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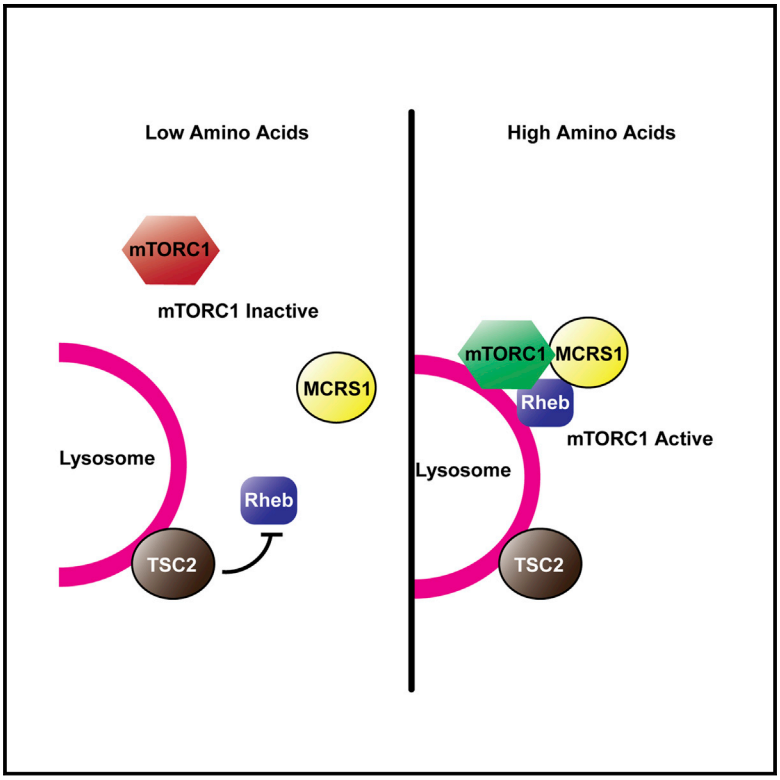
MCRS1 Binds and Couples Rheb to Amino Acid-Dependent mTORC1 Activation

Graphical Abstract



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In Brief

Frequently misregulated in cancers, mTOR signaling can be stimulated by amino acids through Rheb. Fawal et al. identify MCRS1 as the link between Rheb and mTORC1 activation at the lysosome. MCRS1 maintains active Rheb at the lysosome and prevents its interaction with inhibitor TSC2 and localization to recycling endocytic vesicles.

Highlights

- MCRS1 interacts with Rheb and activates mTORC1
- MCRS1 is essential for mTORC1 activity and its associated cellular functions
- MCRS1 maintains Rheb at lysosomes in active GTP-bound form to activate mTORC1
- Absence of amino acids and MCRS1 delocalizes lysosomal Rheb but not lysosomal TSC2

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MCRS1 Binds and Couples Rheb to Amino Acid-Dependent mTORC1 Activation

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SUMMARY

Ras homolog enriched in brain (Rheb) is critical for mechanistic target of rapamycin complex 1 (mTORC1) activation in response to growth factors and amino acids (AAs). Whereas growth factors inhibit the tuberous sclerosis complex (TSC1-TSC2), a negative Rheb regulator, the role of AAs in Rheb activation remains unknown. Here, we identify microspherule protein 1 (MCRS1) as the essential link between Rheb and mTORC1 activation. MCRS1, in an AA-dependent manner, maintains Rheb at lysosome surfaces, connecting Rheb to mTORC1. MCRS1 suppression in human cancer cells using small interference RNA or mouse embryonic fibroblasts using an inducible-Cre/Lox system reduces mTORC1 activity. MCRS1 depletion promotes Rheb/TSC2 interaction, rendering Rheb inactive and delocalizing it from lysosomes to recycling endocytic vesicles, leading to mTORC1 inactivation. These findings have important implications for signaling mechanisms in various pathologies, including diabetes mellitus and cancer.

INTRODUCTION

Growth factor and nutrient signals are integrated through mechanistic/mammalian target of rapamycin (mTOR), a serine/threonine phosphoinositide-related kinase often deregulated in cancer (Laplante and Sabatini, 2012). mTOR complex 1 (mTORC1) integrates growth factors through phosphoinositide-3 kinase (PI3K)/protein kinase B (PKB), which inactivates the tuberous sclerosis complex (TSC1-TSC2) tumor suppressor, a negative mTORC1 regulator. The TSC1-TSC2 complex has guanosine triphosphatase (GTPase)-activating protein (GAP) activity for Ras homolog enriched in brain (Rheb), a Ras-like guanosine triphosphate (GTP)-binding protein required for mTORC1 activation (Garami et al., 2003; Inoki et al., 2003; Saucedo et al., 2003; Stocker et al., 2003; Zhang et al., 2003b). Thus, Rheb is inactivated in guanosine diphosphate (GDP)-bound form, but the GTP-bound form directly stimulates mTORC1 (Avruch et al., 2009). Hence, loss of TSC1 or TSC2 renders Rheb hyperactive, activating mTORC1, which becomes resistant to growth factor, but not AA deprivation, (Kim et al., 2008; Roccio et al., 2006; Sancak et al., 2008; Smith et al., 2005; Zhang et al., 2003b). Accordingly, AA deprivation reduces active GTP-bound Rheb levels (Roccio et al., 2006; Smith et al., 2005), implying that mechanisms controlling mTORC1 by AAs are TSC2-independent.

AAs reportedly activate mTORC1 through the lysosome-associated machinery for AA sensing involving Rag GTP-binding proteins (Sancak et al., 2008), the scaffolding complex Ragulator (Bar-Peled et al., 2012; Sancak et al., 2010; Zoncu et al., 2011a), and vacuolar H^+-adenosine triphosphatase (v-ATPase) (Zoncu et al., 2011b). The first essential step in AA-induced mTORC1 activation is its recruitment to lysosomes by Rag proteins, providing an activation platform on which mTORC1 colocalizes with GTP-bound Rheb (Sancak et al., 2008). Rheb is therefore necessary but insufficient for mTORC1 activation.

We identify microspherule protein 1 (MCRS1) as a molecular link connecting Rheb-GTP to mTORC1 activation at lysosomes in AA responses. MCRS1 depletion promotes Rheb/TSC2 interaction and delocalizes Rheb from lysosomes to recycling endocytic vesicles, resulting in mTORC1 inactivation.

RESULTS

MCRS1 Binds Rheb-GTP in an AA-Dependent Manner

To elucidate Rheb regulation in AA responses, we sought Rheb interactors with mass spectrometry to identify GST-Rheb-associated proteins in human embryonic kidney (HEK)-293T cells overexpressing GST (glutathione-S-transferase) or GST-Rheb. Identified proteins with high peptide scores included mTORC1 components (mTOR, Raptor, mLST8), TSC2, and MCRS1 (Figure 1A). MCRS1 is overexpressed in colorectal cancer (Shi et al., 2009, 2012), so the potential Rheb-MCRS1 interaction was validated in pull-down assays using human colorectal carcinoma HCT-116 and HEK293T cells. Immunoblotting of GST precipitated by specific antibodies identified MCRS1 (Figures 1B and S1A). Similarly, MCRS1 was detected in pull-down assays when purified bacterially expressed recombinant wild-type GST-Rheb was incubated with HEK293T or HCT-116 whole cell lysates (Figure S1B). The specificity of MCRS1 binding to Rheb in vitro was verified using lysates of HCT-116 cells transfected with non-silencing control small interference RNA (siRNA-Ctr) or various MCRS1-depleting siRNAs (siRNA-MCRS1) (Figures S1C and S1D). We also mapped the Rheb-binding part of MCRS1, using bacterially expressed recombinant GST-Rheb and in vitro translated MCRS1 (Figures 1C, 1D, and S1E), a highly conserved C-terminal (Ct) segment. Moreover, using in vitro translated HA-Rheb fragments, we identified Rheb N-terminal (Nt) as the MCRS1-binding part (Figure 1E).

