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Research Article

Inactivation of Ret/Ptc1 oncoprotein and inhibition of papillary thyroid carcinoma cell proliferation by indolinone RPI-1

C. Lanzi[a,*], G. Cassinelli[a], G. Cuccuru[a], N. Zaffaroni[b], R. Supino[a], S. Vignati[b], C. Zanchi[a], M. Yamamoto[c] and F. Zunino[a]

[a] Preclinical Chemotherapy and Pharmacology Unit and
[b] Biomolecular Determinants of Carcinogenesis and Response to Treatments Unit, Department of Experimental Oncology, Istituto Nazionale Tumori, via G. Venezian 1, 20133 Milan, (Italy), Fax: + 39 02 23902692, e-mail: cinzia.lanzi @istitutotumori.mi.it
[c] Department of Neurology, Nagoya University School of Medicine, Nagoya 466 (Japan)

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Abstract. Genetic alterations causing oncogenic activation of the RET gene are recognized as pathogenic events in papillary and medullary thyroid carcinomas. Inhibition of Ret oncoprotein functions could thereby represent a specific therapeutic approach. We previously described the inhibitory activity of the 2-indolinone derivative RPI-1 (formerly Cpd1) on the tyrosine kinase activity and transforming ability of the products of the RET/PTC1 oncogene exogenously expressed in murine cells. In the present study, we investigated the effects of RPI-1 in the human papillary thyroid carcinoma cell line TPC-1 spontaneously harboring the RET/PTC1 rearrangement. Treatment with RPI-1 inhibited cell proliferation and induced accumulation of cells at the G2 cell cycle phase. In treated cells, Ret/Ptc1 tyrosine phosphorylation was abolished along with its binding to Shc and phospholipase Cγ, thereby indicating abrogation of constitutive signaling mediated by the oncoprotein. Activation of JNK2 and AKT was abolished, thus supporting the drug inhibitory efficacy on downstream pathways. In addition, cell growth inhibition was associated with a reduction in telomerase activity by nearly 85%. These findings in a cellular context relevant to the pathological function of RET oncogenes support the role of Ret oncoproteins as useful targets for therapeutic intervention, and suggest RPI-1 as a promising candidate for preclinical development in the treatment of thyroid tumors expressing RET oncogenes.

Key words. Receptor tyrosine kinase Ret; oncoprotein; thyroid neoplasms; c-Jun N-terminal kinase; AKT; telomerase; 2-indolinone.

RET is a receptor tyrosine kinase-encoding gene that plays a critical role in the development of the enteric nervous system and kidney [1]. Genetic alterations in the RET gene causing a deregulated function of the gene products have been described in a set of human diseases. Germline loss-of-function RET mutations are involved in the congenital intestinal malformation known as Hirschsprung's disease (HSCR) [2] whereas oncogenic activation of RET is a specific pathogenic event in thyroid carcinomas [3]. In the vast majority of inherited cancer syndromes of the multiple endocrine neoplasia 2A and 2B type which include medullary thyroid carcinoma (MTC), and in familial MTC, distinct germline point mu-

* Corresponding author.

tations of proto-RET have been identified. Furthermore, somatic point mutations of RET are found in a subset of sporadic MTC. In papillary thyroid carcinomas (PTCs), somatic chromosomal rearrangements of RET (RET/PTC) occur with an incidence of 5–30% in spontaneous cases and 60–70% in radiation-induced PTCs [3]. As a result of such gene recombinations, chimeric oncoproteins are produced harboring the tyrosine kinase domain of Ret fused to protein sequences encoded by the N terminus of other genes typically endowed with dimerizing properties. To date, at least 15 RET/PTC rearrangements involving ten different donor genes have been reported [4].

In physiological cellular contexts, c-Ret participates in the signaling cascades activated by ligands of the glial cell line-derived neurotrophic factor (GDNF) family. Upon ligand binding, mediated by a member of the glycosyl phosphatidyl-anchored-α co-receptor family, the activation of c-Ret tyrosine kinase initiates a signaling network [5]. RET mutations found in thyroid malignancies activate the Ret kinase. The constitutive tyrosine kinase activity of Ret oncoproteins is responsible for their transforming activity in immortalized rodent fibroblasts [6–8]. In addition, transgenic mice with thyroid-targeted expression of oncogenic-activated Ret kinases develop thyroid tumors which mimic features of human carcinomas, thus supporting the clinical relevance of RET activation [9–12].

Although Ret oncoproteins appear causally involved in thyroid carcinomas, the biological consequences of their expression in thyroid tissues are not fully understood. Studies addressing Ret-mediated signaling have been mostly performed with rodent fibroblasts or human cell lines of neural origin expressing exogenous or endogenous forms of RET. These studies indicated that, as in the case of other receptor tyrosine kinases [13], Ret signaling is dependent on the assembly of protein complexes to specific phosphotyrosine residues contributing to the downstream signaling pathways in a cell type-specific way. In these cell systems, transducing and adaptor proteins such as Shc, Grb2, Grb7 and Grb10, phospholipase Cγ (PLCγ), Enigma, IRS1 and FRS2 have been indicated as Ret-binding proteins driving the activation of key pathways such as the Ras/MAPK and PI3K/Akt pathways involved in the regulation of cell proliferation, differentiation and survival [3, 5].

Since RET oncogenes are involved in the molecular pathology of thyroid tumors, their protein products represent exploitable targets for pharmacological intervention. Indeed, the function of inappropriately activated protein tyrosine kinases can be inhibited by specific inhibitors, as already documented [14].

In a previous paper, we described the inhibitory activity of the 2-indolinone derivative RPI-1 (formerly Cpd1) against Ret/Ptc1 kinase and the RET/PTC1 oncogene transforming ability in NIH3T3 mouse fibroblasts [15]. To study the effects of RPI-1 in a cellular context relevant to the pathological function of a Ret oncoprotein, in the present study we investigated the cellular response to the compound of a human PTC cell line (TPC-1) spontaneously expressing one of the most common rearranged forms of RET, RET/PTC1. In this cellular context we then analyzed Ret/Ptc1-mediated signaling and the interference of the drug in involved pathways.

Materials and methods

Chemicals and antibodies

The synthesis and chemical structure of RPI-1 {1,3-dihydro-5,6-dimethoxy-3-[(4-hydrophenyl)methylene)-H-indol-2-one, formerly Cpd1} have been reported previously [17]. Stock solutions were prepared in dimethylsulfoxide. For experiments, the compound was diluted in cell culture medium.

The following monoclonal antibodies were used: anti-p21WAF1 and anti-PKBα/Akt from Trasduction Laboratories, Lexington, Ky.; anti-phospho-p44/42 MAP kinase (Thr202/Tyr204) and anti-phospho-SAPK/JNK (Thr183/Tyr185) from New England Biolabs, Beverly, Mass.; anti-cyclin D1 and anti-cyclin B1 from Santa Cruz Biotechnology, Santa Cruz, Calif.; anti-pRB from PharMingen, San Diego, Calif.; anti-phosphotyrosine antibody clone 4G10 from Upstate Biotechnology, Lake Placid, N. Y. The rabbit polyclonal antibodies used were: anti-Ret recognizing a COOH-terminal sequence (aa 1000–1014) common to the two Ret isoforms [16]; phosphorylation-specific Ret pTyr-1062 antibody [18]; anti-phospho-p38 MAP kinase (Thr180/Tyr182) and anti-SAPK/JNK from New England Biolabs; anti-p44/42 MAP kinase from Upstate Biotechnology; anti phospho-Akt (Ser473) from Cell Signaling, Beverly, Mass.; anti-Shc and anti-PLCγ from Santa Cruz Biotechnology; anti-actin from Sigma, St. Louis, Mo.

