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The splicing factor SC35 has an active role in transcriptional elongation

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Abstract

Mounting evidence suggests that transcription and RNA processing are intimately coupled *in vivo*, although each process can occur independently *in vitro*. It is generally thought that polymerase II (Pol II) C-terminal domain (CTD) kinases are recruited near the transcription start site to overcome initial Pol II pausing events, and that stably bound kinases facilitate productive elongation and co-transcriptional RNA processing. Whereas most studies have focused on how RNA processing machineries take advantage of the transcriptional apparatus to efficiently modify nascent RNA, here we report that a well-studied splicing factor, SC35, affects transcriptional elongation in a gene-specific manner. SC35 depletion induces Pol II accumulation within the gene body and attenuated elongation, which are correlated with defective P-TEFb (a complex composed of CycT1–CDK9) recruitment and dramatically reduced CTD Ser2 phosphorylation. Recombinant SC35 is sufficient to rescue this defect in nuclear run-on experiments. These findings suggest a reciprocal functional relationship between the transcription and splicing machineries during gene expression.

Transcription and pre-mRNA splicing are key steps in gene expression in eukaryotic cells. Previous studies have provided ample evidence for temporal coupling between these two fundamental processes to allow efficient 5' capping and intron removal on nascent transcripts during transcription^{1,2}. Because of such intimate coupling, many spliceosome components are found in close contact with template DNA, detectable by chromatin immunoprecipitation (ChIP) in both yeast^{3–5} and mammalian cells^{6,7}. Functionally, such

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AUTHOR CONTRIBUTIONS

X.-D.F. and S.L. designed the experiment; S.L. developed the modified nuclear run-on assay and performed most initial experiments; G.C.-M. repeated most experiments; D.W. performed the ChIP-DSL analysis; S.P. contributed to analysis of nascent RNA; all authors participated in writing the paper.

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temporal coupling allows the recognition of emerging splicing signals on nascent transcripts in a timely manner by crucial splicing factors in competition with other RNA packaging proteins⁸. As a consequence of co-transcriptional recognition of splicing signals, the rate of transcriptional elongation influences splice-site selection in yeast and mammalian cells^{9,10}.

The CTD of the largest Pol II subunit serves as a loading pad for various RNA processing factors, including those involved in 5' capping, splicing, polyadenylation and RNA export^{11–14}. These physical tethering events are dependent on CTD phosphorylation at Ser5 and/or Ser2 positions, which is concurrent with the transition of the Pol II complex from initial promoter clearance to productive elongation^{15,16}. Ser5 phosphorylation is catalyzed by TFIIF (a complex consisting of CycH–CDK7), which signals 5' capping and histone H3 lysine 4 (H3K4) trimethylation at the initial pausing site ~30 nucleotides (nt) downstream from the transcription start¹⁷; Ser2 phosphorylation is mediated by P-TEFb, which is required for the Pol II complex to enter the mode of productive elongation^{15,18,19}. Other kinases, such as CDK8, are also found to contribute to CTD phosphorylation²⁰. Accordingly, Ser5 phosphorylation peaks early near gene promoters, remains associated with the gene body and declines toward the 3' end, whereas Ser2 phosphorylation is predominantly detected within gene bodies and at the 3' end²¹. It is generally believed that all CTD kinases are recruited at initial promoter-proximal pausing sites, and stably bound kinases have a key role in processive transcriptional elongation and tethering of various machineries to the elongating Pol II complex for co-transcriptional RNA processing^{15,20}.

Whereas most studies focus on co-transcriptional RNA processing, little is known as to whether RNA processing machineries may also influence transcription. We know that the transcription elongation factor TAT-SF1 copurifies with nearly all spliceosome components, which in turn stimulates Tat-dependent transcription elongation²². However, which splicing factor(s) in the complex is responsible for the observed transcription enhancing activity remains undefined. More recently, a previously characterized transcriptional regulator called SKIP was shown to have a crucial role in Tat-dependent transcription⁷. Interestingly, mammalian TAT-SF1 and SKIP correspond to Cus2 and Prp45, respectively, which have been implicated in pre-mRNA splicing in budding yeast^{23,24}, and both co-purify with the spliceosome in mammalian nuclear extracts²⁵. Furthermore, 5' capping was also tied to early transcriptional elongation through the elongation factor Spt5 (refs. 2,26–28). These findings suggest multiple functional ties between transcription and co-transcriptional RNA processing.

Serine/arginine-rich (SR) proteins are a class of RNA binding proteins capable of kinetically committing pre-mRNA to the splicing pathway^{29,30}. Consistent with their crucial role in both constitutive and regulated splicing in higher eukaryotic cells, SR proteins are essential for cell viability in chicken DT40 cells³¹ and primary mouse embryonic fibroblasts (MEFs)^{32,33}. However, inactivation of individual SR proteins does not seem to cause a widespread defect in splicing of most cellular genes^{34,35}, although it has been noted that heterogeneous nuclear RNAs accumulate to some extent in both chicken DT40 cells and *Drosophila melanogaster* S2 cells in response to inactivation of a specific SR protein^{36,37}. The lack of a major impact on splicing has been generally attributed to the functional redundancy of SR proteins *in vivo*, which is thought to protect most constitutive splicing

events against variation or malfunction of any given SR protein in the cell. It is also possible that some important functional requirement for SR proteins in gene expression might have escaped detection during analysis of total RNA in SR protein knockout or knockdown cells. We have therefore been addressing this crucial issue by analyzing nascent transcripts in MEFs derived from conditional SR protein knockout mice.

Here we report an unexpected requirement for the SR family of splicing-commitment factors in Pol II transcription *in vivo*. By analyzing nascent transcripts, we detected a widespread defect in transcription in response to *in vivo* depletion of two prototypical SR proteins, SC35 and SF2 (ASF in mammals). Tiling array analysis revealed Pol II accumulation on the body of selective genes in response to SC35 depletion, suggesting a critical role of the SR protein in transcriptional elongation. Notably, we found that impaired transcriptional elongation could be functionally rescued by recombinant SC35 in an improved nuclear run-on assay. The defect in transcriptional elongation was further attributed to inefficient P-TEFb recruitment to the Pol II complex and diminished CTD Ser2 phosphorylation in SC35-depleted cells. On the basis of these findings and the well-documented function of SR proteins in splicing, we propose a model for further testing where SR proteins scan nascent transcripts to facilitate transcriptional elongation and concurrent commitment of pre-mRNAs to the splicing pathway.

