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Coordinated regulation of neuronal mRNA steady-state levels through developmentally controlled intron retention

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Differentiated cells acquire unique structural and functional traits through coordinated expression of lineage-specific genes. An extensive battery of genes encoding components of the synaptic transmission machinery and specialized cytoskeletal proteins is activated during neurogenesis, but the underlying regulation is not well understood. Here we show that genes encoding critical presynaptic proteins are transcribed at a detectable level in both neurons and nonneuronal cells. However, in nonneuronal cells, the splicing of 3′-terminal introns within these genes is repressed by the polypyrimidine tract-binding protein (Ptbp1). This inhibits the export of incompletely spliced mRNAs to the cytoplasm and triggers their nuclear degradation. Clearance of these intron-containing transcripts occurs independently of the nonsense-mediated decay (NMD) pathway but requires components of the nuclear RNA surveillance machinery, including the nuclear pore-associated protein Tpr and the exosome complex. When Ptbp1 expression decreases during neuronal differentiation, the regulated introns are spliced out, thus allowing the accumulation of translation-competent mRNAs in the cytoplasm. We propose that this mechanism counters ectopic and precocious expression of functionally linked neuron-specific genes and ensures their coherent activation in the appropriate developmental context.

Keywords: coordinated gene expression; neuronal differentiation; presynaptic proteins; polypyrimidine tract-binding protein; intron retention; nuclear mRNA surveillance

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Higher eukaryotes contain multiple cell types that differ in their morphological and physiological properties despite having identical or nearly identical genomes. This phenotypic diversity relies on the expression of distinct repertoires of lineage-specific genes coordinated in a precise spatiotemporal manner. A number of transcriptional and post-transcriptional mechanisms are known to control these elaborate gene networks [Davidson 2006; Moore and Proudfoot 2009; Vaquerizas et al. 2009; Ivey and Srivastava 2010].

Alternative pre-mRNA splicing provides a remarkable example of post-transcriptional regulation operating on a genome-wide scale [Wang and Burge 2008; Nilsen and Graveley 2010]. Recent studies suggest that ~90% of human and mouse genes may give rise to alternatively spliced transcripts, which often encode distinct protein isoforms [Pan et al. 2008; Wang et al. 2008]. This effectively increases genome-coding capacity without expanding the number of the genes. Alternative splicing events often occur in a tissue-specific manner, with the largest number of unique splice forms found in the nervous system [Q Li et al. 2007; Pan et al. 2008; Wang et al. 2008].

We and others have previously shown that the polypyrimidine tract-binding protein [PTBP1 in humans and Ptbp1 in mice; other names: PTB and hnRNP I] functions as a global regulator of a neuron-specific alternative splicing program [Boutz et al. 2007; Makeyev et al. 2007; Spellman et al. 2007]. Although this protein has traditionally been considered a splicing repressor, recent transcriptome-wide surveys have identified examples of both PTBP1-repressed and PTBP1-activated alternative exons [Xue et al. 2009; Llorian et al. 2010]. Ptbp1 tends...
to be expressed at high levels in nonneuronal and neural stem cells (NSCs), whereas its expression is diminished in differentiating neurons by the nervous system-specific microRNA [miRNA] miR-124 (Makeyev et al. 2007).

PTBP1 also autoregulates its own expression through a negative feedback mechanism, in which an excess of this protein induces skipping of an alternative exon within its own pre-mRNA and leads to the appearance of a premature termination codon [PTC] (Wollerton et al. 2004). The PTC-containing PTBP1 mRNAs are then rapidly degraded in the cytoplasm by the nonsense-mediated decay [NMD] machinery (Isken and Maquat 2008; Nicholson and Muhlemann 2010). Using a similar NMD mechanism, Ptbp1 represses the expression of its neuronal paralog [Ptbp2] and at least two other nervous system-specific genes (Gabbr1 and Dlg4) (Boutz et al. 2007; Makeyev et al. 2007; Spellman et al. 2007; Zheng et al. 2012). Thus, in addition to generating distinct protein isoforms, alternative splicing may cooperate with the cytoplasmic RNA surveillance machinery to control gene expression levels (Lareau et al. 2007; Isken and Maquat 2008; Barash et al. 2010).

Several mechanisms are known to control the quality of RNAs in the nucleus (Sommer and Nehrbass 2005; Egecioglu and Chanfreau 2011). One such mechanism blocks the export of unspliced pre-mRNAs from the nucleus to the cytoplasm, thus preventing their translation into aberrant polypeptides (Chang and Sharp 1989; Legrain and Rosbash 1989). A growing body of evidence suggests that, in addition to correcting splicing errors, nuclear mRNA surveillance may contribute to programmed changes in gene expression levels. For example, human SRSF1/ASF/SF2 protein homeostatically autoregulates its expression at least in part by promoting the accumulation of incompletely spliced SRSF1 mRNA species in the nucleus (Sun et al. 2010). Similarly, the retention of intron 3 in the mouse apolipoprotein E transcripts may interfere with their export from the nucleus to the cytoplasm (Xu et al. 2008).

Regulated intron retention is widely used as a general strategy for coordinated expression of meiotic and ribosomal protein genes in budding and fission yeasts (Averbeck et al. 2005; Moldon et al. 2008; Cremona et al. 2010; Munding et al. 2010; Parenteau et al. 2011). However, it is currently unknown whether higher eukaryotes use similar intron-dependent strategies for orchestrating their cell differentiation programs.

Signal transmission from a presynaptic neuron to a postsynaptic cell is a major nervous system function that depends on coexpression of hundreds of neuronal proteins involved in signal-dependent fusion of synaptic vesicles with synaptic terminals [e.g., vesicle-specific v-SNAREs and calcium sensors and terminal-specific t-SNAREs], trafficking of vesicles along the axon, and their recycling following neurotransmitter release (Sudhof 2004; Wojcik and Brose 2007; DeBoer et al. 2008). The genes encoding these important proteins are tightly regulated during neuronal differentiation; however, relatively little is known about the underlying mechanisms (Waites et al. 2005).

