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Epigenetics in alternative pre-mRNA splicing

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Abstract

Alternative splicing plays critical roles in differentiation, development and disease and is a major source for protein diversity in higher eukaryotes. Traditionally, analysis of alternative splicing regulation has focused on RNA sequence elements and their associated factors, but recent provocative studies point to a key function of chromatin structure and histone modifications in alternative splicing regulation. These insights suggest that epigenetic regulation not only determines what parts of the genome are expressed, but also how they are spliced.

Introduction

The tenth anniversary of the publication of the first draft of the human genome sequence has sparked a renewed and expanded interest in alternative pre-mRNA splicing. Alternative splicing explains how the vast mammalian proteomic complexity can be achieved with the limited number of genes found in higher eukaryotes. Current estimates based on deep sequencing methodologies indicate that more than 90% of human genes undergo alternative splicing (Croft et al., 2000; Pan et al., 2008; Wang et al., 2008). Alternative splicing is an integral part of differentiation and developmental programs and contributes to cell lineage and tissue identity as indicated by the mapping of more than 22,000 tissue-specific alternative transcript events in a recent genome-wide sequencing study of tissue-specific alternative splicing (Wang, 2008). The importance of alternative splicing is dramatically highlighted by the numerous diseases that are caused by mutations in either *cis*-acting RNA elements or *trans*-acting protein splicing factors (Caceres and Kornblihtt, 2002; Cooper et al., 2009). Prominent splicing diseases include cystic fibrosis, frontotemporal dementia, Parkinsonism, retinitis pigmentosa, spinal muscular atrophy, myotonic dystrophy, premature aging, and cancer.

Traditionally, alternative splicing has been thought to be predominantly regulated by splicing enhancers and silencers (Chasin, 2007). These short, conserved RNA sequences are typically 10 nt in length, are located either in exons or introns, acting either isolated or in clusters, and stimulate (enhancers) or inhibit (silencers) the use of splice sites through the specific binding of regulatory proteins such as SR proteins (serine/arginine rich proteins) or heterogeneous nuclear ribonucleoproteins (hnRNPs) (Long and Caceres, 2009; Han et al., 2010). In addition, some silencers, instead of recruiting regulatory proteins, act by determining a particular pre-mRNA secondary structure that hinders the recognition of a

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neighboring splicing enhancer by SR proteins (Buratti and Baralle, 2004). Disease mutations often affect the use of constitutive or alternative splice sites by *cis*-acting mutations that disrupt regulatory RNA sequence elements and by *trans*-acting mutations that affect the quality or quantity of alternative or constitutive splicing factors.

It has long been clear that a full understanding of alternative splicing regulation will require the molecular characterization and structural modeling of the spliceosome and the analysis of RNA regulatory elements. However, the emerging complexity of alternative splicing regulation makes it apparent that information from those approaches will not be sufficient to decipher how alternative splicing is regulated. Here we discuss mechanisms and implications of the recently uncovered role of epigenetic components such as chromatin structure and histone modifications to alternative splicing regulation.

Coupling of transcription and splicing

More than twenty years ago, visualization of *Drosophila* embryo nascent transcripts by electron microscopy showed that splicing can occur co-transcriptionally (Beyer and Osheim, 1988) (Figure 1). Co-transcriptional splicing was later directly demonstrated for the human dystrophin gene (Tennyson et al., 1995), where it appears a very intuitive concept given that transcription of this 2,400 kb-gene would take ~16 hours to complete. A quantitative study of the c-*Src* and fibronectin mRNAs, comparing chromatin-bound and nucleoplasmic RNA fractions, shows that most introns are excised efficiently in the chromatin-bound fractions, with a gradient of co-transcriptional splicing efficiency from promoter-proximal to promoter-distal introns, suggesting co-transcriptional splicing (Pandya-Jones and Black, 2009). However, co-transcriptionality of splicing is not strict, in the sense that introns are not necessarily removed in the exact order they are transcribed (Attanasio et al., 2003; Bauren and Wieslander, 1994; Kessler et al., 1993; LeMaire and Thummel, 1990). If that were the case, the competition between splicing sites that leads to alternative splicing would be impossible.

Splicing complexes are recruited to all introns and exons in a time window that begins when the target sequence is transcribed and extends to the moment of splicing catalysis. For the entire splicing reaction to be co-transcriptional, both recruitment and catalysis must occur before transcription termination and transcript release. Alternatively, recruitment of some or all splicing factors may occur co-transcriptionally but the catalysis itself may occur post-transcriptionally. Co-transcriptional pre-mRNA splicing appears to be a general rule for long mammalian genes. It is unclear how prevalent it is in organisms with shorter introns, such as yeast, although several studies support the notion that recruitment of spliceosomal components is also mostly co-transcriptional in this organism (Gornemann et al., 2005; Kotovic et al., 2003; Lacadie and Rosbash, 2005; Tardiff et al., 2006) (Figure 1). Completion of intron removal appears to be post-transcriptional in most cases, and only in genes with relatively long downstream exons does it occur prior to transcript release (Tardiff et al., 2006). The message from these studies is that co-transcriptional recruitment of splicing factors is largely preferred, but that co-transcriptional completion of intron removal is not mandatory and depends on the specific kinetics of transcription and splicing. In other words, the selective pressure in favor of co-transcriptional splicing acts on the association of splicing factors, which can be viewed as the “commitment to splice” rather than on the catalysis itself. This might not apply to other RNA processing events like capping and cleavage/polyadenylation (McCracken et al., 1997a; McCracken et al., 1997b; Hirose et al., 1998; Maniatis and Reed, 2002; Moore and Proudfoot, 2009), where both the recruitment of the factors and enzymes involved as well as the catalysis appear to be co-transcriptional.