RESULTS

Global transcriptional defect in SR protein–depleted cells

We previously engineered two MEF lines for SF2/ASF and SC35 in which each endogenous SR protein gene was deleted and the cell viability complemented by expressing an exogenous SR protein from a tetracycline (tet)-off promoter^{32,33} (Fig. 1a). The exogenous SR protein is largely depleted 4 d after adding a tetracycline analog, doxycycline (Dox), to the culture medium; however, the cells remain viable (assayed by their ability to incorporate 5'-bromo-2'-deoxy-uridine (BrdU)) for 10–12 d³³. We thus chose to analyze the cells 5 d after Dox-induced depletion of SR proteins. To detect nascent transcripts, we labeled the Dox-treated (–SR) and mock-treated (+SR) cells with ³H-uridine followed by trichloroacetic acid (TCA) precipitation to determine the level of ³H-labeled total RNA under each condition. Unexpectedly, we found that the expression of nascent RNA was dramatically attenuated in response to *in vivo* depletion of both SF2/ASF and SC35, with a more severe defect observed on SC35-depleted cells (Fig. 1b). Because TCA precipitates both processed and unprocessed RNA, the reduction in the level of total nascent transcripts suggests a general defect in transcription in SR protein–depleted cells.

To further characterize SR protein–dependent transcription, we analyzed oligo(dT)-selected polyadenylated (poly(A)⁺) mRNAs, revealing that the population of ³H-labeled nascent poly(A)⁺ mRNA was reduced within the total poly(A)⁺ mRNA pool in SR protein–depleted cells relative to mock-depleted cells, suggesting that Pol II transcription was impaired upon *in vivo* depletion of individual SR proteins (Fig. 1c). To visualize directly the transcriptional defect at the single-cell level, we performed *in situ* analysis of bromuridine (BrU)-labeled nascent RNA (Fig. 1d–k). We observed that Pol II transcripts, which are diffusely localized in the nucleus (Fig. 1e), were diminished, whereas Pol I transcription in the nucleolus was

relatively unaffected (Fig. 1g). The lack of effect on Pol I transcription is consistent with the exclusion of SR proteins from the nucleolus³⁸ and with the colocalization of remaining BrU signal with a nucleolar marker in SC35-depleted cells (Fig. 1g, inset). We found further evidence of a reduction in the level of nascent Pol II transcripts in response to SC35 depletion by selectively inhibiting Pol I transcription with a low dose of Actinomycin D (ActD), which highlights the impact of SR protein depletion on Pol II transcription by removing the Pol I transcription signal from the nucleolus (Fig. 1h–k). Together, these results demonstrate that depletion of specific SR proteins causes a widespread defect in Pol II-mediated transcription, indicating that SR proteins may have an unexpected role in transcription, in addition to their established function in pre-mRNA splicing.

SC35 depletion-induced Pol II accumulation on gene bodies

Impaired Pol II transcription might be triggered by catastrophic events due to defective processing of some undefined cellular genes, resulting in the malfunction of the core transcriptional machinery in SR protein-depleted cells. If this were the case, we would expect a widespread defect in the assembly of Pol II-containing transcription complexes on endogenous genes. To test this possibility, we performed tiling array analysis using the sensitive chromatin immunoprecipitation DNA selection and ligation (ChIP-DSL) technology developed in our laboratory³⁹. On a custom tiling array covering ~40 mouse genomic loci, we performed location analysis of hypophosphorylated Pol II, trimethylated H4K4 (H3K4me3) and acetylated histone H3 at lysine 9 (H3K9ac), which localize to promoters and enhancers of active genes, mark the initial promoter-proximal pausing sites and report the action of many transcription coactivators that show histone acetyltransferase (HAT) activities, respectively^{40–42}. Unexpectedly, we detected no defects in histone modifications or the assembly of Pol II-containing complexes on any active gene promoters examined in either SC35 or SF2/ASF-depleted cells (Fig. 2 and Supplementary Figs. 1 and 2 online). Instead, we observed SC35 depletion-induced Pol II accumulation in several, but not all, transcription units on the tiling array, three representatives (two positive and one negative) of which are shown in Figure 2a–c. In this experiment, SC35 and SF2/ASF acted as controls for each other, as depletion had specific effects.

To validate the tiling array results, we performed ChIP and quantitative PCR (qPCR) on the representative genes using several primer sets corresponding to different regions along each gene in SC35-depleted cells (Fig. 2a–c, below). In the case of the poly-pyrimidine tract binding protein 1 (*Ptb1*) gene, although Pol II binding at the promoter was similar, elevated Pol II binding was detected on the gene body (Fig. 2a), consistent with the tiling array result. We also detected elevated Pol II binding near the gene promoter and on the gene body of the ets domain-containing gene 1 (*Ets1*) in SC35-depleted cells relative to mock-depleted cells (Fig. 2b). In contrast, Pol II binding on the promoter of the *Ptb2* gene was unaffected by SC35 depletion, and no Pol II accumulation was induced on the body of the gene (Fig. 2c). These data indicate that the initial shutdown of transcription in SC35-depleted cells may not result from a cascade of indirect defects that lead to a general failure in transcription initiation, although such a failure is expected to occur ultimately in the cell. Instead, the observed Pol II accumulation on gene bodies raises the possibility that depletion of SC35 may have induced Pol II pausing during transcriptional elongation in a gene-specific

manner. Given its impact on Pol II transcription (Fig. 1b,c), SF2/ASF depletion may have caused a similar effect on a distinct group of genes not covered by our existing tiling array. In the present study, we focused on SC35.

Attenuated transcriptional elongation upon SC35 depletion

The accumulation of Pol II on gene bodies implicates SC35 in transcriptional elongation. To test this possibility directly, we analyzed the production of intron-containing nascent transcripts by performing qPCR on DNase I-treated total RNA using several primer pairs that interrogate different intronic regions on the representative *Ptb1* gene to determine potential blockage of transcriptional elongation. We detected a slight reduction at the beginning of the gene (indicated by the p1 primer pair) and dramatic attenuation in the middle and end portions of the gene (indicated by the p2 to p4 primer pairs) in SC35-depleted cells, but not in SF2/ASF-depleted cells (Fig. 3a). A similar result was also obtained with the *Ets1* gene (data not shown).

