A role for the CB-associated SUMO isopeptidase USPL1 in RNAPII-mediated snRNA transcription

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Summary

Cajal bodies are nuclear structures involved in snRNP and snoRNP biogenesis, telomere maintenance and histone mRNA processing. Recently, the SUMO isopeptidase USPL1 was identified as a Cajal body component essential for cellular growth and Cajal body integrity. However, a cellular function for USPL1 is so far unknown. Here, we use RNAi mediated knockdown in human cells in combination with biochemical and fluorescence microscopy approaches to investigate the function of USPL1 and its relation to Cajal bodies. We demonstrate that the levels of RNAPII-transcribed snRNAs are reduced upon knockdown of USPL1 and that downstream processes such as snRNP assembly and pre-mRNA splicing are compromised. Importantly, we find that USPL1 associates directly with U snRNA loci and that it interacts and colocalizes with components of the Little Elongation Complex, which is involved in RNAPII-mediated snRNA transcription. Thus our data indicate that USPL1 plays a key role in the process of RNAPII-mediated snRNA transcription.
Introduction

The eukaryotic cell nucleus contains multiple compartments, or bodies, including the nucleolus, PML bodies, Cajal bodies (CBs) and splicing speckles (reviewed in (Dundr and Misteli, 2010; Handwerger and Gall, 2006; Spector, 2001; Spector, 2006; Spector and Lamond, 2011; Zhao et al., 2009)). By concentrating a defined set of protein and/or RNA components, nuclear bodies have been suggested to act as sequestration or reaction sites for specific factors and/or cellular processes, thereby regulating complex formation and gene expression (Mao et al., 2011).

CBs are dynamic structures and vary in size and number according to cell cycle stage, differentiation and developmental status of the cell (reviewed in (Cioce and Lamond, 2005; Machyna et al., 2013; Morris, 2008)). They have been associated with roles in spliceosomal small nuclear (snRNP) and small nucleolar RNP (snoRNP) biogenesis, telomere maintenance and histone mRNA processing. snRNPs represent the major constituents of the spliceosome, a complex machinery involved in removing introns from pre-mRNA (reviewed in (Wahl et al., 2009; Will and Luhrmann, 2011)). Each snRNP is composed of a unique RNA component in complex with a specific set of proteins (reviewed in (Fischer et al., 2011; Kiss, 2004; Patel and Bellini, 2008)). Most snRNAs are transcribed by RNA polymerase (RNAP) II and require a cytoplasmic maturation step. RNAP II-transcribed snRNA gene loci have been observed in proximity to CBs (Frey and Matera, 1995; Gao et al., 1997; Jacobs et al., 1999; Smith et al., 1995), and several components involved in RNAPII transcription of snRNAs are enriched in CBs (Hu et al., 2013; Polak et al., 2003; Smith et al., 2011). Exceptions are U6 and U6atac snRNAs that are transcribed by RNAP III and pass through the nucleolus for their maturation process (Ganot et al., 1999; Kunkel et al., 1986; Lange and Gerbi, 2000; Reddy et al., 1987; Tycowski et al., 1998).

After snRNA export into the cytoplasm, the SMN complex directs the assembly of snRNAs with the common Sm proteins (B/B’, D1, D2, D3, E, F, G) (Fischer et al., 1997; Kambach et al., 1999; Massenet et al., 2002; Pellizzoni et al., 2002). Subsequently, the 5’-end of the snRNA is hypermethylated to form the TMG-cap and snRNP complexes are re-imported into the nucleus (Fischer and Luhrmann, 1990; Fischer et al., 1993; Hamm et al., 1990; Mattaj, 1986; Mouaikel et al., 2003; Plessel et al., 1994). Here, they initially concentrate within CBs prior to forming the typical “speckled” pattern seen for mature snRNPs (Sleeman and...
In CBs, scaRNAs can mediate post-transcriptional RNA-modifications and additional snRNP assembly steps can take place before snRNP complexes are released into the nucleoplasm (Darzacq et al., 2002; Jady et al., 2003; Kiss, 2001). There is evidence that the formation of di- (U4/6 snRNP) and tri-snRNP (U4/6/5 snRNP) as well as their reassembly after each round of splicing occurs within CBs (Schaffert et al., 2004; Stanek and Neugebauer, 2004; Stanek et al., 2008).

Coilin is generally considered a marker protein for CBs (Raska et al., 1991). Whereas coilin knockdown has been shown to lead to defects in snRNP biogenesis and embryonic lethality in zebrafish (Strzelecka et al., 2010), neither coilin nor the presence of CBs appears to be essential for viability in mammalian cell lines, mice, Drosophila or Arabidopsis (Collier et al., 2006; Lemm et al., 2006; Liu et al., 2009; Tucker et al., 2001). However, coilin-induced CBs may serve to increase the efficiency of complex assembly by providing a structural scaffold to enrich components involved in snRNP biogenesis (Klingauf et al., 2006; Matera et al., 2009; Novotny et al., 2011).

Recently, an essential SUMO isopeptidase (USPL1) has been described that localizes to CBs (Schulz et al., 2012). RNAi-mediated knockdown of USPL1 in HeLa cells leads to CB disruption, relocalization of coilin to the nucleolus and impaired cell proliferation. Strikingly, these effects are not dependent on the catalytic activity of USPL1 as a SUMO isopeptidase, suggesting additional functions for USPL1 linked with the CB. Therefore, we undertook a detailed study on the effects of USPL1 knockdown on nuclear function and architecture.

We show here that upon USPL1 knockdown there are changes in the localization and/or mobility of both snRNA and mRNA associated proteins and the splicing pattern of specific pre-mRNAs is altered. We demonstrate that this phenotype is associated with reduced snRNP biogenesis and low levels of RNAPII- transcribed snRNAs. We further show an interaction of endogenous USPL1 with members of the snRNA-specific transcription complex and an enrichment of USPL1 at snRNA gene loci, suggesting a key role for USPL1 in snRNA transcription.

**Results**

*USPL1 knockdown affects CBs and Splicing speckles*
After siRNA knockdown of USPL1, the CB marker protein coilin relocates into the nucleolus (Schulz et al., 2012). As other CB components form nuclear bodies without coilin (Bauer and Gall, 1997; Jady et al., 2003; Lemm et al., 2006; Tucker et al., 2001), we compared the CB associated protein SMN in control treated cells and upon USPL1 knockdown. USPL1 can often be found colocalized in CBs with both SMN and coilin (Figure 1A, arrows in top panel). Occasionally, we observe USPL1 in nuclear foci that do not obviously label with coilin. Upon siRNA knockdown of coilin, USPL1 still forms nuclear foci, similar to SMN (Figure 1A, arrows in middle panel). As described previously (Schulz et al., 2012), coilin concentrates in the nucleolus after knockdown of USPL1 (Figure 1A, open arrowhead in bottom panel). In these cells SMN localizes in a larger number of nuclear foci (Figure 1A, arrowheads in bottom panel). USPL1 levels were efficiently reduced as detected by immunoblot (Figure S1A)). In contrast, SMN protein levels appeared not or only mildly altered by siRNA against USPL1, despite the drastic changes to SMN foci in the nucleus upon USPL1 knockdown. Controls confirmed no change in the levels of coilin upon USPL1 knockdown (Figure 4A, S1A) (Schulz et al., 2012).