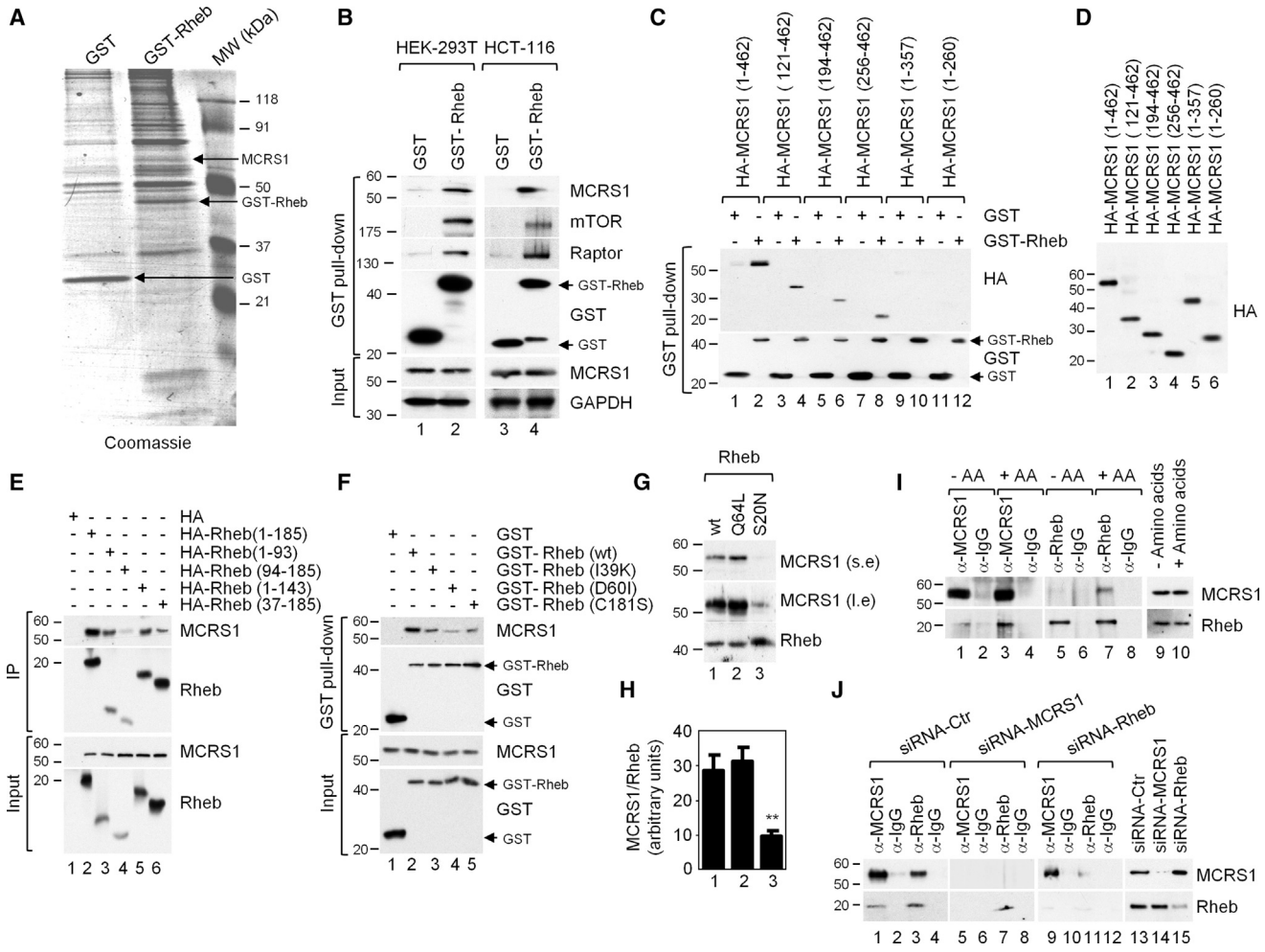


Figure 1. MCRS1 Binds Rheb-GTP in an AA-Dependent Manner

(A) HEK293T cells transiently expressing GST or GST-Rheb were lysed, subjected to GST pull-down assays, and protein complexes were resolved on SDS-PAGE followed by coomassie staining. GST, GST-Rheb and the interacting protein MCRS1 are indicated on the figure.

(B) GST pull-downs from HEK293T and HCT-116 cells transiently expressing GST or GST-Rheb.

(C) GST pull-downs from bacteria purified GST and GST-Rheb incubated with in vitro translated fragments of HA-MCRS1.

(D) In vitro translated HA-MCRS1 fragments used in Figure 1C.

(E) In vitro translated fragments of HA-Rheb were incubated with HCT-116 cell lysate and immunoprecipitated using an HA antibody.

(F) HCT-116 cells transiently expressing either GST, GST-Rheb (wt), GST-Rheb switch 1 mutant (I39K), GST-Rheb switch 2 mutant (D60I), and GST-Rheb mutant unable to undergo prenylation (C181S) were lysed and subjected to GST pull-down assays.

(G) GST pull-downs from HCT-116 cells transiently transfected with either Rheb (wt), the constitutively active form of Rheb (Q64L) or with a nucleotide-deficient Rheb mutant (S20N). Immunoblots of short exposure (s.e) and long exposure (l.e) are shown.

(H) Quantification of (G).

(I) HCT-116 cells were serum-starved for 16 hr followed by 1 hr AA deprivation (lanes 1, 2, 5, 6, and 9) or 1 hr AA deprivation and then AA stimulation for 10 min (lanes 3, 4, 7, 8, and 10). Lysates were subjected to immunoprecipitation assays.

(J) HCT-116 cells transfected either with siRNA control (Ctr) or against MCRS1 or Rheb were lysed and subjected to immunoprecipitation assays.

Statistical analyses were performed using one-way ANOVA test followed by the Bonferroni method (H). Errors bars represent ± SD (n = 3). $^{**}p \leq 0.01$.

Next, we tested the MCRS1-binding ability of three GST-Rheb mutants overexpressed in HCT-116 cells. MCRS1 binding to Rheb (C181S), a mutant unable to undergo the prenylation/farnesylation required for Rheb-membrane interaction and activity, was significantly reduced. Whereas interaction of MCRS1 to Rheb (I39K), carrying a mutation in the Rheb switch 1 region that exhibited similar Rheb-wt GTP binding, was slightly affected, MCRS1 binding was drastically diminished for Rheb (D60I), carrying a mutation in the Rheb switch 2 region interfering selectively with GTP binding in vitro (Long et al., 2005a) (Figure 1F). We therefore tested whether MCRS1 affinity to Rheb depends on Rheb's nucleotide-binding state. The dominant active mutant Rheb-Q64L (~90% GTP charged) showed higher affinity for MCRS1 than both the inactive, strongly nucleotide-deficient mutant Rheb-S20N (Long et al., 2005a, 2005b) and Rheb-wt (Figures 1G and 1H). However, Rheb-wt bound substantial amounts of MCRS1 because of its potent GTP-binding (Inoki et al., 2003) (Figures 1G and 1H). Loading bacterially expressed

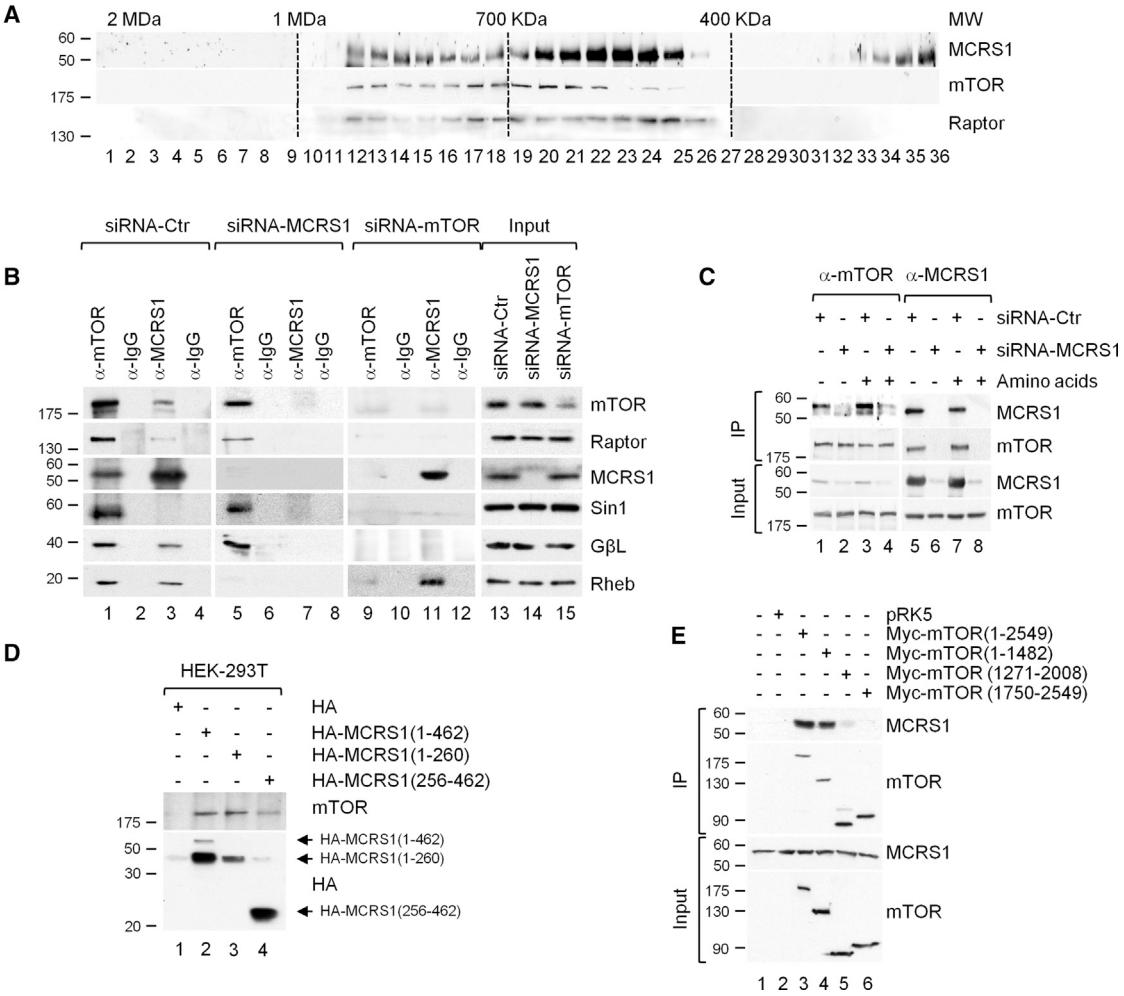


Figure 2. MCRS1 Interacts with mTOR

(A) Size exclusion chromatography of cytoplasmic fraction from HCT-116 cells. The collected fractions were processed for immunoblotting.

(B) HCT-116 cells transfected either with siRNA-Ctr, siRNA-MCRS1, or siRNA-mTOR were lysed and subjected to immunoprecipitation assays.

(C) HCT-116 cells transfected with either siRNA-Ctr or siRNA-MCRS1 were serum-starved for 16 hr followed by 1 hr AA deprivation (lanes 1, 2, 5, and 6) or 1 hr AA deprivation and then AA stimulation for 10 min (lanes 3, 4, 7, and 8). Lysates were subjected to immunoprecipation assays.

(D) HEK293T cells transiently expressing HA-tagged full-length MCRS1 (1–462), N terminal (1–260), or C terminal (256–462) were lysed and subjected to immunoprecipitation using an HA antibody.

