THE ROLE OF HNRNP H IN THE SPLICING RESPONSE TO GENOTOXIC STRESS

by

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University of Pittsburgh, 2015

Submitted to the Faculty of

University Honors College in partial fulfillment

of the requirements for the degree of

Bachelor of Philosophy

University of Pittsburgh

2015

UNIVERSITY OF PITTSBURGH

University Honors College

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Since early in evolutionary history, alternative RNA splicing has been an important method for metazoan organisms to regulate gene expression, allowing for a vastly expanded proteome without the need for significant genome expansion. By tightly regulating the inclusion and exclusion of parts of genes using *cis*-acting elements and *trans*-acting factors, the splicing machinery can create specific isoforms of proteins in response to changes in developmental time, spatiotemporal or environmental factors, etc. Recently, misregulation of this system has been discovered increasingly in various disease states, including many (if not most) cancers. Significantly, aberrant alternative RNA splicing has been implicated in the acquisition of chemotherapy resistance to certain drugs, including cisplatin. Understanding how the splicing machinery fails to act properly in cancer will be important for creating novel gene-based therapies in the future. In this study, I aimed to understand how an important splicing factor implicated in the regulation of cancer-related transcripts, hnRNP H, causes changes in alternative splicing in its own mRNA and of other genes. It was shown that cisplatin causes a dose-dependent decrease in two paralagous exons, HNRNPH1 Exon 4 and HNRNPH3 Exon 3. Structurally similar control compounds did not cause such changes, implying that the effect is specific to cisplatin-induced genotoxic stress. Gel shift assays confirmed the interaction of hnRNP H with its own mRNA in these autoregulatory exons, implying a pathway that allows cancer cells to modulate the levels of this important protein factor.

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1.0   INTRODUCTION

The splicing of pre-mRNA to a mature mRNA transcript is a tightly regulated process directed by the spliceosome in eukaryotic organisms. Acting through two subsequent trans-esterification reactions, intronic sequences are removed and exonic sequences are spliced together. A number of RNA elements and protein factors are responsible for alternative splicing via exon/intron definition, spliceosome assembly, and complex remodeling of snRNPs, enhancing or inhibiting the splicing machinery from continuing. The intricate regulatory networks controlling alternative RNA splicing allow for finely tuned and temporally dynamic reactions to developmental stages, environmental cues, and cell-type dependent processes.

1.1   THE BASICS OF RNA SPLICING: CANONICAL ELEMENTS AND THE SPLICING MACHINERY

While alternative splicing provides cells a significant level of flexibility for gene expression through a complex series of molecular interactions and subsequent decisions, the principles underlying the splicing reaction itself in addition to constitutive splicing also provide insight into how this regulation occurs. Numerous protein and RNA factors are involved, some of which are required to demark the location of intron and exon boundaries on the pre-mRNA, while others are important for aligning these regions properly to allow accurate cutting and

reattaching of the intron-exon borders. While these components are necessary for constitutive splicing, they are also involved in alternative splicing decisions, along with many other important regulators (discussed later). The three main constituents of the splicing reaction are the RNA sequences used to guide the other molecules, the RNA-protein complexes – snRNPs – used to recognize them, and the remainder of the macromolecular machinery used to perform the splicing reaction itself, the spliceosome.

1.1.1 *Cis*-acting Elements Define Introns and Exons

In order to be processed properly, intron-exon boundaries on pre-mRNA must be recognized by the splicing machinery at the appropriate positions. The most important sequences for this recognition are the 5' splice site, the 3' splice site, and the branch point. In humans, the canonical 5' splice site consensus sequence is CAGguragu (R = purine), whereby the capital letters are the final nucleotides of the exon and the lowercase letters represent the beginning of the intron (Mount 1982). The 3' splice site consists of the final bases of the intron, usually AG, the upstream polypyrimidine tract, and the branch point, whose consensus sequence is YNYURAC and contains the catalytically activated adenosine residue essential for splicing chemistry (Zhuang *et al.*1989; Lin and Patton 1995). The splicing reaction itself is a series of two trans-esterification reactions: in the first step, the 2'-OH of the activated branch point adenosine performs a nucleophilic attack on the 5' splice site of the upstream exon, creating a 5'-2' bond. While held in place by RNA-protein interactions, the free 3'-OH of the 5' splice site attacks the 3' splice site, releasing the intronic lariat and the newly spliced RNA.

1.1.2 *Cis*-elements are Recognized by snRNPs

While intronic and exonic sequences are often displayed as distinct units, actual mRNA has no such means by which to differentiate sequences to include and sequences to exclude from the final transcript (Wang and Burge 2008). Therefore, the splicing machinery must decode which regions are to be included and excluded from sequence data. This is accomplished through a series of well-defined RNA-protein interactions via complexes called snRNPs (small nuclear ribonucleoproteins), associations of small nuclear RNAs (snRNAs) and their associated proteins (e.g., Sm proteins, U2AF, etc.). Because nucleic acids readily base pair in a sequence-specific manner, snRNAs are used to identify the consensus sequences discussed above (i.e., 5' splice site, 3' splice site, and branch point). The major-type snRNPs are U1, U2, U4, U5, and U6, each of which recognize a specific region of pre-mRNA or stabilize intermediates in the splicing reaction. The complementarity of pre-mRNAs to snRNAs can partially predict how well an exon will be recognized by the splicing machinery, though several other factors contribute to the "strength" of an exon.

1.1.3 The Spliceosome

While each snRNP has a defined role in properly splicing pre-mRNA to mature mRNA, the process is stepwise and thus requires the formation of a macromolecular machine known as the spliceosome. With the help of the spliceosome, pre-mRNA can proceed through several complexes of snRNPs and associated factors that allow proper intron and exon recognition. The reaction begins with the H complex, which is composed of an assortment of randomly and nonspecifically binding RNA-binding proteins. Next, U1 binds to the 5' splice site, followed by

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U2 binding the branch point in a conformation that allows adenosine to become catalytically active. Next, the U4/5/6 tri-snRNP binds, causing the release of U1 and U4 snRNPs. After this release, U6 replaces U1 but simultaneously binds U2 snRNP. This brings the 5' splice site and branch point sequence in close physical proximity. Finally, the C complex, or catalytically active complex, allows the 2'-OH of the branch point adenosine to attack the 5' splice site. This newly freed upstream RNA molecule is held in place by U5, which allows the second trans-esterification reaction to take place. Following this, the lariat and newly spliced RNAs are released and snRNPs are recycled. This series of molecular exchanges and rearrangements is known as the spliceosome cycle, and it can be used to explain how mutations in snRNPs can have deleterious effects for global splicing (Elliott and Ladomery 2011, Chapter 6, Refs. 19, 23-40).

1.2 ALTERNATIVE SPLICING, THE SPLICING CODE, AND DECISION-MAKING

In order to create the proteomic diversity observed in metazoan organisms from the number of genes contained in the genome, several transcripts must be available to cells when creating mature mRNA from immature mRNA from a majority of genes. Therefore, decisions regarding which portions of nascent transcripts to exclude and include must be made utilizing the splicing machinery described above in addition to several other layers of regulation through a pathway known as alternative RNA splicing. The integration of these signals based on mRNA sequence and interacting proteins is collectively referred to as the "splicing code".

