RET Recognition of GDNF-GFRα1 Ligand by a Composite Binding Site Promotes Membrane-Proximal Self-Association

Graphical Abstract

Highlights

Extracellular architecture of RET ECD is revealed by SAXS

EM structure for a RET ECD-GDNF-GFRα complex reveals a composite ligand-binding site

A GFRα1-binding hotspot contacts the invariant RET CLD2-3 calcium sites

RET CRD couples ligand recognition and receptor homodimerization, exploited by MEN2A

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In Brief

RET is crucial for vertebrate development and causes three different human diseases. The basis for RET recognition of its bipartite GFL-GFRα ligands has been unclear. Goodman et al. describe a flower-shaped structure for a mammalian RET ectodomain-GDNF-GFRα1 complex. The structure reveals a composite binding site in RET that is driven by GFRα1 contacts and able to accommodate multiple GFL ligands. GFL-GFRα binding promotes a homotypic interaction between membrane-proximal regions of RET, a feature hijacked by oncogenic crosslinking mutations found in MEN2A patients.

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RET Recognition of GDNF-GFRα1 Ligand by a Composite Binding Site Promotes Membrane-Proximal Self-Association

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SUMMARY

The RET receptor tyrosine kinase is essential to vertebrate development and implicated in multiple human diseases. RET binds a cell surface bipartite ligand comprising a GDNF family ligand and a GFRα coreceptor, resulting in RET transmembrane signaling. We present a hybrid structural model, derived from electron microscopy (EM) and low-angle X-ray scattering (SAXS) data, of the RET extracellular domain (RETECD), GDNF, and GFRα1 ternary complex, defining the basis for ligand recognition. RETECD envelopes the dimeric ligand complex through a composite binding site comprising four discrete contact sites. The GFRα1-mediated contacts are crucial, particularly close to the invariant RET calcium-binding site, whereas few direct contacts are made by GDNF, explaining how distinct ligand/coreceptor pairs are accommodated. The RETECD cysteine-rich domain (CRD) contacts both ligand components and makes homotypic membrane-proximal interactions occluding three different antibody epitopes. Coupling of these CRD-mediated interactions suggests models for ligand-induced RET activation and ligand-independent oncogenic deregulation.

INTRODUCTION

RET is a single transmembrane-spanning receptor tyrosine kinase (RTK) that plays critical roles in the development of vertebrate central and peripheral (enteric) nervous systems, kidney and Peyer’s patch organogenesis, and spermatogenesis (Ibañez, 2013). RET is directly causal in several human diseases, including Hirschsprung’s disease; congenital anomalies of the kidneys or lower urinary tract; and multiple cancers, including multiple endocrine neoplasia type 2A and 2B (MEN2A and MEN2B) syndromes (Amiel et al., 2008; Mulligan, 2014; Schedl, 2007). RET is the primary signaling receptor for glial-cell-line-derived neurotrophic factor (GDNF) family ligands (also known as GFLs), which are soluble covalent dimeric ligands and members of the cystine-knot superfamily (Airaksinen and Saarma, 2002). However, RET only recognizes GFLs bound to a member of the GDNF receptor alpha (GFRα) family of glycosylphosphatidylinositol (GPI)-linked coreceptors (Treonor et al., 1996; Trupp et al., 1996). There are four human GFLs: GDNF, artemin (ART), neurturin (NTN), and persephin, which combine with four human GFRα coreceptors to form cognate and noncognate pairs. Each pair is capable of binding and stimulating RET autophosphorylation at discrete tyrosine sites. This suggests a remarkable molecular plasticity within RET receptor to accommodate this diverse set of ligands. The RET-GFL-GFRα complex (the RET “ternary” complex) has a 2:2:2 stoichiometry and exhibits positive cooperativity (Schlee et al., 2006).

The ligand-binding RET ectodomain (RETECD) contains four consecutive cadherin-like domains (CLD1–CLD4) followed by a membrane-proximal cysteine-rich domain (CRD) (Figure 1A). RET CLD domains diverge significantly from classical cadherins (calcium-dependent adhesion) in sequence, structure, and arrangement (Anders et al., 2001; Brasch et al., 2012; Kjaer et al., 2010). RETCLD1-2 forms a clamshell arrangement in marked contrast to the linear organization of tandem repeats of cadherin domains (Kjaer et al., 2010). Calcium ions are critical for RET folding consistent with the conservation of classical cadherin calcium-coordinating motifs between CLD2 and CLD3 (Anders et al., 2001; Kjaer and Ibañez, 2003a; van Weering et al., 1998).

Efforts to map the bipartite GDNF-GFRα1-binding site within RETECD have implicated almost the entire RETECD region. Cross-linking studies suggested that the CRD domain makes direct contacts with both the GDNF ligand and GFRα1 molecule (Amoresano et al., 2005). A separate study identified several regions...
within RET\textsuperscript{CLD1} that are important for ligand-coreceptor binding whereas characterization of human/Xenopus RET\textsuperscript{ECD} receptor chimeras implicated human RET\textsuperscript{CLD1-3} in restoration of binding to both mammalian GDNF-GFR\textsubscript{a1} and NTN-GFR\textsubscript{a2} complexes (Kjaer and Ibañez, 2003b). Structure-function analyses on GFL-GFR\textsubscript{a} ligands have implicated residues within domains D2 and D3 of GFR\textsubscript{a} in binding RET (Parkash et al., 2008; Wang et al., 2006). These studies used structures of binary complexes of GFL-GFR\textsubscript{a} that lacked the related domain D1, thought to be dispensable for GDNF and RET binding (Scott and Ibañez, 2001).