To demonstrate directly a transcriptional elongation defect in SC35-depleted cells, we modified the existing nuclear run-on assay⁴³ to analyze nascent transcripts (Fig. 3b). We replaced Nonidet P-40 in the standard nuclear run-on protocol with digitonin, permeabilizing the cells to deplete NTPs but preserve cell integrity and minimize leakage of nuclear factors, such as those involved in transcriptional elongation. We next labeled nascent transcripts with BrUTP, instead of ³²P-labeled UTP, allowing specific and sensitive detection of nascent transcripts by PCR after anti-BrU immunoprecipitation. To demonstrate the specificity of this approach, we pulse-labeled the cell with BrU or uridine by directly adding these chemicals to the culture medium to allow incorporation into nascent transcripts⁴⁴. After transient labeling, anti-BrU-immunoprecipitated RNA (note that the antibody recognizes both bromouridine and bromodeoxyuridine) was analyzed by RT-PCR. We detected the nascent *Ptb1* transcript in BrU-labeled cells, but not in cells labeled with uridine or cells treated with BrU in the presence of the transcription inhibitor ActD (Fig. 3b). This experiment demonstrated the specificity of BrU labeling and anti-BrU immunoprecipitation.

We next prepared both mock-depleted (+SC35) and SC35-depleted (–SC35) cells and subjected them to the nuclear run-on assay (Methods). NTPs and BrUTP were added to the reaction to permit re-elongation of stalled Pol II by depletion of endogenous NTPs. Following immunoprecipitation, we analyzed the BrU-labeled nascent *Ptb1* transcript by RT-PCR using a set of ³²P-labeled primer pairs. In this experiment, we included an internal control for analysis of RNA using a primer pair targeting an upstream region (primer pair A) and additional primer pairs against several locations in the *Ptb1* gene to fine map elongation decrease in response to SC35 depletion. We found that *Ptb1* transcription was indeed attenuated in response to SC35 depletion (Fig. 3c) after a series of Pol II pausing events on the *Ptb1* gene (Fig. 2a). We repeated the experiments several times and quantified the results by RT-qPCR, which unequivocally demonstrated that SC35 is crucial for transcription elongation *in vivo* (Fig. 3d). We note transcript reduction at and after probe p2 when quantifying total RNA (Fig. 3a) and attenuated transcription at and after probe g when

analyzing nascent RNA (Fig. 3c,d). One explanation for this difference might be degradation of aborted transcripts when total RNA was analyzed.

The effect of SC35 depletion on transcriptional elongation may reflect a direct role of this splicing factor in this crucial process of gene expression or an indirect effect on the expression of a general transcription elongation factor(s) in the cell. To distinguish between these possibilities, we attempted to functionally rescue the nuclear run-on assay using recombinant SC35 expressed in baculovirus-infected insect cells. Remarkably, purified SC35 was sufficient to release stalled transcription, restoring elongation of nascent *Ptb1* transcript as measured using radioactive PCR primers (Fig. 4a) and by qPCR (Fig. 4b). Purified SC35 had little effect on the preparation from mock-depleted cells, suggesting that the endogenous SC35 protein associated with the elongating complex is necessary and sufficient for transcriptional elongation and that the functional rescue is not simply due to a general stimulating activity of SC35 in the nuclear run-on assay (Fig. 4c). Finally, rescue is specific to SC35, because other purified SR proteins, such as SF2/ASF and 9G8, did not prevent the defect in transcriptional elongation caused by SC35 depletion (Fig. 4d). Together, these data demonstrate a specific role of SC35 in transcriptional elongation on the *Ptb1* gene.

Mechanism of SC35-dependent transcriptional elongation

Co-immunoprecipitation experiments⁴⁵ and direct proteomics analysis of Pol II-associated proteins⁴⁶ have shown that SR proteins directly or indirectly interact with the Pol II CTD. To confirm and extend these findings, we performed co-immunoprecipitation on SC35 knockout MEFs complemented with a hemagglutinin-tagged SC35. Following anti-hemagglutinin immunoprecipitation, we confirmed the association of SC35 with Pol II by western blotting (Fig. 5). More importantly, we also detected co-immunoprecipitation of SC35 with CDK9, the catalytic component of the P-TEFb kinase, and TAT-SF1, indicating that SC35 is present in the elongating Pol II complex (Fig. 5a). The immunoprecipitation assay is specific, because we did not detect a signal after suppression of SC35 expression using Dox (see –SC35 lanes in Fig. 5a).

To determine whether the status of Pol II phosphorylation might be altered in SC35-depleted cells, we probed both hypo- and hyper-phosphorylated Pol II by western blotting using phosphospecific antibodies. Notably, we found that the level of Pol II Ser2 phosphorylation was reduced by ~40% in SC35-depleted cells, whereas Pol II Ser5 phosphorylation was largely unaffected (Fig. 5b). The reduction of Ser2 phosphorylation strongly suggests impaired recruitment of P-TEFb to the elongating Pol II complex in SC35-depleted cells. We tested this possibility by performing anti-Pol II immunoprecipitation before and after SC35 depletion and then probed CDK9 in the Pol II complex, finding that, indeed, the association of P-TEFb with Pol II was substantially reduced in SC35-depleted cells (Fig. 5c).

Given such a global impact of SC35 depletion on Pol II association with crucial transcriptional elongation factors, we next performed ChIP and qPCR on the *Ptb1* gene to determine whether SC35 depletion affected P-TEFb recruitment to the elongating complex on a specific gene and the phosphorylation state of Pol II in the complex. Our tiling array,

ChIP and qPCR analyses had indicated that hypo-phosphorylated Pol II detected by the antibody 8WG16 accumulated on the body of the *Ptb1* gene in response to SC35 depletion (Fig. 2a replotted in Fig. 5d for comparison). Consistent with the published results, this hypophosphorylated Pol II was largely detected at the promoter whereas Ser2 and Ser5 phosphorylated Pol II was associated with the body and the beginning of the gene, respectively²⁰. In response to SC35 depletion, both Ser2 phosphorylation and CDK9 binding to the body of the *Ptb1* gene were markedly reduced (Fig. 5e,g), whereas Ser5 phosphorylation remained relatively unaltered (Fig. 5f). Because hypophosphorylated Pol II is increased upon SC35 depletion, the reduction in Ser2-phosphorylated Pol II molecules (and, to some extent, Ser5-phosphorylated Pol II as well) might be more dramatic at the p2 position than was apparent. Therefore, opposite to the accumulation of hypophosphorylated Pol II on the gene body, its phosphorylation content at Ser2 positions was probably reduced as a result of impaired P-TEFb recruitment and/or its ability to phosphorylate the CTD in SC35-depleted cells. These findings suggest that P-TEFb may be dynamically recruited in an SC35-dependent manner at specific genes to facilitate transcriptional elongation in mammalian cells.