Although co-transcriptionality of splicing is a pre-requisite for coupling, it does not necessarily mean the two events are coupled. Co-transcriptionality simply means that splicing takes place, or is committed to occur, before the nascent RNA is released from RNA Pol II. Coupling implies that the transcription and splicing machineries interact with each other or that the kinetics of one process determines the outcome of the other. Efficient coordination between transcription and processing may be a specific feature of RNA Pol II and particularly of the carboxy-terminal domain (CTD) of its catalytic subunit given that a phosphorylated CTD is required for co-transcriptional splicing (Bird et al., 2004) (Figure 1). When protein-coding genes are placed under the control of either RNA Pol I, RNA Pol III or T7 RNA polymerase promoters, transcription takes place, but pre-mRNA processing is impaired and the resulting transcripts are poorly spliced (Dower and Rosbash, 2002; McCracken et al., 1998; Sisodia et al., 1987; Smale and Tjian, 1985). In fact, association of splicing factors to sites of transcription is dependent on RNA Pol II CTD (Misteli and Spector, 1999) and deletion of the CTD causes defects in capping, cleavage/polyadenylation, and splicing of the β -globin transcript (McCracken et al., 1997b) (Figure 1). Many splicing factors are able to interact with RNA Pol II *in vivo*, including almost all known SR proteins and U1snRNP and in nuclear extracts that support both transcription and splicing *in vitro*, SR proteins appear to be much more effective in promoting splicing when the latter is co-transcriptional than when it is post-transcriptional (Das et al., 2007). However, SR proteins are not delivered to splicing sites by RNA Pol II alone, but rather require ongoing pre-mRNA synthesis (Sapra et al., 2009), demonstrating that recruitment is not dependent on pre-assembled SR-RNA Pol II complexes. Coupled *in vitro* transcription/splicing assays, although not necessarily reflecting functional coupling as it would occur *in vivo* (Lazarev and Manley, 2007), show that nascent pre-mRNA synthesized by RNA Pol II is stabilized and efficiently spliced (Hicks et al., 2006). This is likely because it is immediately and quantitatively directed into the spliceosome assembly pathway, instead of being assembled into non-specific hnRNP complexes, which are inhibitory for spliceosome assembly (Das et al., 2006).

Strong evidence for functional coupling between transcription and pre-mRNA processing comes from analyzing how modulation of transcription affects alternative splicing events. It has been demonstrated that the outcome of alternative splicing is influenced by the promoter used to drive transcription (Cramer et al., 1999; Cramer et al., 1997; Pagani et al., 2003), by hormone-responsive elements (Auboeuf et al., 2002) or by recruitment of different transcription factors or co-activators to the promoter (Auboeuf et al., 2004a; Auboeuf et al., 2004b; Nogues et al., 2002). The effects are not the trivial consequence of different mRNA levels produced by each promoter, but depend on qualitative properties conferred by promoters to the transcription/RNA processing machinery.

Control of alternative splicing by elongation rate

The standard experimental approach to study splicing mechanisms is by *in vitro* splicing assays. This methodology employs *in vitro* synthesized pre-mRNA substrates in splicing reactions carried out in cell-free nuclear extracts. Although these conditions are appropriate to identify splicing factors and RNA intermediates, they are not ideally suited to obtain an accurate picture of the timing of splicing in relation to the generation of nascent RNA during transcription. These limitations can be overcome by *in vivo* experiments using either transfected reporter minigenes or endogenous genes as templates for splicing reactions. It was in fact differences in the behavior of a splicing event *in vivo* compared to *in vitro* that first hinted at a kinetic role for transcription on splicing. Eperon et al. (1988) found that the use of an alternative 5' splice site sequestered within a short stem of RNA secondary structure was determined by the length of the loop *in vivo*. Above a threshold loop length, the alternative site was used despite the potential structure. In contrast, the alternative site

was not used during splicing *in vitro* with all lengths of loop tested (Eperon et al., 1988). The simplest interpretation of these experiments is that the rate of RNA synthesis affects its secondary structure, which in turn affects splicing. Further evidence for a kinetic link between transcription and splicing came from experiments in which a MAZ sequence, which leads to RNA Pol II pausing, inserted into the *tropomyosin* gene promoted higher inclusion of *tropomyosin* exon 3 (Roberts et al., 1998). Conclusive evidence for a role of elongation on alternative splicing regulation was finally revealed by the finding that the nature of the promoter affects alternative splicing outcome (Cramer et al., 1997, 1999; Kornblihtt, 2005). The original observation of the promoter effect involved transient transfection of mammalian cells with reporter minigenes for the alternatively spliced cassette exon 33 (E33, also referred to as EDI or EDA) of human *fibronectin* (*FN*) under the control of different RNA Pol II promoters. When transcription of the minigene was driven by the β -globin promoter, for example, E33 inclusion levels in the mature mRNA were about 10 times lower than when transcription was driven from the *FN* or *cytomegalovirus* (*CMV*) promoter. These effects were not the consequence of the promoter strength but depended on some qualitative properties conferred by promoters to the transcription/RNA processing machinery. Two non-exclusive mechanisms could explain the promoter effect: differential promoter occupation could affect the recruitment of splicing factors by the transcription machinery (recruitment coupling) or determine different rates of RNA Pol II elongation (kinetic coupling).

Several lines of evidence support the idea that RNA Pol II elongation can affect alternative splicing through kinetic coupling (Figure 2). Replication of reporter plasmids for alternative splicing in transiently transfected cells greatly stimulated E33 inclusion. This effect was counteracted by treating the cells with trichostatin A (TSA), a potent inhibitor of histone deacetylation and therefore a chromatin “opener”, allowing for the possibility that replication conveys a more compact chromatin structure to the template, thus slowing elongation and leading to higher E33 inclusion (Kadener et al., 2001). Furthermore, drugs that inhibit elongation, like DRB (Kadener et al. 2001, Nogués et al. 2002), flavopiridol, or camptothecin (de la Mata et al., 2010), favor E33 inclusion. On the other hand, activation of transcription by Sp1, a transcription factor that promotes initiation, has no effect on E33 inclusion, whereas activation by VP16, a factor that promotes both initiation and elongation, decreases E33 inclusion (Nogués et al. 2002). The strongest evidence for a kinetic role of RNA pol II elongation comes from a slow mutant of RNA Pol II, which increases E33 inclusion in human cells (de la Mata et al., 2003). Interestingly, the homologous mutation in *Drosophila* (C4 pol II) is viable but shows changes in the alternative splicing pattern of *ultrabithorax* (*Ubx*) mRNA, that are consistent with the only conspicuous phenotype of the C4 flies, which is an enlargement of the halteres that resembles the *Ubx* mutants. Why slowing elongation would only affect the *Ubx* gene is not known but a clue might be that this gene has the longest introns in *Drosophila* (17 and 50 kb) flanking the alternative exons affected in the C4 genotype, suggesting that elongation becomes more critical when introns are long. Similar effects of elongation on splicing have been reported in yeast on an artificially created alternative exon when transcription is carried out by a slow RNA Pol II mutant or when the elongation factor TFIIIS is mutated (Howe et al., 2003). Finally, DNA damage signaling following irradiation of cells with UV light affects alternative splicing of *fibronectin*, *caspase 9*, *Bcl-x* and other human genes as a consequence of the inhibition of RNA Pol II elongation caused by UV-dependent hyperphosphorylation of the CTD (Muñoz et al., 2009).

