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

Yuanchao Xue,1,4 Yu Zhou,1,2,4 Tongbin Wu,1 Tuo Zhu,1 Xiong Ji,1 Young-Soo Kwon,2 Chao Zhang,1 Gene Yeo,2 Douglas L. Black,3 Hui Sun,1 Xiang-Dong Fu,1,2,* and Yi Zhang1,4
1State Key Laboratory of Virology, College of Life Sciences, Wuhan University, Wuhan, Hubei 430072, China
2Department of Cellular and Molecular Medicine, University of California, San Diego, La Jolla, CA 92093-0651, USA
3Department of Microbiology, Immunology, and Molecular Genetics, Howard Hughes Medical Institute, University of California, Los Angeles, Los Angeles, CA 90095-1662, USA
4These authors contributed equally to this work
*Correspondence: xdfu@ucsd.edu (X.-D.F.), yizhang@whu.edu.cn (Y.Z.)
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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 (Saulière 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 splicesome 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 RRMAs (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
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.
Association of PTB Binding with Alternative Splicing

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.
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 decay 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 (Saulière 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.

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

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

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.
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). PTB1 in regulated splicing (Wollerton et al., 2001). We found that PTB1 had little activity in rescuing the inclusion of CTNN 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 CTNN 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
Positional Effects of PTB on Regulated Splicing

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 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
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.

**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; Saulière 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 García-Bianco, 2001). PTB dimerization was initially postulated to facilitate RNA looping (Oh et al., 1998; Pérez et al.,
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 sites 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-bound exons as previously proposed (Wagner and Garcia-Blanco, 2001).

Figure 6. Mechanism of PTB-Dependent Exon Inclusion
(A) The reporter construct. pM17 is derived from pZ2W8-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.

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 unclear 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 combinatorial 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% CO₂. 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 sequenced tags longer than 18 nt were mapped to the human genome with the same height were counted only once; (4) control tags were randomly and not extended; (2) peak identification was independently done for each gene cluster of 24,378 clusters, which were grouped by using the program clustalw (-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 peaks. 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 known Genes (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 6480 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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