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SON Controls Cell Cycle Progression by Coordinated Regulation of RNA Splicing

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SUMMARY

It has been suspected that cell cycle progression might be functionally coupled with RNA processing. However, little is known about the role of the precise splicing control in cell cycle progression. Here, we report that SON, a large Ser/Arg (SR)-related protein, is a splicing co-factor contributing to efficient splicing of cell cycle regulators. Down-regulation of SON leads to severe impairment of spindle pole separation, microtubule dynamics, and genome integrity. These molecular defects result from inadequate RNA splicing of a specific set of cell cycle-related genes that possess weak splice sites. Furthermore, we show that SON facilitates the interaction of SR proteins with RNA polymerase II and other key spliceosome components, suggesting its function in efficient co-transcriptional RNA processing. These results reveal a mechanism for controlling cell cycle progression through SON-dependent constitutive splicing at suboptimal splice sites, with strong implications for its role in cancer and other human diseases.

INTRODUCTION

Efficient and proper RNA splicing is one of the critical steps in gene expression and mutations in *cis*-acting splicing elements or *trans*-acting splicing regulators are well known to cause diseases in humans (Cartegni et al., 2002; Cooper et al., 2009; Garcia-Blanco et al., 2004). Interestingly, multiple cell cycle regulators initially identified as cell division cycle (cdc) genes were later found to impair splicing in *S. cerevisiae* and *S. pombe*, suggesting a functional connection between RNA splicing and cell cycle progression (Lundgren et al., 1996; Shea et al., 1994; Vijayraghavan et al., 1989). However, it has been unclear whether the cell cycle defect is a consequence of defective RNA splicing or whether individual cdc

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genes have a direct role in splicing regulation. In at least one case, the impact of the *cdc5* mutant on cell cycle progression could be suppressed by removing the intron in the tubulin-encoding *TUB1* gene, suggesting that cell cycle defects may be manifested by specific splicing defects (Burns et al., 2002). Growing evidence has demonstrated that the control of RNA splicing, especially alternative splicing of apoptotic regulators, contributes to cell survival (Schwerk and Schulze-Osthoff, 2005; Shin and Manley, 2004). However, direct connection, if any, between the control of constitutive splicing and cell cycle progression or cell survival has been lacking.

The RNA splicing process requires an accurate recognition of exon-intron boundaries, which are aided by conserved *cis*-elements, such as the 5′ splice site, 3′ splice site, branch site and polypyrimidine tract, as well as the coordinated action of multiple trans-acting factors, including small nuclear RNPs (snRNPs) and SR proteins (Cartegni et al., 2002). Recent studies demonstrated that SR proteins are recruited to the nascent RNA via direct or indirect interactions with the C-terminal domain (CTD) of RNA polymerase II (RNAP II) to facilitate co-transcriptional RNA processing (Bentley, 2005; Das et al., 2007; Zhong et al., 2009) and defects in this process may cause prolonged association of nascent RNA with template DNA, a configuration known as R-loops responsible for triggering double-stranded DNA breaks (Li and Manley, 2005). As the functional connection between co-transcriptional RNA processing and genome instability may underlie key aspects of tumorigenesis in humans, it has been a major battleground to understand the mechanism of efficient and specific coupling between transcription and RNA splicing.

We have been pursuing a large, poorly characterized protein called SON because of its ability to bind the leukemogenic protein AML1-ETO and modulate its activity in cell growth (Ahn et al., 2008). SON was first cloned from a human embryonic cDNA library and classified as a new member of the *v-mos* Moloney murine sarcoma viral oncogene family (Berdichevskii et al., 1988). Although initially characterized as a DNA binding protein (Sun et al., 2001), SON contains multiple structural features related to RNA processing, including a large arginine/serine-rich (RS) domain, a glycine-rich motif (G-patch), and a double-stranded RNA binding motif (DSRM) (Aravind and Koonin, 1999; Saitoh et al., 2004; Saunders and Barber, 2003). Consistent with a potential role in RNA metabolism, SON has been shown to localize to nuclear speckles, which are enriched in the splicing machinery (Mattioni et al., 1992; Saitoh et al., 2004; Sun et al., 2001; Wynn et al., 2000). These observations raise the possibility that SON may function as an SR-related splicing factor in regulated splicing.

Here, we have uncovered a major function of SON in regulating a large number of genes dedicated to cell cycle progression, as *SON* siRNA caused massive disarray of microtubules and impaired spindle pole separation, thereby arresting the cell at mitotic phase. Mechanistic analysis revealed that SON acts as a co-activator for efficient RNA processing of multiple structural components of the cell cycle apparatus and its signaling molecules. SON-dependent splicing substrates contain weak splice sites, predicting inefficient or unstable spliceosome formation on them. Our data also suggest that SON facilitates splicing through the recruitment of SR proteins, such as SC35, to RNAP II complexes. These results reveal an insight into the regulation of cell cycle progression by cofactor-mediated splicing at suboptimal splice sites associated with a large number of constitutive introns. Our findings, coupled with the recent documentation of another large SR protein-related splicing co-activator (nSR100) required for development of the nervous system (Calarco et al., 2009), have a broad implication for a large number of SR-related proteins in coordinated regulation of RNA processing to govern cell proliferation and differentiation.

RESULTS

SON deficiency causes severe defects in mitotic spindle pole separation, chromosome alignment and microtubule dynamics

Our previous work revealed a functional interaction between SON and the leukemogenic protein AML1-ETO in the regulation of cell proliferation (Ahn et al., 2008). We identified SON as an AML1-ETO NHR4 domain-interacting protein and showed that disruption of the interaction between endogenous SON and AML1-ETO could rescue the cell growth defect induced by the full-length AML1-ETO protein (Ahn et al., 2008). These findings suggest a critical role of SON in the regulation of cell proliferation. To further pursue the function of SON, we performed *SON* siRNA transfection (Fig. S1A), and found a significant growth inhibition (Fig. S1B) and an increase in the 4n population (Fig. S1C). Western blot and flow cytometric analysis for histone H3-Ser10 phosphorylation, a marker of mitotic cells, coupled with Wright-Giemsa staining, revealed that cells were arrested in mitosis after *SON* siRNA transfection (Figs. S1D, S1E and S1F). Collectively, these data demonstrate a critical role of SON in facilitating cell cycle progression through the mitotic phase.

A close examination of mitotically arrested K562 cells revealed a strikingly irregular spread of chromosomes in *SON* siRNA-treated cells (Fig. 1A). A similar phenotype was also evident in HeLa cells where *SON* knockdown appears to arrest the cell at pro-metaphase as indicated by a significant increase in pro-metaphase cells with a concurrent decrease in metaphase, anaphase and telophase cells (Fig. 1B). This early mitotic defect prompted us to examine the status of mitotic spindle poles by staining the cell with an Aurora kinase A antibody, revealing failed mitotic pole separation in ~50% of pro-metaphase cells (Figs. 1B and 1C). We observed similar defects in spindle pole separation and chromosome alignment in K562 cells following *SON* knockdown (Figs. S2A and S2B). Strikingly, many SON-depleted cells exhibited multiple mitotic spindle poles and interphase centrosome amplification (Figs. 1D and S2C). These data revealed severe dysregulation of the spindle pole and centrosomes in SON-deficient cells.

