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Genome-wide Analysis of PTB-RNA Interactions Reveals a Strategy Used by the General Splicing Repressor to Modulate Exon Inclusion or Skipping

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SUMMARY

Recent transcriptome analysis indicates that > 90% of human genes undergo alternative splicing, underscoring the contribution of differential RNA processing to diverse proteomes in higher eukaryotic cells. The polypyrimidine tract-binding protein PTB is a well-characterized splicing repressor, but PTB knockdown causes both exon inclusion and skipping. Genome-wide mapping of PTB-RNA interactions and construction of a functional RNA map now reveal that dominant PTB binding near a competing constitutive splice site generally induces exon inclusion, whereas prevalent binding close to an alternative site often causes exon skipping. This positional effect was further demonstrated by disrupting or creating a PTB-binding site on minigene constructs and testing their responses to PTB knockdown or overexpression. These findings suggest a mechanism for PTB to modulate splice site competition to produce opposite functional consequences, which may be generally applicable to RNA-binding splicing factors to positively or negatively regulate alternative splicing in mammalian cells.

INTRODUCTION

Alternative splicing has been increasingly appreciated as a major mechanism to generate structural and functional diversity of gene products in higher eukaryotic cells (Black, 2003; Maniatis and Tasic, 2002). A recent transcriptome analysis indicated that more than 90% of human genes undergo alternative splicing and that many mRNA isoforms appear to be regulated in a tissue-specific manner (Wang et al., 2008). Differential RNA splicing is controlled by many RNA-binding proteins that recognize intronic and exonic *cis*-regulatory RNA elements, a second code of the genome for posttranscriptional regulation of gene expression (Black, 2003). Characterized *cis*-acting elements can be generally classified into intronic splicing enhancers (ISEs) or silencers (ISSs) and exonic splicing enhancers (ESEs) or silencers (ESSs), which act to positively or negatively influence the selection of alternative splice sites (Fu, 2004). However, splicing regulators can often affect alternative splicing in a position-dependent manner, as has recently emerged from genome-wide analysis of RNA-binding splicing regulators (Licatalosi et al., 2008; Yeo et al., 2009).

The polypyrimidine tract-binding protein PTB (also known as hnRNP I) is a well-characterized splicing repressor on model minigene constructs (Spellman and Smith, 2006). PTB binds to CU-rich elements, often overlapping with the U2AF65-binding sites near the 3′ splice site. Therefore, one of the mechanisms for PTB-mediated splicing repression is thought to compete with U2AF65 binding (Sauliére et al., 2006; Singh et al., 1995). PTB also binds to CU-rich sequences in many exonic and intronic regions to influence splice site selection by interfering with the process of exon definition (Izquierdo et al., 2005), obstructing intron definition (Chou et al., 2000; Sharma et al., 2005) or preventing the transition from exon to intron definition (Sharma et al., 2008).

To explain how PTB prevents spliceosome assembly events across exons or introns, it was initially proposed that PTB homodimers might induce RNA looping to sequester the alternative exon from the splicing machinery (Oh et al., 1998; Pérez et al., 1997b). However, a later study indicates that PTB exists as a monomer in solution, capable of binding to RNA with high affinity (Amir-Ahmady et al., 2005; Monie et al., 2005), and an NMR study suggests that PTB may use different RRMs (PTB has four) to contact CU-rich RNA elements at different locations to induce RNA looping (Oberstrass et al., 2005). Although no direct experimental evidence is available to demonstrate RNA looping mediated either by PTB dimers or by two RRMs within a single PTB molecule, both models predict extensive PTB-mediated RNA networks during regulated splicing, which is also consistent with the observation that mutating one PTB-binding site reduces PTB binding to another site in a model pre-mRNA substrate (Chou et al., 2000).

Although PTB is a well-known splicing repressor, recent splicing array analyses revealed both PTB-dependent exon

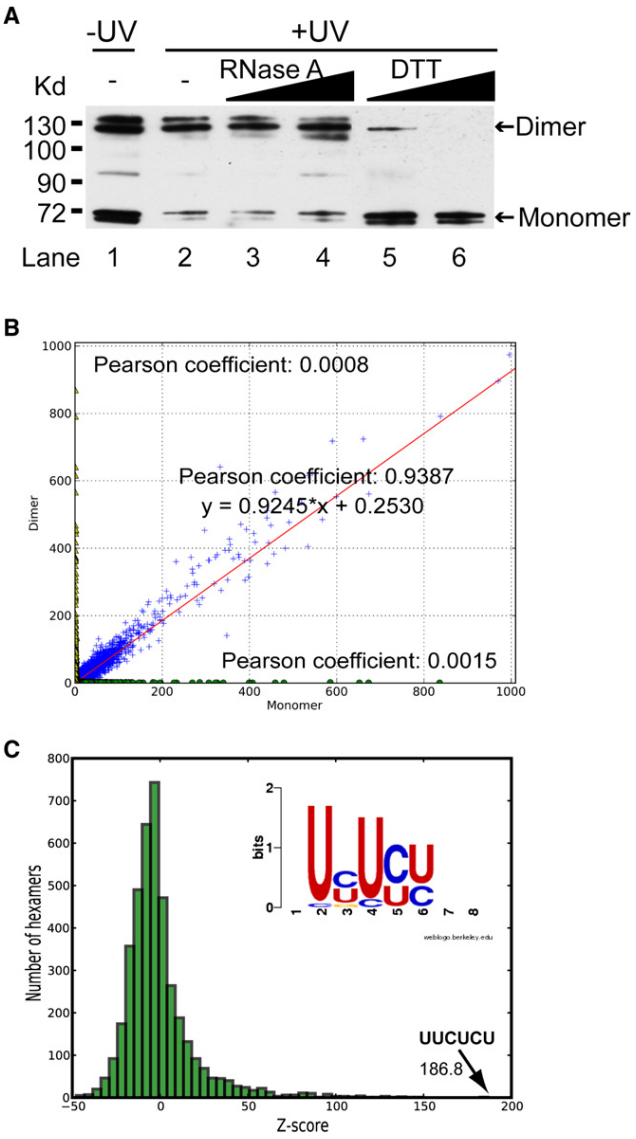

Figure 1. Extensive PTB-PTB and PTB-RNA Interactions In Vivo

(A) Western blotting analysis of immunoprecipitated PTB from mock-treated and UV-treated HeLa cells. The protein was resolved by SDS-PAGE in the presence of either 1 mM (nonreducing) or 10 mM (reducing) DTT as indicated.

(B) Overlapping binding with monomeric and dimeric PTB. The Pearson coefficient between monomeric and dimeric tags in a 500 bp window across the whole genome is high (0.9387), which contrasts with the lack of correlation between randomized tags and monomeric tags (0.0015) or dimeric tags (0.0008) (see Figure S5 for additional details).

