THEMATIC REVIEW

Structure and function of RET in multiple endocrine neoplasia type 2

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Abstract

It has been twenty-five years since the discovery of oncogenic germline RET mutations as the cause of multiple endocrine neoplasia type 2 (MEN2). Intensive work over the last two and a half decades on RET genetics, signaling and cell biology has provided the current bases for the genotype–phenotype and functional correlations within this cancer syndrome. On the contrary, the structural and molecular basis for RET tyrosine kinase domain activation and oncogenic deregulation has remained largely elusive. Recent studies with a strong crystallographic and biochemical focus have started to elucidate key insights into such molecular and atomic details revealing unexpected and private mechanisms of actions and molecular determinants not previously envisioned. This review focuses on the structure and function of the RET receptor, and in particular, on what a more detailed view of the protein itself and what the current structural and molecular information tell us about the genotype and phenotype relationships in the cancer syndrome MEN2.

Introduction

RET is a receptor tyrosine kinase (RTK) that plays key roles during embryonic development of central and peripheral (enteric) nervous systems, kidney and Peyer’s patch organogenesis and spermatogenesis (Mulligan 2014). RET is a remarkable example illustrating how genetic alterations within a single gene have such profound biological and clinical consequences in human disease. Perturbation of RET signaling by both gain- or loss-of-function mutations cause multiple endocrine neoplasia type 2 (MEN2) cancer syndrome and developmental failure in the neural innervation of the gut (Hirschsprung’s disease) (Plaza-Menacho et al. 2006), respectively. Furthermore, genetic rearrangements usually involving the coding sequences of the RET kinase domain and C-terminal segment together with coding amino terminal portion of cytoplasmic proteins with coiled-coil and dimerization motifs are causative/found in several human cancers (Mulligan 2014, Plaza-Menacho et al. 2014b).

RET (Fig. 1) is the signaling receptor for the glial cell-derived neurotrophic factor (GDNF) family of ligands (GFLs): GDNF, Neurturin (NRTN), Persephin (PSPN) and Artemin (ARTN) (de Groot et al. 2006, Plaza-Menacho et al. 2006). To stimulate RET tyrosine kinase activity from the extracellular environment, these GFLs first need to assemble into a complex with their glycosylphosphatidylinositol (GPI)-anchored co-receptors, the GDNF receptor-α family (GFRα1–4), after which the GFL–GFRα complex recruits RET to form cognate and non-cognate heterodimers. The RET–GFL–GFRα complex (the RET ‘ternary’ complex) has a 2:2:2 stoichiometry,
meaning a dimer of GDNF binds two co-receptor molecules that at the same time recruit two RET receptor molecules and exhibits positive cooperativity (Schlee et al. 2006, Goodman et al. 2014). Recently, the architecture of RET extracellular domain (ECD) was revealed by small angle X-ray scattering (SAXS) and electron microscopy (EM). The EM structure for a RET–GDNF–GFRα ternary complex reveals a composite ligand-binding site, and a GFRα1-binding hotspot that contacts the invariant RET cadherin-like domain (CLD) 2–3 containing calcium sites region of RET. Furthermore, the cysteine-rich domain (CRD) couples ligand recognition and receptor homodimerization (Fig. 1); this potentially being exploited by oncogenic mutations targeting RET CRD (Goodman et al. 2014).

Upon ligand and co-receptor binding, RET is recruited and assembled into a multicomponent receptor complex (i.e. ternary complex), followed by activation of the kinase domain and intermolecular autophosphorylation of intracellular tyrosine residues. These phospho-tyrosine sites serve as docking sites for downstream signaling proteins carrying SRC homology 2 (SH2) or phosphotyrosine-binding (PTB) domains mainly, also FERM (4.1 protein, ezrin, radixin and moesin) domains (i.e. FAK), which transmit signals inside the cell via the activation of a wide range of effector proteins (Plaza-Menacho et al. 2006, 2014b). While the latter role for autophosphorylation has been widely demonstrated for RET, the effect of autophosphorylation on catalytic activation is been less clear. At least 14 of the 18 tyrosine residues present in the intracellular region of RET can become phosphorylated (Liu et al. 1996, Kawamoto et al. 2004, Knowles et al. 2006). In vitro, activation loop phosphorylation has little effect on catalytic activity despite they are known to contribute to full-length RET receptor tyrosine kinase activation in cells (Iwashita et al. 1996, Kawamoto et al. 2004, Knowles et al. 2006, Plaza-Menacho et al. 2014a). These data indicate that the cellular effects linked to individual tyrosine phosphosites are not a direct consequence of the lack/or perturbation of kinase activity by the receptor per se, but due to the disruption of downstream signaling pathways and feedback loops at the intracellular level that impact on RET phosphorylation and kinase activity.
Clinical subtypes associated to MEN2

