Ras/ERK1/2-mediated STAT3 Ser\textsuperscript{727} Phosphorylation by Familial Medullary Thyroid Carcinoma-associated RET Mutants Induces Full Activation of STAT3 and Is Required for c-fos Promoter Activation, Cell Mitogenicity, and Transformation*  

The precise role of STAT3 Ser\textsuperscript{727} phosphorylation in RET-mediated cell transformation and oncogenesis is not well understood. In this study, we have shown that familial medullary thyroid carcinoma (FMTC) mutants RET\textsuperscript{Y791F} and RET\textsuperscript{S891A} induced, in addition to Tyr\textsuperscript{705} phosphorylation, constitutive STAT3 Ser\textsuperscript{727} phosphorylation. Using inhibitors and dominant negative constructs, we have demonstrated that RET\textsuperscript{Y791F} and RET\textsuperscript{S891A} induce STAT3 Ser\textsuperscript{727} phosphorylation via a canonical Ras/ERK1/2 pathway and that integration of the Ras/ERK1/2/ELK-1 and STAT3 pathways was required for up-regulation of the c-fos promoter by FMTc-RET. Moreover, inhibition of ERK1/2 had a more severe effect on cell proliferation and cell phenotype in HEK293 cells expressing RET, whereas inhibition of ERK1/2/ELK-1/STAT3 pathways was only partially able to rescue the phenotype of RET\textsuperscript{Y791F} and RET\textsuperscript{S891A}-transfected cells. The transforming activity of RET\textsuperscript{Y791F} and RET\textsuperscript{S891A} in NIH-3T3 cells was also inhibited by U0126, indicating a role of the ERK1/2 pathway in RET-mediated transformation. To investigate the biological significance of Ras/ERK1/2-induced STAT3 Ser\textsuperscript{727} phosphorylation for cell proliferation and transformation, N-Ras-transformed NIH-3T3 cells were employed. These cells displayed elevated levels of activated ERK1/2 and Ser\textsuperscript{727}-phosphorylated STAT3, which were induced by treatment with U0126. Importantly, overexpression of STAT3, in which the Ser\textsuperscript{727} was mutated into Ala (STAT3\textsuperscript{WT}), rescued the transformed phenotype of N-Ras-transformed cells. Immunohistochemistry in tumor samples from FMTC patients showed strong nuclear staining of phosphorylated ERK1/2 and Ser\textsuperscript{727}STAT3. These data show that FMTc-RET mutants activate a Ras/ERK1/2/STAT3 Ser\textsuperscript{727} pathway, which plays an important role in cell mitogenicity and transformation.

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2 The abbreviations used are: GDNF, glial cell line-derived neurotrophic factor; ERK, extracellular signal-regulated tyrosine kinase; HEK, human embryonic kidney; FMTC, familial medullary thyroid carcinoma; MEN, multiple endocrine neoplasia; IRE, interleukin-6 response element; SRE, serum response element; RET, rearranged during transfection; UAS, upstream activating sequence.
transcriptional activation and DNA binding capacity (10). Various kinases have been shown to phosphorylate STAT3 on Ser727, depending on the cytokines and growth factors involved and the cellular context (10–14). However, the precise role of STAT3 Ser727 phosphorylation in cell transformation and in RET-mediated oncogenesis was unknown.

Here we have investigated the different signaling pathways activated by FMTC-RET at the level of STAT3 Ser727 and whether the deregulation of such pathways by oncogenic RET plays a crucial role in cell mitogenicity and transformation. In this study, we have shown that RETY791F and RETS891A activate a Ras/ERK1/2/STAT3 Ser727 pathway required for cell transformation and tumor cell proliferation, and we further extend our findings to tumor samples from patients carrying a germ line-activating RETS891A mutation.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Cell Culture Reagents**—Human embryonic kidney 293 (HEK293) cells were grown as described previously (7). NIH-3T3 cells and the N-Ras-transformed NIH-3T3 cell line 149169 were provided by Prof. C. Marshall (The Institute of Cancer Research, London, UK) and grown in Dulbecco’s modified Eagle’s medium, supplemented with 5% donor calf serum (In Vitrogen), 100 IU/ml penicillin, 1 mg/ml streptomycin, and 2 mm L-glutamine (In Vitrogen). To generate stably transfected cell lines, 1 × 10⁶ HEK293, NIH-3T3, or 149169 cells were plated in 90-mm dishes and transfected with 0.5 μg of expression plasmid by the calcium phosphate method. 24 h after transfection, the cells were rinsed with phosphate-buffered saline and cultured in fresh medium containing 500 μg/ml G418 (Sigma-Aldrich). Clones were picked after 2 weeks and screened by Western blot analysis. GDNF (Peprotech, Rocky Hill, NJ), STI571 (Novartis, Basel, Switzerland), PP2, and U0126 (CalBiochem, La Jolla, CA) were used as indicated.

