

The alternative role of DNA methylation in splicing regulation

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Although DNA methylation was originally thought to only affect transcription, emerging evidence shows that it also regulates alternative splicing. Exons, and especially splice sites, have higher levels of DNA methylation than flanking introns, and the splicing of about 22% of alternative exons is regulated by DNA methylation. Two different mechanisms convey DNA methylation information into the regulation of alternative splicing. The first involves modulation of the elongation rate of RNA polymerase II (Pol II) by CCCTC-binding factor (CTCF) and methyl-CpG binding protein 2 (MeCP2); the second involves the formation of a protein bridge by heterochromatin protein 1 (HP1) that recruits splicing factors onto transcribed alternative exons. These two mechanisms, however, regulate only a fraction of such events, implying that more underlying mechanisms remain to be found.

Alternative splicing and DNA methylation

Introns were identified almost 40 years ago, but we still do not fully understand how the splicing machinery locates short exons embedded between long flanking intron sequences and splices them out to generate a mature mRNA molecule. Splicing is an especially enigmatic process in higher eukaryotes, including humans, in which a large fraction of introns have grown to thousands of nucleotides in length during evolution [1]. By contrast, exons are under selective pressure to maintain a small size-range, with an average length of approximately 147 nt [2]. The tight selection on exon length exists in all multicellular organisms and is likely driven by different packing of exons and introns at the DNA level. Exons have higher nucleosome occupancy levels than do flanking introns, and are apparently under selection to maintain a length which corresponds to the DNA fragment protected by one nucleosome. Several recent studies demonstrate that chromatin structure plays a crucial role in splicing regulation [1–9]. Chromatin organization is influenced by nucleosome density and positioning, as well as by particular histone modifications and DNA methylation. We focus here on the role of DNA methylation in the exon selection process and in the regulation of alternative splicing.

Alternative splicing is an evolutionarily conserved mechanism that increases transcriptome and proteome diversity by allowing the generation of multiple mRNA products from a single gene [10]. More than 90% of human genes were shown to undergo alternative splicing [11,12]. Furthermore, the average number of spliced isoforms per gene is higher in vertebrates [13], implying that the prevalence of alternative splicing in these organisms is important for their greater complexity. The splicing reaction is regulated by various activating and repressing elements such as *cis*-acting sequence signals and RNA-binding proteins [13–15]. Its regulation is essential for providing cells and tissues their specific features, and for their response to environmental changes [16]. Aberrant splicing may result in developmental abnormalities, hereditary diseases, or cancer [17]. Multiple alignments of exon–intron architectures revealed the existence of consensus sequences important for mRNA splicing: the 5' splice site and the 3' splice site located at the 5' and 3' ends of introns, respectively, the branch site sequence, and the polypyrimidine tract located upstream of the 3' splice site [18]. The strength of these sites regulates alternative splicing: the stronger the site is the higher the inclusion level in alternative splicing [18]. Alternative splicing is also regulated by *cis*-acting sequences located on exons or flanking introns which act as binding sites for RNA-binding proteins that can enhance or suppress the inclusion level in alternative splicing.

For a large fraction of exons, splicing is co-transcriptional [6]. This fact opens up new possibilities for epigenetic modifications to regulate alternative splicing: epigenetic modifications can affect chromatin structure, condensing it or opening it up, which affects the elongation rate of Pol II, and as a consequence changes the alternative splicing of 'weak' exons [7]. In addition, chromatin-binding proteins which bind to specific epigenetic modifications can recruit RNA-binding proteins. These RNA-binding proteins are then transferred to the mRNA molecule as it is being transcribed and change its alternative splicing pattern [7].

DNA methylation is an epigenetic modification used by mammalian cells to control the pattern of gene expression and has been implicated in diverse processes including embryogenesis, genomic imprinting, X chromosome inactivation, and regulation of gene transcription [19–22]. More recently, it has also been found to play a role in alternative splicing. DNA methylation occurs predominantly on cytosine in CpG dinucleotides and is achieved by the addition of a methyl group to the 5 position of the cytosine ring by DNA methyltransferases (DNMTs) [23]. CpG-rich regions of

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Keywords: alternative splicing; DNA methylation; CpG; transcription; chromatin organization; nucleosome positioning; histone modifications.

