McDonald, Neil Q. (2016) RET functions as a dual-specificity kinase that requires allostERIC inputs from juxtamembrane elements. Cell Reports 17 (12), pp. 3319-3332. ISSN 2211-1247.

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RET Functions as a Dual-Specificity Kinase that Requires Allosteric Inputs from Juxtamembrane Elements

Highlights
- The JM segment enhances RET catalytic domain activity
- Structural visualization of activation-loop phospho-S909 engaging the HRD motif
- Phospho-S909 arises from an intrinsic RET dual-specificity kinase activity
- RET αC hydrophobic pocket is a potential drug-targetable allosteric site

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In Brief
Receptor tyrosine kinases exhibit a plethora of activation mechanisms despite highly homologous catalytic domains. Plaza-Menacho et al. find that RET tyrosine kinase activation and signaling require allosteric inputs from juxtamembrane elements as well as dual-specificity activity.

Accession Numbers
5FM3
5FM2
RET Functions as a Dual-Specificity Kinase that Requires Allosteric Inputs from Juxtamembrane Elements

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http://dx.doi.org/10.1016/j.celrep.2016.11.061

SUMMARY
Receptor tyrosine kinases exhibit a variety of activation mechanisms despite highly homologous catalytic domains. Such diversity arises through coupling of extracellular ligand-binding portions with highly variable intracellular sequences flanking the tyrosine kinase domain and specific patterns of autophosphorylation sites. Here, we show that the juxtamembrane (JM) segment enhances RET catalytic domain activity through Y687. This phospho-site is also required by the JM region to rescue an otherwise catalytically deficient RET activation-loop mutant lacking tyrosines. 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. We demonstrate that this phospho-S909 arises from an intrinsic RET dual-specificity kinase activity and show that an equivalent serine is required for RET signaling in Drosophila. Our findings reveal dual-specificity and allosteric components for the mechanism of RET activation and signaling with direct implications for drug discovery.

INTRODUCTION
Vertebrates have close to 60 receptor tyrosine kinases (RTKs) that respond to a diverse set of extracellular polypeptide ligands by stimulating their intrinsic tyrosine kinase function. RTKs play key roles during embryogenesis and cellular homeostasis; they are also crucial at the origin and progression of many types of cancer (Lemmon and Schlessinger, 2010). Recent progress on the structural basis for EGFR, IR, and FGFR activation has emphasized the importance of RTK-specific or “private” mechanisms of activation for their catalytic domains involving flanking regions and asymmetrical and symmetrical arrangements of dimeric and higher-order oligomeric states (Bae and Schlessinger, 2010; Cabail et al., 2015; Jura et al., 2011; Lemmon et al., 2014). The activation mechanism operating in RET in these terms is currently unclear.

In the current RET paradigm for ligand-dependent RET activation, autophosphorylation (autoP) of several tyrosine residues within the cytoplasmic domain is required for cell signaling (Airaksinen et al., 1999; Plaza-Menacho et al., 2006). For other RTKs, such as the IR and FGFR2, ligand-dependent stimulation leads to kinase activation and phosphorylation of specific tyrosine residues, which relieve repressive cis-inhibitory interactions to enhance catalytic activity and to promote binding of phosphotyrosine-binding domain (PTB)- and Src homology 2 (SH2)-domain-containing proteins to transmit downstream signals (Chen et al., 2007; Hubbard, 1997). While the latter role for phosphorylation has been demonstrated for RET, its effect on catalytic activation has been only recently elucidated. In vitro, phosphorylation of the canonical RET activation loop has little effect on catalytic activity (Knowles et al., 2006; Plaza-Menacho et al., 2011). Indeed, RET activation-loop tyrosines Y900 and Y905 should not be considered activating, because they undergo delayed autoP and are not catalytically required (Plaza-Menacho et al., 2011, 2014a). A similar situation is found for the EGFR and non-RTK ACK1 (Lougheed et al., 2004; Zhang et al., 2006). In these cases, allosteric mechanisms have been identified to stimulate receptor activity independent of activation segment phosphorylation.

Cell-based studies have revealed the importance of the juxtamembrane (JM) segment in RET-receptor-mediated signaling, in particular Y687, a known phospho-tyrosine binding site for SHP2.
(Perrinjaquet et al., 2010). In addition, phosphorylation at RET S696 by protein kinase A (PKA) has also been reported. Mutation of S696 affected the ability of RET to activate the small GTPase RAC1 and stimulate formation of cell lamellipodia (Fukuda et al., 2002). Homozygous knockin mice carrying this mutation lacked enteric neurons in the distal colon, resulting from a migration defect of enteric neural crest cells (Asai et al., 2006), indicating a physiological role for a PKA-RET functional crossstalk. However, structural and molecular information about allosteric mechanisms promoted by the JM region on RET kinase activity are lacking. Taking into account that the role of the JM segment of EGFR family members is distinct from that of typical RTKs because it enhances, rather than inhibits, the catalytic activity (Li et al., 2003; Thiel and Carpenter, 2007), the nature of this coupling between the JM segment and catalytic domain for RET has not been properly explored.

In this study, we define flanking elements and phospho-sites required for RET catalytic domain activation and signaling. We show that the JM segment functions to increase RET catalytic domain activity through Y687. Structure-function analyses revealed a crossstalk among the JM hinge, zC helix, and serine phosphorylated activation loop. We demonstrate that the previously unreported S909 phospho-site arises from a dual-specificity RET kinase activity, unique among RTKs. We show that an equivalent serine in Drosophila RET is required for signaling in vivo. Further structural and biochemical examination revealed an RET zC hydrophobic pocket as a potential drug-targetable allosteric site.

