Cellular/Molecular

Lipid Rafts Are Physiologic Membrane Microdomains Necessary for the Morphogenic and Developmental Functions of Glial Cell Line-Derived Neurotrophic Factor *In Vivo*

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Glial cell line-derived neurotrophic factor (GDNF) promotes PNS development and kidney morphogenesis via a receptor complex consisting of the glycerophosphatidylinositol (GPI)-anchored, ligand binding receptor GDNF family receptor α1 (GFRα1) and the receptor tyrosine kinase Ret. Although Ret signal transduction *in vitro* is augmented by translocation into lipid rafts via GFRα1, the existence and importance of lipid rafts in GDNF–Ret signaling under physiologic conditions is unresolved. A knock-in mouse was produced that replaced GFRα1 with GFRα1–TM, which contains a transmembrane (TM) domain instead of the GPI anchor. GFRα1–TM still binds GDNF and promotes Ret activation but does not translocate into rafts. In *Gfrα1*$^{TM/TM}$ mice, GFRα1–TM is expressed, trafficked, and processed at levels identical to GFRα1. Although *Gfrα1*$^{+/TM}$ mice are viable, *Gfrα1*$^{TM/TM}$ mice display bilateral renal agenesis, lack enteric neurons in the intestines, and have motor axon guidance deficits, similar to *Gfrα1*$^{-/-}$ mice. Therefore, the recruitment of Ret into lipid rafts by GFRα1 is required for the physiologic functions of GDNF in vertebrates.

Key words: **GDNF; lipid raft; neurotrophic factor; Ret; spinal motor neuron; transgenic**

Significance Statement

Membrane microdomains known as lipid rafts have been proposed to be unique subdomains in the plasma membrane that are critical for the signaling functions of multiple receptor complexes. Their existence and physiologic relevance has been debated. Based on *in vitro* studies, lipid rafts have been reported to be necessary for the function of the Glial cell line-derived neurotrophic factor (GDNF) family of neurotrophic factors. The receptor for GDNF comprises the lipid raft-resident, glycerophosphatidylinositol-anchored receptor GDNF family receptor α1 (GFRα1) and the receptor tyrosine kinase Ret. Here we demonstrate, using a knock-in mouse model in which GFRα1 is no longer located in lipid rafts, that the developmental functions of GDNF in the periphery require the translocation of the GDNF receptor complex into lipid rafts.

Introduction

Glial cell line-derived neurotrophic factor (GDNF) is a growth factor that is critical for the development of the nervous system and kidneys (Airaksinen and Saarma, 2002). The high-affinity binding receptor for GDNF is GDNF family receptor α1 (GFRα1), a glycerophosphatidylinositol (GPI)-anchored cell-surface protein (Treanor et al., 1996; Cacalano et al., 1998). During GDNF binding to GFRα1, which causes the dimerization of two GFRα1 molecules, this complex then binds to and activates signal-transducing receptors (Bespalov and Saarma, 2007). The receptor tyrosine kinase Ret is necessary for the functions of GDNF in the PNS, such as motor neuron axon guidance (Enomoto et al., 2000; Kramer et al., 2006; Runeberg-Roos and Saarma, 2007; Dudanova et al., 2010; Bonanomi et al., 2012). GDNF/GFRα1-mediated Ret activation is also required for kidney morphogenesis and for the proliferation, migration, and differentiation of enteric precursors that form the enteric nervous system (Baloh et al., 2000).

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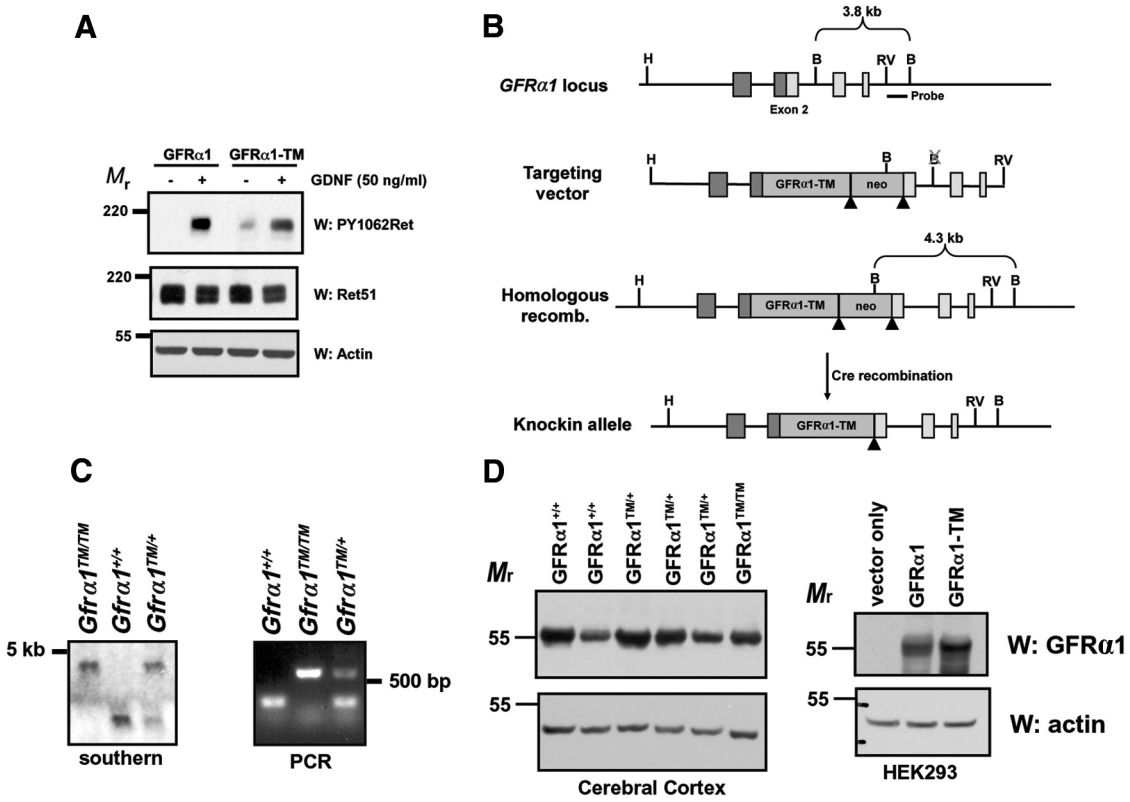

Figure 1. Production of knock-in mice in which GFRα1 is excluded from lipid rafts. ***A***, Stable Neuro2A cell lines expressing either GFRα1 or GFRα1-TM were stimulated with GDNF (50 ng/ml) for 15 min before detergent extraction. The extracts were immunoblotted with antibodies to phosphorylated Ret (PY1062Ret), total Ret51, and actin. GDNF activates Ret to a similar extent in Neuro2A cells expressing GFRα1–TM as those expressing GFRα1. ***B***, Targeting strategy for the production of GFRα1–TM knock-in mice. Insertion of the cDNA for human GFRα1–TM into exon 2 of *Gfrα1* eliminated expression of the native gene. ***C***, Confirmation of homologous recombination of the targeting vector into the *Gfrα1* locus was confirmed by Southern blotting using a probe outside of the targeted region (left). Genotyping of F2 offspring by PCR identified mice that were $Gfr\alpha1^{+/+}$, $Gfr\alpha1^{+/TM}$, and $Gfr\alpha1^{TM/TM}$ (right). ***D***, GFRα1–TM is expressed at levels similar to GFRα1 in knock-in mice. Whole cellular extracts from transfected HEK293 cells (right) or cerebral cortices (left) of $Gfr\alpha1^{+/+}$, $Gfr\alpha1^{+/TM}$, and $Gfr\alpha1^{TM/TM}$ mice were immunoblotted with antibodies to GFRα1 that detects both normal and mutant GFRα1. Actin immunoblotting of the extracts confirmed similar loading of protein among the different samples.

