



RPSAP52 lncRNA is overexpressed in pituitary tumors and promotes cell proliferation by acting as miRNA sponge for HMGA proteins

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Abstract

Long non-coding RNAs (lncRNAs) are emerging as fundamental players in cancer biology. Indeed, they are deregulated in several neoplasias and have been associated with cancer progression, tumor recurrence, and resistance to treatment, thus representing potential biomarkers for cancer diagnosis, prognosis, and therapy. In this study, we aimed to identify lncRNAs associated with pituitary tumorigenesis. We have analyzed the lncRNA expression profile of a panel of gonadotroph pituitary adenomas in comparison with normal pituitaries. Then, we focused on RPSAP52, a novel lncRNA antisense for the *HMGA2* gene, whose overexpression plays a critical role in the development of pituitary adenomas. We report that RPSAP52 expression is highly upregulated in gonadotroph and prolactin-secreting pituitary adenomas, where it correlates with that of HMGA2, compared with normal pituitary tissues. Conversely, its expression showed a variable behavior in somatotroph adenomas. We also demonstrate that RPSAP52 enhances HMGA2 protein expression in a ceRNA-dependent way acting as sponge for miR-15a, miR-15b, and miR-16, which have been already described to be able to target HMGA2. Interestingly, RPSAP52 also positively modulates HMGA1, the other member of the High-Mobility Group A family. Moreover, functional studies indicate that RPSAP52 promotes cell growth by enhancing the G1-S transition of the cell cycle. The results reported here reveal a novel mechanism, based on the overexpression of the lncRNA RPSAP52, which contributes to pituitary tumorigenesis, and propose this lncRNA as a novel player in the development of these tumors.

Key Messages

- RPSAP52 is overexpressed in pituitary adenomas.
- RPSAP52 increases HMGA protein levels.
- A ceRNA mechanism is proposed for the increased HMGA1/2 expression.

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Introduction

Human pituitary adenomas (PAs) are benign neoplasms, accounting for 10% of all diagnosed brain tumors [1]. The driving events are genetic and/or epigenetic alterations determining monoclonal expansion of a cell subset. Then, pituitary hormones and/or growth factors increase cell proliferation eventually leading to pituitary cell transformation and, then, to PA development [2].

Gonadotroph adenomas constitute the vast majority of clinically nonfunctioning pituitary adenomas (NFPA), which accounts for 15–22% of all pituitary adenomas overall [3–5]. These tumors generally come to medical attention because of tumor mass effect causing the compression of critical nervous structures.

Several genetic alterations have been already identified in PAs: activating mutations of guanine nucleotide-binding protein α subunit (GNAS) in somatotroph adenomas [6]; Aryl hydrocarbon receptor-interacting protein (AIP) mutations in sporadic PAs and Familial Isolated Pituitary Adenomas [7]; somatic mutations in Ubiquitin-Specific Protease 8 (USP8) gene in ACTH-secreting PAs [8]. Moreover, overexpression of High-Mobility Group A2 (HMGA2) has been found in almost all of the human PAs [9], and its role in pituitary tumorigenesis is supported by the development of mixed growth hormone/prolactin cell PAs in HMGA2-overexpressing transgenic mice through a HMGA2-mediated E2F1 activation [10].

Recently, epigenetic mechanisms have been also envisaged to account for the development of PAs. Various studies have demonstrated that non-coding RNAs (ncRNAs) play an important pleiotropic role in the development of PAs [11]. Indeed, several microRNAs (miRNAs or miRs), including miR-15a, miR-15b, miR-16 [12], miR-34b, miR-326, miR-432, miR-548c-3p, miR-570, and miR-603 [13], targeting genes involved in the HMGA/E2F pathway [14], are drastically and constantly downregulated in PAs. Moreover, we have shown the downregulation of miR-410 in gonadotroph adenomas with respect to normal pituitary gland and validated *CCNB1* as its target [15].

Recently, other types of ncRNAs, referred as long non-coding RNAs (lncRNAs), have been shown to have dynamic roles in the regulation of gene transcription and translation, and to be involved in several human diseases including cancer [16]. lncRNAs are endogenously transcribed RNA molecules, ranging from 200 to 100,000 nucleotides in length, which lack protein-coding capacity. They are poorly conserved during evolution and regulate gene expression by different mechanisms that have not yet fully understood [17]. Recent studies have revealed that they are deregulated in

several neoplasias, including pituitary tumors [18]. Indeed, maternally expressed gene 3 (MEG3) represents the first recognized tumor suppressor lncRNA in PAs [19]. Loss of MEG3 was also observed in other human neoplasias, including colorectal cancer [20], non-small-cell lung carcinoma (NSCLC) [21], hepatocellular carcinoma (HCC) [22], breast cancer [23], and glioma [24]. More recently, it has been shown that increased expression of Hox transcript antisense intergenic RNA (HOTAIR), a lncRNA involved in cancer progression by remodeling the chromatin landscape, is associated with PA development and invasion [25]. Thus, lncRNAs can be considered as new emerging players in carcinogenesis. Therefore, the search for lncRNAs deregulated in PAs and the definition of their biological function may represent an important aim to better define the molecular mechanisms underlying pituitary tumorigenesis.

In our study, we analyzed the lncRNA expression profile of 12 gonadotroph adenomas with respect to three normal pituitary tissues in order to identify differentially expressed lncRNAs between normal pituitary gland and PAs. Several lncRNAs were found deregulated in PAs. Among the upregulated lncRNAs, we decided to focus on the RPSAP52 (ribosomal protein SA pseudogene 52) gene that represents the antisense of the *HMGA2* gene and the expression of both are correlated in PAs. Consistently, RPSAP52 overexpression increases HMGA protein levels acting as miRNA sponge. Therefore, these results propose RPSAP52 as a novel player in pituitary tumorigenesis.

Materials and methods

lncRNA microarray

Sample information is reported in [supplementary material](#). Expression profiling analysis was performed on RNA from 12 gonadotroph tumors and 3 normal pituitary samples. The microarray analysis was performed using Agilent Array platform as reported in [supplementary material](#).

Reverse transcription and quantitative RT-PCR

The qRT-PCR analyses were performed using the primers reported in [supplementary material](#). To calculate the relative expression levels, we used the $2^{-\Delta\Delta CT}$ method [26]. As internal reference genes for RT-PCR analysis of tumor samples, we chose *PPIA1* since it showed the lower expression variability between normal pituitary and PA samples.

Cell lines, vectors, and transfections

Details for cell lines, vectors, and transfections are reported in [supplementary material](#).

Protein extraction, western blotting, and antibodies

Protein extraction and western blots were performed as previously described [15]. After blotting, membranes were incubated with primary antibodies against HMGA2, HMGA1 [14, 27], or β -actin (sc-1615, Santa Cruz), γ -tubulin (T6557, Sigma-Aldrich), and GAPDH (sc-32233, Santa Cruz). The reaction was detected with a western blotting detection system (Thermo Scientific, Rockville, IL).

Growth curve assay

5×10^4 cells were plated in 96-well plates and transfected with the indicated plasmids. Cell growth was assessed using CellTiter 96® Aqueous One Solution Cell Proliferation Assay (MTS) (Promega), at 0, 24, 48, 72, 96, and 120 h after plating, according to the manufacturer's instructions.

Flow cytometry

HT-29, HCT-116, GH3, ATt-20, MEG01, and BCPAP cells were transfected with RPSAP52 expression vector, RPSAP52 antisense LNA™ GapmeR, and the relative negative controls and collected after 48 h. After trypsinization, cells were washed in phosphate-buffered saline and fixed in 70% ethanol. Staining for DNA content was performed with 2 μ g/ml propidium iodide and 20 μ g/ml RNase A for 30 min. For each measure, 10,000 events were analyzed. We used a FacsARIA III flow cytometer (Becton Dickinson, San Jose, CA). Cell cycle data were analyzed with the ModFit LT program (Verity software house).