DISCUSSION

In studying the coupling between transcription and RNA processing, most studies have focused on understanding how the RNA processing machinery takes advantage of the transcription apparatus to achieve efficient co-transcriptional RNA processing. Here we present evidence that splicing machinery components may impact the transcriptional machinery during gene expression. Our findings provide new insights into a critical role of SC35 (and possibly other SR proteins) in gene expression, aside from its traditionally studied function in pre-mRNA splicing (summarized in Fig. 6).

Dynamic recruitment of P-TEFb to elongating Pol II complex

P-TEFb is responsible for catalyzing Pol II phosphorylation at Ser2 positions in its CTD, which has been linked to transcriptional elongation in many, but not all, genes^{15,20}. This kinase is also known to phosphorylate an elongation inhibitor in the promoter-proximal region, thereby releasing the Pol II complex from initial pausing sites^{47,48}. It has generally been thought that these steps are crucial for promoter escape of the Pol II complex and for the complex that carries the stably bound P-TEFb kinase to enter the mode of productive elongation. Our current work shows that the association of P-TEFb with Pol II and CTD Ser2 phosphorylation are both attenuated in SC35-depleted cells, suggesting that SC35 may have a critical role in directly or indirectly mediating dynamic recruitment of P-TEFb and other elongation factors to the Pol II complex during transcriptional elongation. This dynamic P-TEFb recruitment model also implies that various CTD kinases may be in constant competition with CTD-specific phosphatases, such as FCPs, during the transcriptional elongation phase, in addition to the documented role of the CTD phosphatases in transcription initiation^{17,49}.

Integration of the transcription and splicing machineries

RNA processing is more efficient co-transcriptionally than post-transcriptionally and, importantly, this effect requires Pol II-directed transcription, rather than the process of transcription *per se*, as tested using transcription directed by a phage polymerase⁸. Therefore, the coupling between transcription and splicing probably reflects various functional interactions between the transcription and splicing machineries². It has been extensively documented that multiple RNA processing factors, including SR proteins, interact directly or indirectly with the CTD of the largest subunit of Pol II in a phosphorylation-dependent manner²⁰.

Numerous studies have established that SR proteins initiate pre-mRNA splicing by kinetically committing pre-mRNA to the splicing pathway and facilitating spliceosome assembly²⁹. Reed and colleagues recently showed that SR protein-mediated co-transcriptional splicing is much more efficient than SR protein-dependent post-transcriptional splicing⁴⁶. Our current work suggests that SC35, and possibly other SR proteins, may have an even earlier function in gene expression, possibly acting as single-strand RNA binding proteins, to facilitate transcriptional elongation. SR proteins may initially 'scan' nascent RNA; such an early function may be transient and independent of splicing signals, consistent with splicing-independent function of SR proteins on some intronless genes^{50–52} (Fig. 6). We speculate that these SC35-dependent events may help recruit crucial transcriptional elongation factors to overcome strong Pol II pausing sites in template DNA and thus facilitate transcriptional elongation.

SR proteins have been demonstrated to have a fundamental role in cell proliferation. On the basis of our current finding that SR protein deficiency causes a widespread defect in transcription, we suggest that this defect in gene expression is responsible for cellular mortality in SR protein-depleted DT40 cells and MEFs.

METHODS

Cell culture

SF2/ASF and SC35 tet-repressible MEFs were derived from conditional SC35 and SF2/ASF knockout mice as previously described³². The endogenous SC35 or SF2/ASF gene was deleted by a Cre-expressing virus and the cells complemented by the corresponding cDNA of each gene expressed from a tet-off promoter. The MEFs were cultured in DMEM plus 10% FBS, 1 mM sodium pyruvate and antibiotics. SR protein was largely depleted in ~4 d in the presence of 10 $\mu\text{g ml}^{-1}$ of Dox, but treated cells remained viable up to 10 d. We quantified live cells by fixation with 0.5% glutaraldehyde in PBS followed by staining with 0.1% crystal violet overnight at 37 °C. We performed all assays on cells mock-treated or treated with Dox for 5 d.

Nascent RNA analysis by tritium and bromouridine labeling

In vivo tritium labeling was performed as described⁵³. Briefly, we added ³H-uridine (15 Ci mmol⁻¹) to cells in a 10 cm dish at a concentration of 0.5 $\mu\text{Ci ml}^{-1}$ for 2 h, washed the cell four times with ice-cold PBS followed by the addition of 10 ml cold 5% (v/v) TCA. After 5

min, we harvested the TCA-soluble fraction (TCA-soluble pool) and rinsed the remaining material twice with 5% (v/v) TCA, and hydrolyzed the acid-precipitated material in 10 ml 10% (v/v) TCA at 70 °C for 1 h. Aliquots (1 ml) of both soluble and TCA-precipitated and -hydrolyzed materials were counted in a Beckman scintillation counter. To select ³H-labeled mRNA, we harvested the cell in Trizol (Invitrogen) and purified Poly(A)⁺ mRNA using the Oligotex mRNA extraction kit (Qiagen).

To label nascent RNA with BrU, we incubated cells growing on coverslips with 2 mM BrU (Sigma-Aldrich) for 1 h. After extensive washing, we fixed pulse-labeled cells with 4% (v/v) paraformaldehyde in PBS for 20 min at room temperature, stained for 1 h with anti-BrU antibody (1:200; Sigma-Aldrich), and developed for 1 h with Alexa594-conjugated donkey anti-mouse IgG (1:500; Molecular Probes). Actin was simultaneously labeled with Alexa488-conjugated phalloidin (1 U ml⁻¹; Invitrogen). To inhibit Pol I activity, we treated the cell with 0.04 µg ml⁻¹ ActD for 3 h before adding BrU44. The nucleolus was stained with anti-Nucleophosmin (1:200; Aviva Systems Biology).

