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Chromatin and Alternative Splicing

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Alternative splicing affects more than 90% of human genes. Coupling between transcription and splicing has become crucial in the complex network underlying alternative splicing regulation. Because chromatin is the real template for nuclear transcription, changes in its structure, but also in the "reading" and "writing" of the histone code, could modulate splicing choices. Here, we discuss the evidence supporting these ideas, from the first proposal of chromatin affecting alternative splicing, performed 20 years ago, to the latest findings including genome-wide evidence that nucleosomes are preferentially positioned in exons. We focus on two recent reports from our laboratories that add new evidence to this field. The first report shows that a physiological stimulus such as neuron depolarization promotes intragenic histone acetylation (H3K9ac) and chromatin relaxation, causing the skipping of exon 18 of the neural cell adhesion molecule gene. In the second report, we show how specific histone modifications can be created at targeted gene regions as a way to affect alternative splicing: Using small interfering RNAs (siRNAs), we increased the levels of H3K9me2 and H3K27me3 in the proximity of alternative exon 33 of the human fibronectin gene, favoring its inclusion into mature messenger RNA (mRNA) through a mechanism that recalls RNA-mediated transcriptional gene silencing.

Most mRNA expression processes that take place in the nucleus are highly coordinated and involve multimolecular complexes that couple transcription with pre-mRNA processing. Pre-mRNA capping, splicing, cleavage, and polyadenylation as well as mRNA export are known to occur cotranscriptionally (Kornblihtt et al. 2004; Bentley 2005; Perales and Bentley 2009). Cotranscriptionality seems to be a prerequisite but does not necessarily imply coupling of transcription with processing. However, the fact that cotranscriptional splicing seems to be more prevalent than thought before (Das et al. 2007; Pandya-Jones and Black 2009) suggests that the evolutionary advantage of cotranscriptionality resides in allowing for coupling. One of the advantages of cotranscriptional splicing when compared with posttranscriptional splicing could be that the former preferentially drives the nascent pre-mRNAs to the association with spliceosome components (Lacadie et al. 2006; Listerman et al. 2006) and splicing regulatory factors, such as serine/arginine-rich (SR) proteins (Das et al. 2007). This improves splicing efficiency, allows for different levels of regulation of alternative splicing, and helps to prevent back-hybridization of the nascent pre-mRNA to the DNA template strand, which can cause genome instability due to accumulation of DNA breaks triggered by the single-strand status of the nontemplate strand (Li and Manley 2005; Aguilera and Gómez-González 2008).

The current view is that alternative splicing regulation not only depends on the interaction of splicing factors with splicing enhancers and silencers, but also on the coupling with RNA polymerase II (Pol II) transcription. One of the first indications for this coupling was the finding that promoters affect alternative splicing. The idea that promoter regulation affected only the quantity and not the quality of the gene transcript dominated our conception of gene expression in the past. However, the finding that promoter identity and occupation by transcription factors modulates alternative splicing (Cramer et al. 1997, 1999; Auboeuf et al. 2002; Pagani et al. 2003; Robson-Dixon and García-Blanco 2004; for review, see Kornblihtt 2005) deeply questioned that conception and opened the way to consider that other factors classically restricted to transcriptional regulation, such as chromatin structure, could also be important for splicing regulation. The original observation of the promoter effect involved transient transfection of mammalian cells with reporter minigenes for the alternatively spliced cassette exon 33 (E33, also referred to as EDI or EDA) of human fibronectin (FN) under the control of different Pol II promoters (Cramer et al. 1997, 1999). For example, when transcription of the minigene is driven by the α-globin promoter, E33 inclusion levels in the mature mRNA are about 10 times lower than when transcription is driven by FN or cytomegalovirus (CMV) promoters (Fig. 1). These effects are not the trivial consequence of the promoter strength but instead depend on some qualitative properties conferred by promoters to the transcription/RNA processing machinery.

The finding that promoter structure is important for alternative splicing predicts that factors regulating alternative splicing could be acting through promoters and that cell-specific ones may not simply result from the differential

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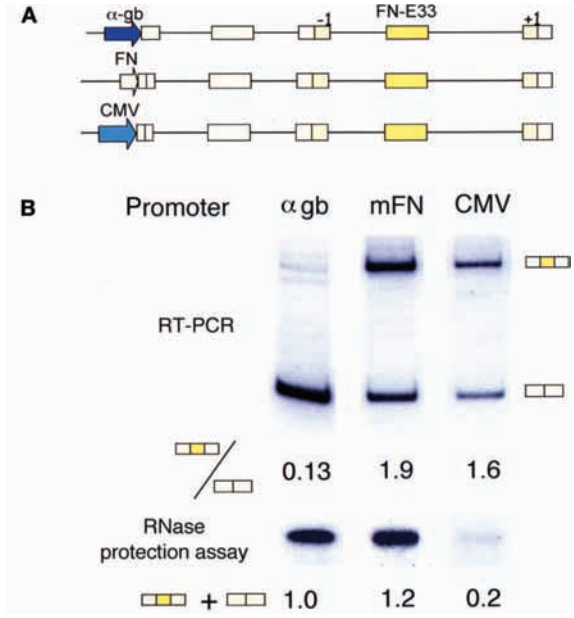

Figure 1. Promoters affect alternative splicing. (*A*) α-globin/FN hybrid minigenes under the control of three different promoters, used in transient transfections of mammalian cells in culture to assess inclusion levels of the alternatively spliced E33 (EDI or EDA) cassette exon (dark yellow). (*B*) Reverse-transcriptase–polymerase chain reaction (RT-PCR) analysis followed by native polyacrylamide electrophoresis shows that inclusion levels with the FN and CMV promoters are >10-fold higher when compared to inclusion levels with the α-globin promoter. Ribonuclease protection assays (*B, bottom*) show that expression levels are higher with α-globin and FN promoters compared with the CMV promoter. (Based on Cramer et al. 1997.)

abundance of ubiquitous SR proteins but from a more complex process involving cell-specific promoter occupation. However, promoters are not swapped in nature and because most genes have a single promoter, the only conceivable way by which promoter architecture could control alternative splicing under physiological conditions should be the differential occupation of promoters by transcription factors of a different nature. Accordingly, it has been found that transcriptional activators and coactivators with different actions on Pol II initiation and elongation affect alternative splicing differentially (Nogués et al. 2002; Auboeuf et al. 2004).

