Supplementary Figure Legends:

Figure S1. Gβγ Enhances GDP-Gαs Binding to 5NT in the Presence and Absence of Aluminum Fluoride. GST or GST-tagged 5NT (2 μM final) was incubated with G-protein subunits, GDP-Gαs and/or Gβ1γ2 (1 μM). GST pull-down assay was performed in the presence or absence of AlF4 (n=2). When present, 30 μM aluminum and 10 mM fluoride was present in all incubation and wash buffers.

Figure S2. Gel Filtration Analysis of Complex Formation between 5NT 60-129 and Gαs·βγ. Proteins (10 μM of each) were applied on a superdex 200 column in buffer containing 0.1% $C_{12}E_{10}$, 75 mM NaCl, and 10 μM GDP and fractions (0.3 ml) were analyzed by SDS-PAGE and immunoblotting. Upper panel: complex of 5NT 60-129/Gαs/Gβγ, middle panel: 5NT 60-129 alone, lower panel: Gαs/Gβγ. The 5NT60-129/Gαs/Gβγ complex is boxed while smaller complexes containing 5NT with Gαs or Gβγ are marked with an asterisk (n=2).

Figure S3. Gβγ and GDP-Gαs Bind to 6NT. GST proteins (2 μM final) were incubated with G-protein subunits, GDP-Gαs or Gβγ and subjected to GST pull-down assay as described in Fig. S1 (n=5).

Figure S4. **The N-terminus of AC5 Does Not Serve As GAP or GEF. (A),** GTPγS filter binding assay was performed as described in (Graziano and Gilman, 1989). Briefly, G-protein subunits, GDP-Gαs·βγ (1 μM) in presence or absence of GST or GST-tagged 5NT (10 μM final) were incubated with 100 uM [^{35}S]GTPγS (~ 2000 cpm/pmol). Bound [^{35}S]GTPγS was detected by binding to nitrocellulose filters in a 96 well format. **(B)** GTPase assay was performed as

described in (Graziano and Gilman, 1989) with slight modification. Briefly, Gαs (12.5 pmol) was incubated with GST or GST-tagged 5NT (10 μM final). The reaction was started by addition of [γ-^{32}P]GTP (~60000 cpm/pmol). An aliquot was taken at indicated time points and ^{32}Pi was separated using activated charcoal and measured by scintillation counting.

Figure S5. 5NT and 5NT60-129 Pull Down a 5C1/5C2/GTPγS-Gαs Complex in the Presence or Absence of Forskolin. 5C1, 5C2, and GTPγS-Gαs (1 μM each) were incubated in presence or absence of 100 μM forskolin for 30 min on ice prior to addition of GST, GST-5NT, or 5NT 60-129 (2 μM). GST pull-down assay was performed as described in the Methods section. Western blot analysis of input and eluted proteins in shown (n=2).

Figure S6. 5NT Has No Effect on C1/C2 Basal AC Activity. Purified AC5 catalytic domains, 5C1 (70 nM) and 5C2 (1 μM), were preincubated with GST or GST-tagged 5NT deletions (5 μM) for 10 min prior to start of the assay (n=3).

Figure S7. Purified 5NT Does Not Alter Full-Length AC5 Activity. *Sf9* membranes expressing AC5 or AC5Δ66-137 were assayed for basal activity, Gαs-stimulated activity (50 nM) or forskolin-stimulated activity (50 μM) in the presence 5 μM GST, 5NT, or 5NT deletions (n=2).

Figure S8. **Gβγ Does Not Enhance 5C1/5C2/5NT Activity. (A),** Purified AC5 catalytic domains, 5C1 (70 nM) and 5C2 (1 μM), were preincubated with GST or GST-tagged 5NT in the presence or absence of Gβ1γ2 as indicated for 10 min prior to stimulation with 400 nM GTPγS-

Gαs (n=3).

References:

Graziano MP and Gilman AG (1989) Synthesis in Escherichia coli of GTPase-deficient mutants of Gs alpha. *J Biol Chem* **264**:15475.

