Regulation of HnRNP LL by the Depolarization/CaMKIV Pathway

by

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ABSTRACT

The RNA binding protein heterogeneous nuclear ribonucleoprotein L-Like (hnRNP LL) is known to regulate the alternative splicing of various physiologically important precursor messenger RNAs (pre-mRNAs). It undergoes a wide range of post-translational modifications (PTMs), including phosphorylation, ubiquitination and acetylation. However, the target amino acids and effects of these PTMs on the functions of hnRNP LL have not been characterized so far. In this study, we show for the first time that the endogenous hnRNP LL is phosphorylated upon depolarization. Using phosphopeptide mapping followed by the generation of a custom-made phospho-site specific antibody, we further show that phosphorylation at Ser308 of hnRNP LL is induced by depolarization though it is probably not the major phospho-amino acid target of depolarization/CaMKIV. The residue is critical for the nuclear localization and its phosphorylation essential for the CaMKIV-caused perinucleolar localization of the hnRNP LL protein in HEK293T cells. The residue is likely also critical in the regulation of nuclear functions like pre-mRNA splicing.

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DEDICATION

I dedicate this work to my parents Mr. Mahmudul Hoque and Mrs. Syeda Jesmin Banu as well as my brother Nafis and sister Nusrat.

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LIST OF ABBREVIATIONS

CaMK	Ca^{2+}/calmodulin-dependent protein kinase
CaRRE	CaMKIV-responsive RNA element
DMEM	Dulbecco's Modified Eagle Medium
EDTA	Ethylenediamine tetraacetic acid
FBS	Fetal bovine serum
GSK-3β	Glycogen synthase kinase 3 beta
HnRNP	Heterogeneous nuclear ribonucleoprotein
HnRNP LL	HnRNP L-like
JMJD6	Jumonji Domain Containing 6
KRG buffer	Krebs-Ringer glucose buffer
NCS	Newborn calf serum
Pre-mRNA	Precursor messenger RNA
PTB	Polypyrimidine tract binding protein
RIPA	Radio immunoprecipitation assay
RRM	RNA recognition motif
RT-PCR	Reverse transcription-polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SR protein	Serine/arginine-rich protein
STREX	Stress axis-regulated exon
TBS	Tris-buffered saline
TSA	Trichostatin A
U2AF	U2 auxiliary factor

µl	Microliter
µm	Micrometer
UV	Ultraviolet

CHAPTER I

Introduction

Alternative splicing is one of the pivotal steps in the regulation of gene expression that greatly contributes to the transcriptomic and proteomic diversity in higher eukaryotes [1, 2]. Several studies have indicated that ~92-95% of the protein coding human genes undergoes alternative splicing [3, 4]. This implies that its regulation impacts almost every aspect of cellular functions [5, 6]. Aberrant splicing leads to many human diseases, such as cystic fibrosis, growth hormone deficiency and spinal muscular atrophy [7-10]. Therefore, it is important to understand the control of alternative splicing.

During alternative splicing, the splice sites are selectively used by the spliceosome. The selection is mediated by various regulatory elements known as splicing enhancers or silencers, which can be in either the introns or exons [11]. It can also be regulated by cell signals through regulatory elements and *trans*-acting factors, which ensures appropriate temporal and spatial expression of splice variants of various genes [11-14].

The Ca^{++}/voltage-sensitive Big Potassium (BK) channels function in various physiological processes, such as hormone secretion, muscle contraction, learning and memory formation [15-17]. Functional diversity of these channels is mainly attributed to the alternative splicing of the *Slo1* gene which is widely expressed in the nervous and endocrine systems of vertebrate species. The human *hSlo1* precursor mRNA consists of at least 27 exons [16], which is depicted in Figure 1. Of the more than 10 alternative exons, the stress axis-regulated exon (STREX) confers higher Ca++/voltage sensitivity on the BK channels [16]. This exon is enriched in the high frequency region of cochleae where it contributes to the fine-tuning of hearing frequencies in turtles and birds [16, 18, 19].

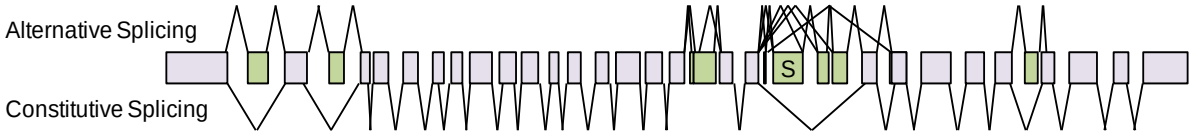

Figure 1: Diagram of human *Slo1* pre-mRNA (based on Xie'08 with modifications [16])**.** The constitutive exons are shown in 'purple' and alternative exons in 'green' boxes. S: STREX. Not drawn to scale. The constitutive and alternative splicing are depicted as described before [16].

Previous studies have shown that STREX is repressed upon depolarization [20]. Briefly, membrane depolarization causes the activation of L-type calcium channels and as a result intracellular Ca^{2+} level increases, which in turn activates Ca^{2+}/calmodulin-dependent protein kinase IV (CaMKIV) [20]. Activated CaMKIV phosphorylates a *trans*-acting splicing regulator heterogeneous nuclear ribonucleoprotein L (hnRNP L) at an evolutionarily conserved Ser513 [21]. The phospho-hnRNP L represses STREX inclusion by binding to the CaMKIV responsive RNA element 1 (CaRRE1) between the polypyrimidine tract (Py) and 3′ AG, thereby interfering with the binding of the large subunit of the U2 auxiliary factor (U2AF65) [21] (Figure 2). However, loss-of-function studies have indicated that hnRNP L knockdown alone cannot abolish the repression of STREX by depolarization [22], which suggests the involvement of other splicing regulator(s). Interestingly, double knockdown of hnRNP L and its paralogue hnRNP L-like (hnRNP LL) almost abolished the effect by depolarization [21]. This demonstrates a critical role for both hnRNP L and LL in STREX splicing regulation upon depolarization, but the molecular basis is not clear [21].

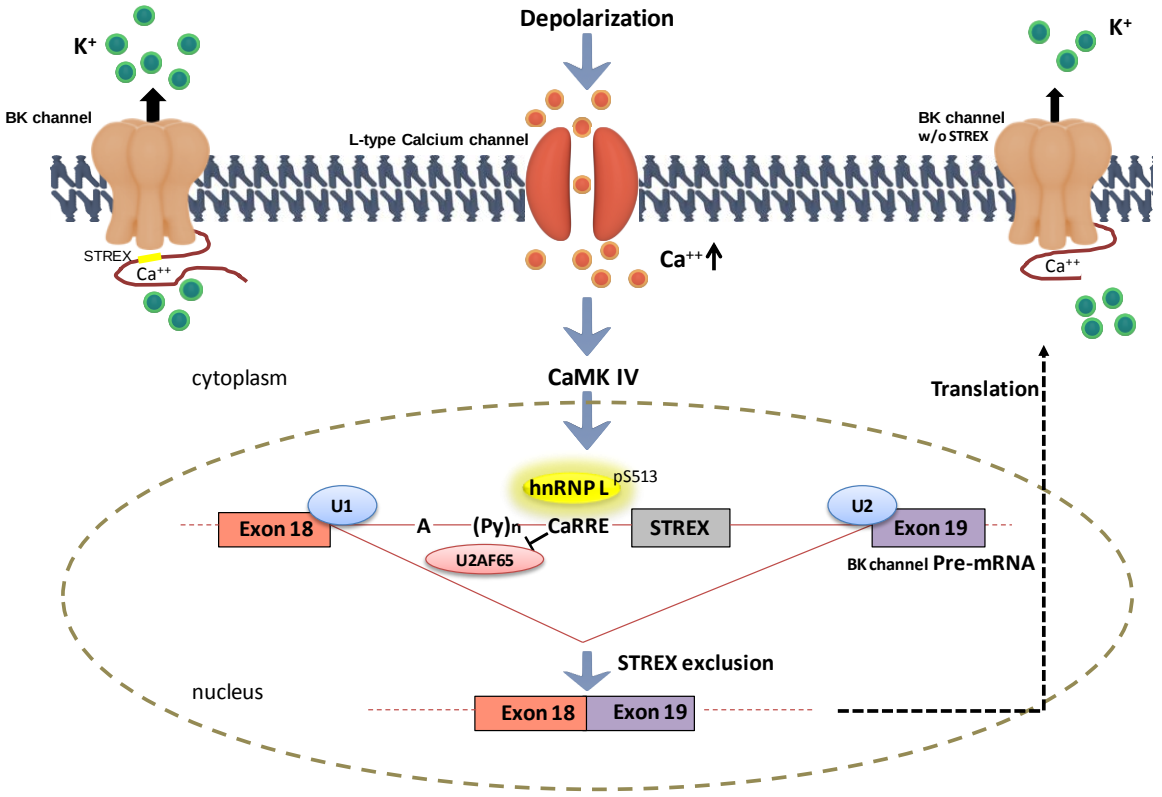

Figure 2: Schematic representation of the mechanism of STREX splicing in response to depolarization. Membrane depolarization causes the phosphorylation of hnRNP L at Ser513 through the CaMKIV pathway, which increases its binding to the CARRE1 element and inhibits the binding of U2AF65, thereby causing the repression of the STREX exon.

The human hnRNP LL protein has 68% sequence similarity with hnRNP L and contains four highly structured globular RNA recognition motifs (RRMs) [23]. Phylogenetic studies have revealed that hnRNP LL emerged at a much later time during the course of evolution compared to hnRNP L, as it is not found in species lower than fish in the evolutionary tree whereas hnRNP L is found in lower species like fruit fly and worm [24]. The hnRNP LL protein is critical for the regulation of alternative splicing in activated T cells [25, 26]. In another study by our lab, my colleagues have found that knocking down hnRNP LL almost abolished the production of growth hormone and prolactin in the culture

medium of GH_3 pituitary cells (Lei and Xie, unpublished data). Therefore, understanding the regulation of hnRNP LL has important physiological implications.

In this project, we mainly investigated the regulation of hnRNP LL by the depolarization/CaMKIV-pathway. Through *in vivo* labeling experiments coupled with phosphopeptide mapping, we have found that the endogenous hnRNP LL is a phosphoprotein, phosphorylation of which is increased upon depolarization in rat GH_3 pituitary cells. Site-directed mutagenesis followed by phosphopeptide mapping indicated that the phosphorylation at Ser308 of hnRNP LL is induced by depolarization though it is probably not the major phosphorylation target of the depolarization/CaMKIV-pathway. In addition, preliminary observations from this study indicate that CaMKIV may affect the intranuclear localization of the hnRNP LL protein and mutation at Ser308 changes the perinucleolar localization of hnRNP LL in HEK293T cells. To our knowledge this is the first reported functional phosphorylation site of hnRNP LL which may also have implication in splicing regulation.

CHAPTER II

Literature Review

2.1 Pre-mRNA Splicing

Pre-mRNA splicing was first described in 1977 [27, 28]. Soon after that Gilbert coined two terms “intron” and “exon” to designate the intragenic and expressed regions respectively [29]. In eukaryotes, most of the protein coding genes consist of short exons interrupted by much longer introns. Splicing is a highly regulated process of gene expression that takes place in the nucleus, by which the introns are removed and exons are joined together to form mature mRNA [30, 31]. This splicing reaction is carried out by a macromolecular complex known as spliceosome [32].

Alternative splicing allows differential usage of exons to produce different protein isoforms that may have similar or opposite functions [33]. For example, skipping of exon 3 of protein arginine methyltransferase 5 (PRMT5) produces an isoform that has totally opposite effect on cell cycle compared to the isoform containing the exon 3 [34]. Therefore, alternative splicing provides cells with the flexibility to produce proteins with diverse functions by the optimal use of the genetic sequences.

With the advent of different high throughput techniques and subsequent genome-wide analyses of different species, it is now clear that the number of genes in metazoans and lower eukaryotes do not vary significantly enough to correlate with the immense differences in structural, behavioral and functional complexities between them. It is rather the proteomic diversity within the metazoans, which is responsible for their evolution as complex organisms (Table 1). Mechanisms pertaining to such increase in the protein

diversity include the use of multiple transcription start sites, the use of multiple polyadenylation sites, alternative pre-mRNA splicing, pre-mRNA editing, and post-translational modifications [31, 35]. Among these mechanisms, alternative splicing is the most potent generator of proteomic diversity [36], presumably contributing to speciation [37]. The prevalence of alternative splicing events among different species shows a high level of variability [24, 38, 39]. Table 1 shows a comparison of alternative splicing among some of the well-studied species.

Table 1: Comparative analysis of genome size, total gene number and alternative splicing events among different vertebrate and invertebrate species: human (*Homo sapiens*), mouse (*Mus musculus*), fugu (*Takifugu rubripes*), stickleback (*Gasterosteus aculeatus*), medaka (*Oryzias latipes*), fly (*Drosophila melanogaster*) and worm (*Caenorhabditis elegans*).

Organism	Genome size	Total # of protein coding of genes	Total # of alternatively spliced genes	% of AS events	Ref
Human	3.3 billion bp	22,180	21,144	*88%	[2]
Mouse	3.3 billion bp	22,740	19,654	*63%	[2]
Fugu	390 million bp	21,600	9,336	43.2%	[40]
Stickleback	446 million bp	23,200	7,513	32.4%	[40]
Medaka	700 million bp	25,400	7,929	31.2%	[40]
Fly	165 million bp	13,937	11,767	*45%	[2]
Worm	100 million bp	20,541	20,008	*25%	[2]

* percentage with 2+ isoforms as described by Lee and Rio [2].

From the Table 1 it is apparent that there is a relationship between alternative splicing and organismal complexity, a phenomenon which has been described by several groups [38, 39].

It is estimated that a human gene on average consists of around 28,000 nucleotides (considering the 5' and 3' untranslated regions, UTRs) and contains 7.8 introns that intervenes between 8.8 exons [41]. The human exons are on average around 150

nucleotides in length and the introns on average are around 3,500 nucleotides long [42]. Removing these large segments of introns by the spliceosome is quite a demanding task since it must be done rapidly and precisely to meet with the physiological requirements of the cells and tissues. The splicing fidelity is achieved through the basal information present in the pre-mRNA known as the 'splicing code' which helps the spliceosomes to recognize the exon–intron boundaries [35]. There are only four small stretches of sequence information that distinguishes the introns from the exons: the 5′ splice site, branch point sequence, polypyrimidine tract and the 3′ splice site [43]. One essential feature of these sequences is that they are highly conserved and changing even one of the nucleotides may cause splicing inhibition [44]. The 5′ and 3′ splice sites are present at the boundary between the exons and the flanking introns with a consensus sequence of AG|GURAGU (Here, "|" designates the boundary between exon and intron; the purines are denoted by "R") and (Y)nNYAG|G ("Y" means pyrimidine, "Yn" designates the polypyrimidine-tract while "N" represents any nucleotide) respectively. The branch point sequence is located around 18-40 nucleotides upstream of the 3′ splice site with a consensus of YNYYRAY ("A'' represents the branch formation site) and the polypyrimidine tract is positioned between the branch point and 3′ splice site [37].

At the biochemical level, the process of splicing involves two transesterification steps where the components of the spliceosomal complex are assembled in a step-wise manner to catalyze the overall splicing reaction (Figure 3B). In the first step, the 2'-hydroxyl group of the adenosine (A) residue at the branch formation site attacks the phosphate at the 5' splice site, which leads to cleavage of the 5' exon and formation of a lariat structure. In the second step, the free 3'-OH of the cleaved 5' exon attacks the

phosphate at the 3' splice site which results in ligation of the two exons and release of the intron as a lariat [31, 45].

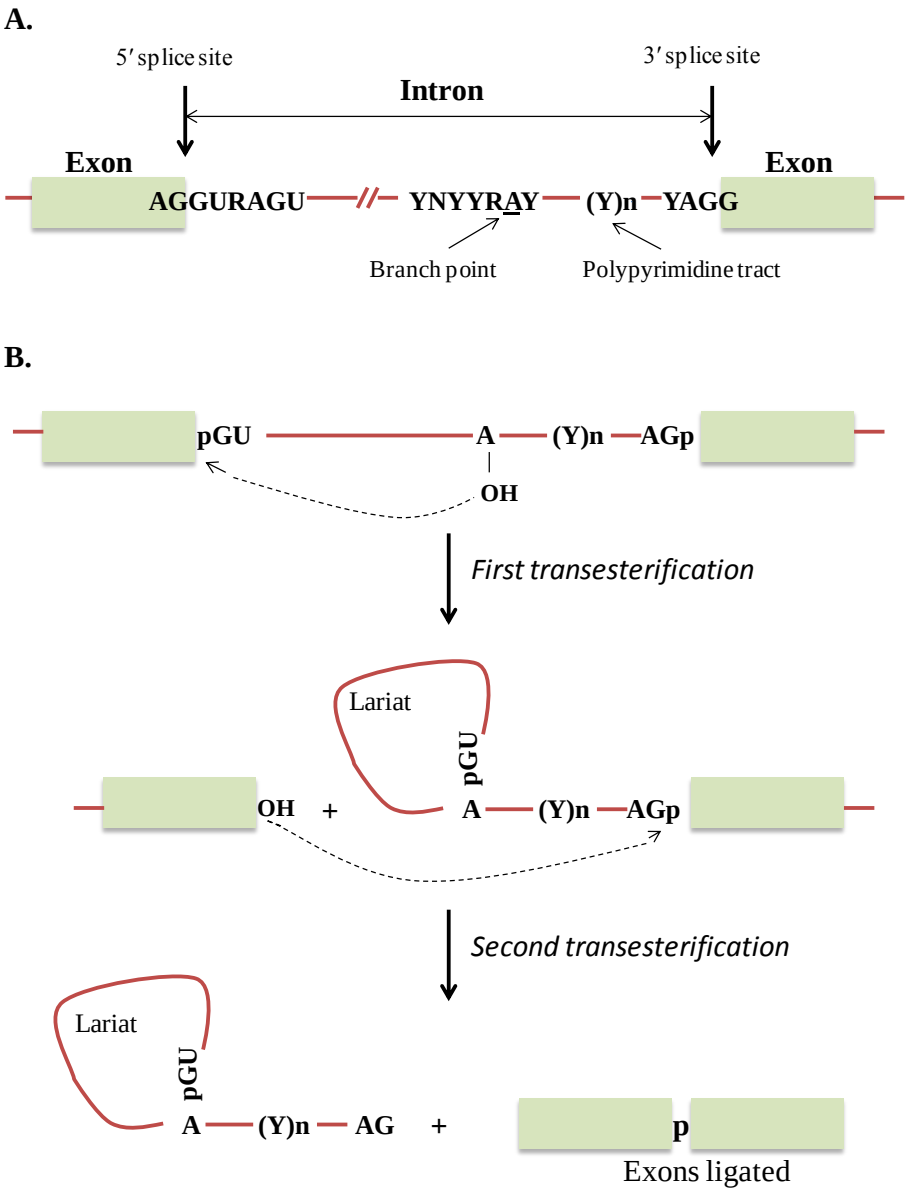

Figure 3: Schematic representation of the two-step transesterification during pre-mRNA splicing. Here, exons are represented by boxes and introns by lines. A. The conserved GU and AG nucleotide sequences of the 5′ and 3′ splice site, the branch point (donated by '**A'**) and the polypyrimidine tract as denoted by (Y)n in the figure act as signals for a splicing reaction to take place. **B**. A lariat is formed at the branch point and the exon at the 5′ end is released during the first transesterification reaction while the lariat intron is released and the exons are joined together by the action of a second transesterification reaction.

2.2 Spliceosome assembly

The splicing reaction is catalyzed in the nucleus by the spliceosome, which is a macromolecular complex comprising five small nuclear ribonucleoproteins (snRNP) and more than 200 auxiliary proteins [32]. The *de novo* assembly of the spliceosome on each intron requires the recognition of 5′ splice site, the branch point, polypyrimidine tract and 3′ AG [46]. Several spliceosomal complexes (named as E, A, B and C-complexes) are formed during the step-wise catalysis of the splicing reaction (Figure 4). The formation of E-complex is initiated by the ATP-independent binding of the U1 snRNP to the 5′ splice site via base pairing interaction between the U1 snRNA and the splice site [47]. On the other hand, the SF1/BBP (splicing factor 1/branch point binding protein) interacts with branch point sequence while the 35-kDa and 65-kDa subunit of U2 auxiliary factors U2AF35 and U2AF65 interacts with the 3′ AG and polypyrimidine tract respectively [31, 32]. Taken together, these interactions mark the formation of E-complex, which is a commitment step for splicing reactions to take place. Afterwards, U2 snRNP is recruited to the complex where it binds to branch point sequence via an ATP-dependent base-pairing interaction between the U2 snRNA and the pre-mRNA sequence and causes the displacement of SF1/BBP from the branch point and thus generates the A-complex [32]. Then U4/U6.U5 tri-snRNP is incorporated into the spliceosome and the B-complex is formed. This B-complex catalyzes the first step of splicing and in this process goes through some rearrangement and releases the U1 and U4 snRNPs. After that the components of B-complex reorganize to form the C-complex that catalyzes the second step of splicing [48]. The spliceosomal complex then dissociates and releases the mRNA. The snRNPs released are recycled and remodeled before taking part in new rounds of splicing [32].

