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## INVITED REVIEW

Prostate Cancer

# The role of mRNA splicing in prostate cancer

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Alternative splicing (AS) is a crucial step in gene expression. It is subject to intricate regulation, and its deregulation in cancer can lead to a wide array of neoplastic phenotypes. A large body of evidence implicates splice isoforms in most if not all hallmarks of cancer, including growth, apoptosis, invasion and metastasis, angiogenesis, and metabolism. AS has important clinical implications since it can be manipulated therapeutically to treat cancer and represents a mechanism of resistance to therapy. In prostate cancer (PCa) AS also plays a prominent role and this review will summarize the current knowledge of alternatively spliced genes with important functional consequences. We will highlight accumulating evidence on AS of the components of the two critical pathways in PCa: androgen receptor (AR) and phosphoinositide 3-kinase (PI3K). These observations together with data on dysregulation of splice factors in PCa suggest that AR and PI3K pathways may be interconnected with previously unappreciated splicing regulatory networks. In addition, we will discuss several lines of evidence implicating splicing regulation in the development of the castration resistance.

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## INTRODUCTION

Expression of a gene is a multistep process and is subject to a precise control by transcription and translation machinery as well as by surveillance mechanisms, such as nonsense mediated decay (NMD). A critical tightly regulated step in gene expression is the pre-messenger ribonucleic acid (mRNA) splicing. In cancer its dysregulation impacts on all hallmarks of cancer including proliferation, apoptosis, angiogenesis, invasion, metastasis, and metabolism.<sup>1</sup>

Splicing produces mature mRNA molecules by excision of introns from pre-mRNA and ligation of exons.<sup>2</sup> Most human genes contain multiple exons and >95% of genes undergo alternative splicing (AS) generating multiple mRNA isoforms with different subsets of exons.<sup>3,4</sup> The functional consequences of AS include amplification of protein diversity; transcription regulation through the introduction of premature stop codons and degradation of mRNA through NMD; mRNA translation efficiency, localization and stability through variability in untranslated regions (UTRs).<sup>5–7</sup> Splicing is tightly regulated in a tissue, developmental stage and condition dependent manner by *cis*-regulatory sequences within pre-mRNAs and in *trans* by a large ribonucleoprotein complex, the spliceosome. This complex contains some 200 regulatory RNAs and proteins and its components recognize *cis*-elements within mRNA, such as splice sites, branch site, exonic and intronic splicing enhancers and silencers.<sup>8,9</sup> The combination of *cis*-elements defines the “splicing code” that, together with the splicing machinery, directs AS.<sup>10</sup> In addition, the splicing machinery is coupled with transcription and epigenetic mechanisms making gene expression extremely complex.<sup>11</sup> This intricate regulatory process provides ample opportunity for disruption,

and cancer cells exploit this through perturbations of both *cis*- and *trans*-regulators. *Cis*-elements are altered through somatic single nucleotide mutations as was observed for a number of genes.<sup>12,13</sup> Although the frequency of mutations in splice sites appears to be quite low accounting for only 1%–5% of total mutations detected in numerous cancers.<sup>14–16</sup> In contrast, mutations in *trans*-regulators are found at high frequency in several neoplasms.<sup>17–19</sup> Further, aberrant transcriptional and/or post-transcriptional regulation of *trans*-factors, such as splice factors, has been widely reported in cancer (reviewed by David and Manley<sup>1</sup>). The result of disrupted splicing regulation in cancer is the aberrant function of important cancer genes/pathways and cellular processes they participate in, such as apoptosis, growth, invasion, and angiogenesis. There are many examples of individual genes expressing cancer specific splice isoforms with functions favorable for tumorigenesis (e.g. anti-apoptotic or pro-invasion and pro-angiogenesis). In addition, there are several examples of aberrant function of splice factors that can behave as oncogenes (for excellent reviews see David and Manley<sup>1</sup> and Pal *et al.*<sup>20</sup>). Further, splicing regulation plays an important role in developmental pathways,<sup>20,21</sup> stem cell fate decisions,<sup>22,23</sup> as well as epithelial-to-mesenchymal transition (EMT),<sup>24</sup> all of which play prominent roles in tumorigenesis.<sup>25,26</sup> It has been observed that cancer cells tend to shift the splice isoform repertoire toward those normally expressed in early developmental stages.<sup>20</sup> EMT has been associated with global changes in splicing programs involving genes important for cellular morphology and movement.<sup>27</sup> This review will summarize the current state of knowledge of aberrant splicing regulation of genes and pathways in prostate cancer (PCa) and will discuss emerging evidence for its role in the development of castration resistance.