Despite the importance of RET in vertebrate development and disease, the molecular basis for RET recognition of its bipartite GFL-GFR\textsubscript{a} ligands is not known. Here, structural models for reconstituted mammalian (mTC) and zebrafish (zTC) RET-GDNF-GFR\textsubscript{a1} ternary complexes are presented and further validated by a Fab-complex reconstruction by zTC mutational assay and by probing monoclonal antibody epitopes in mTC. The flower-shaped structural model identifies how a composite binding site in RET involving multiple CLD domains and the CRD domain can accommodate multiple GFL ligands and drives a homotypic interaction between membrane-proximal regions of RET\textsuperscript{CRD}. Ligand engagement may organize CRD self-association triggering RET signaling, a property that is hijacked by crosslinking oncogenic mutations found in MEN2A patients.

**RESULTS**

**Spatial Organization of the RET Extracellular Domain**

Recombinant zebrafish RET extracellular domain (zRETECD) and cadherin-like domains 1–4 (zRETCLD1-4) were prepared using baculovirus-mediated protein expression in insect cells. By size exclusion chromatography coupled multilateral light scattering (SEC-MALS), zRETECD and zRETCLD1-4 had apparent molecular weights of 83.7 and 67.5 kDa, respectively (Figures S1A and S1B). Partial deglycosylation of zRETECD and zRETCLD1-4 was achieved with endoglycosylase F1, resulting in monodisperse samples with reduced sugar content. To examine whether these proteins were functional in vitro, zRETECD, zRETCLD1-4, and zRETCLD4-CRD proteins were tested for binding to zGDNF-zGFR\textsubscript{a1} (referred to subsequently as zGFR\textsubscript{a1}) using a zTC reconstitution assay (Figure 1B). Only zRETECD bound the ligand complex and only in the presence of calcium. Shorter constructs showed no detectable binding, indicating the entire RET\textsuperscript{ECD} is required for ligand recognition similar to human RET\textsuperscript{ECD} (Kjaer and Ibañez, 2003b).

To obtain information on the molecular shape of both zRETECD and zRETCLD1-4 in solution, low-angle X-ray scattering (SAXS) data were collected (Figures 1C, S1C, and S1D). Pair distance distributions exhibited fine features consistent with a multidomain protein sample. Ab initio envelopes were generated for both zRETECD and zRETCLD1-4 using DAMAVER consistent with the pair distance distributions. Both showed an elongated shape and a similar radius of gyration. Zebrafish RET\textsuperscript{ECD} data generated a twisted “horse-shoe”-shaped object whereas the RET\textsuperscript{CLD1-4} displays an “L”
shape with a short and long arm with a wide “head” (Figures 1D and 1E). The addition of the CRD within RET-ECD did not extend the maximum intramolecular vector length but generated a “bump” adjacent to RETCLD4, which was tentatively attributed to part of the CRD.

The zRETCLD1-4 structure was modeled using the human RETCLD1-2 (hRETCLD1-2) structure (Protein Data Bank [PDB] code 2X2U) and cadherin-based models for hRET CLD3/hRETCLD4. The hRETCLD1-2 structure, with its distinctive clamshell shape, was recognizable at the wider end of the RETCLD1-4 envelope (Figure 1D). A model for the RETCLD3 domain was placed relative to RETCLD2 by using a tandem classic cadherin domain template (PDB code: 1LW3) to preserve both the conserved calcium-coordinating ligand geometry (LDRE, DXX, and DEDD motifs; Figure S5C) and the presumed linear arrangement of RETCLD2 and RETCLD3. A RETCLD4 domain model was added to RETCLD1-3 with a bend angle of 100° consistent with the ab initio SAXS envelopes. The final residuals from fitting the theoretical curves derived from this RETCLD1-4 model against the experimental curves gave a $\chi^2$ value of 3.1, indicating a reasonable agreement. The high-quality SAXS data were therefore sufficient to generate preliminary models for RET-ECD and RETCLD1-4, defining all pairwise CLD interdomain angles and a potential location for the CRD. The CLD2-CLD3 angle of 150° is within the range observed for tandem cadherin domains (130°–170°); however, given the lack of calcium ligands between CLD1-CLD2 and CLD3-CLD4, interdomain angles cannot be reliably predicted. The SAXS model indicates the CLD1-CLD2 clamshell resembles that of a calcium-free T-cadherin (Ciatto et al., 2010) whereas CLD3-CLD4 angle of 100° means CLD4 projects away from the CLD2-CLD3 principal axis.

**Reconstitution of mTC and zTC RET-GDNF-GFR α1 Ternary Complexes**

Two recombinant RET ternary complexes were assembled for structural analysis. The mTC consisted of human RETECD (residues 1–635), rat GFR α1 (residues 1–427, removing the GPI-attachment site), and mature human GDNF (residues 77–211; Figure 2A; Supplemental Information). Previous studies showed that hRETECD produced in a glycosylation-deficient Chinese hamster ovary (CHO) Lec8 cell line binds to hGDNF-rat GFR α1 ligand with a Kd of 15 nM (Kjaer et al., 2010). No interaction between RET-ECD and GDNF or GFR α1 coreceptor individually was detectable (data not shown). The mTC was reconstituted in the presence of 1 mM calcium to give a pure and monodisperse sample with a molecular weight of 343 kDa by SEC-MALS (Figures 2B and S1E). The calculated molecular weight of the reconstituted mTC was 353 kDa, which is close to the theoretical value of 343 kDa, indicating a reasonable agreement. The high-quality SAXS data were therefore sufficient to generate preliminary models for RET-ECD and RETCLD1-4, defining all pairwise CLD interdomain angles and a potential location for the CRD.
weight of mTC, using a stoichiometry of two copies of RET-ECF; two copies of GFRα: one GDNF dimer, was 260 kDa, suggesting a substantial portion of the measured mass is contributed by glycosylation.