0168-9525/

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approximately 1 kb, known as CpG islands, are found in more than half the genes in the vertebrate genome and are depleted in the rest of the genome [24,25]. DNA methylation is dynamically remodeled during the mammalian life cycle through distinct phases of reprogramming and *de novo* methylation. Following fertilization, the zygote undergoes extensive demethylation followed by gradual methylation during later stages of development [26]. The specific genomic regions where dynamic changes in DNA methylation are observed overlap with transcription factor binding sites and are linked to their targeted regulation [27–30].

Almost 20 years ago it was found that methylation has dual and opposing roles in the regulation of gene expression: in promoter regions, DNA methylation is associated with transcriptional repression, whereas in gene bodies DNA methylation is generally associated with high expression levels [8,31–34]. This paradox emphasizes the different involvement of methylation in particular genomic and cellular contexts. The role of intragenic DNA methylation in mammals has recently emerged as a key point of interest because exon sequences tend to have higher methylation levels than the flanking intron sequences. Furthermore, 34% of all intragenic CpG islands are methylated in the human brain [35]. One possible role of intragenic DNA methylation is to prevent spurious transcriptional activation from cryptic internal promoters [36]. We review below new data indicating that DNA methylation is a regulator of mRNA splicing and alternative splicing, revealing yet another important role for DNA methylation in fine-tuning gene expression in higher eukaryotes.

Higher levels of DNA methylation in exons

In genomes of homeothermic organisms (mammals and birds), two classes of exon–intron architecture have evolved: one with higher GC-content level in exons relative to the flanking introns, and another with a similar GC-content level between exons and their flanking introns. These two groups exhibit differences in nucleosome occupancy patterns between exons and introns. There is a higher level of nucleosome occupancy only in the group of genes in which there is a higher level of GC-content in exons compared to the flanking intron sequences. The second group, which displays similar levels of GC-content in both exons and their flanking introns, shows no difference in nucleosome occupancy pattern between exons and introns (Figure 1) [4,37,38].

Several recent studies in humans, honey bees, and *Arabidopsis* using high-definition profiling of DNA methylation by single-molecule-resolution bisulfite sequencing found enriched methylation in exons compared to the flanking introns [39–41]. Furthermore, in the two exon–intron GC content architecture groups there is a differential degree of DNA methylation between introns and exons, with 17% higher DNA methylation levels in exons compared to the flanking introns in the equal GC-content group and 5% differential DNA methylation in the other group (Figure 1). Therefore, DNA methylation is more abundant in exons compared to the flanking introns, regardless of the GC-content environment. Thus, DNA methylation can be considered as another marker,