Results
Ptbp1 regulates a set of genes at the level of mRNA abundance

To understand the Ptbp1-controlled post-transcriptional network, we transfected the mouse neuroblastoma CAD cell line with either Ptbp1-specific [siPtbp1] or control [siControl] siRNAs and analyzed the samples by RNA sequencing [RNA-seq]. In addition to the expected stimulation of the nervous system-specific alternative splicing program [data not shown], an overall abundance of a number of transcripts either decreased [n = 431, >1.5-fold, \( P < 0.001 \)] [Supplemental Table S1] or increased [n = 276, >1.5-fold, \( P < 0.001 \)] [Supplemental Table S2] in the Ptbp1 knockdown sample. As expected, siPtbp1 down-regulated Ptbp1 expression (~5.4-fold, \( P = 0 \)) [Supplemental Table S1] and up-regulated the expression of its neuron-enriched paralog, Ptbp2 (~3.5-fold, \( P = 2.1 \times 10^{-250} \)) [Supplemental Table S2]. Interestingly, these large-scale transcriptome changes were accompanied by an increased propensity of CAD cells to undergo neuron-like differentiation [Supplemental Fig. S1].

The RNA-seq data contained a substantial number of intronic RNA-seq reads likely derived from the nuclear pre-mRNA fraction. We reasoned that the ratio between intronic reads and reads originating from the adjacent exons—the statistic that we refer to as IRENE [intronic reads normalized to exons]—should be a faithful indicator of post-transcriptionally regulated genes. Strikingly, the statistically significant IRENE changes induced by siPtbp1 correlated inversely with statistically significant changes in the corresponding mRNA expression levels [\( P = 3.2 \times 10^{-37} \), Fisher’s exact test] [Fig. 1A, red dots].

We focused on the subset of genes showing increased expression levels and reduced IRENE scores [33 genes] [Fig. 1A, top left quadrant, Supplemental Table S3]. Since Ptbp1 and Ptbp2 may regulate overlapping pre-mRNA sets and the loss of Ptbp1 leads to the Ptbp2 up-regulation [Makeyev et al. 2007], we treated CAD cells with a mixture of Ptbp1- and Ptbp2-specific siRNAs [siPtbp1/2] and repeated the RNA-seq analysis. In this case, increased mRNA expression and reduced IRENE scores were detected for 102 genes (>1.5-fold changes; \( P < 0.001 \)) [Supplemental Table S4], which included 19 of the above 33 genes.

To confirm that these effects were due to increased transcript abundance rather than altered splicing patterns, we analyzed the mRNA expression levels for the 31 Mouse Genome Informatics [MGI]-annotated genes by RT-qPCR using primers specific to constitutively spliced regions. All of these genes were significantly up-regulated upon Ptbp1 or Ptbp1/2 knockout [Fig. 1B, top graph, Supplemental Fig. S2A]. We concluded that Ptbp1 and, possibly, Ptbp2 regulate the expression levels of extensive sets of genes.
Ptbp1 represses the expression of a number of genes in an NMD-independent manner

Ptbp1 protein has previously been shown to reduce the expression of Ptbp2 and Gabbr1 mRNAs through the NMD pathway (Makeyev et al. 2007). Since NMD is thought to function in the cytoplasm without affecting nuclear [pre-mRNA levels, Ptbp1 knockdown was expected to increase the abundance of Ptbp2 and Gabbr1 mRNAs and simultaneously decrease the corresponding IRENE statistics. Both genes were indeed present among the up-regulated genes with reduced IRENE values (Fig. 1B, top graph; Supplemental Table S3).

To examine whether the remaining 29 genes were also regulated by NMD, we treated CAD cells with cycloheximide (CHX), a protein synthesis inhibitor that also blocks NMD-mediated mRNA degradation, and analyzed the effect of this treatment on the gene expression using an Agilent gene expression microarray. To our surprise, only three additional genes (Dusp8, Id1, and Zc3h8) significantly increased their expression levels in response to CHX treatment (Fig. 1B, bottom graph; Supplemental Fig. S2B). Thus, the 26 remaining genes that failed to respond to the CHX treatment were likely regulated by mechanisms other than NMD.

Expression levels of several NMD-independent genes positively correlate with the 3′-terminal intron splicing efficiency

Gene ontology (GO) analysis of the 26 NMD-independent genes returned “neurotransmitter transport” [GO:0006836] as the highest-scoring term (4.3-fold enrichment over the background frequency, Bonferroni-corrected \( P = 3.54 \times 10^{-4} \)) [Supplemental Table S5]. This corresponded to a subset of four neuron-specific genes encoding critical presynaptic proteins: Stx1b (a t-SNARE), Vamp2 (a v-SNARE), Sv2a (a synaptic vesicle-associated regulator of Ca\(^{2+}\) levels), and Naph/\( \beta \)SNAP (a SNARE recycling protein) (Sudhof 2004; Wojcik and Brose 2007). Similar to
the siPtbp1 effect, down-regulation of the Ptbp1 expression by the miRNA miR-124 led to a significantly elevated expression of these genes in CAD cells [Supplemental Fig. S2C; data not shown].

Interestingly, for the Stx1b, Vamp2, and Sv2a genes, the most dramatic decrease in the IRENE scores following Ptbp1 knockdown was observed for the 3′-terminal introns [Supplemental Table S3]. This effect was due to a simultaneous decrease in the density of intronic reads and an increase in the density of exonic reads [Fig. 1C]. At least three other protein-coding genes from the NMD-independent list followed a similar trend: the Kif5a gene encoding a nervous system-specific kinesin heavy chain involved in anterograde axonal transport of synaptic vesicles and other membrane-bound organelles [DeBoer et al. 2008], the Exosc2 gene encoding the Rrp4 subunit of the RNA exosome complex, and a nucleoporin gene, Nup35 [Supplemental Table S3; Supplemental Fig. S2D; data not shown].