**Expression and Reporter Plasmids**—The expression plasmids pRC-CMV-RETWT, -RETs891A, and -RETY791F, pGL5-STAT3α, and pGL5-STAT3β were described previously (7). The expression plasmid pGL5-STAT3S727A, pcDNA3.1, dominant negative (dn) Ras (RasN17), dominant negative Raf (kinase-deleted), dominant negative SEK1 (15), pUAS-luc, and pGAL4-ELK-1 were provided by Drs. A. T. J. Wieringa and J. J. Schuringa of the Department of Hematology (University Medical Center Groningen, Groningen, The Netherlands). The reporter plasmids pTAL-SRE-luc, pIRE-ti-Luc, and pDM2-LacZ were described previously (7, 16). Wild-type c-fos promoter luciferase plasmid, the mutants p18 (containing a mutated TCF binding site at the 5’ end of the serum response element (SRE)) and the SIE mutant (containing a mutated STAT binding site) were previously described (17). The GFRα-1 plasmid was kindly provided by Prof. Lois M. Mulligan (Department of Pathology, Queen’s University, Ontario, Canada).

**Transformation Foci and Soft Agar Assays**—Transformed foci assays were carried out as previously described (18). Briefly, NIH-3T3 and 149169 cells were transfected with 0.5 μg of the indicated plasmid using calcium phosphate. The next day, cells were washed with phosphate-buffered saline, and fresh medium containing 500 μg/ml G418 was added. After 3 weeks, the cells were fixed in 4% paraformaldehyde for 1 h and then washed and stained for 1 h with 0.1% crystal violet.

For soft agar colony formation assays, 1% agar (DNA grade; Invitrogen) was boiled and mixed 1:1 with 2× Dulbecco’s modified Eagle’s medium/F-12 with standard additives and 5 ml added to 90-mm dishes to form the base agar. For the top agar, 2 × 10⁴ cells were mixed with 5 ml of a 1:1 mix of 0.7% agarose and 2× Dulbecco’s modified Eagle’s medium/F-12 and poured on the top of the base agar. Plates were incubated at 37 °C in a humidified incubator for 10–14 days prior to counting of colonies.

**Proliferation Assays**—Established HEK293 (2 × 10⁵) and transformed 149169 cells (5 × 10⁴) were plated in triplicate, and cells were counted at days 1, 2, 4, and 6 using a standard hemocytometer.

**RNA Extraction, cDNA Synthesis, and Reverse Transcription-PCR**—The RNAeasy protect minikit (Qiagen, Crawley, UK) was used to extract total RNA from established HEK293 cell lines. For cDNA synthesis, 200 ng of total RNA were used with Ready-to-go-your-prime first strand beads (Amersham Biosciences) using oligo(dT) primers as indicated by the manufacturer. For reverse transcription-PCR, c-fos (forward 5′-TGCCAACTCTTACATCCAGGT-3′, reverse 5′-TAGTTGTCGCTTCGCTTG-3′), egr-1 (forward 5′-TTGCGAGGGCAGATGAAAC-3′, reverse 5′-CCGACAGGCGCAACACCTT-3′), and actin (forward 5′-GTCGGTGCAACAGGCT-3′, reverse 5′-CAACATGATCTGGGTTCATCTTTCCTC-3′) primers were used, and PCR reactions were performed under standard conditions.

**Luciferase Reporter Assays**—Reporter assays were undertaken as previously described (5, 7, 16). Briefly, 24 h after transfection, cells were washed and treated with ligand or inhibitors as indicated. The following day, cells were harvested in lysis buffer (Promega, Madison, WI), and luciferase activity was determined using the SteadyLite HTS kit (PerkinElmer Life Sciences). In all transfections, a β-galactosidase expression plasmid (pDM2LacZ) was included to normalize luciferase activities. β-Galactosidase activity was determined in 100 mM Na₂HPO₄/NaH₂PO₄, 1 mM MgCl₂, 100 mM 2-mercaptoethanol, and 0.67 mg/ml O-nitrophenylgalactopyranoside. Data represent three independent experiments performed in triplicate.

