

## ORIGINAL ARTICLE

# Heterogeneous nuclear ribonucleoprotein A2 is a SET-binding protein and a PP2A inhibitor

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The oncoprotein SET participates in a diversity of cellular functions including cell proliferation. Its role on cell cycle progression is likely mediated by inhibiting cyclin B-cdk1 and the protein phosphatase 2A (PP2A). On identifying new SET cellular partners, we found that SET interacts *in vitro* and *in vivo* with the heterogeneous nuclear ribonucleoprotein A2 (hnRNPA2); a protein involved in various aspects of mRNA biogenesis. The SET-binding region of hnRNPA2 is the RNP1 sequence that belongs to the RNA-binding domain (RBD) of this protein. We also found that hnRNPA2 has much higher affinity for single-stranded DNA than for SET. On analysing the effect of hnRNPA2 on PP2A inhibition by SET, we observed that hnRNPA2 cooperates with SET on PP2A inhibition. This is because we found that hnRNPA2 is also a PP2A inhibitor. HnRNPA2 interacts with PP2A by the RNP1 sequence; however, to inhibit PP2A activity, the complete RBD is needed. We also observed that overexpression of hnRNPA2 inhibits PP2A activity and stimulates cell proliferation. Interestingly, the overexpression of the complete RBD is sufficient to stimulate proliferation. As hnRNPA2 is overexpressed in a variety of human tumors, our results suggest that hnRNPA2 might participate in oncogenesis by stimulating cell proliferation.

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**Keywords:** SET protein; hnRNPA2; PP2A; cell cycle

## Introduction

The SET protein was firstly identified as a *can*-fusion gene in an acute undifferentiated leukemia (von Lindern *et al.*, 1992). It is a 37 kDa phosphoprotein expressed ubiquitously that participates in a diversity of cellular functions. It has been identified as an specific inhibitor of protein phosphatase 2A (PP2A) (Li *et al.*, 1996b), a major serine–threonine phosphatase that regulates many cellular events including cell proliferation and differ-

entiation. Specifically, it has been shown that SET modulates the effect of PP2A on dephosphorylating the cytochrome *P450c17* and regulating its 17, 20 lyase activity involved in the sex steroids production (Pandey *et al.*, 2003). It has also been reported that both PP2A and SET interact with the HRX leukemic fusion protein in myeloid cell extracts and probably these complexes might participate in the deregulation of cell growth and differentiation in leukemia (Adler *et al.*, 1997).

A number of reports also indicate that SET is a key modulator of DNA replication, chromatin remodeling and gene transcription. It has been shown that SET stimulates the replication of the adenovirus DNA in an *in vitro* system (Matsumoto *et al.*, 1993). SET also stimulates transcription by altering histone–DNA interaction (Okuwaki and Nagata, 1998). SET binds to core histones decreasing the ability of acetyltransferases p300/CBP and PCAF to acetylate them (Seo *et al.*, 2001). More recently, SET has been shown to associate specifically to un- or hypoacetylated histones and to histone deacetylases, thus functioning as a transducer of these signals into transcriptional repression (Kutney *et al.*, 2004). Moreover, it has also been reported that SET and other members of its family might bind to acetyltransferases increasing transcriptional activity (Shikama *et al.*, 2000).

The association of SET with histones is performed as a complex, called inhibitor of acetyltransferases with the protein pp32 (Seo *et al.*, 2001; Kutney *et al.*, 2004). Interestingly, pp32 is a tumor suppressor protein that also has a PP2A inhibitory activity (Li *et al.*, 1996a). SET and pp32 also participate in a 270–420 kDa multi-protein complex termed the SET complex, which also includes the DNA-binding protein HMG-2, the endonuclease Apel and the transcriptional regulator and nucleoside diphosphate kinase NM23-H1 (Beresford *et al.*, 2001; Fan *et al.*, 2002, 2003a, b). The SET complex could also be located in the endoplasmic reticulum, and although its function is still not known, it has been proposed to be involved in the repair response to oxidative stress (Lieberman and Fan, 2003).

A number of reports indicate that SET participates in cell cycle regulation by interacting with components of cyclin–cdk complexes. It has been shown that it binds directly to p21<sup>Cip1</sup> and reverses its inhibitory effect on cyclin E–cdk2 activity (Estanyol *et al.*, 1999). SET also interacts with B-type cyclins (Kellogg *et al.*, 1995), inhibiting cyclin B–cdk1 activity and regulating G2/M

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transition (Canela *et al.*, 2003). Moreover, it has been reported that SET associates with neuronal p35<sup>nck5a</sup> and enhances p35<sup>nck5a</sup>-cdk5 activity (Qu *et al.*, 2002).

Thus, SET is a multifunctional protein that is related to the regulation of cell proliferation, and in the cell, it appears to be associated with other proteins that are also related to proliferation and oncogenic transformation. The aim of our work has been the identification of new cellular partners of SET in order to get new insights on the role of this protein on cell proliferation and oncogenesis. By affinity chromatography and subsequent proteomic analysis, we have identified the heterogeneous nuclear ribonucleoprotein A2 (hnRNPA2) as a SET-binding protein. Our results indicate that similarly to SET protein, hnRNPA2 is also an inhibitor of the activity of the PP2A. We found that overexpression of hnRNPA2 stimulates cell proliferation.

