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Small Molecule Regulation of Alternative Splicing: Characterization of the Cardiotonic Steroid Digitoxin and Identification of BIN1 exon 12a Modulators

A dissertation submitted in partial satisfaction of the requirements for the degree of Doctor of Philosophy in Molecular Biology

by

Erik Steven Anderson

2012

ABSTRACT OF THE DISSERTATION

Small Molecule Regulation of Alternative Splicing:
Characterization of the Cardiotonic Steroid Digitoxin and
Identification of BIN1 exon 12a Modulators

by

Erik Steven Anderson
Doctor of Philosophy in Molecular Biology
University of California, Los Angeles, 2012
Professor Douglas L. Black, Chair

Alternative splicing aberrations can cause disease or promote its progression, including the development of cancer. Reversal of pathologic splicing events is a potential therapy for those cases. Efficient methods for identification and characterization of small molecule modulators of specific splicing events are required to develop therapeutics. The cardiotonic steroids, long prescribed for congestive heart failure, alter splicing of many exons. Two methods are described to define their mechanism of action. First, an unbiased analysis of transcriptome-wide changes in alternative splicing predicted loss of function of two specific splicing factors, SRp20 and Tra2-beta. Both proteins are depleted by the cardiotonic steroid digitoxin, and their restoration blocks digitoxin induced splicing changes. This general method can be applied to any identified drugs in future screens. Second, a candidate approach was taken to identify which of the known

cardiotonic steroid effectors is required for induced splicing changes. An inhibitor to protein phosphatase 1, tautomycin, antagonizes digitoxin induced changes, through a yet to be determined mechanism.

BIN1 exon 12a inclusion is associated with progression of metastatic melanoma. Using a high throughput approach, cDNA expression and small molecule libraries were screened for regulators of exon 12a splicing. Specific RNA-binding proteins including hnRNP-K, hnRNP-LL, Tra2-beta and PCBP1/4 were identified as repressors while hnRNP-R is an enhancer of 12a splicing. Furthermore, expression of cell signaling effectors such as Map3k3 and -11, as well as CLK2 and FAST can modulate 12a inclusion levels. hnRNP-K, which has conflicting roles in the development of cancer, represses 12a splicing through an exonic RNA element. Small molecules that modulate exon 12a include modulators of prostaglandin activity, the antibiotic puromycin, and the psychoactive MAOA inhibitor harmaline. Interaction between these molecules and the identified genetic regulators of 12a splicing could elucidate the mechanism by which BIN1 splicing changes to promote tumorigenesis.

The dissertation of Erik Steven Anderson is approved.

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CHAPTER ONE: INTRODUCTION TO ALTERNATIVE mRNA SPLICING AND DISEASE

Alternative pre-mRNA Splicing

Alternative pre-mRNA splicing is an essential mechanism of post-transcriptional gene regulation. Through this tightly controlled process, different protein-encoding mRNA isoforms are generated from identical pre-mRNA transcripts. Such versatility greatly increases proteomic diversity from genomes with fewer absolute numbers of genes. Recent investigations into the nature of complete eukaryotic transcriptomes have revealed that >95% of multi-exon transcripts contain alternatively spliced regions that give rise to distinct mature mRNA molecules(1).

The core splicing reaction is the process by which two exons are joined together after excision of the non-coding intronic sequence between them(2). This reaction is remarkably conserved throughout evolution in both mechanistic detail and catalytic ribonucleoprotein components that form the spliceosome. The U2-dependent or 'major' spliceosome removes >99% of introns from eukaryotic transcripts while an analogous U12-dependent or 'minor' spliceosome, acts on a rare set of remaining introns(3). Interestingly, spliceosome components that interact with an RNA sequence to define an intron-exon boundary do so with strongly conserved consensus binding sites. These splice sites or signals define all exons that will be targeted by the spliceosome. Through spliceosomal interaction with these sites, and subsequent assembly, rearrangement, and catalysis of two trans-esterification reactions, an intron is excised while the exons are ligated together(2).

Alternative pre-mRNA splicing is the process by which differential splicing choices are made by the spliceosome to ultimately yield mature mRNAs that encode different protein isoforms. Alternative splicing choices range in type, from the inclusion or skipping of an entire

exon to altering the end of an exon by utilizing a different splice site(2). Additional patterns include use of alternate promoter or poly-A sites, or retention of introns in a mature mRNA isoform. Since all exons are ultimately spliced by one of the two spliceosomes, alternative splicing events require additional levels of regulation that influence the ultimate action of the spliceosome. To this end, there are controlling sequences in alternative exons and conserved intronic sequences surrounding them that enhance or repress splicing(4,5). The RNA motifs that govern splice site choice for an alternative exon are termed *cis*-elements that interact with a host of RNA binding proteins called *trans*-factors. *cis*-elements and *trans*-factors act in concert to regulate splicing in a tightly controlled manner. Collectively, *cis*-elements are described by their location and ultimate function, termed exonic splicing enhancers or silencers (ESEs or ESSs, respectively), and intronic splicing enhancers or silencers (ISEs or ISSs)(6). Through a variety of mechanisms, as well as by many that are yet to be determined, the RNA binding proteins that interact with these elements can promote or antagonize spliceosome function to ultimately direct alternative splicing choices.

Alternative splicing events can show distinct developmental and tissue-specific patterns of regulation(1). Often these patterns correspond to regulated expression of the RNA binding splicing factors or changes in the upstream signaling cascades that govern their function. In a growing number of examples, it has been shown that encoded protein function can change coordinately with transitions in splicing to promote development or tissue specificity. For example, the expression of the polypyrimidine tract binding protein (PTBP1) is high in early nervous system development, but gradually decreases during development of mature post-mitotic neurons(7,8). Of note, alternative splicing patterns change over this time of development as well, some of which are controlled by PTBP1 (or its paralog, PTBP2). Interestingly, PTBP1

expression increases again in cells that show a jump in proliferation rate long after development has occurred, such as in rapidly dividing tumor cells(9,10). The alternative splicing pattern for a specific exon can be viewed as a combinatorial competition that is largely influenced by the RNA binding proteins that are present in the cell, as well as their avidity for a specific RNA site in that transcript. In this vein, transcriptome-wide prediction maps for both binding and regulation by specific splicing factors has been recently pursued for a number of RNA binding proteins(11-13). In agreement with studies on specific transcripts, the large-scale maps have revealed that depending on binding context, splicing factors can direct different outcomes for an alternative event. For example, the SR protein family of splicing factors, which in general prefer binding different combinations of purine-rich RNA elements, can both enhance or repress exon inclusion depending on binding site(15-19). They enhance exon inclusion when bound to exonic elements, and repress splicing when bound to intronic sequences. Similar studies have led to general rules for the effect some splicing factors such as the Rbfox family of proteins, while prediction of binding or regulation by other factors has remained unclear(12,14). Ultimately, methods that can detect strong regulators for a specific splicing event will not only aid in characterization of that exon, but also in further development of prediction algorithms as more examples of robustly regulated or non-regulated targets are defined.

Alternative Splicing and Disease

The misregulation of alternative splicing events can cause or promote disease(6,20). Disruption can occur on the RNA level, in the form of genetic mutation, or at the level of the *trans*-acting factors that bind these sequences. The function of the RNA binding proteins can be disrupted by mutation of the genes that encode them or can be misregulated by upstream

signaling cascades(6). Interruption of alternative splicing regulation at any of these points can promote production of aberrant isoforms that disrupt normal cellular function or are themselves toxic. There is a growing list of alternative splicing events that are linked with disease through either mutation of a genetic locus or through inappropriate regulation of a splicing choice.

One example is the alternative splicing of the microtubule associated protein tau (MAPT) transcript. Specific mutations in the MAPT locus have been causatively to the hereditary disorder frontotemporoal dementia with parkinsonism linked to chromosome 17 (FTDP-17)(21,22). In this genetic disease, some of the identified mutations have been mapped to exon 10 and the introns surrounding it. These mutations enhance the inclusion levels of exon 10, which is normally low in childhood and increases to nearly 50% in adults. Of note, exon 10 encodes the fourth of four microtubule binding domains in the tau protein, which is predicted to change the cellular function of the protein. Patients who suffer from FTDP-17 and other MAPT-associated neurodegenerative diseases display neurofibrillary tangles that are composed of aberrantly aggregated proteins. The tangles seen in FTDP-17 contain tau protein that primarily includes exon 10, indicating that increased splicing of the exon does change the cellular properties of the protein(21). The FTDP-17 causing mutations specifically illustrate how genetic disruption of the splicing code, via change in strength of splicing enhancers or silencers can ultimately impact the final protein product. An early study found that 15% of disease-causing mutations lie in consensus splice sites, but MAPT and other cases illustrate that mutations in or even near alternative exons can promote disease as well(23). Indeed, nearly 80% of mutations in the Human Gene Mutation Database lie in exons and have largely been hypothesized to be pathogenic due to an effect on coding sequence(6). It is likely that many of these mutations, as well as those that lie in introns, can be toxic through alteration of splicing choices as well.

Genetic mutation can also occur in the genes that encode specific splicing factors, such as in the case of retinitis pigmentosa, which can result from mutations to multiple members of the spliceosome complex. In these cases, as well as potential mutations in other alternative splicing factor proteins, widespread alterations in splicing would be predicted and cell types that are most sensitive would display the ultimate phenotype(6).

MAPT splicing further illustrates disease-associated alternative splicing by its mis-splicing in other disorders. In progressive supranuclear palsy (PSP), corticobasal degeneration (CBD) and argyrophilic grain disease (AGD), neuropathological sections have revealed neurofibrillary tangles that also primarily contain exon 10 positive tau proteins(24-26). Conflicting evidence exists as to whether MAPT is spliced abnormally throughout the brain in Alzheimer's disease, but it does appear to be most strongly misregulated in the temporal lobe of AD patients(27). Consistent with the neurodegenerative phenotype, an increased inclusion of exon 10 occurs at the RNA level, but without the FTDP-17 causative mutations in these disorders. In fact, the MAPT locus remains genetically intact in these disorders that primarily afflict aging patients. Instead, the regulation of this splicing event has changed in disease, either by dysfunction of the RNA binding proteins that govern the choice, or the signaling cascades that control them. These concepts illustrate the other major way that changes in alternative splicing can occur to result in disease. While the exact cause of aberrant exon 10 splicing is not known in each of these disorders, it is likely that if a controlling factor has changed activity, that a host of other alternative splicing events are changed as well. In these cases, the disease phenotype could be a summation of the total of alternative splicing changes.

Whether through genetic mutation or acquired misregulation, changes in alternative splicing clearly cause and promote disease. Efficient targeting and reversal of these processes

could represent a novel strategy to alleviate these specific diseases, many of which have no current therapeutic standard of care.

Therapeutic Potential of Alternative Splicing Targeting

The goal of modulating alternative splicing for therapeutic purposes has been approached from multiple angles. These include modulation by small molecules, anti-sense oligonucleotides and even delivery of trans-splicing therapeutic exons(6,20). The varying specificity of each of these methods makes them amenable to different types of disease based on their therapeutic target. The work presented in this thesis focuses on identification of small molecules that can modulate splicing for multiple reasons, but does not discount the role that specifically targeted RNA therapeutics play in disease targeting.

RNA-based therapeutics such as antisense oligonucleotides, have inherent advantage over all other types of therapeutics due to their innate specificity for a target. While off-target effects of even unique sequences is possible, these are effects that can be minimized by optimal target sequence selection and some degree of trial and error(20). Through various strategies, oligonucleotides are designed to target an alternative exon and ultimately control its inclusion (whether in a positive or negative manner). Importantly, these types of therapies are tailored to diseases and conditions in which a specific alternative splicing event is altered, whether by mutation or misregulation, such as MAPT in FTDP-17, SMN2 locus in SMA or nonsense mutations in Duchenne Muscular Dystrophy. In each of these cases, restoration of a single splicing event could potentially alleviate all or much of a phenotype of disease. Indeed, antisense oligonucelotides that induce skipping of exons containing premature stop codons in DMD are presently in clinical trials, and case reports show muscle regeneration near local sites

of injection(28,29). These exciting results, coupled with commercial optimization of AON half-life and delivery will undoubtedly drive the development of this therapeutic model in the years to come.

Small molecule splicing modulators offer a different set of advantages for splicing associated diseases. With some exceptions, small molecules tend to target proteins; in these examples, they likely target those that play functional roles in modulation of an alternative splicing event. These targets can include splicing factors themselves, or the upstream proteins that regulate their function(30-34). Since the ultimate goal of small molecule therapeutics will be to change the total function of a splicing program, it is an approach appropriately suited to diseases in which aberrant expression or loss of a splicing factor program is the source of a problem. These diseases include cancers which show abnormal expression of splicing factors such as SRSF1, PTBP1 and HNRNPA1 as well as disorders such as myotonic dystrophy in which a specific splicing factor MBNL1 is sequestered by a (CUG)n repeat expansion(9,10,35-37). In each of these cases, the altered function of a splicing factor will dusrupt the entire splicing program that is controlled by that factor. Ultimately, many of the coordinated splicing changes that follow a change in factor function can all contribute to the disease phenotype in additive or even synergistic fashion. Depending on their structure, small molecules can often provide increased plasma stability and delivery to difficult to reach organs, which may also drive their development along with oligonucleotide based therapy. Finally, the two therapies are not mutually exclusive in their effects, and perhaps the most effective way to therapeutically target alternative splicing will be a combination of a specific antisense oligonucleotide and small molecule that can either enhances its half-life or delivery, or potentially target the splicing event through a complementary means.

High Throughput Screening for Modulation of Alternative Splicing

To achieve therapeutic regulation of alternative splicing, it is necessary to have efficient methods that can identify and rank compounds with specific activity toward the desired target. For the case of small molecules, a typical strategy involves the use of high throughput screens. Screens include small molecules with known function, or large combinatorial libraries that are developed based on the specific chemical properties or structures. The goal of screening molecules that can number well into the hundred of thousands is to identify the most potent regulator of a target of interest. Additional steps are necessary to determine mechanism of action as well as specificity to the target.

Targeting alternative splicing with small molecules is currently a topic of much interest in the field of RNA biology(32,34,38). Demand for splicing regulatory drugs stems from at least two discrete directions. The first, as detailed to above, is for identification of compounds that could reverse disease-associated alternative splicing. The second comes from a need for tools with which to probe and analyze the core splicing reaction itself. Unlike some other well-studied molecular processes such as transcription or translation, the RNA splicing toolkit is not filled with chemicals that can modify specific steps in these processes. For each of these reasons, a diverse range of methods to identify drugs that regulate splicing have been developed. They range from low or moderate throughput to very high throughput, based simply on how many drugs can be assayed confidently in a relatively short amount of time.

Warf and colleagues described an example of a low throughput, high confidence method in 2009(38). In this study, twenty-six small molecules were specifically assayed by gel mobility shift assays for their ability to disrupt binding of a splicing factor MBNL1 to $(CUG)_n$ repeats.

Since MBNL1 is sequestered by $(CUG)_n$ repeats in myotonic dystrophy, the therapeutic route would be a small molecule that can release sequestered splicing factors to alleviate their functional loss at other alternative splicing events. This approach confidently identified a small molecule, pentamidine, which can block this interaction and promote MBNL1 dependent splicing events in the nucleus. Such a screen established a proof-of-principle that this therapeutic strategy could be of value to myotonic dystrophy, but the efficiency of throughput is not ideal for identifying the strongest small molecule regulators, due to the time and labor intensive gel shift assays.

To increase speed and efficiency of small molecule assays, strategies have been developed that have easily assayable readouts, but that remain splicing dependent. O'Brien and colleagues described one such method in 2008(32). Here, stable integration of a splicing reporter was established in a cell line. This reporter, when spliced correctly, yields luciferase expression, which can be easily tracked as a proxy for splicing activity. This approach increases the amount of drugs that can be assayed, as the limiting step becomes luciferase detection, a process that has been largely optimized by commercial approaches. Their experiments identified isoginkgetin as a general inhibitor of splicing; this yielded a tool that can be added to the molecular toolkit for probing RNA splicing.

To assay alternative splicing, as opposed to the general splicing reaction itself, a splicing reporter with two readouts is ideal, i.e. one signal to represent exon skipping and another to represent exon inclusion. This has led to the development of multiple dual fluorescence splicing reporters, including one in the Black laboratory(34). These alternative splicing reporters yield one fluorescent reporter protein when an alternative exon is skipped, and a different fluorescent product when the exon is included. In this way, a dynamic range of two fluorescent protein

represents the continuum of splicing levels in a reporter cell line. Changes in the ratio of fluorescent proteins indicate changes in splicing levels in the reporter. We have successfully used this strategy for high throughput screening in cell lines, and other labs including the Hagiwara group have established similar reporters in *in vivo* settings for both *C. elegans* and *M. musculus* models of alternative splicing regulation(39,40).

Naturally there are caveats and potential sources of error whenever proxy readouts for splicing are used in the reporters that are amenable to high throughput screening. The dual fluorescence strategies described previously and herein are sensitive to treatments that alter transcriptional and especially translation stability or activity(34). For these reasons, effective counter-screening methods have been developed to relieve potential sources of false positives, and to streamline the identification of the most active compounds. Once the strongest compounds have been identified, the next steps to drug characterization are to determine the specificity to the targeted exon, as well as the mechanism by which the drug achieves splicing regulation. These points are the focus of this dissertation work.

CHAPTER 2: THE CARDIOTONIC STEROID DIGITOXIN REGULATES ALTERNATIVE SPLICING THROUGH DEPLETION OF THE SPLICING FACTORS SRSF3 AND TRA2B

Introduction: Characterizing mechanisms by which small molecule regulate splicing

The discovery that small molecule compounds can regulate alternative splicing has application to the investigation of RNA biology as well as therapeutic potential for those diseases that are characterized by alternative splicing changes. Therefore, it is of critical importance to uncover the mechanisms by which such drugs modify the splicing reaction. In the realm of basic investigation, such characterization will allow identification and elucidation of specific molecular processes that underlie splicing regulation. On the other end of the spectrum, those drugs that are destined for therapeutic testing will be greatly aided in development by knowledge of their mechanism of action as well as any potential off-target effects. Especially important to potential therapeutic small molecules will be the ability to manifest the specificity of the drug to its target. Splicing is a critical mechanism of gene regulation and cellular process that is essential to life in metazoan cells, which necessitates the careful analysis of both on and off-target mechanisms of action.

Our lab previously described the identification of the cardiotonic steroid class of small molecules as regulators of alternative splicing(34). In this report, regulation of the MAPT transcript was the primary readout, but small-scale microarrays suggested that a larger set of alternative exons respond to digoxin, the prototypical member of this drug family. We sought to use the cardiotonic steroids as a test case for characterization of the mechanism by which a drug can regulate alternative splicing(41).

This project entailed two approaches with which we planned to characterize the role of digitoxin, a cardiotonic steroid, in regulation of alternative splicing. The first approach, which is described in the publication in the following section, was an unbiased one. Here, 'unbiased' means that the characterization of digitoxin targets was done without *a priori* knowledge of the drug's cellular and physiological effects. We feel that an approach such as this is applicable to the situation in which large scale drug screens identify candidate compounds from randomly generated drug libraries that contain few known or well-characterized drugs. Lead compounds that are brought forward toward research or clinical use can be investigated using these unbiased methods to begin the path toward mechanism discovery.

The second approach lies on previous work that has been performed on cardiotonic steroids. Since these and other described hits have come from libraries of drugs with known functions, we sought to use these known mechanisms of action to determine how alternative splicing is a targeted process by these drugs. This strategy, described in Chapter 3, will be useful in guiding research pursuits that are aimed at elucidating upstream alternative splicing regulation cascades, as well as identifying target pathways or components for increasingly targeted screens. While this strategy did not ultimately inform our characterization of digitoxin, we do feel that it will serve an essential role during future screens for modulators of a specific target exon.

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The cardiotonic steroid digitoxin regulates alternative splicing through depletion of the splicing factors SRSF3 and TRA2B

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ABSTRACT

Modulation of alternative pre-mRNA splicing is a potential approach to therapeutic targeting for a variety of human diseases. We investigated the mechanism by which digitoxin, a member of the cardiotonic steroid class of drugs, regulates alternative splicing. Transcriptome-wide analysis identified a large set of alternative splicing events that change after digitoxin treatment. Within and adjacent to these regulated exons, we identified enrichment of potential binding sites for the splicing factors SRp20 (SRSF3/SFRS3) and Tra2-β (SFRS10/TRA2B). We further find that both of these proteins are depleted from cells by digitoxin treatment. Characterization of SRp20 and Tra2-β splicing targets revealed that many, but not all, digitoxin-induced splicing changes can be attributed to the depletion of one or both of these factors. Re-expression of SRp20 or Tra2-β after digitoxin treatment restores normal splicing of their targets, indicating that the digitoxin effect is directly due to these factors. These results demonstrate that cardiotonic steroids, long prescribed in the clinical treatment of heart failure, have broad effects on the cellular transcriptome through these and likely other RNA binding proteins. The approach described here can be used to identify targets of other potential therapeutics that act as alternative splicing modulators.

Keywords: alternative splicing; cardiotonic steroid; SRp20; Tra2-β

INTRODUCTION

Alternative pre-mRNA splicing is a tightly controlled process of gene regulation with critical roles during both development and disease (Black 2003). Misregulation of alternative splicing can be the molecular cause of disease or can modify its progression and severity (Wang and Cooper 2007). One potential therapeutic strategy to target aberrant alternative splicing is the identification of small molecule modulators that might restore an alternative splicing event to a non-disease-associated pattern (Soret et al. 2005; Kaida et al. 2007; Kotake et al. 2007; O'Brien et al. 2008; Stoilov et al. 2008; Younis et al. 2010). Alternative splicing patterns can be controlled by changes in the core RNA splicing machinery that make up the spliceosome and by a host of *trans*-acting RNA binding proteins that influence spliceosome assembly (Black 2003). A typical alternative splicing event is controlled by multiple regulatory factors that bind to the pre-mRNA and direct a splicing choice in a combinatorial fashion. The flanking intronic sequence of an alternative exon is often conserved throughout mammalian species, and this conservation allows identification of regulatory sequences. For many splicing factors, consensus binding sites have been defined that help to predict alternative splicing regulation at the sequence level (Cartegni et al. 2003). Splicing choices are precisely controlled in a developmental and tissue-specific manner, and aberrant expression of splicing regulatory factors or altering their activity can have pathogenic effects (Wang and Cooper 2007).

We recently identified the cardiotonic steroid class of drugs as modulating the alternative splicing of exon 10 in the microtubule-associated protein tau (MAPT) transcript (Stoilov et al. 2008). Increased splicing of MAPT exon 10 causes frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17) and is associated with other

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nonhereditary dementia syndromes (Caffrey and Wade-Martins 2007; Dawson et al. 2007). A large number of splicing factors and signaling cascades control the splicing of MAPT exon 10, thereby presenting multiple potential therapeutic targets. The splicing factors SRp20, SRp30c, SRp55, Tra2-β, and 9G8 have all been shown to affect exon 10 splicing. Each of these proteins contains an arginine-serine repeat (RS) domain, characteristic of the SR family of splicing factors (Yu et al. 2004; Wang et al. 2005; Gao et al. 2007). SR proteins can act as either alternative splicing enhancers or repressors (Long and Caceres 2009; Shepard and Hertel 2009). SR proteins generally function to stimulate splicing of an exon when bound to exonic RNA motifs (exonic splicing enhancers, or ESEs). This enhancement can occur through recruitment of the spliceosomal machinery or by antagonism of a splicing repressor protein (Long and Caceres 2009; Shepard and Hertel 2009). SR proteins can also repress alternative splicing through multiple mechanisms, including acting through intronic splicing silencer elements (ISSs) adjacent to an alternative exon (Kanopka et al. 1996; Shin et al. 2004; Buratti et al. 2007). Modulation of SR proteins by small molecules has been shown in some studies, and they present interesting therapeutic targets for misregulated alternative splicing events (Soret et al. 2005; Katzenberger et al. 2009).

Cardiotonic steroids are characterized by their ability to bind the Na^+/K^+ ATPase in the plasma membrane and to block its ion exchange activity. In regions where the plasma membrane is juxtaposed with the endoplasmic or sarcoplasmic membrane, the block of Na^+/K^+ exchange promotes Na^+/Ca^{2+} exchange, thereby increasing intracellular Ca^{2+} and the contractile potential of the cardiac myocyte (Schoner and Scheiner-Bobis 2007a). Due to this activity, these drugs have been widely used in the treatment of congestive heart failure. The cardiotonic steroids are also increasingly studied for their ability to activate specific signaling cascades, mainly in the control of vascular tone and tumorigenic potential. Notably, certain cardiotonic steroids have been detected endogenously in mammalian cells (Schoner and Scheiner-Bobis 2007a). These cardiotonic steroids have similar effects as exogenously administered drugs on both subcellular ion concentrations and signaling activation. When bound by drug, the Na^+/K^+ ATPase interacts with the cytoplasmic tyrosine kinase Src, which activates multiple signaling cascades, including those mediated by MAPK, Akt, and CamK (Prassas and Diamandis 2008). The activation of MAPK, Akt, and CamK has all been shown to affect alternative splicing and the activity or expression of specific splicing factors.

As a model for analyzing how small molecules can alter splicing, we have examined the mechanism by which the cardiotonic steroid digitoxin modulates alternative splicing. We find that this drug has a very specific effect on the expression of SRp20 and Tra2-β and that the loss of these proteins mediates part of the splicing effects of digitoxin.

RESULTS

The cardiotonic steroid digitoxin modulates inclusion of a large set of alternative exons

We previously identified digitoxin and other cardiotonic steroids in a small molecule screen for modulators of splicing in the MAPT transcript (Stoilov et al. 2008). In addition to enhancing MAPT exon 10 splicing, we found that these drugs affected additional alternative exons. Cardiotonic steroids are commonly prescribed for heart failure but had not previously been shown to affect splicing. To further characterize these effects, we assessed the transcriptome-wide changes in alternative splicing that are induced by digitoxin. RNA isolated from control and digitoxin-treated HEK293 cells was used to probe the Affymetrix Human Research Junction Array (HJAY) (Yamamoto et al. 2009; Shen et al. 2010). These arrays contain probes for thousands of exon–exon junctions that are subject to alternative splicing. The arrays were analyzed for splicing changes by two previously described methods: MADS+ and OmniViewer (Sugnet et al. 2006; Shen et al. 2010). Each analysis method identified a large set of digitoxin-responsive alternative splicing events, including many changes in cassette exon inclusion (608 repressed exons, 132 enhanced exons by OmniViewer analysis) as well as other changes (Supplemental Fig. S1A; Supplemental Table 5). To confirm the array results, we performed semi-quantitative RT-PCR analysis of 41 high-confidence alternative cassette exons identified by one or both methods (Fig. 1A; Supplemental Fig. S1B). Assays were performed in triplicate (Fig. 1B).

We obtained validation rates of 92% (24 out of 26 cassette exons) and 100% (15 out of 15 exons) for the MADS+ and OmniViewer analysis methods, respectively. Subsequent array analyses used the OmniViewer method. This procedure generates a "sepscore" for each probed alternative event that assesses change in splicing pattern normalized by gene expression. When coupled with a low statistical false-discovery rate ($q < 0.001$), this score can characterize splicing changes that range from quite large (absolute sepscore > 1.0) to small but consistent (absolute sepscore between 0.3 and 0.5) (Sugnet et al. 2006).

Other small molecules have been reported to inhibit general splicing activity or spliceosome assembly (Kaida et al. 2007; Kotake et al. 2007; O'Brien et al. 2008; Roybal and Jurica 2010). We did not observe a general inhibition of splicing upon digitoxin treatment. Instead, a subset of the assayed alternative splicing events was affected by the drug. Consistent with previous smaller-scale array analyses (Stoilov et al. 2008), digitoxin induced both skipping and inclusion of different exons, though increased exon skipping was more frequent. These widespread and differential

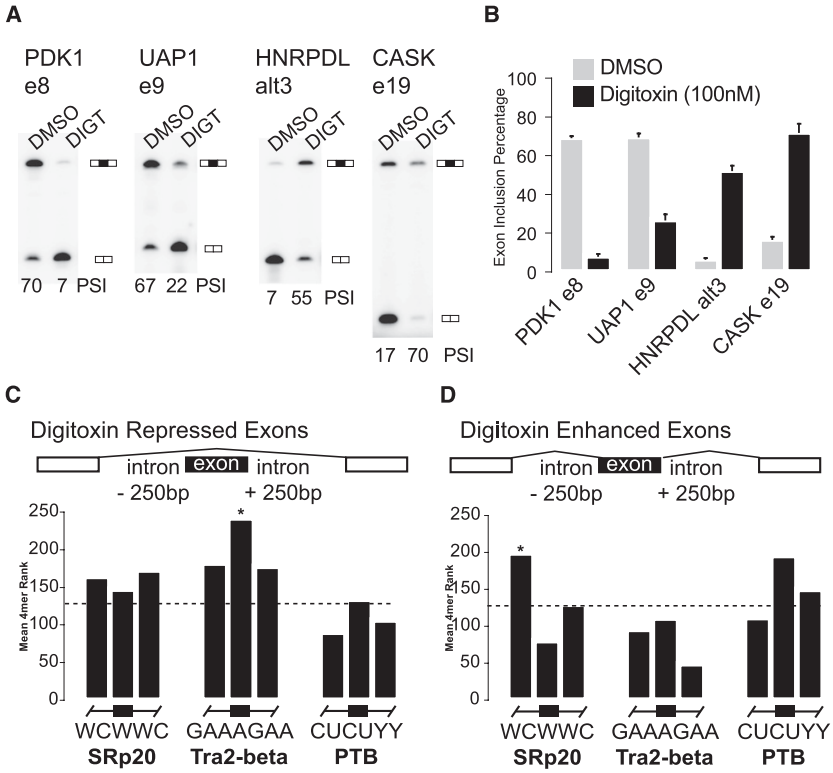

FIGURE 1. Digitoxin regulates a large set of alternative cassette exons that share potential binding sites for SRp20 and Tra2-β. (*A*) Representative RT-PCR gels for four high-confidence hits identified by HJAY. (*B*) Summary of exon inclusion levels for these exons across three independent experiments. Error bars, SEM exon inclusion (PSI). (*C*) For digitoxin repressed exons, Tra2-β binding sites are enriched. Plotted is mean rank of all tetramers that satisfy the given consensus binding sequence. The dashed line denotes the arithmetic mean tetramer rank of 128. Motifs with a significantly altered distribution of ranks (both high and low) are denoted by an asterisk and satisfy a Wilcoxon rank-sum *P*-value cutoff of 0.01. (*D*) The same analysis for digitoxin enhanced exons show an enrichment of SRp20 sites in the upstream intron.

effects on alternative splicing are consistent with the alteration of multiple splicing regulators that have both enhancing and repressive activity.