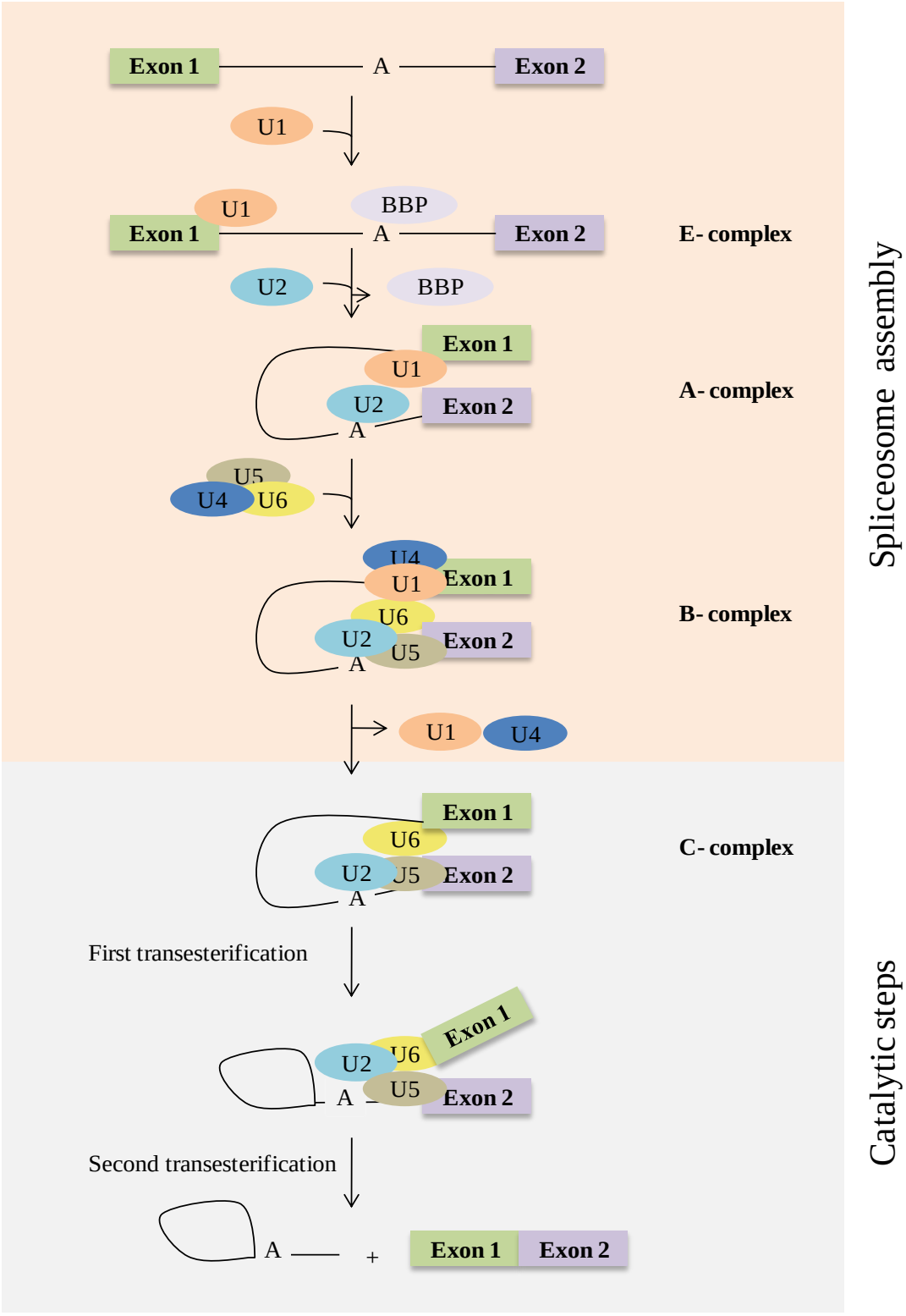

Figure 4: Schematic diagram of the major spliceosome assembly. Several complexes are formed (E, A, B and C) during the removal of intron and joining of two exons.

The spliceosome described above is known as the major (or U2-type) spliceosome. In addition, there is another type known as minor (or U12-type) spliceosome [49]. The snRNP components for minor spliceosome are mostly different from major spliceosome, and includes U11, U12 and U4atac/U6atac, while the U5 snRNP is common for both types of spliceosomes [50]. Even though the snRNAs of major and minor spliceosome have different primary sequence, they have similarity in their secondary structure [50, 51]. It has been found that the minor spliceosome works on introns that have longer consensus sequences at the 5′ splice site and branch point sequence compared to the major spliceosome; and they usually do not contain the polypyrimidine tract [49, 50]. Table 2 shows comparison between the two different types of spliceosomes. There are also some instances where both the major and minor spliceosome work on the same pre-mRNA to catalyze the splicing reaction [52].

Table 2: Comparison between major and minor spliceosomes.

Major Spliceosome	**Minor Spliceosome**
Most introns containing canonical consensus sequences are excised by this pathway.	Certain rare introns that have non-canonical consensus sequences are excised by this pathway.
The snRNPs of major spliceosome includes U1, U2, U4, U5 and U6.	The snRNPs for minor spliceosome includes U11, U12, U4atac and U6atac. U5 snRNP is used by both spliceosomes.
Also known as U2-type spliceosomes because the excision of the introns is dependent on U2 snRNP.	Also known as U12-type spliceosome because the excision of the introns is dependent on U12 snRNP.
They usually contain polypyrimidine tracts.	They usually do not contain polypyrimidine tracts.

In addition to the aforementioned constitutive splicing components, various *trans*-acting splice factors (SR proteins, hnRNPs and other splice factors) which bind to the *cis*-

acting regulatory sites by acting as either enhancers or silencers are necessary for correct alternative splicing [53]. There is also emerging evidence supporting that alternative splicing is also influenced by the transcriptional apparatus. There are two existing models that were proposed to explain the transcriptional regulation of alternative splicing: the kinetic coupling model [54] and the recruitment model [55]. The 'kinetic coupling model' (also called 'kinetic competition') suggests that the efficiency of splice site recognition of spliceosomes is regulated by the variation in the elongation rate of RNA polymerase II [54]. This means that slower RNA pol II elongation rate will have a longer window to allow the inclusion of certain exons compared to faster RNA pol II elongation rate where the window of opportunity to include a exon will be shorter [56]. For example, the inclusion of exon 33 of the human fibronectin 1 gene *(FN1)* is enhanced due to the slower elongation rate of RNA pol II [57] . The 'recruitment model' suggests that the interaction between carboxyl-terminal domain (CTD) of RNA polymerase II and the pre-mRNA processing factors spatially couples transcription to splicing [55]. According to this model, the pre-mRNA processing factors are recruited by the action of CTD of RNA pol II. The advantages of the proximity between CTD and pre-mRNA processing factors has been shown by the fact that the RNA processing steps are inhibited upon the deletion of CTD [58] .

2.3 Evolution of Alternative splicing

Even after almost 40 years of its discovery, the origin and functions of alternative splicing remained equally intriguing. The mechanism of mRNA splicing remained quite conserved throughout the course of evolution and is suggested to be originated from the autocatalytic 'Group II introns' [59]. There are two proposed models to explain the origin

of alternative splicing: one is sequence based and the other is *trans*-acting factor based [37]. The first model suggests that plasticity in the splice sites provided opportunity to the splicing machineries for the sub-optimal recognition of exons, leading to alternative splicing. This helps the cells to produce new transcripts from the gene without compromising the original set of transcripts [37]. Studies have found that the alternative exons, indeed, contain weaker splice sites compared to constitutively spliced exons –thus strengthening the logic behind this model [60]. The second model, an adaptation of the model on evolution of transcription factors (TFs) by Lenny Moss [61], suggests that the expansion of *trans*-acting splice factors like the SR and hnRNP proteins reduced selective pressures on the evolutionarily conserved splice sites and as a result the constitutive exons became alternatively spliced. One important point to be noted is that these two models on the evolution of alternative splicing do not essentially contradict each other. Since SR and hnRNP-like protein homologs are found to be present in early eukaryotes like yeast (*Schizosaccharomyces cerevisiae* and *S. pombe*), it has been suggested that both the protein classes are ancestral [62]. While only two SR proteins are found in *S. pombe*, canonical SR proteins are completely missing in *S. cerevisiae* [62], though three SR-like proteins have been found. Similarly, only one canonical hnRNP protein is present in *S. pombe* and one hnRNP-like protein has been identified in *S. cerevisiae* [63]. On the other hand, multiple families of SR and hnRNP proteins have been found in metazoan lineage. It has been found that alternative splicing is more prevalent in higher eukaryotes compared to lower eukaryotes, and the percentage of alternative splicing is higher in vertebrates than in invertebrates [64, 65]. So it has been proposed that there might be a direct link between the higher occurrence of alternative splicing events and the expansion of SR and hnRNP

proteins in metazoan lineage [62]. This correlation strongly argues for the *trans*-acting factor based model of alternative splicing evolution.

The major step in alternative splicing is the recognition of the boundaries between the exons and introns by the splicing machineries. There are two mechanisms that define such recognition: intron definition and exon definition [66, 67]. The more ancient of these two mechanisms is 'intron definition' where the recognition of a particular intron is defined by the placement of the basal splicing machinery across it. Similarly, the splicing machinery is placed across exons in 'exon definition'; and these exons are under selective pressure to remain short [67]. It is speculated that exon definition probably evolved at a later stage of evolution and became the main mechanism in higher eukaryotes. As a result, several forms of alternative splicing have been generated. While intron retention is the most common form in lower eukaryotes, the prevalence of exon skipping gradually increased in higher eukaryotes during evolution [67]. Plants, on the other hand, show lower levels of alternative splicing compared to other higher eukaryotes with a higher level of intron retention than exon skipping [68]. The use of alternative 5′ and 3′ splice sites are speculated to be derived from exon skipping and therefore are considered as representatives of an intermediate evolutionary stage [67, 69]. Other forms of alternative splicing events include the occurrence of mutually exclusive exons, use of alternative promoters and polyadenylation.

2.4 A snapshot of the multifunctional hnRNP proteins

Heterogeneous nuclear ribonucleoproteins (hnRNPs) are classically defined as a family of proteins that bind to the pre-mRNA produced by RNA polymerase II [70]. Structurally, the hnRNPs are modular proteins; and they contain multiple domains that are

connected by linker regions of variable lengths [57]. Such modularity accounts for the structural variation of the hnRNP proteins and ultimately causes functional diversity [71]. Indeed, the hnRNPs have been associated with a wide variety of cellular functions including pre-mRNA splicing [72], transcriptional regulation [73], mRNA stability [74], 3′-end processing [75, 76], maintenance of telomere length [56, 58], mRNA transport [77] as well as translational regulation [78].

Members of this family were initially identified by ultraviolet crosslinking and immunoprecipitation assays [70]. Later on, large scale sequencing and bioinformatics analysis helped to identify paralogues of many of the hnRNP proteins. In addition, based on structural and functional features, some other proteins like TDP-43, TIA1, TIAR, HuD, HuR, Fox2, Nova, Sam68, QKI, CUG-BP family members and MBNL have been designated as members of the hnRNP family [72]. However, the inclusion of the new members has been unsystematic and non-uniform [57]. In this review, I have limited the focus to the canonical hnRNPs and their paralogues and discuss how post-translational modification of these proteins affects their cellular function.

2.5 Different types of post-translational modifications of hnRNP proteins

Each hnRNP undergoes various types of post-translational modifications (PTMs). Most of the hnRNP PTMs have been identified by mass-spectrometry in recent years and thus functions of only a few of them have been deduced so far (Table 3). Table 4 in the results section contains the list of canonical hnRNPs along with their post-translational modifications according to the *PhosphoSitePlus*® database [79]. The most important issue still remains in validating the functions of the different hnRNP PTMs. Validation is either done by mutating the modification site or by using inhibitors that block the enzymes that

induces the modification. Even though the database suggests that different types of post-translational modification can take place on the hnRNPs (Table 4), functional significance of only five types have been validated till now. These modifications include phosphorylation, methylation, acetylation, SUMOylation and ubiquitination. So hereafter this literature review will focus on these five types of modifications of hnRNP proteins.

2.5.1 Phosphorylation

Reversible protein phosphorylation is one of the main regulatory mechanisms of cell signaling and recent large scale phosphoproteomic studies suggest that around two third of the cellular proteins are phosphorylated [80]. These events are precisely controlled by the action of protein kinases and phosphatases within the cell. Phosphorylation is by far the most studied of the hnRNP PTMs. All the canonical hnRNPs appear to be phosphorylated (Table 4) and thus likely regulate different types of cellular processes. The most common approach to deduce the function of specific phosphorylation site is to mutate the site, knock down that specific gene followed by rescue with wild type and mutant protein. For example, studies in our lab have found that phosphorylation of hnRNP L by CaMKIV at Ser-513 regulates the alternative splicing of stress-axis regulated exon (STREX) of the BK channel pre-mRNA [21]. In this case Ser-513 was mutated to alanine and knockdown effect of hnRNP L was rescued by the wild-type version of the protein while the Ser-513 mutant couldn't rescue the splicing event suggesting the critical role of this specific phosphorylation site. Another example is the phosphorylation of hnRNP I (also known as the polypyrimidine tract-binding protein, PTB) at Ser-16 where the mutation at the site altered the subcellular localization of the protein [81].

2.5.2 Methylation

Methylation is a post-translational modification mediated by different types of methyltransferases where methyl groups are transferred to nitrogen or oxygen residue of the amino acid side chains of proteins. This is known as N- and O-methylation, respectively [82]. Initially it was thought that only O-methylation was reversible [82]; however, later it was demonstrated that N-methylation on arginines is also reversible – it can be demethylated by Jumonji Domain Containing 6 (JMJD6) [83]. Methylation is common for hnRNPs. Even though 79% of the hnRNPs listed in Table 4 are methylated, cellular function of only a few of them have been deduced so far. Use of inhibitors of upstream methylating enzyme and site-specific mutation are mainly done for characterization of cellular function. Methylation of hnRNPs have been shown to regulate functions like transcriptional activity of other proteins [84], subcellular localization [85].

2.5.3 Ubiquitination

Ubiquitination is an ATP-dependent, three-step protein process where a protein known as ubiquitin gets attached to the protein [86]. Such tagging of the proteins for degradation is an important homeostatic mechanism that regulates different cellular processes. All the canonical hnRNPs appear to be ubiquitinated (Table 4). For example, ubiquitination of hnRNP L causes its degradation, which in turn helps to maintain translational repression of vascular endothelial growth factor (VEGF)-A mRNA [87] .

2.5.4 Acetylation

Acetylation is a process by which an acetyl group (-CH_3CO) is attached to the target protein, which is usually catalyzed by lysine acetyl transferases [88]. Similar to

phosphorylation, this modification can also be reversed and the enzymes that are involved in the reversal of the acetylated state are known as deacetylases. It is estimated that 80-90% of the human proteins are acetylated [89] and interestingly through our database search we have found around 90% of the hnRNP proteins appear to be acetylated (Table 4). However, like other proteins, there are only limited examples where the acetylated proteins have been linked to certain cellular function. Our lab has recently shown that the degradation of hnRNP F by the proteasome/ubiquitination pathway is prevented upon treatment with a deacetylase inhibitor trichostatin A (TSA) in MDA-MB-231 breast cancer cells grown in the presence of transforming growth factor beta 1 (TGFβ1) [90]. More interestingly, when the mutually exclusive acetylation or ubiquitination sites (K87, K98 and K224) of hnRNP F were mutated individually, the TSA-mediated stability of the protein was abolished. In addition, mutation of K98 and K224 abolished the TSA mediated regulation of alternative splicing of Bcl-x in minigene reporter assay. Furthermore, TSA also stabilized some other hnRNPs like hnRNP A1, I and L suggesting that similar type of regulation likely renders stability to many other hnRNPs [90].

2.5.5 SUMOylation

SUMOylation is a post-translational modification in which Small Ubiquitin-like Modifier (SUMO) proteins are covalently conjugated to other proteins by a three step mechanism and thereby regulates a wide variety of cellular processes, such as nucleocytoplasmic transport, transcription, signal transduction, apoptosis and cell cycle control [91]. SUMOylation of hnRNP M and lysine 237 of hnRNP C proteins at the nuclear pore complexes (NPCs) regulate the conformation and/or composition of these hnRNPs, and these modifications have also been proposed to regulate the mechanism of

nucleocytoplasmic transport [92]. SUMOylation of hnRNP C has also been shown to influence their binding with nucleic acids [92]. It has been shown that SUMOylated form of hnRNPA2B1 is present in exosomes and this SUMOylation controls the binding of hnRNPA2B1 to miRNAs [93]. Among the other members, hnRNP A1 and F undergo SUMOylation even though their functional significance has not been determined [94].

2.6 Crosstalk between the PTMs of hnRNP

The abundance of hnRNP PTMs makes it likely that there is cross-talk between them within the cell. Indeed, there are examples where a specific type of PTM on hnRNP plays role in either promoting or reducing another type of PTM. For example, methylation of hnRNP K by PRMT1 reduces its interaction with c-Src kinase. This affects the hnRNP K-dependent activation of c-Src and results in reduced tyrosine phosphorylation of hnRNP K [95]. Such reciprocal relationship among the different PTMs facilitates the proteins to switch between different functions depending on the need of the cell.

Interestingly, there are also some other reports that showed the interplay between hnRNP phosphorylation and methylation. It has been shown that methylation of Arg296 and 299 of hnRNP K suppresses the nearby Ser302 phosphorylation by PKCγ and negatively modulates apoptosis [96]. Upon DNA damage, there is a reduction in the methylation of the Arg296 and 299 and an increase in PKCγ-mediated phosphorylation at Ser302 which results in the induction of apoptosis [96]. The Ser302 site of hnRNP K has also been shown to be phosphorylated by PKCγ where it regulates the translation of VEGF mRNA upon angiotensin II-induced renal injury [97]. This is an example that shows the same hnRNP PTM can have different roles in regulating different cellular signaling pathways. The translational regulation of VEGF mRNA is interesting because several

other hnRNP PTMs are also shown to be involved. Yao and colleagues have shown that inflammation causes hnRNP L ubiquitination through prolyl hydroxylation and von Hippel-Lindau (VHL)-mediated proteasomal degradation while hypoxia causes phosphorylation at Tyr359 of hnRNP L protein which increases its binding to hnRNP A2/B1 and thereby stabilize the hnRNP L protein from degradation [87]. This phosphorylation also helps to recruit DRBP76 (double-stranded RNA binding protein 76) to the 3´ UTR of VEGF-A mRNA, thereby allowing its expression to ensure appropriate angiogenesis [87]. Perrotti et al. have shown that protein kinase CbII (PKCbII) mediated phosphorylation of hnRNP P2 (also known as FUS protein) at Ser256 also prevents its degradation by the proteasome-pathway [98]. Taken together, it can be suggested that there is a pattern where phosphorylation of hnRNPs prevents their degradation by proteasome-pathway. This is consistent with the observation that regulation of protein ubiquitination, in many of the cases, is dependent on protein phosphorylation [99].

2.7 Function of hnRNP PTMs in different aspects of gene regulation

HnRNPs have a diverse array of functions ranging from transcription, pre-mRNA splicing to translation and localization of proteins. While doing so, these proteins also undergo different types of post-translational modification. Perturbation of these modifications may hinder the function of these proteins and may also lead to disease.

2.7.1 HnRNP PTMs in transcription

The hnRNP PTMs can either activate or repress transcription. However, there is still not much data available in the literature to predict which modification plays a role in activation and which in repression in case of the hnRNPs. The most widely studied PTMs

in this case are those of the histone proteins. In general, acetylation, methylation, phosphorylation, and ubiquitination of histone proteins are involved in transcriptional activation whereas methylation, ubiquitination and SUMOylation play role in repression [100].

Methylation of HnRNP K has been shown to enhance the transcriptional activity of p53 in response to UV-irradiation mediated DNA damage, which then arrests cell cycle via its target genes namely p21, GADD45 [84]. Through chromatin immunoprecipitation (ChIP) assays it has been shown that suppression of hnRNP K methylation by either MTA treatment or RNA interference (RNAi) against the upstream enzyme PRMT1 reduced the recruitment of p53 to p21 promoter and thereby decreased the expression of p21 [84]. This means recruitment of p53 to the promoter of p21 following DNA damage allows increased expression of the cell-cycle arrest genes like p21, GADD45 which may cause the induction of DNA repair or may even cause cell death by apoptosis and prevent tumorigenesis. However, there are several other types of PTMs that play role in this regulation. Two groups have separately shown that the DNA damage-induced SUMOylation of hnRNP K at lysine 422 is required for the transcriptional activation of p53 [101, 102]. Under normal conditions, hnRNP K and p53 both undergoes ubiquitination and subsequently degraded by the proteasome pathway. Upon DNA damage, hnRNP K becomes SUMOylated which then serves as a cofactor for p53-mediated gene expression [101]. Recently, Yang et al. showed that hnRNP K can also regulate apoptosis independent of the p53 pathway [96].

2.7.2 HnRNP PTMs in alternative splicing

Alternative splicing is a highly elaborate process where the resultant transcriptomic and proteomic diversity is truly enormous [103]. The hnRNPs play critical roles as *trans*-

acting splicing regulatory factors. Recent evidence suggests that post-translational modification of hnRNPs itself can modify their role in splicing regulation. The only well-studied pathway regulated by such a mechanism is that of depolarization/CaMKIV mediated splicing of STREX exon of the *Slo1* gene [104]. As mentioned in the introduction, the inclusion of STREX encoded peptide provides higher calcium and voltage sensitivity to the channel [6, 16, 104] and membrane depolarization represses its inclusion [20]. Upon membrane depolarization, CaMKIV causes hnRNP L phosphorylation at Ser513 which causes its increased binding to the CaRRE1 element located at the 3′ splice site of the STREX exon and hinders the binding of U2AF65 and thereby causes the repression of STREX exon [21]. In this case, hnRNP L phosphorylation regulates a critical step in the assembly of early spliceosome to control the splicing of STREX exon. Mayrand and colleagues have shown that phosphorylation of hnRNP C by casein kinase II decreases its binding to the adenovirus and human β-globin pre-mRNAs while dephosphorylation of the protein increases it's binding to the β-globin pre-mRNAs [105]. This dynamic cycle of phosphorylation and dephosphorylation is proposed to be controlling the splicing of β-globin pre-mRNAs.

