Alternative splicing switches: Important players in cell differentiation

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Alternative splicing (AS) greatly expands the coding capacities of genomes by allowing the generation of multiple mature mRNAs from a limited number of genes. Although the massive switch in AS profiles that often accompanies variations in gene expression patterns occurring during cell differentiation has been characterized for a variety of models, their causes and mechanisms remain largely unknown. Here, we integrate foundational and recent studies indicating the AS switches that govern the processes of cell fate determination. We include some distinct AS events in pluripotent cells and somatic reprogramming and discuss new progresses on alternative isoform expression in adipogenesis, myogenic differentiation and stimulation of immune cells. Finally, we cover novel insights on AS mechanisms during neuronal differentiation, paying special attention to the role of chromatin structure.

Keywords:
alternative splicing; cell differentiation; chromatin structure; neuronal differentiation; RNA binding proteins; spliceosome; splicing factors

Introduction

During the differentiation process, cells undergo striking changes. The size, morphology, metabolism and responsiveness to signals, among other aspects, are continuously developing. These modifications are largely due to major changes in gene expression programs that impact on RNA and protein production. During differentiation from a pluripotent state, cells progressively develop a narrower potential as their gene-expression programs become more defined [1]. Since transition from one type of cell to another involves a switch in gene expression programs, the cell differentiation process can be followed by changes in transcriptome patterns.

Changes in gene expression patterns during differentiation are often accompanied by epigenetic modifications [2]. These epigenetic modifications confer stability of gene expression during mammalian development and are the main cause of the silencing of pluripotent-state genes as well as the turning-on of linage-specific genes [3]. It has been extensively reported that the percentage of methylation on CpG dinucleotides in mammalian genomes greatly varies during development [4]. For instance, during a discrete phase of early development, CpG methylation levels in the mouse decline sharply to approximately 30% of the typical somatic level [5, 6]. Moreover, recent evidence suggests that differentiation-specific genes are held in an epigenetically silenced manner in pluripotent cells [7]. These mechanisms ensure that during the early stages of development, genes that are required later in development are transiently held in a repressed state while during differentiation pluripotency-associated genes are epigenetically inactivated.

In coordination with epigenetic modifications, changes in gene expression during development and cell differentiation are accompanied or caused by changes in the expression levels of transcription factors. It is known that several transcription factors function in the maintenance of pluripotency in both early embryos and ES cells. Moreover, it was demonstrated that pluripotent stem cells can be directly generated from fibroblast cultures by the overexpression of only four transcriptional regulators [8]. However, recent studies reveal that not only regulation of transcription, but also mRNA processing, are crucial for genome reprogramming during development, and for tissue-specific gene expression. An increasing body of evidence shows that alternative splicing (AS) correlates closely to stem cell lineage differentiation, suggesting a key role for splicing in controlling gene regulatory networks required for cell differentiation.

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Abbreviations:
AS, alternative splicing; **RBP,** RNA binding protein.

In this review we focus on the role of AS decisions in the process of cell differentiation, particularly discussing some recent findings introducing AS as an integrated regulator of pluripotency and differentiation. Moreover, we describe how AS plays central roles in the control of differentiation processes and we discuss the new insights into AS mechanisms that operate in neuronal function and differentiation.

Mechanism of splicing leads to alternative splicing

Splicing is the pre-mRNA processing mechanism that removes introns and joins exons. While the term intron refers to non-coding interspersed sequences that in most cases are not included in the mature mRNA, exon refers to the sequences that remain in the mature RNA once the introns have been removed. The boundaries of exons and introns are marked by the splice donor site at the 5′ end of the intron and the splice acceptor site at the 3′ end of the intron. Both splice sites and some other specific regions within the spliced intron are characterized by defined consensus sequences [9]. Splicing is catalyzed by components of a large complex known as the spliceosome (Fig. 1). The spliceosome is a ribonucleoprotein composed of small nuclear RNAs and dozens of protein components [10].

There are multiple ways to alter spliceosome assembly and affect AS patterns. Many protein factors can bind to the pre-mRNA and enhance or repress spliceosome assembly at various steps. It is now widely accepted that most splicing commitment takes place co-transcriptionally. This opens an additional layer of splicing regulation based on the regulation of transcription. Additionally, there are many different modes of AS. However, the most common mode of AS in mammalian cells is a skipped exon: that is, an exon that may be included or excluded from the mature mRNA.

Many alternative isoforms have distinct or even opposing functions, and the regulation of isoform abundance often plays important biological roles. The alternative use of genome regions to codify proteins allows the generation of multiple proteins from a single gene, which increases the coding potential of the genome. Thus, AS enhances proteomic diversity from a limited number of genes (Fig. 1). Indeed, many gene transcripts have multiple splicing patterns, and some have thousands [11]. Furthermore, many genes show multiple AS and alternative mRNA processing events, creating a much more complex scenario and a large number of isoforms.

Overall, AS is one of the major contributors to protein diversity in metazoans, and is fundamental to greatly expanding the coding capacities of genetic information. AS provides a further, and flexible, level of gene expression control that acts in all steps of cellular differentiation.

Alternative splicing acts as a key mode of gene expression

It is well accepted that AS contributes to the diversification of the transcriptome, and is at the base of phenotypic differences among vertebrate species. However, our knowledge of the differential expression of specific splicing events is still limited. One of the first genome-scale expression compendium of human AS events was generated using whole-transcript microarrays [12]. The study monitored the expression of 24,426 events of alternative splicing in 48 diverse human samples and found that more than 9,500 of these events were differentially expressed. Furthermore, the systematic screen of regulatory sequences identified RNA motifs enriched near regulated cassette exons. Overall, this work provided one of the first resources for studying AS regulation in human tissues.