Cell lines and culture conditions

The human PTC cell lines, TPC-1 and NPA, and NIH3T3^{PTC1} cells obtained by transfecting mouse NIH3T3 fibroblasts with human DNA from a PTC, were kindly provided by Dr M. G. Borrello and Dr I. Bongarzone (Milan, Italy). The human PTC cell line K1 and the Nthy-ori 3-1 cell line derived from human thyroid follicular epithelium were purchased from the European Collection of Cell Cultures (Salisbury, U. K.). TPC-1 and NPA cells were grown in Dulbecco's modified Eagle's medium (DMEM); K1 cells were cultivated in DMEM : Ham's F12 : MCDB 104 (2 : 1 : 1), and Nthy-ori 3-1 cells in RPMI 1640 medium. Media of the thyroid cell lines were supplemented with 10% fetal calf serum (FCS). NIH3T3 and NIH3T3^{PTC1} cells were maintained in DMEM with

10% or 5% calf serum, respectively (Colorado Serum Company, Denver, Colo.).

In the cell growth inhibition assays, cells were trypsinized after 72 h of drug treatment and counted by a Coulter Counter (Coulter Electronics, Luton, U. K.). The concentrations able to inhibit cell proliferation by 50% (IC_{50}) were calculated from the dose-response curves. Each experiment was performed in duplicate. Alternatively, cells were subjected to the sulforhodamine B colorimetric assay as described elsewhere [18] at the indicated times after exposure to the drug. Each experiment was performed in eight replicates.

Heat shock was induced in NIH3T3 cells by incubation at 42 °C, for 30 min.

Cell cycle analysis

Cells were trypsinized after 72 h of treatment and fixed overnight in 70% ethanol at –20 °C. After washing in phosphate-buffered saline (PBS), cells were incubated in a solution of 10 µg/ml propidium iodide (PI) (Sigma) and 66 U/ml RNase (Sigma) in PBS, for 16–18 h. The cell cycle distribution was measured by a FACScan flow cytometer equipped with an argon laser (Becton Dickinson, Mountain View, Calif.). For biparametric analysis of cyclin B1 and DNA content, cells were fixed in 70% ethanol and washed with cold PBS. After resuspension in PBS containing 0.25% Triton X-100 and saturation in PBA (1% bovine serum albumin in PBS), cells were incubated with anti-cyclin B1 antibody (1:40), for 1 h, and then with a FITC-conjugated secondary antibody. Counterstaining was performed with 5 µg/ml PI.

Immunoprecipitation and Western blotting

For immunoprecipitation experiments, control and treated cells were processed as previously described [15]. For whole-cell extract preparation, cells were lysed in sodium dodecyl sulfate (SDS) sample buffer [62.5 mM Tris-HCl (pH 6.8), 2% SDS] with 1 mM PMSF, 10 µg/ml pepstatin, 12.5 µg/ml leupeptin, 100 KIU aprotinin, 1 mM sodium orthovanadate, 1 mM sodium molybdate. Protein concentration was determined in an appropriately diluted aliquot by the BCA method (Pierce, Rockford, Ill.). Then samples were adjusted to a final concentration of 10% glycerol, 5% β-mercaptoethanol, 0.001% bromophenol blue.

Immunoprecipitates from 1–3 mg of protein extract or whole-cell lysates (30–60 µg) were separated on SDS-PAGE and transferred to nitrocellulose filters. Membranes were incubated with primary antibodies overnight. Immunoreactive bands were revealed by horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibodies using enhanced chemiluminescence detection systems from Amersham Biosciences (Amersham, U. K.) or Pierce.

Telomerase activity detection assay and RT-PCR analysis of telomerase components

Cell extracts were obtained as previously described [19]. Telomerase activity was measured by the telomeric repeat amplification protocol (TRAP) [20]. After extension of the substrate by telomerase, the products were amplified by PCR in the presence of [^{32}P]-end-labeled TS primer and resolved in 10% polyacrylamide gels. Each reaction product was amplified in the presence of a 36-bp internal TRAP assay standard (ITAS), and each sample extract was tested for RNase sensitivity. A TSR8 quantitation standard (which serves as a standard to estimate the amount of product extended by telomerase in a given extract) was included for each set of TRAP assays. Quantitative analysis was performed with the Image-QuanT software (Molecular Dynamics, Sunnyvale, Calif.), which allowed densitometric evaluation of the digitized image. Telomerase activity was quantified by measuring the signal of telomerase ladder bands, and the relative telomerase activity was calculated as the ratio to the internal standard using the following formula:

$$\text{relative telomerase activity} = [(X - X_0)/C] \cdot [(R - R_0)/Cr]^{-1}$$

where X is the untreated sample, X_0 is the RNase-treated sample, C is the internal control of untreated samples, Cr is the internal control of TSR8, R is the TSR8 quantitation control, and R_0 is the negative control. The effect of RPI-1 on telomerase was expressed as the percentage inhibition of enzyme activity in samples exposed to the drug compared to untreated controls.

For RT-PCR analysis, total cellular RNA was extracted from frozen samples with the TRIzol reagent (Life Technologies, Gaithersburg, Md.) according to the manufacturer's instructions. Total RNA (0.5 µg) from each sample was used for cDNA production using the RT-PCR Core kit (Perkin Elmer) with random hexamers. cDNA samples were then amplified using specific primers for the telomerase RNA component (hTR), telomerase-associated protein (TEP1), and telomerase reverse transcriptase (hTERT) as previously described [21]. All telomerase subunits were co-amplified with β-actin as the internal standard. The PCR products were run on a 3% agarose gel and visualized with ethidium bromide. Amplified products were analyzed by a ScanJET IIcx/T scanner (Hewlett Packard) and quantified by ImageQuant software.

Results

Effects on cell growth and cell cycle progression of human thyroid cell lines

A cell growth inhibition assay was used to detect the antiproliferative effect of RPI-1 on the TPC-1 cell line, on two additional PTC cell lines, NPA and K1, and on the

Table 1. Sensitivity of thyroid cell lines to RPI-1.

Cell line	Origin	IC_{50} (μM)
TPC-1	PTC (RET/PTC1+)	5.1 ± 0.4
NPA	PTC	29.8 ± 4.9
K1	PTC	17.1 ± 4.7
Nthy-ori 3-1	follicular epithelium	25.7 ± 5.1

Data represent the mean values ± SD from two independent experiments.

thyroid follicular epithelial cell line Nthy-ori 3-1. In contrast to TPC-1 cells, NPA and K1 carcinoma cells, as well as Nthy-ori 3-1 cells deriving from normal human thyroid tissue, lack oncogenic activation of RET. As shown in table 1, TPC-1 cells were the most sensitive to the growth inhibitory effect of RPI-1 with an IC_{50} of 5.1 ± 0.4 μM, following 72 h of treatment. A prolonged exposure to drug concentrations in the range of the IC_{50} at 72 h of the other thyroid cell lines (17–30 μM) induced a growth delay in Ret/Ptc1-negative cells, whereas in TPC-1 cells, the growth inhibitory effect persisted for days (TPC-1 and Nthy-ori 3-1 cells in fig. 1) and even weeks (not shown), thus suggesting a cytostatic effect. Such an antiproliferative effect was associated with marked enlargement and flattening of the cellular morphology (fig. 2A), reminiscent of the reversion of the transformed phenotype induced by the drug in NIH3T3^{PTC1} transfectants [15]. The selectivity of RPI-1 for TPC-1 cells was also maintained in low-serum (0.5%) culture conditions (not shown).