Tiling array analysis by chromatin immunoprecipitation DNA selection and ligation, and validation by chromatin immunoprecipitation and quantitative polymerase chain reaction

The ChIP-DSL assay was performed as previously described³⁹. Based on the ChIP-DSL method, we tiled 41 selected mouse genomic loci every 0.5 kb, excluding repeats. About 1×10^6 cells were used for each ChIP-DSL experiment using anti-H3K4me3 (07-473, Upstate), anti-H3K9ac (06-599, Upstate), and anti-RNAP (8WG16, Covance). Both input (5% of total DNA) and antibody-enriched DNA were cohybridized to the tiling array. After data normalization, we plotted the signals corresponding to individual probes against UCSC mouse genome assembly (mm5) as the Log₂ ratio of enriched over input. We carried out standard ChIP using the following antibodies: Anti-RNAP (8WG16, Covance), anti-Pol II phosphoSer2 (H5, Covance), anti-Pol II phosphoSer5 (H14, Covance) and anti-CDK9 (D-7, Santa Cruz). After ChIP, we analyzed the extracted DNA by qPCR using the primer pairs listed in Supplementary Figure 3 online.

Nuclear run-on and reverse transcriptase polymerase chain reaction assays

The nuclear run-on assay was carried out largely as described previously⁴³, with the following modifications. We used Digitonin (40 µg ml⁻¹; Calbiochem) in place of NP-40 to permeabilize cells. Instead of using ³²P-labeled NTPs, we used an NTP mix (1.8 mM ATP, 0.5 mM CTP and GTP, 0.375 mM UTP) plus BrUTP (0.125 mM); the control reactions for monitoring nonspecific binding were carried out with nuclei lacking added NTPs. We performed the run-on reaction at 25 °C for 15 min on $\sim 10^7$ permeabilized cells in the run-on buffer (50 mM Tris-HCl, pH 7.4, 10 mM MgCl₂, 150 mM NaCl, 25% (v/v) glycerol, 0.5 mM PMSF and 25 U ml⁻¹ RNasin) plus the NTP/BrUTP cocktail or no NTPs at all for the control tubes. After the reaction, we purified DNase I-treated total RNA using the Qiagen RNeasy mini kit from nuclei of both labeled and control samples. We preincubated 2 µl of anti-BrU antibody (B2531, Sigma) with 20 µl Protein G Dynabeads (Invitrogen) and 2.5 µg tRNA per sample for 1 h at 4 °C with rotation. After that, the beads were washed three times with RSB-100 buffer (10 mM Tris-HCl, pH 7.4, 100 mM NaCl, 2.5 mM MgCl₂ and 0.4% (v/v) Triton X-100) and resuspended in 150 µl RSB-100 with 40 U RNasin (Promega) and

2.5 μg tRNA. 25 μg of total RNA was then added and beads were incubated for an additional 1 h at 4 °C with rotation. At the end of the incubation period, the beads were washed three times with RSB-100 buffer and the RNA bound to beads was eluted by direct addition of 300 μl RLT buffer (Qiagen RNeasy mini kit) supplemented with 2 μg tRNA. The beads were discarded and eluted, and the RNA was subjected to another round of purification using the Qiagen RNeasy mini kit. RNAs were eluted in 30 μl RNase-free water.

cDNA was synthesized using 10 μl of the eluted RNA and 125 ng random hexamers with the Omniscript RT kit (Qiagen). We used 2 μl of the reverse transcription product for PCR using ^{32}P -labeled primers or qPCR. For semiquantitative PCR, 100 pmol of each forward primer was labeled with 1 μl ^{32}P - γATP (800 Ci mmol^{-1}). We performed PCR for 32 cycles in a 20 μl reaction containing 1 \times Invitrogen Taq buffer plus 2.5 mM MgCl_2 , 2.5 pmol ^{32}P -labeled forward primer and 2.5 pmol unlabeled reverse primer, analyzed 4 μl PCR product on a 6% Tris-borate with EDTA (TBE)-buffered polyacrylamide gel, and quantified on a Phosphorimager (Molecular Dynamics). The primer sets used in these studies are listed in Supplementary Figure 3.

SR protein co-immunoprecipitation

We washed $\sim 5 \times 10^6$ cells with cold PBS and resuspended them in 0.5 ml RSB-100 (10 mM Tris-Cl, pH 7.4, 100 mM NaCl, 2.5 mM MgCl_2) with 0.4% TritonX-100 and proteinase and phosphatase inhibitor cocktails (Sigma). We next incubated the cells on ice for 10 min and lysed them by pipetting up and down and by sonication (Heat Systems XL2005) twice, 5 s each time. We added 20 μl of protein A beads to absorb nonspecific binding followed by centrifugation at 13,000g for 10 min to remove cell debris. We incubated the supernatant with 20 μl anti-hemagglutinin beads (Roche) for 1 h at 4 °C, washed the beads three times with RSB-100 plus 0.4% TritonX-100 and once with RSB-100 without Triton, fractionated proteins in 8% SDS-PAGE and processed for western blotting.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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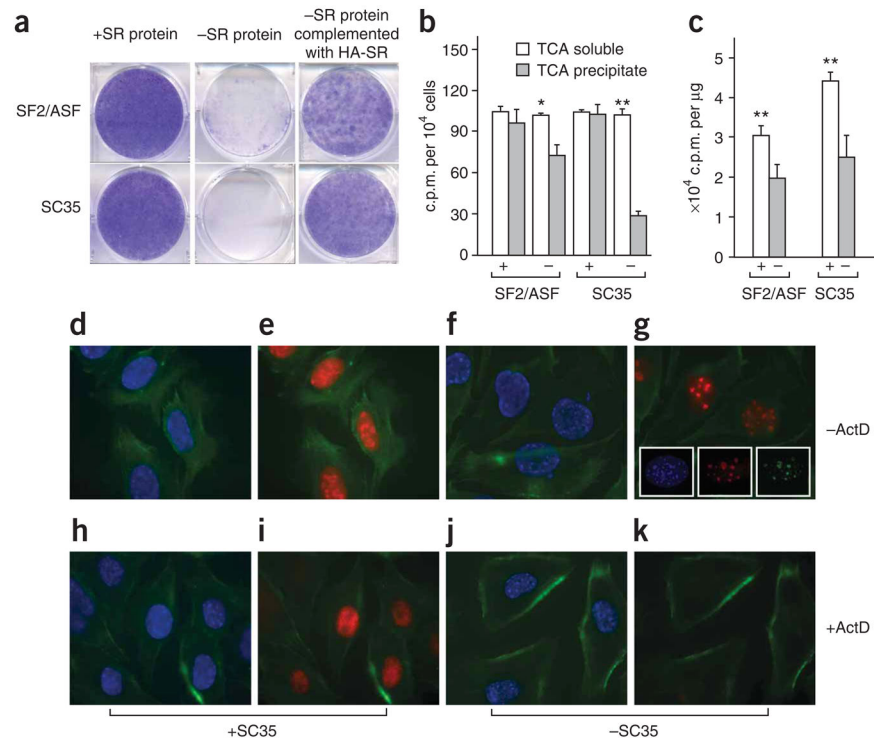


Figure 1.