Because chromosome alignment and segregation during mitosis require a tight control of microtubule assembly and disassembly, we next examined spindle microtubules in SON-depleted cells. By staining the cell with an α-tubulin antibody, we observed complete disruption of mitotic spindle organization. All mitotic spindles were irregular in shape, and microtubules associated with the mitotic spindles were totally disorganized and tangled without polarity with some exhibiting abnormally long fibrils while others were insufficiently elongated (Figs. 1E and S2B).

As microtubules also serve as critical cytoskeletal structures for organizing and maintaining the cell morphology required for various cellular functions, we further determined the functional requirement of SON for microtubule organization in interphase cells. Normally, microtubules are nucleated at the centrosome, and subsequently released from the initial nucleation site and reorganized to form a non-radial microtubule array during interphase. In contrast to this normal morphology observed in control siRNA-treated HeLa cells, microtubules in *SON* siRNA-transfected cells formed a dense aster originating from the centrosome, resulting in a completely radial array without forming any microtubule network/branching (Fig. 1F). This abnormal microtubule pattern failed to define the structural frame of the nucleus or contour of the cell. We performed a microtubule re-growth assay to further characterize the defect in microtubule assembly and disassembly after *SON* knockdown, observing that microtubules retained their ability to nucleate at the centrosome, but were abnormally elongated without forming networks as seen in normal cells (Fig. 1G). These results revealed severe defects in the regulation of microtubule biogenesis, organization, and dynamics throughout the cell cycle in response to *SON* knockdown.

SON is required for cytokinesis and maintaining genome stability

As expected from massive defects in microtubule organization and dynamics and multiple other cellular defects in *SON* knockdown cells, we found that about 10 to 15% cells were induced to commit apoptosis as detected 2 to 3 days after *SON* siRNA treatment (Fig. S1G). Among the cells that made it through mitosis, many exhibited abnormal nuclear structures, including the formation of protruding nuclear buds/lobes and micronuclei (Fig. 2A). We also observed micronuclei on BJ primary fibroblasts cells by DAPI staining (Fig. S2C), and flow cytometric analysis of DNA content revealed a dramatic increase in the population of cells containing 4n or higher DNA content (Fig. 2D). In addition, multinucleated cells were observed typically 6 - 7 days after siRNA transfection in HeLa (Fig. 2B) and U937 human leukemic monocyte lymphoma cells (Fig. 2C), suggesting defective cytokinesis among the cells that escaped the initial block at pro-metaphase.

Because protruding nuclear buds and micronuclei are hallmarks of DNA damage and abnormal chromosome rearrangement, we further examined whether SON deficiency might induce genome instability. We stained *SON* siRNA-treated HeLa cells with an antibody against phosphorylated histone H2A.X (γH2A.X), a marker of double-stranded DNA breaks, revealing a marked increase in γH2A.X foci (Fig. 2E). Together, these data indicate that SON is critical for maintaining genome stability by preventing aneuploidy/polyploidy and DNA breaks.

SON regulates the expression of a specific subset of genes involved in cell cycle progression and genome stability

Given the pleiotropic defects observed in *SON* knockdown cells and the fact that SON does not seem to be a structural component of the affected cellular structures, we hypothesized that SON might affect specific cellular functions through regulating gene expression. We tested this hypothesis by performing microarray analysis on HeLa cells transiently transfected with *SON* siRNA, revealing that expression levels of 659 genes were changed by more than 1.45-fold with statistical significance (p value <0.001) in response to *SON* knockdown.

By analyzing the biological functions of these 659 genes using the Ingenuity Pathway Analysis (IPA) software, we found that the most highly enriched functional categories are related to cancer, cell cycle and DNA replication/recombination/repair (Fig. 3A). Interestingly, down-regulated genes are most associated with DNA replication/ recombination/repair and cell cycle (Fig. 3B), while up-regulated genes are linked to cell death/survival, cell signaling and molecular transport (Fig. S3A). Focusing on down-regulated genes, we noted many genes involved in repair of single-stranded and double-stranded DNA breaks, DNA damage responses, DNA rearrangement, DNA replication, G2/ M checkpoint, cell cycle, mitosis and microtubule dynamics (Fig. 3C), suggesting that down-regulation of these genes may directly contribute to the observed phenotype. Quantitative real-time PCR, immunoblotting and immunostaining analyses validated the microarray results on a panel of down-regulated genes (Figs. 3D, 3E and S3B). These data thus suggest that SON is involved in regulated expression of many genes dedicated to maintaining genome stability and facilitating cell cycle progression.

SON is required for efficient RNA processing of affected genes

SON was previously reported to be present at nuclear speckles enriched with most splicing factors (Ahn et al., 2008; Saitoh et al., 2004; Wynn et al., 2000), suggesting that SON may play a role in RNA metabolism in mammalian cells. We confirmed that the pattern of SON localization exactly matches with that of typical pre-mRNA splicing factors throughout the cell cycle (Fig. S4A), and showed SON co-localizes with the SR splicing factor SC35 and

small nuclear RNAs (snRNAs) (Figs. S4B and S4C). These findings strongly implicated SON as part of the pre-mRNA splicing machinery.

We therefore focused on testing the possibility that down-regulation of gene expression after *SON* knockdown might be due to inefficient pre-mRNA splicing. We designed a series of PCR primer pairs targeting two neighboring constitutive exons on a panel of affected genes, including *TUBG1*, *KATNB1*, *TUBGCP2*, *AURKB*, *PCNT*, *AKT1*, *RAD23A*, and *FANCG*, as well as on a few unaffected genes as controls. RT-PCR analyses revealed multiple intron retention events on genes that were down-regulated by *SON* siRNA, but no change on unaffected genes (Fig. 4A). To further confirm that SON is required for specific intron removal events, we designed PCR primers targeting each exon-intron (5′ splice site) or intron-exon (3′ splice site) junction within the entire *TUBG1* gene (Fig. 4B), demonstrating that SON depletion caused inefficient intron removal at several, but not all, splice sites (Fig. 4C). In all affected cases, intron removal was impaired, but not abolished, suggesting strongly that SON acts as a co-activator for efficient intron removal at constitutive splice site on a selective group of genes and the resulting splicing defects are likely responsible for down-regulation of these genes in *SON* knockdown cells.

Furthermore, we performed UV crosslinking and immunoprecipitation (CLIP) with SON antibody and analyzed associated RNAs by RT-PCR. The results showed that SON is associated with the RNAs from all examined down-regulated genes, including *TUBG1*, *KATNB1*, *TUBGCP2*, *PCNT*, *AKT1* and *AURKB*. The RNAs from unaffected genes, such as *TUBA1B*, *KATNA1*, *AURKA*, *TUBGCP4* and *GAPDH*, were not detected or marginally enriched in the parallel experiment (Fig. 4D and S4D). These results indicate that SON is directly involved in the processing of its target RNAs.

