

# *Drosophila* RET contains an active tyrosine kinase and elicits neurotrophic activities in mammalian cells

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**Abstract** The RET receptor tyrosine kinase controls kidney organogenesis and development of subpopulations of enteric and sensory neurons in different vertebrate species, including humans, rodents, chicken and zebrafish. RET is activated by binding to a ligand complex formed by a member of the glial cell line-derived neurotrophic factor (GDNF) family of neurotrophic factors bound to its cognate GFR $\alpha$  GPI-linked co-receptor. Despite the absence of GDNF or GFR $\alpha$  molecules in the *Drosophila* genome, a RET orthologue (dRET) has recently been described in this organism and shown to be expressed in subpopulations of cells of the excretory, digestive and nervous systems, thus resembling the expression pattern of RET in vertebrates. In this study, we report on the initial biochemical and functional characterization of the dRET protein in cell culture systems. Full-length dRET could be produced in mammalian and insect cells. Similar to its human counterpart (hRET), overexpression of dRET resulted in its ligand-independent tyrosine phosphorylation, indicating that it bears an active tyrosine kinase. Unlike hRET, however, the extracellular domain of dRET was unable to interact with mammalian GDNF and GFR $\alpha$ 1. Self association between dRET molecules could neither be detected, indicating that dRET is incapable of mediating cell adhesion by homophilic interactions. A chimeric molecule comprising the extracellular domain of hRET and the kinase domain of dRET was constructed and used to probe ligand-mediated downstream activities of the dRET kinase in PC12 cells. GDNF stimulation of cells transfected with the hRET/dRET chimera resulted in neurite outgrowth comparable to that obtained after transfection of wild-type hRET. These results indicate significant conservation between the biological effects elicited by the human and *Drosophila* RET kinases, and suggest functions for dRET in neuronal differentiation in the fly.

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## 1. Introduction

The *ret* gene was originally identified as a human oncogene in cellular transformation assays [1]. Subsequent studies revealed this gene to encode a phylogenetically conserved receptor tyrosine kinase, consisting of an extracellular domain, a single-pass transmembrane domain and a typical intracellular tyrosine kinase domain (reviewed in [2]). The extracellular domain of mammalian RET binds soluble ligands belonging to the glial cell line-derived neurotrophic factor (GDNF) family in collaboration with glycosyl phosphatidylinositol (GPI) anchored co-receptors known as GFR $\alpha$  proteins. Upon activation in this complex, RET is thought to form active homodimers that undergo autophosphorylation of specific tyrosine residues in the cytoplasmic domain, leading to downstream signaling [3–5]. During mammalian embryogenesis, RET signaling plays a critical role in determining survival and differentiation of many subpopulations of neurons originating from the neural crest, as well as in kidney morphogenesis [2,6]. Both loss of function and gain of function mutations in the *ret* gene have been associated to human diseases. Heterozygous loss-of-function mutations lead to Hirschprung disease, a hereditary developmental disorder characterized by aganglionosis of the distal part of the intestinal tract [2,7]. On the other hand, activating mutations are associated with multiple endocrine neoplasias MEN2A and MEN2B, medullary thyroid carcinomas (MTC) and pheochromocytomas [2,7,8]. Only one RET paralogue gene has been found in all species investigated so far, and alignments of RET sequences from several vertebrate species and from *Drosophila melanogaster* have revealed phylogenetic relationships between RET orthologues of different metazoan species [9]. Intriguingly, in the developing chick, zebrafish and in *Drosophila*, RET mRNA has been localized in cells that are functionally equivalent to RET-expressing cells in developing mammalian embryos, including portions of the nervous, excretory and digestive systems [10–13], suggesting a significant degree of functional conservation across species.

The tyrosine kinase domain is the most conserved portion of RET, with up to 90% of sequence identity within the vertebrate lineage and overall 65% sequence identity between seven vertebrate species and *Drosophila* [9,13]. The high degree of homology of the catalytic core of the kinase between vertebrate and invertebrate species suggests that *Drosophila* RET (herein dRET, also reported as sequence CG14396, FlyBase ID: Fbgn0011829) is an active tyrosine kinase [14], and may therefore exert functions similar to human RET (herein hRET) with regards to downstream signaling. Although the extracellular

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domain of RET shows a lower sequence identity (only 40% among vertebrate RET species), it contains highly homologous structural motifs which unequivocally identify RET as a unique receptor tyrosine kinase in those species [9,13,14]. One such element is the cysteine-rich domain (CRD). Cysteine residues in this region of RET are believed to be involved in intramolecular disulphide bridges, which are important for the tertiary structure of the molecule [7]. Fourteen of the 28 cysteine residues within this domain are conserved between human, zebrafish and *Drosophila* RET molecules [13]. MEN2A and familial MTC syndromes are associated with mutations of one out of 6 conserved cysteine residues in the CRD of human RET (positions 609, 611, 618, 620, 630 and 634, respectively). These mutations lead to aberrant intermolecular disulphide bonds between RET molecules, dimerization and subsequent constitutive activation of receptor kinase activity [7]. Another characteristic feature of the extracellular domain of RET is the presence of four cadherin-like domains (CLDs) [9]. Cadherins are  $\text{Ca}^{2+}$ -dependent cell–cell adhesion proteins and their adhesive properties depend on a domain of 110 amino acids repeated in tandem in their extracellular region. The presence of four phylogenetically conserved CLDs in the extracellular region of RET has prompted its classification within the cadherin superfamily [15,16], although no cell adhesion activity has been demonstrated so far for RET molecules.

