RET/PTC oncogenic signaling through the Rap1 small GTPase

Valentina De Falco*, Maria Domenica Castellone*, Gabriella De Vita, Anna Maria Cirafici, Jerome M. Hershman, Carmen Guerrero, Alfredo Fusco, Rosa Marina Melillo and Massimo Santoro.

Istituto di Endocrinologia ed Oncologia Sperimentale del CNR ”G.Salvatore”, c/o Dipartimento di Biologia e Patologia Cellulare e Molecolare, Universita’ Federico II, 80131 Naples, Italy (VDF, MC, GD, AMC, AF, RMM, MS). Endocrinology and Metabolism Division, UCLA School of Medicine, Los Angeles, California (JMH); and Instituto de Biologia Molecular y Celular del Cancer, Centro de Investigacion del Cancer (Consejo Superior de Investigaciones Cientı´ficas-Universidad de Salamanca), University of Salamanca, Salamanca, Spain (CG).

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Corresponding Author: Massimo Santoro, Dipartimento di Biologia e Patologia Cellulare e Molecolare, via S. Pansini 5, 80131 Naples, Italy. Ph: 39.081.7463056; Fax: 39.081.7463037; E-mail: masantor@unina.it

*These two Authors contributed equally to the work

Abstract

RET/PTC oncoproteins are activated specifically in human thyroid papillary carcinomas (PTC). They result from the in-frame fusion of the RET receptor tyrosine kinase with protein dimerization motifs encoded by heterologous genes. Here we show that the RET/PTC1 oncoprotein activates the Rap1 small GTPase. The activation of Rap1 was dependent on the phosphorylation of tyrosine 1062, the major RET docking site. By co-immunoprecipitation and pull-down experiments, we show that RET/PTC1 recruited a molecular complex containing Gab1 (Grb2-associated binding protein 1), CrkII (v-crk sarcoma virus CT10 oncogene homolog II) and C3G (Rap guanine nucleotide exchange factor 1). By using dominant negative CrkII (SH2CrkII), C3G (C3GΔCat) mutants and small interfering duplex (siRNA) oligonucleotides for Gab1, we show that RET/PTC1-mediated Rap1 activation was dependent on CrkII, C3G and Gab1. RET/PTC1-mediated activation of Rap1 was involved in the stimulation of the BRAF kinase and the p42/p44 mitogen-activated protein kinases (MAPK). Proliferation and stress fibers formation of RET/PTC1-expressing PC Cl 3 thyroid follicular cells was inhibited by the dominant negative Rap1(N17) and by Rap1GAP (Rap1 GTPase activating protein). Thus, Rap1 is a downstream effector of RET/PTC and may contribute to the transformed phenotype of RET/PTC-expressing thyrocytes.

Introduction

In about 30% of the cases, human papillary thyroid carcinomas (PTC) feature chromosomal aberrations that result in the in-frame fusion of the intracellular kinase domain of the RET receptor with the N-terminus of heterologous proteins, generating the RET/PTC oncoproteins (1, 2). RET/PTC1 (the H4-RET fusion) and RET/PTC3 (the NCOA4-RET fusion) are the most prevalent variants. Thyroid-targeted expression of RET/PTC induces PTC formation in transgenic mice, showing that RET/PTC can initiate thyroid carcinogenesis (1, 2). Fusion with protein partners possessing protein-protein interaction motifs provides RET/PTC kinases with dimerizing interfaces, thereby resulting in ligand-independent autophosphorylation and signaling. The RET intracellular domain contains at least 12 autophosphorylation sites (3). Among the autophosphorylated tyrosines, Y1062 plays an important role in RET-mediated cell transformation (4-6). Tyrosine 1062 is embedded in a consensus sequence (NXXpY) for the binding to PTB (Protein Tyrosine Binding) domains. Accordingly, when phosphorylated, it acts as the binding site for several PTB-containing proteins including Shc family adaptors, IRS1/2, FRS2, and DOK1/4/5 (7). Binding to Shc and FRS2 mediates recruitment of Grb2/SOS complexes to RET so leading to GTP exchange and Ras stimulation (7-8). This results in the activation of the BRAF serine/threonine kinase (6, 9-10). However, the role of the various docking proteins that bind tyrosine 1062 in RET is not limited to Ras stimulation. Indeed, by recruiting Grb2, they are also able to mediate RET binding to Gab (Grb2-associated binding protein) family adaptors (Gab1 and Gab2) (8, 11, 12). Gab1/2 are large docking proteins able to associate to many SH2 or SH3 domain-containing proteins, including Grb2, p85, PLCγ, Shc, Shp2 and Crk (13).

Rap1 is a member of the Ras family of small G proteins and it has been implicated in a wide range of biological processes from cell proliferation to cell differentiation and adhesion (14, 15). Although it was initially isolated as a Ras inhibitor (and called KREV for K-Ras revertant) (16), subsequent studies have shown that Rap1, like Ras, can activate the ERK cascade at least in some cell types (14, 17-20). Ras and Rap1 bind to common effectors, including RAF, BRAF, and RalGDS (14). Moreover, Rap1 signaling leads to integrin activation, F-actin fibers formation and cell adhesion (14, 15). Rap1 is activated by transmembrane receptors including tyrosine kinases, G-protein-coupled receptors, cytokine receptors and cell-adhesion proteins and it is switched-off by specific GTPase activating proteins (GAPs) (14, 15). Recently, it has been shown that Rap1b has mitogenic activity in rat thyroid cells (21). However, microinjection of the Rap1 protein failed to induce proliferation of dog thyrocytes demonstrating that Rap1 alone is not sufficient to trigger thyroid cell growth (22); no mutations in Rap1 have been found in thyroid follicular adenomas (23). Here we show that RET/PTC1 activates Rap1 in a tyrosine 1062-dependent fashion and we provide evidence that Rap1 is required for RET/PTC1 induced BRAF activation, mitogenesis and actin cytoskeleton rearrangement.

Materials and Methods

Cell lines

HEK293 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (Invitrogen Groningen, The Netherlands). Transient transfections were carried out with the lipofectamine reagent according to the manufacturer's instructions (Invitrogen). PC Cl 3 (hereafter referred to as "PC") were cultured in Coon's modified Ham F12 medium supplemented with 5% calf serum and a mixture of 6 hormones (6H), including thyrotropin (TSH, 10 mU/ml), hydrocortisone (10 nM), insulin (10 μg/ml), apo-transferrin (5 μg/ml), somatostatin (10 ng/ml) and glycyl-histidyl-lysine (10 ng/ml) (Sigma Chemical Co., St. Louis, MO, USA) (6). PC RET/PTC1, PC RET/PTC1(Y1062F) and PC BRAF(V600E) cells have been described previously (6). For stable transfections, 5 x10^5 cells were plated 48 h before transfection in 60-mm tissue culture dishes. The medium was changed to DMEM containing 5% calf serum and 6H. Three hours later, calcium phosphate DNA precipitates were incubated with the cells for 1 h. Cells were then incubated with 15% glycerol in HEPES-buffered saline for 2 min. Mass populations of several cell clones and single clones were obtained by puromycin (2 μg/ml) selection. The PC Cl 3 cell line expressing RET/PTC1 in a doxycyline dependent manner was a kind gift of Dr J. A. Fagin and obtained by sequential stable transfection with expression vectors for the tetracycline(tet)-dependent trans-activating rtTA protein and for tet-inducible RET/PTC1 (24). To induce RET/PTC expression, cells were treated for different times with 1 μg/ml doxycycline. The RET/PTC1-positive TPC1, FB2, BHP2-7 and BHP10-3 human papillary thyroid carcinoma cell lines and the P5 primary culture of normal human thyroid follicular cells were grown as described elsewhere (25).

Plasmids

All the RET constructs used in this study were cloned in pCDNA3.1(Myc-His) (Invitrogen); they encode the short (RET-9) RET spliced form and are described elsewhere (6). For simplicity, we numbered the residues of RET/PTC (PTC) proteins as the corresponding residues in unrearranged RET. Briefly, RET/PTC1(K-) is a kinase-dead mutant, carrying the substitution of the catalytic lysine (residue 758 in full-length RET) with a methionine. RET/PTC1(Y1062F) carries the substitution of the indicated tyrosine with phenylalanine. Plasmids encoding Rap1 (Rap1a), the constitutively active Rap1(V12), the dominant-negative Rap1(N17), Rap1GAP, AU5-tagged constitutively activated Rho(QL) and Ras(V12) were kind gifts of J. S. Gutkind. LTR2 vectors encoding full-lenght C3G and C3GΔCAT mutant, lacking the C-terminal half of the protein (last 457 bp) containing the catalytic domain, were obtained from E. Santos and C. Guerrero (26). The pcDNA3 expression vector encoding Gab1 was a kind gift of P. Gual and S. Giordano (27). The pCMV6-CrkII plasmid was from OriGene Technologies, Inc. (Rockville, MD, USA). The plasmid encoding the myc-tagged isolated SH2 domain of CrkII (residues 13-118) (SH2CrkII) was obtained by PCR and subcloning into pCDNA3.1(Myc-His) (Invitrogen). The plasmid encoding enhanced Green Fluorescent Protein (pEGFP) was from Clontech (Mountain View, CA, USA).

Antibodies

Anti-RET is an affinity-purified polyclonal antibody raised against the tyrosine kinase protein fragment of human RET. Anti-phospho p44/42 MAPK (#9102), specific for MAPK (ERK1/2) phosphorylated at Thr202/Tyr204, anti-p44/42 MAPK (#9101), anti-phospho MEK1/2 (MAP kinases 1 and 2) (#9121), specific for MEK1 and MEK2

phosphorylated at Ser217/Ser221, and anti-MEK1/2 (#9122) were purchased from Cell Signaling (Beverly, MA, USA). Anti-Rap1 (sc-65), anti-myc antibody (sc-40), anti-C3G (C-19) (sc-869), anti-BRAF (sc-9002), anti-CrkII (sc-289) and anti-RhoA (sc-179) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-Gab1, anti-phosphotyrosine and anti-Ras (clone 10) antibodies were from Upstate Biotechnology Inc, (Lake Placid, NY, USA). Monoclonal anti-AU5 (MMS135R) was from BabCO (Berkeley Antibody Co Inc, Richmond CA, USA). Monoclonal anti-α tubulin was from Sigma Chemical Co. Secondary antibodies coupled to horseradish peroxidase were from Amersham Pharmacia Biotech (Little Chalfort, UK).