Separately, a zebrafish ternary complex (zRET-ECF, zGDNF-gzGFRα1a) was also prepared consisting of zRET-ECF (residues 1–626), gzGFRα1a (residues 1–352), and zGDNF (residues 135–235; Figure S1F). These constructs were designed to truncate the carboxy-terminal 120-amino-acid tail of gzGFRα1 (referred to as GFRα1dec) and the unstructured amino-terminal 46 amino acids of mature zGDNF (referred to as zGDNFden). The components formed a ternary complex (defined hereafter as zTCmin) that shows a size-exclusion profile similar to mTC (Figure S1F). However, by SEC-MALS, the zTCmin appeared less homogeneous than mTC, with molecular weights ranging from 200 kDa to 420 kDa.

3D Reconstruction of a Mammalian RET-GDNF-GFRα1 Ternary Complex

The mTC complex was placed on electron microscopy (EM) grids, negatively stained, and analyzed by single-particle methods to obtain a 3D reconstruction. Molecular images of mTC revealed a range of different orientations of the complex and a good level of internal detail (Figure S2A). Reference-free classification gave a range of class averages from which two distinct views were extracted: (1) the “leg” view with two densities branching from a more diffuse base (Figure S2B) and (2) a “figure of eight” view (Figure S2B) with apparent 2-fold rotational symmetry. The multivariate statistical analysis performed on the complete non-rotationally aligned single particles data set shows eigen images with 2-fold symmetry (Figure S2C). A starting 3D model was built from two such classes assumed to correspond to almost orthogonal orientations of the particle on the grid. This initial model was further refined to produce the EM map shown in Figure 2C. The reliability of the map is attested by the good agreement between classes and reprojections (Figure S2D) and the even distribution of Euler angles (Figure S2E). The resulting map has a resolution of 24 Å as estimated by the good agreement between classes and reprojections (Figure S2F). The mTC complex measures 190 Å in its longer dimension and is made up of two elliptic domains (“wings”) when viewed down the 2-fold rotational axis (Figure 2C, left), coalescing into a base domain (Figure 2C, right). When the density map is contoured at a threshold chosen to encompass a molecular weight of 264 kDa (calculated molecular weight of mTC), the map generally has good connections between adjoining domains but lacks a connection between the wings and the base. However, bridging density is visible when the threshold used encloses a volume equivalent to a molecular weight of 430 kDa and connects to the inner lobe of the base (Figure S3A).

Generating an EM Structural Model for mTC

To construct an mTC structural model from the EM map, coordinates of the bipartite GFRα1D2-D3:GDNF dimeric ligand (PDB code: 3FUB) were placed into the 3D reconstruction by aligning the dyad symmetry axis of the GDNF-GFRα1D2-D3 crystal structure with the 2-fold symmetry axis of the EM map. Only one of the two possibilities gave a good fit with the EM map, namely GDNF closest to the base (as opposed to GFRα1D2-D3 toward the base; Figure 2D, right-hand view). This placed the bipartite ligand at the center of the mTC complex (Figure 2D). Next, RET-CLD1-4 could be readily identified on the outside (the wings) of the 3D reconstruction using the knowledge of the SAXS-derived model (Figure S3B). Fitting the RET-CLD1-4 SAXS-derived model into the EM 3D reconstruction closely preserved the interdomain CLD angles observed for RET-CLD1-4. The theoretical SAXS scattering curves derived from the EM model of RET-CLD1-4 fit the observed SAXS curves better than the SAXS-derived RET-CLD1-4 model (Figure S3C). This validated the SAXS model and confirmed the identity of this region of the EM 3D reconstruction. Finally, a homology model of the GFRα1D1 domain (derived from GFRα3D3) was placed into a prominent density adjacent to RET-CLD1-2 and next to GFRα1D2-D3 (Figure 2D). This gave a good fit and match to the estimated volume calculated from the D1 domain sequence. The L-shaped GFRα1D1 domain was oriented to place a conserved N-linked glycosylation site into solvent rather than in an opposite arrangement that would place the glycosylation site in an interface with GFRα1D3. The D1 domain placement is made with lower confidence than the rest of the mTC structural model due to this independent fit and orientation into the EM map (see Supplemental Information for a description). It gives a GFRα1 domain organization with GFRα1D3 flanked by both GFRα1D1 and GFRα1D2. The long, highly conserved D1-D2 linker most likely wraps around one side of GFRα1D2-D3. Overall, a correlation coefficient (CC) of 0.84 between the mTC structural model at 24 Å with the EM map was indicative of the good agreement between model and density (RET-CLD1-4: CC = 0.87, GDNF-GFRα1D2-D3: 0.79, and GFRα1D1, 0.79). The EM map was therefore sufficient to develop a structural model for the mammalian RET ternary complex containing either known or readily modeled mTC domain structures.