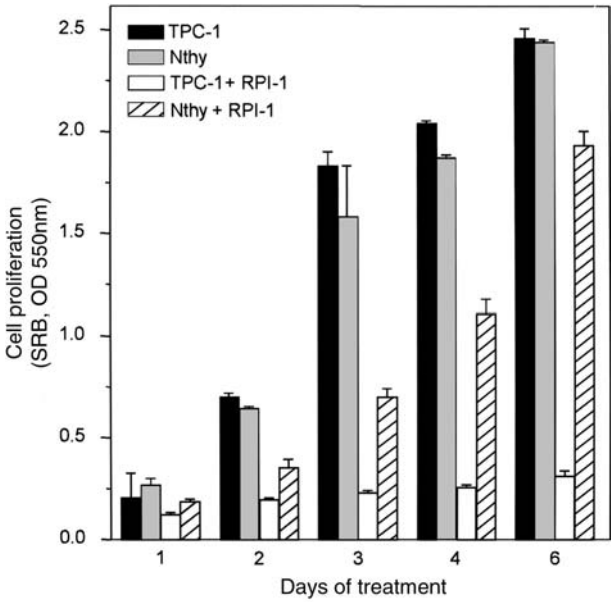

Figure 1. Antiproliferative effects of RPI-1 on human NThy-ori 3-1 and TPC-1 thyroid cell lines. Cells were incubated in the presence of vehicle or 25 μM RPI-1. Cell density, measured daily by the sulforhodamine B colorimetric assay, is expressed as optical density units at 550 nm ± SD. One experiment is shown, representative of two independent experiments.

We next examined whether TPC-1 cell growth inhibition by RPI-1 was reflected by cell cycle perturbation. The flow cytometer analysis of DNA content in TPC-1 cells exposed to RPI-1 (60 μM, 72 h) showed a reduction in the G0/G1 and increase in the G2/M peak when compared to control cells (fig. 2B). According to an accumulation of treated cells at the G2 phase, the expression of cyclins A and B1 was increased and pRb was partly hyperphosphorylated (fig. 2C). The increase in cyclin B1 expression in treated cells was specifically ascribable to G2 cells as demonstrated by biparametric analysis of cyclin B1 and DNA content by flow cytometry (fig. 2D). In addition, mitosis-specific phosphoepitopes were undetectable in both control and treated cells (not shown) thus excluding accumulation of treated cells in mitosis. On the other hand, a strong induction of the cyclin-dependent kinase inhibitor p21WAF1 (fig. 2C) was consistent with the permanence of part of the cell population in G0/G1. Because of the multifunctional role of c-Myc in cell cycle progression and the control of cell growth, including thyroid cancer cell growth [22], we also compared c-Myc expression in control and RPI-1-treated cells. However, no modulation of the overall levels of c-Myc could be detected (data not shown), thereby suggesting that RPI-1 treatment does not affect the expression of this transcription factor.

Inhibition of Ret/Ptc1 autophosphorylation by RPI-1 in TPC-1 cells

To examine the effect of RPI-1 on the activation of Ret/Ptc-1 proteins expressed in PTC cells, tyrosine-phosphorylated proteins were immunoprecipitated from control and treated TPC-1 cells and then analyzed by immunoblotting with anti-Ret antibody. Figure 3A shows that phosphorylation on tyrosine of both Ret/Ptc1 isoforms, p59 and p64, was reduced in a dose-dependent way. Complete dephosphorylation was evident at drug concentrations higher than 15 μM, following 72 h of treatment. However, the inhibition was already detectable after 10 min, thus indicating rapid intake and efficacy of the drug. Crossed immunoprecipitation/immunoblotting, showing dephosphorylation of Ret/Ptc1 proteins immunoprecipitated with anti-Ret antibody from treated cells (fig. 3B), further confirmed the inhibitory effect of RPI-1 on Ret/Ptc1 activation. Overall expression of the two Ret/Ptc1 isoforms, better demonstrated in whole-cell lysates, was not affected by the treatment (fig. 3B).

Effects on Shc proteins

Shc adaptor proteins have been implicated in signaling pathways mediated by Ret tyrosine kinases (23–25). Specific phosphorylated tyrosine residues on Ret proteins have been identified as the docking sites for Shc, i.e., Tyr586 on Ret/Ptc2 corresponding to Tyr1062 of proto-Ret, which appear to play a key role in the transforming ability of exogenously expressed Ret oncoproteins [26–

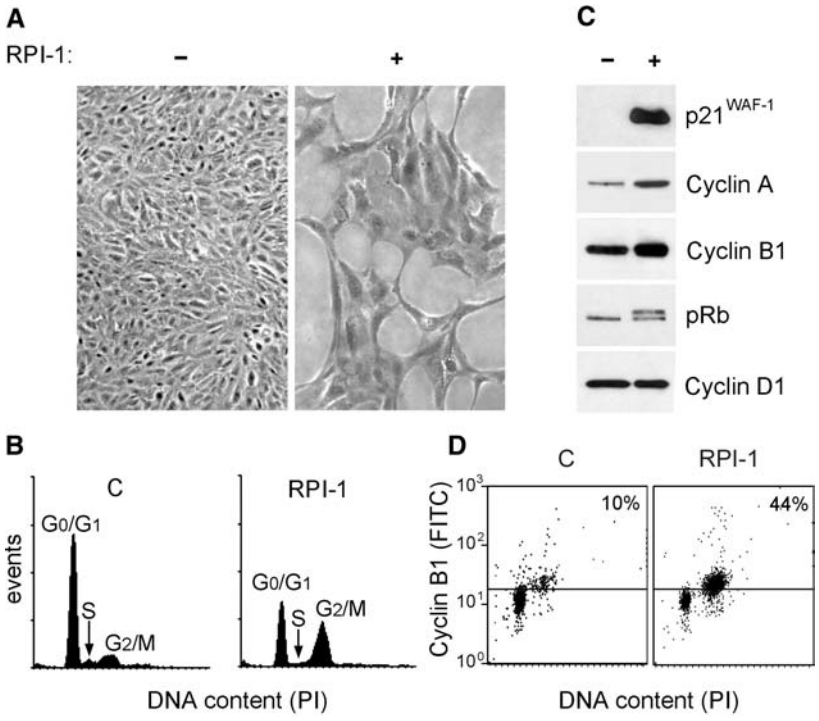

Figure 2. Effects of RPI-1 treatment on TPC-1 cell morphology (*A*), cell cycle progression (*B*), expression of cell cycle-regulating proteins (*C*), and cell cycle distribution of cyclin B1 (*D*). Cells were treated with vehicle (–) or 60 μM RPI-1 (+). After 72 h, cells were photographed under a phase-contrast microscope (original magnification × 100) (*A*), or lysed for whole-cell extract preparation and Western blot analysis (*C*). For Western blotting, equal amounts of protein were run in each lane and probed with the indicated antibodies. Alternatively, cells were fixed in ethanol and then stained with PI for flow cytometry analysis of DNA profiles (*B*) or processed for biparametric analysis of cyclin B1 versus DNA content (*D*). The percentages of FITC-positive cells in control and RPI-1-treated cells are reported.

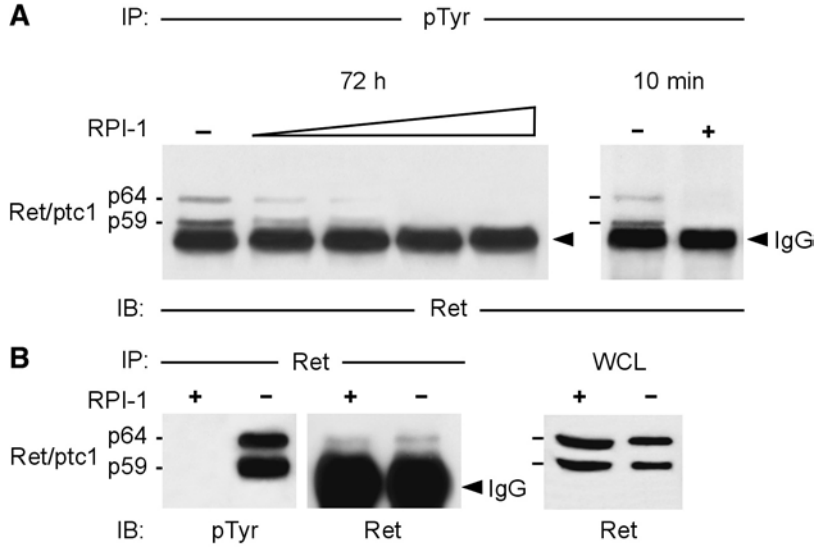

Figure 3. (*A*) Cells were exposed to solvent (–) or to increasing concentrations of RPI-1 (7.5, 15, 30, and 60 μM) for 72 h (left) or to 60 μM RPI-1 (+) for 10 min (right). Equal amounts of protein were used for immunoprecipitation (IP) with anti-phosphotyrosine (pTyr) antibodies. Immunoprecipitated proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes, and subjected to immunoblotting (IB) with anti-Ret antibody. (*B*) Cells were treated with solvent (–) or 60 μM RPI-1 (+) for 72 h. Whole-cell lysates (WCL) were prepared and directly resolved by SDS-PAGE or they were first immunoprecipitated with anti-Ret antibody, followed by immunoblotting with the indicated antibodies.