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## PCa CHALLENGES

PCa is unique among solid tumors in that it has a relatively long natural history, is extremely heterogeneous both between tumors and among multiple tumor foci in a single prostate, and is devoid of histological subtypes that correlate with clinical course, with the exception of a rare small cell carcinoma or neuroendocrine PCa. The main histological subtype is adenocarcinoma. Advanced adenocarcinoma initially responds well to androgen deprivation therapy, however, most patients ultimately develop castrate-resistance prostate cancer (CRPC), an incurable lethal disease which claims ~30,000 lives yearly in the North America alone.<sup>28</sup> Therefore, in the setting of the high-risk PCa challenges are to develop improved biomarkers and therapies to better manage CRPC, distinguish indolent cancers from aggressive ones, and to delineate extensive clinical and molecular heterogeneity of tumors. Microarrays and more recently next generation sequencing (NGS) technology have been instrumental in defining molecular subclasses of prostate tumors. Subclasses have been defined based on the presence of genomic alterations, such as copy number gains and losses, fusions and somatic mutations. Genome copy number has demonstrated prognostic value as tumors with highly altered genomes were associated with extremely unfavorable prognosis.<sup>29,30</sup> Gene fusions between E26 transformation-specific (ETS) family transcription factors and androgen regulated genes (e.g., TMPRSS2-ERG) are found in ~50% of prostate tumors.<sup>31</sup> Among ETS fusion-negative tumors, mutations in the speckle-type POZ protein (SPOP) are found in 6%–13%,<sup>32</sup> mutations in the chromodomain helicase deoxyribonucleic acid binding protein 1 (CHD1) in 5%–10%,<sup>33</sup> and overexpression of the serine peptidase inhibitor, Kazal type 1 (SPINK1) in ~10%.<sup>34</sup> Relatively rare fusions involving RAF kinases are found in ~1% of ETS-negative prostate tumors and are hypothesized to constitute yet another molecular subclass.<sup>35</sup> These alterations provide valuable insights into the molecular basis of PCa heterogeneity, and in some cases may provide diagnostic and prognostic benefit.<sup>36</sup> Unlike genome profiling, transcriptome profiling has not identified molecular subclasses of strong biological or clinical relevance, which may be explained, in part, by the heterogeneity of PCa. For example, the specimens may contain a mixture of prostatic epithelial cells, stromal cells and infiltrating immune cells. Gene expression profiles of prostate tumors have been extensively studied over the last decade emphasizing the importance of this confounding factor.<sup>37–40</sup> In contrast, splice isoform profiling of prostate tumors lags behind many other cancer types for which the subtype specific splicing signatures have been identified<sup>41,42</sup> and splice isoform based biomarkers and therapies have been developed.<sup>43</sup> Nonetheless, as we will discuss below, recent reports bring to light the importance of splice isoform profiling for PCa. Emerging evidence supports the involvement of splicing regulation in oncogenic processes including tumor progression and the development of castration resistance.

## SPLICE ISOFORM PROFILING FOR PCa CLASSIFICATION

Recent microarray and NGS technology-based surveys of global splicing profiles of PCa have demonstrated the potential value for improving classification and development of cancer specific splice isoform-based biomarkers. Using splice-junction microarray platform and DASL assay, Zhang *et al.*<sup>44</sup> profiled ~1500 splice isoforms from over 300 genes in a panel of 22 formalin-fixed paraffin-embedded prostate tumors and matched normal tissues. Over 200 genes showed significant changes in their splice isoforms expression compared to normal tissues and a third of them exhibited no change in the gene expression. Further, the authors developed a 100 isoform signature based on the composite splicing and gene expression information

that yielded 5% improvement in the tumor-normal classification accuracy compared to gene expression alone. Although, this is a modest improvement, it appears that compared to gene expression profiling, splice isoforms provide additional benefit for classification problems.

