

Bipartite functions of the CREB co-activators selectively direct alternative splicing or transcriptional activation

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The CREB regulated transcription co-activators (CRTCs) regulate many biological processes by integrating and converting environmental inputs into transcriptional responses. Although the mechanisms by which CRTCs sense cellular signals are characterized, little is known regarding how CRTCs contribute to the regulation of cAMP inducible genes. Here we show that these dynamic regulators, unlike other co-activators, independently direct either pre-mRNA splice-site selection or transcriptional activation depending on the cell type or promoter context. Moreover, in other scenarios, the CRTC co-activators coordinately regulate transcription and splicing. Mutational analyses showed that CRTCs possess distinct functional domains responsible for regulating either pre-mRNA splicing or transcriptional activation. Interestingly, the CRTC1–MAML2 oncoprotein lacks the splicing domain and is incapable of altering splice-site selection despite robustly activating transcription. The differential usage of these distinct domains allows CRTCs to selectively mediate multiple facets of gene regulation, indicating that co-activators are not solely restricted to coordinating alternative splicing with increase in transcriptional activity.

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Introduction

The complexity and diversity of biological responses that occur following receipt of environmental cues far exceeds the absolute number of genes that are transcriptionally induced or repressed by signalling networks. To circumvent this problem, genes are regulated at additional levels, including precursor mRNA (pre-mRNA) processing. One mechanism to generate protein, and therefore functional, diversity is through alternative splicing of pre-mRNAs to include or

exclude select coding regions. Remarkably, nearly 60% of the transcripts expressed from human genes are alternatively spliced (Lander *et al*, 2001; Sharp, 2005; Moore and Silver, 2008).

The splicing process is carried out by the large multi-subunit spliceosome complex and is responsible for catalysing the two transesterification steps that constitute the splicing reaction (Black, 2003). Although the spliceosome is primarily composed of general components that remove intronic sequences in an unregulated manner from the pre-mRNA, it is now evident that several accessory proteins and *cis*-acting elements in the RNA sequence cooperate with the spliceosome to control splice-site selection (Kornbliht *et al*, 2004; Bentley, 2005). Further, there are several instances in which signalling pathways promote alternative mRNA splice patterns by modulating the function of these accessory proteins. For example, signalling directed by c-Jun N-terminal kinase (JNK), calcium/calmodulin-dependent protein kinase IV, the phosphatidylinositol 3' kinase (PI3K), and mitogen-activated protein kinase/extracellular signal-regulated kinase (MEK) regulate the splicing patterns of several pre-mRNAs (Konig *et al*, 1998; Lynch and Weiss, 2000; van der Houven van Oordt *et al*, 2000; Xie and Black, 2001; Matter *et al*, 2002). In addition, cAMP-dependent protein kinase A (PKA) phosphorylation regulates the nucleo-cytoplasmic localization of the pre-mRNA splicing factor polypyrimidine tract-binding protein (PTB) (Xie *et al*, 2003). In each of these instances, kinases in these signalling cascades directly mediate RNA processing by phosphorylating key components of the spliceosome.

In addition to regulating pre-mRNA processing through protein phosphorylation, signalling-dependent coupling of transcription and alternative splice-site selection is mediated by recruitment of co-activators by nuclear hormone receptors (Auboeuf *et al*, 2007). Here, the complement of proteins assembled on promoters can dramatically influence pre-mRNA processing, and in many instances these two processes seam coordinately regulated (Cramer *et al*, 1997; Monsalve *et al*, 2000; Auboeuf *et al*, 2004; Auboeuf *et al*, 2005). Moreover, transcriptional co-regulators can orchestrate signalling-dependent coupling of transcription and alternative splice-site selection. For example, the nuclear hormone receptor co-activators PGC-1, CoAA and CAPER coordinately regulate transcription and splicing of some target genes through intrinsic RNA recognition motifs (RRM) (Basu *et al*, 1997; Monsalve *et al*, 2000; Auboeuf *et al*, 2002, 2004; Dowhan *et al*, 2005; Fox *et al*, 2005).

The CREB regulated transcription co-activators (CRTCs) are signal-dependent transcriptional co-activators of cAMP-responsive promoters and are key regulators of gluconeogenesis (Koo *et al*, 2005; Dentin *et al*, 2007), adaptive mitochondrial biogenesis (Wu *et al*, 2006) and long-term synaptic plasticity (Kovacs *et al*, 2007). Under basal conditions, CRTCs are phosphorylated by AMP/SNF kinases and

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are bound by 14-3-3 proteins that sequester CRTCs in the cytoplasm (Screaton *et al*, 2004; Koo *et al*, 2005). As cAMP levels rise, PKA_c phosphorylates and inhibits AMP/SNF kinases, which prevents CRTC phosphorylation. Dephosphorylated CRTCs are then released from 14-3-3 proteins, translocate to the nucleus and bind to CREB (Bittinger *et al*, 2004; Screaton *et al*, 2004). The phosphorylation status of CRTCs and their cytoplasmic retention by 14-3-3 integrates converging cellular signals. For example, hormone and energy-sensing pathways converge on CRTC2 phosphorylation to modulate glucose output through CREB-mediated hepatic gene expression (Koo *et al*, 2005) and to regulate incretin hormones and glucose to promote β -cell survival (Jansson *et al*, 2008). Further, CRTC functions as a coincidence detector in excitable cells that funnels cAMP and calcium signalling pathways to CREB-dependent transcription (Screaton *et al*, 2004; Kovacs *et al*, 2007).

Here we report that CRTC co-activators, components of the cAMP signalling pathway, are bipartite regulators of gene expression that selectively control pre-mRNA splicing and/or transcriptional activation of CRE-containing genes. Remarkably, unlike other co-activators that contain RNA-binding motifs, we now provide data showing that the CRTC co-activators can induce alternative splicing without increasing transcriptional activation or direct transcriptional activation without altering splice-site selection.

Results

CRTC co-activators regulate alternative splicing

The CRTC co-activators are robust transcriptional co-activators of cAMP-dependent promoters. However, how they direct this function is not understood as they lack identifiable catalytic domains and homology to other proteins. Two previous studies have hinted that unidentified components of the cAMP pathway can mediate pre-mRNA processing, as a small deletion in the *fibronectin* promoter encompassing a CRE element alters alternative exon usage (Cramer *et al*, 1997), and a genetic screen for CRTC interactors identified a known regulator of pre-mRNA splicing NONO (p54nrb) (Amelio *et al*, 2007). Given these connections, we tested whether CRTCs might also contribute to pre-mRNA processing in response to cAMP signalling.

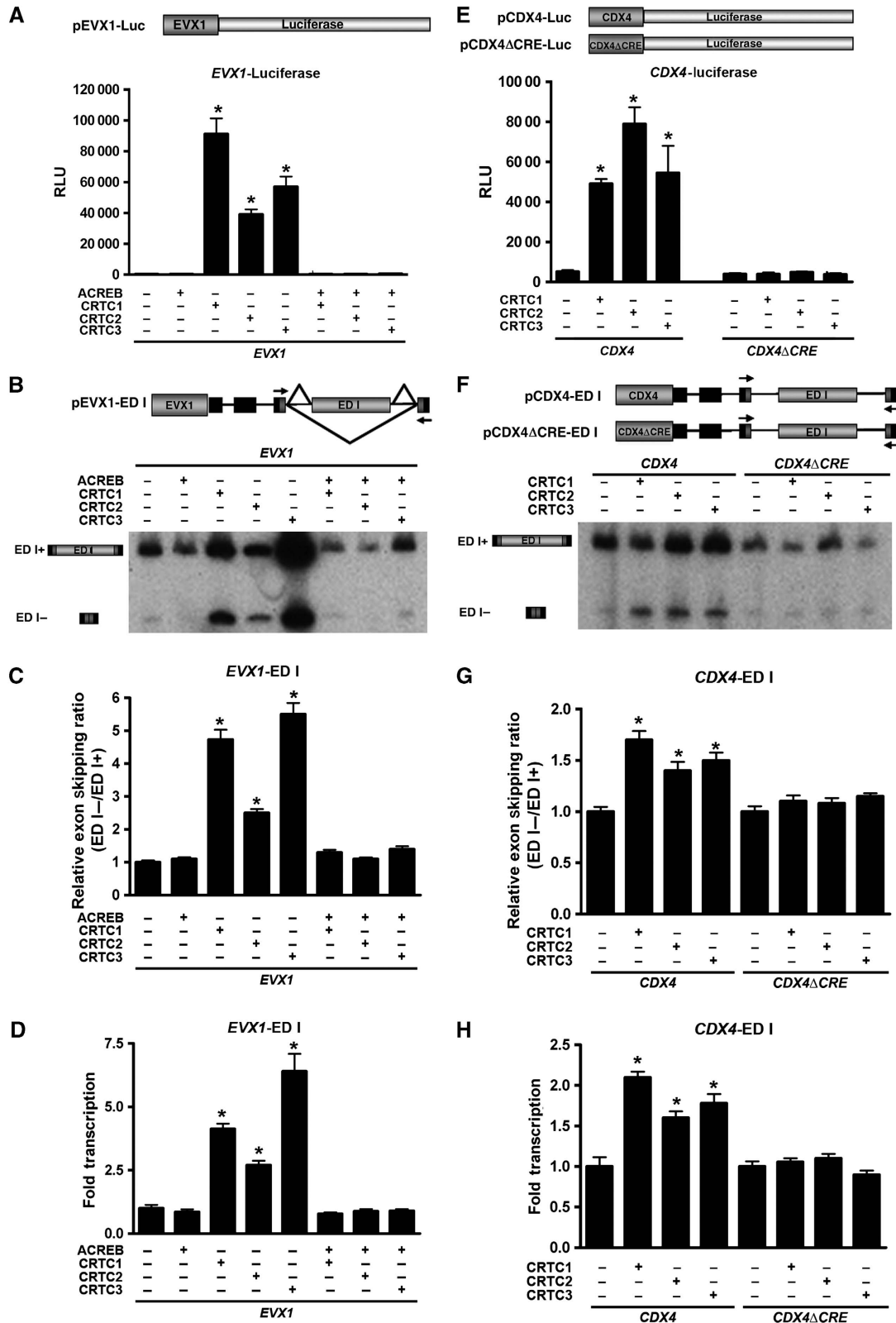
To test the potential dual roles of CRTCs in transcriptional activation and splice-site selection of cAMP-responsive genes, we generated several luciferase reporter genes and minigene splicing reporters with matched promoter fragments containing well-characterized CREB binding sites (Supplementary Figure S1) (Conkright *et al*, 2003b). Although the luciferase reporters were used to monitor transcriptional activity, the minigene splicing reporters measured inclusion or skipping of the alternatively spliced *fibronectin* extra domain I (ED I) exon (Caputi *et al*, 1994; Cramer *et al*, 1997). Important for these studies, ectopic expression of the CRTC co-activators overrides normal regulatory circuits that sequester CRTCs in the cytoplasm, thereby allowing their nuclear localization. As CRTC co-activators are the limiting components of the CREB transcriptional complex, they trigger robust induction of cAMP-responsive genes and, accordingly, overexpression of each CRTC family member (CRTC1-3) induced robust transcriptional activity of the cAMP-responsive *EVX1* promoter (Figure 1A). Further, as expected, the CREB dominant-negative mutant A-CREB, which masks the basic residues of the CREB DNA-binding domain, blocked this response (Figure 1A) (Ahn *et al*, 1998; Conkright *et al*, 2003b). Surprisingly, the CRTC co-activators caused a profound alteration in splice-site selection of the ED I minigene construct and these changes were also ablated by addition of A-CREB (Figure 1B). TaqMan primer/probe sets were designed to quantitatively measure alternative splicing or total transcription by detecting sequences that either include or exclude the alternatively spliced ED I exon (Supplementary Figure S2AB). The quantification of minigene alternative splicing transcripts by real-time qPCR showed that co-expression of CRTCs promoted >5-fold increase in skipping of the ED I exon (Figure 1C), a response robust as those of known regulators of pre-mRNA processing (Cramer *et al*, 1997; Monsalve *et al*, 2000; Auboeuf *et al*, 2002). Analysis of RNA transcripts from the ED I minigene by real-time qPCR showed that all CRTC family members activated transcription of the ED I minigene relative to basal conditions similar to findings observed with luciferase enzymatic assays (Figure 1D). Differences between each CRTC family member, on transcriptional activation and alternative splicing, were probably due to differences in their expression levels (Supplementary Figure S3A). Effects on alternative splicing were also blocked by A-CREB, indicating that CRTCs act in