These data support a “first come, first served” model for regulation of alternative splicing (Aebi, 1987) (Figure 2). In one version of this model, slow elongation favors removal of the intron upstream of an alternative cassette exon before removal of the downstream intron. In an alternative version, slow elongation favors recruitment of splicing factors to the upstream

intron before the downstream intron is synthesized, which in turn would promote exon inclusion. Once commitment is achieved, the order of intron removal becomes irrelevant (Figure 2). The latter model is supported by recent evidence showing that there is a preferential removal of the intron downstream of the *fibronectin* cassette exon 33 before the upstream intron has been removed (de la Mata et al., 2010). Most importantly, whereas cis-acting mutations and trans-acting factors that upregulate E33 inclusion act by changing the relative order of intron removal, reduction of elongation, which also causes higher E33 inclusion, does not affect the order of intron removal, suggesting that slow elongation favors commitment to exon inclusion during spliceosome assembly (de la Mata et al., 2010). According to this, “first served” would not be equivalent to “first excised” but to “first committed”, in agreement with the observed preferential co-transcriptionality of spliceosome recruitment rather than catalysis.

Chromatin and histone modifications as regulators of alternative splicing

As we delve deeper into the regulation of alternative splicing, it is becoming clear that control of splice site choice is far more complex than anticipated. Neither RNA-binding elements nor control by RNA Pol II elongation rate appear sufficient to fully explain the faithful regulation of alternative splicing. RNA binding motifs are not always conserved between genes, and even when motifs are transcribed containing errors, they often still accurately recruit the appropriate set of splicing factors to the exon (Fox-Walsh and Hertel, 2009). Similarly, although RNA Pol II elongation rate affects splicing outcome in different scenarios (de la Mata et al., 2003; Muñoz, 2009), it remains unclear to what extent RNA pol II processivity can be modulated in vivo, how RNA Pol II elongation rate is controlled and whether regulation of alternative splicing patterns through RNA Pol II kinetics is a commonly used mechanism in vivo. These considerations indicate that other mechanisms contribute to the control of alternative splicing. A major recent discovery is that chromatin structure and epigenetic histone modifications act as key regulators of alternative splicing.

Chromatin structure

The first indirect evidence that chromatin structure participates in the regulation of alternative splicing was the finding that *fibronectin* exon E33 inclusion was sensitive to replication-mediated chromatinization status of the plasmid and to the histone deacetylase inhibitor TSA (Kadener et al., 2001; Nogués et al., 2002). Further support came from the study of hormone-sensitive promoters that were tested for their effects on alternative splicing of a CD44 reporter gene (Auboeuf et al., 2002). Treatment with different steroid hormones induced changes in CD44 alternative splicing only if the minigene was under the control of the appropriate steroid-dependent promoter and in the presence of the specific hormone receptor, even though strong constitutive promoters were used (Auboeuf et al., 2002). Importantly, the effect on splicing was not due to changes in transcription rate, the density of the RNA Pol II, the strength of the promoter or saturation of the splicing machinery, but appeared mediated by the recruitment of specific hormone receptor co-regulators that remodeled chromatin (Auboeuf et al., 2002). In addition, the histone acetyltransferase Gcn5 in yeast (Gunderson and Johnson, 2009) and STAGA in humans (Martinez et al., 2001) physically interact with U2 snRNPs, and the histone arginine methyltransferase CARM1 interacts with U1 snRNP proteins (Cheng et al., 2007; Ohkura et al., 2005), suggesting a role of chromatin complexes in facilitating the correct assembly of the pre-spliceosome on pre-mRNA. These effects are independent of elongation rate, arguing for a more direct role for chromatin structure on splicing factor recruitment (Gunderson and Johnson, 2009). Furthermore, chromatin remodelers of the SWI/SNF family in humans and *Drosophila* also have an effect on alternative splicing that is independent of their ATPase remodeling activity and dependent on physical interaction and recruitment of snRNPs U1 and U5 (Batsche et al., 2006; Tyagi et al., 2009).

The recent advent of methods to map chromatin structure at a genome-wide scale further supports a role for chromatin structure in alternative splicing. Genome-wide mapping of nucleosome positioning by micrococcal nuclease (MNase) digestion from various species has shown that nucleosomes are positioned non-randomly along genes and are particularly enriched at intron-exon junctions thus marking exons (Andersson et al., 2009; Chodavarapu et al., 2010; Dhami et al., 2010; Kolasinska-Zwierz et al., 2009; Nahkuri et al., 2009; Ponts et al., 2008; Schwartz et al., 2009; Spies et al., 2009; Tilgner et al., 2009). Nucleosomes, defined as a stretch of ~ 147 bp of DNA wrapped around an octamer of histone proteins, are structural units of chromatin that determine chromatin conformation and compaction. Intriguingly, the average size of a mammalian exon is similar to the length of DNA wrapped around a nucleosome, possibly pointing to a protective role of the nucleosome and a function in exon definition (Schwartz et al., 2009; Tilgner et al., 2009). Indeed, nucleosome enrichment around exons is conserved in evolution from plants to mammals and found both in somatic cells and gametes (Nahkuri et al., 2009), suggesting an essential role of nucleosome positioning in exon definition.