**Immunohistochemistry, Western Blotting, and Antibodies**—Immunohistochemistry was performed on tumor samples of patients carrying germ line RETS891A mutations as described previously (19). For protein analysis, cells were serum-starved for 12 h, unless otherwise stated, followed by lysis in 10 mM Tris-Cl, pH 7.4, 144 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, 2 mM dithiothreitol, 1 mM sodium-vanadate, 87% glycerol, 10 μg/ml aprotinin, 2 μg/ml leupeptin, and 0.2 mM phenylmethysulfonfluoride. Protein lysates were resolved on SDS-PAGE and analyzed by Western blotting and ECL (Roche Applied Science). Primary antibodies were used at 1:1000 dilution and were obtained as follows: RET (H-300), phospho-Tyr1062RET, and STAT3 (C-20) from Santa Cruz Biotechnology (Palo Alto, CA), phospho-ERK1/2, ERK1/2, phospho-STAT3Tyr705, and phos-
pho-STAT3Ser<sup>727</sup> from Cell Signaling Technology (New England Biolabs, UK). Blots shown are from a single experiment and are representative of three independent experiments.

**Statistical Analysis**—Statistical analysis was performed using Prism software. Cell and colony number assays were compared using one-way analysis of variance for repeating measures and the Dunnett test. Differences were considered statistically significant when \( p < 0.05 \).

**RESULTS**

RET<sup>Y791F</sup> and RET<sup>S891A</sup>, two mutations targeting the tyrosine kinase domain of RET and associated with the FMTC phenotype, signal independently of GDNF and induce STAT3 Tyr<sup>705</sup> phosphorylation through a Src- and Janus kinase-dependent pathway (7). To determine whether RET<sup>Y791F</sup> and RET<sup>S891A</sup> contribute to the aberrant transcriptional activity of STAT3, first we investigated whether these mutants also induce STAT3 Ser<sup>727</sup> phosphorylation. Western blot analyses of HEK293 cells expressing RET<sup>WT</sup>, RET<sup>Y791F</sup>, and RET<sup>S891A</sup> indicated that both mutant receptors are activated in the absence of GDNF, as shown by RET Tyr<sup>1062</sup> phosphorylation (Fig. 1A). Furthermore, these mutant receptors induced STAT3 phosphorylation on Tyr<sup>705</sup> and Ser<sup>727</sup> (Fig. 1A). Next, the effect of RET<sup>S891A</sup>-induced STAT3 Ser<sup>727</sup> phosphorylation on the transcriptional activity of STAT3 was investigated. STAT3<sup>C</sup>, STAT3<sup>B</sup>, and a STAT3 point mutant, STAT3<sup>S727A</sup> (Fig. 1B), were expressed in HEK293 cells in combination with RET<sup>S891A</sup> and an interleukin-6 response element (IRE) luciferase reporter. Activation of the IRE-reporter by RET<sup>S891A</sup> was increased by co-expression of STAT3<sup>C</sup> but not by co-expression of either STAT3<sup>B</sup> or STAT3<sup>S727A</sup> (Fig. 1B). The same reporter experiments were performed using the promoter regions of the Cyclin D1, ICAM1 and Bcl-x<sub>L</sub> genes fused to a luciferase reporter gene. In all promoters tested, STAT3<sup>C</sup> potentiated reporter activation by RET<sup>S891A</sup>, whereas co-expression of STAT3<sup>S727A</sup> did not result in increased transcriptional activation (data not shown). These data indicate that STAT3 Ser<sup>727</sup> phosphorylation is important for RET-induced transcriptional activation of STAT3 target genes and may be required for RET-mediated oncogenesis.

STAT3 Ser<sup>727</sup> is located in a consensus sequence Pro-Xaa-(Ser/ Thr)-Pro that can be phosphorylated by ERK1/2 (20). As ERK1/2 can be activated by wild-type RET (21), a number of approaches were taken to investigate whether FMTC-RET mutants aberrantly activate the ERK1/2-MAPK pathway in the absence of GDNF. First, HEK293 cells were transfected with RET<sup>WT</sup>, RET<sup>Y791F</sup>, or RET<sup>S891A</sup> in combination with an SRE-luciferase reporter, as a readout for downstream activation of ERK1/2 (17). In the absence of GDNF, RET<sup>Y791F</sup> and RET<sup>S891A</sup> induced a 3- and 5-fold activation of the SRE reporter, respectively, whereas no induction was observed with RET<sup>WT</sup> (Fig. 2A). To confirm these results, Western blot analysis of HEK293 cells transiently transfected with RET<sup>WT</sup>, RET<sup>Y791F</sup>, or RET<sup>S891A</sup> showed increased levels of ERK1/2 phosphorylation by both mutants (Fig. 2B).

