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Accumulated SET protein up-regulates and interacts with hnRNPK, increasing its binding to nucleic acids, the Bcl-xS repression, and cellular proliferation

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ABSTRACT

SET and hnRNPK are proteins involved in gene expression and regulation of cellular signaling. We previously demonstrated that SET accumulates in head and neck squamous cell carcinoma (HNSCC); hnRNPK is a prognostic marker in cancer. Here, we postulate that SET and hnRNPK proteins interact to promote tumorigenesis. We performed studies in HEK293 and HNSCC (HN6, HN12, and HN13) cell lines with SET/hnRNPK overexpression and knockdown, respectively. We found that SET and/or hnRNPK protein accumulation increased cellular proliferation. SET accumulation up-regulated hnRNPK mRNA and total/phosphorylated protein, promoted hnRNPK nuclear location, and reduced Bcl-x mRNA levels. SET protein directly interacted with hnRNPK, increasing both its binding to nucleic acids and Bcl-xS repression. We propose that hnRNPK should be a new target of SET and that SET–hnRNPK interaction, in turn, has potential implications in cell survival and malignant transformation.

1. Introduction

The heterogeneous nuclear ribonucleoprotein family (hnRNPs) is involved in many biological processes such as cellular signaling, DNA repair, and regulation of gene and protein expression [1]. In particular, hnRNPK bears three K homology (KH) domains that are responsible for DNA–RNA binding, and one K-interactive (KI) region responsible for protein–protein interaction [2]. HnRNPK is predominantly located in the nucleus [3], where it is involved in multiple steps of gene expression such as transcription, RNA splicing, and translation [4,5].

HnRNPK acts in the transcription of the c-myc gene [6], in the p53 pathway [7], and in the decreased expression of the pro-apoptotic Bcl-xS spliced isoform, suggesting an anti-apoptotic action in cancer cells [8]. Changes in the cellular distribution and increases of hnRNPK have been demonstrated in many cancer types, characterizing it as a prognostic marker [9–13]. In head and neck squamous cell carcinoma (HNSCC), hnRNPK protein is up-regulated in association with a poor prognosis [14].

SET (TAF-1β or I2PP2A), a phosphatase 2A (PP2A) inhibitor [15], is a multifunctional protein that interacts with other proteins in the regulation of cellular signaling. SET bears a histone chaperone function by binding to histones H2B and H3 in the organization of the chromatin domains [16], in addition to interacting with proteins involved in the regulation of the cell cycle, such as p21Cip1 [17] and cyclin B [18]. The hnRNPA2 is an established SET-binding protein and the SET–hnRNPA2 complex contributes, in turn, to PP2A inhibition [19]. Recently, we demonstrated that SET accumulates in HNSCC and proposed its involvement in cell survival [20] and DNA damage response [21].

In this context, we postulated that SET and hnRNPK proteins interact to promote tumorigenesis, and we performed studies in HEK293 and HNSCC (HN6, HN12, and HN13) cell lines with SET or hnRNPK overexpression and knockdown, respectively. We found that accumulated SET interacts with hnRNPK, increasing its activity, with potential implications in cell survival and malignant transformation.

2. Materials and methods

2.1. Cell lines and culture conditions

HN6, HN12, and HN13 (HNSCC) and HEK293 (human embryonic kidney; ATCC, Manassas, VA, USA) cell lines were cultured in

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DMEM supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 mg/ml) (Sigma–Aldrich), at 37 °C, in a humidified atmosphere containing 5% CO_2.

2.2. Reagents and antibodies

Anti-fibrilarin (clone C13C3; #2639) (Cell Signaling Technology); anti-BrdU (#B2531) and anti-hnRNPK/J (isoforms with 52/50 kDa, respectively) (Sigma–Aldrich); and anti-SET (E-15; #sc5655), anti-β-actin (clone C4; #sc47778) (Santa Cruz Biotechnology) primary antibodies were used. Anti-rabbit, anti-mouse, and anti-goat secondary antibodies conjugated with horseradish peroxidase (Santa Cruz Biotechnology) were also used. LY294 002 (#L9908) and 5-Bromo-2′-deoxyuridine (BrdU) (#B5002) were purchased from Sigma–Aldrich.

2.3. SET and hnRNPK overexpression in HEK293 cell line

The HEK293 cells were transfected with SET or hnRNPK DNA constructions using the PolyFect Reagent (Qiagen, Valencia, CA, USA), following the manufacturer's instructions. A SET human full-length cDNA clone (pCMV SPORT.6; NM_003011.3) was purchased from Invitrogen and transferred to a pcDNA3.1 vector (Invitrogen). Human hnRNPK cDNA (transcript 2; NM_031263.2), obtained through PCR, was cloned using a Gateway® cloning system (Invitrogen) for pDONR™/ZEO and pcDNA3.1/nV5-DEST™ vectors.