Regions in the mTC map density not included in the structural model are shown in a difference density map (yellow surface, Figure 2E; Table S1). This map was calculated by subtracting the density for the structural model low-pass filtered to a resolution of 25 Å from the mTC map density. Two regions are evident: a globular density sandwiched between CLD4 and GFRα1D2-D3; GDNF ligand (seen in Figure 2E, left panel) and a detached “base” region (Figures 2C and 2E, right panel). The first region is bilobal, the larger lobe forms part of a “shared” GFRα1D2-D3 GDNF RET interface as described later. The smaller lobe projects toward the central density of the base region containing the 2-fold axis (Figure 2E). This difference map volume was estimated to correspond to about 12 kDa, leading to the assignment of the RET-CLD4 domain (CRDC); residues 509–600; Table S1) to this density consistent with its location close to the RET-CLD4, as suggested by the SAXS envelope for RET-ECF (Figure 1E). The base region from the EM map has an inner volume around the 2-fold axis flanked by symmetry-related external volumes. Either could potentially contain the N terminus of mature hGDNF residues 1–39; known to be unstructured), the C-terminal tail of GFRα1 (residues 353–427), or the C-tail of hRET-ECF (CRDC; residues 601–635). The inner volume of the base region was tentatively assigned to the C-tail of RET-ECF based on volume estimates (Table S1) and the weaker connecting density observed at lower thresholds (Figure S3A, right panel).
would indicate that the bottom of the base lies adjacent to the plasma membrane. The flanking outer base density (Figure 2E, right panel) was then assigned to the 120-residue C-tail of GFRα1. This interpretation is consistent with the RETCRD C-terminal residues being in close proximity, allowing the RET transmembrane helices (residues 636–660) to homodimerize as previously shown (Kjaer et al., 2006). Relaxing the C2 symmetry applied to mTC revealed some asymmetry in the base region but did not markedly change the main core of the mTC (Figure S3D).

In parallel to the mTC EM reconstruction, a data set was collected for the zTCmin complex containing 7,510 particles using similar conditions to the mTC. The stability of the zTCmin on EM grids was not as good as mTC, so the sample was cross-linked using glutaraldehyde to improve the sample homogeneity (Strauss and Wagenknecht, 2013). zTCmin-refined class averages and their corresponding reprojections closely resembled those obtained for mTC (Figure S3F). A 3D reconstruction for zTCmin calculated using the mTC model as reference contained the same overall architecture for the ligand/coreceptor/RETCLD1-4 with density for the D1 and CRD domains, but importantly, it lacked density for the base region (Figure S3G). This is consistent with the assignment of the C-terminal 120-residue tail of zGFRα1 within the base region, where it contributes to base stability.

Validation of the mTC EM Structural Model

To further validate the mTC structural model, four anti-RETECD monoclonal antibodies were characterized and their human RETECD epitopes mapped by either immune blot or by immobilized peptide arrays (see Figure S4 for epitope mapping), summarized in Figure 3A. The monoclonal antibody (mAb) 1D9 antibody previously described (Salvatore et al., 2002) has a conformation-sensitive structural epitope between RET CLD3 and RETCLD4-CRD. An ELISA assay indicated that the epitope was accessible within both hRETECD and the mTC complex (Figure 3B). In contrast, three other antibodies were found to have linear epitopes within the hRETCRD, which could be recognized in a RETECD context, but not within the mTC complex (discussed later). Therefore, Fab fragments derived from the mAb 1D9 were used to obtain an EM reconstruction of a Fab-labeled mTC complex. This complex was prepared and applied to EM grids in the same manner as the mTC alone. The Fab 1D9-labeled mTC sample gave rise to distinctive reference-free classes (Figure 3C, right panel), which were matched with references consisting of forward projections calculated from the mTC alone map. The resulting reconstruction showed good density consistent with two symmetrically bound 1D9 Fabs that mapped accurately to the density assigned to RETCLD3 and RETCLD4 on the exterior of the wings (Figure 3C). This demonstrated that the wing was
correctly assigned to RET\textsuperscript{ECD} and was correctly oriented. Validation of the mTC model allows a proper description of the arrangement of its component parts and confirms the placement of the plasma membrane at the bottom of the view of mTC in Figure 2E (right-hand panel).

**A Composite Binding Site for Bipartite Ligand within the mTC Structure**

The mTC structural model reveals how GDNF-GFR\(_{x1}\) ligand is captured by RET\textsuperscript{ECD} and drawn into close proximity to the membrane (Figure 4A). It also shows that the major RET\textsuperscript{ECD} contacts are with the GFR\(_{x1}\) subunit and RET\textsuperscript{ECD} makes very limited interactions with GDNF ligand. There are four major heterotypic contact sites (i.e., between bipartite ligand and RET\textsuperscript{ECD}) within the mTC structure, defined hereafter as sites I, II, III, and the fourth as a shared ligand/coreceptor site. The sites are designated from the RET\textsuperscript{ECD} amino terminus to carboxy-terminal residue R635 and are discussed in more detail below. It is notable that sites I, II, and III contact RET\textsuperscript{CLD1-3}, consistent with the extended ligand-binding surface proposed from human/Xenopus chimeric RET experiments (Kjaer and Ibañez, 2003b). A fifth heterotypic site is inferred between GFR\(_{x1}\) C-tail and RET\textsuperscript{CRD} within the mTC base region, together with a homotypic RET\textsuperscript{CRD} interaction that is discussed later. The contact surfaces are separated by substantial cavities within the mTC, and some regions, such as RET\textsuperscript{CLD4}, appear to make no direct contacts to ligand-coreceptor at all.

