Chapter 4
Differential activation of ERK1/2, STAT3 and SRC by specific Multiple Endocrine Neoplasia type 2 associated RET mutants

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ABSTRACT

The RET proto-oncogene encodes a receptor tyrosine kinase whose dysfunction plays a crucial role in the development of several neural crest-related disorders. Distinct activating RET mutations cause Multiple Endocrine Neoplasia type 2A (MEN2A), type 2B (MEN2B) and Familial Medullary Thyroid Carcinoma (FMTC), respectively. Despite clear genotype-phenotype correlations, the molecular mechanisms connecting the mutated receptors with the distinct clinical subtypes are far from understood. Site-directed mutagenesis, luciferase reporter assays, western analysis in combination with structural modelling studies were performed in order to find RET mutant specific signalling. We show that ERK1/2 and STAT3 are differentially activated by specific MEN2-RET oncoproteins and that the activation level of both pathways strongly correlated with the degree of RET Tyr1062 and Tyr981 phosphorylation, respectively. Moreover, phosphorylation levels of RET Tyr1062 and Tyr918 were up-regulated by co-expression of a constitutive active SRC (v-SRC), suggesting in trans phosphorylation/activation of RET by v-SRC. Receptor activation was stronger for those intracellular point mutations situated in the proximity of or within the WMxxEx motif, also known as the P+1 loop of RET. Furthermore, we modelled the tyrosine kinase domain of RET with MEN2B mutations, RETA883F and RETM918T. Our modelling results confirmed that MEN2B-type mutations could alter the conformation of the RET kinase domain. In particular, the RETM918T mutation display an increased P+1 and P+3 binding pocket, changing the overall shape of the substrate binding site, possibly altering its substrate specificity. Mutation RETA883F was able to induce both, conformational changes in the activation loop resulting in a more open ATP and substrate binding conformation as well as an alteration in the conformation of the catalytic loop thereby shifting the equilibrium from the inactive to the active state of the kinase domain of RET.
INTRODUCTION

Ligand regulated signalling by the receptor tyrosine kinase RET is crucial for the development of neural crest-derived lineages and kidney organogenesis (1). Dysfunction of RET, on the other hand, plays an important role in the development of several neural crest-related disorders (1). Specific germline missense mutations in the RET proto-oncogene that result in constitutive activation of the receptor cause the dominantly inherited cancer syndrome Multiple Endocrine Neoplasia type 2 (MEN2). Depending on the tissues affected and on the mutations found, three different clinical subtypes of MEN2 are being recognized. MEN2A is characterized by Medullary Thyroid Carcinoma (MTC), pheochromocytoma and hyperplasia of the parathyroid. MEN2B is characterized by MTC, pheochromocytoma, but instead of hyperplasia of the parathyroid, patients develop neuromas on the tongue, lips and eyelids, and also intestinal ganglioneuromas. In FMTC, only the c-cells of the thyroid become malignant (1).

Mutations located in the cystein-rich domain of RET give rise to MEN2A and FMTC. Distinct mutations in the tyrosine kinase domain of the receptor can give rise to FMTC or to MEN2B (1). Interestingly, some mutations in the cystein-rich domain are not only found in families with MEN2A/FMTC but also in patients with Hirschsprung’s disease (HSCR), a congenital malformation characterized by an absence of enteric ganglia in the distal part of the colon, or in patients having a combination of both MEN2A and HSCR (2).

Despite clear phenotype-genotype correlations, the molecular mechanisms connecting the mutant receptor with the different clinical subtypes are largely unknown, (3). Different oncogenic mechanisms of activation and patterns of receptor auto-phosphorylation have been shown for the different RET-MEN2A and RET-MEN2B oncoproteins (4, 5). As consequence, different sets of phosphotyrosine-mediated signalling pathways might be activated, resulting in a different tissue-specific pattern of gene expression. In this article we studied the degree of activation of ERK1/2, STAT3 and SRC promoted by specific disease phenotype associated RET mutants in order to obtain new insights into the aberrant signalling properties of different MEN2-mutated RET receptors.
EXPERIMENTAL PROCEDURES

Cell lines and cell culture reagents
HEK293 human embryonic kidney cells were grown in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% Fetal Calf Serum (FCS) (Gibco), 100 µg/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine.