Figure 1 CRTC co-activators regulate alternative exon splicing and transcriptional activation. (A) Effect of CRTCs on transcriptional activation. Transient transfection assays of *EVX1* luciferase reporter co-transfected into HEK293T cells with CRTC 1, 2 or 3 and/or A-CREB were analysed for luciferase activity ($n = 6$ wells; mean \pm s.e.m.; asterisk denotes P -value ≤ 0.05). (B) Effect of CRTCs on alternative exon splicing. Transient transfection assays of *EVX1* ED I minigene reporter co-transfected into HEK293T cells with CRTC 1, 2 or 3 and/or A-CREB. RT-PCR products were separated by PAGE and splice variants were visualized by autoradiography to determine the effect of CRTC expression on splice site selection. (C) TaqMan real-time qPCR analysis of CRTCs affects on *EVX1* ED I minigene exon skipping. The relative exon skipping levels are expressed as the ratio of skipped versus included transcripts ($n = 3$ experiments; mean \pm s.e.m.; asterisk denotes P -value ≤ 0.05). (D) TaqMan real-time qPCR analysis of CRTCs affects on *EVX1* ED I minigene reporter gene expression. Relative amounts of ED I reporter transcripts obtained by each transfection were plotted as folds relative to basal transcription ($n = 3$ experiments; mean \pm s.e.m.; asterisk denotes P -value ≤ 0.05). (E) Effect of CRTCs on CRE-dependent transcriptional activity. Transient transfection assays of *CDX4* luciferase or *CDX4*CRE reporters co-transfected into HEK293T cells with CRTC 1, 2 or 3 were analysed for luciferase activity ($n = 6$ wells; mean \pm s.e.m.; asterisk denotes P -value ≤ 0.05). (F) Effect of CRTCs on CRE-dependent alternative exon splicing. Transient transfection assays of *CDX4* or *CDX4*CRE ED I minigene reporters co-transfected into HEK293T cells with CRTC 1, 2 or 3. RT-PCR products were separated by PAGE and splice variants analysed to determine the effect of CRTC expression on splice site selection. (G) TaqMan real-time qPCR analysis of CRTCs affects on *CDX4* or *CDX4*CRE ED I minigene exon skipping. The relative exon skipping levels are expressed as the ratio of skipped versus included transcripts ($n = 3$ experiments; mean \pm s.e.m.; asterisk denotes P -value ≤ 0.05). (H) TaqMan real-time qPCR analysis of CRTCs affects on *CDX4* or *CDX4*CRE ED I minigene reporter gene expression. Relative amounts of ED I reporter transcripts obtained by each transfection were plotted as folds relative to basal transcription ($n = 3$ experiments; mean \pm s.e.m.; asterisk denotes P -value ≤ 0.05).

conjunction with promoter-bound CREB (Figure 1C). The affects of CRTC on alternative splicing were also observed in cell lines that do not express SV40 large T antigen or adenovirus E1A (Supplementary Figure S3B).

A second cAMP-responsive promoter, *CDX4* (Conkright *et al*, 2003b), was tested to establish that whether this was

a general property of CRE-containing promoters. Similar to the *EVX1-luciferase* and -ED I minigenes, CRTCs promoted transcriptional activation and splicing in the *CDX4* promoter context (Figure 1E-H). To confirm that CRTCs directly affected mRNA processing and that this required a CREB bound to CRE site, a minigene with a deletion in the essential



GC core of the CRE (TGACGTC → TGATCA) (Montminy *et al.*, 1986) was created within the *CDX4* promoter. This CRE-disabling mutation ablates both transcription and mRNA processing induced by CRTCs (Figure 1E–H). Thus, CRTC-mediated induction of transcription and splicing are direct and require functional CRE-containing promoters.

To assess whether the observed effects of CRTCs on alternative splicing could reflect sequestering of splicing factors, assays were conducted using increasing amounts of each reporter substrate (Supplementary Figure S4). Although titration of the *EVX1*-luciferase reporter resulted in near 10-fold increases in transcriptional activity (Supplementary Figure S4A), this did not translate into a change in the ratio of ED I exon skipping (Supplementary Figure S4B). Therefore, CRTC-mediated alternative splicing is not due to a decrease in pools of available splicing factors and there is not a linear relationship between transcription and splicing induced by CRTCs. Therefore, once recruited to CRE-containing promoters by CREB, CRTC co-activators have bipartite roles in gene regulation, by augmenting transcriptional activation and mediating pre-mRNA processing.

Previous studies have suggested a role for PKA activation in the regulation of alternative pre-mRNA splicing, by directly phosphorylating and altering the subcellular localization of the splicing factor PTB (Xie *et al.*, 2003). As expected, expression of the PKA catalytic subunit (PKA_c) induced transcriptional activity of the cAMP-responsive *EVX1* promoter and A-CREB blocked this response (Figure 2A). Interestingly, co-expression of each CRTC family member (CRTC1–3) with PKA_c led to robust transcriptional response. Real-time qPCR analysis of *luciferase* transcripts showed that luciferase activity is in agreement with promoter activity (Supplementary Figure S5). Similar to luciferase assays, PKA_c augmented splicing through CRTCs, leading to a marked increase in ED I exon skipping (Figure 2B). Real-time qPCR analysis of minigene alternative splicing showed a twofold increase in skipping of the ED I exon induced by PKA_c alone, and that co-expression of PKA_c with each CRTC family member induced a robust, 10–25-fold increase in exon skipping (Figure 2C). Real-time PCR analysis of minigene transcription showed that co-expression of each CRTC family member with PKA_c robustly activated transcription relative to