RET has a role in the transduction of signals from all ligands belonging to the GDNF family identified in vertebrates and its function is not genetically redundant. Moreover, the expression pattern of the *Drosophila* RET gene, as determined by whole mount in situ hybridization of *Drosophila* embryos, strongly suggests a function of dRET in triggering developmental signals analogous to those exerted in mammalian embryogenesis [2,6,12,13]. Nonetheless, neither GDNF family ligands nor GFR $\alpha$  co-receptors can be found in the *Drosophila* genome [17,18]. Thus, it is not yet understood whether human

and *Drosophila* RET are functionally related nor which are the counterparts of the mammalian system in *Drosophila*. The paucity in our understanding of the functional properties of dRET is underscored by the absence of mutant alleles of the dRET locus in the FlyBase database. In the present work, we have characterized the expression of dRET in cultured cells and investigated its biochemical and biological functions in vitro in comparison with hRET.

## 2. Results

### 2.1. Full-length dRET is correctly folded and phosphorylated in mammalian cells

As a first step to investigate functional properties of full-length dRET, we generated constructs containing the full length dRET cDNA (classified as CG14396 in the FlyBase database) fused to a C-terminal myc tag for overexpression in mammalian and insect cells. Cell lysates were analyzed by immunoprecipitation with anti-myc antibodies, followed by immunoblotting with antibodies (panRET) directed against a peptide sequence in the activation loop of the RET kinase that is highly conserved between mammalian and *Drosophila* RET proteins. Full-length dRET was consistently expressed at lower levels in either mammalian or insect cells, and required pooling of several confluent plates for immunodetection in cell lysates. Although the calculated molecular weight of the dRET polypeptide was deduced to be around 140 kDa, full-length dRET was expected to migrate slower due to N-glycosylation at eight predicted sites. dRET expressed in mammalian cells appeared primarily as two bands migrating with a molecular weight of approximately 200 kDa in SDS/PAGE, that were likely to represent full-length dRET (Fig. 1A). Additional products were also observed at 120 and 60 kDa (Fig. 1A), which may have arisen by endoproteolytic cleavage of dRET. dRET bands of

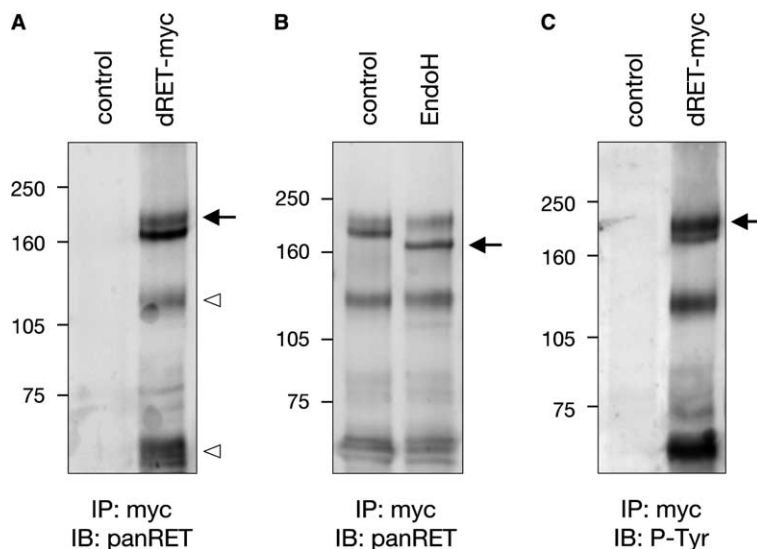


Fig. 1. Full-length dRET is correctly folded and phosphorylated in mammalian cells. (A) Immunoprecipitation (IP) and immunoblot (IB) analysis of full-length dRET C-terminally tagged with a c-myc epitope (dRET-myc) in COS cells. Arrow indicates full-length, correctly folded dRET protein running at about 200 kDa. Proteolytically cleaved dRET products were also observed running at 120 and 60 kDa, respectively (open arrowheads). (B) EndoH digestion confirms correct folding of the upper dRET in the 200 kDa doublet, and indicates that the lower, EndoH-sensitive band (arrow) is a folding intermediate retained in the endoplasmic reticulum (see [20]). (C) Immunoblotting with anti-phosphotyrosine antibodies revealed spontaneous tyrosine phosphorylation of dRET after overexpression in COS cells. Full-length dRET is indicated with an arrow.

