Corepressor CtBP and Nuclear Speckle Protein Pnn/DRS Differentially Modulate Transcription and Splicing of the E-Cadherin Gene

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CtBP is a transcriptional corepressor with tumorigenic potential that targets the promoter of the tumor suppressor gene E-cadherin. Pnn/DRS (Pnn) is a “nuclear speckle”-associated protein involved in mRNA processing as well as transcriptional regulation of E-cadherin via its binding to CtBP. Here, we show that CtBP can recruit Pnn to CtBP-associated complexes, resulting in Pnn-dependent chromatin remodeling at the E-cadherin promoter. In addition, CtBP and Pnn can differentially modulate E-cadherin mRNA splicing, with polymerase II serving as an interface in this event. Therefore, the Pnn/CtBP functional interplay represents a novel mechanism linking the corepressor CtBP and Pnn to the transcription-coupled mRNA splicing of a major tumor suppressor gene. Our findings implicate the existence of the molecular switches involved in tumorigenesis, which coordinate promoter-specific events and mRNA processing, by serving as bridging elements between the regulatory complexes both at gene promoters and within the mRNA splicing machineries.

Gene expression is a complex process involving coordinated events at the promoter regions and during pre-mRNA processing. A number of studies have demonstrated a connection between coactivator or corepressor complexes, operating at the gene promoters, as well as mRNA splicing (2, 3, 12, 60). Furthermore, the mechanism coupling of promoter-associated regulators to mRNA splicing complexes might depend on the promoter architecture and may involve RNA polymerase II (Pol II)-dependent specific interactions (3, 9, 10, 37, 53, 56).

CtBP is a transcriptional corepressor that silences the tumor suppressor gene E-cadherin, which is essential for epithelial cell-cell adhesion (62). CtBP associates with transcriptional repressor complexes involved in gene regulation in varied developmental and oncogenic contexts (7, 15, 17, 19, 22, 29, 30, 39, 40, 41, 46). CtBP is targeted to promoters via sequence-specific DNA-binding transcription factors and, in turn, contributes to gene silencing by recruiting complexes with histone methyltransferase, demethylase, and deacetylase activities (16, 33, 41, 49, 51, 54). For example, CtBP can be recruited to the E-cadherin gene promoter by the repressor ZEB (7, 8, 15, 16, 41, 51, 61). Similarly, the corepressor mSin3A has been demonstrated to interact with CtBP in vivo (23) and target the E-cadherin promoter via its interaction with Snail (38) and as part of the CoREST complex (14, 51).

A distinguishing structural feature of CtBP is a conserved NAD(H)-binding motif. NAD(H) binds to CtBP and regulates its function, thereby promoting tumor cell migration under hypoxic conditions (62). In addition, CtBP-mediated repression and the interaction with transcriptional proteins are regulated by levels of NAD(H), thereby linking CtBP activity to the local metabolic state (4, 13, 22, 26, 32, 55, 57, 61).

Pnn/DRS (Pnn/DRS) is a multifunctional protein that promotes epithelial adhesion properties (20, 35, 36, 47, 50, 52). Pnn also influences the expression of a number of tumor suppressor genes (48, 50). In the nucleus, Pnn interacts with multiple mRNA processing factors such as SRm300, SRp75, SRp130, and RNPS1 and is involved in mRNA splicing (27, 44, 58, 63). Pnn also associates with the components of the basal transcriptional machinery, such as Mediator and CA150 (45, 53). We have shown that Pnn modulates E-cadherin promoter activity through its NADH-dependent interaction with the corepressor CtBP (1), thus supporting Pnn’s role in transcription-specific mechanisms in the context of tumorigenesis.

