The RET receptor tyrosine kinase plays a pivotal role in cell survival, proliferation, and differentiation, and its abnormal activation leads to cancers through receptor fusions or point mutations. Mutations that disrupt the disulfide network in the extracellular domain (ECD) of RET drive multiple endocrine neoplasia type 2A (MEN2A), a hereditary syndrome associated with thyroid cancers. However, structural details of how specific mutations affect RET are unclear. Here, we present the first structural insights into the ECD of the RET(C634R) mutant, the most common mutation in MEN2A. Using electron microscopy, we demonstrate that the C634R mutation causes ligand-independent dimerization of the RET ECD, revealing an unusual tail-to-tail conformation that is distinct from the ligand-induced signaling dimer of WT RET. Additionally, we show that the RET-C634R ECD dimer can form complexes with at least two of the canonical RET ligands and that these complexes form very different structures than WT RET ECD upon ligand binding. In conclusion, this structural analysis of cysteine-mutant RET ECD suggests a potential key mechanism of cancer induction in MEN2A, both in the absence and presence of its native ligands, and may offer new targets for therapeutic intervention.

Multiple endocrine neoplasia type 2A (MEN2A) is a hereditary polyglandular cancer syndrome that is typically characterized by childhood development of medullary thyroid carcinoma with a high probability of additionally suffering parathyroid and adrenal cancers. Genetically, MEN2A syndromes are caused by pathogenic germline RET variants, the most common of which contain activating mutations in one of six cysteine codons in the extracellular domain (codons 609, 611, 618, 620, 630, and 634 account for more than 98% of MEN2A cases) (1–3). Among these, the C634F/G/R/S/W/Y mutations are both the most frequently identified and the most aggressive, being designated high risk by the American Thyroid Association (4). Historically, these cancers have been associated with poor prognoses, developing and progressing throughout childhood, and have had limited treatment options. In 2020, the first highly RET-selective kinase inhibitor (Selpercatinib) was approved for the treatment of RET-mutant medullary thyroid carcinoma, showing marked and durable antitumor activity (5, 6). Cysteine-mutant RET now represents a clinically validated drug target, and a comprehensive understanding of the molecular mechanisms of cysteine-mutant oncogenic activation of RET is required to aid in diagnosis and treatment.

The extracellular domain (ECD) of RET comprises four cadherin-like domains (CLDs) and a cysteine-rich domain (CRD) that collectively coordinate ligand recognition and subsequent signal transduction. A variety of ligands have been identified to signal through RET, including the four glial-cell line–derived neurotrophic factor ligands (GFLs), glial-cell line–derived neurotrophic factor (GDNF), neurturin (NTRN), artemin (ARTN), and persephin (PSPN) (7–11), and the structurally related growth and differentiation factor 15 (GDF15) (12–15). These RET ligands exist as soluble homodimers and their binding to RET is mediated through pairing with specific membrane-bound coreceptors, the expression of which is tissue dependent. Under normal physiology, activation occurs sequentially: the dimeric GFLs or GDF15 first recruit two copies of the GDNF receptors (GDNF α GFRα1–4) or GDNF receptor α-like (GFRAL) and this complex is further coordinated by two copies of RET, yielding a 2:2:2 hexameric tripartite complex that exhibits a “butterfly” conformation (16). Different ligand/coreceptor pairs result in distinct signaling outcomes: for example, the activation of the RET/GDNF/GFRα1 signaling pathway is critically involved in the maintenance of dopaminergic neurons (17), while elevated RET/GDF15/GFRAL signaling suppresses energy intake and is associated with the cancer cachexia syndrome (18). Although the ligands all activate intracellular signaling through dimerization of RET, there are substantial structural and conformational differences of the ligand, coreceptor, and RET in their
complexes. Notably, the angles between the two “wings” of the RET complexes are approximately 60°, 105°, 108°, and 125° for the RET/GDF15/GFRα1, RET/NTRN/GFRA2, RET/ARTN/ GFRA3, and RET/GDNF/GFRA1 complexes, respectively (16). These extracellular structural differences impact the relative conformations of the intracellular kinase domain dimerization and result in differential signaling outcomes (19, 20).

The C634R mutation in RETCRD induces RET dimerization via a C630-C630 intermolecular disulfide bond and is one of the most common mutations in MEN2A cases (3, 21). However, a structural understanding of how this dimerization leads to constitutive activation of the kinase domains has so far been lacking. Previous cellular studies have shown contradictory results regarding the response of RETC634R to ligand stimulation, with downstream phosphorylation being either increased or unchanged upon the addition of GDNF (22, 23) or unchanged (24). Therefore, it remains uncertain whether dimeric RETC634R is able to bind to its ligands and coreceptors extracellularly. Furthermore, if RETC634R does associate with its ligand and coreceptors, does the C630-C630 disulfide bond introduce conformational restrictions that prevent binding in the same stoichiometry and “butterfly” conformation as the WT RET (RETWT)?

In this study, we investigate the function of the RETC634R ECD oncogenic mutant and report electron microscopy (EM) models of both the dimeric RETC634R ECD mutant and its complex with GDF15/GFRα1 as well as a cryo-EM model of the WT RET/GDF15/GFRα1 extracellular domain complex. We report the first structural investigation of the unliganded RETC634R ECD dimer and show that the two RETC634R protomers associate with each other through the CRDs with the N-terminal domains pointing outward. We also observe and describe a unique “twin-butterfly” conformation of GDF15/ GFRα1 binding to RETC634R ECD in a mechanism that differs from that of the RETWT complexes and that of RETC634R/ GDNF/GFRA1, forming a dodecameric tripartite complex. The results suggest the existence of divergent oncosignal activation mechanisms of the RETC634R mutant resulting from conformational changes of unliganded and liganded RETC634R extracellular domain complexes. These findings were further supported by molecular dynamics (MD) simulations, which found that the RETC634R ECD dimer can adopt a diverse range of activating conformations driven by aberrant disulfide stapling.

