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SI Appendix

Materials and Methods

Mice: All mouse strains have been reported previously. All experiments were performed in accordance with the NIH IACUC.

DNA constructs: Supertopflash was provided by Dr. Randall Moon (University of Washington) and Renilla-luciferase plasmid (phRL-null) was purchased from Promega. pIRES-control and pIRES-Wnt3a have been described(1) and pcDNA-hGαs-WT, pcDNA-hGαs-R201C, pcDNA-hGαs-R201H have been reported(2). Myc-Axin was provided by Dr. Frank Constantini (Columbia Univ.). Constructs for HA-Gαs-WT, HA-Gαs-QL, HA-Gαi2-WT, HA-Gαi2-QL, HA-Gαq-WT, HA-Gαq-QL, HA-Gα13-WT, HA-Gα13-QL, β2-AR, Gαi/13 and Rac1-QL were kindly provided by Dr. J. Silvio Gutkind (NIH/NIDCR). Lentiviral shRNA plasmids against Human β-catenin were purchased from Sigma: LV1 (TRCN0000003843) and LV2 (TRCN0000003845). PcDNA-α2A-AR was provided by Dr. Brian Kobilka (Stanford University). PcDNA3.1-M1-AchR, -M2-AchR, -Gαo-QL and -Gαz-QL were purchased from the Missouri S&T cDNA resource. PcDNA-Gs-DREADD and –Gq-DREADD have been reported(3). CRE-luciferase, SRE-Luciferase and Ap1-luciferase were purchased from Stratagene.

Antibodies: mouse anti-β-catenin (BD Biosciences, 610154), rabbit anti-β-catenin (Cell signaling, 9562), mouse anti β-actin(Sigma, A5441), mouse anti-myc (Santa Cruz, sc-40), mouse anti-HA (Santa Cruz, sc-7392), rabbit anti-Gαs(4), anti-Gαq/11 (Santa Cruz, sc-392), anti-Gα12/13 (Santa Cruz, sc-409 and sc-410), rat anti-tubulin (Abcam, ab6160), phospho-CREB and total CREB (Upstate), phospho-LRP6 (Cell Signaling, 2568)

Other Reagents: Lithium chloride, forskolin, 3-isobutyl-1-methylxanthine (IBMX), isoproterenol, carbachol, and clonidine were purchased from Sigma-Aldrich. Clozapine-N-oxide (Enzo Life Sciences) and pertussis toxin (Calbiochem) were also purchased. GFP- and Cre-adenoviral stocks were produced by SAIC (SAIC, Frederick, MD).

Cell culture: hBMSCs were isolated as previously described(5). hBMSCs were grown in Alpha-MEM, 20% lot-selected FBS, 100U/mL penicillin, 100μg/mL streptomycin, 2mM glutamine, 10^{-8}M dexamethasone, 10^{-4}M L-ascorbic acid 2-phosphate. For osteoblast differentation, hBMSCs were grown to confluence and placed in osteogenic media for 3-4 weeks: DMEM, 10% lot-selected FBS, 100U/mL penicillin, 100μg/mL streptomycin, 2mM glutamine, 10^{-8}M dexamethasone, 10^{-4}M L-ascorbic acid 2-phosphate and 10mM β-glycerol phosphate. Mouse BMSC were isolated from 8-10 week old mice and cultured using similar media to hBMSCs, though without dexamethasone. MEF cells were isolated from E12.5 embryos and cultured in DMEM containing 20% FBS, 100U/mL penicillin, 100μg/mL streptomycin, 2mM glutamine, 1mM sodium pyruvate, 1X MEM non-essential amino acids (Gibco). Polyclonal MEF cell lines were formed by spontaneously immortalize. 293T cells (ATCC) were grown in DMEM, 10% FBS, 100U/mL penicillin, 100μg/mL streptomycin, 2mM glutamine. Hela cells (ATCC) were grown in DMEM, 10% FBS, 100U/mL penicillin, 100μg/mL streptomycin, 2mM glutamine.