Germline missense activating mutations of RET cause MEN2, a dominantly inherited cancer syndrome affecting neuroendocrine organs (Mulligan et al. 1993, Hofstra et al. 1994). Depending on the affected tissues and mutations found, three different clinical subtypes can be found (de Groot et al. 2006):

(a) MEN2A is characterized by medullary thyroid carcinoma (MTC), a tumor of the calcitonin-secreting parafollicular c-cells, pheochromocytoma (PC), a tumor of the adrenal chromaffin cells and hyperparathyroidism (HPT). MTC is the common feature in MEN2A patients (100%), PC is observed in 50% of patients and HPT in 15–30% of the cases (Sipple 1984). Occasionally, MEN2A can be associated with cutaneous lichen amyloidosis (a pruritic and pigmented papular lesion of the skin on the upper back) (Verga et al. 2003).

(b) MEN2B patients develop a more complex clinical phenotype including ganglioneuromas in the tongue, lips and eyelids, intestinal ganglioneuromas, thickened corneal nerves as well as a marfanoid habitus in addition to the common features MTC (100%) and PC (approximately 50%) and no HPT. It is considered to be the most aggressive subtype of MEN2 with the earliest age of onset (Carney et al. 1976).

(c) Familial MTC (FMTC) is characterized by the only manifestation of MTC in four or more members of an affected family. FMTC should be considered no longer a ‘stand alone’ disease and it should be recognized as a variant of MEN2A (Wells et al. 2015).

RET oncogenic signaling in MEN2

Mutations affecting the extracellular cysteine-rich domain of RET (associated with MEN2A and FMTC) result in covalent dimerization and constitutively activation of the receptor (Mulligan et al. 1993, Hayashi et al. 2001). Mutations affecting the intracellular domain of RET, usually associated with FMTC and always with MEN2B, signal independently of GDNF apparently as monomeric oncoproteins (Hofstra et al. 1994). These oncoproteins show not only an altered catalytic activity but also an altered substrate specificity because, contrary to wild-type (WT) RET, they tend to phosphorylate substrates that are usually preferred by cytoplasmic tyrosine kinases such as SRC and FAK (Santoro et al. 1995, Plaza-Menacho et al. 2011). While activation loop Y905 is required for the transforming activity and signaling of RET-MEN2A mutations, the transforming activity of RET-MEN2B significantly decreased by replacing tyrosine 864 or 952 to phenylalanine being in this case independent of activation loop Y905 (Iwashita et al. 1996).

This short review will focus on the structural and molecular determinants for RET catalytic domain activity at the atomic and molecular levels as a ground to understand how oncogenic mutations found in MEN2 (many of them targeting the catalytic domain) corrupt these mechanisms. For this purpose, it would be useful to understand first which secondary structural elements and motifs are required for RET catalytic activation and function.

