# Stress-responsive maturation of Clk1/4 pre-mRNAs promotes phosphorylation of SR splicing factor

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It has been assumed that premessenger ribonucleic acids (RNAs; pre-mRNAs) are spliced cotranscriptionally in the process of gene expression. However, in this paper, we report that splicing of *Clk1/4* mRNAs is suspended in tissues and cultured cells and that intermediate forms retaining specific introns are abundantly pooled in the nucleus. Administration of the Cdc2-like kinase–specific inhibitor TG003 increased the level of *Clk1/4* mature mRNAs by promoting splicing of the intron-retaining RNAs. Under stress conditions, splicing of general pre-mRNAs was inhibited by dephosphorylation of SR splicing factors, but exposure to stresses, such as heat shock and osmotic stress, promoted the maturation of *Clk1/4* mRNAs. Clk1/4 proteins translated after heat shock catalyzed rephosphorylation of SR proteins, especially SRSF4 and SRSF10. These findings suggest that *Clk1/4* expression induced by stress-responsive splicing serves to maintain the phosphorylation state of SR proteins.

## Introduction

The appropriate phosphorylation of SR (serine–arginine-rich) proteins, a family of non–small nuclear RNP splicing factors, is essential for constitutive splicing and involved in regulation of alternative splicing (Duncan et al., 1997; Prasad et al., 1999; Lin and Fu, 2007; Long and Caceres, 2009), and both hypo- and hyperphosphorylations cause misregulation of splicing and splicing arrest. Phosphorylation states of SR proteins depend on the balanced activity between SR protein kinases and phosphatases. For example, heat shock–induced activation of PP1 (protein phosphatase 1) was reported to repress splicing through the dephosphorylation of SRSF10 (SRp38; Shin et al., 2004; Shi et al., 2006; Shi and Manley, 2007). SRPKs (SR protein kinases; Gui et al., 1994; Kuroyanagi et al., 1998; Wang et al., 1998), Cdc2-like kinases (Clks; Ben-David et al., 1991; Howell et al., 1991; Johnson and Smith, 1991; Nayler et al., 1997; Duncan et al., 1998), PRP4 (pre-mRNA processing 4; Alahari et al., 1993; Kojima et al., 2001), and dual-specificity tyrosine-regulated kinases (DYRKs; Alvarez et al., 2003; de Graaf et al., 2004) have been reported to phosphorylate SR proteins. To clarify the function of these kinases, we have developed synthetic inhibitors that specifically inhibit each target kinase (Hagiwara, 2005). We first found TG003, a kinase inhibitor specific for Clks (Muraki et al., 2004). TG003 affects splicing both in vitro and in vivo and suppresses influenza virus proliferation (Karlas et al., 2010; Nishida et al., 2011). We next synthesized SRPIN340, a specific inhibitor of SRPKs, which reduces the phosphorylation states of SR proteins (Fukuhara et al., 2006) and affects the splicing pattern of VEGF-A mRNA (Nowak et al., 2010). Recently, we reported on INDY1, a specific inhibitor of DYRKs, which rescues the transcriptional suppression of the NFAT1–regulated genes and abnormal development in *Xenopus laevis* embryo induced by DYRK overexpression (Ogawa et al., 2010).

The Clk family is a group of nuclear kinases for SR proteins and consists of four genes: ubiquitously expressed *Clk1*, *Clk2*, and *Clk4* and testis-specific *Clk3* (Nayler et al., 1997). Clks were demonstrated to be able to modulate splicing in vitro and in vivo (Colwill et al., 1996; Prasad et al., 1999; Yomoda et al., 2008). Clk1 and Clk4 are almost identical in amino acid sequence and considered to be functionally equivalent. After administration of TG003 to cultured cells, SR proteins, especially SRSF4 (SRp75), were dephosphorylated and accumulated in speckles within 60 min but rapidly rephosphorylated when the compound was washed out, indicating that constitutively active Clk1 or Clk4 is required to maintain the phosphorylation state of SR proteins (Yomoda et al., 2008).

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Abbreviations used in this paper: Clk, Cdc2-like kinase; dig, digoxigenin; DYRK, dual-specificity tyrosine-regulated kinase; NMD, nonsense-mediated mRNA decay.



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Splicing of pre-mRNA is regulated by various environmental stresses, such as heat shock, genotoxic agents, osmotic change, and acidosis (Biamonti and Caceres, 2009). Splicing interruption by heat shock was initially found in *Drosophila melanogaster* cells in 1986 (Yost and Lindquist, 1986) and has been observed in other organisms from yeast to humans (Bond, 1988; Yost and Lindquist, 1991). As most metazoan genes contain introns, heat shock–induced splicing interruption results in general inhibition of gene expression; the exceptions to this are heat shock proteins (Yost and Lindquist, 1988). Here, we report that splicing of *Clk1/4* pre-mRNAs is suspended, the intermediate forms retaining specific introns are abundantly pooled in the nucleus, and administration of the Clk-specific inhibitor TG003 or exposure to stresses, such as heat shock and osmotic stress, increases the mature mRNAs of *Clk1/4* by promoting splicing of the intron-retaining RNAs.

## Results

### Introns adjacent to exon 4 of *Clk1* are retained in tissues

When checking the *Clk1* mRNA expression levels in various tissues of mice with Northern blotting using an antisense DNA probe for exons 1 and 2 (Fig. 1 A), we detected an extra band of 3.1 kb in addition to the 1.8-kb band of the expected size of the mature *Clk1* mRNA (1,818 bases) in all tissues tested (Fig. 1 B, left). Although it was not clarified, an upper corresponding band was observed in Northern blot in a previous study (Nayler et al., 1997). The product 1,300 bases longer (Fig. 1 C, arrows) than mature *Clk1* mRNA (Fig. 1 C, arrowheads) could be amplified with PCR using the primer set of exon 1 and exon 13 (the last exon) from embryonic and adult brain cDNA. The longer product could be also amplified with PCR using primer sets of exons 2–5 and exons 3–13, but not of exons 5–13, from cDNA reverse transcribed with a *Clk1*-specific primer (Fig. S1 A), suggesting that this extension was a result of the retention of introns 3 and 4. Actually, cloning and sequence analysis of the longer PCR product revealed that it is derived from *Clk1* pre-mRNA that retains introns 3 (871 nt) and 4 (387 nt) as schematically shown in Fig. 1 A. The 3.1-kb band of the Northern blot corresponds in size to the intron 3/4–retaining *Clk1* RNA (3,076 bases) and was detected both with the intron 3– or intron 4–specific antisense probe of *Clk1* with the same tissue profile as the exon 1/2 probe indicated (Fig. 1 B, middle and right). Thus, we concluded that introns adjacent to exon 4 of *Clk1* are retained in tissues. The ratios of intron-retaining to mature *Clk1* mRNA in each tissue, estimated by the signal intensity of the Northern blot using the exon 1/2 probe (Fig. 1 B, left), were 2.5 (heart), 2.7 (brain), 2.9 (spleen), 2.5 (lung), 1.3 (liver), 1.5 (skeletal muscle), 1.4 (kidney), and 1.3 (testis), indicating that the intron-retaining *Clk1* RNA is the predominant form in every tissue we tested. Although the ratios of cell lines, especially in the proliferation phase, seemed to be lower than those in tissues (unpublished data), the intron retention of *Clk1* was observed in all tested tissues, primary cultures and cell lines of mice and humans (unpublished data), indicating that this is a ubiquitous phenomenon conserved at least from mice to humans. The intron-retaining *Clk1* RNA was detected on the tissue poly(A)$^+$ RNA blot (Fig. 1 B) and amplified by poly(A)-dependent RT-PCR (Fig. S1 B). Thus, the intron-retaining RNA is considered to be already polyadenylated at the 3′ terminus. A similar PCR product was amplified with primers of *Clk4*, which is closely related to *Clk1* among *Clk* family genes (Fig. 1, A and C). In contrast, the specific intron-retaining RNA of *Clk2* was not detected upon PCR analysis (Fig. 1 C) or Northern blotting (not depicted).

### The intron 3/4–retaining RNA of *Clk1* is localized in the nucleus

Next, we examined the intracellular localization of this intron-retaining *Clk1* RNA by semiquantitative and quantitative real-time PCR of the nucleus/cytoplasm subfraction (Fig. 2, A and B) and in situ hybridization (Fig. 2 C). The RT-PCR analysis after subfractionation revealed that mature mRNAs of *Clk1* and *Gapdh* were predominantly enriched in the cytoplasmic fraction of NIH-3T3 cells, whereas the intron 3/4–retaining RNA was mainly enriched in the nuclear fraction (Fig. 2 A). This was confirmed by specific detection of intron-retaining *Clk1* RNA with the sets of an exonic primer and an intronic primer (Fig. 2 A, b and c). The real-time PCR analysis revealed that the nucleus/cytoplasm ratio of intron-retaining *Clk1* RNA was rather higher than that of *Neat1* (Fig. 2 B), a known nuclear-localized noncoding RNA (Hutchinson et al., 2007).

