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*Running title: *Diverse RET/PTC Proinflammatory and Transforming Pathways*

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Background: Follicular cell-derived thyroid carcinomas harboring RET/PTC oncogenes are immunostimulatory and highly curable, despite activating RAS/BRAF/MEK/ERK and PI3K/AKT.
Results: RET/PTC oncoproteins associate with TRAFs to mediate cytokine production but not transformation.
Conclusion: The RET/PTC:TRAF pathway is functionally separable from the RET/PTC-induced MEK/ERK and PI3K/AKT pathways.
Significance: Understanding RET/PTC-mediated immunostimulation could provide new strategies for treating more aggressive forms of thyroid carcinoma.

SUMMARY

Thyroid carcinomas that harbor *RET/PTC* oncogenes are well differentiated, relatively benign neoplasms compared to those expressing oncogenic *RAS* or *BRAF* mutations despite signaling through shared transforming pathways. A distinction, however, is that RET/PTCs induce immunostimulatory programs, suggesting that, in the case of this tumor type, the additional pro-inflammatory pathway reduces aggressiveness. Here we demonstrate that pro-inflammatory programs are selectively activated by TRAF2 and TRAF6 association with RET/PTC oncoproteins. Eliminating this mechanism reduces pro-inflammatory cytokine production without decreasing transformation efficiency. Conversely, ablating MEK/ERK or PI3K/AKT signaling eliminates transformation but not pro-inflammatory cytokine secretion. Functional uncoupling of the two pathways demonstrates that intrinsic pro-inflammatory pathways are not required for cellular transformation and suggests a need for further investigation into the role inflammation plays in thyroid tumor progression.

Inflammation can contribute to late phases of cancer progression by enhancing viability, angiogenesis and metastasis (1, 2). The notion that inflammation can also be an important early event during neotransformation is based upon the positive correlation observed between pre-existing chronic inflammatory conditions and cancer incidence (1-3); and the observed effects of anti-inflammatory compounds modulating cancer development (4) Thus, it has been suggested that activation of the NF-κB family of transcription factors promotes mitogenicity by triggering cytokine secretion from neoplasia and inflammatory infiltrates while supporting tumor viability through inhibition of apoptotic pathways (5). Additionally, inflammatory infiltrates have the potential to induce reactive oxygen species and

such an oxidative environment could promote oncogenic mutations (6). Yet, NF-κB itself is not an oncogene and tissue specific autoimmunity in the absence of cancer often exists in patients for extensive periods. Therefore, the requirement for inflammation during initiation and early stages of tumorigenesis remains unclear (1).

Papillary thyroid carcinoma (**PTC**), the most prevalent endocrine cancer worldwide (7), is particularly attractive for exploration of this issue. PTCs that progress to become more aggressive follicular cell-derived thyroid carcinomas (**FDTC**), such as follicular thyroid carcinoma (**FTC**) and anaplastic thyroid carcinoma (**ATC**) are generally associated with point mutations in RAS small GTPases or BRAF serine/threonine kinases, respectively (2, 8). The more indolent PTCs are associated with radiation-induced RET/PTC oncogenes that encode fusions of the RET receptor kinase domain with one of several different dimerizing proteins, resulting in a constitutively active kinase (9). The most prevalent RET/PTC isoforms are RET/PTC1 (**RP1**) and RET/PTC3 (**RP3**) consisting of either H4/CCDC6 or ARA70/ELE1 as the respective N-terminal dimerizing partner (10)(10)(10) (10-12). RET/PTC oncogenes activate both RAS/BRAF/MEK/ERK and PI3K/AKT pathways that are crucial for thyrocyte transformation (13-15), yet are associated with a high cure rate and low tumor recurrence. In contrast, the more aggressive FDTCs usually harbor oncogenic RAS or BRAF point mutations and are associated with a poorer prognosis and higher recurrence rate (2, 8).

Notably, FDTC harboring RET/PTC oncogenes display an immunostimulatory profile (14, 15) and is associated with the development of autoimmune thyroiditis (16-21). Conversely, the more aggressive and poorly differentiated FDTCs, expressing oncogenic RAS or BRAF point mutations, are characterized by tumor-promoting immune responses such as the infiltration of immunosuppressive macrophages (22). Although the mechanistic basis for RET/PTC-induced immunostimulation is currently unclear, it is thought to involve members of both the classical and alternative pathways of NF-κB through the stabilization of NIK kinase (23-25).

Since RET/PTC oncoproteins activate RAS/BRAF/MEK/ERK, PI3K/AKT, and NF-κB signal transduction pathways, this tumor type might be expected to be highly proliferative and readily progress to a less differentiated cancer such as anaplastic carcinoma. However, RET/PTC-expressing PTCs tend to be rather indolent and expression in poorly-differentiated and anaplastic thyroid carcinomas are rare (26). Thus, in the case of PTC, one interpretation of this process is that the additional immunostimulatory program is detrimental to the progressing tumor. To resolve this conundrum, our primary goal was to determine whether proinflammatory cytokine release and cellular transformation proceed along the same signaling pathways, or whether the two processes are functionally distinct and separable. The latter would permit investigation of whether the RET/PTC-induced proinflammatory program is necessary for transforming events as well as exploration of the mechanistic link between RET/PTC expression and NF-κB activation. The results of our investigations provide insight into the early stages of thyroid oncogenesis that could influence future approaches to the treatment of all types of FDTC.

Experimental Procedures

Chemical Reagents: All cell culture and chemical reagents were purchased from Sigma unless stated otherwise.

Cloning: mRP3.51 (RP3) was previously constructed and cloned into a bacterial expression vector (27). To perform the following studies RP3 was excised from the pET29a vector and cloned into the mammalian expression vectors Rc/CMV and MSCV.IRES.GFP. A Kozak consensus and a TAA stop sequence were placed 5' and 3' of the RP3 cDNA. All RP3 mutants were created by site-directed mutagenesis using Quikchange II (Stratagene) according to manufacturer's instructions. MSCV.IRES.mRFP was created by excising the GFP sequence and replacing it with the sequence for monomeric RFP. The sequences encoding TRAF2 and TRAF6 blocking peptides (**T2pep** and **T6pep**) and control peptides (**T2cntl** and **T6cntl**) were synthesized by IDT, amplified by PCR and cloned into MSCV.IRES.mRFP.