Figure S1

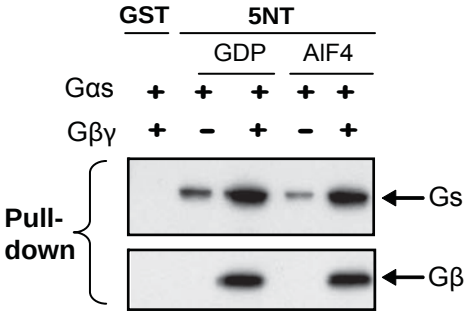

Figure S2

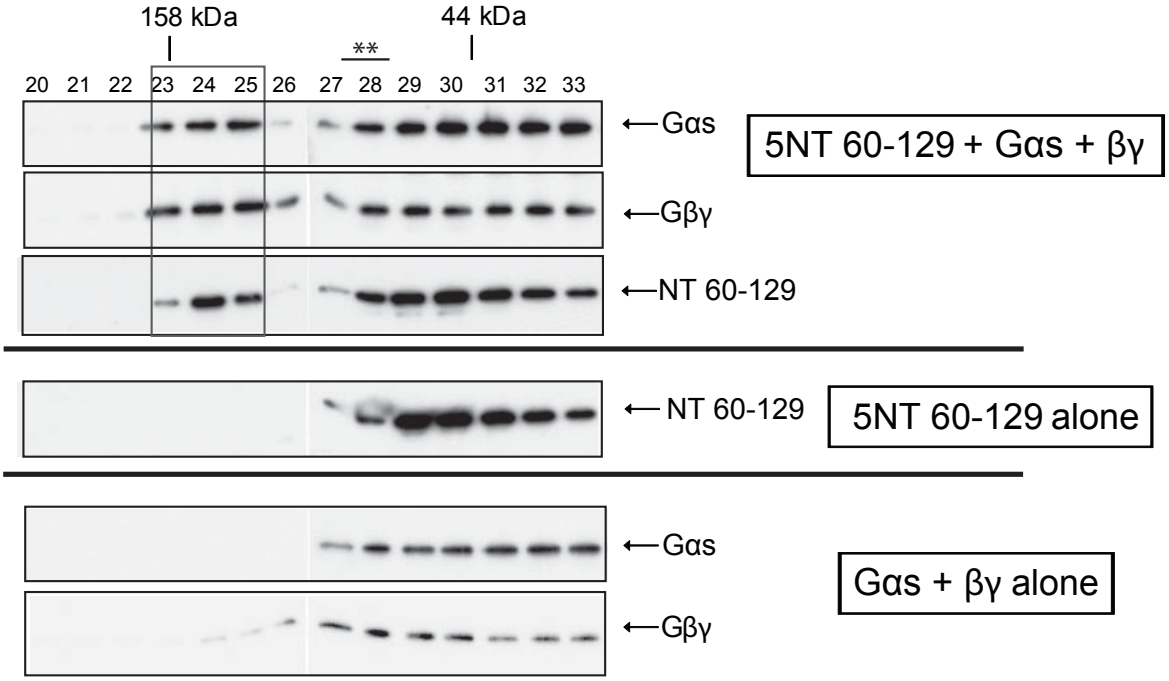

Figure S3

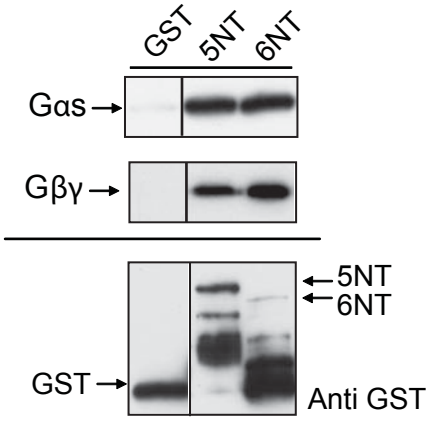

Figure S4