We next tested whether other nuclear bodies might also be affected by knockdown of USPL1. Therefore we investigated the effect of USPL1 knockdown on nucleoli, PML bodies and splicing speckles by immunostaining. While in the nucleoli of some cells UBF and fibrillarin appeared less condensed upon USPL1 depletion (Figure 1B and S1B, respectively), no dramatic loss of nucleolar integrity could be observed, consistent with earlier observations (Schulz et al., 2012). The integrity of PML bodies showed no apparent change upon knockdown of USPL1 (Figure 1C). This suggests that USPL1 knockdown causes only a minor effect on nucleoli and little or no effect on PML bodies.

Splicing factors are often concentrated in interchromatin granule clusters, also called nuclear speckles (reviewed in (Spector and Lamond, 2011)). In control cells, nuclear speckles exhibit an irregular, fine structured pattern, as shown for ASF/SF2 and Sm proteins (Y12 antibody) (arrows in top panel Figure 1D and S1C, respectively). In contrast, USPL1 knockdown cells displayed enlarged, rounded nuclear speckles (Figure 1D and S1C, respectively, arrowheads in bottom panel). In comparison, splicing speckles in cells transfected with siRNA targeting coilin did not differ from control cells, demonstrating this effect on speckles is not caused directly by loss of CBs (Fig 1D, arrows in middle panel).
USPL1 knockdown affects splicing

The observed reorganization of splicing speckles into enlarged, rounded structures, typically occurs upon inhibition of transcription and/or pre-mRNA splicing (Carmo-Fonseca et al., 1992; O'Keefe et al., 1994; Spector et al., 1983). To analyze whether general transcription is affected by the knockdown of USPL1, we visualized nascent RNA synthesis by EU labeling (Jao and Salic, 2008) (Figure 2A). To improve comparison of transcription levels, an excess of cells transfected with the siRNA against USPL1 were mixed with cells treated with control siRNA the day before analysis (Figure 2A). Based on the good correlation of nucleolar localization for coilin with knockdown of USPL1, we used immunostaining for coilin to monitor USPL1 knockdown.

Cells with reduced levels of USPL1 (identified by nucleolar coilin stain; Figure 2A, arrowheads) still exhibited transcriptional activity at levels either comparable with, or only slightly reduced, in comparison to control cells (coilin in CBs, arrows; Figure 2A). This is consistent with the fact that we do not observe nucleolar cap formation upon USPL1 knockdown, which would be expected upon a general inhibition of transcription (Carmo-Fonseca et al., 1992; Shav-Tal et al., 2005).

As a general loss of transcription is not causing the enlarged nuclear speckles upon USPL1 knockdown, we next analyzed pre-mRNA splicing of endogenous pre-mRNAs extracted from either control, or USPL1 knockdown cells (Figure 2B). We detected changes in the alternative splicing pattern of several pre-mRNAs by qualitative RT-PCR and agarose gel electrophoresis (CCNA2, FAS, MCL1, RBM5, ASF/SF2 and Caspase 9 mRNA; Figure 2B). Additionally, using a combination of primers located in introns and exons of pre-mRNA, we detected different ratios of intermediate splicing products of the constitutively spliced pre-mRNAs coding for β-tubulin and β-actin (Figure 2B). This demonstrates that the pattern of pre-mRNA splicing was altered following USPL1 knockdown in this cell system, at least for a subset of pre-mRNAs.

USPL1 knockdown alters localization and abundance of snRNP components

We next analyzed whether there were changes in overall protein abundance and localization after siRNA knockdown of USPL1, combining the siRNA knockdown with stable isotope labeling by amino acids in cell culture (SILAC)-based mass spectrometry (MS) (Ong et al.,
2002) and cellular fractionation. After metabolic labeling, HeLa cells were transfected with the respective siRNAs (R_0K_0/light: siControl; R_10K_0/heavy: siUSPL1). Equal numbers of cells were mixed and fractionated into cytoplasm, nucleoplasm and nucleoli as previously described (Andersen et al., 2002; Boisvert et al., 2010) (Figure 3A). The knockdown and fractionation efficiency was checked by immunofluorescence staining for coilin and immunoblot analysis, respectively (data not shown).

The individual fractions were analyzed by 1D-SDS-PAGE and the peptides obtained from in-gel tryptic digestion analyzed by LC-MS/MS (see supplementary table 1). To relate changes of protein levels in subcellular fractions to possible changes in overall protein levels, total cell lysate (TCL) was analyzed in parallel. When displayed as a histogram of the log_2 ratio H/L (Figure 3B-C and S2A-B), most protein groups from the cellular fractions fall into a distribution centered around zero, indicating that their intracellular localization is unaffected by USPL1 knockdown. However, ~5% of all proteins identified in the cytoplasmic and ~2% of all proteins in the nucleolar fraction were more abundant in these fractions upon USPL1 knockdown (indicated by red box in Figure 3B and 3C). As expected, we found coilin enriched in the nucleolar fraction (log_2 H/L ratio nucleolus ~4, cytoplasm N/A; Figure 3D), confirming our previous immunofluorescence data showing its nucleolar relocalization upon USPL1 knockdown.

An enrichment analysis based on functional annotation for proteins using the bioinformatics database DAVID (Huang da et al., 2009a; Huang da et al., 2009b) revealed a highly significant enrichment for proteins associated with the spliceosome and RNA splicing in the cytoplasmic and/or nucleolar fractions upon USPL1 knockdown (corrected p-value (Benjamini):< 1x10^{-10}; Supplementary table 2). This enrichment is illustrated by highlighting splicing factors in Figure 3D in green (see list of protein group IDs in supplementary table 1).

When analyzed by immunostaining, none of the non-snRNP proteins displaying enrichment in the cytoplasm upon cellular fractionation (such as ASF/SF2 or snRNP A1) showed any detectable relocalization into the cytoplasm of intact cells (see Figure 1D and data not shown). The increase seen by SILAC could either be below the detection limit for microscopy and/or reflect “leaking” of proteins upon cellular fractionation as a result of their increased mobility.

As shown in Figure S2A, a subset of proteins was reduced in overall protein levels upon USPL1 knockdown (~2% of all identified proteins in the TLC; indicated by red box),
including U5 snRNP proteins PRPF6 and snRNP40 and the U1 snRNP protein snRNP70. These MS data were confirmed for PRPF6 by immunoblotting of total cell lysates obtained from USPL1-depleted U2OS cells (see Figure 4A). The blotting data showed PRPF6 protein levels were reduced by ~50%, consistent with the SILAC data. Importantly, siRNA knockdown of coilin had no comparable effect, demonstrating that the changes in total protein levels were specific for USPL1 knockdown (Figure 4A). In comparison, neither coilin nor SMN levels were affected by USPL1 knockdown, in agreement with earlier observations (Figure S1A). In conclusion, knockdown of USPL1 causes dramatic changes in the abundance and nucleo/cytoplasmic distribution of specific spliceosome components.

