Can changes in SR protein acetylation affect alternative splicing?

Heather E. Knupp

West Virginia University

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CAN CHANGES IN SR PROTEIN ACETYLATION AFFECT ALTERNATIVE SPICING?

Heather E. Knupp

Thesis submitted to the
School of Medicine
At West Virginia University
In partial fulfillment of the requirements
for the degree of

Master of Science
in
Biomedical Science

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Morgantown, West Virginia
2011

Keywords: SR proteins, overexpression, acetylation, alternative splicing
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ABSTRACT

Can Changes in SR Protein Acetylation Affect Alternative Splicing?

Heather E. Knupp

Cellular phenotype and function is determined by the cellular proteome. Within the cellular proteome there are processes that have a potential to significantly alter the composition of the proteome. One such process is called alternative splicing. Splicing is the removal of introns from a pre-mRNA sequence and the remaining pre-mRNA sequences, called exons, are combined. Alternative splicing modifies the intron-exon combination, and can form novel products from the different arrangement of the coding sequences. This process, due to the high potential for new, possibly unwanted products, is highly regulated and can occur in a tissue and developmental-specific fashion. The misregulation of alternative splicing has been documented in many human diseases. The regulation of alternative splicing involves a complex network of proteins and RNA. Serine-arginine repeat (SR) proteins are a known family of regulatory proteins that bind within exons to promote exon inclusion into the mature mRNA transcript. SR protein activity is regulated by the post-translational modification, phosphorylation. Another post-translational modification of SR proteins known as acetylation has recently been identified. The acetylation of lysine residues on proteins can result in changes in localization, function, and/or structure of modified proteins. The location and function of this post-translational modification on SR proteins has not been determined. Cellular acetylation status of the HepG2 cell line was increased by histone deacetylase (HDAC) inhibition, using the HDAC inhibitors MS-275 and SAHA. RNA from treated cells was isolated for use with RT-PCR arrays developed by Peter Stoilov. Western blotting was used to determine efficacy of HDAC inhibitors on total cellular acetylation. The future goal is to understand how acetylation affects alternative splicing and characterize a new post-translational modification of SR proteins.
ACKNOWLEDGEMENTS

I would like to thank Dr. Lisa M Salati for her continued support and guidance during my graduate career. She has pushed me to become a better, more thorough scientist, and taught me how to slow down and consider every step carefully. I also want to thank my committee, Dr. Schaller, Dr. Ruppert, Dr. Olson, and Dr. Stoilov for their helpful suggestions and for asking all the hard questions during committee meetings. My continued thanks goes out to them for reading my master’s thesis and giving their feedback on my writing.

I’d also like to thank my labmates and friends, Angie, Amanda, Holly, and Travis for entertainment, assistance, and support throughout my years in the lab. My family and John Montoya deserve special thanks for supporting me every step of the way.
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<tr>
<td>ATP</td>
<td>Adenosine Tri-Phosphate</td>
</tr>
<tr>
<td>bp</td>
<td>Base Pair</td>
</tr>
<tr>
<td>BPS</td>
<td>Branch Point Sequence</td>
</tr>
<tr>
<td>CTD</td>
<td>C-terminal Domain</td>
</tr>
<tr>
<td>ESE</td>
<td>Exonic Splicing Enhancer</td>
</tr>
<tr>
<td>ESS</td>
<td>Exonic Splicing Silencer</td>
</tr>
<tr>
<td>G6PD</td>
<td>Glucose-6-Phosphate Dehydrogenase</td>
</tr>
<tr>
<td>HII4E cells</td>
<td>A rat hepatoma cell line</td>
</tr>
<tr>
<td>HAT</td>
<td>Histone Acetyltransferase</td>
</tr>
<tr>
<td>HDAC</td>
<td>Histone Deacetylase</td>
</tr>
<tr>
<td>HepG2</td>
<td>A human hepatocarcinoma cell line</td>
</tr>
<tr>
<td>hnRNP</td>
<td>Heterogeneous Ribonucleoprotein Particle</td>
</tr>
<tr>
<td>INSR</td>
<td>Insulin Receptor</td>
</tr>
<tr>
<td>ISS</td>
<td>Intronic Splicing Silencer</td>
</tr>
<tr>
<td>kb</td>
<td>Kilo Base</td>
</tr>
<tr>
<td>MBNL-1</td>
<td>Muscleblind-1</td>
</tr>
<tr>
<td>MS-275</td>
<td>Entinostat</td>
</tr>
<tr>
<td>NCS</td>
<td>Newborn Calf Serum</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
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<tr>
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</tr>
<tr>
<td>NLS</td>
<td>Nuclear Localization Signal</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein Kinase C</td>
</tr>
<tr>
<td>pre-mRNA</td>
<td>precursor mRNA</td>
</tr>
<tr>
<td>PTB</td>
<td>Polypyrimidine Tract Binding Protein</td>
</tr>
<tr>
<td>PTM</td>
<td>Post-translational Modification</td>
</tr>
<tr>
<td>RNP</td>
<td>Ribonucleoprotein Particle</td>
</tr>
<tr>
<td>RRM</td>
<td>RNA-Recognition Motifs</td>
</tr>
<tr>
<td>RRMH</td>
<td>RNA-Recognition Motif Homology</td>
</tr>
<tr>
<td>RS domain</td>
<td>Arginine-Serine Rich Domain</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse-Transcriptase Polymerase Chain Reaction</td>
</tr>
<tr>
<td>SAHA</td>
<td>Vorinostat</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>SELEX</td>
<td>Systematic Evolution of Ligands by Exponential Enrichment</td>
</tr>
<tr>
<td>snRNP</td>
<td>Small Nuclear Ribonucleoprotein</td>
</tr>
<tr>
<td>SR</td>
<td>Serine-Arginine</td>
</tr>
<tr>
<td>SRPK</td>
<td>Serine-Arginine Protein Kinase</td>
</tr>
<tr>
<td>ss</td>
<td>Splice Site</td>
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SPECIFIC AIMS

The function and phenotype of a cell is regulated by the cellular proteome. The process most likely to significantly alter the composition of the proteome is called alternative splicing. Splicing is the process in which intronic sequences are removed from pre-mRNA and the remaining exonic sequences combined. Alternative splicing modifies the pattern of combined introns and exons to form unique mRNA products. This process is highly regulated to occur in tissue and developmental-specific manners. Aberrant splice site selection has also been documented in cancers, cystic fibrosis, frontotemporal dementia, Parkinsonism, and myotonic dystrophy (1). These conditions underscore the importance of understanding the regulation of alternative splicing. Much less is known about the role hormones and environmental factors play in regulation of alternative splicing in somatic tissues.

Regulation of alternative splicing is achieved by the combination of \textit{cis}-acting elements and \textit{trans}-acting factors. The \textit{cis}-acting elements are sequences within the pre-mRNA that can contribute to the strength of a splice site, or be critical in recruiting \textit{trans}-acting factors to the pre-mRNA. Classical \textit{trans}-acting factors are ribonucleoprotein members of the spliceosome, as well as their accompanying regulatory proteins, called SR proteins. SR proteins belong to the serine/arginine-rich (SR) protein family, and have been shown to bind to \textit{cis}-elements within the pre-mRNA known as exonic splicing enhancer elements (ESEs), thereby promoting exon inclusion. Activation and localization of SR proteins is regulated by the post-translational modification, phosphorylation. Recently, another post-translational modification of SR
proteins was found, called acetylation (2). This novel post-translational modification has yet to be confirmed and characterized for SR proteins.

Specific Aim #1

Hypothesis

SRp20 is acetylated and this post-translational modification (PTM) plays a functional role in regulating alternative splicing. The interaction may be direct, with acetylation altering affinity of SRp20 for the pre-mRNA, or working in tandem with phosphorylation regulating activity, turnover, and/or degradation of the protein (3). Alternative splicing may be affected indirectly by the acetylation of histones recruiting chromatin-adaptors, or by modifying the chromatin structure itself (4).

Determine the role of SRp20 acetylation in alternative splicing

HepG2 cells were treated with histone deacetylase (HDAC) inhibitors and RNA isolated over several time points. The RNA from HDAC inhibitor treated cells were compared with DMSO control cells by utilizing reverse-transcriptase polymerase chain reaction (RT-PCR) splicing array technology developed by Dr. Peter Stoilov. Changes in alternative splicing were determined by quantifying the amount of transcript isoforms produced. Upon confirmation of alternative splicing changes in HepG2 cells, primary rat hepatocytes will be treated with the HDAC inhibitors, Vorinostat (SAHA) and Entinostat (MS-275), and RNA extracted to evaluate possible splicing effects on metabolic genes. SRp20 acetylation will be confirmed by immunoprecipitation if significant splice variant
changes are documented with HDAC inhibition and RNA analysis with the RT-PCR arrays. Immunoprecipitated SRp20 protein will be analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by Western blotting with an acetyl-lysine antibody, as well as, an anti-SRp20 antibody as a control.
CHAPTER 1
LITERATURE REVIEW

Introduction

Almost all protein-coding genes have pre-mRNAs that are processed in the nucleus by removing introns and ligating protein-coding portions, called exons, together in a mechanism known as splicing. Depending on the gene, exon usage can be variable, such that multiple transcripts are generated from the same protein-coding gene. Recent reports estimate that over 90% of genes undergo alternative splicing (5). The numerous transcripts produced are prime candidates for studies on their regulation, isoform type, and tissue specificity.