RESULTS

The JM Segment Increases RET Tyrosine Kinase Activity

To define the functional impact of the JM segment on RET tyrosine kinase activity, we used purified recombinant RET kinase domain (KD; residues 705–1013) and RET KD with the JM segment (JM-KD; residues 661–1012; see Figure 1A) and performed a series of biochemical experiments. First, we measured the enzymatic parameters of RET JM-KD and RET KD against an exogenous peptide (Figures S1A and S1B). RET JM-KD showed a 5-fold increased catalytic efficiency (kcat/Km constant) toward the substrate, indicating increased RET enzymatic activity promoted by the JM region. To support these results further, we performed in vitro time-course autoP assays using saturating concentrations of ATP (5 mM) and MgCl2 (10 mM) for 0–80 min (Figures 1B, upper panel and S1D). Western blot (WB) analysis demonstrated increased kinetics and total phosphorylation by RET JM-KD, as indicated by levels of phospho-tyrosine 4G10 antibody. The temporal sequence of RET autoP was also evaluated by label-free quantitative mass spectrometry (LFQMS) following a previously described protocol (Plaza-Menacho et al., 2014a). LFQMS analysis identified tyrosine residues—Y687, Y826, Y900, and Y905—which upon RET catalytic activation were efficiently phosphorylated in a time-dependent fashion (Figure 1B, lower panel). Signal log2 ratios of phosphorylated peptides standardized to their non-phosphorylated counterparts were plotted relative to a zero time point (Figures 1C and 1D).

As indicated by the kinetics of saturation, JM segment Y687 undergoes faster autoP than activation-loop Y900 and Y905. Furthermore, enhanced phosphorylation kinetics for Y900 and Y905 by RET JM-KD were observed compared with RET KD. In particular, a significant difference was observed in the kinetics of the double-phosphorylated activation-loop peptide. Examination of the total cumulative phosphorylation for each site demonstrated that fully phosphorylated RET JM-KD was achieved between 20 and 40 min compared with the 80–90 min required for RET KD (Figure 1B). Taken together, these data demonstrated that the JM segment increases RET catalytic activity presumably through an allosteric means. Contrary to the EGFR (Jura et al., 2008), the JM region did not promote the formation of RET dimers in solution at protein concentrations used in the biochemical assays as assessed by dynamic light scattering (DLS; Figure S1C). The JM segment had no appreciable impact on the stability of RET KD as reported by thermal shift experiments (Figure S1C). However, the apparent affinity for ATP measured by isothermal titration calorimetry (ITC) was affected by 2-fold (RET JM-KD Kd = 37.5 ± 3.1 μM, RET KD Kd = 64.3 ± 10 μM; Figure S1C). In line with these results, RET JM-KD also displayed increased enzyme kinetic parameters for ATP (Figures 1E and S1C).

Mapping JM Elements Required for RET Catalytic Activation

To map key residues within the RET JM region, we generated a series of deletions and performed biochemical analyses. First, enzymatic assays were performed using an ABL-derived peptide, used previously as a good surrogate substrate for RET. Comparison of the catalytic efficiency (kcat/Km) among the different RET JM-KD deletions demonstrated that full-length JM segment starting at residue 661 (JM661) was required to achieve maximal catalytic activity (Figures 2A and 2B). Time-course autoP assays (0–80 min) using RET phospho-specific antibodies were performed to validate the enzymatic assays and LFQMS data. More rapid and elevated phosphorylation levels were observed by RET JM661 as indicated by total phosphorylation and phospho-specific antibodies, respectively (Figures 2C and S2C). In this context, the shorter RET JM669 behaved similarly to RET KD, showing slower kinetics. Faster Y905 and Y981 autoP was observed by RET JM661 compared with RET JM678, and even more significantly with RET JM698 or RET KD. These results confirmed an increased RET catalytic activity because of the JM segment and implicate the region between residues 661 and 697. Further truncations targeting the transition toward the RET catalytic core, especially residues 705–712, were evaluated in expression analyses and in autoP assays. While the recombinant RET catalytic domain starting at residue 709 was stable in solution, the construct starting from residue 713 gave rise to an unstable protein (Figure S2A). The RET catalytic domain starting at residue 709 displayed slower kinetics of phosphorylation compared with that beginning at residue 705 (Figure S2B).

Recombinant glutathione S-transferase (GST)-RET KD fusions with different lengths of the JM segment were used to assess the impact of “forced” dimerization on RET catalytic activity in solution. GST–RET fusions displayed faster kinetics and increased levels of phosphorylation than the untagged proteins (Figures S2D and S2D). However, such enhanced kinetics were independent of the length of the JM segment. Although in principle
this artificial system could lead to forced dimerization by the GST modules to dominate and override the effect of JM segment on RET activity, these data indicate that dimerization is not driving the increased activity promoted by the JM region in solution.

**The JM Segment Increases RET Catalytic Activity without Affecting Substrate Presentation**

The JM segment could potentially increase RET autoP by promoting a better substrate. To assess whether the JM segment also influences the substrate presentation properties of RET, we performed phosphorylation rescue experiments in trans using catalytically deficient RET K758M variants as substrates (Plaza-Menacho et al., 2014a). Consistent with earlier experiments, RET JM-KD-containing residues 661–677 were more active against catalytically deficient RET intracellular domain (ICD) K758M (i.e., substrate) than RET KD (Figure S3A). A reciprocal experiment was then performed using an active RET ICD and catalytically deficient RET K758M in either JM-KD or KD context (Figure S3B). No significant differences were observed between the two substrate variants with or without the JM segment, indicating that the JM region makes RET kinase a better enzyme and not a better substrate for autoP.

