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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
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+/Ca2+ exchange, thereby increasing intracellular Ca2+ 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
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 splicing factors (Supplemental Table 2). Though we also observed enrichment of entire degenerate binding sites (Fig. 1C,D).

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 repressed 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) 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 (log2 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 camptothecin (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 exhibited 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 Ca2+ levels, which can activate pathways that require Ca2+ as a second messenger, such as the PLCγ cascade. In addition, binding of cardiotonic steroids to the Na+/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
exon 9 contains binding sites for both SRp20 and Tra2-β and can be regulated by both splicing factor knockdowns as well as digitoxin. 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 through 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 regulates splicing via SRSF3 and TRA2B

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 the regulatory cascades will be needed to reveal the direct mechanism of regulation. It is notable that cardiotonic steroid concentrations in the nano-molar range are similar to tissue concentrations found in patients treated for heart failure (Schoner and Scheiner-Bobis 2007a,b). These concentrations clearly induce tran- 
scription-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 Lipoferm 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 32P-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 PhosphorImage (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.

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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 sescore 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 ranked 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-glycerophosphate, 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; 1:5000), 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, 1:5000), rabbit α-ΔNRPDL (Abcam, 1:5000), and mouse α-U70k (1:1000). Secondary antibodies were goat α-mouse conjugated to AlexaFluor488 (Invitrogen; 1:5000), 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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