I. Overview of Splicing

The mechanism of splicing involves two $S_{N2}$-type trans-esterification reactions. First the phosphodiester bond at the 5’ splice site (ss) is attacked by an adenosine 2’-hydroxyl of the intronic branch point sequence (BPS). This generates a free 5’ exon and an intron lariat-3’ exon. Next, the 3’ hydroxyl of the 5’ exon attacks the phosphodiester bond of the 3’ ss leading to ligation of the exon and excision of the lariat intron (6). The splicing reaction is possible by the formation of a macromolecular ribonucleoprotein complex called the spliceosome along the pre-mRNA. The spliceosome is comprised of five small nuclear ribonucleoprotein particles (snRNPs), U1, U2, U4, U5, and U6, along with many more splicing associated proteins.
Assembly of the spliceosome along the pre-mRNA is initiated by the recognition of the 5’ and 3’ splice sites by the U1 snRNP and the heterodimeric U2AF, forming the E complex (Figure 1; (6)). In the E complex, all consensus elements are bound by proteins; U2AF 65/35 binds the polypyrimidine tract and the 3’ splice site, and SR proteins bind to the exonic splicing enhancer elements (ESEs) and contact U2AF, U1 snRNP, and the branch point. The A complex marks the addition of U2 snRNP to the pre-mRNA BPS in an ATP-dependent manner. Next, the U4/U6 and U5 tri-snRNP is added forming the B complex (Figure 2; (6)). The splicesome remains catalytically inactive until the removal of the U1 and U4 snRNPs forming the B* catalytically active complex. The first catalytic step takes place forming the C complex, and then the second catalytic step occurs, after which the spliceosomal components dissociate, ready to be reassembled on a different pre-mRNA site (6).

The need for many components in spliceosomal complexes may originate from the features surrounding the splicing process. For one, the reactive groups are recognized multiple times by RNA or associated proteins to ensure precision of splicing targets. Many of the interactions between RNA:RNA, protein:protein, and RNA:protein are weak, but are enhanced by combining several of these weak interactions (7). The rearrangement of the RNPs during spliceosomal assembly involves dynamic rearrangement of the molecular components to progress through the splicing reaction. Each step has a different number of RNPs and proteins associated with it, and these interactions are dependent on the RNA, elements within the RNA, and upstream
signaling pathways. Due to the complexity of the process, active spliceosomal complexes may contain upwards of 100 proteins (6).

II. Types of Alternative Splicing

Alternative splicing is the differential combining of exonic and intronic sequences within a pre-mRNA transcript. The combination of exons and introns determines what protein isoform is formed from the mRNA, and contributes to the genetic diversity of a species. Recent estimates from deep sequencing studies indicate that over 90% of genes are alternatively spliced (5). The regulation of alternative splicing depends upon the expression and activity of numerous splicing regulatory factors differentially expressed in tissues and throughout development. The dysregulation in these alternative-splicing processes can contribute to pathogenesis, demonstrated in a growing number of diseases, including cystic fibrosis, frontotemporal dementia, Parkinsonism, retinitis pigmentosa, spinal muscular atrophy, myotonic dystrophy, premature aging, and cancer (8).

There are five general ways in which alternative splicing can change the sequence of mRNA (Figure 3; (9)); exon skipping, 5’ alternative splice site, 3’ alternative splice site, mutually exclusive exons, and intron retention. Intron retention usually introduces a pre-mature stop codon, resulting in the formation of truncated proteins or targeting the transcript for degradation by nonsense-mediated decay, the process by which mRNA containing premature stop codons are recognized and degraded (10).
**Figure 1. Location and components of the E complex bound to pre-mRNA.**

The branch point is bound by SF1/BBP, and U2AF, the subunit U2AF65 binds to SF1/BBP along with the other U2AF subunit, U2AF35, which binds to the 3' splice site.
Figure 2. Spliceosomal components during phases of the splicing reaction.

The dynamic rearrangement of spliceosomal subunits is critical for intron-exon recognition, catalytic activation, and subsequent ligation of the exon-exon product.
Alternate splice site selection can be influenced by a variety of factors, whether those be cis-acting or trans-acting elements or an environmental stimulus. Alternative splicing can lead to different isoforms of the same protein, or form an entirely new protein.
III. Functional Consequences

a. Tissue specific

Within an organism, there are proteins comprised from different splice variants depending on the tissue type. Tissue specific isoforms make up 10-30% of all alternatively spliced genes, and these different isoforms created by alternative splicing allow for specificity of action and localization of the protein within the correct tissue (11). An example of tissue specific alternative splicing is the mitochondrial phosphate transporter gene \textit{SLC25A3}. Exons 3A and 3B of this gene are ‘mutually exclusive’ meaning that one or the other is included, but not both. This kind of splicing, termed ‘switch-like’, results in exons that are normally recognized as exons in one tissue being recognized as introns in another tissue. In an mRNA-seq study, exon 3A of \textit{SLC25A3} was shown to be included in heart and skeletal muscle and 3B was seen almost exclusively in the testes and liver (12).

b. Developmental

Muscleblind-like-1 (MBNL-1) is a protein that exhibits a developmental splicing transition. MBNL-1 has ten coding exons, some of which are alternatively spliced (exons 3, 5, 7, 9), and the expression of these isoforms is developmentally regulated. In humans and mice, the MBNL-1 regulatory factor controls the fetal-to-adult alternative splicing switch by binding to YCGY sequence elements in a key set of pre-mRNAs (13). The binding of MBNL-1 promotes either the inclusion or exclusion of alternative exons.
depending on the 3’ or 5’ placement of cis-regulatory elements within the pre-mRNA transcript, making MBNL-1 an important regulator of splicing in development.

Over ten isoforms of MBNL-1 have been identified and are developmentally regulated. In type 1 myotonic dystrophy the expression of MBNL-1 isoforms is altered, which indicates a role for alternative splicing in regulating the function of the protein. Cassette exons 5 and 7 are included mainly in fetal brain and muscle, and are preferentially included in type 1 myotonic dystrophy patients. One hypothesis of how type 1 myotonic dystrophy progresses, is based on a toxic RNA gain of function, which could lead to dysregulated alternative splicing of targeted transcripts, like the insulin receptor (INSR) gene.

In cellular models of myotonic dystrophy, mutant RNA transcripts with long-CUG repeats form odd double-stranded RNA structures, accumulate in the nucleus, and lead to foci that sequester ribonuclear proteins like MBNL-1 (13-16). In the instance of INSR in myotonic dystrophy, the inactivated MBNL-1 is associated with an increase in CUG-BP1, a CUG-repeat binding protein that has different specificities than MBNL-1 and a binding site in exon 11 of INSR (17). The splicing factor hnRNP-H promotes exon exclusion, and is also increased in myotonic dystrophy myoblasts. The combination of the increase in amount of proteins promoting exon exclusion and the disrupted RNA-protein interaction culminate in a reduction of INSR exon 11 inclusion in models of myotonic dystrophy (17). Additional studies have shown that there is aberrant alternative splicing of several MBNL-1 targets in myotonic dystrophy patients and when
MBLN1 is inactivated, suggesting a loss-of-function mechanism in myotonic dystrophy progression (14).

c. Disease

The changes in alternative splicing documented in disease are primarily manifested in mutations in either the cis-acting RNA elements, or the trans-acting protein splicing factors. The cis-acting mutations disrupt regulatory RNA sequences and affect the binding of splicing regulators. These mutations can result in the localization of trans-acting factors to improper 5’ or 3’ splice sites, creating non-functional protein isoforms, premature stop codons, or out-of-frame transcripts. In some cases, mutations in one of the splicing elements can activate the use of a cryptic splice site, a weak splice site that is only used when a mutation occurs. Any one of these mutations can lead to a variety of diseases including, but not limited to, cystic fibrosis, frontotemporal dementia, Parkinsonism, retinitis pigmentosa, spinal muscular atrophy, myotonic dystrophy, premature aging, and cancer (8).

The trans-acting mutations affect the quality or quantity of alternative or constitutive splicing factors. System lupus erythematosis is the classic example associated with the splicing machinery per se. In this disease, the immune system produces antibodies targeting to snRNPs. Autosomal dominant retinitis pigmentosa is a disorder that eventually results in complete blindness. In this disorder, there are mutations in the splicing factors PRPF31/U4-61k and PRP8. PRP8 is the major enzymatic component of the spliceosome, and interacts with all spliceosomal components (18). Many diseases involve aberrant splice site selection.
d. Environmental stimuli

A change in splicing in response to hormones or nutrients involves activation of signaling cascades containing kinases, which phosphorylate SR proteins. Phosphorylated SR proteins are active and can enter the nucleus to bind to pre-mRNAs and influence splice site selection. The hormone, insulin and the nutrient, arachidonic acid have been shown to regulate amount of glucose-6-phosphate dehydrogenase in opposite ways. Treatment of primary rat hepatocytes with insulin increases the phosphorylation of SR proteins, while treatment with arachidonic acid decreases the phosphorylation of SR proteins in the liver (19). Treatment of hepatocytes or liver with insulin/refeeding results in an increase in the spliced form and amount of G6PD, while arachidonic acid/starvation results in a decrease in the spliced product and amount of G6PD mRNA (20). In hepatocytes and whole liver, SR proteins have been shown to bind to a splicing enhancer element within exon 12 of G6PD.

