HnRNP L represses cryptic exons

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ABSTRACT

The fidelity of RNA splicing is regulated by a network of splicing enhancers and repressors, although the rules that govern this process are not yet fully understood. One mechanism that contributes to splicing fidelity is the repression of nonconserved cryptic exons by splicing factors that recognize dinucleotide repeats. We previously identified that TDP-43 and PTBP1/PTBP2 are capable of repressing cryptic exons utilizing UG and CU repeats, respectively. Here we demonstrate that hnRNP L (*HNRNPL*) also represses cryptic exons by utilizing exonic CA repeats, particularly near the 5'SS. We hypothesize that hnRNP L regulates CA repeat repression for both cryptic exon repression and developmental processes such as T cell differentiation.

Keywords: alternative splicing; cryptic exons; dinucleotide repeats; *HNRNPL*; HnRNP L

INTRODUCTION

RNA splicing in higher eukaryotes helps produce the transcriptional diversity that is required for cellular differentiation. This robust process is governed by RNA-binding splicing factors, many of which bind to degenerate consensus sequences (Wahl et al. 2009; Fu and Ares 2014). Given this limited specificity, how is the spliceosome able to distinguish proper exons from similar intronic sequences? Most introns contain cryptic “pseudoexon” sequences that appear to have all the appropriate splicing motifs, but are never incorporated into mature RNA transcripts (Dhir and Buratti 2010). We previously reported that certain pseudoexons are flanked by repetitive dinucleotide sequences, and that the repression of these cryptic exons is mediated by splicing factors that recognize said dinucleotide repeats (Ling et al. 2015, 2016). Repression of cryptic exons may help maintain splicing fidelity, given that the sequences of cryptic exons are not conserved between species and thus appear to have arisen stochastically.

Transactivation response element DNA-binding protein 43 (TDP-43, *TARDBP*), polypyrimidine tract-binding protein 1 (PTB, *PTBP1*), and polypyrimidine tract-binding protein 2 (nPTB, *PTBP2*) were identified as splicing factors that mediated cryptic exon repression. Specifically, TDP-43 binds to UG repeats (Ling et al. 2015; Jeong et al. 2017; Sun et al. 2017) while PTBP1 and PTBP2 bind to CU repeats (Ling et al. 2016). Other dinucleotide repeats exist in and around cryptic exons, however, aside from UG and CU. This led us to search for other splicing factors that might repress cryptic

exons using other dinucleotide sequences. Here, we demonstrate that heterogeneous nuclear ribonuclear protein L (hnRNP L, *HNRNPL*) is a cryptic exon repressor that binds to CA repeats.

HnRNP L was first identified as a member of the heterogeneous nuclear ribonucleoprotein (HNRNP) family (Pinol-Roma et al. 1989) and has a homolog, hnRNP LL (*HNRNPLL*). Among the HNRNP family members, hnRNP L and hnRNP LL share closest homology with PTBP1 and PTBP2 (Ghetti et al. 1992; Busch and Hertel 2012; Blatter et al. 2015). HnRNP L was shown to play a role in RNA splicing (Hui et al. 2003a) soon after its discovery, and like many other splicing factors, autoregulates the levels of its own transcript (Rossbach et al. 2009). HnRNP L was a leading candidate for cryptic exon repression because strong experimental consensus has indicated that hnRNP L binds to CA dinucleotide repeats (Hui et al. 2003b, 2005; Smith et al. 2013). This preference for CA repeats was further confirmed by UV cross-linking immunoprecipitation (CLIP) (Rossbach et al. 2014; Shankarling et al. 2014) and crystal structure analysis (Zhang et al. 2013a). CA repeats are among the most common repeats in the human genome (Lander et al. 2001; Subramanian et al. 2003; Sawaya et al. 2013). Furthermore, hnRNP L has been reported to act as a splicing repressor in certain contexts (Hui et al. 2005; Rothrock et al. 2005; Hung et al. 2008; Motta-Mena et al. 2010), with several reports suggesting that hnRNP L interferes with appropriate

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assembly of the spliceosome at the 5' splice site (5'SS) (Hui et al. 2005; Heiner et al. 2010; Chiou et al. 2013; Loh et al. 2017). Together, these studies provided strong rationale to study hnRNP L as a cryptic exon repressor candidate.