To further analyze SON-mediated splicing, we constructed several minigenes containing appropriate exonic and intronic sequences. Since the splicing efficiency between exons 7 and 8, and exons 8 and 9 in the endogenous *TUBG1* pre-mRNA was inefficient and sensitive to SON down-regulation (Fig. 4A and 4C), we constructed two minigenes from these regions under the CMV promoter and tested their response to *SON* knockdown. RT-PCR analysis confirmed that splicing of these *TUBG1* minigenes was indeed dependent on SON (Fig. 4E). In contrast, splicing of a minigene derived from the *TUBA1B* gene, which was not responsive to *SON* knockdown based on our microarray data, was independent of SON (Fig. 4E). These results demonstrated that SON-mediated splicing occurs in a gene-specific manner, which can be fully recaptured in the minigene assay.

The C-terminal domain containing the RS domain and the G-patch is necessary for SON's activity in splicing

Taking advantage of this minigene system, we determined the domain requirement for SON-mediated splicing. SON is a large protein of 2,426 amino acids, containing various repeat sequences in the central part of the protein, and interestingly, its C-terminal domain carries a number of features related to the SR superfamily of splicing factors and regulators, including an RS domain and two putative RNA binding motifs, consisting of a G-patch and a double-stranded RNA binding domain (DSRM) (Fig. 5A). We constructed expression vectors for the full-length human *SON* cDNA (Wt-*SON*) and siRNA-resistant *SON* (siRR-*SON*) in which six nucleotides within the *SON* siRNA-target sequence were mutated without altering the coding capacity (Fig. S5A). When co-transfected with the *TUBG1* minigene reporter, we found that siRR-*SON*, but not Wt-*SON*, could fully rescue the splicing defect of the minigene caused by *SON* siRNA (Fig. 5C), thus confirming the specific effect of exogenous SON on splicing of the minigene.

We next generated a series of deletion mutants (Fig. 5B and S5B) and tested their abilities to rescue splicing of the minigene. We found that DSRM deletion (construct 3) does not affect splicing activity of SON, indicating that this double-stranded RNA binding domain is dispensable for SON's splicing activity (Fig. 5C). In contrast, the G-patch deletion mutants (constructs 4 and 5) reduced its activity. Deletion of the RS domain causes a complete loss of splicing activity (constructs 6 and 7). Strikingly, the C-terminal RS/GP/DSRM fragment (construct 8), which is only ~1/3 of the full-length protein, was sufficient to rescue SON-dependent splicing to a significant degree (Fig. 5C). We concluded from this analysis that the RS domain and the G-patch are the core motifs necessary for SON-mediated splicing, and the upstream sequences, which contain highly repetitive amino acid sequences, further support the function of SON in achieving efficient RNA splicing.

SON-dependent genes possess weak splice sites

To elucidate the specificity of SON regulation of RNA splicing, we examined the features of the splice sites associated with either SON-dependent or SON-independent genes, using the matrices for splice sites available in ESEfinder (http://rulai.cshl.edu/tools/ESE/, Cartegni et al., 2003). Interestingly, SON-dependent genes all possess a weak 5′ or 3′ splice site. Some of them showed low motif scores that are below the threshold score of typical constitutive splice sites (Fig. S6). Interestingly, some splice sites score as the standard constitutive splice sites, but correspond to so-called dual-specificity splice sites (Fig. S6), which may "confuse" the splicing machinery as they may be recognized as either 5′ or 3′ splice sites (Zhang et al., 2007). These observations suggest that the presence of weak or ambiguous splice sites may render splicing of those cell cycle genes particularly dependent on SON for their efficient recognition by the splicing machinery.

To experimentally test this hypothesis, we examined which sequence feature(s) contribute to SON dependency on the *TUBG1* minigene model. We created mutant minigenes that possess stronger 5′ splice site with optimal sequences (5′ mt), stronger 3′ splice site with the improved polypyrimidine tract (3′ mt), or stronger splice sites at both ends (5′ mt + 3′ mt) (Figs. 6A and 6B). When a single site was converted to a strong one, the splicing efficiency of the mutant minigene was still SON-dependent. However, when both 5′ and 3′ splice sites were modified to stronger sites, splicing of the mutant minigene was no longer SON-dependent (Fig. 6C). To further confirm that consensus sequences within the intron are sufficient to render the minigene SON-independent, we replaced intron 7 of *TUBG1* with intron 2 of the SON-independent *TUBA1B* gene (α2-Intron), which contains canonical splicing signals (Figs. 6A and 6B). Replacement of the intron indeed abolished SON dependency (Fig. 6C). We also performed a converse experiment by weakening the splice site on a SON-independent *TUBA1B* minigene, finding that weakening either the 5′ or the 3′ splice site impaired the splicing efficient and combination of these mutations abolished splicing; however, none of these mutants showed SON-dependency (Fig. S7). We conclude from these experiments that SON functions as a co-factor to institute correct splice site selection on a specific subset of genes containing weak or suboptimal constitutive splice sites. However, a weak splice site alone is clearly insufficient to confer SON-dependency, indicating that other sequence features in conjunction with weak splice sites contribute to SON regulated splicing.

SON facilitates the interaction of SR proteins with RNA polymerase II

A common mechanism for SR-like factors is to recruit other splicing factors and regulators to the splice sites, which may be an underlying mechanism to achieve regulated splicing on a subset of substrates containing a combination of specific *cis*-acting elements. Interestingly, in SON-depleted cells, we observed that nuclear speckles stained with anti-SC35 rounded up, but the cellular localization of RNAP II was not affected. Localization of the splicing

machinery labeled by the tri-methylguanosine cap was also moderately affected (Fig. 6D). The fact that SC35 speckles become round upon *SON* knockdown suggests impaired shuttling of SR proteins between nuclear speckles and the site of active transcription for co-transcriptional RNA processing (Lamond et al., 2003).

To test this hypothesis, we generated a HEK293 cell line stably expressing V5-tagged SC35 as a model for SR protein recruitment and determined how the recruitment of SC35 to RNAP II is affected by *SON* siRNA. Immunoprecipitation with V5 antibody from cell lysate efficiently pulled down SON, demonstrating that SON is indeed associated with SC35 or SC35-containing complexes. We also detected the association of SC35 with RNAP II, U1-70K, and $U2AF_{65}$ in control siRNA-treated cells (Fig. 6E). In contrast, SC35 interaction with RNAP II (detected by 8WG16; note that this antibody is known to cross-react with both hypo- and hyper-phosphorylated RNAP II, although the former seems to be the preferred antigen) was significantly attenuated in SON-depleted cells (Fig. 6E). Notably, the association of SC35 with Ser2-phosphorylated RNAP II (detected by the H5 antibody), which has been linked to transcriptional elongation, appears to be selectively affected, relative to Ser5-phosphorylated RNAP II (detected by the H14 antibody), which is predominantly associated with transcriptional initiation. The interaction of SC35 with U1-70K and $U2AF_{65}$ was also attenuated in SON-depleted cells (Fig. 6E). Together, these results provide strong support to a model where SON facilitates co-transcriptional assembly of SR proteins and other key spliceosome components, such as U1 and U2AF to elongating RNAP II complexes, thereby ensuring efficient co-transcriptional RNA splicing (Fig. 7).

