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Opposed Reviewers:

Functional coupling of transcription and splicing

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Abstract

The tightly regulated process of precursor messenger RNA (pre-mRNA) alternative splicing is a key mechanism to increase the number and complexity of proteins encoded by the genome. Evidence gathered in recent years has established that transcription and splicing are physically and functionally coupled and that this coupling may be an essential aspect of the regulation of splicing and alternative splicing. Recent advances in our understanding of transcription and of splicing regulation have uncovered the multiple interactions between components from both types of machinery. These interactions help to explain the functional coupling of RNAPII transcription and pre-mRNA alternative splicing for efficient and regulated gene expression at the molecular level. Recent technological advances, in addition to novel cell and molecular biology approaches, have led to the development of new tools for addressing mechanistic questions to achieve an integrated and global understanding of the functional coupling of RNAPII transcription and pre-mRNA alternative splicing. Here, we review major milestones and insights into RNA polymerase II transcription and pre-mRNA alternative splicing as well as new concepts and challenges that have arisen from multiple genome-wide approaches and analyses at the single-cell resolution.

Introduction

The expression of protein-coding genes in eukaryotes is a complex process that involves numerous tightly regulated steps. The genomic coding DNA sequence must be transcribed into a precursor messenger RNA (pre-mRNA) by the nuclear RNA polymerase II (RNAPII). The pre-mRNA must then undergo a number of processing steps to yield a mature, functional messenger RNA (mRNA), which is then ready to be exported from the nucleus and used by the translational machinery of the cell. Thus, RNA transcripts undergo extensive maturation in the nuclei of eukaryotic cells. The complexity of the processing that occurs during the formation of a mature mRNA provides a basis for the multilayered regulation of gene expression. These regulatory networks correlate with the organizational complexity of a given organism and with the specialization of tissues such as the components of the central nervous system and the cardiac muscle (Maniatis and Tasic, 2002; Mendes Soares and Valcarcel, 2006; Taliaferro et al., 2011).

In higher eukaryotes, most protein-coding genes contain long non-coding stretches or intervening sequences (introns) that are transcribed in the pre-mRNA and must therefore be removed in a process called splicing, which leaves the resulting protein coding sequences (exons) appropriately aligned and ligated in the mRNA. The last decade has revealed the massive complexity and fundamental importance of this phenomenon in the regulation of gene expression. Splicing is a complex two-step trans-esterification reaction that is driven by the spliceosome, which is a large ribonucleoprotein complex of five uridine-rich small nuclear RNAs (snRNAs) and approximately 200 additional proteins. Alternative splicing, the phenomenon by which a single pre-mRNA can be potentially spliced in multiple different patterns to yield two or more different mRNA products, contributes significantly to the diversity of cell and

tissue-specific protein expression profiles (Maniatis and Tasic, 2002). The results of deep sequencing-based expression analyses suggest that more than 90% of multi-exon human genes undergo alternative splicing (Pan et al., 2008; Wang et al., 2008). Changes in the *cis*- or *trans*- regulation of this process can cause multiple pathologies as a result of general or specific aberrant pre-mRNA processing underscoring the fundamental importance of this regulatory process. Several reviews covering dysfunctions related to mRNA processing have been published elsewhere (Caceres and Kornblihtt, 2002; Garcia-Blanco et al., 2004; Licatalosi and Darnell, 2006; Wang and Cooper, 2007). Although our understanding of how alternative splicing is controlled in the context of global gene expression regulation and its relevance in specific metabolic processes has greatly advanced during the last decade, the *functional* integration of pre-mRNA processing with other nuclear machineries is not yet well understood.

It is widely accepted that transcription and splicing are physically and functionally coupled and that this coupling might be an important aspect of the regulation of splicing and alternative splicing. This coupling implies that splicing occurs co-transcriptionally (i.e., during transcription). However, this process can also take place post-transcriptionally (i.e., after transcription). Ten years have passed since the co-transcriptional splicing of pre-mRNAs, with a particular emphasis on the connections between transcriptional elongation and splicing, was last thoroughly discussed in this forum (Goldstrohm et al., 2001b). Since then, excellent reviews covering specific aspects of the coupling of transcription and alternative splicing have been published (for recent reviews see (Moore and Proudfoot, 2009; Munoz et al., 2010; Oesterreich et al., 2011)). In this review we aim to revisit current concepts and experimental observations, discuss the recent advances in the field, and highlight the major unanswered questions

regarding the functional coupling of RNAPII transcription and pre-mRNA alternative splicing.

The regulation of alternative splicing: general arguments for functional coupling

The predominant form of the spliceosome (the U2-spliceosome or "major spliceosome") is composed of a core of five snRNPs, termed U1, U2, U4, U5, and U6, and an additional estimated number of ~ 200 protein factors (Kramer, 1996; Wahl et al., 2009). Numerous *in vitro* and *in vivo* studies support the model that spliceosome assembly occurs in an ordered step-wise manner (Will and Luhrmann, 2001; Gornemann et al., 2005; Matlin and Moore, 2007; Wahl et al., 2009; Huranova et al., 2010; Hoskins et al., 2011). This unidirectional multistep assembly initially reflects the orderly recognition of the primary *cis* sequence elements that define the exon/intron boundaries (the 5' and 3' splice sites) and the associated 3' sequences for intron excision (the polypyrimidine (Py) tract, and the branch point sequence (BPS)). The first stable complex formed during the assembly of the spliceosome is the early spliceosome complex (E), also known as the commitment complex. In this step, the 5' splice site of the intron is bound by the U1snRNP, and the splicing factors SF1 and U2AF cooperatively recognize the BPS, the Py, and the 3' splice site (Berglund et al., 1997; Berglund et al., 1998). These two factors promote the recruitment of the U2snRNP to the branch point in an ATP-dependent manner to form complex A (MacMillan et al., 1994). Complex B is formed by the subsequent binding of the U4/U6-U5 tri-snRNP. After RNA-RNA and RNA-protein rearrangements at the heart of the spliceosome, U1 and U4 are released. The release of U1 and U4 produces the C (or catalytic) complex that is ultimately responsible for catalyzing the intron excision and exon-exon ligation reactions (Fig. 1A).

Alternative exons, as opposed to constitutive exons, can be alternatively spliced to give rise to two or more mRNAs from a single pre-mRNA. Interestingly, an essential property of the genome sequence of eukaryotes (especially in higher eukaryotes) is the suboptimal arrangement and poor conservation of the core *cis*-elements that drive the recruitment of the spliceosome (Izquierdo and Valcarcel, 2006; Mendes Soares and Valcarcel, 2006). This "loose" or suboptimal recognition of splicing signals in alternative exonic/intronic sequences is critical because it renders the sequences sensitive to the combinatorial regulation exerted by different elements, including transcription-coupled alternative splicing regulation. Additional *cis* elements, known as exonic and intronic splicing silencers or enhancers (ISS, ISE, ESS, and ESE), participate in the regulation of alternative splicing. These elements are most often recognized and acted on by specific RNA-binding proteins that include heterogeneous nuclear ribonucleoproteins (hnRNPs) and serine/arginine-rich (SR) proteins (Wu and Maniatis, 1993) (Fig. 1B). These positive and negative regulators together with an ever-increasing number of additional auxiliary regulators, act on a given alternatively processed pre-mRNA sequence as defined by the "splicing code". This "splicing code" is still incompletely characterized, and it is highly contextual in nature, varying among different tissues and distinct homeostatic environments (Zhang et al.; Lin and Fu, 2007; Barash et al., 2010).

Initial work supporting the existence of co-transcriptional splicing comes from direct observation. Electron microscopy has shown that intron lariat formation and/or the removal of intronic sequences can occur in nascent transcripts that are still tethered to their DNA templates (Beyer and Osheim, 1988; Osheim et al., 1988). Several high-resolution light microscopy studies using RNA-FISH probes to specifically discriminate between unprocessed and processed mRNA species revealed that in a significant

proportion of genes, either endogenous or transiently expressed synthetic reporter constructs, the processing of intronic sequences was performed at or very close to the active template gene (Johnson et al., 2000; Custodio et al., 2004). Analogous "direct" observation of co-transcriptional pre-mRNA processing has been achieved by comparing the patterns observed in nascent chromatin-associated RNA with those found after the release of the RNA into the nucleoplasm (Pandya-Jones and Black, 2009). Other more indirect approaches have also provided support for a model of co-transcriptional processing for the vast majority of intronic sequences. For example: (1) multiple components of the spliceosome physically interact with the transcription apparatus and components of the chromatin environment, including modified histones (see below); (2) ChIP analysis suggests a highly conserved stepwise assembly of spliceosomal subunits on the chromatin template (Listerman et al., 2006); (3) measurement of the rates of RNAPII transcription and pre-mRNA splicing *in vivo* on endogenous human genes show that splicing occurs long before the ends of the gens are transcribed (Singh and Padgett, 2009), and (4) co-transcriptional pre-mRNA processing can be reproduced *in vitro* in an RNAPII-specific manner (Natalizio and Garcia-Blanco, 2005) (Das et al., 2006; Yu et al., 2010). Consistent with these findings, total RNA sequencing reveals widespread co-transcriptional splicing in the human brain (Ameur et al., 2011). Similarly, measurements of complete and incomplete splicing rates through the analysis of genome-wide RNAseq datasets in various sub-cellular fractions is consistent with widespread co-transcriptional splicing in humans (H. Tilgner, R. Guigo, and the ENCODE CONSORTIUM, personal communication).

