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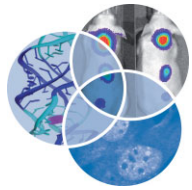

Connections between chromatin signatures and splicing

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Splicing and alternative splicing are involved in the expression of most human genes, playing key roles in differentiation, cell cycle progression, and development. Misregulation of splicing is frequently associated to disease, which imposes a better understanding of the mechanisms underlying splicing regulation. Accumulated evidence suggests that multiple *trans*-acting factors and *cis*-regulatory elements act together to determine tissue-specific splicing patterns. Besides, as splicing is often cotranscriptional, a complex picture emerges in which splicing regulation not only depends on the balance of splicing factor binding to their pre-mRNA target sites but also on transcription-associated features such as protein recruitment to the transcribing machinery and elongation kinetics. Adding more complexity to the splicing regulation network, recent evidence shows that chromatin structure is another layer of regulation that may act through various mechanisms. These span from regulation of RNA polymerase II elongation, which ultimately determines splicing decisions, to splicing factor recruitment by specific histone marks. Chromatin may not only be involved in alternative splicing regulation but in constitutive exon recognition as well. Moreover, splicing was found to be necessary for the proper 'writing' of particular chromatin signatures, giving further mechanistic support to functional interconnections between splicing, transcription and chromatin structure. These links between chromatin configuration and splicing raise the intriguing possibility of the existence of a memory for splicing patterns to be inherited through epigenetic modifications.

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INTRODUCTION

Alternative splicing, the process by which pre-mRNA molecules can be spliced in different ways to generate multiple mRNA isoforms from a single gene, is thought to be the main mechanism of transcriptome and proteome expansion that explain the phenotypic complexity of higher eukaryotes.[1,2] Alternative splicing can generate proteins with different activities or intracellular localization,[3] and also regulate protein levels by generating nonfunctional proteins or mRNA isoforms subjected to nonsense-mediated decay.[4] It is now estimated that more than 90% of the human genes undergo alternative splicing,[5,6] being at least 60% of the human alternative splicing events regulated between tissues.[6] The notion that splicing and alternative splicing must both be very accurate and fine tuned is rather intuitive. As a matter of fact, mutations in *cis*-acting splicing elements and changes in the abundance or activity of splicing regulatory proteins that lead to misregulation of splicing and alternative splicing have been associated with cancer and hereditary disease.[7] Although many questions remain unanswered, it is now well understood that multiple layers of regulation are needed to determine the proper processing of pre-mRNAs.

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The spliceosome is the major ribonucleoprotein complex responsible for splicing catalysis. A single spliceosome forms on each pre-mRNA intron in a stepwise pathway.[3,8,9] The 5′ splice site (5′ss), the 3′ splice site (3′ss), and the branch point sequence (BPS) of every intron constitute the core splicing signals.[3,9] These signals, however, are very short consensus elements whose degree of conservation does not seem to fully explain exon/intron recognition. Proper recognition of both constitutive and alternatively spliced introns is dependent on *trans*-acting factors that bind to pre-mRNA *cis*-acting elements.[3,9] Therefore, splicing regulation depends on the presence of particular sequence elements at the pre-mRNA level and on the abundance of splicing factors that specifically bind to these elements and elicit positive or negative actions over splice site usage. In light of this, splicing must not only be an accurate and precise process but also a highly regulated one in which the spliceosome must be a dynamic and flexible machine,[9] features thought to be achieved by weak binary interactions that are greatly enhanced by the combination of multiple interactions.[10] This is consistent with recent evidence showing that every step during the spliceosome assembly can be potentially reversed, suggesting that regulation can occur at any given stage.[8] An additional challenge in understanding exon–intron boundary recognition appears when analyzing exon–intron architecture in higher eukaryotes: exons are in average 140–150 nucleotides long, whereas introns are very long regions that do not appear to have any constraints in length.[11,12] Accordingly, a mechanism known as exon definition, in which initial spliceosome formation occurs across an exon, has evolved.[13,14] However, keeping in mind that splice sites are very short and the consensus is rather degenerate, how these sequences are recognized within a multitude of similar sequences in huge stretches of RNA is an elusive question.[15] A possible answer comes from the fact that splicing is mostly a cotranscriptional process, meaning that spliceosome is assembled on the pre-mRNA as it is being transcribed, allowing exon recognition as exons are being synthesized. Furthermore, splicing is functionally coupled with transcription in a way that transcriptional features such as RNA polymerase II (RNAPII) elongation rate or splicing factor recruitment by the transcribing machinery influence splicing and, vice versa, splicing factors affect the dynamics of transcription. Finally, we will discuss here recent evidence confirming that chromatin structure, histone marks, and nucleosome positioning act as another layer of splicing regulation.

INSIDE-OUT: LAYERS OF SPLICING REGULATION

RNA Sequence Features Constitute the Basic Code for Splicing and Alternative Splicing

The minimal splice signal motifs (5′ss, 3′ss, and the BPS) present in every intron seem to provide insufficient information to define exon–intron boundaries. This is revealed by the existence of several intronic regions, called pseudoexons, that have the full appearance of exons flanked by apparently strong splice sites and that are never included in mRNAs.[3] The auxiliary information required for splicing to occur comes then from other *cis*-acting RNA elements that recruit positive or negative *trans*-acting splicing factors.[3,9] When considering alternative splicing, these elements and factors become of particular importance: the usage of competing weak splice sites is going to be dependent on the balance between positive and negative factors acting on them. According to their location on the pre-mRNA and their positive or negative action, the RNA elements are classified into exonic splicing enhancers (ESEs) and silencers (ESSs), and intronic splicing enhancers (ISEs) and silencers (ISSs).[3] Most ESEs recruit SR proteins[3] that are thought to stabilize the exon definition complex, whereas most ESSs bind hnRNPs that usually act negatively on exon recognition.[3]

