REVIEW

Nucleus 1:6, 447-459; November/December 2010; © 2010 Landes Bioscience

The function of spliceosome components in open mitosis

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Key words: open mitosis, splicing, spliceosome, RNAi, cell cycle, chromatin, mRNA processing

Abbreviations: cdk1, cyclin dependent kinase; NE, nuclear envelope; NPC, nuclear pore complex; NTC, nineteen (Prp19) containing complex; RNP, ribo-nucleoprotein particle; snRNP, small nuclear RNP

Spatial separation of eukaryotic cells into the nuclear and cytoplasmic compartment permits uncoupling of DNA transcription from translation of mRNAs and allows cells to modify newly transcribed pre mRNAs extensively. Intronic sequences (introns), which interrupt the coding elements (exons), are excised ("spliced") from pre-mRNAs in the nucleus to yield mature mRNAs. This not only enables alternative splicing as an important source of proteome diversity, but splicing is also an essential process in all eukaryotes and knock-out or knock-down of splicing factors frequently results in defective cell proliferation and cell division. However, higher eukaryotes progress through cell division only after breakdown of the nucleus ("open mitosis"). Open mitosis suppresses basic nuclear functions such as transcription and splicing, but allows separate, mitotic functions of nuclear proteins in cell division. Mitotic defects arising after loss-of-function of splicing proteins therefore could be an indirect consequence of compromised splicing in the closed nucleus of the preceding interphase or reflect a direct contribution of splicing proteins to open mitosis. Although experiments to directly distinguish between these two alternatives have not been reported, indirect evidence exists for either hypotheses. In this review, we survey published data supporting an indirect function of splicing in open mitosis or arguing for a direct function of spliceosomal proteins in cell division.

Introduction

During the past 30 years, it has become apparent that the vast majority of all protein-coding primary transcripts in human cells contain intronic sequences and therefore are subject to splicing. Several excellent recent reviews have summarized our current understanding of structure and function of the eukaryotic splicing machinery at the molecular level (reviewed in refs. 1–5). In general, splicing involves two trans-esterification reactions in the pre-mRNA, which seamlessly join the exons and leave behind a lariat-shaped excised intron. Although the chemistry of splicing is rather simple, the biochemical basis for splicing in eukaryotic nuclei is enormously complex. Advances in mass spectrometry techniques have enabled comprehensive proteomic analysis of splicing machineries ("spliceosomes"); these led to estimates that splicing in the nucleus generally involves about 150 different proteins, many of which associate with small nuclear RNAs:[6-10] the central components of spliceosomes are ribo-nucleoprotein particles (RNPs) comprising Uridine rich small nuclear RNAs (UsnRNAs) and proteins. The corresponding snRNPs (U1, U2, U4/U6 and U5 snRNPs) contain proteins common to most snRNPs, named Sm proteins, as well as specific proteins unique to the different snRNPs.[11-14] snRNPs, together with many other proteins or non RNA containing protein complexes, assemble spliceosomes anew, trigger the catalytic events of the splicing reaction, then disassemble, on every splice site in a defined order.[5] More recent proteomic studies surveyed the dramatically changing protein content of intermediates of the human spliceosome during the different stages of spliceosome assembly, catalysis and disassembly.[15-18] They confirmed a large body of biochemical and genetic evidence from yeast and mammalian systems, indicating that the spliceosome dynamically reshapes and exchanges snRNP moieties, accessory proteins and protein complexes concomitant with the splicing reaction (**Fig. 1**).

Our current model of eukaryotic spliceosome assembly and function locates the initial step to the 5' end of the intron, which is recognized by base pairing between the pre mRNA substrate and the RNA moiety of U1snRNP (**Fig. 1**). The heterodimeric U2 auxilary factor (U2AF35/U2AF65) identifies specific sequences within the intron, such as the branch point sequence (BPS), and at the 3' splice site.[19,20] Again, base pairing of U2snRNA and the pre mRNA then enables U2snRNP to take over recognition of the BPS, resulting in formation of a rather stable, biochemically accessible spliceosome intermediate named complex A[21] (**Fig. 1**). Complex A recruits the largest spliceosomal entity, a preassembled tri-snRNP of the U4/U6 di-snRNP and the U5snRNP.[22] Moreover, 35–50 additional, non-snRNP proteins get involved, while several complex-A-proteins are concomitantly released.[17] Biochemical data from different systems indicate that at this point, for instance, the spliceosome recruits the Nineteen-containing complex (NTC): a macromolecular, but non-RNA

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Submitted: 06/30/10; Revised: 08/09/10; Accepted: 08/13/10
Previously published online:
www.landesbioscience.com/journals/nucleus/article/13328
DOI: 10.4161/nucl.1.6.13328