Immunohistochemistry: For β-catenin staining on decalcified human bone sections, slides were deparaffinized and boiled in 0.1M citrate buffer for 15 minutes. Endogenous peroxidase activity was quenched by placing slides in 3%H_2O_2/MeOH for 15 minutes. Slides were blocked with 5% goat serum/TBST for 1 hour and rabbit anti-β-catenin (Cell Signaling #9562; 1:80)

overnight at 4°C. Slides were washed with TBST and staining was detected using the ABC elite detection kit (Vector).

Histology: For H&E staining, mouse tibia were first fixed in 4% paraformaldehyde (PFA) and decalcified with EDTA. For undecalcified sections, mouse tibia were fixed in 4% PFA and embedded in methyl methacrylate. Von Kossa staining was performed using standard protocols. X-gal staining was performed using standard methods.

Co-immunoprecipitation: 293T cells were transiently transfected using Lipofectamine plus (Invitrogen). 24 hours following transfection cells were washed with ice-cold PBS and lysed with cold IP buffer [50mM Tris-HCl (pH 7.4), 150mM NaCl, 2mM MgCl2, 1% NP-40, 10% glycerol, 10mM NaF, 1mM Na3VO4, and complete protease inhibitor cocktail (Roche)]. Cells were rotated at 4°C for 20 minutes, scraped into an eppendorf tube, vortexed for 30 seconds and centrifuged for 30 minutes at 4°C. Supernatant was collected, antibody was added for 2 hours at 4°C followed by protein G sepharose (GE Healthcare) for an additional 30 minutes.

Cytoplasmic β-catenin: MEF cells were cultured in 6 well dishes in 0.2%FBS media overnight and then treated with 25% Wnt3a-conditioned media/75% 0.2% FBS media for 1-2 hours. Cells were washed in ice-cold PBS, 1mL hypotonic lysis buffer (25mM Tris-HCl pH7.5, 1mM EDTA, 25mM NaF, 1mM DTT, protease inhibitors) was added per well and scraped into an eppendorf tube. Tubes were vortexed and rocked at 4°C for 15 minutes then centrifuged at 3000 RPM for 5 minutes to remove nuclei and unlysed cells. Supernatant was then centrifuged at 100,000XG at 4°C for 90 minutes. This second supernatant fraction was considered the cytoplasmic fraction.

Immunofluorescence: Hela cells were transiently transfected using Fugene 6 (Roche). 40ng myc-Axin and 50ng HA-Gα were transfected into wells of a 2- well chamber slide (Nalgene,

LabTek II). Cells were fixed and stained 12-24 hours later. As others have described(6), the relative amount and ratios of plasmids is critical.

Statistics: Experiments were performed a minimum of 3 times. Graphed data are shown as AVG±SD. P values were generated from unpaired student's T-test.

Supplemental Figures and legends:

S1-S4: *Gα_s* removal leads to decreased Wnt/β-catenin signaling in skeletal development. S1) X-gal staining of forelimbs from E16.5 control mice containing a Topgal transgene identifies active Wnt/β-catenin signaling in osteoblastic cells (arrow). S2) X-gal staining of forelimbs from *Prx1-cre; Gα_s^{fl-}; Topgal* mutant littermate mice shows decreased Wnt/β-catenin signaling (arrow) in osteoblastic cells. S3) qPCR analysis of Wnt/β-catenin signaling transcription targets in these limbs demonstrates reduced levels of *Gα_s, Axin2*, *Tcf1* and *Lef1* mRNA (n=3-5; *p<0.05). S4) qPCR data showing reduced expression of endogenous targets of Wnt/β-catenin signaling (*Axin2* and *Tcf1*) following removal of Gα_s during osteoblastic differentiation. BMSCs were isolated from *G$\alpha_s^{fl/fl}$* mice and infected with either a control (GFP-) or Cre-containing adenovirus and grown under osteoblastic conditions for 7 days (n=3; avg±SD; p=0.001 and 0.005 for *Axin2* and *Tcf1*, respectively).

S5: Treatment of BMSCs with shRNA-containing lentiviruses leads to decreased β-catenin protein levels. Human BMSCs were treated with either a control lentivirus (LV) or LVs targeting β-catenin. LV1 leads to a modest (~20%) decrease in β-catenin protein while treatment with LV2 leads to a more substantial (~80%) decrease.