Conserved structural and functional features of RET catalytic domain

We will take as reference the crystal structure of the prototypical protein kinase A catalytic subunit (PDB: 1ATP) bound to both, substrate analog and ATP (Knighton et al. 1991). In general, protein kinases adopt a characteristic twin-lobed catalytic core structure with the ATP-binding site in a deep cleft located between the lobes. The N-terminal small lobe is composed of several antiparallel beta sheets (b1–5) and an important alpha (α) C helix. In general, the N-lobe is important for the suitable positioning and orientation of the ATP molecule into the active site for catalysis. A hinge or linker connects the N-lobe with the larger α-helical C-lobe composed mainly by several α(D–H) helices and some functionally important sequence motifs within flexible unordered segments (loops). In the case of phosphorylated RET catalytic domain (as an example PDB 2IVU), on the N-lobe there is a crucial β3 K758 (K72, in PKA catalytic subunit, hereafter PKA) that forms a catalytically required salt bridge with εC E775 (E91) (Knighton et al. 1991, Meharena et al. 2013). The catalytic K758 coordinates via hydrogen bonds with α and β groups of the nucleotide to optimally orientate the γ phosphate of ATP for the phospho-transfer reaction (Fig. 2). An important motif on the N-lobe is the so-called glycine-rich loop (GRL), nucleotide-binding loop or P-loop (Hanson & Whiteheart 2005, Meharena et al. 2013) that is involved in accommodating the nucleotide (i.e. ATP) into the active site by hydrophobic and electrostatic interactions. In the case of RET, the glycine-rich loop is formed by a sequence motif GEGFGK (consensus G-Xα-GK-T/S) (Hanson & Whiteheart 2005, Knowles et al. 2006). In general, within this motif, there is an important lysine, which is crucial for nucleotide binding by forming hydrogen bonds with β and γ phosphate groups of ATP (Hanson & Whiteheart 2005). In the case of RET such
lysine (K737) is not apparently involved in nucleotide coordination, and this may indicate redundancy due to spatial proximity with catalytic K758. An important RET residue in the ATP-binding loop located at the tip is E734 that forms a key tether with activation loop R912, with important functional connotations, see e.g. section cis-inhibited RET catalytic domain. The region that connects both N- and C-lobes is the hinge; this region forms a series of hydrogen bonds with the adenine ring of ATP between the two lobes of the protein (Meharena et al. 2013). This motif is not only important for binding and positioning of the nucleotide (ATP) but also for binding of tyrosine kinase inhibitors (TKIs) that are ATP competitors (Endicott et al. 2012). Crucially, mutations of the so-called, gate-keeper residue (RET V804M) are often associated with resistance to TKIs due to steric impediments blocking access to the active site (Plaza-Menacho et al. 2007). The catalytic loop is another functionally important element, which is characterized by a HRD motif (YRD in PKA) (Knighton et al. 1991). It is a motif conserved throughout the protein kinase family in all eukaryotic, eukaryotic-like and atypical protein kinases (Meharena et al. 2013), aspartate (D166, PKA and D875, RET) being the most conserved member of the motif. This residue is responsible for nucleophilic attack and the correct orientation and positioning of the phospho-site hydroxyl acceptor group in the peptide substrate. Typically kinases lacking the HRD arginine are not phosphorylated in the activation loop (Kornev et al. 2006). Just before the activation segment, we observe the conserved magnesium-binding loop or DFG motif (Kornev & Taylor 2010), which plays a very important functional role by a Mg\textsuperscript{2+}-coordinating/chelating aspartate D892 (D184) required for the optimal orientation of β- and γ-phosphate of ATP for the phosphorylation reaction; this action comes together with the catalytically required K758 (K72). The orientation of the DFG motif is a surrogate for the conformation adopted by the activation loop whether it is an active (DFG\textsuperscript{in}) or inactive (DFG\textsuperscript{out}) conformation (Steichen et al. 2012, Hari et al. 2013). Many protein kinases are activated by a phosphorylation event on the activation loop that releases cis-inhibitory constrains and at the same time increases catalytic activity (Hubbard 1997, Chen et al. 2007). However, there are a group of kinases (RET among them) that do not require activation loop phosphorylation for catalytic activation in vitro (Louheed et al. 2004, Zhang et al. 2006). The activation loop is a flexible loop spatially close to the catalytic loop. Activation loop phosphorylation can counteract the positive charge of the arginine in the catalytic loop by the HRD motif (Iyer et al. 2005, Steichen et al. 2010), which in the case of RET this is commonly seen by a salt bridge network between αC R770 and activation loop R897 and K907, Fig. 2 and (Knowles et al. 2006, Plaza-Menacho et al. 2014a). A recent crystal structure of a hyper-phosphorylated RET catalytic domain with a proximal juxtamembrane segment showed a distinct arrangement of the salt bridge network with important implications for the activation loop to adopt a more extended conformation having as a consequence an impact on downstream signaling (Plaza-Menacho et al. 2016). Tyrosine kinases usually have one or two tyrosines in the activation loop, in the case of RET there are two, Y900 and Y905, within the RDVYEEDESYVKRSQG peptide, both of which can be phosphorylated, although as early
said not required for catalytic activity in vitro. The region just after the activation loop is called the $P+1$ loop, which often binds the substrate residue just C-terminal of the phosphorylated one (the $P+1$ residue) (Hubbard 1997). Together, the activation loop and $P+1$ loop constitute the activation segment, which runs from the DFG motif to the APE motif.