SR proteins are required for Pol II transcription in MEFs. (a) SR proteins are essential for viability of MEFs. Cells were stained with crystal violet 10 d after mock depletion (+SR protein) and Dox-induced SR protein depletion (–SR protein). HA, hemagglutinin. (b) Reduction of nascent transcripts in SR protein–depleted cells (5 d after Dox treatment), as determined by ³H labeling. Error bars indicate s.d. from three independent experiments, and statistical significance in each pairwise comparison is indicated (**P* < 0.05; ***P* < 0.01). (c) Reduction of ³H-labeled Poly(A)⁺ mRNA. Equal amounts of purified Poly(A)⁺ mRNA were counted. Error bars indicate s.d. from three independent experiments, and statistical significance in each pairwise comparison is indicated (**P* < 0.05; ***P* < 0.01). (d–k) Selective inhibition of transcription in the nucleoplasm of SC35-depleted MEFs. Mock-depleted (+SC35) and SC35-depleted (–SC35) cells were double stained with anti-BrU antibody for nascent RNA (red) or DAPI (blue) in combination with phalloidin for cytoplasmic actin (green). The remaining BrU signals in the nuclei of SC35-depleted cells colocalized with the nucleolar marker Nucleophosmin (NPM), indicating they are due to Pol I transcription (inset in g: blue, DAPI staining of a nucleus; red, anti-BrU staining of nascent RNA; green, anti-NPM staining of the nucleolus). In the presence of a low dose of ActD, Pol I transcription was selectively suppressed in nucleoli, allowing better visualization of SC35 depletion–induced reduction of Pol II transcription in the nucleoplasm (i,k).

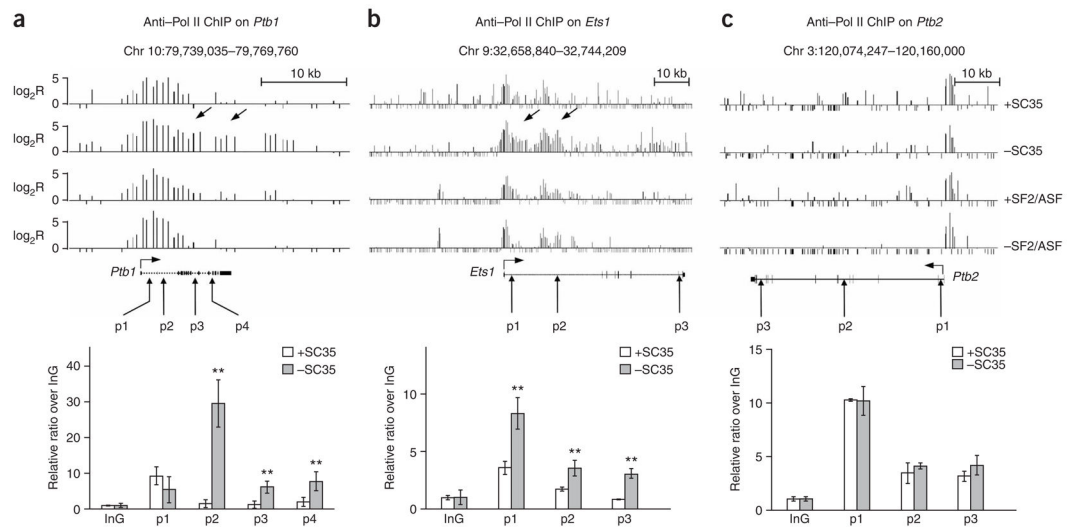


Figure 2.

Induced Pol II accumulation in gene bodies in response to SC35 depletion *in vivo*. Two representative genes (*Ptb1* and *Ets1*) in which Pol II was found to accumulate in specific regions of the gene body detected on a tiling array (a,b), and a representative gene (*Ptb2*) in which Pol II profiling was unaffected by SR protein depletion (c). Above, Pol II tiling array profiling on the representative genes in mock-depleted (+SC35 or +SF2/ASF) and SR protein-depleted (-SC35 or -SF2/ASF) MEFs. Arrows indicate accumulated Pol II on gene bodies. Gene structures and the locations of primer sets for qPCR validation are indicated underneath the profiles. Below, validation by ChIP and qPCR. Primer sets targeting an upstream intergenic region (InG, see the primer sequence in Supplementary Fig. 3); the beginning, middle and end of each gene were used to quantify immunoprecipitated DNA by qPCR. Data were normalized against the signal on InG. Error bars indicate s.d. based on three independent experiments, and statistical significance in each pairwise comparison is indicated (** $P < 0.01$).