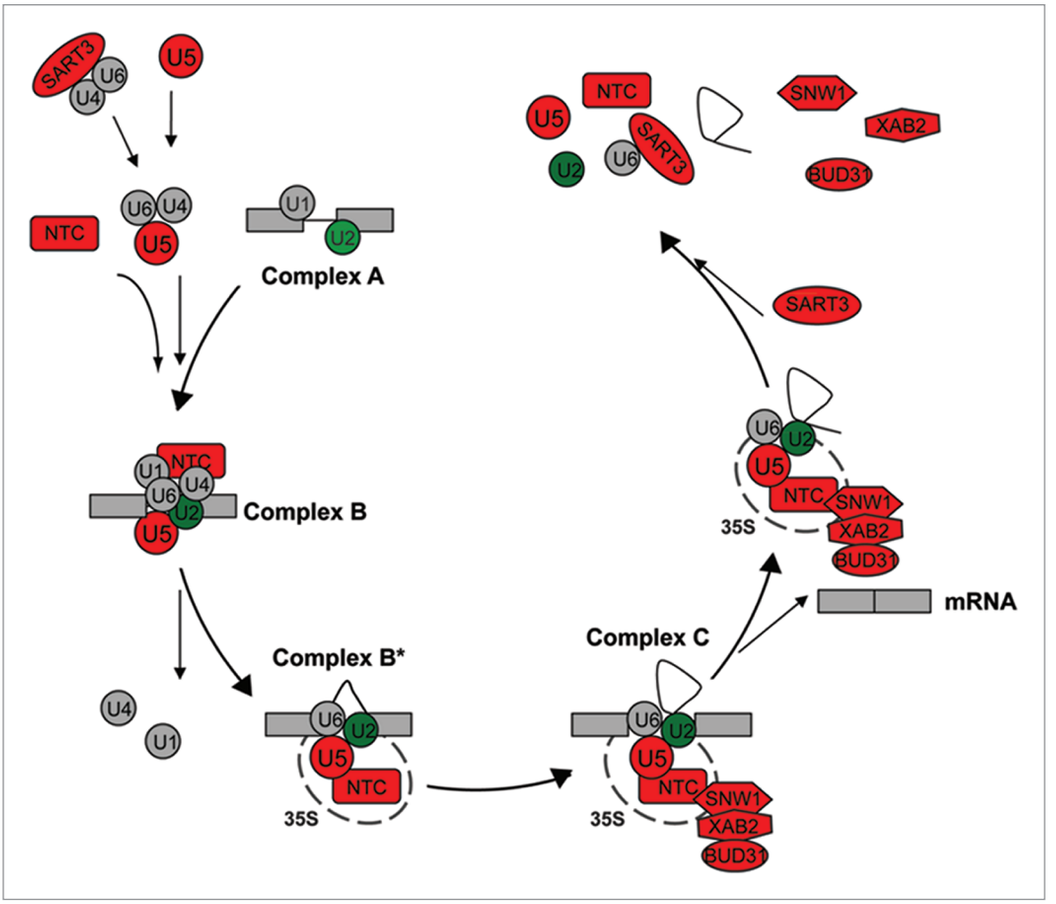

Figure 1. Dynamic changes of spliceosomal components during the splicing reaction. The spliceosome assembles intermediates during recognition of the splice site (complex A), before and after the first catalytic step (complex B and B*), and for the second catalytic event (complex C). Spliceosomal proteins and subcomplexes are indicated: U1, U2, U4/U6 and U5 snRNPs as well as the nineteen containing complex (NTC) and accessory proteins. The RNAi mediated knock-down of red components leads to defects in mitosis in human cells; green color indicates defects in cytokinesis (see also **Table 1**).

containing complex (**Fig. 1**). The name-giving protein of this complex, Prp19p, was initially identified in a genetic screen for factors essential for pre mRNA processing in yeast.[23] Prp19p turned out to be required for splicing, yet not as a core snRNP component.[24] Its human counterpart, PRPF19, forms a tight complex with several other proteins: SPF27/BCAS2, PLRG1 and CDC5L, as well as three additional proteins (CTNNBL1, SNW1, HSP73/HSPA8), which are less tightly associated with the core complex.[25-27] An analogous complex also exists in *S. cerevisiae*[28] and *S. pombe*;[29] it interacts with the spliceosome at a similar stage of the assembly as its human counterpart.

After recruitment of the NTC and the tri-snRNP, the so called complex B spliceosome contains all the snRNPs as well as many other proteins required for catalysis, but it is still not competent to drive the first catalytic step of the splicing reaction (**Fig. 1**). The spliceosome undergoes a series of rearrangements which include loss of U1 and U4 snRNPs[5] (**Fig. 1**) to bring the entire machinery into a form that is capable of catalysis (complex B*, ref. 30, **Fig. 1**). After the first catalytic event, further proteins get associated or released, such as the U2-related proteins and U4/U6 proteins: this accompanies additional defined rearrangements. The resulting complex C then catalyzes the second transesterification reaction, before the spliceosome disassembles and dissociates from the spliced site of the mRNA[2] (**Fig. 1**).

During final disassembly, the U5snRNP leaves the spliceosome together with the NTC and other components as a 35S-supercomplex.[26,31] All components are recycled, with the reassembly of the U4/U6 di-snRNPs to allow renewed tri-snRNP assembly for further rounds of splicing.[26] Meanwhile, after release of the splicing machinery, a non-spliceosome RNA binding protein complex called the exon-junction complex marks the site of the splicing event within the mature mRNA. The exon-junction complex constitutes a label for properly spliced mature mRNAs; it helps to export the mRNA from the nucleus and to control mRNA stability in the cytoplasm.[32,33]

The splicing machinery not only executes splicing catalysis, but also governs faithful identification of splice sites. A large number of proteins associate with the splicing machinery to influence splice site decisions. In particular, the family of highly conserved SR proteins (rich in Serine and Arginine) favor recruitment of the splicing machinery to splice sites.[34-36] In contrast, several hetergenous nuclear ribonucleoprotein particle (hnRNP) proteins reduce the efficiency with which a given splice site is recognized

by spliceosomal components.[37] SR and hnRNP proteins together establish a specific "code" on each splice site.[38,39] The spliceosome decodes this information and translates it into the probability for selecting this site. This provides the basis for regulated changes in the pattern of mature mRNAs by alternative splicing. Alternative splicing mechanisms include a choice between 5' and/or 3' splice sites for an individual intron, the use of novel pairs of 5' and 3' splice sites or omission ("skipping") of an entire exon (reviewed in ref. 40–43). The potential to differentially modify primary transcripts generated from the same genomic DNA by alternative splicing enormously increases the repertoire of proteins that can be produced in a eukaryotic cell. In human cells, for example, roughly 20,000 open reading frames generate far more than 100,000 proteins, which is consistent with estimates that the average number of different mature mRNAs generated from a single primary transcript is between 7 and 10.[44,45]

Changes in alternative splicing frequently occur upon cellular fate decisions and tissue specific expression of splicing regulators such as SR proteins or hnRNP proteins is often involved. hnRNPI/PTB, for instance, is a strong splice site repressor that is generally expressed in cells before neuronal differentiation. When it gives way to its paralogue nPTB (neuronal PTB), this enables the expression of a variety of different genes specific to the nervous system.[46,47] In HeLa cells, PTB can be counteracted by the splicing regulator TIA1, which modulates the sensitivity of cells to apoptotic signals: the activities of PTB versus TIA1 determine the relative amounts of active versus inhibitory forms of the death receptor CD95.[48] Tissue specific splicing regulators such as FOX1 and FOX2,[49] or the antagonizing activities of *muscleblind* and CELF family proteins[50] control alternative splicing upon differentiation in skeletal and heart muscle as well as in brain cells. Splicing regulators of the Nova protein family are required in brain cell differentiation and possibly in identification of subregions of the brain, in which they are specifically expressed.[51-54] Although the mechanisms by which all the cues influencing splice site decisions are decoded are not understood in detail, it is clear that the splicing machinery needs to be highly flexible in order to integrate signals from both its substrates as well as a large number of trans-acting proteins. The need to execute splicing catalysis on a huge number of substrates faithfully, and to react flexibly to changes in the molecular environment of splice sites may explain the complexity of the spliceosome.

