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Supplementary Materials for

Biased signaling by thyroid-stimulating hormone receptor–specific antibodies determines thyrocyte survival in autoimmunity

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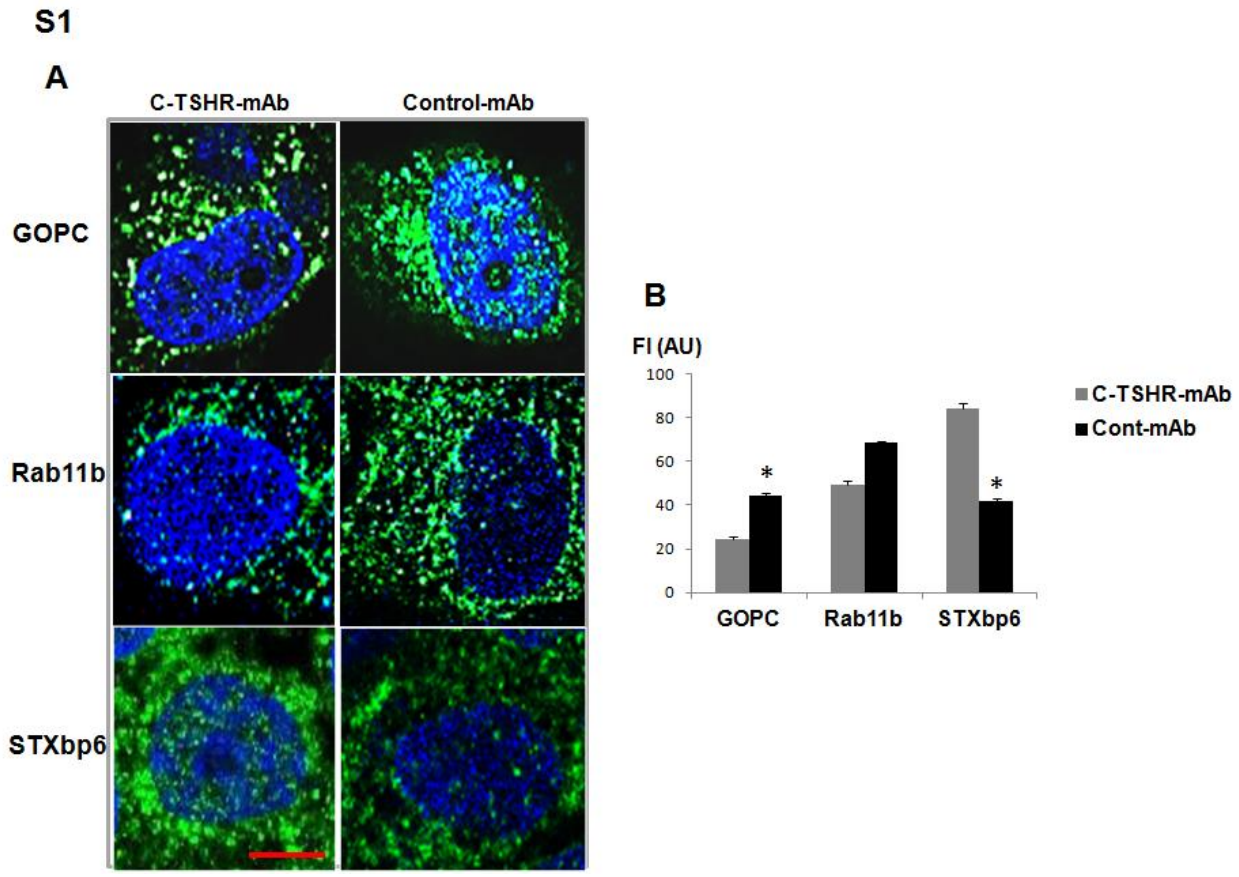

Fig. S1. The C-TSHR-mAb disrupts vesicular trafficking. (**A**) Thyrocytes were treated with anti-C-TSHR monoclonal antibody (C-TSHR-mAb) or isotype control mAb (Control-mAb; both at 1 μg/ml) for 24 hours. The cells were then analyzed by immunohistochemistry with antibodies against GOPC (Golgi-associated PDZ and coiled-coil motif–containing protein), Rab11b (vesicular protein), and STXbp6 (syntaxin-binding protein 6; a vesicular protein). The images are representative of three independent experiments. Green color indicates antibody staining of specific proteins and blue color is nuclear staining by DAPI. Scale bar, 100 μm. (**B**) The fluorescence intensity (FI) values of the staining for the indicated proteins in the images represented in (A) were quantified. Data are means ± SEM of three independent experiments. **P* < 0.05 compared to C-TSHR-mAb.