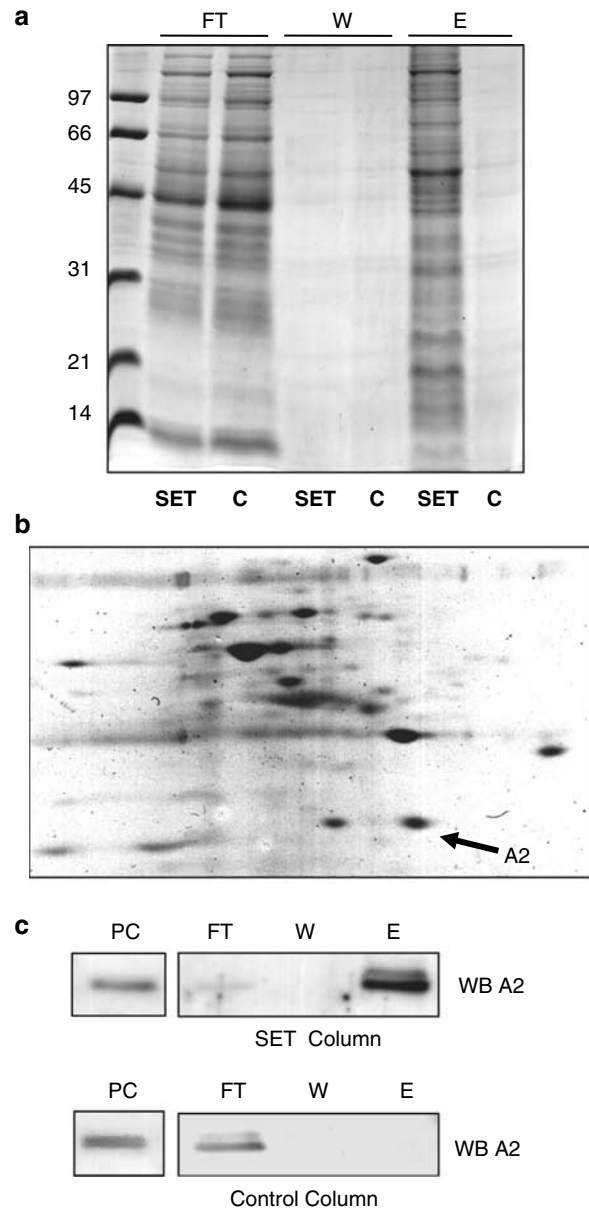
## Results

### Identification of hnRNPA2 as a SET-binding protein

With the aim of identifying new SET-binding proteins, we generated affinity chromatography columns of SET–Sepharose 4B as described under Materials and methods. The columns were loaded with mouse liver lysates, washed and the SET-binding proteins were subsequently eluted with a buffer containing 1 M KCl. The collected proteins were first visualized by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and Coomassie blue staining. As shown in Figure 1a, approximately 20 major proteins were detected in the eluates from the SET columns, whereas in contrast, no proteins were bound to the control columns. To identify the proteins from the eluate, they were first separated by two-dimensional (2-D) gel electrophoresis (Figure 1b). Then, the spots were isolated, trypsinized and the resulting peptides were processed by mass spectrometry using a MALDI-TOF. The spot of 36 kDa pointed out (arrow) in the 2-D gel (Figure 1b) was identified as hnRNPA2. The identification was confirmed by Western blotting of the eluates from the SET columns, using anti-hnRNPA2 antibodies (Figure 1c). As hnRNPA2 is a tumor marker for distinct human malignancies (Fielding *et al.*, 1999), we aimed to study the functional relevance of its interaction with SET protein.

### In vitro and in vivo interaction between hnRNPA2 and SET

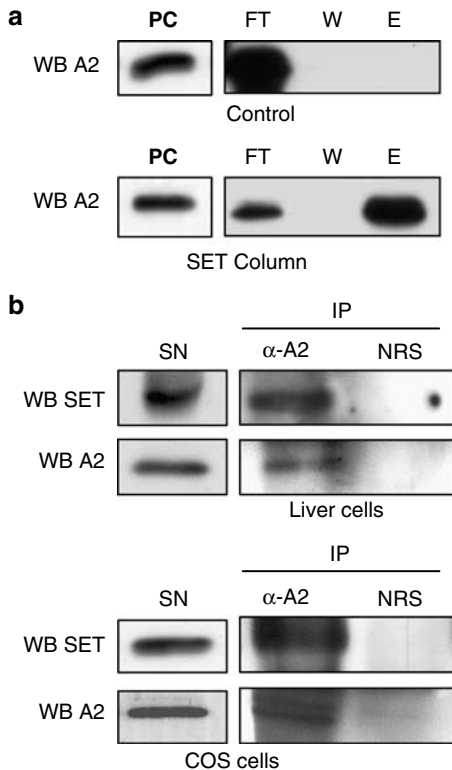
Direct interaction between hnRNPA2 and SET was examined by affinity chromatography using a SET–Sepharose 4B column. This column was prepared by coupling purified recombinant human SET protein to Sepharose 4B beads. HnRNPA2 purified from mouse liver nuclei (Bossler *et al.*, 1995) was loaded onto a SET–Sepharose 4B column or onto a Sepharose 4B column that was used as a control. Then, the binding of hnRNPA2 to the columns was analysed by Western blotting using anti-hnRNPA2 antibodies. Results



**Figure 1** Identification of the heterogeneous nuclear ribonucleoprotein A2 (hnRNPA2) as a SET-binding protein. (a) Mouse liver lysates were loaded onto a SET–Sepharose 4B column (SET) or onto a Sepharose 4B column (C). After extensive washing, the proteins bound to the columns were eluted with a 1 M KCl-containing buffer. Samples from the flow-through (FT), washes (W) and eluates were separated in SDS–PAGE and stained with Coomassie blue. (b) Eluates from a SET–Sepharose 4B column were separated by 2-D electrophoresis and the gels were silver stained. The arrow indicates the protein that was subsequently analysed by mass spectrometry. (c) Samples: pre-column (PC), flow-through (FT), wash (W) and eluate (E) were separated in a SDS–PAGE, transferred onto membranes and analysed by immunoblot with hnRNPA2 antibody.

showed that hnRNPA2 was bound to the SET column but not to the control (Figure 2a).

To investigate whether hnRNPA2 and SET interact *in vivo*, immunoprecipitation experiments were performed. Mouse liver extracts were subjected to immunoprecipitation

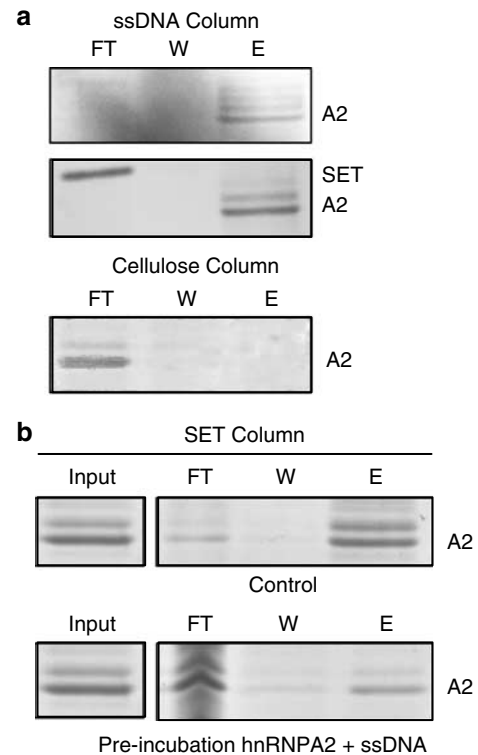


**Figure 2** *In vitro* and *in vivo* interactions between hnRNP2 and SET. **(a)** Purified hnRNP2 was loaded onto a SET-Sepharose 4B column (SET) or onto a Sepharose 4B column (control). The protein bound to the columns was analysed by immunoblot with hnRNP2 antibody. PC, precolumn; FT, flow-through; W, wash; and E, eluate. **(b)** Immunoprecipitation in extracts from mouse liver or COS-7 cells were performed using hnRNP2 ( $\alpha$ -A2) antibody or with normal rabbit serum (NRS) as a control. Then, the presence of SET and hnRNP2 in the immunoprecipitates was analysed by Western blotting using SET or hnRNP2 antibodies, respectively. A sample of cell lysate (SN) was used as a control in the gels.

tation using anti-hnRNP2 antibody or normal rabbit serum that was used as a control. Then, the immunoprecipitates were analysed by Western blotting with anti-SET or anti-hnRNP2 antibodies. Results shown in Figure 2b revealed that endogenous SET and hnRNP2 co-immunoprecipitated. Results were confirmed in similar experiments performed in COS-7 cells (Figure 2b).

#### *HnRNP2 associated with single-stranded DNA (ssDNA) does not bind to SET*

HnRNP2 is an RNA- and an ssDNA-binding protein (Moran-Jones *et al.*, 2005). Thus, we aimed to analyse whether SET could affect the association between hnRNP2 and ssDNA. To test this possibility, a series of binding experiments, based in the capacity of hnRNP2 protein to associate with ssDNA, were performed. First, an ssDNA-cellulose column was loaded with highly purified hnRNP2 that was retained in the column. Then, the column was extensively washed to eliminate the nonbound fraction of hnRNP2. To determine whether SET could interact with hnRNP2



**Figure 3** HnRNP2 associated with ssDNA does not bind to SET. **(a)** Purified hnRNP2 was loaded onto an ssDNA-cellulose column and it was retained in the column. After extensive washing, the column was subsequently loaded with purified SET. Then, after extensive washing, the proteins retained in the column were eluted with a 1M KCl-containing buffer. Samples: flow-through (FT) wash (W) and eluate (E) were electrophoresed and the gels stained with Coomassie blue. A similar experiment, but avoiding the loading of SET onto the column, was performed as a control. An additional control was performed by loading hnRNP2 on a cellulose column. **(b)** Purified hnRNP2 was preincubated with ssDNA, and then the mixture was loaded onto a SET-Sepharose 4B column. After extensive washing, the proteins retained in the column were eluted with a 1M KCl-containing buffer. Samples: flow-through (FT), wash (W) and eluate (E) were electrophoresed and the gels stained with Coomassie blue (bottom panel). A similar experiment but avoiding the preincubation of hnRNP2 with ssDNA was performed (upper panel). In both cases, the input indicates the 15% of the amount of hnRNP2 that was loaded onto the column.

