BioMed Research International

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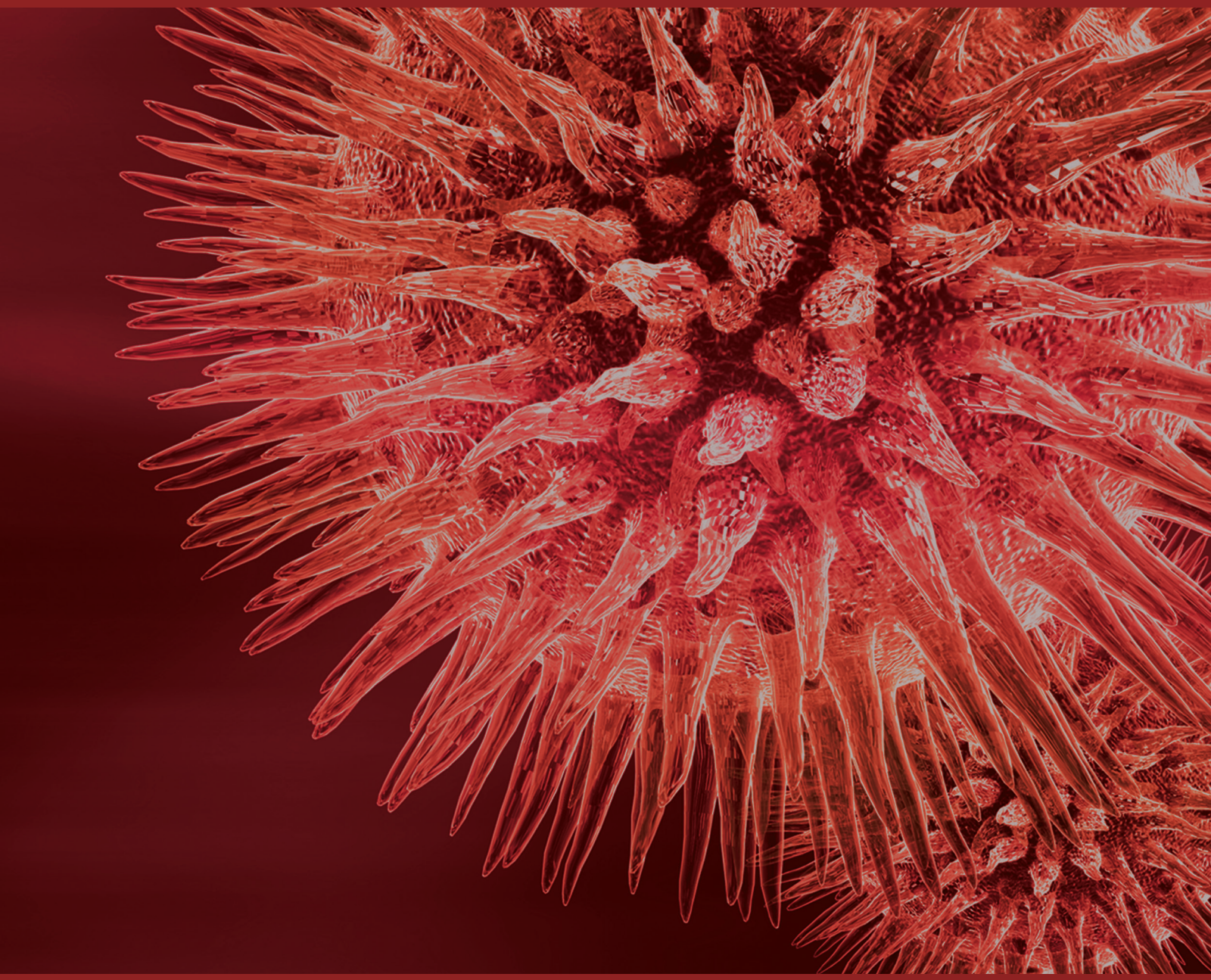
Guest Editors: Claudia Ghigna, Luca Cartegni, Peter Jordan, and Maria Paola Paronetto



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Editorial

Posttranscriptional Regulation and RNA Binding Proteins in Cancer Biology

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Following the completion of the human genome sequence and the concomitant technological innovations required for whole genome analyses, the last decade has witnessed an explosion of data and information concerning the posttranscriptional regulation of gene expression, in both pathological and nonpathological contexts. Among the most notable posttranscriptional events studied are the widespread usage of alternative splicing, the pleiotropic regulatory roles of miRNAs, and breakthroughs in the understanding of the control of gene expression by noncoding RNA transcripts.

In this special issue of this journal, the spotlight is centered on the role that various mechanisms of *posttranscriptional regulation*—and the *RNA binding proteins (RBPs)* that control them—play in *cancer biology*.

Adaptive changes in gene expression programs are crucial during tumor development, in order to allow cancer cells to support growth, survival, and metastasis and to resist to therapeutic treatments. Cancer cells have now been shown to efficiently adapt the expression of their proteome through changes in alternative splicing patterns, modifications of mRNA translational efficiency, or feedback modulators such as miRNAs and noncoding RNAs.

RBPs play a pivotal role in these processes and many of them are found aberrantly expressed in several tumor types. Moreover, each RBP most likely regulates a discrete but often broad subset of target transcripts at the same time, thus leading to an expanding functional network of changes that have important consequences for cancer cell biology.

Several of these changes occur at the level of alternative splicing, with the generation of variants that promote multiple aspects of tumorigenesis. V. Pagliarini et al. in "Splicing Regulation: A Molecular Device to Enhance Cancer Cell Adaptation" review some of the most striking and best-characterized examples of altered splicing events, which allow cancer cells to rapidly adapt to the adverse conditions encountered during the transformation process, leading to chemoresistance. In this regard, the authors discuss the possibility of new therapeutic protocols combining canonical chemotherapy with novel tools targeting this adaptive splicing response.

M. R. da Silva et al. in "Splicing Regulators and Their Roles in Cancer Biology and Therapy" explore the significance of cancer-associated alternative splicing events and then focus on the role of the major family of splicing regulators, SR proteins, and the kinases that regulate their activities. Their impact on cancer progression, as well as their possible use as targets for novel anticancer therapies, are discussed.

V. Gonçalves and P. Jordan in "Posttranscriptional Regulation of Splicing Factor SRSF1 and Its Role in Cancer Cell Biology" zoom in on the specific role played by one of the best-characterized SR-family proteins, SRSF1 (formerly SF2/ASF). Their review encompasses the posttranscriptional modifications and deregulated expression that contribute to transforming this essential splicing regulator into a powerful oncoprotein.

Our current understanding of how SAM68, a multifunctional member of the separate STAR (signal transduction

and activation of RNA metabolism) family of RBPs, affects key cellular regulatory circuitries and promotes cancer development and progression is summarized by P. Frisone et al. in "SAM68: Signal Transduction and RNA Metabolism in Human Cancer." In particular, the authors address how the transcriptional and posttranscriptional regulation of gene expression mastered by SAM68 contributes to changes occurring in cancer cells, thus opening the possibility of new therapeutic approaches targeting SAM68 activities in cancer.

Finally, the original research paper by E. Hong et al., "Unravelling the RNA-Binding Properties of SAFB Proteins in Breast Cancer Cells," sheds light on the RNA-map of SAFB protein in breast cancer cells and highlights the contribution of this relative newcomer to the complex deregulated landscape of RNA processing in tumors.

Another set of manuscripts addresses regulatory events involved in mRNA translation.

M. J. Halaby et al. in "Translational Control Protein 80 Stimulates IRES-Mediated Translation of p53 mRNA in Response to DNA Damage" contribute an original research paper on the regulation of cap-independent p53 protein translation upon genotoxic stress. In particular, they identify two novel regulators of the p53 Internal Ribosome Entry Site (IRES): the translational control protein 80 (TCP80) and the RNA helicase A (RHA). The functional interaction between these two proteins becomes relevant for p53 induction and its tumor suppressive function in response to DNA damage.

In addition, F. Han et al. in "Emerging Roles of MicroRNAs in EGFR-Targeted Therapies for Lung Cancer" review our current knowledge concerning the role of the deregulation of the EGFR signaling pathway in lung cancer. In particular, they discuss the involvement of miRNAs in the development of drug resistance to anti-EGFR agents in lung cancer cells, indicating their possible application as predictive biomarkers for anti-EGFR therapy.

Finally, the original research paper by P. Cremaschi et al., "An Association Rule Mining Approach to Discover lncRNAs Expression Patterns in Cancer Datasets," employs a bioinformatic approach (ARM (Association Rule Mining) methodology) for the meta-analysis of gene expression data. The ARM algorithm was applied for the study of differential expression profile of long noncoding RNAs (lncRNAs) in multiple tumor types and resulted in the identification of lncRNAs patterns differentially expressed in tumor versus normal tissues.

We hope that this special issue will contribute to a more thorough understanding of the role of posttranscriptional regulation and RBPs in tumorigenesis. In particular, a better comprehension of the molecular events that underlie malignant transformation will reveal potential novel drug targets for the development of more selective and effective anticancer therapies or identify novel biomarkers for disease progression or personalized patient stratification.

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Research Article

An Association Rule Mining Approach to Discover lncRNAs Expression Patterns in Cancer Datasets

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In the past few years, the role of long noncoding RNAs (lncRNAs) in tumor development and progression has been disclosed although their mechanisms of action remain to be elucidated. An important contribution to the comprehension of lncRNAs biology in cancer could be obtained through the integrated analysis of multiple expression datasets. However, the growing availability of public datasets requires new data mining techniques to integrate and describe relationship among data. In this perspective, we explored the powerness of the Association Rule Mining (ARM) approach in gene expression data analysis. By the ARM method, we performed a meta-analysis of cancer-related microarray data which allowed us to identify and characterize a set of ten lncRNAs simultaneously altered in different brain tumor datasets. The expression profiles of the ten lncRNAs appeared to be sufficient to distinguish between cancer and normal tissues. A further characterization of this lncRNAs signature through a comodulation expression analysis suggested that biological processes specific of the nervous system could be compromised.

1. Introduction

Cancer is a highly complex disorder characterized by the dysregulation of the expression of several genes preserving cellular identity and differentiation. A comprehensive analysis of gene expression profiles in different cancer types has been performed and numerous expression signatures have been identified [1–4]. In most cases the genes described for their involvement in cancer were protein-coding oncogenes and tumor suppressors. However, in the past few years it has become increasingly clear that the human genome is pervasively transcribed and thousands of genes producing noncoding RNAs (ncRNAs) with regulatory functions were identified [5]. In particular, long noncoding RNAs (lncRNAs), transcripts longer than 200 nucleotides with no significant open reading frames, have been shown as important regulators of transcriptional and posttranscriptional events [6, 7]. This finding has prompted the researchers to investigate their role in cancer [8, 9] and several lncRNAs have been implicated in both cancer development and progression, highlighting the high genetic complexity of the disease [10].

The lncRNAs exert their functional role in cancer through various biological mechanisms and in different stages of the tumorigenic process [11]. For example HOTAIR, one of the most well-known lncRNAs, was reported as a predictor of breast cancer metastasis and poor prognosis. HOTAIR interacts with chromatin-remodeling complexes to induce heterochromatin formation in different genomic loci thus silencing gene expression [12, 13]. lncRNAs have been described also for their direct interaction with negative regulators of transcription, like in the case of lincRNA-p21 that is activated by p53 upon DNA damage and plays its role associating with hnRNP-K which acts as a transcriptional repressor [14]. However, besides these and few other examples, the lncRNAs functional mechanisms are poorly understood and their role in cancer biology remains to be fully elucidated.

An important contribution to the comprehension of lncRNAs biology in cancer could be obtained through the integrated analysis of multiple expression datasets. Traditionally, the methods used to analyze gene expression data are mostly based on the application of clustering algorithms to datasets of specific biological conditions, an approach which leads to

the identification of comodulated groups of genes. However, with the growing availability of publicly available datasets, the use of new data mining techniques to integrate and to describe relationships among different types of data is highly desirable. In this perspective, the Association Rule Mining (ARM) based approaches, looking for frequent patterns in the data, have been proposed as an alternative methodology to analyze expression data [15, 16]. While this technique is commonly used in many research fields, its application in the analysis of gene expression is still limited due to the difficulties to deal with the high level of complexity and interconnection of biological processes despite several customization being proposed to overcome this issue [17–20].

In this paper, we proposed a new implementation of the ARM method for the meta-analysis of gene expression data and, in particular, to study differential expression profile of lncRNAs in multiple tumor types. The application of the ARM algorithm led us to define a total of 102 nonredundant frequent rules in lncRNAs transcriptional levels distinguishing tumor from corresponding normal tissues. We focused on the rule including the highest number of lncRNAs in brain cancers that was confirmed by independent microarray and RNA-seq datasets. Moreover, a comodulation analysis of the lncRNAs rule allowed us to shed light on putative biological processes impaired in brain tumors.

2. Materials and Methods

2.1. Long Noncoding RNA Definition. For the purpose of this study, we employed the list of lncRNAs compiled from Gencode (release 19) [21]. The selected genes corresponded to the following transcript types: 3prime_overlapping_ncrna (21), antisense (5276), lincRNA (7114), processed_transcript (515), sense_intronic (742), and sense_overlapping (202) for a total of 13870 transcripts.

2.2. Expression Datasets Description. For the purpose of the ARM analysis (see Section 2.3), items were represented by differentially expressed genes. Differentially expressed genes from cancer-related datasets were obtained from the CorrelaGenes database [27]. In brief, human-specific datasets were selected from the Gene Expression Omnibus (GEO) [28] Curated DataSets (GDS) and downloaded with the R package GEOquery (ver. 2.32.0) [29]. The datasets were analyzed with R package limma (ver. 3.11.1) [30]. All the results were stored in a PostgreSQL database (http://www.postgresql.org/). For this study we selected those datasets performed on the platform "Affymetrix Human Genome U133 Plus 2.0 Array" and related to cancer tissues. This selection allowed the identification of 26 datasets including 50 comparisons. From each comparison, we selected gene symbols with at least one mapped probe having an absolute value of LFC greater or equal to 1, False Discovery Rate (FDR) corrected p value lower than 0.05 and corresponding to a known lncRNA. This selection allowed the identification of 34 gene lists that were organized in the form of transactions for the application of the Association Rule Mining algorithm.

The ARM analysis results were compared to differentially expressed lncRNAs obtained in an independent dataset including samples from the tissues of interest. To this aim we selected the dataset E-GEOD-16011 (GSE16011) that was not present in the CorrelaGenes database. The expression set was downloaded from the ArrayExpress repository in the form of R expression set (http://www.ebi.ac.uk/arrayexpress/files/E-GEOD-16011/E-GEOD-16011.eSet.r). The expression sets were renormalized with Robust Multiarray Average (RMA) expression measure process (R package affy ver. 1.44.0) [31] and analyzed with R package limma (ver. 3.11.1) using gene annotations from platform "Affymetrix Human Genome U133 Plus 2.0 Array."

2.3. Association Rule Mining Methodology. The identification of frequent patterns was performed using the Association Rule Mining algorithm implemented in the R package arules ver. 1.1.5 [32]. In the ARM formalism, datasets are organized in the form of transactions. Each transaction contains a list of elements, called items, whose nature depends on the application. In our context, each transaction corresponds to a comparison and includes all lncRNAs with at least one differentially expressed probe (absolute value ≥ 1 and FDR adjusted p value ≤ 0.05). The application uses the transactions to identify association rules (ARs) of the form IF A then C (A=>C). In our context, these rules can be interpreted as follows: if Set of Genes 1 is differentially expressed in a comparison then Set of Genes 2 is differentially expressed as well [16].

To measure the quality of the associations, we herein used two indexes: support and confidence. Considering two generic gene sets X and Y the two measures are defined as follows. (i) Support: the probability to find all the genes in sets X and Y differentially expressed in the same comparison. Formally Sup. = $\Pr(X \cup Y)$. (ii) Confidence: the probability to find all the genes in set Y differentially expressed in a comparison where all the genes in set X are differentially expressed. Formally Conf. = $\Pr(X \mid Y)$.

In our study we defined as redundant a set of rules characterized by the same set of genes or a subset of it and with the same support. In order to remove redundancy for each set of redundant rules we retained only the set including the highest number of genes ($X \cup Y$).

2.4. Principal Component Analyses. PCA is a technique that uses an orthogonal transformation to convert a dataset onto a linear space spanned by a number of linearly independent components, named principal components, ordered by decreasing variance. The projection of the observations onto the first few principal components (i.e., PC1 and PC2) allows a reduced dimensionality maximizing the variance retained. PCA was performed with the R package FactoMineR ver. 1.29 [33]. The expression data table (Row: probes; Columns: samples) related to the DataSets GDS1962 and E-GEOD-16011 were extracted from the eSet R object and used for the PCA. In the analysis we used as variables the log2 normalized intensity values of platform probes without scaling. The different samples were used as individuals and they were labeled according to their histological classification.

2.5. RNA-Seq Data Analysis. RNA-seq data were used as an independent approach to validate differential expression of lncRNAs. RNA-seq data used in this study were downloaded from ArrayExpress (https://www.ebi.ac.uk/arrayexpress/) and NCBI SRA (http://www.ncbi.nlm.nih.gov/sra/) repositories. Three samples of normal brain, under the accession number E-MTAB-1733, were downloaded from ArrayExpress (ERR315477, ERR315455, and ERR315432). All tumor samples were downloaded from NCBI SRA (study SRP027383). We used three samples of glioblastoma (SRR934934, SRR934966, and SRR934911), three samples of oligodendroglioma (SRR934990, SRR934971, and SRR934734), and three samples of astrocytoma (SRR934772, SRR934784, and SRR934794). All samples share common sequencing features: they were sequenced using the Illumina HiSeq 2000 platform and a paired-end protocol (2×101 bp) for a total of about 60 million reads each.

Processing of RNA-seq data was performed following the protocol described in Trapnell et al. [34]. In brief, raw sra files were transformed into fastq files using SRA Toolkit available at NCBI. Raw reads were subjected to standard quality control procedures with the NGSQC-toolkit software and aligned to the human genome reference sequence (NCBI37/hg19) by the TopHat alignment software. Genes were annotated using the lncRNAs annotation file coming from Gencode (release 19). lncRNAs genes were quantified according to the TopHat-Cufflinks protocol and differential gene expression analysis was performed by CuffDiff [34]. Visualization of genomic alignments of RNA-seq reads was obtained with the IGV tool [35].

2.6. Comodulation Expression Analysis. The comodulation expression analysis was performed with the CorrelaGenes web application [27]. The tool uses an implementation of the Association Rule Mining algorithm based on three main customizations: (i) it extracts association rules based on two genes; (ii) one of the involved genes is constrained to be the gene selected by the user (target gene); (iii) the association indexes are calculated based on the transitions where both the target and the associated genes were present to account for the heterogeneity of the different platforms. These customizations allow CorrelaGenes to identify sets of genes whose expression appeared altered in different experimental conditions simultaneously with the target gene thus suggesting their coordinated action in the same biological process. The analysis in CorrelaGenes [27] was performed with the default parameters with the exception of copresence ≥ 10, LIFT ≥ 0, χ^2 p value ≤ 1. The gene Target Sign parameter was selected, for each analysis, equal to the LFC sign of the gene in brain cancer tissues (Sign +1 for ncRNA upregulated in brain cancer; Sign −1 for ncRNA downregulated in brain cancer). To improve the significance of the results we further ranked the CorrelaGenes output based on the Correlation index [36] calculated using the standard CorrelaGenes output. Only genes with a Correlation index greater than 0.3 were retained for the next step of the analysis.

2.7. Gene Ontology Term Enrichment Analysis and Network Visualization. The analysis of the Gene Ontology (GO) term enrichment was performed by the GOFunction R package ver. 1.14.0 [37]. The R packages biomaRt 2.20 was used to convert gene symbols into Entrez Gene IDs required by the GOFunction R package. The GO terms definition was obtained by the org.Hs.eg.db 3.0.0 R package [38]. The Benjamini correction was applied to Fisher Exact Test p values of enriched GO terms and considered as significant if lower than 0.05. In order to minimize the Gene Ontology (GO) term overrepresentation we selected the most specific term of each ontology (i.e., marked as "Final" in the GOFunction R package). The lists of genes associated with specific GO terms were downloaded using the QuickGO web tool (http://www.ebi.ac.uk/QuickGO/) [39].

The GeneMANIA (http://www.genemania.org/) [40] and STRING 9.1 (http://string-db.org/) [41] web tools were used to visualize the network of interactions among genes.

3. Results and Discussion

3.1. Association Rule Mining Meta-Analysis. We applied the ARM method to identify common patterns of long noncoding RNAs differential expression distinguishing tumor samples from their respective not affected tissues. For this purpose, we selected 26 microarray datasets from the GEO Datasets Archive (http://www.ncbi.nlm.nih.gov/gds) from which a total of 34 pairwise comparisons (i.e., tumor against normal tissue) showing expression modulation for at least one lncRNA were assessed (see Section 2.2 and Supplementary Table I available online at http://dx.doi.org/10.1155/2015/146250). The lists of differentially expressed lncRNAs were used as input for the ARM algorithm. After applying a support threshold of ≥0.15, ensuring that the identified rules were present in at least 6 out of 34 comparisons tested, and a confidence threshold equal to 1, ensuring that the identified rules were confirmed in all the comparisons where the gene set is differentially expressed (i.e., the rule "if gene X is modulated then gene Y is modulated" is true in all the comparisons where the gene X is modulated), the ARM algorithm identified 59,542 redundant rules each including a number of lncRNAs ranging from 2 to 13. The obtained rules resulted based on the differential expression of 53 lncRNAs assorted in 102 nonredundant rules (Supplementary Tables II and III). In Figure 1 is shown the distribution of the identified 102 nonredundant rules based on (i) the number of ncRNAs contained (Figure 1(a)) and (ii) the threshold of support (Figure 1(b)).

In order to verify the consistency of the results obtained we performed a simulation analysis running the ARM algorithm for 100 times on a comparable set of randomly selected comparisons and applying the same selection thresholds to extract rules. The results of the simulation test were analyzed in terms of the number of rules obtained and of the number of lncRNAs included in each rule. We found that only four simulations generated a number of redundant rules (i.e., >10.000) comparable with those found in the cancer dataset and only 4 simulations produced at least one rule containing more than 10 lncRNAs (Figure 2).

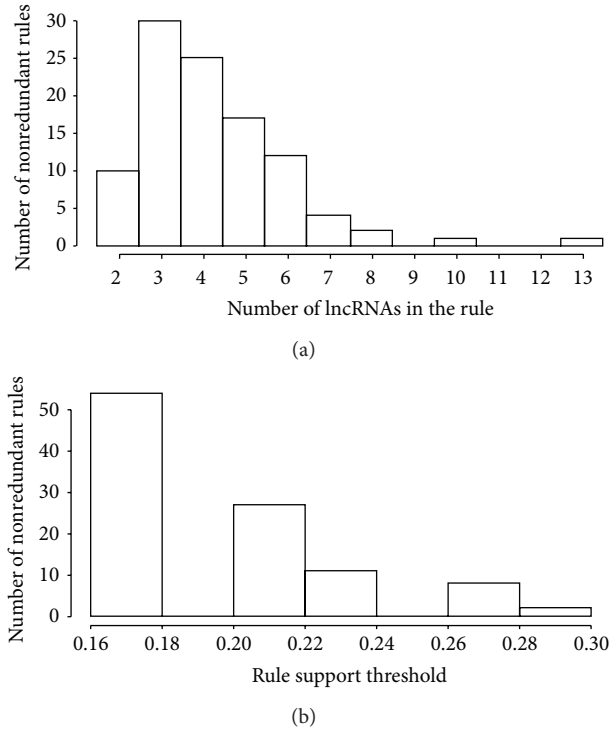


Figure 1: Distribution of the identified 102 nonredundant rules. (a) Distribution of identified rules based on the number of lncRNAs contained; (b) distribution of the identified rules based on support thresholds.

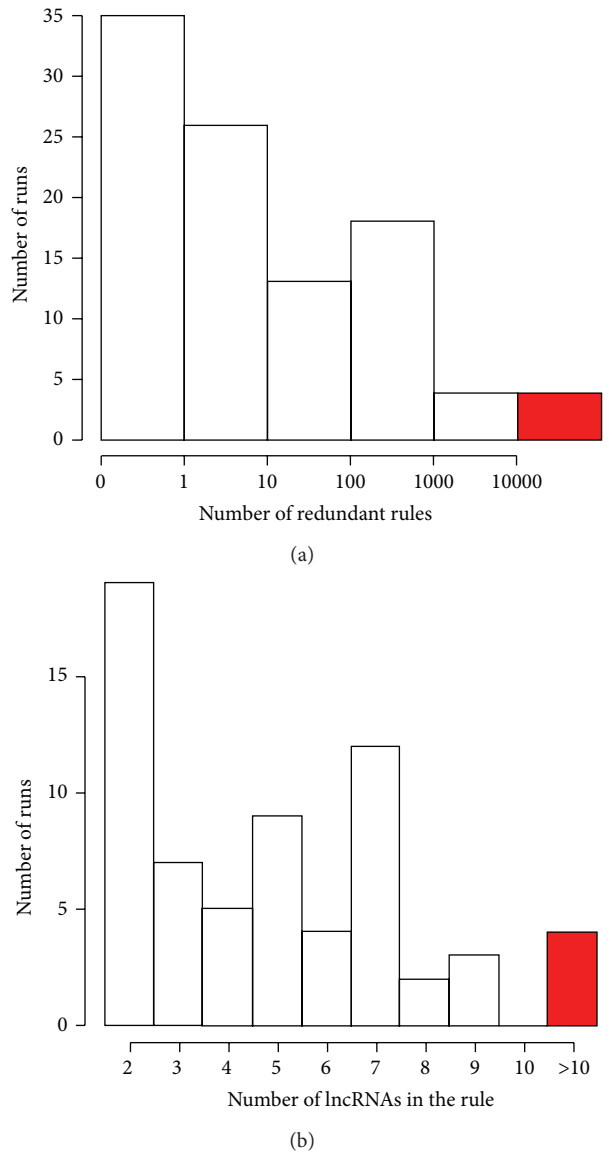


Figure 2: Distribution of the results of the 100 simulation runs. (a) Distribution of the number of redundant rules produced in the simulation runs; (b) distribution of the number of lncRNAs contained in the wider rule in each simulation run.

The implementation of the ARM algorithm we proposed here represents a new way to integrate heterogeneous expression data converting them in transactions that could be then compared to identify frequent patterns of differential expression. This application of the ARM method allowed us to identify 102 nonredundant rules representing frequent patterns of lncRNAs expression potentially elucidating the biological processes involved in tumorigenesis. To reduce the likelihood of generating false hypotheses, we applied a conservative confidence threshold (Conf. = 1) accounting for the limited number of comparisons available for this meta-analysis. The availability of a larger number of datasets would produce informative results even considering a lower confidence threshold. The consistence of our approach was assessed through a 100-run simulation on randomly selected datasets showing that the results obtained were unlikely due to randomness thus supporting further investigation.

3.2. Thirteen-Gene Rule Characterization and Validation. We concentrated our attention on the rule containing the highest number of lncRNAs (i.e., 13 lncRNAs) showing modulation of their expression in a total of six comparisons. Among the 13 lncRNAs of the rule, five (i.e., CRNDE, DLEU2, MEG3, PART1, and RFPL1S) were previously reported as involved in multiple tumor types [22, 24–26, 42] while nothing was known for six of them (i.e., KRTAP5-AS1, LINC00301, OIP5-AS1, PPP1R26-AS1, RUSC1-AS1, and UBL7-AS1). For two of the lncRNAs included in the rule (i.e., SYN2 and UHRF1), the noncoding transcript overlaps with protein-coding isoforms of the same gene thus preventing us to distinguish between the two types of molecules (Table 1).

The 13 lncRNAs rule was identified in five comparisons from the GEO dataset GDS1962 testing different kind of human brain tumors (i.e., astrocytoma grades II and III, glioblastoma grade IV, and oligodendroglioma grades II and III) against normal brain tissues. In the five comparisons, the differential expression of the 13 lncRNAs was highly consistent showing eight lncRNAs always downregulated and five lncRNAs always upregulated (Table 2). The sixth comparison supporting the 13 lncRNAs rule came from GEO dataset GDS3592 in which ovarian cancer epithelial cells were compared to normal tissue. In this comparison, the majority

Table 1: List of the 13 lncRNAs.

Number	lncRNA symbol	lncRNA name	Reference
1	CRNDE	Colorectal neoplasia differentially expressed	Ellis et al., 2012 [22] Zhang et al., 2012 [23]
2	DLEU2	Deleted in lymphocytic leukemia 2	Lerner et al., 2009 [24]
3	KRTAP5-AS1	KRTAP5-1/KRTAP5-2 antisense RNA 1	
4	LINC00301	Long intergenic non-protein coding RNA 301	
5	MEG3	Maternally expressed 3	Wang et al., 2012 [25] Zhang et al., 2012 [23]
6	OIP5-AS1	OIP5 antisense RNA 1	
7	PART1	Prostate androgen-regulated transcript 1	Zhang et al., 2013 [26]
8	PPP1R26-AS1	PPP1R26 antisense RNA 1	
9	RFPL1S	RFPL1 antisense RNA 1	Zhang et al., 2012 [23]
10	RUSC1-AS1	RUSC1 antisense RNA 1	
11	SYN2*	Synapsin II	
12	UBL7-AS1	UBL7 antisense RNA 1	
13	UHRF1*	Ubiquitin-like with PHD and ring finger domains 1	

*lncRNA not distinguishable from the protein coding isoform.

of the lncRNAs (10/13) resulted upregulated and seven lncRNAs (i.e., MEG3, KRTAP5-AS1, LINC00301, PART1, PPP1R26-AS1, SYN2, and CRNDE) appeared modulated in the opposite direction with respect to the brain tumor samples (Table 2).

In order to assess the reliability of our findings, we exploited the E-GEOD-16011 microarray dataset downloaded from the ArrayExpress archive (https://www.ebi.ac.uk/arrayexpress/) and RNA-seq data from NCBI SRA study SRP027383 including brain tumor samples with an histological classification comparable to the ones in the GDS1962 dataset. The validation of ovarian cancer data could not be performed due to the unavailability of comparable expression datasets. From the analysis of expression profiles obtained in the E-GEOD-16011 and in the SRP027383 RNA-seq study, we were able to confirm the altered expression of six lncRNAs (i.e., RFPL1S, KRTAP5-AS1, PART1, and SYN2 which appeared consistently downregulated and DLEU2 and UHRF1 which appeared consistently upregulated). The expression of four of the 13 lncRNAs was considered as consistent with previous findings although they showed less severe modulation of their transcription levels (i.e., OIP5-AS1 and UBL7-AS1) or their expression values could not be assessed in all samples tested (i.e., CRNDE and RUSC1-AS1). Three lncRNAs were not validated: two of them (i.e., LINC00301 and PPP1R26-AS1) resulted not significantly modulated in the RNA-seq analysis and the MEG3 lncRNA appeared modulated in two out of three samples but with discordant values (Table 3). In Figure 3, the expression profiles of the CRNDE and PART1 lncRNAs from RNA-seq data were shown as example (the expression profiles of the eight remaining lncRNAs were shown in Supplementary Figure 1). Thus, we were able to confirm the altered expression of 10 out of the 13 lncRNAs identified by the ARM method on GDS1962.

Among the 10 confirmed lncRNAs, four were previously described as involved in the genesis of different tumors. In particular, CRNDE appeared to be upregulated in colorectal cancer, leukemia, and gliomas concordantly with our observations [22, 26]. DLEU2 was known to be frequently deleted in lymphocytic leukemia [24], while our study revealed an upregulation of its expression in gliomas suggesting a tissue-specific regulation of this gene. Interestingly, three out of 10 lncRNAs were previously identified as part of a signature able to distinguish among different types and grades of gliomas [26, 42]. Consistently with the signatures of Zhang et al., identified using the same datasets of the present analysis, we reported the differential expression of CRNDE, PART1, and RFPL1S. The lack of a complete overlap between the studies could be due to three main factors: (i) different criteria to select probes mapped to lncRNAs; (ii) a different statistical model for the identification of differential expressed genes, or (iii) a different study design to identify gene signatures. These observations, validated in different datasets and confirmed by previous studies, suggest that the ARM method was a suitable approach to identify set of genes whose altered expression is peculiar of brain tumor.

3.3. Principal Component Analysis. In order to investigate the power of the 10 lncRNAs rule to distinguish among brain tumor and normal samples, we performed a Principal Component Analysis (PCA) using the probe intensity values from GEO dataset GDS1962 as variables. Figure 4 showed principal components (PC) 1 and 2 obtained using intensities of all probes (Figure 4(a)) or only probes corresponding to the 10 lncRNAs (Figure 4(b)). In both analyses, the majority of normal brain samples appeared as a separate cluster distinguishable from tumor tissues. This observation was confirmed by the PCA performed on ArrayExpress dataset E-GEOD-16011 (Figures 4(c) and 4(d)) that showed similar pattern of clustering among normal and tumor samples. Moreover, a certain degree of clustering was also appreciable when tumor

Table 2: LFC of the 13 lncRNAs in GEO datasets.

lncRNA symbol	Gene ID	lncRNA name	GDS1962					GDS3592
			Astrocytoma (grade II)	Astrocytoma (grade III)	Glioblastoma (grade IV)	Oligodendroglioma (grade II)	Oligodendroglioma (grade III)	Ovarian cancer epithelial cells
OIP5-AS1	729082	OIP5 antisense RNA 1	−1	−1.3	−1.5	−1	−1.5	−1.3
RFPL1S	10740	RFPL1 antisense RNA 1	−2.5	−2.6	−3.8	−2.3	−3.3	−2.5
MEG3	55384	Maternally expressed 3	−2.4	−2.8	−2.7	−2.6	−2.7	1.2
KRTAP5-AS1	338651	KRTAP5-1/KRTAP5-2 antisense RNA 1	−1.7	−1.7	−2	−1.1	−1.8	1
LINC00301	283197	Long intergenic non-protein coding RNA 301	−2.2	−1.5	−1.9	−1.4	−2.1	1.9
PART1	25859	Prostate androgen-regulated transcript 1	−1.4	−1.7	−2	−1.4	−1.9	2.4
PPP1R26-AS1	100506599	PPP1R26 antisense RNA 1	−1.4	−1.4	−1.2	−1.1	−1.4	1.9
SYN2	6854	Synapsin II	−2.6	−2.6	−4	−2.5	−3.8	2.2
CRNDE	643911	Colorectal neoplasia differentially expressed	3.2	3.6	4.2	1.8	3.7	−4.3
RUSC1-AS1	284618	RUSC1 antisense RNA 1	1.6	1.5	1.2	1.4	1.5	2
UBL7-AS1	440288	UBL7 antisense RNA 1	1.8	1.6	1.5	1.4	1.8	1.6
DLEU2	8847	Deleted in lymphocytic leukemia 2	1	1	1.5	1	1.4	1.5
UHRF1	29128	Ubiquitin-like with PHD and ring finger domains 1	2.5	3.6	4	3.1	3.8	3.4

Table 3: LFC of the 13 lncRNAs in different brain cancer datasets.

		OIP5-AS1	RFPL1S	MEG3	KRTAP5-AS1	LINC00301	PART1	PPP1R26-AS1	SYN2	CRNDE	RUSC1-AS1	UBL7-AS1	DLEU2	UHRF1
GDS1962	Astrocytoma (grade II)	−1.0	−2.5	−2.4	−1.7	−2.2	−1.4	−1.4	−2.6	3.2	1.6	1.8	1.0	2.5
	Astrocytoma (grade III)	−1.3	−2.6	−2.8	−1.7	−1.5	−1.7	−1.4	−2.6	3.6	1.5	1.6	1.0	3.6
	Glioblastoma (grade IV)	−1.5	−3.8	−2.7	−2.0	−1.9	−2.0	−1.2	−4.0	4.2	1.2	1.5	1.5	4.0
	Oligodendroglioma (grade II)	−1.0	−2.3	−2.6	−1.1	−1.4	−1.4	−1.1	−2.5	1.8	1.4	1.4	1.0	3.1
	Oligodendroglioma (grade III)	−1.5	−3.3	−2.7	−1.8	−2.1	−1.9	−1.4	−3.8	3.7	1.5	1.8	1.4	3.8
E-GEOD-16011	Astrocytoma (grade II)	−1.0	−3.8	−2.7	−1.0	−0.3	−2.9	−0.3	−3.8	2.8	n.s.	0.5	1.4	2.9
	Astrocytoma (grade III)	−1.5	−4.6	−3.8	−1.1	−0.3	−3.4	−0.4	−5.4	3.7	0.8	0.9	1.9	3.2
	Glioblastoma (grade IV)	−1.8	−4.8	−3.7	−1.1	−0.3	−3.3	−0.2	−5.4	4.4	n.s.	0.8	1.4	3.6
	Oligodendroglioma (grade II)	−1.0	−3.1	−2.7	−1.0	−0.3	−3.1	−0.3	−3.9	n.s.	1.0	0.7	1.6	3.7
	Oligodendroglioma (grade III)	−1.5	−3.6	−3.7	−1.0	−0.3	−3.3	−0.3	−5.1	2.8	1.1	0.8	1.8	3.4
RNAseq	Astrocytoma	−0.3	−2.2	n.s.	−3.0	n.s.	−3.0	n.s.	−2.5	4.7	1.5	2.0	2.3	2.5
	Glioblastoma	−0.3	−4.7	0.6	−3.2	n.s.	−4.2	n.s.	−2.2	4.7	n.s.	1.8	2.1	1.8
	Oligodendroglioma	−0.4	−1.7	−2.0	−1.4	n.s.	−1.0	n.s.	−3.8	4.9	1.0	2.3	4.0	2.5

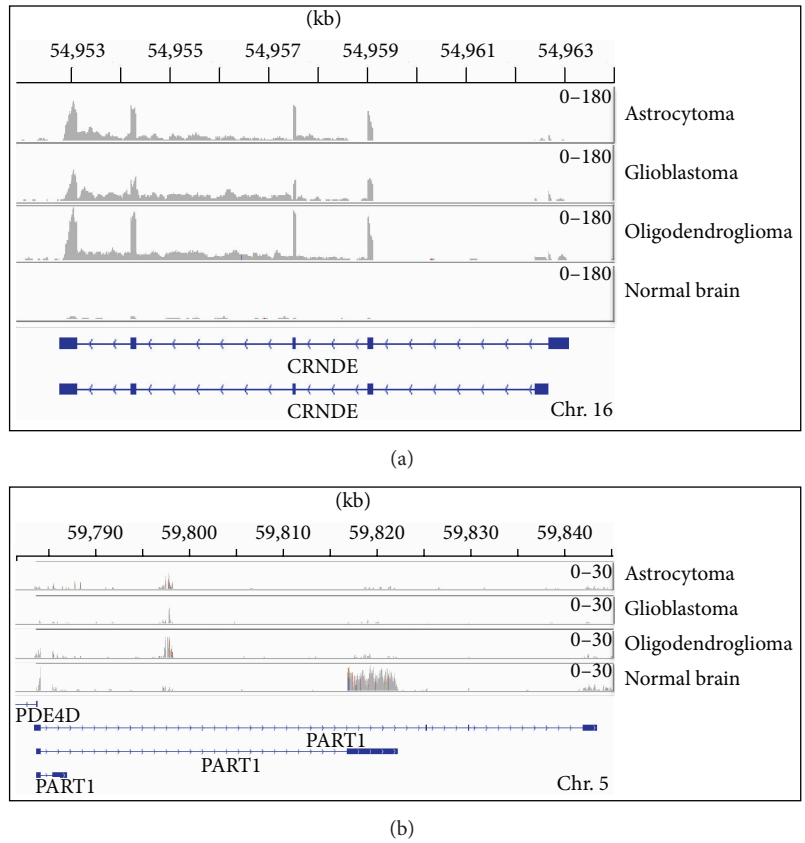


Figure 3: Genomic alignments of RNA-seq reads corresponding to the lncRNAs: (a) CRNDE and (b) PART1 in the three brain tumors types. The visualization of the alignment was obtained with the IGV software.

samples were labeled according to tumor type and grade (Supplementary Figure 2).

The PC analysis performed on the two independent datasets suggested that the 10 lncRNAs expression levels were sufficient to clearly separate samples belonging to the two groups.

3.4. Comodulation Gene Expression Analysis. In order to get insight into the putative involvement of the 10 long noncoding molecules in specific biological processes, we performed a comodulation analysis. For this purpose, we exploited our CorrelaGenes tool [27] looking for set of genes altered in their expression levels simultaneously with the up- or downregulation of each of the 10 lncRNAs. The CorrelaGenes tool (http://www.igm.cnr.it/cabgen/web-correlagenes0/) was queried for each lncRNAs with LFC > +1 or LFC < −1 according to their sign in the rule, in order to identify genes showing significant alteration of their expression (i.e., |LFC| > 1) in a significant proportion of comparisons tested. The analyses resulted in a total of 10 gene lists including a number of genes between 1675 and 6601 (Supplementary Tables S4 and S5). For each gene list, an enrichment analysis for Gene Ontology terms was conducted by means of the R/Bioconductor GOfunction package [37] using up- or downregulated genes separately (Supplementary Tables S6 and S7).