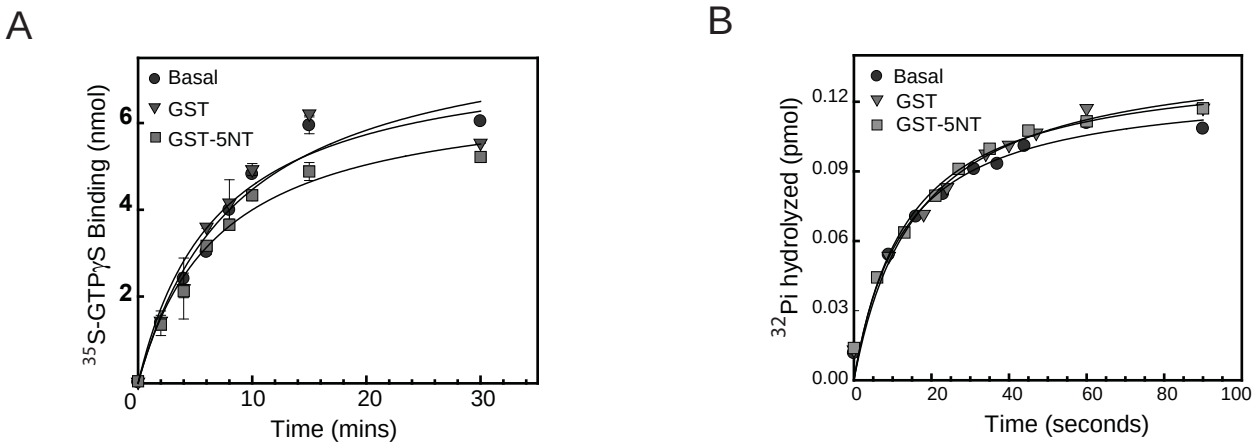

Figure S5

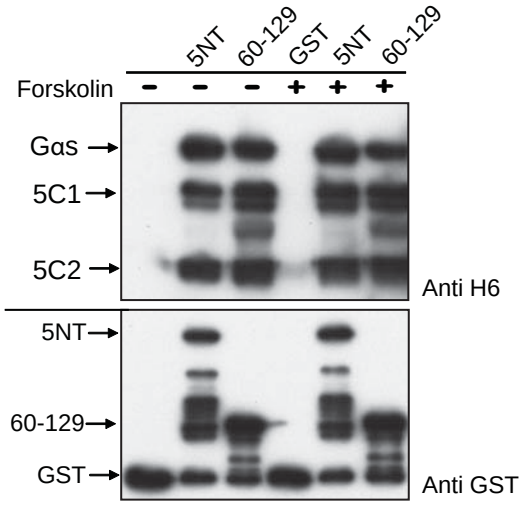

Figure S6

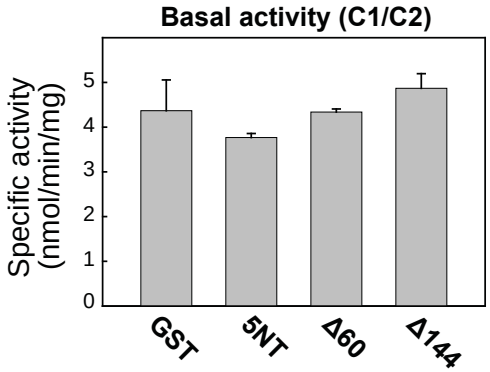

Figure S7

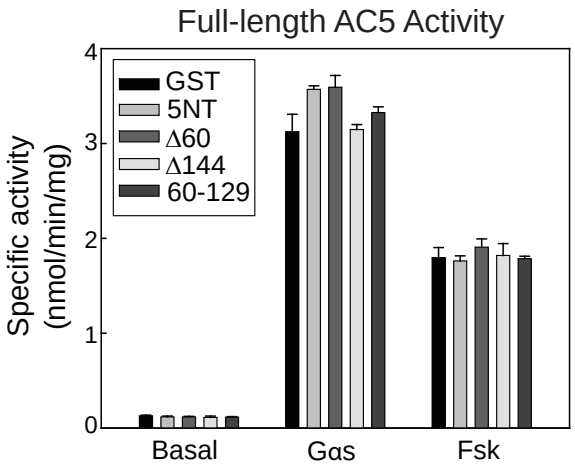

Figure S8