1.2.1 *Cis*-acting Motifs: ESSs, ISS, ESEs, ISEs

The ability of the spliceosome to decode what is supposed to be an exon versus what is supposed to be an intron in any given environment is dependent upon its recognition of regulatory sequences on the RNA molecule itself. While the consensus sequences discussed above for the 3' and 5' splice sites and branch point sequence are crucial for exon and intron definition, other motifs play important roles in aiding or hindering the assembly of the spliceosome. These sequences can be categorized into four types of binding sites: Exonic Splicing Silencers (ESSs), Intronic Splicing Silencers (ISSs), Exonic Splicing Enhancers (ESEs), and Intronic Splicing Enhancers (ISEs). Splicing enhancers tend to favor the inclusion of an exon into a final transcript, whereas silencers tend to inhibit exonic inclusion. Constitutively included and strong alternatively included exons tend to either contain several ESEs, few ESSs, or both. Likewise, weak exons and pseudoexons contain many splicing silencers and few enhancers. The consensus sequences for these sites vary extensively, and can even overlap with each other.

1.2.2 *Trans*-acting Factors: Diverse Roles of hnRNPs and SR Proteins

The *cis*-acting motifs discussed above are recognized by RNA-binding proteins known as splicing factors. While the structural diversity of these proteins is high, the most common domain conserved amongst them is the RNA-recognition motif, or the RRM. These proteins can be divided into two major categories: hnRNPs and SR proteins.

SR proteins all share an RS domain, rich in arginine and serine residues, and often contain one or more RRMs (Graveley 2000; Maniatis and Tasic 2002). The SR domain

stabilizes binding to mRNA while also facilitating protein-protein interactions for regulatory purposes. SR proteins are typically considered to be splicing enhancers, blocking repressors from binding or stabilizing components of the spliceosome, either directly or through cooperative interactions across snRNPs. Phosphorylation of SR proteins often acts as a way to activate their splicing activity, while dephosphorylation often shuttles them to the cytoplasm (Stamm 2007). This added level of regulation allows continuous coupling of environmental cues to levels of SR proteins in the nucleus, titrating binding to nascent mRNA (Long and Ceceres 2009).

The other major type of splicing factor, hnRNPs, are some of the most abundant proteins in the cell, and mammals can have up to 25 different types (Choi *et al.* 1986; Dreyfuss 1993; Ladd and Cooper 2002). With a few notable exceptions (such as hnRNP K and hnRNP E), hnRNPs contain at least one RRM or quasi-RRM (qRRM) and often a protein interaction domain that allows regulation and localization similar to SR proteins. hnRNPs are often thought to be splicing silencers, and can act through one of several methods: direct competition, by which spliceosomal components are physically blocked from binding their RNA motifs; oligomerization and "coating" of an exon to prevent recognition by spliceosomal components; dimerization across RNA molecules, causing looping out of regions to make them invisible to the spliceosome; and preventing interactions between spliceosomal components across an exon during exon definition.

1.3   STRESS AND ALTERNATIVE SPLICING

As discussed previously, alternative splicing is an efficient means for cells to couple environmental factors to regulation of gene expression. Therefore, one may expect alternative RNA splicing to be involved at least partially in the cellular response to environmental stressors. Additionally, these pathways are often misregulated in cancer, so the combined effects of stress on splicing and cancer on splicing must be considered. For example, DNA damage response pathways tend to shut down RNA production while chromosomal lesions are fixed, whereas highly replicative cancers tend to speed up RNA production (Lenzken 2013). This may be significant because modifying concentrations of splicing factors being produced may alter alternative splicing events on a global scale. Likewise, overproducing splicing factors, as is often the case in cancers, could shift splicing events in a different direction. Genotoxic damage, such as cisplatin-induced damage, can indirectly shift alternative splicing patterns by inhibiting transcription. Because splicing is often cotranscriptional, processing of nascent mRNA separate from transcription may produce different splice isoforms. In each of these ways and likely others, chemical stress brought about by chemotherapeutic agents and/or tumorigenic stress likely have long-range effects on splicing, which may directly or indirectly alter the cell's ability to correct genetic/genomic lesions. Interestingly, Hai and colleagues reported that a specific *cis*-acting motif, the G-tract (bound by hnRNP H), modulates splicing patterns in response to apoptotic-inducing agents. Though this example is highly specific, it is reasonable to believe other motifs work similarly using similar compounds (Hai 2008).

The cotranscriptional nature of splicing can also link stress to splicing via RNA polymerase II elongation. For example, UV irradiation of cells leads to hyperphosphorylation of the RNA pol II CTD, leading to downregulation of elongation activity. Muñoz and colleagues

demonstrated that this hyperphosphorylation caused the same alternative splicing effects as UV irradiation in a p53-independent pathway, showing that the alternative splicing response to stress may be linked to physical DNA damage (Muñoz et al. 2009). Similarly, stress-inducing compounds like DRB which slow transcription elongation cause shifts in alternative splicing, though this likely has more to do with regulation of the RNA pol II complex in factors like P-TEFb and CDK9 rather than insults to genomic DNA (Muñoz et al. 2010).

Beyond these factors, it is believed that combinatorial control of splicing factors and other regulators, often alternatively spliced themselves, lead to many shifts in response to stress. In light of this, MDM2, an important gene involved in the regulation of p53, has been shown to respond to stressors through alternative splicing shifts in *cis*-regulated intronic elements, though the upstream regulators of this effect have not been identified (Singh et al. 2009). Though this is only one example of a gene responding to stress through specific RNA elements, it is likely that many others act in similar ways. Additionally, the regulatory pathways leading to these changes are largely mysterious and require further study. High-throughput technologies and systems biology will no doubt be of great use in these experiments, and will contribute greatly to our understanding of global alternative splicing regulation in response to environmental cues.

1.4   HNRNP H

Heterogeneous nuclear ribonucleoprotein H is a member of the ubiquitously expressed hnRNP F/H family of splicing factors (Bent 1995). It binds RNA via three quasi-RRMs (qRRM) and can interact with several other splicing factors (UniProt Consortium 2013). hnRNP H binds G-rich elements when interacting with pre-mRNA, and can act as both a splicing silencer and

enhancer depending on the context (Dominguez 2006). G-rich elements were recently implicated in special importance for cancer-regulated genes, and hnRNP H has indeed been shown to regulate the mRNA of several genes important for the formation/progression of cancer and cell survival, such as Bcl-x and p53 (Sohail 2014; Garneau 2005; Rauch 2010; LeFave 2011; Decorsière 2011). It is of special note that hnRNP H's interaction with p53 is not one of splicing regulation, but of 3' end stabilization in response to stress. This reinforces the idea that hnRNPs and other RNA binding proteins do not always have singular roles, and that post-transcriptional modifications of these molecules (such as splicing or mRNA degradation via nonsense mediated decay) can have downstream effects on other effectors of the proteins (Goldstrohm *et al.* 2001).

1.5   THESIS AIMS

In light of the information presented above, we aim to find the biochemical mechanism by which genotoxic stress modulates hnRNP H alternative splicing. It has been previously noted that hnRNP H responds to stress by regulating other proteins, but no direct connection has yet been described regarding its own transcript regulation. Here, we take the first steps to establish the series of events that are required to relay the stress signal to hnRNP H mRNA splicing modulation. Additionally, we aim to explore the possibility of larger splicing networks that respond to genotoxic stress using the compounds displayed in Figure 1.

