitation of Hes1 with Usp27x deletion mutants revealed that Usp27x interacts with Hes1 at the conserved USP domain (Fig. 2A,B, lanes 13–16) [17]. Because Usp22 and Usp51 also contain the USP domain (Fig. 2D), these DUBs may recognize poly-ubiquitinated Hes1 and interact with Hes1 via this domain.

Usp27x and its homologs stabilize Hes1

To examine whether Usp27x and its homologs stabilize Hes1 by deubiquitinating it, we monitored protein levels of HA-tagged Hes1 in the presence of the translation inhibitor cycloheximide. Over-expression of wild-type Usp27x stabilized HA-tagged Hes1 (Fig. 3A, lanes 4–6), but the inactive mutants of Usp27x did not increase the half-life of HA-tagged Hes1 (lanes 7–9 and 10–12). Usp22 stabilized HA-tagged Hes1, but the catalytically inactive mutants of Usp22 did not (Fig. 3B, lanes 1–3 and lanes 4–9). Usp51 stabilized HA-tagged Hes1, but Usp3 did not (Fig. 3C, lanes 5–8 and lanes 9–12). These results indicate that the catalytic activities of Usp27x, Usp51 and Usp22 are important for stabilizing Hes1 protein, and that Usp3 does not affect the stability of Hes1. The catalytically inactive point mutants Usp27x mt1 and Usp27x mt2 slightly decreased the half-life of HA-tagged Hes1 compared with the control, suggesting that these mutants may destabilize Hes1 by inhibiting interaction with the endogenous DUBs (Fig. 3A).
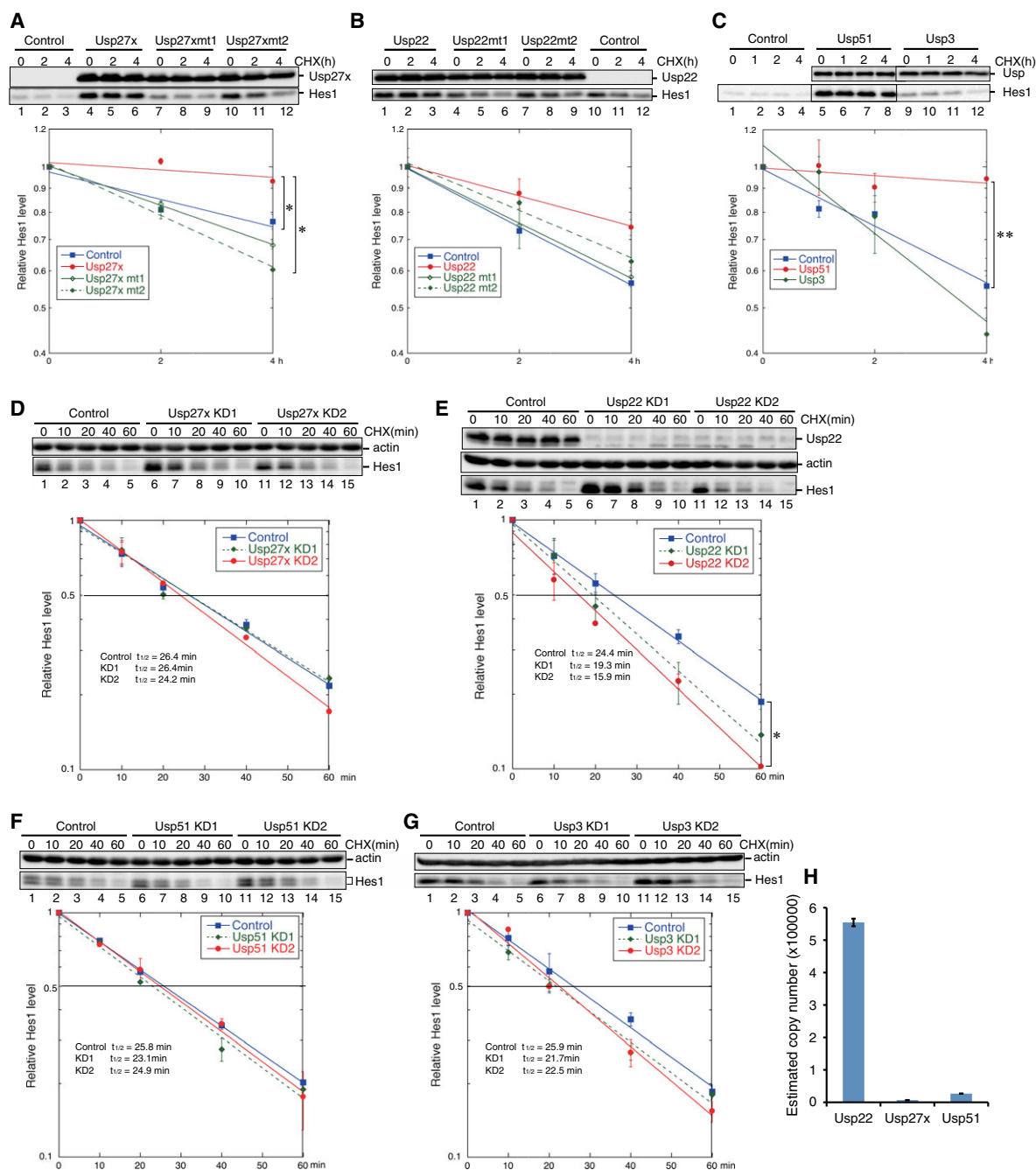
Fig. 3. Usp27x and its homologs stabilize Hes1 protein. (A) Usp27x stabilizes Hes1. HA-tagged Hes1 was co-transfected with FLAG-tagged Usp27x or its derivatives into C3H10T1/2 cells. Hes1 stability was analyzed by a cycloheximide (CHX) chase assays. Protein levels were analyzed by western blotting with HA antibody (upper panels), and the relative amounts of Hes1 were quantified (lower panel). (B,C) Usp27x homologs stabilize Hes1. HA-tagged Hes1 was co-transfected with FLAG-tagged Usp51, Usp3, Usp22 or Usp22 mutants into C3H10T1/2 cells. Hes1 degradation was analyzed as in (A). (D–G) *Usp27x*, *Usp22*, *Usp51* and *Usp3* knockdown. C3H10T1/2 cells were transfected with control, or siRNAs specific to *Usp27x* (D), *Usp22* (E), *Usp51* (F) or *Usp3* (G). Protein levels were analyzed by western blotting with Hes1 and actin antibodies, and quantified. The relative amounts of Hes1 were normalized against the amounts of actin. The half-life of Hes1 protein was estimated from exponential curves fitted using Kaleidagraph software. (H) Estimated copy numbers of Usp22, Usp27x and Usp51. The copy numbers of *Usp22*, *Usp27x* and *Usp51* mRNA in 50 ng total RNA of C3H10T1/2 cells were determined by quantitative PCR with known amounts of plasmids encoding these factors. Values are means ± SEM from two independent experiments. Asterisks indicate statistically significant differences (*$P < 0.05$, **$P < 0.01$; Student's *t* test).