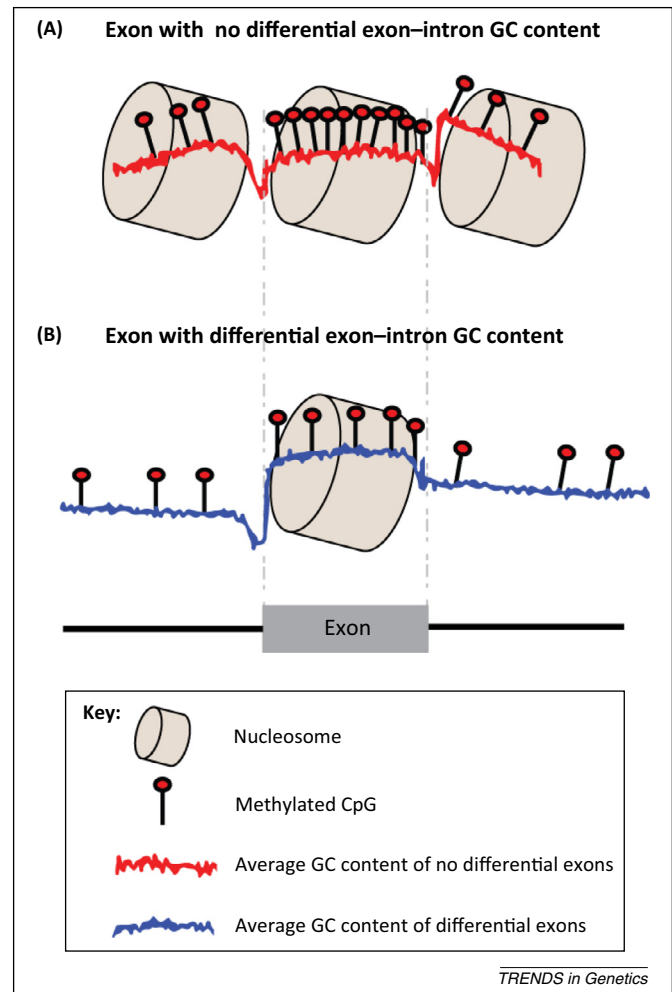


Figure 1. Two different exon–intron architectures (A) The red line represents the mean GC-content for exons flanked by short introns (see [3]) with equal GC-content between exons and their flanking introns. In this group, nucleosomes are spread evenly over the exon–intron architecture and the exons are highly CpG-methylated compared to their flanking introns (see [5]). (B) The blue line represents the mean GC-content for exons flanked by long introns (see [3]). This group shows differential exon–intron GC-content, marked by highly localized exonic nucleosomes, and has slightly higher CpG-methylation on exons compared to their flanking introns (see [5]). The exon–intron architecture is shown at the bottom.

separate from nucleosome occupancy, that distinguishes exons from introns.

The higher nucleosome occupancy found at exons compared to introns might be a factor that drives exonic methylation. Nucleosomes containing methylated DNA were found to stabilize the *de novo* DNA methyltransferases DNMT3A and DNMT3B, and to promote propagation of DNA methylation [42]. This sustained process could eventually result in higher DNA methylation levels in exons, which initially have higher nucleosome occupancy levels. By contrast, this influence might also work in the other direction because dense DNA methylation was found to directly disfavor nucleosomes [43]. These findings suggest that there is a link between methylation and nucleosome occupancy, but it is clear that the nature of the interaction is not yet resolved.

DNA methylation and splicing regulation

Is the higher level of DNA methylation in exons merely a byproduct of other features, such as high nucleosome

occupancy, or does it play a regulatory role? DNA methylation is emerging as an important factor for both exon selection by the splicing machinery and for the regulation of alternative splicing. In the equal exon–intron GC-content group, the presence of CG dinucleotides at specific positions in the 3' splice site and the 5' splice site is correlated with a very high level of DNA methylation (almost 100%) compared to the methylation level of the surrounding regions [5]. In 2009 it was found that the ten-eleven translocation family of enzymes, TET1, TET2, and TET3, mediated oxidation of 5-methyl cytosine to 5-hydroxymethyl cytosine in human and mouse brains [44,45]. 5-Hydroxymethyl cytosine can be further oxidized to 5-formylcytosine and 5-carboxylcytosine [46,47]. A recent study showed enrichment of hydroxymethyl cytosine at exon–intron boundaries in the brain, and DNA methylation is also highly enriched at exon–intron boundaries in non-neuronal cells [48,49]. Moreover, hydroxymethyl cytosine is more abundant in constitutive exons than in exons that are alternatively spliced [49]. A genome-wide study of DNA methylation in mouse retina and brain revealed tissue-specific differentially methylated regions that appear to regulate tissue-specific alternative splicing and transcription [50]. Another genome-wide study of the honey bee demonstrated that inhibition of expression of *DNA methyltransferase 3 (Dnmt3)* causes widespread and diverse changes in alternative splicing that are directly related to decreased methylation levels [51]. These genome-wide studies all made use of correlational observations to link DNA methylation to splicing regulation. A more direct causal connection was provided by a recent study in which a single gene, composed of five exons (two of which are alternatively spliced) separated by four introns, was either *in vitro* CpG methylated or left unmethylated and then inserted into the same genomic target. Thus, DNA methylation was effectively switched on or switched off in that single gene, while keeping the endogenous background unchanged. The addition of DNA methylation to the entire gene increased the inclusion of its alternative exons (while still maintaining a similar level of transcription), thus demonstrating a causal relationship between DNA methylation and alternative splicing [52].