(I)   (II)

(III)   (IV)
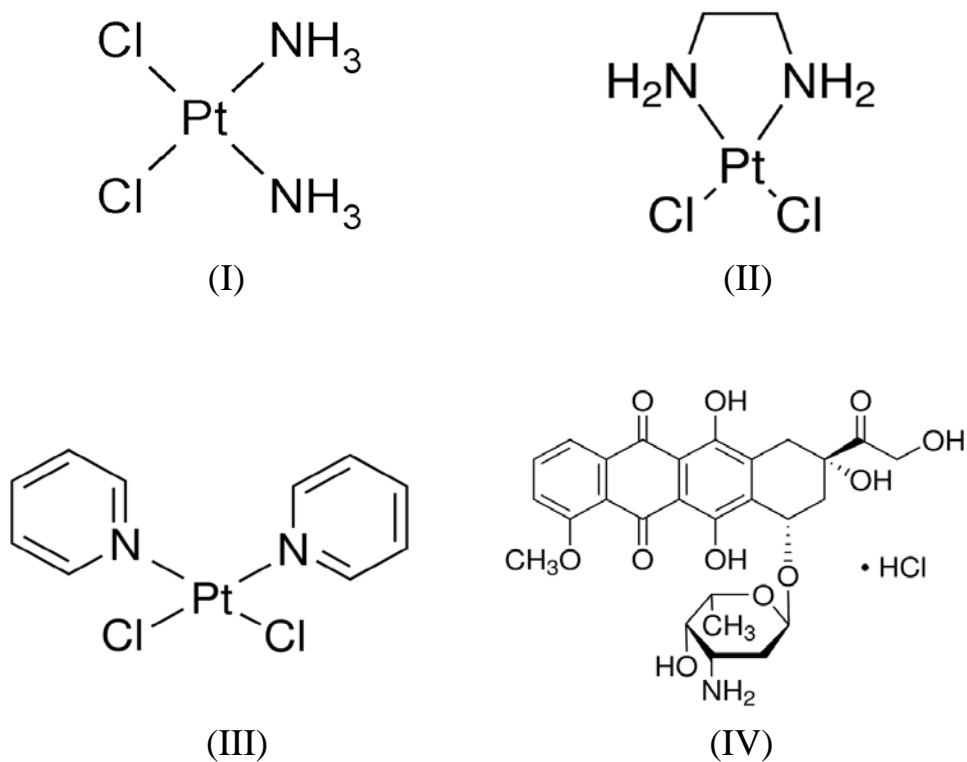
Figure 1. Cisplatin, its analogs, and the unrelated doxorubicin hydrochloride

1. (I) Cisplatin, cis-diamminedichloroplatinum(II), is a chemotherapeutic agent that induces DNA damage by forming inter- and intra-strand DNA alkyl-like adducts, binding preferentially to the N7 position of guanines
2. (II) Dichloro(ethylenediamine)platinum (II), and (III) cis-Dichlorobis(pyridine)platinum (II) are chemical analogs of cisplatin but do not introduce DNA damage
3. (IV) Doxorubicin hydrochloride is another chemotherapeutic agent that has an unrelated structure and mechanism of action – DNA intercalation

2.0   RESULTS

2.1   HUMAN HNRNP H PARALOGS ARE HIGHLY CONSERVED IN TWO EXONS

According to the most recent release of the Ensembl genome browser, the human genome

contains three genuine (i.e., not pseudogenes) hnRNP H paralogs: hnRNP H1, H2, and H3

(Flicek 2013). hnRNP H1 and H3 are multi-exon genes, with 49 and 11 splice variants reported,

respectively. (Though hnRNP H2 is listed as having containing two exons, the first exon

contains only a very small portion of 5' UTR. Additionally, there is only one splice variant,

meaning this small exon is constitutively included in the mature transcript along with the

remainder of the second exon.) Notably, the database shows that the canonical forms of H1 and

H3 contain unusually similar exons both in length (139 bp) and sequence (90.6%): hnRNP H1

Exon 4 (H1 E4) and hnRNP H3 Exon 3 (H3 E3) (Figure 2). Besides containing a high degree of

amino acid similarity, it is also possible that the high level of sequence homology codes for

similar splicing regulatory elements (ESSs or ESEs). It is also of note that the intronic regions

immediately upstream and downstream of the paralagous exons are highly similar, and they also

have the potential to contain important splicing regulatory elements (ISSs or ESEs) (House

2008). Important for splicing regulation, it was previously posited that the skipping of these

exons might create a frameshift leading to nonsense mediated decay (Grabowski 2004). The

possibility for this set of regulatory factors in combination makes hnRNP H especially

interesting in light of its previously described role in the splicing of several important cancer-associated genes.
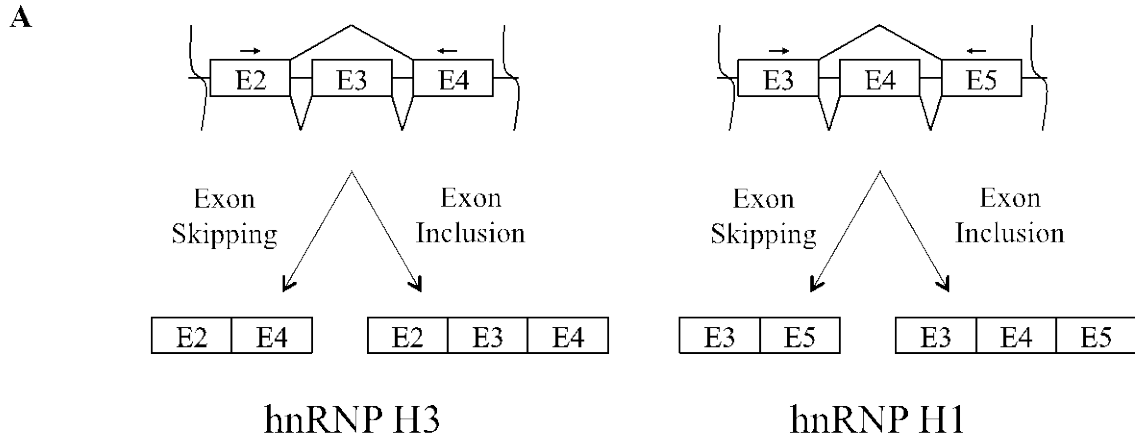
A