Expression and reporter plasmids
The pRC-CMV-RETwt (RETWT) plasmid encoding the short form of the human RET proto-oncogene was used to create RETC620R, RETC634R, RETY791F, RETS891A, RETA883F and RETM918T by site-directed mutagenesis according to the manufacturer’s instructions (QuickChange site-directed mutagenesis kit, Stratagene, La Jolla, USA) using the following forward (F) and reverse (R) primers:

RETC620R F 5’- GGCACCTGCAACTACTTCCCTGAGGAGG -3’
RETC620R R 5’- CCTCCTCAGGGAAGTAGTTGCAGGTGCC -3’
RETC634R F 5’- GTGCGACGAGCTGCGCCGCACGGTGATCG -3’
RETC634R R 5’- CGATCACCCTGCGCCAGCTCGTCGAC -3’
RETY791F F 5’-CCACATGTCAATCAAATTGTTGCTGCGATCCA
GCAAGCCCTGCATGGCCC -3
RETY791F R 5’-GGTGTACAGTAGTTTAACAAACCCCGGACGTCGG
TCGTACCAGG -3’
RETS891A F 5’- GGAAGATGAAGATTGCGGATTTCGGCTTCCC -3’
RETS891A R 5’- GGGACAAGCCGAAATCCGCAATCTTCATCTTCCC -3’
RETA883F F 5’- GCCAGAAACATCCTGGTATTTGAGGGCGGA -3’
RETA883F R 5’- CATCTTCCGCCCCCTCATACTACCAGGTGGT -3’
RETM918T F 5’- CGGATTCCAGTTAAATGGAGGAGCCG -3’
RETM918T R 5’- GGGATTCAATTGCAGGTCGCTCAAGCGAATTT -3’

Following mutagenesis, the entire RET cDNA was checked by sequencing. The pTAL-SRE-Luc, pIRE-Luc and pDM2-LacZ reporter plasmids were described previously (6). The v-SRC plasmid was kindly provided by Dr. J.J. Schuringa (Department of Hematology, UMCG, The Netherlands).
**Luciferase reporter assays**

HEK293 cells were transfected using the calcium phosphate method as described previously (7). Briefly, HEK293 cells were seeded in 6 well plates (250,000/well) and transfected the next day. After transfection (24 hr), cells were washed and new medium was added. The next day (48 hr) cells were harvested in lysis buffer (Promega). Luciferase activity was determined using the SteadyLite HTS kit (Perkin Elmer). In all transfections, a β-galactosidase expression plasmid (pDM2LacZ) was included as an internal standard to normalize luciferase activities. β-galactosidase activity was determined in 100 mM Na₂HPO₄/NaH₂PO₄, 1 mM MgCl₂, 100 mM 2β-mercaptoethanol and 0.67 mg/ml O-nitrophenylgalactopyranoside.

**Western blotting**

Cells were lysed with 10 mM Tris-Cl pH 7.4, 144 mM NaCl, 2 mM EDTA, 1% Nonidet P40, 2 mM DTT, 1 mM Na-vanadate, 10% glycerol, 10 µg/ml aprotinin, 2 µg/ml leupeptin, 0.2 mM PMSF, resolved on a 10% SDS-PAGE and analyzed using western blotting and ECL (Roche). The following antibodies (1:1000) were used: RET (H-300), phospho-Tyr1062RET, STAT3 (C-20) (all of them from Santa Cruz), phospho-Tyr981RET (8), phospho-ERK1/2, ERK1/2 and phospho-STAT3 (Tyr705 and Ser727) (all of them from Cell Signalling Technology, New England Biolabs, UK). Quantification of signal intensity was performed using ImageJ software.

