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DARPP-32 binds to tra2-beta1 and influences alternative splicing

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Abstract

The majority of human genes undergo alternative splicing, which is frequently altered in response to physiological stimuli. DARPP-32 (Dopamine and cAMP regulated phosphoprotein, 32 kD) is a component of PKA-dependent signaling pathways. Here we show that DARPP-32 binds directly to the splicing factor tra2-beta1 (transformer 2). DARPP-32 changes the usage of tra2-beta1 dependent alternative exons in a concentration dependent manner, suggesting that the DARPP-32:tra2-beta1 interaction is a molecular link between signaling pathways and pre-mRNA processing.

Introduction

Splicing

All polymerase II transcripts undergo pre-mRNA processing and at least 95% of the transcriptional units are alternatively spliced [1]. The exact regulation of alternative splicing events is physiologically important as evidenced by an increasing number of diseases shown to result from the selection of the wrong splice site [2,3]. The proper recognition of alternative exons is regulated by the transient formation of protein complexes on the pre-mRNA that mark a sequence for its recognition by the spliceosome [4].

Tra2-beta1

Tra2-beta1 is one of the proteins that mark exonic sequences for inclusion in the mRNA. Tra2 was first discovered in drosophila where it regulates the sex development of flies and has been subsequently identified in all metazoan organisms [5,6]. The protein consists of a central RNA recognition motif flanked by two serine-arginine rich protein domains that promote protein interactions. Tra2-beta1 generally promotes the inclusion of alternative exons in a concentration-dependent manner [7]. The protein contains an evolutionary conserved RVDF-binding motif in the beta4 strand of its RNA recognition motif, which

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allows tra2-beta1 to bind to protein phosphatase 1 (PP1) [8]. This motif corresponds to the consensus sequence found in most PP1 interacting proteins (RVXF) [9,10]. The ability to bind to protein phosphatase 1 is essential for the function of tra2-beta1 in splice site selection [8] and a change in PP1 activity influences the splicing of a subset of alternative exons [8].

DARPP-32

Protein phosphatase 1 catalytic activity is tightly controlled in the cell. This control is achieved by sequestration of PP1 by targeting subunits and regulatory proteins that usually inhibit its activity. Only upon stimulation, the catalytic PP1 activity is released and PP1 dephosphorylates other proteins [11]. One of the inhibiting proteins is DARPP-32, for dopamine and cAMP regulated phosphoprotein, 32 kD. DARPP-32 is phosphorylated by PKA in response to an elevation of cellular cAMP levels and this phosphorylation causes a tight binding and inhibition of PP1. DARPP-32 was originally described as a cytosolic protein [12]. Recent results showed DARPP-32 accumulates in the nucleus after amphetamine or cocaine treatment. This accumulation blocked protein phosphatase 1 in the nucleus, leading to an increase of histone H3 phosphorylation [13].

Splicing and signaling

The usage of alternative exons can be altered by the cell, allowing for adaptations of the gene expression due to a stimulus [14,15]. The molecular pathways that connect a change in gene expression caused by a different usage of alternative exons with cellular signaling pathways are only beginning to emerge. Numerous examples indicate that reversible phosphorylation of splicing factors plays a crucial role in this process [16]. Here, we show that DARPP-32, a well-known signaling molecule, interacts with the splicing factor tra2-beta1 and changes splice site selection of tra2-beta1 dependent exons. These results link DARPP-32 dependent signaling pathways with pre-mRNA processing events.

Material and Methods

Primary neuronal culture

Primary hippocampal cultures were prepared as described [17]. In brief, hippocampi were removed from Wistar rat embryos (E18). Cells were dissociated by mild trypsination (0.25% trypsin) in the presence of DNAse I (EC 3.1.21.1; 0.1 mg/mL) for 30 min. After addition of 10% fetal calf serum, dissociated cells were washed and resuspended in neurobasal medium containing l-glutamine and B-27 supplement (Gibco–BRL, Eggenstein, Germany) and plated on poly-l-lysine-coated (50 μg/mL) 6-well plates (3.5×10^6 cells per well), or on silanated and poly-l-lysine coated glass coverslips in 3-cm plates (1.5×10^5 cells per plate).