RESULTS AND DISCUSSION

In order to determine whether hnRNP L is a cryptic exon repressor, we analyzed RNA-seq data sets from hnRNP L knockdown in three human cell lines: JSL1, HepG2, and K562 (Cole et al. 2015; Sloan et al. 2016; Sundararaman et al. 2016), as well as RNA-seq data sets from genetic knockouts of *Hnrnp1* in mouse fetal liver hematopoietic stem cells (HSC) (Gaudreau et al. 2016). Applying the bioinformatics pipeline previously used to identify TDP-43 and PTBP1/PTBP2 cryptic exons (Ling et al. 2015, 2016), we analyzed the above data sets for novel splice sites and found many cryptic cassette exons, cryptic alternative splice site usage (exon extensions), cryptic terminal exons and cryptic polyadenylation sites (3' end exons) that were only observed after hnRNP L depletion (Fig. 1A–E; Supplemental Fig. S1; Supplemental Excel File). Importantly, we validated by RT-PCR use of many of the cryptic exons in JSL1 cells depleted of hnRNP L protein (Fig. 1F–H; Supplemental Fig. S2). We were also able to identify cryptic cassette exons, cryptic exon extensions, and cryptic 3' end exons in the mouse knockout data sets (Fig. 1I–K). As expected, there was no overlap in cryptic exons between mouse and human, consistent with the lack of conservation of these elements.

Next, we analyzed hnRNP L knockdown in the HepG2 and K562 cell lines utilizing RNA-seq data sets from the ENCODE shRNA library. Although we found that 58% (49/84) of cryptic exons were shared between at least two cell types, 42% (35/84) of cryptic exons were unique to a single cell type (Supplemental Fig. S3). Notably, much of this cell-type specificity appears to be due to the cell-type-specific expression of the gene (Supplemental Excel File). These results are analogous to our previous observations with tissue-specific cryptic exons associated with TDP-43 loss of function (Jeong et al. 2017).

Further inspection of cryptic exons and their flanking sequences confirmed the presence of numerous CA repeat clusters. Interestingly, aligning cryptic cassette exons to the 3'SS

FIGURE 1. Human and mouse hnRNP L cryptic exons viewed in the UCSC Genome Browser (hg38, mm10). Human cryptic cassette exon (A), cryptic exon extension (B), and cryptic 3' end exon (C). Examples of variability: a large cassette exon (D) and a cassette exon flanked by long introns (E). (F–H) Representative RT-PCR gels showing splicing of hnRNP L repressed cryptic exons for both control JSL1 cells (cntrl) and JSL1 cells transfected with short-hairpin RNA against hnRNP L (shL). Splice graphs of each splicing event are shown to the left of each gel with the cryptic exon in gray, constitutive (upstream and downstream) exons in white, and intersecting alternative exons in black. The average percent inclusion of the cryptic exon (% cryptic) and standard error of the mean (SEM) for at least two replicates is shown (see Supplemental Fig. 1 for other examples). Mouse cryptic cassette exon (I), cryptic exon extension (J), and cryptic 3' end exon (K). Genes displayed in UCSC views: RIF1 (A), SRC (B), MED12L (C), BCL11A (D), SH3BGRL (E), Usp16 (I), Lyl1 (J), and Iars2 (K).