S2

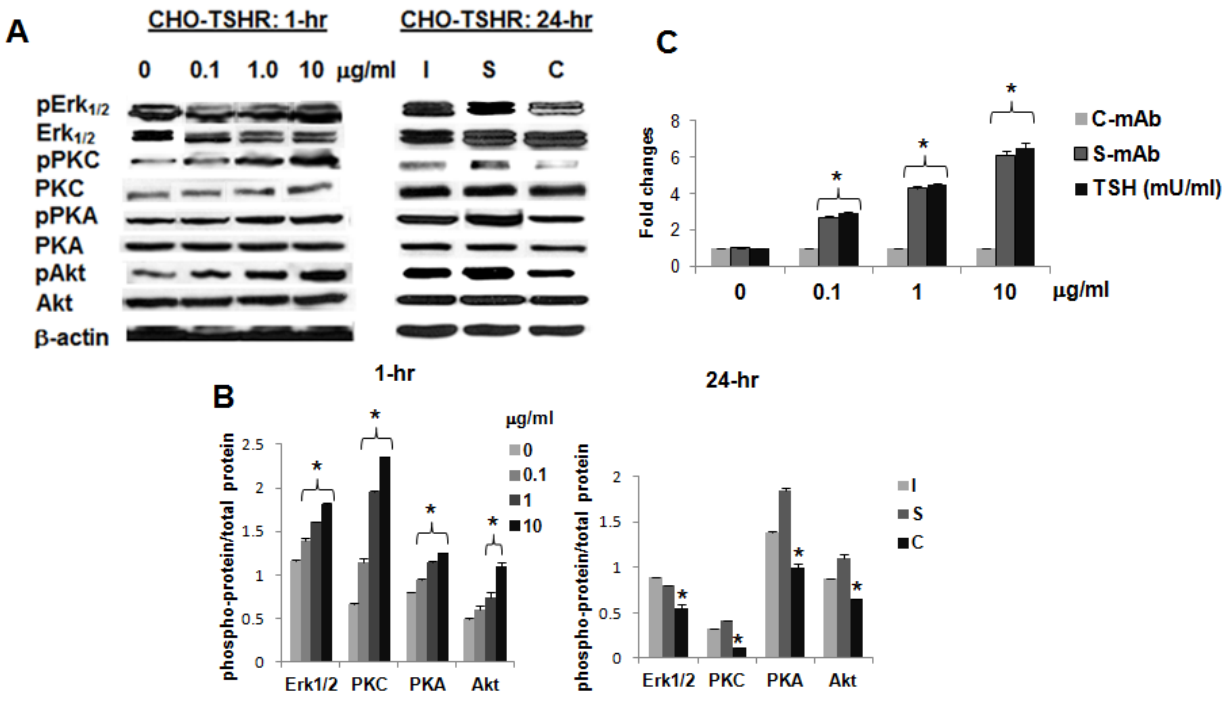

Fig. S2. The C-TSHR-mAb does not stimulate signaling in CHO-TSHR cells. (**A**) CHO-TSHR cells were treated for 1 hour with the indicated concentrations of C-TSHR-mAb (left) or for 24 hours with a fixed concentration (1 µg/ml) of control-mAb (I), S-TSHR-mAb (S), or C-TSHR-mAb (C) (right). Cell lysates were then analyzed by Western blotting with antibodies specific for the indicated proteins. β-actin was used as a loading control. Western blots are representative of three experiments. (**B**) Densitometric analysis of Western blotting data. Data are presented as the ratio between the indicated total proteins and phosphorylated proteins. *$P < 0.05$ compared to control mAb. (**C**) CHO-TSHR cells were left untreated or were treated with the indicated concentrations of C-TSHR-mAb, S-TSHR-mAb (both in µg/ml), or TSH (mU/ml) for 1 hour. The concentrations of cAMP in the cells were then measured by ELISA. Data are means ± SEM of three experiments. *$P < 0.05$ compared to C-TSHR-mAb.