200, 120 and 60 kDa were also observed upon expression of dRET in *Drosophila* Schneider's 2 cells (data not shown), suggesting an intrinsic propensity of dRET to proteolytic cleavage independently of cell type. The fact that dRET fragments of 60 and 120 kDa retained the C-terminal myc tag, together with their apparent molecular weights, pointed to extracellular and/or transmembrane cleavage sites. However, treatment of dRET-expressing cells with the  $\gamma$ -secretase inhibitor L-685,458 [19] had no effect on the profile of dRET products detected in cell lysates (data not shown). Similar to previous observations with hRET [20], only the upper band of the 200 kDa dRET doublet was resistant to treatment with endoglycosidase H (EndoH) (Fig. 1B), indicating that it represented mature, correctly folded, plasma membrane dRET. EndoH cleaves off carbohydrates from glycoproteins which are at the stage of folding intermediates and present in the endoplasmic reticulum (ER). Correctly folded glycoproteins lose sensitivity to EndoH treatment as carbohydrates of higher complexity are added in the Golgi. As with hRET, the lower, EndoH-sensitive band in the doublet (arrow in Fig. 1B) was likely to represent immature, partially glycosylated dRET retained in the ER. Similar to other receptor tyrosine kinases (e.g. [21]), overexpression of dRET in mammalian cells resulted in ligand-independent tyrosine phosphorylation (Fig. 1C). This was likely due to autophosphorylation, as overexpressed kinase-dead mutants of RET proteins do not show tyrosine phosphorylation (data not shown). Intriguingly, tyrosine phosphorylation was observed in all three dRET fragments, suggesting that they all contain an active tyrosine kinase.

### 2.2. Expression and functional analysis of the extracellular domain of dRET

In order to analyze dRET functions, we first generated several mammalian expression plasmids encoding a soluble form of the extracellular domain of dRET (herein dRET<sup>ECD</sup>) alternatively fused to different C-terminal tags, such as c-myc, human Fc and hemagglutinin (HA) epitopes. Upon overexpression of the dRET<sup>ECD</sup> in COS cells, we tested the correct folding and post-translational modification of the secreted product by EndoH and peptide:N glycosidase F (PNGaseF) digestion assays as described above. We performed EndoH and PNGaseF digestions on equivalent amounts of conditioned medium harvested from COS7 cells overexpressing HA-tagged dRET<sup>ECD</sup>, and subsequently detected the products by Western blotting with antibodies directed against the C-terminal HA tag. The band migrating at a molecular weight of about 120 kDa corresponds to the expected size of the fully glycosylated dRET<sup>ECD</sup>. The protein was resistant to EndoH and sensitive to PNGaseF digestion, indicating that it was secreted from COS cells as a fully mature polypeptide (Fig. 2A). COS cell conditioned medium containing dRET<sup>ECD</sup> was then used to probe the ability of this protein to interact with mammalian GDNF and GFR $\alpha$ 1 in an ELISA binding assay. HA-tagged dRET<sup>ECD</sup> was allowed to bind to a solid support that had previously been coated with anti-HA antibodies, and GDNF was subsequently added along with a purified soluble form of GFR $\alpha$ 1 fused to a fragment of human IgG heavy chain (GFR $\alpha$ 1-Fc). The amount of GFR $\alpha$ 1-Fc retained on the support through binding to GDNF and dRET<sup>ECD</sup> was then quantified using a second anti-Fc antibody, and compared to negative (blank) and positive (hRET<sup>ECD</sup>) controls. Unlike its human counterpart, the dRET<sup>ECD</sup> was unable to

form a complex with mammalian GDNF and GFR $\alpha$ 1 (Fig. 2B). In order to investigate whether membrane bound dRET could bind mammalian GDNF in the presence of GFR $\alpha$ 1, we transfected hRET or dRET together with GFR $\alpha$ 1 in COS cells and tested the ability of RET molecules to interact with radiolabeled GDNF (<sup>125</sup>I-GDNF) by chemical cross-linking and immunoprecipitation. This experiment showed that COS cell-expressed hRET, but not dRET, was able to bind GDNF in the presence of GFR  $\alpha$ 1 (Fig. 2C), indicating that the *Drosophila* receptor is unable to interact with mammalian GDNF ligands.

All RET orthologues identified so far are likely to be phylogenetically related, not only by the high degree of sequence identity of the tyrosine kinase domain, but also by the presence of homologous cadherin-like domains in their extracellular regions [9,13]. Given that cadherins have cell adhesion functions, we investigated the possibility that the dRET<sup>ECD</sup> may be able to mediate cell adhesion. In the first set of experiments, we addressed this in vitro by combining equivalent amounts of two differentially tagged soluble ECD constructs, immunoprecipitating the mixture with antibodies directed against either of the two tags, and performing Western blot analysis against the second tag. A similar experiment was also performed using supernatants of COS cells that had been simultaneously co-transfected with the two ECD constructs. Previous studies have shown that *bonafide* cell adhesion molecules are able to engage in homologous interactions in solution (e.g. [22]). However, no co-immunoprecipitation of the two tagged dRET<sup>ECD</sup> proteins could be detected in either system (data not shown), indicating that the dRET<sup>ECD</sup> is not capable of self associating in vitro. In a second set of experiments, we tested the ability of dRET to mediate cell adhesion directly in transfected Jurkat cells. Two days after transfection together with a GFP fluorescent protein reporter, cells that received an expression construct of the neural cell adhesion molecule NCAM formed clusters of 2–20 cells, indicating NCAM-mediated cell adhesion (Fig. 2D). On the other hand, control transfected cells remained as a dispersed cell suspension. In contrast to NCAM-transfected cells, no clusters were observed in cells that received dRET (Fig. 2D), indicating that dRET is unable to mediate cell adhesion in this assay.