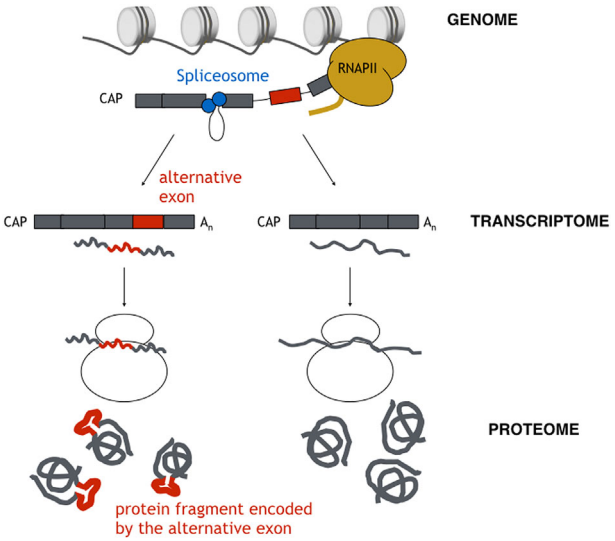


Figure 1. By generating different mature mRNAs from a single pre-mRNA, AS contributes to protein diversity.

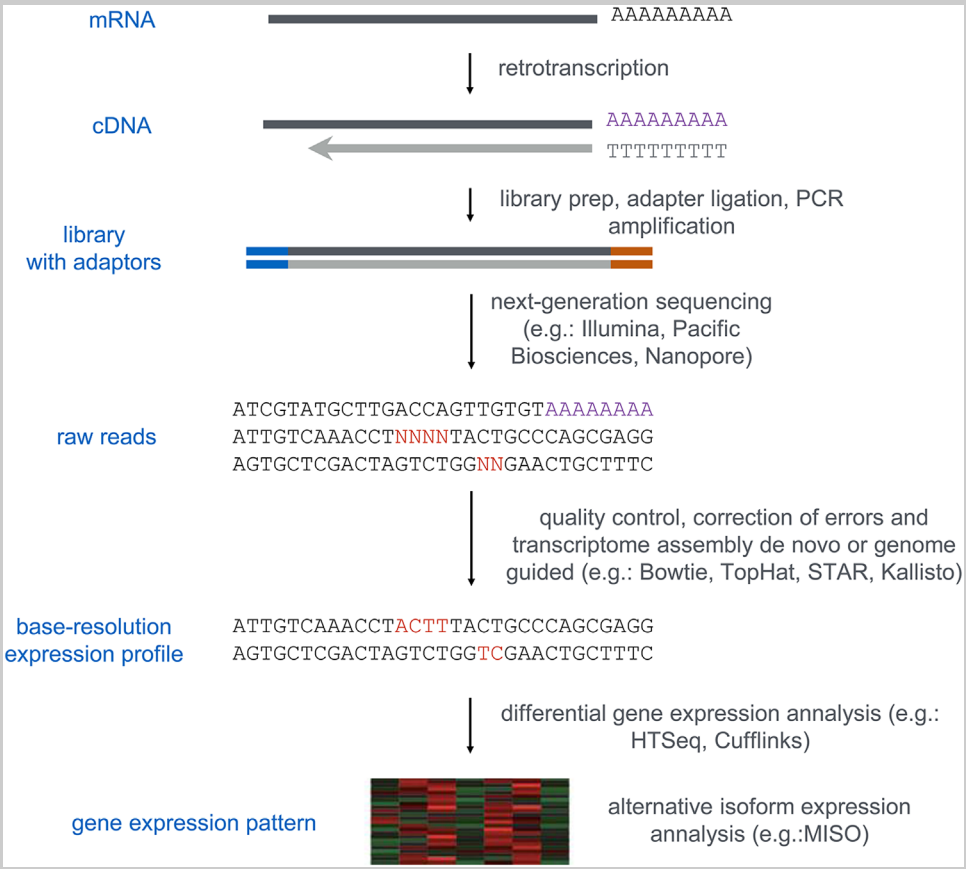
More recently, two independent studies observed significant differences in AS complexity between vertebrate lineages [13, 14] by high-throughput RNA sequencing (RNA-Seq) (see Box 1 for a description of the technique). While tissue-specific gene expression programs were seen to be largely conserved, AS appeared to be well conserved in only a subset of tissues and frequently lineage-specific.

In the past decades, many splicing factors have been discovered. Due to the extensive target set of each regulator, mutations in a particular one often lead to major phenotypic changes [15]. Splice site mutations are among the most common point mutations with major impacts. Mutations that disrupt the consensus sequence of splice sites will produce changes in splicing decisions, leading to protein alterations. Mutations in other regions could also reduce splice site strength, or create new splice sites that lead to altered splicing [15]. For example, the most common adult form of muscular dystrophy, myotonic dystrophy, is caused by reduced levels of the splicing factor MBNL1 [16].

These studies point out that regulation of AS is not only integral for biological processes including cell differentiation, but also that misregulation of AS can cause a variety of human diseases, including muscular dystrophy, cancer, and neurological disorders.

Box 1

RNA sequencing (RNA-seq) analyses the quantity of RNA in a given time point and informs the differential gene expression patterns of biological samples. The first step consists of RNA isolation from samples, DNA contamination is removed, and the RNA is divided into short fragments. The fragments are then retrotran scribed into cDNA. Illumina library prep (as exemplified in the figure) begins with phosphorylation of the cDNA and adapter ligation. The library is then amplified for clustering and sequencing. Next-generation sequencing could be performed by different methods; examples are Illumina (which produces many short reads) and Pacific Bioscience (which can sequence long reads). Different pipelines could achieve the downstream analysis. Computational parameters are established by programs, such as Bowtie, TopHat, Kallisto, STAR, and a variety of sites are now offering standardized analysis, for example Galaxy. The raw reads are processed by removing low-quality reads, and errors are corrected. Typically, a genome of reference was used to map the reads, but de novo assembly methods are starting to emerge and become more popular. The expression level of each transcript is estimated by counting the number of reads that align to each transcript, and these numbers are typically adjusted by the length of genes. The results are often expressed as fragments per kilobase of transcript per million mapped reads (FPKM). Different tools seek to identify isoform expression differences between conditions. These include MISO, JuncBASE and SpliceR among others.