S3

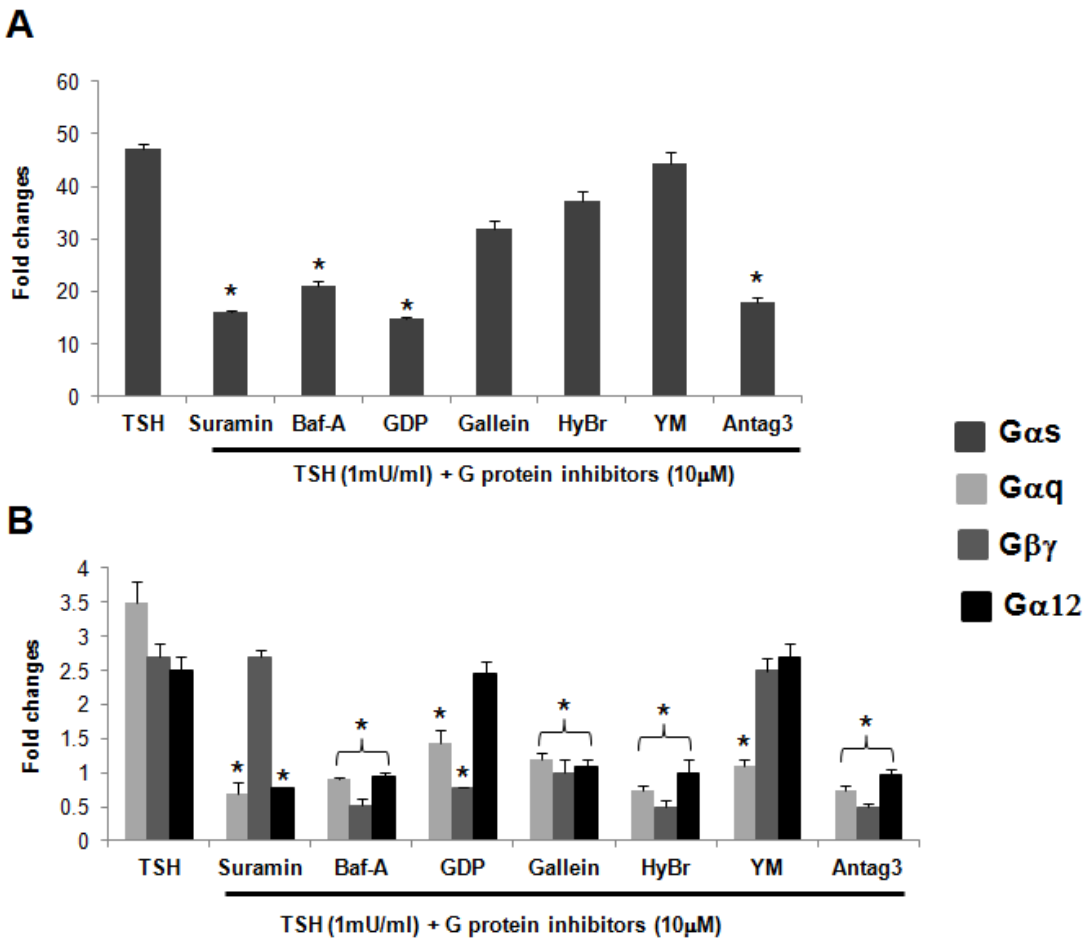

Fig. S3. Effects of G protein perturbations on $G\alpha_s$, $G\alpha_q$, Gβγ, and Gα12 activities on CHO-TSHR cells. (**A** and **B**) CHO-TSHR cells were treated with TSH (1 mU/ml) alone or in the presence of the following inhibitors (all at 10 μM): suramin, bafilomycinA1 (Baf-A), GDP, Gallein (Gβγ inhibitor), Hydrobromide (HyBr), YM ($G\alpha_{q/11}$ inhibitor), or a TSHR selective antagonist (Antag3). After 1 hour of pretreatment, the CHO-TSHR cells were treated with TSH for 4 hours. The luciferase activities of reporters for $G\alpha_s$, (A) and for $G\alpha_q$, Gβγ, and Gα12 (B) were then measured. Data are presented as the fold-change in luciferase activity relative to that in basal CHO-TSHR cells and are means ± SEM of three experiments. *$P < 0.05$ compared to TSH.