(E) HCT-116 cells transfected with either empty plasmid (pRK5) or different Myc-tagged mTOR fragments were lysed and immunoprecipitated using Myc antibody.

recombinant GST-Rheb-wt with GTP also enhanced MCRS1/Rheb interaction (Figure S1F). Importantly, reciprocal co-immunoprecipitation experiments indicated that AAs strongly enhanced endogenous MCRS1/Rheb association (Figures 1I and 1J). In vitro Rheb's GST pull-downs or Rheb endogenous immunoprecipitates in different cell lines confirmed that AA depletion reversibly reduced MCRS1 interaction with Rheb (Figures S1G–S1K). Glucose deprivation in the absence of AAs was not sufficient to promote MCRS1/Rheb interaction (Figures S1I and S1J), suggesting that AAs are essential in MCRS1/Rheb interaction. Inhibiting the v-ATPase and Ragulator by knocking down ATP6V0C and LAMTOR1, respectively, had no effect on Rheb/MCRS1 interaction (Figure S1H). However, both leucine and non-essential AAs promoted MCRS1/Rheb association (Figures S1I and S1J), suggesting that MCRS1/Rheb interaction is influenced by AA-sensing pathways independent of the v-ATPase and Ragulator axis. Furthermore, MCRS1/Rheb association was not further enhanced in the presence of insulin-like growth factor (IGF)-1, although IGF1 alone slightly increased MCRS1/Rheb interaction (Figures S1I and S1J). Interestingly, endogenous Rheb interacted in a similar fashion with full-length and Ct MCRS1 in response to AAs (Figure S1K). Finally, AA or MCRS1 depletion impaired Rheb/mTOR interaction (Figures S1I and S1L) in multiple cell lines. Thus, MCRS1 binds active Rheb in an AA-dependent manner.

MCRS1 Interacts with mTOR

MCRS1 co-eluted in the same fractions as mTORC1 in gel filtration assays (Figure 2A). The ability of MCRS1 to form complexes with mTORC1 was checked with co-immunoprecipitation with

three custom-made MCRS1 antibodies (Figure S2A). Furthermore, reciprocal co-immunoprecipitation assays detected endogenous MCRS1 in a complex with mTOR/Raptor in HCT-116, human colon adenocarcinoma SW-620 (Figures 2B and S2B) and HEK293T (Figure S2C) cells. Notably, Sin1 (an mTORC2 member) did not co-immunoprecitate with MCRS1, suggesting that MCRS1 forms a complex with mTORC1 but not mTORC2 (Figure 2B). Additionally, AAs did not dramatically enhance endogenous mTOR/MCRS1 association (Figures 2C and S2D). Interestingly, MCRS1 binds mTOR and Rheb independently, as shown by co-immunoprecipitation of endogenous MCRS1 in Rheb-depleted HCT-116 cells and by GST pull-downs in mTOR-depleted HCT-116 cells (Figures S2E and S2F).

In vitro binding assays indicated that Nt (1–260) part but not Ct (256–462) segment of MCRS1 specifically bound mTOR (Figures 2D, S2G, and S2H) Notably, URI, a regulator of mTOR/S6K1 axis (Djouder et al., 2007) did not bind to mTOR (Figure S2H). Finally, overexpressing Myc-mTOR fragments in HCT-116 cells and Myc immunoprecipitation assays showed that mTOR bound MCRS1 through its Nt part (Figure 2E). Thus, MCRS1 provides a molecular platform connecting Rheb to mTORC1 in AA responses.

MCRS1 Is Essential for mTORC1 Activation

MCRS1 knockdown impaired mTORC1 activation in HCT-116 cells under normal growth conditions, manifested by dephosphorylations of two mTORC1 targets: S6K1 at Thr-389 and 4EBP1 at Ser-65 (Figure 3A). Similarly, MCRS1 depletion in HEK293T cells using siRNAs targeting various regions of *MCRS1* mRNA (Figure S3A) abolished mTORC1 activation, also in response to AAs and/or IGF1 in HCT-116 (Figure 3B), HeLa (Figure S3B), and SW-620 (not shown) cells. To exclude the possibility that MCRS1 acts on the energy sensor AMP-activated kinase (AMPK), which inhibits mTORC1 signaling under glucose deprivation (Jewell and Guan, 2013), we depleted MCRS1 and analyzed AMPK activation through Thr-172 phosphorylation. MCRS1 suppression dephosphorylated S6K1 without affecting AMPK activity, indicating that MCRS1 regulates mTORC1 independently of energy depletion (Figure S3C). Notably, AAs depletion, independently of MCRS1 suppression, slightly increased AMPK activity but, as with MCRS1 knockdown, did not significantly modify cellular ATP levels (Dennis et al., 2001) (Figure S3D). Interestingly, MCRS1 localizes to minus ends of chromosomal microtubules and K-fibers, protecting them from depolymerization (Meunier and Vernos, 2011). However, MCRS1 knockdown in HCT-116 cells did not depolymerize microtubules (Figure S3E). Moreover, MCRS1 depletion in HCT-116 cells treated with the microtubule-depolymerizing compound nocodazole did not affect (or slightly increased) phosphorylation of 4EBP1 and S6K1 at Thr-389 (Figure S3F). Thus MCRS1 depletion-mediated mTORC1 inactivation is independent of microtubule network changes. Finally, depleting cells of NDE1, a centrosomal MCRS1-interacting protein implicated in cell division and survival (Hirohashi et al., 2006), did not affect S6K1 phosphorylation, excluding the potential nuclear function of MCRS1 in mTORC1 activation (Figure S3G).

In further tests, we generated conditional MCRS1 knockout mice by introducing the *MCRS1* floxed allele by homologous recombination in embryonic stem cells (Figures S3H–S3K). Immortalized mouse embryonic fibroblasts (MEFs) from mice carrying the *Mcrs1* lox allele infected with adenoviruses expressing Cre recombinase (adeno-Cre) for at least 48 hr to delete MCRS1 ($MCRS1^{-/-}$) resulted in severe mTORC1 activity inhibition, as shown by S6K1 dephosphorylation at Thr-389 (Figure 3C). We also isolated MEFs from $MCRS1^{lox/lox}$ mice crossed with Tg-UQ-CreERT2 mice, a transgenic line expressing tamoxifen-inducible Cre recombinase under ubiquitin C promoter control (Ruzankina et al., 2007). Both MCRS1 levels and mTORC1 activation were reduced in tamoxifen-treated MEFs (Figure S3L). Importantly, PKB phosphorylation at Ser-473 slightly increased in MCRS1's absence (Figure S3M), indicating that MCRS1 regulates mTORC1 independently of mTORC2.

Next, we examined MCRS1 depletion effects in $TSC2^{-/-}$ MEFs, which display constitutively active Rheb and chronic mTORC1 activation. $TSC2^{-/-}$ MEFs were stably infected with lentiviral-based particles for integrating and expressing isopropyl β-D-1-thiogalactopyranoside (IPTG)-inducible small hairpin RNA (shRNA) targeting a non-coding sequence (shRNA-Ctr) or two silencing *Mcrs1* mRNA (sh1RNA-MCRS1 and sh2RNA-MCRS1). *Mcrs1* deletion in the presence of IPTG reduced mTORC1 activity, as revealed by S6K1 phosphorylation at Thr-389 (Figures 3D and S3N), confirming the existence of TSC2-independent but MCRS1-specific mechanisms controlling mTORC1 activation in AA responses.

Additionally, MCRS1 depletion decreased cell number and size of HCT-116, HeLa (Figures 3E–3H), and HEK293T cells (Figure S3O). These effects were mostly pronounced in HeLa cells, which showed higher levels of MCRS1 and higher S6K1 activation than HCT-116 or HEK293T cells (Figure S3P). Moreover, MCRS1 knockdown reduced survival of HCT-116 (Figures 3I–3K) and HeLa (Figures S3Q–S3S) cells and decreased cell number in various $MCRS1^{-/-}$ MEFs (Figure S3T). Activated autophagy measured by accumulation of lipidated LC3 was also detected in $TSC2^{-/-}$, HEK293T, and HCT-116 cells (Figures 3L–3N and S3U). Finally, overexpression of MCRS1 fragments Ct (256-462, Rheb-binding) and Nt (1-260, non-Rheb-binding) respectively enhanced and impaired mTORC1 activity, in HeLa, HEK293T, and HCT-116 cells (Figure S3V). These effects correlated with Rheb/mTOR association (Figure S3W) and cell number in HeLa and HEK293T cells (Figure S3X), suggesting that MCRS1 Nt (1–260), which does not bind to Rheb (Figure 1C), acts as a dominant-negative fragment. Thus, MCRS1 regulates cellular functions that may be associated with mTORC1.

MCRS1 Expression Is Enhanced in Human Colorectal Cancer and Correlates with mTORC1 Activation

Finally, enhanced MCRS1 expression in human colorectal cancers, detected with immunohistochemistry using a MCRS1 antibody, correlated with poor patient prognosis and tumor stage, increased proliferation, and mTORC1 activation (revealed by S6K1 and S6 phosphorylation at Thr-389 and Ser-240/244, respectively) (Figures 4 and S4). These results concur with reported deregulation of MCRS1 expression (Shi et al., 2009, 2012) and mTORC1 activation (Gulhati et al., 2009) in colon cancer, and suggest that MCRS1 is oncogenic through mTORC1 activation.