Open Mitosis

Extensive modification and splicing of pre-mRNAs require the integrity of the nuclear compartment. However, most eukaryotic cells disassemble the nucleus at mitotic onset so that cytoplasmic and nuclear components merge in the mitotic cytoplasm.[55,56] The central regulator of mitosis, the cyclin B/cdk1 (cyclin-dependent kinase 1) complex, mediates break down of the nuclear compartment. Activated cyclinB/cdk1 enters the nucleus early in mitosis and directly phosphorylates several proteins of the nuclear pore complex (NPC) and most likely also proteins of the nuclear lamina.[57,58] In interphase, the lamina maintains the structural integrity of the nuclear envelope (NE). Mitotic phosphorylation of lamins, the building blocks of the nuclear lamina, directly promotes lamin depolymerisation.[59-61] Likewise, mitotic phosphorylations of NPC proteins trigger NPC disassembly in a defined, stepwise manner.[62] Together with mechanical forces generated by mitotic microtubules and motor proteins, the above-mentioned modifications of NE and NPC proteins implement breakdown of the nucleus.[63-65]

Apart from NPCs and the NE, intranuclear compartments disassemble and their functions are disabled upon mitotic entry and NE breakdown. For instance, the nucleolus, the organising center for the biogenesis of ribosomal subunits, is dismantled during mitosis of eukaryotes. Cdk1-dependent phosphorylations induce the loss of initiation factors for RNA-polymerase I-dependent transcription from fibrillar centers of nucleoli and stop initiation of polymerase I-dependent transcription.[66,67] Likewise, cyclinB/cdk1 downregulates polymerase II dependent transcription.[68,69] Moreover, the mitotic chromatin is subject to global compaction, which prepares chromosomes for their segregation. Inhibitory phosphorylation of the nuclear transcription machinery and most likely the hypercondensation of mitotic chromosomes are responsible for the largely reduced transcription in mammalian cells after mitotic entry.[70,71] Moreover, mitotic modifications regulate splicing: experiments with mammalian cells and cell extracts showed that the modification state of SRp38, a central splicing regulator, changes at the beginning of mitosis.[72,73] The majority of SRp38 was shown to be phosphorylated in interphase; in this form SRp38 can act as a sequence specific activator of splicing and contributes to splice site selection.[74] However, SRp38 is partially dephosphorylated in mitotic cell extracts and in intact cells preparing for mitosis. Mitotic dephosphorylation turns the splice activator SRp38 into a general, potent repressor of splicing. Consistent with this, cell-free lysates from mitotic cells were inefficient in in vitro splicing reactions, but activity was restored when SRp38 was specifically immunodepleted.[72] Although there is no experimental evidence to date, we speculate that the disassembly of splicing protein complexes by mitotic modifications also contributes to downregulation of splicing in mitosis. In general, the combination of chromosome condensation and mitotic modifications of the nuclear gene expression machinery shuts down basic nuclear functions, including not only transcription, but also RNA modifications such as splicing.

Dual Functions of Nuclear Proteins in Open Mitosis

So far, it seems that many nuclear proteins, RNAs and RNPs stop their specific interphase function in open mitosis, they diffusely distribute throughout the mitotic cytoplasm and await reactivation of their functions upon nuclear reassembly in telophase. Pursuing basic nuclear functions during open mitosis is apparently inefficient or even harmful for the cell. However, several observations challenge this simplistic model. After NE breakdown and NPC disassembly, a fraction of the Nup107/160 subcomplex of NPCs accumulates at kinetochores and colocalizes with spindle poles and spindle microtubules[75,76] (see **Fig. 2**). RNAi experiments in human cells showed that reducing the levels of components of this complex leads to impaired attachment of microtubules to kinetochores, reduced inter-kinetochore tension

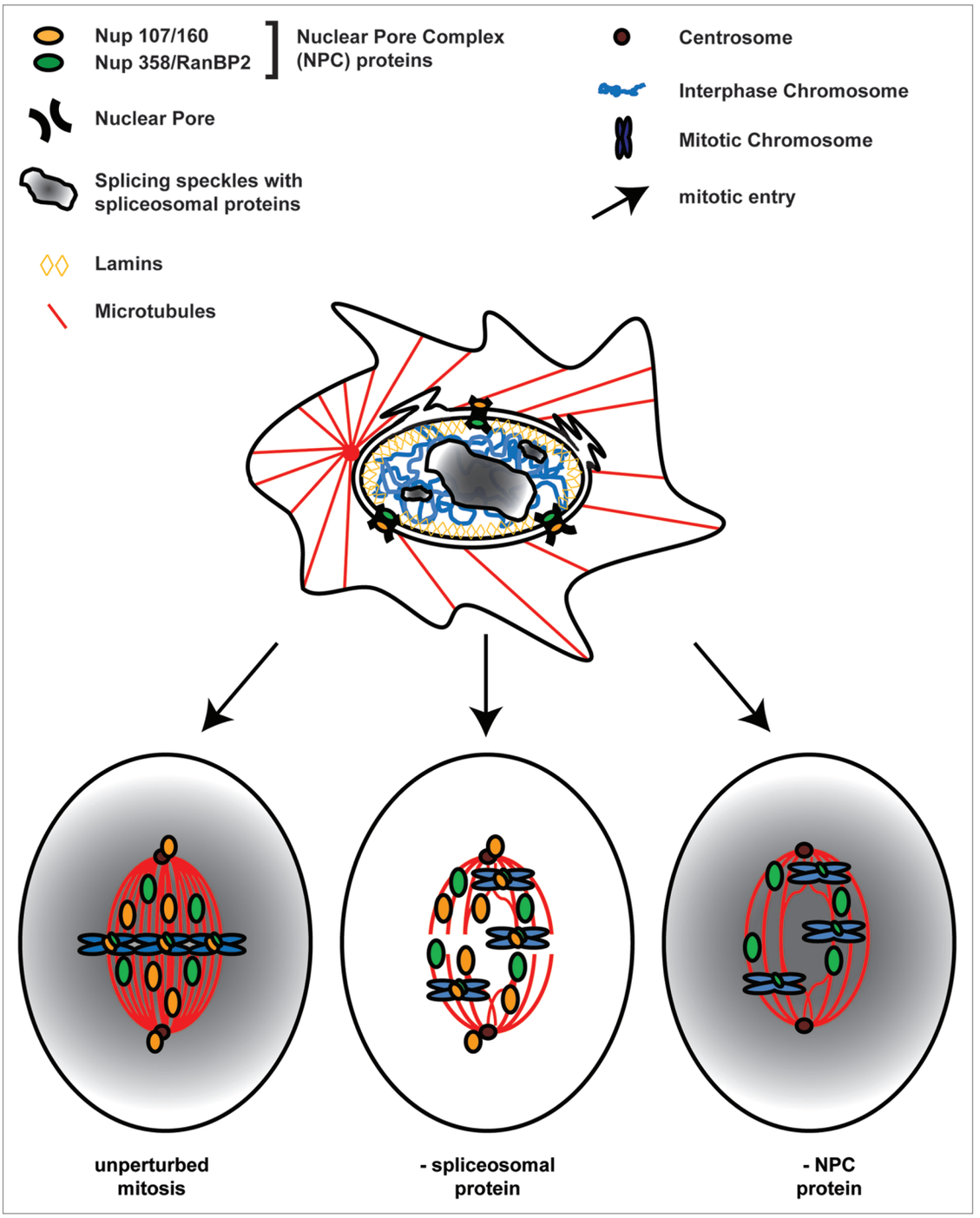

Figure 2. The role of splicing proteins in open mitosis. During Interphase (top) the nuclear envelope ensures spatial separation of nuclear and cytoplasmic processes and NPCs (Nuclear Pore Complex) mediate exchange of molecules between the cytoplasm and the nuclear compartment. Special areas in the nucleus harbor many splicing proteins ("splicing speckles", top, gray gradient). With the onset of mitosis (arrows) phosphorylation of lamins and NPC proteins triggers the disassembly of the nucleus. Some components of the NPC directly contribute to open mitosis; their knock-down results in cell division defects (bottom, right). These are relocalized to the mitotic spindle or kinetochores (bottom). In contrast, splicing factors are evenly distributed in the open mitotic cytoplasm (bottom, gray gradient). However, their knock-down also leads to defective mitoses (bottom, center).