Two nonexclusive models have been proposed to explain the effects of promoters, transcription factors, and coactivators on alternative splicing: The recruitment model, by which different factors associated with the transcription machinery, in particular, with Pol II's carboxy-terminal domain (CTD), regulate splicing choices, and the kinetic coupling model, where the rate of Pol II elongation influences the outcome of the alternative events by providing different windows of opportunity in the usage of weak and strong splice sites, depending on the timing of their emergence

Although there is accumulated evidence supporting both models (for review, see Kornblihtt 2007), we focus here on the kinetic coupling mechanism. The mechanistic framework underlying kinetic coupling is based on the "first come, first served" hypothesis (Aebi and Weissman 1987). Skipping of many cassette exons, such as FN E33, occurs because the 3′ splice site of the upstream intron is suboptimal compared with the 3′ splice site of the downstream intron. In previous interpretations, we have speculated that if the polymerase paused anywhere between these two sites, elimination of the upstream intron would take place first. Once the pause was passed or the polymerase proceeded, there would be no option for the splicing machinery but to excise the downstream intron, which would lead to higher exon inclusion (Fig. 2B). Instead, highly elongating Pol II would favor the simultaneous presentation of both introns to the splicing machinery (Fig. 2A), a situation in which the stronger 3′ splice site of the downstream intron would outcompete the weaker 3′ splice site of the upstream intron, resulting in exon skipping. However, the recent realization that inhibition of elongation does not affect the relative rate of intron removal (de la Mata et al. 2010) prompted us to abandon this interpretation, at least for FN E33. This does not mean that we should abandon the "first come, first served" model, but instead rethink it: Slow elongation would favor recruitment of splicing factors to the pre-mRNA that would favor E33 recognition and commitment to subsequent inclusion. Once commitment is achieved, the order of intron removal becomes irrelevant to guarantee inclusion. According to this interpretation, "first served" would not be equivalent to "first excised" but to "first committed" (Fig. 2C).

A role for Pol II elongation on alternative splicing had been suggested before the finding of the promoter effect (Eperon et al. 1988) but obtained stronger support later from evidence showing that alternative splicing is (1) regulated by transcriptional pauses introduced inside genes (Roberts et al. 1998), (2) differentially affected by transcription factors that stimulate either initiation or elongation of transcription (Nogués et al. 2002), (3) affected when transcription is performed by slow Pol II mutants (de la Mata et al. 2003; Howe et al. 2003), and (4) affected by inhibition of Pol II elongation resulting from Pol II hyperphosphorylation triggered by the DNA damage signaling that follows irradiation of cells with ultraviolet (UV) light (Muñoz et al. 2009). In the cases of both the slow Pol II mutant (Boireau et al. 2007) and UV-mediated Pol II hyperphosphorylation (Muñoz et al. 2009), inhibition of transcriptional elongation was measured in live cells and in real time using imaging techniques. The UV effect constitutes an example of a naturally occurring physiopathological process affecting alternative splicing through its kinetic coupling with transcription and illustrates the regulation of transcriptional elongation through changes in the intrinsic transcribing properties of the enzyme. However, a different way to affect alternative splicing via elongation exists: the modulation of chromatin structure and compaction at intragenic regions that may either inhibit or facilitate the passage of elongating Pol II.

DNA REPLICATION, CHROMATIN, AND ALTERNATIVE SPLICING

A role for chromatin in alternative splicing was suggested almost 20 years ago when it was found that two copies of the same adenovirus genome in the same nu-

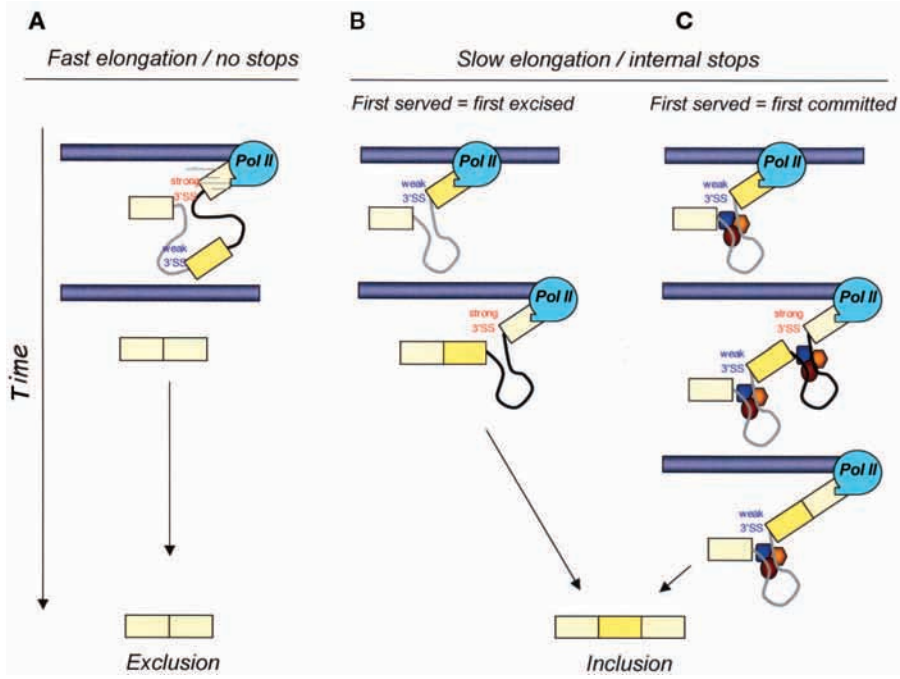

Figure 2. Alternative models for the "first come, first served" mechanism of splice site selection. (*A*) Fast elongation promotes usage of the stronger downstream 3′ splice site. (*B*) Slow elongation causes preferential excision of the upstream intron (first served = first excised). (*C*) Slow elongation causes commitment to E33 inclusion via recruitment of splicing factors (first served = first committed). Both introns are excised individually and in an order that is independent of elongation. (Based on de la Mata et al. 2010.)

cleus gave rise to different alternatively spliced RNAs each (Adami and Babiss 1991). Staggered infections of two discernible viral genomes resulted in the simultaneous presence of unreplicated and replicated genomes in the same nucleus, with each of them eliciting two different mRNA splicing variants of the *E1a* gene. Because there were no sequence differences between the two templates, these investigators speculated that the molecular bases for the change in splicing should be attributed to epigenetics, i.e., to the chromatin organization acquired following replication. If, after viral replication, the template acquired a more compact chromatin structure with the subsequent reduction in Pol II elongation rates, more time would be given to assemble splicing complexes at the upstream E1a 5′ splice site, favoring its use compared with the downstream 5′ splice site, and giving rise to a shorter E1a mRNA. This interpretation predicted that inhibition of elongation by other means would have similar effects on splicing. This is indeed the case because the use of the upstream 5′ splice site is favored when transcription is performed by a slow Pol II mutant (de la Mata et al. 2003).

