SAPIENZA
Università di Roma
Facoltà di Farmacia e Medicina

DOTTORATO DI RICERCA
IN SCIENZE E TECNOLOGIE CELLULARI

XXVI Ciclo
(A.A. 2012/2013)

Alternative splicing regulation in human cancer

Dottoranda
Chiara Naro

Tutor
Prof. Claudio Sette

Coordinatore
Prof. Elio Ziparo

Index

The thesis explained

Gene expression in eukaryotic cells is highly regulated by several co-transcriptional and post-transcriptional mechanisms, which finely tune cellular responses to external and internal cues. Each cellular process is indeed the result of a sequence of regulative events controlling gene transcription, pre-mRNA processing, mRNA translation and finally protein activity. Several different mechanisms, interplaying with each other, take part to this complex regulative network. Among these, pre-mRNA splicing is emerging as a key step involved in cell fate decisions and in physiological and pathological processes (Kornblihtt et al., 2013).
Splicing mediates removal of intronic sequences and joining of exons in the newly synthesized pre-mRNAs, thus ensuring their correct maturation in eukaryotic cells (Black, 2003). The majority of the multiexonic genes of eukaryotic genomes undergoes alternative splicing (AS), thus allowing a single gene to encode for multiple transcripts, potentially having different coding properties and/or pattern of spatial/temporal expression. AS thus represents an important tool for eukaryotic cells to expand the coding potential of their genome and for proper regulation of gene expression (Blencowe, 2006).
Although AS has evolved as a powerful genetic tool conferring advantage to complex organisms, its complex and flexible regulation also represents a risk factor. Indeed, defects in the regulation of this molecular step of pre-mRNA processing may have detrimental consequences for the cellular homeostasis and aberrant regulation of AS has been demonstrated to contribute to the onset of several human diseases, including cancer (Tazi et al., 2009).
High-throughput analyses of transcriptomes have highlighted widespread alterations of AS patterns in several different tumor types (Venables et al., 2008; Misquitta-Ali et al., 2011). Furthermore, it has been shown that production of specific

oncogenic splice variants of cancer-related genes supports the neoplastic transformation process by enhancing cancer cells proliferation, metabolism, motility and drug-resistance (David and Manley, 2010).

Given the well-recognized role of aberrant regulation of AS in cancer development, it is extremely important to characterize the molecular mechanisms underneath this phenomenon, which requires a better characterization of the multiple layers of control of the AS process.

In this PhD project, we investigated the functional relevance of the nuclear localization in cancer cells of the centrosomal kinase NEK2. This led to the discovery that this kinase interacts with several splicing factors and it acts as a splicing factor kinase in cancer cells. In particular, we demonstrated that NEK2 phosphorylates and regulates the activity of the oncogenic splicing factor SRSF1, thereby promoting splice variants that support cell survival. Therefore, our results have uncovered a novel function for the centrosomal kinase NEK2 and suggest that part of its oncogenic activity may be ascribed to its ability to modulate AS.

The second part of my study was aimed at investigating the reciprocal control of AS and chromatin modifications. In particular, we have investigated whether the histone deacetylases inhibitor (HDACi) LBH589, a new generation anti-cancer drug, affects splicing of genes relevant for prostate cancer proliferation. We found that treatment with this HDACi regulates different AS events and in particular the inclusion of the variable exons of the *CD44* gene in prostate cancer cells. The splicing regulative activity of the LBH589 does not rely on the modulation of the expression levels of common splicing regulators, but it appears to affect local changes in the chromatin structure within the *CD44* locus. This may in turn affect the processivity of the RNAPII or the recruitment of splicing regulator, a hypothesis that is currently being evaluated.

Introduction

1. Alternative splicing

Splicing is the molecular process mediating removal of intronic sequences and joining of exons in the newly synthesized pre-mRNAs; it is therefore one of the key event in the multistep process ensuring correct pre-mRNA maturation in eukaryotic cells (Moore and Proudfoot, 2009).
The splicing process is carried out by the spliceosome, a complex macromolecular machinery composed of five small nuclear ribonucleoprotein particles (U1, U2, U4, U5 and U6 snRNP) and more than 200 auxiliary proteins. The spliceosome mediates the recognition of the short consensus sequences defining the 5'-(GU) and 3'- (AG) splice sites and it catalyzes the two transesterification reactions necessary for the junction of the selected exons and the removal of the intervening intron (Will and Lührmann, 2011).
Even though largely occurring co-transcriptionally (Singh and Padgett, 2009), splicing does not remove introns in the same strict 5'-3'order that they are transcribed (Pandya-Jones and Black, 2009). On the contrary, it has been widely demonstrated that the majority of the genes of eukaryotic genomes undergoes alternative splicing (AS), which allows a great expansion of their potential coding (Merkin et al., 2012) (Barbosa-Morais et al., 2012). Exons from a single pre-mRNA can be joined through AS in different combinations, thus allowing a single gene to encode for multiple transcripts, potentially having different coding properties and/or pattern of spatial/temporal expression. AS is therefore one of the main mechanisms finely tuning gene expression in eukaryotic cells (Blencowe, 2006), playing an important role in the regulation of several physiological cellular processes (Kalsotra and Cooper, 2011). As a matter of fact, several reports in the recent literature have highlighted the importance of regulated AS

events during developmentally highly-regulated processes, such as the maintenance of pluripotency of embryonic stem (ES) cells (Han et al., 2013). For example, it has been revealed that the muscleblind-like RNA binding proteins (RBPs), MBNL1 and MBNL2, control a large number of AS events differently regulated between ES and differentiated cells; moreover knocking down of MBNLs was shown to enhance reprogramming of somatic cells in induced pluripotent stem cells (IPSC) (Han et al., 2013).

Due to the important role played by AS in the control of gene expression, any alterations of its regulation can profoundly modify important cellular processes, thus resulting in a potential cause of disease (Cooper et al., 2009). In this regard, several evidence have shown that aberrant splicing regulation of cancer-related genes supports tumoral transformation by producing protein isoforms enhancing cancer cells proliferation, metabolism, motility and drug-resistance (David and Manley, 2010). Moreover high-throughput analyses of transcriptomes have highlighted widespread alterations of AS pattern for several different tumor types, as breast, prostate and lung cancer (Venables et al., 2008; Li et al., 2006; Misquitta-Ali et al., 2011), thus strongly supporting the involvement of dysregulation of AS in the molecular pathogenesis of cancer.

Deciphering the multiple layers of control regulating the AS process is therefore an important step for a comprehensive understanding of the molecular mechanisms regulating pre-mRNA processing, which is essential for the rational design of future therapies targeting the aberrant AS process in cancer and other human diseases (Singh and Cooper, 2012).

1.1 Alternative splicing regulation

Human genes structure is characterized by short exons (approximately 200 bp) interspersed among larger introns

(Sakharkar et al., 2004). Short and degenerate sequences defining splice sites are therefore not sufficient to ensure the accurate recognition and splicing of constitutive and alternative exons, but both *cis*-regulatory sequence elements on the pre-mRNA and *trans*-acting RBPs are necessary to correctly guide spliceosome on selected exons and facilitate splicing catalysis (House and Lynch, 2008).
Moreover, an increasing number of evidence is highlighting the influence exerted by chromatin structure and epigenetic modifications on the splicing process, by both acting on transcription dynamics and recruitment of spliceosome and splicing factors (Schwartz and Ast, 2010).
This multilevel regulation of AS makes it a flexible process, able to efficiently regulate gene expression, but, at the same time, it makes it susceptible to threatening alterations, eventually pathological, from several from multiple fronts (Pal et al., 2012).

1.2 Alternative splicing regulatory sequences and factors

Several *cis*-acting regulatory sequence elements rule selection of constitutive and alternative exons, regulating their recognition by the spliceosome (Hertel, 2008).These elements are known as exonic and intronic splicing enhancer (ESEs and ISEs respectively) and silencer (ESSs en ISSs respectively); their function is to recruit on specific sites RBPs, which in turn can promote or inhibit spliceosome assembly and activity (Hertel, 2008) (Figure 1).
Mutations disrupting splicing regulatory elements and/or canonical splice sites are highly frequent in cancer cells, thus likely favoring the general splicing deregulation that correlates and contributes to tumoral transformation (Ghigna et al., 2008). An illustrative example of this kind of mutations are those disrupting the activity of ESE elements in the coding region of the tumor-suppressor BRCA1 gene (Liu et al., 2001).

Two main classes of splicing factors regulates AS by binding to these *cis*-acting regulatory elements: Ser/Arg rich (SR) proteins, which mainly exert a positive regulation, and heterogeneous nuclear ribonucleoproteins (hnRNPs), which, on the contrary, usually inhibit splicing (Matlin et al., 2005) (Figure 1). Other RBPs not belonging to neither of these two classes can also modulate AS events, as the multifunctional RBP SAM68 (Bielli et al., 2011), thus further intriguing the scenario of AS regulation.
Even if SR proteins are generally described as splicing activators and hnRNPs as splicing repressors, their splicing activity, as well as of the other RBPs, is actually dependent on the location of their binding sites and on the outcome of their competition with other splicing factors (Matlin et al., 2005). For example, it has been shown that the commonly defined splicing repressor PTB (also known as hnRNPI) can also act as splicing activator, depending on the position of its binding site (Llorian et al., 2010). Indeed, a microarray analysis has revealed that PTB acts as a splicing repressor when its target pyrimidine-rich motifs are located within or upstream an alternative exon, whereas it acts as splicing activator on target motifs downstream alternative exons (Llorian et al., 2010). PTB also offers a clear example of regulative competition between different splicing factors, as it has been

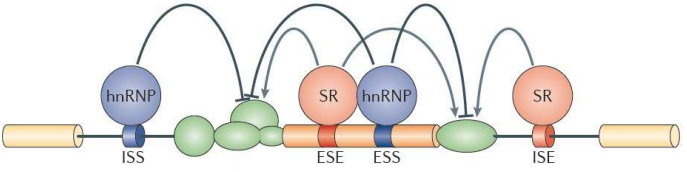


Figure 1. AS regulatory elements: AS is regulated by cis-regulatory sequences in the pre-mRNA that promote (ESE - exonic splicing enhancer, ISE - intronic splicing enhancer or inhibit (ESS - exonic splicing silencer, ISS - intronic splicing silencer splicing. These elements are recognized by two main families of trans-acting factors, the SR proteins (SR) and the heterogeneous nuclear ribonucleoproteins (hnRNP), which respectively enhance or inhibit spliceosome assembly on 5' and 3' splice sites (adapted from Kornblihtt et al., 2013).

shown that its repressive activity is due to the ability to compete out from target exons the auxiliary splicing factor U2AF, thus impairing recruitment of the U2 snRNP component of the spliceosome (Izquierdo et al., 2005) (Sharma et al., 2005).
Activity and expression of splicing factors is frequently deregulated in cancer cells and a proper proto-oncogenic activity has been identified for some of them (Grosso et al., 2008). Indeed, it has been shown that overexpression of the SR protein SRSF1 or of the hnRNPA2/B1 is sufficient to induce malignant transformation of immortalized cells (Karni et al., 2007) (Golan-Gerstl et al., 2011). Interestingly, these splicing factors, as many others RBPs, have been found up-regulated in several different tumor types, strongly supporting suggestions for their pivotal role in carcinogenesis (Grosso et al., 2008).
An important layer of control of the activity of both spliceosome components and auxiliary RBPs is their post-translational modifications, such as ubiquitination (Song et al., 2010), acetylation and phosphorylation (Edmond, et al., 2011).
Importantly, reversible phosphorylation plays an outstanding role in the modulation of both constitutive and AS, with many protein kinases and phosphatases involved in such regulation (Stamm, 2008). Both phosphorylation and dephosphorylation events are required for the proper catalysis of splicing by the spliceosome (Mermoud et al., 1994). Moreover, phosphorylation can modulate the activity of splicing factors by affecting several aspects of their functionality, such as their subcellular localization (Misteli et al., 1998) (van der Houven van Oordt et al., 2000), their binding to target pre-mRNAs (Tacke et al., 1997) and their interaction with other proteins (Cho et al., 2011). On particular, the importance of reversible phosphorylation in the regulation of AS lies on its ability to modulate gene expression in response to both internal and external cues (Shin and Manley, 2004). Phosphorylation of splicing factors by both signaling-activated kinases and splicing-factor specific kinases, as those of the SR-Protein Kinases

(SRPKs) family, allows integration of AS regulation with the other mechanisms of gene expression modulation activated by the cells as a response to specific stimuli (Matter et al., 2002) (Zhou et al., 2012).
Up-regulation of the kinases and aberrant activation of the signal-pathways controlling the phosphorylation status of splicing factors are among the main causes of the altered splicing regulation observed in cancer cells (Amin et al., 2011) (Locatelli and Lange, 2011). For instance, overexpression of the prototypical SR-protein kinase SRPK1 has been documented in several different cancer types (Hayes et al., 2007). In addition, it deserves to be noticed that phosphorylation of splicing factor is also carried out by protein kinases totally unrelated to the splicing process, such as the centrosomal kinase AURKA (Moore et al., 2010), or even by proteins totally unexpected to have a kinase activity, such as the DNA topoisomerase I (Soret et al., 2003); therefore, it cannot be ruled out that other protein kinases up-regulated in cancer cells could be involved in the dysregulation of AS associated to tumoral transformation.

1.3 Transcriptional and epigenetic control of alternative splicing

It is now well-established that pre-mRNA maturation occurs co-transcriptionally, meaning that splicing, as well as capping, termination and polyadenylation, takes place while the RNA polymerase II (RNAPII) is still transcribing (Brown et al., 2012). Co-transcriptionality determines a reciprocal control between the transcriptional apparatus and the splicing machinery (de la Mata et al., 2003) (Lin et al., 2008) and it establishes an intimate connection between the nascent pre-mRNA and the template DNA, which allows chromatin structure and epigenetic modifications to modulate both the transcription and the splicing process (Schwartz and Ast, 2010).

Currently, two non-mutually exclusive models have been proposed to describe the transcriptional and epigenetic control of AS: the “kinetic model” and the “recruitment model” (Figure 2) (Pandya-Jones, 2011) (Luco et al., 2011).
The “kinetic model” states that variation of the transcriptional elongation rate of the RNAPII can influence AS events by modulating the lapse of time during which each exon is accessible to the splicing machinery (Figure 2A) (Carrillo Oesterreich et al., 2011). Indeed, a slow elongation rate of the RNAPII allows the recognition by the splicing machinery of the weak splice sites of alternative exons before the synthesis of competing downstream exons, leading to its commitment to the splicing reaction (de la Mata et al., 2010).

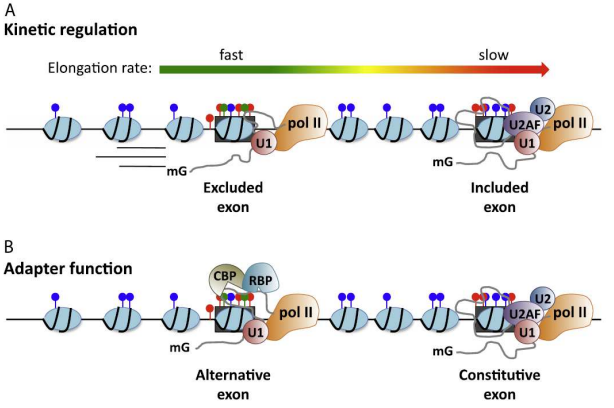


Figure 2. Transcriptional and epigenetic control of AS: Two models, the "kinetic model"(A) and the "recruitment model" (B) describe the transcriptional and epigenetic control of AS. (A) Modulation of the elongation rate of the RNAPII affects AS: a slow elongation rate favors recognition by the spliceosome of weak alternative exons before that downstream exons are transcribed; on the contrary a fast elongation rate favors exon skipping events. Epigenetic modifications can induce local changes in RNAPII processivity. (B) Both the CTD of the RNAPII and histones modification can act as docking site for the recruitment of RBPs, directly or through the mediation of a chromatin binding protein (CBP) (adapted from Shukla and Oberdoerffer, 2012).

In this regard, several reports have recently highlighted the ability of chromatin modifications, such as DNA methylation (Shukla et al., 2011) or histone acetylation (Hnilicová et al., 2011), to influence AS events by affecting RNAPII dynamics. Moreover, it has been displayed that splicing factors can regulate AS by inducing local changes in the chromatin structure and thereby in RNAPII processivity, as observed for HU proteins (Zhou et al., 2011). These RBPs have indeed been shown to promote the skipping of their target exons by locally recruiting HDAC2, which induces a local increase of histone acetylation that in turn causes a local acceleration of the RNAPII (Zhou et al., 2011). Variations of the transcription rate of the RNAPII are also associated to changes in the phosphorylation status of the regulatory C-terminal domain (CTD) of the RNAPII (Muñoz et al., 2010). For example, it has been shown that the modulation of the AS of several apoptotic genes after DNA damage is due to the hyperphosphorylation of the CTD of the RNAPII and the subsequent inhibition of the transcriptional elongation (Muñoz et al., 2009).

According to the "recruitment model" both the CTD of the RNAPII and histone modifications can act as molecular platform for the recruitment of specific splicing factors on their target pre-mRNAs (Figure 2B) (Muñoz et al., 2010) (Luco et al., 2011). The CTD of the RNAPII is indeed reported to interact with several splicing factors, like SRSF3 (previously known as SRp20) (de la Mata and Kornblihtt, 2006) or SAM68 (Cappellari et al., 2013), and to favor their recruitment on target pre-mRNAs. Moreover, it has been shown that binding of splicing factors to target exons can be facilitated by their interaction with chromatin adaptor proteins recognizing specific histone modifications (Luco et al., 2010). For instance, PTB recruitment on its target pre-mRNAs is enhanced by its interaction with the adaptor protein MRG5, which specifically binds to the H3K36me3, a histone mark enriched on PTB target genes (Luco et al., 2010).

Since their impact on transcriptional dynamics and recruitment of splicing factors, epigenetic modifications definitely represent an important layer of control of the AS process, which certainly deserves future intense studies, aimed at discovering to which extent the AS alterations observed in cancer cells can be due to epigenetic aberrations and whether they could be rescued by chromatin modifying drugs.

2. The serine-threonine kinase NEK2

2.1 NEK2 structure and function

NEK2 is a Ser/Thr-kinase belonging to the family of NIMA-related kinases or NEKs. This protein family owes its name to the high homology of its members with the never-in-mitosis A (NIMA) kinase, originally identified as a regulator of mitosis in the filamentous fungus *Aspergillus nidulans* (Fry, 2002). In human cells the NEKs family is composed of eleven members (NEK1-11), which, with the exception of NEK10, share a similar catalytic domain at their N-terminus and important roles in the cell cycle control, in particular during the mitotic progression (Fry et al., 2012).
NEK2 is the member of the NEKs human family most closely related to the NIMA protein, having a 44% of homology within their catalytic domain (Fry, 2002).
NEK2 structure is characterized by the prototypical N-terminal catalytic domain and a C-terminal regulatory region, where a proximal leucine zipper domain and a terminal coiled coil domain are located (Figure 3) (Fry, 2002). The C-terminal domain of NEK2 mediates its interaction with its substrates (Fry et al., 1998a) (Jeong et al., 2007) and, through the leucine zipper motif, NEK2 homodimerization, which is essential for its activating auto-phosphorylation (Fry et al., 1999).

NEK2 is mainly localized in the centrosome (Fry et al., 1998b); both its expression and activity are cell cycle regulated, being low during the M and G1 phase, progressively increasing during the S phase and reaching their maximum levels in the late G2 phase (Fry et al., 1995). This temporal pattern of expression well correlates with the observed NEK2 ability to regulate centrosome separation, a critical event in the G2-M transition (Fry et al., 1998).

Interestingly, the NEK2 transcript undergoes AS, which leads to the formation of three main isoforms, NEK2 A, B and C, having a different temporal and spatial pattern of expression (Hames and Fry, 2002) (Wu et al., 2007). NEK2A is the predominant canonical splice-variant; its expression peaks during the S and G2 phases of the cell cycle and rapidly decreases when entering mitosis. NEK2A is mainly localized in the centrosome and its non-centrosomal pool is equally distributed among nucleus and cytoplasm (Hames and Fry, 2002) (Wu et al., 2007). The selection of an alternative polyadenylation signal within the intron 7 of the NEK2 pre-mRNA gives rise to a shorter isoform, named NEK2B. NEK2B lacks part of the C-terminal region of the canonical variant (Hames and Fry, 2002) and its non-centrosomal pool is mainly localized in the cytoplasm (Wu et al., 2007). In particular, NEK2B lacks of the cyclin A-like D-box and the KEN box, which allow NEK2 targeting to proteasomal degradation by the anaphase promoting complex/cyclosome (APC/C) (Hames et al., 2001) (Figure 3). Therefore NEK2B protein levels remain elevated during the whole M phase and begin to decline just at the re-entry in the G1-phase (Hames and Fry, 2002). The third variant, NEK2C, differs from the canonical NEK2A just for a short deletion of eight residues in the C-terminal domain (Δ371-378), due to the choice of an alternative 3'SS within the terminal exon 8. Lack of these eight residues creates a strong nuclear localization signal in the NEK2C protein that causes its nuclear accumulation (Wu et al., 2007).

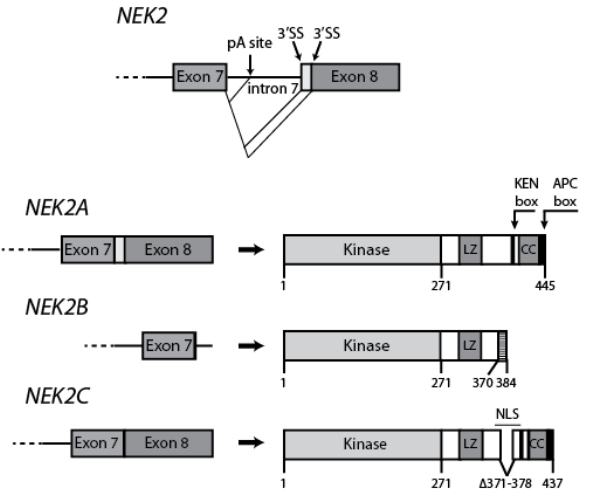


Figure 3. NEK2 protein structure. Three different isoforms of the NEK2 gene are generated through AS: NEK2A, NEK2B, and NEK2C. All three NEK2 variants are identical in their N-terminal kinase domain and leucine zipper (LZ) dimerization motif. They differ in their C-terminal domain: NEK2B is the shortest isoform, lacking of the PP1 binding site, the coiled coil (CC) domain and the APC degradation motifs present in NEK2A and NEK2C. NEK2C shows a short deletion of eight residues in the C-terminus respect to the longest isoform NEK2A, creating a strong nuclear localization signal (NLS). (Alternative polyadenylation signal, alternative 3' splice sites (SS) and aminoacids number are indicated).

The primary function of NEK2 is the regulation of centrosome separation during the G2/M transition, through the phosphorylation of its specific substrates, C-NAP1 and ROOTELIN (Fry et al., 2012). These two proteins are key components of the intercentriolar linkage material, which keeps tethered the duplicated centrioles before splitting (Nigg and Stearns, 2011). Several reports have indeed suggested that C-NAP1 and ROOTELIN dissociation from the centrosome is triggered by their NEK2-mediated phosphorylation (Fry et al., 1998a) (Bahe et al., 2005). The localized activity of NEK2 at the centrosome is regulated by the kinase MST2 and the scaffold

protein hSAV1, which target phosphorylated NEK2 at the centrosome (Mardin et al., 2010). NEK2 activity is counteracted by the protein phosphatase PP1: both isoforms PP1α and PP1γ have been found to interact with NEK2 and to counteract its activity, by mediating C-NAP1 dephosphorylation (Helps et al., 2000) (Meraldi and Nigg, 2001). Timely activation of NEK2 at the G2/M boundary is guided by another centrosomal kinase, the polo-like kinase 1 (PLK1): PLK1 phosphorylation of MST2 weakens the interaction between NEK2 and PP1γ, leading to its dissociation from the complex MST2-NEK2-PP1γ; consequent PP1γ leaving from the centrosome allows NEK2 to phosphorylate C-NAP1 and to induce centrosome splitting (Mardin et al., 2011). A correct balance between the activity of both protein kinases and protein phosphatases is therefore required for a proper centrosome separation (Mardin and Schiebel, 2012).
Other centrosomal proteins, such as NLP and CENTROBIN, are target of the kinase activity of NEK2, strongly suggesting a crucial role for this protein for the proper formation of the bipolar mitotic spindle (O'regan et al., 2007). Moreover, NEK2 has been found to interact with components of the spindle assembly checkpoint (SAC), like the kinetochore proteins MAD1 (Lou et al., 2004), MAD2 (Liu et al., 2010) and HEC1 (also known as NDC80) (Chen et al., 2002). In particular, NEK2, in antagonism with PP1, regulates HEC1 phosphorylation on Ser165 at kinetochores of misaligned chromosomes, which represents a crucial event for the activation of the SAC signaling (Wei et al., 2011).

2.2 Role of NEK2 in cancer

NEK2, as well as several other mitotic kinases, such as AURORA-A or PLK1, is up-regulated in different tumor types, strongly suggesting their involvement in the chromosome

instability and aneuploid phenotype observed in cancer cells (Hayward and Fry, 2006).
NEK2 overexpression has been documented for many different kind of tumors, such as breast carcinomas (Hayward et al., 2004), lung adenocarcinoma (Landi et al., 2008), testicular seminomas (Barbagallo et al., 2009) and diffuse large B cell lymphomas (Andréasson et al., 2009). The causes of NEK2 up-regulation in cancer cells have been suggested to be the amplification of its locus within the chromosomal region 1q32 (Hayward and Fry, 2006) and the up-regulation of its transcriptional regulator Forkhead box M1B (FOXM1) (Calvisi et al., 2009).
Oncogenic potential of NEK2 overexpression is mainly due to the resulting alterations of the centrosome duplication cycle (Fry et al., 1998b). Indeed, it has been shown that NEK2 overexpression in non-transformed epithelial cells induces aberrant amplification of centrosomes and subsequent aneuploidy (Hayward et al., 2004), which is known to contribute to tumoral transformation (Vitre and Cleveland, 2012). Furthermore, a recent report has suggested that overexpression of NEK2 promotes drug-resistance in cancer cells by activating the AKT pathway and increasing the nuclear levels of β-catenin, a key factor of the WNT pathway (Zhou et al., 2013). NEK2 induced deregulation of these two pathways has been demonstrated to induce in myeloma cell lines the up-regulation of the ABC transporter family members (Zhou et al., 2013), which are drug-efflux pumps contributing to the onset of drug resistance in cancer cells (Wu, et al., 2008a). Moreover, by using a *Drosophila melanogaster* model, it has been recently shown that alteration of the AKT and WNT pathways due to NEK2 overexpression alters the expression of cell migration markers such as RHO1, RAC1 and E-CADHERIN, thus suggesting a positive role for this centrosomal kinase in tumor invasion and metastasis (Das et al., 2013). Interestingly, some of these recent studies supporting a prognostic role for NEK2 overexpression in human cancer have highlighted a peculiar

nuclear localization for this kinase in different tumor types (Barbagallo et al., 2009) (Zhou et al., 2013), suggesting the existence of centrosomal unrelated functions for NEK2, whose characterization would allow to shed light on its oncogenic activity.

Given the strong correlation between NEK2 expression and drug-resistance and poor prognosis in human cancer (Zhou et al., 2013), targeting NEK2 activity represents an attractive approach for the development of new anti-cancer therapies. In line with these suggestions, several studies have shown that inhibition of NEK2 activity (Wu et al., 2008b) or suppression of its expression (Tsunoda et al., 2008) negatively affect cancer cells proliferation and invasion, as it has been demonstrated in both *in vitro* and *in vivo* models of cholangiocarcinoma (Kokuryo et al., 2007) and breast cancer (Cappello et al., 2013). These observations strongly suggest that developing new therapies targeting NEK2 activity can be a powerful tool in cancer treatment, highlighting the importance of new studies fully deciphering its oncogenic potential.

Aims

AS is one of the key process taking part to the complex regulative network of co- and post-transcriptional mechanisms regulating gene expression in eukaryotic cells. AS is, indeed, a powerful tool for eukaryotic cells to increase the coding potential of their genomes and to finely regulate their gene expression in order to maintain correct cellular homeostasis.
Aims of this project has been to investigate some of the multiple mechanisms controlling AS and their involvement in the aberrant regulation of the AS concurring to tumoral transformation.
First, we attempted to investigate the nuclear function of the centrosomal kinase NEK2 and its possible involvement in the regulation of the AS through its interaction with several splicing factors.
Secondly, we have investigated whether the histone deacetylases inhibitor (HDACi) LBH589 can affect the splicing of genes relevant for prostate cancer proliferation and we have approached to the study of the mechanisms underlying this regulation, in order to shed light on the reciprocal control between AS and chromatin modifications.

Results and discussion

1. The centrosomal kinase NEK2 is a novel splicing factor kinase involved in cell survival

NEK2 is a serine/threonine kinase belonging to the family of the NIMA kinases, which are well known as key regulators of the cell cycle (Fry et al., 2012). In particular, NEK2 promotes centrosomes splitting and insures correct chromosomes segregation during the G2/M phase of the cell cycle through the phosphorylation of specific substrates, such as the centrosomal protein C-NAP1 (Fry et al., 1998b). Aberrant expression and activity of NEK2 leads to dysregulation of the centrosome cycle and aneuploidy (Hayward and Fry, 2006). Thus, a tight regulation of NEK2 activity and expression is needed during cell cycle progression.
NEK2, as other centrosomal kinases, is up-regulated in several human cancers, such as breast carcinomas, testicular seminomas, and myelomas and its expression levels have been proposed as an accurate prognostic marker. In both testicular seminomas and myelomas, NEK2 overexpression correlates with its nuclear localization. This observation suggests the existence of unknown nuclear functions for NEK2 in cancer cells, which have been object of our further investigation.

1.1 NEK2 is enriched in the nucleus of cancer cells

NEK2 is enriched in the nucleus of human testicular seminoma cells (Barbagallo et al., 2009). Nuclear localization of this kinase was also recently observed in myeloma cells and shown to correlate with poor prognosis (Zhou et al., 2013). To investigate whether other cancer cells that overexpress NEK2 share this nuclear localization, we performed immunohistochemistry analysis of tissue specimens derived from cancer patients. Using a

previously validated antibody (Barbagallo et al., 2009), we observed that NEK2 staining was concentrated in the nucleus of breast and lung cancer cells (Figure 1A). In colon, prostate and cervix cancer cells, although it was also detected in the cytoplasm, NEK2 staining was enriched in nucleus (Figure 1A). Confocal immunofluorescence analyses of NEK2 localization in cell lines derived from breast cancer (MCF7; Figure 1B), seminoma (TCam-2), prostate cancer (PC-3), colon carcinoma (Caco-2) and cervix cancer (HeLa) (Supplementary Figure S1) suggest that nuclear localization of NEK2 is a common feature of human cancer cells.

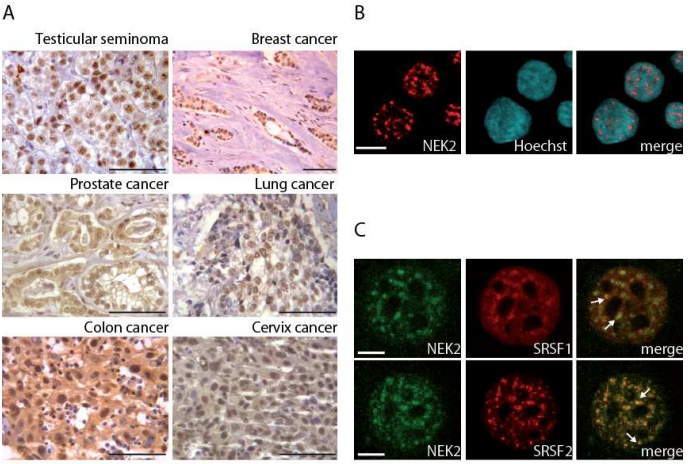
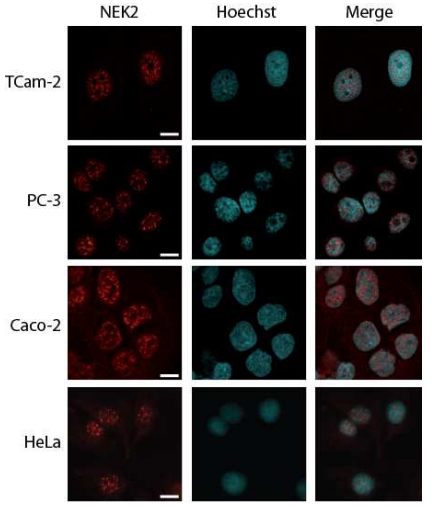
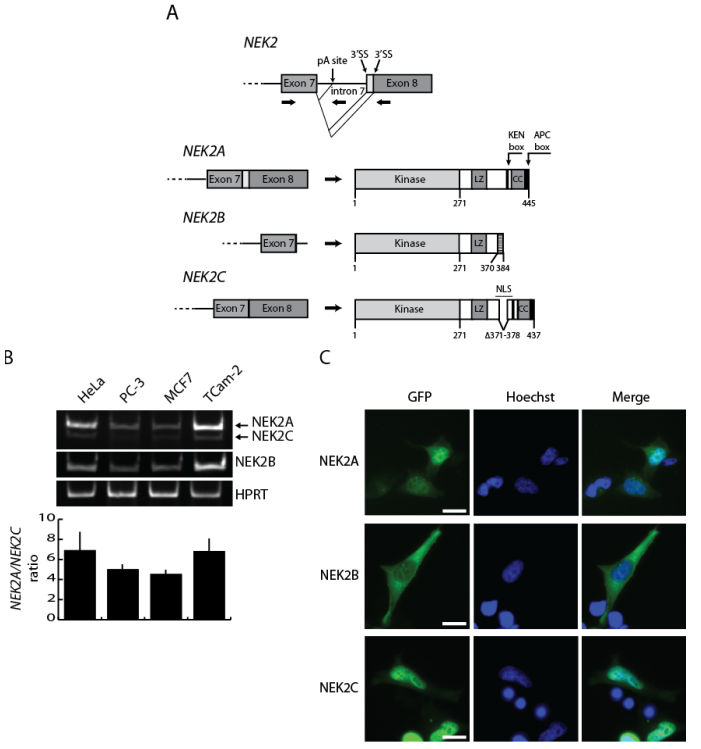


Figure 1. NEK2 localizes in the nucleus of cancer cells. (A) Immunohistochemistry of NEK2 in testicular seminomas, breast, prostate, lung and cervix cancer specimens (scale bar = 25 μm). (B,C) Confocal immunofluorescence analysis of MCF7 cells stained with anti-NEK2 (red) antibody and Hoechst (blue) (B, scale bar = 10 μm) or with anti-NEK2 (green), anti-SRSF1 (red, upper panel) or anti-SRSF2 (red, lower panel) (C, scale bar = 5 μm). White arrows indicate co-localization in speckles.