Based on these observations, it was initially thought that the physical coupling of transcription and downstream maturation events was a constitutive and intrinsic property of the machinery involved in these phenomena and that this coupling was

therefore not necessarily subject to regulation. Early models depicted the spliceosome as being optimally assembled when recruited co-transcriptionally to a ternary complex composed of the RNAPII holoenzyme, the chromatin template, and the nascent transcript. This view prompted pioneering *in vitro* studies demonstrating that indeed, RNAPII-associated co-transcriptional pre-mRNA constitutive splicing is significantly more efficient than the uncoupled processing of a purified, pre-transcribed template or the processing of a transcript that is being synthesized by an enzyme that cannot couple with the spliceosome, such as the T7 phage RNA polymerase (Natalizio and Garcia-Blanco, 2005; Hicks et al., 2006; Das et al., 2007). It is worth noting that these *in vitro* co-transcriptional models required an intact RNAPII CTD, but the addition of a complete CTD to the T7 polymerase did not confer enhanced co-transcriptional processing (Natalizio et al., 2009). However, we know that some pre-mRNA sequences are spliced after the release of the transcript depending on the position of the intron (Bauren and Wieslander, 1994; Pandya-Jones and Black, 2009). In some cases, splicing occurs significantly far away from the chromatin template (Johnson et al., 2000). Recently published work reporting the real-time visualization of intron removal in live mammalian cells has revealed that although constitutive introns are indeed co-transcriptionally removed, there are some particular cases where processing is delayed until after transcription has been completed (Vargas et al., 2011). This result most likely reflects some regulatory effects on gene expression programs. Thus, splicing may occur after transcription is completed and co-transcriptional splicing is not obligatory but is rather a general rule with exceptions.

Although it is generally accepted that the physical co-occurrence of transcription and splicing favors both processes, it is also likely that physical coupling is not absolutely required for either process. Rather, co-transcriptional processing is predominantly

regarded as a widespread property of a significant share of intronic sequences. Co-transcription allows the development of multiple mechanisms that exquisitely co-regulate pre-mRNA synthesis and alternative processing. We use the phrase "functional coupling" to refer to the mutual influence of transcription and pre-mRNA splicing on one another. The functional coupling of transcription and pre-mRNA processing appears to be a major element in the regulation of gene expression programs, driving complex cell functions such as tissue-specific differentiation and development (Barboric et al., 2009) and the integration of DNA damage signaling and apoptosis (see below). The immediate question is: which molecular mechanisms drive these functional coupling events? As we will see in the following sections, the functional coupling of transcription and alternative splicing is largely based on the shared properties of alternative splicing-regulatory sequences and their derived regulatory mechanisms. The available data show that transcription influences alternative splicing by recruiting positive and negative regulators to the nascent pre-mRNA ("physical coupling") or by presenting a specific sequence context to the spliceosome by altering the synthesis rate of the nascent transcript ("kinetic coupling"). These two pathways, which are proposed to explain the link between transcription and splicing, are distinct but not mutually exclusive models.

Physical coupling: interactions between the transcription and splicing machineries

One mechanism that may explain the functional coupling between transcription and splicing is the establishment of physical interactions among components of both machineries. Many examples of these interactions have been described throughout the literature (see below), and components of these macromolecular complexes have been purified using a variety of approaches. These interactions are also believed to underlie

regulatory mechanisms shared by both processes, such as post-translational modifications that transduce signaling cues or the reading of chromatin marks (Sims et al., 2007; Lenasi and Barboric, 2010).

It is believed that the carboxyl terminal domain (CTD) of the large subunit of RNAPII plays a major role in the co-transcriptional coupling of RNA biogenesis and processing. The CTD is comprised of 52 tandem repeats in mammals (26 in yeast) of the heptapeptide YSPTSPS, with a heterogeneous degree of degeneration. The CTD constitutes an extremely versatile and flexible platform for the recruitment of factors to the nascent transcript. This recruitment promotes the co-transcriptional accomplishment of gene expression-related functions, ranging from pre-mRNA capping, 3'-end processing and excision, pre-mRNA splicing, and chromatin remodeling (Phatnani and Greenleaf, 2006).

Specific residues within this repeat are targeted for post-translational modifications in a differential manner that depends on the stage of transcription. The modification of the CTD is believed to constitute an extremely versatile mechanism for encoding and transducing information about the functional status of the transcription complex to the different gene expression machineries. Dynamic changes in post-transcriptional modifications generate a "CTD code" (Fig. 2) that contributes to the recruitment and release of processing factors at specific stages of transcription (Komarnitsky, 2000; Buratowski, 2003; Egloff and Murphy, 2008). Hypophosphorylated RNAPII assembles into pre-initiation complexes positioned at gene promoters, whereas hyperphosphorylated RNAPII associates with the elongation stage. Cyclin-dependent kinase 7 (CDK7), which is part of the general transcription factor IIH (TFIIH), is responsible for the phosphorylation of Ser5. This modification has been linked with transcription initiation and is preferentially associated with the 5'-end of the genes. Ser2

phosphorylation by CDK9, the catalytic subunit of the positive transcription elongation factor (P-TEFb), is associated with the 3'-end of the genes. Classically, P-TEFb was thought to be the sole CTD kinase responsible for the conversion of RNAPII into its processive elongating form; however, recent studies have shown that *Drosophila* CDK12 and human CDK12 and CDK13 are also Ser2-CTD kinases (Bartkowiak et al., 2010). These results impact our understanding of CDK9/CTD phosphorylation and open the door to revisions of the current model on CTD phosphorylation and transcriptional elongation. Other residues within the consensus heptapeptide are substrates for phosphorylation, including threonine, tyrosine, and the less conserved Ser7 (Zhang and Corden, 1991; Baskaran et al., 1993; Zhou et al., 2009; Hsin et al., 2011). Recent genome-wide analysis in yeast detected Ser7 phosphorylation at the 5'-ends of genes and in introns, suggesting that Ser7 marks could facilitate elongation and splicing (Kim et al., 2010; Tietjen et al., 2010). Glycosylation, methylation, ubiquitylation and proline isomerization of residues within the CTD have also been reported (Kelly et al., 1993; Morris et al., 1999; Li et al., 2007b; Sims et al., 2011).

Evidence that the CTD is required for splicing has been observed both *in vivo* and *in vitro*. Truncation of the CTD reduces splicing efficiency *in vivo* (McCracken et al., 1997; Fong et al., 2003; Rosonina and Blencowe, 2004). The transcriptional activation of RNAPII-dependent genes induces the spatial recruitment of splicing factors to the transcription site, and this reaction is dependent on the integrity of the CTD (Du, 1997; Misteli, 1999). *In vitro* assays have shown that purified hyperphosphorylated RNAPII is able to activate pre-mRNA splicing, but hypophosphorylated RNAPII can inhibit splicing (Hirose, 1999). Finally, recombinant CTD can also stimulate the splicing reaction *in vitro*. However, this finding is only valid in the context of exon definition, when both the 3' and 5' splice sites are accessible (Zeng and Berget, 2000).

Despite the significant efforts focused on the characterization of the CTD, the specific role of the CTD in the regulation of splicing is not completely understood. The role of the CTD is likely to be variable and context-specific, and probably depends on the subset of effectors and/or regulators recruited in each condition. In some cases, RNAPII may not be sufficient for splicing factor accumulation and instead a more complex and dynamic mechanism for recruitment that involve direct interactions with the nascent RNAs may occur (Kotovic et al., 2003; Gornemann et al., 2005; Lacadie and Rosbash, 2005; Listerman et al., 2006; Das et al., 2007). It has been surmised that "scaffolding", adaptor or bifunctional proteins may regulate the functional coupling of transcription and splicing in a manner similar to the specification of signaling pathways in other cell systems. Indeed, some nuclear factors, also known as "cross-talk" factors or "coupling mediators" interact with both the transcription and splicing machineries. These nuclear factors have therefore been proposed to regulate the physical coupling of the two processes. Although their differential features are significant, these nuclear factors all share the following properties: first, they are capable of physically interacting with components of both the RNAPII transcription apparatus and the spliceosome, mostly through the CTD of RNAPII. In fact, the specific recognition of hyperphosphorylated forms of the CTD is currently regarded as a significant feature supporting the putative role of a given factor on co-transcriptional pre-mRNA processing. Second, these forms contain multiple protein-protein interaction domains or motifs, which confer the potential ability to act as scaffolding elements. Third, these factors are capable, at least under specific experimental conditions, of modulating transcription, splicing, or both (Kornblihtt et al., 2004; Allemand et al., 2008).