RNA immunoprecipitation coupled to high-throughput sequencing (HITS-CLIP, see Box 1) allowed the identification of several splicing factor-binding sites in a transcriptome-wide manner. Further analysis indicates that splicing factors may exert positive or negative actions depending on the context and their relative binding distance to alternative exons, revealing a rather complex splicing regulation network.[16] Taking into consideration the complex interconnections between RNA regulatory elements in alternative splicing regulation, a 'splicing code' with high predictive capacity of tissue-dependent alternative splicing was built.[17] The aim of this effort was to predict their splicing patterns along several tissues, based solely on their RNA sequence features. To assemble the code, genome-wide RNA expression analysis (RNA-seq, see Box 1) of 27 mouse tissues, grouped in nervous system, muscle tissues, digestive system tissues, and whole embryos, was used to profile over 3500 alternative exons in terms of tissue-specific splicing patterns. In parallel, a collection of RNA features was compiled, being an 'RNA feature' the combination of each of the alternative splicing regulatory motifs considered and their presence along seven RNA regions that span the alternative exon and the flanking introns and exons. The regulatory

BOX 1

HIGH-THROUGHPUT SEQUENCING TECHNOLOGIES IN SPLICING AND CHROMATIN STUDIES

RNA-seq: Double-stranded cDNA fragments are generated from total or polyadenylated RNA fractions and subjected to high-throughput sequencing. RNA-seq allows mRNA expression analysis in a quantitative manner as well as alternative isoform expression characterization and relative quantification. Alternative splicing patterns can be studied across tissues and global changes can be analyzed upon drug treatments or protein knockdown.[5,6]

HITS-CLIP: It is used to study transcriptome-wide RNA–protein interactions. HITS-CLIP (high-throughput sequencing—UV cross-linking and immunoprecipitation) employs *in vivo* UV cross-linking to create covalent bonds between RNA and proteins allowing immunoprecipitation of the RNA bound to specific proteins; this is converted into double-stranded cDNA fragments and subjected to high-throughput sequencing. PAR-CLIP (Photoactivatable-Ribonucleoside-Enhanced Crosslinking and Immunoprecipitation) increases cross-linking efficiency, and iCLIP allows single nucleotide resolution. This approach is used to generate tissue-specific and transcriptome-wide maps of splicing factor RNA-binding sites.[16,18]

MNase-seq: Chromatin extraction is followed by micrococcal nuclease (MNase) digestion, which digests DNA stretches not wrapped around nucleosomes (linker DNA). DNA purification and further high-throughput sequencing allow to map genome-wide occupancy sites of nucleosomes. This was used to study nucleosome positioning in regards to exon–intron architecture.[19]

ChIP-seq: Chromatin immunoprecipitation coupled to high-throughput sequencing allows mapping of protein–DNA interactions genome wide. It can be used to map transcription factor-binding sites and RNAPII occupancy profiles. It is also used to study histone mark signatures across entire genomes in a tissue and differentiation stage comparative manner.[20]

Methyl-seq: Bisulfite DNA treatment can also be coupled to high-throughput sequencing in order to detect specific methylated CpG sites.[21]

motifs compiled here comprise previously known motifs, new motifs (putative or supported by weak evidence), short conserved intronic sequences around alternative exons, and structural features such as exon and intron length and sequences that potentially form secondary structures. The observed splicing patterns along tissues were then correlated with the presence of these RNA features and, by a method that recursively selects features to maximize the code quality, the splicing code was finally assembled with ~200 RNA features. When put to test by comparing predictions with microarray data and RT-PCR results, this code showed high rates of prediction capacity and was sufficient to account for much of the tissue-specific splicing regulation, at least when considering the direction of change between pairs of tissues.[17]

Cotranscriptionality of Splicing: Regulation on Top of RNA

More than 20 years ago, electron microscopy visualization of nascent transcripts of Drosophila embryos[22] demonstrated that splicing can occur cotranscriptionally, meaning that introns are excised from the pre-mRNA before RNAPII reaches the end of the gene. Since then, compelling evidence using various approaches reinforced this notion and showed that transcription and RNA processing are nuclear processes that occur in a coordinated manner and by complexes acting in coordination. Analysis of chromatin-bound and nucleoplasmatic RNA fractions showed that introns are efficiently excised within the chromatin-bound fraction. Moreover, the promoter proximal introns are more efficiently excised than the distal ones,[23] suggesting that introns are removed as they are being transcribed. It should be noted, however, that the sequential order is not strict: introns are not necessarily removed in the exact order they are transcribed. They can be eliminated in different orders, and some can be processed cotranscriptionally, whereas others can be processed post-transcriptionally.[23–25] Two different stages in splicing should be distinguished at this point: recruitment of splicing complexes to pre-mRNA and splicing catalysis itself. Studies revealed that while catalysis can be both cotranscriptional and post-transcriptional, being the prevalence dependent on the organism (in yeast splicing catalysis is mainly post-transcriptional, whereas in mammals it is mainly cotranscriptional),[23,26] recruitment of splicing complexes and splicing commitment is mostly cotranscriptional in all organisms.[27–31] It can be thought then that there is a selective pressure for cotranscriptionality at the 'commitment to splice' level. Interestingly, in a recent work, single-cell and single-molecule imaging technologies were used to assess spatial and temporal associations between

transcription and splicing.[32] It was shown that while constitutive splicing catalysis is tightly cotranscriptional, this is not always the case when analyzing alternative splicing events. Specifically, mutations that mildly disrupt the 3′ss snRNP-binding sites lead to an increase in post-transcriptional splicing. Accordingly, alternative splicing events where skipping is induced by negative factors binding to intronic 3′regions showed the same transition from cotranscriptionality to post-transcriptionality.[32] Altogether, the evidence suggests that the order of intron removal and its cotranscriptionality versus post-transcriptionality both depend on the underlying mechanism of exon recognition.

