Article:
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Supplementary Information

Pull-down assays

Comparing the activity of RET^{ECD} expressed using CHO-K1 and HEK293T cells

In brief, RET^{ECD} expressed using either CHO-K1 or HEK293T cells was added to pre-incubated Fc-GDF15/GFRAL mixture and the final concentration of RET^{ECD}, GFRAL and Fc-GDF15 was 2.5 μM, 2.5 μM and 1.25 μM, respectively. After 1-hr incubation at 4 ˚C, 5 μl protein A resin was added to each sample together with 400 μl binding buffer and the samples were mixed by end-to-end rotation for 2 hr at 4 ˚C. The resin was then washed and incubated with SDS PAGE loading buffer without DTT before electrophoresis.

RET^{ECD} mutants and their binding to GDF15/GFRAL

Single mutations of RET^{ECD} (N336Q, N343Q and N468Q) were introduced using Q5 site directed mutagenesis. The soluble ECDs of RET^{N336Q}, RET^{N343Q} and RET^{N468Q} were expressed as described earlier for the wild-type RET^{ECD} and purified by Ni-NTA affinity chromatography. Pull-down assay was performed as described earlier and samples were analysed under non-reducing conditions using Coomassie-stained SDS-PAGE gel.

Deglycosylation

5 μg of RET^{ECD} (insect) was deglycosylated using Endo H_f or PNGase F under native and denaturing conditions according to the manufacturer's protocol. Phenylmethylsulfonyl fluoride (PMSF) was supplemented to all samples at a final concentration of 1 mM to prevent possible proteolysis. After the addition of the glycosidases, the mixture was incubated at 37 ˚C for 1 hr under denaturing conditions and 4 hr under native conditions. Afterwards, the samples were mixed with SDS PAGE loading buffer with DTT and were subject to electrophoresis.

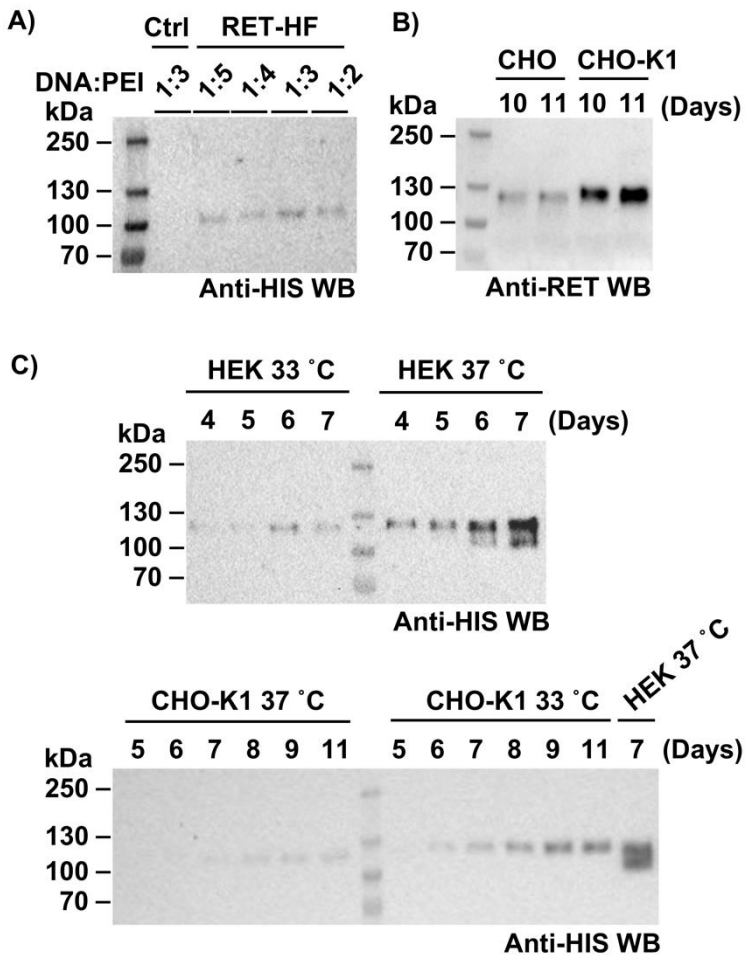
Western blotting

For western blotting, proteins were transferred to 0.2 μm nitrocellulose membrane with the Trans-Blot Turbo transfer system and membranes were blocked with 3% BSA in TBST for 30 min. After being probed with

