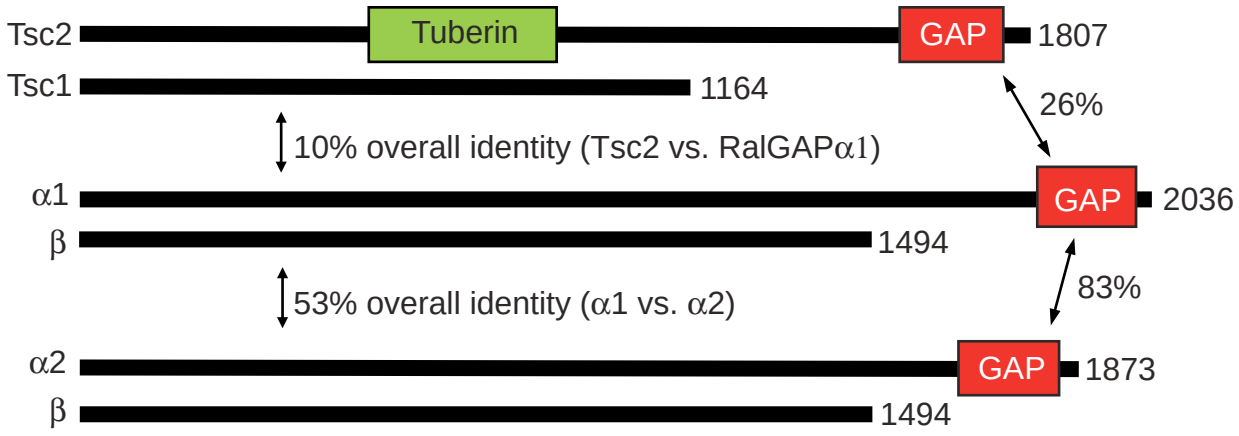
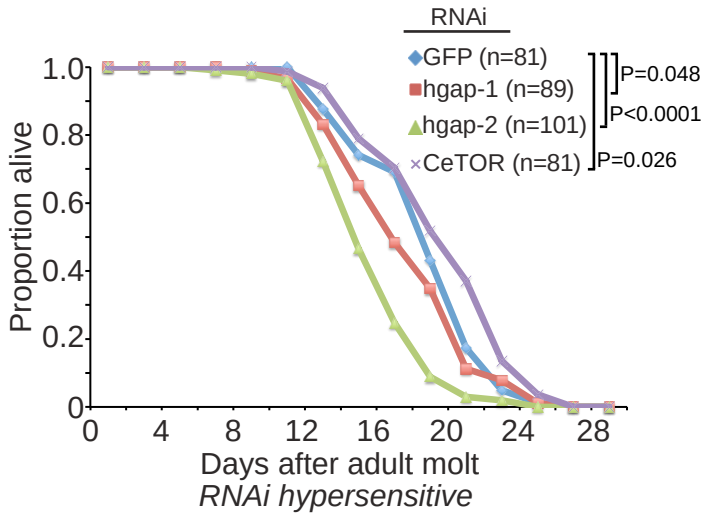
Figure S1

A



B



C

let-363/CeTOR genotype

RNAi	Animal Stage	h98/ h98/+	h98/ h98	ok3018/ +	ok3018/ ok3018
Luciferase	L4A	65	0	31	0
Luciferase	L3	2	57	0	4
hgap-1/ rrf-3	L4A	52	0	36	0
hgap-1/ rrf-3	L3	2	54	0	4

D

background: *hgap-2(578143)*

Animal Stage	h98/ +	h98/ h98	ok3018/ +	ok3018/ ok3018
L4A	123	0	163	0
L3	0	28	2	28

Figure S1. Conservation of RalGAPs, related to Figure 1

(A) Primary structures and sequence identity comparison of the Tsc1-Tsc2 RhebGAP subunits and the α and β subunits of the RalGAP heterodimeric complexes. Tsc1 (harmatin) and Tsc2 (tuberin) form the heterdimeric RhebGAP protein. The two human RalGAPs are comprised of related α catalytic subunits and a common regulatory β subunit. Percentages represent overall sequence identity and GAP domain sequence identity between human Tsc2 and the human RalGAPα1 and α2 subunits. Domains were identified in SMART and ClustalW sequence alignment was done to determine sequence identity. Numbers indicate amino acid size.

(B) *hgap-1(RNAi)* (P = 0.048) and *hgap-2(RNAi)* (P < 0.0001) confer decreased lifespan in the *eri-1(mg366)* RNAi hypersensitive background compared to control *gfp(RNAi)*. *Tor/let-363(RNAi)* animals live longer (P = 0.026).

(C) HGAP-1 loss fails to rescue LET-363/CeTOR requirement for growth. Animals heterozygous or homozygous for two different *let-363* alleles were grown on control luciferase or *hgap-1+rrf-3* RNAi to assess whether *hgap-1* loss could bypass the L3 arrest conferred by LET-363/CeTOR loss. *rrf-3* was targeted to increase RNAi sensitivity. Strains used were KR344 *let-363(h98) dpy-5(e61) unc-13(e450)*; *sDp2(*I;f*)* and VC2312 l*et-363(ok3018)* / *hT2 [bli-4(e937) let-?(q782) qIs48* + / *hT2*. *h98* homozygotes were identified by duplication loss, revealing the Dpy cis marker, while *sDp2*-bearing heterozygotes were Unc. *ok3018* homozygotes were identified by the absence of balancer pharyngeal GFP, while heterozygotes expressed GFP in the pharynx. Animals were scored in synchronous broods.

(D) HGAP-2 loss fails to rescue LET-363/CeTOR requirement for growth. Animals heterozygous or homozygous for two different let-363 alleles with the *hgap-2(gk578143)* mutation in the background *were* assayed for whether HGAP-2 loss could bypass the L3 arrest conferred by LET-363/CeTOR loss. Strains used were DV2869 *let-363(h98) dpy-5(e61) unc-13(e450)* / *hT2 [bli-4(e937) let-?(q782) qIs48* I; *hgap-2(gk578143)* II and DV2870 *let-363(ok3018)* / *hT2 [bli-4(e937) let-?(q782) qIs48* I; *hgap-2(gk578143)* II. *h98* and *ok3018* homozygotes were identified by the absence of pharyngeal GFP and, with *h98*, the presence of Dpy and Unc cis markers, while heterozygotes expressed GFP in the pharynx. Animals were scored in synchronous broods. Animals were grown at 20°C. P values from log-rank test.