Cotranscriptionality of splicing is a prerequisite to the functional coupling between splicing and transcription, meaning that features of one process, such as protein recruitment and kinetics, can influence the other one in two-way interconnections.[33–35] One of the molecular mechanisms of coupling involves splicing factor recruitment to transcription sites by the transcription machinery. A key player in this recruitment appears to be the carboxy-terminal domain (CTD) of the catalytic subunit of RNAPII, as it is known to recruit splicing factors to the sites of transcription in a phosphorylation-pattern-dependent manner.[36–39] Moreover, *in vitro* studies revealed that SR proteins enhance splicing efficiency only when added before transcription.[40] Functional coupling becomes also evident when analyzing alternative splicing events whose patterns are dependent on transcription factor recruitment to the promoter.[41–43] In a recent study, the transcriptional coactivator complex Mediator was shown to interact with the splicing factor hnRNPL, and thus it regulates hnRNPL-dependent events by modulating its promoter occupancy.[41]

Transcription can also be influenced by splicing.[44,45] The SR protein SRSF2 (previously known as SC35), for instance, was found to affect RNAPII elongation.[45] More generally, RNAPII pausing was detected in yeast on the terminal exon of intron-bearing genes, a feature shown to be dependent on splicing.[46,47] The timing of the pause is coincident with that of splicing factor recruitment,[46] suggesting a selective pressure toward cotranscriptionality as this mechanism of splicing-dependent RNAPII stalling apparently favors it. In humans, RNAPII dynamics at the 3′ end of genes was also found to be dependent on splicing.[48] Specifically, it was shown that accumulation of RNAPII at the 3′ end of genes and subsequent retention of the pre-mRNA depend on the stage at which spliceosome assembly is experimentally inhibited.[48]

Alternatively to the splicing factor recruitment model of coupling between transcription and splicing discussed so far, a kinetic model in which RNAPII elongation rates determine the outcome of alternative splicing was also described. Sequences that induce RNAPII pausing, treatment with drugs that inhibit RNAPII elongation by different mechanisms (5,6-Dichlorobenzimidazole 1-β-D-ribofuranoside (DRB), favopiridol, or camptothecin), and transactivators that induce only transcription initiation versus those inducing both initiation and elongation, all were shown to favor the inclusion of several alternative cassette exons.[25,30,49–51] Direct evidence for the role of transcriptional elongation in alternative splicing came from the use of a 'slow' mutant of RNAPII that increases exon inclusion when considering several alternative splicing events.[49,52] Finally, and in a more physiological context, signaling elicited by DNA damage caused by UV irradiation results in changes in alternative splicing patterns of many genes, including the upregulation of the proapoptotic splicing isoforms of *Bcl-X* and *Caspase 9*. The underlying mechanism involves inhibition of RNAPII elongation caused by CTD hyperphosphorylation.[53] The kinetic model of coupling between transcription and alternative splicing can be summarized as follows: slow elongation favors the recruitment of splicing factors to the upstream intron before the downstream intron is synthesized, which in turn promotes exon inclusion. It is worth noting that slow elongation can also lead to higher exon skipping in alternative splicing events in which binding of a negative regulatory factor to the upstream intron is critical.[49,52]

CHROMATIN, SPLICING, AND ALTERNATIVE SPLICING

It is now well established that chromatin structure influences transcription.[54,55] Nucleosomes are a major barrier to RNAPII elongation,[56] and even though promoters and transcription start sites of active genes are naturally depleted of nucleosomes, they occupy the body of most genes. Many mechanisms assist RNAPII in avoiding these obstacles, such as disassembly and assembly of nucleosomes, chromatin remodelers that slide, and evict histones and transcription factors that enhance elongation.[55,57] Histone post-translational modifications (referred here as histone marks) are also involved in the modulation of chromatin compaction.[55,58] Histone acetylation, for instance, directly alters DNA–histone associations, promoting a more loosened nucleosomal structure.[58] Aside from this direct action on chromatin, histone marks may also affect the recruitment of nonhistone proteins to

TABLE 1 | Some Histone Marks and Their Roles in Transcription

Histone Mark	Enzymes Responsible[58]	Associated Transcriptional Activity[54,55]	Gene-Related Distribution[54,55]	Evidence Showing Connections with Splicing
H3/H4Ac	HAT1, CBP/p300, PCAF/GCN5, TIP60	Activation	Promoter region of active genes	Nogues et al.,[50] Schor et al.,[61] Zhou et al.[62]
H3K27me3	EZH2	Repression	Inactive loci	Allo et al.[63]
H3K9me2, me3	Suv39h, G9a, Eu-HMTase I, ESET, SETBD1	Repression	Inactive loci, also in the body of active genes	Allo et al.,[63] Saint-Andre et al.[64]
H3K4me1	MLL, SET1, ASH1	Activation	Enhancer regions and body of active genes	Sims et al.,[65] Luco et al.[66]
H3K4me3	MLL/WRD5, SET1, ASH1	Activation	Promoter and 5′ end of active genes	
H3K36me3	HYPB, SMYD2, NSD1	Repression of internal initiation, cotranscriptional deposition	Gene body, increasing toward the 3′ end of active genes	Kolasinska-Zwierz et al.,[78] Luco et al.,[66] Fernandes de Almeida et al.,[68] Kim et al.[69]
H3K79me1	Dot1	Activation	Gene body of active genes	

chromatin, many of which have enzymatic activities, further modifying chromatin structure.[58] H3K9 methylation, for instance, recruits heterochromatin protein 1 (HP1) that binds the methyltransferase responsible for the H3K9me, thus favoring further recruitment of HP1, which in turn dimerizes and promotes chromatin packaging into facultative heterochromatin.[58,59] Altogether, how tight DNA is wrapped around nucleosomes together with the capacity of the transcribing machinery to overcome nucleosomal barriers constitute a physical basis for transcriptional elongation regulation. Different histone marks may also participate in the recruitment of various complexes involved in transcriptional activation, repression, and DNA replication. This raises the notion of chromatin being an active platform in which histone marks may act in a combinatorial way as a 'histone code' to control gene expression. Particular combinations were found to be associated to distinct genomic and functional regions such as promoters of active genes, intergenic regions of active genes, large-scale repressed regions, and repetitive sequences.[58,60] Some histone marks and their associated functions in transcription are summarized in Table 1. The important underlying concept is that chromatin is a highly dynamic structure and that its regulation determines gene expression both by modulating protein recruitment and RNAPII elongation rates.