S6: Activation of G_s and G_q has opposite effects on Wnt/β-catenin signaling. Either prototypical GPCRs known to have restrictive pathway activation or designer receptors exclusively activated by a designer drug (DREADDs)(7), which activate one G protein pathway, were stimulated with specific agonists. MEF cells were transfected with either a vector expressing a G_s-coupled GPCR (DREADD-G_s or β2-adrenergic receptor), a G_q-coupled

GPCR (DREADD-G_q or M1-acetylcholine receptor) or a control vector, and stimulation of Wnt/β-catenin signaling was quantified using Supertopflash. Activation of G_s-coupled GPCRs did not affect basal Wnt/β-catenin signaling but potentiated Wnt3a-induced activation (^{1}p=0.004; ^{2}p=0.006). Activation of G_q-coupled GPCRs inhibited Wnt3a-induced activation (^{3}p=0.001, ^{4}p=0.02). Clozipine-N-oxide (CNO) and isoproterenol (isopr) were added to a final concentration of 10μM. Carbachol (carb) was added to a final concentration of 100μM. (n=3-5; avg±SD)

S7: Activation of $G_{i/o}$ has no effect on Wnt/β-catenin signaling while activation of G_{13} inhibits this pathway. Transfection of MEF cells with either of the two $G_{i/o}$-coupled GPCRs (α2A- adrenergic receptor or M2- acetylcholine receptor) fails to affect basal or Wnt3a-induced activation of Supertopflash. As no GPCR receptor has yet been identified as being specific for the $G_{12/13}$ pathway we chose instead to use a chimeric $G\alpha_{i/13}$ subunit that allows $G_{i/o}$-coupled GPCRs to activate the $G_{12/13}$ pathway(8). Co-transfection of M2-AchR with the $G\alpha_{i/13}$ construct leads to inhibition of Wnt/β-catenin signaling (*p=0.02). Clonidine (clon) was added to a final concentration of 10μM. Carbachol (carb) was added to a final concentration of 100μM. (n=3-5; avg±SD)

S8: Activated forms of Gα proteins reproduce the effect of GPCR stimulation. Transfection of activated (QL) forms of the Gα proteins does not affect basal or LiCl-induced activation of Wnt/β-catenin signaling in MEF cells, but differentially regulates Wnt3a-induced stimulation similarly to receptor activation. $G\alpha_s$-QL potentiates (p=0.002), while $G\alpha_q$-QL

(p=0.03) and $G\alpha_{13}$-QL (p=0.04) inhibit, signaling. Addition of either 10μM forskolin or 0.5mM IBMX has no effect. (n=3; avg±SD)

S9: Activation with forskolin leads to CRE-luc activation and phosphorylation of CREB. For luciferase assay, MEF cells were transfected and allowed to recover overnight before adding forskolin for 8 hours (n=3, *$p<0.05$). MEFs were serum starved overnight and incubated with 10μM forskolin for the indicated times. A western blot confirms induction of phospho-CREB while total levels of CREB remain constant. (n=3; avg±SD)

S10: $G\alpha_q$ and $G\alpha_{13}$ activate Ap1- and SRE-luciferase. MEFs were transfected with either SRE-luciferase or Ap1-luciferase and a vector encoding the indicated G proteins. After 24 hours the cells were lysed and luciferase levels were quantified (n=3, *$p<0.05$). (n=3; avg±SD)

S11: Levels of cytoplasmic β-catenin correlate with Gα protein effects. Wnt3a-induced accumulation of cytoplasmic β-catenin is similarly affected by the presence of activated Gα proteins with $G\alpha_s$ potentiating Wnt3a-induced β-catenin accumulation and $G\alpha_q$ and $G\alpha_{13}$ inhibiting this response. Experiments were performed in MEF cells.

S12: $G\alpha_s$ and $G\alpha_{13}$ compete for binding to Axin. Cotransfection of 293T cells with Axin and both $G\alpha_s$ and $G\alpha_{13}$ leads to reduced binding relative to binding of Axin to either $G\alpha_s$ or $G\alpha_{13}$ alone.