and 5'SS revealed that most CA repeats are exonic and proximal to the 5'SS (Fig. 2A,B). We confirmed the functionality of these exonic CAs in at least one case in which they were well clustered by transfection of JSL1 cells with an antisense morpholino oligo (AMO) complementary to seven CA dinucleotides located upstream of the cryptic 5'SS in SH3BGRL (Fig. 3A). Blocking of these seven CAs by AMO resulted in a level of exon inclusion that mirrors that of hnRNP L depletion by translation blocking AMO (Fig. 3B). In contrast to the pattern of CA dinucleotides in the hnRNP L cryptic exons, analysis for TDP-43 and PTBP1/2 cryptic exons revealed that each dinucleotide repeat had different frequency distributions. TDP-43 cryptic exons had primarily intronic

A SH3GBRL cryptic exon

agaaagatcaagtaattgattgaattaaattcttcttgatttcttag/GGAAGAGATGGATCAAGATGGTGAAAT
 AGAAGACTCCACCAATCATCCCCCTTCACACGAACACCAATTTAACTATCTACATGCA
CACACACAAAAGCTTCATCAATTTAACTATCTACATGCACACACACAAAACCTTTGT
 AAGAACTCAAAATCAG/gtaagtactcatagtacctgggttaacatcatattggtgaagaggcac

FIGURE 3. Blocking CA repeats mimics depletion of hnRNP L in inclusion of the SH3GBRL cryptic exon. (A) Sequence of the SH3GBRL cryptic exon (uppercase) and flanking intron (lowercase) with sequence complementary to CA blocking AMO (underlined). CA dinucleotides are in bold. (B) RT-PCR gel showing inclusion of SH3GBRL cryptic exon upon transfection of CA blocking AMO, hnRNP L-depleting AMO or an unrelated GT-rich AMO. Western blot confirming depletion of hnRNP L upon treatment with the hnRNP L AMO, but not others, shown on right. HnRNP L depletion by AMO was performed as described previously (Cole et al. 2015). Translation blocking AMO for hnRNP L was used at 2, 5, and 10 nmol. CA and nonspecific AMO were used at 5, 10, and 20 nmol.

UG repeats that were most commonly downstream from the 5'SS, with some repeats upstream of the 3'SS. In contrast, PTBP1 and PTBP2 cryptic exons had mostly intronic CU repeats upstream of the 3'SS but also some exonic repeats as well (Fig. 2B).

In general, recognition and splicing of exons involves the stepwise assembly of various U snRNP components of the spliceosome with the pre-mRNA substrate (Fig. 4A, right). Regulation of any of these steps can alter the efficiency of splicing (Wahl et al. 2009; Fu and Ares 2014). Interestingly, previous work has shown that hnRNP L represses exon inclusion through several distinct mechanisms. Binding of hnRNP L in the intron immediately proximal to the 5' SS results in repression, presumably through steric hindrance of U1 recruitment (Hui et al. 2005; Hung et al. 2008). In contrast, in the case of CD45 exon 4, binding of hnRNP L within the exon functions to recruit hnRNP A1, remodel the U1 snRNP association with the 5'SS, and thereby inhibit association of U6 snRNP (Chiou et al. 2013). Importantly, we showed that a hallmark of the U1-remodeling mechanism of hnRNP L repression is a relatively strong association of the snRNA component of the U1 snRNP with the 5'SS. Specifically, exons for which the 5'SS:U1 snRNA base pairing had a minimal free energy (MFE) of -7 or

lower (more negative) were prone to repression when hnRNP L bound to the exon (Chiou et al. 2013).