(C) Overrepresented PTB-binding motifs identified by CLIP-seq. Histogram of Z scores indicates the enrichment of hexamers in CLIP-seq clusters compared to randomly chosen regions of similar sizes in the same genes. Z score of the top hexamer is indicated. Insert shows the PTB-binding consensus calculated from the top 20 enriched hexamers.

inclusion and skipping (Boutz et al., 2007; Xing et al., 2008). A recent observation indicates that PTB can promote exon inclusion by antagonizing an inhibitory binding event by a different splicing repressor (Paradis et al., 2007). However, it is unclear how widely this "repression-of-repressor" strategy is used by PTB to regulate alternative splicing. It has also been postulated that PTB may act in a similar fashion to the Nova and Fox families of splicing regulators to promote or suppress splice site selection in a location-dependent manner (Boutz et al., 2007). Genome-wide analysis provides a unique opportunity to directly test this hypothesis, which is key to understanding the contribution of PTB to the splicing code in mammals.

Here, we employed CLIP-seq to identify direct RNA targets for PTB in HeLa cells, finding that PTB bound to intronic regions near the 5′ or 3′ splice site, regardless of whether the site is subject to regulation. About one-third of PTB-binding events in the human genome are linked to regulated splicing, consistent with PTB being a major splicing regulator in mammals, but the functional outcomes depend on the relative PTB binding frequency on the competing splice sites. Dominant PTB binding near the alternative splice site is correlated with exon skipping, whereas overriding PTB binding near a competing constitutive splice site is associated with exon inclusion. We further showed that PTB-mediated exon inclusion could be achieved by inserting a PTB-binding site near the flanking constitutive splice sites, thereby elevating the competitiveness of the alternative splice sites. These findings reveal a positional effect of PTB on regulated splicing through modulating the relative strength of competing splice sites, which is fundamentally distinct from the recently elucidated position-dependent activity of the Nova and Fox families of RNA-binding proteins in the regulation of alternative splicing.

RESULTS

Evidence for an Extensive PTB-RNA Interaction Network In Vivo

In preparation for genome-wide analysis of PTB binding by CLIP-seq, we first characterized a monoclonal anti-PTB antibody (BB7, described in Chou et al., 2000) on HeLa cells before and after UV treatment by immunoprecipitation/western blotting. Consistent with a previous study (Pérez et al., 1997b), we detected both PTB monomer and dimer under a nonreducing SDS-PAGE condition but predominantly monomer under a reducing condition (+DTT) (Figure 1A). UV treatment dramatically increased the dimeric fraction of PTB. However, the PTB dimer is not tethered by RNA, as it is resistant to RNase treatment (Figure 1A, lanes 3 and 4) but sensitive to DTT (Figure 1A, lanes 5 and 6), which is consistent with an early observation that the PTB dimer is held together by a specific disulfide bond (Monie et al., 2005).

The induction of PTB dimerization by UV may be interpreted to indicate that a fraction of PTB might exist as dimer before binding to RNA, and UV might catalyze the disulfide bond formation. This would agree with the PTB-PTB interaction detected in the yeast two-hybrid assay (Oh et al., 1998). Alternatively, PTB might bind to RNA as a monomer but become dimerized upon binding to RNA, which could be enhanced and/or stabilized by UV. This possibility would be consistent with the observation that PTB can bind to RNA as monomer with high affinity (Amir-Ahmady et al., 2005), but PTB binding on one site can influence PTB binding on another site in the same pre-mRNA substrate

(Chou et al., 2000). In any case, the ability of PTB to simultaneously engage in protein-protein and protein-RNA interactions suggests that PTB may nucleate extensive RNA-protein interaction networks, which are likely contributed by RNA-binding activities of individual RRMs in PTB (Oberstrass et al., 2005; Clerte and Hall, 2009).

By ^{32}P labeling, we found that both monomeric and dimeric PTB are associated with RNA (Figure S1 available online). To detect potential functional differences between the monomeric and dimeric forms of PTB, we separately isolated the two protein-RNA complexes and constructed two independent libraries for CLIP-seq analysis. We first determined the quality of the libraries by conventional cloning and sequencing, obtaining 341 and 214 unique tags associated with PTB monomer and dimer, respectively. Most of these tags (~80%) were mapped to introns as expected (Figure S2), and the average size is ~30 nt in length (Figure S3), which is consistent with the previous report that the minimal PTB-binding sequence is 30 nt (Amir-Ahmady et al., 2005). This finding suggests that the actual PTB-binding sites are likely to reside within, rather than nearby, the sequenced tags in most cases, therefore eliminating the need to computationally extend the tags for subsequent peak finding (Yeo et al., 2009). We further confirmed the specificity of the CLIP assay by performing RIP-PCR analysis on a panel of anti-PTB enriched RNAs (Figure S4).

Having thoroughly characterized the libraries, we next subjected PCR amplicons to high-throughput sequencing on Solexa, resulting in 2.44 million tags for PTB monomer and 2.37 million for PTB dimer that were uniquely mapped to the human genome (hg18). These high-density reads allowed us to ask first whether PTB monomeric and dimeric tags are differentially distributed in the genome. As shown in Figure 1B, using a 500 bp window, most of the monomeric and dimeric tags are similarly distributed in the genome with a Pearson correlation coefficient of 0.9387, and the coefficient between monomer and dimer increases with the increasing number of tags compared (Figure S5). We conclude that there are no two separate sets of sites for PTB binding as monomer or dimer in the cell.

We therefore used combined tags to determine overrepresented motifs in PTB-binding clusters (see Experimental Procedures), finding that CU-rich hexamers are highly enriched (Figures 1C and S6A). About 21% of clusters (5.17% for random; $p = 0.00$) contain the top-scored motif UUCUCU (Z score, 186.5). The top 20 motifs are all CU enriched; 83.56% of total clusters obtained contain at least one of the top 20 motifs (38.56% for random; $p = 0.00$) (Figure S6B), and the consensus generated from the top 20 hexamers is UYUYU (insert in Figure 1C). In fact, the C/U percentage is broadly elevated surrounding the PTB-binding sites, but not among randomly selected background sequences (Figure S7), which fully corroborates with biochemically defined PTB-binding characteristics (Ashiya and Grabowski, 1997; Pérez et al., 1997a). Of interest, ~90% of identified PTB-binding sites overlap with those predicted by an algorithm based on the biochemical properties of PTB (Gama-Carvalho et al., 2006), but the number of the experimentally detected sites represents only ~1% of ~5 million sites predicted in the human genome by the algorithm (data not shown). Thus, biochemically deduced consensus may not be sufficient to predict true binding sites because the recognition of some consensus motifs may be obstructed by competition of other RNA-binding proteins or by certain RNA secondary structures. Indeed, we did note a few underrepresented, A/G-rich motifs (Figure S6A), indicating that depletion of A/G-rich sequences may help maximize the single strandedness of PTB-binding sites by minimizing potential stem-loop structures due to base pairings between C/U and A/G rich sequences. These observations suggest that experimentally validated binding sites coupled with critical features in local genomic context will help to further improve prediction algorithms for RNA-binding splicing factors.

Genomic Landscape of PTB Binding

By mapping the sequenced tags to the knownGene set from the UCSC genome database, we found that 58.4% of the tags are localized in introns (Figure 2A), with the relative density (counts per kb) 17-fold higher in introns than in exons, indicating that most of the tags are derived from pre-mRNAs (a fraction of PTB-binding events may also be derived from excised lariats). A sizable fraction of tags was mapped to antisense transcripts (8.1%) and intergenic regions (28.4%), implying that PTB may also bind to many noncoding RNA and/or unannotated transcripts, which is subject to future studies.

