RESEARCH

Protein stabilization by RSUME accounts for PTTG pituitary tumor abundance and oncogenicity

M Fuertes[1], M Sapochnik[1], L Tedesco[1], S Senin[1], A Attorresi[1], P Ajler[2], G Carrizo[2], A Cervio[3], G Sevlever[3], J J Bonfiglio[1], G K Stalla[4] and E Arzt[1,5]

[1]Instituto de Investigación en Biomedicina de Buenos Aires (IBioBA) – CONICET – Partner Institute of the Max Planck Society, Buenos Aires, Argentina
[2]Servicio de Neurocirugía, Hospital Italiano, Buenos Aires, Argentina
[3]Departamento de Neurocirugía, Fundación Para la Lucha Contra las Enfermedades Neurológicas de la Infancia (FLENI), Buenos Aires, Argentina
[4]Department of Clinical Research, Max Planck Institute of Psychiatry, Munich, Germany
[5]Departamento de Fisiología y Biología Molecular y Celular, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Buenos Aires, Argentina

Correspondence should be addressed to E Arzt: earzt@ibioba-mpsp-conicet.gov.ar

Abstract

Increased levels of the proto-oncogene pituitary tumor-transforming gene 1 (PTTG) have been repeatedly reported in several human solid tumors, especially in endocrine-related tumors such as pituitary adenomas. Securin PTTG has a critical role in pituitary tumorigenesis. However, the cause of upregulation has not been found yet, despite analyses made at the gene, promoter and mRNA level that show that no mutations, epigenetic modifications or other mechanisms that deregulate its expression may explain its overexpression and action as an oncogene. We describe that high PTTG protein levels are induced by the RWD-containing sumoylation enhancer (RWDD3 or RSUME), a protein originally identified in the same pituitary tumor cell line in which PTTG was also cloned. We demonstrate that PTTG and RSUME have a positive expression correlation in human pituitary adenomas. RSUME increases PTTG protein in pituitary tumor cell lines, prolongs the half-life of PTTG protein and regulates the PTTG induction by estradiol. As a consequence, RSUME enhances PTTG transcription factor and securin activities. PTTG hyperactivity on the cell cycle resulted in recurrent and unequal divisions without cytokinesis, and the consequential appearance of aneuploidies and multinucleated cells in the tumor. RSUME knockdown diminishes securin PTTG and reduces its tumorigenic potential in a xenograft mouse model. Taken together, our findings show that PTTG high protein steady state levels account for PTTG tumor abundance and demonstrate a critical role of RSUME in this process in pituitary tumor cells.

Key Words
- pituitary tumors
- PTTG
- RSUME

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Introduction

Considerable progress has been achieved in identifying mechanisms/factors that are involved in pituitary tumor initiation and progression (Dworakowska & Grossman 2012, Perez-Castro *et al.* 2012, Gatto *et al.* 2013, Farrell 2014). Despite their high prevalence in the general population (Beckers 2010, Fernandez *et al.* 2010), these tumors are invariably benign and exhibit features of differentiated pituitary cell function as well as premature

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proliferative arrest (Melmed 2011, Sapochnik *et al.* 2017). Most pituitary adenomas develop sporadically, and only a small proportion of pituitary adenomas have a hereditary background. Disturbed cell-cycle regulator expression and action resulting in aberrant cell-cycle control is often observed in pituitary tumors (Clayton & Farrell 2004, Quereda & Malumbres 2009, Drouin *et al.* 2010).

Originally isolated from rat pituitary tumor cells (Pei & Melmed 1997), PTTG was subsequently identified as a member of the securin family of proteins (Zou *et al.* 1999). PTTG, a multifunctional protein, is a transcription factor and a well-established cell-cycle regulator (Ramos-Morales *et al.* 2000, Tong *et al.* 2007, Tong *et al.* 2008), with functions in cell replication (Zou *et al.* 1999), DNA damage/repair (Romero *et al.* 2001), organ development, metabolism, cell transformation (Wang *et al.* 2001, Cancer Genome Research Network 2003) and cell senescence (Chesnokova *et al.* 2007, 2008). PTTG has been identified in human tissues with a low expression in normal cells, but overexpression in a wide variety of human solid tumors, especially in endocrine-related tumors such as thyroid, pituitary, breast, ovarian and uterine carcinomas, and also non-endocrine tumors such as pulmonary, gastrointestinal and renal cell carcinomas, malignancies of the CNS, melanoma and blastic leukemia (Dominguez *et al.* 1998, Heaney *et al.* 2000, Solbach *et al.* 2004, Ogbagabriel *et al.* 2005, Ito *et al.* 2008, Tong & Eigler 2009). Its expression positively correlates with tumor invasiveness and recurrence (Zhang *et al.* 1999*a*, Boelaert *et al.* 2003, Amousha *et al.* 2015).

The product of the RWDD3 gene, RSUME, was identified by differential expression also in rat pituitary tumor cells (Carbia-Nagashima *et al.* 2007). RSUME increases protein sumoylation (Carbia-Nagashima *et al.* 2007), a dynamic post-translational modification that regulates, like protein ubiquitination (Ciechanover 1994), many key cellular processes (Hay 2005, Geiss-Friedlander & Melchior 2007). RSUME is expressed in several normal tissues and is induced by heat shock (Druker *et al.* 2013) and hypoxia (Carbia-Nagashima *et al.* 2007, Gerez *et al.* 2013, 2015, He *et al.* 2017). It is differentially expressed in cells with high tumoral and angiogenic potential (Carbia-Nagashima *et al.* 2007, Shan *et al.* 2012, Gerez *et al.* 2013, 2015, He *et al.* 2017) and has been associated with the gene expression signature of breast cancer patients (Huang *et al.* 2013, Schneider *et al.* 2015) and decreased survival in a group of renal cell carcinoma patients (Cancer Genome Research Network 2003, Gao *et al.* 2013).