**Structural modelling analysis**

Structure and sequence databases (PDB and Swissprot) were searched for sequences related to the human RET tyrosine kinase (RETK) domain (Swissprot accession code P07949) using BLAST. Related sequences were aligned with ClustalW using default parameters. The tyrosine kinase domains of human fibroblast growth factor receptor 1 (FGFR1K) and FGFR2K were found to have sequence identities of 50% with RETK. The crystal structure of FGFR1K (PDB accession code 1FGK) determined to 2.0 Å resolution was selected as template to construct the model. The crystal structure of the activated insulin receptor tyrosine kinase domain (PDB accession code 1IR3) was used as template to construct a model of activated RETK. The homology models of inhibitory and activated wild-type RET
and the RETA883F and RETM918T mutants were constructed using MODELLER version 7 as implemented in DS Modelling 1.1 (Acers Inc, San Diego, CA) using standard settings. The kinase insert region of RETK was not included in the final models. The quality of the models was evaluated using Whatif and Procheck. Quality parameters of the models were comparable to those of the template structure. A short energy minimization using constraints on the backbone atoms was performed using the CHARMM module of DS Modeling 1.1.
RESULTS AND DISCUSSION

Activation of RET by GDNF causes trans-phosphorylation of intracellular tyrosine residues triggering downstream signalling pathways required for the development of neural crest derived tissues (such as C-cells of the thyroid gland and enteric neurons) and kidney organogenesis (1). The RET receptor has sixteen tyrosines residues in the intracellular domain, including six tyrosines in the kinase domain (Tyr 864, Tyr 900, Tyr 905, Tyr 928, Tyr 952 and Tyr 981). From these, Tyr 905 has been shown to play a crucial role in the catalytic and cell transforming activity of RET (9) and Tyr981 has been demonstrated to be the docking site for c-SRC and to promote neural survival (8). In the c-terminal tail of RET, Tyr1015 was identified as the docking site for PLC-γ and Tyr1062 as a docking site for SHC/ENIGMA/IRS1/DOK4/5 amongst others (1). Therefore, Tyr1062 seems to be one of the most important docking sites of RET, as mutation of this residue abrogates the RET-mediated activation of the MAPKs (ERK1/2, JNK, P38, ERK5) and PI3-K signalling pathways as well as it able to abrogate the transforming capacity of RET-MEN2A and RET-MEN2B mutants (10). Salvatore and colleagues (11) showed that RETM918T is more active than RETC634R in associating with SHC and that the RETM918T mutation specifically potentiates the ability of RET to auto-phosphorylate Tyr1062 and consequently to trigger higher activation levels of the RAS/MAPK and the PI3K/AKT pathways, respectively (11). In this study we compared not only the most common RET mutations found associated with MEN2A (RETC634R) or MEN2B (RETM918T), but also others disease-phenotype associated RET mutants (Table 1) in order to get a better insight in the molecular mechanism by which the different mutations in the RET proto-oncogene give rise different signalling properties and possibly thereby contributing to the different disease phenotypes.