We next focused on clustered PTB-binding events by identifying peaks above the gene-specific, randomized background as previously described (Yeo et al., 2009). The resulting 64,314 peaks were further merged to 51,394 clusters by placing PTB peaks within a 50 nt window. Of interest, whereas more than half (56.5%) of PTB-binding clusters are separated by 1 to 10 kb, as expected from independent binding events, a significant fraction (43.5%) of PTB clusters appears to be more closely positioned (<1 kb) (Figure 2B), likely reflecting a concert action of multiple PTB-binding events in regulated splicing. Further analysis revealed that PTB binds to 10,372 out of the 24,378 annotated human genes (30,986/66,803 knownGene transcripts). This number might be an underestimate because our current sequencing density has not yet reached saturation according to power analysis (data not shown). Given the fact that most sequence tags contain PTB-binding consensus, indicating that contamination with other nonspecific RNA is minimal, this binding profile suggests that PTB is a major RNA-binding protein that may be widely involved in RNA metabolism in mammals.

Association of PTB Binding with Alternative Splicing Events

We next explored how frequently PTB binding is linked to annotated alternative splicing events. We separately examined PTB association with several major modes of alternative splicing, including cassette exon, alternative 5′ splice site, alternative 3′ splice site, and retained intron, based on the knownAlt track of the UCSC genome browser (Karolchik et al., 2008). This analysis revealed that 28.3% of PTB-binding events are associated with annotated alternative splicing, and 22.2% of all annotated alternative splicing events are linked to PTB binding, thus suggesting a prevalent role of PTB as a splicing regulator in the human genome. PTB is involved in all common modes of alternative splicing (Table 1), with cassette exons being the most frequent

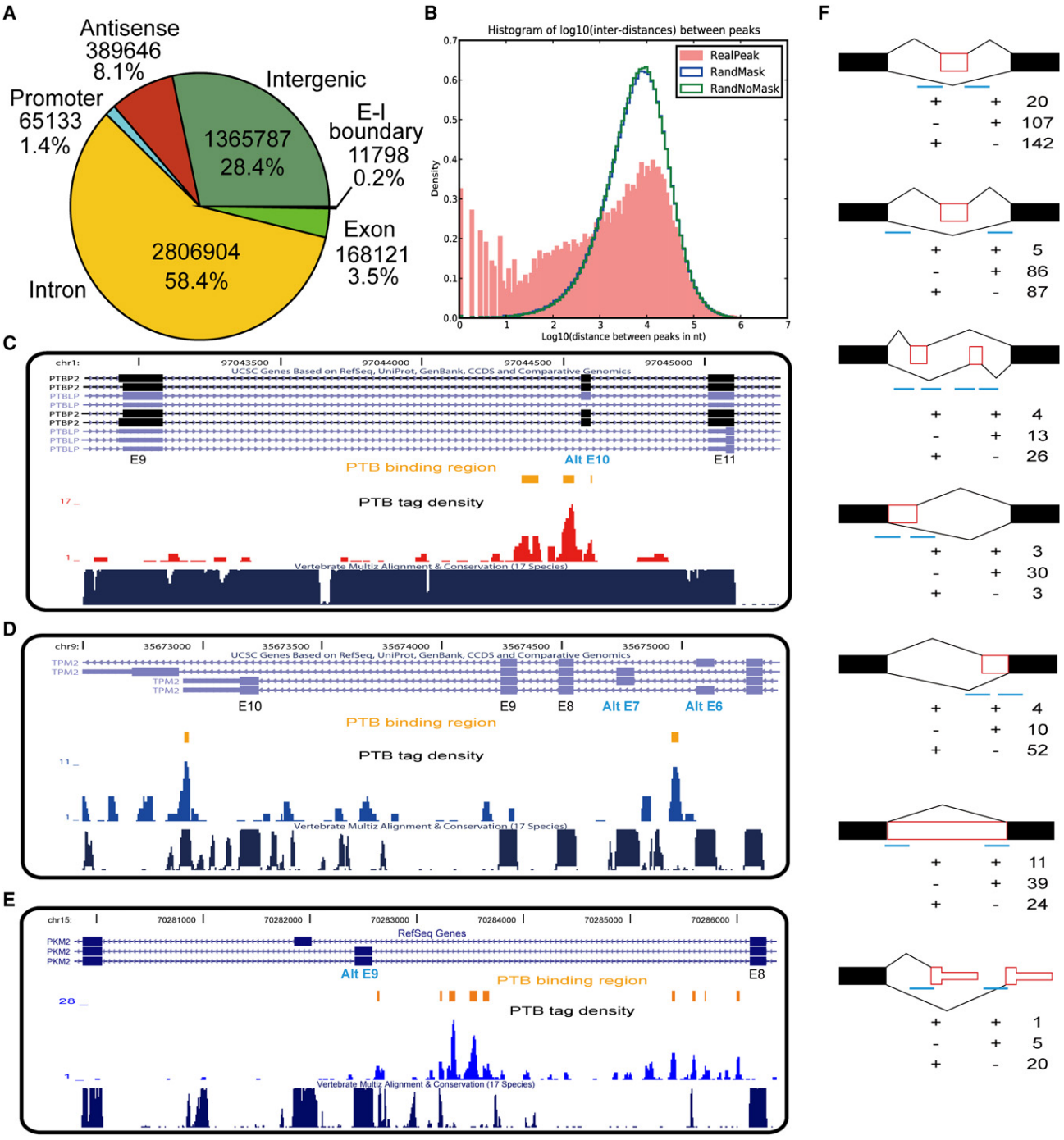

Figure 2. Genomic Landscape of PTB Binding

(A) The distribution of PTB tags in the human genome (hg18).

(B) The distribution of PTB-binding clusters relative to one another in the same genes.

(C) Screen shot of PTB binding around the well-characterized *nPTB* exon 10.

(D) Screen shot of PTB binding around *TPM2* exon 7 and the two alternative polyadenylation sites.

(E) Screen shot of PTB binding in the intron preceding the regulated exon 9 in *PKM2* gene.

(F) PTB-binding clusters associated with six major alternative RNA-processing modes. The patterns of PTB binding in 250 nt intronic and 30 nt exonic regions around splice sites were counted. The filled black boxes indicate constitutive exons or exonic regions, whereas empty red boxes show alternative exons or exonic regions. The short blue lines mark the regions where PTB binding clusters were present (+) or absent (−). The number is the total events of PTB binding at each location.

Table 1. PTB-Binding Clusters Associated with Different Modes of Alternative Splicing

Alt Event	# Total Events	# PTB Cluster Associated		
		Observed	Expected	Z Score
Cassette exon	7449	5824	5053	14.11
Alt terminal	909	815	661	8.56
Retained intron	1446	147	96	6.59
Mulx exon	522	662	581	4.04
Alt5Prime	1970	582	524	3.24
Alt3Prime	3207	805	748	2.7

Column 1 shows the total number of events in each mode extracted from the UCSC knownAlt track. The observed number of PTB clusters associated with each mode is the count of PTB clusters within the region covering the alternative exon, the flanking intron(s), and constitutive exons. The expected number is the averaged number of associations in 100 trials (random placement of PTB clusters). The column Z score shows the significance of association.

targets for PTB regulation (Z score 14.11, compared to 100 trials of randomly placed clusters). Many PTB-binding events are also found on ''constitutive'' introns and exons, which might be associated with alternative splicing events that have not yet been annotated. Alternatively, PTB may function to repress decoy splicing signals within constitutively spliced genes, which deserves a close look in future studies.