No mutations, epigenetic modifications or other mechanisms that explain high PTTG expression and action as oncogene have been described so far. The aim of the study was to identify causes that modify PTTG abundance. We demonstrate in pituitary cells and xenograft transplanted animals that RSUME increases PTTG protein stability, altering its transcription factor and securin activity, resulting in recurrent cell division without cytokinesis and tumor development. Together, these results reveal the regulation of PTTG abundance at the protein level and the interplay of RSUME and PTTG in tumor development of pituitary gland.

Materials and methods

Unless otherwise stated, reagents were obtained from Life Technologies/Thermo Fisher Scientific or Sigma-Aldrich/Merck.

Plasmids and cloning

The following plasmids were kindly provided by: pCEFL, O Coso; Gam1 wt and mut, S Chiocca; c-MYC-LUC, K Mitsuyasu; cycD3-LUC, K Ohtani; CMV-βgal, D Spengler. V5-RSUME and RSUME-Luc were obtained as described (Carbia-Nagashima *et al.* 2007). HA-PTTG was constructed by cloning hPTTG1 cDNA into the Bgl II-Not I sites of the pCEFL expression vector. The nucleotide sequences of all constructs were confirmed by DNA sequencing.

Cell culture and stimulation

Cell lines AtT-20 (mouse corticotroph cell line), GH3 (rat lactosomatotroph cell line), GH4 (rat lactosomatotroph cell line) and COS-7 (African green monkey kidney fibroblast-like cell line) were acquired from ATCC, either directly or by colleagues. Cells were cultured in Dulbecco's modified Eagle's medium (pH 7.3) supplemented with 10% fetal bovine serum, 2.2 g/L $NaHCO_3$, 10 mmol/L HEPES, 2 mmol/L glutamine and 10^5 U/L penicillin/streptomycin, under a 5% CO_2 atmosphere at 37°C. AtT-20 or GH4 clones, generated as indicated in the following section, were cultured under the same conditions but adding Geneticin (G418) to the medium. For cycloheximide (CHX) stimuli, cells were cultured in medium with 100 µg/mL CHX for the indicated times. For estradiol (E2) stimuli, cells were cultured in DMEM without phenol red for a week at least. Cells were treated with 100 nM E2 for the indicated times.

Stable clones generation

AtT-20 or GH4 stable clones were obtained as described (Paez-Pereda *et al.* 2003). Cells were transfected

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with each shRNA plasmids directed against RSUME (5′-GGAGAAGTGGGCTTCAGATTT-3′) or Scramble (5′-GGAATCTCATTCGATGCATAC-3′) from SABiosciences (Hilden, Germany). Clones were selected as a pool with 600 µg/mL Geneticin and maintained with 300 µg/mL Geneticin. PTTG and RSUME expression levels were checked by WB.

Primary cell cultures

Mouse embryonic fibroblast (MEF) primary cell cultures were prepared from CF-1 embryonic day 13.5 (E13.5) embryos. The red organs of embryos were removed, single torsos were minced and cells dispersed by rotation for 30 min in 0.1% trypsin-EDTA solution at 37°C. Cells were washed once in PBS, taken up in DMEM containing 20% FBS, and plating for culture. MEF cells were maintained in DMEM with 20% FBS. Cells at passages 2 or 3 were plated at 100-mm dish, and transfected.

Testis primary cell cultures

Mouse fetal testis primary cell cultures were prepared from CF-1 embryonic day 18.5 (E18.5) embryos. The testes of male embryos were minced, and cells dispersed by rotation for 30 min in 0.05% collagenase at 37°C. Cells were washed once in HBSS (Gibco), taken up in DMEM containing 20% FBS, and plating for culture. Cells were plated at 100-mm dish, and transfected.

Tumor samples

Human pituitary adenoma tissues were obtained from the Neurosurgery Service, Hospital Italiano, Argentina and Neurosurgery Department, FLENI, Argentina. This study complies with the June 1964 Declaration of Helsinki and has been approved by the hospital ethics committee; informed written consent was received from each patient whose tumor tissue was used in the study. A portion of the tumor tissue, after transsphenoidal surgery, was received in sterile medium and treated with standard cracking buffer. A total of 23 pituitary adenomas were diagnosed according to clinical, biochemical, radiological and surgical findings as well as by routine immunohistochemistry and were classified as somatotrophinomas ($n=4$), corticotrophinomas ($n=6$), prolactinomas ($n=3$), plurihormonal adenoma ($n=1$), non-functioning adenomas ($n=6$) and gonadotrophinomas ($n=3$), with the last used for aneuploidy studies. For these aneuploidy analysis, tumor cell cultures were performed as described (Paez-Pereda *et al.* 2003) and incubated in 5 µg/mL Colcemid for 24 h. Further, chromosome analysis was made.

Mouse tissue samples

Mouse pituitary, kidney and testis were dissected from CD-1 mice, put in a tube with standard cracking buffer for proteins, homogenized by ULTRA-TURRAX dispersers PRO sc-250 (IKA) and sonicated for 10 s to 20% potency. For pituitary tissue, we polled 3 animals.

Transfections

Cell transfection with siRNA against RSUME or scramble (Carbia-Nagashima *et al.* 2007) was performed using Lipofectamine 2000; COS-7 and GH4 cells were transfected using Lipofectamine 2000; AtT-20 and GH3 were transfected using Lipofectamine; MEFs cells and mouse fetal testis primary cell cultures were transfected using calcium phosphate method. Transfections with Lipofectamine reagent were performed following the manufacturer's instructions.