Cell culture and transfection

HEK293 cells were maintained in DMEM supplemented with 10% fetal calf serum (GibcoBRL). For immunolabeling experiments, cells were grown on glass coverslips in 3.5 cm cell culture dishes. The day before transfection, 3.0×10^5 HEK293 cells per 3.5 cm plate were seeded in 3 ml of DMEM and 10% FCS and incubated at 37°C in 5% CO_2 for 24 h. Transient transfections of adherent HEK293 cells with cDNAs were performed using the calcium precipitation method as described [18].

Immunocytochemistry

HEK293 cells and primary hippocampal cultures were grown on coverslips. HEK293 cells were transfected with pEGFP-DARPP-32 and Tra2-beta1-Flag constructs overnight, washed in PBS at pH 7.4 and fixed in 4% para-formaldehyde for 20 min at 4°C. Permeabilization

and blocking was for 30 min with 0.5% Triton X-100 and 3% Normal Goat Serum (Dianova) in PBS. Incubation with the anti-Tra2- or anti-DARPP-32 antiserum (1:200 in PBS, 0.3% NGS, 0.5% Triton X-100) was for 1h at 4°C. After washing three times for 10 min with PBS, the cells were incubated with a 1:200 dilution of a Cy3-coupled goat anti-rabbit-IgG antibody (Dianova) for 45 min. Untransfected neuronal cultures were fixed and incubated with primary antiserum as described above. After washing the cells were incubated with a goat anti-rabbit Cy-2 conjugated IgG (Dianova, 1:200) and a rabbit anti-goat Cy-3 conjugated IgG (Dianova, 1:300). Cells were washed three times in 1xPBS prior to mounting on microscope slides with Gel/Mount (Biomeda). The cells were examined by confocal laser scanning microscopy (Leica DMIRE2) using a HCX Plan Appochromat 100×1.4 CS oil immersion objective.

Coimmunoprecipitation and Western blots

were performed as described [19]. The following antibodies were used: polyclonal rabbit anti Tra2 (pan Tra2 beta) 1:1000 [20]; polyclonal goat anti-PP1 (C-19) 1:200 (Santa-Cruz biotechnology); polyclonal goat anti-DARPP-32 (N-19) 1:200 (Santa-Cruz biotechnology).

siRNA knockdown

The siRNA knockdown of DARPP-32 in HEK293 cells was performed using the reverse transfection procedure according to manufacturer's (Qiagen, Hilden) direction on 6 well-plates. siRNAs for DARPP-32 (Santa Cruz sc-35173) were used as a pool of 3 target-specific 20–25 nt oligos at 5 nM final concentration. As a negative control scrambled siRNA-A (Santa Cruz sc-37007) was used. After 48 hours 1000 ng/well cDNA of the minigenes tau or tra2-beta expressing constructs were transfected as described earlier. RNA was isolated after 72 hours siRNA treatment (16–18 h after minigene transfection).

Protein purification and affinity isolation

Tra2-beta1 was purified from baculo virus infected cells as previously described [8]. DARPP-32 was purified from bovine brain as described [21]. Unbound protein was removed by native wash buffer 50mM NaH_2PO_4, 300mM NaCl, 20mM Imidazole, 0,1% Triton X-100, pH 8.0 in pull down assays.