We next examined the effects of DUB knockdown on the protein stability of endogenous Hes1. We analyzed the knockdown of *Usp27x*, and its homologs *Usp22*, *Usp51* and *Usp3*. Knockdown of *Usp22* using two distinct siRNA sequences (KD1 and KD2) significantly shortened the half-life of endogenous Hes1 from 24.4 min to 19.3 min (KD1) and 15.9 min (KD2) (Fig. 3E). The shortened half-lives correlated with the knockdown efficiency (79% in KD1 and 85% in KD2). However, knockdown of *Usp27x*, *Usp51* and *Usp3* using two siRNA sequences each did not have any significant effect on the half-life of endogenous Hes1, although the knockdowns were highly effective: 93.8% (KD1) and 89.8% (KD2) for Usp27x, 80.6% (KD1) and 93.9% (KD2) for Usp51, and 97.3% (KD1) and 94.2% (KD2) for Usp3 (Fig. 3D–G). We found that C3H10T1/2 cells expressed abundant Usp22 but little Usp27x or Usp51 (an approximately 100-fold difference between Usp22 and the others, Fig. 3H). Endogenous Hes1 interacted with endogenous Usp22 (Fig. 1B, lane 3), and Usp22 knockdown increased the amount of polyubiquitinated endogenous Hes1 (Fig. 1F, lanes 2 and 4). Over-expression of Usp22 reduced the level of ubiquitinated endogenous

Hes1 (Fig. 1G, lanes 2 and 4). These results suggest that Usp27x, Usp22 and Usp51 are able to stabilize Hes1 protein, and that Usp22 is the main enzyme stabilizing endogenous Hes1 in C3H10T1/2 fibroblast cells.

Usp22 depletion modulates Hes1 protein oscillation

To examine whether Usp22 modulates Hes1 oscillation in C3H10T1/2 fibroblast cells, we analyzed temporal changes in endogenous Hes1 mRNA and protein every 30 min after serum stimulation (Fig. 4A,B). *Hes1* mRNA first peaked 1 h after serum treatment (Fig. 4A), and Hes1 protein peaked at 1.25 h (Fig. 4B, C, lane 3), which is 15 min after the peak of *Hes1* mRNA, as described previously [3]. Knockdown of *Usp22* did not change the timings of the first peaks of either mRNA or protein, indicating that the rates of both transcription and translation of *Hes1* are unaffected in *Usp22* knockdown cells. On the other hand, the second peaks of both mRNA and protein were affected in *Usp22* knockdown cells. The second peak of *Hes1* mRNA occurred at 3 h in control cells and
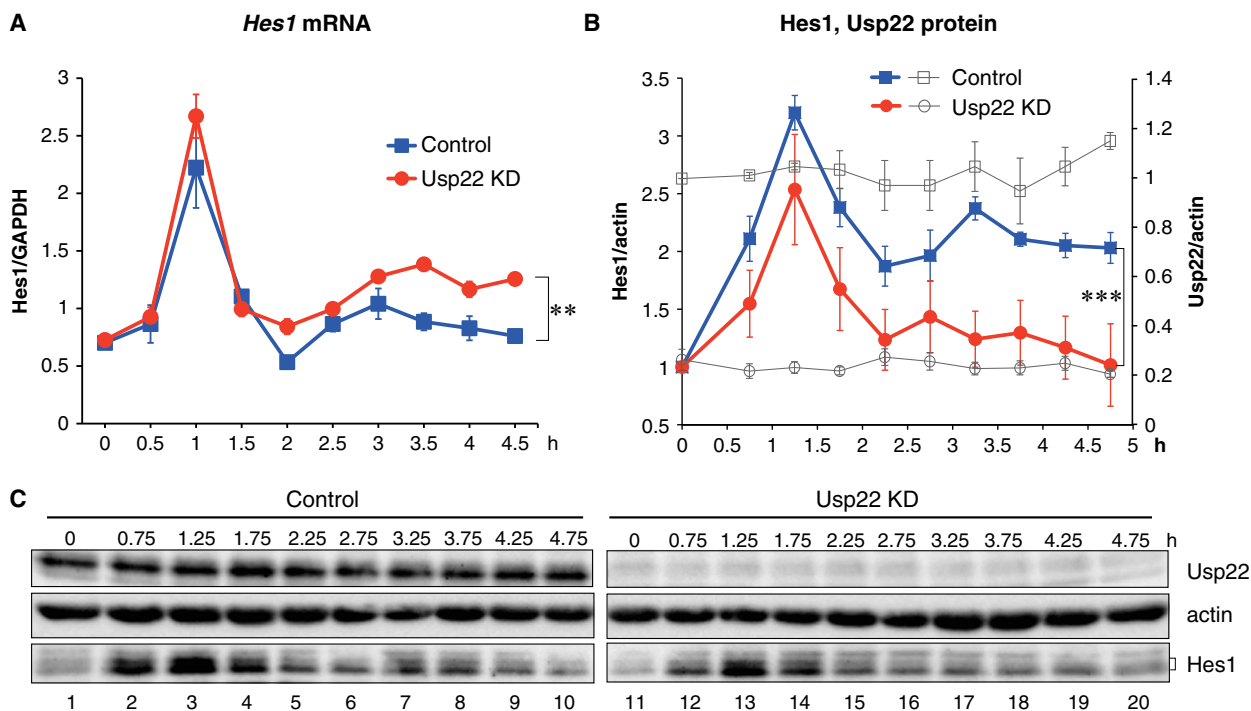