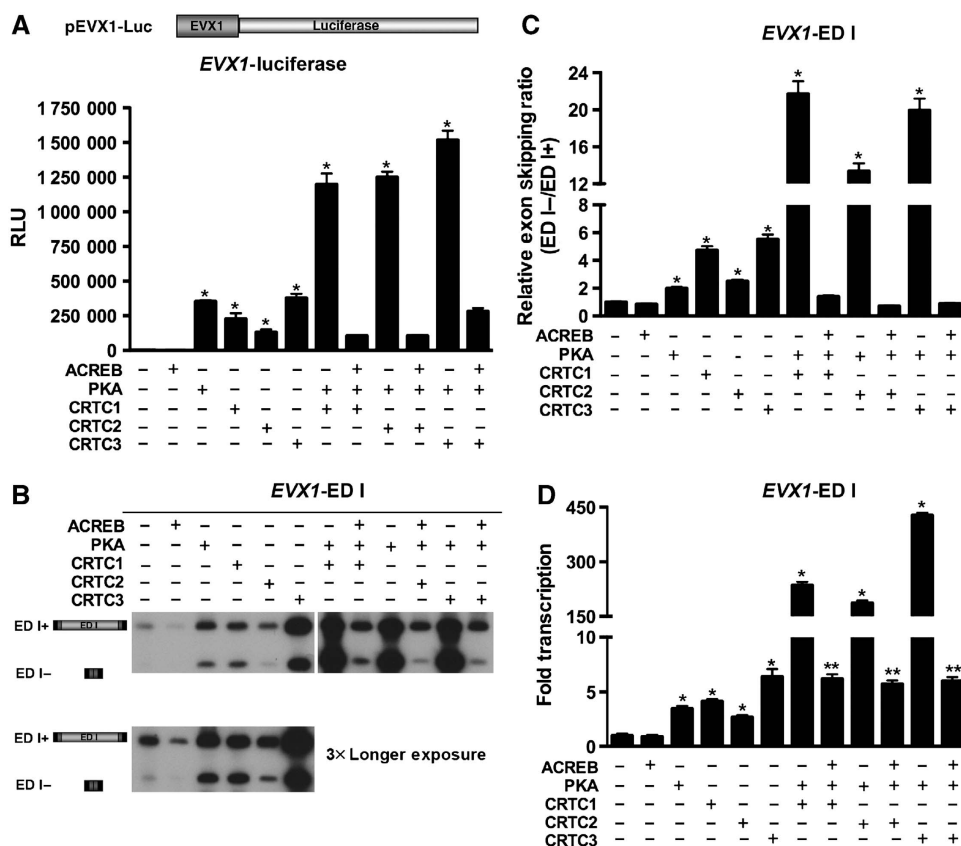


Figure 2 PKA_c activation of the cAMP pathway controls alternative exon splicing. (A) Effect of activating the cAMP pathway on transcriptional activity. Transient transfection assays of *EVX1* luciferase reporter co-transfected into HEK293T cells with PKA_c in the presence or absence of CRTC 1, 2 or 3 and/or A-CREB were analysed for luciferase activity ($n = 6$ wells; mean \pm s.e.m.; asterisk denotes P -value ≤ 0.05). (B) Effect of activating the cAMP pathway on alternative exon splicing. The schematic illustrates the ED I minigene splicing reporter and alternatively spliced transcripts that either include or skip the ED I exon. Transient transfection assays of *EVX1* ED I minigene reporter co-transfected into HEK293T cells with PKA_c in the presence or absence of CRTC 1, 2 or 3 and/or A-CREB. RT-PCR products were separated by PAGE and splice variants analysed to determine the effect of CRTC expression on splice-site selection. Identities of spliced and unspliced amplicons are indicated on the right side of the panel. (C) TaqMan real-time qPCR analysis of ED I exon skipping. The relative exon skipping levels are expressed as the ratio of skipped versus included transcripts ($n = 3$ experiments; mean \pm s.e.m.; asterisk denotes P -value ≤ 0.05). (D) TaqMan real-time qPCR analysis of reporter gene expression. Relative amounts of ED I reporter transcripts obtained by each transfection were plotted as folds relative to basal transcription ($n = 3$ experiments; mean \pm s.e.m.; single asterisk denotes P -value ≤ 0.05 ; double asterisk for A-CREB inhibition denotes P -value ≤ 0.05).

basal conditions (Figure 2D). Notably, co-expression of A-CREB attenuated the shift in splice-site selection mediated by PKA and the CRTC co-activators, indicating that within this setting the role of PKA in alternative splicing is also promoter dependent.

CRTC co-activators activate CRE-dependent 3' splice-site selection

These findings established that CRTC-directed regulation of pre-mRNA splicing is dependent on the promoter. However, the alternatively spliced ED I minigene was derived from the *fibronectin* gene, which can be induced by cAMP signalling (Dean *et al*, 1988, 1989). Thus, some intrinsic component of the minigene or its transcript might facilitate CRTC-mediated pre-mRNA splicing. Furthermore, alternative splicing is complex, in which multiple splice sites within an exon can be selected, in addition to complete exon inclusion or exclusion. To address these caveats, a second minigene reporter derived from the HIV-1 LTR was tested, which is not regulated by CREB/CRTC and measures alternative 3' splice-site selection (Supplementary Figure S6) (Caputi *et al*, 1999). The effect of the CRTC co-activators on 3' splice-site selection was examined using minigenes regulated by either the native HIV-1 LTR, wild-type *EVX1* or mutated CRE *EVX1* promoters (Figure 3A). Each CRTC family member activated transcription and splicing fivefold relative to basal conditions (Figure 3B and C). Moreover, splicing activation is independent of the 3' splice site chosen, as each of the five 3' splice sites within the transcript were induced equally, even though each 3' splice site is activated by different sets of spliceosome factors (Caputi *et al*, 1999; Zahler *et al*, 2004). Mutations of the CREB binding sites again effectively blocked this response, confirming the dependency of the CRE-containing promoter for CRTC function on pre-mRNA processing (Figure 3B and C). Importantly, overexpression of CRTCs with a HIV-1 LTR promoter construct devoid of CRE sites did not affect basal transcription or splicing levels, reaffirming that CRTCs do not affect the general transcriptional or splicing machinery independent of promoter recruitment (Figure 3B and C, right panels).

To determine whether the observed 3' splice-site selection is responsive to promoter strength or saturation of splicing factors, the transcription and splicing ratio of each construct was analysed. Comparison of qRT-PCR products expressed from the *EVX1* promoter with those expressed from the *LTR* promoter shows a nearly 40-fold difference in promoter strength (Supplementary Figure S7A). Increasing each minigene substrate resulted in increasing amounts of the *HIV-1* reporter transcripts (Supplementary Figure 7B), whereas the ratio between spliced and unspliced mRNA isoforms did not change; therefore, available splicing factors are not being saturated (Supplementary Figure S7C). Thus, despite dramatic differences in promoter strength, the CRTC co-activators are capable of promoting efficient splicing of transcripts originating from the weaker *EVX1* promoter. In addition, the ability of the CRTC co-activators to function as mediators of RNA processing is independent of the minigene splicing substrate.

CRTCs direct cell type-specific alternative splicing of endogenous transcripts

Alternatively spliced isoforms exist for ~60% of transcripts of the human genome (Lander *et al*, 2001; Sharp, 2005; Moore and Silver, 2008). As CREB binds to ~10% of all proximal

promoters, it is likely that a subset of CREB/CRTC target genes are also regulated by CRTCs at the level of alternative splicing (Impey *et al*, 2004; Zhang *et al*, 2005). To assess whether CRTCs mediate alternative splicing of endogenous CRE-containing genes, we examined the *Nr4a2* (*Nurr1*) gene, which is CRTC responsive (Conkright *et al*, 2003a). *Nr4a2*, a member of the structurally related nuclear receptor superfamily, is a transcription factor expressed predominantly in the liver, skeletal muscle and nervous tissues that controls key aspects of metabolism and dopamine biosynthesis (Smits *et al*, 2003; Pei *et al*, 2006; Fu *et al*, 2007). Consequently, alternative splicing in exon 3 and exon 7 of *Nr4a2* in nervous tissue (Figure 4A) yields isoforms that encode functionally altered *Nr4a2* proteins (Ohkura *et al*, 1999; Xu and Le, 2004; Michelhaugh *et al*, 2005).

Physiologic cues that regulate cAMP or both cAMP and calcium signalling pathways have been shown to converge on the CRTCs in non-excitabile and excitable cells, respectively, to regulate CRE-responsive genes (Screaton *et al*, 2004). Therefore, we analysed RNA isolated from primary rat hepatocytes or neuronally differentiated PC12 (ND-PC12) cells that were treated with forskolin (FSK) or depolarized with KCl (Figure 4B). Primary hepatocytes treated with KCl or FSK did not show significant changes in *Nr4a2* exon 3 alternative splice site-selection despite FSK-induced transcription. In contrast, both KCl-mediated depolarization and FSK-induction treatments promote alternative splice-site selection in *Nr4a2* exon 3 within ND-PC12 cells, and co-stimulation with both KCl and FSK further potentiates this shift in alternative splicing. This response was attenuated by transduction with adenovirus expressing an shRNA directed against CRTC (Figure 4B, right panel and Supplementary Figure S8A). Induction of the cAMP and calcium signalling pathways may be activating key splicing factors in addition to CRTCs. To show the role of CRTC, we also analysed RNA isolated from either primary rat hepatocytes or ND-PC12 cells that were transduced solely with adenovirus expressing GFP or CRTC (Figure 4C and Supplementary Figure S8B). Alternative splice-site selection in both exon 3 and exon 7 were induced 3.5-fold and 2-fold by CRTC, respectively, in ND-PC12 cells, whereas minimal effects of CRTC on splice-site selection were observed in hepatocytes, despite robust (16-fold) transcriptional activation of the *Nr4a2* gene (Figure 4C). Moreover, these effects were reversed by co-transduction with adenovirus expressing an shRNA directed against CRTC (Supplementary Figure S8C). Endogenous CRTC RNA, and protein levels relative to overexpressed adeno-CRTC were monitored by real-time TaqMan PCR and western blot analysis (Supplementary Figures S8DE, respectively).