28]. Therefore, we examined the ability of the constitutively activated Ret/Ptc1 expressed in TPC-1 cells to bind Shc, and analyzed the interference of RPI-1 on this interaction by co-immunoprecipitation experiments. Cell lysates from control and treated TPC-1 cells (60 µM RPI-1, 72 h) were immunoprecipitated with anti-Shc antibody and immunoblotted with anti-Ret or anti-phosphotyrosine antibodies. As shown in figure 4A, tyrosine-phosphorylated Ret/Ptc1 proteins were present in anti-Shc immunoprecipitates from control but not treated cells.

To determine whether the constitutive association of Ret/Ptc1 with Shc in TPC-1 cells would be disrupted as a result of inhibition of Ret/Ptc1 autophosphorylation by RPI-1, we examined the phosphorylation state of the expected Shc docking site on the Ret/Ptc1 proteins (Tyr451) in whole-cell extracts using a phosphospecific antibody (pTyr1062) recognizing the Ret/Ptc1 pTyr451 which corresponds to pTyr1062 in the Ret receptor. Indeed, in accordance with inhibition of Shc binding, the pTyr1062 antibody detected in immunoblotting the two Ret/Ptc1 isoforms in control TPC-1 cell lysates and not in RPI-1-treated cells. Cell extracts of NIH3T3 and NIH3T3^{PTC1} cells were run in parallel as negative and positive control, respectively (fig. 4B).

Immunoprecipitation experiments with anti-Shc antibody provided additional information concerning the effect of RPI-1 on the three Shc isoforms which, unexpectedly, appeared differently affected by drug treatment (fig. 4A). In fact, tyrosine phosphorylation of p66Shc and p52Shc disappeared in RPI-1-treated cells whereas the p46Shc tyrosine phosphorylation was unaffected. In addition, p66Shc expression, but not the expression of p52Shc and p46Shc isoforms, was reduced after RPI-1 treatment. In a time-course experiment, the latter effect was already appreciable in total cell lysates after 24 h of exposure to the drug and more evident at 48 h and 72 h (fig. 4C).

Inhibition of Ret/Ptc1 association with PLCγ

In addition to Shc, several docking and signaling proteins have been proposed to participate through direct binding in signaling pathways mediated by Ret proteins [5]. Among these, PLCγ was found associated with both Ret/Ptc1 and Ret/Ptc2 oncoproteins in NIH3T3^{PTC} transfectants. Tyr539 on Ret/Ptc2 was identified as a functional PLCγ docking site required for the full RET/PTC2 oncogene transforming potential [16]. As illustrated in figure 5, the Ret/Ptc1 p64 isoform was clearly co-immunoprecipitated with PLCγ in a tyrosine-phosphorylated state, thus supporting an interaction between the on-

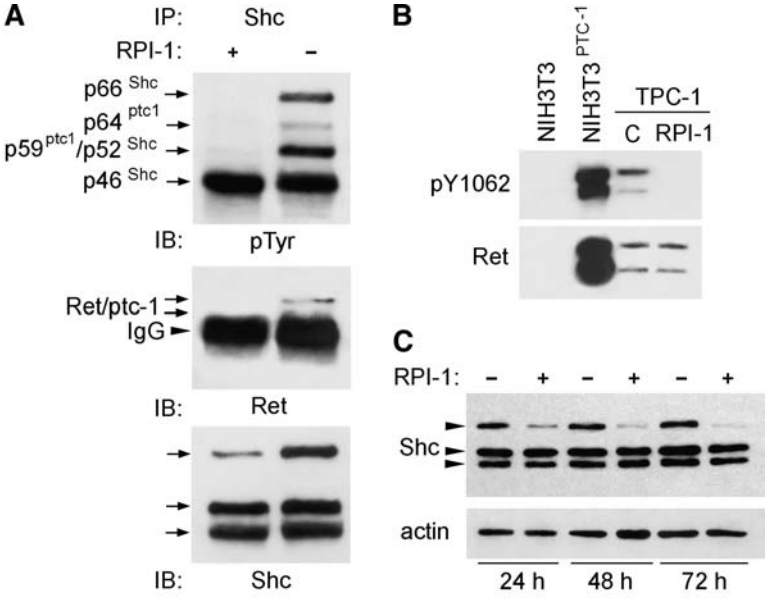

Figure 4. Disruption of Ret/Ptc1-Shc interaction by RPI-1 and effect of the drug on expression and tyrosine phosphorylation of Shc isoforms. (*A*) Abrogation of co-immunoprecipitation of Ret/Ptc1 with Shc. TPC-1 cells were treated with 60 µM RPI-1 for 72 h. Cell lysates were immunoprecipitated (IP) with anti-Shc antibody, resolved by SDS-PAGE followed by immunoblotting (IB) with anti-pTyr, anti-Ret, or anti-Shc antibodies. Arrows indicate the three Shc isoforms p66, p52, and p46 or the two Ret/Ptc1 isoforms p64 and p59. p59^{Ptc1} and p52^{Shc} co-migrate. The arrowhead indicates the IgG heavy chain detected in the anti-Ret blot. (*B*) Dephosphorylation of the Shc docking site on Ret/Ptc1. Cells were treated with RPI-1 as in *A*. Equal amounts of total cell lysate were resolved by SDS-PAGE in parallel with cell lysates from untreated NIH3T3 and NIH3T3^{PTC-1} cells, followed by immunoblotting with the phosphospecific anti-Ret pY1062 antibody (upper blot). Ret/Ptc1 expression in treated cells was confirmed by stripping and reprobing the filter with anti-Ret antibody (lower blot). (*C*) Down-regulation of p66Shc. Cells were exposed to 60 µM RPI-1 (+) or vehicle (–) for 24, 48, or 72 h. Equal amounts of whole-cell lysate were then subjected to Western blot with anti-Shc or anti-actin antibody. Arrowheads indicate the three Shc isoforms.

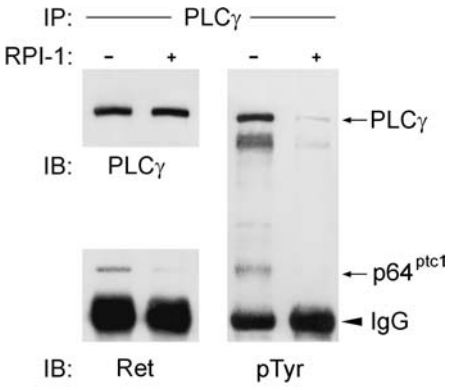

Figure 5. Abrogation of Ret/Ptc1 binding to PLCγ and inhibition of PLCγ tyrosine phosphorylation in RPI-1-treated cells. TPC-1 cells were exposed to the drug (+) or DMSO (–) as in figure 4. Cell lysates were immunoprecipitated (IP) with anti-PLCγ antibody, followed by SDS-PAGE and immunoblotting (IB) with anti-Ret, anti-pTyr or anti-PLCγ antibody. Bands corresponding to PLCγ, $p64^{Ptc1}$ and IgG heavy chain are indicated. $p59^{Ptc1}$ was not detected in these blots.

