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## An Epigenetic View on Alternative Splicing

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### Abstract

Genome-wide analysis indicate that alternative splicing of mRNA precursors (pre-mRNAs) affects the vast majority of human genes. Alternative splicing provides a fundamental mechanism to increase transcriptome complexity, allowing the production of two or more mRNA variants that often encode proteins with different, sometimes opposite functions. Its importance is underscored by the observation that misregulated alternative splicing can lead to human diseases. Pre-mRNA splicing has long been known to be regulated by *cis*-acting sequence elements and *trans*-acting protein factors. In higher eukaryotes, it mostly occurs co-transcriptionally so that it is not surprising that a role for chromatin and epigenetic factors in the regulation of exon inclusion is now emerging. In this review, we will discuss the most recent findings on the roles played by chromatin structure on the modulation of the cotranscriptional splicing reactions. In particular, we will focus our attention on how the modulation of the transcribing RNA polymerase II, the changes in nucleosome architecture and the presence of different histone modifications contribute to the regulation of the splicing process.

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## 1. Introduction

Most eukaryotic mRNAs are generated from their primary transcripts (pre-mRNAs) through capping at the 5' end, removal of introns by splicing and 3' end cleavage and polyadenylation. These processes can lead to transcript diversification through the phenomenon of alternative splicing (AS). AS can lead to the specific inclusion or the skipping of exons (or even parts of an exon), to the selection of different 3' terminal exons, or to intron retention. It is likely that more than 95% of all human genes give rise to alternative mRNAs (Pan et al., 2008; Barash et al., 2010). The effect of AS in expanding the protein repertoire might partially underlie the apparent discrepancy between gene number and the complexity of higher eukaryotes. Given the crucial role of AS in the regulation of gene expression, it is not surprising that alterations in this process are associated with cancer and neurodegenerative pathologies, such as spinal muscular atrophy (SMA) and amyotrophic lateral sclerosis (ALS) (for review, David et al., 2010, Cooper et al., 2009).

RNA splicing occurs in the spliceosome, a large complex composed of five small ribonucleoprotein particles (U1, U2, U4/U6 and U5 snRNPs) and many non-snRNPs splicing factors. SR proteins and hnRNP proteins were the first non-snRNPs splicing factors identified. These proteins are components of the basal splicing machinery but, since their concentration can influence splice site selection, they contribute to AS. In addition, a growing list of tissue-specific AS regulators have been identified in recent years (for review see Wahl et al., 2009).

The mature 3' ends of mRNAs, with the exception of replication-dependent histones transcripts, are generated by endonucleolytic cleavage of the pre-mRNA followed by polyadenylation of the upstream cleavage product. Pre-mRNA 3'-end processing requires also several *trans*-acting protein factors (for review see Proudfoot, 2011). A large proportion of mammalian genes also undergoes alternative polyadenylation generating mRNA variants that differ in their coding sequence and/or in their 3' untranslated regions (UTRs), thereby potentially regulating the stability, localization and translation efficiency (for review see Tian et al., 2013)

Although the general mechanisms of pre-mRNA splicing and 3'-end processing have been well studied, how specific exons are chosen and are included in the mature transcript is still not completely clear. Both the splicing machinery and the 3' end processing complex assemble on conserved sequence elements that define the intron-exon junctions, the so-called splice sites (ss), the branch point sequence (BPS), a poorly conserved sequence located near the 3' end of the intron, and the polyadenylation site (PAs, Figure 1). In addition to these core signals, splicing is influenced by other regulatory elements (Wang et al., 2008). These elements are conventionally classified as exonic splicing enhancers (ESEs) or silencers (ESSs) depending whether they function to promote or inhibit inclusion of the exon they reside in, and as intronic splicing enhancers (ISEs) or silencers (ISSs) if they enhance or inhibit usage of adjacent splice sites or exons from an intronic location. These regulatory elements function by recruiting *trans*-acting factors that activate or inhibit splice site recognition and/or spliceosome assembly.

The early steps of spliceosome assembly, which provide the main targets for regulation, involve recognition of the consensus splice sites at both ends of the intron. Members of the SR family of splicing factors play essential roles in the early steps of splice-site recognition.

These proteins contain one or two N-terminal RNA recognition motifs (RRMs) that function in sequence-specific RNA binding and a C-terminal domain rich in alternating arginine and serine residues, referred to as RS domain that is required for protein-protein interactions with other RS domains. SR proteins bound to specific RNA sequence elements are thought to recruit key splicing factors enhancing the recognition of splice sites and influencing splice site selection in a concentration-dependent manner (for review, see Zhou et al., 2013). This raises the possibility that tissue-specific expression of SR proteins may drive variation in splicing patterns. In addition, members of the family of hnRNP have also been shown to participate in the regulation of AS. Often these proteins have an antagonistic function to SR proteins. So far, only a few systems of regulated splice site choice have been genetically or biochemically dissected and most regulatory proteins and sequence elements have not yet been identified.