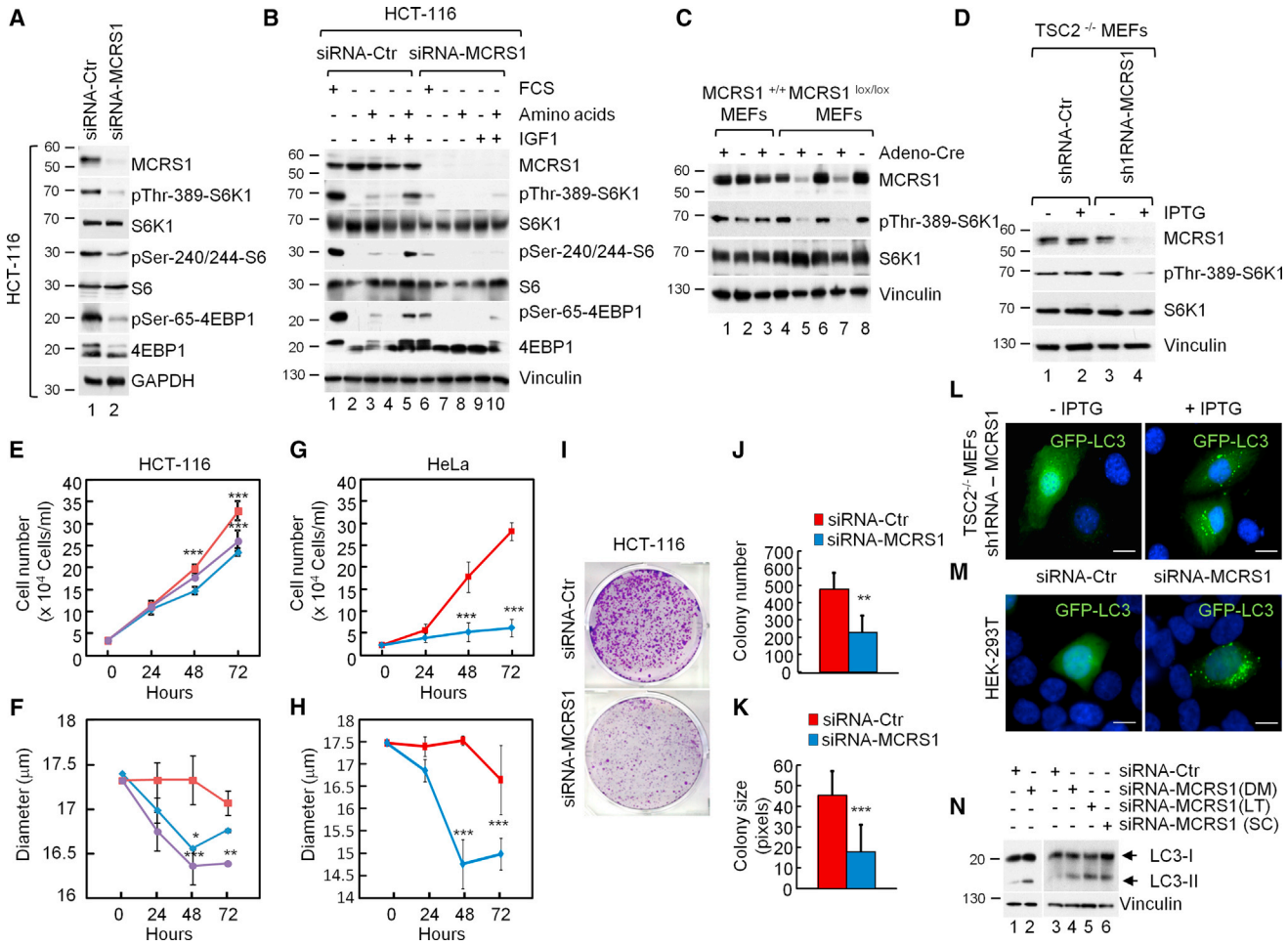


Figure 3. MCRS1 Is Essential for mTORC1 Activation

(A) HCT-116 cells cultured under normal growth conditions were transfected with siRNA-Ctr (control) or siRNA-MCRS1.

(B) HCT-116 cells transfected with either siRNA-Ctr (lanes 1–5) or siRNA-MCRS1 (lanes 6–10) were cultured under normal conditions (lanes 1 and 6) or serum-starved for 16 hr followed by AA deprivation for 1 hr (lanes 1, 2, 4, 6, 7, and 9) or AA deprivation and then re-stimulated by AAs (lanes 3 and 8) or IGF1 (lanes 4 and 9) for 10 min or both (lanes 5 and 10).

(C) MCRS1$^{+/+}$ or MCRS1$^{lox/lox}$ MEFs were infected or not infected with adeno-Cre lentiviral particles for 48 hr.

(D) TSC2$^{-/-}$ MEFs stably expressing either shRNA-Ctr (control) (lanes 1 and 2) or sh1RNA against MCRS1 (Sh1RNA-MCRS1) (lanes 3 and 4) were cultured in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of IPTG for 36 hr.

(E–H) Cell number (E and G) and cell diameter (F and H) was assessed in HCT-116 (E and F) and HeLa (G and H) cells transfected either with siRNA-Ctr (red square) or siRNA-MCRS1 (blue diamond) or treated with 20 nM of rapamycin (purple circle).

(I) Colony formation assay was performed in HCT-116 cells transfected either with siRNA-Ctr or siRNA-MCRS1.

(J and K) Quantification of colony number (J) and size (K) from (I) using ImageJ.

(L and M) Immunofluorescence images of TSC2$^{-/-}$ MEFs, MCRS1 depleted (sh1RNA-MCRS1) (L) and HEK293T cells either treated with siRNA-Ctr or siRNA-MCRS1 (M) and transiently transfected with GFP-LC3.

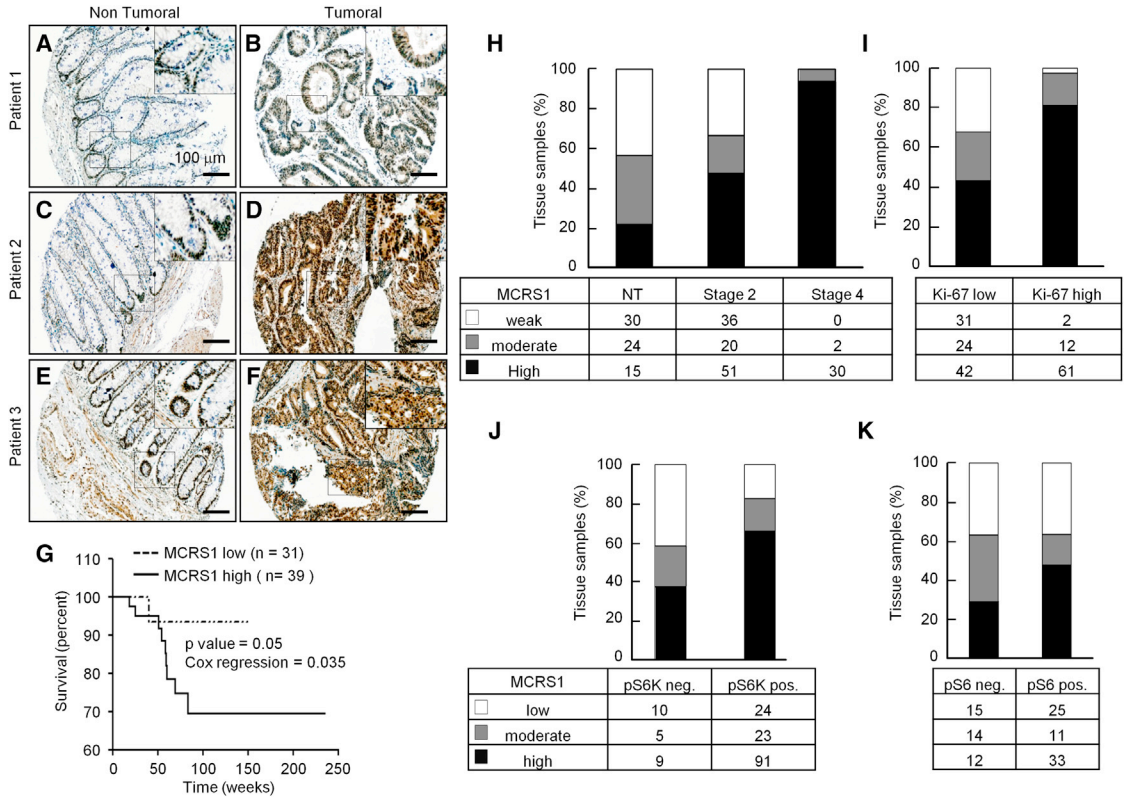
(N) HEK293T (lanes 1 and 2) and HCT-116 (lanes 3 to 6) cultured in normal growth conditions in presence of FCS were transfected with either siRNA–Ctr (control) (lane 1) or three different siRNA targeting *MCRS1* mRNA.

Statistical analysis was performed using two-way ANOVA test followed by the Benferroni method (E–H) and t test (J and K). Errors bars represent ± SD (n = 3). *p ≤ 0.05, **p ≤ 0.01, and ***p ≤ 0.001. Scale bars represent 5 μm.

MCRS1 Regulates Rheb Activity

Hypothesizing that MCRS1 activates mTORC1 through Rheb, we checked whether MCRS1 regulates farnesylation, required for Rheb activity. Fractionations of HCT-116 and HEK293T cells revealed that Rheb was cytoplasmic following AA or MCRS1 depletion, but relocalized to the nucleus when HCT-116 cells were treated with the farnesyltransferase inhibitor FTI-277 (Figures S5A–S5D), a known effect of farnesylation inhibition (Basso et al., 2006; Buerger et al., 2006; Jiang and Vogt, 2008; Takahashi et al., 2005). Thus, neither AA nor MCRS1 depletion affects Rheb farnesylation.

Because MCRS1 binds to active Rheb (Rheb-GTP) in an AA-dependent manner, we asked whether MCRS1 affects its nucleotide-binding state. Use of an immunoprecipitation Rheb-GTP-specific antibody (Figures S5E and S5F) showed that AA or MCRS1 depletion in HCT-116 cells significantly reduced



MCRS1	NT	Stage 2	Stage 4
weak	30	36	0
moderate	24	20	2
High	15	51	30

Ki-67 low	Ki-67 high
31	2
24	12
42	61

MCRS1	pS6K neg.	pS6K pos.
low	10	24
moderate	5	23
high	9	91

pS6 neg.	pS6 pos.
15	25
14	11
12	33

Figure 4. MCRS1 Expression Is Enhanced in Human Colorectal Cancer and Correlates with mTORC1 Activation

(A–F) Immunohistochemistry of MCRS1 expression in paired non tumoral (A, C, and E) and tumoral (B, D, and F) samples from three different patients.

(G) Kaplan Meier curve for high and low MCRS1 expression in human colon tumors. p = 0.05 and Cox regression = 0.035

(H–K) Correlation of MCRS1 expression in human colon tumors with tumor stage (H), Ki-67 (proliferation marker) (I), and mTORC1 activation revealed by pThr-389/412-S6K1 (pS6K1) (J) and pSer-240/244-S6 (pS6) (K).