2.4. SET and hnRNPK knockdown in HNSCC cell lines

HNSCC cell lines were transfected with double-stranded RNA oligonucleotides directed against SET (GS6418; Qiagen) or hnRNPK (SI00300468; Qiagen), using the HiPerFect transfection reagent (Qiagen), following the manufacturer's instructions. The si*CONTROL* AllStars siRNA Negative Control (Qiagen) was used as a negative siRNA control.

2.5. RNA extraction and quantitative real-time PCR

RNA was isolated from cultured cells with the TRIzol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's protocol, and treated with RQ1 RNase-Free DNase (Promega, Madison, WI, USA). The cDNA synthesis was performed using GoScript™ Reverse Transcription System (Promega).

The mRNA sequences were the following: *hnRNPK* (transcript 2-encoding A isoform; NM_031263.2) F – AGTATGCAGATGTTGAAGG ATT; hnRNPK (transcript 3 – encoding B isoform; NM_031262.2) F – TGTGAAGCAGTATTCTGGAAAGT and hnRNPK (encoding A and B isoforms) R – GCAGAACACCTATGAAGCAGAG; β-actin (NM_001 1012) F – GCCTCGCTGTCCACCTTCCA and R – AGAAAGGGTGTAACG CAACTAAG; and GAPDH (NM_0020463) F – GACTTCAACAGCGACA CCCACTC and R – GTCCACCACCCTGTTGCTGTAG. The reactions were performed in a final volume of 10 μL, which contained 5 μL of Fast EvaGreen Master Mix (Uniscience, Cambridge, UK), 0.3 μM per primer, and 100 ng of cDNA. The cycling conditions were 2 min at 96 °C, followed by 40 cycles of 15 s at 96 °C, 10 s at 55 °C, and 25 s at 72 °C.

2.6. Immunofluorescence assay

Cells were fixed with absolute methanol at −20 °C for 6 min, and blocked with 0.5% (v/v) Triton X-100 in phosphate-buffered saline (PBS) and 3% (w/v) bovine serum albumin (BSA). They were then incubated overnight with primary antibodies and washed three times with PBS. After incubation with an Alexa Fluor 546- and a FITC- or TRITC-conjugated secondary antibody for 1 h, the cells were stained with DAPI (Sigma–Aldrich) and visualized using a Zeiss Axiovert 40 CFL Microscope and Zeiss AxioVision 4.8.2 software.

2.7. Cytoplasmic and nuclear protein extraction

Protein subcellular fractions were obtained using ProteoJET™ Cytoplasmic and Nuclear Protein Extraction Kit (Fermentas), according to the manufacturer's instructions.

2.8. Isolation of chromatin-associated proteins

Chromatin was extracted in an acid buffer containing 250 mM NaCl, 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, pH 7.0), 5 mM ethylenediaminetetraacetic acid (EDTA), 0.1% NP-40, 0.5 mM dithiothreitol (DTT), 100 ng/mL TSA (trichostatin A), 5 mM NaF, and 1 mM Na_3VO_4. The pellet was precipitated in 20% trichloroacetic acid (TCA). The chromatin fraction was ressuspended in 100 mM Tris-(hydroxymethyl) aminomethane (Tris–HCl, pH 8.0) and the chromatin-associated proteins were eluted in an SDS buffer (10% glycerol, 5% β-mercaptoethanol, 2.3% SDS, and 62.5 mM Tris–HCl pH 6.8).

2.9. Immunoblotting assay

Protein concentration of the cellular extract was determined through the DC protein assay (Bio-Rad). Total proteins (30–50 μg) were resolved in SDS–PAGE and transferred to the PVDF membrane. The membrane was incubated overnight with primary antibodies, washed and incubated with secondary antibodies conjugated with horseradish peroxidase for 1 h. Bound antibodies were detected using an ECL Western blotting system (GE Health Care).

2.10. Co-immunoprecipitation assay

Cellular proteins were obtained using Buffer A (10 mM Tris–HCl pH 7.9, 60 mM KCl, 1 mM EDTA, and 1 mM DTT) containing 0.1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, and complete protease inhibitors (Sigma–Aldrich). Protein extracts were clarified by centrifugation at 15,000*g* for 10 min and immunoprecipitated using 5 μg of antibodies (against SET and phospho-Thr) and 50 μl of protein G-agarose (Upstate Biotechnology) for 2 h, at room temperature. Then, protein G agarose beads were collected by centrifugation at 15,000*g* and washed four times with Buffer A. A pellet of immunoprecipitated proteins, bound to protein G agarose beads, was eluted in an SDS buffer.