Both sites I and II involve RET\textsuperscript{CLD1}, the most divergent RET CLD, which contains residues and secondary structural elements previously identified as being unique to higher vertebrates (Kjaer et al., 2010). RET\textsuperscript{CLD1} is pinned by independent contacts from loop1 of GFR\(_{x1}\)D\(_1\) (site I—lower confidence, as discussed earlier) and loop1 from GFR\(_{x1}\)D\(_3\) (site II—high confidence; Figures 4B and S5A). Neither of these loops contains invariant GFR\(_{x1}\) residues, and the GFR\(_{x1}\) family member lacks a domain D1 altogether. The GFR\(_{x1}\)D\(_1\) contact centers on residues separating the CLD1 B\(_1\)F strand and the cis-Pro disulfide-constrained loop. This sequence was disordered within the isolated CLD1-CLD2 structure. GFR\(_{x1}\)D\(_3\), with its loop1 in known to be flexible, as it adopts different conformations in two GDNF-GFR\(_{x1}\) structures (PDB codes: 2V5E and 3FUB). Site III is a high-confidence site from the mTC structural model. It contains conserved residues N-X-X-E/D-E/D motif between loop 3 and x10 helix of GFR\(_{x1}\)D\(_3\) and several regions proximal to the calcium-binding site between RET\textsuperscript{CLD2} and RET\textsuperscript{CLD3} (Figures 4B and S5C). GFR\(_{x1}\)D\(_3\) loop 3 is flanked by two disulfide-linked cysteines and is significantly longer than equivalents in domains D1 and D2. Similarly, contacts within RET\textsuperscript{ECD} lie spatially close to the calcium-binding motifs D-E-D-E and E-N (Figure S5C). A second loop in RET\textsuperscript{CLD2} adjacent to the D-X-D motif also faces toward the GFR\(_{x1}\)D\(_3\) surface. Contacts close to these calcium ligands are even more intriguing because calcium is essential for mTC assembly, an observation previously interpreted to reflect calcium’s structural role in RET\textsuperscript{ECD} stability. The shared site (high confidence) consists of surfaces from both protomers of the GDNF dimer as well as loop1 from GFR\(_{x1}\)D\(_2\) (Figure 4C). These regions face toward the larger difference density lobe interpreted as containing part of RET\textsuperscript{CRD}. The GDNF site involves residues G54–E58 (GLGYE) from x1 helix of one protomer and the edge of the GDNF “fingers” from the second protomer (Figure S5B). Loop1 from GFR\(_{x1}\)D\(_2\) spans residues C178–C189 between helices x2 and x3 and are generally poorly conserved among GFR\(_{x1}\)s.

**Figure 4. Bipartite Ligand Recognition by hRET\textsuperscript{ECD} Shows a Composite Binding Site**

(A) Contact surfaces within the EM structural model for mTC labeled with the EM map superposed (beige). Right panel: schematic of the contact sites within RET.

(B) Left panel: close up of site I and site II. Bold black line roughly delineates the contact surface. Right panel: close up of site III.

(C) Left panel: close up of the shared GDNF-GFR\(_{x1}\) contact surface highlighting the difference volume assigned to the CRD domain (yellow). Right panel: close up of the base region difference volume (yellow) assigned to CRD and GFR\(_{x1}\) C-tail.
Figure 5. Mutational Analysis Identifies a Crucial RET-Binding Hotspot within GFRα1 Adjacent to the RETCLD2-CLD3 Calcium-Binding Region

(A) Location of residues chosen for mutation within the zGDNF-zGFRα1 complex. N-linked glycosylation sites were added or deleted within each contact site to perturb binding, except for site III, which was tested by a triple-point mutation. Mutants were assessed for their ability to reconstitute a functional zTC in the presence of calcium.

(B) Quantification of zRETECD binding to immobilized GFRα1-zGDNF or mutant complexes. Three to four independent experiments were performed for each mutant, using three to four separate protein preparations. Nonspecific RETECD binding was assessed in the absence of calcium and was <5% of total binding. The N186D zGDNF mutant was misfolded but is included to show no detectable binding to zRETECD occurs in the absence of zGDNF. Error bars were calculated using the SEM from three to four independent experiments.

(C) Close up of the site III contact, highlighting the three residues mutated that are crucial for mTC assembly.

To probe the contribution of the mTC contact regions on RET ternary complex assembly, N-linked glycosylation sites (N-X-S/T) or point mutations were introduced or deleted within zGDNF ligand or zGFRα1 and assessed in a zTCmin reconstitution assay (Table S2). The zTCmin context was more amenable to a structure-function mutation analysis than mTC, given the simplicity of producing mTC components in stable CHO cell lines. Mutations in zGDNF ligand or zGFRα1 coreceptor were designed to sample each of the four contact surfaces through surface loops or structural elements (Figure 5A). They were tested for their ability to bind equivalent levels of zRETECD and reconstitute a zTCmin complex. Residues tested were mostly conserved between zGFRα1-zGDNF and hGFRα1-hGDNF (Figure S5). The mutant zGFRα1-zGDNF proteins exhibited very different effects on zTCmin complex formation (Figure 5B). A triple-alanine mutation in site III of zGFRα1 (N323A/E326A/E327A) adjacent to the CLD2-CLD3 calcium-binding site essentially abolished interaction with zRETECD (Figures 5B and S6). A site II mutation (S276N) introducing a glycosylation site also significantly reduced binding by 60%. A loop2CD mutant (L305S) also had a markedly lowered affinity for zRETECD. Addition of a glycosylation site in loop1D2 of zGFRα1 (R180N), located within the shared ligand/coreceptor site, actually increased affinity to 130%. Mutations that removed existing N-linked glycosylation sites from either zGDNF (N150D) at the shared site or at the site I interface with GFRα1 (N62D) had essentially wild-type binding consistent with previous studies, indicating sugar is not essential for complex assembly (Jørgensen and Ibañez, 2003b). Loss of these N-linked sites could be observed by SDS-PAGE (Figure S6).