Binding sites for splicing factors SRp20 and Tra2-β are enriched in the digitoxin-regulated alternative exons

Although sodium-potassium pump inhibitors are known to activate particular signal transduction pathways, a mechanism by which cardiotonic steroids might regulate alternative splicing has not been described. Alternative splicing is generally regulated through *trans*-acting splicing factors that bind specific sequences within or adjacent to the target exon to enhance or repress its splicing. The binding elements for these factors range from short and specific (e.g., the Rbfox family of splicing factors binding UGCAUG) to longer and more degenerate (e.g., polypyrimidine tract binding protein [PTB] binding pyrimidine-rich sequences). As a first step in identifying factors responsive to digitoxin, we examined the sequences of the target exons.

We compiled the exon sequences and their adjacent introns (250 bp upstream and downstream) to search for motif enrichment. The data set of all digitoxin-regulated exons exhibiting an absolute sepscore of 1.0 or greater was separated into induced skipping and induced inclusion groups, which contained 608 and 132 exons, respectively. To characterize motif enrichment, we measured the frequency of RNA tetramers and pentamers in the digitoxin sets relative to a control set of exons also assayed by OmniViewer. Significantly enriched motifs were compiled in each of three regions; the upstream intron, the exon, and the downstream intron for both the enhanced and repressed exons (Supplemental Table 1). The intronic sequences included splice sites that have highly skewed motif frequencies. To control for these enrichments, we compared the digitoxin exon set to a set of non-drug-regulated alternative exons from the array. This provided

a background that controlled for splice site enrichment as well as nucleotide composition bias between exons and introns.

We looked for binding sites of known splicing regulators, including SF2/ASF, SRp20, SRp40, SRp55, Tra2-β, 9G8, and PTB (Supplemental Table 2; Tacke and Manley 1995; Liu et al. 1998; Tacke et al. 1998; Cavaloc et al. 1999; Schaal and Maniatis 1999; Spellman and Smith 2006). By searching for enriched tetramers that match portions of known splicing regulator binding sequences, we determined enrichment of entire degenerate binding sites (Fig. 1C,D). Among statistically significant changes, we found that binding sites for SRp20 are enriched in the introns upstream of digitoxin-enhanced exons (Fig. 1D). Binding sites for Tra2-β are enriched in digitoxin-repressed exons (Fig. 1C). We also found enrichment of sites for SF2/ASF and SRp40 in the digitoxin-regulated exons, suggesting that these factors may also play a role in digitoxin regulation of splicing (Supplemental Table 2). Though we also observed variation in motif frequency for binding sites of other factors, these did not always reach statistical significance. SR and SR-like splicing factors most frequently act as enhancers when exon-bound but can repress splicing when intron-bound. This is consistent with digitoxin inducing a loss-of-function of SRp20, Tra2-β, or both.

Examination of pentamers yielded additional enriched motifs in the digitoxin-repressed exons. We identified enrichment of the "AU"-rich pentamers in the introns upstream of digitoxin-repressed exons. These motifs are potentially bound by multiple splicing factors, including hnRNP-A2/B1, hnRNP-D1/2, TIA-1, Sam68, HuR, and TTP (Supplemental Table 1). Though the introns in the digitoxin-enhanced exons did not yield any statistically significant enriched motifs, we did identify potentially enriched pentamer motifs in enhanced exons (Supplemental Table 1). However, the number of occurrences of a specific pentamer was often low in the smaller digitoxin-enhanced data set.

Digitoxin treatment induces a loss of SRp20 and Tra2-β proteins

The positions of observed enrichment of SRp20 and Tra2-β binding sites relative to the digitoxin-responsive exons are consistent with a loss of activity for these two proteins. SRp20 sites can be repressive when found in introns, whereas exonic Tra2-β sites will typically enhance splicing of the exon. To examine these proteins, we probed immunoblots of HEK293 cell lysates after digitoxin or control treatment. Notably, we found that digitoxin causes depletion of both SRp20 and Tra2-β proteins (Fig. 2A). SRp20 was reduced by 58% after digitoxin treatment, compared with DMSO-treated control cells (Fig. 2C). Total Tra2-β was decreased by an average of 36% by digitoxin, and its hyperphosphorylated form (upper band on Western blot)

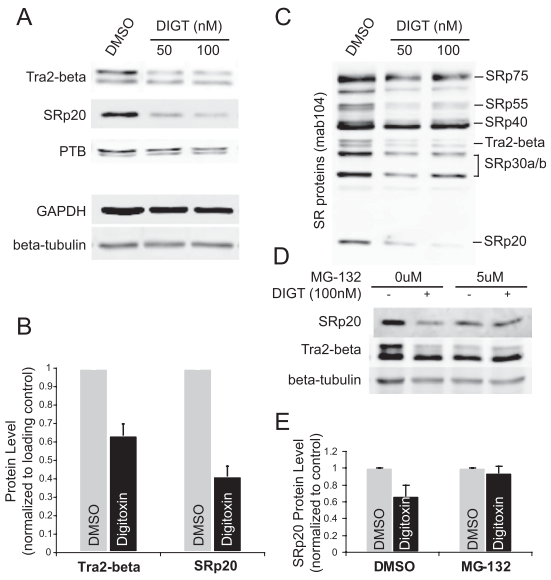

FIGURE 2. Digitoxin induces degradation of SRp20 and Tra2-β proteins. (*A*) Immunoblot analysis of digitoxin-treated HEK293 cells shows decreased expression of SRp20 and Tra2-β proteins. An unrelated splicing factor, PTB, remains unchanged. (*B*) Quantification of SRp20 and Tra2-β protein expression normalized to GAPDH and β-tubulin. Error bars, SE across three independent digitoxin treatments. (*C*) Immunoblot with pan-SR antibody reveals that other members of the SR family are largely unchanged after digitoxin treatment. (*D*) Co-incubation with MG-132 prevents depletion of SRp20 and Tra2-β and is quantified in *E* for three independent experiments. Error bars, SEM protein level.

was more strongly reduced (Fig. 2C). We also assayed for changes in additional splicing factors after digitoxin treatment. SRp30c has been reported to interact directly with Tra2-β and modulate its splicing function (Young et al. 2002). However, SRp30c protein levels were minimally changed after digitoxin treatment (Supplemental Fig. S2C). Similarly, PTB expression was not altered by digitoxin treatment (Fig. 2A).

By using the mab104 monoclonal antibody reactive with the phosphorylated RS domain of many SR proteins, we assayed the phosphorylated forms of additional SR proteins after digitoxin treatment. We confirmed the depletion of SRp20 and Tra2-β, which exhibited reduced reactivity with mab104 (Fig. 2A,B). There were also potential changes in SRp55 and SRp40 phosphorylation, although the total protein level of these two splicing factors did not change (Supplemental Fig. S2C).

The loss of Tra2-β and SRp20 proteins could be mediated by changes in transcription or translation or by changes in mRNA or protein degradation. The array data showed only small changes in the mRNAs for either SRp20 or Tra2-β ($\log_2$ fold changes of 0.1 and 0.4, respectively),

which we confirmed by quantitative RT-PCR (Supplemental Fig. S3). Notably, Tra2-β was previously shown to be regulated in a proteasome-dependent manner by the chemotherapeutic drug camphothecin (Wang et al. 2005). To assess whether the changes in SRp20 and Tra2-β resulted from protein degradation by the proteasome, we treated cells with the proteasome inhibitor MG-132 with or without digitoxin. The loss of SRp20 after digitoxin was suppressed by MG-132 (Fig. 2D,E). MG-132 also suppressed digitoxin-induced loss of the upper Tra2-β band, although its effect on the lower band was less consistent (Fig. 2D). These data indicate that at least part of the action of the cardiotonic steroids is to stimulate the proteasome-mediated degradation of these two splicing factors.

We also found that ''AU''-rich elements were enriched upstream of exons repressed after digitoxin treatment. Multiple RNA binding proteins can interact with these motifs to direct alternative splicing, including TIA-1, Sam68, and the hnRNP-D proteins. We found that both the hnRNP-D and D-like transcripts exhibit altered splicing in their 3′ UTRs after digitoxin treatment. These splicing changes downstream from the coding sequence could potentially lead to nonsense-mediated decay of the transcript (Fig. 1B; Stoilov et al. 2008). However, we found no change in either the hnRNP-D or hnRNP-D–like proteins after drug treatment (Supplemental Fig. S2A). Additionally, we found no changes at the protein level for TIA-1 or Sam68 (Supplemental Fig. S2B) or large alternative splicing changes in the transcripts of TIA-1, hnRNP-A2/B1, Sam68, HuR, and TTP (Supplemental Table 5). Since SRp20 and Tra2-β were clearly responding to digitoxin treatment, we focused on these proteins to determine their contribution to the digitoxin-induced splicing changes.

Defining the SRp20 and Tra2-β target exon sets

Binding of cardiotonic steroids, including digitoxin, to the Na^+/K^+ ATPase activates multiple cell signaling pathways (Schoner and Scheiner-Bobis 2007b). Inhibition of ion exchange leads to a rise in intracellular Ca^{2+} levels, which can activate pathways that require Ca^{2+} as a second messenger, such as the PLCγ cascade. In addition, binding of cardiotonic steroids to the Na^{2+}/K^+ ATPase activates both the PI3K/Akt and the Src/Ras/MAPK cascades, in a manner that does not depend on inhibition of ion exchange. These widespread effects of cardiotonic steroids on cell signaling present many possible mechanisms for control of SRp20 and Tra2-β. Cell signaling control of SR and SR-like proteins has been described (Shin and Manley 2004; Lynch 2007). SR proteins are regulated at the post-translational level by specific protein kinases (SRPK and Clk/Sty families), as well as by cellular phosphatases (Stamm 2008). Notably there is evidence for PI3K/Akt regulation of ASF/SF2, 9G8, and SRp40 (Blaustein et al. 2005; Patel et al. 2005). Additionally, altered splicing patterns after Ras modulation have been reported that include a known Tra2-β target (Weg-Remers et al. 2001). By using chemical inhibitors, we investigated the dependence of digitoxin-induced splicing changes on specific signaling cascades. However, we found no consistent dependence of the digitoxin effect on the PLCγ, PI3K/Akt, or Src/Ras/MAPK signaling cascades (data not shown). Given the diverse actions of digitoxin and the other cardiotonic steroids, it is likely that the observed splicing changes result from effects on multiple signaling pathways that converge on these proteins.

To identify those splicing events attributable to SRp20 or Tra2-β, we knocked down each splicing factor in HEK293 cells and assayed the changes in splicing by microarray (Fig. 3A,B). Knockdown of either factor led to increases or decreases in the splicing of large sets of exons (Fig. 3B), consistent with both repressive and activating roles for the two proteins. At an absolute sepscore of 1.0 or higher, we identified 435 cassette exons whose splicing was altered by SRp20 knockdown (182 induced inclusion; 253 induced skipping). Using an absolute sepscore cutoff of 0.6, Tra2-β knockdown altered the splicing of 193 exons (103 induced inclusion; 90 induced skipping) (Fig 3B). Among these exons were multiple previously identified Tra2-β and SRp20 targets. These included fibronectin exon 33/EIIIA, targeted by SRp20, and survival motor neuron 2 exon 7 and the Tra2-β transcript itself, both regulated by Tra2-β (Hofmann et al. 2000; Lorson and Androphy 2000; Stoilov et al. 2004). Since the regulation of a particular exon depends on multiple splicing factors that vary by cell type, we did not expect every known SRp20 or Tra2-β target exon to change.

We confirmed the changes in multiple alternative exons from each array by RT-PCR (Fig. 3C; Supplemental Fig. S4). Knockdown of SRp20 altered the splicing of more exons than knockdown of Tra2-β, and these changes were on average much larger. The lower effects of Tra2-β may result from the presence of compensatory factors that bind similar purine-rich sequences, such as Tra2-α, a Tra2-β–related protein.

To identify digitoxin target exons that are regulated through SRp20 or Tra2-β, we identified overlapping exons among the three data sets. Of the exons regulated by digitoxin treatment, we found 44 cassette exons to be altered in the same direction by SRp20 knockdown. We found 16 exons to be regulated by both digitoxin and Tra2-β knockdown. From each of the knockdown arrays, we confirmed 10 exons that overlapped with the digitoxin set (Fig. 3D,F; Supplemental Fig. S5A,B). Examples of coregulated exons include amyloid precursor protein (APP) exon 8, which contains a consensus SRp20 binding site and is induced to skip both by digitoxin treatment and SRp20 knockdown (Fig. 3D). Similarly, receptor-interacting serine-threonine kinase 2 (RIPK2) exon 2 contains two potential Tra2-β binding sites and is reduced in splicing by both Tra2-β knockdown and digitoxin (Fig. 3E). ZNF207

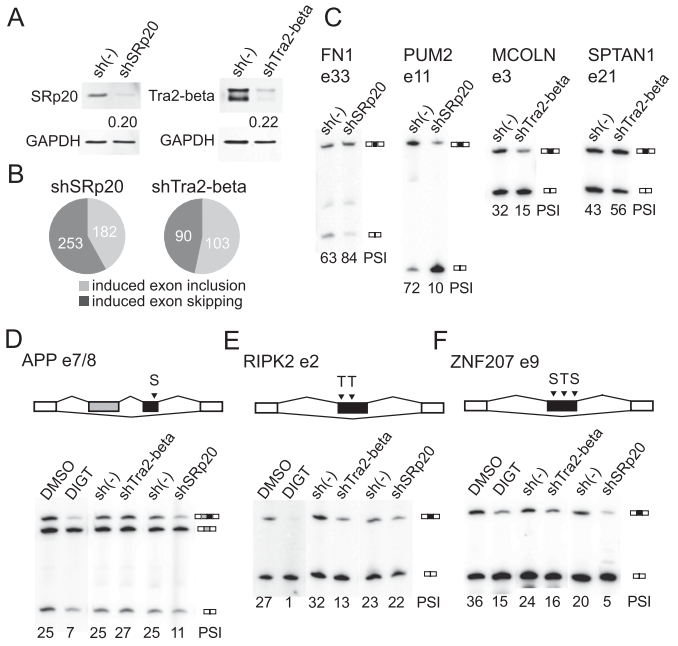

FIGURE 3. Regulation of digitoxin target exons by SRp20 and Tra2-β. (*A*) shRNA-mediated knockdown of SRp20 and Tra2-β in HEK293 cells. Number *below* each blot shows relative expression level normalized to GAPDH and β-tubulin. (*B*) OmniViewer analysis for changes in alternative cassette exons in the two knockdown conditions compared with control. (*C*) Representative RT-PCR gels for alternative splicing changes. (*D*) APP exon 8 is the second exon of a dual cassette exon pair with exon 7. It possesses a consensus SRp20 binding site and is repressed by both digitoxin and SRp20 knockdown, but not Tra2-β knockdown. (*E*) RIPK2 exon 2 contains two potential Tra2-β binding sites and is repressed by digitoxin treatment and Tra2-β knockdown, but is unaffected by SRp20 knockdown. (*F*) ZNF207 exon 9 contains two potential SRp20 binding sites and one potential Tra2-β binding site and is repressed by digitoxin and both knockdowns.

exon 9 contains binding sites for both SRp20 and Tra2-β and can be regulated by both splicing factor knockdowns as well as digitoxin (Fig. 3F).

Many digitoxin-induced splicing changes were not affected by either SRp20 or Tra2-β. These are presumably controlled by other splicing factors affected by digitoxin. They may also require activation of particular signaling pathways in addition to the loss of SRp20 or Tra2-β to manifest a change.

If loss of SRp20 or Tra2-β is the primary mechanism by which digitoxin alters splicing of a particular exon, then re-expression of one or both factors should reverse the effect of digitoxin. We find that expression of recombinant SRp20 blocks digitoxin-induced skipping of two newly identified targets, APP exon 8 and ZNF207 exon 9 (Fig. 4A,C). This is also seen with a previously identified SRp20 target, fibronectin exon 33, which is enhanced by digitoxin treatment but remains repressed when SRp20 is simultaneously overexpressed (Fig. 4D). Similarly, the Tra2-β target RIPK2 exon 2 is reduced in splicing by digitoxin treatment. This loss of splicing can be fully reversed by expression of recombinant Tra2-β (Fig. 4B). Thus, re-expression of SRp20 or Tra2-β is sufficient to reverse the effect of digitoxin on multiple exons. For these exons, the depletion of SRp20 and Tra2-β is the primary cause of digitoxin's effect on splicing.

DISCUSSION

There is great interest in therapeutics that target the splicing reaction in the treatment of diseases caused by aberrant splicing. We recently found that digitoxin and other cardiotonic steroids alter the splicing of MAPT exon 10 and other exons. However, it was not clear how these exons are specifically targeted by the drugs. Exogenous and endogenous cardiotonic steroids are known to activate a variety of signal transduction cascades, notably though the Src kinase, as well as pathways mediated by MAPT, Akt, and CamK. All of these pathways have been shown to affect splicing patterns.

Through analysis of RNA motif frequencies within or adjacent to regulated exons, we identified enrichment of *cis*-acting elements that are known to interact with RNA binding proteins. Specifically, our analysis implicated SRp20 and Tra2-β as potential mediators of the digitoxin effect. Confirming the motif association, we find that the SRp20 and Tra2-β proteins both decrease in a dose-dependent manner after digitoxin treatment. By using exon junction microarrays, we identified alternative splicing events that respond to SRp20 and Tra2-β. Comparison of the digitoxin-regulated events to the SRp20/Tra2-β–regulated events indicates that the loss of these two proteins accounts for many of the splicing changes induced by digitoxin. Overexpression of these factors can reverse the changes induced by digitoxin, indicating that their depletion is one mechanism by which the drug acts. We also observed enrichment of elements that do not fit SRp20 or Tra2-β binding sites, and noted changes in phosphorylation of additional SR proteins. Thus, other digitoxin-induced splicing changes will likely be mediated by additional factors.

Digitoxin and the cardiotonic steroids are widely prescribed for the treatment of heart failure. Their therapeutic effect derives from their ability to block the Na^+/K^+ pump in cardiac cells and subsequently alter local Ca^{2+} concentrations. Cardiotonic steroids are known to alter multiple cell signaling cascades and thus will have widespread effects

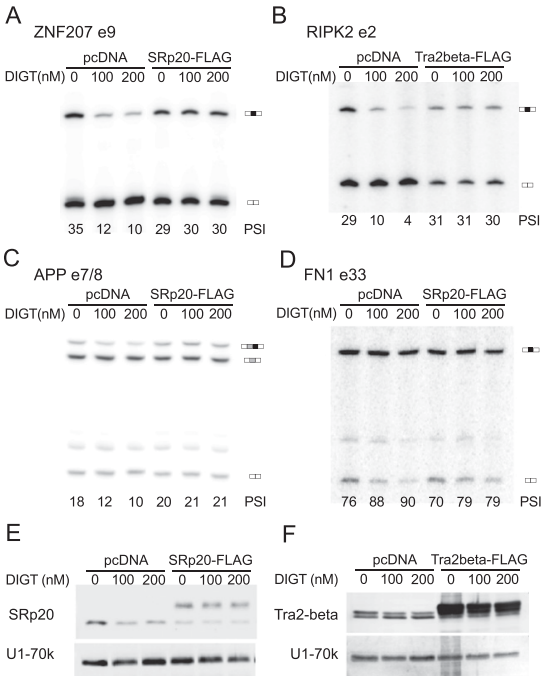

FIGURE 4. SRp20 and Tra2-β overexpression reverses digitoxin effect on coregulated alternative exons. (*A*) Digitoxin-induced repression of ZNF207 exon 9 is reversed by SRp20 expression. (*B*) RIPK2 exon 2 repression is rescued by Tra2-β expression. (*C*,*D*) Digitoxin induced repression of APP exon 8 is rescued by expression of exogenous SRp20, while the same overexpression reduced digitoxin-induced enhancement of FN1 exon 33. (*E*,*F*) Flag-tagged overexpression of SRp20 and Tra2-β in HEK293 cells, respectively.

on cellular function. By using chemical inhibitors, we probed specific cell signaling cascades for their roles in determining the splicing response to digitoxin. Inhibiting the MEK/ERK, PI3k/Akt, or PLCγ pathways had inconsistent effects on Tra2-β and SRp20. It is likely that multiple pathways are contributing to the observed splicing effects of digitoxin. Further investigation of these regulatory cascades will be needed to reveal the direct mechanism of regulation. It is notable that cardiotonic steroid concentrations in the nanomolar range are similar to tissue concentrations found in patients treated for heart failure (Schoner and Scheiner-Bobis 2007a,b). These concentrations clearly induce transcriptome-wide splicing changes in cell culture (Stoilov et al. 2008). Thus, it will be interesting to examine patient tissues for splicing changes after cardiotonic steroid treatment. This altered splicing may contribute to the effects of digitoxin in the heart as well as its off-target effects in other tissues. Such splicing changes may also be controlled by endogenous cardiotonic steroids (Schoner and Scheiner-Bobis 2007a,b).

The approach used here can be generalized to many small molecule modulators of splicing. The whole transcriptome profiling identifies particular alternative splicing events targeted by a drug. The bioinformatics analysis can then be used to predict splicing regulators that are targeted by the drug. These individual regulators can then be tested for affecting the exons in question. In addition to providing new understanding of how signaling pathways communicate with the splicing apparatus, these studies can also identify previously unknown effects of a drug. It is likely that other commonly prescribed drugs have unrecognized effects on post-transcriptional gene regulation.

MATERIALS AND METHODS

Cell culture and reagents

HEK293 cell line was maintained in DMEM supplemented with 10% fetal bovine serum (Atlanta) and 100 μM L-glutamine (GIBCO). Transfections were performed in OPTI-MEM (Invitrogen) either in suspension (knockdown) or on cells plated in monolayer (overexpression), using Lipofectamine 2000 transfection reagent (Invitrogen), per the manufacturer's instructions. Digitoxin (Sigma-Aldrich) was dissolved in DMSO at a concentration of 100 mM and added directly to cell culture media for drug treatment. Digitoxin was applied to HEK293 cells at a concentration of 100 nM for a period of 24 h. Control treatment was DMSO vehicle alone. These conditions replicate the previously reported effective dose of cardiotonic steroids in regulating alternative splicing of MAPT-exon 10 (Stoilov et al. 2008). MG-132 (Enzo LifeSciences) was dissolved in DMSO at 5 mM and added directly to cell culture media for treatment.

SRp20 and Tra2-β knockdown was performed by transient transfection of specific short hairpin RNA vectors. Targeted knockdown of SRp20 was achieved using a single hairpin sequence, whereas Tra2-β was most efficiently decreased using a combination of two hairpins that target its 3′ UTR (Supplemental Table 3). RNA and protein were isolated 72 h after transient transfection of the hairpin vectors. By using these knockdown conditions, we achieved ∼80% reduction of each splicing factor at the protein level compared with transfection of the hairpin vector alone (Fig. 3A). We probed Affymetrix HJAY microarrays in triplicate with RNA from cells in which the two factors had been knocked down.

RT-PCR

RNA was extracted from HEK293 cells using Trizol reagent per the manufacturer's instructions. cDNA for RT-PCR experiments was synthesized from 2 μg of total RNA using random hexamers for priming. PCR reactions contained ^{32}P-end labeled oligonucleotide primers and were run for 22 cycles at the following conditions: 30 sec at 95°C, 45 sec at 58°C, and 60 sec at 72°C. Primer sequences used in these studies are shown in Supplemental Table 4. PCR products were resolved by urea-polyacrylamide gel electrophoresis. Phosphor storage screens were imaged on a Typhoon PhosphorImager (GE) and radioactivity quantified using ImageQuant TL software (Amersham Biosciences).

Microarray preparation and analysis

RNA from three biological replicates for each condition was probed for alternative splicing changes by the Affymetrix HJAY.

Total RNA was extracted with Trizol reagent (Invitrogen) and assayed for integrity using the Agilent Bioanalyzer. Samples were prepared from 100 ng total RNA using the WT Expression Kit (Ambion) and GeneChip Terminal Labeling Kit (Affymetrix), per the manufacturer's instructions. The samples were hybridized to the HJAYs (Affymetrix) and scanned in the UCLA Microarray Core. Array analysis was performed using two software packages: MADS+ (http://www.healthcare.uiowa.edu/labs/xing/madsplus/) (Shen et al. 2010) and the OmniViewer method (http://metarray.ucsc.edu/omniviewer/) (Sugnet et al. 2006). Each analysis assays both gene expression and alternative splicing changes. The OmniViewer method generates a sepscore for changes in specific alternative exons that is a composite representation of probes that detect specific splice isoforms and those that detect general expression level of the transcript. Similarly, MADS+ analysis generates a gene expression normalized *P*-value for significant change in each junction comprising an alternative event as well as for the alternative sequence itself.

Bioinformatics

For each data set, the frequency of each possible nucleotide tetramer and pentamer was calculated from the total number of pentamers in the analyzed region. Frequency was tested against the control frequency generated from the analogous region of all alternative exons assayed by the OmniViewer analysis. *P*-values and significance were established by a binomial test of these two frequencies. For specific binding site analysis, frequency enrichment of all tetramers was calculated as above. Binomial *P*-values were rank ordered for all tetramers, and those that were matches to all or part of a consensus were counted and subjected to the Wilcoxon rank-sum test. *P*-values for consensus enrichment were generated from this test.

Western blots

Total protein was isolated using standard RIPA lysis conditions (25 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 1% deoxycholic acid, 0.1% SDS) and resolved by SDS polyacrylamide gel electrophoresis. Cell lysis was performed in the presence of protease inhibitor cocktail (Roche), as well as phosphatase inhibitor cocktail (50 mM sodium fluoride, 2 mM β-glycerophoshpate, 1 mM EDTA, 1 mM sodium orthovanadate). Secondary detection was fluorescence based and carried out on the Typhoon phosphorimager (GE). Protein levels were quantified using ImageQuant TL software (Amersham Biosciences). Primary antibodies were obtained and used as follows: mouse α-SRp20 (Zymed; 1:3500 dilution), rabbit α-Tra2-β (Sigma-Aldrich; 1:3500), mouse α-SR (clone 1H4, Zymed; 1:1000), mouse α-SRp30c (generous gift from Benoit Chabot), rabbit α-PTB (NT antibody generated in D. Black's laboratory; 1:5000), mouse α-GAPDH (Ambion; 1:10000), rabbit α-β-tubulin (Santa Cruz, 1:1000), goat α-TIA1 (C-20, Santa Cruz, 1:1000), rabbit α-AUF1 (Millipore, 1:500), rabbit α-HNRPDL (Abcam, 1:500), and mouse α-U170k (1:1000). Secondary antibodies were goat α-mouse conjugated to AlexaFluor488 (Invitrogen; 1:3500), goat α-rabbit conjugated to Cy5 (Jackson Laboratories; 1:2500), and rabbit α-goat conjugated to Cy5 (Jackson Laboratories, 1:2500).

DATA DEPOSITION

Data from the two sets of microarray experiments have been deposited in the NCBI Gene Expression Omnibus (GEO) under accession nos. GSE36058 for digitoxin versus DMSO and GSE36069 for Tra2-β and SRp20 knockdowns versus control.

SUPPLEMENTAL MATERIAL

Supplemental material is available for this article.

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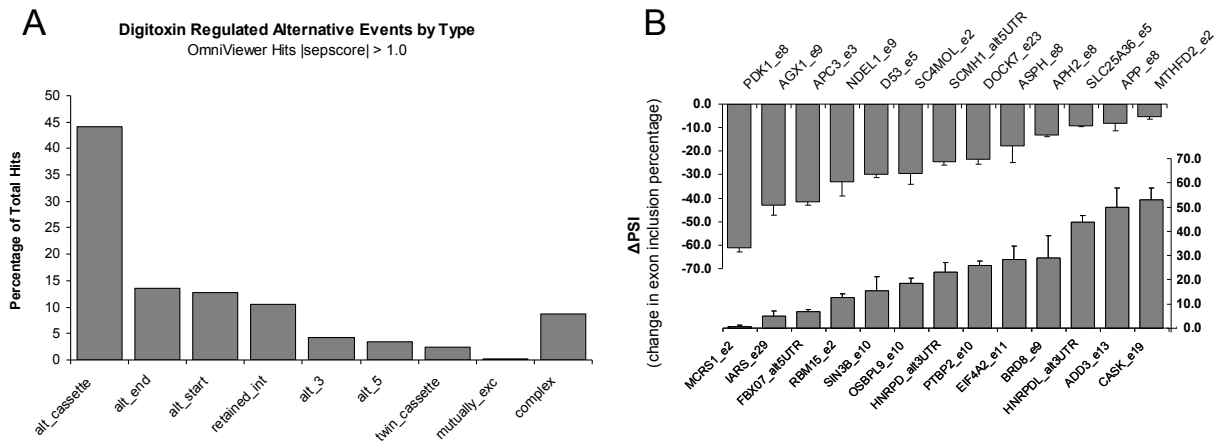

Figure S1: Digitoxin alters many different types of alternative splicing events. (A) OmniViewer analysis reveals that digitoxin changes many different types of alternative splicing events. Treatment conditions were 100nM for 24 hours in HEK293 cell line. Shown here are relative percent of total induced changes for each subtype, as annotated by OmniViewer analysis. (B) Validation of 26 cassette exons identified to be splicing changes by MADS+ method. Error bars are standard error of the mean change in exon inclusion percentage for three independent experiments.

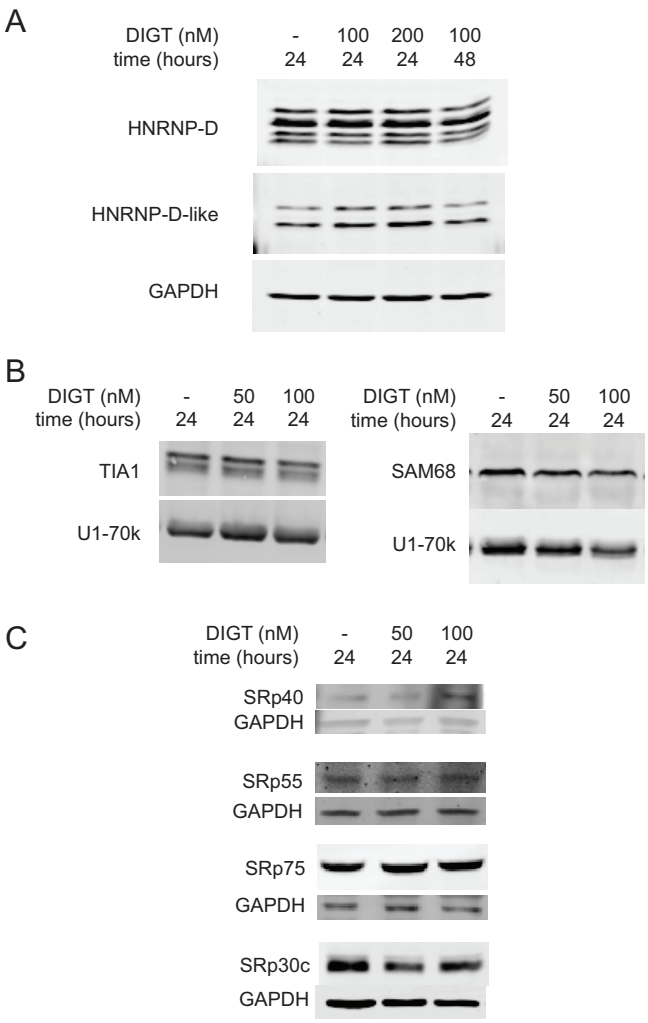

Figure S2: AU motif binding proteins and other SR proteins remain unchanged after digitoxin treatment. (A) hnRNPD and D-like splicing factors can bind AU-rich motifs and showed altered splicing of their own transcripts after digitoxin. Immunoblot analysis reveals no changes in protein level for these two proteins after digitoxin. (B) TIA1 and SAM68 are both known to bind AU-rich elements, but are unchanged at the protein level after digitoxin treatment. (C) Total protein levels of SRp30c, SRp40, SRp55 and SRp75 are unchanged by digitoxin, when probed with specific antibodies by immunoblot.