The adenoviral observation gave us the clue to understand the reason when transfected minigene plasmids that are reporters for E33 alternative splicing were prompted to replicate, and inclusion of E33 into the mature mRNA was greatly increased (Fig. 3). Transiently transfected minigenes and infected viral genomes are known to assemble in a physiological chromatin context (Cereghini and Yaniv 1984; Tong et al. 2006), whose compaction increases following replication (Nahreini and Mathews 1995). We confirmed that replication favored lower Pol II elongation (Kadener et al. 2001), which was the cause of the observed up-regulation of the inclusion of a cassette exon into mature mRNA. This idea was reinforced by finding that trichostatin A (TSA), a potent inhibitor of histone deacetylation, favored skipping of alternative exons, whose inclusion is stimulated by slow elongation (Nogués et al. 2002). Two opposite forces—plasmid replication by creating compaction and TSA by promoting opening—could then regulate elongation and, in turn, alternative splicing at the chromatin level.

CHROMATIN REMODELERS AND ALTERNATIVE SPLICING

Further evidence that chromatin changes could affect alternative splicing of an endogenous gene was provided by the finding that the Brahma (Brm) subunit of the chromatin remodeling factor SWI/SNF (switch/sucrose nonfermentable) regulates alternative splicing of the *CD44* gene (Batsché et al. 2006). Brm promotes inclusion into mRNA of the block of 10 consecutive alternative exons located in the center of the gene by interacting with Pol II, spliceosomal snRNPs U1 and U5, and the RNA-binding protein Sam 68. The latter binds to splicing regulatory elements present in the CD44 variable exons and were known to stimulate their inclusion following activation of the extracellular-regulated kinase (ERK) mitogen-activated protein (MAP) kinases. The macromolecular complex accumulates on the *CD44* gene, peaking at the intragenic alternative splicing region and causing Pol II stalling that, in turn, favors inclusion of the variable exons, in agreement with the kinetic coupling model. These results highlighted the possibility that internal roadblocks

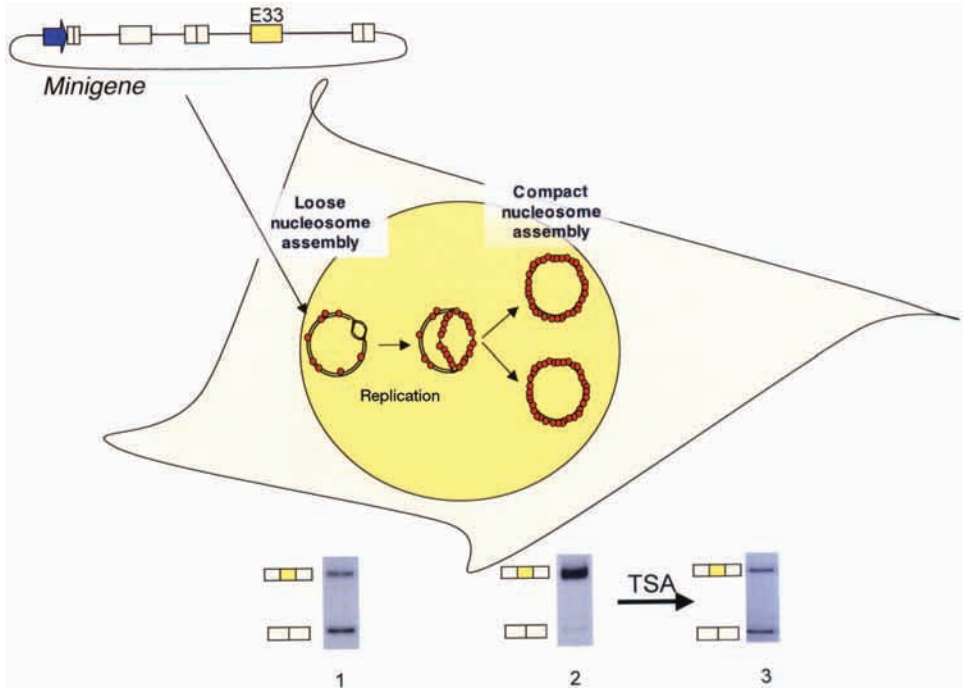

Figure 3. Early evidence of a role for chromatin on splicing: Replication of transfected reporter minigenes affects alternative splicing. (*Lane 1*) Transfected reporter minigenes acquire a loose nucleosome assembly and give rise to ~50% inclusion of the FN alternatively spliced E33 into mature mRNA, as assessed by RT-PCR. (*Lane 2*) After replication, nucleosome organization becomes more compact, promoting much higher E33 inclusion. (*Lane 3*) Inclusion levels become lower if cells are treated with the histone deacetylase inhibitor TSA. (Based on Kadener et al. 2001.)

to Pol II elongation, created by complexes assembling on chromatin, could regulate alternative splicing. This idea is highly consistent with findings of the Groudine laboratory that methylation of a DNA sequence in the middle of a gene causes local decreases in histone acetylation and chromatin accessibility, resulting in a decline in Pol II elongation, without quantitatively affecting transcription levels (Lorincz et al. 2004).

NEURON DEPOLARIZATION, INTRAGENIC CHROMATIN CHANGES, AND ALTERNATIVE SPLICING

The nervous system is a suitable physiological context in which to look at possible alternative splicing events regulated by chromatin structure. Neurons exhibit an unusually large number of functionally relevant alternative splicing events (Ule and Darnell 2006; Li et al. 2007). A well-characterized stimulus that regulates alternative splicing is the depolarization of neural cells with high extracellular potassium concentration, a treatment that triggers calcium signaling through the opening of voltage-dependent channels and a pathway involving activation of calcium/calmodulin-dependent kinase IV; recruitment of *trans*-acting factors to specific RNA elements has been described (Xie and Black 2001; An and Grabowski 2007; Lee et al. 2007). On the other hand, the transcriptional regulation involved in neuron differentiation is dependent on chromatin modifications (Levenson and Sweatt 2005; Fischer et al. 2007) and, in particular, on histone acetylation (Korzus et al. 2004). Using the alternative splicing of exon 18 (E18) of the neural cell adhesion molecule (*ncam*) gene as a model, we found that neuron depolarization promotes E18 skipping through intragenic histone acetylation (Schor and Kornblihtt 2009; Schor et al. 2009). Alternative splicing of E18 originates two isoforms: NCAM140 and 180. Both are integral membrane proteins, and the differential segment corresponds to the cytosolic region. NCAM140 is most abundant in nondifferentiated neurons, whereas NCAM180 increases following neuronal differentiation and is thought to contribute to organize stable synapses through the interaction of its cytosolic domain with the cytoskeleton (Pollerberg et al. 1986).