To examine whether the increase in mRNA abundance of the NMD-independent genes led to the accumulation of the corresponding proteins, we knocked down the Ptbp1 protein in CAD cells by RNAi and analyzed the expression of Stx1b and Vamp2 proteins by immunoblotting [Fig. 2A]. The levels of both SNARE proteins increased noticeably in the Ptbp1 knockdown sample as compared with the siControl-treated cells [Fig. 2A]. Taken together, these data indicated that Ptbp1 might control the expression levels of a subset of genes by modulating the 3′-terminal intron splicing efficiency.

Ptbp1 represses the 3′-terminal intron splicing in the Stx1b, Vamp2, Sv2a, and Kif5 pre-mRNAs

Since the 3′-terminal introns of Stx1b, Vamp2, Sv2a, and Kif5 contained multiple consensus Ptbp1-binding sites [Supplemental Fig. S3], we reasoned that Ptbp1 may directly inhibit splicing of these introns. To begin addressing this hypothesis, we first determined the steady-state splicing efficiency of the four short 3′-proximal introns within the Stx1b pre-mRNA using RT–PCR analysis of the CAD cell nuclear fraction with corresponding exon-specific primers [Supplemental Fig. S4A,B]. Interestingly, the last intron [intron 9] was retained in >75% of the Stx1b transcripts, in contrast to the three upstream introns [5, 6, and 8] that were retained in <25% of the Stx1b transcripts [P = 4.6 × 10^{-5}, single-factor ANOVA test] [Supplemental Fig. S4A,B]. This difference could not be attributed simply to the 5′-to-3′ directionality of the pre-mRNA synthesis, since the steady-state splicing efficiency of the 3′-terminal intron of the β-actin [Actb] pre-mRNA was not significantly different from that of an upstream intron [Supplemental Fig. S4C,D].

We then treated CAD cells with siPtbp1, siPtbp2, siPtbp1/2, or siControl siRNAs and analyzed the Stx1b, Vamp2, Sv2a, and Kif5a 3′-terminal intron splicing status in the whole-cell RNA samples by RT–PCR [Fig. 2B,C; Supplemental Fig. S4E,F; first four samples]. The 3′ intron-retained forms of all four transcripts were readily detectable in the siControl transfected samples. Down-regulation of Ptbp1 or both Ptbp1 and Ptbp2 significantly decreased the relative abundance of the intron-retained species [P < 4 × 10^{-5}, single-factor ANOVA test] [Fig. 2B,C; Supplemental Fig. S4E,F, first four Kif5a samples]. Treating the cells with siPtbp2 only had no detectable effect, consistent with the low background Ptbp2 expression in the presence of Ptbp1 [Makeyev et al. 2007]. We concluded that Ptbp1 represses splicing of the Stx1b, Vamp2, Sv2a, and Kif5a 3′-terminal introns.

Retention of the 3′-terminal introns inhibits mRNA accumulation in the cytoplasm

Incompletely spliced mRNA species often fail to be efficiently exported from the nucleus to the cytoplasm. In line with this trend, the intron-retained forms of the Stx1b, Vamp2, and Sv2a transcripts were up to 10 times more abundant in the nucleus than in the cytoplasm of untreated CAD cells [P < 0.01, t-test] [Fig. 2D,E]. Nucleocytoplasmic fractionation of the siRNA-treated CAD cells further revealed that knocking down Ptbp1 alone or both Ptbp1 and Ptbp2 dramatically reduced the percentage of the intron-retained species in the nucleus [P = 5 × 10^{-9}, single-factor ANOVA] [Supplemental Fig. S4E,F; data not shown]. These treatments also appeared to reduce the fraction of the intron-retained forms in the cytoplasm [P = 0.04, single-factor ANOVA] [Supplemental Fig. S4E,F; data not shown], although the effect was difficult to quantify accurately due to the overall low abundance of the intron-retained form in this cellular compartment. Notably, we failed to detect Actb transcripts retaining the 3′-terminal intron in the whole-cell and cytoplasmic samples [Supplemental Fig. S4E,F; Actb panels]. These species were present in the corresponding nuclear fractions, but their abundance was not affected by changes in the Ptbp1 and Ptbp2 expression [Supplemental Fig. S4E,F; Actb panels].

We further assayed the effect of Ptbp1 and Ptbp2 on mRNA expression levels by RT-qPCR using Gapdh mRNA as a normalization control [Fig. 2F]. This analysis showed that the overall abundance of the cytoplasmic Stx1b mRNA rose more than threefold in the siPtbp1 and siPtbp1/2 samples as compared with the siControl-treated cells [P = 1.7 × 10^{-4}, single-factor ANOVA] [Fig. 2F]. The Stx1b transcript expression also increased in the corresponding nuclear fractions, albeit to a lesser extent [less than two times; P = 1.3 × 10^{-4}, single-factor ANOVA] [Fig. 2F]. Interestingly, the apparent abundance of the Stx1b transcripts was sevenfold to eightfold higher in the nucleus than in the cytoplasm of the siControl- and siPtbp2-treated CAD cells [Fig. 2G]. This difference decreased to approximately fourfold following the Ptbp1 or the double Ptbp1/2 knockdown [Fig. 2G]. The regulation of the Stx1b, Vamp2, Sv2a, and Kif5a mRNA abundance by Ptbp1 was not specific to the CAD cells, since similar results were obtained using a clone of the L929 mouse fibrosarcoma cell line [Supplemental Fig. S4G].
The above results are consistent with the model that the Ptbp1-stimulated retention of 3′-terminal introns inhibits the export of the incompletely spliced Stx1b, Vamp2, Sv2a, and Kif5a mRNAs from the nucleus to the cytoplasm and eventually leads to their nuclear degradation.