The CRTC-mediated shift in transcript splicing of the *Nr4a2* transcription factor generates three distinct open reading frames whose products are either transcriptionally compromised or completely inactive (Ohkura *et al*, 1999; Xu and Le, 2004; Michelhaugh *et al*, 2005). Transient transfection of a luciferase reporter containing multimerized *Nr4a2* binding sites (3xNBRE) into PC12 or HEK293T cells with each respective *Nr4a2* isoform showed a dominant-negative affect of each isoform on full-length *Nr4a2* (Supplementary Figures S9A and B, respectively). Therefore, although CRTCs induce endogenous *Nr4a2* transcription in multiple cell types, their control of splice-site selection of the *Nr4a2* locus is cell type specific. Further, these data suggested that CRTC-mediated

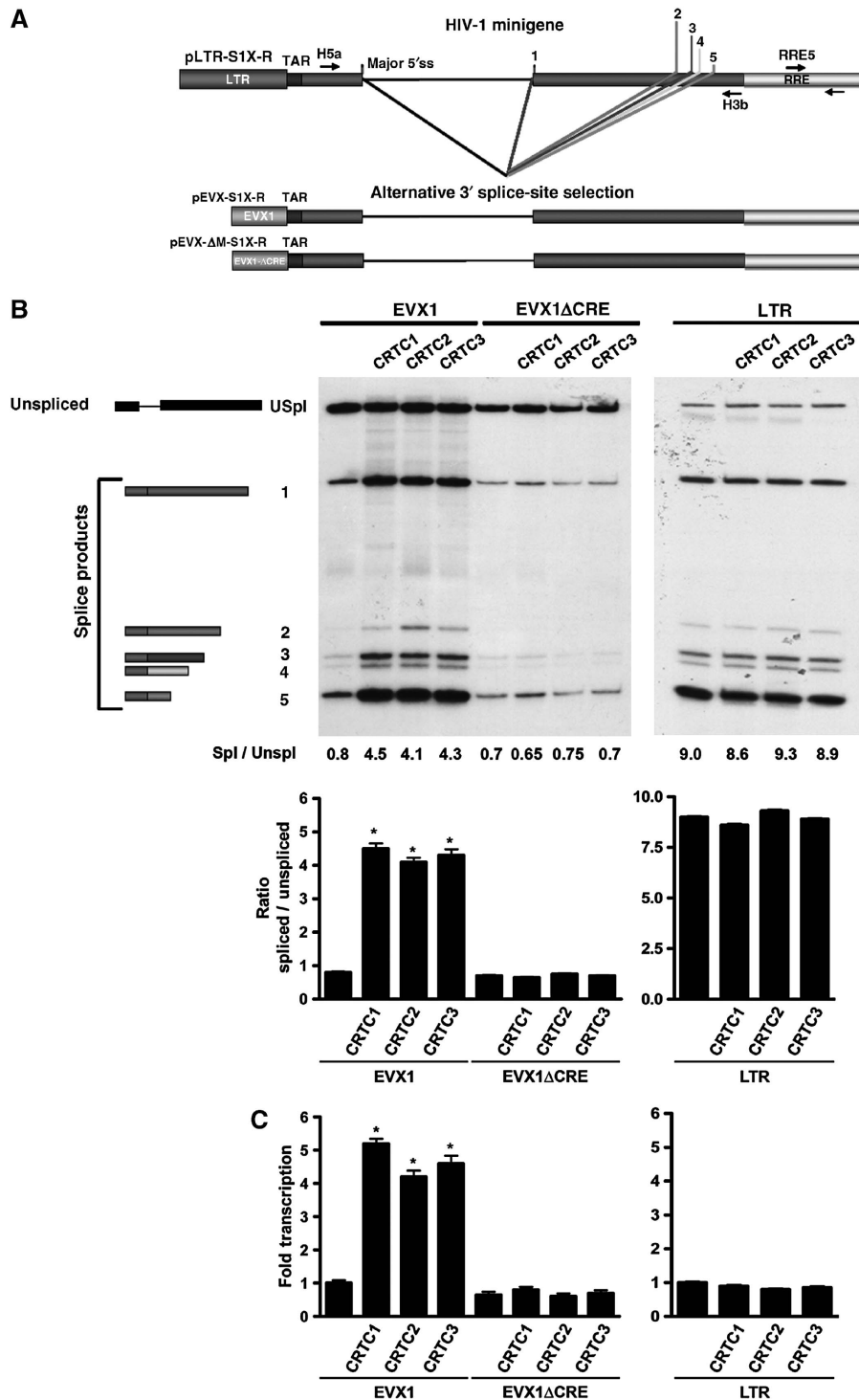


Figure 3 CRTC co-activators facilitate alternative 3' splice-site selection. (A) Effect of CRTC co-activators on alternative 3' splice-site selection. Schematic representation of the pLTR-S1X-R minigene reporter substrates derived from the HIV-1 genome. The major viral 5' and the alternative 3' splice sites specific for the tat, rev, env and nef gene products are indicated. Plasmids that substitute the LTR for the *EVX1* (pEVX1-S1X-R) and *EVX1*-mutated CRE (pEVX-ΔM-S1X-R) promoters are also represented. Location of the primers utilized for the detection of alternatively spliced products (H5a, H3b) and qPCR quantification of the transcripts generated by the reporter constructs (RRE5, RRE3) are indicated. (B) HEK293T cells were transfected with the indicated reporter construct together with a CRTC expression plasmid. RT-PCR products were separated by PAGE and splice variants analysed to determine the effect of CRTCs on splicing. Analysis of the ratio of spliced versus unspliced transcripts expressed by the reporter constructs is plotted. The ratio represents the sum of the total amount of spliced transcripts versus unspliced transcripts for each transfection experiment ($n = 3$ experiments; mean \pm s.e.m.; asterisk denotes P -value ≤ 0.05). (C) qPCR analysis of reporter gene expression. Relative amounts of HIV-1 reporter transcripts obtained by each transfection were plotted as fold of total gene products expressed (unspliced + spliced) versus the amount expressed by the pEVX1-S1X-R or pLTR-S1X-R constructs ($n = 3$ experiments; mean \pm s.e.m.; asterisk denotes P -value ≤ 0.05).

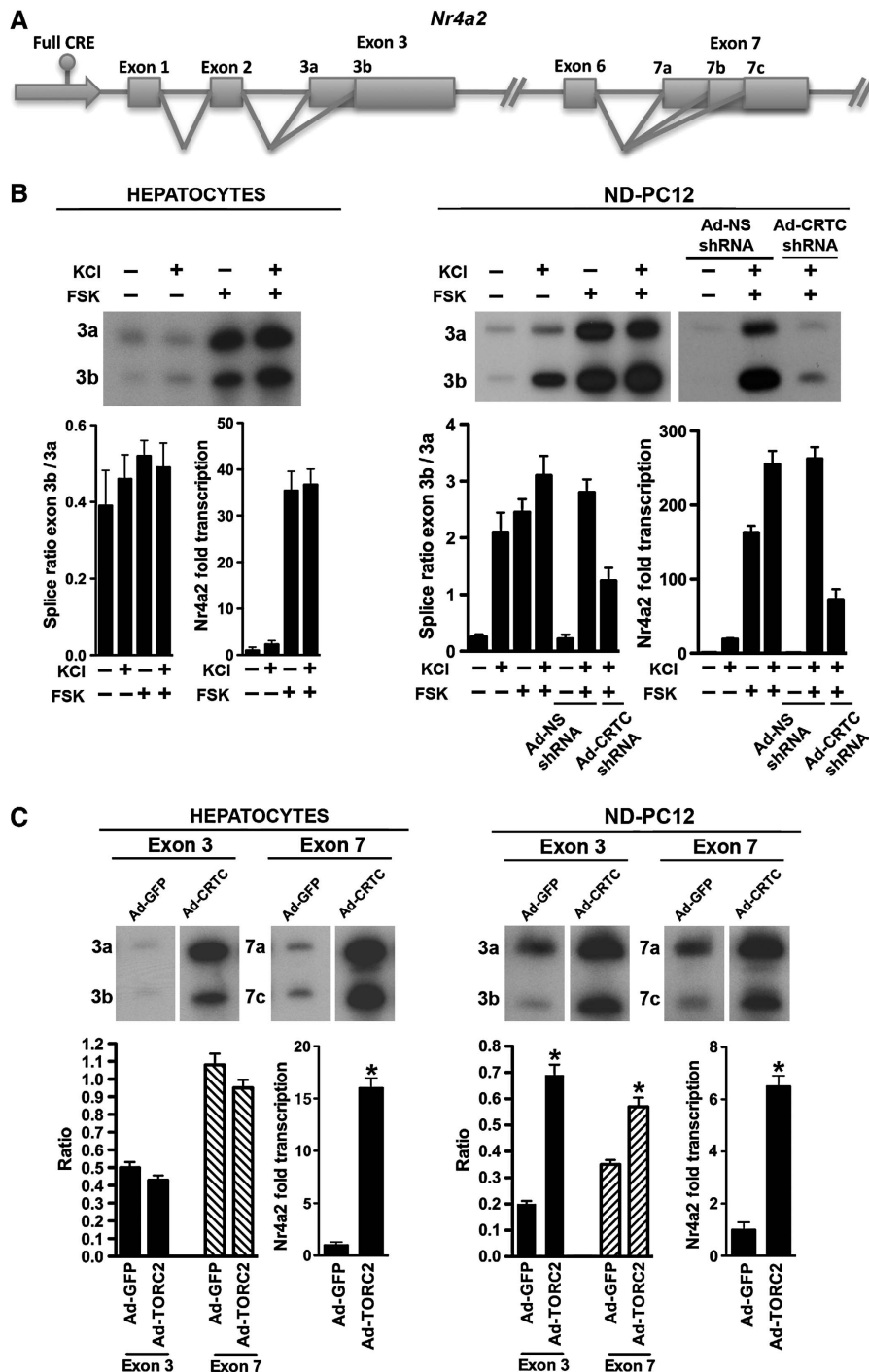


Figure 4 CRTC promotes alternative splicing of endogenous *Nr4a2*. (A) Schematic representation of the *Nr4a2* gene. Possible exon 3 and exon 7 products arising from alternative splicing are depicted. (B) RT-PCR analysis of *Nr4a2* was carried out using exon-specific primers on cDNA from primary rat hepatocytes or neuronally differentiated PC12 (ND-PC12) cells treated with KCl and/or forskolin (FSK). Analysis of ND-PC12 cells transduced with adenovirus expressing either GFP or CRTC2 and either non-specific (NS) or CRTC2-specific shRNAs. (C) RT-PCR analysis of *Nr4a2* was carried out using exon-specific primers on cDNA from primary rat hepatocytes or ND-PC12 cells transduced with adenovirus expressing either GFP or CRTC2. Ratio of alternatively spliced exons are indicated and shown in graphs ($n = 3$ experiments; mean \pm s.e.m.; asterisk denotes P -value ≤ 0.05). Panels of gel images shown here were from the same gel shown in Supplementary Figure 8SB.

splice-site selection is not obligatory to transcriptional activation and is dependent on the cellular context.

