

Downregulation of RalGTPase-activating protein promotes invasion of prostatic epithelial cells and progression from intraepithelial neoplasia to cancer during prostate carcinogenesis

Short title: Loss of RalGAP promotes prostatic carcinogenesis

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Abstract

RalGTPase-activating protein (RalGAP) is an important negative regulator of small GTPases RalA/B that mediates various oncogenic signaling pathways in various cancers. Although the Ral pathway has been implicated in prostate cancer (PCa) development and progression, the significance of RalGAP in PCa has been largely unknown. We examined RalGAP α 2 expression using immunohistochemistry on two independent tissue microarray sets. Both datasets demonstrated that the expression of RalGAP α 2 was significantly downregulated in PCa tissues compared with adjacent benign prostatic epithelia. Silencing of *RalGAP α 2* by shRNA enhanced migration and invasion abilities of benign and malignant prostate epithelial cell lines without affecting cell proliferation. Exogenous expression of wild-type RalGAP, but not the GAP activity-deficient mutant of RalGAP, suppressed migration and invasion of multiple PCa cell lines and was phenocopied by pharmacological inhibition of RalA/B. Loss of *Ralgapa2* promoted local microscopic invasion of prostatic intraepithelial neoplasia without affecting tumor growth in a *Pten*-deficient mouse model for prostate tumorigenesis. Our findings demonstrate the functional significance of RalGAP downregulation to promote invasion ability, which is a property necessary for prostate carcinogenesis. Thus, loss of RalGAP function has a distinct role in promoting progression from prostatic intraepithelial neoplasia to invasive adenocarcinoma.

Summary

RalGAP, a small GTPase RalA/B suppressor, is downregulated in human prostate cancer tissues. RalGAP loss induced RalA/B activation in benign and malignant prostate epithelial cells and increased local invasion, required for prostate carcinogenesis, in a mouse prostate tumor model.

Introduction

Despite recent advances in prostate cancer (PCa) research (1), the molecular mechanisms underlying prostate carcinogenesis have not been completely elucidated. Carcinogenesis is considered to be a multistep process including initiation and progression. Prostate carcinogenesis is characterized by long-term latent status after cancer initiation that has already taken place at a relatively early age (2-4). Considering that pre-malignant and latent PCa lesions are highly prevalent in the elderly population, the progression step seems to be more critical for the development of clinical PCa and the progression of prostate carcinogenesis. In this regard, extraductal invasion of neoplastic cells is a definitive pathological finding that distinguishes PCa from pre-malignant lesions including high-grade prostatic intraepithelial neoplasia (PIN) (1).

Aberrant expression of *ETS* family transcription factors such as *ERG*, *ETV1*, and *ETV4* is recognized as one of the important driver events in the progression of prostate carcinogenesis (5,6) as well as overexpression of *SPINK1* (7). Up to 70% of treatment-naïve PCa harbor chromosomal rearrangements that generate *ETS* gene fusion, such as *TMPRSS2-ERG*, resulting in aberrant expression of *ETS* family genes under the control of androgen-responsive promoters of 5'- (upstream) fusion partners (7,8). Previous studies showed that aberrant expression of *ERG* upregulates cell migration, a property necessary for prostate carcinogenesis in particular, and promotes the progression of high-grade PIN to invasive adenocarcinoma (6). However, approximately 30% of PCa cases do not show these gene alterations, and this frequency is even higher in the Asian population, as *ETS* gene alteration is less frequent in this population compared with African Americans or Caucasians (9-11). These findings suggest the involvement of other events that may promote cell invasion and contribute to prostate carcinogenesis.

The small GTPase Ral subfamily, a branch of the Ras family, consists of two closely related proteins, RalA and RalB, with 80% homology (12,13). These proteins function as important drivers of tumor growth and metastasis (12,13) by regulating various biological processes including cell survival (14), migration (15), malignant transformation (16), and metastasis (17,18). We and others previously reported that activation of the Ral pathway promotes bladder cancer progression (17,19). The Ral pathway was also implicated in PCa development (20) and metastasis (21). Smith and colleagues reported that the Ral-related transcriptional signature was significantly associated with inferior postprostatectomy recurrence-free survival of PCa (20). Several investigators demonstrated that Ral activation promoted growth and metastasis of PC3 human PCa cells *in vivo* (21,22). Another report showed that RalA activation was associated with castration-resistant progression (23). Thus, Ral activation plays important roles in the development and progression of PCa. However, the mechanism by which Ral is regulated in prostate cancer remains unclear.

Like other small GTPases, Ral is activated by guanine nucleotide exchange factors (GEF) and inactivated by GTPase-activating proteins (GAP). GEFs for Ral, including RalGDS, RGL1, RGL2, RGL3, RalGPS1 and RalGPS2, convert RalA/B to the activated GTP-bound form in a Ras-dependent

or -independent manner (24). Recently, our group identified two specific GTPase-activating protein complexes, RalGAP1 and RalGAP2 (25). RalGAPs constitute heterodimers of a common β subunit (RalGAP β) and one of two catalytic α subunits (RalGAP α 1 for RalGAP1 or RalGAP α 2 for RalGAP2). Each form of RalGAP inactivates both RalA and RalB (12). We also demonstrated that RalGAP2 is a major negative regulator of RalA/B in bladder urothelial carcinoma (BUC) by inhibiting RalA/B activities and aggressiveness of human BUC cells (17). Moreover, the expression levels of RalGAP α 2 were inversely correlated with clinicopathological stages and oncological outcomes of human BUC.

Here we investigated the biological and clinical significances of RalGAP in PCa. Our findings demonstrate a previously undescribed role of RalGAP and the Ral pathway in a relatively early stage of human and murine prostate carcinogenesis, namely local progression from PIN to invasive adenocarcinoma.

Materials and methods

Cell lines and treatments

We used three human PCa cell lines, PC3 (CRL-1435), DU145 (HTB-81) and MDA PCa2b (CRL-2422) (26) and a human papillomavirus-18-immortalized, non-tumorigenic prostate epithelial cell line, RWPE-1 (CRL-11609) (27). All cell lines were purchased from American Type Culture Collection (Manassas, VA) within 6 months from the initiation of the present study. Therefore, cells were not authenticated in the present study. PC3 and DU145 cells were cultured in RPMI 1640 (Nacalai Tesque, Kyoto, Japan) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA), 12.5 mM HEPES, penicillin and streptomycin at 37°C in 5% CO₂. MDA PCa2b cells were cultured in HPC1 (AthenaES, Baltimore, MD) supplemented as described elsewhere (26). RWPE-1 cells were cultured in Keratinocyte Serum Free Medium (K-SFM, Invitrogen) supplemented with 10% FBS.

Ral inhibitors, BQU57 and RBC8, were purchased from Sigma-Aldrich (St. Louis, MO) and used in experiments at the final concentration of 10 μ M.

Quantitative RT-PCR was performed as previously described (17) to evaluate gene expression of the prostate cancer cells.

GST-sec5 pull down assay and immunoblotting

The activation levels of small GTPase proteins RalA/B were determined by GST-sec5 pull-down assays as described previously (17,28). Total cell lysate containing 10 μ g of cellular protein separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Immunoblotting was performed using anti-RalGAP α 1, anti-RalGAP α 2, anti-RalGAP β (17), anti-RalA (#610221, BD Transduction Laboratories, San Jose, CA), anti-RalB (#04-037, Merck, Kenilworth, NJ), and anti- β -

actin (#ab6276, Abcam, Cambridge, UK) polyclonal antibodies as described previously (17). Specific bands were quantified using Image-J software.

DNA constructs and lentivirus infection

The full-length cDNA of wild-type RalGAP α 2 (wt-RalGAP α 2) and mutant RalGAP α 2-N1742K (mut-RalGAP α 2) (25), which lacks GAP activity, were subcloned into the lentiviral vector pBOB1 (Addgene, Watertown, MA). The oligonucleotide sequences used for construction of short hairpin RNA (shRNA) vectors targeting RalGAP α 2 and the GFP control are shown in Supplementary Table S1. The oligonucleotides were subcloned into the TRC2-pLKO-puro vector (Sigma-Aldrich).

Lentivirus was produced, purified and used for infection as described previously (17), with institutional approval under P2 biohazard restriction. PC3 and DU145 cells were infected with cDNA-expressing lentivirus. Infected cells were cultured in the presence of blasticidin (10 μ g/ml; Invitrogen). MDAPCa2b and RWPE1 cells were infected with shRNA-expressing lentivirus. The infected cells were cultured in the presence of puromycin (1 μ g/ml; Invitrogen). Infection efficiency was confirmed by GFP expression.

WST-8 cell proliferation assay

Cells were seeded at 1 x 10³ cells/100 μ l (PC3 cells), 2 x 10³ cells/100 μ l (DU145 cells), or 5 x 10³ cells/100 μ l (MDAPCa2b or RWPE1 cells) per well in 96-well plates in triplicate and cultured for 72 h. Next, 10 μ l/well Cell Proliferation Reagent from the Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan) was added and the cells were incubated for another 2 h. The absorbance of the formazan product was detected at 450 and 650 nm with a 96-well spectrophotometric plate reader.

Cell motility and invasion/migration assays

Cell motility assays were performed as described previously (17). Briefly, cells on cultured dishes were scratched with a plastic pipette tip and the width of the scratch was measured. After 18 h (PC3 cells) or 24 h (DU145 cells), the scratch width was measured again.

Cell invasion and migration assays were performed as described previously (17). Briefly, cells were seeded in a 24-well Matrigel-coated transwell cell culture chamber (for invasion assays for PC3 and MDAPCa2b cells) or 24-well non-coated control culture chamber (for migration assay for RWPE-1 cells) (both from BD Falcon, Franklin Lakes, NJ) and cultured under serum-starved conditions for 6 h. The medium in the bottom of the chamber was changed with fresh medium containing 10% FBS. After 24 h, the membrane of the chamber was fixed with 70% ethanol and stained with hematoxylin and eosin. For each membrane, invaded or migrated cells were counted in three distinct high power fields under microscopy.