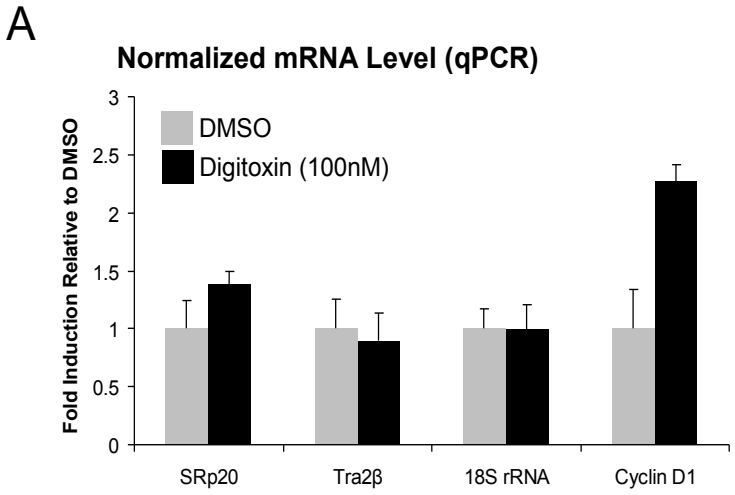

Figure S3: SRp20 and Tra2-beta are unaffected at the transcriptional level, as measured by quantitative real-time PCR.

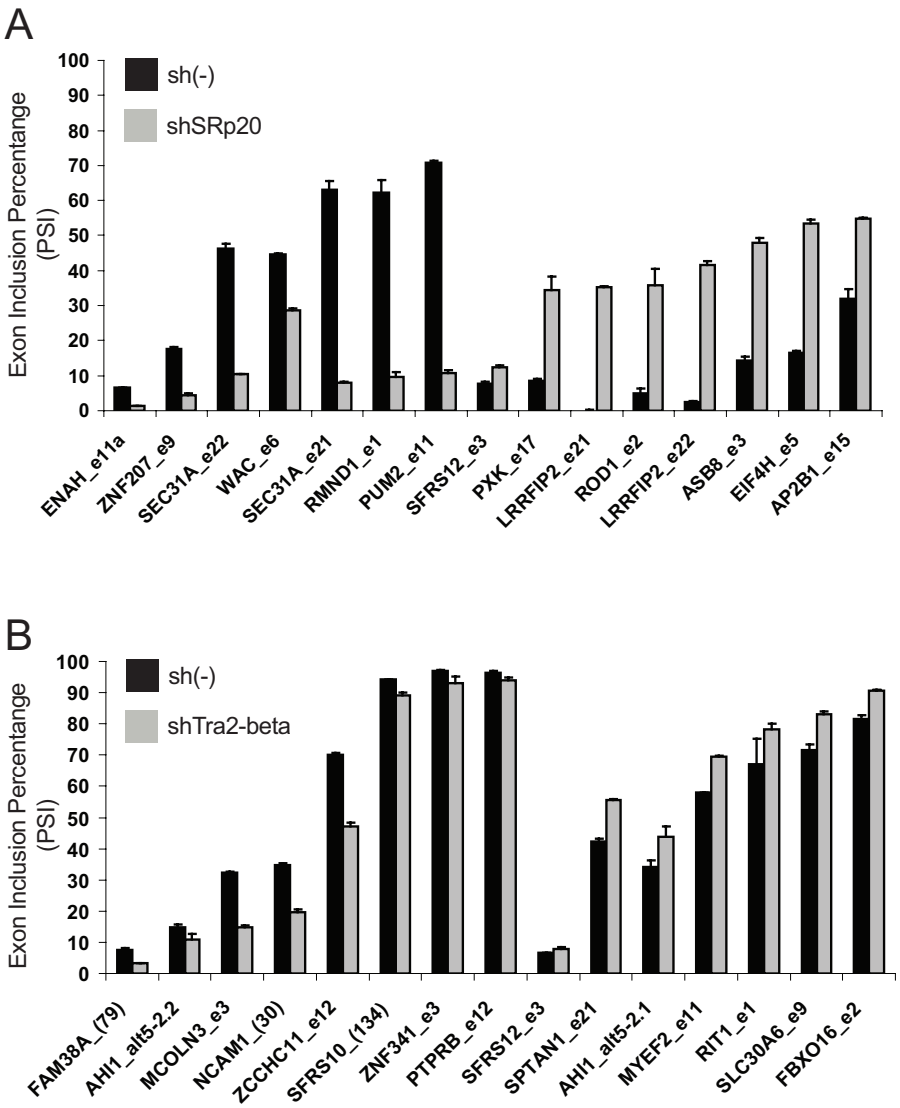

Figure S4: shSRp20 and shTra2-beta HJAY Validation reveals wide range of induced splicing changes. (A) Validation of induced splicing changes by knockdown of SRp20 for 15 exons identified by microarray analysis. Error bars represent standard error of mean exon inclusion percentage for three independent knockdown experiments. (B) The same analysis for array-identified Tra2-beta targets.

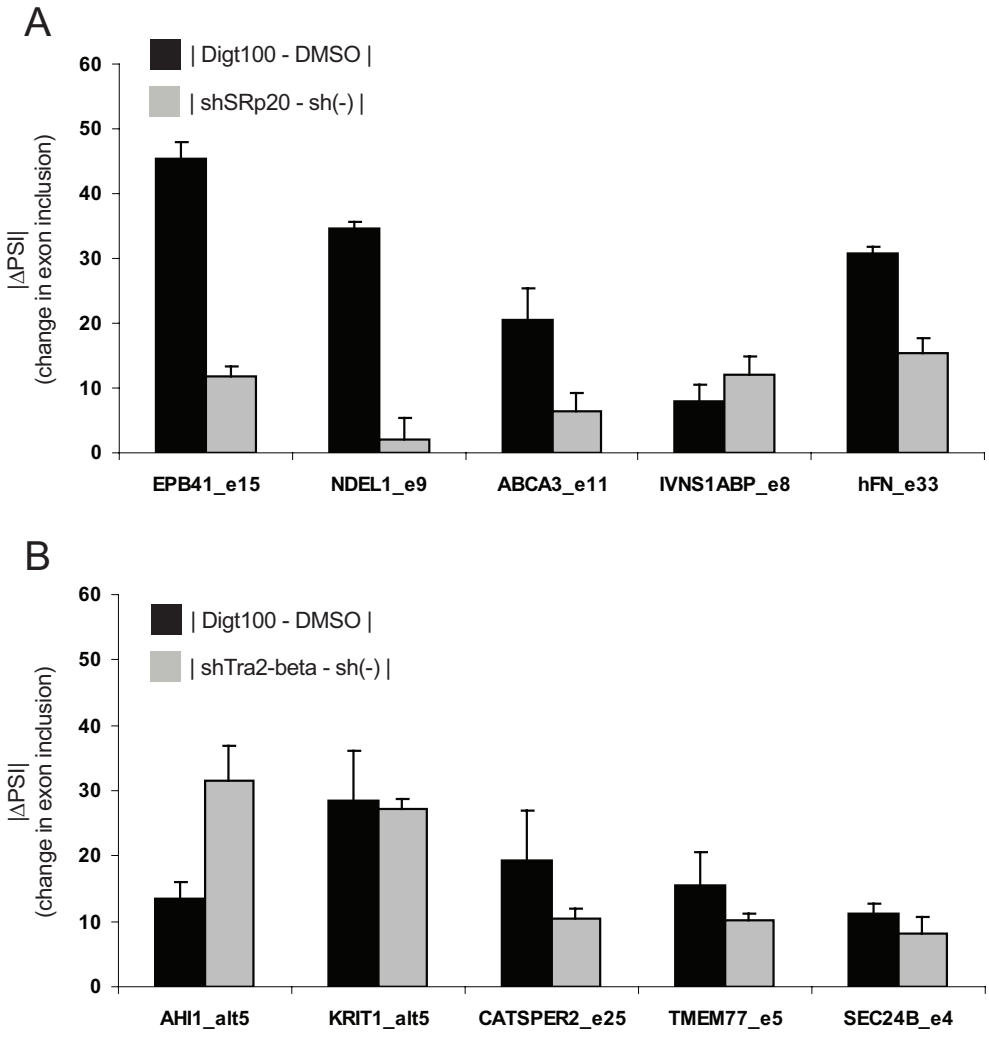

Figure S5: For overlapping exons, loss of SRp20 or Tra2-beta causes similar changes in alternative splicing as digitoxin treatment. (A) For 5 exons that are both digitoxin and SRp20 regulated, the induced splicing changes for the treatments were similar in direction and magnitude. Error bars represent standard error of induced splicing change for three independent experiments. (B) The same analysis for 5 overlapping digitoxin and Tra2-beta.

CHAPTER 3: ANALYSIS OF KNOWN CARDIOTONIC STEROID FUNCTIONS TO DETERMINE THEIR ROLE IN ALTERNATIVE SPLICING REGULATION

Introduction: Cardiotonic steroids

Cardiotonic steroids have long been used in the treatment of congestive heart failure, mainly due to their physiologic function of Na^+/K^+ pump antagonism(42,43). In cardiac myocytes, this effect can increase contractile potential and help alleviate some symptoms associated with this disease. However, it has become clear over time that the effects of cardiotonic steroids are more widespread, and there is some speculation that their therapeutic benefit may not be derived from ion pump inhibition as previously hypothesized. In fact, studies point to the therapeutic effect of the cardiotonic steroids to depress nervous system function, which may could to decrease the sympathetic input into the heart, thereby relieving congestive stress(44). It is likely that combinations of multiple cellular effects exert the therapeutic effect of the drug, but each of these actions could potentially be accompanied by a different set of off-target effects. Detailed analysis of these diverse cellular effects could reveal the mechanism by which cardiotonic steroids regulate a process such as RNA splicing.

Investigation of cardiotonic steroids extends to the roles of low-level endogenous cardiotonic steroids that may have critical roles during development and disease to exogenously administered drugs for heart failure. Specifically, they can modulate the function of cell signaling cascades in both ion-exchange dependent and independent mechanisms (Figure 2.1)(42,45). First, as these drugs inhibit Na^+/K^+ pump function, they ultimately modulate concentrations of intracellular ions, including Ca^{2+}. Fluctuations in intracellular calcium has ionotropic effects on contractile cells, but also will effect cell-signaling cascades that depend on

calcium as an input, such as calmodulin dependent kinase pathways that have known effects on nuclear processes such as RNA splicing(46-48). These induced changes in calcium signaling naturally depend on blockade of ion-exchange, but recent work has described roles for cardiotonic steroids that do not depend on changes in ion-exchange.

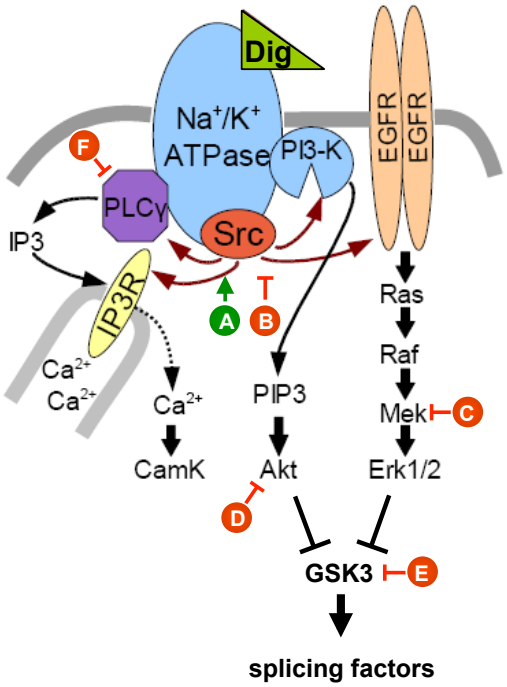

FIGURE 3.1: Cardiotonic steroids activate Src dependent cell signaling cascades. Shown are multiple pathways that are activated by endogenous and exogenous cardiotonc steroids. Letters indicate points of modulation in these studies. (A-B) Molecular genetic manipulation of Src via overexpression of mutant kinases. (C-E) Small molecule inhibition of downstream Src-dependent kinases. (F) Small molecule inhibition of PLCγ, a key enzyme involved in Ca2+ dependent signaling.

Specifically, it has been shown that even at low levels, cardiotonic steroids can activate Src-dependent cell-signaling cascades, with activation of downstream effectors including the kinases Akt and ERK1/2. Activation of Src-dependent signaling cascades results from an interaction between Src and the Na+/K+ ATPase, but importantly proceeds regardless of actual blockade of ion exchange. This finding implies that off-target effects of the cardiotonic steroids could extend far beyond the targeted cells with contractile potential and into any cellular processes that lie downstream of these signaling cascades.

Background information about a drug or a class of drugs can additionally drive investigation of their roles on a new process, such as for cardiotonic steroids on digitoxin. We have already described how an unbiased approach can yield molecular targets of a small molecule, but the wealth of information that exists on many small molecules is not to be discarded. The second approach to characterization of the mechanism by which cardiotonic steroids regulate alternative splicing relied on the previous

work that has been performed on this class of drug. Specifically, we sought to analyze whether the cell signaling cascades that are known to be regulated by cardiotonic steroids have a role in induced splicing changes by the drugs. We find that individual blockade of these pathways did not ultimately have an effect on digitoxin-induced splicing changes. It is likely that modulation of a combination of pathways, both known and unknown, contribute to the global changes in alternative splicing in response to the cardiotonic steroids.

Results

Digitoxin modulates both Akt and ERK1/2 phosphorylation in HEK293 cells

The previous high throughput screens that identified cardiotonic steroids as regulators of alternative splicing used the dual-fluorescence pFlare splicing reporter stably established in an HEK293 cell line. To begin investigation of molecular pathways that could lead from cardiotonic steroid treatment to splicing changes, we first sought to identify which cell signaling cascades are activated by digitoxin in this cell type. Different pathways have been

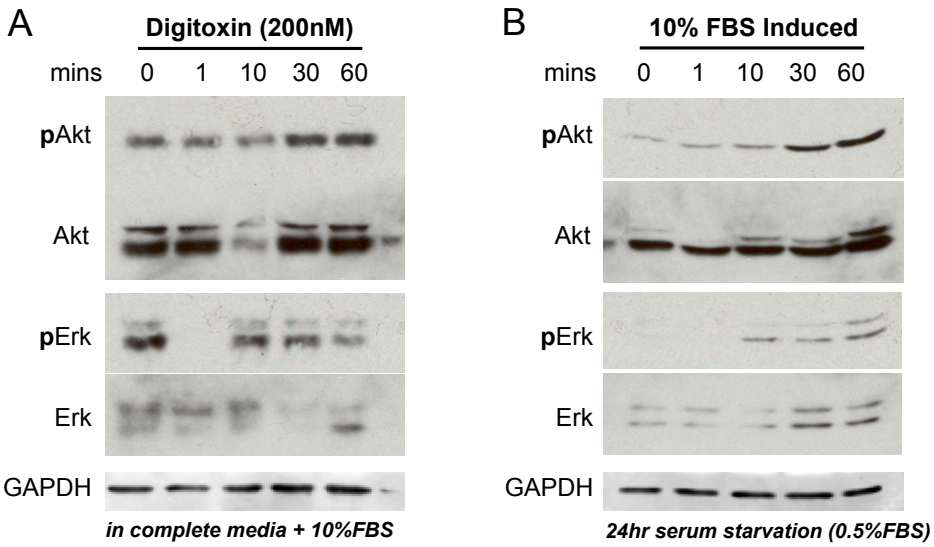

FIGURE 3.2 Digitoxin modulated Akt and ERK1/2 phosphorylation levels in HEK293 cells. (A) In HEK293 cells that are maintained in complete medium + 10%FBS, a 200nM digitoxin treatment further increases Akt phosphorylation levels beginning at 30 minutes after treatment. Conversely, phosphoryalted levels of ERK1/2 decrease after digitoxin treatment. (B) These findings compare to HEK293 cells that were serum starved for 24 hours and then stimulated with complete media containing 10%FBS. In these cells, both phosphorylated Akt and ERK1/2 increase steadily during the time course, from a baseline of near zero phosphorylation for each.

reported to be activated by cardiotonic steroids depending on cell type of study, with changes often detectable at very early timepoints(49). Digitoxin had previously given the most consistent effect on alternative splicing changes; therefore it was used for each of the subsequent experiments. Treatment of HEK293 cells with a 200nM dose of digitoxin robustly increased levels of phosphorylated Akt at serine 473 by 30 minutes after treatment (Figure 2.2A). Phosphorylation at this site is associated with an increase in Akt function, and we therefore predict that digitoxin would positively regulate Akt-dependent processes in the cell. It is important to note that these experiments are performed on HEK293 cells that are maintained in complete cell culture media containing 10% fetal bovine serum. Thus, the effect by digitoxin here on Akt is above the normally increased level of Akt activity already seen in these cells. This is in contrast to what is seen in the same cells after serum starvation, namely 24 hours incubation in media containing 0.5% FBS (Figure 2.2B). In these cells, phosphorylated, or active, Akt has been strongly downregulated, and is induced only upon stimulation with media containing 10% FBS. The net effect on Akt phosphorylation is similar between the two treatments, however, in that phosphorylated Akt is increasing during the timecourse of each experiment.

Digitoxin also had an effect on the ERK1/2 (p44/p42) phosphorylation at residues Thr202 and Tyr204 respectively. During the time course of digitoxin treatment, the level of phosphorylated ERK1/2 steadily decreased, after a complete but temporary loss at 1 minute post treatment, which is yet to be explained. This finding implies that digitoxin treatment is steadily decreasing the activity of ERK1/2 in HEK293 cells that are maintained in complete media containing 10% FBS. This finding is in contrast to what is seen in the serum-starved cells. As expected after starvation, the levels of phosphorylated ERK1/2 are not detectable. Stimulation

by media that contains 10% FBS sharply increases the levels of phosphorylated ERK1/2. This finding led to the prediction that digitoxin has multiple effects on different cell signaling cascades, with the notable changes being activation of Akt signaling and potential repression of ERK1/2 function. Each of these pathways can be a downstream of Src kinase activation, which would be predicted given prior work on cardiotonic steroids.

Modulation of Src activity does not change digitoxin-induced changes in alternative splicing.

The next experiments sought to determine whether Src modulation itself could mimic or block digitoxin-induced changes in alternative splicing. Two genetic overexpression clones for Src were used, each harboring a single amino acid mutation that imparts greatly different effects on Src function. The first, K298M, is a "kinase-dead" mutation, in which a critical lysine residue within the kinase domain has been mutated to methionine.

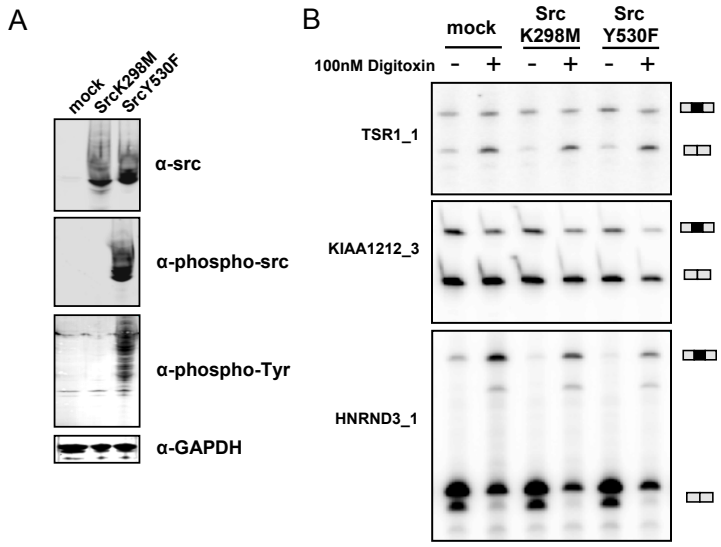

FIGURE 3.3 Overexpression of consititutively active or inactive Src clones has no effect on digitoxin induced alternative splicing changes. (A) Overexpression of two Src clones with different activity in HEK293 cells. SrkK298M is a kinase-dead, inactive form of the Src kinase, while SrcY530F is a consititutively active form of the enzyme. Widespread autophosphorylation as well as general tyrosine phosphorylation is observed after expression of SrcY530F, but not after expression of SrcK298M. (B) Three digitoxin targets retain regulation in the Src modulated cells. TSR1_1 and KIAA1212_3 are repressed by digitoxin treatment while the drug enhances HNRND3_1.

This expressed Src can not function as a kinase, and can potentially act as a dominant negative enzyme, as the remaining regulatory domains of the protein remain intact. The second Src

mutant used here is a Y530F mutant, which displays constitutive active phenotype. This can be seen by the striking Src-autophosphorylation as well as widespread increase in generic tyrosine phosphorylation in transfected HEK293 cells (Figure 3.3A). We overexpressed each of these clones along with a pcDNA control transfection, and performed digitoxin treatments after 48 hours. We found that the baseline inclusion levels of three digitoxin-regulated exons was unchanged with Src overexpression. Furthermore, the induced changes by 100nM digitoxin treatment was unimpeded either by active or inactive Src overexpression. We concluded that if the downstream modulations of Akt or ERK1/2 are responsible for digitoxin-induced changes in alternative splicing, they are occurring in a Src-independent manner.

Chemical inhibition of cardiotonic steroid modulated signaling enzymes does not alter digitoxin effect on alternative splicing

After finding that molecular genetic manipulations of Src kinase function had no effect

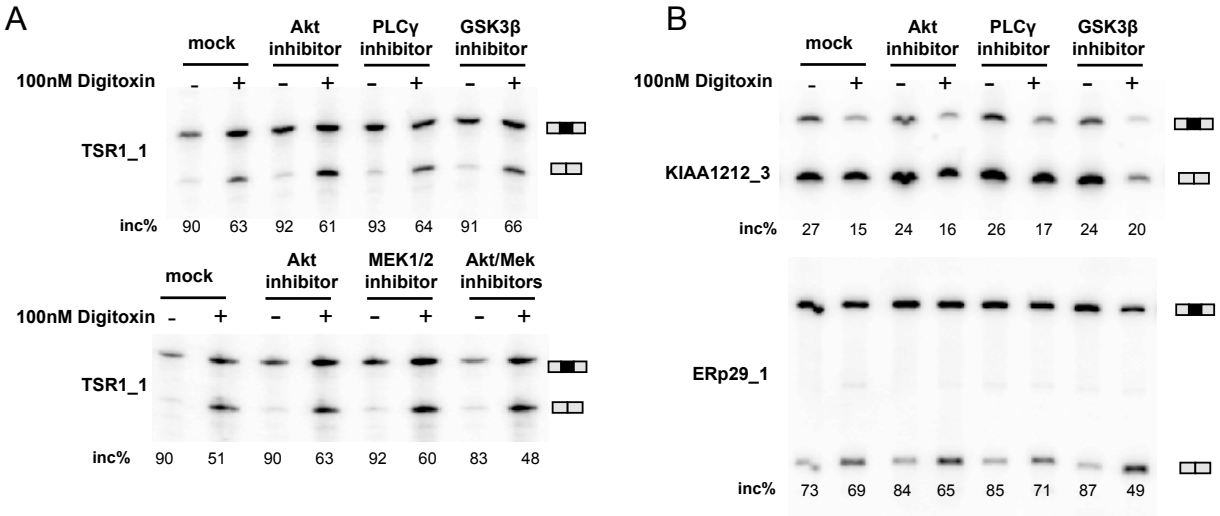

FIGURE 3.4: Chemical inhibition of Src-regulated signaling cascades does not alter digitoxin effect on alternative splicing. (A) Digitoxin-induced skipping of TSR1 alternative event 1 is monitored after blockade of Akt, MEK1/2, GSK3β and PLCγ. Digitoxin treatment is was 100nM for 24 hours in HEK293 cells. (B) To confirm that this result to exons other than TSR1_1, digitoxin regulation of KIAA1212_3 and ERP29_1 were analyzed after blockade of Akt, GSK3β and PLCγ.

on digitoxin induced splicing changes, we sought to analyze enzymes that lie further downstream from Src in cell signaling cascades. We reasoned that if the noted effects by digitoxin on Akt or ERK1/2 were relevant to the splicing changes induced by the drug, then direct inhibition of either those enzymes or those that directly regulate them might prove informative. As noted in Figure 3.1, we used chemical inhibitors to specifically probe four key enzymes in these cell signaling cascades. Independent inhibition of PLCγ, Akt, MEK1/2 and GSK3 each had no significant effect on a strongly digitoxin-regulated alternative exon (Figure 3.4A). We additionally probed for splicing changes in two other digitoxin-regulated exons and found similar results (Figure 3.4B). These results indicate that alternative splicing changes induced by cardiotonic steroids result from a combination of effects or from yet to be identified cellular effects.

Tautomycin treatment blocks digitoxin-induced alternative splicing changes, but loss of PP1 activity does not account for this effect

Our final approach to characterizing the cell signaling modifications that could account for cardiotonic steroid mediated changes in alternative splicing followed from our previous findings. Through an unbiased approach, we found that digitoxin can deplete Tra2-beta and SRp20, two well-known splicing factors. Given that these factors have been well studied for many years, there is some evidence for the cell signaling cascades that regulate them.

Specifically, Tra2-beta is regulated by protein phosphatase 1 (PP1), via a direct protein-protein interaction(50). Therefore, we sought to determine whether PP1 serves a functional role in digitoxin-mediated alternative splicing changes. Using tautomycin, a chemical inhibitor to PP1 as well as other cellular phosphatases, we probed the requirement of PP1 activity after

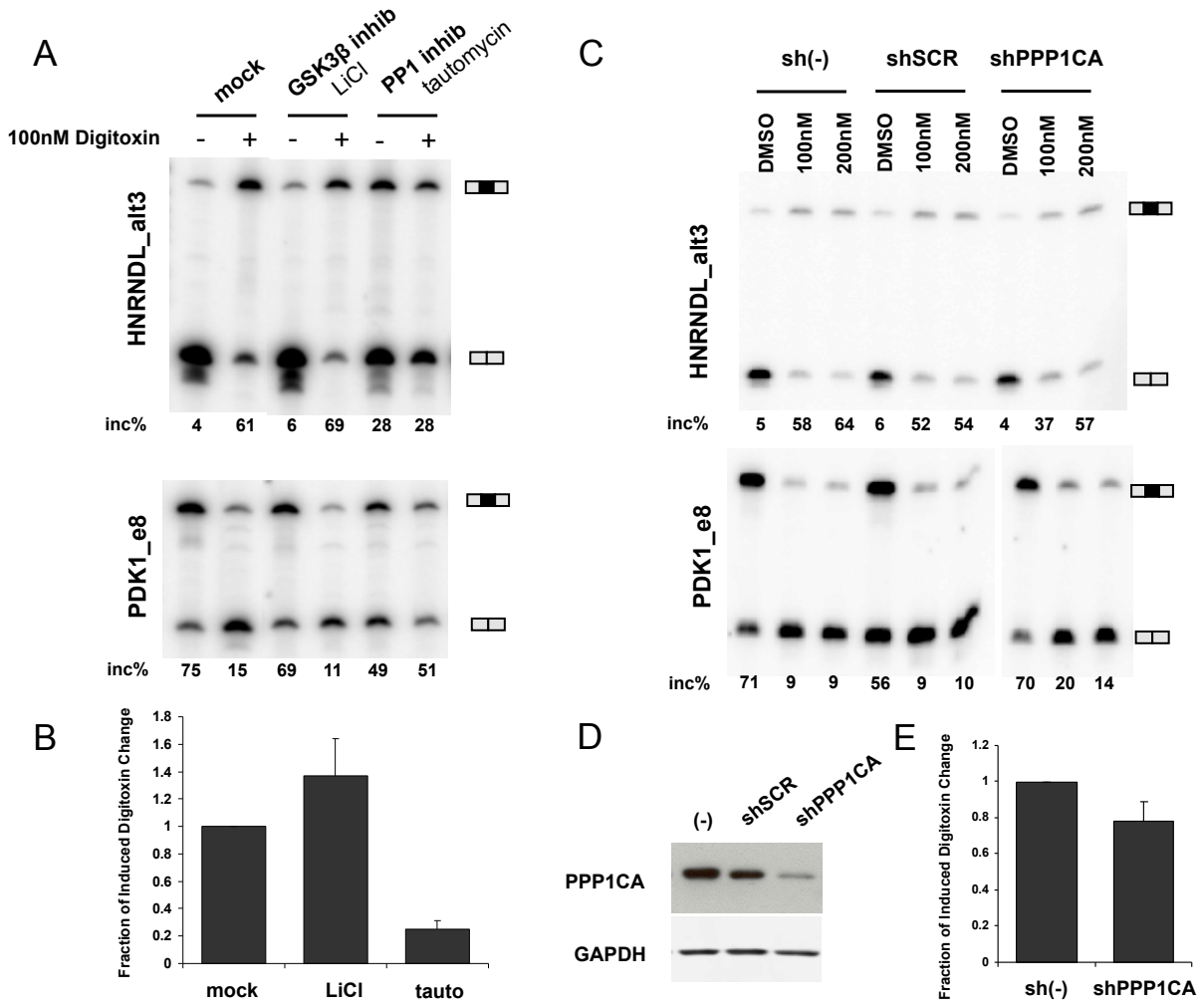

FIGURE 3.5: Tautomycin blocks digitoxin induced splicing changes. (A) Tautomycin pre-treatment changes baseline inclusion level of multiple alternative exons, but blocks further induced changes by digitoxin. (B) For 20 different exons, tautomycin blocks digitoxin induced changes by an average of 75%. (C) Knockdown of PPP1CA, the catalytic subunit has no effect on digitoxin-induced splicing changes. (D) Western blot showing knockdown of PPP1CA and the resulting splicing changes for seven exons in (E) showing at most 20% induced changes.

digitoxin treatment. We found that tautomycin treatment for 24 hours greatly changed the baseline inclusion level of many alternative exons (Figure 3.5A). Importantly, regardless of baseline inclusion level, tautomycin treatment reduced digitoxin-induced alternative splicing changes by an average of 75%, as compared to mock control or inhibition of GSK3, which we have previously shown to have no effect (Figure 3.5A-B). Tautomycin inhibits PP1, but also other cellular phosphatases to a lesser extent. Therefore, we analyzed whether the tautomycin effect was due to PP1 inhibition. We knocked down the catalytic subunit of PP1 (PPP1CA),

using RNAi methods, and tested for modulation of digitoxin effect. We found that the digitoxin effect on alternative splicing was preserved in the PPP1CA knockdown cells. This indicates that either PP1 has residual activity in the knockdown samples, perhaps due to the remaining protein or to functional replacement with a different catalytic subunit, or that PP1 inhibition is not the effect of tautomycin that blocks digitoxin-induced splicing changes.