The SRE element is known to be activated by ELK-1, an ERK1/2 target. Consequently, HEK293 cells were transfected with the RET constructs and a GAL4/ELK-1 reporter system in which ELK-1 is fused to the DNA binding domain of GAL4 in combination with an upstream activating sequence (UAS)-Luc reporter (Fig. 2C). Robust activation of the UAS-reporter by the RET<sup>Y791F</sup> and RET<sup>S891A</sup> mutants (200- and 300-fold, respectively) was observed compared with a lower activation observed with RET<sup>WT</sup> (70-fold). To further investigate the activation of ERK1/2 by mutant FMTC-RET receptors, the mRNA levels of c-fos and Egr-1 genes in established HEK293-expressing RET<sup>Y791F</sup> and RET<sup>S891A</sup> were analyzed using reverse transcription-PCR. Up-regulation of both of these genes was observed in the mutant cell lines when compared with control HEK293 cells (Fig. 2D). Taking these results together, we conclude that both RET<sup>Y791F</sup> and RET<sup>S891A</sup> activate the ERK1/2 pathway independent of GDNF.

To determine whether the STAT3 Ser<sup>727</sup> phosphorylation induced by FMTC-RET was mediated through a Ras/ERK1/2 pathway, established HEK293 cells expressing RET<sup>S891A</sup> were treated during 24 h with the tyrosine kinase receptor inhibitor STI571 (22). A reduction of RET Tyr<sup>1062</sup> phosphorylation accompanied by a reduction of RET expression (22) was observed at 20 μM STI571. Treatment with this inhibitor also

**FIGURE 1.** RET<sup>Y791F</sup> and RET<sup>S891A</sup> induce constitutive STAT3 Ser<sup>727</sup> phosphorylation required for maximal STAT3 transcriptional activity. A, HEK293 cells were mock-transfected (CONT) or transiently transfected with RET<sup>WT</sup>, RET<sup>Y791F</sup>, and RET<sup>S891A</sup>. After 48 h, cell lysates were analyzed by Western blotting with phospho-Tyr<sup>1062</sup> RET, RET, phospho-Tyr<sup>705</sup> STAT3, phospho-Ser<sup>727</sup> STAT3, and STAT3 antibodies, as indicated. B, HEK293 cells were transiently transfected with RET<sup>Y791F</sup>, STAT3<sup>C</sup>, STAT3<sup>B</sup>, and STAT3<sup>S727A</sup> expression plasmids in combination with the IRE (IL-6 response element) luciferase reporter plasmid, as indicated. Data shown are the mean fold activation of normalized luciferase activity ± S.D. C, diagram representation of STAT3<sup>C</sup> and STAT3<sup>B</sup> isoforms: N-terminal domain (ND), coiled-coil domain (CCD), DNA-binding domain (DBD), linker domain (LD), Src-homology 2 domain (SH2), and transcriptional activator domain (TAD). WT, wild type.
**REM791F and RETS891A Activate a Ras/ERK1/2/STAT3 Ser727 Pathway**

A

B

C

D

![Graphs and images](Image)

**Figure 2. RETS891A and REM791F induce constitutive activation of ERK1/2 and ELK-1 and up-regulation of c-fos and Egr-1.** A and C, HEK293 cells were transiently transfected with RET WT, RET Y791F, and RET S891A expression plasmids in combination with SRE-luciferase and ELK1-GAL4/UAS-luciferase reporter plasmids, as indicated. Data shown are the mean fold activation of normalized luciferase activity ± S.D. B, cell lysates from HEK293 cells mock-transfected (CONT) or transiently transfected with RET WT, REM791F, and RET S891A expression plasmids were analyzed by Western blotting using antibodies against RET, phospho-ERK1/2, and ERK1/2. D, reverse transcription-PCR analysis on cDNA from parental HEK293 cells (CONT) or HEK293 cells stably expressing REM791F and RET S891A showing up-regulation of c-fos and Egr-1 mRNA levels in mutant cell lines. Actin mRNA levels are shown as a control. WT, wild type.