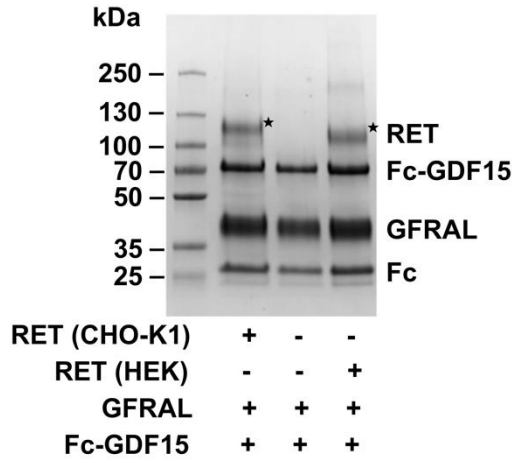
appropriate antibodies, the membranes were washed three times in TBST, developed with enhanced chemiluminescence (ECL) substrate and imaged using a ChemiDoc XRS+ System (Bio-Rad) to detect bound antibodies. Anti-RET(C-3)-HRP conjugated antibody was used at 1:1000 dilution in blocking buffer. Anti-5xHIS antibody was used at 1:5000 dilution in blocking buffer and horseradish peroxidase (HRP) conjugated mouse IgG kappa binding protein (m-IgGκ BP-HRP) secondary antibody was used at 1:5000 dilution in 10% non-fat milk dissolved in TBS.

***Supplementary Table 1**. Approaches used to express RET^{ECD} using mammalian cells.*

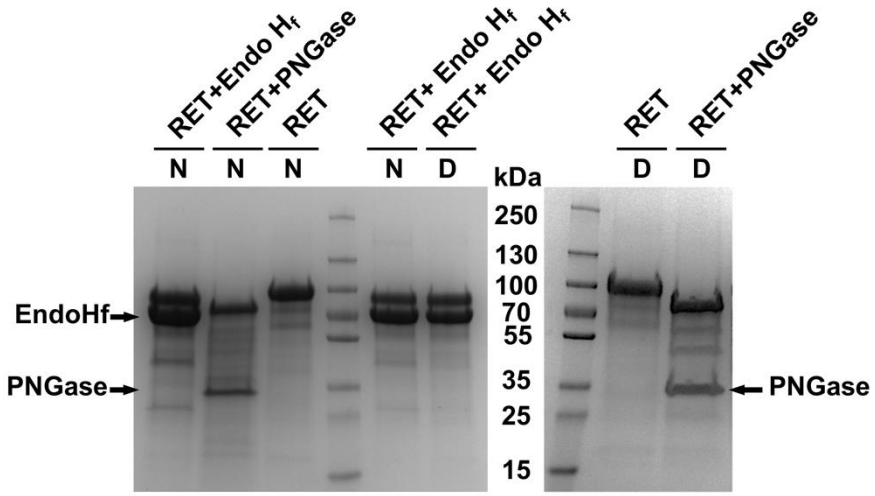
Target protein	**Construct**	**Vector**	**Cell line**	**Expression method**	**Reference**
humanRET^{ECD} (amino acids 1-635)	RET-TEV-Protein A	pcDNA3	CHO Lec8	Stable cell line	[15]
	RET-HA-c-Myc-His_6	pSecTag2AHA	CHO	Stable cell line	[20]
	RET-TEV-His_6	/	CHO	Transient	[17]
	RET-His_8	pEZT-BM	HEK293S GnTI- or FreeStyle 293 F cells	Transient (Baculoviruses)	[16]
	RET-TEV-His_8-Flag	pcDNA3	HEK293T	Transient	(This study)
humanRET^{ECD} (amino acids 29-635)	RET-Fc RET-His	pJSV (CD33 signal peptide)	HEK293 6E cells	Transient	[11]