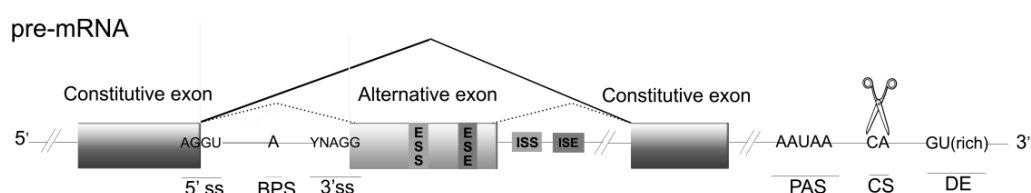


Figure 1. Schematic structure of an eukaryotic pre-mRNA. The pre-mRNA contains different *cis*-acting regulatory elements. The intron sequences are highly variable except for a conserved short region at 5' and 3' ends, called splice sites (ss), and the branch point sequence (BPS) within the intron. Exons and introns also contain splicing enhancers (ESEs and ISE) or silencers (ESSs and ISSs). At the 3' end of the pre-mRNA signals are found that direct cleavage and polyadenylation. The canonical polyadenylation signal consists of a conserved hexameric sequence, termed polyadenylation site (PAS) that precedes the CA dinucleotide where cleavage of the pre-mRNA occurs (cleavage site, CS). The CS is followed by a U- or UG- rich stretch of nucleotides (downstream element, DE).

This review concentrates on recent advances in the study of the cross-talk between chromatin structure and AS regulation. First, it focuses on several recent reports, that found large-scale evidence for a connection between nucleosome positioning and exon-intron architecture. An interesting emerging concept from these studies is that nucleosome positioning may reflect the exon-intron architecture. Then, we review histone modifications that may contribute to splicing regulation. Finally, intragenic DNA methylation and evidence for a role of methylated cytosine (5-mC) in exon definition are reviewed.

## 2. Alternative Splicing and RNA Pol II Elongation Rate

Although initially splicing and 3' end formation have been studied *in vitro* as independent processing events, biochemical, cytological and functional evidence suggest that all the events leading to the synthesis of the mature mRNA are coupled to transcription (for review see Kornblihtt et al., 2013). Several RNA processing factors are recruited on the C-terminal domain of RNA polymerase II (RNA Pol II) and are deposited on the nascent pre-mRNA molecule during transcription elongation. Transcription-coupled AS can be explained

by the differential recruitment of AS factors on the transcribing polymerase. The carboxy-terminal domain (CTD) of the largest RNA Pol II subunit has a key role in the coupling of transcription with the different maturation steps that are required for the biogenesis of the mature mRNA molecule. The CTD undergoes extensive phosphorylation on Ser2 and Ser5 of the heptapeptides repeats (YSPTSPS). These phosphorylation events are associated to the transition from initiation to elongation. It has been proposed that RNA Pol II pausing at promoter proximal sites is phosphorylated on Ser5 but not on Ser2 (de la Mata et al., 2003; Morris et al., 2005). Several AS factors, including SR proteins bind to the phosphorylated CTD (Das et al., 2007).

Recently, a novel mode of splicing factor recruitment by Argonaute proteins has been uncovered (Ameyar-Zazoua et al., 2012). Argonaute proteins are the catalytic components of the cytoplasmic RNA-inducing silencing (RISC) complex responsible for RNAi silencing. In the nucleus, they regulate transcription by inducing gene silencing. Interestingly, silencing of AGO1 and AGO2 was found to influence AS of the CD44 gene. Moreover, both AGO proteins physically interact with components of the splicing machinery, suggesting that they may participate in the recruitment of the splicing machinery to chromatin.

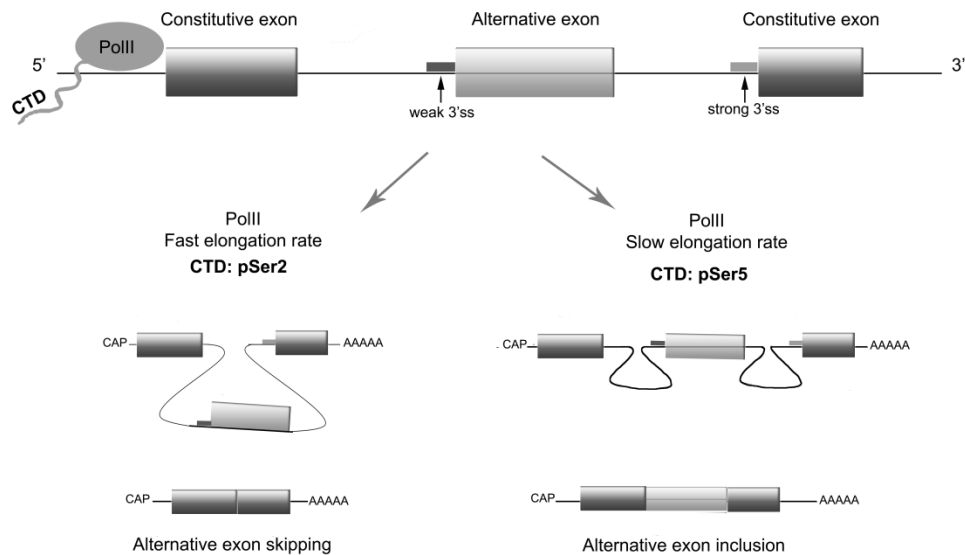


Figure 2. Kinetic coupling between transcription elongation and alternative splicing. Alternative exons are generally characterized by weak splice sites. The inclusion of an alternative exon with a weak 3'ss near a constitutive exon can be modulated by the elongation rate of RNA Pol II. A fast, processive polymerase, which is associated with a CTD phosphorylated in Serine 2 (pSer2), favors the skipping of the alternative exon. In contrast, phosphorylation of Serine 5 (pSer5) is associated with a slow processive Pol II that favor the inclusion of the alternative exon allowing more time for splice site recognition.