Alternative splicing switches contribute to embryonic stem cell differentiation

Recent advances have addressed the contribution of AS patterns to stem cells pluripotency maintenance and differentiation. Advanced technologies such as high-throughput RNA-seq have allowed the profiling of dynamic splicing decisions that are responsible for specific transcriptome changes during cell differentiation at a genome-wide scale (see Box 1). Specific transcriptome changes during the differentiation of embryonic stem cells (ESCs) into different lineages have been extensively reported [17–20]. However, most works have focused on the study of key transcription factors involved in the regulation of transcriptome networks [7, 21, 22] and the role of chromatin structure in the regulation of transcription programs during stem cell differentiation [23–27].

Focusing on AS, one of the first genome-wide approaches revealed that more that 1,000 genes experience alternative

splicing events in ESCs [17]. Using splicing microarrays, it was reported that among 40,000 putative exon-exon junctions, AS patterns of 170 were changed between ES cells and embryonic bodies [19], and it was predicted that 67% of these events altered the protein sequence and domain composition. Moreover, different RNA binding proteins (RBPs) including putative splicing regulators were shown to be differentially expressed and spliced across embryonic developmental stages, suggesting frequent AS variations during early phases of differentiation [28]. Specifically, it was reported that an AS event of FOXP1 changes during ESC differentiation and that different isoforms have distinct DNA-binding affinities and regulate the expression of genes required for ESC differentiation [29].

More recently, it was found that expression of dozens of AS isoforms differed during stem cell differentiation, including the previously known embryonic stem cell-specific event in FOXP1 [30, 31]. Moreover, the authors identified the specific splicing factors that orchestrate the differential AS programs between pluripotent and differentiated cells [30]. In particular, expression levels of two known splicing regulators, the muscleblind-like RBPs MBNL1 and MBNL2 were found to increase during ESC differentiation. Moreover, AS sites in ESC were highly enriched in MBNL1 and MBNL2 binding motifs and knockdown of MBNL proteins in differentiated cells causes switching to an ESC like AS pattern (Fig. 2).

The well-characterized factors that directly control pluripotency also undergo changes in their AS patterns during ESC differentiation. A core set of transcription factors, including OCT4, NANOG, and SOX2, are master genes required for the stemness properties of murine and primate embryonic stem cells [32]. Their function resides in the control of the expression of gene cohorts required for establishment and maintenance of ESC pluripotency. A modest increase or decrease in OCT4 protein levels impairs ESC self-renewal and triggers differentiation [33, 34]. Thus, their AS choices could have a direct impact on ESC differentiation. The classic example is that of the OCT4 gene, which was identified as encoding three different isoforms. While one isoform is able to control genes that are responsible for stemness [35], the other isoforms do not have the ability to maintain ESC self-renewal. There also exist eight processed OCT4 pseudogenes that cannot produce stable proteins [36], suggesting that the specific isoform OCT4A is the only characterized isoform required for stemness. In terms of future directions, it will be very interesting to assess the specific mechanism of how AS of the core set of transcription factors governing pluripotency have a direct impact in regulating embryonic stem cell differentiation.

Alternative splicing profiles are reverted during stem cell reprogramming

In order to study the relevance of AS decisions in ESC, an integrated point of view must also take into account the gene expression programs during the transition of reprogrammed cells. It has been documented that when somatic cells are reprogrammed to pluripotent stem cells, transcription of most genes reverted to an ESC-like state [8]. Moreover, the evolutionarily conserved ESC-specific AS events have been addressed. As mentioned before, Gabut et al. [29] identified a specific ESC isoform of FOXP1 that stimulates the expression of master transcription factors required for the development of pluripotency. Moreover, FOXP1 ESC-specific splicing isoform contributes to efficient reprogramming of somatic cells into induced pluripotent stem cells by controlling fundamental ESC-specific transcriptional programs.

Covering the AS profiles of induced pluripotent stem cells and ESC, a genome-wide study identified the splicing events that changed during the reprogramming process [37]. The authors found that somatic AS profiles revert to pluripotent patterns during reprogramming of induced pluripotent stem cells and identified some regulatory key regions. Furthermore, they showed that genes with changes in AS patterns during reprogramming were relevant for biological processes and pathways as varied as embryonic development and RNA post-transcriptional modification. Finally, the authors demonstrated that the splicing factors U2AF1 and SRSF3 play a crucial role in the efficiency of somatic cell reprogramming. These observations highlight that the drastic alteration in AS patterns represents part of the molecular network involved in the reprogramming process.

In addition, a comprehensive study analyzed AS changes in high- and low-expressed genes during induction of fibroblasts, from