Differences in number of cases among immunohistochemical markers were due to tissue damage (either tissue loss or inadequate tumor tissue). Scale bars represent 100 μm.

Rheb-GTP levels and impaired mTORC1/Rheb interaction (Figures 5A, S5G, and S5H). Importantly, we also measured the GTP/GDP-charged state of ectopically expressed GST-Rheb-wt and endogenous Rheb (Nobukuni et al., 2005; Roccio et al., 2006; Smith et al., 2005; Wolthuis et al., 1997) with GST pull-down and immunoprecipitation assays following ^{32}P-labeling of MCRS1-depleted HEK293T cells in the presence or absence of AAs. In both endogenous Rheb immunoprecipitation and GST-Rheb pull-down assays, the GTP/GDP ratio was decreased when MCRS1 was knocked down, while presence of AAs increased the ratio by ~20% (Figures 5B, 5C, S5I, and S5J). MCRS1 depletion in TSC2$^{-/-}$ MEFs, in which Rheb is not exposed to TSC1-TSC2 GAP activity, caused no significant differences in Rheb-GTP levels (Figures 5D and S5K). Furthermore, GST-Rheb pull-downs in HCT-116 cells and TSC2-immunoblotting showed that AA or MCRS1 depletion significantly enhanced Rheb/TSC2 interaction (Figures 5E, S5L, and S5M). Finally, AA-mediated decreased Rheb/TSC2 interaction was reversed by MCRS1 depletion (Figure 5F). Thus, MCRS1 and AAs reduce TSC1-TSC2 binding to Rheb.

Next, we tested whether MCRS1 binds competitively to Rheb, and, thus, whether MCRS1 and AA depletion increases TSC1-TSC2 GAP activity toward Rheb. For this, we measured Rheb GTP hydrolysis by incubating bacterially expressed [α-^{32}P] GTP-labeled GST-Rheb with TSC2 immunoprecipitated from AA-depleted HEK293T cells and with increased MCRS1 concentrations. Adding increasing amounts of MCRS1 significantly reduced Rheb-GTP hydrolysis by TSC1-TSC2 GAP activity (Figures 5G and 5H), indicating increased Rheb/MCRS1 and decreased Rheb/TSC2 interactions (Figure 5I). However, addition of the Nt or Ct segment of MCRS1 alone was not sufficient to confer protection from TSC2 GAP activity (Figure 5G). These data indicate that while Ct segment of MCRS1 was sufficient to bind Rheb, full-length MCRS1 is required to protect Rheb from TSC2 GAP activity, suggesting that MCRS1 may not exert its protective role by competing with TSC2 for Rheb binding but rather by preventing TSC2/Rheb interaction. Absence of MCRS1 or AAs significantly increased Rheb GTP hydrolysis by TSC1-TSC2 GAP (Figures 5J and 5K). Similarly, when GST-Rheb was charged and labeled with [γ-^{32}P]-GTP, MCRS1 and AA depletion increased GTP hydrolysis (Figures 5L–5N). Thus, TSC2 complexes immunoprecipitated following AA or MCRS1 depletion have increased GAP activity toward Rheb, indicating that AAs and MCRS1 promote active Rheb scaffolding and hinder TSC1-TSC2 GAP activity.

Furthermore, AA requirement was partially bypassed in Rheb-overexpressing HeLa cells, but not HEK293T and SW-620 cells

transiently transfected with GST-Rheb (Figure S5N). This "bypass" of AA effects in Rheb-overexpressing cells apparently depends on mTORC1 downstream targets. For instance, in HeLa cells 4EBP1 phosphorylation at Ser-65 was sensitive to AA withdrawal, even with Rheb overexpression, but not phosphorylation of S6K1 at Thr-389 and S6 at Ser-240/244 (Figure S5N). Impaired mTORC1 activity upon AA withdrawal was also observed following MCRS1 overexpression in all cell types, indicating that AAs are essential for Rheb and MCRS1 to activate mTORC1. Notably, overexpressed Rheb colocalized with mTOR in HEK293T and human osteosarcoma U2-OS cells (Figure S5O). Thus, MCRS1 is apparently required for AA-dependent mTORC1 activation.

Finally, we assayed mTORC1 kinase activity, which is directly stimulated by Rheb-GTP (Long et al., 2005a), in vitro in the presence of recombinant 4EBP1 and immunoprecipitated Raptor from HEK293T cells transfected with siRNA-Ctr or siRNA-MCRS1 (Figures 5O and 5P). Thus, MCRS1 and AA depletion reduced 4EBP1 phosphorylation at Ser-65, establishing a critical role for MCRS1 in mTORC1 activation.

MCRS1 Maintains Rheb at Lysosomes in an AA-Dependent Manner

Next, we verified that MCRS1 is a lysosomal membrane-bound protein, by immunoblotting Rheb, MCRS1, and mTORC1 in fractions separated by a sucrose-density gradient (Figures S6A and S6B). Furthermore, their lysosomal localization appeared to be AA dependent, although some mTOR remained lysosomal following AA deprivation (Figure 6A). Immunofluorescence analysis confirmed this, showing that ~15% of cells retained residual mTOR and Lamp2 colocalization (a lysosomal marker) after AA depletion (Figure S6C). MCRS1 localization to the lysosomes was confirmed by immunofluorescence (Figure S6D). Interestingly, MCRS1 FL (1–462) and MCRS1 Ct (256–462) fragment localized to the lysosomes in presence of AAs but not the dominant-negative part MCRS1 Nt (1–260) (mainly nuclear), consistent with our results shown in Figure S3V.

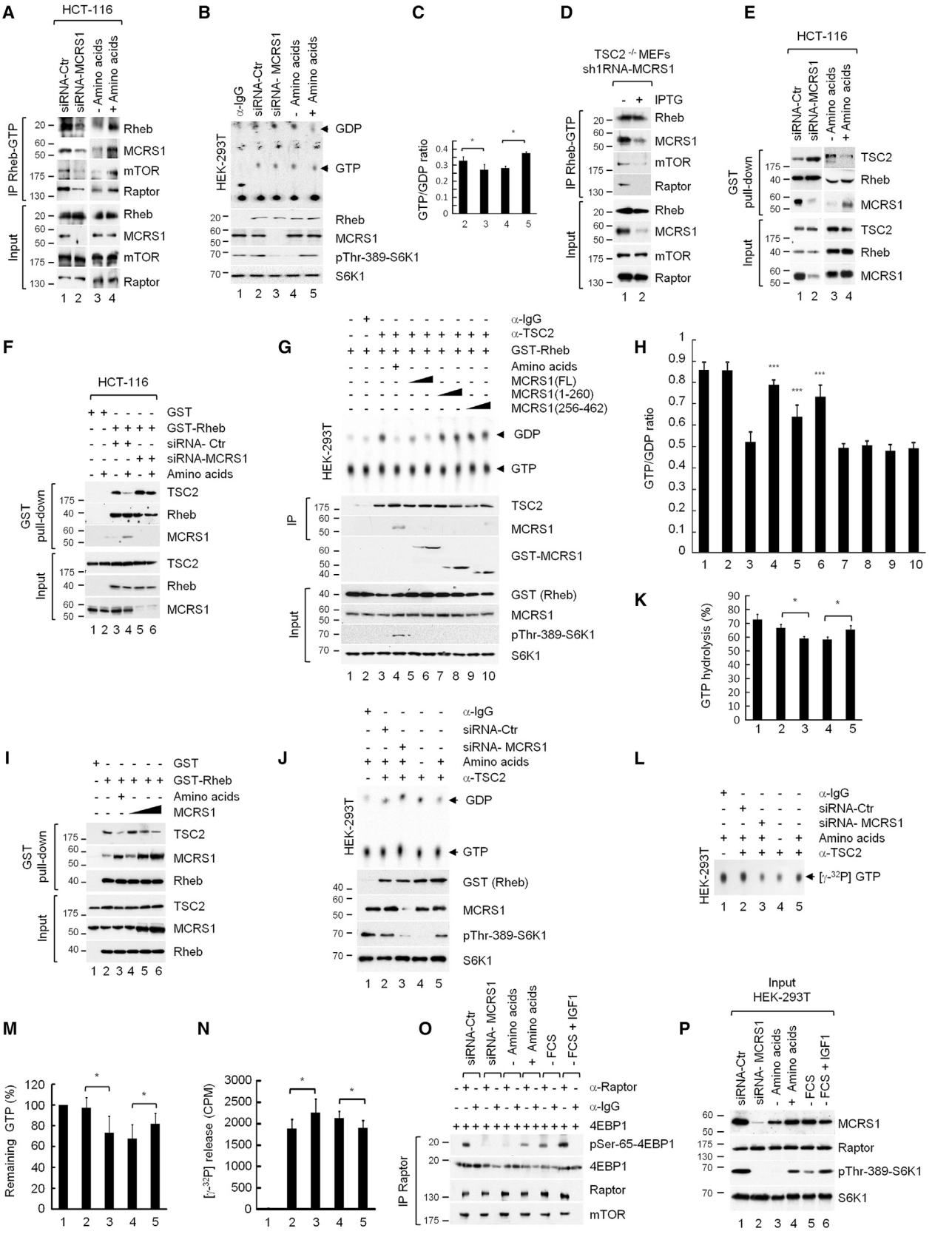
AAs strengthened MCRS1/Rheb and MCRS1/mTOR interactions, suggesting that mTORC1 AA-sensing machinery might target MCRS1 to lysosomes. To test this hypothesis, components of the v-ATPase (ATP6V0C) and Ragulator complexes (LAMTOR1) were knocked down in HCT-116 cells. Cells were also treated with FTI-277 to delocalize lysosomal Rheb. MCRS1 lysosomal localization was reduced by depleting the v-ATPase/Ragulator-AAs sensing machinery and further abolished by FTI-277 addition (Figures 6B, 6C, S6E, and S6F), indicating that AAs via v-ATPase/Ragulator machinery may play a role in MCRS1 recruitment at lysosomal surfaces. Interestingly, overexpression of the dominant active mutant RagB (Q99L) ($RagB^{GTP}$) constitutively targeted mTORC1 and MCRS1 to lysosomes where Rheb was retained despites AA depletion (Figures S6G–S6J). In MCRS1-depleted and RagB (Q99L)-overexpressing HCT-116 cells, AA-dependent mTORC1 activation was dramatically reduced (Figure 6D). Taken together, these data suggest that MCRS1, possibly through its binding to mTOR, is partly targeted to the lysosomes through the AA-sensing v-ATPase machinery.