Transcription-splicing coupling factors

The family of serine/arginine rich-proteins (SR proteins) includes several conserved RNA-binding factors that have been primarily characterized as splicing regulators, although they seem to play a role in several other mRNA metabolism processes such as nucleocytoplasmic export and translation. The activity of SR proteins is tightly regulated through several mechanisms, including tissue-specific differential expression, signal-dependent phosphorylation, subnuclear distribution and conditional competition with other factors (for an extensive discussion of different aspects of these proteins, please refer to (Lin and Fu, 2007)). Physical interactions between SR proteins and the CTD of RNAPII have been reported, but the functional relevance of this association is not yet known (Yuryev et al., 1996; Bourquin et al., 1997). Indeed, this association was proposed to be required for the appropriate regulation of the recruitment of SR proteins from specific subnuclear structures called nuclear speckles to transcriptionally active sites (Misteli, 1999). SR proteins have been found to co-purify with the RNAPII holoenzyme (Das et al., 2007). Moreover, SR proteins partially mediate the enhancement of *in vitro* co-transcriptional pre-mRNA processing presumably by facilitating the recruitment and assembly of the early spliceosome on nascent RNAPII transcripts (Das et al., 2006 and 2007). The elongation-dependent influence of some of these factors, including SRp20 and SRSF1 (formerly called SF2/ASF), on specific alternative splicing minigene models has been shown to be dependent on the integrity of the RNAPII CTD as well as its phosphorylation state (de la Mata and Kornblihtt, 2006; Barboric et al., 2009). In cells, the depletion of the essential splicing factor SC35 led to a diminished recruitment of P-TEFb to the RNAPII complex and, consequently, a lower CTD phosphorylation level and increased accumulation of the RNAPII large subunit on the 5' ends of genes, both of which are hallmarks of impaired elongation (Lin et al., 2008). The specific mechanisms by which SR proteins establish the functional coupling

between transcription and alternative splicing are not yet completely understood. However, interactions involving both the co-transcriptional assembly of pre-spliceosomal complexes and the regulation of the progression towards an elongation-competent state by the RNAPII complex are likely to be important in this regulation.

Other splicing factors participate in the functional coupling of transcriptional elongation and alternative splicing. The splicing factor SKIP interacts with PTEF-b and positively influences transcription elongation by the HIV-1 Tat activator (Bres et al., 2005). Tat-SF1, the mammalian homolog of the yeast U2 snRNP component *CUS1*, has also been proposed to mediate the splicing-dependent regulation of transcription elongation *in vitro* (Fong and Zhou, 2001; Maniatis and Reed, 2002).

An interesting group of factors has been shown to play dual roles both in transcription and splicing regulation and/or to physically interact with components of both the splicing and transcription machinery. These factors include, among others, PSF, p54nrb/NonO, Prp40, FBP11, and TCERG1. Some of these factors contain tandem repeats of WW (Chan, 1996) and FF (Bedford and Leder, 1999) domains; this feature might define a subset of potential coupling factors.

PSF and $p54^{nrb}$/NonO are two RRM domain-containing proteins that are ubiquitously expressed in human tissues. These proteins share 71% identity over a 320-amino-acid region encompassing their RNA recognition motif (RRM) (Dong et al., 1993). Intriguingly, these proteins have been found to interact with each other through their RRM domains, although the functional consequences of this interaction are not known (Peng et al., 2002). PSF and $p54^{nrb}$/NonO interact with either hypophosphorylated or hyperphosphorylated forms of the RNAPII CTD (Emili et al., 2002). It has been shown that $p54^{nrb}$/NonO associates with 5' splice sites in high molecular weight complexes containing the U1 and U2 snRNPs and mostly (but not exclusively) phosphorylated

forms of RNAPII, both *in vitro* and *in vivo* (Kameoka, 2004). Similarly, PSF can be found in higher-order complexes assembled *in vitro* that contain all of the spliceosomal subunits (Peng et al., 2006). The fact that PSF was found to interact with the hypophosphorylated forms of the RNAPII and spliceosomal components involved in the 5' splice site is particularly interesting given that hypophosphorylated RNAPII is detected in the paraspeckle nuclear compartment. Moreover, PSF is enriched at paraspeckles, and mass spectrometry analysis of hypophosphorylated RNAPII complexes isolated both U1 snRNP proteins and SR proteins (Fox et al., 2005; Das et al., 2007; Fox and Lamond, 2010). Based on these data, it is tempting to speculate that PSF may prime interactions between the transcription initiation complex and the early spliceosome. Furthermore, the paraspeckle region might be the location at which these events take place. Consistent with these observations, *in vivo* experiments suggest that the PSF is capable of mediating transcription activator-dependent stimulation of pre-mRNA processing through its binding to the CTD of RNAPII (Rosonina et al., 2005).

The yeast splicing factor Prp40 contains two WW domains followed by four FF domain repeats, and it was first characterized as a splicing regulator. Prp40 is an essential U1 snRNP-associated protein that participates as a scaffold in the earlier steps of spliceosome complex formation (Kao and Siliciano, 1996). The WW domains of Prp40 recognize the consensus sequence PPXY in BBP, which is the yeast ortholog of the splicing factor SF1. BBP interacts directly with Mud2p, which is the yeast homolog of human U2AF^{65}, which binds to the polypyrimidine region at the 3'-end of introns. Based on these interactions, it has been proposed that Prp40 helps to define a bridging interaction that links both ends of the intron (Abovich and Rosbash, 1997). Prp40 also binds to the yeast U5-associated protein Prp8. Therefore, Prp40 might contribute to the recruitment of the U5 snRNP to the early spliceosome during cross-intron bridging.

According to the currently prevailing sequential model, this interaction may destabilize U1 snRNP binding, allowing for the progression towards a mature spliceosome (Abovich and Rosbash, 1997). Intriguingly, a recent study demonstrated that, in the absence of the WW domains of Prp40, no defects in *in vivo* splicing, *in vivo* U1 or U2 snRNP recruitment, or *in vitro* early splicing complex assembly were detected (Gornemann et al., 2011). Moreover, the Prp40 WW domains were dispensable for yeast viability. Delays in U5snRNP recruitment and altered spliceosome formation *in vitro* did not prevent spliceosome maturation or changes in RNAPII distribution along the transcripts. One plausible explanation that may reconcile these apparently opposing observations is that the Prp40 WW domains are required to act as a scaffold for early spliceosome assembly in only a subset of genes or in specific functional contexts. Finally, Prp40 was the first splicing factor that was shown to bind the phosphorylated CTD of RNAPII via its WW domains (Morris and Greenleaf, 2000).

There are two putative mammalian orthologs of Prp40, FBP11 and HYPC. FBP11, also known as HYPA or Prpf40A, comprises two WW and six FF domains. Similar to yeast Prp40, FBP11 interacts with SF1 and $U2AF^{65}$, which suggests that it may play a role in the recognition of the 3'-end of introns (Lin et al., 2004), However, unlike Prp40, FBP11 has not been strictly characterized as a component of the U1snRNP. Structural characterization of the binding mechanism and involved surfaces in the FF domain-containing region of FBP11 revealed that FBP11 is able to recognize phosphorylated sequences such as the CTD of RNAPII (Allen et al., 2002). Intriguingly, this interaction is preferentially established by the FF1 repeat, whereas Prp40 interacts weakly with the phosphorylated CTD through its FF2 repeat (Gasch et al., 2006). This observation illustrates that this family of factors displays significant differences regarding the

binding specificity of their WW and FF domains despite their apparent structural conservation.

HYPC, also known as Prpf40B, is the least well characterized of the putative mammalian Prp40 orthologs. It has an analogous modular structure of two WW domains and four FF domains. HYPC, as well as FBP11, has been implicated in the pathogenesis of neurological disorders, including Huntington's disease and Rett syndrome (Faber et al., 1998; Passani et al., 2000; Buschdorf and Stratling, 2004). High-resolution confocal microscopy analysis revealed that HYPC shows a precise overlap with SC35 in the splicing factor-rich nuclear speckles (S. Becerra and C. Suñé, unpublished data), which suggests that HYPC is involved in pre-mRNA processing.

FBP21 is the only member of the family of potential coupling factors that lacks FF domain repeats. However, similarities between its WW domains and the interdomain spacer length and those of the above-mentioned Prp40 paralogs indicate that FBP21 might play a conserved role in splicing as well. This theory is supported by data showing that FBP21 interacts with the U1 snRNP protein U1C, the core snRNP proteins SmB and SmB', and the branchpoint binding protein SF1, suggesting that FBP21 may play a role in the cross-intron bridging of U1 and U2 snRNPs in the mammalian A complex (Bedford et al., 1998). Importantly, the WW domains of FBP21 are critical for its splicing activator function and for its interaction with SIPP1 (Huang et al., 2009), a splicing factor that associates with polyglutamine-tract-binding protein (PQBP1) and protein phosphatase 1 (PP1).