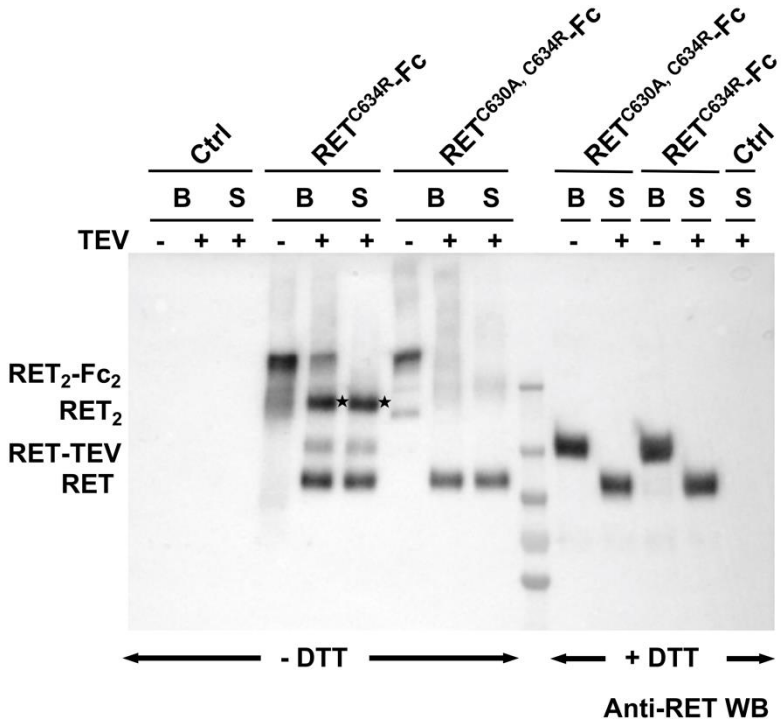
Supplementary Figure 1. Western blot images showing RET^{ECD} expression using HEK293T, CHO-K1 and CHO cells under various conditions. **A**) The expression of RET^{ECD} in HEK293T cells transfected using different DNA:PEI ratios; **B**) The expression of RET^{ECD} in CHO and CHO-K1 cells 10- or 11-day post-transfection at 33 ˚C. **C**) Time dependent expression of RET^{ECD} using HEK293T and CHO-K1 cells at 33 ˚C and 37 ˚C.