Luciferase assay

Cells were co-transfected with the previously described modified firefly luciferase vector [12, 13], along with the miRNA oligonucleotides. Firefly and renilla luciferase activities were measured 48 h after transfection with the dual-luciferase reporter assay system (Promega, Madison, WI). Firefly activity was normalized to renilla activity as control of transfection efficiency.

RNA pulldown assay

The pulldown analysis was performed according to the protocol previously described by Yoon [28]. The RPSAP52 or RPSAP52-MUT-15/16cDNA was cloned in the pMS2 vector (pcDNA 3.1 plasmid containing 24 repeats of the MS2 tag—

ACATGAGGATCACCCATGT) upstream of the MS2 tag in the BamHI and EcoRI site. HT-29 cells were co-transfected with the above-indicated vectors or empty vector and pMS2-GST vector. Forty-eight hours after transfection, cells were collected and lysated and protein concentration was measured. Five hundred microliters (2 μ g/ μ l) of lysate was incubated with 50 μ l GSH agarose beads (GE Healthcare) for 3 h at 4 °C. After incubation, beads were washed twice with cold PBS to remove unspecific binding and the co-precipitated RNAs were purified and detected by RT-PCR.

Nucleus-cytoplasm fractionation

Both nuclear and cytoplasmic RNAs from HT-29 cells or from gonadotroph adenoma tissue sample were isolated by using the PARIS™ Kit (Thermo Fisher Scientific) following the manufacturer's instruction. NEAT1 and RP14 RNAs were used as control for nuclear and cytoplasmic RNAs, respectively. qRT-PCR was carried out to detect abundance of lncRNAs.

Statistical analysis

Statistical analyses were performed using the GraphPad Prism (version 6) software. Student's *t* test or ANOVA was used to determine the significance of quantitative experiments. Error bars represent the standard deviation (S.D) or the standard error of mean (s.e.m). To evaluate the statistical correlation between RPSAP52 and *HMGA2*, the non-parametric Spearman correlation coefficient *R* was used. *p* values < 0.05 were considered significant.

Results

lncRNA expression profile of gonadotroph pituitary tumors

First, we performed the lncRNA expression analysis of 12 tumor samples and 3 normal pituitaries by using the Agilent Array platform. 1467 lncRNAs were upregulated and 1909 downregulated (≥ 2 fold change, *p* < 0.05). In Table 1, we report the list of the most 20 up- and 20 downregulated lncRNAs.

Subsequently, the qRT-PCR analysis confirmed the results of the microarray expression profile, since the expression of RP11-500G22.2 and RPSAP52 was upregulated, while the expression of NR_045196, XIST, and MEG3 was downregulated in gonadotroph PAs (Fig. 1). Then, among the deregulated lncRNAs, we focused on the RPSAP52 (ribosomal protein SA pseudogene 52) gene, since it was among the highest upregulated lncRNAs (fold change 53.186455) and also represents the antisense of the *HMGA2* gene, previously reported to have a critical role in pituitary tumorigenesis [14].

Table 1 Representative list of lncRNAs deregulated in gonadotroph adenomas vs. normal pituitary

Upregulated lncRNAs in gonadotroph adenomas vs. normal pituitary		Downregulated lncRNAs in gonadotroph adenomas vs. normal pituitary							
seqname	Gene symbol	RNA length	chrom	Strand	Sequence	Relationship	Associated gene name	p value	Fold change
1	TCONS_00009149	XLOC_004134	236	chr4	-	GAAAAAATAAAAAATGGTCGAACTTGAAGCTG CAGCAGAAGAAGATATGGAATAAACTAAA	Intergenic	0.001267584	109.01462
2	ENST00000437593	RP11-500G22.2	951	chr10	+	ATTTTGCAACCTACCAGAAAGCACCCTTAGGAA AGTGATGATCTGATGGAGAAATAAGAA	Natural antisense	7.74421E-09	83.39822
3	ENST00000437593	RP11-500G22.2	951	chr10	+	ATTTTGCAACCTACCAGAAAGCACCCTTAGGAA AGTGATGATCTGATGGAGAAATAAGAA	Natural antisense	7.74421E-09	83.39822
4	ENST00000437593	RP11-500G22.2	951	chr10	+	ATTTTGCAACCTACCAGAAAGCACCCTTAGGAA AGTGATGATCTGATGGAGAAATAAGAA	Natural antisense	7.74421E-09	83.39822
5	ENST00000510034	RP11-255I10.2	610	chr4	+	CCTCTACTGGAGTCACCTGTAAAAGAGTTTTGA TTTTAAAAAGAAATTGACGTAGAATTG	Intergenic	4.81343E-06	73.01977
6	ENST00000435884	AC099754.1	454	chr3	-	CCATGAATCCTTTGTACAAAGTGATCCAGTTA ACTACTAATAACAAAACATCCTTAAAACCT	Bidirectional	2.22038E-07	64.34437
7	ENST00000517706	RP11-108E14.1	764	chr8	-	CAGGGAGATGCACATTTTTAGCACAAAATGC CCAGATAGAAITGGTAAAACCTGAAAATA	Intergenic	1.98434E-05	54.46335
8	ENST00000489520	RPSAP52	1144	chr12	-	TCTTGACGGACTCTAAGCAACATGAAAATA AGTTTGACAGAAAATAAACATCAGTTTC	Intronic antisense	1.07138E-07	53.186455
9	ENST00000489520	RPSAP52	1144	chr12	-	TCTTGACGGACTCTAAGCAACATGAAAATA AGTTTGACAGAAAATAAACATCAGTTTC	Intronic antisense	1.07138E-07	53.186455
10	TCONS_00008628	XLOC_004134	395	chr4	-	AAATAITAGAGTAATGTCAITATCTCACCAT GCTCTTTCTGGTTATAAATGCTAG	Intergenic	0.000762855	51.859764
11	ENST00000414699	AL773572.7	336	chr21	-	GTAATATCAGGCACAAAGGAAGGGCACTACAA GTAATATCAGGCACAAAGGAAGGGCACTACAA	Exon sense-overlapping	1.08703E-07	45.709812
12	ENST00000414699	AL773572.7	336	chr21	-	GTAATATCAGGCACAAAGGAAGGGCACTACAA CCACAACCAATATCAGTAAAGCTTTTGGG	Intronic antisense	1.08703E-07	45.709812
13	ENST00000414699	AL773572.7	336	chr21	-	GTAATATCAGGCACAAAGGAAGGGCACTACAA CCACAACCAATATCAGTAAAGCTTTTGGG	Intronic antisense	1.08703E-07	45.709812
14	ENST00000414699	AL773572.7	336	chr21	-	GTAATATCAGGCACAAAGGAAGGGCACTACAA CCACAACCAATATCAGTAAAGCTTTTGGG	Intronic Antisense	1.08703E-07	45.709812
15	uc002now.2	ZNF826P	1109	chr19	-	CTCTTGTAAGAAAGTGAAGTTGAAATCTTGATTT CACAGAATTTTATAAACCCTTTTATTT	Intergenic	1.06862E-07	40.317093
16	ENST00000443469	RP5-1119D9.4	1928	chr20	-	TGACATCTGTAGCCTGATCAATCTGACTCC AGCAAGATGGCCTTGAACCACTATA	Natural antisense	2.23512E-08	38.03677
17	ENST00000443469	RP5-1119D9.4	1928	chr20	-	TGACATCTGTAGCCTGATCAATCTGACTCC AGCAAGATGGCCTTGAACCACTATA	Natural antisense	2.23512E-08	38.03677
18	ENST00000502882	RP11-158J3.2	219	chr5	-	CAAGAATGAGAAAGGAACATCCCAACTGGAGCA TAAATGTCAAGGCAATAGAGAATTTAGA	Intron sense-overlapping	0.006983092	35.015583
19	ENST00000503998	RP11-380D23.1	1447	chr4	-	GAGTATGTTGATGGCTTACATTTCCCAATT ATGCGGGTTTCATTTATAACAGGAAAGG	Natural antisense	1.70598E-08	29.626844
20	ENST00000515782	RP11-588P8.1	526	chr4	+	ATAAATAACATCTACAGACAATTGAAACTAATT GACTATAGAATGACCGCAGTTGTTCAAG	Intronic antisense	2.72944E-05	28.826624