Cell Culture: TPC-1 and PCCL3 cells were kindly provided previously by Dr. Massimo Santoro. NIH-3T3 (kindly provided by Dr. Tschiclis, Tufts University), 293T (ATCC), and TPC-1 cell lines were maintained in DMEM media with 10% FBS (D10). The rat PCCL3 thyroid cell line was maintained in F12 media containing $5x10^{-3}$ IU/ml bovine TSH, 5 μg/ml bovine insulin, 10 ng/ml Gly-His-Lys, somatostatin 10 ng/ml, apo-transferin 5 μg/ml, 10 nM hydrocortisone, and 10% FBS (F12+). PCCL3 cells require thyroid stimulating hormone (TSH) for cell growth under basal conditions and exhibit TSH-independent growth upon expression of RET/PTC. However, since removing TSH can alter the ability of thyrocytes to produce inflammatory cytokines (28-30), all PCCL3 treatments were maintained in the same culture conditions. For all experiments PCCL3 cells were treated in a 1:1 ratio of F12 media and D10. All cells were maintained in a 37°C incubator at 9% CO_2. *For the generation of stable lines:* Retrovirus was prepared by co-transfecting 293T cells with pCL-Eco and indicated MSCV.IRES.GFP or MSCV.IRES.mRFP constructs with Fugene 6 (Roche). At 24 hr post-transfection, fresh medium was replaced and viral supernatant was collected at 24, 48, and 72hr. For viral transduction, cells were plated at 5x10^4 cells/well in 6-well plates and treated with pre-filtered viral supernatant diluted 1:2 in DMEM containing 10% FBS and 8 μg/ml polybrene. At 24 hr post-transduction viral supernatant was replaced with fresh 10% media and sorted for GFP or monomeric RFP expression on day 3. *For maintenance of cell lines:* NIH-3T3 transductants were maintained in D10 while all PCCL3 transductants were maintained in 1:5 ratio of F12+:D10. TSH, insulin, Gly-His-Lys, somatostatin, apo-transferin, and hydrocortisone were all from Sigma. U0126 (Cell Signaling Technology) was chosen based on its high selectivity to both MEK1 and MEK2 (versus PD098059 which inhibits MEK1), and used at 10 μM at all indicated times based on previously published results (31). LY294002 (Cell signaling Technology) was chosen based on its high selectivity to PI3K and used at 10 μM to demonstrate PI3K/AKT inhibition in the absence of apoptosis/toxicity (32, 33).

Transient Transfection and siRNA knockdown: For cytokine analysis of PCCL3 cells, 10^5 cells/well were plated in 6-well tissue culture plates. The next day cells were transfected with Genejuice (EMD Chemicals) at a 3:3 ratio of reagent (μl):DNA (μg). After 48 hr, total RNA (Machery-Nagel) or cytosolic protein was harvested for RT-PCR or western analysis. For TRAF knockdown of TPC cells, TRAF2 siRNA and TRAF6 siRNA (Santa Cruz) were transfected at 25 nM with siRNAMAX (Invitrogen) and on day 2 was harvested for ELISA, RT-PCR, or western analysis or subsequently treated and harvested on day 4 as indicated.

RT-PCR: Total RNA was extracted using the Nucleospin RNA II kit (Macherey-Nagel). Reverse transcriptase-PCR was performed on 0.5-2 μg of total RNA using Superscript II (Invitrogen) according to manufacturer's instructions. PCR was performed for 30 cycles at 60°C.

Western Blot Analysis and Immuno-precipitations: All cells were lysed with 150 mM NaCl, 20 mM Tris-Cl, 1% Triton X 100, pH 7.2 containing 1X HALT protease inhibitors with EDTA (Pierce) and 1X HALT phosphatase inhibitors (Pierce). Lysates were quantified using BCA kit (Pierce). *For immuno-precipitation:* Pre-cleared 500 μg total protein was incubated overnight with anti-RET antibody and Protein G-sepharose beads (Pierce). Subsequently, beads were washed with cell lysis buffer three times and eluted with 100 mM glycine (pH 2.7). Eluted material was neutralized with 1M Tris pH 8.8, combined with 6X loading buffer, and run on 12% SDS-PAGE gel. *For Western analysis:* 10 μg of total protein was loaded onto a 12% SDS-polyacrylamide gel. Gels were blotted for 1 hr at 100V onto nitrocellulose membranes (GE Biosciences) and blocked for 30 min with 5% milk/TBS + 0.1% Tween. All blots were incubated overnight with the indicated antibody, washed and stained with anti-rabbit-HRP (Cell Signaling), donkey anti-mouse-HRP or donkey-anti-goat-HRP (Jackson Immunoresearch). All indicated antibodies were from Cell Signaling Technology aside from anti-phospho-RET (1062),

anti-RET (C-20), and anti-TRAF6 (H274) which were from SantaCruz Biotechnology.

Macrophage Chemotactic Protein MCP-1 ELISA: A 96-well Maxisorp plate (Millipore) was coated at 2 mg/ml with anti-CCL2 antibody (eBioscience), overnight 4°C. The plate was blocked with 1% BSA/PBS and cell culture supernatants were incubated in duplicate wells overnight at 4°C. The next day, plates were washed with PBS/0.05% Tween 20 and incubated with biotinylated anti-CCL2 antibody (eBioscience) for 2 hr, 27°C, washed, stained with 1:1000 Streptavidin-HRP (BD Bioscience) for 1 hr, 27°C, and developed with 2 mg/ml OPD (Sigma) according to manufacturer's instructions and visualized at 405 nm in a spectrophotometer (Victor2 by Wallac 1420 multilabel counter).