Erho *et al.*<sup>45</sup> have reported the analysis of exon level profiling of a large cohort of prostate tumors from the Memorial Sloan-Kettering Cancer Center Prostate Oncogenome Project.<sup>29</sup> The cohort consisted of 131 fresh frozen primary tumors, 29 adjacent normal tissues and 19 metastases that were profiled with Affymetrix Human Exon Array. The authors identified 680 genes with splice isoforms differentially expressed between any pair of primary, benign or metastatic specimens. Several known alternatively spliced genes were detected in this study, including androgen receptor (AR) and fibroblast growth factor receptor-2 (FGFR2), but more than a half of the identified genes had no previous association with PCa. In addition, a set of 28 transcripts demonstrated a continuum of expression change through disease progression from normal through primary, to metastatic disease. Unfortunately, this study did not include experimental validation of AS events and therefore it was not possible to estimate the false positive rate. However, the fact that only a small fraction of differentially expressed isoforms (18 out 92) could be identified also at the gene expression level emphasizes the value of isoform profiling. Finally, the authors evaluated splice isoforms for PCa risk stratification in comparison with gene-based classifiers. Relative to nomograms alone, gene-based classifiers added little benefit for predicting post-operative recurrence consistent with previous reports.<sup>46</sup> In contrast, splice isoform classifiers provided additional power suggesting that biomarker signatures based on splice isoforms may offer unique prognostic information.

Other whole transcriptome screens of tumor-normal pairs with expression microarrays or NGS technology have resulted in identification of additional tumor specific splice isoforms that occur at high frequency. Thorsen *et al.*<sup>47</sup> reported five genes expressing prostate tumor specific splice variants that were predicted to encode for protein isoforms with altered function. These included actinin 1 (ACTN1), caldesmon 1 (CALD1), vinculin (VCL), collagen, type VI, 3 (COL6A3), leucine rich repeat flightless interacting protein 2 (LRRFIP2), phosphatidylinositol 4-kinase, catalytic,  $\beta$ -polypeptide (PIK4CB), and tropomyosin 1 (TPM1). Ren *et al.*<sup>48</sup> identified recurrent AS in prostate specific antigen gene kallikrein (a.k.a. Prostate-specific antigen) in ~60% of Chinese prostate tumors as well as recurrent exon skipping event within lipid metabolism gene AMARC in ~30% of Chinese tumors. The high frequency of these AS events in PCa suggests a functional role. Clearly, the potential functional significance and therapeutic value warrants further investigation. Tumor specific splice isoforms, such as those of ACTN1, CALD1, and VCL genes that are found in several cancers including prostate,<sup>47</sup> may form the basis for pan cancer biomarkers and therapeutic targets.

## THE FUNCTIONAL ROLE OF AS IN PCa

Modulation of protein function by AS has been documented for several genes in PCa with the sentinel example being the AR (**Figure 1**). The AR is a steroid hormone nuclear receptor that mediates transcriptional programs in response to binding its ligand dihydrotestosterone. Blocking the AR signaling axis is the primary therapeutic intervention for advanced PCa.<sup>49</sup> The C-terminal domain of the AR protein contains the ligand-binding domain (LBD) that triggers activation of AR function upon ligand binding. Resistance to AR blockade can be associated with AS that results in multiple truncated variants that lack the LBD and do not require a ligand for an activation of transcriptional programs.<sup>50</sup>

Many truncated isoforms have been identified in model systems and clinical specimens, but the AR-V7 and ARv567es isoforms are the only AR variants (ARVs) demonstrated to be clinically relevant. These isoforms are prevalent in CRPC and associated with biochemical recurrence and short survival.<sup>51-54</sup> Although ARVs function as transcription factors independent of androgens, there is conflicting evidence with regard to their transcriptional output. Some reports suggest that ARVs mediate transcriptional programs distinct from the full length (FL) AR (AR-FL),<sup>51,55</sup> whereas others claim that ARVs re-activate the transcriptional program of the AR-FL.<sup>56</sup> Importantly, the expression of ARVs is increased upon androgen withdrawal and treatment with new generation antiandrogen therapies<sup>55,57,58</sup> and can mediate resistance to enzalutamide in cell culture.<sup>56</sup> However, whether ARVs are mechanistically linked to the emergence of CRPC or represent a transient stress response to treatment remains unknown. Nonetheless, because the AR signaling axis is active in most CRPC and since current drugs cannot bind ARVs efforts to block both FL and truncated ARVs are underway.<sup>59</sup>