Immunofluorescence

PC3 and MDAPCa2b cells (5 x 10³ cells/well) were plated in an 8-well chamber slide (BD Falcon, Franklin Lakes, NJ, USA). After 24 h, the medium was replaced with serum-free medium containing

DMSO or BQU57 (10 μ M) and cells were cultured for 6 h. Cells were then fixed with 4% PFA and then stained with Alexa Fluor 546 Phalloidin (Invitrogen) according to the manufacturer's protocols. Cells were then mounted with Vectashield/DAPI (Vector Labs, Burlingame, CA) and imaged using a Biozero BZ-8000 fluorescence microscope (KEYENCE, Osaka, Japan).

Prostate tumor mouse model

The *PSA-Cre-ERT2* transgenic mouse strain expressing Cre-ERT2 under control of the promoter of the human PSA gene is described elsewhere (29). The floxed allele of *Pten* is described elsewhere (30,31). The *Ralgapa2* knockout strain was described previously (17). The strains were crossbred and *PSA-Cre-ERT2;Pten^{flox/flox};Ralgapa2^{+/+}* (hereafter referred as *Ralgapa2^{+/+}*), *PSA-Cre-ERT2;Pten^{flox/flox};Ralgapa2^{+/-}* (*Ralgapa2^{+/-}*), and *PSA-Cre-ERT2;Pten^{flox/flox};Ralgapa2^{-/-}* (*Ralgapa2^{-/-}*) were generated to evaluate the functional significance of Ralgapa2 in murine prostate epithelia.

Tamoxifen was injected intraperitoneally at a dose of 1 mg/day for 5 days in 8-week-old male mice. Mice were euthanized by CO₂ at 6 months after tamoxifen treatment and dissected as described previously (29). Prostate glands were weighed, fixed in 10% formalin, embedded in paraffin, and sectioned (5- μ m thick). Sections were examined by histopathological evaluation (32).

All mouse experiments were performed in accordance with the policies of the Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions by the Ministry of Education, Culture, Sports, Science and Technology of Japan. The experimental protocol was approved by the Animal Research Committee, Graduate School of Medicine, Kyoto University (MedKyo13551, MedKyo14316, MedKyo15290, MedKyo16161, MedKyo17207, MedKyo18238).

Clinical samples and immunohistochemistry (IHC)

PCa and benign prostate tissue samples were surgically obtained at Kyoto University Hospital and used for the present study with approval by the Institutional Review Board at Kyoto University Graduate School of Medicine (#G0052-17). Disease stage was reported according to TNM Classification of Malignant Tumours, 7th edition, UICC, while histological grading was reported according to the 2014 International Society of Urological Pathology (ISUP) Consensus Conference on Gleason Grading of Prostatic Carcinoma (33,34).

The first tissue microarray (TMA1) was originally built using surgical specimens from 79 patients who underwent radical prostatectomy for clinically localized PCa at Kyoto University Hospital between 1993 and 1997 (35,36). Three spots from the cancer lesion and one spot from an adjacent benign lesion were punched out from each prostatectomy specimen. A representative immunostaining score of the three cancer spots was used for the cancer lesion from each patient. After excluding 12 patients who received preoperative androgen deprivation therapy and another two patients owing to exfoliation of array spots, 65 patients were included in the present analysis (Supplementary Table S2).

The second set (TMA2) was constructed using surgical specimens from 175 patients who underwent radical prostatectomy for clinically localized PCa at Kyoto University Hospital between 2004 and 2012. None of the 175 patients received preoperative androgen deprivation therapy. Samples were arrayed as previously described (37-40), with some modifications. Tissue cylinders 3 mm in diameter were punched out from the selected areas of each block by a tissue arrayer (KIN-2, Azumaya, Tokyo, Japan) and then embedded into paraffin blocks in a systematic fashion. For each case, one spot from the highest grade area of the index cancer lesion was selected and arrayed. For 17 cases, one spot from the adjacent benign lesion was also selected and arrayed. From the 175 patients, 164 malignant and 134 benign lesions were evaluated for RalGAP α 2. The clinicopathological characteristics of the 175 patients are summarized in Supplementary Table S3.

The third set consisted of 20 prostatectomy specimens that contain benign prostatic epithelia, high-grade PIN, and adenocarcinoma lesion on the same histopathological slice. Median (range) age and preoperative PSA 69 (60–78) years and 7.349 (4.32–19.0) ng/ml, respectively. Pathological grade was Gleason group 1 (Gleason score 3+3) in 2, group 2 (Gleason score 3+4) in 10, group 3 (Gleason score 4+3) in 4, group 4 (Gleason score 4+4) in 2 and group 5 (Gleason score 4+5, none showed Gleason score 5+5) in 2. Pathological stage was pT2c in 14, pT3a in 4 and pT3b in 2.

Immunostaining for RalGAP α 2 was performed as described previously (17). Additional immunostaining was performed using anti-ERG rabbit monoclonal (EPR3864, Abcam #ab92513), anti-CK5 rabbit polyclonal (Abcam #ab53121), and anti-CK8 (EP1601Y, Abcam) antibodies. A trained pathologist (Y. T.) and two authors (M. U. and T.K.) independently scored RalGAP α 2 and ERG staining intensity into four grades (G0, none; G1, weak; G2, moderate; G3, strong; based on the majority of each core), and discrepant cases were resolved by discussion with all observers.

Statistical analysis

All experiments were performed in triplicate and the data are presented as mean $\pm$ S.E.M. unless otherwise indicated. Student's t-test was used for the comparison of numerical data while ANOVA was used for the comparison of repeated numerical data. Chi square or Fisher's exact tests were used for contingency analyses. Kruskal–Wallis test was also used for non-parametric comparison between three groups. All statistical analyses were performed using commercially available software (SPSSII, SPSS Japan Inc.). All tests were two-sided and *P* values < 0.05 were considered statistically significant.

Results

Downregulation of RalGAP α 2 is associated with human prostate carcinogenesis

RalGAP α 2 expression in benign prostatic epithelia and PCa. We first evaluated the expression of RalGAP α 2, a subunit of the RalGAP complex (25) that is predominantly expressed in the prostate

gland (17), in benign and malignant human prostate tissues using IHC on two distinct TMAs (see Materials and Methods section for detailed information). As shown in Figures 1A and 1B, RalGAP α 2 expression was significantly stronger in benign prostatic epithelia than in malignant adenocarcinoma tissue in the two TMA sets ($P < 0.0001$ for TMA1 and $P = 0.0008$ for TMA2). Approximately one-third to one-fourth of benign epithelia showed strong RalGAP α 2 expression, whereas PCa tissue scarcely ($<10\%$) exhibited strong expression. Approximately half of PCa tissues were deficient for RalGAP α 2 expression, whereas only $\sim 20\%$ of benign epithelia were negative for RalGAP α 2. We did not find any statistically significant association of RalGAP α 2 expression with Gleason grade or pathological T stage in PCa samples (Supplementary Tables S2 and S3) or expression of ERG (Supplementary Figure S1A), which is a key driver in prostate carcinogenesis (5). Furthermore, we examined RalGAP α 2 expression using 20 prostatectomy specimens that contained adenocarcinoma, adjacent PIN, and benign prostatic epithelia. We observed downregulation of RalGAP α 2 was more prominent between adenocarcinoma and adjacent PIN, rather than between PIN and benign epithelia (Supplementary Figure S2).

Downregulation of RalGAP α 2 confers upregulation of Ral activity and invasion ability to human

prostate epithelial cells. To investigate the functional significance of RalGAP α 2 in prostatic epithelia, we silenced RalGAP α 2 in RWPE1 benign prostatic epithelial cells using shRNA. Knocking down of RalGAP α 2 using either of two shRNAs consistently resulted in upregulation of both RalA and RalB as assessed by the abundance of the GTP forms of Ral proteins (Figure 1C). These cells showed similar proliferation abilities (Figure 1D), suggesting that RalGAP α 2 or Ral activity has little impact on the proliferation of RWPE1 cells in culture. In a migration assay using transwell culture chambers, knockdown of RalGAP α 2 doubled the number of cells migrating across the membrane ($P < 0.05$ for each shRNA vs. control, Figure 1E), suggesting that RalGAP α 2 suppresses migration of RWPE1 cells.

Taken together, these findings suggest that RalGAP negatively regulates the migration ability of prostate epithelial cells and its downregulation is associated with prostate carcinogenesis, in which extra-ductal invasion of the atypical cells is required.

Functional significance of RalGAP α 2 in human PCa cells

RalGAP α 2 regulates Ral activities in human PCa cells. We next investigated the functional significance of RalGAP α 2 in human PCa cells. Exogenous expression of wild-type (wt) RalGAP α 2, but not the GAP activity-deficient mutant RalGAP α 2 N1742K (25), referred to hereafter as mut-RalGAP α 2, suppressed the activities of RalA and RalB as assessed by the abundance of their GTP forms (Figures 2A and 2B). We also examined MDAPCa2b cells, which express endogenous RalGAP α 2 relatively more abundantly than PC3 and DU145 cells. The two RalGAP α 2 shRNAs efficiently

suppressed RalGAP α 2 expression and upregulated RalA and RalB activation in these cells (Figure 2C). Additionally, we examined expression of known RalGEFs (*RalGDS*, *RalGPS1*, *RalGPS2*, *RGL1*, *RGL2* and *RGL3*), upstream activating regulators of RalA/B (Supplementary Figure S2) and found that RalGAP α 2 is the one that reflected RalA/B activity of the benign and malignant prostatic epithelial cells used in the present study. These findings suggested that RalGAP α 2 plays a pivotal role in Ral activation in PCa cells.

RalGAP α 2 does not affect the proliferation of prostate cancer cells in culture. We next examined the impact of expression of wt- or mut-RalGAP α 2 on the proliferation of PC3 and DU145 cells, as well as the proliferation of MDAPCa2b cells with RalGAP α 2 or control shRNA expression using WST-8 cell proliferation assays. None of the cell lines showed any statistically significant differences in cell proliferation upon manipulation of RalGAP α 2 expression (Figures 2D–F), suggesting that RalGAP α 2 has little functional significance in the proliferation of PCa cells.