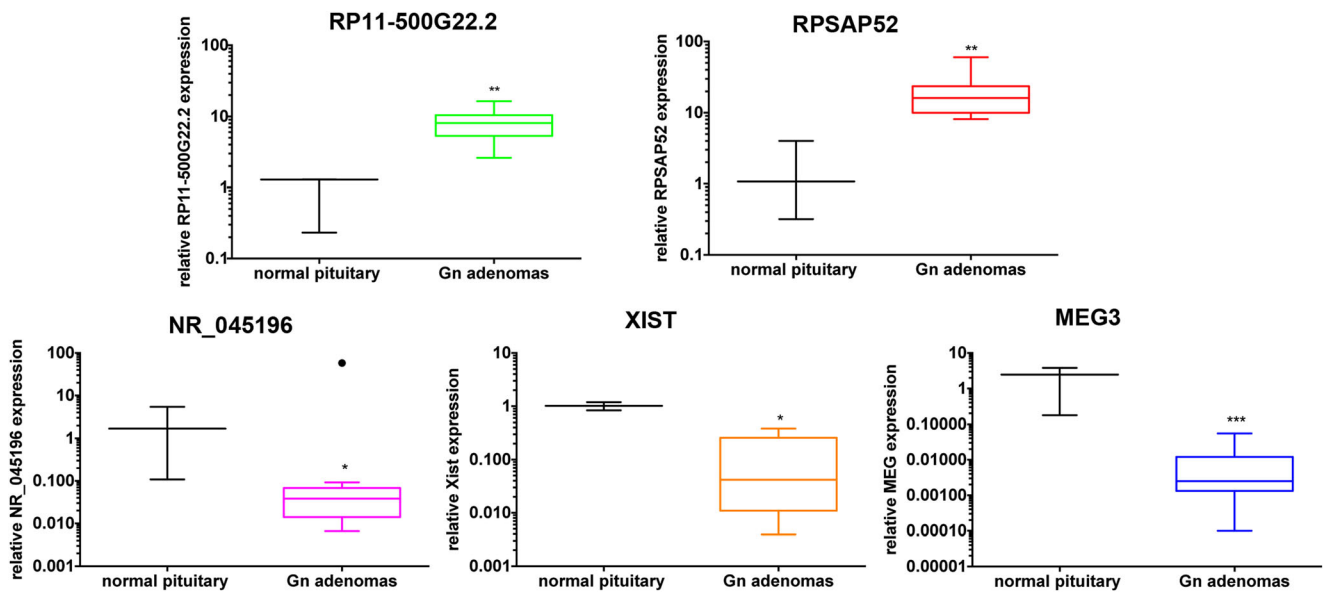


Fig. 1 lncRNAs are dysregulated in PAs: Box plot of RNA expression of RP11-500G22.2, RPSAP52, NR_045196, Xist, and MEG03 in 12 gonadotroph PAs vs. 3 normal pituitaries. XIST expression was evaluated

in 12 gonadotroph pituitary samples from female patients compared with 3 normal female pituitaries. The boxes define the interquartile range and the thick line is the median. Open dots are possible outliers

A qRT-PCR of 32 PA tumors, including the 12 cases used for the microArray analysis, confirmed the upregulation of RPSAP52 compared with 5 normal pituitary samples (Fig. 2a). Moreover, in order to determine whether RPSAP52 was correlated with the expression of *HMGA2*, we also analyzed *HMGA2* mRNA levels: a positive statistically significant correlation between RPSAP52 and *HMGA2* mRNA levels has been also found (Fig. 2b, Spearman r 0.5135, p 0.0016). Consistently, we found an increase in *HMGA2* and *HMGA1* protein levels in 8 gonadotroph PA samples, which showed RPSAP52 overexpression, compared with normal pituitary tissues (Fig. 2c). However, the overexpression of RPSAP52 resulted quite homogeneous in gonadotroph adenomas with no significant association between invasive and non-invasive cases (Supplementary Figure 1A).

Then, we analyzed RPSAP52 expression in PRL adenomas and somatotroph adenoma samples by qRT-PCR. RPSAP52 upregulation was observed in all of the PRL adenoma samples analyzed (Supplementary Figure 1A), where it positively correlates with *HMGA2* mRNA expression. Conversely, its expression showed a variable behavior in somatotroph adenomas (Supplementary Figure 1B). These results suggest that RPSAP52 overexpression is not specific of the gonadotroph adenomas.

RPSAP52 modulates *HMGA2* and *HMGA1* expression and promotes cell proliferation by enhancing the G1-S transition of the cell cycle

In order to verify whether *HMGA2* expression could be affected by overexpression or inhibition of RPSAP52 in vitro, we selected two cancer cell lines (HT-29 and

MEG01) showing low RPSAP52, *HMGA2*, and *HMGA1* expression and two cancer cell lines (HCT-116 and BCPAP) with high RPSAP52, *HMGA2*, and *HMGA1* expression (Fig. 3a).

Then, we transiently transfected HT-29 and MEG01 cells with RPSAP52 expression vector and HCT-116 and BCPAP cells with antisense oligonucleotide inhibitors. The enforced RPSAP52 expression increased *HMGA2*-specific mRNA and protein levels in HT-29 and MEG01 cell lines (Fig. 3b, c and Supplementary Figure 2A, B), while downregulation of *HMGA2* expression followed RPSAP52 silencing in HCT-116 and BCPAP cell lines (Fig. 3d, e and Supplementary Figure 2C, D). Interestingly, RPSAP52 expression also modulates protein but not mRNA levels, of *HMGA1*, the other member of the High-Mobility Group A family (Fig. 3d and Supplementary Figure 2A).

Subsequently, we evaluated the growth rate of the HT-29- and MEG01-RPSAP52-transfected cells and the HCT116- and BCPAP-RPSAP52 antisense-transfected cells. As shown in Fig. 4a and Supplementary Figure 2E, a significant increase in cell growth rate, compared with the empty vector, was observed after transfection with p-RPSAP52, whereas an opposite effect was achieved after transfection of RPSAP52 antisense oligonucleotides. Consistently, RPSAP52 increased the growth rate also of the rat pituitary tumor GH3 and ATt-20 cells (Fig. 4b), confirming our results in the context of pituitary gland.

Then, we analyzed the cell cycle progression of the same cells described above by flow cytometry. As shown in Fig. 4c and Supplementary Figure 2F, RPSAP52-transfected HT-29, GH3, and ATt-20 cells displayed an