Western blot

48 h post-transfection, cell lysates were prepared in standard cracking buffer and boiled for 5 min at 95°C. Cell extracts were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) as previously described (Paez-Pereda *et al.* 2003). Proteins were blotted onto nitrocellulose membranes using standard procedures, and incubated with antibodies to PTTG (34-1500, 1:500) from Zymed; V5 (ab9116, 1:3000), RWDD3 (ab128285, 1:1000) and GAPDH (ab8245, 1:10,000) from Abcam; β-actin (C4 sc-47778, 1:3000) from Santa Cruz Biotechnology.

Immunoprecipitation

48 h post transfection, cells were washed with PBS, lysed on ice with modified RIPA buffer and immunoprecipitated with anti-HA (Covance) antibody as described (Paez-Pereda *et al.* 2003). Mock corresponds to the condition with all the plasmids transfected and immunoprecipitated with unspecific IgG of the same isotype of the antibody used to precipitate.

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Luciferase assay

Cells were harvested and luciferase activity was measured as previously described (Carbia-Nagashima *et al*. 2007).

Chromosome analysis

24 h post transfection, MEF cells were treated with 5 µg/mL Colcemid (to obtain mitotic cells, Metaphase) or serum starvated (to obtain cells in interphase) for 24 h, collected, hypotonized with 0.075 M KCl and fixed with cold methanol/acetic 3:1. Fixed cells were processed by standard cytogenetic procedures with 5% Giemsa or 1 µg/mL DAPI staining. Chromosome number and gross rearrangements were determined in at least 50 metaphase or interphase cells.

Immunofluorescence

For multinucleated cell experiments, MEF cells were analyzed by immunofluorescence using anti-Vimentin VI-10 antibody (Abcam) with Alexa Fluor 647-conjugated secondary antibody; nuclei were stained with Hoescht. For mitotic bridge, MEF cells were analyzed using anti-α-Tubulin TU-01 antibody (Abcam) with Alexa Fluor 647-conjugated secondary antibody; nuclei were stained with DAPI. Images were captured by an inverted AxioObserver Z1 LED Colibrí microscope (Carl Zeiss).

Proliferation assay

5×10^3 cells were plated and 72 h later, a WST-1 assay (Roche) was used to measure viability and proliferation following the manufacturer's instructions. The reaction products were measured in an ELISA plate reader at 450 nm, as previously described (Paez-Pereda *et al*. 2003).

Xenograft assay in nude mice

AtT-20 or GH4 derived clones were harvest by trypsinization, washed twice with PBS, resuspended in DMEM and injected subcutaneously, as described (Paez-Pereda *et al*. 2003), into the flanks of 6- to 8-week-old male nude mice (strain N:NIH (S)-FoxnInu), obtained from Fundación Facultad de Ciencias Veterinarias, National University of La Plata, Argentina. Twelve mice were injected with 3×10^6 cells of AtT-20 (6 for shRSUME and 6 for shSCRAMBLE) or 2×10^6 cells of GH4 (6 for shRSUME and 6 for shSCRAMBLE) in two independent experiments

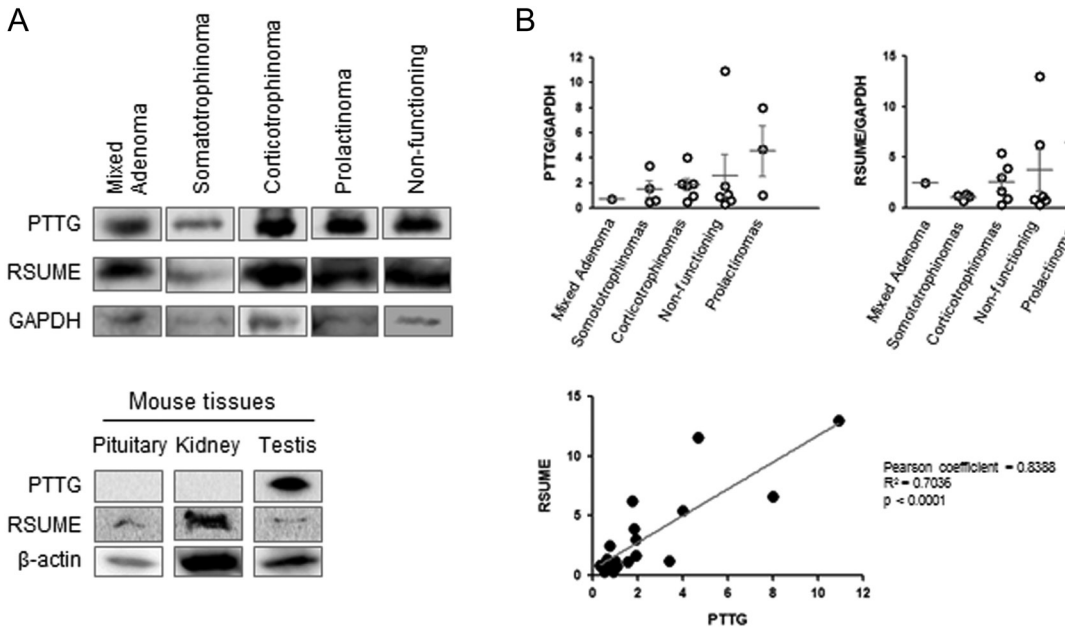

Figure 1
RSUME and PTTG are co-expressed in human pituitary tumors. (A) Representative Western blot (WB) analysis in samples of each type of human pituitary tumors. GAPDH was used as loading control. Normal mouse tissues as negative or positive controls of PTTG or RSUME expression are shown in the bottom panel. (B) Graphs show ratio of intensity between PTTG or RSUME and GAPDH for each group of tumors (1 mixed adenoma, 4 somatotrophinomas, 6 corticotrophinomas, 6 non-functioning adenomas and 3 prolactinomas). Graph of correlation analysis between PTTG and RSUME expression; Pearson correlation with Pearson coefficient = 0.8388, $R^2 = 0.7036$, $P < 0.0001$.