Cell Proliferation: PCCL3 and TPC-1 transductants were cultured in triplicate at 1000 cells per well in 96-well flat bottom plates in D10 media in the absence of hormones. Viable cells were counted daily by trypan blue exclusion.

Soft Agar Assay: $5x10^3$ stable GFP-expressing NIH-3T3 lines were suspended in 0.25% agarose in 1X MEM + 10% FBS with 1 X penicillin/streptomycin (Gibco) and 1X gentamicin (Gibco) and plated in triplicate over a layer of pre-set 0.5% agarose in 1X MEM + 10% FBS with 1 X penicillin/streptomycin and 1X gentamicin in 6-well plates. 20 days after plating the cells were photographed and analyzed for colony number and size or stained for 1 hour at 27°C with crystal violet in PBS and photographed over a light box. For quantification of colony size, 5 photographs were taken per well using a Nikon Eclipse TE 2000-U camera attached to a confocal microscope. 1 photograph/1 cm^2 block was taken using a grid that was partitioned in 1 cm^2 sections was . The longest length of the colonies was measured in μm using the NIS Elements Version 2.3 program. The sum of the number and length of the colonies was calculated per well and the mean and standard deviation of triplicate wells was calculated per condition (total of 15 photographs taken per condition).

RESULTS

Chemical inhibition of PI3K and MEK1/2 does not decrease RET/PTC-mediated cytokine induction. Both RP1 and RP3 activate similar oncogenic and inflammatory programs (14, 15, 25, 34) and this trait is attributed to the constitutively active C-terminal RET kinase domain that is shared by all RET/PTC variants (35, 36). Therefore, we utilized an immortalized rat thyroid cell line, PCCL3, transiently transfected with RP3 to examine early activation of proinflammatory cytokine transcription.. Early phase induction occurs within the first 48 hr of expression and drives ectopic production of MCP1 in abundant quantities and IL6 to a lesser extent (34). To determine whether this early cytokine induction is dependent upon PI3K/AKT or MEK/ERK signaling, PCCL3 cells were transfected with the highly related mouse homologue of RP3 (**$RP3^{WT}$**) and treated with selective pathway inhibitors. As demonstrated in **Fig. 1A**, the PI3K inhibitor LY294002 (**LY**) or with the MEK1/2 inhibitor U0126 (**U0**) did not disrupt early transcriptional induction of MCP1 or IL6 by RP3. As a control for protein expression, $RP3^{K284M}$, which is devoid of all kinase activity (37, 38), was examined and demonstrated no increase in AKT, ERK, or proinflammatory cytokine activity. Furthermore, RP3 transfected cells treated with LY or U0 and cells transfected with $RP3^{K284M}$ exhibited less cell scattering, indicative of increased cell mobilization, and less transformation when phenotypically compared to cells transfected with $RP3^{WT}$ (**Fig. S1A**). The inhibition of DUSP6 induction by U0 and activation of the sodium iodide symporter (NIS) expression by U0 and LY, demonstrate that the inhibitors were effective at disrupting their respective pathways (**Fig. 1A**). Furthermore, western blot analysis revealed that treatments with LY and U0 led to a dramatic decrease in phosphorylation of AKT and ERK, respectively (**Fig. 1B**).

Similar experiments were then performed to determine the influence of the AKT and ERK pathways on late phase cytokine expression. After long-term expression in thyrocytes, RET/PTC induces a milieu of proinflammatory cytokines that include MCP1, IL6, IL8, TNFα, IL1α, IL1β, CXCL10, M-CSF and GM-CSF (14). The TPC-1 cells line is derived from human papillary thyroid carcinoma, constitutively expresses RP1, and produces these same proinflammatory cytokines. TPC-1 cells treated with LY or U0 exhibited no

loss of MCP1, IL6, and TNFα transcript production after 48 hrs (**Fig. 1C**). In fact, it appears that inhibition of ERK activity actually increases cytokine transcription. This phenomenon was also observed at the protein level (**Fig. S1C**), suggesting that a potential negative feedback role of MEK/ERK exists to reduce RET/PTC-mediated late phase cytokine secretion. Again, these compounds effectively disrupted intended components in these signaling pathways as indicated by western blot analysis (**Fig. 1D),** reduced transformed phenotype, and decreased cell proliferation (**Figs. S1B and S1D**). We did observe decreased MCP-1 secretion from LY treated cells (**Fig. S1C**), however this decrease was attributable to a reduction in total cell number (**Fig. S1D**). Together these results support the idea that ectopic cytokine secretion is a common feature of RET/PTC variants (14, 34), and support the notion that this genetic program is not dependent upon PI3K/AKT and MEK/ERK for early or late phases of cytokine secretion.

Proinflammatory and mitogenic signaling pathways originate from distinct regions of the RP3 oncoprotein. The observation that RP3-mediated early and late inflammatory programs are not dependent upon AKT and ERK signaling, led us to investigate whether the inflammatory and mitogenic pathways could be uncoupled through genetic approaches. Mass spectrometric analysis had previously determined that at least 8 of 16 key tyrosine residues are autophosphorylated during RET activation (39), and RP3 contains all 8 proposed signaling tyrosines. Therefore, we generated a panel of Y→F substitutions and screened for the ability of these mutants to induce early cytokine production from PCCL3 cells. One of these mutations, Y588F, is known to be needed for PI3K/AKT and RAS/BRAF/MEK/ERK signaling (40-43). If functional separation is possible, a mutation could demonstrate proinflammatory activity in the absence of AKT or ERK activity. Indeed, both $RP3^{WT}$ and $RP3^{Y588F}$ induced equal amounts of MCP-1 (**Fig. 2A**), indicating that the inflammatory pathway does not initiate from Y588. Since it has been reported that Grb2 binds the Y622 site (44), although it does not appear to contribute to ERK activity (40),, we additionally tested a Y588F/Y622F double mutant and did not detect decreases in MCP1 production (**Fig. 2B**). With respect to the reverse phenotype, (transformation without proinflammatory activity) three substitutions, $RP3^{K284M}$, $RP3^{Y431F}$, and $RP3^{Y478F}$, resulted in ablation of early phase RP3-induced MCP-1 production from transiently transfected PCCL3 cells (**Fig. 2A**). This was somewhat expected for $RP3^{K284M}$ and $RP3^{Y431F}$ since both encode proteins with catalytically inactive kinase domains based on the crystal structure and biochemical analysis of RET (39, 45). In contrast, altered MCP-1 induction by $RP3^{Y478F}$ was not anticipated and the downstream effect of this mutation on other RP3 regulated signaling pathways was investigated by western blot analysis. As shown in **Fig. 2C**, compared to the other mutations, $RP3^{Y478F}$ enabled activation of both AKT and ERK pathways to the same degree as $RP3^{WT}$. These findings prompted a closer evaluation of the protein interactions at or adjacent to the Y478 region.