RalGAP α 2 downregulates mobility and invasion of prostate cancer cells. We next investigated whether RalGAP α 2 affects mobility or invasion ability of PCa cells. Scratch assays showed that exogenous expression of wt-RalGAP α 2, but not mut-RalGAP α 2, inhibited restoration of the scratch gap in PC3 and DU145 cells (Figures 2G and 2H). Similarly, exogenous expression of wt-RalGAP α 2, but not mut-RalGAP α 2, downregulated the invasion ability of PC3 cells, as assessed by Boyden chamber invasion assays (Figure 2I). In comparison, increased numbers of migrated cells (by 2- to 3-fold) were observed in MDAPC2b cells expressing RalGAP α 2 shRNAs compared with cells expressing control shRNA (Figure 2J). Additionally, we evaluated expression of two genes that were reported to be involved in Ral-induced transcriptional signatures associated with aggressive clinicopathologic characteristics in human cancer (20). Downregulation of *FLNA* is known to promote motility and invasion, while *AURKA* is known to be associated with accelerated cell proliferation. As shown in supplementary figure S4, it seemed that Ral pathway affected expression of *FLNA* but not *AURKA*. Upregulation of Ral induced suppression of *FLNA* with a single exception of PC3 cells, whereas inconsistent results were obtained by the same manipulation of RalGAP α 2 for *AURKA*. These findings are consistent with our observation that Ral pathway was related with invasion phenotype but not cell proliferation. These findings collectively suggest that RalGAP α 2 downregulates the mobility and invading ability of PCa cells without affecting their proliferation.

Therapeutic implications of Ral inhibitors

Because we observed a role for RalGAP α 2 as a negative regulator of RalA/B activity and the mobility and invasion ability of PCa cells, we next examined whether RalGAP α 2 and the downstream Ral pathway could be a potential therapeutic target. Recently, Theodorescu's group identified BQU57 and RBC8 as small molecule chemicals that block Ral function by inhibiting the binding of Ral to its effector RALBP1 (41). Treatment with BQU57 or RBC8 downregulated cellular motility as assessed by scratch assay in PC3 (Figure 3A) and DU145 cells (Figure 3B) expressing control or mut-

RalGAP α 2 but not wt-RalGAP α 2. In invasion assays, treatment with BQU57 or RBC8 resulted in fewer number of migrated cells in PC3 cells expressing control or mut-RalGAP α 2 but not wt-RalGAP α 2 (Figure 3C). These drugs also blocked RalGAP α 2 shRNA-mediated upregulation of transwell migration in MDAPCa2b cells, whereas they showed no significant effects in control cells (Figure 3D).

Because Ral was previously shown to regulate cellular mobility and invasion via actin cytoskeleton organization (16), we also evaluated cell shape and structure using phalloidin immunofluorescence imaging (Figure 3E). PC3 cells expressing mut-RalGAP α 2 or control vector maintained a spindle shape. In contrast, PC3 cells expressing wt-RalGAP α 2 or treated with the Ral inhibitor BQU57 showed a rounded or less spread shape. Control MDAPCa2b cells showed a round shape, but showed stress fiber formation and spindle shape upon shRNA silencing of RalGAP α 2. However, these modifications in cytoskeleton organization were completely blocked by treatment with BQU57.

Taken together, these findings suggest that downregulation of RalGAP α 2 promotes mobility and invasion via Ral activation and that pharmacological blockade has the potential to suppress mobility and invasion of PCa cells with downregulated RalGAP α 2.

Loss of Ralgapa2 promotes local microscopic invasion of prostate tumors in mice

To further investigate the functional significance of RalGAP α 2 *in vivo*, we examined local invasion in a genetically engineered mouse model for prostate tumors. Specific gene recombination to prostatic luminal epithelial cells was confirmed by expression of the RFP reporter in *PSA-Cre-ERT2;LSL-RFP* mice at 3 days after tamoxifen treatment (Figures 4A and 4B).

We crossed *PSA-Cre-ERT2;Pten^{flox/flox}* mice, which develop hyperplasia or PIN but not invasive adenocarcinoma at 6 months after tamoxifen induction (29), with *Ralgapa2* knockout mice. *PSA-Cre-ERT2;Pten^{flox/flox};Ralgapa2^{+/+}* (hereafter referred as *Ralgapa2^{+/+}*), *PSA-Cre-ERT2;Pten^{flox/flox};Ralgapa2^{+/-}* (*Ralgapa2^{+/-}*), and *PSA-Cre-ERT2;Pten^{flox/flox};Ralgapa2^{-/-}* (*Ralgapa2^{-/-}*) were generated without a large deviation from Mendelian characteristics. At 6 months after tamoxifen treatment, control 8-month-old mice (wild-type mice with tamoxifen treatment, *PSA-Cre-ERT2;Pten^{fl/fl};Ralgapa2^{-/-}* without tamoxifen treatment, or *PSA-Cre-ERT2;Pten^{+/+};Ralgapa2^{-/-}* with tamoxifen treatment) showed no hyperplasia or PIN (Figures 4C, D and E, respectively). In contrast, *Pten*-deficient *Ralgapa2^{+/+}* (Figures 4F–H), *Ralgapa2^{+/-}* (Figures 4I–K), and *Ralgapa2^{-/-}* mice (Figures 4L–N) showed high-grade PIN, which is consistent with a previous report (29). The lesions showed positive immunostaining for the luminal marker keratin 8 (Ck8, Figures 4H, K and N) but were negative for the basal marker keratin 5 (Ck5, Figures 4G, J and M).

When we further histopathologically examined tumors from *Pten*-deficient mice (*Ralgapa2^{+/+}*, *Ralgapa2^{+/-}*, and *Ralgapa2^{-/-}*), we found that *Ralgapa2^{-/-}* mice often showed neoplastic lesions with microinvasion of Ck8-positive cells into interstitium, which was not observed in *Ralgapa2^{+/+}* or *Ralgapa2^{+/-}* as frequently (Figures 4O–T). We quantified this by counting microscopic loci on a

transverse section of dorsolateral lobes of each mouse. As shown in Figure 4U, *Ralgapa2*^{-/-} mice showed neoplastic lesions with microinvasion of Ck8-positive cells into interstitium approximately 3-fold more often than *Ralgapa2*^{+/+} or *Ralgapa2*^{+/-} mice ($P = 0.0007$ vs. *Ralgapa2*^{+/+} and $P = 0.011$ vs. *Ralgapa2*^{+/-}, Figure 4U). We did not observe statistically significant differences in prostate weight in *Ralgapa2*^{+/+}, *Ralgapa2*^{+/-}, and *Ralgapa2*^{-/-} mice (Figure 4V).

Taken together, these findings suggest that loss of *Ralgapa2* promotes local invasion of prostatic tumor cells in mice, which is consistent with our observation that RalGAP α 2 was downregulated in human PCa, which is defined as invasive to interstitium beyond basement membrane of prostatic epithelia.

Discussion

In the present study, we demonstrated that RalGAP α 2 expression was downregulated in PCa significantly more frequently than in benign prostatic epithelia, implicating the involvement of RalGAP α 2 in prostatic carcinogenesis. In PCa, one of the definitive characteristics that distinguish malignant acinar adenocarcinoma and benign PIN is neoplastic invasion of atypical cells beyond the basement membrane of the acinar duct. Differential expression of RalGAP α 2 between benign and malignant human prostate tissue suggests that functional loss of RalGAP is closely related to the malignant progression from PIN to invasive carcinoma in the multistep prostate carcinogenesis.

Our observations in a genetically engineered mouse model support the hypothesis for the functional significance of RalGAP in prostate carcinogenesis. The mouse model used in the present study recapitulates relatively early steps in prostate tumorigenesis (29). Using the *PSA-CreERT2* allele, loss of the *Pten* tumor suppressor is induced specific to prostatic epithelial cells in the dorsolateral lobe, which is considered to be the most similar to the peripheral zone of human prostate, in which adenocarcinoma is preferentially localized (1,42). The affected mice developed PIN in 3 to 6 months and microinvasive lesions in 12 to 15 months (29), which was reproduced in the present study. At 6 months after induction, we observed significantly more (~3-fold) lesions in *Ralgapa2*-deficient mice exhibiting local microinvasion of atypical cells, which is a hallmark of invasive prostatic adenocarcinoma.

We did not observe significant differences in prostate weights with respect to *Ralgapa2* status. Loss of *Ralgapa2* may in fact represent progression events rather than initiation events in multistep prostate carcinogenesis (1), as considered for ETS genetic rearrangements (6,43). This is consistent with our observation that difference in RalGAP α 2 expression was more prominent between PIN and adenocarcinoma rather than benign prostatic epithelia and PIN. Interestingly, we found that downregulation of RalGAP α 2 expression was not significantly correlated with ERG expression in

human PCa, suggesting mutually independent functional roles for Ral and ERG. This is consistent with a previous report showing that the Ral-related transcription signature was not associated with Gleason score or *TMPRSS-ERG* fusion (20). The prevalence of aberrant expression of ETS family genes was reported to be lower in an Asian population than in other ethnicities (9-11), which is consistent with our results in the present study. These findings suggest that loss of RalGAP and consequent activation of the Ral pathway may play a rather important role in the Asian population and may account for racial differences in the prevalence of clinically manifested PCa.

Despite efforts to reduce unnecessary biopsy, numerous men undergo prostate biopsy and the incidence of precursor lesions including PIN or atypical small acinar proliferation is approximately 15% (44). Men with PIN or atypical small acinar proliferation are considered to be at high risk to eventually develop PCa. Therefore, management similar to active surveillance is recommended for these men with close follow-up but without radical intervention, unless clear evidence of malignant disease is identified (45). Because the present study has shown the possibility that pharmacological inhibition of Ral is an effective option in PCa with RalGAP inactivation, pharmacological inhibition of Ral using novel Ral inhibitors (13,41) may be a promising option for the prevention of PCa development in a selected population.