TCERG1, also known as CA150, is a nuclear protein that has been implicated in transcriptional elongation and pre-mRNA splicing. TCERG1 contains three WW domains in its amino-terminal half and six FF repeat domains in its carboxyl-terminal half (Suñé et al., 1997) and associates with hyperphosphorylated RNAPII and with

transcriptional elongation and splicing components through both its WW and FF domains (Goldstrohm et al., 2001a; Lin, 2004; Smith et al., 2004; Sanchez-Alvarez et al., 2006). TCERG1 has been identified in highly purified spliceosome fractions (Neubauer et al., 1998; Makarov et al., 2002; Rappsilber et al., 2002; Deckert et al., 2006). TCERG1 localizes at the interface of nuclear speckles and what are presumably nearby transcription sites (Sanchez-Alvarez et al., 2006). Several studies have demonstrated that TCERG1 can affect the splicing of several splicing reporters (Carty et al., 2000; Lin, 2004; Cheng et al., 2007; Pearson et al., 2008; Sánchez-Álvarez et al., 2010) and of putative cellular targets identified by microarray analysis following TCERG1 knockdown (Pearson et al., 2008). Given these data, TCERG1 has been observed as a possible factor in coordinating transcriptional elongation and splicing, a suggestion that was in fact proposed in the first Gene review on co-transcriptional splicing (Goldstrohm et al., 2001b) (see below). Like other members of this family of proteins, TCERG1 has been implicated in the pathogenesis of neurodegenerative diseases (Holbert, 2001; Arango et al., 2006; Andresen et al., 2007).

Kinetic coupling: how elongation rate affects splice site choice

RNAPII transcription initiation and pre-initiation complex assembly were initially described as the rate-limiting steps of transcriptional activation. However, transcriptional elongation is also a very highly regulated process. Numerous genes have been shown to be controlled at this stage, and a great variety of factors that affect this step of the transcription cycle have been identified (Sims et al., 2004). Increasing evidence shows that after promoter clearance, which depends on CTD phosphorylation, RNAPII stalls approximately 30-50 nucleotides downstream of the transcription start site. This promoter-proximal pause was first described for the *Drosophila* heat shock

genes and in the c-myc and HIV-1 genes (Bentley and Groudine, 1986; Kao et al., 1987; O'Brien and Lis, 1991; Krumm et al., 1992). More recent, genome-wide studies suggest that the promoter-proximal pause is a global tool for gene regulation (Core and Lis, 2008; Nechaev and Adelman, 2008). The promoter-proximal pause has been proposed as a checkpoint to ensure that only correctly assembled RNAPII complexes continue elongating through the gene (Levine, 2011). Given that different splicing sites of a transcript are synthesized and co-transcriptionally exposed to the splicing machinery, it is then plausible that the RNAPII elongation rate might modulate alternative splicing. This "kinetic" regulation of alternative splicing model proposes that the transcriptional elongation rate is critical for the outcome of competing splicing reactions that occur co-transcriptionally. Some authors have viewed this regulation as a 'window of opportunity' (Perales and Bentley, 2009), referring to the period during which the spliceosome is able to functionally assemble at one splice site before it is subject to competition from a downstream splice site.

The first evidence of this kinetic coupling came from a study by Eperon and colleagues, which showed that the rate of RNA synthesis could affect the secondary structure of the mRNA and, in turn, affect alternative splicing (Eperon et al., 1988). A similar mechanism of regulation was also suggested by experiments in which the insertion of an RNAPII pause site downstream of weak alternative splice sites caused a reduction in the amount of regulated exon skipping by delaying the synthesis of an inhibitory element (Roberts, 1998). These studies hinted that the rate of transcript synthesis itself could define the specific sequence context presented to the co-transcriptional processing machinery.

One of the most studied experimental systems that largely (but not exclusively) fits this kinetic model and can be used to illustrate this model is that of the fibronectin extra

domain I (EDI). This exon is preceded by a rather weak or degenerate 3' splice site, which is best utilized when the processivity of RNAPII is low and the splicing machinery has sufficient time to recognize it as a splice site. When RNAPII processivity is high due to elements such as transcriptional coactivators, open chromatin states or strong upstream promoters, it is more likely that the weak splice site will be presented simultaneously with the downstream, strong 3' splice site. The strong 3' splice site can then outcompete the weak splice site, favoring the skipping of the regulated exon (Cramer et al., 1999; Kadener et al., 2001; Kadener et al., 2002; Nogues et al., 2002). Direct evidence supporting the kinetic model was obtained by the exogenous expression of RNAPII mutant proteins that exhibit intrinsically low processivity. In these elegant, seminal experiments, the RNAPII-dependent transcription of the cell was driven exclusively by different α-amanitin-resistant polymerases; invariably, the polymerases with intrinsically low processivity elicited greater inclusion of the EDI exon (de la Mata et al., 2003). Interestingly, one mutation, termed *C4* (Coulter and Greenleaf, 1982), which provokes a decrease in the rate of the RNAPII processivity, was found to be associated with changes in the alternative splicing patterns of transcripts encoded by the *Drosophila ultrabithorax* gene, underscoring the potential physiological relevance of this phenomenon (de la Mata et al., 2003).

These observations may reflect a more general mechanism. Bentley and coworkers reported several years ago that RNAPII is paused at both 5' and 3' ends of genes and that this pausing correlates with the recruitment of processing factors (Glover-Cutter et al., 2008). More recent studies using high-resolution ChIP methods, demonstrate the preferential accumulation of RNAPII at specific regions within the body of genes (Alexander et al., 2010; Carrillo Oesterreich et al., 2010). Similarly, global analysis of nascent transcripts in yeast have shown a peak of RNAPII accumulation within terminal

exons, located ~ 250 nt upstream of the poly(A) site (Carrillo Oesterreich et al., 2010). Pausing near the 3' end of introns that correlates with the recruitment of splicing factors has also been described in intron-containing genes (Alexander et al., 2010). Thus, it is proposed that transcriptional pausing is imposed by a regulatory checkpoint that is associated with co-transcriptional splicing (Alexander et al., 2010). Therefore, proteins acting at the interface of these processes (the "coupling factors") would act as checkpoint factors to regulate co-transcriptional splicing. Strong data that may support this hypothesis have been reported in recent work showing that the putative coupling factor TCERG1 regulates the alternative splicing of the *Bcl-x* gene by modulating the rate of RNAPII transcriptional elongation (Montes et al., 2012). The authors of the aforementioned study speculate that TCERG1 modulates the elongation rate of RNAPII to relieve pausing, thereby acting as a checkpoint regulator to promote co-transcriptional splicing.

The kinetic coupling model could explain the accurate control of the alternative processing of very specific sequences through the tight control of RNAPII processivity at the boundaries of the region of interest. This scenario has been proposed for the alternatively spliced variable region of the CD44 gene, in which a subset of regulatory RNA-binding factors could participate in the local control of RNAPII through the chromatin remodeling factor SWI/SNF, which in turn determines the processing profile of the resulting transcripts (Batsche et al., 2006). SWI/SNF can also associate with nascent transcripts to regulate alternative pre-mRNA splicing (Tyagi et al., 2009). Generally, RNAPII pausing could be a way to ensure its optimal activity, especially when transcribing large, highly regulated genes (Gilchrist et al., 2010; Levine, 2011). Insufficient time spent at the pause site could therefore result in more error-prone transcription and the production of inadequately processed transcripts.

The influence of chromatin in the regulation of alternative splicing

The fundamental unit of chromatin is the nucleosome, which is composed of DNA wrapped around an octamer of the four core histones (H3, H4, H2A, and H2B) and separated from its neighboring nucleosomes by linker DNA (Izban and Luse, 1991). The reversible modification of histones and the positioning of nucleosomes along the chromatin template regulate transcription (Orphanides and Reinberg, 2000; Hodges et al., 2009). This finding was, to some degree, expected. However, the existence of connections between chromatin state and splicing was rather unexpected. A link between chromatin and splicing was first suggested when different RNA products were obtained when studying transcription from a collection of adenovirus-based templates, with identical trans-acting factors (Adami and Babiss, 1991). These authors hypothesized that changes in elongation rate, transcriptional pauses, or the transcriptional machinery could affect splicing patterns. At the same time, it was reported that the distances between the 5' and 3' splice sites followed a periodic pattern resembling nucleosome phasing, suggesting that nucleosomes are positioned according to exon/intron boundaries and thus reflect connections between chromatin and splicing (Beckmann and Trifonov, 1991). Accumulating evidence supports these hypotheses: cell replication alters alternative splicing by favoring compact chromatin and slowing RNAPII processivity (Kadener et al., 2001); the inhibition of topoisomerase-I by camptothecin promotes RNAPII stalling, which favors the co-transcriptional recruitment of splicing factors (Listerman et al., 2006); SW1/SNF chromatin remodeling factors have been shown to promote the inclusion of a cluster of exons in the endogenous CD44 gene (Batsche et al., 2006) and to regulate the alternative pre-mRNA processing of a specific subset of transcripts in *Drosophila* (Waldholm et al.,

2011); histone modifying enzymes interact with various components of the splicing machinery (Martinez et al., 2001; Ohkura et al., 2005; Gunderson and Johnson, 2009); and high-throughput sequencing-based techniques have revealed that nucleosome positioning and specific histone marks are significantly correlated with the intron/exon structure of genes, underlining the potential impact of chromatin structure on splicing (see below).