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of six animals for each cell clone. Animals were examined for tumor formation every 3 days and tumor growth was determined as described (Paez-Pereda *et al.* 2003). All experimental protocols were approved by the Ethical Committee on Animal Care and Use (CICUAL), University of Buenos Aires, Argentina.

Statistical analysis

Statistics were performed by analysis of variance (ANOVA) in combination with the Scheffé's test. Data are shown as mean ± S.E.M. Correlations between variables were evaluated by Pearson's correlation. Statistical significance was accepted at $P<0.05$.

Results

We first examined a potential relationship between PTTG and RSUME, both originally cloned from the same cell type (Pei & Melmed 1997, Carbia-Nagashima *et al.* 2007). We noticed that RSUME and PTTG are co-expressed in explants of different types of human pituitary tumors and the expression correlates significantly (Fig. 1A and B). Normal mouse pituitary and kidney tissues, negative controls not expressing PTTG (Pei & Melmed 1997, Zhang *et al.* 1999*b*) but RSUME (Carbia-Nagashima *et al.* 2007), are shown (Fig. 1A).

RSUME overexpression increases PTTG protein steady state levels, both in transfected COS-7 cells that do not express PTTG and in pituitary tumor cell line GH4 that do express PTTG endogenously (Fig. 2A). Also, in primary cultures of mouse fetal testis, a known tissue that expresses high levels of PTTG (Pei 1999), RSUME increases PTTG protein levels (Fig. 2B). Consequently, RSUME knockdown with a specific small interfering RNA (siRNA) has the opposite effect on PTTG protein levels (Fig. 2C). In cells in which PTTG protein is not present, such as non-transfected COS-7 cells in Fig. 2A and normal mouse pituitary and kidney tissues in Fig. 1A, RSUME has no action on PTTG. To ascertain that protein stability accounts for the increase in PTTG levels, transfected COS-7 cells were treated for different times with cycloheximide; after treatment, PTTG protein expression levels declined, and RSUME slowed the decay and increased the half-life of PTTG in 50% (from 2 to 4 h) (Fig. 2D). In accordance with PTTG mRNA (Heaney *et al.* 1999), PTTG protein levels increased after the addition of 100-nM estradiol (E2) and downregulation of RSUME resulted in the blockage of this time-dependent induction by E2 (Fig. 2E). As RSUME is a sumoylation enhancer, we analyzed the PTTG

