The spliceosome-activating complex: molecular mechanisms underlying the function of a pleiotropic regulator

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INTRODUCTION

Intron sequences of RNA polymerase II (RNAPII) transcribed nuclear pre-mRNAs in eukaryotes are removed by the spliceosome to produce mature mRNAs. The spliceosome is composed of U-rich U1, U2, U4, U5, and U6 small nuclear snRNAs, which form snRNP particles with specific sets of proteins. Spliceosomal snRNPs undergo in cell extracts timely ordered assembly on model pre-mRNA templates and recruit numerous stage specific auxiliary regulatory factors, the complexity of which increases in metazoan’s evolution (Wahl et al., 2009). Except for plants, where thus far no suitable in vitro splicing assay is available, past studies of in vitro spliceosome assembly have generated a wealth of mass spectrometry, RNA cross-linking, and crystallographic data on basic functions, binding specificities, and interactions of core spliceosomal proteins. Other studies have independently identified the functions of numerous auxiliary factors that co-purify with different snRNPs. The emerging regulatory interactions reveal a huge complexity and gene/intron specific variation of in vivo spliceosome interactions with the RNAPII transcription initiation, capping, elongation, and polyadenylation/cleavage complexes, as well as subunits of the exon junction, pre-mRNA splicing and retention (RES), mRNA export (THO/TREX), exosome, microRNA processing, nuclear pore, nonsense-mediated decay (NMD), and chromatin remodeling/modifying complexes. In fact, accumulating data on transcript specific splicing kinetics and alternative splicing demonstrate that these interactions govern co-transcriptional assembly, selectivity, and progressivity of the spliceosome, which are also remarkably dependent on changes in epigenetic modifications of histones during different phases of transcription (Oesterreich et al., 2011). Co-transcriptional coupling and regulation of the spliceosome activity is further indicated by the fact that introns are removed from the majority of pre-mRNAs before completion of transcription in yeast and mammalian cells. In general, splicing of introns close to the 5′-ends of transcripts is completed earlier compared to the removal of 3′ introns. Due to different sequence features of introns, their splicing does not however follow always the order of their transcription. Some “difficult” introns might remain completely or partially unspliced upon transcription termination. Partially spliced pre-mRNAs accumulate together with hyper-phosphorylated RNAPII, spliceosome components, and auxiliary factors in nuclear speckles, especially in differentiated cells types, where their processing occurs post-transcriptionally (Han et al., 2011). Thus, while spliceosome assembly appears to be regulated...
co-transcriptionally, splicing *per se* does not necessarily depend on active transcription.

**SPLICEOSOMAL snRNPs**

As in other metazoans, the majority of introns are recognized by the U2-type spliceosome in *Arabidopsis*. Regulatory features and targets of *Arabidopsis* U12-specific minor spliceosome have been reviewed recently (Simpson and Brown, 2008). Here we provide a compilation of proteins identified by mass spectrometry in 27 purified yeast, *Drosophila*, and human U2-type spliceosomal complexes and annotation of corresponding *Arabidopsis* homologs (Table S1 in Supplementary Material). Yeast spliceosomal complexes are composed of 50–60 core snRNP subunits and about hundred additional splicing-related factors, most of which are conserved in metazoans (Fabrizio et al., 2009). In comparison, the total number of proteins identified in purified *Drosophila* and human spliceosomal complexes is about 260 and 400, respectively (Rappsilber et al., 2002; Herold et al., 2009; Will and Lührmann, 2011). Based on comparative sequence analysis, Wang and Brendel (2004) predicted 395 genes encoding splicing-related proteins in *Arabidopsis*, including all putative RNA-binding factors identified earlier by Lorkovic and Barta (2002). Our compilation, based on comparative analysis of mass spectrometry data using the TAIR10 version of reannotated genome sequence, indicates a conservation of about 430 spliceosomal factors in *Arabidopsis* (Table S1 in Supplementary Material). However, in some cases the relationships between yeast, *Arabidopsis*, and other metazoan homologs are suggested only by conservation of certain functionally important domains. Thus, verification of composition of *Arabidopsis* core snRNPs and auxiliary factors of various spliceosomal complexes awaits further analysis of their subunits by mass spectrometry.

In yeast and metazoans, the U1, 2, 4, and 5 snRNAs associate through their U-rich PuAU4–6GPu motives to a heptamer of Sm proteins, while U6 is bound to a similar Sm-like LSm complex (Tarrun, 2009; Weber et al., 2010). In *Arabidopsis* all seven Sm proteins and LSm1, 3, and 6 are encoded by duplicated genes (Figure 1). In addition to their U6 snRNP-specific roles, the LSm proteins target aberrant transcripts for decapping and 5′ to 3′ decay. Mutation of *LSm4* (*emb1644*) causes embryonic lethality, while the *lsm5/sad1* point mutation results in a partial loss of function conferring supersensitivity to absicic acid (ABA) and drought (Xiong et al., 2001).