Another way to explain the coordination of splicing with transcription is a kinetic coupling between AS and transcription elongation (for review see Srebrow et al., 2006; Allemand et al., 2008, Figure 2). Slowing down the polymerase may favor the use of weak splice sites by delaying the synthesis of downstream splice sites, thus facilitating the recognition of suboptimal exons. The observation that inhibitors of histone deacetylation

favors skipping of alternative exons (Nogués et al., 2002), possibly by promoting hyperacetylation of core histones thus facilitating transcription elongation, points towards an involvement of chromatin structure in the elongation-dependent regulation of AS. Further support to this idea has been recently provided by the report that the catalytic subunit Brahma (BRM, *SMARCA2*) of the chromatin remodelling complex SWI/SNF modulates AS by affecting RNA Pol II elongation rate (Batsché et al., 2006, see below). Recently, the kinetic coupling of alternative splicing and RNA Pol II elongation was shown to be influenced by DNA damage (Muñoz et al., 2009). These authors found that UV irradiation inhibits transcription elongation by inducing the hyperphosphorylation of RNA Pol II carboxy-terminal domain. This in turn can influence AS decisions, influencing the choice between cell survival and cell death.

### 3. Nucleosomes and Alternative Splicing

Nucleosomes are the basic building blocks of chromatin and consist of 147 base pairs of duplex DNA wrapped around a protein multi-subunit complex called “histone octamer”, which in turn is composed of two copies of each of the four canonical histone proteins H2A, H2B, H3 and H4. The positively charged residues of the histone proteins contact the phosphate backbone of the DNA every 10,4 base pairs, so that the 147 bases stretches of the DNA wrapped around the histone octamer make nearly 14 contacts (Khorasanizadeh, 2004).

During transcription, nucleosomes represent a “roadblock” to gene expression, as their presence may affect the accessibility to the DNA and prevent the polymerase from reading the template strand. Chromatin remodeling complexes are able to overcome these problems, as they displace the nucleosomes starting from the promoter region of a given gene, so that complete transcriptional activation can occur. During elongation, nucleosomes are evicted or remodeled in front of the transcribing polymerase, and are subsequently replaced in the region behind the polymerase (Workman, 2009). The replacement of nucleosomes after the passage of the RNA Pol II is an important step that allows nucleosomes exchange and/or recycling (Kulaeva et al., 2007).

Since 75-90% of genomic DNA is wrapped around nucleosomes (Wu et al., 2009), nucleosome occupancy certainly contributes to the regulation of gene expression. In fact, nucleosomes follow a non-random distribution in the genome, as they mainly cover the coding regions. In contrast, non-coding regions are characterized by a low nucleosome occupancy. These “open” regions are preferentially located at transcription start sites (TSS), which may be depleted of nucleosomes to retain their accessibility for transcription factors binding (Segal et al., 2006; Liu et al., 2011). The differential nucleosome occupancy observed at the level of coding versus non-coding regions possibly depends on specific genomic sequences. As a matter of facts, it has been proposed that coding regions are enriched in nucleosomes because of their relatively high GC content, while non-coding regions, that are characterized by a low GC content, are depleted of nucleosomes (Schwartz et al., 2009). These observations allow the computational prediction of nucleosome binding sites on the genomic sequence, that can be subsequently validated experimentally (Segal et al., 2006; Wu et al., 2009; Liu et al., 2011)

The first evidence of a connection between AS and nucleosome organization dates back to 1991, when Beckmann and colleagues observed that the distance between consecutive 5' and 3' splice sites in the pre-mRNA is very similar to the unit of DNA wrapped around a single nucleosome (Beckmann et al., 1991). Thanks to technological advancements in high-throughput screenings, this initial observation was supported by robust experimental evidence. Schwartz and colleagues found a consistently higher nucleosome occupancy on exons with respect to flanking introns, possibly due to the higher GC-content found in coding regions (Schwartz et al., 2009). Moreover, well-positioned nucleosomes were found in exons with weak splice signals and in isolated exons, suggesting that nucleosomes may facilitate the recognition of these exons. A difference in the levels of nucleosome occupancy was also found between constitutive and alternatively spliced exons. The same observations were independently published by Tilgner and colleagues (Tilgner et al., 2009), who found that a high nucleosome occupancy in genomic regions containing exons with weak splice sites. Moreover, they found a striking connection between nucleosome positioning and exon definition. By analyzing high-throughput data from human and from *C.elegans*, these authors found that high nucleosome occupancy on specific exons is evolutionarily conserved and correlates with the exon inclusion rate. More specifically, exons which are constitutively included in the mature transcript have high nucleosome occupancy within and upstream of their sequence. Those nucleosomes, that are located within the exons, are preferentially localized at the centre of the coding sequence, and not at the level of the splice sites. Tilgner and colleagues finally propose that the nucleosome-dependent exon definition is independent from transcription, as non-expressed genes, as well as actively expressed genes, show a comparable nucleosome occupancy in their exons. The consideration that nucleosomes define the exons independently from transcription is sustained by the study of Andersson and coauthors (Andersson et al., 2009). In this report, the authors reasoned that the presence of nucleosomes in inactive genes suggests that they participate in the exon-intron architecture of the genome in completely opposite way compared to their role in the regulation of transcription. As a matter of fact, they found significant difference in nucleosome occupancy at the level of actively expressed genes versus silenced genes. In particular, they found that low-expressed genes display a relative depletion of nucleosomes around their TSS, while actively transcribed genes show a peculiar nucleosome pattern that defines the region around their TSS. Nucleosomes located around the TSS are highly mobile and prone to be displaced, in accordance with their role in transcription regulation. In contrast, nucleosomes located in the body of the gene are more resistant to displacement (Weiner et al., 2010).

