SUPPLEMENTAL DATA

EXPERIMENTAL PROCEDURES

Analysis of RET endocytosis and recycling by fluorescence microscopy-RET endocytosis and recycling were measured by immunofluorescence methods developed from our previous works (1,2). Briefly, Flag-tagged RET chimera and its mutants were constructed on the backbone of pcDNA3.1 vector and transfected into PC12 cells. To measure RET endocytosis, cells were incubated with Alexa-488-conjugated M1 anti-FLAG antibody for 30 minutes at 4℃ to selectively label FLAG-tagged receptors on the plasma membrane at the beginning of the experiment and then incubated at 37℃ for 30 minutes in the presence of ligand to drive receptor internalization. At the end of this incubation, cells were quickly washed 3 times with ice-cold PBS supplemented with 3 mM EDTA to dissociate fluorescent M1 antibody from residual cell surface receptors. Cells were then fixed with 4% paraformaldehyde, with the intracellular fluorescence representing the internalized receptors. In each experiment, two parallel control groups were included. In one control group, cells were fixed after a 30-minutes incubation in the absence of ligand and without EDTA stripping step (100% surface receptor control). In the other control group, cells were stripped immediately after the feeding step (0% Internalization control). Cells were examined by fluorescence microscopy by using appropriate filter sets to selectively detect Alexa-488, and staining intensities of each fluor in individual cells were integrated using MetaMorph software (Molecular Devices, Sunnyvale, CA). Receptor internalization level was determined by the fluorescence ratio of internalization/surface receptors.

To quantify the extent of recycling observed after ligand removal in individual cells, after the similar feeding and ligand driven receptor internalization steps, transfected cells were quickly washed 3 times with ice-cold PBS/EDTA to dissociate FLAG antibody bound to the remaining receptors in the plasma membrane, thereby leaving fluorescent antibody bound only to the internalized pool of receptors. EDTA-stripped cells were then incubated at 37℃ for 45 minutes in the presence of Alexa-594-anti-mouse IgG to label all receptors that returned back to the cell surface, and then cells were fixed with 4% paraformaldehyde. For each receptor examined, two parallel control groups were included, one in which cells were fixed after a 30-minutes incubation in the absence of ligand and without EDTA stripping step (100% surface receptor control) and one in which cells were fixed immediately after the EDTA-mediated stripping step (0% recycled control). This analysis indicated that the efficiency of the EDTA strip (reduction of Alexa-594 staining intensity in the 0% recycled control relative to the 100% surface receptor control) was >95%. The percentage of receptors recycled in individual cells after ligand washout was then calculated from the red/green ratios determined from the control conditions according to the following formula $(E\text{-}Z)/(C\text{-}Z) \times 100$, where E is the mean ratio for the experimental group, Z is the mean ratio for the zero surface control, and C is the mean ratio for the 100% surface control.

FIGURE LEGENDS

Figure S1. RET and TrkB expression pattern in PC12 cells. PC12 cell lysates were immunoblotted with RET and TrkB antibodies respectively. RET and TrkB levels in DRG extracts were set as a positive control and tubulin levels were determined as protein loading control.

Figure S2. Thr^{675} does not affect RET internalization and recycling. (A) Ligand induced internalization of RET and RET-Thr^{675} mutants were quantified. Flag-tagged RET chimera

and its Thr^{675} mutants were constructed on the backbone of pcDNA3.1 vector and transfected into PC12 cells. The internalization levels of RET and RET-Thr^{675} mutants were quantified by immunofluorescence methods described in EXPERIMENTAL PROCEDURES. (B) Recycling extent of Flag-tagged RET and RET-Thr^{675} mutants were determined as described in EXPERIMENTAL PROCEDURES. (C) Cell surface levels of RET and RET-Thr^{675} mutants were determined when receptor endocytosis was blocked. PC12 cells expressing GFP fused RET and RET-Thr^{675} mutants were treated with dynasore for two hours and then receptor cell surface levels were quantified using ratiometric fluorescence assay. Relative receptor surface levels were normalized to that of RET-GFP in vehicle treated cells. In each experiment more than 30 cells were examined at random. The results are represented as mean ± SEM from three independent experiments (*$p<0.05$ vs. RET surface level in the same group; one-way ANOVA).