For all the 10 lists of downregulated genes, the analysis showed highly significant enrichments mainly concentrated in three categories: (i) "Synaptic transmission" (GO:0007268), (ii) "Ion transport" (GO:0006811) and related terms, and (iii) "Nervous System Development" (GO:0007399). The analysis of a list of 503 "common" genes, found in at least nine out of the 10 lists, confirmed the enrichment for the same categories (Figure 5 and Supplementary Figure 3). Interestingly, these results appeared highly consistent with the neuronal enriched GO categories found in the article of Liu and coauthors [43]. In this paper, authors performed an analysis of miRNAs differential expression in pediatric gliomas together with a GO terms enrichment analysis of miRNA target genes resulting in the identification of several neuronal GO categories belonging to the "Synaptic transmission" and "Nervous System Development" clades. Any GO term related to the "Ion transport" category resulted significantly enriched in the work of Liu and colleagues leading us to speculate about a specific role of lncRNAs in this specific biological process.

Taking into consideration the upregulated transcripts, the number of "common genes" resulted highly reduced (i.e., n = 150) and, as expected, not significantly enriched for any GO term. However, the analysis of single gene lists allowed us to group some recurrent GO terms in three enriched categories: (i) "Cell cycle" (GO:0007049) and related terms such

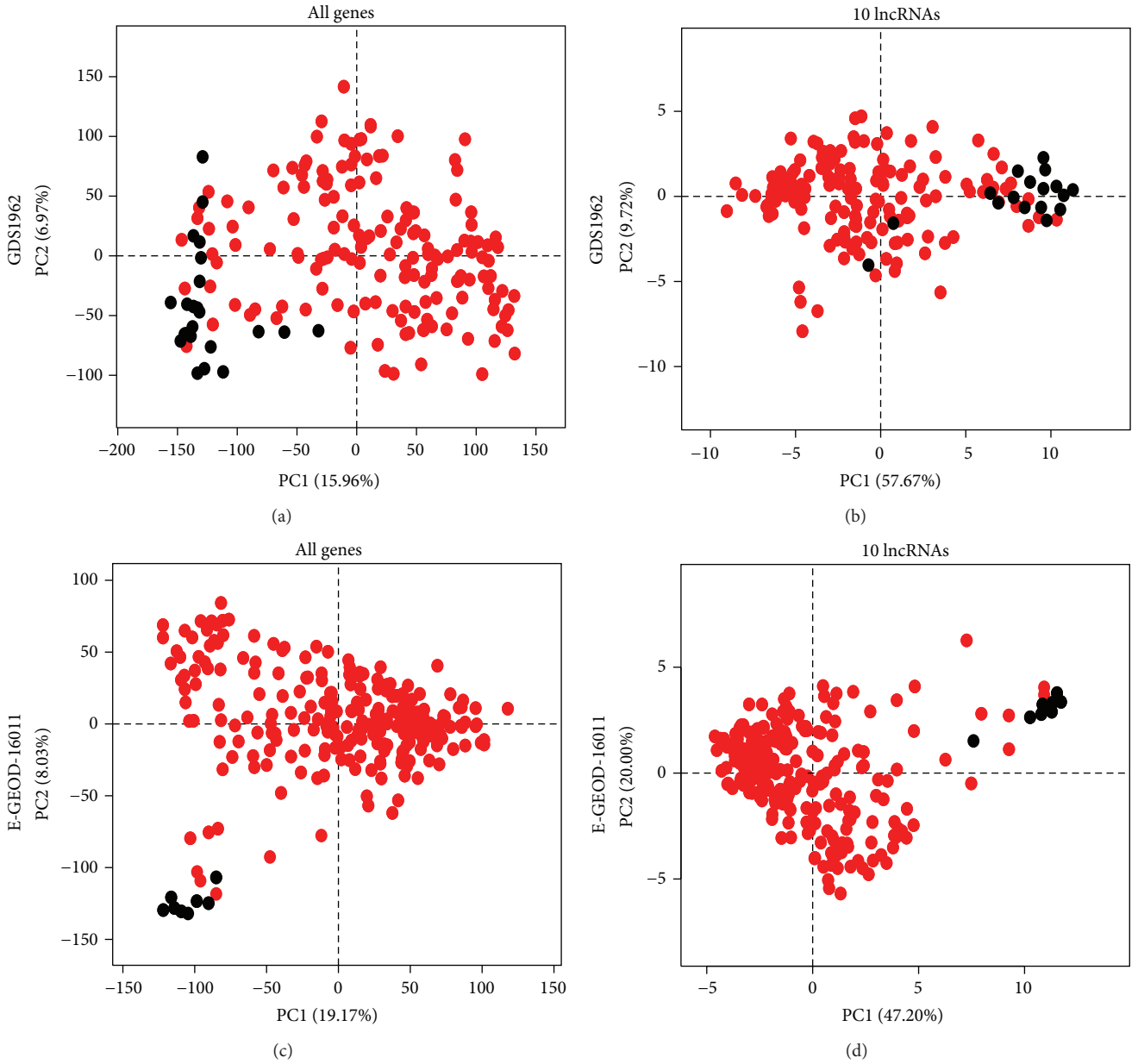


Figure 4: Principal Component Analysis (PCA) performed on the GEO dataset GDS1962 (a and b) and ArrayExpress dataset E-GEOD-16011 (c and d) considering intensity values of all probes (a and c) or only probes corresponding to the 10 lncRNAs (b and d). Red dots correspond to brain tumor samples and black dots correspond to normal brain samples.

as "Mitotic cell cycle" (GO:0000278), "Cell cycle process" (GO:0022402), and "Cell cycle checkpoint" (GO:0000075), enriched in seven out of 10 gene lists (with adjusted p values ranging from 1×10^{-14} to 1×10^{-2}); (ii) the "RNA metabolic process" (GO:0016070) which includes terms such as "mRNA metabolic process" (GO:0016071), "RNA splicing" (GO:0008380), and "Regulation of mRNA stability" (GO:0043488), enriched in five out of 10 gene lists (with adjusted p values ranging from 1×10^{-9} to 1×10^{-3}); (iii) the "Gene expression" (GO:0010467) to which belong terms as "Regulation of transcription from RNA polymerase II promoter" (GO:0006357) and "Positive regulation of gene expression" (GO:0010628), enriched in four out of 10 gene lists (with adjusted p values ranging from 1×10^{-6} to 1×10^{-3}).

Among several other features, we focused on the "RNA metabolic process" category that includes many genes involved in posttranscriptional modification pathways. Taking into account all genes annotated in the "RNA metabolic process" category and all its children terms, a pool of 109 genes were found present in at least seven out of the 10 lists of upregulated genes. A functional analysis performed using both STRING and GeneMANIA tools allowed us to select

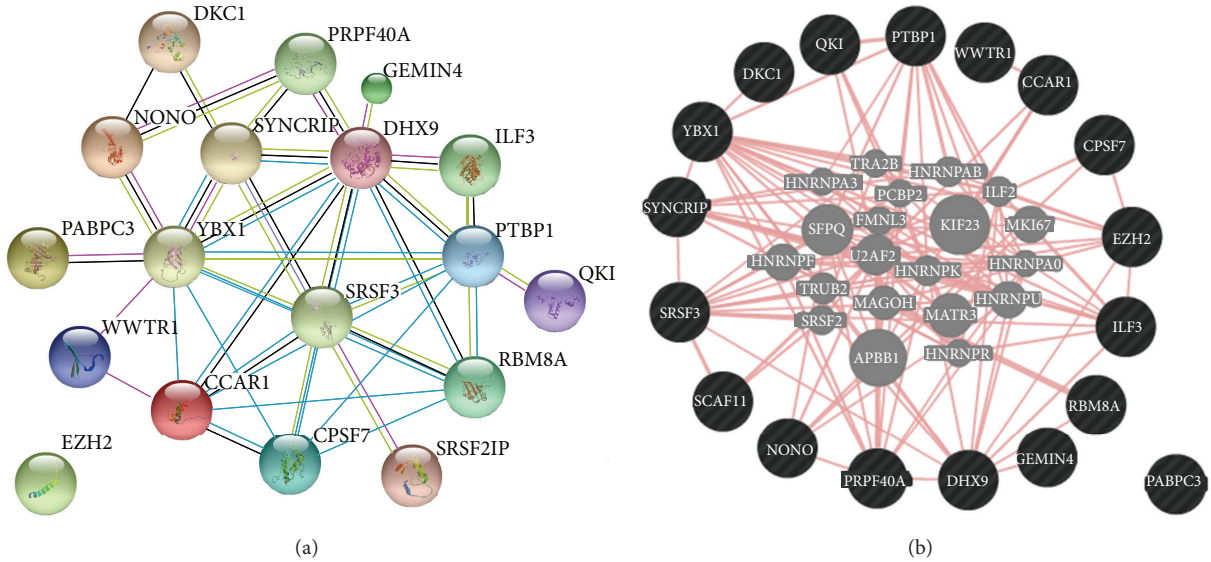
lncRNA	n	Rank 1	2	3	4	5
CRNDE	2467	GO:0007268 <1.00$E-14$ 241	GO:0007399 <1.00$E-14$ 392	GO:0043269 9.21$E-11$ 96	GO:0006887 3.33$E-09$ 86	GO:0006836 3.45$E-09$ 51
DLEU2	1852	GO:0007268 9.82$E-11$ 120	GO:0007269 1.51$E-03$ 27	GO:0031175 3.62$E-02$ 100	– – –	– – –
KRTAP5-AS1	1810	GO:0006836 <1.00$E-14$ 58	GO:0007268 <1.00$E-14$ 250	GO:0007399 <1.00$E-14$ 334	GO:0007626 <1.00$E-14$ 57	GO:0030001 <1.00$E-14$ 127
OIP5-AS1	2778	GO:0007268 <1.00$E-14$ 215	GO:0006396 3.63$E-06$ 160	GO:0034660 1.01$E-04$ 83	GO:0007399 3.58$E-03$ 345	GO:0006996 7.88$E-03$ 448
PART1	1181	GO:0006812 <1.00$E-14$ 100	GO:0007268 <1.00$E-14$ 146	GO:0034220 <1.00$E-14$ 89	GO:0007399 1.75$E-12$ 179	GO:0043269 3.74$E-10$ 55
RFPL1S	4108	GO:0006836 <1.00$E-14$ 73	GO:0007268 <1.00$E-14$ 316	GO:0007399 <1.00$E-14$ 524	GO:0034220 <1.00$E-14$ 195	GO:0044708 <1.00$E-14$ 143
RUSC1-AS1	2123	GO:0006811 <1.00$E-14$ 251	GO:0007268 <1.00$E-14$ 238	GO:0007399 <1.00$E-14$ 385	GO:0050890 <1.00$E-14$ 70	GO:0007186 7.60$E-12$ 138
SYN2	3117	GO:0006836 <1.00$E-14$ 62	GO:0007268 <1.00$E-14$ 268	GO:0007399 <1.00$E-14$ 422	GO:0034220 <1.00$E-14$ 163	GO:0006813 2.45$E-11$ 60
UBL7-AS1	2266	GO:0006836 <1.00$E-14$ 55	GO:0007268 <1.00$E-14$ 261	GO:0007399 <1.00$E-14$ 392	GO:0050877 <1.00$E-14$ 179	GO:0006812 3.34$E-13$ 165
UHRF1	1500	GO:0007268 <1.00$E-14$ 149	GO:0007399 2.00$E-09$ 222	GO:0032940 7.76$E-09$ 113	GO:0007611 5.24$E-08$ 42	GO:0060341 1.06$E-07$ 113
Common genes	503	GO:0007268 <1.00$E-14$ 84	GO:0034220 1.66$E-09$ 45	GO:0007399 3.67$E-07$ 88	GO:0006836 4.83$E-06$ 19	GO:0023061 4.83$E-06$ 30

GO:0007268 Synaptic transmission
GO:0044708 Single-organism behavior
GO:0023061 Signal release
GO:0007600 Sensory perception
GO:0032940 Secretion by cell
GO:0006396 RNA processing
GO:0042391 Regulation of membrane potential
GO:0043269 Regulation of ion transport
GO:0017157 Regulation of exocytosis
GO:0060341 Regulation of cellular localization
GO:0006813 Potassium ion transport
GO:0006996 Organelle organization
GO:0006836 Neurotransmitter transport
GO:0007269 Neurotransmitter secretion
GO:0031175 Neuron projection development
GO:0050877 Neurological system process
GO:0007399 Nervous system development
GO:0034660 ncRNA metabolic process
GO:0030001 Metal ion transport
GO:0007626 Locomotory behavior
GO:0007611 Learning or memory
GO:0006811 Ion transport
GO:0034220 Ion transmembrane transport
GO:0007186 G-protein coupled receptor signaling pathway
GO:0006887 Exocytosis
GO:0050890 Cognition
GO:0007154 Cell communication
GO:0006812 Cation transport

Figure 5: Enrichment analysis of downregulated genes from comodulation results.

a core of 18 genes highly interconnected on the basis of experiments/database or physical interactions annotations, respectively, implemented in the two tools (Figure 6).

The investigation of downregulated genes resulted highly concordant in the 10 gene lists and highlighted the putative impairment of neuronal development and functionality according to brain tumors characteristics. The analysis of the 10 lists of upregulated genes showed the enrichment of a wider range of biological processes. In agreement with the tumorigenic model, many genes showing an increase of their



(a) (b)

Figure 6: Gene networks of the selected 18 genes obtained by the tools: (a) STRING 9.1 and (b) GeneMANIA.

transcriptional levels were related to different aspects of the cell cycle. Moreover, the involvement of posttranscriptional regulation mechanisms was demonstrated by a relative enrichment of the "RNA metabolic process" GO category. A detailed characterization of upregulated genes belonging to this clade allowed us to identify a subset of 18 genes whose correlations were independently supported by different kind of studies as, for example, between YBX1 and SYNCRIP [44–46] or between CCAR1 and WWTR1 [47]. The 18 genes selected appeared to operate in several mechanisms of posttranscriptional regulation such as ILF3 in pre-mRNA splicing, mRNA cytoplasmic export, and mRNA stability [48] or QKI in alternative splicing [49]. Remarkably, some studies already demonstrated the impact of expression alterations on cell cycle and proliferation of some of these genes like SRSF3 [50] and EZH2 [51].

4. Conclusions

In this paper, we described the implementation of the Association Rule Mining methodology for the meta-analysis of gene expression data. The application of the ARM method resulted in the identification of a 10 lncRNAs pattern that was validated in two independent datasets of brain tumors expression data. Throughout a Principal Component Analysis, we assessed the potential of the 10 lncRNAs rule to distinguish between cancer and normal tissues. Moreover, by a comodulation analysis, we were able to outline some specific biological processes that could be putatively related to the altered expression of the 10 lncRNAs. In conclusion, we proposed this new ARM-based approach as a valuable tool to extract relevant biological information in the form of common expression patterns.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Paolo Cremaschi and Roberta Carriero equally contributed to this work.

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Review Article

Posttranscriptional Regulation of Splicing Factor SRSF1 and Its Role in Cancer Cell Biology

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Over the past decade, alternative splicing has been progressively recognized as a major mechanism regulating gene expression patterns in different tissues and disease states through the generation of multiple mRNAs from the same gene transcript. This process requires the joining of selected exons or usage of different pairs of splice sites and is regulated by gene-specific combinations of RNA-binding proteins. One archetypical splicing regulator is SRSF1, for which we review the molecular mechanisms and posttranscriptional modifications involved in its life cycle. These include alternative splicing of SRSF1 itself, regulatory protein phosphorylation events, and the role of nuclear versus cytoplasmic SRSF1 localization. In addition, we resume current knowledge on deregulated SRSF1 expression in tumors and describe SRSF1-regulated alternative transcripts with functional consequences for cancer cell biology at different stages of tumor development.

1. Introduction

The expression of a gene is initiated by its transcription into a precursor messenger RNA (mRNA), which is then further processed and spliced into a mature mRNA. Splicing is regulated through the interaction between RNA-binding proteins (RBPs) and their cognate splicing regulatory sequence elements (SREs) in the mRNA. This is especially important for alternative splicing where multiple mRNAs can be generated from the same pre-mRNA through the joining of selected exons or usage of different pairs of splice sites [1].

The number of genes encoding RBPs in the human genome is currently estimated to be around 860 [2, 3], far below the number of around 200 000 transcripts that can be produced from the roughly 21 000 human protein-coding genes. Therefore, a key principle in splicing regulation is that the interaction of RNA-binding proteins with SREs is not a one-to-one relationship: each SRE motif can be recognized by multiple alternative RBPs and most splicing factors can recognize two or more SRE motifs. This is particularly relevant for alternative splicing events, the regulation of which involves a complex network of competing protein-RNA interactions so that individual exons can be controlled by multiple factors [4, 5]. For example, multiple RNA-binding proteins with similar splicing regulatory activities might bind the same motif and this functional redundancy creates robustness in a splicing decision. Also, some factors compete with or displace another factor with opposite activity and confer functional antagonism. These overlapping binding specificities allow regulatory plasticity, which underlies tissue-specific splicing patterns, subtle fine-tuning of splice variant levels, and regulatory relationships between splicing regulators and upstream signaling pathways.

Among the RBPs, the major classes of splicing factors that control splice site recognition are the families of Serine/Arginine-rich (SR) proteins and heterogeneous nuclear ribonucleoproteins (hnRNPs). These proteins act by selecting splice sites for recognition by the spliceosome through binding to intronic or exonic splice enhancer and silencer elements and promoting or destabilizing protein interactions with spliceosome components. One of the best studied factors is SRSF1, formerly known as ASF or SF2 [6]. SRSF1 is a prototypical splicing factor mostly recruited to SREs classified as exonic splicing enhancers (ESEs). SRSF1

recognizes degenerate purine-rich sequence motifs [7, 8] and its binding promotes recognition of both constitutive and alternative exons during spliceosomal assembly. The current knowledge about its regulation will be the focus of this review. The described principles of regulation also apply to many other SR proteins and RBPs.

2. Posttranscriptional Regulation of SRSF1 mRNA

The SRSF1 gene is essential for normal embryonic development that is constitutively expressed and tightly regulated at the posttranscriptional level. In particular, SRSF1 recognizes SREs in its own transcripts, leading to alternative splicing, with some transcript forms being degraded by nonsense-mediated mRNA decay (NMD). In case of SRSF1, alternative splicing occurs in the 3′ untranslated region following excision of an additional intron and thus introduction of a new exon-exon junction. In consequence, the original stop codon is recognized as premature and the transcript targeted for NMD [9]. This mechanism is highly conserved and shared by other SR proteins. It serves both as a negative feedback loop, in which increased SR protein levels promote an increase in unproductive splice variants of their own transcripts, and as a target for regulation, for example, depending on the ERK1/2-mediated phosphorylation status of the splicing regulator Sam68 [10].

In addition, other posttranscriptional mechanisms of SRSF1 autoregulation were described such as nuclear retention of alternative SRSF1 transcript variants or regulation of the translational efficiency of its transcripts [11, 12]. Furthermore, miRNAs targeting SRSF1 translation have begun to be identified, including miR-28, miR-505, miR-10a, and miR-10b [13, 14]. Thus, SRSF1 transcript levels are fine-tuned by various posttranscriptional mechanisms but the quantitative contribution of each step and their orchestration in response to different cellular stimuli remains undetermined.

3. SRSF1 Regulation by Protein Phosphorylation

Following translation of SRSF1 transcripts into protein, constitutive phosphorylation steps occur. First, the predominantly cytoplasmic SR-specific protein kinases (SRPKs) phosphorylate part of the C-terminal Arg-Ser-rich (RS) domain, which contains 20 serine residues. SRPK1 was shown to phosphorylate the proximal first 12 residues [15] and this promotes nuclear import through interaction of phospho-SRSF1 with the import factor transportin-SR2 [16] and subsequent localization into nuclear speckles [17, 18].

Once in the nucleus the Cdc2-like kinases (CLKs) phosphorylate the remaining serine residues in the distal RS domain which leads to dispersed nuclear localization of SRSF1 and is required for its function in splicing [19–21] through cotranscriptional association with RNA polymerase II (pol2). Upon transcription inhibition SRSF1 is translocated from the nucleoplasm back to nuclear speckles [22] (see Figure 1 for a graphic summary).

Recently, it was found that SRPK1 can also shuttle into the nucleus [23–26] where then SRPK1 and CLK1 display similar activities toward Arg-Ser repeats in the distal RS domain, suggesting that these kinases no longer operate in a strict linear manner along the RS domain. Instead, CLKs appear to recognize preferentially the three Ser-Pro dipeptides in the RS domain, the phosphorylation of which has been proposed to change the conformation of the RS domain and regulate SRSF1 contact sites required in the spliceosome [27]. Nuclear translocation of SRPK1 can be induced, for example, by stress conditions, and involves disruption of its binding to a cytoplasmic Hsp70/Hsp90 complex [28].

Besides these phosphorylation events considered to be constitutive, other protein kinases have been reported to regulate SRSF1 through phosphorylation.

Protein kinase A (PKA) can phosphorylate SRSF1 on serine 119 *in vitro* and modulate its activity as a splicing factor [29, 30]. This phosphorylation occurs in the so-called pseudo-RNA recognition motif (RRM) and was described to change the RNA-binding properties of SRSF1 and reduce its capacity to activate splicing.

Human DNA topoisomerase I (topo I) has also been described to phosphorylate SRSF1 [31, 32], most likely in the RS domain. This phosphorylation promotes the binding of SRSF1 to cognate ESEs during alternative splicing events [33]. The DNA damage signal poly-ADP ribose forms a complex with SRSF1, and this promotes Topo I to switch from its protein kinase to DNA relaxation activity [34]. A further connection between SRSF1 and Topo I is their role in preventing R-loop formation, stable mRNA:DNA hybrids that can form following transcription [35, 36].

Another kinase reported to phosphorylate SRSF1 *in vitro* is AKT [37, 38], which also targets serine residues in the RS domain, leading to altered splicing decisions. A subsequent study reported that AKT1 interacts with and promotes SRPK1 and SRPK2 autophosphorylation and their subsequent translocation into the nucleus [26] with simultaneous formation of a phosphatase containing complex to downregulate AKT activity [39]. It remains to be established whether AKT phosphorylates SRSF1 directly or whether the described activity of immunoprecipitated AKT to phosphorylate SRSF1 originates from associated SRPKs.

The serine/threonine kinase NEK2 is also a splicing factor kinase that colocalizes with SRSF1 in nuclear speckles. It interacts with and phosphorylates SRSF1, affecting the splicing activity of SRSF1 in a SRPK1-independent manner [40].

4. SRSF1 Regulation through Nuclear-Cytoplasmic Distribution

Ample experimental evidence showed that SRSF1 is a shuttling protein that localizes to both the nucleus and the cytoplasm, depending on the phosphorylation state of its RS domain [37, 41]. Furthermore, experimental blocking of SRPK (by either depleting its expression level or inhibiting its kinase activity) revealed that the cytoplasmic pool of SRSF1 increased, identifying phosphorylation as a major factor for

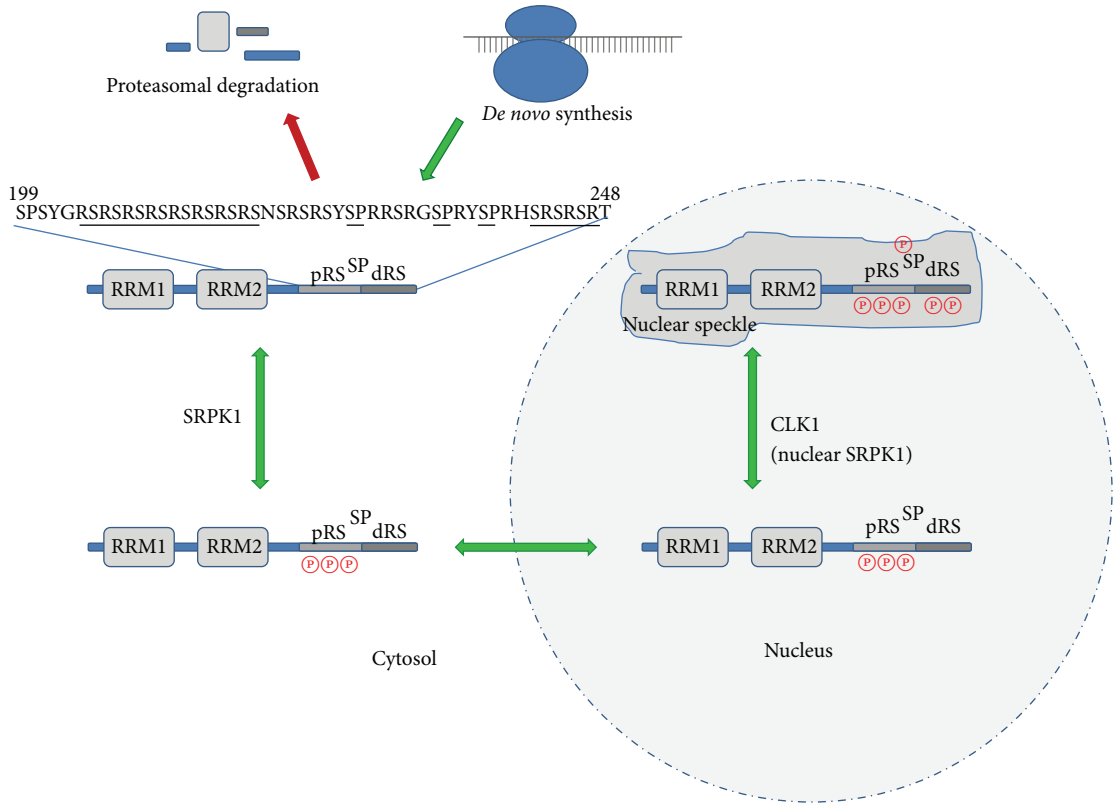

Figure 1: Life cycle and posttranslational modifications of the SRSF1 protein. Following its *de novo* synthesis at ribosomes, the cytoplasmic SRSF1 protein is constitutively phosphorylated by the cytosolic protein kinase SRPK at serine residues in the proximal RS domain (pRS). This first phosphorylation step is required for nuclear import of SRSF1, followed by a second phosphorylation step at the distal RS domain (dRS), including several serine-proline (SP) motifs. This step is usually catalyzed by the nuclear protein kinase CLK1 but can also be performed by SRPK if induced to translocate into the nucleus. Nuclear translocation is further modulated through methylation of the three arginine residues R93, R97, and R109 located between the two RNA recognition motif (RRM) domains (not shown). SRSF1 with a completely phosphorylated RS domain accumulates in nuclear speckles from where it is recruited to the spliceosome. SRSF1 dephosphorylation induces its nuclear to cytoplasmic translocation and lack of phosphorylation by SRPK in the cytosol leads to its proteolytic degradation.

SRSF1 nuclear translocation [42, 43]. The contribution of nuclear phosphatase activity to cytoplasmic export of SRSF1 has not been directly demonstrated *in vivo* but protein phosphatase 1 can dephosphorylate the proximal RS domain of SRSF1 *in vitro* or in permeabilized cell nuclei [44–46].

One physiological condition modulating SRSF1 localization is stress response, when general splicing is inhibited but specific alternative splicing events continue to occur [47]. For example, replicative senescence or induced stress stimuli of the vascular endothelium result in preferential cytoplasmic localization of SRSF1 and the underlying mechanism was postulated to involve nuclear import of SRPK1 and consequently lack of constitutive cytosolic SRSF1 phosphorylation [48]. In contrast, hyperphosphorylation of SRSF1 was observed during the DNA damage response and caused altered subnuclear distribution and changes in alternative splicing pattern of target genes [49].

Another posttranslational modification involved in SRSF1 localization is the methylation of three arginine residues (R93, R97, and R109) located in a region between the two RRM domains [50]. Lack of methylation in a triple-Ala mutant turned SRSF1 predominantly cytoplasmic, whereas a triple-Lys substitution maintaining the positive charge localized to nuclear speckles, as the wild-type protein. How the respective protein arginine methyltransferases (PRMTs) are regulated and contribute to the nuclear-cytoplasmic transitions of SRSF1 is poorly understood.

Once in the nucleus, the long noncoding RNA MALAT1 (metastasis-associated lung adenocarcinoma transcript 1) interacts with SRSF1, which is important for the recruitment of other SR proteins into nuclear speckles [51].

5. Functional Consequences of Nuclear versus Cytoplasmic SRSF1 Concentrations

Relative concentrations of antagonizing or competing SFs are important determinants in alternative splicing regulation. For example, SRSF1 generally displays a stimulatory role in splicing when bound to exons and its function in alternative splicing *in vitro* can be antagonized by the activity of hnRNP A proteins in a concentration-dependent manner. *In vivo*, competition between SFs can originate from the relative ratios of such antagonists expressed in different tissues or developmental stages, creating tissue or stage-specific patterns of

splicing. In addition, the dynamic regulation of subcellular SF localization allows cells to modulate the effective nuclear concentration of a given SF and alter the pattern of expressed splicing variants in response to external stimuli. For example, the subnuclear distribution of SRSF1 changes during the DNA damage response following hyperphosphorylation and results in a shift in the alternative splicing pattern of target genes that control cell survival [49]. Also, drug-induced disruption of nuclear speckles with concomitant release of SRSF1 into the nucleoplasm induced changes in alternative splicing events [52]. And endothelial senescence is associated with a scattered distribution of SRSF1 throughout the cytoplasm. This leads to the expression of alternative isoforms of target genes such as endoglin (ENG), vascular endothelial growth factor A (VEGFA), tissue factor (T3), or lamin A (LMNA) that integrate into a common molecular senescence program [48]. Vice versa, epithelial cells treated with insulin-like growth factor-1 (IGF-1) displayed nuclear translocation of SRSF1, which was dependent on SPRK1/2 activity.

6. Regulation of SRSF1 by Cytosolic Protein Degradation

A specific decrease in SRSF1 protein levels was observed in SRPK1-depleted or SRPIN340-treated colorectal cells, without changes in the corresponding SRSF1 mRNA. This suggests that cytoplasmic SRSF1 localization leads to protein degradation. Indeed, the SRSF1 protein remained stable in such treated cells when incubated with inhibitors MG132 or lactacystin, indicating degradation by the proteasome [43]. It should be noted that studying SFs with proteasome inhibitors needs to be well controlled at the corresponding transcript level because the inhibitors are likely to affect other SFs or transcription factors in the cell. For example, the gene encoding SRSF3 (former SRp20) is a direct transcriptional target of β-catenin/TCF4 [53] so that inhibition of β-catenin degradation will increase expression of SRSF3, which in turn can promote unproductive alternative SRSF1 transcripts [54].

SRSF1 protein expression levels did also not correlate with mRNA expression levels following T cell stimulation. Immunoprecipitation studies showed increased ubiquitylation of SRSF1 in activated T cells and proteasomal but not lysosomal degradation was shown to be involved by blocking with specific inhibitors MG132 and bafilomycin, respectively. Interestingly, T cells from patients with SLE (systemic lupus erythematosus) showed increased ubiquitylation of SRSF1 when compared to those from healthy individuals [55].

Downregulation of SRSF1 protein level was further found to occur following inhibition of activity or siRNA-mediated depletion of GSK3β in U87 or U373 glioblastoma cells [56]. Similarly, GSK3β depletion in HT29 colorectal cancer cells led to a reduction in both SRSF1 and SRPK1 protein levels, suggesting an indirect effect of GSK3β on SRSF1 via SRPK1 [43].

It has been described that the RS domain, which is common to all SR proteins, is required for their proteolytic degradation by the proteasome [57] but further mechanistic details remain to be determined.

7. Impact of Posttranslational Modification on Other RNA-Related Functions of SRSF1

SRSF1 has been shown to facilitate the nuclear export of spliced mRNAs to which it is bound through its interaction with the TAP/NXF1 receptor [58]. Interestingly, this adaptor function implies partial dephosphorylation of its RS domain for cytoplasmic translocation [59], suggesting the phosphorylation status of SRSF1 serves to regulate nuclear export of some mRNPs.

The subsequent ribosomal translation of transcripts containing a SRSF1-targeted ESE is also stimulated, both *in vivo* and *in vitro* [60]. Thus, SRSF1-mediated alternative splicing, mRNA export, and translational efficiency of its target transcripts are coupled, and their number has been identified experimentally to be around 500 [61].

In addition, SRSF1 overexpression was found to increase the ratio between cap-dependent and internal ribosome entry site-dependent translation initiation [62], probably by suppressing the activity of 4E-BP, a competitive inhibitor of cap-dependent translation [63]. Probably related to these properties is the observation that SRSF1 enhances nonsense-mediated mRNA decay (NMD) [64] because SRSF1 overexpression can promote the pioneer round of translation required for NMD to occur [58].

Another class of RNA pol2 transcripts is miRNAs and SRSF1 overexpression in HeLa cells promoted the maturation step of miR-7 and other miRNAs. SRSF1 (and also other splicing factors) directly interacts with primary miR transcripts and promotes the Drosha cleavage step generating mature miRNA [65].

Another recently discovered function of SRSF1 is to enhance protein sumoylation [66]. SRSF1 associates with the SUMO E2 conjugating enzyme Ubc9 and enhances SUMO conjugation to RNA processing factors but further details on the regulation or consequences of this modification remain to be identified.

It should also be noted that SRSF1 was shown to be involved in chromatin organization and histone modifications such as H3K36me3, which are relevant for splicing decisions [67, 68].

8. Role of SRSF1 in Cancer Cell Biology and Tumorigenesis

Malignant changes in the cellular genome can either be tumor-initiating driver events or subsequent adaptations required for tumor cell progression. Such changes either alter the expression level of critical genes or their nucleotide sequence to generate gain- or loss-of-function mutant gene products. Although point mutations in core components of the spliceosome were recently discovered using whole-genome sequencing approaches [69], reports from various tumor types revealed that splicing factors mostly show increased expression levels [70–72]. Concerning SRSF1, overexpression was reported in tumors from colon, thyroid, small intestine, kidney, lung, liver, pancreas, and breast [73, 74]. In childhood acute lymphoblastic leukemia SRSF1

was further found to be upregulated together with protein arginine methyltransferase PRMT1 [75], which is involved in promoting SRSF1 nuclear localization [50].

The overexpression of SRSF1 in tumors has been related to several alternative mechanisms. First, in breast tumors and breast cancer cell lines amplification of the *SFRS1* gene at chromosomal location 17q23 was detected and the increased DNA copy number correlated with elevated SRSF1 mRNA levels [73].

Second, the *SRSF1* gene is a target of MYC, a potent oncogenic transcription factor overexpressed in many different tumor types that has pleiotropic effects on cancer cell biology [76]. MYC binds directly to the SRSF1 promoter and activates transcription. Both genes were found coexpressed in lung and breast carcinomas and MYC depletion downregulates SRSF1 expression in lung cancer cell lines [77].

Third, the above-mentioned negative feedback loop, in which SRSF1 promotes an increase in unproductive splice variants of its own transcripts, can be subverted in the presence of splicing regulator SAM68. Changes in expression or phosphorylation of SAM68 were found to promote the formation of full-length SRSF1 transcripts, thus leading to increased SRSF1 protein levels [10]. SAM68 phosphorylation depends on ERK/MAP kinase activity, which is frequently augmented in human tumors.

Together, this indicates that SRSF1 overexpression is in general the consequence of other preceding tumor-initiating genetic changes but contributes to further tumor progression.

Two apparently opposing consequences of SRSF1 overexpression on cancer cell biology have been described: the induction of oncogene-induced senescence and the malignant transformation of cells. On the one hand, SRSF1 overexpression leads to the formation of a nucleoplasmic complex with the ribosomal protein RPL5 and the E3-ubiquitin ligase MDM2, which normally ubiquitylates the p53 tumor-suppressor protein leading to its proteolytic degradation [78]. Complex formation inhibits MDM2 and thus p53 protein levels increase and trigger a cellular senescence response, which normally is part of a ribosomal stress pathway. Because the ability of SRSF1 overexpression to activate a tumor-suppressing senescence response is dependent on an intact p53 pathway, the identified SRSF1-overexpressing tumor types revealed characteristics of p53 inactivation [78].

On the other hand, SRSF1 can act as an oncogene since a twofold increase in expression can transform immortalized rodent fibroblasts [73, 79] and human mammary epithelial cells [73, 79]. In these models, SRSF1 overexpression promoted cell proliferation and antiapoptotic pathways, mainly reflecting the combined effects of several alternative splicing variants which were activated by the concentration-dependent changes in SRSF1 availability. Some of these specific variants have been characterized, as detailed below, but probably represent just the tip of the iceberg.

One group of identified target genes is formed by the apoptosis regulators BIN1, BCL2L11 (BIM), BCL-XL, ICAD, and MCL1, with SRSF1 overexpression in cancer cells promoting the formation of their respective antiapoptotic splice variants. Several target genes belong to the Bcl-2 family of proteins, which regulate whether the Bak and Bax proteins can cause mitochondrial outer membrane permeabilization and cytochrome c release as the trigger for intrinsic apoptosis induction. The Bcl-2 family comprises both proapoptotic and antiapoptotic proteins, depending on their BH domain composition, and it is the balance between both types of proteins that determines whether the mitochondrial pathway to apoptosis is activated [80].

Regarding BIM, several SRSF1-induced transcript variants were described lacking exons 2, 3, or 4 (BIM γ1, γ2, ES) which encode the BH3 domain. This domain binds antiapoptotic Bcl-2 family members and is necessary for induction of apoptosis by BIM [79, 81]. Similarly, SRSF1 expression promotes inclusion of exon 2 of the BH3 domain-containing gene MCL-1 (myeloid cell leukemia-1) giving rise to the antiapoptotic MCL-1L isoform in both breast cancer and choriocarcinoma cells [82]. Overexpression of SRSF1 also promotes generation of the antiapoptotic isoform BCL-XL [83].

BIN1 has tumor-suppressor activity by interacting with and activating MYC-mediated apoptosis, except when exon 12A is included by SRSF1-mediated alternative splicing, because the resulting antiapoptotic BIN1+12A isoform is unable to interact with MYC. Furthermore, SRSF1 was shown to modulate exclusion of exon 5 of the mRNA encoding the inhibitor of caspase-activated DNase (ICAD), a regulator of the DNase responsible for DNA fragmentation during apoptosis [84].

A parallel group of SRSF1-regulated target genes is involved in cellular signaling pathways related to proliferation and cell cycle progression. Examples of genes from this group are *CCND1*, *RPS6KB1*, *RON*, *RAC1,* and *MKNK2* genes.

SRSF1 increases expression of the cyclin D1b oncogene which arises from alternative splicing of the *CCND1* transcript, and harbors enhanced oncogenic functions not shared by full-length cyclin D1 (cyclin D1a) [85]. In this case, SRSF1 blocks recognition of the *CCND1* exon 4-intron 4 boundary, thus repressing inclusion of exon 5 so that a nuclear protein with a unique C-terminus is generated. SRSF1 also promotes the inclusion of exon 5 into the pre-mRNAs of *TEAD-1* (TEF-1 or TCF13), a transcription factor normally involved in cell differentiation and cell cycle arrest in myoblasts [86].

The *RPS6KB1* gene encodes the protein S6 kinase 1, a substrate for the cell growth regulating kinase mTOR. Excess SRSF1 promoted an increase in S6K1 variants by including one to three alternative cassette exons between exons 6 and 7 that are normally skipped [73] and include a proper stop codon. These short S6K1 isoforms have a truncated kinase domain and lack the mTOR-regulated C-terminus but are able to bind to and activate the mTORC1 complex. This activation of the mTORC1 complex occurs independent of the classical PI3K/AKT pathway and leads to phosphorylation of eIF4EBP1, releasing its inhibitory effect on cap-dependent translation [87, 88]. In addition, there is evidence that SRSF1 itself participates in a complex with mTORC1 to enhance translation efficiency of its target transcripts [63], for example, survivin [89] and β-catenin [90].

RON encodes a receptor tyrosine kinase in breast and colon tumors and SRSF1 promotes skipping of exon 11 by binding to an enhancer element in the competing exon 12.

The resulting isoform ΔRon is constitutively active and promotes cell motility [91] as part of an epithelial-mesenchymal transition program, which tumor cells may use to escape from adverse local growth conditions.

Breast and colon tumors are further characterized by overexpression of Rac1b [92, 93], a hyperactivated splice variant of the small GTPase Rac1, which is involved in gene transcription and cell motility [94, 95]. In colorectal cells, SRSF1 was shown to be required for inclusion of an additional exon 3b to generate Rac1b [96] and increased expression of Rac1b contributes to cell survival [97, 98].

Finally, SRSF1 overexpression enhances the inclusion of the alternative 3′-terminal exon 13b of the gene *MKNK2* encoding the protein kinase Mnk2, an effector in the ERK/MAPK pathway [73]. The corresponding isoform Mnk2b lacks a C-terminal MAPK-binding domain and does not phosphorylate and activate the p38-MAPK required for stress-induced cell death. In contrast, it sustains phosphorylation of the translation initiation factor eIF4E, thus promoting cap-dependent protein translation and cell growth [99].

Curiously, splicing of the Mnk2b isoform was not induced when a chimeric nucleus-retained SRSF1 protein (SRSF1-NRS1), fused to the nuclear retention signal of the non-shuttling protein SRSF2, was overexpressed, suggesting an indirect effect of SRSF1 for this splicing event. Nevertheless, SRSF1-NRS1 was as competent as wild-type SRSF1 in inducing mammary cell transformation in 3D cultures but requires presence of the RRM1 domain, revealing a significant contribution of the nuclear functions of SRSF1 to cell transformation [79].