Overall, these data indicate that site II and site III mutations impact significantly on zRETECD binding affinity.

CRD Is Buried within mTC and Couples Ligand Recognition with Receptor Self-Association

In the mTC structural model, the CRD domain participates in both a shared ligand/coreceptor contact surface and a homotypic interaction with a second CRD domain apparently stabilized by a flanking C-tail from the GFRα component. To investigate the RETCRD further, three anti-hRETECD antibodies whose linear epitopes were mapped to the CRD were used to probe epitope accessibility within mTC (Figure 3A). Antibody mAb 421R25 was found to recognize a linear epitope within hRETECD that mapped to residues 540–545 (RCLEWRQ) in the amino-terminal part of RETCRD (Figure S4). This epitope mapped to part of the hRETECD packed against CLD4 and the shared site contacting GDNF-GFRα1 (Figure 2E). Using an ELISA-based assay, the mAb 421R25 epitope was found to be inaccessible within the mTC but exposed in hRETECD, indicating that this part of the CRD is buried upon ligand engagement (Figure 3B). A second antibody mAb m123 produced in house recognized a linear epitope containing residues 600–604 (RGIKA) from within the mTC but exposed in hRETECD, indicating that it selectively recognizes a C630–C634 disulfide epitope (Figure 6A). Therefore, evidence from three different
antibody epitope probes consistently indicates that the CRD is fully accessible within hRET<sup>ECD</sup> but is entirely buried within the mTC, up to and including its C-terminal residues prior to the transmembrane region.

To examine the ability of CRD to promote homotypic RET self-association independent of ligand, both hRET<sup>ECD</sup> and hRET<sup>ECD</sup> MEN2A (C634R), the most common RET mutation found in MEN2A patients (thought to generate crosslinked

Figure 6. A Membrane-Proximal CRD Region Mediates Homotypic Interactions Driven by RET Ligand Engagement

(A) Western blot analysis of mAb 93A binding to a hRET<sup>ECD</sup> disulfide epitope (residues 630–635) in the presence or absence of reducing agents. Load from left to right was 0.5 µg, 0.25 µg, 0.125 µg, 0.0625 µg, and 0.031 µg hRET<sup>ECD</sup>. DTT, dithiothreitol.

(B) SDS-PAGE gel of a pull-down of zRET<sup>ECD</sup> or zRET<sup>ECD</sup>-D using immobilized zGDNF-GFR<sub>a1</sub>, showing enhanced binding in the absence of residues 591–627. Gel quantification indicates 135% ± 10% relative to normalized binding by zRET<sup>ECD</sup>. Three independent experiments were performed.

(C) Selected RET<sup>CRD</sup> sequences close to the transmembrane region, highlighting the cysteine residues targeted for oncogenic mutation in MEN2A/FMTC and the location of antibody epitopes for mAb 93A and m123.

(D) A schematic model for bipartite ligand (green, labeled L) interaction with RET, promoting homotypic dimerization of the RET CRD domain and activation. The GFR<sub>a1</sub> tail contacts with CRD are omitted for clarity.

antibody epitope probes consistently indicates that the CRD is fully accessible within hRET<sup>ECD</sup> but is entirely buried within the mTC, up to and including its C-terminal residues prior to the transmembrane region.
disulfide-linked dimers), were examined for evidence of dimerization in solution. However, neither hRETECD nor hRETECD MEN2A (C634R) spontaneously formed dimers, even at high concentrations (Figure S1B; data not shown). To test whether the RETCRD C-terminal region influenced ligand interaction, residues 591–627 were deleted from zRETECD (zRETECD-D), and this construct was tested in the in vitro pull-down assay (Figure 6B). Elimination of residues 591–627 significantly enhanced binding to zGDNF-GFRx1 (135% relative to normalized binding to wild-type zRETECD), indicating CRD homotypic interactions reduced the overall binding affinity for zGDNF-GFRx1 complex. This suggests that bipartite ligand binding must overcome resistance to self-associate mediated by the RETCRD C-terminal region (CRD²). Overall, these data provide evidence that ligand recognition serves to crosslink two REVECD molecules driving receptor self-association between CRD domains and most likely the transmembrane helix.

**DISCUSSION**

The RETECD organization described here indicates a substantial interdomain interface not only between domains CLD1 and CLD2 but between CLD4 and CRD. Both interfaces are consistent with observed mutual folding dependencies for each domain pair (i.e., they only fold correctly when expressed together; Kjaer and Ibáñez, 2003a). Interdomain angles for RET deviated significantly from those previously predicted (Anders et al., 2001), except for the CLD2-CLD3 angle, which resembles a classical calcium-binding cadherin arrangement. The SAXS-derived model for REVECD aided an EM-derived structural model of a reconstituted mammalian RET-GFRx1-GDNF complex ("RET ternary complex," abbreviated mTC), revealing the basis for bipartite ligand recognition. The mTC structural model was validated in several ways. These include EM single-particle reconstructions of a Fab-labeled ternary complex and a RET ternary complex from *Danio rerio* (2TC), together with site-specific mutational analysis and an ELISA probing anti-RET antibody epitope accessibility.