**JM Segment Y687 Promotes RET Catalytic Activity**

The activating JM segment spanning residues 661–697 contains Y687, a known autoP site. To evaluate the functional role of this phospho-site in RET catalytic activity, we made Y687F mutant variants. AutoP assays of wild-type (WT) or Y687F mutants in a RET JM661 or JM678 context showed a significant detrimental effect for Y687F (Figure S3C). A control experiment was then performed using a RET JM698 context (Figure S3D). No significant differences were observed between the two substrate variants, indicating that the JM region makes RET kinase a better enzyme and not a better substrate for autoP.
mutants using both total phospho-tyrosine and phospho-specific RET Y905 and Y981 antibodies, respectively. In RET JM661, the effect of the Y687F mutant was reduced, suggesting 661–678 could partially compensate for the loss of Y687. Next, a phospho-specific polyclonal antibody was raised against a phospho-Y687 peptide. As expected, the phospho-specific Y687 antibody showed no signal for Y687F mutants nor RET JM698, but increased signal for WT RET JM661 compared with RET JM678 (Figures 3A and S3C). Previous data showed no impact of Y687 on RET ICD activity (Plaza-Menacho et al., 2014a). A dependency on Y687 is seen only in the absence of RET C-terminal (CT) sequences. One explanation would be if the JM and CT segments were in a spatially close proximity and could exhibit a compensatory effect masking a Y687 functional role (see Figure 5 and
Crystallographic Identification of an Unexpected Activation-Loop Phospho-S909

We have determined two similar crystal structures of a construct containing the RET JM region and KD (amino acids 859–1013) at 3.3 and 2.95 Å, respectively (Table 1). Both crystal structures contained the PP1 tyrosine kinase inhibitor in the nucleotide-binding pocket, had an ordered proximal portion of the RET JM segment, and had a hyper-phosphorylated status with four sites phosphorylated (Y809, Y905, S909, and Y928) (Figures 4A and 4B). The structures differ slightly in resolution and in the occupancy of the phospho-S909 site. The enhanced multi-site phosphorylation status was surprising when compared with previously solved crystal structures of mono-phosphorylated RET catalytic domain (see PDB: 2IVT, 2IVU, 2IVV, and 4CKI), but consistent with biochemical data, indicating higher RET JM-KD levels of tyrosine kinase activity compared with RET KD (Figures 1 and 2). Residue Y809 is located within the RET hinge connecting the N-lobe and C-lobe of the RET KD, whereas Y905 and S909 are within the RET activation loop, and Y928 follows the WMAE motif at the end of the activation segment between helices z4 and z5 (Hanks et al., 1988). The presence of these phosphorylation sites impacts mainly on the activation-loop conformation detaching it from the body of the catalytic core without affecting the conformation of the hinge, as described later (Figures 4A, 4B, and S4A). Previously solved phosphorylated RET KD crystal structures (PDB: 2IVT, 2IVU, and 2IVV) showed phospho-Y905 tethers several basic side chains including R770 from the αC helix and residues R687 and K907 from the activation loop. In the crystal structures presented in this study, phospho-S909 displaces phospho-Y905 and adopts an approximate equivalent position by engaging activation segment residues R897 and R912, as well as R873 from the HRD motif instead (Figures 4B, 4C, and S4B). In this situation, Y905 does not engage the side chain of the αC helix R770; as a consequence, phospho-Y905 projects away from the body of the RET kinase to mimic a fully solvent-accessible conformer. The second unexpected phosphorylation site at Y928 is positioned beneath the tethered phospho-S909 and is likely to further disrupt interactions of phospho-Y905 with the activation loop. Phospho-Y928 forms hydrogen bonds with side chains of R673 (HRD motif) and activation loop R897 at the top and with H926 from beneath. Its partially buried position indicates the activation loop must have adopted an accessible conformation to fully expose Y928 to undergo phosphorylation. These data are consistent with a recent study where we showed 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., 2014a).

Phospho-S909 Arises from an Intrinsic RET Dual-Specificity Kinase Activity

Full-length RET and RET ICD are known to be serine phosphorylated in cells and in vitro, respectively (Plaza-Menacho et al., 2014a; Takahashi et al., 1993). S909 is invariant in all RET sequences and is found only within a minority of RTKs in the human kinome (e.g., FGFR4, ROR1, and HER3). We did not detect S909 phosphorylation by mass spectrometry; however, when we used a specific antibody against an RET phospho-S909 epitope (pSQG), weak basal serine-serine activity was observed for

**Table 1. Data Collection and Refinement Statistics**

|                        | RET JM-KDd3 | RET JM-KDd1 |
|------------------------|-------------|-------------|
| Space group            | P 63 2 2    | P 63 2 2    |
| Cell dimensions        |             |             |
| a, b, c, Å             | 98.5, 98.5, 146.3 | 98.4, 98.4, 144.5 |
| α, β, γ                | 90.0°, 90.0°, 120.0° | 90.0°, 90.0°, 120.0° |
| Resolution (outer resolution shell), Å | 40 – 2.95 (3.11 – 2.95) | 50 – 3.30 (3.48 – 3.30) |
| R_work (%)             | 10.9 (79.5) | 0.17 (0.89) |
| R_free (%)             | 4.1 (30.1)  | 0.05 (0.28) |
| Completeness (%)       | 99.9 (100.0) | 100 (100) |
| Redundancy             | 7.9 (8)     | 10.9 (11.3) |
| Resolution (outer resolution shell), Å | 40 – 2.95 (3.37 – 2.95) | 55.0 – 3.30 (3.75 – 3.33) |
| No. of unique reflections | 9,360       | 6,686       |
| R_work (%)             | 19.9 (25.0) | 23.4 (25.7) |
| R_free (%)             | 22.4 (28.9) | 25.3 (29.4) |
| No. of atoms           | 2,151       | 2,105       |
| Wilson B factor        | 76.8        | 79.4        |
| Average isotropic B factors, Å² | 74.4       | 75.9        |
| Rmsds                   |             |             |
| Bonds, Å               | 0.002       | 0.002       |
| Angles, °              | 0.66        | 0.65        |
| Ramachandran plot (favored/allowed/disallowed), % | 94.9/4.7/0.4 | 96.3/3.7/0.0 |