Ptbp1 directly inhibits splicing of the Stx1b 3′-terminal intron

To test whether Ptbp1 repressed the 3′-terminal intron splicing directly, we synthesized a transcript comprising...
the Stx1b exon 9, intron 9, and exon 10 sequences and assayed splicing of this substrate in vitro followed by RT-PCR and RT-qPCR analyses (Fig. 2H–K). Splicing of this RNA was fairly inefficient in a control-treated HeLa S3 nuclear extract but was dramatically activated by immunodepleting the endogenous PTBP1 protein (Fig. 2H,I, Supplemental Fig. 5A). In contrast, PTBP1 depletion had no effect on splicing of a control adenovirus-specific RNA substrate [AdV] (Fig. 2H,I). To ensure that the stimulation of the Stx1b intron 9 splicing following PTBP1 immunodepletion was a specific effect, we added various amounts of purified recombinant PTBP1 to the immunodepleted splicing reactions and repeated the analysis (Fig. 2J,K). Satisfyingly, increasing the PTBP1 concentrations efficiently repressed splicing of the Stx1b but not AdV RNA substrates. Similar results were obtained using in vitro splicing assays with recombinant radioactive RNA substrates (Supplemental Material; Supplemental Fig. S5B–F). Overall, these data indicated that Ptbp1 directly represses splicing of the Stx1b intron 9.

**Stx1b intron 9 in its natural context is sufficient for the regulation**

To identify cis-elements involved in the regulation of the intron 9 splicing and the Stx1b mRNA abundance, we turned to the minigene approach. Since transiently transfected Stx1b constructs failed to recapitulate the regulation, possibly as a result of titrating out a saturable nuclear factor [data not shown], we took advantage of the single-copy transgene knock-in technology based on high-efficiency and low-background recombination-mediated cassette exchange (HILO-RMCE) [Khandelia et al. 2011]. Using this approach, we generated a population of CAD cells expressing a “minitransgene” cassette in which a short intron 9-containing fragment of the Stx1b gene was placed under the control of a doxycycline [Dox]-inducible promoter element containing a recombinant constitutive intron (TREI) (Fig. 3A).

We then knocked down the expression of Ptbp1 or/and Ptbp2 in the transgenic cells [Supplemental Fig. S6] and assayed the minitransgene expression under the conditions in which it was either fully activated [Dox+] or transcribed at a low background level [Dox−] (Fig. 3B–D). RT-qPCR analysis using transgene-specific primers showed that, both with and without Dox, the expression of the Stx1b minitransgene was significantly up-regulated following Ptbp1 or double Ptbp1/2 knockdown as compared with the siControl-treated cells (Fig. 3B). RT–PCR analysis of the same samples indicated that splicing of the transgenic intron 9 was noticeably more efficient in the Ptbp1 or double Ptbp1/2 knockdown samples (Fig. 3C,D). Thus, intron 9 within its natural context is sufficient for Ptbp1-dependent regulation of Stx1b mRNA expression.

**Polypyrimidine sequences within the Stx1b intron 9 are necessary for the regulation**

To test whether intron 9 was necessary for Stx1b gene regulation, we integrated an ~170-kb transgenic fragment containing the entire mouse Stx1b gene into the CAD cell genome using an RMCE protocol modified for bacterial artificial chromosome (BAC)-encoded transgenes (Supplemental Fig. S7A,B). K Yap, ZQ Lim, and EV Makeyev, in prep.). To distinguish between the recombinant and endogenous copies, we marked the 3′ untranslated region (UTR) of the transgenic Stx1b with a 68-nucleotide [nt] sequence encoding an FRT site (Supplemental Fig. S7A). Stx1b-FRT transgene expression was up-regulated following Ptbp1 or Ptbp1/2 knockdown, similar to the endogenous Stx1b gene (Supplemental Fig. S7C).

Importantly, deletion of either the entire intron 9 (∆intron 9, 226-nt deletion) or its fragment containing Ptbp1 consensus binding sites (∆PS, 125-nt deletion) from the Stx1b transgene (Supplemental Fig. S7A,B) completely abolished the regulation of mRNA abundance in response to siPtbp1 or siPtbp1/2 (Supplemental Fig. S7D). Notably, splicing of the wild-type transgenic intron 9 was regulated by Ptbp1 similarly to the endogenous intron 9, whereas the ∆PS intron was spliced in a constitutive manner (Supplemental Fig. S7E,F). As expected, no retention products were detected for the ∆intron 9 transgene (Supplemental Fig. S7E).

A more detailed bioinformatic analysis of the mouse Stx1b intron 9 sequence revealed two putative Ptbp1-binding surfaces: one highly conserved across placental mammals [PS1], and the other one poorly conserved [PS2] (Fig. 3E). To examine the significance of these elements, we mutated them in the minigene context. Interestingly, mutating PS1 and PS2 individually reduced the response of minitransgene expression to Ptbp1 or Ptbp1/2 knockdown, whereas the mutPS1/mutPS2 double mutation abolished the regulation completely (Fig. 3F). We concluded that intron 9 is necessary for mRNA abundance control by Ptbp1 and Ptbp2 and that both PS1 the PS2 elements are likely required for optimal regulation.

**Intron 9 recognition by the splicing machinery is required for nuclear retention and degradation of the incompletely spliced Stx1b mRNA**

Two models could account for the repressive effect of intron 9 retention on mRNA export: [1] Intron-bound Ptbp1 completely inhibits the intron interaction with the splicing machinery and simultaneously hinders the export of the incompletely spliced mRNA to the cytoplasm. [2] Alternatively, the intron is recognized by the early components of the splicing machinery [e.g., by U1 snRNP, U2AF, and/or U2 snRNP], but the subsequent splicing and mRNA export steps are arrested in the presence of intron-bound Ptbp1.