Intensive work over the last two and a half decades on RET genetics, signaling and cell biology have provided the current paradigm for the functional, biological and clinical correlations associated with distinct RET oncogenic variants. However, the structural and molecular bases for RET catalytic domain activation and oncogenic deregulation remained largely elusive. Recent crystallographic and biochemical studies have started to provide key insights into the molecular and atomic details revealing unexpected and private mechanisms of actions and molecular determinants not previously envisioned (Knowles et al. 2006, Plaza-Menacho et al. 2014a, 2016). This information is crucial as current compounds used in the clinic to treat RET-positive cancers are not RET specific and do not provide a significant survival response (Sherman 2013); hence, better therapeutic options are needed (e.g. allostERIC inhibitors for personalized treatment). This is also relevant for oncogenic RET kinase domain fusions and other types of RET-positive human cancers (lung, thyroid, colon).

**Early and late RET phospho-sites reveal allostERIC inputs from adjacent elements to the catalytic core**

Auto-phosphorylation is one of the most important cellular processes by which RTK transmit signal inside the cell from external cues in a spatial and temporal regulatory manner. Hence, this process has to be remarkably controlled, not only from the extracellular compartment (i.e. ligand availability) but also from the intracellular compartment (i.e. catalytic domain) where the tyrosine kinase domain activity has to be kept under tight control. Recently, a quantitative proteomic approach to define the temporal sequence of RET autophosphorylation by label-free quantitative mass spectrometry was applied to provide insights into the molecular bases for RET kinase activation and oncogenic deregulation (Plaza-Menacho et al. 2014a). In this experimental setting using recombinant purified RET ICD, the earliest detectable autophosphorylation sites upon kinase domain activation map to regions flanking the core catalytic domain, in particular, C-segment Y1062 and JM-region Y687, while sites within the activation loop (e.g. Y900 and Y905) and other sites of the catalytic domain core (Y981) only form at later time-points. This process was tightly regulated and was protein concentration dependent in line with an intermolecular mechanism (in trans) (Plaza-Menacho et al. 2014a). Furthermore, primary sequences flanking phospho-tyrosine acceptor sites did not determine the order of autophosphorylation, and activation loop Y900 and Y905 were not found to be ‘de facto’ activating, as they are late phosphorylation events and are not catalytically required. A similar situation is found for the EGFR and non-RTK ACK1 (Lougheed et al. 2004, Zhang et al. 2006), in these cases, allostERIC mechanism of activation and regulation have been described. In the case of RET, the definition of the kinetics of autophosphorylation revealed functional roles for flanking elements in the regulation of RET catalytic domain activity, pointing at the importance of phospho-sites within flanking elements outside the RET catalytic core prior to activation loop phosphorylation, as potential allostERIC component for the mechanism of RET activation and signaling (Plaza-Menacho et al. 2014a). This comes in line with a closed to open transition of the RET ICD upon catalytic domain activation and autophosphorylation. Interestingly, phospho-tyrosine site mutants did not have any detrimental impact on catalytic activity (as indicated by lack of effect on the autophosphorylation reaction) in the context of the full RET ICD. These data indicate that phosphosites are catalytically redundant, and as a consequence must act as substrate or docking platform for downstream interactors. The fact that in cells, phospho-sites mutants do have an impact on RET signaling (Kato et al. 2002) reveal the existence of complex intracellular mechanisms of RET catalytic domain regulation in trans (Plaza-Menacho et al. 2014a,b).