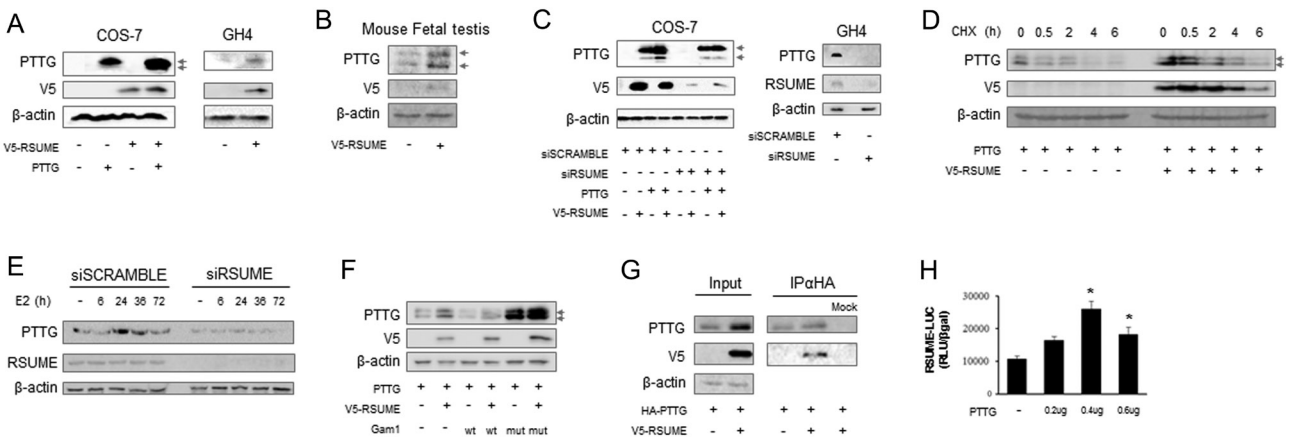

Figure 2

RSUME causes PTTG protein stabilization. (A) Representative WB analysis of COS-7 or GH4 cells transfected with a RSUME-expressing or empty vector ($n=3$). COS-7 cells were also transfected with a PTTG-expressing plasmid. (B) Representative WB analysis in primary cultures of mouse fetal testis at embryonic day 18.5 transfected with a RSUME-expressing or empty vector ($n=2$). (C) Representative WB analysis of COS-7 or GH4 cells transfected with a small interference RNA (siRNA) against RSUME or Scramble ($n=3$). COS-7 cells were also transfected with PTTG and/or RSUME-expressing plasmids. (D) Representative WB analysis of COS-7 cells transfected with PTTG- and/or RSUME-expressing vectors and treated with cycloheximide (CHX) (100 µg/mL) for the indicated times ($n=4$). (E) Representative WB analysis of GH3 cells transfected with an siRNA against RSUME or Scramble, and stimulated with estradiol (E2) (100 nM) for the indicated times ($n=3$). (F) Representative WB analysis of COS-7 cells transfected with PTTG, RSUME and Gam1 wt or mutant-expressing vectors ($n=4$). (G) Representative WB analysis for PTTG and RSUME after immunoprecipitation (IP) of HA-PTTG, with anti-HA, from COS-7 cells transfected with PTTG and/or RSUME ($n=3$). (H) Luciferase activity of COS-7 cells transfected with 0.2–0.6 µg PTTG-expressing vector and RSUME reporter vector ($n=3$). First column are cells transfected with the corresponding empty vectors. Error bars represent mean ± S.E.M. *$P<0.05$ compared with cells with empty vector (ANOVA with Scheffé's test). In WB, β-actin was used as loading control. Arrows, phosphorylated (upper) and unphosphorylated (lower) forms of PTTG. Note that in some cells, the increase in protein stability was observed also in the phosphorylated (active) PTTG, probably due to the expression level attained, which is not observed at endogenous levels in GH4 cells.

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stability in the presence of Gam1 (a viral enzyme that inhibits sumoylation (Boggio *et al*. 2004)) and we found diminished protein levels of PTTG, even in the presence of RSUME (Fig. 2F). With a Gam1 mutant that lacks activity on the SUMO-pathway (Boggio *et al*. 2004), this effect was reversed (Fig. 2F). Analyzing *in silico* the sequence of PTTG looking for potential sumoylation sites with the software SUMOplot and SUMOsp, we obtained that human PTTG1 protein has many potentially sumoylable lysines, but both software predicted that the lysine with greater likelihood of sumoylation is K168, which is part of a nonconsensus sumoylation site (sequence -VKMP-). The SUMOsp software also predicts that residues 4–8 constitute a SUMO interaction motif.

We observed by co-inmunoprecipitation that PTTG and RSUME proteins interact (Fig. 2G), which provides the structural basis for the action of RSUME on PTTG stability. Interestingly, PTTG increases the induction of RSUME (Fig. 2H).

To understand the functional consequences of PTTG stability, we initially studied PTTG transcription factor activity. In correspondence to increased levels of endogenous PTTG protein, an increment on promoter activation of c-MYC (Fig. 3A) and cyclin D3 (Fig. 3B) is observed in response to increasing amounts of RSUME. At high RSUME concentrations, without exogenous PTTG, we observed an increase of c-MYC activation concomitant with an endogenous PTTG protein increase. Downregulation of RSUME resulted in a significant decrease in the transcriptional activity of PTTG on both promoters (Fig. 3C).

Second, we investigated the action of RSUME on the function of PTTG as securin. For this, we used primary cultures of MEF given the impossibility to study aneuploidy in cell lines. MEF cells transfected with empty vector showed a normal appearance in interphase, count of $2n=40$ chromosomes in metaphase (Fig. 4A, Vectors), and very few binucleated cells (Fig. 4B, Vectors), while MEF cells co-expressing PTTG and RSUME, in concurrence with the observed PTTG abundance (Fig. 4C), resulted in increased genomic instability. We observed an increase in the frequency of aneuploid cells (Fig. 4A), in the detection of chromosomal abnormalities (micronuclei, chromosome fusions, mitotic bridge) (Fig. 4A), and in the frequency of occurrence of binucleated cells (Fig. 4B). Notably, some cells from human pituitary tumor samples had aneuploidies (Fig. 4D).

In a corticotroph cell line AtT-20, RSUME overexpression increases PTTG protein steady state levels (Fig. 5A). We established stable shRSUME clones of the pituitary cell lines AtT-20 and GH4 and confirmed the silencing of RSUME (shRSUME) and consequent decreased levels of PTTG protein, compared to shSCRAMBLE clones (Fig. 5B). shRSUME clones presented a significant inhibition of cell proliferation with respect to the parental cells and shSCRAMBLE clones (Fig. 5C). Nude mice injected with AtT-20 shRSUME stable clones did not develop tumors compared to mice injected with AtT-20 shSCRAMBLE clones (Fig. 5D). Meanwhile, the GH4 shRSUME clones developed slower growing tumors than GH4 shSCRAMBLE clones (Fig. 5D). In AtT-20 and GH4 shRSUME clones in which PTTG was re-expressed,