Another example of regulated splicing by insulin involves an isoform of protein kinase C (PKC), protein kinase CβII (PKCβII). The Cooper lab has shown that Akt2 stimulation by insulin promotes βII exon inclusion through the phosphorylation of SRp40 (21). Binding of SRp40 introduces a stop codon within the βII exon, resulting in a truncated isoform of PKC. The insulin regulation of PKCβII has been shown in several cell types including: primary rat hepatocytes, 3T3-L1 adipocytes, HepG2 cells, and L6 cells. There are two alternative sites that allow for inclusion of the βII exon, one is directly at the 5’ splice site (I), and the other is a little farther downstream of the 5’ splice site, in the intron (II). The regulation of splice site selection in insulin sensitive tissues
has been shown to vary in splice site selection (I vs. II) and amount of time after insulin treatment to the increase in isoform PKCβII (22). The different cellular responses to insulin stimulation show splice site preference and temporal regulation depending on the context and cellular uses for insulin. The examples of nutrient and hormonal regulation of metabolic genes suggest that SR proteins are directly involved in mediating the changes in splicing in metabolic tissues, making them prime candidates for the regulation of other lipogenic genes in the liver.

IV. Molecular Mechanisms

Alternative splicing is controlled by targeted sequences within the pre-mRNA known as cis-acting elements, and complexes of proteins and ribonuclear proteins known as trans-acting factors. Many of the cis-acting elements contain additional information in the form of 6-8 nucleotide sequences known as splicing enhancers or silencers. These elements within the pre-mRNA serve to recruit trans-acting splicing factors that can activate or suppress splice site selection as well as spliceosomal formation (9; 23).

The cis-acting elements bind splicing enhancers like SR proteins when the sequence is an enhancer element, or splicing silencers, like heterogeneous ribonuclear particles (hnRNPs), in the case of a silencing element. These binding sequences were determined using SELEX (systematic evolution of ligands by exponential enrichment) and were shown to have a high degree of degeneracy, meaning a single factor can regulate a large number of genes by binding to more than one sequence (24). Further
more, because SELEX is an *in vitro* technique, these results do not prove that the sequence has biological activity. The variation in mRNA binding sequences leads to the question of whether the sequences alone determine SR protein/hnRNP binding, or if other *trans*-acting factors or RNA secondary structures contribute to SR protein/hnRNP association with mRNA.

a. Splicing enhancers

SR proteins are a family of at least seven RNA binding proteins (SF2/ASF, SC35, 9G8, SRp20, SRp40, SRp55, and SRp75). They bind to sequences within the pre-mRNA known as exonic splicing enhancers (ESEs)). Bound SR proteins block the binding of other regulatory proteins, like hnRNPs that may favor exon exclusion, while the SR proteins themselves promote exon inclusion. SR proteins bind to the RNA using a RNA recognition motif (RRM) at their N-terminus that determines RNA-binding specificity. Their C-terminal RS domain facilitates protein-protein interactions and recruitment of the spliceosomal components (Figure 4, (25; 26)). SR proteins effectively promote spliceosome assembly on nascent pre-mRNAs to catalyze intron removal during the processing of actively transcribed genes. The RS domain can also contact the pre-mRNA directly via the branch point and the 5’ ss, which may indicate another method of spliceosomal assembly.

There are two main models proposed for how SR proteins direct exon inclusion. The recruitment model proposes that bound SR proteins can recruit and stabilize interactions between the U1 snRNP at the 5’ ss and U2AF65 and the 3’ ss. The other
model proposes that SR proteins form a network of protein-protein interactions across introns to connect the 5’ and 3’ ss early in spliceosomal assembly, as shown by the interaction of SF2/ASF and SC35 with U1-70K at the 5’ ss and with U2AF at the 3’ splice site in an RS domain-dependent manner (25). SR proteins may facilitate the recruitment of the U4/U6, U5 tri-snRNP to the inactive spliceosome through other SR-related proteins.

The main mode of SR protein regulation is reversible phosphorylation of the RS domain. This serine-arginine rich domain is extensively phosphorylated on serine residues and plays an important role in regulating the localization and activity of SR proteins. For example, phosphorylation of SF2/ASF enhances protein-protein interactions with other RS domain-containing splicing factors, like U1-70K, and the dephosphorylation of all SR proteins is required for splicing catalysis to proceed (27; 28). There are several protein kinase families that phosphorylate the RS domain of SR proteins, the SR protein kinase (SRPK) family, the Clk/Sty family of dual specificity kinases, Akt, and topoisomerase I (25). These kinases are required for SR protein activation and the majority of their functional ability.

SR proteins are localized in the nucleus, specifically in the nuclear speckles. When the SR proteins are hyperphosphorylated, they move out of the nuclear speckles and are recruited to sites of RNA polymerase II (RNAPII) transcription where at least some bind to the CTD (29).
The RRM is the RNA-recognition motif, while the RS is the serine-arginine repeat domain. Some of the SR proteins also have a second RNA binding domain known as the RNA-recognition motif homology domain (RRMH).

**Figure 4. Structural and functional domains of serine-arginine (SR) proteins** (25).

SRp20  
SC35  
SF2/ASF  
SRp30c  
SRp40  
SRp55  
SRp75
The binding of SR proteins to RNAPII and the pre-mRNA can occur co-transcriptionally (30). Localization on the CTD allows binding to ESEs coincident with transcription. In addition to shuttling to and from nuclear speckles, the SR protein family members, with the exception of SC-35, have been shown to shuttle between the nucleus and the cytoplasm.

The shuttling activity suggests a role in transporting mRNA out of the nucleus (31), and the splicing factors SRp20, 9G8, and SF2/ASF have been shown to interact with the mRNA nuclear export receptor TAP/NFX1 with a higher affinity when hypophosphorylated, linking mRNA transport with dephosphorylation (32-34). These studies have demonstrated the dynamic regulation of SR proteins by phosphorylation and dephosphorylation and lead the way for studies on other posttranslational modifications.

In mouse model systems, knockouts of SRp20, SC35, SF2/ASF all have been shown to be embryonic lethal. The lethality of knocking out these splicing factors indicates the diversity of their functions and necessity for sustaining cellular viability.

b. Splicing inhibitors

hnRNPs bind to exonic and intronic splicing silencer elements (ESSs and ISSs) within the pre-mRNA. Their function has primarily been shown to be opposite of SR proteins. They repress exon inclusion by promoting distal splice site selection, possibly by looping out the pre-mRNA in such a way that internal 5’ splice sites are hidden within the hnRNP complex.
hnRNPs have an RNA recognition motif (RRM), that contacts the RNA using the degenerate consensus sequences RNP-1 and RNP-2. The contact is mediated by hydrophobic interactions between four aromatic side chains and two bases, however, not all hnRNPs bind RNA with RRM domains. hnRNPs E/K bind RNA via the KH (K homology) domain. This domain can only fit four bases within its narrow groove, and the interaction with RNA is facilitated by hydrogen bonding and electrostatic interactions. hnRNPs F/H contain qRRMs which have an extra $\beta_3$ loop as compared to classical RRM and lack the RNP consensus sequence. There are RRM s in hnRNP I, referred to as atypical RRM s (aRRMs), that lack canonical RNP motifs (Figure 5, (35)). With the exception of hnRNP U, all hnRNPs contain RNA-binding domains (RBDs). Splice variants have been identified for the majority of hnRNPs and they frequently undergo post-translational modifications. Primarily, the post-translational modifications are phosphorylation of serine/threonine residues and methylation of arginine residues. The function of these post-translational modifications is currently unknown. The hnRNPs exhibit nucleoplasmic shuttling, and some do so with nuclear localization signals while others, like hnRNPs A1 and I do so without classical nuclear localization signals (35).

As a way to identify the precise roles of hnRNPs in mRNA regulation, Konig et al. (2010) developed a technique that provided individual-nucleotide resolution by UV cross-linking and immunoprecipitation (iCLIP). Using this technique, hnRNPs were found to have enhanced incorporation on silenced exons and proximal intronic regions.
Figure 5. Structural and functional domains of hnRNPs (35).

The hnRNPs have diverse functions in the process of mRNA splicing regulation. Part of this diversity is due to the combination of the structural and functional domains that make up each hnRNP.
The authors showed that hnRNPs enhance splicing when they bind in the intron preceding the alternative exon (36). The resulting hypothesis is that hnRNPs may act as ‘RNA nucleosomes’ by binding the pre-mRNA, but maintain the integrity of the splice sites and access of the splicing machinery to the pre-mRNA. Their findings suggest that hnRNPs can serve dual purposes in splicing regulation, and their purpose depends on the positioning of the particles on the pre-mRNA (36). The observation that hnRNPs have a dual enhancer/repressor role adds another layer of complexity to the mechanism of mRNA splicing.