We next determined how PTB binding might affect splice site selection on both known and newly identified PTB target genes. As expected, a significant number of tags were mapped to the *PTBP2* (also known as *nPTB*) gene, a well-known PTB target in which the alternative exon 10 is repressed by PTB. Of note, PTB binds preferentially to the sequences upstream of the 3′ splice site of exon 10 in *nPTB* as previously characterized (Boutz et al., 2007; Spellman et al., 2007). We also identified a binding cluster near the downstream 5′ splice site and some distributive PTB binding in the upstream intron (Figure 2C), suggesting that PTB binds to multiple locations surrounding the regulated exon, which may collectively contribute to PTB-mediated exon repression, a situation similar to the well-characterized *c-Src* N1 exon (Sharma et al., 2005).

In another example (Figure 2D), we identified two PTB-binding clusters between the two mutually exclusive exons (exon 6 and 7) in the *TPM2* gene, which is consistent with the observed repression of exon 7 in nonmuscle cells (Sauliére et al., 2006; Spellman et al., 2007). We also detected prevalent PTB binding near the polyadenylation site for E10, in agreement with the observed utilization of the E11 polyadenylation site in nonmuscle cells. Of interest, we note multiple PTB-binding events between the regulated exons and polyadenylation sites, suggesting a potential RNA network that may underlie the coordinated regulation of both events as reported (Spellman et al., 2007).

The high-quality PTB-RNA interaction map also helps to assign PTB as a regulator to previously uncharacterized alternative splicing events. For example, the pyruvate kinase 2 (*PKM2*) gene expresses two mutually exclusive isoforms, and such regulated splicing appears to be critical for cancer metabolism and tumor growth (Christofk et al., 2008). Although PTB has been implicated in the regulation of *PKM2* splicing, critical *cis*-acting regulatory elements has remained undefined (Spellman et al., 2007). We found extensive PTB-binding clusters in the intron preceding the alternative exon 9 (Figure 2E), and RT-PCR confirmed PTB-dependent repression of *PKM2* exon 9 in HeLa cells (data not shown). This finding raises the possibility that PTB may contribute to certain cancer phenotypes by regulating the alternative splicing of *PKM2*.

PTB binding appears to associate with regulated cassette exons more significantly than do other modes of alternative splicing (Table 1). On the well-characterized *c-Src* gene, PTB binds to both sides of the regulated exon N1 (Amir-Ahmady et al., 2005). To estimate how frequently PTB binds to both sides of alternative exons or exonic sequences, we analyzed a large number of annotated alternative splicing/polyadenylation events in comparison with mapped PTB-binding events (Figure 2F). This analysis revealed several interesting trends. First, whereas the bracket binding mode of PTB is clearly associated with many regulated RNA-processing events, PTB appears to bind either up- or downstream of the alternative splice site in the majority of cases. Second, among regulated cassette exons, PTB has the same tendency to bind to one of the competing (constitutive versus alternative) splice sites, implying that PTB does not always target the alternative splice site, which has distinct functional consequences (see below). Third, in most cases of regulated 5′ and 3′ splice site choices, PTB appears to prefer binding on the intronic side, predicting that PTB may favor the distal splice site by repressing the proximal site in general.

PTB-Dependent Repression or Enhancement of Alternative Splicing In Vivo

Most minigene-based analysis focused on the consensus PTB-binding motif near a regulated exon(s), which leaves a general impression that PTB preferentially targets alternative splice sites for regulation. The PTB CLIP-seq data now offer an unbiased view on the actual location of PTB binding on PTB-regulated genes. We found both known and new locations for PTB binding on all 13 previously documented PTB-regulated exons. This prompted us to examine additional candidates based on prevalent PTB-binding events. Of 32 targets assayed, we found that 22 altered splicing in response to PTB knockdown by RNAi (>5% absolute change), and among these, 10 showed PTB-dependent inclusion, and 12 exhibited PTB-dependent exon skipping (Table S1). This finding confirmed the previous observation that PTB regulates both exon inclusion and skipping in vivo (Boutz et al., 2007; Xing et al., 2008).

Although the mechanism for PTB-mediated exon skipping is well characterized on multiple minigene models in literature, it has been unclear how PTB enhances exon inclusion. We first wished to establish sequence-dependent regulation of exon inclusion by PTB. For this purpose, we selected the *CTTN* gene that showed positive regulation by PTB to construct a minigene for analysis in transfected cells. The minigene, which contains the cassette exon 11 and flanking introns and exons, was expressed from the CMV promoter in pcDNA3 (Figure 3A; note that this minigene was spliced less efficiently than was the endogenous gene [see Figure 4B], likely because the

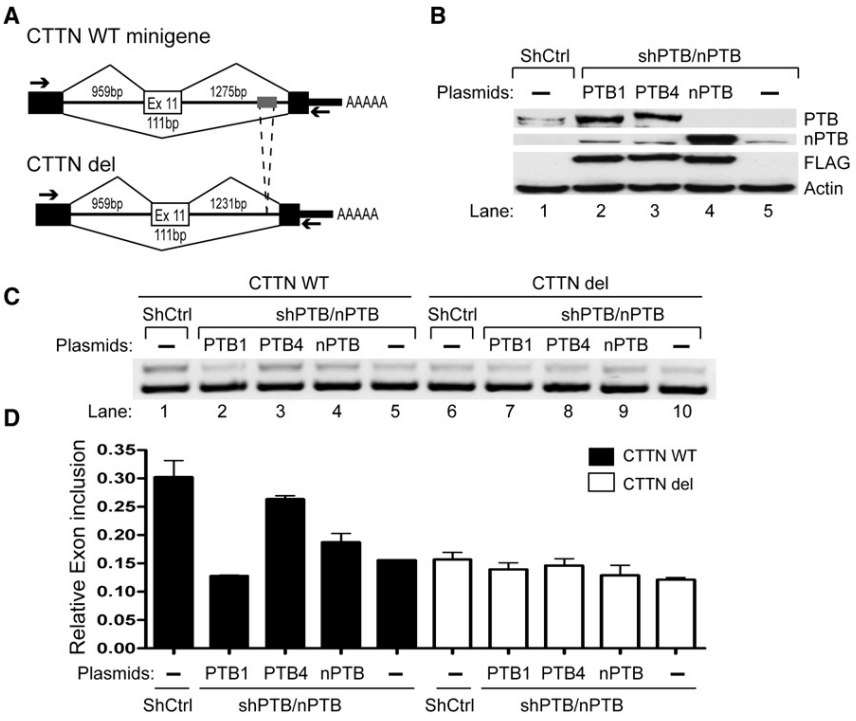

Figure 3. PTB-Dependent Inclusion of Alternative Exon

(A) Schematic representation of the *CTTN* minigene constructs, showing both wild-type and the mutant that lacks the 44 nt PTB-binding cluster. The mapped PTB-binding site is marked in gray, and the PCR primers used to detect alternatively spliced products are indicated by arrows.