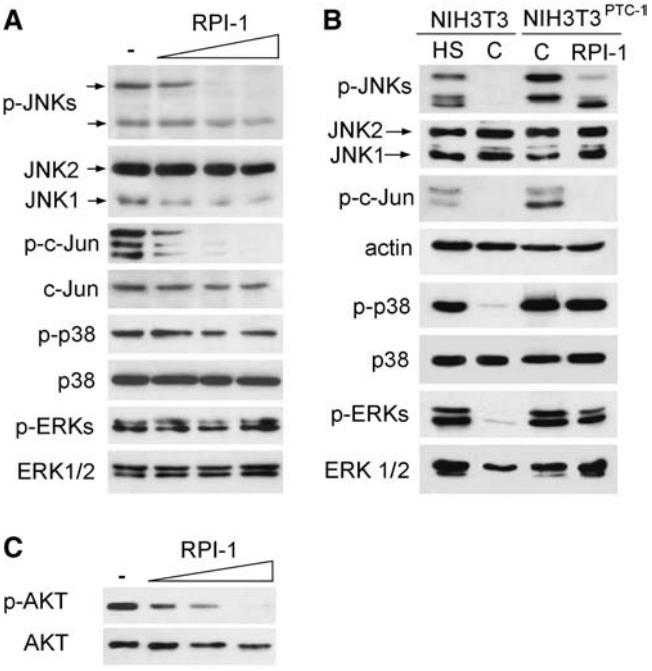

Figure 6. Effects of TPC-1 cell treatment with RPI-1 on MAPKs and AKT signaling pathways. (*A*) Inhibition of JNK2 activation in TPC-1 cells. Whole-cell extracts were prepared from TPC-1 cells exposed to vehicle (–) or increasing concentrations of RPI-1 (15, 30 and 60 µM), for 72 h. Equal amounts of protein were separated by SDS-PAGE and analyzed by immunoblotting with phosphorylation-specific antibodies: anti-phospho JNKs (p-JNKs), anti-phospho Jun (p-c-Jun), anti-phospho p38 (p-p38), anti-phospho ERK1/2 (p-ERKs). Blots were then stripped and reprobed with antibodies directed against the respective proteins. (*B*) Inhibition of JNK activation by RPI-1 in NIH3T3^{PTC1} transfectants. NIH3T3^{PTC1} cells were exposed to RPI-1 (30 µM) or solvent (*C*), for 72 h. Equal amounts of protein were separated by SDS-PAGE. Extracts from confluent untreated NIH3T3 cells (*C*) or cells subjected to heat shock (HS) were run in parallel as negative and positive controls for activation of MAPKs, respectively. Proteins were transferred to nitrocellulose and probed with the antibodies designated as in *A*. Control for protein loading by actin is shown. (*C*) Inhibition of AKT activation. TPC-1 cells were treated with RPI-1 and processed as in *A*. Immunoblotting was performed with anti-phospho AKT (Ser473) antibody followed by stripping and immunoblotting with anti-AKT antibody.

coprotein and the phospholipase in the PTC cell line. However, this association was abolished and tyrosine phosphorylation of PLCγ was heavily reduced in cells treated with RPI-1. In these co-immunoprecipitation experiments, the shortest Ret/Ptc1 isoform p59 was not detected.

Effects on downstream Ret/Ptc1-mediated signaling pathways

As TPC-1 cell treatment with RPI-1 resulted in dissociation from Ret/Ptc1 of two key components of signaling pathways, Shc and PLCγ, along with their tyrosine dephosphorylation, an inhibitory effect by the drug on downstream signaling pathways could be predicted. We thus investigated the impact of drug treatment on the MAPK and PI3K/Akt pathways which have been implicated in the signaling induced by Ret proteins in several cell systems [5]. The state of activation of protein kinases involved in these pathways was examined in control and treated cell extracts by immunoblotting with phosphospecific antibodies recognizing the activated forms of the kinases. RPI-1 inhibited the activation of the 54-kDa c-Jun N-terminal kinase 2 (JNK2) in a dose-dependent way. Accordingly, the transcription factor c-Jun, a major substrate of the JNK family proteins, became partially dephosphorylated following exposure to the drug (fig. 6A). In contrast, activation of the 46-kDa JNK1 was not affected. Reprobing the blots with anti-JNK1/2 or anti-Jun antibodies established that the protein levels of JNKs and Jun were not altered in treated cells (fig. 6A). Regarding the other members of the MAPK family, unlike JNK2, neither p42/p44 extracellular signal-regulated kinases (ERKs) nor p38 kinase were affected by RPI-1 treatment (fig. 6A). Similar results were observed with NIH3T3^{PTC1} transfectants although, in this case, the activation of JNK1 was also impaired (fig. 6B). Since JNKs are constitutively active in both cell systems [29] (data not shown), inhibition of JNK2 activation could conceivably be a consequence of Ret/Ptc1 inhibition by RPI-1. The signaling pathway dependent on Akt was also affected by RPI-1 treatment in TPC-1 cells. In fact, phosphorylation at Ser473, a known activating modification of this kinase [30], was reduced in treated cells in a dose-dependent way (fig. 6C). Together, these findings indicate that RPI-1 inhibitory effects in the TPC-1 cell culture conditions lead to inhibition of pathways involving JNK2 and AKT.

Effects on telomerase activity and expression of telomerase components

A variety of oncogenes positively regulate telomerase activity [31] and such ribonucleoprotein polymerase enzyme activity is repressed when cultured cell lines become quiescent [32]. This prompted us to examine telomerase activity in growth-arrested TPC-1 cells following exposure to RPI-1. The effect exerted by a 72-h exposure to RPI-1 was assessed by the TRAP assay. A marked reduction (–85 ± 10%) in enzyme catalytic activity was observed in drug-treated compared to control cells (fig. 7A). Such an inhibition was reversible upon drug removal. In fact, a progressive increase in TRAP signal was observed in treated cells by prolonging the incubation time in drug-free medium, and the level of telomerase activity approached that of control cells after 24 h (fig. 7B).