Here, we investigated the potential mechanism by which the CtBP/Pnn functional interaction can influence E-cadherin gene expression. We demonstrate that CtBP can recruit Pnn to CtBP-associated silencing complexes, in turn resulting in Pnn-dependent effects on chromatin remodeling at the E-cadherin promoter. We also demonstrate that Pnn interacts with the transcriptionally competent form of Pol II and positively influences E-cadherin mRNA splicing, whereas CtBP negatively affects the splicing possible through its modulatory effect on the amount of initiation- and elongation-specific Pol II. These findings carry intriguing implications, that CtBP and Pnn may be involved in tumor progression through both promoter-dependent regulatory events and posttranscriptional mRNA processing.

MATERIALS AND METHODS

Cell lines, cell culture, and transfections. Suspensory HeLa (sHeLa) cells stably expressing a Pnn-Flag-hemagglutinin (HA) fusion protein (HeLa Pnn-Flag-HA) or a CtBP-Flag-HA fusion protein (HeLa CtBP-Flag-HA) were designed as described previously (51). They expressed exogenous Pnn and CtBP proteins at the levels comparable to the levels of the wild-type proteins. Control HeLa cells contained the Flag-HA vector alone.

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Cells were transfected by utilizing 3 μl of 1 mg/ml of 25-kDa branched polyethyleneimine (Sigma-Aldrich) per 1 μg of DNA. Polyethyleneimine and DNA were incubated in serum-free Dulbecco’s modified Eagle’s medium for 10 min in separate tubes. Following incubation, the contents of two tubes were combined, incubated for an additional 10 min, and applied onto cells.

**Expression vectors.** pCMV-Flag, expressing human CtBP1, and pCMV-1.3-P promoter-(myc-His, expressing human Pnn (hPnn), were based on pCMV-Flag (Stratagene) and pCMV-1.3-myc-His (Invitrogen), respectively. The Pnn-His-glutathione S-transferase (GST) fusion construct was based on the Pet 42b (+) vector (Novagen). The ZEB1-myc vector was a gift from A. A. Postigo and D. C. Dean (Washington University). The mSin3A-myc vector was a gift from E. Seto (University of South Florida).

**DNA Pol II antibodies.** Mouse anti-RNA Pol II H4 antibody recognizes the phosphohistone 5 form of initiation-specific Pol II (Covance), mouse anti-RNA Pol II H5 antibody recognizes the phosphohistone 2 form of elongation-specific Pol II (Covance), mouse anti-RNA Pol II SW46 antibody recognizes the hypophosphorylated form of preinitiation Pol II (Covance), and rabbit N-20 antibody recognizes the N terminus of Pol II (Santa Cruz).

**Coimmunoprecipitation and immunoblotting.** HEK293 cells were transfected with 4 μg of the expression plasmid ZEB1-myc or mSin3A-myc and CtBP1-Flag in the presence or absence of 4 μg of Pnn-green fluorescent protein (GFP). Twenty-four hours posttransfection, cells were washed with cold phosphate-buffered saline and lysed in immunoprecipitation buffer (20 mM HEPES [pH 7.9], 200 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 10 mM KCl, 25% glycerol [vol/vol], 2 mM phenylmethylsulfonyl fluoride, and complete cocktail of protease inhibitors [Roche]).

**ChIP assays.** Chromatin immunoprecipitation (ChIP) was performed according to guidelines from Upstate Biotechnology. ChIPs were performed using Flag M2 agarose (Sigma-Aldrich) or the following antibodies: rabbit anti-acetylated histone H3 at lysine 9 (Upstate), rabbit anti-acetylated histone H4 at lysine 14 (Upstate), N-20 anti-Pol II (Santa Cruz), and rabbit anti-CBP (Abcam). For the real-time PCR, primers spanning the E-cadherin promoter region, the GAPDH (glyceraldehyde-3-phosphate dehydrogenase) promoter region, and the GAPDH intragenic region were utilized.