This study had several limitations. We evaluated ERG expression using immunohistochemistry, which might not accurately reflect *TMPRSS2-ERG* gene fusion status. ~~Because there were a limited number of adjacent PIN lesions in our TMA sets of human PCa, we were unable to compare RalGAP α 2 expression between PIN and PCa.~~ The present study focused on the functional significance of RalGAP in early PCa, but its role in advanced PCa, particularly in metastatic progression, is still unclear and should be examined in future studies. Since *Ralgapa2* deletion in the mouse model used in this study was not prostate-specific but systemic, we cannot exclude the possibility that loss of *Ralgapa2* in extra-prostate organs affected the results. Since our mouse prostate tumorigenesis was modeled solely based on *Pten* loss of function, the findings of the mouse model should be interpreted in the context of *Pten*-loss, although it is one of the most frequently altered oncogenic signals in prostate cancer.

In conclusion, our results highlighted the functional significance of RalGAP in human and murine prostate carcinogenesis. Loss of RalGAP and consequent activation of the Ral pathway may promote prostate carcinogenesis independently from aberrant activation of ETS family genes. The present study provides novel insights into our understanding of prostate carcinogenesis and may facilitate the development of novel strategies for the prevention and management of PCa.

Supplementary Material

Supplementary Figures S1-8 and Tables S1-3 can be found at <http://carcin.oxfordjournals.org/>.

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Figure legends

Fig. 1. RalGAP α 2 is frequently downregulated in human prostate adenocarcinoma compared with benign prostatic epithelia. **A.** Representative hematoxylin and eosin staining (left) and RalGAP α 2 immunostaining (right) in human benign prostatic epithelium (top) and adenocarcinoma lesions (the remaining lower panels). RalGAP α 2 staining (moderate, weak and none) is indicated on the left. **B.** Distribution of RalGAP α 2 immunostaining patterns in benign and malignant prostatic lesions in the two independent tissue microarrays (TMA). **C.** Western blot analysis of RWPE1 cells expressing shRNAs against RalGAP α 2 (#1 and #2) using the indicated antibodies. Quantification of RalGAP α 2 protein levels relative to control levels in GFP shRNA are shown. Abundances of GTP-RalA and GTP-RalB relative to levels in controls are indicated. **D.** Proliferation abilities of cells expressing the indicated shRNAs were evaluated using WST-8 assays. **E.** Representative images (left) and quantified results (right) for cell migration assays using RWPE1 cells expressing indicated shRNAs against RalGAP α 2 (#1 and #2) compared with control (GFP shRNA).

Fig. 2. Functional roles of RalGAP α 2 in Ral activity and cell proliferation, motility and invasion in human prostate cancer cells. **A–C.** Western blot in PC3 (**A**), DU145 (**B**) and MDAPCa2b (**C**) prostate cancer cells expressing constructs or shRNAs using indicated antibodies. Abundances of GTP-RalA and GTP-RalB relative to levels in control cells are indicated. **D–F.** Proliferation ability of PC3 (**D**), DU145 (**E**) and MDAPCa2b (**F**) prostate cancer cells expressing constructs or shRNAs were evaluated using WST-8 assays. **G, H.** Representative images (left) and quantified results (right) for cell motility assays using PC3 (**G**) and DU145 (**H**) cells expressing indicated constructs. **I, J.** Representative images (left) and quantified results (right) for cell invasion assays using PC3 (**I**) and MDAPCa2b (**J**) cells expressing constructs or shRNAs.

Fig. 3. Effect of Ral inhibitors on cellular motility and invasion promoted by loss of function of RalGAP α 2. **A, B.** Quantified results of cell motility assays using PC3 (**A**) and DU145 (**B**) cells expressing constructs in the presence or absence of Ral inhibitors BQU57 (10 μ M) and RBC8 (10 μ M) as indicated. Original scratch distance was set as 100%. **C, D.** Quantified results of cell invasion assays using PC3 (**C**) and MDAPCa2b (**D**) cells expressing constructs or shRNAs in the presence or absence of Ral inhibitors BQU57 and RBC8 as indicated. **E.** Morphology and actin cytoskeleton organization of PC cells were evaluated by immunostaining phalloidin (red) for F-actin and DAPI (blue) for nuclei. Cells were infected with the indicated lentiviral constructs or treated with the Ral inhibitor BQU57 (Control + BQU57). **F.** Morphology and actin cytoskeleton organization of MDA PCa2b cells were evaluated by visualizing phalloidin (red) for F-actin and DAPI (blue) for nuclei. Cells

were infected with the indicated lentiviral constructs alone or also treated with the Ral inhibitor BQU57.

Fig. 4. Functional deletion of *Ralgapa2* promotes local invasion of prostatic tumors in *PSA-Cre-ERT2;Pten^{fl/fl}* mice. **A, B.** Representative immunofluorescence images for prostatic epithelium-specific gene recombination in *PSA-Cre-ERT2;LSL-RFP* mice. Mice were untreated (**A**) or treated with tamoxifen (**B**) and RFP expression was evaluated by immunohistochemistry on day 3 after the last tamoxifen treatment. **C–N.** Representative histological images of prostate glands obtained from 8-month-old mice of indicated genotypes and treatment. **C–E:** H&E staining in control mice (**C**: wild-type, tamoxifen-untreated; **D**: *PSA-Cre-ERT2;Pten^{fl/fl}*, tamoxifen-untreated; **E**: *PSA-Cre-ERT2;Pten^{+/+}*, tamoxifen-treated). H&E (**F, I, L**), Ck5 (**G, J, M**), and Ck8 (**H, K, N**) immunohistochemical staining in *PSA-Cre-ERT2;Pten^{fl/fl};Ralgapa2^{+/+}* (**F–H**), *PSA-Cre-ERT2;Pten^{fl/fl};Ralgapa2^{+/-}* (**I–K**), and *PSA-Cre-ERT2;Pten^{fl/fl};Ralgapa2^{-/-}* (**L–N**) mice. **O–T.** Evaluation for microinvasion using H&E and Ck8 immunohistochemical staining. Arrowheads indicate microinvasion loci. **U.** Quantification of numbers of microinvasion loci at 6 months after induction in *PSA-Cre-ERT2;Pten^{fl/fl};Ralgapa2^{+/+}* (n = 10), *PSA-Cre-ERT2;Pten^{fl/fl};Ralgapa2^{+/-}* (n = 8), and *PSA-Cre-ERT2;Pten^{fl/fl};Ralgapa2^{-/-}* mice (n = 7). **V.** Quantification of weight of the prostate gland at 6 months after induction. Controls indicate either of wild-type, tamoxifen-untreated; *PSA-Cre-ERT2;Pten^{fl/fl}*, tamoxifen-untreated; or *PSA-Cre-ERT2;Pten^{+/+}*, tamoxifen-treated mice (n = 4). *PSA-Cre-ERT2;Pten^{fl/fl};Ralgapa2^{+/+}* (n = 10), *PSA-Cre-ERT2;Pten^{fl/fl};Ralgapa2^{+/-}* (n = 8), and *PSA-Cre-ERT2;Pten^{fl/fl};Ralgapa2^{-/-}* (n = 7).

Supplementary Table S1. Sequences of shRNAs used in this study.

Supplementary Table S2. Clinicopathological characteristics of the 65 prostate cancer patients in tissue microarray #1.

Supplementary Table S3. Clinicopathological characteristics of the 164 prostate cancer patients in tissue microarray #2.

Supplementary Fig. S1. Loss of RalGAP occurs independently from aberrant expression of the prostate cancer driver ERG. **A.** Representative images of ERG immunostaining in benign epithelium (top) and adenocarcinoma (ERG-negative, middle; ERG-positive, bottom). None of the benign

epithelia showed positive ERG expression in our TMAs. Among PCa tissues, 14 (23.7%) of 59 evaluable spots in TMA1 and 19 (11.7%) of 162 spots in TMA2 were positive for ERG. **B.** Distribution of RalGAP α 2 immunostaining patterns (as shown in Figure 1A) in adenocarcinoma lesions based on ERG expression (as shown in **A**). No significant association between RalGAP α 2 and ERG expression was observed in the two TMAs.

Supplementary Fig. S2. RalGAP α 2 is frequently downregulated in human prostate adenocarcinoma compared with prostatic intraepithelial neoplasia (PIN) and benign prostatic epithelia. **A, B.** Radical prostatectomy specimens (n = 20) that contained adenocarcinoma (AC), PIN, and benign epithelia were subject to H&E (**A**) and RalGAP α 2 immunostain (**B**). Bars represent 500 μ m. **C-H.** Representative H&E (**C, E** and **G**) and RalGAP α 2 immunostaining (**D, F** and **H**) in human benign prostatic epithelium (**C** and **D**), PIN (**E** and **F**) and adenocarcinoma lesions (**G** and **H**). Distribution of RalGAP α 2 immunostaining patterns in benign, PIN and adenocarcinoma lesions in the 20 specimens.

Supplementary Fig. S3. Results of quantitative RT-PCR of *RalGAP α 1* (**A**), *RalGAP α 2* (**B**), *RalGAP β* (**C**), *RalGDS* (**D**), *RalGPS1* (**E**), *RalGPS2* (**F**), *RGL1* (**G**), *RGL2* (**H**) and *RGL3* (**I**) in the indicated benign and malignant prostate epithelial cells. The expression levels were normalized by that of *GAPDH*.

Supplementary Fig. S4. Results of quantitative RT-PCR of *FLNA* (**A–D**) and *AURKA* (**E–H**) in the indicated benign and malignant prostate epithelial cells. The expression levels were normalized by that of *GAPDH*.

Supplementary Fig. S5. Original gel images for Fig. 1C.

Supplementary Fig. S6. Original gel images for Fig. 2A.

Supplementary Fig. S7. Original gel images for Fig. 2B.