Protein studies

Protein extractions and immunoblotting experiments were performed according to standard procedures. Protein concentration was estimated with a modified Bradford assay (Bio-Rad, Munich, Germany). Immune complexes were detected with the enhanced chemiluminescence kit (ECL, Amersham). Signal intensity was analyzed at the Phosphorimager (Typhoon 8600, Amersham Pharmacia Biotech) interfaced with the ImageQuant software.

BRAF kinase assay

The BRAF kinase assay was performed as previously described (6). Briefly, HEK293 cells were transiently transfected with the indicated vectors, cultured for 18 h in serum-deprived medium and harvested. BRAF kinase was immunoprecipitated with the anti-BRAF antibody and resuspended in a kinase buffer (20 mM MOPS pH 7.2, 25 mM β-glycerol phosphate, 5 mM EGTA, 1 mM sodium orthovanadate, 1 mM dithiothreitol) containing

100 mM $MgCl_2$, 20 μM ATP and 0.5 μg of recombinant GST MEK (Upstate Biotechnology Inc.). After 15 min incubation at 30˚C, reactions were stopped by adding 2X Laemmli buffer. Proteins were then subjected to 10% SDS gel electrophoresis and immunoblotted with anti-phospho-MEK1/2 antibodies. Filters were then reprobed with anti-MEK and anti-BRAF.

Small GTPases activity assays

Small GTPases activity assays were performed as described elsewhere (28). Briefly, cells were serum starved for 8h and lysed at 4°C in a buffer containing 20 mM HEPES, pH 7.4, 0.1 M NaCl, 1% Triton X-100, 10 mM EGTA, 40 mM β-glycerophosphate, 20 mM MgCl2, 1 mM Na3VO4, 1 mM dithiothreitol, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride. Lysates were incubated for 30 min with glutathione sepharose beads coupled to GST (glutathione-S-transferase) fusion proteins containing either the Rap1-binding domain (RalGDS), or the Ras-binding domain (RafRBD), or the Rho-binding domain (GST-RhotekinGDS). Total Rap1, Ras and RhoA levels and levels of their GTP-loaded forms (bound to GST–fusion proteins) were determined by immunoblot.

Cell growth and staining

For growth curves, $0.5x10^5$ cells were seeded in complete medium and counted at the indicated time points. DNA synthesis was measured by the 5'-bromo-3'-deoxyuridine (BrdU) Labeling and Detection Kit from Boehringer Mannheim (Germany). Briefly, cells were seeded on glass coverslips, incubated for 1 h with BrdU (final concentration of 10 μM), fixed and permeabilized with ethanol-glycine solution. Coverslips were incubated with anti-BrdU mouse monoclonal antibody and with a rhodamine-conjugated secondary

antibody (Jackson ImmunoResearch Laboratories, Philadelphia, Pennsylvania) and mounted in Moviol on glass slides. Cell nuclei were identified by Hoechst 33258 (final concentration 1 μg/ml; Sigma Chemicals Co) staining. The fluorescent signal was visualized with an epifluorescent microscope (Axiovert 2, Zeiss) (equipped with a 100X lens) interfaced with the image analyzer software KS300 (Zeiss). At least 100 GFP-positive cells were counted in five different microscopic fields.

For actin staining, cells were seeded on glass coverslips, fixed with paraformaldeyde (4%) and permeabilized with Triton X-100 (0.2%). Actin rearrangements were revealed by treating coverslips with Texas Red-X-conjugated phalloidin (Molecular Probes) for 1 h. For transient experiments, cells were transiently transfected as described above. Briefly, various combinations of plasmids were co-transfected with pEGFP in a 1:10 ratio. After 36 h, cells were plated, fixed at different time points and stained. All coverslips were counterstained with PBS containing Hoechst 33258 (final concentration 1 μg/ml; Sigma Chemicals Co), rinsed and mounted in Moviol. The fluorescent signal was visualized with an epifluorescent microscope (Axiovert 2, Zeiss) (Oberkochen, Germany) interfaced with the image analyzer software KS300 (Zeiss). Experiments were perfomed in triplicate and at least 60 EGFP-positive cells were scored for each transfection. To assess statistical significance, Bonferroni multiple comparisons test was applied and the Instat software program (Graphpad Software Inc). Differences were significant at $P < 0.01$.

Reporter assay

The pSRF-luc plasmid contains the luciferase (*Firefly* luciferase) reporter gene driven by a basic promoter element (TATA box) joined to tandem repeats of SRF binding elements (Stratagene, La Jolla, CA) (29). HEK293T cells were transfected with the required

expression vector together with 0.1μg of the pSRF-luc reporter and 0.01μg of phRL, which expresses *Renilla* luciferase from *Renilla reniformis* for normalization. The total amount of plasmid DNA was adjusted with pcDNA3-β-Galactosidase. *Firefly* and *Renilla* luciferase activities were assayed using the Dual-Luciferase Reporter System (Promega Corporation, Madison, WI). Light emission was quantitated using a Berthold Technologies luminometer (Centro LB 960). Experiments were performed in triplicate and data were represented as average ± S.D.

RNA silencing

Small inhibitor duplex RNAs targeting Gab1 were as described elsewhere (30) and were chemically sinthesized by PROLIGO (Boulder, CO). The scrambled control oligo was: 5'-AGGAUAGCGUGGAUUUCGGUTT-3'. For siRNA transfection, cells were grown under standard conditions. The day before transfection, cells were plated in six-well dishes at 30–40% confluency. Transfection was performed using 5 μg of duplex RNA and 6 μl of Oligofectamine reagent (Invitrogen). Cells were harvested 48h after transfection.

Results

RET/PTC1 activates Rap1 in a kinase- and Y1062-dependent manner-Vectors expressing wild-type and mutant RET/PTC1 were transiently transfected along with Rap1 into HEK293 cells. The RET/PTC1 mutants used were a kinase dead mutant, PTC1(K-), and PTC1(Y1062F), carrying the substitution of tyrosine 1062 to phenylalanine (Fig. 1A). Clarified protein lysates were pulled-down with GST-RalGDS, which binds specifically to GTP-loaded activated Rap1. Immunoblotting with anti-Rap1 specific antibodies revealed that Rap1 activity was stimulated by RET/PTC1 (> 5- folds) (Fig. 1B). Rap1 stimulation required RET/PTC1 kinase activity as well as the integrity of tyrosine 1062 (Fig. 1B).

A Gab1-CrkII-C3G complex mediates RET/PTC1-induced Rap1 activation-Rap1 is activated by numerous GEFs (Guanine-nucleotide Exchange Factors) like C3G, cAMP-GEFs (Epac), CalDAG-GEF and PDZ-GEF (14, 15). C3G forms a complex with the Crk adaptor proteins by binding to the N-terminal SH3 domain of Crk (31). The C3G-Crk complex is activated via the interaction between the Crk SH2 domain and phosphorylated tyrosines. The Gab1 docking protein contains six potential binding sites for the SH2 of Crk (31, 32). Since it has been reported that RET binds to Gab proteins (8, 11, 12), we hypothesized that Gab1, Crk and C3G may be involved in RET/PTC1-mediated Rap1 activation. To investigate this possibility, we examined whether RET/PTC1 was able to form a molecular complex with Gab1. Co-expression of RET/PTC1 and Gab1 in HEK293 cells, followed by RET/PTC immunoprecipitation and Gab1 immunoblot, showed that the two proteins interacted *in vivo* (Fig. 2A, upper panel). Gab1 immunoprecipitation and phosphotyrosine immunoblot showed that the binding resulted in Gab1 phosphorylation

(Fig. 2A, middle). This was demonstrated also by the mobility shift of Gab1 in the presence of RET/PTC1 (Fig. 2A). RET/PTC1-Gab1 binding was dependent on the integrity of tyrosine 1062, since the amount of co-immunoprecipitated Gab1 (Fig. 2A, upper panel), its mobility shift (Fig. 2A, upper panel) and its tyrosine phosphorylation levels (Fig. 2A, middle) were affected by the Y1062F mutation.

Then, HEK293 cells were co-transfected with Gab1, CrkII and RET/PTC1. Protein lysates were immunoprecipitated with anti-CrkII antibodies and blotted with phosphotyrosine (Fig. 2B). The various phosphorylated bands co-precipitated with CrkII were identified by subsequent reprobing of the filters with specific antibodies. CrkII co-precipitated with tyrosine phosphorylated Gab1 and RET/PTC1. As expected, the complex did not form in the absence of RET/PTC1 or in the presence of the RET/PTC1(Y1062F) mutant (Fig. 2B).

Thus, HEK293 cells were transfected only with RET/PTC1 and protein lysates were immunoprecipitated with anti-CrkII and blotted with phosphotyrosine. The protein complex was detectable also when Gab1 and CrkII were expressed at endogenous levels (Fig. 2C).

We asked whether C3G (Rap1 GEF) was recruited to the RET/PTC1-Gab1-CrkII complex. HEK293 cells were transfected with CrkII alone or together with RET/PTC1 and C3G. As shown in Figure 2D, a complex containing tyrosine phosphorylated C3G and Gab1 was formed on wild type RET/PTC1 but not on the Y1062F mutant (Fig. 2D, upper panel). By immunoprecipitating CrkII, we could demonstrate that C3G complexed with CrkII constitutively and that this interaction was enhanced in the presence of RET/PTC1 (Fig. 2D, middle).