Accordingly, MCRS1 suppression abolished Rheb detection in lysosomal fractions, without affecting mTORC1 levels (Figures 6E and S6K), independently of its reported interaction with Nde1 and nuclear function, because Nde1 depletion did not affect Rheb lysosomal localization (Figure S6L). Notably, no Rheb was detected in lysosomal fractions of $TSC2^{-/-}$ cells following MCRS1 suppression (Figures 6F and S6M), in accordance with mTOR inhibition and Rheb colocalization and binding by AA depletion (Long et al., 2005b). Overexpression of a Rheb construct that was constitutively targeted to the lysosomes (LAMTOR1-HA-Rheb) maintained mTOR activation upon AA depletion (Figures 6G–6I). However, this effect was lost upon MCRS1 depletion, confirming the role of MCRS1 to bridge mTOR/Rheb interaction (Figure 6G). Finally, overexpression of MCRS1 construct constitutively targeted to the lysosomes (LAMTOR1-HA-MCRS1) prevented mTOR inhibition following AA depletion (Figures 6H and 6J). Thus, MCRS1 is essential for maintaining Rheb on lysosomes and, couples Rheb to mTORC1 activation in AA responses.

Endogenous mTOR localization to lysosomes following AA stimulation was unaffected by MCRS1 depletion, as shown by its colocalization with the lysosomal marker Lamp2 (Figure 6K). Using a validated antibody in immunofluorescence and recognizing endogenous Rheb on the lysosomes (Figure S6N), we demonstrated that endogenous Rheb delocalized from lysosomes upon AA withdrawal and MCRS1 suppression in HCT-116 (Figures 6L and 6M) and U2-OS (Figures S6O and S6P) cells, apparently to another membrane-bound vesicle-like structure, indicated by its reduced co-staining with lysotracker. Similar results were obtained upon Rheb overexpression (Figures S6Q–S6S). Notably, RagC colocalized with Lamp2, indicating that RagC is lysosomal (Figures S6T and S6U). These results suggest that MCRS1 maintains Rheb at the lysosmal surfaces in an AA-dependent manner.

AAs and MCRS1 Protect Rheb from Endosomal Recycling

The lack of effects of MCRS1 depletion on Rheb farnesylation suggested that Rheb relocalized to another membrane-bound compartment, we investigated Rheb localization during the endocytic cycle. Endogenous Rheb colocalization with Rab5A, an early endosome marker, was not detected in HCT-116 cells (Figures 7A–7D). However, endogenous Rheb partially colocalized with Rab7A, a late endosome and lysosome marker (Figures 7E–7H), and AA or MCRS1 depletion reduced Rheb/Rab7A colocalization (Figures 7E–7I). Furthermore, 10% of endogenous Rheb colocalized with Rab11A, a marker of recycling endosomes, following AA or MCRS1 depletion in HCT-116 (Figures 7J–7N). Overexpressed HA or GST-tagged Rheb following AA or MCRS1 depletion showed similar patterns to endogenous Rheb in HCT-116 cells (Figures S7A–S7D) and, similar results were obtained in HEK293T and U2-OS (Figures S7E–S7M) cells. Notably, siRNA-mediated Nde1 depletion had no effect on Rheb localization (Figure S7N). In addition, in TSC2-depleted cells, Rheb did not colocalize with Rab5A (Figures S7O and S7P), but colocalized with Rab7A in the presence of AAs and MCRS1 (Figures S7Q and S7R) and partially shuttled on recycling endosomes, following AA and MCRS1 depletion (Figures S7S and S7T). Finally, AA depletion, MCRS1, or mTOR knockdown significantly decreased Rab5A and Rab11A but increased Rab7A vesicles number in HCT-116 cells (Figure S7U),



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suggesting a role of MCRS1 in the endocytic cycle that may also depend on mTORC1 activity. We also observed that Rab5A, Rab7A, and Rab11A vesicle size increased upon AA stimulation and decreased upon MCRS1 and mTOR knockdown (Figure S7V), suggesting that increased lysosomal platform is required for fully operative mTORC1/Rheb/MCRS1 complexes.

TSC2 Is Lysosomal and Does Not Shuttle in Response to AAs

Interestingly, regardless of the presence of AAs and MCRS1, TSC2 colocalized with Lamp2 and Rab7A, but not Rab5A or Rab11A (Figures 8A–8D), indicating that TSC2 resides on lysosomes and does not circulate during endocytic cycles in AA responses. While lysosomal TSC2 localization in response to AAs was recently debated (Demetriades et al., 2014; Menon et al., 2014), serum starvation followed by IGF1 addition spatially delocalized TSC2 from lysosomes, as previously reported (Menon et al., 2014) (Figure S8). These results indicate that AA stimulation recruits mTORC1 with MCRS1 to late endosomes/lysosomes. MCRS1 maintains Rheb on lysosome surfaces and protects it from lysosomal TSC1-TSC2 GAP activity, connecting Rheb/mTORC1. The absence of MCRS1 or AAs favors TSC2/Rheb interaction and inactivated Rheb, but not TSC2, delocalizes from the lysosomes leading to mTORC1 inactivation (Figure 8E).

DISCUSSION

Full mTORC1 activation requires AAs for its translocation to lysosomes, and growth factors that inhibit TSC1-TSC2 GAP activity toward Rheb (Menon et al., 2014). Loss of TSC1 or TSC2 renders Rheb hyperactive, constitutively activating mTORC1, which becomes resistant to growth factor (but not AA) deprivation (Kim et al., 2008; Roccio et al., 2006; Sancak et al., 2008; Smith et al., 2005; Zhang et al., 2003a, 2003b), indicating that AAs may be essential to convert Rheb to its active (mTOR kinase activity-stimulating) GTP-bound form. Rheb-GTP binds to the mTOR catalytic domain's amino-terminal lobe and activates mTOR kinase GTP-dependently (Sato et al., 2009). AA depletion reduces proportions of active GTP-bound Rheb (Roccio et al., 2006; Smith et al., 2005), and reversibly inhibits Rheb/mTOR binding (Long et al., 2005b).

Rheb overexpression can override effects of AA starvation on mTORC1 inhibition (Garami et al., 2003; Im et al., 2002; Inoki et al., 2003; Long et al., 2005a, 2005b; Saucedo et al., 2003; Smith et al., 2005; Stocker et al., 2003; Tee et al.,

Figure 5. MCRS1 Regulates Rheb Activity

(A) HCT-116 cells transfected with siRNA-Ctr (control) or siRNA-MCRS1 were serum-starved for 16 hr followed by AA deprivation for 1 hr or AA deprivation for 1 hr and then re-stimulated with AAs for 10 min. Cell lysates were processed for Rheb-GTP immunoprecipitation using a configuration-specific Rheb-GTP monoclonal antibody.

(B) HEK293T cells were either not transfected or transfected with siRNA-Ctr (control) or siRNA-MCRS1 and grown overnight in 1.5% serum (lanes 1, 2, and 3). Cells were then cultured in phosphate-free media for 3.5 hr supplemented with ^{32}p radionuclide. Cells were AA deprived for 1 hr in HBSS supplemented with ^{32}P radionuclide and then re-stimulated for 10 min with 10% dialyzed fetal calf serum (FCS) either in the absence (lane 4) or presence (lane 5) of AAs. Endogenous Rheb was immunoprecipitated with Rheb C-19 antibody and radioactive GTP/GDP eluted nucleotides were resolved with TLC.

(C) Quantification of (B). The ratio of GTP to GDP was detected by PhosphorImager and quantified by ImageJ following the formula $\%^{32}P{-}GTP = {}^{32}P{-}GTP/({}^{32}P{-}GTP + 1.5 \times {}^{32}P{-}GDP)$.

(D) TSC2$^{-/-}$ MEFs stably expressing sh1RNA against MCRS1 were cultured in the absence or presence of IPTG for 36 hr to deplete MCRS1. Cell lysates were subjected to Rheb-GTP immunoprecipitation assay.

(E) HCT-116 cells transiently transfected with GST Rheb and treated as in (A). Cell lysates were subjected to GST pull-downs.

(F) GST pull-downs in HCT-116 cells transiently expressing GST-Rheb and transfected with either siRNA-Ctr or siRNA MCRS1. Cells were serum-starved for 16 hr followed by AAs for 1 hr or starved and then re-stimulated by AAs for 10 min.

(G) HEK293T cells were serum-starved for 16 hr followed by AA deprivation for 1 hr or starved and then re-stimulated by AAs for 10 min. Cells were lysed and subjected for TSC2 immunoprecipitation and assayed for GAP activity toward [α-^{32}P] GTP-loaded bacterial GST-Rheb in the absence or the presence of increasing amount of GST-MCRS1 (full-length [FL]), Nt-MCRS1 (1–260) or Ct-MCRS1 (256–462). Radioactive GTP and GDP were resolved with one-dimensional TLC.

(H) Quantification of (G) following the formula $\%^{32}P{-}GTP = {}^{32}P{-}GTP/({}^{32}P{-}GTP + 1.5 \times {}^{32}P{-}GDP)$. Radioactive GTP and GDP were detected by using PhosphorImager and the ratio of GTP to GDP was quantified by ImageJ.

(I) HEK293T cells transfected with either GST or GST-Rheb were treated as in (F). GST pull-downs were performed with increased concentrations of in vitro translated HA-MCRS1.