To distinguish between these possibilities, we inactivated the 5′ splice site [5′ss] within the Stx1b(MCS) minitransgene intron 9 by changing the consensus GU sequence to CC and analyzed the expression of the mutated mRNA [Stx1b[mut5′ss]] in CAD cells treated with siControl or siPtbp1/2 siRNAs (Fig. 4A). RT–PCR analysis confirmed that the mutation completely inactivated intron splicing [Fig. 4B]. Notably, in siControl transfected cells, overall Stx1b[mut5′ss] expression increased 3.6-fold as compared with the Stx1b(MCS)
minitransgene ($P = 0.04$, $t$-test) [Fig. 4C]. Moreover, Ptbp1/2 knockdown had no effect on Stx1b[mut5’ss] transcript levels, unlike Stx1b[MCS], whose expression increased 2.2-fold following this treatment ($P = 0.03$, $t$-test) [Fig. 4C].

siPtbp1/2 also had little effect on the apparent abundance of the Stx1b[mut5’ss] transcript in the cytoplasmic and nuclear fractions. In contrast, Stx1b[MCS] transcript levels were significantly up-regulated in both the cytoplasm [3.3-fold; $P = 0.008$, $t$-test] and the nucleus [1.3-fold; $P = 0.04$, $t$-test] [Fig. 4D]. The apparent abundance of the Stx1b[mut5’ss] transcripts was comparable between the nuclear and cytoplasmic fractions in both siControl- and siPtbp2-treated samples, whereas siPtbp1 and siPtbp1/2 dramatically stimulated the intron splicing.

Figure 3. Stx1b intron 9 is sufficient and necessary for the Ptbp1-dependent regulation. (A) Integration of the Stx1b minitransgene into the CAD-A13 cell genome using RMCE. (B–D) Recombinant CAD-A13 cells containing a single copy of the Stx1b minitransgene were treated with the indicated siRNAs for 48 h, followed by 24-h incubation with or without Dox. (B) RT-qPCR analysis showing that the minitransgene expression levels increase following the Ptbp1 or the double Ptbp1/2 knockdown. (C) Top The splicing status of the transgenic intron 9 was assayed by RT–PCR. (Bottom) No PCR signal was detected when reverse transcriptase was omitted from the RT reactions. (D) Quantification of the data in C indicates that a large fraction of transgenic transcripts retain intron 9 in siControl- and siPtbp2-treated samples, whereas siPtbp1 and siPtbp1/2 dramatically stimulate the intron splicing. (E) Diagram of the exon 9–intron 9–exon 10 fragment of the mouse Stx1b gene. The phastCons plot (green) shows the probability of sequence conservation across placental mammalian species (Siepel et al. 2005). Note that a fragment of intron 9 is conserved across species. The orange rectangle marks the position of a poorly conserved [C/T]n repeat predicted by RepeatMasker [http://www.repeatmasker.org]. The wild-type PS1 and PS2 sequences containing consensus Ptbp1-binding sites (underlined) and the corresponding mutations are indicated at the bottom. (F) Recombinant CAD-A13 cells containing a single copy of either the wild-type minitransgene or minitransgenes containing the PS1 and/or PS2 mutations were treated with the indicated siRNAs for 72 h, and the expression levels of the transgenic transcripts were analyzed by RT-qPCR. Note that the individual mutations reduce the minitransgene response to the Ptbp1 or the Ptbp1/2 knockdown, whereas the PS1/PS2 double mutation completely abolishes the regulation. Data in B and F are averaged from three experiments, ±SD.
nuclei of siPtbp1/2-treated cells [Fig. 4E]. Similar to the Stx1b(mut5’ss) behavior, Stx1b(mut5’ss/mut3’ss) transcripts with mutations in both the 5’ss and the 3’ss were efficiently expressed in the nucleus and the cytoplasmic compartments regardless of the Ptbp1 and Ptbp2 concentrations [Fig. 4A–E]. These results suggested that the nuclear retention and nuclear degradation of incompletely spliced Stx1b mRNAs require a functional interaction between intron 9 and the splicing machinery.

Stx1b expression is regulated by the nuclear mRNA surveillance machinery

The data presented above are consistent with the model that incompletely spliced mRNAs encoding presynaptic proteins are retained in the nucleus and degraded by the nuclear mRNA quality control machinery [e.g., see Fig. 2F]. To identify factors involved in this process, we knocked down several known nuclear RNA surveillance components, including catalytic [Exosc10/Rrp6 and Dis3/Rrp44] and structural [Exosc1/Csl4 and Exosc9/Rrp45] subunits of the exosome complex; a nuclear 5’-to-3’ exonuclease, Xrn2; a nuclear pore protein, Tpr [Mlp1/2 homolog]; and a nuclear poly(A)-binding protein, Pabp2 [Pab2 homolog] [Fig. 5A; Galy et al. 2004; Houseley and Tollervey 2009; Tomecki and Dziembowski 2010; Coyle et al. 2011; Kiss and Andrulis 2011; Lemieux et al. 2011].

Strikingly, the most prominent increase in the Stx1b mRNA expression was detected in the Tpr knockdown samples [Fig. 5B]. This was not an off-target effect, since an shRNA against a distinct Tpr-specific sequence stimulated Stx1b expression in a similar manner (data not shown). Stx1b expression was also stimulated by the Dis3 and Exosc9 knockdowns and, to a lesser extent, the Exosc10 and Exosc1 knockdowns (Fig. 5B). No significant changes in the Stx1b levels were detected in the samples expressing Xrn2- and Pabp2-specific shRNAs (Fig. 5B). Similarly similar effects were observed for the Vamp2, Sv2a, and Kif5a mRNAs, except for the lack of significant effect of the Exosc9 knockdown in the case of Vamp2 and the Exosc1 knockdown in all three cases [Supplemental Fig. S8]. Importantly, the levels of the Hprt and Actb “housekeeping” mRNA controls were not significantly up-regulated in the knockdown samples [Fig. 5C; Supplemental Fig. S8]. We concluded that intron-retained RNAs are specifically destabilized in the nucleus in a Tpr- and exosome-dependent manner.