We used a dominant-negative plasmid encoding the isolated myc-tagged CrkII-SH2 domain (SH2CrkII) to test whether C3G recruitment to the complex was dependent on CrkII. HEK293 cells were co-transfected with Gab1 and C3G and, where indicated, RET/PTC1 and the SH2CrkII plasmid. Protein lysates were immunoprecipitated with an anti-CrkII that does not react with its SH2 domain and blotted with phosphotyrosine, C3G or Gab1 antibodies. The expression of SH2CrkII decreased the amount of both Gab1 and RET/PTC1 co-immunoprecipitated with endogenous CrkII (Fig. 3A). Also C3G binding to endogenous CrkII decreased in the presence of SH2CrkII (Fig. 3A).

Finally, to investigate whether both CrkII and C3G were required for RET/PTC1-induced Rap1 activation, we used the isolated SH2CrkII domain and C3GΔCat, a C3G mutant devoid of the GEF activity. In the presence of RET/PTC1, the amount of active Rap1 precipitated with GST-RalGDS was clearly inhibited by both SH2CrkII and C3GΔCat (Fig. 3B).

Rap1 is involved in RET/PTC1-induced BRAF/MAPK activation-RET/PTC activates the MAPK (mitogen-activated protein kinases) (p42/p44 ERKs) through its tyrosine 1062 and BRAF activation (6). We reasoned that although BRAF activation by RET/PTC was dependent on Ras (6), it was possible that Rap1 contributed to this activation, as well. Therefore, we performed *in vitro* BRAF kinase assays by immunoprecipitating BRAF and measuring its ability of phosphorylating *in vitro* GST-MEK (MAP kinase kinase or ERK kinase). As shown in Figure 4A, the activity of BRAF increased in HEK293 cells transfected with RET/PTC1. BRAF stimulation was blunted when RET/PTC1 was co-expressed with Rap1GAP (Rap1-specific GTPase activating protein), SH2CrkII or C3GΔCAT (Fig. 4A). As a control, Rap1(V12) expression was also able to stimulate

BRAF activity (Fig. 4A). To exclude that the overexpression of Rap1GAP, SH2CrkII or C3GΔCAT exerted off-target effects on Ras that may, in turn, be responsible for BRAF inhibition, we also controlled GTP-loading on Ras by pull-down with GST-RafRBD. Ras activation by RET/PTC was not affected by Rap1GAP, SH2CrkII or C3GΔCAT expression (Fig. 4B).

MEK and MAPK activation by RET/PTC1 was measured with phosphospecific antibodies (Ser217/Ser221 and Thr202/Tyr204, respectively). Phosphorylation was only reduced but not abrogated by the co-expression of Rap1GAP, SH2CrkII or C3GΔCAT (Fig. 4C, left panel). This was consistent with the possibility that mutiple pathways (including Rap1 and Ras) lead to BRAF/MAPK stimulation by RET/PTC. Thus, we compared the relative contribution of Ras and Rap1 on MEK/MAPK phosphorylation. As shown in Fig. 4C (right panel), both Rap1(V12) and Ras(V12), when transiently expressed in HEK293 cells, were able to trigger MEK and MAPK phosphorylation, with Ras(V12) being about 2-fold more active than Rap1(V12).

Rap1 activation in RET/PTC-positive human thyroid cancer cells.-We used 4 human thyroid papillary carcinoma cell lines endogenously expressing RET/PTC1 (FB2, TPC1, BHP2-7, BHP10-3) to verify RET/PTC-mediated Rap1 activation. High levels Rap1 activation were found in RET/PTC1-positive cell lines compared to a primary culture of normal thyroid cells (P5) used as a control (Fig. 5A). By co-immunoprecipitation, we could also show that a RET/PTC1-Gab1-CrkII-C3G protein complex was present in the RET/PTC1-positive cell lines (Fig. 5B). Small interfering duplex (siRNA) oligonucleotides (si Gab1) were used to knock-down Gab1 expression. The transfection in two different cancer cell lines (BHP10-3 and BHP2-7) of the Gab1 siRNA, but not of the scrambled

control (si scr), reduced Gab1 protein levels; this, in turn, resulted in the suppression of Rap1 activation (Fig. 5C, lower panel) and MEK/MAPK phosphorylation (Fig. 5C, upper panel).

Rap1 is required for cell growth and stress fibers formation in RET/PTC1-expressing thyrocytes -PC Cl 3 (hereafter referred to as "PC"), a continuous line of follicular thyroid cells, constitutes a model system with which to study growth regulation in an epithelial thyroid cell setting. These cells require a mixture of 6 hormones (6H), including TSH (thyrotropic hormone) for proliferation. RET/PTC1 expression in these cells induces hormone-independent proliferation in a Y1062-dependent manner (6). We initially verified whether the pathway described above was active in PC cells. Fig. 6A (upper panel) shows that, indeed, in PC cells stably expressing RET/PTC1 (PC PTC1), but not in those expressing the Y1062F mutant, endogenous CrkII and Gab1 co-immunoprecipitated with RET/PTC1. Furthermore, Fig. 6A (lower panel) shows that Rap1 activity, assessed by the GST-RalGDS pull-down assay, was increased in RET/PTC1- but not in RET/PTC1(Y1062F)-expressing cells. To further support these findings, we used a PC cell line, in which the expression of RET/PTC1 was under the control of a doxycyline dependent promoter (24). As shown in Figure 6B, Rap1 activation followed doxycyline-induced RET/PTC1 expression, and peaked at 48h, when RET/PTC1 levels were the highest. The wash-out of doxycycline decreased both RET/PTC1 expression and Rap1 activation (Fig. 6B).

PC PTC1 cells were transiently co-transfected with plasmids encoding Rap1GAP or the dominant negative Rap1(N17) together with trace amounts of EGFP (enhanced Green Fluorescent Protein) to track transfected cells. Cells were kept in the absence of

TSH and BrdU incorporation was assessed by epifluorescence to monitor DNA synthesis rate. As shown in Figure 6C, both Rap1(N17) and Rap1GAP exerted a marked inhibitory effect on DNA synthesis of PC PTC1 cells (Fig. 6C, left). We established a mass population of several clones of PC cells stably co-expressing RET/PTC1 (PC PTC1) and Rap1(N17). Cell growth was measured by counting cells at different time points. Also in this setting, Rap1(N17) co-expression blunted PC PTC1 cell proliferation in the absence of 6H (Fig. 6C, right).

One of the well-known effects of Rap1 is its ability to induce cell adhesion to the substrate, cell-cell adhesion, cell spreading and cell migration. This is likely mediated by its ability of promoting integrin activation (15). RET/PTC-expressing PC cells feature prominent actin bundles organized in stress fibers and this depends on the integrity of Y1062 (33). We used Texas Red X-conjugated phalloidin staining to analyse actin rearrangement in PC PTC1 cells transiently transfected with Rap1GAP, Rap1(N17), SH2CrkII, or C3GΔCat. Representative micrographs are shown in Fig. 6D (upper panel). As previously reported (33), PC PTC1 cells were highly adhesive to the plastic dish and formed a remarkable amount of stress fibers few hours (4 h) after plating. All the mutants interfering with Rap1 activation, but not the active Rap1(V12) (not shown), inhibited stress fibers formation (Fig. 6D, upper panel). PC PTC1 cells positive for stress fibers were 82.6 ± 6.7% in vector-transfected cells and 12.8 ± 4.2% ($P< 0.001$), 29.4 ± 4.2% ($P< 0.001$), 16.7 ± 3.3% ($P< 0.001$), and 30.6 ± 2.5% ($P< 0.001$) in cells transfected with Rap1GAP, Rap1(N17), SH2CrkII or C3GΔCat, respectively. Accordingly, PC PTC1 cells stably expressing Rap1(N17) displayed a rounded morphology and a remarkably reduced number of cells (< 25%) positive for stress fibers even several days after plating (Fig. 6D, lower panel). In stark contrast, PC cells stably expressing the constitutively active Rap1(V12)

rapidly (4 h after plating) formed tick actin bundles (> 65% of the cells) (Fig. 6D, lower panel). Constitutively active BRAF(V600E)-expressing PC cells also formed stress fibers after plating (~40% of the cells) (Fig. 6D, lower panel).

It is well established that the RhoA GTPase is necessary for stress fibers formation (34). RhoA activates gene transcription *via* the activation of the Serum Responsive Factor (SRF) (35); this is believed to be a consequence of the actin reorganization induced by RhoA resulting in a decrease in the cellular pool of unpolymerized actin (G-actin) (36). Thus, we measured RhoA activation by a pull-down assay with a GST-RhotekinGDS bait. In transiently transfected HEK293, RET/PTC stimulated RhoA activation and this was dependent on the integrity of Y1062 and was obstructed by Rap1GAP and Rap1(N17); Rap1(V12) stimulated RhoA as well (Fig. 7A). Then, we used a luciferase reporter gene driven by tandem repeats of SRF binding elements; as a positive control activated RhoA(QL) was used (29). Average results ± SD are reported in Fig. 7B. According to their ability to signal actin polymerization through RhoA, both Rap1(V12) (~10 fold) and RET/PTC1 (~4 fold) stimulated pSRF-luc activity in HEK293 cells. Constitutively active BRAF(V600E) also stimulated pSRF-luc. In the case of RET/PTC1, this was obstructed by the co-expression of Rap1GAP, SH2CrkII or C3GΔCat (Fig. 7B).

Discussion

In this study, we show that a large molecular complex forms on tyrosine 1062, the major RET/PTC1 docking site, and mediates stimulation of the Rap1 small GTPase (Fig. 7C). Such a complex includes Gab1, CrkII and C3G. Gab1 is recruited to tyrosine 1062 of RET oncoproteins *via* its binding to the SH3 domain of Grb2 (8, 11, 12). We show that, in the presence of RET/PTC1, CrkII is recruited to Gab1 and CrkII, in turn, recruits C3G. Crk proteins exist in constitutive complexes with C3G in different cell types (31) and the Crk-C3G binding can be further enhanced by Crk recruitment to membrane receptors (37, 38). By the use of dominant negative CrkII (SH2CrkII) and C3G (C3GΔCat) mutants, we have found that the Gab1-CrkII-C3G complex was responsible for RET/PTC1-mediated Rap1 activation. Such a molecular mechanism is similar to that described for Rap1 stimulation mediated by Met, the hepatocyte growth factor receptor (39). It has been recently reported that Y1062 in RET/PTC is necessary for the activation of Ras and the BRAF/MAPK pathway; this in turn is essential for RET/PTC-mediated thyroid cell growth (6, 9, 10). Here we add further complexity to this cascade by showing that Rap1 may function in parallel with Ras by mediating the activation of BRAF and the MAPK pathway by RET/PTC.