V. SRp20

In a global analysis of the SR proteins, SRp20 and SRp75, in neural cells, a distinct separation was made in the mRNA transcript specificity of these two SR proteins. There was little overlap in the mRNA bound by SRp20 and SRp75, and there were more mRNAs that were associated with, and depended on SRp20 for proper expression (37). In neural cells, the importance of SRp20 in alternative splicing is validated by the transcript specificity, and number of transcripts bound by SRp20. Studies of this nature have not been attempted regarding SRp20’s role in regulating splicing in the liver. There have been three SRp20 target mRNAs identified in the liver, glucose-6-phosphate dehydrogenase (G6PD), insulin receptor (IR), and pyruvate kinase-M (PK-M) (37-39).

SRp20 binds to exon 12 in G6PD leading to exon inclusion in the fed state. In the fasted state, hnRNPs bind to G6PD and prevent SRp20 from binding, leading to intron
inclusion and degradation of the transcript. G6PD is a lipogenic gene, and is coordinately regulated with the other lipogenic genes in the liver. The binding of SRp20 to G6PD in response to nutritional status can mean that SRp20 may regulate other lipogenic genes in a similar manner.

The splicing of IR results in two isoforms, IR-A and IR-B. The difference between the two isoforms is a 12-amino-acid insertion in the hormone-binding domain of the receptor, which is determined by the alternative splicing of exon 11. IR-A is primarily expressed in the embryo and lacks exon 11. This isoform promotes growth by being able to bind insulin and insulin-like growth factor II. IR-B contains exon 11 and is expressed in adult insulin-sensitive tissues, like the liver, muscle, and adipocytes, but only binds insulin. The inclusion of exon 11 has been shown to be dysregulated in a number of diseases, including type II diabetes, myotonic dystrophy, and cancer (40). The dysregulation stems from the aberrant binding of splicing enhancers and inhibitors to their compatible enhancer and silencer elements within alternatively spliced exon 11 and intron 10 of the human \textit{INSR} gene (38). There are overlapping binding sites for CUG-BP1, an exonic silencer and SF2/ASF, an exonic enhancer in the stem-loop structure. There is a SRp20 binding site in the unstructured loop preceding the stem-loop. Point mutations made disrupting the base pairing of the stem-loop favor exon inclusion, while mutations that strengthen the base-pairing favor exon exclusion. The thought is, that the binding of CUG-BP1 sequesters exon 11 within the stem-loop excluding exon 11, while the binding of SF2/ASF and SRp20 prevent the formation of the stem-loop, favoring exon 11 inclusion (38).
SRp20 is involved in the splicing of another important metabolic gene, pyruvate kinase M (PK-M). Pyruvate kinase catalyzes the last step in glycolysis, forming pyruvate and ATP from phosphoenolpyruvate (PEP) and ADP. Exons 9 and 10 of the PK-M gene are alternatively spliced and referred to as mutually exclusive, forming two isoforms PK-M1, which includes exon 9 and PK-M2, which includes exon 10. PK-M1 is constitutively active and present in most terminally differentiated tissues, and PK-M2 is expressed in embryonic and undifferentiated tissues, as well as cancer cells (39). PK-M2 is allosterically regulated by fructose-1,6-bisphosphate (FBP) and has been shown to interact with tyrosine-phosphorylated signaling proteins. In cancer, PK-M2 is inhibited by growth-factors, which stops flux through the glycolytic pathway, and accumulates upstream intermediates (39). The intermediates are then shunted to anabolic pathways and can contribute to increased cell proliferation. In an effort to understand the mechanism of cancer growth, the features of PK-M splicing were studied.

First, Wang et al. (39) assessed the relative strength of exon 9 and 10 splice sites, by transfecting 293 cells with mutant PK-M minigenes. The authors found that when the 5’ or 3’ splice sites of exon 10 are inactivated, exon 9 inclusion is only marginally rescued. When exon 9 splice sites were strengthened, a new transcript was produced including exon 9 and exon 10. The double exon product suggests inclusion of exon 10 is an active process and largely independent of exon 9 splicing. Next, the cis-elements of PK-M were analyzed, and it was shown that exon 10 is included if placed before exon 9, indicating that the cis-elements in exon 10 are sufficient enough to activate exon 10 and repress exon 9 (39). The authors then analyzed the ESE
sequences within exon 10 and found a strong SRp20 consensus sequence. Analysis of the ESE by mutation and RBP-immunoprecipitation assay confirmed binding of SRp20 to exon 10 of PK-M, and that SRp20 binding is necessary for exon 10 inclusion. Interestingly, overexpression of SRp20 increases exon 10 inclusion in a neuroblastoma model preferentially including exon 9 and expressing low levels of SRp20. Also, knockdown of SRp20 in HEK-293 cells increases lactate production and decreases proliferation, suggesting a switch from PK-M2 to PK-M1. The discovery of another alternatively spliced metabolic gene regulated by SRp20, in addition to G6PD and INSR, suggests other metabolic genes may be regulated in the same fashion. Wang et al. (39) provides more evidence of SRp20 as an oncogene by facilitating splicing that favors increased growth and proliferation.

VI. Role of Acetylation

Splicing is coupled to transcription, and signals changing transcription rates or chromatin structure appear to influence splice site selection. In addition, splicing factors can be posttranslationally modified by phosphorylation, glycosylation, methylation, and now, acetylation (2; 7; 41; 42). Thus, acetylation of both histones and splicing factors may be coupled to regulate gene expression.

Acetylation of the ε-amino group of lysine functions to neutralize the positive charge carried by the nitrogen. The modification is reversible and changes the protein function in many diverse and interesting ways. Lysine acetylation was originally discovered as a post-translational modification of core histone tails and the extent of
acetylation is regulated by histone acetyltransferases (HATs) or histone deacetylases (HDACs) (43). The first evidence of SR proteins being directly acetylated comes from Edmond et al. (3), who identified SC35 as being a target of Tip60. Tip60 is the catalytic subunit of the NuA4 histone acetyltransferase complex, and has shown to be involved in DNA damage repair and transcriptional regulation. The authors showed that loss of Tip60 increases the amount of SC35 protein and enhances the half-life of the protein. They conclude that Tip60 regulates degradation of SC35 through a mechanism involving the proteasomal pathway. Next, they wanted to look at what happened to SC35 when it was deacetylated. HDAC6 is a histone deacetylase that possesses ubiquitin-linked functions, and was chosen because of its link to the proteasome (44; 45). Overexpression of HDAC6 resulted in the accumulation of SC35 protein. Overexpression of Tip60 significantly reduced SC35 phosphorylation, while the knockdown of Tip60 enhanced SC35 phosphorylation. The results suggest interplay between Tip60 negatively regulating SC35 phosphorylation to control SC35 protein activity.

VII. Histone Modifications in Alternative Splicing

Histone modifications determine the availability of DNA to be replicated, transcribed into mRNA, and translated into protein. Recent observations along sites of histone modifications include; the presence of cotranscriptional pre-mRNA splicing, alternative splice site choice influenced by RNA polymerase II elongation rate, the presence of chromatin remodelers, and lysine deacetylase (KDAC) activity (30; 46; 47).
Fibroblast growth factor receptor 2 (FGFR2) is a receptor tyrosine kinase gene with an established alternative-splicing pattern where exons IIIb and IIIc undergo mutually exclusive and tissue-specific alternative splicing. Studies have shown that exon IIIb is preferentially included in epithelial cell types and IIIc is included in mesenchymal cell types. The switch from one cell type to another is nearly perfect in models of epithelial-to-mesenchymal transition (EMT), linking the switch to metastatic, highly invasive cancers (48). In the prostate, exon IIIb is included, while in human mesenchymal stem cells, exon IIIc is included. Exon choice was found to be regulated by polypyrimidine-tract binding protein (PTB) binding to silencing elements around exon IIIb. This phenomenon was mapped by qChIP and identified the histone modifications in the alternatively spliced regions of FGFR2 Overexpression of the H3-K4 methylase increased the usage of the normally excluded PTB-dependent exon, and not of PTB-independent exons or constitutively spliced exons. These findings demonstrated that histone modifications can influence alternative splicing (4).

Histone acetylation is classically linked to an increase in gene activity. In cancer models, treating HepG2 and Huh6 cells with HDAC inhibitors like suberoylanilide hydroxamic acid (SAHA), has been effective in activating the extrinsic apoptotic pathway, increasing the expression of FasL, FasL receptor, and activating caspase-8 (49). SAHA enhanced the level of Bim proteins, stimulated the alternative splicing of Bcl-X to its proapoptotic form, Bcl-Xs, induced degradation of Bid into the apoptotic factor t-Bid, and dephosphorylated Akt. These effects were seen in the transformed cell
lines, HepG2 and Huh6, but not in primary human hepatocytes (PHH), indicating a potential role of HDAC inhibitors for treatment in liver cancer.