Given our observation that hnRNP L cryptic exons have a high frequency of CA repeats near the 5'SS (Fig. 2), we sought to determine if the U1 remodeling mechanism may account for some of the repression of cryptic exons by assessing the MFE of 5'SS:U1 snRNA interaction as done in the previous study (Chiou et al. 2013). We first identified the cryptic exons with a strong presence of CA repeats (three or more dinucleotide repeats) in the final 50 nucleotides (nt) of the exon. We then separated these into exons which had additional CA dinucleotide repeats in the intronic 50 nt downstream from the 5'SS, which we reasoned would result in multimerization of hnRNP L across the 5'SS (Fig. 4A, left). Notably, the 5'SS flanked only by exonic CA repeats had a median MFE of -7.0 , while those flanked by both intronic

and exonic repeats had a median MFE of -5.4 (Fig. 4B; Supplemental Table S1). These results are consistent with a model whereby hnRNP L mediates repression of these cryptic exons by mechanisms described previously; namely, binding of hnRNP L across a 5'SS can sterically block recruitment of the U1 snRNP (especially when this binding is already weak), while binding of hnRNP L to the exon hyperstabilizes an already strong interaction of the U1 snRNP with the 5'SS to

FIGURE 4. Model for different mechanisms of hnRNP L repression of weak and strong 5' splice sites. (A) Schematic of normal spliceosome assembly (right) and mechanisms by which hnRNP L represses normal assembly. Exons are designated as gray boxes, introns are black lines. The U snRNP components of the spliceosome are indicated by blue and purple ovals. HnRNP L is indicated by a red oval. HnRNP L can either block accessibility of the 5'SS to U1 (exon and intron), or inhibit exchange of U1 for U6 (exon only). In either case, assembly of the final spliceosomal complex is prevented. (B) Minimal free energy (MFE) of the association of the 5'SS with the U1 snRNA for hnRNP L-repressed exons that have CA dinucleotides in the exon and/or intron flanking the 5'SS. See Supplemental Table 1 for details. As described in the text, binding of hnRNP L to the exon only is predicted to repress U1-U6 exchange and is typically marked by a strong 5'SS:U1 interaction. P-value is a two-tailed *t*-test.

prevent association of the U6 snRNP (Fig. 4A). Likewise, the demonstration that TDP-43 and PTBP1/PTBP2 repress cryptic exons from upstream and downstream intronic positions is consistent with the known activities of these proteins (Ashiya and Grabowski 1997; Pérez et al. 1997; Ayala et al. 2005; Oberstrass et al. 2005; Boutz et al. 2007; D'Ambrogio et al. 2009; Kuo et al. 2009; Xue et al. 2009; Chiang et al. 2010; Polymenidou et al. 2011; Tollervy et al. 2011; Kafasla et al. 2012; Keppetipola et al. 2012; Licatalosi et al. 2012; Lukavsky et al. 2013; Zhang et al. 2013b; Humphrey et al. 2017). Therefore, our results suggest that cryptic pseudoxons are repressed by mechanisms similar to standard exons, and that a widespread activity of hnRNP L is repression of strong 5'SSs, presumably through inhibiting replacement of U1 by U6.

Finally, in order to investigate the potential functional importance of cryptic exon repression by hnRNP L, we looked at consequences of cryptic exon inclusion on protein production. Given that cryptic exons are not conserved across species, we predict that their inclusion is likely to disrupt reading frame and protein expression. Almost half (244/550) of annotated exons repressed by hnRNP L in human JSL1 cells maintain reading frame (Cole et al. 2015). In contrast, 97% (60/62) of human cryptic exons and 86% (25/29) of mouse cryptic exons alter reading frame and are predicted to induce premature termination codons (Supplemental Excel File). For example, the gene encoding ZAP70, a critical signaling protein downstream from the T-cell receptor, contains a cryptic exon between exons 13 and 14 that introduces several stop codons. Upon knockdown of hnRNP L, this ZAP70 cryptic exon is included in almost half of the ZAP70 transcripts (Supplemental Fig. S2). Importantly, inclusion of the ZAP70 cryptic exon correlates with a marked decrease in ZAP70 protein levels (Supplemental Fig. S2, bottom right). This result indicates that repression of cryptic exons by hnRNP L serves to protect the fidelity of the proteome.