*All of 5% of the data were set aside to compute R_free.*

Discussion. Next, we assessed the effect of single Y900F, Y905F, and Y981F and double Y900/905F mutants on RET JM-KD activity. In contrast with the detrimental effect observed for Y687F mutants, replacement of the other phospho-sites (Y/F) did not disrupt RET autoP (Figures 3B and S3D). Of note, double activation-loop RET JM-KD Y900/905F mutant showed significant lower levels of Y981 phosphorylation despite no effect on total phosphorylation. More importantly, RET JM661-KD Y900/905F showed WT levels of phospho-Y687 and total phosphorylated RET kinase, indicating the JM segment is able to rescue the activity in cis of the catalytically deficient RET KD Y900/905F mutant (Plaza-Menacho et al., 2011, 2014a). These data demonstrate that crosstalk (i.e., rescue) between the JM segment and the activation-loop is required for RET catalytic function. Further evidence of coupling between the JM and activating segments was obtained by testing a triple RET JM-KD Y687F/Y900/905F mutant for tyrosine kinase activity (Figures 3C and S3E). Crucially, RET JM-KD Y687F was not able to rescue the catalytically deficient Y900/905F mutation. Altogether, these data demonstrate that Y687 is required for a proper allosteric input by the JM segment on RET catalytic activity able to overcome and stabilize a Y900/905F-deficient activation-loop mutant.
Figure 4. Crystallographic Identification of an Unexpected Activation-Loop Phospho-S909 Reveals Intrinsic RET Dual-Specificity Activity

(A) Cartoon representation of phosphorylated RET JM-KD structure bound to PP1 inhibitor. Selected residues (including phosphorylated side chains) and secondary structure elements are depicted with discrete colors: JM-segment residues D707 to K716 (magenta), αC helix (purple), hinge residues (yellow), and activation segment residues (green) are shown.

(B) Close-up of the activation-loop conformation and side chains in (A) (green) superposed with RET KD (tint wheat, PDB: 2IVV).

(legend continued on next page)
RET WT (Figure 4D, upper panel). RET S909 phosphorylation was dependent on the catalytic status of the receptor as indicated by lack of signal of a kinase-dead K758M mutant and was highly specific for S909 (i.e., no signal by a S909A mutant). These data suggested that, contrary to a constitutive phosphorylation event in trans on S909 by an unknown serine-threonine kinase as we initially hypothesized, RET could be a dual-specificity kinase that can autophosphorylate on S909. To test this hypothesis, we performed time-course autoP assays with RET ICD WT versus K758M and S909A mutants (Figure 4D, lower panel). As predicted from previous results, RET K758M showed no tyrosine kinase activity compared with RET WT and S909A mutant, which showed similar time-dependent tyrosine autoP (Figure 4D, lower panel). Crucially, when a phospho-specific RET S909 epitope antibody was used, a time-dependent effect was seen only for RET WT, which showed phospho-serine levels saturating at 60–90 min after stimulation. In contrast, no signal was seen in the case of RET K758M or S909A mutants. Taken together, these data demonstrated that phospho-S909 arises from an intrinsic RET dual-specificity kinase activity not previously reported for an RTK. 

**Structure-Function Validation of RET JM-KD Crystal Structure**

To interpret the increased JM-KD kinase activity from the new structure, we considered whether zC R770 side chain, which coordinates phospho-Y905 in the RET KD structure, could instead make contacts with the JM segment, thereby stabilizing a more active conformer independently of phospho-Y905. Assessing the functional impact of an R770A mutant in the context of both RET JM-KD and RET KD, we found the mutant was selectively impairing RET JM-KD activity but had not a measureable detrimental effect on RET KD (Figures 4E and S4F). These data implicate R770 in engaging the JM segment to increase RET catalytic domain activity. Second, we evaluated whether S909 was required for RET tyrosine kinase activity in vitro. Surprisingly, we did not observe any significant effect of the S909A mutant on RET activity in either enzyme kinetics using peptide substrates or in autoP assays (Figures S4C and S4D). Further enzymatic experiments using purified recombinant RET KD with increasing concentrations of activation-loop-derived S909 phospho- and non-phospho-peptides confirmed these results further (Figure S4E). These data indicate that analogously to RET KD tyrosine autoP sites, S909 is not intrinsically required for catalytic activity. We also considered that redundancy of phospho-S909 with phospho-Y905 could mask such a critical role. The latter possibility was further excluded by the lack of any functional effect observed by an RET JM-KD Y905F/S909A double mutant (Figure S5A). An equally plausible explanation is that multi-site phosphorylation of the RET activation segment could play a role in releasing phospho-Y905 or even phospho-S909 acting as a docking or adaptor site required for downstream signaling (see Discussion).

**Structural Identification and Functional Validation of RET zC Hydrophobic Patch**

The JM-KD structure revealed contacts from a short segment of the proximal JM region (residues D707 to W717) with a hydrophobic patch composed of residues from different structural elements including β4 (L790), β5 (L800, L801, L802), and zC helix (L769, L772, L773, F776, L779) (Figures 5A and 5B). This zC hydrophobic patch is present in many tyrosine and serine-threonine kinases and is frequently a site of regulation to assemble a functional regulatory (R) spine (Kannan et al., 2007; Kovacs et al., 2015; Thompson et al., 2009). Intramolecular contacts with this hydrophobic patch arise from interaction with either N- or C-terminal sequences flanking the KD (Jura et al., 2011). We noticed a passing similarity between RET zC hydrophobic patch–JM-segment interaction and the PIF pocket-hydrophobic motif interaction found in AGC kinases first described for the PDK1 serine-threonine kinase (where PIF is defined as the PDK1-interacting fragment) and PKA (Kannan et al., 2007; Biondi et al., 2000). Superposition of the RET JM-KD crystal structure with the PKA catalytic domain (PDB: 1ATP) suggests an equivalence between residues of the PKA hydrophobic motif located at its C-terminus to contact the zC helix with those observed in the RET JM segment that engage the hydrophobic zC patch (Figures 5A and 5B). Our interest in this similarity was stimulated by the development of selective drugs against the PDK1 PIF pocket, suggesting the potential for targeting the same region of RET by chemical inhibitors as an alternative route to RET nucleotide pocket inhibition. 