Conclusions and Future Directions

The first approach to characterize the mechanism by which digitoxin regulates alternative splicing was an unbiased one described above. Essentially, the transcriptome-wide changes in alternative splicing in response to drug treatment were analyzed at the sequence level to implicate specific splicing factors. This method identified two splicing factors, SRp20 and Tra2-beta, which mediate part of digitoxin-induced changes in alternative splicing. We hypothesize that other splicing factor mediators could be identified in similar fashion, as noted above.

In addition to the unbiased approach of digitoxin target identification, we also sought to use a hypothesis-driven approach. For small molecules such as the cardiotonic steroids, previous studies have established many cellular effects of the compounds, including specific perturbations of cell signaling cascades. Specifically the cardiotonic steroids have been shown to activate PLCγ, Akt and MAPK pathways often in a Src-dependent manner(42). Since these pathways have previously been implicated in cell signaling control of alternative splicing, we investigated them first(46,47). Through a combination of molecular genetic and chemical inhibition studies, we found that perturbation of the baseline function of each of these pathways had no effect on the ability of digitoxin to induce robust changes in alternative splicing. Though these experiments did not exhaustively rule out that activation of these cascades has a role in induced

changes, individual modulation of one of them does not appear to account for the bulk of the changes. These findings could potentially result from insufficient perturbation of the pathways (e.g. incomplete inhibition by small molecule antagonists), or the functioning of all pathways in concert could represent combinatorial control over alternative splicing. If this is the case, functional redundancy from other pathways after digitoxin treatment could mask roles of the specifically investigated pathways. Regardless, the use of prior knowledge about a drug mechanism of action can inform the investigation of molecular targets that impact alternative splicing.

The final approach to characterization of digitoxin-induced changes was motivated by the finding that digitoxin treatment specifically depletes SRp20 and Tra2-beta. Previously, it has been reported that interaction with protein phosphatase 1 (PP1) can have effects on Tra2-beta function(50). We investigated a role for PP1 after digitoxin treatment, and we found that chemical inhibition of the enzyme with tautomycin has multiple effects on alternative splicing. First, treatment with tautomycin altered baseline inclusion levels of many digitoxin-regulated alternative exons, and importantly, pre-treatment with tautomycin before digitoxin treatment decreased digitoxin induced changed by an average of 75%. This result was not replicated with knockdown of the catalytic subunit of PP1, PPP1CA, implying that PP1 could have multiple catalytic subunits, or the pleiotropic effects of tautomycin could be responsible for the blockade. The link between a target identified with an unbiased approach and previous work that enabled an hypothesis-driven finding about those targets is strong, and will facilitate the characterization of small molecule alternative splicing regulators that are found in future pursuits. Taken together, we have developed multiple effective methods to discover the mechanism by which small molecules can regulate alternative splicing. These methods will be particularly useful as

more drugs with splicing regulation function are discovered, and will be especially important as such compounds move toward therapeutic development.

CHAPTER 4: TUMORIGENESIS-ASSOCIATED ALTERNATIVE SPLICING: THERAPEUTIC POTENTIAL AND A STRATEGY FOR IDENTIFCATION OF SMALL MOLECULE REGULATORS

Introduction: Cancer and alternative splicing

Misregulation of alternative splicing plays an important role as both a cause of disease and in modification of the course of the illness(6). Evidence of alternative splicing misregulation has become increasingly described during tumorigenesis and the progression of many cancer subtypes. Perhaps the most striking example of a role for alternative splicing changes in cancer is the almost ubiquitous switch in splicing of the PKM transcript from PKM1 to PKM2, a change that enables solid tumors to grow advantageously under anaerobic conditions(51). Other alternative splicing changes accompany progression of cancers with specific molecular signatures, enabling their growth advantage over neighboring non-diseased tissue(52). In many of these cases, a switch in splicing of some transcripts imparts invasive potential unto the tumor and accompanies transitions to metastatic states of the cancer(53). For these reasons, characterization of these alternative splicing changes and the signaling cascades that regulate them have been the subject of active research.

Cancer is often viewed as collection of mutations in a specific cell or tissue that ultimately results in progression to an advantageous growth state. Interestingly, misregulation of many tumorigenesis-associated splicing events can occur in the absence of mutation in the spliced transcript itself. This implies that upstream regulation of the splicing choice or downstream stabilization of the encoded transcripts has changed in the tumor. Importantly, given the lack of mutation, it is possible to restore the correct regulation of the splicing event,

and potentially decrease the growth potential of the cancer cell. Restoration of correct splicing profiles can be achieved in the laboratory by standard molecular genetic approaches, but as a viable therapeutic, it would be ideal to target the splicing event with commonly used types of medical intervention. These can include, but are not limited to, anti-sense oligonucleotide therapy as well as small molecule or chemical treatments.

Results

Generation of dual fluorescence reporters for six tumorigenesis-associated alternative splicing events

We assembled a short list of alternative splicing events that are specifically altered in multiple cancer types. Selection of alternative splicing events that are appropriate for high throughput screening and drug targeting entailed meeting three specific criteria. The first is that the alternative splicing change must be correlated with tumorigenic progression either as an associative or causative event. The second is that there should be either known or hypothesized functional change between the protein isoforms that are encoded by alternative transcripts. In this way, the alternative splicing change can potentially aid in tumor advantage over neighboring cells. Finally, the association of known splicing factors or signaling cascades on the alternative splicing event is useful, but not required. Prior knowledge of the splicing regulators enables downstream investigation of drug targets. As shown in Chapter 6, however, we can identify the regulators of a splicing event with unbiased high throughput screens as well.

The alternative splicing events that met these criteria are summarized in Table 4.1, showing the transcript, splicing event type and type of cancer with which the change is associated. Additionally, it is noted whether the appropriate counter reporter was created, and whether either

reporter was established as a stable cell line. Multiple exons were chosen to be studied, because

Table 4.1: Tumorigenesis-associated alternative splicing events investigated in these studies

Transcript	*BIN1*	*FAS*	*RAC1*	*MAX*	*FGFR1*	*FGFR2*
Function	maintenance of membrane curvature, regulation of endocytosis, and regulation of c-myc transcription factor through protein-protein interation	receptor for FAS ligand, a pro-apoptotic signal	small GTPase that promotes activation of NFκB gene programs	forms a heterodimer with c-myc transcription factor to promote myc-dependent transcription	membrane bound receptor-tyrosine kinase for FGF family of ligands	membrane bound receptor-tyrosine kinase for FGF family of ligands
Exon	12a (13)	6	3b	5	α	IIIb / IIIc
AS Type	cassette exon	cassette exon	cassette exon	cassette exon	cassette exon	mutually exclusive exons
Tumorigenic Direction	exon inclusion	exon skipping	exon inclusion	exon inclusion	exon skipping	skipping / inclusion
Function Switch	exon 12a inclusion disrupts myc interaction and sequestration, promoting myc dependent transcription	exon 6 skipping eliminates transmembrane domain, creating a soluble receptor	exon 3b inclusion blocks RAC1 activation of RelB dependent transcription without disrupting activation of NFκB signaling	exon 5 inclusion encodes a truncated isoform, deltaMAX that increases metabolic advantage for tumor cells	exon α skipping results in β isoform of FGFR1 that has increased affinity for FGF	mutually exclusive switch from IIIb to IIIc accompanies epithelial-mesenchymal transition, changing FGF ligand affinity from FGF7/10 to FGF2. In prostate cancer, the switch may promote androgen-indepdendent growth
Splicing Regulators	enhancers: SRSF1, hnRNPA2B1	enhancer: TIA1 repressors: PTBP1,HuR	enhancer: SRSF1 repressor: SRSF3	enhancer: hnRNPA1	enhancer: SRSF6 repressor: PTBP1	IIIb repressors: PTBP1, hnRNPA1 IIIb enhancers: TIA1, ESRP1/2 IIIc repressors: TRA2B, hnRNPM IIIc enhancers: Rbfox1/2
pFlareA clone	YES	YES	YES	YES	YES	YES
pFlareA stable line	HEK293, WM239A	HEK293	HEK293	-	HEK293	HEK293
pFlareG clone	YES	-	-	-	-	-
pFlareG stable line	HEK293	-	-	-	-	-

replication of endogenous splicing regulation is not always possible in a reporter construct. Each alternative exon and its flanking intronic sequence was cloned into the pFlare dual fluorescence alternative splicing reporter. For some events, such as the FGFR2 mutually exclusive splicing event, there is evidence in the literature for specific intronic regulatory elements. In these cases, the cloned area included known regulatory sequences. For exons with no identified regulatory elements, such as BIN1 exon 12a, the closest approximation for potential regulatory elements was used, namely mammalian conservation of intronic residues. Isolation of an alternative exon and insertion into a mini-gene construct can disrupt regulation of exon inclusion. This was evident for the FAS exon 6 mini-gene, which showed no exon inclusion in the pFlare reporter constructs. If an alternative splicing reporter does not include the alternative exon in its mature mRNA transcript, it is less appropriate for screening and investigation. This result can also reflect the baseline regulation in the cell type in which the reporter is expressed. We utilized the HEK293 cell line for all high throughput screens, so strong repression can preclude identification of more subtle enhancers in screens. This selection step excluded the FAS exon 6 and RAC1 exon 3b reporters.

The next step in characterization of the alternative splicing reporters is to verify that the reporter responds to known splicing regulators, if they exist. For example, as described in the following chapter, the FGFR2 splicing reporters displayed differential response to known factors. The FGFR2-IIIb reporter did respond to PTB modulation as described previously, while FGFR2-IIIc reporter displayed cryptic splice site activation when Rbfox proteins were expressed. In a case such as FGFR2-IIIc, the cryptic splice site indicates an altered state of splicing regulation, and would potentially disrupt the readout of the dual fluorescence reporter. This selection step excluded the FGFR2-IIIc reporter from further study.

As a proof-of-concept study, we performed a focused screen on the pFlareA-FGFR2-IIIb reporter cell line to identify small molecules that modulate the splicing event. In this screen, described in the following chapter, we identified drugs that fall into classes of known function (e.g. inhibitors of cellular kinases and phosphatases), and that have been previously reported to affect alternative splicing. FGFR2 exon IIIb is repressed during the epithelial-mesenchymal transition that is displayed in many types of tumor progression, and the drugs identified here represent a step toward finding treatments that can reverse molecular consequences of EMT and potentially inhibit tumor progression.

Conclusions and Future Directions

Alternative splicing changes accompany both development and disease. Misregulation of critical alternative splicing events can fundamentally alter the nature of the cells, disabling normal development or promoting disease pathogenesis. Splicing diseases that result from genetic mutation of a critical splicing event, can potentially be reversed by exciting interventions such as antisense oligonucleotide therapy aimed at a specific sequence. In other diseases,

however, misregulation of a splicing factor enables a transcriptome wide disruption of alternative splicing, which en masse can contribute to the disease phenotype. In these cases, the targeting of the splicing factor or the pathways that regulate it present an alternative and perhaps more wide-ranging therapeutic potential. In fact, the coupling of targeting a specific RNA sequence and a factor that binds to it may present the most efficient targeting possible. Small molecule modulators are one avenue to inducing changes of a splicing factor function, and have recently been investigated for their ability to alter alternative splicing.

Here, we have focused our studies on specific alternative splicing changes that promote cellular proliferation or impart a growth advantage to tumor cells over neighboring normal tissue. We have identified multiple alternative exons that show aberrant splicing patterns during tumorigenesis. Importantly, these events are misregulated despite an absence of mutations in their specific sequences. This implies that the upstream cascades are inappropriately regulating these exons and likely many others as well. For each of the exons investigated, we constructed dual fluorescent alternative splicing reporters that are amenable to high throughput screens for both genetic and small molecule modulators. We highlight in the following manuscript that a focused screen of small molecules identified drugs that can modulate the alternative splicing of FGFR2 exon IIIb. This exon is aberrantly skipped in multiple cancer types, and the therapeutic potential lies in reversal of its repression. Interestingly, these drugs inhibit known cell signaling regulators, such as protein phosphatase 2A, tyrosine kinases TrkA and PDGFR, and CaM kinase(54).

Targeting of a splicing factor or its regulation cascades will almost certainly have many effects within a cell, especially with respect to alternative splicing. Though each of these described screens has focused on finding regulators of a specific alternative event, the hits are

likely also modulating many alternative splicing events within the transcriptome. This result is not entirely unexpected, given the fact that splicing factors typically control an entire network of targets. However, for some diseases including cancer, it is becoming increasingly clear that the misregulation of an alternative splicing factor has broad ranging effects that in summation promote the disease phenotype. PTB has been implicated in the misregulation of the alternative splicing of many transcripts, including FAS, FGFR1/2 and PKM(10,55-57). Strikingly, the effect of PTB expression on each of these events serves to enhance tumorigenic potential of the cell. Therefore, the therapeutic effect of inhibiting PTB function would spread over each of these targets and not just a single exon. This inherent difference between small molecule targeting and anti-sense oligonucleotide targeting may facilitate their independent development for different types of diseases. Development of each method is also attractive for the potential synergy that the two therapies present for an alternative exon.

Finally, the application of these high throughput screens as described in Chapter 6 highlight their ability to identify novel regulators of an alternative splicing event. Through genetic screens, elucidation of the splicing factors and cascades that regulate them will provide an insight into the cellular changes that promote tumorigenesis. Drug discovery and target identification will be facilitated when coupled to small molecule identification strategies, and the route to therapeutic intervention will be expedited.

CHAPTER 5: HIGH THROUGHPUT SCREENING FOR SMALL MOLECULE MODULATORS OF FGFR2-IIIb PRE-mRNA SPLICING

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High Throughput Screening for Small Molecule Modulators of FGFR2-IIIb pre-mRNA Splicing

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ABSTRACT

Splicing of pre-mRNA transcripts is an essential mechanism of gene regulation that is often mis-regulated in human disease. Aberrant splicing can result from disruption of specific RNA sequences or from changes in the function of proteins that control the splicing reaction. Identification of small molecules that target the splicing process is one approach to development of therapeutics that reverse disease-associated splicing aberrations. Additionally, drugs that modulate the splicing reaction or machinery are useful research tools for investigation of splicing mechanism and regulation. We previously reported a high-throughput screening method for identification of small molecule modulators of alternative splicing. Here, we apply this method to find drugs that regulate inclusion of the alternative exon IIIb in the FGFR2 pre-mRNA transcript. We describe development of a suitable FGFR2 splicing reporter and perform a pilot drug screen. From a screen of bioactive compounds, we identify drugs that can change the splicing dependent readout of the reporter, including inhibitors of protein phosphatase I/II, Cam kinase and other tyrosine kinases. We highlight the capacity of this assay to facilitate identification of general splicing modulators and discuss potential applications and adaptations.

INTRODUCTION

pre-mRNA splicing is an essential mechanism of gene regulation

Pre-mRNA splicing is the process in which a ribonucleoprotein complex called the spliceosome removes introns from a primary transcript and ligates its exons together [1]. The spliced exons produce an mRNA containing mature protein coding sequence. The splicing process is remarkably consistent in its choice of splice sites, and these choices can be regulated to allow different mRNA sequences to arise from the same pre-mRNA transcript, in a process called alternative splicing. In this way, genes often yield multiple mRNAs and encoded proteins to increase diversity of eukaryotic proteomes. Alternative splicing patterns often show developmental and tissue-specific expression. Misregulation of splicing is seen in human disease and can be a direct cause of pathology [2]. Splicing regulatory mechanisms thus present a promising target for therapeutic intervention.

Splicing is controlled by *cis* and *trans*-acting elements

In the canonical splicing reaction, consensus 5' and 3' splice sites and the intronic branch point sequence define the sites of chemistry at the intron ends [1]. These sites bind core components of the spliceosome and their disruption can repress splicing of the associated exon. Alternative exons contain *cis*-acting elements that alter the splicing choice through the binding of splicing regulatory proteins. These regulatory elements are characterized by their location (exonic or intronic), and their effect on the exon (enhancer or repressor). The proteins that bind these RNA motifs can interact with the spliceosome or alter its assembly to stimulate or inhibit the use of particular splice sites [1].

The misregulation of splicing can result from disruption of either *cis*-acting elements or *trans*-acting splicing factors [2]. One example of disease caused by aberrant splicing is

frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17), in which the microtubule associated protein tau (MAPT) locus is mutated [3]. These mutations cause increased inclusion of MAPT exon 10 and lead to disease [3]. Misregulation of *trans*-acting factors can also promote disease which may occur in the increased MAPT exon 10 inclusion seen in non-hereditary causes of dementia [3]. Aberrant splicing regulation is also seen during tumor and cancer progression [2]. An example is splicing of the fibroblast growth factor receptor 2 (FGFR2). FGFR2 contains a pair of mutually exclusive alternative exons (IIIb and IIIc) whose differential inclusion changes the ligand affinity of the receptor [4]. Inclusion of exon IIIb is seen in cells of epithelial origin and responds primarily to ligands in the FGF7 sub-family of growth factors as well as FGF10. Exon IIIc inclusion occurs in cells of mesenchymal origin and does not share affinity for the FGF7 family, instead preferring FGF2 as a ligand. Importantly, a switch of exon IIIb to IIIc inclusion accompanies the epithelial to mesenchymal transition exhibited by many tumor types, including prostate cancer [5,6]. In prostate cancer, the IIIb to IIIc switch is one potential mechanism for tumor transition from androgen-dependence to independence, a hallmark of anti-androgen therapeutic resistance.

Perhaps due to the tightly controlled nature of the FGFR2 splicing event, IIIb/IIIc exon choice is modulated by a number of splicing factors. Previous studies have elucidated *cis*-elements and their associated *trans*-acting factors that can regulate either IIIb or IIIc exon choice. Exon IIIb contains a weakened 3' splice site and splicing factors hnRNP-A1 and polypyrimidine tract binding protein (PTB) repress IIIb inclusion in mesenchymal cells [7-9]. In epithelial cells, multiple proteins have been implicated in enhancing the inclusion of IIIb, including TIA-1 and the recently characterized epithelial splicing regulatory proteins (ESRPs) [10,11]. Other splicing factors such as Tra2-beta and hnRNP-M can repress IIIc inclusion [12,13]. The RbFox family of

splicing regulators has also been implicated in regulation of exon IIIc [14], and their functions may depend on interacting partners such as hnRNP-H and –F [15]. Additionally, fluorescence-based screens have yielded insight into the tissue-specific splicing patterns of FGFR2 and expression of the factors that control it [16,17,9].

Small molecule splicing modulators

Recent reports have described identification of small molecule compounds that can modulate pre-mRNA splicing [18-22]. We described identification of drugs that regulate inclusion of MAPT exon 10, using a dual fluorescence spicing reporter [20]. We found that these small molecules do affect MAPT splicing, though their effects were not restricted to just this splicing event. These compounds may target the splicing by interfering with splicing factors directly or by modulation of upstream cell signaling pathways. Applications for such small molecules are widespread. At a basic science level, identification of compounds such as isoginkgetin, pladienolide and spliceostatin, which inhibit assembly of the spliceosome, are valuable for elucidation of the molecular mechanisms underlying this process [22,21,18,23]. Drugs that allow the exogenous modulation of specific splicing events have therapeutic potential for many human diseases, including cancers that display splicing aberrations [2,19,20]. Here we describe a screening strategy to target the mutually exclusive exons IIIb/IIIc in the FGFR2 transcript.

RESULTS

Defining the FGFR2 exon IIIb/c regulatory regions

Exons IIIb and IIIc are spliced in a mutually exclusive manner in which only one exon is chosen for the mature transcript, while transcripts containing both or neither exon are rarely seen (Fig 1a,1c). One hallmark of alternative exons is increased sequence conservation in the

adjacent introns. These conserved sequences often correspond to important regulatory elements for splicing of the alternative exon. In the case of FGFR2 exons, IIIb is surrounded by approximately of 600 base pairs of highly conserved intronic sequence, while IIIc lies within 1.6 kilobases of conserved sequence (Fig 1b). Within these conserved regions are previously characterized splicing regulatory elements, including binding sites for ESRPs, PTB, RbFox2, and the hnRNP-H and –M proteins. To ensure that any potential intronic regulatory sequences are included in the screening reporter, we amplified these regions from genomic DNA for insertion into the vector.

Creation of dual-fluorescence FGFR2 splicing reporters

To detect splicing changes in cell based screens, we developed FGFR2 splicing reporters in the pFlare splicing-dependent dual fluorescence vector [20] (Fig 1d). We first inserted the conserved genomic region encompassing both exon IIIb and exon IIIc and their adjacent introns into the splicing reporter. However this reporter showed significant amount of double exon inclusion and double exon skipping, which does not appropriately replicate the regulation of the two exons and would confound the analysis (data not shown). We therefore created single cassette exon reporters for each of the mutually exclusive exons (Fig 1e). We selected HEK293 cells stably expressing each reporter and single cell clones were isolated and expanded.

Confirmation of appropriate splicing by each reporter clone

In HEK293 cells, endogenous FGFR2 transcripts show nearly 100% inclusion of exon IIIc, and exclusion of exon IIIb as measured by RT-PCR (Fig 1c). Similarly, pFlare:FGFR2-IIIb shows less than 10% exon inclusion by RT-PCR. This yields predominantly green fluorescence from cells expressing the IIIb reporter (Fig 1e-f). In contrast, the pFlare:FGFR2-IIIc reporter line shows greater than 90% exon inclusion resulting predominately red fluorescence from the

cells. After confirming that splicing of the reporters mirrors the pattern of the endogenous transcript, we sought to confirm that the reporters respond to known regulators FGFR2 splicing. Since PTB has been reported to induce repression of the IIIb exon, we expect its activity to be high in the reporter cells [8]. We tested the effect of PTB depletion on splicing in the pFlare:FGFR2-IIIb reporter. Indeed, we find that the reporter increases exon IIIb inclusion after double knockdown of PTB and its homolog, nPTB (Fig 2a). Conversely, transient expression of additional PTB induced further exon skipping of the pFlare:FGFR2-IIIb reporter (Fig 2a). These findings confirm that the pFlare:FGFR2-IIIb reporter is subject to the expected splicing regulation.

Additionally, we sought to confirm that the pFlare:FGFR2-IIIc reporter cells respond to known regulators of exon IIIc. RbFox2 has been reported to be required for repression of exon IIIc in cells of epithelial origin [14]. Rbfox1, a homolog with an identical RNA recognition motif to Rbfox2, has a similar effect. We found that expression of Rbfox1 represses exon IIIc in the pFlare:FGFR2-IIIc reporter line. Rbfox1 also alters choice of the IIIc 5' splice site to an alternative splice site within the exon (Fig 2b). This may be a difference in the splicing of the reporter and the endogenous transcripts. Since the pFlare:FGFR2-IIIb reporter did not show cryptic splicing activation, we used this line for subsequent small molecule screens.

Small molecule screen identifies compounds that modulate FGFR2-IIIb splicing

To provide a characterization of the signaling pathways affecting exon IIIb splicing, we screened two libraries of drugs. We screened the BioMol bioactive compound library and the Prestwick FDA-approved drug library for modulators of FGFR2-IIIb inclusion. These libraries contain a total of 1624 unique small molecules, many of which have substantial information available on the drug target and mechanism. The cell line was cultured in 384 well plates.

Compounds from the BioMol and Prestwick libraries were robotically added at Day 0. Live fluorescent detection of the splicing reporters was performed daily for four days, after which the HEK293 cell line had reached confluence. We performed scans of the treated plates on a Typhoon Phosphorimager (GE). We did an initial fluorescence detection scan approximately 60 minutes after drug addition to provide a baseline fluorescence measurement and to identify autofluorescent small molecules. Autofluorescent compounds were discarded from this analysis. If needed, they can be manually validated in the secondary RT-PCR steps. Screening over multiple days produced five time points of splicing detection. This allows the identification of molecules inducing a rapid change in splicing (a potential primary effect) and molecules whose effect on splicing is slower (possibly secondary or indirect effects).

Non-fluorescent cells without the reporter and 'vehicle only' controls were used for appropriate background correction and treatment normalization (diagrammed in Fig 3a). The 'non-fluorescent cell' control allows for accurate quantification of background fluorescence from media, plate and cells. The vehicle only control (DMSO) serves as negative control for drug treatment. Placement of these controls in many rows across the plates aids in detection of variation on the scanning platform of the phosphorimager (Fig 3a). The dual fluorescent nature of the reporter allows detection of increased skipping as well as increased inclusion of the exon (Fig 3b). The IIIb reporter used here detects changes primarily in one direction due to its baseline inclusion profile (i.e. detection of increased skipping of the pFlare:FGFR2-IIIb reporter is less likely due to its <10% inclusion ratio at baseline). We defined the effect of compounds on splicing based on the slope of a linear best fit to the fluorescence ratios plotted over time (Fig 3c). We defined hits as compounds having RFP/GFP slopes lying outside three times the standard deviation of the DMSO controls across the libraries. For this pFlare:FGFR2-IIIb

screen, this cut-off yielded 63 compounds that induce IIIb exon inclusion (increased red fluorescence) and 23 compounds that decrease IIIb inclusion (as measured by increase in green fluorescence). Using other exon reporters, the cutoff can be made more or less stringent to adjust for how many compounds are testable in secondary screens.

Small molecule regulators of FGFR2-IIIb splicing fall into categories of known function

Small molecules identified in this screen include many that were previously identified in the screen for modulators of MAPT exon 10 (Table 1) [20]. As expected, more compounds were detected that increase exon IIIb inclusion, presumably due to the low baseline exon inclusion in the reporter cell line. Compounds that increase exon IIIb splicing fall into categories also seen in previous screens. The largest groups of drugs include antibiotics and anticancer drugs. Drugs with antiproliferative effects may induce global changes in cell signaling that alter splicing regulation. Compounds in this category include inhibitors of translation, oxidative phosphorylation and essential catabolic processes. In addition to drugs that globally alter cellular function, we identified compounds with more specific molecular effects. These include inhibitors of tyrosine kinases (AG-879), p38 MAP kinase (SB-202190) and Cam kinase (KN-93). These are interesting given the known switch from exon IIIb to IIIc inclusion during tumor progression, implying that the activation of growth-inducing cell signaling cascades could trigger the FGFR2 splicing switch. Another class of molecules altering fluorescence readout of the FGFR2-IIIb reporter contains protein phosphatase inhibitors, including cantharidin which tested positively in both libraries.

Finally, we also identified molecules that induce exon IIIb skipping. One well-represented class of these drugs is the cardiotonic steroids. We previously reported that cardiotonic steroids induce inclusion of MAPT exon 10 [20]. The cardiotonic steroids can also

cause translational inhibition that reduces RFP production from the dual fluorescence reporter. Thus, the effect of cardiotonic steroids on FGFR2-IIIb will need further validation. In addition to the drug classes mentioned, we found other small molecules that have been identified previously, shown in Table 1. These hits are likely regulators of essential cellular processes that have downstream effects on alternative splicing.

CONCLUSIONS

We aimed to identify small molecules that can modulate alternative splicing of the FGFR2 pre-mRNA transcript. Through construction of a dual-fluorescence splicing reporter containing FGFR2 exon IIIb and its intronic control sequences, we were able to faithfully detect FGFR2 splicing regulation in HEK293 cells. We performed a targeted small molecule screen with two libraries of known bioactive compounds. We identified drugs that induced inclusion of the IIIb exon, resulting in increased red fluorescence, as well as drugs that repressed IIIb splicing resulting in enhanced green fluorescence. We will continue to characterize the specificity of these drugs for exon IIIb splicing, as the screen likely detected small molecules of varying degrees of specificity.

Many of the small molecules that we identified are regulators of cell signaling cascades that are essential for cell growth and proliferation. Fitting with the repression of exon IIIb in proliferating tumors, we found that inhibition of tyrosine kinases, p38 MAP kinase and protein phosphatases 1/2 can all increase red fluorescence in the pFlare:FGFR2-IIIb reporter cell line. Further characterization of these small molecules will enhance our understanding of the cell signaling pathways that alter FGFR2 exon choice during tumorigenesis. Additionally, though investigation of the known splicing factors that control this splicing choice, we hope to identify the factors that play key roles during the epithelial-mesenchymal transition and thus present

potential therapeutic targets. Finally, the pFlare:FGFR2-IIIb reporter will allow much more comprehensive screens of large combinatorial libraries to produce leads for therapeutic development.

METHODS

Construction of pFlare:FGFR2 reporters and stable HEK293 cell line

FGFR2 DNA sequence was amplified from HEK293 genomic DNA, and cloned into the pFlare vector as described previously [20]. Stable transfection in HEK293 cells was carried out by lipofectamine-mediated transfection of linearized plasmid and subsequent antibiotic selection. RT-PCR primers for the reporter were (DupE1Bgl-F: 5'-AAACAGATCTACCATTGGTGCACCTGACT-3' and EGFP-N: 5'-CGTCGCCGTCCAGCTCGACCAG-3') and primers for endogenous FGFR2 were (E7-F: 5'-TGGTCGGAGGAGACGTAGAG-3' and E10-R: 5'- TACGTTTGGTCAGCTTGTGC-3'). Each PCR used a reverse primer with 5' terminal FAM fluorophore for fluorescent detection after denaturing PAGE. Before electrophoreseis, FGFR2 products were cleaved with restriction enzymes AvaI, EcoRV or both to distinguish IIIb and IIIc exon inclusion. Gels were imaged directly on a Typhoon Phosphorimager (GE) and bands quantified with ImageQuant TL (Amersham/GE).

Transient transfection of expression clones was performed using lipofectamine 2000. RNA from expression experiments was harvested after 48 hours and from knockdown experiments after 72 hours. PTB modulation studies were performed as described previously [24]. For Rbfox1 expression, a full length mouse Rbfox1 cDNA was expressed under the CMV promoter.

Small molecule screen

Reporter cell lines were maintained in OPTI-MEM + 4%FBS + L-glutamine. Media for screens lacked phenol red to minimize background fluorescence. As a note, culturing in media that contains decreased serum can decrease proliferation rates and enable longer timecourse. Here we use media containing 4% fetal bovine serum, as opposed to the 10% that is typically used to culture HEK293 cells. Another consideration for timecourse length is the differential stability of the fluorescent proteins. In our screens with HEK293 cells, we find that increased stability of RFP will begin to skew fluorescent ratios after growth for approximately 4 days.