**Cis-inhibition of RET catalytic domain by a tether between phosphate-binding and activation loops**

A recent crystal structure of a phosphorylated RET catalytic domain at 1.65 Å resolution (Plaza-Menacho et al. 2014a) revealed the remarkable feature of the presence of two distinct conformations of the glycine-rich loop (PDB 4CKJ). Glycine-rich loop or P-loop (i.e. phosphate- or ATP-binding loop), also known as walker motif is a sequence motif in proteins that is associated with phosphate binding. The motif has the pattern G-X(4)-GK-[TS], it is present in many ATP- or GTP-utilizing proteins; it normally binds the β phosphate of the nucleotide. In general, the lysine
(K) residue in the P-loop, together with the main chain amino atoms, is crucial for nucleotide-binding (Hanson & Whiteheart 2005). In the case of RET, the phosphate-binding loop is formed by a sequence motif G-x-G-x-x-GK (Knowles et al. 2006), not followed by TS indicating that the conformation of this loop is not subjected potentially to post-translational modification as shown for the case of some bacterial tyrosine kinases (Schumacher et al. 2012) and also human DYRK2 (Soundararajan et al. 2013). Furthermore, lysine (K737) is not apparently involved in nucleotide coordination, due to redundancy caused by spatial proximity with catalytic K758. In the ‘closed’ conformation, the phosphate-binding loop is anchored over the kinase cleft with F735 side chain packed against the inner part of αC helix and the side chain of E734 from the phosphate-binding loop forming a salt bridge with the side chain of activation loop R912 (which also interacts with side chain of αC helix D771) forming together a tether that precludes ATP binding (Plaza-Menacho et al. 2014a). In the open (ATP competent) conformer the phosphate-binding loop moves away from the activation loop and αC helix showing a solvent accessible F735 side chain. The two different conformers are defined by mutually exclusive side chain rotamers of residues E768 from the αC helix and F735 from the phosphate-binding loop. A detailed analysis at the ATP-occluded conformer revealed an autoinhibitory mechanism (cis-inhibition) to block nucleotide binding and perturb substrate interaction, involving three key structural elements: the phosphate-binding loop, activation loop and αC helix. Functional evaluation of residues implicated in the tether E734A, R912A and I913A (to a lesser extend) showed a significant increase in the kinetics of RET autophosphorylation by oncogenic catalytic domain mutants compared to WT, consistent altogether with elevated enzymatic activity for both mutants. In these experiments, it was observed that RET M918T displayed some degree of phosphorylation at specific sites even at zero time point; this indicated that this mutant may overcome cis-inhibitory mechanisms of regulation or use a trans mechanism for oncogenic activation.

Furthermore, another crystal structure of oncogenic RET catalytic domain M918T (PDB: 4CKI) showed despite a double-phosphorylated activation loop on Y900 and Y905, paradoxically a ‘closed’ phosphate-binding loop, not competent for ATP binding. A clue to this apparent paradox (i.e. high activity in solution vs a cis-inhibited state in the crystal) comes from the proteomic and biochemical characterization of RET M918T and V804M in solution, that showed late activation loop phospho-sites become phosphorylated much earlier than in the case of RET WT, which in turn affected the balance between enzymatic activity and substrate presentation in the process of autophosphorylation. These changes in the kinetics of RET autophosphorylation by oncogenic catalytic domain mutants are linked to changes in substrate and signaling partners specificity (Plaza-Menacho et al. 2007), although the structural and molecular mechanisms for these interactions are yet unknown.

**Oncogenic RET M918T subverts cis-inhibition by promoting intermolecular substrate phosphorylation**

Further biophysical and biochemical characterization based on intrinsic fluorescence (IF), differential scanning fluorimetry (Tm) and calorimetry showed that the M918T mutation in solution influences the accessibility of the activation loop to be presented as a substrate to a second RET kinase molecule in trans. By destabilizing an ‘activation loop-in’ conformer, a surrogate for the closed phosphate-binding loop, and hence, the auto-inhibited state, the M918T oncogenic mutation increases the exposure of phospho-acceptor tyrosine sites presented to the catalytic cleft of another RET kinase molecule (Plaza-Menacho et al. 2014a). Crystallographic evidence for such mechanism came from a recent set of crystal structures (PDB 5FM2, 5FM3) of hyper-phosphorylated RET catalytic domain (4P) with a partial portion of the JM-segment and an extended activation loop conformation (Plaza-Menacho et al. 2016). In these structures, phospho-Y928 forms hydrogen bonds with side chains of R873 (HRD motif) and R897 (activation loop) at the top and with flanking elements displayed even faster phosphorylation kinetics compared to WT, consistent altogether with elevated enzymatic activity for both mutants. In these experiments, it was observed that RET M918T displayed some degree of phosphorylation at specific sites even at zero time point; this indicated that this mutant may overcome cis-inhibitory mechanisms of regulation or use a trans mechanism for oncogenic activation.