In hepatocellular xenografts, however, SRSF1-NRS1 protein had a much lower effect on tumor formation than SRSF1. In this model, SRSF1 overexpression also promotes activation of ERK/MAPK, probably by increasing B-RAF mRNA and protein levels. Although the mechanism remains to be explained, the RRM1 domain is required and sufficient to induce activation of this oncogenic pathway, indicating that the effect occurs at the mRNA level [81]. These findings reveal that SRSF1 can exert its oncogenic role through both nuclear and cytosolic pathways depending on the cellular contexts.

More recently, SRSF1 overexpression was also reported in lung cancer and novel SRSF1 target transcripts were identified, including the genes, ATP11C, IQCB1, TUBD1, proline-rich coiled-coil 2C (PRRC2C) [100], and survivin [89].

Apart from the genetic changes affecting proliferation and survival of the transformed cancer cells themselves, another important aspect of cancer cell biology is their cellular communication with the surrounding stroma. It is imperative for the growth of epithelial tumors to gain access to nutrient supply via blood vessels so that cancer cells release angiogenic signals to endothelial cells, for instance, the vascular endothelial growth factor VEGF. SRSF1 is involved in promoting proximal splice site selection in C-terminal exon 8 of VEGF, resulting in the generation of proangiogenic isoforms [101, 102]. Besides overexpression, this can result from SRSF1 activation in epithelial cells following oncogenic signaling through IGF-1, EGF, or TNF-α. These factors lead to activation of SRPKs [26, 42], which then phosphorylate SR proteins including SRSF1. SRPK1 inhibition has been used to manipulate the local balance of pro- and antiangiogenic in eye pathologies caused by neovascularization [103] and might be interesting for cancer therapy. In addition, SRSF1-mediated alternative splicing of both Ron and TEAD-1 has been linked to increased expression of angiogenic growth factors [77, 104]. SRSF1 may thus impact VEGF expression through both direct and indirect regulation to promote angiogenesis.

The role of fibroblasts in the stroma is to deposit or remodel extracellular matrix components and this is important for tumor cell migration. For example, a dense fibronectin meshwork favors epithelial cell invasion and results from inclusion of the EDA exon through SRSF1-regulated alternative splicing of the unique fibronectin-encoding gene [105, 106]. This occurs during embryogenesis but also in adult fibroblasts during tissue repair, tumor progression, and inflammation when expression levels of SRSF1 increase. It remains to be established whether tumor cells can release signals that induce increased SRSF1 expression in tumor-associated fibroblasts.

Following therapeutic challenge of tumor cells with DNA-damaging agents, resistant cells can eventually emerge. In one report hyperphosphorylation of SRSF1 was observed in the presence of DNA damage, causing altered subnuclear distribution and changes in alternative splicing pattern of target genes that promote cell survival [49]. Similarly, treatment of pancreatic tumor cells with the nucleoside analogue gemcitabine induced SRSF1 overexpression, and the resulting splicing of MNK2b with consequent phosphorylation of the translation initiation factor eIF4E was identified as the cause for drug resistance [107]. Furthermore, non-small cell lung cancer (NSCLC) cells respond to daunorubicin or cisplatin with an antiapoptotic caspase 9b splice variant. SRSF1 regulates this alternative splicing event by binding to a splicing enhancer in intron 6 and subsequent exclusion of an exon 3,4,5,6-cassette, generating caspase 9b [108]. SRSF1 is activated following hyperphosphorylation at serines 199, 201, 227, and 234 [109], mediating the therapeutic resistance of NSCLC. Another study in NSCLC observed that SRSF1 protein accumulates when cells were treated with carboplatin and paclitaxel and that cells stably overexpressing SRSF1 were more resistant to these chemotherapeutic drugs [74].

9. Conclusions

SRSF1 is an important protein for the regulation of constitutive and alternative splicing of cellular pre-mRNAs. Its activity as splicing regulator depends on the relative expression level of SRSF1 compared to other antagonistic or synergistic splicing factors as well as on its posttranslational modifications. In particular, the phosphorylation state of SRSF1 determines its nuclear or cytoplasmic localization and proteolytic degradation. Overexpression of SRSF1 has been reported in various tumors types and this has consequences for the alternative splicing profile expressed in tumor cells. Clear experimental evidence for tumor-promoting effects of SRSF1-induced alternative splicing variants has been provided but the genome-wide scale of its effects on cancer cell biology remains to be described.

Similar studies on other splicing factors are beginning to emerge and will likely reveal comparable complex effects on cancer cell transcriptomes as part of an adaptive response to activate survival pathways in tumor cells. A more comprehensive knowledge of these pathways may allow designing therapeutic interventions based on a combination of inhibitory drugs targeting simultaneously various pathways to reduce the selection of therapy-resistant tumor cell clones.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Translational Control Protein 80 Stimulates IRES-Mediated Translation of p53 mRNA in Response to DNA Damage

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Synthesis of the p53 tumor suppressor increases following DNA damage. This increase and subsequent activation of p53 are essential for the protection of normal cells against tumorigenesis. We previously discovered an internal ribosome entry site (IRES) that is located at the 5′-untranslated region (UTR) of p53 mRNA and found that the IRES activity increases following DNA damage. However, the mechanism underlying IRES-mediated p53 translation in response to DNA damage is still poorly understood. In this study, we discovered that translational control protein 80 (TCP80) has increased binding to the p53 mRNA *in vivo* following DNA damage. Overexpression of TCP80 also leads to increased p53 IRES activity in response to DNA damage. TCP80 has increased association with RNA helicase A (RHA) following DNA damage and overexpression of TCP80, along with RHA, leads to enhanced expression of p53. Moreover, we found that MCF-7 breast cancer cells with decreased expression of TCP80 and RHA exhibit defective p53 induction following DNA damage and diminished expression of its downstream target PUMA, a proapoptotic protein. Taken together, our discovery of the function of TCP80 and RHA in regulating p53 IRES and p53 induction following DNA damage provides a better understanding of the mechanisms that regulate IRES-mediated p53 translation in response to genotoxic stress.

1. Introduction

The tumor suppressor protein p53 inhibits cell transformation by stopping cell growth or triggering apoptosis. It is mutated in more than half of all human cancers, and the inactivation of the p53 pathway plays a major role in the process of oncogenesis [1]. Under unstressed conditions, p53 protein levels are usually low, and this protein exists in an inactive form. The level of p53 increases only when the cells are stressed or damaged [1, 2]. Induced p53 is then activated through multiple posttranslational modifications. The accumulation and activation of p53 allow it to function as a tumor suppressor. Activated p53 protein binds to specific target DNA sequences and stimulates transcription of a variety of downstream target genes. The upregulation of the proteins encoded by these genes results in cell growth arrest to maintain genetic integrity of the cell or apoptosis to eliminate the damaged cell.

Since elevated levels of p53 protein are known to be important in initiating the events leading to cell growth arrest or apoptosis after cellular stress [1, 2], regulation of p53 induction has been a major area of cancer research over the last three decades. Although it is known that p53 is stabilized and therefore accumulates in the cell after DNA damage, there is also clear evidence showing that an increase in p53 synthesis in response to DNA damage, such as ionizing radiation (IR) or ultraviolet (UV) irradiation, also contributes to increased p53 levels in the cell [2–5]. It was demonstrated that p53 biosynthesis increases rapidly in response to IR in mouse 3T3 cells, even after treating the cells with the transcription inhibitor actinomycin D [6]. Also, exposure to IR or etoposide was found to lead to an increase in the association of p53 mRNA with polysomes, which further suggests an increase in p53 translation [7, 8]. The mechanism underlying translational regulation of p53 induction via its 5′-UTR has started to emerge.

It is known that cap-dependent initiation of protein translation is used by the majority of mRNAs, since almost all eukaryotic mRNAs have an N^7-methylguanosine cap structure at their 5′-ends [9]. eIF-4E is a translation initiation protein that binds to the cap structure. A translation repressor, eIF4E-binding protein 1 (4E-BP1, also called PHAS-I), inhibits cap-dependent translation by binding to eIF-4E [10, 11]. In quiescent cells, 4E-BP1 is hypophosphorylated and binds tightly to eIF-4E. Binding between 4E-BP1 and eIF-4E blocks the assembly of the eIF-4F protein translation initiation complex. Addition of growth hormones, such as insulin and IGF-I, induces phosphorylation of 4E-BP1 and causes the release of eIF-4E from 4E-BP1, which facilitates the translation of capped mRNA by making eIF-4E available for the formation of the eIF-4F complex.

In situations where cap-dependent translation is compromised by cyto- or genotoxic stress, cap-independent protein translation, promoted by internal ribosome entry sites (IRES), is required to maintain expression of critical proteins [12, 13]. This is an alternate mode of translation initiation in which ribosomal subunits are recruited to the IRES by a subset of initiation factors without the participation of eIF-4E. It is thought that IRES-mediated translation is required in eukaryotes for the synthesis of key regulatory proteins in situations where cap-dependent translation is impaired, such as apoptosis or DNA damage [14, 15]. Indeed, it was shown that IRES activity of several mRNAs encoding for proteins involved in cell cycle regulation and apoptosis increases under conditions of cellular stress, which includes DNA damage caused by etoposide treatment [16] or UV irradiation [17].

We and others discovered that an IRES sequence is present in the 5′-untranslated region (UTR) of the p53 mRNA [8, 18]. We also found that the IRES activity of the p53 mRNA increases following DNA damage in MCF-7 cells [4, 8]. MCF-7 is a breast cancer cell line that contains wild-type p53 and has increased synthesis of p53 following DNA damage [8]. This result suggests that this IRES sequence plays a key role in regulating p53 synthesis following DNA damage or other cellular stress.

The presence of an IRES sequence in an isoform of p53, p47 (also known as p53/p47, Δ40p53, and ΔNp53), and a p53 homologue, p73, has also been discovered [18–20]. The increase of p53 IRES activity following genotoxic or other cellular stress was further confirmed by a number of other reports [21–28]. For instance, it was found that during DNA damage or oncogene induced senescence (OIS), the p53 IRES exhibits enhanced activity to facilitate p53 translation [22], which provides further evidence that the p53 IRES plays a key role in regulation of p53 synthesis following DNA damage and OIS. More recently, it was shown that IRES activity of p53 increases in response to glucose deprivation, which links p53 IRES activity with metabolic stress [28].

Control of translational initiation at cellular IRESs requires the presence of auxiliary factors that are known as IRES-*trans* acting factors or ITAFs [12, 29]. ITAFs are proteins that can positively or negatively affect IRES activity [14]. A number of proteins have been identified as binding to the p53 5′-UTR *in vitro* [30]. Many of them are also known to be involved in multiple critical cellular events, including protein translation and ribosomal biogenesis. Therefore, some of these proteins could be potential p53 ITAFs that regulate p53 IRES activity and p53 synthesis. However, to date, there are no reports on whether any of these proteins are potential ITAFs of the p53 IRES. In this study, we discovered two novel, positive regulators of the p53 IRES, translational control protein 80 (TCP80) and RNA helicase A (RHA), from these proteins. Our results also suggest that the interaction between these two proteins is important for the p53 induction and its tumor suppressive function in response to DNA damage.

2. Experimental Procedures

2.1. Materials. Etoposide was from Calbiochem. The antibodies include anti-DRBP76 (TCP80) antibody (BD Transduction Laboratories), anti-DHX9 (RHA) antibody (Bethyl Laboratories), and anti-β-actin antibody (Sigma). The HRP-conjugated p53 antibody for immunoblotting was from Santa Cruz Biotechnology. The original pcDNA3.1/HisB/TCP80 expression vector was from Dr. Michael B. Matthews. TCP80 Δ401-702 and TCP80 Δ640-702 were obtained by regional deletion of the TCP80 vector. Vectors containing altered first or second dsRBM of TCP80 were mutated using the Quick Change site-directed mutagenesis kit from Stratagene. The TCP80 mdsRBM1 vector was obtained by mutating two lysines to glutamate (K450E and K451E) in the first dsRBM, while the TCP80 mdsRBM2 vector was obtained by mutating two lysines to glutamate (K573E and K574E) of the second dsRBM. The pcDNA3.1/RHA expression vector was from Dr. Suisheng Zhang.

2.2. Cell Culture and Transfection. MCF-7 and H1299 cells were grown in DMEM medium supplemented with antibiotics and 10% fetal bovine serum (FBS). All plasmid transfections were performed using Fugene 6 transfection reagent (Roche). Cells were seeded in six-well plates and allowed to grow overnight. They were then transfected with 1.5 μg of DNA. Between 24 and 48 hours following transfection, the cells were lysed.

2.3. Dual-Luciferase Assays. Cells were lysed with 1x passive lysis buffer (Promega). The Dual-Luciferase Reporter Assay System (Promega) was then used in conjunction with a Berthold luminometer to determine Firefly and Renilla luciferase activities according to manufacturer's instructions.

2.4. Immunoprecipitation and RT-PCR. Immunoprecipitation and RT-PCR was performed using the method as previously described [31]. Briefly, MCF-7 cells were lysed in a polysome lysis buffer [31]. Protein G-plus agarose beads (Calbiochem) were coated with an anti-Xpress antibody overnight. The beads were then washed several times and incubated with MCF-7 cell lysate for 2 hours at room temperature. The immunoprecipitated messenger ribonucleoprotein (mRNP) complexes were then washed extensively and treated with proteinase K. The mRNA was extracted using Tri-LS reagent (MRC) and further purified using RNeasy mini

columns (Qiagen). The purified RNA was reverse-transcribed using the SuperScript First-Strand synthesis system for RT-PCR (Invitrogen) and the cDNA was amplified using the Expand High Fidelity PCR system (Roche) using primers flanking the p53 IRES (~145 bp). The resulting PCR fragments were run on a 1% agarose gel containing ethidium bromide and visualized using a transilluminator.

2.5. Cell Extract Preparation, SDS-PAGE, and Western Blot. Cells were washed twice with phosphate buffered saline and lysed with TGN lysis buffer [8] containing 1% NP-40 and a protease inhibitor cocktail tablet (Roche). Protein concentration was measured using the Lowry assay method. Equal amounts of protein from each cell lysate were loaded onto an SDS-PAGE gel. After electrophoresis, proteins were transferred onto either a nitrocellulose or PVDF membrane.

2.6. Coimmunoprecipitation. Subconfluent MCF-7 cells were lysed by TGN lysis buffer and RHA was immunoprecipitated by mixing cell lysate containing equal amounts of protein with an antibody against RHA and protein A/G agarose beads overnight. The mixture was then centrifuged and the precipitated beads were washed three times with TGN lysis buffer followed by addition of SDS sample loading buffer.

3. Results

As stated earlier, a previous study has identified multiple proteins that bind to the p53 5′-UTR using an *in vitro* RNA pull-down assay [30]. We wanted to determine whether some of these proteins can act as activators of the p53 IRES to stimulate p53 IRES activity in response to DNA damage. Translational control protein 80 (TCP80), also known as nuclear factor 90 (NF90) or double-stranded RNA binding protein 76 (DRBP76), was one of the proteins that were found to bind to the p53 5′-UTR *in vitro*. It is a double stranded-RNA binding protein [32] and has documented roles in the regulation of protein translation [33]. TCP80 is also involved in IRES-mediated protein translation by acting as an ITAF of the rhinovirus type 2 IRES [34]. Therefore, the ability of TCP80 to associate with p53 IRES *in vivo* was investigated. The binding between TCP80 and p53 IRES in the presence of DNA damage was also assessed.

3.1. TCP80 Binds to the p53 mRNA In Vivo. An Xpress-tagged TCP80 protein was overexpressed in MCF-7 cells, and TCP80/mRNA complexes were subsequently immunoprecipitated with the anti-Xpress antibody. RT-PCR was then used to amplify the p53 IRES sequence from the immunoprecipitated TCP80/mRNA complexes. Amplification of the p53 IRES mRNA was observed in the immunoprecipitate derived from MCF-7 cells treated with etoposide, a DNA damage agent that induces DNA double-stranded breaks (Figure 1(a)). In contrast, no amplification was observed in immunoprecipitate obtained from the untreated control samples. These results suggest that TCP80 associates with p53 mRNA *in vivo* and binding of TCP80 to p53 mRNA increases in response to etoposide-induced DNA damage.

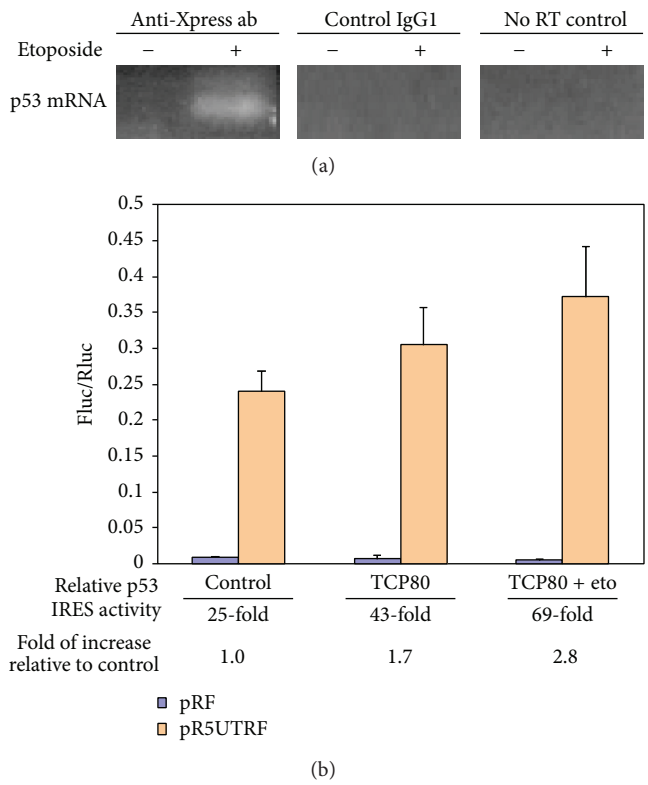

Figure 1: (a) TCP80 has increased binding to the p53 mRNA following DNA damage. MCF-7 cells were transfected with pcDNA3.1/HisB/TCP80 that encodes for the Xpress-tagged TCP80 protein. Twenty-four hours following transfection, the cells were treated with or without 10 μM etoposide for 2 hours. They were then lysed in polysome lysis buffer and incubated with protein G-plus agarose beads coated with the anti-Xpress antibody. The TCP80 and mRNA complexes (mRNP) were immunoprecipitated and mRNA was extracted from the immunoprecipitate as described in Experimental procedures. RT-PCR was then performed to reverse-transcribe and amplify the p53 IRES sequence (~145 bp). (b) TCP80 positively affects the p53 IRES activity in response to DNA damage. MCF-7 cells were cotransfected with pRF or pR5UTRF along with either pcDNA3.1 or pcDNA3.1/HisB/TCP80. Twenty-four hours following the transfection, the cells were treated with or without etoposide for 2 hours. The cells were then lysed and a dual-luciferase assay was performed to detect firefly (Fluc) and renilla (Rluc) luciferase activities as described in Experimental procedures. The results presented are average ± SEM from three individual experiments.

3.2. TCP80 Upregulates p53 IRES Activity in Response to DNA Damage. TCP80 affects protein translation and has increased binding with p53 mRNA following DNA damage. Therefore, it is conceivable that TCP80 may modulate p53 IRES activity in response to DNA damage. The bicistronic dual-luciferase reporter vector pR5UTRF, which contains the p53 IRES sequence, was used to determine p53 IRES activity in cellular systems [8]. Additionally, the empty vector (pRF) was used as a negative control for pR5UTRF. To determine whether or not TCP80 can affect p53 IRES activity, MCF-7 cells were cotransfected with either pRF or pR5UTRF along with a plasmid expressing TCP80. p53 IRES activity was

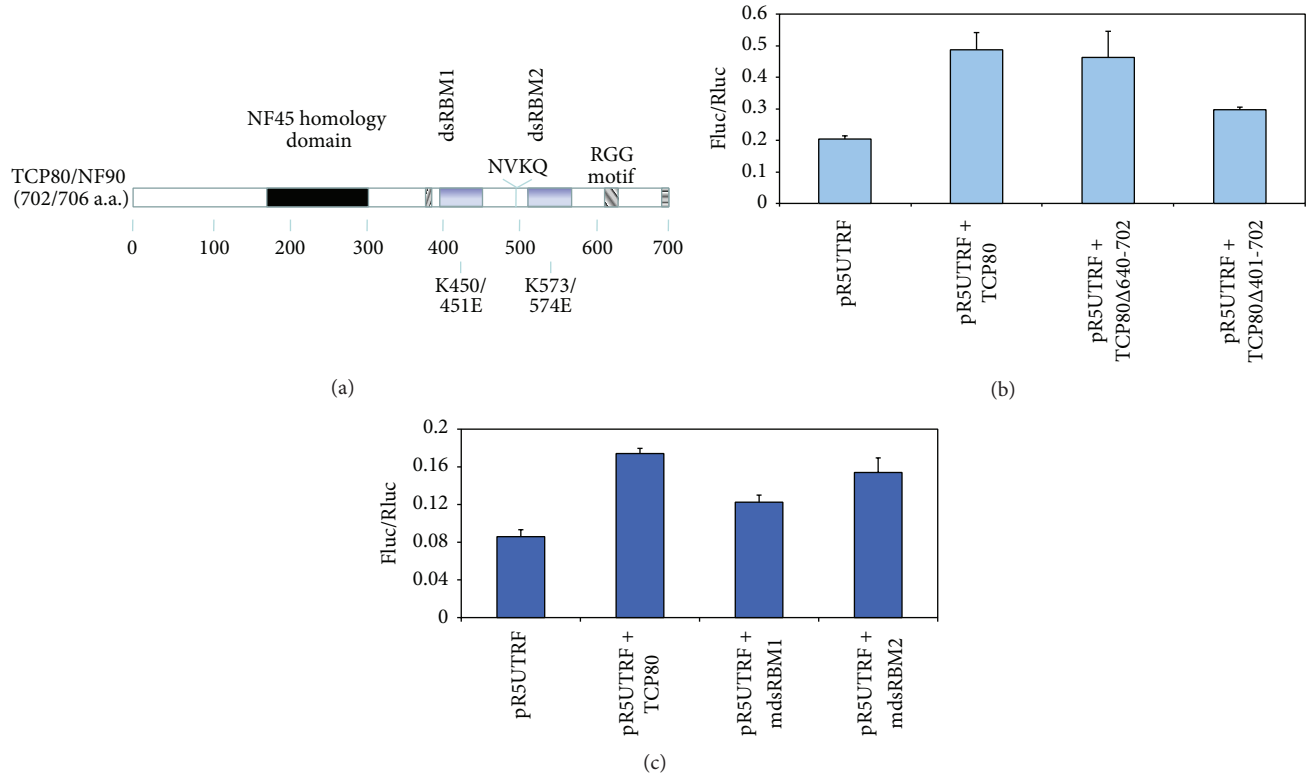

Figure 2: TCP80 dsRBMs are important for the induction of the p53 IRES activity. (a) Map of TCP80/NF90 functional domains. TCP80 contains three RNA binding sites: the RGG domain and two dsRBMs. The mutated residues in the mdsRBM1 and mdsRBM2 constructs, respectively, are also shown in this diagram. (b) Effect of deletion of the RGG domain and the dsRBMs on TCP80-mediated p53 IRES induction. MCF-7 cells were transfected with pR5UTRF along with wild-type TCP80, TCP80Δ640-702 (minus RGG domain), or TCP80Δ401-702 (minus RGG and dsRBMs). Twenty-four hours after transfection, cells were lysed, and a dual-luciferase assay was performed. (c) Effect of mutations in the dsRBMs on TCP80-mediated p53 IRES induction. MCF-7 cells were transfected with pR5UTRF along with plasmids encoding wild-type TCP80, mutant dsRBM1 TCP80 (mdsRBM1), or mutant dsRBM2 TCP80 (mdsRBM2). Firefly and renilla luciferase activities were determined. The results presented in both (b) and (c) are average ± SEM from three individual experiments.

then measured as the ratio of firefly luciferase (Fluc), which is controlled by the p53 IRES, to renilla luciferase (Rluc) activity [8]. Renilla luciferase is controlled by cap-dependent translational machinery and is used as the internal control. A nearly 2-fold increase in the relative p53 IRES activity was observed in MCF-7 cells overexpressing TCP80 as compared to the control cells (Figure 1(b)). More importantly, when the MCF-7 cells overexpressing TCP80 were also treated with etoposide, a nearly 3-fold increase of relative IRES activity was observed as compared to the control cells (Figure 1(b)). Considering the fact that etoposide treatment alone only leads to nearly 2-fold increase of p53 IRES activity in MCF-7 cells [4, 8], these results indicate that TCP80 not only is a positive modulator of p53 IRES activity but also causes an increase of p53 IRES activity in response to DNA damage.

3.3. TCP80 dsRBMs Are Important for the Induction of the p53 IRES Activity. The TCP80 protein contains three RNA binding domains (Figure 2(a)). It has two double-stranded RNA binding motifs (dsRBMs) and one RGG (arginine-glycine-glycine) domain located at its C-terminus [35]. These domains have been known to play an important role in RNA-protein interactions [36]. Therefore, we wanted to determine which of the three domains of TCP80 is important for its interaction with the p53 IRES.

We tested the ability of mutants lacking the TCP80 RGG motif (TCP80 Δ640-702) or the TCP80 RGG motif plus the two dsRBMs (TCP80 Δ401-702) to stimulate p53 IRES activity in MCF-7 cells. We found that the deletion of the RGG domain alone did not affect TCP80's ability to stimulate p53 IRES activity, whereas the additional deletion of both dsRBMs did result in a 40% decrease in p53 IRES stimulation as compared to wild-type TCP80 (Figure 2(b)). These results suggest that the two dsRBMs of TCP80 are important for the interaction between TCP80 and the p53 IRES. We then used plasmids containing mutations in either the first dsRBM (mdsRBM1) or the second dsRBM (mdsRBM2) of TCP80 to determine which of the two dsRBMs is more important for the p53 IRES activity, using full-length TCP80 as the control. These mutants have been known to be important for the interaction between TCP80 and its associated RNAs (Jiang and Miskimins, unpublished observations). Our results revealed

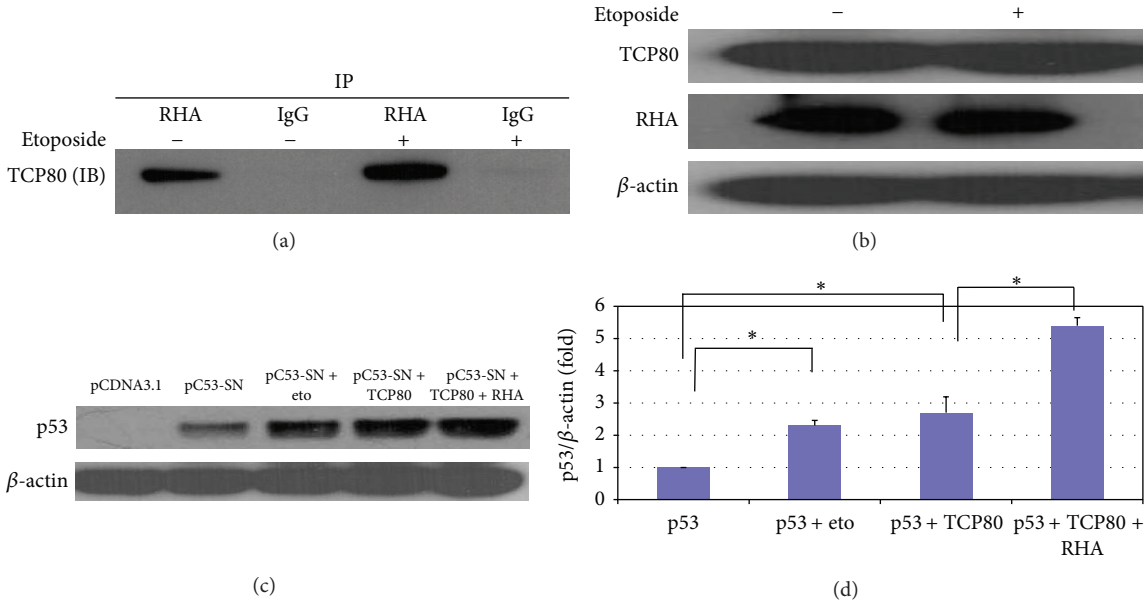

Figure 3: TCP80 and RHA interact *in vivo* and cooperatively stimulate p53 expression in MCF-7 cells. (a) TCP80 has increased binding with RHA following DNA damage in MCF-7 cells. Subconfluent MCF-7 cells were treated with or without 10 μ M etoposide for 2 hours and then lysed with TGN buffer [8]. RHA was immunoprecipitated from the cell lysate as described in experimental procedures. The precipitated beads were then washed three times with TGN lysis buffer and SDS sample loading buffer was added. The samples were subjected to SDS-PAGE. An immunoblotting experiment was then performed to detect the TCP80 protein. The results presented are representative of three individual experiments. (b) Levels of TCP80 and RHA protein do not change following exposure to DNA damage in MCF-7 cells. MCF-7 cells were treated with 10 μM etoposide for 2 hours and then lysed with TGN lysis buffer. The samples were subjected to SDS-PAGE. TCP80, RHA, and β-actin were detected by their respective antibodies. The results presented in (a) and (b) are representative of three individual experiments. (c) Overexpression of TCP80 and RHA leads to increased p53 expression in H1299 cells transfected with the pC53-SN3 vector. H1299 lung carcinoma cells (p53-null) were cotransfected with the p53 expression vector pC53-SN3 along with the empty pCDNA 3.1 vector, the TCP80 expression vector, or the TCP80 plus RHA expression vector. Twenty-four hours after transfection, the cells were treated with or without etoposide for 2 hours. Cells were then lysed, and equal amounts of protein were subjected to SDS-PAGE and transferred to a nitrocellulose membrane. The p53 protein and β-actin were then detected by their respective antibodies. (d) Statistical analysis of the expression levels of p53 (p53/β-actin) between individual groups as shown in (c) was performed using one-way ANOVA with a Newman–Keul post hoc test from 4 sets of experimental results. Significance was assumed at $^*P < 0.05$.

that mutations in dsRBM1 led to a 30% decrease in p53 IRES stimulation as compared to wild-type TCP80, but mutations in dsRBM2 led to a 10% decrease in the p53 IRES stimulation as compared to wild-type TCP80 (Figure 2(c)). These results suggest that the first dsRBM is more important for TCP80-induced p53 IRES activation.

3.4. TCP80 and RHA Bind to Each Other In Vivo. In addition to TCP80, RNA helicase A (RHA) or nuclear DNA helicase II (NDH II) was also identified to bind to the p53 5′-UTR *in vitro* [30]. Interestingly, RHA is known to associate with TCP80 *in vitro* [37] and has a known role in the regulation of protein translation as well [38]. We performed an immunoprecipitation experiment to pull down RHA in MCF-7 cells treated with etoposide and look at the binding between RHA and TCP80. Interestingly, we found increased binding of TCP80 to RHA following DNA damage (Figure 3(a)), while levels of both TCP80 and RHA stay the same before and after DNA damage (Figure 3(b)). Since TCP80 also has increased binding to p53 mRNA following DNA damage (Figure 1(a)), these results suggest that the interaction between TCP80 and RHA may be important for p53 IRES activity and p53 induction in response to DNA damage.

3.5. RHA Cooperates with TCP80 to Stimulate p53 Expression. Next, we examined the effect of overexpression of TCP80 on levels of p53 in H1299 (p53-null) lung carcinoma cells. Transfection of the pC53-SN3 vector, which contains the p53 IRES sequence (~140 bp) and p53 ORF, in H1299 cells resulted in expression of the p53 protein. When H1299 cells were cotransfected with the pC53-SN3 vector and a plasmid encoding TCP80, a significant increase in p53 levels was observed when compared to cells cotransfected with the pC53-SN3 and the empty vector (Figure 3(c)). The level of increase in p53 expression was similar to that observed in cells transfected with pC53-SN3 and treated with etoposide (Figure 3(c)). Interestingly, when both TCP80 and RHA were overexpressed in H1299 cells transfected with the pC53-SN3 vector, a much greater increase of p53 expression was observed (Figures 3(c) and 3(d)), suggesting a cooperative effect of TCP80 and RHA on p53 expression.

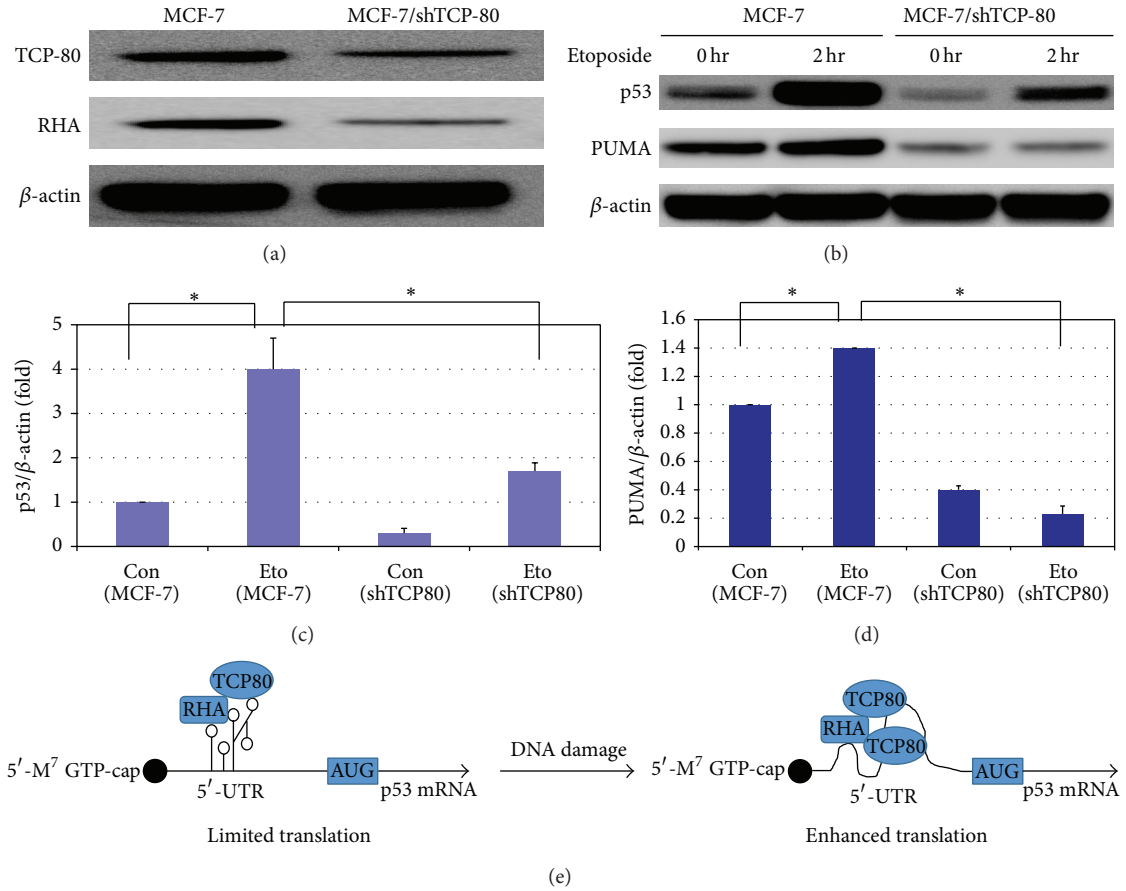

Figure 4: (a) MCF-7/shTCP80 cells express lower levels of TCP80 and RHA as compared to MCF-7 cells. MCF-7 and MCF-7/shTCP80 cells were grown to subconfluency. Cells were then lysed and equal amounts of protein were subjected to SDS-PAGE and western blotting. TCP80, RHA, and β-actin were detected by immunoblotting. (b) MCF-7/shTCP80 cells exhibit reduced induction of p53 and its downstream target PUMA following DNA damage. MCF-7 and MCF-7/shTCP80 cells were grown to subconfluency. Cells were then treated with 10 μM etoposide for 2 hours. After the treatment, cells were lysed and equal amounts of protein were subjected to SDS-PAGE and transferred to PVDF membranes. p53, PUMA, and β-actin proteins were detected with their respective antibodies. (c) Statistical analysis of the expression levels of p53 (p53/β-actin) between individual groups as seen in (b) was carried out using one-way ANOVA with a Newman–Keul post hoc test from 3 sets of experimental results. Significance was assumed at $^*P < 0.05$. (d) Statistical analysis of the expression levels of PUMA (PUMA/β-actin) between individual groups as shown in (b) was performed using one-way ANOVA with a Newman–Keul post hoc test from 3 sets of experimental results. Significance was assumed at $^*P < 0.05$. (e) A diagram showing proposed regulation of p53 IRES activity by TCP80 and RHA. During the basal conditions, the secondary structure of the p53 IRES is largely stabilized and has limited translational activity due to inadequate interaction between TCP80/RHA and the p53 IRES. Following DNA damage, increased binding of TCP80 to the p53 IRES and enhanced interaction between TCP80/RHA and the p53 IRES facilitate the unwinding of the secondary structure of the p53 IRES, allowing increased translation of the p53 mRNA in response to DNA damage.

3.6. Decreased Expression of TCP80 and RHA in MCF-7 Cells Leads to Diminished p53 Induction following DNA Damage and Decreased Expression of Its Downstream Target PUMA. To further determine the functional link between positive regulators of p53 IRES, such as TCP80 and RHA, and p53 induction following DNA damage, we created a MCF-7 cell line that is stably transfected with a plasmid containing a shRNA against TCP80. Our results indicate that TCP80 expression is markedly reduced in MCF-7/shTCP80 cells as compared to control MCF-7 cells (Figure 4(a)). Since expression levels of TCP80 and RHA are known to be correlated in various cell lines [35], we tested whether a decrease in TCP80 expression would also result in decreased cellular levels of RHA. Our results showed that this is indeed the case, as the MCF-7/shTCP80 cell line also exhibits reduced expression of RHA (Figure 4(a)).

Next, we examined the expression of p53 in MCF-7/shTCP80 and MCF-7 cells after treating the cells with or without etoposide. We found that p53 expression is reduced in MCF-7/shTCP80 cells as compared to MCF-7 cells. More importantly, we also observed a dramatically decreased p53 induction in MCF-7/shTCP80 cells following etoposide treatment as compared to MCF-7 cells (Figures 4(b) and 4(c)). The p53 upregulated modulator of apoptosis (PUMA) is a proapoptotic protein whose transcription is stimulated by the tumor suppressor p53. Our results show that PUMA

expression is reduced in MCF-7/shTCP80 cells as compared to MCF-7 cells. Moreover, while we observed significantly increased expression of PUMA in MCF-7 cells in response to DNA damage, the induction of PUMA following DNA damage is essentially abrogated in MCF-7/shTCP80 cells (Figures 4(b) and 4(d)). As a key regulator of apoptotic process, p53 can induce apoptosis by upregulating the expression of PUMA following DNA damage; our results thus suggest that TCP80 and its binding protein RHA may play important roles in IRES-mediated p53 induction and in regulating p53's tumor suppressive function in response to DNA damage.

4. Discussion

TCP80 is known to regulate the translation of the acid beta-glucosidase mRNA by binding to its coding sequence [33]. We found that binding of TCP80 to the p53 mRNA *in vivo* increases following DNA damage. Our results also show that TCP80 is a positive regulator of the p53 IRES and its overexpression enhances the p53 IRES activity following DNA damage. Furthermore, TCP80 stimulates p53 IRES activity in great part through its first double-stranded RNA binding domain (dsRBM), indicating that interaction between TCP80's dsRBM and p53 IRES's secondary structure could be important for increased p53 IRES activity following DNA damage. The involvement of TCP80 and its RBM in cellular IRES-mediated protein translation is further supported by a previous report indicating that TCP80 is an ITAF of the rhinovirus type 2 IRES [34].

RHA plays a crucial role in the translation of some viral and cellular mRNAs that contain a posttranscriptional control element (PCE) within their 5′-UTR [38]. The PCE typically forms a complex secondary structure that hinders 40S ribosomal subunit scanning and efficient translation. However, RHA can bind to the PCE and disrupt or open up its secondary structure by modifying RNA-RNA or RNA-protein interactions. This allows for a more efficient scanning of the 40S ribosomal subunit and translation initiation [38]. The region containing the p53 IRES is predicted to have a strong secondary structure [30]. RHA therefore could exert a positive effect on the p53 IRES by aiding in the unwinding of its secondary structure.

TCP80 and RHA proteins were found to bind to each other *in vitro* [19]. We have further confirmed that these two proteins associate with each other in MCF-7 cells. More interestingly, we observed increased binding of TCP80 to RHA and the p53 mRNA following DNA damage, and overexpression of TCP80, along with RHA, leads to increased expression of p53. Interestingly, expression levels of TCP80 and RHA are correlated in various cell lines [35]. We also found that levels of both TCP80 and RHA are low in MCF-7/shTCP80 cells, which leads to decreased expression of p53 and diminished p53 induction following DNA damage.

Our results suggest that the interaction between TCP80 and RHA is important for the stimulation of p53 IRES activity and p53 induction following DNA damage. It is thought that the dsRBMs of TCP80 are also needed for its interaction with RHA [32, 39]. Although we observed that overexpression of RHA leads to enhanced p53 IRES activity, overexpression of RHA alone cannot lead to a further increase in p53 IRES activity following DNA damage (Figure S1) (see Supplementary Material available online at http://dx.doi.org/10.1155/2015/708158). This result suggests that RHA could be mediating its effect on the p53 IRES activity through its interaction with TCP80. One explanation for the cooperative effect of TCP80 and RHA could be that RHA, as an RNA helicase, utilizes its ability to remodel RNA-RNA or RNA-protein interactions to facilitate increased binding of TCP80 to the p53 IRES following DNA damage, therefore leading to increased p53 IRES activity. Additionally, once more TCP80 are bound to p53 IRES, it could further facilitate the interaction between RHA and the p53 IRES so RHA can help unwind the secondary structure of the p53 IRES (Figure 4(e)).