Figure S2

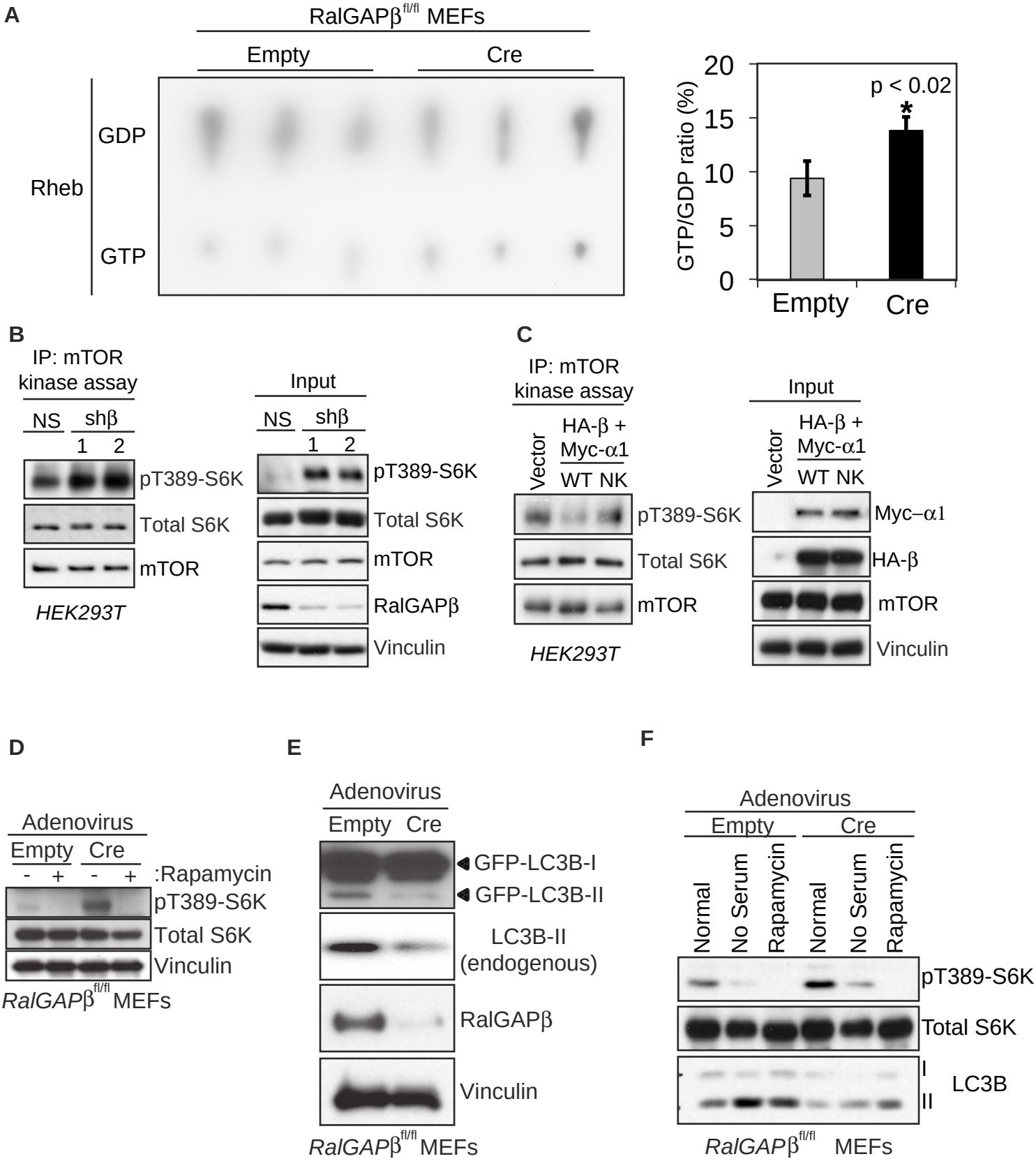

A
RalGAPβ fl/fl MEFs
Empty
Cre
Rheb
GDP
GTP
GTP/GDP ratio (%)
20
15
10
5
0
p < 0.02
*
Empty
Cre
B
IP: mTOR kinase assay
NS
shβ
1
2
pT389-S6K
Total S6K
mTOR
HEK293T
Input
NS
shβ
1
2
pT389-S6K
Total S6K
mTOR
RalGAPβ
Vinculin
C
IP: mTOR kinase assay
Vector
HA-β + Myc-α1
WT
NK
pT389-S6K
Total S6K
mTOR
HEK293T
Input
Vector
HA-β + Myc-α1
WT
NK
Myc–α1
HA-β
mTOR
Vinculin
D
Adenovirus
Empty
Cre
- + - +
:Rapamycin
pT389-S6K
Total S6K
Vinculin
RalGAPβ fl/fl MEFs
E
Adenovirus
Empty
Cre
GFP-LC3B-I
GFP-LC3B-II
LC3B-II (endogenous)
RalGAPβ
Vinculin
RalGAPβ fl/fl MEFs
F
Adenovirus
Empty
Cre
Normal
No Serum
Rapamycin
Normal
No Serum
Rapamycin
pT389-S6K
Total S6K
I
II
LC3B
RalGAPβ fl/fl MEFs

Figure S2. RalGAP β loss does significantly alter Rheb activation and RalGAPs contribute to the regulation of mTOR activity, related to Figure 2

(A) Loss of RalGAP results in limited Rheb activation. *RalGAP* $\beta^{fl/fl}$ MEFs expressing FLAG-Rheb were treated with either empty or Cre expressing adenovirus. FLAG-Rheb was immunoprecipitated and the [^{32}P]GTP and GDP nucleotide-loaded state was resolved by thin layer chromatography.

(B) Knockdown of RalGAP results in enhanced in mTOR kinase activity. RalGAPβ was knocked down with shRNA in HEK293T cells and mTOR kinase activity was assessed by in vitro kinase assays.

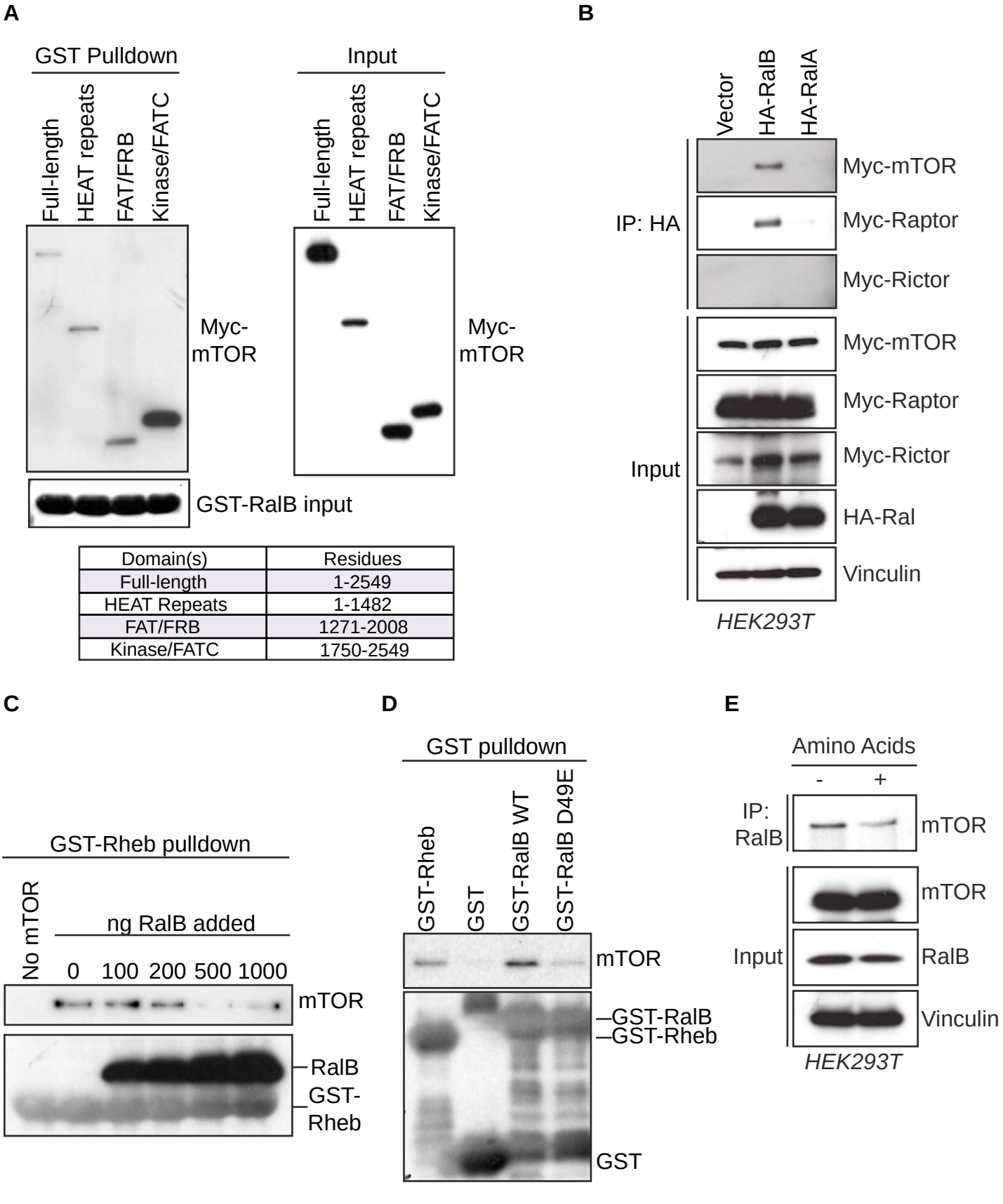
(C) Overexpression of wildtype but not GAP-dead RalGAP reduces mTOR kinase activity. HEK293T cells were transfected with HA-RalGAP β and either wildtype or GAP-dead (N1703K) Myc-RalGAP α 1. mTOR kinase activity was assessed by in vitro kinase assays.