Fig. 4. Effect of *Usp22* knockdown on Hes1 oscillation. (A,B). Hes1 oscillation was induced by serum stimulation in C3H10T1/2 cells. Levels of *Hes1* mRNA (A) and Hes1 and Usp22 proteins (B) were measured every 30 min after serum stimulation by quantitative PCR and western blotting, respectively. Values are means ± SEM of four (A) and three (B) independent experiments. Asterisks indicate statistically significant differences (**$P < 0.01$, ***$P < 0.001$; Student's *t* test and two-way ANOVA). (C) Representative blots of Usp22, Hes1 and actin. There are two bands for Hes1 protein because of modification. The knockdown efficiency of *Usp22* mRNA was 96.2%.

3.5 h in *Usp22* knockdown cells (Fig. 4A). The second peak of Hes1 protein was observed at 3.25 h in control cells, but Usp22 depletion weakened the oscillation such that no clear second peak of protein was observed (Fig. 4B,C, lane 7 and lanes 16–20). Usp22 was stably expressed after serum treatment (Fig. 4B, C). Although Usp22 is reported to regulate gene transcription by removing ubiquitin from histones H2B, H2A and others [21–26], we found that level of *Hes1* mRNA was unaffected by *Usp22* knockdown before serum stimulation ($t = 0$). After the first peak ($t = 1$ h), the level of *Hes1* mRNA was higher in

Usp22 knockdown cells than in control cells (Fig. 4A). However, Hes1 protein levels were lower in *Usp22* knockdown cells (Fig. 4B,C). These findings suggest that *Usp22* knockdown decreases the level of Hes1 protein and thereby weakens its own auto-inhibition, which results in delay and up-regulation of the second peak of *Hes1* mRNA, and dampening of Hes1 oscillation at the population level.