DISCUSSION

A large number of SR-related proteins are encoded in mammalian genomes. Although a few large SR-related proteins have been shown to act as splicing co-activators, little is known about how various SR-related proteins are involved in specific cellular processes. In this study, we identified SON as an SR-related protein specifically devoted to RNA processing of many cell cycle/DNA repair-related genes. Although SON has recently been implicated as a regulator of alternative splicing (Moore et al., 2010), our findings uncover a link between SON-dependent fine-tuning of constitutive splicing and cell cycle progression.

SON as a mediator of specific cellular processes via regulated splicing

It has been documented that SR-related proteins, such as SRm160 and SRm300, function as co-activators of pre-mRNA splicing via interactions with SR family proteins and snRNPs (Blencowe et al., 1998; Eldridge et al., 1999). However, the biological significance of individual SR-related proteins in specific cellular processes or diseases is poorly understood. A recent study on an SR-related protein, nSR100, which is required for development of the nervous system (Calarco et al., 2009), suggests that SR-related proteins may play critical roles in specific gene expression programs. The requirement of SON for efficient intron removal from pre-mRNA of the genes associated with mitotic progression revealed SON as a critical mediator for the cell cycle machinery. Consistent with our finding on the importance of SON in cell cycle progression, SON was reported as one of the genes that showed elevated expression in proliferating areas of embryonic and postnatal brain as well as in postnatal developing teeth (McKee et al., 2005). Our data now unveil the underlying mechanism of cell cycle regulation via SON-mediated splicing. Our findings, together with the elucidated role of nSR100 in the nervous system (Calarco et al., 2009), reveal an emerging role of SR-related splicing co-activators in the regulation of specific cellular programs through coordinating the RNA splicing process.

Microarray analysis also revealed a set of genes up-regulated in response to *SON* knockdown (Fig. S3A). However, upon examining *SON* knockdown at an earlier point, we

found that SON predominantly down-regulates gene expression without causing significant up-regulation of many genes (data not shown), indicating that most of those up-regulated genes may indirectly result from *SON* knockdown.

SON-mediated constitutive splicing of weak splice sites as a sensor for cell cycle progression

In eukaryotic cells, it is generally believed that weak or suboptimal splice sites are critical features for alternative splicing (Keren et al. 2010). However, little is known about the functional importance of processing suboptimal splicing signals present at constitutive splice sites. Interestingly, our results now reveal that such suboptimal splicing signals are built on a set of cell cycle-related genes, which renders their splicing particularly sensitive to SON levels, thus constituting a SON-mediated sensory system for the regulation of cell proliferation.

Our results demonstrate that SON is required for efficient processing of a specific group of genes with weak splice sites during constitutive splicing. The splicing of the *TUBG1* minigene lost SON-dependency when both the 5′ and 3′ splice sites were modified to consensus sequences. This is entirely consistent with SON-independent splicing of the *TUBG1* minigene when its less optimal intron was replaced with strong intron 2 of the *TUBA1B* gene, indicating that a weak splice site is necessary for modulation by SON. We attempted a converse experiment in which the *TUBG1* intron 7 containing a weak splice site was inserted between the SON-independent *TUBA1B* exons 2 and 3, finding that this minigene could not undergo splicing at all even in wild type cells, indicating that the *TUBG1* intron 7 in combination with the *TUBA1B* exons generates a pre-mRNA that is too weak to be recognized by the splicing machinery (data not shown). We thus took a different strategy to address whether a weak splice site is sufficient to confer SON-dependency by directly converting the strong splice sites in the SON-independent *TUBA1B* minigene to weak ones. The data clearly showed that a weak splice site alone is insufficient, indicating that a combination of other factors, such as the ratios of ESE/ESS/ISE/ISS, the length of polypyrimidine tract, and other protein modulators, may collectively contribute to SON-dependency. Although it is currently unclear which of these features are most critical, our data suggest that the association of SON on specific gene transcripts, instead of all pre-mRNAs with a weak splice site, may reflect mutually beneficial interactions between SON and other critical splicing factors, including SR proteins (Fig. 7A).

Structural features of SON for efficient coupling between transcription and splicing

SON is a large multi-domain protein, suggesting that it may serve as a landing pad for multiple protein-protein interactions to facilitate co-transcriptional splicing in the cell. Our data demonstrated that the RS domain and the G-patch in SON play important roles in splicing, but the DSRM is dispensable (Fig. 5). The G-patch is a conserved domain found in type D retroviral polyproteins and several eukaryotic RNA-binding proteins, but its precise role in RNA binding and/or protein-protein interactions remains a subject of future investigation (Aravind and Koonin, 1999). SON has also been implicated in DNA binding through a region upstream of the RS domain (Sun et al., 2001), raising an intriguing possibility that SON may use this function to provide a tight connection between transcription and RNA splicing.

It is also important to emphasize our finding that SR proteins become partially aggregated in rounded nuclear foci without impairing the general splicing machinery. It was previously reported that nuclear speckles become round up in response to inhibition of splicing and transcription (Mintz and Spector, 2000; O'Keefe et al., 1994). In our current study, we found that SR proteins became aggregated but the cellular distribution of snRNPs detected by a tri-

methylguanosine cap antibody was not severely affected in *SON* knockdown cells, suggesting inefficient recruitment of SR proteins to nascent transcripts for co-transcriptional splicing. Recently, Sharma *et al*. reported that SON is required for correct localization of SR proteins and snRNPs in nuclear speckles, which appears to depend on the N-terminal region with repetitive amino acid sequences (Sharma et al. 2010). Our data indicate that this N-terminal fragment alone does not possess any RNA splicing activity; however, it is clearly important for the full activity of the protein in splicing (Fig. 5C). The multi-domain nature of SON, including its ability to bind to RNA and a potential role in inducing modification of SR proteins (i.e. phosphorylation), suggests that SON may nucleate diverse protein-protein and protein-RNA interactions to enhance the process of co-transcriptional splicing (Fig. 7B).