Genome-Wide Analysis of Chromatin Signatures and Their Connections to Splicing

A panoply of reports using high-throughput data analysis to assess the distribution of chromatin signatures and their cross-talk to splicing have been recently published. They revealed some unexpected and intriguing correlations between the chromatin structure and RNA processing fields. Genome-wide mapping of nucleosome density (MNase-seq, see Box 1) in human cells strikingly revealed that nucleosome positioning is higher on exons compared to introns.[70–73] Interestingly, this distribution was also found to be the case in *Caenorhabditis elegans* and *Drosophila melanogaster*,[70–73] suggesting that nucleosome preference to exons is conserved at least from worms to humans. Further analysis of nucleosome density data of human cells showed that the intensity of nucleosome occupancy on exons is inversely correlated to splice site strength[72,73] and that this biased nucleosome distribution is not dependent on transcription.[70,73] Together with the fact that pseudoexons flanked by strong splice sites appeared to be depleted of nucleosomes,[73] evidence suggests a correlation between nucleosome occupancy and exon recognition, supporting a novel functional role for nucleosomes. In *Arabidopsis thaliana*, nucleosomes also show a preference for exons,[21] allowing to speculate that this feature might be the result of functional convergence in organisms with complex genomes. As in higher eukaryotes exons are in average short sequences of approximately 140–150 bp in length surrounded by long introns variable in length,[11,12] and being 147 bp the length of DNA that is wrapped around a nucleosome, speculations can be made on evolutionary constraints that may have acted on exon length so that exon recognition could be enhanced by nucleosomes.[74] In support of this hypothesis, exons flanked by long introns were observed to be more enriched in nucleosomes

than clustered exons.[72] Other similar speculations can be done in regards to base composition of exons in comparison to intronic sequences.[71–73] Nucleosomes are preferently positioned on GC-rich regions[75,76] and, accordingly, exons have higher GC content.[73] Moreover, exons surrounded by weak splice sites have higher GC content than exons surrounded by strong splice sites and pseudoexons, correlating with the observed nucleosome positioning. Supporting these observations, it was also observed that noncoding exons have higher GC content and nucleosome occupancy than introns, ruling out the idea that GC content of exons might only be dependent on codon usage.[73] Keeping in mind that in higher eukaryotes introns are much longer than exons and that RNA sequences are thought to be insufficient to define exons, the findings that average exon length correlates with that of nucleosome-wrapping DNA stretches and that DNA sequences that favor nucleosome positioning are prevalent in exons, while introns are enriched in disfavoring sequences, add new relevant features to explain exon recognition.[73,74] Two alternative mechanisms were suggested to explain how nucleosomes might enhance exon recognition during cotranscriptional splicing: nucleosomes might recruit splicing factors through specific histone marks or they might mediate changes in transcription elongation rates, as it is known that nucleosomes greatly impair RNAPII elongation.[56,57] RNAPII stalling around nucleosome-enriched exons might further enhance splicing factor recruitment, by bringing the transcription and splicing machineries in close temporal and spatial proximity. Accordingly, nucleosome enrichment on exons was found to positively correlate with RNAPII accumulation, suggesting reduced elongation rates in these regions.[21,71] Nevertheless, further experimental approaches aimed at assessing causal effects of nucleosome positioning on exon recognition are needed. Other possible explanations for a functional role of nucleosome positioning on exons were proposed, e.g., nucleosomes could have a protective role over exonic sequences upon DNA damage exerted by UV light.[77] Nucleosome occupancy and its putative regulatory relevance in pre-mRNA processing becomes even more relevant when being analyzed across alternative splicing events and correlated to their inclusion/exclusion levels. Indeed, nucleosome occupancy on exons positively correlates with their inclusion levels.[71] High nucleosome positioning was also found on some intronic sequences previously typified as pseudoexons. This accurately predicted that many of them are actually alternative exons that are included in at least one tissue sample.[73] Therefore, nucleosomes might not only reinforce exon recognition in those cases in which splice sites are weak but they could have regulatory roles in alternative splicing as well. How this regulation takes place in physiologically relevant circumstances and which alternative splicing events are modulated by changes in nucleosome positioning remain to be studied.

A striking finding concerning chromatin and splicing is that there is a differential distribution of histone marks according to the exon–intron architecture of genes. H3K36me3, for instance, was found to be enriched in exons of active genes both in *C. elegans* and in human cells.[70,72,78] This is consistent with the fact that this histone mark is enriched in the body of active genes because the methyltransferase responsible for its deposition, HYPB2/Setd2, is associated to the CTD of the elongating RNAPII.[79] Even though there is some discussion about whether the enrichment observed on exons is a mere reflection of the underlying nucleosome occupancy or if it is independent of it, an interesting observation was made in regards to splicing and alternative splicing: in *C. elegans*, mouse, and human cells, alternatively spliced exons bear lower H3K36me3 enrichments in comparison to the flanking constitutive exons.[78] In another analysis, a positive correlation was found between H3K36me3 enrichment and alternative exon inclusion.[70] These observations suggest that the accumulation of H3K36me3 on exons is somehow related to the splicing process. Many other histone marks were found to be enriched in exons, some also being dependent on the transcriptional status of the gene.[70,72] Among them, H3K79me1 shows a similar distribution as the one observed for H3K36me3 in regards to splicing.[70,72] DNA methylation of CpG islands was also found in humans and in *A. thaliana* to be enriched at exons in comparison to introns.[21,67] This is consistent with the fact that DNA methylation correlates with nucleosome positioning.[21]

Much of the above evidence relies on correlations, which calls for cautiousness at the moment of interpretations in terms of functionality and biological relevance. Further experimentation is needed to leave the speculation area. Nevertheless, the evidence strongly suggests a widespread functional interconnection between chromatin, transcription, splicing, and alternative splicing, and, in light of these observations, it becomes clear that regulation of gene expression and pre-mRNA processing mediated by the underlying chromatin structure constitutes a general mechanism of regulation.