The intracellular localization of the intron-retaining *Clk1* RNA was visualized under the microscope by fluorescence in situ hybridization of HeLa cells using a digoxigenin (dig)-labeled RNA probe for human *Clk1* intron 4. Hybridized with the antisense probe, the signals were detected mainly in the nucleus (Fig. 2 C, top). Thus, all these results indicated that intron-retaining *Clk1* RNA was predominantly localized in the nucleus. In addition, we observed that administration of the Clk1/4-specific inhibitor TG003 decreased the nuclear signal for the intron 4 of *Clk1* (Fig. 2 C, middle), whereas the faint signals in the cytoplasm were still detectable

### TG003 promotes splicing of the intron 3/4–retaining *Clk1* RNA

As previously reported, *Clk1* mRNA including exon 4 encodes an active form of the Clk1 protein, whereas the spliced product excluding exon 4 was targeted by the nonsense-mediated mRNA decay (NMD) pathway (Duncan et al., 1997), and administration of TG003 promoted exon 4 inclusion and suppressed exon 4 exclusion in the cultured cells (Muraki et al., 2004). Previously, the increase of the exon 4 exclusion form of *Clk1* was attributed to the Clk1 kinase activity-dependent suppression of alternative splicing, but the data shown in Fig. 2 C indicate the possibility that TG003 may promote the splicing from the intron-retaining *Clk1* RNA abundantly stored in the nucleus. As expected, treatment with TG003 of NIH-3T3 cells reduced the amount of intron 3/4–retaining *Clk1* RNA and promoted production of mature *Clk1* mRNA within 30 min (Fig. 3 A, a). This observation supports the specificity of the nuclear signal with the *Clk1* intron 4 antisense probe

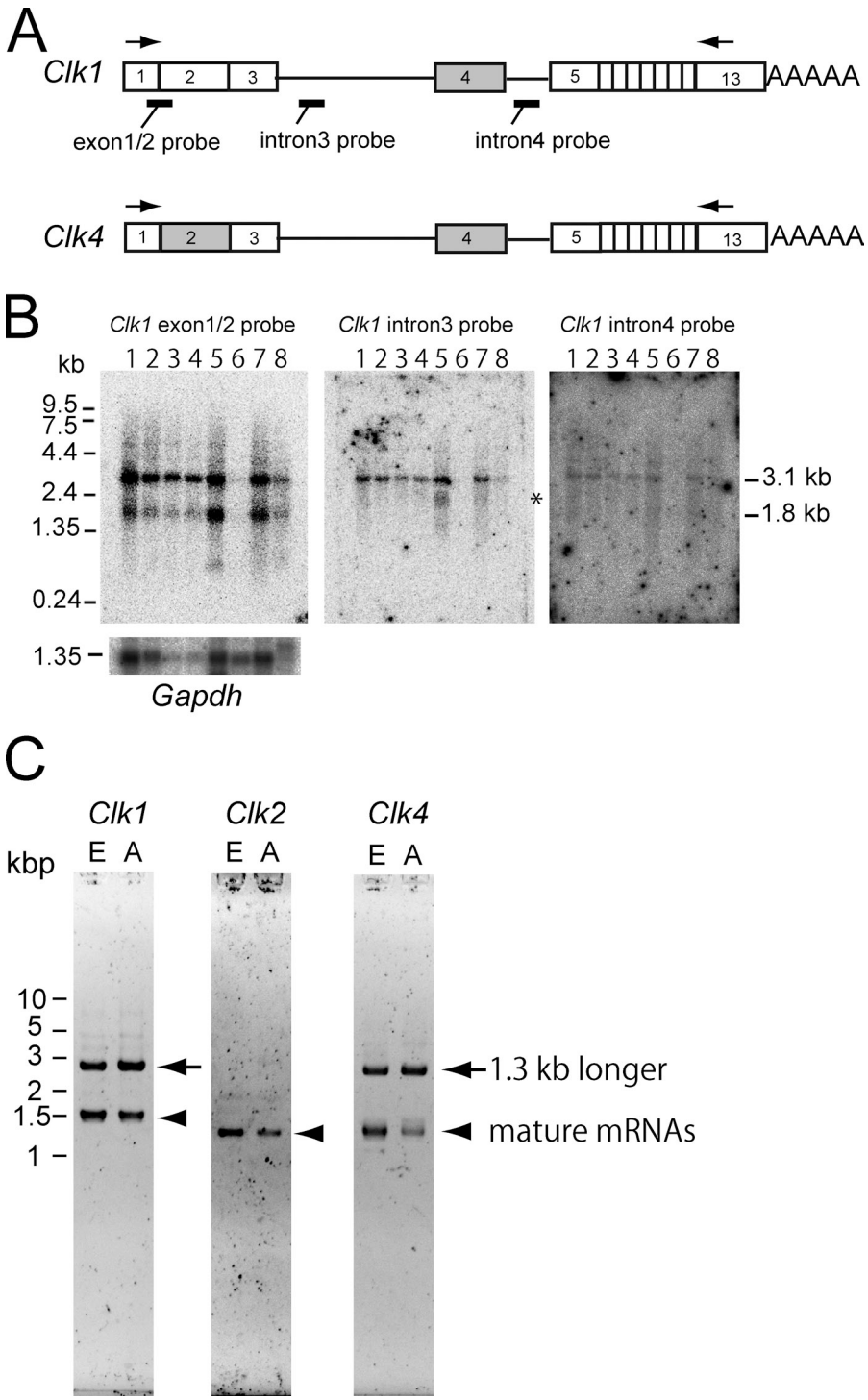


Figure 1. **Identification of *Clk1/4* intron 3/4–retaining RNAs.** (A) Schematic illustration of the intron-retaining RNAs of *Clk1* and *Clk4*. Open boxes indicate constitutive exons, and gray boxes indicate selective exons. Positions of Northern blot antisense probes (bars) and PCR primers (arrows) used in the following assays are indicated. (B) Tissue expression profile of *Clk1* pre-mRNA and mature mRNA in Northern blot analysis. mRNAs were detected by radiolabeled antisense probes. Lanes: 1, heart; 2, brain; 3, spleen; 4, lung; 5, liver; 6, skeletal muscle; 7, kidney; 8, testis. The asterisk shows that a faint band at 2.4 kb of lane 5 of the intron 3 probe is nonspecific. *Gapdh* was used as a control. (C) RT-PCR analysis of *Clk* family genes. Almost the entire regions of *Clk1*, *2*, and *4* were amplified by RT-PCR from mouse embryonic (E) or adult (A) brains. The arrowheads and arrows indicate the mature and the premature mRNAs, respectively.

of in situ hybridization, which disappeared by TG003 treatment (Fig. 2 C). To further analyze this process, we checked the maturation intermediates by RT-PCR with the forward primer of exon 2 and the reverse primer of intron 4 (Fig. 3 A, b). Administration of TG003 increased the maturation intermediate (Fig. 3 A, b, arrow), in which intron 3 was spliced, but intron 4 was retained, indicating that the retained introns of *Clk1* were spliced stepwise from the 5′ side when Clk1 activity was inhibited. In support of this, siRNA knockdown against mature *Clk1/4* mRNA resulted in the decrease of the intron retention form of *Clk1/4* (Fig. S2, A and B, indicated by arrows). TG003-induced splicing of intron-retaining *Clk1* RNA was not affected by cotreatment of a transcription inhibitor, α-amanitin (Fig. 3 B), suggesting that this transient increase of mature *Clk1* mRNA resulted primarily from the splicing promotion of the reserved intron-retaining RNA.

After the intron-retaining RNA was completely consumed by 50 μM TG003 treatment for 30 min (Fig. 3 C, a, lane 2), the drug was removed by washing with fresh medium. Then, within 1 h after drug removal, the pool of intron-retaining