TRAF2 and TRAF6 associate with RP3 in a kinase-independent manner. To identify potential candidates as binding partners at or near Y478, the full length sequences of human, mouse, and rat RP3 were screened online at the Eukaryotic Linear Motif (ELM) site (http://elm.eu.org/) (46) using protein structure, cell compartment (cytosol), and taxonomic (human, mouse, and rat) filters. Consequently, the sequence 482-487 (GI**P**P**E**RL**F**), adjacent to Y478, was identified as a potential binding site for TRAF6 and 560-564 (**S**E**E**E) was designated as a site for TRAF2. TRAF2 and TRAF6 are members of the TRAF (TNF Receptor Associated Factor) family of E3 ubiquitin ligases that initiate either classical or alternative NF-κB signaling cascades from TNFR family members, TLRs, and other cell surface receptors such as NGFR and TGFβR to promote proinflammatory cytokine production (47).

The GIPPERLF site is situated within a surface soluble disordered loop (45) suggesting that this region is accessible to cytosolic binding partners, and the SEEE motif also appears to be located within disordered regions according to the ELM site and the online program GlobPlot (http://globplot.embl.de/) using Deleage/Roux or Russell/Linding set parameters (48). To determine whether TRAF proteins associate with RET/PTC, 293T cells were transiently transfected with RP3 and cell lysates were subjected to

immunoprecipitation with an anti-RET antibody specific for the last 51 amino acids of the C-terminus. As shown in **Fig. 3B**, TRAF2 and TRAF6 co-precipitate with $RP3^{WT}$ without additional cross-linking indicating a specific association. Additionally, TRAF2 and TRAF6 binding to $RP3^{Y588F}$ is equivalent to that of $RP3^{WT}$, while $RP3^{Y478F}$ exhibits decreased association to TRAF6 (**Figs. 3C&D**). Of note, TRAF2 and TRAF6 bind equally well to the kinase dead mutant, $RP3^{K284M}$, indicating that binding is independent of kinase activity (**Figs 3B-D**).. To determine the requirements for TRAF interaction, we created truncated mutants of RP3 (**Fig. 3A**). $RP3^{\Delta N}$ lacks the autodimerizing N-terminal segment (residues 1-238) and $RP3^{\Delta TK2}$ lacks the second tyrosine kinase domain of RET (residues 338-589) in which the TRAF-binding motifs are predicted to reside. No association was detected with $RP3^{\Delta TK2}$, while association of TRAF2 and TRAF6 was detectable with $RP3^{\Delta N}$, only after prolonged exposure of the blot but beyond the linear range of detection compared to the $RP3^{WT}$ signal (unpublished data) (**Fig. 3B**). These findings suggest that, although TRAF binding is independent of kinase function, TRAF association is still dependent upon RP3 dimerization (model in **Fig. 7**). Furthermore, the absence of TRAF association with $RP3^{\Delta TK2}$ suggests that TRAF or TRAF-binding adaptor proteins associate within residues 338-589. We confirmed the specificity of the association by examining the endogenous interaction of TRAF2 and TRAF6 to RP1 in the TPC-1 cell line. **Fig. 3G** demonstrates that immunoprecipitation of RP1 can pull down both TRAF2 and TRAF6; suggesting that TRAF association is most likely occurring in the C-terminus derived from RET.

To dissect the specificity of TRAF2 and TRAF6 binding, the putative sites were altered from SEEE to SE**AA** ($RP3^{T2mut}$) and GIPPERLF to GI**Q**P**A**RL**A** ($RP3^{T6mut}$). Examination of $RP3^{T2mut}$ showed decreased binding to TRAF2 compared to $RP3^{WT}$ with no effect on TRAF6 association (**Fig. 3D)**, while $RP3^{T6mut}$ demonstrated decreased binding to both TRAF2 and TRAF6 (**Fig. 3E)**. In contrast to the ΔN and ΔTK2 truncations, none of the tested mutations completely ablated TRAF association, suggesting that there are additional points of contact within the binding sites or that RP3 contains additional binding sites for TRAFs and/or TRAF-associated adaptors. Overall these findings further support the idea that proinflammatory signaling is initiated at TRAF-binding sites through a mechanism that is distinct from Y588-mediated pathways.

The RP3-induced TRAF-mediated production of proinflammatory cytokines is independent of the RAS/BRAF/MEK/ERK and PI3K/AKT pathways. Since TRAF oligomerization is known to activate NF-κB family members and subsequent cytokine induction, we investigated whether TRAF signaling was responsible for production of cytokines from RET/PTC-transformed cells. As shown in **Figs. 4A-C**, siRNA knockdown of either TRAF2 or TRAF6 substantially decreased MCP1 production. Induction of other cytokines was also reduced, GMCSF > IL8 > IL6 > TNFα. This change in inflammatory profile was regulated mainly, if not entirely, at the transcriptional level as demonstrated by RT-PCR (**Figs. 4A-B**). To confirm the reduction of cytokine secretion, supernatants were harvested from TPC-1 cells on day 2 and day 4 after siRNA treatment and tested in a specific MCP1 ELISA. As shown in **Fig. 4C**, MCP1 protein secretion by TRAF siRNA-treated cells was greatly reduced in a time dependent manner compared to siRNA control.