(D) Elevated pS6K in *RalGAP6* deficient MEFs is dependent on mTORC1 activity. *RalGAP6*$^{fl/fl}$ MEFs treated with empty or Cre expressing adenovirus were treated with either vehicle control or 20 nM rapamycin for 16 h. Cell lysates were analyzed by immunoblotting with the indicated antibodies.

(E) Depletion of RalGAPβ results in reduced autophagy. *RalGAP6*$^{fl/fl}$ MEFs in growth medium with and without serum or treated with 100 nM rapamycin were treated with empty or Cre recombinase expressing adenovirus followed by stable expression of GFP-LC3. Cell lysates were immunoblotted with the indicated antibodies.

(F) Inhibition of mTOR restores autophagy induction in RalGAPβ depleted cells. *RalGAP6*$^{fl/fl}$ MEFs were treated with empty or Cre-expressing adenovirus were either serum-starved or treated with 20 nM rapamycin for 16 h. Cell lysates were immunoblotted with the indicated antibodies.

Figure S3



Domain(s)	Residues
Full-length	1-2549
HEAT Repeats	1-1482
FAT/FRB	1271-2008
Kinase/FATC	1750-2549

Figure S3. RalB but not RalA engages mTORC1 and the association is unperturbed by amino acids, related to Figure 3

(A) RalB interacts with the kinase domain of mTOR. HEK293T cells were transfected with the indicated Myc-tagged mTOR truncation mutants. GST-RalB pulldowns were performed on lysate from each Myc-mTOR mutant to assess binding. Total cell lysate was immunoblotted to determine relative levels of Myc-mTOR mutant expression.

(B) RalB only interacts with mTORC1 while RalA does not associate with either mTORC1 or mTORC2. HEK293T cells transiently expressing the indicated HA-tagged Ral and Myc-tagged mTORC1/2 proteins were subjected to anti-HA immunoprecipitation followed by anti-Myc immunoblotting to determine co-precipitation of Myc-tagged mTOR, Rictor, or Raptor.

(C) RalB and Rheb compete for mTOR binding. GST-Rheb was allowed to bind mTOR from 293T cells. Following GST-Rheb binding, the indicated amounts of purified recombinant RalB was added and allowed to bind for 2 h. GST-Rheb bound to glutathione resin was washed and association with mTOR was determined by western blotting.

(D) RalB requires exocyst association to interact with mTOR. GST, GST-Rheb, and GST-RalB wildtype and exocyst binding-deficient D49E proteins were incubated with 293T cellular lysate for 2 h. mTOR association was determined by western blotting.

(E) The stimulation of cells by amino acids does not affect the RalB-mTOR complex. HEK293T cells were starved of amino acids, serum, and glucose for 1 h followed by either treatment with 2X essential amino acids or no amino acid control for 20 min. Cells were harvested and subjected to endogenous RalB immunoprecipitation followed by immunoblotting to determine co-precipitation of endogenous mTOR.

Figure S4

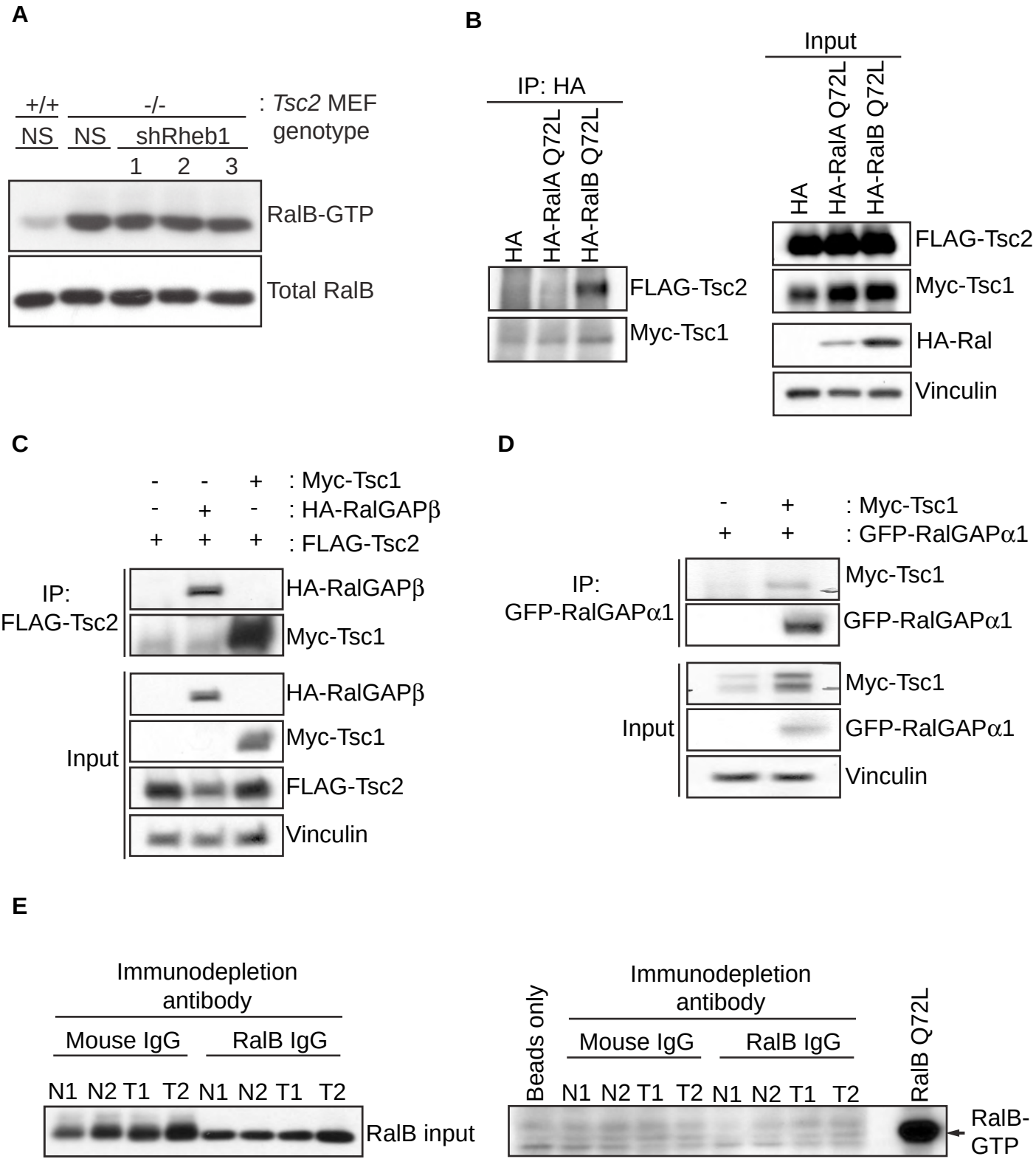

A
+/+
-/-
: Tsc2 MEF genotype
NS
NS
shRheb1
1 2 3
RalB-GTP
Total RalB
B
IP: HA
HA
HA-RalA Q72L
HA-RalB Q72L
FLAG-Tsc2
Myc-Tsc1
Input
HA
HA-RalA Q72L
HA-RalB Q72L
FLAG-Tsc2
Myc-Tsc1
HA-Ral
Vinculin
C
- - + : Myc-Tsc1
- + - : HA-RalGAPβ
+ + + : FLAG-Tsc2
IP: FLAG-Tsc2
HA-RalGAPβ
Myc-Tsc1
Input
HA-RalGAPβ
Myc-Tsc1
FLAG-Tsc2
Vinculin
D
- + : Myc-Tsc1
+ + : GFP-RalGAPα1
IP: GFP-RalGAPα1
Myc-Tsc1
GFP-RalGAPα1
Input
Myc-Tsc1
GFP-RalGAPα1
Vinculin
E
Immunodepletion antibody
Mouse IgG
RalB IgG
N1 N2 T1 T2 N1 N2 T1 T2
RalB input
Beads only
Immunodepletion antibody
Mouse IgG
RalB IgG
RalB Q72L
N1 N2 T1 T2 N1 N2 T1 T2
RalB-GTP