We also examined Hes1 oscillation periods in individual cells by real-time imaging, using a destabilized luciferase reporter gene under the control of the *Hes1* promoter in NIH3T3 fibroblast cells (Fig. 5A). This
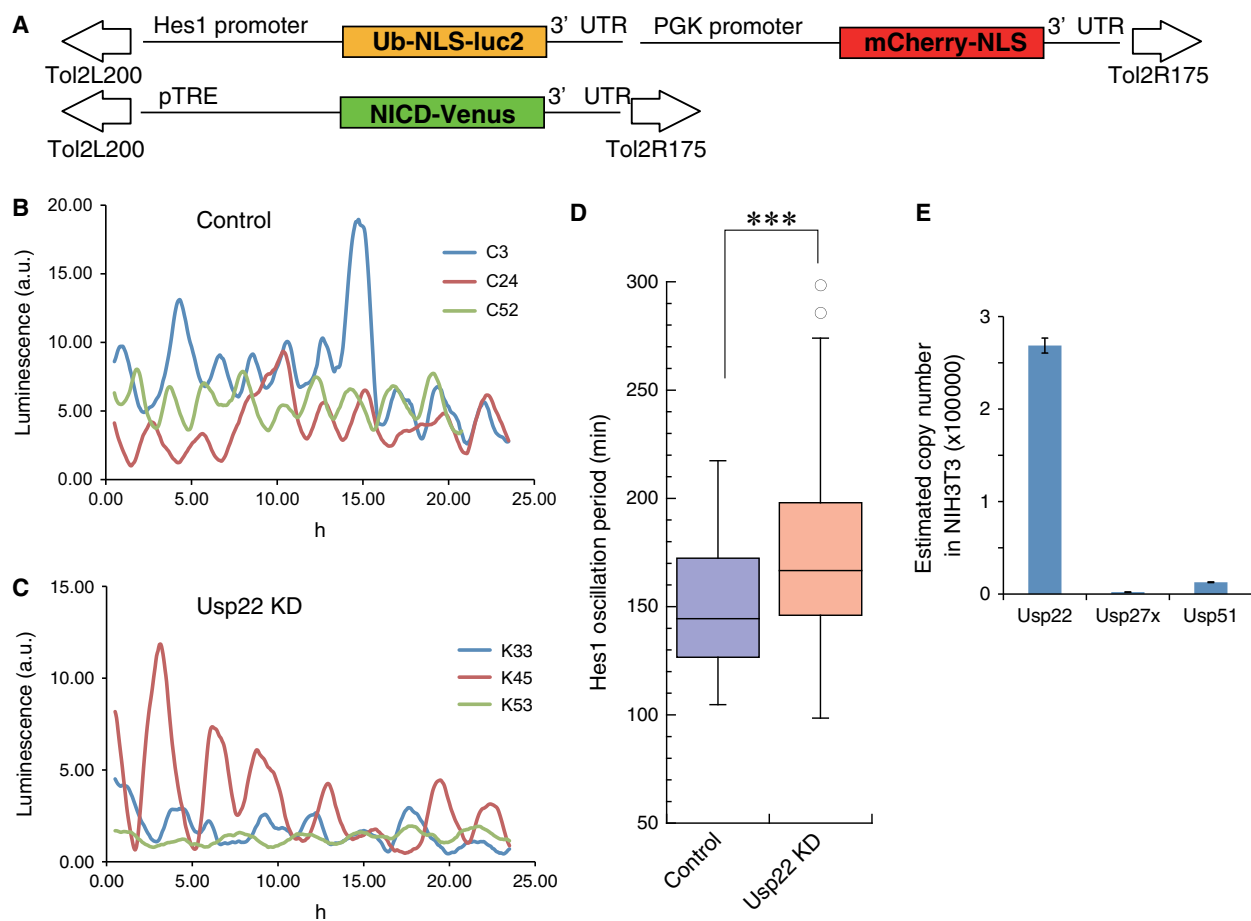

Fig. 5. The period of *Hes1* oscillation in individual fibroblast cells. (A) Schematic structures of reporter constructs used to establish the stable cell line for real-time imaging. The reporters are flanked by the Tol2 transposon sequence (L200 and R175). Cells with these transgenes were selected by fluorescence-activated cell sorting of mCherry-expressing cells. Continuous expression of the Notch intracellular domain (NICD) is required to induce *Hes1* expression in NIH3T3 cells. (B,C) Quantification of *Hes1* expression dynamics in individual cells. Hes1 expression was quantified by real-time imaging of the *Hes1* promoter-driven luciferase reporter in individual control cells (B) and *Usp22* knockdown cells (C) for 24 h. Representative plots for three individual cells of each type are shown. a.u., arbitrary units. (D) Oscillation periods of *Hes1*. The oscillation periodicity of the *Hes1* promoter-driven luciferase reporter in individual cells was estimated by Fourier transform using the maximum entropy method [5]. The results are displayed as box plots for oscillation periods of the bioluminescence in control cells ($n = 74$) and *Usp22* knockdown cells ($n = 73$). Asterisks indicate a statistically significant difference (***$P < 0.001$; Student's *t* test). The mean oscillation periods were 151.2 min in control cells and 175.8 min in *Usp22* knockdown cells. Circles indicate outliers. (E) Estimated copy numbers of Usp22, Usp27x and Usp51 in this cell line. Values are means ± SEM for two independent experiments.

reporter is able to monitor the timing of *Hes1* transcription [27]. As found for C3H10T1/2 cells, NIH3T3 cells expressed abundant Usp22 but little Usp27x and Usp51 (Fig. 5E). We found that the oscillation periods of individual cells were elongated by approximately 25 min, with greater variations in *Usp22* knockdown cells compared with those in control cells (Fig. 5B–D). The knockdown efficiency of Usp22 was 81.8% in this cell line. These results suggest that Hes1 was destabilized by *Usp22* knockdown, resulting in the delay of *Hes1* oscillation in *Usp22* knockdown cells, and that Usp22 contributes to control of the post-translational delay in Hes1 oscillation by inhibiting its rapid degradation. Elongated periods with greater variations probably make the oscillation out of synchrony between cells and thereby dampen Hes1 oscillation at the population level (Fig. 4).

Usp22 and Usp27x regulate neuronal differentiation of stem cells in the developing mouse neocortex