Subunits of snRNPs show weaker sequence conservation but similar redundancy in *Arabidopsis*. From the U1 snRNP-associated proteins, several are encoded by at least two genes, while *Arabidopsis* lacks Prp42 and Snu56 homologs (Figure 1). Similarly, the U2AF35, U2AF65, and polyypyrimidin tract-binding (PTB) proteins from the splice-site selecting U2 components, as well as numerous U2 snRNP subunits and U2-related factors are encoded by duplicated genes in *Arabidopsis*. Spliceosome association of other factors that share only domain homology with the U2 snRNP components is nonetheless uncertain and should be confirmed by further proteomics analyses. Genetic studies indicate that inactivation of *PRP39a* component of U1 snRNP confers late flowering by upregulating the transcription of *FLC* flowering time repressor (Wang et al., 2007). In contrast, the elf9 mutation of *Arabidopsis* ortholog of *tat-SF1* U2 snRNP component upregulates transcription of a defective splice isoform of suppressor of overexpression of CO1 (SOCI) causing early flowering (Song et al., 2009). On the other hand, the U2 SPF45 subunit is implicated in DNA recombination and repair both in *Arabidopsis* and *Drosophila*, where it is an interacting partner of RAD201, a member of the RecA/Rad51 family (Chaouki and Salz, 2006).

In the case of U5 snRNP, there are multiple *Arabidopsis* homologs of Prp8 and Brz2 RNA helicases and Snu114 GTPase that play important roles in both activation and dissociation of the spliceosome (Wahl et al., 2009). Nonetheless, analysis of the *Arabidopsis* NineTeen Complex (NTC) complex (Monaghan et al., 2009) and genetic data suggest that BRR2 is represented probably by only a single locus, while PRP8 and SNU114 are encoded by two differentially regulated genes. The *prp8a/sus2* mutation results in a cell division defect of embryonic suspensor, while the *emb1507* embryo lethal mutation is located in the BRR2 gene. Different genetic screens led to the identification of *mutant effect of embryo arrest 5* (*me5*), *clotho* (*clo1*), and gametophytic factor 1 (*gfl1*) mutations of SNU114, which is required for specification of egg cell fate and floral organ number and identity (Liu et al., 2009; Yagi et al., 2009). Similarly, the *atropos* (*ato*) mutation of U2 snRNP SF3a60/PRP9 gene compromises egg and central cell fate, and SNU114 is necessary for tissue specific expression of *Lachesis* (*LIs*) that encodes the U4/U6 snRNP subunit PRP4 (Moll et al., 2008). A common consequence of all these mutations is the abortion of gametophyte. A potential link to cell death regulation is suggested by high level induction of *Yellow-Leaf Specific Gene 8* (*U5-15/YLS8*) during late leaf senescence (Yoshida et al., 2001). Expression of the U5 snRNA subunit gene *U5-102/PRP6* is cold stimulated and its stabilized 1 (*stat1*)/emb2770 mutation results in
defective splicing and stabilization of the cold-induced COR15A and other unstable transcripts leading to a range of pleiotropic developmental and stress response defects (Lee et al., 2006). Like the ELF9/tat-SF1 subunit of U2 snRNP, PRP6 might function in interaction with the exosome and exon junction complex (EJC) implicated in the recruitment of NMD regulators (Gehring et al., 2005).

The U4/U6 subunits PRP3 and Tri-20 are represented by two Arabidopsis homologs, while U4/U6-15.5/SNU13 is likely encoded by three genes as the Tri65 subunit of U4/U6.U5 tri-snRNP. Mutation of U4/U6 subunits Prp24 and Prp31 lead to embryo lethality (emb140 and emb1220), while inactivation of the RS-domain of Tri120/SNU66 by the meristent defective (mdf) mutation stimulates ectopic meristem formation in vegetative tissues but inhibits root and shoot meristem activities. The mdf mutation reduces the transcript levels of PIN2 and PIN4 auxin influx carriers, as well as those of Plethora, Scarecrow, and Shortroot in the root and Wusche (WUS) in the shoot meristem (Casson et al., 2009, likely due to splicing defects and destabilization of these transcripts. The mdf mutation is allelic with defectively organized tributaries 2 (dot2) that causes altered vein differentiation pattern in juvenile leaves consistently with defects of auxin transport (Petricka et al., 2008).

**SPliceosome Catalytic CYCLE**

The U1 and U2 snRNPs recognize the 5′ and 3′ splice-sites (5′SS and 3′SS) and conserved branch sites (BS: AG) of introns. BS is followed by a polypyrimidine tract (PPT) upstream of the 3′SS in metazoans. BS and PPT are poorly conserved in plants (Brown and Simpson, 1998; Simpson et al., 2002), although orthologs of BS-interacting U2AF and PTB proteins are present in Arabidopsis. In vitro assembly studies indicate that the U1 snRNP binds first through U1-C and U1-70K to the 5′SS. Subsequently, PPT and 3′SS are bound respectively by the U2 auxiliary factors U2AF65 and U2AF35 that interact with SF1 at the BP, as well as with a range of SR, hnRNP, Transformer (Tra), and EJC proteins that recognize exonic and intronic splicing enhancer and silencer sequences, contributing to the definition of exons’ positions (for review see Will and Lührmann, 2011). The SR-related Arabidopsis SR45a/Tra-2b factor interacts, for example, with U1-70K and U2F53a to assist splice-site selection, as well as with PRP38 during spliceosome activation (Tanabe et al., 2009). From the 19 Arabidopsis SR-proteins classified into seven subfamilies (Barta et al., 2010), yeast and human orthologs of SR1, SC35, RS33, and RSZ33, as well as the SR-related proteins Tra-1A, B1/2, and SRm160 were identified in purified spliceosomal complexes (Table S1 in Supplementary Material). Transcription and alternative splicing of SR-protein genes is regulated by a multitude of stress and hormonal stimuli, and their known mutations result in pleiotropic regulatory defects (Reddy, 2007). The activity and stability of SR-proteins is regulated by phosphorylation including the Lammer/CLK, SRPK1, and SRPK2 kinases families, as well as by several PRMT arginine methylases that also recognize other classes (e.g., Sm, Lsm, hnRNP, etc.) of spliceosomal proteins (Flühr, 2008). Arabidopsis PRMT5 was recently demonstrated to methylate several Sm and Lsm factors (Deng et al., 2010). The prmt5 mutation results in defective splicing of FLK/hnRNP-E pre-mRNA and late flowering by increasing the FLC transcript level, as well as alters 3′SS recognition leading to aberrant processing of pre-mRNAs encoding components of the circadian clock (Sanchez et al., 2010).