The cells were added to transparent black bottom 384 well plates and allowed to adhere to the plate surface (approximately 2 hours). The small molecules were added robotically at the UCLA Molecular Screening Shared Resource (MSSR). Plates were maintained under normal tissue culture conditions during the 96 hour time course except during scans on the Typhoon phosphorimager (GE).

Data analysis

Fluorescence intensity was quantified using ImageJ software and SpotFinder3.1.1. After background subtraction of fluorescence from the 'non-fluorescence cell control' wells, red and green fluorescence at each timepoint was fit to a linear model for each well using R statistical software. The slope of the fluorescence change for each well was normalized to the median of all DMSO control wells in the screen. Wells that contained slope differences that were greater than 3 times the standard deviation of the DMSO wells were scored as positive hits.

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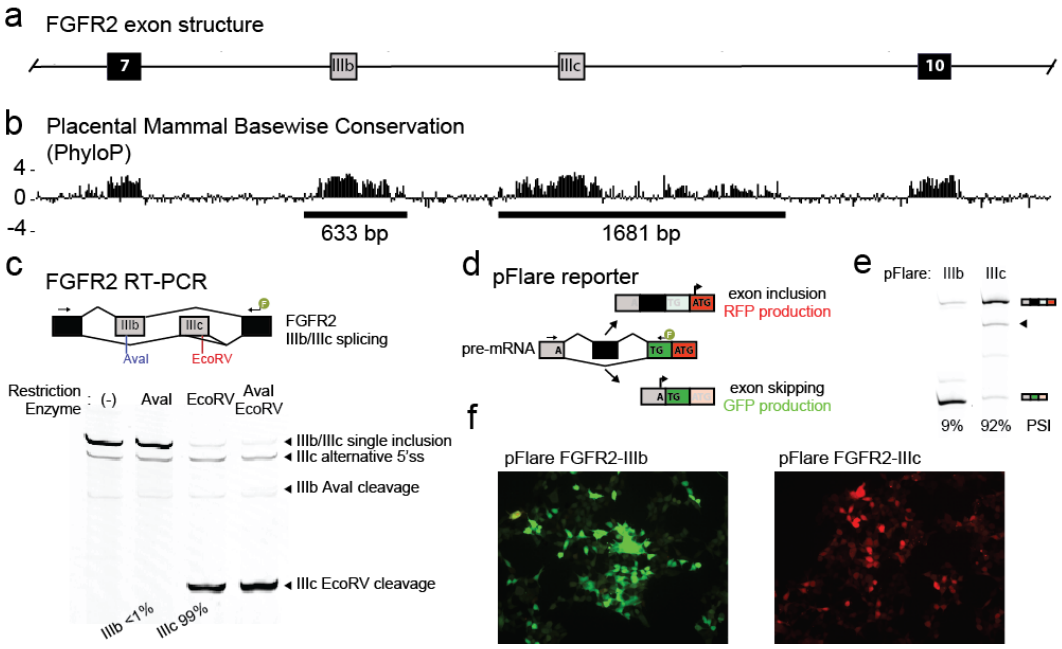

Figure 1: Insertion of FGFR2 exons IIIb / IIIc into pFlare reporter. (a) Exon structure of FGFR2 including the two mutually exclusive alternative exons IIIb and IIIc. (b) Mammalian conservation plot as calculated by PhyloP and the UCSC Genome Browser. Regions of increased conservation surround the two alternative exons. (c) In HEK293 cells, exon IIIc is exclusively included in mature mRNA transcripts as shown by specific restriction enzyme cleavage by EcoRV. (d) The pFlare reporter changes fluorescence output based on alternative inclusion of a cassette exon. (e-f) Insertion of FGFR2 exon IIIb into pFlare shows almost complete exon skipping and green fluorescence while pFlare:FGFR2-IIIc generates red fluorescence, indicating exon inclusion.

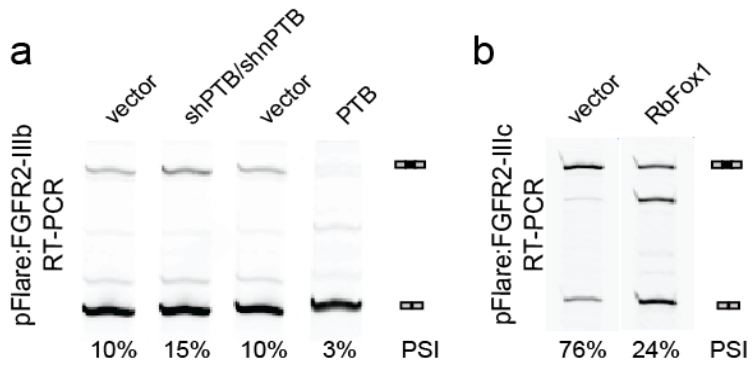

Figure 2: pFlare:FGFR2 reporters respond to known splicing factor regulators. (a) Knockdown of PTB and its homolog nPTB induces IIIb exon inclusion while expression of PTB decreases IIIb inclusion (b) Expression of RbFox1 represses exon IIIc inclusion. The PSI for IIIc is given as percent of full-length exon inclusion

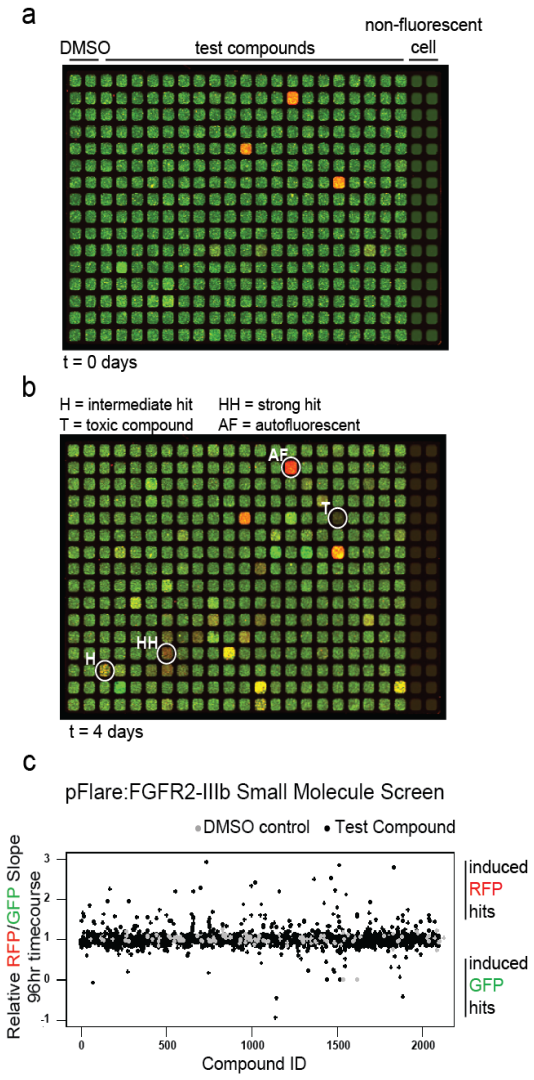

Figure 3: High throughput screen for small molecule modulators of FGFR2-IIIb inclusion. (a) Experimental set up of a screening plate. Image taken on Day 0, 60 minutes after addition of small molecules. Important controls in each row are the no cell controls in columns 23 and 24 as well as DMSO treatment controls in columns 1 and 2. (b) The same screening plate after 4 days incubation. Highlighted are compounds that exhibit toxicity (T), autofluorescence (AF) and hits that switch from GFP to RFP production (H, HH). (c) Summary of BioMol and Prestwick screens, with highlight of compounds that change fluorescence readout to increased RFP or GFP. DMSO controls are plotted in gray.

INDUCTION OF RFP (exon IIIb inclusion)

COMPOUND	LIBRARY	ΔRFP/GFP†	TYPE / TARGET
Camptothecine (S,+)	Prestwick	6.243	DNA topo I inhibitor (cytotoxic)
Cephaeline dihydrochloride	Prestwick	2.912	translational inhibitor
Cantharidin	BioMol	2.399	PP2A inhibitor (also PP1)
Cantharidin	Prestwick	2.322	PP2A inhibitor (also PP1)
Gossypol	Prestwick	2.187	proapoptotic, calcineurin inhibitor
5-iodotubercidin	BioMol	1.880	kinase inhibitor (ERK2, adenosine kinase, CK1/2)
Ethoxyquin	Prestwick	1.870	quinoline-based antioxidant, food preservative
Tyrphostin 9	BioMol	1.681	PDGF receptor tyrosine kinase inhibitor.
AG-879	BioMol	1.651	tyrosine kinase inhibitor (TrkA)
KN-93	BioMol	1.576	CaM kinase inhibitor

INDUCTION OF GFP (exon IIIb skipping)

COMPOUND	LIBRARY	ΔRFP/GFP†	TYPE / TARGET
Nicardipine hydrochloride	Prestwick	-0.089	calcium channel antagonist
Ouabain	BioMol	0.161	cardiotonic steroid (Na/K channel inhibitor)
Digoxin	Prestwick	0.182	cardiotonic steroid (Na/K channel inhibitor)
Digitoxigenin	Prestwick	0.250	cardiotonic steroid (Na/K channel inhibitor)
Lanatoside C	Prestwick	0.308	cardiotonic steroid (Na/K channel inhibitor)

† **ΔGFP/RFP** values are normalized to median of DMSO controls

TABLE 1: Selected hits identified in the FGFR2-IIIb high throughput small molecule screen.

CHAPTER 6: HIGH THROUGHPUT SCREEN FOR REGULATORS OF BIN1 EXON 12a ALTERNATIVE SPLICING

Introduction

Alternative splicing aberrations accompany many disease processes, including tumorigenesis and the development of cancer(52). The changed splicing of certain targets imparts a growth advantage to developing cancer cells. The wider implication from these specific splicing changes is that altered activity of the splicing regulatory pathways has a multi-fold effect on a cell's tumorigenic potential. For example, increased expression of polypyrimidine tract binding protein 1 (PTBP1) changes splicing of many targets, and often results in expression of protein isoforms that facilitate growth advantages. For PTBP1, these target exons include FAS exon 6, as well as the mutually exclusive exon in both FGFR2 and PKM transcripts(10,55-57). For specifically altered exons, identification of the upstream regulation cascades will enable an increased understanding of routes to cancer development and present molecular targets with therapeutic potential.

BIN1 (AMPH2/bridging integrator 1) is a gene with a nearly ubiquitous expression pattern that includes multiple alternatively spliced variants(58). The BIN1 protein contains at least five discrete domains with diverse functions including a canonical BAR (BIN1-amphiphysin-Rvs167) domain, a phosphoinositide-binding domain, a clathrin associated protein-binding domain, a myc-binding domain and an SH3 (src homology type 3) domain (Figure 5.1A). Consistent with these different protein domains, the protein displays a wide range of functions within the cell, including regulation of membrane invagination as required for endocytosis and specifically vesicle formation in the nervous system(59). It is also required for the creation of T tubules in muscle cells, which are specialized membrane structures that

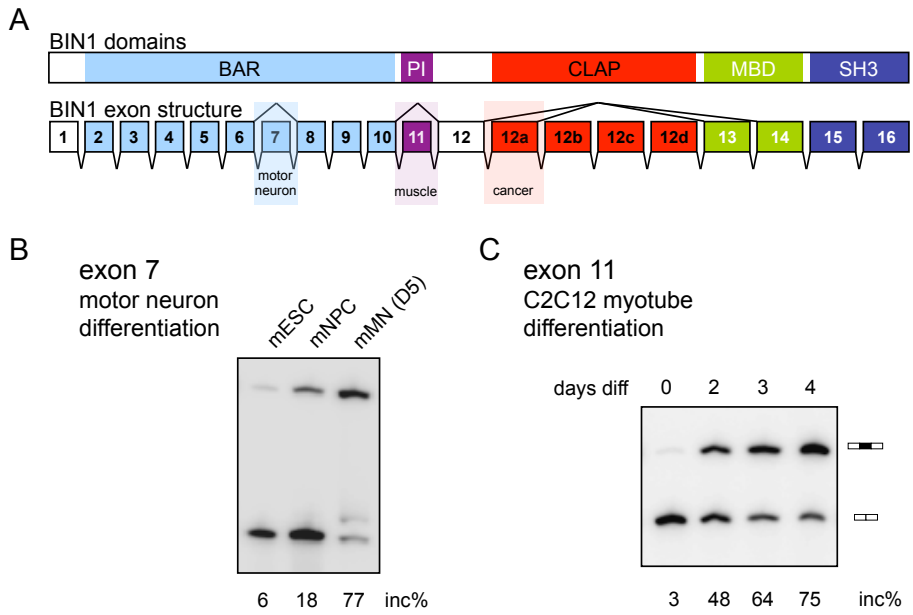

FIGURE 6.1: BIN1 alternative splicing patterns. (A) BIN1 protein and mRNA structure. Alternative exons encode a portion of the BAR domain and CLAP domains, and the entire PI domain. (B) Exon 7 is enriched in motor neuron development from mouse ES cells (with help of Anthony Linares. (C) PI encoding exon 11 is included only in muscle cells as is seen during myotube differentiation from C2C12 myoblasts.

facilitate coupling of neuronal excitation and muscle contraction (58). Moreover, it has a distinct role in tumor suppression due to its ability to bind c-myc and sequester it from its roles in transcriptional activation (60). Lastly, single nucleotide polymorphisms in BIN1 have been linked by genetic studies to a predisposition to development of Alzheimer's disease, perhaps indicating additional roles in the nervous system that are essential to maintenance of neuronal circuits specifically relating to memory and cognition(61).

BIN1 function is intricately linked to its alternative splicing patterns, which are largely regulated in a tissue-specific manner. Specifically, exon 7 in the BAR domain, exon 11 in the PI domain and exons 13-17 spanning the CLAP and MBD all display tissue-specific expression, and some change the function of the protein in those tissues (Figure 5.1A). Exon 11 encodes the entire PI domain, and is only expressed in mature muscle cells to enable the role of BIN1 in T-tubule formation. Similarly, exon 7 is largely skipped in embryonic stem cells and precursor motor neurons, but shows a robust increase in inclusion in mature motor neurons, where it could potentially have a role in the differentiated cells. Exon 12a (which has been alternatively called

exon 13 in the literature) is one of four cassette exons that encodes the CLAP, but its inclusion disrupts BIN1 interaction with c-myc. In this way, inclusion of exon 12a eliminates the tumor suppressive roles of the BIN1 protein.

In previous work, it has been shown that exon 12a inclusion correlates strongly with invasive and metastatic potential of multiple melanoma samples and cell lines. In these cases, exon 12a inclusion yields a BIN1-12a protein isoform whose expression correlates with increased proliferation rate and survival of the tumor cells(60). The current model holds that c-myc has a protein-protein interaction with the SH3 domain of the BIN1 protein, when exon 12a is not included. Encoded in the exon 12a amino acid sequence is a peptide that mimics c-myc, and can compete for binding to the SH3 domain, either through intra- or inter-molecular interaction. This competition releases c-myc, which then drives a gene expression program that enhances cell proliferation and tumorigenesis. Interestingly, re-expression of a BIN1 isoform lacking 12a decreases viability of the same melanoma cells, indicating that a reversal in splicing could have a therapeutic impact for tumor types that display this molecular characteristic. To this end, multiple splicing factors have been identified that have the ability to regulate this alternative event. SRSF1 (SF2/ASF) has been reported to be an enhancer of exon 12a, consistent with its oncogenic role in multiple cancer types(36). Additionally, hnRNP-A2B1 knockdown induces skipping of the 12a exon, though it is unclear whether this is a direct or indirect effect(62).

Here, we use high throughput genetic and small molecule screens to identify potent regulators of exon 12a inclusion and to begin characterization of drugs that can modulate the same event, presumably through modification of the genetic regulators. To carry out these screens, we utilized the pFlare dual fluorescence splicing reporters in both a screen and counter

screen format(34). Through use of these reporters, we found that hnRNP-K and other splicing factors that bind C-rich sequences have a distinct repressive role for exon 12a inclusion. Additionally we identify other factors, both RNA-binding and some upstream, which influence 12a inclusion in the reporter cell lines.

Results

Creation of two dual fluorescence BIN1-exon 12a splicing reporters

The region surrounding BIN1 exon 12a was amplified from HEK293 genomic DNA. The amplified region contains the 129 base pair alternative exon, along with 233 base pairs from the proximal upstream intron and 225 base pairs downstream of the alternative exon. These intronic regions contain three blocks of conserved sequences (FIGURE 6.2A), one extending from 110 base pairs upstream of the exon to the exons start and two downstream. The downstream conserved elements extend from the 5'splice site to 105 base pairs away, and the second lies +160 to +212 base pairs downstream from the 5' splice site. This region was cloned into the pFlareA dual fluorescence splicing reporter (described in Chapter 4, Figure 6.2B). The pFlareA-BIN1-12a reporter splices exon 12a

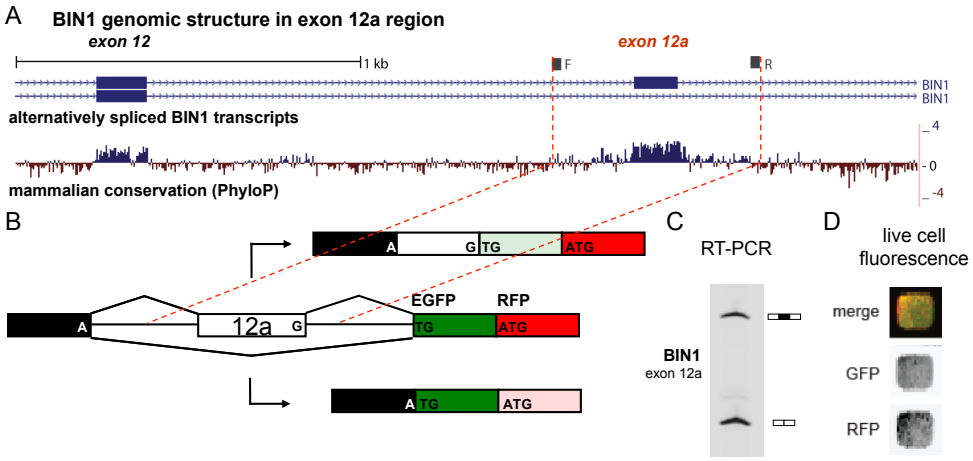

FIGURE 6.2: Amplification and cloning of the pFlareA-BIN1-12a dual fluorescence reporter. (a) Genomic structure of the BIN1 locus showing exon 12a, along with region of amplification. Three conserved regions of intronic sequence are included in the amplicon. (b) Cloning of exon 12a region into pFlareA reporter. Skipping of exon 12a will result in GFP while inclusion produces RFP. (c) RT-PCR of transiently transfected HEK293 cells shows baseline inclusion and production of both fluorescent proteins (d).

at in intermediate level, both by transient transfection in HEK293 cells, as well as after stable transfection in both HEK293 and WM239A cell lines (Figure 6.2C). These intermediate levels of inclusion result in production of both EGFP and RFP from the reporter, which can be detected by the Typhoon phosphorimager (Figure 6.2D).

The dual fluorescence splicing reporter is amenable to high throughput screens and faithfully detects alternative splicing changes. In addition to alternative splicing modulation, however, it can also change its fluorescent output for other reasons. As described in Zheng and Black (unpublished, 2012), these can include treatments that change reporter transcription rates, that alter translation of the reporter proteins, or that affect stability of the fluorescent protein products. To identify true positive hits, a counter reporter can be used that switches fluorescent output that results from a specific splicing change. In the case of the BIN1 exon 12a reporter, the initial reporter was made in the pFlareA backbone, in which exon inclusion results in RFP production while skipping results in GFP. In the counter reporter, pFlareG, the terminal adenosine in the upstream exon is changed to a guanosine. With only this change, both inclusion and skipping of exon 12a would result in RFP production. However, two single nucleotide mutations (C123A, C125G) in exon 12a create in in-frame start codon for the GFP open reading frame. This change hypothetically results in GFP with exon inclusion and RFP after exon skipping, effectively switching the fluorescence readout (FIGURE 6.3a). Once constructed, the fluorescent output of the pFlareG-BIN1-12a-ATG1 counter reporter yielded only red fluorescence (FIGURE 6.3b). Potential sources for total red fluorescence were: 1) mutation in the EGFP open reading frame, which was confirmed to be mutation-free by sequencing, 2) an alternate 5' splice site used in the mutant reporter, which was determined to not be the case by additional mutagenesis studies that shift the frame of the exon, and 3) the start site was not strong

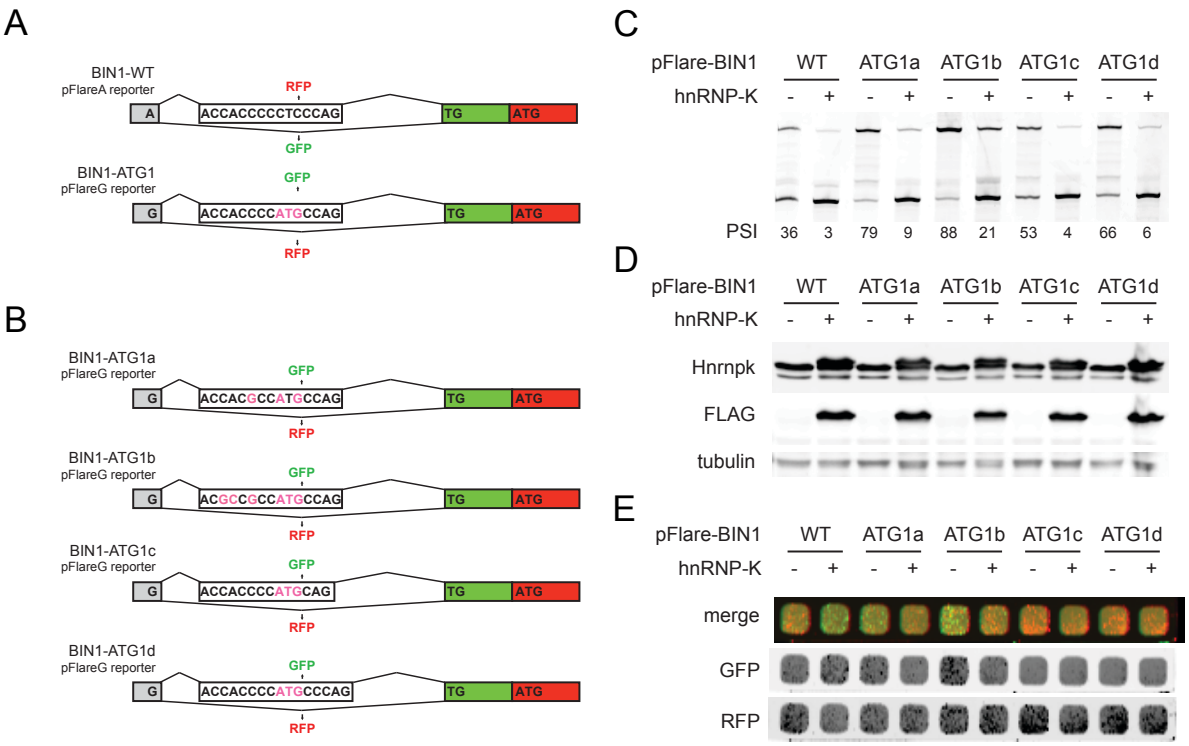

FIGURE 6.3 Creation of the pFlareG-BIN1-12a counter reporter. (A) Schematic of pFlareA and pFlareG reporters. Two substitutions in the wild type sequence yields an ATG that is in frame with the downstream EGFP open reading frame. (B) Four variations on pFlareG-BIN1: -a and –b contain base pair substitutions to create a stronger Kozak sequence. –c and –d delete and add one base to the exon respectively to change reading frame of the ATG. (C) All four pFlareG reporters retain repressible regulation by hnRNP-K as shown in RT-PCR with Western blot (D) for protein expression. (E) Fluorescence of cells transfected with indicated reporter with or without hnRNP-K. pFlareA-BIN1-WT switches from RFP to GFP, while pFlareG-BIN1-ATG1a and –b switch from GFP to RFP, all consistent with splicing change. pFlareG-BIN1-ATG1c and –d display only red fluorescence, consistent with out-of-frame ATG start for EGFP.

enough to initiate translation at the exonic ATG. This third source of total red fluorescence was addressed by making two additional mutants that contained a C120G mutation (pFlareG-BIN1-12a-ATG1a) or C117G, A118C and C120G (pFlareG-BIN1-12a-ATG1b). Each of these changes were designed to create a Kozak sequence that more closely resembles the consensus Kozak sequence of $(GCC)_n$. Each of these ATG-1a and -1b reporters showed differential fluorescence (FIGURE 6.3d), and ultimately the ATG1a reporter was chosen as the BIN1-12a counter reporter because it contains the minimum number of mutations from the wild type sequence (3 total point mutations).

When making such changes to the exon of an alternative exon, it is critical to confirm that a known regulatory site has not been created or eliminated by the mutations. The three

known splice factor regulators of BIN1-12a alternative splicing are Srsf1, Hnrnp-A2B1 and Hnrnp-K. By transient transfection in HEK293 cells, we validated the responses of both the pFlareA and pFlareG mutant reporters to these known at the RNA splicing level and at the fluorescence output level factors (FIGURE 6.3C-E, and data not shown). Additionally, we used existing prediction software to identify any potential exonic sequence enhancers that the ATG1a mutations might have created(63). ESEfinder, the exonic splicing enhancer prediction software, found that the mutations minimally disrupted a potential SRp40 ESE and one of three potential SC35 sites in the terminal 25 exonic bases. Since there is no evidence supporting regulation of exon 12a by these factors, and because the mutations did not create any artificial enhancers that could give rise to false positives, we proceeded to prepare the lines for high throughput screening.

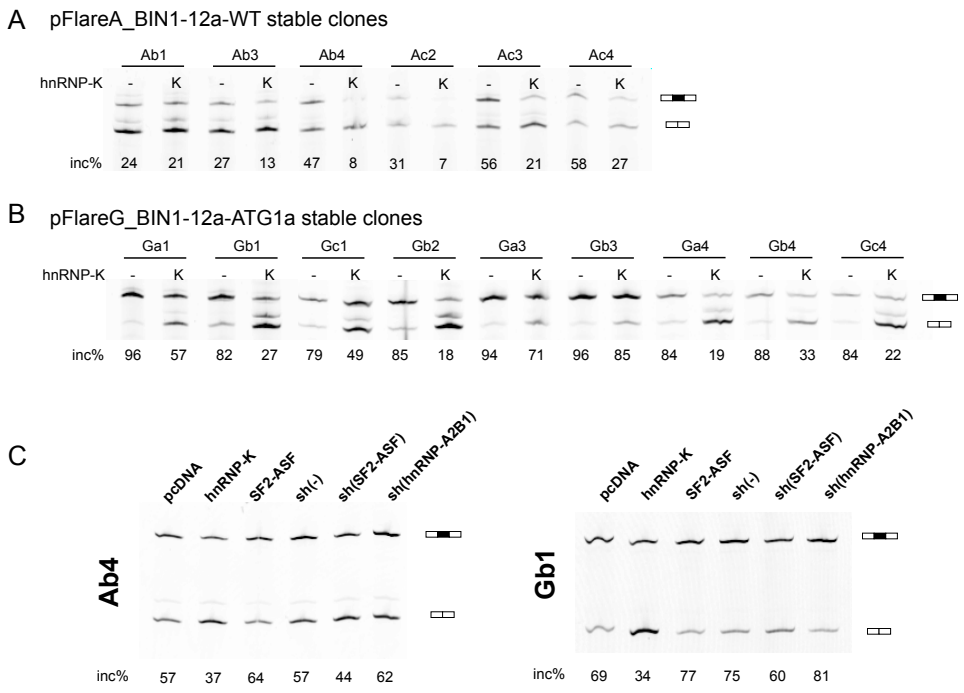

FIGURE 6.4 Stable integration of pFlareA and pFlareG-BIN1-12a reporters. (A-B) Stable integration of pFlareA and pFlareG reporters in HEK293 cell lines yields multiple clones with varying degrees of baseline inclusion and repression by hnRNP-K. (C) Two selected reporters respond to modulation of all three known BIN1-exon 12a regulators, hnRNP-K, SF2-ASF (SRSF1) and hnRNP-A2B1.

Stable integration of the two reporters into HEK293 cells produced the two screening lines that would be used for high throughput screening. At least 6 clonal lines of each stable reporter were isolated, as integration location

can greatly affect alternative splicing choices and patterns. Indeed, we found that baseline exon inclusion varied in clonal cell lines for each reporter (Figure 6.4A). In addition to baseline variation in exon inclusion level, the clones showed varying response to transient transfection of a known regulator, hnRNP-K (Figure 6.4A-B). We sought to choose clones that responded most robustly to hnRNP-K, and also that displayed splicing regulation by SRSF1 and hnRNP-A2B1 as well (Figure 6.4C). Next we sought to optimize the fluorescence readout of the reporter lines and find the line that would be most amenable to high throughput screening.

Optimization of molecular genetic screening conditions

The first screens performed on the pFlare-BIN1-12a reporters were cDNA overexpression screens. In this library, a combination of nearly 16,000 full-length mouse and human cDNAs are transiently expressed in high throughput format and imaged over a course of four days. To select reporter clones for the high throughput screen, we tested multiple clones of each type for responsiveness to known regulators in 384-well reverse transfection format. As we find that each clonal line responds differently to known regulators, this will ensure a high sensitivity screen is used for the large scale screen.