Supplementary Figure S1. NEK2 nuclear localization in cancer cells. (A) Immunofluorescence analysis of TCam-2 (seminoma), PC-3 (prostate), Caco-2 (colon) and HeLa (cervix) cancer cells stained with rabbit anti-NEK2 (red) antibody and Hoechst (blue) and analysed by fluorescence microscope. Scale bar = 10 μm.

NEK2 is expressed as three alternative splice variants, named NEK2A, B and C (Supplementary Figure S2A) (Wu et al., 2007). NEK2A and B differ in the C-termini because an alternative polyadenylation signal in intron 7 is used in NEK2B, thus preventing inclusion of the last exon 8 (Hayward and Fry, 2006). Notably, exon 8 encodes for protein degradation motifs in NEK2A, which mediate its degradation in mitosis (Hayes et al., 2006). NEK2C is identical to NEK2A with the exception of a small internal deletion of 8 amino acids (Δ371-378), due to usage of a downstream splice acceptor site in exon 8 (Wu et al., 2007). Although the biochemical features of NEK2C are undistinguishable from those of NEK2A, deletion of this sequence creates a nuclear localization signal (NLS) that promotes NEK2C accumulation in the nucleus (Wu et al., 2007). To investigate which splice variant was prevalently expressed in cancer cell



Supplementary Figure S2. NEK2 spliced-variants expression and localization. (A). Schematic representation of the *NEK2* gene and of its alternative splice variants, NEK2A, NEK2B, and NEK2C (see introduction for details). Black arrows indicate primers used for the RT-PCR analysis. (B) RT-PCR amplification of *NEK2* spliced-variants from HeLa (cervix), MCF7 (breast), PC-3 (prostate), TCam-2 (seminoma) cancer cells (upper panel). PCR products were separated on 10% polyacrylamide gel and stained with ethidium bromide. *HPRT* expression levels were evaluated as loading control. Bar graph (lower panel) represents densitometric analysis for the NEK2A/NEK2C ratio (mean ± SD, n=3). (C) HeLa cells were transfected with expression vectors for the GFP-tagged NEK2 spliced-variants and fixed 24h after transfection for fluorescence-microscopy analysis. Nuclei were stained with Hoechst (blue). Scale bar = 20 μm.

lines, we performed RT-PCR analyses using variant-specific primers (Supplementary Figure S2A). NEK2A and B were readily detected in all cell lines analysed, whereas NEK2C was barely detectable (Supplementary Figure S2B), suggesting that its expression is unlikely to account for the nuclear localization of NEK2 in cancer cells. Previous results indicated that a substantial fraction of NEK2A localizes in the nucleus when the protein is overexpressed (Wu et al., 2007). In line with this report, we observed that overexpression of NEK2A was sufficient to allow its accumulation in the nucleus of HeLa cells, with a localization pattern that closely resembled that of NEK2C (Supplementary Figure S2C), whereas NEK2B remained mainly cytoplasmic (Supplementary Figure S2C). These results suggest that up-regulation of NEK2A is likely responsible for the nuclear localization of NEK2 in cancer cells.

1.2 NEK2 localizes in nuclear splicing speckles and co-fractionates with splicing factors

We found that NEK2 accumulated in nuclear granules of variable size and irregular shape (Figure 1B, Supplementary Figure S1), which resembled the splicing speckles, the interchromatin regions enriched in splicing factors (Spector and Lamond, 2011). Confocal immunofluorescence analysis in MCF7 cells confirmed that NEK2 co-localizes in the nuclear speckles with SRSF1 and SRSF2 (Figure 1C), two SR proteins commonly used as markers of these structures (Spector and Lamond, 2011). We next used subcellular fractionation experiments to confirm the association of NEK2 with splicing factors. By using this technique, it was documented that splicing factors accumulate in the nuclear matrix-attached insoluble fraction (Lin et al., 2005). Analyses of cytosolic (S), nuclear soluble (NS), and nuclear matrix-attached insoluble (NI) fractions confirmed that splicing factors were enriched in the NI fraction isolated from MCF7, PC-3 and TCam-

2 cells (Figure 2A). In addition, we observed that NEK2 was also enriched in both the NS and the NI fraction in all cell lines tested (Figure 2A). The molecular weight of this band corresponded to that of NEK2A and C. A faster migrating band corresponding to the molecular weight of NEK2B was instead detected in the cytosolic fraction.

NEK2 interacts with substrates and activators through the carboxyl terminal regulatory region (residues 273-445, Supplementary Figure S2A) (Hayward and Fry, 2006). Thus, we used purified GST-NEK2A (271-445) fusion protein as bait in affinity chromatography of nuclear extracts isolated from MCF7 and PC-3 cells. GST-NEK2A (271-445) selectively associated with some splicing regulators, as SRSF1, hnRNPA1, hnRNPF and SAM68, but not others, as SRSF3 and hnRNPC1/C2 (Figure 2B).

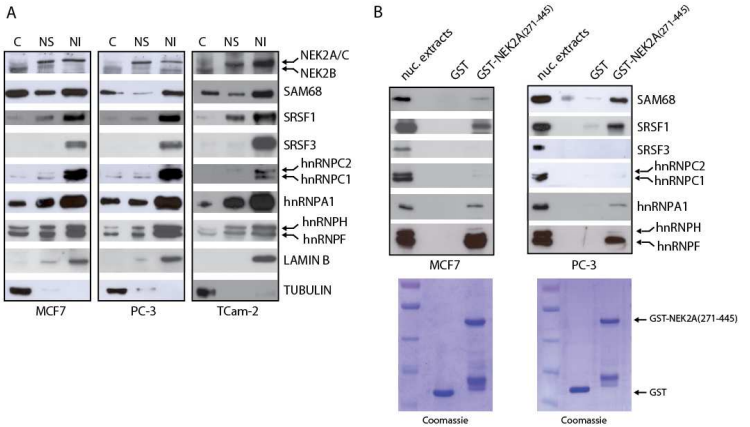


Figure 2. NEK2 associates with splicing factors. (A) Cytosolic (C), nuclear soluble (NS) and nuclear insoluble matrix-associated (NI) fractions of MCF7, PC-3, TCam-2 cells were analysed by Western blot using antibodies for NEK2 and indicated splicing factors. LAMIN B and TUBULIN were evaluated as nuclear matrix and cytosolic markers. (B) Western blot analysis for the indicated splicing factors in pull-down assays of MCF7 and PC-3 nuclear extracts with GST-NEK2A (271-445) fusion protein and GST (as negative control). Coomassie staining shows the purified GST and GST-NEK2A (271-445) fusion protein (lower panels).

These results suggest that NEK2 interacts with specific splicing factors in the cell nucleus.

1.3 NEK2 is a splicing factor kinase

Next, we set out to determine whether splicing factors were substrates for NEK2. We focused on SR proteins because their splicing activity is finely tuned by phosphorylation (Long and Caceres, 2009) (Zhou and Fu, 2013). As first step, we determined whether SRSF1 and SRSF7 were directly phosphorylated by NEK2. Kinase assays using purified full-length HIS- or GST-fusion proteins of these splicing factors showed that purified NEK2 efficiently phosphorylates SRSF1 and SRSF7 in vitro (Figure 3A). We focused the rest of our study on SRSF1 because it was more efficiently phosphorylated by NEK2. Furthermore, this splicing factor is a bona fide oncogene (Karni R., 2007) and it is upregulated in several human cancers, including breast and prostate carcinomas, where it modulates cancer-relevant AS events (Anczuków et al., 2012) (Olshavsky et al., 2010).
First, we investigated whether the interaction between NEK2 and SRSF1 also occurred in live cells. We found that GFP-NEK2C (Figure 3B) and GFP-NEK2A (Supplementary Figure S3A) were efficiently co-immunoprecipitated with FLAG-SRSF1 in HEK293T cells, whereas the cytosolic GFP-NEK2B was not (Supplementary Figure S2C and S3B). Next, to verify the ability of NEK2 to phosphorylate SRSF1 in live cells, we co-expressed wild type or kinase-dead GFP-NEK2C with GFP-SRSF1. When expressed alone, GFP-SRSF1 yielded two bands, with the faster migrating band being more abundant, indicating that the bulk of the protein is hypophosphorylated (Figure 3C). Expression of wild type NEK2C increased the amount of the higher molecular weight band of SRSF1, suggesting its hyperphosphorylation, whereas kinase-dead NEK2C was ineffective (Figure 3C). A similar result was obtained with NEK2A (Supplementary Figure S3C),

indicating that both NEK2 splice variants can phosphorylate SRSF1 in live cells.

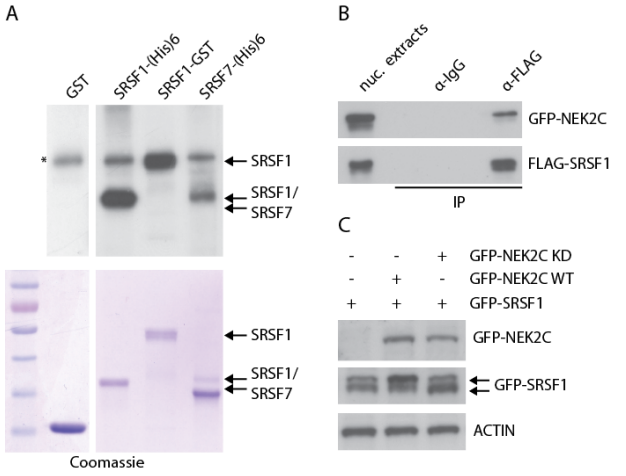
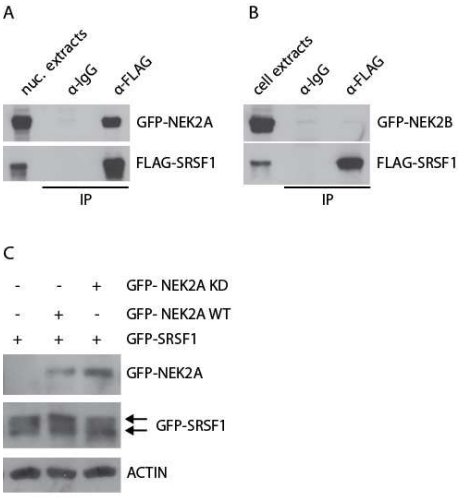


Figure 3. NEK2 phosphorylates splicing factors. (A) Purified NEK2 was incubated with purified GST (as negative control), SRSF1-$(His)_6$, SRSF1-GST or SRSF7-$(His)_6$ proteins and γ-[^{32}P]ATP. Phosphorylation levels were detected by autoradiography (* indicates autophosphorylated NEK2). Coomassie staining shows the purified proteins (lower panel). (B) Western Blot analysis with anti-GFP and anti-FLAG antibodies of the immunoprecipitation assay performed with control mouse IgG or anti-FLAG antibody of nuclear extracts of HEK293T expressing FLAG-SRSF1 and GFP-NEK2C. (C) Western Blot analysis using anti-GFP antibody of HEK293T cells transfected with GFP-SRSF1, wild-type NEK2C (WT) or kinase-dead NEK2C (KD). ACTIN was used as loading control.



Supplementary Figure S3. NEK2A phosphorylates SRSF1 in vivo. (A,B) Western Blot analysis with anti-GFP and anti-FLAG antibodies of the immunoprecipitation assay performed with control mouse IgG or anti-FLAG antibody of nuclear extracts of HEK293T expressing FLAG-SRSF1 and GFP-NEK2A (A) or of total extracts of HEK293T expressing FLAG-SRSF1 and GFP-NEK2B (B). (C) HEK 293T cells were transfected with GFP-SRSF1, wild-type NEK2A (WT) or kinase-dead NEK2A K37R (KD) vectors. Cell lysates were resolved on SDS-PAGE and expression of recombinant proteins was detected by Western Blot using anti-GFP antibody. ACTIN was used as loading control.

1.4 NEK2 behaves as an SR protein kinase in live cells

Having established that NEK2 phosphorylates SRSF1, we asked whether it behaves as an SR protein kinase (SRPK). For these studies, we focused on NEK2C, which was chosen for its almost exclusive localization in the nucleus (Wu et al., 2007). Overexpression of NEK2C induced a pattern of SR proteins phosphorylation similar to that elicited by SRPK1, a prototypical SRPK (20), leading to phosphorylation of SRSF6 (SRp55), SRSF5 (SRp40) and SRSF2/SRSF9 (SRp30) (Figure 4A).

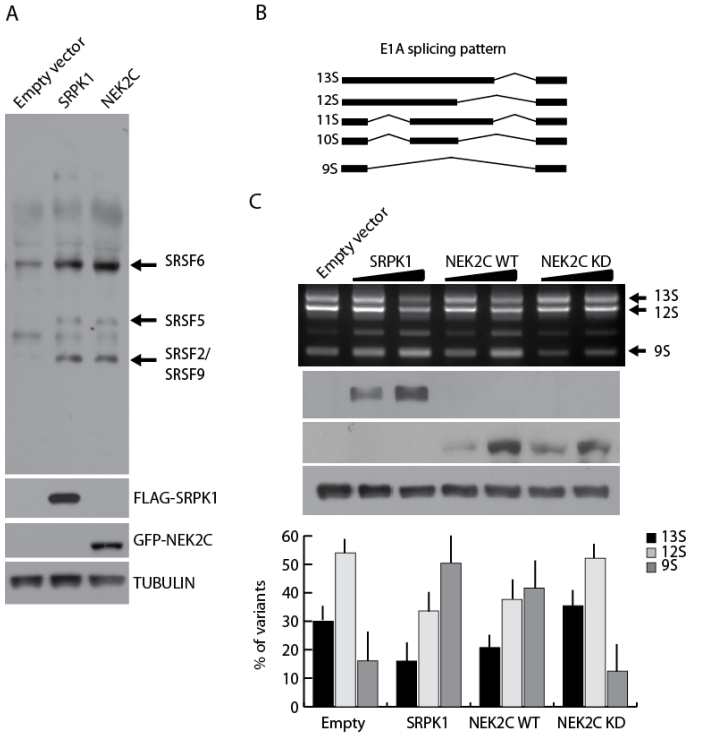


Figure 4. NEK2 phosphorylates SR proteins and modulates E1A splicing. (A) Western Blot analysis of SR proteins phosphorylation in HeLa cells transfected with FLAG-SRPK1 or GFP-NEK2C using the anti-SR proteins 1H4 antibody. TUBULIN was detected as loading control. (B) Schematic representation of the alternative splice variants of the E1A minigene. (C) RT-PCR analysis (upper panel) of the in vivo splicing assay in HeLa cells transfected with the E1A minigene and increasing doses of FLAG-SRPK1, GFP-NEK2C WT or GFP-NEK2C KD. Over-expression efficiency was assessed through anti-FLAG and anti-GFP Western Blot. TUBULIN was evaluated as loading control. Bar graph (lower panel) represents densitometric analysis for the major E1A splice variants (13S, 12S, 9S) evaluated in presence of the highest dose of vectors (mean ± SD, n=3).

Next, we tested whether NEK2 functionally regulates the activity of SR proteins. Previous reports demonstrated that overexpression of SRPK1 modulates splicing of the E1A reporter minigene (Zhong et al., 2009), a commonly used splicing target that contains several 5' and 3' alternative splice sites (ss) (Figure 4B). As expected (Zhong et al., 2009), we observed that increasing the expression of SRPK1 in HeLa cells caused a dose-dependent switch from the 13S to the 9S 5' splice site of the E1A minigene (Figure 4C). Remarkably, upregulation of NEK2C caused a similar switch in E1A splicing. This effect required the kinase activity of NEK2, as the kinase-dead mutant had no effect (Figure 4C). Thus, NEK2 displays features of an SRPK in live cells.

1.5 NEK2 expression modulates SRSF1-dependent BCL-X splicing

Phosphorylation of SR proteins regulates their splicing activity (Long and Caceres, 2009) (Zhou and Fu, 2013). Thus, we asked whether NEK2 could modulate the AS of an endogenous target of SRSF1, such as the *BCL-X* gene (Paronetto et al., 2007) (Moore et al., 2010). Selection of two alternative 5' ss in exon 2 of *BCL-X* leads to the production of two splice variants: the anti-apoptotic BCL-X_L and the pro-apoptotic BCL-X_S (Boise, 1993). SRSF1 promotes selection of the proximal 5' ss leading to expression of BCL-X_L (Paronetto et al., 2007) (Moore et al., 2010). By performing real time quantitative PCR (qPCR) using exon junction-specific primers for BCL-X_L and BCL-X_S (Figure 5A), we found that overexpression of NEK2C in HeLa cells increased the BCL-X_L/BCL-X_S ratio to a similar extent as overexpression of SRSF1 (Figure 5B).
Importantly, this effect was not due to activation of SRPK1. Indeed, while knockdown of SRPK1 promoted the pro-apoptotic BCL-X_S variant, indicating that SRPK1 also modulates this AS event, NEK2C was still capable to enhance splicing of the anti-

apoptotic BCL-X_L variant in SRPK1-depleted cells as observed in control cells (Figure 5C).

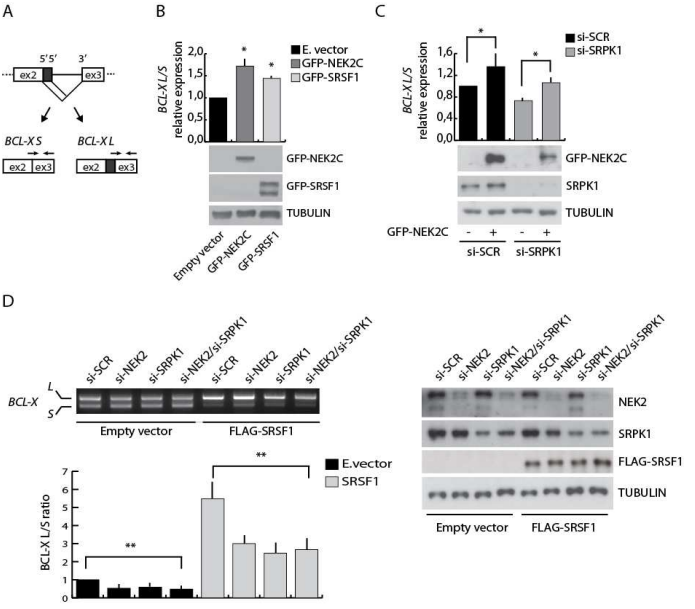


Figure 5. NEK2 modulates *BCL-X* AS affecting SRSF1 activity. (A) Schematic representation of the *BCL-X* AS. Black arrows indicate exon-junction primers used for qRT-PCR analysis. (B,C) qRT-PCR analysis of endogenous *BCL-X* splice-variants. Bar graphs represent BCL-X_L/BCL-X_S ratio (mean ± SD, n=3, *p<0,05). HeLa cells were transfected with mock, GFP-NEK2C or GFP-SRSF1 (B) or with either scramble (si-SCR) or SRPK1 (si-SRPK1) siRNAs and then with or without GFP-NEK2C (C). Silencing and over-expression efficiency was assessed by Western blot analysis. (D) RT-PCR analysis of the in vivo splicing assay of BCL-X minigene in HEK293T cells transfected with scramble (si-SCR), NEK2 (si-NEK2), SRPK1 (si-SRPK1) or both NEK2 and SRPK1 (si-NEK2/si-SRPK1) siRNAs and with or without FLAG-SRSF1. Bar graph represents the densitometric analysis of the BCL-X_L/BCL-X_S ratio, normalized for the value obtained in cells transfected with scramble siRNA and empty vector, set to 1 (mean ± SD, n=3, **p<0,01). Silencing and over-expression efficiency was assessed by Western Blot analysis (right panel).

To determine whether NEK2 expression affected the ability of SRSF1 to modulate *BCL-X* AS, we employed a minigene that recapitulates the splicing of the endogenous gene (Massiello A. S., 2004). We found that knockdown of NEK2 in HEK293T cells slightly enhanced splicing of the pro-apoptotic BCL-X_S variant (Figure 5D). Moreover, while transfection of suboptimal amounts of SRSF1 efficiently promoted splicing of the anti-apoptotic BCL-X_L variant in control cells (si-SCR), this effect was partially impaired when NEK2 was silenced (Figure 5D). Importantly, similar effects were also observed when SRPK1 was knocked down, even though silencing of both NEK2 and SRPK1 did not exert additive effect on SRSF1-induced *BCL-X* splicing (Figure 5D).

1.6 NEK2 is involved in the regulation of apoptosis

Next, we sought out to determine whether AS of known endogenous targets of SRSF1 was affected by the knockdown of NEK2 in HeLa cells. We examined the splicing pattern of SRSF1 target transcripts from three genes with roles in cancer and for which AS variants have been characterized: *BCL-X*, *MKNK2* and *BIN1* (Karni R., 2007) (Paronetto et al., 2007) (Moore et al., 2010) (Adesso et al., 2013). For comparison, we also knocked down SRSF1 and SRPK1 in parallel experiments (Figure 6A,B). Transient knockdown of NEK2 in HeLa cells resulted in decreased ratio of BCL-X_L/BCL-X_S and MNK2b/MNK2a, and induces skipping of exon 12A in BIN1 mRNA variants, without affecting SRSF1 expression. All the splicing changes exerted by NEK2 depletion favoured pro-apoptotic splice variants and were recapitulated by knockdown of either SRSF1 or SRPK1, although to different extent for the three genes (Figure 6A,B).

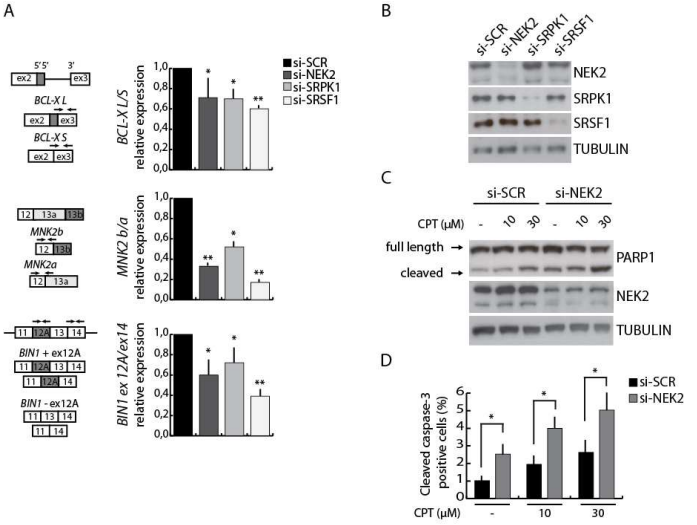
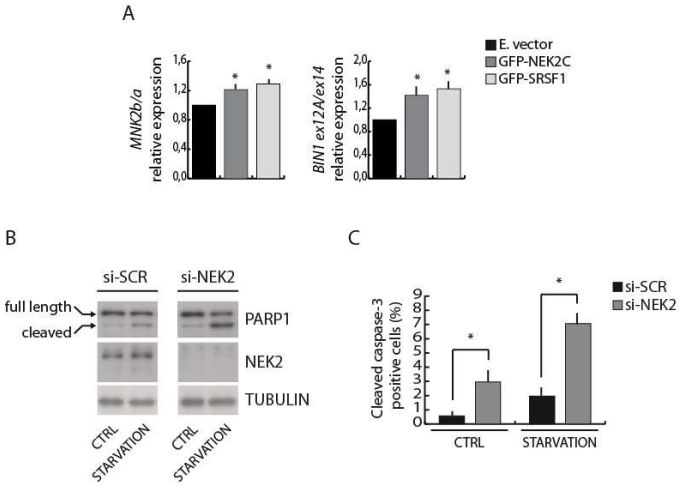


Figure 6. NEK2 silencing affects splicing of SRSF1 target genes and sensitize cells to apoptosis. (A) Schematic representation of the SRSF1-regulated of *BCL-X*, *MKNK2* and *BIN1* AS events (left panel). Black arrows indicate primers used for the qRT-PCR analysis performed in HeLa cells transfected with scramble (si-SCR), NEK2 (si-NEK2), SRPK1 (si-SRPK1) or SRSF1 (si-SRSF1) siRNAs. Ratio of the AS variants are represented in the bar graph (mean ± SD, n=3, *p<0,05, **p<0,01) (right panel). (B) Western Blot analysis assessing NEK2, SRPK1 and SRSF1 silencing efficiency. (C,D) Western Blot analysis of PARP1 cleavage (C) and quantitative analysis of cleaved-CASPASE 3 immunofluorescence (bar graph represents mean ± SD, n=3, *p<0,05) (D) in HeLa cells transfected with either scramble (si-SCR) or NEK2 (si-NEK2) siRNAs and treated for 24h with increasing doses of cisplatin (CPT). Western Blot analysis assessing NEK2 silencing efficiency was performed (C).

Moreover, as observed for BCL-X_L, overexpression of either NEK2C or SRSF1 promoted splicing of anti-apoptotic *MKNK2* and *BIN1* variants (Supplementary Figure 4A). These results indicate that NEK2 contributes to the regulation of SRSF1 splicing activity.

Since NEK2 knockdown induced expression of pro-apoptotic splice variants, we asked whether it also plays a role in cell viability. In line with its effect on AS, depletion of the endogenous NEK2 in HeLa cells significantly increased the basal level of apoptosis, as monitored by cleavage of PARP1 (Figure 6C) and CASPASE 3 (Figure 6D), and enhanced the apoptotic response of cells to stress, such as treatment with cisplatin (Figure 6C,D) or starvation (Supplementary Figure 4B,C). These results suggest that the effect of NEK2 on AS events regulated by SRSF1 is physiologically relevant.



Supplementary Figure S4. NEK2 expression promotes cell survival. (A,B) Western Blot analysis of PARP1 cleavage (A) and quantitative analysis of cleaved-CASPASE 3 immunofluorescence (bar graph represents mean ± SD, n=3, *P<0,05) (B) in HeLa cells transfected with either scramble (si-SCR) or NEK2 (si-NEK2) siRNAs and serum-starved for 8h. Western Blot analysis assessing NEK2 silencing efficiency was performed (A).

1.7 Discussion

NEK2 is a centrosomal kinase involved in centrosome duplication in mitosis that is frequently upregulated in human cancers (Hayward and Fry, 2006). Recent evidence suggests that nuclear localization of NEK2 is a predictor for drug resistance and a marker of poor prognosis in patients (Zhou et al., 2013). Nevertheless, the specific nuclear functions of NEK2 are still completely obscure. The present study indicates that NEK2 acts as a regulator of AS events by modulating SRSF1 activity, thus uncovering a previously unknown nuclear function for this oncogenic kinase.

NEK2 overexpression has been extensively described in several types of tumours (Hayward and Fry, 2006) (Hayward and Fry, 2006) (Landi et al., 2008) (Barbagallo et al., 2009) (Andréasson et al., 2009) (Zhou et al., 2013). Its oncogenic activity has been primarily ascribed to the ability to induce aneuploidy by perturbing centrosome duplication and its segregation dynamics (Hayward and Fry, 2006). However, it was recently demonstrated that in testicular seminomas (Barbagallo et al., 2009), myelomas and other types of cancer (Zhou et al., 2013), NEK2 is primarily localized in the nucleus of neoplastic cells. We now document that the nuclear localization of NEK2 is also observed in several carcinomas and cancer cell lines in which the kinase is upregulated. Thus, although our analysis is too limited to draw conclusions, these results suggest that nuclear localization of NEK2 is a common feature of neoplastic cells.

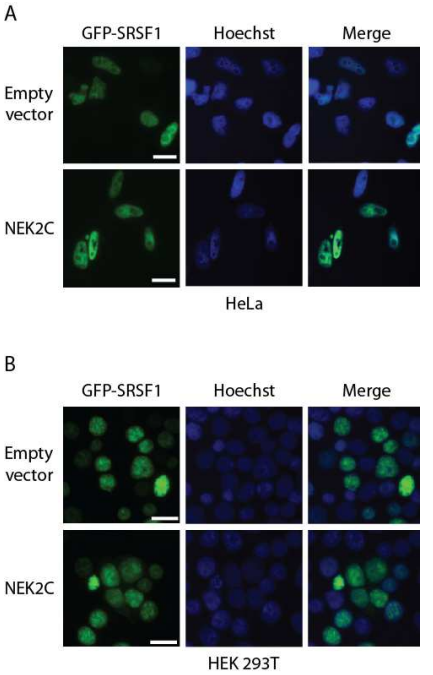
The NEK2C splice variant was reported to localize prevalently in the nucleus (Wu et al., 2007), suggesting that its selective up-regulation in cancer cells might account for the observed localization of the kinase. However, our study indicates that NEK2C is expressed at very low levels in all cancer cells analysed, raising doubts on its contribution to the localization of NEK2 in primary tumours and in cell lines. Conversely, up-

regulation of NEK2A, but not NEK2B, is sufficient to induce its nuclear localization, suggesting that NEK2A is the prevalent isoform in the nucleus of cancer cells.

Characterization of the subcellular distribution of NEK2 pointed out its co-fractionation with several splicing factors in the nuclear-insoluble material of cancer cells. Moreover, NEK2 co-localized with two SR-proteins in nuclear splicing speckles. These inter-chromatin granules are particularly enriched in SR-proteins and are supposed to function as nuclear storage sites for pre-mRNA processing regulators (Spector and Lamond, 2011). Assembly of splicing speckles and active recruitment of SR-proteins from these sites to the newly synthesized pre-mRNA is strictly regulated by reversible phosphorylation (Misteli et al., 1998). Phosphorylation represents one of the main mechanisms by which subtle regulation of the splicing process, and especially of AS, is achieved (Stamm, 2008) (Naro and Sette, 2013). These observations led us to hypothesize the existence of a functional interaction between NEK2 and splicing. Several results of our study support this hypothesis. First, NEK2 interacts with and phosphorylates SRSF1. Second, we found that the splicing activity of SRSF1 is modulated by NEK2. Lastly, silencing of NEK2 negatively affects AS events that are target of SRSF1 in live cells. Collectively, these results point to NEK2 as a novel direct regulator of SRSF1 phosphorylation and activity.

The cellular localization and splicing activity of SRSF1 are regulated by reversible phosphorylation (Zhou and Fu, 2013) (Cao et al., 1997) (Sanford et al., 2005) (Prasad et al., 1999). We observed that NEK2 did not influence the nuclear localization of SRSF1 in HeLa and HEK293T cells (Supplementary Figure S5). By contrast, our study suggests that NEK2 can modulate SRSF1 splicing activity similarly to SRPK1, the prototype member of the SRPK family of kinases that mediate phosphorylation of SR-proteins (Zhou and Fu, 2013).Indeed, overexpression of either

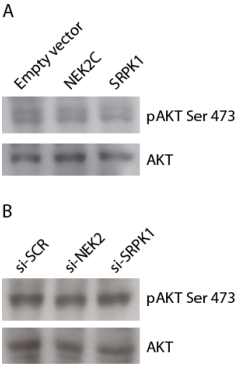
NEK2 or SRPK1 induced a similar pattern of SR proteins phosphorylation and caused a similar modulation of E1A AS.



Supplementary Figure S5. NEK2C overexpression does not affect SRSF1 subcellular localization. Immunofluorescence analysis of HeLa (A) and HEK 293T (B) cells transfected with the GFP-SRSF1 vector in presence or not of the pCDNA3N2myc-NEK2C WT vector. Nuclei were stained with Hoechst (blue). Scale bar = 20 μm.

Likewise, knockdown of NEK2 or SRPK1 similarly reduced the splicing activity of SRSF1 toward the BCL-X minigene. Although the effects of NEK2 might be indirect (i.e. mediated by another kinase), three lines of evidence support a direct action. First, our in vitro kinase assays were performed using highly purified proteins, strongly indicating that NEK2 can directly phosphorylate SRSF1 and SRSF7. Second, although NEK2 was recently reported to induce activation of AKT in myeloma cells (Zhou et

al., 2013), a signaling kinase known to directly and indirectly modulate SR proteins phosphorylation (Patel et al., 2005) (Zhou et al., 2012), overexpression or knockdown of NEK2 did not alter the activity of AKT in HeLa cells (Supplementary Figure S6).



Supplementary Figure S6. Modulating NEK2 expression levels does not affect AKT activation. (A,B) Western Blot analysis for AKT phosphorylation status, using anti-AKT and anti-pAKT Ser473 antibodies. (A) HeLa cells were transfected with FLAG-SRPK1 or GFP-NEK2C WT vectors and harvested 24h after transfection. (B) HeLa cells were transfected with scramble (si-SCR), *NEK2* (si-NEK2) or *SRPK1* (si-SRPK1) siRNAs and harvested 48h after transfection.

Third, the effect of NEK2 on *BCL-X* splicing was not affected by knockdown of SRPK1. Thus, these experiments suggest that NEK2 behaves as a bona fide splicing factor kinase in live cells.

Our study implicates NEK2 in AS regulation of several SRSF1 target genes involved in cell viability. We found that knockdown of NEK2 mimicked that of SRSF1, or SRPK1, and induced expression of pro-apoptotic *BCL-X*, *BIN1* and *MKNK2* splice variants. Consistently, NEK2 depletion sensitized HeLa cells to spontaneous and stress-induced apoptosis, suggesting a pro-survival function for this kinase. Although protection from cell death may also involve other splicing-unrelated functions of NEK2 (Zhou et al., 2013), it is likely that enhanced splicing of the

anti-apoptotic variants of *BCL-X*, *BIN1* and *MKNK2* contributes to this pro-survival effect.
As NEK2, SRPK1 is also overexpressed in human cancers (Hayes et al., 2007), suggesting that these kinases may act in concert to modulate SRSF1 activity. In line with this hypothesis, we found that NEK2 affected *BCL-X* splicing independently of SRPK1. Thus, even if apparently redundant, SRPK1 and NEK2 could regulate SR-protein phosphorylation and subsequent AS events in a coordinate manner. In fact, the subcellular localization of SRPK1, mainly cytosolic (Zhong et al., 2009), and NEK2, predominantly nuclear (this study), suggest a possible coordinated activity of these kinases in different cellular compartments, as previously reported for SRPKs and CLKs (Cdc2-like kinases) (Ngo et al., 2005). Notably, activation of SRPK1 in response to AKT-mediated signalling was reported to modulate gene expression by regulating AS programs (Zhou et al., 2012). Since activation of NEK2 by the ERK1/2 pathway was observed in germ cells (Di Agostino et al., 2002), and this pathway is often activated in cancer cells, it is possible that NEK2 also participates to AS regulation in response to environmental cues. Finally, since NEK2 expression and activity peak during the late S-G2 phase (Fry et al., 1995), we cannot exclude the possibility that it could affect SR proteins function in a cell-cycle dependent manner, thus contributing to coordinate gene expression regulation with cell cycle progression. Noteworthy, another centrosomal kinase, AURKA, was shown to modulate the apoptotic response to mitotic arrest of the cell cycle by regulating the stability of SRSF1 (Moore et al., 2010).In conclusion, our study identifies NEK2 as a novel regulator of AS, which promotes SRSF1-dependent splicing of anti-apoptotic variants, thus contributing to cell survival.