### 2.3. The dRET kinase domain can mediate ligand-dependent neuronal differentiation of PC12 cells

In order to characterize the functional capabilities of the kinase domain of dRET, and in the absence of cognate dRET ligands, we generated a chimeric molecule consisting of the extracellular and transmembrane regions of human RET fused to the kinase domain of *Drosophila* RET (herein termed hRET<sup>ECD</sup>/dRET<sup>KIN</sup>) (Fig. 3A). A similar approach – albeit using the ectodomain of the human EGF receptor – has previously been used to functionally characterize the hRET receptor prior to the discovery of its endogenous ligands [23]. We speculated that activation of such chimeric molecule by GDNF and GFR $\alpha$ 1 should allow us to evaluate the functional output of the dRET kinase domain. After overexpression in COS cells, the hRET<sup>ECD</sup>/dRET<sup>KIN</sup> chimera could be detected by immunoblotting with an apparent molecular weight similar to that of dRET (Fig. 3B). A kinase dead chimeric construct carrying a substitution of Lys-805 into Met (h/dRET<sup>K805M</sup>) was also generated. Wild type dRET and hRET as well as the hRET<sup>ECD</sup>/dRET<sup>KIN</sup> chimera showed high levels of Tyr

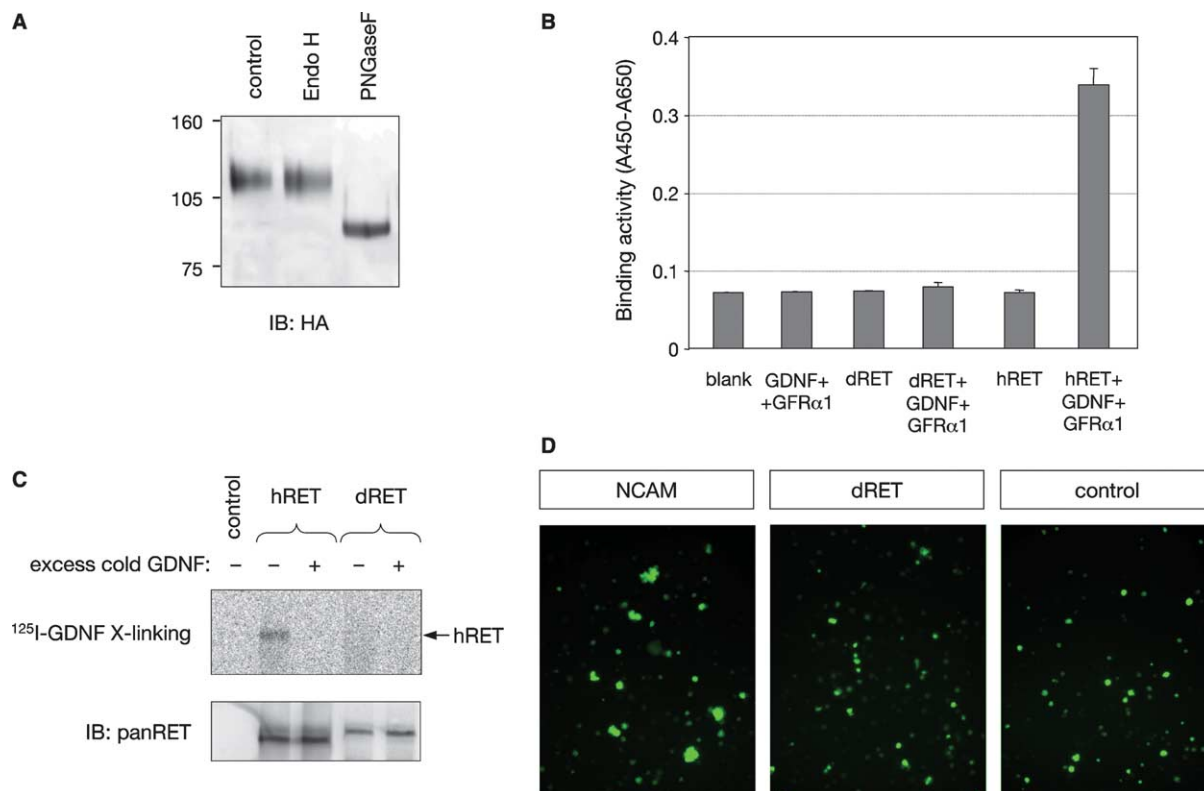


Fig. 2. dRET does not bind to the GDNF/GFR $\alpha$ 1 complex of mammalian origin and nor does it mediate cell adhesion. (A) Soluble dRET<sup>ECD</sup> from COS cell conditioned medium was digested with the indicated glycosidases as previously described [20], and subsequently analyzed by immunoblotting (IB) with antibodies against the C-terminal HA tag. dRET<sup>ECD</sup> was resistant to EndoH digestion and therefore correctly folded. Negative (control) and positive (PNGaseF) control digestions are shown. (B) Solid phase binding assay of mammalian GDNF and GFR $\alpha$ 1 to human and *Drosophila* RET<sup>ECD</sup> proteins. Significant binding was only observed using hRET. No binding above background could be observed with dRET<sup>ECD</sup>. (C) Chemical cross-linking of <sup>125</sup>I-GDNF in COS cells transfected with GFR $\alpha$ 1 and the indicated RET constructs. Mock transfected COS cells were included as control. Excess unlabeled GDNF (cold) was used to verify the specificity of the binding. Following ligand binding and cross-linking, cell lysates were immunoprecipitated with anti-hRET or anti-myc antibodies – as appropriate – and processed by autoradiography after SDS/PAGE. The bottom panel shows a reprobing with the panRET antibody of the same membrane. (D) Cell adhesion assay in Jurkat cells transfected with NCAM, dRET or a control construct along with the fluorescence protein marker GFP. Note that cell clusters – indicative of cell adhesion – can only be seen in cells that received NCAM but not dRET.