The marking of exons by nucleosomes may play a role in splicing regulation given that they are positioned irrespective of gene expression levels (Andersson et al., 2009; Tilgner et al., 2009). Moreover, isolated exons in the middle of long introns display higher nucleosome positioning than clustered exons separated by small introns (Spies et al., 2009), whereas pseudo-exons, which are non-included intronic sequences flanked by strong splice sites, are depleted of nucleosomes (Tilgner et al., 2009). More tellingly, included alternatively spliced exons are more highly enriched in nucleosomes than excluded ones (Schwartz et al., 2009) and nucleosome density varies according to splice site strength with stronger positioning at exons defined by weaker splice elements (Spies et al., 2009; Tilgner et al., 2009), arguing for a role of nucleosome positioning not only in exon definition but also in the regulation of splicing.

Along with nucleosomes, RNA Pol II is also differentially distributed along genes in plants and humans with preferential accumulation at exons relative to introns (Brodsky et al., 2005; Chodavarapu et al., 2010; Schwartz et al., 2009). Nucleosomes have been shown to behave as barriers that can locally modulate RNA Pol II density by inducing its pausing (Hodges et al., 2009a). Together with the ability of RNA Pol II to interact with histone modifiers, such as the histone 3 lysine 36 (H3K36) methyltransferase Set2 (Xiao et al., 2003), and to recruit splicing regulators, such as SR proteins or U2 snRNP subunits (de la Mata and Kornblihtt, 2006; Listerman et al., 2006), nucleosome positioning may be modulating RNA Pol II density at exons and therefore splicing efficiency. In agreement, RNA Pol II is more highly enriched at alternatively spliced exons than at constitutive ones (Brodsky et al., 2005). Furthermore, overexpression of the ATPase-dependent chromatin remodeling complex SWI/SNF subunit Brm in human cells induces accumulation of phospho-RNA Pol II in a central block of alternative exons of the CD44 gene, and causes increased inclusion of these exons into mature mRNA (Batsche et al., 2006).

Although these observations point to a role of nucleosome positioning and chromatin structure in alternative splicing regulation, a caveat of these studies is their correlative nature. Directed experiments to test the effect on alternative splice site selection upon modulation of chromatin and nucleosome positioning in a targeted fashion will be required to distinguish direct from indirect effects on alternative splicing.

Histone modifications in alternative splicing regulation

Histone modifications are also emerging as potential regulators of alternative splicing. Genome-wide analysis of the distribution of 42 histone modifications reveals that histone marks are non-randomly distributed in the genome and that several modifications are

enriched specifically in exons relative to their flanking intronic regions (Kolasinska-Zwierz et al., 2009; Spies et al., 2009; Andersson et al., 2009; Schwartz et al., 2009). Even though the enrichment of many histone modifications is a reflection of the higher density of nucleosomes at exons, some histone marks such as trimethylated H3K36 (H3K36me3), H3K4me3 and H3K27me2, are elevated even after normalization for nucleosome enrichment, whereas others, such as H3K9me3, are depleted (Dhami et al., 2010; Spies et al., 2009). In support of a splicing regulatory role of histone marks, histone modification levels do not correlate with transcriptional activity (Spies et al., 2009) and in active genes the transcription-associated H3K36me3 modification is less prominently enriched in alternatively spliced exons than in constitutive exons (Andersson et al., 2009; Kolasinska-Zwierz et al., 2009).

An additional indication for a role of histone modifications in alternative splicing is the observation that treatment of cells with the histone deacetylase inhibitor TSA induces skipping of the alternatively spliced *fibronectin* E33 and the *neural cell adhesion molecule* (NCAM) exon 18 (Nogués et al., 2002; Alló et al., 2009; Schor et al., 2009). In a more physiological context, depolarization of human neuronal cells increases H3K9 acetylation and H3K36 methylation locally around the alternatively spliced exon 18 of NCAM and induces exon skipping (Schor et al., 2009). Noticeably, no changes in histone acetylation are observed at the NCAM promoter. This reversible effect is likely due to an intragenic and local modulation of the RNA Pol II elongation rate (Schor et al., 2009). Furthermore, targeting of an intronic sequence upstream of the alternatively spliced E33 of *fibronectin* with small interfering RNAs induces local heterochromatinization and increased E33 inclusion without affecting general transcription levels (Allo et al., 2009). Consistently, inhibition of histone deacetylation, DNA methylation, H3K9 methylation and downregulation of heterochromatin protein 1 α (HP1 α) abolishes the siRNA-mediated effect on exon E33 splicing (Allo et al., 2009), suggesting a role of these modifications in alternative splicing regulation.

Further evidence for histone-mediated alternative splicing control comes from observations on the *human fibroblast growth factor receptor 2* (FGFR2) gene. FGFR2 is alternatively spliced into two mutually exclusive and highly tissue-specific isoforms, FGFR2-IIIb and -IIIc. According to the pattern of splicing, the gene is enriched in a particular subset of histone modifications with H3K36me3 and H3K4me1 accumulating along the alternatively spliced region in mesenchymal cells, where exon IIIc is included, and H3K27me3 and H3K4me3 enriched in epithelial cells, where exon IIIb is used (Luco et al., 2010). Importantly, modulation of H3K36me3 or H3K4me3 levels by overexpression or downregulation of their respective histone methyltransferases changes the tissue-specific alternative splicing pattern in a predictable fashion (Luco et al., 2010). Taken together these observations suggest that localized changes in chromatin conformation and histone modification signatures along an alternatively spliced region can change splicing outcome.

Although there is no experimental evidence at present, it is possible that DNA methylation may also, directly or indirectly via histone modifications, affect splice site choice. DNA methylation patterns correlate better with histone methylation patterns than with genome sequence context (Meissner et al., 2008). Mapping in plants and human cells of DNA methylation levels by single-molecule whole-genome bisulfite-sequencing reveal that DNA methylation is also non-randomly distributed along the genome, specifically marking exons (Chodavarapu et al., 2010; Hodges et al., 2009b) and correlating well with H3K36me3, but inversely correlating with H3K4me2 levels (Hodges et al., 2009b).