**snRNP assembly and recycling are compromised upon USPL1 knockdown**

We next investigated whether snRNP formation was affected by USPL1 knockdown. Considering that PRPF6 is required for the formation of the tri-snRNP complex (Makarov et al., 2000; Schaffert et al., 2004), we first investigated tri-snRNP formation. For this, we immunoprecipitated PRPF4, a component of the U4/6 di-snRNP, from nuclear extracts prepared from either control, coilin- or USPL1 knockdown HeLa cells, respectively. All immunoprecipitates were tested for the U5 snRNP components PRPF8, snRNP200 and EF-TUD, by immunoblot analysis (Figure S2C). All these proteins were reduced in the PRPF4-immunoprecipitates from USPL1 knockdown cells (last lane, Figure S2C), demonstrating a reduction in tri-snRNP formation. In contrast, little or no reduction in the amount of co-precipitated U5 snRNP proteins could be detected upon siRNA treatment against coilin. This is consistent with previous reports showing that tri-snRNP formation still occurs in the nucleoplasm in the absence of coilin, albeit with reduced efficiency compared with CBs (Stanek and Neugebauer, 2004). This suggests that reduced tri-snRNP formation is a phenotype specifically associated with the knockdown of USPL1.

The altered localization pattern for spliceosome components seen upon cellular fractionation in USPL1 knockdown cells might indicate either a reduction in complex assembly, and/or a change in the stability of snRNP complexes (Figure 3D). We therefore analyzed whether nascent snRNP production in general was affected upon knockdown of USPL1. It has been reported previously that Sm proteins that are N-terminally tagged with fluorescent proteins localize correctly and assemble into the heptameric Sm complex (Sleeman et al., 1998). We took advantage of this to distinguish newly assembled snRNPs, formed after we reduced
levels of USPL1 by siRNA, from the pool of previously assembled snRNPs. Thus, we generated a U2OS cell line stably expressing GFP-SmB under a doxycycline (dox)-inducible promoter. When analyzed by immunoblot, a major band of the predicted size for GFP-SmB was detectable upon 18h of dox-induction (Figure 4B). GFP-SmB localized correctly in CBs and nuclear speckles, as shown by immunostaining for coilin in combination with the Y12 anti Sm antibody as markers for CBs and snRNPs, respectively (Figure 4C). Equal amounts of protein lysate prepared from either control, or USPL1 knockdown cells upon 18h of dox-induction were subjected to immunoprecipitations (IPs) with either empty beads (control), or beads pre-coupled to anti TMG-antibody, which recognizes the 5’-terminal snRNA trimethyl cap structure. Precipitated snRNP complexes were eluted from the beads using 7-methylguanosine (7-mG) and GFP-SmB detected by immunoblotting (Figure 4D). GFP-SmB was detected in TMG-eluates from control treated cells, but was reduced in cells transfected with siRNA against USPL1. This indicates that the production of nascent snRNPs is reduced upon USPL1 knockdown.

**USPL1 is involved in maintenance of snRNA levels**

Having detected reduced snRNP assembly upon knockdown of USPL1, we wondered whether snRNA levels in general were affected. To address changes in snRNA expression upon USPL1 knockdown quantitatively, we performed qRT-PCR for the major snRNAs that are transcribed by either RNAP II, or RNAP III. In both, HeLa and U2OS cells, primarily all RNAPII-transcribed snRNAs tested (U1, U2, U4 and U5 snRNA) exhibited reduced levels upon USPL1 knockdown as compared with control cells (Figure 5A and S3A, respectively). U1 and U2 snRNAs are transcribed as longer precursor snRNAs that get trimmed at their 3’ end by the Integrator complex before their export into the cytoplasm (Baillat et al., 2005; Chen and Wagner, 2010; Cuello et al., 1999; Egloff et al., 2008; Medlin et al., 2003). To differentiate between the possibility of USPL1 knockdown affecting either 3’-processing, or directly snRNA transcription, we used primers specific for the 3’-precursor U2 snRNA ((Broome and Hebert, 2012); U2pre, Figure 5A-B and S3A). Interestingly, also the levels of this nascent transcribed form of the U2 snRNA appear reduced in a similar manner to the final processed form. This indicates that the transcription of snRNAs is compromised upon knockdown of USPL1.
In addition, we analyzed snRNA localization by RNA-FISH. This shows, snRNAs localize in nuclear speckles and in CBs in control cells (Figure 5C and S3B, arrowheads and arrows, respectively). In agreement with our qRT-PCR data, the signal for RNAPII-transcribed snRNAs was reduced upon USPL1 knockdown. Moreover, it concentrated in enlarged nuclear speckles, similar to the structures detected by immunostain for ASF/SF2 and Sm proteins after USPL1 knockdown (compare Figure 5C and S3B with 1D and S1C).

**USPL1 interacts with LEC components involved in RNP II mediated snRNA transcription**

Previously, ELL and Ice1/ KIAA0947 were identified in a large-scale screen for human de-ubiquitinating enzymes as two good candidate interactors for overexpressed USPL1 (Sowa et al., 2009). Both proteins have recently been shown to be involved in the regulation of snRNA transcription as components of the little elongation complex (LEC) in Drosophila and mammalian cells (Hu et al., 2013; Smith et al., 2011). To confirm the interaction of USPL1 with members of the LEC, we first used transient transfection of FLAG-ELL and HA-USPL1. As shown in Figure 6A, HA-USPL1 was detected by protein immunoblotting following IP of FLAG-ELL, but was not detected following IP from extracts expressing the FLAG-tag alone. We next employed affinity-purified USPL1 antibodies to immunoprecipitate endogenous USPL1 from both HEK293 and HeLa cells. In both cell lines we detected endogenous ELL by immunoblotting following IP of USPL1 (arrow, Figure 6B), but not following the control IP.

USPL1, ELL and Ice1 have all been reported to localize to CBs (Hu et al., 2013; Polak et al., 2003; Schulz et al., 2012; Smith et al., 2011). As shown in Figure 6C (arrows), both Ice1 and ELL co-localized with USPL1 in CBs. To address the possible interdependence of these proteins with respect to their recruitment to nuclear bodies, HeLa cells were transfected with either siRNA against Ice1, or against USPL1, and their localization analyzed by immunostaining (Figure 7A). As seen upon reduced levels of USPL1, knockdown of Ice1 resulted in disassembly of CBs and redistribution of coilin into the nucleolus (open arrowheads, Figure 7A). We did not detect any remaining nuclear foci containing USPL1 after transfection of siRNAs against Ice1, suggesting that the recruitment of USPL1 into foci might depend on the presence of Ice1. However, knockdown of USPL1 did not lead to disappearance of Ice1 containing nuclear foci, at least in HeLa cells. Instead some HeLa cells
showed an increase in the number of nuclear foci containing Ice1 (Figure 7A, arrows bottom panel). However, we were unable to detect persistent Ice1 nuclear foci after transfection of siRNA against USPL1 in U2OS cells.

Interestingly, enlarged, rounded nuclear speckles could be detected for ASF/SF2 (Figure 7B arrows middle panel) upon efficient knockdown of Ice1, similar to USPL1 knockdown (Figure 7B, arrows in bottom panel). Additionally, the number of nuclear SMN foci increased as compared with control cells (Figure 7C, arrows in projection). Therefore, knockdown of the LEC component Ice1 results in similar phenotypes regarding nuclear localization of SMN and splicing speckles as seen following knockdown of USPL1.