Supplementary Fig. S8. Original gel images for Fig. 2C.

Supplementary Table S1. Sequences of shRNA used in this study.

shRNA code	Sequence
RalGAP α 2#1	5'- GGTACCGGGACCGAACCGATGCGATTAGCTCGAGCTAAATCGCATCGGTTCGGTCTTTTTGAATTC-3'
RalGAP α 2#2	5'- GGTACCGGACTTGTCATCCACGGATTATGCTCGAGCATAATCCGTGGATGACAAGTTTTTTGAATTC-3'
GFP	5'- GGTACCGGTACAACAGCCACAACGTCTATCTCGAGATAGACGTTGTGGCTGTTGTATTTTTGAATTC-3'

Supplementary Table S2. Clinicopathological characteristics of the 65 prostate cancer patients in tissue microarray #1.

	Overall		RalGAP α 2 stain = 0		RalGAP α 2 stain = 1 or 2*		P value
Number	65		40		25		
Age, years, median (range)	68	(50 – 76)	67	(50–76)	71	(57–75)	0.095 [§]
Preoperative PSA, ng/ml, median (range)	9.9	(3.2 – 120)	11.7	(3.2–120)	9.0	(3.8–40.5)	0.181 [§]
Prostatectomy Gleason grade group, number (%)							
1 (Gleason sum $\leq$ 6)	16	(24.6)	12	(30.0)	5	(20.0)	
2 (Gleason score 3+4)	24	(36.9)	13	(32.5)	11	(44.0)	0.407 [†]
3 (Gleason score 4+3)	16	(24.6)	7	(17.5)	9	(36.0)	
4 (Gleason sum 8)	3	(4.6)	3	(7.5)	0	(0.0)	
5 (Gleason sum $\geq$ 9)	5	(7.7)	5	(12.5)	0	(0.0)	
Prostatectomy T stage, number (%)							
pT2	42	(64.6)	25	(62.5)	17	(68.0)	
pT3	23	(35.4)	15	(37.5)	8	(32.0)	0.791 [‡]

PSA, prostate-specific antigen.

*In tissue microarray 1, only one prostate cancer case showed RalGAP α 2 stain of 2, and no case showed RalGAP α 2 stain of 3.

[§]Unpaired t test, [†]Chi-square test for trend, [‡]Chi square test.

Supplementary Table S3. Clinicopathological characteristics of the 164 prostate cancer patients in tissue microarray #2.

	Overall		RalGAP α 2 stain = 0		RalGAP α 2 stain = 1		RalGAP α 2 stain = 2		<i>P</i> value
Number	164		70		75		19		
Age, years, median (range)	66	(47–77)	66	(47–75)	67	(51–77)	65	(56–74)	0.848*
Preoperative PSA, ng/ml, median (range)	7.7	(4.1–28.4)	8.0	(4.1–26.7)	7.7	(4.1–28.4)	7.2	(4.1–22.4)	0.654*
Prostatectomy Gleason grade group, number (%)									
1 (Gleason sum $\leq$ 6)	33	(20.1)	22	(31.4)	9	(12.0)	2	(10.5)	0.214†
2 (Gleason score 3+4)	67	(40.9)	27	(38.6)	36	(48.0)	4	(21.1)	
3 (Gleason score 4+3)	32	(19.5)	6	(8.6)	18	(24.0)	8	(42.1)	
4 (Gleason sum 8)	14	(8.5)	4	(5.7)	7	(9.3)	3	(15.8)	
5 (Gleason sum $\geq$ 9)	18	(11.0)	11	(15.7)	5	(6.7)	2	(10.5)	
Prostatectomy T stage, number (%)									
pT2	99	(61.1)	48	(68.6)	38	(50.7)	13	(68.4)	0.661‡
pT3	65	(38.9)	22	(31.4)	37	(49.3)	6	(31.6)	

PSA, prostate-specific antigen.

In tissue microarray #2, no case had a RalGAP α 2 stain of 3.

*Kruskal-Wallis test, †Chi-square test for trend, ‡Chi square test.

Figure 1.

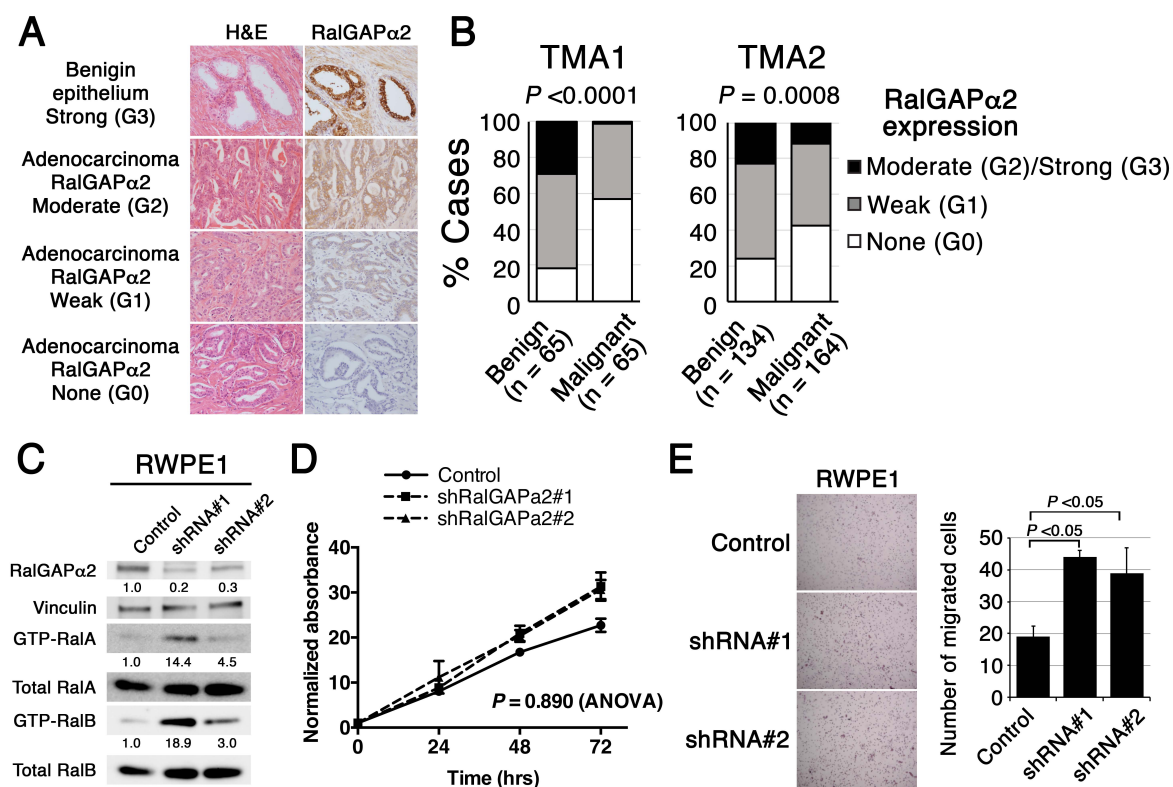


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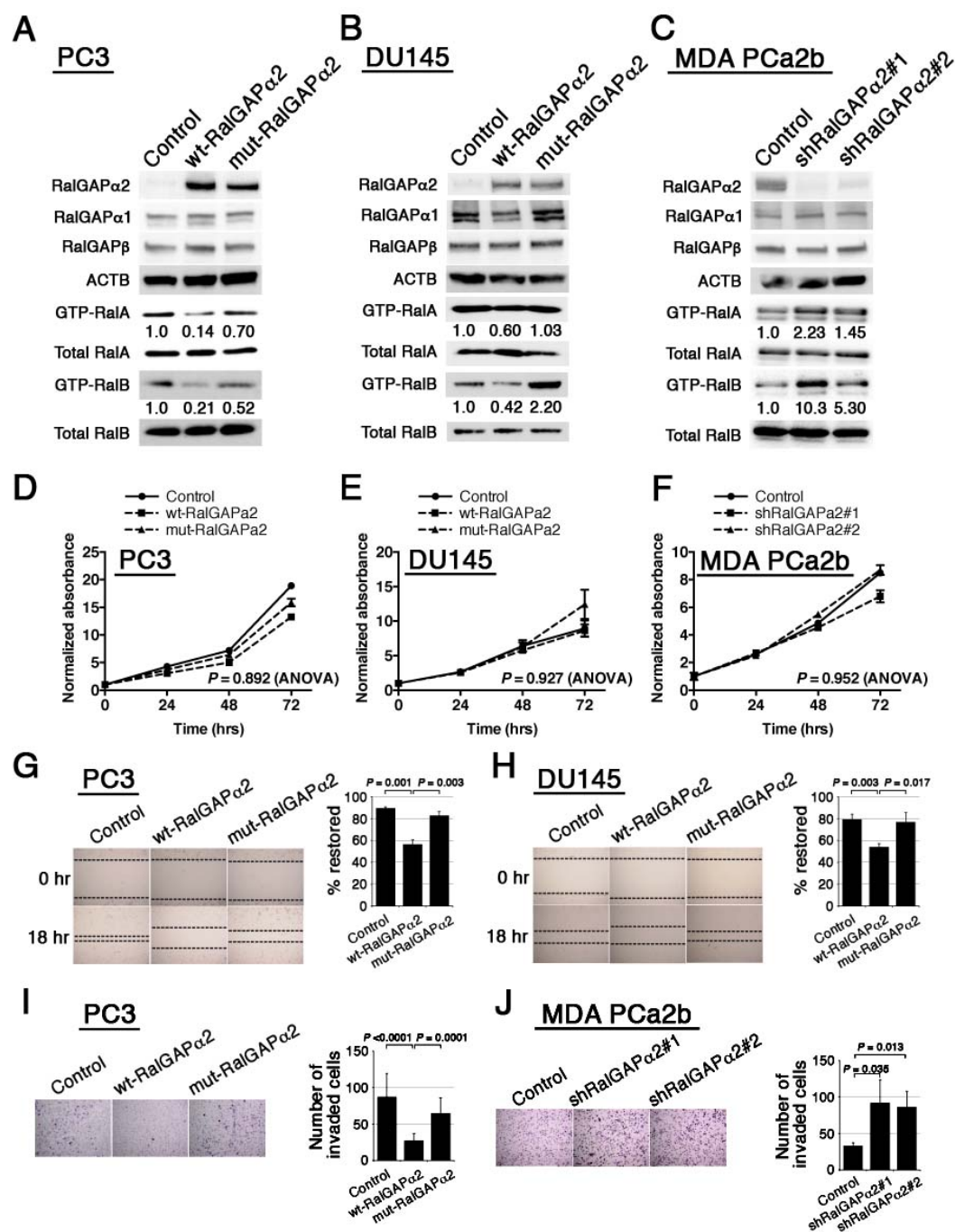


Figure 3.