Ptbp1 expression correlates with 3’-terminal intron splicing and mRNA abundance of neuronal genes in vivo

To test whether the above mechanisms functioned in the context of development, we analyzed the expression of
the Ptbp1 protein and its SNARE targets, Stx1b and Vamp2, in embryonic day 13.5 (E13.5) mouse brains [Fig. 6A]. Ptbp1 was expressed predominantly in the neuroepithelial layer containing proliferating NSCs, whereas Stx1b and Vamp2 showed reciprocal staining patterns, with the maximal expression in the mantle layer containing neurons at different stages of differentiation [Fig. 6A]. The expression of mature miR-124 miRNA in the developing neural tube was reciprocal to the Ptbp1 expression pattern, as reported previously [Makeyev et al. 2007], and overlapping with the Stx1b expression pattern (Supplemental Fig. S9A,B).

We further examined 3′-terminal intron splicing for the Stx1b, Vamp2, Sv2a, and Kif5a transcripts in adult and embryonic mouse tissues [Fig. 6B–F; Supplemental Fig. S9C,D]. Although the four transcripts were expressed in virtually all samples (except for the lack of the Sv2a expression in the adult liver), the percentage of the transcripts retaining the 3′-terminal introns varied dramatically across the tissues. Virtually no intron-retained products were detected in the total adult brain or its nuclear and cytoplasmic fractions [Fig. 6B,C; Supplemental Fig. S9C,D]. We also failed to detect intron-retained Vamp2, Sv2a, and Kif5a RNAs in unfractonated E12.5 brain samples [Fig. 6B,C; Supplemental Fig. S9]. However, these species were detected in the corresponding nuclear fractions [Fig. 6B; Supplemental Fig. S9C]. Substantial amounts of the intron 9-retained Stx1b transcripts were expressed in both the total E12.5 brain samples and the nuclear fraction, while being noticeably depleted from the cytoplasmic fraction [Fig. 6B,C]. Strikingly, all three nonneural tissues contained readily detectable amounts of the intron-retained transcripts [Fig. 6D,E; Supplemental Fig. S9D].

Quantitative analyses showed that the Ptbp1 expression levels correlated positively with the relative abundance of the retained mRNA species in the corresponding tissues [Spearman's correlation coefficient $r = 0.8$, $P = 4.0 \times 10^{-6}$] and negatively with the overall expression levels of the Stx1b, Vamp2, Sv2a, and Kif5a genes ($r = -0.83$, $P = 7.0 \times 10^{-7}$) [Fig. 6G; Supplemental Fig. S9E]. These results are consistent with the role of Ptbp1 as a negative regulator of the Stx1b, Vamp2, Sv2a, and Kif5a genes in vivo.

Ptbp1 regulates Stx1b expression in primary mouse cells

As a direct test of the Ptbp1 repressor activity in primary mouse cells, we knocked down Ptbp1 expression in embryonic cortical NSCs by RNAi. Immunoblot analysis of the siPtbp1- and siControl-treated samples suggested that the reduction in the Ptbp1 protein levels led to a noticeable increase in the Stx1b protein expression [Fig. 7A]. This was accompanied by a 2.1-fold decrease in the relative abundance of the intron 9-retained Stx1b transcripts [Fig. 7B] and a modest but significant increase in the overall Stx1b mRNA abundance ($P = 6.5 \times 10^{-5}$) [Fig. 7C]. The increased Stx1b expression was not due to siPtbp1-induced differentiation, since virtually all cells in the siControl- and siPtbp1-treated samples expressed the NSC marker nestin [Supplemental Fig. S10A]. Ptbp1 knockdown did not change the expression of Stx1a, a Stx1b paralog lacking extensive Ptbp1-binding motifs within its last intron [Fig. 7C]. Similarly, transfection of primary mouse embryonic fibroblasts (PMFs) with siPtbp1/2 decreased the relative abundance of the intron 9-retained species 10-fold [Fig. 7D] and increased the

**Figure 5.** Nuclear RNA surveillance machinery is involved in the Stx1b regulation. (A) CAD-A13 cells encoding stably integrated shRNAs under the control of a Dox-inducible promoter (Khandelia et al. 2011) were treated with 2 μg/mL Dox for 72 h, and the knockdown efficiencies were analyzed by RT-qPCR. Shown are residual mRNA expression levels normalized to the expression of the corresponding mRNAs in the presence of a firefly luciferase-specific shRNA control. Values are averaged from three amplification experiments, ±SD. Note that in all five cases, mRNA expression is noticeably diminished by the gene-specific shRNAs. (B) Stx1b mRNA expression levels were assayed by RT-qPCR following the knockdown of the indicated components of the RNA surveillance machinery. Note that shRNA specific to the nuclear pore-associated protein Tpr and the exosome subunit Dis3 leads to statistically significant accumulation of the Stx1b mRNA. (C) None of the shRNAs tested in this experiment led to significant up-regulation of the control Hprt mRNA. In A and C, the $P$-values were calculated using a two-tailed $t$-test assuming unequal variances and shown only for the samples showing significant ($P \leq 0.05$) up-regulation of mRNA expression in response to shRNA treatment as compared with the luciferase shRNA control. Data are normalized to the expression levels of Gapdh mRNA and are averaged from at least three amplification experiments, ±SD.
Figure 6. Ptbp1 expression pattern is consistent with its role as a regulator of presynaptic genes in vivo. (A) Immunofluorescence analyses of E13.5 medulla sections of the hind brain show that the Ptbp1 protein is expressed in the NSC-containing neuroepithelial layer (NL) lining the fourth ventricle (FV) but not in the mantle layer (ML) containing neurons at different stages of differentiation. Stx1b and Vamp2 proteins are expressed in a strictly reciprocal manner. The sections are additionally stained with antibodies against Map2, a marker of mature neurons. (B) Total RNAs were purified from the entire adult and E12.5 embryonic mouse brains or the corresponding cytoplasmic and nuclear fractions, and the Stx1b, Vamp2, and Sv2a 3′-terminal intron splicing was analyzed by RT–PCR. Nuclear [45S pre-rRNA] and cytoplasmic [7SL] RNA markers were analyzed by RT–PCR to confirm the nucleocytoplasmic fractionation quality. (C) Quantification of the results in B. (D,E) The analyses in B and C were repeated for six adult and embryonic tissue samples. (F,G) RT-qPCR analyses of Stx1b, Vamp2, Sv2a, Kif5a, and Ptbp1 expression in vivo.
expression of the Stx1b [2.1-fold, \( P = 1.0 \times 10^{-4} \), t-test] but not the Stx1a mRNA (Fig. 7E).