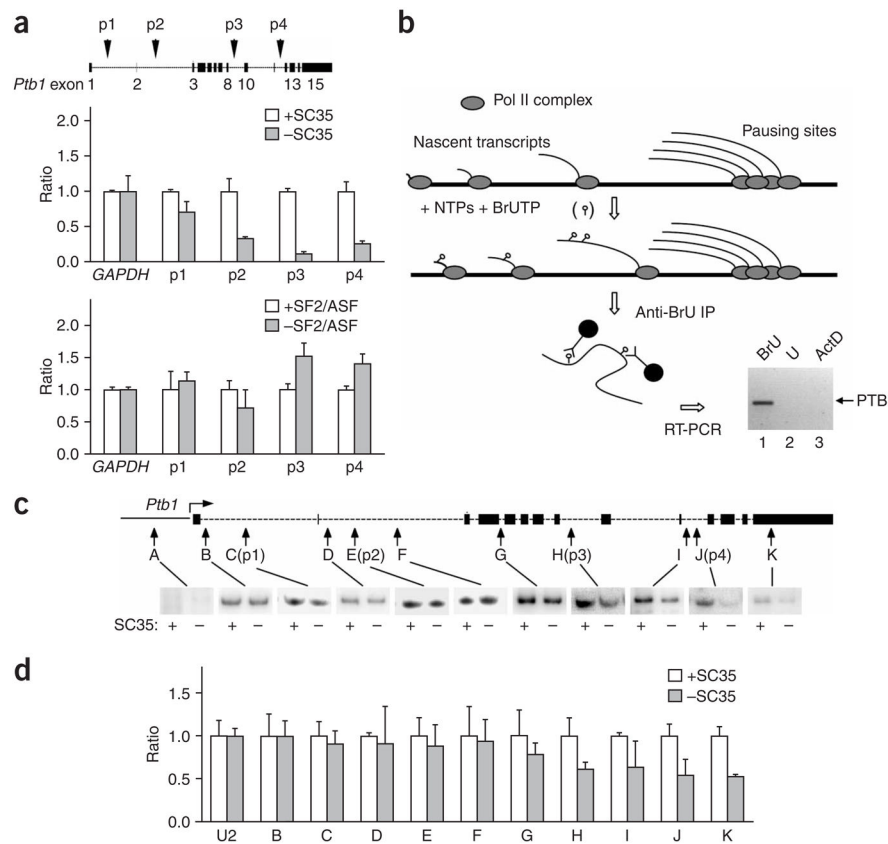


Figure 3. Requirement for SC35 in transcription elongation. **(a)** Total RNA extracted from mock-depleted (+SC35 or +SF2/ASF) and SR protein-depleted (–SC35 or –SF2/ASF) cells was analyzed by RT-qPCR using the indicated primer sets, which target different intronic locations in the *Ptb1* gene. The GAPDH mRNA level was unaffected and served as a control. No signal was detected if the reverse transcription step was omitted (not shown). All data from mock-depleted cells were normalized to 1.0 and the ratio reflects the fold change of the RNA from SC35-depleted cells over mock-depleted cells. **(b)** Diagram for the nuclear run-on assay based on the use of BrUTP. The specificity of BrU-labeled nascent *Ptb1* transcript detection was demonstrated by analyzing total RNA from wild-type MEFs labeled with BrU, uridine (U) or BrU in the presence of ActD. **(c)** Nuclear run-on assay on the nascent *Ptb1* transcript. The gene structure and the probe positions (A to K) are indicated on the top. Note that the four probes against *Ptb1* used in **a** (p1 to p4) are included in this expanded probe set to achieve fine mapping of transcription attenuation in the nuclear run-on assay. A representative set of RT-PCR data are shown. RT-PCR products from mock-depleted (+SC35) and SC35-depleted (–SC35) MEFs are indicated below each set. No signal was detected without reverse transcription in these reactions. **(d)** RT-qPCR analysis of the nuclear run-on assay as in **c**. U2 small nuclear RNA (snRNA) was unaffected by SC35 depletion, which was assayed as a control. Error bars indicate s.d. based on three independent experiments.

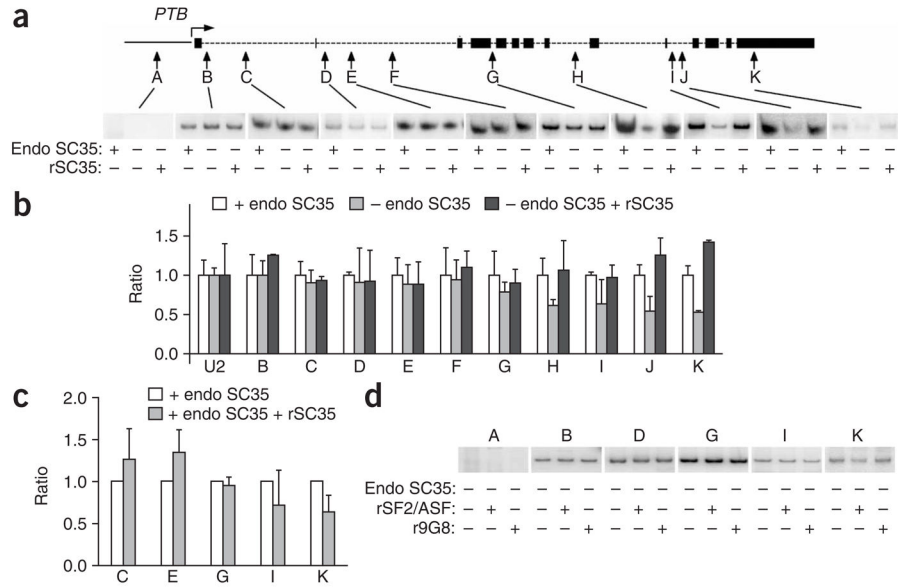
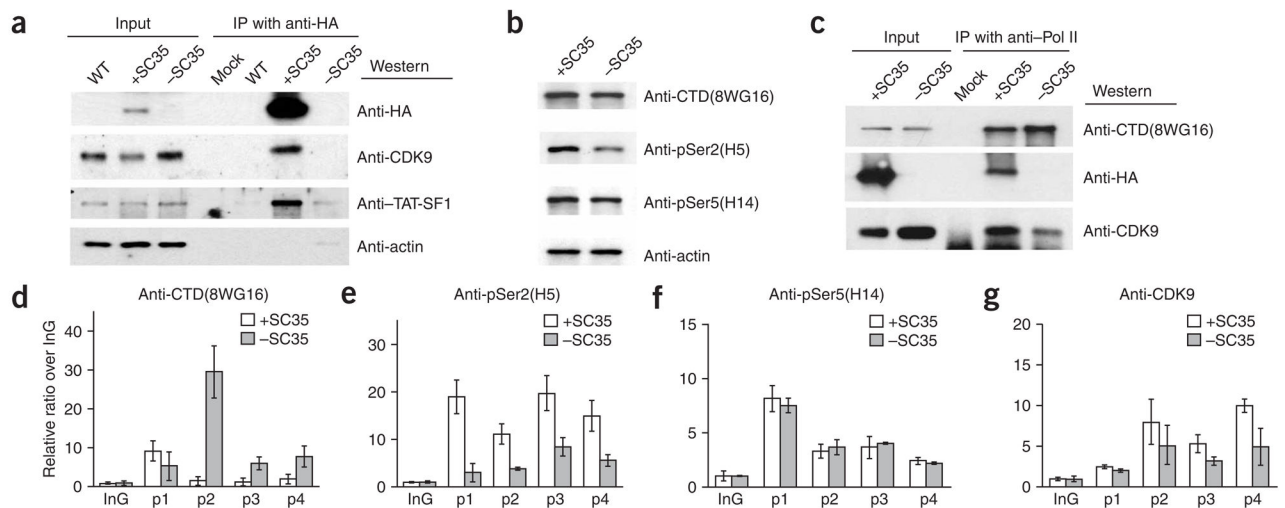