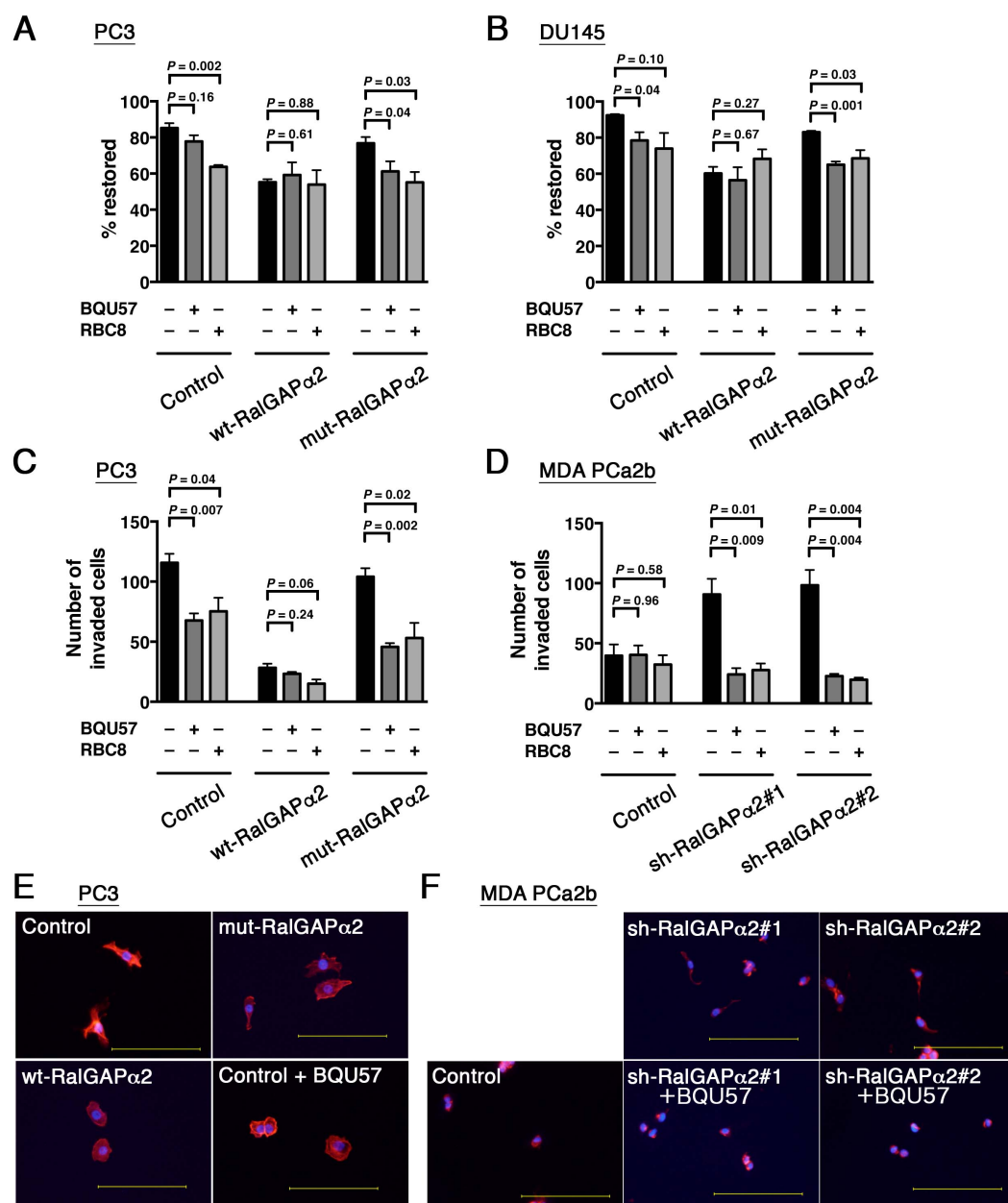


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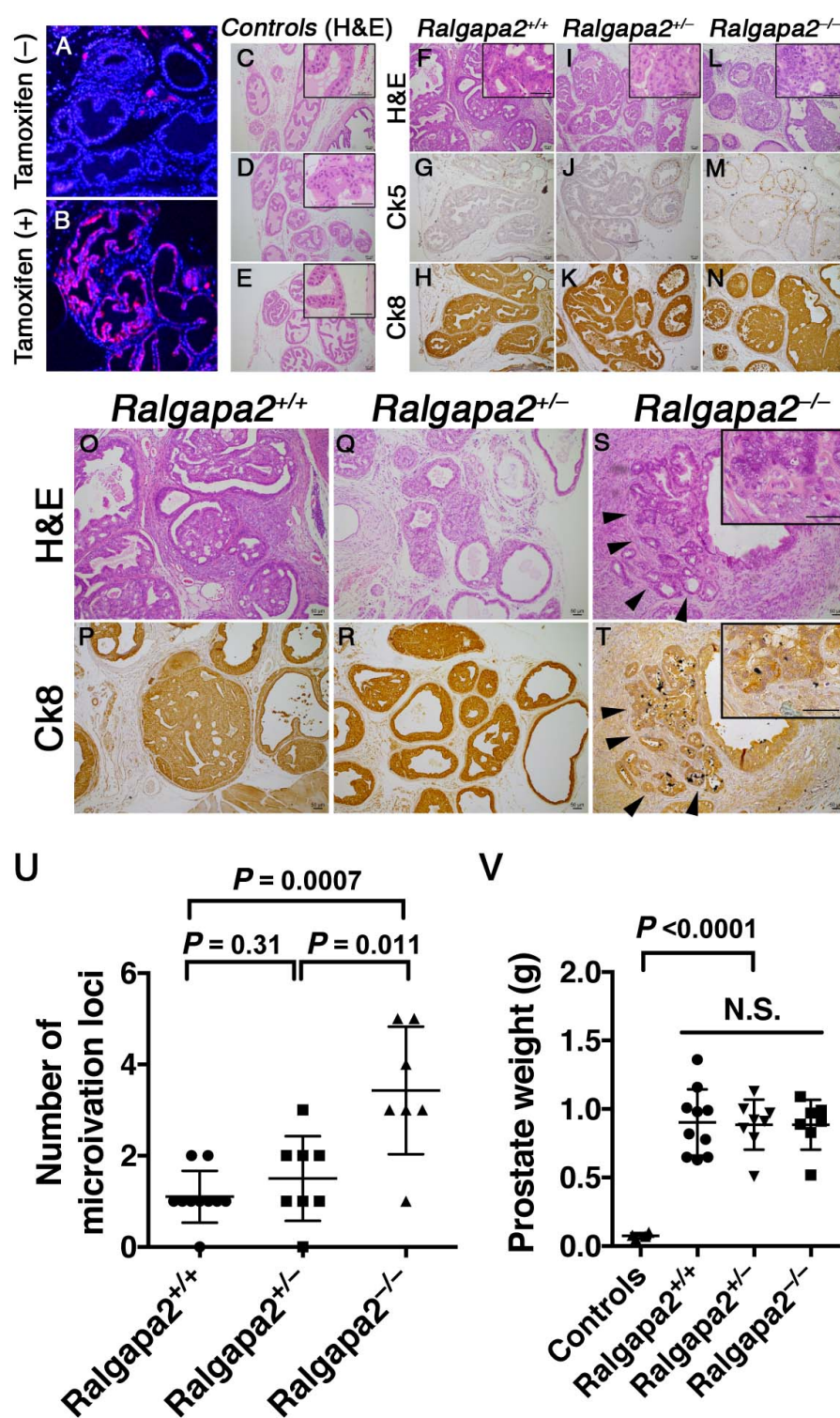


Figure S1.