Lipid rafts are membrane microdomains that are enriched in cholesterol and sphingolipids, forming more ordered lipid bilayers than the surrounding plasma membrane (Simons and van Meer, 1988; Patra, 2008; Simons and Sampaio, 2011). Lipid rafts are enriched with GPI-anchored proteins and proteins that are modified with saturated lipids, such as Src family kinases. In cultured cells, GFRα1 is enriched highly in lipid rafts because of its GPI anchor, but Ret is excluded from lipid rafts under basal, unactivated conditions (Tansey et al., 2000; Paratcha et al., 2001). During the exposure of primary neurons to GDNF *in vitro*, Ret is translocated rapidly into lipid rafts in a GFRα1-dependent manner, which is important for downstream signal transduction (Tansey et al., 2000; Encinas et al., 2001; Paratcha et al., 2001; Pierchala et al., 2006). Investigations of the importance of lipid rafts in Ret signal transduction is based on biochemical experiments using detergent insolubility or buoyancy on density gradients. However, it has been argued that cooling cells down and using detergents for cell lysis may coalesce membrane microdomains that do not exist normally (Munro, 2003). Direct evidence for the existence of lipid rafts in living cells using imaging techniques, such as fluorescence energy transfer, have provided varied results (Simons and Ikonen, 1997; Munro, 2003; Simons and Sampaio, 2011). Identifying the cellular functions of lipid rafts typically relies on their disruption via the depletion of cholesterol or sphingomyelin in the plasma membrane of cultured cells, which likely also affects non-raft-dependent functions (Brown and London, 1998; Galbiati et al., 2001; Kenworthy, 2002; Munro, 2003). Because these *in vitro* methods and treatments cannot be applied to complex, multicellular organisms, evidence for the physiological relevance of lipid rafts at an organismal level remains lacking.

We report here the production of a knock-in mouse containing a GFRα1 gene replacement such that GDNF still promotes Ret activation but not its translocation into lipid rafts. These knock-in mice displayed renal agenesis, a loss of the enteric nervous system, and motor neuron axon pathfinding defects reminiscent of GFRα1 knock-out mice, providing evidence for the physiologic importance of lipid rafts in neurotrophic factor signaling *in vivo*.

Materials and Methods

Production of GFRα1–TM knock-in mice. The cDNA for human GFRα1–TM was kindly provided by Jeffrey Milbrandt (Washington University, St. Louis, MO) and subcloned into the second exon of the *Gfrα1* gene. The plasmid encoding the genomic region flanking exon 2, along with other constructs for homologous recombination, were generously provided by Hideki Enomoto (RIKEN Center for Developmental Biology, Kobe, Japan). Homologous recombination of ES cell clones was confirmed by Southern blotting using a probe outside of the targeting construct region, and subsequent offspring were genotyped by PCR (Fig. 1*C*). The PCR primers are as follows: common forward, 5′-CTTCCAGGTTGGGTCGGAACTGAACCC; wild-type reverse, 5′-AGAGAGCTCAGCGTGCAGAGATC; and mutant reverse, 5′-CATGCTCCAGTAG ATACGCAGACA.

Two independent knock-in lines from two different ES cell clones were analyzed and had identical phenotypes. An AC–Cre cassette was used in the targeting construct that allowed the expression of Cre in somatic cells but self-excises in the male germ line (Bunting et al., 1999). The original two founder lines were backcrossed seven generations into C57BL/6 mice, and these mice were used for all the studies described. For the examination of spinal motor neuron projections, *Gfrα1*$^{TM/+}$ mice were bred with Hb9–GFP mice (The Jackson Laboratories), and embryos were imaged directly under a stereoscope with fluorescent illumination (Discovery V8; Zeiss).

Primary sympathetic neurons. Sympathetic neurons of the superior cervical ganglion (SCG) were dissected, enzymatically dissociated, and maintained *in vitro* as described previously (Tsui and Pierchala, 2010). The SCGs from each mouse were isolated and cultured separately, and their genotype ascertained from extracted tail DNA. Thus, individual conditions typically represented extracts produced from sympathetic neurons derived from individual mice. For the pharmacologic inhibition of matrix metalloproteinases and phospholipases, TNF-α protease inhibitor 1 (TAPI-1; 10 μM; Calbiochem) and U73122 (1-[6[[(17β)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1*H*-pyrrole-2,5-dione; 2 μM; Sigma), respectively, were applied to the cultures for 48 h. Vehicle alone (DMSO; Sigma) served as the negative control, and phosphatidylinositol-specific phospholipase C enzyme (PI-PLC; 2 U/ml; Sigma) served as a positive control.

Tissue extractions and immunoprecipitations. Whole embryos [embryonic day 14.5 (E14.5) to E18.5] or organs dissected from E19.5 mouse pups were homogenized using a Tissuelyser II with steel grinding jars (Qiagen). These homogenates were then detergent extracted (10% glycerol, 1% Nonidet P-40, and sodium orthovanidate in Tris-buffered saline, pH 7.4). Insoluble debris was removed by centrifugation, and the supernatant was then used for immunoprecipitations or was used directly for immunoblotting. For immunoprecipitations, crude extracts were precleared by incubation with protein A and protein G (Roche) for 1 h at 4°C. The protein A/G was then removed by centrifugation, and Ret antibodies (Ret C19-G and Ret C20 mixed; Santa Cruz Biotechnology) or GFRα1 antibodies (Cell Sciences), along with fresh protein A/G, were added and allowed to incubate in the extracts overnight at 4°C with gentle agitation. Immunoprecipitates were then washed, and the protein complexes were denatured by boiling them in SDS-PAGE sample buffer (2% SDS, 1% β-mercaptoethanol, 10% glycerol, and bromophenol blue in Tris, pH 6.8). For the analysis of soluble and secreted proteins, sympathetic neurons were maintained in culture for 5–7 d. The medium was then replaced with serum-free medium, and the neurons were maintained for an additional 4 d with two medium changes (4 ml total). The conditioned medium was centrifuged for 30 min at 4°C (16,000 × *g*) to remove cellular debris, and the proteins in the conditioned medium supernatants were precipitated using saturated trichloroacetic acid (TCA). The protein pellets were washed twice with ice-cold ethanol/ether (1:1 v/v) to remove any remaining TCA and were then analyzed by immunoblotting. Equal volumes of conditioned medium from equal numbers of neurons were analyzed in each condition.

Cell-surface biotinylation. Biotinylation of cell-surface proteins of primary neurons was performed as described previously (Tsui and Pierchala, 2010). Biotinylated proteins were isolated from intracellular proteins by detergent extraction of the neurons, followed by the selective isolation of biotinylated proteins using streptavidin agarose (Pierce Thermo Fisher Scientific). Isolated proteins were then analyzed by immunoblotting.

Immunoblotting analysis. Denatured protein extracts were subjected to SDS-PAGE and blotted onto PVDF membranes (Immobilon P; Millipore). Membranes were incubated in either 5% milk or 3% BSA for 1 h before an overnight incubation in the primary antibody at 4°C. The blots were then washed, incubated in the appropriate HRP-linked secondary antibody, and visualized by using a chemiluminescent substrate (Pierce Thermo Fisher Scientific). The antibodies used, and their working dilutions from the stock, were as follows: anti-GFRα1 (1:1000; R&D Systems), anti-phosphotyrosine (1:2000, clone 4G10; Millipore), anti-actin and anti-Ret51 (1:1000 each; Santa Cruz Biotechnology), anti-caveolin-1 (1:500; Sigma), anti-transferrin receptor (1:1000; Invitrogen), and anti-multi-ubiquitin (1:1000, clone FK2; Enzo Life Sciences). Quantification of immunoblots was performed using NIH ImageJ.

Acetylcholinesterase histochemistry and tyrosine hydroxylase immunohistochemistry. Sections of small and large intestine were dissected from E18.5 embryos, and the mesenteric attachments were removed. These tissues were incubated in a glycine buffer, pH 5.6, containing ethopropazine HCl, aceytlthiocholine iodide, cupric sulfate, and sodium acetate (all chemicals from Sigma) for 30 min. The color was developed by incubation in sodium sulfide for 2–3 min. The intestines were then mounted in glycerol and imaged using a stereoscope with polarized lighting (Discovery V8; Zeiss). Whole-mount immunohistochemistry for tyrosine hydroxylase was performed as described previously (Enomoto et al., 2001). After immunostaining, the embryos were dissected into an "open-book" preparation for stereoscope imaging to reveal the sympathetic chain ganglia and projections.

Results

Production of mice that selectively lack lipid raft-mediated GDNF signaling

To determine whether lipid rafts are required for the developmental functions of GDNF, knock-in mice were produced in which the GFRα1 gene was replaced with a cDNA encoding GFRα1–TM. GFRα1–TM is a fusion protein in which GFRα1 lacking the amino acids that target GPI anchorage were replaced with the transmembrane (TM) domain of HLA–B44 (Hansbrough et al., 1991; Tansey et al., 2000). This results in a GFRα1 molecule that is not GPI anchored and instead has a TM domain that targets GFRα1 to the cell surface but not to lipid rafts (Hansbrough et al., 1991; Tansey et al., 2000). GFRα1–TM still binds to GDNF and promotes efficient Ret activation but not the translocation of this signaling complex into lipid rafts (Fig. 1*A*; Tansey et al., 2000). For the generation of the targeting construct, GFRα1–TM was inserted into exon 2 of *Gfrα1*, thereby disrupting exon 2 and resulting in a complete loss of GFRα1 expression (Fig. 1*B*, *C*), as was done previously to make GFRα1 knock-out mice (Enomoto et al., 1998).