**Isolation of the endogenous Pnn-Pol II complex.** Hela nuclear (200 μl) lysates were brought up to 500 μl with equilibration buffer (20 mM HEPES [pH 7.9], 150 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 10 mM KCl, 25% glycerol [vol/vol]) and incubated with rabbit N-20 (Santa Cruz) or control immunoglobulin G (IgG) for 2 h, followed by a 1-h incubation with protein A-Sepharose Fast Flow (Amersham Biosciences). Beads were then washed with equilibration buffer and resuspended in SDS loading buffer. Proteins were resolved by 8% SDS-PAGE followed by Western blotting.

**Pnn and RNA Pol II complex isolation.** Nuclear extracts from the HeLa Pnn-Flag-HA cells expressed tagged Pnn at a ratio to the endogenous Pnn of 1:1 and were prepared as described previously (34). Nuclear extracts from 4 liters of cells were incubated with anti-Flag M2 monoclonal antibody-conjugated agarose beads (Sigma-Aldrich), followed by washes until no Pnn was released and elution with SDS loading buffer. The samples were subjected to SDS-PAGE followed by Western blotting.

**Lysate-dependent kinase and binding reactions.** Kinase reactions were performed as described previously (31), with modifications. Isolated nuclei were resuspended in kinase buffer (20 mM HEPES [pH 7.4], 10 mM MgCl₂, 0.02% NP-40, 1 mM diethiothreitol) and briefly sonicated. Lysates, which also served as the “input” for pull-down experiments, were centrifuged and resuspended in kinase reaction buffer and incubated at 30°C for 1 h with shaking. Control reaction mixtures contained kinase buffer instead of nuclear lysates. Beads were washed three times in kinase buffer and resuspended in SDS buffer. The samples were subjected to SDS-PAGE followed by Western blotting.

**Splicing reporters and splicing assays.** The E-cadherin exon 4-intron-exon 5 segment was PCR amplified using Pfu polymerase (Stratagene) and genomic DNA as a template. Splicing reporter constructs were based on the pGL-3 basic luciferase reporter (Promega) carrying the −427 to +53 E-cadherin basal promoter or the simian virus 40 (SV40) promoter. Luciferase cDNA was excised and replaced with the cassette, which included intact exon 4 (144 bp) and exon 5 (156 bp) of the E-cadherin gene linked by the native intronic sequence (124 bp).

**RESULTS**

Pnn is recruited to repressor complexes in a CtBP-dependent manner. CtBP binds to transcriptional repressors and recruits chromatin-modifying enzymes, thereby achieving gene silencing (Fig. 1A). In order to investigate the mechanisms behind the Pnn-dependent modulation of CtBP-mediated silencing, we entertained two possible models, which could account for the Pnn-mediated derepression of the E-cadherin promoter (Fig. 1B and C). Pnn may sequester CtBP from the CtBP-associated proteins, which target the E-cadherin promoter (Fig. 1C). Alternatively, Pnn may be brought to the silencing complexes via its interaction with CtBP and attenuate CtBP-mediated repression (Fig. 1B). To explore these possible outcomes, we focused on two transcriptional silencers, ZEB and mSin3A, which interact with CtBP and target the E-cadherin promoter.

HEK 293 cells were cotransfected with vectors expressing either ZEB1-myc (Fig. 1D) or mSin3A-myc (Fig. 1E) along with CtBP1-Flag in either the absence (lanes 1 and 3) or presence (lanes 2 and 4) of hPnn-GFP vector. Subsequently, immunoprecipitations were performed using anti-Flag agarose. Immunoprecipitated material was then subjected to Western blotting using anti-Pnn-143, anti-myc, and anti-Flag antibodies. These experiments revealed that hPnn-GFP expression did not result in changes in the amount of ZEB1 or mSin3A associated with CtBP1, leading us to conclude that Pnn does not drastically affect the interaction of CtBP with either ZEB or mSin3A. Furthermore, both endogenous as well as exogenous Pnn were detected in the CtBP-Flag immunoprecipitates, suggesting that Pnn may be present in the CtBP-dependent silencing complexes (Fig. 1D and E).