Results

Characterization of the extracellular domain of dimeric RETC634R

The expression of correctly folded and functional human RET requires the use of mammalian expression systems and we previously reported that the addition of a cleavable C-terminal Fc tag facilitates the expression of RETC634R ECD in its dimeric form, mediated by an intermolecular C630-C630 disulfide bond (25). For this study, we further optimized the expression construct (see Experimental procedures for details) and were able to isolate dimeric RETC634R ECD in high homogeneity (Fig. 1, A and B). To investigate the structural configuration of dimeric RETC634R ECD, we used single particle negative stain EM (NS-EM) to generate a three-dimensional model. With a total of 3490 particles, both the single particles and the two-dimensional (2D) class averages (Fig. 1C) showed a clear “S” shape, suggesting a rotational C2 symmetry of the model. Initial model building and further model refinement performed with C2 symmetry agreed well with the 2D classes (Fig. 1, D and E). The three-dimensional (3D) refinement of the model converged at 22 Å resolution. The dimensions of the reconstructed model of the dimeric RETC634R are 60 Å × 190 Å × 80 Å and the shape resembles a nonplanar “S”, comprising two “C”-shaped RETC634R protomers that are connected tail-to-tail at the CRDs via a C630- C630 disulfide linkage. Segmentation of the 3D map (26) resulted in seven components, representing 2 × CLD1/2, 2 × CLD3, 2 × CLD4 and joint density in the center for 2 × CRDs (Fig. 1D). We docked one copy of RETECD (modified from PDB ID: 6Q2J) into each half of the density map, which gave a good overall match of both size and shape and defined the location of the individual domains in the segmented maps (Fig. 1F). There are no regions of joint density other than the CRDs, indicating that none of the CLDs interact across the dimer interface. Because the C-terminal residues E623-R635 of RETECD are not resolved in the previously published structures (i.e., PDB ID: 6Q2J) (16), we could not define the position of the C630-C630 disulfide bond precisely. However, the measured distance between the two P622 residues of each RETC634R protomer is ~18 Å, leaving enough space to accommodate the missing region in the docked structures.

The extracellular domain of RETC634R binds to its ligands through a novel mechanism

Dimeric RETC634R is generally considered to be constitutively activated in the absence of ligand binding. However, previous studies have shown that RETC634R signaling is further upregulated in respond to GDNF stimulation (22, 23), which may play a role in oncogenesis. It is currently unclear whether GDNF binds RETC634R in the same manner as RETWT and whether other GFL/coreceptor pairs share this activation capacity. In order to probe the mechanism of ligand-induced hyperactivation of RETC634R, we compared how RETWT ECD and dimeric RETC634R ECD bind two distinct ligand/coreceptor pairs (GDNF/GFRA1 and GDF15/GFRα1) using Blue Native (BN) PAGE. As expected, we found that RETWT forms a stable 2:2 tripartite complex with GDNF/GFRA1, as indicated by the formation of a major band that migrates more slowly than any of its components (Fig. 2A). Interestingly, we found that dimeric RETC634R also complexes with GDNF/ GFRA1 to produce a major band that migrates similarly, suggesting that it too assembles predominantly as a 2:2:2 tripartite complex. However, RETC634R/GDNF/GFRA1 also forms a band with an even slower migratory shift, which is probably a higher-order complex (red star, lane 4). The RETWT/GDNF/ GFRA1 mixture contains a substantial amount of uncomplexed component proteins unlike the RETC634R/GDNF/GFRA1 complex.
mixture, suggesting that the RET C634R/GDNF/GFRα1 complex, especially 2:2:2, may be more favored. In support of this, we found that both the 2:2:2 and higher-order RET C634R/GDNF/GFRα1 complexes are more thermally stable than the RET WT/GDNF/GFRα1 complex (Fig. 2B, left panel and Fig. S1), indicating that the C630–C630 disulfide bond has a stabilizing effect.

RET WT forms a major complex with GDF15/GFRAL that migrates similarly to RET WT/GDNF/GFRα1, which is consistent in that both complexes are known to share a 2:2:2 tripartite composition. Surprisingly, however, we found that RET C634R/GDF15/GFRAL forms one major higher-order complex that migrates substantially more slowly than the RET WT/GDF15/GFRAL complex but similarly to the weaker band in the RET C634R/GDNF/GFRα1 sample (Fig. 2A). We found that this higher-order complex is moderately thermally stabilized ($T_m = 60.6$ °C) relative to the WT complex ($T_m = 57.8$ °C) (Fig. 2B, right panel). These results suggest that dimeric RET C634R may be conformationally restricted from forming the 2:2:2 complex with GDF15/GFRAL but not with GDNF/GFRα1 or that the higher-order species is more favored in the case of GDF15/GFRAL.

To characterize these higher-order RET C634R complexes further, we investigated the oligomeric state of RET C634R/GDF15/GFRAL using size-exclusion chromatography-coupled multiangle static laser light scattering (SEC-MALS). Analysis of the RET C634R/GDF15/GFRAL complex gave a calculated molecular weight of 805 kDa (Fig. 2C), approximately twice the size of the heterohexameric RET WT/GDF15/GFRAL (395 kDa, Fig. S2). To investigate whether the RET WT- and RET C634R-ligand complexes have the same molar ratio of all three components under reducing conditions, we compared the composition of the individual subunits using nonreducing and reducing SDS-PAGE (Fig. 2D). Under reducing conditions, the RET WT- and RET C634R-complexes have the same molar ratio of RET:GDF15:GFRα1, which is consistent in that both complexes are known to share a 2:2:2 tripartite composition. Surprisingly, however, we found that RET WT forms a major complex with GDF15/GFRAL that migrates similarly to RET WT/GDNF/GFRα1, which is consistent in that both complexes are known to share a 2:2:2 tripartite composition. Surprisingly, however, we found that RET C634R/GDF15/GFRAL forms one major higher-order complex that migrates substantially more slowly than the RET WT/GDF15/GFRAL complex but similarly to the weaker band in the RET C634R/GDNF/GFRα1 sample (Fig. 2A). We found that this higher-order complex is moderately thermally stabilized ($T_m = 60.6$ °C) relative to the WT complex ($T_m = 57.8$ °C) (Fig. 2B, right panel). These results suggest that dimeric RET C634R may be conformationally restricted from forming the 2:2:2 complex with GDF15/GFRAL but not with GDNF/GFRα1 or that the higher-order species is more favored in the case of GDF15/GFRAL.