Figure S4. Crosstalk between the Ral and Tsc signaling pathways, related to Figure 4

(A) Rheb is not required for the enhanced RalB-GTP observed in Tsc2-deficient cells. *Tsc2* wildtype (+/+) or null (-/-) MEFs stably expressing NS or Rheb1 shRNAs from Figure 4D were subject to GST-Sec5-RBD pulldown assays to determine Ral GTPase activity. Total cellular lysates were also analyzed with an anti-RalB antibody to determine total RalB protein expression.

(B) Active RalB but not RalA can complex with overexpressed Tsc2. HEK293T cells were transfected with either vector, HA-RalA(Q72L), and HA-RalB(Q72L) along with Myc-Tsc1 and FLAG-Tsc2. HA-tagged proteins were immunoprecipitated and Tsc1/2 association was determined by western blot.

(C) and (D) Tsc and RalGAP proteins can heterodimerize. HEK293T cells were transfected with the indicated RalGAP and Tsc constructs. Co-immunoprecipitation was performed as indicated and association was determined by western blotting.

(E) Eker renal tumors have elevated RalB activity. Normal (N1, N2) and tumor (T1, T2) renal tissue lysates from Eker rats were immunodepleted with either mouse IgG or RalB IgG. Lysates were then subject to GST-Sec5 RalBD pulldowns to analyze RalB-GTP levels.

Figure S5

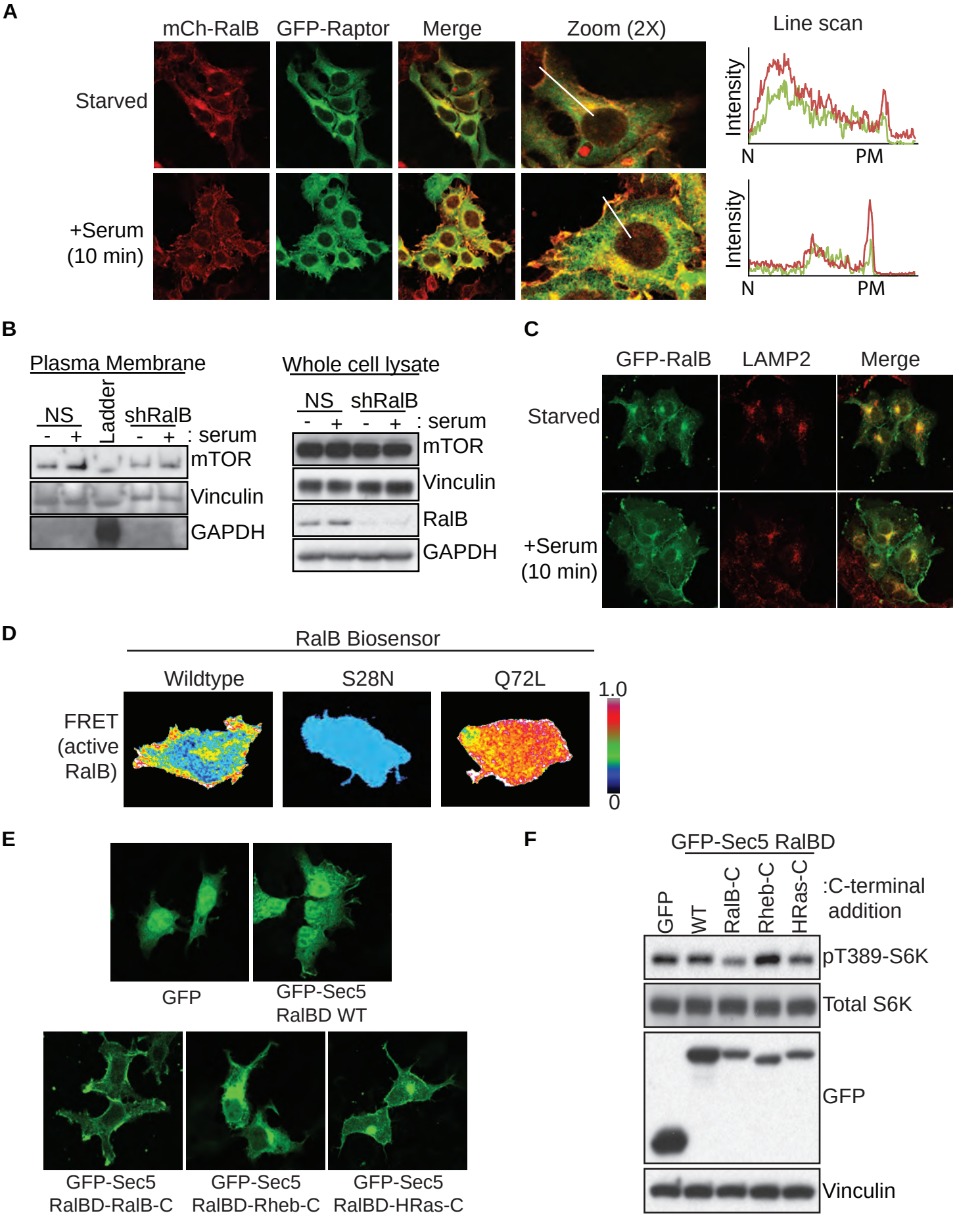

A
mCh-RalB
GFP-Raptor
Merge
Zoom (2X)
Line scan
Starved
+Serum
(10 min)
Intensity
N
PM
B
Plasma Membrane
Whole cell lysate
NS
shRalB
Ladder
- + - + : serum
mTOR
Vinculin
GAPDH
RalB
C
GFP-RalB
LAMP2
Merge
Starved
+Serum
(10 min)
D
RalB Biosensor
Wildtype
S28N
Q72L
FRET
(active
RalB)
1.0
0
E
GFP
GFP-Sec5
RalBD WT
GFP-Sec5
RalBD-RalB-C
GFP-Sec5
RalBD-Rheb-C
GFP-Sec5
RalBD-HRas-C
F
GFP-Sec5 RalBD
GFP
WT
RalB-C
Rheb-C
HRas-C
:C-terminal
addition
pT389-S6K
Total S6K
GFP
Vinculin

Figure S5. Serum stimulation results in mTORC1 plasma membrane recruitment, related to Figure 5

(A) Serum stimulation leads to Raptor recruitment to the plasma membrane. HEK293T cells expressing mCh-RalB and GFP-Raptor were serum starved overnight. Localization of fluorescent proteins in starved cells and cells stimulated with serum for 10 min were analyzed by confocal microscopy. Line scans indicate protein localization along the line from the nucleus (N) to the plasma membrane (PM).

(B) RalB is required for the recruitment of mTOR to the plasma membrane. HEK293T cells expressing either NS or RalB shRNA were serum starved overnight. Plasma membrane fractionation was performed on starved cells and cells stimulated with serum for 10 min and immunoblots were performed. Whole cell lysates were also immunoblotted to determine protein expression levels.

(C) Lysosomal localization of RalB is unchanged by serum stimulation. HEK293T cells expressing GFP-RalB were serum starved overnight. GFP-RalB colocalization with the lysosomal protein LAMP2 was examined in both starved cells and cells stimulated with serum for 10 min.