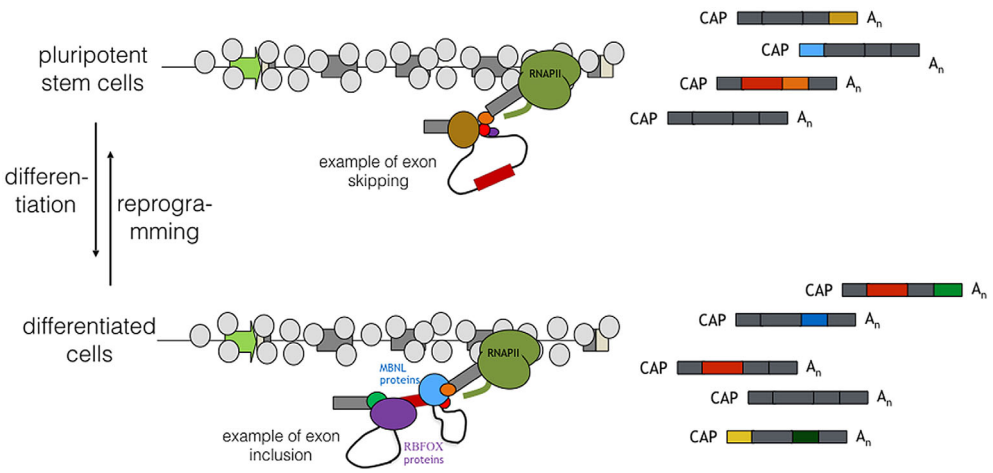


Figure 2. AS isoforms greatly change their expression patterns during differentiation of pluripotent stem cells. Changes in expression levels of regulators of AS, as MBNL and RFOX, orchestrate the distinct AS outputs observed in differentiated cells compared to stem cells. During reprograming of somatic cells, the reverse mechanism is activated, and AS patterns revert to those of undifferentiated cells.

several donors, into pluripotent stem cells and their subsequent re-differentiation [38]. The authors observed a reversible program of AS events between the fibroblastic and pluripotent states. Moreover, in order to identifying the splicing regulators involved in pluripotency and stem cell reprogramming, they monitored 47 specific alternatively spliced regions in different genes upon knockdown of 81 potentially involved splicing factors. Surprisingly, MBNL1 and RBFOX2 were found to be associated with the regulation of AS events during induction of fibroblasts into the pluripotent state. Also, expression levels of MBNL1 and RBFOX2 were downregulated in pluripotent stem cells, suggesting that both factors could be involved in the control of the reversible reprogramming of splicing events observed in pluripotent transition (Fig. 2).

Finally, a recent study profiled transcriptome-wide AS changes during reprogramming of fibroblasts and performed a comprehensive temporal analysis of the dynamic regulation of AS during the acquisition of pluripotency [39]. Using RNA-seq, the authors defined the AS programs that accompany the distinct temporal phases during fibroblasts reprograming, and found additional splicing regulators that play important roles. Analyzing distinct patterns of AS changes across the time course of reprograming, they identify 95 RBPs as candidate regulators of splicing. Moreover, the authors go deeply into possible mechanisms and asses the role of particular splicing regulators. Specifically focusing in changes in the expression of the AS factor Esrp1, they show that by modulating the AS of the epithelial specific transcription factor Grhl1, Esrp1 enhances fibroblast reprogramming.

Alternative splicing contributes to adipogenesis

Adipose tissues are endocrine organs that play crucial roles in energy homeostasis pathways of organisms [40]. AS profiles, as in other tissues, constitute a fundamental mechanism to modulate adipogenesis development. Some AS variants have been reported to have different or even opposite effects on adipogenesis [41, 42]. Although genome-wide analyses on splicing factors that regulate AS in differentiating adipocytes have rarely been documented, some examples of splicing factors were identified to influence adipocyte differentiation by regulating particular AS events.

For example, Huot et al. [43] identified that the RNA-binding protein Sam68 regulates energy expenditure and adipogenic differentiation by regulating specific AS events as the retention of intron 5 of the mTOR transcript. When this intron is retained, it introduces a premature termination codon that leads to an unstable mRNA that in turn triggers defects in adipogenesis.

Furthermore, the role of AS during differentiation of adipose tissues has been extended and connected to other mRNA processing events such as the demethylation of RNA N6-methyladenosine (m6A). Enhanced levels of m6A in response to depletion of Fat Mass and Obesity-associated protein (FTO) promote the RNA binding ability of certain splicing factors, leading to increased inclusion of target exons [44]. Specifically, FTO ablation was demonstrated to reprogram the splicing profiles of Runt-related transcription factor 1 (RUNX1T1) gene. Gradual reduction of FTO protein with a concomitant decrease in the inclusion of RUNX1T1 exon 6 was observed during the adipogenesis. Taken together, these results support a role for FTO-dependent m6A demethylation in regulating RNA AS during adipogenesis.

Another biologically relevant adipocyte-related AS event involves the Pref-1 gene, which participates in the different stages of adipogenesis. Upregulation of the multifunctional RNA-binding protein RBM4a changes the AS patterns of Pref-1 relieving its repressive effect on adipogenesis [45].

However, much remains to be done to characterize the spectrum of genes that are regulated at the level of splicing in adipogenesis and to identify the underlying mechanism of this trends.

Alternative splicing plays a role in myogenic differentiation

Apart from the roles of AS in stem cell differentiation discussed above, reprogramming and adipogenesis, other cell fate transitions such as myogenic differentiation seems to be affected by AS modulation. To assess the extent and significance of AS events in myogenesis, an original work used splicing-sensitive microarray analysis of differentiating C2C12 myoblasts [46]. Analyzing four temporal points during myogenic differentiation, the authors identified 95 different AS changes. Moreover, the AS events that underwent modulation during myogenic transition were often enriched in binding sites of splicing regulatory factors. Finally, some splicing regulators showed significant differences in their expression patterns, suggesting that their modulation is an integral component of myogenic differentiation.

More recently, Rbfox proteins were found to control AS patterns of Mef2D that represent a key step during muscle differentiation [47]. The authors showed that Rbfox1 and Rbfox2 proteins cooperate in promoting Mef2D splicing, because its overexpression caused a significant increase in Mef2D $\alpha 2$ exon inclusion. As Mef2D splicing is essential for myogenesis, this work provides evidence of splicing factors directly regulating muscle differentiation. Another study also identified Rock2 as another important Rbfox2 splicing target [48] and showed that Rbfox2 is required for the specific step of myoblast fusion during myogenesis and is responsible for the regulation of 30% of the splicing transitions observed.