Interaction of U1 with the U2AF-recruited U2 snRNP is stabilized by the ATP-dependent DExH/D-box RNA helicase Prp5. Displacement of SF1 by the SF3b14a subunit of PPT-binding U2-SF3a/b complex is stimulated by Prp5 leading to the formation of prespliceosome complex A (Rezhadin et al., 2007; Figure 2). Prp5 also facilitates annealing the U2 snRNA with BS, which bulges out an A residue of the intron for the first transesteverification reaction. Subsequent recruitment of the U4/U6.U5 tri-snRNP results in the assembly of U1/2/4/5/6 penta-snRNP in the precatalytic complex B (Deckert et al., 2006). Penta-snRNP can be purified from yeast but it is inactive and requires additional factors, in particular the NTC, to form an activated BACT complex (Stevens et al., 2002).

During complex B to BACT transition, interaction of U1 and U1-C with the 5′SS is interrupted by the Prp28/U5-100 helicase, which is activated by the U4/U6.U5 tri-snRNP-associated kinase SRPK2. Subsequently, unwinding the based-paired U4/U6 snRNAs by the Br2/U5-200 helicase facilitates U6 snRNA interactions with the 5′SS and U2 snRNA. This stimulates the release of U1 and U4 snRNPs, U6 specific Lsm, and Prp24 proteins, as well as the formation of an intramolecular stem-loop (ISL) in U6, which represents the metal-binding catalytic center of the spliceosome. Br2r is controlled by the interacting Snu114 GTPase and Prp8 U5 subunit. Removal of U2-associated SF3a/b proteins by the Prp2 helicase exposes BS in the remodeled catalytically active complex B+. During step I of splicing, the 2′-OH of BS adenosine residue

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**FIGURE 2** | Schematic presentation of spliceosomal assembly and catalytic cycle. The scheme is drawn according to Wahl et al. (2009) indicating the assembly phase specific regulatory roles of key ATP-dependent DExH/D-box RNA helicases (in red) and the spliceosome-activating NTC complex. 5′ and 3′ splice site (5′SS and 3′SS), branch point (BP), and polypyrimidine tract (PPT). Exons are indicated by gray boxes, while thin black lines show intron and intron lariat.
attacks and forms a covalent bond with the U6 ISL-cleaved 5′SS yielding the 5′-exon and lariat intron-3′ exon intermediates.

Catalytic activation of the spliceosome critically depends on its association with the NTC before or during unwinding of the U4 and U6 snRNAs. The NTC regulates the interactions of U5 and U6 with the pre-mRNA before and after step I, as well as formation of the spliceosome’s catalytic center (Chan et al., 2003; Chan and Cheng, 2005). Nucleophilic attack of 3′-OH of 5′ exon at the 3′SS in step II requires further structural rearrangements by multiple factors leading to the formation of complex C. Prp8, anchoring the 5′ exon and lariat intron-3′ exon intermediates (Graniero and Beggs, 2005), the Prp16 ATPase, and the NTC subunit Isy1 are involved in monitoring completion of step I and displacing U6 from the 5′SS to liberate it for the catalytic step II. The Prp18 helicase and loop 1 of U5 snRNA juxtapose for ligation of the 5′ exon and 3′SS bound by the interacting NTC subunits Slu7 and Prp22 helicase (Smith et al., 2008). Next, Prp22 deposited downstream of the exon–exon junction disrupts the interaction of Prp8 and U5 with exon sequences, releasing the spliced mRNA from complex C. Dissociation of U2, U5, and U6 is catalyzed by the Prp43 helicase, which is encoded by three candidates genes in Arabidopsis, while Br2 and Snu114 are thought to unwind and separate the U2 and U6 components of the post-splicing complex (Valadkhan and Jaladat, 2010).