We also report that some phenotypic effects induced by RET/PTC1 in PC thyroid cells, e.g. mitogenesis and stress fibers formation, require Rap1 activation. Several reports show that BRAF is a key player in thyroid cells growth, as it regulates the RET/PTC-mediated MAPK activation and TSH-independent proliferation (6, 9, 10). Furthermore, about 45% of human PTC feature activating mutations in BRAF (40). Therefore, it is likely that the role of Rap1 in RET/PTC-mediated thyrocyte proliferation relies on its ability of participating to BRAF stimulation. Although the mitogenic activity of Rap1 for rat thyroid

cells has been described (21) it should be noted that activated Rap1 was itself insufficient to induce cell-cycle entry of dog thyroid cells; therefore, it is likely that Rap1 exerts its growth-promoting ability only in conjunction with additional stimuli (22).

As far as the role of Rap1 in the actin cytoskeleton remodeling of RET/PTC-expressing PC cells, we show that this results in RhoA-mediated actin polymerization, formation of actin stress fibers and SRF-stimulated gene transcription. It it well-known that Rap1 regulates, from inside the cell, all integrins (β1, β2 and β3 families) that are associated with the actin cytoskeleton, by controlling their activity and clustering (15). Effectors of Rap1 that act in these processes are among the others: AF-6 (a multidomain adaptor protein that binds various cell junction proteins), RapL (a small protein binding both Rap1 and integrins), Riam (a protein that binds Rap1 and induces integrin-mediated cell adhesion), Arap3 (a Rap1 effector protein that contains a RhoGAP domain) and some GEFs (Vav2 and Tiam1) for Rho-family GTPases (15). Given the ability of Rap1 in stimulating cell adhesion in many cell types, it is likely that its effects on the actin cytoskeleton observed in PC PTC cells are, at least in part, indirect and mediated by stimulation of cell adhesion and spreading. However, since some of the proteins interacting with Rap1 directly modulate Rho-family smallGTPases, a more direct biochemical link between Rap1 and RhoA activation in RET/PTC-transformed thyrocytes could also be envisaged. This pathway (RET/PTC-Rap1-RhoA) could be distinct to that mediating BRAF and MAPK stimulation, as suggested by the notion that PC cells transformed by Ras (another potent trigger of the MAPK pathway) are negative for stress fibers formation (33). However, the two pathways may cross-talk at some levels; indeed, we observed that also PC cells expressing BRAF(V600E) contain stress fibers and BRAF has been previously described as a regulator of stress fibers formation in other cell systems (41).

Recent studies have shown that Rap1 can be implicated in tumorigenesis. Mutations in one RapGEF, CalDAG-GEF1, have been found secondary to a proviral insertion in myeloid leukemia in BXH-2 mice (42) and Rap1GAP has been identified as a putative tumor suppressor gene in pancreatic cancer (43). Moreover, mice deficient in SPA-1, a second Rap1GAP, developed disorders that resemble chronic myeloid leukemia (44). Finally, another Rap1GAP, E6TP1, is targeted for ubiquitin-mediated degradation by the papillomavirus E6 oncoprotein (45). Although Rap1 mutations have not been found in human thyroid tumors (23), given the role of Rap1 in the RET/PTC signaling cascade, it is conceivable that Rap1 or proteins involved in Rap1 signaling might be implicated in PTC tumorigenesis.

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References

1. Nikiforov YE. RET/PTC rearrangement in thyroid tumors. Endocr Pathol 2002;13:3-16.
2. Fagin JA. How thyroid tumors start and why it matters: kinase mutants as targets for solid cancer pharmacotherapy. J Endocrinol 2004;183:249-56.
3. Kawamoto Y, Takeda K, Okuno Y, et al. Identification of RET autophosphorylation sites by mass spectrometry. J Biol Chem 2004;279:14213-24.
4. Asai N, Murakami H, Iwashita T, Takahashi M. A mutation at tyrosine 1062 in MEN2A-Ret and MEN2B-Ret impairs their transforming activity and association with shc adaptor proteins. J Biol Chem 1996;271:17644-9.
5. Segouffin-Cariou C, Billaud M. Transforming ability of MEN2A-RET requires activation of the phosphatidylinositol 3-kinase/AKT signaling pathway. J Biol Chem 2000;275:3568-76.
6. Melillo RM, Castellone MD, Guarino V, et al. The RET/PTC-RAS-BRAF linear signaling cascade mediates the motile and mitogenic phenotype of thyroid cancer cells. J Clin Invest 2005;115:1068-81.
7. Ichihara M, Murakumo Y, Takahashi M. RET and neuroendocrine tumors. Cancer Lett 2004;204:197-211.
8. Hayashi H, Ichihara M, Iwashita T, et al. Characterization of intracellular signals via tyrosine 1062 in RET activated by glial cell line-derived neurotrophic factor. Oncogene 2000;19:4469-75.
9. Mitsutake N, Knauf JA, Mitsutake S, Mesa C Jr, Zhang L, Fagin JA. Conditional BRAFV600E expression induces DNA synthesis, apoptosis, dedifferentiation, and chromosomal instability in thyroid PCCL3 cells. Cancer Res 2005;65:2465-73.

10. Mitsutake N, Miyagishi M, Mitsutake S, et al. BRAF mediates RET/PTC-induced mitogen-activated protein kinase activation in thyroid cells: functional support for requirement of the RET/PTC-RAS-BRAF pathway in papillary thyroid carcinogenesis. Endocrinology 2006;147:1014-9.
11. Besset V, Scott RP, Ibanez CF. Signaling complexes and protein-protein interactions involved in the activation of the Ras and phosphatidylinositol 3-kinase pathways by the c-Ret receptor tyrosine kinase. J Biol Chem 2000;275:39159-66.
12. De Falco V, Guarino V, Malorni L, et al. RAI(ShcC/N-Shc)-dependent recruitment of GAB 1 to RET oncoproteins potentiates PI 3-K signalling in thyroid tumors. Oncogene 2005;24:6303-13.
13. Gu H, Neel BG. The "Gab" in signal transduction. Trends Cell Biol 2003;13:122-30.
14. Stork PJ. Does Rap1 deserve a bad Rap? Trends Biochem Sci 2003;28:267-75.
15. Bos JL. Linking Rap to cell adhesion. Curr Opin Cell Biol 2005;17:123-8.
16. Kitayama H, Sugimoto Y, Matsuzaki T, Ikawa Y, Noda M. A ras-related gene with transformation suppressor activity. Cell 1989;56:77-84.
17. Vossler MR, Yao H, York RD, Pan MG, Rim CS, Stork PJ. cAMP activates MAP kinase and Elk-1 through a B-Raf- and Rap1-dependent pathway. Cell 1997;89:73-82.
18. York RD, Yao H, Dillon T, et al. Rap1 mediates sustained MAP kinase activation induced by nerve growth factor. Nature 1998;392:622-6.
19. Mishra S, Smolik SM, Forte MA, Stork PJ. Ras-independent activation of ERK signaling via the torso receptor tyrosine kinase is mediated by Rap1. Curr Biol 2005;15:366-70.

20. Mizuchi D, Kurosu T, Kida A, et al. BCR/ABL activates Rap1 and B-Raf to stimulate the MEK/Erk signaling pathway in hematopoietic cells. Biochem Biophys Res Commun 2005;326:645-51.

21. Ribeiro-Neto F, Urbani J, Lemee N, Lou L, Altschuler DL. On the mitogenic properties of Rap1b: cAMP-induced G(1)/S entry requires activated and phosphorylated Rap1b. Proc Natl Acad Sci U S A 2002; 99:5418-23.

22. Dremier S, Vandeput F, Zwartkruis FJ, Bos JL, Dumont JE, Maenhaut C. Activation of the small G protein Rap1 in dog thyroid cells by both cAMP-dependent and -independent pathways. Biochem Biophys Res Commun 2000;267:7-11.

23. Vanvooren V, Allgeier A, Nguyen M, et al. Mutation analysis of the Epac-Rap1 signaling pathway in cold thyroid follicular adenomas. Eur J Endocrinol 2001;144:605-10.

24. Saavedra HI, Knauf JA, Shirokawa JM, et al. The RAS oncogene induces genomic instability in thyroid PCCL3 cells via the MAPK pathway. Oncogene 2000;19:3948-54.

25. Vitagliano D, Carlomagno F, Motti ML, et al. Regulation of p27Kip1 protein levels contributes to mitogenic effects of the RET/PTC kinase in thyroid carcinoma cells. Cancer Res 2004;64:3823-9.

26. Guerrero C, Fernandez-Medarde A, Rojas JM, Font de Mora J, Esteban LM, Santos E. Transformation suppressor activity of C3G is independent of its CDC25-homology domain. Oncogene 1998;16:613-24.

27. Gual P, Giordano S, Anguissola S, Comoglio PM. Differential requirement of the last C-terminal tail of Met receptor for cell transformation and invasiveness. Oncogene 2001;20:5493-502.

28. Servitja JM, Masgrau R, Pardo R, et al. Metabotropic glutamate receptors activate phospholipase D in astrocytes through a protein kinase C-dependent and Rho-independent pathway. Neuropharmacology 2003;44:171-80.

29. Marinissen MJ, Chiariello M, Tanos T, Bernard O, Narumiya S, Gutkind JS. The small GTP-binding protein RhoA regulates c-jun by a ROCK-JNK signaling axis. Mol Cell 2004;14:29-41.

