Published in final edited form as:
Mol Endocrinol. 2005 September ; 19(9): 2371–2379.

Pituitary hypoplasia in *Pttg*$^{-/-}$ mice is protective for *Rb*$^{+/-}$ pituitary tumorigenesis.

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Abstract

Pituitary tumor transforming gene *(Pttg)* is induced in pituitary tumors and associated with increased tumor invasiveness. *Pttg*–null mice do not develop tumors, but exhibit pituitary hypoplasia, while mice heterozygous for the retinoblastoma (*Rb)* deletion develop pituitary tumors with high penetrance. *Pttg*-null mice were therefore cross-bred with *Rb*$^{+/-}$ mice to test the impact of pituitary hypoplasia on tumor development. Prior to tumor development, *Rb*$^{+/-}$*Pttg*$^{-/-}$ mice have smaller pituitary glands with fewer cycling pituitary cells and exhibit induction of pituitary p21 levels. *Pttg* silencing in vitro with specific shRNAi in AtT20 mouse corticotrophs led to a marked induction of p21 mRNA and protein levels, decreased RB phosphorylation, and subsequent 24% decrease in S-phase cells. Eighty six percent of *Rb*$^{+/-}$*Pttg*$^{+/+}$ mice develop pituitary adenomas by 13 months, in contrast to 30% of double-crossed *Rb*$^{+/-}$*Pttg*$^{-/-}$ animals ($P<0.01$). Pituitary hypoplasia, associated with suppressed cell proliferation, prevents the high penetrance of pituitary tumors in *Rb*$^{+/-}$ animals, and is therefore a protective determinant for pituitary tumorigenesis.

Keywords

Pituitary adenoma; corticotroph cell; *Pttg*

Introduction

Pituitary tumors are invariably benign and exhibit high prevalence and potential for significant morbidity (1). Like other differentiated neuroendocrine tissues, the pituitary gland displays trophic hormone cell plasticity in response to physiological and homeostatic demands (2,3). Intrapituitary growth factors, hypothalamic hormones, inactivating tumor suppressor genes or activated oncogene mutations have been implicated in the spectrum of pathogenetic events leading to pituitary hyperplasia and adenoma development (1,4,5).

Mice bearing a single *Rb* mutant allele develop pituitary tumors with almost complete penetrance (6-8). Analysis of mutant mouse strains for the retinoblasoma gene (*Rb1*) has underscored the importance of retinoblastoma for tumor suppression. In mammalian cells, proliferation control is primarily achieved in the G1-phase of the cell cycle. RB is phosphorylated in a cell cycle dependent manner, and G1 cyclin/cyclin dependent kinase (Cdk) complexes phosphorylate RB and RB-related pocket binding proteins. RB hyperphosphorylation promotes subsequent release of E2F transcription factors resulting in S

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Supported by NIH grant CA 75979 (S.M.) and The Doris Factor Molecular Endocrinology Laboratory

phase cell cycle progression (9). Cyclin-dependent kinases integrate extracellular signals into the cell-cycle machinery (10-12). Cyclin/Cdk complexes are regulated by multiple mechanisms, including cyclin-dependent kinase inhibitors (CdkI) (11). Cdk4(6) actions are regulated specifically by Ink4-type inhibitors (p16, p15, p18, p19) whereas Cdk2 is inhibited by Cip/Kip-type p21, p27 and p57 inhibitors (12,13). By inhibiting cyclin/Cdk activity CdkIs govern the G1-to-S transition. Perturbed G1 control is a critical step for cellular transformation and tumorigenesis (14-16).

Pituitary tumor transforming gene (*Pttg*) behaves as a mammalian securin homolog facilitating sister chromatid separation during metaphase (17). *Pttg* exhibits oncogene properties as over-expression causes cell transformation, induces aneuploidy (18,19), promotes tumor formation in nude mice, induces basic fibroblast growth factor (bFGF) and activates angiogenesis (20, 21). *Pttg* initially isolated from pituitary tumor cells, is over-expressed in pituitary tumors and correlates with tumor invasiveness (22). Mice lacking *Pttg* are viable and fertile, and exhibit testicular and splenic hypoplasia, thymic hyperplasia, and pancreatic ß cell hypoplasia (23, 24), while pituitary-directed transgenic *Pttg* over-expression results in focal pituitary hyperplasia and adenoma formation (25) To elucidate the PTTG role in tumorigenesis, we generated compound *Rb* x *Pttg* mutant mice to determine effects of deficient PTTG on tumor development in $Rb^{+/-}$ animals.

Results

Pttg deletion results in selective decreased organ weight

Compound $Rb^{+/-}Pttg^{-/-}$ mutant mice have lower body weights as compared to $Rb^{+/-}Pttg^{+/+}$ animals ($P<0.05$), but similar to single $Pttg^{-/-}$-deficient animals. At 4 months of age, before tumor development these animals weighed 32 g as compared to $Rb^{+/-}Pttg^{+/+}$ (41.3 ± 2.4 g, $P<0.05$) littermates (Table 1). *Pttg* deficiency resulted in organ-specific decreased $Rb^{+/-}$ weights. Spleen ($P<0.01$), pancreas ($P<0.05$), testis ($P<0.05$) and pituitary ($P<0.05$) dry weights were lower in compound $Rb^{+/-}Pttg^{-/-}$ mutant mice than in $Rb^{+/-}Pttg^{+/+}$ and WT mice, and did not differ from single *Pttg* mutant animals. Similar organ-specific weight patterns were apparent when determined as a percentage of body mass (Table 1). Liver, brain and heart weights did not differ between genotypes (data not shown).