2.11. Preparation of recombinant human hnRNPK protein

Recombinant hnRNPK protein was obtained using human hnRNPK cDNA (transcript 2; NM_031263.2) through PCR using *Pfu* DNA polymerase (Promega). The PCR product was cloned into PCR2.1-TOPO (Invitrogen) and clones were submitted to DNA sequencing. The selected clone was digested using *NheI* and *XhoI* restriction enzymes. Rosetta (DE3) *Escherichia coli* was transformed by the plasmid pET-28a expression vector (Novagen) containing hnRNPK cDNA. Transformants were grown at 37 °C in LB medium with 25 μg/mL kanamycin. Recombinant human hnRNPK protein was purified by affinity chromatography using a Ni Sepharose™ High Performance resin (GE HealthCare). The elution was performed using a 25 mM phosphate buffer (pH 7.4) containing 60–500 mM imidazole and 500 mM NaCl. Each fraction was analyzed using SDS–PAGE. The gel was stained with Coomassie brilliant blue. The eluted proteins were dialyzed against 100 mM Tris buffer (pH 7.4) containing 1 mM EDTA. Human recombinant hnRNPK protein was confirmed by immunoblotting assay.

2.12. Electrophoretic mobility shift assay (EMSA)

Total proteins (10 μg) were incubated with 2 μL of 5× EMSA binding buffer (50 nM Tris–HCl pH 7.5, 5 mM EDTA, 0.5 mM DTT, and 50% glycerol) and 2 μL (60 ng) of CT2–30 (AGCTAACCCTCCCCA GATCAAGCCTGCGATGATTTATACTCACAGCCCTCCCCATAG) oligonucleotide [22]. CT2–30 oligonucleotide (60 ng) incubated with 2 μL of EMSA binding buffer was used as a negative control, and a mix of 1 μL (630 ng/12.25 μM) of purified recombinant human hnRNPK and 2 μL (60 ng) of CT2–30 oligonucleotide incubated with 2 μL of EMSA binding buffer was used as a positive control. Each mix was homogenized and stored at 0 °C for 20 min. Reactions were resolved in 7% polyacrylamide gel. Samples were stained with SYBR® Gold (Invitrogen) and visualized by ultraviolet light. The images were obtained using a Gel Doc™ XR + System (Bio-Rad, Hercules, CA).

2.13. BrdU cell proliferation assay

Cells were treated with 0.03 mg/mL BrdU for 6–12 h, at 37 °C, fixed with 4% paraformaldehyde, washed in 0.1 M PBS (phosphate-buffered saline, pH 7.4) with 1% Triton X-100, and incubated with 1 M HCl (hydrochloric acid) and 2 M HCl. A borate buffer (0.1 M) was added and cells were blocked with 0.1 M PBS in the presence of 1% Triton X-100, 1.0 M glycine, and 5% normal goat serum, and incubated in the sequence with anti-BrdU and secondary anti-mouse HRP conjugated with diaminobenzidine (DAB).

2.14. Quantifying and statistical analysis

Relative quantifying of real-time PCR assays was performed using the $2^{-\Delta\Delta CT}$ method ($2^{-[(CT\ sample - CT\ sample\ housekeeping\ gene) - (CT\ calibrator - CT\ calibrator\ housekeeping\ gene)]}$). Immunobloting and EMSA assays were quantified using ImageJ 1.44o software (National Institutes of Health, USA). Statistical analysis was performed using the GraphPad Prism software (version 5.0, USA). A student's *t*-test or ANOVA was used to examine the association between media and treatments. *P* values <0.05 were considered significant.

3. Results and discussion

3.1. SET and/or hnRNPK protein accumulation increases cellular proliferation and SET accumulation increases total/phosphorylated hnRNPK protein levels

Considering previous reports on the association between hnRNPK and pancreatic cancer cell growth [22], and between SET and cell survival in HNSCC [20], we assessed the influence of the accumulation of these proteins in the proliferation of HEK293 and HN13 cells. BrdU assay was performed in the HEK293 cell line overexpressing either hnRNPK or SET, or both proteins, as well as in the HN13 cell line with knockdown of either hnRNPK, SET, or both. The hnRNPK or SET overexpression in the HEK293 cell increased the cellular proliferation, and simultaneous overexpression displayed an additive effect (Fig. 1A). Accordingly, hnRNPK or SET knockdown in the HN13 cell reduced cellular proliferation (Fig. 1B). Immunoblotting of SET-overexpressing HEK293 cell and HN13 cell with knockdown of either SET or hnRNPK revealed that SET overexpression increased the hnRNPK level and the SET knockdown reduced hnRNPK, whereas hnRNPK knockdown did not reduce SET (Fig. 1C). Therefore, SET accumulation increases hnRNPK protein level, in an apparent association with cell proliferation/survival.