Our results have also shown that reduced expression of TCP80 and RHA can lead to diminished induction of a p53 downstream target PUMA, a proapoptotic protein, following DNA damage. Since PUMA plays a critical role in p53's ability to induce apoptosis and prevent malignant transformation, this finding suggests that defective IRES-mediated p53 translation is involved in tumorigenesis [4]. The expression of TCP80 is known to be greatly reduced in malignant brain tumors of glial origin, and the subcellular localization of TCP80 is altered in these malignant tumors as well [40]. These results suggest abnormal expression or subcellular localization of TCP80 is linked to malignant transformation of normal cells. In addition, it was found that RHA maps to chromosome band 1q25, which is the site of a major prostate cancer susceptibility locus [41]. RHA also upregulates activity of several other tumor suppressors, such as Werner Syndrome Helicase (WRN), that are involved in DNA repair process through interaction with proteins in the DNA damage foci [42, 43]. Therefore, it is possible that alteration or deletion of this locus may result in abrogated RHA function or expression and prevent induction of the p53 IRES and/or other tumor suppressors, thereby increasing the risk of malignant transformation of prostate tumors. The roles of TCP80 and RHA in regulating p53 IRES activity and their involvement in oncogenesis require further investigation.

5. Conclusions

To date, the majority of research on p53 and oncogenesis has been aimed at characterizing the genetic mutations or posttranslational modifications that alter the p53 protein and lead to the loss of its transcriptional activity or induction in cancer cells [1, 44]. The mechanisms underlying translational regulation of the p53 tumor suppressor and the role of p53 translation in the prevention of tumorigenesis are significantly understudied. Our discovery of the function of TCP80 and RHA in regulating p53 IRES and p53 induction following DNA damage has provided a better understanding of the mechanisms that regulate IRES-mediated p53 translation in response to genotoxic stress. Given the importance of p53 in preventing tumorigenesis, the results obtained from this study may also provide important insights regarding

defective IRES-mediated p53 translation in the pathogenesis of cancer.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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Review Article

Splicing Regulators and Their Roles in Cancer Biology and Therapy

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Alternative splicing allows cells to expand the encoding potential of their genomes. In this elegant mechanism, a single gene can yield protein isoforms with even antagonistic functions depending on the cellular physiological context. Alterations in splicing regulatory factors activity in cancer cells, however, can generate an abnormal protein expression pattern that promotes growth, survival, and other processes, which are relevant to tumor biology. In this review, we discuss dysregulated alternative splicing events and regulatory factors that impact pathways related to cancer. The SR proteins and their regulatory kinases SRPKs and CLKs have been frequently found altered in tumors and are examined in more detail. Finally, perspectives that support splicing machinery as target for the development of novel anticancer therapies are discussed.

1. Introduction

Alterations in the alternative splicing pattern are essential for cellular development, differentiation, and response to physiological stimuli. However, abnormal splicing events can generate variants that contribute to different types of diseases, including cancer [1, 2]. Normally, the affected genes encode proteins involved in the main biological aspects of cancer cells such as cell cycle control, proliferation, differentiation, signal transduction pathways, cell death, angiogenesis, invasiveness, motility, and metastasis [3].

Alternative splicing offers the plasticity to reshape the proteome. It provides opportunity for the cancerous cells to subvert the production of protein isoforms for the benefit of tumor growth and spreading needs. Many of these processes represent a genomic return to isoforms normally expressed in a tightly controlled manner during development but repressed in most adult cells. Therefore, the regulation of these events in cancer can be understood as a consequence of the disruption of important developmental pathways [4].

The causing mechanisms of changes in the mRNA processing pattern involve both alteration of primary transcript regulatory sequences (*cis*-acting elements) and modifications in the activity of splicing factors (*trans*-acting elements). As the later ones can act in multiple pre-mRNAs, they have the capability of modifying the expression of multiple genes [5] and may then impact widely the cellular splicing pattern. Among the splicing factors that have been shown with abnormal activity in tumors, the SR proteins have received considerable attention [6]. This class of proteins is extensively phosphorylated in their SR domain mainly by Serine Arginine Protein Kinases (SRPKs) and CDC-like kinases, which affect their subcellular localization and splicing activity [7, 8]. When looking at neoplasia, the lack

of control in phosphorylation processes has a causative effect on protooncogenes as well as on splicing activity. It is the case of kinases SRPKs and CLKs which have also been found with altered activity in different types of cancer [9, 10].

Therefore, a better understanding of the regulatory mechanisms of these splicing regulatory elements in cancer biology is essential to support the development of new therapies. In this review, key findings on the roles of alternative splicing and its main regulators in tumor biology are discussed. In addition, pharmacological intervention possibilities that can impact the abnormal processing of pre-mRNAs in tumor cells are also examined.

2. Splicing Activity in Cancer Related Pathways and Processes

2.1. Apoptosis. Eukaryotic cells are constantly exposed to external and internal stress factors that cause damage to the integrity of the cell of their genome and other molecular components. Numerous cellular adaptive strategies involving pathways that control cell cycle and apoptosis were developed during evolution to ensure the organism survival [11]. As cancerous cells display a behavior that normally tries to avoid apoptosis, in various types of tumors the transcripts of a number of genes related to apoptosis are processed abnormally in order to prevent cell death [12, 13].

A well-known example of apoptosis regulator modulated by alternative splicing refers to the *BCLX* gene. It encodes two isoforms with opposite functions, BCL-XL (antiapoptotic) and BCL-XS (proapoptotic) [14]. The overexpression of the antiapoptotic BCL-XL isoform is related to both poor prognosis in acute myeloid leukemia [15] and chemotherapeutic resistance and poor prognosis in breast, prostate, and hepatocellular carcinomas [16–18]. BCL-XS/BCL-XL expression has been shown to be controlled by a number of splicing factors [19–22] as well as by the activity of a long intronic noncoding RNA named *INXS*, which acts by interacting with the splicing factor SAM68 [23]. *INXS* induces apoptosis by favoring the expression of the proapoptotic BCL-XS. The BCL-XS was found downregulated in kidney, liver, breast, and prostate human cancer cell lines in comparison to nontransformed cells, consistent with the observation of elevated levels of the antiapoptotic BCL-XL isoform [23].

The proper activity of the apoptosis regulator FAS has been shown to be an important determinant for clinical outcomes and chemotherapy effectiveness [24]. Besides its transmembrane proapoptotic isoform, the *FAS* gene can also be expressed as a soluble prosurvival variant (sFAS) due to the skipping of exon 6 which encodes the FAS transmembrane domain [25, 26]. Associated with poor overall survival and disease-free survival rates, sFAS levels have been found increased in serum of patients with malignant lymphoma and chronic lymphocytic leukemia [27–30]. Mechanistically, a long intronic noncoding RNA known as *FAS-AS1* is involved in sFAS levels control. *FAS-AS1* binds to and sequesters the RNA binding protein RBM5, inhibiting, in turn, exon 6 skipping and reducing sFAS expression. Moreover, it has been shown that when *FAS-AS1* is expressed, the levels of sFAS are decreased which sensitizes lymphoma cells to FAS-mediated apoptosis [31].

Other splicing events important for apoptosis regulation include the genes *BIN1* and *CASP2*. BIN1 is a tumor suppressor absent in solid cancers including melanoma, neuroblastoma, breast, colon, and prostate cancers [32]. *BIN1* gene encodes multiple alternatively spliced isoforms important for DNA repair, cell-cycle control, apoptosis, and membrane dynamics. Some isoforms such as BIN1 +10 and BIN1 +13 have antiproliferative and proapoptotic roles, acting through caspase-independent pathways. In cutaneous T-cell lymphoma, the proapoptotic function of BIN1 isoforms occurs through downregulation of c-FLIP, an important inhibitor of apoptosis mediated by FAS/FASL [33]. However, abnormal splicing of *BIN1* can generate the BIN1 +12A which lacks the tumor suppressor activity [34, 35] (Figure 1).

Considering the *CASP2*, the activity of the RNA binding protein RBM5 increases the synthesis of mRNAs encoding the proapoptotic CASP-2L compared to the antiapoptotic CASP-2S [36]. In ovarian cancer cells, the cisplatin-induced apoptosis was inhibited by CASP-2S overexpression or promoted by its knockdown [37]. The antiapoptotic action of CASP-2S has been shown to be related to its interaction with cytoskeletal membrane associated proteins such as α-actinin and fodrin 4. Moreover, CASP-2S has been demonstrated to be responsible for inhibiting DNA damage-induced cytoplasmic fodrin cleavage, independent of cellular p53 status [37].

All these observations reinforce the idea that alternative splicing dysregulation in genes related to apoptosis is an important aspect in cancer research. For additional information about the relationship between apoptosis and alternative splicing, readers are referred to the recent specific reviews [4, 38].

2.2. Cell Migration, Adhesion, and Invasiveness. Splicing activity has been found to be important in different steps of metastatic process. It is the case of the cellular alternative splicing reprogramming observed during the epithelial to mesenchymal transition (EMT) in metastatic tumors [39], and the protein isoforms involved in cell migration, adhesion, and invasiveness generated by abnormal splicing [40, 41] (Figure 1). Specific examples are described below.

It has been demonstrated that the CD44 standard isoform (CD44s) plays an important role during EMT in bone breast cancer metastasis [42]. The expression of this isoform has been proved to be controlled by hnRNPM during tumor metastasis, attesting the concept that splicing regulatory networks is a crucial mechanism for cancer phenotypes [43]. Importantly, hnRNPM has been found associated with aggressive breast cancer and correlated with increased CD44s in patient specimens [44]. Mechanistically, ubiquitously expressed hnRNPM can act in a mesenchymal-specific manner to precisely control CD44s splice isoform switching during the EMT observed in tumor metastasis [44].

Other alternative splicing events important during the EMT that occurs in metastatic tumors involve the genes *BCLX* and *RON*. Overexpression of the BCL-XL isoform not only is associated with antiapoptotic function but also is

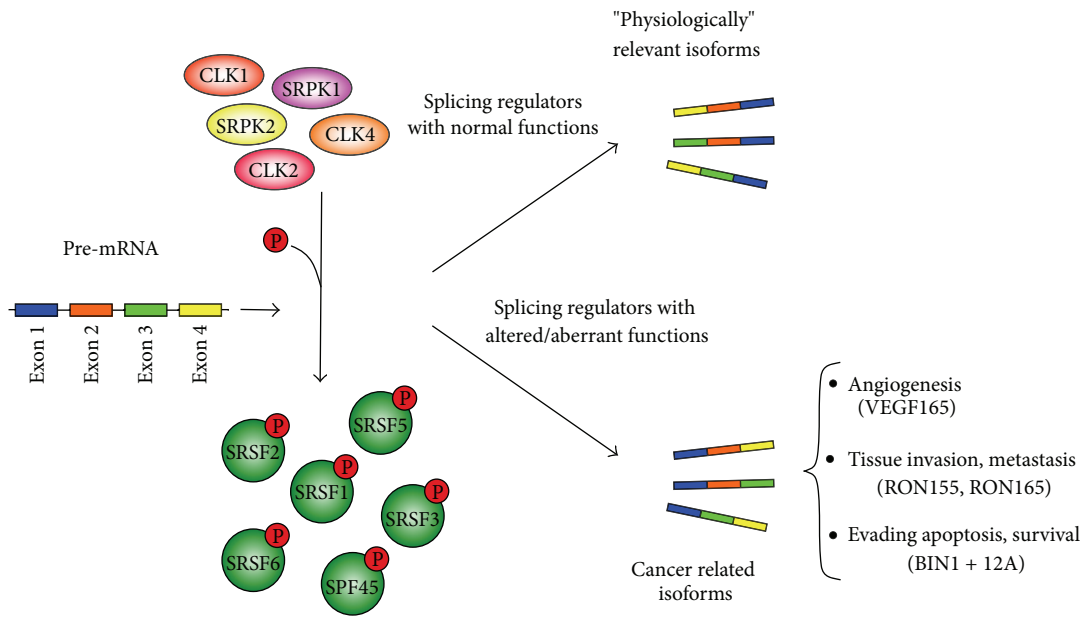

Figure 1: Dysregulation of splicing factors activity in cancer cells. Alternative splicing can generate physiological relevant transcripts in nontumor cells. Alterations in the splicing machinery, such as overexpression or dysregulation of function in regulatory splicing factors, that is, SRPKs, CLKs, or SR proteins, promote angiogenesis, tissue invasion, metastasis, apoptosis evasion, or survival in cancer. These aspects of cancer biology are supported by isoforms that predominate in tumor cells [57, 61–69].

correlated with increased risk of metastasis in breast tumors and multiple myeloma [45]. Moreover, isoforms derived from *RON* alternative splicing, which are involved in the control of cell motility, adhesion, proliferation, and apoptosis, are also related to EMT [46–48]. In this case, isoforms such as RON155 and RON165 are favored by overexpression of the splicing regulator SRSF2, resulting in cell morphology alterations that lead to increased activation in EMT and cell motility [49].

It has also been described that the RNA helicases DDX17 and DDX5 contribute to tumor cell invasiveness by regulating alternative splicing of several DNA and chromatin binding factors, including the macroH2A1 histone. The macroH2A1 splicing isoforms regulate the transcription of a set of genes involved in redox metabolism, such as the extracellular superoxide dismutase 3 (*SOD3*) gene, involved in cell migration [50].

Also, alternative splicing of *KAI1* gene leads to the generation of an isoform lacking exon 7 (*KAI1-SP*) which has been detected in metastatic tissues of gastric cancer patients with poor prognosis [51]. When ectopically expressed, contrarily to the tumor suppressive KAI1, this variant can increase *in vitro* invasiveness and *in vivo* tumorigenicity. These observations suggest that functional differences between these two proteins exist in events such as cell adhesion, spreading, and migration [51]. In ovary cancer, KAI1-SP has been detected with increased expression in metastatic tissues in comparison to primary tumors. Its role in reducing cell adhesion and increasing cell migration was demonstrated to be mediated by integrin ctVp3 [52]. Therefore, splicing activity over the *KAI1* gene leads to the expression of an isoform that favors tumor progression and metastasis [52].

Thus, considering the examples described above it is possible to notice that splicing activity provides critical isoforms for cellular processes that culminate in tumor metastasis.

2.3. Angiogenesis. As the tumor mass and size increase, the formation of new blood vessels is required to meet the needs for nutrients, oxygen, and elimination of the diverse metabolic waste. The important role of splicing events in angiogenesis can be fully demonstrated when looking at the control exerted on *VEGFA* gene. *VEGFA* splicing variants are produced due to proximal or distal splicing sites selection at exon 8, resulting in the expression of proangiogenic or antiangiogenic VEGF165 and VEGF165b, respectively [53–55]. Normal tissues can generate both isoforms [55]. Antiangiogenic isoforms have dominant expression in nonangiogenic tissues such as normal colon, whereas proangiogenic isoforms have been found prevalent in cancerous tissues such as colon and skin and in pediatric neuroblastoma [56–58]. Additionally, VEGF antiangiogenic isoforms levels have been found reduced in primary melanoma samples from patients who subsequently developed tumor metastasis compared with those who did not. This data suggests that there is a switch in splicing as part of the metastatic process from antiangiogenic to proangiogenic VEGFA isoforms [57]. This favoring of proangiogenic VEGF165 expression depends on the activity of SRSF1 upon control by the kinases SRPK1/2 [59] (Figure 1).

In colorectal cancer, a novel mechanism for VEGFA isoform expression has been shown to involve the T-cell Intracellular Antigen (TIA-1) activity [60]. A *TIA-1* splice variant encodes for a truncated form called short TIA-1 (sTIA-1). sTIA-1 has been found with elevated expression

in colorectal carcinomas and in *KRAS* mutant colon cancer cells and tissues, having its expression increased depending on the tumor development stage. Knockdown of sTIA-1 or overexpression of the full length TIA-1 induced expression of the antiangiogenic isoform VEGFA165b. Interestingly, the increased VEGFA165b translation promoted by TIA-1 is counteracted by sTIA-1, due to prevention of TIA-1 binding to *VEGFA165b* mRNA. sTIA has likewise been demonstrated to impact tumor development in mouse xenograft model by forming bigger, more vascularized, and resistant tumors during treatment with antiVEGF antibodies. Therefore, the finding that aberrant splicing of a translation regulator can modulate differential expression of VEGFA variants certainly adds a new layer of complexity to the angiogenic profile of colorectal cancer and their resistance to antiangiogenic therapy [60].

3. Splicing Regulators Related to Cancer: The SR Proteins

Among factors that regulate alternative splicing, the SR proteins family is essential to control and regulate various aspects of mRNA splicing as well as other RNA metabolism events [70–72]. Several studies have reported that changes in the expression or phosphorylation of SR proteins lead to expression of isoforms that stimulate resistance to apoptosis and cell proliferation and migration (Figure 1). These events have been identified in multiple types of cancers such as leukemia, glioma, breast, colon, pancreas, and lung, among others [62–64, 73, 74].

SRSF1 is a SR protein prototype that has been extensively characterized functionally and biochemically. It corresponds to the first splicing factor described as oncogenic and it has been implicated in a number of cancer related mechanisms [65, 75]. For instance, overexpression of SRSF1 in MCF-7 breast cell line has been linked to elevated levels of the isoforms BIN1 +12A (Figure 1) and S6K1-p31 which are involved in decreased tumor suppressor activity and increased oncogenic activity, respectively [61, 65]. Furthermore, SRSF1 has been found to regulate the expression of MNK2a and MNK2b, both splice isoforms of the MAPK pathway component MNK2 [65]. The expression of the isoform MNK2b, for instance, is implicated in the resistance of pancreatic cancer cells to treatment with gemcitabine [76]. Moreover, SRSF1 overexpression has been related to expression of two isoforms of the BCL-2 family proapoptotic BIM, BIM γ1 and BIM γ2. As they both lack the BH3 domain and the C-terminal hydrophobic regions, proapoptotic functions cannot be performed [77, 78]. Increased SRSF1 phosphorylation induced by hyperactivation of AKT can also result in the production of CASP9 prosurvival isoforms in nonsmall cell lung cancers [79]. In addition, SRFS1 along with the protein SAM68 [80, 81] regulates the expression of the cyclin D1 isoform CD1b which is involved in cell transformation [82, 83]. As previously mentioned (Section 2.3), SRSF1 has also been found to play a crucial role in angiogenesis since its knockdown prevents angiogenesis and tumor growth [59]. Regardless of the examples herein cited, readers may find additional information about the role of SRSF1 activity in cancer in two recently published specific reviews [75, 84].

Other SR protein family members have also been linked to cancer. SRSF3 and SRSF5 overexpression, for instance, have been found oncogenic by means of increasing the levels of the MCL-1 L isoform, which is involved with antiapoptotic response in MCF-7 and MDA-MB-231 cells [85]. Increased SRSF3 expression in colon and ovary cancers has been related to cell transformation and tumor growth maintenance [86–89]. In addition, SRSF6 and SRSF2 have been found engaged in the control of the ratio between the pro- and antiangiogenic VEGFA isoforms VEGFA165 and VEGFA165b, respectively [66, 67, 90, 91]. Also, SRSF2 can control *RON* transcription and splicing due to the exon 11 physical interaction and inclusion [49]. As RON is a protooncogene constitutively active if exon 11 is skipped, when SRSF2 is downregulated it may favor tumorigenesis by generating a prooncogenic RON isoform [49]. In skin cancer, SRSF6 is overexpressed and it can bind to alternative exons of the extracellular-matrix protein *tenascin C* pre-mRNA. This interaction promotes the expression of isoforms related to invasive and metastatic cancer independently of cell type [92].

Based on these examples described above, it is clear that SR proteins have critical roles in tumorigenesis when its normal activity is disturbed.

4. Splicing Regulatory Kinases and Their Roles in Cancer

A diverse number of kinases have been reported to transfer phosphate groups to SR proteins [93]. In the next sections, the main players of this context will be analyzed, that is, Serine-arginine Protein Kinases (SRPKs) and CDC-like kinases (CLKs), both responsible for phosphorylating SR proteins *in vivo* [73, 91, 94, 95].

4.1. SRPKs. The SRPKs are serine/threonine kinases that specifically recognize and phosphorylate SR proteins at Ser/Arg dipeptide in a processive manner [96–99]. Until now, four members of this protein family have been described in mammalian cells, that is, SRPK1, SRPK1a (spliced form of the previous one), SRPK2, and SRPK3 [100–102]. Whereas SRPK1 is found predominantly expressed in testicles and pancreas, SRPK2 is mainly found in the brain. Both are found moderately expressed in other human tissues such as skeletal muscle and heart and slightly expressed in the lung, liver, and kidney [102]. The expression of SRPK3 seems to be restricted to muscle cells [100, 102] and it has not been linked to cancer so far.

SRPK1 and SRPK2 have been found overexpressed in different types of cancer including breast, colon, pancreatic carcinomas, leukemia, nonsmall cell lung carcinoma, squamous cell lung carcinoma, gliomas, ovary, and hepatocellular carcinoma [62–64, 74, 103, 104]. Increased SRPK1 expression in breast and colonic cancer has been coordinately correlated to the enhancement of tumor grade [63]. Furthermore, targeting SRPK1 using small interfering RNA (RNAi) in

cell lines of these two tumors resulted in both increased apoptotic potential and enhanced cell killing after treatment with gemcitabine and cisplatin. These findings seemed to be accompanied by reduced phosphorylation of MAPK3, MAPK1, and AKT [63]. In breast cancer cells, increased levels of SRPK1 and the RNA binding protein RBM4 have been related to apoptosis resistance [105]. In leukemia, SRPK2 overexpression has been shown to result in increased cell proliferation due to SR protein acinus phosphorylation and cyclin A1 upregulation. These data have been complemented by knockdown experiments whose cyclin A1 expression attenuation and cell arrest at G_1 phase were both observed [64].

Overexpression of SRPK1 and SRPK2 has also been found in lung tumors samples in percentages as high as 92% and 94% for lung adenocarcinoma and 72% and 68% for squamous cell lung carcinoma, respectively [62]. Additionally, SRSF2 overexpression has been shown to mostly accumulate under its phosphorylated form in these patient samples in agreement with the observed overexpression of SRPK1 and SRPK2 [62]. In patients with ovarian cancer, SRPK1 has been found upregulated in 55% of tumor samples. *In vitro* experiments conducted with ovarian cell lines revealed that SRPK1 knockdown can lead to reduced cell proliferation rate, slower cell cycle progression, and compromised anchorage-independent growth and migration ability. Yet, it can lead to a decreased level of phosphorylation of multiple SR proteins, P44/42 MAPK and AKT. Finally, it enhances sensitivity to cisplatin similarly to that observed in breast and colonic cells [63].

SRPK1 has been found upregulated in low-grade gliomas and related to patient prognosis. Moreover, SRPK1 knockdown inhibited glioma cells growth, invasion, and migration in normoxic condition [74]. In clinical samples of hepatocellular carcinoma, SRPK1 has been found upregulated at both mRNA and protein levels [103]. In further *in vitro* and *in vivo* studies, SRPK1 appeared to influence hepatocellular cell growth and malignancy suggesting that SRPK1 plays an oncogenic role and might be a potential therapeutic target in these cancer cells [103].

Interestingly, it has been demonstrated that depending on the context SRPK1 can act as either oncogene or tumor suppressor [106] (Figure 2). SRPK1 presented tumor suppressor activity since its inactivation in mouse embryonic fibroblasts could induce cell transformation. This phenotype has been related to the impairing of PHLPP recruitment which leads to hyperactivation of AKT by maintaining its phosphorylated form. Furthermore, the overexpression of SRPK1 was observed to be tumorigenic as excess of SRPK1 squelches PHLPP1 and leads to a marked AKT phosphorylation. Therefore, it was concluded that both under- and overexpression of SRPK1 are tumorigenic since both induce constitutive AKT activation [106]. Taken together, these findings could mechanistically explain previous observations that SRPK1 could be found downregulated in some cancer contexts.

4.2. CLKs. CLKs comprise a nuclear kinase group that phosphorylates SR proteins. This family is also implicated in the control of splicing and consists of four members, *CLK1–CLK4*. While CLK1, CLK2, and CLK4 are ubiquitously expressed, CLK3 is specifically expressed in testicles [107]. The CLKs are characterized by presenting a C-terminal kinase domain with dual specificity, which is closely related to serine-threonine kinases, and an N-terminal RS domain that allows interaction with SR proteins. CLKs colocalize with SR proteins in nuclear speckles. Overexpression of CLKs leads to hyperphosphorylation of SR proteins and induces the redistribution of proteins SR within the nucleus [108]. Although CLKs and SRPKs share common substrates, they have different specificities and act coordinately to regulate splicing properly [109]. For instance, SRPK1 phosphorylates SRSF1 which, in turn, is assembled in nuclear speckles. The release of SRSF1 from speckles depends on phosphorylation by CLK1, also called CLK/STY [9]. CLKs and SRPKs correlated activity can also be demonstrated during the regulation of *VEGFA* splicing. While IGF-1 growth factors and TNF-α induce the production of VEGF165 through SRPKs activation, TGF-β1 increases the expression of VEGF165b through the activation of CLKs [67].

CLKs have also been related to cancer. For example, CLK1 phosphorylates the alternative splicing factor 45 (SPF45) at eight serine residues (Figure 1). The SPF45 expression is low in normal tissues but high in breast, ovarian, and prostate cancers [68]. In a CLK1 phosphorylation dependent way, the overexpression of SPF45 induces ovarian cancer cells migration and invasion, fibronectin expression, and splicing and phosphorylation of cortactin—a protein that regulates actin polymerization. Another example is the tumorigenic CLK2 which has been found amplified and overexpressed in a significant fraction of human breast tumors [110]. Its downregulation also inhibits breast cancer cell growth and tumorigenesis *in vitro* as well as in a mouse tumor model [110, 111].

5. Splicing Activity Related to PI3K/AKT/mTOR and Ras/MAPK Pathways

The PI3K/AKT/mTOR and RAS/RAF/MEK/ERK pathways (Figure 2) are the most frequently impaired signaling pathways in cancer [111, 112]. Alternative splicing machinery dysregulation has been demonstrated to impact the proper physiological signal flow across these pathways, contributing to cell transformation, tumor development, and maintenance [113]. Several examples of abnormal alternative splicing events that affect components of these pathways have been shown in cancerous cells including the tyrosine kinase receptors EGFR, FGFR, INSR, VEGFR, MET, and RON as well as the cytosolic SRC, RAS, and RAF. The alternative splicing events related to these components have been accordingly revised by Siegfried et al. [114]. However, some examples of how alternative regulators can be linked to the abnormal isoform generation or involved in these pathways dysfunction will be discussed below.

As previously mentioned in Sections 3 and 4.1, SRSF1 and SRPK1 have been shown to influence MAPK pathways activity in tumor cells due to their activities as splicing regulators [63, 65] (Figure 2). In addition to the dysregulation of

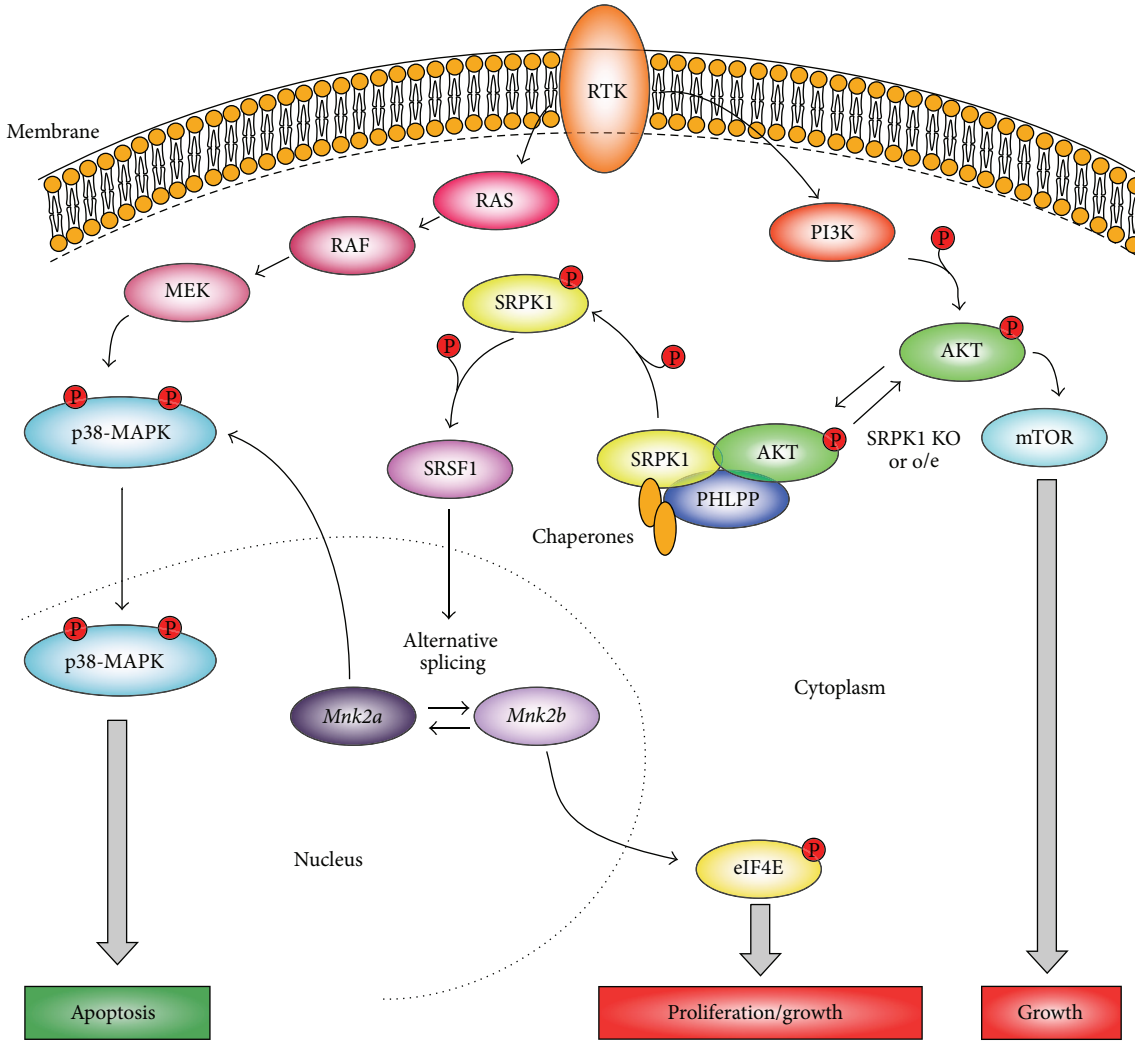

FIGURE 2: Cellular pathways related to SRPK1 activity. SRPK1 has been connected to important pathways of tumor biology. For instance, it can control alternative splicing events due to the activation of Receptor of Tyrosine Kinases (RTK). In this case, AKT activation can lead to SRPK1 nuclear translocation (not shown), activation of SRSF1, and generation of isoforms such as MNK2b, involved in promoting cell growth and proliferation. On the other hand, MNK2a variant expression, which is disfavored by SRSF1, can promote apoptosis. The phosphatase PHLPP is a key regulator in this process since its activity is necessary for AKT inactivation. Reduced expression of SRPK1 has been shown to decrease PHLPP recruitment to AKT leading to cellular growth increasing. Higher SRPK1 levels, however, may titrate PHLPP away from AKT complex which can also result in AKT/mTOR axis activation. Thus, either overexpression or downregulation of SRPK1 may be oncogenic, explaining why it can be found overexpressed in some tumors but also downregulated in others [106].

MAPK pathways in colon and breast cancers owing to activity changes in MAP2K1 and MAP2K2, SRPK1 overexpression can also affect the splicing of the MAPK signaling pathway component PYK2 which, in turn, has been associated with cancer development [115]. Considering the regulation performed by SRFS1 on *MKNK2* gene [116], *MKNK2* can be expressed as mRNA two spliced isoforms with differences in the last exons, *MNK2a*, which encodes for a MAPK binding domain, and *MNK2b*, which does not do that [117, 118]. MNK2a interacts and translocates p38α-MAPK into the nucleus leading to the activation of target genes, increasing cell death, and suppressing induced transformation by RAS [119]. Alternatively, MNK2b is prooncogenic as it cannot activate p38α-MAPK [76] (Figure 2). Thus, downregulation of MNK2a due to SRSF1 activity controlled by SRSF1 constitutes a tumor suppressor mechanism that is lost in tumors such as breast, lung, and colon [119].

Other examples on how splicing activity can affect or be affected by MAPK pathways include the activity of the splicing factor SPF45 and the protein lysyl oxidase-like 2 (LOXL2). SPF45 has been found overexpressed in cancer cells and can be phosphorylated by MAP kinases such as ERKs, JNK, and p38 MAPK in response to phorbol myristate acid (PMA), H_2O_2, UV, and anisomycin stimulation [68, 120]. It has

been suggested that SPF45 activation via MAP kinases may connect extracellular stimuli to alternative splicing events that may impact cancer. It is the case of the decrease of SPF45-dependent *FAS* exon 6 exclusion, which is a phenomenon observed under ERK and p38 activation. These findings point out that a splicing factor such as SPF45 may be regulated by multiple MAP kinase pathways which can result in alterations in splicing programs relevant to cancer cells.

The LOXL2 protein has also been described as a poor prognosis indicator in human squamous cell carcinomas [121] and as a contributor to tumor cell invasion and metastasis during gastric carcinoma progression [122]. It has been demonstrated that a LOXL2 isoform produced due to lack of exon 13 (LOXL2 Δe13) modulates cancer cell migration and invasion through a different mechanism from that of full-length LOXL2. LOXL2 Δe13 affects MAPK8 expression without affecting the FAK, AKT, and ERK signaling pathways. Differently from the full-length LOXL2, MAPK8 seems to be a downstream component of LOXL2 Δe13, as RNAi-mediated knockdown of MAPK8 results in cell migration blockage promoted by LOXL2 Δe13, but not by the full-length LOXL2 activity [123]. These observations suggest how an abnormal alternative splicing event may affect the activity of MAPK pathway components.

Regarding the pathway PI3K/AKT/mTOR, *S6K1* variants controlled by the splicing factor SRSF1 possess oncogenic properties able to assist breast epithelial cells transformation, motility, and anchorage-independent growth [65]. For example, SRSF1 increases the expression of a shorter oncogenic S6K1 isoform capable of transforming immortal mouse fibroblasts [65]. This small isoform can bind to mTOR and activate mTORC1 leading to an increased 4E-BP1 phosphorylation, cap-dependent translation, and upregulation of the antiapoptotic protein MCL-1 [124].

6. Targeting Pre-mRNA Splicing Machinery in Cancer and Its Challenges

Not so long ago, several drugs acting on specific cellular targets started to be approved as anticancer agents. Medicines such as herceptin, gleevec, EGFR inhibitors (gefitinib, erlotinib, and cetuximab), and avastin are now being clinically used to target specific proteins in order to block subcellular pathways relevant to cancer cells [125, 126]. Nevertheless, how patients respond to these drugs is still a puzzle and the answer may rest in the alternative molecules expressed in different individuals when the tumor is under attack during treatment. Thus, although great improvements involving the understanding of cancer mechanisms have been achieved, the treatment and prognosis of tumors remain a big challenge and still require a permanent investigation by academia.

In this review, we discussed the most recent findings regarding how splicing machinery alterations may affect the expression of genes relevant to cancer. As we presented, the findings herein described with focus on the SR proteins and their regulatory kinases, SRPKs and CLKs, highlight the mammalian RNA metabolism as a new source of subcellular targets for the development of anticancer therapies [72]. Despite the availability of a plenty of reports corroborating such idea in the literature, at least two main questions may intrigue scientists in the field: first, are splicing regulators good targets for cancer therapy even if they are expressed in every kind of tissue? Second, how can these drugs be specific for cancer cells?

With our current understanding, these questions cannot be yet fully answered by the available published data. However, some promising experimental results involving pharmacological *in vitro* and *in vivo* inhibition of splicing regulators may help to think over these questions. It is the case of the small molecule inhibitor of SRPK1/2 named SRPIN340. It seems that this compound is effective in blocking angiogenesis and preventing tumor growth in nude mice [59, 127]. Also, SRPIN30 possesses antimelanoma effect *in vitro* and *in vivo* [128]. In addition to this SRPKs inhibitor, pharmacological inhibition of CLKs also seems to be a plausible strategy towards control of tumor growth. This statement can be corroborated taking into account three small CLKs-inhibiting molecules which have been found to modulate *S6K* splicing and suppress breast, lung, and colorectal cancer cell growth *in vitro* [129]. Other CLKs inhibitors that have already been published include the dichloroindolyl enaminonitrile KH-CB19, a potent and highly specific inhibitor for CLK1 and CLK4 [130], and the amino-substituted pyrimidine, a dual specificity inhibitor which targets CLK1, CLK4, and the dual-specificity tyrosine-regulated splicing regulatory kinase DYRK [131]. Furthermore, a 2,4-bis-heterocyclic substituted thiophenes compound has been found to inhibit DYRK1A and 1B, showing a moderate selectivity for DYRK2. Since central nervous system penetration of this compound may occur, it has been believed that it might be used to the development of therapeutic agents against glioblastoma [132].

Even though these reports are encouraging since they suggest novel therapeutic opportunities for fighting cancer, the low pharmacological capacity of some splicing machinery inhibitors (SRPIN340, for instance) has already been noticed *in vivo* [128]. This points to the fact that the search for novel compounds with increased drug-like properties is desirable. Moreover, not all the splicing machinery inhibitors have been evaluated *in vivo* limiting the perception of their real chemotherapeutic potential. Nonetheless, the availability of these *in vitro* and *in vivo* data for the research community *per se* would be considered as an interesting opportunity to guide further studies. The rationalization of these data along with the use of already solved crystallographic structures and deposited in the protein data bank certainly may favor further structure guided efforts to design more favorable substances in the light of the medicinal chemistry knowledge.

Finally, it is not worthless to affirm that cancer treatment is still a great challenge. It is imperative to keep searching for alternative approaches in order to stop the growing list of cancer death cases globally. As a multifactorial disease, cancer demands a better look at patient molecular signatures and predictors in order to pursue an efficient therapeutic regime for each individual who will receive a treatment as specific

as the available drug arsenal increases. Thus, cancer control depends on a constant effort toward the discovery of novel and efficient therapeutic strategies [125, 133, 134].

7. Conclusions

In recent years, there have been significant advances in research areas that link alternative splicing to cancer. Certainly, there is still a lot to learn about the role of splicing activity within the context of this disease. It is hoped that future studies in the field may favor the development of alternative therapeutic approaches. The recognition of the splicing regulatory kinases SRPKs and CLKs as signal transducers in mammalian cells has opened the doors not only for the understanding of regulatory factors behind abnormal splicing found in tumor cells but also for the development of novel targeting therapies. Thus, based on the investigations herein discussed, it is clear that pharmacological interventions based on regulatory splicing pathways may represent a promising antitumor alternative and should be explored by the scientific community.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Splicing Regulation: A Molecular Device to Enhance Cancer Cell Adaptation

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Alternative splicing (AS) represents a major resource for eukaryotic cells to expand the coding potential of their genomes and to finely regulate gene expression in response to both intra- and extracellular cues. Cancer cells exploit the flexible nature of the mechanisms controlling AS in order to increase the functional diversity of their proteome. By altering the balance of splice isoforms encoded by human genes or by promoting the expression of aberrant oncogenic splice variants, cancer cells enhance their ability to adapt to the adverse growth conditions of the tumoral microenvironment. Herein, we will review the most relevant cancer-related splicing events and the underlying regulatory mechanisms allowing tumour cells to rapidly adapt to the harsh conditions they may face during the occurrence and development of cancer.

1. Introduction

The transcription units of most eukaryotic genes are characterized by exons, short regions of approximately 200 bp containing untranslated and coding sequences, interspersed between large noncoding introns (generally ≥ 1000 bp) [1]. Removal of intronic sequences and joining of exons is one of the key events in the multistep process ensuring maturation of pre-mRNAs into mRNAs [2]. This process, called splicing, 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 a large number of auxiliary proteins [3]. Alternatively, a small proportion of introns (≈1%) are processed by the minor spliceosome comprising U11, U12, U4atac/U6atac, and U5 snRNPs [4]. The main spliceosome mediates the recognition of short consensus sequences defining the 5′ (GU) and 3′ (AG) splice sites (ss) and catalyses the two transesterification reactions necessary for the junction of exons and removal of introns [5]. Beside the conserved dinucleotide sequence that marks the ss, exon-intron junctions are not characterized by a stringent consensus and their short and degenerate nature is not sufficient to ensure perfect recognition by the spliceosome. Thus, additional factors are required to assist the spliceosome in its critical and essential function.