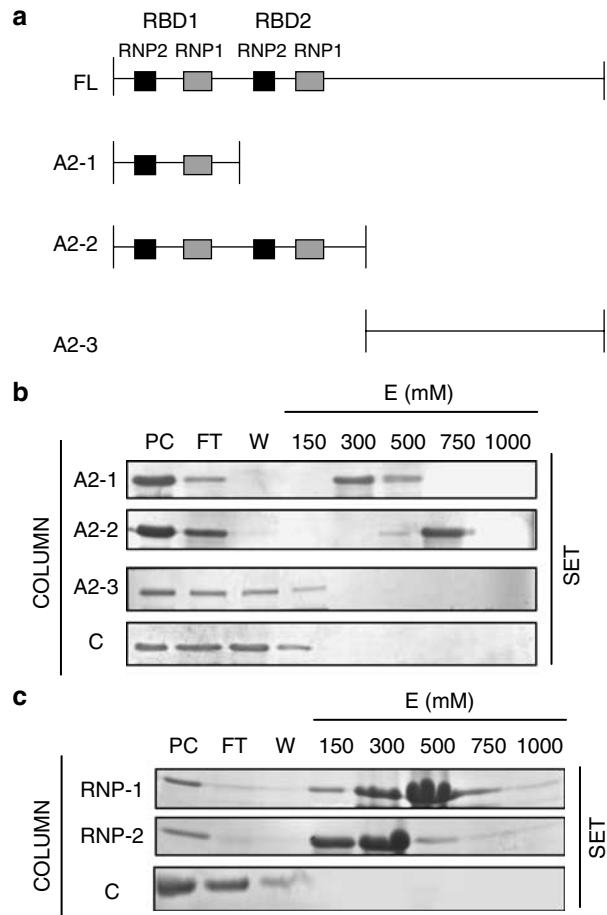
when it was associated with ssDNA, the ssDNA column, containing hnRNP2, was loaded with purified SET and then the binding was analysed. Results showed that SET was not retained into the column, but it appeared in the flow-through fraction (Figure 3a). This result was confirmed by experiments in which purified hnRNP2 was preincubated with SET and then the complex was loaded onto an ssDNA-cellulose column. In these experiments, it was observed that hnRNP2 was able to bind to ssDNA, and that this binding disrupted the interaction between hnRNP2 and SET, with the former but not the latter being retained into the column (data not shown). Finally, hnRNP2 was preincubated with ssDNA and then the mixture was subsequently loaded onto a SET-Sepharose 4B column. After washing, proteins bound to the column were

eluted, electrophoresed and visualized with Coomassie blue staining. Results indicate that preincubation of hnRNPA2 with ssDNA avoided its association with the SET–Sephacrose 4B column (Figure 3b). All these results revealed that hnRNPA2 bound to ssDNA is not able to interact with SET, indicating that hnRNPA2 has much higher affinity for ssDNA than for SET and suggest that ssDNA and SET could interact within the same domain of hnRNPA2.

*SET interacts with the RNP1 sequence of the RNA-binding domain (RBD) of hnRNPA2*

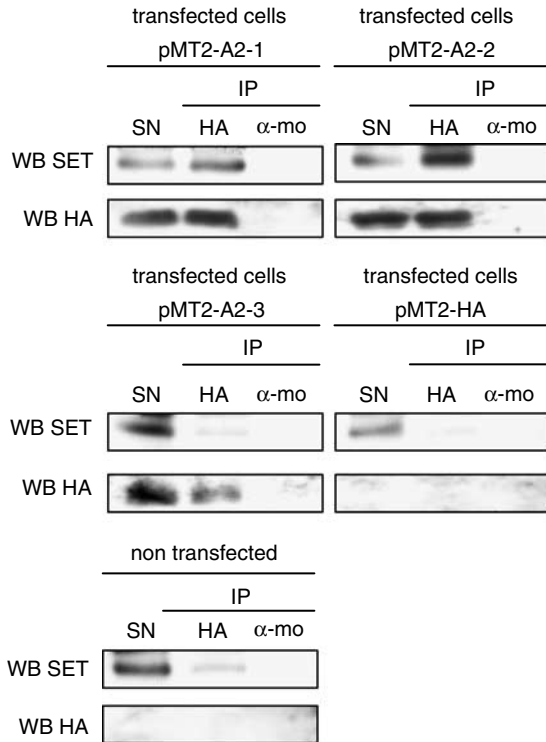
To identify the domain of hnRNPA2 that interacts with SET, we first generated three hnRNPA2 protein fragments named A2-1 (amino acid (aa) 1–86), A2-2 (aa 1–177) and A2-3 (aa 178–340) (Figure 4a). We subsequently prepared affinity chromatography columns by coupling each one of these fragments to Sepharose 4B beads. Then, purified recombinant SET was loaded onto the columns. After extensive washing, the SET bound to the hnRNPA2 fragments was eluted with a buffer containing growing amounts of KCl. Then, the eluates were subjected to SDS–PAGE and the proteins visualized with Coomassie blue staining. Results indicate that SET bound to A2-1 and A2-2 fragments but not to A2-3 (Figure 4b). Interestingly, SET bound to A2-2 fragment with higher affinity than to A2-1. As fragment A2-2 contains two RBDs and fragment A2-1 only one, we further aimed to analyse whether SET directly interacts with the RBD regions of hnRNPA2. The RBDs of hnRNPA2 includes 80- to 90-aa residues containing two short sequences, RNP1 and RNP2, highly conserved in all eukaryotes. RNP1 and RNP2 from both RBD1 and RBD2 are highly homologous. To test whether RNP1 or/and RNP2 sequences were able to interact with SET, the binding of this protein to two synthetic peptides corresponding to RNP1 and RNP2 from RBD1 was analysed. These peptides were coupled to Sepharose 4B and the capacity of purified SET to bind to these columns was tested. Purified SET was loaded onto the columns, and after extensive washing, the protein bound to the columns was eluted with a buffer containing increasing concentrations of KCl. The collected fractions were electrophoresed and the gels stained with Coomassie blue. Results showed that SET binds to both RNP1 and RNP2 sequences but with different affinities, the affinity of SET for RNP1 being much higher (Figure 4c).

To identify the domain of hnRNPA2 that might interact *in vivo* with SET, cells were transfected with HA-tagged expression vectors harboring anyone of the three previously mentioned hnRNPA2 fragments (A2-1, A2-2 and A2-3). Then, cell extracts were immunoprecipitated with anti-HA antibodies and the immunoprecipitates were analysed by Western blotting using anti-HA and anti-SET antibodies. Results indicated that fragments A2-1 and A2-2, which contain RBD domains, are able to interact with SET *in vivo*, whereas fragment A2-3 lacking RBD domains did not (Figure 5).