HEK293 cells expressing either RET wild type or a series of MEN2-mutated RET receptors were analyzed by western blotting using an antibody against RET phospho Tyr 1062. The highest level of phosphorylated Tyr1062 was observed with the M918T mutant (MEN2B) (11). Interestingly, RETS891A (a mutation affecting the tyrosine kinase domain and associated with FMTC) showed higher levels of Tyr 1062 phosphorylation than RETC634R and other MEN2B-associated mutation (A883F). RET mutants C620R (MEN2A/FMTC) and Y791F (FMTC) showed increased levels of phosphorylated compared to the wild type receptor but lower than the previously mentioned mutants (Fig. 1A)
Next, we tested the levels of ERK1/2 phosphorylation induced by these mutant RET receptors. A similar pattern of ERK1/2 activation, as seen for RET Tyr1062 phosphorylation, was observed. These results suggest a strong correlation between the ERK1/2 pathway and RET Tyr1062 in MEN2 (Fig. 1A). Remarkably, the high levels of RET Tyr1062 phosphorylation displayed by RETS891A did not correlate with the levels of ERK1/2 activity. This could possibly mean that RETS891A, through Tyr1062, interacts with other signalling pathways with a higher affinity than with ERK1/2. Reporter assays in HEK293 cells, in which mutant RET receptors were coexpressed with a GAL4-ELK fusion/UAS-luciferase reporter system, further supported these findings (Fig. 1B).

We also analysed the levels of Tyr981 phosphorylation induced by specific MEN2-RET mutant receptors. RET Tyr981 was reported to be the docking site for c-SRC, and it was shown that a mutation of this residue reduced neural survival promoted by RET (8). Higher levels of phosphorylated RET Tyr981 were shown for RET mutants S891A, M918T and A883F, indicating higher levels of SRC activation for intracellular mutations located in the proximity of or within the WMxxEx motif, also known as the P+1 loop (Fig. 2A, 12). This loop represents a small motif immediately C-terminal of the activation loop. It plays an important role in recognising the residues flanking the target tyrosine in the substrate (12).

**Table 1.**

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Bases substitution</th>
<th>Amino acid change</th>
<th>Exon</th>
<th>Phenotype</th>
<th>Domain*</th>
</tr>
</thead>
<tbody>
<tr>
<td>C620R</td>
<td>TGC to CGC</td>
<td>Cys to Arg</td>
<td>10</td>
<td>MEN 2A</td>
<td>CRD</td>
</tr>
<tr>
<td>C634R</td>
<td>TGC to CGC</td>
<td>Cys to Arg</td>
<td>11</td>
<td>MEN 2A</td>
<td>CRD</td>
</tr>
<tr>
<td>Y791F</td>
<td>TAT to TTT</td>
<td>Tyr to Phe</td>
<td>13</td>
<td>FMTC</td>
<td>TKD</td>
</tr>
<tr>
<td>S891A</td>
<td>TCG to GCG</td>
<td>Ser to Ala</td>
<td>15</td>
<td>FMTC</td>
<td>TKD</td>
</tr>
<tr>
<td>A883F</td>
<td>GCT to TTT</td>
<td>Ala to Phe</td>
<td>15</td>
<td>MEN 2B</td>
<td>TKD</td>
</tr>
<tr>
<td>M918T</td>
<td>ATG to CTG</td>
<td>Met to Thr</td>
<td>16</td>
<td>MEN 2B</td>
<td>TKD</td>
</tr>
</tbody>
</table>

*CRD (cystein rich domain), TKD (tyrosine kinase domain) and CTD (C-terminal tail).
Extracellular mutants RET C620R and C634R, as well as RETY791F, a mutation in the β-4 strand of the N-terminal lobe, distantly situated from the P+1 loop, showed lower levels of phosphorylated RET Tyr981, suggesting a lower SRC-dependency of the oncogenic signalling mechanism of the Y791F mutant compared with the S891A mutant (6). Next, we wanted to investigate the levels of STAT3 (one of the main targets of SRC kinase) activation (Tyr705 phosphorylation) promoted by different MEN2-RET mutants. A comparable pattern of STAT3 Tyr705 phosphorylation, as seen for RET Tyr981, induced by different specific RET oncoproteins was observed (Fig. 1A). These results corroborate a previous report in which the RETM918T triggered higher levels of Tyr705 STAT3 phosphorylation than RETC634R (12). Luciferase reporter assays in HEK293 expressing mutated RET receptors with the pIRE-Luc reporter supported these results (Fig.1C). As predicted by the Westerns analyses, higher levels of STAT3 reporter activation were observed by intracellular point mutations M918T, A883F and S891A, followed (in decreasing order) by C634R, Y791F and C620R, respectively (Fig.1C). These results indicate an interaction and signalling cooperation between RET and SRC in STAT3 activation. Some studies indeed have addressed this cooperation between members of the SRC family of kinases and the receptor tyrosine kinase RET (6, 8, 9).