Several recent studies have revealed a specific, genome-scale nucleosome occupancy pattern in exon-intron regions. High-throughput immunoprecipitation and sequencing of nucleosome-associated chromatin have provided evidence that nucleosomes are predominantly enriched in exons (Andersson et al., 2009; Schwartz et al., 2009; Spies et al., 2009; Tilgner et al., 2009). The preferential occupancy of nucleosomes on exons is independent of transcription because it is also observed in non-expressed genes (Tilgner et al., 2009). Exons have higher GC content compared to introns, and GC-enriched regions have been suggested to be preferred for the assembly of nucleosomes (Peckham et al., 2007). Therefore, some of these studies suggested a correlation between GC content and nucleosome occupancy. However, GC-richness is likely not the only factor that determines nucleosome patterning. Data obtained from bioinformatics studies strongly suggest a functional link between nucleosome occupancy and splicing. Notably, the average length of the DNA wrapped around a histone octamer is 147 nucleotides in length, which is approximately the length of an average mammalian internal exon, suggesting a conserved role for the nucleosome in the exon definition process (Schwartz et al., 2009; Tilgner et al., 2009). Moreover, nucleosome density in exons correlates with splice site strength and the properties of flanking introns. The correlation is stronger in weak exons as well as in exons that are surrounded by long introns (Spies et al., 2009; Tilgner et al., 2009). Intriguingly, alternatively spliced exons

are more enriched in nucleosomes than constitutively spliced exons (Schwartz et al., 2009), and longer exons are more enriched in nucleosomes than shorter exons (Schwartz et al., 2009; Spies et al., 2009; Tilgner et al., 2009). These observations might suggest that exonic nucleosomes do not act as a mark for spliceosome recognition. Despite the discrepancies, these data strongly support a role for nucleosome patterning in the regulation of RNA splicing. In the absence of further experimental evidence, two non-mutually exclusive hypotheses have been proposed. First, nucleosomes may act as "speed-bumps" for RNAPII, which would affect transcription rates and in turn modulate alternative splicing through the kinetic model discussed above (Schwartz et al., 2009; Spies et al., 2009). Secondly, nucleosomes could interact with splicing factors during transcription or facilitate interactions between splicing factors during exon definition and other splicing assembly steps (Tilgner et al., 2009). Additional experiments are required to elucidate the mechanisms by which nucleosomes affect splicing.

Histones are substrates for numerous post-translational modifications, and their N-terminal tails are subjected to acetylation, methylation, phosphorylation, ubiquitylation or sumoylation (Kouzarides, 2007). Most of these modifications are reversible, and the enzymes responsible of their removal have been characterized extensively (Bannister et al., 2002; Goto et al., 2002; Sugiyama et al., 2002; Yang and Seto, 2007). The combinatorial and dynamic nature of the post-translational modifications of histones enables them to be an extremely versatile and complex combinatorial system. The "histone code" is capable of encoding vast amounts of information, which regulates the expression of the underlying DNA sequences, by affecting the recruitment, assembly and function of multiple nuclear complexes.

Histone methylation, acetylation and phosphorylation have recently emerged as major splicing regulators. For example, treatment with deacetylase inhibitors has been shown

to affect the alternative splicing of several reporter and endogenous genes (Nogues et al., 2002; Hnilicova et al., 2011), and histone acetylation is important for co-transcriptional spliceosome assembly (Gunderson and Johnson, 2009); histone H3 phosphorylated on serine 10 releases SR proteins from chromatin during the cell cycle (Loomis et al., 2009); and histone H3 lysine 4 tri-methylation (H3K4me3) enhances the recruitment of spliceosomal components to gene units (Sims et al., 2007). These data support a model in which histone modifications actively participate in the coupling of transcription and splicing. However, further study is required to elucidate their functional impact in the cell and the extent to which these potential mechanisms function throughout the genome.

Genome-wide analyses revealed specific histone modification patterns within exons and introns. One early study revealed that histone H3 lysine 36 tri-methylation (H3K36me3) was globally enriched in exons (Kolasinska-Zwierz et al., 2009). Parallel studies have identified additional chromatin modifications in exons and introns (Andersson et al., 2009; Schwartz et al., 2009; Spies et al., 2009; Tilgner et al., 2009; Dhami et al., 2010; Huff et al., 2010; Luco et al., 2010). It remains controversial whether these marks reflect nucleosome occupancy within exons (Schwartz et al., 2009; Tilgner et al., 2009) or constitute independent, relevant patterns with an intrinsic functional significance (Kolasinska-Zwierz et al., 2009; Spies et al., 2009). In support of the latter hypothesis, the average enrichment landscape for the H3K36me3 mark is actually contradictory to that of nucleosome occupancy because H3K36me3 is more highly enriched in constitutive exons than in alternative exons. Importantly, the relative enrichment of a number of histone marks differs between intronic and exonic sequences. The enrichment of some of these modifications depends on the relative positioning of the exon within the gene. For example, histone H4 lysine 20 methylation (H4K20me1) and

histone H2B lysine 5 methylation (H2BK5me1) are found preferentially at exons located at the 5' end of genes. In contrast, H3K36me3 tends to accumulate at the 3' end of the gene (Hon et al., 2009). Other marks exhibit a relative enrichment throughout the body of a gene reflective of its activation state, such as the histone H3 lysine 27 tri-methylation (H3K27me3), which is highly enriched at the exons of weakly expressed genes and at the introns of highly expressed genes (Dhami et al., 2010). These observations suggest that histone modifications play an active role in the regulation of alternative splicing because they constitute potentially informative hallmarks of the identity of an exon or an intron within a specific activation context (Fig. 3).

How can chromatin labels modulate co-transcriptional alternative splicing? From our current knowledge, mainly gathered from the fields of transcription and genome maintenance, these post-translational modifications are recognized with a striking degree of specificity by highly specialized modules. Following recognition, these modifications either exert a direct influence on the gene expression machinery or, more often, play a role as adaptors or scaffolding molecules for different groups of effector complexes. This model seems to be valid for the regulation of alternative splicing through the control of histone marks. In a recent pioneering study based on the mutually exclusive alternative splicing of the exons IIIb and IIIc of the *FGFR2* gene (Carstens et al., 2000), it was observed that the H3K36me3 and H3K4me1 modifications are enriched when exon IIIc is included, but H3K27me3 and H3K4me3 accumulate preferentially when exon IIIb inclusion is favored (Luco et al., 2010). In this experimental system of alternative splicing, the RNA binding protein PTB functions as a major regulator by reducing the inclusion of IIIb and favoring the inclusion of IIIc. The PTB effect is mediated by MRG15, an adaptor protein that specifically binds H3K36me3, which is relatively enriched in cell types that exhibit greater IIIc inclusion

(Luco et al., 2010). Detailed integrative studies merging the available information about the alternative splicing profiles elicited when manipulating PTB levels, the preferential genome-wide binding sites for PTB and the H3K36me3 landscape might further explain the specificity of these mechanisms for PTB-mediated alternative splicing regulation.

DNA methylation has also been shown to be enriched in exonic regions, although it remains unknown how this modification affects the splicing outcome. Methylation could affect splicing in combination with other factors. For example, in the *CD45* gene, DNA methylation inhibits the inclusion of exon 5, and this methylation pattern opposes that of CTCF, which promotes the inclusion of exon 5. Genome-wide data indicate that CTCF binding downstream of alternative exons correlates with local RNAPII accumulation, suggesting that chromatin structure affects alternative splicing by modulating the RNAPII elongation rate (Shukla et al., 2011). This finding is especially relevant because CTCF acts as a general chromatin organizer and may be involved in numerous chromatin-dependent molecular processes. It is an exciting possibility that methylation patterns, which fluctuate during development, might modulate tissue-specific alternative splicing events through the differential recruitment of CTCF. Further studies are required to reveal whether other DNA binding proteins are involved in the regulation of alternative splicing through a similar mechanism.

Overall, histone modifications may elicit the modulation of pre-mRNA alternative processing both through the control of RNAPII kinetics and the co-transcriptional recruitment of splicing regulators. Histone modifications regulate the conversion between ‘closed’ (silent) and ‘open’ (active) chromatin conformations (Li et al., 2007a; Lee et al., 2010). Histone modifications can thus potentially modulate alternative splicing choices by regulating the RNAPII elongation rate. Supporting this model, intragenic hyperacetylated chromatin induced after neuronal depolarization increases

the RNAPII elongation rate and favours exon skipping in the neural cell adhesion molecule *(NCAM)* pre-mRNA (Schor et al., 2009). However, modifications related to transcriptional repression induce exon inclusion (Saint-Andre et al., 2011). Furthermore, targeting intronic sequences with siRNAs generates a closed chromatin context enriched in heterochromatin marks, blocking RNAPII elongation and affecting the splicing outcome (Allo et al., 2009).