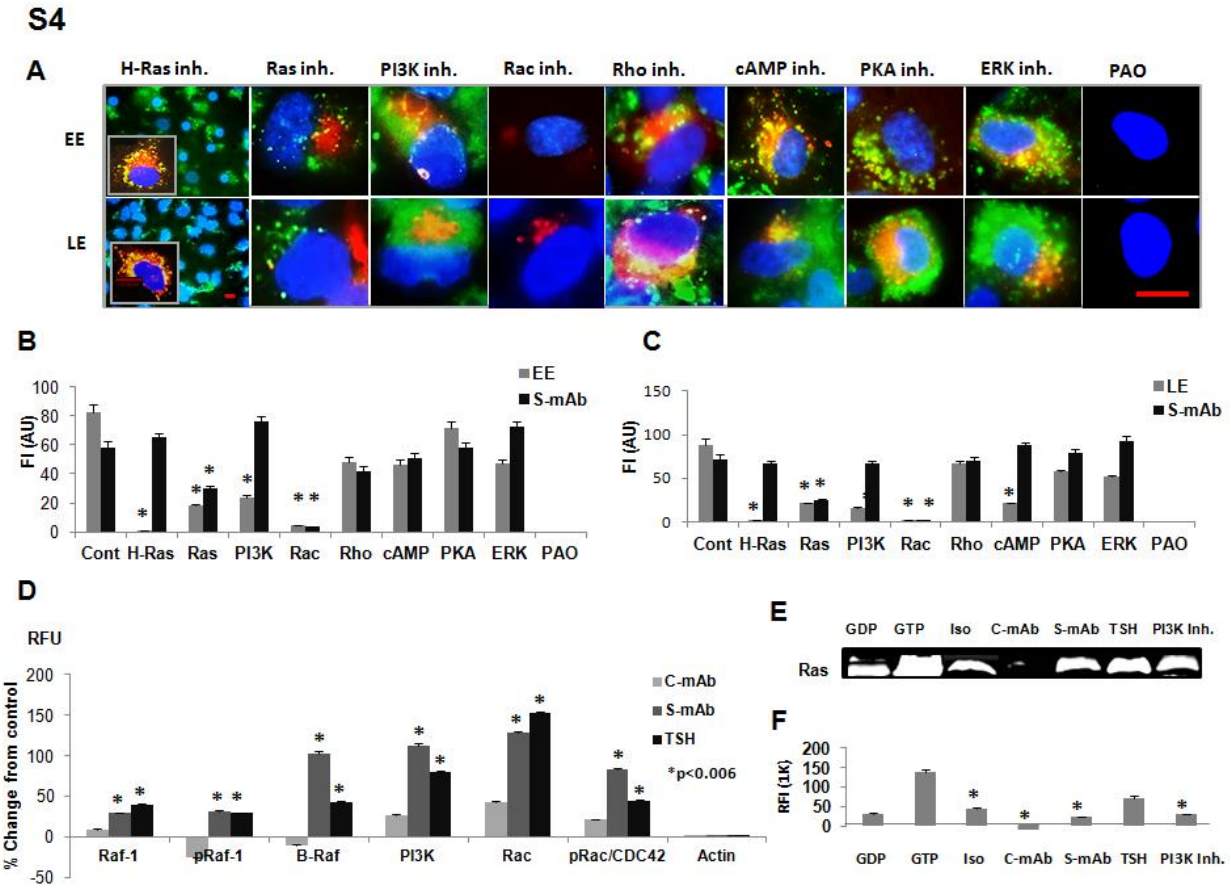

Fig. S4. The Ras-PI3K-Rac pathway downstream of G proteins is a key signaling pathway for vesicle formation. (**A**) Live-cell imaging of early endosomes (EE) and late endosomes (LE) in thyrocytes pretreated with different inhibitors (all at 10 μM) in the presence of labelled (green) S-TSHR-mAb (1 μg/ml); inset shows antibody-treated cells without inhibitors. Malformed endosomes (red) were also present in the different treatments. Scale bar, 100 μm. (**B** and **C**) Representative images were analyzed quantitatively by measuring the fluorescence intensity (FI) of EE (red), LE (red), and labeled TSH (green). Data are means ± SEM of three independent experiments. **P* < 0.006 compared to control-mAb after exposure to the same series of inhibitors as those shown in (A). (**D**) Percentage change in protein as RFU compared to control-mAb (hamster IgG2, κ chain) was determined by proteomic array analyses of Raf-1, pRaf-1, B-Raf, PI3K, Rac, and pRac/CDC42 in cells treated as indicated. Data are means ± SEM of three independent experiments. **P* < 0.006 compared to C-mAb. (**E**) Immunoprecipitated active Ras-GTP was detected by LI-COR Western blotting. Thyrocytes were treated with GDP (100 μM), GTP (10 mM), the indicated antibodies (all at 1 μg/ml), TSH (1 mU/ml), or PI3K inhibitor (10 μM) for 24 hours and then immunoprecipitated with mAb against Ras-GTP. (**F**) Relative fluorescence intensity (RFI) of the Western blotting bands was quantified with Image Studio software. Data are means ± SEM of three independent experiments. **P* < 0.005 compared to GTP or TSH.