autophosphorylation when overexpressed in COS cells (Fig. 3B). As expected, the kinase dead construct was not phosphorylated (Fig. 3B), confirming that Tyr phosphorylation of the dRET kinase requires an activate kinase and is therefore most likely a consequence of autophosphorylation.

GDNF has been shown to elicit neuronal differentiation of the rat pheochromocytoma cell line PC12 expressing RET and GFR $\alpha$ 1 receptors [24,25]. Although PC12 cells do express low levels of RET endogenously, the effect of GDNF is significantly more robust after introducing exogenous RET into these cells. Thus, we overexpressed wild type and chimeric receptors in PC12 cells by transient transfection together with a construct encoding green fluorescent protein (GFP) to identify transfected cells. Immunostaining with the panRET antibody showed reactivity in RET-expressing cells coinciding with GFP expression (Fig. 4A). Negligible background signal was observed in untransfected cells, indicating too low levels of endogenous RET expression for detection by the panRET antibody. Transfected cells were stimulated with GDNF and soluble GFR $\alpha$ 1, and the percentage of differentiated cells was assessed in mock-transfected cells as well as cells that received either full-length hRET, dRET or the hRET<sup>ECD</sup>/dRET<sup>KIN</sup> chimeras. Low levels of ligand-mediated differentiation were

observed in cells transfected with a control plasmid (Fig. 4B), a response presumably produced by endogenous RET receptors present in PC12 cells. On the other hand, transfection of either hRET, dRET or the hRET<sup>ECD</sup>/dRET<sup>KIN</sup> chimera induced low levels of ligand-independent differentiation (Fig. 4B), probably caused by the spontaneous activation of overexpressed receptors. Ligand stimulation, however, produced a robust increase in the percentage of differentiated cells (about 5–6-fold) that received hRET or hRET<sup>ECD</sup>/dRET<sup>KIN</sup> but not dRET or the kinase dead chimera h/dRET<sup>K805M</sup> (Fig. 4B). Importantly, the levels of cell differentiation achieved by hRET or the hRET<sup>ECD</sup>/dRET<sup>KIN</sup> chimera were comparable. This indicated that the binding of GDNF and soluble GFR $\alpha$ 1 to the extracellular portion of hRET was able to activate neuronal differentiation pathways in PC12 cells through the kinase domain of dRET, and suggested significant conservation between the signaling pathways activated by the two species of RET molecules. In order to test this notion further, we investigated the role of the MAP kinase pathway in the effects of the dRET kinase on PC12 differentiation. For this purpose, we used the MEK1 inhibitor PD98059 which blocks upstream activation of the pathway in PC12 transfected with wild type and chimeric RET constructs as above. The inhibitor