In light of this, we studied the levels of RET phosphorylation in trans by co-expression of v-SRC (a constitutive active Src kinase) in HEK293 cells expressing MEN2-associated RET mutations (Fig. 1A). Up-regulation of RET phosphorylation on Tyr981 and Tyr1062 was observed in cells co-expressing RET mutants. Interestingly, the levels of phosphorylated RET were much higher in the case of intracellular point mutants proximal to or within the P+1 loop of the kinase domain of RET (S891A, A883F and M918T) (Fig. 1A). These results suggest that phosphorylation of RET by SRC kinase in trans could be an oncogenic mechanism of activation specific for those intracellular point mutations situated in the proximity or within the P+1 loop.

Taken all together, these results shown that signaling differences between different MEN2-associated RET mutations do exist and that these differences most likely will contribute to the clinical differences found associated with the distinct MEN2 subtypes. A table resuming the genotype-signalling-phenotype correlation is depicted in table 2.
Figure 1

ERK1/2, STAT3 and SRC signalling profile by specific MEN 2 disease phenotype associated RET mutants

A) HEK293 cells were transfected with wild type RET or various RET mutants, in combination with v-SRC as indicated. Whole cell protein lysates were resolved (20 µg/lane) on SDS-PAGE and analyzed by Western blotting using antibodies against: phospho-Tyr1062RET, phospho-Tyr981RET, RET phospho-Tyr705STAT3, STAT3, phospho-ERK1/2 and ERK1/2 as depicted.

B) HEK293 cells were transfected with wild type RET and mutant RET receptors in combination with ELK-1/GAL4, UAS-Luc and pDM2-LacZ reporter plasmids. The mean fold activation of the luciferase reporter with the SD of two independent experiments, each of them performed in triplicate, is depicted. Quantification of ERK1/2 phosphorylation was performed using ImageJ software. The data represent the analysis of two independent experiments, scale (1/2).

C) HEK293 cells were transfected with wild type RET and mutant RET receptors in combination with the IRE-Luc and pDM2-LacZ reporter plasmids. The mean fold activation of the luciferase reporter with the SD of three independent experiments, each of them performed in triplicate, is depicted. Quantification of Tyr705 STAT3 phosphorylation was performed using ImageJ software. The data represent the analysis of three independent experiments, scale (1/20).
We previously modelled the RET kinase domain (RETK) using the already solved crystal structure of the fibroblast growth factor receptor 1 and 2 tyrosine kinase domains (FGFR1 and 2) as a template (6). To model the conformational changes in the activation loop caused by MEN2B mutations as well as the consequence of these in the receptor’s peptide substrate binding properties, we used the crystal structure of the Insulin receptor kinase (IRK) domain as a template (13). Although the percentage in sequence identity between RETK and IRK is smaller (~38%) compared to FGFRK1 (~50%), the overall structural characteristics of receptor tyrosine kinase domains are highly conserved (Fig. 2A), allowing us to use the crystal structure of the activated IRK (13) in complex with a substrate peptide to construct an activated conformation of the RETK model (13). The residues creating the P+1 and P+3 substrate binding pockets are highly conserved between IRK and RETK. The P+1 binding pocket of the insulin receptor consist of Val1173, Leu1219 and the aliphatic portions of Asn1215 and Glu1216 side chains (Fig. 2A). The P+3 pocket is created by residues Leu1171, Val1173, Met1176, Leu1181 and Leu1219 (13). The corresponding P+1 binding pocket residues of RETK are Ile913, Val915 and the aliphatic portion of the side chain of Glu958; however the corresponding residue to Asn1215 of IRK is a proline in RETK (Pro957) (Fig. 2A). The P+3 pocket of RETK comprise of residues Ile913, Val915, Met918, Leu923 and Phe961 (Fig. 2A).