Next, we assessed whether the phosphorylated hnRNPK level could also be affected by SET accumulation; phosphorylation has been reported in the hnRNPK Ser, Tyr, and Thr residues [4,22–25], and it has been associated with hnRNPK cellular location and function [4,25–27]. The co-IP with an antibody against phospho-Thr in the HEK293 cell, followed by immunoblotting with anti-hnRNPK/J, showed that SET accumulation increased the phosphorylated hnRNPK level; LY294, a PI3K inhibitor, did not cause a significant effect (Fig. 1D). In this regard, it was proposed that phosphorylation

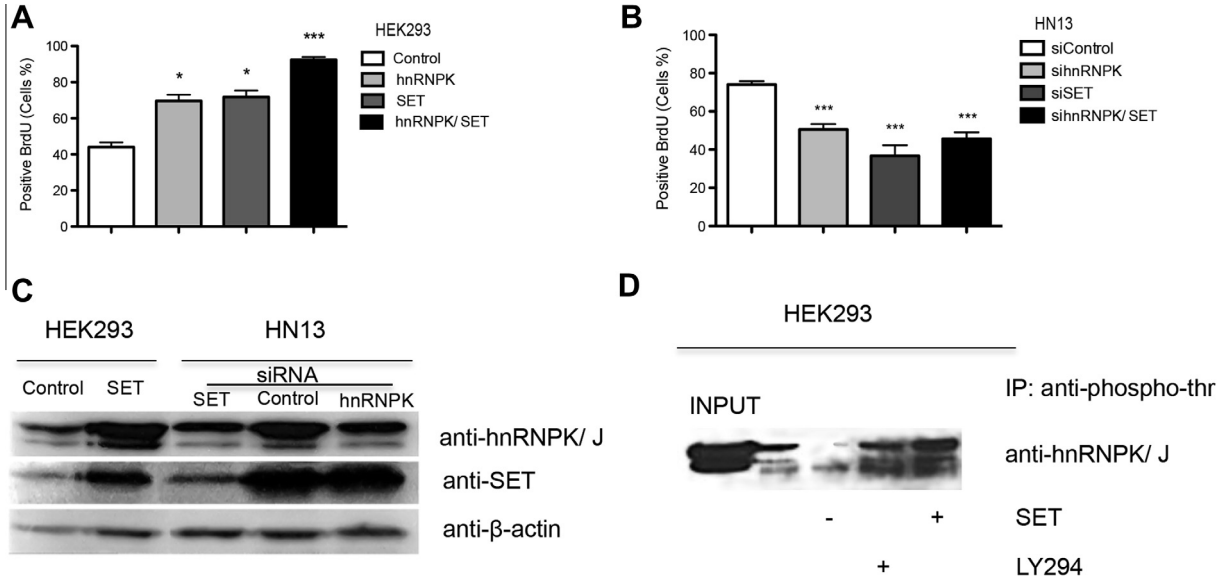

Fig. 1. SET and hnRNPK protein accumulation increases cellular proliferation and SET accumulation increases total and phosphorylated hnRNPK protein levels. (A) BrdU cell proliferation assay performed in HEK293 cell overexpressing either hnRNPK (HEK293/hnRNPK), SET (HEK293/SET), or hnRNPK + SET (HEK293/hnRNPK/SET), compared to HEK293 control. (B) BrdU cell proliferation assay in HN13 cell with knockdown of hnRNPK (HN13 sihnRNPK), SET (HN13 siSET), or hnRNPK + SET (HN13 sihnRNPK/SET), compared to HN13 siRNA negative control (siControl). (C) Immunoblotting for hnRNPK and SET proteins extracted from HEK293/SET cell, and from HN13 cell with knockdown of SET (HN13 siSET) or hnRNPK (HN13 sihnRNPK), as well as respective controls; β-actin was used as a loading control. (D) Detection of phosphorylated hnRNPK in HEK293 cell with SET knockdown (−SET) or overexpressing SET (+SET), in relation to the respective controls. Proteins were immunoprecipitated (IP) using primary antibody for phospho-Thr; anti-hnRNPK was used in the immunoblotting. Effect of LY294, after 24 h (+LY294). In A and B: The data are presented as mean ± SD of three independent experiments performed in triplicate; significantly different from controls: $^{*}p < 0.05$; $^{***}p < 0.001$. In C and D: Samples were resolved in polyacrylamide gel and transferred to PVDF membrane; pictures are representative of three independent experiments.