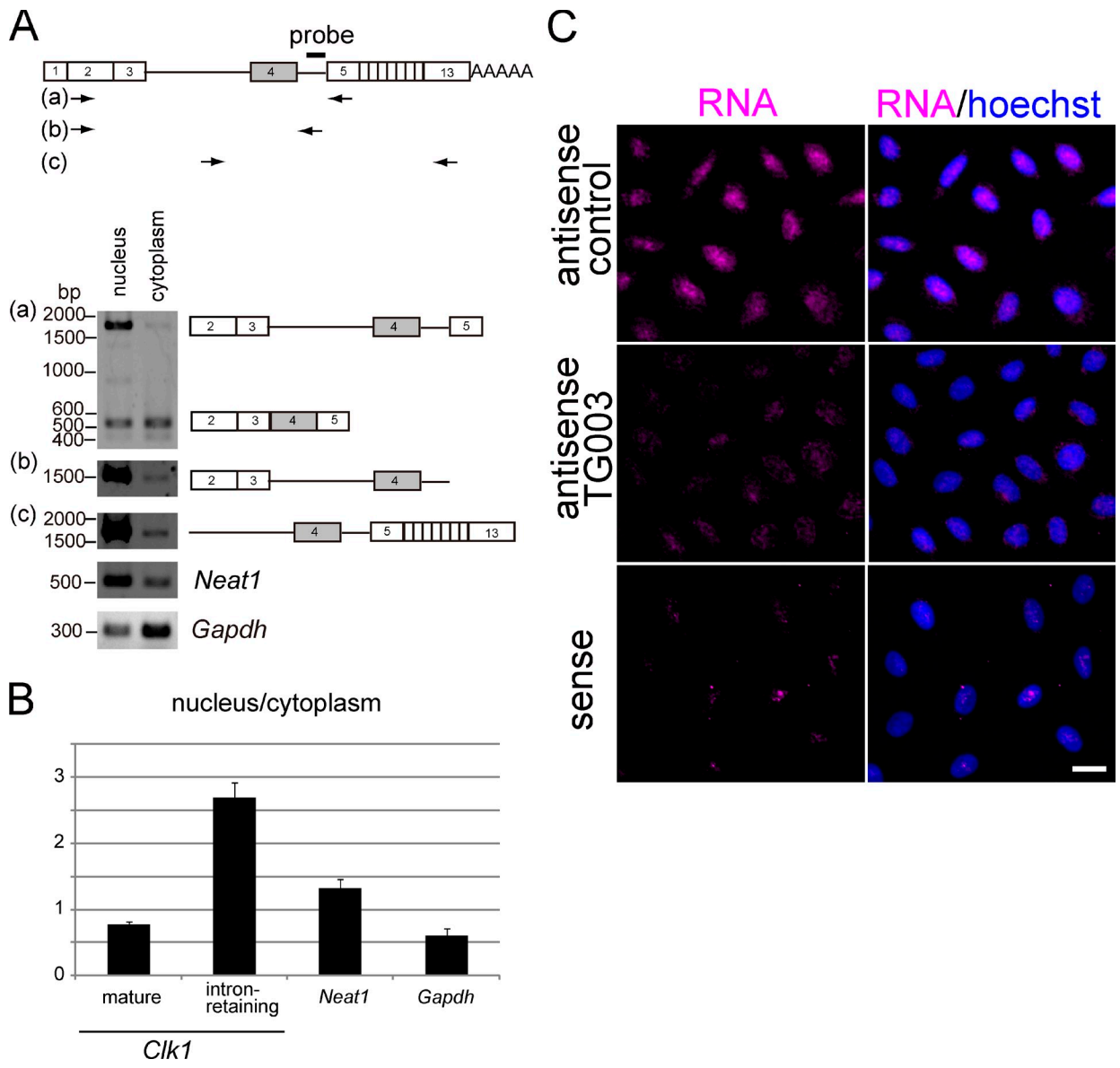


Figure 2. **Nuclear localization of the *Clk1* intron-retaining RNA.** (A) RT-PCR analysis of nucleus/cytoplasm subfraction. Positions of the primers used in a, b, and c and the hybridization probe used in C are indicated in the schema by arrows and a bar, respectively. The same primer pairs were used in the PCR analyses of Fig. 3, Fig. 5, Fig. 6, and Fig. 7. Intron 3/4–retaining *Clk1* RNA (a band at 1,804 bp in a and bands shown in b and c) and *Neat1* RNA were enriched in the nuclear fraction, whereas mature *Clk1* (546 bp in a) and *Gapdh* mRNAs were enriched in the cytoplasm. (B) The ratio of nuclear to cytosolic mRNAs quantitated by real-time PCR. Each bar shows the mean with SEM of three independent experiments. (C) In situ hybridization of HeLa cells using a human *Clk1* intron 4 probe. Nuclear signals of the *Clk1* intron 3/4–retaining RNA, visualized by the antisense probe, were detected in control cells and disappeared in the cells fixed after 50 µM TG003 treatment for 30 min. Nuclei were stained with Hoechst 33342. Bar, 20 µm.

*Clk1* RNA was restored almost to the control level (Fig. 3 C, lane 4), whereas the intron-retaining *Clk1* RNA was not detected in cells incubated in the drug-containing medium (Fig. 3 C, lane 3).

### Cis-element for intron retention and TG003 responsivity are highly conserved

Splicing of intron-retaining *Clk4* was also promoted by TG003 (unpublished data). This suggested the possibility that *Clk1* and *Clk4* have common cis-regulatory elements for the intron retention and TG003-induced splicing. Moreover, the cis-element should be conserved between the *Clk1* genes of mice and humans. The sequence of exon 4 and the proximal region in the adjacent introns of *Clk1* are homologous to that of *Clk4* and highly conserved in mice and humans (Fig. S3). When we inserted the conserved region of *Clk1* (Fig. S3, blue line) into the second intron of the *β-globin* minigene, a well-established splicing reporter (for *β-globin* minigene, see Yamashita et al., 2001), the splicing-suspended pre-mRNA was expressed from the minigene construct, and the splicing of the inserted region was promoted by the administration of TG003 (Fig. 4, A and B). This suggests that the cis-regulatory region for the intron retention and TG003-sensitive splicing are highly conserved between *Clk1* and *Clk4* and between species.

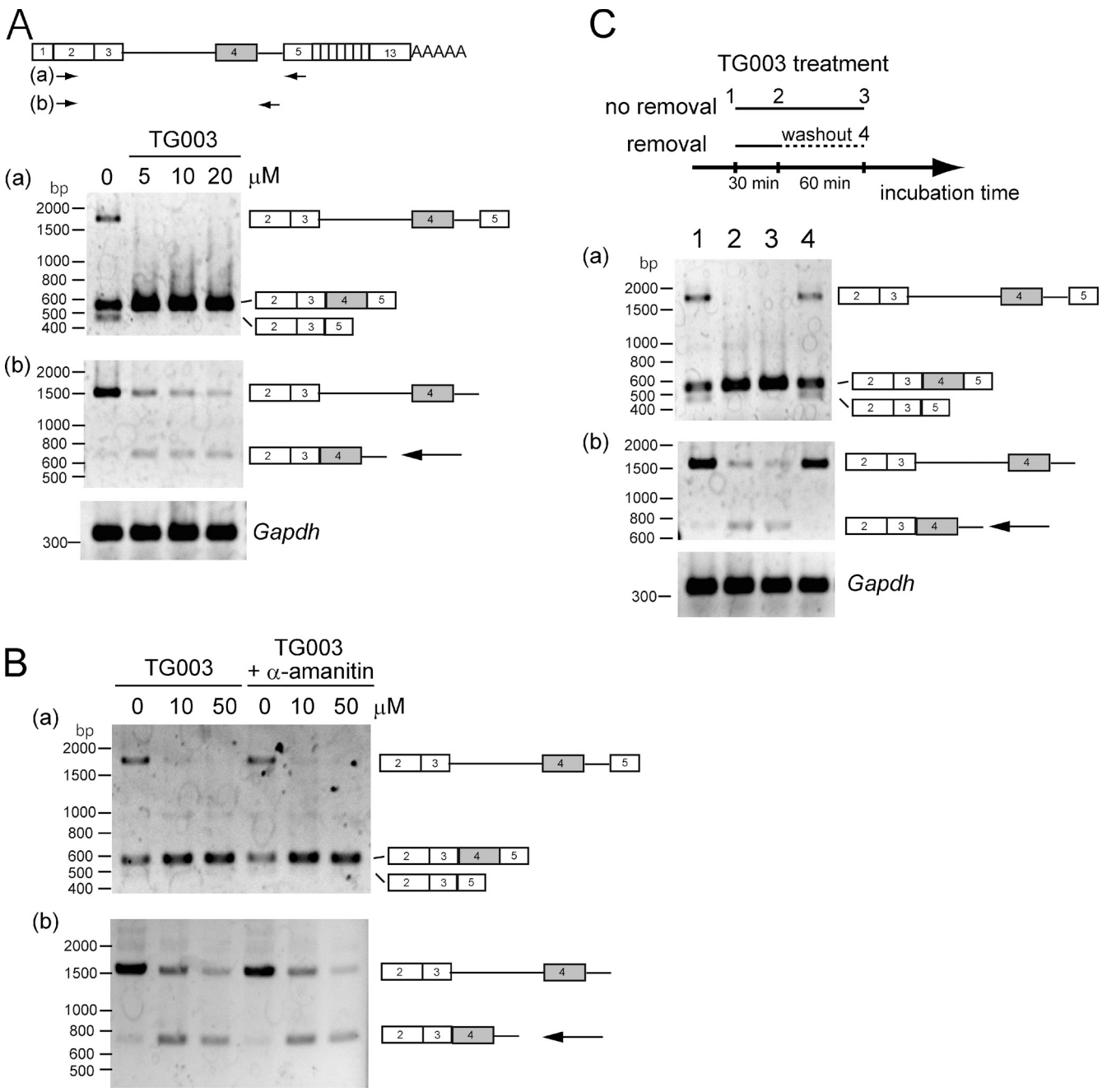


Figure 3. **Maturation of *Clk1* intron 3/4–retaining RNA induced by the inhibition of Clk1.** (A) Clk1 inhibitor treatment. Positions of primer sets are illustrated above. TG003 was administered to NIH-3T3 cells at the concentrations of 0, 5, 10, and 20 μM for 30 min followed by RT-PCR analysis. In the drug-treated cells, the amount of *Clk1* intron-retaining RNA was decreased (1,804 bp in a and 1,550 bp in b) as the mature mRNA was increased (546 bp in a). In addition, a maturation intermediate product, which retained only intron 4, was detected in the presence of TG003 (arrow in b). (bottom) *Gapdh* was used as an internal control. (B) Effect of a transcription inhibitor, α-amanitin, on the maturation of *Clk1* mRNA. Maturation induced by TG003 (0, 10, and 50 μM for 30 min) was not influenced in the presence of a transcription inhibitor, α-amanitin (10 μg/ml at final concentration). The maturation intermediate product, which retained only intron 4, was also detected in the presence of TG003 (arrow in b). (C) Effect of the removal of Clk1 inhibitor. A scheme of the experimental time course and corresponding lane numbers are indicated above. NIH-3T3 cells (control; lane 1) were treated with 50 μM TG003 for 30 min (lane 2) and then washed with fresh medium twice. Compared with the culture without drug removal (lane 3), intron 3/4–retaining *Clk1* RNA was restored to the control level within 60 min after the removal of TG003 (lane 4).