VIII. Acetylation and metabolism

ATP-citrate lyase provides acetyl groups for histone acetylation through citrate-derived acetyl CoA. Silencing ATP-citrate lyase with siRNA decreased the amount of histone acetylation for all core histones (50). Histone acetylation was rescued with supraphysiological levels of acetate, the substrate used by acetyl CoA synthetase, and occurred in a dose-dependent manner. Upon ATP-citrate lyase silencing the expression of Glut4, hexokinase 2, phosphofructokinase-1, and lactate dehydrogenase A, along with a gene involved in fatty acid synthesis, carbohydrate responsive element-building protein (ChREBP) were suppressed and then able to be rescued by acetate. The transcriptional effects correlated well with the glycolytic activity with a reduction of 32% in glucose consumption (50).
CHAPTER 2
MATERIALS AND METHODS

Cell Culture

The human hepatocarcinoma cell line, HepG2, was used to study the effect of acetylation and alternative splicing. The cells were plated and cultured in Dulbecco’s Modified Eagle’s Medium (GIBCO®) with 10% FBS (Atlanta Biologicals) and 1% antibiotic-antimycotic (GIBCO®). When confluent, the cells were passaged at a 1:3 or 1:4 ratio. The media was changed every 2-3 days.

Western Blot Analysis

Western blot analysis was done as described in Walsh et al. submitted. Acetylated proteins were detected using a polyclonal acetyl-lysine antibody (Cell Signaling). The PVDF membrane was incubated overnight in polyclonal rabbit acetyl-lysine IgG antibody diluted 1:1000 in 5% bovine serum albumin (BSA). The antibody was synthesized by (Cell Signaling Technology®). The membrane was incubated in a goat anti-rabbit secondary antibody (Zymed®) diluted 1:2000 in 5% non-fat dry milk. After washing in TBS-T, antibody bound acetyl-lysine was visualized using ECL-Plus™ (General Electric Healthcare®). Chemi-fluorescent signals were detected by X-ray film or by the Typhoon scanner using ImageQuant™ software.
RT-PCR arrays

Total RNA was isolated from the DMSO, SAHA, and MS-275 treated HepG2 cells after 16 hours. The RNA was reverse transcribed (RT) in 200μl reactions containing 10μg of RNA per reaction. Table 1 shows the components of the RT reaction and the PCR parameters. The cDNA was amplified using primer sets for 84 different alternatively spliced genes developed by the Stoilov lab using 96 or 364 well plates. The cDNA was subjected to acrylamide-urea electrophoresis (7.5M urea, 6% acrylamide, 10X Tris/Borate/EDTA (TBE)) in large sequencing gel apparatuses and visualized on the Typhoon phosphoimager (See Appendix I for gel-setup). Quantitation of spliced products was done using the ImageQuant software.
CHAPTER 3
ACETYLATION AND ALTERNATIVE SPlicing

Introduction

Acetylation has primarily been associated with regulation of histone function and chromatin structure. Until recently, the fields of chromatin biology and pre-mRNA splicing were thought to be completely independent of one another. More recent studies have shown that the two fields are intimately connected by demonstrating that DNA associated with nucleosomes is preferentially located in exons, and that the SR proteins, SRp20 and ASF/SF2 bind to the tail of histone H3 to control cell cycle progression (51; 52). The observation of SR proteins associated with chromatin suggests the SR proteins may undergo some of the same reversible posttranslational modifications that histones do, like acetylation. Lysine acetylation is a highly regulated process that is directed by the opposite actions of histone acetyltransferases (HATs) and histone/lysine deacetylases (HDACs or KDACs).

A proteomics study by Choudhary et al. (2) identified many new proteins as targets for lysine acetylation, including SR proteins. Adding an acetyl-group to a lysine neutralizes the positive charge and can change the structure and function of the modified target protein, making acetylation of SR proteins a worthwhile topic to study. The function and location of acetylation of SR proteins has not been identified, but the RRM domain is lysine rich and a candidate region. If acetylation of SR proteins has any functional relevance, then there is potential for alternative splicing to be altered as a result. The Choudhary et al. (2) study examined acetylated targets in MV4-11, Jurkat,
and A549 cells, all myeloid adenocarcinoma cell lines. We sought to determine if acetylation of SR proteins could occur in liver cells and if this regulated alternative splicing. The first step was to identify if HDAC inhibition has an effect on alternative splicing.

Results

Acetylation of SR proteins has been detected in cancer cell lines, and due to our interest in the liver, HepG2 cells were chosen as the primary model for our investigations. The cells are derived from a human hepatoma, and the use of human cells permitted the use of the PCR arrays developed by Peter Stoilov to detect cancer specific alternative splicing events. These arrays contained primer sets that amplified the RNA products of 84 genes and permitted detection of the alternatively spliced isoforms of their mRNAs. Modification of these arrays for use in primary rat hepatocytes would be difficult and time consuming. The primary rat hepatocytes are also sensitive, are only viable for 5 days in culture before de-differentiating, and may not withstand treatment with HDAC inhibitors.

Cell lysates from HepG2 cells incubated with 2 μM SAHA or 5 μM MS-275 for 16 hours were subjected to western blotting with an anti-acetyl lysine antibody to detect changes in acetylation (Figure 6). HDAC inhibition resulted in an increase in acetylation of histone H3 (H3) and histone H4 (H4) indicated by arrows (Figure 6) when compared with DMSO.
Figure 6. Total acetylation in HepG2 cells treated with the HDAC inhibitors SAHA or MS-275.

Total protein was isolated from HepG2 cells at the times indicated. The left blot shows no treatment (NA) and vehicle (DMSO) as controls compared with HepG2 cell lysate from cells treated with HDAC inhibitors, 2μM SAHA or 5μM MS-275. The highest acetylation was visible from histone H3 at ~17kDa and H4 at ~11kDa. MW, molecular weight markers.
With confirmation of the increase in acetylation, a RT-PCR screen was performed to identify changes in splicing associated with HDAC inhibition. RNA isolated from HepG2 cells treated with 2 μM SAHA or 5 μM MS-275 for 16 hours was reverse transcribed, and the resulting cDNA amplified using the PCR arrays. Several genes exhibited changes in alternative splicing due to HDAC inhibition when compared with the DMSO controls, as determined by visual confirmation from gel images (Appendix I; Figure A1, A-F). Figure 7 shows a representative group of genes with no change (Figure 7A, lanes 1, 5, 9), increase (Figure 7B, lanes 5 and 9), or decrease in isoform in response to HDAC inhibition (Figure 7B, lanes 5, 8, 9 and 12). Densitometry was performed on the isoform bands to confirm visual changes observed in comparing the DMSO treated and HDAC inhibitor treated groups. The raw quantification of bands using ImageQuant software is represented in pixels per band. The subsequent normalization of the data, by dividing pixels per band by total number of pixels in all bands per gene, in some cases, does not match to the ratios, normalized to DMSO, or bar graph representation (Table 1; Figure 7B, lanes 1, 5, 9, and Figure 8, RPS6KB1). The differences from visual to numeric data may be a result of high background, lack of product, or unequal loading of the amplified product. A solution would be to replicate the experiment (n=3) and analyze genes listed in Table 1 again to confirm the changes in isoform presence with HDAC inhibition.
Figure 7. Isoform expression of genes from HepG2 cells treated with DMSO, SAHA, or MS-275.

Total RNA extracted from HepG2 cells treated with DMSO, 2 μM SAHA, or 5 μM MS-275 for 16 hours was reverse transcribed and the resulting cDNA was amplified with primers designed to genes with alternatively spliced products. Each lane represents the results from RNA isolated from 3 plates/treatment. (A) Bands with boxes are examples of genes with no change in isoform with inhibitor treatment (lanes 1, 5, and 9). (B) Red arrows below bands indicate an increase in isoform with inhibitor treatment (lanes 9, 5), and blue arrows indicate a decrease in isoform with inhibitor treatment (lane 9). Refer to Table 1 for densitometry pixel values of noted bands. Bands not referenced are mis-primed products, artifacts, or primer dimers.
The RT-PCR array showed the largest changes in decreasing isoform presence in the genes *RPS6KB1, ENAH, MKNK2, and Fas1*. *RPS6KB1* isoforms were generated from regions between exon 9 and exon 10. There are two alternative exons identified by the UCSC Genome Browser database EST collection. The inclusion of both exons results in a product size of 441 bp. Inclusion of one exon, but not the other, results in products with sizes of 366 bp and 188 bp. When both exons are skipped the product size is 113 bp. Figure 8 and Table 1 show a decrease in the isoforms containing only one of the alternative exons (366 bp or 188 bp). The splice variants detected have not been referenced in the literature, and may be novel products induced by HDAC inhibition.

The gene *ENAH* showed a decrease in expression of the 212 bp and 155 bp isoforms with SAHA and MS-275 treatment. The decrease in the 212 bp isoform was the most pronounced and similar with both inhibitors. The decrease in the 155 bp isoform was not as pronounced with SAHA treatment, as it was with MS-275 treatment (Figure 8, *ENAH*). The literature suggests that the amplified isoforms correspond to the cassette exons 3a and 3b, also known as INV (+++) and (++), respectively (53).