2. Co-transcriptional control of genic expression in cancer cells

Mounting evidence has recently highlighted a novel important layer of control for the AS process exerted by the chromatin structure (Gómez Acuña et al., 2013). As most of the different steps of the pre-mRNA processing, AS largely occurs co-transcriptionally and it is significantly modulated by variations in the dynamics of the transcription process (Pandya-Jones, 2011). Therefore, by affecting the accessibility of the DNA template to the transcriptional apparatus, epigenetic modifications can influence RNAPII processivity, thereby modulating the co-transcriptional AS process (Schwartz and Ast, 2010). Several studies have shown that recognition and inclusion of weak exons in the mature transcript is facilitated by a slow elongation rate of the RNAPII, as it increases the lapse of time during which these exons are accessible to the splicing machinery. On the contrary, a faster elongation rate favors exon skipping events (de la Mata et al., 2010). Moreover, it has been shown that specific histone modifications can act as docking sites for spliceosome components and accessory RBPs, thus directly affecting their recruitment on the nascent pre-mRNA (Luco et al., 2010). In line with the contribution of epigenetic modifications to regulation of the AS process, it was shown that the AS pattern of several genes can be modulated by treatment with agents that modulate chromatin structure, such as histone deacetylases inhibitors (HDACi) (Hnilicová et al., 2011).
Herein, we have investigated the effect of the pan-HDACi LBH589 on the splicing of genes relevant for prostate cancer proliferation and we have approached the study of the molecular mechanisms underlying this regulation.

2.1 HDACi induce specific changes in alternative splicing events

In order to assess whether treatment with HDACis could significantly affect the AS process in prostate cancer cells, we treated PC-3 cells with different HDACis: LBH589, SAHA, trichostatin A (TSA) and valproic acid (VPA). The splicing pattern of cancer-related genes, known to be relevant for tumoral proliferation, such as the HGFL-receptor *RON* (Lu et al., 2007), the apoptotic regulator *BCL-X* (Mercatante et al., 2001) and the adhesion molecule *CD44* (Zöller, 2011), was then analyzed through RT-PCR. In particular, we analyzed the effect of the treatment with HDACis on the splicing of the pro-tumoral isoforms of these genes, such as the exon-11 skipped isoform of

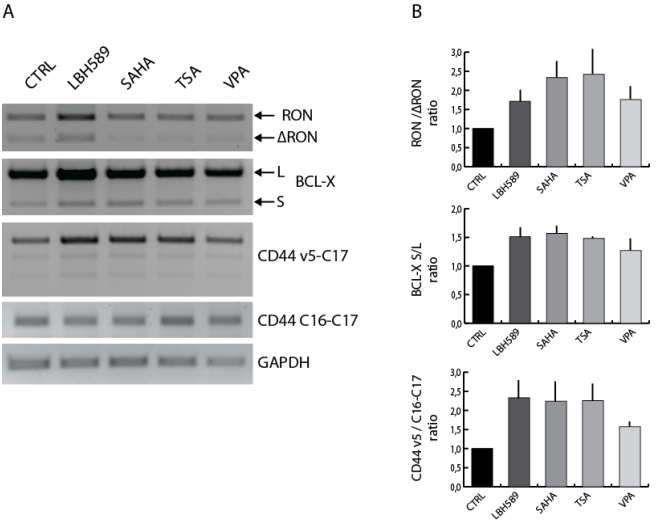


Figure 1. HDACis treatment induces specific changes in AS. (A) RT-PCR analysis of AS of *RON*, *BCL-X* and *CD44* genes in PC3 cells treated for 24h with different HDACis at following concentrations: 10 nM LBH589, 2,5 µM SAHA, 300 nM trichostatin A (TSA), 5 mM valproic acid (VPA). *GAPDH* was evaluated as loading control. (B) Densitometric analysis of the ratio between the splice variants analyzed (means ± SD, n=2).

the *RON* gene, known as *Δ*RON (Ghigna et al., 2005), the antiapoptotic *BCL-X$_S$* (Boise et al., 1993) and the exon v5 containing isoforms of the *CD44* gene (Cheng and Sharp, 2006).
Interestingly, we found that almost all HDACis tested affected splicing of the analyzed genes in a similar manner, albeit with different efficiency, by promoting splicing of the *BCL-X$_S$* isoform and inclusion of the RON exon11 and *CD44* exon v5 (Figure 1). These results confirm the previously reported ability of HDCAIs to induce specific changes in AS events (Hnilicová et al., 2011).

2.2 LBH589 selectively affects CD44 splicing.

Having highlighted the general ability of HDACis to modulate AS events, we then focused our study on the characterization on the splicing regulatory ability of the pan-HDACi LBH589, which among the inhibitors used is the more innovative and displays the broader spectrum of activity (Atadja, 2009). As model gene we selected *CD44*, whose peculiar exonic structure makes it a suitable model for AS studies: the human *CD44* gene is characterized by nine variable exons (v2-v10) embedded between two sets of constitutive exons (c1-c5 and c6-c9) (Figure 2A) (Zöller, 2011). Alternative inclusion of the variable exons in the mature transcript of the *CD44* gene is strictly regulated by different mechanisms, including the activity of co-transcriptional factors, such as the BRM subunit of the SWI-SNF complex (Batsché et al., 2006) or the transcriptional activator SND1 (Cappellari et al., 2013), the binding of transacting RBPs, such as SAM68 (Matter et al., 2002), and the presence of epigenetic modifications in the *CD44* locus, such as the trimethylation of Lys9 in histone H3 (H3K9me3) (Saint-André et al., 2011).
We initially analyzed the expression of *CD44* v5-containing splice variants in PC-3 cells treated with increasing doses of LBH589. Both conventional (Figure 2B) and quantitative RT-PCR (qPCR) (Figure 2C) analysis showed that LBH589 treatment promotes the

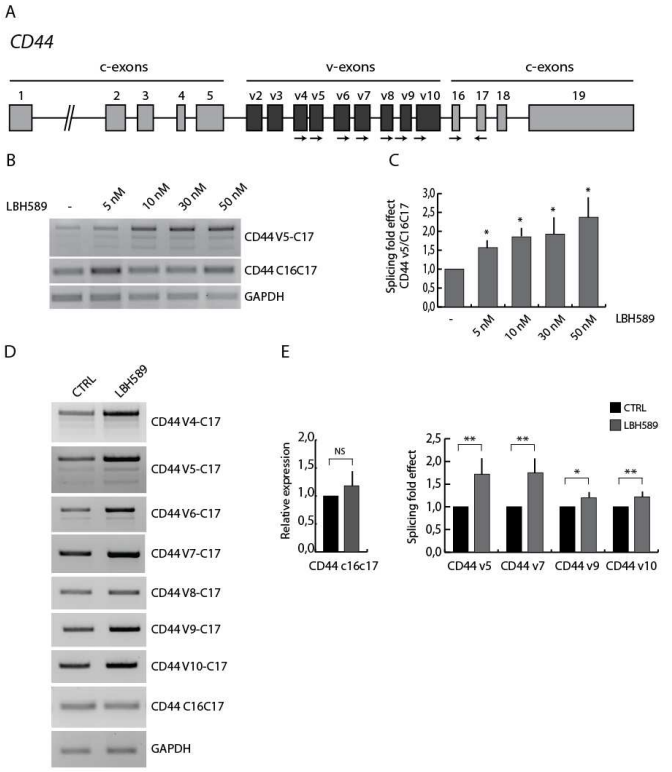


Figure 2. LBH589 treatment selectively affects CD44 splicing. (A) Schematic representation of the *CD44* gene; constant and variable exons are shown in light and dark grey respectively. Black arrows indicate primers used for the PCR analyses. (B,C). RT-PCR (B) and qPCR (C) analysis of the expression of *CD44* C16C17 constant region and of the *CD44* v5 variable exon in PC3 cells treated with indicated doses of LBH589. *GAPDH* was evaluated as loading control. In (C) the splicing fold effect was evaluated by measuring the enrichment of *CD44* v5 exon versus the C16C17 constant region, set to 1 in control cells (means ± SD, n=3, *P≤0,05). (D) RT-PCR analysis of the expression levels of *CD44* v4-v10 variable exons and of C16C17 constant region in PC3 cells treated for 24h with 10 nM LBH589. *GAPDH* was evaluated as loading control. (E) qPCR analysis of total *CD44* expression (left bar graph) and of *CD44* variable exons (v5, v7, v9, v10) inclusion (right graph) in PC3 cells treated for 24h with 10 nM LBH589. Total levels of CD44 mRNA were evaluated by normalizing values obtained for the *CD44* constant region C16C17 to those of *GAPDH* (means ± SD, n=4, *P≤0,05).

inclusion of the variable v5 exon in the *CD44* transcript in a dose-dependent manner. Moreover these results suggested that the 10 nM dose is already sufficient to induce a significant effect on v5 inclusion. Therefore, this dose was chosen for all subsequent experiments. Conventional RT-PCR analysis of the variable exons using primer in the v4-v10 variable exons indicated that LBH589 treatment preferentially promotes the inclusion of upstream variable exons (v4-v7) with respect to those located downstream in the *CD44* gene (v8-v10) (Figure 2D). This selective effect was confirmed by quantitative real-time PCR (qPCR) analysis, which showed that the expression of variable exons v5 and v7 was significantly increased with respect to downstream exons v9 and v10. Importantly, exon inclusion was not accompanied by an overall increase of the transcriptional levels of the *CD44* gene (Figure 2E). These observations suggest that the increased levels of *CD44* exons v5 and v7 induced by LBH589 treatment are not due to a general transcriptional activation following HDAC inhibition, but it is instead a proper AS response elicited by the treatment. The molecular mechanisms underneath the AS modulation of the *CD44* gene induced by LBH589 treatment have been therefore object of our further studies.

2.3 LBH589 does not alter expression of splicing factors.

First of all, we investigated if LBH589 modulates AS events in PC-3 cells by altering the expression levels of general and auxiliary splicing factors. Proteins involved in the RNA splicing process have been indeed identified as a prominent part of the cellular “acetylome” (Choudhary et al., 2009) and it has been widely demonstrated that LBH589, as well as other HDACis, can affect the acetylation status of several of non-histone proteins thereby directly or indirectly modulating their stability (Sadoul et al, 2008). Nevertheless, our Western Blot analysis of total extracts

obtained from cells treated with LBH589 did not shown any significant variation in the expression levels of the splicing regulators analyzed, except for a mild reduction in the expression levels of the hnRNPF/H and hnRNPI (Figure 3). Although there is no evidence in the literature concerning the involvement of these RBPs in the regulation of *CD44* AS, direct experiments of overexpression of these recombinant proteins in cells treated or not with LBH589 need to be performed to rule out that the mild reduction of these two proteins could be correlated to the observed modulation of *CD44* AS.

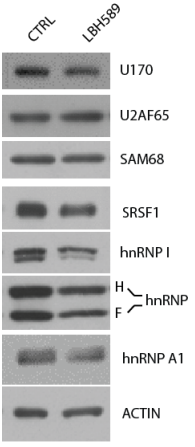


Figure 3. LBH589 treatment does not alter expression of splicing factors. Western Blot analysis of indicated splicing factors in PC3 cells treated for 24 h with LBH589. ACTIN was evaluated as loading control.

2.4 LBH589 locally affects RNAPII processivity in the CD44 locus.

One of the main consequences of treatment with HDACis is the global increase in histone acetylation, which usually correlates with an open chromatin conformation, thus rendering it more accessible to the transcriptional apparatus (Clayton et al., 2006). Indeed, previous studies have shown that other HDACis induce a

global acceleration of the RNAPII processivity in different genes, thus favoring exon-skipping splicing events (Hnilicová et al., 2011). This result is in line with several other reports showing that a higher elongation rate of the RNAPII correlates with the exclusion of weak alternative exons (Nogues et al., 2002; de la Mata et al., 2003). Differently from these previous results about other HDACis, our observations show that LBH589 promotes inclusion of the variable weak exons of the *CD44* gene. We therefore decided to investigate whether the modulation of *CD44* splicing by LBH589 was correlated to changes in RNAPII processivity along the locus. We initially evaluated whether treatment with the reversible inhibitor of transcription 5,6-Dichlorobenzimidazole 1-b–D-ribofuranoside (DRB) could impairs the splicing effect of LBH589. We thus evaluated the inclusion of variable exons v5 and v6 in the transcripts newly synthesized after the release from a transcriptional block induced by DRB treatment. For this analysis, nascent pre-mRNAs were labeled with BrU for 1h after the release from DRB block and then purified by immunoprecipitation with an anti-BrdU antibody. Analysis of the nascent transcripts revealed higher inclusion of the *CD44* variable exons in LBH589-treated cells respect to the control (Figure 4A), confirming the ability of LBH589 to positively regulate *CD44* variable exons splicing, even after DRB treatment.

Then, we investigated whether LBH589 could affect RNAPII processivity along the entire *CD44* gene. As previously reported, RNAPII processivity can be indirectly evaluated by measuring the ratio between a promoter-proximal and a promoter-distal region of the same pre-mRNA, as a slower elongating RNAPII leads to the accumulation of the proximal over the distal pre-mRNA (de la Mata et al., 2003). Thus we evaluated through qPCR the accumulation of *CD44* pre-mRNA in intron 1 with respect to intron 18, 60 minutes after DRB release. This analysis highlighted

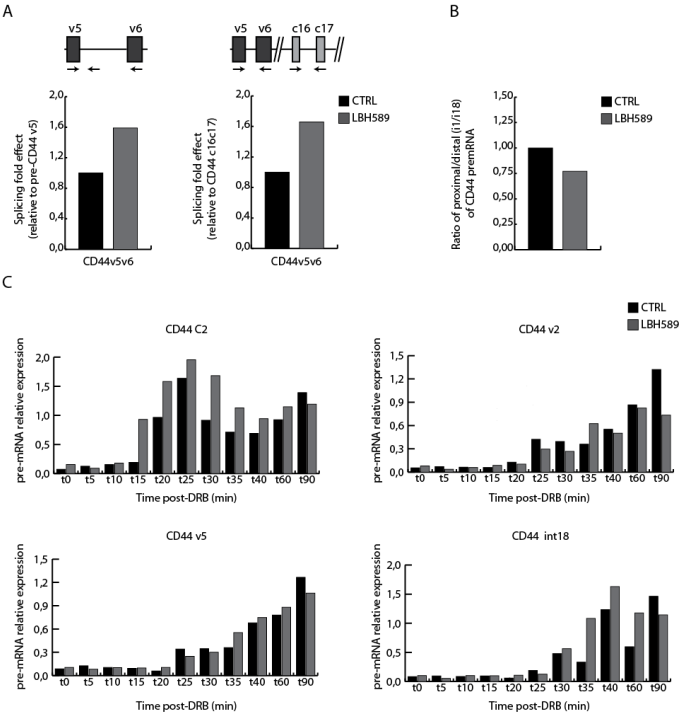


Figure 4. LBH589 affects *CD44* splicing by affecting RNAPII processivity. (A) qPCR analysis of nascent *CD44* transcripts labeled in the presence of BrU. Novel transcripts were immunoprecipitated by using specific anti-BrU antibody and analyzed with the indicated primers (schematic representations over the bar graphs). Cells not treated with BrU were used as control of the immunoprecipitation. Left graph represents the ratio between mRNA versus pre-mRNA of the *CD44* v5 region, right panel represents the ratio between *CD44* v5v6 mRNA versus the *CD44* C16C17 constant region. The value of control cells was set as reference. (B) qPCR analysis of the relative abundance of *CD44* proximal and distal pre-mRNAs after 60' of release from DRB block in PC3 cells treated or not with LBH589 10 nM. Data are presented as the ratio between *CD44* intron 1 and 18 set to 1 in control cells. (C) qPCR analysis of *CD44* nascent transcripts in PC3 cells treated with 10 nM LBH589, harvested at 5 minutes intervals after the release from the transcriptional block with DRB. The expression of different portions of *CD44* pre-mRNA was analyzed at the indicated times and normalized for the expression levels of *GAPDH*. Values of the no-treatment control were set to 1 for both CTRL and LBH589 treated cells.

that LBH589 treatment decreases by approximately 20% this ratio (Figure 4B), suggesting that it induces a global acceleration of the RNAPII along the *CD44* gene.

As a faster elongating RNAPII usually correlates with enhancement of exon skipping events, we then investigated whether LBH589 treatment induces local variations of the RNAPII processivity along specific regions of the *CD44* gene. Our approach was to analyze the effect of this drug on the timing and extent of accumulation of the nascent *CD44* pre-mRNA after DRB treatment (Singh and Padgett, 2009; Cappellari et al., 2013). Using primers spanning different exon-intron junctions or intronic regions of the *CD44* locus, we analyzed the pre-mRNA expression in control and LBH589 treated cells at different time points from the removal of the drug. This analysis revealed higher and earlier levels of recovery of the transcription of the constitutive *CD44* C2 exon in LBH589-treated cells versus control cells, indicative of an accelerated RNAPII. However, despite this initial burst of transcription, LBH589 did not induce a higher elongation rate of the RNAPII along the variable region of the *CD44* locus, as no differences in the timing and extent of the recovery of transcription were observed between control and treated cells in this portion of the gene (*CD44* exons v2 and v5) (Figure 4C). On the contrary, along the distal region of *CD44* int18 LBH589-treated cells showed again higher levels of transcription, with an earlier time of recovery (Figure 4C). These preliminary results suggest that LBH589 can induce a global acceleration of the RNAPII along the *CD44* locus, possibly by increasing histone acetylation levels and facilitating its recruitment on the promoter region.

However, despite this global increase in the elongation rate, RNAPII appears to slow-down when entering the variable region of the *CD44* gene, wherein no differences are observed in the transcription dynamics between control and LBH589-treated cells. These observations suggest that LBH589-mediated increase of the

inclusion of *CD44* alternative exons correlates with a local slow-down of the RNAPII in the variable region of the gene, which may allow more time for recognition of these weak exons by the spliceosome. Further experiments will be aimed at identifying the factors that slow-down the RNAPII polymerase along the *CD44* variable gene and the mechanisms by which LBH589 can promote them, while concomitantly accelerating RNAPII in the rest of the gene.

2.5 Discussion

AS represent one of the best example of integrative connection between the different molecular processes regulating gene expression in eukaryotic cells, from transcription to translation, (Kornblihtt et al., 2013). In particular, it is really intriguing the reciprocal influence that AS and transcription exert on each other and the role that chromatin structure plays in this mutual regulation (Shukla et al., 2012).
In this scenario, our characterization of the ability of the pan-HDACi LBH589 to modulate the AS of genes in prostate cancer cells represents an attempt to dissect the molecular mechanisms underlying this reciprocal regulation. Treatment with HDACis is a powerful tool in the investigation of the epigenetic control of AS; indeed, modulation of histone acetylation can affect dynamics of the transcriptional process (Hnilicová et al., 2011) and probably also the deposition of other histone modifications, due to the extensive crosstalk existing between different histone marks (Suganuma and Workman, 2008).
Our results showed that LBH589 treatment affects AS of different genes in prostate cancer cell lines, similarly to what previously reported in other cellular systems for different HDACis (Hnilicová et al., 2011; Schor et al., 2012). Among the HDACis tested, LBH589 was selected as the agent exerting an effect on AS of all three genes tested, even at the lowest dose employed. These

results are in agreement with the wide spectrum of action and high activity of LBH589, which is one of the most innovative HDACi available (Atadja, 2009). Interestingly, despite the global impact that treatment with LBH589 could have on gene expression and its possible impact on protein stability through modulation of their acetylation, our observations showed no significant variations on the expression of several different splicing regulators, suggesting that its splicing effect could be associated more strictly to epigenetic mechanisms. Notably, it has been recently shown that treatment with TSA induces a substantial redistribution of several splicing factors within the nucleus, leading to their dissociation from chromatin and accumulation in nuclear speckles (Schor et al., 2012). It would be therefore interesting to investigate if LBH589 also modulates the subcellular distribution of splicing regulators in prostate cancer cells, especially of those RBPs known to be involved in the regulation of splicing events that are affected by the treatment.

Among the splicing targets of LBH589 treatment, the *CD44* gene certainly represents the most attractive because of its relevance for cancer cells proliferation (Zöller, 2011) and of the extensive regulation of its splicing pattern (Batsché et al., 2006; Cheng et al., 2006; Cappellari et al., 2013; Saint-André et al., 2011). Notably, our results show that LBH589 treatment enhances the inclusion of specific variable exons in the *CD44* transcript, without affecting its global transcriptional level.

Deciphering the mechanisms underneath the modulation of *CD44* AS by LBH589 needs a comprehensive dissection of the multiple layers of control of AS and it could lead to the identification of novel mechanisms involved in the regulation of this process. In particular, it will be interesting to understand how LBH589 can enhance, at the same time, inclusion of the *CD44* variable exons and an acceleration of the RNAPII along the *CD44* gene, which is instead usually associated with exon-skipping events (Nogues et al., 2002; de la Mata et al., 2003). Our preliminary studies about

the kinetics of the transcription process suggest that LBH589 induces a local slow-down of the RNAPII in proximity of the variable region of the *CD44* gene, which could allow variable exons recognition by the spliceosome. This hypothesis should be further confirmed by ChIP experiments analyzing the distribution of the RNAPII along the *CD44* gene before and after LBH589 treatment. In particular, ChIP experiments for the differently phosphorylated forms of the RNAPII C-terminal domain (CTD) should be also performed. Indeed, it has been shown that modulation of the CTD phosphorylation status can largely affects splicing events (Muñoz et al., 2010) and that stimuli promoting *CD44* variable exons inclusion induce a local accumulation of the CTD-Ser5 phosphorylated form of the RNAPII (Batsché et al., 2006). If we will detect a stalling of the RNAPII along the *CD44* variable region after LBH589 treatment, we could also evaluate whether it correlates with an increased recruitment of splicing factors known to be recruited by the CTD of the RNAPII along their target genes (Hsin and Manley, 2012), such as for example the RBP SAM68 (Paronetto et al., 2011; Cappellari et al., 2013).
Another possibility that will be worth investigating is the effect of the LBH589 treatment on the epigenetic status of the *CD44* locus and its involvement in the splicing effect observed. First of all, ChIP experiments will be performed to investigate the impact of LBH589 on the acetylation status of the *CD44* locus. Notably, it has been recently found that BRD2, a protein of the BET (Bromodomain Extra Terminal) family, is able to modulate AS events through a mechanism that depends on the interaction between its two bromodomains and the acetylated chromatin (Hnilicová et al., 2013). It would be therefore interesting to analyze the effect of LBH589 on the recruitment of BRD2 or other BET proteins on the *CD44* locus and to verify if treatment with inhibitors of their binding to acetylated chromatin, such as JQ1 (Filippakopoulos et al., 2010), can counteract the effects of LBH589 on splicing.

Recent evidence has highlighted a widespread crosstalk between histone modifications (Suganuma and Workman, 2008), which can strongly impact on the transcriptional process (Zippo et al., 2009; Zhao et al., 2013). It would be thus interesting to analyze if other histone marks, beyond histone acetylation, are affected by LBH589 treatment, in particular those modifications which have already been correlated with increased inclusion of *CD44* variable exons, such as H3K9me3 (Saint-André et al., 2011) or trimethylation of Lys36 in histone H3 (H3K36me3) (de Almeida et al., 2011).

Finally it could be interesting to investigate whether the activity of some of the known splicing regulators of *CD44*, such as SAM68 (Matter et al., 2002; Cappellari et al., 2013) or ESRP1/2 (Warzecha et al., 2009), is modulated by LBH589 treatment and if its effect relies on the activity of these proteins. Analysis of the effect of LBH589 treatment on *CD44* splicing in cells overexpressing or silenced for these factors and analysis of the recruitment on the *CD44* pre-mRNA of these factors after LBH589 treatment should be performed in order to address this issue.

In conclusion, our observations have highlighted the ability of LBH589 to modulate the AS of an intriguing gene, *CD44*, paving the way for further studies which will hopefully contribute to the characterization of the chromatin control of the AS process.

Material and methods

Immunohistochemistry and immunofluorescence analysis.

Cancer patient's tissues (14 cases of cryopreserved tissue from seminoma, breast, lung, prostate, cervix and colon cancer) were obtained from the National Cancer Institute "G. Pascale". Ethical Committee approval was given in all instances. 5-µm sections were processed for immunohistochemistry with antibodies against NEK2 (Abgent) as described (Barbagallo et al., 2009). Immunofluorescence was performed as described (Barbagallo et al., 2009) (Paronetto et al., 2007) using the following primary antibodies (1:500): rabbit anti-NEK2 (Abgent), mouse anti-SRSF1, anti-SRSF2 (Santa Cruz Biotechnology) and rabbit anti-cleaved CASPASE 3 (Sigma Aldrich). Confocal analyses were performed using a Leica confocal microscope as described (Busà et al., 2010). Images in Figure 6D, S2 and S5 were taken using a Leica inverted microscope as described (Barbagallo et al., 2009). Images were saved as TIFF files and Photoshop (Adobe) was used for composing panels.

Cell culture, transfections and treatment.

TCam-2 cells were grown in RPMI 1640 (Lonza), HEK293T, HeLa, MCF7, PC-3 cells were grown in DMEM (Sigma Aldrich), all supplemented with 10% FBS, gentamycin, penicillin and streptomycin. Transfection with the indicated expression vectors was performed using Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions. After 24h, cells were harvested for protein and RNA analyses. For RNA interference, cells were transfected with siRNAs (Sigma-Aldrich) using Lipofectamine RNAiMAX (Invitrogen) according to manufacturer's instructions and harvested 48h later for protein and RNA analyses. Sequences for SRPK1 siRNAs were previously described (Zhong et al.,

2009). NEK2, SRSF1 siRNAs and scrambled siRNA sequences are listed in Table S1. For apoptosis, HeLa cells were treated for 24h with the indicated doses of cisplatin or starved for 8h in Earle's balanced salt solution (Sigma). For treatment with HDACis, PC3 cells were treated for 24h with following agents: 10 nM LBH589, 2,5 μM SAHA, 330 nM trichostatin A – TSA, and 5 mM valproic acid – VPA).

Protein extracts and Western blot analysis.

Total cellular extracts and cellular fractionations were processed and analysed by Western Blot as described (Lin et al., 2005) (Paronetto et al., 2007) using the following primary antibodies (1:1000): rabbit anti-NEK2 R31 (generously provided by Prof. A.M. Fry); mouse anti-GFP, anti-MYC, anti-SRSF1, anti SRSF3, rabbit anti-SAM68; goat anti-lamin B, anti-hnRNPI, anti-U2AF65 and anti-U170K (Santa Cruz Biotechnology); rabbit anti-ACTIN, mouse anti-FLAG, anti-hnRNPA1, anti-hnRNPC1/C2 and anti-TUBULIN (Sigma-Aldrich); mouse anti-hnRNPF/H (Abcam); mouse anti-SR proteins (1H4) (Invitrogen), mouse anti-SRPK1 (BD Pharmingen); rabbit anti-AKT (Novus Biologicals); rabbit anti-pAKT Ser 473 and anti-PARP1 (Cell Signaling).

In vitro kinase assay.

Glutathione S-transferase (GST)-fusion proteins were expressed in *Escherichia coli* cells (strain BL21-DE3) and purified as previously described (Sette et al., 1998). $(His)_6$-tagged proteins were expressed in Sf9 cells using a baculovirus system and purified on a TALON affinity resin (CLONETECH), as described (Dreumont et al., 2010). *In vitro* kinase assays were performed as described (Di Agostino S, 2004), using purified NEK2 active protein (Millipore).

GST-pull-down and co-immunoprecipitation assays.

Nuclear extracts were incubated with GST or GST-NEK2A (271-445) adsorbed on glutathione-agarose beads; bound proteins were eluted and analyzed as described (Sette et al., 1998). For co-immunoprecipitation, nuclear extracts or total extracts from HEK293T cells, transfected with the indicated vectors, were incubated with mouse anti-FLAG or mouse anti-IgG antibodies adsorbed on Dynabeads protein A (Invitrogen) and immunocomplexes were eluted and analysed as described (Paronetto et al., 2007).

Extraction of RNA, RT-PCR and real-time PCR analysis.

RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. After digestion with RNase-free DNase (Roche), 1 μg of total RNA was retrotranscribed using M-MLV reverse transcriptase (Promega), used as template for PCR (GoTaq, Promega) and reactions were analysed on agarose or acrylamide gels. Quantitative real-time PCRs (qPCR) were performed using LightCycler 480 SYBR Green I Master and the LightCycler 480 System (Roche), according to the manufacturer's instructions. Control reactions omitting M-MLV reverse transcriptase were also carried out. All primers used are listed in the Table S1.

Transcriptional elongation analysis and RNA immunoprecipitation

PC-3 cells were treated for 24h with 10 nM LBH589; in the last 6h of treatment 75 mM DRB was added to inhibit transcription and synchronize cells. The cells were washed twice with PBS to remove the DRB and incubated in fresh medium for various time periods. Following the incubation period, cells were directly lysed

and total RNA was isolated using TRIzol reagent. To label nascent pre-mRNAs, after the removal of DRB, 2mM of BrU was added to the fresh medium for 60 min. Labeled pre-mRNAs were immunoprecipitated with 1 mg of anti- BrdU antibody (Becton Dickinson) and isolated as previously described (Zhou et al., 2011).

Statistical analysis.

Statistical analysis was performed by the t-Student test as described in the figure legends.

Table 1: Sequence of si-RNAs and primers used in this study

siRNAs	
Oligo name	**Sequence (5'→3')**
si-SCR	UCUUUCUUCUGCUUUGCGG
si-NEK2	GCAGACAGAUCCUGGGCAU
	GGAUCUGGCUAGUGUAAUU
	GGACCUACUUAGAUGAAGA
	GCUAGAAUAUUAAACCAUG
si-SRSF1	CCAAGGACAUUGAGGACGU
Primers	
Oligo name	**Sequence (5'→3')**
NEK2 FW H1077	GGAACGGAAGTTCCTGTC
NEK2A/C REV H1229R	CACTTGGACTTAGATGTGA
NEK2B REV AF008	GGCGAATTCCATACCGTTAC
HPRT FW	TGACCAGTCAACAGGGGACA

HPRT REV	TTCGTGGGGTCCTTTTCACC
E1A-569 FW	ATTATCTGCCACGGAGGTGT
E1A-1315 REV	GGATAGCAGGCGCCATTTTA
BCL-X Exon2 FW (BCL-X minigene)	CCATGGCAGCAGTAAAGCAA
BGH REV (BCL-X minigene)	CAGTGGGAGTGGCACCTTC
GAPDH FW	CCCTTCATTGACCTCAACTACATG
GAPDH REV	TGGGATTTCCATTGATGACAAGC
BCL-X SHORT FW (endogenous BCL-X, qPCR)	GGGACAGCATATCAGAGCTTTGAACAGGATAC
BCL-X LONG FW (endogenous BCL-X, qPCR)	AGGAGAACGGCGGCTGGGATAC
BCL-X REV (endogenous BCL-X, qPCR)	TCATTTCCGACTGAAGAGTGAGCCCA
hBIN1 ex12 FW	GGAAAGGCCCACCAGTCC
hBIN1 ex 12 REV	AACGTGTCCTCAAACAGGCT
hBIN1 ex 14 FW	CTCTCTTCCTGCTGTCGTGG
hBIN1 ex 14 REV	GGGGGCAGGTCCAAGCG
MNK2 ex 12 FW	GCGCTGCCAAAGACCTCATCTCC
MNK2a REV	ACGTGAGGTCTTTGGCACAGCT
MNK2b REV	GGAAGTGACTGTCCCACCTCTGC
BCL-X/ATG/FW (Endogenous BCL-X, RT-PCR)	ATGTCTCAGAGCAACCGGGAGCTG

BCL-X/TGA/REV (Endogenous BCL-X, RT-PCR)	TCATTTCCGACTGAAGAGTGAGC C
RON 2507 FW	CCTGAATATGTGGTCCGAGACCC CCAG
RON 2291 REV	CTAGCTGCTTCCTCCGCCACCAGT A
CD44 v5 FW	TGTAGACAGAAATGGCACCACTG
CD44 v5 REV	TTGTGCTTGTAGAATGTGGGGTC
CD44 C17 REV	CCAGAGGTTGTGTTTGCTCCACC
CD44 C16 FW	AGACACATTCCACCCCAGTG
CD44v4 FW	GGCTTTTGACCACACAAAACA
CD44 v6 FW	GAGGCAACTCCTAGTAGTAC
CD44 v6 REV	CTGTTGTCGAATGGGAGTC
CD44 v7 FW (RT-PCR)	CAGCCTCAGCTCATACCAGC
CD44 v7 FW (qPCR)	CCAATGCAAGGAAGGACAACA
CD44 v7 REV (qPCR)	GGTTGAAGAAATCAGTCCAGGAA
CD44 v8 FW	TCCAGTCATAGTACAACGCT
CD44 v9 FW	CAGAGCTTCTCTACATCACA
CD44 v9 REV	ATGTCAGAGTAGAAGTTGTTGGA T
CD44 v10 FW	GGTGGAAGAAGAGACCCAAA
CD44 v10 REV	TCGTGTGTGGGTAATGAGAGGTA
CD44 C2 FW	CCACGTGGAGAAAAATGGTC
preCD44 C2 REV	GTCGGGTGCTGGTCTCTTA
CD44v2 FW	GGTTGTTTCTACCATCAGAGTCA

preCD44v2 REV	AGGCTGTCAAGAAAACATACGC
preCD44v5 REV	TCCCTATTGCTGGAATGTCTTT
CD44 int 1 FW	AGAAGGGATCACAATTCCAAGTG
CD44 int 1 REV	CCAGGACCCCTTTCTGGAAA
CD44 int18 FW	CACTTTGAGGAGTGCTGGTTGT
CD44 int18 REV	GATTGCAGTGACTCAGGCATCA

References

Adesso, L., Calabretta, S., Barbagallo, F., Capurso, G., Pilozzi, E., Geremia, R., Delle Fave, G., Sette, C. (2013). Gemcitabine triggers a pro-survival response in pancreatic cancer cells through activation of the MNK2/eIF4E pathway. *Oncogene*. 32, 2848-28.

Amin, E.M., Oltean, S., Hua, J., Gammons, M.V., Hamdollah-Zadeh, M., Welsh, G.I., Cheung, M.K., Ni, L., Kase, S., Rennel, E.S., et al. (2011). WT1 mutants reveal SRPK1 to be a downstream angiogenesis target by altering VEGF splicing. *Cancer Cell.* 20(6):768-80.

Anczuków, O., Rosenberg, A.Z., Akerman, M., Das, S., Zhan, L., Karni, R., Muthuswamy, S.K., Krainer, A.R. (2012). The splicing factor SRSF1 regulates apoptosis and proliferation to promote mammary epithelial cell. *Nat Struct Mol Biol*. 19(2):220-8.