2.7.3 HnRNP PTMs in translation

HnRNP A0 and A1 phosphorylation is involved in the post-transcriptional regulation of several inflammatory mediators. Cytokines are small signaling molecules that play a crucial part in mediating immune responses. It has been shown that lipopolysaccharide, a type of inflammatory stimulus, causes the activation of downstream MAPKAP-K2 kinase which phosphorylates the *trans*-acting factor hnRNP A0 at specific serine 84 residue [106]. This phosphorylation increased the binding of hnRNP A0 to the 3´-untranslated region (3´-

UTR) of inflammatory mediators like tumor necrosis factor alpha (TNFα), Cox-2 and macrophage inflammatory protein-2 (MIP-2). The LPS-induced productions of the inflammatory mediators were suppressed in MAPKAP-K2 deficient mouse and also when the kinase inhibitors (SB203580 and PD184352) were used. When hnRNP A0 is not phosphorylated its interaction with the mRNAs of these three inflammatory mediators is prevented. In case of TNFα, the interaction of hnRNP A0 with its mRNA is proposed to relieve translational repression while the interaction of hnRNP A0 with Cox-2 and MIP-2 mRNAs increases their mRNA stability. Similarly hnRNP A1 phosphorylation at Ser192 & Ser 312 is also involved in the post-transcriptional regulation of TNFα synthesis [107]. When hnRNP A1 is phosphorylated, there is decreased binding of hnRNP A1 to the 3´-untranslated region (3´-UTR) of TNFα which removes the repression of TNFα synthesis. Treatment with the inhibitor of the upstream Mnk kinase resulted in the reduced synthesis of TNFα. These studies suggest the importance of hnRNP PTMs in regulation of immune response via regulating the synthesis of several inflammatory mediators [107].

HnRNP A2 & hnRNP F phosphorylation relieves translational repression of MBP to facilitate myelination. Myelination helps the nerve impulses to rapidly move along the axon and it requires the production of large amounts of myelin basic protein (MBP) at the axon – glial contact site [108]. HnRNPs A2, E1 and F remain bound at the 3´ untranslated region (UTR) of MBP mRNA (also known as the A2 response element, A2RE) causing the translational repression of the protein [109]. Upon the contact of axon and glial cells through the interaction of L1 and F3 receptors found on their respective surface, Fyn kinase is activated inside the glial cell which then phosphorylates hnRNP A2 and F. Phosphorylation liberates the MBP mRNA from the bound hnRNP A2, F as well as

hnRNP E1 and thereby allows localized MBP synthesis for the generation of the myelin sheath [110]. In this case, phosphorylation of hnRNP A2 and F relieves the translational repression of MBP.

There are several other examples where phosphorylation causes translational activation. hnRNP K has been shown to be phosphorylated at different tyrosine residues by c-Src upon its activation which inhibits its binding to the differentiation control element (DICE) located at the 3´ UTR of the LOX mRNA *in vitro* and subsequently relieves the translational repression of LOX [111]. This is an example of differentiation-dependent translation of mRNA since LOX mRNA remains silenced in early stages of life and needs to be activated during the later stages. Chaudhury and colleagues have shown that transforming growth factor-β (TGF-β) causes protein kinase Bβ/Akt2 mediated phosphorylation of hnRNP E at Ser 43, which in turn results in the relieve of translational inhibition of Dab2 and ILEI mRNAs [112]. Similarly, Pak1 mediated phosphorylation of hnRNP E relieves the translational inhibition on L1CAM mRNA [113].

2.7.4 HnRNP PTMs in the regulation of subcellular localization

Some of the hnRNP proteins are known to be involved in shuttling between the nucleus and cytoplasm depending on the need of the cell. It has been shown that hnRNP PTMs play role in their subcellular localization, which may spatially regulate the production of other proteins. HnRNP A1 is a nuclear protein, which upon the induction of stress becomes phosphorylated at the serine residues within a region located in its C-terminus, known as the "F-peptide". This stress-induced phosphorylation increases its cytoplasmic localization and thereby changes its activity as a splicing regulator [114-116].

In addition, this phosphorylation modulates the interaction of hnRNP A1 with transportin Trn1 and mutation in the serine residues of the "F-peptide" resulted in the cytoplasmic accumulation of the protein [115]. This suggests that the phosphorylation of F-peptide is necessary to regulate the rate of hnRNP A1 nuclear import. Such alteration of the localization changes upon post-translational modification has also been reported for several other hnRNPs. Methylation of hnRNP A2 and Q by PRMT1 [85, 117], phosphorylation of hnRNP K at Ser116, Ser284 and Ser353 [118, 119] have also been shown to regulate subcellular localization pattern of the protein.

2.8 Role of HnRNP-PTM's in disease

HnRNP K phosphorylation in prostate cancer. It has been shown that a phosphorylated form of hnRNP K is associated with the androgen receptor (AR), which plays a key role in the development and progression of prostate cancer [118]. Treatment of prostate cancer cells with anti-androgen drugs like bicalutamide (BIC) caused hypo-phosphorylation of hnRNP K as well as its decreased co-localization with AR whereas treatment with agonist like cyproterone acetate (CPA) resulted in hyperphosphorylation of hnRNP K and increased co-localization with AR.

HnRNP K phosphorylation in motor neuron disease. Preliminary observation from a recent study revealed that HnRNP K phosphorylation also has role in the accumulation of TAR-DNA binding protein 43 (TDP-43), which is a major component of the cytosolic inclusions in spinal cord of patients with motor neuron disease [120]. In this study, the authors claimed that inhibition of upstream kinase blocks hnRNP K phosphorylation and thereby prevents the incorporation hnRNP K along with its interacting partner TDP-43 into

the stress granules. This may provide a good therapeutic strategy to prevent TDP-43 accumulation.

HnRNP L phosphorylation in non–small cell lung cancer (NSCLC) tumors. Lung cancer is the leading cause of mortality amongst all form of cancer [121]; and there is a need to understand this deadly disease from a molecular perspective so as to develop therapeutic interventions to combat it. It has been shown that phosphorylation at Ser52 of hnRNP L is increased in non–small cell lung cancer (NSCLC) cells compared to non-transformed lung epithelial cells [122]. Phosphorylation causes increased binding of hnRNP L to the *cis*-acting exonic splicing silencer (ESS) positioned within the exon 3 and thereby modulates the splicing of caspase-9 pre-mRNA [122, 123]. Caspase-9 has two splice variants: pro-apoptotic caspase-9a and anti-apoptotic caspase-9b [124]. The Ser52 phosphorylation promotes caspase-9b production and is required for the tumorigenic capacity of NSCLC cells [122]. This modification can be used as a potential therapeutic target for NSCLC treatment.

HnRNP U phosphorylation at Ser59 as a potential marker of DNA damage. Even though there is no direct link with disease/ treatment, some hnRNP PTMs are increased in response to DNA damage. Berglund et al. have shown that DNA-PK can induce increased hnRNP U phosphorylation at Ser59 upon the induction of DNA damage [125, 126]. This may serve as a potential marker for cellular DNA-PK activity. As discussed in section 2.6 of this literature review, several other hnRNP PTMs have also been reported to be either increased or decreased upon DNA damage.

Table 3: List of hnRNP PTMs with biological functions (by experimental evidence). Here, Phosphorylation =P, Methylation=M, SUMOylation=Sm, Acetylation=Ac, Ubiquitination=Ub

Pro-tein	PTM	Amino acid modified	How the PTM was identified	Modification is done by	Functional impact	Evidence for function	Ref
A0	P	Ser84	Mass-fingerprinting, phospho antibody	MAPKAP-K2	LPS-induced post-transcriptional regulation of TNF-α,COX-2 and MIP2	RNA-IP with and without kinase inhibitor (SB203580) treatment	[106]
A1	P	F-peptide	Phospho-amino acid mapping, MALDI-TOF	p38 kinase, Mnk1/2	Alters subcellular distribution and thereby regulates splicing changes	Mutagenesis, Immunoprecipitation and Immunostaining	[114-116]
	P	Ser192 & Ser 312	HPLC, Phospho-peptide mapping,	Mnk	Regulates the translation of TNFα	Surface plasmon resonance (SPR) binding assay with TNFα RNA	[107]
	P	Ser199	Mutagenesis	AKT	Inhibits IRES-mediated translation initiation	Knock-down followed by rescue with wild type and Ser199 mutant	[127]
A2	P	tyrosine	Mass spectrometry	Fyn kinase	Increases the localized translation of myelin basic protein (MBP) mRNA	Immunostaining & Immunoblotting	[109, 110]
	Me	Arginine	Adenosine dialdehyde treatment	PRMT1	Regulates localization	Mutagenesis, Immunostaining	[85]
A2/B1	Sm	-	Mass-spectrometry	-	Controls its binding to miRNAs	IP-qPCR w/wo anacardic acid	[93]
C	P	Serine/ threonine	2,3-diphosphoglycerate and quercetin treatment	Casein kinase 2	Regulates binding to adenovirus /7 human β-globin pre-mRNAs	Binding of biotinylated pre-RNAs to streptavidin-agarose beads	[105]
	Sm	K237	Mutagenesis	-	Reduced binding to ssDNA	ssDNA binding assays	[92]
D	P	S83,S87	Mutagenesis	cAMP-kinase, GSK-3β	Ser87 enhances while Ser83 inhibitsits transactivator activity	Overexpressing GSK-3β inhibited transactivation by hnRNP D	[128]
E1	P	Ser43	Mutagenesis	protein kinase Bβ/Akt2	Induces EMT via transcript-selective translational induction of Dab2 & ILEI	RNA affinity pull-down, Immunobloting	[112]
	P	T60, T127	Mutagenesis, phospho-antibody	Pak1	Relieves the translational inhibition on L1CAM	EMSA, Luciferase assay	[113]
F	P	tyrosine	Phospho-tyrosine antibody	Fyn kinase	Increases the localized translation of myelin basic protein (MBP) mRNA	Overexpression of Fyn kinase increases cytosolic hnRNP F and relieves MBP mRNA	[129]

F	Ac	K87, K98, K224	Mutagenesis	-	Provides stability to the protein	TSA stabilizes the protein while mutations in either of the lysine residues destabilizes the protein even after TSA treatment	[90]
I	P	Ser16	Mutagenesis, peptide mapping	PKA	Modulates the subcellular localization	Heterokaryon assay, Immunostaining	[81]
K	P	tyrosine	Phospho-tyrosine antibody, mutagenesis	c-Src	Translational activation of LOX mRNA	Northwestern blot	[111, 130]
	Me	Arginine	MTA treatment	PRMT1	Enhances p53 transcriptional activity	ChIP assay, RNAi of PRMT1	[84]
	Me	Arginine	Mass spectrometry, Edman degradation	PRMT1	Reduces c-Src mediated phosphorylation of hnRNP K	Co-transfection with hnRNP K, PRMT1 and c-Src	[95]
	Me	Arg 296, Arg299	Mutagenesis	PRMT1	Inhibits serine302 phosphorylation , negatively modulates apoptosis	Mutation and inhibitor treatment	[96]
K	Sm	K422	Mutagenesis	E3 ligase Pc2/CBX4	Required for transcriptional activation of p53	Knockdown and rescue with WT and mutant of hnRNP K	[101, 102]
L	P	Ser52	Mutagenesis, phospho-specific antibody	phosphoinositide 3-kinase/AKT	Interferes with the binding of hnRNP U at exon 3 of caspase 9 and represses splicing of exon 3,4,5,6 cassette	knockdown followed by rescue with wildtype and Ser52 mutant	[122, 123]
	P	Y359	Mutagenesis	not known	Regulates VEGFA mRNA translation	Co-IP with hnRNP A2/B1 using wildtype & Y359 mutant	[87]
	P	Ser513	Phosphopeptide Mapping, phospho-Ab	CaMKIV	Regulates depolarization-induced alternative splicing of STREX exon	Knockdown followed by rescue with wildtype and Ser513 mutant	[21]
	Ub	-	proteasome inhibitor MG132	IFN-γ and von Hippel-Lindau	Maintains repression of VEGFA mRNA by degrading hnRNP L	siRNA-mediated knockdown of VHL	[87]
P2	P	Ser256	Mutagenesis	Protein Kinase CβII	prevents its degradation by the proteasome	Mutation caused degradation of the protein which was restored by the treatment of lactacystin	[98]
Q	P	Tyrosine	MALDI mass spectrometry	insulin receptor tyrosine kinase	Proposed function in regulation of mRNA translation/ stability by insulin	-	[131]
	Me	C-terminal RGG box	Treatment with adenosine dialdehyde	PRMT1	Regulates cellular localization of hnRNP Q	Mutagenesis, Immunoblotting and Immunostaining	[117]
U	P	Ser59	Mass spectrometry	DNA-PK	Marker for DNA damage	Drug-induced DNA damage followed by IB with phospho-Ab	[125]

CHAPTER III

Statement of Hypothesis

Previous knockdown studies have indicated an essential role for hnRNP LL in depolarization-induced alternative splicing of the STREX exon [21], but how it is regulated upon depolarization remains unknown. Different types of stimuli can cause changes in signal transduction pathways, which in turn can cause changes in splicing patterns of a gene through modulation of the splicing regulatory proteins. There are very few examples where the regulation of splicing protein by upstream stimuli has been studied. The main focus of this study was to understand the regulation of hnRNP LL by the depolarization/CaMKIV pathway.

Based on some of the preliminary results obtained during the initial stages of this study, **I hypothesize that hnRNP LL is phosphorylated upon depolarization/CaMKIV activation.**

CHAPTER IV

Materials and Methods

Materials

List of chemicals/reagents used

Name of chemical/reagent	Manufacturer/Source
32P-orthophosphoric acid (37000MBq/0.1mL)	PerkinElmer
Agarose	American Bioanalytical
BLUeye Prestained Protein Ladder	FroggaBio
Calcium chloride ($CaCl_2$)	Fisher
Dimethyl Sulfoxide ($(CH_3)_2SO$)	Fisher
Dithioreitol	Fisher
ECL Western Blotting Detection Reagents	VWR
Ethanol	Fisher
Ethidium bromide	Sigma-Aldrich
Ethylenediamine Tetraacetic Acid (EDTA)	Fisher
Formic Acid (Aldehyde-Free/Sequencing)	Fisher
Glucose	Sigma-Aldrich
Glacial acetic acid	Mallinckrodt AR
Glycerol	Fisher
Glycine	Fisher
Hydrogen chloride	Fisher
Isopropanol (C_3H_7OH)	Fisher
Lipofectamine 2000	Life Technologies
Magnesium chloride	Fisher
Methanol	Fisher

Nitrocellulose paper	GE Healthcare/Whatman
Phenylmethylsulfonyl fluoride (PMSF)	Fisher
PBS tablets	Fisher
Polybrene (Hexadimethrine bromide)	Sigma
Ponseau S	Fisher
Potassium chloride (KCl)	Fisher
Protease inhibitor cocktail	Sigma
Protein G sepharose beads	Fisher (GE Health Care)
PVDF membranes	Millipore
Sodium chloride (NaCl)	Fisher
Sodium dodecyl sulfate (SDS)	Fisher
Sodium Orthovanadate (Na_3VO_4)	Fisher
Sodium Fluoride (NaF)	Fisher
TLC plates	EMD Chemicals
TEMED	Fisher
Tris base	VWR (J.T.Baker)
Tween 20	Fisher

List of enzymes, inhibitors and buffers used for various experiments

Name of enzyme/buffer	Manufacturer/Source
5x First strand buffer	Invitrogen
5x Forward buffer	Invitrogen
5X T4 DNA ligase Buffer	Invitrogen
Age I	New England Biolabs
Benzonase® Nuclease	Sigma-Aldrich
Calf intestinal alkaline phosphatase (CIAP)	Invitrogen
Dnase I	Roche
dNTPs	Invitrogen
KN-93	VWR

M-MLV Reverse Transcriptase	Life Technologies
Phusion High-Fidelity DNA Polymerase	New England Biolabs
Reaction Buffer H	New England Biolabs
Sal I	New England Biolabs
SUPERase• In™ RNase Inhibitor	Ambion
T4 DNA ligase	Invitrogen
Taq DNA polymerase	Xie lab
TLCK-chymotrypsin	Worthington Biochemical Corp
TPCK-trypsin	Worthington Biochemical Corp
TSA	Sigma-Aldrich

List of Antibodies used in this study

Name of Antibody	Source
Anti-hnRNP LL (4783)	Cell Signaling Technology
Anti-c-Myc (789 and 40)	Santa Cruz
Anti-C23 (Nucleolin) (H-6)	Santa Cruz
ANTI-FLAG® M2 (F1804)	Sigma-Aldrich
Anti-phospho-Ser513-hnRNPL	Xie lab
Anti-phospho-Ser308-hnRNPLL	Xie lab
Goat anti-Mouse IgG HRP (358914)	Santa Cruz
Goat anti-rabbit IgG-HRP (2054)	Santa Cruz

List of mammalian cell lines used in this study

Cell Line	Organism and disease	Culture properties
GH_3	Rat pituitary tumor cells	Loosely adherent/Suspension
HEK293T	Human embryonic kidney cells	Adherent

Bacterial strain used during transformation
DH5α (from Xie Lab)

List of materials used for mammalian cell culture

Name of chemical/media/product	Commercial Source
Ham's F-10 Nutrient Mix	Life Technologies
Horse Serum (HS)	Invitrogen
Fetal Bovine Serum (FBS)	Sigma
Penicillin-Streptomycin (10,000 U/mL)	Life Technologies
L-Glutamine (200 mM)	Life Technologies
DMEM, powder, high glucose	Life Technologies
Newborn Calf Serum (NCS)	Life Technologies
Trypsin	Life Technologies
Cell culture plate	BD FALCON
6-well and 12-well plates	VWR
T175 Flasks	VWR
Disposable Serological Pipettes	VWR

Recipe for the buffers and solutions (as described in [21, 81, 132-134]**)**

KRG buffer	
Component	**Concentration**
NaCl	128 mM
$CaCl_2$	2.1 mM
$MgSO_4$	29 mM
KCl	5.2 mM
HEPES (pH 7.5)	10 mM
pH 1.9 buffer	
Component	**Amount per 1 Liter**
Formic acid	25 mL
Glacial acetic acid	78 mL
ddH_2O	879 mL

Phospho chromatography buffer	
Component	**Amount per 100 mL**
Butanol	37.5 mL
Glacial acetic acid	7.5 mL
Pyridine	25 mL
ddH_2O	30 mL
NP-40 buffer (Nonidet-P40) [mild]	
Component	**Concentration or percentage**
NaCl	150 mM
Tris-HCl (pH 7.5)	10 mM
EDTA	1 mM
Igepal	0.325%
NP-40 buffer (Nonidet-P40) [Harsh]	
Component	**Concentration or percentage**
NaCl	150 mM
Tris-HCl (pH 7.5)	10 mM
EDTA	1 mM
Igepal	0.65%
RIPA (RadioImmunoPrecipitation assay) buffer	
Component	**Concentration or percentage**
NaCl	150 mM
Tris-HCl (pH 7.5)	50 mM
Triton X-100	1 %
Deoxycholic Acid	0.5%
SDS	0.1%

Methods

4.1 Mammalian cell culture and transfection

Rat pituitary GH_3 cells were cultured and maintained in F-10 media containing 10% horse serum (HS), 2.5% fetal bovine serum (FBS) and 1% penicillin-streptomycin-glutamine solution (PSG) as described previously [21]. HEK293T cells were grown in Dulbecco's Modified Eagle Medium (DMEM) containing 10% newborn calf serum (NCS) and 1% PSG [22].

Sub-culturing or passaging of the cells was done when the cells reached their mid-logarithmic phase, at which time the cells were about 70-80% confluent.

Treatment with either 50mM or 25 mM KCl was done for at least 3 hours to depolarize the GH_3 cells. There was no apparent change in the cell morphology after KCl treatment. To detect acetylation of the hnRNP LL protein, 5μM of trichostatin A (TSA) was used.

For transfection of plasmids into the cells, overnight cultures of HEK293T were used and transfection was done using Lipofectamine 2000 (Invitrogen) reagent following the protocol supplied by the manufacturer. Transfection was done when the cells were about 80-85% confluent. For minigene assay, 22 hours transfection was done. For all other cases, at least 24 hours transfection was done before the cells were harvested.

4.2 Cytoplasmic RNA preparation

To extract cytoplasmic RNA, the established protocol in our lab was followed [132]. The cells were spun down and afterwards washed with ice-cold PBS containing

1mM EDTA for two times. Then the cells were spun down, resuspended in NP-40 buffer (recipe is described in the materials part) and kept on ice for 5 minutes. Then the samples were spun at 14,000 rpm for 2 minutes and the supernatant was transferred into new tubes for cytoplasmic RNA preparation while the pellet was resuspended in RIPA buffer containing the phosphatase and proteinase inhibitors, sonicated and kept for further western blot analysis. As for the supernatant, lysis solution with β-mercaptoethanol and 100% ethanol were added and applied to the QIAquick spin column and centrifuged at 14,000 rpm for 1 minute. Then the column was washed once with PE buffer containing ethanol and twice 75% ethanol and at the last step water is applied to the column and centrifuge at 14,000 rpm for 1 minute to collect the cytoplasmic RNA. The concentration and quality of the RNA were then checked by spectrophotometer.

4.3 Reverse transcription-PCR

Semi-quantitative RT-PCR was done according to the previously described procedure [20, 21]. Briefly, about 200 ng of RNA, M-MLV Reverse Transcriptase (Life Technologies), SUPERase• In™ RNase Inhibitor (Ambion) and Oligo(dT)18 primers were used in a 10 μL reverse transcription reaction. After that, 1 μL from the RT product was used in 12.5 μL PCR reaction for 28 cycles at an annealing temperature of 60°C. Then the PCR products were run on a 2.5% agarose gel and bands were visualized from Alpha Innotech gel documentation systems by using the FluorChem 8900 software. The intensities of the obtained bands were then quantified using ImageJ software (NIH).