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resulted in a reduction in phosphorylation of STAT3 Ser727 and ERK1/2 (Fig. 3A). These results suggest that RET signaling is required for STAT3 Ser727 and ERK1/2 phosphorylation. To delineate the pathway from RET to ERK1/2 activation, GALA4-ELK-1/UAS-Luc reporter assays were used. Co-expression of either dn-Ras (RasN17) or dn-Raf (a kinase-dead Raf construct) resulted in a complete loss of UAS-reporter activation, indicating that Ras and Raf are required for ELK-1 activation by RET WT and RET S891A (Fig. 3B). To integrate the Ras/ERK1/2 pathway with STAT3 signaling, the IRE-Luc reporter was used in combination with a series of dominant negative constructs (Fig. 3B). dn-Ras and dn-Raf partially inhibited reporter activation by RET S891A, whereas expression of a dn-SEK1 (15) had no effect on RET S891A-induced IRE-Luc activity, indicating that Ras and Raf (but not SEK1) are involved in ERK1/2-mediated STAT3 activation. To test whether MEK1/2 (a MAPK kinase upstream of ERK1/2) was activating ERK1/2 in response to RET S891A, the effect of the MEK1/2 inhibitor U0126 on RET S891A-induced UAS-Luc and IRE-Luc activation was assessed. U0126 (10 and 40 µM) reduced both UAS-Luc and IRE-Luc activation by RET S891A (Fig. 3C). To further confirm these results, Western analysis demonstrated that U0126 was able to inhibit RET S891A-induced ERK1/2 and STAT3 Ser727 phosphorylation (Fig. 3D). Together these data demonstrated that RET S891A-induced STAT3 Ser727 phosphorylation is mediated by a canonical Ras/Raf/MEK1/2/ERK1/2 pathway. To elucidate whether FMTC-associated RET mutant signaling differs from GDNF-stimulated wild-type RET, Western analyses were performed (Fig. 4). RET WT, when stimulated with GDNF in the presence of GFRA1, was able to induce ligand-dependent receptor activation followed by ERK1/2 and slight STAT3 Ser727 phosphorylation, which was independent of STAT3 Tyr705 phosphorylation (Fig. 4). In contrast, RET S891A induced complete activation of the STAT3 pathway as promoted by both Tyr705 and Ser727 STAT3 phosphorylation and also ERK1/2 activation (Fig. 4) in the absence of the ligand. When established RET WT cells were treated with the Src and RET kinase inhibitor PP2, a decrease in receptor phosphorylation was accompanied by a decrease in ERK1/2 and STAT3 Ser727 phosphorylation (Fig. 4).

FMTC-RET mutants induced c-fos up-regulation (Fig. 2). The c-fos promoter contains various transcription factor binding sites; among them there are STATs (SIE) and ELK-1 (TCF) binding elements (17). To determine a possible cooperativity between STAT3 and ERK1/2-ELK-1 pathways in c-fos promoter activation by FMTC-RET, luciferase reporter assays were performed in established cell lines expressing vector alone (control), RET WT, and RET WT transiently transfected with GFRA1 and stimulated with GDNF and RET S891A (Fig. 5). Activation of the wild-type c-fos promoter was observed by RET S891A and GDNF-stimulated RET WT but not by RET WT or vector (alone)-expressing cells (Fig. 5). A partial reduction in promoter activity by RET S891A was observed with the p18 mutant (in which the TCF binding site required for ELK-1 binding is mutated) and the SIE mutant (in which the STAT binding site is mutated) c-fos promoter constructs. However, when both transcriptional binding sites were mutated in the c-fos promoter (p18/SIE mutant), reporter activation by RET S891A was completely abolished (Fig. 5). GDNF-stimulated RET WT induced a ligand-dependent activation of the c-fos promoter but to a lesser extent compared with RET S891A. When the p18 mutant c-fos promoter was used, a 2-fold decrease in promoter activation was seen, whereas a weak effect was observed with the SIE mutant (Fig. 5). This supports the data presented in Fig. 4; hence we concluded that activation of the Ras/ERK1/2/ELK-1 and STAT3 pathways is required for ligand-independent c-fos promoter up-regulation by FMTC-RET.

To further investigate the biological significance of the functional interaction between ERK1/2 and STAT3, we determined the effect of the MEK1/2 inhibitor U0126 on the morphology and proliferation of established HEK293 cells. First, we examined the phenotype of established HEK293 cells transfected with vector alone (control), RET WT, or RET S891A (Fig. 6A). HEK293-RET WT cells displayed epithelial morphological features, a low number of cell extensions, and thin distal cell processes. They were indistinguishable from vector (alone)-transfected HEK293 cells. In contrast, HEK293-RET S891A cells showed a less differentiated phenotype, being more scattered and with a higher number of neural-like extensions and thin distal cell processes (Fig. 6A, arrows). When HEK293-RET S891A cells were cultured for 5 days in the presence of U0126 (10 µM), a lower number of neural-like extensions and thin distal cell processes was observed, as well as a lower degree of cell scattering (Fig. 6A). Next, we inhibited STAT3 activation by RET S891A with PP2 (7). Treatment of cells with PP2 (3 µM) reverted the phenotype of the mutant cell lines as well (Fig. 6A). No
effect of either inhibitor was seen in HEK293 control and HEK293-RET<sup>WT</sup> (data not shown). In addition to the cell morphology analysis, proliferation assays demonstrated that HEK293-RET<sup>S891A</sup> cells grew faster than HEK293 control and HEK293-RET<sup>WT</sup> cells. Furthermore, HEK293-RET<sup>S891A</sup> cells had a higher sensitivity for the MEK1/2 inhibitor, as the proliferation rate was significantly more impaired by U0126 compared with HEK293 control and HEK293-RET<sup>WT</sup> cells (Fig. 6B).