The activity of the spliceosome is regulated by both *cis*-acting sequences on the pre-mRNA and transacting factors, which may enhance or inhibit both recognition of the ss and splicing catalysis [5]. The *cis*-acting regulatory elements are classified according to their location and activity into exonic and intronic splicing enhancers (ESEs and ISEs, resp.) or silencers (ESSs and ISSs, resp.) [6]. These sequence elements are recognized by transacting RNA binding proteins (RBPs), which in turn promote or inhibit spliceosome assembly and activity. Two main classes of RBPs that regulate splicing by binding to these *cis*-acting regulatory elements are the Ser/Arg rich (SR) proteins, which mainly exert a positive regulation on the spliceosome, and the heterogeneous nuclear ribonucleoproteins (hnRNPs), which often act antagonistically and inhibit splicing [7].

Chromatin signatures on the template DNA also participate in splicing regulation. Higher levels of nucleosome occupancy and specific histone modifications, such as trimethylation of H3K36, were found to be enriched in exons [8]. These observations suggest that epigenetic marks may facilitate exon recognition during splicing, perhaps by slowing down

RNA polymerase II (RNAPII) in proximity of exons. Indeed, splicing largely occurs cotranscriptionally when the nascent pre-mRNA is still bound to the DNA template [9] and is likely affected by the elongation rate of the polymerase [10]. In addition, some splicing factors (SFs) interact with chromatin-binding proteins (i.e., MRG15, Gcn5, CHD1, and HP1α) and are recruited to histone marks enriched nearby exons, thereby modulating their selection [10, 11].

An additional layer of complexity to the splicing process is provided by the presence of exons characterized by even weaker elements defining exon-intron boundaries. Although this feature makes these exons weaker, it also represents a flexible resource for the gene as it allows their variable inclusion into mature transcripts through the alternative splicing (AS) process. Indeed, through differential assortment of weak or variable exons, a gene can yield multiple mRNA splice variants, potentially encoding proteins with different or even opposite function and/or displaying different patterns of spatial/temporal expression [6, 12].

The advent of high-throughput sequencing technologies has revealed the unexpected pervasive nature of AS. It is now clear that the vast majority of higher eukaryotic genes undergo AS [13, 14]. In some cases, the combinatorial nature of AS allows a single gene to encode for up to thousands of mRNA variants. This extreme flexibility of the splicing process contributes to the great expansion of the coding potential and plasticity of the genome [15, 16]. In support of this notion, eukaryotic cells have been documented to promptly modulate their splicing program in response to different intra- and extracellular cues [17], thus making AS a key tool to fine-tune gene expression. AS plays a pivotal role in controlling core cellular processes, such as proliferation, metabolism, and apoptosis, and fundamental physiological decisions, such as maintenance of pluripotent state or induction of a specific differentiation lineage [18]. Nevertheless, although AS represents a key tool to control gene expression in higher organisms, the extreme flexibility and multilayer nature of its regulation render it error prone and susceptible to alterations that threatens the maintenance of cellular homeostasis. As a proof of this concept, aberrant regulation of AS contributes to the onset or progression of several human diseases, including cancer [19, 20].

In the last decade, high-throughput analyses of transcriptomes have highlighted widespread alterations of AS patterns in human cancer [21, 22]. When identified, the causes of these alterations were attributed to almost all the regulatory steps controlling AS [23–25]. Mutations in the *cis*-acting splicing regulatory elements, altered expression of SFs, and aberrant regulation of proteins and signalling pathway regulating their activity have all been documented in cancer cells and identified as factors promoting oncogenic splice variants and contributing to neoplastic transformation or later stages of carcinogenesis [23, 24]. Thus, cancer cells can rapidly adapt to stimuli received from both extracellular and intracellular cues by finely regulating AS in order to shape gene expression. Herein, we will discuss examples of how AS contributes to the enhanced adaptation capability of cancer cells towards the adverse conditions occurring during the tumorigenic process or triggered by therapeutic intervention.

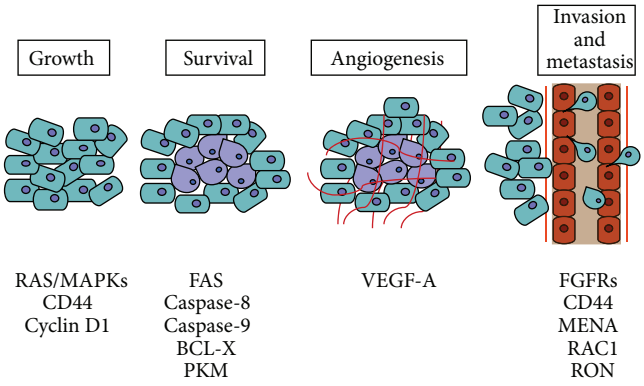

Figure 1: AS events that contribute to the adaptive response of cancer cells during tumorigenesis. Most relevant cancer-related genes undergoing AS misregulation are shown below the key events of tumoral transformation.

2. Functional Impact of Splicing in Cancer Adaptation

Cancer is a complex disease associated with a variety of genetic and epigenetic aberrations. As illustrated by Hanahan and Weinberg [26], during carcinogenesis cells acquire ten common traits: sustained proliferative signalling, resistance to death, evasion from growth suppressors, ability to invade normal tissues and metastasize, replicative immortality, induction of angiogenesis, genetic diversity generated by genome instability, inflammation, reprogramming of energy metabolism, and escape from immune destruction. A number of studies have now documented that aberrant regulation of AS in cancer cells contributes to many of these traits by allowing the production of oncogenic splice variants from multiple genes (Figure 1). Specific splice variant signatures are strongly associated with particular types of cancers, representing valuable diagnostic and prognostic markers [27, 28]. Although their functional/mechanistic roles are still largely uncharacterised, these splice variants likely contribute to the acquisition of therapeutic resistance and to the increased adaptability of cancer cells to adverse environments. Herein, we will review some of the most important cancer-related AS events that play a functional role in the adaptation process set in motion by a tumour cell during both the early stages of development and progression of the pathology (Figure 2). Although cancer cells do not act "on purpose," we present a figurative writing style to stress the dynamic nature of cancer cell adaptation.

2.1. Sustained Proliferative Signalling. A critical feature of tumorigenesis is uncontrolled cell proliferation, including the ability to grow in the absence of external stimuli. This skill is acquired through a myriad of abnormal modifications of growth factor signalling cascades and expression of their messengers and effectors. It is therefore not surprising that the powerful combinatorial effect conferred by AS is hijacked by cancer cells to increase the expression of isoforms whose activity promotes and sustains cell proliferation.

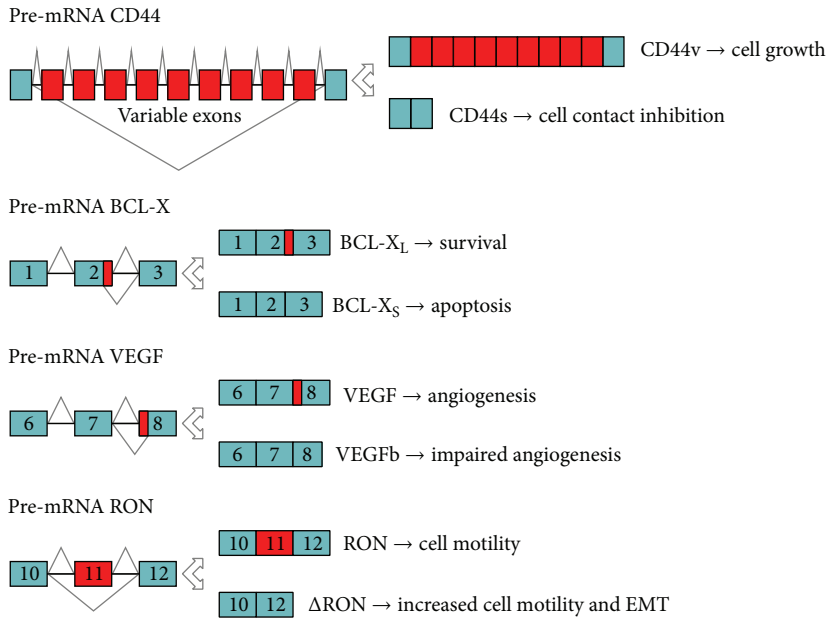

Figure 2: AS events that characterize specific phases of tumour occurrence and development. Green boxes and red boxes indicate constitutive and variable exons, respectively. Black lines indicate the intron sequences. The splice variants produced and their cellular functions are illustrated to the right of the gene schematic representation.

Homeostasis of growth control in cancer is often disrupted by constitutive activation of the RAS/MAPKs signalling pathway that plays a central role in cell proliferation, differentiation, and migration. Aberrant RAS activity may occur by several regulatory mechanisms that disrupt negative feedback loops or establish aberrant positive feedback loops in the pathway. The misregulation of AS of genes involved in the RAS pathway contributes to its activation in cancer, thus enhancing cell proliferation. An example is represented by the RAS-activated A-RAF kinase. AS of *A-RAF* is modulated by the splicing factor (SF) hnRNP A2, which represses the production of a short dominant-negative isoform in favour of the full-length transcript. Aberrant regulation of this splicing event leads to constitutive activation of the RAS/MAPKs pathway, cellular transformation, and increased proliferation [29]. Similarly, AS of *B-RAF* (V600E), a mutation present in 50% of metastatic melanomas, might result in being critical for this cancer and its treatment. A recent study involving experimental cell culture models and patient samples showed the existence of B-RAF (V600E) splice isoforms that lack the RAS binding domain and promote resistance to chemotherapy [30].

Hyperactivation of transmembrane receptors upstream of RAS can also contribute to favouring cancer-related AS events through positive feedback loops that modulate the activity of specific SFs. A typical example is represented by the epidermal growth factor receptor (EGFR), a tyrosine kinase receptor that plays a central role in cell proliferation and motility. EGFR pre-mRNA is alternatively spliced to generate a variant lacking exon 4 (EGFRΔ4). Skipping of this exon yields a receptor that is constitutively active and promotes proliferation. Notably, the EGFRΔ4 isoform is abundantly expressed in several cancers, such as glioma, prostate, and ovarian cancer [31]. Furthermore, a recent work documented that an active EGF signalling *per se* induces a massive reprogramming of AS. This effect was attributed to AKT-induced nuclear translocation of the SR protein kinase 1 (SRPK1). AKT binding to SRPK1 induces its autophosphorylation and dissociation from the HSP70 chaperone, which normally holds SRPK1 into the cytoplasm, thus favouring its nuclear translocation [32]. Once in the nucleus, SRPK1 can phosphorylate SR proteins and modulate the splicing pattern of several genes [32]. Since SRPK1 is usually localized in the cytoplasm in the absence of an extracellular signal and phosphorylates shuttling SR proteins in this cellular compartment [33, 34], stress signals might expand the effect of SRPK1 activation to the nucleus and influence also SR proteins that mostly reside in this compartment.

AS of the *CD44* gene also serves as a critical mechanism for a feed-forward loop that sustains activation of RAS/MAPK signalling [35]. CD44 is a transmembrane glycoprotein mediating the response of cells to their cellular microenvironment. CD44 is expressed in most tissues, where it functions in lymphocyte homing, adhesion, migration, and regulation of cell growth [36]. This variety of roles is favoured by the existence of multiple CD44 splice variants. The *CD44* gene is composed of 10 constitutively spliced exons and 10 variable exons, residing between constitutive exons 5 and 6. Upon mitogenic activation, the RAS/MAPK pathway positively regulates the activity of SAM68 and SRm160, two SFs that promote inclusion of variable exons in CD44 [37, 38]. The newly synthesized CD44v6 isoform, containing variable

exon 6, forms complexes with receptor tyrosine kinases (RTKs) that promote RAS/MAPK activation and cell cycle progression [35].

Another oncogenic AS event that sustains uncontrolled proliferation of cancer cells affects the cyclin D1 (*CCND1*) protooncogene. Cyclin D1 associates with the cyclin-dependent kinase 4 (CDK4) to drive progression through the G1 phase of the cell cycle. Importantly, cyclin D1 expression is often deregulated in cancer cells [39, 40]. This gene encodes for two alternative transcripts: the common cyclin D1a isoform and the prooncogenic cyclin D1b isoform. In prostate epithelial cells, the canonical cyclin D1a isoform is involved in a negative feedback loop that controls proliferation. Cyclin D1a interacts with and represses the transcriptional activity of the androgen receptor (AR), which orchestrates the proliferation and activity of prostate cells [41]. By contrast, although the cyclin D1b isoform is capable of driving the G1/S transition of the cell cycle and to interact with AR, it does not repress its transcriptional activity, thereby interrupting this negative feedback [41]. Notably, two SFs that are often upregulated in cancer cells [42, 43], SRSF1 and SAM68, promote cyclin D1b splicing in prostate cancer cells [44, 45]. SAM68-dependent regulation of cyclin D1b splicing represents another clear example of how activated signalling pathways modulate cancer-related AS events by influencing the activity of specific SFs. Indeed, activation of the RAS/MAPKs pathway enhanced SAM68 binding affinity for cyclin D1 RNA and SAM68-dependent cyclin D1b splicing, whereas SAM68 phosphorylation by SRC-family kinases (SFKs) counteracted these activities [45]. Thus, these studies indicate how upregulation of two oncogenic SFs can unleash prostate cancer cells from the cyclin D1a/AR negative feedback that limits excessive proliferation of the epithelial cells in the normal organ.

2.2. Induction of Angiogenesis. Angiogenesis is the physiological process yielding new blood vessels. Neoangiogenesis normally occurs during embryogenesis and fetal development in response to the need for oxygen and nutrients of the growing mass of cells forming new tissues and organs. A similar situation occurs during tumorigenesis, when cancer cells begin to proliferate within a steady-state adult tissue. Growth of the tumour mass depletes the host tissue of nutrients and oxygen, causing starvation and promoting the formation of new vessels as an adaptive response. Tumour-associated neoangiogenesis provides cancers cells with access to blood circulation, thus facilitating tumour growth.

The main growth factors promoting angiogenesis are the vascular endothelial growth factors (VEGF) [46]. In humans, the VEGF family consists of five ligands and three signalling receptors. The ligands, VEGF-A-D and placental growth factor, are all alternatively spliced to yield isoforms with opposite or, in some cases, unknown function. Since *VEGF-A* AS is altered in a number of cancers, such as metastatic melanoma, neuroblastoma, and renal, prostate, colorectal, and bladder cancers [47], this gene is also the most studied of the family.

The alternative splice variants of *VEGF-A* exert different effects on tissue and tumour growth due to their opposing effects on angiogenesis. Several regulatory mechanisms that are critical for the splicing of this gene have now been identified. The best-known one consists in alternative usage of two 3′ ss in *VEGF-A* exon 8 [48]. SRSF1 and SRSF5 (SRp40) promote usage of the proximal 3′ ss, thus favouring the production of mRNAs encoding proangiogenic proteins [49]. By contrast, SRSF6 (SRp55) and SRSF2 (SC35) facilitate the selection of the distal 3′ ss, resulting in production of the anti-angiogenic VEGFb isoform [49].

Signalling pathways evoked in cancer cells by the surrounding environment can mediate the balance between anti-angiogenic and proangiogenic VEGF isoforms [50, 51]. Such regulation occurs either by direct control of their phosphorylation status by signalling kinases or by indirectly regulating splicing factor kinases involved in their posttranslational modifications. An example of indirect regulation is illustrated by insulin-like growth factor-1- (IGF-1-) mediated activation of protein kinase C (PKC) signalling, which in turn positively regulates SRPK1-dependent phosphorylation of SRSF1 and SRSF1-dependent *VEGF-A* AS [51]. A similar regulatory mechanism is also observed in prostate cancer, where selective upregulation of proangiogenic VEGF is under the direct control of SRPK1-regulated SRSF1 activity [50]. Importantly, genetic or pharmacological interference with SRPK1 activity caused a switch in the expression of proangiogenic towards antiangiogenic VEGF splice isoform, resulting in decreased microvessel density and reduced tumour growth [50]. Thus, the upregulation of SRPK1 and SRSF1 activity frequently observed in human cancers might contribute to the ability of the tumour mass to promote neoangiogenesis and redirect the blood stream towards itself. Alternatively, SRSF1-dependent *VEGF-A* AS may be indirectly regulated by the transcription factor WT1, encoded by the Wilms' tumour gene (*WT1*) [52]. It was shown that WT1 represses the transcription of *SRPK1* by directly binding to its promoter. This effect results in reduced SRPK1-dependent SRSF1 phosphorylation and inhibition of the production of prooncogenic VEGF isoform. Importantly, the authors demonstrated that in WT1 mutant cells *SRPK1* is highly expressed, SRSF1 is hyperphosphorylated, and VEGF prooncogenic isoforms are abundant, causing abnormal angiogenesis [52].

Recent evidence describes other novel regulatory circuits underlying the *VEGF-A* gene regulation that do not depend on the activity of SR proteins and/or on different usage of the 3′ ss in exon 8. For instance, *VEGF-A* AS is modulated by the alternatively spliced isoforms of the splicing factor T cell intracellular antigen (TIA-1). AS of *TIA-1* leads to expression of a truncated protein, called short TIA-1 (sTIA-1) in some cancer cells [53]. sTIA-1 competes with the binding of full-length TIA-1 to VEGF-A mRNA, thus favouring the production of the prooncogenic VEGF-A isoform, angiogenesis, and tumour growth in animal models. Notably, sTIA-1 expression positively correlates with advanced tumour stage in colorectal carcinoma (CRC) patients, supporting its prooncogenic function [53].

In addition to the well-studied antiangiogenic (VEGFb) and proangiogenic (VEGF) *VEGF-A* isoforms, a novel isoform named VEGF-A "extended" (VEGFAx), which displays antiangiogenic activity, was recently described [54]. In line

with its inhibitory function on angiogenesis, VEGFAx expression levels are reduced in high-grade CRC tumours with respect to normal human colon mucosa. This isoform is produced by an uncommon regulatory mechanism, called programmed translational readthrough (PTR). This process is due to the presence of a *cis*-acting element that directs protein translation to continue beyond the canonical stop codon, with translation stopping at an alternative downstream stop codon. A recognition element for hnRNP A2/B1 was identified in the Ax region and loss of this recognition site, by either mutation of the sequence or knockdown of hnRNP A2/B1, reduced expression of VEGFAx [54].

AS of the *VEGF-A* gene may also be affected by epigenetic mechanisms. Chromatin features can directly affect splicing outcome by physically coupling the transcription machinery with the splicing apparatus via chromatin-binding adaptor proteins. The latter recognize exons or introns enriched in particular histone modifications and, in turn, recruit splicing regulators to nascent pre-mRNAs [10]. Using a high-throughput screen, *VEGF-A* was identified as a main target for chromatin-mediated AS regulation [55]. The authors showed that H3K9 methylation operated by the methyltransferase EHMT2 favours recruitment of the chromatin-binding protein HP1γ and its associated partner SRSF1 with the VEGF pre-mRNA, thus modulating its AS.

These examples illustrate the complexity of the regulation underlying VEGF-A pre-mRNA processing and translation and highlights how this process amplifies the escape routes available for cancer cells to adapt to an adverse environment.

2.3. Invasion and Metastasis. More than 90% of cancer-related deaths are due to metastasis and spread of cancer cells to multiple tissues and organs. The ability to form metastasis is probably the most complex task for cancer cells, which need to migrate from the primary tumour, intravasate, survive in blood, extravasate, and colonise different new environments. This implies an incredible phenotypic plasticity, which is largely due to a process called epithelial to mesenchymal transition (EMT) and the reverse, mesenchymal to epithelial transitions (MET) [56]. Through EMT, epithelial cells undergo an extensive reorganization of cytoskeletal architecture, with loss of intercellular junctions and cell polarity and acquisition of an elongated, fibroblast-like shape, thus acquiring invasive capabilities. EMT physiologically pertains to embryogenesis, when cells migrate to shape new organs, but it is adopted by cancer cells to generate metastases.

The ability of cancer cells to undergo EMT relies on the activation of a specific gene expression program in response to extracellular cues. Several interconnected regulatory networks drive EMT and modulation of any of them elicits profound effects on the others. The most extensively studied network is built around the transcription factors SNAIL, SLUG, ZEB1/2, and TWIST. Cues from the tumour microenvironment favour the expression of these factors and trigger a global change in gene expression that underlies EMT [57]. Nevertheless, other regulatory layers, including co- and post-transcriptional control by AS and small noncoding RNAs, interconnect with the transcriptional program and in some case can substitute or activate it, setting in motion critical aspects of EMT-associated phenotypic changes [57].

Many EMT-related genes generate AS variants encoding for proteins with essential functions in EMT and this topic has been recently reviewed elsewhere [57–59]. Herein, we wish to highlight few of the most relevant and well-described events. During EMT, several adhesion molecules specific of epithelial or mesenchymal cells are regulated through AS, such as CD44, p120-catenin (*CTNND1*), and MENA (*ENAH*) proteins [57]. For instance, AS of the *CD44* gene is tightly regulated during EMT in breast cancer cells. *CD44* AS is governed by the epithelial splicing regulatory protein 1 (ESRP1), a SF that stimulates inclusion of variable exons (CD44v isoforms). During EMT, ESRP1 levels drastically decrease, leading to the upregulation of the standard isoform (CD44s), which contributes to the formation of EMT-associated recurrent breast cancer in mice [60]. ESPR1 also positively regulates the production of the epithelial isoform MENA11a, which inhibits the migratory ability of breast cancer cells and is able to counteract the invasive activity of the mesenchymal MENAΔv6 isoform of the same gene [61].

The ESRP family members (ESRP1 and ESRP2) are so far the only known SFs exhibiting epithelial cell-type-specific expression and that undergo pronounced changes in expression during EMT [62, 63]. High-throughput experimental approaches revealed a high-affinity ESRP-binding motif (with UGG as a core motif) and a predictive "RNA map" that governs ESRP1/2 activity [64]. Importantly, downregulation of ESRP proteins during EMT affects splicing of a large number of these target genes, indicating that ESRPs are key players in this cancer-related cellular transition.

Other tissue-specific and more ubiquitously expressed SFs, such as the RBP FOX1 homologue (RBFOX), CUGBP Elav-like family (CELF), muscleblind-like protein (MBNL), SR proteins, and hnRNPs, also play a role in EMT [58]. For instance, hnRNP A1 has been recently implicated in the induction of the RAC1b isoform of the GTPase RAC1 [65], which is known to induce EMT through generation of reactive oxygen species (ROS) and induction of SNAIL expression [66]. hnRNP A1 negatively regulates RAC1b splicing by binding to RAC1 alternative exon 3b and inhibiting its inclusion; treatment with matrix metalloproteinase-3 (MMP-3) inhibits hnRNP A1 binding to exon 3b, thus relieving its repressive activity and favouring RAC1b splicing in mammary epithelial cells [65]. Conversely, in colorectal cells RAC1b splicing is positively regulated by the SR protein SRSF1 in a SRPK1-regulated manner [67]. Recent evidence shows indeed that the knockdown of SRPK1 or inhibition of its catalytic activity reduced phosphorylation and subsequent translocation of SRSF1 to the nucleus, limiting its availability to promote the inclusion of alternative exon 3b into the RAC1b pre-mRNA [67]. Thus, although a direct competition between SRSF1 and hnRNP A1 in RAC1b splicing regulation has not been demonstrated, it is tempting to speculate that epithelial or mesenchymal phenotype of a cancer cell could be modulated by the balance in the activity of these SFs and the consequent effect on RAC1b splicing.

SRSF1 also regulates the splicing of the tyrosine kinase receptor RON by inhibiting inclusion of exon 11 [68].

The resulting ΔRON isoform is unable to undergo proteolytic cleavage, rendering the protein constitutively active and conferring increased motility to cancer cells [68, 69]. Importantly, cancer cells modulate the expression levels of SRSF1, splicing of the ΔRON isoform, and induction of EMT in response to external cues from the surrounding environment. This process is orchestrated by a splicing cascade relying on phosphorylation/activation of the SAM68 by the extracellular regulated protein kinases (ERK1/2). Once activated, SAM68 promotes inclusion of a cryptic intron in the 3′ untranslated region of SRSF1 mRNA, thus inhibiting its degradation by nonsense mediated decay (NMD) [70].

Altogether, these observations indicate that AS plays a major role in EMT by establishing a specific program of splice variants of genes important for epithelial and mesenchymal cell morphology and motility. These observations raise the intriguing possibility that abnormal changes in splicing can steer cancer cells towards malignant progression through a partial EMT, without the need for canonical transcriptional reprogramming.

2.4. Resisting Cell Death. Apoptosis (also called programmed cell death) is a death process characterized by shrinkage of the cell and its nucleus. The apoptotic machinery is composed of both upstream regulators and downstream effector components. These players receive and integrate extracellular or intracellular cell death-inducing signals, giving rise to extrinsic and intrinsic apoptotic programs [26]. Both pathways culminate in a proteolytic cascade exerted by caspases. Once an insult hits a cell, it is the balance between pro- and antiapoptotic factors that determines cell fate.

Although apoptosis serves as a natural barrier to eliminate cells that develop aberrant features, transformed cells have developed a variety of strategies to limit or circumvent it. One of such strategies consists in modulation of AS to shift expression from pro- to antiapoptotic isoforms of several genes. Below, we will summarize some examples of apoptosis-related AS events that occur in the tumour microenvironment. As can be inferred from the list, each AS event is finely regulated by many SFs exhibiting synergistic or opposing functions. The cancer cell exploits the cooperation or competition between them to establish regulatory mechanisms that favour the production of the splicing isoform suitable for survival.

The death receptor *FAS* (an upstream regulator that receives extracellular death signals induced by the FAS ligand) and *CASP9* and *CASP8* (initial executioners of apoptosis) genes are regulated by AS, giving rise to splice isoforms with pro- or antiapoptotic roles. For instance, inclusion or skipping of FAS exon 6, respectively, generates two functionally distinct receptors, a membrane-bound protein with proapoptotic function and a soluble form with antiapoptotic function [71, 72]. TIA-1, TIAR-1 (TIA-1 related) [73], and EWS (Ewing sarcoma protein) [74] positively regulate *FAS* splicing by favouring the assembly of the spliceosome on the 5′ and 3′ ss of exon 6, resulting in the generation of the proapoptotic isoform. By contrast, PTB/hnRNP I [73], RBM5 [75], HuR [76], and hnRNP C1/C2 [77] negatively regulate exon 6 splicing in favour of the antiapoptotic FAS isoform.

Caspase-9 is the most studied family member in terms of AS. The *CASP9* gene generates two splice variants, the proapoptotic caspase-9a and the antiapoptotic caspase-9b, which differ for the inclusion or exclusion of a four-exon cassette (exons 3, 4, 5, and 6), respectively [78, 79]. SRSF1 promotes the inclusion of the exon cassette contributing to the generation of caspase-9a proapoptotic isoform in non-small cell lung cancer (NSCLC) cells [80]. However, constitutive activation of the PI3K/AKT pathway in these cells repressed this activity [80]. On the other hand, hnRNP L promotes skipping of the exon cassette to generate the antiapoptotic caspase-9b protein [81]. Interestingly, the expression level and the phosphorylation status of hnRNP L strongly influence the outcome of this AS event. Overexpression of hnRNP L in NSCLC cells, but not in nontransformed cells, lowers the caspase-9a/9b ratio, favouring the oncogenic isoform. The physiological relevance of this mechanism was confirmed by the complete loss of tumorigenic capacity in a mouse xenograft model of NSCLC cells depleted of hnRNP L [81].

The cancer-restricted role of hnRNP L in caspase-9 AS is apparently due to NSCLC-specific phosphorylation of hnRNP L on Ser52, suggesting that cancer cell developed a device to switch an ubiquitous RBP into a prooncogenic protein through a specific posttranslational modification.

Many other apoptosis-related genes are also subjected to AS regulation. The *BCL-X* (*BCL2L1*) gene contains 3 exons and encodes two splice variants [82]. Two alternative 5′ ss are present in exon 2: selection of the canonical one at the end of the exon yields the long, antiapoptotic variant BCL-X_L, whereas selection of the distal one located upstream in the exon produces the short, proapoptotic variant BCL-X_S [82]. Several SFs have been shown to modulate *BCL-X* splicing. HnRNP H, F, and I (PTB) [83, 84], SAM68 [85], the RBPs RBM25 [86], and RBM11 [87] were all shown to promote splicing of the proapoptotic BCL-X_S variant. By contrast, the SFs SAP155 [88], SRSF9 [89], hnRNP K [90], and SRSF1 [85, 91] enhance splicing of the antiapoptotic BCL-X_L. The balance of BCL-X isoforms is affected in a large number of cancer cell lines and human cancer samples, and fine-tuned regulation of this AS event can determine the cell fate in response to various stresses [85, 92, 93].

These examples highlight how different families of SFs are employed by cancer cells to coordinate splicing regulation and to promote cell survival in response to the hazards imposed by the variable environmental conditions, gaining an advantage with respect to nontransformed cells.

2.5. Deregulating Cellular Energies. The uncontrolled cell proliferation that characterizes cancer cells involves adjustments of energy metabolism in order to favour a rapid growth and division of tumour cells even in adverse microenvironments. Under aerobic conditions, cells produce energy via glycolysis in the cytosol (this reaction allows the conversion of glucose to pyruvate) and then via oxidative phosphorylation in the mitochondria (this reaction allows the conversion of pyruvate to carbon dioxide). Under anaerobic conditions, glycolysis is favoured compared to oxygen-consuming mitochondrial oxidative phosphorylation. Cancer cells, however, primarily use glycolysis, by reprogramming their glucose

metabolism and energy production regardless of oxygen supply. This cancer-related process, called "aerobic glycolysis," was already discovered in 1930 by Warburg [94, 95]. The efficiency of ATP production insured by glycolysis is lower than that provided by mitochondrial oxidative phosphorylation. However, an increased glycolysis provides advantages to cancer cells by allowing a more efficient utilization of glycolytic intermediates in other biosynthetic pathways that favour proliferation also in presence of limited amounts of nutrients [96]. This reliance on glycolysis can be further accentuated under the hypoxic conditions occurring within the growing tumour mass.

AS of key metabolic enzymes partially governs the metabolic switch that characterizes cancer cell metabolism. A well-studied example is that of pyruvate kinase (PKM), an enzyme that catalyses the conversion of phosphoenolpyruvate (PEP) to pyruvate [97]. The *PKM* gene encodes two alternative splice variants through usage of mutually exclusive exons [97]. The PKM1 isoform, produced when exon 9 is included in the mature transcript, is normally expressed in adult life and stimulates mitochondrial oxidative phosphorylation. PKM2, generated by inclusion of exon 10, is exclusively expressed during embryonic development and promotes aerobic glycolysis. However, PKM2 is typically reexpressed in cancer cells where it confers oncogenic features [97–99]. Indeed, replacement of PKM2 with PKM1 in lung tumour cells correlated with impaired tumour occurrence in mouse xenografts [97]. *PKM* splicing in cancer cells is modulated by hnRNP A1, hnRNP A2, and hnRNP I/PTB, which cooperate to promote splicing of PKM2 by binding to sequences flanking exon 9 and repressing its inclusion [100, 101]. Notably, all three hnRNPs are overexpressed in several cancers [23, 24] and their expression can be coordinated by the oncogenic transcription factor MYC [101]. Thus, during neoplastic transformation upregulation of MYC activity and of these SFs might predispose the cell to alter its energy metabolism through modulation of *PKM* AS. This transition would render the cancer cell less susceptible to starvation and/or other unfavourable metabolic conditions occurring in the tumour microenvironment.

2.6. Chemotherapy Resistance. Surgery, radiation, and chemotherapeutic drugs are the standard approaches for cancer treatment. Radiation and chemotherapy mainly act by inducing cancer cell death. Although most tumours respond to chemotherapy at first, some cancer cells often survive treatments, expand, and acquire chemoresistance causing disease relapse. The mechanisms by which cancer cells adapt or are selected for their resistance to treatments vary with cancer type and from patient to patient. Most of these mechanisms causing chemotherapy resistance have been elegantly described elsewhere and mainly involve mutations and/or altered expression of genes and proteins [102]. Among these, AS participates in the process of acquired chemoresistance by controlling the expression of cancer-related splice variants that contribute to cancerous phenotype (Figure 3). Herein, we illustrate some examples of how AS allows cancer cells to adapt to tumour microenvironment, under conditions where normal cells would undergo cell death, and to overcome the chemotherapy-mediated selective pressures.

An interesting example of AS adaptive response driven by chemotherapy is provided by the switch from cyclin D1a to cyclin D1b in breast cancer cells. Upon treatment of MCF-7 cells with cisplatin and the estrogen receptor antagonists 4-hydroxy tamoxifen and ICI 182780, endogenous protein cyclin D1a expression is strongly reduced, whereas oncogenic cyclin D1b splice variant is maintained and confers chemoresistance [103].

The HER2-targeted therapy using trastuzumab is widely used for the treatment of patients with metastatic breast tumours overexpressing HER2, a member of EGFR family of receptor tyrosine kinases. Although the search for a somatic *HER2* oncogenic mutation in *HER2*-amplified breast tumours has failed to identify a promising activating genetic lesion [104–106], the existence of HER2 isoforms that may influence trastuzumab response in breast tumours evidenced the key role of AS in chemoresistance [107–109]. A new *HER2* splice variant (HER2Δ16) with potent transforming activity was detected in several *HER2*-overexpressing breast cancer cell lines [108, 109] and primary tumours [107, 109]. Furthermore, the expression of HER2Δ16 is a tumour-specific molecular event and the vast majority of women with expression of HER2Δ16 develop locally disseminated node-positive breast cancer. Furthermore, tumour cell lines expressing HER2Δ16 are resistant to the HER2-targeted therapy trastuzumab [110]. The critical effector of HER2Δ16 tumorigenic properties is represented by SRC kinase. In fact, SRC kinase appears to function as a "master regulator" stabilizing HER2Δ16 protein expression and coupling HER2Δ16 to multiple mitogenic and cell motility pathways [110]. Cotargeting of HER2Δ16 and SRC kinase with the single agent tyrosine kinase inhibitor dasatinib resulted in SRC inactivation, destabilization of HER2Δ16, and suppressed tumorigenicity [110]. An important issue will be to characterize the cancer-specific splicing event leading to HER2Δ16 expression in breast cells. Understanding these mechanisms might indeed offer therapeutic perspective to counteract the activity of this oncogenic splice variant in breast cancers with poor prognosis.

Another SF involved in drug resistance is SPF45, a 45 kDa nuclear protein [111, 112]. SPF45 is highly expressed in numerous carcinomas including bladder, breast, colon, lung, ovarian, pancreatic, and prostate. Forced overexpression of SPF45 in HeLa cells demonstrated a 4–7-fold increase in resistance to doxorubicin. Ectopic SPF45 expression in the A2780 ovarian cancer cells induced a multidrug resistant phenotype, inducing 3–21-fold resistance to a variety of chemotherapeutics with differing mechanisms of action, including carboplatin, vinorelbine, doxorubicin, etoposide, mitoxantrone, and vincristine [111]. The mechanism underlying the multidrug resistant phenotype acquired upon SPF45 overexpression is still unknown but probably relies on misregulation of AS of its targets [113, 114]. Few splicing targets of SPF45 are currently known. SPF45 promotes the proapoptotic transmembrane receptor FAS pre-mRNA [114], but this activity is repressed by both mitogenic (ERK1/2) and stress-response (p38 and JUN N-terminal kinases) MAPK-dependent phosphorylation in cancer cells [113]. SPF45 is

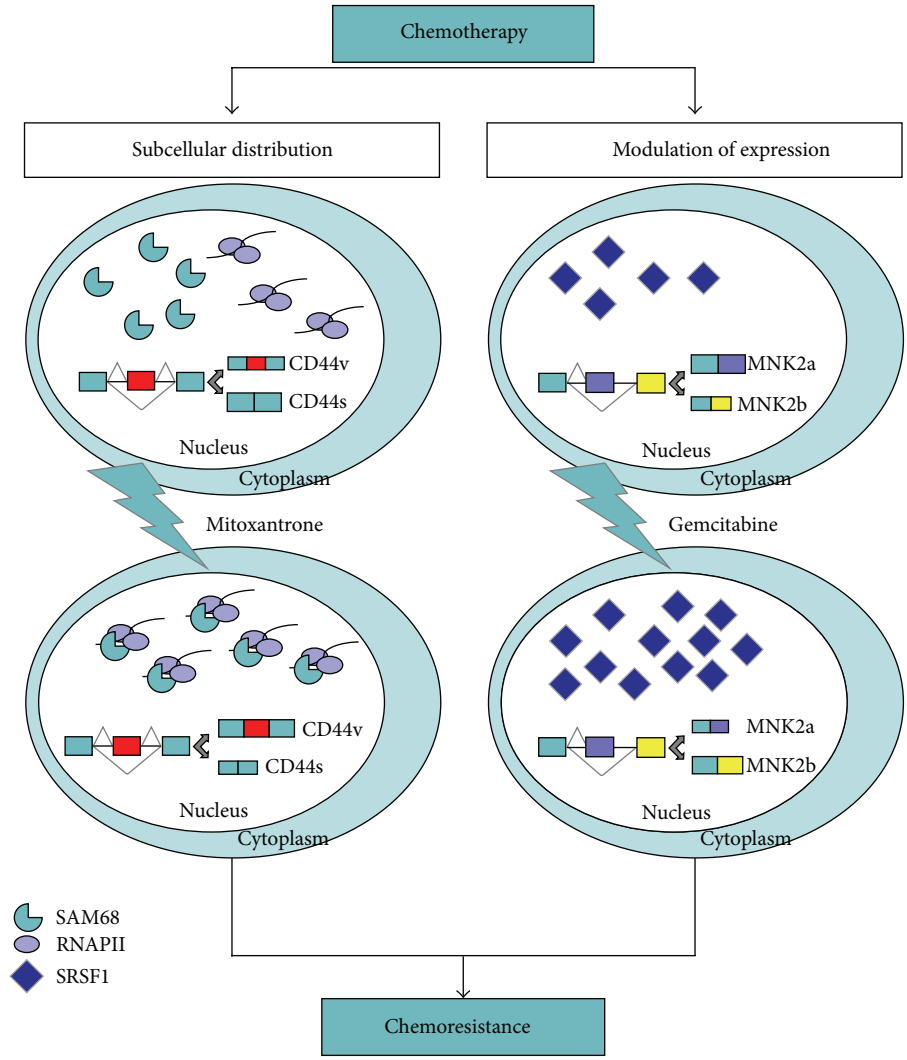

Figure 3: Regulation of chemoresistance via alternative messenger RNA splicing. Chemotherapy can affect the subnuclear distribution of SAM68 (left panel) and the expression of SRSF1 (right panel), thus modulating AS of cancer-related variants (CD44v and MNK2b, resp.) and contributing to chemoresistance. Green boxes indicate constitutive exons; red, yellow, and violet boxes indicate variable exons.

also phosphorylated by CLK1 on multiple serine residues and this posttranslational modification regulates alternative ss utilization by SPF45 and its intranuclear localization [115]. Furthermore, stable SPF45 overexpression in SKOV-3 cells induces enhancement of fibronectin 1 expression and regulates fibronectin 1 AS by enhancing inclusion of the EDA region into fibronectin transcripts [113]. Since inclusion of EDA region in fibronectin enhances the migratory capacity of embryonic cells and tissue, SPF45 overexpression might contribute to promote metastasis *in vivo* by modulating this AS event. Thus, full elucidation of the spectrum of AS events regulated by this SF in cancer cells might reveal pathways involved not only in acquisition of chemoresistance but also in other key oncogenic features.

Although a large spectrum of AS events associated with chemoresistance has been described [116–118], much less is known about the mechanisms activated during chemotherapy that result in the observed splicing changes. In this regard, genotoxic stress may cause a subcellular redistribution of many RBPs and/or modify their activity through posttranslational modifications as an attempt of the cancer cell to adapt to the hostile environment [119–121]. The subcellular localization of SAM68 and other RBPs was affected by treatment of prostate cancer cells with mitoxantrone (MTX), a topoisomerase II inhibitor used in chemotherapy, partially altering the cellular AS pattern [119]. Another regulatory mechanism through which tumour cells acquire resistance involves the modulation of expression of specific SFs or of cancer-related splicing variants and/or of their counterparts. Treatment of pancreatic ductal adenocarcinoma (PDAC) cells with gemcitabine induced the upregulation of SRSF1 that, in turn, regulates AS of mitogen activated protein kinase (MAPK) interacting kinase 2 (*MNK2*) in favour of MNK2b isoform [122]. SRSF1-dependent AS of MNK2b following gemcitabine treatment conferred increased resistance of PDAC cells to chemotherapeutic drug, identifying a novel chemotherapy-mediated adaptation response through AS in PDAC cells [122]. Notably, a recent report showed that MNK2a behaves as

tumour suppressor in breast cancer, whereas the alternative MNK2b splice variant was prooncogenic [123]. Thus, it appears that upregulation of SRSF1 in response to genotoxic stress confers resistance to treatments by switching this splicing event in favour of the prooncogenic MNK2b variant.

These observations suggest that AS changes induced by chemotherapeutic treatment represent an important side-effect, which may contribute to therapy resistance. These aspects need to be taken into account for the development of new therapeutic protocols that could exploit the combined usage of canonical chemotherapy with novel pharmaceutical tools targeting the adaptive splicing response associated with treatments.