Pttg deletion results in decreased cell proliferation in the pre -tumorous pituitary gland

Pituitary weight of $Pttg^{-/-}$ animals was low (1.5 ± 0.07 mg, $P<0.01$ vs WT), and at 4 months, before tumor development, pituitary dry weight was lower in $Rb^{+/-}Pttg^{-/-}$ than in $Rb^{+/-}Pttg^{+/+}$ mice (2.1 ± 0.8 vs 5.0 ± 0.6 mg, $p<0.05$) and did not differ from WT (2.8 ± 0.08 mg) (Table 1). Pituitary cell proliferation rate evaluated by BrdU incorporation and Ki67 immunolabeling was attenuated in mice deficient in PTTG. In young 1- month single mutant $Rb^{+/+}Pttg^{-/-}$ mice pituitary BrdU incorporation was lower (0.1 ± 0.04%) than in $Rb^{+/-}Pttg^{-/-}$ and WT animals (0.76 ± 0.037% and 0.8 ±0.09% respectively), and lower than in $Rb^{+/-}Pttg^{+/+}$ pituitary glands (1.2 ± 0.22%, $P<0.05$) (Fig. 1a). These results were supported by finding low immunolabeled Ki67 expression in pituitary sections derived from $Rb^{+/+}Pttg^{-/-}$ mice (2.7 ± 0.09% $P<0.01$). In double mutant mice the Ki67 count did not differ from WT animals (13.2 ± 3.2% and 7.8 ± 1.3% respectively) but was markedly lower than in $Rb^{+/-}Pttg^{+/+}$ controls (26.1 ± 5.3%, $P<0.05$) (Fig. 1b).

Pttg is up-regulated in pre-tumorous $Rb^{+/-}Pttg^{+/+}$ pituitary gland

Pituitary *Pttg* mRNA levels were 2-fold higher ($p<0.05$) and PTTG immunoreactivity was stronger in $Rb^{+/-}Pttg^{+/+}$ compared to WT pituitary gland (Fig.2 a,b) when tested at 2-4 months of age.

Pttg deletion increases p21 expression in pre -tumorous pituitary gland

p21 belongs to the Cip/Kip family of cyclin-dependent kinase (Cdk) inhibitors which regulate cell cycle progression (12,26,27). p21 restrains Cdk2 activity, and decreased phosphorylation of Cdks leads to decreased RB phosphorylation (11,12,26,27), which consequently slows cell cycle progression. Young 2 month old $Rb^{+/+}Pttg^{-/-}$ and compound $Rb^{+/-}Pttg^{-/-}$ mice exhibit increased pituitary p21 mRNA (Fig.3a) and protein levels, while phosphorylated Cdk2 is reduced. Pituitary glands derived from *Pttg*-deficient animals demonstrate abundant p21 nuclear staining relative to WT and $Rb^{+/-}Pttg^{+/+}$ mice (Fig.3b). Conversely, in transgenic mice over-expressing pituitary PTTG, p21 protein levels are very low, and phoshorylated Cdk2 is increased (Fig.3c).

PTTG regulates p21 expression in murine corticotroph cells

To confirm that the observed decreased cell proliferation in PTTG deficient mice is a result of *Pttg* deficiency, two short hairpin interfering RNAs were specifically designed from residues 497 to 521 (shRNAi I) and from residues 394-413 (shRNAi II) of the mouse *Pttg* mRNA coding region. AtT20 corticotroph cells were transfected with shRNAi I or shRNAi II or mismatched control shRNAi. Both specific shRNAi constructs suppressed *Pttg* expression by ~90% suggesting that most cells were successfully transfected. In cells where *Pttg* was silenced, p21 mRNA and protein levels were up-regulated, indicating an apparent inverse relationship between PTTG and p21 expression (Fig.4a). p21 induction was associated with decreased levels of phosphorylated RB. Silencing *Pttg* in AtT20 cells with shRNAi I resulted in ~24% reduction in the number of BrdU- incorporated cells (cells in S-phase) as assessed by FACS analysis (30.3 ± 2.1% cells transfected with mismatched shRNAi vs 23.3 ± 3.1% cells transfected with specific *Pttg* shRNAi, $p<0.05$). Transfection with shRNAi II resulted in ~20% reduction in the number of BrdU-incorporated cells (32.3±1.1% cells transfected with mismatched shRNAi vs 26.3±2.6% cell transfected with specific *Pttg* shRNAi, $p<0.05$) (Fig. 4b). Thus, disrupted *Pttg* expression results in decreased murine pituitary cell proliferation rates.

The abundance of endogenous *Pttg* in experimental pituitary cell lines makes it difficult to accurately interpret tissue-specific effects of *Pttg* on p21 promoter activity. To explore the possibility that *Pttg* transcriptionally suppresses p21, CHO cells were therefore tested and transfected with murine a p21 promoter- luciferase reporter construct and co-transfected with increasing amounts of human wild type *Pttg*. p21 promoter activity was suppressed dose-dependently by the *Pttg* expression plasmid but not by either control plasmids. Mutated human *Pttg* (28) partially suppressed p21 promoter activity relative to wt *Pttg* (33% in wt *Pttg* vs 59% in mutant *Pttg* vs 100% in basic vector, $P<0.05$ between wt *Pttg* and basic vector) (Fig.4c).

These results suggest that PTTG restrains p21 expression in pituitary corticotrophs, and *Pttg* deletion decreases pituitary cell proliferation in young $Rb^{+/-}Pttg^{-/-}$ animals prior to visible tumor development by inducing pituitary p21.