B

```
H3E3   tgggttaaagGGTTGGAAATCGTGCCAAATGGGATAACATTGCCGGTGGACTTCCAGGGGGAGGAGTACGGGGGAGGCCTTCGTGCAGTTT
H1E4   tgggttgaagGGTTGGAAATCGTGCCAAATGGGATAACATTGACGATGGACTACCAGGGGCAGAAGCACAGGGGAGGCCTTCGTGCAGTTT
       *****  **************************************  ****** ********  **  ** *******************

H3E3   GCTTCACAGGAAATAGCTGAAAAGGCTCTAAAGAAACACAAGGAAACAATAGGGCACAGgtggggatggagagtttggga
H1E4   GCTTCAAACGAGATAGCAGAAAATGCTCTGGCGAAACACAAGGAAACAATAGGGCACAGgtggggatggatggttggttg
       ****** **** ***** ***** *****    **************************************  *** *
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Figure 2. Human hnRNP H Paralogs are alternatively spliced

A) Two paralagous pre-mRNAs of human hnRNP H – H3 and H1 – are alternatively spliced as cassettes in exons 3 and 4, respectively; positions for primers used in PCR are indicated by arrows above the flanking exons

B) Sequence alignment of hnRNP H3 exon 3 and hnNRP H1 exon 4 by Clustal Omega. Exonic bases are in capital letters, while intronic bases are lowercase. Homology analysis by LALIGN reveals 90.6% sequence homology in the exon, 90.0% homology in the final 10 nucleotides of the upstream intron, and 83.3% similarity in the first 21 nucleotides of the downstream intron. Positions with identical nucleotides are indicated by an asterisk (*) below the alignment

2.2 HNRNP H3 EXON 3 IS DIFFERENTIALLY SPLICED IN RESPONSE TO CISPLATIN IN MOUSE NEUROBLASTOMA CELLS

In our initial experiments, we tested the effects of cisplatin on hnRNP H3 Exon 3 (H3 E3), in the mouse neuroblastoma cell line N18TG2. Optimization experiments were performed in order to obtain a proper dose and time course for drug treatment. Because of the cytotoxic nature of cisplatin, an excess dose would lead to massive cell damage via apoptosis, making RNA quantification and subsequent results difficult to interpret. On the other hand, a small dose could potentially render splicing effects negligible due to an insufficient amount entering cells or inactivation of cisplatin by endogenous nucleophiles (Sidik 2003).

Exon-specific PCR primers were used to quantify the ratio of exon inclusion to exclusion (Figure 2A, arrows above splicing schematics). Several optimization reactions revealed that 15 μM cisplatin applied for 9 hours was sufficient to cause a splicing shift in hnRNP H3 Exon 3. Notably, the effect was dose-dependent and time dependent, as longer time courses using higher cisplatin doses caused a larger exclusion in Exon 3. The extent of splicing change is reported as a change in Ψ-value (PSI, or "**P**ercent-**S**pliced-**I**n"), calculated by subtracting the percent exon inclusion without treatment from the percent exon inclusion post-treatment (i.e., $\Delta\Psi$) (Wang 2008). That is, a more negative Ψ is indicative of more exon skipping. Experiments were performed in biological triplicate, and were used to calculate standard deviation (shown as error bars) and statistical significance (Student's t-test, where *p<0.05* is significant).

Though the longer time point showed a more significant change in Exon 3 inclusion compared to the shorter experiments (Ψ = -11.6% versus -14.7%), a greater degree of cell death was observed for this extended period of time. Therefore, it was decided that future tests would utilize a cisplatin treatment of 9 hours.
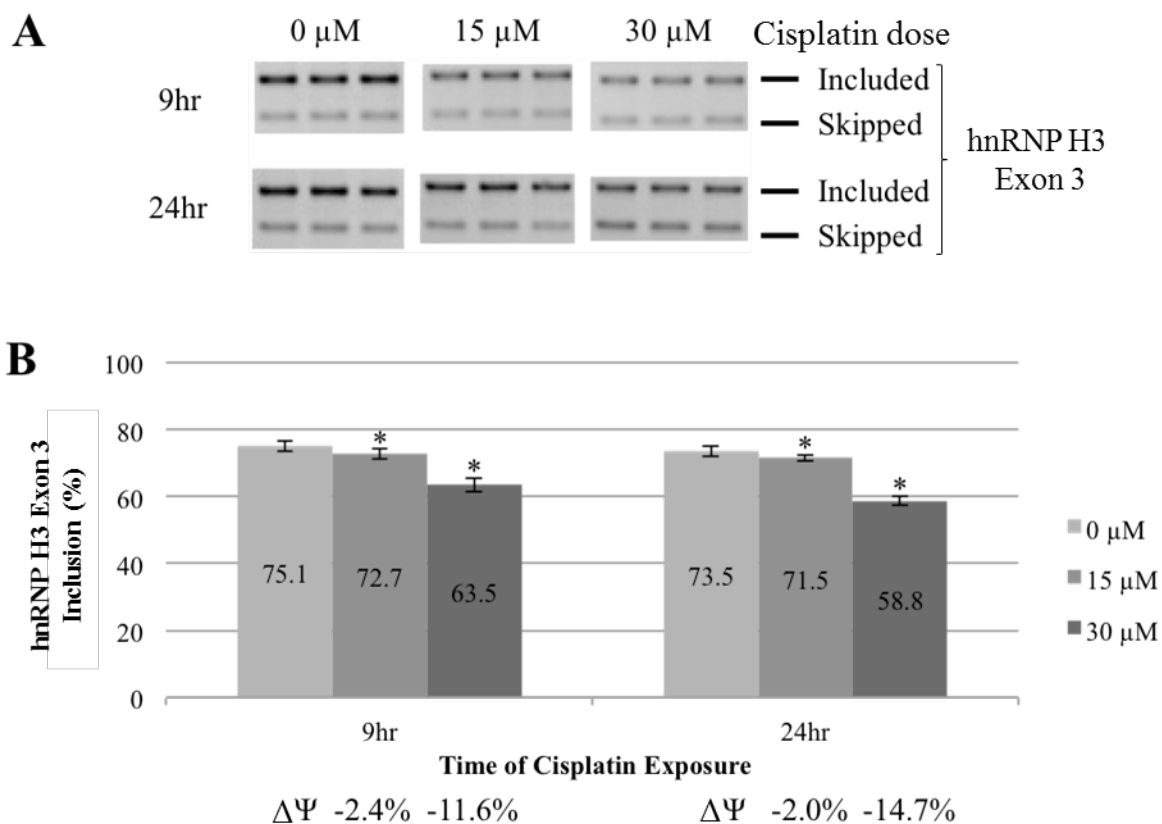
Figure 3. Optimization of cisplatin treatment in N18TG2, mouse neuroblastoma cells

A) Agarose gel electrophoresis of PCR products using hnRNP H3 Exon 3-specific primers reveals bands corresponding to transcripts including and excluding the cassette exon
B) hnRNP H3 Exon 3 is skipped in a dose-dependent and time-dependent manner upon cisplatin treatment

In order to rule out the possibility that cisplatin causes wide-scale changes in multiple cellular processes, we probed two features that could lead to confounding results and difficult interpretation of later experiments. First, we performed Western Blots on HeLa 2CCL whole cell lysates and immunoblotted for phosphorylated γH2A.X, a species formed at points of DNA damage. We observed a large increase in γH2A.X in cisplatin-treated HeLa cells, whereas cells treated with one of the inert platinum-based compounds did not display this dramatic increase (Figure 4A). To determine whether or not cisplatin causes alternative splicing changes in all

cellular transcripts, we assayed the splicing pattern of an exon unrelated to hnRNP H, MEN1 Exon 8. When HeLa 2CCL cells were subjected to 24 hour cisplatin treatments of 0, 15, and 30 µM, we observed no significant change in included:skipped ratios (Figure 4B). This helped to show that the splicing effects observed in this study stemming from cisplatin treatment are likely due to a specific response rather than a general misregulation of splicing.