3. Concluding Remarks

As shown by the several examples illustrated in this review, AS plays a key role in the rearrangement of gene expression, thus enabling cancer cells to adapt to the adverse conditions encountered during the transformation process and to evade different therapeutic approaches. At the same time, these observations suggest that the splice variants aberrantly expressed by cancer cells might represent suitable targets for the development of new antitumor therapies, in particular those whose prognostic or diagnostic values have already been demonstrated [124]. Redirecting aberrant splicing events or inhibiting the activity of oncogenic splice variants can represent a valuable approach to increase cancer cells sensitivity to canonical chemotherapies, which could be exploited in new combined therapies. As an example, susceptibility of NSCLC cells to different chemotherapeutic drugs can be enhanced by RNA interference of the expression of the antiapoptotic splice variant caspase-9b of the *CASP9* gene [125]. Notably, one of the advantages of therapeutically targeting alternative splice variants is the possibility to act on two different fronts: on one hand, therapies targeting the specific activity of the oncogenic splice variant could be developed; on the other hand, the mechanisms driving the aberrant splicing event could also be targeted. In light of this, great interest has arisen for studies exploiting antisense oligonucleotide (ASO) to redirect splicing of tumoral variants towards a nontumoral isoform. This approach has been recently shown to be possible for the PKM2/PKM1 [126] and the BCL-$X_{L/S}$ [93] splicing switch. The high therapeutic value of the ASO approach is strongly supported by recent studies demonstrating the good bioavailability and efficacy of an ASO redirecting *SMN2* splicing for the treatment of SMA animal models [127].

Thus, it is certainly possible to envision the development in the near future of new personal anticancer therapies targeting the specific splicing-alterations of each patient, whose identification will be ensured by the novel and rapidly evolving high-throughput sequencing techniques that allow genome-wide profiling of cellular transcriptomes, even at a single cell-resolution [21].

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

SAM68: Signal Transduction and RNA Metabolism in Human Cancer

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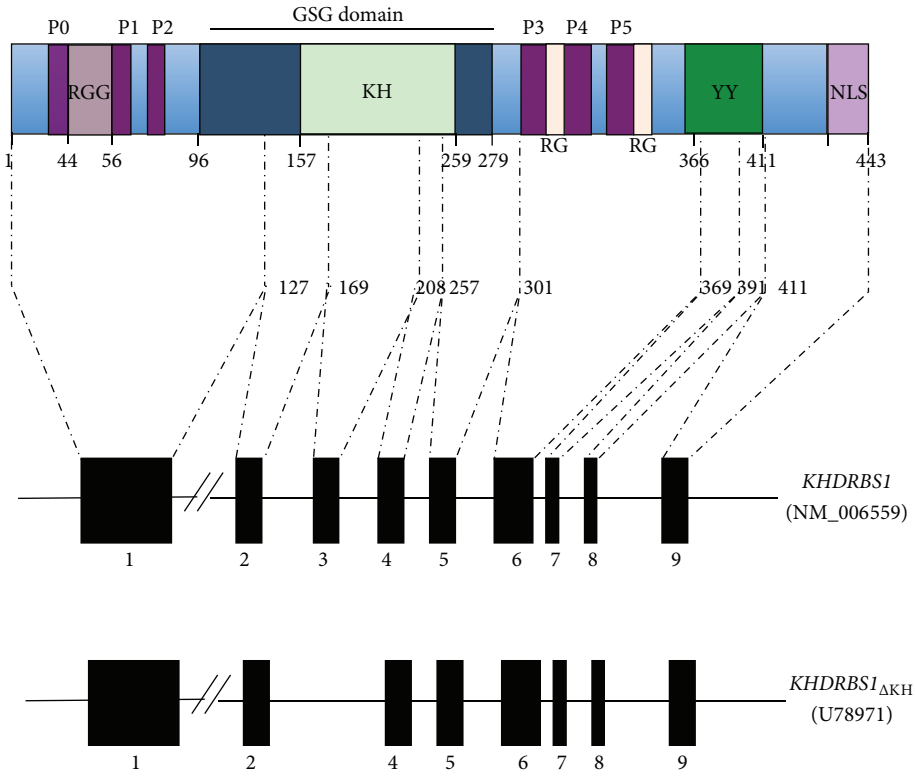
Alterations in expression and/or activity of splicing factors as well as mutations in *cis*-acting splicing regulatory sequences contribute to cancer phenotypes. Genome-wide studies have revealed more than 15,000 tumor-associated splice variants derived from genes involved in almost every aspect of cancer cell biology, including proliferation, differentiation, cell cycle control, metabolism, apoptosis, motility, invasion, and angiogenesis. In the past decades, several RNA binding proteins (RBPs) have been implicated in tumorigenesis. SAM68 (SRC associated in mitosis of 68 kDa) belongs to the STAR (signal transduction and activation of RNA metabolism) family of RBPs. SAM68 is involved in several steps of mRNA metabolism, from transcription to alternative splicing and then to nuclear export. Moreover, SAM68 participates in signaling pathways associated with cell response to stimuli, cell cycle transitions, and viral infections. Recent evidence has linked this RBP to the onset and progression of different tumors, highlighting misregulation of SAM68-regulated splicing events as a key step in neoplastic transformation and tumor progression. Here we review recent studies on the role of SAM68 in splicing regulation and we discuss its contribution to aberrant pre-mRNA processing in cancer.

1. Introduction

SAM68 (SRC associated in mitosis of 68 kDa) was originally identified as a protein physically associated with and phosphorylated by the tyrosine kinase c-SRC during mitosis [1, 2], opening the interesting possibility of a signaling circuitry driven by c-SRC and affecting RNA processing and trafficking in a cell-cycle-dependent manner.

SAM68 belongs to the STAR (signal transduction and activation of RNA metabolism) family of RNA binding proteins (RBPs) that link signaling pathways to RNA processing [3, 4]. STAR proteins include *Artemia salina* GRP33 [5], *C. elegans* GLD-1 [6], mammalian QKI [7], SAM68 [8, 9], SLM-1 and SLM-2 [10, 11], *Drosophila* HOW [12], KEP1 and Sam50 [13], and the evolutionary conserved splicing factor SF1 [14]. All STAR proteins, from worms to mammals, share common architecture (Figure 1). They contain a GRP33/SAM68/GLD-1 (GSG) domain for RNA binding and homodimerization, flanked by regulatory regions harboring motifs for protein-protein interactions (Figure 1), often mediated by conserved amino acid residues targeted by posttranslational modifications [15]. SAM68 contains six proline-rich sequences and a tyrosine-rich region at the C-terminus, which form docking sites for signaling proteins containing SRC homology 3 (SH3) and 2 (SH2) domains (Figure 1) [1, 2, 9, 16]. Notably, tyrosine phosphorylation by SRC-related kinases impairs SAM68 homodimerization [17] as well as its affinity for RNA both *in vitro* [16, 18] and *in vivo* [19]. Additional posttranslational modifications were also reported to affect the functions of this RBP. SAM68 binds to and is methylated by the arginine methyltransferase PRMT1 [20], thus affecting SAM68 interaction with SH3 domains [21] and its nuclear localization [20]. SAM68 acetylation, described in tumorigenic breast cancer cell lines [22], by



GSG: GRP33/SAM68/GLD-1 domain
KH: heterogeneous nuclear ribonucleoprotein particle K (hnRNP K) homology domain
P0–P5: proline-rich motifs
RGG and RG: arginine/glycine/glycine and arginine/glycine boxes
YY: tyrosine-rich domain
NLS: nuclear localization signal

Figure 1: Schematic representation of SAM68 domains. In the upper part, schematic model representing the structural/functional domains of SAM68 protein as a prototype of a STAR protein. SAM68 protein is composed of the GRP33/SAM68/GLD-1 (GSG) domain, formed by a single heterogeneous nuclear ribonucleoprotein particle K (hnRNP K) homology domain (KH) embedded in two flanking regions, six consensus proline-rich motifs (P0–P5), arginine/glycine/glycine (RGG) and arginine/glycine (RG) boxes, C-terminal tyrosine-rich domain (YY), and a nuclear localization signal (NLS). In the lower part, the two protein coding mRNA isoforms of human *KHDRBS1* are represented. Black boxes indicate exons (numbered from 1 to 9). The sizes of exons and the protein domains encoded by each exon are indicated.

the acetyltransferase CBP increases SAM68 binding to RNA *in vitro*. Furthermore, SAM68 can be SUMOylated by the SUMO E3 ligase PIAS1, which enhances its transcriptional repression activity [23]. Thus, posttranslational modifications greatly influence the biochemical properties of SAM68 and finely tune its subcellular localization, interaction with signaling proteins, and RNA binding affinity.

Despite the growing interest in STAR proteins, their physiological role has not been completely elucidated yet. Nevertheless, recent mouse models of genetic ablation of STAR proteins are now greatly helping in pursuing this goal. In this review, we discuss the functional properties of SAM68 in signaling and RNA metabolism, with particular emphasis on malignant transformation. In particular, we highlight recent advances and new insights into SAM68-based signaling that have been made in the last two decades, which expand our understanding of STAR-mediated signaling in cancer cells.

2. SAM68 Biological Role(s): Lessons from Mouse Models

The first indication of the involvement of STAR proteins in tumorigenesis came from studies in *C. elegans*. Critical missense mutations in the *gld-1* gene caused germ-line tumors, thus suggesting an important role for *GLD-1* as a tumor suppressor [24]. These null mutations in hermaphrodites caused female germ cells to exit from the meiotic prophase and to start proliferating, thus leading to the formation of a germ-line tumor [3]. In this regard, it is important to notice that the function and localization of GLD-1 appear quite different from the SAM68 subfamily of STAR proteins. Indeed, GLD-1 is localized exclusively in the cytoplasm of germ cells and it does not contain the protein domains flanking the GSG of SAM68, which are involved in cell signaling [3]. Nevertheless, an initial observation seemed to suggest a similar tumor suppressor role also for SAM68.

A random homozygous knockout (RHKO) screen in NIH3T3 murine fibroblasts indicated that functional inactivation of the *Sam68* gene induces tumorigenesis and allows NIH3T3 cells to form metastatic tumors in nude mice [25]. These studies suggested that SAM68 negatively affects neoplastic transformation, like its *C. elegans* ortholog *GLD-1*. However, in contrast to this proposed function, disruption of the *Sam68* gene in chicken DT40 cells showed reduced growth rate, indicating that SAM68 plays a positive role in cell proliferation [26]. Moreover, a natural alternative isoform of *SAM68* with deletion of the KH (RNA binding) domain ($SAM68_{\Delta KH}$) was specifically expressed during growth arrest in normal cells, but absent in SRC-transformed cells (Figure 1) [27]. Importantly, transfection of the $SAM68_{\Delta KH}$ isoform inhibited serum-induced DNA synthesis and Cyclin D1 expression, thus highlighting for the first time the involvement of SAM68 RNA binding activity in cell proliferation [27]. Thus, despite the initial putative role as a tumor suppressor gene, subsequent studies appeared to suggest a positive role of SAM68 in tumorigenesis. These findings were also supported by investigation of the *Sam68* knockout mouse model, which has recently unveiled the physiological processes in which SAM68 is involved.

Sam68-deficient mice displayed high lethality soon after birth [28]. Nevertheless, mice that survived beyond weaning showed a normal lifespan. Importantly, surviving *Sam68*−/− mice lived to old age (~2 years) and were not prone to tumor formation, clearly indicating that SAM68 is not a tumor suppressor *in vivo* [28]. Moreover, haploinsufficiency of SAM68 delayed mammary tumor onset and reduced metastasis [29]. Although the authors reported higher activation of SRC and FAK in the mammary gland of *Sam68* heterozygote females, indicating altered regulation of the SRC signal transduction pathway [29], whether or not this effect was related to the lower tumorigenicity of *Sam68* haploinsufficient cells was not investigated.

Additional phenotypes of the *Sam68*−/− mice revealed the important role played by this RBP in a number of physiological processes. Adult knockout females displayed defects in bone metabolism [28] and delayed development of sexual organs [29]. *Sam68*−/− mice were protected against age-induced osteoporosis and were characterized by preserved bone density. This phenotype was linked to the preferential differentiation of knockout mesenchymal stem cells toward osteoblasts instead of adipocytes [28]. Furthermore, *Sam68*−/− females displayed a reduction in the number of developing ovarian follicles, alteration of estrous cycles, and impaired fertility [30]. Similarly, spermatogenesis and fertility were impaired in *Sam68*−/− males, due to the involvement of both nuclear RNA processing events [31] and translational regulation of a subset of mRNAs during spermiogenesis [32]. Although almost exclusively nuclear in the majority of normal cells, SAM68 localized in the cytoplasm of secondary spermatocytes and associated with polysomes, thus playing a role in translational regulation of target mRNAs [32, 33]. Notably, this function in male germ cells closely resembles that of its orthologue in *C. elegans* GLD-1.

Aberrant regulation of splicing events also contributes to the phenotypes of *Sam68*−/− mice. For instance, stimulation of *Sam68*−/− cerebellar neurons was dramatically attenuated due to the impaired regulation of *Nrxn-1* alternative splicing [34]. *Nrxn-1* encodes a synaptic cell surface receptor that contributes to the assembly of functional presynaptic terminals, and a severe perturbation of *Nrxn-1* splice variants was observed in *Sam68*−/− brains [34]. Moreover, *Sam68*−/− mice exhibited a lean phenotype due to a dramatic reduction in adiposity. The decreased commitment to early adipocyte progenitors and defects in adipogenic differentiation were attributed to aberrant splicing of *mTOR* described in *Sam68*−/− mice [35].

Collectively, the defects documented in *Sam68* knockout mice reflect the multiple roles played by SAM68 in signal transduction and RNA processing and emphasize how aberrant regulation of SAM68 function(s) might contribute to oncogenic transformation [28, 29, 36]. Nevertheless, to what extent SAM68 RNA binding activity contributes to the mouse defects and to neoplastic transformation has not been unraveled yet, and, in this context, knock-in or transgenic mouse models displaying *Sam68* gene with mutations in the RNA binding domain would really help to answer this question.

3. SAM68 Signaling in Human Cancer

SAM68 acts as a scaffold protein in response to different signal transduction pathways [36, 41]. Through its proline-rich motifs, SAM68 interacts with the SH3 domains of different SRC kinases [1, 2], like BRK [42], FYN [18], and Itk/Tec/BTK [43], all involved in different aspects of cell transformation. Importantly, the interaction of SAM68 with the SRC SH3 domain enables SRC kinases to phosphorylate their substrates [9].

The interaction of SAM68 with FYN induces the assembly of a protein complex containing also PLCγ1 (phospholipase C gamma) [18], triggering its phosphorylation and activation [18, 44]. Interestingly, a truncated form of the tyrosine kinase receptor c-KIT, named tr-KIT, stimulates the formation of this complex [18]. Tr-KIT is aberrantly expressed in a subgroup of prostate cancer (PCa) patients and its expression correlates with enhanced activation of SRC and elevated expression and high tyrosine phosphorylation of SAM68 [45]. Moreover, SAM68 is frequently upregulated in PCa patients and promotes PCa cell proliferation and survival to chemotherapeutic agents [46], suggesting a role for this pathway in prostate cancer biology.

The breast tumor kinase BRK, a nonreceptor tyrosine kinase, is also responsible for the tyrosine phosphorylation of SAM68 in cancer cells, which has been associated with SAM68 increased nuclear localization and cell cycle promotion [47, 48]. Importantly, both SAM68 and BRK are upregulated in breast cancer cells and breast tumors [39, 48, 49]. In addition, in the transformed HT29 adenocarcinoma cell line, endogenous BRK colocalized in SAM68

nuclear bodies (SNBs), and BRK-mediated phosphorylation of SAM68 impaired its ability to bind RNA molecules [50]. Consistent with these results, nuclear BRK was also detected in differentiated androgen-responsive LNCaP human PCa cell line, while it was mainly cytoplasmic in the undifferentiated and more aggressive androgen-unresponsive PC3 prostate cancer cell line [50]. Thus, relocalization of the BRK kinase during PCa development and progression may indicate disruption of a signaling pathway important for maintaining the normal phenotype of prostate epithelial cells.

Proteomic analyses revealed that SAM68 is able to form two (large and small) protein complexes, interacting with several RBPs and with regulators of cytoskeletal organization and signal transduction pathways [51, 52]. In accordance with this, *SAM68*-deficient fibroblasts displayed defects in cell migration [53] and an increase in SRC kinase activity [53]. These observations suggest that SAM68 is required for a negative feedback inhibition of SRC and that deregulated SRC activity could be responsible for the defects in actin cytoskeleton and cell migration observed in *SAM68*-deficient fibroblasts. Interestingly, epidermal growth factor (EGF) treatment induced a change in the size of the SAM68-containing complexes, from the large to the smaller one, the latter containing splicing activity [51]. Since EGF receptor (EGFR) stimulation triggers signaling cascades controlling cellular proliferation, migration, differentiation, and survival, and EGFR overexpression has been associated with poor prognosis in several types of epithelial cancers, such as lung, head and neck, colorectal, and breast cancer [54], EGFR-SAM68 signaling could be targeted to attenuate the oncogenic features of cancer cells.

In addition to PCa [46, 52], aberrant expression of SAM68 was detected in several other tumors. In particular, SAM68 was shown to be upregulated in colorectal cancer [55] and in patients with non-small cell lung cancer [56]. Moreover, in patients with renal cell carcinoma high SAM68 expression was inversely associated with overall survival while SAM68 cytoplasmic localization significantly correlated with pathologic grade and outcome of this tumor [57]. Furthermore, in breast cancer patients expression and cytoplasmic localization of SAM68 significantly correlated with clinical characteristics of patients, including clinical stage, tumour-nodule-metastasis classification, histological grade, and ER expression [39]. In line with an oncogenic role played by SAM68 in this tumor type, silencing of SAM68 inhibited proliferation and tumourigenicity of breast cancer cells [39]. Finally, SAM68 was shown to be significantly upregulated in cervical cancer at both mRNA and protein levels [58]. SAM68 upregulation and its cytoplasmic localization were significantly associated with risk factors and correlated with lymph node metastasis and poor prognosis in patients with early-stage cervical cancer [58]. Consistently, downregulation of SAM68 in cervical cancer cells inhibited cellular motility and invasion by the inhibition of the AKT/GSK-3 β/Snail pathway [58].

Collectively, these reports strongly suggest that high SAM68 expression and its cytoplasmic localization are associated with poor overall survival in different types of tumors. Moreover, the deregulation of SRC and AKT pathways could be involved in the oncogenic function of SAM68 in the cytoplasm.

4. SAM68 and Transcriptional Regulation in Human Cancer

The first evidence of the involvement of SAM68 in transcriptional regulation came out in 2002 when Hong and colleagues documented the repressive effect of SAM68 on different mammalian and viral promoter constructs [37]. Direct recruitment of SAM68 to a promoter region resulted in strong transcriptional repression and mutation of the SAM68 RNA binding domain had no influence on this effect, thus suggesting that SAM68 transcriptional activity occurs in a RNA-independent fashion [37]. Mechanistically, the authors described the functional association of SAM68 with the acetyl-transferase CBP, which caused modulation of CBP transcriptional activity (Figure 2(a)) [37].

Other reports confirmed the role of SAM68 as a transcriptional repressor. SAM68 was shown to interact with hnRNP K, leading to inhibition of the *trans*-activating effects of hnRNP K on c-myc target genes [59]. Moreover, overexpression of SAM68 in mouse fibroblasts inhibited accumulation of *Cyclin D1* and *E* transcripts [60], whereas SAM68 SUMOylation by PIAS1 further enhanced repression of Cyclin D1 expression (Figure 2(b)) [23].

In PCa cells, SAM68 was proposed to function as a transcriptional coregulator and to promote the transcriptional activity of the androgen receptor (Figure 2(c)) [38]. Furthermore, in hematopoietic stem cells SAM68 was shown to form an oncogenic transcriptional complex with mixed lineage leukaemia (MLL) and PRMT1 [61]. Chimeric fusion of MLL with PRMT1 or SAM68 enhanced self-renewal of primary hematopoietic cells; conversely, specific knockdown of PRMT1 or SAM68 suppressed MLL-mediated oncogenic transformation [61]. Similarly, SAM68 depletion in breast cancer cells impaired cell proliferation and their tumorigenic features through the upregulation of cyclin-dependent kinase inhibitors p21 (Cip1) and p27 (Kip1). Thus, in this context SAM68 depletion might lead to suppression of AKT phosphorylation and subsequent activation of FOXO factors, which in turn promote the upregulation of p21 (Cip1) and p27 (Kip1) (Figure 2(d)) [39].

In normal and transformed human T cells SAM68 was shown to bind the *CD25* promoter and facilitate p65 recruitment, thus suggesting a novel role for SAM68 in NF-κB regulation of gene expression in human T cell signaling (Figure 2(e)) [40]. In this context, *CD25* expression and aberrant NF-κB signaling led to increased proliferation, expression of antiapoptotic proteins, and drug resistance, while *SAM68* knockdown markedly impaired CD25 upregulation. Remarkably, elevated expression of CD25 has been detected in a large variety of hematopoietic malignancies and solid tumors [62]; thus the p65-SAM68 association might be strategically used to target CD25 expression in those particular tumors that depend on CD25 for survival [40].

Transcription and RNA processing machineries are tightly coupled. Temporal coupling not only provides efficient

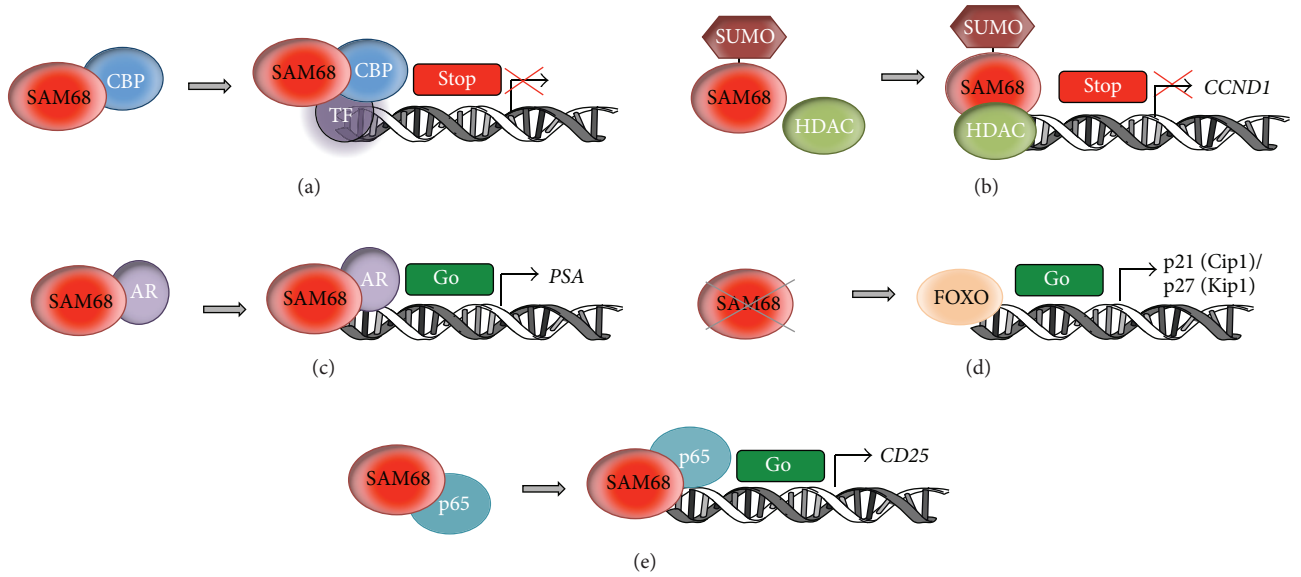


Figure 2: Transcriptional regulation by SAM68. (a) SAM68 forms a complex with CBP and transcriptional repressor factors (TF), thus negatively regulating CBP targets transcription [37]. (b) The PIAS1 complex SUMOylates SAM68, which interacts with a histone deacetylase (HDAC) and represses *CCDN1* transcription [23]. (c) SAM68 directly interacts with the androgen receptor (AR) and binds to androgen-responsive elements (AREs) leading to AR targets activation (i.e., *PSA* gene) [38]. (d) SAM68 depletion in breast cancer cells leads to activation of FOXO factors thus inhibiting cell proliferation and tumourigenicity through the upregulation of cyclin-dependent kinase inhibitors p21 (Cip1) and p27 (Kip1) [39]. (e) SAM68 binds the *CD25* promoter and facilitates p65 recruitment, thus contributing to NF-κB regulation of gene expression [40].

gene expression to accomplish rapid growth and proliferation, but also allows rapid response to diverse signaling events [63]. Many splicing regulators are recruited to nascent pre-mRNAs by their interaction with the phosphorylated carboxyl-terminal domain (CTD) of RNAPII thus affecting splicing decisions [64]. Interestingly, SAM68 was shown to interact directly with RNA polymerase II (RNAPII) in meiotic spermatocytes [31] and with the RNAPII associated Brahma (Brm) subunit of the SWI/SNF chromatin-remodeling complex [65]. These observations strongly suggest the involvement of SAM68 in cotranscriptional splicing. Thus, on one hand, SAM68 binding to transcription factors and to the RNAPII itself can affect transcriptional regulation of gene expression; on the other hand, through the cooperation with chromatin remodelers, SAM68 can impact cotranscriptional splicing events. In this regard, interaction of the protooncogenic transcription factor FBI-1 with SAM68 in PCa cells was shown to inhibit SAM68 recruitment on the *BCL-X* pre-mRNA, thus affecting apoptosis [66]. By contrast, binding of SAM68 to the transcriptional coactivator SND1 was required for the efficient association of SAM68 with RNAPII and for the recruitment of SAM68 on the *CD44* pre-mRNA [67]. Remarkably, *CD44* alternative splicing isoforms are associated with tumor progression and metastasis [68]. Thus, the SND1/SAM68 complex might be an important determinant of PCa progression and the concomitant upregulation of these proteins might provide an advantage for cancer cells to invade other tissues, consequently favoring the spreading of metastatic cells [67].

Hence, depending on the cellular partner, SAM68 displays different effects on target genes, modulating in this way different or even antagonistic functions within the cell.

In summary, growing evidence documents the involvement of SAM68 in the transcriptional regulation of gene expression of cancer related genes, both by direct binding to the chromatin and by recruitment of specific transcription factors, which in turn affect its splicing activity.

5. SAM68-Regulated Alternative Splicing Events in Cancer

SAM68 preferentially binds A/U-rich sequences in RNA [16]. SELEX experiments identified the UAAA consensus motif bound with Kd ~12–60 nM. Importantly, a single A to C mutation within this motif abolished SAM68 binding [69], indicating that this motif is involved in high affinity direct binding or in a specific RNA structure. Indeed, SAM68 was then shown to bind cellular RNAs enriched in such U/A-rich sequences [70] and to directly modulate alternative splicing events in target genes [71]. Interestingly, the UAAA motif matches with the last four bases of the mammalian polyadenylation signal AAUAAA, thus opening the hypothesis of SAM68 involvement in RNA stability.

During tumor progression, a variety of oncogenic signaling pathways induce modifications of the downstream effectors of key biological functions [76]. Notably, SAM68 was the first identified "hub factor" able to translate extracellular stimuli to pre-mRNA processing of specific target

genes in the nucleus [71]. As mentioned above, several posttranslational modifications regulate the function and/or localization of SAM68. In particular, serine-threonine and tyrosine phosphorylation of SAM68, which often occurs in cancer cells, are important for SAM68 homodimerization and RNA affinity (Figure 3(a)) [2, 72, 73].

The *CD44* gene represents an interesting example of SAM68-mediated coupling between signal transduction cascades and alternative splicing. *CD44* pre-mRNA is affected by complex alternative splicing events occurring in 10 adjacent exons (v1–v10) to produce multifunctional transmembrane glycoprotein isoforms implicated in cell-cell and cell-matrix adhesion, migration, and invasion [77] and with crucial roles in cancer progression and metastasis [78]. By binding to A/U-rich enhancer element located within exon *v5*, SAM68 promotes the production of the oncogenic *CD44v5* variant (Figure 3(b), (A)) [71], which is upregulated in several cancers [78, 79] and bears prognostic value in gastric and renal carcinoma [80–82].

Several molecular mechanisms (not mutually exclusive) have been proposed to explain the ability of SAM68 to stimulate *CD44* exon *v5* inclusion: (i) SAM68 competes or displaces the antagonistic splicing repressor hnRNP A1 that binds a specific splicing silencer element located within exon *v5* [83]; (ii) SAM68 affects the dynamic recruitment of spliceosomal components, including U2AF65, an auxiliary factor involved in the recognition of the 3′ splice site during the splicing reaction [84]; upon SAM68 phosphorylation this interaction is disrupted and U2AF65 dissociates from pre-mRNA allowing the subsequent spliceosome remodeling and exon *v5* inclusion [85]; (iii) SAM68 interacts with the splicing coactivator SRm160 and they functionally cooperate to simulate *CD44* exon *v5* inclusion [86].

Aberrant regulation of alternative splicing is emerging as a key step in oncogenesis [87]. Recent data demonstrated that genotoxic stress widely modulates alternative splicing events in cancer cells [88, 89]. This regulation is exerted in part through reduced transcription elongation rates as a consequence of RNA polymerase II (RNAPII) phosphorylation [90] and in part through direct involvement of specific RBPs in the repair process or by specific regulation of DNA damage response gene expression [91], also accomplished by RBP relocalization [92]. *CD44* exon *v5* splicing is also influenced by genotoxic stress induced by chemotherapeutic drugs, such as the topoisomerase II inhibitor mitoxantrone (MTX) [93]. Specifically, MTX causes relocalization of SAM68 from nucleoplasm to transcriptionally active nuclear granules and this correlates with changes in alternative splicing of *CD44* exon *v5*. This effect is independent of signal transduction pathways activated by DNA damage [93]. Nevertheless, it appears to be functionally relevant for the cells, as SAM68 was found overexpressed in prostate carcinoma where it promotes resistance and survival to chemotherapeutic treatments [46].

In addition to *CD44*, changes in alternative splicing of other transcripts, including *Caspase 2* (*CASP2*) [94], *BCL-2* [90], the p53 negative modulators *MDM2* and *MDM4* [95], and *Cyclin D1* (*CCND1*), have been observed in cancer cells after treatment with chemotherapy drugs [96, 97]. Notably, *CCND1* pre-mRNA was also identified as a novel alternative splicing target of SAM68 [74]. *CCND1* is a protooncogene that is frequently deregulated in several human cancers through different mechanisms, such as chromosomal translocations, amplification of the *CCND1* locus, and intragenic mutations [97–99]. Alternative splicing also plays an important role in aberrant Cyclin D1 expression. The *CCND1* gene encodes two alternatively spliced transcripts: the canonical *Cyclin D1a* and the alternative *Cyclin D1b*, which results from the retention of intron 4 and premature termination of the transcript [100]. These isoforms display different biological properties and cellular localization [96]. In particular, Cyclin D1b is exclusively nuclear and displays stronger oncogenic potential than Cyclin D1a [74, 100, 101] and its upregulation correlates with poor prognosis in several tumor types [96]. At the molecular level, SAM68 was observed to bind to the proximal region of intron 4 and to interfere with the recruitment of the U1 snRNP, in this way promoting intron 4 retention (Figure 3(b), (B)) [74]. Signal transduction pathways affecting SAM68 phosphorylation status, such as those conveyed by ERK1/2 and SRC kinases, regulate alternative splicing of *CCND1* pre-mRNA by modulating SAM68 affinity for this target [74]. Notably, SAM68 expression positively correlates with levels of Cyclin D1b, but not D1a, in human PCa cells [97], suggesting that increased levels of SAM68 in human PCa contribute to tumorigenesis by elevating the expression of Cyclin D1b in this tumor type.

Recent studies have demonstrated an important contribution of alternative splicing regulation in the cascade of events characterizing the morphological conversion of tumor cells during epithelial-to-mesenchymal transition (EMT) [102], one of the major routes through which cancer cells acquire migratory and invasive potentials [103, 104]. SAM68 phosphorylation by ERK1/2 plays an important role during neoplastic progression of epithelial cells through activation of EMT. This is illustrated by the ability of SAM68 to repress alternative splicing-activated nonsense-mediated mRNA decay (AS-NMD) [105] of a splicing factor of the serine arginine (SR) family, SRSF1 [75]. AS-NMD of *SRSF1* pre-mRNA, which involves a cryptic intron in the 3′ UTR region of the gene, decreases *SRSF1* mRNA stability and protein levels (Figure 3(b), (C)) and, notably, this event is altered in colon cancer [75]. In mesenchymal cells, phosphorylation of SAM68 is controlled by soluble factors expressed by epithelial cells that act through the activation of ERK1/2 kinase [75]. SRSF1, an oncogenic splicing factor upregulated in many human cancers [106], severely impacts on cell physiology. For instance, its overexpression stimulates skipping of exon 11 of the *RON* protooncogene increasing the production of the constitutively active Δ*RON* isoform, which in turn promotes the acquisition of an invasive cellular phenotype [107]. Interestingly, inhibition of ERK activity by small molecules or by using conditioned medium from epithelial cells reverts SAM68 phosphorylation, decreases *SRSF1* mRNA and protein levels, promotes inclusion of *RON* exon 11, and induces the reversal program named mesenchymal-to-epithelial transition (MET) [75]. MET occurs at the final metastatic sites where redifferentiation of mesenchymal cells to an epithelial state is required for the colonization of distant organs [103, 104].

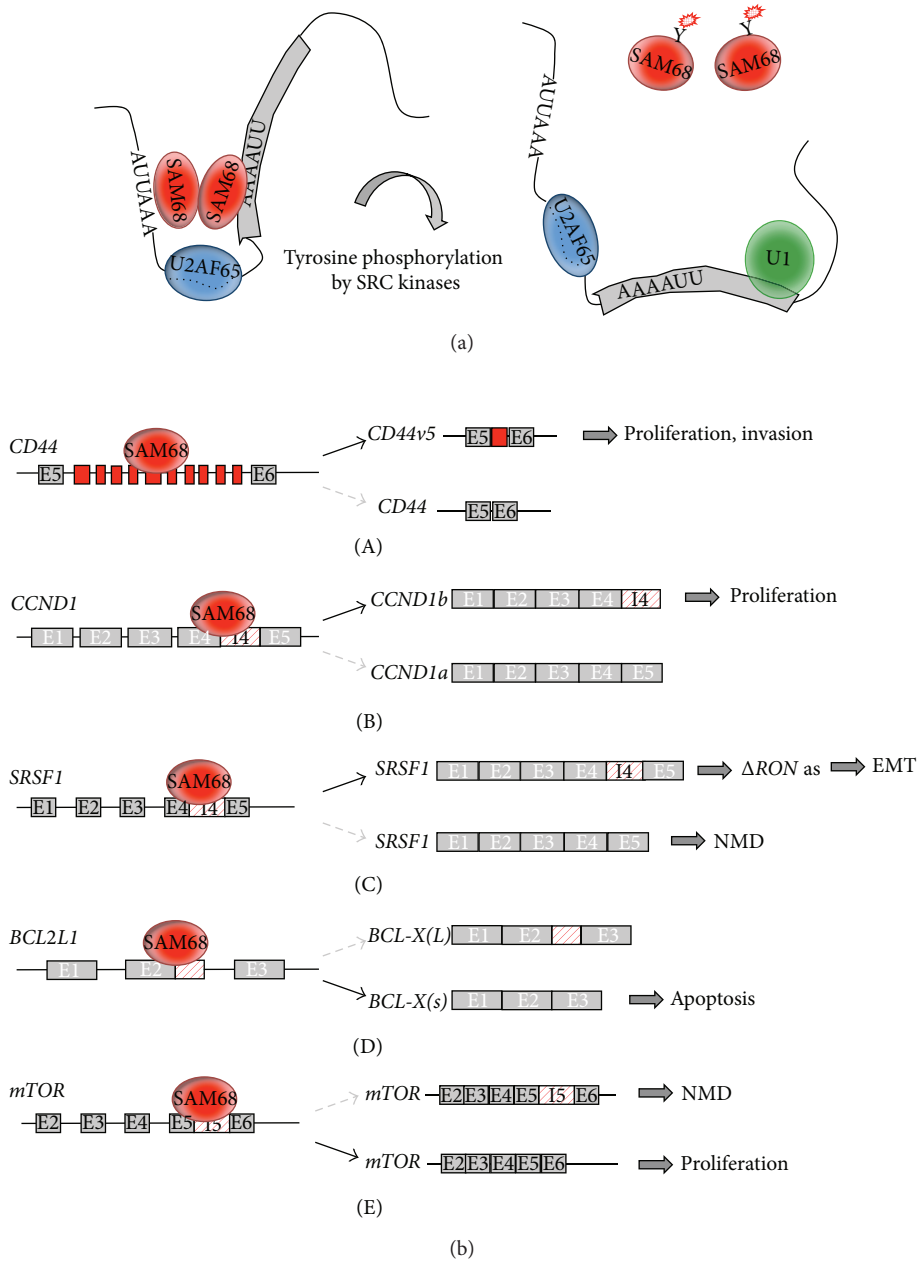


Figure 3: Model of SAM68 interaction with pre-mRNAs and splicing regulation. (a) SAM68 recognition of A/U-rich bipartite sequence in the pre-mRNA homodimerization allows simultaneous binding to the pre-mRNA and to U2AF65 [69–73]. Tyrosine phosphorylation of SAM68 reduces the RNA binding affinity and thus releases SAM68 from the pre-mRNA. (b) Model of alternative splicing events regulated by SAM68 in cancer cells. (A) SAM68 promotes inclusion of *CD44* variable exon *v5*. Inclusion of variable exons in the *CD44* pre-mRNA is specific to cancer cells and correlates with cancer progression and invasiveness [71]. (B) SAM68 promotes splicing events that regulate cell proliferation. Binding of SAM68 to *CCND1* intron 4 interferes with the correct recruitment of U1 snRNP at the exon 4 5′ splice sites, thus enhancing retention of intron 4 and generating the *Cyclin D1b* isoform. In prostate cancer, the expression of Cyclin D1b interrupts a negative feedback in the regulation of androgen receptor (AR) transcriptional activity, thereby promoting cell proliferation [74]. (C) As for *CCND1*, SAM68 promotes retention of *SRSF1* intron 4, thus stabilizing *SRSF1* pre-mRNA and inhibiting its degradation by nonsense-mediated decay (NMD) [75]. Accumulation of SRSF1 in turn favors the splicing of Δ*RON*, an oncogenic variant of *RON* that triggers epithelial-mesenchymal transition (EMT). (D) SAM68 regulates the alternative splicing of *BCL2L1* leading to the short (*BCL-X(s)*) proapoptotic isoform [19]. This activity can be reverted by tyrosine phosphorylation of SAM68 from SRC family kinases, thereby switching the role of SAM68 from being proapoptotic to being antiapoptotic and allowing cells to differentially react to external cues. (E) SAM68 regulates *mTOR* alternative splicing thus leading to the correct mRNA isoform and avoiding retention of intron 5 that generates a premature termination codon and the consequent reduction of mTOR protein levels [35]. Notably, mTOR is a critical effector in cell-signalling pathways commonly deregulated in human cancers and overexpression of the components involved in the PI3K/AKT/mTOR pathway has been shown to induce malignant transformation.

A paradigmatic example of the central role of SAM68 in apoptosis is represented by the regulation of *BCL-X* (*BCL2L1*), a member of the *BCL-2* gene family. *BCL-X* pre-mRNA is alternatively spliced to generate two isoforms with opposite functions in promoting apoptosis. Selection of the proximal 5′ splice site (5′ SS) in exon 2 causes the production of the antiapoptotic long *BCL-X(L)* variant, while the proapoptotic short *BCL-X(s)* variant is produced by the use of the distal alternative 5′ SS [108]. In several cancer types, the *BCL-X(L)* isoform is upregulated thus increasing resistance to chemotherapeutic agents [109, 110]. Targeting this mechanism and switching the splicing of *BCL-X* gene toward the production of the proapoptotic variant thereby offer the opportunity to revert cancer cells resistance to chemotherapeutic drugs and to promote tumor cell death [111, 112]. Due to its relevance in cancer, *BCL-X* alternative splicing has been extensively investigated in the past years and several RBPs were shown to regulate this specific splicing event [19, 113–119]. Among these, SAM68 exerts a proapoptotic function, leading to production of *BCL-X(s)* variant [19]. In particular, SAM68-mediated splicing regulation of *BCL-X* depends on its specific binding to *BCL-X* pre-mRNA and on its ability to interact with the splicing repressor hnRNP A1, thus antagonizing SRSF1, a positive regulator of *BCL-X(L)* splicing (Figure 3(b), (D)) [19, 110]. However, in PCa cells, high levels of SAM68 do not correlate with high levels of *BCL-X(s)* [38, 46, 110]. This apparently contradictory observation can be explained by the fact that tyrosine phosphorylation of SAM68 by the SRC-related kinase FYN counteracts its splicing activity, promoting the antiapoptotic *BCL-X(L)* isoform [19, 120]. In tumors, SRC activity is often increased [121] and it correlates with SAM68 phosphorylation in different cancer types, including prostate cancer [45, 47, 122]. Recently, an additional layer of complexity to the regulation of SAM68-mediated *BCL-X* splicing in cancer has been revealed. This mechanism involves the direct interaction of the transcriptional factor FBI-1 with SAM68, reducing its binding to *BCL-X* pre-mRNA and therefore promoting the production of the antiapoptotic *BCL-X(L)* variant and cell survival [66]. Fascinatingly, FBI-1 function in *BCL-X* splicing regulation is dependent on the activity of histone deacetylases [66], suggesting an important link between this alternative splicing event and dynamic organization of chromatin structure.