Hes1 is highly expressed in neural stem cells, and its oscillation plays a crucial role in neuronal differentiation of the developing brain [1,4,9]. Neural stem cells and intermediate progenitors exist in the ventricular zone (VZ) and the sub-ventricular zone (SVZ), respectively, while differentiated neurons migrate out into the cortical plate via the intermediate zone during neural development. Previous studies showed that mis-expression of Hes1 in neural stem cells inhibits neuronal differentiation, and the transfected cells are retained in the VZ [28]. We examined the function of Hes1 DUBs *in vivo* following *in utero* electroporation of knockdown or mis-expression vectors. This was performed by co-electroporation with nuclear-localizing GFP into neural stem cells of the VZ in embryonic day (E)13.5 mouse brain. The electroporated cells were examined at E15.5. In control samples, the majority of GFP-positive cells were present in the VZ or the SVZ. Approximately 20% of GFP-positive cells had migrated and were present in the intermediate zone and the cortical plate, which were positive for the neuronal marker neuron-specific class III β-tubulin (TUJ-1) (Fig. 6A–E). *Usp22* knockdown led to a significant increase in the number of electroporated cells in the intermediate zone and the cortical plate (42.2%, representing a 24.1% absolute increase compared with the control), but a significant decrease in the progenitor cell population in the VZ and SVZ (Fig. 6A,B,E). However, mis-expression of *Usp22* did not have significant effects on neuronal differentiation (data not shown), probably because *Usp22* was abundantly expressed in the developing brain (Fig. 6F). On the other hand, mis-expression of *Usp27x*, which was not expressed in the developing brain (Fig. 6F), significantly reduced the number of electroporated cells in the intermediate zone and the cortical plate (1.7%, representing a 17.9% decrease compared with the control), and most GFP-positive cells remained in the VZ and SVZ as undifferentiated cells (negative for TUJ-1) (Fig. 6C–E). These data suggest that both Usp22 and Usp27x negatively regulate neuronal differentiation *in vivo*.

Discussion

In this study, we identified new regulators of Hes1 stability and function. We found that Hes1 was deubiquitinated and stabilized by the DUBs Usp27x, Usp22 and Usp51. These DUBs physically interact with Hes1 and remove ubiquitin molecules from Hes1 by their catalytic activities. Over-expression of these DUBs stabilizes Hes1 and increases its half-life. Knockdown of *Usp22* significantly shortens the half-life of endogenous Hes1, suggesting that Usp22 is the main DUB for Hes1. Moreover, *Usp22* knockdown affects Hes1 oscillation and enhances neuronal differentiation in the mouse developing brain, while Usp27x mis-expression maintains the progenitor state. These results suggest that deubiquitination regulates Hes1 functions by stabilizing it *in vivo*.

We first identified Usp27x as an interacting partner of Hes1 in ES cells. However, *Usp27x* knockdown did not significantly alter either the half-life or the poly-ubiquitination level of endogenous Hes1 in fibroblasts (Figs 1E and 3D), and we did not detect any significant phenotypes in *Usp27x* knockout mouse embryos (data not shown). Usp22 is required for embryonic development and is strongly expressed in the mouse embryonic brain (Fig. 6F) [23,29]. We observed that the levels of both Usp27x and Usp51 were much lower than that of Usp22 in fibroblast cells and the mouse developing brain. These results suggest that Usp22 may compensate for the loss of its homologs.