To test whether rescue of the Ptbp1 levels in post-mitotic neurons—where it is naturally diminished by miR-124 [Makeyev et al. 2007]—would reduce presynaptic gene expression levels, we nucleofected primary cortical neurons from E15.5 mouse embryos (Supplemental Fig. S10B) with a plasmid encoding a constitutively
expressed EGFP marker and a Dox-inducible Ptbp1 cDNA lacking its natural 3′ UTR to bypass miR-124 regulation [Fig. 7F]. Nucleofected EGFP-positive neurons were then isolated from neuronal cultures by fluorescence-activated cell sorting (FACS) and analyzed by RT-qPCR and RT–PCR. Notably, steady-state levels of Stx1b and Vamp2 but not Actb mRNA decreased significantly in the presence of exogenous Ptbp1 [Fig. 7G,H]. Moreover, Ptbp1 overexpression arrested splicing of the regulated Stx1b and Vamp2 introns, as expected [Fig. 7I]. We concluded that Ptbp1 represses presynaptic gene expression in vivo.

Discussion

Intron retention is a major form of alternative splicing in metazoan organisms that contributes to proteome diversity, regulates the efficiency of translation, and modulates intracellular mRNA localization [Galante et al. 2004; Denis et al. 2005; Mansilla et al. 2005; Marinescu et al. 2007; Wang and Burge 2008; Bell et al. 2010; Nilsen and Graveley 2010; Buckley et al. 2011]. Here we show that the RNA-binding protein Ptbp1 coordinately regulates the expression levels of at least four functionally linked neuron-specific genes (Stx1b, Vamp2, Sv2a, and Klf5a) by inhibiting the 3′-terminal intron splicing and thus promoting nuclear retention and nuclear degradation of the incompletely spliced mRNAs [Fig. 7J].

This regulation mechanism adds a compelling example to the growing list of RNA-binding proteins that function as post-transcriptional master regulators coordinating the expression of functionally linked genes [Keene 2007]. There is also an interesting parallel between the Ptbp1-controlled pathway uncovered in our study and the transcriptional repression of neuronal genes in nonneuronal cells by the REST/NRSF complex [Ballas and Mandel 2005]. Similar to Ptbp1, components of this transcriptional repressor complex are expressed at high level in nonneuronal cells, and reduced expression of these proteins in mature neurons is essential to activate a number of neuron-specific genes containing the REST/NRSE-binding motifs [Ballas and Mandel 2005].

While further work will be required to address the functional significance of the 3′-terminal location of the regulated introns, at least one additional gene (Klc1) encoding a Klf5a-interacting kinesin light chain appears to be regulated through a related mechanism. Klc1 encodes multiple splice forms, some of which are restricted to the nervous system [DeBoer et al. 2008]. Our RNA-seq data indicate that the effect of Ptbp1 and, possibly, Ptbp2 on Klc1 mRNA is threefold: [1] reduced overall abundance, [2] retention of the two 3′-terminal introns flanking the neuron-specific cassette exon, and [3] repression of the neuron-specific penultimate cassette exon and the 3′-terminal alternative exon [Supplemental Fig. S11].

Notably, introns neighboring alternative exons regulated by the TDP-43 and NOVA splicing regulators have recently been shown to change their retention status during brain development [Ameur et al. 2011]. This effect might be explained by a largely post-transcriptional splicing of introns adjoining repressed exons demonstrated by Tyagi and colleagues [Vargas et al. 2011]. Although not shown experimentally, we predict that regulation of intron splicing in these cases may also be used as a mechanism for regulating the abundance of the corresponding mRNAs and that the mechanistic link between intron retention status, utilization of alternative exons, and mRNA steady-state expression levels in mammalian cells might be substantially more prevalent than currently thought.

Of note, the Stx1b splice form retaining intron 9 has previously been hypothesized to generate a truncated Stx1b protein [Pereira et al. 2008]. While we do not exclude the possibility that minute amounts of this protein may indeed be produced under some circumstances, the significant nuclear enrichment of the intron 9-containing Stx1b mRNA species in both neuroblastoma cells and embryonic tissues argues that the main function of this intron is to regulate mRNA abundance.

Several lines of evidence argue that the Stx1b, Vamp2, Sv2a, and Klf5a genes are controlled by a mechanism distinct from the NMD. [1] These genes are not up-regulated by CHX [Fig. 1B]. [2] The 3′ position of the regulated introns makes NMD an unlikely scenario, since the 3′-terminal exon–exon junction is located upstream of a translation termination codon regardless of intron splicing status. [3] The Stx1b minitranseqe lacking a functional translation initiation codon is regulated at the mRNA abundance level [Fig. 3A–D], and this regulation is abolished by mutation of the intronic 5′s, which is not expected to result in a PTC [Fig. 4]. Moreover, translocation of the intron-retained Stx1b transcripts to the cytoplasm by inactivating the intronic splice sites [Fig. 4] leads to a significant increase in the mRNA levels, which rules out models invoking any form of cytoplasmic instability of intron-containing transcripts.