To biochemically probe the role of this zC hydrophobic patch on RET tyrosine kinase activity in vitro, we engineered individual mutants L769A, L772A, and L773A and double mutants L769/772A and L769/773A, and assessed the effect on autoP and enzyme kinetic assays (Figures 5C and 5D). Out of the three single-point mutants, L772A had a profound detrimental effect compared with WT and L769A, whereas L773A had a marked gain-of-function effect on RET kinase activity. The proximity of L772 and L773 on the zC helix and their opposing effects suggests a subtle conformational alteration of zC would be important for R-spine assembly and hence RET activation. We note that the insulin receptor kinase L1045 (structural equivalent to L773 of RET) directly contacts JM segment Y984 side chain stabilizing an auto-inhibited form (Li et al., 2003). By analogy, L773 could also potentially engage Y687 bound in a similar manner; this would explain why a L773A mutant stimulates RET activity (see Discussion). In contrast, L772A gave rise to a loss-of-function effect. We hypothesize that an RET L772A mutant would not create a constitutively active RET (based on
Figure 5. Structure-Function Analysis of RET αC Hydrophobic Patch

(A) Upper panel shows a cartoon representation of RET JM-KD structure, colored according to Figure 4A, with the superposition of PKA C-terminal residues (PDB: 1ATP). Lower panel shows two views of a surface representation of PKA catalytic subunit together with one of the RET JM-KD structures. The PKA C-terminal segment (pale brown), αC helix (purple), and activation loop (green) are depicted together with selected residues.

(B) Close-up of a superposition of RET αC hydrophobic patch contact residues from the proximal JM-residues (D707 to W717) together with the C-terminal hydrophobic motif (FTDF) from PKA. Selected residues and secondary structural elements are depicted as in (A); some residues have been omitted for clarity. Alignment of RET sequences from different species indicating secondary structural elements and key residues (*) implicated in the αC hydrophobic patch (lower panel).

(C) WB analysis of in vitro time-course phosphorylation assay using RET JM661-KD WT and indicated mutants after addition of ATP (5 mM) and MgCl2 (10 mM) for 0–80 min using the indicated antibodies.

(D) Enzymatic assay performed with recombinant purified (1 μM) RET JM661-KD WT and indicated mutants with increasing concentrations of ATP using the ABL peptide at a fixed concentration (4 mg/ml). The corresponding fold-difference in $k_{cat}/K_m$ values is shown in the lower panel. Data represent mean ± SEM, n = 2.
the BRAF paradigm, see Discussion), but would rather impact on the catalytically required K758-E775-D892 tether and would therefore have a similar impact to the loss-of-function K758M mutant. Double mutants L769/772A and L769/773A were both impaired in their catalytic activity. The contribution of residues from the proximal JM region (i.e., D707, F709, and I711) was also evaluated, revealing lack of functional effect (Figures S6A and S6B). These data suggest that other N-terminal residues from the JM segment not captured in the crystal structure may be relevant for this interaction (e.g., Y687). Alternatively, residues proximal to the transition between JM segment and catalytic domain boundary could also be implicated (see RET W717 Contributes to the Assembly of the JM Hinge and R-Spine and Discussion sections). These data implicate residues L772 and L773 from the xC helix as key determinants in achieving an active conformer and may potentially, by analogy to PDK1, provide an alternative druggable pocket to target within RET.

**RET W717 Contributes to the Assembly of the JM Hinge and R-Spine**

Further structural examination of the xC hydrophobic patch highlighted W717, a highly conserved residue preceding the β-1 strand in many protein kinases includes SRC, BTK, EGFR, and BRAF that separates the JM segment from the core catalytic domain (Figure 1A). In the case of BRAF (W342), this residue is important for capping the R-spine in an active conformation and is preceded by a short sequence that engages the catalytic core (Figure 3). Alternatively, W717 would be required for docking onto and proper alignment of the R-spine in the active conformation, following the BRAF paradigm (Figure 6A). We hypothesize that mutating W717 by alanine and not by phenylalanine will perturb the R-spine side chain stacking and, as a consequence, impact on RET activity. Examination of the crystal structure (Figure 6B) revealed that in RET the R-spine is composed of four hydrophobic residues originating from the xF helix connecting N- and C-lobes; these residues include H872 (from the catalytic HRD motif), F893 (from the DFG motif), L779 (xC helix), and L790 (β-4 strand). W717 caps from the top the R-spine in a linear tetrad compatible with an active DFG in conformation of the kinase (Taylor and Kornev, 2011). It is further preceded by D714 adjacent to the fusion site between L712 and E713, which forms an important salt bridge with xC K780, a specific feature lacking in previously solved RET catalytic domain crystal structures. The combined effect of both W717 docking and the D714-K780 tether locks the hinge between the proximal JM segment and N-terminal residues of the catalytic core. In this scenario it is plausible also to hypothesize that perturbation of the hinge by the W717A mutant, contrary to hydrophobic motif DxLxIx alanine mutants, results in a non-compatible JM-proximal segment alignment with N-terminal residues of the catalytic core and a consequent alteration of the R-spine. How perturbation of the R-spine linear architecture results in catalytic inefficiency is likely an indirect effect on both the catalytic (C)-spine and the catalytically required K758 (β-3 strand)-E775 (xC helix)-D892 (DFG motif) tether, which links both spines and the nucleotide moiety (Figure 6C). From these data we conclude that W717 is an important residue for RET function by playing a role in the assembly of the JM hinge and R-spine.