Results

Tra2-beta1 binds to DARPP-32 in vivo

We previously showed that tra2-beta1 binds protein phosphatase 1 (PP1) via an evolutionary conserved PVXF motif in the RNA recognition motif of tra2-beta1 [8]. These findings suggested that PP1 is present in a complex with splicing factors. We next asked whether other PP1 interacting proteins could also interfere with splicing regulatory proteins. Unexpectedly, during these experiments we found that purified DARPP-32, a known PP1 interactor, binds to tra2-beta1. This was surprising, as a DARPP32:tra2-beta1 interaction has not been detected previously in yeast-two hybrid assays [6,22]. We next confirmed these results by co-immunoprecipitation experiments and determined which protein domains are necessary for the interaction. Tra2-beta1 is composed of a central RNA binding motif flanked by two RS domains (Figure 1A). The RS domains are characterized by clusters of arginine-serine repeats and allow interaction between SR proteins. The RRM of tra2-beta1 binds to RNA and PP1. To investigate which tra2-beta1 protein parts are responsible for binding to DARPP-32 we performed immunoprecipitation experiments using tra2-beta1 variants lacking the first, second or both RS domains. As shown in Figure 1B, deletion of the first RS domain has no effect on DARPP-32 binding, whereas deletion of the second RS domain prevents binding.

To determine whether DARPP-32 can interact with endogenous tra2-beta1, we analyzed immunoprecipitates made from HEK239 cells that transiently express EGFP-DARPP-32. As shown in Figure 1C, we could detect endogenous tra2-beta1 in these immunoprecipitates, suggesting that the interaction can take place under physiological tra2-beta1 concentrations.

We next ask whether other SR-proteins could interact with DARPP-32 and coexpressed EGFP-tagged DARPP-32 with Flag-tagged SRp30c, SF2/ASF and SC35. As shown in Figure 1D-F, we could not detect these SR-proteins in the DARPP-32 immunoprecipitates.

Together, this data indicate that DARPP-32 binds to tra2-beta1 and that the second RS domain of tra2-beta1 is necessary for DARPP-32 binding. In contrast to tra2-beta1, several other SR-proteins do not interact with DARPP-32.

DARPP-32 colocalizes with tra2-beta1 in the nucleus of primary neurons

To investigate whether the interaction between DARPP-32 and tra2-beta1 could occur in cells, we performed double immunohistochemistry staining experiments using primary neuronal cultures. We used hippocampal neurons, which express low levels of DARPP-32 *in vivo*, but in which we found a significant labeling in culture. Neurons derived from E18 embryos were stained for endogenous DARPP-32 and tra2-beta1 after 14 days of culture. As shown in Figure 2, A–C both proteins show an overlapping staining in the nucleus. To determine whether there is a colocalisation in another cell type, we tested the localization of overexpressed DARPP-32 and endogenous tra2-beta1 in HEK293 cells. As shown in Figure 2E–G, in these cells DARPP-32 is primarily cytosolic, but can also be found in the nucleus, where DARPP-32 colocalizes with tra2-beta1. No staining was observed when the primary antisera were omitted (Figure 2D). Together, these results argue that both proteins colocalize *in vivo* and could indeed interact with each other.

PP1 antagonizes the binding of DARPP-32 to tra2-beta1

We next tested whether the interaction between DARPP-32 and tra2-beta1 is direct and performed pull-down experiments using purified proteins. His-tra2-beta1 generated in insect cells [8] was coupled to Ni-agarose. This affinity matrix was incubated with purified DARPP-32. To rule out nucleic acid mediated interaction, benzonase was present in the binding reaction. The protein mixture was washed three times in Native Wash Buffer. After washing, protein was removed by boiling in 1% SDS and analyzed by PAGE followed by Western blot. As shown in Figure 3A, lane 4, this affinity resin bound DARPP-32. In contrast, no DARPP-32 was retained when no tra2-beta1 was prior bound to the resin (Figure 3A, lane 6). This experiment supports the interaction indicated by immunoprecipitation and shows that the binding between DARPP-32 and tra2-beta1 is based on direct protein:protein interaction.