S5

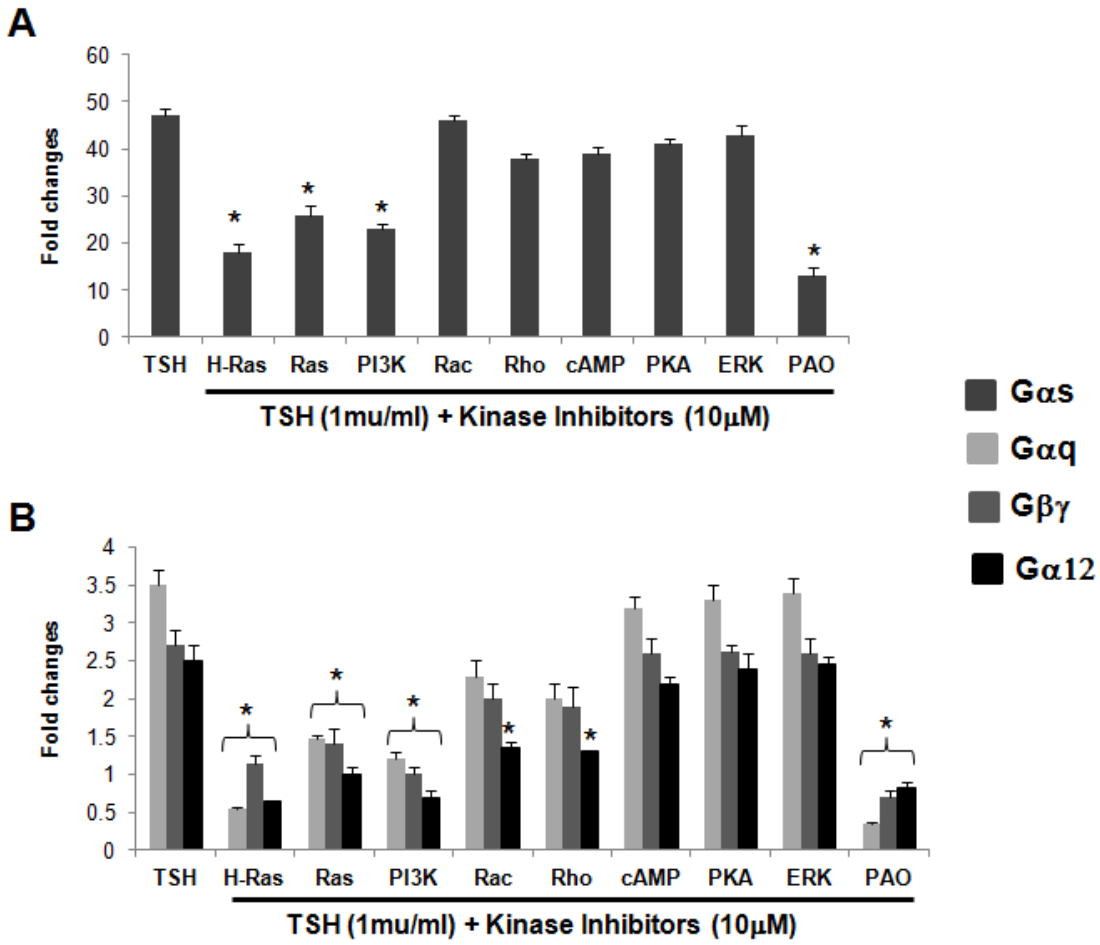

Fig. S5. Perturbation of major G proteins by different pharmacological inhibitors in CHO-TSHR cells. (**A** and **B**) In the presence of TSH (1 mU/ml) and multiple inhibitors (all at 10 μM) including those against H-Ras, Ras, PI3K, Rho, cAMP, PKA, ERK1/2 (ERK), and PAO (endocytosis inhibitor), luciferase activities for $G\alpha_s$, $G\alpha_q$, $G\beta\gamma$, and $G\alpha12$ were determined. Data are presented as the fold-change in luciferase activity relative to that in CHO-TSHR cells and are means ± SEM of three experiments. **P* < 0.05 compared to TSH.