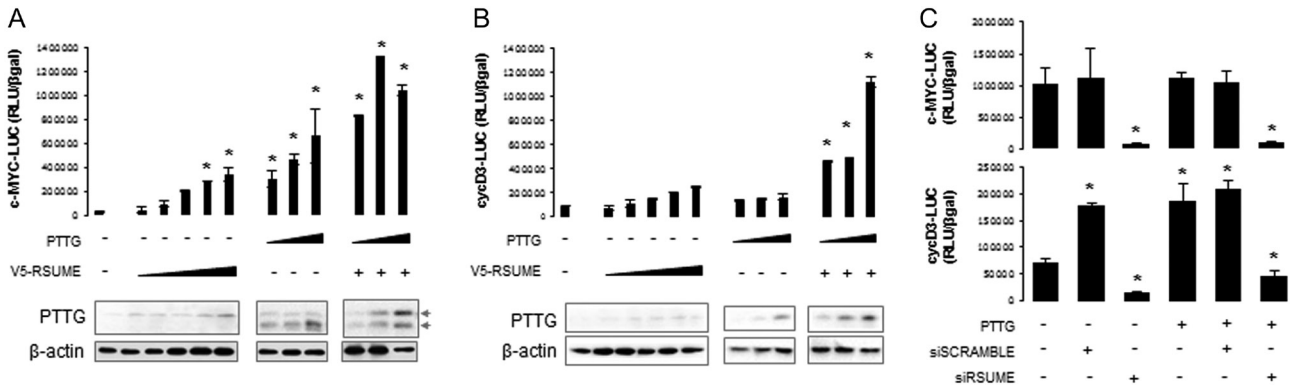

Figure 3
RSUME increases PTTG transcription factor activity. (A and B) Luciferase activity of GH4 cells transfected with 0.1–0.5 µg PTTG and/or 0.1–0.9 µg RSUME-expressing vectors, for c-MYC (A) or cycD3 (B) reporter vector ($n=3$). WB of PTTG expression is shown, in which arrows indicate phosphorylated (upper) and unphosphorylated (lower) forms of PTTG. Error bars represent mean ± S.E.M. (C) Luciferase activity of GH4 cells transfected with siRNA against RSUME or Scramble, and/or PTTG-expressing vector ($n=3$). First column are cells transfected with the corresponding empty vectors. Error bars represent mean ± S.E.M. *$P<0.05$ compared with cells with the corresponding empty vector (ANOVA with Scheffé's test).

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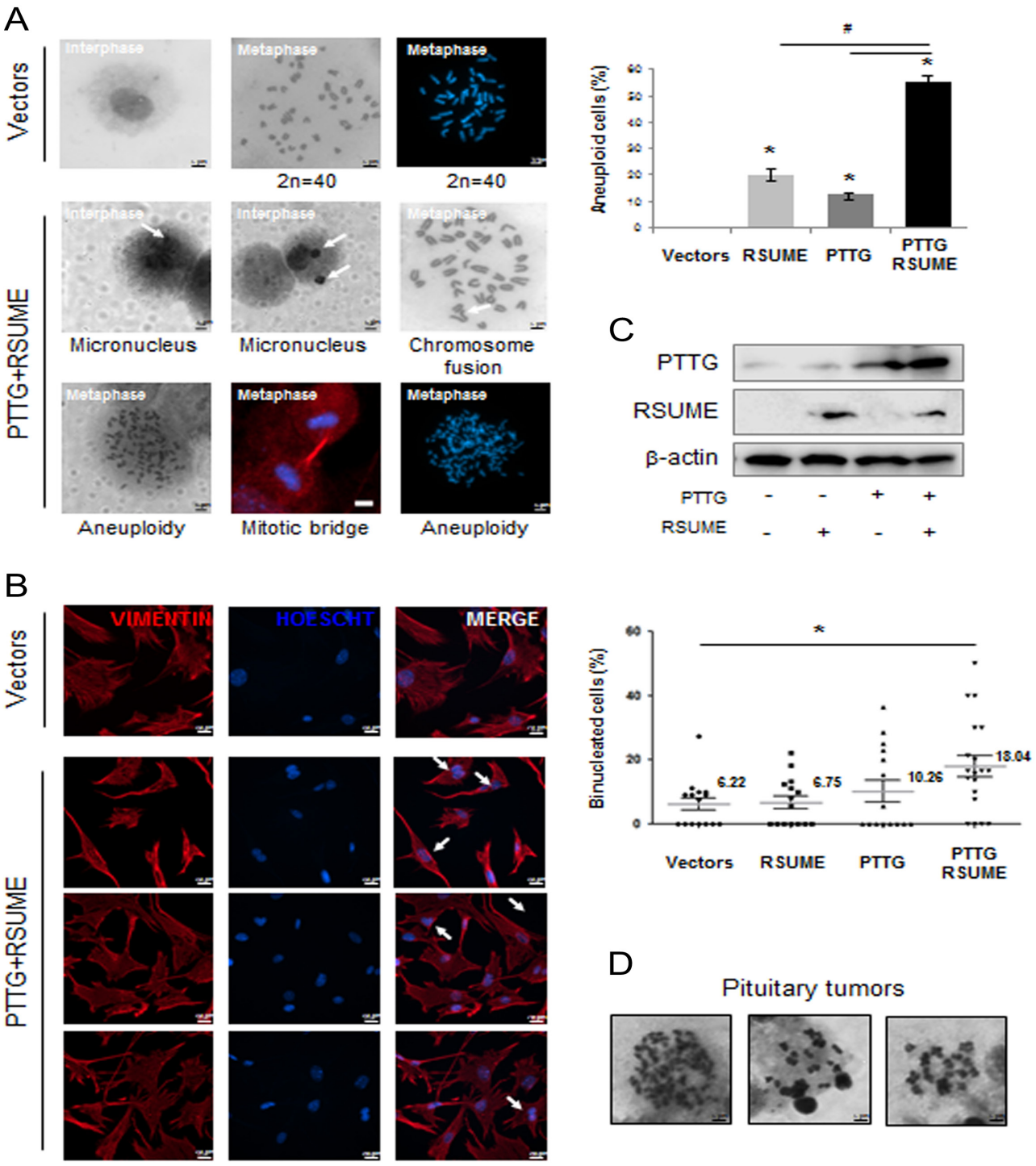

Figure 4

RSUME regulates PTTG securin function. (A) Representative cytochemistry images showing staining of Giemsa (5%) or DAPI (1 μg/mL) in MEF cells transfected with PTTG- and RSUME-expressing plasmids or empty vectors, and arrested on interphase or metaphase. Representative image of mitotic bridge was obtained by immunohistochemistry of α-tubulin (red) and DAPI (1 μg/mL) (blue). Scale bar, 5 μm. Graph shows quantification of percentage of aneuploidy cells ($n=3$). Arrow, chromosome abnormalities (B) Representative immunofluorescence images showing staining of Vimentin (red) and Hoescht (blue) in MEF cells transfected with PTTG- and RSUME-expressing plasmids or empty vectors ($n=3$). Scale bar, 20 μm. Arrow, binucleated cells. Graph shows quantification of percentage of binucleated cells per field. For all the experiments: $^*P<0.05$ compared with cells with the corresponding empty vector (ANOVA with Scheffé's test). (C) Representative WB analysis of MEF cells transfected with PTTG- and RSUME-expressing plasmids or empty vectors ($n=2$). β-actin was used as loading control. (D) Representative chromosome images showing staining of 5% Giemsa in human pituitary tumor samples (3 gonadotrophinomas). Scale bar, 5 μm.