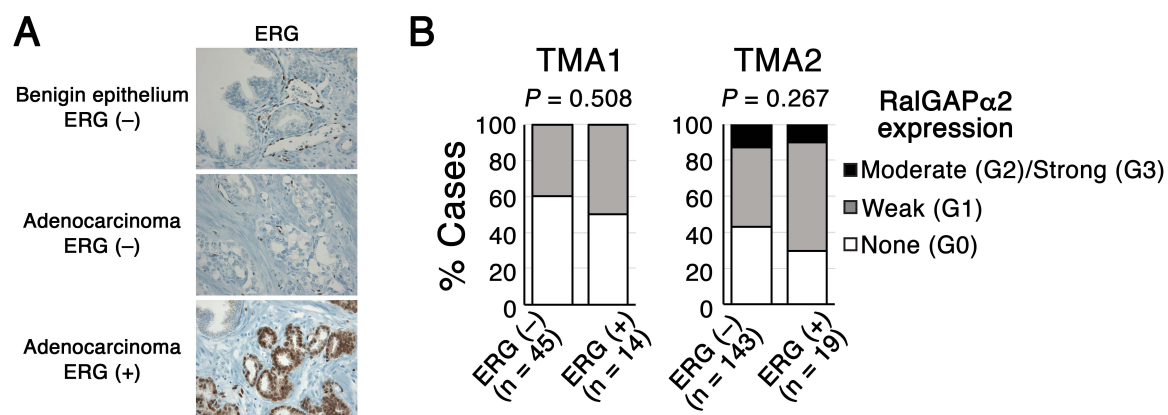


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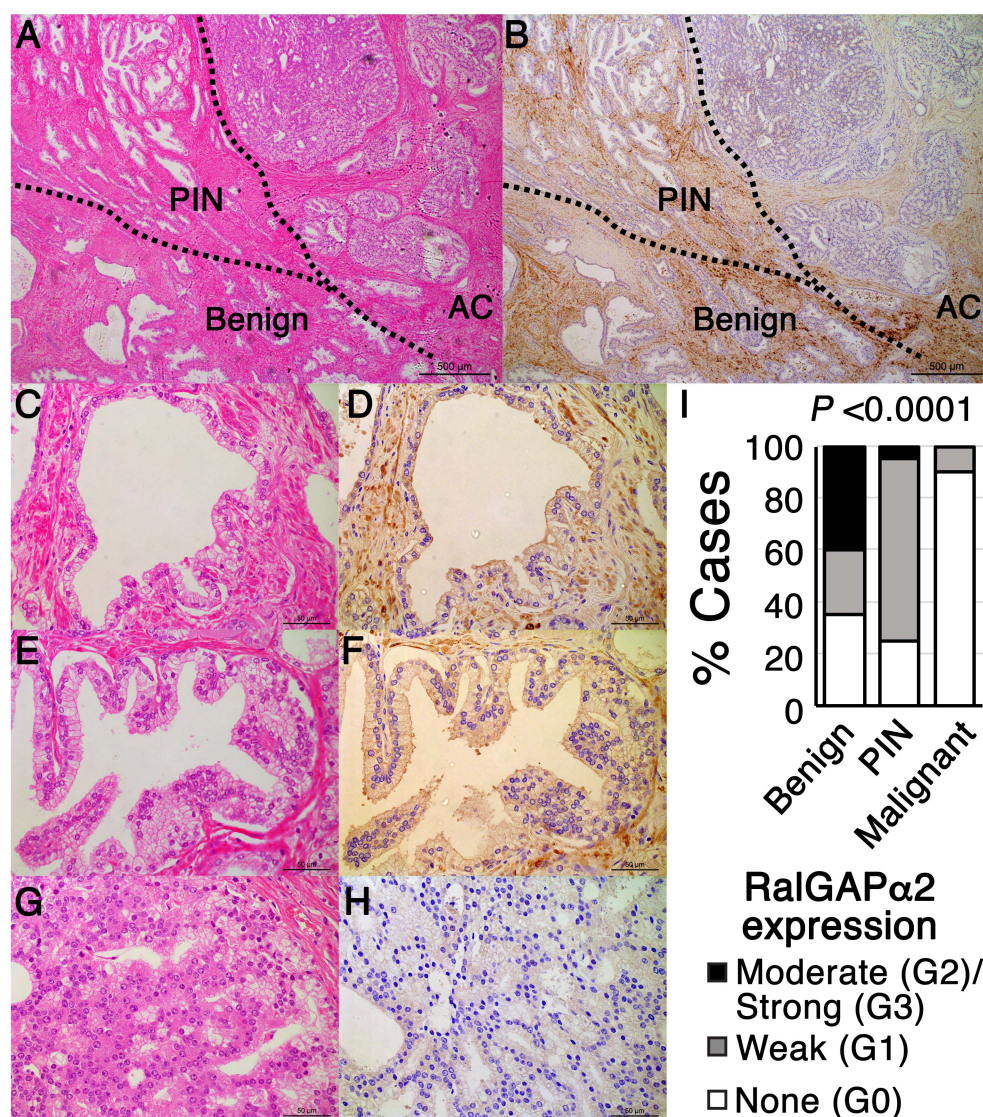


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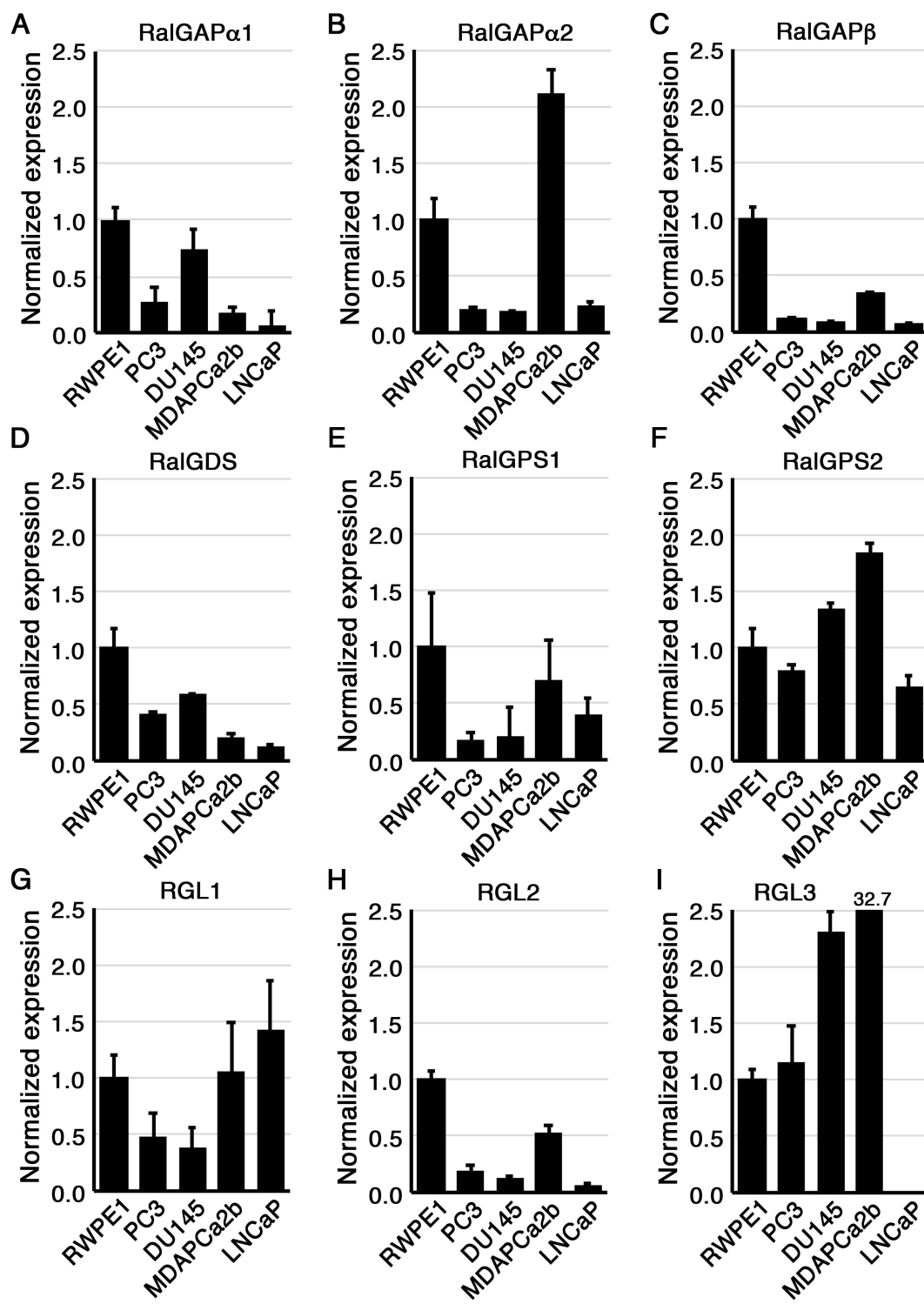


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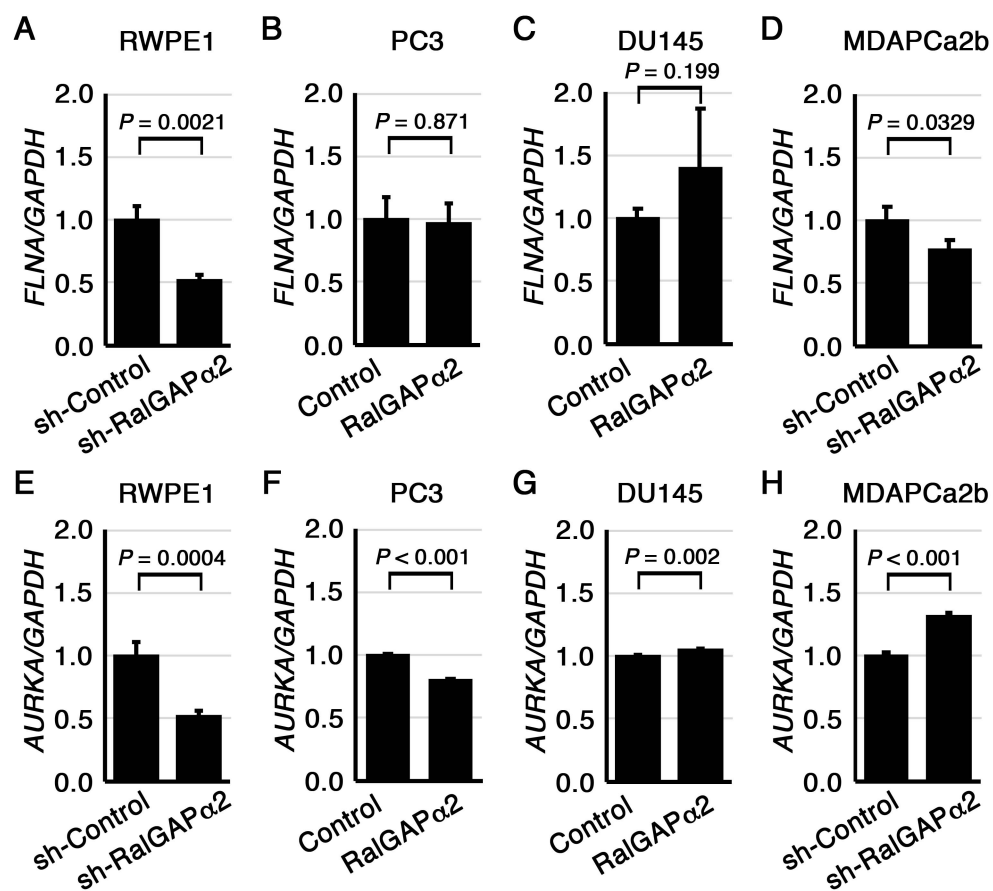