**Figure 1.** Characterization of the extracellular domain of the RET C634R dimer. A, coomassie-stained SDS-PAGE image showing the dimeric RET C634R mutant under reducing and nonreducing conditions. B, sample homogeneity assessed using SEC-MALS. C, a representative micrograph and 2D class averages of RET C634R. Scale bar for 2D class images represents 10 nm. D, top and front views of the segmented maps of the NS-EM model of RET C634R dimer docked with two RET ECD (yellow and brown, modified from PDB ID: 6Q2J). An enlarged view of the density between the C-termini of RET C634R’s is shown (left side, boxed), showing the distance between the two P622 residues in the docked structures. CLD, cadherin-like domain; CRD, cysteine-rich domain; EM, electron microscopy; ECD, extracellular domain; SEC, size-exclusion chromatography; SEC-MALS, size exclusion chromatography-coupled multiangle static laser light scattering.
weaker than that of RET WT ECD (KD = 3.2 μM) (25) (Fig. S3). We had initially anticipated that the dimeric nature of RETC634R might offer an entropic advantage compared to monomeric RETWT in forming a 2:2:2 hexameric ligand complex and that this would instead result in a stronger binding affinity. We inferred from this loss of affinity that dimeric RETC634R is likely to be geometrically restricted from binding the ligands in the same conformation as two independent copies of monomeric RETWT, supporting the hypothesis that RETC634R forms a complex with GDF15/GFRAL through a novel mechanism.

Structural comparison of the RETWT and RETC634R extracellular domain complexes

In order to compare the character of the RETWT and RETC634R ligand complexes, we used EM to interrogate their structures. Accordingly, we first reconstituted the RETWT/GDF15/GFRAL complex and analyzed it by cryo-EM. We performed 3D model reconstruction with 142,083 particles of RETWT/GDF15/GFRAL using an initial model built from the 2D class averages, and the resulting refined model had an overall resolution of 8 Å (Fig. 3). Comparing our map to a previously published cryo-EM structure of the same complex (PDB ID: 6Q2J) (16), we observed only minor differences, most notably extra density, consistent with the additional glycosylation at certain sites. RETECD is heavily glycosylated, containing 11 glycosylation sites that influence the structure and function of RET (25, 27, 28). In our study, we used HEK293T cells to produce RETECD bearing near-native glycans and, at this resolution, densities of the base glycans at all the glycosylation sites could be observed (Fig. S4). Overall, two of the glycosylation site densities point toward the inside of the complex (N361 and N336) while the rest point outward.

Figure 2. Extracellular domain complex formation of RET/GDNF/GFR α1 and RET/GDF15/GFRAL. A, RETC634R formed a higher order complex with GDNF/GFRα1 and GDF15/GFRAL (marked with red stars) compared with RETWT in BN PAGE. The complexes of RETWT/GDNF/GFRα1, RETC634R/GDNF/GFRα1, and RETWT/GDF15/GFRAL with a molar ratio of 2:2:2 are marked with black stars. The Fc-GDF15 showed two major bands on the gel with the strongest band being the dimeric Fc2-GDF15 and the lower fainter band being the monomeric Fc-GDF15 as a result of Fc dissociation. B, stability measurement of the tripartite complexes of GDNF/GFRα1 (left panel) and GDF15/GFRAL (right panel) with RETC634R and RETWT. Above: an example temperature challenge BN PAGE image of the 2:2:2 RETWT/GDNF/GFRα1 complex (please refer to Fig. S1 for full image). Band intensity of the complexes treated at various temperatures was quantified using ImageJ (43). Normalized band intensity was used to plot the melting curves, fitted by nonlinear regression to a sigmoidal model. C, characterization of RETC634R/GDF15/GFRAL (yellow), RETC634R (blue) using SEC-MALS. The right Y-axis represents the calculated molecular weight (MW), while the left Y-axis is the differential refractive index (dRI). They are plotted against elution volume (X-axis). The calculated average molecular weights of the peaks are plotted (black), showing MWs of 185 kDa for RETC634R dimer and 805 kDa for RETC634R/GDF15/GFRAL. D, coomassie-stained SDS-PAGE image showing purified RETC634R, RETC634R/GDF15/GFRAL, and RETWT/GDF15/GFRAL under reducing and nonreducing conditions. BN, Blue Native; GFRAL, GDNF receptor α-like; SEC-MALS, size exclusion chromatography-coupled multangle static laser light scattering.
class 3 had the widest (94°) and narrowest (102°) angles. Class 2 shared similar angles with class 1 along the x (90°) and y axes (109°). To assess these changes of conformation, the WT complex structure was fitted individually into the three refined cryo-EM maps from 3D classification using rigid-body flexible fitting. The resulting structures were then superimposed along one half of the complex for structural comparison. Overall, RET<sup>ECD</sup> showed the highest degree of conformational change relative to GDF15 and GFRAL (Fig. 4B). Morphing of the structures suggests that the two “wings” of the complex are flexible, showing an “out-in” bend and “front-back” twist movement (Movie S1).