There were two isoform changes associated with the gene *MKNK2*. In response to SAHA treatment there was a decrease in the expression of a 193 bp isoform and an increase in the expression of a 170 bp isoform. The 193 bp isoform is associated with MKNK2a, and the 170 bp isoform is associated with MKNK2b (Table 1, Figure 8, *MKNK2*).
Expression of the 130bp isoform of *Fas1* decreased with SAHA and MS-275 treatment (Figure 8, *Fas1*). This isoform represents a decrease in the exon 6 deletion isoform of Fas1 with HDAC inhibition.

The largest increase in isoform appearance with HDAC inhibition was in the 156 bp product of PTK2 (FAK) (Table 1, Figure 8, *PTK2*) This area corresponds to an increase in the inclusion of exon 31, an exon not normally found in FAK transcripts.

Based upon the data generated from the RT-PCR arrays, genes represented in the literature with identifiable changes in isoform presence comparing DMSO to HDAC inhibitor treated groups were; *PTK2, ENAH (hMENA), MKNK2, and Fas1*. Genes with stars in Table 1 also show changes in splicing with HDAC inhibition and warrant further investigation by qRT-PCR.
Figure 8. Changes in isoform abundance of genes in HepG2 cells treated with SAHA or MS-275 from Table 1.

Changes in alternative splicing were assessed as in Figure 7. The pixel density of the isoform bands was quantified using the ImageQuant software for the Typhoon imager. Values from Table 1 were used to populate the bars on the graph. The bars represent pixels per band/pixels of total # of bands per gene normalized to DMSO with a log₂ transformation (y-axis). Each bar represents the results from RNA isolated from 3 plates/treatment. The blue bars represent change in isoform presence with SAHA treatment, relative to DMSO treatment. Red bars represent isoform presence with MS-275 treatment relative to DMSO treatment.
Table 1. Quantification of isoform expression in HepG2 cells in response to DMSO, SAHA, or MS-275 treatment.

*Genes showing changes with HDAC inhibition not described in the text.

**Alternative exon locations were determined by assembly Mar. 2006 (NCBI36/hg18)

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1; expressed as pixels per band/pixels of total # of bands per lane

2; log₂ (SAHA¹ or MS-275¹/DMSO¹)

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Discussion

Treatment of HepG2 cells with HDAC inhibitor altered the splicing of several genes that have diverse roles in maintaining normal cellular function (Figure 8). These include; Fas1, PTK2 (FAK), MKNK2, and ENAH (mENA) (Figure 9). The overall changes in splicing are small, but could be sufficient enough to significantly affect cellular processes (Figure 8).

The HDAC inhibitors chosen for the study, SAHA (Vorinostat) and MS-275 (Entinostat) differ in their affinities for the varying HDACs and specificity. MS-275 inhibits HDAC1 (IC$_{50}$ = 300nM) over HDAC3 (IC$_{50}$ = 8$\mu$M), and demonstrates no inhibitory activity to HDAC8 or HDAC6 (IC$_{50}$ >100$\mu$M). SAHA inhibits Class I deacetylases, HDAC1, HDAC2, and HDAC3, as well as, Class II deacetylase HDAC6 (IC$_{50}$ < 86nM). Neither SAHA nor MS-275 inhibit the Class III HDACs, also known as sirtuins (SIRT1-7). Class I HDACs have one catalytic site, while Class II HDACs have two catalytic sites. Both classes of HDACs are zinc-dependent enzymes. The differences in affinity and specificity of the HDAC inhibitors may explain the change in splicing; by ruling out deacetylases based on comparing the altered splicing pattern between SAHA and MS-275 treated groups.

The two inhibitors chosen have been studied in depth and are either in use for the treatment of cancer, in the case of SAHA (Vorinostat), or in clinical trials. MS-275 (Entinostat) is in many phase II clinical trials in the treatment of breast and lung cancers. SAHA, now more commonly known as Zolinza, is used in the treatment of cutaneous T-cell lymphoma that has gotten worse, not responded to treatment, or recurred after
taking other medications. Normal cells are relatively resistant to HDAC inhibitor induced cell death, perhaps because normal cells have regulatory mechanisms in place that prevent HDACs from deacetyllating inappropriate substrates (49).

The change in expression of Fas receptor isoforms is an example of an effect of HDAC inhibition that may have functional consequences. The bands represent the presence of exon 6 or absence of exon 6. In cancers, the Fas receptor is a way for tumor cells to evade detection by the immune system. Normally, Fas is expressed on the cell surface, and when bound to Fas ligand on cells can initiate a signaling cascade leading to cell death. The alternative splicing of Fas, leads to the deletion of exon 6, eliminating the transmembrane domain, and leading to an unbound soluble Fas (Figure 9A). The soluble Fas is thought to inhibit Fas-mediated cell death by binding Fas ligand before it can recognize membrane bound Fas. In light of this, we see a decrease in exon 6 exclusion with HDAC inhibitor treatment, which indicates that more exon 6 included transcripts present in the cells treated with HDAC inhibitors (Figure 8, (48)). The HDAC inhibitors appear to be increasing the presence of membrane bound Fas, which would lead to cell death, and is advantageous in a cancer model.

MAP kinase interacting serine/threonine kinase 2 (MKNK2) is another RNA whose splicing may have functional significance. The activation of MKNK2 by Erk or p38 MAP kinase α/β allows it to interact with the scaffold protein eIF4G and phosphorylate eIF4E. eIF4E is responsible for binding to the 5’ cap of mRNA and is part of the pre-initiation complex regulating translation. The different splice variants of MKNK2 are the result of two alternative 3’ exons that encode proteins with the same N-
terminal and catalytic domain, but vary at their extreme C-termini (54). MKNK2a uses
the first 3'-splice site while MKNK2b utilizes the second, which eliminates the MAPK
binding domain (Figure 9B). MKNK2b is localized in the cytoplasm and nucleus, while
MKNK2a is primarily cytoplasmic. MKNK2b has been shown to bind to the estrogen
receptor β and associates with promyelocytic leukemia protein (PML). PML has been
shown to bind eIF4E and decrease its affinity for capped mRNA, impairing translation.
The association of MKNK2b with PML may cooperate in restricting the transport of
cancer promoting (cell cycle, adhesion, motility) transcripts into the cytoplasm (54).

Alternative splicing for PTK2 (FAK) exons 13, 14, and 16 have been
characterized in the brain and are the main isoforms studied in mammalian systems
(Figure 9C, (55)). Our data shows an increase in the presence of a small 9 nucleotide
exon, exon 31, with MS-275 treatment. Exon 31 is localized immediately before the
focal adhesion-targeting domain. This exon is referred to as PWR, which corresponds to
a Pro-Trp-Arg amino acid insertion, and is in a proline rich region of FAK. FAK isoforms
containing exon 31 have been identified in mouse, but no functional mechanisms have
been identified for exon 31. A review has recently been published noting an interaction
of HEF1, ASAP1 and GRAF around this proline rich region, which calls for further
examination, as the modification of exon 31 may enhance or discourage molecular
interactions (Personal communication, Schaller lab, (56; 57)).

The alternative splicing changes of ENAH in response to HDAC inhibitor
treatment correspond to the exons known in the literature as the INV (+++) and (++)
exons. The exons, 3a and 3b, are located between the Ena/VASP homology domain
(EVH1) and the unstructured proline rich domain of the Enah (Mena) protein. The EVH1 domain is responsible for protein-protein interactions and the localization and regulation of Ena/VASP. The unstructured proline rich domain interacts directly with the actin monomer binding protein, profilin, along with G- and F-actin. The +++ and ++ exons have been shown to be included in the most invasive, metastatic cancer cells (58). The RT-PCR array data shows a decrease in the inclusion of both exons (first set of ENAH bars; Figure 8), but an increase in the inclusion of only the INV (+++) form (second set of ENAH bars; Figure 8). The changes in expression are small, and need to be confirmed by RT-PCR analysis of the exon 3a – 3b region upon HDAC inhibition. The presence of the INV and (++) exons within the ENAH transcript have been associated with poor prognosis in breast cancer models, due to the role ENAH plays in actin cytoskeletal remodeling. Rapid and unchecked actin remodeling has been documented in metastatic and aggressive cancers, leaving ENAH as an attractive target gene to study in the context of cancer and metastasis (58).

Even the largest changes in isoform expression were small and an opposite response was often detected using the alternative inhibitor. The lack of more or larger responses suggest that at least in HepG2 cells, changes in the acetylation status may not produce large effects. There are several possibilities. First, HepG2 cells are from hepatomas and may not exhibit acetylation related regulatory events because acetylation is already at a high level, and changes may only be seen when deacetylation occurs. Second, the HDAC inhibitors chosen for this study differ in their binding and specificity, which may explain opposite responses in alternative splicing. Other inhibitors
may need to be tested to determine if there are alternative splicing differences linked to the type of HDAC inhibitor used. This information would be valuable as HDAC inhibitors are in use for the treatment of cancer, and many more are in clinical trials. Third, while we saw large increases in histone acetylation, we did not determine if in fact SR proteins were similarly affected and the experiments have not been replicated to determine if these changes are reproducible. Fourth, a non-transformed cell type might be appropriate for these studies, as a normal cell type control. Primary human hepatocytes can be purchased and could be a possible model for these studies.