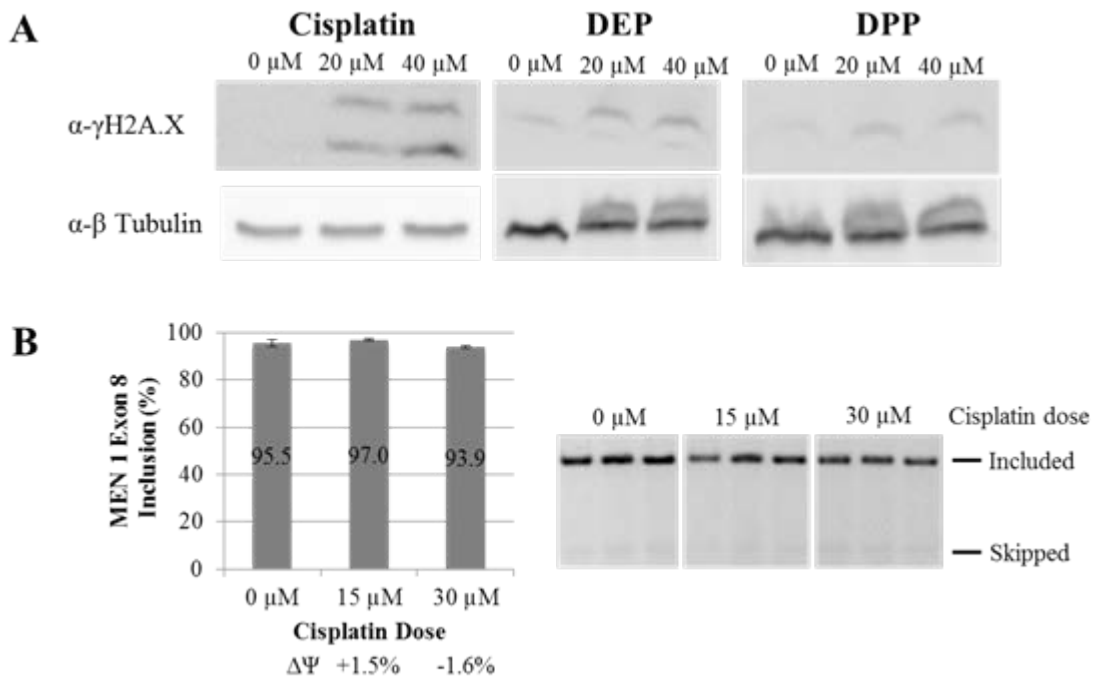
Figure 4. Cisplatin specificity for DNA damage and splicing in HeLa 2CCL

A) Cisplatin causes a large dose-dependent increase in γH2A.X protein levels *in vivo* in HeLa cells, indicative of DNA damage. The two control compounds, DEP and DPP, do not show the same large increases in γH2A.X, and have consistently low levels over the three doses
B) 24 hour cisplatin treatment does not cause significant splicing changes in MEN1 Exon 8 splicing. This data in conjunction with data from Figure 3 demonstrates that only specific transcripts react to cisplatin treatment in HeLa cells

2.3   CISPLATIN CAUSES DOSE-DEPENDENT SKIPPING OF HNRNP H3 EXON 3

IN HUMAN CELLS

In order to further validate the splicing changes observed in mouse cells, we repeated the previously described experiments using human cervical cancer cells, HeLa 2CCL (ATCC). After 9 hours of treatment by 20 or 40 μM cisplatin, similar trends in hnRNP H3 Exon 3 splicing were observed, though the effect was more mild than in neuroblastoma cells (Figure 5A). That is, increasing cisplatin doses caused a decrease in H3 E3 inclusion ($\Delta\Psi$ = -8.4% for the highest dose). It should be noted that the inclusion of H3 E3 was significantly lower in mice in the untreated condition than in human cells (approximately 74% versus 95%). These differences in PSI values are likely explained by the tissue-specific nature of alternative splicing patterns, which has been well documented (Xu, Modrek, and Lee 2002). However, species-specific effects on splicing cannot be ruled out.
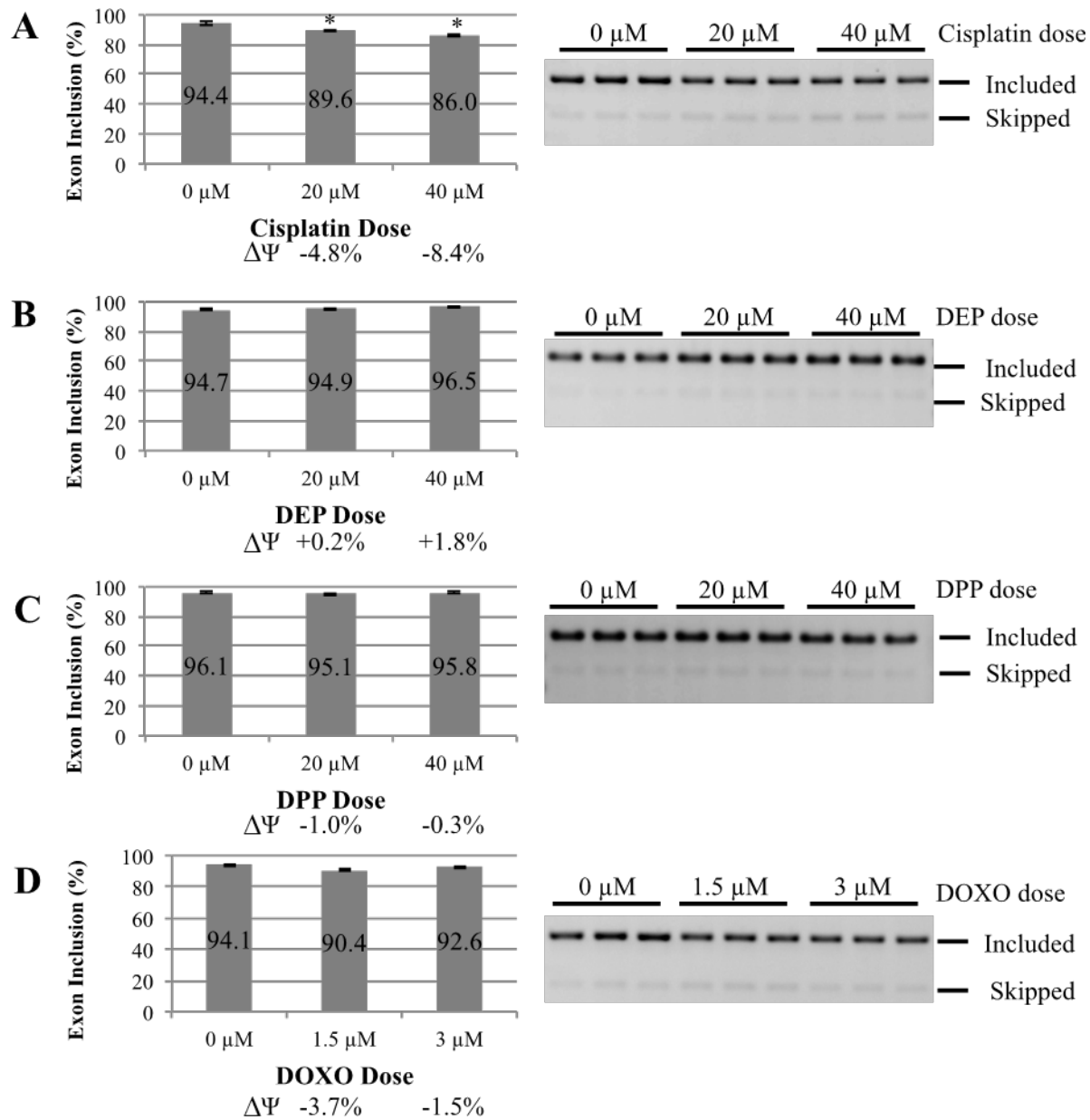
Figure 5. Dose-dependent exon skipping of hnRNP H3 Exon 3 in HeLa 2CCL

A) After 9 hours of treatment with cisplatin, hnRNP H3 Exon 3 skipping increased by 4.8% and 8.4% in the lower and higher doses, respectively; analysis was performed based on biological triplicate, and a representative gel is shown

B) The cisplatin analogue DEP was treated and analyzed as for cisplatin, and no change in splicing was observed

C) The cisplatin analogue DPP was treated and analyzed as for cisplatin, and no change in splicing was observed

D) The structurally unrelated Doxorubicin hydrochloride was used in the doses indicated; no changes in splicing were observed

$\Delta\Psi$ indicates percent change in exon inclusion from untreated

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In order to determine whether the shifts in exon inclusion were specific to cisplatin or were simply an artifact of sample treatment, we utilized several structural analogs of cisplatin to see if these control compounds would produce similar or contrasting results. Two biochemically inert platinum-based cisplatin analogs, dichloro(ethylenediamine)platinum(II) (DEP) and (III) cis-dichlorobis(pyridine)platinum(II) (DPP), were applied using treatment conditions identical to the samples in Figure 5A. As shown in Figure 5B and 5C, neither compound produced significant decreases in exon inclusion, and in some cases resulted in small, insignificant increases in hnRNP H3 Exon 3 inclusion. Similarly, a structurally distinct yet biochemically active chemotherapeutic drug, doxorubicin hydrochloride (DOXO), was used as another control to determine whether the observed changes in H3 Exon 3 splicing is generalized to all stressors or specific to cisplatin-induced genotoxic stress. Doxorubicin is a DNA intercalating agent and therefore interferes with DNA and RNA synthesis (Momparler 1976). Unlike cisplatin, no change in H3 E3 splicing was caused by DOXO, demonstrating the specificity of this effect to cisplatin (Figure 5D). As in the neuroblastoma experiments, all procedures were performed in biological triplicate and subjected to the Student's t-test (i.e., *p<0.05* is significant).

2.4   HNRNP H3 EXON 3 AND HNRNP H1 EXON 4 ARE SPLICED DIFFERENTLY IN RESPONSE TO CISPLATIN

As mentioned previously, hnRNP H has three paralogs in humans, two of which share a homologous exon: hnRNP H1 Exon 4 and hnRNP H3 Exon 3. With such a high degree of sequence homology (90.6%), one would expect to observe similar splicing patterns between the two exons. In order to confirm this suspicion, we used PCR primers specific to H1 E4 to

compare the level of exon inclusion in the presence of cisplatin relative to H3 E3's. Surprisingly, H1 E4 was significantly more sensitive to cisplatin treatment than H3 E3 ($\Delta\Psi$ = -16.7% versus -8.4%, respectively) (Figure 6A). The differential regulation of exon inclusion could come from a variety of sources, and an attractive hypothesis contributes this observation to differences in regulatory *cis*-acting RNA elements such as E/ISSs and E/ISEs.

A)

B)