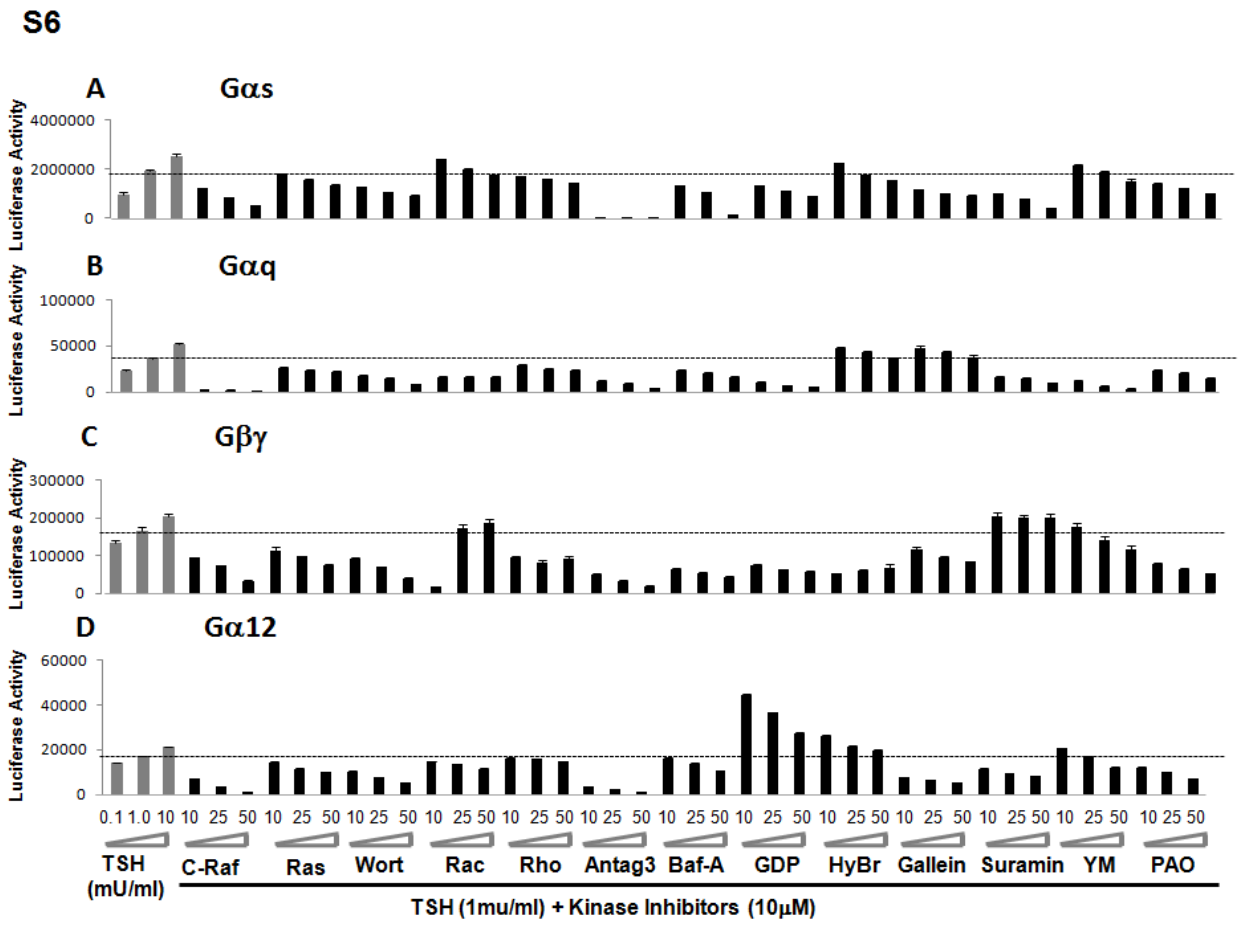

Fig. S6. Dose-dependent suppression of the TSH-induced activities of $G\alpha_s$, $G\alpha_q$, Gβγ, and Gα12 by pharmacological inhibitors in CHO-TSHR cells. (**A** to **D**) CHO-TSHR cells treated with TSH with or without the indicated inhibitors were analyzed for luciferase activity to determine the activation of $G\alpha_s$ (A), $G\alpha_q$ (B), Gβγ (C), and Gα12 (D) signaling. Data are presented as luciferase activity relative to that in CHO-TSHR cells and are means ± SEM of three experiments. Dashed lines indicate luciferase activity at a TSH concentration of 1 mU/ml.