Alternative exons display lower levels of DNA methylation than exons that are constitutively spliced [5,53]. This led to the initial hypothesis that DNA methylation promotes exon inclusion. Recent results challenge this idea and paint a more complex picture. A genome-wide analysis comparing exon inclusion in methylated versus unmethylated mouse embryonic stem cells showed that DNA methylation can either enhance or suppress the inclusion of a distinctly characterized population of alternative exons in a context-specific manner [52]. Lack of DNA methylation did not significantly alter splicing of constitutive exons but had a major influence on the splicing of alternative exons. There, the outcome was exactly the opposite of what was expected, namely high methylation levels repressed exon recognition, whereas low methylation levels enhanced it [52]. The emerging picture is that constitutive exons might have high inclusion 'despite' and not 'because of' high DNA methylation levels because these exons are controlled by much

stronger intrinsic factors (such as strong splice sites) that overshadow the weaker suppressing effects of DNA methylation. In fact, many model organisms, such as *Caenorhabditis elegans*, *Drosophila melanogaster*, *Saccharomyces cerevisiae*, and *Schizosaccharomyces pombe*, that engage in mRNA splicing (with the former two organisms exhibiting also widespread alternative splicing), lack DNA methylation altogether [54]. Therefore, DNA methylation does not appear to be essential for splicing, but instead has a more subtle 'fine-tuning' regulatory role which is dispensable in some species.

The molecular mechanisms that link DNA methylation and alternative splicing

There are three known factors that can transmit information from the DNA methylation level to the regulatory level of alternative splicing: (i) CTCF, (ii) MeCP2, and (iii) HP1. The first two proteins affect alternative splicing by modulating the elongation rate of Pol II, whereas HP1 recruits splicing factors from the methylated DNA onto the mRNA precursor.

CTCF

CTCF is a DNA-binding factor that is responsible for many functions in the cell. When the binding site of CTCF is methylated, binding of CTCF is prevented. In 2012, the first link was demonstrated between DNA methylation and alternative mRNA splicing [55]. The authors analyzed splicing of the human *CD45* gene that is alternatively spliced during lymphocyte differentiation. When CTCF binds to exon 5 it serves as a roadblock that slows the elongation rate of Pol II; this elevates the inclusion level of the alternative exon. DNA methylation inhibits CTCF binding, which enables Pol II to traverse the pre-mRNA more rapidly than when CTCF is bound, resulting in exon 5 exclusion [55,56]. Because CTCF binding sites are also located in exons and introns of genes other than *CD45*, the authors performed a genome-wide analysis in three cell lines (BL41, BJAB, and CD4⁺ T cells) in which CTCF levels were depleted or lowered. Their analysis revealed that, for alternative exons possessing downstream CTCF binding sites, depletion of CTCF released downstream Pol II pausing and lowered exon inclusion levels through kinetic coupling (Figure 2A) [6].

MeCP2

Multifunctional protein MeCP2 is involved in gene regulation at the post-transcriptional level [57]. MeCP2 was the first identified member of a family of proteins with methyl-CpG-binding domains and transcriptional repressor domains. MeCP2 is capable of binding to a single symmetrically methylated CpG both in naked DNA and within chromatin [57–59]. It has also been shown to bind to YB-1, a component of messenger ribonucleoprotein particles and to play a role in splicing regulation [60]. In 2013, it was found that MeCP2 can regulate alternative splicing of particular exons [61]. Knockdown of MeCP2 or treatment that reduces DNA methylation (lowering MeCP2 binding to the DNA) results in aberrant exclusion of alternative exons. This study demonstrates that MeCP2 is enriched at the DNA level in a specific fraction of alternative exons and