S13: Gα_s is not required for Wnt/β-catenin signaling. Accumulation of cytoplasmic β-catenin and phosphorylation of Lrp6 are not affected in MEFs lacking Gα_s protein. Cells were treated with 30ng/mL rWnt3a for 1 hour.

S14: Removal of G$\alpha_{q/11}$ and G$\alpha_{i/o}$ does not affect Wnt/β-catenin signaling. Experiments in which G$\alpha_{q/11}$ and G$\alpha_{i/o}$ signaling were removed either singly or doubly from MEF cells demonstrated normal accumulation of cytoplasmic β-catenin and phosphorylation of Lrp6. Cells were treated with 30ng/mL rWnt3a for 1 hour. These results suggest that G$\alpha_{q/11}$ and G$\alpha_{i/o}$ are not required for Wnt/β-catenin signaling in MEFs. Cells were pretreated with 100ng/mL PTX.

S15: Removal of G$\alpha_{12/13}$ does not affect Wnt/β-catenin signaling. Removal of G$\alpha_{12/13}$ from MEF cells failed to affect accumulation of cytoplasmic β-catenin or phosphorylation of Lrp6 in MEFs. These results suggest that G$\alpha_{12/13}$ activity is not essential for Wnt/β-catenin signaling.

Supplemental Table 1: Primer sets used for qPCR

18S	CGT TGA TTA AGT CCC TGC CCT T	TCA AGT TCG ACC GTC TTC TCA G
B-ACTIN	GGC ACG AAG GCT CAT CAT TC	CCG ACA GGA TGC AGA AGG AG
AXIN2	TTA TGC TTT GCA CTA CGT CCC TCC A	CGC AAC ATG GTC AAC CCT CAG AC
TCF1	CGG GAC AGA GGA CCA TTA CAA CTA GA	CCA CCT GCC TCG GCC TGC CAA AGT
ALK PHOS	GAG ACA CTG AAA TAT GCC CTG G	CTC ATT GGC CTT CAC CCC ACA CAG
BSP	AGG CAG AAA ACG GCA ACG GCA	GTC CCC ACG AGG TTC CCC GTT
OC	GAG CCC CAG TCC CCT ACC CG	GAC ACC CTA GAC CGG GCC GT
OPG	CTG TAC AGC AAA GTG GAA GAC CGT G	GCA GCT TCA GGA TCT GGT CAC TGG
RANKL	CAG GCC TTT CAA GGA GCT GTG CA	AAG GAG GGG TTG GAG ACC TCG ATG C
Tubulin	CAA CGT CAA GAC GGC CGT GTG	GAC AGA GGC AAA CTG AGC ACC
B-actin	CAC AGC TTC TTT GCA GCT CCT T	CGT CAT CCA TGG CGA ACT G
Gs	GCA GAA GGA CAA GCA GGT CT	CCC TCT CCG TTA AAC CCA TT
Axin2	ATG TGT GGA TAC GCT GGA CTT	TTC TTG ATG CCA TCT CGT ATG
Tcf1	ACA TGA AGG AGA TGA GAG CCA	CTT CTT CTT TCC GTA GTT ATC
Lef1	TCT CAA GGA CAG CAA AGC TC	CAC TTG AGG CTT CAT GCA CAT

References

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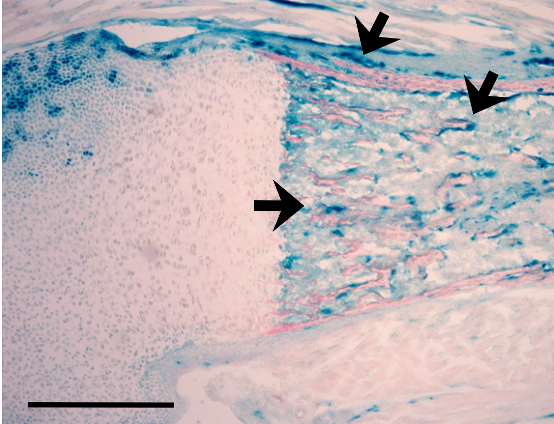
S1)