(B) Exogenously expressed *PTB* isoforms and *nPTB* in double *PTB*/*nPTB* knockdown cells.

(C) Semiquantitative RT-PCR analysis of WT and mutant *CTTN* pre-mRNA splicing in response to *PTB*/*nPTB* knockdown with or without complementation with exogenously expressed *PTB* isoforms or *nPTB*.

(D) Quantification of the data as in (C) based on three independent experiments. Error bars are based on SEM; the statistical significance is determined by Student's t test ($p < 0.05$).

minigene might miss some positive regulatory elements in the construct and/or impair efficient transcription/splicing coupling as on the endogenous gene). In response to simultaneous knockdown of *PTB* and *nPTB* by shRNAs (Figure 3B, lane 5), we detected a significant reduction of exon 11 inclusion in cells cotransfected with the minigene reporter (Figure 3C, compare lane 1 treated with control shRNA with lane 5 treated with combined shRNAs against *PTB* and *nPTB*).

We next attempted to rescue the splicing defect by cotransfecting the cell with a plasmid expressing Flag-tagged PTB or nPTB, each of which contains a synonymous mutation that disrupts the shRNA target. By western blotting, these exogenous genes were robustly expressed (Figure 3B). We observed that the full-length PTB (PTB4; see below) was able to fully rescue the inclusion of the alternative exon 11 (Figure 3C, lane 3). nPTB was also capable of rescuing exon 11 inclusion to a significant degree (Figures 3C and 3D). Previous studies showed that the *PTB* gene expresses two major isoforms, PTB4 and PTB1, which differ by the presence or absence of the alternative exon 9 (Wollerton et al., 2001). We found that PTB1 had little activity in rescuing the inclusion of *CTTN* exon 11 in comparison with the exon 9-containing PTB4, even though both proteins were expressed at comparable levels in transfected cells. This observation is consistent with the previous study that reported a stronger activity of PTB4 than PTB1 in regulated splicing (Wollerton et al., 2001). In these rescue experiments, we did not detect a further increase in exon 11 inclusion even though the exogenous *PTB* or *nPTB* was overexpressed, indicating that PTB or nPTB is involved in the regulation but is not the only regulator(s) for this alternative splicing event (as a result, it is no longer a rate-limiting factor in *PTB*-overexpressed cells).

To determine whether the regulation is dependent on the mapped PTB-binding site in the intron, we deleted the 44 nt PTB-binding site in the reporter and found that the mutation abolished the response to exogenous *PTB* or *nPTB* (Figures 3C and 3D). Deletion of the PTB-binding site renders levels of exon inclusion in the *CTTN* minigene similar to those caused in the wild-type by depletion of PTB/nPTB, further supporting the involvement of these proteins in regulation. Deletion of the PTB-binding sites also abolished the functional rescue by any PTB isoforms. We conclude from these experiments that PTB/nPTB is also directly involved in regulated exon inclusion in addition to its widely perceived role in exon skipping.

Mechanistic Insights into PTB-Regulated Alternative Splicing

In order to understand the mechanisms for PTB-dependent exon inclusion or skipping, we analyzed the PTB-binding pattern with respect to the functional consequence of alternative splicing and realized some general trends for PTB-regulated splicing (Figure 4). Among PTB-mediated exon repression events, we note that PTB binding typically takes place near the alternative exon. This is clearly the case with both the *MINK1* and *EIF4G2* genes (Figure 4A, rows 1 and 2). However, PTB also binds to other intronic locations besides around the alternative exon, as seen on the *RBM27* and *FAM38A* gene (Figure 4A, rows 3 and 4). The remaining two examples (*CCDC138* and *RBM15*, rows 5 and 6 in Figure 4A) illustrate PTB binding on both sides of the regulated exon, although the upstream PTB-binding sites appear to vary in distance from the regulated exon. In these cases, we notice a relatively short intron after the alternative exon, indicating that PTB binding in the intron might obstruct the intron definition process (Fox-Walsh et al., 2005), thus resulting in PTB-dependent skipping of the alternative exon. These examples agree in general with the established principle of PTB-dependent exon skipping, wherein PTB appears to mainly

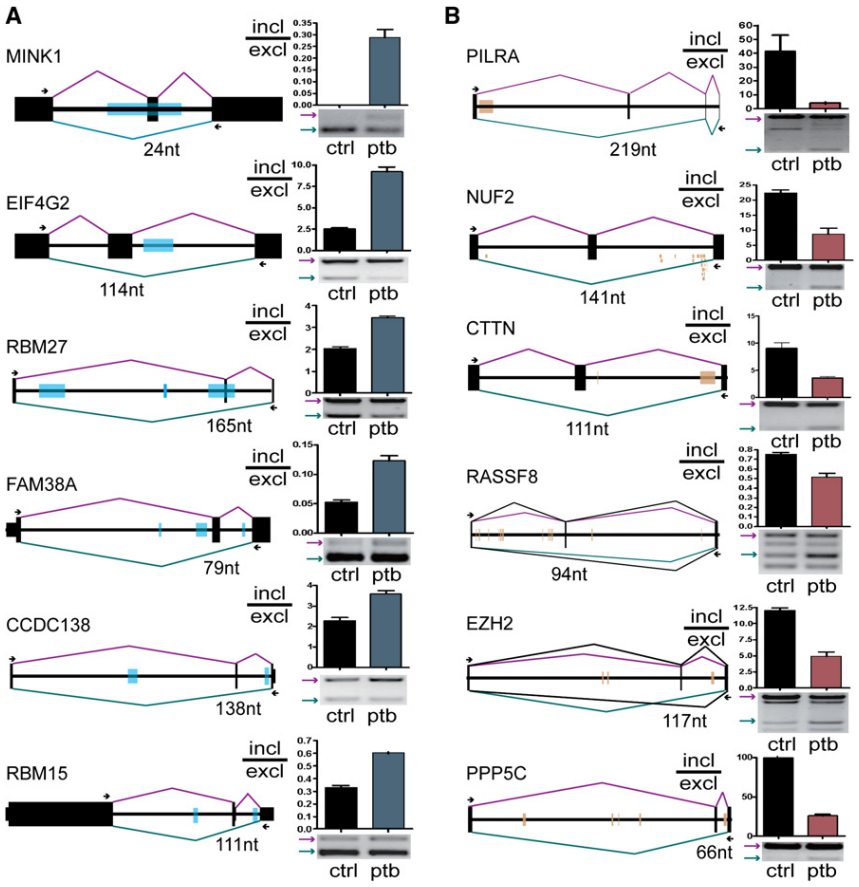

Figure 4. PTB Can Either Represses or Enhance Alternative Splicing In Vivo

(A) Examples of PTB-dependent exon skipping. Each is schematically diagramed (exon, black box; intron, black line) with mapped PTB-binding clusters as marked by blue boxes. PTB RNAi-induced splicing changes are shown on the right. Error bars are based on SEM from three independent experiments. All detected changes are significant as determined by the Student's t test ($p < 0.05$).