Furthermore, microRNAs had also been involved in the regulation of AS during myogenic differentiation. MicroRNA-222 was shown to be downregulated during muscle differentiation and to affect two targets, Ahnak and Rbm24, involved in the regulation of myogenic differentiation [49]. This finely regulated pathway impairs the production of muscle-specific isoforms of Coro6, Fxr1, and NACA transcripts.

In addition, a model in which CELF and MBNL proteins regulate muscle-specific AS patterns by an antagonistic mechanism has been proposed [50]. CELF1 and MBNL1 bind independently to 3′UTRs of hundreds of mRNAs and functionally compete to specify down-regulation or localization/stabilization, respectively, of their targets. For instance,

the levels of MBNL are elevated in myotonic dystrophy type 1 causing an aberrant splicing and the consequent lost of the muscle-specific chloride channel (ClC-1) mRNA and protein [51]. Aberrant regulation of MBNL proteins has also been described to disrupt important functions of muscle cells as insulin receptors due to altered splicing of the insulin receptor (IR) pre-mRNA [52], and T tubule proteins caused by alternations in the splicing patterns of the bridging integrator-1 (BIN1) pre-mRNA [53].

However, the molecular mechanisms regulating AS and ensuring proper temporal control of myogenesis had been poorly described for other essential muscle-specific AS events. New studies in the field would shed light on this outstanding question, and help to predict the general properties of these and other examples.

Global changes in alternative splicing profiles occur during immune cell differentiation and stimulation

Because AS contributes to many cell differentiation pathways and represents a major mechanism regulating transcript isoform expression, it is easy to imagine that splicing decisions could play a role during immune responses. Given the diversity and plasticity required for immune effector functions it seems inevitable that cells of the adaptive immune system rely on regulated pre-mRNA splicing to at least some extent to control protein expression and function [54].

The first large analysis of AS in T cells was performed by microarray profiling [55]. A more recent large scale study using RNA-seq technology identified more than 150 AS events that exhibit robust changes upon stimulation of a human T-cell line [55]. Moreover, these regulated alternative exons were found to be significantly enriched in genes related to the immune response. Importantly, expression of the splicing factor CELF2 was found to increase in T cells during development and in response to antigen-induced signaling events, and to be responsible for the regulation of specific splicing events as the alternative exons of LEF1 and MKK7 [56, 57]. 300 CELF2-regulated splicing events change upon stimulation of T cells [57] depending on the position of CELF2. Thus, the location of CELF2 binding around alternative exons can be considered as a primary predictor of CELF2 function [56]. Moreover, another splicing factor, hnRNP L, was found to control the inclusion of a broad spectrum of alternative cassette exons in T cells [58]. Specifically, the authors observed 826 cassette exons in unstimulated cells and 635 exons in stimulated cells that differ upon hnRNP L depletion, including splicing events in MYBL1, TURD1, PUF60, and SRRM1. Although these studies represent the basis of our knowledge of global AS changes during T-cell activation, many more regulatory factors and splicing events with their specific mechanism of regulation remain to be investigated.

Additionally, another mechanism of AS known as intron retention, represents a major step of gene expression regulation in the immune system. Global intron retention levels were significantly reduced upon T cell activation [59]. The majority of the genes upregulated in activated T cells are regulated at the intron retention level, and highly enriched in the proteasome pathway, which is essential for proper T cell proliferation and cytokine release. Furthermore, an analyses of transcriptomics data of white blood cell differentiation revealed that intron retention of 86 functionally related genes plays a crucial role during granulopoiesis [60]. Included among the regulated genes are the ones that determine the nuclear shape of granulocytes. Moreover, the authors suggest that intron retention in specific genes is associated with downregulation of splicing factors and higher GC content. One particular mechanism elucidated in this work refers to the intron retention of Lmnb1 transcript that leads to downregulation of Lmnb1 protein levels. Because the reduced expression of intron-retaining genes, including Lmnb1, was not explained by altered transcription, the authors suggest that the mechanism underlying this downregulation at the protein level could be nonsense-mediated decay triggered by intron retention.

Although some examples of AS regulation in the differentiation of the immune system, as the ones we discussed here, are emerging, many more events and regulatory pathways have yet to be investigated and rigorously explored.

Alternative splicing regulates neuronal differentiation

Despite its complexity, splicing regulatory programs greatly affect neuronal development, and some examples points out the importance of particular RNA-binding proteins [61].

The AS mechanism generating either calcitonin or the calcitonin gene related peptide (CGRP) was the first neuronal-specific AS events to be described. The alternative isoform of the calcitonin mRNA is produced in the C cells of the thyroid, while CGRP mRNA is made in hippocampal neurons and is crucial for their maturation [62].

High-throughput genome-wide approaches revealed that AS is particularly widespread in the mammalian nervous system [10, 13, 14]. Many different splicing choices are induced during neuronal differentiation and play key regulatory functions in neuronal development. The most complex patterns of splicing described occur in molecules important for the differentiation of neurons and the formation of their intricate connections [10].

A recent study analyzed the differentially spliced exons during neuronal differentiation of P19 mouse embryonic carcinoma cells [63] and found that 262 exons were alternatively spliced in neuronal cells compared with undifferentiated cells. Moreover, many of the genes containing these differentiation exons were suggested to be involved in neural events. From the 262 events, the authors studied 241 that are predicted to change the amino acid sequences between the alternatively spliced protein isoforms. Conserved domain searches indicated that 49 genes have differentially spliced in exons that affect their main functions, and identified the EGFR and mTOR signaling pathway as being involved [64].