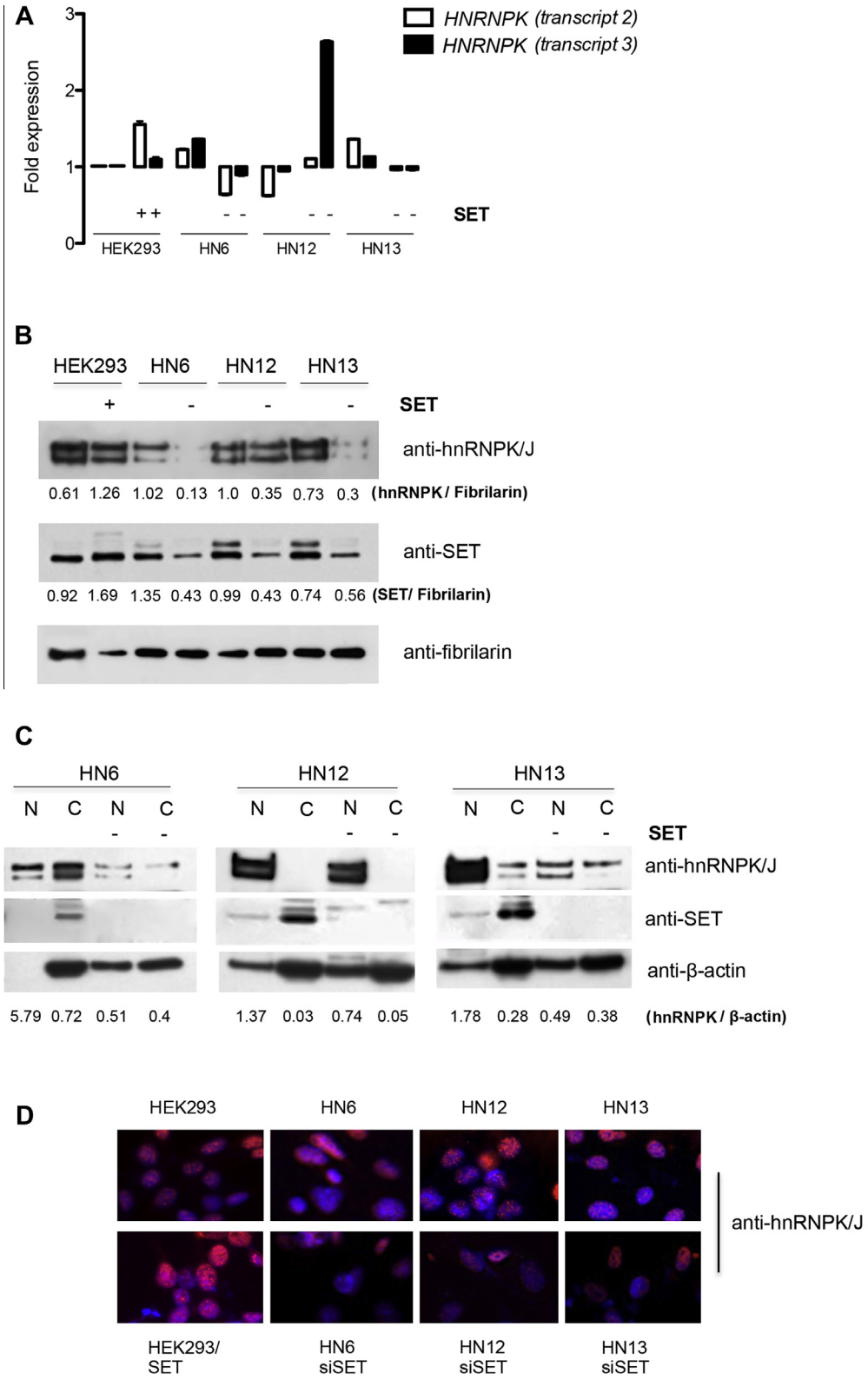

Fig. 2. SET accumulation up-regulates hnRNPK mRNA/protein and promotes nuclear location of hnRNPK protein. Studies in SET-overexpressing HEK293 cell (+SET or HEK293/SET) and HNSCC cell lines (HN6, HN12, and HN13) with knockdown of SET (−SET or siSET). (A) Real-time PCR of hnRNPK transcripts 2 and 3 in HEK293 cell overexpressing SET (+SET) or control, and HNSCC cells with SET knockdown (−SET) or siRNA negative control. The relative mRNA expression levels were calculated by the $2^{-\Delta\Delta CT}$ method. β-Actin and GAPDH were used as housekeeping. The values are presented as fold change expression. The data are representative of three independent experiments performed in triplicate. (B) Immunoblotting for hnRNPK and SET in HEK293/SET cell (+SET) and HNSCC siSET cells (−SET); fibrilarin was used as a loading control. (C) Immunoblotting for SET and hnRNPK in the nuclear (N) and cytoplasmic (C) subcellular fractions of HNSCC siSET cells (−SET); β-Actin was used as a loading control. (D) Immunofluorescence assay (×400 magnification) for hnRNPK (red) in HEK293 and HEK293/SET cells, HNSCC control and HNSCC siSET cells; nuclei were stained with DAPI (blue). Pictures are representative of at least two independent experiments; original magnification, ×200. Numbers in B represent hnRNPK/fibrilarin and SET/fibrilarin ratios, and in C, hnRNPK/β-actin ratio, determined by densitometry analysis. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