Pttg deletion suppresses pituitary tumor development in $Rb^{+/-}$ mice

Rb heterozygous mice die mostly from pituitary tumors at 8-12 months of age depending on their genetic background (6,29-31). $Rb^{+/-}Pttg^{+/+}$ mice developed pituitary tumors starting from 4 months of age, and by 13 months 25 of 29 (86%) $Rb^{+/-}Pttg^{+/+}$ mice had pituitary tumors. The appearance of pituitary tumors was delayed in $Rb^{+/-}Pttg^{-/-}$ mice; of 57 doubly mutant mice, only 20 % harbored tumors at 13 months, and by 17 months 30% had tumors. These adenomas did not differ morphologically from $Rb^{+/-}$ tumors. In WT mice spontaneous pituitary tumors were observed in 4 of 28 animals (14%) starting at 9 months of age. Of 23 $Rb^{+/+}Pttg^{-/-}$ mice, three animals (13%) harbored pituitary tumors at 16 months. While $Rb^{+/-}$ mice do not survive more than 13 months, compound $Rb^{+/-}Pttg^{-/-}$ animals have now survived

for more than 18 months (Fig. 5a). Kaplan-Meier survival analysis (log-rank test) of the time of death with evidence of pituitary tumor in the different genotypes showed significant differences between $Rb^{+/-}Pttg^{-/-}$and $Rb^{+/-}Pttg^{+/+}$ (P <0.01), between $Rb^{+/-}Pttg^{-/-}$ and $Rb^{+/+}Pttg^{-/-}$ (P< 0.05), and between $Rb^{+/-}Pttg^{+/+}$ and $Rb^{+/+}Pttg^{-/-}$ mice (P < 0.01).

Consistent with results previously shown for $Rb^{+/-}$ tumors (32), *Rb* LOH was observed in five of seven compound tumors analyzed, indicating that loss of the single *Rb* allele in $Rb^{+/-}Pttg^{-/-}$ animals is present in these tumors (Fig.5b).

Discussion

The pathogenesis of pituitary neoplasms has been extensively studied to identify oncogene or growth factor mutations, or signaling defects (33,34). *Pttg* originally isolated from experimental pituitary tumor cells, is expressed in several malignancies (21), is associated with increased tumor invasiveness of pituitary adenomas (22), epithelial neoplasias (35) and colorectal carcinomas (22), and in the pituitary is especially induced by estrogen (36). *Pttg* is also a component of a 17- gene expression signature marker of metastatic potential for human tumors (37). As mice heterozygous for *Rb* show enhanced predisposition to pituitary and thyroid tumors (8,29,31), $Rb^{+/-}Pttg^{-/-}$ compound mutant mice were employed to determine the impact of PTTG loss on development and progression of pituitary tumors in *Rb*-deficient animals.

Pituitary *Pttg* mRNA and protein levels were induced in pre-tumorous *Rb* +/- mice. *Pttg* deletion leads to slowing of pituitary cell proliferation and induction of the Cdk inhibitor, p21, in young pre-tumorous pituitary glands, and in mouse AtT20 corticotroph cells. Conversely, mice with pituitary directed *Pttg* overexpression exhibit very low pituitary p21 levels. Compound mice with deleted *Pttg* develop pituitary tumors with markedly lower frequency than *Rb* heterozygous animals. High p21 levels likely restrain tumor initiation and progression in *Pttg*-deficient compound animals. The results suggest that pituitary cell proliferation capacity is required for early high penetrance of pituitary tumor formation in *Rb* heterozygous mice.

Pre-tumorous compound $Rb^{+/-}Pttg^{-/-}$ animals had lower selective organ weights consistent with splenic, testicular and pancreatic ß cell hypoplasia observed in $Pttg^{-/-}$ mice (23,24), indicating the requirement for PTTG in post-development growth control of selected cell types. Hypoplastic organs appeared developmentally normal with appropriate differentiated gene expression; although the testes are hypoplastic, males are fertile (23). *Pttg*-disrupted MEF or pancreatic ß cells do not exhibit higher rates of apoptosis (23,24). The relation between PTTG and apoptosis is not clear. PTTG overexpression caused p53-dependent and p53 independent apoptosis (18), and p53 suppresses *Pttg* promoter activity in response to DNA damage (38). While pituitary weights were lower in PTTG-deficient mice, apoptosis rates were extremely low in young pre-tumorous pituitary glands and no differences in apoptotic rates were noted between genotypes as assessed by TUNEL assay (data not shown).

Low pituitary and other selected organ weights in animals lacking PTTG might result from a proliferation defect. Slow pituitary cell proliferation is evident by low pituitary BrdU incorporation as well as low immunolabeling with Ki67. Ki67 is expressed during both G1 and S phases of proliferation, but not in quiescent cells (39). Additional support for a proliferative defect was derived from experiments showing that *Pttg* suppression in AtT20 cells by shRNAi decreased the percentage of cells in S-phase. Thus, PTTG deletion slows pituitary cell proliferation, while up-regulated *Pttg* mRNA and protein levels observed in the pre-tumorous $Rb^{+/-}Pttg^{+/+}$ pituitary gland may promote cell cycle entry.

Mechanisms underlying organ-specific decreased *Pttg*$^{-/-}$ cell proliferation are not clear. In humans, two additional *Pttg* homologs have been identified (40): the index *Pttg*, and homologous *Pttg2* and *Pttg3.* Although *Pttg* is most abundantly expressed in normal testes, *Pttg2* is preferentially expressed in spleen, liver, heart and pituitary, and *Pttg3* in the kidney and prostate. PTTG may be important for neuro-endocrine cell proliferation, while in other tissues PTTG requirement could be less essential, or *Pttg* function may be substituted by other *Pttg* family members. Similarly, acute RB loss in quiescent pituitary cells is compensated by *Rb*-related associated pocket binding protein p107 (41).

Negative regulation of cell cycle progression, particularly during development, could depend on cell-specific combinations of Cdk inhibitors (42). No differences in the expression of Cdk inhibitors p27 and p18 were found between genotypes (data not shown). Therefore, a mechanism for decreased pituitary cell proliferation in mice lacking PTTG could be induced specifically by pituitary p21 expression. Cell proliferation control is primarily achieved in G1, when RB and p21 are critical components (26). Sequential activation of cyclin/Cdk complexes regulates progression through the cell cycle. In vitro, p21 has a high affinity for cyclin E/Cdk2 complexes and 95% of active Cdk2 in normal fibroblasts is associated with p21(27,43). A recent model describes G1 progression as occurring in two discrete stages controlled by Cdk4 (6) under RB regulation and Cdk2 under p21 regulation. Inhibition of either stage attenuates cell progression (26). *Rb*$^{+/-}$*p21*$^{-/-}$ mice exhibit alteration of both stages and have accelerated pituitary tumor development compared to *Rb* heterozygous animals (26). In our experiments, induced p21 leads to a decline in phosphorylated Cdk2 levels which likely affect pituitary cell proliferation. These results indicate that p21 function limits tumor cell growth and that the delay in tumor progression observed in compound *Rb*$^{+/-}$*Pttg*$^{-/-}$ animals might arise as a consequence of pituitary p21 overexpression. Mutually exclusive patterns of Ki67 and p21 occur in gastrointestinal epithelium with p21 apparently restraining epithelial proliferation (39). Similarly, our data showing high p21 and low Ki67 expression suggest a restraining role for p21 in pituitary cell proliferation in the young PTTG-deficient pituitary gland.