S7

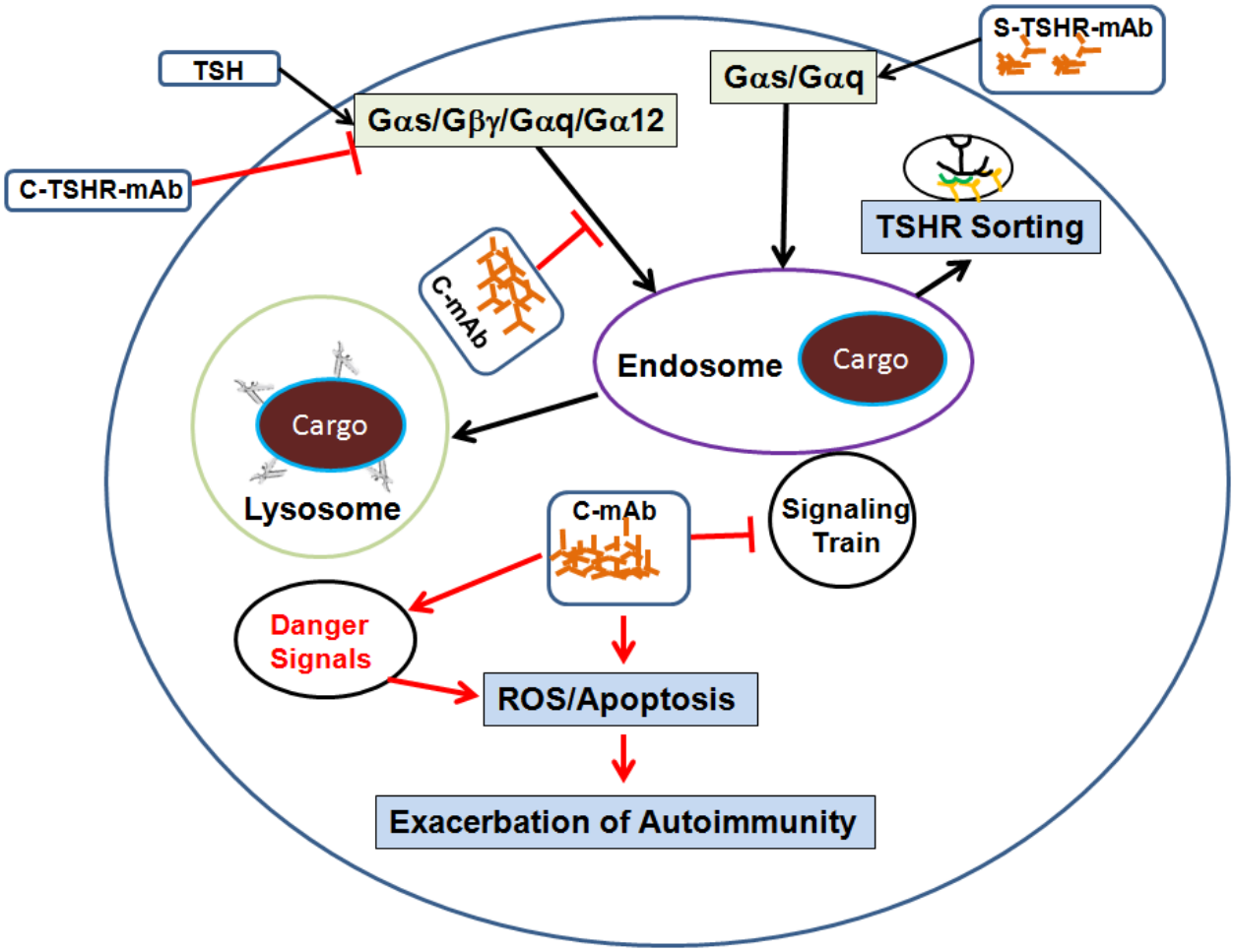

Fig. S7. Model depicting the mechanism of action of C-TSHR-mAb in FRTL-5 thyrocytes. Whereas the S-TSHR-mAb activates some of the major G proteins and behaves similarly to TSH, the C-TSHR-mAb functions differently. It binds to the receptor and is endocytosed, but fails to activate G proteins and does not undergo endosomal and lysosomal vesicular trafficking, which leads to TSHR-biased danger signaling. Intracellular accumulation of C-TSHR-mAbs induces cellular stress and the generation of ROS. These signaling events activate apoptotic signaling cascades that may lead to the exacerbation of thyroid autoimmunity.