CRTC-directed transcription and alternative splicing are independent functions

The activation of gene expression by the CREB/CRTC complex is dictated by promoter context (Conkright *et al*,

2003a, b). Specifically, in contrast to genes with core promoters containing TATA box elements, TATA-less core promoters such as those possessing GC-rich or initiator (INR) elements do not respond to cAMP stimuli or to CRTC overexpression despite containing a CRE that is bound *in vivo* by CREB and CRTCs (Conkright *et al*, 2003a, b). Moreover, mutation of the CRE motif results in significant loss of basal transcriptional

activity indicating that this site is regulatory (Conkright *et al*, 2003b).

Our data showing that CRTC co-activators augment splicing in a cell context-specific manner suggested that transcriptional activation and splice-site selection might be independent functions of these co-activators. As CREB and CRTCs cannot induce transcription when recruited to CRE-containing TATA-less promoters, we tested whether CRTCs could still mediate splice-site selection in this context. To test this idea, we compared the effects of promoter context on CRTC-mediated transcription and pre-mRNA processing on a natural promoter and in a completely defined system (Figure 5 and Supplementary Figure S10). We generated luciferase and splicing reporters possessing the TATA-less CRE-containing *CDC37* promoter, or having promoter fragments derived from previously described constructs containing multimerized GAL4 UAS binding sites upstream of either an INR element or a consensus TATA box (Emami *et al*, 1995). As expected, PKA or CRTC expression does not direct transcriptional induction of the TATA-less *CDC37* promoter (Figure 5A). However, the TATA-less *CDC37* promoter is alternatively spliced in response to PKA or CRTC, and co-expression of PKA and CRTC further potentiates this effect, despite a lack of transcriptional activation (Figure 5B and C). Similarly, CRTC had only marginal effects on the INR-containing GAL4 UAS promoter, yet robustly induced transcription of the TATA-containing GAL4 UAS promoter when co-transfected with full-length CREB fused to the GAL4 DBD (Supplementary Figure S10A). In contrast to CRTCs core promoter-dependent affects on transcription, analysis of the ED I minigene reporters showed similar exon skipping ratios (Supplementary Figures S10B and C). Thus, core promoter context does not affect CRTC regulation of pre-mRNA splicing, suggesting that CRTC-mediated transcriptional activation and splicing are separable functions.

The observed effects of CRTC on pre-mRNA processing are also specific for this co-activator, as overexpression of the CBP co-activator had no effect (Supplementary Figures S10B and C). Furthermore, the phospho (Ser 133) CREB mutant, defective for CBP recruitment (Gonzalez and Montminy, 1989), did not block CRTC-mediated splice-site selection, indicating a CBP-independent mechanism for splice-site selection. To minimize the possible recruitment of splicing factors by other regions of CREB, we tested a GAL4-DBD fusion containing only the CREB basic leucine zipper (bZIP) domain. This 70-residue peptide is sufficient to bind the CRTC co-activators (Conkright *et al*, 2003a; Sreaton *et al*, 2004) and indeed CRTC directed robust transcriptional activation of the TATA-containing construct (Supplementary Figure S10D), and this was blocked by a single amino-acid substitution previously shown to be critical for CREB-CRTC interaction (R314A; relative to full-length CREB) (Sreaton *et al*, 2004). Importantly, alternative ED I exon splicing is also CRTC specific, as it is also blocked by the R314A mutation (Supplementary Figures S10E and F). Direct recruitment of GAL4-CRTC not only resulted in a robust transcription from the TATA-containing construct, but also yielded similar effects on alternative splicing for both promoter configurations (Supplementary Figures 10G-I). Collectively, these data show that the ability of the CRTC co-activators to regulate pre-mRNA processing is not a linear relationship with

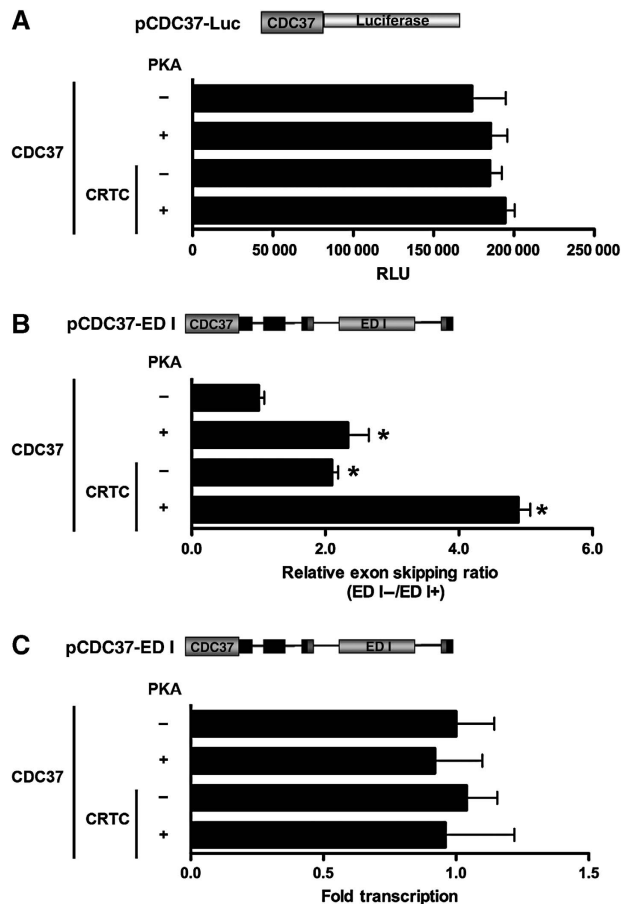


Figure 5 CRTCs elicit alternative ED I splicing independent of core promoter context. **(A)** Effect of PKA and CRTCs on transcriptional activity of a TATA-less CRE-containing promoter. Transient transfection assays of *CDC37* luciferase reporter co-transfected into HEK293T cells in the presence or absence of PKA or CRTC were analysed for luciferase activity ($n = 6$ wells; mean \pm s.e.m.; asterisk denotes P -value ≤ 0.05). **(B)** TaqMan real-time qPCR analysis of PKA and CRTCs effects on *CDC37* ED I minigene exon skipping. The relative exon skipping levels are expressed as the ratio of skipped versus included transcripts ($n = 3$ experiments; mean \pm s.e.m.; asterisk denotes P -value ≤ 0.05). **(C)** TaqMan real-time qPCR analysis of PKA and CRTCs affects on *CDC37* ED I minigene reporter gene expression. Relative amounts of ED I reporter transcripts obtained by each transfection were plotted as folds relative to basal transcription ($n = 3$ experiments; mean \pm s.e.m.; asterisk denotes P -value ≤ 0.05).

increases in transcriptional activity, suggesting these events may not be mechanistically linked.

CRTC recruitment to non-cAMP responsive promoters is sufficient to augment alternative splicing but not transcription

The finding that CRTCs can selectively regulate splicing independent of transcription activation led us to question whether these effects were specific to the configuration of the core promoter and/or to the splicing substrate. To address these alternatives, we tested whether tethering CRTC to a non-cAMP responsive promoter would affect the processing of pre-mRNA (Figure 6A). Neither CRTC nor CREB affect splicing of the HIV-1 minigene when regulated by the β -globin promoter (Figure 6B). However, inserting two tandem GAL4 UAS sites at position -275 in the β -globin promoter and

