Structure and Physiology of the RET Receptor Tyrosine Kinase

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The identification of the *ret* oncogene by Masahide Takahashi and Geoffrey Cooper in 1985 was both serendipitous and paradigmatic (Takahashi et al. 1985). By transfecting total DNA from a human lymphoma into mouse NIH3T3 cells, they obtained one clone, which in secondary transformants yielded more than 100-fold improvement in transformation efficiency. Subsequent investigations revealed that the *ret* oncogene was not present as such in the primary lymphoma, but was derived by DNA rearrangement during transfection from normal human sequences of the *ret* locus. At the time, activation by DNA rearrangement had not been previously described for a transforming gene with the NIH3T3 transfection assay. The discovery of *ret* opened a field of study that has had a profound impact in cancer research, developmental biology, and neuroscience, and that continues to yield surprises and important insights to this day.

AN UNUSUAL RECEPTOR TYROSINE KINASE WITH CADHERIN REPEATS

Isolation of *ret* cDNA clones revealed a carboxy-terminal domain with high homology with members of the tyrosine kinase gene family preceded by a hydrophobic sequence characteristic of a transmembrane domain, suggesting that the *ret* oncogene encoded a cell-surface receptor (Takahashi and Cooper 1987). The characterization of the human (Takahashi et al. 1988) and mouse (Iwamoto et al. 1993; Pachnis et al. 1993) *ret* proto-oncogenes revealed the full primary structure of the RET protein and the unusual presence in its extracellular region of a sequence with similarity to cadherins, transmembrane proteins that mediate Ca^{2+}-dependent homophilic cell adhesion (Nollet et al. 2000). A molecular modeling study of the extracellular domain of RET later revealed four cadherin repeats—termed cadherin-like domains or CLDs 1–4—each of about 110 residues, and one Ca^{2+}-binding site between CLD2 and CLD3 (Anders et al. 2001). Ca^{2+} binding is required for the functional integrity of the RET protein and for its ability to interact with ligand. Following the four CLDs, the extracellular domain of RET contains a Cys-rich region of 120 residues connected to the transmembrane domain. The intracellular region of RET begins with a juxtamembrane portion of 50 residues, a tyrosine kinase domain split by a 14-residue linker, and a 100-residue-long carboxy-terminal tail, which comes in two flavors as a result of alternative splicing. After position 1063, the "short" RET isoform contains nine unique

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carboxy-terminal residues (RET9), whereas the "long" contains 51 (RET51).

ONE GENE, MANY DISEASES

Mutations in the *RET* gene have been found in a number of human diseases, including several different cancers of neuroendocrine origin and a gut syndrome characterized by intestinal obstruction known as Hirschsprung's disease. Four different human cancers carry mutations in the *RET* gene, including papillary thyroid carcinoma (PTC) (Grieco et al. 1990), medullary thyroid carcinoma (familial and sporadic) (Donis-Keller et al. 1993; Hofstra et al. 1994), and the multiple endocrine neoplasias type 2A (MEN2A) (Donis-Keller et al. 1993; Mulligan et al. 1993) and 2B (MEN2B) (Hofstra et al. 1994). Dozens of different substitutions and rearrangements in the *RET* gene underlie these syndromes, a complexity that has profound implications for our understating of genotype/phenotype relationships and the molecular mechanisms of signal transduction (for an in-depth review of the cancer biology of *RET*, see the article by Santoro and Carlomagno 2013). Although *RET* mutations that lead to tumor formation have in most cases been described as gain of function, mutations that result in Hirschsprung's disease—of which more than 50 are known so far—invariably result in loss of RET function by targeting its kinase activity (Iwashita et al. 1996; Pelet et al. 1998), docking sites for intracellular signaling effectors (Geneste et al. 1999), or residues in the RET extracellular domain that affect RET processing in the endoplasmic reticulum and prevent RET expression at the cell surface (Iwashita et al. 1996; Cosma et al. 1998; Kjaer and Ibanez 2003b).