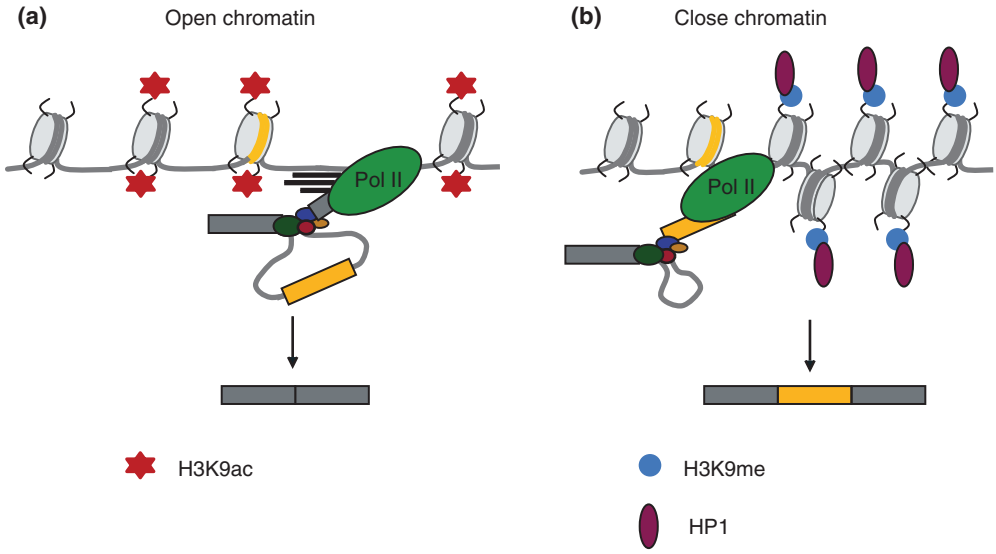

FIGURE 1 | Models of alternative splicing regulation through chromatin structure. (a) Intragenic histone acetylation induces chromatin relaxation favoring high RNA polymerase II (RNAPII) elongation rates and ultimately inducing exon skipping. (b) Intragenic H3K9 methylation induces DNA compactation into chromatin by HP1 recruitment. Accordingly, RNAPII elongation rates decrease and alternative exon inclusion is favored.

Chromatin Control of Alternative Splicing through Modulation of RNAPII Elongation

The first evidence that linked chromatin structure to alternative splicing regulation through RNAPII elongation emerged about 10 years ago in our laboratory. In these early studies, it was shown that when a plasmid bearing a splicing reporter minigene is allowed to replicate in the nucleus of a human cell, and thus to acquire a more physiological chromatin structure in terms of nucleosomes being more properly assembled,[80] there is more inclusion of the alternative cassette exon in the mRNA product of the minigene.[51] Further experiments, in which this effect is reverted by factors that stimulated RNAPII elongation, gave rise to the hypothesis that alternative exon inclusion is enhanced by reduced RNAPII elongation rates due to template DNA compactation into chromatin.[51] Furthermore, the effect is reverted by trichostatin A (TSA) treatment, a histone deacetylase inhibitor that enhances histone acetylation and subsequent chromatin relaxation.[50,51]

Further research shed light to direct functional links between post-translational modifications of histone tails and alternative splicing. Regulation of alternative splicing of the *ncam* gene, encoding the neural cell adhesion molecule (protein), and its functional association to the chromatin context was studied in a murine neuronal depolarization model.[61] It was shown that upon membrane depolarization of N2a cells with increased KCl concentrations, H3K9 acetylation (H3K9ac) increases around the alternative cassette exon 18 (E18), correlating with its increased skipping. In contrast, no modulation of histone acetylation was observed at the promoter region.[61] The effect of H3K9ac on E18 skipping was confirmed by treating cells with TSA, which mimics and even potentiates the effect of depolarization on E18 skipping.[61] Most importantly, the effect of H3K9 hyperacetylation on exon skipping is mediated by a mechanism of kinetic coupling between transcription and alternative splicing: histone hyperacetylation induces the relaxation of the chromatin structure, shown by an increase in the chromatin accessibility to the restriction endonuclease MspI, that ultimately leads to an increased processivity of RNAPII[61] (Figure 1(a)). The importance of intragenic histone acetylation levels in alternative splicing regulation was also revealed in a murine neuronal differentiation model.[62] In neurons differentiated from mouse embrionic stem (ES) cells, the alternative exon 23a of the *Nf1* gene and the alternative exon 6 of the *Fas* gene are almost skipped, whereas their inclusion is favored in ES cells, a difference that can be explained by intragenic H3 and H4 acetylation during differentiation.[62] Such an increase is not paralleled at promoter regions and depends on the recruitment of Hu proteins to chromatin, where they directly inhibit histone deacetylases activity in neurons only, as they are not expressed in ES cells.[62] Interestingly, Hu proteins bind to pre-mRNAs specific binding sites, being this binding a prerequisite to their chromatin association and further histone deacetylase inhibition.[62] As observed in the neuronal

depolarization model, changes in splicing patterns were associated to changes in RNAPII elongation rates.[62] Hu proteins are also direct alternative splicing regulators that induce exon skipping by blocking the positive regulator TIA1-binding site.[81] A positive feedback mechanism of alternative splicing regulation is then suggested: Hu proteins act directly at the RNA level inducing exon skipping and, as they bind to the pre-mRNA, they also stimulate changes in the chromatin structure that reinforce the splicing decision.