(B) Examples of PTB-dependent exon inclusion with mapped PTB-binding clusters marked by brown boxes. For *NUF2*, PTB tags (not clusters) are shown under the intron line.

act to interfere with the recognition of the splice sites associated with the alternative exon.

PTB-dependent exon inclusion seems to exhibit a different trend. As illustrated in Figure 4B, the first three examples exhibited PTB-binding events that are far away from the alternative exon and close to the competing constitutive 5′ (*RILRA*, row 1) or 3′ splice site (*NUF2*, row 2). This trend may also be applicable to the *CTTN* gene (Figure 4B, row 3), despite a minor PTB-binding site near the alternative exon, and our mutagenesis study showed that the major site near the downstream constitutive 3′ splice site was responsible for PTB-dependent exon inclusion (Figure 3). The remaining three examples (*RASSF8*, *EZH2*, and *PPP5C*, rows 4 to 6) are not clear-cut. PTB clearly binds to both sides of the alternative exon in each case, which is similar to the situation with PTB-dependent exon skipping events. However, both PTB-binding sites appear closer to the competing constitutive 5′ and 3′ splice sites than to the alternative exon. Together, these examples appear to point to the trend that the PTB-binding sites associated with PTB-dependent exon inclusion events are associated with competing constitutive splice sites.

To generalize the trend for both PTB-regulated exon inclusion and skipping, we collected a number of PTB-regulated exons, including 22 identified in the present study and 11 that have been previously reported in humans (Table S1). In addition, we found that the CLIP tags are generally mapped to the conserved regions on PTB-regulated mouse genes as reported previously (Boutz et al., 2007), which strongly implicates similar regulation between mice and humans. We directly tested a subset of the human orthologs of several PTB-regulated genes previously characterized on mouse cells, including *SPAG9*, *PTB*, *nPTB*, *TPM1*, *KTN1*, *TPM2*, and *MINK1*, and found that these genes all similarly responded to *PTB* knockdown in HeLa cells. We therefore included additional PTB-regulated splicing events in mouse cells (Table S1), resulting in a total of 55 PTB-regulated splicing events (41 PTB-dependent exon skipping and 14 PTB-dependent exon inclusion) for further analysis. As controls, we selected 100 groups of randomly sampled constitutive exons (each group contains 50 exons) for similar analysis.

By integrating all PTB-binding events, we generated an RNA map associated with PTB-repressed, -enhanced, and -nonregulated (constitutive) exons on a scaled pre-mRNA model, an approach that has been recently used for analysis of position-dependent activities of Nova (Licatalosi et al., 2008). Of interest, the map revealed that PTB binds to both the 5′ and 3′ splice sites of constitutive exons, as well as to both the 5′ and 3′ splice sites of alternative exons (Figure 5). Though PTB binding to the 3′ splice site is expected (because of the polypyrimidine tract as part of the splicing signal at the 3′ splice site), we were surprised by equally frequent PTB binding at the 5′ splice site. Most PTB-dependent exon-skipping events (bottom portion of Figure 5A) are associated with PTB binding near either side of the alternative exon, which is fully consistent with functional studies conducted so far on model minigenes. In contrast, the RNA map associated with PTB-dependent exon inclusion events (top portion of Figure 5A) suggests that PTB binds prevalently to the flanking constitutive splice sites, especially at the downstream constitutive 3′ splice site (Figure 5A). This most likely reflects PTB interference with the recognition of the competing constitutive 3′ splice site, therefore in favor of the selection of the upstream alternative exon. On nonregulated exons, we found no clear bias in PTB binding to intronic regions

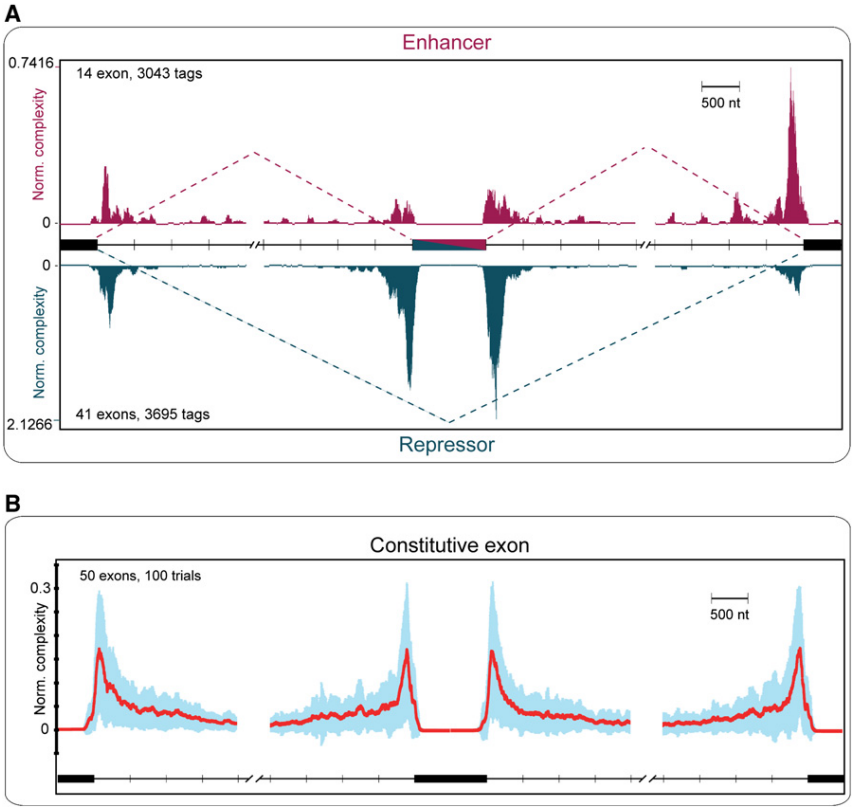

Figure 5. Composite Functional Map of PTB-Regulated Splicing

(A) PTB-regulated cassette exons are collected from previously reported cases and those that are validated in the present study. Among 55 PTB-regulated splicing events compiled, 14 exhibited PTB-dependent splicing inclusion, and 41 showed PTB-dependent exon skipping.

(B) RNA map on constitutive exons. Red line shows the average of normalized complexity of 100 sets (each contains 50 randomly selected exons) of constitutive exons; the upper and lower light-blue boundaries show the one standard deviation (see Experimental Procedures for further details).

near any upstream or downstream splice sites (Figure 5B). Together, these findings formally suggest a PTB-mediated splice site titration mechanism by which the relative binding frequency near the competing constitutive and alternative splice sites dictates the functional outcome, which appears to be neutralized on nonregulated constitutive exons (see further in Discussion).

Induction of Exon Inclusion by Engineered PTB-Binding Sites

We demonstrated that the PTB-binding site near the constitutive 3′ splice site of the *CTTN* gene is responsible for PTB-dependent inclusion of the upstream alternative exon (Figure 3). To further test the hypothesis that PTB induces the inclusion of the alternative exon by weakening the competing constitutive splice site(s), we engineered a different minigene containing a *SIRT1* exon (Figure 6A), which was previously used to screen for *cis*-acting splicing suppressors (Wang et al., 2004). To improve the PTB response range of the reporter, we made a minor modification on the sequence in the *SIRT1* exon to reduce its inclusion level and selected four regions to insert a PTB-binding site (Figure 6B). In order not to directly interfere with U1 binding, the positions for insertion in the upstream exon (UpE) or intron (UpI) are both ~15 nt away from the constitutive 5′ splice site. To avoid obstruction of 3′ splicing signals, the position for insertion in the downstream intron (DoI) is 15 nt upstream of the branchpoint, whereas the position for insertion in the downstream exon (DoE) is 10 nt from the 3′ AG dinucleotide.