In activated IRK, the side chain of Met1176 forms hydrophobic bonds with residues Leu1171, Val1173 and Phe1186. In receptor tyrosine kinases, this methionine is highly

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Phenotype</th>
<th>pTyr1062RET</th>
<th>pTyr981RET</th>
<th>pERK1/2</th>
<th>pTyr705STAT3</th>
</tr>
</thead>
<tbody>
<tr>
<td>C620R</td>
<td>MEN2A/FMTC/HSCR</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>C634R</td>
<td>MEN2A</td>
<td>++</td>
<td>+</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>Y791F</td>
<td>FMTC</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>S891A</td>
<td>FMTC</td>
<td>++</td>
<td>+++</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>M918T</td>
<td>MEN2B</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>A883F</td>
<td>MEN2B</td>
<td>++</td>
<td>+++</td>
<td>++</td>
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</table>

We previously modelled the RET kinase domain (RETK) using the already solved crystal structure of the fibroblast growth factor receptor 1 and 2 tyrosine kinase domains (FGFR1 and 2) as a template (6). To model the conformational changes in the activation loop caused by MEN2B mutations as well as the consequence of these in the receptor’s peptide substrate binding properties, we used the crystal structure of the Insulin receptor kinase (IRK) domain as a template (13). Although the percentage in sequence identity between RETK and IRK is smaller (~38%) compared to FGFRK1 (~50%), the overall structural characteristics of receptor tyrosine kinase domains are highly conserved (Fig. 2A), allowing us to use the crystal structure of the activated IRK (13) in complex with a substrate peptide to construct an activated conformation of the RETK model (13). The residues creating the P+1 and P+3 substrate binding pockets are highly conserved between IRK and RETK. The P+1 binding pocket of the insulin receptor consist of Val1173, Leu1219 and the aliphatic portions of Asn1215 and Glu1216 side chains (Fig. 2A). The P+3 pocket is created by residues Leu1171, Val1173, Met1176, Leu1181 and Leu1219 (13). The corresponding P+1 binding pocket residues of RETK are Ile913, Val915 and the aliphatic portion of the side chain of Glu958; however the corresponding residue to Asn1215 of IRK is a proline in RETK (Pro957) (Fig. 2A). The P+3 pocket of RETK comprise of residues Ile913, Val915, Met918, Leu923 and Phe961 (Fig. 2A).
conserved, whilst in cytosolic tyrosine kinases like SRC, ABL and LCK this residue is a threonine (12). In the active RETK model, the side chain of the corresponding Met918 forms hydrophobic contacts with residues Ile913, Val915 and with Tyr928 (Fig. 2B). The conformational change of the activation loop from the inactive to the active state changes the packing around this methionine residue. For example, in the inactive RETK model, the activation loop residue Ile913 is substantially displaced and does no interact with Met918. Mutation of Met918 to the smaller hydrophilic threonine, the packing around the P+1 and P+3 binding pocket, in both the inactive and the activated state, will be substantially altered. The threonine is almost completely buried, with the hydrogen of the hydroxyl gamma bound to a backbone oxygen atom (Fig. 2B). This threonine will not contribute to direct substrate binding, however due to its smaller size it allows the neighbouring residues to readjust. This probably will result in an increased P+3 binding pocket which can change the overall shape of the substrate binding sites resulting in a change of substrate specificity. Additionally, the change of packing around Ile913 might also cause an increase in constitutive RETK activity. Replacing Met918 for a smaller threonine and subsequent change of packing around Ile913 possibly permits the activation loop to change its conformation more easily from the inactive to the active conformation, thereby shifting the equilibrium between inactive and active state of RETK (Fig. 2B).