```
H3 E3   tgggttaaagGGTTGGAAATCGTGCCAAATGGGATAACATTGCCGGTGGACTTCCAGGGGAGGAGTACGGGGGAGGCCTTCGTGC

H1 E4   tgggttgaagGGTTGGAAATCGTGCCAAATGGGATAACATTGACGATGGACTACCAGGGGAGAAGCACAGGGGAGGCCTTCGTGC


H3 E3   AGTTTGCTTCACAGGAAATAGCTGAAAAGGCTCTAAAGAAACACAAGGAAAGAATAGGGCACAGgtgggatggagagtttggga

H1 E4   AGTTTGCTTCAAAGGAGATAGCAGAAAATGCTCTGGGGAAACACAAGGAAAGAATAGGGCACAGgtgggatggatggttggttg
```

Figure 6. hnRNP H1 Exon 4 displays increased sensitivity to cisplatin treatment in HeLa

A) Inclusion of hnRNP H1 Exon 4 decreased significantly in both cisplatin treatments, and both were more dramatic than the response of hnRNP H1 Exon 3

B) H3 E3 and H1 E4 vary in putative binding sites for possibly relevant splicing factors, including hnRNP H; putative hnRNP H sites are shown in blue, SC35 in red, and hnRNP A1 in orange (where overlapping sites exist, colored underlines are shown)

In order to explore this possibility, the two exons in question were aligned using ClustalW, including intronic regions immediately upstream and downstream of the splice sites.

Each sequence was applied to bioinformatics programs that predict regulatory motifs for several splicing factors (Human Splicing Factor 2.4.1, Desmet 2009; SFmap 1.8, Paz 2010, Akerman 2009). The RNA maps produced for each revealed a great deal of similarity, though a few important differences were seen. For example, H3 E3 contains one exonic and three intronic putative hnRNP H binding sites not found in H1 E4. Additionally, H3 E3 contains an extended hnRNP A1 RNA element compared to H1 E4 and an unshared SC35 site. On the other hand, H1 E4 contains two exonic hnRNP H binding sites not found in H3 E3 in addition to a unique hnRNP A1 site. Because RNA binding proteins (such as splicing factors) are often autoregulatory (Buratti 2011), and the two exons show several dissimilarities in hnRNP H binding sites, we hypothesized that the differences in regulatory motifs may help to explain the significantly different splicing changes in addition to hnRNP H's response to cisplatin.

2.5   HNRNP H BINDS TO ITS OWN MRNA

Before exploring the role of individual regulator motifs on hnRNP H's autoregulation, we first sought to confirm that H in fact interacts with its own transcript in the exon described. To test this, an electrophoretic mobility shift assay (EMSA) was performed, using recombinant hnRNP H protein and fluorescently labeled hnRNP H1 E4 RNA transcript. Because RNA-protein complexes are heavier than RNA or protein alone, interactions between hnRNP H and its mRNA would be expected to reduce the mobility of RNA in a polyacrylamide gel. This effect was indeed observed, as RNA migrated significantly faster in a native gel compared with RNA plus protein mixtures. This interaction was further confirmed by the fact that increasing amounts of

protein created a more significant upward shift in mobility relative to a sample with half the concentration of protein.

In order to determine the sequence specificity of this interaction, *in vitro* binding assays with competitor oligonucleotides will need to be performed. If the interaction were truly specific for any or all of the putative sites, competitor sequences would reduce the protein's interactions with the labeled probe, binding to the unlabeled molecule instead. However, if this binding is unspecific and is a more general RNA-protein interaction, competitor oligo would compete for binding less effectively. Additionally, it would be important to determine where exactly on the exon hnRNP H binds, especially to determine the effects of nucleotide sequence within codons (as discussed above).

[MBP-H] (µM)		1.5	0	1.5	3.0
H1E4 RNA (ng)		0	300	300	300
Lane		1	2	3	4

hnRNP H1 E4 RNA
shifted upward by protein

← hnRNP H1 E4 RNA only

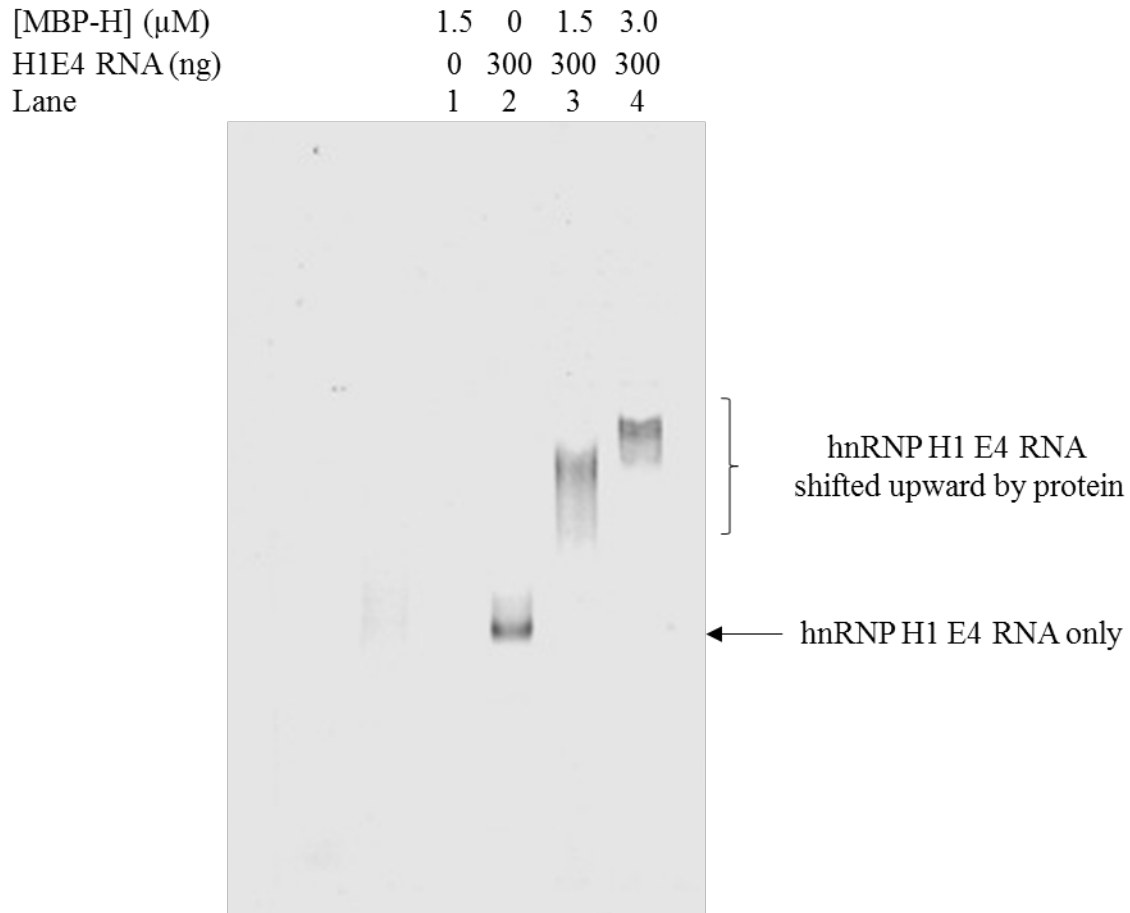**Figure 7. Recombinant hnRNP H binds to its own *in vitro* synthesized transcript**

An electrophoretic mobility shift assay shows that recombinant hnRNP H causes an upward mobility shift when incubated with RNA encoding hnRNP H1 Exon 4. Control lane 1 containing only protein does no fluoresce because it does not contain labeled UTP. Control lane 2 shows basal mobility of the H1 E4 transcript in the polyacrylamide gel.

3.0   DISCUSSION

Since its discovery approximately 40 years ago, alternative RNA splicing and its regulation have been studied extensively. It has more recently been discovered that aberrant alternative RNA splicing can be attributed to causing several – if not many – human diseases that span a range of organ systems over varying developmental time (Faustino 2003; Wang 2007; Poulos 2011). Perhaps unsurprisingly, it has also come to light that many instances of cancer – a disease associated with high mutational loads and genomic instability – are either caused by, sustained by, or lead to aberrant alternative splicing (Venables 2004; David 2010; Sette 2013; Ladomery 2013).

In light of new sequencing technologies, advances in bioinformatics, and improved methods for large-scale analyses of alternative splicing decisions, many interesting patterns in the relationship between splicing and disease have emerged (Lee 2005). For example, Wang and colleagues demonstrated on an exome-wide scale that members of the hnRNP H/F family of splicing regulators can both modulate exon inclusion and exclusion in a context-dependent manner through several modes of alternative splicing (e.g., cassette exons, alternative 5'/3' splice

sites, etc.). Functionally, the authors demonstrated a global link to a developmental shift from oligodendrocyte progenitor cells to oligodendrocytes (Wang 2012). This study exemplifies a marked shift from previous studies addressing a single exon, single gene, or single pathway (Gonçalves 2009, for example).

Despite this recent glut of genome-wide, exome-wide, and transcriptome-wide information, there is strikingly little reporting on comprehensive splicing pathways or programs from this data. One of the most important and well-understood examples of such a program involves two isoforms of pyruvate kinase M (PKM): PKM1 and PKM2. PKM1 is expressed in normal cells, while PKM2 is preferentially selected in cancer cells due to its shift toward anaerobic respiration. The difference between these isoforms is as simple as the exchange of one mutually exclusive exon for another, facilitated at least partially by hnRNPs A1, A2, and I (or PTB) (Christofk 2008; Chen 2010). Several other studies approach similar phenomena with more global perspectives generalizable either to certain types of cancers or cancer in general. Such projects often identify families of splicing factors, *cis*-acting regulatory elements, or modes of splicing events that are differentially regulated in cancers such as SRSF proteins, FOX proteins, and hnRNPs (Bradmard 2009; Lapuk 2010; Shapiro 2011). While each of these reports approaches alternative splicing regulation from a relatively large-scale view, they mention several specific mechanisms by which each splicing switch works.