To determine whether GFRα1–TM protein in *Gfrα1*$^{TM/TM}$ mice was expressed at levels similar to native GFRα1, cellular extracts were produced from the cerebral cortex (Fig. 1*D*) of postnatal day 0 mice and analyzed by immunoblotting. GFRα1–TM from *Gfrα1*$^{TM/TM}$ mice was expressed at levels similar to native GFRα1 from *Gfrα1*$^{+/+}$ mice compared with the levels of actin. In transfection studies, the vast majority of GFRα1–TM migrates at a similar molecular weight to GPI-anchored GFRα1 by SDS-PAGE (Fig. 1*D*). Thus, the production of *Gfrα1*$^{TM/TM}$ knock-in mice using this strategy did not result in a hypomorphic allele that could confound the phenotypic analysis.

Plasma membrane targeting is normal in *Gfrα1*$^{TM/TM}$ mice

To confirm that the replacement of the GPI anchor of GFRα1 with a TM domain did not impair its trafficking to the plasma membrane, primary sympathetic neurons from *Gfrα1*$^{+/+}$, *Gfrα1*$^{+/TM}$, and *Gfrα1*$^{TM/TM}$ mice were subjected to cell-surface biotinylation. Cell-surface proteins were then isolated from other proteins using immobilized streptavidin and analyzed by immunoblotting (Fig. 2*A*). This analysis revealed that a similar amount of GFRα1–TM was localized to the plasma membrane of *Gfrα1*$^{TM/TM}$ neurons compared with GPI-anchored GFRα1 from *Gfrα1*$^{+/+}$ neurons, indicating that intracellular trafficking of GFRα1–TM to the plasma membrane was not altered.

Ret activation is unaffected in *Gfrα1*$^{TM/TM}$ mice

The GFRα1–TM fusion does not alter the region of GFRα1 that binds to GDNF and, therefore, does not affect formation of the

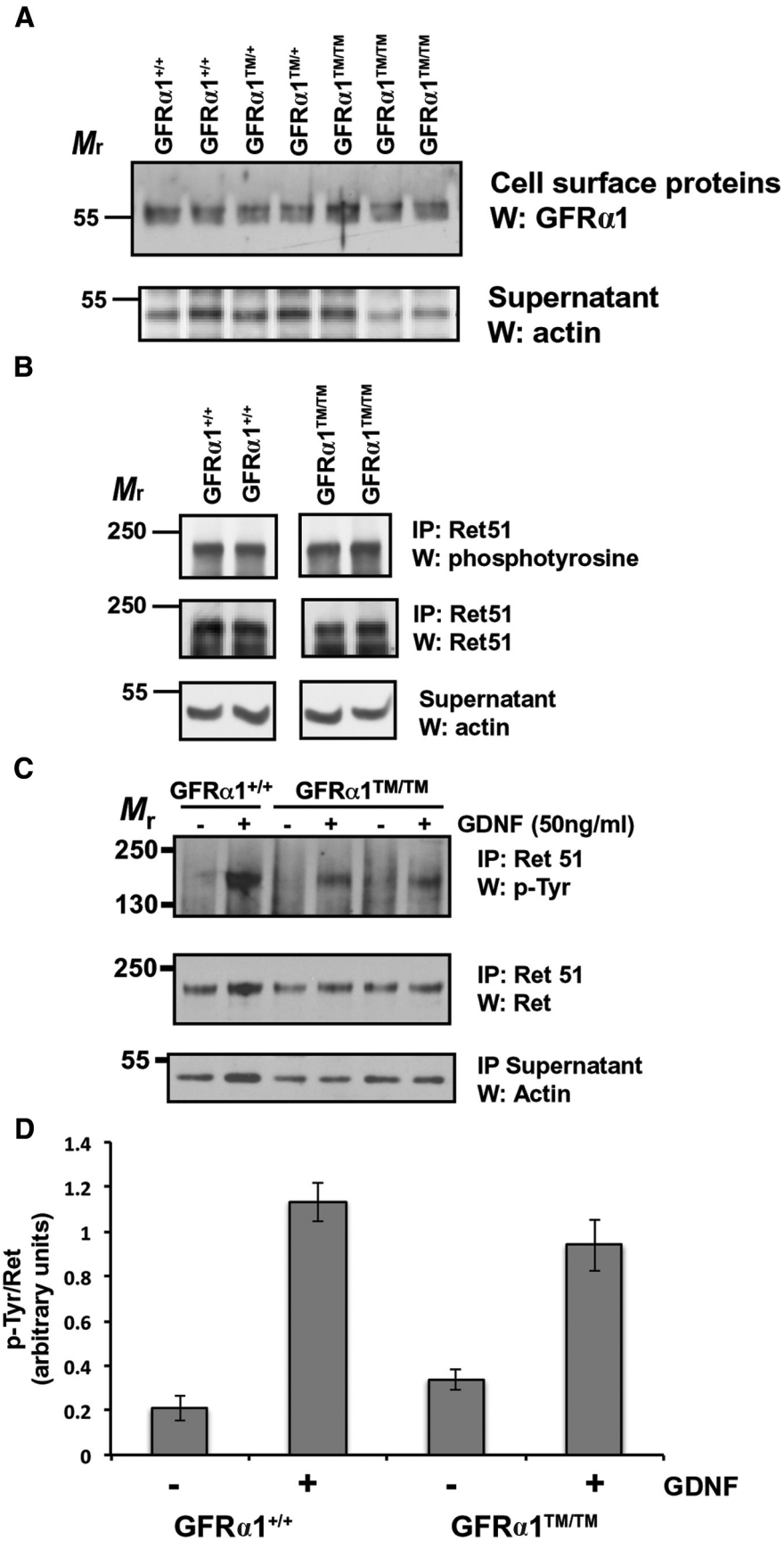

Figure 2. The localization of GFRα1 in lipid rafts is not necessary for the subcellular trafficking or activation of Ret. ***A***, Primary neurons were subjected to cell-surface biotinylation, followed by detergent extraction and isolation of the biotinylated, cell-surface proteins. The cell-surface proteins were subjected to GFRα1 immunoblotting (top), and actin immunoblotting of the remaining supernatant fractions served as protein loading controls (bottom). ***B***, E15.5 $Gfr\alpha1^{+/+}$ and $Gfr\alpha1^{TM/TM}$ mice were homogenized and detergent extracted. Ret was isolated from these extracts by immunoprecipitation, and the level of activation was determined by phosphotyrosine immunoblotting. The amount of Ret in each sample was ascertained by reprobing the blots with Ret51 antibodies, and actin immunoblotting of supernatants served to confirm the analysis of equal amounts of protein. These experiments were performed three times with similar results. ***C***, Primary neurons from $Gfr\alpha1^{+/+}$ and $Gfr\alpha1^{TM/TM}$ mice were stimulated with GDNF (50 ng/ml) or medium alone for 10 min. The neurons were then detergent extracted, Ret51 was immunoprecipitated from the extracts, and its level of activation was determined by phosphotyrosine immunoblotting (top). The level of Ret in each condition was ascertained by reprobing these blots with Ret51 antibodies (middle), and actin immunoblotting of the immunoprecipitation supernatants served as a loading control (bottom). ***D***, The experiments shown in ***C*** were quantified and graphed as the mean ± range ($n = 2$) or SEM ($n = 3$). Similar results were obtained from neurons derived from two $Gfr\alpha1^{+/+}$ and three $Gfr\alpha1^{TM/TM}$ mice from two separate cultures.

GDNF/GFRα1/Ret complex or the autophosphorylation of Ret (Fig. 1*A*; Tansey et al., 2000). To confirm that this was also true in GFRα1–TM knock-in mice, whole E15.5 $Gfr\alpha1^{+/+}$ and $Gfr\alpha1^{TM/TM}$ mice were homogenized, and Ret was isolated from these extracts (Fig. 2*B*). Phosphotyrosine immunoblotting of the Ret immunoprecipitations revealed that there was no difference in the amount of autophosphorylated Ret between these two genotypes, suggesting that GFRα1–TM is fully capable of forming an activated GDNF/Ret complex *in vivo*. To further confirm that GDNF family ligand (GFL)-initiated Ret autophosphorylation was not impaired in neurons expressing GFRα1–TM, sympathetic neurons from the SCG were isolated and cultured from $Gfr\alpha1^{+/+}$ and $Gfr\alpha1^{TM/TM}$ mice. These neurons were then stimulated with GDNF, or medium alone, and the extent of Ret activation was determined by Ret immunoprecipitation, followed by phosphotyrosine immunoblotting (Fig. 2*C*, *D*). GDNF activated Ret to a similar extent in neurons derived from $Gfr\alpha1^{TM/TM}$ mice as it did neurons from $Gfr\alpha1^{+/+}$ mice (Fig. 2*C*, *D*), indicating that GFRα1–TM is capable of activating Ret during GDNF stimulation.