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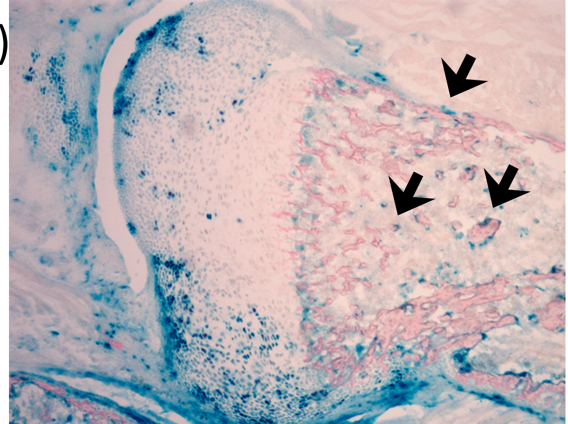

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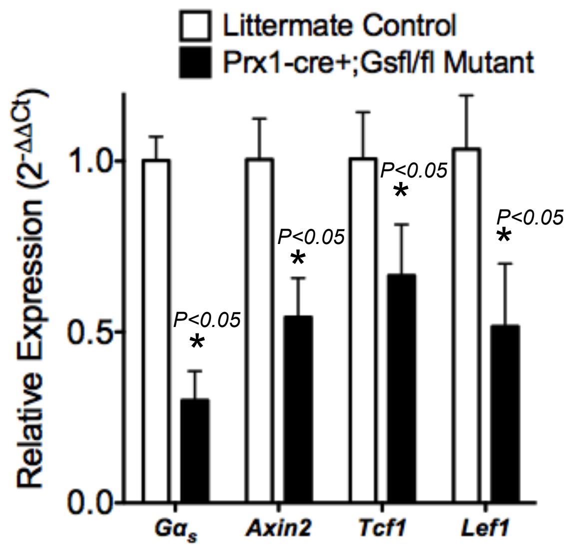

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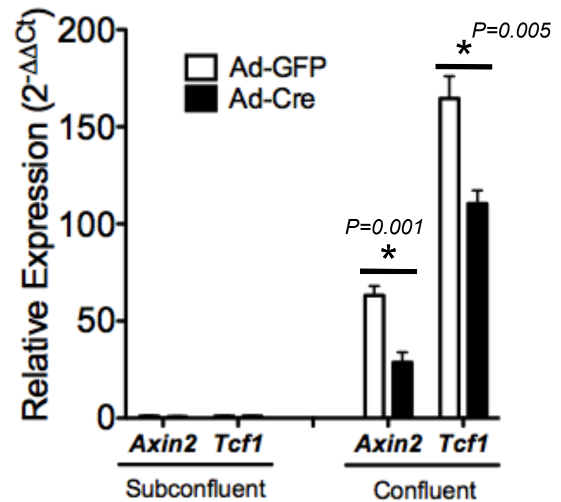