and activation of the spindle assembly checkpoint.[76-78] Consistent with this, spindles assembled in Xenopus egg extracts depleted of the Nup107/160 subcomplex displayed severe assembly defects, which were likely due to the loss of stable microtubule-to-kinetochore attachments.[78] Likewise, a macromolecular entity consisting of the large nucleoporin Nup358/RanBP2 localizes to spindle microtubules and kinetochores in mitosis. Compromising its function by RNAi led to defects in chromosome congression and sister chromatid separation in human somatic cells[79,80] (**Fig. 2**). RanBP2 operates as an E3 ligase of the SUMOylation system[81] and modifies several target proteins specifically in mitosis, including Topoisomerase II (TopoII).[82,83] TopoII starts to disentangle sister chromatids after replication, and is also required in mitosis to resolve catenation between sister chromatids, in particular at centromeric regions. Targeting of TopoII to the centromere requires RanBP2 mediated SUMOylation. Consequently, mice expressing reduced levels of RanBP2 displayed defects in sister chromatid separation leading to severe aneuploidy.[84] The question arises whether the mitotic function of NPC proteins is just an exotic exception, or whether this example underlines a general theme of nuclear proteins actively contributing to open mitosis.

Splicing factors in mitosis. In 2004, Frank Buchholz and colleagues (Kittler et al., ref. 85) initiated a screen to identify gene products required for proper cell division on the basis of RNAi in human cells. Kittler et al. analyzed 5,305 genes, which they specifically knocked down by transfection of in vitro transcribed and endonuclease processed siRNAs. After screening and validation using time lapse microscopy to follow cells through the cell cycle, the authors found 37 genes, the knock-down of which caused severe cell cycle progression or cell division abnormalities. Seven of the products had a well-established function in splicing. Downregulation of snRNP proteins, like SmB/B' or U2A', the tri-snRNP assembly protein SART1 or the NTC protein SKIP/SNW1, led to severe problems in mitotic spindle assembly and caused accumulation of cells in mitosis.[85] Later, the same group investigated functions of human gene products in cell cycle progression and cell division on a genome-wide scale. They determined the DNA content of human cells to analyze cell cycle progression after knock-down of single gene products. Targeting of more than 2,000 gene products by siRNAs resulted in cell cycle defects, suggesting that roughly 10% of all gene products may be required for proper cell cycle progression and cell division in humans. Out of the approximately 150 gene products involved in splicing (see **Supp. Table 1** and ref. 6), the knock-down of 18 (i.e., 14%) resulted in delays in cell cycle entry (G_1), S-phase or at several stages of M-phase.[86,87] It therefore transpired that the group of mitotic gene products may contain a considerable number of splicing proteins. Yet, the repression of splicing in mitosis of higher eukaryotes argues against a direct function of splicing in cell division. Were the identified splicing factors therefore just false positives?

Phenotypic Analysis of Splicing Proteins in the MITOCHECK Screen

Very recently, the MITOCHEK consortium finished a more detailed screen focused on human cell division. Jan Ellenberg and colleagues used time lapse imaging in human cells stably expressing histone H2B as a chromatin marker.[88] They systematically knocked-down human gene products by synthetic siRNAs and tracked chromatin dynamics throughout two cell cycles. This screen identified candidates for gene products required for cell division.[89,90] In addition, through the observed phenotypes, it suggested different functional classes: gene products involved in mitotic entry (i.e., prometaphase), metaphase progression and metaphase chromosome alignment, chromosome segregation or cytokinesis. The knock-down phenotypes included chromosome congression and alignment defects—often resulting in subsequent apoptosis—lagging chromosomes or binucleated cells. Quality, penetrance and occurrence time of phenotypes seen after each individual knock-down generated a characteristic signature. Comparing the signature of well-characterized mitotic gene products with novel potential mitotic effectors may predict their potential mitotic functions.

The MITOCHECK screen found 27 out of ca. 150 core components of the human spliceosome to be required for proper mitosis[89,90] (see **Table 1 and Supp. Table 1**). The screen indicated, for instance, that the Sm proteins SmF and SmD3, as well as the general splicing promotor SF2/ASF are required for proper mitosis in human tissue culture cells.[89] The same applied for the human ortholog of yeast *prp17*, PRPF17 and Slu7/human SLU7.[89] Both of these proteins are Complex C proteins and only associate with the spliceosome in preparation for the second catalytic event.[91,92] Although *prp17* is not essential in *S. cerevisiae*, mutants of yeast *prp17* previously turned out to be allelic with *cdc40* (*cell division cycle* mutant 40). These mutants accumulated splicing intermediates, but also displayed severe defects in spindle assembly as well as hypersensitivity to DNA damaging agents and defects in entry into replication.[93-98] Recently, also, the analysis of a ts-allele of the putative splicing regulator Smu1 revealed defects in chromosomal stability and proper cell division in mammalian cells.[99] The MITOCHEK screen identified human SMU1 as a potential mitotic regulator: its knock-down caused a penetrant delay upon mitotic entry, defects in chromosome alignment and in establishing metaphase and chromosome segregation problems[89] (**Table 1**). Moreover, the human Red/IK protein was indispensable for mitosis[89] (**Table 1**). Red/IK associates with the spliceosome in human cells[6] although its molecular mode of action in splicing is unclear.

Notably, most "mitotic hits" were associated with functions of a few specific subcomplexes of the spliceosome.[89] Five out of seven components of the PRPF19/NTC complex (PRPF19, CDC5L, BCAS2/SPF27, PLRG1 and CTNNBL1,[26,27]; see **Fig. 1**) were apparently required for proper cell division (**Table 1 and Fig. 1**). In addition, reducing the levels of the SKI-interacting protein (SKIW), XAB2/Syf1 and BUD31/G10, which all associate during late stages of spliceosome function with the NTC,[5,26] resulted in a similar pattern of mitotic defects as was observed upon reduced levels of PRPF19 or BCAS2 itself[89] (**Table 1 and Fig. 1**). The NTC joins the spliceosome after the association of the tri-snRNP, which contains U5snRNP. All the high molecular weight proteins of the U5snRNP (U5-220/PRPF8; U5-200/Brr2/SNRNP200/ASCC3L1; U5116/EFTUD2 and U5102/PRPF6,[13])