GFRα1–TM is shed from the plasma membrane to a similar extent as wild-type GFRα1

GFRα1 can be shed from the plasma membrane of cells maintained *in vitro* and from tissues *in vivo*, and soluble GFRα1 can participate in signaling events *in trans*

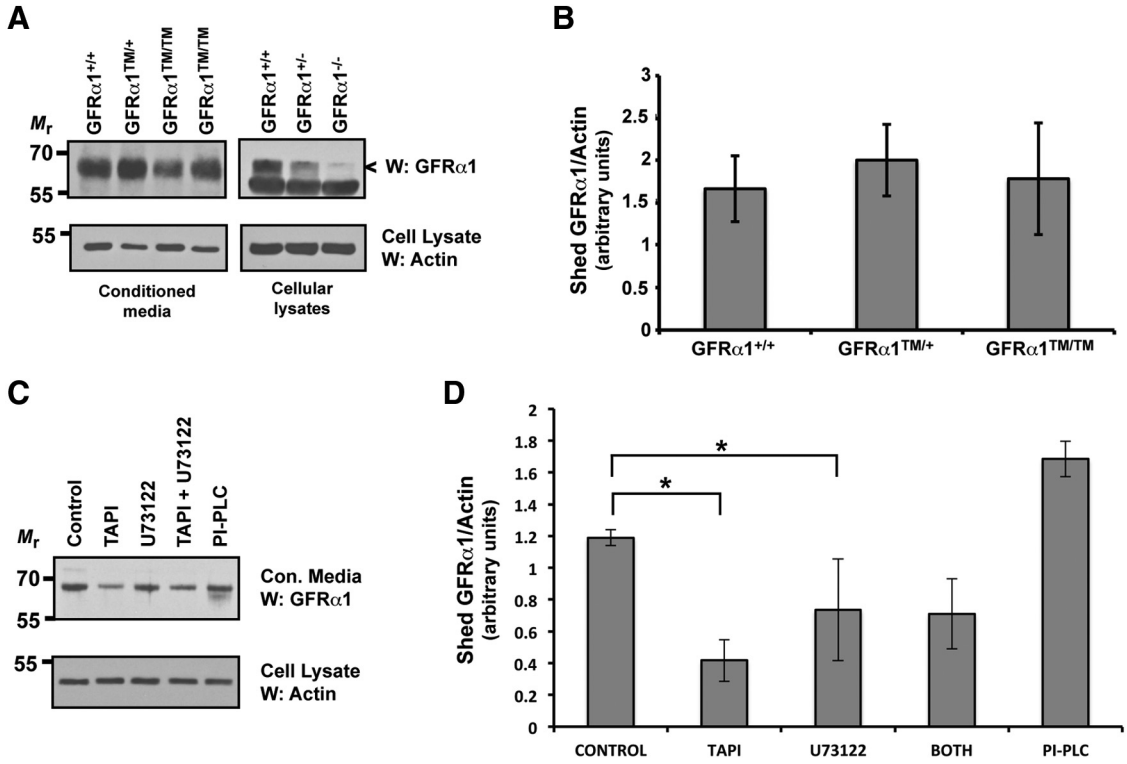

Figure 3. Shedding of GFRα1 is dependent on matrix metalloproteinases. ***A***, The conditioned medium from *Gfrα1*$^{+/+}$, *Gfrα1*$^{+/TM}$, and *Gfrα1*$^{TM/TM}$ neurons was collected and cleared of cellular debris, and the proteins were precipitated. The presence of soluble GFRα1/GFRα1–TM was analyzed by GFRα1 immunoblotting (top). Actin immunoblotting of cellular extracts from the neurons that conditioned the medium confirmed the analysis of similar amounts of neurons (bottom). GFRα1 immunoblotting of brain extracts from *Gfrα1*$^{+/+}$, *Gfrα1*$^{+/-}$, and *Gfrα1*$^{-/-}$ mouse embryos confirmed the specificity of the band that we identified as GFRα1 from conditioned medium (right). ***B***, The immunoblots shown in ***A*** were quantified and graphed as the mean ± SEM. There were no significant differences among any of the genotypes. Three to five mice of each genotype from at two separate cultures were analyzed. ***C***, Primary sympathetic neurons were exposed to TAPI-1 (metalloproteinase inhibitor), U73122 (phospholipase inhibitor), both TAPI-1 and U73122, or medium containing vehicle alone, for 48 h. As a positive control, a separate set of neurons was exposed to PI-PLC for 2 h. The amount of released GFRα1 was determined as in ***A***. ***D***, The experiments displayed in ***C*** were quantified and graphed as the mean ± SEM. There was a significant reduction in the amount of released GFRα1 during TAPI-1 and U73122 treatment compared with neurons exposed to vehicle alone ($p < 0.05$). Exposure to both TAPI-1 and U73122 did not have any additional inhibitory effect. This analysis represents three to four separate dishes of each condition from three independent cultures.

(Paratcha et al., 2001). If cleavage from the membrane occurs via a lipase that cleaves the GPI anchor of GFRα1, then *Gfrα1*$^{TM/TM}$ mice would be deficient in the membrane shedding of GFRα1–TM. Therefore, the extent of membrane shedding was examined in primary sympathetic neurons from *Gfrα1*$^{+/+}$, *Gfrα1*$^{+/TM}$, and *Gfrα1*$^{TM/TM}$ mice. Conditioned medium was not collected until after 7 d *in vitro*, well after the initial period of cellular injury and death attributable to the enzymatic dissociation of the ganglia. On day 8 *in vitro*, conditioned medium was collected every 2 d for 4 d and was centrifuged thoroughly to remove any cellular debris. Soluble GFRα1 or GFRα1–TM was then analyzed from the medium by immunoblotting (Fig. 3*A*, *B*). Interestingly, there was just as much secreted GFRα1 from the *Gfrα1*$^{TM/TM}$ neurons as there was GFRα1 secreted from the *Gfrα1*$^{+/+}$ and *Gfrα1*$^{TM/+}$ neurons, and quantification of the immunoblots confirmed that there were no significant differences between the three genotypes (Fig. 3*B*). Immunoblotting of brain extracts from *Gfrα1*$^{+/+}$ and *Gfrα1*$^{-/-}$ mice with the GFRα1 antibody confirmed the specificity of our immunoblotting analysis (Fig. 3*A*). These data suggest that GFRα1 was shed from the plasma membrane by proteolysis, most likely by an extracellular protease, such as metalloproteinases, rather than by a phospholipase. To examine this possibility, wild-type primary sympathetic neurons were exposed to an inhibitor of matrix metalloproteinases (TAPI-1) or an inhibitor of phospholipases (U73122). After 48 h, the culture medium was collected, and the amount of GFRα1 shedding was analyzed by immunoblotting, as before. Inhibition of either metalloproteinases or phospholipases reduced significantly the amount of GFRα1 that was shed into the culture medium (Fig. 3*C*, *D*). Exposure of the cells to purified PI-PLC for 2 h resulted in GFRα1 cleavage and release into the culture medium as well, confirming this immunoblotting assay. Treatment of neurons with both TAPI-1 and U73122 did not have an additive effect on the inhibition of GFRα1 membrane shedding, suggesting that there may be other enzymes that can liberate GFRα1 from the cell surface (Fig. 3*C*, *D*). Thus, matrix metalloproteinases contribute to the shedding of GFRα1 from the surface of primary neurons. Together, *Gfrα1*$^{TM/TM}$ mice appeared to have normal levels of cell-surface GFRα1–TM shedding and would presumably be capable of normal levels of signaling *in trans*.

Ret does not translocate into lipid rafts during GDNF stimulation in *Gfrα1*$^{TM/TM}$ mice

As a proof of concept that the GDNF/GFRα1/Ret complex does not translocate into lipid rafts in *Gfrα1*$^{TM/TM}$ mice, lipid raft translocation experiments were performed. Detergent-resistant membranes were isolated from primary sympathetic neurons from *Gfrα1*$^{+/+}$ and *Gfrα1*$^{TM/TM}$ mice. When *Gfrα1*$^{+/+}$ neurons were stimulated with GDNF, Ret translocated rapidly into lipid rafts (Fig. 4*A*). This was in dramatic contrast to *Gfrα1*$^{TM/TM}$ neurons in which GDNF exposure resulted in a markedly reduced movement of Ret into the detergent-resistant, lipid raft fraction

(Fig. 4*A*). The small portion of Ret that did translocate into lipid rafts during GDNF stimulation may be attributable to Ret kinase-dependent translocation of Ret into rafts that occurs with slower kinetics (Tansey et al., 2000; Paratcha et al., 2001). Overall, quantification of these experiments indicated that there was a significant, 75% reduction in the movement of the Ret receptor complex into lipid rafts during GDNF exposure in *Gfrα1*$^{TM/TM}$ neurons (Fig. 4*B*).