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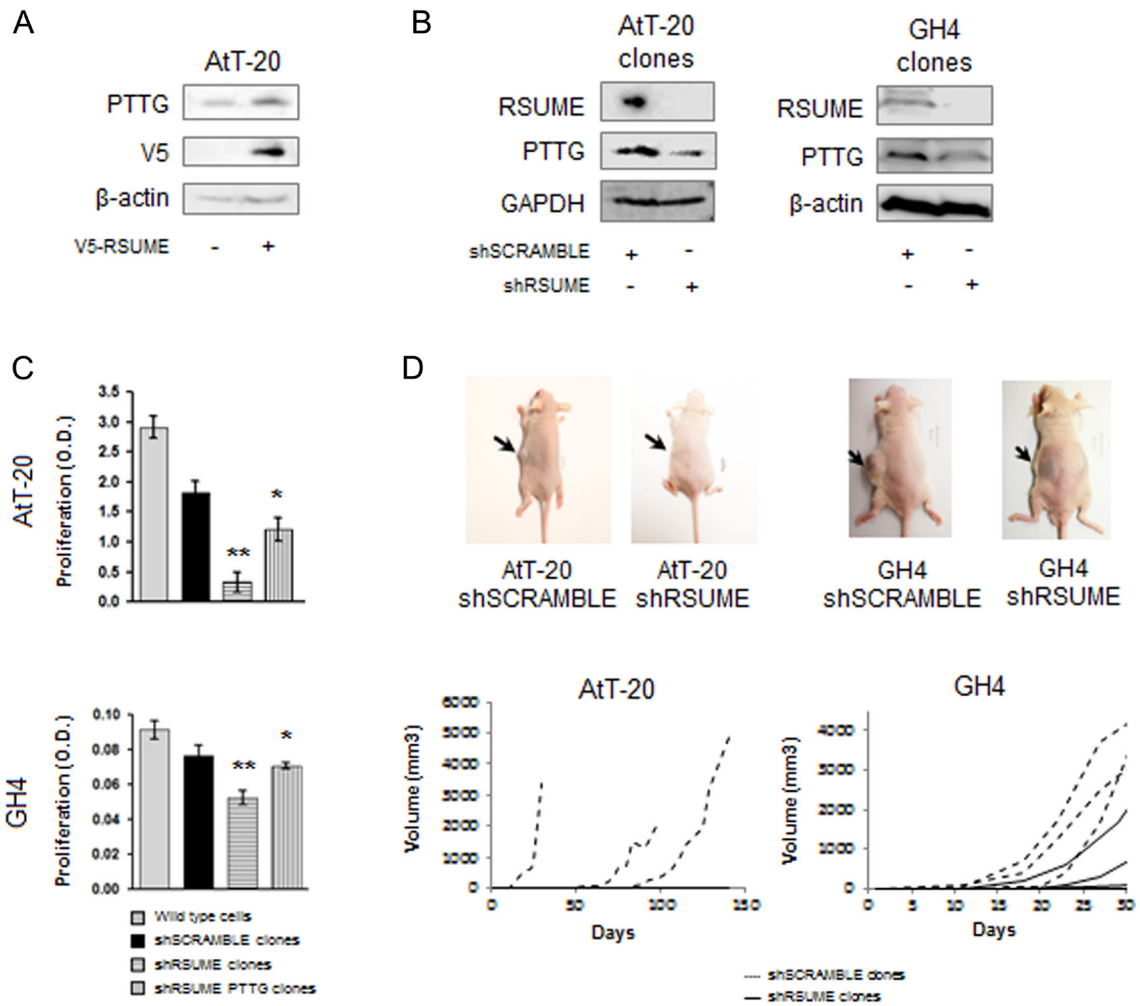

Figure 5
RSUME knockdown abolishes tumorigenic and proliferative capacity of PTTG. (A) Representative Western blot (WB) analysis of AtT-20 cells transfected with a RSUME-expressing or empty vector ($n=3$). (B) Representative WB analysis of AtT-20 or GH4 stable clones ($n=3$). (C) Wst-1 cell proliferation assay of AtT-20 or GH4 stable clones. Error bars represent mean ± S.E.M.($n=3$). shRSUME PTTG, clones transfected with PTTG-expressing vector. $^{**}P<0.05$ compared with wild type or shSCRAMBLE cells; $^{*}P<0.05$ compared with shRSUME cells (ANOVA with Scheffé's test). (D) Xenograft tumor assay in male athymic nude mice injected subcutaneously with AtT-20 or GH4 stable clones. Representative images were taken at 60 days (AtT-20) or 20 days (GH4) after injection. Graph shows tumoral volume vs days after injection. One of two independent experiments with 3 mice in each group in each experiment with similar results is shown ($n=12$). shSCRAMBLE clones, dotted line; shRSUME clones, full line. In WB, GAPDH or β-actin was used as loading control. A full color version of this figure is available at https://doi.org/10.1530/ERC-18-0028.

the decrease in proliferation was reversed (Fig. 5C, bar 'shRSUME PTTG').