(J) HEK293T cells were either transfected or not with siRNA-Ctr (control) (lane 2) or siRNA-MCRS1 (lane 3) or were serum-starved for 16 hr followed by AA deprivation for 1 hr or AA deprivation and then re-stimulated by AAs for 10 min. TSC2 was immunoprecipitated from cell lysates and assayed for GAP activity toward [α-^{32}P] GTP-loaded bacterial GST-Rheb. Radioactive GTP and GDP were resolved by one-dimensional TLC. Radioactive GTP and GDP were detected by using PhosphorImager and the ratio of GTP to GDP was quantified by ImageJ.

(K) Quantification of (J).

(L) TSC2 was immunoprecipitated from HEK293T cells, which were serum-starved for 16 hr followed by AA deprivation for 1 hr or deprived and then re-stimulated by AAs for 10 min, as indicated. Immunoprecipitates were assayed for GAP activity toward purified bacterial [γ-^{32}P] GTP-loaded GST-Rheb (see Experimental Procedures). [γ-^{32}P] GTP was eluted from Rheb after a 60 min incubation with the specified immune complexes and resolved by one-dimensional TLC.

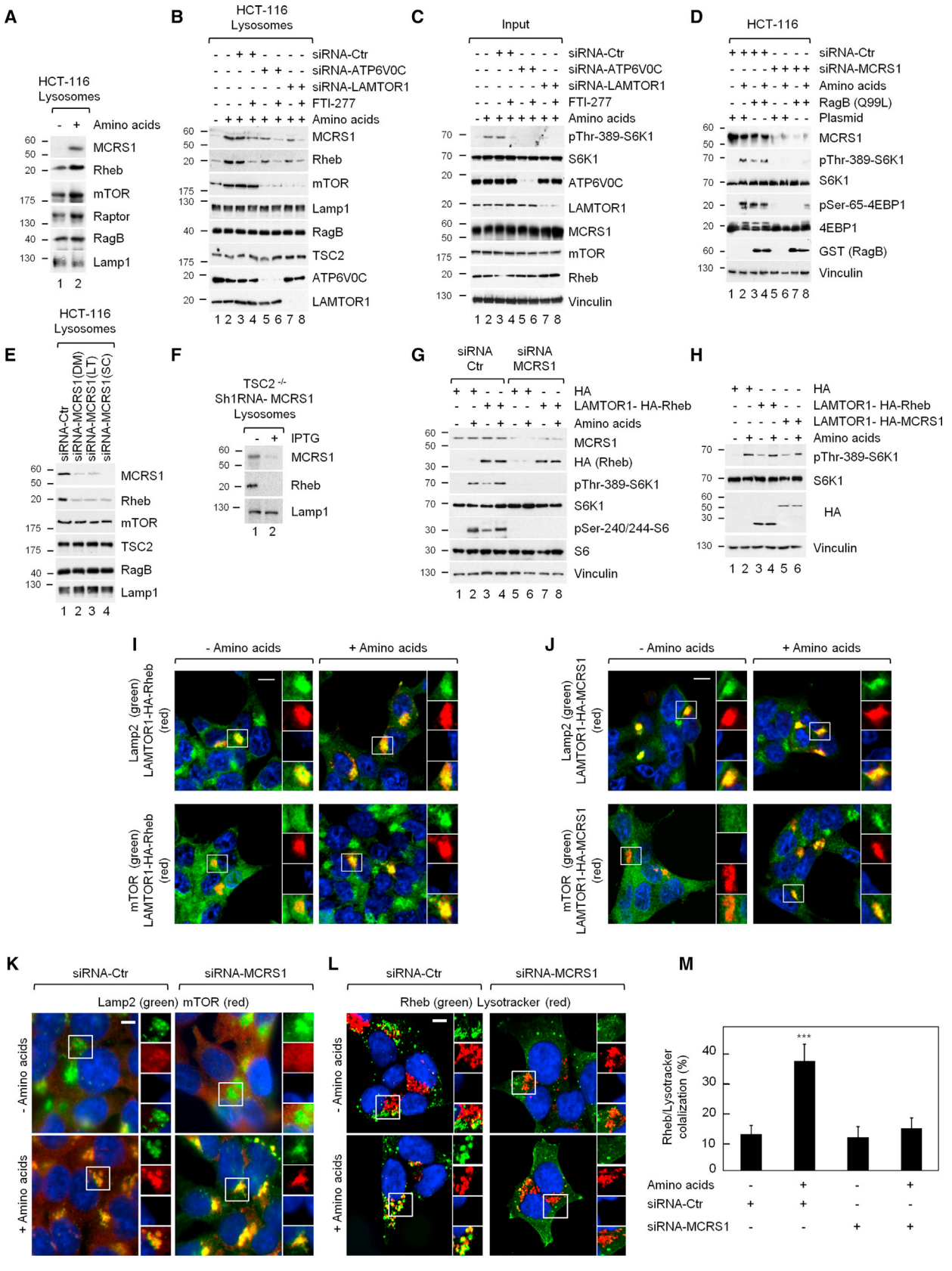
(M) Quantification of (L) using ImageJ. Data were normalized to IgG.

(N) Scintillation counts of [γ-^{32}P] GTP released after hydrolysis.

(O) HEK293T cells were transfected with either siRNA-Ctr (control) (lanes 2 and 3) or siRNA-MCRS1 (lanes 4 and 5) or, were AA-deprived for 1 hr in presence of dialyzed 10% FCS and then re-stimulated for 10 min with dialyzed 10% FCS either in the absence (lanes 6 and 7) or presence (lanes 8 and 9) of AAs or starved from FCS for 24 hr (lanes 10 and 11) or FCS starved and stimulated with IGF1 for 10 min (lanes 12 and 13) as indicated. Whole-cell extracts were processsed for immunoprecipitation using Raptor antibody. Immunoprecipitates were subjected to in vitro kinase assay using recombinant 4EBP1 protein as a substrate. Samples were processed for immunoblotting with the indicated antibodies.

(P) Cell lysates used for immunoprecipitation and kinase assays shown in (O).

Statistical analysis was performed using one-way ANOVA test followed by the Benferroni method (C, H, K, M, and N). Errors bars represent ± SD (n = 3). *$p \leq 0.05$ and ***$p \leq 0.001$.



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2003). Notably, Rheb overexpression or forced localization of mTORC1 to lysosomal surfaces (by fusing a lysosomal localization signal to Raptor) bypasses requirements for Rag-mediated recruitment of mTORC1 and allows mTORC1 activation in the absence of AAs (Zoncu et al., 2011a). Our results indicate that mTORC1 responses to AA withdrawal are weak even with Rheb overexpression, which may ectopically stabilize Rheb/mTORC1 complexes, thereby overriding MCRS1 requirement. However, these complexes are only partially activated upon AA depletion, depending on cell type and downstream targets modulated by mTORC1 stimulation. AAs may putatively activate mTORC1 Rheb-independently via conformational changes in mTOR/Raptor complexes that impair their ability to phosphorylate substrates (Hay and Sonenberg, 2004), but our findings indicate that AAs target MCRS1 to lysosomes, maintaining and connecting active Rheb to mTORC1 activation.

It is currently uncertain which specific AAs are sensed to activate mTORC1, although leucine, L-glutamine, and arginine have been implicated in mTORC1 activation (Jewell et al., 2015; Rebsamen et al., 2015; Wang et al., 2015). The AA-sensing v-ATPase lysosomal machinery is necessary to target mTOR to the lysosomes. This same machinery is partly required to localize MCRS1 to the lysosomes, possibly through its binding to mTOR. Additionally, leucine and non-essential AAs containing L-glutamine and arginine are necessary in maintaining MCRS1/Rheb interaction, independently of v-ATPase and Ragulator, suggesting that different AA-sensing pathways channel to MCRS1. Recent studies reportedly described that the cytoplasmic leucyl-tRNA synthetase (LeuRS) acts as a direct sensor of leucine and is implicated in mTORC1 activation (Bonfils et al., 2012; Han et al., 2012), and mTORC1 is differentially regulated by glutamine and leucine through distinct mechanisms that require Arf1 or Rag GTPases, respectively (Jewell et al., 2015). Further work is needed to better understand how different AAs signal to MCRS1 and to regulate mTORC1 activity.

Nutrient regulation of mTORC1 signaling is functionally conserved across eukaryotes. Database searches detected no potential MCRS1 orthologs in yeasts, despite high MCRS1 conservation among multicellular eukaryotes. Furthermore, TSC1, TSC2 (Hay and Sonenberg, 2004; Long et al., 2002), and Ragulator (Sancak et al., 2010) are not conserved in *Saccharomyces cerevisiae*. Although a Rheb homolog has been identified in *S. cerevisiae*, phylogenetic analyses indicate that the Rheb ortholog in budding yeast does not appear to regulate TOR pathway (Aspuria and Tamanoi, 2004; Berchtold and Walther, 2009). Notably, even following AA depletion, TOR appears to remain bound to vacuoles (lysosome counterparts) in *S. cerevisiae*. In contrast to budding yeast, conservation of the TSC/Rheb/TOR signaling pathway in fission yeast (*Schizosaccharomyces pombe*) and mammalian cells (Aspuria et al., 2007) argues that MCRS1 evolved in multicellular organisms later after TSC and Rheb proteins, acquiring an mTORC1-activation regulatory role.

Interestingly, mice lacking components described to be critical in mTORC1 activation, such as Rheb (Goorden et al., 2011), Ragulator (Nada et al., 2009; Teis et al., 2006), Raptor (Guertin et al., 2006), or mTOR itself (Gangloff et al., 2004), die at early days of embryonic development (between 6 and 8 days). Although further analysis is required, the complete knockout of MCRS1 also results in embryonic lethality (not shown). Because MCRS1 is essential for mTORC1 function, which is necessary to support normal development in embryos, we would therefore not be surprised that mice lacking MCRS1 may die at an earlier time than other mTORC1 components.