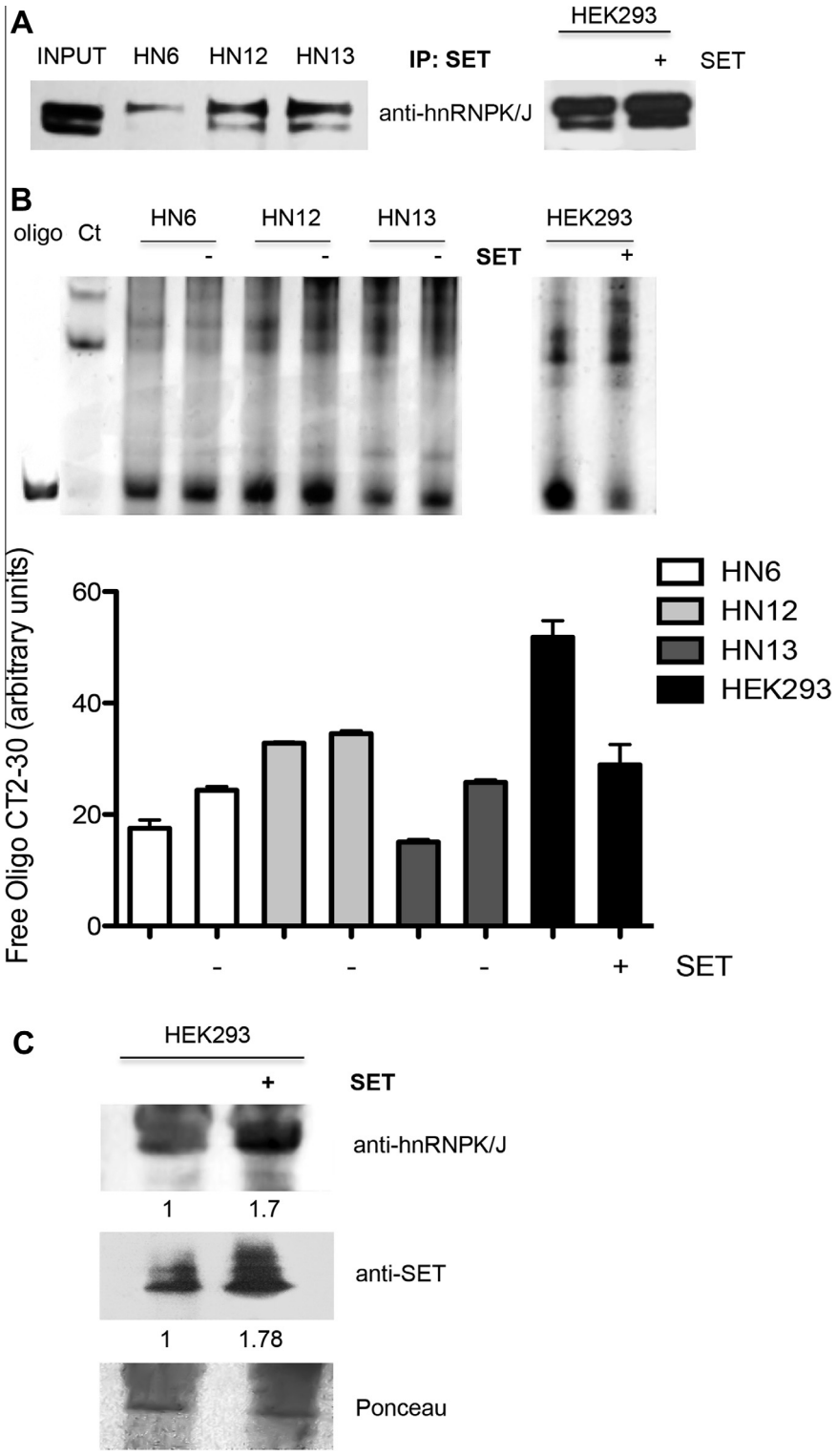

Fig. 3. SET protein interacts with hnRNPK and enhances the binding of hnRNPK with nucleic acids. HNSCC cell lines with knockdown of SET (−SET) and SET-overexpressing HEK293 cell (+SET). (A) Co-immunoprecipitation assay using anti-SET and immunoblotting with anti-hnRNPK. (B) Electrophoretic mobility shift assay (EMSA), performed with 10 μg of total cell protein extract. In the lines, are shown: free DNA oligo CT2–30 (60 ng), oligo CT2–30 + purified recombinant human hnRNPK (positive control, Ct), and binding reactions of oligo CT2–30 with total proteins extracted from HNSCC cell lines with SET knockdown (−SET), and siRNA negative control, or SET-overexpressing HEK293 cell (+SET), and control. The ssDNA (both free DNA oligo and complexed DNA oligo-protein) was stained with SYBR Gold and visualized by UV. Graphic is representative of densitometric analysis of free DNA oligo CT2-30. (C) Immunoblotting for hnRNPK and SET proteins, performed in the chromatin protein sub-fraction, from SET-overexpressing HEK293 cell (+SET), and control; numbers represent the hnRNPK or SET/constitutive protein ratio (Ponceau staining), determined by densitometry analysis. Pictures are representative of three independent experiments.