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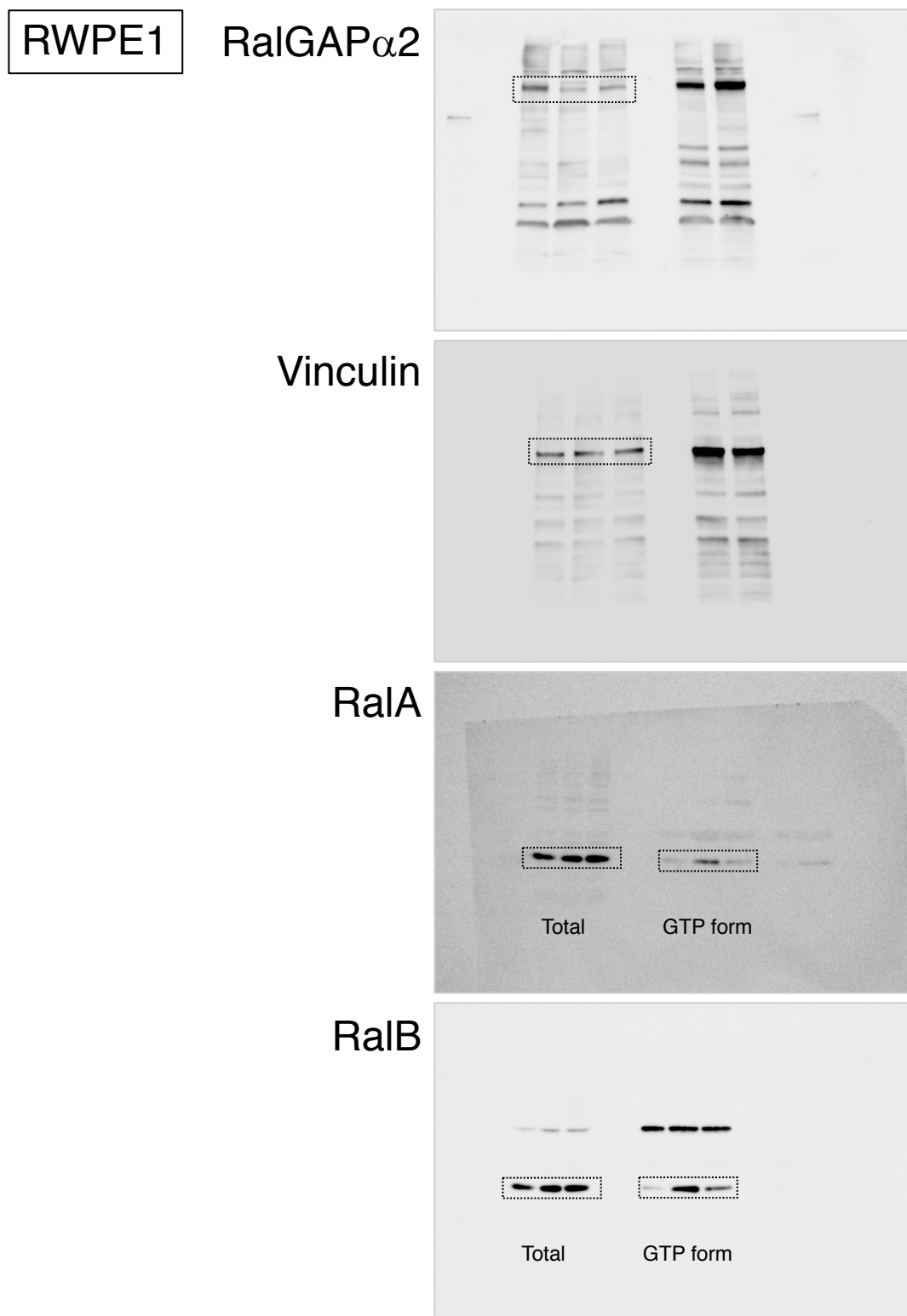


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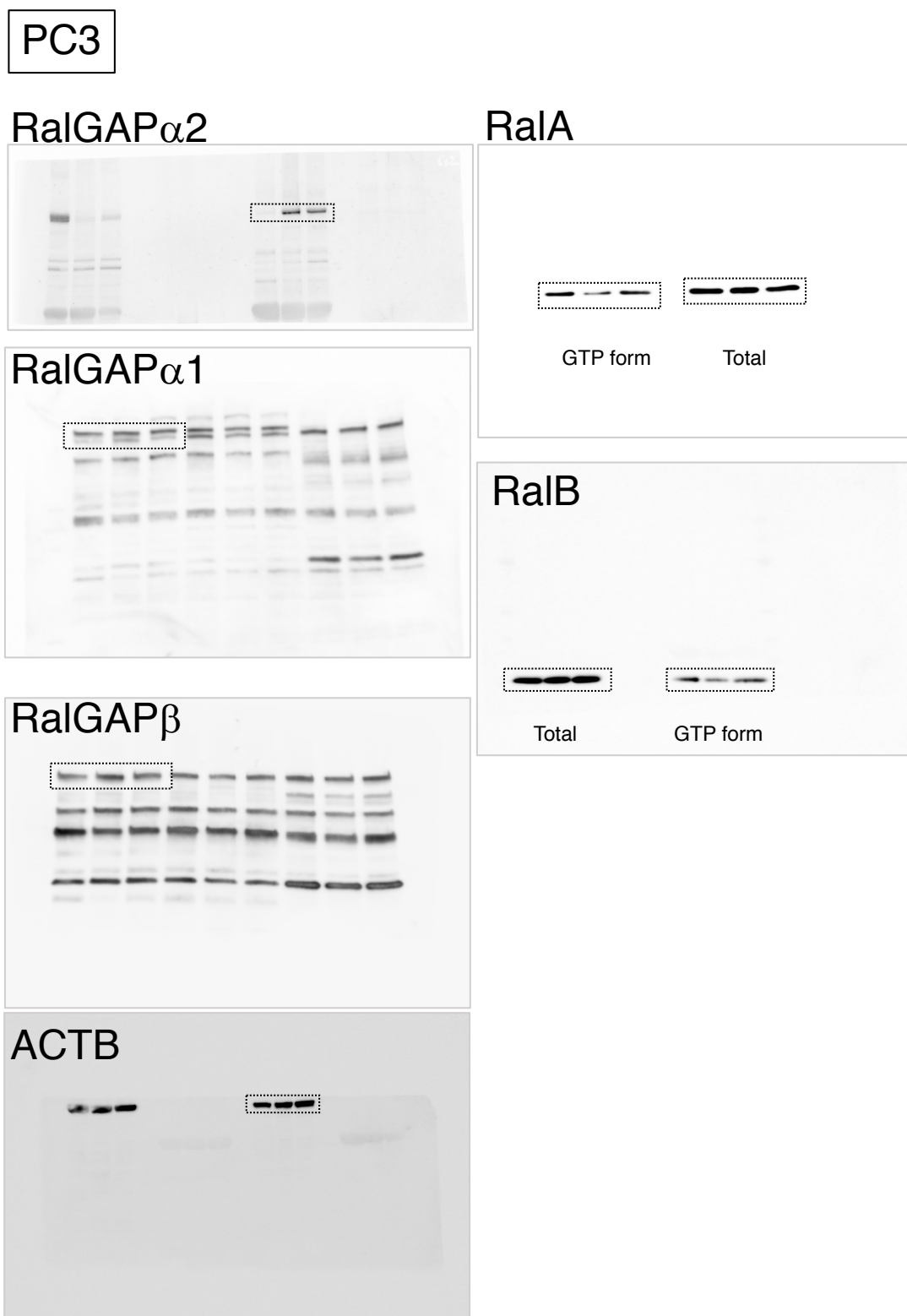


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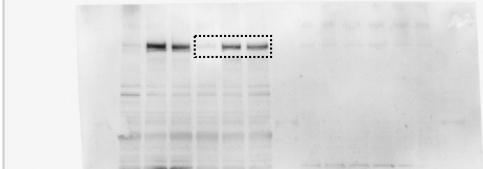
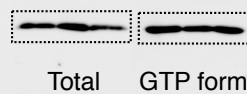
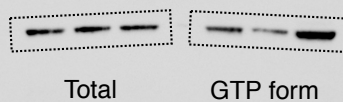
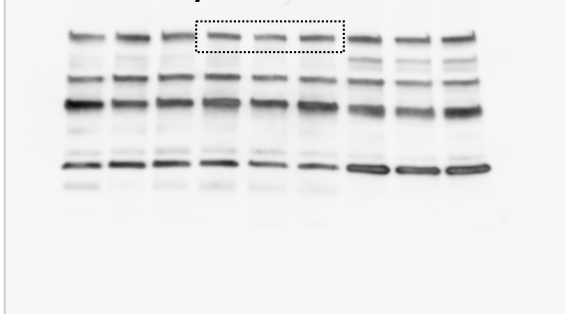
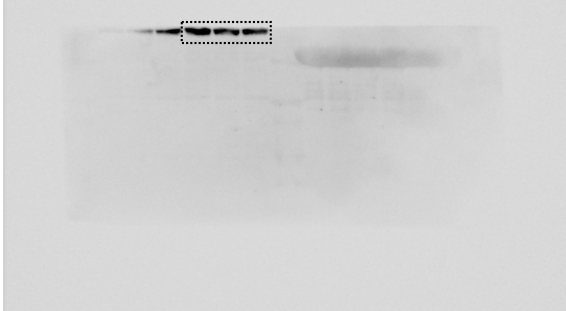
DU145**RalGAP α 2****RalA****RalGAP α 1****RalB****RalGAP β** **ACTB**

Figure S8.