To investigate the importance of the Ras-ERK1/2 pathway in the transforming activity of RET<sup>Y791F</sup> and RET<sup>S891A</sup>, NIH-3T3 cell transformation foci assays (18) were performed in the presence or absence of the inhibitors U0126 or PP2 (Fig. 7). RET<sup>Y791F</sup> and RET<sup>S891A</sup> were able to induce transformed foci when compared with cells transfected with RET<sup>WT</sup>. As a positive control, RET<sup>C634R</sup> was used. When cells were cultured in the presence of U0126 or PP2, the transforming capacity of both FMTC mutants was significantly reduced (Fig. 7). These data, in combination with the previous results, indicate that ERK1/2 activation is required for STAT3 Ser<sup>727</sup> phosphorylation and for the proliferation and transforming activity of FMTC-RET mutants and it further points toward the ERK1/2 and STAT3 pathways as targets for therapeutic intervention in MTCs (see "Discussion").

To demonstrate that integration of the ERK1/2 and STAT3 pathways is required for cell transformation and that STAT3 Ser<sup>727</sup> phosphorylation plays a crucial role in this process, experiments were undertaken using an NIH-3T3 cell line expressing the N-Ras oncogene (149169 cell line). First, Western blot analysis of cell lysates from NIH-3T3 and 149169 cells demonstrated that, in the N-Ras-transformed cells, high levels of phosphorylated ERK1/2 correlated with high levels of Ser<sup>727</sup> phosphorylated STAT3 (Fig. 8A). When cells were treated with the MEK1/2 inhibitor U0126, a reduction in phosphorylated ERK1/2 and STAT3 on Ser<sup>727</sup> was observed (Fig. 8A). Second, the transforming activity of 149169 cells transfected with STAT3<sup>/H9251</sup>, STAT3<sup>/H9252</sup>, or STAT3<sup>S727A</sup> was scored by transformed foci. Cells transfected with STAT3<sup>/H9251</sup> resulted in a significantly increased number of transformed foci when compared with 149169 cells transfected with STAT3<sup>/H9252</sup> or STAT3<sup>S727A</sup> mutant (Fig. 8B). Next, the transformed phenotype of established 149169 cells expressing the distinct STAT3 molecules was assessed. Vector (alone)-transfected 149169 cells showed typical transformed features, such as long neural-type elongations, poorly differentiated shape, and high polarization with a small cytoplasm and nucleus (Fig. 8C, arrows). 149169 cells expressing STAT3<sup>α</sup> displayed large filaments and a poorly differentiated phenotype (see arrows) with a small cellular body, and they showed reduction in contact inhibition compared with vector
**FIGURE 4.** FMTC-RET<sup>S891A</sup> but not GDNF-stimulated RET<sup>WT</sup> induces full activation of the STAT3 pathway. Protein lysates of established HEK293 cells expressing RET<sup>WT</sup>, RET<sup>S891A</sup> transiently transfected with GFRα-1 and stimulated with GDNF (20 ng/ml, 10 min), RET<sup>S891A</sup> and RET<sup>WT</sup> transiently transfected with GFRα-1 and treated with PP2 (Src tyrosine inhibitor; 3 μM, 120 min) prior to stimulation with GDNF (20 ng/ml, 10 min) were analyzed by Western blotting using the indicated antibodies.

**FIGURE 5.** Integration of the STAT3 and Ras/ERK1/2/ELK-1 pathways is required for ligand-independent c-fos promoter up-regulation by FMTC-RET<sup>S891A</sup>. Luciferase reporter assay was performed in established HEK293 cells expressing empty vector (293CONT), RET<sup>WT</sup> or RET<sup>S891A</sup> transiently transfected with GFRα-1 and stimulated with GDNF (25 ng/ml) overnight, and RET<sup>S891A</sup> and transfected with the c-fos wild-type promoter luciferase construct (wt), a c-fos promoter with a mutated TCF binding site (p18), a c-fos promoter with a mutated STAT binding site (SIE) and a c-fos promoter double mutant (p18/SIE), respectively, as described under “Experimental Procedures.” Data shown are the mean fold activation of normalized luciferase activity ± S.D.