Increased p21 expression in *Rb*$^{+/+}$*Pttg*$^{-/-}$ and *Rb*$^{+/-}$*Pttg*$^{-/-}$ animals is probably due to *Pttg* ablation. Our in vitro experiments demonstrate that silencing *Pttg* in AtT20 mouse corticotrophs by shRNAi leads to marked p21 gene and protein induction. High p21, in turn, is associated with decreased RB phosphorylation with subsequent diminished S phase cell number. PTTG might also directly affect the p21 promoter as PTTG over-expression dose-dependently decreased p21 promoter activity.

An alternative explanation of our results would be that PTTG-derived mitotic alteration could activate checkpoint signals, leading to p53 stimulation and consequent p21 induction (39,44, 45). Indeed, PTTG has been shown to interact with p53 and inhibit its transcriptional ability after DNA damage (46). In this and previous (23,24) studies however we did not observe p53-dependent increased pituitary apoptosis in PTTG-deficient mice. In undamaged cells, p21 may negatively control proliferation in a p53-independent manner(39). Thus, our results indicate that PTTG deficiency has significant consequences for cell proliferation, and imply that PTTG regulation of the pituitary cells involves p21-dependent mechanisms.

Striking similarities are apparent between *Pttg*$^{-/-}$ and Cdk4–deficient mice. *Cdk4*$^{-/-}$ animals have hypoplastic pituitary glands and develop diabetes mellitus associated with pancreatic islet degeneration (47). At least in part, Cdk4 controls S-phase transition via negative regulation of p27, another Cdk inhibitor (42,48). PTTG negatively regulates p21, and similar to Cdk4 promotes cell cycle entry. Cooperation of p27 and p21 appears critical for tissue-specific withdrawal from the cell cycle (42).

High p21 levels likely restrain tumor formation and progression in compound double mutant mice. In this study we show that by 12 months pituitary tumors were evident in 86% of $Rb^{+/-}Pttg^{+/+}$ mice. *Pttg* absence suppresses and delays progression of *Rb*-related tumors resulting in extended murine life span. Thus, while $Rb^{+/-}Pttg^{+/+}$ mice invariably die by 13 months, only 30% of $Rb^{+/-}Pttg^{-/-}$ develop tumors by 18 months.

Both humans and mice harboring a germ line *Rb* mutation develop tumors with almost complete penetrance, and tumor development is accompanied by tumor loss of the wild type allele (30, 32,49). In the absence of PTTG, the proportion of individual cells that eliminate the remaining WT allele of *Rb* during tumor development could be lower. However, as 5 of 7 tumors derived from $Rb^{+/-}Pttg^{-/-}$ compound mice do in fact exhibit *Rb* LOH, it is unlikely that PTTG regulates the frequency of loss of the remaining *Rb* allele in these tumors. However, we cannot exclude the effect of PTTG as a securin protein on chromatin exchange, leading to accelerated LOH and tumor formation. Aneuploidy is a ubiquitous feature of human solid tumors, causes genetic instability, and also promotes further aneuploidy. PTTG is a mammalian securin, localizes in the interphase nucleus and mitotic spindles and binds to and inhibits separin, which cleaves cohesin binding of sister chromatids (17). At the end of metaphase, PTTG is degraded, allowing equal separation of sister chromatids. PTTG overexpression induces aneuploidy by inhibiting equal chromatid segregation (19) and increasing the number of aneuploid cells leading to genomic instability. Paradoxically, abnormal nuclei, increased aneuploidy and premature centromere division are also observed in fibroblasts derived from $Pttg^{-/-}$ mice (23). Therefore, both *Pttg* excess as observed in tumors, and *Pttg* loss lead to cell cycle disruption and aneuploidy. These features point to *Pttg* as a caretaker gene ensuring genomic stability (50, 51). It is not yet apparent whether aneuploidy is a contributing cause or secondary consequence of cell transformation (51). Chromosamal instability can also arise from defects in cell cycle transformation (52). Despite increased aneuploidy, the incidence of pituitary tumors in *Pttg*-null mice are notably lower than in *Rb* heterozygous animals.

Our results are in contrast with an earlier in vitro study showing that PTTG overexpression induced growth arrest in human lung cancer cells by a p21-dependent mechanism (53). However, low pituitary weight, decreased cell proliferation, induction of pituitary p21 in PTTG-deficient mice, very low p21 protein levels in mice with pituitary-directed PTTG-overexpression, high levels of PTTG in pre-tumorous pituitary glands of *Rb*-heterozygous mice and marked decrease in tumor incidence in $Rb^{+/-}$ mice with *Pttg* deletion, all observed in our study indicate that in vivo PTTG promotes the pituitary cell cycle via p21 arrest and thus may induce or potentiate pituitary tumor formation. The contrasting results could be explained by strong tissue-specific properties of p21 (for example, RB stimulates p21 promoter in epithelial cells, but not in fibroblasts) (54). Thus the effect of PTTG-deficiency on p21 over-expression and cell cycle arrest may also be pituitary-specific. The extent to which such tissue specificity underlies the relations hip between PTTG and p21 requires further study.