Additionally, we examined the ability of TRAF-inhibiting peptide sequences to knockdown cytokine secretion over a longer period of time. The TRAF2 peptide sequence designed from CD40, AYPIQETA (T2pep), and the TRAF6 sequence designed from RANK, APTEDEYA (T6pep), as well as their controls (AYAIAATA and AATADAYA) were overexpressed using MSCV.IRES.mRFP viral vectors and stable transductants were selected by cell sorting for RFP. **Fig. 4D** demonstrates that inhibition of TRAF2 oligomerization via overexpression of the TRAF2 binding motif greatly reduce cytokine expression in TPC-1 cells. Overexpression of T6pep did not reduce MCP1 induction to the same extent as T2pep and may reflect a decreased affinity of this peptide for its target.

We further examined the cytokine profile of PCCL3 cells stably expressing the RP3 mutants. As observed in **Fig. 4E**, it appears that ablation of MCP1 production was not maintained with

sustained expression of $RP3^{Y478F}$ or $RP3^{T2mut}$; however, this is in contrast to the $RP3^{T6mut}$, in which the long-term production of MCP1 was greatly reduced. This result may reflect the fact that the $RP3^{Y478F}$ or $RP3^{T2mut}$ mutations do not completely ablate TRAF binding although the association is reduced.

We next determined whether TRAF2 or TRAF6 contribute to either AKT or ERK signaling. As predicted by our original findings, TPC-1 cells treated with siRNA specific for TRAF2 or TRAF6 showed no decrease in the phosphorylation levels of AKT or ERK (**Fig. 5A**). Indeed, it appears that TRAF2 signaling negatively regulates PI3K/AKT and RAS/RAF/MEK/ERK pathways since there is increased phosphorylation of AKT and ERK in the absence of TRAF2 (**Fig. 5A**). In addition, long-term expression of TRAF2 and TRAF6 blocking peptides does not affect AKT and ERK activity or cellular proliferation relative to the peptide controls **(Figs. 5B-C)**. Furthermore, it appears that TRAF2 knockdown (**Fig 5A**) or T2pep expression (**Fig 5C**) leads to decreased NIK kinase stability. This finding is consistent with recent studies showing that RP3 promotes the stabilization of NIK to activate the canonical pathway of NF-κB (23). Together these findings suggest that RET/PTCs induce a TRAF-dependent proinflammatory program in thyroid cells and this pathway is independent of PI3K/AKT and RAS/BRAF/MEK/ ERK activity.

TRAF-deficient RP3 mutants maintain transforming properties. Our data indicate that the TRAF-mediated pathway is spatially and functionally distinct from the PI3K/AKT or RAS/BRAF/MEK/ERK pathways. Previous results have reported that point mutations within RAS or BRAF are sufficient to drive transformation of thyroid follicular cells (8). Based on our results above, we hypothesized that disrupting the TRAF pathway should have little to no impact on transformation. To test this prediction, we examined whether the TRAF-deficient mutants were capable of maintaining hormone independent growth. In accordance with our observation that TRAF-deficient mutants maintained ERK and AKT activity, PCCL3 cells stably transduced with $RP3^{Y478F}$, $RP3^{T6mut}$, and $RP3^{T2mut}$ demonstrated hormone-independent growth comparable to RP3-transduced cells (**Fig 5D**). We further examined the ability of the TRAF-deficient mutants to promote anchorage-independent growth. NIH-3T3 cells were transduced with GFP-tagged retroviral constructs encoding wild-type or mutated RP3, sorted for GFP expression, and examined for colony growth in soft agar. After 20 days of growth, the colonies were stained with crystal violet (**Fig. S2**) or photographed for measurement of colony size (**Figs. 6A-B**). Most notably, the TRAF-deficient mutants, $RP3^{T6mut}$ and $RP3^{T2mut}$ induced colonies in size and number comparable to wild type RP3 (**Figs. 6A-B**). In contrast, $RP3^{K284M}$, $RP3^{Y431F}$, and $RP3^{Y588F}$ produced little to no colonies. We confirmed that the lack of colony formation was not due to loss of gene expression (**Fig. S3**). Interestingly, the Y478 mutation resulted in transductants with compromised transformation properties compared with $RP3^{WT}$ (**Figs. 6A-B**).

The lack of inflammatory cytokine production by $RP3^{T6mut}$ and $RP3^{T2mut}$ transfectants may limit their capacity to form tumors *in vivo* due to the lack of induced stromal support (49). To test this notion, immunodeficient RAG-/- mice were subcutaneously injected with the NIH3T3 transductants described above and monitored for tumor growth. **Fig. S6** shows that mice injected with $RP3^{WT}$, $RP3^{T6mut}$, or $RP3^{T2mut}$ transfectants rapidly developed palpable tumors after 13 days, while mice injected with NIH3T3 expressing GFP alone showed no tumor growth. Mice that received $RP3^{K284M}$ or $RP3^{Y588F}$ cells did not grow significantly measurable tumors. Interestingly it appears that the $RP3^{T6mut}$ transductants produced smaller tumors which may reflect the smaller colony size in **Fig. 6B**, a decreased influx of innate cells, or decreased stromal support and angiogenesis. Further investigation that includes immunocompetent mice and increased animal numbers may allow for more detailed insight into the role that an intrinsic TRAF pathway plays on *in vivo* tumor progression. In summary, the results indicate that RET/PTC-induced TRAF-mediated proinflammatory cytokine production is not required for cellular transformation driven by the PI3K/AKT and RAS/BRAF/MEK/ERK pathways.