### Stress also induces splicing of the intron-retaining *Clk1* RNA

Environmental stresses, such as heat shock and osmotic stress, cause dephosphorylation of SR proteins and inhibit pre-mRNA splicing (Shin et al., 2004; Shi et al., 2006; Shi and Manley, 2007; Zhong et al., 2009). Then, we examined whether heat shock or osmotic stress affects the amount of the intron 3/4–retaining *Clk1* RNA. Surprisingly, heat shock (incubated at 43°C for 60 min) and osmotic stress (treatment with 600 mM sorbitol for 60 min) induced maturation of intron-retaining *Clk1* RNA (Fig. 5 A). To further examine the stress-induced maturation process, we performed an RT-PCR–based time course analysis

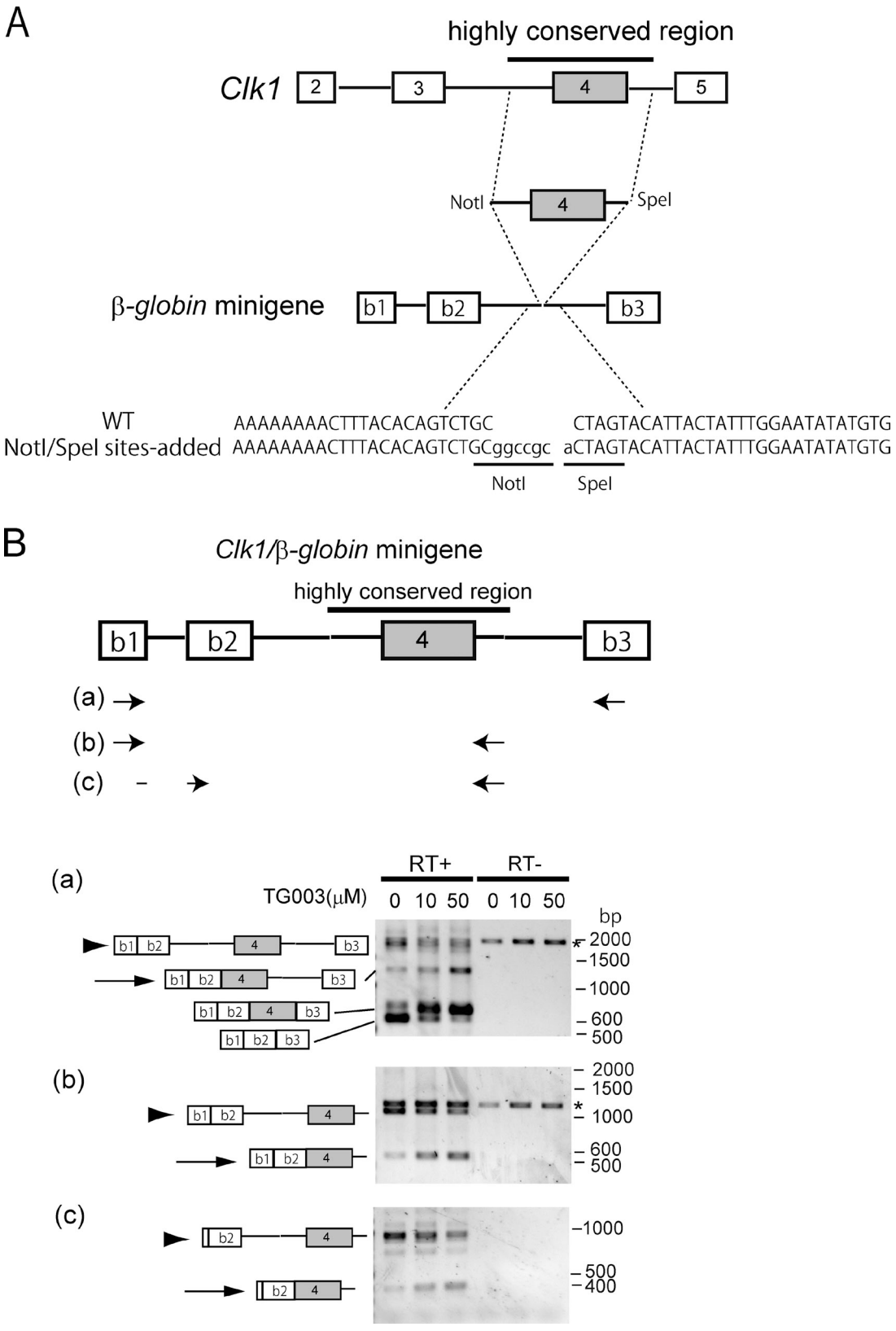


Figure 4. **The cis-regulatory elements required for the intron retention of *Clk1* RNA.** (A) Scheme of *β-globin* minigene construction. Exons 1, 2, and 3 of *β-globin* are indicated by b1, b2, and b3, respectively. The highly conserved region of mouse *Clk1* (indicated by a blue line in Fig. S3) was inserted into NotI–SpeI sites added in the second intron of the *β-globin* minigene expression vector. The sequence of insertion site in *β-globin* is indicated. The added sequence is indicated in the lowercase letters. (B) RT-PCR analysis of the reporter RNA splicing. Arrows indicate primer position. Retention of introns adjacent to the translocated exon 4 was observed (arrowheads). In the presence of TG003, a Clk1/4 inhibitor, the intron-retaining pre-mRNA (arrowheads) was decreased, and the splicing intermediate (arrows) and the exon 4–included spliced product were increased. Asterisks show the PCR products derived from the primary transcript and/or contaminated plasmid DNA. RT, reverse transcription; WT, wild type.

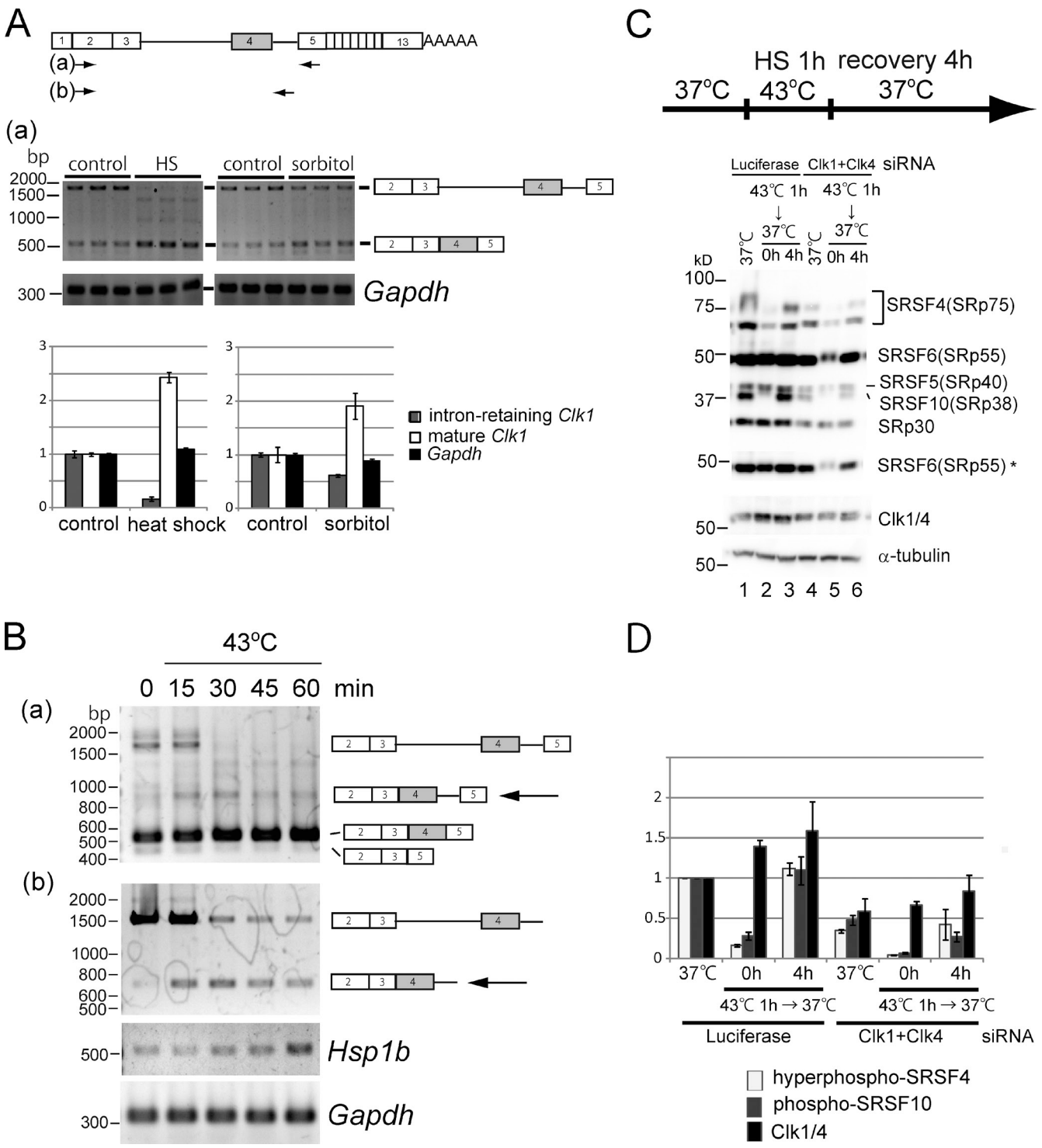


Figure 5. **Stress-induced expression of Clk1.** (A) Stress-induced maturation of intron 3/4–retaining *Clk1* RNA. NIH-3T3 cells were incubated under heat shock (HS) condition (incubated at 43°C) for 60 min (left) or osmotic stress condition (treatment with 600 mM sorbitol) for 60 min (right). Splicing pattern of *Clk1* was analyzed by RT-PCR using the same primers as in Fig. 2 A (a). The experiment was performed in triplicate. (bottom) Graphs represent the means with SEM ($n$ = 3) of changes in the intensities after the stresses. (B) Time course analysis of heat shock–induced maturation of *Clk1*. NIH-3T3 cells were exposed to heat shock at 43°C for the indicated times. Splicing pattern of *Clk1* was analyzed by RT-PCR using the same primers as Fig. 2 A (a and b). The maturation intermediate retaining intron 4 is indicated by arrows in a and b. (bottom) *Hsp1b* mRNA was used as a heat shock control, and *Gapdh* mRNA was used as an internal control. (C) Effect of Clk1/4 knockdown on SR protein phosphorylation. The cells were incubated with the siRNA against Clk1/4 from 3 d before the assay (lanes 4–6) with the same protocol as in Fig. S2 A. As a control, siRNA against luciferase was used (lanes 1–3). The cells were incubated at a normal temperature (37°C; lanes 1 and 4), incubated at 43°C for 1 h (lanes 2 and 5), and further incubated at 37°C for 4 h (lanes 3 and 6) as indicated in the schema. Phosphorylation states of SR proteins were estimated by immunoblotting using an antiphospho-SR protein antibody. An asterisk shows the shorter exposure for SRSF6. (bottom) The immunoblot with an antibody against α-tubulin was shown as an internal control. (D) Quantification of results in C. Phospho-SRSF4 was quantified by measuring the intensity of the top band (at ∼80 kD) of SRSF4 in C. Each bar shows the mean with SEM of three independent experiments.