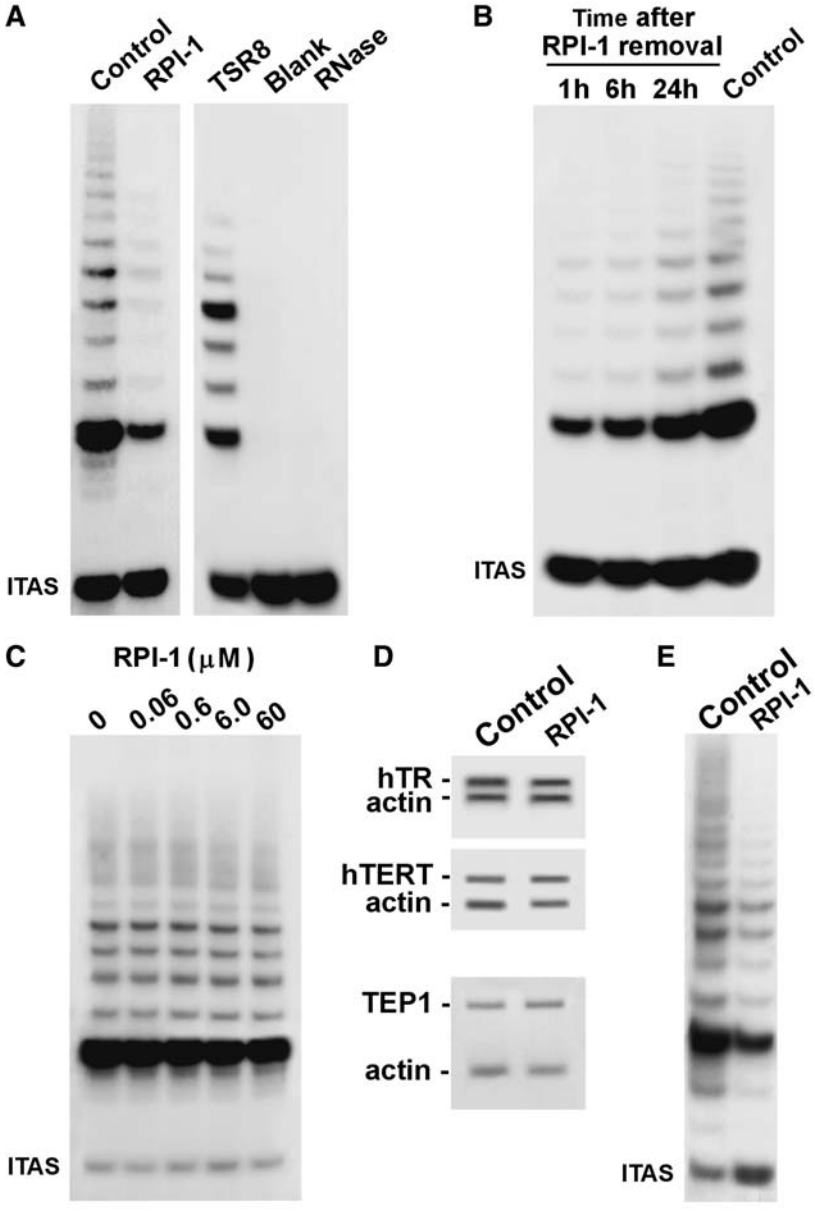

Figure 7. Effects of RPI-1 cell treatment on telomerase activity. Representative experiments. (*A*) Inhibition of telomerase activity, measured by TRAP assay, after exposure of TPC-1 cells to 60 μM RPI-1, for 72 h. The lane indicated TSR8 represents an external quantitative standard. Blank represents a negative control to which no cell extract was added. RNase represents a negative control containing TPC-1 cell extract pretreated with RNase. (*B*) telomerase activity (TRAP assay) in TPC-1 cells exposed for 72 h to 60 μM RPI-1 at different intervals after drug removal. (*C*) Telomerase activity (TRAP assay) in cell extracts obtained from untreated TPC-1 cells and exposed for 12 h to different concentrations of RPI-1. (*D*) RT-PCR experiment showing the expression of hTR, hTERT mRNA and TEP1 mRNA in TPC-1 cells exposed for 72 h to 60 μM RPI-1. All telomerase subunits were co-amplified with β-actin as the internal standard. (*E*) Telomerase inhibition measured by TRAP assay telomerase inhibition in NPA cells treated with 60 μM RPI-1 for 72 h. The position of the internal amplification standard (ITAS) is indicated.

RPI-1-induced inhibition of telomerase activity was not ascribable to a direct interaction of the drug with the oloenzyme. In fact, when protein extracts of untreated TPC-1 cells were exposed in vitro to different concentrations (ranging from 0.06 to 60 μM) of RPI-1 for 12 h, no appreciable reduction in telomerase activity was observed (fig. 7C).

To understand whether the inhibition of telomerase catalytic activity in intact TPC-1 cells exposed to RPI-1 was a consequence of a reduced expression of telomerase subunits, we assessed the abundance of the telomerase RNA component (hTR) and of the reverse transcriptase (hTERT) and associated protein (TEP1) mRNAs, after 72 h of treatment. Results from RT-PCR experiments showed no evidence for a significant reduction in the levels of these transcripts in drug-treated compared to control cells (fig. 7D). Telomerase inhibition by RPI-1 was not restricted to TPC-1 cells. Inhibition of enzyme activity was also observed, although to a lower extent, in Ret/Ptc1-negative PTC cells (–65 ± 5% in NPA cells; fig. 7E) and in cells of different histological types (–0-70%, not shown). These results suggest that Ret/Ptc1 expression is not mandatory but could contribute to the indirect inhibition of telomerase activity by RPI-1.

Discussion

In the present study, we investigated the response of the human PTC cell line TPC-1 to the 2-indolinone RPI-1, the first agent reported to be a specific Ret inhibitor. The compound inhibited TPC-1 cell proliferation and, at the molecular level, abolished tyrosine phosphorylation of the Ret/Ptc1 oncogenic fusion protein and its association with the signaling molecules Shc and PLCγ. In addition, activation of JNK2, Akt, and telomerase were inhibited in treated cells.

TPC-1 cells represent a good model to study the effects of Ret inhibitors in a proper cellular context. Indeed, they have been used as a positive control for RET activation in several studies since the description of the chimeric RET/H4 transcript expressed in this cell line [33, 34]. Like another more recently established RET/PTC1-positive cell line (FB-2) [35], TPC-1 cells are not tumorigenic, a feature that is in agreement with the low aggressiveness of most PTCs of the thyroid but that hampers in vivo antitumor activity studies. Indeed, only a few cellular models of thyroid cancer characterized for the expression of oncogenic Ret proteins are available.

RPI-1 inhibited TPC-1 cell proliferation by a cytostatic effect characterized by accumulation of cells at the G2 cell cycle phase, concomitant with a marked induction of the cyclin-dependent kinase inhibitor p21WAF1. The role of p21WAF1 as a negative regulator of cell cycle progression at both G1 and G2 phases is well established. Moreover, its involvement in multiple biological functions is increasingly recognized. For example, p21WAF1 was shown to associate with and to inhibit activation of the JNK and p38 members of the MAPK family [36]. Such an effect could contribute to the inhibition of JNK2 activation observed in both NIH3T3^{PTC1} fibroblasts and thyroid TPC-1 cells following exposure to RPI-1. Notably, in thyroid cells, JNK activity has been associated, physiologically, with mitogenesis [29] and exogenously expressed oncogenic forms of RET were shown to induce JNK activity in different cell lines including thyroid cells [37–39], thus suggesting that constitutive JNK activation could participate in Ret-mediated transformation processes in human thyroid cells.

A relevant role in the pathway of JNK activation has been ascribed to the multifunctional docking site Tyr1062 of proto-Ret. In fact, the RET mutation Y1062F was shown to impair the activation of MAPK family members (JNK, ERKs, and p38), as well as AKT, induced by GDNF in human neuroblastoma cells [40]. All these pathways, constitutively active in TPC-1 cells, were inhibited following RPI-1 treatment in the absence of serum (not shown). However, in normal cell culture conditions, RPI-1-induced dephosphorylation of Tyr451 on Ret/Ptc1 (Tyr1062 of membrane Ret proteins) and prevention of cell proliferation were associated with inhibition restricted to activation of Akt and JNK2. Together with the previously reported observation that microinjection of an antibody directed against Tyr1062 significantly reduced the proliferation of TPC-1 cells [41], our findings support a main role of JNK2 and Akt pathways in the Ret/Ptc1-triggered signaling driving PTC cell growth, although we cannot rule out that inhibition of additional protein tyrosine kinases might also contribute to the down-regulation of JNK2 and AKT activation by RPI-1.