Andréasson, U., Dictor, M., Jerkeman, M., Berglund, M., Sundström, C., Linderoth, J., Rosenquist, R., Borrebaeck, C.A., Ek, S. (2009). Identification of molecular targets associated with transformed diffuse large B cell lymphoma using highly purified tumor cells. *Am J Hematol.* 84(12):803-8.

Atadja, P. (2009). Development of the pan-DAC inhibitor panobinostat (LBH589): successes and challenges. *Cancer Lett.* 280(2):233-41.

Bahe, S., Stierhof, Y.D., Wilkinson, C.J., Leiss, F., Nigg, E.A. (2005). Rootletin forms centriole-associated filaments and functions in centrosome cohesion. *J Cell Biol.* 171(1):27-33.

Barbagallo, F., Paronetto, M.P., Franco, R., Chieffi, P., Dolci, S., Fry, A.M., Geremia, R., Sette, C. (2009). Increased expression and nuclear localization of the centrosomal kinase Nek2 in human testicular seminomas. *J Pathol.* 217(3):431-41.

Barbosa-Morais, N.L., Irimia, M., Pan, Q., Xiong, H.Y., Gueroussov, S., Lee, L.J., Slobodeniuc, V., Kutter, C., Watt, S., Colak, R., et al. (2012). The evolutionary landscape of alternative splicing in vertebrate species. *Science.* 338(6114):1587-93.

Batsché, E., Yaniv, M., Muchardt, C. (2006). The human SWI/SNF subunit Brm is a regulator of alternative splicing. *Nat Struct Mol Biol.* 13(1):22-9.

Bielli P., Busà., R., Paronetto, M.P., Sette, C. (2011). The RNA-binding protein Sam68 is a multifunctional player in human cancer. *Endocr Relat Cancer.* 18(4):R91-R102.

Black, D.L. (2003). Mechanisms of alternative pre-messenger RNA splicing. *Annu Rev Biochem.* 72:291-336.

Blencowe, B. (2006). Alternative splicing: new insights from global analyses. *Cell.* 126(1):37-47.

Boise, L., González-García, M., Postema, C.E., Ding, L., Lindsten, T., Turka, L.A., Mao, X., Nuñez, G., Thompson, C.B. (1993). Bcl-x, a bcl-2-related gene that functions as a dominant regulator of apoptotic cell death. *Cell*. 74, 597-608.

Brown, S.J., Stoilov, P., Xing, Y. (2012). Chromatin and epigenetic regulation of pre-mRNA processing. *Hum Mol Genet.* 21(R1):R90-6.

Busà R, Geremia, R., Sette, C. (2010). Genotoxic stress causes the accumulation of the splicing regulator Sam68 in nuclear foci of transcriptionally active chromatin. *Nucleic Acids Res.* 38(9):3005-18.

Calvisi, D.F., Pinna, F., Ladu, S., Pellegrino, R., Simile, M.M., Frau, M., De Miglio, M.R., Tomasi, M.L., Sanna, V., Muroni, M.R., et al. (2009). Forkhead box M1B is a determinant of rat susceptibility to hepatocarcinogenesis and sustains ERK activity in human HCC. *Gut*. 58(5):679-87.

Cao, W., Jamison, S.F., Garcia-Blanco, M.A. (1997). Both phosphorylation and dephosphorylation of ASF/SF2 are required for pre-mRNA splicing in vitro. *RNA.* 3,1456-1467.

Cappellari, M., Bielli, P., Paronetto, M.P., Ciccosanti, F., Fimia, G.M., Saarikettu, J., Silvennoinen, O., Sette, C. (2013). The transcriptional co-activator SND1 is a novel regulator of alternative splicing in prostate cancer cells. *Oncogene.* doi: 10.1038/onc.2013.360.

Cappello, P., Blaser, H., Gorrini, C., Lin, D.C., Elia, A.J., Wakeham, A., Haider, S., Boutros, P.C., Mason, J.M., Miller, N.A. et al. (2013). Role of Nek2 on centrosome duplication and aneuploidy in breast cancer cells. *Oncogene*. doi: 10.1038/onc.2013.183.

Carrillo Oesterreich, F., Bieberstein, N., Neugebauer, K.M. (2011). Pause locally, splice globally. *Trends Cell Biol.* 21(6):328-35.

Chen, Y., Riley, D.J., Zheng, L., Chen, P.L., Lee, W.H. (2002). Phosphorylation of the mitotic regulator protein Hec1 by Nek2 kinase is essential for faithful chromosome segregation. *J Biol Chem.* 277(51):49408-16.

Cheng, C., Sharp, P.A. (2006). Regulation of CD44 alternative splicing by SRm160 and its potential role in tumor cell invasion. *Mol Cell Biol.* 26(1):362-70.

Cho, S., Hoang, A., Sinha, R., Zhong, X.Y., Fu, X.D., Krainer, A.R., Ghosh, G. (2011). Interaction between the RNA binding domains of Ser-Arg splicing factor 1 and U1-70K snRNP protein determines early spliceosome assembly. *Proc Natl Acad Sci U S A.* 108(20):8233-8.

Choudhary, C., Kumar, C., Gnad, F., Nielsen, M.L., Rehman, M., Walther, T.C., Olsen, J.V., Mann, M. (2009). Lysine acetylation targets protein complexes and co-regulates major cellular functions. *Science.* 325(5942):834-40.

Cooper, T.A., Wan, L., Dreyfuss, G. (2009). RNA and disease. *Cell*. 136(4):777-93.

Clayton, A., Hazzalin, C.A., Mahadevan, L.C. (2006). Enhanced histone acetylation and transcription: a dynamic perspective. *Mol Cell.* 23(3):289-96.

Das, T.K., Dana, D., Paroly, S.S., Perumal, S.K., Singh, S., Jhun, H., Pendse, J., Cagan, R.L., Talele, T.T., Kumar, S. (2013). Centrosomal kinase Nek2 cooperates with oncogenic pathways to promote metastasis. *Oncogenesis.*2:e69 doi: 10.1038/oncsis.2013.34..

David, C.J., Manley, J.L. (2010). Alternative pre-mRNA splicing regulation in cancer: pathways and programs unhinged. *Genes Dev.* 24(21):2343-64.

de Almeida, S.F., Grosso, A.R., Koch, F., Fenouil, R., Carvalho, S., Andrade, J., Levezinho, H., Gut, M., Eick, D., Gut, I., et al. (2011). Splicing enhances recruitment of methyltransferase HYPB/Setd2 and methylation of histone H3 Lys36. *Nat Struct Mol Biol.* 18(9):977-83.

de la Mata, M., Alonso, C.R., Kadener, S., Fededa, J.P., Blaustein, M., Pelisch, F., Cramer, P., Bentley, D., Kornblihtt, A.R. (2003). A slow RNA polymerase II affects alternative splicing in vivo. *Mol Cell.* 12(2):525-32.

de la Mata, M., Kornblihtt, A.R. (2006). RNA polymerase II C-terminal domain mediates regulation of alternative splicing by SRp20. *Nat Struct Mol Biol.* 13(11):973-80.

de la Mata, M., Lafaille, C., Kornblihtt, A.R. (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(5):904-12.

Di Agostino, S., Rossi, P., Geremia, R., Sette, C. (2002). The MAPK pathway triggers activation of Nek2 during chromosome condensation in mouse spermatocytes. *Development*. 129, 1715-1727.

Di Agostino, S., Fedele, M., Chieffi, P., Fusco, A., Rossi, P., Geremia, R., Sette, C. (2004). Phosphorylation of high-mobility group protein A2 by Nek2 kinase during the first

meiotic division in mouse spermatocytes. *Mol Biol Cell*. 15(3):1224-32

Dreumont, N., Hardy, S., Behm-Ansmant, I., Kister, L., Branlant, C., Stévenin, J., Bourgeois, C.F. (2010). Antagonistic factors control the unproductive splicing of SC35 terminal intron. *Nucleic Acids Res*. 38(4):1353-66.

Edmond, V., Moysan, E., Khochbin, S., Matthias, P., Brambilla, C., Brambilla, E., Gazzeri, S., Eymin, B. (2011). Acetylation and phosphorylation of SRSF2 control cell fate decision in response to cisplatin. *EMBO J.* 30(3):510-23.

Filippakopoulos, P., Qi, J., Picaud, S., Shen, Y., Smith, W.B., Fedorov, O., Morse, E.M., Keates, T., Hickman, T.T., Felletar, I., et al. (2010). Selective inhibition of BET bromodomains. *Nature*. 468(7327):1067-73.

Fry, A.M., Schultz, S.J., Bartek, J., Nigg, E.A. (1995). Substrate specificity and cell cycle regulation of the Nek2 protein kinase, a potential human homolog of the mitotic regulator NIMA of Aspergillus nidulans. *J Biol Chem.* 270(21):12899-905.

Fry, A.M., Mayor, T., Meraldi, P., Stierhof, Y.D., Tanaka, K., Nigg, E.A. (1998)a. C-Nap1, a novel centrosomal coiled-coil protein and candidate substrate of the cell cycle-regulated protein kinase Nek2. *J Cell Biol*. 141(7):1563-74.

Fry, A.M., Meraldi, P., Nigg, E.A. (1998)b. A centrosomal function for the human Nek2 protein kinase, a member of the NIMA family of cell cycle regulators. *EMBO J.* 17(2):470-81.

Fry, A.M., Arnaud, L., Nigg, E.A. (1999). Activity of the human centrosomal kinase, Nek2, depends on an unusual leucine zipper dimerization motif. *J Biol Chem*. 274(23):16304-10.

Fry, A.M. (2002). The Nek2 protein kinase: a novel regulator of centrosome structure. *Oncogene*. 21(40):6184-94.

Fry, A.M., O'Regan, L., Sabir, S.R., Bayliss, R. (2012). Cell cycle regulation by the NEK family of protein kinases. *J Cell Sci.* 125(Pt 19):4423-33.

Ghigna, C., Giordano, S., Shen, H., Benvenuto, F., Castiglioni, F., Comoglio, P.M., Green, M.R., Riva, S., Biamonti, G. (2005). Cell motility is controlled by SF2/ASF through alternative splicing of the Ron protooncogene. *Mol Cell.* 20(6):881-90.

Ghigna C., Valacca, C., Biamonti, G. (2008). Alternative splicing and tumor progression. *Curr Genomics.* 9(8):556-70.

Golan-Gerstl, R., Cohen, M., Shilo, A., Suh, S.S., Bakàcs, A., Coppola, L., Karni, R. (2011). Splicing factor hnRNP A2/B1 regulates tumor suppressor gene splicing and is an oncogenic driver in glioblastoma. *Cancer Res.* 71(13):4464-72.

Gómez Acuña, L.I., Fiszbein, A., Alló, M., Schor, I.E., Kornblihtt, A.R. (2013). Connections between chromatin signatures and splicing. *Wiley Interdiscip Rev RNA.* 4(1):77-91.

Grosso, A.R., Martins, S., Carmo-Fonseca, M. (2008). The emerging role of splicing factors in cancer. *EMBO Rep.* 9(11):1087-93.

Hames, R.S., Wattam, S.L., Yamano, H., Bacchieri, R., Fry, A.M. (2001). APC/C-mediated destruction of the centrosomal kinase Nek2A occurs in early mitosis and depends upon a cyclin A-type D-box. *EMBO J.* 20(24):7117-27.

Hames, R.S., Fry, A.M. (2002). Alternative splice variants of the human centrosome kinase Nek2 exhibit distinct patterns of expression in mitosis. *Biochem J.* 361(Pt 1):77-85.

Han, H., Irimia, M., Ross, P.J., Sung, H.K., Alipanahi, B., David, L., Golipour, A., Gabut, M., Michael, I.P., Nachman, E.N., et al. (2013). MBNL proteins repress ES-cell-specific alternative splicing and reprogramming. *Nature.* 498(7453):241-5.

Hayes, M.J., Kimata, Y., Wattam,S.L., Lindon, C., Mao, G., Yamano, H., Fry, A.M. (2006). Early mitotic degradation of Nek2A depends on Cdc20-independent interaction with the APC/C. *Nat Cell Biol*. 8(6):607-14.

Hayes, G.M., Carrigan, P.E., Miller, L.J. (2007). Serine-arginine protein kinase 1 overexpression is associated with tumorigenic imbalance in mitogen-activated protein kinase pathways in

breast, colonic, and pancreatic carcinomas. *Cancer Res.* 67(5):2072-80.

Hayward, D.G., Clarke, R.B., Faragher, A.J., Pillai, M.R., Hagan, I.M., Fry, A.M. (2004). The centrosomal kinase Nek2 displays elevated levels of protein expression in human breast cancer. *Cancer Res*. 64(20):7370-6.

Hayward, D.F. and Fry, A.M. (2006). Nek2 kinase in chromosome instability and cancer. *Cancer Lett.* 237(2):155-66.

Helps, N.R., Luo, X., Barker, H.M., Cohen, P.T. (2000). NIMA-related kinase 2 (Nek2), a cell-cycle-regulated protein kinase localized to centrosomes, is complexed to protein phosphatase 1. *Biochem J.* 349(Pt 2):509-18.

Hertel, K.J. (2008). Combinatorial control of exon recognition. *J Biol Chem*. 283(3):1211-5.

Hnilicová, J., Hozeifi, S., Dušková, E., Icha, J., Tománková, T., Staněk, D. (2011). Histone deacetylase activity modulates alternative splicing. *PLoS One.* 6(2):e16727.

Hnilicová, J., Hozeifi, S., Stejskalová, E., Dusková, E., Poser, I., Humpolícková, J., Hof, M., Stanek, D. (2013). The C-terminal domain of Brd2 is important for chromatin interaction and regulation of transcription and alternative splicing. *Mol Biol Cell.* 24(22):3557-68.

Hsin, J.P. and Manley, J.L. The RNA polymerase II CTD coordinates transcription and RNA processing. *Genes Dev.* (2012). 26(19):2119-37.

House, A.E,, Lynch, K.W. (2008). Regulation of alternative splicing: more than just the ABCs. *J Biol Chem.* 283(3):1217-21.

Izquierdo, J.M., Majós, N., Bonnal, S., Martínez, C., Castelo, R., Guigó, R., Bilbao, D., Valcárcel, J. (2005). Regulation of Fas alternative splicing by antagonistic effects of TIA-1 and PTB on exon definition. *Mol Cell.* 19(4):475-84.

Jeong, Y., Lee, J., Kim, K., Yoo, J.C., Rhee, K. (2007). Characterization of NIP2/centrobin, a novel substrate of Nek2, and its potential role in microtubule stabilization. *J Cell Sci.* 120(Pt 12):2106-16.

Kalsotra, A., Cooper, T.A. (2011). Functional consequences of developmentally regulated alternative splicing. *Nat Rev Genet.* 12(10):715-29.

Karni, R., de Stanchina, E., Lowe, S.W., Sinha, R., Mu, D., Krainer, A.R. (2007). The gene encoding the splicing factor SF2/ASF is a proto-oncogene. *Nat Struct Mol Biol.* 14(3):185-93.

Kokuryo, T., Senga, T., Yokoyama, Y., Nagino, M., Nimura, Y., Hamaguchi, M. (2007). Nek2 as an effective target for inhibition of tumorigenic growth and peritoneal dissemination of cholangiocarcinoma. *Cancer Res.* 67(20):9637-42.

Kornblihtt, A.R., Schor, I.E., Alló, M., Dujardin, G., Petrillo, E., Muñoz M.J. (2013). Alternative splicing: a pivotal step between eukaryotic transcription and translation. *Nat Rev Mol Cell Biol.* 14(3), 153-65.

Landi, M.T., Dracheva, T., Rotunno, M., Figueroa, J.D., Liu, H., Dasgupta, A., Mann, F.E., Fukuoka, J., Hames, M., Bergen, A.W., et al.(2008). Gene expression signature of cigarette smoking and its role in lung adenocarcinoma development and survival. *PLoS One.* 3(2):e1651.

Li, H.R., Wang-Rodriguez, J., Nair, T.M., Yeakley, J.M., Kwon, Y.S., Bibikova, M., Zheng, C., Zhou, L., Zhang, K., Downs, T., et al. (2006). Two-dimensional transcriptome profiling: identification of messenger RNA isoform signatures in prostate cancer from archived paraffin-embedded cancer specimens. *Cancer Res*. 66(8):4079-88.

Lin, S., Xiao, R., Sun, P., Xu, X., Fu, X.D. (2005). Dephosphorylation-dependent sorting of SR splicing factors during mRNP maturation. *Mol Cell*. 20(3):413-25.

Lin, S., Coutinho-Mansfield, G., Wang, D., Pandit, S., Fu, X.D. (2008). The splicing factor SC35 has an active role in transcriptional elongation. *Nat Struct Mol Biol.* 15(8):819-26.

Liu, H.X., Cartegni, L., Zhang, M.Q., Krainer, A.R. (2001). A mechanism for exon skipping caused by nonsense or missense mutations in BRCA1 and other genes. *Nat Genet.* 27(1):55-8.

Liu, Q., Hirohashi, Y., Du, X., Greene, M.I., Wang, Q. (2010). Nek2 targets the mitotic checkpoint proteins Mad2 and Cdc20: A mechanism for aneuploidy in cancer. *Exp Mol Pathol.* 88(2):225-33.

Llorian, M., Schwartz, S., Clark, T.A., Hollander, D., Tan, L.Y., Spellman, R., Gordon, A., Schweitzer, A.C., de la Grange, P., Ast, G., et al. (2010). Position-dependent alternative splicing activity revealed by global profiling of alternative splicing events regulated by PTB. *Nat Struct Mol Biol.* 17(9):1114-23.

Locatelli, A, and Lange, C.A. (2011). Met receptors induce Sam68-dependent cell migration by activation of alternate extracellular signal-regulated kinase family members. *J Biol Chem.* 286(24):21062-72.

Long, J.C., Caceres, J.F. (2009). The SR protein family of splicing factors: master regulators of gene expression. *Biochem J.* 417(1):15-27.

Lou, Y., Yao, J., Zereshki, A., Dou, Z., Ahmed, K., Wang, H., Hu, J., Wang, Y., Yao, X. (2004). NEK2A interacts with MAD1 and possibly functions as a novel integrator of the spindle checkpoint signaling. *J Biol Chem.* 279(19):20049-57.

Lu, Y., Yao, H.P., Wang, M.H. (2007). Multiple variants of the RON receptor tyrosine kinase: biochemical properties, tumorigenic activities, and potential drug targets. *Cancer Lett*, 257(2):157-64.

Luco, R.F., Pan, Q., Tominaga, K., Blencowe, B.J., Pereira-Smith, O.M., Misteli, T. (2010). Regulation of alternative splicing by histone modifications. *Science.* 327(5968):996-1000.

Luco, R.F., Allo, M., Schor, I.E., Kornblihtt, A.R., Misteli, T. (2011). Epigenetics in alternative pre-mRNA splicing. *Cell.* 144(1):16-26.

Mardin, B.R., Lange, C., Baxter, J.E., Hardy, T., Scholz, S.R., Fry, A.M., Schiebel, E. (2010). Components of the Hippo pathway cooperate with Nek2 kinase to regulate centrosome disjunction. *Nat Cell Biol*. 12(12):1166-76.

Mardin, B.R., Agircan, F.G., Lange, C., Schiebel, E. (2011). Plk1 controls the Nek2A-PP1γ antagonism in centrosome disjunction. *Curr Biol*. 21(13):1145-51.

Mardin, B.R., Schiebel, E. (2012). Breaking the ties that bind: new advances in centrosome biology. *J Cell Biol*. 197(1):11-8.

Massiello, A., Salas, A., Pinkerman, R.L., Roddy, P., Roesser, J.R., Chalfant, C.E. (2004). Identification of two RNA cis-elements that function to regulate the 5' splice site selection of Bcl-x pre-mRNA in response to ceramide. *J Biol Chem.* 279(16):15799-804.

Matlin, A.J., Clark, F., Smith, C,W. (2005). Understanding alternative splicing: towards a cellular code. *Nat Rev Mol Cell Biol.* 6(5):386-98.

Matter, N., Herrlich, P., König, H. (2002). Signal-dependent regulation of splicing via phosphorylation of Sam68. *Nature.* 420(6916):691-5.

Meraldi, P.N., Nigg, E.A. (2001). Centrosome cohesion is regulated by a balance of kinase and phosphatase activities. *J Cell Sci.* 114(Pt 20):3749-57.

Mercatante, D.R., Bortner, C.D., Cidlowski, J.A., and Kole R. (2001). Modification of alternative splicing of Bcl-x pre-mRNA in prostate and breast cancer cells. analysis of apoptosis and cell death. *J Biol Chem*. 276(19):16411-7.

Merkin, J., Russell, C., Chen, P., Burge, C.B. (2012). Evolutionary dynamics of gene and isoform regulation in mammalian tissues. *Science.* 338(6114):1593-9.

Mermoud, J.E., Cohen, P.T., Lamond, A.I. (1994). Regulation of mammalian spliceosome assembly by a protein phosphorylation mechanism. *EMBO J.* 13(23):5679-88.

Misquitta-Ali, C.M., Cheng, E., O'Hanlon, D., Liu, N., McGlade, C.J., Tsao, M.S., Blencowe B.J. (2011). Global profiling and molecular characterization of alternative splicing events misregulated in lung cancer. *Mol Cell Biol.* 31(1):138-50.

Misteli, T., Cáceres, J.F., Clement, J.Q., Krainer, A.R., Wilkinson, M.F., Spector, D.L. (1998). Serine phosphorylation of SR proteins is required for their recruitment to sites of transcription in vivo. *J Cell Biol*. 143(2):297-307.

Moore, M.J., Proudfoot, N.J. (2009). Pre-mRNA processing reaches back to transcription and ahead to translation. *Cell*. 136(4): 688-700.

Moore, M.J., Wang, Q., Kennedy, C.J., Silver, P.A. (2010). An alternative splicing network links cell-cycle control to apoptosis. *Cell.* 142(4):625-36.

Muñoz, M.J., Pérez Santangelo, M.S., Paronetto, M.P., de la Mata, M., Pelisch, F., Boireau, S., Glover-Cutter, K., Ben-Dov, C., Blaustein, M., Lozano, J.J. et al. (2009). DNA damage regulates alternative splicing through inhibition of RNA polymerase II elongation. *Cell.* 137(4):708-20.

Muñoz, M.J., de la Mata, M., Kornblihtt, A.R. (2010). The carboxy terminal domain of RNA polymerase II and alternative splicing. *Trends Biochem Sci.* 35(9):497-504.

Naro, C., Sette, C. (2013). Phosphorylation-Mediated Regulation of Alternative Splicing in Cancer. *Int J Cell Biol.* 2013:151839.

Ngo, J.C., Chakrabarti, S., Ding, J.H., Velazquez-Dones, A., Nolen, B., Aubol, B.E., Adams, J.A., Fu, X.D., Ghosh, G. (2005). Interplay between SRPK and Clk/Sty kinases in phosphorylation of the splicing factor ASF/SF2 is regulated by a docking motif in ASF/SF2. *Mol Cell.* 20(1):77-89.

Nigg, E.A., Stearns, T. (2011). The centrosome cycle: Centriole biogenesis, duplication and inherent asymmetries. *Nat Cell Biol.* 13(10):1154-60.

Nogues, G., Kadener, S., Cramer, P., Bentley, D., Kornblihtt, A.R. (2002). Transcriptional activators differ in their abilities to control alternative splicing. *J Biol Chem.* 277(45):43110-4.

Olshavsky, N.A, Comstock, C.E., Schiewer, M.J., Augello, M.A., Hyslop, T., Sette, C., Zhang, J., Parysek, L.M., Knudsen, K.E.(2010). Identification of ASF/SF2 as a critical, allele-specific effector of the cyclin D1b oncogene. *Cancer Res.* 70(10):3975-84.

O'regan, L., Blot, J., Fry, A.M. (2007). Mitotic regulation by NIMA-related kinases. *Cell Div.* 2:25.

Pal, S., Gupta, R., Davuluri, R.V. (2012). Alternative transcription and alternative splicing in cancer. *Pharmacol Ther.* 136(3):283-94.

Pandya-Jones, A., Black, D.L. (2009). Co-transcriptional splicing of constitutive and alternative exons. *RNA.* 15(10):1896-908.

Pandya-Jones, A. (2011). Pre-mRNA splicing during transcription in the mammalian system. *Wiley Interdiscip Rev RNA.* 2(5):700-17.

Paronetto, M.P., Achsel, T., Massiello, A., Chalfant, C.E., Sette, C. (2007). The RNA-binding protein Sam68 modulates the alternative splicing of Bcl-x. *J Cell Biol.* 176(7):929-39.

Paronetto, M.P., Messina, V., Barchi, M., Geremia, R., Richard, S., Sette, C. (2011). Sam68 marks the transcriptionally active stages of spermatogenesis and modulates alternative splicing in male germ cells. *Nucleic Acids Res.* 39(12):4961-74.

Patel, N.A., Kaneko, S., Apostolatos, H.S., Bae, S.S., Watson, J.E., Davidowitz, K., Chappell, D.S., Birnbaum, M.J., Cheng, J.Q., Cooper, D.R. (2005). Molecular and genetic studies imply Akt-mediated signaling promotes protein kinase CbetaII alternative splicing via phosphorylation of serine/arginine-rich splicing factor SRp40. *J Biol Chem.* 280(14):14302-9.

Prasad, J., Colwill, K., Pawson, T., Manley, J.L. (1999). The protein kinase Clk/Sty directly modulates SR protein activity: both hyper- and hypophosphorylation inhibit splicing. *Mol Cell Biol.* 19, 6991-7000.

Sadoul, K., Boyault, C., Pabion, M., Khochbin, S. (2008). Regulation of protein turnover by acetyltransferases and deacetylases. *Biochimie*. 90(2):306-12.

Saint-André, V., Batsché, E., Rachez, C., Muchardt, C. (2011). Histone H3 lysine 9 trimethylation and HP1γ favor inclusion of alternative exons. *Nat Struct Mol Biol.* 18(3):337-44.

Sakharkar, M.K., Chow, V.T., Kangueane, P. (2004). Distributions of exons and introns in the human genome. *In Silico Biol*. 4(4):387-93.

Sanford, J.R., Ellis, J.D., Cazalla, D., Cáceres, J.F. (2005). Reversible phosphorylation differentially affects nuclear and cytoplasmic functions of splicing factor 2/alternative splicing factor. *Proc Natl Acad Sci U S A.* 102,15042-15047.

Schwartz, S., Ast, G. (2010). Chromatin density and splicing destiny: on the cross-talk between chromatin structure and splicing. *EMBO J.* 29(10):1629-36.

Schor, I.E., Llères, D., Risso, G.J., Pawellek, A., Ule, J., Lamond, A.I., Kornblihtt, A.R. (2012). Perturbation of chromatin structure globally affects localization and recruitment of splicing factors. *PLoS One*. 7(11):e48084.

Sette, C., Bevilacqua, A., Geremia, R., Pellegrino, R. (1998). Involvement of phospholipase Cgamma1 in mouse egg activation induced by a truncated form of the C-kit tyrosine kinase present in spermatozoa. *J Cell Biol.* 142(4):1063-74.

Sharma, S., Falick, A.M., Black, D.L. (2005). Polypyrimidine tract binding protein blocks the 5' splice site-dependent assembly of U2AF and the prespliceosomal E complex. *Mol Cell.* 19(4):485-96.

Shin, C., Manley, J.L. (2004). Cell signalling and the control of pre-mRNA splicing. *Nat Rev Mol Cell Biol.* 5(9):727-38.

Shukla, S., Kavak, E., Gregory, M., Imashimizu, M., Shutinoski, B., Kashlev, M., Oberdoerffer, P., Sandberg, R., Oberdoerffer, S. (2011). CTCF-promoted RNA polymerase II pausing links DNA methylation to splicing. *Nature.* 479(7371):74-9.

Shukla, S., Oberdoerffer, S. (2012). Co-transcriptional regulation of alternative pre-mRNA splicing. *Biochim Biophys Acta.* 1819(7): 673-83.

Singh, J., Padgett, R.A. (2009). Rates of in situ transcription and splicing in large human genes. *Nat Struct Mol Biol.* 16(11):1128-33.

Singh, R.K., Cooper, T.A. (2012). Pre-mRNA splicing in disease and therapeutics. *Trends Mol Med.* 18(8):472-82.

Song, E.J., Werner, S.L., Neubauer, J., Stegmeier, F., Aspden, J., Rio, D., Harper, J.W., Elledge, S.J., Kirschner, M.W., Rape, M. (2010). The Prp19 complex and the Usp4Sart3 deubiquitinating enzyme control reversible ubiquitination at the spliceosome. *Genes Dev.* 24(13):1434-47.

Soret, J., Gabut, M., Dupon, C., Kohlhagen, G., Stévenin, J., Pommier, Y., Tazi, J. (2003). Altered serine/arginine-rich protein phosphorylation and exonic enhancer-dependent splicing in Mammalian cells lacking topoisomerase I. *Cancer Res*. 63(23):8203-11.

Spector, D.L., Lamond, A.I. (2011). Nuclear speckles. *Cold Spring Harb Perspect Biol.* 3(2). pii: a000646.

Stamm, S. (2008). Regulation of alternative splicing by reversible protein phosphorylation. *J Biol Chem.* 283(3):1223-7.

Suganuma, T., Workman, J.L. (2008) Crosstalk among Histone Modifications. *Cell*. 135(4):604-7.

Tacke, R., Chen, Y., Manley, J.L. (1997). Sequence-specific RNA binding by an SR protein requires RS domain phosphorylation: creation of an SRp40-specific splicing enhancer. *Proc Natl Acad Sci U S A.* 94(4):1148-53.

Tazi, J., Bakkour, N., Stamm, S. (2009). Alternative splicing and disease. *Biochim Biophys Acta.*, 1792(1):14-26.

Tsunoda, N., Kokuryo, T., Oda, K., Senga, T., Yokoyama, Y., Nagino, M., Nimura, Y., Hamaguchi, M. (2009). Nek2 as a novel molecular target for the treatment of breast carcinoma. *Cancer Sci*. 100(1):111-6.

van der Houven van Oordt, W., Diaz-Meco, M.T., Lozano, J., Krainer, A.R., Moscat, J., Cáceres, J.F. (2000). The MKK(3/6)-p38-signaling cascade alters the subcellular distribution of hnRNP A1 and modulates alternative splicing regulation. *J Cell Biol.* 149(2):307-16.

Venables, J.P., Klinck, R., Bramard, A., Inkel, L., Dufresne-Martin, G., Koh, C., Gervais-Bird, J., Lapointe, E., Froehlich, U., Durand, M., et al. (2008). Identification of alternative splicing markers for breast cancer. *Cancer Res.* 68(22):9525-31.

Vitre, B.C., Cleveland, D.W. (2012). Centrosomes, chromosome instability (CIN) and aneuploidy. *Curr Opin Cell Biol*. 24(6):809-15.

Warzecha, C.C., Sato, T.K., Nabet, B., Hogenesch, J.B., Carstens RP. (2009). ESRP1 and ESRP2 are epithelial cell-type-specific regulators of FGFR2 splicing. *Mol Cell*. 33(5):591-601.

Wei, R., Ngo, B., Wu, G., Lee, W.H. (2011). Phosphorylation of the Ndc80 complex protein, HEC1, by Nek2 kinase modulates chromosome alignment and signaling of the spindle assembly checkpoint. *Mol Biol Cell.* 22(19):3584-94.

Will, C.L., and Lührmann, R. (2011). Spliceosome structure and function. *Cold Spring Harb Perspect Biol.* 3(7): pii: a003707.

Wu, W., Baxter, J.E., Wattam, S.L., Hayward, D.G., Fardilha, M., Knebel, A., Ford, E.M., da Cruz e Silva, E.F., Fry, A.M. (2007). Alternative splicing controls nuclear translocation of the cell cycle-regulated Nek2 kinase. *J Biol Chem.* 282(36):26431-40.

Wu, C.C., Calcagno, A.M., Ambudkar, S.V. (2008)a. Reversal of ABC drug transporter-mediated multidrug resistance in cancer

cells: evaluation of current strategies. *Curr Mol Pharmacol*. 1(2): 93-105.

Wu, G., Qiu, X.L., Zhou, L., Zhu, J., Chamberlin, R., Lau, J., Chen, P.L., Lee, W.H. (2008)b. Small molecule targeting the Hec1/Nek2 mitotic pathway suppresses tumor cell growth in culture and in animal. *Cancer Res*. 68(20):8393-9.

Zippo, A., Serafini, R., Rocchigiani, M., Pennacchini, S., Krepelova, A., Oliviero, S. (2009). Histone crosstalk between H3S10ph and H4K16ac generates a histone code that mediates transcription elongation. *Cell*. 138(6):1122-36.

Zhao, X., Su, J., Wang, F., Liu, D., Ding, J., Yang, Y., Conaway, J.W., Conaway, R.C., Cao, L., Wu, D. et al. (2013). Crosstalk between NSL Histone Acetyltransferase and MLL/SET Complexes: NSL Complex Functions in Promoting Histone H3K4 Di-Methylation Activity by MLL/SET Complexes. *PLoS Genet.*, 9(11):e1003940.

Zhong, X.Y., Ding, J.H., Adams, J.A., Ghosh, G., Fu, X.D. (2009). Regulation of SR protein phosphorylation and alternative splicing by modulating kinetic interactions of SRPK1 with molecular chaperones. *Genes Dev.* 23(4):482-95.

Zhou, H.L., Hinman, M.N., Barron, V.A., Geng, C., Zhou, G., Luo, G., Siegel, R.E., Lou, H. (2011). Hu proteins regulate alternative splicing by inducing localized histone hyperacetylation in an RNA-dependent manner. *Proc Natl Acad Sci U S A.* 108(36):E627-35.

Zhou, Z., Qiu, J., Liu, W., Zhou, Y., Plocinik, R.M., Li, H., Hu, Q., Ghosh, G., Adams, J.A., Rosenfeld, M.G., et al. (2012). The Akt-SRPK-SR axis constitutes a major pathway in transducing EGF signaling to regulate alternative splicing in the nucleus. *Mol Cell*. 47(3):422-33.

Zhou, Z.F., Fu, X.D. (2013). Regulation of splicing by SR proteins and SR protein-specific kinases. *Chromosoma.* 122(3):191-207.

Zhou, W., Yang, Y., Xia, J., Wang, H., Salama, M.E., Xiong, W., Xu, H., Shetty, S., Chen, T., Zeng, Z., et al., (2013). NEK2 induces drug resistance mainly through activation of efflux drug pumps and is associated with poor prognosis in myeloma and other cancers. *Cancer Cell*. 23(1):48-62.

Zöller, M. (2011). CD44: can a cancer-initiating cell profit from an abundantly expressed molecule? *Nat Rev Cancer*. 11(4):254-67.

List of Publications

Naro, C., Sette, C. (2013). Phosphorylation-Mediated Regulation of Alternative Splicing in Cancer. *Int J Cell Biol*. 2013:151839.