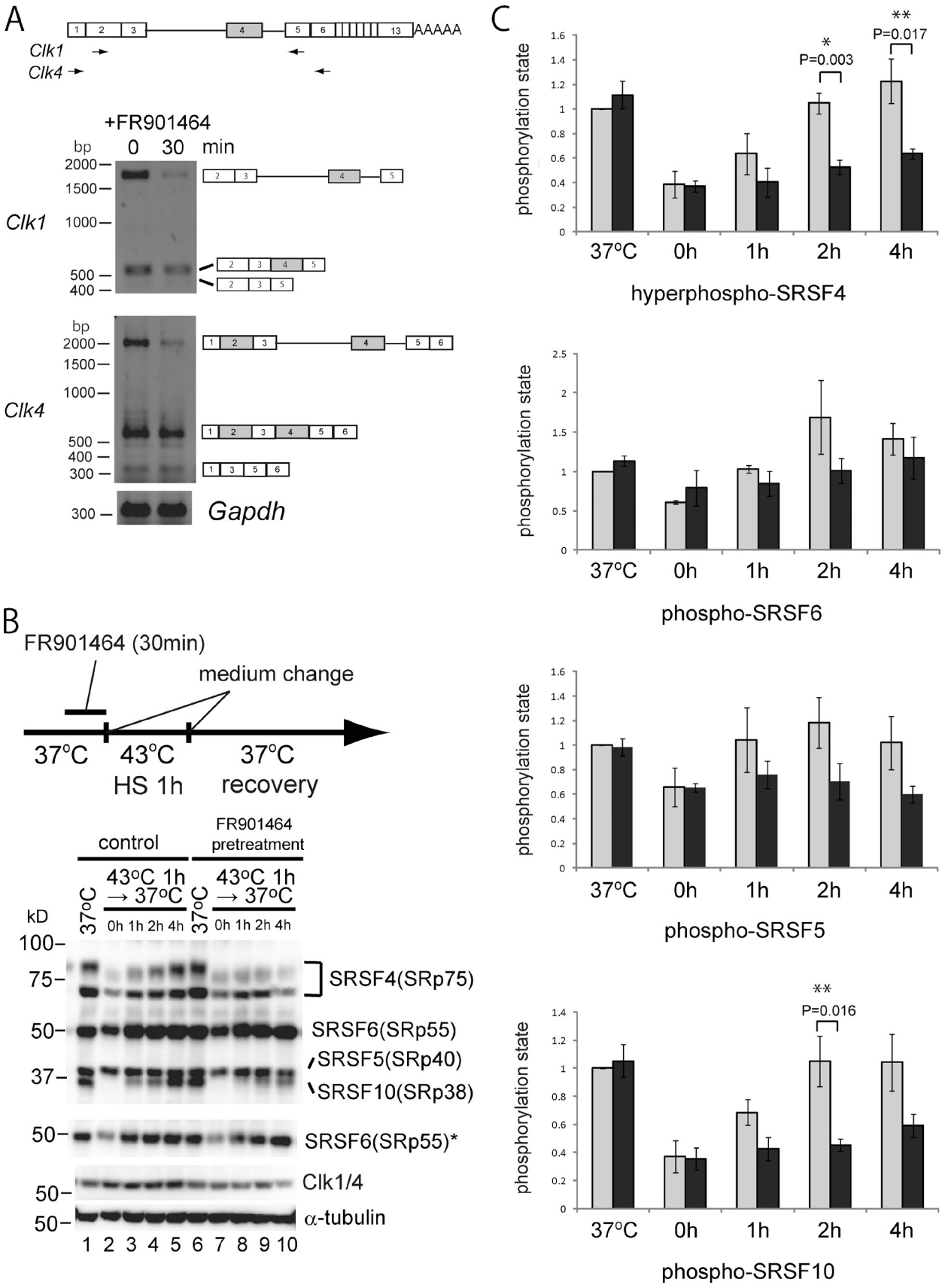


Figure 6. **Delay of SR protein rephosphorylation after heat shock by pretreatment with an SF3b inhibitor.** (A) Effect of the SF3b inhibitor FR901464 on *Clk1/4* RNAs. The effect of FR901464 treatment on NIH-3T3 cells (20 nM for 30 min) was analyzed by RT-PCR using primer sets of exons 2–5 (*Clk1*; same as shown in Fig. 2 A [a]) or exons 1–6 (*Clk4*) as illustrated above. *Gapdh* was used as an internal control. (B) Time course analysis of phosphorylation states of SR proteins during and after heat shock (HS). Recovery of the phosphorylation states were compared between NIH-3T3 cells of control (no drug-treated cells;

of the intron-retaining RNA after heat shock induction at 43°C. As a positive control of heat shock response, induction of *Hsp1b* mRNA, one of the heat shock response genes, was observed from 30 min after the induction (Fig. 5 B). The splicing of the intron 3/4–retaining *Clk1* RNA was almost completed within 30 min (Fig. 5 B, a). The maturation intermediate, which retained only intron 4, appeared immediately after heat shock induction (Fig. 5 B, a and b, arrows). The results obtained raised a hypothesis that the stress-induced production of Clk1/4 proteins was required for the quick recovery of SR protein phosphorylation. To explore this possibility, first, we examined the effect of siRNA knockdown of *Clk1/4* on SR protein phosphorylation and Clk1/4 protein expression under normal and stressful conditions (Fig. 5, C and D). In the control cells (the cells transfected with luciferase siRNA), Clk1 and/or Clk4 protein expression was induced after heat shock (Fig. 5 C, lane 2), and the increased protein level was maintained for 4 h after stress removal (Fig. 5 C, lane 3). SR proteins were dephosphorylated after heat shock (43°C for 60 min; Fig. 5 C, lane 2) but rephosphorylated within 4 h at the normal temperature (Fig. 5 C, lane 3). Administration of siRNAs against *Clk1* and *Clk4* mRNAs, which almost completely depleted the mature mRNAs (Fig. S2 A) and decreased the protein level to 59% of the control (Fig. 5 C, lane 4), resulted in a reduced phosphorylation state of SR proteins, especially SRSF4 and SRSF10, in normal and stressful conditions (Fig. 5 C, compare lanes 4, 5, and 6 with lanes 1, 2, and 3, respectively). The quantitated data of phosphorylated SRSF4, phosphorylated SRSF10, and Clk1/4 proteins are shown in Fig. 5 D.

### The intron 3/4–retaining *Clk1/4* RNAs contribute to the rephosphorylation of SR proteins after heat shock

To further examine the role of the intron-retaining *Clk1/4* RNAs on the rephosphorylation of SR proteins, we attempted to predeplete the intron-retaining *Clk1/4* RNAs with FR901464, a drug equivalent to spliceostatin. Spliceostatin and FR901464 specifically, but noncovalently, target SF3b, a component of spliceosome, and consequently, inhibit spliceosome formation and nuclear retention of premature RNA (Kaida et al., 2007). We found that the brief and transient treatment of the NIH-3T3 cells with 20 nM FR901464 for 30 min could reduce the amount of the intron 3/4–retaining *Clk1/4* RNAs without any effect on the levels of mature mRNAs (Fig. 6 A and Fig. S4 A, lane 4). The intron-retaining RNA released from nuclear retention by FR901464 should be transported to the cytoplasm and degraded by NMD because several termination codons are located in introns 3 and 4 of *Clk1* RNA. In support of this, cotreatment of FR901464 and cycloheximide, an inhibitor of translational elongation and NMD, increased the intron 3/4–retaining *Clk1* RNAs (Fig. S4 A, compare the bands indicated by arrowheads in lane 3 with those in lane 4). The inhibitory effect of cycloheximide on NMD was confirmed by stabilization of the *Clk1* mRNA that lacks exon 4, which is continuously degraded by NMD because of premature termination codons (Fig. S4, A and B, +CHX lanes, double asterisk).

We next examined the effect of pretreatment with 20 nM FR901464 for 30 min on the rephosphorylation of SR proteins after heat shock. To analyze the time-dependent alteration of SR protein phosphorylation states after heat shock, the NIH-3T3 cells of control (Fig. 6 B, lanes 1–5, no drug-treated cells) or FR901464 pretreated (Fig. 6 B, lanes 6–10, cells treated with 20 nM FR901464 for 30 min before heat shock) were exposed to heat shock at 43°C for 60 min and then returned to the normal temperature (Fig. 6 B). In the control cells, SR proteins, especially SRSF4 and SRSF10, were dephosphorylated after heat shock (Fig. 6 B, lane 2) and gradually rephosphorylated to the control level within 4 h (Fig. 6 B, lanes 3–5). Pretreatment with 20 nM FR901464 for 30 min significantly delayed the recovery of phosphorylation states of SRSF4 and SRSF10 (Fig. 6, B [lanes 7–10] and C). Rephosphorylation of SRSF6 (SRp55) and SRSF5 (SRp40) also seemed to be delayed (Fig. 6, B and C). Basal phosphorylation levels of SR proteins or the heat shock–induced dephosphorylation was not affected by pretreatment with FR901464 (Fig. 6 B, compare lanes 1 and 2 with lanes 6 and 7, respectively). Clk1/4 proteins were induced after heat shock in the control cells (Fig. 6 B, lanes 1–5 of Clk1/4), whereas they were not increased after FR901464 pretreatment (Fig. 6 B, compare lanes 6–10 with lanes 1–5 of Clk1/4). This could be attributed to the depletion of the intron-retaining *Clk1/4* RNAs in the FR901464-pretreated cells. The FR901464 itself did not suppress SR protein phosphorylation or reduce the amounts of Clk1/4 proteins throughout the time course in the cells cultured consistently at 37°C (Fig. S4 C). These results indicate that stress-induced *Clk1/4* expression from the intron-retaining *Clk1/4* RNAs, which are sensitive to FR901464, contributes to rephosphorylation of SR proteins SRSF4, SRSF5, SRSF6, and SRSF10 after heat shock.