Recent data demonstrate that splicing can also modulate histone modification. Splicing activation enhances the recruitment of methyltransferase HYPB/Setd2 and the subsequent methylation of histone H3 at lysine 36 (H3K36me3) (de Almeida et al., 2011), and splicing inhibition through splice site mutations or spliceostatin A (SSA) treatment produces a redistribution of H3K36me3 towards the 3' end of the gene (Kim et al., 2011). These data again suggest extensive bidirectional communication between epigenetic marks and splicing regulation. The characterization of genome-wide RNAPII elongation profiles compared with global alternative splicing programs and the distribution of the different histone marks will shed light on the functional relevance of these mechanisms in the regulation of gene expression.

Functional impact of the coupling between transcription and splicing

The functional coupling of transcription and alternative splicing provides a number of potential advantages when implemented in a given gene expression program. The first aspect promoted by co-transcriptional pre-mRNA processing is the coordination of these two simultaneous mechanisms of control, enabling the specification of a transcriptional regulation program that is tailored to each splicing isoform and increasing the available possibilities to correctly tune gene expression. The functional coordination of transcription and splicing is a mechanism that would help to restrict a specific alternative processing pattern to the subpopulation or bulk of transcripts whose

synthesis is promoted by a transcriptionally driven response. Coupling would decrease the leakage of a specific alternative splicing mechanism beyond the time window during which the coupled transcriptional response of the cell is activated. Thus coupling would minimize gene expression noise while maximizing the efficiency of the whole process, as recently predicted using mathematical modeling approaches (Aitken et al., 2011; Kalsotra and Cooper, 2011). This aspect may be especially relevant to adaptive mechanisms based on regulatory switches that can radically affect cell fate, such as cell cycle or apoptosis control.

The transitions between two distinct gene expression profiles that are associated with different contexts, such as consecutive developmental stages, are best implemented when the global coordinated regulation of the different involved mechanisms is ensured (Mendes Soares and Valcarcel, 2006; Keene, 2007; Kalsotra and Cooper, 2011). The coupling of transcription and post-transcriptional processing is likely essential for achieving this aim. Alternative splicing has been recently shown to influence the nonsense-mediated decay (NMD) and the usage of different 3' UTR stretches that in turn determine the miRNA sensitivity, subcellular localization, or stability of the resulting transcripts. Alternative splicing is extensively coupled to NMD because certain alternative exons introduce premature termination codons in their mature transcripts upon inclusion, and this can constitute a mechanism for diverting a subset of transcripts toward NMD-dependent degradation. A significant number of the targets of alternative splicing-determined NMD are splicing regulators themselves, potentially creating extensive arrays of regulatory feedback loops (Saltzman et al., 2008; Ip et al., 2010). These findings might support the evolution of an intricate NMD-mediated feedback regulation of splicing factors to achieve a high degree of robustness in post-transcriptional gene expression control, similar to what has been suggested for other

RNA-processing networks (Mittal et al., 2009). Importantly, NMD-eliciting alternative splicing events, which are overrepresented in RNA-binding protein-encoding genes, are affected by pharmacological treatments that alter RNAPII elongation (Ip et al., 2010) (see below).

Coupling mechanisms also enable the establishment of checkpoints or quality controls that would promote each step of pre-mRNA synthesis and processing while ensuring the accomplishment of the essential preceding stages. One example is the significant influence of co-transcriptional pre-mRNA 5' capping on the establishment of processive elongation itself and vice versa (Schroeder et al., 2000; Moteki and Price, 2002). In a similar scenario, a highly processive elongation rate would elicit the recruitment of components of the splicing machinery and other pre-mRNA processing modules, such as 3' end processing complexes (Glover-Cutter et al., 2008). For a number of experimental models, the splicing components and the proximity of the splicing sequences to the 5' terminus of the gene or other regulatory sequences are required for, or at least significantly influence, the activity of the RNAPII complex (Fong and Zhou, 2001; Lin et al., 2008). A potential function for this link is that splicing could be used as a quality checking mechanism in the control of transcription through a given gene. Although it does not fully overlap with the regulation of alternative splicing through co-transcriptional coupling, it is probable that similar molecular events govern this reverse coupling.

Another advantage of the functional coupling is the unification or simplification of the architecture of the signaling networks upstream of the coupled transcriptional and alternative splicing programs. The most intuitive example is represented by the proposed "kinetic model" of functional coupling: if the transcriptional elongation rate of RNAPII determines the alternative processing pattern of the transcripts, a single

signaling input primarily aimed at controlling the transcription of a given gene can also be used to specify the processing profile of the resulting transcripts. This option could be especially relevant in the case of regulatory programs that require rapid and efficient activation. As mentioned above, recent studies have demonstrated the widespread pausing or stalling of RNAPII complexes at gene promoters, relying on transcription elongation activation and release from pausing for induced expression (Muse et al., 2007; Zeitlinger et al., 2007; Gilchrist et al., 2008; Adelman and Rogatsky, 2010; Gilchrist et al., 2010; Nechaev et al., 2010). Importantly, a number of these elongation-driven genes encode regulators of stress-related responses, the innate immune response, and regulators of cell proliferation and apoptosis (Nechaev and Adelman, 2008; Adelman et al., 2009). Transcription elongation control during development may also be more important than previously thought (Levine, 2011). The production of a coherent alternative processing pattern that is regulated in part by transcription elongation control not only ensures the appropriate temporal coordination of transcription and splicing but also simplifies and equally prioritizes the triggering of the alternative splicing when necessary. The regulation of transcription-coupled alternative splicing seems to be deeply ingrained in the gene expression programs associated with such responses, including at least two different branches of the DNA damage response (DDR) and the regulation of apoptosis and cell cycle progression.

The DDR encompasses a heterogeneous group of signaling pathways that have evolved to detect errors introduced in the genomic DNA by different exogenous or endogenous agents. The DDR triggers functional programs to avoid (1) the transmission of damage to the offspring cell population and (2) the disruption of essential processes, such as DNA replication. Alternative splicing is one target of the DDR (Katzenberger et al., 2006; Matsuoka et al., 2007). To date, two different mechanisms that regulate different

control points of the DDR through transcription-coupled alternative splicing have been identified. One recent study identified a specific molecular and functional interaction between the Ewing Sarcoma EWS transcription factor and the stress-associated transcription factor YB-1. This interaction is essential for the appropriate co-transcriptional recruitment of the spliceosome and thus for the timely inclusion of a number of alternative exons of the MDM2 gene (Dutertre et al., 2010). The full-length mRNA sequence yields a functional MDM2 protein, which is in turn capable of down-regulating p53, one of the master regulators of DDR-triggered programs. Triggering a canonical DDR with different genotoxic agents, such as cisplatin and camptothecin, abrogated the interaction between EWS and YB-1. As a result, genome-wide alterations in the alternative splicing pattern of the transcriptome of the cell, including the splicing of the MDM2 gene, were observed. The mechanism of action of EWS/YB-1 in alternative splicing is currently poorly understood. Recently, EWS was found to interact co-transcriptionally with its target RNAs and to be redistributed to nucleoli upon UV light exposure (Paronetto et al., 2011). However, the molecular details of these regulatory mechanisms are currently unknown. Importantly, both inclusion and exclusion events were detected as specific consequences of camptothecin treatment, which suggests that an EWS/YB-1-based mechanism is unlikely to be the sole transcription-coupled effector of the DDR.

A second potential mechanism that links DDR with transcription-coupled alternative splicing was proposed when analyzing RNAPII activity and alternative splicing in UV-treated cells (Munoz et al., 2009). UV exposure elicits a significant global increase in the phosphorylation level of the RNAPII CTD, through an as yet not understood mechanism. Interestingly, UV exposure also elicits a decrease in RNAPII processivity on different gene templates. This change in processivity, in turn, influences pre-mRNA

processing, including that of two essential regulators of apoptosis, *Bcl-x* and *caspase-9*. This mechanism was found to be independent of other DNA damage signaling events, such as BRAD DNA damage-labeling proteins, ATM-dependent signaling, and p53-dependent regulation. This mechanism could thus constitute a novel "pro-apoptotic checkpoint" in UV-mediated DNA damage and cell fate determination.