**NTC SUBUNIT COMPOSITION**

NTC is a regulatory non-snRNP complex that is essential for catalytic activation of the spliceosome (Hogg et al., 2010). When isolated under stringent conditions from *in vitro* assembly reactions, yeast NTC contains eight core subunits (Prp19, Cef1/Ccd5, Snt309/Spf27, Syf1, Syf2, Syf3/Clf1, Isy1, and Ntc20 listed in Figure 3; Fabrizio et al., 2009). In contrast, tandem affinity purification of NTC by its tagged Cef1/Cdc5 subunit from budding and fission yeast cell extracts indicates a conservation of at least 26 NTC-associated proteins (Ohi et al., 2002). Recent mass spectrometry analysis of NTC complexes purified by the help of TAP-tagged Prp19, Prp17, and Cwc2 subunits also shows that the eight core subunits are associated *in vivo* with several Sm proteins, components of the U2 and U5 snRNPs, and at least 30 non-snRNP proteins (Ren et al., 2011). Many of the latter NTC-associated factors were previously found to co-purify with spliceosome A, B, B*, or C, as well as with the RES and THO/TREX complexes. The human core NTC assembled on RNA templates and Gould, 2002; Grote et al., 2010). Nucleophilic attack of 3′-OH of 5′ exon at the 3′SS in step II requires further structural rearrangements by multiple factors leading to the formation of complex C. Prp8, anchoring the 5′ exon and lariat intron-3′ exon intermediates (Graniero and Beggs, 2005), the Prp16 ATPase, and the NTC subunit Isy1 are involved in monitoring completion of step I and displacing U6 from the 5′SS to liberate it for the catalytic step II. The Prp18 helicase and loop 1 of U5 snRNA juxtapose for ligation of the 5′ exon and 3′SS bound by the interacting NTC subunits Slu7 and Prp22 helicase (Smith et al., 2008). Next, Prp22 deposited downstream of the exon–exon junction disrupts the interaction of Prp8 and U5 with exon sequences, releasing the spliced mRNA from complex C. Dissociation of U2, U5, and U6 is catalyzed by the Prp43 helicase, which is encoded by three candidates genes in Arabidopsis, while Br2 and Snu114 are thought to unwind and separate the U2 and U6 components of the post-splicing complex (Valadkhan and Jaladat, 2010).

**FIGURE 3 | Conserved components of NTC core and associated subunits detected in purified yeast, human, and Arabidopsis complexes.** Proposed NTC core subunits are highlighted in bold, brackets indicate factors that are not present in purified NTC complexes but encoded by corresponding homologous genes in the yeast, human, and Arabidopsis genomes.
the yeast NTC core [CDC5/Cef1 (MAC1), Prp19a/b (MAC3A/B), SPF27 (MOS4), SYF1 (MAC9), SYF3/CRN1c (MAC10), and ISY1 (MAC8) listed in Figure 3]. In PRL1 prp19a/b mutants, Prp3 prp19a/b scns suggest that these factors cooperate with Prp19 and NTC components for spliceosome activation (Gräub et al., 2008). Human CDC5 gene (Burns et al., 2002). Cell cycle arrest of the yeast CDC5 homolog snt309 (Snt309/BCAS2) is required for stable interaction of Prp19 with the α-tubulin (Tub1) gene (Burns et al., 2002). Cell cycle dependent phosphorylation of CDC5 appears to be an important modulator of NTC-mediated activation of the spliceosome (Gräub et al., 2008). Human CDC5 interacts with NIP11 (nuclear inhibitor of protein phosphatase 1 absent from Arabidopsis), which recognizes the SF3b155 subunit of U2-SF3a/b complex that becomes hyperphosphorylated during the first splicing reaction. Interaction of CDC5 with NIP11 probably targets a PP1 phosphatase to the SF1a/b complex leading to its destabilization, which is required for the second step of splicing (Tanuma et al., 2008). Furthermore, human CDC5 directly interacts with hLodestar/HuF2, which is an SF2-like ATP-dependent helicase subunit of SWI/SNF chromatin remodeling complex implicated in transcription termination (Leonard et al., 2003).

C-terminus of CDC5 binds the C-terminal WD-40 domain of Prp46/PRL1 in yeast and human NTCs (Ajuh et al., 2001; Oh and Gould, 2002). PRL1 encoded by the Pleiotropic Regulatory Locus 1 was first identified in Arabidopsis by a T-DNA insertion mutation causing highly pleiotropic defects characterized by altered regulation of root and leaf development, flowering time, sugar, cold, ethylene, cytokinin, and auxin responses (Németh et al., 1998). Arabidopsis has a PRL1 paralog, PRL2, which is transcribed at low levels in vegetative tissues but shows much higher expression compared to PRL1 during embryogenesis and seed development. Low PRL2 expression in the prl1 mutant thus appears to be sufficient for maintaining plant viability but leads to highly pleiotropic defects. Nuclear import of PRL1 is aided by...
its interaction with a specific member of the α-importin family, ATHKAP2/IMPA-3 (Németh et al., 1998). Inactivation of IMPA-3 in the Arabidopsis mos6 mutant suppresses constitutive activation of innate immunity by the sncl mutation similarly to the mos4 (snt309), cdc5 (mac1), and prl1 (mac2) mutations (Palma et al., 2005, 2007). In comparison, human CDC5 is critical for the nuclease by the CTNNBL1 (catenin-β-like 1) armadillo repeat protein, which remains in CDC5L-bound form in the human core NPC and mediates interaction with the PRP31 subunit of U4/U6 snRNP (Grote et al., 2010). Temperature sensitive mutations of fission yeast Cwf1/CDC5 and Prp5/PRL1 show strong negative genetic interactions (i.e., synthetic lethality at permissive temperature) suggesting overlapping functions (McDonald et al., 1999). Removal of the C-terminus of CDC5 results in dissociation of PRL1 from the yeast NPC (Ohi and Gould, 2002) but it is yet unclear whether this leads to overall destabilization of the complex. Virus-induced gene silencing of Arabidopsis CDC5 is reported to activate early senescence causing accelerated cell death independently of salicylic acid (SA) signaling (Lin et al., 2007a). According to Palma et al. (2007), however, the cdc5, prl1, and mos4 mutations stimulate SA production but show npr1-independent activation of the PR genes. RNAi-silencing of CDC5 was also suggested to result in a G2/M cell cycle defect by affecting Shoot Meristemless (STM) and WUS transcription and meristem development. Furthermore, the GABI_278B09 cdc5 T-DNA insertion mutation was found to cause embryo lethality by Lin et al. (2007b). The latter phenotypic trait is however probably caused by an unrelated mutation, since Palma et al. (2007) found that removal of C-terminal PRL1-binding domain from the CDC5 coding sequence by the same T-DNA insertion mutation results in viable plants showing a partial loss of function phenotype similar to that of the prl1 mutant.