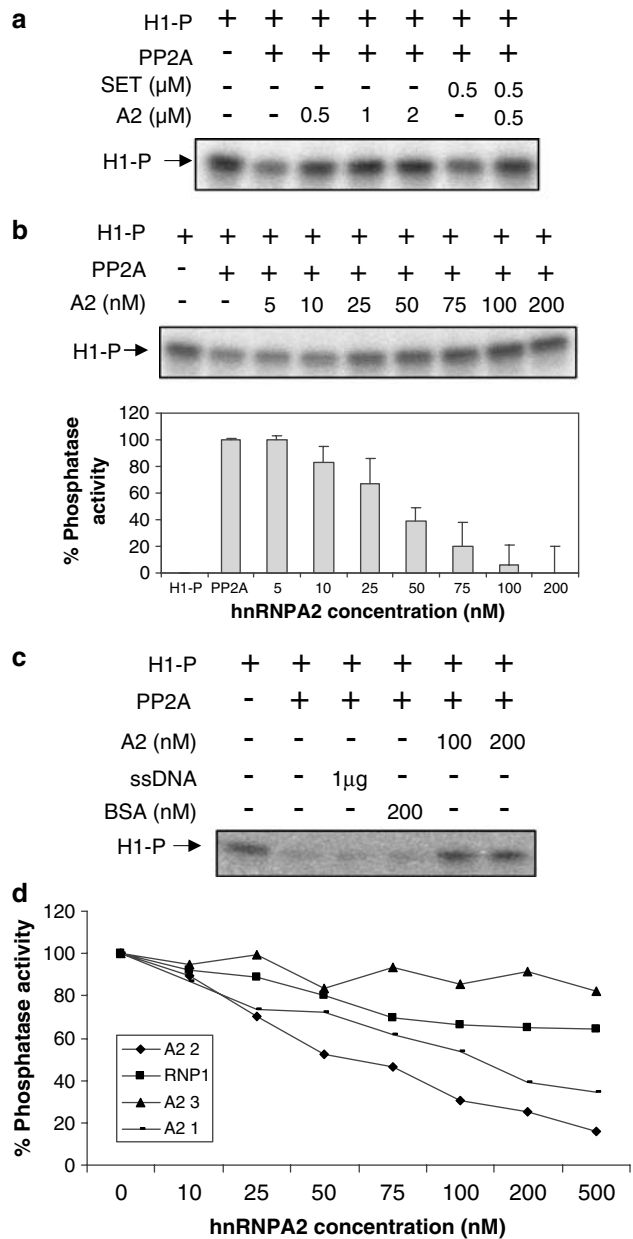
**Figure 4** SET binds to the RNP1 sequence of the RNA-binding domain (RBD) of hnRNPA2. (a) Three hnRNPA2 protein fragments, A2-1 (aa 1–86), A2-2 (aa 1–177) and A2-3 (aa 178–340) were generated. (b) Fragments A2-1, A2-2 or A2-3 were coupled to Sepharose 4B beads to construct affinity chromatography columns. A Sepharose 4B column was used as a control (C). The columns were subsequently loaded with purified SET. Then, after extensive washing, the protein retained in the columns was eluted with a buffer containing growing concentrations of KCl as indicated. Samples: precolumn (PC) representing the 15% of the amount loaded onto the column, flow-through (FT), wash (W) and eluates (E) were electrophoresed and the gels stained with Coomassie blue. (c) Peptides containing the sequence RNP1 or RNP2 of the RBD of hnRNPA2 were coupled to Sepharose 4B to generate affinity chromatography columns. A Sepharose 4B column was used as a control (C). Then, purified SET was loaded onto each column and incubated for 30 min at 4°C. Then, after extensive washing, the protein retained in the column was eluted with a buffer containing growing concentrations of KCl as indicated. Samples: precolumn (PC) representing the 15% of the amount loaded onto the column, flow-through (FT), washes (W) and the distinct eluates were electrophoresed and stained with Coomassie blue.

*HnRNPA2 is an inhibitor of PP2A phosphatase activity*  
SET is an inhibitor of the phosphatase PP2A. Thus, we aimed to analyse whether hnRNPA2 could affect the inhibition of PP2A activity produced by SET. To check this possibility, *in vitro* PP2A phosphatase assays in the presence of SET or SET plus hnRNPA2 were performed. In these experiments, the decrease in the amount of phosphorylated histone H1 was determined.



**Figure 5** *In vivo* interaction between SET and the different fragments of hnRNPA2. COS-7 cells were transfected with pMT2-HA expression vectors harboring the A2-1, A2-2 or A2-3 fragments of hnRNPA2 or with a pMT2 empty vector that was used as a control. At 20h after transfection, cell lysates were immunoprecipitated with anti-HA antibodies or with a normal mouse serum ( $\alpha$ -mo) that was used as a control. Then, the presence of SET and the HA-tagged fragments in the immunoprecipitates was analysed by Western blotting using anti-SET or anti-HA antibodies, respectively. A sample of cell lysate (SN) was used as a control in the gels.

As described previously, we found that SET inhibited PP2A activity (Figure 6a). Interestingly, when both hnRNPA2 and SET were present in the reaction media, the inhibition was significantly increased (Figure 6a). These results suggested that hnRNPA2 could promote the inhibitory effect of SET on PP2A activity or alternatively that hnRNPA2 by itself could be a PP2A inhibitor. To discern between these two possibilities, we analysed whether hnRNPA2 alone was able to inhibit PP2A activity. Thus, experiments to determine PP2A activity in the presence of increasing amounts of hnRNPA2 were performed. Our results indicate that hnRNPA2 inhibited PP2A activity, being the  $IC_{50} \approx 35$  nM (Figures 6a and b). This effect is specific for hnRNPA2 because neither an unrelated protein (BSA) nor ssDNA affected PP2A activity (Figure 6c). We further aimed to identify the hnRNPA2 domain involved in the inhibition. Thus, we assayed the effect of hnRNPA2 fragments on *in vitro* PP2A activity. Results indicate that fragments of hnRNPA2 containing RBDs (A2-1 and A2-2) inhibited PP2A activity, whereas in contrast, fragment A2-3 did not (Figure 6d). These results suggested that RBDs of hnRNPA2 could be responsible for the inhibition of PP2A activity. To test