Given the importance of ARVs, the mechanisms underlying their production have been under intense investigation. Models of CRPC, including cell lines and xenografts often express truncated variants and in some of them ARVs have been linked to genomic rearrangements of the AR gene locus.<sup>60</sup> LuCaP xenograft models express the variant ARv567es that lacks exons 5, 6 and 7. These models contain either deletion or inversion of the region spanning exons 5, 6 and 7 that provide a rational explanation for the ARv567es.<sup>61</sup> In the CRPC cell line 22Rv1 that expresses high levels of the AR-V7 the AR gene locus contains a tandem duplication of the region encompassing alternative exons.<sup>62</sup> In another CRPC model CWR-R1, that also expresses high levels of AR-V7, the region of the first intron was found to be deleted. Importantly, a fraction of CRPC tissues from patients also contained focal amplifications of the alternative exons region, suggesting the presence of AR intragenic rearrangements.<sup>60</sup> Interestingly, individual models and CRPC tissues harbored different rearrangements and did not share the same breakpoints. Moreover, the splice sites were found to be intact in CWR-R1 and 22Rv1 models. This suggests that although genomic rearrangements predispose to the production of truncated variants, ARVs are likely the result of aberrant splicing. The regulatory sequences within intronic regions, as well as the spatial location of constitutive downstream exons in pre-mRNA may influence the splicing machinery. Indeed, a recent report on the mechanisms of AR pre-mRNA splicing demonstrated that the production of AR-V7 was coupled with the transcription rate of the AR gene and depended upon essential components of the spliceosome, U2AF65 and SRSF1 splice factors.<sup>63</sup> These factors recognize specific enhancer sequences around splice sites of the AR-V7 alternative exon. Under androgen depletion condition as the AR transcription rate increases, the recruitment of these splice factors to enhancer elements is also dramatically increased. It results in the enhanced splicing of AR-V7 without changes in protein levels of these splice factors. Therefore, it is possible that the focal amplifications observed in CRPC models lead to the relative increase in abundance of pre-mRNA species with alternative exons harboring enhancers for U2AF65 and SRSF1. This can enhance spliceosome assembly at these *cis*-elements resulting in increased expression of AR-V7.

**Figure 1** summarizes several other notable AS events in PCa. The BCL-X gene (a.k.a. BCL2L1) regulates apoptosis and encodes for two isoforms with opposing functions. The pro-apoptotic short isoform, BCL-XS, is down-regulated in many cancers including prostate, compared with the anti-apoptotic long isoform, BCL-XL.<sup>1</sup> Modulation of splicing with antisense synthetic oligonucleotides

to favor production of the BCL-XS isoform sensitizes PCa cells to radiation and chemotherapeutic agents.<sup>64</sup> Thus, BCL-X represents a good example of therapeutic targeting of AS in PCa.

The pre-mRNA of the FGFR2 undergoes AS during EMT.<sup>65</sup> Isoforms IIIb and IIIc are cell type specific with IIIb being expressed in epithelial cells and IIIc in mesenchymal cells. During EMT these isoforms are completely switched. In PCa, this switch is associated with castration resistance, with isoform IIIb being expressed in castrate sensitive cells and isoform IIIc in castrate resistant cells.<sup>66</sup> The functional consequence of this is a change in ligand binding specificity. Isoform IIIb has high affinity to FGF7 that is predominantly expressed by stromal cells, and isoform IIIc has high affinity to a distinct ligand FGF8b.<sup>66</sup> In addition, the expression of FGF8b has been associated with Gleason grade and clinical stage of PCa.<sup>67</sup> Targeted inhibition of FGF signaling has been proposed as a potential therapeutic approach for PCa and knowledge of splicing regulation of this pathway will be critical.