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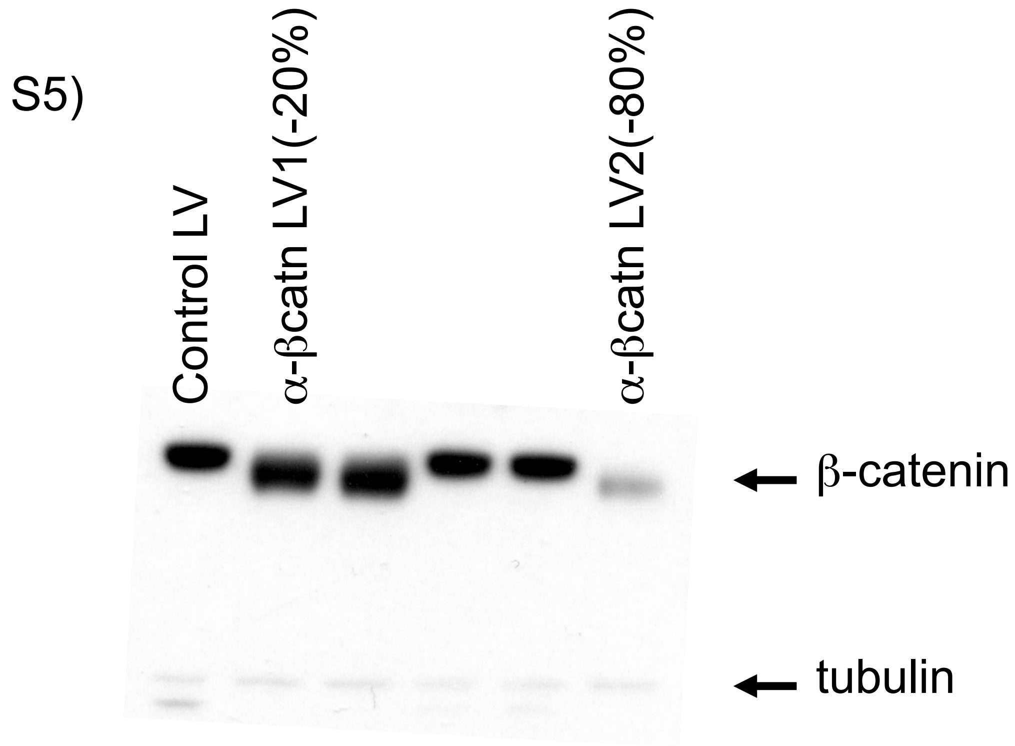

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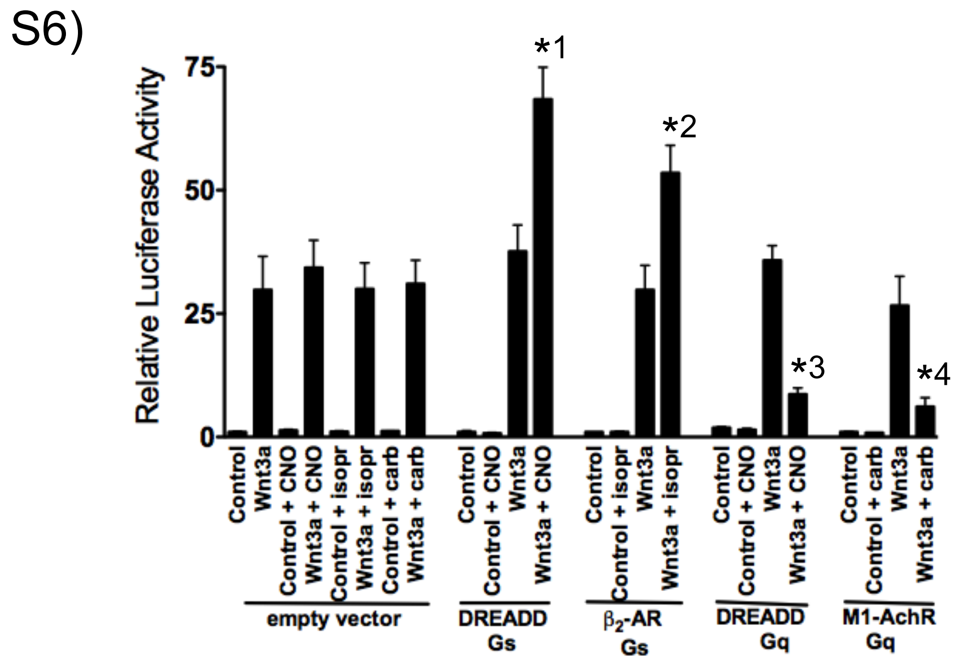