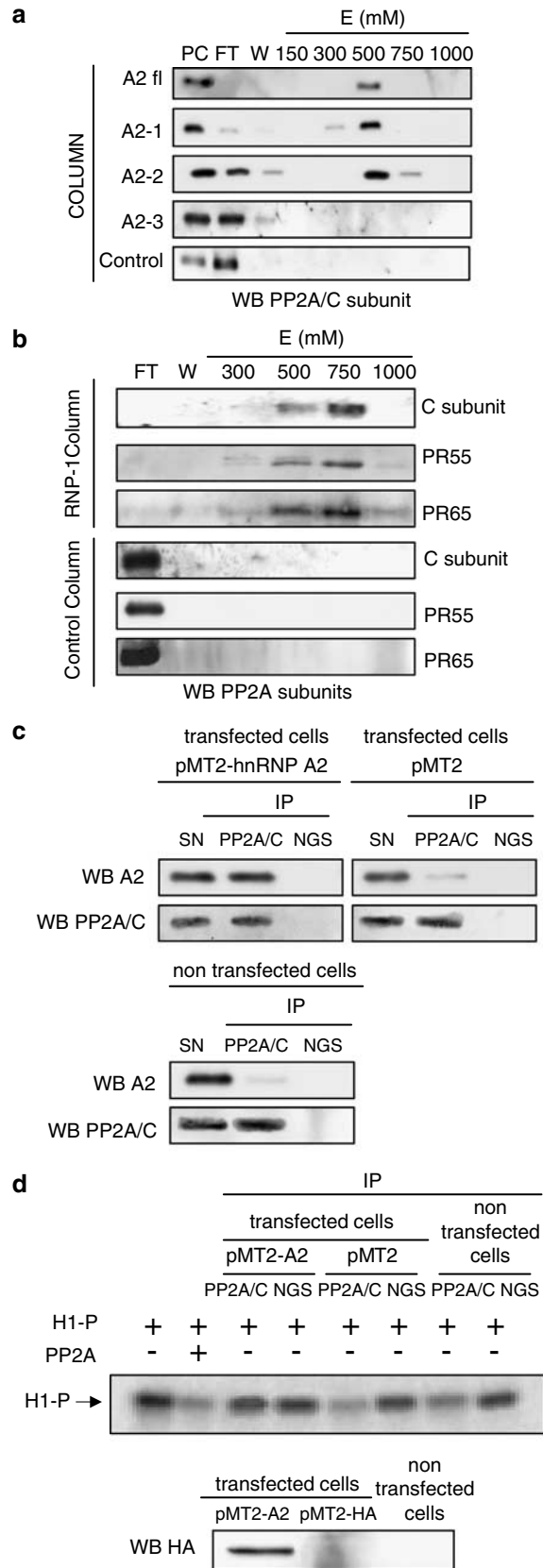
**Figure 6** hnRNPA2 is an inhibitor of PP2A phosphatase activity. (a) PP2A activity was measured *in vitro* by determining the decrease of the substrate phosphorylation (histone H1-P<sup>32</sup>) in the presence of SET, hnRNPA2 or SET plus hnRNPA2. After reaction, the samples were separated in a SDS-PAGE and dried. The radioactive bands were visualized by phosphorimaging. (b) PP2A activity was determined in the presence of increasing concentrations of hnRNPA2 (5–200 nM) (upper panel). The activity was quantified by the mass densitometry program Quantity One and represented as the mean value  $\pm$  s.d. of three independent experiments. Results were expressed as percentage of the highest activity observed in the assay performed in the absence of hnRNPA2 (bottom panel). (c) PP2A activity was determined in the presence of different concentrations of hnRNPA2 (100, 200 nM), or in the presence of ssDNA (1  $\mu$ g) or BSA (200 nM). (d) PP2A activity was determined in the presence of increasing concentrations of hnRNPA2 fragments. The activity was quantified by the mass densitometry program Quantity One and represented as the mean value  $\pm$  s.d. of three independent experiments. Results were expressed as percentage of the highest activity observed in the assay performed in the absence of hnRNPA2.

this possibility, the effect of the synthetic RNP1 peptide on PP2A activity was assayed. Results indicate that purified RNP1 peptide only partially inhibited PP2A activity (Figure 6d). These results indicate that the RNP1 domain of hnRNPA2 is not sufficient to achieve the full inhibition of PP2A activity.

*HnRNPA2 interacts with PP2A through the RNP1 sequence of the RBD*

As we found that hnRNPA2 inhibits PP2A activity, we further aimed to identify the hnRNPA2 domain involved in the interaction with this phosphatase. Thus, affinity chromatography experiments using full-length hnRNPA2–Sepharose 4B columns or columns with fragments A2-1, A2-2 and A2-3 of this protein were performed. Then, purified trimeric PP2A, containing the catalytic subunit and the regulatory subunits, was loaded onto the different hnRNPA2 columns and the PP2A bound to the columns was eluted with a buffer containing increasing concentrations of KCl. The collected fractions were electrophoresed and immunoblotted with antibodies against the PP2A catalytic subunit. Results indicate that PP2A was retained in the columns of the full-length hnRNPA2 and in the columns of the hnRNPA2 fragments containing RBDs (fragments A2-1 and A2-2). In contrast, PP2A was not bound either to the A2-3 column or to the control (Figure 7a). Results also revealed that trimeric PP2A complex was not disrupted by its association with hnRNPA2 because all three subunits were simultaneously eluted from the columns (data not shown).

These results suggested that the RBD of hnRNPA2 might be involved in the interaction with PP2A. To confirm this possibility, the binding of PP2A to a



**Figure 7** The RNP1 domain of hnRNPA2 interacts with protein phosphatase PP2A. **(a)** Purified PP2A (Calbiochem) containing the three subunits: the catalytic subunit (C), the regulatory subunit B (PR55) and the regulatory subunit A (PR65) was loaded onto hnRNPA2 (A2 fl), A2-1, A2-2, A2-3 or Sepharose 4B (control) affinity chromatography columns. Then, after extensive washing, the protein retained in the column was eluted with a buffer containing growing concentrations of KCl as indicated. Samples: pre-column (PC), flow-through (FT) washes (W) and the distinct eluates were electrophoresed and visualized by Western blotting using antibodies against the catalytic subunit of PP2A. **(b)** Purified PP2A was loaded onto a RNP1–Sepharose 4B column. Then, after extensive washing, proteins retained in the column were eluted with a buffer containing growing concentrations of KCl as indicated. Samples: flow-through (FT), washes (W) and the distinct eluates (E) were electrophoresed and visualized by Western blotting using antibodies against the three subunits of PP2A. Control experiments were performed using a column of Sepharose 4B. **(c)** To analyse the *in vivo* interaction between hnRNPA2 and PP2A, COS-7 cells were transfected with a pMT2-HA expression vector harboring hnRNPA2 or with a pMT2-HA empty vector that was used as a control. At 20h after transfection, cell lysates were immunoprecipitated with anti-PP2A (catalytic subunit) antibodies or with a normal goat serum (NGS) that was used as a control. Then, the presence of hnRNPA2 or PP2A in the immunoprecipitates was analysed by Western blotting using anti-hnRNPA2 or anti-PP2A (catalytic subunit) antibodies, respectively. A sample of cell lysate (SN) was used as a control in the gels. **(d)** PP2A activity was analysed in the immunoprecipitates (upper panel). As a control of the assay in the two first lanes, PP2A activity was measured using histone H1-P<sup>32</sup> as a substrate in the presence of purified PP2A. The expression of ectopic HA-hnRNPA2 in transfected cells was analysed by Western blotting using anti-HA antibodies (bottom panel).

column of RNP1 peptide was analysed. Results revealed that purified RNP1 peptide interacted with PP2A and that the PP2A trimeric complex was not disrupted by the interaction with RNP1 peptide (Figure 7b).

Finally, we analysed whether hnRNPA2 and PP2A interact in the cells. Thus, cells were transfected with a HA-tagged expression vector for hnRNPA2 or with an HA-empty vector as a control. Then, cell extracts were immunoprecipitated with antibodies against the catalytic subunit of PP2A and the immunoprecipitates were analysed by Western blotting using anti-hnRNPA2 and antibodies against the catalytic subunit of PP2A. Results indicated a co-immunoprecipitation of hnRNPA2 and PP2A in nontransfected cells, in cells transfected with the empty vector and in cells overexpressing hnRNPA2. However, the association between both proteins was much higher in cells overexpressing hnRNPA2 (Figure 7c). As hnRNPA2 was able to inhibit PP2A activity *in vitro*, we aimed to study whether the overexpression of hnRNPA2 in cells could affect PP2A activity *in vivo*. Thus, COS-7 cells were transfected with HA-hnRNPA2 expression vector or with HA-empty vector as a control. Then, cell extracts were immunoprecipitated with antibodies against the catalytic subunit of PP2A and the immunoprecipitates were analysed for PP2A activity. Results revealed that immunoprecipitates from nontransfected cells or from cells transfected with the HA-empty vector displayed a significant phosphatase activity, whereas in immunoprecipitates from cells overexpressing hnRNPA2, the activity of PP2A was clearly reduced (Figure 7d).