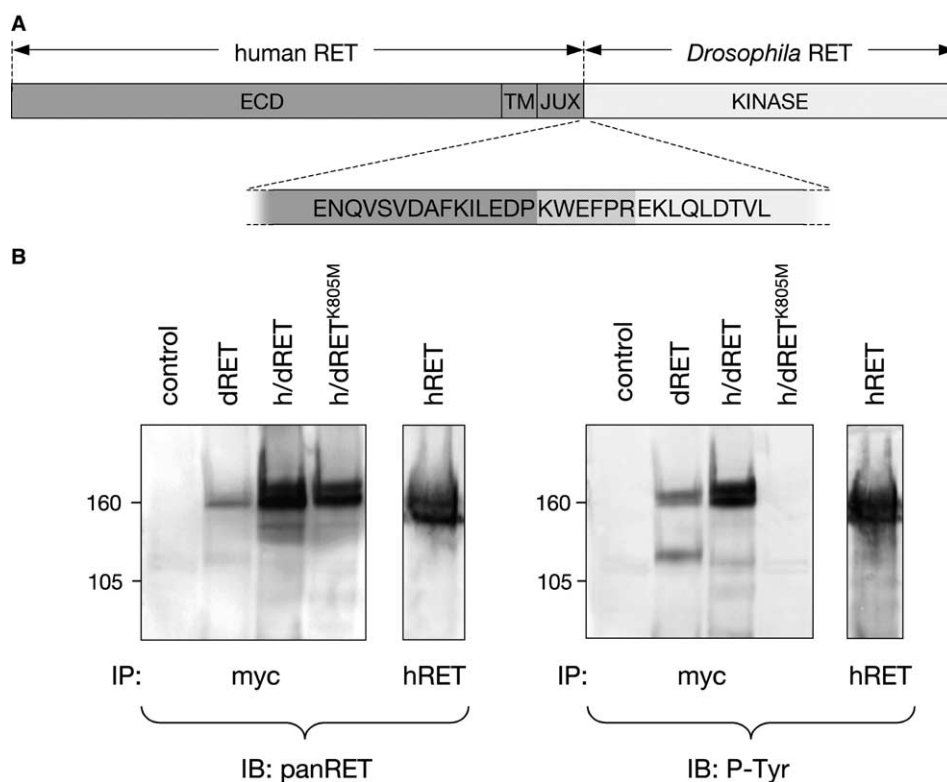


Fig. 3. Generation of a chimeric hRET<sup>ECD</sup>/dRET<sup>KIN</sup> receptor. (A) Schematic representation of the hRET<sup>ECD</sup>/dRET<sup>KIN</sup> chimeric molecule showing the sequence at the junction between human and *Drosophila* fragments. The conserved motif (KWEFPR) common to both proteins is highlighted. ECD, extracellular domain; TM, transmembrane domain, JUX, juxtamembrane domain. (B) Immunoprecipitation (IP) and immunoblot (IB) analysis of hRET<sup>ECD</sup>/dRET<sup>KIN</sup> C-terminally tagged with a c-myc epitope in COS cells. hRET and dRET are also shown for comparison. Note lack of Tyr phosphorylation of the mutant chimera h/dRET<sup>K805M</sup>.

dramatically reduced the effects of either the hRET or dRET kinase in PC12 cells differentiation (Fig. 4B), indicating that *Drosophila* RET, like its human counterpart, utilizes the MAP kinase pathway to induce neuronal differentiation.

### 3. Discussion

In this work, we set out to characterize biochemically and functionally the dRET tyrosine kinase receptor in cell culture systems. Our findings support the hypothesis that dRET can trigger pathways that are to some degree comparable to those elicited by its human counterpart, and are in line with the previously suggested similarity between the kinase domains of the two receptors [9,13], and by the resemblance between the expression patterns of the two proteins in embryos [10–13].

In addition to the full-length protein, overexpression of dRET resulted in the appearance of two smaller fragments both in mammalian and insect cells. The functional significance of such cleavage is at present unknown. Protease cleavage could represent a strategy to regulate dRET expression at the post-translational level. In vertebrates, for example, a folding bottleneck during RET biosynthesis has been proposed to regulate the levels of RET at the plasma membrane [20]. It is also possible that endogenous dRET forms part of a larger complex in vivo, so that cleavage may be inhibited in cells co-expressing physiological interactors of the receptor. These possibilities suggest that the regulation of the expression of

the RET protein in *Drosophila* might be as complex as in vertebrates. Although hRET has been reported to have pro-apoptotic activities following caspase cleavage of its intracellular domain [26], we were not able to detect increased cell death upon transfection of dRET in mammalian cells (C.A., unpublished observations). Moreover, neither of the two consensus sites that have been reported to be required for hRET cleavage by caspase-3, namely VSVD<sup>707</sup> and DYLD<sup>1017</sup> [26], are conserved to any significant degree in the primary sequence of dRET. In any case, cleavage of intracellular sequences would not be able to explain the dRET fragments of 120 and 60 kDa that we detected in transfected cells. Overexpression of RET in mammalian cells results in autophosphorylation of tyrosine residues in a ligand-independent manner [27]. The fact that both human and *Drosophila* RET showed similar tyrosine phosphorylation after overexpression suggests they may share a common mechanism of activation mediated by receptor dimerization, similar to other receptor tyrosine kinases, and in agreement with the high sequence similarity between the kinase domains of human and *Drosophila* RET.

The divergence of the first three CLDs of human and *Drosophila* RET explains the inability of dRET to interact with the mammalian GDNF/GFR $\alpha$ 1 complex, as that region of the RET receptor has been shown to be involved in ligand binding [28]. No neurotrophic molecule has to date been identified in the *Drosophila* genome [17,18,29,30], which could be taken to imply that cognate dRET ligands might be “non-neurotrophic” signaling factors. On the other hand, this receptor