Changes in repressive histone mark levels within intragenic regions were implicated in alternative splicing regulation through RNAPII elongation rate changes as well[63,64] (Figure 1(b)). It was first shown that the transfection of exogenous small interfering RNAs (siRNAs) targeting the sequence in the downstream intron of the alternative exon EDI (exon 33) of the endogenous *fibronectin* gene induced EDI inclusion through local facultative heterochromatin formation around the alternative cassette exon.[63] The mechanism involved here resembles that of transcriptional gene silencing, first described around promoter regions,[82] and depends on Argonaute 1 and 2 proteins, part of the nuclear RNAi silencing pathway.[83] siRNAs targeting regions upstream of EDI have no effect on its inclusion, suggesting a mechanism that acts locally and downstream of the alternative exon.[63] It was clearly shown that upon transfection of the intronic siRNAs, H3K9me2 and H3K27me3 levels increase in these regions, being this enrichment reduced when analyzing further downstream regions.[63] This effect was abolished by treating cells with drugs that favor chromatin relaxation, implying a causal relationship between the local repressive chromatin structure and the splicing outcome.[63] This work not only demonstrates that repressive histone mark profiles in intragenic regions can determine and modulate alternative splicing patterns but also raises the possibility of regulation by endogenous siRNAs and other noncoding RNAs, an expanding intriguing field.[84] Observations in a more physiological context further support the role of intragenic repressive histone marks in alternative splicing regulation. This is the case of the *CD44* gene that is composed by a cluster of alternative exons flanked by constitutive exons.[64] Upon activation of the MAP kinase signal transduction pathway, known to induce variant exons inclusion, levels of H3K9me3 increase intragenically with the appearance of a clear peak of HP1γ over the variant exons region.[64] HP1γ was found to be in part responsible for the RNAPII accumulation around the cluster of alternative exons and for an enhanced recruitment of U2AF65, with the concomitant exon inclusion, all of which is bypassed when knocking down the histone lysine methyltransferases EHMT1 and EHMT2.[64] Besides binding to H3K9me through its chromodomain,[59] HP1γ was also found to bind RNA through a different domain,[85] raising the possibility of HP1γ acting as a bridge between chromatin and the pre-mRNA of *CD44*. Accordingly, HP1γ was found to promote increased retention to chromatin of the pre-mRNA sequences comprising the variant region, correlating this with an increased association between those RNA regions and the transcribing RNAPII machinery.[64] This last evidence not only supports the notion that closed chromatin structures elicited intragenically by repressive histone marks may influence alternative splicing by modulating RNAPII elongation but also supports the idea that histone marks can modulate how close the association between the pre-mRNA and the transcribing RNAPII is. How changes in this association may affect the inclusion levels of the alternative exons into the CD44 mRNA is yet to be elucidated.

DNA methylation in alternative splicing regulation was recently demonstrated by Shukla et al.[86] In a lymphocyte cell line model, inclusion of the alternative exon 5 (E5) of the *CD45* gene is favored by the accumulation of CTCF specifically around the exon.[86] In addition to the well-established roles in transcriptional insulation,[87] CTCF was shown here to reduce elongation of the active RNPII without affecting global *CD45* expression.[86] As CTCF is ubiquitously expressed, a mechanism must exist to target it to specific gene regions. DNA methylation is known to impair CTCF association to chromatin.[87] Accordingly, a DNA methylation site was found downstream of *CD45* E5 and a negative correlation was observed between methylation of this DNA site and CTCF accumulation around E5.[86] Furthermore, inhibition of the methyltransferase DNMT1 in lymphocytes that naturally have low levels of E5 inclusion showed higher binding of CTCF and higher E5 inclusion levels[86] (Figure 2). A genome-wide analysis showed that this model of regulation seems to operate in other genes: the comparison of cell-type-specific CTCF-binding sites and high-throughput RNA sequencing data of two different cell lines revealed a strong correlation between CTCF depletion, exon inclusion and RNAPII pausing, if CTCF-binding sites are downstream not upstream of the alternative exons.[86]

Altogether, the evidence discussed here provides a clear association between histone mark profiles, DNA methylation, and alternative slicing regulation through changes in RNAPII elongation. Importantly, changes in chromatin landscapes were observed in

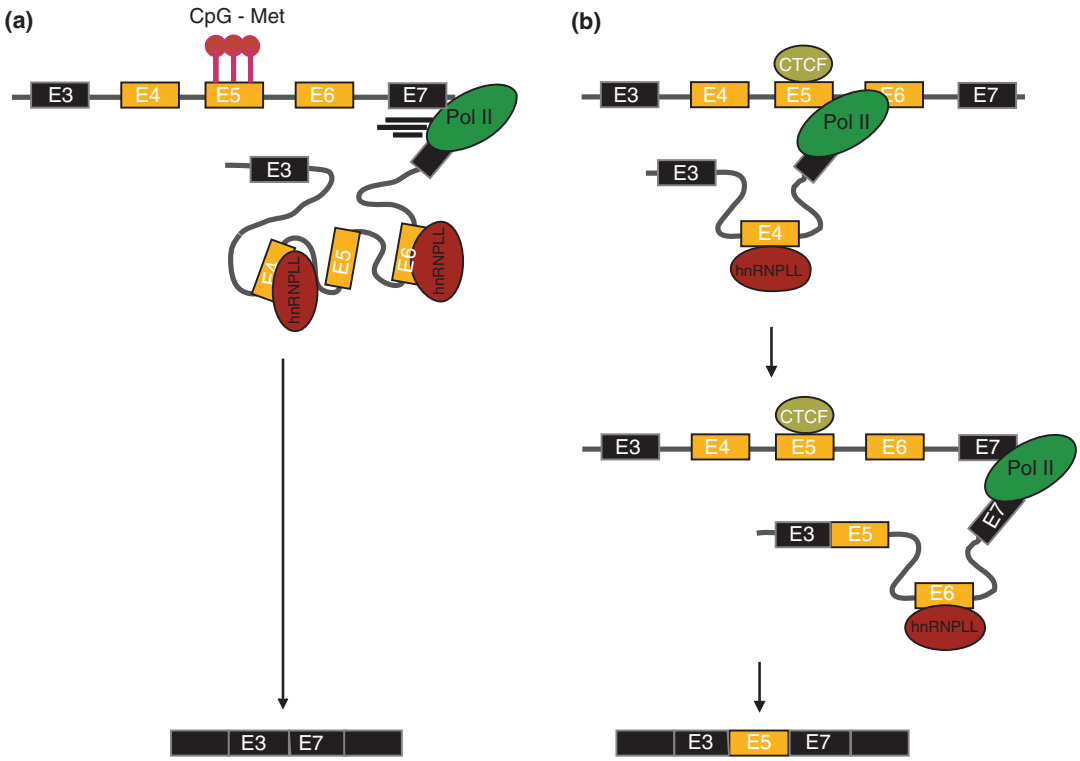

FIGURE 2 | Model of alternative splicing regulation by DNA methylation and CTCF accumulation described for exon 5 (E5) of the *CD45* gene by Shukla et al.[86] (a) E5 skipping is favored by fast RNA polymerase II (RNAPII) elongation rates promoted by specific DNA methylation in the exonic region that inhibits CTCF binding. E4 and E6 skipping, on the other hand, is promoted by hnRNPL binding to pre-mRNA. (b) In the absence of DNA methylation, CTCF binds to E5 DNA where it creates roadblocks to RNAPII elongation favoring E5 recognition and inclusion. E4 and E6 skipping is not affected by this mechanism.

intragenic regions and around the alternative exon regions in particular, modulating RNAPII elongation locally and without affecting the global transcriptional status of the host gene.