We tested 6 clonal lines for each reporter type for responsiveness to hnRNP-K, shSRSF1 and shHNRNP-A1 in high throughput format. Each cell line has a different baseline level of fluorescence, both in intensity as well as GFP/RFP ratio. This is illustrated by comparing lines Ac3 and Ac4 (Figure 6.5). In the hours after transfection, line Ac3 displays strikingly more RFP expression than line Ab4, despite similar levels of exon 12a splicing in the reporters (58% inclusion in Ac3 vs. 48% inclusion in Ab4; Figure 6.5A). These differences could be due to differences in the integration sites of the reporters such as local chromatin structure, as well as

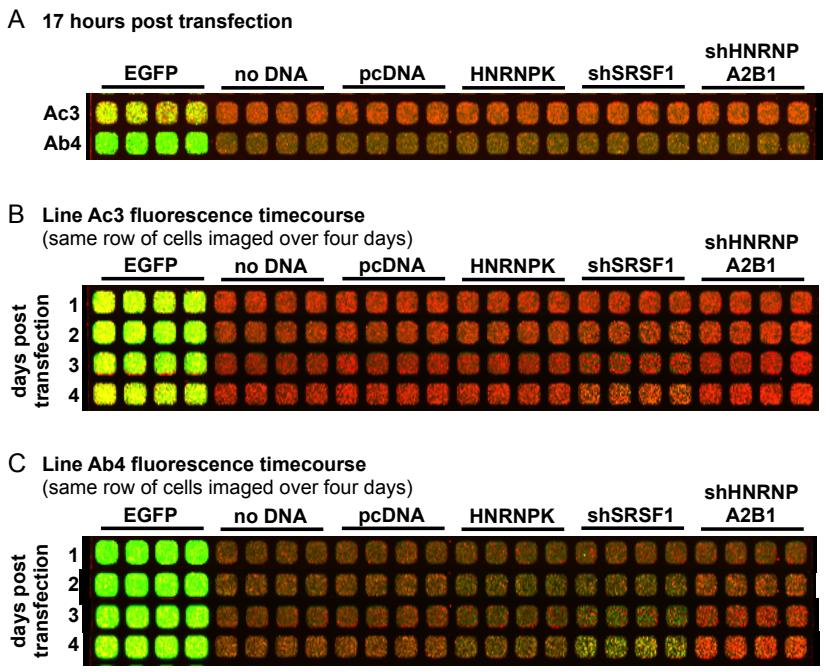

FIGURE 6.5 Varying fluorescent responses of pFlare reporters. (A) For two clonal pFlareA-BIN1-12a cell lines, the baseline fluorescence less than 24 hours after transfection varies widely. (B) Modulation of known exon 12a splicing regulators has no observable effect on fluorescence output of line Ac3 (C) Line Ab4 robustly changes fluorescence after modulation of all three known splicing regulators over the course of 96 hours.

number of times integration occurred. Regardless of baseline fluorescence ratio, the critical parameter to measure was ratio change after expression of a known regulator. Clonal line Ab4 changed fluorescence more robustly than the other clones tested (Figure 6.5B-C). Significant changes in fluorescence were detectable as early as 48 hours after transfection for hnRNP-K cDNA overexpression experiments, and at 72 hours after transfection for shRNA mediated knockdown of SRSF1 (Figure 6.6). For the cDNA overexpression screens here, we identified hits at 48 hours post transfection with the hypothesis that we would be enriching for factors that have a direct effect on BIN1 exon 12a splicing rather than potentially indirect mechanisms that would become more evident with time. One clear advantage to the cell based screening assay is the ability to segregate hits into early and late in this way. Hits that only appear after 72 or even 96 hours post transfection could represent indirect effects, but these expressed proteins could also represent master regulators that lie far upstream of the ultimate alternative event. Understanding both direct and indirect

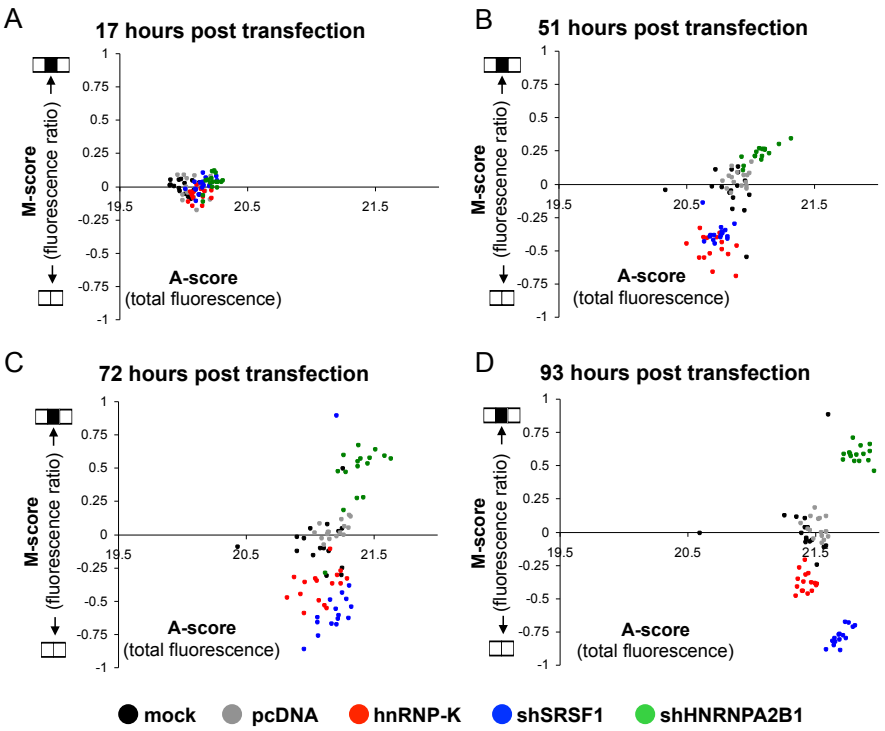

FIGURE 6.6 M-score and A-score parameters identify splicing regulators by fluorescence readout (A-D) Over four days, RFP and GFP measurements were made for control plates containing treatments that can modulate known exon 12a regulators. M-score is a normalized deviation from control fluorescence ratio, while A-score is an log score based on total fluorescence that is a proxy for cell viability. Repressive treatments (hnRNP-K expression, SRSF1 knockdown) result in M-score < 0 while enhancing treatment of hnRNP-A2B1 knockdown results in M-score > 0.

regulators is a route to characterization of entire pathways that display aberrant function in disease. Identical experiments identified line Gb1 as the most suitable pFlareG-BIN1-12a reporter to use for the counter screen (data not shown), and we proceeded to the high throughput screen using lines pFlareA-BIN1-12a_Ab4 and pFlareG-BIN1-12a_Gb1.

High throughput screen of mammalian MGC cDNA expression library identifies genetic regulators of BIN1 exon 12a splicing

We screened the MGC cDNA expression library that contains nearly 16,000 cDNA expression clones for mouse and human genes for their effect on BIN1 exon 12a splicing. To identify a hit, a cDNA had to change fluorescence of both the pFlareA-BIN1-12a reporter as well as the pFlareG-BIN1-12a reporter. This screen and counter screen method has been shown to effectively filter out hits that have primary effect on reporter transcription or translation, or that

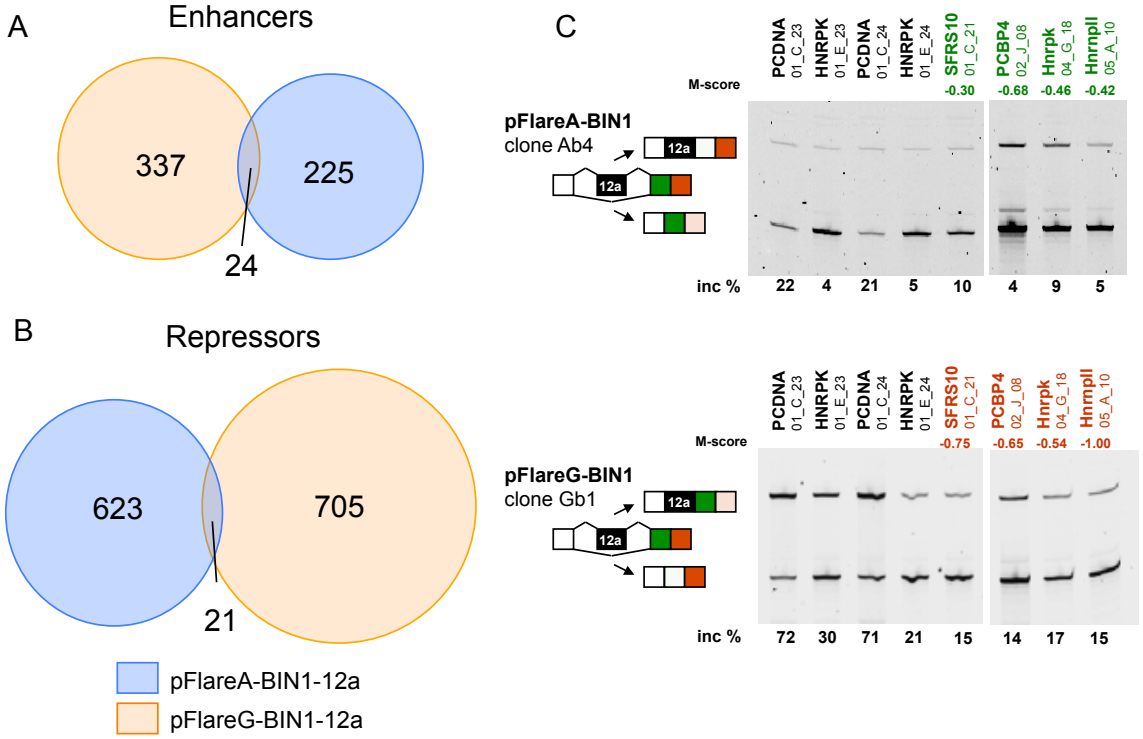

FIGURE 6.7 MGC cDNA expression library screen identifies both enhancers and repressors of BIN1 exon 12a splicing (A-B) Both pFlareA and pFlareG screens identify large sets of treatments that modify fluorescent readout, but only small overlapping sets significantly change fluorescence readout in both. (C) Validation of four repressors that were identified in the overlapping hit set. Shown are controls in black, and hits with associated M-score for each reporter.

have an effect on stability of the fluorescent protein outputs. While the relatively strict requirement of requiring a hit in both screens could potentially increase a false negative rate, we believe that the benefit of enriching for true positive hits will effectively outweigh this effect.

The first analyzed set of hits came from 48 hour timepoint data. Based on the optimization plates, we expected to identify factors that have direct effects on the BIN1 exon 12a splicing event, such as the optimized control, hnRNP-K. The fluorescent values were normalized with a cross-plate normalization method described in Zheng et al. (*in preparation*), in which intra-well variation in fluorescence is normalized to the expected variation for each reporter line. Log scores were generated for each well for both total fluorescence ('A-score') and fluorescence ratio ('M-score'). Wells with total A-scores that were less than two standard deviations from the mean were discarded, as total fluorescence is a proxy readout for cell viability. After this correction, there remained 12235 non-control cDNA transfections for

analysis. For each well, fluorescence ratio was corrected by the median ratio in a given plate yielding the M-score. A positive M-score indicates increased exon inclusion and negative score indicates exon skipping. Significant hits were chosen by a cutoff determined to be 1.5 standard deviations away from the mean M-score. Each independent analysis identified large sets of cDNAs that could modify the fluorescence ratio given this relatively relaxed cutoff. However, comparing the two screens for overlapping hits yielded small sets of high confidence regulators: 21 repressors and 24 enhancers (Figure 6.7A).

Table 6.1 Overlapping hits from two BIN1-exon 12a cDNA overexpression screens

BIN1 exon 12a repression

Gene Symbol	RNA-binding	Gene Desription
Brunol4	Y	Bruno-like 4, RNA binding protein (Drosophila)
Hnrpll	Y	Heterogeneous nuclear ribonucleoprotein L-like
SFRS10	Y	Splicing factor, arginine/serine-rich 10 (transformer 2 homolog, Drosophila)
Pcbp1	Y	Poly(rC) binding protein 1
Pcbp4	Y	Poly(rC) binding protein 4
Csda	Y	Cold shock domain protein A
Ints9	int	Integrator complex subunit 9
Shisa4	-	Shisa homolog 4 (Xenopus laevis)
Hnrnpk, Hnrpk	Y	Heterogeneous nuclear ribonucleoprotein K
Necap1	-	NECAP endocytosis associated 1
Rbm38	Y	RNA binding motif protein 38
Fastk	-	Fas-activated serine/threonine kinase
LASS5	-	LAG1 homolog, ceramide synthase 5
Zc3h10	pot	Zinc finger CCCH type containing 10
MFAP4	-	Microfibrillar-associated protein 4

BIN1 exon 12a enhancement

Gene Symbol	RNA-binding	Gene Desription
EFR3B	-	EFR3 homolog B (S. cerevisiae)
LECT1	-	Leukocyte cell derived chemotaxin 1
CLK2	int	CDC-like kinase 2
RAB3IL1	-	RAB3A interacting protein (rabin3)-like 1
IQCK	-	IQ motif containing K
MGAT4B	-	Mannosyl (alpha-1,3-)-glycoprotein beta-1,4-N-acetylglucosaminyltransferase, isozyme B
Pxmp2	-	Peroxisomal membrane protein 2
Dhdds	-	Dehydrodolichyl diphosphate synthase
CXorf26	-	Chromosome X open reading frame 26
Map3k11	-	Mitogen-activated protein kinase kinase kinase 11
Map3k3	-	Mitogen-activated protein kinase kinase kinase 3
Hnrnpr	Y	Heterogeneous nuclear ribonucleoprotein R
Mrps23	-	Mitochondrial ribosomal protein S23
Kif1c	-	Kinesin family member 1C
Nadsyn1	-	NAD synthetase 1

Y = yes, int = RNA/RNA factor interacting, pot = potential nucleic acid binding

Of note, ten of the top fifteen repressors are known RNA binding or RNA interacting proteins (Table 6.1). Many of these are also known splicing factors including Hnrnpll, Hnrnpk, SFRS10 and Brunol4. Hits were validated by isolating RNA from the hit wells and assaying BIN1-exon 12a splicing in the reporter (Figure 6.7B). hnRNP-A2B1 cDNA was not present in

this library, and therefore not identified as a repressor of BIN1-12a splicing. Reassuringly, hnRNPK was identified as a hit, as it had previously been shown to be a strong repressor of BIN1-12a splicing. Each of the other repressors remains to be validated, but there are notable signaling molecules as well, including Fas-activated serine threonine kinase (Fastk), which has previously been shown to regulate FAS alternative splicing(64,65).

The list of identified enhancers contains fewer cDNAs that have known RNA binding or interacting properties. The most notable are hnRNP-R, which is an established RNA-binding protein and CLK2, a kinase that has been shown to regulate the SR family of splicing factors. Noticeably absent from the hits is SRSF1, a known enhancer of exon 12 a function. It was a hit in the pFlareA-BIN1-12a screen, but was discarded for not overlapping with the pFlareG-BIN1-12a hits. This could be the result of mutation in the pFlareG reporter, though it did retain SRSF1 regulation in control experiments. Alternatively, though the protein can enhance 12a inclusion, it may not be the strongest enhancer in an unbiased screen. The remaining enhancer hits contain proteins that can have a positive impact on cell growth and proliferation, such as Map3k3 and Map3k11, or otherwise affect cellular metabolism. These hits will be interesting to investigate as it could place oncogenic BIN1 alternative splicing downstream of growth signals which ultimately synergize to enable tumorigenic growth advantage.

Characterization of hnRNP-K as a regulator of BIN1 exon 12a alternative splicing

Srsf1 and hnRNP-A2B1 have been established in the literature to regulate exon 12a of the BIN1 pre-mRNA transcript. The cDNA overexpression screens identified hnRNP-K as a strong repressor of exon 12a, and we sought to confirm and characterize this finding. hnRNP-K contains three highly conserved RNA-binding motifs, termed KH domains for their initial

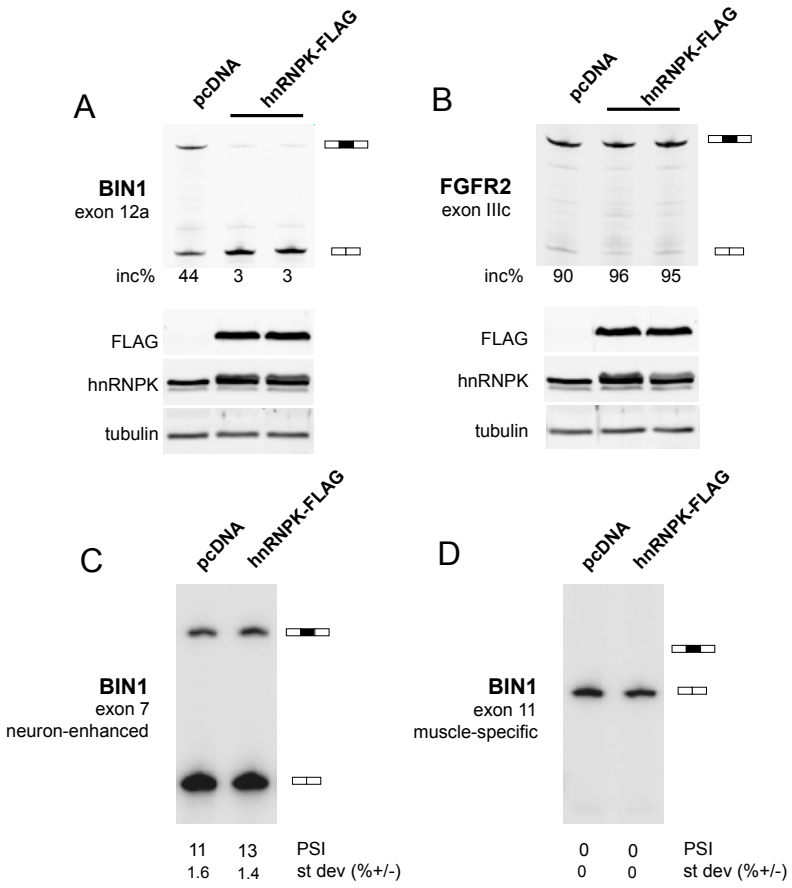

FIGURE 6.8 hnRNP-K is a potent repressor of BIN1 exon 12a splicing (A) hnRNP-K expression represses exon 12a inclusion in BIN1 reporter in HEK293 cells after 48 hours. (B) FGFR2-IIIc reporter shows no response to expression of hnRNP-K. (C-D) Endogenous BIN1 alternative exons 7 and 11 are not hnRNP-K regulated. Standard deviation is calculated from three transfections.

characterization in this protein. Despite not being extensively reported to be a splicing factor, it is clear that modulation of hnRNP-K levels can have widespread effects on alternative splicing patterns(66). It is likely that hnRNP-K modulates the function of other splicing factors, and it is possible that it can directly bind RNA to regulating splicing as well. Evidence of hnRNP-K in the literature has been largely described roles in altering translation and stability of target mRNA transcripts. Interestingly, hnRNP-K can interact with eIF4E to stabilize the c-myc transcript(67). This makes its potential role in regulation of c-myc function through BIN1 alternative splicing even more intriguing, as it could have dual levels of control over myc-dependent gene expression. Furthermore, hnRNP-K has been extensively researched for its role in tumorigenesis and malignant transformation. Surprisingly, it has been strongly implicated in both tumor suppressive and oncogenic roles(68-70). For example, it is a cofactor for p53 and is required for DNA-damage induced gene expression, a tumor suppressive role. However, it has also been reported to be overexpressed in colorectal cancer, among others, where its expression

is correlated with decreased survival. Clearly its role in tumor cells is complex and will require more study to fully understand.

We found that hnRNP-K is a potent repressor of BIN1-12a alternative splicing when overexpressed in cell culture. We performed hnRNP-K overexpression experiments in both human HEK293 cells and mouse N2A cells, and found that the splicing of the BIN1-exon 12a reporter was repressed (Figure 6.8A, and data not shown). To determine whether hnRNP-K directly regulates BIN1 exon 12a or works through in indirect mechanism, we sought to identify a *cis*-acting RNA motif that is required for hnRNP-K induced repression. First, we verified the effect of hnRNP-K was specific to the BIN1-12a reporter construct by co-transfecting a reporter for the FGFR2 exon IIIc cassette exon. This reporter vector is identical to the pFlareA-BIN1-12a reporter, except with the FGFR2 IIIc alternative region cloned instead. We found that overexpression of hnRNP-K had no effect on splicing of this reporter, and had minimal effect on two other splicing reporters (MAPT exon 10 in the pFlareA vector and Dup-51 in the previously described Dup minigene reporter, data not shown). In human cell lines, we have been unable to detect this region of the endogenous BIN1 transcript via standard RT-PCR techniques. Therefore, experiments focused on 12a studies have utilized the BIN1-12a reporter construct to determine the mechanism of hnRNP-K regulation.

Importantly, hnRNP-K expression does not influence splicing of either of the other alternative exons in the BIN1 transcript (Figure 6.8C-D). Exon 8, which is enhanced in motor neurons, is not changed by hnRNP-K expression and exon 11, which is muscle-specific, is not detectable in HEK293 cell line. We conclude that the effect on BIN1 is specific to the alternative region including exon 12a, and next sought to implicate specific sub-regions of this nearly 1000 base pair region.

The regulation of BIN1 pre-mRNA by hnRNP-K can either be direct or indirect depending on whether hnRNP-K binds specifically to the RNA or whether it affects a different splicing factor through an intermediate protein or cascade. We first sought to determine whether a specific motif in the RNA sequence is required for hnRNP-K induced repression of exon 12a. The identification of such a motif would not directly implicate hnRNP-K as the RNA binding factor, it would determine the region that is bound by the specific splicing factor that mediates the repression.

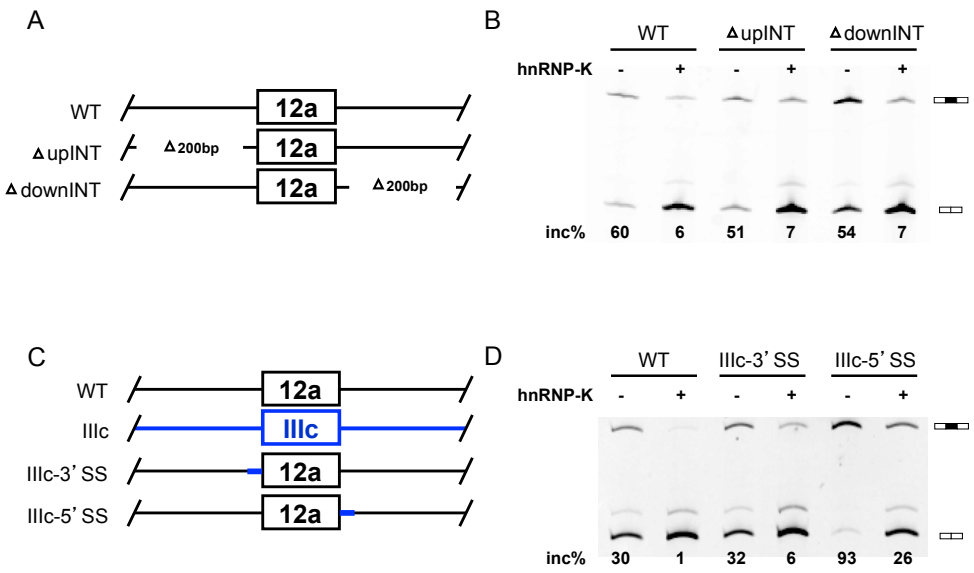

FIGURE 6.9 hnRNP-K repression of BIN1-exon 12a is not mediated through a proximal intronic element (A-B) Deletion of upstream and downstream BIN1 intronic sequence has no effect on repression by hnRNP-K (B) FGFR2-IIIc reporter shows no response to expression of hnRNP-K. (C-D) Replacement of 5' and 3' splice sites with those of FGFR2-IIIc does not abrogate hnRNP-K repression, though baseline splicing of exon 12a does change. All experiments are RT-PCRs for transiently transfected reporter in HEK293 cells for 48 hours.

As a first line analysis, we deleted the entire BIN1 specific intronic regions both upstream and downstream of the exon. These deletions did not include the proximal 25 intronic bases to the splice site, so that splicing of the reporter would not be eliminated. These two intronic deletions had no effect on repression by hnRNP-K (Figure 6.9A-B). Next, to determine whether the responsible *cis*-element was in the proximal introns in the splice site region, we swapped the flanking 25 base pairs of the BIN1 introns for the analogous sequence that flanks FGFR2 exon IIIc, an exon that we have already shown is unaffected by hnRNP-K expression. We find that exchanging either of these regions also does not affect hnRNP-K induced 12a repression (Figure

6.9C-D). Thus, the adjacent intronic sequences are not solely responsible for regulation of exon 12a alternative splicing by hnRNP-K. It is likely that mediation of the hnRNP-K effect is through an exonic splicing silencer alone or in combination with intronic sequences.

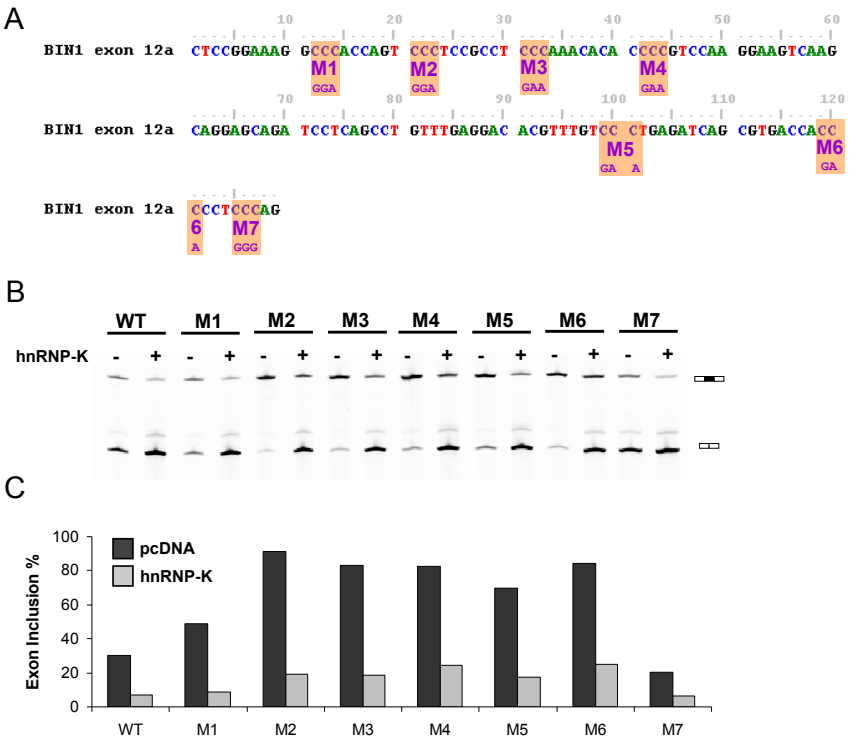

FIGURE 6.10 hnRNP-K repression of exon 12a is not mediated by a single CCC motif in the exon (A) Mutagenesis of each CCC run in exon 12a. (B) Representative RT-PCR gels show retained repression by hnRNP-K by each mutant. (C) Quantification of RT-PCR gels shows variation in baseline inclusion for many reporters, but increased skipping with hnRNP-K expression for all. All experiments are RT-PCRs for transiently transfected reporter in HEK293 cells for 48 hours.

One approach to characterize the exonic hnRNP-K responsive element was through site directed mutagenesis of potential hnRNP-K binding sites. hnRNP-K has been shown to bind poly-cytidine sequences preferentially and exon 12a contains 51 cytosine nucleotides including 7 runs of three consecutive residues. We made mutations in each of the CCC runs (to either GAA or GGA, Figure 6.10A). None of the single CCC mutations had a significant impact on splicing repression by hnRNP-K (Figure 6.10B-C). This result is not completely unexpected, as hnRNP-K has three RNA binding domains, and can potentially mediate splicing repression without three optimal binding sites. Further analysis of RNAs that are bound by hnRNP-K has elucidated some potential sequence and spacing requirements for efficient binding. In a yeast three-hybrid study, Ostrowsi and colleagues identified a motif conforming to (C/U)(C/U)AUCN$_{2-5}$C(C/U)ACC(C/A)N$_{11-}$

$_{17}$UCA(C/U) in forty three true positive hits(14). While this consensus does not exactly match the BIN1 pre-mRNA sequence, there are two regions that adhere to the consensus with few mismatches: from exonic nucleotides 27-69 and exonic nucleotide 104 to intronic residue +5 (spanning the 5' splice site). Additionally, the degenerate nature of this binding sequence suggests that hnRNP-K can tolerate alterations to this consensus. Future experiments will focus on combinatorial mutation of these potential binding sites, as well as *in vitro* hnRNP-K binding assays using specific exonic sequences.

High throughput screen of three small molecule libraries for BIN1-12a regulators

After defining the sets of splicing factors and some upstream signaling molecules that can change alternative splicing of the BIN1-12a alternative splicing reporters, we next sought to identify small molecules that can act in similar fashion. We performed a focused screen using three drug libraries at the UCLA Molecular Screening Share Resource. The three libraries screened were the BioMol, Prestwick FDA

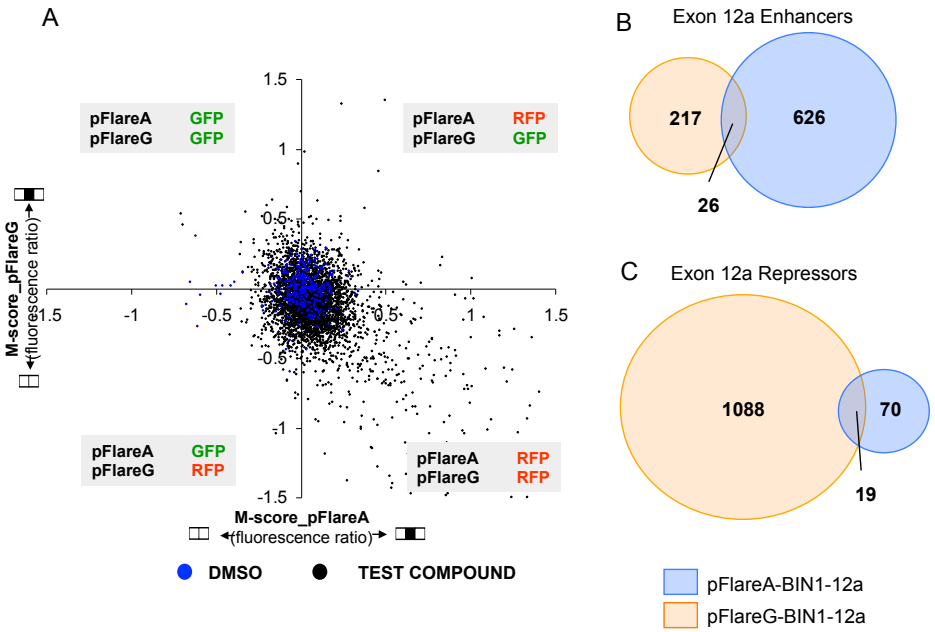

FIGURE 6.11 Small molecule screens identify overlapping regulators of BIN1 exon 12a alternative splicing. (A) All assayed small molecules with M-score plotted for both pFlareA and pFlareG screens. Hits would be in the quadrants I and III with induction of different fluorescence proteins and M-scores in similar direction, while drugs with an intrinsic effect on the fluorescent proteins would like in quadrants II and IV. (B-C) Large sets of fluorescence modifying drugs are picked up by each screen, but only small numbers of high confidence overlapping drugs are found.

Approved Drug Library and the Microsource Spectrum Collection. The same two pFlare-BIN1-12a reporter lines were used to probe these libraries that contain more than 15000 compounds, some of which are duplicated in different libraries. In previous screens, such as those reported for MAPT and FGFR2 splicing regulators, the hits contained a high rate of false positive compounds that affected fluorescent readout without largely changing splicing. The use of the counter reporter screen should eliminate many of the drugs that cause change in the same fluorescent direction, whether by regulation of the reporter or due to intrinsic fluorescence of the compound itself.