4.4 Immunoblotting

Immunoblotting was done according to the procedure described previously [132]. In all the immunoblotting experiments, 1 mM Na_3VO_4 and 1mM NaF were mixed with milk suspension in order to inhibit the activity of protein phosphatases. For stripping the western blot membrane, eraser buffer (2% SDS, 62.5 mM Tris-HCl (pH 6.8) and 100 mM β-mercaptoethanol) was used. Briefly, the membrane was put into western blot pouch and then eraser buffer was added and incubated at 50°C for 1 hour. Then the membrane was rinsed twice in TBS buffer for 10 minutes each time. Blocking was done by putting the membrane in milk suspension for 2.5 hours and then standard immunoblotting procedure was followed.

4.5 Immunoprecipitation

Immunoprecipitation was done according to the method described previously [21] except that the beads incubated with antibody and the cell lysates were washed five times with RIPA buffer (containing 150 mM NaCl) before gel run.

4.6 Phosphopeptide mapping

The two dimensional phosphopeptide mapping experiment was done according to the previously described procedure [21, 81, 133]. Briefly, the cells were spun down and washed with KRG buffer four times before they were resuspended thoroughly in KRG buffer; and then they were plated onto 6-well plates and incubated at 37°C, 5% CO_2 for 1 hour. Then ^{32}P orthophosphoric acid was added to the wells and swirled gently. For depolarization, KCl was added just after the addition of ^{32}P orthophosphoric acid. The cells were then incubated for 3-4 hours. The 6-well plate was then taken out carefully, put on ice and the cells were transferred to 2ml screw-cap tubes using filtered pipettes,

and then spun down with the caps wrapped with parafilm. After removing the KRG buffer, the cells were resuspended in RIPA buffer containing phosphatase- and proteinase-inhibitors. Then DNase was added and incubated for about 30 minutes and the samples were spun at 14,000 rpm for 15 minutes at 4°C. Then the supernatants were added onto pre-packed antibody-protein G beads mixture and incubated overnight at 4°C. For immunoprecipitation, anti-hnRNP LL was used for the endogenous hnRNP LL protein from GH_3 cells and anti-Myc for Myc-hnRNP LL/mutants in HEK293T cells. The next day, the samples were spun down using a horizontal centrifuge, the supernatants were removed and the beads along with protein-antibody complex was washed 5 times using RIPA buffer containing phosphatase inhibitors. Then the precipitates were boiled at 95°C for 5 minutes in SDS protein loading buffer and loaded on a 10% SDS-PAGE gel, and then transferred to a nitrocellulose membrane, which was air-dried briefly, wrapped in saran wrap and exposed to phosphorimager cassette. Then the plates were scanned and the band corresponding to the size of the protein of interest was cut and put into a 1.5 ml eppendorf tube. Then 500 ul of 0.5% PVP in 0.1 M acetic acid was added in each eppendorf tube containing the membrane strips and blocked at 37°C for 1 hour. The PVP was then removed, and the membrane strips were washed 5 times with 500 ul ddH_20 and one time with 500 ul 50 mM ammonium biocarbonate. Then 200 ul of 50 mM ammonium biocarbonate along with 20 ul of each TPCK-trypsin and TLCK-chymotrypsin (1 mg/ml in 0.1 mM HCl) was added to each tube and digested overnight at 37°C. The next day, the digest solutions were put into new tubes, and the strips were washed with 500 ul of ddH_20 and combined with the digestion solution so that the final volume of the tube is 700 ul (200 ul digestion solution + 500 ul

ddH_20). Then the peptides were dried in a Speedvac. After completion of the first round of speedvac, the peptides were resuspended in 500ul of ddH_20 and dried, which was repeated twice. Then the peptides were resuspended in 300 ul ddH_20, spun at 14,000 rpm for 2 minutes. This spin makes the debris to stay in the bottom, which will not form a very hard pellet, and then transfer of all but 10-20 ul of the phosphopeptides solution was done into a new tube. Speedvac was done again to dry the peptide solution. Then the peptides were resuspended in 9 ul of ddH_20 and load onto TLC plates. The loading spot was 1.2 cm from the bottom and 1.4 cm from the left side of the TLC plate. The spot was very faint and did not leave a hard-pressed print in the plate. The peptides were loaded at multiple times: 0.5 ul or less peptides were loaded at a time using a specialized tip without touching the TLC plate and the spot was dried each time after loading the peptides. This process was repeated until the entire sample was loaded. A very faint yellow line appears around the outline of the spot after drying. Then the plates were placed in the center of Hunter unit and a wet Whatman paper having a hole cut in the corner around the location of the loading spot was placed on top of the TLC plates to compress the loading sample to the spot. Then electrophoresis was done by running at 1000 V for 20 minutes with anode (+) on the left and the cathode (-) on the right. The samples will move from the left to the right. After electrophoresis, the plates were allowed to dry in the fume hood. Then the plates were placed inside a tank containing chromatography buffers and the chromatography step was allowed to proceed until the solvent buffer reaches ~1 to 0.5 cm from the top of the TLC plate. Then the TLC plates were removed from the tank and allowed to air-dry inside the fume hood. The edge of the plates were marked using some 32P containing solutions. The TLC plates were

wrapped and exposed to phosphorimager cassette and scanned afterwards to visualize the differential migration of the phosphopeptides.

4.7 Plasmid construction

Different plasmids were used during the course of this study. Some plasmids were already available in the lab and some had to be made. The plasmids such as Cppt2E-Myc-hnRNP LL, pLKO.1-shLL ,Flag-CaMKIV-dCT (CaMKIV), kinase dead mutant of CaMKIV (CaMKIVm) were already available in the lab [20, 21].

Three truncation mutants that have different RRM domains deleted as well as the plasmids containing point mutations in different positions in hnRNP LL were made following the mutagenesis PCR strategy. Briefly, this is a two-step PCR process. In the first step, PCR was done with the two mutation primers and their respective flanking primers with Phusion High-Fidelity DNA Polymerase (New England Biolabs). The samples were run on gel, cut and gel purified. In the next step, the gel purified DNA samples from first step PCR was used as primers for each other and amplified for the first 8 steps at 52 ° C and then the two flanking primers were added and the PCR was continued. Then the PCR products were cut by Sal I restriction enzyme and ligated back to digested Cppt2E vector.

For making shRNA resistant Ser308 mutant, one step long overlapping PCR was done. After that the PCR product was digested with Dpn I enzyme. This step ensures that only newly synthesized DNA is left in the tube. After that transformation was done and positive clones were sent and confirmed by sequencing.

Primers used during this study

Primer	Sequence
HhnRNPLL1A	GAATTCCGCGGGCCCGTCGACTTACTGTTGACCAGCAATGTACACG
HhnRNPLL2A	GAATTCCGCGGGCCCGTCGACTTAACCAGAGGGATTTCCTCCATGCATG
HhnRNPLL3A	GAATTCCGCGGGCCCGTCGACTTAATTCTTAGATGCTTGGCCAGCACTTG
Cppt2EseqF	CAGCCCTCACTCCTTCTCTAGGC
Cppt2EseqR	CAGCTGCCTTGTAAGTCATTGGTC
MycLLAgeIF	CTGAACCGGTAGCTGCGGAATTGTACCCGC
MycLLSalIR	CGATGTCGACTTATAAATGGGATGATGTAG
LLS308A F	TCGTTACAGAATGGATACACCTGAAC
LLS308A R	GCCCATTCTGTAACGACTTGG
mutant_LL_ATG F	CATTTAAACGACAGGCACTAGTTGAGTTTGAAAACATAGATAGTGC
mutant_LL_ATG R	CTATCTATGTTTTCAAACTCAACTAGTGCCTGTCGTTTAAATGGCATC
Oligo(dT)18	TTTTTTTTTTTTTTTTT

4.8 Production of phospho-Ser-308-specific antibody

Two synthetic peptides were made from the Alpha Diagnostics Inc. The control peptide (SRYRMGSRDTPEL) didn't have phosphophate group at Ser308 while the phosphopeptide (SRYRMG(p-S)RDTPEL) contained a phosphate group at the Ser308 residue. The antibody was generated by injecting the phosphopeptide with an immunogenic carrier into two rabbits and all these were done at the Alpha Diagnostics Inc. In total, ~50ml of anti-serum was obtained from two rabbits. The specificity of the antibody was confirmed by dot blot and western blot.

4.9 Immunostaining

Immunostaining was done following an established protocol of our lab [132] using anti-c-Myc primary antibody for Myc-hnRNP LL or its Ser308A mutant and anti-Flag antibody for CaMKIV or its mutant. To visualize the DNA, 4', 6-diamidino-2-phenylindole (DAPI) was used.

CHAPTER V

Results

5.1 HnRNP LL protein and RNA levels did not change upon depolarization in GH_3 cells

Regulation of splicing factors through cell signaling can happen at multiple levels. Some of the most commonly observed mechanisms include changes in the cellular level of splicing factors either by promoting their expression or down-regulation through protein degradation, post-translational modification as well as changes in the subcellular localization of the splice factors [11].

To elucidate how hnRNP LL is regulated, we first checked whether the protein level of hnRNP LL was changed upon depolarization. Immunoblot analysis showed that when a time course experiment was done the protein level of hnRNP LL did not change 3 and 6 hours after KCl treatment compared to the non-treated control samples from rat pituitary GH_3 cells (Figure 5A).

We then checked the gene expression of hnRNP LL upon depolarization by semi-quantitative RT-PCR and found that the hnRNP LL gene expression also did not change upon depolarization compared to the non-treated samples from GH_3 cells (Figure 5B, $p=0.35$).

Therefore, the RNA and protein levels of hnRNP LL are not altered upon depolarization, suggesting the involvement of other modes of regulation.

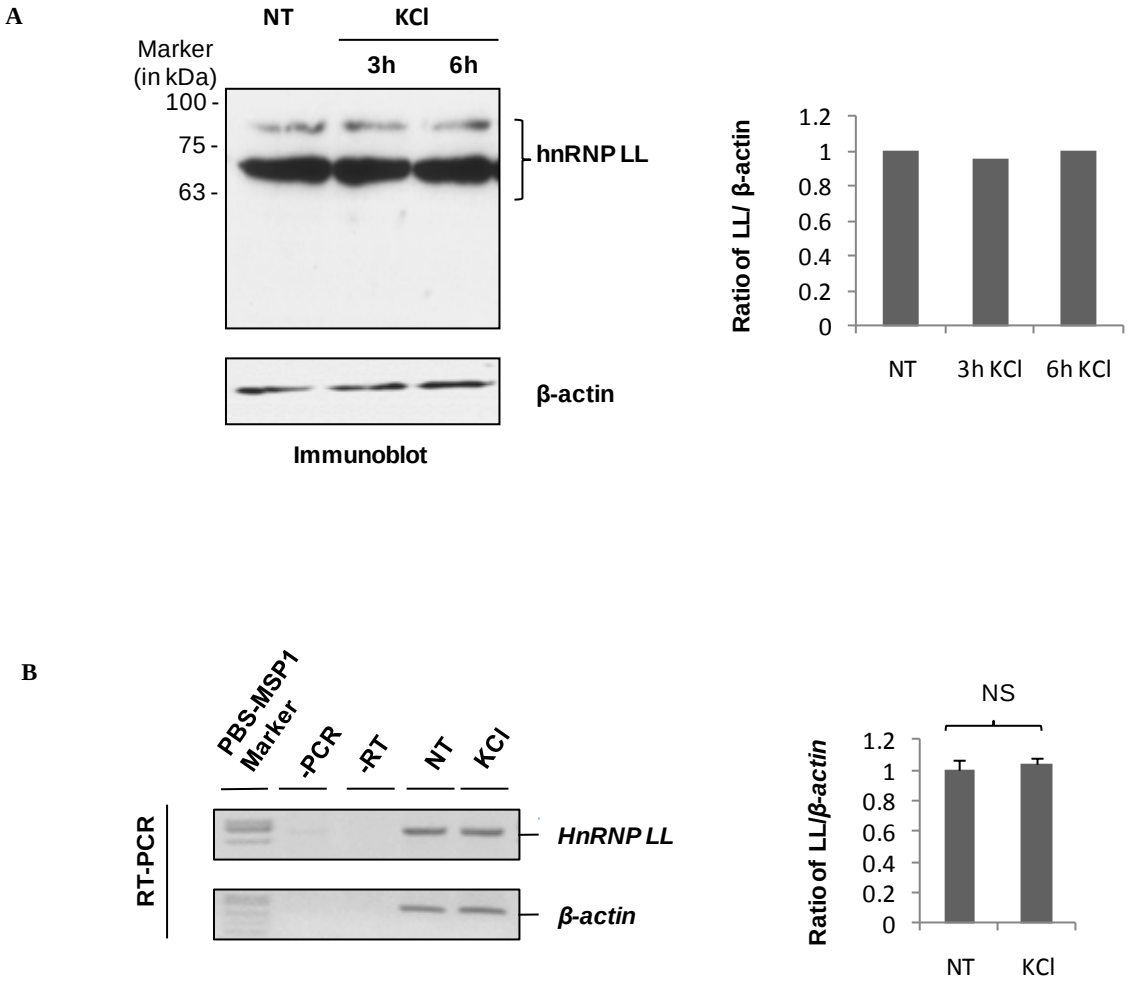

Figure 5: Assessment of the changes in hnRNP LL protein and RNA level upon depolarization in GH3 cells. A. Western blot from non-treated and KCl treated samples (3 and 6 hours after treatment) of GH_3 cells. Whole cell lysates were used in this experiment. Two bands (100 and 70 kD) were detected by the hnRNP LL antibody (Cell Signaling Technology®, catalogue # 4783). While the detection of 70 kD band was consistent in all the experiments, the 100 kD band was not always observed during the immunoblot experiments. **B.** Gene expression analysis of hnRNP LL upon KCl-mediated depolarization. The agarose gel image on the left side is showing the level of hnRNP LL and β-actin in the samples after 28 cycles of PCR. β-actin was used as an internal control. '-PCR' and '-RT' stands for the PCR and RT negative controls respectively. The band intensities were then quantified by ImageJ software and ratio of LL/β-actin was plotted in the bar diagram shown in the right panel (mean ± standard deviation, n=4, P=0.35).

5.2 Database search identified different types of hnRNP PTMs

Post-translational modifications have been shown to regulate the function of many hnRNP proteins. So we then carried out a database search to list all the known post-translational modification identified for hnRNP LL as well as all other hnRNP proteins. Table 4 (page 47) contains the list of canonical hnRNPs along with their post-translational modifications according to the PhosphoSitePlus® database [79]. Phosphorylation and ubiquitination is found in all of the hnRNP proteins listed, while acetylation and methylation occurs in 90% and 79% of the listed hnRNP proteins respectively. Other types of modifications like Succinylation (45%), SUMOylation (38%), Glycosylation (14%) and Caspase cleavage site (21%) are also observed. More importantly, we have found that the hnRNP LL protein undergoes three known types of post-translational modifications. These are phosphorylation, acetylation and ubiquitination (Table 4). This helped us to narrow down which of the post-translational modifications to be examined. We then proceeded to test the changes in the phosphorylation status of hnRNP LL upon depolarization.

5.3 HnRNP LL is phosphorylated upon depolarization

Phosphorylation is known to modulate the activity of many of the splicing factors including hnRNP L and PTB [21, 81]. Previous studies in our lab have shown that depolarization/CaMKIV-mediated phosphorylation of hnRNP L is critical for splicing regulation of the *Slo1* gene [21]. Since hnRNP LL is a paralogue of hnRNP L, it is possible that hnRNP LL is also phosphorylated upon depolarization and the phosphorylated form of hnRNP LL protein might be involved in different functions like the regulation of the alternative splicing of STREX exon.

Table 4: PTMs of hnRNP according to PhosphoSitePlus® database/literature search. The check mark symbol (✓) denotes the presence of that specific modification and the minus symbol (-) denotes not present/ not identified yet. Here, P= Phosphorylation, Me= Methylation, Ac=Acetylation, Ub= Ubiquitination, Sm = SUMOylation, Sc= Succinylation, Gly= Glycosylation and Ca = Caspase cleavage site.

Protein	**P**	**Me**	**Ac**	**Ub**	**Sm**	**Sc**	**Gly**	**Ca**
HnRNP A0	✓	✓	-	✓	-	-	-	-
HnRNP A1	✓	✓	✓	✓	✓	✓	-	-
HnRNP A1-Like 2	✓	✓	✓	✓	-	-	-	-
HnRNP A2/B1	✓	✓	✓	✓	✓*	✓	-	-
HnRNP A3	✓	✓	✓	✓	✓	-	-	✓
HnRNP A/B	✓	✓	✓	✓	-	✓	-	-
HnRNP C	✓	✓	✓	✓	✓	✓	-	-
HnRNP C-Like 1	✓	-	✓	✓	-	-	-	-
HnRNP C-Like 2	✓	✓	✓	✓	-	-	-	-
HnRNP C-Like 3	✓	-		✓	-	-	-	-
HnRNP D0	✓	✓	✓	✓	-	✓	-	✓
HnRNP D-Like (HnRNP DL)	✓	✓	✓	✓	✓	-	-	-
HnRNP E1	✓	-	✓	✓	-	-	-	✓
HnRNP F	✓	✓	✓	✓	✓	✓	-	-
HnRNP H1	✓	✓	✓	✓	✓	✓	✓	-
HnRNP H2	✓	✓	✓	✓	-	-	-	-
HnRNP H3	✓	✓	-	✓	-	-	-	-
HnRNP G	✓	✓	✓	✓	-	✓	✓*	-
HnRNP I	✓	-	✓	✓	-	✓	✓	-
HnRNP K	✓	✓	✓	✓	✓	✓	-	-
HnRNP L	✓	✓	✓	✓	-	✓	-	-
HnRNP L-Like (HnRNP LL)	✓	-	✓	✓	-	-	-	-
HnRNP M	✓	✓	✓	✓	✓	✓	-	-
HnRNP P2	✓	✓	✓	✓	-	-	✓	✓
HnRNP Q	✓	✓	✓	✓	-	-	-	-
HnRNP R	✓	✓	✓	✓	-	-	-	-
HnRNP U	✓	✓	✓	✓	✓	✓	-	-
HnRNP U-Like 1	✓	✓	✓	✓	✓	-	-	✓
HnRNP U-Like 2	✓	-	✓	✓	-	-	-	✓
Percentage	**100%**	**79%**	**90%**	**100%**	**38%**	**45%**	**14%**	**21%**

*Not found in *PhosphoSitePlus*®

To determine if hnRNP LL is phosphorylated, *in vivo* labeling of rat pituitary GH_3 cells was done by culturing cells in a phosphate-free medium supplemented with ^{32}P-orthophosphoric acid. Immunoprecipitation using hnRNP LL antibody showed that the endogenous hnRNP LL is a phospho-protein as it was labeled with ^{32}P-orthophosphate (Figure 6). More interestingly, the phosphorylation status of the protein changed upon depolarization. An increase in the level of phosphorylation signal of hnRNP LL was observed upon depolarization (by treating with 50 mM KCl for 3 hours) compared to that of non-treated (non-depolarized) samples (Figure 6), suggesting that the protein phosphorylation is increased upon depolarization in rat pituitary GH_3 cells.

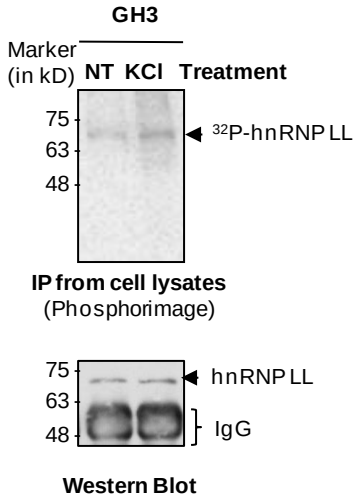

Figure 6: Metabolic labeling of the endogenous hnRNP LL in rat pituitary GH_3 cells reveals it as a phospho-protein. Phosphorimage of immunoprecipitated hnRNP LL protein from non-depolarized and depolarized (KCl-treated) GH_3 cell extracts (using the same amount of cell lysate and hnRNP LL Antibody). There is a slight increase in the phosphorylation signal in the KCl-treated sample compared to the non-treated sample. The figure below shows western blot by probing the membrane with Anti-hnRNP LL antibody where the protein loading seems to be almost the same for both the samples. A band corresponding to the heavy chain of 'Ig' also showed up in the bottom as the same antibody was used for both immunoprecipitation and western blotting.

To determine whether there are any differentially phosphorylated peptides between the depolarized and non-depolarized samples of hnRNP LL, we performed 2D-phosphopeptide mapping. Briefly, endogenous hnRNP LL protein from 32-P labeled non-treated and depolarized GH_3 cell lysates were first pulled down by immunoprecipitation, run on a 10% SDS-PAGE gel and transferred to a nitrocellulose membrane (Figure 7). The band corresponding to the size of hnRNP LL protein (~70 kD) was cut from the membrane followed by proteolytic cleavage using L-1-Tosylamide-2-phenylethyl chloromethyl ketone (TPCK) treated trypsin and 1-chloro-3-tosylamido-7-amino-2-heptanone (TLCK) treated chymotrypsin enzymes. The digested peptides were loaded onto TLC plates and subjected to electrophoresis followed by chromatography (Figure 7). We observed the presence of one major spot along with one minor spot just below the major spot in case of the depolarized sample (shown by arrow marks in Figure 7) both of which were not as distinct in the non-treated samples, suggesting their increased phosphorylation upon depolarization in GH_3 cells (Figure 7).

5.4 Phosphopeptide mapping using the truncation mutants identified RRM2 and its linker region as a potential CaMKIV-target

We next searched the PhosphoSitePlus® database (http://www.phosphosite.org) and found more than 15 phosphorylation sites of hnRNP LL as determined by the mass-spectrometry approach (Figure 8A). In addition to these sites there might be some other unknown sites of phosphorylation. So rather than making individual point mutations, we proceeded by making different truncation mutants to narrow down the potential site(s) of phosphorylation.