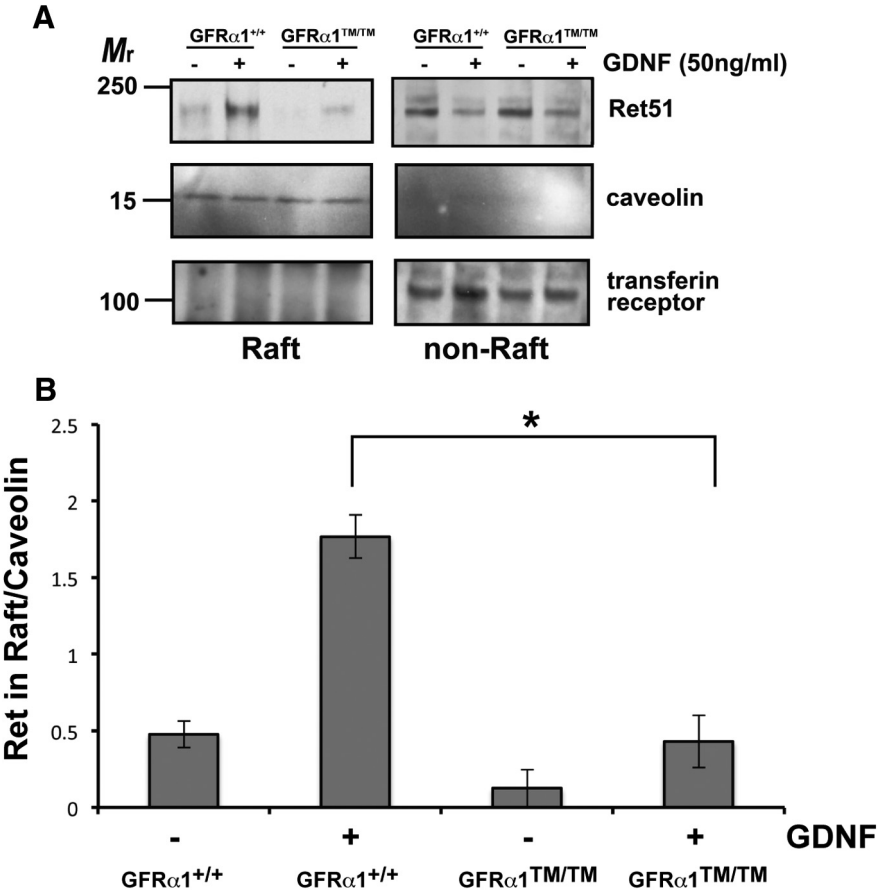

Figure 4. Ret does not translocate into lipid rafts during GFL activation in *Gfrα1*$^{TM/TM}$ neurons. ***A***, Primary sympathetic neurons isolated from *Gfrα1*$^{+/+}$ and *Gfrα1*$^{TM/TM}$ mice were maintained *in vitro* for several days. The neurons were then treated with GDNF or medium alone for 15 min. Detergent-resistant membranes were isolated from the neurons and analyzed by immunoblotting for Ret51. Immunoblotting for caveolin and transferrin receptor confirmed relative purity of the detergent-resistant and detergent-soluble fractions, respectively. ***B***, The experiments shown in ***A***, were quantified and graphed as the mean ± SEM. There was a statistically significant decrease in the amount of Ret51 that translocated into lipid rafts during GDNF stimulation in *Gfrα1*$^{TM/TM}$ neurons compared with *Gfrα1*$^{+/+}$ neurons ($p < 0.05$). This experiment was performed four times with similar results.

Gfrα1$^{TM/TM}$ mice have deficits in kidney morphogenesis and enteric nervous system development

We observed that *Gfrα1*$^{TM/TM}$ mice, but not *Gfrα1*$^{TM/+}$ or *Gfrα1*$^{+/+}$ littermate mice, died perinatally, with <10% of *Gfrα1*$^{TM/TM}$ mice surviving >24 h after birth. This was reminiscent of *Gfrα1*$^{-/-}$ mice that die perinatally as a result of renal agenesis (Enomoto et al., 1998). To determine whether there were deficits in kidney development, the urogenital tracts of *Gfrα1*$^{+/+}$, *Gfrα1*$^{TM/+}$, and *Gfrα1*$^{TM/TM}$ mice were analyzed. *Gfrα1*$^{TM/TM}$ mice displayed bilateral renal agenesis, in contrast to *Gfrα1*$^{TM/+}$ and *Gfrα1*$^{+/+}$ mice that had apparently normal kidneys (Fig. 5*A*, *B*). This renal morphogenesis phenotype was completely penetrant, and no *Gfrα1*$^{TM/TM}$ mice had kidneys, although other urogenital structures, such as the bladder and male and female gonads, appeared macroscopically normal. The examination of *Gfrα1*$^{TM/+}$ mice did not reveal any abnormalities, and renal development appeared grossly normal (Fig. 5*A*, *B*), suggesting that GFRα1–TM does not act in a dominant inhibitory manner in the presence of wild-type GFRα1. These data suggest that the presence of GFRα1 in lipid rafts is required for the ureteric bud branching initiated by GDNF and Ret to promote kidney morphogenesis.

A second dramatic developmental abnormality in *Gfrα1*$^{-/-}$ mice is the loss of enteric neurons distal to the stomach (Enomoto et al., 1998). During embryonic development, neural crest derivatives populate the gut, initiating their migration and proliferation in the stomach and continuing through both the small and large intestines. This process requires GDNF, which signals via GFRα1 and Ret. GDNF, GFRα1, and Ret null mice all display an identical loss of enteric neurons in both the small and large intestines (Schuchardt et al., 1994; Moore et al., 1996; Pichel et al., 1996; Sánchez et al., 1996; Cacalano et al., 1998; Enomoto et al., 1998). Acetylcholinesterase staining of both the small and large intestines of E18.5 *Gfrα1*$^{+/+}$, *Gfrα1*$^{TM/+}$, and *Gfrα1*$^{TM/TM}$ mice indicated that there was a dramatic loss of enteric neurons in the small and large intestines of *Gfrα1*$^{TM/TM}$ mice (Fig. 5*C*). All of the *Gfrα1*$^{TM/TM}$ mice analyzed lacked enteric neurons in the large intestine compared with the normal presence of neurons in G*frα1*$^{+/+}$ and *Gfrα1*$^{TM/+}$ mice. The small intestines of *Gfrα1*$^{TM/TM}$ mice were also typically devoid of enteric neurons, although some *Gfrα1*$^{TM/TM}$ mice (18%, 2 of 11 *Gfrα1*$^{TM/TM}$ mice) displayed initial enteric nervous system development in the proximal small intestine closest to the stomach. Overall, the enteric nervous system deficits of the *Gfrα1*$^{TM/TM}$ mice were somewhat less severe than in *Gfrα1*$^{-/-}$ mice, which have a complete loss of enteric neurons in both the small and large intestines (Cacalano et al., 1998; Enomoto et al., 1998). These data suggested that GDNF-mediated proliferation and migration of enteric precursors requires signaling of the Ret complex in lipid rafts.