Naro, C., Barbagallo F., Chieffi, P., Bourgeois, C., Paronetto, M.P., Sette, C. (2013). The centrosomal kinase NEK2 is a novel splicing factor kinase involved in cell survival. *Nucleic Acids Res.* doi: 10.1093/nar/gkt1307

Hindawi Publishing Corporation
International Journal of Cell Biology
Volume 2013, Article ID 151839, 15 pages
http://dx.doi.org/10.1155/2013/151839

Review Article

Phosphorylation-Mediated Regulation of Alternative Splicing in Cancer

Chiara Naro[1,2] and Claudio Sette[1,2]

[1] *Department of Biomedicine and Prevention, University of Rome "Tor Vergata", 00133 Rome, Italy*
[2] *Laboratories of Neuroembryology and of Cellular and Molecular Neurobiology, Fondazione Santa Lucia IRCCS, 00143 Rome, Italy*

Correspondence should be addressed to Claudio Sette; claudio.sette@uniroma2.it

Received 31 May 2013; Accepted 26 July 2013

Academic Editor: Michael Ladomery

Alternative splicing (AS) is one of the key processes involved in the regulation of gene expression in eukaryotic cells. AS catalyzes the removal of intronic sequences and the joining of selected exons, thus ensuring the correct processing of the primary transcript into the mature mRNA. The combinatorial nature of AS allows a great expansion of the genome coding potential, as multiple splice-variants encoding for different proteins may arise from a single gene. Splicing is mediated by a large macromolecular complex, the spliceosome, whose activity needs a fine regulation exerted by *cis*-acting RNA sequence elements and *trans*-acting RNA binding proteins (RBP). The activity of both core spliceosomal components and accessory splicing factors is modulated by their reversible phosphorylation. The kinases and phosphatases involved in these posttranslational modifications significantly contribute to AS regulation and to its integration in the complex regulative network that controls gene expression in eukaryotic cells. Herein, we will review the major canonical and noncanonical splicing factor kinases and phosphatases, focusing on those whose activity has been implicated in the aberrant splicing events that characterize neoplastic transformation.

1. Introduction

In eukaryotic cells, the expression of each gene is finely tuned by a complex network of regulative processes affecting all steps of transcript maturation, from nuclear transcription to cytosolic export and utilization of the mRNA. A crucial step in this regulative network is represented by pre-mRNA splicing, the molecular process that mediates the removal of intronic sequences and the joining of exons. What makes splicing an outstanding player in controlling gene expression is its flexibility, which allows a remarkable increase of the coding potential of the genome through alternative selection of exons. Indeed, alternative splicing (AS) allows each gene to encode for several coding and noncoding mRNA variants, which often display different activities and/or patterns of expression. AS is, therefore, one of the principal mechanisms underlying the well-known discrepancy between increasing organismal complexity and content of genes contained in the genome [1]. In line with its central contribution to genome complexity, it is estimated that up to 90% of human multiexon genes undergo AS [2], and the importance of this regulative mechanism for both developmentally regulated and pathological cellular processes is now well recognized (reviewed in [3]).

The splicing process is carried out by the spliceosome, a complex macromolecular machinery composed of five small nuclear ribonucleoprotein particles (U1, U2, U4, U5, and U6 snRNPs) and more than 200 auxiliary proteins. The spliceosome mediates the recognition of the short consensus sequences surrounding the 5′-(GU) and the 3′-(AG) splice site and catalyzes the two transesterification reactions necessary for the removal of the intron and ligation of the selected exons (reviewed in [4]). Due to the degenerate nature of the sequence elements recognized by the spliceosome, its recruitment to the maturing pre-mRNA requires the action of both *cis*-acting RNA sequence elements and *trans*-acting RNA binding proteins (RBPs), such as Ser/Arg (SR) rich proteins, heterogeneous nuclear ribonucleoproteins (hnRNPs), and splicing factors belonging to other RBP families. In addition, AS is also regulated by mechanisms acting both co- and posttranscriptionally, through epigenetic modifications of the chromatin, regulation of the RNA polymerase II (RNAPII)

transcription rate, and posttranslational modifications of both spliceosome components and auxiliary splicing factors, among which reversible phosphorylation acts as a major player.

2. Impact of Phosphorylation on the Catalysis of Splicing

A proper regulation of the phosphorylation status of the spliceosomal proteins and of accessory splicing factors is crucial for the correct regulation of both constitutive and AS events. Early studies already described the importance of a correct balance between phosphorylation and dephosphorylation events in the splicing process by showing that both activation [5] and inhibition [6] of the PP1 and PP2A phosphatases are required for splicing catalysis. Several reports have then further highlighted the importance of regulated phosphorylation events for the correct assembly and catalytic activation of spliceosomal components, such as PRP28 [7], PRP6, or PRP31 [8]. Equally, dephosphorylation events, such as those regarding the U5 and U2 snRNP component, U5-156 kDa and SAP155 [9, 10], were shown to be essential for spliceosome activity, proving the importance of subsequent rounds of phosphorylation and dephosphorylation events in the regulation of the splicing process.

Regulative phosphorylation and dephosphorylation events concern not only the spliceosomal components but also some accessory RBPs that cooperate with the spliceosome in the selection of splice sites. For example, phosphorylation of the splicing factors SF1 and SRSF1 (prototypic SR protein previously known as ASF/SF2) modulates their interaction with U2AF65 and U1snRNP, respectively, thus modulating spliceosome assembly [11, 12]. The dynamic phosphorylation/dephosphorylation of SR proteins is particularly relevant for the regulation of their functions, as both hypo- and hyperphosphorylation can inhibit splicing [13]. For instance, phosphorylation of SRSF1 promotes spliceosome assembly, whereas its dephosphorylation is necessary for the catalysis of the first transesterification reaction [14].

3. Phosphorylation and Splicing Factors

SR proteins are a family of nuclear RBPs involved in the regulation of both constitutive and AS, whose activity is greatly modulated by reversible phosphorylation. Their structure is generally characterized by two N-terminal RNA recognition motif (RRM) a C-terminal region enriched in Arg-Ser residues (RS domain), which are the main targets of regulative phosphorylation. Phosphorylation of the RS domain of SR proteins has a great impact on their functionality, as it may affect their binding to target mRNAs, their interaction with other proteins and their intracellular localization. As an example, binding of SRSF5 (previously reported as SRp40) to its high-affinity RNA-binding site is strictly dependent on the phosphorylation of its RS domain [15]. One of the most significant examples of how phosphorylation may affect the splicing activity of SR proteins is represented by SRSF10 (previously known as SRp38). This SR protein acts as a specific splicing activator in its RS-phosphorylated form [16], whereas dephosphorylation converts it into a potent splicing repressor [17]. Notably, dephosphorylation of SRSF10 occurs during the M phase of the cell cycle [17] or under stress condition [18], when general inhibition of splicing occurs. In particular, it was demonstrated that, under normal conditions, phosphorylated SRSF10 is a sequence-specific splicing activator, which promotes U1 and U2 snRNP assembly on target pre-mRNAs endowed with SRSF10-dependent exonic splicing enhancer (ESE) sequences [16]. Conversely, under stressful cellular conditions, as during heat shock, SRSF10 is rapidly dephosphorylated by PP1, while other SR proteins are maintained in phosphorylated state by SR protein kinases (SRPKs) [19]. Interestingly, during the stress response all SR proteins are similarly dephosphorylated by PP1. However, they are rapidly rephosphorylated by SRPKs, while SRSF10, which is a poor substrate for SRPKs, remains dephosphorylated. Under this condition, SRSF10 can still interact with the U1 snRNP, but in this case the interaction impairs its ability to recognize the 5′ splice site, thus resulting in splicing inhibition [18].

Phosphorylation of the RS domain can also dictate SR protein subcellular localization, by affecting both their intranuclear localization and their nucleocytoplasmic shuttling. In interphase cells, SR proteins are enriched in interchromatin granules called nuclear speckles, which are enriched in factors involved in pre-mRNA processing and RNA transport (reviewed in [20]). The recruitment of SR proteins to nascent pre-mRNAs from these sites of storage is regulated by their phosphorylation (**Figure 1**); indeed, it has been shown that phosphorylation of the RS domain is a prerequisite for their recruitment to transcription sites *in vivo* [21]. This modification plays also an important role in the regulation of nucleocytoplasmic shuttling of SR proteins. For instance, phosphorylation of SR proteins in the cytoplasm is required for their nuclear import [22]. On the other hand, dephosphorylation of the RS domain is essential for their translocation to the cytoplasm during mRNP maturation (**Figure 1**) [23]. Interestingly, dephosphorylation of SRSF1 and SRSF7 (previously known as 9G8) enhances their interaction with the export receptor TAP, thereby favoring also the export of their target mRNAs [24, 25]. Furthermore, SRSF1 translational activity is increased by dephosphorylation of its RS domain (**Figure 1**) [26]. Phosphorylation has therefore a great impact also on the splicing unrelated functions in which many SR proteins are involved (reviewed in [27]).

Ser/Thr phosphorylation represents an important regulative process not only for SR proteins but also for hnRNPs and other splicing factors. For instance, hnRNP A1 is phosphorylated by the mitogen-activated protein kinase (MAPK) p38 in response to stress conditions (**Figure 2**), thus causing its cytoplasmic translocation and consequent modulation of hnRNP A1-sensitive AS events [28, 29]. Similarly, phosphorylation of the SR-like protein TRA2-β by CLK2 induces its relocalization into the cytoplasm, thus reducing its ability to bind its own mRNA and regulate its splicing [30]. For other splicing factors, instead, Ser/Thr phosphorylation affects the splicing activity by modulating their interaction with other proteins.

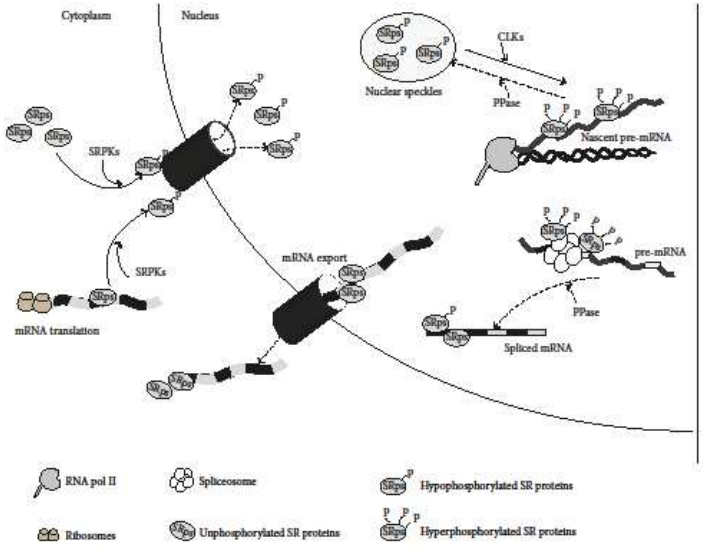


FIGURE 1: Phosphorylation-mediated regulation of SR proteins activity. Reversible phosphorylation of their RS domain profoundly affect SR protein (SRps) activity and subcellular localization. Newly synthesized SRps need SRPK-mediated phosphorylation in order to enter the nucleus and assemble in nuclear speckles. CLKs mediate SRps hyperphosphorylation and induce their release from nuclear speckles and their recruitment to transcription sites. Dephosphorylation of SRps is successively required for proper splicing catalysis. Moreover, dephosphorylated SRps facilitate export of spliced mRNA in the cytosol, where they enhance protein translation.

For instance, phosphorylation of hnRNP L reduces its interaction with the U2AF65 subunit of the U2 auxiliary factor [31, 32]. The function of some splicing factors can be influenced also by Tyr phosphorylation. A well-documented example in this sense is SAM68, a member of the signal transduction and activation of RNA (STAR) family of RBPs (reviewed in [33]). Tyr phosphorylation by SRC-family kinases (SFKs) caused the accumulation of SAM68 in nuclear granules, named SAM68 nuclear bodies (SNBs) [34, 35]. Moreover, it was shown that this posttranslational modification negatively affected the interaction of SAM68 with hnRNP A1 and with the *BCL-X* pre-mRNA, thus impairing its ability to promote splicing of this target gene [35]. On the other hand, Ser/Thr phosphorylation of SAM68 by the MAPKs ERK1/2 was reported to increase splicing of the variable exons in the *CD44* gene [36, 37]. Notably, SAM68 represents an interesting example of how Ser/Thr and Tyr-phosphorylation may have opposite impact on the splicing activity of an RBP toward a target pre-mRNA (Figure 2). This was formally shown by studying its effect on the *CCND1* gene. Increased expression of SAM68 promotes splicing of the cyclin D1b variant of the *CCND1* gene in prostate cancer cells. This activity is further enhanced by activation of the RAS/ERK pathway and counteracted by SFKs [38]. In both cases, the effect was due to modulation of the affinity for RNA, as ERK-dependent phosphorylation increased binding of SAM68 to intron 4, whereas SFK-dependent phosphorylation abolished it (Figure 2) [38]. Thus, activation of signaling pathways can indirectly modulate AS events through posttranslational modification of selected splicing factors (see also later).

4. Splicing Factor Kinases

Phosphorylation of spliceosomal components and splicing factors is mediated by numerous protein kinases. Some of these kinases, such as the SRPK and CLK families, are

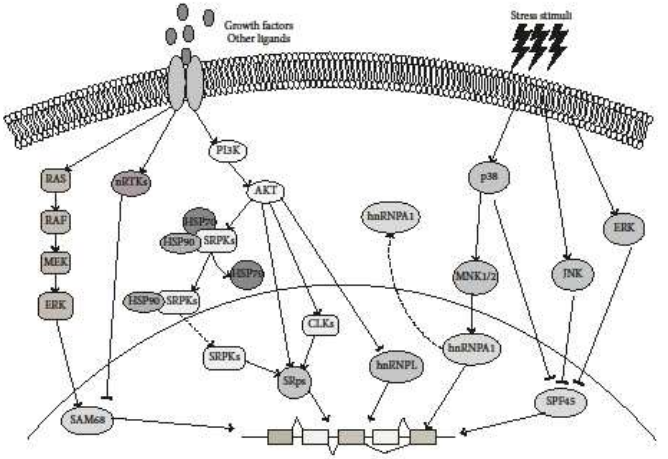


FIGURE 2: Signaling-activated kinases regulate splicing factor activity. Various extracellular cues, like growth factors or stress stimuli, activate different signal-transduction cascades impinging on protein kinases that in turn phosphorylate RBPs, thereby modulating their splicing activity. SAM68 splicing activity is inversely regulated by ERKs and nRTKs, which, respectively, activate and inhibit its splicing activity. The PI3 K-AKT pathway regulates the activity of several SR proteins both directly or by phosphorylating and modulating the activity and localization of CLKs and SRPKs. Stress signal-activated kinases, like JNK or p38, can both modulate splicing factor localization, like for hnRNPA1, or activity, like for SPF45 (see text for details).

specifically devoted to this function, whereas others also participate to signal transduction pathways or phosphorylate distinct primary substrates in addition to the splicing factors. Herein, we will review the kinases whose ability to influence splicing decisions has been better characterized. For convenience, we will classify them as SR-protein specific kinases; signaling-activated splicing kinases, and "atypical" splicing factor kinases.

5. SR-Protein Specific Kinases

5.1. SR-Protein Kinases (SRPKs). The first SR protein kinase identified was SRPK1, which was isolated from mitotic cells, and it was described to phosphorylate SR proteins and to promote their release from nuclear speckles during the G2/M phase of the cell cycle [39]. However, SRPK1 is present and active also in interphase cells. SRPK1 is the prototype of the SRPK family, which also includes the two homologous SRPK2 and SRPK3 proteins. SRPKs are characterized by a bipartite catalytic domain separated by a unique spacer sequence (reviewed in [40]) and are mainly localized in the cytoplasm of mammalian cells. This is due to the presence of a strong cytoplasmic retention signal localized in the spacer domain [41] and of their interaction with the molecular chaperones HSP70 and HSP90, which in complex seem to favor the folding of SRPKs into an active state [42]. However, SRPKs can translocate into the nucleus of cells under several conditions, such as during the G2/M phase of the cell cycle [39], or after osmotic stress [42], or as a consequence of activation of the epidermal growth factor (EGF) signal transduction pathway [43]. Due to this dual localization, SRPKs can phosphorylate SR proteins both in the nucleus and in the cytoplasm, thus affecting several aspects of their function. SRPK-mediated phosphorylation of SR proteins in the cytoplasm is necessary to ensure SR proteins nuclear import (**Figure 1**) [44], as it enhances their interaction with the specific transportin SR2 [22, 45]. SRPK nuclear activity promotes release of SR proteins in the nucleoplasm from the nuclear speckles [46]. For instance, several reports suggest that SRPK-mediated phosphorylation of SRSF1 is essential for its nuclear localization and the resulting splicing activity triggered by stimulation of specific signaling pathways (i.e., IGF-1 and EGF treatments) [43, 47]. However, under conditions that strongly increase nuclear localization of SRPKs, such as under cellular stress, they can also induce nuclear speckles enlargement [42, 48]. Indeed,

Zhong and colleagues showed that osmotic stress induced by sorbitol treatment can lead to a massive nuclear translocation of SRPK1, which causes hyperphosphorylation of SR proteins and inhibits their splicing activity toward the reporter *EIA* minigene [42]. These studies indicated that SRPK-mediated phosphorylation of SR proteins can finely tune their splicing activity in response to external and internal cues.

5.2. Cyclin-Dependent Like Kinases (CLKs). The cyclin-dependent like kinases (CLK1-4) represent the other prototypical family of SR protein kinases. They are characterized by a C-terminal kinase domain, with dual specificity, and an N-terminal RS domain, which allows their interaction with the SR proteins. CLKs colocalize with SR proteins in nuclear speckles, and their overexpression leads to hyperphosphorylation of SR proteins and induces speckles disassembly [49]. Several studies reported the ability of CLKs to influence splicing events by regulating the subnuclear localization of SR proteins (Figure 1). In particular, the release of SR proteins from nuclear speckles induced by CLKs overexpression has been reported to modulate splicing of the *EIA* reporter minigene [50] and of the exon 10 of the *TAU* gene [51], whose aberrant regulation has been implicated in several neurodegenerative diseases. Recently it has been shown that CLKs also modulate the activity of splicing factors not related to the SR-protein family, such as SPF45. CLK-mediated phosphorylation of SPF45 interferes with its proteasomal degradation and enhances exon 6 inclusion of *FAS* by promoting binding of this splicing factor to the *FAS* pre-mRNA [52]. The nuclear localization of CLKs is one of the major differences between them and SRPKs, which are instead mainly cytosolic. Because of their different localization, CLKs and SRPKs can cooperate in regulating SR proteins subcellular localization. Indeed, it has been shown that SRPK1 interacts with SRSF1 and phosphorylates the N-terminal part (RS1) of its RS domain, a posttranslational modification that is essential for its assembly into nuclear speckles, whereas CLKs phosphorylate the C-terminal part (RS2) of its RS domain, thereby causing release of SRSF1 from the speckles [53]. Moreover, SRPKs and CLKs have also distinct substrate specificity, as SRPKs preferentially phosphorylate Ser-Arg sites, while CLKs have a broader specificity and can phosphorylate also Ser-Lys or Ser-Pro sites [54]. Therefore, even if apparently redundant, the coordinated activity of SRPKs and CLKs is crucial for correct splicing regulation. This was well illustrated by Nowak and colleagues, whose work highlighted how SR-proteins phosphorylation induced by these two families of kinases may differently control a single splicing event [55]. The vascular endothelial growth factor A (*VEGFA*) gene, a key regulator of angiogenesis, produces several isoforms by alternative splice-site selection in the terminal exon 8: proximal splice-site selection results in proangiogenic *VEGFxxx* isoforms, whereas distal splice-site selection results in antiangiogenic isoforms *VEGFxxxb*. Different growth factors inversely influence these splicing events by inducing in both cases phosphorylation of SR proteins. However, IGF-1 and TNF-α induced production of *VEGFxxx* through activation of SRPKs, whereas TGF-β1 enhanced *VEGFxxxb* production by activating CLKs [55].

6. Signaling-Activated Splicing Factor Kinases

AS represents a crucial step in the regulation of gene expression in eukaryotic cells. Therefore, its regulation needs to be finely integrated in the complex network of regulative mechanisms that allows the cell to modulate gene expression in response to the different physiological and pathological stimuli that are received from both the internal and external environment. In support of this notion, activation of signal transduction pathways has been shown to modulate AS in a large number of situations. However, while in some cases the mechanism(s) has been described, in other cases the transacting factors mediating the response are unknown. Here we will review signaling-activated kinases that can modulate AS by directly phosphorylating splicing factors or their regulators, such as the SRPKs or CLKs.

6.1. AKT. The Ser/Thr kinase AKT, also known as PKB, is the hinge molecule of the phosphoinositide-3-kinase-protein kinase (PI3 K) signaling pathway, which transduces the signal of several growth factors and cytokines. Through the phosphorylation of its many nuclear and cytosolic targets, AKT can regulate a multitude of cellular processes, such as cell metabolism, proliferation, and survival (reviewed in [56]). To exert its multiple functions, AKT regulates different steps of the gene expression network, from transcription, to AS and translation. Indeed, several reports have highlighted the ability of AKT to directly and indirectly modulate the function of many RBPs. AKT phosphorylates both hnRNPs and SR proteins, which contain within their RS domain multiple AKT phosphorylation consensus sequences: RXRXX(S/T) [57]. By modulating their phosphorylation status, AKT regulates both splicing and splicing independent functions of hnRNPs and SR proteins. For example, AS of a four-exon cassette in the *CASPASE-9* gene allows expression of a proapoptotic splice variant (exon inclusion, *CASPASE-9a*) or an antiapoptotic splice variant, (exon skipped, *CASPASE-9b*). AKT-dependent phosphorylation of hnRNP L increases its affinity for exon 3 and induces expression of the antiapoptotic variant. Indeed, AKT-phosphorylated hnRNP L competes with hnRNP U for the binding to the mRNA and impairs its ability to promote the pro-apoptotic *CASPASE-9a* [58] (Figure 2). This is a clear example of how AKT may promote cell survival by regulating a key AS event. On the other hand, phosphorylation of hnRNP A1 by AKT has no effects on splicing, but it modulates the translational activity of this RBP. Following phosphorylation, hnRNP A1 loses its ability to promote IRES dependent translation of the *CCND1* and the *c-MYC* mRNAs [59]. In the case of SR proteins, AKT was shown to modulate both splicing and translational activity through phosphorylation. Growth factor-induced phosphorylation of SRSF1 and SRSF7 by AKT enhanced their ability to promote the inclusion of the *EDA* exon in the fibronectin mRNA and translation of the spliced mRNA [60]. One of the most characterized AS events regulated by AKT is affecting *PKCβ* pre-mRNA after insulin stimulation. This hormone induces splicing of the *PKCβ II* isoform, which enhances insulin-stimulated glucose transport better than the *PKCβ I* variant, even if they differ only for two residues in their C-terminus

[61]. Insulin stimulation induces PI3K-dependent activation of AKT, which phosphorylates SRSF5 [62, 63], thus promoting *PKCβ II* splicing. Furthermore, AKT phosphorylates CLK1 and enhances its activity (Figure 2). In turn, CLK1 phosphorylates SRSF4 (previously named SRp75) and SRSF6 (previously named SRp55), thus contributing to the *PKCβ II* splicing regulation [64]. Recently, it has been suggested that AKT and CLK may also regulate SRSF5 splicing activity by affecting its nuclear localization, which was impaired when a CLK mutant that cannot be phosphorylated by AKT is expressed [65]. Importantly, this concerted regulation of SRSF5-dependent *PKCβ II* splicing by AKT and CLK was essential for adypogenetic differentiation, thus providing physiological relevance for this signaling route.

AKT was also shown to regulate the activity of SRPKs. A recent work documented that EGF signaling induces a massive reprogramming of AS that depends on AKT-induced nuclear translocation of SRPKs [43]. In fact, AKT binding to SRPKs induces their autophosphorylation and dissociation from the HSP70 chaperone, which normally holds SRPKs into the cytoplasm, thus favoring their nuclear translocation guided by HSP90 (Figure 2). Once in the nucleus, SRPKs can phosphorylate SR proteins and modulate the splicing pattern of several genes. Thus, given its ability to modulate the activity of both regulators (SRPKs and CLKs) and effectors (SR proteins and hnRNPs) of AS, AKT stands up as a crucial player in the modulation of splicing in response to external cues, and this activity might represent a primary function of AKT in the regulation of multiple cellular processes.

6.2. Mitogen-Activated Protein Kinases (MAPKs). MAPKs are a family of Ser/Thr kinases that transduce external signals into the cell and regulate many different cellular processes, such as metabolism, proliferation, survival, differentiation, and motility (reviewed in [66]). The MAPK family includes the extracellular regulated kinases (ERK 1/2), the c-Jun amino terminal kinases (JNK 1–3), p38 (α, β, γ, and δ), and ERK5 family. The role of MAPKs in these cellular processes is mediated by regulation of protein activity and stability and by modulation of gene expression, which also occurs through AS. The first evidence of MAPK-modulated splicing came from studies on the regulation of AS of the CD44 gene, which encodes for the extracellular receptor for hyaluronic acid, a key component of the extracellular matrix. The *CD44* gene is characterized by a block of variable exons (v2–v10) embedded between ten constant exons; the inclusion of the variable exons into the mature transcript modulates CD44 protein interaction with its substrate, thus significantly affecting cell adhesion, migration, and proliferation (reviewed in [67]). The inclusion of the variable exon v5 in the mature mRNA of *CD44* upon T-cell activation is dependent by the RAS-RAF-MEK-ERK signaling cascade [68]. The target of this pathway is SAM68, whose ability to promote exon v5 inclusion is increased by ERKs-mediated phosphorylation [36]. SAM68 interacts with the splicing factor U2AF65, and this interaction seems to enhance the recognition of the 3′ splice-site. Phosphorylation by ERKs reduces the affinity of the SAM68/U2AF65 complex to the *CD44* pre-mRNA, probably favoring the subsequent recruitment of other spliceosomal components [69]. SAM68 is not the only RBP regulated by ERK1/2. Furthermore, other MAPKs, like p38 or JNKs, are known to phosphorylate and modulate the activity of splicing factors. For example, it has been recently demonstrated that phosphorylation of the splicing factor SPF45 can be mediated by all the three families of MAPKs in response to different stimuli (e.g., oxidative stress activates ERK1/2 and JNK mediated phosphorylation, whereas UV-light induces p38 and JNK activity) [70] (Figure 2). These kinases phosphorylate SPF45 on two residues, Thr 71 and Ser 222; these posttranslational modifications inhibit SPF45-dependent exon 6 inclusion in the *FAS* gene, thus leading to the production of a dominant negative isoform of this death receptor [70].

Modulation of hnRNP A1 activity by p38 is another well characterized regulative phosphorylation event operated by a MAPK. Environmental stresses, such as osmotic stress or UV irradiation, induce p38 activation and phosphorylation of hnRNP A1, leading to the relocalization of this nuclear RBP into the cytoplasm, where it concentrates into discrete phase-dense particles, called stress granules (SGs) [29] (Figure 2). The nuclear exclusion of hnRNP A1 is the result of its reduced interaction with the transportin Trn1, which under normal conditions mediates its nuclear translocation [71], and leads to consequent modulation of hnRNP A1-dependent splicing events, which were tested using the *E1A* minigene reporter [28]. HnRNP A1 phosphorylation is mediated by the p38 effectors MAP kinase signal-integrating kinases (MNK1/2) [28], which can also regulate the translational activity of this splicing factor. It was observed that the increase in TNF-α protein production following T-cell activation relies on MNK-mediated phosphorylation of hnRNP A1. However, in this cellular context, phosphorylation of hnRNP A1 does not affect its localization, but it rather lowers its affinity for the AU-rich element (ARE) in the 3′UTR of the *TNF-α* mRNA, thus probably relieving a translation repressive control and allowing enhanced TNF-α production [72].

Thus, MAPKs can regulate different steps of mRNA processing through phosphorylation of several splicing factors, integrating in this way this complex regulative step of gene expression with the response of the cell to external cues.

6.3. Tyrosine Kinases. Protein tyrosine kinases (PTKs) catalyze the transfer of a phosphate group from ATP to a tyrosine residue of their target proteins. PTKs may be classified in two different classes: the transmembrane receptors tyrosine kinases (RTKs) and the nonreceptor tyrosine kinases (nRTKs). PTKs mediate the phosphorylation of several proteins in response to both internal and external cues, leading to the modification of their activity or affecting their interaction with other proteins. Transduction pathways triggered by PTK activation affect gene expression, also at the level of AS, even though only a small number of splicing factors have been shown to be regulated by Tyr-phosphorylation. Among these few RBPs, the members of the STAR proteins family, and in particular SAM68, stand out (Figure 2). In many STAR family members, the RNA binding domain is flanked by regulatory regions, like proline-rich or tyrosine-rich sequences, which mediate their interaction with the Src Homology 3 (SH3)

and SH2 domains of other proteins, including PTKs. For instance, the breast tumor kinase (BRK) is a nRTKs that interacts in the nucleus with a proline rich region of SAM68 through its SH3 domain. BRK-dependent phosphorylation of SAM68 reduces its RNA binding affinity [73]. Analogously, BRK phosphorylates also the SAM68 homologous proteins SLM-1 and SLM-2, reducing their affinity to the RNA also in this case [74]. SAM68 is also substrate of FYN, another soluble nRTKs. In this case, it was also shown that Tyr-phosphorylation interfered with SAM68-dependent splicing of the *BCL-X* and *CCND1* genes [35, 38]. FYN-dependent phosphorylation reduced the affinity of SAM68 for these target RNAs and affected its interaction with different proteins, such as hnRNP A1, thereby affecting the outcome of AS events [35, 38]. Tyr-phosphorylation also influences the splicing activity of the nuclear RBP YT521-B, which can also be elicited by several nRTKs such as FYN, SRC, or c-ABL [75, 76]. This posttranslational modification induces translocation of YT521-B from the nuclear YT bodies, where it normally resides, to the nucleoplasm. Phosphorylated YT521-B shows reduced ability to modulate splice-site selection of different targets, in association with a reduced binding to their mRNA, possibly because the nucleoplasmic translocation distances YT521-B from the effective sites of pre-mRNA processing [76].

6.4. cAMP-Dependent Protein Kinase (PKA). Increased intracellular levels of the second messenger cyclic adenosine 3′,5′-monophosphate (cAMP) lead to the activation of the cAMP-dependent protein kinase (PKA), which transduces the signals of many hormones, growth factors, and neurotransmitters [77]. PKA is a tetrameric protein, composed of two regulatory subunits (R) and two catalytic subunits (C): binding of cAMP to the R subunits leads to their dissociation from the C subunit and activation of the kinase [77]. Activated PKA phosphorylates several effectors, including transcription factors, ion channels, and metabolic enzymes, thus influencing multiple cellular functions. PKA activity is also regulated by interaction of the R subunits with the PKA-anchoring proteins (AKAPs); AKAPs maintain PKA in specific subcellular compartments and in proximity of its substrates, thus retaining PKA activity where it is needed. The first evidence of a possible involvement of PKA in the regulation of AS came from the observation that a fraction of the C subunit translocates into the nucleus, colocalizes with SRSF2 (previously reported as SC35) in splicing speckles, and phosphorylates several SR proteins, at least *in vitro* [78]. Localization of the C subunit in nuclear speckles seems to be related to its interaction with the C-subunit binding protein HA95 [78] and to the SR protein SRSF17A, which was shown to be a novel AKAP required to anchor PKA C subunit in splicing speckles [79]. Importantly, modulation of *EIA* reporter minigene splicing by SRSF17A is dependent on its interaction with PKA [79]. Moreover, nuclear PKA itself is able to modulate AS of the *EIA* reporter minigene, even in the absence of the cAMP stimulation [78].

Several stimuli that increase the cAMP intracellular levels were shown to affect AS events through phosphorylation of both SR proteins and hnRNPs by PKA. For example, it was demonstrated that forskolin, which stimulates the synthesis of cAMP, modulates AS of exon 10 of the *TAU* gene [80, 81]. Notably, activated PKA affects the activity of two SR proteins, SRSF1 and SRSF7, which inversely modulate exon 10 splicing: SRSF1 promotes exon 10 inclusion, whereas SRSF7 prevents it. However, PKA-dependent phosphorylation of SRSF1 enhances its activity [80] whereas it inhibits SRSF7 [81], thus globally favoring exon 10 inclusion.

PKA is also able to modulate AS of genes that are crucial for neuronal differentiation, through the phosphorylation of hnRNP K. After phosphorylation by PKA, hnRNP K shows higher binding activity to its target mRNAs with respect to its competitor U2AF65; this mechanism impairs the recognition of the 3′ splice site and leads to the skipping of its target exons [82]. On a broader view, hnRNP K target motifs are found in many genes involved in neuronal differentiation and in neurological diseases [82]. These pieces of evidence suggest that PKA mediated regulation of hnRNPs and SR proteins activity may be an important player in the complex network of regulative mechanisms that finely control AS events during neuronal development (reviewed in [83]). Although cAMP and PKA are usually involved in cell differentiation, their contribution to cancer has also been demonstrated. It will be interesting to investigate whether PKA-dependent modulation of AS also occurs in genes with relevance to human cancer.

7. Other Kinases

In this section, we will describe the regulative activity of some proteins that showed an unexpected kinase activity towards splicing factors, so that they cannot be included in any of the classes described previously. Some of these kinases were known to have other specific substrates different from splicing factors, for others, instead, the kinase activity was totally unpredicted.

7.1. DNA Topoisomerase I. The first of these atypical kinases to be described was the DNA topoisomerase I, whose best known function is to relieve both positive and negative DNA supercoils ensuring correct DNA topology during transcription, DNA replication and repair (reviewed in [84]). Despite the absence of a canonical ATP binding site, DNA topoisomerase I was shown to phosphorylate SR proteins, in particular the prototypic SRSF1, within their RS domain [85]. This phosphorylation event can significantly affect SR proteins modulatory activity on AS events. Indeed, it has been demonstrated that cells deficient for this enzyme show a general status of hypophosphorylation for the SR proteins, which correlates with an impaired regulation of several AS events, whereas constitutive splicing results unaffected [86]. Moreover, treatment with a selective inhibitor of the kinase activity of DNA topoisomerase results in reduced phosphorylation levels for SR proteins, which in turn leads to a defective spliceosome assembly and alterations in the splicing pattern of several genes [87]. As it is now well established that pre-mRNA splicing occurs cotranscriptionally, it has been suggested that this double activity of DNA topoisomerase I

could be one of the mechanisms ensuring the correct coordination between DNA transcription and splicing [88]. Indeed, DNA topoisomerase I activity is fundamental to solve DNA supercoils generated by RNA pol II progression along the DNA template and might simultaneously ensure a regulated splicing factor activity through their phosphorylation.