The mTC structural model reveals that the GFL ligand is captured beneath the GFRx coreceptor, close to the membrane. RETECD wraps around and shields both the GFL ligand and coreceptor rather than projecting away from the plasma membrane. Limited contacts observed between RET and GFL ligand could accommodate any of the four GFLs when combined with a GFL-dimerized GFRx component. The mTC structural model rationalizes several previous studies indicating all RETECD domains are required for a functional GDNF-GFRx1-binding site. The explanation is a synergy of distributed binding hotspots with mutual domain-folding dependencies. A binding hotspot between CLD2 and CLD3 is necessary (but not sufficient) to engage bipartite ligand, whereas the CRD is also required but is not sufficient for mTC assembly. CLD1 is essential for CLD2 folding, and CLD4 is required for CRD folding. Combining both RETECD domain-folding dependencies with the location of ligand-binding hotspots prevents any single domain being dispensable for ligand interaction. The observed composite ligand-binding site contrasts sharply with other RTK-ligand structures that show a much more continuous, often domain-confined, surface to engage ligands (Lemmon and Schlessinger, 2010). It is more analogous to cytokine-receptor systems where individual cytokines have specialized receptors, a shared/common signaling receptor component, and composite binding sites (Stauber et al., 2006; Wang et al., 2005). Furthermore, these systems also offer precedents for the recognition of divergent ligands, such as the degenerate gp130 receptor that recognizes ciliary neurotrophic factor, leukemia inhibitory factor, and interleukin-6 cytokine ligands (Boulanger et al., 2003). The mTC structure suggests an unusual RTK recruitment mechanism more akin to cytokine-receptor complexes but driven by coreceptor GFRx1 engagement.

The mTC structural model also explains how hRETECD CLD1–3 consists of hGDNF-hGFRx1 binding specificity onto a human/Xenopus RETECD chimera by preserving determinants for sites I, II, and III whereas permitting the self-associating CRD to come from a Xenopus RET origin (Kjaer and Ibáñez, 2003b). Binding determinants for hGDNF-hGFRx1 binding within CLD1 are particularly important (Kjaer and Ibáñez, 2003b). Satisfyingly, all but one of these CLD1 determinants appears to contribute directly to ligand-binding sites I/II/III in the mTC structural model (Figure 3). The exception is the amino-terminal β strand (residues 32–37) that is buried within the CLD1-CLD2 interface (Kjaer et al., 2010). Despite the composite nature of the interaction, a critical and conserved GFRx-binding energy “hotspot” is identified at site III, involving the motif N-X-X-E/D-E/D from domain D3. The GFRx contacts lie adjacent to the hRETECD calcium-binding site between CLD2 and CLD3. This suggests the calcium dependence is not only crucial for RET folding but is also critical for ligand recognition, a feature that has been previously overlooked. Evolutionary pressure to preserve calcium ligands in RET only between CLD2 and CLD3 may reflect a need to retain a functional ligand-binding site. Previous studies predicted a RET-binding site based on an ART-GFRx3 structure but lacked supporting experimental evidence (Wang et al., 2006) or identified many potential RET-binding residues from different regions of the GFRx1 coreceptor (Parkash et al., 2008). These data need revisiting in view of the EM structural model described here. For example, GFRx1 D3 residues R190/R197, R257/R259, and K194/Q198/K202 (human GFRx1 numbering) were all implicated in RET binding but map to an interface with the GFRx1 D1 domain in the EM structural model. These residues constituted a putative heparin-binding site, leading the authors to suggest that heparin could inhibit RET signaling by binding to this surface. Alternatively, perturbing the D1 interface could affect RET engagement. Other residues such as E323/D324 (equivalent to zRET E326/E327, the site III hotspot) were correctly proposed but only now have a proper understanding as to how they contact RET (Parkash et al., 2008; Wang et al., 2006).

Probing three separate RETCRD epitopes with different monoclonal antibodies indicates they are buried within the mTC complex. The different locations of these RETCRD epitopes, two within CRD² (CDELKR and RGKIA), suggested that joint recognition of coreceptor and ligand promotes RETCRD C-tail self-association, assisted in part by the C-tail of GFRx1. The RETCRD C-tail homotypic interaction is the only source of direct dimerization contacts visible between RET dimers in the mTC. Deleting the CRD² increases ligand interaction, suggesting the
membrane-proximal region of RET may negatively influence ternary complex formation. It could act as a failsafe to block inappropriate ligand-independent receptor activation, similar to findings reported for the VEGFR2 receptor tyrosine kinase (Brozzo et al., 2012). EM class averages suggest the RET<sup>CRD</sup> is a relatively flexible region that is discernably separated from the core of the mTC complex. Previous data showed a strong association of the hRET transmembrane region (Kjaer et al., 2006), placing a tight constraint that the last CRD domain residue, R635, that precedes the transmembrane region must be in close proximity to a second RET receptor in the ternary complex. The absence of a base region in zTC<sup>CRM</sup> containing a GFR<sub>x1</sub> C-tail truncation mutant uncovers a detectable but noncritical role for the GFR<sub>x1</sub> C-tail in zTC assembly, warranting further characterization.