30. Jin ZG, Wong C, Wu J, Berk BC. Flow shear stress stimulates Gab1 tyrosine phosphorylation to mediate protein kinase B and endothelial nitric-oxide synthase activation in endothelial cells. J Biol Chem 2005;280:12305-9.

31. Feller SM. Crk family adaptors-signalling complex formation and biological roles. Oncogene 2001;20:6348-71.

32. Kao S, Jaiswal RK, Kolch W, Landreth GE. Identification of the mechanisms regulating the differential activation of the mapk cascade by epidermal growth factor and nerve growth factor in PC12 cells. J Biol Chem 2001;276:18169-77.

33. Barone MV, Sepe L, Melillo RM, et al. RET/PTC1 oncogene signaling in PC Cl 3 thyroid cells requires the small GTP-binding protein Rho. Oncogene 2001;20:6973-82.

34. Ridley AJ, Hall A. The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. Cell 1992;70:389-99.

35. Hill CS, Wynne J, Treisman R. The Rho family GTPases RhoA, Rac1, and CDC42Hs regulate transcriptional activation by SRF. Cell 1995;81:1159-70.

36. Sotiropoulos A, Gineitis D, Copeland J, Treisman R. Signal-regulated activation of serum response factor is mediated by changes in actin dynamics. Cell 1999;98:159-69.

37. Okada S, Matsuda M, Anafi M, Pawson T, Pessin JE. Insulin regulates the dynamic balance between Ras and Rap1 signaling by coordinating the assembly states of the Grb2-SOS and CrkII-C3G complexes. EMBO J 1998;17:2554-65.

38. Ichiba T, Hashimoto Y, Nakaya M, et al. Activation of C3G guanine nucleotide exchange factor for Rap1 by phosphorylation of tyrosine 504. J Biol Chem 1999; 274:14376-81.

39. Sakkab D, Lewitzky M, Posern G, et al. Signaling of hepatocyte growth factor/scatter factor (HGF) to the small GTPase Rap1 via the large docking protein Gab1 and the adapter protein CRKL. J Biol Chem 2000;275:10772-8.

40. Xing M. BRAF mutation in thyroid cancer. Endocr Relat Cancer 2005;12:245-62.

41. Pritchard CA, Hayes L, Wojnowski L, Zimmer A, Marais RM, Norman JC. B-Raf acts via the ROCKII/LIMK/cofilin pathway to maintain actin stress fibers in fibroblasts. Mol Cell Biol 2004;24:5937-52.

42. Dupuy AJ, Morgan K, von Lintig FC, et al. Activation of the Rap1 guanine nucleotide exchange gene, CalDAG-GEF I, in BXH-2 murine myeloid leukemia. J Biol Chem 2001;276:11804-11.

43. Zhang L, Chenwei L, Mahmood R, et al. Identification of a Putative Tumor Suppressor Gene Rap1GAP in Pancreatic Cancer. Cancer Res 2006;66:898-906.

44. Ishida D, Kometani K, Yang H, et al. Myeloproliferative stem cell disorders by deregulated Rap1 activation in SPA-1-deficient mice. Cancer Cell 2003;4:55-65.

45. Singh L, Gao Q, Kumar A, et al. The high-risk human papillomavirus type 16 E6 counters the GAP function of E6TP1 toward small Rap G proteins. J Virol 2003;77:1614-20.

Figure legends

Figure 1

A) Schematic representation of the RET/PTC1 constructs used in this study. SP: RET signal peptide, EC: extracellular domain, Cys: cysteine-rich, TM: transmembrane, JX: juxtamembrane, TK: tyrosine kinase. The RET/PTC breakpoint is indicated. RET tyrosine 1062 (corresponding to Y451 in RET/PTC1) and lysine 758 (corresponding to K147 in RET/PTC1) are indicated. B) Affinity precipitation of Rap1 with immobilized GST-RalGDS fusion protein from HEK293 transfected with the indicated RET/PTC1 constructs or the empty vector (-). Bound Rap1 was revealed by staining blot with anti-Rap1. Whole cell lysates were analyzed for Rap1 protein expression for normalization. The signal was analyzed at the Phosphorimager. This experiment is representative of at least four independent assays.

Figure 2

A) Gab1 recruitment to RET/PTC1 and tyrosine phosphorylation depends on tyrosine 1062. Protein lysates (500μg) from HEK293 transfected with Gab1 and the indicated RET/PTC plasmids were immunoprecipitated with anti-RET and immunoblotted with anti-Gab1 or anti-RET for normalization (upper panel). Alternatively, protein lysates were immunoprecipitated with anti-Gab1 and immunoblotted with anti-phosphotyrosine antibodies (middle). Total Gab1 levels were determined for normalization (lower panel). B) CrkII co-immunoprecipitates with tyrosine-phosphorylated RET/PTC1 and Gab1. Protein lysates (500μg) from HEK293 transfected with CrkII, and where indicated Gab1 and RET/PTC1, were immunoprecipitated with anti-CrkII and immunoblotted with anti-

phosphotyrosine. RET/PTC1 and Gab1 were identified by reprobing with specific antibodies (not shown). CrkII levels are shown for normalization. C) Endogenous CrkII and Gab1 co-immunoprecipitate with RET/PTC1. Protein lysates (2mg) from HEK293 transfected with RET/PTC1 or its mutant Y1062F were immunoprecipitated with anti-CrkII and immunoblotted with anti-phosphotyrosine. RET/PTC1 and Gab1 were identified by reprobing with specific antibodies (not shown). D) RET/PTC1-stimulated CrkII-C3G binding. Protein lysates (500μg) from HEK293 transfected with the indicated plasmids, in the presence or not of RET/PTC1, were immunoprecipitated with anti-RET and blotted with phosphotyrosine, C3G or RET (upper panel) or immunoprecipitated with anti-CrkII and immunoblotted with C3G or phosphotyrosine antibodies (middle). In the lower panel, total cell lysates were immunoblotted with the indicated antibodies for normalization. These findings are representative of several independent assays.

Figure 3

A) Gab1 and C3G recruitment by RET/PTC1 depends on CrkII. Cells were transfected with C3G and Gab1 with or without the other plasmids where indicated. Proteins were immunoprecipitated with CrkII and blotted with the indicated antibodies (upper panel). Total cell lysates were immunoblotted with C3G, Gab1 and RET antibodies for normalization. Anti-myc (tag) was used to ascertain SH2CrkII protein expression (lower panel). B) Rap1 activation by RET/PTC1 depends on CrkII and C3G. Rap1 from HEK293 transfectants was affinity precipitated with immobilized GST-RalGDS fusion protein. Bound Rap1 was revealed by immunoblot. These findings are representative of several independent assays.

Figure 4

A) HEK293 were transfected with the indicated plasmids, kept overnight in serum-deprived medium, and harvested. Cell lysates were subjected to an *in vitro* BRAF kinase assay using GST-MEK as a substrate. Kinase reactions were analyzed with anti-phospho-MEK antibody. Filters were reprobed with anti-BRAF. Total cell extracts were immunoblotted with anti-RET or with anti-AU5 (the Rap1 tag) for normalization. B) HEK293 were transfected with RET/PTC1 and the indicated plasmids. Rap1 or Ras activity was measured by affinity precipitation with immobilized GST-RalGDS or GST-RafRBD, respectively. Total cell lysates were analyzed for Rap1 and Ras protein for normalization. The signal was analyzed at the Phosphorimager. C) MEK and MAPK phosphorylation was evaluated in HEK293 cells transiently transfected with the indicated plasmids by immunoblotting with phosphospecific antibodies. The AU5 tag (Rap1 and Ras constructs) was used for normalization. The experiments are representative of at least three independent assays.

Figure 5

A) GST-RalGDS pull-down assay of Rap1 in RET/PTC1-expressing FB2, TPC1, BHP10-3, BHP2-7 cells compared to normal P5 cells. B) RET/PTC1/Gab1/CrkII/C3G complex in thyroid cancer cells. Total lysates (4 mg) of the indicated cell lines were immunoprecipitated with CrkII and blotted with the indicated antibodies. C) si Gab1 or control scrambled oligonucleotide (si scr) were transiently transfected in the indicated cells. Gab1 levels were measured by immunoblot (upper panel); levels of MEK and MAPK

phosphorylation were measured by immunoblot (middle); Rap1 activation was measured by the GST-RalGDS pull-down assay (lower panel).