7.2. Dual-Specificity Tyrosine-(Y)-Phosphorylation Regulated Kinase 1A (DIRK1A). Another protein kinase able to modulate the splicing activity of SR proteins is DIRK1A (dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 1A). This dual-specificity protein kinase autophosphorylates on Tyr, Ser, and Thr residues but phosphorylates substrates only on Ser or Thr residues (reviewed in [89]). The human DYRK1A gene maps to chromosome 21, and it is ubiquitously expressed in adult and fetal tissues, with high levels of expression in the brain. DYRK1A is supposed to play a major role during neuronal development, through its interaction with several cytoskeletal, synaptic, and nuclear proteins (reviewed in [89]). Several SR proteins were shown to interact with DYRK1A. Indeed, DYRK1A has been reported to colocalize with SRSF2 in nuclear speckles, and its overexpression induces the disassembly of this subnuclear structures [90]. Alteration of subcellular localization of the SR proteins phosphorylated by DYRK1A seems to be the main mechanism by which this kinase regulates the splicing activity of its target factors. For instance, phosphorylation of SRSF1 and SRSF7 by DYRK1A induces their cytoplasmic translocation [91, 92], whereas phosphorylation of SRSF2 and SRSF6 causes their dissociation from nuclear speckles [93, 94]. For each of these splicing factors the mislocalization induced by DYRK1A impaired their ability to modulate the inclusion of exon 10 of the *TAU* gene, thus shifting the splicing balance toward the exclusion of this exon.

7.3. Fas-Activated Serine/Threonine Kinase (FAST). Fas-activated serine/threonine kinase (FAST) is a constitutively phosphorylated Ser/Thr kinase, which undergoes rapid dephosphorylation after the binding of Fas ligand to its receptor Fas, an interaction that triggers T-cell apoptosis. It was known that dephosphorylated FAST was able to interact with and phosphorylate the RBP TIA1 [95], but the functional relevance of this interaction in the regulation of the splicing process remained unknown for a long time. It was later discovered that phosphorylation of TIA1 by FAST regulates its ability to promote the inclusion of exon 6 of the *FAS* gene [96]. Phosphorylated TIA1 enhances U1 snRNP recruitment to *FAS* pre-mRNA, thus favoring the recognition of this variable exon. Inclusion of exon 6 into the *FAS* mRNA favors the production of a proapoptotic isoform of this gene, suggesting that FAST and TIA1 take part to a positive regulative circuitry that enhances Fas-dependent apoptosis once activated. Furthermore FAST is also endowed with an intrinsic splicing activity, independent from TIA1 [97]. It was observed that FAST can modulate the splicing of the *FGFR2* reporter gene in the same direction of TIA1, favoring the inclusion of exon III b but independently from this RBP. Thus, FAST can directly and indirectly affect splicing, and it would be interesting to determine how many targets are influenced by this kinase in T cells.

7.4. Aurora Kinase A (AURKA). AURKA was identified in a high-throughput siRNA screening for factors involved in the regulation of AS of two apoptotic genes: *BCL-X* and *MCL1* [98]. Among several regulators identified by the screen, authors noticed a peculiar enrichment for proteins involved in the regulation of the cell cycle. They focused their study on AURKA, a kinase involved in the regulation of centrosomal splitting that is frequently upregulated in cancers, where it is supposed to promote aneuploidy [99]. AURKA was demonstrated to positively regulate splicing of the antiapoptotic variant *BCL-X_L* through stabilization of SRSF1. Cells depleted of AURKA showed reduced levels of SRSF1, which then resulted in increased levels of the *BCL-X_S* pro-apoptotic variant. Moreover, since AURKA is activated at the G2/M phase of the cell cycle, the authors suggested that this kinase links *BCL-X* splicing regulation to cell cycle progression. These observations suggest that, in addition to the effects on centrosome duplication, upregulation of AURKA can favor neoplastic transformation also by promoting antiapoptotic splice variants.

8. Splicing Factor Kinases in Cancer and Other Human Diseases

Due to the important role played by the AS process in the control of gene expression, any alterations of its regulation can profoundly modify important cellular processes, thus resulting in a potential cause of disease (reviewed in [100]). Altered expression, activity, or subcellular localization of splicing factor kinases can be among the causes of the aberrant splicing events associated to several diseases, particularly neurodegenerative pathologies and cancer.

Aberrant inclusion of exon 10 of the *TAU* gene is a well-known example of pathogenetic splicing event, caused by the deregulated activity or expression of splicing factor kinases. TAU protein is a microtubule associated protein, which controls assembly and stability of microtubules. Exon 10 of the *TAU* gene encodes for one of the four microtubule binding domain repeats (R) of the TAU protein and regulates its affinity for microtubules and, consequently, its ability to induce their polymerization. Alternative inclusion of exon 10 leads to the production of either 4R-tau (inclusion) or 3R-tau (exclusion), and equal levels of these two isoforms seem to be essential for normal function of the human brain. Alteration of the normal ratio 1:1 between the 4R and the 3R isoform, in both directions, has been observed in several cases of Alzheimer's disease (AD); moreover, nearly half of the mutations in the *TAU* gene associated with FTDP-17 (frontotemporal dementia with parkinsonism linked to chromosome 17) affects exon 10 splicing, both inhibiting or promoting its inclusion, strongly suggesting that a proper regulation of this splicing event is essential for the maintenance of the healthy balance between 4R and 3R isoforms (reviewed in [101]). Several reports have highlighted or suggested a strong correlation between aberrant splicing of

the exon 10 of the *TAU* gene in tauopathies and deregulated activity of the kinases regulating this splicing event. Stamm's group, for example, observed an increased production of an inactive isoform of the CLK2 kinase in the brain of AD patients, which correlated with increased inclusion of *TAU* exon 10. This observation suggested that the CLK2-dependent phosphorylation of SR proteins and the SR-like protein TRA2-β is required for the correct regulation of this splicing event [102]. Another kinase supposed to be involved in the altered regulation of *TAU* splicing is PKA, which also promotes the inclusion of the exon 10 of this gene through the phosphorylation of different SR proteins [80]. As reduced levels of PKA-Cα have been observed in AD brains [103], it has been speculated that the lack of its activity may participate in the alteration of the normal balance between the 3R and 4R splice variants of *TAU* [81]. As described in previous section, the kinase DYRK1A exerts an important regulation on the AS of the *TAU* gene, thus strongly suggesting that its increased dosage due to the trisomy of chromosome 21 could be the main cause of the early onset of tauopathies in patients with Down syndrome [103]. Modulation of *TAU* gene splicing is a very attractive potential therapeutic target for treatment of tauopathies (reviewed in [104]); since protein kinases regulate this splicing event and are involved in tauopathy pathogenesis, targeting the activity of these kinases should be certainly considered in the development of future approaches for the treatment of these pathologies.

Upregulation and/or misregulated activity of splicing kinases are often associated to cancer development. This has been widely reported for SRPK1, which is overexpressed in several cancer types, such as pancreatic carcinomas [105], breast and colon carcinomas [106], and lung cancer [107]. Moreover, increased SRPK1 levels positively correlate with tumor grade [106] and are associated with higher resistance to chemotherapeutic treatments [105, 108]. Through modulation of selective splicing events, SRPK1 may allow cancer cells to enhance their proliferative, invasive, and angiogenetic potential. For example, in pancreatic, breast, and colon cancer cells SRPK1 promotes the generation of specific splice variants of the *MAP2K2* gene, which sustained higher activity of the MAPK pathway [106]. Recently, SRSF1 mediated splicing of the *MNK2b* isoform of the *MKNK2* gene has been correlated with resistance to gemcitabine treatment in pancreatic cancer cells [109]; since SRPK1 is upregulated in this cancer type and promotes cell survival, it would be interesting to evaluate whether this kinase contributes to the SRSF1-induced prosurvival pathway. A similar regulation has been described in Wilms Tumor, wherein SRPK1 promotes the production of the proangiogenic isoform *VEGF165* of the *VEGFA* gene through the phosphorylation and nuclear translocation of SRSF1 [48]. In these nephroblastomas tumors SRPK1 transcriptional upregulation is driven by the mutated transcription factor WT1, and its splicing activity is fundamental for the high levels of vascularization required by these tumors [48]. Importantly, the physiological relevance of SRPK1 for angiogenesis has been demonstrated, as injection of an SRPK1/2 inhibitor reduced it in a mouse model of retinal neovascularization, suggesting that targeting AS through their upstream regulator could be a potential tool to target pathological angiogenesis in cancer [48].

Several signal transduction kinases, whose activity is often deregulated by neoplastic transformation, exert their oncogenic activity in part through the aberrant regulation of splicing events. For instance the MAPK pathway, which is frequently hyperactivated in tumors, can promote the acquisition of an invasive and migratory phenotype by modulating the AS pattern of the cell adhesion molecule *CD44*. In fact, it has been shown that hepatocyte growth factor (HGF) can induce cell migration of cancer cells by promoting this splicing event, as a consequence of induced ERK1/2-mediated phosphorylation of SAM68, induced by the MET receptor signaling pathway [110]. Also epithelial-to-mesenchymal transition (EMT), which is crucial for the invasiveness of cancer cells, is regulated by AS events that are sensitive to activation of the MAPK pathway. Indeed, production of the constitutively active Δ*RON* splice variant of the *RON* oncogene, the extracellular receptor for HGF, leads to EMT in colorectal cancer cells [111]. This splicing event is promoted by the upregulation of SRSF1. Remarkably, under conditions that favor EMT, epithelial cells release soluble factors that activate the ERK1/2 pathway. This in turn causes phosphorylation and activation of SAM68, which causes retention of a cryptic intron in the 3′UTR of the SRSF1 mRNA, reducing the amount of the nonsense-mediated-decay (NMD) targeted splice variant and enhancing expression of SRSF1 [112]. Thus, activation of the ERK1/2 pathway triggers a cascade of splicing events that culminate in a cellular response favoring cancer cell invasion.

Activation of the AKT pathway has also been suggested to promote cancer cell survival through the regulation of specific splicing events. For example, it has been observed that hyperactivation of AKT through the RAS signaling pathway is implicated in the production of pro-survival splice variants of the *KLF-6* and *CASPASE-9* genes in nonsmall-cell lung cancer and hepatocellular carcinoma, respectively, [113, 114]. In both cases, AKT induces SRSF1 phosphorylation, enhancing its ability to promote *KLF-6SV1* and *CASPASE-9b* isoforms. These observations strongly suggest a primary role for this splicing regulatory activity in the oncogenic potential of AKT.

9. Protein Phosphatase Regulating Splicing Factors

In the previous paragraphs, we have broadly described the importance of a proper balance between phosphorylation and dephosphorylation events in the regulation of the pre-mRNA splicing process. Therefore, even if this review focuses primarily on the activity of the numerous kinases involved in this regulation, a brief description of the protein phosphatases counteracting their activity is also required for a comprehensive overview of the phosphorylative regulation of splicing.

PP1 and PP2A were the first Ser/Thr phosphatases whose activity was demonstrated to be necessary for splicing catalysis [5, 6]. PP1 and PP2A are required for the later steps of the splicing reaction, in particular for the

second transesterification reaction, whose accomplishment is favored by dephosphorylation of U5 (U5-156 kDa) and U2 (SAP155) snRNP components [9]. In particular, PP1-mediated dephosphorylation of SAP155 is favored by the nuclear inhibitor of PP1 (NIPP1). NIPP1 is a nuclear regulatory subunit of PP1, enriched in nuclear speckles [115], known to interact with several splicing regulators, like CDC5L or the same SAP155 [116, 117]. In particular, NIPP1 stimulates PP1-mediated dephosphorylation of SAP155 by facilitating the interaction between the phosphatase and its substrate [10]. In subsequent studies, PP2Cγ was also demonstrated to be important for the splicing process, as it was shown to be physically associated with the spliceosome, and its enzymatic activity was necessary for the early steps of spliceosome assembly [118].

Ser/Thr phosphatases are important regulators of both constitutive and AS events, as it was suggested by pioneering studies showing alternative 5′ splice selection after addition of PP1 in splicing assay *in vitro* [119]. Furthermore, PP2Cγ was shown to interact with the RBP YB-1 and to modulate AS of the *CD44* gene [120], while PP1 was demonstrated to interact with a short motif RVXF motif within the RRM of several splicing factors, like SRSF1, SRSF9 (previously known as SRp30C), and the SR-like protein TRA2-β [121]. Dephosphorylation of TRA2-β by PP1 positively modulates its dimerization and its interaction with partner proteins, like SRSF1. Moreover, PP1 regulates alternative splice selection in TRA2-β target mRNAs like the *SMN2* gene [121]. Exclusion of the exon 7 of *SMN2* gene, combined with the primary deletion of *SMN1* gene, is the cause of the spinal muscular atrophy (SMA) [122]. TRA2-β promotes the inclusion of the exon 7 of *SMN2* favoring the production of a functional full length protein. TRA2-β splicing activity is enhanced by inhibition of PP1 activity [121] and, surprisingly, by activation of PP2A [123]. Indeed, the Stamm's group found that a class of compounds derivative from cantharidin (a well-known phosphatase inhibitors) activates PP2A, which in turn dephosphorylates TRA2-β on Thr33, favoring inclusion of exon 7 [123]. These observations suggest the possibility to develop new protein phosphatase inhibitors that could be used for the therapeutic correction of the splicing defects occurring in neurodegenerative diseases like SMA.

Modulating protein phosphatases' activity in order to manipulate pathogenetic splicing events has been suggested as a potential therapeutic tool also for cancer treatment. Indeed, it has been shown that genotoxic agents inducing apoptosis in cancer cells act through the generation of ceramide and activation of PP1, which in turn promotes the formation of the proapoptotic *BCL-X$_S$* and *CASPASE-9b* splice variants [124]. On the other hand, it has been shown that the proapoptotic activity of synthetic ceramides, like C6 pyridinium ceramide, is instead associated with activation of PP1 and the consequent reduced phosphorylation of several splicing factors and modulation of several splicing events [125]. These observations underline the importance of the regulated activity of protein phosphatases for proper regulation of the splicing process and strongly suggest the possibility to develop new molecules targeting their activity, which could be used for the therapeutic correction of the splicing defects occurring in several human diseases.

10. Concluding Remarks

Increasing evidence points out to a key role of misregulation of AS in the cellular transformation process. Cancer-specific splice variants can potentially be used as accurate diagnostic and prognostic markers, as it was recently highlighted by genome-wide studies [126, 127]. Targeting the splicing process represents, therefore, an attractive therapeutic target for cancer treatment, and it is currently under intense investigation. Therapeutic modulation of AS is mainly realized through RNA-based technologies (reviewed in [128]) or through chemical reagents inhibiting spliceosome activity (reviewed in [129]). The RNA-based technologies exploit antisense oligonucleotide masking specific sequence elements to splicing factors and/or the spliceosome [130], whereas chemical approaches make use of drugs that directly target the activity of spliceosomal components, as for examples spliceostatin A, which inhibits the SF3b subunit of the U2 snRNP, thereby modulating the AS of genes important for cell cycle control [131].

Considering the important control exerted by protein kinases on AS, modulation of their activity represents a potential approach for the development of new drugs targeting RNA splicing in cancer therapy. These suggestions are supported by recent reports highlighting the high efficacy of SRPK1/2 inhibitors in reducing angiogenesis through the negative modulation of the AS of the proangiogenic splice variant *VEGFxxx* gene [48]. Considering the great impact that SRPKs have on the splicing activity of SR proteins and the large number of AS events that they regulate, modulation of SRPK activity could be a powerful tool in the emerging field of splicing-modulating therapies. It is also important to mention that SRSF1, a well-known target of SRPKs, is upregulated in human cancers and functions as an oncogene [132]. For the same reasons, CLKs are a fascinating chemotherapeutic target too, and important efforts are being made for the realization of selective and efficient CLKs inhibitors [133].

Signal-transduction pathways able to modulate the phosphorylation status of SR proteins or the activity of other RBPs represent another potential druggable target for RNA splicing modulation. For example, it has been recently shown that amiloride, a well-known diuretic, can reduce proliferative and invasive properties of both hepatocellular carcinoma and leukemia cancer cells by inducing hypophosphorylation of SR-proteins [134, 135]. Genome-wide exon array analysis has demonstrated that amiloride treatment induces the modulation of a large number of AS events, and, in particular, it negatively regulates protumoral splice variants of several genes, such as the antiapoptotic *BCL-X$_L$* or proinvasive *ΔRON*. Reduced phosphorylation levels of AKT and ERKs were observed after amiloride treatment, suggesting that this drug reduces SR protein phosphorylation through inactivation of these kinases [135].

Deregulation of signal-transduction pathways in cancer cells is a general feature, and much effort has been made in order to develop chemotherapeutic agents that efficiently

inhibit the activity of the kinases mediating the intracellular transduction of these signals, such as AKT or kinases of the MAPK and SRC families (reviewed in [136–138]). As many of these inhibitors are already in clinical practice, and many of them are undergoing promising clinical trials, it would be very interesting to understand whether their antiproliferative and cytotoxic effects could be partly due to their ability to interfere with AS events regulated by these kinases. Even more attractive is the possibility to exploit protein kinase inhibitors to selectively affect splicing decisions in order to restore in cancer cells a normal, nonpathological AS pattern.

Shedding light on the expression, structure, and functions of the kinases regulating the activity of splicing factors is therefore an important step for a comprehensive understanding of the molecular mechanisms regulating pre-mRNA processing, which is essential for the rational design of future therapies targeting the aberrant AS process in cancer and other human diseases.

Conflict of Interests

The authors declare no conflict of interests.

Acknowledgments

The work in the laboratory of C. Sette was supported by Association for International Cancer Research (AICR Grant no. 12-0150), the Associazione Italiana Ricerca sul Cancro (AIRC), and Istituto Superiore della Sanità (ISS Grant Convenzione 11US/6A).

References

[1] N. L. Barbosa-Morais, M. Irimia, Q. Pan et al., "The evolutionary landscape of alternative splicing in vertebrate species," *Science*, vol. 338, no. 6114, pp. 1587–1593, 2012.

[2] M. Sultan, M. H. Schulz, H. Richard et al., "A global view of gene activity and alternative splicing by deep sequencing of the human transcriptome," *Science*, vol. 321, no. 5891, pp. 956–960, 2008.

[3] M. Irimia and B. J. Blencowe, "Alternative splicing: decoding an expansive regulatory layer," *Current Opinion in Cell Biology*, vol. 24, no. 3, pp. 323–332, 2012.

[4] C. L. Will and R. Lührmann, "Spliceosome structure and function," *Cold Spring Harbor Perspectives in Biology*, vol. 3, no. 7, 2011.

[5] J. E. Mermoud, P. Cohen, and A. I. Lamond, "Ser/Thr-specific protein phosphatases are required for both catalytic steps of pre-mRNA splicing," *Nucleic Acids Research*, vol. 20, no. 20, pp. 5263–5269, 1992.

[6] J. E. Mermoud, P. T. W. Cohen, and A. I. Lamond, "Regulation of mammalian spliceosome assembly by a protein phosphorylation mechanism," *EMBO Journal*, vol. 13, no. 23, pp. 5679–5688, 1994.

[7] R. Mathew, K. Hartmuth, S. Möhlmann, H. Urlaub, R. Ficner, and R. Lührmann, "Phosphorylation of human PRP28 by SRPK2 is required for integration of the U4/U6-U5 tri-snRNP into the spliceosome," *Nature Structural and Molecular Biology*, vol. 15, no. 5, pp. 435–443, 2008.

[8] M. Schneider, H.-H. Hsiao, C. L. Will, R. Giet, H. Urlaub, and R. Lührmann, "Human PRP4 kinase is required for stable tri-snRNP association during spliceosomal B complex formation," *Nature Structural and Molecular Biology*, vol. 17, no. 2, pp. 216–221, 2010.

[9] Y. Shi, B. Reddy, and J. L. Manley, "PP1/PP2A phosphatases are required for the second step of Pre-mRNA splicing and target specific snRNP proteins," *Molecular Cell*, vol. 23, no. 6, pp. 819–829, 2006.

[10] N. Tanuma, S.-E. Kim, M. Beullens et al., "Nuclear inhibitor of protein phosphatase-1 (NIPP1) directs protein phosphatase-1 (PP1) to dephosphorylate the U2 small nuclear ribonucleoprotein particle (snRNP) component, spliceosome-associated protein 155 (Sap155)," *Journal of Biological Chemistry*, vol. 283, no. 51, pp. 35805–35814, 2008.

[11] V. Manceau, M. Swenson, J.-P. Le Caer, A. Sobel, C. L. Kielkopf, and A. Maucuer, "Major phosphorylation of SF1 on adjacent Ser-Pro motifs enhances interaction with U2AF65," *FEBS Journal*, vol. 273, no. 3, pp. 577–587, 2006.

[12] S. Cho, A. Hoang, R. Sinha et al., "Interaction between the RNA binding domains of Ser-Arg splicing factor 1 and U1-70K snRNP protein determines early spliceosome assembly," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 108, no. 20, pp. 8233–8238, 2011.

[13] J. Prasad, K. Colwill, T. Pawson, and J. L. Manley, "The protein kinase Clk/Sty directly modulates SR protein activity: both hyper- and hypophosphorylation inhibit splicing," *Molecular and Cellular Biology*, vol. 19, no. 10, pp. 6991–7000, 1999.

[14] W. Cao, S. F. Jamison, and M. A. Garcia-Blanco, "Both phosphorylation and dephosphorylation of ASF/SF2 are required for pre-mRNA splicing in vitro," *RNA*, vol. 3, no. 12, pp. 1456–1467, 1997.

[15] R. Tacke, Y. Chen, and J. L. Manley, "Sequence-specific RNA binding by an SR protein requires RS domain phosphorylation: creation of an SRp40-specific splicing enhancer," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 94, no. 4, pp. 1148–1153, 1997.

[16] Y. Feng, M. Chen, and J. L. Manley, "Phosphorylation switches the general splicing repressor SRp38 to a sequence-specific activator," *Nature Structural and Molecular Biology*, vol. 15, no. 10, pp. 1040–1048, 2008.

[17] C. Shin and J. L. Manley, "The SR protein SRp38 represses splicing in M phase cells," *Cell*, vol. 111, no. 3, pp. 407–417, 2002.

[18] C. Shin, Y. Feng, and J. L. Manley, "Dephosphorylated SRp38 acts as a splicing repressor in response to heat shock," *Nature*, vol. 427, no. 6974, pp. 553–558, 2004.

[19] Y. Shi and J. L. Manley, "A complex signaling pathway regulates SRp38 phosphorylation and pre-mRNA splicing in response to heat shock," *Molecular Cell*, vol. 28, no. 1, pp. 79–90, 2007.

[20] D. L. Spector and A. I. Lamond, "Nuclear speckles," *Cold Spring Harbor Perspectives in Biology*, vol. 3, no. 2, 2011.

[21] T. Misteli, J. F. Cáceres, J. Q. Clement, A. R. Krainer, M. F. Wilkinson, and D. L. Spector, "Serine phosphorylation of SR proteins is required for their recruitment to sites of transcription in vivo," *Journal of Cell Biology*, vol. 143, no. 2, pp. 297–307, 1998.

[22] M.-C. Lai, R.-I. Lin, and W.-Y. Tarn, "Transportin-SR2 mediates nuclear import of phosphorylated SR proteins," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 98, no. 18, pp. 10154–10159, 2001.

[23] S. Lin, R. Xiao, P. Sun, X. Xu, and X.-D. Fu, "Dephosphorylation-dependent sorting of SR splicing factors

during mRNP maturation," *Molecular Cell*, vol. 20, no. 3, pp. 413–425, 2005.

[24] Y. Huang, T. A. Yario, and J. A. Steitz, "A molecular link between SR protein dephosphorylation and mRNA export," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 101, no. 26, pp. 9666–9670, 2004.

[25] M.-C. Lai and W.-Y. Tarn, "Hypophosphorylated ASF/SF2 binds TAP and is present in messenger ribonucleoproteins," *Journal of Biological Chemistry*, vol. 279, no. 30, pp. 31745–31749, 2004.

[26] J. R. Sanford, J. D. Ellis, D. Cazalla, and J. F. Cáceres, "Reversible phosphorylation differentially affects nuclear and cytoplasmic functions of splicing factor 2/alternative splicing factor," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 42, pp. 15042–15047, 2005.

[27] X.-Y. Zhong, P. Wang, J. Han, M. G. Rosenfeld, and X.-D. Fu, "SR proteins in vertical integration of gene expression from transcription to RNA processing to translation," *Molecular Cell*, vol. 35, no. 1, pp. 1–10, 2009.

[28] W. V. D. H. Van Oordt, M. T. Díaz-Meco, J. Lozano, A. R. Krainer, J. Moscat, and J. F. Cáceres, "The MKK(3/6)-p38-signaling cascade alters the subcellular distribution of hnRNP A1 and modulates alternative splicing regulation," *Journal of Cell Biology*, vol. 149, no. 2, pp. 307–316, 2000.

[29] S. Guil, J. C. Long, and J. F. Cáceres, "hnRNP A1 relocalization to the stress granules reflects a role in the stress response," *Molecular and Cellular Biology*, vol. 26, no. 15, pp. 5744–5758, 2006.

[30] P. Stoilov, R. Daoud, O. Nayler, and S. Stamm, "Human tra2-beta1 autoregulates its protein concentration by influencing alternative splicing of its pre-mRNA," *Human Molecular Genetics*, vol. 13, no. 5, pp. 509–524, 2004.

[31] R. W. Goehe, J. C. Shultz, C. Murudkar et al., "hnRNP L regulates the tumorigenic capacity of lung cancer xenografts in mice via caspase-9 pre-mRNA processing," *Journal of Clinical Investigation*, vol. 120, no. 11, pp. 3923–3939, 2010.

[32] G. Liu, A. Razanau, Y. Hai et al., "A conserved serine of heterogeneous nuclear ribonucleoprotein L (hnRNP L) mediates depolarization-regulated alternative splicing of potassium channels," *The Journal of Biological Chemistry*, vol. 287, no. 27, pp. 22709–22716, 2012.

[33] C. Sette, "Post-translational regulation of star proteins and effects on their biological functions," *Advances in Experimental Medicine and Biology*, vol. 693, pp. 54–66, 2010.

[34] K. E. Lukong, D. Larocque, A. L. Tyner, and S. Richard, "Tyrosine phosphorylation of Sam68 by breast tumor kinase regulates intranuclear localization and cell cycle progression," *Journal of Biological Chemistry*, vol. 280, no. 46, pp. 38639–38647, 2005.

[35] M. P. Paronetto, T. Achsel, A. Massiello, C. E. Chalfant, and C. Sette, "The RNA-binding protein Sam68 modulates the alternative splicing of Bcl-x," *Journal of Cell Biology*, vol. 176, no. 7, pp. 929–939, 2007.

[36] N. Matter, P. Herrlich, and H. König, "Signal-dependent regulation of splicing via phosphorylation of Sam68," *Nature*, vol. 420, no. 6916, pp. 691–695, 2002.

[37] C. Cheng and P. A. Sharp, "Regulation of CD44 alternative splicing by SRm160 and its potential role in tumor cell invasion," *Molecular and Cellular Biology*, vol. 26, no. 1, pp. 362–370, 2006.

[38] M. P. Paronetto, M. Cappellari, R. Busà et al., "Alternative splicing of the cyclin D1 proto-oncogene is regulated by the RNA-binding protein Sam68," *Cancer Research*, vol. 70, no. 1, pp. 229–239, 2010.

[39] J.-F. Gui, W. S. Lane, and X.-D. Fu, "A serine kinase regulates intracellular localization of splicing factors in the cell cycle," *Nature*, vol. 369, no. 6482, pp. 678–682, 1994.

[40] G. Ghosh and J. A. Adams, "Phosphorylation mechanism and structure of serine-arginine protein kinases," *FEBS Journal*, vol. 278, no. 4, pp. 587–597, 2011.

[41] J.-H. Ding, X.-Y. Zhong, J. C. Hagopian et al., "Regulated cellular partitioning of SR protein-specific kinases in mammalian cells," *Molecular Biology of the Cell*, vol. 17, no. 2, pp. 876–885, 2006.

[42] X.-Y. Zhong, J.-H. Ding, J. A. Adams, G. Ghosh, and X.-D. Fu, "Regulation of SR protein phosphorylation and alternative splicing by modulating kinetic interactions of SRPK1 with molecular chaperones," *Genes and Development*, vol. 23, no. 4, pp. 482–495, 2009.

[43] Z. Zhou, J. Qiu, W. Liu et al., "The Akt-SRPK-SR axis constitutes a major pathway in transducing EGF signaling to regulate alternative splicing in the nucleus," *Molecular Cell*, vol. 47, no. 3, pp. 422–433, 2012.

[44] C. Y. Yun and X.-D. Fu, "Conserved SR protein kinase functions in nuclear import and its action is counteracted by arginine methylation in Saccharomyces cerevisiae," *Journal of Cell Biology*, vol. 150, no. 4, pp. 707–717, 2000.

[45] C. Y. Yun, A. L. Velazquez-Dones, S. K. Lyman, and X.-D. Fu, "Phosphorylation-dependent and -independent nuclear import of RS domain-containing splicing factors and regulators," *Journal of Biological Chemistry*, vol. 278, no. 20, pp. 18050–18055, 2003.

[46] H.-Y. Wang, W. Lin, J. A. Dyck et al., "SRPK2: a differentially expressed SR protein-specific kinase involved in mediating the interaction and localization of pre-mRNA splicing factors in mammalian cells," *Journal of Cell Biology*, vol. 140, no. 4, pp. 737–750, 1998.

[47] D. G. Nowak, E. M. Amin, E. S. Rennel et al., "Regulation of Vascular Endothelial Growth Factor (VEGF) splicing from pro-angiogenic to anti-angiogenic isoforms: a novel therapeutic strategy for angiogenesis," *Journal of Biological Chemistry*, vol. 285, no. 8, pp. 5532–5540, 2010.

[48] E. M. Amin, S. Oltean, J. Hua et al., "WT1 mutants reveal SRPK1 to be a downstream angiogenesis target by altering VEGF splicing," *Cancer Cell*, vol. 20, no. 6, pp. 768–780, 2011.

[49] K. Colwill, T. Pawson, B. Andrews et al., "The Clk/Sty protein kinase phosphorylates SR splicing factors and regulates their intranuclear distribution," *EMBO Journal*, vol. 15, no. 2, pp. 265–275, 1996.

[50] P. I. Duncan, D. F. Stojdl, R. M. Marius, and J. C. Bell, "In vivo regulation of alternative pre-mRNA splicing by the Clk1 protein kinase," *Molecular and Cellular Biology*, vol. 17, no. 10, pp. 5996–6001, 1997.

[51] A. M. Hartmann, D. Rujescu, T. Giannakouros et al., "Regulation of alternative splicing of human tau exon 10 by phosphorylation of splicing factors," *Molecular and Cellular Neuroscience*, vol. 18, no. 1, pp. 80–90, 2001.

[52] Y. Liu, L. Conaway, J. Rutherford Bethard et al., "Phosphorylation of the alternative mRNA splicing factor 45 (SPF45) by Clk1 regulates its splice site utilization, cell migration and invasion," *Nucleic Acids Research*, vol. 41, no. 9, pp. 4949–4962, 2013.

[53] J. C. K. Ngo, S. Chakrabarti, J.-H. Ding et al., "Interplay between SRPK and Clk/Sty kinases in phosphorylation of the splicing factor ASF/SF2 is regulated by a docking motif in ASF/SF2," *Molecular Cell*, vol. 20, no. 1, pp. 77–89, 2005.

[54] K. Colwill, L. L. Feng, J. M. Yeakley et al., "SRPK1 and Clk/Sty protein kinases show distinct substrate specificities for serine/arginine-rich splicing factors," *Journal of Biological Chemistry*, vol. 271, no. 40, pp. 24569–24575, 1996.

[55] D. G. Nowak, J. Woolard, E. M. Amin et al., "Expression of pro- and anti-angiogenic isoforms of VEGF is differentially regulated by splicing and growth factors," *Journal of Cell Science*, vol. 121, no. 20, pp. 3487–3495, 2008.

[56] T. F. Franke, "PI3K/Akt: getting it right matters," *Oncogene*, vol. 27, no. 50, pp. 6473–6488, 2008.

[57] T. Obata, M. B. Yaffe, G. G. Leparc et al., "Peptide and protein library screening defines optimal substrate motifs for AKT/PKB," *Journal of Biological Chemistry*, vol. 275, no. 46, pp. 36108–36115, 2000.

[58] N. T. Vu, M. A. Park, J. C. Shultz et al., "hnRNP U enhances caspase-9 splicing and is modulated by AKT-dependent phosphorylation of hnRNP L," *The Journal of Biological Chemistry*, vol. 288, no. 12, pp. 8575–8584, 2013.

[59] O. D. Jo, J. Martin, A. Bernath, J. Masri, A. Lichtenstein, and J. Gera, "Heterogeneous nuclear ribonucleoprotein A1 regulates cyclin D1 and c-myc internal ribosome entry site function through Akt signaling," *Journal of Biological Chemistry*, vol. 283, no. 34, pp. 23274–23287, 2008.

[60] M. Blaustein, F. Pelisch, T. Tanos et al., "Concerted regulation of nuclear and cytoplasmic activities of SR proteins by AKT," *Nature Structural and Molecular Biology*, vol. 12, no. 12, pp. 1037–1044, 2005.

[61] C. E. Chalfant, H. Mischak, J. E. Watson et al., "Regulation of alternative splicing of protein kinase Cβ by insulin," *Journal of Biological Chemistry*, vol. 270, no. 22, pp. 13326–13332, 1995.

[62] N. A. Patel, C. E. Chalfant, J. E. Watson et al., "Insulin regulates alternative splicing of protein kinase C beta II through a phosphatidylinositol 3-kinase-dependent pathway involving the nuclear serine/arginine-rich splicing factor, SRp40, in skeletal muscle cells," *Journal of Biological Chemistry*, vol. 276, no. 25, pp. 22648–22654, 2001.

[63] N. A. Patel, S. Kaneko, H. S. Apostolatos et al., "Molecular and genetic studies imply Akt-mediated signaling promotes protein kinase CβII alternative splicing via phosphorylation of serine/arginine-rich splicing factor SRp40," *Journal of Biological Chemistry*, vol. 280, no. 14, pp. 14302–14309, 2005.

[64] K. Jiang, N. A. Patel, J. E. Watson et al., "Akt2 regulation of Cdc2-like kinases (Clk/Sty), serine/arginine-rich (SR) protein phosphorylation, and insulin-induced alternative splicing of PKCβII messenger ribonucleic acid," *Endocrinology*, vol. 150, no. 5, pp. 2087–2097, 2009.

[65] P. Li, G. Carter, J. Romero et al., "Clk/STY (cdc2-like kinase 1) and Akt regulate alternative splicing and adipogenesis in 3T3-L1 pre-adipocytes," *PLoS ONE*, vol. 8, no. 1, Article ID e53268, 2013.