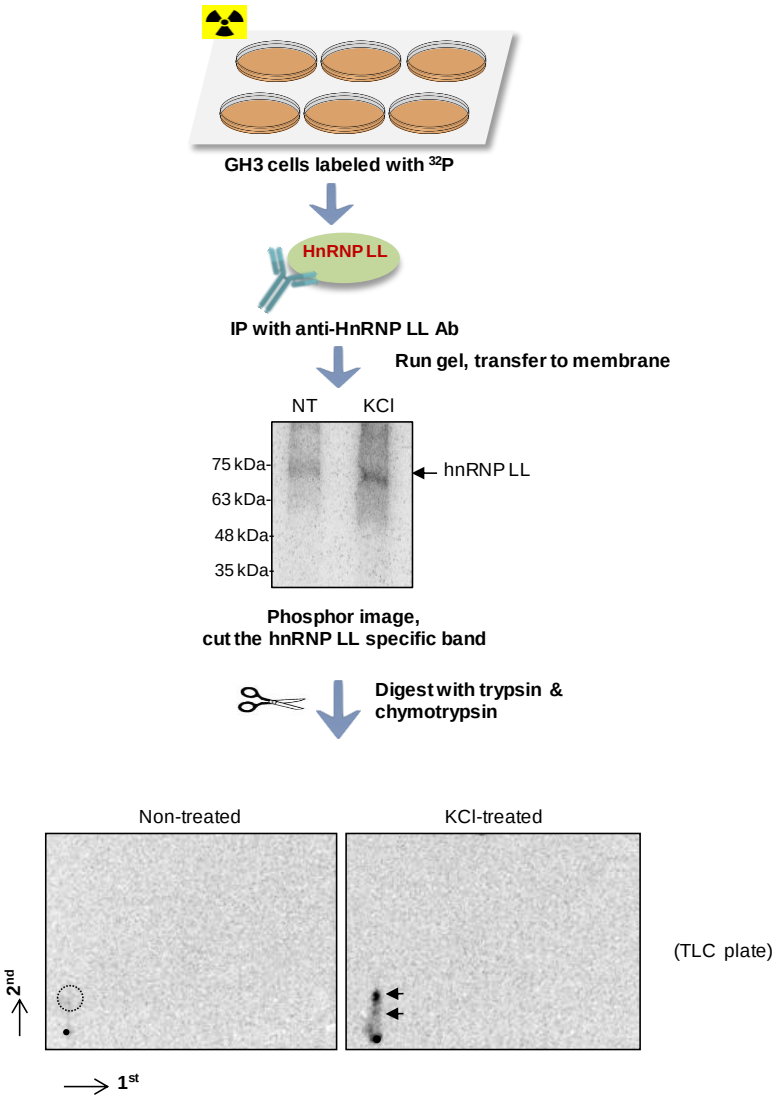

Figure 7: Schematic diagram of 2D-Phosphopeptide mapping experiment. The 32-P labeled hnRNP LL protein from non-treated and KCl treated samples of GH_3 cells were used in this experiment. In case of the KCl-treated samples, the top arrow indicates the major phosphopeptide which has a distinct spot while the bottom arrow indicates the presence of a weaker spot just below the major phosphopeptide both of which are distinct for depolarized sample compared to the non-treated sample. The sequence and direction of the two dimensions are as marked beside. The black dot at the bottom left corner of the plates marks the loading spot.

HnRNP LL is a modular protein that has four highly structured globular RNA recognition motifs (RRMs). The truncation mutants were constructed by deleting different RRM domains. The Myc-tagged full length hnRNP LL plasmid was previously made in the lab and is described in a previous paper of the lab [21]. Using primers designed from the linker regions, I made three different truncation mutants with different RRMs being deleted (Figure 8B). The mutants were first confirmed by sequencing. Then the mutant plasmids were transfected in HEK293T cells and their expression was confirmed by western blotting using c-Myc-antibody where different bands correspond to the proteins encoded by the truncation mutants (Figure 8C).

Next, another round of phosphopeptide mapping experiment for wild type hnRNP LL and its mutants was performed in HEK293T cells. From the previous studies of the lab, it was known that CaMKIV is the major kinase that plays role in this pathway by phosphorylating hnRNP L. Since HEK293T cells do not express CaMKIV, which is the major kinase involved depolarization-induced splicing regulation [20], we used a plasmid that encodes a constitutively active CaMKIV kinase to phosphorylate hnRNP LL and its truncation mutants in these cells. The immunoprecipitated proteins (wild type and mutants) were subjected to phosphopeptide mapping and the spots obtained from the 2D-TLC plates of the Myc-hnRNP LL and its truncation mutants were compared with the phosphopeptide map of endogenous hnRNP LL of depolarized sample of GH_3 cells. As a control, a kinase inactive mutant of CaMKIV (designated as CaMKIVm) was co-transfected with the full length Myc-hnRNP LL protein. Figure 8D shows the phosphopeptide maps obtained from these samples.

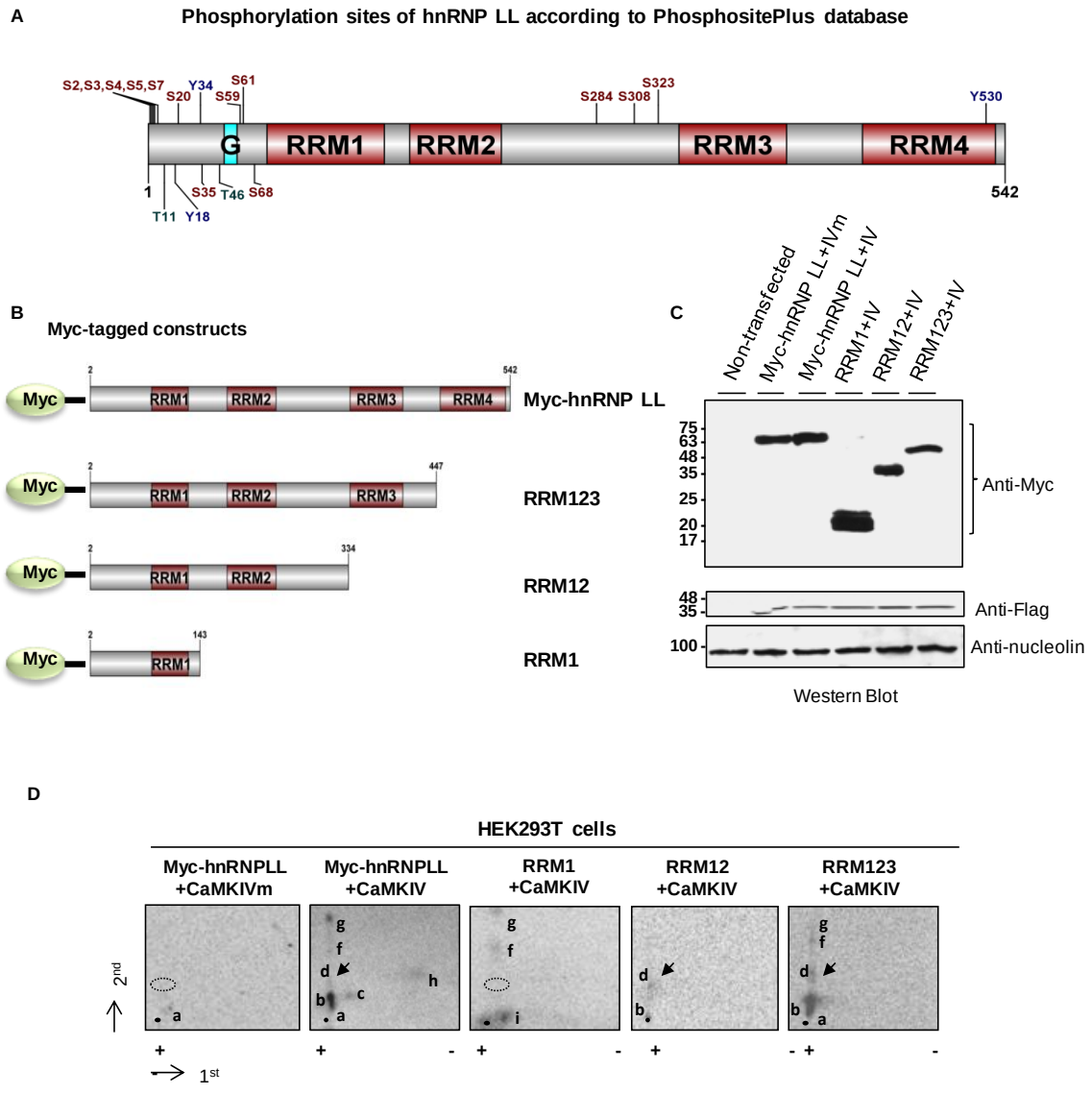

Figure 8: Phosphopeptide mapping using the truncation mutants. A. Known phosphorylation sites of the hnRNP LL proteins as found at the PhosphoSitePlus® database. **B.** Schematic representation of the Myc-tagged wild type and truncation mutants of hnRNP LL. **C.** Western blotting to confirm the expression of proteins by the truncation mutants. Anti-nucleolin was used as a control. **D.** 2D-phosphopeptide mapping of ^{32}P-labeled c-Myc-hnRNP LL and its different truncation mutant proteins co-expressed with either constitutively active CaMKIV or kinase inactive CaMKIV mutant proteins in HEK293T cells. The phosphopeptide that migrated at the similar location like that of the major phosphopeptide from the depolarized sample of GH_3 cell lysates, indicated by the arrow, showed up in full length Myc-tagged hnRNP LL (spot 'd') and also in the truncation mutants RM12 and RRM123, but not in RRM1. The sequence and direction of the two dimensions are as marked beside the first panel. The black dot at the bottom of the plates marks the loading spot.

It seems that the full length Myc-tagged-hnRNP LL co-expressed with CaMKIV in HEK293T cells showed several spots in the TLC plate which also included a phosphopeptide (spot 'd') that had similar migration like that of the phosphopeptides from endogenous hnRNP LL in GH_3 cells in the vertical axis. For the truncation mutant that had only RRM1, spot 'd' was absent. However, both RRM12 and RRM123 containing plasmids showed a spot that migrated in the same location as spot 'd'. Taken together, these observations suggest that the depolarization/CaMKIV-induced phosphopeptide is likely located in the region covering RRM2 and its flanking linkers.

5.5 Evolutionarily conserved Ser308 of hnRNP LL is a target of depolarization/CaMKIV-induced phosphorylation

Next we searched for the potential CaMKIV targets with the RRM2 and its flanking linker region and found that there are two potential sites within CaMKIV consensus (Arg/Lys-x-x-Ser/Thr), Ser308 and Thr154. We mutated these two sites to alanine and another round of phosphopeptide mapping was done by cotransfection of each of the mutants with CaMKIV in HEK293T cells (Figure 9A & 9B). In addition, phosphopeptide mapping for depolarization induced endogenous hnRNP LL from GH_3 cells was conducted in parallel to compare the spots obtained from the mapping of the mutant proteins. It seemed that the spot 'd' is abolished in the S308A mutant while the T154A mutant retained the spot (Figure 9C), suggesting that S308 might be the possible site of phosphorylation. To confirm the exact identity of spot 'd' that is abolished in S308A mutant in HEK293T cells, we mixed peptides of endogenous hnRNP LL from depolarized GH_3 cells along with the peptides obtained from the T154A mutant in HEK293T cells; and then the mixed peptides were subjected to another round of 2D-

phosphopeptide mapping. Then we quantified the intensity of the target (spot 'd') by ImageJ before and after mixing the two samples; and compared the ratio of the spot intensity with another spot (spot 'f') on the plate whose intensity didn't change before and after mixing the two samples. The ratio of f/d changed from 0.301 to 0.0839. It thus seems that the spot 'd' mixed with phosphopeptides from the endogenous hnRNP LL from depolarized samples (Figure 9C). However, from the migration pattern of the phophopeptides it seems more likely that spot 'd' corresponds to the relatively weaker phosphopeptide of the endogenous hnRNP LL from depolarized samples. Taken together, these observations suggest that even though Ser308 is a potential site where phosphorylation may takes place, it is probably not the major phosphorylation site in depolarized sample from GH_3 cells.

In addition, we also tested the migration pattern of the peptides of hnRNP LL using an online tool known as '2D-Mobilty Plot', where one can calculate the migration coefficient of the phosphopeptides digested by specific proteolytic enzymes. We compared the output generated from the '2D-Mobilty Plot' tool to the experimental data obtained from the peptide maps. The peptide composition of the Ser308-containing peptide remains the same when cut by either trypsin alone or a combination of trypsin (cleavage after K/R) and chymotrypsin (cleavages after F/W/Y). This allowed us to compare our experimental results to that of computer-generated prediction. From the prediction it seems that the Ser308 containing peptide mainly migrates in the vertical axis and it has a retention factor (Rf) value of 0.366 which is similar to that of the Rf value (0.32) of spot 'd'. This suggests that spot 'd' more likely contains Ser308 containing phosphopeptides.

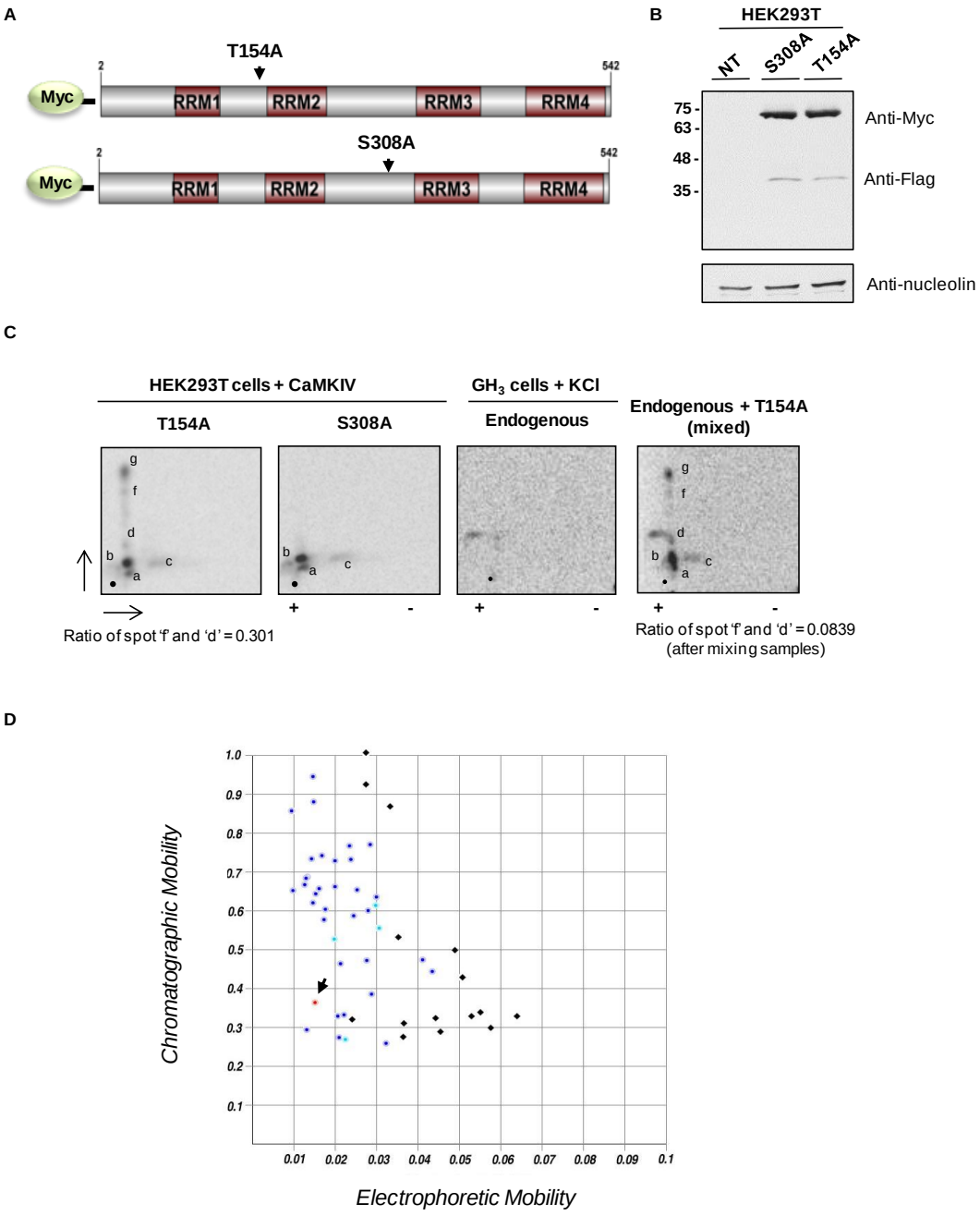

Figure 9: Phosphopeptide mapping using point mutations of hnRNP LL. A. Two potential CaMKIV consensus site within the region covering RRM2 and its flanking linker regions are Ser308 and Thr154 which were mutated. **B.** Western blot showing the expression of the mutant proteins in HEK293T cells. **C.** 2D- phosphopeptide mapping of mutants coexpressed with CaMKIV in HEK293T cells (plate 1 and 2 from the left), endogenous hnRNP LL from GH_3 cells and the last TLC plate from the left side contains a mixture of peptides from T154 and endogenous hnRNP LL from GH_3 cells . **D.** *In silico* determination of the mobility of the Ser308 containing peptide when digested by trypsin which shows a similar migration pattern as spot 'd'.

Since Ser308 phosphorylation is induced by depolarization, we went on to do further studies on this site. We then constructed a multiple sequence alignment of hnRNP LL proteins from thirty species and found that the Ser308 as well as its CaMKIV consensus site remained highly conserved throughout the course of evolution of these species (Figure 10). Such evolutionary conservation suggests for a possible functional significance of this site.

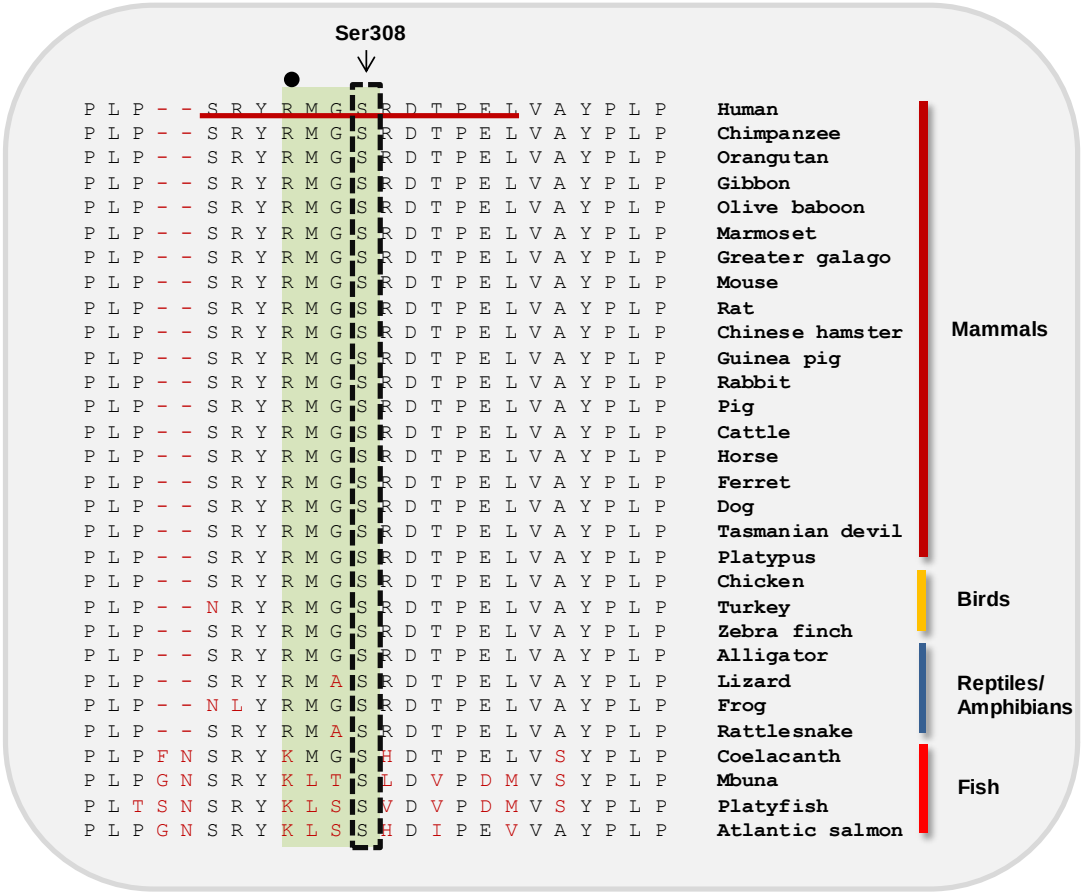

Figure 10: Alignment of hnRNP LL proteins of different species. The Ser308 of hnRNP LL protein is highly conserved in thirty different species ranging from fish to higher mammals. The alignment shown here constitutes amino acid sequences of hnRNP LL around the Ser308 residue in different species where the Ser-308 residue is shown within a *box of dotted lines*. The CaMKIV consensus sequence (Arg/Lys-X-X-Ser/Thr) is shown within a *green box*. Amino acids with similarity across the different species are shown in *black text color* whereas those with differences are shown with *red color*. The *red color heavy line* indicates the region used to synthesize the peptide for generating the

anti-pSer-308 antibody, which is also conserved from human to alligator. Shown at the right hand side of the alignment are the common names of the species. The binomial nomenclatures and NCBI accession numbers (shown within brackets) are as follows: Human, *Homo sapiens* (NP_612403); Chimpanzee, *Pan troglodytes* (BAK62427); Orangutan, *Pongo abelii* (XP_002812155); Gibbon, *Nomascus leucogenys* (XP_003262804); Olive baboon, *Papio anubis* (XP_003908576); Marmoset, *Callithrix jacchus* (XP_002757889); Greater Galago, *Otolemur garnettii* (XP_003795724); Mouse, *Mus musculus* (AAH12849); Rat, *Rattus norvegicus* (XP_233805); Chinese hamster, *Cricetulus griseus* (XP_003506620); Guinea pig, *Cavia porcellus* (XP_003473082); Rabbit, *Oryctolagus cuniculus* (XP_002709957); Pig, *Sus scrofa* (XP_003361415); Cattle, *Bos Taurus* (NP_001070368); Horse, *Equus caballus* (XP_001918049); Ferret, *Mustela putorius furo* (AER99845); Dog, *Canis lupus familiaris* (XP_003639639); Tasmanian devil, *Sarcophilus harrisii* (XP_003758397); Platypus, *Ornithorhynchus anatinus* (XP_001507993); Chicken, *Gallus gallus* (XP_414998); Turkey, *Meleagris gallopavo* (XP_003203845); Zebra finch, *Taeniopygia guttata* (XP_002199365); *Taeniopygia guttata* (XP_002199365); Alligator, *Alligator sinensis* (XP_006025000); Lizard, *Anolis carolinensis* (XP_003216160); Rattlesnake, *Crotalus adamanteus* (AFJ51180); Frog, *Xenopus (Silurana) tropicalis* (NP_001120904); Coelacanth, *Latimeria chalumnae* (XP_006007837); Atlantic salmon, *Salmo salar* (NP_001133491); Mbuna, *Maylandia zebra* (XP_004572082); Platyfish, *Southern platyfish* (XP_005812137).