We note that our results raise the question of the role of hnRNP L's homolog hnRNP LL, given that hnRNP L is alone sufficient to repress cryptic exons. In contrast to the ubiquitous expression of hnRNP L, hnRNP LL is highly expressed only under certain conditions such as following T-cell activation, where it regulates the alternative splicing of genes such as CD45 (Hung et al. 2008; Oberdoerffer et al. 2008; Topp et al. 2008; Wu et al. 2008; Preußner et al. 2012). Therefore, we assume that hnRNP LL is not expressed at sufficient levels to functionally compensate for loss of hnRNP L cryptic exon repression in most cell types and conditions, including those analyzed in this study.

In summary, we have demonstrated that hnRNP L plays an important role in repressing nonconserved cryptic exons by utilizing exonic CA repeats. Together with our earlier studies, these results suggest that at least three out of six possible dinucleotide repeats are utilized to repress cryptic exon splicing (Supplemental Table S2). Of the remaining repeats, AG repeats may not be utilized because exonic splicing enhancers

are often purine rich; a broad splicing repressor with an AG repeat consensus motif may interfere with normal splicing. Alternatively, GC repeats may not be useful for a cryptic exon repressor because GC repeats are extremely rare within the genome due to cytosine methylation and spontaneous deamination. However, other homopolymers and tri/tetranucleotide repeats may also be used to repress cryptic exons. For example, hnRNP C binds to continuous uridine tracks to repress cryptic exons derived from Alu elements (Zarnack et al. 2013). Future work will be required to identify additional splicing factors and their cognate sequences that may function to guard the transcriptome against cryptic splicing.

MATERIALS AND METHODS

RNA-seq data sets analyzed

To identify cryptic exons, we accessed RNA-seq data sets from the following cell types: human JSL1 Jurkat T cells—wild-type and hnRNP L knockdown (SRP059357, PMID: 26437669), human HepG2 cells—wildtype and hnRNP L knockdown wild-type and hnRNP L knockdown (ENCODE Project Accession: ENCSTR155 BMF), human K562 cells—wild-type and hnRNP L knockdown (ENCODE Project Accession: ENCSTR563YIS), mouse embryo—wild-type and hnRNP L knockout (SRP042164, PMID: 27271479).

Bioinformatics analysis of cryptic exons

Fastq files were aligned to mouse and human genomes using HISAT2 (Kim et al. 2015), transcript assemblies were generated using StringTie (Pertea et al. 2015, 2016), and RPKM values were calculated using feature Counts (Liao et al. 2014) on Galaxy (Afgan et al. 2016). Cryptic exons were identified as previously described (Ling et al. 2015, 2016). Briefly, relative read coverage and split read counts were calculated between control and experimental condition for each exon in StringTie generated transcript assemblies. Data were visualized on the UCSC Genome Browser and false positives were removed following manual curation.

Calculation of repeat frequency around hnRNP L-repressed cryptic exons in Figure 2B was done by (i) Masking all "CA" and "AC" as "YY"; (ii) replacing all "A," "C," "T," "G" as "N"; (iii) identifying all pentamer and longer repeat sequences allowing for a single N insertion (i.e., "YYYYY," "YYYYYY," "YYYYYYY," "YNNYYYY," "YYNNYYY," "YYYNNYY," "YYYYNNY," ...); (iv) assigning the "Y"s in sequences from step 3 a value of 1 and all other sequences a value of 0; (v) aligning all sequences to the 3'SS and 5'SS and calculating the repeat frequency for each base pair position (i.e., sum vertically and divide by the total number of sequences). Repeat frequencies for UG repeats (TDP-43) and CU repeats (PTBP1/PTBP2) were derived in a similar fashion.

Calculation of minimal free energy of the 5'SS region of hnRNP L-repressed cryptic exons was done using RNAcofold (<http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAcofold.cgi>) to assess the free energy of the interaction of the 5'SS interacting nucleotides of U1: (5' ACUUACCUG) with the 9 nt encompassing positions +3 to -6 around the 5'SS of the cryptic exons (cleavage bond is designated as 0).