#### *Overexpression of hnRNPA2 accelerates cell cycle progression*

The observation that hnRNPA2 inhibits PP2A activity together with the fact that PP2A downregulation accelerates cell cycle progression suggested that the overexpression of hnRNPA2 could stimulate cell cycle progression. Thus, to address this issue, COS-7 cells were transfected with HA-hnRNPA2 expression vector or with HA expression vector as a control. Then, at 24 h after transfection, cells were plated at low density and the number of cells counted at different times after plating. As shown in Figure 8a, cells overexpressing hnRNPA2 proliferated much faster than control cells. Similar results were observed in experiments performed with HCT116 cells (Figure 8b). We further aimed to identify the hnRNPA2 domain involved in the stimulation of cell proliferation. Thus, similar experiments were performed in COS-7 cells transfected with HA-tagged expression vectors harboring any one of the three hnRNPA2 fragments (A2-1, A2-2 and A2-3). Results indicated that fragments A2-1 and A2-2 stimulated cell proliferation, whereas in contrast fragment A2-3 did not.

#### **Discussion**

On identifying new SET-binding proteins, we found that hnRNPA2 is able to interact with the SET protein both

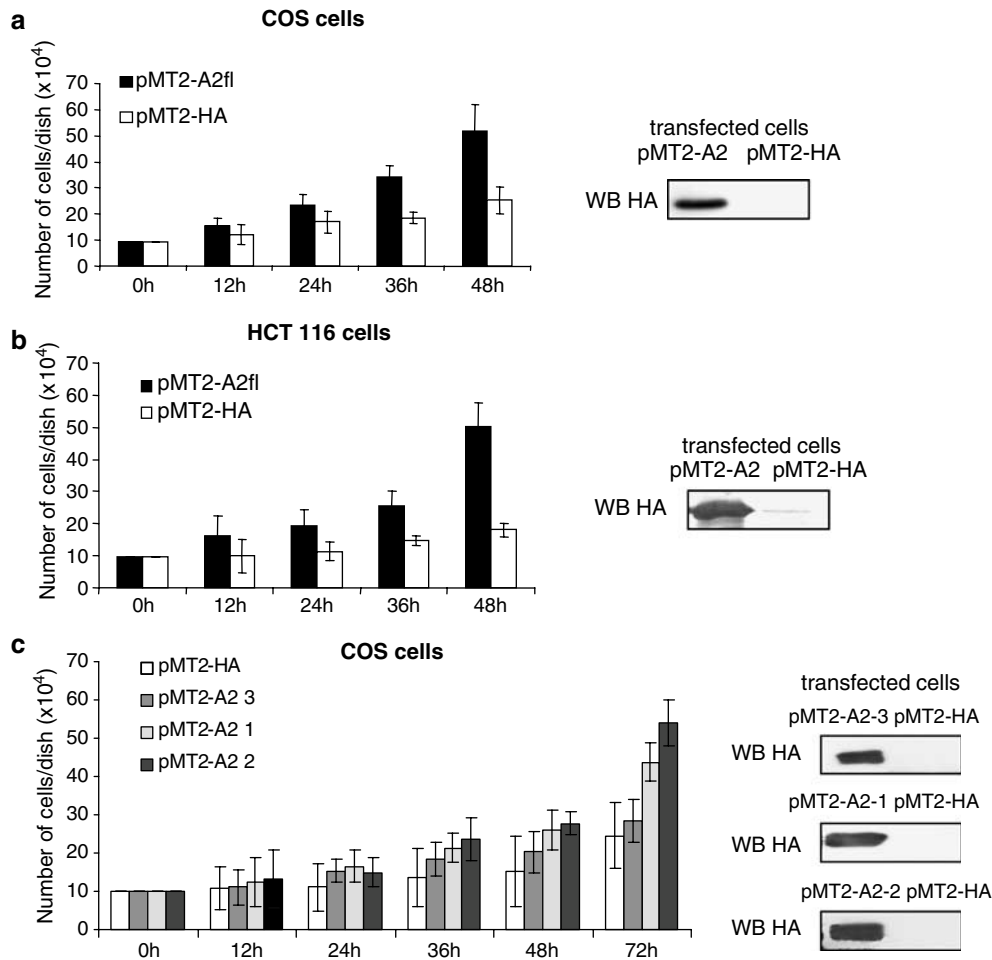
*in vitro* and *in vivo*. HnRNPA2 belongs to a large family of RNA-binding proteins involved in various aspects of mRNA biogenesis (Krecic and Swanson, 1999; Dreyfuss *et al.*, 2002). HnRNPA2 shuttles between the nucleus and the cytoplasm where it participates in the regulation of translation of specific mRNAs that contain the specific *cis*-acting ARE as, for instance, the myelin basic protein mRNA (Ainger *et al.*, 1997; Kwon *et al.*, 1999). However, hnRNPA2 is mostly nuclear and there it is involved in several functions as splicing and telomere length maintenance (Krecic and Swanson, 1999; Dreyfuss *et al.*, 2002; Ford *et al.*, 2002). A growing number of evidence indicates that hnRNPA2 is overexpressed in a variety of cancers, including tumors from lung, breast and pancreas cancer (Tockman *et al.*, 1997; Zhou *et al.*, 2001; Yan-Sanders *et al.*, 2002; Patry *et al.*, 2003). In some cases, the overexpression of hnRNPA2 has a prognostic value and this protein is now considered to be the lead marker for early diagnosis of lung cancer (Mulshine *et al.*, 2000).

Our results indicate that the domain of hnRNPA2 that interacts with the SET protein is the sequence RNP1 that belongs to the RBD of hnRNPA2. We also observed that when hnRNPA2 is associated with ssDNA, the ability of SET to interact with hnRNPA2 is lost, indicating that hnRNPA2 has much more affinity for ssDNA than for SET. Thus, ssDNA-binding to hnRNPA2 regulates the interaction of this protein with SET.

To analyse the functional relevance of hnRNPA2–SET interaction, we studied the effect of hnRNPA2 on PP2A inhibition by SET. Results reported here indicate that SET and hnRNPA2 cooperate in PP2A inhibition. This is because we found that hnRNPA2 is also a PP2A inhibitor. Thus, it appears that hnRNPA2 might be a putative regulator of SET and PP2A functions.

The putative role of hnRNPA2 in the regulation of PP2A activity might have special interest because PP2A has recently been defined as a tumor suppressor gene (Chen *et al.*, 2004; Van Hoof and Goris, 2004). PP2A is a family of abundantly expressed Ser/Thr phosphatases implicated in many cellular functions including cell proliferation and apoptosis (Millward *et al.*, 1999). The PP2A core structure consists of a catalytic subunit and a scaffold protein. This core dimer can recruit a third regulatory subunit. The regulatory subunits are encoded by three multigene families referred to as B/PR55, B'/PR61 and B''/PR72 (Janssens and Goris, 2001). They confer distinct properties to the PP2A holoenzyme. The generation of a diversity of PP2A holoenzymes that dephosphorylate distinct substrates in different cellular compartments results in diverse cellular functions of PP2A and might explain the important role of PP2A in a variety of signaling pathways (Millward *et al.*, 1999).