For what the position occupied by nucleosomes respect to the 5' and 3' splice sites is concerned, the debate is still open, as observations differ. Some reports (see for example Kogan et al., 2005) indicate that nucleosome patterns are evolutionarily conserved and that they mirror the distribution of the splice sites. In this scenario, nucleosomes would participate to the splicing reaction by "covering" and protecting the splice sites from possible mutations. Instead, other reports (see for example Andersson et al., 2009; Tilgner et al., 2009) found a specific enrichment of nucleosomes within the exons, and not at the level of the 5' and 3' splice sites. Despite these differences, there is a general consensus that nucleosomes are recruited on the specific genomic regions based on their sequences.

The correlation between nucleosome positioning and AS has been very recently addressed by Keren-Shaul and colleagues (Keren-Shaul et al., 2013). Based on the evidence that loss of RNA Pol II causes a relaxation of chromatin (Weiner et al., 2010), and that

transcription causes massive rearrangements in nucleosomes positioning (Kulaeva et al., 2007), the authors investigated whether the AS reaction could also participate in regulating chromatin structure. They found that overexpression of mutated forms of the U1 snRNA, either with low or strong efficiency in binding to the 5' splice sites present in the pre-mRNA, has an impact on chromatin structure. Specifically, a more efficient binding of U1 snRNA induces an increase in exon inclusion and a concomitant increase in nucleosome occupancy at the level of internal alternative exons. This is paralleled by a local increase in the amount of pausing RNA Pol II at the level of the alternative exon, a feature which is linked to exon inclusion (de la Mata et al., 2003). Even if the mechanism underlying the AS-dependent increase in nucleosome occupancy is still unclear, it appears to be independent from transcription, as both the low-efficient and the strong-efficient form of U1 have the same effects in terms of RNA Pol II recruitment.

But how nucleosomes positioning influences the cotranscriptional AS reaction? The mechanism remains elusive, but some possible explanations have been proposed. First, nucleosomes may affect the RNA Pol II processivity, which in turn may have an impact on exon inclusion (Keren-Shaul et al., 2013). This putative connection may be true, even if several reports agree that nucleosome positioning on exons is independent from transcription (Tilgner et al., 2009; Keren-Shaul et al., 2013). Second, nucleosomes may influence AS thanks to post-translational modifications (PTMs) present in the tails of the histones that constitute them. The growing body of evidence connecting histone PTMs and AS suggests that this explanation is highly probable, and it is sustained by the observation that the nucleosomes that define the exons in the genome are not only positioned in a predictable way but they also carry specific histone PTMs (Andersson et al., 2009, see below). Third, nucleosomes, either directly or indirectly, may recruit the splicing factors on the splice sites of the nascent pre-mRNA. Even if the splicing reaction can occur *in vitro* without the presence of nucleosomes, the same reaction is more efficient *in vivo* when it is coupled to transcription (Das et al., 2006). This consideration, together with the robust evidence that nucleosomes are positioned more stably on alternative exons with weak splice sites (Tilgner et al., 2009), indicates that nucleosomes are a crucial player in defining the exon-intron architecture in the genome.

## 4. Chromatin Remodeling and Alternative Splicing

Eukaryotic cells have evolved a number of enzymatic complexes that are able to change the chromatin architecture. These protein complexes, known as chromatin remodelers, are divided in four main families: SWI/SNF, ISWI, CHD and INO80. These families share common features, such as the ability to disrupt the contacts between nucleosomes and DNA, their multi-subunit composition, and the presence of a constitutive and evolutionarily conserved ATPase subunit (Clapier et al., 2009). The ATP-driven chromatin remodelling activity of these complexes regulates the accessibility of the different regions of the chromosome (Hargreaves et al., 2011). The “remodelers” play crucial roles in regulating DNA replication, repair, and recombination, as well as gene expression. In particular, chromatin remodelers are involved in the regulation of transcription, by controlling the accessibility of transcription factors (TFs) at the level of the promoters and by facilitating

transcription elongation in the body of the gene. Chromatin remodelling complexes are recruited to specific sites of the chromatin thanks to presence, in their subunits, of protein domains (bromodomains and chromodomains) that recognize specific histone modifications (such as acetylation, methylation and phosphorylation) (Clapier et al., 2009).