A WEALTH OF RET LIGANDS AND CORECEPTORS

The RET ligand has been identified as glial cell line-derived neurotrophic factor (GDNF) (Durbec et al. 1996; Trupp et al. 1996), a dimeric growth factor-like protein distantly related to members of the transforming growth factor-β (TGF-β) superfamily. Three additional proteins highly related in sequence to GDNF, known respectively as Neurturin (Kotzbauer et al. 1996), Persephin (Milbrandt et al. 1998), and Artemin (Baloh et al. 1998b), were subsequently also identified as ligands of RET. However, this ligand–receptor relationship proved to be a little more unusual than initially expected. Neither ligand is able to bind RET on its own, but require a ligand-binding subunit acting as coreceptor, known as the GDNF family receptor α (GFRα) component. Four different GFRαs have been described (GFRα1–4), each with selectivity—although not absolute specificity—for each of the four distinct members of the GDNF ligand family (Jing et al. 1996; Baloh et al. 1997, 1998a; Buj-Bello et al. 1997; Klein et al. 1997; Sanicola et al. 1997; Naveilhan et al. 1998; Trupp et al. 1998; Worby et al. 1998; Masure et al. 2000). GDNF can be chemically cross-linked to RET (Trupp et al. 1996), indicating that it does make direct contact with the receptor, although its binding affinity is too low to stabilize a complex. On the other hand, GDNF has high affinity for GFRα1 independently of RET. A model initially proposed had GDNF forming first a complex with GFRα1 and subsequently recruiting RET to the complex (Massagué 1996). An alternative model, in which GFRα1 and RET are preassociated to some extent before ligand binding, was later proposed based on binding and site-directed mutagenesis studies (Eketjäll et al. 1999; Cik et al. 2000). It should be noted that two additional receptors for GDNF have also been described, namely, the neural cell adhesion molecule NCAM (Paratcha et al. 2003) and syndecan-3 (Bespalov et al. 2011), which are able to transmit GDNF signals independently of RET.

EVOLUTIONARY RELATIONSHIPS

Only one *ret* gene is known to exist in higher organisms. RET, GDNF family ligands, and GFRα proteins have been found in all vertebrate species investigated so far. A *ret* orthologue has also been found in the genome of the cephalochordate *Amphioxus*, along with sequences corresponding to one GDNF-like and one GFRα-like encoded protein product. RET is also found in *Drosophila melanogaster*. Intriguingly, its ex-

pression pattern in the fly is to some extent reminiscent of the one found in vertebrates (Hahn and Bishop 2001). However, no GDNF or GFRα proteins appear to be encoded in the fly genome. *Drosophila* RET is unable to interact with GDNF or GFRα1 of mammalian origin, nor is it capable of mediating cell adhesion (Abrescia et al. 2005). A chimeric approach was used to show that *Drosophila* RET contains an active tyrosine kinase that is competent to induce neuronal differentiation on activation in PC12 cells (Abrescia et al. 2005). The physiological function of RET in *Drosophila* remains unknown.

STRUCTURE–FUNCTION STUDIES OF RET EXTRACELLULAR AND KINASE DOMAINS

Loss-of-function mutations in RET cause abnormal development of the enteric nervous system, leading to Hirschsprung's disease. Hirschsprung mutations in the extracellular domain of RET (RET^{ECD}) affect processing in the endoplasmic reticulum (ER) and prevent RETexpression at the cell surface. Most Hirschsprung mutations examined prevent the maturation of RET^{ECD} in the ER, indicating defects in protein folding (Kjaer and Ibanez 2003b). Maturation of RET^{ECD} mutants can be rescued by allowing protein expression to proceed at 30°C, a condition known to facilitate protein folding, regaining their ability to bind to the GDNF/GFRα1. Analysis of autonomous folding subunits in the RET^{ECD} has indicated an intrinsic propensity to misfolding in the amino-terminal CLDs 1–3 (Kjaer and Ibanez 2003b), which also concentrate the majority of Hirschsprung mutations affecting the RET^{ECD}. A recent crystal structure of the two amino-terminal CLD1–2 domains of the RET^{ECD} has revealed these two CLDs folded onto each other in a compact clam-shell arrangement distinct from that of classical cadherins (Kjaer et al. 2010). CLD1 structural elements and disulfide composition are unique to mammals, indicating an unexpected structural diversity within higher and lower vertebrate RET CLD regions. The same study identified two unpaired cysteines that predispose human RET to maturation impediments in the ER. The intrinsic susceptibility to misfolding of mammalian RET^{ECD} may be the result of a trade-off that helps to avoid an increased incidence of tumors, at the expense of a greater vulnerability to Hirschsprung's disease.