The data discussed above link the functional coupling of transcription and splicing to the control of apoptosis-regulating networks. A number of genes that control apoptosis exhibit alternative splicing-driven functional switches (Akgul et al., 2004). A typical example is the *Bcl-x* gene, which encodes a master regulatory switch of apoptosis through the modulation of mitochondrial permeability. Alternative splicing yields the large, antiapoptotic isoform Bcl-x_L or the short, proapoptotic regulator Bcl-x_S upon the usage of two alternative 5' splice sites. An analogous example is *Mcl-1*, another Bcl-2 family-related gene. A pioneering genome-wide siRNA survey for regulators of alternative splicing of both genes uncovered the extensive integration of cell cycle progression with these alternative splicing regulation events through aurora kinase A (AURKA) and SRSF1 (Moore et al., 2010). Suggesting a coherent, global apoptosis control pathway based on alternative splicing regulation, Bcl-x and Mcl-1 shared a large number of regulators with a disproportionate enrichment of alternative splicing and cell cycle regulators. Intriguingly, large subsets of RNAPII-dependent transcription regulators scored as positive modulators of the alternative splicing of *Bcl-x*, but not of *Mcl-1*. As mentioned before, the putative coupling factor TCERG1 has been shown to regulate alternative splicing of the *Bcl-x* gene by modulating the rate of RNAPII processivity (Montes et al., 2012). Regardless of the precise mechanisms underlying these differences, these data support an additional tuning mechanism specific for Bcl-x and other apoptosis-control genes, which would appropriately regulate apoptosis

sensitivity in different contexts. These observations lead to several interesting questions. (1) Why is the alternative splicing of Bcl-x, but not Mcl-1, functionally coupled to transcription control? (2) Is the transcription-coupled alternative splicing observed for Bcl-x related to the previously identified AURKA-SRSF1-driven, cell cycle-dependent axis? (3) What is the transcriptional coupling status for other apoptotic regulators that exhibit alternative splicing-dependent switches, such as *Fas* (Izquierdo et al., 2005; Bonnal et al., 2008)?

To date, a small but rapidly growing number of studies have attempted to characterize global transcription-coupled alternative splicing profiles. A recently published study describes the transcriptome landscape in cells treated with either camptothecin or 5,6-dichloro-1-β-D-furanosylbenzimidazole (DRB; a potent and specific inhibitor of the RNAPII CTD kinase CDK9). Both treatments inhibit RNAPII elongation and promote partially overlapping alternative splicing profiles. In both cases, enrichment for alternative splicing events associated with downstream NMD was noted, with a clear preference for splicing factor encoding-genes. The set of genes with alternative splicing patterns induced by camptothecin treatment were enriched in apoptosis-related and cell cycle-related genes, independently from RNA-binding proteins. These observations indicate that transcription-coupled alternative splicing likely depends on specific, conserved molecular mechanisms with functional significance. These observations also suggest that transcription-coupled alternative splicing (and most likely general alternative splicing) has been highly developed and evolutionary conserved in genes encoding RNA binding proteins and RNA splicing regulators. Alternative splicing in these genes most likely serves as a mechanism to regulate and ensure the robustness, stability and versatility of RNA-processing regulatory networks.

Importantly, these reports provide substantial data suggesting that uncoupled alternative splicing may be a significant component of the global regulation of downstream functions. Indeed, subsets of genes are found to either display changes only in their total transcript levels (regulation of transcription without significant change in splicing isoform) or changes in their alternative splicing (alternative splicing without apparent changes in transcriptional output). Similar observations have been reported by different surveys of global alternative splicing profiles associated with other biological mechanisms. These mechanisms include heart development (Kalsotra et al., 2008), neuron differentiation (Jelen et al., 2007; Ule and Darnell, 2007), or the triggering of specific major signaling routes, such as the insulin and wingless pathways (Hartmann et al., 2009). It must be noted that the transcriptome profiling in UV light-induced coupled alternative splicing regulation study discussed above (Munoz et al., 2009) also identified genes that are alternatively spliced but that are not affected at the level of expression. These genes represent approximately 15% of the total number of affected genes. However, it is conceivable that local changes in transcription elongation are capable of profoundly affecting the processing of a specific alternative exon but do not significantly (or detectably) impact the total output of that gene. Therefore, even in these documented models, transcription-dependent alternative splicing regulation may be more common than currently estimated. As discussed above, certain molecular mechanisms may have been implemented to attain precisely this localized effect on alternative splicing through transcriptional modulation without significantly affecting the total output of transcripts. Novel approaches exploiting unbiased, high-resolution technologies based on deep sequencing and integrative analysis with detailed information about RNAPII positioning, chromatin context and bioinformatics analysis of *cis*-features may help to further elucidate this question.

Concluding remarks

The functional coupling of transcription and alternative splicing is emerging as an essential component of gene expression regulation. Despite considerable efforts, numerous questions remain regarding the functional significance and global impact of this coupling on cellular and organismal homeostasis as well as its underlying molecular mechanisms. High-throughput transcriptome profiling techniques are beginning to fill the gaps in our knowledge of the genes that undergo alternative processing. We are learning when and where this coupling is happening, and the co-transcriptional transcriptome is being characterized. A further step of considerable importance consists of the integrated analysis of these datasets together with information about the genome-wide distribution of different forms of RNAPII, specific chromatin marks, and potential mediators of coupling. Due to its intrinsic potential as a mechanism for tissue-specific gene expression regulation, comparative analysis of co-transcriptional alternative splicing across distinct experimental models, such as panels of different cell lines, will also shed light on the global understanding of this exciting phenomenon.

Last, but not least, the spatial organization of the eukaryotic genome within the nucleus is an additional layer of regulation to be taken into account. Chromosomes are not randomly organized but occupy defined "territories" within the nucleus, and this distribution often results in the intermingling of specific subsets and combinations of genes that need to be co-regulated in a given situation (Branco and Pombo, 2006; Cavalli, 2007; Zhao et al., 2009; Sánchez-Álvarez et al., 2011). The functional coupling of transcription and pre-mRNA processing in the context of the highly compartmentalized eukaryotic nucleus still needs to be characterized. The recent purification and exhaustive analysis of the "transcription factory" (Melnik et al., 2011), the proposed structural unit for RNAPII transcription in the nucleus (Cook, 2010), will

substantially contribute to this goal. Notably, spliceosome components and associated factors were identified together with essential components of the RNAPII machinery as integral components of these higher-order structures (Melnik et al., 2011). It is also imperative to address the exact role of nuclear speckles in co- and post-transcriptional splicing. The recent observation showing the accumulation of intron-containing transcripts near these structures (Dias et al., 2010) has been strongly supported by single-molecule imaging studies, which revealed released unspliced pre-mRNA congregating around nuclear speckles (Vargas et al., 2011). This finding has important implications for our understanding of the mechanisms that regulate alternative splicing. This and other studies (Lionnet et al., 2010; Brody et al., 2011; Larson et al., 2011; Martins et al., 2011; Schmidt et al., 2011; Vargas et al., 2011; Gurskaya et al., 2012) that have used innovative microscopy and spectroscopy tools coupled with powerful statistical analyses and modeling allow us to monitor and quantify transcript biogenesis as well as co- and post-transcriptional processing at single-cell resolution *in vivo*. It is likely that, in the near future, numerous new insights and questions regarding the functional coordination of RNAPII transcriptional activity and pre-mRNA processing will continue to capture our attention.

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Figure Legends

Fig. 1. A. Schematic representation of spliceosome assembly and the splicing of a pre-mRNA. The spliceosome is composed of a core of five small ribonucleoprotein particles (snRNPs), U1, U2, U4, U5, and U6, and ~ 200 additional proteins. The first step in spliceosome assembly is the formation of complex E (the commitment complex). The 5' splice site (GU, 5'ss) is bound by the U1 snRNP, and the splicing factors SF1 and U2AF cooperatively recognize the branch point sequence (BP), the Py, and the 3' splice site (AG, 3'ss). In an ATP-dependent manner, the pairing of the U2snRNP with the branchpoint results in the pre-spliceosomal complex A. Subsequent steps lead to the binding of the U4–U5–U6 tri-snRNP and the formation of the complex B. The catalytic complex C, which performs two trans-esterification reactions at the splice sites, is formed after rearrangements that detach the U1 and U4 snRNPs. These reactions result in the ligation of the exons and the excision of the intron, which is removed as the lariat RNA. B. Alternative splicing regulation by *cis*-elements and *trans*-acting factors. The core *cis* sequence elements that define the exon/intron boundaries (5' and 3' splice sites, GU-AG in red) and associated 3' sequences (polypyrimidine tract, Py; and branch point sequence, A) are poorly conserved. Additional enhancer and silencer elements in exons and in introns (ESE/ESS: exonic splicing enhancers/silencers, ISE/ISS: intronic splicing enhancers/silencers) add another layer of alternative splicing regulation. *Trans*-acting splicing factors, e.g., serine/arginine-rich (SR) family proteins and heterogeneous nuclear ribonucleoprotein particles (hnRNPs), bind to enhancers and silencers and interact with spliceosomal components. In general, SR proteins bound to enhancers facilitate exon recognition (green arrows) but hnRNPs inhibit this process (black arrows).