As reported by a paper from Muchardt's lab (Batsché et al., 2006), it has been proposed that chromatin remodelling complexes play also a role in the AS regulation. Specifically, the authors report that Brahma (BRM), one of the two mutually exclusive ATPase subunits of the human SWI/SNF chromatin remodelling complex, is able to modulate the AS reaction by enhancing the inclusion of alternative exons which reside in the body of the genes. In particular, when BRM is present inside the SWI/SNF complex, it interacts with components of the splicing machinery such as U1 and U5 snRNPs. BRM is also able to cooperate with Sam68, a well-known exon inclusion enhancer. This interaction, as well as the shared interaction between Brm, Sam68 and U5, increases the inclusion of alternative exons. Concomitant with the presence of this complex, accumulation of RNA Pol II-pSer5 was observed in the same genomic regions. The pSer5 modification of the CTD is linked to a slow processive form of the polymerase and to exon inclusion (de la Mata et al., 2003). Most importantly, the positive effect played by BRM on exon inclusion seems to be specifically connected to exons with weak splice sites, which, without a molecular mechanism able to increase their inclusion, would be instead excluded from the mature transcript. In conclusion, this paper suggests a intriguing link between chromatin remodelling complexes and cotranscriptional AS. The observation that BRM plays a role in the exon inclusion process is substantially confirmed by an independent paper, in which Ito and colleagues (Ito et al., 2008) reported that Brm is involved in the inclusion of *TERT* exon 7, by an interaction with p54<sup>nrb</sup>. Interestingly, modulation of exon 7 inclusion may have an impact on the activity of the telomerase, the protein which is encoded by the *TERT* gene,

Interestingly silencing of different subunits of the SWI/SNF complex in *Drosophila* cells indicate that this complex is not only involved in the AS of internal exons, but also in the choice of different polyadenylation sites. A first report provides evidence that the *Drosophila* SWI/SNF complex is associated with the nascent pre-mRNPs (Tyagi et al., 2009). A more recent paper from the same group then demonstrated that selective knock-out of the *Drosophila* core SWI/SNF subunits (Brm, Snr1 and Mor) affects the alternative processing of a subset of transcripts, changing the relative abundance of the isoforms produced (Waldholm et al., 2011).

## **5. Histone Post-Translational Modifications and Alternative Splicing**

All the four major histone types that are included in the nucleosome have an amino-terminal region that protrudes beyond the nucleosome surface. This "tail" is the target of a wide variety of post-translation modifications (PTMs). In contrast to the DNA methylation, the only chemical modification that occurs on DNA so far identified, histones have at least 100 different PTMs, which include methylation, acetylation, phosphorylation and ubiquitination (Bernstein et al., 2007). Table 1 summarizes the major PTMs affecting transcription and splicing.



From a mechanistic point of view, histones PTMs have an impact on the stability of the interaction between nucleosomes and DNA, modulate the protein-protein interactions involving histones, and/or generate the platform that triggers other subsequential histone PTMs. Acetylation of the histone tails, catalyzed by histone acetylases (HATs), removes the positive charges present on the lysine residues, thereby decreasing the interactions between the tails and the negatively charged phosphate groups present in the DNA. As a consequence of HATs activity, chromatin structure becomes more relaxed and transcription is generally facilitated. On the contrary, de-acetylation of the histone tails, catalyzed by the histone deacetylases (HDACs), results in a more closed and compacted chromatin structure associated with transcriptional silencing. Compared to acetylation, methylation, the second major histone PTM, has a different effect as the addition of a methyl group does not alter the relative charge of lysine and/or arginine residues. For this reason, methylation (catalyzed by histone methylases, HMTs) and demethylation (catalyzed by histone demethylases, HDMs) of histone tails have different outcomes on chromatin compaction depending on their target residue and on the presence of other methyl or acetyl groups in close proximity (Zentner et al., 2013).

The advent of epigenetic studies focused on genome-wide mapping of histone PTMs, has allowed to correlate the presence of these modifications to different gene expression outcomes. It has now become clear that the combination of the modifications targeting the histone tails residing in a specific genomic region creates an “histone code” that can be read by evolutionarily conserved chromatin-interacting proteins, that contain specific interaction domains. For example, the methylation mark is known to recruit proteins containing the chromodomain module (Eissenberg, 2012) while histone acetylation can be read by proteins containing bromodomains (Filippakopoulos et al., 2012). These protein-protein interactions are strictly regulated by “histone writers” (such as the already mentioned HATs/HDACs and HMTs/HDMs) that modulate both the relative abundance of a specific modification in a given genomic region and the amount of PTMs targeting a single residue.

Compared to acetylation, the patterns of methylation are even more complex, because a single lysine can harbor one (me1), two (me2) or three (me3) methyl groups, and the relative level of methylation of a single lysine may also lead to completely opposite effects. For example, it has been proposed that mono-methylation of lysine 9 of histone 3 (H3K9me1) recruits proteins that facilitate transcription (Barski et al., 2007), while di- (H3K9me2) and tri-methylation (H3K9me3) of the same residue are associated to transcriptional silencing (Barski et al., 2007, Rosenfeld et al., 2009). Specific methylases are dedicated to the task of adding the methyl group to a lysine which already harbors one modifications, while other enzymes are able to add this PTM if two methyl groups are already present, thus enhancing the complexity of the regulation of this process. Moreover, whole-genome mapping of histones modifications has revealed that different histone PTMs map in different regions of the body of a gene and are linked to specific outcomes. Some modifications, like H3K4me3 are enriched at the level of transcription start sites (Kolasinska-zwierz et al., 2009), whereas others, such as H3K36me3 and H3K79me3, are instead present in the body of the gene (Spies et al., 2009); moreover, marks such as H3K27me2, have been mapped at the level of intergenic heterochromatin regions (Kolasinska-zwierz et al., 2009). Table 1 is an attempt to simplify the great complexity of the histone codes that has so far been identified, highlighting their proposed functions.