A growing number of evidence indicates a role of PP2A in cell proliferation. It appears to have stimulatory effects during the early phases of the cell cycle by affecting the activity of G1-specific cdk complexes (Schonthal and Feramisco, 1993; Yan and Mumby, 1999). Moreover, PP2A regulates the loading of Cdc45 to the prereplicative complexes at the replication origins



**Figure 8** Overexpression of hnRNPA2 stimulates cell proliferation. (a) COS-7 cells were transfected with the HA-hnRNPA2 expression vector pMT2-A2 (black bars), or with the HA expression vector pMT2-HA (white bars). At 20 h after transfection, cells were plated and subsequently their number was determined at different times as indicated. Results are expressed as the mean value  $\pm$  s.d. of three independent experiments (left panel). The expression of ectopic HA-hnRNPA2 in transfected cells (24 h) was analysed by Western blotting using anti-HA antibodies (right panel). (b) Similar experiments were performed in HCT116 cells. (c) To analyse the effect of the different fragments of hnRNPA2, COS-7 cells were transfected with the pMT2 expression vector harboring the fragments A2-1, A2-2 or A2-3 of hnRNPA2. Transfection with the pMT2 empty vector was used as a control. At 24 h after transfection, cells were plated and subsequently their number was determined at different times as indicated. Results are expressed as the mean value  $\pm$  s.d. of three independent experiments (left panel). The expression of ectopic hnRNPA2 fragments in transfected cells (24 h) was analysed by Western blotting using anti-HA antibodies (right panel).

and in that way participates in the triggering of DNA synthesis (Chou *et al.*, 2002). However, these positive functions of PP2A in the regulation of cell cycle progression contrast with its well-established negative role during G2/M transition (Schonthal, 2001). Specifically, it has been shown that PP2A is required to maintain cyclin B-Cdc2 complexes inactive. On the one hand, PP2A dephosphorylates and inactivates the cdk-activating kinase, responsible for the phosphorylation of Thr-161 of Cdc2 that activates cyclin B-Cdc2 complexes. On the other hand, PP2A dephosphorylates and activates the Wee 1 kinase, responsible for the inhibitory Tyr-15 phosphorylation of Cdc2 (Kinoshita *et al.*, 1993; Elder *et al.*, 2001). Finally, PP2A dephosphorylates and inactivates Cdc25 (the protein phosphatase that eliminates the inhibitory phosphorylations on Thr-14 and

Tyr-15 of Cdc2) (Clarke *et al.*, 1993). The molecular basis for these opposing functions of PP2A still remain unclear, but it could be due to the actions of distinct PP2A holoenzymes that are targeted at different G1 versus G2/M-phase substrates.

Interestingly, it has recently been shown that suppression of the PP2A B56 $\gamma$  expression in cells inhibited PP2A-specific phosphatase activity and conferred the ability to growth in an anchorage-independent manner and to form tumors (Chen *et al.*, 2004). These results support the idea that PP2A plays a role as a tumor suppressor and indicate that the negative role of PP2A in cell cycle regulation dominates over the positive regulatory pathways.

Our results indicate that hnRNPA2 interacts with PP2A by the RNP1 sequence of the RBD that also binds

to RNA and SET. However, we observed that the binding of this sequence to PP2A is not sufficient to fully inhibit the activity of PP2A. Thus, a more extensive domain of hnRNP2 is required for inhibition of PP2A activity. The minimal hnRNP2 fragment we found to be able to inhibit PP2A activity is fragment A2-1 containing a complete RBD. Thus, whereas RNP1 sequence is sufficient for the interaction with PP2A, likely the complete RBD domain would be needed to inhibit PP2A activity.

We report here that hnRNP2 overexpression results in a decrease of PP2A activity and in a stimulation of cell proliferation. Moreover, the overexpression of the different fragments of hnRNP2 in the cells indicates that fragment A2-1 containing a complete RBD is sufficient to stimulate cell proliferation. Thus, the same domain of hnRNP2 that inhibits PP2A activity is able to stimulate cell proliferation. These results suggest that cell proliferation induced by hnRNP2 might be mediated by PP2A activity inhibition.

HnRNP2 is overexpressed in different types of tumors having a prognostic value in some cases. However, the role of hnRNP2 overexpression in tumor development remains to be established. Our results reported here suggest the possibility that tumor cells overexpressing hnRNP2 would proliferate faster than normal cells and in that way tumorigenesis could be facilitated.

## Materials and methods

### Plasmids

pGEX-KG-SET and GFP-SET were obtained as described in Canela *et al.* (2003). pET9c-hnRNP2 was a gift from Dr Adrian R Krainer (Cold Spring Harbour Laboratory, New York, USA). HnRNP2 cDNA was amplified by PCR from pET9c-hnRNP2 vector, using the following specific primers: sense, 5'-CCGGAATTCTCTGCAGTCATGGAGAGAGAA AAG-3'; and antisense, 5'-CCGCTCGAGCCGCGGTCAG TATCGGCTCCT-3'. The PCR product was cloned into the pMT2 vector by *EcoRI* and *XhoI*. The different hnRNP2 fragments were generated by PCR using pET9c-hnRNP2 full-length vector as a template. The following primers were used to generate the PCR products: A2-1 (aa 1–86), sense 5'-ccggaattctctcgatcatggagagagaaaag-3' and antisense 5'-ctacc gctccagcccggtgctacagcagcttttg-3'; A2-2 (aa 1–177), sense 5'-cc ggaattctctcgatcatggagagagaaaag-3' and antisense 5'-ctaccgctc gagccgagagacaagcctttcttac-3'; and A2-3 (aa 178–340), sense 5'-ccggaattctctcgatcagacaagaatcgag-3' and antisense 5'-ctacc gctcagcgcggtcagtcagtcctct-3'. After amplification, the PCR products were digested with *EcoRI* and *XhoI*, and cloned into pGEX-4T1 and pMT2.

### Protein expression and purification

To obtain recombinant glutathione *S*-transferase (GST)-SET protein and hnRNP2 fragments (A2-1, A2-2 and A2-3), the Codon Plus<sup>®</sup> strain of *Escherichia coli* was transformed with the vectors pGEX-KG-SET, pGEX-4T1-A2-1, pGEX-4T1-A2-2 or pGEX-4T1-A2-3. Purification of GST-fusion proteins was performed as described (Sanchez-Piris *et al.*, 2002) and GST was separated from the fusion proteins by digestion with thrombin protease following the manufacturer's instructions (Sigma).

To produce recombinant hnRNP2, the BL21 strain of *E. coli* was transformed with the pET9c-hnRNP2 vector. Bacteria were grown in LB-kanamycin to saturation and protein expression was induced at 1 mM IPTG for 3 h at 37°C. Cells were washed in 10 mM Tris-HCl, pH 8.0, 0.1 M NaCl, centrifuged and resuspended in buffer A (20 mM HEPES, pH 8.0, 5% (v/v) glycerol, 0.2 mM EDTA and 1 mM DTT). After sonication and centrifugation at 12 000 *g* at 4°C for 20 min, the supernatant obtained was dialysed with buffer A containing 2 M NaCl. Then, 5 mg of the dialysed protein were loaded on a phenyl-Sepharose column previously washed with buffer A containing 2 M NaCl. Elution was performed with a lineal gradient of buffer A containing from 2 to 0 M NaCl. HnRNP2 eluates were dialysed with buffer A containing 150 mM NaCl and loaded onto an ssDNA-cellulose column previously washed with buffer A containing 150 mM NaCl. Elution was performed with 15 ml of a lineal gradient of buffer A containing from 150 mM to 2 M NaCl. Finally, hnRNP2 eluates were concentrated by Centricon<sup>®</sup> YM-10 (Millipore).