These observations point to a role for histone modifications in the regulation of alternative splicing and this regulation may involve the modulation of RNA Pol II elongation rate.

However, an additional mechanism has recently emerged involving direct physical crosstalk between chromatin and the splicing machinery *via* an adaptor complex (Sims et al., 2007; Luco et al., 2010) (Figure 3).

Chromatin-splicing adaptor systems

A hint towards a direct role for histone modifications in alternative splicing regulation came from comparative mapping of a set of histone modifications along several genes whose alternative splicing is dependent on the polypyrimidine tract binding protein (PTB) splicing factor. These studies revealed a strong correlation between several histone modifications across the alternatively spliced regions and splicing outcome (Luco et al., 2010). PTB-dependent genes were found to be enriched in H3K36me3 and depleted in H3K4me3 in the alternatively spliced regions. Modulation of these histone marks was sufficient to switch the pattern of PTB-dependent exon inclusion (Luco et al., 2010). The molecular mechanism by which H3K36me3 acts in this case does not appear to involve modulation of RNA Pol II elongation rate, but rather the creation of a platform on chromatin for the recruitment of PTB to the nascent RNA (Figure 3) (Luco et al., 2010). This occurs via an adaptor protein, MRG15, that specifically binds to H3K36me3. The high levels of H3K36me3 along the alternatively spliced region of the gene attract MRG15, which in turn interacts with PTB recruiting it to the nascent RNA (Luco et al., 2010). In contrast, in cell types where H3K36me3 levels are low, the splicing repressor PTB is only poorly recruited to the newly forming RNA favoring inclusion of the PTB-dependent exon (Luco et al., 2010) (Figure 3). H3K36me3, MRG15 and PTB thus establish a chromatin-splicing adaptor system. In line with this interpretation, increasing H3K36me3 levels in the absence of the MRG15 adaptor protein has no effect on *FGFR2* alternative splicing (Luco et al., 2010).

Although histone modifications clearly play a direct role in splicing regulation in this system, interestingly, the histone modifications do not appear to be the sole determinant of splicing outcome, but rather appear to act as a modifier. Genome-wide analysis of PTB-dependent alternative splicing patterns reveal that the splicing events most sensitive to changes in histone modifications relied on weak PTB-binding sites whereas alternative splicing events involving strong PTB-binding sites are not dependent on H3K36me3, suggesting that epigenetic modifications act in concert with RNA-binding elements to strengthen their effect (Luco et al., 2010).

There is reason to believe that the combination of H3K36me3/MRG15/PTB is not the only chromatin-splicing adaptor system in mammalian cells (Figure 4). It is known that H3K4me3 levels play a role in the recruitment of the early spliceosome to human cyclin D1 pre-mRNA via binding of the chromatin-adaptor protein CHD1 (Sims et al., 2007). CHD1 contains a chromo-domain that specifically recognizes H3K4me3 and interacts with components of the U2 snRNP complex, but not U1 snRNP (Sims et al., 2007). Consistent with a role in splicing regulation, downregulation of H3K4me3 or CHD1 alters the efficiency of pre-mRNA splicing and reduces association of splicing factor 3a (SF3a) subcomplexes and U2 snRNP with pre-mRNA *in vitro* and *in vivo* (Sims et al., 2007). Interestingly, CHD1 is also a component of the histone acetyltransferase SAGA complex (Pray-Grant et al., 2005) in which Gcn5, which binds to acetylated H3 (Li and Shogren-Knaak, 2009), also interacts and recruits U2 snRNP components to the exon (Gunderson and Johnson, 2009; Figure 4). Another example of a possible chromatin-splicing adaptor system is H3K9 tri-methylation and HP1 proteins, which appear to recruit hnRNPs in *Drosophila* (Piacentini et al., 2009; Figure 4). Mass spectrometry analysis of proteins that bind to H3K9me identified the chromatin-binding protein HP1 α/β and the splicing factors SRp20 and ASF/SF2 as interaction partners (Loomis et al., 2009). Co-immunoprecipitation experiments confirmed that HP1 β interacts with ASF/SF2 in humans (Loomis et al., 2009).

and HP1 α with hnRNP proteins in *Drosophila* (Piacentini et al., 2009). These results point to a possible role for H3K9me3 in the regulation of recruitment of splicing factors mediated by the chromatin-adaptor protein HP1, although their functional relevance to splice site selection remains to be determined (Figure 4). Finally, other combinations of interacting histone modifications, chromatin-binding proteins and splicing factors may exist, possibly constituting a complex network of communication between chromatin and RNA. Genome-wide mapping of histone modifications and comparison to alternative splicing patterns should reveal such additional chromatin-splicing adaptor systems.

An integrated model for alternative splice site selection

Regulation of alternative splicing has long been thought to involve mostly *cis*-acting RNA elements. However the picture becomes far more complex when one considers that RNA processing is coupled to transcription (Figure 5). The ultimate driving factor in determining splicing outcome will always be the recruitment of splicing regulators to the target RNA (Barash et al., 2010). However, when and which factors are recruited is not only dependent on the combination of RNA motifs, the tissue- or developmental-specific pattern of expression of the splicing factors or their post-translational modifications, but is also greatly influenced by chromatin architecture and histone modifications. The contribution of transcription regulators and histone modifiers to splicing regulation is likely two-fold: On the one hand, they remodel and open chromatin for the recruitment of elongation factors that activate RNA Pol II elongation kinetics. On the other hand, the positioning of nucleosomes along exons, plus the enrichment in particular subsets of histone modifications, may modulate the recruitment of splicing regulators (Kolasinska-Zwierz et al., 2009; Schwartz et al., 2009; Spies et al., 2009). This could occur either through pausing of RNA Pol II, which favors the formation of the spliceosome by protein-protein interactions (de la Mata and Kornblihtt, 2006; Listerman et al., 2006), or through specific recruitment of splicing factors via adaptor complexes to weak RNA binding sites (Luco et al., 2010; Sims et al., 2007; Gunderson and Johnson, 2009; Piacentini et al., 2009). These observations suggest an integrated model for the regulation of transcription and splicing in which the factors involved in the transcription control and chromatin maintenance are also involved in the recruitment and assembly of the spliceosome (Figure 5).