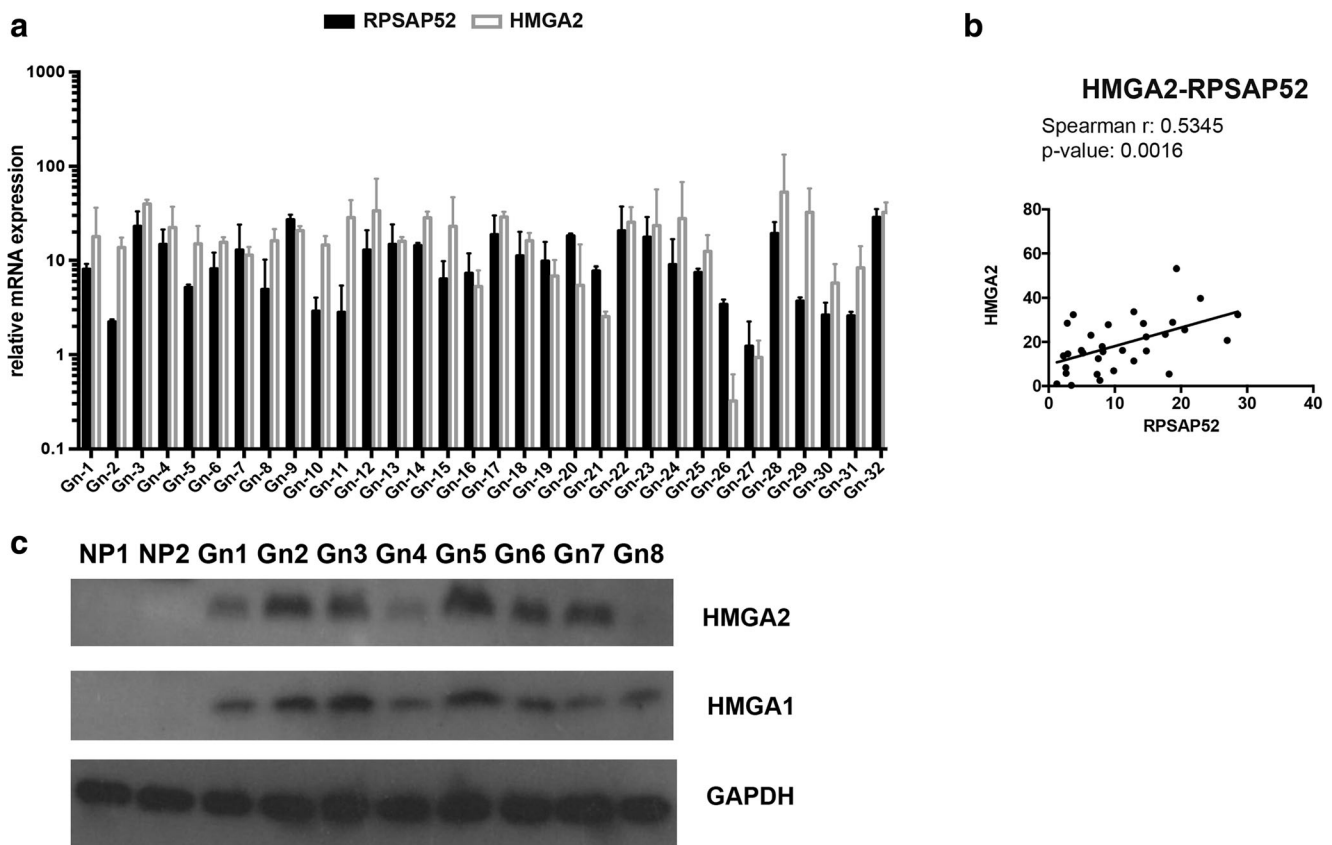


Fig. 2 RPSAP52 positively correlates with HMGA2 and HMGA1 expression. **a** qRT-PCR analysis of RPSAP52 and *HMGA2* expression in a larger set of gonadotroph PAs than those analyzed in panel **a** ($N = 32$). The relative expression values indicate the relative change in the expression levels between gonadotroph adenoma vs. five normal pituitary samples, assuming that the mean value of the normal samples was equal to 1. Each bar represents the mean value \pm s.e. from three independent

experiments performed in triplicate. **b** Spearman rank correlation graph shows the positive relationship between RPSAP52 and *HMGA2* on gonadotroph adenomas of panel **c** (Spearman r 0.5345, p value 0.0016). **c** Western blot analysis of HMGA2 and HMGA1 in 8 gonadotroph PAs compared with two normal pituitary samples. GAPDH expression was analyzed as loading control of western blot analysis

increase in the S-phase population and a decrease in the G1-phase of the cell cycle compared with empty vector-transfected ones, whereas an opposite effect was observed when RPSAP52 expression was silenced. These results indicate that the overexpression of RPSAP52 affects the G1-S transition of the cell cycle progression, consistently with its ability to modulate the levels of HMGA2 and HMGA1 proteins that have been already reported to promote E2F1 activation [14].

RPSAP52 overexpression leads to increased HMGA protein levels by a miRNA “sponge” mechanism

Subsequently, we investigated whether RPSAP52 could act as an endogenous “sponge,” protecting *HMGA1*- and *HMGA2*-specific mRNAs from the activity of their miRNAs.

Indeed, it has been previously reported that lncRNAs and their related coding genes can communicate through

a ceRNA (competing endogenous RNA) language, as already shown for the *PTENP1* pseudogene, which acts as “decoy” by protecting *PTEN* mRNA from common miRNA binding [29].

Then, we searched for potential common MRES (miRNA response elements) in both RPSAP52 and *HMGA* genes by using the online software <http://www.mircode.org>. As shown in Supplementary Table 2, we identified in *HMGA2*, *HMGA1*, and RPSAP52 MRES sequences complementary to miR-15a, miR-15b, and miR-16, which have been previously demonstrated to target the *HMGA1* and *HMGA2* mRNAs [12].

Consequently, we investigated whether RPSAP52 was able to directly bind these miRNAs by performing a RNA pulldown assay. Briefly, we co-transfected a vector expressing a chimeric RNA containing the RPSAP52 lncRNA followed by MS2 RNA hairpins (pRPSAP52-MS2) along with a plasmid expressing a chimeric protein comprising a glutathione-S-transferase domain fused to a