### SET-Sepharose affinity chromatography

To prepare SET and the different hnRNP2 fragments-Sepharose 4B columns, 3 mg of purified protein were coupled to 1 ml of CNBr-activated Sepharose 4B (Amersham Biosciences). Cell extracts were obtained by homogenizing mice livers in lysis buffer (50 mM HEPES-KOH, pH 7.6, 50 mM KCl, 1 mM EGTA and 1 mM MgCl<sub>2</sub>) containing 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml aprotinin and 10 μg/ml leupeptin. Homogenates were filtered through four layers of cheesecloth and sedimented at 14 000 *g* for 10 min. The supernatant was collected and the protein content measured. In all, 20 mg of the lysate were then loaded on the SET or A2 fragments-Sepharose or Sepharose control columns. After washing in 200 vol of lysis buffer, the bound proteins were eluted with the same buffer but containing 1 M KCl instead of 50 mM KCl. In some cases, elution was performed with increasing concentrations of KCl (300–500–1000 mM KCl).

### RNP1/2-Sepharose affinity chromatography

Peptides RNP1 and RNP2 with the following sequences H-RKKREKEQFRKLFIGGLS FE-NH<sub>2</sub> and H-MRDPAS KRSRGGFVTFSSM-NH<sub>2</sub>, respectively, were synthesized owing to the *Unitat de Síntesi de Pèptids* (University of Barcelona). The affinity chromatography columns were performed at a concentration of 2 mg/ml as described previously.

### HnRNP2-ssDNA-cellulose affinity chromatography

SsDNA-cellulose affinity chromatography was carried out essentially as described by (Bossler *et al.*, 1995). Briefly, 30 μg of purified hnRNP2 was diluted 1:1 with buffer C (50 mM Na phosphate, pH 7.4) containing 100 mM NaCl and then loaded onto an ssDNA-cellulose column (Sigma) pre-equilibrated with the same buffer. The column was washed with 10 vol of the same buffer. Finally, hnRNP2 was eluted with buffer C containing 1 M NaCl.

For the binding experiments, 30 μg of purified SET protein were loaded onto an hnRNP2-ssDNA-cellulose column and incubated for 30 min at 4°C before elution.

### Gel electrophoresis and immunoblotting

Protein samples were electrophoresed on SDS-PAGE as described (Estanyol *et al.*, 1999). The gels were further stained with Coomassie blue G-250 or with silver nitrate or transferred onto Immobilon-P membranes (Millipore). Immunoblotting

was performed essentially as described (Estanyol *et al.*, 1999) by using the following antibodies anti-SET ( $\alpha$ -I2A-PP2A, AbCam), anti-hnRNPA2 (Bossler *et al.*, 1995), anti-PP2A/B $\alpha$  (14–27 PR55, Calbiochem), anti-PP2A/A (7–19 PR65, Calbiochem), anti-PP2A/C subunit (SC G-20, Santa Cruz) and anti-HA (12CA5, Roche).

#### 2-D gel electrophoresis and MALDI-TOF characterization

Eluates obtained from the SET–Sepharose affinity chromatography experiments were precipitated with 10% trichloroacetic acid (TCA). Samples were analysed by 2-D gel electrophoresis (isoelectrofocusing). Second dimension was performed in SDS–PAGE gels using 10% acrylamide. Gels obtained were visualized by silver stain. The spots were sliced from the gel and digested with trypsin. The resulting peptides were analysed in a MALDI-TOF mass spectrometer. The masses of mono-isotopic peaks were used for comparison to a theoretical digestion of the protein by trypsin. This was carried out using the protein prospector (MS)-Fit software.

#### Cell culture and transfection

COS-7 cell line was cultured in Dulbecco's modified Eagle's medium (DMEM) (Biological Industries). HCT116 cells were cultured in DMEM/F-12 (HAM). Both were supplemented with 10% fetal calf serum. Cells were seeded in culture dishes at 90% confluence and transfected with pMT2, pMT2-hnRNPA2, pMT2-A2-1, pMT2-A2-2 and pMT2-A2-3 using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's instructions. After growing for different hours at 37°C, Western blot or proliferation analyses were performed.

#### Immunoprecipitation

Cells were lysed in buffer A (50 mM Tris-HCl, pH 7.4, 250 mM NaCl, 5 mM EDTA, 0.1% Triton X-100) containing 50 mM NaF, 1 mM PMSF, 10  $\mu$ g/ml leupeptin, 0.5  $\mu$ g/ $\mu$ l aprotinin and 0.1 mM Na<sub>3</sub>VO<sub>4</sub> for 30 min on ice. Lysates were clarified by centrifugation at 10 000 g for 10 min at 4°C. The supernatants (0.5 mg) were incubated with 3  $\mu$ g of anti-hnRNPA2 or 3  $\mu$ g of anti-PP2A/C subunit overnight at 4°C, followed by incubation with protein A beads for 1 h at 4°C. After washing in buffer A, the immunocomplexes were subjected to immunoblotting. To immunoprecipitate the HA-tagged proteins, the clarified

lysates were incubated with 40  $\mu$ l of 1:1 suspension of the anti-HA agarose conjugate (Sigma) following the manufacturer's instructions.

#### PP2A phosphatase assay

To determine PP2A phosphatase activity, a first step was the preparation of [<sup>32</sup>P]histone H1. Labeling was performed by incubating 5  $\mu$ g of histone H1 with 800 nM cyclin A–cdk2, 15  $\mu$ M ATP, 2 mM DTT and 10  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P]ATP (Amersham; 3000 Ci/mmol) in a total volume of 50  $\mu$ l of a buffer containing 25 mM HEPES (pH 7.4) and 10 mM MgCl<sub>2</sub> at 37°C for 30 min. The reaction was stopped by adding 200  $\mu$ l of 100% TCA for 5 min on ice. Histone H1 was collected by centrifugation at 12 000 g for 15 min. After washing once with 20% TCA and three times with ether, [<sup>32</sup>P]histone H1 was resuspended in 8  $\mu$ l of 0.1% NP-40 in 0.01 N HCl.

PP2A phosphatase assay consisted in the incubation of 10  $\mu$ l of phosphatase buffer (50 mM Tris-HCl, pH 7.4, 1 mM benzamidine, 0.1 mM PMSF, 14 mM  $\beta$ -mercaptoethanol, 0.25 mM MnCl<sub>2</sub>), appropriate units of PP2A (Calbiochem), <sup>32</sup>P-labeled histone H1 and different concentrations of hnRNPA2, A2-1, A2-2, A2-3 or SET proteins or RNP1 peptide. Reaction was stopped by adding 2  $\times$  Laemmli sample buffer, and then the samples were electrophoresed in 10% SDS–PAGE gels. The gels were dried and the phosphorylated proteins were analysed with a phosphor imaging machine.

#### Cell proliferation assay

HCT116 and COS-7 cells were transfected with pMT2, pMT2-hnRNPA2, pMT2-A2-1, pMT2-A2-2 or pMT2-A2-3 vectors. After 24 h, cells were cultured into P35 dishes (100 000 cells/dish). Then at 12, 24, 36, 48 and 72 h of growth, cells were trypsinized and counted.

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