The RET<sup>C634R</sup>/GDF15/GFRAL complex was reconstituted similarly to the RET<sup>WT</sup> complex and purified by gel filtration (Fig. 5A). Analysis of the RET<sup>C634R</sup>/GDF15/GFRAL complex by negative stain EM showed that the particles were monodisperse with a distinct change in morphology with both single particles and the resultant 2D class averages showing an overall “X” shape with apparent two-fold rotational symmetry (Fig. 5B). An initial model built without symmetry (Fig. 5C) agreed well with the overall “X” shape observed in the 2D classes and was roughly twice the size of the RET<sup>WT</sup> complex. Further refinement was performed with no symmetry assigned (Fig. 5D). The dimensions of the reconstructed model of the RET<sup>C634R</sup>/GDF15/GFRAL complex are 190 Å × 130 Å × 140 Å, showing an “X” shape as the front view. Reprojections of the density match well with the 2D class averages, supporting the quality of the reconstructed model (Fig. 5D). In agreement with our hypothesis, two copies of the RET<sup>WT</sup>/GDF15/GFRAL structure could be docked back-to-back into the RET<sup>C634R</sup>/GDF15/GFRAL density map (Fig. 5E), confirming our observations in the biochemical and biophysical assays (Fig. 2). In particular, the top
half of the density provides a much better fit for the complex than the lower half, with extra densities observed in the top half, which could be from Domain 1 (D1) in GFRAL that was not previously resolved. This conformational heterogeneity likely also explains the poorer resolution in the lower half. Given that the RETWT/GDF15/GFRAL complex has 2:2:2 stoichiometry and possesses C2 symmetry, we hypothesized that the RETC634R/GDF15/GFRAL complex, which has 4:4:4 stoichiometry and an "X" shaped 2D projection, would have D2 symmetry. However, assigning C2 or D2 symmetry in further refinements did not improve the resolution of the map (data not shown), likely due to conformational flexibility between the domains. At this resolution, we were able to assign the overall conformation of the complex with moderate confidence but were unable to model the disulfide linked loops fully. Since the two RETWT/GDF15/GFRAL particles in a RETWT dimer are linked by a disulfide bond and favor the formation of a 4:4:4 complex, it is likely that a pair of disulfide-linked RETC634R dimers form two semi-independent 2:2:2 complexes in trans (Fig. 5E). Although the disulfide linkage itself cannot be resolved at this resolution, the distance between the P622 residues in the two RETC634R protomers is ~40 Å, which is longer than that of the unliganded RETC634R dimer but could be spanned by residues E623-R635 that are missing from the model and known to be flexible (Fig. 1F). This suggests that ligand binding drives a conformational change of the C-terminals of the ECDs of dimeric RETC634R, separating them from each other to accommodate the second heterohexamer.

To obtain higher resolution information regarding the structure of the RETC634R/GDF15/GFRAL ECD complex, cryo-EM grids were prepared using the same sample that was used for negative stain EM. The particles in the cryo-EM micrographs exhibit a similar "X" shape to that seen in negative stain EM (Fig. 6A). With a set of 288,927 RETC634R/GDF15/GFRAL particles, 2D class averages were processed and several classes looked similar to those of the RETWT complex (Fig. 6, B and C). Additionally, several 2D class averages were observed that are unique to the RETC634R complex (Fig. 6B, red stars). These unique 2D classes generally contain a region which is better resolved with the rest of the view blurred, suggesting flexible particles within the classes, so that good alignment was achieved only with part of the particles. 3D classification and refinement with C1 point symmetry result in a 3D model similar to that of a RETWT complex with a final resolution of 10 Å, and the structure of RETWT/GDF15/GFRAL (PDB ID: 6Q2J) could be fitted into the density (Fig. 6D). Notably, after docking one copy of the RETWT/GDF15/GFRAL complex into the map, extra density was observed below one of the two
“wings” (Fig. 6D, dotted circle), suggesting the presence of additional structurally flexible components to the complex. However, further processing did not help to improve the resolution of the complex by either masked classification and refinement (29), using the NS-EM model as an initial model or by applying D2 symmetry.

Conformational flexibility of the extracellular domains of RET<sup>C634R</sup> and the RET<sup>WT</sup>/GDF15/GFRAL complex from MD simulations

To further investigate the conformational flexibility of the covalently linked RET<sup>C634R</sup> dimer and the RET<sup>WT</sup>/GDF15/GFRAL complex, we performed atomistic MD simulations. MD simulations of the RET<sup>WT</sup>/GDF15/GFRAL complex showed that the angle between the two wings oscillates in a normal distribution fashion with the peak in the range of 45 to 50° (Fig. 7). Alignment of the simulation trajectory by backbone revealed that the two RET protomers in the tripartite complex exhibit a higher degree of movement than GDF15 and GFRAL (Fig. S7, and Movie S2), in close agreement with our observations in the cryo-EM maps as described earlier (Fig. 4C). MD simulations of the RET<sup>C634R</sup> mutant dimer predicts a high degree of conformational flexibility, with the angle between the two RET<sup>C634R</sup> protomers ranging primarily from 110 to 140° (Fig. 7 and Movie S3) but without unraveling of the secondary structure (Fig. S6). Such observation is not surprising as the terminal region of the RET<sup>ECD</sup> is unstructured and flexible. During the entire simulation runs, the RET<sup>C634R</sup> dimer did not once adopt a conformation similar to that of RET<sup>WT</sup> protomers in the RET<sup>WT</sup>/GDF15/GFRAL complex, suggesting that the RET<sup>WT</sup> conformation is not favored for RET<sup>C634R</sup> in the absence of its ligands. The further implication of this is that activation may well occur by a different mechanism. It is thus consistent with the formation of the 4:4:4 highly flexible RET<sup>C634R</sup>/GDF15/GFRAL complex described above (Figs. 5 and 6). However, binding of certain ligands, for example GDNF/GFRα1, to RET<sup>C634R</sup> can apparently force the receptor into a WT-like signaling complex (Fig. 2A).

Discussion

WT RET signaling is regulated by ligand-induced dimerization of the RET ECD, bringing the two intracellular kinase domains close to each other and thus mediating proximity-induced autophosphorylation and activating downstream signaling. The dynamic control of RET complex association and disassociation is essential for normal cellular functions. In MEN2A, RET mutations such as C634R reorganize the disulfide network of the RET ECD such that an intermolecular disulfide bond occurs, enforcing persistent and ligand-independent dimerization of RET. Despite its clinical importance, little is known about the molecular basis for the oncogenic activation of the RET C634R mutant due to a lack of structural studies. In this work, we structurally characterized the extracellular domain of the RET<sup>C634R</sup> mutant both with and without ligands and showed that the RET<sup>C634R</sup> dimer can further complex with the different canonical RET-activating ligands in divergent modes that are structurally distinct from RET<sup>WT</sup> and have the potential to assemble high-order signaling clusters. These discrete structures define the molecular mechanisms through which the RET<sup>C634R</sup> mutation exerts its oncogenicity. The RET<sup>C634R</sup> ECD dimer adopts an activating conformation in the absence of ligands

In the absence of activating ligands, the extracellular domains of RET<sup>WT</sup> either exist as inactive monomers or
dimerize in an inactive head-to-head conformation (16) (Fig. 8A). Our work and other EM studies have shown that activation of RETWT ECD is mediated by the formation of a 2:2:2 "butterfly" complex with its ligands, driving a tail-to-tail conformational rearrangement that promotes proximal kinase domain activation (Fig. 3B). We reveal that the aberrant disulfide bond of the oncogenic RET C634R ECD mutant enforces a dimeric "S" conformation that presumably results in constitutive activation of RET signaling in the full-length receptor (Figs. 8B, 1, C and E). The angle between the two docked RET C634R ECD protomers is approximately 140°, which is wider than that of the two RETWT protomers in complex with any of its ligands (PDB ID: 6Q2O, 6Q2N, and 6Q2J) but consistent with the modal value in our MD simulations (Fig. 7, A and C). Conversely, the distance measured between the P622 residues (ca. 18 Å) is three times shorter (42–50 Å). This implies that the unliganded RET C634R dimer may result in more efficient autoactivation than for a liganded RETWT complex, although the lack of structural information regarding full length RET precludes a comprehensive understanding of how these distances and angles affect the intracellular domains.