We transfected the parental splicing reporter and the PTB site insertion derivatives into HeLa cell and analyzed the splicing products by semiquantitative RT-PCR. As shown in Figure 6B, insertion of a PTB-binding site near either the 5′ or 3′ constitutive splice site significantly enhanced the inclusion of the alternative exon. RNAi knockdown of *PTB* and *nPTB* completely abolished the exon inclusion induced by inserted PTB-binding sites (Figure 6C), and overexpression of *PTB4* further enhanced exon inclusion in a PTB binding site-dependent manner (Figure 6D). These observations provide unequivocal support to the splice site titration mechanism for PTB-dependent exon inclusion in which weakening the constitutive 5′ or 3′ splice site enhances the competitiveness of the alternative 5′ or 3′ splice site. These findings have therefore documented a positional effect for a general splicing repressor to positively regulate alternative splicing in mammalian cells.

DISCUSSION

Our global analysis of PTB-RNA interactions in the human genome provides mechanistic insights into PTB-regulated RNA processing. Besides competing directly with U2AF65 binding to interfere with 3′ splice site recognition (Lin and Patton, 1995; Sauliére et al., 2006; Singh et al., 1995), PTB has been shown to use multiple mechanisms to regulate alternative RNA processing by binding to regions other than the core splicing signals on minigene models (Izquierdo et al., 2005; Sharma et al., 2008; Spellman and Smith, 2006). We have now generalized and significantly extended these findings at the genome level.

Interference of Splice Site Recognition and Communication by PTB-Mediated RNA Networks

RNA looping has been proposed as one of the mechanisms for PTB-mediated splicing repression to sequester the alternative exon from the splicing machinery (Chou et al., 2000; Wagner and Garcia-Blanco, 2001). PTB dimerization was initially postulated to facilitate RNA looping (Oh et al., 1998; Pérez et al.,

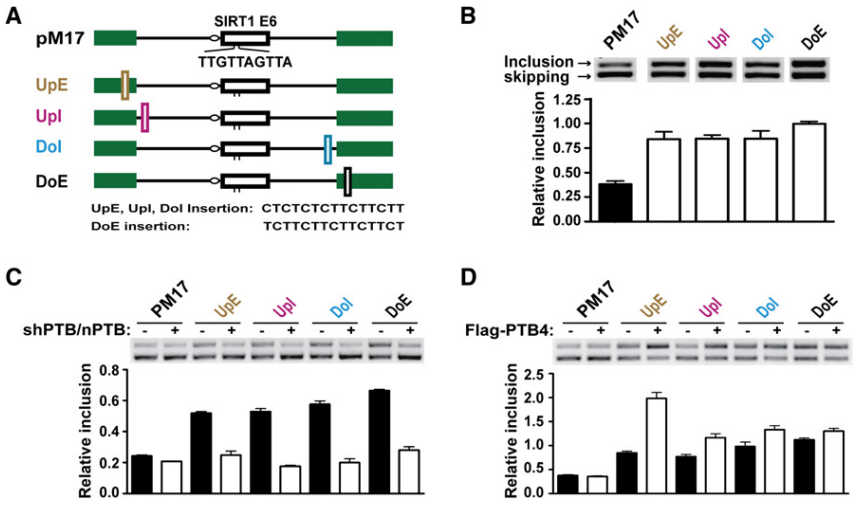

Figure 6. Mechanism of PTB-Dependent Exon Inclusion

(A) The reporter construct. pM17 is derived from pZW8-ESS17 (Wang et al., 2004) with a *cis*-acting regulatory element disrupted by the inserted sequence in the alternative *SIRT1* exon. Four positions are selected for inserting a PTB-binding site as diagrammed.

(B) Splicing of the parental and mutant reporters in transfected HeLa cells was determined by RT-PCR (representative gel images shown as inserts) and quantified.

(C and D) PTB-dependent exon inclusion through the inserted PTB-binding sites. The effect on exon inclusion was diminished by the shRNAs against *PTB* and *nPTB* in cotransfected HeLa cells (C). The effect on exon inclusion could be further enhanced by *PTB* overexpression (D). Together, these results demonstrate that PTB is directly involved in enhancing exon inclusion through the inserted binding sites. Error bars are based on SEM derived from three independent experiments.

1997b), but a later structural analysis suggests potential induction of RNA looping via RRM3 and RRM4 in the same PTB molecule to simultaneously bind to *cis*-acting RNA elements (Oberstrass et al., 2005). However, these two modes of RNA looping induced by inter- or intramolecular interactions do not have to be mutually exclusive. Although purified PTB exists predominantly as a monomer in solution, which can bind to RNA with high affinity, it has been suggested that PTB binding to RNA may create the spatial proximity for enhanced PTB-PTB interactions on RNA, which may be stabilized by the induced formation of a disulfide bond (Amir-Ahmady et al., 2005; Monie et al., 2005; Oberstrass et al., 2005). Our data are fully consistent with PTB binding to RNA as monomer and subsequent disulfide bond formation on closely spaced PTB molecules on target RNA. Of interest, we found that UV can further enhance or stabilize PTB-PTB interactions. Importantly, our data indicate that there are no separate sets of binding sties for monomeric and dimeric PTB in the human genome. However, this does not undermine the potential synergy between protein-protein and protein-RNA interactions that may be critical for induced RNA looping surrounding PTB-regulated exons as previously proposed (Wagner and Garcia-Blanco, 2001).

Given frequent PTB binding in multiple locations in a single intron in many cases (e.g., Figure S6B), we may envision an extensive RNA network nucleated by PTB, which may be the underlying mechanism for the observed interference of both exon definition and the transition from exon definition to intron definition during spliceosome assembly (Izquierdo et al., 2005; Sharma et al., 2005, 2008). As there is no reason to believe that PTB-mediated RNA network has to be restricted within a single regulatory unit, we may further speculate that the network may spread on multiple intronic and exonic locations in the same pre-mRNA molecule, thereby allowing a coordinated regulation of multiple RNA processing events as evidenced on the *TPM2* gene (Spellman et al., 2007). Such a network may be more prevalent than what we can imagine at this point because initial PTB-RNA interactions may induce additional PTB binding to other sites that may not even contain a motif for high-affinity binding by PTB, which may be further enhanced by other PTB cofactors, such as Raver1 (Gromak et al., 2003).