Using the same cells as a neural differentiation model, another work observed a temporal correlation between RBM4 expression and a change in splicing isoforms of the cell-fate determination gene Numb [65]. The authors demonstrated that, by regulating Numb AS patterns, RBM4 controls the expression of the proneural gene Mash1. Moreover, they found that this regulation of RBM4 is essential for neurite outgrowth, indicating that RBM4 modulates exon selection of Numb to generate isoforms that promote neuronal cell differentiation and neurite outgrowth. Moreover, the splicing switch in Numb can also be regulated by RBFOX3. Using the developing chicken spinal cord as a model, RBFOX3 was found to be required during neuronal differentiation by regulating Numb AS patterns [66].

In addition, a recent report demonstrate that repression of the RBP known as PTB is sufficient to induce trans-differentiation of fibroblasts into functional neurons [67]. PTB is down-regulated during normal brain development via the action of miR-124, causing a de-repressing of a large array of neuronal genes, including multiple neuronal-specific transcription factors. When this mechanism is induced in non-neuronal cells, it triggers a positive feedback loop to elicit cellular reprogramming to the neuronal lineage. Furthermore, PTBP2 was shown to be required for neuronal maturation, as its depletion leads to degeneration of mouse cortex. Although depletion of PTBP2 did not affect neurotic outgrowth and neuronal marker expression, the mutant cells failed to mature and die over the first week in culture [44]. Furthermore, the authors found many AS events misregulated in mutant brains, defining a genetic regulatory program in which PTBP2 repress the expression of mature neuronal isoforms until the final maturation of neurons. The significant role of PTBP2 in the nervous system was previously defined because it was discovered to be essential for postnatal survival [68]. The authors present evidence supporting a direct role of PTBP2 in inhibiting adult-specific alternative exons by binding pyrimidine-rich sequences. These works present more examples of changes in the protein levels of splicing factors during cell differentiation that can be directly linked to the regulation of crucial AS outcomes.

On the other hand, the neuron-specific AS factor Nova was found to bind specific intronic clusters and enhance spliceosome assembly and exon inclusion, resulting in a local action to regulate spliceosome assembly and AS in neurons [69]. Apart from their important role in neuronal differentiation, neuron-specific RBPs were seen to control excitation-inhibition balance in the brain [70]. Neuronal Elav-like (nElavl), which binds preferentially to GU-rich sequences, were found to bind intronic sequences in a position-dependent manner to regulate AS and to 3′UTR sequences to regulate mRNA levels. Their regulation of AS and mRNA levels in the brain is required to maintain neurotransmitter glutamate levels [70].

The splicing phenotype of a cell not only affects neuronal differentiation, but also contributes to its identity as a mature neuron [71]. Transcripts from the gene for the transcription factor neuron restrictive silencer factor (NRSF/REST) undergo AS, generating two different isoforms. In non-neuronal cells, the NRSF/REST transcripts encode a transcriptional repressor of neuron-specific genes [72, 73], while in mature neurons, a truncated NRSF/REST protein is produced [74]. By analyzing the role of the AS decisions of REST during neurogenesis, Raj et al. demonstrated that the neural specific factor nSR100 favors the expression of the neural specific REST isoform that is characterized by a reduced repressive activity [75]. The expression of this neural isoform of REST triggers the activation of REST targets in neural cells, directly regulating the specific expression programs required for neurogenesis.

Although many studies have been pointing out the relevance of AS in neurogenesis, much remains unknown about the specific mechanism governing the association between splicing decisions and neurological disorders. Of particular importance is the role of RBPs RBPs in the nervous system and how their expression and activity patterns could influence global AS choices. The complex overlap between RBP modulation programs allows for a high degree of regulation in global and specific splicing events. It will be very interesting to assess how the expression of RBPs and spliced isoforms contribute to defining different neurological diseases, and how they affect the differentiation of specific neuronal subtypes.

Chromatin structure regulates alternative splicing in neuronal differentiation

Of particular importance, the neural cell adhesion molecule (NCAM) is encoded by a single gene that gives rise to at least

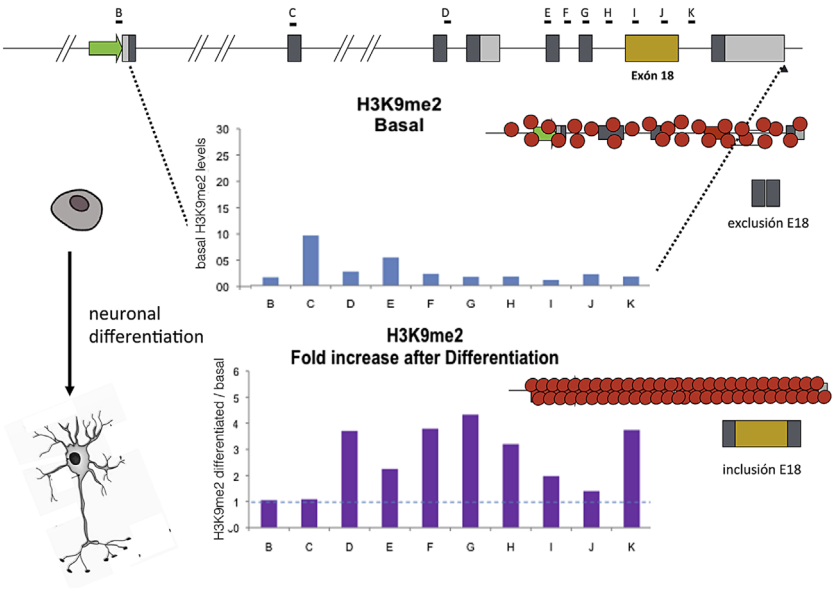


Figure 3. During neuronal differentiation, chromatin structure changes from an open state (top) in the region of NCAM exon 18 to a more close state (bottom), indicated by increased levels of dimethylation on lysine 9 of histone H3 (H3K9me2). Analyzed regions for H3K9me2 are shown with letters B through K in the upper panel. Concomitantly, RNA AS pattern changes in favor of higher inclusion of exon 18 into the mature mRNAs.