(D) Controls for the RalB biosensor. Shown are the controls used to verify that the RalB biosensor was functioning properly. These included constitutively GDP-bound RalB(S28N) constitutively GTP-bound RalB(Q72L).

(E) Addition of C-terminal membrane targeting sequences from different GTPases to GFP-Sec5-RBD leads to expected subcellular localization. HEK293T cells were transfected with the indicated constructs. Cells were serum-starved overnight and then stimulated with 10% serum for 10 min prior to fixation.

(F) Blocking Ral signaling at plasma membrane but not the lysosome via GFP-Sec5-RBD expression leads to impaired mTORC1 signaling. HEK293T cells were treated as in panel S5E and mTORC1 activation was examined by western blotting. For all images scale bars are 20 μm.

Figure S6

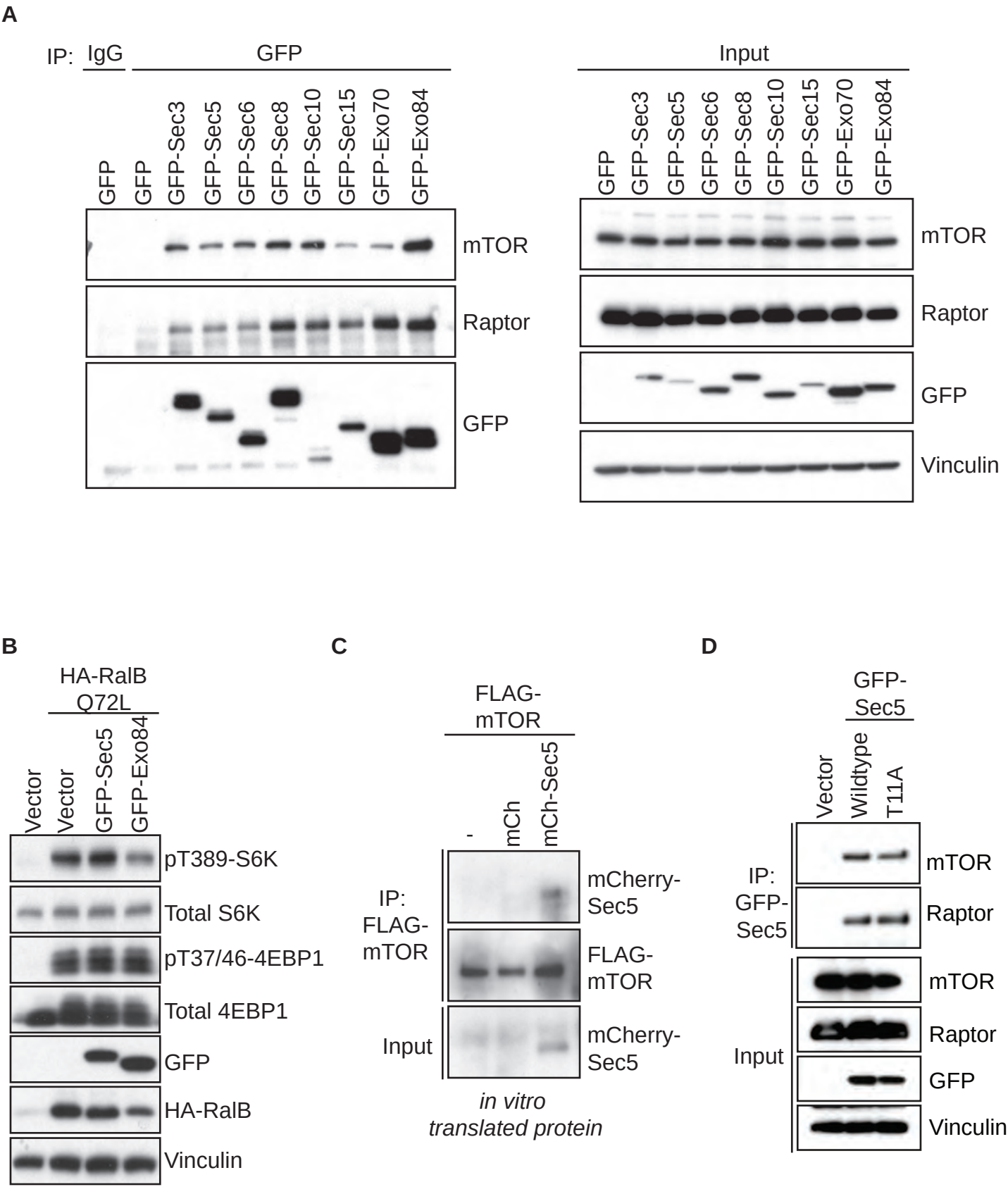

A
IP:
IgG
GFP
GFP
GFP
GFP-Sec3
GFP-Sec5
GFP-Sec6
GFP-Sec8
GFP-Sec10
GFP-Sec15
GFP-Exo70
GFP-Exo84
mTOR
Raptor
GFP
Input
Vinculin
B
HA-RalB Q72L
Vector
Vector
GFP-Sec5
GFP-Exo84
pT389-S6K
Total S6K
pT37/46-4EBP1
Total 4EBP1
GFP
HA-RalB
Vinculin
C
FLAG-mTOR
-
mCh
mCh-Sec5
IP: FLAG-mTOR
mCherry-Sec5
FLAG-mTOR
Input
mCherry-Sec5
in vitro translated protein
D
GFP-Sec5
Vector
Wildtype
T11A
IP: GFP-Sec5
mTOR
Raptor
Input
mTOR
Raptor
GFP
Vinculin

Figure S6. The exocyst associates with mTORC1, related to Figure 6

(A) All eight components of the exocyst associate with mTORC1. HEK293T cells were transfected with either GFP or the indicated GFP-tagged exocyst proteins followed by GFP immunoprecipitation. mTORC1-exocyst association was determined by immunoblot.

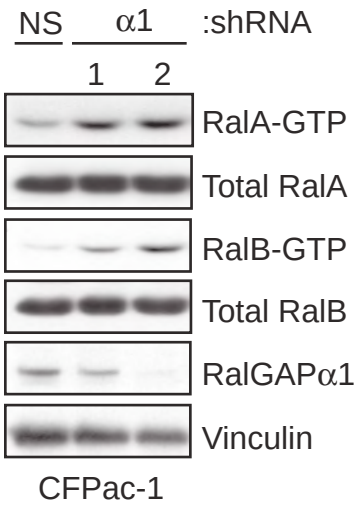
(B) Sec5 but not Exo84 co-expression enhances active RalB driven mTORC1 activity. HEK293T cells were transfected with the indicated constructs and were serum starved overnight prior to harvest 48 h later. mTORC1 activity was determined by immunoblotting with the indicated antibodies.

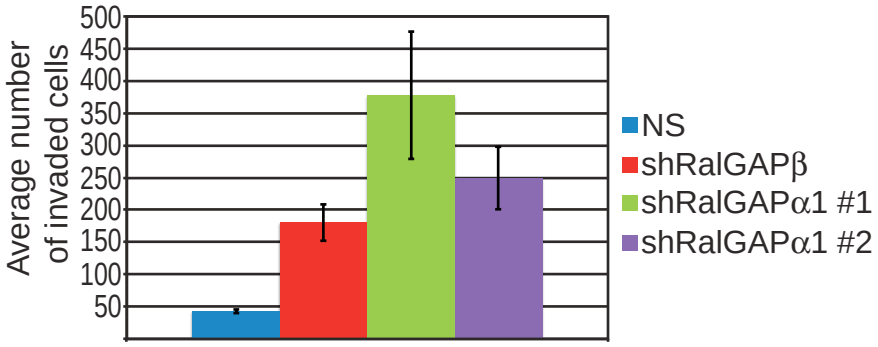
(C) Sec5 can associate directly with mTOR. Rabbit reticulocyte lysate was used to prepare FLAG-mTOR and mCherry, and mCherry-Sec5 and FLAG-mTOR was immunoprecipitated. mCherry and mCherry-Sec5 were allowed to bind with the FLAG-mTOR IP for 2 h. Association was determined by western blotting.