Spinal motor neuron, but not sympathetic neuron, axonal projections are aberrant in *Gfrα1*$^{TM/TM}$ mice

The axonal projections of motor neurons into the hindlimb is a highly stereotyped process. Neurons from the lateral division of the lateral motor column (LMC_L) project into the dorsal portion of the limb and contribute to the peroneal nerve, whereas the medial division of the LMC projects into the ventral limb. The axon guidance mechanism that, at least in part, directs LMC_L neurons dorsally is the combinatorial activation of Ret via both a low level of GFRα1–GDNF signaling along with reverse signaling of EphA receptors expressed in the dorsal mesenchyme onto Ephrin-As expressed on the motor axons (Kramer et al., 2006; Dudanova et al., 2010, 2012; Bonanomi et al., 2012). Although it has been proposed that the "coincidence detection" via Ret from both GFRα1 and Ephrin-As requires their localization in lipid

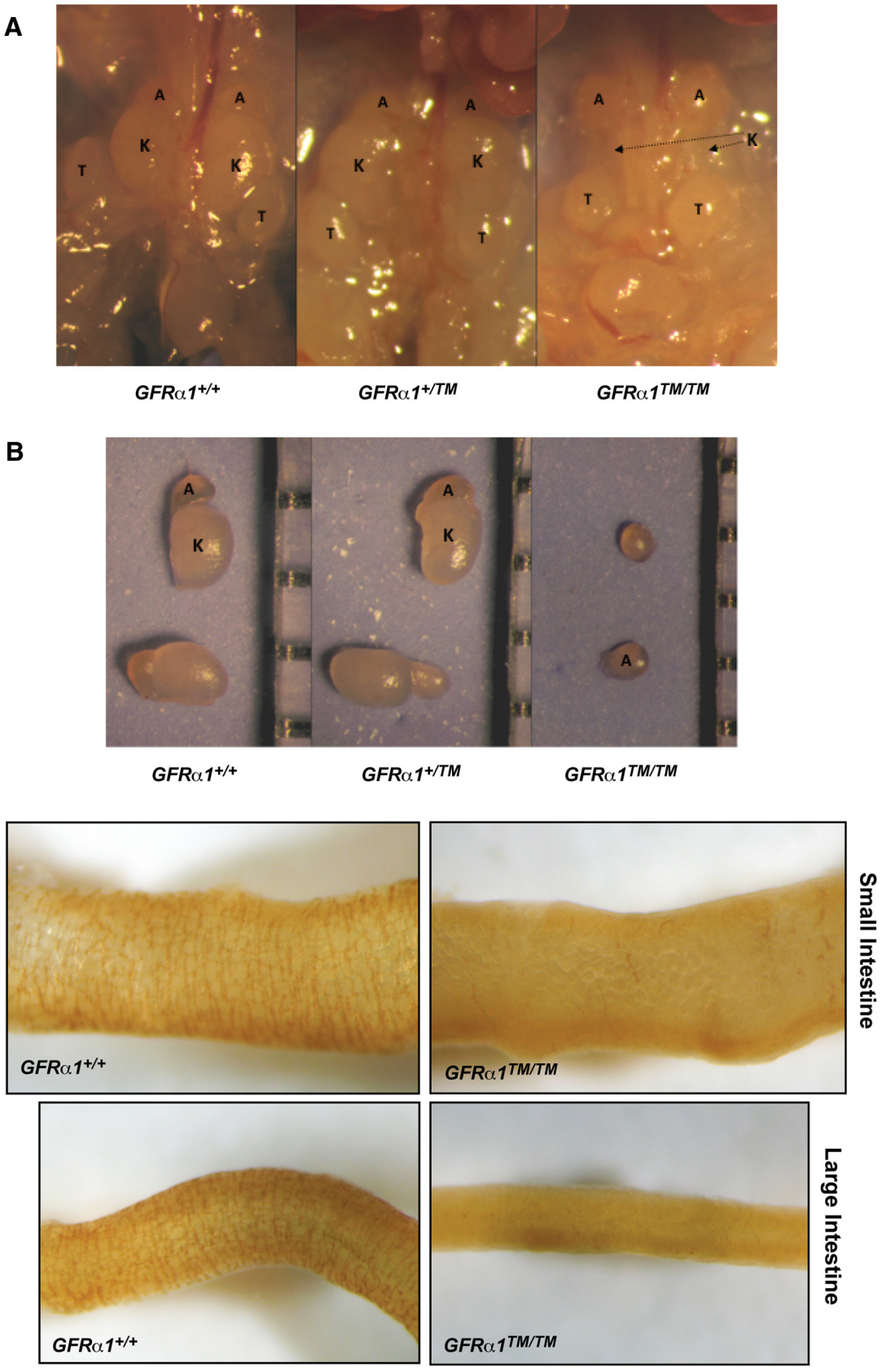

Figure 5. Localization of the GDNF signaling complex in lipid rafts is required for kidney morphogenesis and enteric nervous system development. ***A***, The urogenital tracts of $Gfr\alpha1^{+/+}$, $Gfr\alpha1^{+/TM}$, and $Gfr\alpha1^{TM/TM}$ mice were examined at P0. All $Gfr\alpha1^{TM/TM}$ mice lacked kidneys, whereas $Gfr\alpha1^{+/+}$ and $Gfr\alpha1^{+/TM}$ mice had normal, bilateral kidney development. ***B***, The size and pallor of the kidneys from $Gfr\alpha1^{+/TM}$ mice were similar to $Gfr\alpha1^{+/+}$ mice. Note that development of the adrenal glands appeared normal in all genotypes. The renal agenesis of $Gfr\alpha1^{TM/TM}$ mice was highly penetrant (128 of 128 $Gfr\alpha1^{TM/TM}$ mice had bilateral renal agenesis). In both ***A*** and ***B***, A in the images denotes the adrenal glands, K denotes the kidneys, and T indicates the testes. ***C***, Visualization of the myenteric plexes was performed using acetylcholinesterase histochemistry of the small intestines (top 2 panels) and large intestines (*Figure legend continues.*)

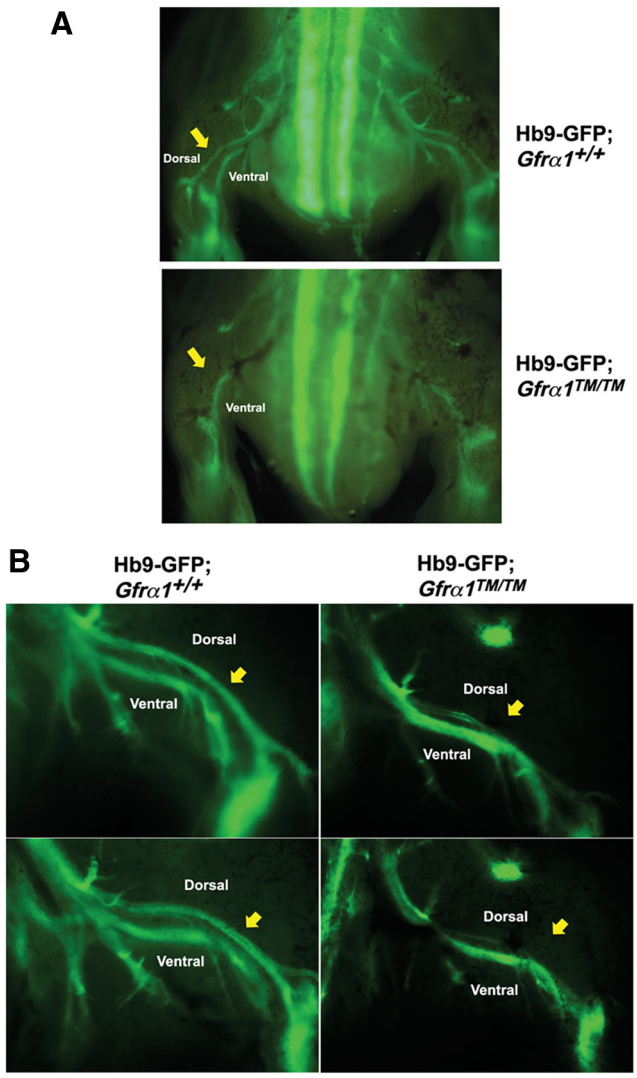

Figure 6. Spinal motor projections are deficient in $Gfr\alpha1^{TM/TM}$ mice. ***A***, Hb9–GFP; $Gfr\alpha1^{TM/TM}$ and Hb9–GFP;$Gfr\alpha1^{+/+}$ embryos were imaged directly in an open-book preparation. Dorsal and ventral projections in the hindlimb are clearly visible, and yellow arrows indicate the dorsal projection that is absent in Hb9–GFP;$Gfr\alpha1^{TM/TM}$ mice. ***B***, Sagittal view of the hindlimbs of Hb9–GFP;$Gfr\alpha1^{+/+}$ and Hb9–GFP;$Gfr\alpha1^{TM/TM}$ embryos with the dorsal hindlimb motor projection indicated by yellow arrows.