Regarding the connections between histone's PTMs and co-transcriptional AS, it has been observed that not only specific histone marks, such as H2BK5me1, H3K27me3 (Andersson et al., 2009) and H3K36me3 (Spies et al., 2009), are enriched in the gene body and at the level of the exons, but that different modifications also lead to diverse splicing outcomes. For example, it has been proposed that H3K36me3, one of the most studied and debated histone modification, is enriched at the level of the genomic regions containing exons, which are constitutively included in the mature transcript, while it is generally less present at the level of alternative exons (Kolasinska-zwierz et al., 2009).

**Table 1. Histone codes and their link with genes expression and alternative splicing**

Histone	Site	Modification	Proposed functions	References
H1	Lys26	Me1	Transcriptional silencing	Daujat et al., 2005; Garcia et al., 2004
	Ser27	Pho	Transcriptional activation	Daujat et al., 2005
H2A	Lys5	Ac	Transcriptional activation	Cuddapah et al., 2008
	Ser1	Pho	Transcriptional repression	Zhang et al., 2004
H2B	Lys5	Ac	Transcriptional activation	Karlič et al., 2010
		Me1	Transcriptional activation Enriched in exons	Barski et al., 2007 Schwartz et al., 2009
		Me3	Transcriptional silencing	Rosenfeld et al., 2009
H3	Lys4	Me1	Transcriptional activation	Benevolenskaya et al., 2007
		Me2	Transcriptional activation	Kornblihtt et al., 2013
		Me3	Transcriptional activation Alternative splicing	Spies et al., 2009 Koch et al., 2007; Kornblihtt et al., 2013
	Lys9	Me1	Transcriptional activation	Barski et al., 2007
		Me2	Transcriptional silencing	Rosenfeld et al., 2009; Kornblihtt et al., 2013
		Me3	Transcriptional silencing	Barski et al., 2007
		Ac	Transcriptional activation	Koch et al., 2007 ; Kornblihtt et al., 2013
	Lys14	Ac	Transcriptional activation	Koch et al., 2007
	Lys27	Me1	Transcriptional activation	Barski et al., 2007
		Me2	Transcriptional silencing Heterochromatin marker	Rosenfeld et al., 2009 ; Kolasinska-zwierz et al., 2009
		Me3	Transcriptional silencing Enriched in internal exons	Barski et al., 2007; Kornblihtt et al., 2013 Andersson et al., 2009
	Lys 36	Me3	Transcriptional activation Marks constitutively included exons	Schwartz et al., 2009 Kolasinska-zwierz et al., 2009
	Lys 79	Me1	Transcriptional activation	Barski et al., 2007
		Me2	Transcriptional activation	Steger et al., 2008
		Me3	Transcriptional activation Enriched in internal exons	Spies et al., 2009 Andersson et al., 2009
	Ser10	Pho	Transcriptional activation	Lo et al., 2000
H4	Lys20	Me1	Transcriptional activation	Barski et al., 2007
		Me3	Transcriptional activation	Schwartz et al., 2009

How can the presence of specific histone marks influence the pattern of AS? Two mechanisms have been proposed so far. First, similarly to transcription regulation, histone modifications can provide a platform for the recruitment of specific splicing regulators. For example, it has been shown that H3K36me3 recruits proteins which are able to modulate the splicing outcomes, such as MRG15 (and adaptor protein), PTB (a splicing factor which interacts with MRG15) and SRSF1 (a SR protein which enhances exon inclusion) (Pradeepa et al., 2003). Second, histone modifications can influence RNA Pol II processivity. For example, Schor and colleagues demonstrated that depolarization of neurons decreases the inclusion of the murine *Ncam* exon 18 via local changes in specific histone PTMs which in turn influence the Pol II elongation rate (Schor et al., 2009). Specifically, an increase in the acetylation of H3K9 (either triggered by depolarization or by Trichostatin A, TSA) in the genomic region surrounding the variable *Ncam* exon 18 causes an increase in the chromatin accessibility, which locally allows to the polymerase to proceed faster, resulting in exon skipping. Interestingly, this hyperacetylation is paralleled by the increase in the H3K36me3 mark in the all the actively transcribed *Ncam* gene region, indicating a more general change in the chromatin landscape and a possible crosstalk between the histone PTMs. A more recent report by Chen and colleagues demonstrated that the H3K27me2 mark induces an increase in the elongation rate of the polymerase (Chen et al., 2012). This is possible because H3K27me3 recruits the JMJD3 and KIAA1718 demethylases on a subset of genes. These genes are marked at their promoter-proximal regions with the H3K27me3 and H3K4me3 marks, and are also associated with a promoter-proximal, paused RNA Pol II. The JMJD3/ KIAA1718 complex demethylates the H3K27me3 mark, and this event releases the Pol II, triggering the productive elongation phase. This is possible because the JMJD3 complex recruits elongation factors, such as SPT6, SPT16, CDC73, and SETD2. The silencing of JMJD3 and of KIAA1718 reduces the Pol II elongation rate in the bodies of the monitored genes, a feature previously linked to exon inclusion (de la Mata et al., 2003; Batsché et al., 2006). Interestingly, the H3K27me3 mark has been mapped in the bodies of transcribed genes (Schwartz et al., 2006), suggesting that it may play a role in regulating the intragenic RNA Pol II processivity.