PP1 binds to both tra2-beta1 and DARPP-32 ([8], Figure 1,3). To test whether both proteins compete for PP1 binding, we incubated both PP1 and DARPP-32 with the tra2-beta1-affinity column. We found that the presence of PP1 reduced the amount of DARPP-32 bound to the column, (Figure 3A, lane 5). To further test whether DARPP-32 and PP1 compete for tra2-beta1 binding, we used different PP1 concentrations. The tra2-beta1 affinity matrix was loaded with purified DARPP-32 (50 μM), and subsequently half and equal molar amounts of PP1 were added. As shown in Figure 4B, lane 2 and 3, the added PP1 replaced DARPP-32 bound to tra2-beta1. Finally, we tested whether the PP1 binding site in the RRM of tra2-beta1 is involved in DARPP-32 binding and used tra2-beta1-RATA protein as an interacting partner. In this protein, the PP1 binding site in the beta4 strand of the RRM is changed from RVDF to RATA, which completely abolished PP1 binding [8]. As shown in Figure 3B, lane 4, DARPP-32 binds to tra2-beta1-RATA, indicating the PP1

binding site is not involved in DARPP-32 binding. Together, these data indicate that PP1 competes with DARPP-32 to bind to tra2-beta1.

DARPP-32 changes alternative splicing of tra2-beta1 dependent exons

Tra2-beta1 binds to GAA-rich sequences and generally promotes inclusion of alternative exons that contain such motifs [7]. We therefore determined whether DARPP-32 influences alternative splice site selection of two of such exons, the alternatively spliced exon 2 of the tra2-beta1 pre-mRNA and the tau exon 10 [7]. First, we transfected an increasing amount of DARPP-32 expression construct together with splicing reporter genes into HEK293 cells. As shown in Figure 4A–C, an increased amount of DARPP-32 caused skipping of the alternative exon in both the tau and tra2-beta1 reporter constructs, that paralleled the increase of DARPP-32 protein caused by the overexpression (Figure 4D).

We next performed the reverse experiment and removed DARPP-32 by siRNA treatment. As shown in Figure 5A–C, siRNA treatment promoted inclusion of both alternative exons, which again paralleled the decrease of DARPP-32 caused by siRNA treatment.

These experiment indicates that the relative concentration of DARPP-32 influences alternative splice site selection.

Discussion

DARPP-32 is a new interactor for tra2-beta1

Alternative splice site selection is regulated by reversible formation of protein complexes on pre-mRNA. Tra2-beta1 is a splicing regulatory protein that is present in all metazoa. Tra2-beta1 binds to other proteins via two domains that are rich in serine and arginine residues, the RS-domains. Previous studies showed interaction of tra2-beta1 with other typical RNA processing proteins, such as SR-proteins [6], hnRNP G and hnRNP G-like proteins [23,24] as well as SAM68/p62 [25]. Recently, it was demonstrated that tra2-beta1 binds to protein phosphatase 1 via an evolutionary conserved binding site in its RNA recognition motif [8]. PP1 influences the phosphorylation status of tra2-beta1 and its ability to change splice site selection.

Here we describe that the PP1 interacting protein DARPP-32 binds to tra2-beta1. Both DARPP-32 and tra2-beta1 shuttle between nucleus and cytosol [13,26] and staining of both proteins reveals a nuclear colocalisation, indicating that the interaction between the two proteins is physiologically relevant. The interaction between DARPP-32 and tra2-beta1 cannot be observed in yeast two hybrid systems (our unpublished data), which could indicate that post-translational modifications, such as proper phosphorylation patterns are required for the interaction. The binding between the two proteins is direct and most likely involves the C-terminus of tra-beta1. The tra2-beta gene generates two main proteins: tra2-beta1 and tra2-beta3. Tra2-beta1 contains two RS domains and tra2-beta3 lacks the first RS domain. Whereas tra2-beta1 has been shown to influence numerous alternative exons, the tra2-beta3 isoform does not change alternative exon usage. Our immunoprecipitation experiments indicate that tra2-beta3 binds DARPP-32. Similar to DARPP-32, the tra2-beta3 isoform is predominantly expressed in brain [6]. We therefore postulate that the function of this isoform is to sequester DARPP-32 without influencing splice site selection.