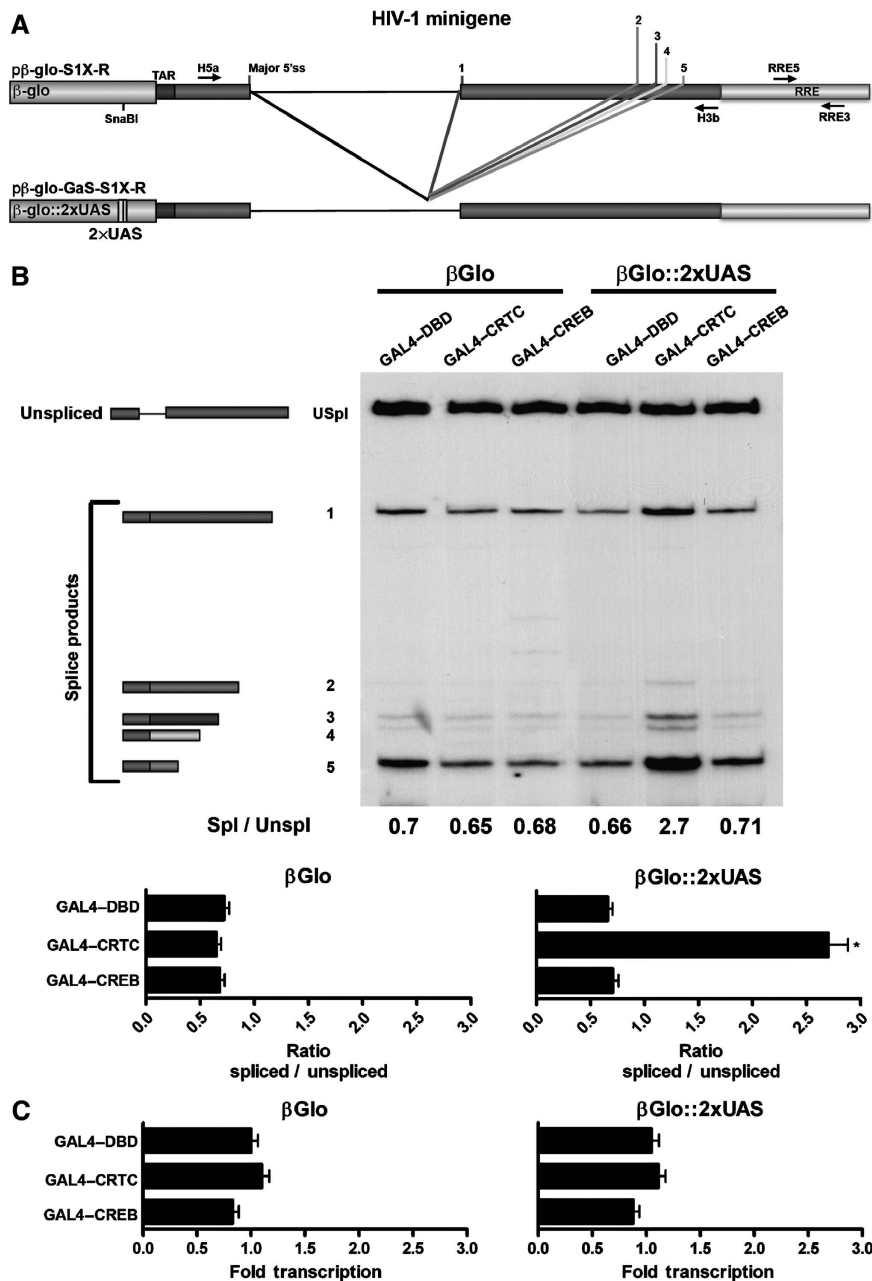


Figure 6 CRTC recruitment to promoters is sufficient to augment splicing but not transcriptional activation. (A) Schematic representation of the S1X-R minigene reporter substrates that substitute the LTR for the wild-type β -globin promoter or a β -globin promoter-containing tandem GAL4-UAS binding sites. (B) HEK293T cells co-transfected with the indicated reporter construct together with GAL4-CRTC1 or GAL4-CREB expression plasmids. RT-PCR products were separated by PAGE and splice variants analysed to determine the effect of CRTC recruitment to the β -globin promoter on splicing. Ratio of spliced versus unspliced exons are graphically displayed ($n = 3$ experiments; mean \pm s.e.m.; asterisk denotes P -value ≤ 0.05). (C) qPCR analysis of reporter gene expression. Relative amounts of HIV-1 reporter transcripts obtained by each transfection were plotted as fold of total gene products expressed (unspliced + spliced) versus the amount expressed by the pβGlo-S1X-R or pβGlo-GaS-S1X-R constructs ($n = 3$ experiments; mean \pm s.e.m.; asterisk denotes P -value ≤ 0.05).

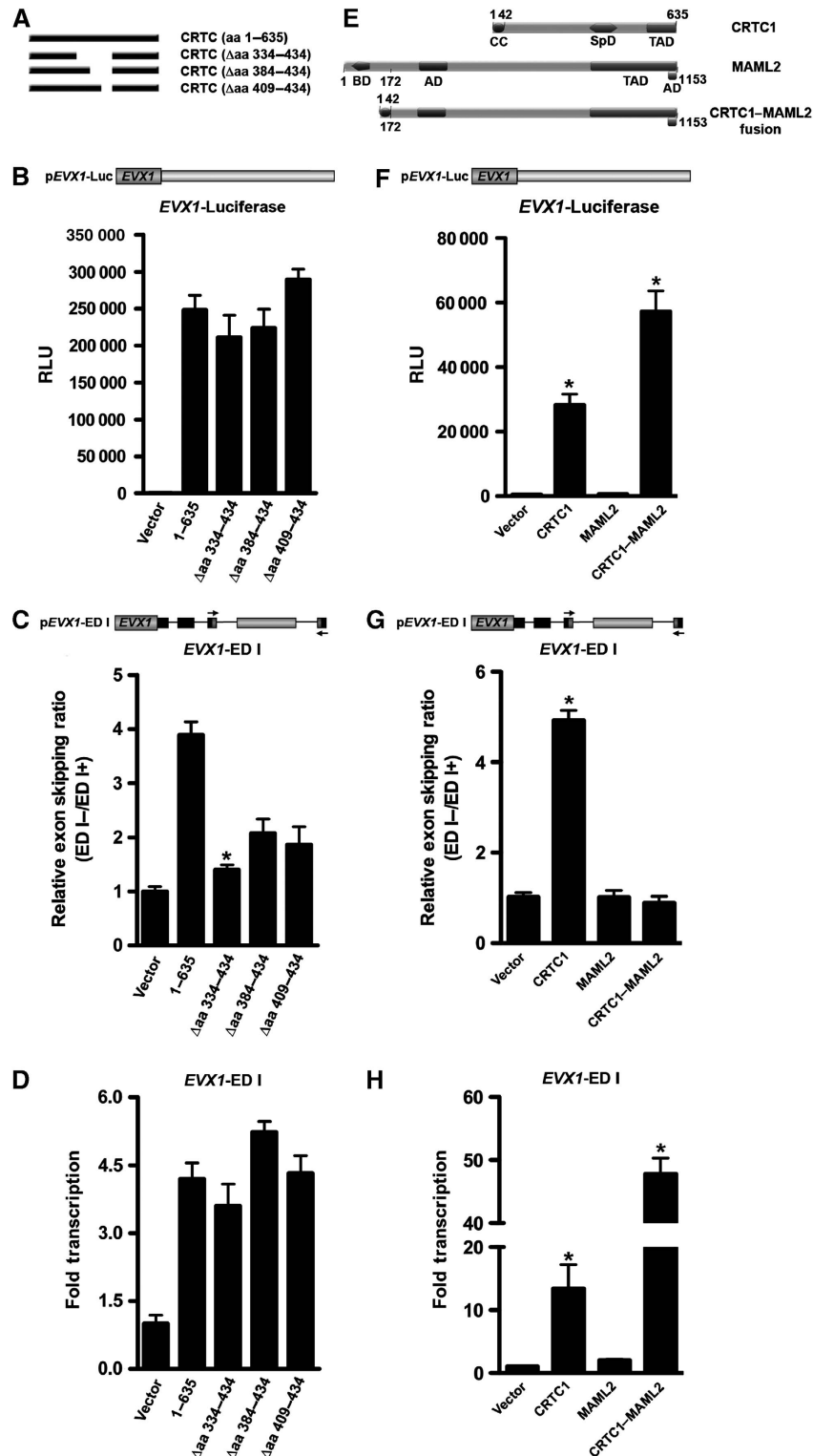
ectopically expressing heterologous GAL4-CRTC was sufficient to provoke a fourfold increase in spliced mRNAs relative to expression of the control GAL4-DBD. In contrast, GAL4-CREB failed to affect splicing confirming that CRTCs role in pre-mRNA processing is independent of CREB. Finally, although CRTC promotes alternative splicing, analysis of transcript levels by qPCR indicates that tethering CRTC to the β -globin promoter does not augment transcription (Figure 6C). CRTC recruitment to promoters is sufficient to augment splicing but not transcriptional activation.

CRTC-dependent alternative splicing is mediated by a domain separable from transcriptional activation

To identify the domain within CRTCs that facilitates splicing and to test whether splicing is separable from transcription, we generated a series of CRTC1 deletion mutants (Figure 7A). Previous experiments defined residues 435–635 as the trans-activation domain, whereas a clustal alignment of all three CRTCs showed a conserved proline-rich region, spanning residues 334–434, that shares considerable identity to several proteins including known splicing factors. Analysis of

internal deletion mutants encompassing this domain (residues 334–434) ablated alternative splicing activity despite comparable transcriptional output (Figure 7B–D). Analysis of smaller deletions within this domain maintained transcriptional activity and showed attenuated alternative splicing compared with wild-type CRTC1, although the level of attenuation was not as effective as the larger internal deletion (Figure 7B–D).

Several connections have been made between aberrant pre-mRNA splicing and tumour development (Srebrow and Kornblihtt, 2006; Fackenthal and Godley, 2008). Salivary gland tumours possess a t(11;19) translocation that fuses the 42-amino-acid N-terminal portion of CRTC1 (MECT1) to a 981-amino-acid C-terminal portion of MAML2 to generate the chimeric oncoprotein (Figure 7E) (Tonon *et al*, 2003; Wu *et al*, 2005). Although CRTC1 functions as a potent



co-activator for CREB, MAML2 (mastermind-like 2) is a key component of the NOTCH signalling pathway and functions as a co-activator for RBPJ (Wu *et al*, 2002). Consequently, studies suggest that the CRTC1–MAML2 translocation induces the expression of multiple CREB and NOTCH target genes, thereby contributing to transforming activity (Tonon *et al*, 2003; Wu *et al*, 2005). Transformation relies on the formation of the chimeric oncoprotein, as overexpression of either parent protein does not promote tumourigenesis. As the CRTC1–MAML2 oncoprotein lacks the identified CRTC splicing domain, we tested whether this fusion oncoprotein leads to aberrant splicing of CREB-dependent targets. Analysis of the CREB-dependent *EVX1* luciferase reporter shows robust transcriptional activity with both CRTC1 and CRTC1–MAML2, but not with MAML2 alone (Figure 7F). Notably, TaqMan qPCR analysis of *EVX1* ED I alternative splicing shows that the CRTC1–MAML2 oncoprotein is not capable of influencing splice-site selection of the *EVX1* minigene reporter (Figure 7G and H). Collectively these data show that the region of CRTCs that recruits factors capable of mediating splice-site selection functions on cAMP-responsive genes is independent of the transcriptional activation domain present.

Discussion

The findings presented herein show that the CRTC co-activators are capable of coupling activated transcription of cAMP-responsive genes to alternative pre-mRNA splicing. Remarkably and unique to the CRTCs, these robust transcriptional co-activators can also direct alternative splicing independent of CRTC-induced transcriptional activation. These autonomous activities of the CRTC co-activators include cell type-specific modes of regulation and support the idea that co-regulators involved in pre-mRNA splicing are not restricted solely to mechanisms requiring transcriptional activation.