Cyclin D1 (CCND1), the key regulator of cell cycle expresses two isoforms: normal D1a and truncated D1b. D1b isoform is up-regulated in PCa and unlike its normal counterpart possesses oncogenic properties.<sup>68</sup> Furthermore, D1b isoform cooperates with AR to promote transcription of genes involved in metastasis.<sup>69</sup> In this study, the authors have shown that CCND1b isoform's main effector is a transcription factor SLUG, suggesting the cooperation of oncogenic D1b/SLUG and AR pathways to promote metastatic progression of AR-positive, castration-resistant cancers.

Kruppel-like factor 6 (KLF6) is a tumor suppressor gene that regulates cell proliferation through transcriptional regulation of the cell cycle inhibitor p21.<sup>70</sup> A truncated splice variant-1 (SV1) is up-regulated in PCa and is associated with poor prognosis.<sup>71</sup> A common germline polymorphism underlying expression of the SV1 was associated with increased PCa risk in a large multi-institutional study of 3411 men.<sup>72</sup> The SV1 isoform is functionally active, antagonizing the tumor suppressor function of the FL KLF6 and promoting tumor growth and dissemination through repression of p21 in PCa models.<sup>72</sup> The targeted inhibition of SV1 isoform reduces tumor growth, invasion and angiogenesis.<sup>73</sup>

Vascular endothelial growth factor (VEGF) is a key signaling protein expressed by tumor cells in response to hypoxia to induce angiogenesis. VEGF transcripts are extensively alternatively spliced and the two isoforms, which differ in six terminal amino acids, have antagonistic properties.<sup>74,75</sup> The VEGF165 isoform promotes angiogenesis mainly through VEGFR2 receptor signaling and is the predominant isoform expressed in cancer. The VEGF165b isoform inhibits angiogenesis because it cannot signal through VEGFR2 and is down-regulated in several cancers including prostate.<sup>74</sup> *In vivo* administration of the anti-angiogenic 165b isoform causes shrinkage of tumors formed by prostate-cancer cells xenotransplanted into nude mice.<sup>76</sup> Noteworthy, VEGF inhibition by the bevacizumab, a pan-isoform binding antibody, has been evaluated in PCa clinical trials. It showed no benefit as a single agent, but showed promising results in a combination therapy.<sup>77</sup> It is tempting to speculate that instead of the pan-isoform inhibition of VEGF, a therapeutic strategy to restore the ratio of pro-and anti-angiogenic isoforms in cancer cells might be more productive. Collectively, these data suggest that splice isoforms may be important for understanding the response to targeted drugs and illustrate the potential benefits of more precise splice sensitive therapeutics.

## THE REGULATION OF AS IN PCa

The number of individual splice isoforms with relevance to PCa is growing, however, relatively little is known about the regulatory

mechanisms responsible for their generation. Genomic aberrations or mutations in *cis*-elements have been linked to AS in only a few genes, including AR and KLF6. This is in line with recent studies focused on the mutational spectra of prostate tumors indicating that mutations in splice sites constitute the minority of all somatic mutations (as low as ~0.6%).<sup>78</sup> Therefore, it is conceivable that *trans* regulatory factors may play a larger role. Indeed in other cancers, the aberrant expression and/or function of splice factors has been well-documented and, importantly, can be exploited therapeutically.<sup>79,80</sup> In PCa, two splice factors have been implicated: Sam68 and SRSF1. Sam68 is frequently up-regulated in PCa, is involved in regulation of CCND1 splicing<sup>81</sup> and promotes growth and survival of prostate tumor cells.<sup>82</sup> The recently identified cofactor of Sam68, SND1, was also found to be up-regulated in PCa cells and was shown to coordinate splicing of CD44 to promote cell motility.<sup>83</sup> SRSF1 is up-regulated in many cancers and has been shown to act as a proto-oncogene.<sup>84</sup> In non-small lung cancer, SRSF1 was shown to act as oncogene in mTOR pathway dependent manner.<sup>85</sup> In PCa, SRSF1 expression correlates with the CCND1b isoform expression.<sup>86</sup> Taken together, it appears that SRSF1 is an interesting target for investigation. In addition, since this splice factor can regulate splicing of many genes, understanding of its wider role in PCa will be of interest.