[66] M. Cargnello and P. P. Roux, "Activation and function of the MAPKs and their substrates, the MAPK-activated protein kinases," *Microbiology and Molecular Biology Reviews*, vol. 75, no. 1, pp. 50–83, 2011.

[67] H. Ponta, L. Sherman, and P. A. Herrlich, "CD44: from adhesion molecules to signalling regulators," *Nature Reviews Molecular Cell Biology*, vol. 4, no. 1, pp. 33–45, 2003.

[68] S. Weg-Remers, H. Ponta, P. Herrlich, and H. König, "Regulation of alternative pre-mRNA splicing by the ERK MAP-kinase pathway," *EMBO Journal*, vol. 20, no. 15, pp. 4194–4203, 2001.

[69] A. Tisserant and H. König, "Signal-regulated pre-mRNA occupancy by the general splicing factor U2AF," *PLoS ONE*, vol. 3, no. 1, Article ID e1418, 2008.

[70] A. M. Al-Ayoubi, H. Zheng, Y. Liu, T. Bai, and S. T. Eblen, "Mitogen-activated protein kinase phosphorylation of splicing factor 45 (SPF45) regulates SPF45 alternative splicing site utilization, proliferation, and cell adhesion," *Molecular and Cellular Biology*, vol. 32, no. 14, pp. 2880–2893, 2012.

[71] E. Allemand, S. Guil, M. Myers, J. Moscat, J. F. Cáceres, and A. R. Krainer, "Regulation of heterogenous nuclear ribonucleoprotein A1 transport by phosphorylation in cells stressed by osmotic shock," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 10, pp. 3605–3610, 2005.

[72] M. Buxadé, J. L. Parra, S. Rousseau et al., "The Mnks are novel components in the control of TNFα biosynthesis and phosphorylate and regulate hnRNP A1," *Immunity*, vol. 23, no. 2, pp. 177–189, 2005.

[73] J. J. Derry, S. Richard, H. V. Carvajal et al., "Sik (BRK) phosphorylates sam68 in the nucleus and negatively regulates Its RNA binding ability," *Molecular and Cellular Biology*, vol. 20, no. 16, pp. 6114–6126, 2000.

[74] A. Haegebarth, D. Heap, W. Bie, J. J. Derry, S. Richard, and A. L. Tyner, "The nuclear tyrosine kinase BRK/Sik phosphorylates and inhibits the RNA-binding activities of the Sam68-like mammalian proteins SLM-1 and SLM-2," *Journal of Biological Chemistry*, vol. 279, no. 52, pp. 54398–54404, 2004.

[75] A. M. Hartmann, O. Nayler, F. W. Schwaiger, A. Obermeier, and S. Stamm, "The interaction and colocalization of Sam68 with the splicing-associated factor YT521-B in nuclear dots is regulated by the Src family kinase p59(fyn)," *Molecular Biology of the Cell*, vol. 10, no. 11, pp. 3909–3926, 1999.

[76] I. Rafalska, Z. Zhang, N. Benderska et al., "The intranuclear localization and function of YT521-B is regulated by tyrosine phosphorylation," *Human Molecular Genetics*, vol. 13, no. 15, pp. 1535–1549, 2004.

[77] P. Sassone-Corsi, "The cyclic AMP pathway," *Cold Spring Harbor Perspectives in Biology*, vol. 4, no. 12, 2012.

[78] A.-K. Kvissel, S. Ørstavik, S. Eikvar et al., "Involvement of the catalytic subunit of protein kinase A and of HA95 in pre-mRNA splicing," *Experimental Cell Research*, vol. 313, no. 13, pp. 2795–2809, 2007.

[79] E. Jarnæss, A. J. Stokka, A.-K. Kvissel et al., "Splicing factor arginine/serine-rich 17A (SFRS17A) is an A-kinase anchoring protein that targets protein kinase A to splicing factor compartments," *Journal of Biological Chemistry*, vol. 284, no. 50, pp. 35154–35164, 2009.

[80] J. Shi, W. Qian, X. Yin et al., "Cyclic AMP-dependent protein kinase regulates the alternative splicing of tau exon 10: a mechanism involved in tau pathology of Alzheimer disease," *Journal of Biological Chemistry*, vol. 286, no. 16, pp. 14639–14648, 2011.

[81] J. Gu, J. Shi, S. Wu et al., "Cyclic AMP-dependent protein kinase regulates 9G8-mediated alternative splicing of tau exon 10," *FEBS Letters*, vol. 586, no. 16, pp. 2239–2244, 2012.

[82] W. Cao, A. Razanau, D. Feng, V. G. Lobo, and J. Xie, "Control of alternative splicing by forskolin through hnRNP K during neuronal differentiation," *Nucleic Acids Research*, vol. 40, no. 16, pp. 8059–8071, 2012.

[83] S. Zheng and D. L. Black, "Alternative pre-mRNA splicing in neurons: growing up and extending its reach," *Trends in Genetics*, vol. 29, no. 8, pp. 442–448, 2013.

[84] J. C. Wang, "Cellular roles of DNA topoisomerases: a molecular perspective," *Nature Reviews Molecular Cell Biology*, vol. 3, no. 6, pp. 430–440, 2002.

[85] E. Labourier, F. Rossi, I.-E. Gallouzi, E. Allemand, G. Divita, and J. Tazi, "Interaction between the N-terminal domain of human DNA topoisomerase I and the arginine-serine domain of its substrate determines phosphorylation of SF2/ASF splicing factor," *Nucleic Acids Research*, vol. 26, no. 12, pp. 2955–2962, 1998.

[86] J. Soret, M. Gabut, C. Dupon et al., "Altered serine/arginine-rich protein phosphorylation and exonic enhancer-dependent splicing in Mammalian cells lacking topoisomerase I," *Cancer Research*, vol. 63, no. 23, pp. 8203–8211, 2003.

[87] B. Pilch, E. Allemand, M. Facompré et al., "Specific inhibition of serine- and arginine-rich splicing factors phosphorylation, spliceosome assembly, and splicing by the antitumor drug NB-506," *Cancer Research*, vol. 61, no. 18, pp. 6876–6884, 2001.

[88] M. Malanga, A. Czubaty, A. Girstun, K. Staron, and F. R. Althaus, "Poly(ADP-ribose) binds to the splicing factor ASF/SF2 and regulates its phosphorylation by DNA topoisomerase I," *Journal of Biological Chemistry*, vol. 283, no. 29, pp. 19991–19998, 2008.

[89] F. J. Tejedor and B. Hämmerle, "MNB/DYRK1A as a multiple regulator of neuronal development," *FEBS Journal*, vol. 278, no. 2, pp. 223–235, 2011.

[90] M. Álvarez, X. Estivill, and S. de la Luna, "DYRK1A accumulates in splicing speckles through a novel targeting signal and induces speckle disassembly," *Journal of Cell Science*, vol. 116, no. 15, pp. 3099–3107, 2003.

[91] J. Shi, T. Zhang, C. Zhou et al., "Increased dosage of Dyrk1A alters alternative splicing factor (ASF)-regulated alternative splicing of tau in down syndrome," *Journal of Biological Chemistry*, vol. 283, no. 42, pp. 28660–28669, 2008.

[92] S. Ding, J. Shi, W. Qian et al., "Regulation of alternative splicing of tau exon 10 by 9G8 and Dyrk1A," *Neurobiology of Aging*, 2011.

[93] W. Qian, H. Liang, J. Shi et al., "Regulation of the alternative splicing of tau exon 10 by SC35 and Dyrk1A," *Nucleic Acids Research*, vol. 39, no. 14, pp. 6161–6171, 2011.

[94] X. Yin, N. Jin, J. Gu et al., "Dual-specificity tyrosine phosphorylation-regulated kinase 1A (Dyrk1A) modulates serine/arginine-rich protein 55 (SRp55)-promoted Tau exon 10 inclusion," *The Journal of Biological Chemistry*, vol. 287, no. 36, pp. 30497–30506, 2012.

[95] Q. Tian, J.-L. Taupin, S. Elledge, M. Robertson, and P. Anderson, "Fas-activated serine/threonine kinase (FAST) phosphorylates TIA-1 during Fas-mediated apoptosis," *Journal of Experimental Medicine*, vol. 182, no. 3, pp. 865–874, 1995.

[96] J. M. Izquierdo and J. Valcárcel, "Fas-activated Serine/Threonine Kinase (FAST K) synergizes with TIA-1/TIAR proteins to regulate Fas alternative splicing," *Journal of Biological Chemistry*, vol. 282, no. 3, pp. 1539–1543, 2007.

[97] M. Simarro, D. Mauger, K. Rhee et al., "Fas-activated serine/threonine phosphoprotein (FAST) is a regulator of alternative splicing," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 104, no. 27, pp. 11370–11375, 2007.

[98] M. J. Moore, Q. Wang, C. J. Kennedy, and P. A. Silver, "An alternative splicing network links cell-cycle control to apoptosis," *Cell*, vol. 142, no. 4, pp. 625–636, 2010.

[99] R. Reiter, P. Gais, U. Jutting et al., "Aurora kinase A messenger RNA overexpression is correlated with tumor progression and shortened survival in head and neck squamous cell carcinoma," *Clinical Cancer Research*, vol. 12, no. 17, pp. 5136–5141, 2006.

[100] T. A. Cooper, L. Wan, and G. Dreyfuss, "RNA and disease," *Cell*, vol. 136, no. 4, pp. 777–793, 2009.

[101] A. Andreadis, "Tau splicing and the intricacies of dementia," *Journal of Cellular Physiology*, vol. 227, no. 3, pp. 1220–1225, 2012.

[102] D. C. Glatz, D. Rujescu, Y. Tang et al., "The alternative splicing of tau exon 10 and its regulatory proteins CLK2 and TRA2-BETA1 changes in sporadic Alzheimer's disease," *Journal of Neurochemistry*, vol. 96, no. 3, pp. 635–644, 2006.

[103] Z. Liang, F. Liu, I. Grundke-Iqbal, K. Iqbal, and C.-X. Gong, "Down-regulation of cAMP-dependent protein kinase by over-activated calpain in Alzheimer disease brain," *Journal of Neurochemistry*, vol. 103, no. 6, pp. 2462–2470, 2007.

[104] J. Zhou, Q. Yu, and T. Zou, "Alternative splicing of exon 10 in the tau gene as a target for treatment of tauopathies," *BMC Neuroscience*, vol. 9, no. 2, article S10, 2008.

[105] G. M. Hayes, P. E. Carrigan, A. M. Beck, and L. J. Miller, "Targeting the RNA splicing machinery as a novel treatment strategy for pancreatic carcinoma," *Cancer Research*, vol. 66, no. 7, pp. 3819–3827, 2006.

[106] G. M. Hayes, P. E. Carrigan, and L. J. Miller, "Serine-arginine protein kinase 1 overexpression is associated with tumorigenic imbalance in mitogen-activated protein kinase pathways in breast, colonic, and pancreatic carcinomas," *Cancer Research*, vol. 67, no. 5, pp. 2072–2080, 2007.

[107] S. Gout, E. Brambilla, A. Boudria et al., "Abnormal expression of the pre-mRNA splicing regulators SRSF1, SRSF2, SRPK1 and SRPK2 in non-small cell lung carcinoma," *PLoS ONE*, vol. 7, no. 10, Article ID e46539, 2012.

[108] K. Odunsi, P. Mhawech-Fauceglia, C. Andrews et al., "Elevated expression of the serine-arginine protein kinase 1 gene in ovarian cancer and its role in Cisplatin cytotoxicity in vitro," *PLoS ONE*, vol. 7, no. 12, 2012.

[109] L. Adesso, S. Calabretta, F. Barbagallo et al., "Gemcitabine triggers a pro-survival response in pancreatic cancer cells through activation of the MNK2/eIF4E pathway," *Oncogene*, vol. 32, no. 23, pp. 2848–2857, 2013.

[110] A. Locatelli and C. A. Lange, "Met receptors induce Sam68-dependent cell migration by activation of alternate extracellular signal-regulated kinase family members," *Journal of Biological Chemistry*, vol. 286, no. 24, pp. 21062–21072, 2011.

[111] C. Ghigna, S. Giordano, H. Shen et al., "Cell motility is controlled by SF2/ASF through alternative splicing of the Ron protooncogene," *Molecular Cell*, vol. 20, no. 6, pp. 881–890, 2005.

[112] C. Valacca, S. Bonomi, E. Buratti et al., "Sam68 regulates EMT through alternative splicing-activated nonsense-mediated mRNA decay of the SF2/ASF proto-oncogene," *Journal of Cell Biology*, vol. 191, no. 1, pp. 87–99, 2010.

[113] S. Yea, G. Narla, X. Zhao et al., "Ras promotes growth by alternative splicing-mediated inactivation of the KLF6 tumor suppressor in hepatocellular carcinoma," *Gastroenterology*, vol. 134, no. 5, pp. 1521–1531, 2008.

[114] J. C. Shultz, R. W. Goehe, D. S. Wijesinghe et al., "Alternative splicing of caspase 9 is modulated by the phosphoinositide 3-kinase/Akt pathway via phosphorylation of SRp30a," *Cancer Research*, vol. 70, no. 22, pp. 9185–9196, 2010.

[115] L. Trinkle-Mulcahy, P. Ajuh, A. Prescott et al., "Nuclear organisation of NIPP1, a regulatory subunit of protein phosphatase 1 that associates with pre-mRNA splicing factors," *Journal of Cell Science*, vol. 112, no. 2, pp. 157–168, 1999.

[116] A. Boudrez, M. Beullens, P. Groenen et al., "NIPP1-mediated interaction of protein phosphatase-1 with CDC5L, a regulator of pre-mRNA splicing and mitotic entry," *Journal of Biological Chemistry*, vol. 275, no. 33, pp. 25411–25417, 2000.

[117] A. Boudrez, M. Beullens, E. Waelkens, W. Stalmans, and M. Bollen, "Phosphorylation-dependent interaction between the splicing factors SAP155 and NIPP1," *Journal of Biological Chemistry*, vol. 277, no. 35, pp. 31834–31841, 2002.

[118] M. V. Murray, R. Kobayashi, and A. R. Krainer, "The type 2C Ser/Thr phosphatase PP2Cγ is a pre-mRNA splicing factor," *Genes and Development*, vol. 13, no. 1, pp. 87–97, 1999.

[119] B. Cardinali, P. T. W. Cohen, and A. J. Lamond, "Protein phosphatase 1 can modulate alternative 5′ splice site selection in a HeLa splicing extract," *FEBS Letters*, vol. 352, no. 3, pp. 276–280, 1994.

[120] E. Allemand, M. L. Hastings, M. V. Murray, M. P. Myers, and A. R. Krainer, "Alternative splicing regulation by interaction of phosphatase PP2Cγ with nucleic acid-binding protein YB-1," *Nature Structural and Molecular Biology*, vol. 14, no. 7, pp. 630–638, 2007.

[121] T. Novoyatleva, B. Heinrich, Y. Tang et al., "Protein phosphatase 1 binds to the RNA recognition motif of several splicing factors and regulates alternative pre-mRNA processing," *Human Molecular Genetics*, vol. 17, no. 1, pp. 52–70, 2008.

[122] C. L. Lorson, E. Hahnen, E. J. Androphy, and B. Wirth, "A single nucleotide in the SMN gene regulates splicing and is responsible for spinal muscular atrophy," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 96, no. 11, pp. 6307–6311, 1999.

[123] Z. Zhang, O. Kelemen, M. A. Van Santen et al., "Synthesis and characterization of pseudocantharidins, novel phosphatase modulators that promote the inclusion of exon 7 into the SMN (survival of motoneuron) pre-mRNA," *Journal of Biological Chemistry*, vol. 286, no. 12, pp. 10126–10136, 2011.

[124] C. E. Chalfant, K. Rathman, R. L. Pinkerman et al., "De novo ceramide regulates the alternative splicing of caspase 9 and Bcl-x in A549 lung adenocarcinoma cells. Dependence on protein phosphatase-1," *Journal of Biological Chemistry*, vol. 277, no. 15, pp. 12587–12595, 2002.

[125] C. Sumanasekera, O. Kelemen, M. Beullens et al., "C6 pyridinium ceramide influences alternative pre-mRNA splicing by inhibiting protein phosphatase-1," *Nucleic Acids Research*, vol. 40, no. 9, pp. 4025–4039, 2012.

[126] J. P. Venables, R. Klinck, A. Bramard et al., "Identification of alternative splicing markers for breast cancer," *Cancer Research*, vol. 68, no. 22, pp. 9525–9531, 2008.

[127] K. Lenos, A. M. Grawenda, K. Lodder et al., "Alternate splicing of the p53 inhibitor HDMX offers a superior prognostic biomarker than p53 mutation in human cancer," *Cancer Research*, vol. 72, no. 16, pp. 4074–4084, 2012.

[128] R. Kole, A. R. Krainer, and S. Altman, "RNA therapeutics: beyond RNA interference and antisense oligonucleotides," *Nature Reviews Drug Discovery*, vol. 11, no. 2, pp. 125–140, 2012.

[129] S. Bonnal, L. Vigevani, and J. Valcárcel, "The spliceosome as a target of novel antitumour drugs," *Nature Reviews. Drug Discovery*, vol. 11, no. 11, pp. 847–859, 2012.

[130] F. Zammarchi, E. De Stanchina, E. Bournazou et al., "Antitumorigenic potential of STAT3 alternative splicing modulation," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 108, no. 43, pp. 17779–17784, 2011.

[131] A. Corrionero, B. Miñana, and J. Valcárcel, "Reduced fidelity of branch point recognition and alternative splicing induced by the anti-tumor drug spliceostatin A," *Genes and Development*, vol. 25, no. 5, pp. 445–459, 2011.

[132] R. Karni, E. De Stanchina, S. W. Lowe, R. Sinha, D. Mu, and A. R. Krainer, "The gene encoding the splicing factor SF2/ASF is a proto-oncogene," *Nature Structural and Molecular Biology*, vol. 14, no. 3, pp. 185–193, 2007.

[133] O. Fedorov, K. Huber, A. Eisenreich et al., "Specific CLK inhibitors from a novel chemotype for regulation of alternative splicing," *Chemistry and Biology*, vol. 18, no. 1, pp. 67–76, 2011.

[134] W.-H. Chang, T.-C. Liu, W.-K. Yang et al., "Amiloride modulates alternative splicing in leukemic cells and resensitizes Bcr-AblT315I mutant cells to imatinib," *Cancer Research*, vol. 71, no. 2, pp. 383–392, 2011.

[135] J.-G. Chang, D.-M. Yang, W.-H. Chang et al., "Small molecule Amiloride modulates oncogenic RNA alternative splicing to devitalize human cancer cells," *PLoS ONE*, vol. 6, no. 6, Article ID e18643, 2011.

[136] S. K. Pal, K. Reckamp, H. Yu, and R. A. Figlin, "Akt inhibitors in clinical development for the treatment of cancer," *Expert Opinion on Investigational Drugs*, vol. 19, no. 11, pp. 1355–1366, 2010.

[137] L. Santarpia, S. M. Lippman, and A. K. El-Naggar, "Targeting the MAPK-RAS-RAF signaling pathway in cancer therapy," *Expert Opinion on Therapeutic Targets*, vol. 16, no. 1, pp. 103–119, 2012.

[138] S. Zhang and D. Yu, "Targeting Src family kinases in anticancer therapies: turning promise into triumph," *Trends in Pharmacological Sciences*, vol. 33, no. 3, pp. 122–128, 2012.

Nucleic Acids Research Advance Access published December 24, 2013

Nucleic Acids Research, 2013, 1–10
doi:10.1093/nar/gkt1307

The centrosomal kinase NEK2 is a novel splicing factor kinase involved in cell survival

Chiara Naro[1,2], Federica Barbagallo[1,2], Paolo Chieffi[3], Cyril F. Bourgeois[4], Maria Paola Paronetto[2,5] and Claudio Sette[1,2,*]

[1]Department of Biomedicine and Prevention, University of Rome 'Tor Vergata', Rome, Italy, [2]Laboratories of Neuroembryology and of Cellular and Molecular Neurobiology, Fondazione Santa Lucia IRCCS, 00143 Rome, Italy, [3]Department of Psychology, II University of Naples, Caserta, Italy, [4]Department of Functional Genomics and Cancer, Institut de Genetique et de Biologie Moleculaire et Cellulaire, F-67400, INSERM U964, F-67400 Illkirch, France and [5]Department of Health Sciences, University of Rome Foro Italico, Rome, Italy

Received August 8, 2013; Revised November 14, 2013; Accepted November 25, 2013

ABSTRACT

NEK2 is a serine/threonine kinase that promotes centrosome splitting and ensures correct chromosome segregation during the G2/M phase of the cell cycle, through phosphorylation of specific substrates. Aberrant expression and activity of NEK2 in cancer cells lead to dysregulation of the centrosome cycle and aneuploidy. Thus, a tight regulation of NEK2 function is needed during cell cycle progression. In this study, we found that NEK2 localizes in the nucleus of cancer cells derived from several tissues. In particular, NEK2 co-localizes in splicing speckles with SRSF1 and SRSF2. Moreover, NEK2 interacts with several splicing factors and phosphorylates some of them, including the oncogenic SRSF1 protein. Overexpression of NEK2 induces phosphorylation of endogenous SR proteins and affects the splicing activity of SRSF1 toward reporter minigenes and endogenous targets, independently of SRPK1. Conversely, knockdown of NEK2, like that of SRSF1, induces expression of pro-apoptotic variants from SRSF1-target genes and sensitizes cells to apoptosis. Our results identify NEK2 as a novel splicing factor kinase and suggest that part of its oncogenic activity may be ascribed to its ability to modulate alternative splicing, a key step in gene expression regulation that is frequently altered in cancer cells.

INTRODUCTION

NEK2 is a member of the NIMA-related family of serine/threonine protein kinases, which share structural relationships and the involvement in cell cycle regulation (1). NEK2 displays constitutive catalytic activity and phosphorylates proteins involved in centrosome duplication and cell cycle regulation (2). Consistently, NEK2 binds to microtubules and is enriched in the centrosome, where it contributes to centrosome splitting during the G2/M phase of the cell cycle (2). Upregulation of NEK2 in human cells causes premature splitting of this organelle (3), whereas overexpression of a NEK2 kinase-dead mutant induces centrosome abnormalities and aneuploidy (4). Hence, a tight regulation of NEK2 abundance and activity is essential to ensure correct centrosome duplication and timely progression of the cell cycle.

Similar to other kinases involved in spindle assembly or duplication (5), overexpression of NEK2 was reported in several neoplastic diseases, such as preinvasive and invasive breast carcinomas (6), lung adenocarcinomas (7), testicular seminomas (8) and diffuse large B cell lymphomas (9). More recently, NEK2 expression has been proposed as a strong predictor for drug resistance and poor prognosis in human cancer, suggesting that it might represent a key therapeutic target (10). In line with this hypothesis, pharmacologic or genetic interference with NEK2 activity strongly reduced proliferation and invasiveness of cancer cells (10–12). Mechanistically, the oncogenic activity of NEK2 has been mainly linked to its ability to regulate centrosome duplication (3,6,13), whose

*To whom correspondence should be addressed. Tel: +3906 72596260; Fax: +3906 72596268; Email: claudio.sette@uniroma2.it
Present address:
Cyril F. Bourgeois, Centre de Recherche en Cancerologie de Lyon, INSERM U1052, CNRS UMR 5286, Universite de Lyon, Centre Leon Berard, Lyon, France.

The authors wish it to be known that, in their opinion, the first two authors should be regarded as Joint First Authors.

aberrant amplification frequently leads to aneuploidy and neoplastic transformation (6). Overexpression of NEK2 in non-transformed breast epithelial cells was shown to induce centrosome overduplication (6), and increased expression of endogenous NEK2 caused centrosome amplification in breast cancer lesions expressing the oncogenic K-RAS(G12D) mutant protein (13). Furthermore, NEK2-dependent phosphorylation was required for proper localization at the kinetochores of HEC1, a protein involved in faithful chromosome segregation (14). These observations strongly suggest that NEK2-dependent centrosome amplification and aneuploidy can favour neoplastic transformation.

We previously reported that increased expression of NEK2 in human testicular seminomas correlated with its accumulation in the nucleus (8). This observation suggested that nuclear functions of NEK2 might also contribute to its role in cancer cells. Herein, we have studied in further detail the nuclear localization and function of this kinase. We found that nuclear localization of NEK2 occurs in cancer cells derived from several tissues. NEK2 localizes to splicing speckles and phosphorylates the oncogenic splicing factor SRSF1. Moreover, we found that NEK2 regulates SRSF1 activity and alternative splicing (AS) of SRSF1 target genes similarly to the SR protein kinase SRPK1. In particular, NEK2 promotes anti-apoptotic splice variants and knockdown of its expression enhanced apoptosis. Our results uncover a novel function for NEK2 in splicing regulation and suggest that phosphorylation of splicing factors and modulation of AS might contribute to its oncogenic activity.

MATERIALS AND METHODS

Immunohistochemistry and immunofluorescence analysis

Cancer patient's tissues (14 cases of cryopreserved tissue from seminoma, breast, lung, prostate, cervix and colon cancer) were obtained from the National Cancer Institute 'G. Pascale' Ethical Committee approval was given in all instances. Five-micrometer sections were processed for immunohistochemistry with antibodies against NEK2 (Abgent) as described (8). Immunofluorescence was performed as described (8,15) using the following primary antibodies (1:500): rabbit anti-NEK2 (Abgent), mouse anti-SRSF1, anti-SRSF2 (Santa Cruz Biotechnology) and rabbit anti-cleaved CASPASE 3 (Sigma Aldrich). Confocal analyses were performed using a Leica confocal microscope as described (16). Images in Figure 6D, S2 and S5 were taken using a Leica inverted microscope as described (8). Images were saved as TIFF files and Photoshop (Adobe) was used for composing panels.

Cell culture, transfections and treatment

TCam-2 cells were grown in RPMI 1640 (Lonza), HEK293T, HeLa, MCF7, PC-3 cells were grown in Dulbecco's modified Eagle's medium (Sigma Aldrich), all supplemented with 10% FBS, gentamycin, penicillin and streptomycin. Transfection with the indicated expression vectors was performed using Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions. After 24 h, cells were harvested for protein and RNA analyses. For RNA interference, cells were transfected with siRNAs (Sigma-Aldrich) using Lipofectamine RNAiMAX (Invitrogen) according to manufacturer's instructions and harvested 48 h later for protein and RNA analyses. Sequences for SRPK1 siRNAs were previously described (17). NEK2, SRSF1 siRNAs and scrambled siRNA sequences are listed in Supplementary Table S1. For apoptosis, HeLa cells were treated for 24 h with the indicated doses of cisplatin or starved for 8 h in Earle's balanced salt solution (Sigma).

Protein extracts and western blot analysis

Total cellular extracts and cellular fractionations were processed and analysed by western blot as described (15,18) using the following primary antibodies (1:1000): rabbit anti-NEK2 R31 (generously provided by Prof. A.M. Fry); mouse anti-GFP, anti-MYC, anti-SRSF1, anti-SRSF3, rabbit anti-SAM68 and goat anti-lamin B (Santa Cruz Biotechnology); rabbit anti-ACTIN, mouse anti-FLAG, anti-hnRNPA1, anti-hnRNPC1/C2 and anti-TUBULIN (Sigma-Aldrich); mouse anti-hnRNPF/H (Abcam); mouse anti-SR proteins (1H4) (Invitrogen), mouse anti-SRPK1 (BD Pharmingen); rabbit anti-AKT (Novus Biologicals); rabbit anti-pAKT Ser 473 and anti-PARP1 (Cell Signaling).

In vitro kinase assay

Glutathione S-transferase (GST)-fusion proteins were expressed in *Escherichia coli* cells (strain BL21-DE3) and purified as previously described (19). $(His)_6$-tagged proteins were expressed in Sf9 cells using a baculovirus system and purified on a TALON affinity resin (CLONETECH), as described (20). *In vitro* kinase assays were performed as described (21), using purified NEK2 active protein (Millipore).

GST–pull-down and co-immunoprecipitation assays

Nuclear extracts were incubated with GST or GST-NEK2A(271–445) adsorbed on glutathione-agarose beads; bound proteins were eluted and analyzed as described (19). For co-immunoprecipitation, nuclear extracts or total extracts from HEK293T cells, transfected with the indicated vectors, were incubated with mouse anti-FLAG or mouse anti-IgG antibodies adsorbed on Dynabeads protein A (Invitrogen) and immunocomplexes were eluted and analysed as described (15).

Extraction of RNA, reverse transcriptase-polymerase chain reaction and real-time polymerase chain reaction analysis

RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. After digestion with RNase-free DNase (Roche), 1 μg of total RNA was retrotranscribed using M-MLV reverse transcriptase (Promega), used as template for polymerase chain reaction (PCR; GoTaq, Promega) and reactions were analysed on agarose or acrylamide gels. Real-time quantitative PCRs (qPCR) were performed using LightCycler

480 SYBR Green I Master and the LightCycler 480 System (Roche), according to the manufacturer's instructions. Control reactions omitting M-MLV reverse transcriptase were also carried out. All primers used are listed in the Supplementary Table S1.

Statistical analysis

Statistical analysis was performed by the Student *t*-test as described in the figure legends.

RESULTS

NEK2 is enriched in the nucleus of cancer cells

NEK2 is enriched in the nucleus of human testicular seminoma cells (8). Nuclear localization of this kinase was also recently observed in myeloma cells and shown to correlate with poor prognosis (10). To investigate whether other cancer cells that overexpress NEK2 share this nuclear localization, we performed immunohistochemistry analysis of tissue specimens derived from cancer patients. Using a previously validated antibody (8), we observed that NEK2 staining was concentrated in the nucleus of breast and lung cancer cells (Figure 1A). In colon, prostate and cervix cancer cells, although it was also detected in the cytoplasm, NEK2 staining was enriched in nucleus (Figure 1A). Confocal immunofluorescence analyses of NEK2 localization in cell lines derived from breast cancer (MCF7; Figure 1B), seminoma (TCam-2), prostate cancer (PC-3), colon carcinoma (Caco-2) and cervix cancer (HeLa) (Supplementary Figure S1) suggest that nuclear localization of NEK2 is a common feature of human cancer cells.

NEK2 is expressed as three alternative splice variants, named NEK2A, B and C (Supplementary Figure S2A) (22). NEK2A and B differ in the C-termini because an alternative polyadenylation signal in intron 7 is used in NEK2B, thus preventing inclusion of the last exon 8 (2). Notably, exon 8 encodes for protein degradation motifs in NEK2A, which mediate its degradation in mitosis (23). NEK2C is identical to NEK2A with the exception of a small internal deletion of 8 amino acids (missing residues 371–378), due to usage of a downstream splice acceptor site in exon 8 (22). Although the biochemical features of NEK2C are undistinguishable from those of NEK2A, deletion of this sequence creates a nuclear localization signal that promotes NEK2C accumulation in the nucleus (22). To investigate which splice variant was prevalently expressed in cancer cell lines, we performed reverse transcriptase-polymerase chain reaction (RT-PCR) analyses using variant-specific primers (Supplementary Figure S2A). NEK2A and B were readily detected in all cell lines analysed, whereas NEK2C was barely detectable (Supplementary Figure S2B), suggesting that its expression is unlikely to account for the nuclear localization of NEK2 in cancer cells.

Previous results indicated that a substantial fraction of NEK2A localizes in the nucleus when the protein is overexpressed (22). In line with this report, we observed that overexpression of NEK2A was sufficient to allow its accumulation in the nucleus of HeLa cells, with a localization pattern that closely resembled that of NEK2C

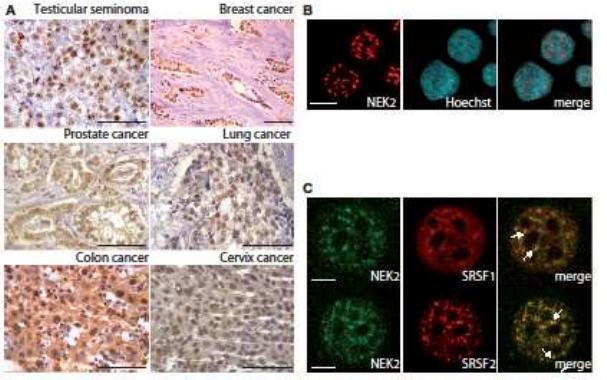


Figure 1. NEK2 localizes in the nucleus of cancer cells. (**A**) Immunohistochemistry of NEK2 in testicular seminomas, breast, prostate, lung and cervix cancer specimens (scale bar = 25 μm). (**B** and **C**) Confocal immunofluorescence analysis of MCF7 cells stained with anti-NEK2 (red) antibody and Hoechst (blue) (**B**, scale bar = 10 μm) or with anti-NEK2 (green), anti-SRSF1 (red, upper panel) or anti-SRSF2 (red, lower panel) (**C**, scale bar = 5 μm). White arrows indicate co-localization in speckles.

(Supplementary Figure S2C), whereas NEK2B remained mainly cytoplasmic (Supplementary Figure S2C). These results suggest that upregulation of NEK2A is likely responsible for the nuclear localization of NEK2 in cancer cells.

NEK2 localizes in nuclear splicing speckles and co-fractionates with splicing factors

We found that NEK2 accumulated in nuclear granules of variable size and irregular shape (Figure 1B, Supplementary Figure S1), which resembled the splicing speckles, the interchromatin regions enriched in splicing factors (24). Confocal immunofluorescence analysis in MCF7 cells confirmed that NEK2 co-localizes in the nuclear speckles with SRSF1 and SRSF2 (Figure 1C), two serine/arginine-rich (SR) proteins commonly used as markers of these structures (24). We next used subcellular fractionation experiments to confirm the association of NEK2 with splicing factors. By using this technique, it was documented that splicing factors accumulate in the nuclear matrix-attached insoluble fraction (18). Analyses of cytosolic (S), nuclear soluble (NS), and nuclear matrix-attached insoluble (NI) fractions confirmed that splicing factors were enriched in the NI fraction isolated from MCF7, PC-3 and TCam-2 cells (Figure 2A). In addition, we observed that NEK2 was also enriched in both the NS and the NI fraction in all cell lines tested (Figure 2A). The molecular weight of this band corresponded to that of NEK2A and C. A faster migrating band corresponding to the molecular weight of NEK2B was instead detected in the cytosolic fraction.

NEK2 interacts with substrates and activators through the carboxyl terminal regulatory region (residues 273–445, Supplementary Figure S2A) (2). Thus, we used purified GST-NEK2A(271–445) fusion protein as bait in affinity chromatography of nuclear extracts isolated from MCF7 and PC-3 cells. GST-NEK2A(271–445) selectively associated with some splicing regulators, as SRSF1, hnRNPA1, hnRNPF and SAM68, but not others, as SRSF3 and hnRNPC1/C2 (Figure 2B). These results suggest that NEK2 interacts with specific splicing factors in the cell nucleus.