Sequence and functional divergences between the ectodomains of mammalian and amphibian RET molecules have been exploited to map binding determinants in the human RET^{ECD} responsible for its interaction with the GDNF-GFRα1 complex through homolog-scanning mutagenesis. It was found that Xenopus RET^{ECD} was unable to bind to GDNF-GFRα1 complexes of mammalian origin. However, a chimeric molecule containing CLD1, 2, and 3 from human RET^{ECD}, but neither domain alone, had similar binding activity than full-length human RET^{ECD} (Kjaer and Ibanez 2003a). This suggested the existence of an extended ligand-binding surface within the three amino-terminal cadherin-like domains of human RET^{ECD}. Subsequently, a study using chemical cross-linking of a reconstituted GDNF/GFRα1/RET complex followed by matrix-assisted laser desorption/ionization (MALDI) mass spectrometry analysis, indicated that CLD4 and the carboxy-terminal cysteine-rich domain (CRD) of the RET^{ECD} are in direct contact with GFRα1 in complex with GDNF (Amoresano et al. 2005). This study failed to identify any direct contacts between RET and GDNF, although, as mentioned earlier, those were known to exist from previous cross-linking experiments (Trupp et al. 1996). These discrepant sets of results could be reconciled if the role of the amino-terminal CLD1–3 in ligand binding was indirect, rather than in establishing physical contact with the GDNF/GFRα1 complex. In this scenario, CLD1–3 would be necessary for RET to adopt a conformation that is competent for binding and complex assembly but not directly involved in contacting the RET ligands (Amoresano et al. 2005). At the time of this writing, efforts to solve the three-dimensional structure of the tripartite GDNF/GFRα1/RET complex are still ongoing, but preliminary results would seem to offer support for this latter model.

The crystal structures of the nonphosphorylated (inactive) and phosphorylated (active) RET

kinase have been determined and shown to adopt the same active kinase conformation competent to bind ATP and substrate (Knowles et al. 2006). Both structures show a preorganized activation loop conformation that is independent of phosphorylation status. In agreement with the structural data, enzyme kinetic data showed that autophosphorylation produces only a modest increase in activity (Knowles et al. 2006). Longer forms of the RET intracellular domain containing the juxtamembrane domain and carboxy-terminal tail showed similar kinetic behavior as the isolated kinase, indicating the absence of a *cis*-inhibitory mechanism within the RET intracellular domain (Knowles et al. 2006). Unlike the situation of most other receptor tyrosine kinases, these results suggest the existence of alternative inhibitory mechanisms, possibly in *trans*, for the autoregulation of RET kinase activity.

RET SIGNALING MECHANISMS

Dimerization of receptor tyrosine kinases is known to be required, although most likely not sufficient, for ligand-induced kinase transphosphorylation and activation. Several receptor tyrosine kinases are found as preformed homodimers at the plasma membrane independently of ligand binding. Using a transmembrane (TM) domain self-association assay, Kjaer et al. observed strong self-association of the RET-TM in a biological membrane (Kjaer et al. 2006). These investigators found that mutagenesis of specific residues in the RET-TM domain reduced receptor homodimerization and abolished the transforming activity of MEN2A RET, one of the strongest oncogenic variants of the RET protein, suggesting that self-association of RET TM domains contributes to the mechanism of activation of RET (Kjaer et al. 2006).