The biological consequences and the possible contribution to tumor progression associated with the aberrant splicing in other relevant SAM68-regulated genes have also been recently described. For example, SAM68 is able to promote the production of the oncoprotein E6 of the human papilloma virus (HPV) type 16 [123], which is a known etiological agent for human cervical cancer [124]. *E6* alternative splicing is controlled by EGF through activation of ERK1/2-kinase that promotes SAM68 phosphorylation, suggesting a possible implication of SAM68 in HPV *E6* splicing during differentiation and the viral life cycle processes of cervical cancer.

More recently, SAM68 has been linked to regulation of alternative splicing of the mammalian target of rapamycin (mTOR) [35], which regulates cell size and cell proliferation in response to nutrients and various growth factors [125, 126]. SAM68-depleted cells display intron 5 retention in the *mTOR* mRNA, which generates a premature termination codon and the consequent reduction of mTOR protein levels (Figure 3(b), (E)) [35]. Notably, mTOR is a critical effector in cell-signaling pathways commonly deregulated in human cancers and overexpression of the components involved in the PI3K/AKT/mTOR pathway has been shown to induce malignant transformation [127]. Interestingly, loss of SAM68 reduces breast and PCa incidence [29, 46], suggesting that in cancer cells SAM68 activation may also regulate the expression of PI3K downstream kinases, such as mTOR.

Collectively, these findings indicate that an evaluation of SAM68-associated splicing signatures in diverse sets of tumors can be of medical relevance.

6. SAM68 and Noncoding RNAs

Recent reports have revealed the involvement of SAM68 in noncoding RNAs (ncRNAs) metabolism. ncRNAs are classified into small (18–200 nt) and long ncRNAs (lncRNAs; 200 nt to >100 kb) [128, 129] and play a role in a wide variety of biological processes, including almost all levels of gene expression regulation, from epigenetic to transcriptional and posttranscriptional control [130]. Coimmunoprecipitation studies documented the interaction between SAM68 and key proteins involved in microRNA (miRNA) biogenesis [131]. miRNA genes are transcribed by either RNA polymerase II or RNA polymerase III into long primary miRNA transcripts (pri-miRNAs) [132]. The cleavage of the pri-miRNAs into stem-loop precursors of ~70 nucleotides (pre-miRNAs) is mediated by DROSHA [133], whereas the cytoplasmic processing of pre-miRNAs into mature miRNAs is mediated by DICER [134]. Coimmunoprecipitation experiments performed in male germ cells indicated that SAM68 interacts with both DICER and DROSHA and that the knockout of *Sam68* leads to changes in expression of specific miRNAs in germ cells [131]. Remarkably, a similar functional interaction with components of the miRNA machinery was shown for Quaking (QKI), another member of the STAR family. In the U343 glioblastoma cell line and in primary rat oligodendrocytes QKI interacts with AGO2, a component of the RISC complex involved in miRNA-dependent translational repression, within stress granules [135]. Collectively, these findings suggest a general role for STAR proteins in the regulation of miRNAs.

Interaction between SAM68 and noncoding RNAs might also affect the splicing activity of this RBP. Recently, a long noncoding RNA (named *INXS*) has been described as a novel mediator of SAM68-dependent regulation of *BCL-X* splicing. *INXS* is transcribed from the antisense genomic strand of *BCL-X* gene and is downregulated in various tumor cell lines and in kidney tumor tissues, whereas its expression is induced by treatments that trigger apoptosis [136]. *INXS* interacts with SAM68 and favors its splicing activity, thus increasing the levels of *BCL-X(s)* isoform and enhancing apoptosis [136]. Notably, in favor of a possible role of *INXS* in anticancer therapy, *INXS* overexpression in a mouse xenograft model was sufficient to induce tumor regression and increase *BCL-X(s)* isoform [136].

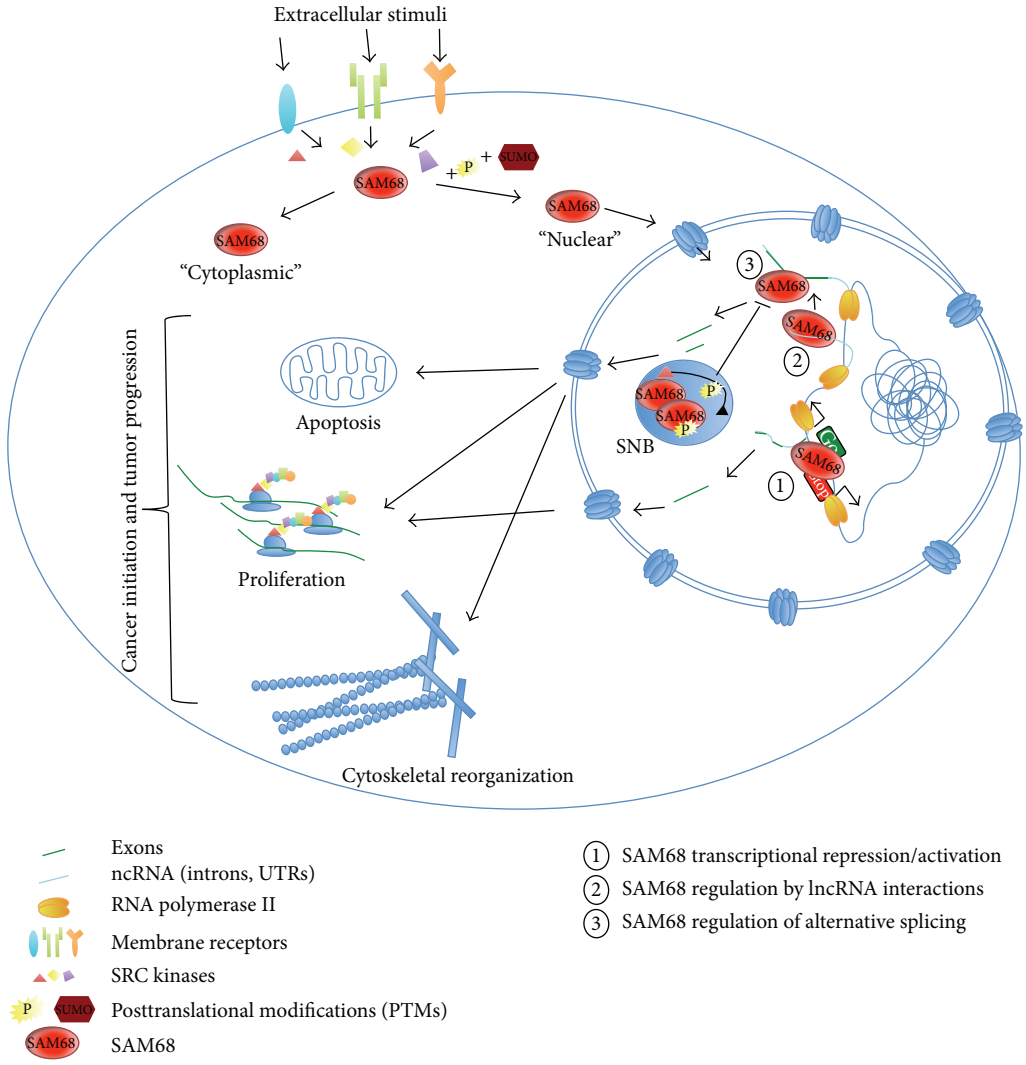


FIGURE 4: Role(s) of SAM68 in transcriptional and posttranscriptional regulation of gene expression in cancer cells. SAM68 and its regulatory networks contribute to important process involved in cancer initiation and progression, such as apoptosis, proliferation, and cytoskeletal reorganization, through different mechanisms. After posttranslational modifications (PTMs) induced by extracellular stimuli and mediated by SRC family kinases, SAM68 is committed to the nucleus where it is able to (1) promote or repress transcription of different targets (see Figure 2 for more details) and (2-3) regulate alternative splicing events through several molecular mechanisms, some of them mediated by lncRNAs (see Figure 3 for more details). In the nucleus, SAM68 can localize in specific bodies (SNB) and associate with other proteins (i.e., BRK kinase) that modify its phosphorylation status, thus affecting its RNA binding activity.

Thus, the complex regulatory network of proteins and ncRNAs orchestrated by SAM68 greatly contributes to the cellular signature in higher eukaryotes and plays a pivotal role in the regulation of gene expression in normal conditions and in oncogenic transformation.

7. Concluding Remarks

Misregulation of cancer-associated alternative splicing events is often correlated with unbalanced expression of splicing factors. SAM68 is a clear example of this concept, as it is upregulated in different types of tumors and it directly affects cancer initiation and progression. Transcriptional and posttranscriptional regulation of gene expression mastered by SAM68 chiefly contributes to changes in gene expression occurring in cancer cells. Moreover, SAM68 orchestrates transcript fate and function (Figure 4). Thus, depicting SAM68 signatures in normal and cancer cells would greatly help in understanding how SAM68 and its regulatory networks contribute to key features of tumor initiation and progression. Although the functional significance of SAM68-regulated alternative splicing events in human cancer has been clearly established, future studies unraveling the positional effect of SAM68 binding to pre-mRNAs would be instrumental for the development of new therapeutic approaches to target SAM68 activities in cancer.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Emerging Roles of MicroRNAs in EGFR-Targeted Therapies for Lung Cancer

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Lung cancer is a leading cause of cancer mortality worldwide. Several molecular pathways underlying mechanisms of this disease have been partly elucidated, among which the epidermal growth factor receptor (EGFR) pathway is one of the well-known signaling cascades that plays a critical role in tumorigenesis. Dysregulation of the EGFR signaling is frequently found in lung cancer. The strategies to effectively inhibit EGFR signaling pathway have been mounted for developing anticancer therapeutic agents. However, most anti-EGFR-targeted agents fail to repress cancer progression because of developing drug-resistance. Therefore, studies of the mechanisms underpinning the resistance toward anti-EGFR agents may provide important findings for lung cancer treatment using anti-EGFR therapies. Recently, increasing numbers of miRNAs are correlated with the drug resistance of lung cancer cells to anti-EGFR agents, indicating that miRNAs may serve as novel targets and/or promising predictive biomarkers for anti-EGFR therapy. In this paper, we summarize the emerging role of miRNAs as regulators to modulate the EGFR signaling and the resistance of lung cancer cells to anti-EGFR therapy. We also highlight the evidence supporting the use of miRNAs as biomarkers for response to anti-EGFR agents and as novel therapeutic targets to circumvent the resistance of lung cancer cells to EGFR inhibitors.

1. Introduction

Lung cancer is a heterogeneous disease, which is the leading cause of cancer-related mortality worldwide. It can be broadly classified into non-small-cell lung cancer (NSCLC) and small cell lung cancer based on clinical, histological, molecular, and endocrinological characteristics. Using histological features, NSCLC can be further subdivided into large-cell carcinoma, bronchoalveolar lung cancer, adenocarcinoma, squamous carcinoma, and mixed histological types (e.g., adenosquamous carcinoma) [1, 2]. NSCLC accounts for more than 85% of all patients with lung cancer. To date, platinum-based doublets remain the mainstay in the treatment of patients with advanced NSCLC [2]. With an increased understanding of the mechanisms underpinning lung cancer development and progression, a number of novel agents specifically targeting oncogenic pathways have been developed and applied to treat lung cancer [3], among which the inhibitor targeting epidermal growth factor receptor (EGFR) signaling cascades is one of the most broadly used agents implemented in clinical practice [3–5].

EGFR is a receptor of tyrosine kinase (RTK), which consists of an N-terminus extracellular ligand-binding site, a hydrophobic transmembrane domain, and a C-terminus intracellular region with tyrosine kinase activity [5]. The EGFR signaling network plays a central role in the growth and maintenance of epithelial tissues. The dysregulation and/or hyperactivation of the EGFR signaling pathway are frequently found in epithelial lung tumor entities, in which the hyperactivated EGFR signaling is associated with advanced lung

cancer and poor prognosis [6]. Therefore, EGFR and its downstream signaling components can be used as major targets in developing novel agents for cancer treatment, such as chimeric monoclonal antibodies (cetuximab and panitumumab) [2] and tyrosine kinase inhibitors (TKIs) (gefitinib, erlotinib, and afatinib) [7–9]. However, the clinical benefits of these anti-EGFR agents are often limited, mainly due to the heterogeneity of lung cancer and the drug resistance to anti-EGFR therapy [10]. Consequentially, a large number of studies focus on the mechanisms underpinning the resistance toward anti-EGFR agents.

MicroRNAs (miRNAs) are a class of small noncoding RNAs that act as key posttranscriptional regulators of gene expression. They can functionally impact cell fate determination by regulating the expression of critical proteins; thus miRNAs play a pivotal role in the diverse processes of human cancer, acting as either tumor suppressors or oncogenes [11–13]. Recently, several studies have demonstrated that EGFR is a target of a number of miRNAs, and *vice versa* a mutation or activity of the EGFR signaling pathway can alter the expressions of miRNAs in lung cancer (Table 1) [14–17]. The involvement of miRNAs in the EGFR signaling pathway of lung cancer development and target therapy has recently gained increasing attentions [16]. In this review, we summarize the emerging role of miRNAs as modulators to regulate the EGFR signaling pathway and mainly focus on miRNAs as predictive biomarkers for anti-EGFR therapy and as novel targets to reverse the resistance of lung cancer cells to EGFR inhibitors.

2. miRNAs Target EGFR in Human Lung Cancers

miRNAs have gained increasing interest owing to their widespread occurrence and diverse functions as regulatory molecules in many signaling pathways, including the EGFR signaling pathway. Accumulating evidence has revealed that miRNAs are oncomirs or tumor suppressors by targeting the EGFR signaling pathway in different types of cancer. Table 1 lists several miRNAs that regulate the EGFR signaling pathway in lung cancer and Figure 1 shows the miRNAs that target the EGFR signaling pathway.

Using bioinformatics tools, Chan et al. predicted 138 miRNAs that potentially target EGFR in NSCLC, some of these miRNAs were confirmed experimentally [18]. Among these miRNAs, miR-7 has been demonstrated to downregulate EGFR in different cancer cells. Mechanistically, miR-7 could directly target EGFR by binding three seeding sequences in human EGFR mRNA 3′-UTR [19, 20]. Apart from its ability to directly target EGFR mRNA, miR-7 can also target several downstream effectors of the EGFR signaling pathway, including the proto-oncogene serine/threonine protein kinase RAF-1, protein kinase B Akt, and extracellular signal-regulated kinase 1/2 (ERK1/2), sequentially inhibits cancer cell migration, invasion, and metastasis [16, 20]. In lung cancer, miR-7 was upregulated in 60% of NSCLC fine-needle aspirates, which could be induced by both wild type and mutant EGFR L858R, and plays an oncogenic role by activating the rat sarcoma viral oncogene homolog (RAS)/ERK/c-Myc signaling axis to promote lung carcinogenesis by repressing the transcriptional regulator Ets2 transcriptional repression factor (ERF) [16, 21]. In this context, an activation of EGFR or ectopic expression of RAS and c-Myc could induce miR-7 transcription in an extracellular signal-regulated kinase- (ERK-) dependent manner. This notion was supported by findings of that c-Myc bound to the miR-7 promoter and enhanced its activity, and an enforced expression of miR-7 promoted cell growth and tumor formation in lung cancer cells and significantly increased the mortality of nude mice orthotopically implanted with lung cancers. Molecular analysis further revealed that miR-7 could directly target ERF, a seeding sequence of miR-7 that was confirmed in the coding sequence of ERF, suggesting that miR-7 may act as an important regulator of EGFR-mediated oncogenesis and can be served as a novel prognostic biomarker and therapeutic target in lung cancer [21].

miRNA profiling of lung cancer cell lines and lung tissues has demonstrated that miRNAs are emerging as unique effectors of the EGFR signaling pathway in lung cancer, in which miRNAs are correlated with the expression of EGFR and/or the EGFR mutant status or signaling activities [22, 23]. Analyzing miRNA expression profiling of lung cancer, Dacic et al. observed a correlation of miRNAs with mutational status of EGFR in lung adenocarcinomas, in which miR-155 was upregulated only in EGFR/KRAS-negative samples, and miR-25 was upregulated only in EGFR-positive group and miR-495 was upregulated only in KRAS-positive adenocarcinomas. Conversely, let-7g was dramatically downregulated in EGFR/KRAS negative adenocarcinomas [23]. Such a correlation was also found in other mRNAs. For examples, miR-542-5p could downregulate EGFR mRNA and protein expression in human lung cancer H3255, A549, and HCC827 cells and inhibit the growth of these cancer cells. Interestingly, an inverse correlation of miR-542-5p transcript and EGFR protein levels was found in human lung cancer tissues [24]. Such an inverse correlation of miRNA expression and the EGFR signaling pathway was also found in miR-133a whose expression was negatively correlated with cell invasiveness in lung cancer cell lines, by targeting insulin-like growth factor 1 receptor (IGF-1R), TGF-beta receptor type-1 (TGFβR1), and EGFR [25]. Similarly, Chan et al. also demonstrated that miR-146a inhibited cell growth and induced cell apoptosis by suppressing the EGFR downstream signaling components and the migratory capacity in various NSCLC cell lines (H358, H1650, H1975, HCC827, and H292), through an EGFR mutation status independent mechanism of directly targeting the EGFR and nuclear factor kappa beta (NF-κB) signaling pathways [18].

In line with the regulatory role of miRNAs in the EGFR signaling pathway, the aberrant expression and/or mutation(s) of EGFR may also alter the expression of miRNAs in lung cancer. For instance, the expression of some miRNAs, such as miR-21 was altered more remarkably in a lung cancer with EGFR mutations relative to those without these mutations [26], suggesting that the EGFR signaling pathway is not only regulated by tumor-suppressive miRNAs, but also has potential to regulate some miRNAs acting as oncogene. In a recent study by Guo et al., the authors found that aberrant

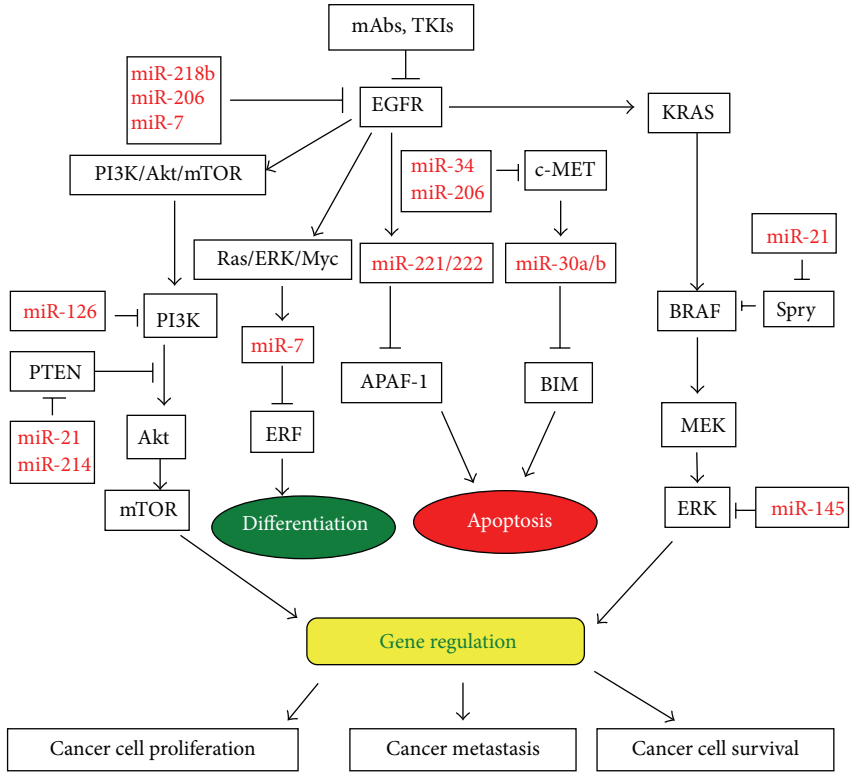

Figure 1: An illustration representing microRNAs (miRNAs) and their targets involved in EGFR signaling pathway in lung cancer and anti-EGFR therapy. The depicted miRNAs target important signaling pathways in lung cancer development and resistance to anti-EGFR agents.

Table 1: MicroRNAs that target EGFR signaling pathway involved in lung cancer.

MicroRNA(s)	Regulation	Potential function(s)	Reference(s)
let-7g	Down	Dramatically downregulated in EGFR/KRAS negative lung adenocarcinomas	[23]
miR-7	Down	Inhibits EGFR-PI3K-AKT signaling and reverses radio-resistance in various cancer cells	[20]
miR-27a	Down	Directly targets EGFR and contributes to mutant p53 gain-of-function	[36, 37]
miR-34a	Down	Regulates Axl receptor tyrosine kinase by targeting SIRT1 and MEK1	[38]
miR-128b	Down	Directly regulates EGFR expression in NSCLC	[15, 18]
miR-133a	Down	Repress EGFR signaling by directly targeting IGF-1R, TGFβR1, and EGFR	[25]
miR-133b	Down	Suppresses EGFR pathway signaling and enhances susceptibility to EGFR-TKI in lung cancer cells by directly targeting EGFR	[39]
miR-145	Down	Negatively regulates EGFR expression in lung cancer cells	[31]
miR-146a	Down	Inhibit EGFR in NSCLC cancer cells	[40, 41]
miR-146b-5p	Down	Suppressed EGFR expression in glioblastoma cell lines	[42]
miR-200	Down	Regulates EMT in anaplastic thyroid cancer cells and bladder cancer cells and reverses resistance of EGFR therapy	[43, 44]
miR-206	Down	Suppresses EGFR signaling in squamous lung cancer cells by directly targeting EGFR and MET	[45]
miR-542-5p, 1203, 1237, 541, 1911	Down	Downregulates EGFR in human lung cancer cells	[20, 24]
miR-21	Up	Regulate the EGFR/AKT pathway in a PTEN independent manner	[46]
miR-24	Up	Activates EGFR signaling by targeting PTPN9 and PTPRF	[47]
miR-25	Up	Upregulated in EGFR positive lung cancer	[23]
miR-214	Up	Regulate acquired resistance to EGFR-TKIs in cancer cells through a PTEN/AKT signaling pathway	[48]

activation of the EGFR signaling pathway downregulated miR-145 expression in NSCLC, an addition of EGFR inhibitor AG1478 that could restore the expression of miR-145 in lung cancer cells [27]. Using an Agilent microarray, Bjaanaes et al. examined the expression of miRNAs in 154 surgically resected lung adenocarcinomas and 20 corresponding normal lung tissue samples; they found that 129 miRNAs were strikingly differentially expressed in lung adenocarcinomas in comparison with normal lung tissues, among which 17 miRNAs were differentially expressed between tumors with EGFR-mutation and wild-type [22]. These studies imply a feedback regulatory mechanism between the EGFR signaling pathway and miRNAs in the development and progression of lung cancers.

3. miRNAs Alter EGFR-TKI Responses in Lung Cancer

Targeting therapy to the EGFR signaling pathway leads to development of EGFR tyrosine kinase inhibitors (TKIs), namely, gefitinib, erlotinib, and afatinib, for the treatment of patients with NSCLC who have EGFR mutations. Different from wild-type EGFR, mutations of EGFR may confer hypersensitivity to TKIs in advanced NSCLC [28], since cells with mutant EGFR transduce survival signals but have no effect in proliferative signals [8]. However, the clinical benefit of TKIs was limited as patients eventually develop resistance to these agents. 70% of this acquired resistance (AR) may be caused by a secondary mutation in the EGFR gene, such as T790M or amplification of the proto-oncogene hepatocyte growth factor receptor (c-MET). In gefitinib or erlotinib resistant tumor samples, about 50% samples have been found to bear T790M mutation and the other 20% cases have c-MET amplification. Mechanically, T790M can increase GTP affinity in the tyrosine kinase domain or block TKI binding to the tyrosine kinase domain of EGFR [29]. Other mechanisms, including the involvement of Anexelekto- (Axl-) kinase and a number of miRNAs in the AR of lung cancer to TKIs [10, 16], and several miRNAs have been demonstrated to be associated with EGFR mutations in lung cancer (Table 2) [30].

Increasing number of studies has revealed a correlation of the clinical responses to TKIs and the expressions of miRNAs. For example, loss of heterozygosity (LOH) at miR-128b is one of the most frequent genetic events in lung cancer. Weiss et al. found that LOH at miR-128b was frequent detected in lung cancer tissues and was positively correlated with clinical response and survival to gefitinib treatment [15]. Other studies showed that the restoration of miR-145 and miR-7 inhibited cancer cell growth in lung adenocarcinoma patients with EGFR activating mutation and could effectively target EGFR addicted and EGFR-TKI resistant tumors [21, 31]. In a recent study, Garofalo et al. demonstrated that RTK of EGFR and c-MET could induce miR-30b/30c/221/222 expressions, and an upregulation of miR-30b/30c/221/222 induced resistance to gefitinib in lung cancer cells by the regulation of BCL2-like 11 (BIM), phosphatase and tensin homolog (PTEN), and apoptotic peptidase activating factor 1 (APAF-1) expressions. In contrast, miR-103/203 could induce gefitinib resistant cell apoptosis and promote mesenchymal to epithelial transformation (MET) by targeting protein kinase C varepsilon (PKC-ε) and sarcoma viral oncogene homolog (SRC), but the ectopic expressions of miR-30b/30c/221/222 conferred resistance to TKIs [32]. Such AR to TKIs could also be induced by miR-214 and miR-21 in lung cancer cells [33]. miR-214 expression was elevated in gefitinib resistant HCC827 lung cancer cells (HCC827/GR), which was inverse with PTEN expression. A knockdown of miR-214 in HCC827/GR showed a restoration of PTEN expression and resensitized HCC827/GR to gefitinib. Similar to miR-21, which was also more aberrantly expressed in EGFR-TKI-resistant lung cancer cell line PC9R relative to its parent cell PC9. The increased level of miR-21 was inversely correlated with the abundance of PTEN and programmed cell death protein 4 (PDCD4) proteins and positively correlated with the phosphatidylinositol-3-kinase (PI3K)/Akt signaling pathway. An inhibition of miR-21 induced apoptosis in PC9R cells and suppressed tumor growth in nude mice treated with EGFR-TKI. Clinically, circulating miR-21 level in EGFR-TKI-treated NSCLC patients was significantly higher at the time of acquiring resistance over the baseline [26, 33]. Recent studies in lung adenocarcinoma of female nonsmokers also revealed that the expression of miR-183-3p, miR-195, and miR-122 was in plasma and associated with EGFR mutations in lung cancer [34, 35].

Intriguingly, several tumor-suppressive miRNAs exhibited an ability to enhance the cytotoxicity of EGFR-TKI to lung cancer cells, such as miR-126, miR-145, and miR-146a [40, 54, 61]. Lung cancer cells H460 and A549 are significantly resistant to gefitinib, forced expression of miR-126 and miR-145 showed an inhibited growth of cells and tumor xenografts and an enhanced cytotoxicity induced by gefitinib [54]. miR-146a is an extensively studied tumor-suppressive miRNA in many types of cancer, the polymorphism of the rs2910164CNG in pre-miR-146a was recently identified to be associated with the genetic susceptibility to lung cancer development in a Korean population [61]. In addition, a downregulation of miR-146a was reported in lung cancers, overexpression of miR-146a was found to suppress cell growth and migration, induce cellular apoptosis, and inhibit the EGFR downstream signaling components in lung cancer cell lines H358, H1650, H1975, HCC827, and H292. Importantly, forced expression of miR-146a could enhance the ability of EGFR-TKIs (gefitinib, erlotinib, and afatinib) and monoclonal antibody (cetuximab) to inhibit cell proliferation by targeting of the EGFR and NF-κB signaling pathways [40].

The activation of c-MET is associated with both primary and acquired resistance to EGFR-TKIs in patients with NSCLCs [62]. Both EGFR and c-MET are RTKs that have been implicated in tumor progression as regulators of miRNA cluster 23a/27a~24-2 in lung cancer, in which miR-27a can regulate both c-MET and EGFR [36]. Such a dual inhibitory role of miRNAs to the c-MET and EGFR oncogenic signaling pathways was also recently identified for miR-206 in lung squamous cell carcinoma [45]. Therefore, simultaneous inhibition of these RTKs might improve disease treatment. The evidence of miRNA participating in the

Table 2: Alteration of EGFR mutation related microRNAs in lung cancer.

MicroRNA(s)	Regulation	Chromosome locus	Reference(s)
miR-10b	Up	2q31.1	[30]
miR-21	Up	17q23.1	[26, 30]
miR-122	Up	18q21.3	[34]
miR-134/487b/655 cluster	Up	14q32	[49]
miR-183-3p	Up	7p32	[35]
miR-200b	Up	1p36.33	[30]
miR-210	Up	11p15.5	[30]
miR-30a	Down	6q13	[30]
miR-30b	Down	8q24.22	[30, 50]
miR-30c	Down	1p34.2	[50]
miR-34a	Down	1p36.23	[51]
miR-126	Down	9q34.3	[30]
miR-145	Down	5q32-33	[31]
miR-451	Down	17q11.2	[30]

Table 3: MicroRNAs regulate chemoresistance in lung cancer.

MicroRNA(s)	Regulation	Agent	Target(s)	Reference(s)
let-7	Down	Erlotinib	Hedgehog	[52]
miR-34	Down	Gefitinib	c-MET/HGF	[51]
miR-103	Down	Gefitinib	PKC-ε	[32]
miR-128b	Down	Gefitinib	EGFR	[15]
miR-138-5p	Down	Gefitinib	GPR124	[53]
miR-145	Down	TKIs	ERK, AKT, OCT4, c-MYC, EGFR, and NUDT1	[31, 54]
miR-146a	Down	TKIs	EGFR and NF-κB	[40]
miR-147	Down	Gefitinib	ZEB1 and AKT	[55]
miR-200	Down	Erlotinib	Hedgehog, MIG6, and TGFβ1	[52, 56, 57]
miR-203	Down	Gefitinib	SRC	[32]
miR-424	Down	TKIs	Not applicable	[15]
miR-548b	Down	TKIs	CCNB1	[58]
miR-7	Up	TKIs	EGFR, RAF1, and IRS-1	[21, 59, 60]
miR-21	Up	Gefitinib	PTEN, MDR1, Bcl-2, and PDCD4	[33]
miR-30b/30c	Up	Gefitinib	BIM	[32]
miR-126	Up	Gefitinib	AKT, EGFL7, PI3KR2, ERK, CRK, and VEGF	[54]
miR-134/487b/655 cluster	Up	Gefitinib	MAGI2	[49]
miR-221/222	Up	Gefitinib	APAF-1	[32]
miR-214	Up	TKIs	PTEN, MAPK, and p38	[46]
miR-374a	Up	TKIs	Wnt5a	[58]

EGFR/c-MET network in lung cancer thus provides a new clue to overcoming EGFR-TKI resistance in lung cancer [63].

4. miRNAs as Biomarkers for Predicting EGFR-TKI Response in Lung Cancer

Given the fact that only small portion of patients with lung cancer benefit from a treatment of EGFR-TKIs, the benefits of these agents to patients are ultimately limited by the emergence of drug resistance [64]. Therefore, great efforts have been made to identify new biomarkers for predicting responses to TKI treatment in lung cancer. The ability to alter EGFR-TKIs responses makes miRNAs as potential predictive biomarkers for EGFR-TKIs in lung cancer treatment, and several miRNAs could be served as biomarkers to predict response to EGFR-TKIs in lung cancer patients have been recently well documented (Table 3) [9, 65].

In a miRNA profiling analysis with retrospective cohorts consisted of 128 radically resected NSCLC patients (60 were EGFR mutation positive, 68 were negative, and 32 healthy controls), Shen et al. found that the expression of miR-21 and miR-10b in radically resected NSCLC patients with EGFR mutation were much higher relative to those without mutation. A Cox proportional-hazards regression analysis

further demonstrated that a reduced expression of miR-21 was associated with a significant improvement in overall survival in patients treated with gefitinib; that is, a patient who had upregulated miR-21 expression might have poor overall survival, but a better response to gefitinib. This data suggests that miR-21 expression may be an independent predictor of the response to gefitinib in lung cancer [30]. Of interest, an aberrant expression of miR-21 was also significantly correlated with platinum-based chemotherapy resistance in NSCLC patients, and an increased miR-21 expression was associated with the shorter disease-free survival [62].

Other miRNAs that targeting the EGFR signaling pathway, such as miR-128b [15], miR-30b, and miR-30c [32], were also significantly correlated with clinical EGFR-TKI responses in lung cancer, in which LOH at miR-128b is frequent in NSCLC [15], and the expressions of miR-30b and miR-30c have been reported to be prognostic predictors in NSCLC patients who underwent first line treatment with EGFR-TKIs [50]. Gu et al. retrospectively examined expression of miR-30b and miR-30c in 41 NSCLC samples of patients who used TKIs as first line of therapy. They found that there is a significant correlation of miR-30b and miR-30c levels and the short-term TKI responses, suggesting that miR-30b and miR-30c may be useful in predicting TKI response in NSCLC patients [50]. Another study using lung epithelial cancer cell line model has identified 13 miRNA genes to predict response to EGFR inhibitors, among which the miR-200c was able to target epithelial-to-mesenchymal transition (EMT) transcription factor, zinc finger homeodomain enhancer-binding protein 1 (ZEB1) and altered the sensitivity to erlotinib, and migration in lung cells [56]. The transforming growth factor-β (TGF-β) is able to induce EMT in cancers [49, 57]. A negative regulator of EGFR, mitogen-inducible gene 6 (MIG6) is a target of miR-200, the ratio of the expression of MIG6/miR200c was found to be tightly correlated with EMT and resistance to erlotinib in lung cancer *in vivo*, in which the MIG6 (mRNA)/miR200 ratio was inversely correlated with response to erlotinib, indicating that the ratio of MIG6/miR200 may be a predictive biomarker of the response of lung cancer to EGFR-TKIs [57].

5. miRNAs as Therapeutic Targets for Sensitizing EGFR-TKI-Resistant Lung Cancer

The emerging role of miRNAs in regulation the EGFR signaling pathway and therapeutic responses to EGFR-TKIs has provided a new avenue for developing novel agents and approaches to resensitize TKI resistance and improve the overall clinical outcomes of TKI-treatment in patients with lung cancer. Clinically, majority of EGFR-TKI resistance is induced by a secondary T790M mutation of EGFR or c-MET. In this regard, a T790M mutation is able to enhance the GTP affinity and block TKI binding to the tyrosine kinase domain of EGFR [29]. Therefore, a strategy by targeting G protein-coupled receptor or c-MET may reverse lung cancer cells to EGFR-TKI resistance. In order to overcome the EGFR-TKI resistance in T790M mutant NSCLC treatment, Rai et al. delivered miR-7 expressing plasmid to NSCLC cells and xenografts by liposomal transfection and found that the miR-7 could inhibit the growth of both TKI sensitive and resistant NSCLC cells *in vitro* and *in vivo* [59]. This finding was supported by a late study using Lewis lung cancer (3LL) cells with a downregulated miR-7; this study demonstrated that a restoration of miR-7 inhibited 3LL cell proliferation, induced cell apoptosis *in vitro,* and reduced tumorigenicity *in vivo* by targeting the EGFR signaling pathway [60].

Gao et al. recently identified miR-138-5p was strikingly downregulated, which was inversely correlated with the expression of G protein-coupled receptor 124 (GPR124) in a gefitinib-resistant lung cancer cell line PC9GR. Bioinformatics analysis suggested that the GPR124 was a direct target of miR-138-5p, which was further validated experimentally. Intriguingly, forced expression of miR-138-5p was sufficient to resensitize the PC9GR cells and gefitinib resistant NSCLC H1975 cells to gefitinib, and knockdown of GPR124 with small RNA mimics exhibited similar effects of miR-138-5p, suggesting that the acquired gefitinib resistance was in part attributed by a downregulation of miR-138-5p and that restoration of miR-138-5p level might be a potential therapeutic approach for sensitizing gefitinib resistance in NSCLC [53]. Using a similar approach, Zhou et al. found that an ectopic expression of miR-34a could inhibit cell growth and induce apoptosis in hepatocyte growth factor- (HGF-) induced gefitinib-resistant HCC827GR and PC-9GR lung cancer cells and in HGF-induced gefitinib resistant mouse xenograft model, partly by targeting MET [51]. In this context, an upregulation of HGF has been demonstrated as an important mechanism involved in the AR to EGFR-TKIs by activation of PI3K/Akt pathway through phosphorylation of c-MET [32, 36, 62]. This notion was supported by the evidence of that the total and phosphorylated of c-MET proteins were partially decreased in gefitinib-sensitive HCC827 and PC-9 cells, but the total and phosphorylated status of the downstream PI3K/Akt or ERK signaling pathway was not affected by the transfection of miR-34a. This result indicated that miR-34a had an inhibitory effect on MET rather than its downstream signaling components, which also implies that the tumor suppressive effects of miR-34a alone in gefitinib-sensitive EGFR mutant NSCLC cells might mainly be dependent on mechanisms other than c-MET inhibition. This was different from in the gefitinib-resistant HCC827GR and PC-9GR cells, in which a combination of miR-34a and gefitinib could efficiently induced cell death and apoptosis with an inhibition of the phosphorylation of c-MET, EGFR, Akt, and ERK [51]. Such a synergistic effect between the miR-34a and EGFR-TKIs was also reported in study of a combination of erlotinib and miR-34a in NSCLC cells with primary and acquired erlotinib resistance, in which a strong synergistic interaction between the erlotinib and miR-34a mimics was observed [51]. These studies clearly suggest that a synergistic strategy using miRNAs and TKIs in a combination may effectively reverse the EGFR-TKI resistance in lung cancer treatment.

Both experimental and preclinical studies have demonstrated that persistent activation of PI3K/Akt and/or Ras/Erk pathways is associated with EGFR-TKI resistance in NSCLC, in which they play pivotal roles in TKI sensitivity [66].

Indeed, the overexpression of miR-21 led to a significant decrease of gefitinib sensitivity in PC9 lung cancer cells through a mechanism by inhibiting PTEN expression and activating the Akt/Erk signaling pathway, while knockdown of miR-21 dramatically reversed gefitinib sensitivity in PC9GR cells by upregulating PTEN and inactivating the Akt/Erk pathway, suggesting modulation of miR-21/PTEN expression may be a promising strategy for resensitizing EGFR-TKI resistance in NSCLC [33].

Apart from targeting the PI3K/Akt/Ras/Erk pathway, EMT is involved in the AR to therapy, which is often activated during the progression of lung cancer [52, 56, 57, 67]. Several lines of evidence have demonstrated that miRNAs are involved in MET and reverse EGFR-TKI resistance in NSCLC [55, 57, 67]. For instances, miR-147 was downregulated in NSCLC, and overexpression of miR-147 could induce the MET of lung cancer cells, sequentially resensitize the resistance to EGFR-TKIs by inhibiting the Akt signaling pathway, and the MET phenotype of lung cancer cells could be attenuated by TGF-β [55]. Exposure NSCLC cells with EMT to TGF-β1 was found to significantly induce miR-134/487b/655 cluster by targeting membrane-associated guanylate kinase, WW domain- and PDZ domain-containing protein 2 (MAGI2), a scaffold protein required for PTEN. Ectopic expression of miR-134 and miR-487b enhanced the EMT potential and the drug resistance to gefitinib of the cells, whereas reduction of the transcripts of these miRNAs led to an inhibition of EMT process and a restoration of drug sensitivity of TGF-β1-induced resistance to gefitinib, implying that the miR-134/miR-487b/miR-655 cluster may be a novel therapeutic target in patients with advanced lung adenocarcinoma [49]. By modulating EMT-regulating miRNAs, Ahmad et al. demonstrated that both specific siRNA to the Hedgehog (HH) signaling pathway and GDC-0449 (a small molecule antagonist of G protein coupled receptor smoothened in the HH pathway) were able to resensitize TGF-β1-induced erlotinib resistant A549 (A549M) cells with an upregulation of miR-200b and let-7c. Ectopic expression of these miRNAs also led to diminish the erlotinib resistance of A549M cells [52]. In another study, Cufí et al. discovered that flavonolignan silibinin could suppress the EMT-driven erlotinib resistance by restoring a high miR-21/low miR-200c signature in EGFR-mutant NSCLC xenografts [67]. A combination of erlotinib and silibinin led a completely abrogate tumor growth in the NSCLC xenograft model. Mechanistically, the silibinin could fully restore the EMT-related high miR-21/low miR-200c signature and inhibit the expression of mesenchymal markers snail family zinc finger 1 (SNAI1), ZEB, and N-cadherin in erlotinib-refractory tumors. In addition, the silibinin was sufficient to fully activate a reciprocal c-MET in erlotinib-refractory cells [67].

In addition to T790M and c-MET amplification, Axl kinase is found to be upregulated in humans with acquired resistance to EGFR-TKI, and the involvement of Axl kinase in acquired resistance of NSCLC to RGFR-TKIs gefitinib or erlotinib has been reported [38, 58, 68]. In order to interrogate the role of miRNAs in the Axl-mediated acquired TKIs resistance in lung cancer, Wang et al. identified a panel of Axl kinase-altered miRNAs in lung cancer cells and experimentally validated that the Axl-induced miR-374a and miR-548b play a crucial role in cell cycle arrest, gefitinib-induced apoptosis, and EMT of gefitinib-resistant lung cancer cells by targeting Wnt5a and CCNB1 genes, respectively. Clinically, a high expression of Axl and miR-374a and low abundance of miR-548b are associated with poor disease-free survival. These observations suggest a promising strategy by targeting miRNAs to reverse gefitinib resistance in NSCLC with high expression of Axl [58, 68].