Next, we determined whether the binding to CtBP is essen-
tial for Pnn’s presence at the silencing complexes. HEK293 cells were cotransfected with vectors expressing ZEB1-myc (D) or mSin3A-myc (E) and CtBP1-Flag in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of hPnn-GFP. Immunoprecipitations (IP) were performed using Flag affinity agarose, followed by Western blotting using anti-Pnn-143, anti-myc, and anti-Flag antibodies. Exogenous hPnn-GFP is indicated by an arrowhead, and endogenous Pnn is indicated by an arrow. An increase in Pnn levels did not appreciably affect the amount of ZEB1 or mSin3A coprecipitated with CtBP1 (D and E, respectively). Exogenous and endogenous Pnn could be detected in CtBP-Flag immunoprecipitates, indicating that Pnn may be present in ZEB1/CtBP1 and mSin3A/CtBP1 complexes.

**Pnn can be present at the E-cadherin promoter.** Because the expression of Pnn results in the enhanced activity of the E-cadherin promoter (1), we investigated whether or not Pnn can be detected at the promoter region and if the expression of Pnn can affect local chromatin modifications. HeLa cells, both control cells and those stably expressing Pnn-Flag-HA, were subjected to ChIPs utilizing anti-Flag agarose to precipitate chromatin associated with Pnn. The ChIPs were subsequently subjected to real-time quantitative PCR (qPCR) with E-cadherin promoter-specific primers or GAPDH promoter primers as controls (Fig. 3A and B, lane 1). These experiments revealed that Pnn was associated with the E-cadherin but not the GAPDH promoter. Next, HeLa Pnn-Flag-HA or control HeLa cells were subjected to quantitative ChIP (qChIP) analysis utilizing antibodies against CBP acetyltransferase and RNA Pol II, which are frequently enriched at the promoters of actively transcribed genes. We used antibodies against histone 3 acetylated at lysine 9 (AcH3K9) and against histone 3 acetylated at lysine 14 (AcH3K14), which are markers of transcriptionally active chromatin. We also used an antibody against dimethylated histone 3 at lysine 9 (diMeH3K9), which is a marker of silenced chromatin. E-cadherin promoter qChIPs from cells expressing Pnn-Flag-HA revealed enhanced H3K9 and H3K14 acetylation (Fig. 3A, lanes 4 and 5, respectively),
an increased presence of CBP and Pol II (Fig. 3A, lanes 2 and 3, respectively), and a decrease in H3K9 dimethylation (Fig. 3A, lane 6) at the E-cadherin promoter compared to those from extracts from control HeLa cells. This was not observed in case of the GAPDH promoter. These results suggest that the presence of Pnn may indeed affect local chromatin modifications in the vicinity of the E-cadherin promoter. Therefore, the presence of Pnn at the E-cadherin promoter appears to positively influence the local chromatin modifications responsible for driving the promoter to a transcriptionally favorable state.

Pnn can associate with transcriptionally competent Pol II.

The observation that Pnn expression correlates with an increased presence of RNA Pol II at the E-cadherin promoter prompted us to investigate the potential involvement of Pnn in basal transcriptional machinery. In order to determine whether endogenous Pnn and Pol II can be coisolated, we utilized HeLa nuclear extracts to perform immunoprecipitations with anti-myc agarose, followed by Western blotting with anti-Pnn-143 and anti-myc antibodies. Pnn was detected in the Pol II precipitates, indicating that these two components may indeed associate in vivo (Fig. 4A).

Next, we assessed if Pnn preferentially associates with a particular form of Pol II. Functionally unengaged (preinitiation) Pol II is hypophosphorylated at its CTD, while initiation-specific Pol II is phosphorylated predominantly at Ser-5 of the CTD, and elongation-specific Pol II is phosphorylated predominantly at Ser-2 of the CTD (11, 18, 28, 42, 43, 64). Nuclear extracts from the HeLa Pnn-Flag-HA cells were subjected to immunoprecipitations with anti-HA antibody, followed by Western blotting using anti-HA and antibodies directed against different forms of Pol II (Fig. 4B). Pnn immunoprecipitates contained both initiation- and elongation-specific Pol II but not the preinitiation form of Pol II. These data thus suggest that Pnn associates predominantly at the transcriptionally active RNA Pol II complexes.