Discussion

The causes of PTTG upregulation in pituitary adenomas remain still an open question. In this study, we demonstrate that PTTG and RSUME are both overexpressed in human pituitary tumors with expression levels that correlate positively. In accordance, RSUME increases PTTG protein in pituitary tumor cell lines, improves the half-life of PTTG protein and regulates the PTTG induction by estradiol. Consequently, RSUME enhances PTTG transcription factor and securin activities. RSUME knockdown reverts its actions on PTTG abundance and transcriptional activity and reduces PTTG tumorigenic potential in a xenograft animal model.

In summary, we found that PTTG overexpression in tumoral cells is explained by its protein stabilization, is regulated by RSUME and accounts for PTTG tumor abundance and pathogenic action. There is no evidence so far that this mechanism operates in normal cells. This finding puts in a central role the protein stabilization-

overexpression of proto-oncogenes as major regulators of cellular dysfunction.

Increased stability of PTTG induced by RSUME may affect the activation of cell cycle by at least two ways: induction of entry to cycle by c-MYC and progression through the G1/S by cyclin D3, but also exacerbation of the activity of PTTG as securin, resulting in recurrent and unequal divisions (without proper separation of sister chromatids) without cytokinesis.

PTTG overexpression triggers a state of unscheduled proliferation, favoring the appearance of chromosomal abnormalities and the consequent tumor development. In endocrine tumors, where PTTG is abundant, it is not uncommon to find pleomorphic nuclei isolated without definite prognostic implication and that are probably aneuploid (Mastronardi *et al*. 2001, Yu *et al*. 2003, Uccella *et al*. 2005). The design of antitumor drugs that target PTTG abundance would be an interesting therapeutic tool for many tumors which manifest PTTG upregulation. Particularly, C-terminal proline-proline-serine-proline motif auto-regulating human PTTG1, although probably not involving RSUME as it is transcriptional, is an interesting target that could be effective to reduce the function and growth of pituitary tumor cells (Horwitz *et al*. 2003).

RSUME was identified as a sumoylation regulator that enhances overall SUMO conjugation to target proteins (Carbia-Nagashima *et al*. 2007). Here, we observed declined PTTG protein levels when the sumoylation pathway was inhibited by the viral protein Gam1 (Boggio *et al*. 2004), and a consequent reversion of this effect when an inactive mutant was used, which suggests that SUMO signaling is involved in PTTG stabilization by RSUME. For many proteins, sumoylation appears to protect target proteins from proteasomal degradation in addition to enhancing protein stability, changing subcellular localization or distribution, and/or modifying molecular interactions of target substrates (Hay 2005).

This work is the first report of interaction of two key pituitary factors, PTTG and RSUME, both originally identified following a screen of somatolactotroph pituitary tumor cell line. In the case of RSUME, the somatolactotroph cells overexpressed gp130 (Castro *et al*. 2003), a transmembrane glycoprotein that belongs to the family of interleukin-6 cytokine receptors (Arzt *et al*. 1999) that signal through JAKs and STATs proteins. Fowkes and Vlotides described that RSUME has at least two putative JAK-STAT sensitive tyrosine phosphorylation sites (Fowkes & Vlotides 2012). It was described that STAT3 induces PTTG expression to facilitate tumor growth and metastasis in colorectal cancer (Zhou *et al*. 2014) and that PTTG expression is regulated by IL-6 via activated STAT3 in prostate cancer (Huang *et al*. 2018). These experimental evidence increases the interest to further study the link between these three proteins, PTTG, RSUME and STAT3. Activation of these pathways involving IL-6/gp130/JAK/STAT3, either through elevated cytokine signaling during inflammation or during stress conditions, could lead to increased RSUME expression or activity, resulting in enhanced PTTG stability and transcriptional activation. Interestingly, in addition to the gp130/IL6 pathway, enhanced RSUME in tumoral pituitary cells could be reached by other mechanisms, including hypoxia (Carbia-Nagashima *et al*. 2007) or heath shock (Druker *et al*. 2013), but also a regulatory positive feedback loop by PTTG.

All types of pituitary adenomas exhibit high PTTG levels (Saez *et al*. 1999, Musat *et al*. 2004). Increased RSUME levels in pituitary tumors has also been described (Carbia-Nagashima *et al*. 2007, Shan *et al*. 2012, Gerez *et al*. 2013, He *et al*. 2017). The interplay of these two proteins opens interesting future research avenues for further understanding of pituitary tumor pathogenesis.

In addition to pituitary tumors, PTTG was found to be overexpressed in many other tumors from different origins like thyroid (Lewy *et al*. 2012, Read *et al*. 2017), salivary gland (Liu *et al*. 2015), esophagus (Zhang *et al*. 2014), testis/ovary (Pei 1998, Panguluri *et al*. 2008), pancreatic islets, brain (Genkai *et al*. 2006, Salehi *et al*. 2013), breast (Solbach *et al*. 2004, Smith *et al*. 2010), prostate (Zhu *et al*. 2006), uterus (Tsai *et al*. 2005), lung/lymph/skin (McCabe & Gittoes 1999) and others. This makes it interesting to deepen into the interaction PTTG/RSUME in other tumor tissues where PTTG is upregulated, and also the search for other factors that could be regulating the protein stabilization that leads to the abundance of tumoral PTTG.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

E A and M F conceived of and designed the experiments. M F, M S, L T, S S, A A and J J B carried out experiments. P A, G C, A C and G S collected biopsies and diagnosed subjects. M F, G K S and E A analyzed the data. M F and E A wrote the manuscript.

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