It is well-established that the AR interacts with transcriptional cofactors, such as a cofactor of BRCA1 (COBRA1) and DDX5, as well as with the splice factor polypyrimidine tract binding (PTB)-associated splicing factor (PSF) (reviewed in detailed by Sette<sup>87</sup>). Through these interactions AR can modulate transcription and splicing of nascent transcripts, such as those of CD44 gene. These findings support the concept that the gene transcription and splicing are coupled.<sup>11</sup> Thus, deeper investigation of how AR signaling may modulate AS is clearly warranted.

## CRPC AND THE REGULATION OF AS

### *AS and the AR and phosphoinositide 3-kinase (PI3K) pathways*

Development of castration resistance is a central challenge in PCa management and significant efforts are directed toward understanding the molecular events driving it. Recent progress in this field points to the role of two critical pathways, the AR and PI3K/Akt/mTOR. AR signaling remains active in CRPC despite targeted inhibition of androgen synthesis and blockade of the AR.<sup>88</sup> The PI3K/Akt/mTOR pathway plays a key role in tumorigenesis and resistance to therapy in PCa and is being evaluated as a target for CRPC treatment.<sup>89</sup> Additionally, there is a cross-talk between the PI3K pathway and the AR signaling axis.<sup>90</sup> AS is involved in regulation of a number of key components of these two signaling cascades in PCa (**Figure 2**; for details see above). The splice isoform IIIc of the FGFR2, the receptor tyrosine kinase upstream of PI3K, is associated with castration resistance and plays a role in EMT and invasion. Truncated isoforms of the AR are linked to castration resistance. An alternative isoform of the tumor suppressor gene TSC2 lacks a functional domain required for mTOR inhibition.<sup>91</sup> In addition, the AR can effect splicing of nascent transcripts through interaction with several cofactors, such as COBRA1 and DDX5. Further the Sam68 and SRSF1 splice factors are implicated in PCa (see above) and in other cancers they are connected to PI3K signaling. Sam68 can be activated through phosphorylation by ERK.<sup>92</sup> SRSF1 activates mTORC1 in lung cancer<sup>85</sup> and its function can be regulated through phosphorylation by AKT.<sup>93</sup> Altogether, based on data from prostate and other cancers, it is tempting to speculate that the AR and PI3K pathways may be densely populated with splicing regulatory networks. Importantly, since these pathways are currently

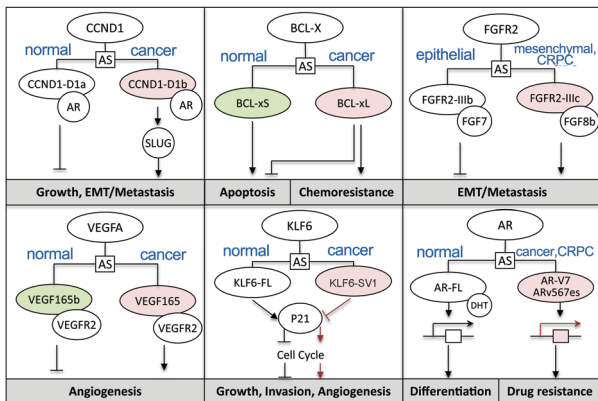
the main targets for treatment of advanced PCa, understanding of the potential splicing networks will be critical.

Understanding the interplay between splicing regulation and various signaling pathways remains a work in progress. Recent studies provide support for additional splicing regulatory connections to the AR and PI3K pathways in human cells, and it will be important to explore their possible relationship to the CRPC. Alternative splice isoforms of the A-RAF kinase have different abilities to transduce signal through ERK and therefore they have different roles in apoptosis and cell transformation.<sup>94</sup> The switch in splicing is regulated by the splice factor hnRNPH (HNRNPH 1) that is a direct transcriptional target of c-Myc. The expression level of c-Myc and hnRNPH has a direct effect on A-RAF splicing (**Figure 2**). c-Myc is an important oncogene in PCa where it is frequently amplified and up-regulated, and its protein level is associated with poor prognosis.<sup>95</sup> It can also induce androgen-independent growth of PCa cells *in vitro*.<sup>96</sup> c-Myc mediates transcription of many genes, including several splice factors, such as PTB, hnRNPA1 and hnRNPA2.<sup>1</sup> It will be interesting to determine whether c-Myc up-regulation can affect splicing programs in CRPC through transcriptional regulation of these or other splice factors.