**CtBP can affect the Pol II phosphorylation status.** Because Pnn interacts with both CtBP and Pol II, we wanted to determine whether or not CtBP can affect the Pnn/Pol II association. We performed coimmunoprecipitations using antibodies against initiation- and elongation-specific Pol II from the nuclear extracts of HeLa or HeLa CtBP-Flag-HA cells. However, we consistently found less phosphorylated Pol II in the precipitates from the HeLa CtBP-Flag-HA cells than in control HeLa cells (data not shown). Therefore, in order to determine whether CtBP can affect the degree of Pol II phosphorylation, we lysed the same number of nuclei from HeLa cells or HeLa CtBP-Flag-HA cells in SDS-containing buffer and subjected samples to SDS-PAGE. Interestingly, lysates of HeLa CtBP-Flag-HA nuclei contained moderately smaller amounts of initiation-specific Pol II and significantly less elongation-specific Pol II than did lysates of control HeLa nuclei (Fig. 4C). N-20 antibody against the N terminus of Pol II, which recognizes both hyperphosphorylated (Ilo) and hypophosphorylated (IIa) forms of Pol II, demonstrated an increase in the ratio of hypophosphorylated to hyperphosphorylated forms of Pol II in HeLa CtBP-Flag-HA nuclei compared to control HeLa cell nuclei (Fig. 4C).

Next, we determined if CtBP has an effect on the phosphorylation status of the C-terminal domain of Pol II in vitro. We adopted the protocol based on the in vitro phosphorylation reaction of the C-terminal domain of Pol II, which utilizes the recombinant CTD as a substrate and nuclear extract as a source of kinase activity (31). We incubated recombinant CTD-GST and Pnn-GST, which served as substrates, with either HeLa or HeLa CtBP-Flag-HA nuclear extracts, which served as sources of kinase activity, in the presence of kinase buffer. We chose Pnn as a control substrate because it is a
known SR-like phosphoprotein that carries a C-terminal stretch of serine residues, which are heavily phosphorylated on SR protein family members. Antibody against GST was used on Western blots to detect a CTD molecular weight shift after incubation with nuclear extracts due to lysate-dependent post-translational modifications (Fig. 4D). Interestingly, incubation with HeLa CtBP-Flag-HA nuclear extracts resulted in a smaller shift of the CTD than that elicited by control HeLa nuclear extracts. Consistent with the SDS-PAGE gel shift, Western blots for CTD phosphorylated at Ser-5 detected less Ser-5 CTD phosphorylation subsequent to incubation with HeLa CtBP-Flag-HA nuclear extracts than did control HeLa nuclear extracts. Unfortunately, anti-phosphorylated Ser-2 antibody exhibited possible cross-reactivity to GST and was therefore omitted during the experiments. In contrast, when we utilized recombinant Pnn-GST as a substrate, we detected a more prominent molecular weight shift of Pnn in reactions utilizing HeLa CtBP-Flag-HA nuclear extracts than in those utilizing control HeLa nuclear extracts. These data suggest that CtBP might have a specific negative effect on the Pol II-CTD phosphorylation status.

Most interestingly, when CTD-bound material from the nuclear lysate-dependent kinase reactions was Western blotted for Pnn and members of the SR family of splicing factors, we found less Pnn and SR proteins associated with Pol II-CTD in HeLa CtBP-Flag-HA lysates than in control HeLa lysates (Fig. 4E). Because Pnn and SR proteins target predominantly phosphorylated CTD, it is tempting to speculate that CtBP negatively affects these interactions by either directly or indirectly inhibiting CTD phosphorylation.