**Oncogenic RET V804M and M918T catalytic domain mutants enhance autophosphorylation kinetics**

Autophosphorylation experiments with RET catalytic domain V804M and M918T mutants revealed much faster kinetics for both oncogenic variants compared to the wild-type (WT) protein. In particular, we observed that late phospho-sites within the catalytic domain of RET WT (Y900, Y905 and Y981) become phosphorylated much earlier than in the case of RET WT (Plaza-Menacho et al. 2014a). While early phospho-sites Y1062 and Y687 from
H926 from beneath. Its buried position indicates the activation loop must have adopted an accessible and hence extended conformation to fully expose Y928 to undergo phosphorylation (Plaza-Menacho et al. 2016). These data are consistent with an enhanced substrate presentation in trans (i.e., activation loop out conformer) in solution by an oncogenic RET M918T mutant targeting the P+1 substrate-binding pocket (Plaza-Menacho et al. 2016).

To dissect the two functions of RET kinase domain during autophosphorylation (enzyme vs substrate activities), phosphorylation rescue experiments in trans using catalytically impaired RET (K758M) variants were performed. These set of experiments showed that oncogenic mutations in the kinase domain enhance the catalytic properties of RET, but at the same time and unexpectedly also enhance the substrate presentation properties of the receptor. This was the first study demonstrating that oncogenic kinase activity is not only driven by the enzymatic properties of the kinase but also by the substrate presentation properties, in other words, by how good as a substrate of itself a kinase is (Plaza-Menacho et al. 2014a). These findings can have important implication in targeted therapy as an alternative to ATP-competitive and allosteric inhibitors.

**Mapping and functional prediction of MEN2 mutations targeting the RET catalytic domain**

While it seems straighter to rationalize from the crystal structure the effects of RET catalytic domain loss-of-function mutations found in Hirschsprung’s disease, less obvious is however to predict the effect of the gain-of-function mutations found in MEN2. In the case of HSCR, there are mutations of residues within important structural and catalytic motifs, such as R873Q (HRD catalytic loop), F893L and G894S (DFG motif), all these having very likely a detrimental effect, if not abolishing the catalytic activity when mutated (Knowles et al. 2006). Mutations of basic side chains that coordinate with activation loop phospho-Y905, including R897Q and K907E, will perturb the optimal active conformation of the activation segment and lower affinity for phospho-tyrosine (Knowles et al. 2006). Another HSCR mutation E734K at the tip of the conformationally sensitive nucleotide-binding loop may disrupt the tether with activation loop R912. Despite apparently having a gain-of-function effect in solution, as indicated by increased kinetics of RET E734A and R912A autophosphorylation compared to WT (Plaza-Menacho et al. 2014a); these mutants do not have impact on ATP affinity or catalytic activity contrary to oncogenic RET M918T, which enhanced both catalytic and substrate presentation properties of the RET kinase.

In order to provide structural insights into the best functionally characterized RET kinase domain mutations found in MEN2 (Iwashita et al. 1996, 1999) and taken as a template the crystal structure of RET catalytic domain bound to Vandetanib (PDB: 2IVU), we rationalized about the mechanisms behind the more common (and best-characterized) disease phenotype-specific mutations found in MEN2: E768D, L790F, Y791F, S891A, V804M/L (FMTC) and A883F, M918T (MEN2B) (Iwashita et al. 1999, Plaza Menacho et al. 2005, Mise et al. 2006), Fig. 3A.

αC helix E768 is in close proximity to the nucleotide-binding loop, and in fact is one of the two residues together with F735 whose side chain rotamer orientations define the open-closed conformation of the RET glycine-rich loop, Fig. 3B (Plaza-Menacho et al. 2014a). Substitution by a shorter acidic (E768D) chain can influence the dynamics of the nucleotide-binding loop and increased nucleotide (ATP/ADP) turnover rates, having as a consequence a gain-of-function effect (Fig. 3B).