(D) Sec5 does not require Ral association to interact with mTORC1. HEK293T cells were transfected with GFP, GFP-Sec5 wildtype, or Ral-binding deficient GFP-Sec5(T11A). Anti-GFP immunoprecipitations were performed 48 h later and association with mTORC1 was determined by western blotting.

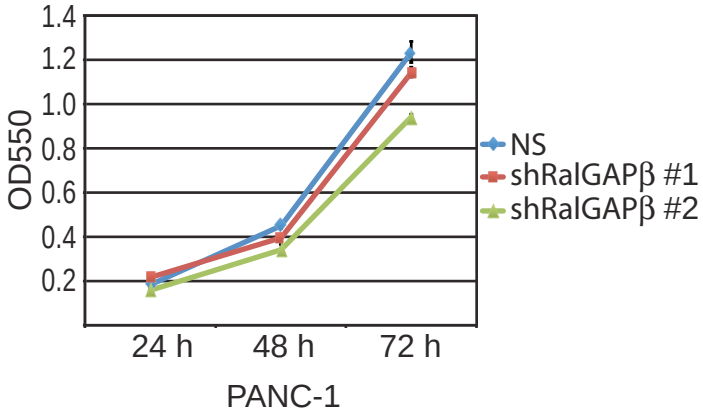
Figure S7

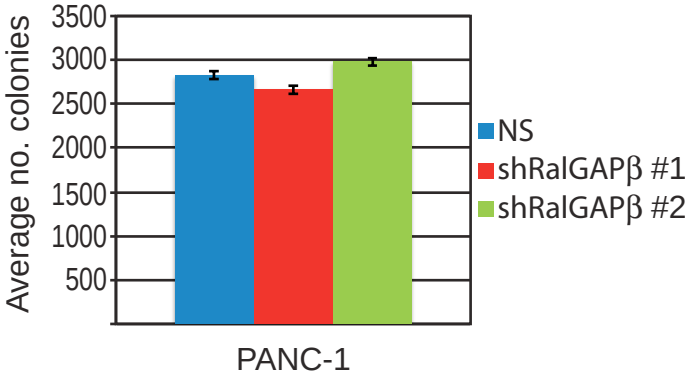
A



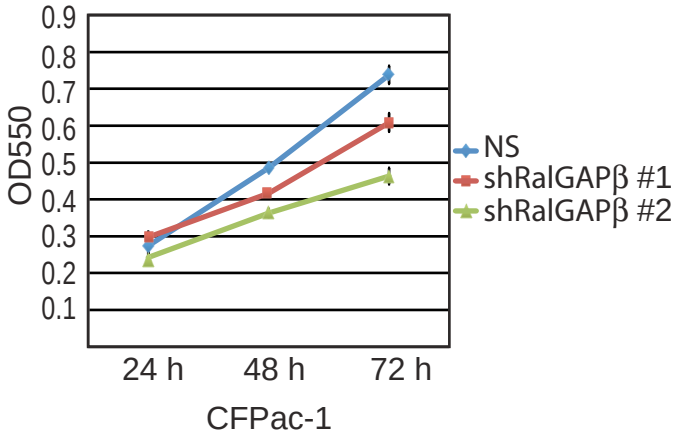


B





C



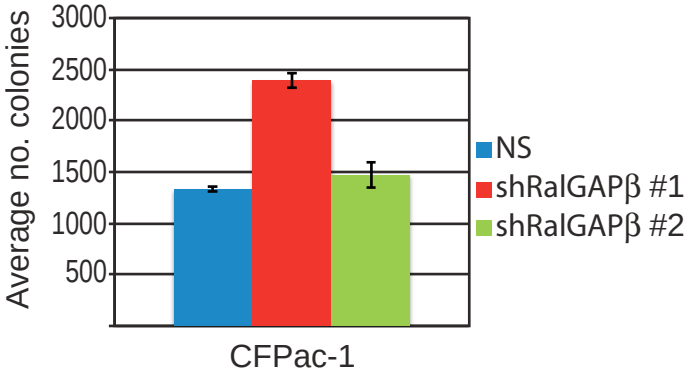


Figure S7. RalGAPα1 knockdown results in enhanced PDAC cell invasion and RalGAPβ loss does not affect the anchorage-independent or dependent growth of PDAC cells, related to Figure 7

(A) Knockdown of RalGAPα1 enhances Ral activation and PDAC tumor cell invasion. CFPac-1 cells were stably transduced with the indicated RalGAPβ, RalGAPα1, and NS shRNAs. Lysates were subject to GST-Sec5-RBD pulldown assays to determine Ral-GTP levels (left panel). Total cellular protein was analyzed with the indicated antibodies to determine protein expression and verify knockdown. Blot analysis for vinculin was used to verify equivalent loading of total cellular protein. These same CFPac-1 cells were placed in Matrigel chambers (right panel) to monitor invasion in vitro. Numbers represent the average number of invaded cells per well in an assay performed in triplicate (+/- standard error from the mean, S.E.M).

(B) Knockdown of RalGAPβ does not affect the growth properties of PANC-1 tumor cells. PANC-1 cells stably expressing either NS or RalGAPβ shRNAs were subject to MTT colorometric viability assays to monitor anchorage-dependent growth (left) and soft agar colony formation assays to measure anchorage-independent growth (right). Numbers represent the averages of eight (MTT) or three (soft agar) wells (+/- standard error from the mean, S.E.M).

(C) Knockdown of RalGAPβ does not effect the growth properties of CFPac-1 tumor cells. CFPac-1 cells stably expressing either NS or RalGAPβ shRNAs were subject to MTT proliferation assays to measure anchorage-dependent growth (Left) and soft agar assays to measure anchorage-independent growth (Right). Numbers represent the averages of eight (MTT) or three (soft agar) wells (+/- standard error from the mean, S.E.M).

SUPPLEMENTAL TABLE S1. A phylogenetic summary of RalGAP and Tsc subunits amongst nematodes, related to Figure 1 and Figure S1

BLAST searches based on human RalGAP and Tsc subunits identified C. elegans and Drosophila orthologs of the RalGAP complex, but only Drosophila orthologs of the Tsc complex. The Treefam Project includes complete genomes from many Drosophilid species and related insects, all of which encode both RalGAP and Tsc complex orthologs. In contrast, Treefam includes limited nematode species, which encode RalGAP complex proteins but not Tsc complex. Using D. melanogaster Tsc components (gigas/TSC2 and TSC1) and C. elegans HGAP-1 and HGAP-2 for BLAST analysis, we analyzed all nematode genome sequences available in update WS238, the results of which are shown. We are confident that all sequenced Caenorhabditids lack the Tsc complex. More distantly related nematodes have genome sequences of variable completion or annotation, making it difficult to definitively determine which orthologs they contain. However, based on our analysis we hypothesize that more distantly related nematode species may encode Tsc complex-like molecules.