Arabidopsis PRL1 interacts with and inhibits in vitro the activity of Snf1-related protein kinase AKIN10 (Bhalerao et al., 1999), a functional homolog of yeast Snf1 that represents a regulatory partner of GCN5 histone acetylase in the yeast RNAIPI SAGA co-activator complex (Lo et al., 2001; Liu et al., 2010). Whereas association of SnRK1 with SAGA in plants awaits further confirmation, SnRK1 AKIN10 was found to interact in vivo with the common SKP1 subunits of SCF (Skp1-cullin1-F-box) E3 ubiquitin ligases by targeting them to the α4/PAD1 subunit of 20S proteasome catalytic cylinder (Farrás et al., 2001).

Arabidopsis α4/PAD1 is an ortholog of human proteasome subunit a7, which is the closest neighbor of β7 subunit targeted by Prp19 (Löschler et al., 2005). Recently, PRL1 was found to function as substrate receptor subunit of a Cul4–DDB1 ubiquitin ligase, and the prl1 mutation was reported to stabilize SnRK1 AKIN10. It is remarkable that in addition to PRL1, WD-40 repeats of spliceosome-associated THOC6 and CY subunits of the THO/TREX mRNA export, and polyadenylation/cleavage complexes also contain DDB1-binding DWD motives are detected in association with Cul4 and DDB1 in Arabidopsis (Lee et al., 2008). Knockout of mouse PRLG1/PRL1 results in early embryonic lethality as inactivation of mouse Prp19/Pso4 (Löschler et al., 2005; Kleinridders et al., 2009), whereas no homozygous prl1, prl2 and cdc5 null mutants can be obtained in Arabidopsis. These results are consistent with the observations of Ohi et al. (2002) indicating that Cef1/Cdc5, Prp46/PRL1, and Prp19 are essential genes in yeast. In contrast, the yeast snt309 and Arabidopsis mos4 mutations do no cause lethality (Palma et al., 2007). Ecm2/Slt1 is also not essential in yeast but its mutation causes cold sensitivity (i.e., as the prl1 mutation in Arabidopsis; Németh et al., 1998) and impaired splicing (Xu and Friesen, 2001). However, a combination of mutations of corresponding Arabidopsis homologs ECM2A and B (MAC5A/B) is lethal (Monaghan et al., 2010). Ecm2, as the homologous human RBM22 zinc-finger RNA-binding protein is controlled by sumoylation and involved in U2/U6 helix II formation required for spliceosome activation. Ecm2/RBM22 interacts with the step II splicing factors Shu7, Prp16, and Prp17 and its inactivation in zebrafish leads to early embryol lethality as in Arabidopsis (He et al., 2009).

From the remaining conserved NPC components, Syf3/CRN1/MAC10 tetratricopeptide repeat (TRP) protein is one of the earliest acting NPC proteins, which is essential for loading the U4/U6.U5 tri-snRNP during complex A to B transition. In Drosophila, mutation of CRN1/Syf3 (crooked neck) causes early embryonic lethality (Chung et al., 2002). Isy1/MAC8 interacts with the Prp16 ATPase required for proper recognition of 3’SS and implicated in remodeling of U6. Isy1 also interacts with Cwc21/SRm300, as well as a with the trimethylguanosine synthase capping enzyme during early stage of spliceosome assembly (Villa and Guthrie, 2005; Hausmann et al., 2008; Khanna et al., 2009). The PRL1-binding non-essential second step factor Prp17/CDC40/MAC17 is a WD-40-protein, which functions together with Prp16, Prp8, and the SR protein kinase Sky1 in the recognition of 3’SS. Prp17 is essential for splicing of introns longer than 200 nucleotides and shows co-immunoprecipitation with lariat intron–exon intermediates (Derger and Fu, 2001; Sapra et al., 2008). Finally, from the conserved NPC-associated proteins Aquarius/AQR/MAC7 is found to bind intron sequences in the vicinity of BP and it is identified by the Arabidopsis by the embryo defective 2765 mutation (Hirose et al., 2006). The key step II RNA helicase Prp2 is represented by three potential homologs in Arabidopsis, from which the eps3 mutation of Prp2/MAC5 confers enhanced gene silencing, probably due to destabilization of aberrantly spliced pre-mRNAs and subsequent generation of derived siRNAs (Herr et al., 2006). The maternal embryo effect

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29 (mec29) mutation in its paralog Prp2b results in deficient gametophyte/embryo development (Pagnussat et al., 2005).