of hnRNPK is an important mechanism that regulates the accumulation of hnRNPK in cytoplasm, in association with reduced hnRNPK activity, aimed at silencing mRNA translation [4] and at increasing mRNA stability and protein level [26]. Additionally, it was proposed that splicing in cancer cells can be influenced by the phosphorylation status of proteins [27], including hnRNPK [4,23].

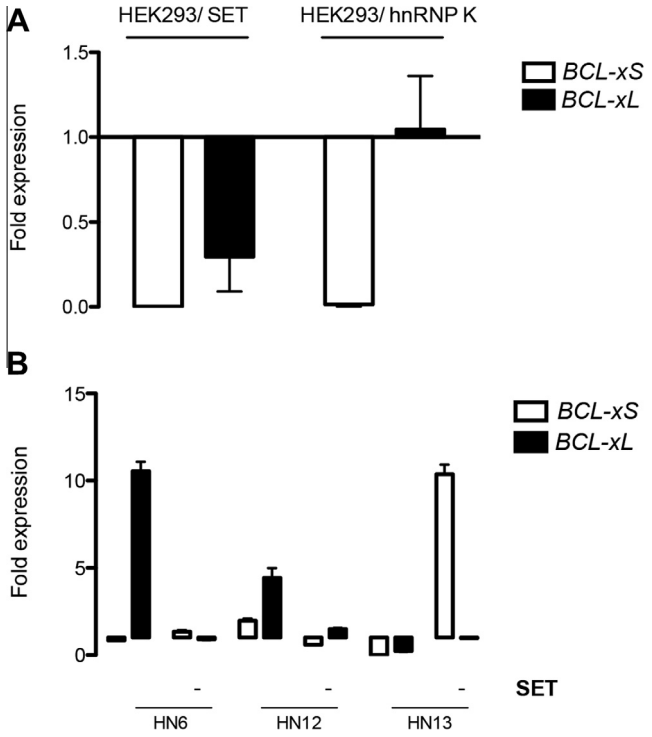

Fig. 4. SET accumulation increases Bcl-xS repression. Quantitative real-time PCR of Bcl-xL and Bcl-xS transcripts in (A) HEK293 cell overexpressing SET (HEK293/SET) or hnRNPK (HEK293/hnRNPK), and (B) HNSCC cells with SET knockdown (−SET) or siRNA negative control. The values are presented as fold change expression. The data are representative of three independent experiments performed in triplicate.

3.2. SET accumulation up-regulates hnRNPK mRNA and protein and may promote its nuclear location

To confirm the results of Fig. 1C regarding the increased hnRNPK level, we assessed hnRNPK mRNA in a SET-overexpressing HEK293 cell (HEK293/SET) and in the HNSCC cell lines HN6, HN12, and HN13 with SET knockdown (siSET), by quantitative real-time PCR. The HEK293/SET cell had an increased level of hnRNPK, as well as transcript 2 (isoform a) and 3 (isoform b); consistent with this effect, SET knockdown in the HNSCC cell lines, in which SET is accumulated, had reduced the level of hnRNPK transcripts (Fig. 2A). Accordingly, immunoblotting showed an increase of hnRNPK protein level in the HEK293/SET cell and a decrease of it in the HNSCC/siSET cell (Fig. 2B).