**Activation-Loop Serine Phosphorylation Is Required for RET Signaling In Vivo**

S909 is a novel autoP site that arises from intrinsic dual-specificity kinase activity exhibited by RET in vitro (Figure 4D). Functional assays of a S909A mutant excluded a direct role of S909 on RET catalytic activity (Figures S4C and S4D). These results are consistent with data for mutants targeting RET KD tyrosine autoP sites (Figure 3B) and suggest phospho-S909 could act as a docking or alternatively a substrate site for effector proteins important for RET signaling. Given the high conservation of S909 in all RET sequences and its consistent occupancy, we assessed whether S909 had a role in RET downstream signaling. We therefore used a Drosophila Ret2B (dRet2B, dRet M955T) fly model to assess whether mutation at residue S946 (equivalent to human RET S909) could influence in vivo the aberrant phenotype promoted by oncogenic dRet2B. We employed a pUAST-attB fly vector system to allow specific site insertion of the transgene into the fly genome (Bischof et al., 2007). Oxoverexpression of dRet2B in the developing eye using the glass multiple reporter (GMR) Gal4-815 promoter (GMR-Gal4-815 > dRet2B) led to extensive mispatterning and positioning of ommatidia resulting in a “rough eye” phenotype in the adult fly compared with the driver-line control. When we generated a transgenic fly expressing a double mutant dRet2B (dRet M955T/S946A) (dRet M955T/S946A), the aberrant rough eye phenotype was completely rescued (Figure 7A). Further ectopic oxoverexpression of dRet2B in the peripodial cells of the developing wing epidermis under the control of the 765 promoter (765>dRetM955T) led to an increase in the number of aberrant veins (Figure 7B, arrows) within the adult wing (Figures 7B and 7C). As anticipated, ectopic expression of the double mutant in the developing wing (pct765>dRet M955T/S946A) resulted in the abolition of the aberrant phenotype. To assess whether a signaling defect was associated with S946A (S909A in humans), we expressed ectopically dRET WT, M955T, and M955T/S946A in S2 insect cells, together with an Actin promoter-driven Gal4 construct, and performed WB analyses. We observed a significant detrimental effect on the double mutant dRet M955T/S946A compared with oncogenic dRet M955T in downstream signaling as indicated by total phospho-tyrosine antibody, and also on dRet phosphorylation, as indicated by RET Y1015 and Y1062 phospho–specific antibodies (Figure 7D). These data indicate that an S909 phosphorylation event plays a crucial role in RET signaling in vivo. These results point
toward further complexity through interplay with RET phospho-
tyrosine sites, or alternatively as a docking and/or phospho-site
for a yet unknown effector that impacts on RET signaling.

**DISCUSSION**

To identify unique features of RET tyrosine kinase activation, we
have applied biochemical, structural, and biophysical analyses,
together with an in vivo model for RET hyper-activation. We
show that the JM segment functions to increase RET tyrosine
KD activity without affecting substrate presentation in trans. Fully
phosphorylated RET JM-KD appears rapidly, between 20 and
40 min, compared with the 80–120 min required for core RET
KD (Figure 1B). This increased activity promoted by the JM
segment, contrary to the EGFR (Jura et al., 2009; Red Brewer
et al., 2009), does not appear to result from stable dimer formation
in solution (Figure S1C) and is independent of “forced” dimeriza-
tion through the presence of a GST tag (Figure 2D). Our results are
consistent with VEGFR2, where in solution the kinetics of autoP is significantly enhanced by the JM region (Solowiej et al., 2009). Comparing these data with the slower overall phosphorylation kinetics of RET ICD (Plaza-Menacho et al., 2014a) suggests the C-terminal (CT) segment could act as a negative regulator of RET catalytic domain activity and restrain by competition JM segment activating input. The RET JM region appears not to play a cis-inhibitory role as observed for KIT and MET (Chan et al., 2003; Hubbard, 2004) but is likely to stabilize an active form of RET in a manner that is dependent on Y687. We argue that autoP is not prevented by a non-phosphorylated conformer of JM segment on Y687, but that timely phosphorylation of JM segment on Y687 leads to a conformation contributing to a more active RET kinase. This is supported by the observation that Y687 is required for the JM segment to rescue a catalytically deficient RET KD lacking both activation-loop tyrosines (Y900/905F). Furthermore, a RET JM-KD Y687E mutant (mimicking a constitutive phospho-Y687) showed a significant decrease on tyrosine kinase activity, which indicated that Y687 is a tightly regulated autoP site. We hypothesize that constitutive phosphorylation on Y687 results in a detrimental effect on activity because of the lack of required contacts between JM segment and RET catalytic core prior and during kinase activation (Figure S5B). Furthermore, the dependency seen by RET JM-KD, but not RET KD, on αC R770 implicates its side chain in engaging the JM segment to increase RET catalytic domain activity. Further evidence for the cis effect of the JM segment in RET activation includes: (1) phosphorylation rescue experiments in trans using as substrate catalytically deficient versions (i.e., K758M) of RET JM-KD and RET KD (Figure S3B) did not show significant differences because of the JM segment, and (2) the presence of the RET JM-segment effectively rescues an otherwise catalytically-deficient RET mutant bearing a double-tyrosine (Y900/905F) substitution in the activation-loop. Note this is contrary to the rescue experiment in trans of catalytically deficient K758M kinase versions, which cannot be rescued in cis by either JM segment (Figure S3) or oncogenic mutations (Plaza-Menacho et al., 2014a).

The trajectory of the proximal part of the RET JM-segment resembles to some extent that seen in the IR JM-KD crystal structure (PDB: 1P14) (Li et al., 2003). In the IR crystal structure, JM-Y984 docks into the αC hydrophobic patch in cis (adopting an equivalent position to RET F776) and forms a network of hydrogen bonds between residues from the αC helix and proximal JM segment. These interactions provide steric restraints preventing αC from assuming a catalytically competent position. A recent study has shown, however, that the JM-IR can also adopt a JM-out conformer contacting the αC of a second receptor molecule and is able to stabilize an active catalytic dimer (Cabail et al., 2015). This role for the JM-segment in trans tethers a quite distinctive symmetric active dimer compared with that observed for the asymmetric EGFR dimer (Jura et al., 2009). This recent JM-IR structure (PDB: 4XLV) shows how the JM-segment pivots about the equivalent residue to RET W717.
Our data pointed also at the critical RET hydrophobic patch from a second molecule. This resembles in trans an extended stretch of the proximal JM region seen in our RET JM-KD crystal structure. Our interpretation is that in the absence of a stable RET JM-KD dimer in solution or in the crystal, the JM-segment collapses onto the αC helix in cis in the crystal lattice. Our data pointed also at the critical RET αC hydrophobic pocket as being sensitive to allosteric input from JM-segment elements, possibly including Y687. This hydrophobic pocket has the potential to be targeted by small molecules, because there are precedents for PDK1 where allosteric inhibitors against an equivalent site are already available (Busschots et al., 2012).