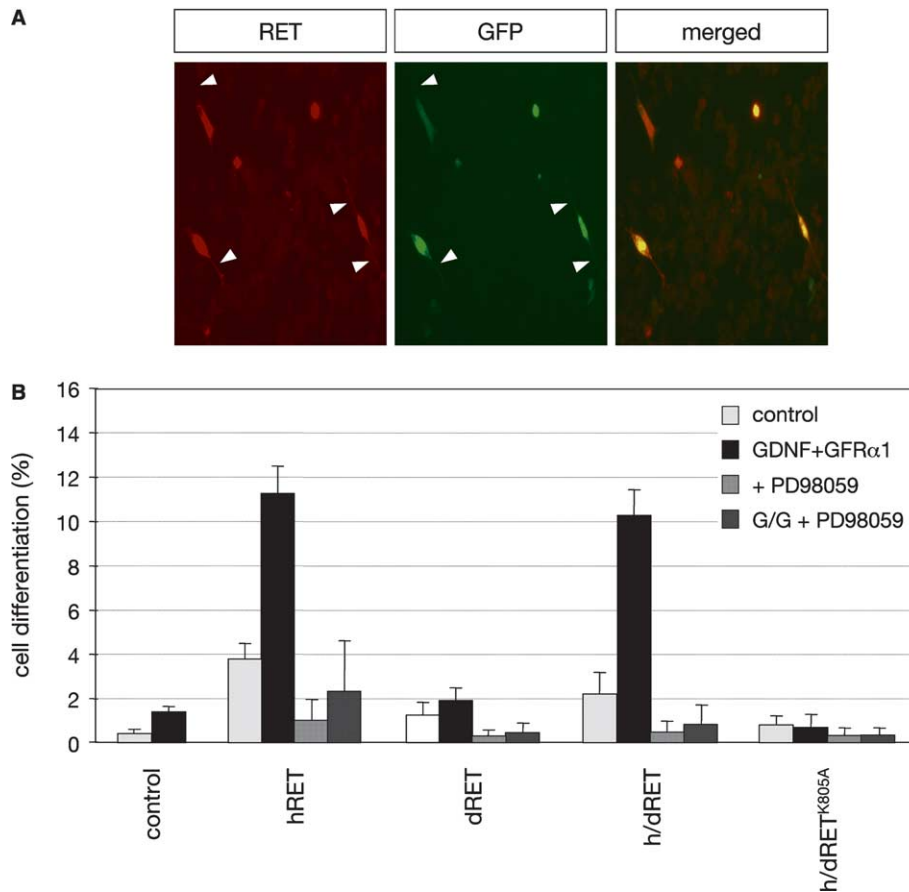


Fig. 4. The dRET kinase domain can mediate ligand-dependent neuronal differentiation of PC12 cells. (A) Expression of hRET<sup>ECD</sup>/dRET<sup>KIN</sup> in transiently transfected PC12 cells stimulated for three days with GDNF and GFR $\alpha$ 1 as analyzed by immunofluorescence with anti-panRET antibodies (left panel). The middle panel shows GFP expression in the same field, confirming that GFP positive cells express the hRET<sup>ECD</sup>/dRET<sup>KIN</sup> chimera. Neuritic processes in differentiated cells are indicated by arrowheads. A merged image is shown to the right. (B) Quantitative analysis of neuronal differentiation induced by GDNF and soluble GFR $\alpha$ 1 in PC12 cells transiently transfected with the indicated constructs. The number of GFP positive cells bearing neuritic processes longer than two cell diameters were counted and expressed as percentage of the total number of GFP-expressing cells. The MEK1 inhibitor PD98059 was used at 50  $\mu$ M as indicated.

has been detected in the developing retina of *Drosophila*, where the control of cell survival has been shown to be dependent on trophic support from target-derived molecules [30,31]. Despite their sequence divergence, the presence of CLDs across metazoan RET molecules identified suggested that these domains might have mediated cell adhesion functions in ancestral RET-like molecules, prior to the appearance of GDNF ligands. However, our combined in vitro association analyses with the dRET<sup>ECD</sup> and cell adhesion assays in Jurkat cells gave no indication that such activity is present in the dRET molecule.

It has been reported that a number of eukaryotic genes originally annotated as kinases based on sequence homologies actually correspond to pseudogenes and do not encode enzymatically active kinases [14]. Although cognate ligands of dRET are presently unknown, our analysis of the hRET<sup>ECD</sup>/dRET<sup>KIN</sup> chimera confirmed that the intracellular portion of the dRET receptor features an active kinase, and indicated that the dRET kinase domain may be activated in a ligand-dependent manner. The ability of the dRET kinase to mediate ligand-dependent neuronal differentiation of PC12 cells to a similar extent than hRET indicated a certain degree of conservation of functional and biochemical properties of the two

receptors. In agreement with this, both kinases required the MAP kinase pathway to induce neuronal differentiation in PC12 cells. The expression of dRET in sensory neurons of the fly suggests that pathways activated by this receptor may contribute to the differentiation of these cells. In this context, identification of endogenous dRET ligands may contribute to elucidate novel mechanisms of developmental regulation of specialized cell types in the fly.

In conclusion, our findings reinforce the hypothesis of an evolutionary link between human and *Drosophila* RET, not only from a structural point of view, but also based on the functional analysis of the dRET protein. In addition, the biochemical tools developed in this study may help to identify the endogenous ligand(s) of dRET and further our understanding of the physiological roles of this protein at the molecular level.

#### 4. Materials and methods

##### 4.1. Plasmid constructs

The LCL12 plasmid containing a full-length dRET cDNA was a kind gift from Dr. Michael Bishop (UCSF, San Francisco). The dRET<sup>ECD</sup> was isolated from this plasmid by PCR and subcloned in a modified pSec-Tag2A mammalian expression vector (Invitrogen)

containing HA, myc or human Fc tags as described [20]. HA-tagged hRET<sup>ECD</sup> has been described elsewhere [20]. Full-length dRET expression plasmids were constructed using expression vectors for insect (pIB-V5HIS, Invitrogen) or mammalian (pCDNA3, Invitrogen) cells and a dRET cDNA amplified from LCL12. Full-length hRET plasmid (long isoform) has been described elsewhere [32] (see Fig. 3A). In order to generate the hRET<sup>ECD</sup>/dRET<sup>KIN</sup> chimeric construct, a fragment corresponding to the dRET kinase domain flanked by a C-terminal myc tag was amplified by PCR and subcloned into the corresponding position of the hRET cDNA (see Fig. 3A for details). The K805M mutation was introduced in the wild-type sequence of the intracellular domain of dRET by site-directed mutagenesis. All constructs were verified by automated DNA sequencing. Enhanced green fluorescence plasmid (GFP) was from Clontech.