Figure 4. Functional rescue of SC35 depletion–induced blockage of transcriptional elongation. (a) Reconstitution of nuclear run-on using recombinant SC35. Each set of three PCR products represents the analysis of RNA from mock-depleted MEFs (+endogenous (endo) SC35), SC35-depleted MEFs (–endo SC35) and SC35-depleted MEFs plus recombinant SC35 (rSC35). (b) RT-qPCR analysis of the nuclear run-on assay as in **a**. U2 snRNA was unaffected by SC35 depletion, which was assayed as a control. Error bars indicate s.d. based on three independent repeats of each reaction. (c) Addition of recombinant SC35 did not generally stimulate transcriptional elongation in mock-depleted cells. Error bars indicate s.d. based on three independent repeats of the experiment using a subset of radioactive PCR primers used in **a**. qPCR analysis based on the use of the entire primer set gave rise to the same result (data not shown). (d) Specificity in the reconstituted nuclear run-on assay. SC35-depleted MEFs (–endo SC35) treated with digitonin were supplemented with recombinant 9G8 and SF2/ASF. This experiment was carried out alongside the experiment in **a**, but using a subset of radioactive PCR primers as indicated.

**Figure 5.**

Dynamic and SC35-dependent recruitment of P-TEFb to the elongating Pol II complex. **(a)** Co-immunoprecipitation of hemagglutinin (HA)-tagged SC35 with Pol II and key factors (CDK9, the kinase component of P-TEFb and TAT-SF1) involved in transcriptional elongation. **(b)** The phosphorylation state of Pol II in mock-depleted and SC35-depleted MEFs. Specific antibodies used in western blotting are indicated on the right. **(c)** Impact of SC35 depletion on P-TEFb association with Pol II. Immunoprecipitation (IP) with anti-Pol II was carried out in mock-depleted and SC35-depleted MEFs followed by western blotting using anti-CDK9. **(d–g)** ChIP and qPCR analysis of the Pol II phosphorylation state and CDK9 binding in the *Ptb1* gene. The data were normalized against the background signal from an intergenic region (InG) upstream of the *Ptb1* promoter. Error bars indicate s.d. based on three independent experiments.

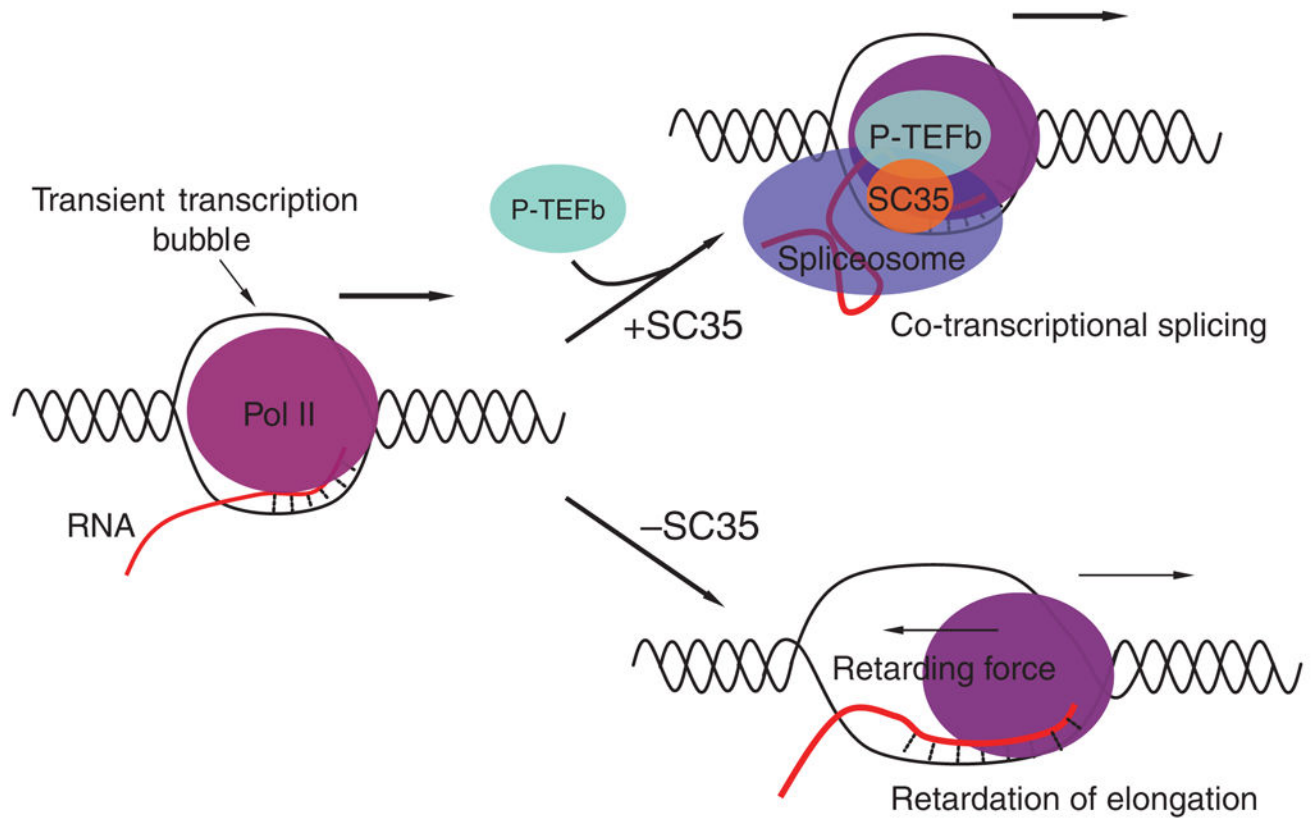


Figure 6. Proposed model for the role of SR proteins in transcriptional elongation and co-transcriptional RNA splicing. During transcription, a transient bubble forms, which collapses quickly during transcriptional elongation. In wild-type cells (above right), SC35 and SC35-recruited factors may travel with the Pol II complex to scan emerging *cis*-acting RNA elements in nascent transcripts, which may contribute to the displacement of RNA from template DNA. Such initial scanning may help nucleate the assembly of the spliceosome on nascent transcripts after additional cooperative *cis*-acting splicing signals become available. In the absence of SC35 (below right), dynamic P-TEFb recruitment and Pol II CTD Ser2 phosphorylation are inefficient or impaired, thereby retarding the elongation of the Pol II complex in general and/or at certain locations within gene bodies.