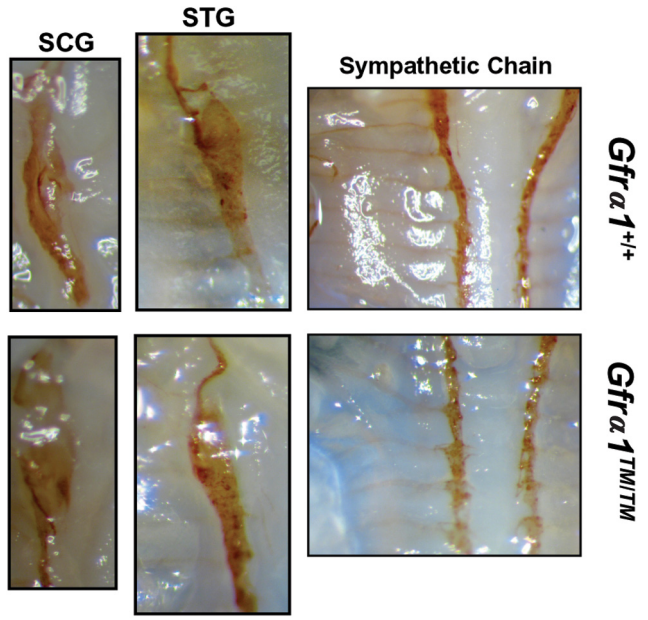

Figure 7. Development of the sympathetic nervous system is phenotypically normal in $Gfr\alpha1^{TM/TM}$ mice. Whole-mount tyrosine hydroxylase immunohistochemistry of P0 mice demonstrates normal positioning of the SCG (left) and the normal positioning and caudal axonal projections to structures of the STG (middle). The sympathetic chain ganglia (right) in $Gfr\alpha1^{+/+}$ and $Gfr\alpha1^{TM/TM}$ mice are also phenotypically normal and display similar axonal projections. Identical phenotypes were observed from three to five individual mice of each genotype from two to three independent litters.

rafts (Bonanomi et al., 2012), there has not been a means to test this *in vivo*. To determine whether the dorsal projection of LMC_L neurons require the localization of GFRα1 in lipid rafts, $Gfr\alpha1^{TM/TM}$ mice were crossed with an Hb9–GFP reporter mouse that selectively labels all spinal motor neuron cell bodies and projections (Bonanomi et al., 2012). The dorsal–ventral projections of LMC motor neurons from the limbs of E13.5 mice were examined via direct imaging of GFP fluorescence of motor projections in the hindlimbs. In Hb9–GFP;$Gfr\alpha1^{+/+}$ mice, both dorsal and ventral projections into the hindlimbs were clearly observed, in contrast to Hb9–GFP;$Gfr\alpha1^{TM/TM}$ mice in which the dorsal projection was either greatly reduced or absent entirely (Fig. 6*A*, *B*). The peroneal nerves of Hb9–GFP;$Gfr\alpha1^{TM/+}$ mice appeared normal (data not shown), suggesting that GFRα1–TM was not dominantly inhibitory to the remaining allele of GFRα1 in regards to spinal motor axon guidance. Therefore, GDNF requires GFRα1/Ret signaling from lipid rafts for coincidence detection via ephrin-A reverse signaling that is necessary for the axonal guidance of spinal motor neurons in the hindlimb.

During embryonic development of sympathetic neurons, migration of the sympathetic chain ganglia, as well as axonal projections along the vasculature, requires Ret signaling in response to artemin via GFRα3 (Nishino et al., 1999; Enomoto et al., 2001; Honma et al., 2002). To determine whether GFRα1–TM could impair the signaling of other GFLs, we examined the development of the sympathetic nervous systems of $Gfr\alpha1^{+/+}$ and $Gfr\alpha1^{TM/TM}$ mice. No abnormalities were observed in the migration or the positioning of the sympathetic chain, including the SCG and stellate ganglion (STG), in $Gfr\alpha1^{TM/TM}$ mice (Fig. 7). Furthermore, axonal projections of the sympathetic chain ganglia were also normal in $Gfr\alpha1^{TM/TM}$ mice. Last, the size and projections of the SCG and STG were not altered in $Gfr\alpha1^{TM/TM}$ mice compared with $Gfr\alpha1^{+/+}$ mice (Fig. 7). Therefore, eliminating the localization of GFRα1 from lipid rafts did not alter GFRα1-independent functions, suggesting that the GFRα1–TM knock-in strategy did not globally affect the signaling of other GFLs *in vivo*.

Ret ubiquitination is altered in the brains of $Gfr\alpha1^{TM/TM}$ mice

Signal transduction studies of primary neurons *in vitro* suggest that lipid rafts sequester activated Ret away from the ubiquitination/degradation machinery present in non-ordered regions,

←

(*Figure legend continued.*) (bottom panels) of E18.5 $Gfr\alpha1^{+/+}$ and $Gfr\alpha1^{TM/TM}$ mice. There was no enteric nervous system in the large intestines of $Gfr\alpha1^{TM/TM}$ mice (100%, 11 of 11 $Gfr\alpha1^{TM/TM}$ mice). The small intestines of most $Gfr\alpha1^{TM/TM}$ mice also lacked enteric neurons (82%, 9 of 11 mice), although a small minority of $Gfr\alpha1^{TM/TM}$ mice (18%, 2 of 11 mice) appeared to have enteric nervous system development in the proximal third of the small intestine. Eight to 11 mice of each genotype from four different litters were analyzed.

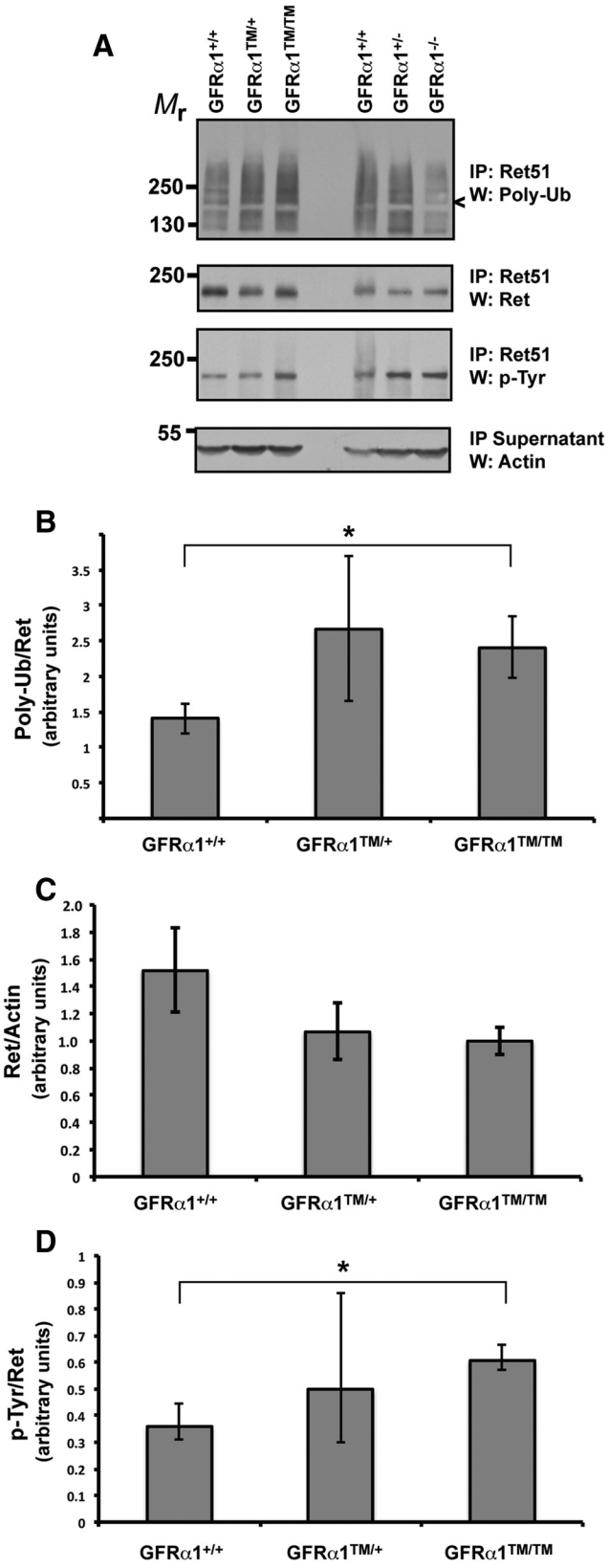

Figure 8. Ret ubiquitination is increased in the brains of $Gfr\alpha1^{TM/TM}$ mice. ***A***, The whole brains of E15.5 $Gfr\alpha1^{+/+}$, $Gfr\alpha1^{TM/+}$, and $Gfr\alpha1^{TM/TM}$ mice were detergent extracted, and Ret was immunoprecipitated from them. The extent of ubiquitination and Ret protein levels were ascertained by poly-ubiquitin and Ret immunoblotting, respectively. The level of Ret autophosphorylation was determined by phosphotyrosine immunoblotting of the immunoprecipitates, and actin immunoblotting of the supernatants served as a loading control. These experiments were quantified and graphed as the mean ± SEM for Ret ubiquitination (***B***), Ret protein levels (***C***), and Ret autophosphorylation (***D***). *$p < 0.05$. These experiments were conducted on three to five mice of each genotype from three separate litters.