## 6. Intragenic DNA Methylation and Alternative Splicing

DNA methylation consists in the addition of a methyl group to the C5 position of the cytosine in a cytosine-phosphate-guanine (CpG) dinucleotide by a DNA-methyltransferase enzyme of the *Dnmt* family (Hattori et al., 2004). In vertebrate genomes the frequency of CpG dinucleotides is lower than expected based on random chance. This is due to the intrinsic instability of the methyl-cytosine that can spontaneously deaminate to thymine. For this reason, CpGs are evolutionarily lost over time, resulting in a progressive general depletion of this dinucleotide (Bird et al., 1980). The CpGs display a completely non-uniform distribution along several genomes, and this dinucleotide appears restricted to clusters termed “CpG islands” (CGIs). CGIs are defined as stretches of interspersed DNA sequences, both enriched in cytosine/guanine content and in the presence of several CpG dinucleotides. CGIs are usually 200 base pairs long, display a lower CpG depletion compared to other genome

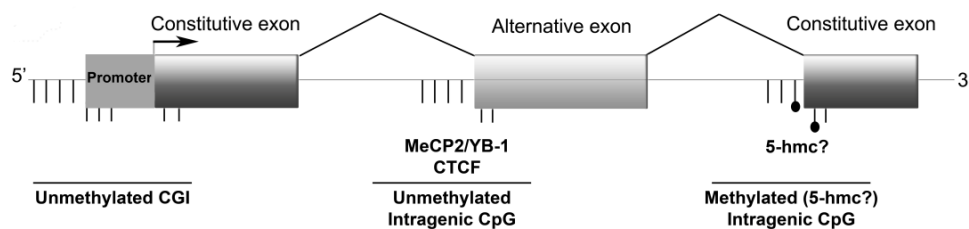
regions, and harbor unmethylated cytosines (Ponger et al., 2002). These clusters of CpG dinucleotides tend to localize in the genomic regions near to the transcription start sites (TSS) of the majority of housekeeping and/or ubiquitously expressed genes, where they constitute a key element defining eukaryotic promoter regions (Gardiner-Garden et al., 1987). A recent report (Vavouri et al., 2010) indicates that CpG-containing promoters have a peculiar transcription-associated chromatin organization, which can be depicted as an ordered and conserved distribution of nucleosomes containing specific histone marks.

CGIs are important elements that epigenetically regulate the expression of eukaryotic genes during differentiation and development. Genome-wide studies revealed that unmethylated CGIs are prominent in undifferentiated cells and in the embryo, and that this methylation-free state is associated with active transcription. During differentiation, some CpG dinucleotides contained in the CGIs acquire the methylation mark in a tissue and cell-specific fashion. This methylated state is associated with silencing of the downstream genes, as the methyl-cytosine directly inhibits the binding of transcription factors and recruits epigenetic modifiers, such as HDACs and Polycomb (PcG) proteins, which are associated with a long-term gene silencing (Deaton et al., 2011).

An enrichment of the CpG dinucleotide has also been detected in intragenic regions and their evolutionary conservation suggests that these sequences hold the potential to epigenetically regulate other “layers” of gene expression, such as AS. It has been observed that the content of the CpG dinucleotide is significantly higher in the introns localized upstream to alternative cassette exons compared to introns preceding constitutive exons or localized downstream to alternative cassette exons. In particular, the CpG frequency is higher in the region flanking the acceptor site of the alternative cassette exons, and is not linked to the relative intron length and/or to the presence of *Alus* sequences (Malousi et al., 2008). An intriguing correlation between the peculiar localization of the CpG dinucleotide at the level of internal cassette exons and the regulation of AS is provided by data published in 2005 by Zoghbi’s group (Young et al., 2005). The paper provides evidences that methyl-CpG-binding protein 2 (MeCP2), the protein containing mutations linked to the emergence of the Rett syndrome, is involved in the regulation AS. MeCP2 was previously described to specifically bind the DNA at the level of methylated CpGs (Nan et al., 2001). By protein co-immunoprecipitation assays, Young and collaborators discovered that MeCP2 directly interacts with the Y box-binding protein 1 (YB-1), a protein which mediates the DNA-RNA interactions involved in the regulation of transcription, translation (Khono et al., 2003) and AS (Stickeler et al., 2001). The MeCP2/YB-1 interaction is RNA-dependent, but independent from methylated DNA. This observation suggests that YB-1 is a RNA-dependent MeCP2 binding protein and that the interaction specifically occurs during transcription and it is separated from the previously reported roles of YB-1 in DNA repair and replication. By using splicing reporter minigenes, the authors demonstrated that the MeCP2/YB-1 complex directly modulates the inclusion of internal alternative exons. This observation was then confirmed by a splicing-sensitive, genome-wide survey of the AS events of endogenous genes expressed in the cerebral cortex of the MeCP2 model mice. Interestingly, this mouse model of Rett syndrome exhibits AS alterations relative to the inclusion of cassette exons containing the YB-1-binding ACE domain, such as the genes encoding the NR1 subunit of the NMDA receptor and the *Dlx5* gene. While this paper demonstrated that MeCP2 can contribute to AS regulation, it did not explore the correlation between cytosines methylation and MeCP2-engagement at the level of intragenic CpGs. However, a very recent report established a link

between intragenic CpG methylation and regulation of AS. Khare and collaborators (Khare et al., 2013) focused their attention on 5-hydroxymethylcytosine (5-hmC), a derivative of methylated cytosine (5-mC), which is highly abundant in human and mouse brain tissues. Using assays able to discriminate between 5-hmC and 5-mC, the authors detected a striking brain-specific enrichment of 5-hmC in genomic regions containing genes involved in synaptic functions. In this specific class of genes, 5-hmC marks the exonic side of the exon-intron boundaries in a brain-specific fashion and has a direct effect on the splicing outcomes. In fact, the authors found that an increase in the 5-hmC content in the region proximal to the exon and/or within the exon is prominent in constitutively included exons, while the 5-hmC modification is less abundant in exons which are subjected to AS.