Moreover, we assessed whether SET accumulation could influence the hnRNPK cellular distribution. We performed immunoblotting for nuclear and cytoplasmic sub-fractions (Fig. 2C) and immunofluorescence (Fig. 2D) assays using anti-hnRNPK/J. Fig. 2C and D show that in all HNSCC cell lines, the level of hnRNPK was higher in the nucleus when compared to the respective siSET controls; this was quite evident in HN13 cells. Accordingly, Fig. 2D shows a higher nuclear level of hnRNPK resulting from SET overexpression in the HEK293 cell line. In this context, a nuclear hnRNPK accumulation has been associated with poor prognosis in cancer and splicing function [28]. Therefore, in addition to up-regulating hnRNPK, SET accumulation may promote its nuclear location.

3.3. SET protein interacts with hnRNPK and enhances the binding of hnRNPK with nucleic acids

Considering the SET-promoted hnRNPK nuclear location, we assessed whether SET accumulation could influence the binding of hnRNPK with nucleic acids, as well as whether SET itself could interact with hnRNPK. Co-immunoprecipitation assay indicated that SET interacts with hnRNPK in both HEK293/SET and HNSCC cells (Fig. 3A). Interestingly, the protein–protein interaction was weaker in the HN6 cell, the same one in which the nuclear hnRNPK level was low (Fig. 2C). These results suggest that SET directly interacts with hnRNPK and that this interaction is occurring in the nucleus.

In this context, we performed a gel electrophoresis mobility shift assay (EMSA) in HNSCC and HEK293 cells, using an oligo ssDNA. HNSCC cell lines, in particular the HN13, as well as the HEK293/SET cell, presented a lower amount of free oligo in relation to the respective controls (Fig. 3B); this indicates that SET enhances the binding of hnRNPK to ssDNA. Moreover, immunoblotting of chromatin-associated proteins (Fig. 3C) showed that both SET and hnRNPK proteins are associated with chromatin, as well as that SET accumulation increases the chromatin-associated hnRNPK level. These results suggest that SET accumulation promotes SET interaction with hnRNPK, which in turn increases the binding of hnRNPK with nucleic acids. This has potential implications in the hnRNPK function regarding gene expression and splicing.

3.4. SET accumulation increases Bcl-xS repression

The hnRNPK protein controls the alternative splicing of Bcl-x and represses the pro-apoptotic Bcl-xS isoform [8]. In this regard, we assessed whether SET could influence the hnRNPK splicing function by regulating the Bcl-xS transcript level. We quantified the Bcl-xS and Bcl-xL mRNAs by real-time PCR in the HEK293 cell overexpressing SET or hnRNPK proteins. A similar reduction of the Bcl-xS mRNA level was observed in either case; it is worth observing that SET accumulation in the HEK293 cell reduced the level of the Bcl-x transcripts (Fig. 4A). The mRNA expression of Bcl-x transcripts was also assessed in the HNSCC cell lines with SET knockdown (Fig. 4B). HNSCC cells presented a low Bcl-xS level, in line with the high SET [20] and hnRNPK [11] levels present in cancer cells, as well as with the reported repression of Bcl-xS [8]. Moreover, an up-regulation of Bcl-xS mRNA was observed in the HN13siSET cell, in agreement with the observed lack of action of SET and hnRNPK on Bcl-xS splicing. These results suggest that SET accumulation influences the hnRNPK function with respect to Bcl-xS splicing, and it could explain the SET/hnRNPK-promoted cell proliferative response (Fig. 1A). This is the first study to demonstrate a SET effect in Bcl-x transcripts (X and L), and it suggests a SET action in association with hnRNPK in the gene expression.

SET and hnRNPK share functions to promote tumorigenesis and cell transformation aiming to improve cell survival, in agreement with the literature's data on the involvement of hnRNPK in these actions [3,5,29], including repression of the pro-apoptotic protein Bcl-xS [8,30]. hnRNPK is a new target of SET, and SET–hnRNPK interaction, in turn, has potential implications in cell survival and malignant transformation, constituting a potential therapeutic target in cancer. Moreover, SET accumulation is a potential model for the study of SET–hnRNPK targets with respect to splicing and transcription control.

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