Mechanisms for Positive and Negative Regulation of Splice Site Selection by PTB

The PTB-RNA interaction map also suggests a potential mechanism for positive and negative regulation of splice site selection by PTB, depending on its binding relative to competing constitutive and alternative splice sites. Of interest, PTB not only binds to intronic locations near the 3′ splice site, but also to sites closer to the 5′ splice site regardless of whether the splice site is subjected to alternative choices. If predominant PTB binding occurs near a constitutive splice site, it may weaken the site, thereby raising the competitiveness of the competing alternative site. A minor modulation of splice site recognition may be translated into a major functional consequence as demonstrated by a recent kinetic analysis of splice site competition (Yu et al., 2008). This principle may be generally applicable to RNA-binding splicing regulators to give rise to either a positive or negative functional outcome that depends on where the factor binds.

A positional effect has clearly emerged from recent genome-wide studies of splicing regulators (Licatalosi et al., 2008; Yeo et al., 2009). However, the positional effect that we observed with PTB-regulated splicing appears to be fundamentally distinct from that exerted by Nova and Fox2. In those cases, Nova and Fox2 binding to their *cis*-acting elements upstream and downstream of the alternative exon generally represses or enhances the selection of the exon, respectively, but it is presently unclearly how such opposite effects on splice site selection are achieved. In contrast, PTB appears to be sampling multiple intronic locations in a pre-mRNA to exert a negative effect on the selection of the nearby splice site. PTB binding close to the intronic region near the 3′ and 5′ alternative splice sites likely results in skipping of the alternative exon, whereas PTB binding to sequences adjacent to constitutive exons tends to induce the inclusion of the alternative exon.

It is important to point out that potential composite effects may account for some apparent exceptions to this general trend. For

example, the PTB-binding pattern was similar on both the *CCDC138* and *EZH2* genes, but *PTB* knockdown had opposite effects on these two genes. The PTB-binding events on the *CCDC138* gene are both far away from the regulated exon, yet the net effect is PTB-dependent exon skipping, perhaps because the exon skipping effect, due to strong PTB binding on both sides of the alternative exon, might be dominant over its influence on the downstream constitutive exon. Therefore, the final functional outcomes, in many cases, may be determined by the sum of those competing binding events. In addition, most alternative splicing events are likely subjected to regulation by multiple different splicing regulators, which may act synergistically or antagonistically. Therefore, the possibility that other regulators may override the effect of PTB binding on certain regulated exons may account for various exceptions to the positional effect observed, thus emphasizing the combinatory control of alternative splicing that likely operate in mammalian cells.

EXPERIMENTAL PROCEDURES

Cell Culture, Plasmids, and RNAi

HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% newborn bovine serum plus 100 U penicillin /streptomycin (Gibico) at 37°C in 5% CO_2. Oligofectamine and Lipo2000 (both from Invitrogen) were used for siRNA and plasmid transfection, respectively, according to manufacturer's instructions. To construct the expression plasmids for PTB1, PTB4, and nPTB, FLAG-tagged primer sets were used to amplify the coding region of individual genes, and the PCR products were inserted into pcDNA3 between the EcoR1 and Not1 sites. The *CTTN* minigene was constructed by amplifying Exon 10 to Exon 12 regions, which were inserted into pcDNA3 between the EcoR1 and Xho1 sites. Validated siRNAs against *PTB* and *nPTB* were purchased from Ribbobio.

CLIP-seq, RIP-PCR, and Validation of PTB-Regulated Splicing

HeLa cells were UV irradiated at 400 mj and collected by scraping the cells from 15 cm plates. The CLIP procedure was performed as described (Yeo et al., 2009). Immunoprecipitated RNA was extracted using Trizol, and after DNase I (Promega) treatment, RNA was reverse transcribed using MMLV with N9 random primer at 37°C followed by inactivation of the reverse transcriptase at 70°C for 15 min. The resulting cDNA was analysis by PCR. Table S2 lists PCR primers for validation of PTB-dependent splicing.

Bioinformatics Analysis

The sequenced tags longer than 18 nt were mapped to the human genome sequence by allowing two mismatches and two insertions or deletions, and only those with maximal identity ($\geq$ 90%) were kept. To analyze genomic distribution of tags, known human genes (knownGene track from the UCSC genome browser) were chosen as the gene set, which contained 66,803 entries. We arbitrarily defined promoter regions as 5 kb upstream of the transcriptional start site of the gene. For genes having multiple isoforms, the transcript with the longest length, the largest number of exons, the longest CDS, or the maximum length of all exons was chosen to analyze the tag distribution in exon, intron, 5′ UTR, CDS, and 3′ UTR.

The PTB-binding sites enriched of tags were detected by using the similar strategy as previously described (Yeo et al., 2009). The differences were: (1) tags were not extended; (2) peak identification was independently done for each gene cluster of 24,378 clusters, which were grouped by using the program *clusterGenes* (-joinContained) on all known genes; (3) consecutive positions with the same height were counted only once; (4) control tags were randomly placed on gene cluster with repetitive elements masked; (5) for each height level *h*, the p value was assigned as the ratio of the number of heights higher than *h* divided by the total number of heights in 100 random placements, and the p values for all heights were adjusted by using Bonferroni correction to account for multiple hypotheses testing. The smallest height that gave an FDR < 0.001 was defined as the threshold height. Consecutive nucleotide positions with height higher than the threshold were identified as significant PTB-binding peak. If multiple peaks were detected less than 50 nt from one another, they were merged to represent a single PTB-binding site or cluster.

The sequences extracted from genome according to PTB-binding clusters were used to detect overrepresented motifs (Defrance et al., 2008). For each cluster, it was extended to the two sides by 25 nt, as some clusters had small lengths. Background sequences include those from randomly selected intervals in genes or random sequences generated with respect to order 0 and 1 Markov models (same single and dinucleotide frequencies) built from knownGenes (Ponty et al., 2006). Identification of overrepresented k-mers (k = 2, 3, 4, 5, 6, and 7) was based on random intervals. The pictogram was plotted according to WebLogo (http://weblogo.berkeley.edu/), which is based on the alignment of the top 20 motifs by ClustalW (http://www.ebi.ac.uk/Tools/clustalw/).

Normalized complexity map of PTB-RNA interactions was generated as described in (Licatalosi et al., 2008). The composite pre-mRNA was made by joining the longest upstream exon, upstream intron, middle exon (PTB-regulated exon), downstream intron, and downstream exon. The tags around PTB-regulated individual exons were mapped to the composite pre-mRNA according to their positions relative to the nearest splice site. The tags in one transcript were first normalized to their number across the region covering the PTB-regulated exon and flanking introns and exons and then to the number of different transcripts with tags at a given position as described (Licatalosi et al., 2008). For comparison, we extracted 6460 sets of three constitutive internal exons that are associated with PTB binding from knownGene set (hg18). Normalized complexity map was similarly created on 50 randomly selected constitutive exons to deduce both averaged PTB-binding events with standard deviation.

The analysis used the programs from Jim Kent's source code (http://www.soe.ucsc.edu/~kent), bx-python library (http://bitbucket.org/james_taylor/bx-python/), pygr library (http://code.google.com/p/pygr/), and homemade python codes.

ACCESSION NUMBERS

The CLIP-seq data for PTB monomer and dimer are available at the Gene Expression Omnibus under the accession number GSE19323.

SUPPLEMENTAL DATA

Supplemental Data include seven figures and two tables and can be found with this article online at http://www.cell.com/molecular-cell/supplemental/S1097-2765(09)00907-1.

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