Involvement of SON in tumorigenesis and other diseases

Genome instability and deregulation of the cell cycle are hallmarks of cancer and fundamental to many types of human diseases. Our discovery of SON's function in regulating the mitotic machinery, such as centrosome components and genes critical for microtubule dynamics, as well as the DNA repair machinery, provides mechanistic insights into the role of SON in tumorigenesis and other human diseases. For example, SON has been shown to be important for trafficking of H1N1 influenza virions to late endosomes during the early infection stage (Karlas et al. 2010). Because virus trafficking is a microtubule-dependent process (Marsh and Helenius, 2006), disruption of normal microtubule organization and dynamics may underlie the observed defects in viral infection in *SON* knockdown cells. Our findings also predict SON to be a master regulator of multiple cellular processes that depend on microtubules. In addition to the significant role of SON in RNA processing of cell cycle regulators, it is clear from our microarray experiments that SON has a broad role in multiple cellular processes. Because of its function as a splicing sensor for cell cycle-related genes, it may serve as a target for developing therapeutic strategies against cancer.

EXPERIMENTAL PROCEDURES

Microarray and Functional Analysis

HeLa cells were grown in 100 mm dishes and transfected with negative control siRNA or *SON* siRNA (400 pmol). Then, cells were harvested after 66 hours and RNAs were isolated, and microarrays were performed using GeneChip Human Genome U133A 2.0 (Affymetrix). Data were obtained from three independent transfections and sample preparations, and analyzed by The Scripps Research Institute DNA Array Core Facility by two-sample *t*-test with random variance model. The data have been deposited in Gene Expression Omnibus (GEO) as the series accession number GSE26888. Functional analysis of the genes significantly changed by *SON* siRNA was performed using Ingenuity Pathway Analysis (IPA) software.

Construction of minigenes and analysis of minigene splicing

For construction of minigenes, the following regions were amplified by PCR from HeLa genomic DNA; *TUBG1 e*xon 7- intron 7- exon 8, *TUBG1* exon 8 – intron 8 – exon 9, and *TUBA1B* exon 2 – intron 2 – exon 3. These fragments were inserted into the pcDNA3.1(+) vector. The minigene fragment α2-Intron was synthesized (Integrated DNA Technologies), and other mutant minigenes were created by QuikChange site-directed mutagenesis (Stratagene). To analyze splicing of minigenes, HeLa cells were transfected with control siRNA or *SON* siRNA, and then minigene alone or minigene plus various SON constructs were transfected. RT-PCR was performed to detect spliced or unspliced forms.

Detection of SON-RNA interaction by UV corsslinking and immunoprecipitation (CLIP) and PCR

HeLa cells irradiated with UV and then SON antibody or control IgG were used to precipitate associated RNA. Details are described in Supplemental Experimental Procedures.

Immunoprecipitation of SC35 complexes

HEK293 cells stably expressing V5-tagged SC35 were generated by Flp-In System (Invitrogen). These cells were transfected with control siRNA or *SON* siRNA. After 48 hours, cells were harvested, lysed in RSB lysis buffer (10 mM Tris-HCl pH 7.6, 100 mM NaCl, 2.5 mM $MgCl_2$) with 0.4% Triton X-100, protease inhibitors (Roche) and Phosphatase inhibitors (PhosSTOP, Roche), incubated on ice for 20 min and sonicated (5 sec × 3 times). After centrifugation, lysates were incubated with V5-antibody-conjugated agarose beads (Sigma) for 3 hours at 4 °C. Beads were washed 4 times with RSB with 0.4% Triton X-100 and twice with RSB without detergent.

Further detailed experimental procedures are described in the Supplemental Information.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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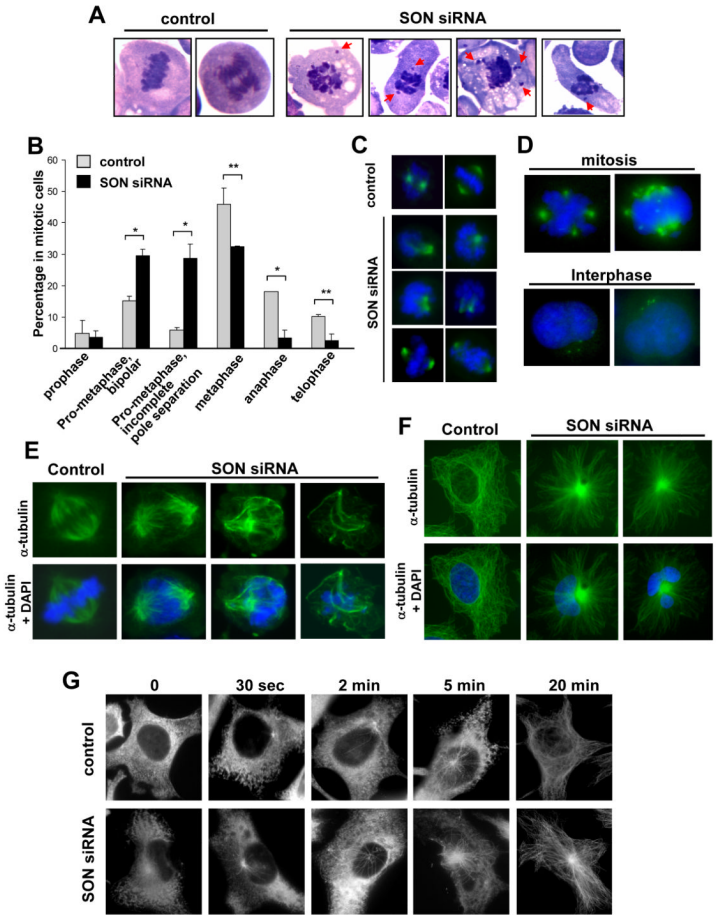

Figure 1. *SON* knockdown causes multiple defects in mitotic chromosome arrangement, spindle pole separation and microtubule dynamics

(A) Mitotic chromosome misalignment in SON-depleted cells. Wright-Giemsa staining revealed misaligned chromosomes in *SON* siRNA-transfected K562 cells. Chromosomes that have shifted to an abnormal location are marked with red arrows.

(B) Increase in pro-metaphase cells and incomplete separation of mitotic poles. HeLa cells were transfected with control siRNA or *SON* siRNA, and immunostained with an Aurora kinase A antibody and DAPI. Then, mitotic cells at different phases and with different mitotic pole status were counted under the fluorescence microscope. Bars represent mean ± SD from three independent experiments ($n > 300$ cells per experiment, $*P < 0.005$, $**P < 0.02$, *t*-test).

(C) Incomplete mitotic spindle pole separation and failure in chromosome alignment caused by *SON* knockdown. HeLa cells were prepared and immunostained as described above (green, Aurora kinase A for mitotic spindle poles; blue, DAPI for DNA).

(D) Abnormal spindle pole/centrosome amplification in *SON* siRNA-transfected K562 cells during mitosis and interphase. Cells were stained with an Aurora kinase A antibody (green) and DAPI (blue).

(E) Defects in mitotic spindle formation in *SON* siRNA-transfected cells. HeLa cells transfected with control siRNA or *SON* siRNA were fixed (day 3), and stained with anti-α-tubulin for microtubules (green) and DAPI for DNA (blue).

(F) Abnormal microtubule organization in interphase cells after SON depletion. Cells were prepared and stained as described in (E).