We observed that sustained depolarization of the membrane potential of mouse neuronal (N2a) cells in culture with KCl caused elevated histone acetylation (H3K9ac) and increased chromatin relaxation around exons 17–19 of the *ncam* gene. These chromatin changes do not take place at the promoter region and are accompanied by an increase in Pol II processivity in the alternative E18 region, which we showed can promote NCAM E18 skipping, in full agreement with the kinetic coupling model (Fig. 4). Consistently, NCAM E18 skipping is also promoted when cells are treated with the hyperacetylating drug TSA, and E18 inclusion is stimulated when transcription is performed by the "slow" mutation of RNA Pol II. Preliminary results (IE Schor et al., unpub.) indicate that at the same *ncam* gene region in which histone H3 results in hyperacetylated (H3K9ac) and chromatin is relaxed following depolarization, there is an increase in histone H3 dimethylation

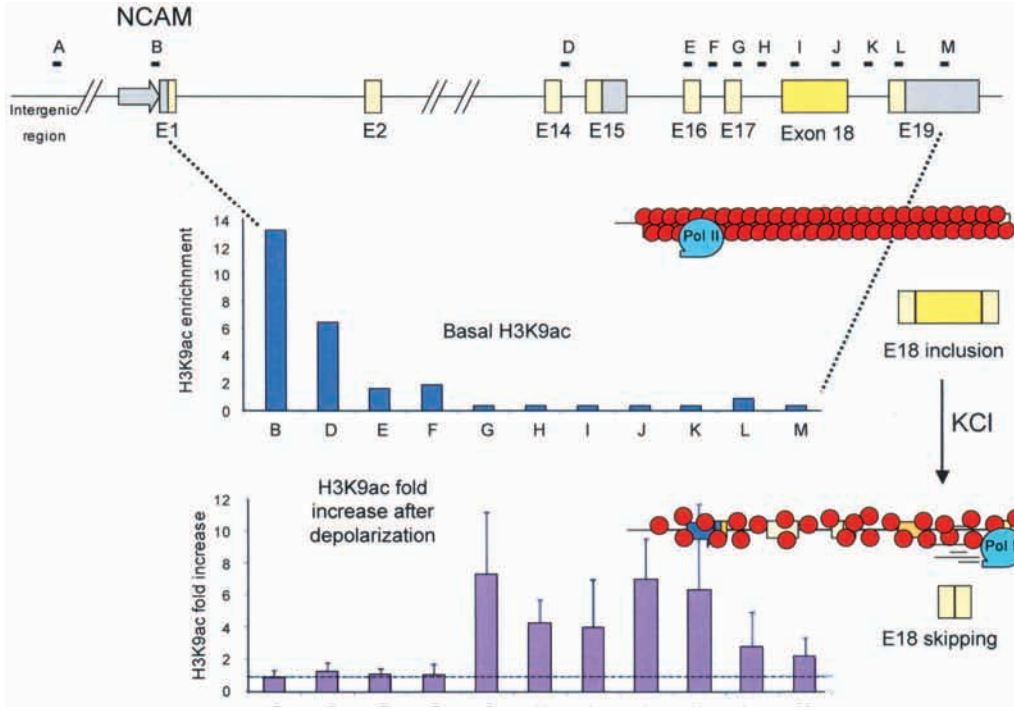

Figure 4. Depolarization of neuronal cells triggers intragenic histone acetylations of the *ncam* gene that cause exons 18 (dark yellow, E18) skipping. (*Top*) A scheme of the *ncam* gene, showing the distribution of qPCR amplicons used for chromatin immunoprecipitation (ChIP) analysis. (Blue bars) Representative levels of H3K9 acetylation in untreated N2a cells at the regions of the different amplicons. (Violet bars) Increase of H3K9 acetylation in the same regions in response to depolarization with 60-mM KCl for 4-6 h. Scheme showing compact and looser nucleosome (red) compaction and different Pol II elongation states (*right*). (Based on Schor et al. 2009.)

(H3K9me2) following neuron differentiation. Recall that H3K9me2 is a typical silencing mark that promotes facultative heterochromatin, a situation that would cause lower Pol II elongation and is consistent with higher E18 inclusion levels characteristic of the differentiation state. How specific chromatin changes are directed against intragenic regions is not known. Histone acetyltransferases, or deacetylases changing the acetylation landscapes away from promoter regions, can be involved because these enzymes, in addition to being part of transcriptional activator or inhibitor complexes, can have genome-wide activities (Shahbazian and Grunstein 2007). Other possibilities are the "piggybacking" of histone-modifying enzymes by elongating Pol II complexes or their recruitment to nascent pre-mRNA (Sjolinder et al. 2005).

CREATING REGION-SPECIFIC SILENCING HISTONE MARKS TO CONTROL ALTERNATIVE SPLICING

Increasing evidence for the role of chromatin in alternative splicing prompted us to wonder whether we could "write" specific silencing marks at certain gene regions that could affect alternative splicing by creating roadblocks to Pol II elongation. For this purpose, we took advantage of evidence that it is possible to affect chromatin in a directed manner using double-strand small RNAs (Morris et al. 2004). Exogenously applied siRNAs directed to promoter regions can trigger transcriptional gene silencing (TGS) in human cells through heterochromatin formation at DNA target sequences. The process involves recruitment of chromatin-modifying enzymes resulting in specific histone 3 methylations usually associated with facultative heterochromatin (H3K9me2 and H3K27me3), DNA methylation, and histone deacetylation (Morris et al. 2004; Kim et al. 2006). We hypothesized that if instead of targeting promoters, siRNAs were directed to sequences in the body of a gene mapping close to an alternative exon, the siRNA leading strand could base pair with the nascent pre-mRNA and subsequently generate a closed chromatin structure and prevent efficient Pol II elongation, affecting alternative splicing (Fig. 5). Accordingly, we recently reported that exogenous siRNAs targeting sequences of the intron immediately downstream from the alternative E33 (EDI or EDA) exon of the endogenous FN gene stimulate E33 inclusion into mature mRNA (Alló et al. 2009). We engineered the siRNA sequences in a way to favor that either the sense or the antisense strand enters the putative silencing complexes, mediating the effect on splicing, and we observed that in human hepatoma Hep3B cells, only the antisense siRNA strand was effective. Most interestingly, in HeLa cells, both strands were equally effective in promoting E33 inclusion. This apparent paradox was solved when we found that HeLa cells, unlike Hep3B cells, express an FN antisense endogenous transcript spanning the target region of our siRNAs. This surprising result reinforced the idea that to control alternative splicing, the siRNA guide strand must

hybridize with nascent RNAs at the region where chromatin modification will take place. Indeed, we showed that siRNA transfection augments H3K9me2 and H3K27me3 marks at the endogenous targeted intragenic region, with no changes observed at the promoter. The siRNA effect on splicing needs AGO1, the argonaute protein necessary to guide small RNAs to their genomic targets leading to chromatin modifications characteristic of TGS. Consistent with a model in which local heterochromatinization affects alternative splicing by partially blocking elongation, the siRNA effect on E33 splicing is abolished by treatments that promote chromatin relaxation, such as inhibition of histone deacetylases by TSA or inhibition of H3K9 methylation with the drug BIX-01294 [2-(hexahydro-4-methyl-1H-1,4-diazepin-1-yl)-6,7-dimethoxy-*N*-[1-(phenylmethyl)-4-piperidinyl]4-quinazolinamine]]. Furthermore, the siRNA effect is abrogated by depletion of the heterochromatin protein HP1α. In view of the multiple and various evidence for the involvement of chromatin in siRNA effects on splicing, we decided to name this mechanism TGS-AS for transcriptional gene silencing–regulated alternative splicing. It is worth noting that because mutations which affect alternative splicing patterns are frequent causes of hereditary disease and cancer, siRNAs may represent a new therapeutic tool to correct gene and exon-specific alternative splicing defects via TGS-AS.