We then checked the mRNA expression of known SR protein kinases, such as *Clks* (*Clk1–4*), *Srpks* (*Srpk1* and *Srpk2*), *Dyrks* (*Dyrk1a* and *Dyrk2*), *Prp4*, and other genes (*Srsf4*, *Srsf10*, and *Hsp1b*) during and after heat shock (Fig. 7 A). Among SR protein kinases, only *Clk1/4* mRNAs were increased after heat shock in the control cells (Fig. 7 A, lanes 1–5 of *Clk1* and *Clk4*). However, in the cells pretreated with FR901464, the *Clk1/4* mRNAs with the exon 4 disappeared after heat shock (Fig. 7 A, lanes 6–10 of *Clk1* and *Clk4*), whereas the mRNA levels of the other kinases were not affected with FR901464.

lanes 1–5) and FR901464 pretreated (cells transiently pretreated with 20 nM FR901464 for 30 min; lanes 6–10) by immunoblotting using the antiphospho-SR protein antibody. The cells were incubated at a normal temperature (37°C; lanes 1 and 6), incubated at 43°C for 1 h (lanes 2 and 7), and further incubated at 37°C for 1 h (lanes 3 and 8), 2 h (lanes 4 and 9), and 4 h (lanes 5 and 10) as indicated in the schema. An asterisk shows the shorter exposure for SRSF6. Clk1/4 was detected using a Clk1 antibody which recognizes Clk1 and Clk4. (bottom) The immunoblot with an antibody against α-tubulin was shown as an internal control. (C) Quantification of the results in B. Gray bars and closed bars indicate the control and the FR901464-pretreated cells, respectively. Phospho-SRSF4 was quantified by measuring the intensity of the top band (at ∼80 kD) of SRSF4 in B. Each bar shows the mean with SEM of three independent experiments. Asterisks indicate a significant difference (*, $P < 0.01$; **, $P < 0.05$; each actual p-value is also indicated).

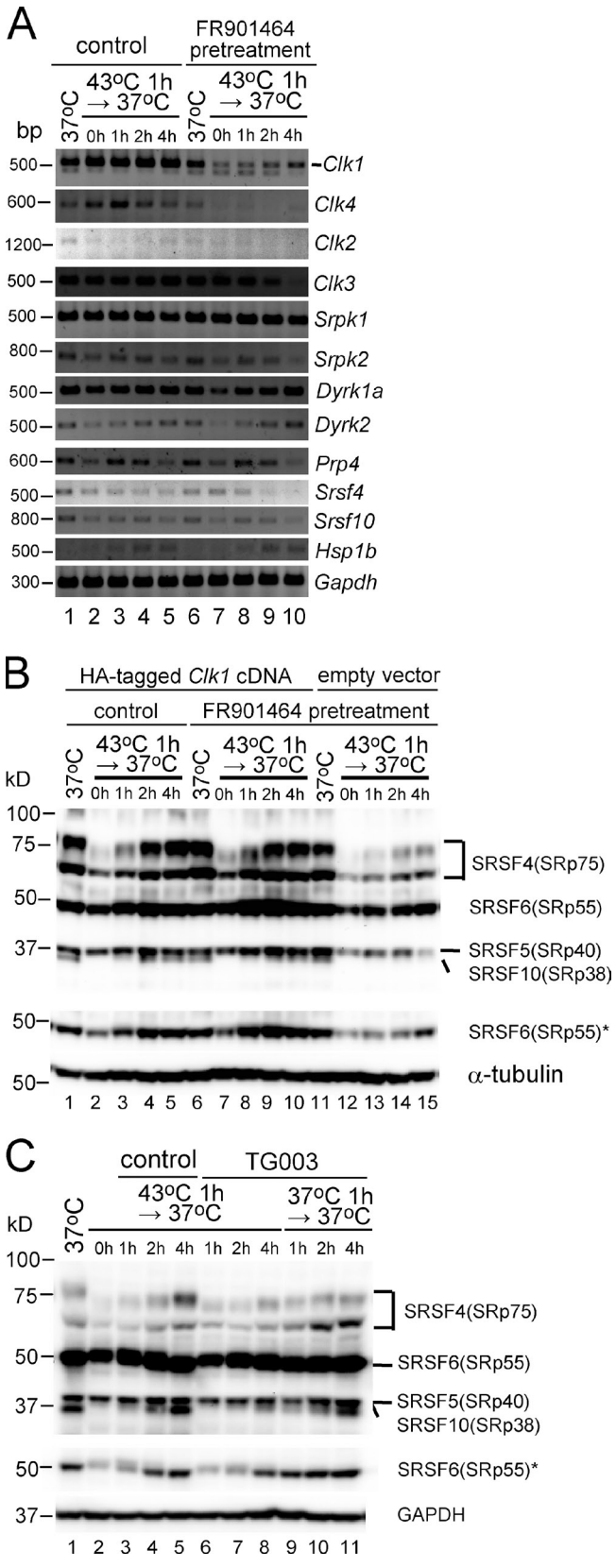


Figure 7. **Requirement of Clk1/4 intron retention for rapid recovery of SR protein phosphorylation.** (A) mRNA levels of SR protein kinases. mRNA expressions of SR protein kinases and other genes during and after heat shock in the control or FR901464-pretreated cells (prepared by the same protocol as for Fig. 6 B) were analyzed by RT-PCR. Primers of *Clk2*, *Hsp1b*, and *Clk1/4* were the same as for Fig.1 C, Fig. 5 B, and Fig. 6 A, respectively. Detailed information of primers of other mRNAs is described in Materials and methods. *Clk2* mRNA was rarely detected in NIH-3T3 cells. (B) Rescue experiment with exogenous *Clk1*. The NIH-3T3 cells on a 35-mm dish were transfected with 1 µg pME-HA-mClk1 vector, which expresses HA-tagged mClk1, or a pME-HA empty vector 16–18 h before the time course experiment same as in Fig. 6 B. The cells were incubated at a normal temperature (37°C; lanes 1, 6, and 11), incubated at 43°C for 1 h (lanes 2, 7, and 12), and further incubated at 37°C for 1 h (lanes 3, 8, and 13), 2 h (lanes 4, 9, and 14), and 4 h (lanes 5, 10, and 15). An asterisk shows the shorter exposure for SRSF6. (bottom) The immunoblot with an antibody against α-tubulin was shown as an internal control. (C) Cancelation of the rephosphorylation by Clk1/4 inhibitor. TG003-mediated inhibition of Clk1/4 activity during the recovery phase delayed the recoveries of the SR protein phosphorylations, estimated by immunoblotting using the antiphospho-SR protein antibody of NIH-3T3 cells without the drug treatment (control; lanes 1–5), and the cells treated with 10 µM TG003 during the recovery phase (TG003; lanes 6–11). The cells were incubated at a normal temperature (37°C; lane 1), incubated at 43°C for 1 h (lane 2), and further incubated at 37°C for 1 h (lanes 3, 6, and 9), 2 h (lanes 4, 7, and 10), and 4 h (lanes 5, 8, and 11). The cells of lanes 9–11 were not exposed to heat shock. An asterisk shows the shorter exposure for SRSF6. (bottom) GAPDH was used as an internal control.

To further confirm that *Clk1/4* play major roles on the rephosphorylations of SR proteins, we examined the effect of *Clk1* cDNA expression, which has no introns and is not affected by FR901464, on the time course of the rephosphorylation in the FR901464-pretreated cells. As we expected, the rephosphorylation of SR proteins was not affected by FR901464 in the cells transfected with *Clk1* cDNA (Fig. 7 B, compare lanes 6–10 with lanes 11–15). In support of this, when the cells were treated with the Clk1/4 inhibitor TG003 just during the recovery phase, rephosphorylation of SRSF4 and SRSF10 was clearly delayed compared with the control (Fig. 7 C, compare lanes 6–8 with lanes 3–5).

## Discussion

### A unique cis-regulatory element is responsible for the intron 3/4 retention of *Clk1/4*

Under the stress condition, several processes of gene expression, transcription, splicing, mRNA export, and translation are suppressed (Biamonti and Caceres, 2009). Therefore, stress-responsive genes should have bypass mechanisms for their expression. For example, many of the major *Hsps* (*heat shock proteins*) lack introns, and thus, their expressions were not intercepted by splicing arrest. Some exceptional *Hsp* genes contain heat-resistant and temperature-sensitive introns (Takechi et al., 1994; Yasuda et al., 1995), although the heat-resistant molecular mechanism has not been elucidated. In this study, we found that intron 3/4 of *Clk1/4* RNAs was retained in all tissues we examined and that the intron-retaining *Clk1* pre-mRNA was anchored in the nucleus but rapidly matured, exported to the cytoplasm, and translated to the protein after the heat shock. The translated Clk1 from the splicing-suspended pre-mRNA seems to be essential for rephosphorylation of SR proteins, especially SRSF4 and SRSF10. The observed fact that the transient expression of *Clk1* induced by heat shock or osmotic stress does not require de novo transcription, splicing of constitutive exons, and polyadenylation suggests a novel stress-resistant gene expression mechanism.

As shown in Fig. 4, the cis-regulatory elements required for the intron retention and TG003-sensitive splicing are suggested to be located in ~400 bases of exon 4 and proximal intronic regions of *Clk1/4* (Fig. S3). No sequence similarity was found between this region of *Clk1/4* and the sequences of the intron-containing heat shock genes, suggesting that the regulatory mechanism of splicing of *Clk1/4* should be unique in the heat shock–responsive genes.