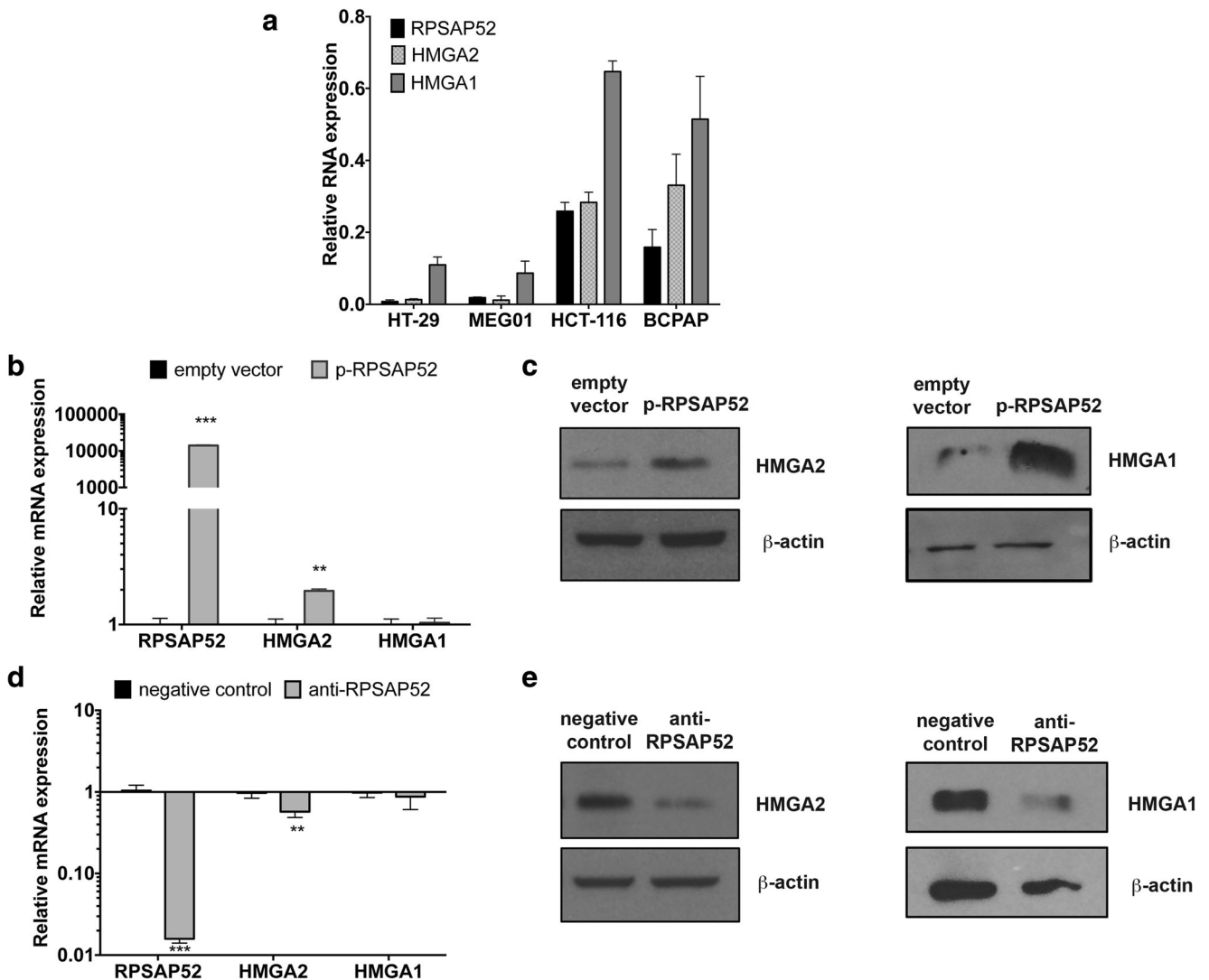


Fig. 3 RPSAP52 induces HMGA2 and HMGA1 expression. **a** qRT-PCR of RPSAP52, *HMGA2*, and *HMGA1* RNA expression in HT-29, MEG01, HCT116, and BCPAP cell lines. Relative change in RNA expression levels was normalized with G6PD. **b** qRT-PCR of RPSAP52, *HMGA2*, and *HMGA1* in HT-29 cells transfected with p-RPSAP52 or relative empty vector. Relative change in RNA expression levels was normalized with G6PD, assuming that the value of the empty vector-transfected sample was equal to 1. The error bars represent the mean value ± s.e. from three independent experiments performed in triplicate (** $p < 0.01$; *** $p < 0.001$). **c** Western blot analysis of HMGA2 and HMGA1 in HT-

29 cells transfected with p-RPSAP52 or relative empty vector. β-Actin expression was analyzed as loading control of western blot analysis. **d** qRT-PCR of RPSAP52, *HMGA2* and *HMGA1* in HCT-116 cells transfected with anti-RPSAP52 or negative control oligonucleotide. G6PD mRNA was used to normalize the RNA expression level. Significance values (** $p < 0.01$; *** $p < 0.001$) relative to cells transfected with negative control. **e** Western blot analysis of HMGA2, HMGA1, and β-actin expression in HCT-116 cells transfected with anti-RPSAP52 or negative control oligonucleotide

MS2 capsid protein which recognizes MS2 hairpins (MS2-GST) leading to the formation of the complex RPSAP52-MS2/MS2-GST. Then, the cells were lysed and miRNAs associated with the chimeric RNA were pulled down by using glutathione (GSH)-coated beads. qRT-PCR assay was performed to determine whether miR-15a, miR-15b, and miR-16 were enriched in the RPSAP52-MS2-pulled down samples. As shown in Fig. 5a, an enrichment of miR-15a, miR-15b, and miR-16 was observed in

RPSAP52-MS2-pulled down samples compared with those derived from the empty vector-transfected cells, supporting the ability of these miRNAs to bind the RPSAP52 sequences.

In order to demonstrate the specific binding of miRNAs to pRPSAP52, we performed the same experiment using a vector expressing RPSAP52-MS2 RNA hairpins with mutated miRNA-binding sites (pRPSAP52-MUT-15/16-MS2), whose sequence is reported in Fig. 5b. As shown

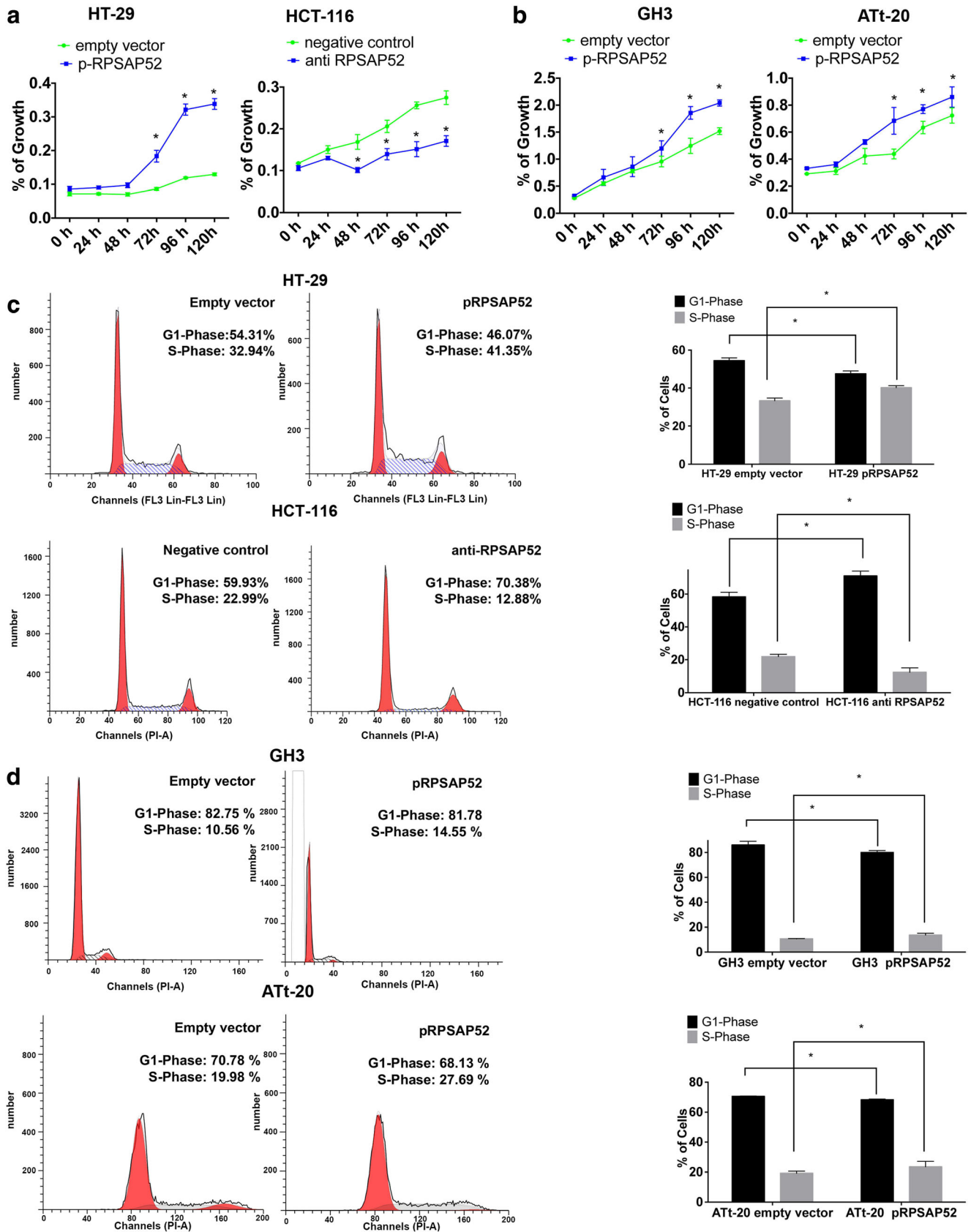


Fig. 4 RPSAP52 affects cell growth and cell cycle progression. Cell growth curve of HT-29 and HCT-116 cells transfected with p-RPSAP52 or empty vector and with anti-RPSAP52 or negative oligonucleotide (**a**) and GH3 and ATt-20 cells transfected with p-RPSAP52 or empty vector (**b**) counted each 24 h for 120 h after plating. The mean values \pm s.e. derive from three independent experiments performed in triplicate. Significance values $*p < 0.05$ relative to cells transfected with empty vector or negative control. **c** Cell cycle analysis of cells in **a** and **b** harvested at 48 h and analyzed by flow cytometry. The histograms show the percentages of cells in the different phases of cell cycle. Values shown represent mean and standard deviations of pooled results from 3 independent experiments; $*p < 0.05$ compared with control cells

in Fig. 5a, the site-directed mutagenesis of the miRNA-binding sites prevented the binding of miR-15a, miR-15b, and miR-16 to RPSAP52 lncRNA.