Hes1 is reported to be important for maintenance of the undifferentiated state of both tumor cells and quiescent cells [30,31]. *Usp22* has been identified as a member of an 11-gene cancer stem cell signature, and may stabilize Hes1 levels in cancer stem cells and inhibit their differentiation in malignant tumors [32,33]. Recently, Usp22 was reported to be the transcriptional repressor of the SRY (sex-determining region Y)-box 2 gene (*Sox2*) in ES cells, whereby Usp22 is required for efficient differentiation into all three germ layers [25]. Sox2 is also expressed in neural stem cells and is
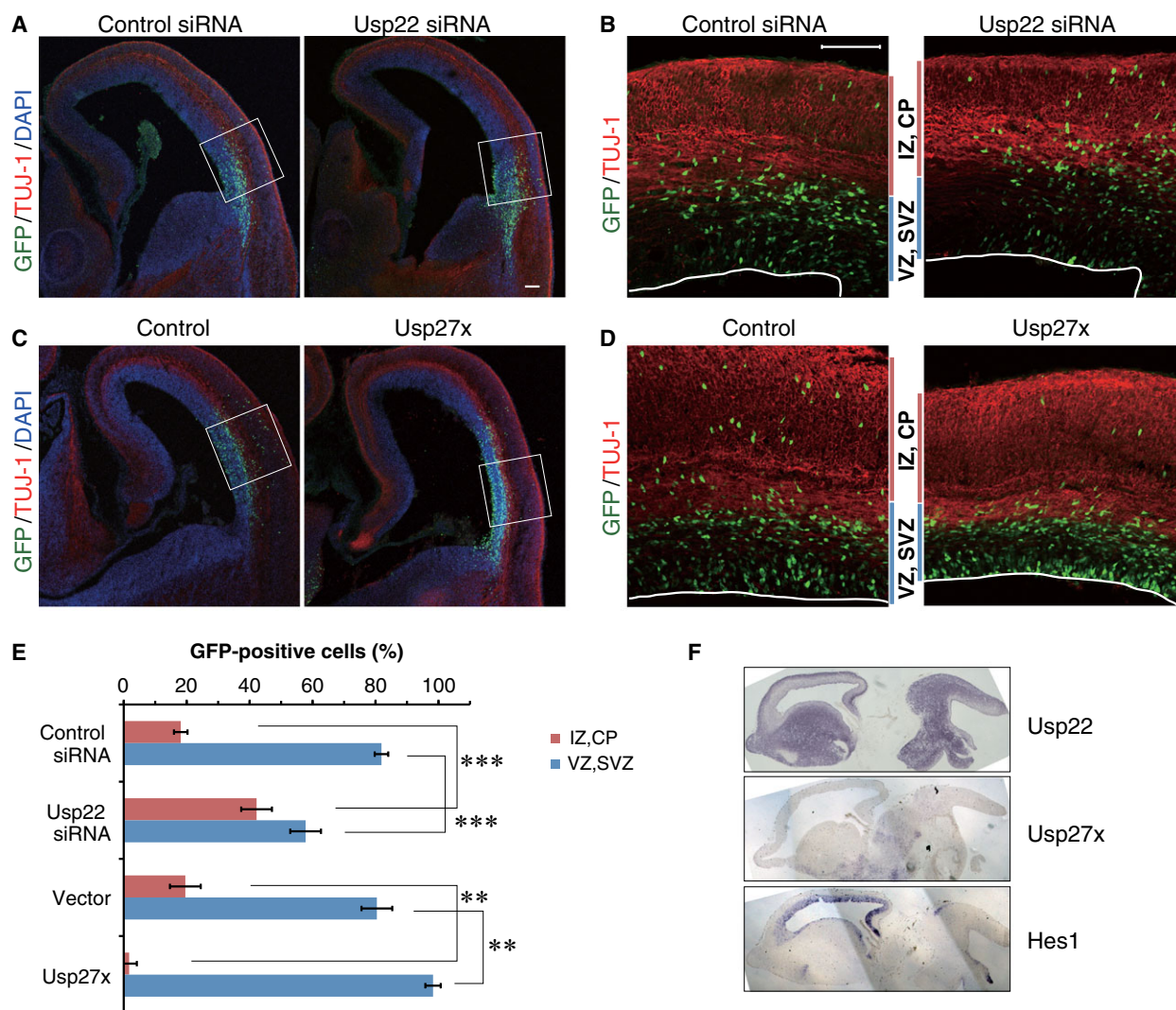
Fig. 6. Usp27x and Usp22 repress neuronal differentiation in the developing brain. (A,B) *Usp22* knockdown in neural stem cells. Control siRNA or *Usp22* siRNA were electroporated with the nuclear-localizing GFP vector into the neocortex of E13.5 mouse embryos in the same mother uterus, and harvested at E15.5. Sections were stained with GFP antibody (green), TUJ-1 antibody (red) and 4′,6-diamidino-2-phenylindole (DAPI) (blue). (C,D) Mis-expression of Usp27x in neural stem cells. Control vector or Usp27x expression vector were electroporated as described in (A). Boxed regions in (A) and (C) are enlarged in (B) and (D), respectively. White lines in (B) and (D) indicate the surface of the ventricle. Scale bars = 100 μm. (E) Quantification of cell distribution, showing the percentage of GFP-positive cells in various cortical layers (CP, cortical plate; IZ, intermediate zone; MZ, marginal zone; SVZ, sub-ventricular zone; VZ, ventricular zone). Values are means ± SEM from four independent experiments. Asterisks indicate statistically significant differences (***$P < 0.001$, **$P < 0.01$; Student's *t* test). Two embryos and at least two sections from each embryo were analyzed for each independent experiment. (F) Expression of *Hes1*, *Usp22* and *Usp27x* in the developing brain. *In situ* hybridization of *Hes1*, *Usp22* and *Usp27x* in sagittal brain sections of E13.5 mouse embryo. Sections were hybridized with dioxygenin-labeled antisense RNA probes, and stained using the nitro blue tetrazolium 5-bromo-4-chloro-3-indolyl-phosphate detection system as described previously [28]. Sequence information for the RNA probes is available upon request.

required for maintenance of neural stem/progenitor cells and their neuronal differentiation [34]. It is possible that Usp22 also regulates expression of other genes required for neural stem cell maintenance at the transcriptional and post-translational levels, and maintains the stem cell pool. Further studies are required to clarify the mechanisms of the detailed regulation by

Usp22 for neuronal differentiation in the developing brain.

Our results demonstrate that Usp22 regulates the oscillation period of Hes1. *Usp22* knockdown elongates the period of *Hes1* oscillation in fibroblast cells. The first peaks of *Hes1* mRNA and protein occur at the same time points in both control and *Usp22*

knockdown cells after serum stimulation, indicating that the rates of *Hes1* transcription and translation are not affected by Usp22 depletion. However, the second peak of *Hes1* mRNA was delayed, and Hes1 protein gradually decreased, resulting in damped oscillation (Fig. 4A,B). Single cell imaging revealed that the oscillation periods of individual cells were elongated with greater variations by *Usp22* knockdown compared with those of control (Fig. 5C,D). These results suggest that Usp22 depletion destabilizes Hes1 protein and leads to more rapid degradation, which elongates the delay of auto-repression and slows or dampens oscillation. Thus, deubiquitination of Hes1 modulates auto-regulation of its own gene expression and thereby regulates the dynamics of oscillatory expression.

In the developing central nervous system, stable Hes1 oscillation is required to maintain neural stem cells; Hes1 oscillation represses the proneural genes required to promote neuronal differentiation, expression of some of which has been reported to oscillate in neural stem cells [4,9]. During neuronal differentiation, Hes1 oscillation is damped, which causes up-regulation of proneural genes and directs the neural stem cells toward neuronal differentiation. Usp22 depletion elongates the Hes1 oscillation period or dampens its oscillation, which may give neural stem cells more opportunity to express proneural genes continuously, resulting in a significant increase in differentiating cells. On the other hand, Usp27x mis-expression inhibited neuronal differentiation of progenitor cells, suggesting that deubiquitination of Hes1 allows Hes1 to suppress proneural genes continuously. To our knowledge, this is the first report demonstrating that post-translational modification of Hes1 modulates its oscillation and function *in vitro* and *in vivo*.