Table 1. Knock-down phenotypes of spliceosomal components in human cells

Protein name	mitotic delay	PM delay	M alignment delay	m-phase delay	Nuclei close/ binuclear	Lagging chromos.	Nuclear shape	Cell death	HUGO name	Acce. # with link	Function in splicing
Sm D3	x		x				x	x	SNRPD3	P43331	Sm/Lsm core
Sm F	x		x		x	x	x		SNRPF	Q15356	"
ASF/SF2	x		x	x					SFRS1	Q07955	SR proteins
U2AF65					x	x	x		U2AF2	P26368	U2snRNP
U2AF35			x	x	x		x		U2AF1	Q01081	"
SAP155	x		x	x	x	x	x	x	SF3B1	AF054284	"
SAP114	x		x	x	x	x	x	x	SF3A1	Q15459	"
U5-220 kD	x		x	x		x	x	x	PRPF8	AB007510	U5snRNP
U5-200 kD (Brr2, (DExD/H))	x		x	x	x	x	x		ASCC3L1	O05643	"
U5-116 kD	x		x	x	x		x	x	EFTUD2	BC002360	"
U5-102 kD	x		x	x			x		PRPF6	BC001666	"
U4/U6-15.5 kD	x		x	x				x	NHP2L1	P55769	U4/U6
Tri-snRNP 110 kD	x		x	x		x	x		SART1	T00034	trisnRNP assembly
SART3*	x		x	x		x	x		SART3	AB020880	"
MFAP1	x		x	x			x	x	MFAP1	P55081	"
CDC5L	x		x		x	x	x	x	CDC5L	NP_001244	NTC/NTC related
PLRG1	x	x	x	x			x	x	PLRG1	AF044333	"
hPrp19	x		x	x	x	x	x	x	PRPF19	BC008719	"
SPF27	x		x	x			x		BCAS2	AF081788	"
NAP	x		x	x	x	x	x	x	CTNNBL1	NP_110517	"
SKIP	x		x		x	x	x	x	SNW1	Q13573	"
fSAP17	x		x						BUD31	P41223	"
XAB2	x		x	x				x	XAB2	BC007208	"
hSlu7	x		x			x	x	x	SLU7	BC010634	Complex C proteins
hPrp17 (hCDC40)	x	x	x				x		CDC40	AF038392	poorly characterized
fSAP57	x	x	x	x		x	x		SMU1	BC002876	"
RED	x		x				x	x	IK	Q13123	"

Spliceosomal proteins, which were identified by comprehensive proteomic analysis[6] were listed and assigned to respective subcomplexes (right column). Their knock-down phenotypes were listed according to the MITOCHECK database (www.mitocheck.org). "Hits" (red) are defined as gene products the knock-down of which resulted in mitotic delay and at least one specific additional category. Categories were defined according to reference 89. The knock-down of U2snRNP associated proteins (green) caused very penetrant defects in chromosome segregation resulting in frequently occurring binuclear cells. *(not in Zhou et al.).

turned out to be important for proper cell division (**Table 1 and Fig. 1**). During spliceosomal rearrangements that enable the catalytic steps of splicing, U4/U6 and U5snRNPs loose some of their proteins concomitantly, such as human NHP2L/snu13, the smallest di-snRNP protein; this turned out to be important for proper mitosis as well[89] (**Table 1**). The human orthologs of yeast *snu66* and *prp24*, SART1 and SART3, are required for tri-snRNP function and recycling. SART3 specifically interacts with the di-snRNP of U4 and U6 but is not directly associated with the tri-snRNP. Consistent with that, SART3 accumulates in Cajal bodies and not in splicing speckles, the storage compartments of spliceosomes, from where they become recruited to sites of active splicing.[100-104] However, reducing the levels of SART3 triggered metaphase alignment problems similar to those after loss of PRPF19, BCAS2 or of snu66/SART1,[89] (**Table 1 and Fig. 1**). Snu66 associates with the tri-snRNP throughout the splicing

reaction, and is directly required for loading the tri-snRNP to the spliceosome.[105,106] In addition, the knock-down of human microfibril associated protein (MFAP1) led to mitotic defects[89] (**Table 1**). The Drosophila ortholog of human MFAP1 was previously shown to associate with the fly homolog of the tri-snRNP associated factor Prp38. Mutations in dMFAP1 and dPrp38 or knock-down of either protein in Drosophila tissue culture cells, consistently demonstrated that both proteins are necessary for proper cell division.[107] This indicates that most of the splicing factors (14 of 27, **Table 1**), which are also indispensable for mitosis, are functionally linked to the NTC and U5snRNP dynamics during and after the splicing reaction.

Finally, three proteins of the U2 snRNP also seem to be essential for proper mitosis. SF3A1, SF3B1 and U2AF35/U2AF1 are U2 snRNP specific proteins or functionally interact with U2 snRNP.[5,13] In contrast to the majority of the other "mitotic" splicing proteins, the functional signature all these U2 associated proteins was different: Their knock-resulted just in penetrant occurrence of binuclear cells or chromosome segregation problems[89] (**Table 1 and Fig. 1**). Binuclear cells consistently arise as a consequence of compromised cytokinesis, like seen after knock-down of essential effectors of the contractile ring such the RhoA regulators RACGAP and ECT2.[108] In this respect, the phenotype observed after knock-down of U2 snRNP proteins is a remarkably exact phenocopy of proteins with central functions in cytokinesis.

Sequential Order of Phenotypes

The majority of the "mitotic splicing proteins" shared a similar signature: they displayed mitotic phenotypes, including prometaphase delay and metaphase alignment problems leading to an arrest before and in metaphase, presumably as a consequence of mitotic checkpoint activation. Most likely due to slippage from this arrest in metaphase, cells subsequently showed cell death or chromosome segregation problems. This "event order" was reminiscent of phenotypes after knock-down of key mitotic regulators and effectors, such as Polo like kinase 1 (PLK1), Aurora A, TPX2 or the motor protein Eg5/KIF11.[89,90] However, phenotypes of knocking-down splicing proteins often became apparent after 30–40 hours, i.e., ca. 10 hours later than mitotic defects were displayed in cells depleted of known mitotic gene products.[85,89] The knock-down of TPX2, for instance, started to cause metaphase alignment problems 20 hours after seeding out cells on the siRNA-containing transfection mix and affected ca. 25% of all cells after 30 hours,[89] while the knock-down of PRPF8 initiated metaphase alignment defects in mitotic cells only after 30 hours[89] and reached a maximum penetrance of 10–15% after 45 hours. This raises the question if patterns of mature mRNAs and protein isoforms of key mitotic regulators are altered after RNAi-mediated knock-down of splicing proteins. It would, for instance, be interesting to see if mRNA and protein levels of TPX2 are reduced upon knock-down of PRPF8. So far, the mitotic defects seen in the genome wide screens could clearly have been secondary to a failure to splice the mRNAs encoding known mitotic effectors. The similar, but less penetrant and delayed phenotypes of splicing proteins compared to mitotic regulators would be consistent with that. The subset of "mitotic hits" might be the selection of splicing proteins, which are simply most sensitive to the siRNA knock-down. This could be due to different RNAi efficiencies or to different protein half lives: the levels of long-lived splicing proteins after RNAi treatment for 4 days might still be too high to see phenotypic consequences. The observations described above are therefore consistent with an indirect contribution of splicing proteins to mitosis. Indeed, splicing is an essential function in all eukaryotes and required for expression of housekeeping as well as cell cycle regulated genes. The knock-down of single splicing components in higher eukaryotes may quickly lead to changes in the complicated pattern of mature mRNAs and therefore of proteins and protein isoforms critical for mitosis. It looks like an obvious possibility that their reduced levels cause many of the observed phenotypic abnormalities upon downregulation of splicing proteins in mitosis (**Fig. 3A**). Are there additional arguments to support this scenario?