Like the majority of receptor tyrosine kinases studied, signaling pathways initiated by the RET receptor include the Ras/MAP kinase, PI3 kinase/AKT, and phospholipase C-γ (PLCγ) pathways. On activation, RET undergoes autophosphorylation of intracellular tyrosine residues, which then serve as docking sites for downstream signaling effectors carrying Src homology 2 (SH2) or phosphotyrosine-binding (PTB) domains. Previous studies have indicated that at least 14 of the 18 tyrosine residues present in the intracellular region of RET can become phosphorylated (Liu et al. 1996; Kawamoto et al. 2004; Knowles et al. 2006). Among those, Tyr^{900} and Tyr^{905} are present in the kinase activation loop and are known to contribute to full kinase activation (Knowles et al. 2006). Autophosphorylation of the key residue Tyr^{1062} is required for activation of Ras/MAP kinase and PI3 kinase/AKT (Besset et al. 2000; Hayashi et al. 2000; Segouffin-Cariou and Billaud 2000; Coulpier et al. 2002). This residue appears to be critical for RET function, and mice with a point mutation in Tyr^{1062} show a severe loss-of-function phenotype (Jijiwa et al. 2004; Wong et al. 2005; Jain et al. 2006a). Phosphorylation of Tyr^{1096}, present only in the long RET51 isoform, also contributes to these pathways. On ligand stimulation, at least two distinct protein complexes assemble on phosphorylated Tyr^{1062} of RET via Shc, one leading to activation of the Ras/MAP kinase pathway through recruitment of Grb2 and Sos, and another to the PI3K/AKT pathway through recruitment of adaptors Grb2 and Gab2 followed by $p85^{PI3K}$ and the SHP2 tyrosine phosphatase (Besset et al. 2000).

The adaptor protein FRS2 can also bind to phosphorylated Tyr^{1062} in the activated RET receptor (Kurokawa et al. 2001; Melillo et al. 2001). FRS2 competes with Shc for binding to Tyr^{1062}, and it has been shown that Shc but not FRS2 is responsible for cell survival effects of RET in neuroblastoma cells (Lundgren et al. 2006). This differential signaling may be mediated from different compartments in the plasma membrane, as RET has been shown to interact with FRS2 in lipid rafts, but with Shc outside lipid rafts (Paratcha et al. 2001). A series of adaptor molecules from the p62dok family have also been shown to interact with the activated RET receptor. Dok-1, -2, -4, -5, and -6 all interact with phosphorylated Tyr^{1062} via their PTB domains (Grimm et al. 2001; Crowder et al. 2004; Kurotsuchi et al. 2010) and are thought to contribute to neuronal differentiation (Grimm et al. 2001; Crowder et al. 2004).

The Grb2/Gab2 complex can also assemble directly onto phosphorylated Tyr[1096], offering an alternative route to PI3K activation by GDNF. Regarding the remaining autophosphorylation sites, it has been found that phosphorylation of Tyr[1015] leads to activation of PLCγ (Borrello et al. 1996), and phospho-Tyr[981] binds the Src cytoplasmic tyrosine kinase (Encinas et al. 2004). Recently, a yeast-two-hybrid screen led to the identification of the GTPase-activating protein (GAP) for Rap1, Rap1GAP, as a novel RET-binding protein (Jiao et al. 2011). Like Src, Rap1GAP was also found to require phosphorylation of Tyr[981] for RET binding and suppressed GDNF-induced activation of ERK and neurite outgrowth. A recent study has established a biochemical function and a physiological role for the phosphorylation of Tyr[687] in the juxtamembrane region of the RET intracellular domain (Perrinjaquet et al. 2010). Using a phage display strategy, Perrinjaquet et al. found that the phosphotyrosine phosphatase SHP2 binds to phospho-Tyr[687] on ligand-induced RET activation. SHP2 is recruited to activated RET in a cooperative fashion, such that both interaction with Tyr[687] and association with components of the Tyr[1062] signaling complex are required for stable recruitment of SHP2 to the receptor. SHP2 recruitment was found to contribute to the ability of RET to activate the PI3K/AKT pathway and promote survival and neurite outgrowth in primary neurons (Perrinjaquet et al. 2010).