REFERENCES

1. Chen, Z. Y., Ieraci, A., Tanowitz, M., and Lee, F. S. (2005) *Mol Biol Cell* **16**, 5761-5772

2. Huang, S. H., Zhao, L., Sun, Z. P., Li, X. Z., Geng, Z., Zhang, K. D., Chao, M. V., and Chen, Z. Y. (2009) *J Biol Chem* **284**, 15126-15136

Figure S1

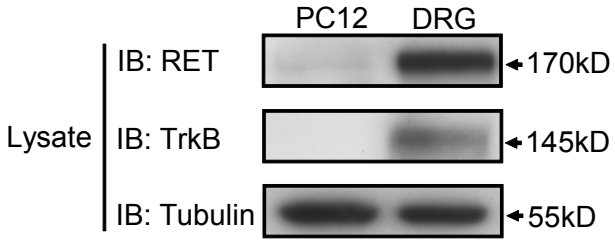
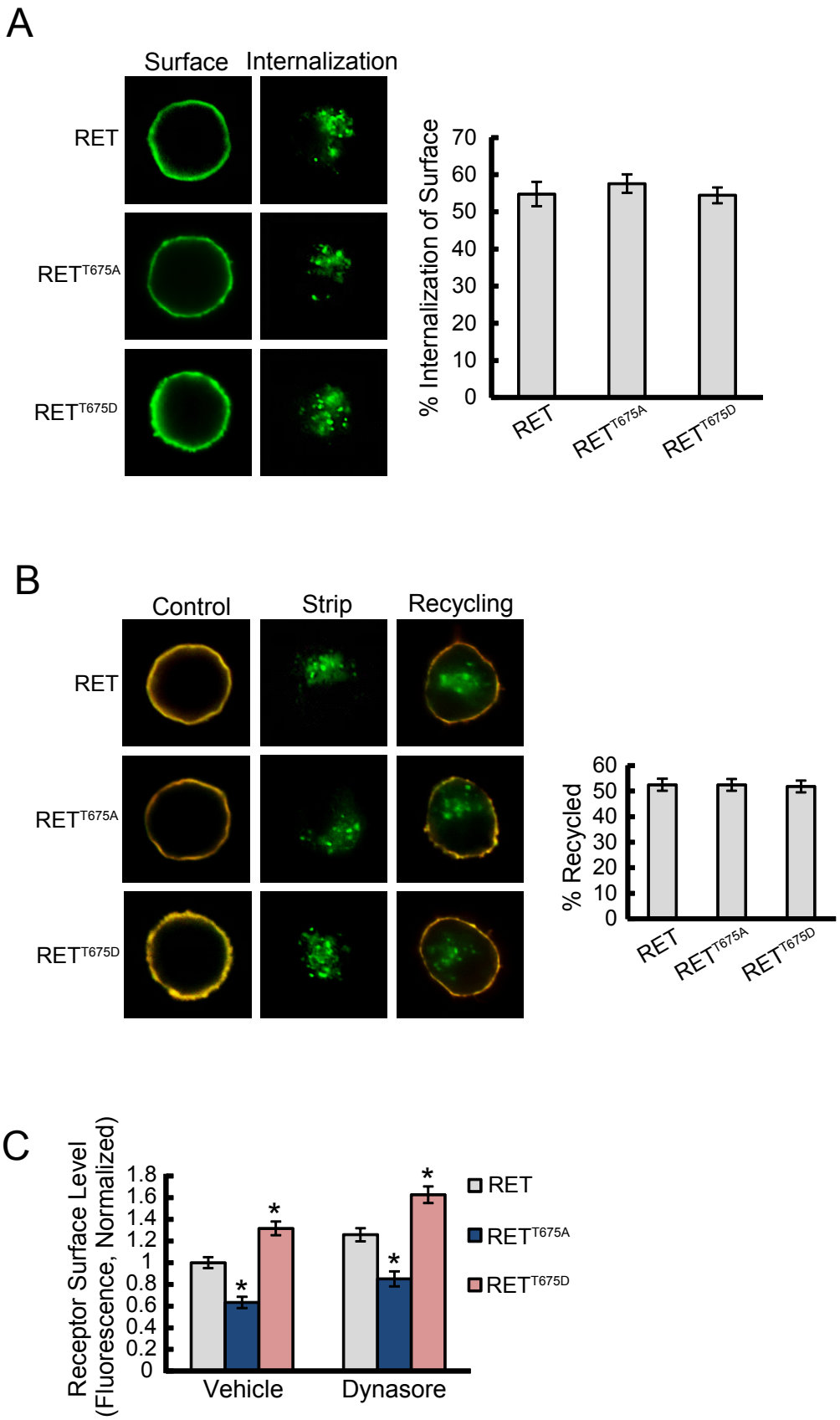


Figure S2


A
Surface
Internalization
RET
RET^T675A
RET^T675D
% Internalization of Surface
70
60
50
40
30
20
10
0
RET
RET^T675A
RET^T675D
B
Control
Strip
Recycling
RET
RET^T675A
RET^T675D
% Recycled
60
50
40
30
20
10
0
RET
RET^T675A
RET^T675D
C
Receptor Surface Level
(Fluorescence, Normalized)
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
*
*
*
Vehicle
Dynasore
RET
RET^T675A
RET^T675D