Experimental procedures

Cell culture and transfection

C3H10T1/2 mouse fibroblast cells, NIH3T3 cells and HEK293T cells were grown in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. To examine Hes1 oscillation, C3H10T1/2 cells cultured in Dulbecco's modified Eagle's medium with 5% fetal bovine serum were stimulated with the same medium (5% fetal bovine serum) at $t = 0$ as described previously [35]. For half-life measurement, 20 μg/mL cycloheximide (Sigma, St. Louis, MO, USA) was added to the culture medium at $t = 0$. Plasmids and siRNA were transfected with polyethyleneimine (Polysciences, Inc., Warrington, PA, USA) and Lipofectamine RNAiMAX (Invitrogen, Life Technologies, Carlsbad, CA, USA), respectively, according to the manufacturers' instructions. siRNA

duplexes of negative control, Silencer® Select Validated siRNA and MISSION® predesigned were purchased from Ambion (Life Technologies) or Sigma.

Plasmids

We cloned DUBs from cDNA of mouse MG1.19 ES cells into the expression vector under the control of the human elongation factor promoter. HA-tagged Hes1, His_6-tagged Hes1, His_6/FLAG-tagged Hes7 and HA-tagged ubiquitin were cloned into the pCI vector (Promega, Madison, WI, USA). Expression of Usp27x shRNA was driven by the h7SK promoter in the puromycin resistance plasmid [35]. The knockdown sequence for Usp27x shRNA was 5′-GGCGCAAGATCACTACGTACATT-3′.

Measurement of Hes1 protein and mRNA

For protein analysis, each sample was lysed in lysis buffer (0.5% Nonidet P-40 (Nacalai Tesque, Kyoto, Japan), 100 mM NaCl, 5 mM $MgCl_2$, 10 mM imidazole, 50 mM Tris/HCl, pH 8.0) at the time points indicated in figures, and 10–20 μg total protein at each time point was analyzed by western blotting with Hes1 antibody (1:2000; a kind gift from Tetsuo Sudo, TORAY Industries Inc., Tokyo, Japan), Usp22 antibody (1:2000; Novus Biologicals, Littleton, CO, USA) and actin antibody (1:2000; Sigma); actin was used as a loading control. The bands of Hes1, actin and Usp22 were quantified using a LAS3000mini luminescent image analyzer (GE Healthcare UK Ltd, Buckinghamshire, UK), and the intensities of Hes1 and Usp22 were divided by that of actin to calculate relative levels. For mRNA analysis, total RNA was extracted using RNeasy Plus mini kits (Qiagen GmbH, Hilden, Germany), and analyzed by real-time PCR (Applied Biosystems, Life Technologies) as described previously [35]. Primer sequences are available upon request.

Immunoprecipitation

For identification of DUBs, proteins associated with endogenous Hes1 in mouse ES cells (MG1.19) were immunoprecipitated using rabbit Hes1 antibody described previously [5] and identified by LC/MS/MS as described previously [36]. The antibody or control IgG was cross-linked to Protein A beads (GE Healthcare) by treatment with dithiobis (succinimidyl propionate), and *Hes1* knockdown cells were used as negative controls [5]. After washing the beads, five times with binding buffer (1% Nonidet P-40, 150 mM NaCl, 1 mM EDTA, 20 mM Tris/HCl, pH 8.0) and then three times with washing buffer (150 mM NaCl, 1 mM EDTA, 20 mM Tris/HCl, pH8.0) at 4° immunoprecipitated proteins were eluted with guanidine hydrochloride buffer (7 M guanidine hydrochloride, 10 mM dithiothreitol, 50 mM Tris/HCl, pH 8.0) and analyzed by LC/MS/MS

[36]. To detect the interaction of DUBs with Hes1, HEK293T cells were transfected with plasmids encoding FLAG-tagged DUBs and HA-tagged Hes1, and lysed in binding buffer containing both protease inhibitors [Complete Protease Inhibitor Cocktail, Roche, (Basel, Switzerland) and 1mM phenylmethylsulfonyl fluoride, Nacalai Tesque] and phosphatase inhibitors (10mM beta-Glycerophosphate, 1mM Sodium Orthovanadate, 1mM Sodium Fluoride, 1mM Sodium Pyrophosphate, Nacalai Tesque). Proteins immunoprecipitated with FLAG M2 agarose (Sigma) were washed three times with binding buffer and twice with washing buffer, and then eluted using $3 \times$ FLAG® peptide (Sigma) and analyzed by western blotting using horseradish peroxidase-conjugated FLAG® M2 antibody (1:5000; Sigma) and HA antibody (3F10, 1:2000; Roche). To detect interaction between endogenous Hes1 and Usp22, the nuclear fraction was extracted from C3H10T1/2 cells and subjected to immunoprecipitation with Hes1 antibody [5] using a Nuclear Complex Co-IP kit (Active Motif, Carlsbad, CA, USA) according to the manufacturer's instructions, and analyzed by western blotting using Hes1 and mouse monoclonal Usp22 antibodies (1:1000, Santa Cruz Biotechnology Inc., Dallas, TX, USA). All signals obtained in western blotting experiments are in the linear range of the LAS3000mini quantification.