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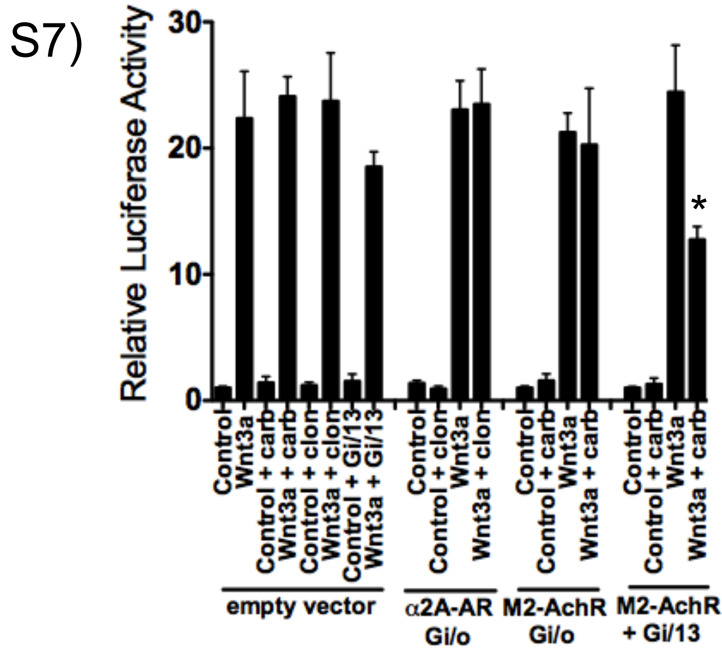

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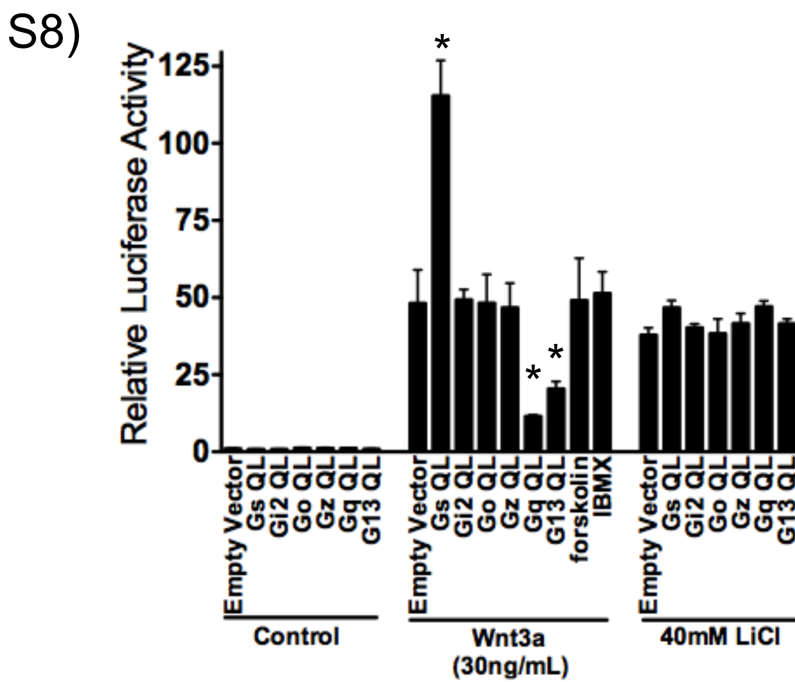

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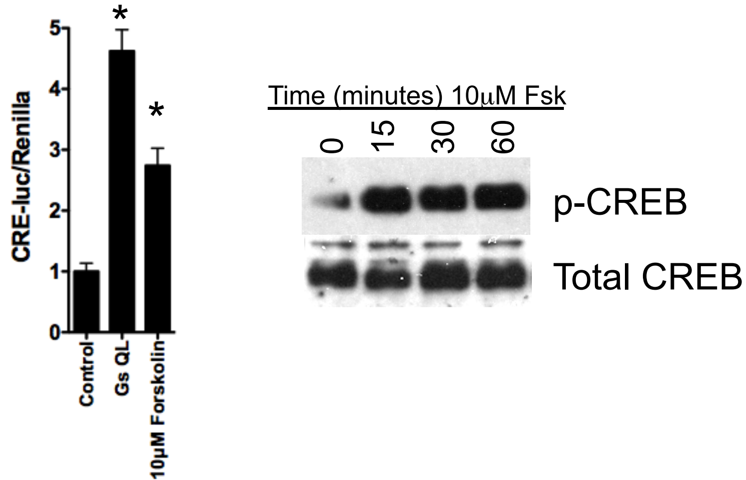