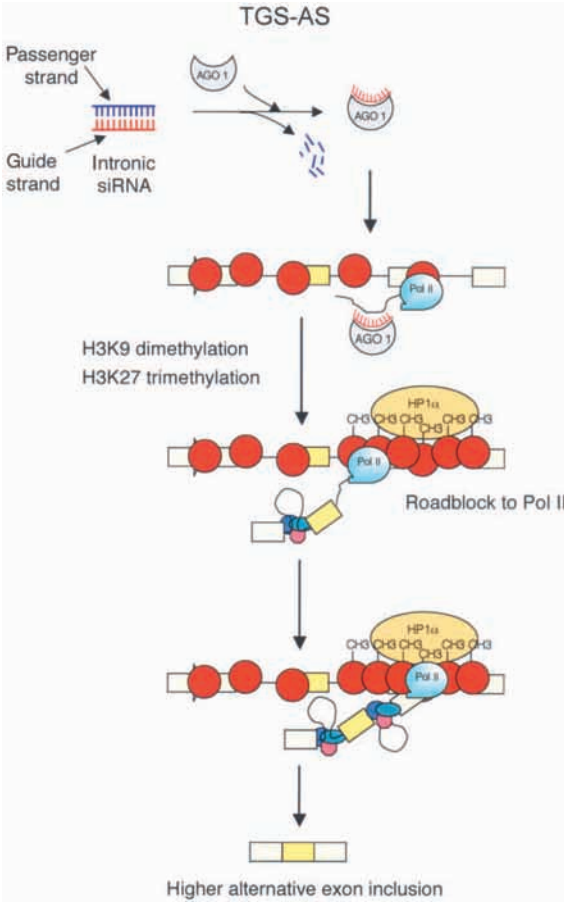

Figure 5. Model for TGS-AS. Transfection with siRNAs targeting the intron mapping downstream from the alternative exon (dark yellow) promotes dimethylation and trimethylation of H3K9 and H3K27 (red, nucleosomes), respectively, triggered by siRNA guide strand entering a silencing complex containing AGO1. HP1α is recruited and the resulting condensed chromatin structure generates roadblocks to Pol II elongation, causing higher inclusion of the alternative exon according to the kinetic coupling model. (Based on Alló et al. 2009.)

The next question, still unanswered, is whether there exists a physiological TGS-AS mechanism, i.e., endogenous double-strand RNAs that regulate alternative splicing through histone modifications in mammalian cells. Potential candidates are Piwi RNAs (Kuramochi-Miyagawa et al. 2008) and endogenous siRNAs (Zhang et al. 2008) shown to regulate gene expression in mammalian cells through histone modifications and DNA methylation. microRNAs (miRNAs), recently shown to trigger TGS in the moss *Physcomitrella patens* (Khraiwesh et al. 2010) and in mammalian cells (Kim et al. 2008), and sense–antisense transcription couples, resulting from regions with bidirectional and overlapping transcription that triggers epigenetic silencing, are also good candidates (Yu et al. 2008).

As a first step aimed at identifying an endogenous TGS-AS pathway, we performed ChIP-seq using antibodies against AGO1 (a key player in TGS) and histone H3 covalent modifications (H3K9me2, H3K27me3, H3K36me3, and H3). Preliminary data allowed for the identification of 24,000 target regions for AGO1, 23,000 for H3K9me2, and 51,000 for H3K27me3 in the genome of the human mammary cell line MCF7. Approximately 50% of the targets for each mark map within genes and, among these, AGO1 targets are enriched in exons, preferentially in genes with low expression levels. Approximately 17% of the H3K9me2 targets overlap with H3K27me3 targets when analyzed genome wide. However, the overlapping increases to 55% when only AGO1 target regions are considered. These results indicate that AGO1 is found in the nucleus, located at specific regions of the genome, and preferentially associated with histone silencing marks. Whether this association is mediated by RNA or is causative of TGS remains to be determined.

CHROMATIN MODIFICATIONS AND RECRUITMENT OF SPLICING FACTORS

Revealing an elongation-independent role for chromatin on splicing, recent reports suggest that histone modifications can affect both constitutive and alternative splicing, by either directly or indirectly recruiting splicing factors. H3K4 trimethylation, a mark observed in the 5′-end proximal regions of active genes, can recruit components of early spliceosomal complexes through the chromodomain-containing protein CHD1, enhancing splicing efficiency (Sims et al. 2007). A more straightforward indication of the role of particular histone modifications on alternative splicing has been reported by the Misteli laboratory (Luco et al. 2010). These investigators found that high levels of H3K36 trimethylation in the intragenic region of active genes were able to recruit the splicing factor polypyrimidine tract-binding (PTB) protein through the chromatin-binding adapter protein MRG15, resulting in inhibition of

inclusion of alternative exons. Moreover, this chromatin-assisted regulation of alternative splicing seems to affect preferentially alternative exons with relatively weak PTB-binding sites, suggesting that it can enhance classical RNA-mediated splicing regulation by concentrating splicing factors in the sites of pre-mRNA synthesis. A role for H3K36 trimethylation had been suggested before in a ChIP-Chip high-throughput analysis primarily performed in *Caenorhabditis elegans* and extended to mouse (Kolasinska-Zwierz et al. 2009). This histone mark, characteristic of intragenic regions with productive transcription, appeared significantly enriched in exons with respect to introns, and most interestingly, the mark is lower in alternative exons compared with constitutive exons. This observation was already suggestive of a relationship between the presence of the H3K36me3 mark and the mechanism of the alternative splicing process, which was confirmed later by Luco et al. (2010).

Another histone mark that might be involved in the control of alternative splicing is H3S10p, which was shown to interact with the serine/arginine-rich (SR) proteins SRSF1 and SRSF3 (formerly known as SF2/ASF and SRp20) (Loomis et al. 2009).