In addition to tyrosine autophosphorylation, RET has been found to undergo serine phosphorylation at Ser[696] by protein kinase A (PKA) (Fukuda et al. 2002). Mutation of Ser[696] affected the ability of RET to activate the small GTPase Rac1 and stimulate formation of cell lamellipodia (Fukuda et al. 2002). Homozygous knock-in mice carrying this mutation lacked neuronal elements of the enteric nervous system in the distal colon, resulting from a migration defect of enteric neural crest cells (Asai et al. 2006), indicating a physiological role for PKA-dependent modification of RET function. Interestingly, the signaling deficits of the Ser[696] RET mutant could be alleviated—at least in vitro—by simultaneous mutation of the nearby residue Tyr[687] (Fukuda et al. 2002). In line with this, activation of PKA by forskolin was found to impair the recruitment of SHP2 to RET and negatively affected ligand-mediated neurite outgrowth (Perrinjaquet et al. 2010). Moreover, mutation of Ser[696] enhanced SHP2 binding to the receptor and eliminated the effect of forskolin on ligand-induced neurite outgrowth. Together, these findings established Tyr[687] as a critical platform for integration of RET and PKA signals.

Among the interactions not mediated by phosphorylation, the PDZ domain-containing Shank3 protein was found to interact with a PDZ-binding motif present in the RET9 but not in the RET51 isoform (Schuetz et al. 2004). Shank3 was shown to mediate sustained MAP kinase and PI3 kinase signaling, and the formation of branched tubular structures in three-dimensional cultures of epithelial cells.

RET FUNCTION IN KIDNEY DEVELOPMENT

Knockout studies have shown that RET inactivation results in renal agenesis or severe hypodysplasia, owing to failure of the ureteric bud to evaginate from the Wolffian duct and branch normally (Schuchardt et al. 1994, 1996). RET expression defines a population of ureteric bud tip cells that proliferate under GDNF stimulation from the metanephric mesenchyme. In the absence of RET, tip cells change fate and instead contribute to the ureteric bud trunk (Shakya et al. 2005). Studies in knock-in mice have provided evidence for differential and isoform-specific roles of RET phospho-Tyr docking sites in kidney development. One earlier study initially reported that mice expressing only RET9 were normal, whereas those expressing only RET51 showed kidney hypodysplasia (de Graaff et al. 2001). In contrast, a later study reported that mice monoisomorphic for either RET9 or RET51 were viable and showed normal kidneys, indicating redundant roles of RET isoforms in kidney development (Jain et al. 2006a). As discussed elsewhere, a possible reason for this discrepancy may lie in the use of chimeric mouse–human knock-in cDNAs in the first study. It has also been reported that wild-type

human RET51 and RET9 are both able to promote branching morphogenesis to a similar extent, but mutation of Tyr^{1062} abrogates this activity only in RET9 and not in RET51, presumably because of redundancy through Tyr^{1096} (Jain et al. 2006a). In contrast, mutation of Tyr^{1015} produced clear defects in ureteric bud outgrowth in the context of either isoform, providing evidence for the importance of PLCγ signaling downstream from RET in renal development. Despite the prominent role of RET signaling in kidney development, no human *RET* mutations have yet been uncovered in children suffering from renal tract malformations.

RET FUNCTIONS IN NERVOUS SYSTEM DEVELOPMENT

Enteric Nervous System

Hirschsprung's disease is a genetic disorder of neural crest development characterized by the absence of enteric parasympathetic neurons in the lower regions of the gut. In agreement with a role in neural crest development, RET is expressed in several neuronal subpopulations derived from this structure, including cells in the enteric, sensory, and sympathetic nervous systems (Pachnis et al. 1993). Mice that are homozygotes for a targeted mutation in the RET gene lack enteric neurons throughout the digestive tract (Schuchardt et al. 1994). A subpopulation of enteric neural crest was found to undergo apoptotic cell death specifically in the foregut of embryos lacking the RET receptor (Taraviras et al. 1999). Together with defects in kidney organogenesis, this leads to the death of *RET* null animals at birth. It has more recently been found that conditional ablation of *RET*, or the GFRα1 coreceptor, in postmigratory enteric neurons causes widespread neuronal death in the colon, leading to colonic aganglionosis that resembles Hirschsprung's disease (Uesaka et al. 2007, 2008).