TABLE 1. Primer sequences used in this study

Gene symbol	Forward primer	Reverse primer
BLOC1S5	AGAGACACAATGCGGGACAGCCTCAG	TCTGTGCTCTTCATCCACTTCAGCCC
BPTF	CCCCAACAAGCAGTACAACCAGCACC	CCTGGGCTGGAATGAAGTAAAGGTTTGC
GEN1	AGTCCCTGGAGTTGAAAAGAGCAAGC	TGCCTATCATGTTCTGTACGGTGCCAC
HDAC7	ACAGCAGAGCAAGGCCAGCAAGATCC	TGGCGATGCAGGGAGATGTAGAGCAC
LIN7C	TAGGTTAAGGGAGAAGATGGCGGCGC	TTCACTTTGAAGGACTCTTTGCAAAGCCTG
LRP8	GCTCCTCAGATCTCCAGCCACTCTCCC	TCAGGCTTGGTGTCTCTGTGCTGTGG
MUTYH	GAAAGCCGGGCGCGCTAGAGCTC	CAATACCACCTCTCCGGCTGCCTGG
RIF1	GGCCAAAGAATCCATACCATGCCCAAC	AGTCCACGAGTCTTCACTGCTGCTC
SGSM2	CACACCATGGGCAGCGCAGAGGAC	GGCACGTCTTCCCCACCTTGGTGAAC
SH3BGRL	CTGGCGAGTTCTCCTTCCACCTTCCC	CCACTTCCGATTCTCTTATTGGCTGC
SLIT1	AGGCCATCCCCAGGAAAGCTTTTCGG	ATGGTTGAAGCTGGACACGGGGATGG
SPATS2	TTCCTCTTCTCAGACCCGGGAGCGTC	ATCCTTTTGCCTTGTCTGGGTTTGTAGTG
ZAP70	CTCAGCAGGGAAGTGGCCGCTCAAG	AGCCTTCTGTGTGCTGCTGGGG
BCL11A	GTGGATAAGCCACCTTCCCCTTCAAC	AATGGTGGTGGTGGTGTAGTGGTGGTGG and GCAAGTTGTACATGTGTAGCTGCTGGGC (three primer system)

hnRNP L knockdown, AMO transfection, RT-PCR validation, and western blots

RNA from JSL1 Jurkat cells (Lynch and Weiss 2000) was generated as previously described (Cole et al. 2015) using RNA-Bee (Tel-Test). HnRNP L depletion was achieved using a stably integrated, doxycycline-inducible short hairpin RNA against the hnRNP L mRNA as described in Cole et al. (2015). The CA-blocking or hnRNP L-depletion antisense morpholino oligos (AMO) were purchased from GeneTools and transfected at the indicated concentrations by electroporation as described previously (Cole et al. 2015). CA-blocking AMO is TTGTGTGTGTGCATGTAGATAGTTG. Nonspecific GT AMO is CATGTAGTGTGCAGGTTAAGAGTGA. hnRNP L-depleting AMO was described previously in Cole et al. (2015) (CGCCCGC CGCCGCCATCTTCACCAT). Cells were incubated following transfection for 24 h and RNA was harvested using RNA-Bee reagent. Low cycle RT-PCR was performed as previously described (Lynch and Weiss 2000), using radiolabeled primers. For genes where the cryptic exon was >1000 nt, two reverse primers were used. Following resolution of products on a 5% denaturing polyacrylamide gel, results were imaged using a Typhoon 9200 (GE) and analyzed using ImageQuant (GE). Percent spliced in of the cryptic exon was determined as the ratio of the signal from PCR product or products containing the cryptic exon sequence to the total PCR product signal. Western blots were performed as described previously (Table 1; Lynch and Weiss 2000).

SUPPLEMENTAL MATERIAL

Supplemental material is available for this article.

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