Chromatin in Splicing Factor Recruitment

Chromatin is a huge platform to protein recruitment through histone marks.[58] The cotranscriptional nature of splicing raises the possibility of this also being the case for spliceosome components and splicing factors. While the pre-mRNA is still in close association to chromatin, recruitment of these factors might be greatly enhanced if they are previously bound to chromatin. Multiple examples illustrate the association of spliceosome components and splicing factors to chromatin through other proteins that bind specific histone marks:

- The chromatin remodeling complex SWI/SNF is recruited to the alternative exon region of the CD44 gene, regulating its inclusion levels. SWI/SNF acts independently of its ATPase remodeling activity but it depends on its chromatin binding capacity. When bound to chromatin it interacts with snRNPs, U5snRNP specifically, and with the hnRNP protein Sam68,[88] and thus it enhances exon inclusion.
- The human histone acetyltransferase STAGA complex interacts with SF3b, a subunit of the U2snRNP.[89]
- SR proteins SRSF1 (formerly known as SF2/ASF) and SRSF3 (SRp20) were found in close association to chromatin in a H3S10 hypophosphorylation-dependent manner.[90]
- The H3K4me3-binding protein CHD1 interacts with the U2 snRNP subunit SF3A1. Upon depletion of CHD1 or of the H3K4 methyltransferase, with the concomitant reduction in SF3A1 recruitment to chromatin-associated pre-mRNA, the rate of the splicing reaction of an inducible splicing reporter minigene was shown to be greatly reduced, without affecting the steady-state splicing efficiency.[65]

Luco et al.[66] uncovered an adaptor system of splicing factor recruitment to specific histone

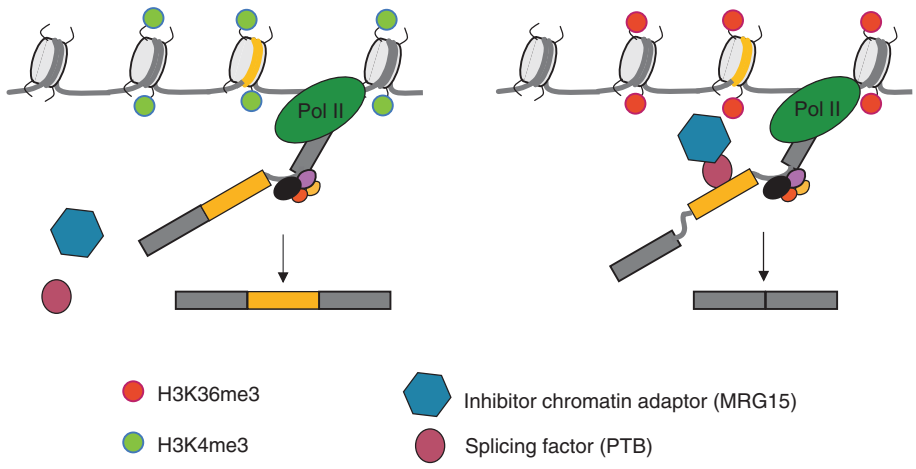

FIGURE 3 | Model of alternative splicing regulation by splicing factor recruitment to specific histone marks described by Luco et al.[66] for PTB-dependent exon skipping. The inhibiting splicing factor binding to the pre-mRNA is favored when it also binds H3K36me3 via an adaptor protein. When H3K36me3 intragenic levels are low and H3K4me3 are high (left panel), binding of the inhibitor factor to the pre-mRNA is disfavored and exon inclusion occurs. Conversely, when H3K36me3 intragenic levels are high and H3K4me3 are low (right panel), the inhibiting splicing factor is recruited to chromatin so its binding to pre-mRNA is favored and exon skipping occurs.

marks involved in alternative splicing regulation. A comparative analysis between chromatin signatures and alternative splicing patterns of genes that bear polypyrimidine tract-binding protein (PTB)-dependent alternative spliced exons revealed a positive correlation between specific histone mark distributions and PTB-dependent repression of alternatively spliced exons, correlation not observed in PTB-independent alternative splicing events and constitutive exons.[66] Whenever PTB-dependent repression of exon inclusion is observed, H3K36me3 is found to be enriched around the alternative exons. Conversely, H3K4me3 was found to be depleted in these regions.[66] Revealing a functional association, downregulation of the H3K36 methyltransferase promoted the inclusion of the normally repressed PTB-dependent exons, but not of PTB-independent or constitutive ones. Alternatively, overexpression of the H3K4 methyltransferases leads to similar results.[66] Further experiments showed that PTB associates to H3K36me3 through binding to MRG15, an adaptor protein that specifically binds to this histone mark. Accordingly, overexpression of H3K36 methyltransferase in the absence of MRG15 had no effect on the inclusion levels of PTB-dependent exons[66] (Figure 3). Interestingly, genome-wide analysis revealed that the H3K36me3-MRG15 adaptor system of PTB recruitment is more relevant in those cases in which the PTB-binding sites on the pre-mRNA are weak,[66] reinforcing the idea that the histone code acts in concert with the RNA code strengthening or weakening RNA-binding sites usage and thus modulating alternative splicing patterns.