Chromosomal translocations involving the RET exons 12–21 are found in human thyroid and lung cancers (Nikiforov and Nikiforova, 2011; Plaza-Menacho et al., 2014b). These gene rearrangements fuse a variety of unrelated coiled-coil proteins within the same RET intron, thereby removing exons 1–11, including the JM segment. Our data are consistent with a scenario in which removing the JM segment, rather than eliminating an autoinhibitory element, replaces it with a more potent dimerizing motif that stabilizes a RET dimer independently of ligand and transmembrane region. For the IR, its JM segment extends away from the kinase core pivoting about a conserved VPDEWE motif to engage a second kinase molecule via contacts to the αC helix. A network of salt-bridge interactions at the pivot point involves the VPDEWE motif to help stabilize a conformation associated with an activated IR tyrosine kinase. The equivalent sequence for RET, EDPKWE, contains the fusion site (between L712 and E713) of many RET translocations that eliminate RET exons 1–11 (Nikiforov and Nikiforova, 2011). Such fusions add a dimeric coiled-coil region just prior to D714 and W717 that would lock permanently the JM hinge and R-spine into a DFG-in conformer resulting in a hyperactive RET, no longer localized at the plasma membrane. These findings have important drug discovery and therapeutic implications as perturbation of the JM hinge and consequent effect on adequate R-spine assembly could be a new drug-targetable strategy against oncogenic RET.

The JM-KD crystal structure shows two unexpected phosphorylation sites, Y928 and S909, both invariant RET residues. Both are in close proximity and engage basic residues otherwise found in the core RET KD structures engaged by phospho-Y905 (Figures 4A and 4B; Figure S4B). In particular, the unconventional activation-loop S909 phosphate-site engages the HRD motif in what it seems to be a unique active conformation promoting both regulatory-spine assembly and accessibility to phosphotyrosine binding modules. As a consequence, Y905 is displaced, adopting a solvent-accessible conformer competent for a signaling function rather than playing an activating role.

Analyses of RET sequences flanking the invariant protein kinase RD motif establish it as tyrosine kinase (HRDLAARN or HRDLRAAN) rather than a serine-threonine kinase (H/YRDLXXN) (Hanks et al., 1988; Lindberg et al., 1992). There are precedents for dual-specificity kinase activity among the cyclin-dependent kinase (CDK), mitogen-activated protein kinase (MAPK), glycogen synthase kinase (GSK3), CDC-like kinase (CLK) group of protein kinases (CMGC) that include the mitogen-activated protein kinase (MAPK) and DYRK kinase members. The latter examples phosphorylate exclusively serine and threonine side chains in their substrates but are able to autoP on tyrosine (Himpel et al., 2000). More recently, a non-receptor tyrosine kinase Syk has been shown to exhibit dual-specificity kinase activity (Heizmann et al., 2010). In our study, robust biochemical examination revealed that S909 is an autoP site that arises from an intrinsic RET dual-specificity kinase activity. This is consistent with a recent study reporting that RET can phosphorylate AP2 in trans on threonine (Bagheri-Yarmand et al., 2015). This dual-specific activity can generate phospho-S909 in vitro. However, phosphorylation on RET S909 was not catalytically required (Figures S4C–S4E), consistent with other serine-to-alanine mutants targeting the JM segment (Figure S5C). Further efforts are needed to establish whether phospho-S909 can act as a docking for a yet-unknown effector protein involved in RET signaling. Interestingly, when we evaluated an RET S909D mutant, a marked increase in RET JM-KD phospho-tyrosine activity was observed (Figure S5B). These data suggest that a phosphorylation event on S909 in trans can contribute to RET tyrosine kinase activity and signaling in vivo. To explore further this hypothesis, we employed an in vivo model and found that mutating this residue in Drosophila Ret (dRet) has a deleterious effect on the dRet<sup>W955G</sup> (dRet M955T) oncogenic phenotype, a well-established model of transformation. In particular, dRet M955T/S946A efficiently rescued the phenotype of dRet<sup>W955G</sup> using alternative promoters.

Overall, our structure-function analyses and in vivo experiments have revealed complex elements in the mechanism of RET activation and signaling. Allosteric inputs from the JM-segment and activation-loop S909 contribute to kinase function. We show that phospho-S909 is an autoP site arising from an intrinsic dual-specificity RET kinase activity and appears to play key roles in oncogenic signaling. Our study also suggests that targeting the αC hydrophobic pocket together with the JM hinge using small molecules to manipulate RET kinase activity may be a productive approach for either blocking oncogenic forms of RET or stimulating RET activity in Hirschsprung’s disease (HSCR) and neurodegenerative Parkinson’s disease (PD).

**EXPERIMENTAL PROCEDURES**

Expression and Purification of Recombinant Protein

Protein expression was carried out using S9 insect cells following a previously described protocol (Knowles et al., 2006). Codon optimized human RET<sub>9</sub> isoform intracellular domain (iCD residues 661–1072), different lengths versions of the RET JM-KD (661 to 698–1012) and RET KD core (705–1013) WT, and the indicated mutants proteins were purified following a protocol previously described (Plaza-Menacho et al., 2014a).

Mass Spectrometric Label-free Quantitation

Mass spectrometry procedures were performed as previously described (Plaza-Menacho et al., 2014a).

Autophosphorylation Assays, SDS-PAGE, and Western Blotting

Unless otherwise indicated, time-course autoP assays were performed with recombinant purified protein as previously described (Plaza-Menacho et al., 2014a). Western blotting was performed with the indicated antibodies as previously described (Plaza-Menacho et al., 2010, 2011). A specific antibody against RET phospho-S909 epitope (pSGG) was from Cell Signaling. Data represent at least two to six independent experiments (n) using different protein preparations. In addition to the quantitation of WB data shown on Figures 4D and 6A, further quantitation of the indicated WB analyses can also be found in the Supplemental Information.
Enzymatic Kinase Assays
Enzyme kinetic experiments were performed as previously described (Plaza-Menacho et al., 2014a).