6. Perspectives and Challenges

As a class of regulators at the posttranscription level, miRNAs display different expression patterns in various types of cancer, in which some miRNAs are dysregulated and they play crucial roles in the initiation, progression, and therapeutic responses of cancer. The involvement of miRNAs in the mutant status of EGFR and the emerging role of these molecules in the regulation of the EGFR signaling pathway and drug resistance to anti-EGFR agents have made miRNAs potential biomarkers for the diagnosis and prognosis of lung cancer, as well as potential predictive markers for the therapeutic outcome using anti-EGFR agents or regimens. In a therapeutic standpoint, in addition to restore the functions of tumor suppressor genes or inhibiting oncogenes by targeting miRNAs in anticancer therapy, the modulation of miRNA profiles is also a plausible therapeutic strategies to resensitizing chemoresistance in cancer treatment. In terms of anti-EGFR therapy, an intervention of specific miRNAs that involved in the EGFR signaling pathway and/or EGFR-TKI resistance has shown a promising effect to reverse the resistance of lung cancer cells to anti-EGFR therapy by enhancing the sensitivity of tumor cells to chemotherapy or inhibiting cancer cell stemness.

Although recent studies in lung cancer and miRNAs have significantly extended our understanding of the EGFR signaling pathway and its involvement in the pathogenic processes of lung cancer, our understanding of the underlying mechanisms that integrate the activity of this pathway remains fragmentary. Therefore, intensively exploring the regulatory roles of miRNAs in this pathway may contribute to the possible implementation of miRNAs as predictive and prognostic biomarkers [69, 70]. Particularly, the application of miRNAs as predictive biomarkers may also be beneficial for predicting therapeutic response to anti-EGFR agents in advanced lung cancer patients and lead to a higher level of personalized therapy. However, challenges for the development of miRNA in therapy remain to be addressed; these include tissue specific delivery, potential off-target effects, and safety. An improvement of the specificity of miRNAs and the development of efficient systemic delivery approaches will facilitate the use of miRNAs for the treatment of patients with lung cancer.

Abbreviations

AKT: Protein kinase B
APAF-1: Apoptotic peptidase activating factor 1

Axl:	Anexelekto
BIM:	BCL2-like 11
CCNB1:	Cyclin B1
CCND1:	Cyclin D1
c-MET:	The transmembrane tyrosine kinase cell surface receptor for HGF
c-Myc:	A regulator gene that codes for a transcription factor
CRK:	CT10 regulator of kinase (Crk)
EGFR:	Epidermal growth factor receptor
EMT:	Epithelial to mesenchymal transition
ERF:	Ets2 transcriptional repression factor
ERK:	Extracellular signal-regulated kinase
GPR124:	G protein-coupled receptor 124
HGF:	Hepatocyte growth factor
HH:	Hedgehog
IGF1R:	Insulin-like growth factor 1 receptor
IRS-1:	Insulin receptor substrate-1
K-Ras:	Kirsten rat sarcoma viral oncogene homolog
LOH:	Loss of heterozygosity
MAGI2:	Membrane-associated guanylate kinase, WW, and PDZ domain-containing protein 2
MAPK:	Mitogen-activated protein kinase
MDR-1:	Multidrug resistance 1
MET:	Mesenchymal to epithelial transition
MIG6:	Mitogen-inducible gene 6
NF-κB:	Nuclear factor kappa beta
NSCLC:	Non-small-cell lung cancer
NUDT1:	Nucleoside diphosphate linked moiety X-type motif 1
PDCD4:	Programmed cell death protein 4
PDZ domain:	An interaction motif that recognizes and binds the C-terminal peptides of target proteins
PI3K:	Phosphatidylinositol-3-kinase
PKC-ε:	Protein kinase C varepsilon
PTEN:	Phosphatase and tensin homolog
PTPN9:	Phosphatases tyrosine-protein phosphatase nonreceptor type 9
PTPRF:	Receptor-type tyrosine-protein phosphatase F
RAF-1:	Proto-oncogene serine/threonine protein kinase
RAS:	Rat sarcoma viral oncogene homolog
RTK:	Receptor of tyrosine kinase
SIRT1:	Sirtuin 1
SNAI1:	Snail family zinc finger 1
SRC:	Sarcoma viral oncogene homolog
TGFβ1:	Transforming growth factor beta 1
TGFβR1:	Transforming growth factor beta receptor 1
VEGF:	Vascular endothelial to growth factor
Wnt:	Wingless-type MMTV integration site family
WW domain:	A short conserved protein domain mediates interactions with protein ligands
ZEB:	Zinc finger homeodomain enhancer-binding protein.

Conflict of Interests

The authors declare they have no conflict of interests.

Authors' Contribution

Fei Han and Jinxi He contributed equally to this paper.

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Research Article

Unravelling the RNA-Binding Properties of SAFB Proteins in Breast Cancer Cells

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Scaffold attachment factor B1 (SAFB1) and SAFB2 proteins are oestrogen (ER) corepressors that bind to and modulate ER activity through chromatin remodelling or interaction with the basal transcription machinery. SAFB proteins also have an internal RNA-recognition motif but little is known about the RNA-binding properties of SAFB1 or SAFB2. We utilised crosslinking and immunoprecipitation (iCLIP) coupled with high-throughput sequencing to enable a transcriptome-wide mapping of SAFB1 protein-RNA interactions in breast cancer MCF-7 cells. Analysis of crosslinking frequency mapped to transcript regions revealed that SAFB1 binds to coding and noncoding RNAs (ncRNAs). The highest proportion of SAFB1 crosslink sites mapped to ncRNAs, followed by intergenic regions, open reading frames (ORFs), introns, and 3′ or 5′ untranslated regions (UTR). Furthermore, we reveal that SAFB1 binds directly to RNA and its binding is particularly enriched at purine-rich sequences not dissimilar to the RNA-binding motifs for SR proteins. Using RNAi, we also show, for the first time, that single depletion of either SAFB1 or SAFB2 leads to an increase in expression of the other SAFB protein in both MCF-7 and MDA-MD231 breast cancer cells.

1. Introduction

The growing interest in SAFB1 and SAFB2 proteins in relation to cancer is generated from their well described ability to bind to and modulate ER-α, a central player in breast cancer development. Moreover, a role for SAFB1 in RNA splicing and metabolism has also been proposed. Nayler et al. [1] first described interactions between SAFB1 with RNA polymerase II and a subset of serine/arginine-rich RNA processing factors (SR proteins) suggesting that SAFB1 serves as a molecular base for the assembly of a transcriptome complex that couples chromatin organising S/MARs elements with transcription and pre-mRNA processing [1]. Protein-protein interactions between SAFB1 and a range of RNA-binding proteins including hnRNP A1, hnRNP D, hnRNP G, SR splicing regulatory protein 86 (SRrp86), SR protein kinase 1 (SRPK1), and Src-associated substrate in mitosis of 68 kDa (Sam68) provide reasonable evidence to implicate a role in alternative splicing [2–6]. However, it is still not known whether these SAFB proteins exert their effects on pre-mRNA splicing through direct RNA interaction or by tethering to other splicing factors.

SAFB1 and SAFB2 proteins share a highly conserved RNA-recognition motif (RRM) with 98% similarity in the central region, although until now their direct RNA-binding potential has remained unclear. SAFB1 has also been labelled as a novel hnRNP protein due to its similarity to the highly conserved RBD found in the hnRNP protein family [6]. Subsequent studies have implicated both SAFB proteins in alternative splicing, as overexpression of SAFB1 and SAFB2 inhibits splicing of a *TRA2B* variable exon [5, 7]. However, further investigation using mutants lacking the RRM domain revealed that SAFB1's ability to inhibit *TRA2B* exon skipping was independent of its RNA-binding ability [7]. This evidence

suggests that SAFB1 may not bind directly to *TRA2B* pre-mRNA to regulate exon skipping but could possibly mediate an indirect effect through its interaction with various splicing factors [2, 4–6]. In an unrelated study, *in vitro* evidence has shown that the RRM domain of SAFB1 was able to bind RNA isolated from MCF-7 breast cancer cells, although the identity of the RNA targets was not described [8]. The current study was designed to establish whether SAFB proteins exert their RNA processing functions through direct RNA interaction as well as by tethering to other protein factors.

2. Material and Methods

2.1. iCLIP. CLIP with individual nucleotide resolution (iCLIP) was performed for SAFB1 using MCF-7 breast cancer cells based on a published protocol [9]. In brief, MCF-7 cells were irradiated with 150 mJ/cm^2 of UV at 254 nm and cell pellets resuspended in lysis buffer treated with Turbo DNase I (Ambion) and high (1 : 10 dilution) or low (1 : 500 dilution) RNase I (Ambion). Dynabeads Protein A or Dynabeads Protein G (Invitrogen) were resuspended in lysis buffer containing 5 μg SAFB1 antibody (Sigma-Aldrich) and precleared lysate was added to the magnetic beads for immunoprecipitation at 4°C for 2 hours. RNA 3′ ends were dephosphorylated and RNA linkers ligated. Magnetic beads were then resuspended in PNK mix containing ^{32}P-γ-ATP to radioactively label the RNA 5′ ends, as previously described [9]. Protein-RNA complexes were isolated following electrophoresis (see Supplementary Figure 1 in Supplementary Material available online at http://dx.doi.org/10.1155/2015/395816). Precipitated RNA was reverse transcribed in RNA/primer mix containing different Rclip primers with individual barcode sequences for each replicate. Three gel fragments corresponding to cDNA size were cut at 120–200 nucleotides (high), 85–120 nucleotides (medium), and 70–85 nucleotides (low) (Supplementary Figure 1(B)). Three independent biological replicates were prepared for sequencing using the TruSeq Sample Preparation kit (Illumina) and sequenced on the Genome Analyser II system (GAIIx, Illumina). Bioinformatic analyses were performed on the web-based iCount software (http://icount.biolab.si/). Mapping of SAFB1 crosslink sites to regions of respective genes was visualised in UCSC Genome Browser (http://genome.ucsc.edu/) and a graphical representation of the novel SAFB1 consensus binding motif was designed using the web-based WebLogo software (http://weblogo.berkeley.edu/). Potential target genes that contain SAFB1 binding sites were selected for further validation using qRT-PCR with TaqMan gene expression assays.

2.1.1. Transient Transfections. MCF-7 and MDA-MB-231 cells were reverse transfected with two independent sets of Silencer Select siRNA for SAFB1 and SAFB2, *Silencer* Negative Control siRNA, *Silencer* Select GAPDH, and β-actin Positive Control siRNA (Life Technologies) using INTERFERin Transfection Agent (Polyplus transfection) according to the manufacturer's instructions. Following optimisation, cells were seeded at 3.5×10^4 cells/well in 24-well plates or 1.75×10^5 cells/well into 6-well plates. After 24 hours, cells were transfected with 5 nM siRNA with 4 μL/mL of INTERFERin transfection agent. RNA was collected 48 and 72 hours after transfection and protein collected 72 hours after transfection. In all experiments, levels of knockdown by RNAi were assessed at the RNA and protein level by PCR and immunoblotting.

2.1.2. RNA Isolation and PCR. Total RNA from cultured cells was extracted with RNeasy spin columns (Qiagen) or the SV Total RNA Isolation System (Promega) according to manufacturer's instructions. One μg of total RNA was reverse transcribed using oligo (dT) primers and Superscript II Reverse Transcriptase (RT) (Invitrogen, Life Technologies) following manufacturer's instructions. Conventional PCR was performed using PCR master mix (Promega). qPCR was performed using TaqMan gene expression assays (Life Technologies) according to the manufacturer's instructions. Validated TaqMan probes were selected to target specific genes as follows: SAFB1 (Hs01561652_g1), SAFB2 (Hs01006796_g1), ITGB4 (Hs00236216_m1), SHF (Hs00403125_m1), MALAT-1 (Hs00273907_s1), and β-actin (Hs99999903_m1). Data analysis was performed using the comparative C_t method normalised against β-actin expression. Experiments were performed in triplicate and statistical analysis was performed using Student's t-test or Repeated Measures ANOVA with Dunnett's Multiple Comparison Test. All effects at $P < 0.05$ are reported as significant.

2.2. RNA Immunoprecipitation. MCF-7 cells were washed in ice cold PBS and then collected in lysis buffer (10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5% NP-40, and 1% Triton X-100) containing protease inhibitors (Sigma-Aldrich) and RNase OUT (Invitrogen). Equal amounts of cell lysate were then incubated for 3 hours at 4°C with 2 μg of either rabbit anti-SAFB1 antibody (Genetex) or rabbit IgG control (Santa Cruz). Cell lysates and antibodies were then incubated with Dynabeads Protein G (Invitrogen) for a further hour at 4°C. Beads were then washed five times with lysis buffer before the immunoprecipitated RNA was collected in Trizol reagent (Invitrogen) according to manufacturer's instructions. The RT-PCR was performed as before but half of the RNA obtained from the immunoprecipitation was used in each reaction. Fold enrichment of target mRNA was determined after normalization to the input and rabbit IgG controls.

3. Results

3.1. Identification of RNA-Binding Sites for SAFB1 in Breast Cancer Cells. Although the role of SAFB proteins in RNA processing has been speculated, the function of their highly homologous internal RRM has not been examined. We sought to identify possible direct RNA targets for SAFB1 in breast cancer cells, using iCLIP technology [9–12] combined with high-throughput sequencing and mapping to generate a transcriptome-wide binding map for SAFB1. SAFB1 protein-RNA complexes were successfully generated by immunoprecipitation and RNA recovered and purified from 3 independent iCLIP replicates (Supplementary Figure 1). High-throughput sequencing and bioinformatics generated a total

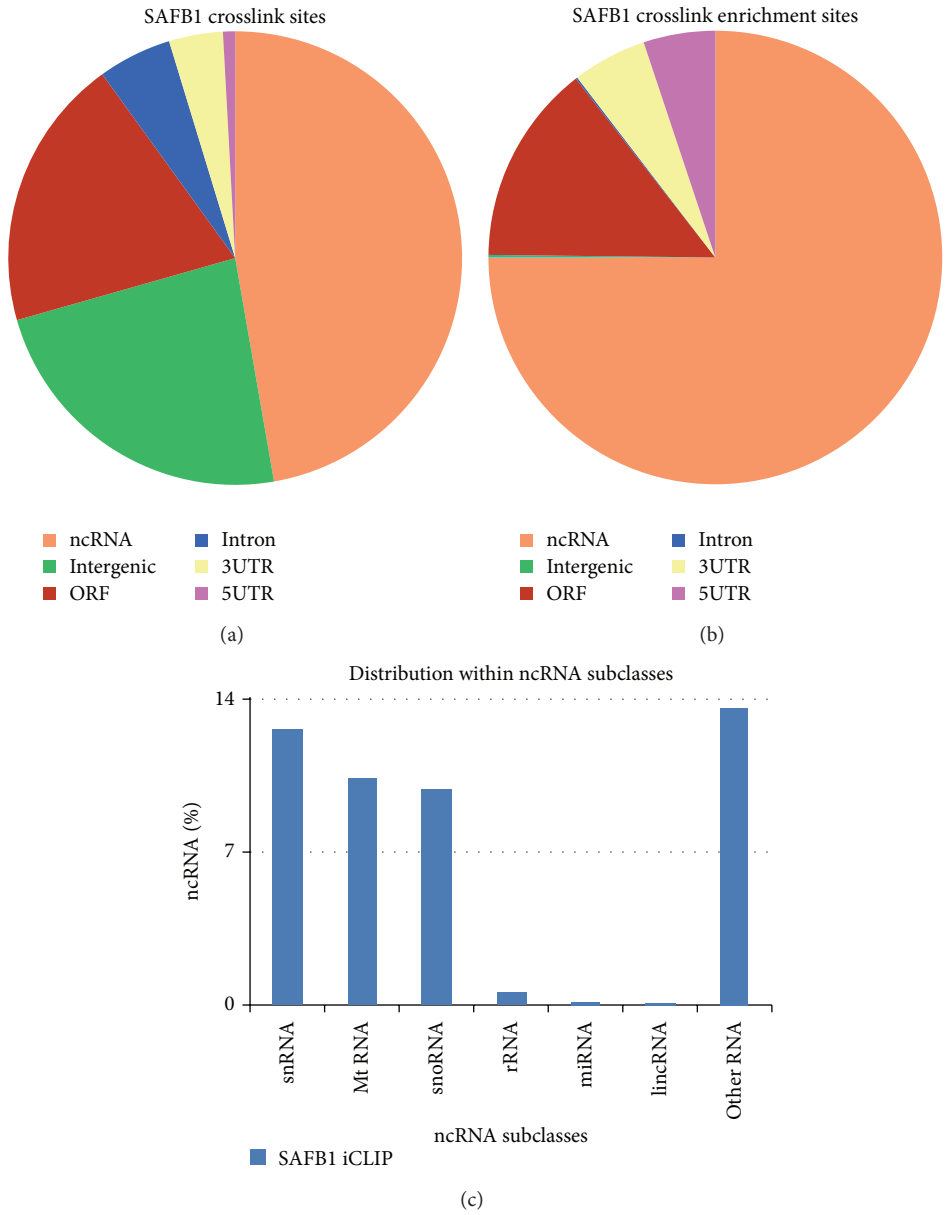


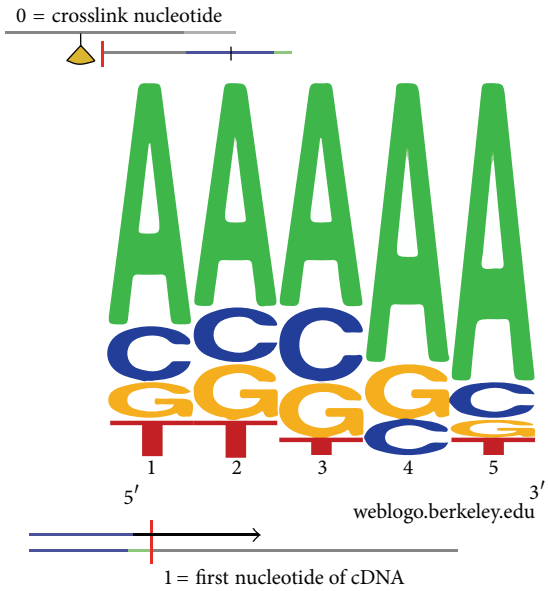
Figure 1: Distribution of significant SAFB1 crosslink sites within RNA segment types. (a) The proportion of cDNAs mapped to different transcript regions relative to the total number of cDNA reads revealed that the highest percentage of cDNAs was mapped to ncRNA (47.08%), followed by intergenic regions (23.24%), ORFs (19.38%), introns (5.23%), 3′ UTRs (3.83%), and 5′ UTRs (0.86%). (b) The fold enrichment of cDNA density in different types of RNAs relative to cDNA density in the whole genome highest density enrichment in ncRNAs. (c) The distribution of SAFB1 crosslink sites within different ncRNA subclasses revealed significant abundance in snRNA, Mt RNA, and snoRNA. "Other RNA" consists of pseudogenes and processed transcripts with no known ORF or function.

of 1,145,271 unique cDNA reads with single-hits mapping to the human genome which were subsequently filtered down to 587,119 significant unique cDNAs distributed over 127308 binding sites in 25207 SAFB1 crosslink clusters (FDR < 0.05). A snapshot of the view for SAFB1 crosslink sites on the UCSC Genome Browser (http://genome.ucsc.edu/) is shown in Supplementary Figure 1(C).

iCLIP identified binding sites for SAFB1 across the whole transcriptome, where 100% of significant cDNA reads mapped to the sense orientation in annotated genes. This confirms the high strand specificity of iCLIP also observed in other studies [11, 13]. Analysis of crosslinking frequency mapped to transcript regions revealed that SAFB1 binds to coding and noncoding RNAs (ncRNAs). Notably, the highest proportion of 127308 SAFB1 crosslink sites from significant clusters map to ncRNAs followed by intergenic regions, open reading frames (ORFs), introns, and 3′ or 5′ untranslated regions (UTRs) (Figure 1(a)). When the cDNA density for each transcript region was analysed relative to the cDNA density in the whole genome, the highest density enrichment

Pentamer	Frequency
GAAAA	34.644
AGAAA	31.201
AAACA	30.533
AACAA	30.072
AAAAC	29.955
ATGAA	29.857
AAGAA	28.988
GAAGA	26.496
AAAGA	26.144
TGAAA	25.850
AACCA	25.815
ACAAC	25.653
AATGA	25.522
ATCAA	25.514
ACCAA	25.450
ACAAA	25.227
CAGAA	24.147
CAAAT	23.917
CAAAA	23.660
TGAAG	23.323

(a)



(b)

Base	Base position 1	2	3	4	5
A	65%	60%	60%	75%	80%
C	15%	15%	20%	10%	10%
G	10%	15%	15%	15%	5%
T	10%	10%	5%	0%	5%

(c)

Figure 2: *In vivo* consensus binding motif of SAFB1. (a) The frequency of pentamers surrounding SAFB1 crosslink sites was determined. Adenine represents 68% of the 20 pentamers that has the highest frequencies. (b) WebLogo showing base frequencies of each base at respective positions of the pentamer. SAFB1 binds to adenine-rich motifs. (c) The frequency of each base relative to its position within the pentamer was summarised in this table. The highest frequency of adenine was observed at base position 5; thymine was excluded at base position 4 of the consensus binding motif. This consensus binding motif was predicted from iCLIP cDNA libraries; therefore the uracil base is referred to as thymine in these sequences.

was detected in ncRNAs (Figure 1(b)). The distribution of SAFB1 crosslink sites within ncRNA subclasses was also analysed. SAFB1 crosslink sites were most abundant in small nuclear RNA (snRNA), mitochondrial RNA (Mt RNA), and small nucleolar RNA (snoRNA) (Figure 1(c)).

3.2. Identification of an RNA-Binding Motif for SAFB1. The *in vivo* binding specificity of SAFB1 is still currently unknown. The advantage of single nucleotide resolution provided by iCLIP method enabled the assessment of sequence specificity for SAFB1 binding. To derive whether a consensus binding motif exists for SAFB1, enriched pentamer sequences surrounding the crosslink sites were identified. The frequencies of each pentamer were analysed to determine the top 20 pentamers for SAFB1. Strikingly, adenine appeared as the most frequent nucleotide in the top 20 pentamers and represents 68% of the enriched pentamers (Figure 2(a)). The predicted SAFB1 consensus binding motif contains adenine-rich sequences derived from the pentamers (Figure 2(b)). When the frequency of each nucleotide in the cDNA libraries was analysed relative to its base position, a strong inclusion of adenine at base position 5 was observed (80%) while thymine (uracil in RNA) was excluded at base position 4 of the putative RNA-binding motif (Figure 2(c)). The consensus binding motif for SAFB1 has not been described before; therefore, this novel finding is likely to be of significance to further our current understanding of SAFB1 RNA-binding specificity.

3.3. Identification of Novel RNA Targets from Data Generated by iCLIP. Data analysis of bound RNAs revealed the number of SAFB1 crosslink sites within each RNA target. When the top 10 RNA targets with the largest number of crosslink sites were listed according to each RNA segment, the position of SAFB1 binding within each gene was visualised using the UCSC Genome Browser (Supplementary Figure 2). This enabled the identification of several interesting RNA targets that were selected for validation. Further experimentations were performed using qRT-PCR or conventional PCR on RNAi transfected MCF-7 and MDA-MB-231 cells to verify the effect of loss of SAFB1 on the expression of these selected RNA

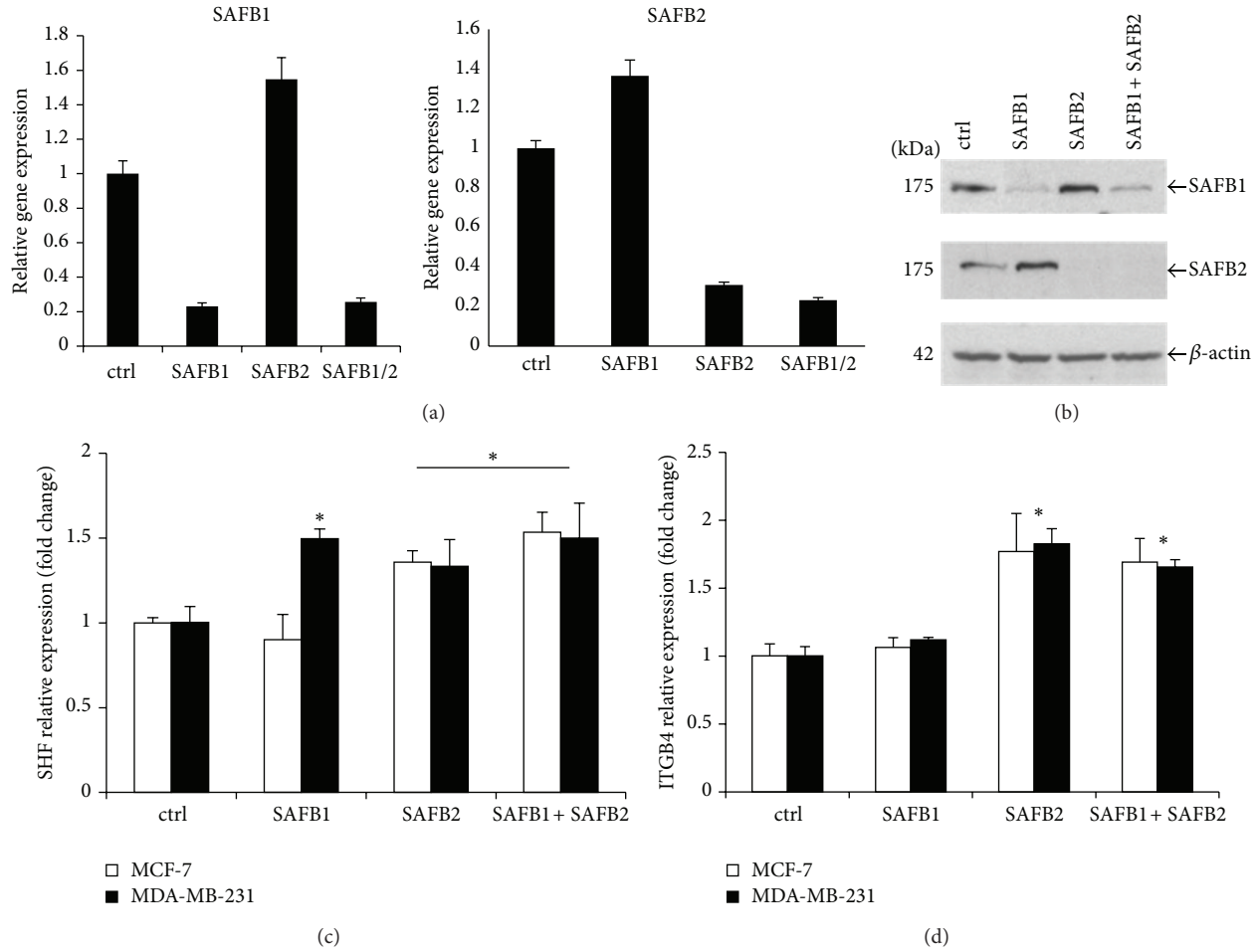


Figure 3: Loss of SAFB proteins affects expression of *SHF* and *ITGB4*. Knockdown of SAFB1 in MCF-7 cells increases mRNA and protein expression of SAFB2 and similarly knockdown of SAFB2 leads to increased expression of SAFB1. MC7-7 cells were transiently transfected with negative, SAFB1, SAFB2 or SAFB1 and SAFB2 siRNA. mRNA levels were measured by qRT-PCR. Data represents the average of three biological replicates ± SD. Statistical significance of mRNA expression was calculated using Student's *t*-test; * = $P < 0.05$. (b) Protein levels were analysed by immunoblotting using SAFB1 and SAFB2 antibodies. (c) The effect of SAFB knockdown on *SHF* and *ITGB4* expression by qRT-PCR using validated TaqMan probes specifically targeting *SHF* (c) or *ITGB4* (d). Data represents the average of three biological replicates ± SD. Statistical significance of mRNA expression was calculated using Student's *t*-test; $P < 0.05$.

targets. Since SAFB2 shares 98% sequence homology to the RRM of SAFB1, these cells were also depleted of SAFB2 and double knockdown of SAFB1 and SAFB2 was also included; interestingly, data shows that when MCF-7 cells are reduced of SAFB1 by RNAi, the levels of SAFB2 mRNA and protein increase (Figure 3(a)). Likewise, levels of SAFB1 increase after knockdown of SAFB2 (Figure 3(b)). A similar pattern was observed when the breast cancer MDA-MB-231 cells were used (Supplementary Figure 3).

Analysis of the RNA map revealed a large number of SAFB1 binding sites on the *SHF* mRNA, particularly accumulated around the alternative promoter (Supplementary Figure 3); the use of alternative promoters plays a significant role in gene expression control (reviewed in [14–16]). More importantly, the aberrant use of alternative promoter has been linked to a number of diseases, including cancer [17]. Therefore, identification of *SHF* as a potential RNA target for SAFB1 warrants further investigation. In MCF-7 noninvasive breast cancer cells, a reduction in SAFB1 did not appear to significantly alter *SHF* mRNA expression, whereas in MDA-MB-231 invasive breast cancer cells there was a significant increase in *SHF* mRNA expression when SAFB1 was reduced (Figure 3(c)). Loss of SAFB2 and both SAFB proteins by RNAi increased *SHF* expression, again supporting their role as transcriptional repressors. Another potential RNA target for SAFB proteins is *ITGB4*. The observed loss of SAFB1 in both breast cancer cell lines does not have an effect on *ITGB4* mRNA expression whereas loss of SAFB2 and both SAFB proteins significantly increased *ITGB4* expression (Figure 3(d)).

3.4. Malat-1: A ncRNA Target for SAF2? Another interesting observation from the iCLIP dataset revealed significant SAFB1 binding sites to metastasis associated lung adenocarcinoma transcript 1 (*MALAT-1*). *MALAT-1* is a highly conserved long ncRNA enriched in nuclear speckles that

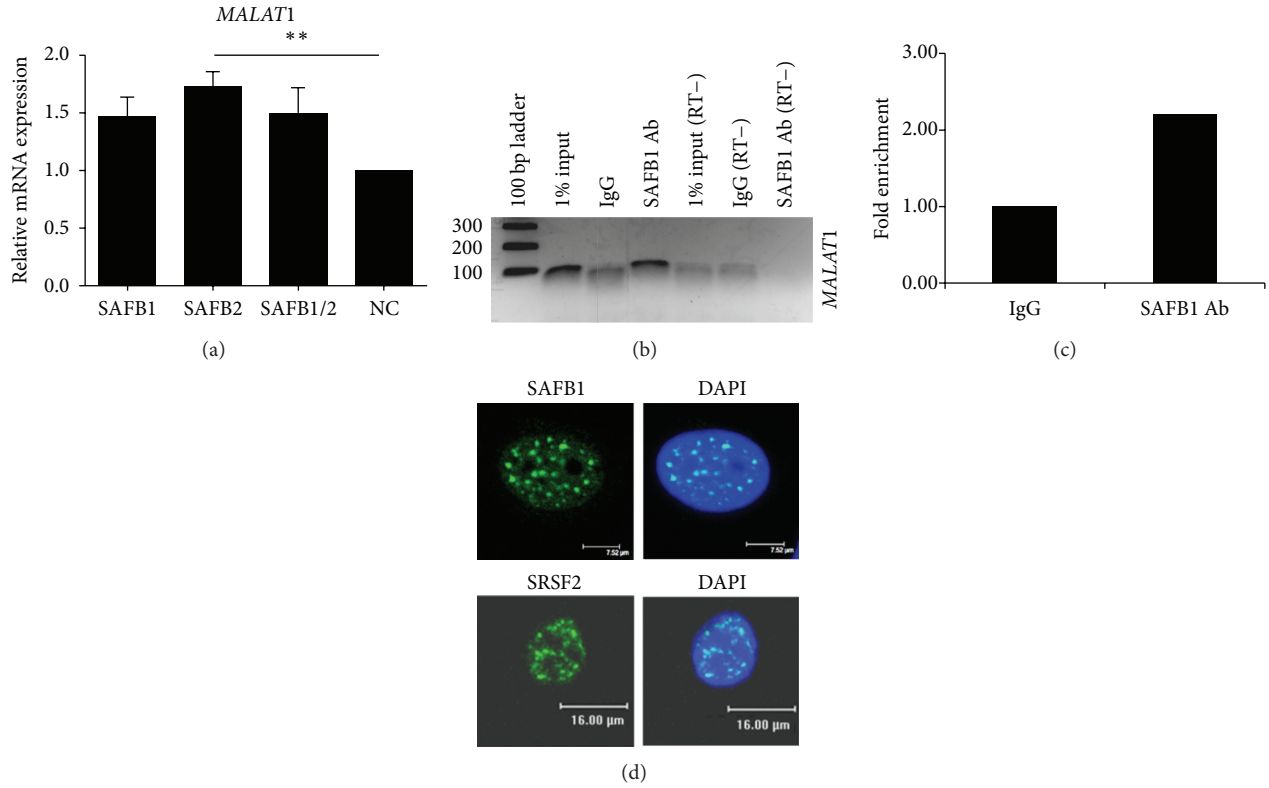


Figure 4: SAFB2 regulates expression of *MALAT-1*. (a) Expression of *MALAT-1* was measured by qRT-PCR using RNA from MCF-7 and MDA-MB-231 cells transfected with negative, SAFB1, SAFB2 or SAFB1 and SAFB2 siRNA using validated TaqMan probes specifically targeting *MALAT-1*. Data represents the average of three biological replicates ± SD. Repeated Measures ANOVA with Dunnett's Multiple Comparison Test statistical significance of mRNA expression was calculated using Student's *t*-test; $P < 0.01$. Enrichment of *MALAT1* RNA by conventional PCR (b) and qPCR (c) after RNA immunoprecipitation with anti-SAFB1 in MCF-7 cells. No enrichment was observed using IgG. (d) Intranuclear distribution of SAFB1 and SRSF2 in MCF-7 cells by immunofluorescent staining. Confocal laser microscopy revealed a punctate pattern for SAFB1 and SRSF2 in nuclear speckles.

regulates alternative splicing by modulating splicing factor phosphorylation [18]. *MALAT-1* is overexpressed in many different cancers including breast and is considered an oncogenic long ncRNA [19, 20]. We show that, in MCF-7 cells, loss of SAFB2 resulted in an increase in the levels of *MALAT-1* expression (Figure 4(a)). Moreover, we also tested the ability of SAFBI to immunoprecipitate *MALAT-1* RNA in MCF-7 cells; enrichment of *MALAT-1* RNA was observed and determined by conventional PCR and qPCR (Figures 4(b) and 4(c)).

4. Discussion

The presence of the highly conserved RRMs within SAFB1 and SAFB2 proteins has been a subject of interest since their discovery, especially in relation to their RNA-binding potential. Despite the fascination, very little has been undertaken until now to describe their RNA-binding capabilities. Initial *in vitro* evidence showed that the RRM of SAFB1 is able to bind RNA when glutathione S-transferase- (GST-) tagged SAFB1 protein combined with total RNA from MCF-7 cells generated a PCR product when reverse transcribed and PCR amplified [8]. However, the identity of the RNA targets was not described and important questions with respect to the role of SAFB1 and SAFB2 in RNA processing remained unanswered.

iCLIP has been proven as a powerful method to determine protein-RNA interactions *in vivo* on a global scale and identify the positions of crosslink sites at nucleotide resolution [11]. The random barcode incorporated to individual cDNA molecules addresses the problem of PCR artifacts faced by all high-throughput sequencing methods. iCLIP has generated a huge dataset and this is an initial analysis of the RNA-binding data for SAFB1. In this study, a global view comparison of the complete dataset from each individual biological replicate showed that all datasets generated consistent and reproducible results, underlining the high quality iCLIP data achieved by high stringency purification and library preparation.

The identification of *in vivo* targets by iCLIP enabled the mapping of transcript regions and RNA classes bound by SAFB1. An overview of the iCLIP results showed that the important class of RNAs bound by SAFB1 was ncRNAs. Interestingly, this binding distribution of SAFB1 is similar to the RNA-binding distribution of splicing factors SRSF3 and SRSF4 rather than hnRNP C protein; SAFB1 was initially classified as a novel member of the hnRNP protein family

[6]. Recent work by Änkö et al. utilised iCLIP to reveal that concentrated SRSF3 and SRSF4 binding sites were also in ncRNAs [21], while König et al. [11] showed that hnRNP C binding sites were most abundant within introns [11]. This observation raises the possibility that SAFB1 protein may have similar characteristics to SR proteins rather than hnRNP protein members, although at this stage this is only speculative.

The term ncRNA is commonly used for RNA that does not encode a protein but appears to comprise internal signals that control various levels of gene expression, including chromatin organisation, transcription, RNA splicing, editing, translation, and turnover (reviewed in [22]). Consistent with already known functions of SAFB proteins, concentrated SAFB1 binding in ncRNAs observed from the iCLIP data could possibly contribute to its various role in chromatin organisation, transcription, and RNA metabolism. Analysis of SAFB1 distribution within ncRNA subclasses revealed most abundant SAFB1 binding in snRNAs. snRNAs are a class of small RNA molecules found to be uridylate-rich and localised within the nucleus [23]. The most common members of snRNAs are the U1, U2, U4, U5, and U6 snRNAs that form the spliceosome along with many other protein factors and primarily function in pre-mRNA splicing (reviewed in [24]). The high distribution of SAFB1 binding sites in snRNAs observed in this study supports previously identified interactions between SAFB1 with various RNA processing factors and splicing machinery [1, 4, 6, 25].

The genome-wide, single nucleotide resolution of iCLIP data enabled the prediction of *in vivo* consensus binding sequences for SAFB1 based on the enriched pentamer sequences surrounding the crosslink sites. This study is the first to report that SAFB1 binds a consensus adenine-rich sequence *in vivo.* Closer examination of the putative consensus binding sequence revealed the exclusion of thymine (uracil in RNA) at base position 4 and a strong inclusion of adenine at base position 5. Interestingly, the predicted SAFB1-binding motif is not dissimilar to purine-rich sequences found in RNA-binding motifs for other SR proteins (reviewed in [26]).

When analysing SAFB1 crosslink sites within protein-coding transcripts, SAFB1 binding density was also enriched in regions encompassing the ORF and 3′ and 5′ UTR. The list of RNA targets was filtered according to the region and density of SAFB1 binding to identify targets that are relevant to tumourigenesis. Several interesting genes were highlighted in this study including *SHF, ITGB4*, and *MALAT-1.*

SHF is a member of a family of adaptor protein characterised by their ability to mediate protein-protein interactions through their Src homology 2 domain [27, 28]. Although the function of SHF is not fully understood, evidence has shown that overexpression of *SHF* significantly decreases the rate of growth factor-induced apoptosis in neuroblastoma cells [27]. Subsequently, Ohira et al. showed that *SHF* mRNA was highly expressed in nonmetastatic neuroblastoma compared to metastatic tumour samples [29]. Another recent study provided evidence that loss of SHF increased cellular mobility and the invasive capability of neuroblastoma cells [30].

Initial iCLIP data from this study revealed enriched SAFB1 binding sites at the alternative promoter of *SHF*. As the aberrant expression of alternative promoters is linked to cancer, SAFB1 binding surrounding this region gathered an interest for further examination. Interestingly, the knockdown of SAFB1 in MCF-7 cells did not significantly alter *SHF* expression while the knockdown of SAFB2 or both SAFB proteins significantly increased *SHF* expression. This suggests that direct SAFB1 binding to the alternative promoter did not affect the expression of this gene. MDA-MB-231 cells were included in this part of the study for comparison and, in this cell type, increased *SHF* expression was observed in the loss of SAFB1 or SAFB2 and both SAFB proteins.

Multiple alternatively spliced transcript variants encoding distinct isoforms have been found for *ITGB4*, although the full function of most variants remains to be defined [31–33]. Alternative splicing mechanism has been indicated to subtly regulate the ligand binding and signalling activity of many integrin subunits (reviewed in [34]). Although the mechanism and significance of alternative splicing in *ITGB4* have not been elucidated, the discovery of SAFB1 binding sites in its exonic regions may provide a new perspective to further understand the mRNA processing of *ITGB4*.

We also identified another potential novel target for SAFB2—*MALAT-1.* Previous work shows that *MALAT-1* colocalises with SRSF2 in nuclear speckles [35]. Furthermore, other splicing factors that localise in nuclear speckles such as SRSF1, SRSF3, and SRSF4 also bind to *MALAT-1* [21, 36]. We, and others [1, 5], observe that SAFB1 distribution has a similar punctate pattern to SRSF2 (Figure 4(d)); it is therefore conceivable that SAFB1 may possess other typical characteristics of a splicing factor which supports its observed function in pre-mRNA splicing [1, 5, 7].

Interestingly, single depletion of either SAFB1 or SAFB2 led to an increase in expression of the other; this pattern was mirrored at both mRNA and protein levels. Our study also suggests that SAFBI and SAFB2 may themselves have different and overlapping RNA targets. This observation supports previous speculations regarding the distinct molecular roles between SAFB1 and SAFB2 [5, 37, 38]. We conclude that SAFB proteins may share multiple similarities in RNA-binding pattern and characteristics with SR proteins. Analysis of SAFB1 crosslink regions and RNA targets confirms previous reports regarding its interaction with other RNA processing machinery and function. Further work will now be undertaken to define whether SAFB1 and SAFB2 function synergistically or compensatory as RNA-binding proteins in breast cancer cells.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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