Then, we transfected a luciferase reporter vector containing RPSAP52 (p-Luc-RPSAP52) or RPSAP52 with mutated miRNA-binding sites (p-Luc-RPSAP52-Mut-15/16) along with miR-15a, miR-15b, or miR-16. We did not find significant differences in the luciferase activity after transfection of miR-15a, miR-15b, or miR-16 compared with scrambled transfected cells or with p-Luc-RPSAP52-Mut-15/16 (Fig. 5c), indicating that these miRNAs do not induce RPSAP52 RNA degradation. Consistently, the RPSAP52 RNA levels do not vary after transfection with the above indicated miRNAs (Fig. 5d).

Moreover, to validate the endogenous miRNA sponge function of RPSAP52, we evaluated the HMGA1 and HMGA2 mRNA and protein levels after transfection of the HT-29 cells with miR-15a, miR-15b, and miR-16 in the presence or absence of RPSAP52. As shown in Fig. 6, the transfection of RPSAP52 increased the HMGA mRNA (Fig. 6a) and protein (Fig. 6b) levels also in the presence of the miRNAs able to target the *HMGA* mRNAs.

The miRNA-induced repression occurs in the cytoplasm and is mediated by RISC (RNA-induced silencing complex). Consistently, the cytoplasmic localization of RPSAP52 supports its sponge function. Indeed, we performed a subcellular fractionation of RPSAP52 in HT-29 cell line and in a gonadotroph adenoma tissue sample. The lncRNA level in nuclear and cytoplasmic fraction was determined by RT-PCR. As shown in Fig. 6c, the HT-29 cells showed an equal distribution of RPSAP52 lncRNA between both compartments (left panel), while RPSAP52 has a higher accumulation in the nucleus versus the cytoplasm in gonadotroph adenoma (right panel).

Finally, to confirm that increased growth rate induced by RPSAP52 overexpression was due to its ability to protect *HMGA* mRNAs from miRNA able to affect the expression of these proteins, we evaluated the growth rate of the HT-29, GH3, and ATt-20 cells transfected with p-RPSAP52 vector containing mutated binding sites for miR-15a, miR-15b, and

miR-16. As shown in Fig. 7, the mutated RPSAP52 vector did not induce any enhancement of the cell growth rate at variance from the RPSAP52 wild-type vector (Fig. 4a, b), suggesting that the RPSAP52-induced proliferative effect is dependent on the presence of these miRNA-binding sites.

Taken together, these findings suggest that the RPSAP52 lncRNA causes an increase in HMGA2 and HMGA1 protein levels by a miRNA sponge mechanism.

Discussion

Recent studies have revealed that lncRNAs, a novel class of gene expression regulators are deregulated in several neoplasias, including pituitary tumors [18]. These evidences suggest that lncRNAs can be considered new emerging molecules involved in the mechanisms leading to the onset of PAs. Therefore, we have first analyzed the lncRNA expression profile of a panel of gonadotroph adenomas and normal pituitary tissues. This analysis revealed 1467 upregulated and 1909 downregulated lncRNAs. Among the lncRNAs upregulated with the highest fold change, we focused our attention on RPSAP52. This lncRNA, positioned on chromosome 12, represents the lncRNA antisense of the *HMGA2* gene, coding for a non-histone chromatinic protein causally involved in pituitary tumors. Indeed, increased *HMGA2* gene copy number was found in human prolactinomas [9] and its overexpression, even in the absence of gene amplification, was detected in the majority of PA histotypes [30]. Consistently, HMGA2-transgenic mice developed prolactin and GH-secreting PAs [14, 31]. Expression analysis of PA tissues confirmed that RPSAP52 was overexpressed in the vast majority of gonadotroph and PRL-secreting PAs and significantly correlated with the expression of HMGA2 and also HMGA1, which shares with HMGA2 most of its biological and oncogenic activities [32, 33].

Even though RPSAP52 is increased only in 8 out of 15 GH adenoma samples, this does not exclude the potentiality of RPSAP52 overexpression to lead to an increased growth rate of a GH3 cell line as reported in our study. This result is consistent with the ability of RPSAP52 to increase HMGA protein levels whose enhancing effect on the cell growth is not restricted to particular cells, but is general event. Consistently, the correlation between RPSAP52 and HMGA protein levels in PAs was further supported by the ability of the lncRNA to increase the HMGA protein levels in vitro. Even though previous results have shown a correlation between HMGA2 overexpression and an aggressive behavior of PAs [34], RPSAP52 overexpression did not seem to correlate with PA clinical parameters (data not shown). Subsequently, we found that RPSAP52 increased cell growth and promoted cell cycle, while its inhibition showed an opposite effect. These data are consistent with the ability of HMGA