Upon proper phosphorylation, DARPP-32 inhibits PP1, which generally promotes exons that are dependent on splicing factors that contain PP1 binding sites, such as SF2/ASF and SRp30c [8]. In contrast to tra2-beta1, these two SR-proteins did not interact with DARPP-32 when analyzed by co-immunoprecipitation. This suggests that DARPP-32 has a special

influence on tra2-beta1 dependent exons, as it sequesters tra2-beta1, but not other SR-proteins.

Functional implication

To test whether the binding of the two proteins has functional implications, we changed the concentration of DARPP-32 by overexpression and siRNA mediated knock down and tested the effect on two splicing reporter genes. We investigated the effect on tau exon 10 and exon 2 of the tra2-beta1 pre-mRNA, which are regulated by tra2-beta1 via a purine-rich motif. We found the DARPP-32 overexpression promotes alternative exon skipping upon overexpression, but favored exon inclusion when it is reduced by siRNA.

A change in intracellular DARPP-32 concentration does not affect every tra2-beta1 dependent alternative exon, as we did not see an effect when testing the CLK2, and SMN2 reporter genes that were previously shown to be regulated by tra2-beta1 [27,28], (data not shown). This indicates that DARPP-32 concentrations influence a subset of alternative exons, which most likely share an similar arrangement of splicing regulatory factors. The exact mechanism of DARPP-32 action remains to be determined. We did not observe a shift in tra2-beta1 mobility on PAGE gels upon DARPP-32 overexpression, which argues against a global effect on the phosphorylation status, but it is possible that specific phosphorylation events are targeted.

The intracellular localization of DARPP-32 and its affinity to PP1 is regulated by protein kinase A in response to cellular stimulation. Our finding that DARPP-32 binds to tra2-beta1 and changes the processing of specific pre-mRNA connects cAMP-dependent signaling pathways emanating from the cell membrane with the regulation of nuclear RNA processing.

Acknowledgments

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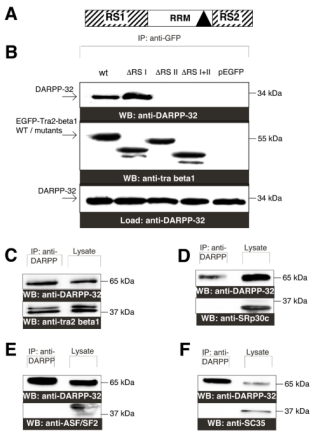

Figure 1. Tra2-beta1 binds to DARPP-32 in vivo

A. Domain structure and sequence of tra2-beta1. The two RS domains (RS), the RNA recognition motif (RRM) are indicated. The PP1 binding site in the RRM is shown as a triangle.

B. Analysis of tra2-beta1 immunoprecipitates. EGFP-Tra2-beta1 was overexpressed in HEK293 and immunoprecipitated with anti tra2-beta1. DAPRP-32 was detected with anti-DARPP-32.

C. Analysis of EGFP-DARPP-32 immunoprecipitates. EGFP-DARPP-32 was expressed in HEK239 cells and immunoprecipitated with anti DARPP-32. Endogenous tra2-beta1 bound in the immunprecipitates was detected by the anti-tra2-beta1 antiserum.

D–F. Coimmunoprecipation of DARPP-32 with SR-proteins. EGFP-DARPP-32 was coexpressed with FLAG-tagged expression constructs for SRp30c, ASF/SF2 and SC35. The immunoprecipitates were analyzed with anti-Flag antiserum.

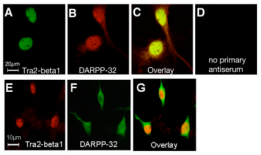

Figure 2. tra2-beta1 colocalizes with DARPP-32

A–D. Primary neuronal cultures were stained with an antiserum against endogenous DARPP-32 and tra2-beta1.

A. Staining of DARPP-32

B. Staining of tra2-beta1

C. Overlay of both fields

D. Negative control where both primary antisera were omitted.

E–G. HEK293 cells were transfected with EGFP-DARPP32 and endogenous tra2-beta1 was visualized with an anti-tra2-beta1 serum.