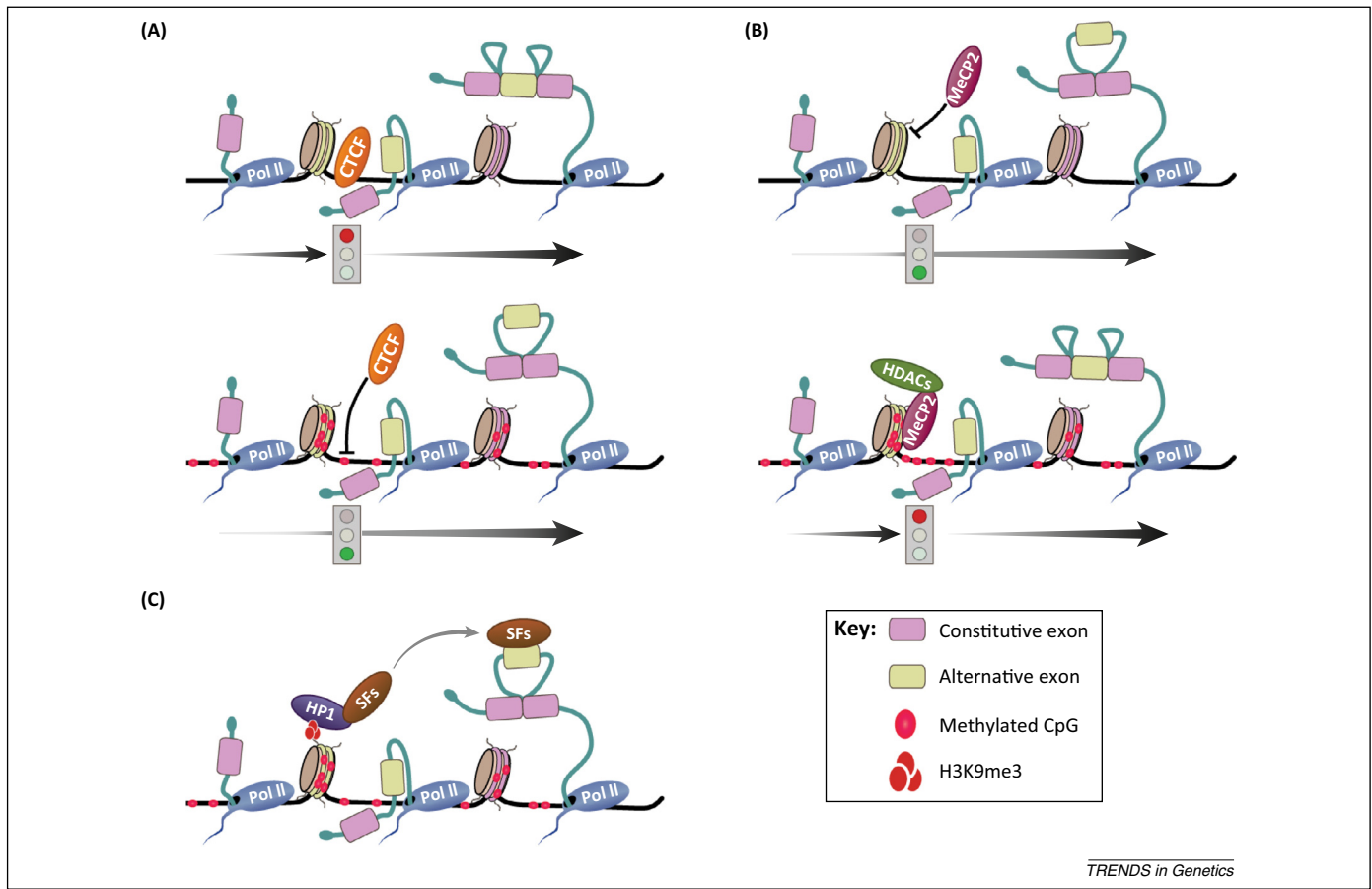


Figure 2. DNA methylation affects alternative splicing by three different factors. **(A)** Upper panel, when DNA is unmethylated, CTCF binds downstream to the alternative exon and creates a roadblock for Pol II elongation which results in exon inclusion on the mature mRNA. Lower panel, DNA methylation downstream of the alternative exon prevents CTCF binding and promotes fast Pol II elongation and exon skipping on the mature mRNA. **(B)** Upper panel, in the absence of DNA methylation, MeCP2 does not bind to the alternative exon, which enables fast Pol II elongation and leads to exon skipping in the mature mRNA. Lower panel, methylation of the alternative exon causes MeCP2 to bind to the exon and recruit enzymes with HDAC activity, which slows Pol II elongation and results in exon inclusion in the mature mRNA. **(C)** Methylated DNA stimulates the histone modification H3K9me3. HP1 binds to this modification and recruits splicing factors (SFs). During transcription, the SFs transfer to the pre-mRNA and modify exon inclusion. Abbreviations: CTCF, CCCTC-binding factor; H3K9me3, histone H3 trimethylated on lysine 9; HDAC, histone deacetylase; HP1, heterochromatin protein 1; MeCP2, methyl-CpG binding protein 2; Pol II, RNA polymerase II.

enhances their inclusion. MeCP2 mediates the effect on alternative splicing by altering the kinetics of Pol II elongation. Specifically, MeCP2 recruits histone deacetylase (HDAC) activity, promoting local histone hypoacetylation, which presumably leads to Pol II pausing and exon inclusion. For MeCP2-regulated exons, the presence of DNA methylation results in exon inclusion, whereas for CTCF-regulated exons the situation is reversed: the presence of DNA methylation results in exon skipping (Figure 2B).