5.6 Phospho-Ser308-specific antibody confirmed the physical presence of the Ser308 site upon depolarization:

Next phospho-Ser308-specific antibody was made to verify the physical presence of the endogenous Ser308 phosphorylation site. The antibody was generated from Alpha Diagnostic International Inc. against a synthesized phosphopeptide (Figure 11A). The specificity of the antibody was first validated by ELISA and dot blot from control (SRYRMGSRDTPEL) and phosphor-Ser308 containing peptide (SRYRMG(p-S)RDTPEL) that was used to generate the antibody. The dot blot showed higher phosphorylation signal in case of the phospho-308 containing peptide compared to the control peptide (data not shown). Interestingly, western blot showed a comparatively stronger phosphorylation signal after 1 and 3 hours of KCl-induced depolarization in pituitary GH_3 cells compared

to the non-treated cell lysates of GH_3 cells (Figure 11B). Taken together, these observations suggest that the phosphorylation at Ser308 is likely to be induced by depolarization in GH_3 cells.

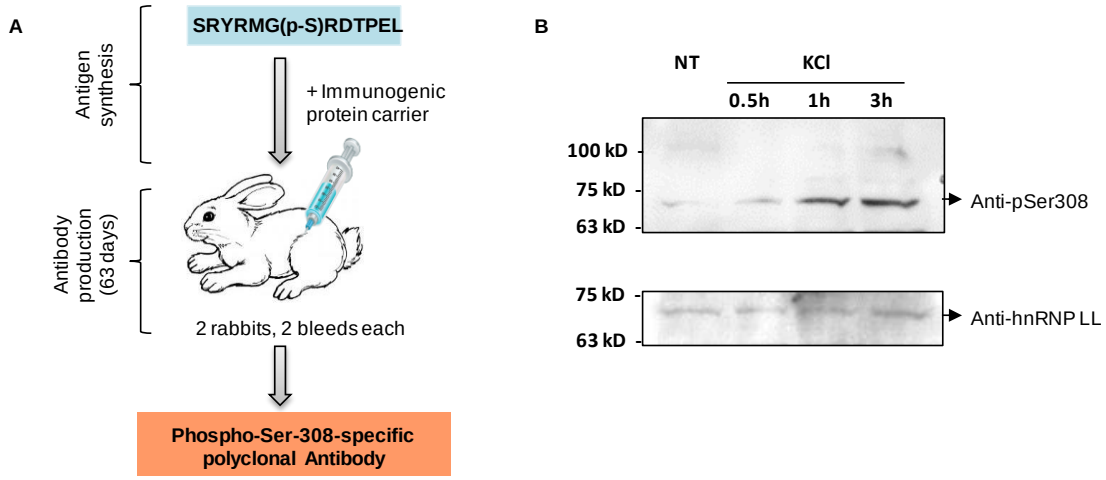

Figure 11: Phospho-Ser308-specific antibody. A. Schematic diagram depicting the steps for the generation of phospho-Ser308-specific antibody. **B.** Western Blot using the phospho-Ser308-specific antibody showed increased phosphorylation status 1 and 3 hours after KCl (25mM) treatment compared to the non-treated control sample. The figure below shows a western using the same membrane probed with hnRNP LL antibody, which is a protein loading control.

5.7 Ser308 plays a role in the perinucleolar localization of hnRNP LL in the presence of CaMKIV

We then investigated the functional effect of Ser308 phosphorylation. Mutation in the phosphorylation sites of many of the hnRNP proteins have been reported to have effect on their localization [81]. Therefore, we tested to see if the localization of the hnRNP LL protein changes due to the Ser308 mutation. First, Myc-tagged hnRNP LL proteins were cotransfected with Flag-tagged CaMKIVm plasmid (Figure 12). It seemed that 61% (43 out of 70) cells that have Myc-staining also showed the staining for anti-Flag antibody in

HEK293T cells. For this experiment only, the amount of Flag-tagged CaMKIVm plasmid doubled the amount of Myc-tagged hnRNP LL. However, for all other experiments, the amount of CaMKIV or its mutant plasmid transfected was always three times more than the Myc-tagged hnRNP LL or its mutants. This would help to increase the efficiency of the cotransfection.

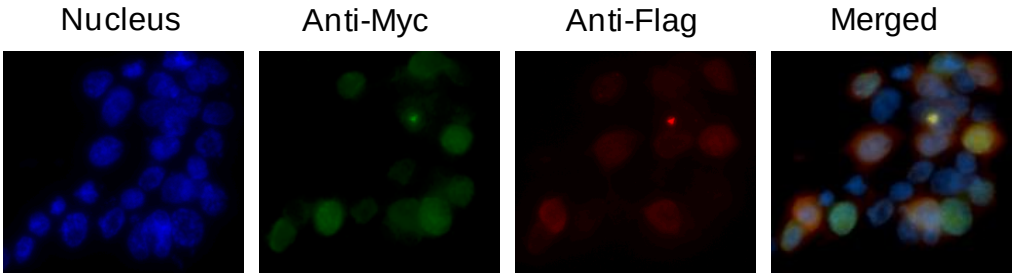

Figure 12: Cotransfection of Myc-tagged hnRNP LL protein with Flag-tagged CaMKIVm plasmid in HEK29T cells.

Next, the Myc-tagged wild-type hnRNP LL or its Ser308 mutants were coexpressed with either CaMKIV or its kinase dead mutant in HEK293T cells and their localization pattern was observed by immunostaining with anti-Myc antibody (Figure 13). This time the amount of Flag-tagged CaMKIV or its mutant used was three times of the amount of Myc-tagged hnRNP LL and its mutant. We observed that both the Myc-tagged hnRNP LL and its Ser308A mutant showed some differences in their nucleocytoplasmic distribution when cotransfected with CaMKIV or its mutant (Figure 13A to 13D). The top bar graph in the right side of Figure 13 shows the percentage of cells with more cytoplasmic staining in the four different groups of transfected cells. A small percentage (<4%) of the wild type hnRNP LL cells showed predominantly cytoplasmic staining but without any significant difference between the CaMKIV or IVm cotransfected cells (Figure 13, top bar graph, two bars on the left). When Ser308A mutant was co-transfected

with CaMKIVm, the cell population with predominantly cytoplasmic staining was increased to about 13% (Figure 13C bottom panel of the immunostaining), suggesting that the Ser308 is critical for the nuclear localization of hnRNP LL. Interestingly, the increase in cytoplasmic localization was reversed by CaMKIV cotransfection, suggesting that phosphorylation of the other CaMKIV target sites compensated the effect of Ser308A mutation on the localization. The major staining for all four groups of transfected cells was in the nuclear region (Figure 13). However, there are differences in their intranuclear localization. When wild-type hnRNP LL was cotransfected with CaMKIVm, the distribution of Myc-signal was homogenous throughout the nucleus. However, in case of Myc-hnRNP LL+ IV, predominantly a more perinucleolar staining of the Myc-hnRNP LL protein was observed (Figure 13, bottom bar graph). The cotransfection with CaMKIV kinase, in this case, changes the intranuclear localization of the protein. However, when hnRNP LL is mutated at Ser308 the protein no longer showed predominant perinucleolar staining even after cotransfection with CaMKIV (Figure 13C & D). When Ser308 was cotransfected with CaMKIVm, it also showed staining throughout the nucleus but the staining was not predominant in the perinucleolar region. Taken together, these observations suggest that the Ser308 is critical for the perinucleolar staining of the hnRNP LL protein in the presence of CaMKIV. Moreover, it is also critical for limiting the hnRNP LL protein inside the nucleus. However, to confirm the perinucleolar localization of Myc-LL+IV, a marker for perinucleolar staining like PTBP1 is needed. (My colleague in the lab Dr. Wenguang Cao performed this experiment)

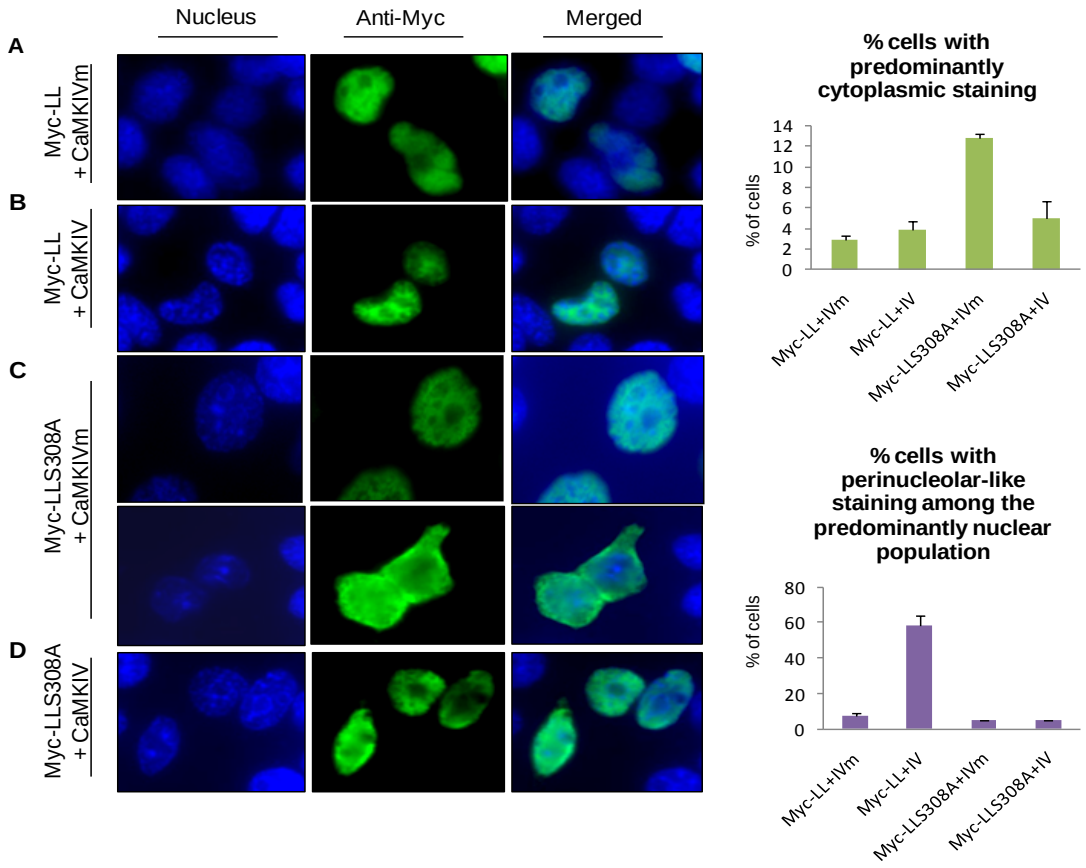

Figure 13: Localization of hnRNP LL and its mutant. Representative images of Myc-tagged hnRNP LL cotransfected with either CaMKIVm **(A)** or CaMKIV (**B**), Myc-tagged Ser308A mutant cotransfected with either CaMKIVm (**C**) or CaMKIV (**D**) captured by fluorescent microscope after immunostaining using Myc antibodies. In most of the cells, the protein mainly localizes in the nucleus. For MycLLS308A+ CaMKIVm cotransfected cells, two panels are shown (in C) where the top panel shows that they are also predominantly nuclear. However, some of the cells in this category also showed predominantly cytoplasmic staining (bottom panel in C). The top bar graph in the right panel shows the percentage of cells with predominantly cytoplasmic staining among different groups of cells. The bar graph at the bottom shows the percentage of cells within the nuclear population that shows more perinucleolar staining within the nucleus.

5.8 Ser308A mutation affects splicing repression in a minigene reporter assay

We next investigated the effect of the Ser308A mutant on splicing by using the DUP175ST splicing reporter. This reporter plasmid was previously described where 3' splice site of the middle exon is replaced with the sequences from the 3' splice site of

STREX exon [20]. The reporter plasmid was cotransfected with two more plasmids in the following combinations: wild type hnRNP LL (Myc-LL) with either CaMKIV (IV) or CaMKIV (IVm), Ser308A mutant (Myc-LLS308A) either CaMKIV (IV) or its mutant (IVm) plasmid in HEK293T cells using lipofectamine reagent. Then semi-quantitative RT-PCR was done and splicing changes were quantified using ImageJ. It seems that inclusion of the middle exon is significantly repressed when the minigene reporter is coexpressed with Myc-LL+IV, Myc-LL+IVm and Myc-LLSer308A+IV compared to the DUP175ST reporter only sample (Figure 14C; $n=3$, $p<0.05$). However, the group of cells containing Myc-LLSer308A+IVm could not significantly repress the inclusion of the middle exon in the minigene reporter (Figure 14C). This suggests a possible role for Ser308A mutant in splicing of the DUP175ST minigene reporter. More specifically, Ser308A abolishes splicing repression in the minigene reporter. On the other hand, cotransfection of Myc-LLS308A with active CaMKIV changed exon repression pattern compared to the DUP175ST only control, which suggests that CaMKIV phosphorylates some other sites than Ser308 which likely compensates for revival of splicing repression. Previously it was observed that overexpression of CaMKIV alone can cause splicing repression of DUP175ST reporter [20]. However, in the previous studies, hnRNP LL was not overexpressed along with CaMKIV. It is possible that the splicing repression by CaMKIV may take place through hnRNP LL and it cannot show further repression when hnRNP LL is overexpressed. This might be a reason why individual splicing repression effect of CaMKIV beyond hnRNP LL expression was not observed. It should be noted that this minigene assay is a one shot experiment done in triplicates. To confirm the effect of Ser308A mutant on the minigene reporter, more experimental repeats are necessary.

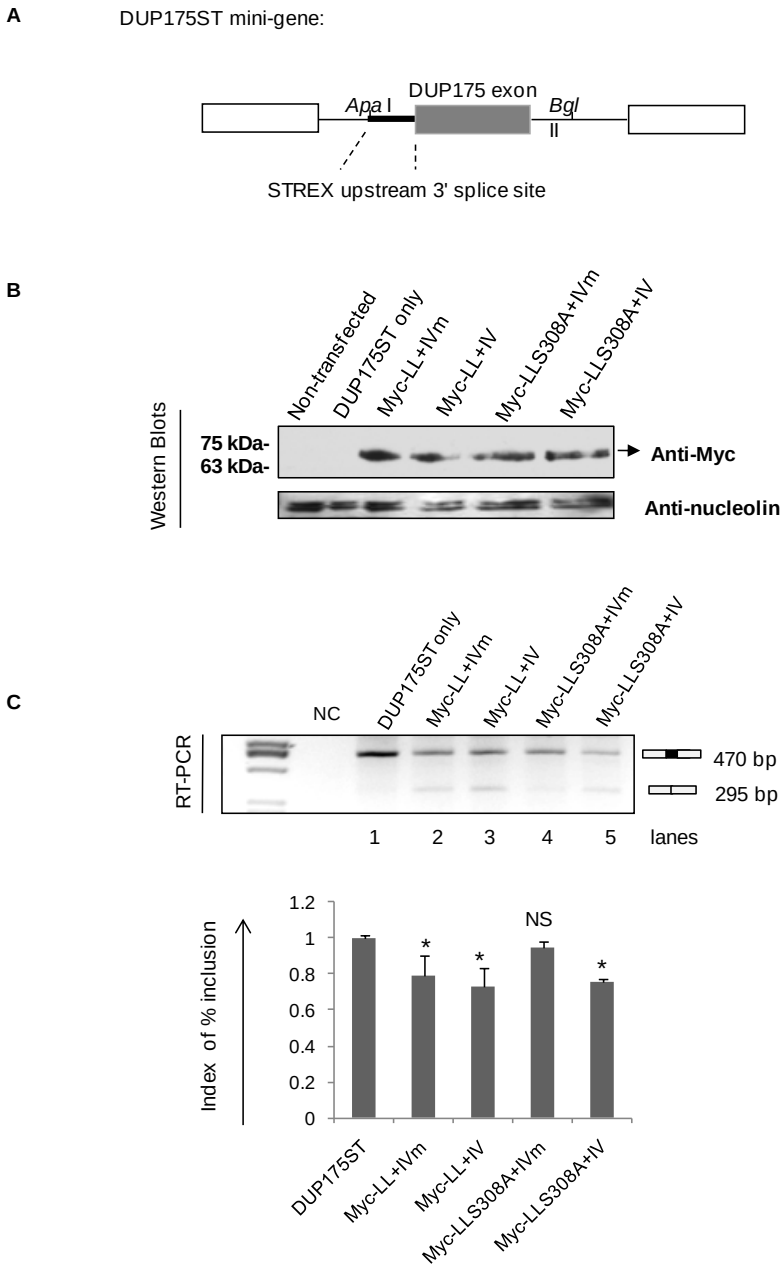

Figure 14: Effect of Ser308 mutation on splicing using DUP175ST minigene reporter. **A.** Diagram of DUP175ST reporter plasmid. **B.** Western blot showing the expression of Myc-LL and Myc-LLSer308A proteins coexpressed with CaMKIV or its kinase dead mutant (CaMKIVm). Here, nulceolin is used as control antibody. **C.** Agarose gel showing the RT-PCR products of the DUP175ST splicing reporter. The panel below shows the bar graph of the relative changes in the middle exon inclusion in the PCR products of the reporter assay (mean ± SD, n = 3) normalized to DUP175ST only lane. Except the S308+IVm (lane 4), the other three lanes (lane 2, 3 and 5) showed significant change in splicing compared to DUP175ST only (lane 1). *, $p<0.05$; two tailed student's *t* test. Here, NC: negative control for PCR.

The figure below shows a summary of observations from this study:

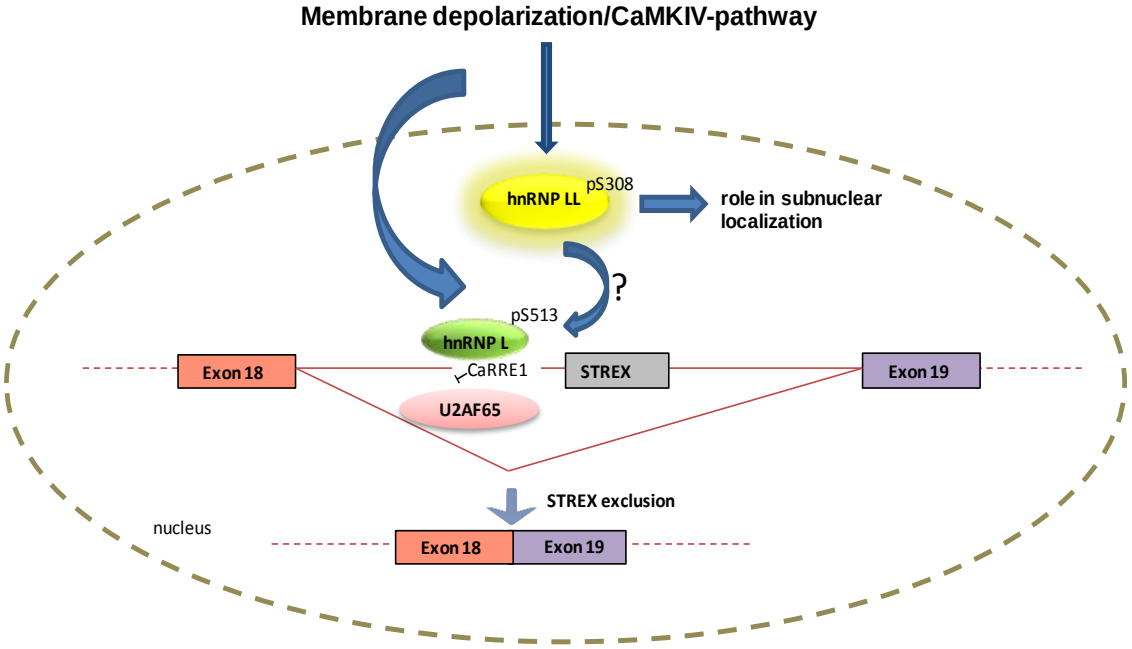

Figure 15: Summary of the regulation of hnRNP LL by depolarization/CaMKIV and the possible functional significance of the Ser308 phospho-amino acid. Membrane depolarization/CaMKIV-pathway mediates phosphorylation at Ser308, which is essential for perinucleolar localization of the hnRNP LL protein. In minigene splicing reporter assay, Ser308A mutation almost abolished the exon repression pattern when expressed with a kinase dead mutant of CaMKIV suggesting its possible role in nucleocytoplasmic location and splicing. However, the exact role of Ser308 site in STREX splicing needs to be confirmed by future loss-of-function of hnRNP LL followed by rescue experiments in GH_3 cells.