Table 6.2 Overlapping hits from two BIN1-exon 12a small molecule screens

BIN1 exon 12a enhancement

Name	Library	Function
PUROMYCIN HYDROCHLORIDE	Microsource	aminonucleoside antibiotic, translation terminating protein synthesis inhibitor
Puromycin dihydrochloride	Prestwick	aminonucleoside antibiotic, translation terminating protein synthesis inhibitor
PINACIDIL	BioMol	cyanoguanidine that opens ATP-sensitive potassium channels
POMIFERIN	Microsource	histone decacetylase inhibitor
PHENETHYL CAFFEATE (CAPE)	Microsource	phenolic ester of caffeic acid and phenethyl alcohol
LACTULOSE	Microsource	nondigestible sugar, osmotic laxativef
THIOGUANINE	Microsource	guanine analog, antimetabolite drug
LIQUIRITIGENIN DIMETHYL ETHER	Microsource	selective estrogen receptor beta agonist
Digoxigenin	Prestwick	cardiotonic steroid
HYDRALAZINE HYDROCHLORIDE	Microsource	increases GMP, decreases IP_3, limits Ca^{2+} release resulting in vasodilation
SAPPANONE A 7-METHYL ETHER	Microsource	antibacterial and anticoagulent activity
CLOBETASOL PROPIONATE	Microsource	anti-inflammatory corticosteroid
FLUPROSTENOL	BioMol	PGF2 analog, potent prostaglandin F receptor agonist
BETAHISTINE HYDROCHLORIDE	Microsource	histamine H3 receptor antagonist, weak H1 agonist
Dirithromycin	Prestwick	macrolide glycopeptide antibiotic

BIN1 exon 12a repression

Name	Library	Function
QX-314	BioMol	lidocaine derivative, voltage-gated Na^+ channel blocker
HARMALINE	Microsource	psychoactive indole alkaloid, MAO-A inhibitor
Levobunolol hydrochloride	Prestwick	nonselective beta-adrenergic antagonist
ACEXAMIC ACID	Microsource	structural lysine analog, used with zinc to treat ulcers
SODIUM MECLOFENAMATE	Microsource	prostaglandin synthesis inhibitor, anti-inflammatory
AVOCADENE	Microsource	fatty alcohol found in avocado fruit
Eicosapentaenoyl ethanolamide	BioMol	omega-3 fatty acid ethanolamide, antiproliferative
Tacrine hydrochloride hydrate	Prestwick	anticholinesterase and indirect cholinergic agonist, AD therapeutic
BACITRACIN	Microsource	topical polypeptide antibiotic
Nipecotic acid	Prestwick	GABA reuptake inhibitor
Sulfaguanidine	Prestwick	sulfonamide antibacterial
Cephalothin sodium salt	Prestwick	first generation cephalosporin antibiotic
Phenylpropanolamine hydrochloride	Prestwick	stereoisomer of norephedrine, psychoactive stimulant and decongestant
HARMALINE HCl	BioMol	psychoactive indole alkaloid, MAO-A inhibitor
URIDINE TRIPHOSPHATE TRISODIUM	Microsource	pyrimidine nucleoside triphospate, may have signaling role

The drugs were added to media in a 384-well plate format, and the screening lines were added to the drug containing media. Cells were incubated for 96 hours, with images taken on the Typhoon phosphorimager taken every 24 hours. Presented here are the data from the 72 hour

timepoint, though the data does further stratify the drugs in to early and late acting during the timecourse. Strikingly, in each individual screen, many small molecules robustly changed the M-score (fluorescent ratio score) of the reporters (Fig 6.11A). The tendency for a given small molecule to always enhance the same fluorescent protein was striking, as a large number of compounds caused an increase in red fluorescence at 72 hours. However the overlapping set of molecules was relatively small, a similar finding to the cDNA overexpression screen, and will have successfully discarded many of the drugs that have increased false positive rates previously (Figure 6.11B-C). In the set of drugs that were discarded in the intersecting analysis were some that are known to affect translation or cell survival as their primary action (and thus likely are false positives), including translational inhibitors cycloheximide and emetine and cardiac glycosides lanatoside C and ouabain(34).

Importantly, the nature of the drugs that remain in the intersecting set generate hypotheses for signaling networks that may regulate BIN1 exon 12a splicing through the splicing factors identified above. For example, in the exon 12a enhancer compounds, we identified fluprostenol, a potent prostaglandin F receptor agonist. In the repressor molecules we identified sodium meclofenamate, a prostaglandin synthesis inhibitor that would have the opposite cellular effect as fluprostenol. These complementary actions may imply that prostaglandin induced signaling activates cell signaling cascades that control BIN1 exon 12a splicing, and ultimately, Myc dependent gene transcription. Also of note are drugs that were selected twice out of different libraries, namely puromycin as an exon 12a enhancer and harmaline as an exon 12a repressor. Significant hits that are identified multiple times are likely to be potent regulators of this alternative splicing event.

BIN1 exon-12a splicing in WM239A cell line

One limitation to the screening strategies set forth to this point is that they have all been carried out in HEK293 cells. The choice of HEK293 cells enabled high sensitivity hits from the cDNA expression library, as well as an shRNA library because of their high rate of transfection with lipofectamine based methods. Similarly for small molecule screens, the choice of HEK293 cells enabled rapid development and efficient execution of the screening methods. The utility of such screens, however, can be decreased if identified compounds have effects that are restricted to the HEK293 cell type. Indeed, compounds that have been identified in an HEK293 cell based screen for modulators of SMN2 alternative splicing displayed validated dose-dependent effects on both reporter and endogenous transcript, but did not cross validate to neurons in culture (P. Stoilov, personal communication).

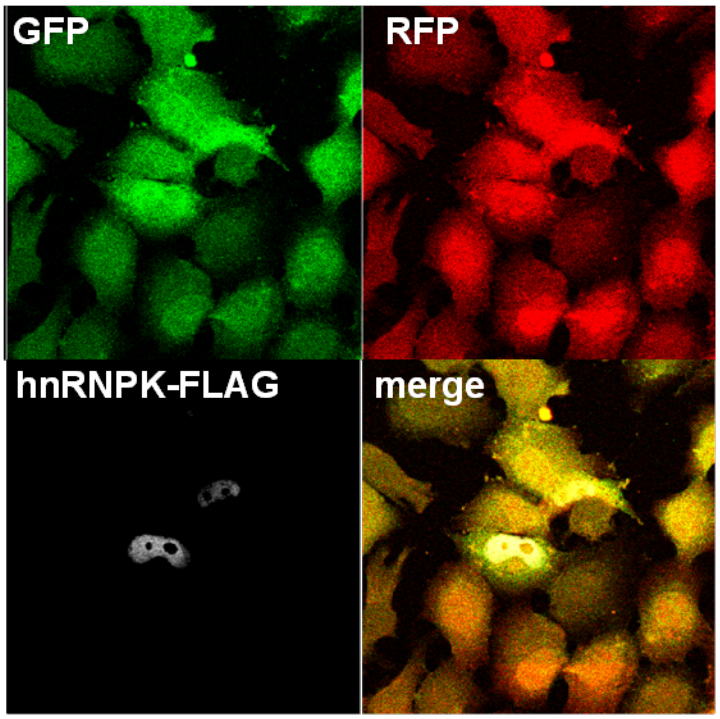

FIGURE 6.12 pFlareA-BIN1-12a stable WM239A line maintains regulation by hnRNP-K. pFlareA-BIN1-12a was established in WM239A melanoma cell line. Cells express both GFP and RFP, consistent with alternative splicing of the reporter, and are switched to green by exogenous expression of hnRNPK.

For this reason, we created a BIN1 alternative splicing reporter in a specific cell type of interest, the invasive melanoma line WM239A (Figure 6.12). The line was created in an identical fashion to the HEK293 reporter line, and one clonal integration of the pFlareA-BIN1-12a reporter showed detectable fluorescence of both RFP and GFP, consistent with the alternative splicing of the reporter, and the described splicing of the endogenous transcript in the

literature. Though minimally transfectable with lipfectamine based means, we did observe that regulation by hnRNP-K is maintained on the pFlareA-BIN1-12a reporter. This line will be particularly useful to cross validate the effect of genetic and small molecule manipulation of BIN1 alternative splicing. Additionally, in WM239A cells, Bin1 protein containing the 12a encoded CLAP domain is detectable by Western blot, and can be used to validate effects on the protein level after confirming regulation of the RNA.

Conclusions and future directions

We have described the development, optimization and use of a dual fluorescence splicing reporter for regulators of BIN1 exon 12a alternative splicing. Exon 12a of BIN1 is implicated in tumorigenesis and shows aberrant inclusion in multiple types of cancers including melanoma. Expression of exon 12a has been reported to disrupt Myc sequestration by BIN1, and its release is thought to drive the Myc-dependent oncogenic gene expression program. Using this screening reporter system, we sought to characterize both the molecular regulation pathways of exon 12a splicing, and also to identify small molecules that can modulate its splicing.

Creation of the dual fluorescence splicing reporters entailed cloning exon 12a and its conserved flanking intronic sequence into the pFlare dual fluorescence construct. We created two versions, one harboring three base pair substitutions in the 3'end of the exon. These mutations created in in-frame start codon in an exon included transcript which effectively reversed fluorescent readout for splicing choice between the two reporters. Isolation of stable clones containing the two reporters revealed that they maintain regulation by known splicing factors.

High throughput cDNA expression screen using the reporter cell lines identified many repressor and activator genes with high confidence due to obtaining significance in both

screens. We found multiple RNA-binding protein for exon 12a splicing, including repressors hnRNP-K, SFRS10 and hnRNP-LL and enhancer hnRNP-R. We also found that expression of multiple cell signaling cascade components such as CLK2, MAP3K3 and -11, and FAST are able to modulate alternative splicing of BIN1 exon 12a.

Further experiments are needed to elucidate how modulation of these cell signaling cascades can modulate the implicated splicing factors. Further, it will be interesting to determine whether expression and modulation of these regulators has a downstream effect on Myc function, as hypothesized in the literature. Each of these regulatory proteins is a potential therapeutic target if their function truly does alter oncogenic gene expression patterns.

Since hnRNP-K is a significant hit, and because of its controversial role in the literature with respect to tumorigenesis, we sought to clarify its role as a regulator of BIN1 exon 12a alternative splicing. We found that hnRNP-K mediates repression specifically for exon 12a, and that this repression occurs through an exonic silencing element, though the exact motif is yet to be determined. Determination of direct binding by hnRNP-K will also be exciting, as this protein has not been extensively reported to be a splicing factor *per se*, despite its canonical RNA binding domains. Increased analysis of the relationship between hnRNP-K and tumorigenesis is necessary, as multiple studies have reported both critical tumor suppressive and oncogenic roles for the protein in different cancers. It is likely that its regulation of BIN1 contributes to an entire set of cellular functions, but its role could represent a novel target pathway for anti-cancer therapy.

We additionally performed high throughput screens for small molecule regulators of the BIN1 exon 12a alternative splicing event. We found that most small molecules had more of an effect on fluorescent protein production than on alternative splicing, but we did find small sets of

drugs that can enhance and repress exon 12a splicing. Since the screen consisted of drug with known function, we are able to generate hypotheses for how they may be working. For instance, a prostaglandin agonist, fluprostenol, activates exon 12a splicing, while a prostaglandin synthesis inhibitor, meclofenamate induces skipping of the exon. The cell signaling cascades that are activated downstream of prostaglandin signaling may be acting through the identified splicing regulators of this event to ultimately direct splicing choice. Further study is required to link these observations together.

The strategy presented here represents a comprehensive method to identify molecular regulators as well as potential therapeutics for alternative splicing events that are aberrantly regulated in disease. While the full potential of the entire method is yet to be realized, the coupling of these identification and characterization steps with the previously described methods for target mechanism identification (Chapters 2-3) will hopefully facilitate the advancement of small molecule based alternative splicing therapies.

CHAPTER 7: CHARACTERIZING THE ROLE OF THE SPLICING FACTOR RBFOX1 AFTER KAINATE INDUCED SEIZURE

Introduction: Neuronal ablation of Rbfox1 in the brain predisposes mice to death from kainate induced seizure

The Rbfox family of alternative splicing factors plays essential role during neuronal development and also regulate alternative splicing in tissues such as embryonic stem cells(12,71,72). The Rbfox family contains three homologs in mice (Rbfox1/2/3), which are highly similar to each other, especially in the RNA recognition motifs (RRM) where they share identical primary sequence and bind an identical RNA motif, (U)GCAUG. Rbfox1 proteins are expressed at varying times during neuronal development, and are associated with different degrees of maturation during this process(73). For example, Rbfox3 (alternatively called NeuN) is expressed only by mature postmitotic neurons, while Rbfox1 and Rbfox2 are expressed during neuronal migration at late and early phases, respectively. The total expression pattern of these proteins during embryonic nervous system development is still to be fully determined. Interestingly, each of the proteins directs splicing of slightly different sets of alternative exons that often lie in transcripts that encode different processes that are essential to neuronal maturation. Furthermore, cell culture work has suggested that Rbfox1 can play a specific role in regulation of depolarization induced splicing changes, and may act to maintain homeostatic control over the proteins that are encoded by transcripts containing regulated exons(74).

To further characterize the total role of the Rbfox1 protein in the mouse brain, we recently reported a complete neuronal ablation of Rbfox1 in which deletion is driven by the nestin promoter ($Rbfox1^{-/-}$)(72). In $Rbfox1^{-/-}$ mice, neuronal cell lineages displayed a phenotype

of increased spontaneous seizures, and an increased susceptibility to kainate acid induced seizures. Specifically when challenged with kainate, Rbfox1$^{-/-}$ mice, all died of status epilepticus while wild-type controls rarely exhibited the same result. These results could be attributable to the many alternative exons that show baseline misregulation in the Rbfox1 knockout mouse, to the absence of a critical role for Rbfox1 after seizure, or a combination of both.

Kainate is a glutamate analog that specifically activates one type of glutamate receptor in the mammalian nervous system. Glutamate, as a primary excitatory neurotransmitter, binds to multiple subtypes of ligand-gated Na^{+}/Ca^{2+} channels(75). Activation of these channels begins depolarization of a post-synaptic neuron and activation of downstream signaling cascades, including those that are dependent on Ca2+ as a second messenger. Excessive or prolonged cationic influx can result in cell injury or death, as well as uncontrolled excitation of the postsynaptic neuron(76). Kainate specifically activates kainate type glutamate receptors, but depolarization of the postsynaptic neuron will induce activation of voltage-gated ion channels as to generate an action potential. Notably, kainate administration is used in research purposes to model temporal lobe epilepsy(77). In rodents, injection of kainate can produce a stereotyped progression of increased neuronal-muscular excitation, ultimately resulting in full tonic-clonic seizure. With controlled dosing, wild type mice recover from induced seizures, and over time can develop recurrent spontaneous seizures. The development of recurring seizures can be used as an epilepsy model.

Kainate administration is thought to mimic temporal lobe epilepsy due to the specific early changes that are seen after treatment. Striking induction of immediate early genes such as c-fos and c-myc are seen in a characteristic pattern in the CA1 and CA3 regions of the dentate gyrus within the hippocampus(78). The genesis of seizure activity after kainate is therefore

thought to focally begin in the temporal lobe and spread to other areas of the brain until complete brain involvement produces a tonic-clonic seizure. This progression is thought to mimic temporal lobe epilepsy, which is one of the most common forms of human epilepsy and is characterized by hippocampal atrophy(79).

Here, we sought to characterize the alternative splicing changes that are observed after kainate induced seizures in wild type mice. These findings can provide clues as to the essential targets that are regulated by Rbfox1 after seizure and that are critical to recovery from full tonic-clonic seizure.

Results

Kainate administration in wild-type mice induces seizures and immediate early gene expression

We initially characterized wild type mice for their response to kainate treatment on a transcriptome wide level. We chose one month aged male Sv129 mice for these experiments, as this background of mouse gave a more consistent progressive seizure response than Black 6 background (data not shown). Mice were administered kainate at a dose of 20mg/kg body weight and it was delivered via intraperitoneal injection. As a control, sterile saline was injected in equal volume in matched mice. A total of six wild type mice were administered kainate for the results presented here (Figure 7.1A). Seizure score was assessed using the Racine scale, which is a subjective scoring system in which a score of 0 represents no stereotyped or repeated movements and 5 represents tonic-clonic seizure and 6 is status epilepticus or death. Mice display key movements at each scoring point to accurately assess the development of seizures (e.g. 1=mouth/facial movement, 2=head bobbing, 3=forearm clonus and 4=rearing). The wild type mice were noted for their highest Racine score(80) in each 10 minute window for two hours

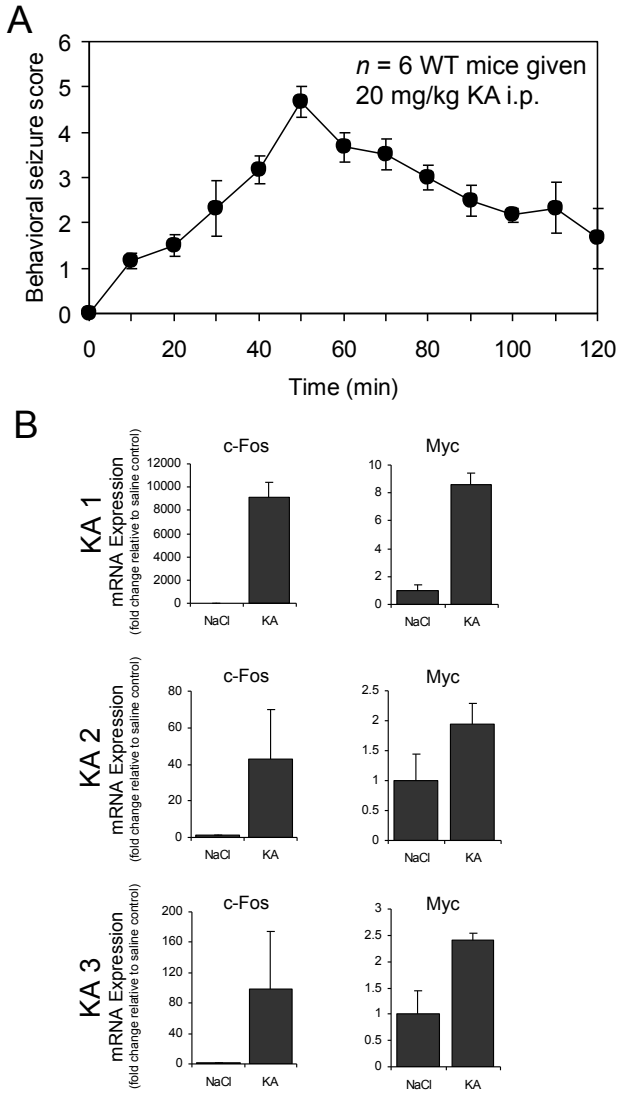

FIGURE 7.1 Kainate administration in Sv129 mice elicits consistent tonic-clonic seizures. (A) Composite seizure scores on Racine scale for mice that received kainate intraperitoneal injection. Animals were observed for 120 minutes and during each 10 minute window, the highest score on Racine scale was noted. (B) Quantitative PCR analysis of c-Fos and Myc expression for each pair of kainate and control treated mice at 6 hours. Signal was normalized to 5S rRNA transcript and presented as fold induction over NaCl treatment. Error bars represent standard deviation for each of 3 technical qPCR replicates.

(Figure 7.1A). All mice proceeded to have at least 1 tonic-clonic seizure, and the mean time to tonic-clonic seizure was 41 minutes. Importantly, all mice recovered to relatively quiet or relaxed baseline by the end of a two hour observation window. Mice were allowed to recover for 6 or 16 hours (3 mice per group), and were then sacrificed. For each pair of control and kainate treated mice, RNA from hippocampal dissection revealed striking induction of both c-fos and c-myc six after kainate (Figure 7.1B). The isolated RNA was analyzed for global alternative splicing and gene expression changes by Affymetrix MJAY (Mouse Research Junction Array).

Alternative splicing changes occur in neuronal transcripts after kainate treatment

The Affymetrix MJAY platform simultaneously assays gene expression and alternative splicing profiles. We probed each of three control and kainate treated mice on individual arrays to identify the most consistently changed transcripts. The RNA that was probed on array was

Table 7.1 Genes showing significantly altered expression after kainate treatment

KA Enhanced Gene Expression

Gene Symbol	log_2(KA/ctrl)	Gene Name/Function
Inhba	4.73	inhibin beta-A
Gadd45b	3.19	growth arrest and DNA-damage-inducible 45 beta
Blnk	3.08	B-cell linker
Cda	3.04	cytidine deaminase
Sult2b1	2.90	sulfotransferase family, cytosolic, 2B, member 1
Pcdh8	2.41	protocadherin 8
Rgs4	2.34	regulator of G-protein signaling 4
Tll1	2.34	tolloid-like
Igsf9b	2.28	immunoglobulin superfamily, member 9B
Ppapdc1a	2.26	phosphatidic acid phosphatase type 2 domain containing 1A

KA Repressed Gene Expression

Gene Symbol	log_2(KA/ctrl)	Gene Name/Function
Gpr12	-2.32	G-protein coupled receptor 12
Fam163b	-2.04	family with sequence similarity 163, member B
Stxbp6	-1.42	syntaxin binding protein 6 (amisyn)
Dbp	-1.35	D site albumin promoter binding protein
Lrrn2	-1.23	leucine rich repeat protein 2, neuronal
Lrrn3	-1.21	leucine rich repeat protein 3, neuronal
Neurod6	-1.16	neurogenic differentiation 6
Lrrn1	-1.15	leucine rich repeat protein 1, neuronal
P2ry12	-1.14	purinergic receptor P2Y, G-protein coupled 12
Ccdc85a	-1.10	coiled-coil domain containing 85A

that of wild type mice six hours after either control or kainate treatment. The MJAY arrays were analyzed using the Omniviewer software package that calculates both gene expression data as well as assigns a score to all annotated alternative splicing events(81). Top gene expression changes included induction of immediate early genes that have been previously shown to follow seizure activity, such as Inhba and Gadd45b (Table 7.1). Interesting downregulated genes included all three members of the Lrrn (leucine rich repeat neuronal) family, some of which have been linked to neuronal function and autism. Notably, none of the significantly changed genes on expression level included known splicing factors or RNA-binding proteins.

Table 7.2: GO analysis of transcripts with altered splicing after kainate

GO Classification Term	Fold Enrichment	p-value
synaptic vesicle	7.3	3.80E-04
actin cytoskeleton organization	4.1	7.80E-04
synapse	4	2.60E-06
synapse part	4	2.00E-04
cell junction	3.2	1.00E-05
vesicle	2.6	3.80E-04
intracellular signaling cascade	2.3	3.20E-05

Alternative splicing changes were widespread six hours after kainate treatment. Detectable splicing changes occurred mostly in cassette exons, but there were also significantly altered events of other types such as retained introns and altered splice sites (Figure 7.2A). Cassette exons displayed changes

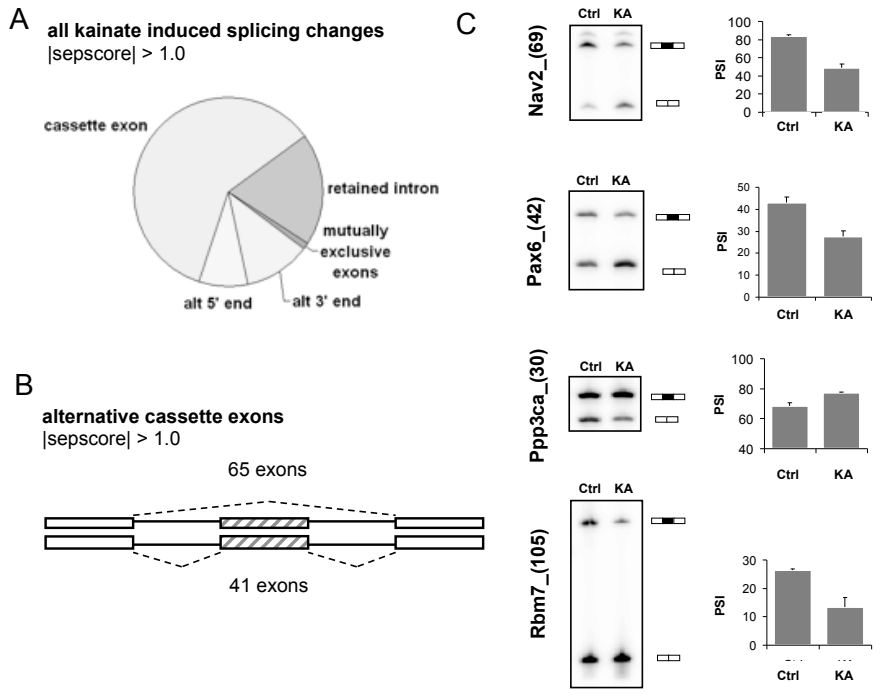

FIGURE 7.2: Kainate induces changes in many neuronal alternative splicing events. (A) All significant induce splicing changes as assayed by Omniviewer and set at a cutoff of |sepscore| > 1.0, and q-value < 0.001. (B) Cassette exons are induced to both skipping and inclusion after kainate. (C) Splicing of four Omniviewer-identified exons 6 hours after kainate (KA) or NaCl (Ctrl) treatment. Bar graphs represent average inclusion level for n=3 mice in each group, and error bars represent standard error of the mean.

both toward more skipping and more inclusion, indicating that there was not a general inhibition of alternative splicing after treatment (Figure 7.2B). High confidence exons were validated by RT-PCR for induced splicing changes, and notably many of these alternative splicing changes occurred in transcripts with known neuronal function (Figure 7.2C). We performed a gene ontology analysis for both biological function and cellular compartment classification. Transcripts with altered splicing clustered into cell signaling classification located in axons and other neuronal compartments (Table 7.2). This suggests that these subsets of neuronal transcripts are showing particular sensitivity to alternative splicing changes induced by seizure.

We chose significantly changed alternative exons for validation by RT-PCR (Figure 7.2C). Alternative exons in critical transcripts such as Pax6, Nav2 and Ppp3ca all displayed changed inclusion levels after kainate treatment. For many transcripts, particular alternative isoforms do not yet have function attributed to them, but for some transcripts different isoforms have been characterized. For example, the changed exon in Pax6, termed exon 5a in the

literature, changes DNA binding properties for the transcription factor. This change could activate a different gene program by Pax6 in the time following seizure activity. Additionally, there were distinct changes in splicing of transcripts that encode RNA binding proteins, such as Rbm14 (Figure 7.2C), and Hnrnpk (data not shown). Often, splicing factors can autoregulate their own splicing, and these could be a potential clue to splicing factors with modified function after kainate treatment.

(U)GCAUG sites are enriched downstream of kainate-repressed exons, and the same exons show significant recovery in splicing over time

To determine whether Rbfox1 has a potential role in regulation of kainate induced alternative splicing changes, we performed a bioinformatics analysis of the altered exons. We separated the significantly altered cassette exons (absolute sepscore > 1.0) into enhanced and repressed groups (containing 41 and 65 exons, respectively). For each group we performed motif frequency analysis for RNA pentamers in the exon and adjacent intronic sequence. The observed frequency was tested for significance by comparison to a first-order Markov model for expected pentamer frequency based on mono- and di-nucleotide content in the respective regions. We found a striking enrichment of UGCAU and GCAUG pentamers in the introns downstream of kainate repressed exons. Since Rbfox1 typically enhances exons that have downstream UGCAUG sequences, these results are consistent with a lack of function of Rbfox1 on these targets. Interestingly, when we analyzed exons with downstream Rbfox1 sites, we did indeed find repression at 6 hours after kainate, but for multiple exons we found subsequent enhancement by 16 hours after kainate (Figure 7.3C-F). This result implies that Rbfox1 could have a restorative effect on critical alternative splicing targets that are first modified by

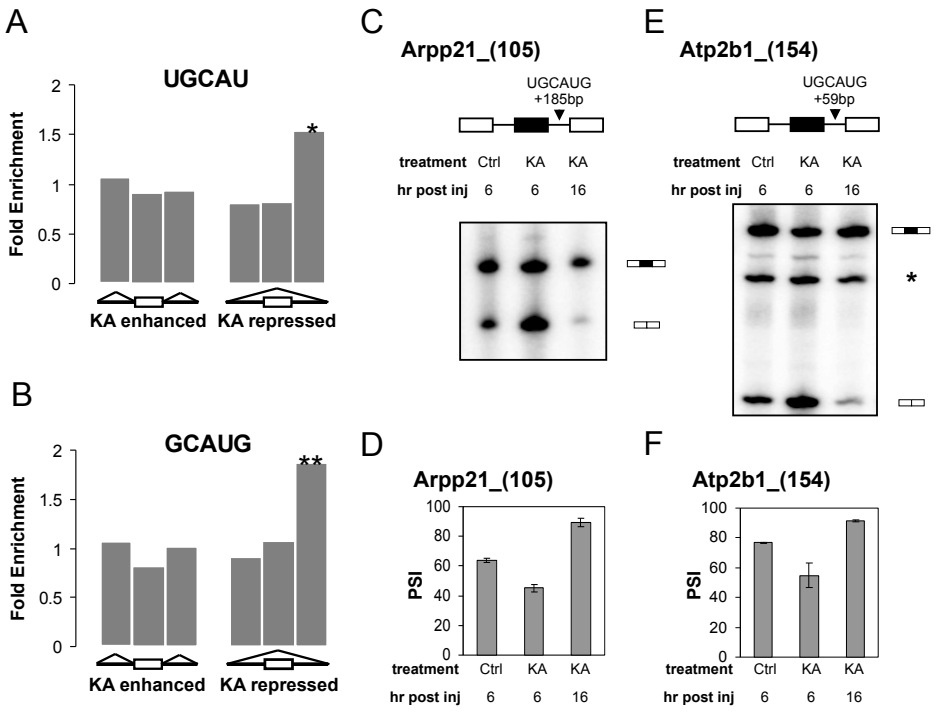

FIGURE 7.3: KA-repressed exons that contain downstream UGCAUG motifs have restored inclusion levels after 16 hours. (A-B) Fold enrichment for pentamers that match UGCAUG motif in three regions of KA-regulated exons. Frequency was tested against an expected frequency derived from a first-order Markov model for each given region. Significance was tested by binomial test (* p-value < 1E-3, ** p-value < 1E-4). (C,E) UGCAUG location and splicing at 6 and 16 hours after KA for two exons that contain downstream motif. (D,F) Quantification of exon inclusion levels for n=3 mice at each time point. Error bars represent standard error of the mean.

depolarization. An alternative interpretation is that Rbfox1 exhibits a loss of function immediately after kainate treatment and is subsequently restored to normal function. We tested this in part by assaying the alternative splicing level of Rbfox1 exon 19. The total transcript level of Rbfox1 is not greatly affected by kainate treatment nor is splicing of exon 19 at 6 hours after treatment, but the splicing of exon 19 does shift to more skipped product 16 hours after kainate (Figure 7.4A-B). The exon 19-skipped isoform of Rbfox1 encodes a nuclear protein, while the exon 19 included isoform encodes a cytoplasmic isoform that has less ability to function as a splicing factor. Though there are significant amounts of each isoform at baseline, the shift to increased exon 19-skipped isoform is consistent with an increase of Rbfox1 dependent splicing that is seen 16 hours after kainate.