The role of chromatin in other RNA processing events

The role of chromatin and histone modifications likely goes beyond alternative splicing. There are some indications that other RNA processing events are similarly modulated by chromatin and epigenetic modifications.

In *S. cerevisiae*, the 3' region near the polyadenylation site is depleted of nucleosomes (Mavrich et al., 2008) and in human T-cells nucleosome density dips noticeably within ~200nt of the canonical polyadenylation signal (Spies et al., 2009). Functional relevance for nucleosome density in polyadenylation is suggested by the fact that in genes containing multiple polyadenylation sites, the most highly used site preferentially falls within a nucleosome-depleted region (Spies et al., 2009). Bioinformatic analysis also suggests that nucleosome affinity is reduced near highly used polyadenylation sites but markedly increases just downstream of them (Spies et al., 2009). Altered nucleosome density may affect RNA Pol II elongation kinetics, which is known to affect polyadenylation, or the recruitment of the polyadenylation machinery to the nascent transcript (McCracken et al., 1997b).

Further evidence for a role of chromatin in RNA processing comes from the surprising finding in *Drosophila* that several histone variants including the core histones H3.3A, H3.3B, H2a.V and the H3-histone chaperone Asf1 are required for processing of the

metazoan histone RNAs (Marzluff et al., 2008). Histone RNAs are unique in that they lack introns, are not polyadeny-lated and their 3' ends are processed in a single step by formation of a 3' stem-loop structure (Wagner et al., 2007). Loss of H2a.V, the functional ortholog of human H2A.X and H2A.Z, results in read-through and failure to process histone pre-mRNAs. The reason appears to be failure to recruit histone RNA processing factors to nascent histone RNAs (Marzluff et al., 2008). Although it is currently unknown how H2a.V affects processing factor recruitment, one intriguing possibility is that the altered chromatin structure at the histone loci interferes with the proper assembly of histone RNA processing machinery, thus decreasing RNA processing efficiency.

Another RNA metabolic event that appears to be influenced by chromatin is RNA degradation. In an attempt to uncover novel functions for the *S.pombe* histone variant H2A.Z, Grewal and colleagues noted that absence of H2A.Z leads to an increase in antisense transcripts in 5–8% of loci, whereas the level of sense-transcripts is only modestly affected (Zofall et al., 2009). The accumulating antisense messages were mostly read-through transcripts, and run-on experiments indicated that the elevated transcript levels are not due to increased transcription but are the consequence of failed degradation by the exosome (Zofall et al., 2009). In support of these conclusions, deletion of the exosome subunit rrp6 resulted in an antisense RNA profile similar to that observed upon loss of H2A.Z. These observations point to a role of chromatin structure in antisense RNA degradation by the exosome. The absence of H2A.Z may directly, or indirectly through loading of factors involved in maintaining chromatin structure, change chromatin conformation, which interferes with RNA Pol II progression, leading to RNA Pol II stalling and consequent degradation of transcripts. However this view is not fully consistent with the observation of read-through transcripts generated in the absence of H2A.Z. Alternatively, H2A.Z may play a role in communicating to an RNA Pol II-associated exosome that read-through transcripts have been generated. Intriguing questions are whether similar mechanisms are also at play in higher eukaryotes and whether H2A.Z, other histone variants and chromatin structure in general, play a more universal role in stability and controlled degradation of regular sense-transcripts.

Chromatin as a memory of alternative splicing patterns

Many alternative splicing events occur in a tissue- and/or cell-type specific fashion. How tissue- and cell-type specific alternative splicing patterns are established, propagated and maintained is only poorly understood. One mechanism involves the tissue-specific expression of alternative splicing regulators, the classical example being the neuron-specific splicing factor NOVA-1/2, which regulates an extensive network of alternatively spliced target genes (Ule et al., 2005). However, given the scarcity of dedicated alternative splicing master controllers such regulation is likely the exception rather than the rule.

Cell- and tissue-specific alternative splicing patterns are obviously not determined solely by RNA-binding motifs, as they are present identically in all the cell types. Although cell- and tissue-specific differences in expression of constitutive splicing factors have been reported (Hanamura et al., 1998), it is difficult to envision how global changes in abundance of general splicing factors in a cell-type or tissue can account for the intricate regulation of individual exons, some of which need to be included, some skipped and others requiring activation of cryptic splice sites, even within the same gene. Given the realization that chromatin structure and histone modifications can affect alternative splicing, it is attractive to speculate that, in analogy to the histone indexing mechanisms used to specify which genes are expressed and which ones are silenced, the histone-based system may also encode information that specifies alternative splicing patterns in cell types and tissues (Figure 6). Such a system may involve marking of chromatin stretches encoding alternatively spliced

regions by histone modifications to either alter RNA Pol II elongation rate locally as the polymerase passes through, or to recruit splicing factors via adaptor complexes. An advantage of such a histone-based alternative splicing regulatory system is that it would provide an epigenetic memory for splicing decisions which could be passed on during proliferation of a cell population and could be modified during differentiation without the requirement to establish a new set of alternative splicing rules at each step of differentiation. Obviously, an epigenetic alternative splicing memory would still require the proper expression of splicing factors, a process which itself may be controlled by epigenetic mechanisms. Regardless of mechanisms, it appears that epigenetic regulation is not limited to controlling what regions of the genome are expressed, but also how they are spliced.