Taken together, the available genetic data indicate that mutations of essential Arabidopsis NTC components result in lethality. In those cases, where one of the duplicated genes is preferentially expressed during embryo and seed development whereas the other shows higher expression in vegetative tissues, the corresponding single gene mutations yield embryo lethality and pleiotropic effects in seedlings, respectively. Although not all NTC mutants were characterized so far for their pleiotropic defects in such a detail as prl1, all studied NTC deficiencies appear to function as mos suppressors of sncl-induced innate immunity, which is coupled to the induction of cell death in pathogen infected tissues. Recently, the prl1 mutation was also demonstrated to suppress the induction of cell death by singlet oxygen generated through the accumulation of free protochlorophyllide in the fluorescent in blue light (flu) mutant (Baruah et al., 2009). Furthermore, the Arabidopsis mos2 mutation, which has not been connected so far to NTC, is located in a gene encoding a spliceosome complex C specific factor Sp2/GPKOW, which is a binding partner of yeast Prp2 and human protein kinase Cβ2 that shows interaction with PRL1 in human cells (Roy et al., 1995; Németh et al., 1998; Zhang et al., 2005; Aksaas et al., 2011). Another candidate MOS factor is the complex C specific protein RUVBL1, a chromatin remodeling ATPase inhibitor of apoptosis (Taniue et al., 2011). The Arabidopsis RUVBL1 homolog, TIP49A/RIN1, is an interacting partner of RPM1 receptor required for disease resistance against Pseudomonas syringae pv. maculicola (Holt et al., 2002). It is also apparent that some pathogen produced effector molecules could directly modulate the activity of the spliceosome. Thus, a natural antitumor compound isolated from Pseudomonas functions as spliceostatin A that specifically targets the U2 snRNP SF3b complex and thereby inhibits splicing and nuclear retention of unspliced pre-mRNAs (Kaida et al., 2007).

**NTC PROVIDES A LINK BETWEEN SPlicing AND REGULATION OF DNA REPAIR, RECOMBINATION, AND CELL DEATH**

In addition to controlling spliceosome assembly and activation, NTC is emerging to play a central role in the regulation of DNA damage responses, which is consistent with the effects of NTC subunit mutations observed in Arabidopsis. In yeast, Prp19 was identified as Ps04, mutation of which causes hypersensitivity to the DNA cross-linking agent psoralen, as well as to a broad range of other DNA damaging agents (see for review Legerski, 2009). Expression of human PRP19/PSO4/SNEV is induced by DNA damage and its down-regulation by RNAi results in the accumulation of double-stranded DNA breaks and apoptosis (Mahajan and Mitchell, 2003). Using a DNA interstrand cross-link repair assay, Ps04 was purified together with the NTC subunits CDC5, PRL1, and SPF27, from which CDC5 directly interacts with the Werner syndrome DNA helicase (WRN) in complex with the replication protein A (RPA, Zhang et al., 2005b). RPA is essential for stabilization of single-stranded gap structure arising from dual incision of damaged DNA strand, while WRN assists DNA repair replication. DNA damage causing an arrest of transcription elongation and DNA replication leads to activation of the DNA repair checkpoint, which prevents cells entering or leaving the S-phase. Mutations inactivating the regulators of this checkpoint lead to persistent activation of p53 tumor suppressor stimulating cell death and apoptosis in mammals. Activation of transcription-independent global genome and transcription-coupled nucleotide excision repair (GG-NER and TC-NER) pathways is mediated by different sensory but common executing components (Lagerwerf et al., 2011). In GG-NER, various DNA lesions are recognized by the UV-DDB (Cul4–DDDB1–DDDB2) and XPC–Rad23B complexes. In TC-NER, the hyperphosphorylated RNAp1 stalled by the DNA damage is recognized the Cockayne Syndrome CB2 chromatin remodeling ATPase, which subsequently recruits the Cul4–DDDB1–CSA E3 ubiquitin ligase and Gcn5/p30 histone acetylase complexes. Remarkably, DDDB2 and CSA/ERCC8 are WD–40 proteins that in analogy to PRL1 are recruited to DDB1. This suggests that PRL1 in the Ps04 complex might perform a similar substrate receptor function although its target is unknown so far. Cul4–DDDB1–DDB2 ubiquitinates histones H3 and H4 contributing to opening the damaged chromatin site. On the other hand, the CSA WD–40 protein is a substrate receptor of CSB, which is being released and degraded before subsequent loading of the TFIIH RNAPII transcription initiation/DNA repair complex to the damaged site. In the case of TC-NER, loading of TFIIH requires the recognition of CSA/CSB by the XAB2/Syf1 NTC subunit, as well as interaction of the XAB2 complex with the transcription elongation factor TFIIIS and high mobility group chromatin factor HMGN1 (Fousteri et al., 2006).