**USPL1 is associated with U snRNA gene loci**

Components of the LEC have been shown to be enriched on RNAP II transcribed snRNA genes (Hu et al., 2013; Smith et al., 2011). Our findings, together with data from (Sowa et al., 2009), indicate an association of USPL1 with components of the LEC. Therefore, we decided to investigate whether USPL1 itself could be found associated with snRNA gene loci. Previously, CBs defined by the marker protein coilin had been shown to be associated with several snRNA gene loci by DNA-FISH (Frey and Matera, 1995; Gao et al., 1997; Jacobs et al., 1999; Smith et al., 1995). The U2 genes are located in a 120kb cluster of ~6 kb long repeat units on the q-arm of chromosome 17 (Lindgren et al., 1985; Van Arsdell and Weiner, 1984; Westin et al., 1984). We designed a set of fluorescently labeled FISH probes consisting of four individual fragments, covering the entire U2 gene locus. The probes hybridized with exactly two loci on two different chromosomes on metaphase spreads of normal human male lymphocytes, consistent with specificity for the U2 gene locus (Figure 8A, arrows). When tested on HeLa interphase cells, the U2 FISH probe frequently associated with CBs, as judged by co-staining for coilin (data not shown), consistent with previous reports (Frey and Matera, 1995; Smith et al., 1995). When we performed the U2 DNA-FISH on HeLa cells in conjunction with immunostaining for USPL1, nuclear foci containing USPL1 were detected in close association with the FISH signal for the U2 gene locus (Figure 8B, arrows).

As a complementary approach to test for association of USPL1 with U snRNA genes, we employed chromatin immunoprecipitation (ChIP) (Figure 8C). USPL1 protein was precipitated from formaldehyde-crosslinked HeLa cells and associated DNA tested for the presence of U
snRNA gene sequences using U1 and U2 specific primers. We detected a significant enrichment for U1 and U2 gene regions using the USPL1 antibody compared with the IgG control. In contrast, no significant enrichment for either the GAPDH gene promoter, or a region 2000bp upstream of the U2 promoter (U2 -2kb), was detected (Figure 8C). To further test whether USPL1 is directly involved in RNAPII-mediated snRNA transcription, we performed ChIP for RNAPII upon USPL1 knockdown. The results obtained by two different antibodies clearly show a significant reduction in RNAPII occupancy at the U1 and U2 promoter regions, respectively, in cells treated with the siRNA against USPL1 (Figure 8D and S4). We conclude that USPL1 is required for RNAPII-dependent snRNA transcription and is a component of complexes that can bind to U snRNA genes.

**Discussion**

In this study we have presented data investigating a functional role for the CB-associated, SUMO isopeptidase USPL1. Using a combination of fluorescence microscopy, molecular biology and proteomic approaches, we demonstrate that USPL1 interacts with components of the RNAPII-associated little elongation complex (LEC) and is associated with the U1 and U2 snRNA gene loci. Efficient knockdown of USPL1 by RNAi leads to reduced RNAPII-mediated snRNA transcription, diminished production of snRNPs and altered pre-mRNA splicing. Our data suggest that USPL1 is involved in snRNA transcription and support the view that CBs have role in snRNP biogenesis.

By using RNAi in combination with cellular fractionation and MS-based protein analysis we detect a striking effect of reduced USPL1 levels on proteins associated with snRNP biogenesis and/or pre-mRNA splicing. This is supported by our accompanying immunofluorescence studies in cells with reduced levels of USPL1, showing changes for CBs and splicing speckles, but not for either PML bodies, or the nucleolus. We observe a decrease in RNAPII-transcribed snRNA levels upon USPL1 knockdown and subsequent defects in snRNP production. Although it is transcribed by RNAPIII, U6 snRNA levels were also slightly affected by USPL1 knockdown. However, it is known that U6 snRNA assembles into a U4/U6 di-snRNP complex with the RNAPII transcribed U4 snRNA and non-incorporated U6 snRNA has a higher turnover rate than the assembled form (Sauterer et al., 1988). A link between RNPIII-mediated U6 transcription and RNAPII has also been suggested (Listerman et al., 2007). The disruption of snRNP biogenesis likely explains both the small effect of
USPL1 knockdown on U6 snRNA levels and the increase of snRNP proteins in the cytoplasm detected upon USPL1 knockdown by our cellular fractionation study (Figure 3C). This increase could either reflect “leaking” of nuclear proteins into the cytoplasm during the cell fractionation procedure and/or proteins that under normal conditions are imported into the nucleus only in complex with their respective snRNA.

Interestingly, we also detected changes in the pattern of specific pre-mRNA splicing after USPL1 knockdown. Previously, defects in splicing and the composition of the pool of snRNA species have been reported upon SMN deficiency, dependent on the severity of the SMN reduction (Boulisfane et al., 2011; Campion et al., 2010; Gabanella et al., 2007; Gao et al., 1997; Zhang et al., 2008). However, we find that SMN levels are either not, or only mildly, affected by USPL1-knockdown, indicating that a distinct mechanism is involved. This raises the intriguing possibility that splicing could be regulated in vivo, either during development, or in distinct human tissues, by varying levels of USPL1.

Transcription of snRNA genes by RNAPII differs significantly from the transcription of most protein-coding genes (for recent reviews see (Egloff et al., 2008; Jawdekar and Henry, 2008)). However, the mechanism of snRNA transcription is still not understood in detail. Most recently, Shilatifard and colleagues show by a genome-wide ChIP and RNA-sequencing approach that ELL and Ice1/2, as part of the LEC, is enriched at RNAPII-snRNA genes and that Ice1 and ELL are required for initiation and elongation of snRNA expression, respectively (Hu et al., 2013; Smith et al., 2011).

Here, we show that human USPL1 not only colocalizes and interacts with components of the LEC, but also demonstrate that endogenous USPL1 is present at snRNA gene loci in human cells. Our interaction data are supported by the findings of Harper and colleagues, who identified human ELL and Ice1 in a large-scale proteomic approach upon USPL1 overexpression (Sowa et al., 2009). Importantly, knockdown of either USPL1, or the LEC member Ice1, causes similar phenotypes regarding decreased snRNA levels (compare our data with (Hu et al., 2013; Smith et al., 2011)) and nuclear architecture (Figure 7A-C). This suggests that USPL1, similar to the LEC, has an important role in snRNP biogenesis at the transcriptional level. This is supported by our data showing a significantly reduced association of RNAPII with snRNA gene loci upon USPL1 knockdown. We therefore speculate that USPL1 might either be a component of the LEC, or else is functionally associated with it. Future studies will show whether USPL1 is involved in transcription
initiation and/or elongation. In Drosophila, CG8229, a protein with limited homology to the N-terminus of USPL1, was identified as a binding partner for Ice1/2 in *Drosophila* (Smith et al., 2011), but lacks a catalytic domain. Therefore it will be interesting to analyze whether and to what extent the desumoylating activity of USPL1 contributes to snRNA transcription in human cells.