CHAPTER VI

Discussion

Post-translational modification adds an extra layer of complexity to the repertoire of proteins. Like other proteins, the hnRNPs themselves can also undergo different types of post-translational modification of which the most common are phosphorylation, ubiquitination, SUMOylation, methylation, acetylation and glycosylation. These modifications play roles in different cellular processes like the regulation of hnRNP functions [128] , their cellular localization [81, 85, 117] and formation of macromolecular complexes with other proteins and RNAs [92, 93]. So studying the different types of hnRNP PTMs is becoming increasingly important to decipher their regulatory roles in different cellular contexts. Large-scale identification of hnRNP PTMs has mostly been conducted by the mass-spectrometry approach. These studies have identified a myriad of PTMs on each members of the hnRNP family. However, the field is still lagging behind in linking these modifications to appropriate cellular function. Nevertheless, a great deal of effort has been made to study the functional significance of different hnRNP PTMs.

In this study, we have identified a phospho-amino acid (Ser308) of hnRNP LL, which is induced by depolarization/CaMKIV but probably is not the major target of the pathway. We further show that this site is important for perinucleolar localization of the protein when coexpressed with CaMKIV in HEK293T cells. In addition, minigene reporter assay showed that the mutation in Ser308 changes splicing repression pattern of the protein when cotransfected with a kinase dead version of CaMKIV.

Depolarization/CaMKIV-mediated phosphorylation of hnRNP LL:

Reversible protein phosphorylation, which plays critical role in the regulation of many cellular processes ranging from cell-cycle to signal transduction, is the most extensively studied post-translational modification. Previous estimation suggested that around 30% of the cellular proteins are covalently modified by phosphorylation [135]. More recent studies suggest that at least 70% of the cellular proteins can be phosphorylated [136]. There are several methods by which the phospho-amino acids of a protein can be identified. Mass-spectrometry (LC-MS/MS approach) is popular due to its rapid and high-throughput nature. However, identification of phosphoprotein is not always straightforward because of low stoichiometry of phosphorylation [137]. On the other hand, the identified phospho-amino acid needs to be mutated later on to determine the functional effect. Therefore, to avoid these issues, targeted mutagenesis experiments followed by 2D-phosphopeptide mapping were done to identify the depolarization/CaMKIV-mediated phosphorylation site(s) of hnRNP LL. This method is also very well-established in our lab and have been used previously to identify a phospho-amino acid of hnRNP L that is critical for splicing regulation [21].

Even though the phosphopeptide mapping is a powerful approach for the identification of phosphorylation sites, we are aware of that it is not beyond any limitation. Apart from the hazards associated with the use of radioactive materials, it is time-consuming and has several critical steps and even a minor error in any of the steps may limit the identification of the phosphopeptide. To narrow down the region of potential depolarization/CaMKIV-mediated phosphorylation site of hnRNP LL, we first made three truncation mutants by cleaving the protein from different linker regions keeping the

individual RRM domains intact to minimize the effect of conformational changes of the mutants since the protein is modular and contains highly structured globular RRM domains. The RRM1 containing mutant showed more spots in the phosphopeptide map compared to the RRM12 mutant that have both RRM1 and 2 (Figure 8D). This might be due to changes in their conformation due to mutation and the phosphorylation sites are differentially exposed to the kinase. To overcome this limitations, mutagenesis of single amino acid of full length hnRNP LL followed by phosphopeptide mapping was done later on to confirm the possible phosphorylation site (Figure 9). It was observed that when Ser308 site was mutated the spot 'd' was abolished (Figure 9C). However, from the mixing experiment it was observed that the spot 'd' more likely corresponds to the weaker phosphopeptide of the hnRNP LL from depolarized GH_3 cells (Figure 9C). This implies that Ser308 is probably not the major target of depolarization/CaMKIV pathway. When Myc-LLS08A mutant was coexpressed with CaMKIV (Figure 14C), the middle exon of the DUP175ST minigene reporter was repressed which means that phosphorylation of some other site(s) of hnRNP LL may be critical for splicing repression. So the major phospho-amino acid induced by depolarization still needs to be examined.

CaMKIV is a serine-threonine kinase which is expressed in a tissue specific manner specifically in the brain, thymus and testis [138]. This kinase has a requirement for Ca++/calmodulin complex along with the phosphorylation of its own Thr200 residue for its full activation [139, 140]. Previous studies have shown that CaMKIV but not the other CaM kinases like CaMKI or CaMKII plays role in the repression of STREX exon [20]. This splicing regulation by CaMKIV have been seen both in cell culture and animal models [141]. When an inhibitor of CaM kinase called KN93 was used, the depolarization

mediated repression of STREX was blocked suggesting the involvement of CaMKIV since the other CaM kinases cannot repress the STREX splicing as seen by the over-expression studies [20]. Like hnRNP L, its paralogue hnRNP LL is also phosphorylated when CaMKIV is over-expressed in HEK293T cells (Figure 8). Interestingly, CaMKIV also has potential role in mediating the interaction of endogenous hnRNP LL with hnRNP L in GH_3 cells (data not shown). When KN93 was used, the interaction between the two proteins decreased compared to the KCl-treated samples suggesting a CaMKIV-regulated interaction of the two hnRNP proteins. Previously, CaMKIV mediated effects have been shown to regulate the RNA binding of the hnRNP proteins. For example, upon depolarization the interaction of hnRNP L protein with RNA element known as CaMK IV-responsive RNA element 1 (CaRRE1) is increased which plays role in the splicing of STREX repression [20, 21]. So the CaMKIV-mediated regulation of the interaction of two hnRNP proteins possesses a great potential for further exploration.

Phospho-Ser308-specific antibody validated the existence of the conserved Ser308:

Even though the phosphopeptide mapping experiment showed that mutation in Ser308 abolishes the potential spot containing the phosphopeptide which is differentially phosphorylated in GH_3 cells (Figure 9C), phospho-Ser308-specific antibody was generated to confirm the physical existence of the site. There is 68% sequence similarity between hnRNP L and hnRNP LL at the amino acid level. In addition, an equivalent serine (Ser357) is also present in hnRNP L (alignment not shown). This is why the peptide used to generate the phospho-Ser308-specific antibody was designed from a region having less than 50% similarity with hnRNP L and thereby reducing the possibility for any epitope-paratope interaction between hnRNP L and the phospho-Ser308-specific antibody.

The western blot results showed that the generated phospho-antibody is specific in the detection of Ser308 phosphorylation site (Figure 11B). A residual phospho-Ser308 signal was seen in the western blot for the non-treated samples of hnRNP LL (Figure 11B). It is possible that the Ser308 of some of the hnRNP LL proteins are also phosphorylated at the non-treated stage and upon depolarization the magnitude of phosphorylation increases which accounts for the higher phosphorylation signal in the KCl-treated samples. The phospho-Ser308-specific antibody also recognizes some of the non-phosphorylated regions since the only difference between the non-treated and depolarized sample is the presence of a phosphate group. The dot blot experiment done using the control and phosphopeptide for determining the specificity of the phospho-Ser308-specific antibody also had some signals in the control lane (data not shown). So the possibility of the antibody to recognize a small fraction of non-phosphorylated peptides cannot be over-ruled. However, the antibody loading control (Figure 8B, bottom gel) indicated that the level of hnRNP LL protein was similar between the non-treated and KCl treated samples, but the phospho-Ser308 signal was different which indicates that the level of phosphorylation increases upon depolarization even though the Ser308 site is probably not the major target of this pathway.

Changes in localization pattern of the hnRNP LL and its S308A mutant protein upon coexpression with CaMKIV and its mutant:

Subcellular redistribution of splicing factors has been shown to play important roles in cellular functions. One interesting example relevant to this study is the subcellular redistribution of Rbfox1 upon depolarization in P19 cells [142]. Upon prolonged depolarization, the exon 19 of Rbfox1 is repressed and the resultant protein shows different

composition of the C-terminus that changed its localization from the cytoplasm to nucleus. This accumulation promotes the inclusion of several exons that were repressed by depolarization/CaMKIV at the initial stages. In this study we have found that the intranuclear localization of Myc-hnRNP LL changes when co-expressed with a constitutively active CaMKIV and its kinase dead mutant (CaMKIVm) in HEK293T cells. When hnRNP LL is coexpressed with CaMKIVm a more homogenous distribution of the protein was seen throughout the nucleus (Figure 13A). On the other hand, coexpression with CaMKIV showed a perinucleolar staining of the protein (Figure 13B). Such localization is not uncommon for splicing regulatory proteins. In fact the presence of perinucleolar compartment (PNC) was itself initially described by Ghetti and colleagues in 1992 while they were studying the localization of the a known splicing protein called PTB /hnRNP I [143]. Evolutionarily PTB, hnRNP L and hnRNP LL are descendant from a common ancestor as shown by the phylogenetic tree constructed from their amino acid sequence [24]. Previously the hnRNP L protein has also been described to be present in discrete perinucleolar compartments [144, 145]. However, later studies described the discrete perinucleolar compartments of hnRNP L localization as Sam68 nuclear body (SNB) [146]. Unlike hnRNP L, hnRNP LL does not localize in the SNBs in HeLa cells [146]. As mentioned earlier, the perinucleolar localization needs to be confirmed further by the use of markers (for example, PTB) for perinucleolar compartment.

In this study we have observed that coexpression of Myc-hnRNP LL with CaMKIV shows a predominantly perinucleolar staining and mutation at Ser308 altered the perinucleolar staining of the hnRNP LL protein when coexpressed with CaMKIV in HEK293T cells (Figure 13), which suggests a possible role of this site in the perinucleolar

distribution of the protein. Some other splicing proteins have also been shown to change its intranuclear localization following phosphorylation. Tyrosine phosphorylation of YT521-B, a splice factor, by Src-kinase changes its intranuclear localization [147]. This protein is localized in the YT body of the nucleus and upon phosphorylation by Src-kinase it is redistributed throughout the nucleus and this also caused changes in the splicing of the minigene reporter constructs [147].

To examine whether the Ser308A mutation has any role in splicing, we performed minigene reporter assay using DUP175ST reporter. When hnRNP LL or Ser308A mutant was coexpressed with CaMKIV it causes repression in the minigene reporter. However, there was a significant decrease in splicing repression when Ser308A mutant was coexpressed with CaMKIV mutant. In the immunostaining experiment (Figure 13), it was observed that a fraction of the cells cotransfected with Myc-LLSer308A+CaMKIVm plasmids showed more cytoplasmic distribution (Figure 13B). Splicing is a nuclear process and the change in the cellular distribution of these cells may contribute to the splicing change observed in the minigene reporter assay where the splicing repression is almost abolished for the cells transfected with Ser308A+CaMKIVm plasmids (Figure 14C, lane 04). Moreover, when the Myc-LLSer308A construct is expressed with CaMKIV some other sites of hnRNP LL are phosphorylated which might compensate for the loss of Ser308 for splicing repression. The immunostaining data also shows consistency with the minigene results in this case. When Myc-LLSer308A construct is expressed with CaMKIV (Figure 13D), the majority of the cells mainly showed nuclear staining and the percentage of cells with more cytoplasmic staining was less compared to that of the cells cotransfected with Myc-LLSer308A+CaMKIVm (Figure 13, top bar graph). This suggests a possible

role of Ser308 in splicing. However, the reporter did not show much effect by CaMKIV itself compared to CaMKIVm when coexpressed with Myc-hnRNP LL (Figure 14C, lane 2 and 3). The splicing repression effect was mainly from the Myc-hnRNP LL expression regardless of its expression with either CaMKIV or its mutant. One possible explanation may be due to the fact that CaMKIV effect on splicing of the minigene reporter mainly happens through hnRNP LL and as result individual effect of CaMKIV cannot be observed when hnRNP LL is overexpressed. Taken together, this minigene reporter assay suggests that Ser308 mutation abolishes the splicing repression in the reporter when it is coexpressed with CaMKIVm. However, the main caveat of the experiment is the presence of endogenous hnRNP LL in the cells. In order to confirm the effect of Ser308, endogenous hnRNP LL needs to be knocked down and then overexpression with either hnRNP LL or Ser308 mutant can draw definitive conclusion on the role of Ser308 in STREX splicing. Also, this experiment lacked controls with only CaMKIV or hnRNP LL transfected, which would help to compare the individual effect of these proteins on the minigene reporter.

How hnRNP LL is involved in the regulation of STREX splicing is still an open question. One interesting feature of the hnRNP paralogues is that they bind to sequences that are highly similar [148]. For example, pyrimidine-rich sequences are preferred by both PTB and nPTB [149], while CA-repeats or C/A-rich sequences are preferentially bound by hnRNP L and hnRNP LL [150, 151]. Therefore, the interaction between hnRNP LL and CA-rich element could provide further insights into the regulation of STREX splicing. In a previous study of our lab, hnRNP LL was not detected when nuclear extracts from HEK293 and GH_3 cells were cross linked with CARRE1 element [22]. It is possible that

hnRNP LL interacts to some other protein(s) and thereby regulates splicing. During this study, my colleague and I have found that hnRNP L and LL interacts in GH_3 cells and this interaction is dependent on depolarization (data not shown). Further insights into the interaction between hnRNP L and LL may reveal a potential mechanism for STREX splicing.

CHAPTER VII

CONCLUSIONS

01. The hnRNP LL mRNA and protein levels do not change upon depolarization compared to the non-treated controls.
02. Endogenous hnRNP LL is a phosphoprotein and the phosphorylation signal is enhanced upon depolarization in GH_3 cells.
03. CaMKIV expression causes phosphorylation of hnRNP LL in HEK293T cells.
04. The Ser308 phosphorylation is induced by depolarization though it is probably not the major phospho-amino acid of depolarization/CaMKIV.
05. Preliminary observation from this study suggests that Ser308A mutation has effect on the intranuclear localization of hnRNP LL. The Ser308 site is more likely to be essential for perinucleolar localization of the protein upon CaMKIV coexpression.
06. The minigene reporter assay showed that splicing repression is almost abolished when Ser308A mutant is coexpressed with CaMKIV mutant in HEK293T cells suggesting a possible role of this serine on splicing.

REFERENCES

1. Nilsen, T.W. and B.R. Graveley, *Expansion of the eukaryotic proteome by alternative splicing.* Nature, 2010. **463**(7280): p. 457-463.
2. Lee, Y. and D.C. Rio, *Mechanisms and Regulation of Alternative pre-mRNA Splicing.* Annual Review of Biochemistry, 2015. **84**(1): p. 291-313.
3. Wang, E.T., et al., *Alternative isoform regulation in human tissue transcriptomes.* Nature, 2008. **456**(7221): p. 470-476.
4. Pan, Q., et al., *Deep surveying of alternative splicing complexity in the human transcriptome by high-throughput sequencing.* Nature genetics, 2008. **40**(12): p. 1413-1415.
5. Evsyukova, I., et al., *Alternative splicing in multiple sclerosis and other autoimmune diseases.* RNA biology, 2010. **7**(4): p. 462-473.
6. Razanau, A. and J. Xie, *Emerging mechanisms and consequences of calcium regulation of alternative splicing in neurons and endocrine cells.* Cellular and Molecular Life Sciences, 2013. **70**(23): p. 4527-4536.
7. Tazi, J., N. Bakkour, and S. Stamm, *Alternative splicing and disease.* Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease, 2009. **1792**(1): p. 14-26.
8. Orengo, J.P. and T.A. Cooper, *Alternative splicing in disease.* Advances in experimental medicine and biology, 2008. **623**: p. 212-223.
9. Shariat, N., et al., *Isolated growth hormone deficiency type II caused by a point mutation that alters both splice site strength and splicing enhancer function.* Clinical genetics, 2008. **74**(6): p. 539-545.
10. Feng, D. and J. Xie, *Aberrant splicing in neurological diseases.* Wiley Interdisciplinary Reviews: RNA, 2013. **4**(6): p. 631-649.
11. Chen, M. and J.L. Manley, *Mechanisms of alternative splicing regulation: insights from molecular and genomics approaches.* Nature reviews Molecular cell biology, 2009. **10**(11): p. 741-754.
12. Heyd, F. and K.W. Lynch, *Degrade, move, regroup: signaling control of splicing proteins.* Trends in biochemical sciences, 2011. **36**(8): p. 397-404.
13. Shin, C. and J.L. Manley, *Cell signalling and the control of pre-mRNA splicing.* Nature reviews Molecular cell biology, 2004. **5**(9): p. 727-738.
14. Wang, Z. and C.B. Burge, *Splicing regulation: from a parts list of regulatory elements to an integrated splicing code.* Rna, 2008. **14**(5): p. 802-813.
15. Wang, Z.-W., et al., *SLO-1 Potassium Channels Control Quantal Content of Neurotransmitter Release at the C. elegans Neuromuscular Junction.* Neuron, 2001. **32**(5): p. 867-881.
16. Xie, J., *Control of alternative pre-mRNA splicing by Ca++ signals.* Biochimica et Biophysica Acta (BBA)-Gene Regulatory Mechanisms, 2008. **1779**(8): p. 438-452.
17. Mirkowski, M.N., *Cognitive Function in BK Channel Knock-Out Mice.* 2013.
18. Jones, E., C. Laus, and R. Fettiplace, *Identification of Ca2+–activated K+ channel splice variants and their distribution in the turtle cochlea.* Proceedings of the Royal Society of London. Series B: Biological Sciences, 1998. **265**(1397): p. 685-692.
19. Ramanathan, K., et al., *A molecular mechanism for electrical tuning of cochlear hair cells.* Science, 1999. **283**(5399): p. 215-217.

20. Xie, J. and D.L. Black, *A CaMK IV responsive RNA element mediates depolarization-induced alternative splicing of ion channels.* Nature, 2001. **410**(6831): p. 936-939.
21. Liu, G., et al., *A conserved serine of heterogeneous nuclear ribonucleoprotein L (hnRNP L) mediates depolarization-regulated alternative splicing of potassium channels.* Journal of Biological Chemistry, 2012. **287**(27): p. 22709-22716.
22. Yu, J., et al., *The heterogeneous nuclear ribonucleoprotein L is an essential component in the Ca2+/calmodulin-dependent protein kinase IV-regulated alternative splicing through cytidine-adenosine repeats.* Journal of Biological Chemistry, 2009. **284**(3): p. 1505-1513.
23. Preußner, M., et al., *HnRNP L and L-like cooperate in multiple-exon regulation of CD45 alternative splicing.* Nucleic acids research, 2012. **40**(12): p. 5666-5678.
24. Xie, J., *Differential evolution of signal-responsive RNA elements and upstream factors that control alternative splicing.* Cellular and Molecular Life Sciences, 2014. **71**(22): p. 4347-4360.
25. Oberdoerffer, S., et al., *Regulation of CD45 alternative splicing by heterogeneous ribonucleoprotein, hnRNPLL.* Science, 2008. **321**(5889): p. 686-691.
26. Topp, J.D., et al., *A cell-based screen for splicing regulators identifies hnRNP LL as a distinct signal-induced repressor of CD45 variable exon 4.* Rna, 2008. **14**(10): p. 2038-2049.
27. Sambrook, J., *Adenovirus amazes at Cold Spring Harbor.* Nature, 1977. **268**(5616): p. 101.
28. Berget, S.M., C. Moore, and P.A. Sharp, *Spliced segments at the 5'terminus of adenovirus 2 late mRNA.* Proceedings of the National Academy of Sciences, 1977. **74**(8): p. 3171.
29. Gilbert, W., *Why genes in pieces?* Nature, 1978. **271**(5645): p. 501.
30. Braunschweig, U., et al., *Dynamic integration of splicing within gene regulatory pathways.* Cell, 2013. **152**(6): p. 1252-1269.
31. Black, D.L., *Mechanisms of alternative pre-messenger RNA splicing.* Annual review of biochemistry, 2003. **72**(1): p. 291-336.
32. Wahl, M.C., C.L. Will, and R. Lührmann, *The spliceosome: design principles of a dynamic RNP machine.* Cell, 2009. **136**(4): p. 701-718.
33. Graveley, B.R., *Alternative splicing: increasing diversity in the proteomic world.* TRENDS in Genetics, 2001. **17**(2): p. 100-107.
34. Sohail, M. and J. Xie, *Evolutionary emergence of a novel splice variant of opposite effect on cell cycle.* Molecular and cellular biology, 2015: p. MCB. 00190-15.
35. Maniatis, T. and B. Tasic, *Alternative pre-mRNA splicing and proteome expansion in metazoans.* Nature, 2002. **418**(6894): p. 236-243.
36. Black, D.L., *Protein Diversity from Alternative Minireview Splicing: A Challenge for Bioinformatics and Post-Genome Biology.* Cell, 2000. **103**: p. 367-370.
37. Ast, G., *How did alternative splicing evolve?* Nature Reviews Genetics, 2004. **5**(10): p. 773-782.
38. Kim, E., A. Magen, and G. Ast, *Different levels of alternative splicing among eukaryotes.* Nucleic acids research, 2007. **35**(1): p. 125-131.
39. Kim, H., et al., *Estimating rates of alternative splicing in mammals and invertebrates.* Nature genetics, 2004. **36**(9): p. 915-916.