CtBP and Pnn differentially affect mRNA splicing. Because Pnn is involved in pre-mRNA processing (27, 58), we considered the possibility that Pnn expression can impact the efficiency of E-cadherin mRNA splicing. To examine this, we created splicing reporter constructs, which carried the intact E-cadherin exon 4-intron-exon 5 cassette driven by either the E-cadherin promoter or the SV40 promoters (Fig. 5). HEK293 cells were cotransfected with splicing reporters along with in-
Increasing amounts of Pnn. Increasing the expression of Pnn resulted in a greater ratio of spliced to total mRNA when the reporter was driven by the E-cadherin promoter (Fig. 5A, left). However, no Pnn-induced increase of splicing was observed with SV40 promoter-driven constructs (Fig. 5A, right). Consistent with these observations, splicing assays conducted in the presence of Pnn RNAi demonstrated a reduction in the ratio of spliced to total mRNA with the E-cadherin-driven vectors (Fig. 5B, left) but not with SV40 promoter-containing constructs (Fig. 5B, right). These data suggest that Pnn may be capable of modulating E-cadherin mRNA splicing efficiency in a promoter-specific manner. Next, we wanted to determine if Pnn has an effect on endogenous E-cadherin splicing. For this purpose, we utilized mouse ES cells carrying the insertion of a neomycin resistance cassette into intron 8 of Pnn (3f/3f cells), which results in a hypomorphic knockdown of Pnn (approxim-
FIG. 5. Pnn can modulate E-cadherin mRNA splicing efficiency. (A) HEK293 cells were cotransfected with E-cadherin[E-cadEx4-Ex5] (left) or SV40[E-cadEx4-Ex5] (right) splicing reporters and increasing amounts of Pnn. The splicing efficiency was calculated as a ratio of intensities of the amplicons corresponding to the spliced mRNA to the sum of unspliced and spliced mRNA (total mRNA). Increasing Pnn expression resulted in an increased ratio of spliced mRNA to total mRNA in the context of the E-cadherin promoter but not the SV40 promoter. (B) Transfection of the Pnn RNAi vector resulted in a decreased ratio of spliced mRNA to total mRNA in the context of the E-cadherin promoter but not the SV40 promoter. (C) RT-PCR of E-cadherin intron 2 and spliced message in wild-type (wt/wt) and 3f/3f cells with and without reverse transcriptase (RT). GAPDH was used as a control. (D) ChIP analysis of E-cadherin and GAPDH at the intragenic region.
Splicing assays were performed using RT-PCR, where unspliced message was detected using primers against E-cadherin intron 2, whereas total spliced E-cadherin message was detected using primers spanning the region between exons 4 and 8 (Fig. 5C). The 3f/3f cells exhibited lower levels of E-cadherin message than did wild-type (wt/wt) ES cells, supporting the role of Pnn in the regulation of E-cadherin expression. Furthermore, the 3f/3f cells displayed an accumulation of the intronic amplicon, which resulted in an increased ratio of unspliced to total mRNA message compared to wt/wt cells (Fig. 5C, bottom). These data suggest that in 3f/3f cells, splicing is hindered compared to that in wt/wt cells. For cotranscriptional splicing to occur, splicing factors are recruited to the processive RNA Pol II as it progresses along the transcribed gene. Thus, we determined whether Pnn is present within the E-cadherin open reading frame. HeLa Pnn-Flag-HA cells were subjected to ChIP utilizing anti-Flag affinity agarose followed by qPCR using E-cadherin intragenic primers or GAPDH intragenic primers as a control (Fig. 5D). Pnn was enriched in the E-cadherin intragenic region but not in the GAPDH intragenic region. These data, together with Pnn/Pol II interaction studies, support Pnn’s role in E-cadherin mRNA processing, possibly through its interaction with transcribing Pol II.