E. Signal EGFP-DARPP-32

F. Staining of tra2-beta1

G. Overlay of both fields

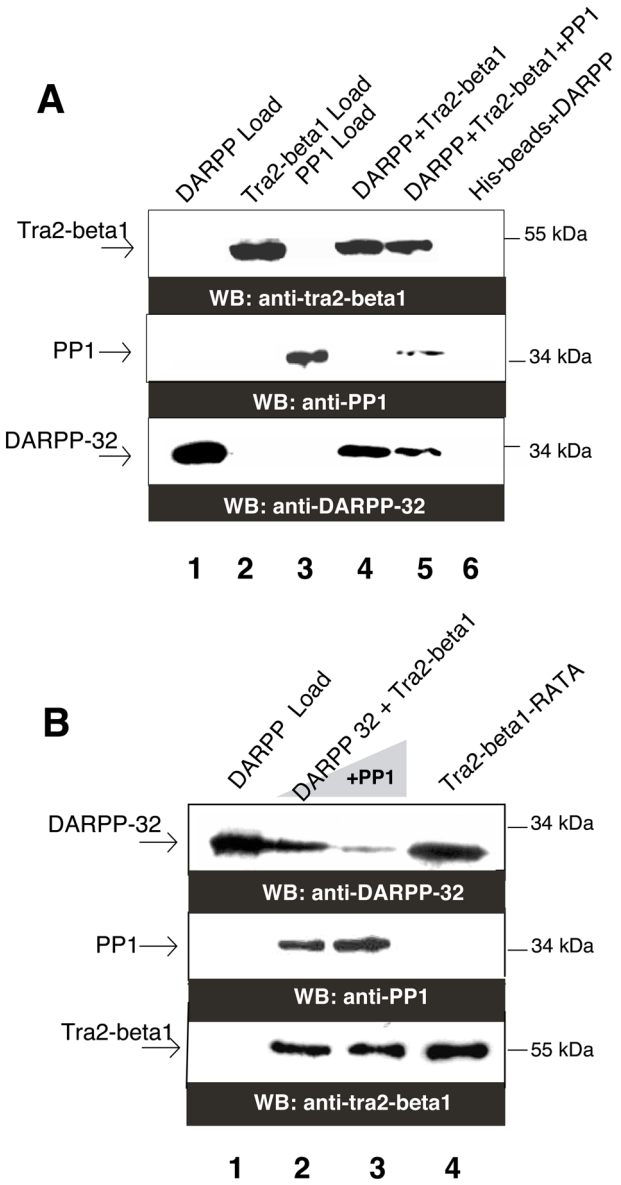

Figure 3. DARPP-32 binds directly to tra2-beta1 and antagonizes PP1 binding to tra2-beta1

His-tra2-beta1 was loaded on Ni-agarose. This affinity column was incubated with recombinant DARPP32 and PP1 alpha.

A. Detection of the bound proteins by Western blot, using antisera against PP1, tra2-beta1 and DARPP-32.

B. DARPP-32 was bound to the tra2-beta1 affinity resin and incubated with an increasing amount of PP1. The bound protein was detected by Western blot.