### Dephosphorylation of SR proteins promotes the suspended splicing and releases the nuclear retention of the *Clk1* pre-mRNA

Considering that all of the Clk1/4 inhibitor, Clk1/4 depletion, heat shock (Fig. 5, C and D), and osmotic stress (Zhong et al., 2009) promote both dephosphorylation of SR proteins and splicing of *Clk1/4* intron-retaining RNAs (Fig. 3 A and Fig. 5), the dephosphorylation of SR proteins may be a trigger for promotion of the suspended splicing of *Clk1/4* pre-mRNA. In support of this, an in vitro cross-linking experiment indicated that some SR proteins bind to the highly conserved region of *Clk1* mRNA (unpublished data). Dephosphorylation of RS domains alters the protein–protein and protein–RNA interactions and/or localization of SR proteins (Cáceres et al., 1997; Xiao and Manley, 1997; Misteli et al., 1998; Lai et al., 2000; Lin et al., 2005). Heat shock stress causes relocalization of some SR proteins to nuclear stress body (Biamonti and Vourc'h, 2010), and the subnuclear architectures are affected by various kinds of stresses. SRSF1 (SF2/alternative splicing factor), SRSF9 (SRp30c), and SRSF7 (9G8) are reported to be recruited to nuclear stress bodies by heat shock (Denegri et al., 2001; Metz et al., 2004). As we reported, SRSF4 was accumulated in speckles by Clk1 inhibition (Yomoda et al., 2008). Thus, relocalization of SR proteins induced by their dephosphorylation may trigger restart of the suspended splicing of *Clk1* intron-retaining RNA.

The recruitment of SF3b to a premature spliceosome is required for its maturation (Roybal and Jurica, 2010). Therefore, the SF3b inhibitor FR901464 usually suppresses a premature spliceosome from reaching the mature stage, resulting in inhibition of splicing and nuclear retention of pre-mRNA (Kaida et al., 2007). On the other hand, the release of SF3b from a mature spliceosome is also needed to initiate splicing reaction (Lardelli et al., 2010). We currently hypothesize that the mature spliceosome is already assembled on intron 3/4 of the intron-retaining *Clk1/4* RNAs. This hypothesis can explain our observation that the *Clk1/4* intron-retaining RNAs are anchored in the nucleus until the splicing reaction is completed because SF3b is required not only for splicing but also for nuclear retention of pre-mRNA (Kaida et al., 2007).

### Clk1/4 plays a key role on rephosphorylation of SR proteins after stress

Depletion of *Clk1/4* pre-mRNAs and a consequent shortage of *Clk1/4* mRNAs delayed the recovery of SRSF4 and SRSF10 phosphorylation reduced by heat shock (Fig. 6, B and C). This is well consistent with the results of Clk1/4 knockdown

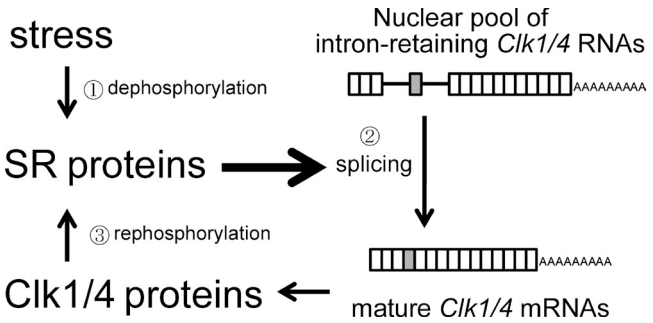


Figure 8. **ReSCUE (rephosphorylation of SR proteins by Clk urgent expression), a model for homeostatic control of SR protein phosphorylation.** (1 and 2) A stress induces dephosphorylation of SR proteins (1) followed by promotion of stress-responsive splicing of intron-retaining *Clk1/4* RNAs (2). (3) Clk1/4 proteins translated from newly matured *Clk1/4* mRNAs rephosphorylate SR proteins.

(Fig. 5, C and D) and our previous study showing that Clk1 and Clk4 are unique kinases for hyperphosphorylation of SRSF4 among known SR protein kinases, such as Clks, SRPKs, DYRKs, and Prp4 (Yomoda et al., 2008). Dephosphorylation of SR proteins, such as SRSF10, mediates heat shock–induced splicing arrest (Shin et al., 2004; Shi et al., 2006; Shi and Manley, 2007). Thus, Clk1/4-dependent rephosphorylation of SR proteins is considered to play a key role in resumption of splicing activity after stress removal. In comparison with SRSF4 and SRSF10, rephosphorylation of SRSF6 and SRSF5 just seems to partially depend on the Clk1/4 activity (Fig. 6, B and C), suggesting that other SR kinases also serve in rephosphorylation of these SR proteins.

Previous studies about SR protein kinases and their stress-induced regulation were mainly predicated on the idea that the stress-induced regulation of kinase activity is directed to phosphorylation changes of SR proteins and alteration of splice site selections under stress conditions (Jang et al., 2009; Zhong et al., 2009). Here, we show that stress-induced *Clk1/4* expression serves in recovery from stressed conditions, providing new insights into the role of SR protein kinases in stress response. For instance, viral infection, like heat shock, activates protein phosphatase PP1 and dephosphorylates SR proteins (Kanj et al., 2006), resulting in interruption of viral RNA processing. This is assumed to be a protective response of host cells by inhibiting viral replication and propagation. Actually, overexpression of SRPK2 or SR protein facilitated viral production (Fukuhara et al., 2006), and administration of TG003, a Clk1/4 inhibitor, also suppressed influenza virus proliferation (Karlas et al., 2010). In contrast, for host cell reactivation at a period of latent infection, the elevation of SR protein kinase activity should be required.

Here, we advocate a novel molecular mechanism named ReSCUE (rephosphorylation of SR proteins by Clk urgent expression) for the recovery of SR protein phosphorylation after stress removal. Our experimental data indicate that Clk1/4 proteins supplied through stress-induced splicing of the intron-retaining pre-mRNAs are essential for rapid recovery of the phosphorylation state of SR proteins and contribute to the restart of splicing reactions after stress removal (Fig. 8). Considering the unique induction system and enzymatic properties of

Clk1/4, they may serve as a guardian to maintain the phosphorylation state of SR proteins to expand the survival chance of cells after the stress.

## Materials and methods

**Cell culture, stress response experiment, and transfection**

Mammalian cells were cultured in Dulbecco's modified Eagle's medium (Nacalai Tesque) supplemented with antibiotics (100 U/ml streptomycin and 100 µg/ml penicillin; Sigma-Aldrich) and 10% fetal calf serum at 37°C in 5% $CO_2$. For heat shock induction, the medium was changed to prewarmed (43°C) medium and incubated at 43°C in an incubator with 5% $CO_2$. To perform time course analysis of heat shock recovery, after heat shock for 60 min, the medium was changed to a new one at 37°C, and the cells were cultured at 37°C. For a control experiment of heat shock in Fig. 7 C (lanes 9–11) and Fig. S4 C, the medium was changed to a new one at 37°C instead of a prewarmed (43°C) one, cultured for 60 min at 37°C, and changed again to a new medium at 37°C. Osmotic stress was induced by treatment with 600 mM sorbitol (Nacalai Tesque). FR901464 was provided by H. Nakajima (Astellas Pharma, Tsukuba-shi, Ibaraki, Japan). For transfection, a plasmid was transfected into the cell using Lipofectamine 2000 (Invitrogen) 16–18 h before the experiment. pME-HA-mCLK1 vector, in which HA-tagged *mClk1* is induced by the SR-β promoter, was prepared by cloning of *mClk1* ORF cDNA into the pME-HA empty vector as previously described (Kojima et al., 2001).

**siRNA knockdown**

For siRNA knockdown, we used Stealth siRNA with Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's manual. The cells were reverse transfected with a mixture of three kinds of siRNAs against each of Clk1 or Clk4 (totaling six kinds of siRNAs for double knockdown of Clk1 and Clk4 at 10 nM each) or siRNA against 60 nM luciferase as a control for 3 d before an assay. Product names of the siRNAs used were as follows: Clk1 (Clk1-MSS273707, Clk1-MSS273708, and Clk1-MSS273709), Clk4 (Clk4-MSS203021, Clk4-MSS203022, and Clk4-MSS203023), and luciferase (Luciferase Reporter Control).

**Northern blot analysis**

A single-strand oligonucleotide probe was radiolabeled by an end labeling reaction using T4 polynucleotide kinase with [$^{32}$P]ATP. The probe was hybridized on mouse multiple tissue Northern blot membrane (Takara Bio Inc.) in hybridization buffer (PerfectHyb Plus; Sigma-Aldrich) at 42°C for 16 h and washed twice in 2× SSC with 0.1% SDS and twice in 0.2× SSC with 0.1% SDS at 42°C. Then, the membrane was exposed to an imaging plate (Fujifilm) and analyzed using the Bio-image Analyzer system (Fujifilm). Probe sequences were as follows: *mClk1* exon 1/2 probe, 5′-TGAATGTCTCATCTTTCCAACCCTGAAGACAAAGCTTGCAAG-3′; *mClk1* intron 3 probe, 5′-ATCTGTGGCATTCACTCAAACATAGTGTCCTAAATTCATACTACA-3′; *mClk1* intron 4 probe, 5′-ATGAGAACAATGTACTTATAAGTATTATCAATGCAGATTATTCTAGGTG-3′; and *Gapdh*, 5′-GGCAGTGATGGCATGGACTGTGGTCATGAGCCCTTCCACAATGCCAAAG-3′.

**Nucleus/cytoplasm fractionation**

Nucleus/cytoplasm subfraction was prepared according to Mili's method (Mili et al., 2001) with minor modification. Culture cells on a 35-mm dish were suspended in 200 µl RSB-100 (10 mM Tris-HCl, pH 7.4, 100 mM NaCl, and 2.5 mM $MgCl_2$) containing 0.1% Triton X-100 and centrifuged at 2,000 *g* for 2 min at 4°C. 100 µl supernatant (cytoplasmic fraction) was lysed in 1 ml Sepasol-RNA I Super (Nacalai Tesque). Pellet (nuclear fraction) was resuspended in 200 µl RSB-100 buffer, and 100 µl of this was lysed in 1 ml Sepasol-RNA I Super. Then, the samples were subjected to RT-PCR as described in the Semiquantitative and quantitative RT-PCR section.