NUCLEOSOMES ARE PREFERENTIALLY POSITIONED IN EXONS

In 2009, an avalanche of articles based on genome-wide analyses, demonstrated that nucleosomes are preferentially positioned in exons (Andersson et al. 2009; Hon et al. 2009; Nahkuri et al. 2009; Schwartz et al. 2009; Spies et al. 2009; Tilgner et al. 2009). These reports made use of computationally based predictions and experimental data on nucleosome positioning within the human genome derived from deep sequencing of DNA fragments attached to mononucleosomes, obtained from chromatin digestion with micrococcal nuclease. The studies found distinct peaks of nucleosome occupancy within exons that are paralleled by nucleosome depletion from introns. Although this subject was extensively covered in recent review articles and commentaries (Kaplan 2009; Kornblihtt et al. 2009; Schor et al. 2010; Schwartz and Ast 2010; Tilgner and Guigó 2010), we just speculate here on putative implications of these findings. Because mammalian exons are much shorter than introns (on average, 140–150 bp versus 8 kbp), one of the most striking puzzles in the field of splicing is how splicing machinery recognizes short exons "floating" in a "sea" of introns. The exon definition concept, originally postulated by Berget (1995), states that spliceosome and auxiliary factors binding the 3′ and 5′ consensus splice sites that flank an exon at the pre-mRNA level physically interact with one another, favoring exon recognition and acting as a selective force for short exon size. Without this mechanism becoming dispensable, nucleosome positioning on exons at the DNA level may help in exon definition by creating roadblocks or "speed bumps" for Pol II elongation that provide longer time for cotranscriptional recognition of splice signals by splicing factors in the nascent pre-mRNA (Fig. 6). To demonstrate this hypothesis, global ChIP or run-on analyses should yield higher Pol II densities at exons or at intron/exon junctions. In the transient absence of such evidence, the hypothesis is fully consistent with a recent study that uses optical tweezers to follow individual Pol II complexes as they transcribe nucleosomal DNA that reveal a bumpy nature of Pol II elongation, where the nucleosome behaves as a fluctuating barrier that locally increases pause density, slows pause recovery, and reduces the apparent pause-free velocity of Pol II (Hodges et al. 2009).

CONCLUSIONS

Different and various experimental approaches point out a major role for chromatin structure in the control of splicing and alternative splicing. The original findings of more than a decade ago, that promoters affect alternative splicing, might have acted as the "tip of the iceberg" by driving our attention to the importance of transcriptional regulation on alternative splicing. However, the overwhelming amount of evidence published since last year, concerning both genome wide and individual genes, strongly suggests that the "bulk of the iceberg" in the transcriptional regulation of splicing is concerns changes in chromatin structure. Most interestingly, these changes seem to occur inside

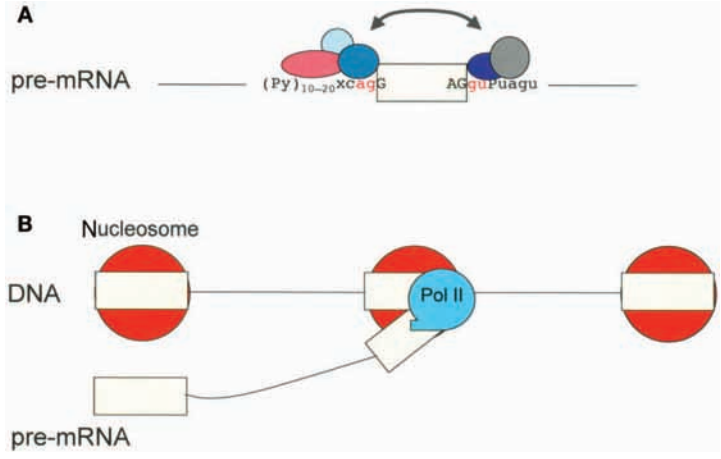

Figure 6. A second possible mechanism for exon definition. (*A*) Exon definition is achieved at the pre-mRNA level. Spliceosomal and auxiliary factors are recruited to the splice sites flanking an exon on the mRNA precursor. Direct and indirect interactions between the 3′ and 5′ complexes favor exon recognition and splicing and exert selective pressure for a conserved exon length of 140–150 nucletotides. (*B*) Nucleosomes (red) are preferentially bound to exons. Exons are therefore marked at the DNA level by nucleosome positioning, which may act as "speed bumps" for RNA Pol II, helping in the cotranscriptional recruitment of splicing factors to the nascent pre-mRNA and improving exon definition. Because nucleosomes accommodate DNA stretches of ~147 nucleotides, their preferential location on exons may act as the selective pressure factor for the conservation in exon length. (Based on Kornblihtt et al. 2009.)

genes, far from promoters and around alternative splicing exons, i.e., in regions usually neglected by most classical chromatin studies, usually focused on promoter and regulatory regions. The ways by which histone modifications and changes in chromatin compaction might affect alternative splicing are not restricted to the creation or elimination of roadblocks to Pol II elongation but also involve nonexclusive mechanisms of recruitment of splicing factors to covalently modified histones. Furthermore, the preferential positioning of nucleosomes in exons appears to be a dogma-breaking and provocative finding that might reveal a dialog between the chromatin and splicing codes whose words we are just starting to decipher.

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REFERENCES

Adami G, Babiss LE. 1991. DNA template effect on RNA splicing: Two copies of the same gene in the same nucleus are processed differently. *EMBO J* **10:** 3457–3465.

Aebi M, Weissman M. 1987. Precision and orderliness in splicing. *Trends Genet* **3:** 102–107.

Aguilera A, Gómez-González B. 2008. Genome instability: A mechanistic view of its causes and consequences. *Nat Rev Genet* **9:** 204–217.

Alló M, Buggiano V, Fededa JP, Petrillo E, Schor I, de la Mata M, Agirre E, Plass M, Eyras E, Elela SA, Klinck R, Chabot B, Kornblihtt AR. 2009. Control of alternative splicing through siRNA-mediated transcriptional gene silencing. *Nat Struct Mol Biol* **16:** 717–724.

An P, Grabowski PJ. 2007. Exon silencing by UAGG motifs in response to neuronal excitation. *PLoS Biol* **5:** e36.

Andersson R, Enroth S, Rada-Iglesias A, Wadelius C, Komorowski J. 2009. Nucleosomes are well positioned in exons and carry characteristic histone modifications. *Genome Res* **19:** 1732–1741.

Auboeuf D, Honig A, Berget SM, O'Malley BW. 2002. Coordinate regulation of transcription and splicing by steroid receptor coregulators. *Science* **298:** 416–419.