S8

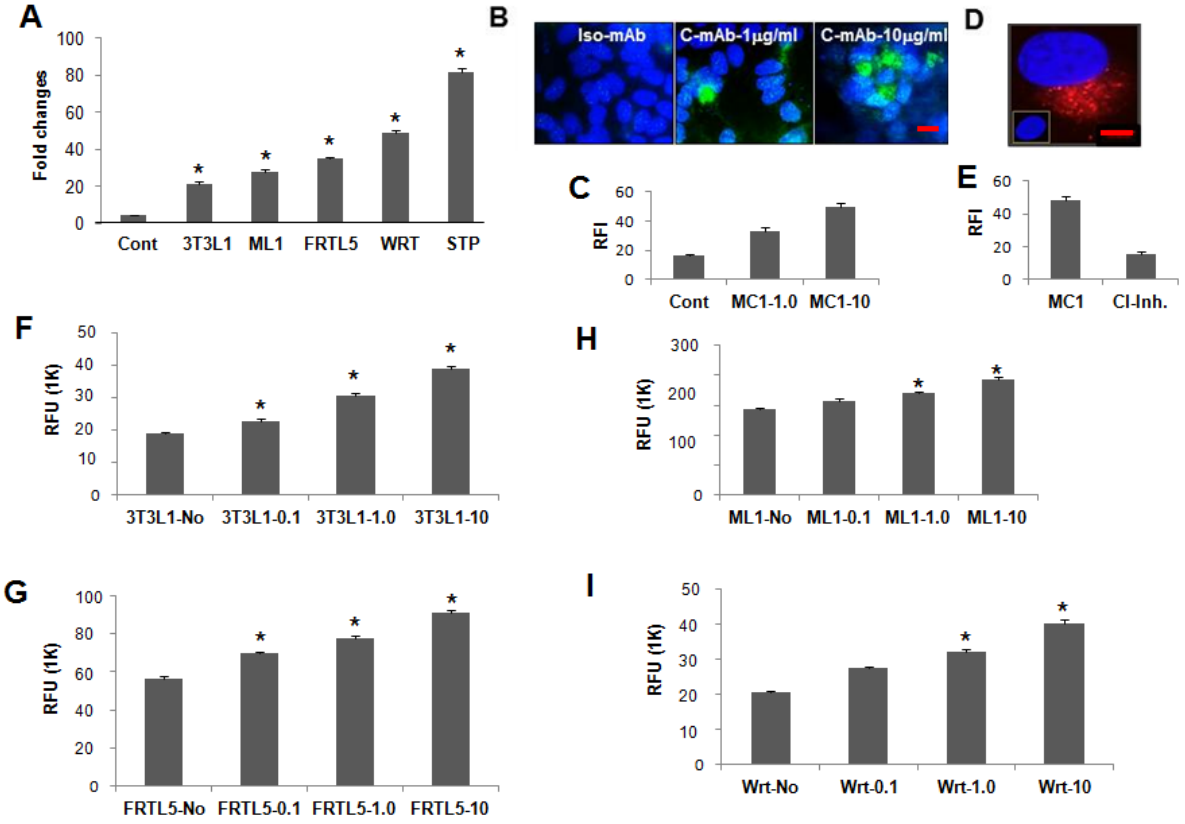

Fig. S8. The C-TSHR-mAb induces apoptosis in thyrocytes and fibroblasts. (**A**) After 24 hours of treatment with C-TSHR-mAb, fluorometric inhibitor of caspases (FLICA) or caspase activities by FAM caspase assay were measured in 3T3L1, ML-1, FRTL5, and WRT cells, as indicated. Data are presented as the percentage increase in caspase activity and are means ± SEM of three experiments. **P* < 0.01 compared to the IgG2 control-mAb (cont). Cells treated with staurosporine (STP, 5 µM) for 4 hours were used as a positive control for apoptosis. (**B**) Representative images from the immunohistochemical analysis of cells showed the activation of caspase 3A (active catalytic unit) by C-TSHR-mAb. Scale bar, 100 µm. (**C**) Quantitative analysis of the images represented in (B). Data are presented as means ± SEM of three experiments. **P* < 0.01 compared to the isotype (IgG2) control mAb. (**D**) Representative live-cell images of endocytosed labelled (red) C-TSHR-mAb in ML-1 thyrocytes after 12 hours of treatment. Data are presented as means ± SEM of three experiments. The clathrin inhibitor (Cl-inh.) suppressed the endocytosis of labelled (red) C-TSHR-mAb in ML-1 thyrocytes (inset). Scale bar, 100 µm. (**E**) Quantitative analysis of the images represented in (D). Data are presented as means ± SEM of three experiments. **P* < 0.01 compared to C-TSHR-mAb (MC1). (**F** to **I**) Dose-dependent activation of total caspases (as assessed by FLICA assay) induced by C-TSHR-mAb in the indicated cell lines. Data are presented as means ± SEM of three experiments. **P* < 0.05 compared to control-mAb (cont) after 24 hours.