The requirement for functional splice sites [Fig. 4] suggests that the regulation circuitry identified in our study involves interaction of the retained introns with the pre-mRNA splicing machinery. Interestingly, the U1 snRNP and U2AF complexes interacting with the 5′ss and the 3′ss have been shown to facilitate nuclear retention of incompletely spliced transcripts [Rain and Legrain 1997; Takemura et al. 2011]. Nuclear retention of incompletely spliced transcripts has additionally been shown to be modulated by cis-regulatory elements. Some intron-containing retroviral and cellular transcripts are known to be efficiently translocated to the cytoplasm due to their interaction with the Tap/NXF1 or Crm1 export factors [Cullen 2003; Li et al. 2006]. It is possible that similar strategies are used by other incompletely spliced mRNA species that have been shown to accumulate in the cytoplasm [Denis et al. 2005; Mansilla et al. 2005; Marinescu et al. 2007; Buckley et al. 2011]. On the other hand, specialized elements have been described that promote RNA retention in the nucleus [Taniguchi et al. 2007]. Further work will be required to understand the cis-regulation underlying nuclear retention of incompletely spliced presynaptic mRNAs.

Our RNAi experiments [Fig. 5; Supplemental Fig. S8] argue that the incompletely spliced Stxl1b mRNA retained...
in the nucleus is cleared by a branch of the nuclear RNA surveillance machinery comprising the exosome complex and Tpr, a mammalian homolog of the yeast nuclear pore basket proteins Mlp1/2 previously implicated in nuclear surveillance of unspliced mRNA in *Saccharomyces cerevisiae* [Galy et al. 2004; Houseley and Tollervey 2009; Tomecki and Dziembowski 2010; Coyle et al. 2011]. The involvement of Tpr in the surveillance of natural intron-retained mRNAs species is an important finding, since the role of this protein in the metabolism of incompletely spliced RNAs in the mammalian system has thus far been documented only for recombinant transcripts containing intron Tap/NXF1-dependent nuclear export signals [Coyle et al. 2011].

Interestingly, of the four exosome subunits analyzed in our study, knockdown of Dis3/Rrp44, Exosc10/Rrp6, and Exosc9/Rrp45 led to comparable up-regulation of the intron-retained transcripts [except for the lack of significant effect of shExosc9 on Vamp2 expression], whereas down-regulation of Exosc1/Cal4 resulted in noticeably less-pronounced effects [Fig. 5; Supplemental Fig. S8]. These data suggest that only a subset of exosomal components may function in the nuclear surveillance of incompletely spliced RNAs, consistent with the recently proposed “exozyme” hypothesis [Kiss and Andrilis 2011].

In conclusion, this study suggests that Pebp1-controlled intron retention synchronizes the expression of critical presynaptic proteins during neuronal differentiation and counters their aberrant expression in nonneuronal cells. We predict that similar mechanisms might contribute to the regulation of other functionally linked gene networks in higher eukaryotes.

**Materials and methods**

**RNAi**

The parental CAD cells [Y Li et al. 2007] and their derivatives were transfected with Pebp1- and/or Pebp2-specific or control ON-TARGETplus siRNAs (Dharmacon) using Lipofectamine 2000 [Invitrogen] and analyzed 72 h post-transfection. Expression of shRNAs was induced by treating corresponding CAD-A13 cell pools with 2 µg/mL Dox for 72 h as described [Khandelia et al. 2011]. To knock down Pebp1 and Pebp2 expression in PMEF cells, they were transfected with the corresponding siRNAs and RNAi MAX [Invivogen] twice over a 46-h interval so that the cells were exposed to siRNA for 72 h in total. To deliver siRNAs into neurosphere cultures, we used the Amaxa mouse NSC nucleofection protocol with minor modifications. Briefly, 4 × 10^6 cells were nucleofected with 100 pmol of corresponding siRNA and plated onto 6-cm culture dishes coated with polyornithine and fibronectin. The cells were harvested 72 h following the nucleofection. Alternatively, we used two rounds of nucleofection, each with 50 pmol of siRNA. In this case, the cells were maintained as neurospheres for 36 h following the first round and as adherent culture for 36 h following the second round of nucleofection.

**RNA-seq**

RNA-seq libraries were prepared in principle as described [Mortazavi et al. 2008]. Total RNAs were extracted from siRNA-treated CAD cells with Trizol [Invitrogen], and the poly(A)^+ mRNA fractions were prepared using a Dynabeads mRNA Direct kit [Invitrogen]. The purified mRNA samples were partially hydrolyzed for 2.5 min at 94°C in the presence of MgCl2, which was followed by the first strand cDNA synthesis with the SuperScript III and random primers [Invitrogen]. The second strand cDNA synthesis and adapter ligation steps were carried out as recommended by Illumina. DNA fragments of 350 bp ± 50 bp were then purified with SizeSelect E-gels [Invitrogen] and amplified by 15 cycles of PCR using Phusion DNA polymerase (New England Biolabs) and Illumina primers. Sequencing was carried out using a Genome Analyzer IIX [Illumina]. The RNA-seq data were analyzed using the ExpressionPlot pipeline [Friedman and Maniatis 2011]. The RNA-seq data have been deposited in NCBI Gene Expression Omnibus (accession no. GSE37933).

**RMCE**

CAD-A13 cells expressing Stx1b minitransgenes and shRNAs against RNA surveillance components were generated as described [Khandelia et al. 2011]. Briefly, 2 × 10^5 CAD-A13 cells were transfected with a mixture containing 99% of a recombination donor plasmid and 1% of the Cre expression plasmid [pEM784] using Lipofectamine 2000 [Invitrogen]. Transgenic cell populations were obtained by treating the transfected cells with 2.5–5 µg/mL puromycin for 7–10 d and pooling the puromycin-resistant colonies. Single-copy integration of BAC-encoded Frt-marked Stx1b transgenes was carried out similarly, except five to six individual puromycin-resistant colonies were picked up in this case and propagated independently. Clones with correctly integrated Stx1b transgenes were used in the subsequent experiments. A complete description of the BAC RMCE protocol will be published elsewhere (K Yap, ZQ Lim, and EV Makeyev, in prep.).

See the Supplemental Material for additional details.

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**References**