Tyr1062 of Ret has been proposed as a multifunctional docking site essential for the transforming ability of Ret oncoproteins [26]. Shc is a main signaling protein recruited at this site and its activation has a relevant role in the transforming pathways triggered by Ret/Ptc fusion proteins [27, 28]. Here we show that, in accordance with RPI-1-induced dephosphorylation of the Shc docking site on Ret/Ptc1 (Tyr451), RPI-1 treatment also abrogated the co-immunoprecipitation of Ret/Ptc1 with Shc in TPC1 cells, thereby indicating interruption of the Ret/Ptc1 signaling through Shc. Worth noting is that the effect of drug treatment on the three Shc isoforms was not uniform. In particular, p66Shc was down-regulated, p52Shc was tyrosine dephosphorylated, whereas p46Shc was not affected. These findings raise the question of the functional role of the single Shc isoforms in Ret signaling, an aspect that has not been addressed up to now. The drug-induced down-regulation of p66Shc was an unexpected finding since it was not observed in NIH3T3^{PTC1} cells [15]. Such an Shc isoform, indeed, does not appear to be involved in

the Ras/ERK pathway but has been proposed as a cytoplasmic signal transducer involved in stress apoptotic responses and life span in mammals [42, 43]. Further investigation is required to understand this cellular response to RPI-1 and the relevance of the cellular context. An additional novel finding in the present study was the indirect RPI-1 inhibitory effect on telomerase activity. Ret/Ptc1 expression appeared to be dispensable to such an effect since telomerase inhibition was also observed to some degree in cells not expressing Ret proteins. Nonetheless, the marked inhibition obtained in TPC-1 cells (85 ± 10%) was not reached in other cell systems. Forced overexpression of p21WAF1 CDK inhibitor was reported to inhibit telomerase activity in immortalized human keratinocytes as a consequence of a reduced expression of the enzyme RNA subunit hTR [44]. However, results from our RT-PCR experiments did not support such an interpretation, since no significant reduction in the abundance of the different telomerase components was observed in TPC-1 cells exposed to the drug, despite up-regulation of p21WAF1, compared to control cells. Moreover, results from time-course experiments carried out in RPI-1-treated cells at different intervals after drug removal showed that telomerase activity recovered almost completely when p21WAF1 was still overexpressed, thus excluding a causal relationship between the two cellular events (data not shown). Our study indicated that activation of Akt, a protein kinase involved in the pathway of telomerase activation through the phosphorylation of the telomerase reverse transcriptase hTERT [45], was inhibited by RPI-1, thus suggesting that RPI-1-induced telomerase inhibition in TPC-1 cells could be ascribable, at least in part, to a reduced level of Akt-mediated hTERT phosphorylation. In addition, the possibility that the high level of inhibition of telomerase catalytic activity observed in TPC-1 cells was dependent on a reduced level of phosphorylation of the holoenzyme could be related to the inhibitory effect induced by RPI-1 on PLCγ. This, in fact, could indirectly compromise protein kinase C (PKC) activity and, as a consequence, attenuate telomerase activity, since PKCα has been implicated in the activation of telomerase through direct or indirect phosphorylation of its protein components hTERT and TEP1 [46]. Although telomerase reactivation has been suggested to play a role during the development of thyroid cancer [47], the clinical relevance of telomerase expression in tumors derived from the thyroid follicular cell remains controversial [48]. Further studies are needed to elucidate the role of telomerase inhibition in the PTC cell response to RPI-1.

In conclusion, our findings, indicating specific modulation of proliferation/transformation-associated signaling pathways by the Ret inhibitor RPI-1, support a role for oncogenic Ret proteins as therapeutic targets in neoplastic thyroid diseases. Such a conclusion is consistent with the recently reported results obtained with the use of the tyrosine kinase inhibitor PP1 on NIH-RET/PTC3 transfectants [49]. In addition to being a useful research tool for studying the physiological and pathological functions of Ret proteins, RPI-1 is a prototype drug candidate for preclinical antitumor activity studies on RET-driven thyroid tumors. Such studies will be crucial for evaluating the therapeutic potential of the drug in advanced thyroid cancers, particularly MTC, for which efficacious chemotherapeutic agents are lacking.

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1 Schuchardt A., D'Agati V., Larsson-Blomberg L., Costantini F. and Pachni V. (1994) Defects in the kidney and enteric nervous system of mice lacking the tyrosine kinase receptor Ret. Nature **367:** 319–320
2 Pasini B., Borrello M. G., Greco A., Bongarzone I., Luo Y., Mondellini P. et al. (1995) Loss of function effect of *RET* mutations causing Hirschsprung disease. Nat. Genet. **10:** 35–40
3 Jhiang S. M. (2000) The *RET* proto-oncogene in human cancers. Oncogene **19**: 5590–5597
4 Tallini G. and Asa S. L. (2001) *RET* oncogene activation in papillary thyroid carcinoma. Adv. Anat. Pathol. **8:** 345–354
5 Takahashi M. (2001) The GDNF/Ret signaling pathway and human diseases. Cytokine Growth Factor Rev. **12:** 361–373
6 Grieco M., Santoro M., Berlingieri M. T., Melillo R. M., Donghi R., Bongarzone I. et al. (1990) PTC is a novel rearranged form of the *ret* proto-oncogene and is frequently detected in vivo in human thyroid papillary carcinomas. Cell **60:** 557–563
7 Ito S., Iwashita T., Asai N., Murakami H., Iwata Y., Sobue G. et al. (1997) Biological properties of Ret with cysteine mutations correlate with multiple endocrine neoplasia type 2A, familial medullary thyroid carcinoma, and Hirschsprung's disease phenotype. Cancer Res. **57:** 2870–2872
8 Iwashita T., Kato M., Murakami H., Asai N., Ishiguro Y., Ito S. et al. (1999) Biological and biochemical properties of Ret with kinase domain mutations identified in multiple endocrine neoplasia type 2B and familial medullary thyroid carcinoma. Oncogene **18:** 3919–3922
9 Santoro M., Chiappetta G., Cerrato A., Salvatore D., Zhang L., Manzo G. et al. (1996) Development of thyroid papillary carcinomas secondary to tissue-specific expression of the *RET*/*PTC1* oncogene in transgenic mice. Oncogene **12:** 1821–1826
10 Cho J.-Y., Sagartz J. E., Capen C. C., Mazzaferri E. L. and Jhiang S. M. (1999) Early cellular abnormalities induced by *RET/PTC1* oncogene in thyroid-targeted transgenic mice. Oncogene **18:** 3659–3665
11 Michiels F.-M., Chappuis S., Caillou B., Pasini A., Talbot M., Monier R. et al. (1997) Development of medullary thyroid carcinoma in transgenic mice expressing the *RET* protooncogene altered by a multiple endocrine neoplasia type 2A mutation. Proc. Natl. Acad. Sci. USA **94:** 3330–3335
12 Acton D. S., Velthuyzen D., Lips C. J. and Hoppener J. W. (2000) Multiple endocrine neoplasia type 2B mutation in human *RET* oncogene induces medullary thyroid carcinoma in transgenic mice. Oncogene **19:** 3121–3125
13 Schlessinger J. (2000) Cell signaling by receptor tyrosine kinases. Cell **103:** 211–225

14 Shawver L. K., Slamon D. and Ullrich A. (2002) Smart drugs: tyrosine kinase inhibitors in cancer therapy. Cancer Cell **1:** 117–123

15 Lanzi C., Cassinelli G., Pensa T., Cassinis M., Gambetta R. A., Borrello M. G. et al. (2000) Inhibition of transforming activity of the ret/ptc1 oncoprotein by a 2-indolinone derivative. Int. J. Cancer **85:** 384–390

16 Borrello M. G., Alberti L., Arighi E., Bongarzone I., Battistini C., Bardel A. et al. (1996) The full oncogenic activity of Ret/ptc2 depends on tyrosine 539, a docking site for phospholipase Cγ. Mol. Cell. Biol. **16:** 2151–2163

17 Yamamoto M., Li M., Mitsuma N., Ito S., Kato M., Takahashi M. et al. (2001) Preserved phosphorylation of Ret receptor protein in spinal motor neurons of patients with amyotrophic lateral sclerosis: an immunohistochemical study by a phosphorylation- specific antibody at tyrosine 1062. Brain Res. **912:** 89–94

18 Skehan P., Storeng R., Scudiero D., Monks A., McMahon J., Vistica D. et al. (1990) New colorimetric cytotoxicity assay for anticancer-drug screening. J. Natl. Cancer Inst. **82:** 1107–1112