Whereas C630 and C634 are located at the end of the flexible C-terminal loop of RET ECD, the other cysteine residues, such as C609, C611, C618, and C620 whose mutations are also associated with MEN2A and are cancer-inducing, albeit with better prognosis, sit in two strands of an antiparallel β-sheet. In WT RET, C609-C620 and C611-C618 form two intramolecular disulfide bonds. Interestingly, we found that expression of RET C620R ECD using the same approach and conditions as for RET C634R ECD also yielded a disulfide-crosslinked dimer after Fc-tag removal (Fig. S8). However, the extent of dimerization was less than for RET C634R, which correlates with the reduced oncogenic potency of RET C620R (3). We hypothesize that the formation of the C609-C609 intermolecular disulfide bond in RET C620R is less favored due to structural constraints, which may be the same for other cysteine mutation-induced oncogenic dimers of RET and explain why C634 mutations are the most aggressive. Equally, some of these dimerized RET mutants may not pass through the protein production machinery as efficiently, in agreement with previous reports that the C609, C611, C618, and C620 mutations impair RET folding and maturation (30–32).

**Divergent activation of RET C634R is mediated by ligand-dependent complexation**

We show that RETWT ECD readily forms 2:2:2 tripartite complexes with both GDF15/GFRAL and GDNF/GFRα1 (Figs. 2 and 3), in accordance with previous reports (16, 33). We observed multiple conformations of the RETWT/GDF15/GFRAL complex using cryo-EM, revealing that the "wings" Figure 7. MD simulation of the extracellular domain complex of RETWT/GDF15/GFRAL and RET C634R dimer. A, distribution of the angles between the two "wings" of the RETWT/GDF15/GFRAL complex (P161GFRAL-C77GDF15-P161GFRAL, PDB ID: 6Q2J) and the two RET C634R protomers of the RET C634R dimer (V262RET-C630RET-V262RET). Three representative frames (F1, F2, and F3) of the RETWT/GDF15/GFRAL complex (B) and the RET C634R dimer (C) aligned along one protomer. GDF15 dimer is colored in green, GFRAL in light blue, and different RET protomers in yellow and orange. GFRAL, GDNF receptor α-like.
undergo a concerted twisting and bending motion (Fig. 4). Our MD simulations corroborate this finding and predict that the degree of these movements would be even larger at biologically relevant temperatures (Fig. 7). In particular, we found that, within the tripartite complex, the RET-ECD undergoes the greatest degree of movement (Fig. 4C), which is consistent with findings from previous studies of the RET-WT/NTRN/GFRα2 (27) and the zebrafish RET-WT/GDFN/GFRα1 (34) complexes. Such conformational flexibility might be anticipated for a protein capable of binding as diverse range of ligands as RET but may also have limited the resolution of the cryo-EM map reconstructed with the data used in this study.

We found that mutant RET-C634R ECD is capable of forming both the archetypal 2:2:2 tripartite RET-ligand complex but also higher order complexes that have not been previously reported. We have assigned the major higher order complex as a 4:4:4 tripartite complex with a back-to-back twin butterfly configuration. Our studies suggest that, in contrast to RET-WT, the preference for RET-C634R to adopt these distinct complex configurations is ligand dependent. In the published structures of the RET-WT/GDF15/GFRAL (PDB ID: 6Q2J) and RET-WT/GDNF/GFRα1 (PDB ID: 6Q2N) complexes, the distance between the last resolved residues (P622) of each RET-ECD protomer are ~50 Å and ~44 Å, respectively. In the case of RET-C634R/GDNF/GFRα1, we were able to model in the unresolved C-terminal residues of RET-ECD (622–632) as disordered chains containing the mutant C630-C630 disulfide bond. However, this is not at all possible for RET-C634R/GDF15/GFRAL, on account of the increased RET-ECD-protomer separation, explaining the ligand-dependent architecture of the RET-C634R complexes that we observed (Fig. 9). Furthermore, the RET-C634R/GDNF/GFRα1 complex is more thermally stable relative to RET-WT/GDNF/GFRα1 and this is likely explained by the entropic benefit provided by the C630-C630 RET-stapling (Fig. 2B). If these distinctive arrangements of the RET-C634R ECD complexes translate to its full-length form in vivo, they could impact on the extent of its activation in different tissues. We anticipate that the complexes characterized here are specific to the C634R mutation and that the other oncogenic cysteine mutations of RET may impact on the ligand binding in different ways.

Relevance to cancer

Antibody-based studies have identified that GFRAL is expressed in multiple cancer tissues (Human Protein Atlas available from http://www.proteinatlas.org/) (35) and its signaling via RET-C634R may be biologically relevant under disease conditions. The ECD of RET-C634R clearly forms a twin-butterfly complex with GDF15/GFRα1 with 4:4:4 stoichiometry (Figs. 2 and 5), and we speculate that about 30% of the RET-C634R/GDNF/GFRα1 also forms a 4:4:4 complex. The structures seen in this study obviously lack the spatial constraints imposed by the cell membrane. In our low-resolution NS-EM model, the CRDs of the docked dual-hexameric complex sit directly opposite from each other (Fig. 5E). However, given how flexible the RET-C634R cross-linked dimer is (Fig. 7), the hexameric complexes in the membrane-bound full length 4:4:4 complex might be able to bend toward each other to allow more efficient anchoring of the TM domains.