Conclusions and outlook

The realization that chromatin and histone modifications contribute to RNA processing, particularly alternative pre-mRNA splicing, has recently inspired many avenues of investigation, but at the same time raises many key questions. At this point, we do not even have a comprehensive view of how histone modifications relate to alternative splicing outcome. For that, histone modifications must be comprehensively mapped across the genome in as many cell types and tissues as possible and compared to genome-wide alternative splicing patterns. This information should be readily forthcoming from genome-wide histone modification mapping projects and the parallel characterization of the transcriptome in those systems by deep sequencing. These approaches will also answer the fundamental question of whether histone modifications that have been implicated in alternative splicing regulation act alone or in a combinatorial fashion with other epigenetic marks. Systematic genome-wide studies should also resolve the issue of how extensive the regulation of alternative splicing by histone modifications is. Are all alternative splicing events sensitive to histone modifications, or is only a subset of exons affected? If so, what are their characteristics? An intriguing extension of these considerations is the possibility that non-coding RNAs might play a role in alternative splicing regulation (Kishore and Stamm, 2006; Khanna and Stamm, 2010). ncRNAs are now known to be involved in heterochromatin structure and it is possible that some ncRNAs are specifically transcribed and associate with alternatively spliced regions of genes.

Having established a role for histone modifications in alternative splicing, and given the intimate linkage between transcription and RNA processing, the question of whether splicing in turn also affects histone modifications must be asked. It is possible that in the same way histone modifications modulate recruitment of splicing factors, splicing regulators also modulate the recruitment of histone modifiers and chromatin remodelers to the nucleosomes regulating chromatin conformation in a feedback mechanism. In support of such cross-talk, inhibition of splicing abolishes transcription and splicing factors stimulate elongation (O'Keefe et al., 1994; Fong and Zhou, 2001; Lin et al., 2008), suggesting that transcription, chromatin and splicing are intimately dependent on each other. Considering the still preliminary but tantalizing evidence that chromatin may also affect other RNA processing events, it will be important to probe in more detail the effect of chromatin on 3' processing, RNA stability and other RNA processing steps.

Finally, we must consider the possible physiological and pathological consequences and opportunities of histone mediated RNA processing effects. First and foremost, it will be important to determine whether histone modification effects on RNA processing are heritable and therefore truly epigenetic or whether they are merely transient modulators. To address this question, we will have to systematically analyze changes in RNA processing patterns during differentiation and development and compare them to histone modification fingerprints of cells and tissues. We should also comprehensively probe for aberrant RNA

processing in diseases caused by epigenetic defects. In addition, we may have to reconsider the expected effects of drugs targeting epigenetic mechanisms such as the clinically used histone deacetylase and DNA methyltransferase inhibitors and we might want to think about designing new drugs targeting chromatin for the treatment of splicing-diseases. Clearly the emerging role of epigenetics in RNA processing provides many new challenges, and even more opportunities.

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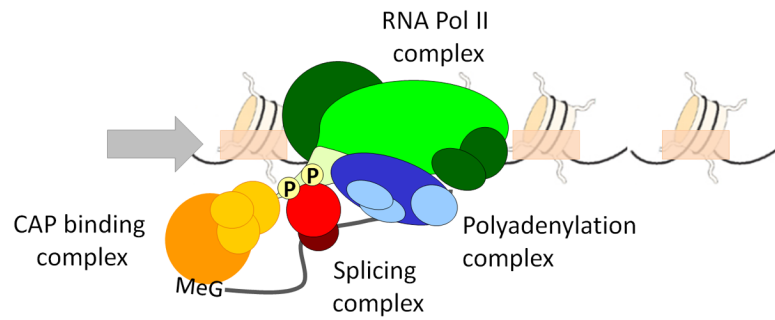


Figure 1. Coupling of transcription and RNA processing

RNA polymerase II (green) recruits RNA processing factors such as the 5' cap-binding complex (CAP) (yellow), splicing and pre-spliceosome factors (red) and the polyadenylation complex (blue) in the context of nucleosome-containing chromatin. Recruitment of RNA processing factors occurs via the RNA Pol II C-terminal domain (CTD; light green) and much of RNA processing occurs co-transcriptionally.

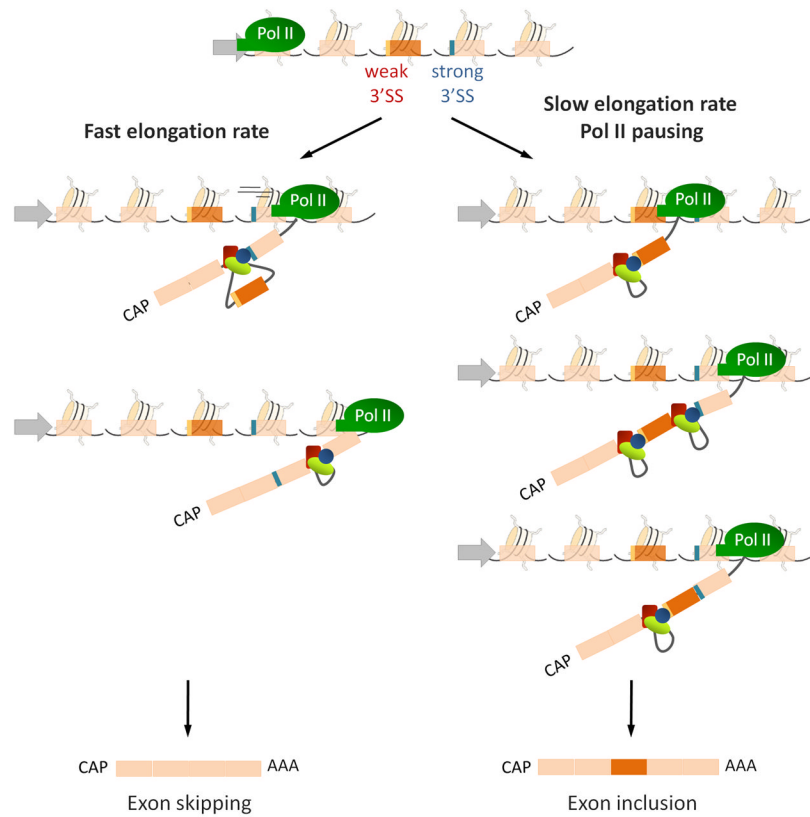


Figure 2. The RNA Polymerase II kinetic model for alternative splicing

Rapid elongation of RNA Polymerase II (Pol II) leads to simultaneous availability to the splicing machinery of a weak (red) and a strong (blue) splice site which compete for the recruitment of splicing factors (red, blue and green ovals) resulting in skipping of the weaker exon (orange rectangle). Pausing or slowing down of the RNA Pol II favors the recruitment of the splicing machinery to the first transcribed, weaker exon leading to its subsequent inclusion in a “first served, first committed” model.