Auboeuf D, Dowhan DH, Kang YK, Larkin K, Lee JW, Berget SM, O'Malley BW. 2004. Differential recruitment of nuclear receptor coactivators may determine alternative RNA splice site choice in target genes. *Proc Natl Acad Sci* **101:** 2270–2274.

Batsché E, Yaniv M, Muchardt C. 2006. The human SWI/SNF subunit Brm is a regulator of alternative splicing. *Nat Struct Mol Biol* **13:** 22–29.

Bentley DL. 2005. Rules of engagement: Co-transcriptional recruitment of pre-mRNA processing factors. *Curr Opin Cell Biol* **17:** 251–256.

Berget SM. 1995. Exon recognition in vertebrate splicing. *J Biol Chem* **270:** 2411–2414.

Boireau S, Maiuri P, Basyuk E, de la Mata M, Knezevich A, Pradet-Balade B, Backer V, Kornblihtt A, Marcello A, Bertrand E. 2007. The transcriptional cycle of HIV-1 in real-time and live cells. *J Cell Biol* **179:** 291–304.

Cereghini S, Yaniv M. 1984. Assembly of transfected DNA into chromatin: Structural changes in the origin-promoter-enhancer region upon replication. *EMBO J* **3:** 1243–1253.

Cramer P, Pesce CG, Baralle FE, Kornblihtt AR. 1997. Functional association between promoter structure and transcript alternative splicing. *Proc Natl Acad Sci* **94:** 11456–11460.

Cramer P, Caceres JF, Cazalla D, Kadener S, Muro AF, Baralle FE, Kornblihtt AR. 1999. Coupling of transcription with alternative splicing**:** RNA pol II promoters modulate SF2/ASF and 9G8 effects on an exonic splicing enhancer. *Mol Cell* **4:** 251–258.

Das R, Yu J, Zhang Z, Gygi MP, Krainer AR, Gygi SP, Reed R. 2007. SR proteins function in coupling RNAP II transcription to pre-mRNA splicing. *Mol Cell* **26:** 867–881.

de la Mata M, Alonso CR, Kadener S, Fededa JP, Blaustein M, Pelisch F, Cramer P, Bentley D, Kornblihtt AR. 2003. A slow RNA polymerase II affects alternative splicing in vivo. *Mol Cell* **12:** 525–532.

de la Mata M, Lafaille C, Kornblihtt AR. 2010. First come, first served revisited: Factors affecting the same alternative splicing event have different effects on the relative rates of intron removal. *RNA* **16:** 904–912.

Eperon LP, Graham IR, Griffiths AD, Eperon IC. 1988. Effects of RNA secondary structure on alternative splicing of pre-mRNA: Is folding limited to a region behind the transcribing RNA polymerase? *Cell* **54:** 393–401.

Fischer A, Sananbenesi F, Wang X, Dobbin M, Tsai LH. 2007. Recovery of learning and memory is associated with chromatin remodelling. *Nature* **447:** 178–182.

Hodges C, Bintu L, Lubkowska L, Kashlev M, Bustamante C. 2009. Nucleosomal fluctuations govern the transcription dynamics of RNA polymerase II. *Science* **325:** 626–628.

Hon G, Wang W, Ren B. 2009. Discovery and annotation of functional chromatin signatures in the human genome. *PLoS Comput Biol* **5:** e1000566.

Howe KJ, Kane CM, Ares M, Jr. 2003. Perturbation of transcription elongation influences the fidelity of internal exon inclusion in *Saccharomyces cerevisiae*. *RNA* **9:** 993–1006.

Kadener S, Cramer P, Nogués G, Cazalla D, de la Mata M, Fededa JP, Werbajh SE, Srebrow A, Kornblihtt AR. 2001. Antagonistic effects of T-Ag and VP16 reveal a role for RNA pol II elongation on alternative splicing. *EMBO J* **20:** 5759–5768.

Kaplan CD. 2009. Revealing the hidden relationship between nucleosomes and splicing. *Cell Cycle* **8:** 3633–3634.

Khraiwesh B, Arif MA, Seumel GI, Ossowski S, Weigel D, Reski R, Frank W. 2010. Transcriptional control of gene expression by microRNAs. *Cell* **140:** 111–122.

Kim DH, Villeneuve LM, Morris KV, Rossi JJ. 2006. Argonaute-1 directs siRNA-mediated transcriptional gene silencing in human cells. *Nat Struct Mol Biol* **13:** 793–797.

Kim DH, Saetrom P, Snove O, Jr., Rossi JJ. 2008. MicroRNA-directed transcriptional gene silencing in mammalian cells. *Proc Natl Acad Sci* **105:** 16230–16235.

Kolasinska-Zwierz P, Down T, Latorre I, Liu T, Liu XS, Ahringer J. 2009. Differential chromatin marking of introns and expressed exons by H3K36me3. *Nat Genet* **41:** 376–381.

Kornblihtt AR. 2005. Promoter usage and alternative splicing. *Curr Opin Cell Biol* **17:** 262–268.

Kornblihtt AR. 2007. Coupling transcription and alternative splicing. *Adv Exp Med Biol* **623:** 175–189.

Kornblihtt AR, de la Mata M, Fededa JP, Muñoz MJ, Nogués G. 2004. Multiple links between transcription and splicing. *RNA* **10:** 1489–1498.

Kornblihtt AR, Schor IE, Alló M, Blencowe BJ. 2009. When chromatin meets splicing. *Nat Struct Mol Biol* **16:** 902–903.

Korzus E, Rosenfeld MG, Mayford M. 2004. CBP histone acetyltransferase activity is a critical component of memory consolidation. *Neuron* **42:** 961–972.

Kuramochi-Miyagawa S, Watanabe T, Gotoh K, Totoki Y, Toyoda A, Ikawa M, Asada N, Kojima K, Yamaguchi Y, Ijiri TW, et al. 2008. DNA methylation of retrotransposon genes is regulated

by Piwi family members MILI and MIWI2 in murine fetal testes. *Genes Dev* **22:** 908–917.

Lacadie SA, Tardiff DF, Kadener S, Rosbash M. 2006. In vivo commitment to yeast cotranscriptional splicing is sensitive to transcription elongation mutants. *Genes Dev* **20:** 2055–2066.

Lee JA, Xing Y, Nguyen D, Xie J, Lee CJ, Black DL. 2007. Depolarization and CaM kinase IV modulate NMDA receptor splicing through two essential RNA elements. *PLoS Biol* **5:** e40.

Levenson JM, Sweatt JD. 2005. Epigenetic mechanisms in memory formation. *Nat Rev Neurosci* **6:** 108–118.

Li X, Manley JL. 2005. Inactivation of the SR protein splicing factor ASF/SF2 results in genomic instability. *Cell* **122:** 365–378.