DISCUSSION

The unusual pleiotropic signaling properties of the RET/PTC oncogenes (14, 24, 34) provide an attractive model for studying the mechanistic relationships between oncogene-induced cellular transformation and proinflammatory pathways. RET/PTC translocation is thought to be one of the earliest events in the initiation of papillary thyroid cancer (9, 50) and ectopic pro-inflammatory mediators have been presumed to contribute to the cellular transformation process (2, 3), but this has not been formally tested for this cancer type. We report here that TRAF-mediated cytokine induction and cellular transformation are functionally distinct in neoplastic thyrocytes, a finding that will enable a finer resolution of the components necessary to drive cancer progression. These data also point to one explanation for why oncogenes such as mutant RAS or BRAF do not exhibit the same degree of immunostimulatory phenotype and progress along different pathologies despite utilizing the same transforming pathways as RET/PTC-based tumors. Although stimulatory crosstalk between the RAS/AKT and NF-κB pathways has been suggested for lung epithelial cell tumorigenesis (51), we did not observe positive feedback mechanisms in our experimental systems. If anything, TRAF2 mediated signaling appears to suppress the AKT and ERK signaling pathways (**Fig. 5A**). This is in accordance with the observation that TRAF6 negatively regulates PI3K activity during T cell activation (52). Additionally the signaling dichotomy we demonstrate here has recently been shown for MIG-6 tumor suppressor expression in PTC. These studies demonstrate that MIG-6 overexpression in PTC tumor samples or the TPC-1 cell line activates NF-κB and inhibits ERK phosphorylation. Conversely knockdown of MIG-6 decreased NF-κB nuclear localization and enhanced ERK activity (53).

PTC rarely harbors coexisting RET/PTC, RAS, and/or BRAF mutations suggesting that there is limited functional complementation between these oncogenes with respect to thyroid tumor initiation/progression (54-56). The experiments reported here demonstrate that RET/PTC-induced TRAF-mediated inflammation is not required for transformation. Evidently only the PI3K/AKT and RAS/BRAF/MEK/ERK pathways are required to establish and maintain follicular cell transformation (57), while the inflammatory pathway that is unique to RET/PTC translocation may serve to modulate the tumor environment. This conclusion is supported by recent evidence demonstrating that the MIG-6 tumor suppressor expression correlates directly with increased NF-κB activity and inversely with tumor recurrence, survival, and oncogenic BRAF mutations (53, 58).

Other studies have demonstrated functional separation in other cell types such as fibroblasts. For instance, the NF-κB members, p65 (Rel A) and c-REL, are not required for RAS-mediated transformation in fibroblasts, although p65 and c-REL signaling can potentiate anchorage independent growth (59). Furthermore, our studies showed that blockade of MEK/ERK and PI3K/AKT actually enhances inflammatory cytokine production from thyroid papillary carcinoma, compatible with the published finding that oncogenic RAS blocks TNF-induced NF-κB signaling (60).

Our investigation has revealed that TRAFs associate with RP3 and activate an inflammatory program that is independent from the PI3K/AKT and RAS/BRAF/MEK/ERK pathways. TRAF2 and TRAF6 appear to associate independently of kinase action (as observed with $RP3^{K284M}$), while pro-inflammatory TRAF complexes are formed only upon a catalytically active conformational change of the kinase domain (as observed with $RP3^{WT}$). This type of interaction has been described for TGFβR in which wild type, kinase dead, and constitutively active TGFβR, all associate with TRAF6 (61). Since RET/PTC is constitutively expressed in transformed cells, it is possible that RET:TRAF complexes will be present at all times leading to aberrant regulation of NF-κB. This is distinct from the conditional activation observed with RET and its ligand GDNF under physiological conditions. A general model of RET/PTC signaling, based on our results, is depicted in **Fig. 7**. A key feature is the association of TRAF2 and TRAF6 only after dimerization of RP3. This is similar to TNFR family members BAFFR and CD40 which exhibit TRAF association only after ligand binding (62). In the case of RP3, conversion to the active

dimeric conformation (**Fig. 7**) may result in increased surface exposure of the TRAF-binding segments. The observation that mutation of the putative TRAF6 binding site ($RP3^{T6mut}$) resulted in reduced association with both TRAF2 and TRAF6 (**Figs. 3D&F**) was also of interest. TRAF2 often forms hetero-complexes with other TRAF family members (63). Therefore it is possible that TRAF2 interacts directly with TRAF6 at this site or that this location contains binding motifs for both TRAF2 and TRAF6. This type of TRAF2:TRAF6 interaction has also been described for the proinflammatory receptor CD40 (64).

We observed that both TRAF2 and TRAF6 associate with RP3 independent of the Y588 site. Previous reports suggest that proinflammatory cytokine production initiates `via a Y588-dependent signaling cascade (14, 25). It is interesting that in the absence of TRAF mediated pathways, we observed the greatest difference in MCP1 production compared with other cytokines whereas MCP1 was the least affected using Y588 mutants (25). Further investigation is required to assess the relative involvement of a TRAF-, AKT-, and ERK-independent/Y588-dependent pathway in cytokine induction. The RAS/BRAF pathway has also been implicated in secretion of IL-24, IL-8, and other chemokines (65, 66), it is possible that the quality of the proinflammatory cytokine induction from a RAS-mediated pathway may lead to a more immune-suppressive/tumor-promoting environment. While the additional RET/PTC-induced TRAF-mediated pathway skews the cytokine profile towards a more immunostimulatory phenotype. This change in cytokine profile may strongly contribute to the autoimmune thyroiditis that is associated with RET/PTC-expressing tumors.

Activation of NF-κB by RET/PTC involves members of the classical and alternative pathways through the stabilization of NIK kinase (23). NIK was originally cloned and described as a binding partner of TRAF2 through two-hybrid screening of a human B cell cDNA library (67). Subsequently, TRAF2 and TRAF6 were shown to activate NF-κB by inhibiting the constitutive proteolytic degradation of NIK (63, 67-70). Additionally, the deregulated constitutive expression of NIK results in a predominant classical activation of NF-κB and production of proinflammatory mediators *in vivo* (71). In our studies we observed that knockdown of either TRAF2 or TRAF6 in PTC abrogates the accumulation of NIK (**Fig. 5**), suggesting that RET/PTC activation of TRAF complexes mediates a pro-inflammatory state through NIK kinase. The observation that TRAF knockdown results in depletion of NIK kinase, with no impact on phosphorylation of AKT and ERK argues further for functional divergence of the inflammatory and oncogenic pathways.