Treefam Project trees:

Alpha subunits (both Tsc2 and RalGAPα):
http://www.treefam.org/family/TF324484#tabview=tab1

Tsc1: http://www.treefam.org/family/TF325466#tabview=tab1

RalGAPβ: http://www.treefam.org/family/TF324460#tabview=tab1

Supplemental Table S1

Wormbase WS238				
Species	RalGAP alpha	RalGAP beta	TSC2 (alpha)	TSC1 (beta)
Ascaris suum	ASU06793 (0.0)	ASU10047(e-104)	ASU13651 (1e-35)	ASU13669 (weak: 2e-19)
Brugia malayi	NA	BM3056 (1e-93)	BM9134 (2e-31)	NA
Bursaphelenchus xylophilos	BUX s00294 (0.0)	BUX s01254 (9e-90)	NA	NA
Caenorhabditis angaria	Cang g12587 (0.0)	Cang g3767 (0.0)	NA	NA
C. brenneri	CBN32568 (0.0)	CBN30422 0.0)	NA	NA
C. briggsae	CBG05152 (0.0)	CBG11128 (0.0)	NA	NA
C. elegans	*hgap-1* (Y18H1A.3)	*hgap-2* (D2085.5)	NA	NA
C. japonica	CJA04561 (0.0)	CJA00590 0.0)	NA	NA
C. remanei	CRE20789 (0.0)	CRE01530 (0.0)	NA	NA
C. sp11	g8138 (0.0)	g15069 (0.0)	NA	NA
C. sp5	g35647 (0.0)	g311173 (0.0)	NA	NA
Heterorhabditis bacteriophora	HBA_03857 (2e-90)	HBA_15983 (7e-46)	NA	NA
Haemonchus contortus	NA	NA	NA	NA
Loa loa	EFO27774 (e-143)	EJD76283 (e-106)	EJD76451 (1e-34)	NA
Meloidogyne hapla	NA	NA	NA	contig130.frx3.gene47 (8e-85)
Pristionchus pacificus	NA (Rap1GAP at 1E-09)	PPA22794 (7E-45)	NA	PPA20476 (weakish:: 2e-29)
Strongyloides ratti	g3693 (0.0)	NA	NA	NA
Trichinella spiralus	EFV54610 (2e-33)	EFV55615 (4e-30)	EVF52050 (2e-46)	NA
	Human RalGAP alpha 1	Human RalGAP beta		
C. elegans	Y18H1A.3 (5e-37)	D2085.5 (1e-55)		
D. melanogaster	CG5521 (2e-168)	CG34488 (0.0)		

EXTENDED EXPERIMENTAL PROCEDURES

C. elegans strains used in this study:

DV2824 *hgap-1(gk101481)* I
DV2862 *hgap-1(gk101481)* I; *eri-1(mg366)* IV
DV2965 *hgap-2(gk578143)* II
DV2869 *let-363(h98) dpy-5(e61) unc-13(e450) / hT2* [*bli-4(e937) let-?(q782))qIs48*] (I;III); *hgap-2(gk578143)* II
DV2870 *let-363(ok3018) / hT2* [*bli-4(e937) let-?(q782))qIs48*] (I;III); *hgap-2(gk578143)* II
GR1373 *eri-1(mg366)* IV
KR344 *let-363(h98) dpy-5(e61) unc-13(e450)* I; *sDp2* (I;f)
VC2312 *let-363(ok3018) / hT2* [*bli-4(e937) let-?(q782))qIs48*] (I;III)

cDNA expression and shRNA constructs

Expression vectors for HA-RalGAPβ and Myc-RalGAPα1 have been described previously ((Chen et al., 2011). GFP-LC3 was obtained from Addgene (plasmid 24920) and the GFP-LC3 cDNA was subcloned into pCDH EF1 IRES puro (Systems Biosciences) into the EcoRI and BamHI sites. Myc-mTOR WT (plasmid 1861), Myc-mTOR 1-1482 (plasmid 21745), Myc-mTOR 1271-2008 (plasmid 21746), Myc-mTOR 1750-2549 (plasmid 21747), Myc-Raptor (plasmid 1859), Myc-Rictor (plasmid 1860), HA-GST-S6K1 (plasmid 15511), Raptor shRNA 1 (plasmid 1857), Raptor shRNA 2 (plasmid 1858), Rictor shRNA 1 (plasmid 1853), Rictor shRNA 2 (plasmid 1854), Myc-Tsc1 (plasmid 12133), and FLAG-Tsc2 (plasmid 8996) were obtained from Addgene,

GFP-Raptor was prepared by amplifying Raptor cDNA from Myc-Raptor followed by subcloning into pEGFP-C3 using the XhoI and BamHI sites. GTPase C-termini were added to a GFP-Raptor with no stop codon using the BamHI and XbaI sites. Sequences were as follows: Rheb-C FWD: GATCCATGGACGGGGCAGCTTCACAAGGCAAGTCTTCATGCTCGGTGATGT GAGCGGCCGCGCCT, Rheb-C REV: CTAGAGGCGCGGCCGCTCACATCACCGAGCATGAAGACTTGCCTTGTGAA GCTGCCCCGTCCATG, RalB-C FWD: GATCCATGTCAGAAAACAAAGACAAGAATGGCAAGAAAAGCAGCAAGAACA AGAAAAGTTTTAAAGAAAGATGTTGCTTACTATGAGCGGCCGCGCCT, and RalB-C REV: CTAGAGGCGCGGCCGCTCATAGTAAGCAACATCTTTCTTTAAAACTTTTCTT GTTCTTGCTGCTTTTCTTGCCATTCTTGTCTTTGTTTTCTGACATG. pBabe HAII puro RalB expression vectors, pLKO.1 puro RalA and RalB, GFP expression vectors for Sec5, Exo84, and RalBP1 have been described previously (Martin et al., 2012). GFP-Sec5 T11A was prepared by site-directed mutagenesis.

All other shRNA plasmids were obtained from the UNC lentiviral RNAi core facility including: shRalGAPα1 #1 (TRCN0000047348), shRalGAPα1 #2 (TRCN0000047351), shRalGAPβ #1 (TRCN0000150848), shRalGAPβ #2 (TRCN0000156117), mouse shRalB #1 (TRCN0000077743), mouse shRalB #2 (TRCN0000077746), mouse shRalB #3 (TRCN0000077747), mouse shRheb #1

(TRCN0000075603), mouse shRheb #2 (TRCN0000075604), and mouse shRheb #3 (TRCN0000075605).

cDNA sequences encoding GFP-exocyst components were PCR amplified and subcloned into pEGFP-C3 into the following sites as follows: Sec3 (XhoI and BamHI), Sec6 (EcoRI and BamHI), Sec8 (XhoI and BglII), Sec10 (XhoI and BamHI), Sec15 (XhoI and BamHI), and Exo70 (XhoI and BamHI). mCherry-Sec5 was prepared by subcloning the Sec5 cDNA sequence into pcDNA3-mCherry between the XhoI and ApaI sites.

GFP-Sec5 RalBD was prepared by amplifying the first 140 residues of Sec5 and cloning into pEGFP-C3 using the XhoI and HindIII sites. Additions of different GTPase C-termini were added to GFP-Sec5 (no stop codon) using the HindIII and BamHI sites. Sequences added were as follows: Rheb-C FWD: AGCTTATGGACGGGGCAGCTTCACAAGGCAAGTCTTCATGCTCGGTGATGT GAGCGGCCGCGCCG, Rheb-C REV: GATCCGGCGCGGCCGCTCACATCACCGAGCATGAAGACTTGCCTTGTGAA GCTGCCCCGTCCATA, RalB-C FWD: AGCTTATGTCAGAAAACAAAGACAAGAATGGCAAGAAAAGCAGCAAGAACA AGAAAAGTTTTAAAGAAAGATGTTGCTTACTATGAGCGGCCGCGCCG, RalB-C REV: GATCCGGCGCGGCCGCTCATAGTAAGCAACATCTTTCTTTAAAACTTTTCTT GTTCTTGCTGCTTTTCTTGCCATTCTTGTCTTTGTTTTCTGACATA, HRas-C FWD: AGCTTCAGCACAAGCTGCGGAAGCTGAACCCTCCTGATGAGAGTGGCCCC GGCTGCATGAGCTGCAAGTGTGTGCTCTCCTGAGCGGCCGCGCCG, and HRas-C REV: GATCCGGCGCGGCCGCTCAGGAGAGCACACACTTGCAGCTCATGCAGCCG GGGCCACTCTCATCAGGAGGGTTCAGCTTCCGCAGCTTGTGCTGA.