Another gap in the literature that the current study aims to fill is in understanding how splicing programs interact with environmental cues besides tumorigenic effects. Many reports demonstrate how specific splicing factors either alter or are altered by diseased states, but do not consider the dynamic nature of alternative splicing with respect to integration of spatiotemporal and chemical signals. Here we have demonstrated an exon-specific and signal-specific reaction

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of cancer cells to genotoxic stress. Our findings show that homologous exons from two human paralogs of hnRNP H, H1 E4 and H3 E3, react to treatment with cisplatin via a dose-dependent exclusion of the exon. This reaction was likely dependent on the presence of genotoxic stress in the form of alklyation, as neither inert control compounds nor a DNA intercalating agent elicited this effect. Additionally, hnRNP H1 Exon 4 is more sensitive to cisplatin treatment, as it experiences a more significant decrease in inclusion as a result of identical drug dosages. A map of *cis*-acting regulatory elements within these exons and the immediately adjacent intronic regions suggest possible mechanisms both for this effect in general in addition to the basis for H1 E4's heightened reaction to cisplatin. Though the exact pathway that transduces the signal from cisplatin to hnRNP H1 and H3 splicing shifts has yet to be elucidated, the observation that hnRNP H binds to this exon with moderate affinity suggests a possible autoregulatory feedback loop. This type of autoregulation is certainly precedented, and can be found in the autoregulation of other splicing factors such as SRSF1 mRNA stability by NMD, SC35 mRNA by alternative splicing in the 3' UTR, and other RNA-binding proteins (Sun 2010; Sureau 2001; Brenner 2007; Wollerton 2007).

Though the splicing shifts of H1 E4 and H3 E3 were observed throughout numerous experiments, both in data shown and not shown, it is arguable that the extent of exclusion for these exons is rather small compared to similar systems. For example, SRSF1 mRNA experiences up to a 30% decrease in exon inclusion in response to autoregulation compared to a maximal value of 16% in this study. However, the cytotoxic effects of cisplatin were the ultimately limiting factors in this study: while much higher doses of cisplatin may have produced a larger modulation in H1 E3 and H3 E3 exon exclusion, they almost certainly would have led to massive cell death and degradation of RNAs of interest. Additionally, we believe that the

reproducibility of experiments utilizing the two control platinum-based compounds DEP and DPP further validates our observations to a level sufficient to support our conclusions. We hope to conduct further experiments that demonstrate the specificity of this effect not only to cisplatin, but to exons regulated by hnRNP H. In showing this, we would have more evidence for an autoregulatory mechanism of action in cisplatin-induced reactions.

While we have collected data suggesting the beginnings of a larger splicing program, genome-wide and transcriptome-wide studies need to be performed before drawing larger conclusions about cisplatin's effects on hnRNP H and splicing in general. In line with this thinking, a 2012 proteomic study by Zhang and colleagues suggested that cisplatin causes an upregulation in nuclear hnRNP H, though the methods used do not make this obvious (Zhang 2012). In utilizing conventional wisdom about hnRNPs, this presents possibly counterintuitive logic. Specifically, hnRNPs are typically considered to repress exon inclusion; if hnRNP H regulates the inclusion of H1 or H3, increasing nuclear concentration of the protein would create a larger exclusion of the exon, in line with the data presented in this study. However, because of the premature termination codon transcribed when these exons are excluded, one would expect nuclear concentrations of hnRNP H to decrease through the nonsense mediated decay (NMD) pathway. These seemingly paradoxical conclusions need to be explored further, and will come with further analysis of hnRNP H's reaction to cisplatin and possible contributions from NMD.

The goals of this thesis originally presented were partially fulfilled, and require further work. The basis for a possible autoregulatory loop in hnRNP H splicing has been established through assays involving stressors. While genome-wide studies have become increasingly important, especially in light of so-called splicing networks, several preliminary steps must be completed before grander correlations can be explored. Direct evidence of hnRNP H's binding

occupancy on its own transcript in several stressful contexts will be an important step in bridging this gap. Additionally, the biochemical connection between DNA damage and hnRNP H splicing changes will need to be explored in order to predict other upstream and downstream consequences of these changes.

Though this study only fills a very small gap in the data about environmental reactions to stress through splicing mechanisms, it begins to approach a rather profound question regarding cellular decision making: How is the sum of all cues, internal and external, chemical and physical, spatial and temporal, processed by biomolecules, and how does this converge on alternative splicing? As small pieces of questions like these are answered, we can not only begin to understand how biology of normal cells works, but how diseased states change this homeostasis and how we can return it to a healthy condition.

4.0   MATERIALS AND METHODS

4.1   PRIMER SEQUENCES

Table 1. List of Primers Used

Primer Name	Sequence	Organism (Cell Line)
H3_E3_F	5'-AAT GGT CCA AAT GAC GCT AG-3'	Mouse (N18TG2), Human (HeLa 2CCL)
H3_E3_R	5'-CCA GCA ATC TTC TTG GTG G-3'	Mouse (N18TG2), Human (HeLa 2CCL)
hH1_E4_F	5'-TTG GGT GTT GAA GCA TAC TGG-3'	Human (HeLa 2CCL)