Figure 6

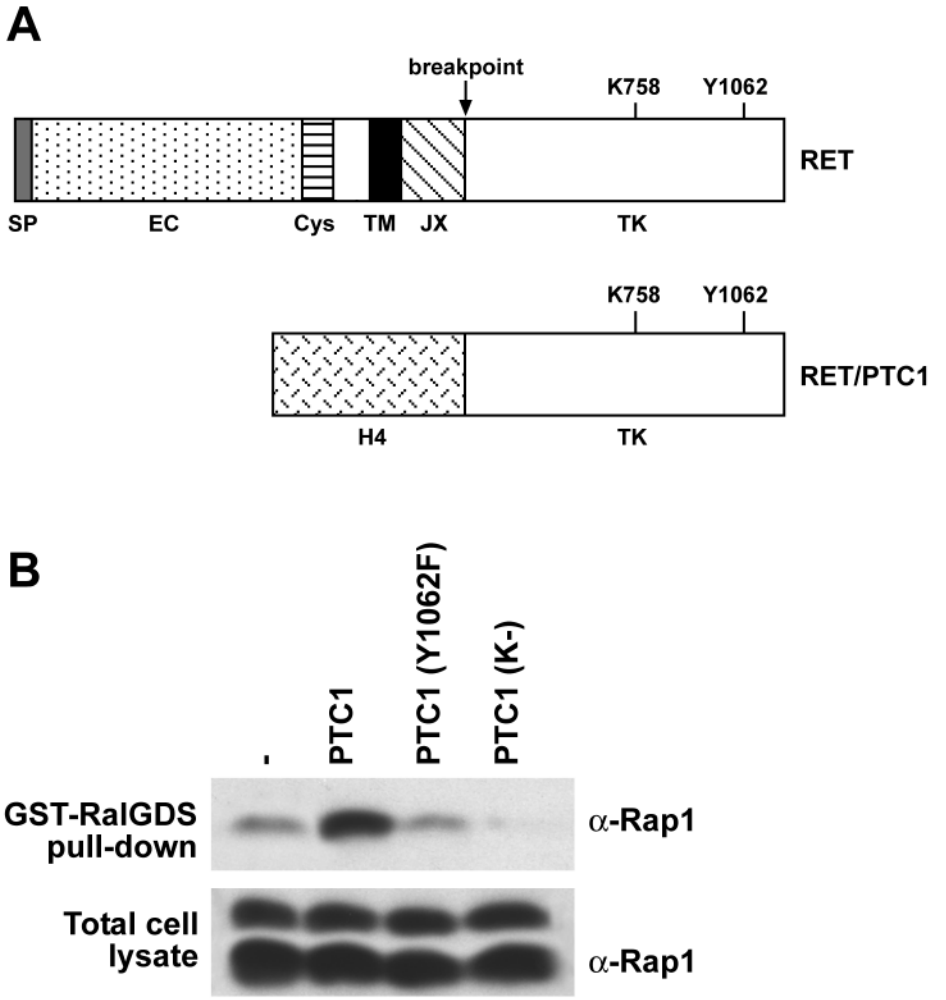
A) RET/PTC1 associates with Gab1 and CrkII in PC cells. Total lysates (2 mg) of PC cells stably transfected with RET/PTC1 or its Y1062F mutant were immunoprecipitated with CrkII and blotted with the indicated antibodies (upper panel). Rap1 activity was measured by the GST-RalGDS pull-down assay in RET/PTC1 or RET/PTC1(Y1062F) transfectants (lower panel). B) PC cells expressing a doxycycline-responsive RET/PTC1 were stimulated with doxycycline and harvested at the indicated time points. Cell lysates were subjected to the GST-RalGDS affinity precipitation. C) RET/PTC1-expressing PC cells were transiently transfected with the indicated plasmids together with EGFP. Cells were kept in the absence of 6 hormones. BrdU incorporation was evaluated in EGFP-positive cells; at least 100 cells were counted per field and the average results ± SD of three independent experiments in which at least 500 cells were scored are reported (left). PC PTC1 and PC PTC1-Rap1(N17) cells were plated at the same density, kept in the absence of 6 hormones and counted at the indicated time points. The average results of three independent determinations ± SD are reported (right). D) PC PTC1 cells were transiently co-transfected with the indicated dominant interfering mutants and EGFP; 36 h after transfection, cells were plated and stained after 4 h with Texas Red X-conjugated phalloidin. Actin organization in EGFP-positive cells was evaluated. Representative fields are shown. The experiment was performed in triplicate and at least 60 EGFP-positive cells were scored (upper panel). The indicated stable transfectants were fixed with

paraformaldehyde (4%) and stained with Texas Red X-conjugated phalloidin (lower panel).

Figure 7

A) RET/PTC1-mediated RhoA activation. Affinity precipitation of RhoA with immobilized GST-RhotekinGDS fusion protein from HEK293 transiently transfected with the indicated constructs or the empty vector (-). Precipitated RhoA was revealed by anti-RhoA immunoblot. Total cell lysates were analyzed for RhoA protein expression for normalization. The signal was analyzed at the Phosphorimager. This experiment is representative of three independent assays. B) SRF-luc activation by RET/PTC. HEK293T cells were co-transfected with pSRF-luc and the indicated plasmids. Luciferase activity was measured as fold induction with respect to the control. Average results of three independent determinations ± SD are reported. C) Recruitment of Grb2 to RET/PTC tyrosine 1062 is mediated by numerous docking proteins, including Shc and FRS2. Grb2 is able to recruit Gab1, that, in turn, associates to the SH2 domain of Crk. Crk *via* its SH3 domain bridges the complex to the Rap1 GEF C3G. Rap1 activation is negatively controlled by Rap1GAP. Rap1 contributes to RET/PTC1-mediated stimulation of BRAF.

Fig. 1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TK
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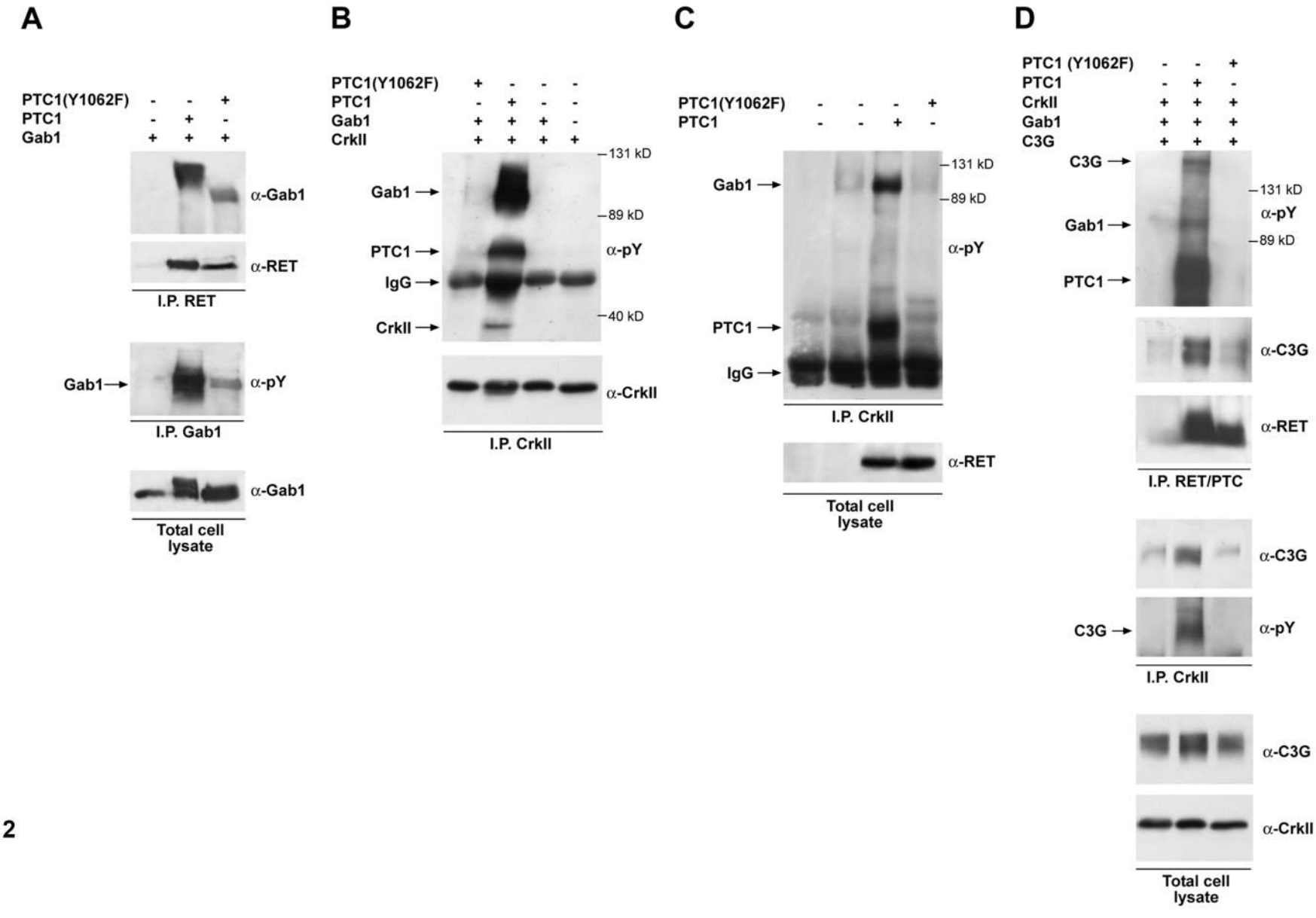
α-Rap1
Total cell lysate
α-Rap1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Gab1 + + +
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α-pY
I.P. Gab1
α-Gab1
Total cell lysate
B
PTC1(Y1062F) + - - -
PTC1 - + - -
Gab1 + + + -
CrkII + + + +
131 kD
89 kD
40 kD
Gab1
PTC1
IgG
CrkII
α-pY
α-CrkII
I.P. CrkII
C
PTC1(Y1062F) - - - +
PTC1 - - + -
131 kD
89 kD
Gab1
PTC1
IgG
α-pY
I.P. CrkII
α-RET
Total cell lysate
D
PTC1 (Y1062F) - - +
PTC1 - + -
CrkII + + +
Gab1 + + +
C3G + + +
C3G
Gab1
PTC1
131 kD
α-pY
89 kD
α-C3G
α-RET
I.P. RET/PTC
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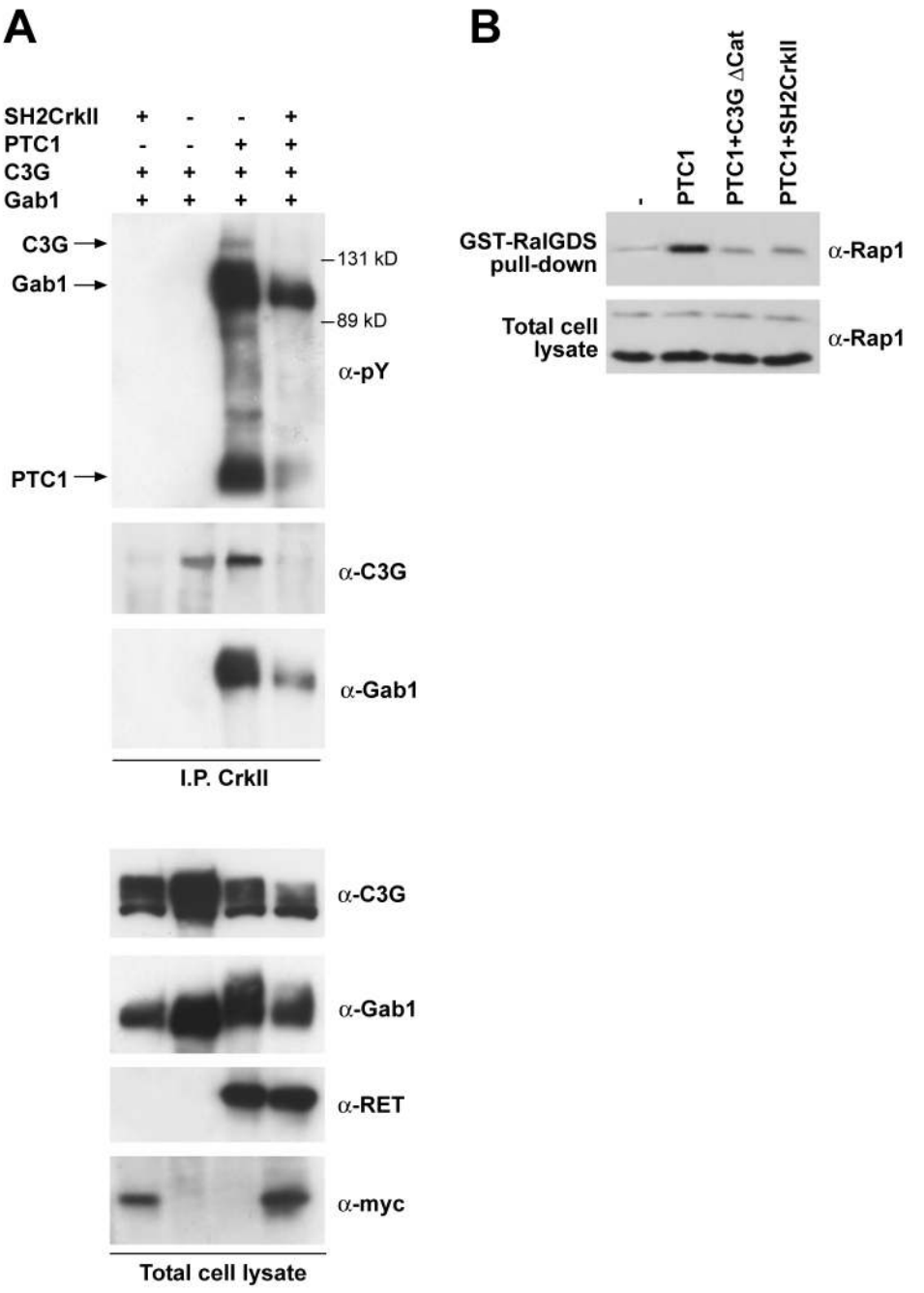
α-CrkII
Total cell lysate