The human XAB2 complex contains Aquarius, PRP19, CCDC16/ZNF830 (a component of spliceosome complex B, Table S1 in Supplementary Material), ISY1 and the NTC-associated PP1e peptidyl-prolyl cis–trans isomerase (Kuraoka et al., 2008). Differences between subunit composition of the Ps04 and XAB2 complexes is probably explained by the observation that in response to DNA damage PRP19 undergoes self-ubiquitination, which stimulates the release of CDC5 and PRL1 (Lu and Legerski, 2007). Whether this event leads to switching the role of NTC between splicing and DNA repair remains however to be determined.

Mutations inactivating essential components of the DNA repair pathways lead to prolonged presence of the ATR checkpoint kinase at the damaged DNA sites. Chronic activation of ATR, as well as inhibition of transcription elongation, causes a dramatic increase in phosphorylated forms of p53 and histone H2AX, and ubiquitination of H2A (Lagerwerf et al., 2011). Although not detected in either Ps04 or XAB2 complex, the NTC subunit Syf2 is found in association with the MCM3 and PCNA DNA replication factors and is required for activation of ATR (Chu et al., 2006). The yeast NTC subunit Gfl1/CRN1 is similarly found in a complex with the DNA replication factor Orc2 and its mutation results in delayed entry into the S-phase, indicating a direct role in DNA replication (Zhu et al., 2002). Human CDC5 interacts with and phosphorylated by ATR, and is required for the activation of components of downstream S-phase checkpoint pathway blocking cell cycle progression (Zhang et al., 2009). Knockouts of mouse and zebrafish PRLG1/PRL1 result in nuclear to cytoplasmic translocation of CDC5, stimulation of p53 and histone H2AX phosphorylation, and induction of apoptosis (Kleinridders et al., 2009). This indicates that, despite their lack in the XAB2 complex, both PRL1
and CDC5 are essential for activation of the DNA repair checkpoint and their absence results in stimulation of default cell death pathway in mammals.

The human NTC subunit BCAS2/SFP27 (MOS4) directly interacts with and inhibits the activity of p53. As the prl1 mutation, inactivation of BCAS2 stimulates nuclear retention and phosphorylation of p53 leading to apoptosis, while in p53 mutant cells permits normal G2/M cell cycle arrest (Kuo et al., 2009). Another NTC subunit, SKIP/Prp45 is specifically required for proper splicing of the cell cycle arrest factor p21Cip1. Thus, inactivation of SKIP leads to deregulation of the cell cycle and activation of p53 even in the absence of DNA damage (Chen et al., 2011). Remarkably, leads to deregulation of the cell cycle and activation of p53 even in the absence of DNA damage (Chen et al., 2011). Remarkably, PRP19/SNEV/PSO4 overexpression confers tolerance to reactive oxygen species and DNA damaging agents prolonging the life span of human cells (Voglauer et al., 2006). Whether this overexpression effect is due to accumulation of free PRP19, which is not incorporated into NTC, remains a question to answer. In any case, in Arabidopsis that lacks a functional p53 homolog the effects of NTC mutations are just opposite compared to yeast and mammals, as instead of stimulating apoptosis they function as suppressors of induced cell death. It is thus important to clarify how these mutations affect the DNA repair pathways and how do they modulate splicing of downstream effectors of signaling pathways that are activated by the srrl and flu mutations of pathogen and oxidative stress response pathways.

**ROLE OF NTC IN CO-TRANSCRIPTIONAL SPLICEOSOME ASSEMBLY**

While splicing defects caused by the Arabidopsis NTC mutations are poorly studied so far, it was observed early on that the Arabidopsis prl1 mutation results in both up and down-regulation of transcription of many stress regulated genes, which is also observable in nuclear run-on transcription assays (Németh et al., 1998). The conclusion that NTC plays a direct role in the regulation of RNAPII transcription and simultaneous co-transcriptional spliceosome assembly is supported now by an overwhelming amount of data (see for reviews e.g., Perales and Bentley, 2009; Muñoz et al., 2010; Oesterreich et al., 2011).

From the core NTC subunits, BCAS2/Snt309, and SKIP/Prp45 are directly recruited to promoters of RNAPII transcribed genes. BCAS2/Snt309 is a transcription co-activator of several nuclear receptors and a binding partner of multiple histone deacetylases and nuclear co-repressors. SKIP plays a pivotal role in the regulation of transcription in response to Notch and Wnt/β-catenin signaling, and acts as an inhibitor of NADH+ -dependent SIRT deacetylase by controlling senescence and aging in conjunction with the retinoic acid receptor (Zhou and Hayward, 2001; Kang et al., 2010; Wang et al., 2010).