In summary, the results show that placing $Rb^{+/-}$ mice into a *Pttg*- deficient background reduces and delays the progression of pituitary tumors. Absent PTTG allows expression of p21. The observed results, taken together with the in vivo finding that pituitary-directed transgenic *Pttg* overexpression causes focal hyperplasia (25) suggest that overexpressed pituitary PTTG in $Rb^{+/-}$ mice influences tumor initiation and progression by enhancing cell proliferation. We conclude that pituitary hypoplasia is an important determinant for protection against pituitary tumor formation.

Materials and Methods

Animals

Experiments were approved by the Institutional Animal Care and Use Committee. *Pttg*$^{-/-}$ mice were generated on a mixed C57BL/6x129/Sv genetic background and backcrossed to a C57BL/6 parental genotype. *Rb*$^{+/-}$ mice on a 129/Sv genetic background were purchased from Jackson Laboratory. Compound *Rb*$^{+/-}$*Pttg*$^{-/-}$ mice were bred by crossing *Rb*$^{+/-}$*Pttg*$^{+/-}$ females and *Rb*$^{+/-}$*Pttg*$^{+/-}$ males. Four genotypes were obtained from the same breeding: *Rb*$^{+/-}$*Pttg*$^{-/-}$, *Rb*$^{+/-}$*Pttg*$^{+/+}$, *Rb*$^{+/+}$ *Pttg*$^{-/-}$, and *Rb*$^{+/+}$*Pttg*$^{+/+}$ (wild type, WT). Animals were genotyped by PCR for *Pttg* (23) and *Rb* loci as described (6). Transgenic mice with aGSU promoter driving PTTG expression (25) were cross-bred with *Rb*$^{+/-}$ animals.

Anatomic and histological analysis

Animals were sacrificed and subjected to necropsy at the first indication of morbidity (weight loss, dehydratation or ataxia). Others were sacrificed as age- matched controls. For histological analyses tissues were fixed, paraffin-embedded, and sections stained with hematoxylin-eosin and periodic acid-Schiff.

Immunohistochemistry

The streptavidin-biotin-peroxidase complex technique was used with polyclonal PTTG antibodies (rabbit anti-human, Zymed, san Francosco, California) (55), and for p21 detection goat anti- mouse p21 polyclonal antibodies conjugated with Alexa 488 fluorescent dye was used (Molecular Probe, Eugine, Oregon). Antigen retrieval was performed by heating; control reactions lacked primary antibodies or were stained with blocking antibodies.

BrdU and Ki67 labeling

One month old mice were injected with BrdU (50 μg/g BW, Sigma, St Louis, Missouri) and sacrificed 24 h later. Pituitary sections were stained for BrdU (mouse anti-BrdU antibody, Becton Dickinson, Franklin Lake, New Jersey), counterstained with hematoxylin, and positive cells detected with ABC peroxidase system (Vector, Burlingame, California). Five to seven randomly chosen visual fields/per section were counted, and three sections/per animal derived from three animals of each genotype were analyzed.

Ki67 labeling index (MIB-1 antibody, Immunotech, Westbrook, Minnesota) was determined based on the number of positively stained nuclei divided by the total number of nuclei counted. Ten fields containing approximately 100 cells were counted from each animal and three animals from each genotype were analyzed.

Loss of heterozygosity (LOH)

Rb loss was determined by Southern blotting of DNA prepared from tumor tissues derived from either *Rb*$^{+/-}$*Pttg*$^{+/+}$ or *Rb*$^{+/-}$*Pttg*$^{-/-}$ animals. DNA was digested with Pst1/Kpn1, and hybridized with a probe spanning exon 3 of the *Rb* locus (generous gift of Dr. T. Jacks, MIT, Cambridge).

Quantitative PCR

Quantitative real time PCR was performed (56) to detect p21 and *Pttg* mRNA expression. The following specific primers were used: p21 forward 5'-CAGTACTTCCTCTGCCCTGC-3', p21 reverse 5'-AATCTGTCAGGCTGGTCTGC-3'. *Pttg* forward 5′-CGTCCTCAATGCCAATATCC-3′, reverse 5′-TCAACCCATCCTTAGATGCC-3′; 18S forward 5′-AAACGGCTACCACATCCAAG-3′, reverse 5′-CCTCCAATGGATCCTGGTTA-3′. Relative quantification of each gene in experimental

samples was determined from the corresponding standard curve, normalized to 18S, and expressed as arbitrary units.

Short hairpin interfering RNA (shRNAi)

For suppression of cellular *Pttg* expression, two shRNAis that specifically targeted *Pttg* mRNA were designed according to the manufacturer's protocol (Epicentra, Madison, Wisconsin). The sense sequence of shRNAi I spanning residues 497 -521 of mouse *Pttg* coding region was 5'-GGACAGTCAACAGAGTTGCCGAAAC-3'. The sense sequence of shRNAi II spanning residues 394 -413 was 5'-CTAGTGTCAAGGCCTTAGATC-3'. AtT20 murine corticotroph cells (American Type Culture Collection, Rockville, Maryland) were transfected with 100 nM *Pttg* shRNAi or mismatched shRNA using Oligofectamine (Invitrogen, Gaithersburg, Maryland), and cellular expression analyzed 24 hours later.

Northern and Western blot analysis

Northern analysis of pituitary *Pttg* and p21 expression was performed as described (56). Membrane was hybridized with ^{32}P-labeled fragment of murine *Pttg* (23), stripped and re-hybridized with a murine p21 fragment (obtained by PCR, gene bank accession number U24173).

For Western blot, pituitaries or cells were processed according to manufacturer's instruction (Immunoprecipitation Kit, Roche Diagnostics, Germany). Proteins were separated by SDS-PAGE, electroblotted onto Millipore membranes (Millipore, Massachusetts), and incubated with anti-PTTG (Zymed, San Francisco, California) or anti-p21, p18, p27 (Santa Cruz, California) or anti-phosphoCdk2 (Thr160) and -phosphorRB (Ser807/811) (Cell Signaling Technology, 'Massachusetts) antibodies overnight, and then with corresponding secondary antibodies. Immunoreactive bands were detected by ECL immunodetection system.