The Molecular Basis for Defective Mitosis

Microarray analysis of gene expression in yeast previously demonstrated that while proceeding through the cell cycle, *S. cerevisiae* actively increases the expression of 10–20% of its genes by periodic transcription.[109,110] Similarly, transcriptional profiling of the human cell cycle revealed periodic changes correlated with the cell cycle in 700 out of 6,800 tested transcripts.[111] Many of the cell cycle regulated gene products in human cells shared common temporal regulation patterns, e.g., they were upregulated on cell cycle entry, at the onset of replication or in preparation for mitosis.[111] Gene products that have functions in cell mobility and remodelling the extracellular matrix turned out to be over represented among the mitotically upregulated transcripts, while the transcription of genes involved in DNA replication and DNA damage response rose in S-phase. For example, the transcription of mRNAs encoding PLK1, which is essential for a variety of M-phase functions, like centrosome maturation, the timing of chromatid cohesion, as well as cytokinesis, was strongly upregulated in M-phase in human fibroblasts.[111] Since the newly transcribed pre-mRNAs must be processed in order to function, splicing will be indispensible for cell cycle progression, and defects in spliceosomal functions in the interphase nucleus might only become visible in subsequent mitosis. The simplest model of the function of splicing proteins in mitosis would be based on the known functions as spliceosomal components: splicing of a single or a few newly transcribed pre-mRNAs is essential for proper mitosis. What could be those critical mitotic effectors?

In *S. pombe*, mutants of *cdc5*, a conserved component of the NTC, displayed corrupted spindle formation and arrested in large budded cells as a consequence of defective nuclear division.[112,113] In this particular case, reduced levels of α-tubulin were sufficient to explain the phenotype: pre-mRNAs encoding for α-tubulin contain a single intron, whose splicing became inefficient upon loss of function of *cdc5*. Rapid degradation of the intron containing α-tubulin mRNA led to reduced levels of α-β-tubulin dimers, which at first affected the highly dynamic spindle microtubules. Strikingly, re-engineering the α-tubulin gene

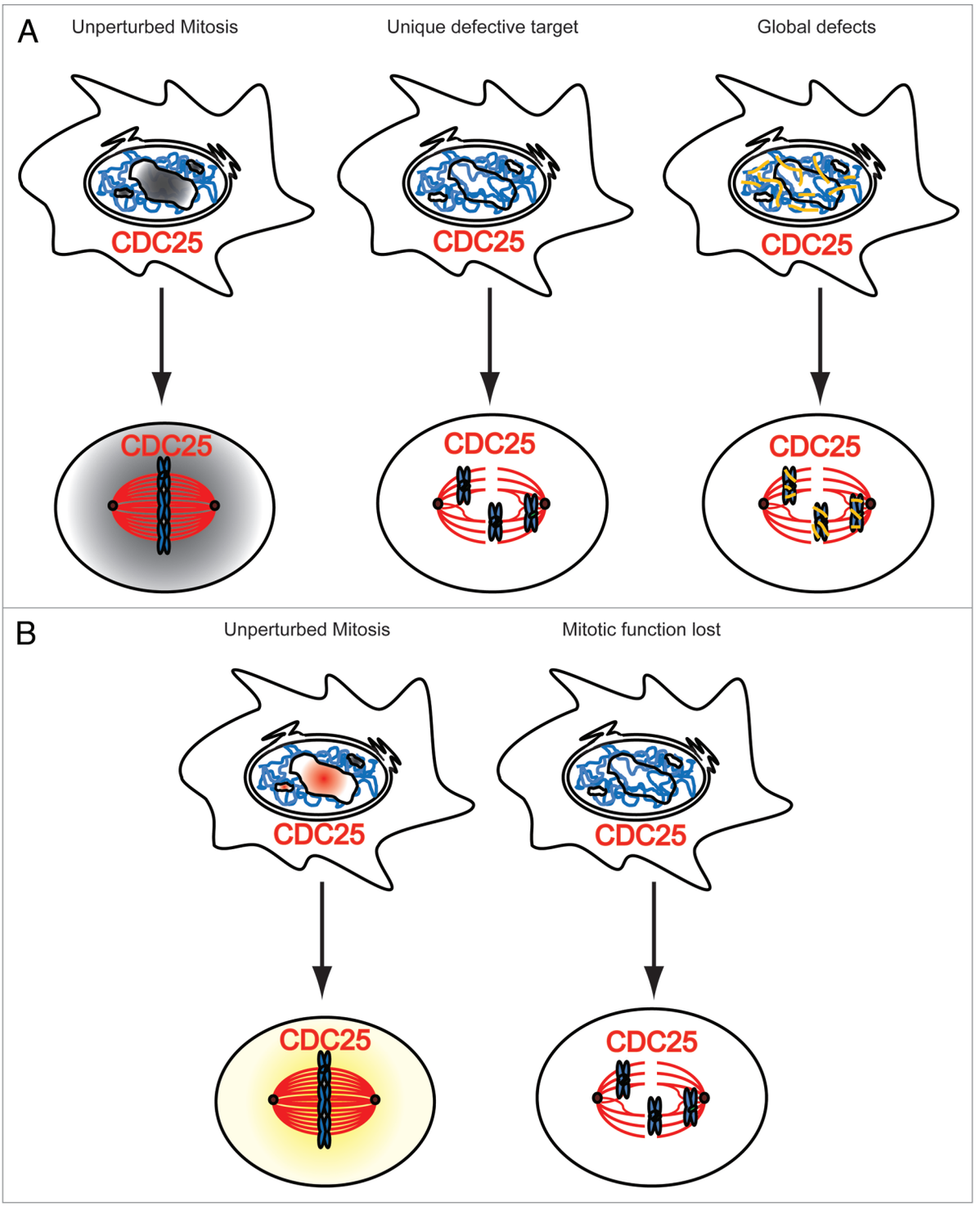

Figure 3. The Molecular basis for mitotic defects after downregulation of splicing proteins. (A) The knock-down of spliceosomal proteins (gray gradient) causes splicing defects, which are causative for mitotic defects. Left: unperturbed mitosis; all mitotic gene products, like e.g., the cell cycle regulator CDC25, are properly spliced and active in mitosis. Middle: Impaired splicing and subsequent loss-of-function of a single gene product, exemplified by CDC25, causes mitotic defects. Right: Impaired splicing leads to the accumulation of improperly processed bulk mRNAs (yellow), which in turn compromise chromatin organization and chromatin condensation in mitosis, and interfere with mitotic progression. (B) Mitotic defects after knock-down of splicing proteins (red) arise as a consequence of direct functions of splicing proteins in open mitosis. Left: unperturbed mitosis; splicing proteins work in the interphase nucleus (red color) and independently in mitosis (yellow color). Right: Their knock-down leads to mitotic defects directly.