L790 just after the β4 is part of the R-spine and hydrophobic pocket (Plaza-Menacho et al. 2016). Recent work has described protein kinase activation in terms of the linear alignment of two sets of spines, the catalytic (C-) and the regulatory (R-) spines (Kornev et al. 2008). These spines are anchored to the αF-helix and secure adequate position of ATP, substrate and amino acid residues that are important for catalysis. The spines are atypical structural motifs composed of amino acids coming from different parts of the protein kinase sequence. One of the main features of the C-spine is that the adenine moiety of ATP is part of the spine itself. The smaller R-spine comprises four residues originating from the αC helix. In RET, these residues include H872 (HRD motif), F893 (DFG motif), L779 (αC helix) and L790 (a site of RET oncogenic activation through mutation L790F) (Plaza-Menacho et al. 2016). Within the N-lobe of RET, we observe an important W717 that not only contributes to the integrity of a hydrophobic αC helix pocket and JM hinge of allosteric nature (Plaza-Menacho et al. 2016), but also structurally caps the regulatory tetrad in analogy to W342 in BRAF, which is required for activation and BRAF dimerization (Hu et al. 2013). RET L790 is caped from the top by W717 and points to αC helix F776 (i.e. hydrophobic pocket) and K789 and to the catalytically required K758–E775 pair. Mutation by a larger and hydrophobic phenylalanine (L790F) will enhance staking of R-spine and potentially
cause a more stable (DFG\textsuperscript{in}) active conformation of the kinase. Alternatively, or simultaneously, a structural displacement of the αC helix caused by the mutation could also have a detrimental effect by disrupting the catalytically required K758–E775 salt bridge (Fig. 3C).

Y791 is located on the β4, its side chain is solvent exposed and it points toward the hinge region (Fig. 3C). It has also been shown to be an autophosphorylation site by mass spectrometry (Kawamoto et al. 2004). Regardless of its phosphorylation status, substitution by a large phenylalanine with a different side chain orientation can also impact directly favoring the staking/linear architecture of the R-spine in conjunction with L746. At the same time, we cannot exclude a phosphorylation event on this site (whether in cis or in trans) having a detrimental impact of the activity by perturbation of the overall N-lobe architecture, mainly αC helix and hinge elements.

S891 precedes the DFG motif at the beginning of the activation segment and mutation by a small Ala
(S891A) would in theory stabilize the motif by the Mg²⁺-coordinating D892 in a DFG⁹ kinase domain conformation. At the same time, S891 forms hydrogen bonds between its main chain oxygen and nitrogen from side chain of catalytic H873. As a consequence, substitution by alanine (S891A) may interfere with the adequate arrangement of the catalytic loop (HRD motif) potentially having at the same time detrimental effects on activity.

In the hinge region of RET between the N- and C-lobes on the β5, we found the gate-keeper residue V804. This residue can restrict nucleotide access to the active site (Fig. 3D), in fact substitution by a bulkier methionine (V804M) or even a leucine (V804L) is associated with resistance to several type I (DFG⁹) TKIs (Plaza-Menacho et al. 2007), which is evident from the modeling analyses (Plaza-Menacho et al. 2014b). However, less obvious is to anticipate the mechanism by which gain of function may be acquired (Plaza-Menacho et al. 2014a). Substitution by methionine (V804M) or leucine (V804L) can interfere in the stabilization of the linear architecture of the R-spine to adopt an active DFG⁹ kinase domain conformation by e.g. in the case of V804L favoring side chain staking nearby R-spine L790 together with I788. How V804M can enhance intermolecular substrate presentation via release of the activation loop is not clear from the structural analyses (Plaza-Menacho et al. 2014a). One can speculate it may be linked to a compensatory mechanism triggered by a detrimental effect on nucleotide binding, independent of the conformation of the hinge itself as seen in a recent crystal structure, where phosphorylated Y809 did not have any impact of the conformational state of the hinge (Plaza-Menacho et al. 2016).

In the case of A883F, a rare MEN2B-associated RET mutation (Iwashita et al. 1999), replacement of the β9 alanine 883 by a larger side chain hydrophobic residue phenylalanine will have a similar impact as the gatekeeper mutants do. On one hand, it will stabilize R-spine linear assembly of L790 from the back by pointing to side chain I788 and below V804, this probably requiring a different side chain rotamer for K889. On the other hand, A883F could impact on the conformation of the hinge with gain-of-function effect by an unknown mechanism.