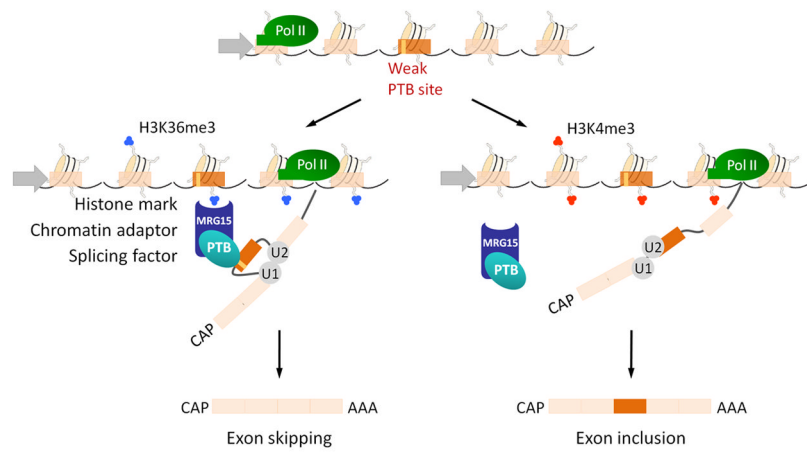
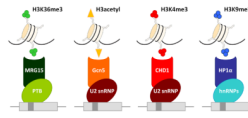


Figure 3. The chromatin-adaptor recruiting model of alternative splicing

Histone modifications along the gene determine the binding of an adaptor protein which reads specific histone marks and in turn recruits splicing factors. In the case of exons whose alternative splicing is dependent on polypyrimidine tract binding protein (PTB) splicing factor, high levels of trimethylated histone 3 lysine 36 (H3K36me3, blue) attract the chromatin-binding factor MRG15 that acts as an adaptor protein and by protein-protein interaction helps to recruit PTB to its weaker binding site inducing exon skipping. If the PTB-dependent gene is hypermethylated in H3K4me3 (red), MRG15 does not accumulate along the gene, and PTB is not recruited to its target pre-mRNA, thus favoring exon inclusion.

**Figure 4. Chromatin-adaptor complexes**

Several histone modification-binding chromatin proteins interact with splicing factors (Luco et al., 2010; Sims et al., 2007; Gunderson and Johnson, 2009; Piacentini et al., 2009; Loomis et al., 2009).

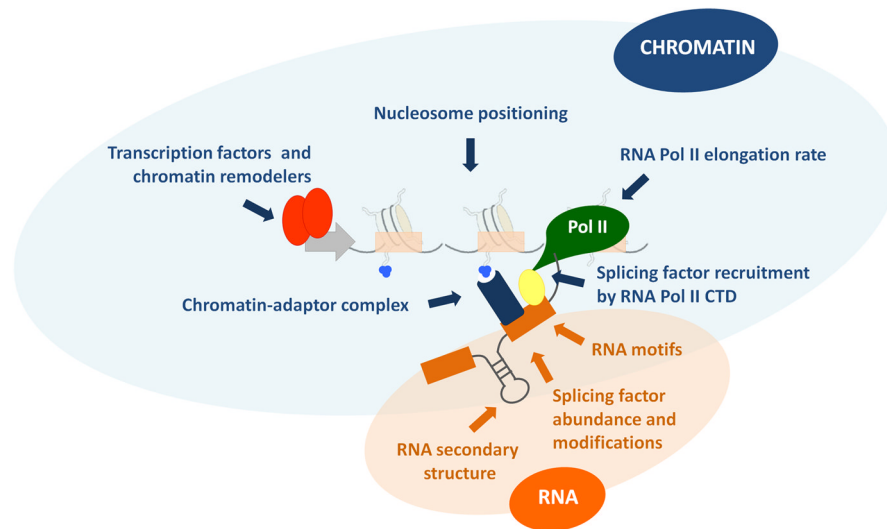


Figure 5. An integrated model for the regulation of alternative splicing

Alternative splicing patterns are determined by a combination of parameters including cis-acting RNA regulatory elements and RNA secondary structures (highlighted in orange) together with transcriptional and chromatin properties (highlighted in blue) that modulate the recruitment of splicing factors to the pre-mRNA.

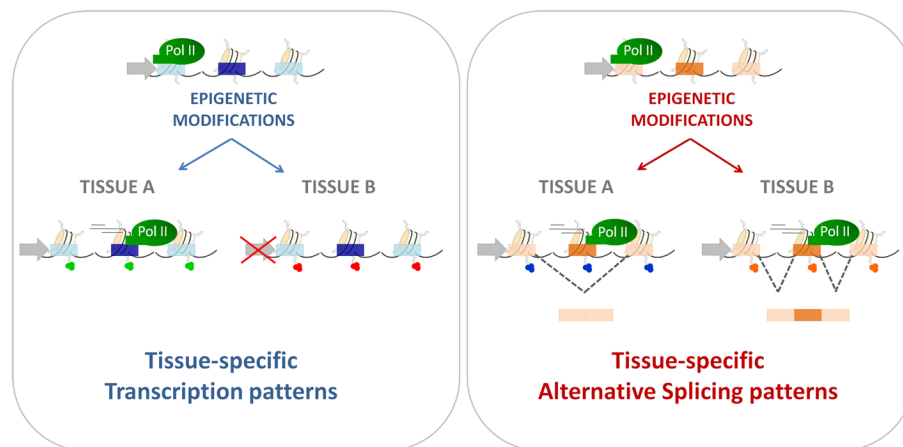


Figure 6. The epigenetics of alternative splicing

The combination of histone modifications along a gene establishes and maintains tissue-specific transcription patterns (left panel), as well as heritable tissue-specific alternative splicing patterns (right panel).