Li Q, Lee JA, Black DL. 2007. Neuronal regulation of alternative pre-mRNA splicing. *Nat Rev Neurosci* **8:** 819–831.

Listerman I, Sapra AK, Neugebauer KM. 2006. Cotranscriptional coupling of splicing factor recruitment and precursor messenger RNA splicing in mammalian cells. *Nat Struct Mol Biol* **13:** 815–822.

Loomis RJ, Naoe Y, Parker JB, Savic V, Bozovsky MR, Macfarlan T, Manley JL, Chakravarti D. 2009. Chromatin binding of SRp20 and ASF/SF2 and dissociation from mitotic chromosomes is modulated by histone H3 serine 10 phosphorylation. *Mol Cell* **33:** 450–461.

Lorincz MC, Dickerson DR, Schmitt M, Groudine M. 2004. Intragenic DNA methylation alters chromatin structure and elongation efficiency in mammalian cells. *Nat Struct Mol Biol* **11:** 1068–1075.

Luco RF, Pan Q, Tominaga K, Blencowe BJ, Pereira-Smith OM, Misteli T. 2010. Regulation of alternative splicing by histone modifications. *Science* **327:** 996–1000.

Morris KV, Chan SW, Jacobsen SE, Looney DJ. 2004. Small interfering RNA-induced transcriptional gene silencing in human cells. *Science* **305:** 1289–1292.

Muñoz MJ, Perez Santangelo MS, Paronetto MP, de la Mata M, Pelisch F, Boireau S, Glover-Cutter K, Ben-Dov C, Blaustein M, Lozano JJ, et al. 2009. DNA damage regulates alternative splicing through inhibition of RNA polymerase II elongation. *Cell* **137:** 708–720.

Nahkuri S, Taft RJ, Mattick JS. 2009. Nucleosomes are preferentially positioned at exons in somatic and sperm cells. *Cell Cycle* **8:** 3420–3424.

Nahreini P, Mathews MB. 1995. Effects of the simian virus 40 origin of replication on transcription from the human immunodeficiency virus type 1 promoter. *J Virol* **69:** 1296–1301.

Nogués G, Kadener S, Cramer P, Bentley D, Kornblihtt AR. 2002. Transcriptional activators differ in their abilities to control alternative splicing. *J Biol Chem* **277:** 43110–43114.

Pagani F, Stuani C, Kornblihtt AR, Baralle FE. 2003. Promoter architecture modulates CFTR exon 9 skipping. *J Biol Chem* **278:** 1511–1517.

Pandya-Jones A, Black DL. 2009. Co-transcriptional splicing of constitutive and alternative exons. *RNA* **15:** 1896–1908.

Perales R, Bentley D. 2009. "Cotranscriptionality"**:** The transcription elongation complex as a nexus for nuclear transactions. *Mol Cell* **36:** 178–191.

Pollerberg GE, Schachner M, Davoust J. 1986. Differentiation state-dependent surface mobilities of two forms of the neural cell adhesion molecule. *Nature* **324:** 462–465.

Roberts GC, Gooding C, Mak HY, Proudfoot NJ, Smith CW. 1998. Co-transcriptional commitment to alternative splice site selection. *Nucleic Acids Res* **26:** 5568–5572.

Robson-Dixon ND, García-Blanco M. 2004. MAZ elements alter transcription elongation and silencing of the fibroblast growth factor receptor 2 exon IIIb. *J Biol Chem* **279:** 29075–29084.

Schor IE, Kornblihtt AR. 2009. Playing inside the genes: Intragenic histone acetylation after membrane depolarization of neural cells opens a path for alternative splicing regulation. *Commun Integr Biol* **2:** 341–343.

Schor IE, Rascovan N, Pelisch F, Alló M, Kornblihtt AR. 2009. Neuronal cell depolarization induces intragenic chromatin modifications affecting NCAM alternative splicing. *Proc Natl Acad Sci* **106:** 4325–4330.

Schor, IE, Alló, M, Kornblihtt, AR. 2010. Intragenic chromatin modifications: A new layer in alternative splicing regulation. *Epigenetics* **5:** 3.

Schwartz S, Ast G. 2010. Chromatin density and splicing destiny: On the cross-talk between chromatin structure and splicing. *EMBO J* **29:** 1629–1636.

Schwartz S, Meshorer E, Ast G. 2009. Chromatin organization marks exon-intron structure. *Nat Struct Mol Biol* **16:** 990–995.

Shahbazian MD, Grunstein M. 2007. Functions of site-specific histone acetylation and deacetylation. *Annu Rev Biochem* **76:** 75–100.

Sims RJ III, Millhouse S, Chen CF, Lewis BA, Erdjument-Bromage H, Tempst P, Manley JL, Reinberg D. 2007. Recognition of trimethylated histone H3 lysine 4 facilitates the recruitment of transcription postinitiation factors and pre-mRNA splicing. *Mol Cell* **28:** 665–676.

Sjolinder M, Bjork P, Soderberg E, Sabri N, Farrants AK, Visa N. 2005. The growing pre-mRNA recruits actin and chromatin-modifying factors to transcriptionally active genes. *Genes Dev* **19:** 1871–1884.

Spies N, Nielsen CB, Padgett RA, Burge CB. 2009. Biased chromatin signatures around polyadenylation sites and exons. *Mol Cell* **36:** 245–254.

Tilgner H, Guigó R. 2010. From chromatin to splicing: RNA-processing as a total artwork. *Epigenetics* **5:** 180–184.

Tilgner H, Nikolaou C, Althammer S, Sammeth M, Beato M, Valcárcel J, Guigó R. 2009. Nucleosome positioning as a determinant of exon recognition. *Nat Struct Mol Biol* **16:** 996–1001.

Tong W, Kulaeva OI, Clark DJ, Lutter LC. 2006. Topological analysis of plasmid chromatin from yeast and mammalian cells. *J Mol Biol* **361:** 813–822.

Ule J, Darnell RB. 2006. RNA binding proteins and the regulation of neuronal synaptic plasticity. *Curr Opin Neurobiol* **16:** 102–110.

Xie J, Black DL. 2001. A CaMK IV responsive RNA element mediates depolarization-induced alternative splicing of ion channels. *Nature* **410:** 936–939.

Yu W, Gius D, Onyango P, Muldoon-Jacobs K, Karp J, Feinberg AP, Cui H. 2008. Epigenetic silencing of tumour suppressor gene p15 by its antisense RNA. *Nature* **451:** 202–206.

Zhang MX, Zhang C, Shen YH, Wang J, Li XN, Chen L, Zhang Y, Coselli JS, Wang XL. 2008. Effect of 27nt small RNA on endothelial nitric-oxide synthase expression. *Mol Biol Cell* **19:** 3997–4005.