NEK2 is a splicing factor kinase

Next, we set out to determine whether splicing factors were substrates for NEK2. We focused on SR proteins because their splicing activity is finely tuned by phosphorylation (25,26). As first step, we determined whether SRSF1 and SRSF7 were directly phosphorylated by NEK2. Kinase assays using purified full-length HIS- or GST-fusion proteins of these splicing factors showed that purified NEK2 efficiently phosphorylates SRSF1 and SRSF7 *in vitro* (Figure 3A). We focused the rest of our study on SRSF1 because it was more efficiently phosphorylated by NEK2. Furthermore, this splicing factor is a bona fide oncogene (27) and it is upregulated in several human cancers, including breast and prostate carcinomas, where it modulates cancer-relevant AS events (28,29).

First, we investigated whether the interaction between NEK2 and SRSF1 also occurred in live cells. We found

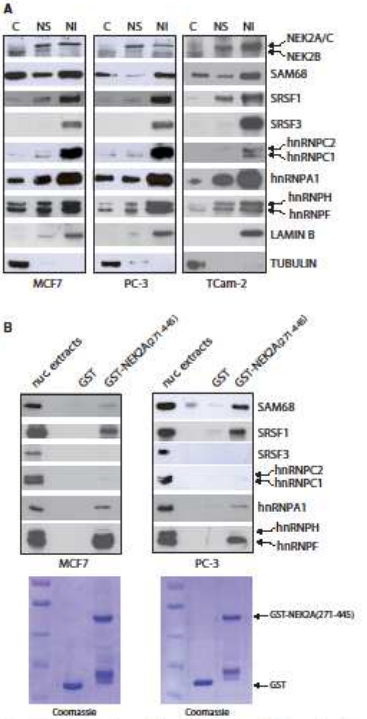


Figure 2. NEK2 associates with splicing factors. (**A**) Cytosolic (C), nuclear soluble (NS) and nuclear insoluble matrix-associated (NI) fractions of MCF7, PC-3, TCam-2 cells were analysed by western blot using antibodies for NEK2 and indicated splicing factors. LAMIN B and TUBULIN were evaluated as nuclear matrix and cytosolic markers. (**B**) Western blot analysis for the indicated splicing factors in pull-down assays of MCF7 and PC-3 nuclear extracts with GST-NEK2A(271–445) fusion protein and GST (as negative control). Coomassie staining shows the purified GST and GST-NEK2A(271–445) fusion protein (lower panels).

that GFP-NEK2C (Figure 3B) and GFP-NEK2A (Supplementary Figure S3A) were efficiently co-immunoprecipitated with FLAG-SRSF1 in HEK293T cells, whereas the cytosolic GFP-NEK2B was not (Supplementary Figure S2C and S3B). Next, to verify the ability of NEK2 to phosphorylate SRSF1 in live cells, we

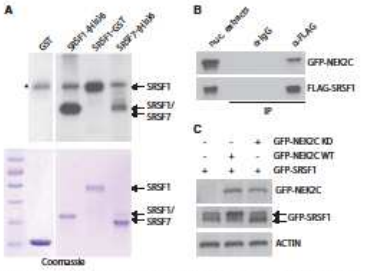


Figure 3. NEK2 phosphorylates splicing factors. (**A**) Purified NEK2 was incubated with purified GST (as negative control), SRSF1-$(His)_6$, SRSF1-GST or SRSF7-$(His)_6$ proteins and γ[^{32}P]ATP. Phosphorylation levels were detected by autoradiography (asterisk indicates autophosphorylated NEK2). Coomassie staining shows the purified proteins (lower panel). (**B**) Western blot analysis with anti-GFP and anti-FLAG antibodies of the immunoprecipitation assay performed with control mouse IgG or anti-FLAG antibody of nuclear extracts of HEK293T expressing FLAG-SRSF1 and GFP-NEK2C. (**C**) Western blot analysis using anti-GFP antibody of HEK293T cells transfected with GFP-SRSF1, wild-type NEK2C (WT) or kinase-dead NEK2C (KD). ACTIN was used as loading control.

co-expressed wild-type or kinase-dead GFP-NEK2C with GFP-SRSF1. When expressed alone, GFP-SRSF1 yielded two bands, with the faster migrating band being more abundant, indicating that the bulk of the protein is hypophosphorylated (Figure 3C). Expression of wild-type NEK2C increased the amount of the higher molecular weight band of SRSF1, suggesting its hyperphosphorylation, whereas kinase-dead NEK2C was ineffective (Figure 3C). A similar result was obtained with NEK2A (Supplementary Figure S3C), indicating that both NEK2 splice variants can phosphorylate SRSF1 in live cells.

NEK2 behaves as an SR protein kinase in live cells

Having established that NEK2 phosphorylates SRSF1, we asked whether it behaves as an SR protein kinase (SRPK). For these studies, we focused on NEK2C, which was chosen for its almost exclusive localization in the nucleus (22). Overexpression of NEK2C induced a pattern of SR protein phosphorylation similar to that elicited by SRPK1, a prototypical SRPK (26), leading to phosphorylation of SRSF6 (SRp55), SRSF5 (SRp40) and SRSF2/SRSF9 (SRp30) (Figure 4A).

Next, we tested whether NEK2 functionally regulates the activity of SR proteins. Previous reports demonstrated that overexpression of SRPK1 modulates splicing of the E1A reporter minigene (17), a commonly used splicing target that contains several 5′ and 3′ alternative splice sites (ss) (Figure 4B). As expected (17), we observed that increasing the expression of SRPK1 in HeLa cells caused a dose-dependent switch from the 13S to the 9S 5′ splice site of the E1A minigene (Figure 4C). Remarkably, upregulation of NEK2C caused a similar switch in E1A splicing. This effect required the kinase activity of NEK2, as the kinase-dead mutant had no effect (Figure 4C). Thus, NEK2 displays features of an SRPK in live cells.

NEK2 expression modulates SRSF1-dependent *BCL-X* splicing

Phosphorylation of SR proteins regulates their splicing activity (25,26). Thus, we asked whether NEK2 could modulate the AS of an endogenous target of SRSF1, such as the *BCL-X* gene (15,30). Selection of two alternative 5′ ss in exon 2 of *BCL-X* leads to the production of two splice variants: the anti-apoptotic BCL-X_L and the pro-apoptotic BCL-X_S (31). SRSF1 promotes selection of the proximal 5′ ss leading to expression of BCL-X_L (15,30). By performing real-time qPCR using exon junction-specific primers for BCL-X_L and BCL-X_S (Figure 5A), we found that overexpression of NEK2C in HeLa cells increased the BCL-X_L/BCL-X_S ratio to a similar extent as overexpression of SRSF1 (Figure 5B). Importantly, this effect was not due to activation of SRPK1. While knockdown of SRPK1 promoted the pro-apoptotic BCL-X_S variant, indicating that SRPK1 also modulates this AS event, NEK2C was still capable to enhance splicing of the anti-apoptotic BCL-X_L variant in SRPK1-depleted cells as observed in control cells (Figure 5C).

To determine whether NEK2 expression affected the ability of SRSF1 to modulate *BCL-X* AS, we used a minigene that recapitulates the splicing of the endogenous gene (32). We found that knockdown of NEK2 in HEK293T cells slightly enhanced splicing of the pro-apoptotic BCL-X_S variant (Figure 5D). Moreover, while transfection of suboptimal amounts of SRSF1 efficiently promoted splicing of the anti-apoptotic BCL-X_L variant in control cells (si-SCR), this effect was partially impaired when NEK2 was silenced (Figure 5D). Importantly, similar effects were also observed when SRPK1 was knocked down, even though silencing of both NEK2 and SRPK1 did not exert additive effect on SRSF1-induced *BCL-X* splicing (Figure 5D).

NEK2 is involved in the regulation of apoptosis

Next, we sought out to determine whether AS of known endogenous targets of SRSF1 was affected by the knockdown of NEK2 in HeLa cells. We examined the splicing pattern of SRSF1 target transcripts from three genes with roles in cancer and for which AS variants have been characterized: *BCL-X*, *MKNK2* and *BIN1* (15,27,30,33). For comparison, we also knocked down SRSF1 and SRPK1 in parallel experiments (Figure 6A and B). Transient knockdown of NEK2 in HeLa cells resulted in decreased ratio of BCL-X_L/BCL-X_S and MNK2b/MNK2a, and induces skipping of exon 12A in BIN1 mRNA variants, without affecting SRSF1 expression. All the splicing changes exerted by NEK2 depletion favoured pro-apoptotic splice variants and were recapitulated by knockdown of either SRSF1 or SRPK1, although to different extent for the three genes (Figure 6A and B). Moreover, as observed for BCL-X_L, overexpression of either NEK2C or SRSF1 promoted splicing of

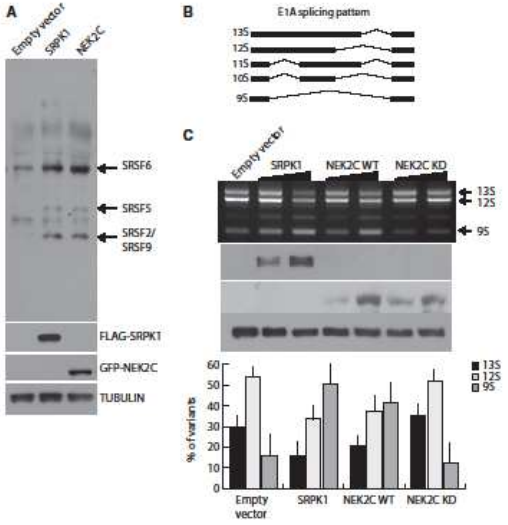


Figure 4. NEK2 phosphorylates SR proteins and modulates E1A splicing. (**A**) Western blot analysis of SR protein phosphorylation in HeLa cells transfected with FLAG-SRPK1 or GFP-NEK2C using the anti-SR proteins 1H4 antibody. TUBULIN was detected as loading control. (**B**) Schematic representation of the alternative splice variants of the E1A minigene. (**C**) RT-PCR analysis (upper panel) of the *in vivo* splicing assay in HeLa cells transfected with the E1A minigene and increasing doses of FLAG-SRPK1, GFP-NEK2C WT or GFP-NEK2C KD. Overexpression efficiency was assessed through anti-FLAG and anti-GFP western blot. TUBULIN was evaluated as loading control. Bar graph (lower panel) represents densitometric analysis for the major E1A splice variants (13S, 12S, 9S) evaluated in presence of the highest dose of vectors (mean ± SD, n = 3).

anti-apoptotic *MKNK2* and *BIN1* variants (Supplementary Figure 4A). These results indicate that NEK2 contributes to the regulation of SRSF1 splicing activity.

Because NEK2 knockdown induced expression of pro-apoptotic splice variants, we asked whether it also plays a role in cell viability. In line with its effect on AS, depletion of the endogenous NEK2 in HeLa cells significantly increased the basal level of apoptosis, as monitored by cleavage of PARP1 (Figure 6C) and CASPASE 3 (Figure 6D), and enhanced the apoptotic response of cells to stress, such as treatment with cisplatin (Figure 6C and D) or starvation (Supplementary Figure 4B and C). These results suggest that the effect of NEK2 on AS events regulated by SRSF1 is physiologically relevant.

DISCUSSION

NEK2 is a centrosomal kinase involved in centrosome duplication in mitosis that is frequently upregulated in human cancers (2). Recent evidence suggests that nuclear localization of NEK2 is a predictor for drug resistance and a marker of poor prognosis in patients (10). Nevertheless, the specific nuclear functions of NEK2 are still completely obscure. The present study indicates that NEK2 acts as a regulator of AS events by modulating SRSF1 activity, thus uncovering a previously unknown nuclear function for this oncogenic kinase.

NEK2 overexpression has been extensively described in several types of tumours (2,6–10). Its oncogenic activity has been primarily ascribed to the ability to induce aneuploidy by perturbing centrosome duplication and its segregation dynamics (2). However, it was recently demonstrated that in testicular seminomas (8), myelomas and other types of cancer (10), NEK2 is primarily localized in the nucleus of neoplastic cells. We now document that the nuclear localization of NEK2 is also observed in several carcinomas and cancer cell lines in which the kinase is upregulated. Thus, although our

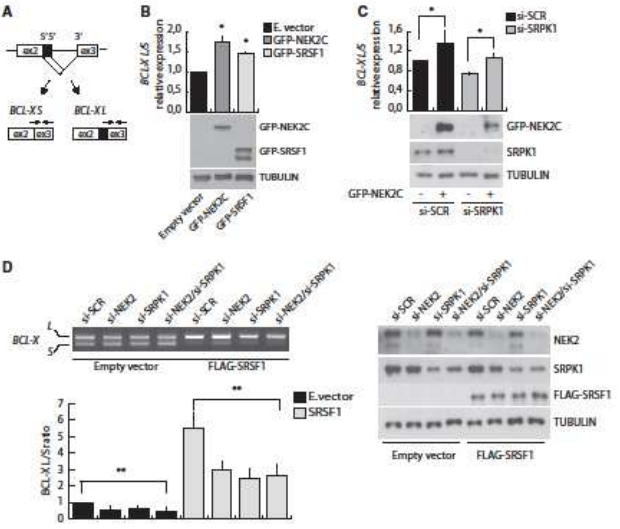


Figure 5. NEK2 modulates BCL-X AS affecting SRSF1 activity. (**A**) Schematic representation of the BCL-X AS. Black arrows indicate exon-junction primers used for qRT-PCR analysis. (**B** and **C**) qRT-PCR analysis of endogenous BCL-X splice-variants. Bar graphs represent BCL-X_L/BCL-X_S ratio (mean ± SD, n = 3, $*P < 0.05$). HeLa cells were transfected with mock, GFP-NEK2C or GFP-SRSF1 (**B**) or with either scramble (si-SCR) or SRPK1 (si-SRPK1) siRNAs and then with or without GFP-NEK2C (**C**). Silencing and overexpression efficiency was assessed by western blot analysis. (**D**) RT-PCR analysis of the *in vivo* splicing assay of BCL-X minigene in HEK293T cells transfected with scramble (si-SCR), NEK2 (si-NEK2), SRPK1 (si-SRPK1) or both NEK2 and SRPK1 (si-NEK2/si-SRPK1) siRNAs and with or without FLAG-SRSF1. Bar graph represents the densitometric analysis of the BCL-X_L/BCL-X_S ratio, normalized for the value obtained in cells transfected with scramble siRNA and empty vector, set to 1 (mean ± SD, n = 3, $**P < 0.01$). Silencing and overexpression efficiency was assessed by western blot analysis (right panel).

analysis is too limited to draw conclusions, these results suggest that nuclear localization of NEK2 is a common feature of neoplastic cells.

The NEK2C splice variant was reported to localize prevalently in the nucleus (22), suggesting that its selective upregulation in cancer cells might account for the observed localization of the kinase. However, our study indicates that NEK2C is expressed at low levels in all cancer cells analysed, raising doubts on its contribution to the localization of NEK2 in primary tumours and in cell lines. Conversely, upregulation of NEK2A, but not NEK2B, is sufficient to induce its nuclear localization, suggesting that NEK2A is the prevalent isoform in the nucleus of cancer cells.

Characterization of the subcellular distribution of NEK2 pointed out its co-fractionation with several splicing factors in the nuclear-insoluble material of cancer cells. Moreover, NEK2 co-localized with two SR proteins in nuclear splicing speckles. These inter-chromatin granules are particularly enriched in SR proteins and are supposed to function as nuclear storage sites for pre-mRNA processing regulators (24). Assembly of splicing speckles and active recruitment of SR proteins from these sites to the newly synthesized pre-mRNA is strictly regulated by reversible phosphorylation (34). Phosphorylation represents one of the main mechanisms by which subtle regulation of the splicing process, and especially of AS, is achieved (35,36). These observations led us to hypothesize the existence of a functional interaction between NEK2 and splicing. Several results of our study support this hypothesis. First, NEK2 interacts with and phosphorylates SRSF1. Second, we found that the splicing activity of SRSF1 is modulated by NEK2. Lastly, silencing of NEK2 negatively affects AS events that are target of SRSF1 in live cells. Collectively, these results point to NEK2 as a novel direct regulator of SRSF1 phosphorylation and activity.

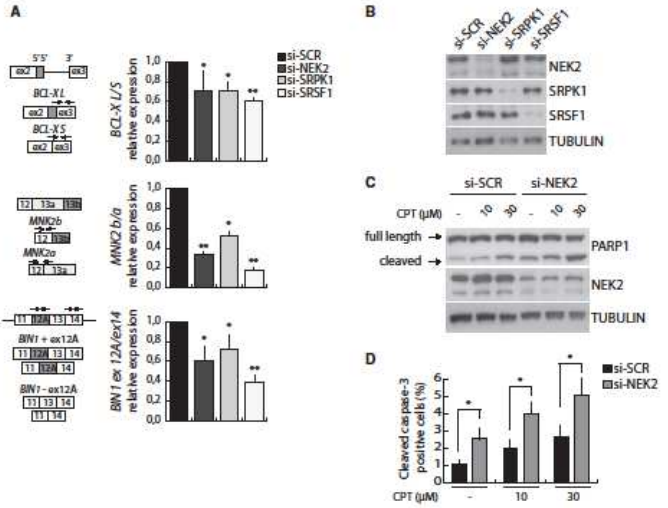


Figure 6. NEK2 silencing affects splicing of SRSF1 target genes and sensitizes cells to apoptosis. (**A**) Schematic representation of the SRSF1-regulated of *BCL-X*, *MKNK2* and *BIN1* AS events (left panel). Black arrows indicate primers used for the qRT-PCR analysis performed in HeLa cells transfected with scramble (si-SCR), NEK2 (si-NEK2), SRPK1 (si-SRPK1) or SRSF1 (si-SRSF1) siRNAs. Ratio of the AS variants are represented in the bar graph (mean ± SD, n = 3, $*P < 0.05$, $**P < 0.01$) (right panel). (**B**) Western blot analysis assessing NEK2, SRPK1 and SRSF1 silencing efficiency. (**C** and **D**) Western blot analysis of PARP1 cleavage (C) and quantitative analysis of cleaved-CASPASE 3 immunofluorescence (bar graph represents mean ± SD, n = 3, $*P < 0.05$) (D) in HeLa cells transfected with either scramble (si-SCR) or NEK2 (si-NEK2) siRNAs and treated for 24h with increasing doses of cisplatin (CPT). Western blot analysis assessing NEK2 silencing efficiency was performed (C).

The cellular localization and splicing activity of SRSF1 are regulated by reversible phosphorylation (26,37–39). We observed that NEK2 did not influence the nuclear localization of SRSF1 in HeLa and HEK293T cells (Supplementary Figure S5). By contrast, our study suggests that NEK2 can modulate SRSF1 splicing activity similarly to SRPK1, the prototype member of the SRPK family of kinases that mediate phosphorylation of SR proteins (26). Overexpression of either NEK2 or SRPK1 induced a similar pattern of SR proteins phosphorylation and caused a similar modulation of E1A AS. Likewise, knockdown of NEK2 or SRPK1 similarly reduced the splicing activity of SRSF1 toward the BCL-X minigene. Although the effects of NEK2 might be indirect (i.e. mediated by another kinase), three lines of evidence support a direct action. First, our *in vitro* kinase assays were performed using highly purified proteins, strongly indicating that NEK2 can directly phosphorylate SRSF1 and SRSF7. Second, although NEK2 was recently reported to induce activation of AKT in myeloma cells (10), a signalling kinase known to directly and indirectly modulate SR proteins phosphorylation (40,41), overexpression or knockdown of NEK2 did not alter the activity of AKT in HeLa cells (Supplementary Figure S6). Third, the effect of NEK2 on *BCL-X* splicing was not affected by knockdown of SRPK1. Thus, these experiments suggest that NEK2 behaves as a bona fide splicing factor kinase in live cells.

Our study implicates NEK2 in AS regulation of several SRSF1 target genes involved in cell viability. We found that knockdown of NEK2 mimicked that of SRSF1, or SRPK1, and induced expression of pro-apoptotic *BCL-X*, *BIN1* and *MKNK2* splice variants. Consistently, NEK2 depletion sensitized HeLa cells to spontaneous and stress-induced apoptosis, suggesting a pro-survival function for this kinase. Although protection from cell death may also involve other splicing-unrelated functions of NEK2 (10), it is likely that enhanced splicing of the anti-apoptotic variants of *BCL-X*, *BIN1* and *MKNK2* contributes to this pro-survival effect.

As NEK2, SRPK1 is also overexpressed in human cancers (42), suggesting that these kinases may act in concert to modulate SRSF1 activity. In line with this hypothesis, we found that NEK2 affected *BCL-X* splicing independently of SRPK1. Thus, even if apparently redundant, SRPK1 and NEK2 could regulate SR proteins phosphorylation and subsequent AS events in a coordinate manner. In fact, the subcellular localization of SRPK1, mainly cytosolic (17), and NEK2, predominantly nuclear (this study), suggest a possible coordinated activity of these kinases in different cellular compartments, as previously reported for SRPKs and CLKs (Cdc2-like kinases) (43). Notably, activation of SRPK1 in response to AKT-mediated signalling was reported to modulate gene expression by regulating AS programs (41). Because activation of NEK2 by the ERK1/2 pathway was observed in germ cells (44), and this pathway is often activated in cancer cells, it is possible that NEK2 also participates to AS regulation in response to environmental cues. Finally, as NEK2 expression and activity peak during the late S-G2 phase (45), we cannot exclude the possibility that it could affect SR protein function in a cell-cycle-dependent manner, thus contributing to coordinate gene expression regulation with cell cycle progression. Noteworthy, another centrosomal kinase, AURKA, was shown to modulate the apoptotic response to mitotic arrest of the cell cycle by regulating the stability of SRSF1 (30).

In conclusion, our study identifies NEK2 as a novel regulator of AS, which promotes SRSF1-dependent splicing of anti-apoptotic variants, thus contributing to cell survival.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEDGMENTS

We wish to thank Professors J. Stevenin for the purification of SR proteins, insightful suggestions and critical reading of the manuscript, A. Fry, F. Cecconi and G. Biamonti for the generous gift of reagents, Drs R. Busà and R. Franco for help with confocal microscopy and immunohistochemistry, P. Bielli for helpful suggestions and critical reading of the manuscript.

FUNDING

Funding for open access charge: Association for International Cancer Research (AICR) [12-0150]; the Associazione Italiana Ricerca sul Cancro (AIRC); the Fondazione Santa Lucia 'Ricerca Corrente'.

Conflict of interest statement. None declared.

REFERENCES

1. Fry,A.M., O'Regan,L., Sabir,S.R. and Bayliss,R. (2012) Cell cycle regulation by the NEK family of protein kinases. *J. Cell Sci.*, **125**, 4423–4433.
2. Hayward,D.G. and Fry,A.M. (2006) Nek2 kinase in chromosome instability and cancer. *Cancer Lett.*, **237**, 155–166.
3. Fry,A.M., Meraldi,P. and Nigg,E.A. (1998) A centrosomal function for the human Nek2 protein kinase, a member of the NIMA family of cell cycle regulators. *EMBO J.*, **17**, 470–481.
4. Faragher,A.J. and Fry,A.M. (2003) Nek2A kinase stimulates centrosome disjunction and is required for formation of bipolar mitotic spindles. *Mol. Biol. Cell*, **14**, 2876–2889.
5. Fukasawa,K. (2007) Oncogenes and tumour suppressors take on centrosomes. *Nat. Rev. Cancer*, **7**, 911–924.
6. Hayward,D.G., Clarke,R. B., Faragher,A.J., Pillai,M.R., Hagan,I.M. and Fry,A.M. (2004) The centrosomal kinase Nek2 displays elevated levels of protein expression in human breast cancer. *Cancer Res.*, **64**, 7370–7376.
7. Landi,M.T., Dracheva,T., Rotunno,M., Figueroa,J.D., Liu,H., Dasgupta,A., Mann,F.E., Fukuoka,J., Hames,M., Bergen,A.W. *et al.* (2008) Gene expression signature of cigarette smoking and its role in lung adenocarcinoma development and survival. *PLoS One*, **3**, e1651.
8. Barbagallo,F., Paronetto,M.P., Franco,R., Chieffi,P., Dolci,S., Fry,A.M., Geremia,R. and Sette,C. (2009) Increased expression and nuclear localization of the centrosomal kinase Nek2 in human testicular seminomas. *J. Pathol.*, **217**, 431–441.
9. Andréasson,U., Dictor,M., Jerkeman,M., Berglund,M., Sundström,C., Linderoth,J., Rosenquist,R., Borrebaeck,C.A. and Ek,S. (2009) Identification of molecular targets associated with transformed diffuse large B cell lymphoma using highly purified tumor cells. *Am. J. Hematol.*, **84**, 803–808.
10. Zhou,W., Yang,Y., Xia,J., Wang,H., Salama,M.E., Xiong,W., Xu,H., Shetty,S., Chen,T., Zeng,Z. *et al.* (2013) NEK2 induces drug resistance mainly through activation of efflux drug pumps and is associated with poor prognosis in myeloma and other cancers. *Cancer Cell*, **23**, 48–62.
11. Wu,G., Qiu,X.L., Zhou,L., Zhu,J., Chamberlin,R., Lau,J., Chen,P.L. and Lee,W.H. (2008) Small molecule targeting the Hec1/Nek2 mitotic pathway suppresses tumor cell growth in culture and in animal. *Cancer Res.*, **68**, 8393–8399.
12. Tsunoda,N., Kokuryo,T., Oda,K., Senga,T., Yokoyama,Y., Nagino,M., Nimura,Y. and Hamaguchi,M. (2009) Nek2 as a novel molecular target for the treatment of breast carcinoma. *Cancer Sci.*, **100**, 111–116.
13. Zeng,X., Shaikh,F.Y., Harrison,M.K., Adon,A.M., Trimboli,A.J., Carroll,K.A., Sharma,N., Timmers,C., Chodosh,L. A., Leone,G. *et al.* (2010) The Ras oncogene signals centrosome amplification in mammary epithelial cells through cyclin D1/Cdk4 and Nek2. *Oncogene*, **29**, 5103–5112.
14. Chen,Y., Riley,D.J., Zheng,L., Chen,P.L. and Lee,W.H. (2002) Phosphorylation of the mitotic regulator protein Hec1 by Nek2 kinase is essential for faithful chromosome segregation. *J. Biol. Chem.*, **277**, 49408–49416.
15. Paronetto,M.P., Achsel,T., Massiello,A., Chalfant,C.E. and Sette,C. (2007) The RNA-binding protein Sam68 modulates the alternative splicing of Bcl-x. *J. Cell Biol.*, **176**, 929–939.
16. Busà,R., Geremia,R. and Sette,C. (2010) Genotoxic stress causes the accumulation of the splicing regulator Sam68 in nuclear foci of transcriptionally active chromatin. *Nucleic Acids Res.*, **38**, 3005–3018.
17. Zhong,X.Y., Ding,J.H., Adams,J.A., Ghosh,G. and Fu,X.D. (2009) Regulation of SR protein phosphorylation and alternative splicing by modulating kinetic interactions of SRPK1 with molecular chaperones. *Genes Dev.*, **23**, 482–495.
18. Lin,S., Xiao,R., Sun,P., Xu,X. and Fu,X.D. (2005) Dephosphorylation-dependent sorting of SR splicing factors during mRNP maturation. *Mol. Cell*, **20**, 413–425.
19. Sette,C., Bevilacqua,A., Geremia,R. and Rossi,P. (1998) Involvement of phospholipase Cgamma1 in mouse egg activation induced by a truncated form of the C-kit tyrosine kinase present in spermatozoa. *J. Cell Biol.*, **142**, 1063–1074.
20. Dreumont,N., Hardy,S., Behm-Ansmant,I., Kister,L., Branlant,C., Stévenin,J. and Bourgeois,C.F. (2010) Antagonistic factors control the unproductive splicing of SC35 terminal intron. *Nucleic Acids Res.*, **38**, 1353–1366.
21. Di Agostino,S., Fedele,M., Chieffi,P., Fusco,A., Rossi,P., Geremia,R. and Sette,C. (2004) Phosphorylation of high-mobility group protein A2 by Nek2 kinase during the first meiotic division in mouse spermatocytes. *Mol. Biol. Cell*, **15**, 1224–1232.

22. Wu,W., Baxter,J.E., Wattam,S.L., Hayward,D.G., Fardilha,M., Knebel,A., Ford,E.M., da Cruz e Silva,E.F. and Fry,A.M. (2007) Alternative splicing controls nuclear translocation of the cell cycle-regulated Nek2 kinase. *J. Biol. Chem.*, **282**, 26431–26440.
23. Hayes,M.J., Kimata,Y., Wattam,S.L., Lindon,C., Mao,G., Yamano,H. and Fry,A.M. (2006) Early mitotic degradation of Nek2A depends on Cdc20-independent interaction with the APC/C. *Nat. Cell Biol.*, **8**, 607–614.
24. Spector,D.L. and Lamond,A.I. (2011) Nuclear speckles. *Cold Spring Harb. Perspect. Biol.*, **3**, pii: a000646.
25. Long,J.C. and Caceres,J.F. (2009) The SR protein family of splicing factors: master regulators of gene expression. *Biochem. J.*, **417**, 15–27.
26. Zhou,Z. and Fu,X.D. (2013) Regulation of splicing by SR proteins and SR protein-specific kinases. *Chromosoma*, **122**, 191–207.
27. Karni,R., de Stanchina,E., Lowe,S.W., Sinha,R., Mu,D. and Krainer,A.R. (2007) The gene encoding the splicing factor SF2/ASF is a proto-oncogene. *Nat. Struct. Mol. Biol.*, **14**, 185–193.
28. Anczuków,O., Rosenberg,A.Z., Akerman,M., Das,S., Zhan,L., Karni,R., Muthuswamy,S.K. and Krainer,A.R. (2012) The splicing factor SRSF1 regulates apoptosis and proliferation to promote mammary epithelial cell transformation. *Nat. Struct. Mol. Biol.*, **19**, 220–228.
29. Olshavsky,N.A., Comstock,C.E., Schiewer,M.J., Augello,M.A., Hyslop,T., Sette,C., Zhang,J., Parysek,L.M. and Knudsen,K.E. (2010) Identification of ASF/SF2 as a critical, allele-specific effector of the cyclin D1b oncogene. *Cancer Res.*, **70**, 3975–3984.
30. Moore,M.J., Wang,Q., Kennedy,C.J. and Silver,P.A. (2010) An alternative splicing network links cell-cycle control to apoptosis. *Cell*, **142**, 625–636.
31. Boise,L.H., Gonzalez-Garcia,M., Postema,C.E., Ding,L., Lindsten,T., Turka,L.A., Mao,X., Nuñez,G. and Thompson,C.B. (1993) bcl-x, a bcl-2-related gene that functions as a dominant regulator of apoptotic cell death. *Cell*, **74**, 597–608.
32. Massiello,A., Salas,A., Pinkerman,R.L., Roddy,P., Roesser,J.R. and Chalfant,C.E. (2004) Identification of two RNA cis-elements that function to regulate the 5′ splice site selection of Bcl-x pre-mRNA in response to ceramide. *J. Biol. Chem.*, **279**, 15799–15804.
33. Adesso,L., Calabretta,S., Barbagallo,F., Capurso,G., Pilozzi,E., Geremia,R., Delle Fave,G. and Sette,C. (2013) Gemcitabine triggers a pro-survival response in pancreatic cancer cells through activation of the MNK2/eIF4E pathway. *Oncogene*, **32**, 2848–2857.
34. Misteli,T., Cáceres,J.F., Clement,J.Q., Krainer,A.R., Wilkinson,M.F. and Spector,D.L. (1998) Serine phosphorylation of SR proteins is required for their recruitment to sites of transcription *in vivo*. *J. Cell Biol.*, **143**, 297–307.
35. Stamm,S. (2008) Regulation of alternative splicing by reversible protein phosphorylation. *J. Biol. Chem.*, **283**, 1223–1227.
36. Naro,C. and Sette,C. (2013) Phosphorylation-mediated regulation of alternative splicing in cancer. *Int. J. Cell Biol.*, **2013**, 151839.
37. Cao,W., Jamison,S.F. and Garcia-Blanco,M.A. (1997) Both phosphorylation and dephosphorylation of ASF/SF2 are required for pre-mRNA splicing *in vitro*. *RNA*, **3**, 1456–1467.
38. Sanford,J.R., Ellis,J.D., Cazalla,D. and Cáceres,J.F. (2005) Reversible phosphorylation differentially affects nuclear and cytoplasmic functions of splicing factor 2/alternative splicing factor. *Proc. Natl Acad. Sci. USA*, **102**, 15042–15047.
39. Prasad,J., Colwill,K., Pawson,T. and Manley,J.L. (1999) The protein kinase Clk/Sty directly modulates SR protein activity: both hyper- and hypophosphorylation inhibit splicing. *Mol. Cell. Biol.*, **19**, 6991–7000.
40. Patel,N.A., Kaneko,S., Apostolatos,H.S., Bae,S.S., Watson,J.E., Davidowitz,K., Chappell,D.S., Birnbaum,M.J., Cheng,J.Q. and Cooper,D.R. (2005) Molecular and genetic studies imply Akt-mediated signaling promotes protein kinase CbetaII alternative splicing via phosphorylation of serine/arginine-rich splicing factor SRp40. *J. Biol. Chem.*, **280**, 14302–14309.
41. Zhou,Z., Qiu,J., Liu,W., Zhou,Y., Plocinik,R.M., Li,H., Hu,Q., Ghosh,G., Adams,J.A., Rosenfeld,M.G. *et al.* (2012) The Akt-SRPK-SR axis constitutes a major pathway in transducing EGF signaling to regulate alternative splicing in the nucleus. *Mol. Cell*, **47**, 422–433.
42. Hayes,G.M., Carrigan,P.E. and Miller,L.J. (2007) Serine-arginine protein kinase 1 overexpression is associated with tumorigenic imbalance in mitogen-activated protein kinase pathways in breast, colonic, and pancreatic carcinomas. *Cancer Res.*, **67**, 2072–2080.
43. Ngo,J.C., Chakrabarti,S., Ding,J.H., Velazquez-Dones,A., Nolen,B., Aubol,B.E., Adams,J.A., Fu,X.D. and Ghosh,G. (2005) Interplay between SRPK and Clk/Sty kinases in phosphorylation of the splicing factor ASF/SF2 is regulated by a docking motif in ASF/SF2. *Mol. Cell*, **20**, 77–89.
44. Di Agostino,S., Rossi,P., Geremia,R. and Sette,C. (2002) The MAPK pathway triggers activation of Nek2 during chromosome condensation in mouse spermatocytes. *Development*, **129**, 1715–1727.
45. Fry,A.M., Schultz,S.J., Bartek,J. and Nigg,E.A. (1995) Substrate specificity and cell cycle regulation of the Nek2 protein kinase, a potential human homolog of the mitotic regulator NIMA of *Aspergillus nidulans*. *J. Biol. Chem.*, **270**, 12899–12905.