thus prolonging the half-life of activated Ret (Pierchala et al., 2006). To determine whether Ret degradation was increased in $Gfr\alpha1^{TM/TM}$ mice, Ret immunoprecipitation studies were conducted from the brains of E15.5 $Gfr\alpha1^{+/+}$, $Gfr\alpha1^{TM/+}$, and $Gfr\alpha1^{TM/TM}$ mice. We chose to examine the brain because other anatomic structures that lipid raft-mediated Ret signaling appears to be important for, such as the kidneys and enteric nervous system, are completely lost in $Gfr\alpha1^{TM/TM}$ mice. Several CNS populations express Ret, such as cholinergic basal forebrain neurons and dopaminergic midbrain neurons, but are not lost in $Ret^{-/-}$ mice, making them tractable populations to study. When the levels of Ret ubiquitination were compared between $Gfr\alpha1^{+/+}$, $Gfr\alpha1^{TM/+}$, and $Gfr\alpha1^{TM/TM}$ brains, Ret isolated from the brains of $Gfr\alpha1^{TM/TM}$ mice was significantly more highly ubiquitinated than Ret isolated from $Gfr\alpha1^{+/+}$ brains (Fig. 8*A*, *B*). A comparison of the level of Ret ubiquitination between $Gfr\alpha1^{+/+}$ mice and $Gfr\alpha1^{-/-}$ mice confirmed that most of the Ret ubiquitination we observed in the brain was attributable to activation by GDNF/GFRα1 (Fig. 8*A*). However, the total level of Ret was not significantly different between $Gfr\alpha1^{+/+}$ and $Gfr\alpha1^{TM/TM}$ mice (Fig. 8*A*, *C*). The detection of a decrease in Ret protein may be difficult if only a small proportion of the total amount of Ret expressed in the brain, presumably activated Ret, is actively being degraded. Surprisingly, there was a higher level of Ret autophosphorylation in the brains of $Gfr\alpha1^{TM/TM}$ mice compared with $Gfr\alpha1^{+/+}$ mice (Fig. 8*A*, *D*). Together, these results suggest that Ret may be degraded more rapidly, or may be trafficked aberrantly after its activation, when associated in a receptor complex with GFRα1–TM.

Discussion

Lipid rafts are proposed to be nanoscale membrane microdomains that are enriched in cholesterol and sphingolipids, which endows them with greater order and rigidity than non-raft regions of the lipid bilayer (Simons and Sampaio, 2011). By genetically replacing GFRα1 with GFRα1–TM, which is incapable of translocating the GDNF/Ret signaling complex into lipid rafts (Tansey et al., 2000), the hypothesis was tested that lipid rafts are physiologic membrane structures in vertebrates that are critical for the developmental functions of GDNF. $Gfr\alpha1^{TM/TM}$ mice displayed a markedly reduced translocation of activated Ret into lipid rafts during GDNF stimulation, while at the same time having normal protein levels and cell-surface localization of GFRα1–TM. Phenotypic analysis of $Gfr\alpha1^{TM/TM}$ mice revealed that they have bilateral renal agenesis, a nearly complete lack of enteric nervous system development, and spinal motor axon guidance abnormalities. All of these deficits are observed in $Gfr\alpha1^{-/-}$ mice (Cacalano et al., 1998; Enomoto et al., 1998; Kramer et al., 2006; Bonanomi et al., 2012), suggesting that lipid rafts are critical for the GDNF/GFRα1/Ret signal transduction cascades that are necessary for the morphogenesis, proliferation, migration, and axon guidance functions of GDNF *in vivo*. $Gfr\alpha1^{TM/+}$ mice were similar to $Gfr\alpha1^{+/+}$ mice and had no abnormalities in any of these peripheral structures. These data suggest that GFRα1–TM does not interfere with the formation of GFRα1/Ret signaling complexes, likely because of their separation between raft and non-raft domains. However, we consistently observed a behaviorally aggressive phenotype of $Gfr\alpha1^{TM/+}$ mice compared with littermate

Gfrα1$^{+/+}$ mice, suggesting that there may be CNS defects in neural circuits involved in reward and/or aggressive behaviors.

The severity of the phenotypes we observed in *Gfrα1*$^{TM/TM}$ mice raises the question of whether the use of a TM domain in GFRα1 causes other signaling deficits that cannot be explained by the lack of lipid raft signaling via Ret. GFRα1–TM was examined thoroughly for alterations in its normal function and processing. The overall expression, trafficking to the cell surface, and membrane shedding of GFRα1–TM all occurred to a similar extent as GFRα1. Although the observation that GFRα1–TM was shed from primary neurons was initially surprising, the fact that a significant proportion of GFRα1 shedding is attributable to the activity of metalloproteinases explains this observation. Importantly, the three developmental processes that were examined, kidney morphogenesis, enteric nervous system development, and spinal motor axon guidance, are not impaired in mice lacking GFRα1 expressed *in trans* (Enomoto et al., 2004), making it unlikely that GFRα1 shedding would be important for these developmental processes. The initial Ret autophosphorylation by GDNF/GFRα1–TM also appeared to be similar to GDNF/GFRα1, which was reported previously for the GFRα1–TM fusion protein (Tansey et al., 2000). However, it is still a possibility that yet other aspects of GFRα1–TM functions are aberrant because of this TM addition, rather than lipid raft localization, making it premature to say definitively that lipid rafts are required for these developmental functions of GFRα1.

Lipid rafts enhance the signal transduction capacity of many receptor complexes by providing a platform for clustering and concentrating signaling molecules close to activated receptors (Simons and Toomre, 2000; Tsui-Pierchala et al., 2002). Indeed, in the case of GFL-mediated Ret signal transduction, lipid rafts enhance downstream signaling cascades by bringing Ret within close proximity to raft-enriched signaling molecules, such as Src and fibroblast growth factor receptor substrate 2 (Tansey et al., 2000; Encinas et al., 2001; Paratcha et al., 2001). Lipid rafts also enhance GFL/Ret signal transduction by sequestering activated Ret away from ubiquitin ligases and other molecules involved in receptor degradation, which are primarily absent from lipid rafts (Pierchala et al., 2006). The examination of Ret ubiquitination revealed that Ret isolated from the brains of *Gfrα1*$^{TM/TM}$ mice was more highly ubiquitinated than Ret from *Gfrα1*$^{+/+}$ mice (Fig. 8). Whether altered Ret ubiquitination, which would ultimately lead to changes in the internalization, trafficking, and degradation of Ret, contributes to the developmental abnormalities observed in these mice requires additional investigation. There are other potential functions of lipid rafts that may be critical for GDNF/GFRα1/Ret signal transduction. One possibility is that intracellular trafficking is altered, given that the internalization of receptors from lipid rafts is typically not clathrin mediated, as it often is outside of rafts (Nichols, 2003; Kirkham and Parton, 2005). A simpler explanation for the importance of rafts in Ret function is as a means of segregating GFRα1 receptors away from Ret in the absence of ligand, to avoid the precomplexing of these receptors. Indeed, overexpression of GFRαs and Ret in cell lines results in their extensive interaction in the absence of ligand, which may have deleterious effects on signaling, such as ligand-independent activation or plasma membrane clearance. It is likely that several of these mechanisms work in a combinatorial manner to promote Ret-dependent signaling that accounts for the importance of lipid rafts in GDNF function. A limitation to this signaling analysis is the general absence of anatomic structures that require GDNF/Gfrα1/Ret signaling for development, such as kidneys and the enteric nervous system, in *Gfrα1*$^{TM/TM}$ mice, precluding their biochemical examination. The observation that *Gfrα1*$^{TM/TM}$ mice were deficient in several GDNF/GFRα1-dependent developmental events in the periphery emphasizes the need to identify signaling properties of Ret *in vivo* that are raft dependent and those that are not and what their role is in Ret ubiquitination, signal transduction, and turnover. In addition to Ret, GDNF/GFRα1 complexes can signal via NCAM and syndecan-3 (Paratcha et al., 2003; Pozas and Ibanez, 2005; Bespalov et al., 2011), which are critical for synaptogenic and migration functions of GDNF in the brain. The extent to which lipid rafts are necessary for these functions *in vivo* are not known and represents an important future direction as a means of determining whether lipid rafts are required for all GDNF/GFRα1-dependent developmental functions, regardless of the signal-transducing receptor. In conclusion, we propose that the function of the GPI anchor, as opposed to TM domains, is to assemble receptor complexes in lipid raft microdomains to maximize their signaling functions.

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