using a version without intron made the cells largely resistant to the loss of *cdc5*. They proceeded through cell division with near wild-type levels of α-tubulin and apparently normal nuclear division, although the cells accumulated global defects in pre-mRNA splicing as demonstrated by intron microarray analysis.[114] This indicates that splicing of the α-tubulin pre-mRNA is indeed the most critical if not the only absolutely essential splicing event for mitosis in *S. pombe*. Likewise, reduced levels of Anc1p explain the cell cycle defects of yeast *prp17/cdc40* mutants: the deletion of the *anc1* intron in yeast cells relieved cell cycle arrest and temperature sensitivity of *cdc40* mutants. Anc1p is part of several complexes involved in transcription regulation and chromatin remodelling and is apparently also required for the transcription of genes that are important for cell cycle progression.[97] These two examples from yeast are clearly consistent with the idea that loss-of-function of splicing proteins leads to splicing defects, which finally compromise cell division. They also illustrate that targeting different splicing proteins can affect very different mitotic effectors, possibly providing a general explanation for the heterogeneity of mitotic phenotypes observed after knock-out or knock-down of splicing proteins.

However, the situation in yeasts is considerably less complicated than in metazoan cells, where far more splicing events per pre-mRNA occur. Moreover, transcription, splicing and translation continue throughout closed mitosis in many yeasts, but not in metazoan cells. Nevertheless, the mitotic defects in Drosophila mutants of dPrp38 and MFAP1 correlated with reduced levels of *string* mRNA, the orthologous gene product of yeast *cdc25*, a central cell cycle regulator.[107] Microarray analysis in human HeLa cells likewise revealed that the RNAi-mediated knock-down of the major isoform of U2AF1/U2AF35 resulted in reduced mRNA levels of several hundred mRNAs, including the mRNAs of CDC25A and B. At the same time cells accumulated in prometaphase and metaphase-like states with defective spindle assembly and delayed metaphase chromosome alignment.[115] Cdc25 is a highly conserved dual specific protein phosphatase, which directly acts on cdks and dephosphorylates cdks to activate them.[116] Human cells express three CDC25 genes (A–C), the transcripts of two of which were affected upon knock-down of U2AF1/U2AF35.

In contrast to the knock-down of U2AF1/U2AF35, however, downregulation of the NTC component SKIW did not correlate with changes in the splicing pattern of Cdc25.[115] Likewise, in *S. cerevisiae*, mutant alleles of conserved core components of the spliceosome affected transcripts very differently: microarray data showed that some transcripts readily display intron retention, while others are still largely unaffected.[117] On the other hand, the introduction of the intron-less α-tubulin gene into *S. pombe* cells compensated mitotic defects in CDC5 mutants, but not of mutant alleles of other spliceosome components.[114] Taken together, this may mean that reduced levels of functionally important spliceosome components cause selective changes in splicing pattern and that the cell cycle and mitotic defects are due to the decrease of a few critical mitotic factors. The complex changes in splice patterns after loss-of-functions of splicing proteins may differ depending on the splicing component affected, on the kinetics of knock-down and on the cell type investigated. Does this exclude that splicing factors may exert a direct function in open mitosis, which is entirely independent of their nuclear function (**Fig. 3B**)?

The analysis of the sequential order of phenotypes in the MITOCHECK screen revealed an interesting feature displayed by cells devoid of splicing components: Before the mitotic defects arose, cells with reduced levels of e.g., BCAS2 or PRPF19 showed abnormalities in nuclear size (category "large", ref. 89) or irregular nuclear shape (category "polylobed", ref. 89; see **Table 1**). The "polylobed" phenotype was defined as the frequent appearance of nuclei, which were "frayed" with multiple extra bulges. Gene knock-downs causing the polylobed phenotype included several that led to defects in chromosome segregation, cytokinesis or nuclear reassembly,[89] such as components of the chromosome passenger complex like Aurora B kinase or the Inner Centromere Protein, INCENP.[118] In these cases, the polylobed phenotype frequently occurred as a consequence of mitotic defects. In contrast, the knock-down of splicing proteins caused the appearance of nuclear abnormalities preceding mitotic defects: a very rare pattern that was almost exclusively observed in this group of proteins.[89] This may suggest that splicing proteins might contribute to mitosis in a way different from their established splicing function (**Fig. 3B**).

Direct Functions of Splicing Proteins in Cell Cycle Progression and Cell Division

The genetic analysis of mutants that are hypersensitive to DNA damaging conditions previously suggested a function of yeast *prp19* independent of splicing. This screen used psoralen DNA crosslinking to induce DNA damage and identified pso mutant alleles with reduced tolerance to the genotoxic stress. One allele, *pso4-1*, turned out to carry a mutation in prp19,[119] and consequently accumulated splicing defects at elevated temperature. However, at lower temperature, splicing was apparently normal, while the hypersensitivity to psoralen persisted.[120,121] This indicates that Prp19 has a specific function in the DNA damage response. Various additional observations suggest an essential function of other NTC components besides their well-established and evolutionary conserved role in the spliceosomal activation: RNAi-mediated knock-down of the NTC component CDC5L made human cells hypersensitive to replication stress.[122] PLRG1, another PRPF19 complex protein, also seems to be important for the DNA damage response: PLRG1 knock-out mouse embryonic fibroblasts displayed hypersensitivity to replication stress and DNA damage and readily went into apoptosis. This explained the dramatic effects seen in PLRG1 knock-out mice, which died as early as 1.5 days after fertilization,[123] reminiscent of PRPF19 knock-out animals.[124] Loss of PLRG1 also affected stability and nuclear accumulation of CDC5L, indicating that the overall integrity of the NTC was impaired. Yet, the level of mRNAs encoding critical cell cycle regulators such as p53, cyclin D1 and E1, as well as tubulin, did not change in these PLRG1 knock-out mouse embryonic fibroblasts. This argues for a spliceosome-independent and essential function of the NTC.

Data from Drosophila and mammalian cells also support a splicing independent function of the NTC-associated protein SKIP/SNW1 in transcriptional regulation. SNW1 was initially named as an interaction partner of the proto-oncogene c-Ski.[125-127] The two proteins can counteract the function of Retinoblastoma protein in the repression of E2F transcription factors required for cyclin D synthesis and cell cycle entry.[128] In general, SKIP can function as a suppressor or activator of transcription. Its role as a negative regulator of transcription is consistent with the observed interaction of SNW1 with multisubunit complexes involved in Histone deacetylation. However, SNW1 also helps to recruit the transcriptional activator P-TEFb to RNA Polymerase II.[129] P-TEFb comprises cyclinT1 and cyclin-dependent kinase 9 and mediates hyperphosphorylation of the C-terminal domain of the RNA polymerase II subunit Rbp1 to increase processivity of RNA polymerisation.[130] Knock-down of SNW1 will not only interfere with proper splicing but certainly affect the transcription of many genes including important cell cycle progression.