Overall, the changes seen in alternative splicing in genes associated with cancer are promising. The RT-PCR array should be replicated to increase the strength of the data, and the genes from Table 1 verified using traditional qRT-PCR.
Figure 9. Schematics of alternatively spliced genes with the greatest change in isoform presence due to HDAC inhibitor treatment (55; 57-59).

Exon maps depicting the alternative exons of genes identified in the RT-PCR array. Fas1 human gene (A); FAK and the corresponding regions in the protein affected by exon selection (B); The two isoforms of MKNK2 (Mnk2) resulting from an alternative 3’ splice site in exon 13. (C); The mouse mENA gene, with alternative splicing sites marked (D). Red boxes highlight the areas exhibiting alternative splicing changes with HDAC inhibitor treatment.
Table A1. List of genes included in the RT-PCR array.

The genes included in the array were chosen for their involvement in cancer and tumor formation; including but not limited to vascularization, metabolism, cell motility, and gene regulation.

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<th>Gene Name</th>
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<td>AKAP1</td>
<td>A kinase (PRKA) anchor protein</td>
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<tr>
<td>AKAP8L</td>
<td>A kinase (PRKA) anchor protein B-like</td>
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<tr>
<td>ANXA7</td>
<td>annexin A5</td>
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<tr>
<td>APC</td>
<td>adenomatous polyposis coli</td>
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<td>ASPH</td>
<td>Aspartate beta-hydroxylase</td>
</tr>
<tr>
<td>BCL2L1</td>
<td>BCL-xS, BCL-xL</td>
</tr>
<tr>
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<td>bridging integrator</td>
</tr>
<tr>
<td>BRCA1</td>
<td>breast cancer 1, early onset</td>
</tr>
<tr>
<td>CASC4</td>
<td>cancer susceptibility candidate 4</td>
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<tr>
<td>CASK</td>
<td>calcium/calmodulin-dependent serine kinase</td>
</tr>
<tr>
<td>CASP1</td>
<td>caspase 1, apoptosis-related cysteine peptidase</td>
</tr>
<tr>
<td>CCND1</td>
<td>cyclin D1</td>
</tr>
<tr>
<td>CD44</td>
<td>CD44 molecule</td>
</tr>
<tr>
<td>CDH1</td>
<td>cadherin, E-cadherin</td>
</tr>
<tr>
<td>CEACAM1</td>
<td>carcinoembryonic antigen-related cell adhesion</td>
</tr>
<tr>
<td>CLK1</td>
<td>CDC-like kinase 1 (CLK/STY)</td>
</tr>
<tr>
<td>CNM2</td>
<td>Cyclin M2</td>
</tr>
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<td>CTNNB1</td>
<td>catenin (cadherin-associated protein) beta 1</td>
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<td>Max</td>
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<td>MYO9A</td>
<td>myosin IXA</td>
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<td>ZEB2</td>
<td>zinc finger E-box binding homeobox 2</td>
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Figure A1. Gene products from primers targeting alternative exons in HepG2 cells treated with DMSO, SAHA, or MS-275.
APPENDIX II
IDENTIFICATION OF NOVEL RNA TRANSCRIPTS REGULATED BY SRp20

Specific Aim #2:

*The identification of transcripts regulated by SRp20 in the liver is an ongoing specific aim in the lab.

Hypothesis

Increase in SRp20 activity will affect other lipogenic genes in the liver, providing means for dysregulation of gene expression and/or alternative splicing. These changes could potentiate the use of pathways involved in growth, differentiation, motility, and tumorigenesis in hepatocytes, which could lead to the formation of hepatomas.

Identification of novel RNA transcripts regulated by SRp20

Primary rat hepatocytes will be infected with an adenovirus expressing SRp20. RNA extracted from these and control hepatocytes infected with empty adenovirus will be used to probe Affymetrix splice-junction arrays. The data obtained will be screened for transcripts altered by the overexpression of SRp20. Pathways increased or decreased by overexpression of SRp20 will be identified and the cause of these changes, alternative splicing or overall expression of existing isoforms will be further studied.

Introduction

Glucose 6-phosphate dehydrogenase (G6PD), a nutrient responsive gene in the liver, has been shown to have an ESE element within exon 12 (3). The ESE is bound by SRp20 and this binding is regulated by nutritional status (Suchanek, unpublished). In addition to changes in binding activity, the phosphorylation of SRp20 is also regulated.
Insulin increases and arachidonic acid decreases SRp20 phosphorylation in primary rat hepatocytes. Little is known about other targets of SRp20 in liver and if it plays a larger role in the regulation of alternative splicing by nutritional status. Increases in SRp20 amount are also observed in ovarian and cervical tumors, as well as in hepatomas (45; 46, Cyphert and Salati, unpublished). The effect of SRp20 overexpression on gene expression and isoform switching in primary rat hepatocytes will be evaluated by the lab.

**Progress**

The construct for SRp20 overexpression has been successfully cloned into the pacAd5-NpA-CMVK adenoviral vector by Amanda Suchanek. The pacAd5-CMVK-FLAG-SRp20 construct was transfected into HEK 293 cells, the virus released, and amplified.

**Adenoviral Materials and Methods: Subcloning Strategy For PacAd5-FLAG-SRp20 (developed by Amanda Suchanek)**

The FLAG-SRp20 construct in pCS2 was a generous gift from the Roz Sandri-Golden lab. The adenoviral vector (pacAd5-CMVK) and vector-containing insert (pCS2-FLAG-SRp20) were digested with XbaI and XhoI to linearize the DNA. The DNA was cleaned up using QIagen Gel Extraction Kit modified protocol for reaction cleanup. The vector and insert were then treated with Klenow to create blunted ends. The Klenow reactions were cleaned up using the Qiagen Gel Extraction Kit reaction cleanup protocol. The blunted vector and insert were digested with BamHI, and run on an 0.7% agarose gel to isolate desired fragments. Removal of agarose and purification of vector
and insert DNA was done with the Qiagen Gel Extraction Kit and eluted in EB. Next, the vector was phosphatase treated and inactivated to prepare for ligation.

Ligation calculations:

\[
\frac{25 \text{ng vector} \times 0.950 \text{kb insert}}{6.2 \text{kb vector}} = 3.8 \times \text{ratio of insert to vector (e.g. 3:1, 5:1, 8:1)}
\]

The ratio chosen was 8:1 insert to vector, respectively. The ligation reactions were run overnight at room temperature. The next day, 5μl of the ligation reaction was electroporated into ~40μl of XL1-Blue electro-competent \textit{E. coli} in 0.2μm-gap cuvettes. The pulse controller was set at 2.5kV, 25μF capacitor, 200Ω pulse control. Next, a pulse was delivered to each sample, and 950μl of Super Optimal broth with Catabolite repression (SOC) was immediately added to each cuvette and transferred to 1.5mL eppendorf tubes. The tubes were incubated at 37°C for 1 hour and 100μl of each plated onto LB/Amp100 plates to grow overnight in a 37°C incubator. The colonies of the 8:1 ligation were selected, grown-up and checked for presence of insert by enzymatic digestion and sequencing.

**Cell Culture Conditions**

**Transfection, Purification, and Titer of PacAd5-FLAG-SRp20**

The pacAd5-CMVK-FLAG-SRp20 plasmid was transfected into 70-80% confluent 293 cells with FuGENE 6 Transfection Reagent (Roche Applied Science). First, 16-24 hours before transfection, 2X10^6 cells were plated on 60mm dishes in antibiotic free
MEM medium. Next, to prepare the DNAs for transfection, the pacAd 9.2-100 backbone, pacAd-GFP, and the pacAd5-CMVK-FLAG-SRp20 plasmid were digested with *PacI* to linearize the DNAs. The cut pacAd5-CMVK-FLAG-SRp20 and pacAd-GFP were cleaned-up using the Qiagen Reaction Clean-Up kit, while the pacAd 9.2-100 backbone had to be phenol-chloroform extracted due to its large size of 34.9kb. All DNAs were eluted in ddH$_2$O and stored at -20°C.

To prepare the transfection reagents, 9μl of FuGENE6 was added to 97μl of serum-free, antibiotic free MEM media for each DNA, and incubated at room temperature for 5 minutes. Next, the DNAs for transfection were added to the medium and transfection reagent. As instructed by the Cell Bio Labs© protocol, 4μg of pacAD-CMVK-FLAG-SRp20 and 1μg of pacAd 9.2-100 backbone were added to one tube and 4μg of pacAd5-GFP and 1μg of pacAd 9.2-100 backbone added to another. The mixtures were incubated for 20 minutes at room temperature and applied dropwise to 293 cells with swirling for complete coverage of the cells. After 24 hours the medium was aspirated amplified in 293 cells, and the virus purified using the ViraBind™ purification kit (Cell Bio Labs). Briefly, the media containing the viral particles will be passed over a purification filter, washed twice, and eluted. Next, the virus will be titered using the QuickTiter™ adenovirus titer immunoassay kit (Cell Bio Labs). The titers are determined by preparing serial dilutions of adenovirus from 10$^{-3}$ to 10$^{-7}$, infecting the 293 cells for 2 days, fixing them with methanol, and incubated with an anti-Hexon primary antibody, followed by incubation with a HRP-conjugated secondary antibody. The virus will be visualized using 3,3’-diaminobenzidine (DAB), and the cells will be a dark
brown color if infected with adenovirus. The titer will be calculated by counting the infected cells on a hemacytometer using the formula for a 12-well plate.