HP1

HP1 proteins are part of the chromodomain superfamily of proteins which contain the methyl-lysine binding chromodomain. There are three HP1 protein family members in humans, each encoded by a separate gene: HP1 α , encoded by *CBX5* (chromobox protein homolog 1); HP1 β , encoded by *CBX1*; and HP1 γ , encoded by *CBX3*. The structure and function of these genes are highly homologous from *Drosophila* to humans, suggesting a very important and evolutionarily conserved function [62]. Several studies have shown that HP1 associates with splicing factors: HP1 β colocalizes and co-immunoprecipitates with serine/arginine-rich splicing factor SRSF1 in mitotic HeLa cells

[63], whereas HP1 α associates with several hnRNPs in *Drosophila* euchromatin [64]. In HeLa S3 cells the Ago protein is found in a complex (or complexes) containing HP1 γ together with several SR proteins, hnRNPs, and other splicing factors [65]. In addition, HP1 α and HP1 γ are clearly linked to splicing regulation: HP1 α mediates both the effect of antisense siRNA on splicing in the fibronectin *FN1* gene [66] and the effect of neuronal depolarization on splicing in the *NCAM* gene [67]. HP1 γ mediates the effect of H3K9 methylation [68] and the effect of Ago proteins [65] on splicing in the *CD44* gene. Furthermore, depletion of HP1 γ from HCT116 cells causes genome-wide aberrant splicing [69].

Recently, a genome-wide approach was used in mouse cells in which expression of each of the HP1 isoforms was inhibited individually or together [52]. This study showed that all three HP1 isoforms regulate alternative splicing. Importantly, the three isoforms display a high level of overlap of about 70–80% of the regulated alternative exons, suggesting that they share similar modes of action. About 20% of the overall effect of DNA methylation on splicing can be explained by an interaction with an HP1 protein [52]. In addition, there appears to be a localization-specific effect of HP1 on alternative splicing. Specifically, HP1

enhances exon recognition when bound (at the DNA level) immediately upstream of an exon, and lowers exon recognition when bound to the exon itself. This position-specific effect is observed for multiple RNA binding proteins such as PTB [70], RBM [71], several hnRNP proteins [72], Nova [73], Fox [74], and Mbnl [75].

A mechanism has been suggested by which HP1 proteins serve as a linker between DNA methylation and the regulation of alternative splicing: at the chromatin level, DNA methylation induces the H3K9 trimethylation (H3K9me3) histone modification on methylated alternative exons. This modification acts as a substrate for HP1 α and HP1 β binding, recruiting HP1 to the alternative exons (at the chromatin level). The HP1 proteins in turn bring several splicing factors to the DNA that encodes the alternative exons [52]. For the fibronectin EDI (extra domain I) alternative exons, SRSF3 binding is modulated by the absence of HP1, affecting its splicing outcome. A similar model was recently demonstrated for the alternatively spliced vascular endothelial growth factor *VEGFA* gene where HP1 γ recognizes H3K9me3 and recruits the splicing factor SRSF1 [76]. These new data reveal a mechanism connecting DNA methylation and alternative splicing by recruitment of splicing factors (Figure 2C).

Concluding remarks

Alternative splicing is regulated on at least three different levels. Most research has been devoted to the RNA level. At this level, splicing factors bind to pre-mRNA and modulate the recruitment of the basal splicing machinery onto the splice sites. Alternative splicing is also regulated by the transcription machinery: for a particular fraction of exons, changes in the elongation rate of Pol II elevates or lowers the inclusion level. Very recently it has become clear that chromatin organization, epigenetic markers, and chromosomal looping are also regulators of alternative splicing. At the DNA level, exons have higher levels of nucleosome occupancy compared to flanking introns, and specific histone modifications tend to mark exons and introns. Pol II pauses before nucleosomes located over exon sequences (exonic nucleosomes), thus regulating the inclusion level of particular exons. Histone modifications that occur at higher levels in 'exonic nucleosomes' provide binding sites for proteins that recruit splicing factors to that specific region of the chromatin or that decrease the elongation rate of Pol II.