Fig. 2

Fig. 3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α-pY
α-C3G
α-Gab1
I.P. CrkII
α-C3G
α-Gab1
α-RET
α-myc
Total cell lysate
B
-
PTC1
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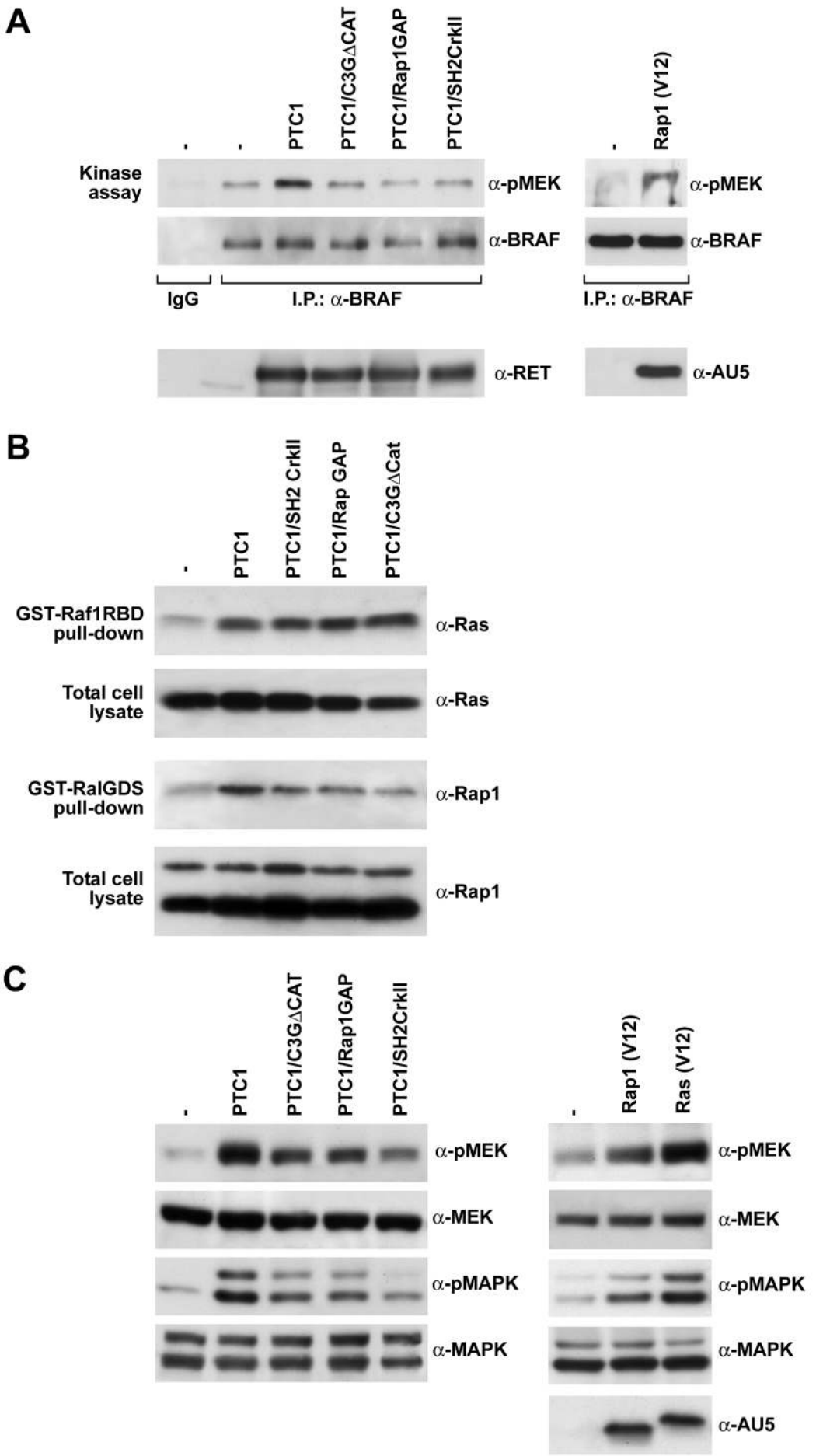
α-Rap1