Equation 1: Viral Titer (ifu/mL) = \( \frac{\text{average positive cells/field} \times (150 \text{ fields/well}) \times \text{(dilution factor)}}{(0.1 \text{mL})} \)

Primary Rat Hepatocyte Isolation and Culturing

Hepatocytes will be isolated from male Sprague-Dawley rats as previously described (20). Briefly, rats are anesthetized using Isoflurane and a catheter is inserted into the inferior portal vein to deliver calcium-free buffer (0.14 M NaCl, 6.7 mM KCl, 0.02 M HEPES, pH 7.4, 25mM glucose, 250 μM EGTA) at 40ml/min followed by 100ml of collagenase buffer (67 mM NaCl, 6.7 mM KCl, 0.1 M HEPES, pH 7.4, 6 mM CaCl₂ * 2H₂O, 25 mM glucose, 1mg/ml collagenase H, 0.05 mg/ml trypsin inhibitor). Following collagenase treatment, the liver will be removed, combined with Waymouth's medium supplemented with 20 mM Hepes, 0.5 mM serine, 0.5 mM alanine, penicillin (100 μg/ml), streptomycin (100 μg/ml), gentamicin (50 mg/ml), and bovine serum albumin (BSA), and filtered through a nylon mesh. Cells will be counted and plated at 8 x 10⁶ cells per 60mm collagen coated plate in Waymouth's medium supplemented with BSA and newborn calf serum (NCS). After 24 hours the media will be changed, and the hepatocytes infected with adenovirus for a pre-determined amount of time.
Adenoviral Infection of 1° Rat Hepatocytes

ViraDuctin™ adenovirus transduction reagent. The reagent and adenovirus will be added to culture medium without serum, and placed on the cells for 4 hours, then medium containing serum will be added. The amount of time for infection of the hepatocytes for RNA isolation will be determined by using a time course to follow the expression of the flag tag. The time point chosen will be at an optimum overexpression level of exogenous SRp20 as determined by Western blot analysis with a Flag specific antibody, in addition to an SRp20 specific antibody. As a control, hepatocytes will be infected with an adenovirus lacking the SRp20 insert. In addition, hepatocytes will be treated with and without insulin to stimulate phosphorylation of SRp20 (Table A2).

Cell Lysate Preparation for Western Blot Analysis

Media will be aspirated from the cells and washed twice with ice-cold 1XPBS. The cells will be incubated in 200ul of RIPA lysis buffer (150 mM NaCl, 1.0% IGEPAL® CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0) supplemented with protease inhibitors and Benzonase nuclease (Sigma-Aldrich). The cells will be scraped and pipetted into eppendorf tubes and subjected to sonication (15 pulses at 50% output). The cell lysates will be centrifuged at 13,000 rpm for 10 minutes and the supernatant will be transferred to a new tube and stored at -80°. The protein concentration of the extracts will be determined by the BCA assay (Pierce).
Table A2. Experimental conditions for overexpression of SRp20 in primary rat hepatocytes. Nuclear RNA will be isolated from the hepatocytes and used for Affymetrix microarray analysis (n=3).

<table>
<thead>
<tr>
<th>1° Rat Hepatocyte Treatment Groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
</tr>
<tr>
<td>Empty adenovirus</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>Empty adenovirus + insulin</td>
</tr>
<tr>
<td>3</td>
</tr>
<tr>
<td>SRp20-Flag adenovirus</td>
</tr>
<tr>
<td>4</td>
</tr>
<tr>
<td>SRp20-Flag adenovirus + insulin</td>
</tr>
</tbody>
</table>
Western Blot Analysis

Western blot analysis will be done as described in Walsh et al. submitted. SRp20 protein will be detected using a mouse anti-SRp20 antibody and an anti-FLAG antibody. The membrane will be incubated overnight in SRp20 antibody (Invitrogen) diluted 1:5000 in 5% bovine serum albumin (BSA). The SRp20 antibody was made by injecting a synthetic peptide corresponding to amino acids 84-104 of the human SRp20 protein into mice. The region of SRp20 from 84-104 has yet to be resolved in a crystal structure, but the amino acids are part of the area between the RRM and the RS domain. The membrane will be incubated in a goat anti-mouse secondary antibody (Zymed) diluted 1:2000 in 5% non-fat dry milk. After washing in TBS-T, antibody bound SRp20 will be visualized using ECL-Plus (GE). Chemi-fluorescent signals will be detected by X-ray film or by the Typhoon scanner using ImageQuant software. The antibody, mAb104, will be used to detect changes in SR-protein activity. mAb104 recognizes phosphorylated serine residues on the SR proteins, SRp20, SRp30a/b, SRp40, SRp55, and SRp75. The mAb104 hybridoma cell line was created by injecting mice with germinal vesicles from *Xenopus laevis* (52). The mouse spleen cells were harvested and incubated with SP2/0 cells and the conditioned medium was collected after 7 days and analyzed for the presence of antibodies. Only the conditioned medium with antibodies that recognized lampbrush loops were selected for further characterization, because the goal was to create antibodies to proteins associated with nascent RNA transcripts. Mouse mAb104 primary antibody will be detected with a goat anti-mouse IgM secondary antibody, and
visualized with ECL-Plus. A proper loading control will be determined, and is likely to be β-tubulin, lamin, or actin.

Nuclear RNA Isolation

Isolation of nuclear RNA begins with the isolation of nuclei before extracting the RNA. Cells will be washed with 1X PBS-A, pelleted, and lysed in lysis buffer (10mM Tris, pH 7.5, 150mM NaCl) containing 0.15% NP-40. After incubation in the lysis buffer for 5 minutes, and sheared with a 1ml syringe to ensure complete rupture, the lysed cells will be layered over a 24% sucrose buffer (10mM Tris, pH 7.5, 150mM NaCl) and centrifuged at 4°C for 10 minutes at 13,000 rpm to separate the nuclei from the other unwanted cell matter. The nuclei will be rinsed with 1xPBS/EDTA and gently resuspended in a 50% glycerol buffer (20mM Tris, pH 7.9, 75mM NaCl, 0.5mM EDTA). Next, the nuclei will be lysed in nuclei lysis buffer (20mM HEPES, pH 7.6, 7.5mM MgCl₂, 0.2M EDTA, 0.3M NaCl, 1M urea, and 1% NP-40) and sheared with a syringe to ensure lysis. The chromatin will be spun down at 13,000 rpm for 2 min, rinsed with 1xPBS/EDTA, and dissolved in 1ml Tri-reagent (MRC) to extract the remaining nuclear RNA. Briefly, 100μl of 1-bromo-3-chloropropane (BCP) will be added to every 1ml Tri-reagent sample and mixed vigorously. After centrifuging the samples for 13,000 rpm for 15 minutes the organic phase will be transferred to a new tube and mixed with an equal volume of isopropanol. After a 30 minute incubation on ice the samples will be centrifuged for 8 minutes in 4°C to collect the RNA pellet. The pellet will be washed with cold 75% ethanol, centrifuged again for 5 minutes, and resuspended in DEPC water. To
ensure the quality of the RNA, and to remove any possible contaminating DNA, it will be 
DNase treated by a 37°C incubation with TURBO DNA-free™ (Ambion), quantitated and 
stored at -80°C until analysis with the Affymetrix arrays.

**Microarray data analysis**

Microarrays will be run and processed by Dr. Wioletta Szeszel-Fedorowicz in the 
WVU Health Sciences Center Genomics Core. Raw data from the analysis will be 
extracted to a text file and analyzed using the Affymetrix Power Tools (APT) and 
R/Bioconductor software to detect changes in gene expression and exon splicing. APT 
implements the pre-processing steps of Robust Multichip Average (RMA) and Iter-
PLIER as well as Detection Above Background (DABG), to normalize and summarize 
raw signals, as well as, to correct for the background (62). The DABG algorithm 
calculates P-values for each probe set signal, which can be used to eliminate data from 
filters in the following steps. Probe sets can be filtered out by variance, signal, and 
Affymerix Microarray Suite (MAS), and should control for the false discovery rate (FDR) 
as defined by Benjamini and Hochberg (63). The gene signals will be estimated with 
APT and statistical analysis done using the Linear Model for Microarray Analysis 
(LIMMA) framework in Bioconductor (64). Changes in splicing will be evaluated using 
The Splice Index, which can identify new isoforms, and the GenASAP software, which 
can quantify the spliced isoforms. The data can also be imported into MySQL tables to 
display results and generate graphics (65).
Real-time PCR of Candidate Genes

The amount of mRNA of candidate genes identified from the Affymetrix array will be measured using the prepared nuclear RNA samples and real-time RT-PCR analysis with the Qiagen Sybr Green Quantitect system as previously described (19). Ct values obtained from each gene will be converted to ng using the standard curve established for each primer set. Results will be expressed as a ratio to the reference gene cyclophilin or other suitable control.


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