**Experimental Procedures**

**Plasmids**

To generate GFP-SmB, the coding sequence for GFP and SmB were PCR amplified and inserted into the pcDNA5-FRT/TO vector (Invitrogen) via HindIII/ KpnI and KpnI/ NotI sites, respectively. For the generation of the FLAG-ELL, ELL was amplified from pCMV-ELL (Imagene) and inserted via BamHI/EcoRI sites into pcDNA3.1 (Meulmeester et al., 2008).

**Cell Culture, Transfection and Metabolic labeling with EU**

Clonal U2OS GFP-SmB were selected and maintained using 150 µg/ml hygromcin B and 15 µg/ml blasticidin-HCl. Expression of GFP-SmB was induced by 10 ng/ml doxycyclin for 18h. Transfection of DNA was performed using either Effectene (Qiagen), Jet Prime (Polyplus transfection) or Polyethylenimine (PEI) (Durocher et al., 2002). siRNA-transfections (20nM final concentration; see supplementary Table 4 for siRNA sequences) were performed using Lipofectamine RNAiMaxx (Invitrogen) omitting antibiotics and experiments were analyzed after 2 days. For the siRNA transfection in SILAC media, OptiMem was replaced with the respective SILAC medium lacking serum and antibiotics. EU-labeling was performed using the EU-Click-iT kit (Invitrogen).

**Antibodies**

A list of all primary antibodies used in this study can be found in the supplementary material (Table 3). Except for goat anti Chicken-FITC (Sigma, F-8888), Alexa488/594/647-dye
conjugated secondary antibodies from donkey (Molecular Probes) were used for immunofluorescence. For immunoblotting, either a Li-Cor Odyssey CLx (Li-Cor Biosciences) in combination with Alexa 680/800 conjugated secondary goat antibodies (Tebu-Bio/Invitrogen) (Figure 4 and S2C) or HRP conjugated secondary antibodies (Jackson Immunoresearch) with ECL detection (Thermo) (Figure 6A, B; S1A) were used.

**Immunofluorescence and Fluorescence Microscopy**

Immunofluorescence and image acquisition was essentially performed as described in (Hutten et al., 2011) except that 1% normal donkey serum was used as blocking buffer. Images were acquired with a DeltaVision Core Restoration microscope (Applied Precision) mounted on an Olympus IX71 stand with a ×60 1.42 NA oil immersion objective lens using 1×1 bin with a section spacing of 0.2 µm. Exposure time was set to provide an intensity of at least ~1,000 counts on a 12-bit CoolSnap HQ2 camera at gain 4 (Roper, USA) for control cells. Note, that for reason of visibility of nucleolar coilin, longer exposure times and different picture processing were used on siRNA treated cells versus control cells. Coilin knockdown cells were treated identically to siIce1 or siUSPL1 transfected cells for image acquisition and processing. Images were corrected by flat field calibration, deconvolved and corrected for chromatic aberration using SoftWorx (Applied Precision). Processing and image analysis was performed using SoftWorx and Adobe Photoshop/Illustrator. Unless stated otherwise, a single z-stack is shown in the figures. Maximum intensity projections were adjusted differently than corresponding single z-sections for clarity of the staining.

For Figure S1C, cells were fixed using 3.7% formaldehyde, permeabilized for 10min in 0.2% Triton-100 on ice and blocked in 3% bovine serum albumin/0.1% Tween-20/PBS for 1h. Antibody incubation was performed in blocking buffer for 1.5h at RT. Cells were mounted in mounting medium (Dako) and images acquired using an Axioskop (Observer zl, Zeiss) and an AxioCam Mrm camera (Zeiss).

**RNA and DNA Fluorescence-in-situ-Hybridization (FISH)**

RNA-FISH: The protocol for FISH against U snRNAs was adapted from (Taneja et al., 1992) with the following modifications: Cells were fixed and permeabilized as described above for
immunofluorescence. The hybridization buffer contained 0.02% BSA and additional 0.4 mM ribovanadyl complex. Probes were obtained as 5’-Alexa488-derivate (sequences according to (Schaffert et al., 2004); see supplementary table 8). After hybridization, cells were washed with 0.5% TX-100 in PBS, re-fixed with PFA and subjected to immunofluorescence.

**DNA-FISH:** A pool of 4 different, Cy5-dCytosine nick-labeled (nick-translation kit; GE Healthcare), 1.5-2 kb long PCR-products (see supplementary table 9) covering the whole 6kb-U2 gene locus was used as probe. The labeled probe (~130 ng) was ethanol-precipitated in the presence of sodium acetate and human cot-1 DNA and resuspended in hybridization buffer (Hybrisol, Abbot Molecular). Before hybridization, cells grown on microscopy glass slides were subjected to immunofluorescence using antibodies twice (primary) to four times (secondary) as concentrated, re-fixed and dehydrated in 70%, 90% and absolute ethanol. Dehydration was repeated after denaturing genomic DNA for 5min in 2x SSC/70% formamide at 80°C (adapted from (Matera et al., 1995)). The probe was pre-warmed to 37°C, added to air-dried, prewarmed slides, denatured for 2min at 80°C and hybridization was performed overnight in a humified chamber at 37°C. After successive washing steps in 2x SSC/50% formamide (5min at 40°C) and 1X SSC (3 times 5min at RT), cells were briefly rinsed in PBS and nuclei were counterstained with Hoechst before mounting as above. Metaphase spreads of normal human male lymphocytes were obtained from Abbot Molecular (Maidenhead, UK).

*Non-quantitative and Real-Time quantitative RT-PCR (qRT-PCR)*

Total RNA was isolated from siRNA treated cells using either the miRNeasy kit (Qiagen; qRT-PCR) or NucleoSpin RNA kit (Macherey & Nagel; qualitative RT-PCR) including a DNase digest. Equal amounts of RNA from control or USPL1 knockdown cells were subjected to qualitative RT-PCR using the 1-Step RT-PCR kit from Qiagen with gene-specific primers (see supplementary table 5) and analyzed by ethidium bromide agarose gel electrophoresis. qRT-PCR was performed on 0.4 ng of RNA per reaction (or 4 ng RNA in case of U2pre-snRNA) in duplicate using the Quantifast RT-PCR SYBR-Green Mix (Qiagen) in a Roche Light Cycler 480. Primers (see supplementary table 6) were tested for their PCR-efficiency and the formation of primer dimers under qRT-PCR conditions.
**Chromatin Immunoprecipitation (ChIP)**

ChIP using the RNAPII and USPL1 antibody was performed as described in (Yin et al., 2012) using ~1-2x10⁷ HeLa cells. ChIP for USPL1 was performed using 1μg of antibody in the presence of 0.3% Brij-35 overnight. For comparison of RNAPII promoter occupancy in siRNA treated cells, total protein concentration was determined by a bicinchoninic acid assay (Thermo Scientific) and a SpectraMax MSe (Molecular Devices) and equal amounts of total cell lysate were subjected to ChIP using either 1μg (mouse) or 2μg (rabbit) anti RNAPII antibody in the presence of 0.1% Brij-35 overnight. In general, 10% of the lysate was retained as input, the rest was split for incubation with either control IgG (Goat: Santa Cruz Biotechnology, rabbit/mouse: Jackson ImmunoResearch) or USPL1/RNAPII antibody. Antibodies were captured using protein G-dynabeads, After RNase treatment, reversal of crosslinks and protein K digest (125 μg/ml) of input and eluates, DNA was purified using the PCR purification kit (Qiagen) and enriched sequences analyzed by qPCR using a Roche 480 Light cycler and the Quantitect SYBR-Green PCR mix (Qiagen) (primers see supplementary table 7). For quantification, crossing point (Cp) values were calculated using the Absolute Quantification Analysis/Fit Points method (Light cycler 480 Software module) and ChIP values were normalized for the input for each primer pair.