Because CtBP can negatively affect the amount of elongation-specific Pol II as well as decrease the binding of positive regulators of splicing, such as Pnn and SR proteins, to Pol II-CTD, we next sought to determine whether CtBP can also affect E-cadherin mRNA splicing. In contrast to the Pnn-dependent positive impact on splicing, splicing reactions performed in the presence of CtBP resulted in a reduction of the ratio of spliced to total E-cadherin mRNA, indicating that CtBP can negatively affect splicing efficiency (Fig. 6A).

We next examined whether CtBP can impact the splicing pattern of the alternative exons. We reasoned that because CtBP can modulate Pol II phosphorylation and inhibit constitutive splicing, CtBP may also exert an effect on alternative splicing, as those events are functionally linked (11, 42, 43). Therefore, we examined human CD44, a gene regulated through alternative splicing and involved in metastasis. This gene consists of 10 constitutive exons and a cluster of nine variable exons, which confer significant variability to the CD44 splice variants, often resulting in enhanced metastasis (5, 6). Examination of control HeLa and HeLa CtBP-Flag-HA cells for CD44 splicing patterns using primers against variable exons v4, v5, v6, v7, and v8 (Fig. 6B) revealed a CtBP-dependent differential modulation of various CD44 isoforms (Fig. 6C). For example, CtBP expression promoted the inclusion of v4 and v5 exons but not v6, v7, and v8 exons (Fig. 6C). These data demonstrate that CtBP is capable of modulating alternative splicing events. However, the functional impact of this CtBP-dependent alteration of splicing on the metastatic potential of cells awaits further investigation.

**DISCUSSION**

Our results provide a novel insight into the regulatory cascade involving corepressor CtBP and Pnn, which modulate the expression of a tumor suppressor gene at different control points via basal transcriptional and splicing machineries. These data support the contention that various aspects of gene expression are affected by a number of multifunctional proteins and that the expression level of each protein can have profound consequences on diverse processes and complex interconnections within the gene expression machinery of the living cell.

In the complex set of events which govern the regulation of E-cadherin gene expression, we were able to track functional interactions of the corepressor CtBP and Pnn at the promoter via the repressors ZEB1 and mSin3A, which are known to associate with CtBP, and target E-cadherin (7, 8, 14, 15, 16, 23, 38, 41, 51, 61).

The fact that Pnn, a protein with no known role in transcriptional repression, is recruited to the CtBP-dependent complexes implies that the composition of the repression apparatus may not be limited to proteins involved directly in gene silencing but rather may include proteins that function in mRNA processing and even factors capable of reversing the silencing effect. Indeed, we demonstrate that the presence of Pnn at the E-cadherin promoter correlated with increased histone H4 acetylation, a decrease in histone H3K9 dimethylation, as well as the increased presence of RNA Pol II, which are correlated with transcriptionally active chromatin. These findings provide indirect support for the context-dependent effect of regulatory complexes on a target gene. For example, a protein complex can deliver a silencing or activating effect on the gene promoter, depending on its factor composition. Indeed, this postulate parallels a newly described model showing a cofactor-mediated specificity for histone demethylation exerted by a CtBP-associated factor, LSD1 (49). For instance, LSD1 may act as an activator or repressor depending on the constituents of the LSD1-associated protein complex (59). Therefore, Pnn might be capable of attenuating CtBP-mediated repression by being recruited to the CtBP complex, where CtBP serves as a bridging molecule.