Cell proliferation assay

Asynchronized AtT20 cells were pulsed with 10μM BrdU (Sigma, St Louis, Missouri) in PBS for 10 min at 37°C. Cells were washed three times with 1% BSA in PBS, harvested, fixed in 75% ethanol and analyzed by FACScan (Becton Dickinson, Mountain View, California). The results depict the mean of three independent experiments ± SE.

Transfection and luciferase assay

Hamster ovarian carcinoma cells (CHO, ATCC, Rockville, Maryland) were plated in 6-well plates 12 hours before transient transfection in triplicate with 0.225 μg murine p21 promoter-luciferase reporter construct in pGL 3 (kindly provided by Dr. J. Pelling, University of Kansas) and co-transfected with increasing amounts of wild type or mutated human *Pttg* in pCI-neo. As a control, cells were co-transfected with reporter and expression vectors and each sample was co-transfected with LacZ control plasmid (Promega, San Louis Obispo, California). 0.5 μg cDNA (including 0.05 μg LacZ) was transfected using Effectin (Qiagen, Valencia, California). Total DNA was kept constant by adding the required amount of pGL 3. Cells were harvested 24 hours after transfection, assayed for luciferase activity, results were normalized to ß-galactosidase activity and represent the average of three independent transfections ± SE. Luciferase activity in cells co- transfected with p21 and pGL3 basic vector is represented as 100%.

Statistical analysis: Comparisons of pituitary tumor incidences in the respective genotypes were made by Kaplan-Meier survival analysis (log-rank test). Body and organ weights, quantitative PCR, BrdU-and Ki67 labeling indices were analyzed using ANOVA followed by

non-parametric t-test (Mann-Whitney) or Student t-test with a probability of $P<0.05$ considered significant.

Acknowledgements

We thank Fabio Rotondo, Anastasia Kariagina, Ph.D and Dana Alon for technical assistance, and Dr. I. Donangelo for providing $Rb^{+/+}$ *aGSU.Pttg* mice

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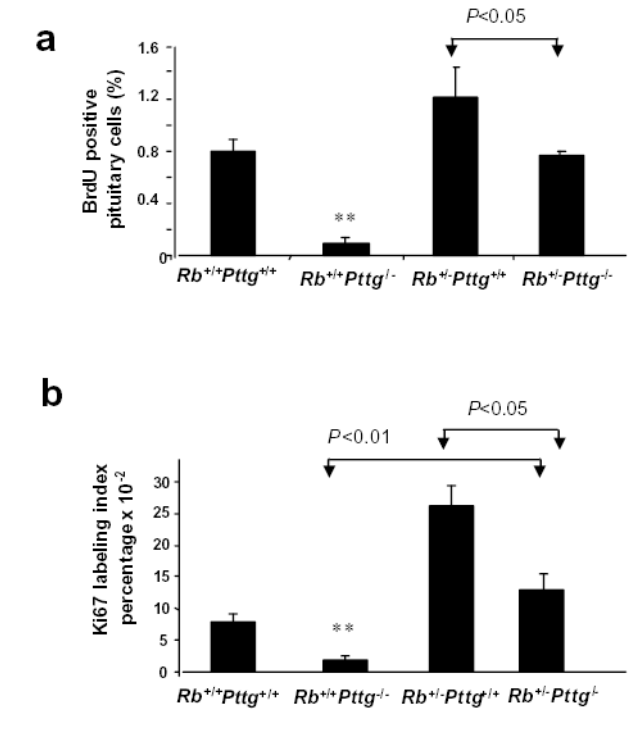

Fig 1. *Pttg* deletion results in decreased cell proliferation in pre-tumorous pituitary

a) BrdU positive pituitary cells in 4 week old $Rb^{+/+}Pttg^{+/+}$, $Rb^{+/-}Pttg^{+/+}$, $Rb^{+/+}Pttg^{-/-}$ and $Rb^{+/-}Pttg^{-/-}$ mice sacrificed 24 hours after BrdU injection. Each value represents mean percentage of positive cells ± SE (5-7 fields/section, 3 sections/animal, *n*=3 animal/genotype analyzed); **b)** Ki67 labeling index in 4 week old $Rb^{+/+}Pttg^{+/+}$, $Rb^{+/-}Pttg^{+/+}$, $Rb^{+/+}Pttg^{-/-}$ *and* $Rb^{+/-}Pttg^{-/-}$ mice. Each value represents mean ± SE (10 fields/animal *n*=3 animal/ genotype analyzed); In **a** and **b**: **, $P<0.01$ in $Rb^{+/+}Pttg^{-/-}$ mice vs three other genotypes.

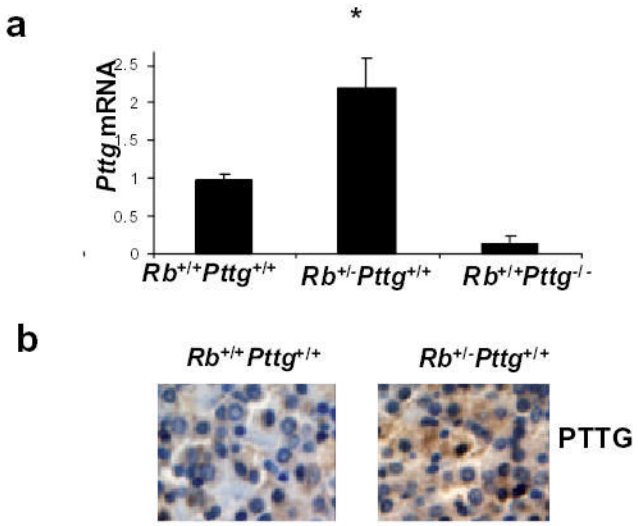

Fig 2. *Pttg* is overexpressed in pre-tumorous $Rb^{+/-}$ pituitary gland

a)Real time PCR analysis of pituitary *Pttg* mRNA in 2-4 month old pre-tumorous mice. Values are expressed as mean ± SE of triplicate measurements for each experimental group (*n*=4-6 animals per group). *, $P<0.05$ vs WT; **b)** Pituitary immunohistochemistry for PTTG expression in WT and $Rb^{+/-}Pttg^{+/+}$mice. Representative sections are shown.