**Splicing analysis of *β-globin* minigene reporter**

*β-globin* minigene expression vector was constructed by replacing EGFP of pEGFP-C1 (Takara Bio Inc.) with *β-globin* minigene (provided by A. Yamashita and S. Ohno, Yokohama City University, Kanazawa-ku, Yokohama, Japan; Yamashita et al., 2001) using NheI–BglII sites. Then, the highly conserved region of mouse Clk1 (as shown in Fig. S3) was inserted to the second intron of *β-globin* minigene expression vector using NotI–SpeI sites made by inverted PCR as schematically illustrated in Fig. 4 A. NIH-3T3 cells on a 12-well plate were transfected with 0.2 µg reporter vector and cultured in the presence or absence of TG003 for 16 h. Then, the splicing pattern of the reporter RNA was analyzed by RT-PCR with (Fig. 4 B, RT+) or without (Fig. 4 B, RT–) reverse transcription. Primers for the reporter were as follows: *β-globin* exon forward primer, 5′-TCGAGATCTGGCAGAATCCAGATGCTCAAG-3′; *β-globin* exon reverse primer, 5′-GCTAGCAATCTACTCCCAGGAGCAG-3′; *β-globin* exon junction forward primer, 5′-AGGCCCTGGGCAGGCTGC-3′; and *Clk1* intron reverse primer, 5′-TTACTTTAGTACCCTGTGGC-3′.

**Semiquantitative and quantitative RT-PCR**

Total RNAs were prepared from culture cells, mouse tissues, and subcellular fraction with Sepasol-RNA I Super according to the manufacturer's instructions. If necessary, the RNAs were treated with RNase-free DNase (RQ1; Promega) according to the manufacturer's manual. First-strand cDNA was synthesized using reverse transcription (Superscript II RNase H−; Invitrogen), usually with oligonucleotide deoxythymidylic acid primer, and amplified by PCR using an appropriate set of primers. In stress induction experiments, total RNA was reverse transcribed with random hexamer because stress, such as heat shock, sometimes causes degradation of the poly(A) tail. For semiquantitative RT-PCR, PCR products were separated by electrophoresis and stained with ethidium bromide. Images were obtained with a gel imaging system (ChemiDoc; Bio-Rad Laboratories) and analyzed with ImageJ software (National Institutes of Health). Primers for semiquantitative RT-PCR were as follows: *Clk1* exon 1 forward, 5′-TTCTCGCCGTCGCCTTGTAAGC-3′; *Clk1* exon 13 reverse, 5′-TCACGTATGCTTTTTAAGTGG-3′; *Clk1* exon 2 forward, 5′-ATGAGACATTCAAAGAGAACTTACTG-3′; *Clk1* exon 5 reverse, 5′-CACTTTATGATCGATGCATTCC-3′; *Clk1* intron 3 forward, 5′-ATGTGTTTATGTTTTGTTGAC-3′; *Clk1* intron 4 reverse, 5′-AACTTGATTTACTGCTGTGG-3′; *Clk2* exon 1 forward, 5′-CACGGGCCTCGCCGCCAGAACG-3′; *Clk2* exon 13 reverse, 5′-TCTGCCTCTGAGGTCAGATACC-3′; *Clk4* exon 1 forward, 5′-AAGGCCGCTTGCTTTTGCGGAG-3′; *Clk4* exon 13 reverse, 5′-TCATGCTCTTCGTCATGACACAGC-3′; *Clk4* exon 6 reverse, 5′-ACTGCTACATGTAAGCCATCC-3′; *Neat1* forward, 5′-TTGGCAGACTCTTCTGGGTCTC-3′, and reverse, 5′-TCACAAAACAACCACATATACAG-3′; *Gapdh* forward, 5′-CCATCACCATCTTCCAGGAGCGAG-3′, and reverse, 5′-GTGATGGCATGGACTGTGGTCATG-3′; and *Hsp1b* forward, 5′-AAGGAGCCTCTGGCTCAGGAC-3′, and reverse, 5′-AACCTACTTAGATTAAATGC-3′. Primers used in Fig. 7 A were as follows: *Clk3* forward, 5′-ATCTTGTTTGTGAATTCTGAG-3′, and reverse, 5′-AACTCTAACATCCTCCTCATCAG-3′; *Srpk1* forward, 5′-AGCTCCAAGTGAAGATCGCAG-3′, and reverse, 5′-TTAGGAGTTTAGCCAAGGATGC-3′; *Srpk2* forward, 5′-TTATACAGAGACAGCCTTGG-3′, and reverse, 5′-TCAATATGGCCATTGGCTTTCG-3′; *Dyrk1a* forward, 5′-TCATCCTGTTCAAGAGACAACC-3′, and reverse, 5′-AACTCCTGTCATGGGAGACTC-3′; *Dyrk2* forward, 5′-ACTCCTGGATGCTTCCAAACGAGC-3′, and reverse, 5′-TCTTCAGATTGCCAGCATCAG-3′; *Prp4k* forward, 5′-TACAGTCAGCAGTTGTTCCTGG-3′, and reverse, 5′-TTGATGCTAATTCGCTTAGCTG-3′; *Srsf4* forward, 5′-ATGCCGCGGGTGTACATCGG-3′, and reverse, 5′-AACCTGGCTTGTCTTCAACTAATCTGA-3′; and *Srsf10* forward, 5′-CCATGTCCCGATACCTGCGCC-3′, and reverse, 5′-TCAGTGGCCACTGGACTTGG-3′. For quantitative RT-PCR, we performed real-time PCR using FastStart Universal SYBR Green Master (Roche) with Eco Real-Time PCR System (Illumina) according to the manufacturer's instructions. Quantification data are represented as means ± SEM of three independent experiments. Primer sets for quantitative RT-PCR were as follows: *Clk1* mature forward, 5′-ACCATTCACACGGGAAGAGTCAC-3′, and reverse, 5′-TTACTGCTACACGTCTACCTCC-3′; intron-retaining *Clk1* forward, 5′-TTGCAGCATCACCTAGAATAATCTG-3′, and reverse, 5′-TTACTGCTACACGTCTACCTCC-3′; *Neat1* forward, 5′-TTGGCAGACTCTTCTGGGTCTC-3′, and reverse, 5′-TGTGTTTTGAAGGCATGGCTCAC-3′; and *Gapdh* forward, 5′-AGACAAAATGGTGAAGGTCGGTG-3′, and reverse, 5′-AACATGTAGACCATGTAGTTGAG-3′.

**In situ hybridization**

Probe templates (356 nt) were amplified from HeLa genome DNA by PCR using primers for *hClk1* intron 4 forward, (5′-GTATAGAATATTTTTCAACAC-3′) and reverse (5′-AGTCATATCAAGAAGTGG-3′) and cloned into a pGEM-T easy vector (Promega). Then, dig-labeled RNA probes were synthesized by in vitro transcription with T7 (sense probe) or SP6 (antisense probe) RNA polymerase from the linealized plasmid using a dig-labeling kit (Roche). The cells cultured on a cover glass were fixed with 10% formaldehyde neutral buffer (Nacalai Tesque) for 15 min and treated with 3% $H_2O_2$ for 30 min. After washing with RNase-free water, the samples were gradually dehydrated with ice-cold ethanol and dried. Then, the dig-labeled probe was hybridized in hybridization buffer (50% formamide, 2× SSC, Denhardt's solution, 0.2% SDS, 5% dextran sulfate, 1 mM EDTA, and 100 µg/ml tRNA)

at 42°C for 16 h. After hybridization, the samples were washed in 2× SSC/50% formamide twice at 42°C and in 2× SSC twice at 42°C. Subsequently, the dig-labeled probe was visualized with the anti-dig monoclonal antibody (Roche) and Alexa Fluor 555–conjugated goat anti–mouse IgG antibody (Invitrogen). The nuclei were stained with Hoechst 33342. Images were obtained at RT with fluorescence microscopy (IX81; Olympus) using a camera (DP30BW; Olympus) and DP-BSW software (Olympus) with a 20×, NA 0.45 objective lens (LucPlanFLN; Olympus) and processed with Photoshop (Adobe).

**Immunoblot and analysis of phosphorylation states of SR proteins**

Phosphorylation states of SR proteins were estimated by immunoblotting using the antiphospho-SR protein antibody (mAb1H4), which is reactive to a phosphorylated RS domain. Culture cells were suspended with the same volume of 2× SDS sample buffer (200 mM dithiothreitol, 100 mM Tris-HCl, pH 6.8, 4% SDS, and 20% glycerol) and boiled for 5 min. The samples were separated by SDS-PAGE and transferred to a polyvinylidene fluoride membrane (Millipore) by electroblotting. After the membranes had been incubated with primary and HRP-conjugated secondary antibodies, the signals were developed by chemiluminescence reaction with ImmunoStar Kit (Wako Chemicals USA), detected with ChemiDoc, and analyzed using ImageJ software. For quantification, the phosphorylation state of each SR protein and the amount of Clk1/4 proteins were quantified by their intensity ratios to α-tubulin and normalized to that of the control cells at 37°C (for example, Fig. 6 B, lane 1). The means and SEM were calculated from three independent experiments. The significance of differences was determined by Student's *t* test. The antibodies used were as follows: antiphospho-SR protein monoclonal antibody (1H4; Santa Cruz Biotechnology, Inc.), anti-Clk1 polyclonal antibody, which is considered to cross react with Clk4 (ab74044; Abcam), anti–α-tubulin monoclonal antibody (NeoMarkers), anti-GAPDH monoclonal antibody (Invitrogen), and HRP-conjugated secondary antibodies (Abcam).

**Online supplemental material**

Fig. S1 shows RT-PCR analyses of *Clk1* intron-retaining RNA. Fig. S2 shows Clk1/4 knockdown with siRNAs. Fig. S3 shows alignment of mouse and human *Clk1* and mouse *Clk4* in the region of intron 3, exon 4, and intron 4. Fig. S4 shows treatment of NIH-3T3 cells with FR901464 and cycloheximide. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201107093/DC1.

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