Fig. 4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α-pMEK
α-BRAF
α-BRAF
IgG
I.P.: α-BRAF
I.P.: α-BRAF
α-RET
α-AU5
B
-
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PTC1/Rap GAP
PTC1/C3GΔCat
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α-Ras
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PTC1/SH2CrkII
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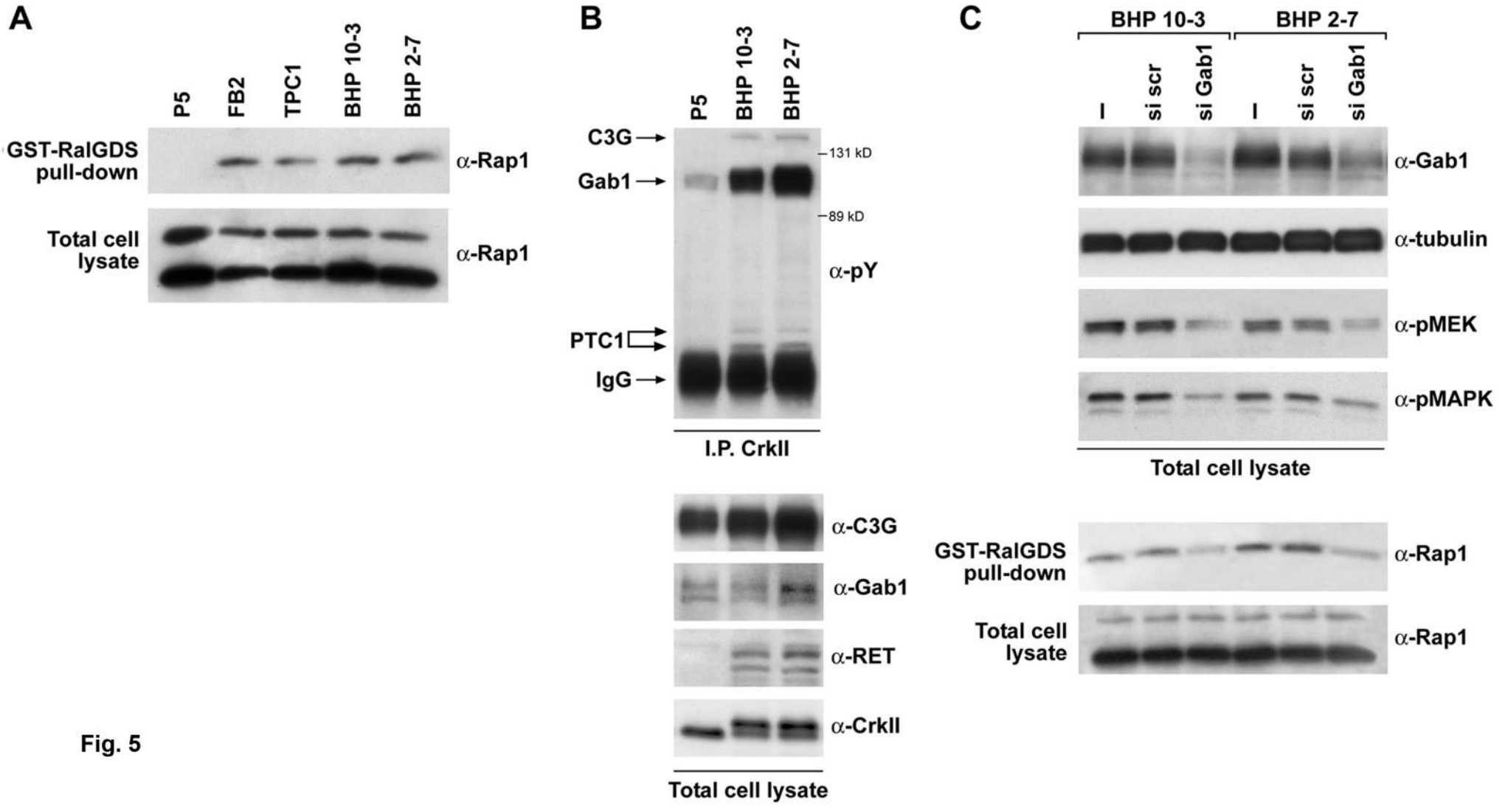
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si Gab1
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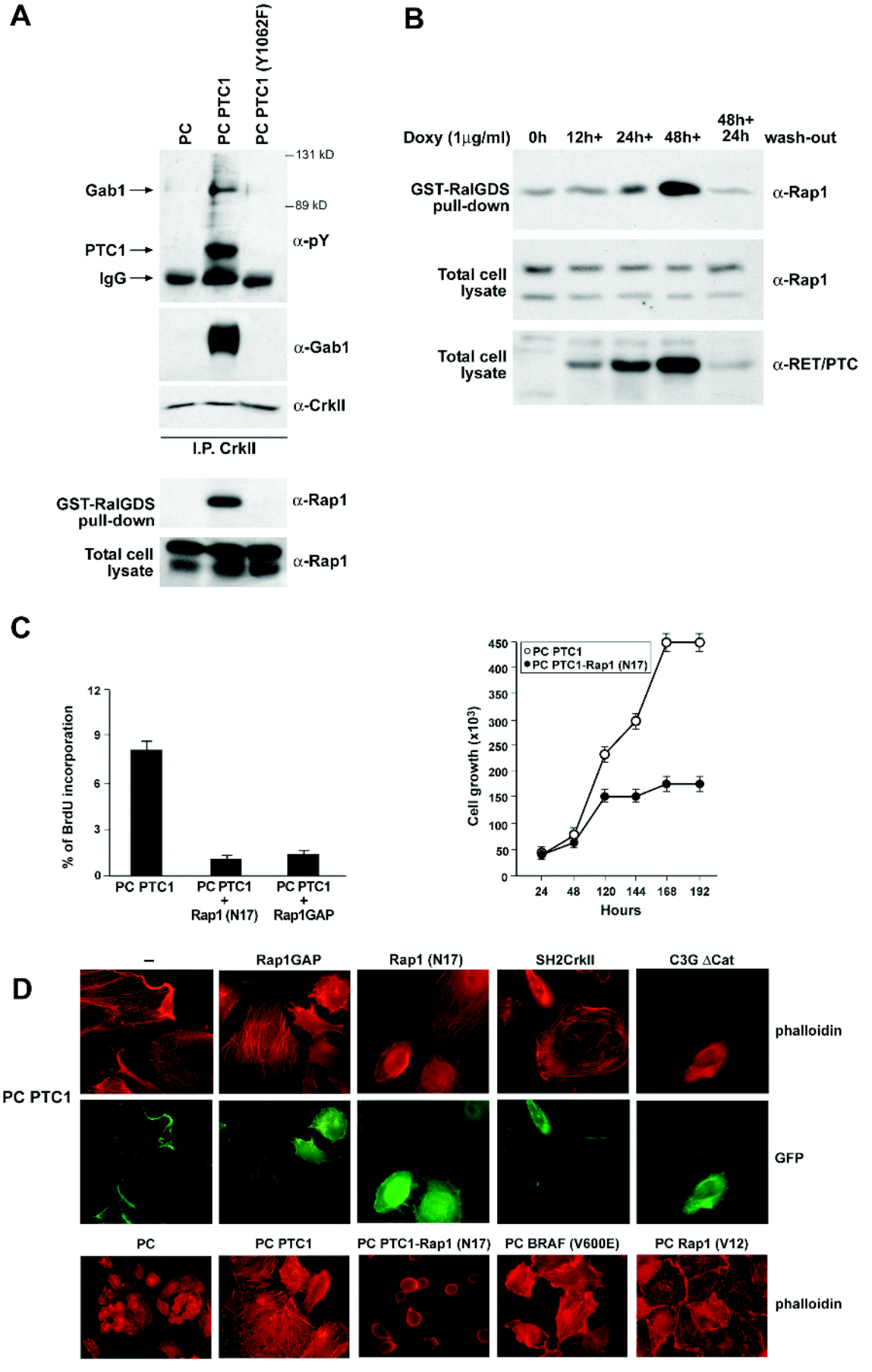
Fig. 5

Fig. 6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I.P. CrkII
GST-RalGDS pull-down
α-Rap1
Total cell lysate
α-Rap1
B
Doxy (1μg/ml)
0h
12h+
24h+
48h+
48h+ 24h
wash-out
GST-RalGDS pull-down
α-Rap1
Total cell lysate
α-Rap1
Total cell lysate
α-RET/PTC
C
% of BrdU incorporation
PC PTC1
PC PTC1 + Rap1 (N17)
PC PTC1 + Rap1GAP
PC PTC1
PC PTC1-Rap1 (N17)
Cell growth (x10³)
Hours
D
–
Rap1GAP
Rap1 (N17)
SH2CrkII
C3G ΔCat
phalloidin
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GFP
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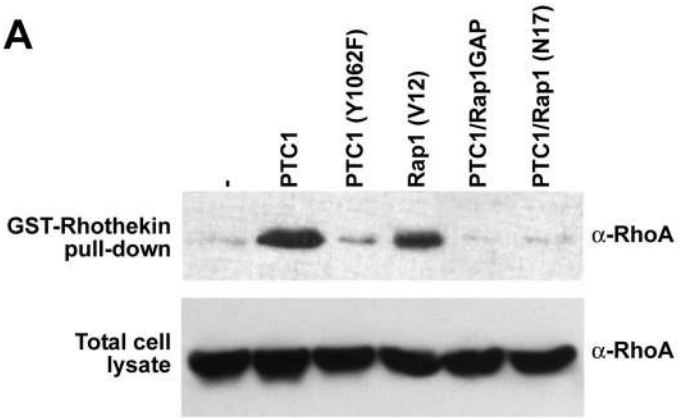
PC BRAF (V600E)
PC Rap1 (V12)
phalloidin

Fig. 7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-
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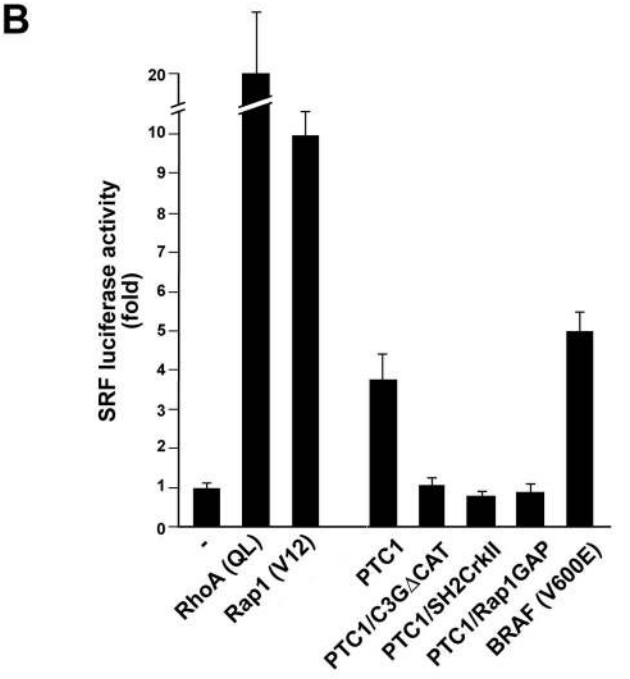
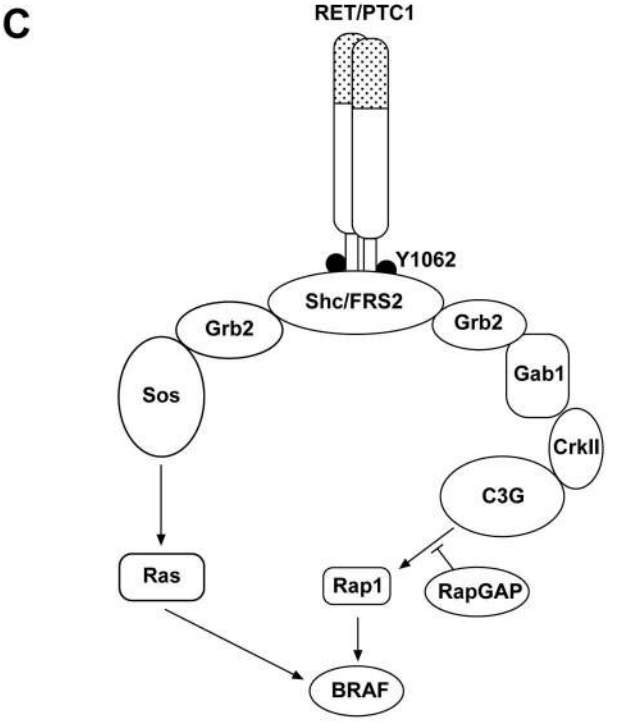
GST-Rhothekin pull-down
α-RhoA
Total cell lysate
α-RhoA


B
SRF luciferase activity (fold)
0
1
2
3
4
5
6
7
8
9
10
20
-
RhoA (QL)
Rap1 (V12)
PTC1
PTC1/C3GΔCAT
PTC1/SH2CrkII
PTC1/Rap1GAP
BRAF (V600E)


C
RET/PTC1
Y1062
Shc/FRS2
Grb2
Grb2
Gab1
Sos
CrkII
C3G
Ras
Rap1
RapGAP
BRAF