20–30 mRNA isoforms by AS [76]. The two more abundant protein isoforms are named according to their apparent molecular weight (NCAM180, NCAM140). NCAM140 only differs from NCAM180 by the absence of exon 18, which causes the shortening of the cytoplasmic domain. During neuronal differentiation, the AS pattern of NCAM changes, favoring the expression of NCAM180 to the detriment of NCAM140. As a consequence of this splicing regulation, NCAM140 expression is more abundant in neuronal precursors where it is homogeneously distributed in the cell membrane and favors neurite growth. Conversely, NCAM180 is more specific of mature neurons, is enriched in cell to cell contacts, and contributes to organized stable and mature synapses [77, 78]. Our laboratory investigated the basis of NCAM AS regulation by membrane depolarization of neuronal cells [79]. Treatment of the murine neuroblastoma cell line N2a with increasing concentrations of extracellular KCl caused depolarization of N2a cells and induced NCAM exon 18 skipping. Moreover, the authors demonstrated that NCAM exon 18 splicing is affected by the elongation rate of RNA polymerase II (RNAPII), and found that when transcription is driven by a slow mutant of RNAPII, inclusion of NCAM exon 18 is increased. Then, they investigated if NCAM exon 18 splicing could be modulated by changes in the histone acetylation patterns, and found that intragenic patterns of H3 lysine 9 and H4 acetylation around exon 18 are lower in untreated cells compared to depolarized cells, suggesting that a localized change in intragenic chromatin structure is triggered by the treatment. The more relaxed chromatin configuration around exon 18 caused by membrane depolarization correlated with skipping of the exon 18 during pre-mRNA processing by a mechanism that presumably involves enhanced transcriptional elongation.

In addition, our lab further analyzed the regulation of NCAM AS by chromatin structure during neuronal differentiation [80]. Using a previously established neuronal differentiation model, we showed that during the differentiation of N2a cells into mature neurons inclusion of NCAM exon 18 in the mature mRNA increases. This up-regulation correlated with the acquisition of repressive histone heterochromatin marks along the NCAM gene body with a concomitant reduction in RNAPII elongation rate at the region of E18. Moreover, we demonstrated that the change in exon 18 splicing is reverted by treatments with drugs that promote chromatin relaxation, showing that the general repressive chromatin conformation being deployed during differentiation inside the NCAM gene plays an important role in the NCAM AS regulation. Our results complete a picture in which differentiation promotes intragenic chromatin silencing, localized decrease in elongation and higher exon 18 inclusion. Overall, these results support a general model in which cells subjected to two different stimuli (membrane potential depolarization or differentiation signals) can modulate the chromatin surrounding a single AS event in opposite ways to have opposite effects on localized RNAPII elongation. In this way, this mechanism gives rise to two different AS patterns, each of them characteristic of a functional and differentiation status of the neuronal cells (Fig. 3).

Additionally, more recent work points out at an important role of the alternative patterns of chromatin modifying enzymes in the regulation of neuronal differentiation. In particular, one specific isoform of the lysine-specific demethylase 1 (LSD1), the enzyme responsible for H3K4me1/2 and H3K9me1/2 demethylation has been shown to play a key role in nervous system development [81]. The inclusion of an alternative exon in LSD1 promotes the acquisition of selective histone substrate specificities and regulates important transcriptional programs in neurons. Specifically, inclusion of the alternative exon mediates H3K9me2 demethylation in collaboration with other protein partners. Knockdown of the isoform that includes the alternative exon compromises neuronal differentiation and increases H3K9me2 levels at its target promoters. Overall, these results suggest that AS pattern of LSD1 is fundamental for the regulation of specific expression programs that promote neuronal differentiation.

On the other hand, G9a, the enzyme responsible for mono- and di-methylation of lysine 9 on histone H3 (H3K9me1 and H3K9me2) in mammals, has been widely implicated in the differentiation of a variety of cell types and has been characterized as a key factor in the nervous system. In our recent publication [82], the role of AS of G9a in neuronal differentiation has been addressed. We found that neuronal differentiation of the N2a cell line in culture positively correlates with the inclusion of G9a alternative exon 10 (E10) in the mature mRNA and upregulated levels of H3K9me2. Although E10 inclusion does not affect G9a intrinsic catalytic activity, it results in increased H3K9me2 global levels due to higher nuclear localization of the enzyme. Because E10 does not encode a nuclear localization signal (NLS), we demonstrated that inclusion of E10 promotes G9a nuclear localization probably by enhancing the exposure of the neighboring constitutive NLS due to its intrinsically unstructured nature. Moreover, we showed that G9a regulates its own AS, and that the isoform including amino acids encoded by E10 promotes more inclusion of E10 in the mature mRNA. Finally, we found that the change in the AS pattern of G9a is required for an efficient neuronal differentiation, suggesting a positive feedback loop that reinforces cellular commitment to differentiation and highlights the key role of alternative isoforms during neuronal differentiation (Fig. 4).

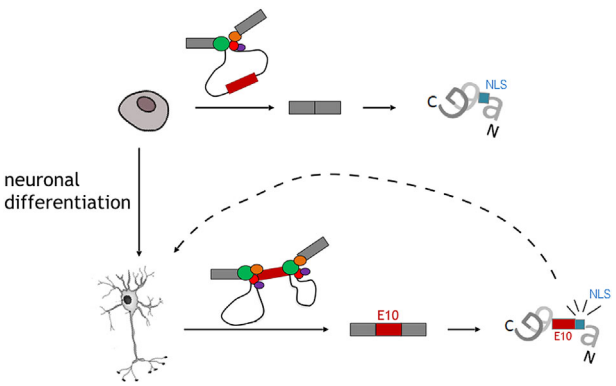


Figure 4. AS of the histone methyl transferase G9a controls neuronal differentiation. In neuronal precursors, G9a exon 10 (E10) is predominantly skipped. During neuronal differentiation, E10 inclusion is upregulated. Modulation of G9a AS, as well as neuronal differentiation, requires G9a methyltransferase activity, suggesting a positive feedback loop in which G9a regulates its own AS during differentiation.