**RNA and Protein Co-Immunoprecipitations**

For the TMG-IP, siRNA transfected U2OS cells (~1-2x10⁷ cells) were harvested 18h after induction of GFP-SmB expression and lysed in RIPA buffer (50 mM Tris pH 7.5/ 150 mM NaCl/ 1% NP40/ 0.5% deoxycholate/ protease inhibitors (Roche)). The lysate was sonicated and cleared by centrifugation at 17,000 g. Equal amounts of protein lysate from control or USPL1 depleted cells was incubated overnight with 30 μl (slurry) TMG-agarose (Calbiochem) or protein-G agarose beads as control. After several washes, bound snRNP complexes were eluted using 25 mM 7-methylguanosine in PBS (Sigma) and analyzed by immunoblotting for the presence of newly incorporated GFP-SmB.

For IP of tri-snRNP complexes, nuclear extracts from ~2-6x10⁷ siRNA transfected HeLa cells were prepared as follows: Cells were harvested and incubated in 1 packed cell volume (PCV) of NE1 buffer (10 mM Hepes pH 7.6/ 1.5 mM MgCl₂/ 10 mM KCl/ 1 mM DTT) for 15min on ice. Afterwards, cells were sheared using a 23 gauge needle and the nuclei extracted by
incubation in 2/3 PCV of NE2 buffer (20 mM Hepes pH7.6/ 1.5 mM MgCl2/ 25% glycerol/ 420 mM NaCl/ 0.2 mM EDTA/ 1 mM DTT/ 0.5 mM PMSF) for 30min at 4°C. Nuclear extracts were cleared by centrifugation at 17,000g. 250-500µg of nuclear extract were diluted 10-16fold in IPP150 buffer (10 mM Tris pH7.5/ 150 mM KCl/ 0.1% NP40 (Blencowe et al., 1993) and subjected to IP using 2 µg of control rabbit IgG (Jackson ImmunoResearch) or anti Prp4 antibody (HPA/Sigma) overnight. Antibodies were captured using protein-G dynabeads (Invitrogen) and bound proteins were eluted using 2x LDS-sample buffer (Invitrogen) and analyzed by immunoblotting.

For IP of FLAG-ELL and USPL1, HeLa or HEK293 cells were harvested in lysis buffer (50 mM Tris-Cl pH 7.5/ 150 mM NaCl/ 1% NP40/ 5 mM EDTA/ 5 mM EGTA/ 1 mM DTT/ protease inhibitors) for 30min on ice. Cells were sheared using a 21 gauge needle and the extracts were cleared by centrifugation at 10,000g. 5mg of lysate were pre-cleared and subjected to IP for 3h using beads pre-blocked with 1% ovalbumin protein. Beads were either loaded with 5µg of anti FLAG (for the transfected FLAG and FLAG-ELL) or anti USPL1 antibodies (for endogenous USPL1). Empty protein-G agarose beads (Roche) served as control in the IP of endogenous USPL1. After several washes, bound proteins were eluted using 2x SDS-sample buffer and analyzed by immunoblotting.

Cellular Fractionation, SILAC and LC-MS/MS

Hela cells were fully metabolically labeled by growing for at least 6 doublings in lysine- and arginine-deficient DMEM SILAC-medium (Fisher) supplemented with dialyzed FBS, 100U/ml penicillin and 100µg/ml streptomycin in addition to labeled amino acids (42 µg/ml arginine and 73 µg/ml lysine; Cambridge Isotope Lab) as follows: R0K0 (L-arginine and L-lysine) or R10K8 (L-arginine 13C/15N and lysine 13C/15N). Two days after siRNA transfection, an equal number of cells were mixed and cytoplasmic, nucleoplasmic and nucleolar fractions prepared according to (Andersen et al., 2002; Boisvert et al., 2010). The cytoplasmic fraction was cleared from cellular debris by centrifugation at 9,600g at 4°C. Lysates were prepared by adding RIPA buffer (final concentration: 50 mM Tris pH7.5/ 150 mM NaCl/ 1% NP40/ 0.5% sodium-deoxycholate/ protease inhibitors) to the individual fractions. Protein concentration was determined by a bicinchoninic acid assay (Thermo Scientific) and a SpectraMax MSe (Molecular Devices). 20 µg protein of each fraction in loading buffer were separated by one-dimensional SDS-PAGE (4-12% Bis-Tris Novex mini-gel) and visualized using colloidal
Coomassie staining (Novex, Invitrogen). The individual gel lanes were cut into 8 slices as indicated (Figure 3A), destained, reduced with 10mM DTT and alkylated in 50 mM iodoacetamide prior to in-gel trypsin digest (Shevchenko et al., 1996). Tryptic peptides were extracted using equal volumes of 5% formic acid and acetonitrile, dried in a speedvac and resuspended in 5% formic acid. Peptides were analyzed by LC/MS-MS on a Orbitrap Velos mass spectrometer over a 156min gradient (Thermo Fisher Sc.) and data analyzed with MaxQuant (version 1.2.2.5) (Cox and Mann, 2008; Cox et al., 2011; Ong et al., 2002; Ong and Mann, 2006) and the Human UniProtKB and TrEmbl Database (retrieval date December 2011). Carbamidomethylation was set as a fixed modification, and oxidation of methionine, N-acetyl protein, glutamine to glutamic acid conversion and deamidation were searched as variable modifications. The match-between runs-function was enabled and the maximum false discovery rate set to 1%. Those protein identifications that were derived from the decoy database, listed as common contaminant by MaxQuant or those only identified by a modification site were excluded from further data analysis. The mean of the log2 of the normalized H/L ratio was calculated based only on protein quantification data with >1 unique peptide for an individual fraction. In order to identify outliers, the arbitrary threshold was defined as two-fold standard deviation (2σ) of the mean.

Protein enrichment analysis was performed using the Bioinfomatics database DAVID (v. 6.7; (Huang da et al., 2009a; Huang da et al., 2009b)). Protein groups being significantly enriched according to the MaxQuant analysis (>2σ) in either cytoplasm or nucleolus were analyzed by functional annotation analysis against the background of all proteins identified with >1 unique peptide in the respective fraction.

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**Figure Legends**

**Figure 1: The consequences of USPL1 knockdown on nuclear architecture**

A: Immunofluorescence of siRNA transfected U2OS cells against USPL1, SMN and coilin. Closed arrows (top panel) point towards CBs with USPL1, SMN and coilin present. Open arrows (middle panel) highlight USPL1 nuclear foci in the absence of coilin, arrowheads (middle and bottom panel) point towards SMN nuclear foci upon siCoilin or siUSPL1 treatment. A maximum intensity projection in the very right panel demonstrates the increase in SMN nuclear foci (foci indicated by arrowheads, nucleus indicated by the dotted line). Similar effects were observed with HeLa cells (data not shown). Bar, 10 µm.