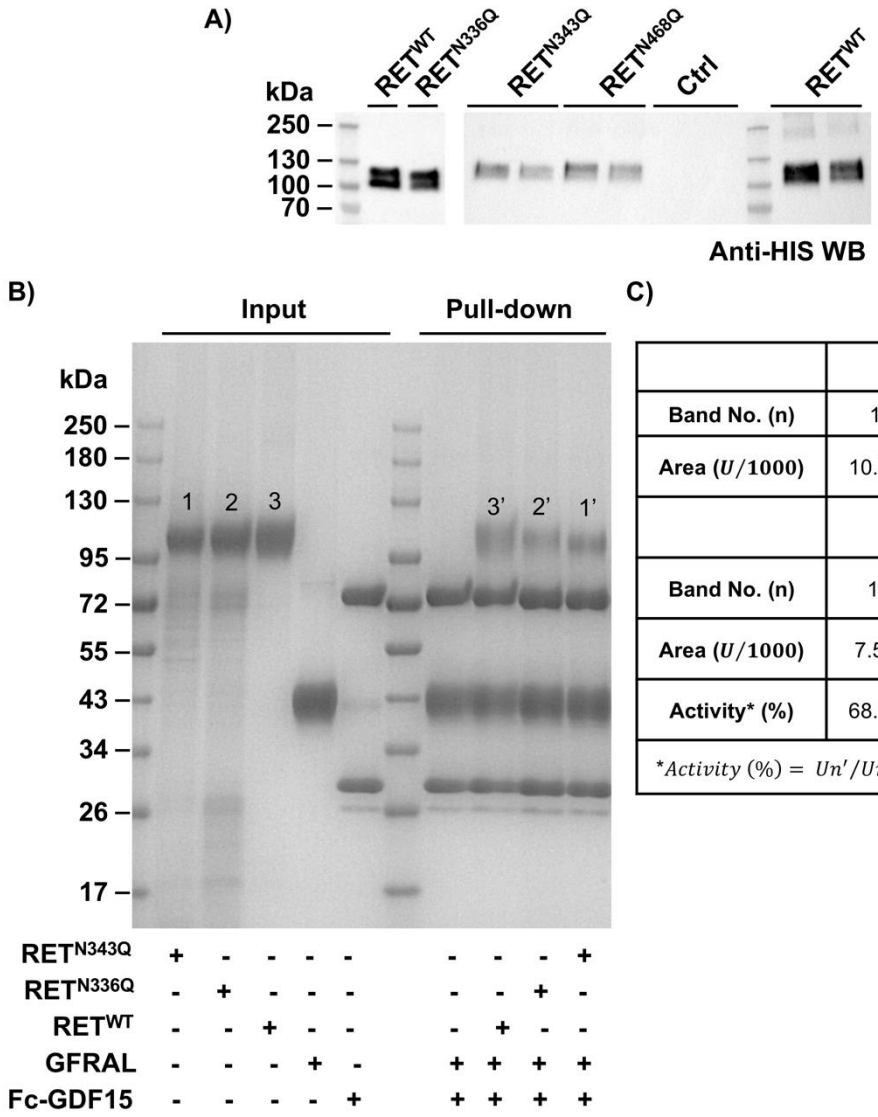
Supplementary Figure 2. Coomassie-stained SDS PAGE gel image showing RET^{ECD} expressed in CHO-K1 and HEK293T cells pulled down by GDF15/GFRAL. RET pulled down is marked with a star. All samples were treated with SDS loading dye without DTT.



Supplementary Figure 3. Deglycosylation of RET^{ECD} (insect) by Endo H_f and PNGase F under native and denaturing conditions. Non-treated RET^{ECD} has a MW of 95kDa while the calculated MWs of RET^{ECD} treated by EndoHf and PNGase are 90 and 75 kDa, respectively. All samples are treated with SDS loading dye with DTT for electrophoresis. N: Native condition; D: Denaturing condition.



Supplementary Figure 4. Anti-RET WB showing the expression of RET^{C634R}-Fc, $RET^{C630A,C634R}$-Fc and RET^{C634R} dimer after Fc tag removal (marked by the black stars). Samples were prepared under non-reducing (- DTT) and reducing conditions (+ DTT). B: Protein A beads; S: Supernatant sample after spinning down the beads.



C)

	Input		
Band No. (n)	1	2	3
Area ($U/1000$)	10.96	13.73	14.82
	Pull-down		
Band No. (n)	1'	2'	3'
Area ($U/1000$)	7.53	6.58	8.24
Activity* (%)	68.70	47.92	55.60
*$Activity\ (\%) = Un'/Un \times 100\ (n = 1,2,3)$			

Supplementary Figure 5. Expression of RET^{ECD} mutants in HEK293T cells and their binding to GDF15/GFRAL. All samples are treated with SDS loading dye without DTT. **A**) Anti-HIS western blot showing the expression of different RET^{ECD} mutants N336Q, N343Q and N468Q. Expression was done in duplicates. **B**) Coomassie-stained gel image showing the protein A resin pull down of different RET^{ECD} mutants by Fc-GDF15/GFRAL. **C**) The table shows the band intensity as measured by ImageJ (Area (U)). Bands correspond to the wild-type RET^{ECD} and mutants are labelled (1 for N343Q, 2 for N336Q and 3 for wild-type). U: units.