(G) Microtubule re-growth assay in HeLa cells transfected with *SON* siRNA or control siRNA (day 2). Cells were cold-treated to depolymerize microtubules and fixed at different time points after incubation in warm media (0, 30 sec, 2 min, 5 min, and 20 min). Microtubules were stained with α-tubulin antibody and analyzed by fluorescence microscopy.

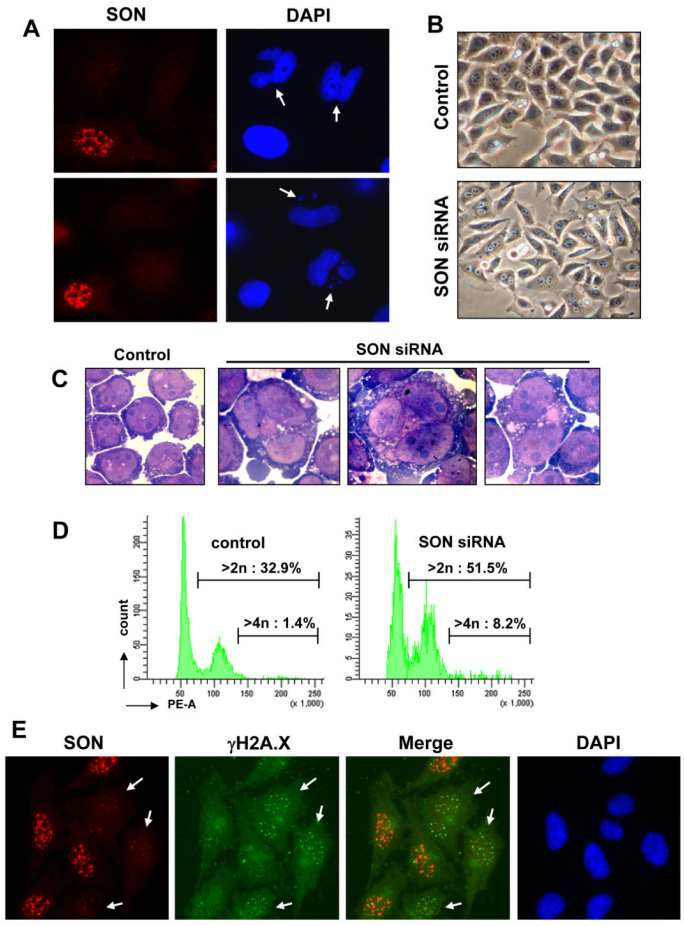

Figure 2. *SON* knockdown causes abnormal nuclear structures, aneuploidy/polyploidy and DNA breaks

(A) Abnormal nuclear structure caused by *SON* knockdown. HeLa cells were transfected with *SON* siRNA, and after 3 days, immunostained with SON antibody together with DAPI. *SON* knockdown causes nuclear buds/lobes and micronuclei (marked with arrows).

(B) Multinuclear HeLa cells were observed after transient *SON* knockdown. HeLa cells were transfected with *SON* siRNA and shown is a photo of representative multinuclear cells observed 7 days after *SON* siRNA transfection.

(C) Multinuclear cells were observed after *SON* siRNA transfection in U937 human leukemic monocyte lymphoma cell line (day 6).

(D) *SON* knockdown causes aneuploidy. BJ human primary fibroblasts were transfected with control siRNA or *SON* siRNA, and DNA content was measure by propidium iodide staining and flow cytometric analysis (day 7), showing that *SON* knockdown increases cells with >2n and >4n DNA.

(E) *SON* knockdown causes double-stranded DNA breaks. HeLa cells were transfected with *SON* siRNA, and stained with γH2A.X antibody. Cells with *SON* knockdown (indicated by arrows) showed γH2A.X foci.

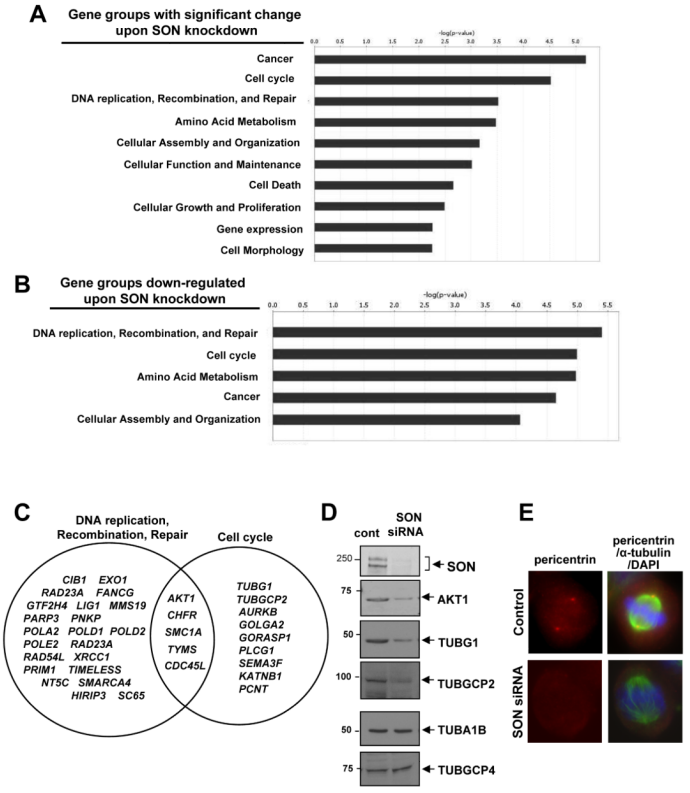

Figure 3. *SON* knockdown causes down-regulation of genes involved in DNA maintenance/ integrity and cell cycle progression

(A) Analysis of top functions of 659 genes that showed significant changes after *SON* knockdown by Ingenuity Pathway Analysis (IPA) software. Fischer's exact test was used to calculate a p-value determining the probability that each biological function and/or disease assigned to that network is due to chance alone.

(B) Analysis of top function of 472 genes that are significantly down-regulated after *SON* knockdown using IPA, as described in (A).

(C) Representative genes that are down-regulated by *SON* knockdown. Down-regulated genes which belong to the functional groups for DNA replication/recombination/repair and cell cycle were determined by IPA, and representative genes were listed in the Venn diagram.

(D) Decrease in protein levels of TUBG1, TUBGCP2 and AKT1 after *SON* knockdown. Whole cell lysates from control or *SON* siRNA-transfected HeLa cells were prepared 3 days after transfection, and immunoblotted for SON, TUBG1 (γ-tubulin), TUBGCP2 (γ-tubulin complex protein 2) and AKT1. TUBA1B (α-tubulin) and TUBGCP4 (γ-tubulin complex protein 4) were also blotted as unaffected controls.

(E) Decrease in pericentrin (PCNT) level in the centrosome after *SON* knockdown detected by immunostaining (red for pericentrin, green for α-tubulin, blue for DNA).