B: Immunofluorescence of siRNA transfected U2OS cells against USPL1, coilin and UBF. Similar effects were observed with HeLa cells. Bar, 10 µm.

C: A maximum intensity projection of siRNA treated Hela cells stained with antibodies against PML and coilin. Similar effects were observed with U2OS cells (data not shown). Bar, 10 µm.

D: Immunofluorescence of siRNA treated HeLa cells against ASF and coilin. Arrows highlight splicing speckles in control and coilin-depleted cells (top and middle panel, respectively), arrowheads indicate enlarged splicing speckles upon USPL1 knockdown (bottom panel). Similar effects were observed with U2OS cells (Figure 7). Bar, 10 µm.

**Figure 2: Knockdown of USPL1 affects pre-mRNA splicing**

A: An excess of HeLa (top panel) or U2OS (bottom panel) cells treated with siRNA against USPL1 were mixed with control treated cells and analyzed by EU-pulse labeling to monitor nascent RNA transcription. siControl and siUSPL1 treated cells can be distinguished by a parallel immunostain for coilin: Arrows point to control cells with coilin in CBs, whereas arrowheads highlight USPL1 knockdown cells with coilin in nucleoli. Note, to allow visibility of nucleolar coilin, CB-localized coilin was allowed to saturate the camera during image acquisition. Bar, 10 µm.

B: Changes in pattern of pre-mRNA splicing detected by qualitative RT-PCR on total RNA isolated from siRNA transfected U2OS cells and subsequent ethidiumbromide agarose gel
electrophoresis. Similar effects were observed with HeLa cells (data not shown). Splicing products from either HeLa or U2OS USPL1 knockdown cells were sequenced and their position are indicated next to the gel. White boxes indicate exons, filled black boxes introns, with the position of respective primers corresponding to the first and last box, respectively (size of box not to scale). An asterisk indicates an unspecific band.

Figure 3: SILAC-MS based analysis reveals impact of USPL1 knockdown on localization and mobility of spliceosome-associated proteins

A: Experimental workflow of the cellular fractionation process and 1D SDS-PAGE for subsequent SILAC-MS analysis upon USPL1 knockdown.

B-D: SILAC analysis of cellular fractionation of HeLa cells upon USPL1 knockdown. Only hits identified with >1 peptide in an individual fraction are displayed here. The log₂ ratio heavy (H; siUSPL1)/ light (L; siControl) of each cellular fraction is displayed as frequency histogram for cytoplasm (B) and nucleolus (C). Protein groups above/below the arbitrary threshold are indicated by a red lined box (B, C). The log₂ ratios (heavy (siUSPL1)/light (siControl)) of the cytoplasmic versus nucleolar fraction are displayed as a scatter plot with each dot representing an individual protein (D). Spliceosome proteins (green, based on (Hegele et al., 2012)) and ribosomal proteins (purple) are highlighted as indicated. The red lined box highlights protein groups enriched in the cytoplasmic fraction. Note, for clarity, the three major environmental contaminants (keratins) are not displayed in (D).

Figure 4: Defects in snRNP production upon USPL1 knockdown

A: Total cell lysates of siRNA treated U2OS were analyzed by immunoblotting using antibodies as indicated. Different amounts of control lysate (100%, 50%, 25%) provide an internal standard for protein levels. Similar effects were observed with HeLa cells (data not shown).
B: Immunoblot of total cell lysate of U2OS GFP-SmB cells without (-dox) or 18h after (+dox) induction using the Y12 or an anti GFP-antibody showing expression of GFP-SmB at its expected molecular weight. Alpha tubulin stain serves as loading control.

C: U2OS GFP-SmB cells were analyzed without (-dox) or 18h after (+dox) induction for expression and localization of GFP-SmB by immunostaining using the Y12 and anti coilin antibody as marker for nuclear speckles (arrowhead) and CBs (arrows), respectively. Bar, 10 μm.

D: Formation of nascent snRNPs was analysed by TMG-IP in siRNA treated U2OS GFP-SmB cells. Empty protein-G agarose beads (-) serve as control. Bound proteins were eluted from the beads using 25 mM 7-methylguanosine (7-mG) and analyzed for the presence of GFP-SmB by anti-GFP immunoblotting. Input corresponds to 0.75% of the material used in the IP.

**Figure 5: Cellular snRNA levels are reduced upon knockdown of USPL1**

A: qRT-PCR for different major U snRNA species in HeLa cells treated with siRNA against USPL1. U2pre represents the unprocessed U2 snRNA. Respective expression levels were normalized for levels of β-actin and snRNA levels of siControl treated cells were set to 1. Bars represent the standard error of the mean of 4 independent experiments, each measured in technical replicates of 2.

B: Schematic representation of the initial U2 snRNA transcript (adapted from (Broome and Hebert, 2012)) depicting additional 634bp after the end of the U2 snRNA coding sequence (CDS) and the position of the respective primers for qRT-PCR used for U2 snRNA in (A).

C: Control- or USPL1 siRNA treated HeLa cells were subjected to RNA-FISH for U2, U5 and U6 snRNA using Alexa488-labeled probes. The immunostaining against coilin was used to monitor the efficiency of the siRNA treatment against USPL1. CBs are indicated by arrows, splicing speckles by arrowheads and nucleolar coilin by an open arrowhead. Bar, 10 μm.

**Figure 6: USPL1 co-localizes and interacts with members of the LEC complex**
A: Total cell lysate obtained from HEK293 cells 24h after transfection with the indicated plasmids were subjected to IP using an anti-flag antibody. 1.5% of the total lysate was loaded as input (left panel). Bound proteins were analyzed by immunoblotting using an anti-HA or anti-flag antibody (right panel).

B: Total cell lysate from either HEK293 (293) or HeLa cells was subjected to an IP using the USPL1 antibody. Input corresponds to 1% of the total cell lysate (left panel) and a cell lysate sample of HEK293 transfected with either HA-USPL1 or FLAG-ELL was loaded onto the same gel as positive control for USPL1 or ELL, respectively. Empty protein-G agarose beads served as IP control. Bound proteins were analyzed using either the USPL1 antibody or ELL antibody (right panel). The arrow points to endogenous ELL, non-specific bands recognized by the ELL antibody are marked with asterisks.

C: Colocalization of either ELL (top panel) or Ice1 (bottom panel) with USPL1 and coilin in CBs (arrows) shown by immunostaining of HeLa cells. Bar, 10µm.

Figure 7: Knockdown of Ice1 has comparable consequences for nuclear architecture as USPL1 knockdown

A: Immunofluorescence of siRNA treated HeLa cells treated against USPL1, Ice1 and coilin. The arrowhead indicates a CB in control cells with all three proteins present (top panel). Nucleolar coilin upon Ice1 or USPL1 knockdown is indicated by open arrowheads (middle and bottom panel, respectively). Arrows in the bottom panel highlight nuclear foci containing Ice1 in the absence of USPL1. Bar, 10 µm.