Transformed cells are characterized by their capacity to proliferate faster and grow in an anchorage-independent manner. To assess these oncogenic features, proliferation and soft agar assays were performed. As expected, proliferation of N-Ras-transformed 149169 cells was higher than the parental NIH-3T3 (Fig. 8D). In 149169 cells expressing STAT3α, the proliferation rate was slightly enhanced, whereas for 149169 cells expressing STAT3<sup>S727A</sup>, the proliferation rate was significantly reduced compared with 149169 cells expressing STAT3α (Fig. 8D). In the same line of evidence, N-Ras-transformed cells were able to grow in an anchorage-independent manner when compared with the parental NIH-3T3, whereas 149169 cells expressing STAT3<sup>S727A</sup> showed no increase in colony formation in soft agar. In contrast, the 149169 cells overexpressing STAT3α (alone)-transfected 149169 cells (Fig. 8C). Cells expressing STAT3<sup>S727A</sup> showed different morphological features, such as less pronounced elongations and thin distal cell processes, a more differentiated mesenchymal phenotype, less polarization with an evident cytoplasm and nucleus, and a fibroblast-type shape typical of parental NIH-3T3 cells, suggesting a reversal in the transformed features (Fig. 8C, asterisks).
showed a significantly increased anchorage-independent growth when compared with 149169 cells expressing STAT3 WT (Fig. 8D). Similar results were obtained with two independent clones for each cell line (data not shown). Taking these results together, we conclude that the Ras/ERK1/2/STAT3 Ser727 pathway plays an important role in cell transformation and tumor cell proliferation.

To investigate the in vivo relevance of these findings, tumor samples from five patients carrying a RET S891A germ line mutation were analyzed. Data from one patient are shown in Fig. 9. Similar results were obtained with the samples of the other four patients (data not shown). All tumor samples displayed high levels of RET expression at the plasma membrane and in the cytoplasm (Fig. 9, A and B). A section containing both normal (N) and tumor (T) tissue was analyzed as control (Fig. 9C). In the area containing normal tissue, highly organized thyroid follicles with low levels of RET expression were observed (Fig. 9C, arrows). In the area containing the tumor cells, disorganized tissue with loss of the follicular pattern and high levels of RET expression were detected (Fig. 9C). Strong nuclear staining for Ser727-phosphorylated STAT3 was seen in all MTC tumors (Fig. 9, D and E). As a control, a biopsy section combining normal and tumor tissue was shown (Fig. 9F). Weak staining was observed for Ser727-phosphorylated STAT3 in areas lacking tumor tissue, as seen in the follicular cells (see arrows), whereas strong staining was detected in the area where the hyperplasia of the C cell localizes (Fig. 9F). Finally, strong nuclear and cytoplasmic phospho-ERK1/2 staining was observed in the tumor samples (Fig. 9, G and H). As a control, a section showing normal tissue is depicted (Fig. 9I). In areas where normal follicular epithelium was seen, low levels of phosphorylated ERK were detected (Fig. 9I, arrows). Controls performed with depletion of the primary antibody showed no staining in any of the tumors tested (data not shown).

**DISCUSSION**

RET signaling and the molecular genetics of RET-associated diseases are among the best examples of phenotypic heterogeneity and cross-talk of signaling pathways. However, despite a clear phenotype-genotype correlation, the molecular mechanisms connecting the different mutant receptors with their associated clinical phenotypes are not well understood (3). FMTC is characterized only by tumors arising from the C-cells of the thyroid, and hence it is considered the less aggressive clinical subtype of the cancer syndrome MEN2 (3). However, the understanding of the signaling pathways activated by intracellular point mutations targeting the tyrosine kinase domain of RET, in particular those mutations associated with the FMTC phenotype, is very limited. Iwashita and co-workers (18) show that RET S891A displays constitutive tyrosine phosphorylation and was able to induce transformed foci in NIH-3T3 cells. In another study, Pasini et al. (23) show similar results with RET E768D and RET Y780D (two mutations associated with FMTC). Recently, a work by Mise et al. (24) has shown that RET Y791F increased the proliferative properties, as indicated by cells in S-phase, colony formation in soft agar, and tumor growth in nude mice. RET Y791F also increased apoptotic resistance, which was accompanied by enhanced levels of active AKT and BCL2 expression. The downstream signaling of FMTC-RET mutants was also investigated by Plaza-Menchache et al. (7). We showed that RET Y791F and RET S891A signaled independently of GDNF as monomeric oncoproteins and that RET Y791F and RET S891A induced STAT3 Tyr705 phosphorylation via Src- and Janus kinase 1/2-dependent mechanism (7). This study has addressed whether STAT3 Ser727 phosphorylation was induced by RET Y791F and RET S891A, the pathway connecting these FMTC-RET receptors with STAT3 Ser727 phosphorylation, and whether phosphorylation of this residue contributes to aberrant transactivation and cell mitogenicity and transformation.

First, RET Y791F and RET S891A are able to induce STAT3 Ser727 phosphorylation in the absence of GDNF. To prove the importance of this phosphorylation event, STAT3 Ser727A reduced the levels of RET-mediated STAT3 transcriptional activity. These results are comparable with those obtained by Huang et al. (25) in which the activation of a STAT3-responsive reporter by the papillary thyroid carcinoma-RET variant RET/PTC1 was reduced by STAT3 Ser727A. However, despite activating STAT3 on Tyr705, RET/PTC1 did not induce phosphorylation of STAT3 on Ser727 (25).