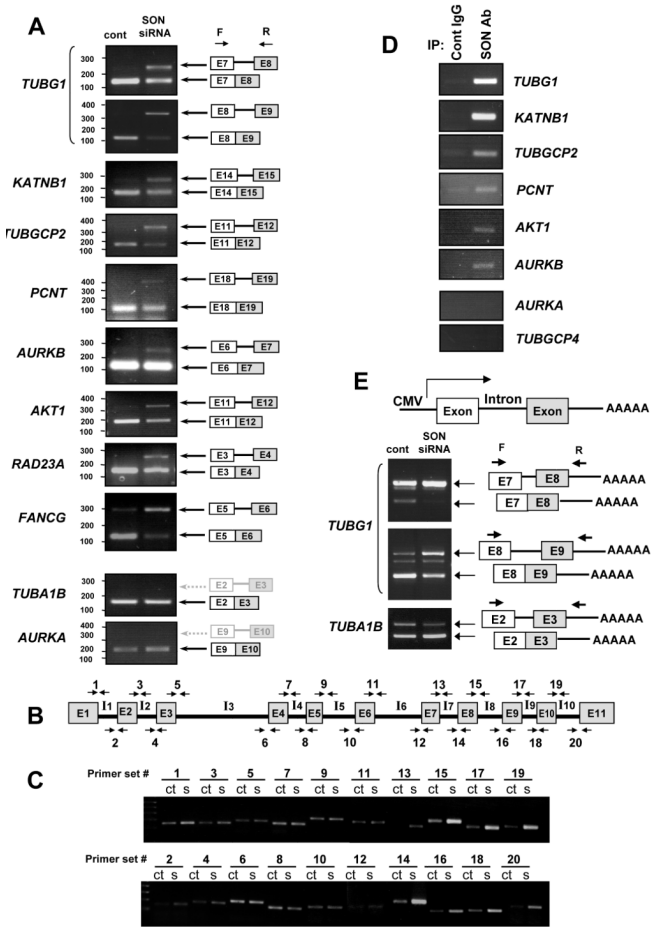

Figure 4. SON is required for efficient intron removal at constitutive splice site on a selective group of genes and binds to the RNA of those genes

(A) Detection of the unspliced form of RNAs in SON-depleted cells by PCR analysis. RNAs were prepared from control or *SON* siRNA transfected HeLa cells, and RT-PCR analyses were done using primers targeting two neighboring constitutive exons for indicated genes (E, exon; F, forward primer; R, reverse primer). PCRs for *TUBA1B* and *AURKA* were done as controls that were not down-regulated by *SON* siRNA. Arrows with solid lines in black indicate detected bands and dotted lines in gray indicate the expected size of undetected bands.

(B) A schematic diagram of exons (E1 – E11) and introns (I1 – I10) of the *TUBG1* gene and the primer sets designed for PCR shown in (C).

(C) PCR analysis using primer sets shown in (B) to compare splicing efficiencies of *TUBG1* splicing junctions in control (ct) and *SON* siRNA-transfected (s) cells.

(D) Interaction of SON with RNA. UV-crosslinking and immunoprecipitation (CLIP) was performed with control IgG or SON antibody and associated RNAs were analyzed by RT-PCR.

(E) SON depletion causes impaired splicing of *TUBG1* minigene. An intron and the flanking exons were cloned from the *TUBG1* gene (exon 7 – 8 region and exon 8 – 9 region) and the *TUBA1B* gene (exon 2 – 3 region) to downstream of the CMV promoter to make minigene constructs. HeLa cells pre-treated with control or *SON* siRNA were transfected with the minigenes, and RT-PCR was performed to detect unspliced and spliced RNA.

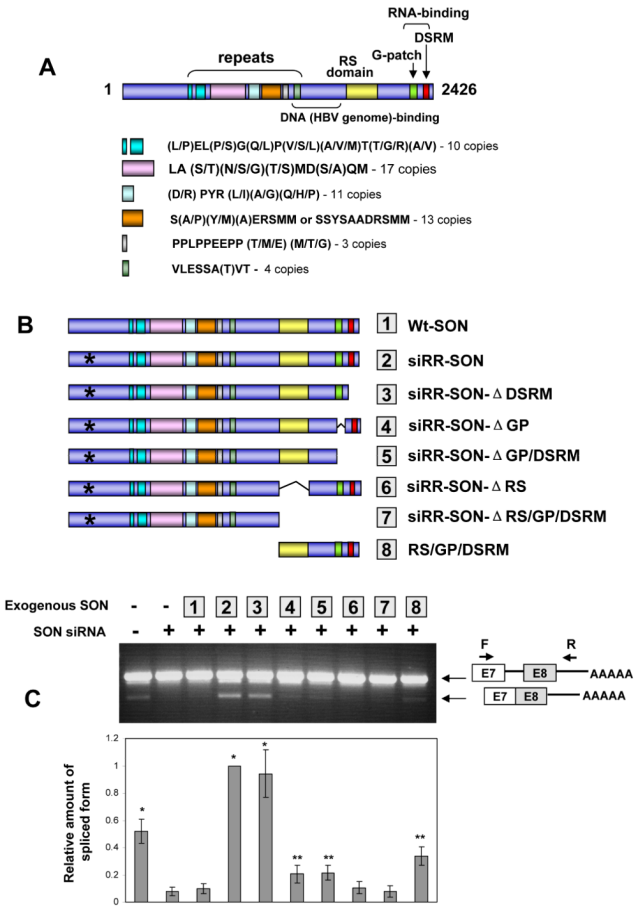

Figure 5. The C-terminal domain containing the RS domain and the G-patch is necessary for SON's activity in splicing

(A) Domains and unique amino acid repeats in the SON protein. Full length SON (known as isoform f) is composed of 2,426 amino acids. It contains an RS domain and two RNA-binding motifs at the C-terminus and a putative DNA-binding domain. In addition, SON contains unique amino acid repeats that span most of the N-terminal region. The features of each repeat are presented.

(B) Various SON fragments generated for the splicing rescue experiment. siRNA-resistant *SON* (siRR-*SON*) was generated by mutagenesis (marked by *), and various deletion mutants were generated from siRR-*SON*.

(C) Splicing rescue by different SON fragments. HeLa cells were transfected with control or *SON* siRNA, and then transfected with *TUBG1* minigene (exon 7 - 8) together with various SON cDNA fragments listed in (B). RT-PCR was performed to detect unspliced and spliced RNA and the photo is the representative result. Relative amount of spliced form was calculated by measuring the density of spliced form (the amount of spliced form in the siRR-*SON* lane was set as 1). Bars represent mean ± SD from 5 independent experiments (*$P <$ 0.005, **$P < 0.03$, *t*-test, when compared to *SON* siRNA only, the 2nd lane).