B: Immunofluorescence of siRNA treated U2OS cells against coilin and ASF. Arrows highlight enlarged, rounded nuclear speckles for ASF upon siIce1 (middle panel) or siUSPL1 (bottom panel) transfection. Bar, 10 µm.

C: Immunofluorescence of siRNA treated U2OS cells against coilin and SMN. A maximum intensity projection illustrates the total number of SMN-containing nuclear foci (arrows right panel). Bar, 10 µm.

Figure 8: USPL1 is associated with U snRNA gene loci by DNA-FISH and ChIP
A: Specificity of the probe for the U2 gene array (Cy5, shown in green; arrows) demonstrated on a metaphase spread from normal human male lymphocytes.

B: HeLa cells were subjected to DNA-FISH for the U2 gene locus (Cy5, shown in red) in combination with immunostaining for USPL1 (Alexa 488, shown in green). Arrows indicate nuclear foci containing both, USPL1 and the U2 gene locus. Bar, 10 µm.

C: ChIP from HeLa cells using the USPL1 antibody is displayed in comparison to IgG control for the respective gene promoter (U1 pr., U2 pr. or GAPDH pr.). Bars represent the standard error of 6 independent experiments, each analyzed as technical replicate of 2 in the qPCR-reaction. Statistical significance was determined using an unpaired, heteroscedastic Student’s t-test.

D: RNAPII occupancy detected by ChIP against the CTD of RNAPII (4H8 antibody) in comparison to IgG control for the respective gene region (U1 pr., U2 pr., U2 -2kb) upon USPL1 knockdown. Bars represent the standard error of 4 independent experiments, each analyzed as technical replicate of 2 in the qPCR-reaction. Statistical significance was determined using an unpaired, heteroscedastic Student’s t-test.

Figure S1: USPL1 knockdown affects factors associated with pre-mRNA splicing

A: Immunoblot for USPL1, coilin and SMN on total cell lysate obtained from HeLa and U2OS cells upon control- or USPL1-knockdown. Alpha tubulin stain serves as loading control.

B: Immunofluorescence of siRNA treated HeLa cells against fibrillarlin, USPL1 and coilin. In a subset of cells, fibrillarlin appears less condensed in the nucleolus upon USPL1 knockdown (indicated by arrow). Bar, 10 µm.

C: Immunofluorescence of siRNA treated HeLa cells against coilin and Sm proteins (Y12 antibody). Arrows highlight splicing speckles in control cells (top panel), arrowheads enlarged nuclear speckles for Sm proteins upon USPL1 knockdown. An open arrowhead highlights nucleolar coilin upon siUSPL1. Bar, 10 µm.
Figure S2: Effects of USPL1 knockdown on the cellular proteome and tri-snRNP formation

A, B: SILAC analysis of total cell lysate and nucleoplasm upon fractionation of HeLa cells and USPL1-knockdown. Only hits identified with at least 2 peptides in an individual fraction are displayed here. The log₂ ratio heavy (H; siUSPL1)/ light (L; siControl) is displayed as frequency histogram for total cell lysate (A) and nucleoplasm (B). Proteins groups above/below the arbitrary threshold are indicated by red lined boxes. C: Control- (IgG) or PRPF4-immunoprecipitations (IP) of nuclear extracts prepared from siRNA treated HeLa cells were analyzed by immunoblot analysis with antibodies as indicated. Input corresponds to ~3% of nuclear extract used in the IP.

Figure S3: USPL1 knockdown affects U snRNA transcription by RNAPII.

A: qRT-PCR for different U snRNA species in U2OS cells upon knockdown of USPL1. U2pre represents the unprocessed U2 snRNA. Respective expression levels were normalized for β-actin and snRNA levels of siControl treated cells were set to 1. Bars represent the standard error of 4 independent experiments, each measured in technical replicates of 2.

B: Control- or USPL1-depleted U2OS cells were subjected to RNA-FISH for U2, U5 and U6 snRNA using Alexa488-labeled probes. The immunostaining against coilin serves as control for the efficiency of the siRNA treatment against USPL1. CBs are indicated by arrows, splicing speckles by arrowheads and nucleolar coilin by an open arrowhead. Bar, 10 µm.

Figure S4: Reduced RNAPII promoter occupancy at snRNA loci

RNAPII occupancy detected by ChIP using an antibody against RNAPII (N20; sc-899) is significantly reduced at U1 and U2 snRNA gene loci upon USPL1 knockdown. Bars represent the standard error of 4 independent experiments, each analyzed as technical replicate of 2 in the qPCR-reaction. Statistical significance was determined using an unpaired, heteroscedastic Student’s t-test.
Supplementary table 1: The first sheet contains protein group table with the total experimental dataset of the SILAC-MS based cellular fractionation upon USP11 knockdown. T = TCL; C = cytoplasm; np = nucleoplasm; nol = nucleolus. The second sheet lists all splicing factors identified in SILAC-MS dataset with >1 peptide according to (Hegele et al., 2012).

Supplementary table 2: Result of DAVID analysis for proteins identified with >1 peptide in cytoplasmic and nucleolar fraction of SILAC-MS dataset.

Supplementary table 3: Antibodies

Supplementary table 4: siRNA sequences

Supplementary table 5: primer for qualitative RT.PCR

Supplementary table 6: primer for qRT-PCR

Supplementary table 7: primer for qPCR (ChIP)

Supplementary table 8: RNA-FISH probes

Supplementary table 9: primers for generation of DNA-FISH probe

Author Contributions
S.H. performed most experiments, G.C. performed some experiments and U.W. contributed to experimental design. All authors participated in writing the manuscript. A.I.L. and F.M. mentored the project.

References


Figure 2

A

EU       coilin       merge

HeLa

U2OS

B

siControl   siUSPL1

Casp 9

CCNA2

CCNB5

TUBB

ASF

FAS

MCL1

actin
Figure 4

A

siControl 100% 50% 25% siCollin siUSPL1

<table>
<thead>
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<td>SMN</td>
<td>38</td>
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</tr>
</tbody>
</table>

B

\( \alpha \) GFP

\( \alpha \) TUB

\( \alpha \) GFP

\( \alpha \) TUB

C

GFP-SmB Y12 coilin DNA merge

-dox

+dox

D

siControl siUSPL1 siControl siUSPL1 siControl siUSPL1

input 7-mG eluate

GFP-SmB
Figure 5

(A) Normalized snRNA expression levels for different siUSPL1 treatments. The graph shows a significant increase in U6 snRNA expression compared to the other snRNAs.

(B) Schematic representation of U2 and U2pre snRNA coding regions. The U2-F and U2pre-F are transcripts of U2 and U2pre, respectively. The 3' box region is highlighted.

(C) Immunofluorescence images showing the effects of siUSPL1 treatment on U2, U5, and U6 snRNAs. The images indicate reduced snRNA expression in the siUSPL1-treated samples compared to the control samples.
Figure 7

A

USPL1    coilin    Ice1    DNA    merge

siControl

silce1

sIOUSPL1

B

ASF    coilin    merge

siControl

silce1

sIOUSPL1

C

SMN    coilin    merge    SMN/DNA pr.

siControl

silce1

sIOUSPL1