Cell type- and developmental stage-specific transcript variations have been documented in several genome-wide mRNA splicing studies (Johnson *et al*, 2003; Ule *et al*, 2005; Kwan *et al*, 2008). These exonomic approaches have posed many questions regarding regulation of gene expression at the exon level and concerted efforts are underway to define the diverse mechanisms that regulate transcript identity (Blencowe, 2006; Moore and Silver, 2008). For example,

some transcripts seem to be governed by the promoter-dependent coordination of transcription and alternative splicing (Auboeuf *et al*, 2007). Our data and a previous study clearly show that promoters containing CRE elements are capable of mediating alternative splicing and here we have shown that the CRTC co-activators regulate this process (Cramer *et al*, 1997). The idea that the CRTC co-activators perform more than one function is in accord with those of other co-regulators that possess multiple activities (Rosenfeld *et al*, 2006). For example, the CREB co-activator CBP mediates gene regulation through its intrinsic and associated histone acetyl transferase activity and by promoting the formation of the pre-initiation complex (PIC) through interactions with components of the core transcriptional machinery (Bannister and Kouzarides, 1996; Nakajima *et al*, 1997). Moreover, the transcriptional co-activators PGC (Monsalve *et al*, 2000), COAA (Auboeuf *et al*, 2004) and CAPER (Dowhan *et al*, 2005) function as transcriptional activators, as well as regulators of transcriptionally coupled pre-mRNA processing. However, in contrast to other co-activators involved in splicing, the CRTCs lack any conserved, definable RNA-binding domain (e.g., an RNA recognition motif (RRM), an arginine–serine (RS) domain, or a K homology (KH) domain). Rather, we hypothesize that CRTCs provide a scaffold for the assembly of a larger complex of proteins that may directly bind the transcript. This idea is supported by our CRTC IP MS/MS experiments that identified several proteins involved in pre-mRNA processing (Amelio *et al*, 2007). If the CRTCs form the basis for a protein scaffold that mediates RNA processing by recruiting other proteins, then the ability of CRTCs to recruit slightly different complexes (i.e. cell type or promoter specific) may contribute to these independent bipartite functions. Moreover, PKA may contribute to TORC-dependent alternative splicing through phosphorylation of components within the recruited splicing complex (Xie *et al*, 2003).

There is now overwhelming evidence that the subcellular localization, and thus function, of the CRTC2 co-activator is mediated by several cues, including glucagon and insulin (Figure 8). CRTC2 increases fasting blood glucose levels and the induction of key genes directly involved in gluconeogenesis (Canettieri *et al*, 2005; Koo *et al*, 2005; Dentin *et al*, 2007). Collectively, these studies showed that CRTC2 is the rate-limiting step of cAMP signalling and that it alone is sufficient to initiate the early stages of the gluconeogenic

Figure 7 Transcriptional activation is not required for CRTC-dependent alternative splicing. (A) Schematic illustration of full-length and internal CRTC deletion proteins with corresponding deleted amino acids indicated in parentheses. (B) Transient co-transfection of CRTC deletion mutants with *EVX1* luciferase reporter into HEK293T cells were analysed for luciferase activity ($n=6$ wells; mean \pm s.e.m.; asterisk denotes P -value ≤ 0.05). (C) TaqMan real-time qPCR analysis of CRTC deletion mutants on *EVX1* ED I minigene exon skipping. The relative exon skipping levels are expressed as the ratio of skipped versus included transcripts ($n=3$ experiments; mean \pm s.e.m.; asterisk denotes P -value ≤ 0.05). (D) TaqMan real-time qPCR analysis of CRTC deletion mutants on *EVX1* ED I minigene reporter gene expression. Relative amounts of ED I reporter transcripts obtained by each transfection were plotted as folds relative to basal transcription ($n=3$ experiments; mean \pm s.e.m.; asterisk denotes P -value ≤ 0.05). (E) Schematic illustration of key CRTC1 and MAML2 protein domains relative to the CRTC1–MAML2 chimeric oncoprotein. Corresponding amino acids derived from CRTC1 are indicated above, whereas those derived from MAML2 are indicated below. Abbreviations are as follows: AD, acidic domain; BD, basic domain; CC, coiled-coil; SpD, splicing domain; TAD, transactivation domain. (F) Transient co-transfection of CRTC1–MAML2 chimeric oncoprotein with *EVX1* luciferase reporter into HEK293T cells were analysed for luciferase activity ($n=6$ wells; mean \pm s.e.m.; asterisk denotes P -value ≤ 0.05). (G) TaqMan real-time qPCR analysis of CRTC1–MAML2 chimeric oncoprotein on *EVX1* ED I minigene exon skipping. The relative exon skipping levels are expressed as the ratio of skipped versus included transcripts ($n=3$ experiments; mean \pm s.e.m.; asterisk denotes P -value ≤ 0.05). (H) TaqMan real-time qPCR analysis of CRTC1–MAML2 chimeric oncoprotein on *EVX1* ED I minigene reporter gene expression. Relative amounts of ED I reporter transcripts obtained by each transfection were plotted as folds relative to basal transcription ($n=3$ experiments; mean \pm s.e.m.; asterisk denotes P -value ≤ 0.05).

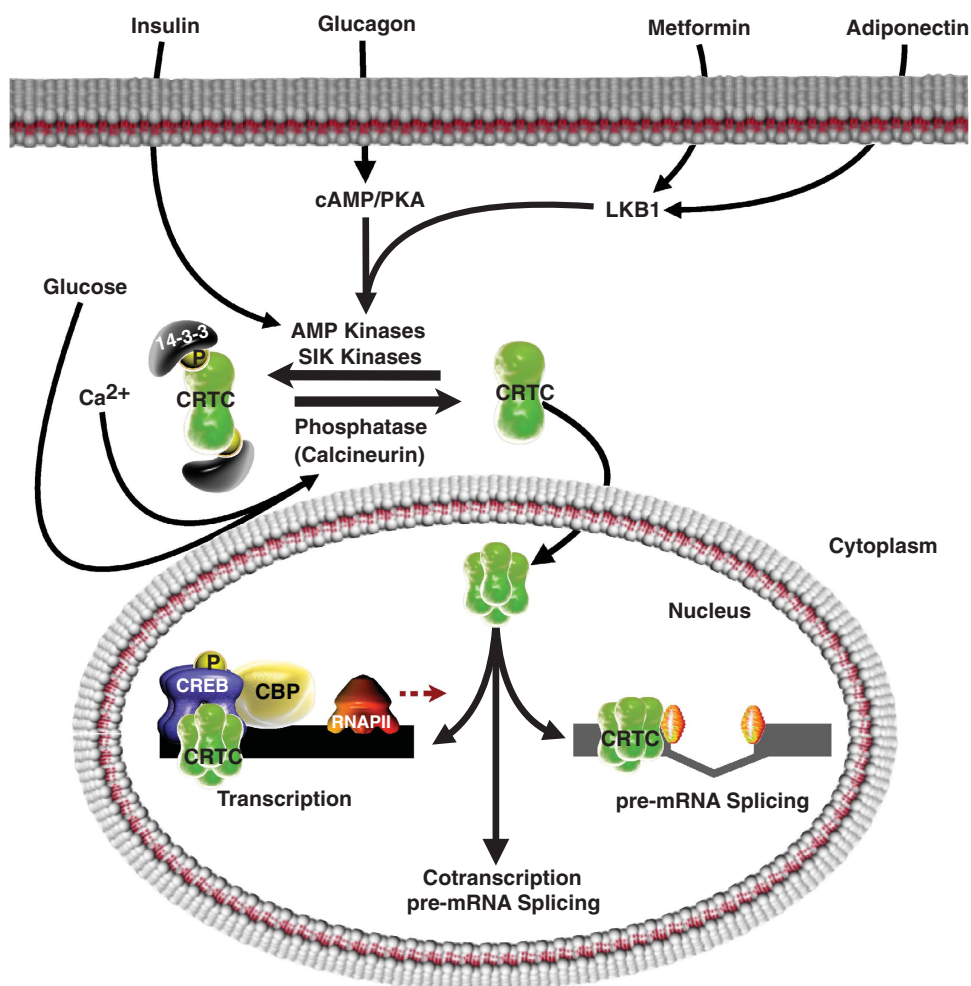


Figure 8 Model of CRTC cytoplasmic and nuclear functions. Schematic representation of environmental signalling cues that converge onto the CRTC co-activators to regulate pre- and post-transcriptional processes.

program. Interestingly, several of the genes within the gluconeogenic program such as *Nurr1* (*Nr4A2*), as well as those encoding proteins that mediate later stages of glucose production such as *PGC-1* (*Ppargc1a*), are alternatively spliced and we show here that CRTC2 can promote alternative splicing of *Nr4a2*. Thus, CRTCs function as integrators of extracellular signals to influence transcript diversity by acting either as a conduit between components of the transcription and splicing machineries or as autonomous regulators of each process (Figure 8). However, although TORCs can selectively activate splicing in some promoter contexts without activating transcription, these promoters possess basal transcriptional activity, therefore, the effects of the TORCs on pre-mRNA splicing could still formally be considered co-transcriptional despite the absence of co-activator-induced transcription.

There are two prevailing models of promoter-dependent coordination of transcription and pre-mRNA processing that could apply to CRTC-mediated alternative splicing. These include splicing factor recruitment to the carboxyl terminal domain (CTD) of RNA polymerase II (RNAP II) (recruitment model) and regulation of RNAP II elongation rates (kinetic model) (Bentley, 2005; Kornblihtt, 2005). These models embody a ‘forward coupling’ mechanism that links RNAP II

transcription to pre-mRNA splicing. However, ‘reverse coupling’ mechanisms have been described, whereby pre-mRNA splicing exerts an influence back on transcription (Brinster *et al*, 1988; Furger *et al*, 2002). Indeed, promoter-proximal 5’ splice sites seem to promote reverse coupling by increasing transcription initiation through recruitment of the PIC (Damgaard *et al*, 2008). CRTCs interact with TAF_{II}130, a component of the PIC (Conkright *et al*, 2003a). Therefore, the finding that CRTCs can induce splicing independent of their effects on transcription may reflect the failure of CRTCs to recruit components of the PIC to TATA-less promoters. Accordingly, although parts of both models help to explain mechanistically how the CRCT co-activators mediate alternative splicing, neither model can fully account for our observations, suggesting that further refinement to the current working models may be necessary.