Figure 8. Proposed models of the dimeric RET-C634R and its interaction with its ligands. A, cartoon representation of the activation mechanism of full-length RET-WT under normal physiology; B, two configurations of the reconstituted extracellular domain complexes of RET-C634R/GDF15/GFRAL and RET-C634R/GDF15/GFRAL representing ligand-dependent activation mechanisms of RET-C634R. The two RET monomers are colored in yellow and brown. Instead of forming a “butterfly” conformation seen in the RET-WT complexes, RET-C634R complexes with the coreceptors (red) and GFLs (pink) adopt either a “butterfly” conformation or a novel “twin-butterfly” conformation. C, cartoon representation of our proposed model for full-length RET-C634R activation by extrapolation from our extracellular domain structures. RET protomer 1 and 1’ (yellow and brown) forms one RET-C634R dimer, while RET protomer 2 and 2’ (blue and light blue) forms another. GFRAL, GDNF receptor α-like.
The oncogenic RET C634R mutant complexes

into the membrane (Fig. 8C). Such a collapsed “twin-butterfly” 4:4:4 complex might even lead to the formation of locally clustered higher-order oligomers (16, 36) that have been characterized for related receptors such as of Eph receptors (37) and endothelial growth factor receptor (38). We speculate that the change of conformation from “butterfly” to “twin-butterfly” leads to different signaling outcomes and intensities (39) and thus provides a structural explanation for oncogenesis. However, as we have only observed these configurations in the context of the isolated extracellular domains, it remains to be seen whether such complexes do form in vivo and how they might influence signaling in cancer. Nonetheless, these insights pave the way for further investigations into RET gain-of-function activation under disease conditions and potentially for the discovery of cancer therapies targeting the extracellular domain of RETC634R as well as the other dimerization-inducing oncogenic cysteine mutations.

Experimental procedures

DNA constructs, protein expression, and purification

The gene encoding human RETECD (residues 1–635) bearing the C634R, C866R, and C216S mutations (RET-C634R) with a C-terminal Thrombin protease cleavage site, Fc, and His8-Flag tags (RET-C634R-Fc) was cloned into pcDNA3.1 vector in frame with the cytomegalovirus promoter (Tables S1 and S2). WT RETECD (residues 1–635) was subcloned into pcDNA3.1 vector with a Tobacco Etch Virus protease cleavage site and His6-Flag tags (RETWT). Human GDF15 (residues 195–308) was cloned into the same vector with a modified N-terminal Fc tag, followed by a thrombin cleavage site, and this construct was used for cotransfection with a separate construct for the expression of modified Fc as described previously (25, 40). The construct generation and expression procedures of RETWT, Fc-GDF15, and human GFRAL (residues 19–351) with C-terminal His6-twin Strep tags were the same as previously described (25). Human GDFN (residues 142–275) and human GFRα1 (residues 25–429) were subcloned into pk503.9 vector with N-terminal Flag-His6 tags followed by a thrombin cleavage site. The over-expression of recombinant RET-C634R-Fc, RETWT, and Fc-GDF15 were achieved using transient expression in adherent HEK293T cells (American Type Culture Collection, CRL-3216), while GFRAL, GDNF, and GFRα1 were expressed using the baculovirus-infected insect cell system in *Trichoplusia ni* High Five (Hi5) and *Spodoptera frugiperda* (Sf 9) insect cells (Thermo Fisher Scientific) maintained as suspension cultures. All proteins were secreted into the cell culture medium and the culturing medium containing the secreted proteins was harvested 7- and 5-days post-transfection for RET-C634R-Fc or RETWT and Fc-GDF15, respectively and after 3 days for GFRAL, GDNF, and GFRα1. The reconstruction of the GDNF/GFRα1 complex was achieved either by coexpression and purification or the assembly of the separately purified proteins.

To purify RETC634R, Protein A resin (GenScript) was used to immobilize RETC634R-Fc for 2 hours, followed by a thorough washing step using washing buffer containing 20 mM Hapes pH 7.5, 150 mM NaCl, and 1 mM CaCl2. Biotinylated thrombin (Merck Millipore) was added to the resin and the sample was incubated at 4 °C for 12 h without agitation. After protease cleavage, the flow-through containing the cleaved proteins was collected and incubated with pre-equilibrated Strep-Tactin resin (IBA Lifesciences) to remove any unbound thrombin for 30 min at room temperature (RT). The supernatant was then collected and concentrated using Amicon concentrators (50 kDa). A final polishing step was performed by size-exclusion chromatography (SEC) on a Superdex 200 increase 10/300 Gl column (GE Healthcare) in 20 mM Hapes pH 7.5, 100 mM NaCl, and 1 mM CaCl2. Each elution fraction was subjected to nonreducing SDS PAGE for purity evaluation and the peak fraction was directly used to prepare EM grids. For storage, fractions containing the dimeric RET-C634R were combined and concentrated with Amicon concentrators (50 kDa). The final concentrated samples were stored at −70 °C in SEC buffer containing 7.5% glycerol.

The purification of RETWT, Fc-GDF15, and GFRAL was performed as described before (25). In brief, RETWT was purified using a two-step purification protocol, which included Ni-NTA (Qiagen) and anti-Flag (GenScript) affinity purifications, and the final sample was eluted from anti-Flag resin using poly-Flag peptide (Bimake). The peptide was removed by buffer exchange using micro bio-spin columns (Bio-Rad). To purify Fc-GDF15, medium containing the secreted protein was incubated with Protein A resin and eluted using low-pH buffer
containing 100 mM sodium citrate pH 3.1 and 100 mM NaCl, followed by immediate neutralization to pH 7.5 using 1 M Tris pH 8.8. For storage, purified Fc-GDF15 was buffer-exchanged into Tris-buffered saline containing 7.5% glycerol. Strep-Tactin resin was used to affinity purify GFRAL and the bound protein was eluted using 5 mM d-desthiobiotin. The eluate was further purified by SEC on a HiLoad Superdex 200 column with a final concentration of 750 ml/min at RT and was then concentrated using Amicon concentrators (10 kDa or 5 kDa) and purified on a Superdex 200 10/300 G1 column (GE Healthcare) in 20 mM Hepes pH 7.2, 150 mM NaCl and buffer exchanged into storage buffer containing 20 mM Hepes pH 7.2, 150 mM NaCl, and 7.5% glycerol. To purify coexpressed GDNF/GFRα1 or GDNF, Ni-NTA resin was used to capture His-tagged proteins and the bound protein was eluted with TBS containing 300 mM imidazole. The eluate was incubated with thrombin protease for tag cleavage overnight at RT and was then concentrated using Amicon concentrators (10 kDa or 5 kDa) and purified on a Superdex 200 increase 10/300 G1 column (GE Healthcare) in 20 mM Hepes pH 7.5, 100 mM NaCl. The peak fractions containing the desired proteins were combined, concentrated, and were stored at −70 °C in SEC buffer containing 7.5% glycerol. GFRα1 was purified similarly to that of the GDNF/GFRα1 complex but without protease cleavage.