Motoneurons

RET is expressed in all spinal cord motoneurons from the earliest stages of their development (Pachnis et al. 1993; Trupp et al. 1997; Garcès et al. 2000). GDNF has potent survival activities in spinal motoneurons (Henderson et al. 1994) and is required for their in vivo survival during late embryogenesis (Oppenheim et al. 2000). At early stages of development, RET is required for the topographic projection of hind limb-innervating axons, functioning as an instructive guidance signal for motor axons (Kramer et al. 2006). In this case, RET was shown to cooperate with the EphA4 receptor tyrosine kinase to enforce the precision of this binary choice in motor axon guidance. More recent studies using conditional alleles have shown that the effect of RET on motoneuron survival during programmed cell death is restricted to the early neonatal development of the subpopulation of γ-motoneurons that innervates muscle spindles (Gould et al. 2008). RET signaling would thus appear to have multiple effects on motoneuron survival and connectivity.

Ventral Midbrain Dopaminergic Neurons

GDNF was identified on the basis of its survival-promoting effects on ventral midbrain dopaminergic neurons, which are important in the pathogenesis of Parkinson's disease (Lin et al. 1993). RET is expressed at high levels in adult ventral midbrain dopaminergic neurons of the substantia nigra, and exogenous application of GDNF was shown to protect RET-expressing neurons in this structure from cell death induced by 6-hydroxydopamine, an animal model of Parkinson's disease (Trupp et al. 1996). The robust effects of GDNF/RET signaling on dopaminergic neuron survival in several lesion paradigms naturally raised expectations of an important physiological function for RET in dopaminergic neurons, where it is expressed from very early stages of development. In agreement with this, knock-in of a constitutive allele of *RET* resulted in increased numbers of dopaminergic neurons and profound elevation of brain dopamine concentration, suggesting that RET signaling can have a direct biological effect in the brain dopaminergic system (Mijatovic et al. 2007). Despite the successes of gain-of-function approaches, the results from loss-of-

function studies have been less clear-cut with regard to the importance of RET activity in dopaminergic neurons. Conditional ablation of RET in dopaminergic neurons has failed to reveal a prominent role for RET signaling in dopaminergic neuron development or maintenance, at least during the average life span of the mouse (Jain et al. 2006b; Kramer et al. 2007). One of these two studies, however, examined aging mutant mice and found that RET ablation caused progressive, but moderate, adult-onset loss of dopaminergic neurons in the substantia nigra and reduced dopaminergic nerve terminals in striatum, reaching 38% reduction by 2 years of age (Kramer et al. 2007). In contrast to those mild effects, another study induced adult deletion of a conditional allele of *Gdnf* and found widespread deficits in dopaminergic and noradrenergic neurons (Pascual et al. 2008). As discussed elsewhere (Ibanez 2008), the striking discrepancy between these studies could be explained by the presence of alternative receptors for GDNF in dopaminergic neurons, compensatory effects in developing RET-deficient neurons, or the possibility that RET functions as a "dependence receptor" in dopaminergic neurons. Dependence receptors make cells that express them dependent on their ligands, and evidence from transformed cell lines has been provided suggesting that RET might indeed function in this way (Bordeaux et al. 2000). At the time of this writing, independent efforts are underway to replicate several of the above-mentioned studies and so it is likely that the controversy over the physiological role of RET signaling in dopaminergic neuron survival and maintenance will become clarified before long.

CONCLUDING REMARKS

Nearly three decades after its discovery, unique aspects of RET function and physiology continue to fascinate biologists and biochemists. Unlike other receptor tyrosine kinases, autophosphorylation has only a modest effect on kinase activity, and so the mechanism of activation of the RET kinase remains elusive. The possibility of autoinhibition in *trans* is tantalizing and would mechanistically set RET apart from the bulk of other receptor tyrosine kinases. Its ligand system, with the GFRα coreceptors, is also rather unique, and upcoming crystal structures of the full ternary complex promise to reveal the mechanism of complex formation, and perhaps explain how a distant TGF-β superfamily member ended up binding a receptor tyrosine kinase. These insights may also find applications in drug discovery. Indeed, although its physiological significance in dopaminergic neurons remains unclear, the robust effects of RET gain-of-function on the survival and function of these neurons has encouraged efforts to identify agonists for treatment of Parkinson's disease (Aron and Klein 2011). Such compounds may circumvent the intrinsic problems of protein delivery of current GDNF-based approaches.

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