Here we propose the possibility that RAS/BRAF and/or PI3K/AKT pathways are required for cellular transformation and that the additional inflammatory property of RET/PTCs shapes the formation of a relatively indolent tumor by providing an immunostimulatory/tumor-suppressive inflammatory microenvironment. This could occur by several potentially cooperative mechanisms: (a). Autocrine stimulation by cytokines may retard tumor growth. RET/PTC-expressing human thyroid cell lines and thyroids from transgenic animals show substantial up-regulation of many inflammatory mediators including IL1α, IL1β, IL6, TNFα, IL8, GMCSF, and MCP1 (14, 24, 34) and it has been reported that many of these cytokines can inhibit thyroid tumor cell growth (72). (b). The composition of the infiltrate itself may lack the factors required for progression. More aggressive carcinomas that harbor RAS or BRAF mutations display a heavy influx of inflammatory cells but these tend to be immunosuppressive in nature (22). Indeed, tumor-associated macrophages (TAMs) generally exhibit alternate ("M2") activation and are mitogenic, angiogenic, and pro-metastatic through mechanisms that include IL10, TGFβ, VEGF, and matrix metalloprotease secretion, respectively (73). In contrast, the lymphocytic infiltration associated with indolent PTC is usually characterized by a Th1 phenotype (74, 75) that is not conducive to the production of immunosuppressive TAMS (73) and infiltrating TAMs are rarely found in less aggressive well-differentiated thyroid carcinoma (22). (c). The proinflammatory environment may cause a break in peripheral tolerance and aid the adaptive arm in controlling/eliminating the tumor. For example, CXCR4, STAT1, CIITA and MHC Class II are also up-regulated by RET/PTC expression in

human thyroid cells *in vivo* (34, 76, 77). Together these molecules provide signals for leukocyte migration, dendritic cell differentiation, and antigen presentation. Consistent with this, tumors derived from RP3 transgenic animals are capable of rapid growth in SCID mice but not immunocompetent hosts (78).

Clinically, PTCs harboring active RET translocations are found co-incident with autoimmune Hashimoto's thyroiditis (HT) (16-21). This longstanding observation has led to the hypothesis that HT is a risk factor for PTC and, furthermore, that HT is a precancerous condition (2, 3). For the various reasons discussed, we suggest the converse, that the autoimmune component is the result, rather than the cause of RET/PTC transformation. Activation of innate and adaptive immunity via the TRAF signaling component of RET/PTC would explain the intense and persistent peritumoral lymphocytic infiltration and indolent growth that is often associated with PTC. The TRAF-mediated proinflammatory signaling linked to RET/PTC may also help explain the concurrent expression of RET/PTC (21) and the better overall prognosis of thyroid cancer in HT patients (79).

Although data indicate that TRAF-mediated pathways are not required for early events in transformation, the question remains whether these same TRAF-mediated pathways are required for later stages of tumor progression. Indeed the $RP3^{T6mut}$, or $RP3^{T2mut}$ tumors were smaller than the comparable $RP3^{wt}$ tumors suggesting that inflammatory mediators may contribute to, but are not necessary for, tumor growth. Accordingly, the lack of these cytokines may limit the recruitment of stromal elements and suppress the likelihood of growth variants (80). Consistent with this, TRAF-mediated NF-κB activation is known to control cell viability and angiogenesis in many tumor types (1).

Elucidating the influence of RET/PTC induced inflammation on thyroid tumorigenesis may lead to more effective therapeutic strategies for PTC. For example, following surgery, any residual RET/PTC harboring tumors may be best approached by targeting only the PI3K/AKT- and RAS-mediated pathways, while preserving the inflammatory component. Conversely, induction of a "RET/PTC-like" TRAF signaling pathway within the more aggressive PTCs that feature only oncogenic RAS and BRAF mutations may convert such tumors to a more benign form. Alternatively, if TRAF-mediated NF-κB pathways are found to play a greater role in angiogenesis and escape during progression then the therapeutic strategy should be to knockdown both proinflammatory and oncogenic arms of RET/PTC signaling. Experiments are currently underway to test these possibilities.

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FOOTNOTES

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The abbreviations used are: FDTC, follicular cell-derived thyroid carcinoma; FTC, follicular thyroid carcinoma; HT, Hashimoto's thyroiditis; LY, LY294002; PTC, papillary thyroid carcinoma; RP1, RET/PTC1; RP3, RET/PTC3; TAM, tumor-associated macrophage; TRAF, TNF receptor associated factor; U0, U0126

FIGURE LEGENDS

Fig. 1. Inhibitors of PI3K or MEK do not inhibit RP3-mediated proinflammatory cytokine transcription. (A) PCCL3 cells were transiently transfected with $RP3^{WT}$ or $RP3^{K284M}$ in the presence of 0.1% DMSO alone, 10 μM of LY294002 or 10 μM of U0126 for 48 hr and cells were lysed for total RNA followed by reverse transcription and PCR for rat specific gene products. (B) PCCL3 cells were treated as in (A) and harvested at 48 hr for total protein and western blot analysis. (C) TPC-1 cells were treated for 48 hr with 0.1% DMSO alone, 10 μM of LY294002 or 10 μM of U0126 and subsequently harvested for total RNA followed by reverse transcription and PCR for human specific gene products. (D) TPC-1 cells were treated as in (C) and harvested at 48 hr for total protein and western blot analysis. (A-D) Data representative of two independent experiments. RevT: Reverse transcriptase; LY: LY294002; U0: U0126

Fig. 2. (A-B) $RP3^{Y478F}$ exhibits reduced MCP-1 expression and maintains AKT and ERK activation. (A) PCCL3 cells were transiently transfected with Rc/CMV vector alone, $RP3^{WT}$ or $RP3^{Y \rightarrow F}$ mutants for 48 hr

and cells were lysed, total RNA extracted and RNA reverse transcribed and cDNA amplified using rat GAPDH and MCP1 specific primers. Error bars indicate SEM from at least three independent experiments. (B) PCCL3 cells were transiently transfected with MSCV.IRES.GFP vector alone, $RP3^{WT}$, $RP3^{K284M}$, or $RP3^{Y588F/Y622F}$ mutants for 48 hr and cells were lysed, total RNA extracted and RNA reverse transcribed and cDNA amplified using rat GAPDH and MCP1 specific primers. (C) Representative western blot of two individual experiments of PCCL3 cells that were treated as in (A) and harvested at 48 hr for total protein.