ITC
ITC experiments were performed as previously described (Plaza-Menacho et al., 2014a).

Dynamic Light Scattering and ThermoFluor Assays
To determine protein stability, we performed thermal shifts assays as previously described (Plaza-Menacho et al., 2010, 2014a). Molecular weight determination in solution was performed by DLS using different RET protein concentrations.

Crystallization, Diffraction, Data Collection, and Processing
Crystals of the phosphorylated RET JM-catalytic domain (residues 659–1013) were grown at 22°C by vapor diffusion in sitting drops containing crystal 1 (5FM2), 1 μL protein stock solution (6 mg/ml) mixed with 1 μL reservoir solution (1.5 M ammonium sulfate, 0.1 M Bis-TRIS propane [pH 7.0]); the protein stock solution also contained 2.5 mM ATP and 5 mM MgCl₂. Crystal 2 (5FM3) comprised 0.8 μL protein stock solution (6 mg/ml) mixed with 0.8 μL reservoir solution (1.2 ammonium sulfate, 0.1 M tri-sodium citrate [pH 5.43]). The crystals were cryoprotected in 25% glycerol in reservoir solution for several minutes and flash frozen in liquid nitrogen, and X-ray datasets were collected at the I-24 beamline of the Diamond Light Source Synchrotron (Oxford, UK). Data collection and refinement statistics are summarized in Table 1. The dataset was indexed with MOSFLM and scaled with SCALA (Winn et al., 2011). Molecular replacement was carried out using the atomic coordinates of the phosphorylated RET KD (PDB: 2IVT) in PHASER (McCoy et al., 2007). Refinement was carried out by using Phenix (Adams et al., 2010). Model building was carried out in COOT (Emsley et al., 2010). Model validation used PROCHECK (Vagune et al., 1999), and figures were prepared using the graphics program PYMOL (http://www.pymol.org).

**Drosophila Experiments**

pUASTattB-dRetM955T (dRet²β) and double mutant pUASTattB-dRetM955T/M955S946A constructs were generated by site-directed mutagenesis using the following primers: M955T forward 5'-GTGCCCGTCAATGGAGACGGCTCCGGA-3'; M955S946A reverse 5'- TCCGGAGCCGGCTGGCTTCGCGACGAGCGGAC-3'; S946A forward 5'- GCCATTAAAGAGAGCCCGAGATCGTGTGCCC-3', and S946A reverse 5'- GGCGACAGATCTGCGCTTCATTAAATAGGGC-. Transgenic flies were generated using P-element-mediated (P-UAST) transgene expression of the developing eye, the transgenic Drosophila stocks and crosses were maintained at 25°C, unless stated otherwise. For ectopic expression of the various transgenes in the developing Drosophila wing, dRet transgenic flies were crossed with the Gal4-C-765 driver (36523, Bloomington). Adult wings were dissected, mounted, and imaged at 400X magnification using the EVOS cell imaging system. For ectopic expression of the dRet²β and dRet⁵β/S946A in the developing eye, the transgenic flies were crossed with the GMR-Gal4 815 (weak) driver and maintained at 18°C. Eye phenotypes were analyzed by light microscopy of whole mounts.

**Ectopic Expression in S2 Cells**
pUASTattB-dRet WT, M955T, and M955T/S946A constructs (400 ng) were co-transfected together with an Actin-promoter-driven Gal4 plasmid (400 ng) as indicated into S2 cells using Effectene and following manufacturer’s instructions. Data represent three independent experiments.

**Statistical Analyses**
Graphs and statistical analyses were done using Prism GraphPad.

**ACCESSION NUMBERS**
The crystallographic coordinates and structure factors for the RET JM-KD crystal structures reported in this paper are PDB: 5FM3 and 5FM2, respectively (http://www.pdb.org).

**SUPPLEMENTAL INFORMATION**
Supplemental Information includes six figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.11.061.

**AUTHOR CONTRIBUTIONS**
I.P.-M. and N.Q.M. planned the project, designed experiments, analyzed the data, and wrote the paper. I.P.-M. performed all biochemical and crystallographic experiments, assembled the initial draft of the paper, and prepared all figures. K.B. performed the mass spectrometry analyses. A.B. and R.C. assisted directly with baculovirus production. R.B. and M.O. performed Drosophila experiments under supervision of I.P.-M. and P.M. S.M. assisted with data processing and structure determination. R.J.M.-T. performed the ITC experiments.

**ACKNOWLEDGMENTS**
We thank our Lincoln’s Inn Fields Laboratories (Francis Crick Institute) colleagues Sara Kisakye Nambozo, Roger George, and Svend Kjaer (Protein Production Facility) for their technical assistance in the production of baculoviruses; Nicola O’Reilly (Peptide Production Facility) for timely supply of peptides; and Andrew Purkiss-Trew (Structural Biology Laboratory) for helping with data collection and structure determination. We also thank Timlan Schirmer (Biocentre, University of Basel) for helpful comments on the manuscript and Ross Cagan (Jahns College of Medicine, Mount Sinai, NY) for providing initial pUAST-dRet plasmids. We thank Diamond Light Source Synchrotron (Oxford, UK) for allowing X-ray dataset collection at the I-24 beamline. N.Q.M. acknowledges that this work was supported by the Francis Crick Institute, which receives its core funding from Cancer Research UK (FC001115), the UK Medical Research Council (FC001115) and the Wellcome Trust (FC001115); by the NCI/NIH (grant reference SR01CA197178); by the Association for Multiple Endocrine Neoplasia Disorders MTC Research Fund. P.M. acknowledges NHR funding to the NIHR Biomedical Research Centre.

Received: March 22, 2016
Accepted: November 20, 2016
Published: December 20, 2016

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