hH1_E4_R	5'-CAT AAG CTT TCG TCG TGG ATC A-3'	Human (HeLa 2CCL)

4.2   HELA CELLS, CISPLATIN TREATMENT, AND RNA ANALYSIS

HeLa 2CCL (ATCC) cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum and grown in a sterile tissue culture incubator at 37^0C with 5% atmospheric CO_2. Drug treatments were performed in 6-well plates once cells reached 60% to 90% confluence. Cisplatin stock solutions were prepared fresh from powder using 0.9% NaCl in ddH_2O as the solvent. Stock solutions were added to DMEM + 10% FBS immediately before treatments to produce the desired final

experimental dosage (20 μM or 40 μM) for 9 hours. Mock treatments without cisplatin were performed in parallel. Control drug treatments were performed using dichloro(ethylenediamine)platinum(II) (DEP), cis-Dichloro(dipyridine)platinum(II) (DPP), and doxorubicin. Control drug treatments were performed in identical conditions and concentrations as cisplatin, except for doxorubicin, for which we used 0 μM (mock), 1.5 μM, and 3 μM doses for 9 hours. Total RNA was collected by pooling identical treatments and using TRIzol according to the manufacturer's instructions. RNA concentrations were measured using A_{260} spectrophotometry. cDNA was synthesized from RNA using random primers and SuperScript III reverse transcriptase. Exon inclusion/exclusion data was obtained by performing PCR on cDNA using primers flanking the exon being quantified. PCR products were run on agarose gels and bands were detected using EtBr staining. ImageGauge software was used to quantify band intensity and ratios were taken to calculate the percentage of exon inclusion:exclusion in each treatment.

4.3   WHOLE CELL LYSATE PREPARATION, SDS-PAGE AND WESTERN BLOTTING

SDS-PAGE and Western Blotting protocols were performed similarly to Dembowski 2009. HeLa 2CCL cells were grown and treated as described above. Whole cell lysates were obtained by decanting cell media prior to extraction followed by treatment with RIPA buffer (supplemented with protease inhibitor cocktail and phosphatase inhibitor in the form of sodium orthovanadate). After incubating for at least 10 minutes in a cold room, lysates were transferred to Eppendorf tubes and subjected to sonification, three times per sample for 10 seconds per

round, making sure to return to ice after each pulse. Cellular debris was removed by centrifugation, and the supernatant was flash frozen until ready for use.

Samples were treated with SDS, boiled, mixed with running buffer, and loaded onto a polyacrylamide gel for electrophoresis-mediated protein separation. After the bromophenol blue band ran to the bottom of the gel, a wet blotting procedure was performed to transfer the contents of the gel to a PVDF membrane. After electrophoretic transfer, the membrane was blocked in milk, washed in PBST, and incubated in the primary monoclonal antibody indicated in Figure 4. After incubation for one hour, membranes were washed in PBST four times each for 10 minutes per wash. Membranes were next incubated in polyclonal secondary antibody conjugated to horseradish peroxidase for one hour. Following the same washing procedure as followed primary antibody incubation, membranes were treated with Western Blotting detection solutions as per the manufacturer's instructions. Proteins were detected via chemiluminescence imaging over several time points to obtain images with optimal signal:background ratios.

4.4 *IN VITRO* TRANSCRIPTION

pIDT_H1E4 plasmid was transformed into DH5α *E. coli* and extracted using QIAprep Spin Miniprep Columns (Qiagen). 25 µg purified DNA was restriction digested in 10 µL EcoRI (NEB) and EcoRI Buffer (NEB) in a reaction volume of 200 µL. The reaction was carried out for 3-4 hours at 37^0C followed by a 20 minute heat killing stage at 65^0C. Complete digestion was confirmed using a 0.8% agarose gel stained with EtBr. Both supercoiled and nicked bands were detectable in the uncut control lane, while only a linearized band was seen in the digested sample. Digested DNA was purified by buffered phenol-chloroform extraction followed by

ethanol precipitation. 0.75-1 μg pure DNA was used for each transcription reaction, in addition to 500 μM each NTP (UTP, ATP, GTP, CTP), 1x Transcription Buffer, and T3 RNA Polymerase (Ambion) in a total volume of 20 μL. The reaction was carried out for one hour at room temperature for unlabeled transcripts and two hours at 37^0C for fluorescently labeled samples. 1 μL TURBO DNase (Ambion) and 1 μg 0.5 M EDTA were added and incubated at 37^0C for 15 minutes to stop the reaction and to degrade DNA. Unincorporated nucleotides were removed using NucAway Spin Columns (Ambion). Remaining protein contaminants were removed by ethanol precipitation using 2 volumes 100% ethanol and 1/10 volume 3 M sodium acetate, pH 5.2. RNA was resuspended in 25-30 μL ddH_2O to give concentrations of between 61 and 150 ng/μL.

For fluorescently labeled probes, an identical procedure was performed, except 750 ng template DNA was used and instead of running the transcription reaction with 0.5 mM unlabeled UTP, ChromaTide Alexa Flour 488-5-UTP (Life Technologies) was combined with unlabeled UTP at a ratio that gave quantifiable labeling of RNA molecules without inhibiting T3 polymerase activity.

4.5   ELECTROPHORETIC MOBILITY SHIFT ASSAYS

The protocol for electrophoretic mobility shift assays was modified from Dembowski 2009. Secondary structures in the RNA transcript were removed by heating an aliquot to 85^0C for 5 minutes and cooling to 37^0C for 5 minutes. Protein-containing samples were prepared on ice in binding buffer (50 mM Tris pH8.0, 150 mM NaCl, 0.1 mg/ml tRNA, 2 mM DTT), and then heated to 37^0C for 5 minutes. After incubating the RNA and protein samples separately for

the indicated times, appropriate amounts of RNA were added to the protein-buffer mixtures. All samples were prepared to a final volume of 25 μL. The samples were allowed to incubate on ice for 1 hour, followed by the addition of 2 μL native loading dye (50% glycerol, 0.1% xylene cyanol, 0.1% bromphenol blue). After 5 minutes of pre-running a TBE-buffered 6% polyacrylamide (80 acrylamide : 1 bisacrylamide ratio) gel at 120V in a cold room, 13 μL of each sample were loaded onto gel. To align samples in the wells, 180V was applied to the gel for 5 minutes, followed by a 1 hour run at 120V. Reactions were kept out of light and were imaged using a 473 nm laser at 400V.

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