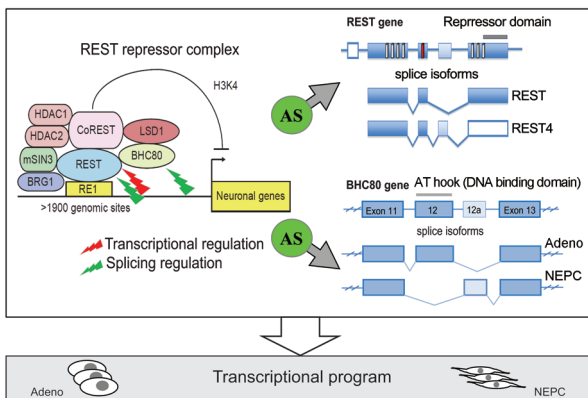
Another interesting connection between splicing regulatory signals and the PI3K/AKT/mTOR pathway was revealed in the study of Zhou *et al.*<sup>97</sup> where authors investigated mechanisms of splicing regulation by the SR family of splice factors (serine/arginine-rich protein family). The function of these proteins is regulated post-transcriptionally by SR protein kinases (SRPKs), which in turn are regulated through phosphorylation by AKT. Zhou *et al.*<sup>97</sup> have shown that a cascade of AKT-SRPKs-SRs constitutes the major signaling branch in the nucleus driving splicing changes in human cells in response to epidermal growth factor signaling. Interestingly, this branch was mediated by HSP70 and HSP90 chaperones, both of which are implicated in PCa.<sup>98,99</sup> Taken together, although these additional connections are observed in other cell types and may be tissue specific, their plausible role in PCa and CRPC deserves attention.

### *AS and the neuroendocrine transdifferentiation*

A significant number of CRPCs lose addiction to AR signaling and undergo transdifferentiation from adenocarcinoma (Adeno) to a lethal small cell carcinoma phenotype or neuroendocrine prostate cancer (NEPC).<sup>101</sup> NEPC is characterized by a distinct transcriptional signature of neuronal genes.<sup>40,102</sup> We have shown that NEPC is also characterized by a distinct splicing signature composed of a number of genes involved in neuronal biology.<sup>40</sup> The transcription of many neuronal genes is governed by the RE1-silencing transcription factor (REST) (a.k.a., neural-restrictive silencer factor), which represses these genes in non-neuronal cells.<sup>103</sup> We have shown that in NEPC tumors, REST is down-regulated and its cofactor, BHC80, is alternatively spliced. The likely consequence of this is a relief of the repression by the complex which enables expression of the neuronal gene signature (**Figure 3**). The transcriptional attenuation of REST was also found in ~50% of tumors with NEPC signature from a large independent cohort, suggesting that this may represent an important recurrent mechanism of transcriptional control in NEPC. We and others have demonstrated that siRNA knock down of REST in LNCaP cells results in induction of neuroendocrine markers, supporting its role in transdifferentiation.<sup>40,104</sup> Recently, REST was found to mediate AR-dependent gene repression and its expression level is attenuated after exposure of cells to the new generation anti-androgen enzalutamide.<sup>104</sup> Therefore, Svensson *et al.*<sup>104</sup> proposed a conceptual framework whereby treatment with new anti-androgens can promote de-repression by REST and induction of neuronal gene expression. Interestingly, in several cancer types REST



**Figure 1:** Functional alternative splicing in prostate cancer. Splice isoforms differentially regulated in prostate cancer are indicated with light red (up-regulated) and light green (down-regulated).