Recent experiments in yeast further support the idea of a direct mitotic function of the NTC and other spliceosome proteins: *S. pombe* cells harboring mutant alleles of several splicing components, including the PLRG1 ortholog *prp5*, showed dramatic defects in mitotic progression.[131,132] These defects arise as a consequence of defective heterochromatin establishment in the centromeric DNA. Proper assembly of centromeric chromatin in *S. pombe* requires expression of non-coding RNAs from outer repeat sequences, which are processed into siRNAs and then target the RNAi machinery to centromeric transcripts. This signals recruitment of H3K9 Histone methylase and contributes to the establishment of centromeric chromatin and functional kinetochore assembly. Although the role of splicing factors in this process could not be fully elucidated, Bayne et al. suggest that they may provide a platform for the recruitment of the RNAi machinery to centromeric transcripts: spliceosomal proteins were shown to interact directly with the RNAi machinery. Notably, however, some splicing factor mutant alleles affected centromeric silencing, while others did not.[132] This indicates that splicing and centromeric silencing are independent functions but share some common factors, like the PLRG1 orthologs gene product Prp5p.

Accumulation of Bulk, Unspliced RNAs Causes Mitotic Defects

Despite evidence for the direct involvement of splicing factors in mitosis, it seems reasonable to assume that loss-of-function of splicing components leads to the accumulation of unspliced or unprocessed RNA species. Li and Manley recently reported that chicken DT40 cells depleted of ASF/SF2 display severe viability defects and at the same time are hypermutagenic.[133,134] Interestingly, increasing levels of RNAse H in cells or cell free assays compensated these defects.[134] RNAse H specifically cleaves the double strand of a DNA:RNA hybrid. Observations in *E. coli*, yeast and higher eukaryotes are consistent with the latter experiments and support the idea that the accumulation of DNA:RNA hybrids (R-loops) can cause genomic instability and lead to defects in DNA replication and cell cycle progression.[135] R-loops potentially arise upon DNA transcription and hybridisation of the nascent transcripts with the DNA template strand. They are counteracted by different mechanisms in bacteria and eukaryotes. In yeast, coupling of transcription to RNP assembly and mRNA export may be a key mechanism to prevent R-loop formation. Higher eukaryotes recruit early components of the splicing machinery such as ASF/SF2 to nascent transcripts;[133,134] besides efficient mRNA processing this will interfere with R-loop formation and protect cells from genomic instability. R-loop formation therefore explains the chromatin instability phenotype of ASF/SF2 knock-out cells.[134] One might further speculate that R-loops also lead to problems in mitotic chromosome condensation or centromeric DNA establishment and therefore contribute the mitotic defects observed in HeLa cells upon knock-down of ASF/SF2 or possibly other splicing proteins. Although the majority of "mitotic" splicing components interact with nascent transcripts at a rather late stage during splicing (complexes B and C), the overall accumulation of unspliced pre-mRNAs upon their knock-down might titrate out early acting spliceosome components and finally also lead to R-loop formation and consequently to hypermutation, genomic instability and chromosomal defects in mitosis.

Splicing components might have evolved in eukaryotic evolution to manage nascent transcripts in general, and therefore could have functions not only in intron removal, but also in preventing reassociation of transcripts with the DNA template strand, or to help processing transcripts into small interfering RNAs. The latter functions could be particularly important for replication and nuclear division. If that were the case, the phenotypic abnormalities observed in mitosis would be a consequence of many different species of non-processed transcripts accumulating in the interphase nucleus. The fatal consequence becomes eminent only after NE breakdown (**Fig. 3**). Such a model is consistent with the nuclear abnormalities that preceded the mitotic defects after knock-down of many splicing proteins in the MITOCHECK screen: the knock-down first led to the accumulation of improperly processed transcripts, which then resulted in compromised chromatin organization.[89] This may still be tolerated in interphase but will not allow proper chromosome condensation and segregation in mitosis.

Concluding Remarks

Uncoupling interphase and M-phase. To understand the function of splicing proteins in mitosis it will be necessary to experimentally differentiate between indirect and direct scenarios. One would need a system in which splicing, or at least processes occurring during interphase in the closed nucleus, can be uncoupled from mitosis. One possibility might be to mistarget spliceosomal components required for mitosis from the nucleus to the cytoplasm. The cytoplasmic components would not be able to function in nuclear splicing, but would retain function in mitosis after NE breakdown. However, most of the splicing proteins form complexes with RNAs and other proteins. This will make it difficult to replace and mislocalize a single spliceosomal protein to the cytoplasm without affecting the overall integrity of the RNP or protein complex it is part of. Biochemical experiments in cell-free

extracts might help to differentiate between splicing defects and direct effects on mitosis. For example, in Xenopus egg extracts, mitosis can be recapitulated even in the absence of ongoing transcription and presumably splicing.[136] Recent experiments from the Heald and Weiss labs using this system indicated an important function of RNA for mitotic spindle assembly. The authors carried out immunodepletion and RNAse A experiments to demonstrate that RNA in general and a ribonucleoprotein complex containing the RNA binding protein RAE1 together with bound RNAs in particular, localize to the spindle and are required for spindle formation in the Xenopus cell free system. Consistent with this, the same authors identified numerous RNAs localising to spindles assembled in cell free extracts.[137,138] Using specific immunodepletions in the Xenopus system, the hypothesis of a direct function of spliceosomal components in mitosis could be directly addressed.

Additional information might also come from large scale localisation and modification analyzes.[139] Despite the notion that SRp20 and ASF/SF2 associate with chromatin after metaphase to anaphase transition,[140] data on the particular localisation of splicing proteins on chromatin, kinetochores or spindle microtubules in early mitotic stages and cell cycle-regulated posttranslational modifications of splicing factors, are largely missing.

Finally, it should be emphasized that the described scenarios of direct and indirect effects are not mutually exclusive. It may be that at least some splicing components have a direct function in mitosis, but their knock-down will also affect the pattern of mature mRNAs. The observed phenotypes might therefore be complicated and caused by a combination of directly compromised mitotic functions of splicing components and the partial loss of mitotic gene products with high turnover.

Acknowledgements

We thank Beate Faba-Neumann and Thomas Held from the MITOCHECK group at EMBL for continuous help with the MITOCHECK database and the interpretation of phenotypes, and we are grateful to Annette Flotho, Björn Boysen, Bernhard Dobberstein, Georg Stöcklin and Christine Clayton for critically reviewing the manuscript.

Note

Supplementary materials can be found at:
www.landesbioscience.com/supplement/HofmannNUC1-6-sup.pdf

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