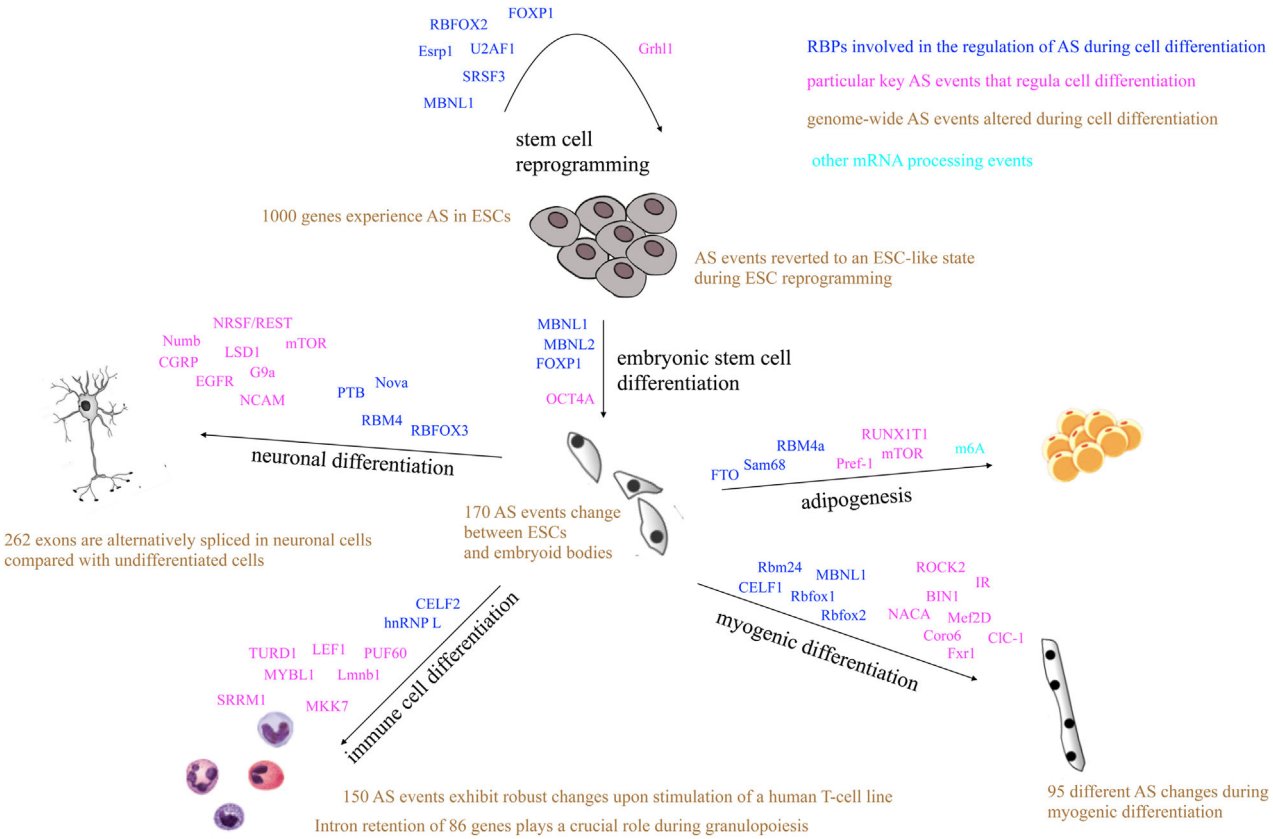


Figure 5. Integrated overview of AS changes during the cell differentiation processes reviewed here. In brown the genome-wide AS events are shown that are regulated during a particular cell differentiation process (depicted by the black arrows). Blue indicates the various RBPs involved in the regulation of the observed AS changes. mRNAs whose AS patterns play a crucial role in cell differentiation are shown in pink and other mRNA processing events that take place during differentiation are pictured in cyan.

Conclusions and outlook

It is now evident that AS is a tightly regulated process that, through the expansion of the coding capacity of the genome, participates in the regulation of different crucial processes including cellular differentiation. Accumulated evidence highlights the role of AS decisions as key factors in the modulation of complexity of organisms. Focusing not only on specific examples, but also analyzing genome-wide approaches, we have discussed here how cell differentiation process can be followed by changes in the AS patterns. In this review, we integrate the genome-wide analyses that characterized the spectrum of AS events that are regulated during different types of cell differentiation with some particular examples of RBPs whose regulation contribute to changes in specific AS patterns that are required for cell differentiation (Fig. 5). An important conclusion of the recent studies is that in many cases changes in AS detected during differentiation are not the consequence, but the cause, of the differentiation process. This is particularly well illustrated by the various splicing roles played in neuronal differentiation.

However, much remains to be done in order to identify the spectrum of genes, the regulation of which, at the level of AS have a major effect on cell differentiation. Although the detailed characterization of individual AS events is essential to ultimately understand the full functional consequence of their regulation during cell differentiation, we need to have a broader understanding of the networks of genes that are coordinately regulated to achieve a concerted functional outcome. Moreover, because AS allows for regulation of expression of specific isoforms in a developmental and tissue-specific manner, and we know that many AS events are conserved across mammalian or vertebrate species, it would be very interesting to asses the effect of these splicing changes on protein activity. One of the challenges of the field is to truly characterize the set of protein isoforms expressed from each gene and understand their different roles in cell differentiation.

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