#### 4.2. Cell culture, transfection, immunofluorescence and cell adhesion assay

*Drosophila* Schneider's 2 (SL2) and mammalian cells were cultured under standard conditions and transfected with Fugene6 (Roche) according to the manufacturer's instructions. PC12 cells growing on PDL-coated 48-well plates that had been differentiated for 3–4 days with GDNF (50 ng/ml) and GFR $\alpha$ 1-Fc (150 ng/ml) were fixed in 4% paraformaldehyde in PBS, washed, permeabilized with 0.1% Triton X-100 and blocked with 1% Goat serum. They were then incubated with primary antibody (panRET anti-Y905 or anti-myc) and washed before addition of biotin-SP conjugated anti-mouse antibody (Jackson Immunoresearch). The reaction was then developed with Cy3-conjugated streptavidin (Jackson Immunoresearch) and visualized with an inverted fluorescence microscope. Cell adhesion assays in transiently transfected Jurkat cells were performed as previously described [33].

#### 4.3. Expression of soluble dRET<sup>ECD</sup> and in vitro binding assay

COS cells were transiently transfected with the dRET<sup>ECD</sup> pSec-Tag2A plasmid and, 24 h after transfection, complete medium was replaced by serum-free medium as previously described [20]. At this time, cultures were moved to 30 °C to enhance production yields [20] and left for 3–4 days. Conditioned medium was concentrated by ultrafiltration (Amicon) and protein concentration was normalized by immunoblotting with HA antibodies. hRET<sup>ECD</sup> was produced, purified and normalized in the same way following previously published procedures [20]. In order to assess binding of RET<sup>ECD</sup> constructs to the GDNF/GFR $\alpha$ 1 complex, a solid-phase immunosorbent assay was utilized as previously described [20]. Briefly, blocked ELISA wells were coated with anti-HA antibodies (Covance) and HA-tagged RET<sup>ECD</sup> proteins were added. The samples were then washed, and subsequently a complex of GDNF and Fc-tagged GFR $\alpha$ 1 was added. After washing, the reaction was developed with goat anti-human Fc antiserum, HRP-conjugated anti-goat antibodies (Jackson Immunoresearch), and the 3,3',5,5'-tetramethyl benzidine (TMB) substrate according to the instructions of the manufacturer (Pierce).

#### 4.4. Immunoprecipitation, immunoblotting, deglycosylation and chemical cross-linking assays

Immunoprecipitations and immunoblottings were performed as previously described [20] using antibodies as follows: anti-myc, anti-phosphotyrosine (PY99) from Santa Cruz Biotechnology; anti-HA from Covance Biosite, anti-panRET (Y905) monoclonal was kindly provided by Anatoly Sharipo (University of Latvia, Riga). All blots were developed with enhanced chemifluorescence (Amersham Biosciences), scanned in a Storm 840 (Molecular Dynamics) and analyzed with ImageQuant software. For deglycosylation assays, immunoprecipitates were eluted by boiling in glycosidase denaturing buffer (New England Biolabs), EndoH or PNGaseF digestion buffer was added to the samples, and digestions with either EndoH or PNGaseF were performed according to the instructions of the manufacturer (New England Biolabs). Soluble dRET<sup>ECD</sup> contained in the conditioned medium harvested from transiently transfected COS cells was assayed as described [20]. GDNF was radioiodinated by the lactoperoxidase method. Chemical cross-linking assays in transfected COS cells were performed with 20 ng/ml <sup>125</sup>I-GDNF in the presence or absence of 500-fold excess unlabeled (cold) GDNF using EDAC and Sulfo-NHS as previously described [33].

#### 4.5. PC12 differentiation assay

Twelve hours after transfection, PC12 cells growing on PDL-coated 48-well plates were transferred to serum-free medium, left for 24 h, and then stimulated with GDNF (80 ng/ml) and GFR $\alpha$ 1-Fc (140 ng/ml) in medium supplemented with 0.5% fetal calf and 1% horse serum. For the MAP kinase pathway inhibition experiments, the MEK1 inhibitor PD98059 (Sigma) was used to a final concentration of 50  $\mu$ M. After 3–4 days, the number of GFP expressing cells was determined in 10 different fields, and the fraction of cells displaying a neurite longer than two cell diameters was calculated. At least three independent determinations were performed after transient transfection of each construct, and in each experiment values were assessed in 3–6 duplicates per sample. Levels of expression of hRET, dRET or the hRET<sup>ECD</sup>/dRET<sup>KIN</sup> chimera were confirmed by immunofluorescence as described above.

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