Second, we demonstrated that FMTC-RET was also able to
constitutively activate a Ras/ERK1/2/ELK-1 pathway (Fig. 2).

Levels of ERK1/2 phosphorylation and SRE and ELK-1 reporter activation displayed by RET<sup>S891A</sup> were higher than RET<sup>Y791F</sup>. These results correlated with the levels of receptor activation and the degree of STAT3 activation previously observed (Fig. 1A). These data suggest that the RET<sup>S891A</sup> mutant has a higher capacity to trigger proliferative signals. Transformation foci assays performed in NIH-3T3 cells indeed showed increased transforming capacity of RET<sup>S891A</sup> than RET<sup>Y791F</sup>. Interest-

ingly, a study by Jimenez et al. (26) shows that patients with the germ line RET<sup>S891A</sup> mutation develop both MTC and pheochromocytoma, indicating that this mutation could be considered as MEN2 and hence associated with a stronger phenotype. The transforming activity of both FMTC-RET mutants was affected by U0126, suggesting an important role of the Ras/ERK1/2 MAPK pathway in RET-mediated transformation (Fig. 7). Recent studies support this conclusion; for example, a report by Sawai et al. (27) demonstrates that the polymorphism RET<sup>G691S</sup> increases the levels of ERK1/2 activation and induces pancreatic cell invasion. Another recent study by Melillo et al. (28) shows that activation of the RET/PTC-Ras/Raf/ERK1/2 pathway induces cell proliferation and invasion of thyroid follicular cells via up-regulation of the CXCL1 and CXCL10 chemokines.

Third, we delineated a Ras/ERK1/2/STAT3 Ser727 pathway triggered by RET<sup>S891A</sup> (Fig. 3). To find specific signaling profiles between wild-type RET and oncogenic RET, we showed that GDNF-stimulated wild-type RET signaled preferentially via the Ras/ERK1/2 pathway rather than the STAT3 route, in contrast to oncogenic FMTC-RET, which was able to induce full activation of the STAT3 pathway as well constitutive activation of ERK1/2 (Fig. 4). Furthermore, we have shown that the integration of both STAT3 and Ras/ERK1/2/ELK-1 pathways was required for c-fos promoter activation by RET<sup>S891A</sup> (Fig. 5). These data suggest that c-fos could be one of the key early genes implicated in the pathogenesis of FMTC. Indeed, cDNA microarray studies using various established cell lines expressing FMTC-mutated RET support this hypothesis.3

The biological importance of the Ras/ERK1/2/STAT3 Ser727 pathway in cell mitogenicity and transformation was analyzed in a model based on an N-Ras NIH-3T3-transformed cell line (149169). These cells displayed higher levels

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of ERK1/2 and STAT3 Ser727 phosphorylation than the parental NIH-3T3 cell line (Fig. 8A). Inhibition of MEK1/2 by U0126 resulted in a decrease in ERK1/2 and STAT3 Ser727 phosphorylation (Fig. 8A). Furthermore, stable expression of a STAT3 Ser727 mutant in 149169 cells showed a reversion of the transformed phenotype (Fig. 8C). The cells showed less transforming activity, less anchorage-independent growth, and less proliferation rate compared with 149169 cells or 149169-overexpressing STAT3α (Fig. 8D). These results suggest that (i) STAT3, in particular when phosphorylated on Ser727, is one of the transcription factors required by Ras for cell transformation; (ii) these two independent pathways (Ras/ERK1/2 and STAT3) can cooperate during signaling and, when deregulated, play an important role in cell mitogenesis and transformation; (iii) targeting both pathways with small inhibitor molecules should be considered as a therapeutic strategy for RET-related tumors.

Immunohistochemical analyses of tumor samples from patients carrying the germ line RETs891A mutation supported our in vitro data, as high levels of RET expression at the plasma membrane were observed in combination with strong nuclear staining of both phospho-Ser727 STAT3 and phospho-ERK1/2 (Fig. 9). These data, in combination with previous studies (7), suggest that activation of both the ERK1/2 and STAT3 pathways occurs in vivo in MTCs.

In conclusion, our data give new insights into the signaling networks implicated in FMTC. We show that aberrant activation of STAT3 by FMTC-RET not only involves the constitutive activation of STAT3 by Tyr705 phosphorylation but that these mutants further enhance the transcriptional activity of STAT3 by constitutive phosphorylation of Ser727 via a Ras/ERK1/2 pathway. These data show that deregulation of the Ras/ERK1/2/STAT3 Ser727 pathway plays an important role in cell transformation and in the development of MTC and points toward the inhibition of this pathway as a potential therapeutic strategy for patients with RET-related neuroendocrine tumors.

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