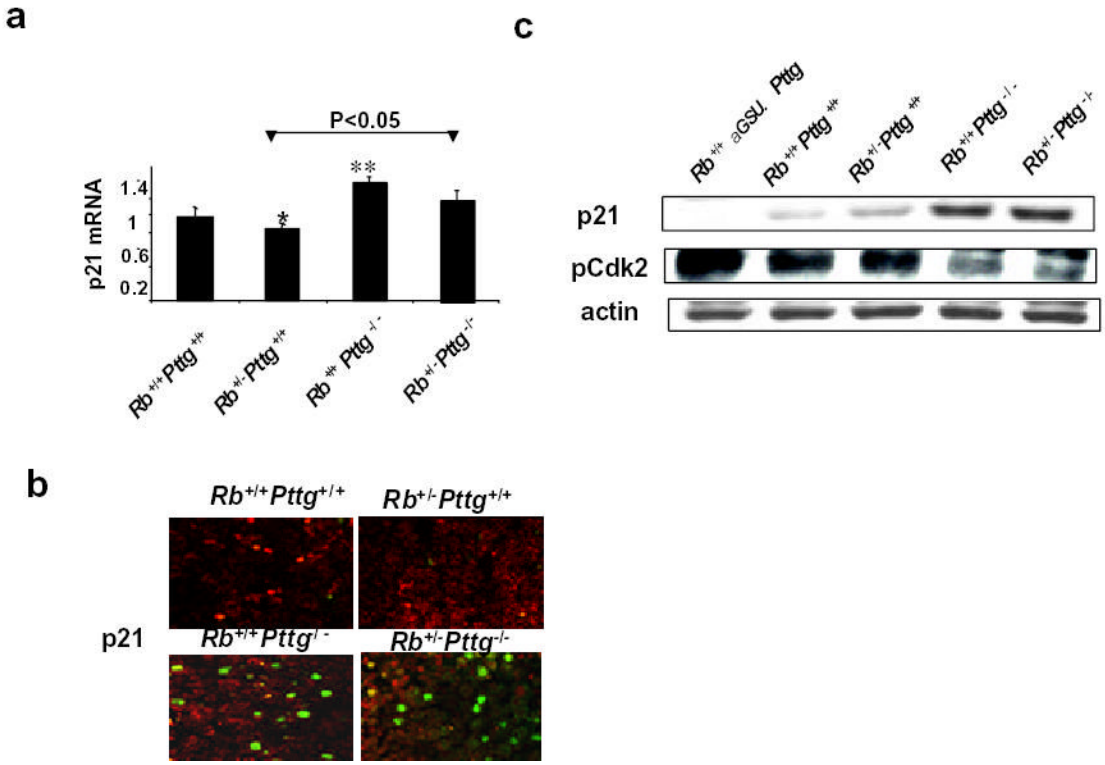

Fig 3. *Pttg* deletion increases pituitary p21expression in PTTG-deficient mice

2-4 month old pre-tumorous $Rb^{+/+}Pttg^{+/+}$, $Rb^{+/-}Pttg^{+/+}$, $Rb^{+/+}Pttg^{-/-}$ and $Rb^{+/-}Pttg^{-/-}$ mice are analyzed**. a)** Real time PCR of p21 mRNA. 1- $Rb^{+/+}Pttg^{+/+}$, 2- $Rb^{+/-}Pttg^{+/+}$, 3- $Rb^{+/-}Pttg^{-/-}$, 4- $Rb^{+/-}Pttg^{-/-}$. Values are expressed as mean ± SE of triplicate measurements for each experimental group (n=4-6 animals per group).*, P<0.05; **, *P*<0.01 vs $Rb^{+/+}Pttg^{+/+}$; **b)** immunohistochemistry for pituitary p21 expression. Green fluorescence indicate intra-nucleus localization of p21. Representative sections are shown; **c)** Western blot analysis of pituitary p21 and phosphoCdk2 protein levels.