#### Undifferentiated cells



#### Differentiated cells

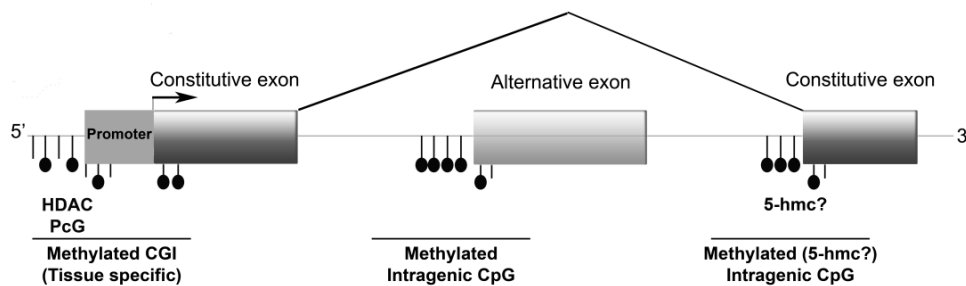


Figure 3. Epigenetic regulation of CpG islands (CGIs) during eukaryotic differentiation and development. Alternative splicing can be modulated during differentiation in a methylation-dependent manner. In undifferentiated cells when intragenic CpG are unmethylated, a MeCP2/YB-1 complex is able to directly modulate inclusion of the alternative exons of CD44, and of CT/CGRP (Young et al., 2005). In differentiated cells, instead a preferential skipping of the alternative exon is observed due to an increase of 5-hydroxymethylcytosine (5-hmC) in the proximal region of constitutive exons, and of methylated intragenic CpGs.

From the mechanistic point of view, it has been proposed that the presence of intragenic CpG methylation inhibits the binding of proteins involved in AS regulation and/or induces the formation of peculiar chromatin structures. Shukla and collaborators (Shukla et al., 2011) focused their attention on the *CD45* gene, which is a widely used model gene to study the regulation of AS. During lymphocyte differentiation the regulated inclusion/skipping of *CD45* variable exon V5 gives rise to protein isoforms that can be easily monitored. As most alternative exons, exon V5 has weak splice sites, so that it is usually skipped. The authors

reported that the genomic region surrounding exon V5 displays an accumulation of the CCCTC-binding factor (CTCF), a protein that has been described for its roles in insulating inactive genomic regions and promoting long-range interactions between distant genomic regions. The accumulation of CTCF on the variant V5 exon promotes its inclusion in the nascent transcript by inducing a localized pausing in the RNA Pol II. An inverse correlation between CTCF accumulation and methylation of the cytosines present in exon V5 was instead observed, indicating that methyl-cytosine inhibit CTCF binding. The DNA methylation patterns change during lymphocyte differentiation, and this dynamic methylation at the level of intragenic sites provide an astonishing mechanistic correlation between exon V5 inclusion and the differentiation process.

To extend this observation, the authors took advantage of CTCF ChIP-seq data, that showed that the great majority of the binding sites for this protein reside in intragenic regions. Other reports, basing on sequencing data, focused their attention on the correlation between intragenic cytosine methylation and the formation of peculiar chromatin structures (see for example Chodavarapu et al. 2011). Figure 3 is a schematic representation of the current knowledge regarding the intragenic methylation-dependent regulation of AS.

## Conclusion

It is well-established that chromatin structure plays an important role in the transcriptional control of eukaryotic gene expression, but only recently it has become clear that chromatin organization can also contribute to AS regulation. AS participate in critical biological processes including cell growth and differentiation, cell death, pluripotency, development (Cooper et al., 2009; Kalsotra et al, 2011). The importance of AS is underscored by the fact that 95% of human genes produce alternatively spliced transcripts (Pan et al., 2008; Wang et al., 2008). However, the functional impact of the vast majority of AS events has not been characterized in any way, nor the molecular mechanisms underlying the selection of specific alternative exons are fully understood. These remain major challenges for future research. Moreover, a greater understanding of the structure-to-function relationship – that is, how chromatin organization can influence specific splicing events and what is the biological function of the different mRNA variants – will be required. In this respect, recent studies have revealed a novel layer of complexity. Although this review focuses on the cross-talk between chromatin proteins and splicing factors, it should be mentioned that the emerging class of long non-coding RNAs (lncRNAs) was shown to contribute to epigenetic regulation of gene expression. LncRNA can guide the organization of higher-order ribonucleoprotein complexes thereby modulating the activity of chromatin-modifying complexes (for review Mercer et al., 2013). No doubt that future work will likely uncover new, unexpected mechanisms that connect lncRNAs and chromatin states to regulate AS.

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