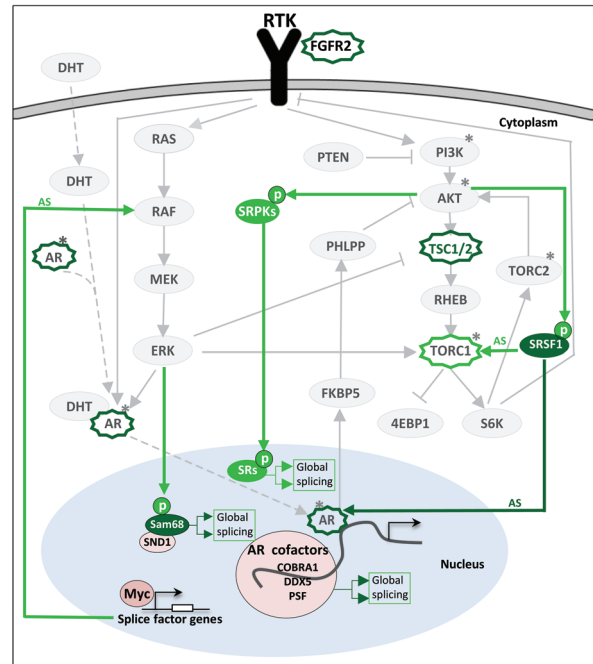


**Figure 3:** REST-mediated transcriptome reprogramming in neuroendocrine transdifferentiation. The REST transcriptional complex represses transcription of neuronal genes through binding to the regulatory RE1 sites located within promoter or enhancer regions of genes. This complex undergoes transcriptional and post-transcriptional changes during neuroendocrine transdifferentiation. REST transcripts are transcriptionally attenuated and alternatively spliced and the REST cofactor BHC80 is alternatively spliced. The resulting REST and BHC80 isoforms lack important functional domains necessary for the repression (REST4 isoform and the NEPC-specific BHC80 isoform). Loss of REST-mediated repression leads to the emergence of the neuronal transcriptional program, including NEPC-specific splicing.

transcripts undergo frequent and complex AS,<sup>105</sup> and in lung cancer this is regulated by the neuronal splice factor SRRM4.<sup>106</sup> In neuronal cells, REST and SRRM4 form a regulatory feedback loop<sup>107</sup> and it will be interesting to explore the interplay between these proteins in PCa. Since NEPC is extremely aggressive with survival of <1 year,<sup>101</sup> it will be important to explore the functional role of AS in NEPC, the mechanisms of its regulation and the role of REST transcriptional complex in NEPC biology (**Figure 3**).

**CONCLUSIONS**

A comprehensive understanding of the PCa transcriptome complexity is made challenging by many factors, including heterogeneity. AS represents one relatively underexplored aspect of this complexity. A current concept in mRNA biology is that splicing and transcriptional mechanisms are coupled, and in this review we have highlighted recent research supporting this concept for AR signaling. It appears that two critical pathways in PCa, AR and PI3K/AKT/mTOR, are subject to splicing control, which should have important therapeutic implications.



**Figure 2:** Splicing regulation of the androgen receptor (AR) and phosphoinositide 3-kinase (PI3K) signaling pathways. Members of the pathway whose transcripts are alternatively spliced in cancer are indicated with green decagons. Splice factors Sam68, SRSF1, and SRs as well as their regulators (SRPKs) that are altered in cancer transcriptionally or post-transcriptionally are marked with green ovals. Dark green indicates that altered function of a splice isoform or a splice factor has been observed in prostate cancer whereas light green—in other cancers. SRs: splice factors of the serine/arginine-rich protein family; SRPKs: SR protein kinases; p: phosphorylation; AS: alternative splicing. The signaling pathways of AR and PI3K have been adapted from Barlow and Shen.<sup>100</sup> \*Pathway components for which new blocking agents are under investigation for advanced prostate cancer.

Additional evidence, from other cell types, suggests that these pathways may be interconnected with splicing regulatory networks. Given the therapeutic importance of these pathways, delineating the splicing networks will be critical. Further, in line with the role of splicing in resistance to therapy,<sup>108</sup> AS contributes to CRPC as exemplified by the AR truncated variants and by the global splicing shift during neuroendocrine transdifferentiation. Also, it is highly probable that the number of splicing events with functional consequences in PCa will greatly expand. Thus, it is becoming increasingly clear that splicing regulation plays an important role in many processes in PCa and can provide a rich source for diagnostically and therapeutically exploitable novel targets.

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