Other NTC components are recruited to RNAPII by their contacts with initiation and elongation factors, and spliceosome components. The carboxy-terminal Y1S2P3T4S5S6P7 heptapeptide repeat domain of RNAPII largest subunit (RNAPII CTD) serves as platform for binding and assembly of these regulatory factors (Buratowski, 2009). During transcription initiation, the RNAPII CTD undergoes S5/S7 phosphorylation by the TFIIH-associated Kin28/CDK7 kinases (Akhtar et al., 2009). Phosphorylation of the S5 CTD-residue mediates the substrate of the capping enzyme, which interacts with the NTC component Isy1 (Hausmann et al., 2008). Subsequent formation of the cap-binding complex CBC and interaction of the U1 snRNP subunit Prp40 with the phosphorylated CTD mediates RNAPII loading of the U1 snRNP through the CBC-binding of U1 subunit Luc7. At the same time, PRP40 recruits the NTC subunit Syf3/Cli1 (Fortes et al., 1999). Arabidopsis PRP40 homologs show similar recognition of RNAPII CTD (Kang et al., 2009) and mutations of the CBC subunits result in the accumulation of retained introns in pre-mRNAs (Laubinger et al., 2008). As U1 snRNP interaction can also be observed with the RNAPII on intronless pre-mRNAs, the U1 snRNP-5′SS commitment complex is only stabilized when the branch site is bound by the U2AF complex. Interaction of the NTC subunit Syf3/Cli1 with U2AF65 and recognition of BP-bound factor SF1 by Prp40 forms a platform for further loading of the U2 snRNP (Chung et al., 2011). In addition, U2AF65 and its interacting SR protein partner SC35 directly bind to RNAPII CTD, and thus play a role in co-transcriptional assembly of the spliceosome (Spiluttini et al., 2010).

Recently, U2AF65 was reported to co-purify with the NTC components PRP19, CDC5, PRL1, and SPF27 and shown to facilitate RNAPII CTD-dependent NTC-mediated activation of splicing (David et al., 2011). The U2 subunit SF3b130 and Cul4–DDB1 are associated with GCCN5/p300 histone acetylases of yeast and human SAGA/STAGA RNAPII co-activator complexes. Similarly to activation of NTC-modulated DNA repair, histone acetylation is essential for stabilization of the U2 snRNP in the prespliceosome (Martínez et al., 2001; Gunderson and Johnson, 2009).

Phosphoserine-5 mark of the RNAPII CTD also provides a signal for recruitment of SET1-type histone methyltransferases (Buratowski, 2009). H3K4-trimethyl histone marks deposited by Set1 are recognized by the human SAGA-associated chromodomain protein CHD1, which specifically interacts with the SF3a subcomplex of U2 snRNP (Sims et al., 2007). The U2 snRNP SF3b complex recruits the NTC-associated factor Bud31 and the Ist3 subunit of mRNA retention RES complex, which remain then associated with the spliceosome (Wang et al., 2005). The U2- associated human factor tat-SF1 together with the cap-binding complex plays a role in recruitment of the CDK9/P-TEFb kinase, which stimulates transcription elongation by phosphorylating the S2 residues of RNAPII CTD.

P-TEFb directly interacts with human SKIP and Menin H3K4 methyltransferase (Brés et al., 2009), as well as with exonic splicing enhancer- and silencer-binding SR-proteins, such as SF2/ASF and SRp20. In complex with the HP1 adaptor protein, SRp20 is involved in the recognition of histone H3K9 trimethylation and regulation of alternative splicing (for review see Lenasi and Barbörö, 2010). In addition, P-TEFb-mediated phosphorylation of RNAPII CTD S2 residue results in the recruitment of Set2-type histone methyltransferases that deposit H3K36-trimethyl histone marks preferentially on exon sequences. The H3K36me3 chromatin mark is recognized by the adaptor protein MRG15, which in complex with the PPT-binding protein PTB acts as important regulator of alternative splicing (see for review Luco et al., 2011). Interaction of NTC with RNAPII also critically affects transcriptional elongation by recruiting components of the THO/TREX
complex required for the formation and nuclear export of messenger ribonucleoproteins (mRNPs, Chanarat et al., 2011).

CONCLUSION AND PERSPECTIVES
In this review, we took a glimpse at molecular mechanisms underlying the emerging central regulatory functions of NTC in coordination of spliceosome assembly/activation with transcription, DNA repair/rePLICATION, and stress responses related to aging and cell death. In particular, we wished to highlight recent advances in understanding the regulatory functions of Arabidopsis NTC and spliceosome components embedded in a frame of current knowledge derived from similar yeast, Drosophila, and human studies. In the absence of corresponding Arabidopsis data on NTC connections to the regulation of mitotic and meiotic recombination, chromosome segregation, chromatin remodeling, temperature sensing, Polycomb and small RNA-mediated gene silencing, nuclear mRNA export, nonsense mediated decay, and several other essential processes, these subjects remained to be covered by a next timely overview. Compilation of available data on yeast, Drosophila, and human NTC and spliceosome-associated proteins and their conserved plant homologs in the Supplement intends to assist further research to fill this gap.

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SUPPLEMENTARY MATERIAL
The Supplementary Material for this article can be found online at http://www.frontiersin.org/Plant_Physiology/10.3389/fpls.2012.0009/abstract

Table S1 | Compilation of mass spectrometry data on protein composition of purified yeast, Drosophila, human, and Arabidopsis spliceosome and NTC complexes. Phosphorylated human proteins reported by Agafonov et al. (2011) are highlighted in red. Arabidopsis factors with indentified regulatory functions are indicated in bold. Core subunits of yeast, human, and Arabidopsis NTC complexes are shaded in gray. References describing the purification of various spliceosomial and NTC complexes are given at the end of the Table corresponding to numbers in bracket indicated in the heading. Brackets within the table mark unique fission yeast factors, which have either Drosophila or human or Arabidopsis homologs. For each factor, the corresponding NCBI gene accession number is depicted and in all cases frequently used alternative designations are listed.
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