40. Lu, J., et al., *Alternative splicing in teleost fish genomes: same-species and cross-species analysis and comparisons.* Molecular Genetics and Genomics, 2010. **283**(6): p. 531-539.
41. Lander, E.S., et al., *Initial sequencing and analysis of the human genome.* Nature, 2001. **409**(6822): p. 860-921.
42. Michael, D. and L. Manyuan, *Intron—exon structures of eukaryotic model organisms.* Nucleic acids research, 1999. **27**(15): p. 3219-3228.
43. Will, C.L. and R. Lührmann, *Spliceosome structure and function.* Cold Spring Harbor perspectives in biology, 2011. **3**(7): p. a003707.
44. Clancy, S., *RNA splicing: introns, exons and spliceosome.* Nature Education, 2008. **1**(1): p. 31.
45. Moore, M.J. and P.A. Sharp, *Evidence for two active sites in the spliceosome provided by stereochemistry of pre-mRNA splicing.* Nature, 1993. **365**(6444): p. 364-368.
46. Reed, R., *Initial splice-site recognition and pairing during pre-mRNA splicing.* Current opinion in genetics & development, 1996. **6**(2): p. 215-220.
47. Zhuang, Y. and A.M. Weiner, *A compensatory base change in U1 snRNA suppresses a 5′ splice site mutation.* Cell, 1986. **46**(6): p. 827-835.
48. Konarska, M.M., J. Vilardell, and C.C. Query, *Repositioning of the reaction intermediate within the catalytic center of the spliceosome.* Molecular cell, 2006. **21**(4): p. 543-553.
49. Sharp, P.A. and C.B. Burge, *Classification of introns: U2-type or U12-type.* Cell, 1997. **91**(7): p. 875-879.
50. Patel, A.A. and J.A. Steitz, *Splicing double: insights from the second spliceosome.* Nature Reviews Molecular Cell Biology, 2003. **4**(12): p. 960-970.
51. Tarn, W.-Y. and J.A. Steitz, *Pre-mRNA splicing: the discovery of a new spliceosome doubles the challenge.* Trends in biochemical sciences, 1997. **22**(4): p. 132-137.
52. ROESSER, J.R., *Both U2 snRNA and U12 snRNA are required for accurate splicing of exon 5 of the rat calcitonin/CGRP gene.* Rna, 2004. **10**(8): p. 1243-1250.
53. Pistoni, M., C. Ghigna, and D. Gabellini, *Alternative splicing and muscular dystrophy.* RNA biology, 2010. **7**(4): p. 441-452.
54. Cáceres, J.F. and A.R. Kornblihtt, *Alternative splicing: multiple control mechanisms and involvement in human disease.* TRENDS in Genetics, 2002. **18**(4): p. 186-193.
55. Kornblihtt, A.R., et al., *Multiple links between transcription and splicing.* Rna, 2004. **10**(10): p. 1489-1498.
56. Bentley, D.L., *Coupling mRNA processing with transcription in time and space.* Nature Reviews Genetics, 2014. **15**(3): p. 163-175.
57. de la Mata, M., et al., *A slow RNA polymerase II affects alternative splicing in vivo.* Molecular cell, 2003. **12**(2): p. 525-532.
58. McCracken, S., et al., *The C-terminal domain of RNA polymerase II couples mRNA processing to transcription.* Nature, 1997. **385**(6614): p. 357-361.
59. Sharp, P.A., *Five easy pieces.* Science, 1991. **254**(5032): p. 663-663.
60. Carmel, I., et al., *Comparative analysis detects dependencies among the 5′ splice-site positions.* Rna, 2004. **10**(5): p. 828-840.

61. Moss, L., *What genes can't do*. 2004: The MIT Press.
62. Busch, A. and K.J. Hertel, *Evolution of SR protein and hnRNP splicing regulatory factors.* Wiley Interdisciplinary Reviews: RNA, 2011. **3**(1): p. 1-12.
63. Kessler, M.M., et al., *Hrp1, a sequence-specific RNA-binding protein that shuttles between the nucleus and the cytoplasm, is required for mRNA 3'-end formation in yeast.* Genes & development, 1997. **11**(19): p. 2545-2556.
64. Alekseyenko, A.V., N. Kim, and C.J. Lee, *Global analysis of exon creation versus loss and the role of alternative splicing in 17 vertebrate genomes.* Rna, 2007. **13**(5): p. 661-670.
65. Artamonova, I.I. and M.S. Gelfand, *Comparative genomics and evolution of alternative splicing: the pessimists' science.* Chemical Reviews-Columbus, 2007. **107**(8): p. 3407-3430.
66. Berget, S.M., *Exon recognition in vertebrate splicing.* Journal of Biological Chemistry, 1995. **270**(6): p. 2411-2414.
67. Keren, H., G. Lev-Maor, and G. Ast, *Alternative splicing and evolution: diversification, exon definition and function.* Nature Reviews Genetics, 2010. **11**(5): p. 345-355.
68. Kim, E., A. Goren, and G. Ast, *Alternative splicing: current perspectives.* Bioessays, 2008. **30**(1): p. 38-47.
69. Koren, E., G. Lev-Maor, and G. Ast, *The emergence of alternative 3' and 5' splice site exons from constitutive exons.* PLoS Computational Biology, 2007. **3**(5): p. e95.
70. Ohkubo, Y., et al., *Intensive insulin therapy prevents the progression of diabetic microvascular complications in Japanese patients with non-insulin-dependent diabetes mellitus: a randomized prospective 6-year study.* Diabetes research and clinical practice, 1995. **28**(2): p. 103-117.
71. Busch, A. and K.J. Hertel, *Evolution of SR protein and hnRNP splicing regulatory factors.* Wiley Interdisciplinary Reviews: RNA, 2012. **3**(1): p. 1-12.
72. Michelle, L., J. Barbier, and B. Chabot, *hnRnP and hnRnP‐Like Proteins in Splicing control.* RNA Binding Proteins, 2012: p. 1-25.
73. Kukalev, A., et al., *Actin and hnRNP U cooperate for productive transcription by RNA polymerase II.* Nature structural & molecular biology, 2005. **12**(3): p. 238-244.
74. Gupta, S.K., et al., *The hnRNP F/H homologue of Trypanosoma brucei is differentially expressed in the two life cycle stages of the parasite and regulates splicing and mRNA stability.* Nucleic acids research, 2013. **41**(13): p. 6577-6594.
75. Decorsière, A., et al., *Essential role for the interaction between hnRNP H/F and a G quadruplex in maintaining p53 pre-mRNA 3'-end processing and function during DNA damage.* Genes & development, 2011. **25**(3): p. 220-225.
76. Mikula, M., et al., *Heterogeneous Nuclear Ribonucleoprotein (HnRNP) K Genome-wide Binding Survey Reveals Its Role in Regulating 3'-End RNA Processing and Transcription Termination at the Early Growth Response 1 (EGR1) Gene through XRN2 Exonuclease.* Journal of Biological Chemistry, 2013. **288**(34): p. 24788-24798.
77. Doyle, M. and M.A. Kiebler, *Mechanisms of dendritic mRNA transport and its role in synaptic tagging.* The EMBO journal, 2011. **30**(17): p. 3540-3552.
78. Bonnal, S., et al., *Heterogeneous nuclear ribonucleoprotein A1 is a novel internal ribosome entry site trans-acting factor that modulates alternative initiation of*

translation of the fibroblast growth factor 2 mRNA. Journal of Biological Chemistry, 2005. **280**(6): p. 4144-4153.
79. Hornbeck, P.V., et al., *PhosphoSitePlus: a comprehensive resource for investigating the structure and function of experimentally determined post-translational modifications in man and mouse.* Nucleic acids research, 2011: p. gkr1122.
80. Olsen, J.V., et al., *Quantitative phosphoproteomics reveals widespread full phosphorylation site occupancy during mitosis.* Science signaling, 2010. **3**(104): p. ra3.
81. Xie, J., et al., *Protein kinase A phosphorylation modulates transport of the polypyrimidine tract-binding protein.* Proceedings of the National Academy of Sciences, 2003. **100**(15): p. 8776-8781.
82. Walsh, C., *Posttranslational modification of proteins: expanding nature's inventory*. 2006: Roberts and Company Publishers.
83. Chang, B., et al., *JMJD6 is a histone arginine demethylase.* Science, 2007. **318**(5849): p. 444-447.
84. Chen, Y., et al., *Arginine methylation of hnRNP K enhances p53 transcriptional activity.* FEBS letters, 2008. **582**(12): p. 1761-1765.
85. Nichols, R.C., et al., *The RGG domain in hnRNP A2 affects subcellular localization.* Experimental cell research, 2000. **256**(2): p. 522-532.
86. Ciechanover, A., *The ubiquitin–proteasome pathway: on protein death and cell life.* The EMBO journal, 1998. **17**(24): p. 7151-7160.
87. Yao, P., et al., *The HILDA complex coordinates a conditional switch in the 3'-untranslated region of the VEGFA mRNA.* PLoS biology, 2013. **11**(8): p. e1001635.
88. Yang, X.-J. and E. Seto, *Lysine acetylation: codified crosstalk with other posttranslational modifications.* Molecular cell, 2008. **31**(4): p. 449-461.
89. Arnesen, T., et al., *Proteomics analyses reveal the evolutionary conservation and divergence of N-terminal acetyltransferases from yeast and humans.* Proceedings of the National Academy of Sciences, 2009. **106**(20): p. 8157-8162.
90. Koumbadinga, G.A., et al., *Increased stability of heterogeneous ribonucleoproteins by a deacetylase inhibitor.* Biochimica et Biophysica Acta (BBA)-Gene Regulatory Mechanisms, 2015. pii: S1874-9399(15)00091-7.
91. Melchior, F., *SUMO-nonclassical ubiquitin.* Annual review of cell and developmental biology, 2000. **16**(1): p. 591-626.
92. Vassileva, M.T. and M.J. Matunis, *SUMO modification of heterogeneous nuclear ribonucleoproteins.* Molecular and cellular biology, 2004. **24**(9): p. 3623-3632.
93. Villarroya-Beltri, C., et al., *Sumoylated hnRNPA2B1 controls the sorting of miRNAs into exosomes through binding to specific motifs.* Nature communications, 2013. **4**: 2980.
94. Li, T., et al., *Sumoylation of heterogeneous nuclear ribonucleoproteins, zinc finger proteins, and nuclear pore complex proteins: a proteomic analysis.* Proceedings of the National Academy of Sciences of the United States of America, 2004. **101**(23): p. 8551-8556.
95. Ostareck-Lederer, A., et al., *Asymmetric arginine dimethylation of heterogeneous nuclear ribonucleoprotein K by protein-arginine methyltransferase 1 inhibits its*

interaction with c-Src. Journal of Biological Chemistry, 2006. **281**(16): p. 11115-11125.
96. Yang, J.-H., et al., *Arginine methylation of hnRNPK negatively modulates apoptosis upon DNA damage through local regulation of phosphorylation.* Nucleic acids research, 2014: p. gku705.
97. Feliers, D., et al., *Heterogeneous nuclear ribonucleoprotein K contributes to angiotensin II stimulation of vascular endothelial growth factor mRNA translation.* American Journal of Physiology-Renal Physiology, 2007. **293**(2): p. F607-F615.
98. Perrotti, D., et al., *BCR-ABL prevents c-jun-mediated and proteasome-dependent FUS (TLS) proteolysis through a protein kinase CβII-dependent pathway.* Molecular and cellular biology, 2000. **20**(16): p. 6159-6169.
99. Gao, M. and M. Karin, *Regulating the regulators: control of protein ubiquitination and ubiquitin-like modifications by extracellular stimuli.* Molecular cell, 2005. **19**(5): p. 581-593.
100. Kouzarides, T., *Chromatin modifications and their function.* Cell, 2007. **128**(4): p. 693-705.
101. Pelisch, F., et al., *DNA damage-induced heterogeneous nuclear ribonucleoprotein K sumoylation regulates p53 transcriptional activation.* Journal of Biological Chemistry, 2012. **287**(36): p. 30789-30799.
102. Lee, S.W., et al., *SUMOylation of hnRNP-K is required for p53-mediated cell-cycle arrest in response to DNA damage.* The EMBO journal, 2012. **31**(23): p. 4441-4452.
103. García-Baños, J., et al., *Maltulose and furosine as indicators of quality of pasta products.* Food chemistry, 2004. **88**(1): p. 35-38.
104. Xie, J. and D.P. McCobb, *Control of alternative splicing of potassium channels by stress hormones.* Science, 1998. **280**(5362): p. 443-446.
105. Mayrand, S.H., P. Dwen, and T. Pederson, *Serine/threonine phosphorylation regulates binding of C hnRNP proteins to pre-mRNA.* Proceedings of the National Academy of Sciences, 1993. **90**(16): p. 7764-7768.
106. Rousseau, S., et al., *Inhibition of SAPK2a/p38 prevents hnRNP A0 phosphorylation by MAPKAP-K2 and its interaction with cytokine mRNAs.* The EMBO journal, 2002. **21**(23): p. 6505-6514.
107. Buxadé, M., et al., *The Mnks are novel components in the control of TNFα biosynthesis and phosphorylate and regulate hnRNP A1.* Immunity, 2005. **23**(2): p. 177-189.
108. Simons, M. and J. Trotter, *Wrapping it up: the cell biology of myelination.* Current opinion in neurobiology, 2007. **17**(5): p. 533-540.
109. Kosturko, L.D., et al., *Heterogeneous nuclear ribonucleoprotein (hnRNP) E1 binds to hnRNP A2 and inhibits translation of A2 response element mRNAs.* Molecular biology of the cell, 2006. **17**(8): p. 3521-3533.
110. White, R., et al., *Activation of oligodendroglial Fyn kinase enhances translation of mRNAs transported in hnRNP A2–dependent RNA granules.* The Journal of cell biology, 2008. **181**(4): p. 579-586.
111. Ostareck-Lederer, A., et al., *c-Src-mediated phosphorylation of hnRNP K drives translational activation of specifically silenced mRNAs.* Molecular and cellular biology, 2002. **22**(13): p. 4535-4543.

112. Chaudhury, A., et al., *TGF-β-mediated phosphorylation of hnRNP E1 induces EMT via transcript-selective translational induction of Dab2 and ILEI.* Nature cell biology, 2010. **12**(3): p. 286-293.
113. Meng, Q., et al., *Signaling-dependent and coordinated regulation of transcription, splicing, and translation resides in a single coregulator, PCBP1.* Proceedings of the National Academy of Sciences, 2007. **104**(14): p. 5866-5871.
114. van Oordt, W.v.d.H., et al., *The MKK3/6-p38–signaling cascade alters the subcellular distribution of hnRNP A1 and modulates alternative splicing regulation.* The Journal of cell biology, 2000. **149**(2): p. 307-316.
115. Allemand, E., et al., *Regulation of heterogenous nuclear ribonucleoprotein A1 transport by phosphorylation in cells stressed by osmotic shock.* Proceedings of the National Academy of Sciences of the United States of America, 2005. **102**(10): p. 3605-3610.
116. Guil, S., J.C. Long, and J.F. Cáceres, *hnRNP A1 relocalization to the stress granules reflects a role in the stress response.* Molecular and cellular biology, 2006. **26**(15): p. 5744-5758.
117. Passos, D.O., A.J. Quaresma, and J. Kobarg, *The methylation of the C-terminal region of hnRNPQ (NSAP1) is important for its nuclear localization.* Biochemical and Biophysical Research Communications, 2006. **346**(2): p. 517-525.
118. Barboro, P., et al., *Androgen Receptor activity is affected by both nuclear matrix localization and the phosphorylation status of the heterogeneous nuclear ribonucleoprotein K in anti-androgen-treated LNCaP cells.* PLoS ONE, 2013. **8**(11): p. e79212.
119. Habelhah, H., et al., *ERK phosphorylation drives cytoplasmic accumulation of hnRNP-K and inhibition of mRNA translation.* Nature cell biology, 2001. **3**(3): p. 325-330.
120. White, A., et al., *Phosphorylation of hnRNP K controls cytosolic accumulation of TDP-43.* Molecular Neurodegeneration, 2013. **8**(Suppl 1): p. P46.
121. Ferlay, J., et al., *Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012.* International Journal of Cancer, 2015. **136**(5): p. E359-E386.
122. Goehe, R.W., et al., *hnRNP L regulates the tumorigenic capacity of lung cancer xenografts in mice via caspase-9 pre-mRNA processing.* The Journal of clinical investigation, 2010. **120**(120 (11)): p. 3923-3939.
123. Vu, N.T., et al., *hnRNP U enhances caspase-9 splicing and is modulated by AKT-dependent phosphorylation of hnRNP L.* Journal of Biological Chemistry, 2013. **288**(12): p. 8575-8584.
124. Seol, D.-W. and T.R. Billiar, *A caspase-9 variant missing the catalytic site is an endogenous inhibitor of apoptosis.* Journal of Biological Chemistry, 1999. **274**(4): p. 2072-2076.
125. Berglund, F.M. and P.R. Clarke, *hnRNP-U is a specific DNA-dependent protein kinase substrate phosphorylated in response to DNA double-strand breaks.* Biochemical and Biophysical Research Communications, 2009. **381**(1): p. 59-64.
126. Müller, B., et al., *DNA-activated protein kinase functions in a newly observed S phase checkpoint that links histone mRNA abundance with DNA replication.* The Journal of cell biology, 2007. **179**(7): p. 1385-1398.

127. Martin, J., et al., *Phosphomimetic substitution of heterogeneous nuclear ribonucleoprotein A1 at serine 199 abolishes AKT-dependent internal ribosome entry site-transacting factor (ITAF) function via effects on strand annealing and results in mammalian target of rapamycin complex 1 (mTORC1) inhibitor sensitivity.* Journal of Biological Chemistry, 2011. **286**(18): p. 16402-16413.
128. Tolnay, M., Y. Juang, and G. Tsokos, *Protein kinase A enhances, whereas glycogen synthase kinase-3β inhibits, the activity of the exon 2-encoded transactivator domain of heterogeneous nuclear ribonucleoprotein D in a hierarchical fashion.* Biochem. J, 2002. **363**: p. 127-136.
129. White, R., et al., *Heterogeneous nuclear ribonucleoprotein (hnRNP) F is a novel component of oligodendroglial RNA transport granules contributing to regulation of myelin basic protein (MBP) synthesis.* Journal of Biological Chemistry, 2012. **287**(3): p. 1742-1754.
130. Amanchy, R., et al., *Identification of c-Src tyrosine kinase substrates using mass spectrometry and peptide microarrays.* Journal of proteome research, 2008. **7**(9): p. 3900-3910.
131. Hresko, R.C. and M. Mueckler, *Identification of pp68 as the tyrosine-phosphorylated form of SYNCRIP/NSAP1 a cytoplasmic RNA-binding protein.* Journal of Biological Chemistry, 2002. **277**(28): p. 25233-25238.
132. Ma, S., et al., *Relocalization of the polypyrimidine tract-binding protein during PKA-induced neurite growth.* Biochimica et Biophysica Acta (BBA)-Molecular Cell Research, 2007. **1773**(6): p. 912-923.
133. Cao, W., et al., *Differential effects of PKA-controlled CaMKK2 variants on neuronal differentiation.* RNA biology, 2011. **8**(6): p. 1061-1072.
134. Li, H., et al., *In vivo selection of kinase-responsive RNA elements controlling alternative splicing.* Journal of Biological Chemistry, 2009. **284**(24): p. 16191-16201.
135. Cohen, P., *The Croonian Lecture 1998. Identification of a protein kinase cascade of major importance in insulin signal transduction.* Philosophical Transactions of the Royal Society B: Biological Sciences, 1999. **354**(1382): p. 485-495.
136. Olsen, J.V., et al., *Quantitative phosphoproteomics reveals widespread full phosphorylation site occupancy during mitosis.* Science signaling, 2010. **3**(104): p. ra3-ra3.
137. Mann, M., et al., *Analysis of protein phosphorylation using mass spectrometry: deciphering the phosphoproteome.* Trends in biotechnology, 2002. **20**(6): p. 261-268.
138. Anderson, K.A., et al., *Components of a Calmodulin-dependent Protein Kinase Cascade molecular cloning, functional characterization and cellular localization of Ca2+/calmodulin-dependent protein kinase kinase β.* Journal of Biological Chemistry, 1998. **273**(48): p. 31880-31889.
139. Selbert, M.A., et al., *Phosphorylation and Activation of Ca-Calmodulin-dependent Protein Kinase IV by Ca-Calmodulin-dependent Protein Kinase Ia Kinase phosphorylation of threonine 196 is essential for activation.* Journal of Biological Chemistry, 1995. **270**(29): p. 17616-17621.

140. Chatila, T., et al., *A unique phosphorylation-dependent mechanism for the activation of Ca2+/calmodulin-dependent protein kinase type IV/GR.* Journal of Biological Chemistry, 1996. **271**(35): p. 21542-21548.
141. Xie, J., *Control of alternative pre-mRNA splicing by Ca++ signals.* Biochimica et Biophysica Acta (BBA)-Gene Regulatory Mechanisms, 2008. **1779**(8): p. 438-452.
142. Lee, J.-A., Z.-Z. Tang, and D.L. Black, *An inducible change in Fox-1/A2BP1 splicing modulates the alternative splicing of downstream neuronal target exons.* Genes & development, 2009. **23**(19): p. 2284-2293.
143. Ghetti, A., et al., *hnRNP 1, the polyprimidine tract-binding protein: distinct nuclear localization and association with hnRNAs.* Nucleic acids research, 1992. **20**(14): p. 3671-3678.
144. Pinol-Roma, S., et al., *A novel heterogeneous nuclear RNP protein with a unique distribution on nascent transcripts.* The Journal of cell biology, 1989. **109**(6): p. 2575-2587.
145. Huang, S., *Review: perinucleolar structures.* Journal of structural biology, 2000. **129**(2): p. 233-240.
146. Rajan, P., et al., *Proteomic identification of heterogeneous nuclear ribonucleoprotein L as a novel component of SLM/Sam68 Nuclear Bodies.* BMC cell biology, 2009. **10**(1): p. 82.
147. Rafalska, I., et al., *The intranuclear localization and function of YT521-B is regulated by tyrosine phosphorylation.* Human molecular genetics, 2004. **13**(15): p. 1535-1549.
148. Smith, S.A., et al., *Paralogs hnRNP L and hnRNP LL Exhibit Overlapping but Distinct RNA Binding Constraints.* PLoS ONE, 2013. **8**(11): p. e80701.
149. Spellman, R., M. Llorian, and C.W. Smith, *Crossregulation and functional redundancy between the splicing regulator PTB and its paralogs nPTB and ROD1.* Molecular cell, 2007. **27**(3): p. 420-434.
150. Martinez-Contreras, R., et al., *hnRNP proteins and splicing control.* Advances in Experimental Medicine & Biology, 2008. **623**: p. 123-147.
151. Rahman, M.A., et al., *HnRNP L and hnRNP LL antagonistically modulate PTB-mediated splicing suppression of CHRNA1 pre-mRNA.* Scientific reports, 2013. **3**:2931.