Ubiquitination assay

Plasmids encoding DUB, His_6-tagged Hes1 or His_6/FLAG-tagged Hes7 and HA-tagged ubiquitin were co-transfected into C3H10T1/2 cells, which were lysed in guanidine hydrochloride lysis buffer (0.5% NP-40, 100 mM NaCl, 5 mM $MgCl_2$, 10 mM imidazole, 6 M guanidine hydrochloride, 50 mM Tris/HCl, pH 8.0) and sonicated in an ice-cold water bath to decrease viscosity. His_6-tagged Hes1 or Hes7 were purified using Ni-NTA agarose beads (Qiagen), washed three times with guanidine hydrochloride lysis buffer and twice with lysis buffer, and then eluted using SDS sample buffer. To detect ubiquitinated endogenous Hes1, proteins were immunoprecipitated using rabbit Hes1 antibody-conjugated Protein A beads or control rabbit IgG-conjugated Protein A beads from C3H10T1/2 cells that were co-transfected with both HA-tagged ubiquitin and the corresponding USPs, or both HA-tagged ubiquitin and shRNA or siRNA. For shRNA knockdown and Usp overexpression, transfected cells were selected using 2 μg/mL puromycin. Cells were lysed in binding buffer containing 2 mM N-Ethylmaleimide after MG132 treatment for 2 h. Precipitated proteins were washed five times with binding buffer and twice with washing buffer, and eluted using SDS sample buffer. Purified samples were analyzed by western blotting using the following antibodies: rabbit Hes1 antibody, horseradish peroxidase-conjugated HA antibody and FLAG M2 antibody at the same dilutions as described above.

Real-time imaging

We used NIH3T3 cells carrying two reporters, ubiquitin–nuclear localization signal (NLS)–luc2 under the control of the Hes1 promoter to monitor Hes1 transcription, and mCherry–NLS under the control of the human elongation factor promoter to track single cells. The Notch intracellular domain (NICD) was expressed under the control of the tetracycline-responsive promoter (Tet-On®, Takara Bio Inc., Shiga, Japan) to induce Hes1 expression. These transgenes were incorporated into NIH3T3 cells using the Tol2 transposon system [37]. Cells were cultured in Dulbecco's modified Eagle's medium, 10% fetal bovine serum, 1 mM luciferin, 1 μg/mL doxycycline. Bioluminescence was measured for 20–24 h as described previously [9]. Oscillation periodicity was estimated by the maximum entropy method of spectrum analysis using ImageJ software (http://rsb.info.nih.gov/ij/index.html) [5]. Samples with very low signals, short periods of less than 30 min, or long periods of more than 400 min were excluded as experimental noise and outliers. This cell line exhibited robust Hes1 oscillation in individual cells because of exogenous Notch intracellular domain expression. Our previous report demonstrated that the oscillation period of Hes1 in C3H10T1/2 cells was more variable among cells and oscillation cycles without serum stimulation, because cell–cell interaction probably induces unpredictable activation of Notch signaling, resulting in unstable and fragile Hes1 oscillation in individual cells [27]. The NIH3T3 cell line was much better than C3H10T1/2 cells for the purpose of accurately evaluating the change in oscillation periods by *Usp22* knockdown in individual cells.

In utero electroporation, immunohistochemistry and quantification

In utero electroporation was performed as described previously [4]. siRNA (50 μM) or 2 μg/μL plasmid encoding Usp27x with 0.5 μg/μL reporter plasmid encoding Histone H2B (H2B)–green fluorescent protein (GFP) under the control of the CAG promoter (chicken beta-actin promoter with CMV enhancer) was electroporated into neural stem cells after it was injected into the lateral ventricle of E13.5 mouse embryos. Approximately five embryos were electroporated for each sample, and all embryos were sectioned. Embryos at E15.5 were fixed in 4% paraformaldehyde, cryoprotected, embedded in Tissue-Tek® O.C.T.™ Compound (Sakura Finetek Japan Co., Ltd., Tokyo, Japan), and cryosectioned at 16 μm. Fixed cryosections were blocked using 5% normal goat serum and incubated with the primary antibodies chicken GFP (1:500; Abcam, Cambridge, UK), rabbit TUJ-1 (1:500; Covance Research Products Inc., Denver, PA, USA) and rat T-box brain protein 2 (Tbr2) (1:500; eBioscience, San Diego, CA, USA), and then incubated with secondary antibodies conjugated with Alexa488 or Alexa594 (1:500; Invitrogen) and 4′,6-diamidi-

no-2-phenylindole. GFP-positive cells were counted within an area 200 μm wide perpendicular to the apical/basal axis of the cortical layers in at least four sections in two embryos. Tbr2 is a marker of intermediate progenitor cells, and is expressed in the SVZ [38]. We considered that the Tbr2-positive region represents the SVZ, and distinguished two regions of the cortical layers: the apical region (the inner 41.4% region of the brain wall), which includes the VZ and the SVZ, and the basal region, which includes the cortical plate and the intermediate zone.

Statistical analyses

Statistical analyses and curve fitting were performed using Kaleidagraph software (Synergy Software, Reading, PA, USA). Statistical differences were examined using Student's *t* test and two-way ANOVA, and *P* values < 0.05 was considered significant.

Acknowledgements

Author contributions

TK and RK planned the experiments; TK, YI, KT, AI and TN performed the experiments and analyzed the data; Koichi Kawakami and Kozo Kaibuchi contributed materials and equipment; YK discussed the experiments; TK and RK wrote the paper.

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