A RalB FRET-based biosensor was prepared similar to the previously described RhoA biosensor (Pertz et al., 2006). mCerulean was inserted into pcDNA3 into the HindIII and KpnI sites. DNA sequences encoding a protease resistant linker of the amino acid sequence GSTSGSGKPGSGEGSTK and mCitrine were amplified from the RhoA biosensor (Addgene plasmid 12602) and subcloned into the pcDNA3-mCerulean vector using the KpnI and XhoI sites. The Sec5-RBD sequence was then inserted into the HindIII site to yield pcDNA3 Sec5 RalBD-mCerulean-linker-mCitrine. Finally wildtype, S28N, and Q72L RalB cDNA sequences were subcloned into the XhoI and XbaI sites to yield the full unimolecular biosensor.

Transfections for all cDNAs were performed using the HBS $CaCl_2$ method and cells were examined 48 h post-transfection.

Cell lines and reagents

All cell lines except HPDE and conditional RalGAPβ$^{fl/fl}$ MEFs were obtained from ATCC and maintained at 37°C and 5% CO_2. HEK293T, *RalGAPβ*$^{fl/fl}$ MEFs, HPDE, MiaPaCa-2, HPAC, and Panc1 cells were maintained in DMEM-H. AsPC-1, Capan-1, CFPAC-1, HPAFII, and SW1990 cells were maintained in RPMI-

1640. All media was supplemented with 10% fetal calf serum (FCS) and 1X penicillin/streptomycin.

Antibodies for pT389-S6K, S6K, mTOR, Raptor, Rictor, pS235/236-S6, S6, pT37/46-4EBP1, 4EBP1, Tsc1, Tsc2, and LC3B were obtained from Cell Signaling. Antibodies for Rheb-1 and Ralb were from Millipore. Antibodies for GFP was from Clontech, for RalA from BD Biosciences, vinculin and actin from Sigma, Myc from Roche and HA from Covance. Antibodies for RalGAPα1 and RalGAPβ have been described previously (Chen et al., 2011). Antibodies for Sec8 was from Stressgen, for Sec5 and Exo84 were provided by Charles Yeaman (University of Iowa). Empty and Cre recombinase expressing adenovirus was obtained from the Gene Vector Core at the University of Iowa. Phosphatase and protease inhibitor cocktails were from Sigma and Roche, respectively.

All siRNAs described were obtained from Thermo and are part of their ON-TARGETplus SMARTpools of siRNA. siRNAs were transfected using RNAiMAX (Invitrogen).

Immunoprecipitations and western blotting

Cells were treated as indicated in the figure legends, harvested, and lysed in 0.3% CHAPS lysis buffer (0.3% CHAPS, 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 10 mM $MgCl_2$, and 50 mM glycerophosphate) supplemented with phosphatase and protease inhibitors. Two to five µg of the indicated antibodies were allowed to bind to their targets for 2-3 h at 4°C. After incubation, 20-50 µL of Protein G Dynabeads (Invitrogen) were added and allowed to rock for 1 h at 4°C. Beads were washed three times with lysis buffer and resuspended in protein sample buffer containing β-mercaptoethanol and examined by western blotting.

Western blots were performed by first separating proteins on either 6% (for proteins larger than 150 KDa) or 10% SDS-PAGE followed by transfer to PVDF membranes for 2 h at 4°C. For proteins larger than 150 KDa, proteins were transferred overnight at 20V and 4°C. All western blots were blocked in a 5% milk/TBST solution for 1 h and all primary antibodies were incubated overnight at 4°C. Blots were then washed three times with TBST and given secondary HRP-conjugated antibodies for 1 h at room temperature. Blots were washed three times with TBST and developed by ECL/chemiluminescence.

mTOR kinase assays

Cells were treated as indicated in the figures, harvested and lysed in 0.3% CHAPS lysis buffer. Three µg of anti-mTOR (Cell Signaling) antibody was added to between 500 µg-1mg of total cellular lysate and allowed to bind for 2 h at 4°C. After 2 h, 30 uL of Protein G Dynabeads were added and allowed to rock at 4°C for an additional hour. Beads were washed three times with CHAPS lysis buffer and twice with mTOR kinase buffer without ATP (25 mM HEPES pH 7.4, 50 mM KCl, 10 mM $MgCl_2$, and 4 mM $MnCl_2$). Beads were resuspended in 7.5 µL 2X mTOR kinase buffer and 150 ng HA-GST-S6K and ATP (final concentration of 250 µM) were added to a final volume of 15 µL. Beads were incubated at 30°C

for 30 min, resuspended in 2X protein sample buffer containing β-mercaptoethanol, and analyzed by western blotting.

Microscopy

All cells were treated as indicated, split onto 0.01% poly-L-lysine (Sigma) coated coverslips, fixed in 4% paraformaldehyde and permeabilized with 0.2% Triton X-100. Cells were then blocked with a mix of 5% normal goat serum (Cell Signaling) and 5% BSA in PBS. Coverslips were allowed to stain overnight at 4°C and then washed three times with PBS. Alexa Fluor-conjugated (Invitrogen) secondary antibodies were added for 1 h followed by three washes with PBS. Coverslips were then mounted with Fluorsave (Calbiochem).

All confocal images were obtained on a Zeiss 710 spectral confocal laser scanning confocal microscope equipped with 405, 458, 488, 514, 543, 594, 633 nm excitation lines and either a 40X or 63X oil plan/Apo objective. Multicolored images were acquired using sequential scanning. All images were manipulated using ImageJ and Photoshop to crop, subtract background, and equalize intensities between channels.

FRET analysis was performed on an Olympus IX81 inverted light microscope equipped with Hammatsu ORCA RC and QImaging RETIGA 4000R cameras. HEK293T cells were first transfected with the indicated RalB biosensor, plated onto glass-bottomed Bioptech dishes coated with 0.01% poly-L-lysine, and allowed to attach overnight. Live cells were imaged using a 60X/1.42 Oil DIC PlanApo objective. Images were acquired using the Velocity software every 10 s for 10-30 min. Images were processed as described for the RhoA biosensor by Pertz et al using an automated script (Biosenprocess1-9develop16-bit-v3) for ImageJ developed by Dr. Bob Bagnell (UNC Chapel Hill, Microscope Services Laboratory). Plugin is freely available at http://www.med.unc.edu/microscopy/resources/imagej-plugins-and-macros/biosensor-fret.

Matrigel invasion, MTT proliferation, and soft agar assays

Cells stably expressing the indicated shRNA constructs were serum starved overnight. The following day they were placed into Matrigel invasion chambers (BD Biosciences) in triplicate and allowed to invade for 48-72 h. Non-invaded cells were removed from the top layer by a cotton swab and invaded cells were stained with crystal violet. Images of each well were taken and cells quantitated by ImageJ. For rapamycin treatment, rapamycin was added during the overnight starvation and while cells were in the Matrigel chambers. Cells were also split into replicate plates for western blotting.

For MTT proliferation assays, 2×10^3 cells expressing for each indicated shRNA were plated onto 96-well plates in eight identical wells. Proliferation was measured by addition of MTT for the indicated time points. Measurements were taken at an OD550 on a spectrophotometer.

For soft agar assays, 10^4 cells expressing the indicated shRNA were suspended between layers of 0.6% (bottom) and 0.3% (top) bacto-agar in triplicate in 6-well plates. Cells were fed normal growth medium and allowed to

form colonies for 14-21 days. Colonies were stained with MTT, imaged, and quantitated using ImageJ.

Plasma membrane fractionation

Plasma membrane proteins were isolated using the Qproteome plasma membrane protein kit (Qiagen). Isolated membrane proteins were precipitated with -20°C acetone and resuspended in protein sample buffer. Proteins were analyzed by western blotting.

Ral GTPase pulldowns

Ral GTPase pulldowns were performed as described previously (Martin et al., 2012). Briefly, GST-Sec5-RBD was prepared in BL21 *E. coli* cells. One hundred µg of total cell lysate was added to 30 µg of GST-Sec5-RBD bound to glutathione and allowed to rock at 4°C for 1 h. Beads were washed three times with lysis buffer and resuspended in 2X protein sample buffer. Active Ral was analyzed by western blotting.