Fig. 2. The CTD-code and pre-mRNA processing. A. Hypophosphorylated RNAPII (IIa) is recruited to the preinitiation complex (PIC) at the promoter. B. Transcription is initiated upon the phosphorylation of the serines at position 5 (Ser5-P, green) and position 7 (Ser7-P, red) of the CTD by CDK7, the kinase component of the TFHII factor. Ser5-P helps to recruit the machinery necessary for the capping of the 5' end of the transcript (pink). C. During active elongation, the CTD is phosphorylated at Ser2 (Ser2-P, blue) by CDK9 and CDK12/13 (see text for more details) to produce the hyperphosphorylated form of RNAPII (IIo). Numerous splicing factors are recruited to the hyperphosphorylated RNAPII (orange). D. During elongation towards the 3' end of the gene, Ser5 phosphorylation decreases by the action of Ser5-specific phosphatases. Polyadenylation and cleavage factors (blue) bind to the CTD to allow transcription termination and the release of the mature transcript. E. After dissociation from the DNA template, Ser2-specific phosphatases remove Ser5-P before recycling or reinitiation.

Fig. 3. The role of chromatin in alternative splicing. A. Changes in chromatin organization due to the presence of chromatin remodeling factors (blue ovals), histone tail modifications (stars), or nucleosome positioning can affect the rate of RNAPII elongation and in turn affect the alternative splicing outcome. Slow elongation allows the binding of splicing factors to the nascent pre-mRNA and the recognition of weak splices sites, favoring exon inclusion (yellow box), while fast elongation favors exon exclusion (red box). B. Histone marks can also influence splicing decisions independently of the rate of RNAPII transcription by recruiting splicing factors via interaction with chromatin adaptors.

Figure(s)

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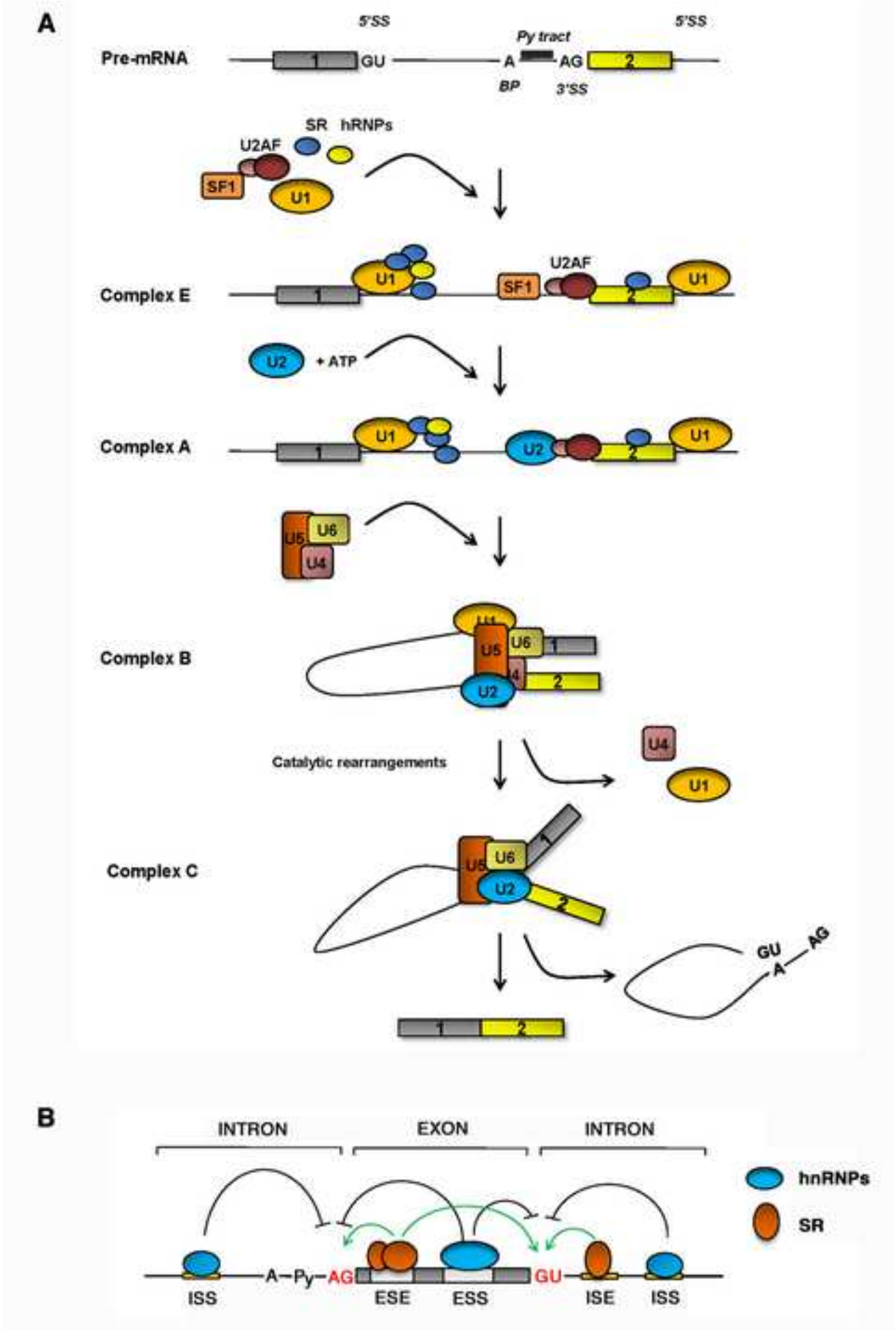

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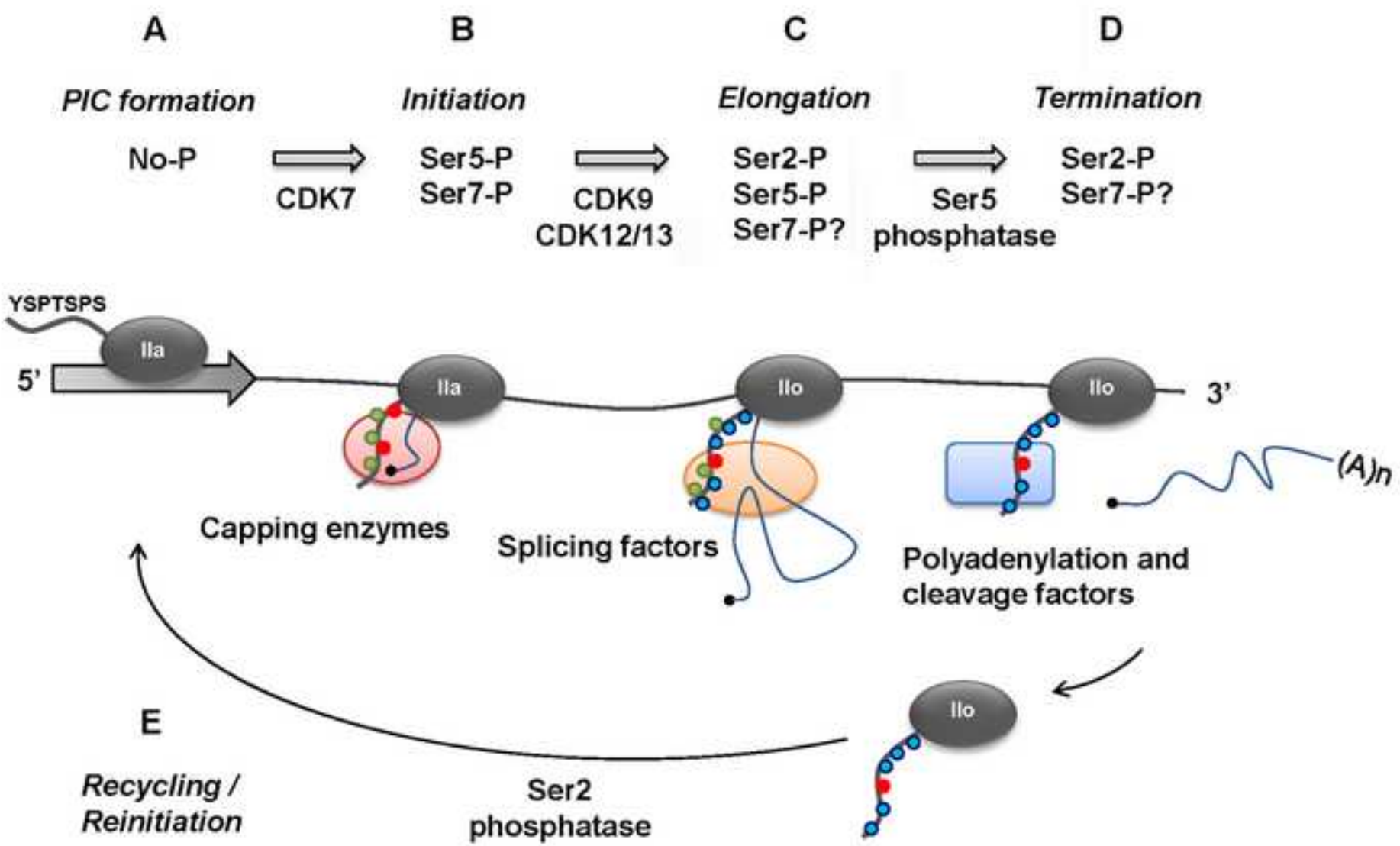

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