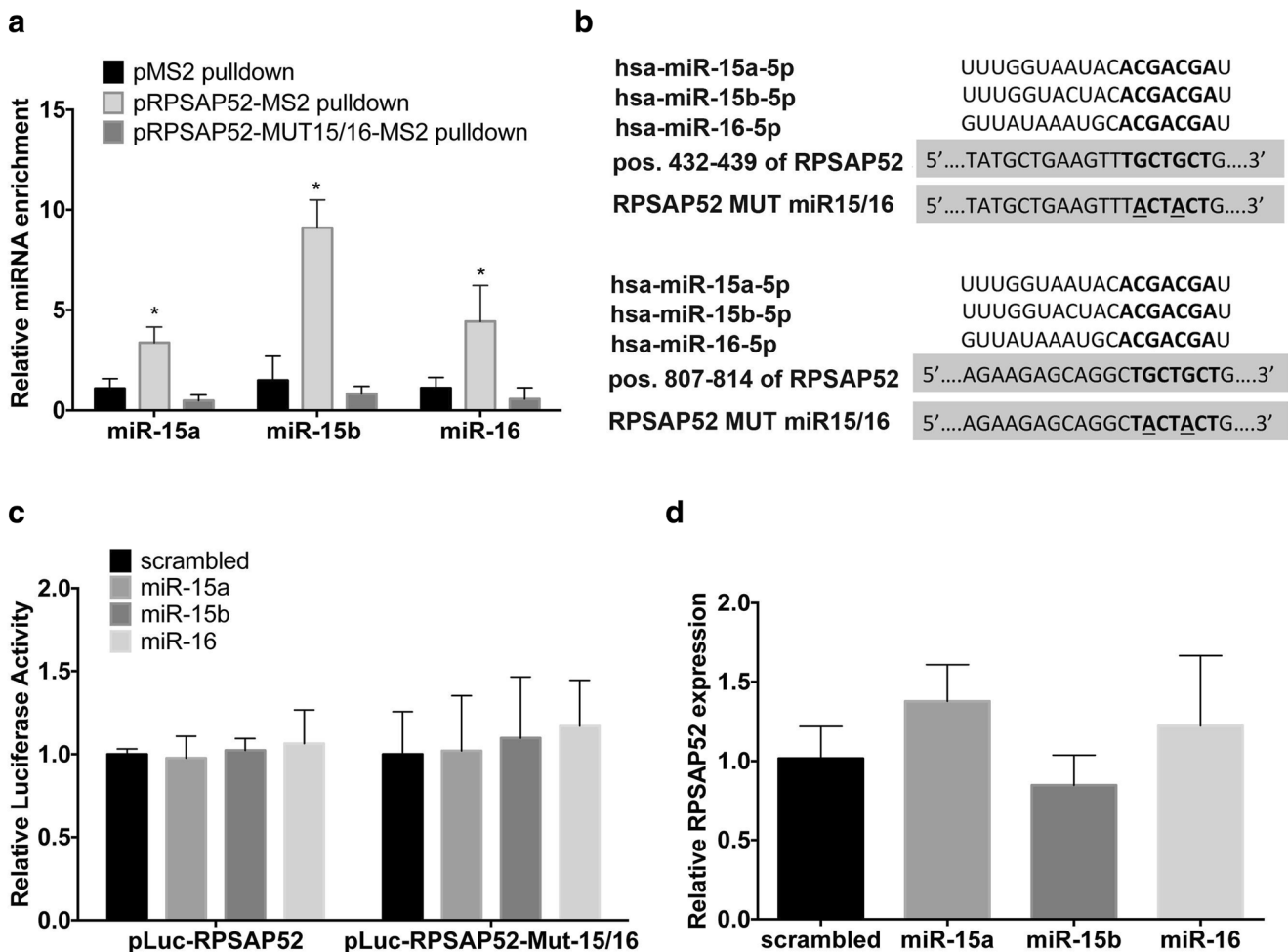


Fig. 5 miR-15a, miR-15b, and miR-16 bind RPSAP52 but do not induce RPSAP52 RNA degradation. **a** HT-29 cells transfected with pMS2, pRPSAP52-MS2, and pRPSAP52-MS2-MUT15/16 plasmids and used for pulldown analysis using GSH beads to test the miRNA binding on RPSAP52. The levels of miRNAs associated with each MS2-tagged RNA was measured by RT-qPCR analysis. **b** The seed sequences of miR-15a, miR-15b, and miR-16; the sites of the RPSAP52 lncRNA targeted by miR-15a, miR-15b, and miR-16; and the sites of the RPSAP52 carrying the deletion in miR-15a, miR-15b, and miR-16 recognition sites. **c** Luciferase

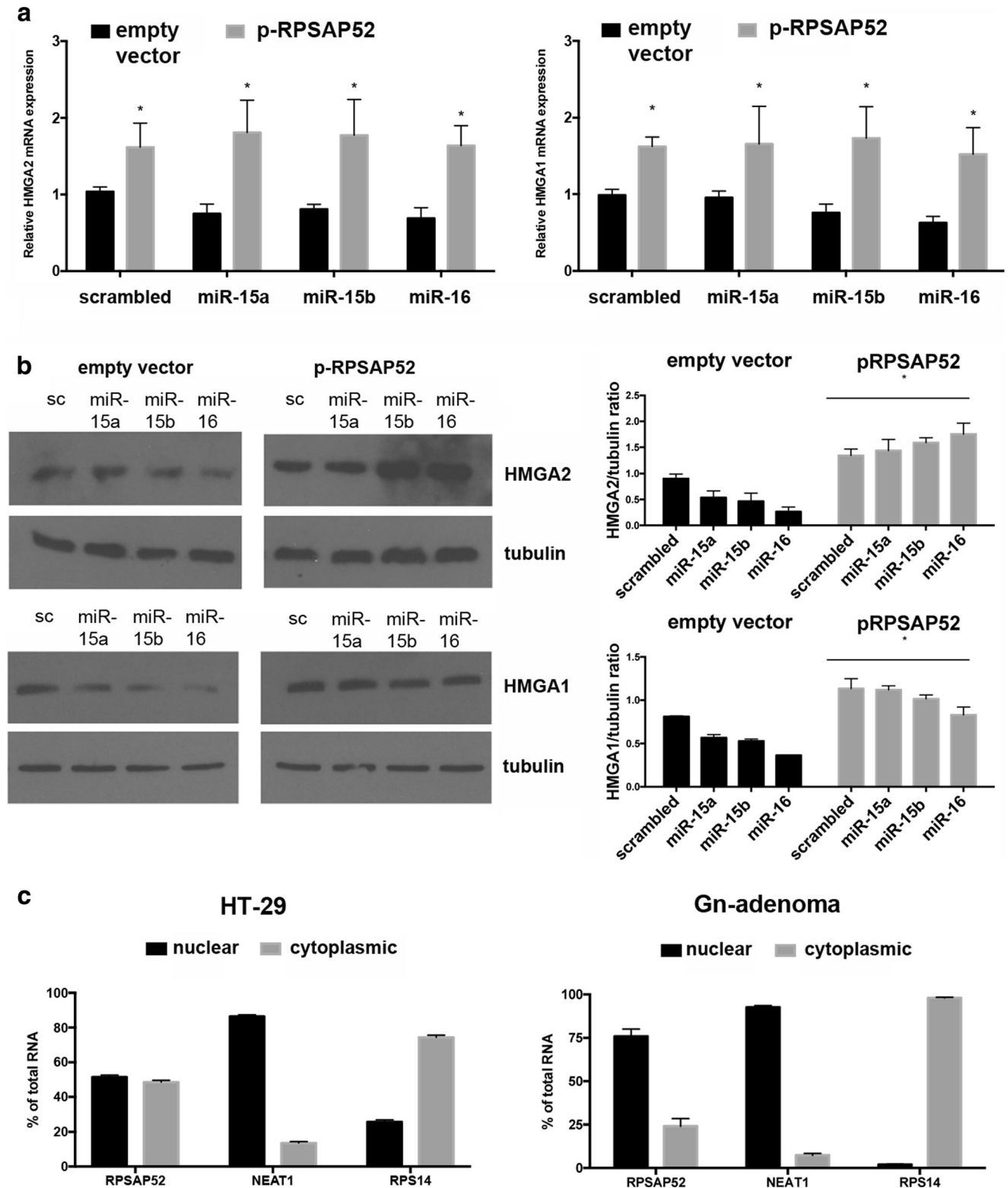
assay of HT-29 cells transfected with p-Luc-RPSAP52 and p-Luc-RPSAP52-MUT15/16 along with miR-15a, miR-15b, and miR-16 or with a scrambled oligonucleotide. The relative luciferase activity was standardized using Renilla luciferase as transfection control. The scale bars represent the mean \pm s.e. of three independent experiments performed in triplicate. **d** qRT-PCR analysis of RPSAP52 RNA levels in HT-29 cells transfected with miR-15a, miR-15b, and miR-16 or the relative scrambled. U6 RNA was used to normalize the RNA expression level. Each bar represents the mean value \pm s.e. from three independent experiments performed in triplicate

proteins to promote cell proliferation. Then, we looked for the molecular mechanisms by which RPSAP52 overexpression increases HMGA protein levels and may contribute to pituitary tumorigenesis. Since recent findings suggest that lncRNAs and their related coding genes can communicate through a ceRNA language [29, 35], we identified, by bioinformatic prediction of MREs (miRNA response elements) present in both RPSAP52 and *HMGAs*, the seed sequences for miR-15a, miR-15b, and miR-16, previously demonstrated to target HMGA1 and HMGA2 proteins [12]. We confirmed the specific binding of such miRNAs to RPSAP52 performing a RNA pulldown assay and demonstrated that the presence, on RPSAP52, of miR-15a, miR-15b, and miR-16-targeting sequences is required for the RPSAP52 biological effects. In fact, the mutagenesis of these sites prevented the binding of