Recent results have demonstrated the effect of DNA methylation on alternative splicing. There is a positive correlation between gene body DNA methylation and transcription level. Furthermore, levels of DNA methylation are higher on exons than introns in diverse organisms, even for exons that have a similar GC content compared to the flanking intron sequences. This observation implies that DNA methylation aids in distinguishing exons from introns; more research is necessary to examine this point in detail.

Three proteins are now known to communicate the information encoded in DNA methylation to the splicing machinery. CTCF binding to DNA decreases the elongation rate of Pol II and facilitates exon inclusion. CTCF binding is inhibited by DNA methylation, therefore

methylation results in exon exclusion. MeCP2 binds to methylated DNA and decreases the elongation rate of Pol II to enhance exon inclusion; in this case, DNA methylation results in exon inclusion. The last identified mechanism involves the formation of a protein bridge in which DNA methylation leads to the histone modification H3K9me3. HP1 binds to this histone modification and also binds to several splicing factors. At the chromatin level, HP1 proteins accumulate on the regulated alternative exons, and the splicing factors that bind to HP1 exert their effect on the regulation of these alternative exons differently when they bind or do not bind to HP1s. Thus, whereas CTCF and MeCP2 modulate the elongation rate of Pol II, and thus regulate alternative splicing of the exons located in proximity to their binding sites, HP1 affects the binding of splicing factors to chromatin. These splicing factors are presumably transferred onto the transcribed mRNA precursor and thus regulate alternative splicing.

One of the remaining open questions is – what dictates which of the three mechanisms applies for each alternative exon that is regulated by DNA methylation? We assume that CTCF regulation is probably the most limited because it is the most directly sequence-dependent, whereas MeCP2 and HP1 are more abundant regulators. It will be interesting to find out if there are alternative exons that are regulated by more than one mechanism; for example, one that enhances their inclusion level and another that suppresses it. In such cases, the ratio of all affecting mechanisms will determine the combinatorial effect and the outcome of alternative splicing. Interestingly, DNA methylation is often tissue- or developmental-stage specific, and differentially methylated loci are found in many regulatory regions across the genome. This offers a platform to achieve this type of differential alternative splicing.

Are there additional mechanisms that convey DNA methylation information into exon selection and alternative splicing regulation? Inclusion of about 22% of alternatively spliced exons in mouse embryonic stem cells is affected by DNA methylation. About 20% of the regulatory effect of DNA methylation on splicing can be explained by HP1 proteins [52]. Shukla *et al.* [55] found approximately 100 alternative exons which are regulated via induction of Pol II pausing, resulting in increased exon inclusion via the CTCF pathway. Also, Maunakea *et al.* [61] found that knockdown of MeCP2 affected several thousands of alternative exons. The combined number of alternative exons affected via the CTCF, MeCP2, and HP1 mechanisms probably represents only a fraction of all methylation-regulated exons. There are many other proteins containing a methyl-CpG binding domain (MBD) such as MBD1, MBD2, and MBD4. These proteins are obvious candidates to test for involvement in communicating the information embedded within DNA methylation to the splicing machinery and should be investigated in the future.

Can we pinpoint the DNA methylation effect? It would be interesting to identify the loci where DNA methylation is crucial for the regulation of alternative splicing. Is the signal on the alternative exon itself most important or are the upstream or downstream signals paramount? Several nucleotides were identified in the 5' and 3' splice sites that are especially marked by DNA methylation [5]. These

could be significant in regulating splice-site selection by the splicing machinery.

Though it has a major effect on splicing of alternative exons, DNA methylation has only a minor influence on the regulation of splicing of constitutive exons [52]. Although the influence is much less significant than for alternative exons, DNA methylation may be a regulatory mechanism that even impacts upon constitutive splicing of exons with strong recognition features. This effect on constitutive exons should be carefully examined and may reveal cases where the effect of methylation on splicing is preeminent.

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