Bio-layer interferometry technology system

A bio-layer interferometry technology system instrument (FortéBio) was used to measure the binding affinity between RETC634R and Fc-GDF15/GFRAL, and the experimental set up was as previously described (25). Fc-GDF15 (15 μM) was first immobilized on anti-hlgG Fc capture biosensors (FortéBio) and the biosensors bound with Fc-GDF15 were then saturated by binding to GFRAL (3 μM). Various concentrations of RETC634R (0.3, 1.2, 2.8, 7, 14, 28, and 42 μM) were used for the second binding step and the saturation signals at the end of the second binding steps were analyzed as a function of RETC634R concentration on a logarithmic scale. The dissociation constant (Kd) was obtained through fitting a nonlinear regression sigmoidal model using GraphPad Prism 8.

BN PAGE

The RET/Fc-GDF15/GFRAL tripartite complexes were prepared by incubating Fc-GDF15 dimer (2 μM) with GFRAL (4 μM) for 15 min, followed by the addition of either RETWT monomer (4 μM) or RETC634R dimer (2 μM) to a final volume of 10 μl. Similarly, the RET/GDF15/GFRAL complex was prepared by adding 0.01 U thrombin protease to the GDF15/GFRAL complex prior to the addition of RET. The formation of the RET/GDNF/GFRα1 complex was performed by incubating RET and copurified GDNF/GFRα1 at a molar ratio of 1:1 at the same concentrations. The mixtures were kept at RT for 30 min to allow complex formation. BN PAGE loading buffer was added to each sample before electrophoresis, which was performed at 100 V and 4 °C for 3.5 h (25, 41, 42). Gels were destained with 20% methanol and 10% acetic acid.

For the temperature-dependent stability measurement, the RET/GDF15/GFRAL and RET/GDNF/GFRα1 complexes were reconstituted as described above, aliquoted into PCR tubes, and heated for 5 min at 50 °C, 54 °C, 57 °C, 59 °C, 60 °C, 64 °C, 70 °C and 54 °C, 60 °C, 64 °C, 67 °C, 70 °C, 72 °C, 76 °C, 80 °C, 85 °C, respectively. After incubation, the samples were immediately placed on ice for 10 min, centrifuged, and mixed with BN PAGE loading buffer. The electrophoresis was performed as described above. The band intensities of the complexes were measured using ImageJ (43). Normalized intensities were analyzed against the different temperature points, and the melting temperature (Tm) was obtained through fitting a nonlinear regression sigmoidal model using GraphPad Prism 8.

Complex reconstitution and purification for structural studies

To form the RETWT/Fc-GDF15/GFRAL complex, Fc-GDF15 and GFRAL were first incubated at a molar ratio of 1:2 for 15 min at RT. RETWT was then added to the mixture so that the final molar ratio of RETWT, Fc-GDF15 dimer, and GFRAL was 2:1:2. The sample was purified using SEC after 30 min postincubation on a Superose 6 increase 10/300 G1 column in 20 mM Hepes pH 7.5, 100 mM NaCl, and 1 mM CaCl2. For the preparation of the RETC634R/GDF15/GFRAL complex, Fc-GDF15 and GFRAL at a molar ratio of 1:2 were first incubated together with 0.055 U thrombin per 100 μg Fc-GDF15 for 15 min at RT before the addition of the RETC634R dimer. The final molar ratio of RETC634R dimer, GDF15 dimer, and GFRAL was 1:1:2. The protein mixture was kept at RT for an additional 30 min and the complex separated from thrombin using SEC on a Superdex 200 increase 10/300 G1 column in 20 mM Hepes pH 7.5, 100 mM NaCl, and 1 mM CaCl2. For both complexes, the purity of each fraction from SEC was assessed using BN PAGE and the peak fractions were directly used to prepare electron microscopy grids without concentrating. For protein storage or other applications, fractions containing the complexes were pooled and concentrated with Amicon concentrators (50 kDa).

Size exclusion chromatography-coupled multiangle static laser light scattering

SEC-MALS was used to characterize the particle distribution and the oligomeric state of RETC634R dimer, RETC634R/GDF15/GFRAL, and RETWT/GDF15/GFRAL complexes. All measurements were performed with a HPLC system (Shimadzu) on a Superdex 200 10/300 G1 column with a flow rate of 0.25 ml/min at RT in SEC buffer containing 20 mM Hepes pH 7.5, 100 mM NaCl, and 1 mM CaCl2. The MALS system was equipped with MiniDAWN TREOS light scattering and Optilab rEX refractive index detectors (Wyatt Technology Corp) The data was first analyzed using ASTRA 6 software (Wyatt Technology Corp) and was exported and replotted using GraphPad Prism 8.

NS-EM sample preparation

Negative stain grids were glow-discharged using either a Pelco Easiglow unit with 10 mA for 30 s or a Quorum Emitech K100X Glow discharge unit with 30 mA for 45 s. For negative stain EM experiments, 3 μl of protein samples at 0.01 mg/ml
**The oncogenic RET C634R mutant complexes**

For RET complexes or 0.005 mg/ml for RET\textsuperscript{C634R} dimer were first applied to the glow-discharged 300 mesh copper grids with carbon coating for 1 min. The grids were blotted, washed once quickly with a 10 μl drop of 2% (w/v) uranyl acetate (UA) solution, blotted again, and incubated with a second 10 μl drop of UA for 45 s, followed by a final blotting step to remove the stain. The grids were left to air dry for 5 min at RT before imaging or storage.