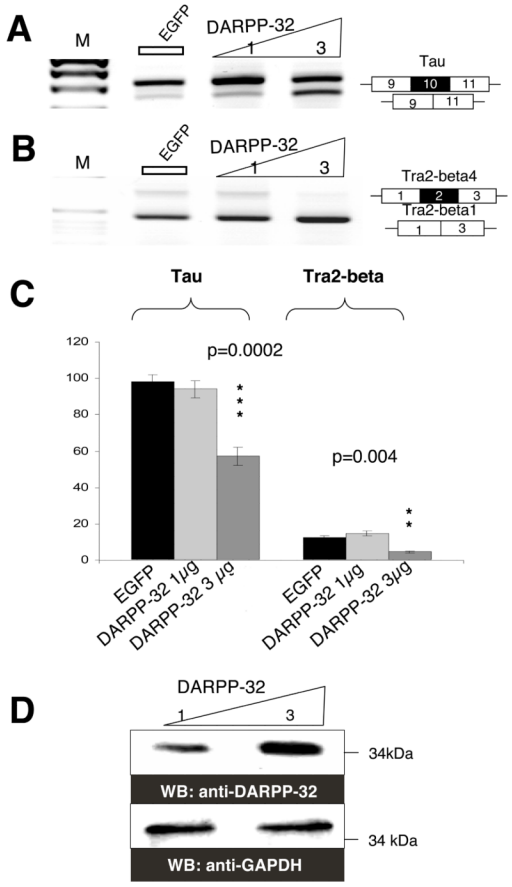

Figure 4. DARPP-32 overexpression changes alternative splicing of reporter genes

The tau and tra2-beta1 reporter minigenes were transfected into HEK293 cells with an increasing amount of DARPP-32 expression constructs. The splice products were analyzed by RT-PCR. Structures of the RNA isoforms are indicated schematically on the right.

A. Effect on the tau minigene

B. Effect on the tra2 minigene

C. Statistical analysis of three independent experiments.

D. Western Blot detecting DARPP-32 overexpression after transfection.

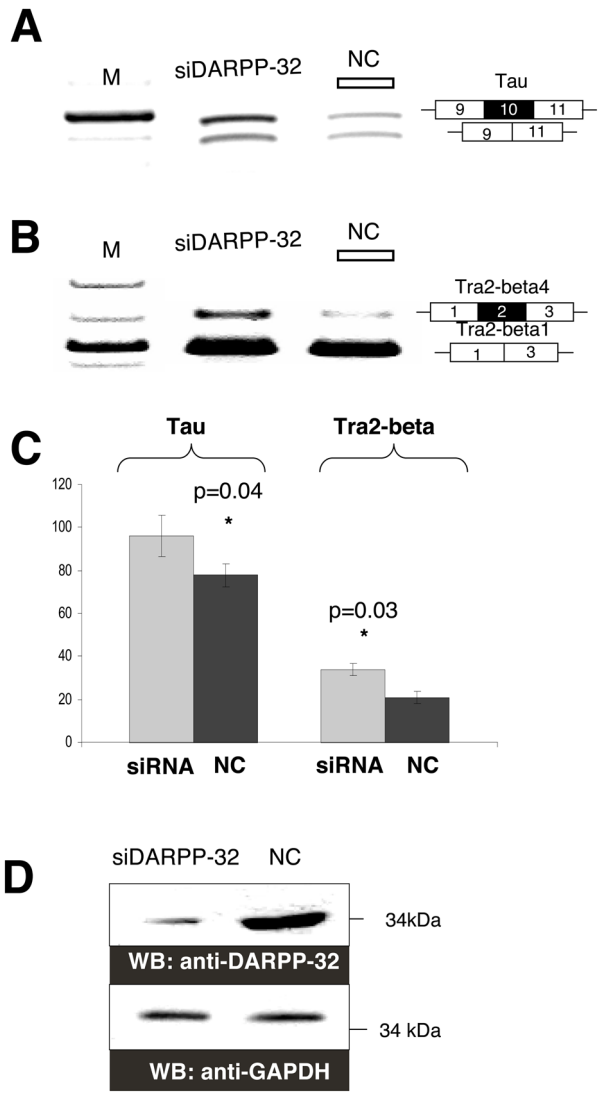

Figure 5. siRNA mediated DARPP-32 knock down changes alternative splicing of reporter genes

The tau and tra2-beta1 reporter minigenes were transfected into HEK293 cells in the presence of DARPP-32 siRNA, and the products analyzed by RT-PCR as in Figure 4.

A. Effect on the tau minigene

B. Effect on the tra2 minigene

C. Statistical analysis of three independent experiments.

D. Western Blot detecting DARPP-32 overexpression after transfection.