The other activating mutation of RETK described here is A883F. Although Ala883 is not highly conserved (Fig. 2A), many tyrosine kinases have a preference for relatively small hydrophilic or charged residues (Ser, Thr, Asp, Asn) or small hydrophobic residues (Gly, Ala) at this position. Residue Ala883 is located at the opposite site of the peptide binding site, in the loop connecting β-strand 7 with β-strand 8 (Fig. 2A, C). These two β-strands connect the catalytic-loop residues with the activation loop residues. Even though this Ala883 is not in direct contact with important catalytic features of RET, mutation to Phenylalanine could indirectly result in increased catalytic efficacy. The side chain of phenylalanine makes contact with Glu884 and Lys889 (Fig. 2C); this lysine is located just three residues N-terminal from the conserved DFG motive at the beginning of the activation loop (Fig. 2A). The phenyl alanine side chain is also in contact with the side chain of Glu805, located just proximal of Tyr806 and with the main chain oxygen atom of Tyr806. This Tyr806 is a known autophosphorylation site of RETK (4). Modelling this A883F mutation did not reveal major
RET signalling in MEN2
Figure 2
Amino acid sequence alignment and predicted secondary structure of the c-terminal tyrosine kinase domain of RET and structural model of RET affected by MEN2B mutations M918T and A883F.

A) ClustalW amino acid sequence alignment of RET with the human RTK homologues FGR1 and IG1R. Legend: white and black boxes are secondary structure elements, α helices and β strands, respectively in FGR1. White-grey bar: catalytic loop; dark-grey bar: activating loop; black bar (*): P+1 loop core.Filled black squares represent identical amino acid and red filled squares represent mutated amino acids. The ruler shows the sequence numbering of RET.

B) Structural model analysis of activating mutation M918T in P+1 and P+3 peptide binding pocket. The wild type RETK (left panel) and M918T RETK (right panel) models are depicted. Residues forming the P+1 and P+3 peptide binding pocket in the activated state are shown. Represented in mesh is residue 918 and C-terminal part of activation loop is colored in orange. Active loop is colored in red.

C) Structural model analysis of activating mutation A883F located between N-terminal lobe and C-terminal lobe. The wild type RETK (left panel) and A883F RETK (right panel) models are depicted. Represented in mesh is residue 883. Selected residues interacting with residue 883 are numbered, in addition residue F893 of the DFG motive is shown as reference. Activation loop is colored red. The αC helix, β4-strand and the interconnecting loop, located in the N-terminal lobe, are colored orange.

Structural disturbances upon substitution of a small residue with a more bulky one. Even so, small structural adjustments will probably happen in order to accommodate this more bulky residue. Adjustments of the glutamic side chain and the main chain oxygen atom of Tyr806 can result in a small change in backbone configuration, which can result in altered accessibility of Tyr806 (Fig. 2C). The same can be proposed for the interaction with Lys889; a small readjustment of backbone configuration can cause a change of the local configuration near the DFG motive and cause a shift in equilibrium of the activation loop from an inhibited state to the active.

In conclusion, combining functional (luciferase reporter assays and Western blotting analysis) and the structural modelling studies, we have shown: i) specific activation of the ERK1/2 and STAT3 pathways by specific MEN2 disease phenotype associated RET mutants. Furthermore we showed that v-SRC can phosphorylate RET in trans, and this phosphorylation is more accentuated for those mutations targeting the P+1 loop in the kinase domain of RET, suggesting a novel oncogenic mechanism of RET activation for RETS891A, RETA883F and RETM918T mutants. ii) predicted the structural changes caused by MEN2B
mutants, RETA883F and RETM918T, and thereby their possible oncogenic mechanism of activation.

All together, we have brought new insights into the signalling properties associated with specific MEN2-RET oncoproteins in order to connect them with their associated disease-phenotypes.
REFERENCES