**NS-EM data processing**

For the RET\textsuperscript{C634R} dimer, 40 micrographs were collected using a Tecnai F20 TEM (FEI, 200 kV) at a nominal magnification of 50,000 magnification resulting in 2.25 Å/pixel. Grids of RET\textsuperscript{C634R}/GDF15/GFRAL were imaged using the same microscope and the same settings with a total of 68 micrographs collected. NS-EM data processing was performed using EMAN2.3 (44). After micrograph evaluation, contrast transfer function (CTF) estimation and structure factor calculation were carried out. A total of 3490 and 11,278 particles were processed routes were performed for the 3D model reconstruction. In one route, 21 images from 2D class averages with 302,004 particles were selected for initial model building and one of the five initial models was used as a reference map for 3D classification. In the other route, a 30 Å map was generated based on the published cryo-EM structure of RET\textsuperscript{WT}/GDF15/GFRAL (PDB ID: 6Q2I) using Chimera (48) which was then 60 Å low-pass filtered and used as a reference map for subsequent 3D classification and refinement with C2 symmetry. The structure of RET\textsuperscript{WT}/GDF15/GFRAL (PDB ID: 6Q2I) was fitted into the final density map using Chimera. Additional glycans (N-acetylgalactosamine) at different glycosylation sites, not present in the published structure (PDB ID: 6Q2I), were added using Coot (49) through manual model building to fit in the densities of the glycans in the refined density map. Analysis of different 3D classes was performed in Chimera.

**Cryo-EM sample preparation**

Cryo-EM grids were glow discharged using the same method as for the NS-EM grids. To prepare the specimens for data collection, 3 μl of protein samples at 0.12 mg/ml for both RET\textsuperscript{WT}/GDF15/GFRAL and RET\textsuperscript{C634R}/GDF15/GFRAL were applied to glow-discharged Quantifoil R1.2/1.3300 mesh gold grids for 5 s before blotting. The grids were prepared using either a Vitrobot Mark IV with 6 s blot time (blot force 6) or a Leica EM GP with 1.5 s blot time at 4 °C with 80% humidity.

**RET\textsuperscript{WT}/Fc-GDF15/GFRAL cryo-EM data processing**

For RET\textsuperscript{WT}/Fc-GDF15/GFRAL, the micrographs were collected on a Titan Krios electron microscope (300 kV) with a BioQuantum967 (K2 summit camera, Gatan) in counting mode at 1.07 Å/pixel at 130,000 nominal magnification. A total of 2852 movies were collected with a defocus range of -1.6 to -3 μm with a dose per fraction of 1.26 e/Å\textsuperscript{2} and a total dose of 62.88 e/Å\textsuperscript{2} (Table S3). Following motion correction and CTF estimation as described earlier using RELION 3, 309,003 particles were autopicked using cryolo, rescaled, and extracted with 2.675 Å pixel size. The bad particles were removed through four rounds of 2D class averaging, and 18 images from 2D classes with 281,224 particles were selected for 3D classification (C1 point symmetry) using the low-pass–filtered map of RET\textsuperscript{WT}/GDF15/GFRAL (PDB ID: 6Q2I) as described earlier as a reference map. One of the three maps from 3D classification was selected as the reference map for auto-refinement (C1 point symmetry).

**MD simulation**

We performed classical atomistic MD simulations on the WT RET/GDF15/GFRAL complex (PDB ID: 6Q2I) and the RET\textsuperscript{C634R} dimer. The cross-linked RET\textsuperscript{C634R} dimer was constructed by docking two copies of RET monomers adapted from the RET/GDF15/GFRAL complex (PDB ID: 6Q2I) in the
reconstructed NS-EM map of the RETC634R dimer. Thirteen additional amino acids (62,63) were added to the C-terminal region of RETEC (101) using Coot, which are absent in the PDB file used, with a disulfide bond between the C630 residues of the two RETC634R protomers. Web-based CHARMM-GUI tools were used to construct the model systems (53–55). Each model system consisted of protein, structurally resolved calcium ions and paucimannose modeled at the known glycosylation sites (56–58) and was solvated in TIP3P water molecules, containing sodium and chloride ions at a concentration of 100 mM. The model system sizes ranged from ~550,000 to ~1,000,000 atoms depending on the simulation box sizes. Due to the irregular shape of the protein, the simulation box was rectangular and protein rotation was prevented by keeping harmonic constraints on individual Cα-atoms of residues: 432, 496, and 506 from RET protomers. The strength of the constraints varied from 2000 kJ mol⁻¹ nm⁻² to 50,000 kJ mol⁻¹ nm⁻² to ensure the constraints do not affect the results. Multiple independent 100 to 400 ns MD simulations of WT (~1.18 μs) and mutant (~1.49 μs) model systems were performed using simulation software GROMACS 2020.2 (59–61) and CHARMM36 (62, 63) forcefield. After minimization and equilibration of the systems, the pressure and temperature were kept stable using Nose-Hoover thermostat (64, 65) and Parrinello-Rahman barostat (66, 67). The timestep of simulations was 2 fs, which was achieved with LINCS algorithm (68), and the long-range electrostatics was handled with Particle-mesh-Ewald (69) as implemented in GROMACS. The simulation trajectories were analyzed using software VMD (70).

Data availability

All representative data are contained within the article.

Supporting information—This article contains supporting information.

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Abbreviations—The abbreviations used are: BN, Blue Native; CLD, cadherin-like domain; CRD, cysteine-rich domain; CTF, contrast transfer function; ECD, extracellular domain; EM, electron microscopy; GDF15, growth and differentiation factor 15; GDNF, glial-cell line–derived neurotrophic factor; GFL, glial-cell line–derived neurotrophic factor ligand; GFRαs, GDNF receptor α; GFRAL, GDNF receptor α-like; MEN2A, multiple endocrine neoplasia type 2A; MD, molecular dynamics; NS-EM, negative stain-electron microscopy; SEC, size-exclusion chromatography; SEC-MALS, size exclusion chromatography–coupled multiangle static laser light scattering; WT, wild type.

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The oncogenic RET C634R mutant complexes
The oncogenic RET C634R mutant complexes

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