Ligand-driven self-association of RET<sup>CRD</sup> within mTC may account for the observed positive cooperativity (>80-fold) for recruiting a second RET molecule into the ternary complex after ligand engagement (Schlee et al., 2006). This is consistent with a stepwise assembly of mTC. RET<sup>CRD</sup> C-tail self-association is also fatally exploited in oncogenic forms of RET in MEN2A patients. Many RET mutations found in MEN2A patients lie in the C-terminal region prior to the membrane (C609Y/W, C611S/W, C618S/R/G/F/Y, C620R/W/F/S/Y, C630F, and C634R/W/F/S/Y; see Figure 6B), the most common being C634R at the end of hRET<sup>CRD</sup> (Waguespack et al., 2011). This mutant readily forms sulfonamide adducts in cells, leading to constitutive RET activation (Santoro et al., 1995). Covalent crosslinking of CRD<sup>C</sup> would potentially bypass a ligand requirement for self-association. However, neither hRET<sup>ECO</sup> nor its MEN2A equivalents form covalent or noncovalent dimers in solution. This can be rationalized by the lack of a cell membrane environment (3D versus a 2D diffusion), as the RET transmembrane region in known to promote self-association (Kjaer and Ibáñez, 2003a; Kjaer et al., 2006). Alternatively, it could also be explained by a ligand-dependent conformational change within RET<sup>CRD</sup> (Figure 6C). Such an allosteric model would require engagement of all four RET contact sites by ligand and coreceptor in order to reorient CRD correctly to promote self-association and activation. Whether such ligand-driven changes can alter the arrangement of RET transmembrane dimers requires investigation but is plausible. Although RET<sup>CLD<sub>1-4</sub></sup> does not appear to grossly alter its conformation on ternary complex formation (comparing the SAXS-derived and EM-derived RET<sup>ECO</sup> model; Figure S3C), the CRD domain conformation appears more labile and could be susceptible to conformational changes. The mTC structural model and knowledge of antibody epitopes buried within the mTC suggests that specific reagents targeting the RET<sup>CRD</sup> may have therapeutic application in a subset of RET-driven cancers.

**EXPERIMENTAL PROCEDURES**

**Protein Production and TC Assembly**

Human RET<sup>ECO</sup> (residues 1–635; hRET<sup>ECO</sup>) was expressed in stably transfected CHO Lec8 cells as a cleavable protein A fusion protein as described in Kjaer et al. (2010). Rat GFR<sub>x1</sub> (residues 19–427, following signal sequence cleavage; rGFR<sub>x1</sub>) was also expressed in CHO cells. Mature human GDNF (residues 77–211; hGDNF) was from Amgen. For mTC assembly, excess hGDNF was added to immobilized GFR<sub>x1</sub> with purified hRET<sup>ECO</sup> added subsequently. The mTC was eluted by tobacco etch virus protease cleavage and was subsequently purified by size-exclusion chromatography. Zebrafish RET<sup>ECO</sup> (residues 1–626) and zRET<sup>CLD<sub>1-4</sub></sup> (residues 1–502) were prepared in insect cells as recombinant baculoviruses with a protein A tag using standard protocols. Zebrafish GFR<sub>x1a</sub> (residues 1–352) and zGDNF (residues 135–235) were prepared in the same manner. For zTC assembly, a similar protocol to mTC was followed with a final size-exclusion chromatography purification step.

**SAXS Data Collection and Processing**

SAXS data were collected on the SWING beamline at synchrotron SOLEIL. Data were processed using both in-house and external SAXS software (see Supplemental Experimental Procedures). Data fitting used GNOM (Svergun, 1992) and ab initio models came from DAMMIF (Franke and Svergun, 2009) and DAMAVER (Volkov and Svergun, 2003).

**Epitope Mapping of mAbs and ELISA to Measure mAb Epitope Accessibility**

The locations of mAb epitopes recognized within hRET<sup>ECO</sup> were determined by either immuno-dot blotting (for 1D9) or by peptide arrays spotted onto cell−/− membranes (m123, 93A, and 421R25). An ELISA assay was employed to measure antibody binding to hRET<sup>ECO</sup>−/− GDNF−/− GFR<sub>x1</sub>, or mTC as previously described (Kjaer and Ibáñez, 2003b).

**Electron Microscopy and Single-Particle Analysis Methods**

Molecular images of the mTC and zTC<sup>CRM</sup> complexes were recorded after negative staining using an FEI Tecnai TF20 electron microscope operating at 200 kV and were used to determine the 3D structures by single-particle analysis procedures. A structural model of the mTC complex was built by fitting atomic coordinates from published crystal structures and the SAXS model obtained in this study. Further details are given in the Supplemental Information.

**ACCESSION NUMBERS**

A negative-stain EM map for the mTC has been deposited in the EMDataBank under accession code EMD-2712 and EMD-2713 for the zTC. The respective coordinates for the EM-based structural model are deposited in the Protein Data Bank under ID code 4ux8.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures, six figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.08.040.

**AUTHOR CONTRIBUTIONS**

K.M.G., S.K., and N.Q.M. designed the study. K.M.G. prepared the zTC complex and carried out all the SAXS and EM experiments on the mTC and zTC complexes. S.K. prepared the mTC complex, characterized the antibody epitopes, and performed the ELISA assays. K.M.G. and F.B. processed and interpreted the EM structural models. P.P.K. purified zGDNF−/− GFR<sub>x1</sub> mutants and performed the biochemical assays. K.M.G., A.N., and E.M.B. generated zGDNF−/− GFR<sub>x1</sub> mutants and expression constructs. A.G.P. assisted with the SAXS data acquisition, processing, and interpretation. R.G. assisted with recombinant baculovirus production. M.S. provided large quantities of 1D9 monoclonal antibody. E.P.M. assisted the mTC single-particle reconstruction and interpretation. K.M.G., S.K., F.B., E.P.M., and N.Q.M. interpreted results and wrote the paper.

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