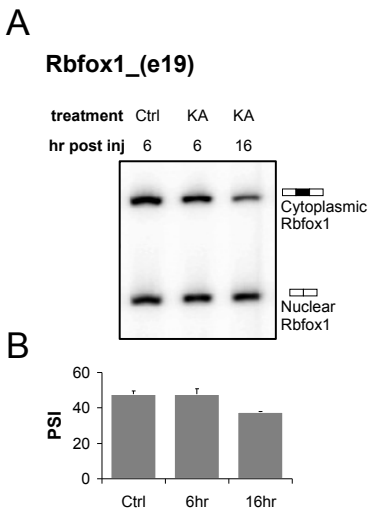

FIGURE 7.4: Alternative splicing of Rbfox1 exon 19 produces more nuclear Rbfox1 isoform 16 hours after kainate. (A) RT-PCR analysis of Rbfox1 exon 19 inclusion. The skipped isoform encodes nuclear Rbfox1 while the cytoplasmic protein is encoded by the included isoform. (B) Quantification exon 19 inclusion of n=3 mice in each group. Error bars represent standard error of the mean.

Conclusions and future directions

The coordinated changes in alternative splicing after depolarization and seizure activity indicate at global response by neurons at the post-transcriptional level. This result is not unexpected, as other depolarization strategies have been shown to induce splicing changes. Here, we specifically assayed for splicing changes after kainate treatment and subsequent seizures in wild-type mice. Previously it has been shown that mice lacking neuronal expression of Rbfox1 are prone to spontaneous seizures and exhibit enhanced sensitivity to kainate-induce seizures. Using an exon-junction microarray approach, we profiled transcriptome wide changes in alternative splicing that are detectable six hours after kainate injection. We found that many transcripts exhibit changed splicing, and they tend to cluster into gene ontology categories related to neuron and synaptic function.

Sequence analysis of the changed alternative exons at 6 hours revealed that many kainate-repressed exons contain UGCAUG RNA motifs in their downstream introns. These sites would typically be predicted to facilitate Rbfox1 dependent enhancement of alternative splicing. We further found that at 16 hours after treatment, the UGCAUG containing alternative exons displayed restored and even enhanced inclusion levels. These findings are consistent with an upregulation of the splicing activity of Rbfox1 to restore homeostatic control over alternative transcripts that could have potentially altered the function of the proteins they encode. The

increase in splicing of these transcripts is accompanied by an increase in the nuclear isoform of the Rbfox1 protein, again indicating that it could have enhanced activity as a splicing factor.

It remains to be determined whether Rbfox1 is playing a critical role in maintenance of these alternative exons. Further, it will be informative to determine whether altered splicing of these targets modifies the response to kainate and depolarization in general. If the splicing of some exons is critical to recovery, they could potentially account for the enhanced response that Rbfox1$^{-/-}$ mice display when challenged with kainate. Finally, specific investigation of Rbfox1 localization and function under different types of neuronal modulation (both excitation and inhibition) will ultimately elucidate the molecular functions of this RNA-binding protein in baseline control of neuronal cellular processes.

CHAPTER 8: CONCLUSIONS

From the outset, the goal of this dissertation work has been to further develop and successfully use methods that will aid in understanding of disease-associated alternative splicing and begin the route toward effective therapy. The therapeutic targets presented in this work represent a wide range of human biology and specific changes that cause or promote disease progression. The diverse nature of targets strengthens the global impact that these data support. Regardless of specific alternative splicing event, these strategies can be used to understand its regulation, target it therapeutically and ultimately elucidate the mechanism by which it is targeted.

With the advent of high throughput sequencing, it has become firmly established that nearly all multi-exon mammalian pre-mRNAs generate multiple mature transcripts through the process of alternative splicing. The depth of analysis that high throughput sequencing permits shows not only that these splicing events exist, but that they are strongly regulated during development and some display striking misregulation in disease. The continued analysis of total transcriptome character from disease samples will further illustrate this key point that is an integral mechanism of gene regulation. Indeed, specific changes in alternative splicing events can cause and promote disease pathogenesis. The roles of splicing factors and their upstream regulators are correlated with disease progression. Characterization of their targets and specific mechanisms of action, both as they relate to alternative splicing and not, will help elucidate the molecular mechanism by which disease can occur. There will be a decided need for efficient and effective strategies that can characterize and when possible, therapeutically target their regulation. In this dissertation research, I have worked to further develop the paradigm of RNA

splicing associated disease, and the ability to modulate its activity it with small molecule therapeutics.

The cardiotonic steroid digitoxin modulates alternative splicing in part through depletion of the splicing factors of SRp20 and Tra2-beta.

Previous work in our lab showed that the cardiotonic steroid family of small molecules can modulate alternative splicing choices, specifically they induce the splicing of MAPT exon 10. Small scale microarray experiments suggested that the effect of cardiotonic steroids on alternative splicing is widespread, but a mechanism of action had yet to be established. Furthermore, the same array experiments implied that despite being widespread, there was a degree of specificity to cardiotonic steroid targets, as they did not entirely overlap other small molecule modulators target sets. The next logical direction for this project was to determine the mechanism by which cardiotonic steroids can control alternative splicing choices.

The first approach to answer this question was to develop an unbiased method that can identify splicing factors that are involved in the drug's regulatory function. Alternative splicing events are controlled by RNA binding proteins interacting with core regulatory RNA motifs in and around alternative exons. It was hypothesized that sequence analysis of the exons that are regulated by cardiotonic steroids could implicate specific RNA binding proteins by enrichment of their consensus binding sites. The power to this method is its unbiased nature. Transcriptome-wide analysis reveals the specificity of a given compound, and the targeted exons can be analyzed for sequence enrichment of specific RNA motifs. In the case of the cardiotonic steroids, digitoxin regulates a large set of alternative exons, some enhanced and some repressed by drug treatment. Further, sequence analysis of these exons revealed context dependent

enrichment of SRp20 and Tra2-beta biding sites, namely intronic SRp20 sites were enriched near digitoxin-enhanced exons while Tra2-beta sites were enriched in digitoxin-repressed exons. Subsequent protein level analysis revealed that digitoxin treatment depletes SRp20 and Tra2-beta, and that those exons that contain binding sites for those proteins can be rescued from drug treatment by overexpression of the splicing factors.

This analysis represents the first step toward understanding how digitoxin and other cardiotonic steroids can regulate alternative splicing. Importantly, the involvement of SRp20 and Tra2-beta was predicted without using *a priori* knowledge about digitoxin and the other cardiotonic steroids. It remains to be determined how the drug targets SRp20 and Tra2-beta for degradation. Tra2-beta for example, is regulated by protein phosphatase 1, and we did find that PP1 inhibition could antagonize the effect of digitoxin on alternative splicing. It is possible that t the cardiotonic steroids are activating specific signaling cascades including cellular phosphatases to promote the degradation of SRp20 and Tra2-beta. Further experiments will be required to identify which cascades are at the root of these and other the other drug-induced changes. Additionally, the effects of digitoxin appear to be widespread on other splicing factors as well, as these findings could not account for all digitoxin-induced alternative splicing changes. Indeed, the sequence analysis did identify other enriched motifs in targeted exons, but the experiments performed here did not implicate a specific factor or factors to act through them. It is likely that digitoxin modifies the functions of many splicing factors at once, accounting for the pleiotropic role on the transcriptome wide splicing choices.

A second candidate approach was attempted with limited success, but remains a viable complement to the unbiased method described above. Screening small molecule libraries of drugs with known function enables specific hypotheses to be generated about the cellular effects

that are ultimately resulting in altered splicing. Specifically, the cardiotonic steroids activate multiple cell signaling cascades in both ion exchange dependent and –independent manners. Probing these activated pathways with chemical inhibitors and molecular genetic modulations ultimately had no effect on digitoxin-induced splicing changes. It is likely that digitoxin activates many pathways (both known and unknown) to ultimately direct splicing regulation, and that a candidate approach is not the most efficient method to identify it. One potential route to uncovering the pathways that lead from digitoxin to splicing changes is to re-screen a focused small molecule library of known cell signaling modulators for their ability to block digitoxin treatment. This data could be coupled with molecular genetic screens as described for the BIN1 alternative splicing event to confidently identify cell signaling effector proteins that are responsible for digitoxin-induced splicing changes.

High throughput characterization of the regulation cascades of BIN1 exon 12a, an exon with oncogenic properties

Modulation of certain alternative splicing events can cause or promote disease. BIN1 encodes a protein with many cellular functions, including roles in establishing and maintaining membrane curvature, endocytosis and T-tubule formation in muscle cells. It can also bind to the transcription factor c-Myc and block its activity as a transcription factor. BIN1 functions are dependent to large extent on the alternative splicing of its pre-mRNA transcript. Importantly, inclusion of a specific alternative exon, 12a, disrupts its ability to sequester c-Myc. Inclusion of exon 12a therefore inactivates the tumor suppressor nature of BIN1 and promotes c-Myc dependent processes that are related to an increase of cellular growth and proliferation. Some splicing factors have been linked to regulation of exon 12a, including enhancement by SRSF1,

an oncogenic splicing factor. However, the regulation pathways that lie upstream of this and the other factors that likely regulate exon 12a are unknown. Elucidation of these pathways will further characterize a mechanism by which tumor cells acquire c-Myc dependent function and provide targets for therapeutic intervention.

To understand the key factors that regulate exon 12a inclusion, both directly and indirectly, a high throughput cDNA expression library was screened for clones that were able to modulate exon 12a splicing. To accommodate a high throughput screen, a dual fluorescence alternative splicing reporter was used that contained exon 12a and its adjacent intronic sequences fused to GFP or RFP which are alternatively produced depending on the splicing choice of exon 12a. Through a screen and counter screen approach in which fluorescent readout is switched for the given regulation event, multiple high confidence hits were identified that can robustly regulate inclusion of exon 12a. Interestingly, these include many RNA binding proteins, as well as upstream cell signaling molecules. These findings will enable generation of hypotheses linking function of cell signaling cascades to specific splicing factors, and ultimately BIN1 alternative splicing. These processes are also linked to the oncogenic activity of the c-Myc transcription factor through this axis of control.

One RNA binding protein that was a robust hit as a repressor of BIN1 exon 12a splicing was hnRNP-K. hnRNP-K is a protein that has been implicated in regulation of mRNA stability, through binding of RNA by its three KH domains. Repression of exon 12a by hnRNP-K is mediated by an exonic element, though the exact motif is yet to be determined. Given its role as a repressor of the oncogenic 12a exon, it was interesting to note that hnRNP-K has been reported to function as a tumor suppressor by alternative mechanisms. Specifically, it is recruited with p53 to p53-responsive genes immediately after DNA damage. An essential role in response to

DNA damage coupled with tumor suppressive control of an oncogenic splicing event argues for a critical role for hnRNP-K in prevention of tumorigenesis. These roles may be balanced by additional roles that contribute to hnRNP-K dependent growth and transformation, including stabilization of the c-Myc transcript itself. It is clear that the true nature of hnRNP-K activity in tumor cells remains to be fully elucidated.

Efficient method to identify drugs that modulate BIN1 exon 12a alternative splicing

Therapeutic targeting of an alternative splicing event requires knowledge of the regulation cascades that govern it, as well as a method to identify small molecules that can modulate it. The approach described above details the elucidation of genetic regulation pathways for the BIN1 exon 12a alternative splicing event. This data will be joined to an additional set of screens that sought to identify drugs that can have similar or antagonistic effects. The screen was performed on the same dual fluorescence reporter lines as the cDNA overexpression screen, but with three libraries of small molecules instead. These drugs all have known functions in the cell, which will enable hypothesis-driven investigation of the cascades that govern exon 12a inclusion. It will also be interesting to determine whether drugs that can target BIN1 alternative splicing do so though different cascades and RNA binding proteins, which would argue for multiple routes to BIN1 splicing misregulation in tumor cells. Specifically interesting drugs that were identified include opposing regulators of prostaglandin function, as well as multiple psychoactive drugs that modulate the function of monoamine neurotransmitters in the nervous system. The BIN1 genomic locus has recently been linked to risk of development of Alzheimer's disease by multiple studies, which motivates work to elucidate neuronal and nervous system regulation of BIN1. Tacrine, one of the only drugs

licensed to treat Alzheimer's disease, was also picked up as a potent repressor of BIN1 exon 12a inclusion.

The intersection of high throughput data from cDNA expression and small molecule screens reveals the power with which we can address biological questions as technology progresses. While the mountain of data that results can be daunting, the results themselves will truly facilitate hypothesis driven experiments as we work to uncover the aberrant regulation of mRNA splicing in disease states. Remarkably, the same experiments that inform this basic science research can act as the lead studies to identification of therapeutics to treat splicing associated diseases.

Widespread alternative splicing changes occur after kainate-induced seizure in the mouse, and are returned to baseline levels after recovery

In addition to work to establish regulation cascades of disease-associated alternative splicing via high throughput screens, a complementary line of experiments sought to define a role for an alternative splicing factor that has an integral role in recovery from a disease process. The disease process in these experiments was seizure activity, which is a component to epilepsy and other neurological disorders. This work stemmed from the initial observation that neuronal deletion of the Rbfox1 splicing factor in mice led to an increased rate of spontaneous seizure and an inability to recover from kainate-induced seizure, a model for temporal lobe epilepsy. The working hypothesis is that specific alternative splicing events that are controlled by Rbfox1 contribute to homeostatic control of neuronal cells and that loss of Rbfox1 contributes to baseline aberrations in these transcripts, as well as their control after an induced seizure. Transcriptome-wide analysis of induced splicing changes in wild-type mice revealed a

potential role for Rbfox1 after seizures. Six hours after recovery from a tonic-clonic seizure, many alternative exons are changed, that are consistent with no regulation by Rbfox1. These same targets show a restoration of splicing profile 16 hours after tonic-clonic seizure, suggesting that Rbfox1 has resumed its role in regulating the transcripts. Interestingly, these regulated transcripts cluster into categories that are related to neuronal function including axon growth and synaptic plasticity. Further work will be required to fully understand the roles for Rbfox1 and induced alternative splicing changes after seizure, but the strength of phenotype in Rbfox1 knockout mice, coupled with genetic linkage of the Rbfox1 locus to many neurological disorders argues for a critical Rbfox1 role in the brain. A draft of a manuscript detailing these findings is included as Chapter 7 in this dissertation.

CHAPTER 9: METHODS USED IN THESE STUDIES

Molecular cloning

Amplification of genomic HEK293 and HeLa DNA for insertion into pFlare reporters was performed using Phusion high fidelity polymerase. Similarly, site-directed mutatgenesis was performed using Phusion polymerase and verified by traditional sequencing. shRNA clones were designed based on existing targeting sequences available in the Broad Institute shRNA library. cDNA expression clones were amplified from plasmid clones purchased from Open Biosystem, except for Tra2-beta-FLAG (generous gift of Stefan Stamm and Peter Stoilov), and SRp20-FLAG (amplified from HeLa cDNA). Src mutant clones were a generous gift from Ivan Babic and shPTB clones were described previously(7).

Cell culture

HEK293 (human embryonic kidney) and N2A (mouse neuroblastoma) cells were maintained in DMEM + 10%FBS (Atlanta) and 100mM L-glutamine. Transfection was performed using Lipofectamine 2000 (Invitrogen) and Opti-MEM media per manufacturer instructions. Cell lines harboring stable integration of the pFlare reporters were selected for 14 days using geneticin and subsequently maintained in media lacking antibiotic. Screening media was different than cell maintenance media. It was OPTI-MEM lacking phenol red, supplemented with 4% FBS (Atlanta) and 100mM L-glutamine. C2C12 (mouse myoblast) cells were maintained in DMEM + 10%FBS and were induced to differentiation by switching to media containing 2% donor horse serum. Differentiation occurred over the course of 4 days with myotubes first visible after approximately 48 hours in differentiation media.

RT-PCR

RNA was isolated using Trizol reagent (Invitrogen). cDNA synthesis was performed using random hexamers as a primer source, and using lab-made reverse transcriptase following SuperScript III instructions (Invitrogen). When possible, 2ug of total cellular RNA was input in RT reactions. PCR experiments were performed two different ways. For reporter transcripts, a fluorescently labeled reverse primer was used in the PCR mix, and the program contained 20 cycles. Fluorescent products were resolved by 8% denaturing PAGE and quantified using a Typhoon phosphorimager. For endogenous products, specific primers were designed in the exons flanking an alternative exon of interest. The reverse primer was P^{32} 5'-end labeled using T4 polynucleotide kinase and γP^{32}-ATP as a substrate. Standard PCR conditions were 24 cycles of 94°C/58°C/72°C for denaturing/annealing/extension respectively. Products were resolved on 8% denaturing PAGE and dried gels were exposed to phosphorescent collection screens overnight. Product quantities were detected using a Typhoon phosphorimager. For all statistical measurements of exon inclusion percentage, experiments were performed in biological triplicate and error was estimated by standard error of the mean for the data. RT-PCR primers for all experiments are listed in Appendix A.

Immunoblotting

Cell culture samples were resuspended in RIPA buffer and lysed on ice for 30 minutes. Proteins were resolved on 10% SDS-tris-glycine gels and transferred to PVDF membrane. Primary antibody dilutions and source are listed in Appendix B. Secondary antibodies for all immunoblots except for Akt, pAkt, Erk1/2, pErk1/2 were fluorescent: (AF488 goat anti-mouse and Cy5 goat anti-rabbit, Invitrogen and Jackson lab respectively) used at 1:2500 dilution.

Detection was via Typhoon phosphorimager or film using HRP conjugated secondary for the cell signaling antibodies.

Immunofluorescence

Samples were fixed for 10 minutes in 4% PFA and permeabilized using Triton detergent. Primary antibody dilutions are listed in Appendix B. Secondary antibodies were used at 1:200 and slides were mounted using ProLong Gold + DAPI mounting medium. Images were taken via confocal microscopte (Zeiss).

High throughput screens

The cDNA overexpression screen is the MGC collection available at the UCLA MSSR. 32ng of DNA was pre-spotted into each well of a plate using a liquid handling robot. 10uL of OPTI-MEM media (Gibco) was dropped into each well using a multi-drop system. The DNA was permitted to incubate at room temperature in the OPTI-MEM for 5 minutes. 96nL of lipofectamine 2000 (Invitrogen) in 10uL OPTI-MEM was added to each well and allowed to incubate for 20 minutes. 5000 cells was dropped into each well in a volume of 35 uL of screening medim (OPTI-MEM + 4%FBS + L-glutamine –phenol red). For small molecule screens, drug was added to 25uL screening media in each well and then 5000 cells were added on top in a volume of 25uL.

Analysis proceeded according to Zheng et al (in preparation). Briefly, cells were allowed to settle for 2 hours before the initial scan on the Typhoon phosphorimager. Plates were imaged every 24 hours for 96 hours. Images were quantified using ImageQuant TL software on the 'Array' setting. Values were normalized by a cross-plate control. This was a plate that

contained only reporter cells in every well and was used to determine the fluorescence skewing in each well. Each plate contained 4 wells with no cells added. The average background intensity for every plate was calculated from these wells and subtracted from every signal on the plate. Final signal was called XFP_{corr}. To calculate total fluorescence: A-score = $0.5*(\log_2(GFP_{norm}) + \log_2(RFP_{norm}))$. Wells were discarded with an $A_{test} < (A_{mean} - 2\sigma)$ where A_{mean} is the mean A-score for the entire screen and σ is one standard deviation from the mean. To calculate fluorescence ratio: pFlareA_M-score = $\log_2(RFP_{test}/GFP_{test})-\log_2(RFP_{back}/GFP_{back})$ where RFP_{back} and GFP_{back} are the median signals for the entire plate. pFlareG_M-score = $\log_2(GFP_{test}/RFP_{test})-\log_2(GFP_{back}/RFP_{back})$. For each screen, hits were called by being either 1.5σ higher or lower than the mean M-score for the entire screen.

Bioinformatics

As described in:

Anderson E.S., Lin C.H., Xiao X., Stoilov P., Burge C.B. and D.L. Black. (2012) 'The cardiotonic steroid digitoxin regulates alternative splicing through depletion of the splicing factors SRSF3 and TRA2B.' RNA 18(5):1041-9.

Microarray experiments

As described in:

Anderson E.S., Lin C.H., Xiao X., Stoilov P., Burge C.B. and D.L. Black. (2012) 'The cardiotonic steroid digitoxin regulates alternative splicing through depletion of the splicing factors SRSF3 and TRA2B.' RNA 18(5):1041-9.

Kaiante-induced seizure assay

Kainaite (20nm/kg) was injected in 30 day old male mice via intraperitoneal injection. Mice were observed for 120 minutes. During each 10 minute interval, the highest score obtained on Racine scale was noted. Scores are: 0-normal movement, 1=facial/whisker twitch, 2=head

bobbing, 3=forearm clonus, 4=rearing, 4=tonic-clonic seizure, 6=status epilepticus). Six hours after injection, mice were sacrificed and hippocampus was isolated by gross dissection. RNA was extracted via homogenization in Trizol. RT-PCR experiments were performed as previously. qPCR measurements were performed on an ABI 96-well qPCR machine, using 5S rRNA primers as control.

Gene ontology analysis (GO)

Gene ontology scores were generated using the DAVID online software suite. Gene names were submitted with background of all detected transcripts on the microarray. Stringency was 'Medium' and GO category terms from 'BP','MF', and 'CC' were noted that had a p-value < 0.001, generated from a modified Fisher exact test (the EASE score).

APPENDIX A: PRIMERS

Appendix A: Primers used in these studies

Chapter 2

as described in Anderson E.S. et al *RNA* (2012)

Chapter 3

TSR E1 F	GCAGTGCTGACTCCGTACAC	394/280	RT-PCR
TSR E3 R	TTCTGGGTGTTTCCCAATTC		
ERP29 E1 F	CCCTGGATACGGTCACTTTC	444/305	RT-PCR
ERP29 E3 R	GGTTATCTTGCCCCTGCTTC		
KIAA1212 E11 F	CGCTCAGACTCCAGTGAAGG	325/242	RT-PCR
KIAA1212 E13 R	AAGGCCATAGCTCCCAATTC		
PDK1_e8_F	TGCTAGGCGTCTGTGTGATT	222/145	RT-PCR
PDK1_e8_R	TTACCCAGCGTGACATGAAC		
HNRPD_3_F	GCAGAGTGGTTATGGGAAGG	267/160	RT-PCR
HNRPD_3_R	GCTATTAGCAGGTGGCAGGA		

Chapter 4

Rac1b-F	CAGCAATTGGCTTTTGGATCTCTCCGGAGGGTTAAGAC	pFlare cloning
Rac1b-R	CGAGGATCCGCATGCTTCATCCATTCTGTATTTTGTGTC	pFlare cloning
FASexo6-F	GACGAATTCCCAATCACTCTTGATTACTAGAAAG	pFlare cloning
FASexo6-R (2)	GACTGATCAGTCACCTTTGTATAACATGTGTCC	pFlare cloning
BIN1_12a-F	CGAGAATTCAGCGTTTCTGGACCGCCTGCCT	pFlare cloning
BIN1_12a-R	CGAGGATCCAGAGGCGGCACCTTCCCCTCTGTG	pFlare cloning
FGFR1_A-F	GACGAATTCTGAGGGTGTGCCCAGTCAGAGGGCT	pFlare cloning
FGFR1_A-R	GACGGATCCACCCCACCTAGTCACCTCTCTGAGA	pFlare cloning
FGFR2_3bF	GACGAATTCCACCAGGTCTTTCCGTTGTCCAC	pFlare cloning
FGFR2_3bR	GACGGATCCGTGCAGTAATGATGGGAAATGTG	pFlare cloning
FGFR2_3cF	GACGAATTCGTCTGCAGCCAACAGGACCAGAAC	pFlare cloning
FGFR2_3cR	GACGGATCCTCTACAAGCAGTGTATTTCCAAATAG	pFlare cloning
Mfe1_MAXe5-F	GGCAATTGcctgcagtagggatcacacc	pFlare cloning
BamH1_MAXe5-R	GCGGATCCtgacaagctggacacttgga	pFlare cloning

Chapter 5

As described in Anderson E.S. et al *Chembiomoleular Science* (2012)

Chapter 6

Dup-E1-Bgl-F	AAACAGATCTACCATTGGTGCACCTGACT	reporter	RT-PCR
EGFP-N	CGTCGCCGTCCAGCTCGACCAG	reporter	RT-PCR
BIN1-E10-F_h	AGAACCTCAATGATGTGCTGG	endo	RT-PCR
BIN1-E12-R_h	TCGTGGTTGACTCTGATCTCGG	endo	RT-PCR
BIN1-E10-F_m	TCAATGATGTCCTGGTCAGC	endo	RT-PCR
BIN1-E12-R_m	GCTCATGGTTCACTCTGATC	endo	RT-PCR
BIN1-E7-F_h	CAAGCTGGTGGACTACGACA	endo	RT-PCR
BIN1-E7-F_m	GAAGCTGGTGGACTATGACA	endo	RT-PCR
BIN1-E9-R	ACACCTTCTGGGCTTTGATG	endo	RT-PCR

Appendix A: Primers used in these studies (continued)

BIN1-M1_F	gCTCCGGAAAGGGGAACCAGTCCCTCC	exon mut	SDM
BIN1-M1_R	GGAGGGACTGGTTCCCCTTTCCGGAGc	exon mut	SDM
BIN1-M2_F	GGAAAGGCCCACCAGTGGATCCGCCTCCC	exon mut	SDM
BIN1-M2_R	GGGAGGCGGATCCACTGGTGGGCCTTTCC	exon mut	SDM
BIN1-M3_F	GTCCCTCCGCCTGAAAAACACACCCCG	exon mut	SDM
BIN1-M3_R	CGGGGTGTGTTTTTCAGGCGGAGGGAC	exon mut	SDM
BIN1-M4_F	CCTCCCAAACACACGAAGTCCAAGGAAGTC	exon mut	SDM
BIN1-M4_R	GACTTCCTTGGACTTCGTGTGTTTGGGAGG	exon mut	SDM
BIN1-M5_F	GGACACGTTTGTGAATGAGATCAGCG	exon mut	SDM
BIN1-M5_R	CGCTGATCTCATTCACAAACGTGTCC	exon mut	SDM
BIN1-M6_F	CAGCGTGACCACGAACTCCCAGgtcagc	exon mut	SDM
BIN1-M6_R	gctgacCTGGGAGTTCGTGGTCACGCTG	exon mut	SDM
BIN1-M7_F	CGTGACCACCCCCGGGCAGgtcagccgcg	exon mut	SDM
BIN1-M7_R	cgcggctgacCTGCCCGGGGGTGGTCACG	exon mut	SDM
IIIcBP_BIN1-12a_F	tgcttcccttgttttctagCTCCGGAAAGGCCCACC	int swap	SDM
IIIcBP_BIN1-12a_R	aaagaaaaggctagacggcgggaaggcacagc	int swap	SDM
IIIcDS_BIN1-12a_F	CTTTCTCTCTGGGctctcctctcttcctgcc	int swap	SDM
IIIcDS_BIN1-12a_R	AACAGTATATACCTGGGAGGGGGTGGT	int swap	SDM
BIN1_EcoR1-3ss-F	ggGAATTCcccctcacccgccgccgacc	int del	DEL
BIN1-5ss-BamH1-R	ccGGATCCctgggccgcggcggccgcgg	int del	DEL
BIN1-ATG1-F	GCGTGACCACCCCaTgCCAGgtcagcc	pFlareG	SDM
BIN1-ATG1-R	ggctgacCTGGcAtGGGGTGGTCACGC	pFlareG	SDM
BIN1-ATG1a-F	GCGTGACCACgCCaTgCCAGgtcagcc	pFlareG	SDM
BIN1-ATG1a-R	ggctgacCTGGcAtGGcGTGGTCACGC	pFlareG	SDM
BIN1-ATG1b-F	GCGTGACgcCgCCaTgCCAGgtcagcc	pFlareG	SDM
BIN1-ATG1b-R	ggctgacCTGGcAtGGcGgcGTCACGC	pFlareG	SDM
BIN1-ATG1c-F	GCGTGACCACCCCaTgCAGgtcagcc	pFlareG	SDM
BIN1-ATG1c-R	ggctgacCTGcAtGGGGTGGTCACGC	pFlareG	SDM
BIN1-ATG1d-F	GCGTGACCACCCCaTgCCCAGgtcagcc	pFlareG	SDM
BIN1-ATG1d-R	ggctgacCTGGGcAtGGGGTGGTCACGC	pFlareG	SDM
Chapter 7			
Nav2_69-F	GCCAGTTTCACATCAGCAAG	249/180	RT-PCR
Nav2_69-R	GGAGAGGTGACAGGTTTGGA		RT-PCR
Arpp21_105-F	AACGCCTGCGATATACAACC		RT-PCR
Arpp21_105-R	ACTCTGTGGCTGAGCCGTTT		RT-PCR
Ppp3ca_30-F	CTGACACTGAAGGGCCTGAC		RT-PCR
Ppp3ca_30-R	GAGGTGGCATCCTCTCGTTA		RT-PCR
Pax6_(42)-F	TCAGCTTGGTGGTGTCTTTG		RT-PCR
Pax6_(42)-R	TGATGGAGCCAGTCTCGTAA		RT-PCR
Rbm7_(105)_F	GTGGGTAACCTGGAGACGAA	233/128	RT-PCR
Rbm7_(105)_R	TTCACGAATGCAAACTGCTT		RT-PCR
RPLP0-qF	ACTGGTCTAGGACCCGAGAAG		qPCR
RPLP0-qR	TCCCACCTTGTCTCCAGTCT		qPCR
MYC-qF	CCTAGTGCTGCATGAGGAGA		qPCR
MYC-qR	TCCACAGACACCACATCAATTT		qPCR
FOS-qF	CGGGTTTCAACGCCGACTA		qPCR
FOS-qR	TTGGCACTAGAGACGGACAGA		qPCR

APPENDIX B: ANTIBODIES

Appendix B: Antibodies used in these studies

Chapter 2

as described in Anderson E.S. et al *RNA* (2012)

Chapter 3

Name	Dilution	Source
Akt	1/1000	Cell Signaling
pAkt_S473	1/1000	Cell Signaling
Erk1/2	1/1000	Cell Signaling
pErk1/2 T202/Y204	1/1000	Cell Signaling
Src	1/1000	Cell Signaling
pSrc	1/1000	Cell Signaling
pTyr	1/500	Cell Signaling
Gapdh	1/10000	Ambion
PPP1CA	1/500	Novus

Chapter 4

none

Chapter 5

As described in Anderson E.S. et al *Chembiomoleular Science* (2012)

Chapter 6

Name	Dilution	Source
hnRNP-K	1 in 5000	Abnova
FLAG (western)	1 in 5000	Sigma-Aldrich
FLAG (IF)	1/500	Sigma-Aldrich
B tubulin	1/750	Santa Cruz

Chapter 7

none

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