From Splicing to Chromatin: Splicing as Determinant of Chromatin Signatures

As discussed above, distinct distributions of H3K36me3 were found within the body of active genes as this mark is not only enriched on exons but also positively correlates with the inclusion levels of alternative exons and with constitutive versus alternative exons.[70,78] Most interestingly, a genome-wide analysis demonstrated that intron-less genes have lower levels of H3K36me3 compared to intron-bearing genes, independently of their transcriptional activity.[68] This suggests that the actual process of splicing might have a relevant role in the writing of this mark. This was demonstrated by functional studies in which disruption of splicing, either by knocking down spliceosome components or by treating cells with splicing inhibitors (spliceostatin A or meayamycin), caused a reduction in H3K36me3 deposition that can be explained by a reduction in the intragenic recruitment of HYPB/Setd2, the enzyme responsible for H3K36 methylation, without affecting RNAPII occupancy levels.[68] Another report showed that splice site mutation and global splicing inhibition cause a redistribution of H3K36me3 toward the 3′ end of active genes with a reduction at 5′ regions without affecting global transcriptional activity,[69] supporting a role of splicing in H3K36me3 pattern determination (Figure 4). Altogether, this line of evidence suggests that both transcription and splicing can shape the chromatin landscape, maybe in a way to further influence splicing decisions and thus establishing positive or negative regulatory circuits.

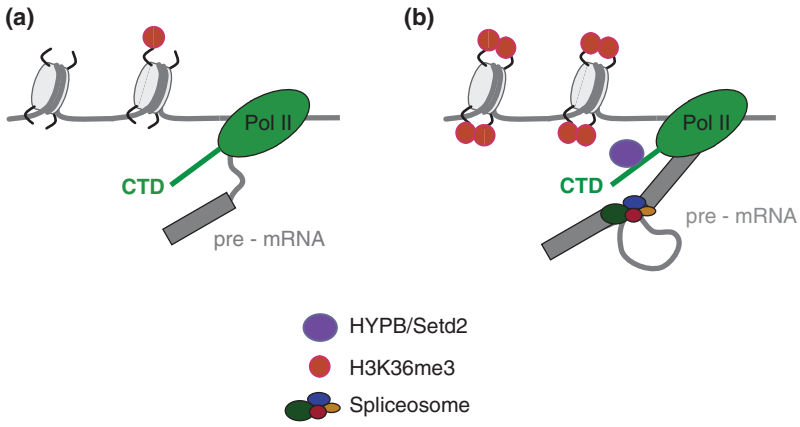

FIGURE 4 | Model of splicing-dependent H3K36me3 deposition.[68] As RNA polymerase II (RNAPII) transcribes and productive spliceosomes are assembled, recruitment of HYPB/Setd2 to the CTD is enhanced and intragenic H3K36me3 levels increase.

CONCLUSIONS

At present, regulation of both constitutive and alternative splicing can be understood as the result of multiple elements and biological processes acting in concert. This is evidenced by the fact that the presence of conserved sequence elements at the RNA level and the coexpression of specific splicing factors, even when considering them in a combinatorial manner, cannot explain all the observed splicing patterns and tissue-dependent splicing regulation. Transcription and splicing act in close proximity and factor recruitment to either of the two machineries is highly dependent on the other one. This also applies to their kinetics: splicing is dependent on RNAPII elongation as well as RNAPII dynamics is dependent on splicing. Considering that both transcription and splicing are highly dynamic and flexible processes, the interacting layers of splicing regulation have a high degree of complexity. Chromatin structure, conceived as the combination of histone marks, nucleosome occupancy, and DNA methylation patterns, is a key actor among these layers. One way in which chromatin was found to influence splicing decisions is through RNAPII elongation. Particular histone marks are known to determine the compaction state of chromatin through various mechanisms, and, when located at intragenic regions, they influence splicing choices by modulating RNAPII elongation rates. In addition, RNAPII stalling caused by chromatin roadblocks enhances the temporal association between the transcription and splicing machineries, favoring splicing factor binding to the pre-mRNA. Chromatin signatures are responsible for recruiting transcription factors that might in turn influence splicing choices. In a similar way, histone marks were also found to regulate splicing by directly binding to splicing factors and hence mediating their binding to pre-mRNA. All the described mechanisms of splicing regulation through chromatin are of special relevance when analyzing alternative splicing events that are regulated by ubiquitously expressed splicing factors: binding to the pre-mRNA might be dependent on the presence of particular histone marks, independently of their high expression levels. Accordingly, the information needed for tissue-dependent alternative splicing regulation is now thought to be encoded both at the chromatin level as well as the RNA/splicing factor level. The observation that nucleosomes and several histone marks are enriched in exons over introns supports the idea that chromatin structure is not only involved in alternative splicing regulation but that it might be involved in constitutive exon recognition as well, through mechanisms that could also span from RNAPII stalling around exons to splicing factor recruitment. Comparative histone mark and DNA methylation mapping across the genomes of different tissues and developmental stages coupled to their transcriptome characterization will be necessary to have a comprehensive picture of the influence of chromatin on tissue-specific alternative splicing. More importantly, how chromatin signatures may act in a combinatorial way to determine 'splicing states' is a matter of great interest still unsolved. Other questions that arise involve the possible mechanisms by which chromatin structure is modulated to regulate splicing. One mechanism might involve interconnections between the splicing and transcription machineries with the enzymatic complexes responsible for histone marks deposition. Examples have been described in which the splicing process determines histone mark distributions maybe influencing future splicing and transcriptional decisions. Many experiments, however, are required to explore this hypothesis.

Regulation by chromatin structure also raises the intriguing possibility of splicing patterns being propagated throughout the cell cycle, in the sense that

given splicing outcomes could be durably maintained in time through epigenetic information encoded in chromatin signatures (note that 'epigenetic' is used here in *sensu stricto* meaning inheritable changes made above the DNA sequence). Some histone marks and DNA methylation are thought to carry epigenetic information as they were shown, for instance, to be important in inheritable transcriptional regulation.[91] An important goal for future research would be to determine whether the chromatin signatures shown to be important in alternative splicing regulation are truly epigenetic or if they are merely transient modulators.

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