Apart from CRTCs mechanistic role in pre-mRNA processing, CRTC-mediated splicing probably has physiological significance. This is supported by the vast number of genes that are alternatively spliced, the large cast of promoters that contain CRE elements (roughly 10%), and by the fact that the CRTC recruitment to promoters (e.g. through interactions with CREB) is sufficient to augment alternative splicing. The inability of the CRTC1–MAML2 oncoprotein to mediate

CREB-dependent splice-site selection, despite retaining robust transcriptional activation properties, suggests that this fusion product may also promote cellular transformation through aberrant pre-mRNA splicing of CRE-containing genes. However, transcriptional activators have been shown to promote pre-mRNA splicing, therefore, it is possible that the MAML2 transactivation domain in the CRTC1–MAML2 fusion can mediate splicing in other promoter contexts. Furthermore, although our data clearly show that the CRTC co-activators have the potential to regulate alternative splicing, this does not mandate that transcripts from CRE-containing genes will be alternatively spliced in a CRTC-dependent manner. Indeed, several studies have shown that the significance of the CREB co-activators, CBP/p300, is dictated by gene context and varies among tissues (Cha-Molstad *et al*, 2004; Zhang *et al*, 2005; Ravnskjaer *et al*, 2007; Xu *et al*, 2007). Similar, cell-type specificity is also observed in CRTC-mediated alternative splicing of the endogenous *Nr4a2* transcript. We speculate that the generation of select transcript isoforms may differ between cell types, in part, due to the many diverse signalling events that converge on the CRTC co-activators (Figure 8).

Traditional methods, such as expression arrays or TaqMan PCR focus on gene expression to examine gene regulation in response to signalling cascades. In these technologies, the probes and primer sets intentionally do not account for alternatively spliced products. As a result the canonical analysis of cAMP responsive genes has been restricted to the analysis of transcription induction effectively missing any effects of cAMP activation on pre-mRNA processing. Our findings establish that CRTCs direct alternative splicing independently or in coordination with transcriptional activation, thereby, linking the cAMP signalling cascade with the regulation of pre-mRNA processing. Future experiments will monitor alternative splicing in addition to transcriptional activation when identifying tissue-specific CRTC target promoters.

Materials and methods

Cell culture

Primary rat hepatocytes (Cellz Direct) were maintained according to manufacturer's instructions. Rat pheochromocytoma (PC12) cells were maintained at 37°C in DMEM containing 2 mM GlutaMax (Invitrogen) supplemented with 10% heat-inactivated horse serum (Gibco), 5% FCS (Gemini Bio-Products) and antibiotics (100 units/ml penicillin and 100 mg/ml streptomycin). For NGF-induced differentiation, 5×10^5 cells were plated on 10 cm collagen-coated plates (BD Biosciences) in DMEM supplemented with 100 ng/ml NGF-2.5S (Sigma), 0.05% FCS and antibiotics. Medium was replaced every 2 days. After 4–5 days of exposure to NGF, all cells showed a differentiated morphology and experiments were carried out 6–7 days after the initial plating. HEK293T cells were cultured in DMEM (Invitrogen) supplemented with 10% FCS and antibiotics.

Adenoviruses

The control GFP-expressing or CRTC2-expressing adenoviruses and the non-specific (NS) or CRTC2 RNAi adenoviruses have been described previously by Koo *et al* (2005). Cells were infected with either GFP-expressing or CRTC2-expressing adenoviruses for 16–18 h. RNAi studies were carried out by co-transducing cells with a 4:1 ratio of adenoviruses expressing either NS or CRTC2 shRNAs relative to CRTC2-expressing adenovirus.

Plasmid construction

ED I minigene. The *EVX1* and *CDX4* ED I minigene splicing reporters were obtained by PCR amplification of previously described plasmid templates (Conkright *et al*, 2003b) using primers tagged with *ScaI* and *BssHII* restriction enzyme sequences on the forward and reverse primer, respectively, (primer sequences available on request). The pSVEDA_Tot minigene plasmid was digested with *ScaI* and *BssHII*, the 7150-bp fragment, used to directionally clone the promoter fragments, and clones were confirmed by sequencing. To obtain the $5 \times$ UAS minigene splicing reporters, a short multiple cloning sequence (MCS) was generated by annealing two oligomers with 5' *ScaI* and 3' *BssHII* restriction enzyme sequences. The MCS sequence was kinase treated and cloned into the *ScaI*- and *BssHII*-digested pSVEDA_Tot minigene. The *5xUAS::INR* and *5xUAS::TATA* promoter sequences were obtained by PCR amplification of previously described plasmid templates (Emami *et al*, 1995), using primers tagged with *AscI* and *NotI* restriction enzyme sequences on the forward and reverse primer, respectively, and subsequently cloned into the new pSVEDA_Tot MCS.

HIV-1 minigene. Splicing reporter plasmid pLTR-S1X-R was obtained by inserting the HIV-1 splicing cassette derived from the plasmid pHS1-X (Amendt *et al*, 1994) into pcDNA3 (Invitrogen). A PCR-amplified fragment, comprehensive of the viral *LTR* promoter, was inserted to (Amendt *et al*, 1994) complete the viral sequence upstream of the major 5' splice site. Plasmids pEVX1-S1X-R and pEVX-DM-S1X-R were obtained by substituting the *LTR* promoter in pLTR-S1X-R with a 400-nt fragment containing the wild-type and mutated *EVX1* promoter derived from the plasmids pEVX1 and pEVX1DCRE, respectively (Conkright *et al*, 2003b). The pβglo-S1X-R plasmid was obtained by substituting the *LTR* promoter in pLTR-S1X for a 700-bp genomic fragment containing the *β-globin* gene promoter region. The pβglo-GaS-S1X-R plasmid containing the gal4 $2 \times$ UAS was generated by annealing, Klenow treating and inserting the following primers into the *SnaBI* restriction site (–275 nt upstream of +1 nt):

Ga5Ba, GATCC cggagtactgtcctccg cggagtactgtcctccg TACGTA G;
Ga3Ba, GATCC TACGTA cggaggacagtactcccgaggacagtactccg G.

Cell transfections and reporter gene analyses

Luciferase assays were carried out as previously described (Amelio *et al*, 2007). Reverse transfection was carried out in 96-well plates using 50 ng of luciferase reporter per well. Serum-free DMEM (60 ml) containing 25 ng test cDNA constructs and Lipofectamine 2000 (Invitrogen) was allocated into each well. After a 30-min incubation at room temperature, DMEM supplemented with 20% FBS (60 ml) containing 3×10^4 HEK293T cells was dispensed into each well. Cells were cultured for 24 h in a humidified incubator at 37°C in 5% CO₂. BrightGlo (Promega) reagent (120 ml) was added to each well and luciferase luminescence was measured with an Acquest plate reader (LJL Biosystems).

Splicing assays were carried out as previously described (Cramer *et al*, 1997) with minor modifications. HEK293T cells were maintained at below 80% confluence in DMEM (Gibco BRL) supplemented with 5% FCS and gentamicin (0.5 mg/ml). Cells were seeded 2 h before transfection at 70% confluence in fresh media containing 5% FCS. Transfections were carried out with 2 mg of the minigene plasmid and 500 ng of each additional expression plasmid (PKA, A-CREB and/or CRTCs 1–3) using Lipofectamine 2000 as the transfection reagent. All transfections were DNA balanced using pSport6 plasmid DNA (Invitrogen) as the control and an equal amount of plasmid pEGFP-N1 (Clontech), expressing EGFP, was added to each transfection mixture as a transfection normalizer. Cells were collected 24 h after transfection and total RNA was isolated. Equal amounts of each total mRNA sample (2 mg) were used in subsequent RT-PCR reactions and reverse transcribed using either oligodT or random pd(N)₆ primers. The alternatively spliced ED I isoforms were identified by PCR using the previously described pSV5'j and pSV3'j primers (Cramer *et al*, 1997), whereas the alternatively spliced HIV-1 minigene mRNAs were detected using the primers H5a (AAGTAGTGTGCCCCGTCTGT) and H3b (TCCTGCGTCGATCGATTGTTT). Primers, H5a and H3b, are located upstream of the major HIV-1 5' splice site and thus detect all splice products. Quantitative TaqMan real-time PCR was carried out to measure ED I alternative splicing and total transcription. The

TaqMan primer/probe sequences are listed in Supplementary figure 1D. Quantitative SYBR green PCR analysis was carried out to measure transcription by amplifying unspliced HIV-1 minigene sequences expressed by the reporter construct with the primers RRE5 and RRE3. Each sample was normalized by quantifying the pEGFP-N1 gene product with the primers E5a (ACCACATGAAGCAGCAGCACTTCT) and E5b (TCACCTTGATGCCGTTCTCTGCT). End-labelled ATP- γ - 32 P PCR products were fractionated on polyacrylamide gels, exposed to film respectively, visualized and the band intensities were quantified utilizing either Bio-Rad Quantity One v4.6.2 or Kodak 1D software.

Endogenous gene expression analysis

RNA was extracted from HEK293T cells using TRIzol (Invitrogen) and reverse transcription was conducted with SuperScript III reverse transcriptase (Invitrogen). PCR amplification reactions were run in triplicate in 96-well optical microplates in an ABI Prism 7900 HT SDS instrument (Applied Biosystems). Differences in mRNA expression levels were measured by relative quantification using the comparative $\Delta\Delta$ Ct method (Applied Biosystems) and were normalized against *GAPDH* levels. Probe sequences have been previously described by Amelio *et al* (2007).

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Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Conflict of interest

The authors declare that they have no conflict of interest.

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