19 Villa R., Zaffaroni N., Folini M., Martelli G., De Palo G., Daidone M. G. et al. (1998) Telomerase activity in benign and malignant breast lesions: a pilot prospective study on fine-needle aspirates. J. Natl. Cancer Inst. **90:** 537–539

20 Kim N. W., Piatyszek M. A., Prowse K. R., Harley C. B., West M. D., Ho P. L. et al. (1995) Specific association of human telomerase activity with immortal cells and cancer. Science **266:** 2011–2015

21 Villa R., Della Porta C., Folini M., Daidone M. G. and Zaffaroni N. (2001) Possible regulation of telomerase activity by transcription and alternative splicing of telomerase reverse transcriptase in human melanoma. J. Invest. Dermatol. **116:** 867–873

22 Cerutti J., Trapasso F., Battaglia C., Zhang L., Martelli M. L., Visconti R. et al. (1996) Block of c-*myc* expression by antisense oligonucleotides inhibits proliferation of human thyroid carcinoma cell lines. Clin. Cancer Res. **2:** 119–126

23 Borrello M. G., Pelicci G., Arighi E., De Filippis L., Greco A., Bongarzone I. et al. (1994) The oncogenic versions of the Ret and Trk tyrosine kinases bind Shc and Grb2 adaptor proteins. Oncogene **9:** 1661–1668

24 Van Weering D. H. J. and Bos J. L. (1997) Glial cell line-derived neurotrophic factor induces Ret-mediated lamellipodia formation. J. Biol. Chem. **272:** 249–254

25 Lorenzo M. J., Gish G. D., Houghton C., Stonehouse T. J., Pawson T., Ponder B. A. et al. (1997) *RET* alternate splicing influences the interaction of activated Ret with the SH2 and PTB domains of Shc, and the SH2 domain of Grb2. Oncogene **14:** 763–771

26 Asai N., Murakami H., Iwashita T. and Takahashi M. (1996) A mutation at tyrosine 1062 in MEN2A-Ret and MEN2B-Ret impairs their transforming activity and association with shc adaptor proteins. J. Biol. Chem. **271:** 17644–17649

27 Arighi E., Alberti L., Torriti F., Ghizzoni S., Rizzetti M. G., Pelicci G. et al. (1997) Identification of Shc docking site on Ret tyrosine kinase. Oncogene **14:** 773–782

28 Mercalli E., Ghizzoni S., Arighi E., Alberti L., Sangregorio R., Radice M. T. et al. (2001) Key role of Shc signaling in the transforming pathway triggered by Ret/ptc2 oncoprotein. Oncogene **20:** 3475–3485

29 Shklyaev S. S., Namba H., Mitsutake N., Alipov G., Nagayama Y., Maeda S. et al. (2001) Transient activation of c-Jun NH_2-terminal kinase by growth factors influences survival but not apoptosis of human thyrocytes. Thyroid **11:** 629–636

30 Toker A. (2000) Protein kinases as mediators of phosphoinositide 3-kinase signaling. Mol. Pharmacol. **57:** 652–658

31 Elenitoba-Johnson K. S. J. (2001) Complex regulation of telomerase activity: implications for cancer therapy. Am. J. Pathol. **159:** 405–410

32 Holt S. E., Aisner D. L., Shay J. W. and Wright W. E. (1997) Lack of cell cycle regulation of telomerase activity in human cells. Proc. Natl. Acad. Sci. USA **94:** 10687–10692

33 Ishizaka Y., Itoh F., Tahira T., Ikeda I., Ogura T., Sugimura T. et al. (1989) Presence of aberrant transcripts of ret proto-oncogene in a human papillary thyroid carcinoma cell line. Jpn. J. Cancer Res. **80:** 1149–1152

34 Jossart G. H., Greulich K. M., Siperstein A. E., Duh Q., Clark O. H. and Weier H. U. (1995) Molecular and cytogenetic characterization of a t(1;10;21) translocation in the human papillary thyroid cancer cell line TPC-1 expressing the *RET/H4* chimeric transcript. Surgery **118:** 1018–1023

35 Basolo F., Giannini R., Toniolo A., Casalone R., Nikiforova M., Pacini F. et al. (2002) Establishment of a non-tumorigenic papillary thyroid cell line (FB-2) carrying the *RET/PTC1* rearrangement. Int. J. Cancer **97:** 608–614

36 Dotto G. P. (2000) $p21^{WAF1/Cip1}$: more than a break to the cell cycle? Biochim. Biophys. Acta **1471:** 430–56

37 Chiarello M., Visconti R., Carlomagno F., Melillo R. M., Bucci C., De Franciscic V. et al. (1998) Signaling of the Ret receptor tyrosine kinase through the c-Jun NH_2-terminal protein kinases (JNKs): evidence for a divergence of the ERKs and JNKs pathways induced by Ret. Oncogene **16:** 2435–2445

38 Marshall G. M., Peaston A. E., Hocker J. E., Smith S. A., Hansford L. M., Tobias V. et al. (1997) Expression of multiple endocrine neoplasia 2B *RET* in neuroblastoma cells alters cell adhesion in vitro, enhances metastatic behavior in vivo, and activates Jun kinase. Cancer Res. **57:** 5399–5405

39 Xing S., Furminger T. L., Tong Q. and Jhiang S. M. (1998) Signal transduction pathways activated by Ret oncoproteins in PC12 pheochromocytoma cells. J. Biol. Chem. **273:** 4909–4914

40 Hayashi H., Ichihara M., Iwashita T., Murakami H., Shimono Y., Kawai K. et al. (2000) Characterization of intracellular signals via tyrosine 1062 in Ret activated by glial cell line-derived neurotrophic factor. Oncogene **19:** 4469–4475

41 Salvatore D., Barone M. V., Salvatore G., Melillo R. M., Chiappetta G., Mineo A. et al. (2000) Tyrosines 1015 and 1062 are in vivo autophosphorylation sites in Ret and Ret-derived oncoproteins. J. Clin. Endocrinol. Metab. **85:** 3898–3907

42 Ravichandran K. S. (2001) Signaling via Shc family adapter proteins. Oncogene **20:** 6322–6330

43 Migliaccio E., Giorgio M., Mele S., Pelicci G., Reboldi P., Pandolfi P. P. et al. (1999) The $p66^{shc}$ adaptor protein controls oxidative stress response and life span in mammals. Nature **402:** 309–313

44 Kallassy M., Martel N., Damour O., Yamasaki H. and Nakazawa H. (1998) Growth arrest of immortalized human keratinocytes and suppression of telomerase activity by $p21^{WAF1}$ gene expression. Mol. Carcinog. **21:** 26–36

45 Kang S. S., Kwon T., Kwon D. Y. and Do S. I. (1999) Akt protein kinase enhances human telomerase activity through phosphorylation of telomerase reverse transcriptase subunit. J. Biol. Chem. **274:** 13085–13090

46 Li H., Zhao L., Yang Z., Funder J. W. and Liu J. P. (1998) Telomerase is controlled by protein kinase Cα in human breast cancer cells. J. Biol. Chem. **273:** 33436–33442

47 Cheng A. J., Lin J. D., Chang T. and Wang T. C. (1998) Telomerase activity in benign and malignant human thyroid tissues. Br. J. Cancer **77:** 2177–2180

48 Matthews P., Jones C. J., Skinner J., Haughton M., Micco C. de and Wynford-Thomas D. (2001) Telomerase activity and telomere length in thyroid neoplasia: biological and clinical implications. J. Pathol. **194:** 183–193

49 Carlomagno F., Vitagliano D., Guida T., Napolitano M., Vecchio G., Fusco A. et al. (2002) The kinase inhibitor PP1 blocks tumorigenesis induced by *RET* oncogenes. Cancer Res. **62:** 1077–1082