Fig. 6 RPSAP52 increases HMGA protein levels by a miRNA “sponge” mechanism. **a** qRT-PCR analysis of *HMG2* and *HMG1* RNA levels in HT-29 cells transfected with miR-15a, miR-15b, and miR-16 and the negative oligonucleotide together with pRPSAP52 expression vector or the relative empty vector. G6PD RNA was used to normalize the RNA expression level. Each bar represents the mean value \pm s.e. from three independent experiments performed in triplicate. * $p < 0.05$ of RPSAP52-transfected HT-29 cells compared with HT-29-empty vector-transfected cells. **b** Western blots analysis of the HMGA2 and HMGA1 protein expression level in the same cells of **a**. β -Actin expression was analyzed as loading control. Densitometric analysis performed using ImageJ software and normalizing to β -actin is reported on the right. The error bars represent the mean value \pm SD. * $p < 0.05$ of RPSAP52 transfected HT-29 cells compared with HT-29-empty vector-transfected cells. **c** Subcellular distribution of RPSAP52 in HT-29 cells (left panel) and in a gonadotroph adenoma tissue sample (right panel). Cytoplasmic and nuclear RNA levels were normalized to NEAT1 (control for nuclear fraction) and RP14 (control for cytoplasmic fraction) respectively

these miRNAs to RPSAP52 and inhibited its biological effects. Then, we demonstrated the ability of RPSAP52 to increase HMGA2 and HMGA1 protein levels through a miRNA sponge function, as supported by its partial localization in the cytoplasm. Interestingly, a ceRNA-sponge mechanism has

been recently reported by our group for the HMGA1 pseudogenes *HMGA1P6* and *HMGA1P7* that protect *HMGA*-specific mRNAs from miRNAs able to target the *HMGA* genes, and are highly overexpressed in growth hormone and nonfunctioning PAs [35, 36]. Thus, the results



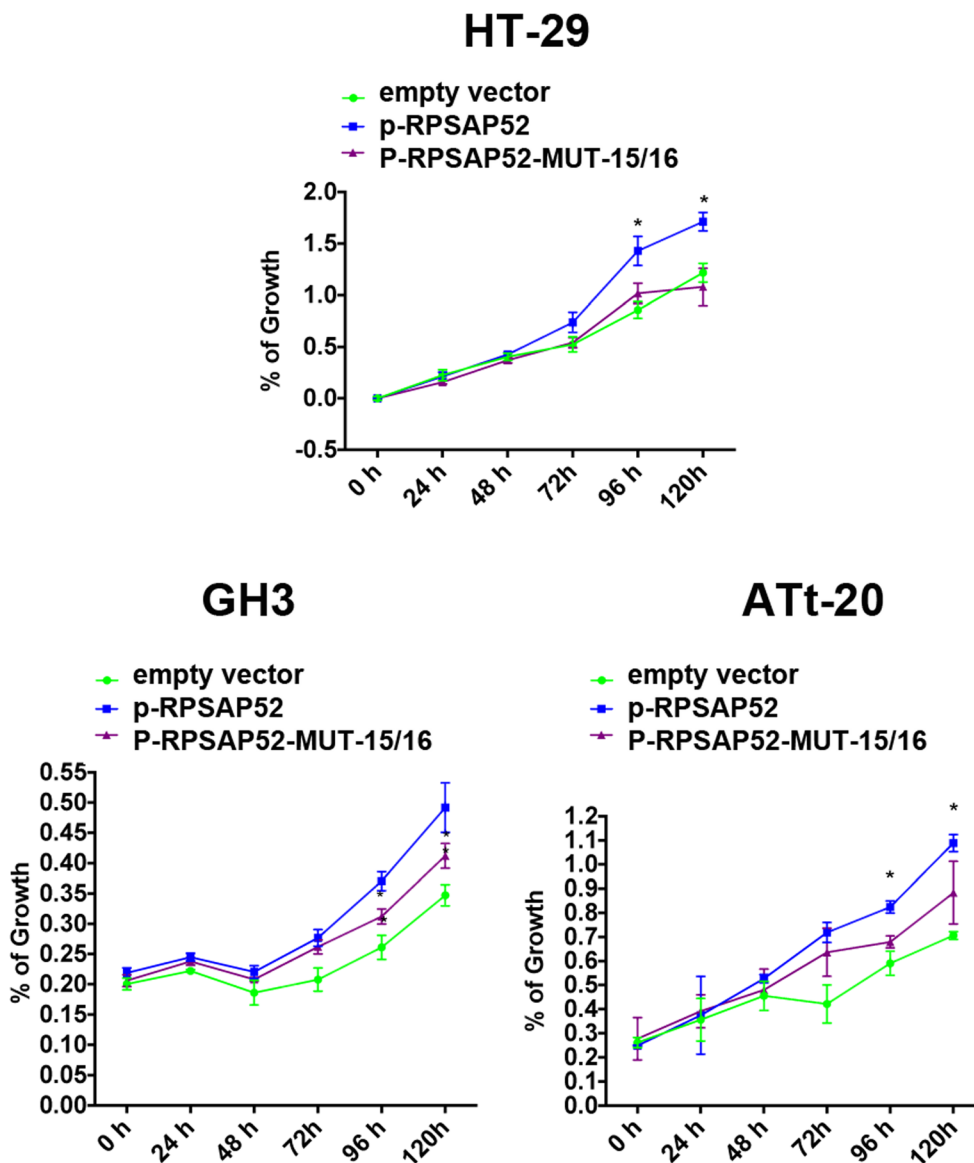


Fig. 7 RPSAP52 mutated in miRNA-binding sites confers less growth ability compared with RPSAP52 wild-type transfected cells. Cell growth curve of HT-29, GH3, and ATt-20 cells transfected with p-RPSAP52, p-RPSAP52-MUT-15/16, or empty vector counted each 24 h for 120 h after

plating. The mean values \pm s.d. derive from two independent experiments performed in triplicate. Significance values $*p < 0.05$ relative to cells transfected with empty vector or negative control

reported here further support the view of the activation of several pathways in pituitary tumors leading to deregulation of cell cycle, and in particular of the G1-S transition. Indeed, we have previously demonstrated the downregulation of miRNAs that are able to target HMGA and/or E2F1 [12]. On the other hand, the HMGA proteins per se are able to enhance E2F activity [14], cyclin E expression [37], and cyclin B expression [38]. Interestingly, our preliminary results demonstrate that RPSAP52 contributes to HMGA1 and HMGA2 overexpression in pituitary tumors, by a mechanism of action that is not limited to the miRNA sponge activity.

Therefore, these results indicate the existence of a complex network involving RNA-binding proteins (RBPs), miRNAs,

and coding genes whose combinatorial effects would act at transcriptional and post-transcriptional levels to enhance the G1-S transition in PAs playing a critical role in the development of these neoplasias (D'Angelo, unpublished results), as also suggested by several reported evidences [30].

It is noteworthy that lncRNA expression profile of the gonadotroph adenomas also revealed some other interesting lncRNAs dysregulated in pituitary tumors. Among the downregulated lncRNAs, we found the X-inactive specific transcript (XIST) RNA, a mammalian lncRNA that is required for the X chromosome inactivation (XCI) process in females. Reactivation of X-linked genes, due to the loss of XIST in adult tissues, has been

found in cancer cells [39], in particular in female malignancies [40], where the involvement of XIST in gene expression changes and heterochromatin stability has been proposed. Among the upregulated genes, we found also RP11-500G22.2, the lncRNA natural antisense of the gene Arginyltransferase 1 (*Ate1*), catalyzing protein arginylation, a post-translation modification involved in stress response, which often leads to cell cycle arrest or cell death [41]. Further studies of these transcripts with unclear function in pituitary gland will likely contribute to the comprehension of the mechanisms underlying the development of pituitary tumors.

Moreover, studies are in progress in our laboratory to verify whether RPSAP52 overexpression is a general event in tumor progression as it has been widely described for the HMGA proteins [32]. Preliminary results suggest a correlation between RPSAP52 overexpression and thyroid cancer progression, since a gradual increase in RPSAP52 expression tightly associated with HMGA1 and HMGA2 protein expression has been observed in the progression from the differentiated papillary and follicular carcinomas to the highly invasive and undifferentiated anaplastic histotype. These results are consistent with the ability of RPSAP52 to enhance the transcription of several genes involved in the epithelial-mesenchymal transition (D'Angelo, unpublished results). However, further studies are required to evaluate RPSAP52 expression in other neoplastic tissues and define its role in human malignancies.

In conclusion, our results clearly show a drastic lncRNA deregulation in PAs and propose the lncRNA RPSAP52 as a novel critical player in the development of human PAs enhancing the expression of the HMGA1 and HMGA2 oncogenic proteins.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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