Fig. 3. RP3 associates with TRAF2 and TRAF6 in a dimer-dependent and kinase-independent manner. (A) Schematic representation of $RP3^{WT}$, $RP3^{K284M}$, and truncation mutants. (B-F) 293T cells were transiently transfected with GFP, $RP3^{WT}$ or $RP3^{K284M}$, or truncation (Δ) mutants for 48 hr, lysed, and subsequently harvested for immunoprecipitation using an anti-RET.51 specific antibody. Immunoprecipitated protein was subjected to western blot analysis using antibodies specific for TRAF2, TRAF6, RET or Actin. (G) TPC-1 cell were lysed and subsequently harvested for immunoprecipitation using an anti-RET.51 specific antibody. Immunoprecipitated protein was subjected to western blot analysis using antibodies specific for TRAF2, TRAF6, RET or Actin. Representative of at three independent experiments.. IP: immunoprecipitation; UB: unbound fraction; hIgG: goat IgG heavy chain.

Fig. 4. Knockdown of TRAF2 and TRAF6 abrogates cytokine expression. (A-B) TPC-1 cells were transfected with non-targeting control, TRAF2, or TRAF6 siRNA and harvested after 48 hours for total RNA followed by reverse transcription and PCR using human GAPDH, MCP1, IL6, IL8, TNF, GM-CSF, TRAF2, TRAF6, and RET/PTC1 specific primers. (A) Representative experiment of cytokine transcript induction of at least 3 individual experiments. (B) Graphs are represented as densitometric analysis of band intensity relative to GAPDH and the fold induction of that ratio over scrambled siRNA control. Error bars indicate SD of samples run in triplicate, in three independent experiments. (C) MCP1 secretion was measured on day 4 by ELISA using culture supernantants derived from TPC-1 cells transfected with scrambled control, TRAF2, or TRAF6 siRNAs treated on day 0 and retreated on day 2. (D) TPC-1 cells were transduced with MSCV.IRES.RFP constructs expressing T2 and T6 binding motifs and their peptide controls. Following selection for RFP, cells were harvested for total RNA followed by reverse transcription and PCR using human GAPDH and MCP1 specific primers. (E) PCCL3 cells were stably transduced with $RP3^{WT}$ or $RP3^{Y\rightarrow F}$ mutants and after a month of passage in culture, cells were plated overnight and lysed for total RNA followed by reverse transcription and PCR with rat GAPDH and MCP-1 specific primers. Represented as densitometric measurements of band intensity relative to GAPDH and the fold induction of that ratio over RP3. (C-E) Error bars indicate SD of samples run in duplicate, in two independent experiments. Cntl: non-targeting siRNA control; T2: TRAF2; T6: TRAF6

Fig. 5. Disruption of TRAF2 and 6 pathways does not inhibit AKT and ERK pathways. (A) TPC-1 cells were transfected with scrambled control, TRAF2, or TRAF6 siRNA and re-treated with siRNA on day 2 and on day 4 were harvested for western blot analysis. (B) TPC-1 cells were transduced with MSCV.IRES.RFP constructs expressing T2 and T6 binding motifs and their peptide controls. Following selection for RFP, cells were harvested for western blot analysis. (C) Cell proliferation assay of transduced TPC-1cells from (B) that were plated at 1000 cells/well in 96-well culture dishes and counted daily. (D) ERK-dependent hormone-independent cell proliferation assay of transduced PCCL3cells expressing wild type RP3 and mutants. Cells were plated at 1000 cells/well in 96-well culture dishes in the absence of 6 hormones (TSH, insulin, Gly-His-Lys, somatostatin, apo-transferin, and hydrocortisone) and counted daily. Data are representative of three independent experiments. Cntl: non-targeting siRNA control; T2: TRAF2; T6: TRAF6

Fig. 6. TRAF-deficient RP3 mutants maintain transforming activity. (A) Representative photographs of anchorage-independent colony growth of NIH-3T3 cells stably transduced with $RP3^{WT}$ and mutants used

for the analysis in (B). (B) Number of colonies counted from a defined grid that were produced from NIH-3T3 transductants as in (A) that were greater than 100 μm in length and averaged over triplicate wells. The table below the graph represents the average colony length and standard deviation of the triplicate wells. Representative of two independent experiments.

Fig. 7. Model of RET/PTC-mediated TRAF activation.
PET/ΠΤΧ διμερσ εξηιβιτ α γρεατερ αφφινιτψ φορ ΤΡΑΦ2 ανδ ΤΡΑΦ6 τηαν PET/ΠΤΧ μονομερσ. Τηε ΤΡΑΦ ιντεραχτιον ωιτη PET/ΠΤΧ δοεσ νοτ ρεθυιρε αν αχτιϖε χονφορματιον οφ τηε κινασε δομαιν. Ηοωεϖερ, ΤΡΑΦ αχτιϖατιον δοεσ ρεθυιρε αν αχτιϖε κινασε δομαιν χονφορματιον ωηι χη μοστ λικελψ ινδυχεσ ΤΡΑΦ ολιγομεριζατιον το προμοτε NIK ανδ ΝΦ−κΒ-mediated proinflammatory signaling. The RET/PTC:TRAF:NIK:NF-κB pathway is functionally distinct from Y588-mediated RAS and PI3K signaling cascades that promote transformation.