M918T is the most and perhaps the best functionally characterized MEN2 mutation, and it correlates with the most aggressive and consistent disease phenotype (i.e. MEN2B). This is relevant as several FMTC-associated mutations e.g. Y791F, V804M/L show a more variable penetrance and onset of disease phenotype among different studies (Berndt et al. 1998, Jindrichova et al. 2004, Lesueur et al. 2005, Toledo et al. 2015). RET M918 is located just after the activation loop in the so-called P+1 loop, which often binds the substrate residue just C-terminal of the phosphorylated side chain (the P+1 residue) (Hanks et al. 1988). Together, the activation loop and P+1 loop constitute the activation segment, which runs from the DFG motif to the APE motif. The crystal structure of the catalytic domain of RET M918T (PDB 2CKI) displayed an activation loop phosphorylated on both Y900 and Y905, compared to the singly phosphorylated AL on Y905 observed in RET WT previously solved catalytic domain structures (PDB ID codes 2IVV, 2IVT and 2IVU). Furthermore, T918 forms a network of hydrogen bonds with a water molecule and main chain carbonyl from I913 and P914. Overall, the crystal structure of oncogenic RET was similar to the WT; paradoxically, we also observed a closed glycine-rich loop conformer defined by a cluster of tethered residues via hydrogen bonds involving E734 (P-loop), R912 (activation loop) and D771 (αc) helix (Plaza-Menacho et al. 2016). This glycine-rich loop conformer is not competent for ATP binding and despite its high activity in solution, the protein appears in a cis-inhibited state in the crystal. This is important to be considered, as in crystallography sometimes the conformation captured in the crystal may not represent the true nature of the protein in solution, or just simply captures one of the multiple steps (i.e. snapshots) of a complex and dynamic process/mechanism. A clue to this apparent paradox comes from a robust proteomic and biochemical characterization of RET M918T in solution (Plaza-Menacho et al. 2014a), that revealed RET late phosphosites (e.g. activation loop) become phosphorylated much earlier than in the case of RET WT. This enhanced kinetics of autophosphorylation reflected a perturbation in the balance between enzymatic and substrate presentation activities. In fact, RET M918T in solution impacted on the accessibility of the activation loop to be presented as a substrate to a second RET kinase molecule in trans. By destabilizing an ‘activation loop-in’ conformer, the M918T oncogenic mutation would increase the exposure of phospho-acceptor tyrosine sites (e.g. activation loop tyrosine) presented to the catalytic cleft of another RET kinase molecule (Plaza-Menacho et al. 2014a).

Interestingly, the majority of all these oncogenic MEN2 associated mutations, if not all, map to the core on the RET catalytic domain on well-defined secondary structural elements and never on the unstructured flanking segments. It is plausible that these mutations perturb or corrupt allosteric inputs by adjacent elements e.g. juxtamembrane (Plaza-Menacho et al. 2016) and c-tail segments (work in progress). This has important
connotations for drug discovery and design, as the mechanistic information about such allostERIC inputs can be exploited therapeutically.

**Conclusion**

Recent studies indicate that RET belongs to the group of RTKs whose catalytic activity is not regulated by activation loop phosphorylation. Autophosphorylation in trans appears in the case of RET to be more complex than a simple recognition of primary sequence motifs containing an accessible phospho-site. Remarkably, oncogenic RET mutations promote autophosphorylation by increasing catalytic activity and generating at the same time a better intermolecular substrate (i.e. non-catalytic function) caused by a greater flexibility, lower stability and higher ATP affinity. It is evident that regions flanking the catalytic domain also play important roles in the allosteric control of RET catalytic activity, e.g. JM-segment (Plaza-Menacho et al. 2016), and crucially, these mechanisms can be therapeutically exploitable. Late activation loop phospho-sites that are catalytically redundant indicate a role in docking and/or effector recruitment rather than playing a role in catalysis (Plaza-Menacho et al. 2014a). This is evidenced by a recent crystal structure where structure–function analyses identified interactions between the JM hinge, αC helix and an unconventional activation loop serine phosphorylation site that engages the HRD motif and promotes phospho-tyrosine conformational accessibility and regulatory spine assembly, acting altogether as a multi-phospho-site signaling platform. The study of RET non-catalytic function as well as R- and C-spine arrangements and dynamics will be important to fully understand the molecular bases of MEN2. Uncovering the structural and molecular determinants for RET catalytic domain activation and oncogenic deregulation, together with an emphasis on focused drug-design and drug discovery projects, will be crucial for the development of more potent and specific RET kinase inhibitors. In order to be successful, these RET inhibitors must exploit private mechanisms of activation and regulation that are not shared among other protein kinases, which should lead to better therapeutic strategies clinically successful to treat patients with RET-driven tumors.

**Declaration of interest**

The author declares that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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