The idea that CtBP and Pnn may directly or indirectly co-
ordinate various aspects of gene expression is substantiated by our findings that Pnn and CtBP have differential effects on the mRNA splicing efficiency. By utilizing a splicing reporter construct driven by the basal promoter of E-cadherin and containing the exon 4-intron-exon 5 cassette, we demonstrated that Pnn expression enhances E-cadherin mRNA splicing efficiency. Unexpectedly, yet perhaps more interestingly, we found that CtBP inhibits the correct splicing of E-cadherin mRNA.
and promotes the inclusion of alternative exons of the CD44 gene. Furthermore, CtBP inhibited Ser-5 and Ser-2 phosphorylation of the C-terminal domain of RNA Pol II, leading to the depletion of the nuclear pool of initiation-specific and elongation-specific Pol II. We speculate that in addition to its silencing effect through chromatin remodeling, CtBP can affect gene expression through altering Pol II phosphorylation and preventing the assembly of regulators of splicing, such as Pnn, on the elongating Pol II. The fact that Pnn has indeed been described as being part of the Pol II-associated complexes involved in transcriptional initiation and elongation, such as Mediator and CA150 (45, 53), further supports the idea of the CtBP-dependent influence on basal transcriptional machinery. Therefore, CtBP and Pnn might have differential effects on E-cadherin gene expression through the association with regulatory complexes at the E-cadherin promoter as well as through their effect on splicing, linking promoter-related events and transcription-coupled mRNA processing. These findings resonate recent data describing the promoter-dependent control of splicing decisions exerted by hormone receptor coregulators (3, 24) as well as regulation of splicing by the chromatin-remodeling factor Brm (5, 25).

The conceptual mechanism of transcription-coupled splicing involving CtBP and Pnn can be visualized as efficiency-driven equilibrium, where CtBP modulates gene expression through transcriptional as well as splicing-related protein interactions. In this situation, gene repression can be coordinated through a CtBP-mediated effect at the gene promoters along with its negative influence on the recruitment of the splicing machinery by Pol II. This coupling delivers an efficient “brake” control to the CtBP target genes. It is unclear at this point whether the CtBP-mediated recruitment of the silencing factors to the gene promoters proceeds with a similar or equal rate compared to the dissociation of splicing factors from Pol II. However, it is likely that because CtBP serves as a common interface between these two events, these processes may progress in a compatible manner and are subject to orchestrated regulation by CtBP. The CtBP-mediated effect on the splicing apparatus carries an important implication with respect to the mechanisms involved in the control of tumor suppressor genes such as E-cadherin and CD44. The fact that CtBP inhibits mRNA processing of a major tumor suppressor gene, E-cadherin, stresses the importance of thorough examinations of tumor-promoting transcriptional repressors that might in fact confer metastatic potential to the transformed cells through both transcriptional inhibition and modulation of mRNA splicing. In addition, by promoting the inclusion of the CD44 alternative exons, CtBP might also influence tumor progression. It is widely accepted that CD44 splice variants heavily influence the metastatic states of numerous tumors (5, 6). Our observations that CtBP influences the inclusion of some of the alternative CD44 exons provide additional evidence of CtBP-mediated posttranscriptional events known to be involved in tumorigenesis. Further detailed analysis of CtBP’s impact on the splicing of CD44 and its consequences with respect to cellular metastasis is certainly in order. Importantly, in this context, gene expression profiles of various tumors based on microarray technology alone, which may not provide significant insight into the differential gene expression patterns, might not adequately address the possibility that a significant number of genes may also be regulated through posttranscriptional mRNA processing events. Therefore, approaches such as splice variant-specific microarrays might become increasingly relevant in correlative gene expression profiling, which links transcriptional repressors, such as CtBP, and the tumor-specific potential to metastasize.

Taken together, our data suggest that the differential regu-
lation of tumor suppressor genes, especially under pathological conditions such as cancer, might be heavily influenced by the transcription-associated bridging proteins that are capable of coordinating promoter-specific and mRNA splicing events.

In conclusion, we propose the concept of the functional coupling of processes governing gene expression where CtBP and Pnn serve as an example of unique protein factors that contribute to promoter-related complex interactions as well as impact mRNA processing (Fig. 7). This idea provides an exciting opportunity for pharmacological targeting of various cancers, whereby a carefully designed targeting strategy directed toward a single key regulatory molecule will have a cumulative and hopefully beneficial effect at multiple control points.

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