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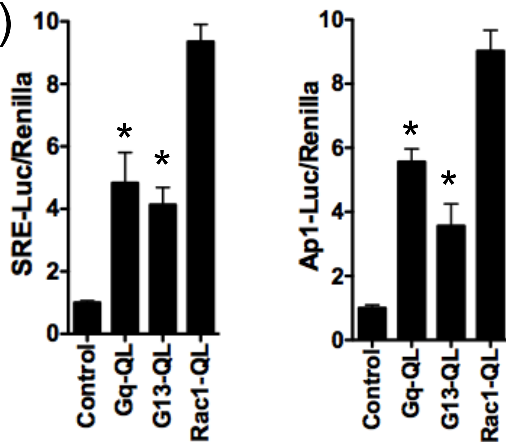

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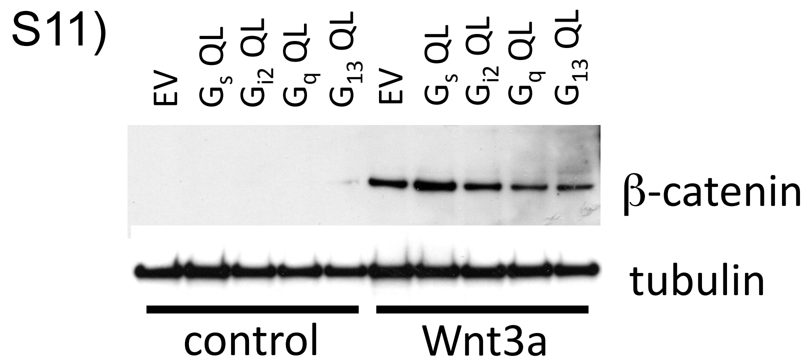

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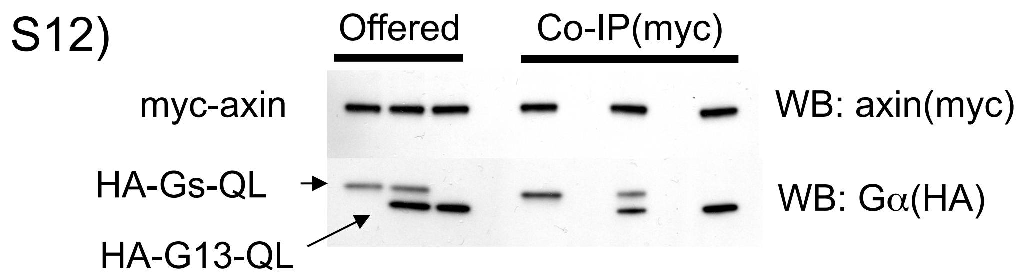

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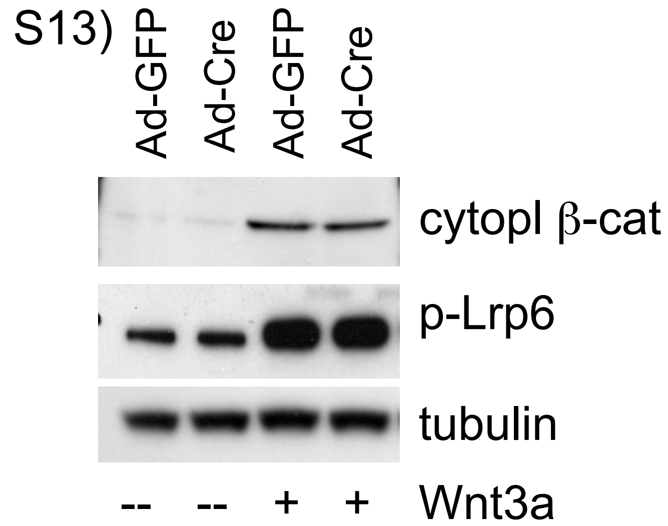

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S14)

-PTX +PTX

control Wnt3a Wnt3a

GFP GFP Cre Cre GFP GFP Cre Cre GFP GFP Cre Cre

cyto β-cat

tubulin

-PTX +PTX

control Wnt3a control Wnt3a

GFP Cre GFP Cre GFP Cre GFP Cre

p-Lrp6

tubulin

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