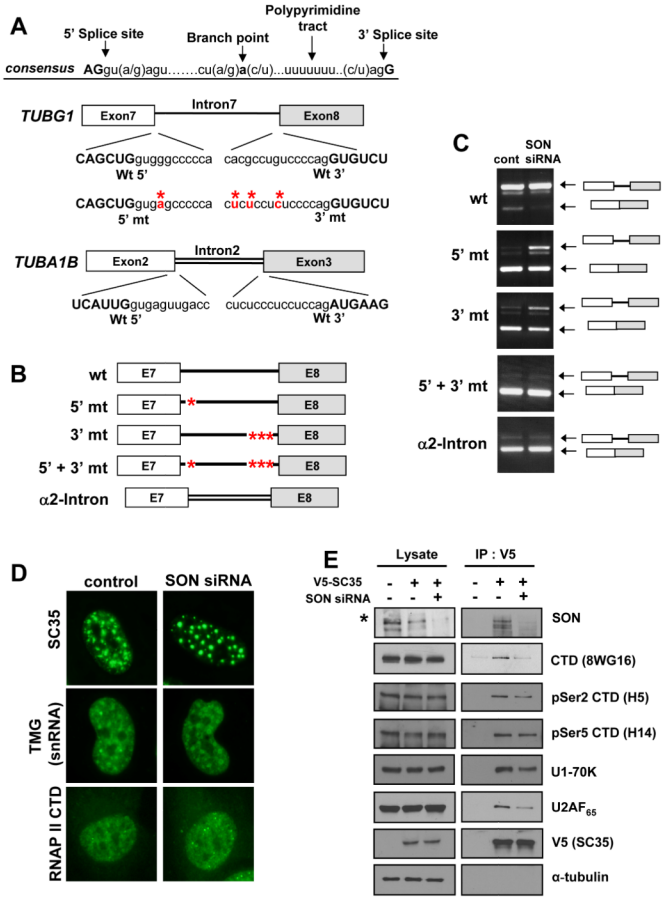

Figure 6. SON is required for processing of weak constitutive splice sites and facilitates the interaction of SR proteins with RNA polymerase II

(A) Splice site sequences in primary transcripts of wild type and mutant minigenes. Consensus sequences in splice sites are presented. The wild type and modified 5′ and 3′ splice site sequences between exon 7 and exon 8 of *TUBG1*, as well as 5′ and 3′ splice site sequences between exon 2 and exon 3 of *TUBA1B*, are presented. Upper cases indicate exon sequences, lower cases indicate intron sequences. Mutated nucleotides are marked in red with an aster.

(B) Various mutant minigene constructs generated for splicing assay. Asters indicate nucleotide changes shown in (A). Intron 7 of *TUBG1* was replaced by intron 2 of *TUBA1B* to generate α2-Intron.

(C) SON is required for processing of weak splice sites. Minigenes shown in (B) were transfected into HeLa cells pre-treated with control or *SON* siRNA. RT-PCR was performed to detect unspliced and spliced RNA.

(D) *SON* knockdown altered SC35 localization, resulting in completely round shaped SC35 dots, and moderately affects snRNP localization, but does not affect localization of the CTD of RNAP II. Cells were immunostained for SC35, 2,2,7-tri-methylguanosine (TMG for snRNA) and the CTD of RNAP II (detected by 8WG16).

(E) SON facilitates SC35 interaction with the CTD of RNAP II, U1 snRNP and $U2AF_{65}$. HEK293 cells expressing V5-tagged SC35 were transfected with control or *SON* siRNA, and V5-immunoprecipitation was performed to pull down SC35 complex. HEK293 cells without V5-SC35 expression were included as a control. Cell lysates and V5-immunoprecipites were immunoblotted with antibodies indicated. Due to the weak affinity

of SON antibody in immunoblot, more concentrated lysates were used to detect SON from the lysates (panel marked with *).

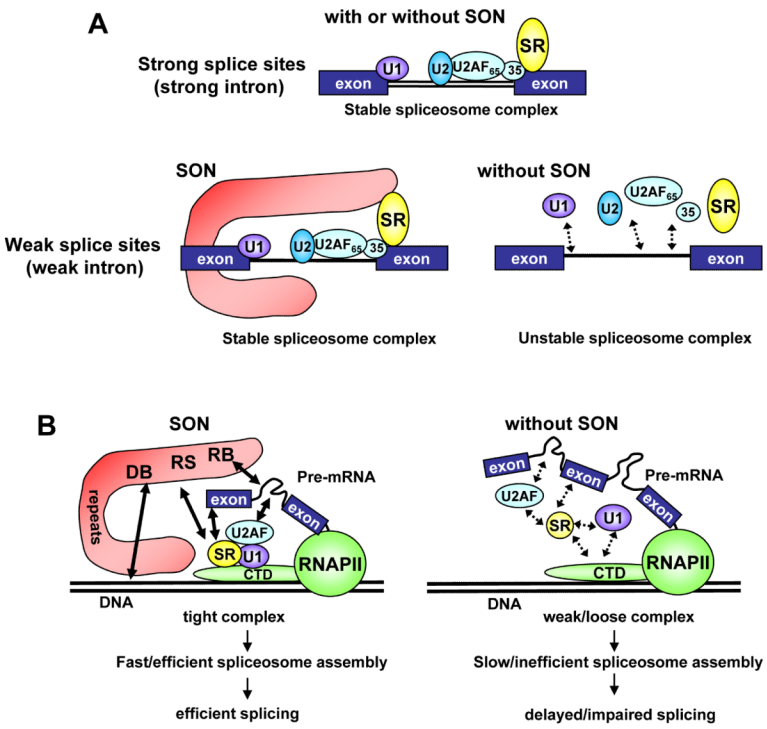

Figure 7. Proposed models for the role of SON as a splicing co-factor

(A) If splice sites are strong (which can be due to optimal sequences in the intron), interactions between pre-mRNA and spliceosome components are strong and stable. Therefore, spliceosome complex is formed for splicing regardless presence or absence of SON. When splice sites are weak (due to suboptimal sequences), spliceosome components form weak and unstable interactions (represented by dotted lines) with pre-mRNA in the absence of SON, while stable interactions can be assured by presence of SON. The process may involve interaction(s) between SON and other critical splicing factors, including SR proteins.

(B) A model for the role of SON as a co-factor in efficient transcription-splicing coupling. During transcription in wild type cells (left), SON interacts with DNA, RNA, SR proteins and other early spliceosome components through its DNA-binding domain (DB), RNA-binding motifs (RB) and RS domain (RS), thereby facilitating recruitment of early spliceosome components to the CTD of RNAP II. The long and unique amino acid repeats in SON may help this protein stretch and make contact with multiple components. Such organization and connection by SON may help efficient and immediate spliceosome assembly on the nascent pre-mRNA with a weak splice site, resulting in efficient splicing. In the absence of SON (right), SR proteins and other early spliceosome components are not efficiently recruited to the CTD of RNAP II, and DNA, RNA and proteins are not closely associated with each other. Therefore, co-transcriptional spliceosome assembly is not efficient, and splice site recognition/selection on a weak splice site is not accurate, resulting in delayed or impaired splicing.