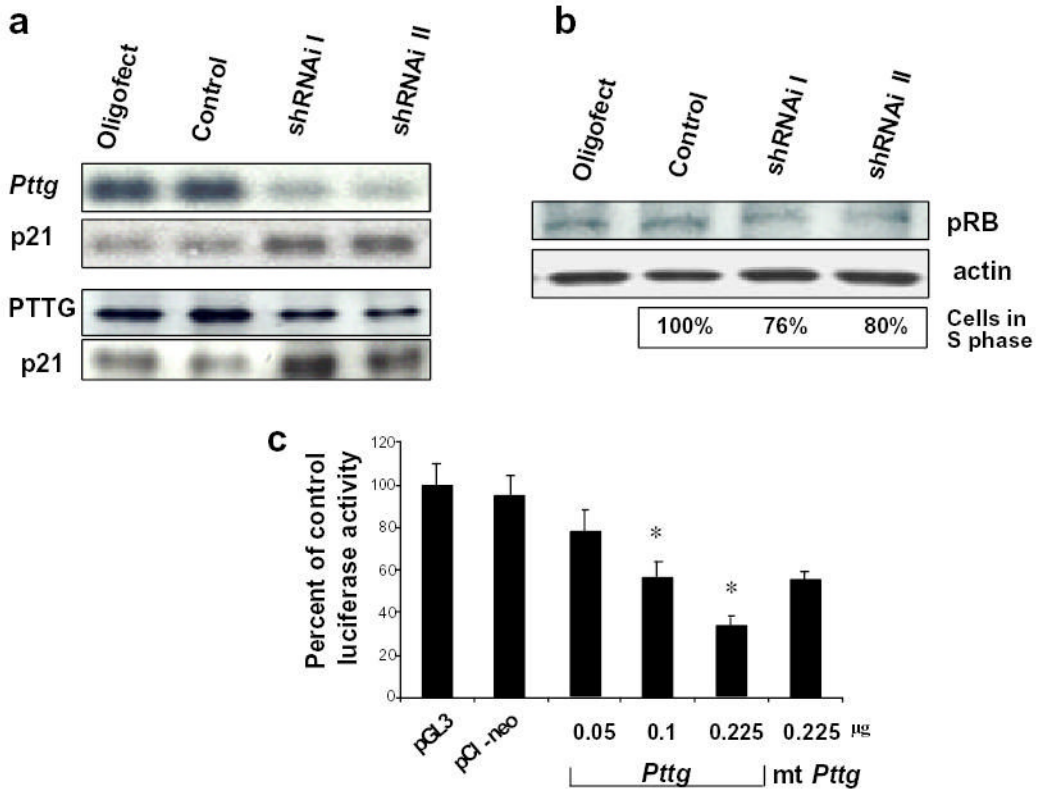

Fig 4. PTTG regulates pituitary corticotroph p21 expression

a) Northern (upper panel) and Western (lower panel) blot analysis of AtT20 cells transfected with anti-*Pttg* shRNAi. After hybridization with *Pttg* probe (Northern) or anti-PTTG antibodies (Western) membranes were stripped and re-blotted with p21 probe (Northern) or anti-p21 antibodies (Western); **b)** Western blot analysis of AtT20 cells transfected with anti-*Pttg* shRNAi and hybridized with anti-phosphoRB antibodies. Lower panel: decreased number of cells in S phase (% of control) after transfection with shRNAi. **c)** Suppression of murine p21 promoter activity by increasing doses of human *Pttg* in CHO cells; mt- mutant *Pttg* *, $P<0.05$ vs basic vector.

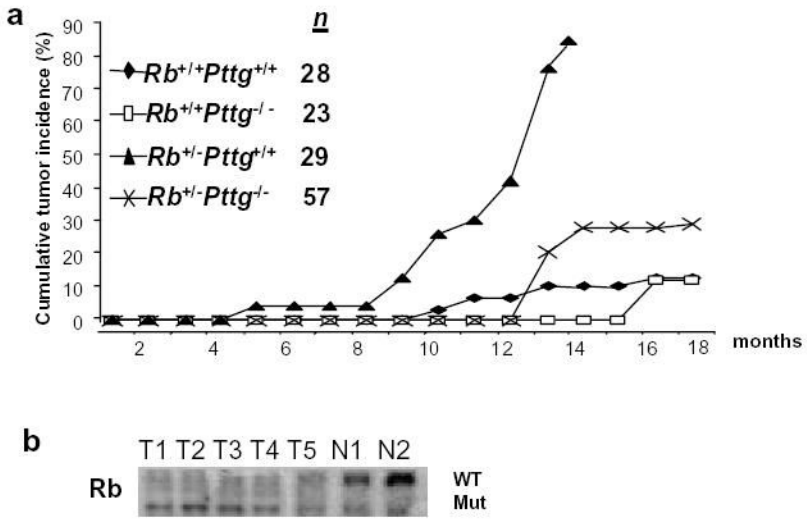

Fig 5. *Pttg* deletion suppresses pituitary tumor development in $Rb^{+/-}$mice

a) Development of pituitary tumors in $Rb^{+/+}Pttg^{+/+}$, $Rb^{+/-}Pttg^{+/+}$, $Rb^{+/+}Pttg^{-/-}$ *and* $Rb^{+/-}Pttg^{-/-}$ mice over time, *n*=total number of animal sacrificed; **b)** Southern-based Rb LOH analysis of DN A extracted from either tumor or tail tissue; mut-mutant, WT-wild type allele. T1, T3, T5-tissue derived from $Rb^{+/-}Pttg^{-/-}$ pituitary tumors, N1, N2-tail tissue from WT mice.

Table 1

Body and organ dry weight in male mice at 4 months.

	$Rb^{+/+}Pttg^{+/+}$	$Rb^{+/+}Pttg^{-/-}$	$Rb^{+/-}Pttg^{+/+}$	$Rb^{+/-}Pttg^{-/-}$
BW (g)	36.5 ±1.7	32.1 ± 1.6§	41.3 ± 2.4	31.9 ± 2.0§
Pituitary (mg)	2.8 ± 0.1	1.5 ± 0.1§	5.0 ± 0.6^{a*}	2.1 ± 0.3§
Pituitary (%BWx10^{-4})	7.5 ± 0.4	4.6 ± 0.4§*	12 ± 1.5*	6.5 ± 1.5§
Spleen (g)	0.18 ± 0.004	0.07 ± 0.05§**	0.11 ± 0.01**	0.05 ± 0.01§*
Spleen (% BW)	0.50 ± 0.02	0.20 ± 0.03§*	0.27 ± 0.03*	0.14 ± 0.04§**
Testes (g)	0.17 ± 0.03	0.10 ± 0.03§**	0.19 ± 0.0013	0.12 ± 0.02§**
Testes (% BW)	0.46 ± 0.01	0.34 ± 0.02§**	0.45 ± 0.01	0.28 ± 0.01§**
Pancreas (g)	0.27 ± 0.02	0.19 ± 0.02§**	0.28 ± 0.02	0.20 ± 0.02§**
Pancreas (% BW)	0.74 ± 0.01	0.62 ± 0.01*	0.67 ± 0.01	0.62 ± 0.01*

n=7-8 animals/group

$^{*}P<0.05$,

$^{**}P<0.01$ vs $Rb^{+/+}Pttg^{+/+}$

$^{\S}P<0.05$,

$^{\S\S}P<0.01$ vs $Rb^{+/-}Pttg^{+/+}$

^{a}, some $Rb^{+/-}Pttg^{+/+}$ pituitary glands may already exhibit pre-tumorous hyperplasia