MCRS1 was initially described as a nucleolar protein p120 interactor (Ren et al., 1998), associated with transcription regulation (Andersen et al., 2010; Ivanova et al., 2005; Lin and Shih, 2002; Wu et al., 2009). However, it participates in other processes, including cellular senescence (Hsu et al., 2012), cell division, and survival by interacting with the centrosomal protein

Figure 6. MCRS1 Maintains Rheb at Lysosomes in an AA-Dependent Manner

(A) Immunoblots of lysosomal fractions of HCT-116 cells that were serum-starved for 16 hr followed by AA deprivation for 1 hr or AA deprivation for 1 hr and then re-stimulated with AAs for 10 min.

(B) HCT-116 cells were transfected either with siRNA-Ctr, siRNA-ATP6V0C, or siRNA-LAMTOR1 or treated with FTI-277 farnesyl inhibitor or both. For AA stimulation, cells were treated as in (A) and were subjected to lysosomal fractionation.

(C) Cell lysates of (B) used for loading control.

(D) HCT-116 cells were transiently transfected with either an empty plasmid or RagB (Q99L) and with either siRNA-Ctr (control) or siRNA-MCRS1. For AA stimulation, cells were treated as in (A).

(E) Lysosomal fractions of HCT-116 cells cultured under normal conditions and treated with either siRNA-Ctr (control) or siRNA-MCRS1 from Dharmacon (DM), Life Technologies (LT), or Santa Cruz (SC).

(F) Lysosomal fractions of $TSC2^{-/-}$ MEFs stably expressing sh1RNA against MCRS1 cultured for 36 hr in the presence of IPTG to deplete MCRS1.

(G) HCT-116 cells were transfected with either siRNA-Control or siRNA-MCRS1 and transiently expressing LAMTOR1-HA-Rheb. For AA stimulation, cells were treated as in (A).

(H) HCT-116 cells transiently expressing either HA empty vector, LAMTOR1-HA-Rheb or LAMTOR1-HA-MCRS1. For AA stimulation, cells were treated as in (A).

(I) Immunofluorescence images of staining for Lamp2 (green), mTOR (green) and HA (red) in HCT-116 cells transiently expressing LAMTOR1-HA-Rheb. For AA stimulation, cells were treated as in (A).

(J) Immunofluorescence images of staining for Lamp2 (green), mTOR (green), and HA (red) in HCT-116 cells transiently expressing LAMTOR1-HA-MCRS1. For AA stimulation, cells were treated as in (A).

(K and L) Staining for Lamp2/mTOR (K) or endogenous Rheb/Lysotracker (L) in HCT-116 cells transfected either with siRNA-Ctr or with siRNA-MCRS1. For AA stimulation, cells were treated as in (A). DNA was labeled with 4′,6′-diamidino-2-phenylindole (DAPI) (blue). In all images, insets show selected magnified fields that overlay.

(M) Quantification of Rheb's colocalization with lysotracker from (L).

Statistical analysis was performed using one-way ANOVA test followed by the Benferroni method (M). Errors bars represent ± SD (n = minimum of ten cells). ***$p \leq 0.001$.

Scale bars represent 5 μm.

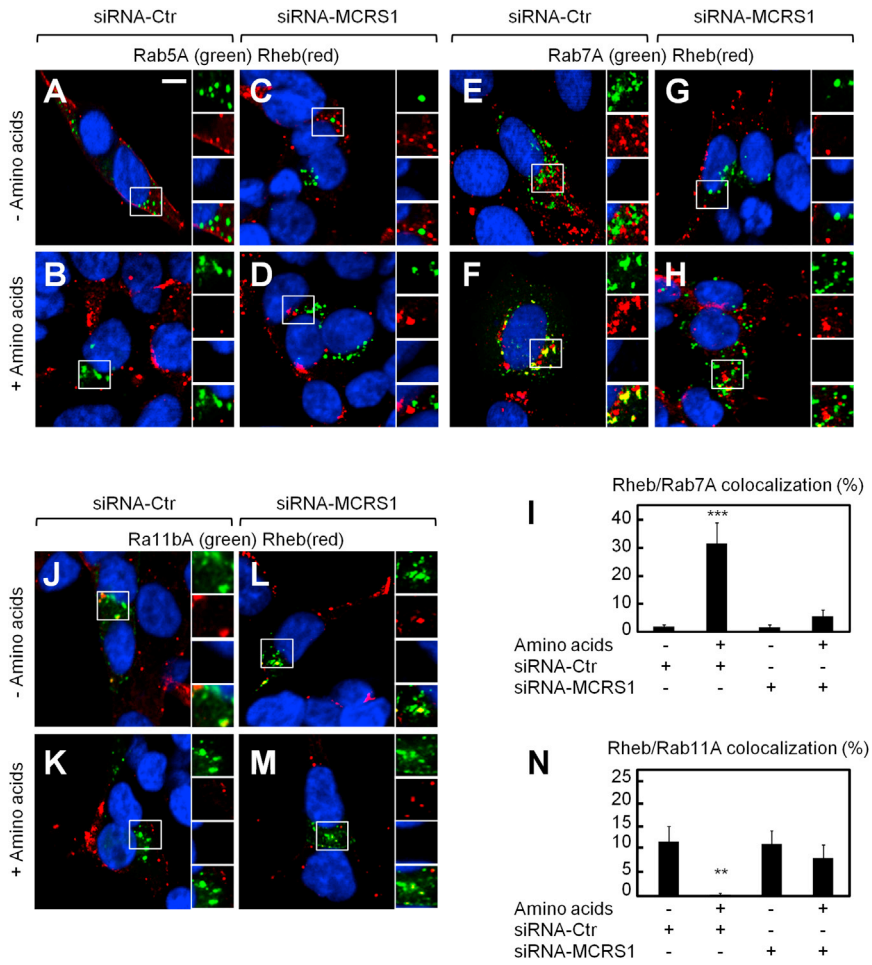

Figure 7. AAs and MCRS1 Protect Rheb from Its Endosomal Recycling

(A–D) HCT-116 cells transiently expressing GFP-Rab5A and transfected with siRNA-Ctr (control) or siRNA-MCRS1 were serum-starved for 16 hr followed by 1 hr AA deprivation or by 1 hr AA deprivation and subsequently re-stimulated by addition of AAs for 10 min. Endogenous Rheb is detected using a specific antibody. Representative immunofluoresence images are shown. Inset shows a higher magnification of a selected field.

(E–I) HCT-116 cells transiently expressing GFP-Rab7A were treated as in (A)–(D). Endogenous Rheb is detected using a specific antibody. Immunofluorescence images are shown (E–H) as well as quantification of the pixel colocalization using ImageJ (I).

(J–N) HCT-116 cells transiently expressing GFP-Rab11A were treated as in (A)–(D). Endogenous Rheb is detected using a specific antibody. Immunofluorescence images are shown (J–M) as well as quantification of the pixel colocalization using ImageJ (N).

Statistical analysis was performed using one-way ANOVA test followed by the Benferroni method (I and N). Errors bars represent ± SD (n = 3). $^{**}p \leq 0.01$ and $^{***}p \leq 0.001$.

Scale bars represent 5 μm.

Nde1 (Hirohashi et al., 2006) and is reportedly an essential RanGTP-regulated factor for bipolar spindle assembly protecting them from depolymerization (Meunier and Vernos, 2011MCRS1 regulates mTORC1 independently of microtubule networks and its nuclear function argues that cells may contain several MCRS1 pools with different functions, without excluding a general role of MCRS1 in scaffolding small GTPase proteins. The potential of farnesyltransferase inhibitors to treat cancer by impairing membrane-bound small GTPases functions is being tested (Berndt et al., 2011). However, inhibiting MCRS1 might offer an attractive and innovative option for treating various pathologies, including diabetes mellitus and cancer.

EXPERIMENTAL PROCEDURES

Materials, Antibodies, siRNA, shRNAs, Plasmids, Cell Culture, Transfections, Cell Number, and Size

All details are provided in the Supplemental Experimental Procedures.

Immunoblotting, Immunoprecipitation, and GST Pull-Down Assays

All assays were performed as previously described (Djouder et al., 2007, 2010) and detailed in the Supplemental Experimental Procedures.

In Vitro Protein Binding Assays

Assays were performed as detailed in the Supplemental Experimental Procedures.

In Vitro Kinase Assays

Detailed protocol is provided in the Supplemental Experimental Procedures.

Measurement of Rheb Nucleotide State

The activated/active state of Rheb, i.e., the percent of Rheb molecules in the active GTP bound state, was determined for the endogenous protein using the Rheb Activation Assay Kit utilizing a configuration-specific monoclonal antibody that recognizes and immunoprecipitates Rheb-GTP but not Rheb-GDP. The measurement of GTP/GDP charged state of Rheb was assayed as described earlier (Nobukuni et al., 2005; Roccio et al., 2006; Smith et al., 2005; Wolthuis et al., 1997). Detection was performed using a PhosphorImager and quantification using ImageJ. Assays were performed as detailed in the Supplemental Experimental Procedures.

Measurement of TSC2 GAP Activity

TSC2 GAP-stimulated Rheb GTP hydrolysis was measured as described previously (Li et al., 2006). Briefly, GST-Rheb GTP loaded was incubated with TSC2 immunoprecipitates in GAP assay buffer (20 mM Tris-HCl pH 8, 10 mM $MgCl_2$, and 1 mM DTT) for 60 min at room temperature. GTP and GDP nucleotide were eluted, and resolved with thin layer chromatography (TLC) on PEI cellulose with KH_2PO_4 as the solvent. Specific details are provided in the Supplemental Experimental Procedures.

Immunofluorescence

Immunofluorescence assays were performed as detailed in the Supplemental Experimental Procedures.

Histopathology and Immunohistochemistry

Histopathology and immunohistochemistry were performed as detailed in the Supplemental Experimental Procedures.

Subcellular Fractionation

Nuclear, cytoplasmic, mitochondrial, and lysosomal fractionations were performed as described in the Supplemental Experimental Procedures. To obtain the lysosomal fraction, the supernatant from the mitochondrial fractionation

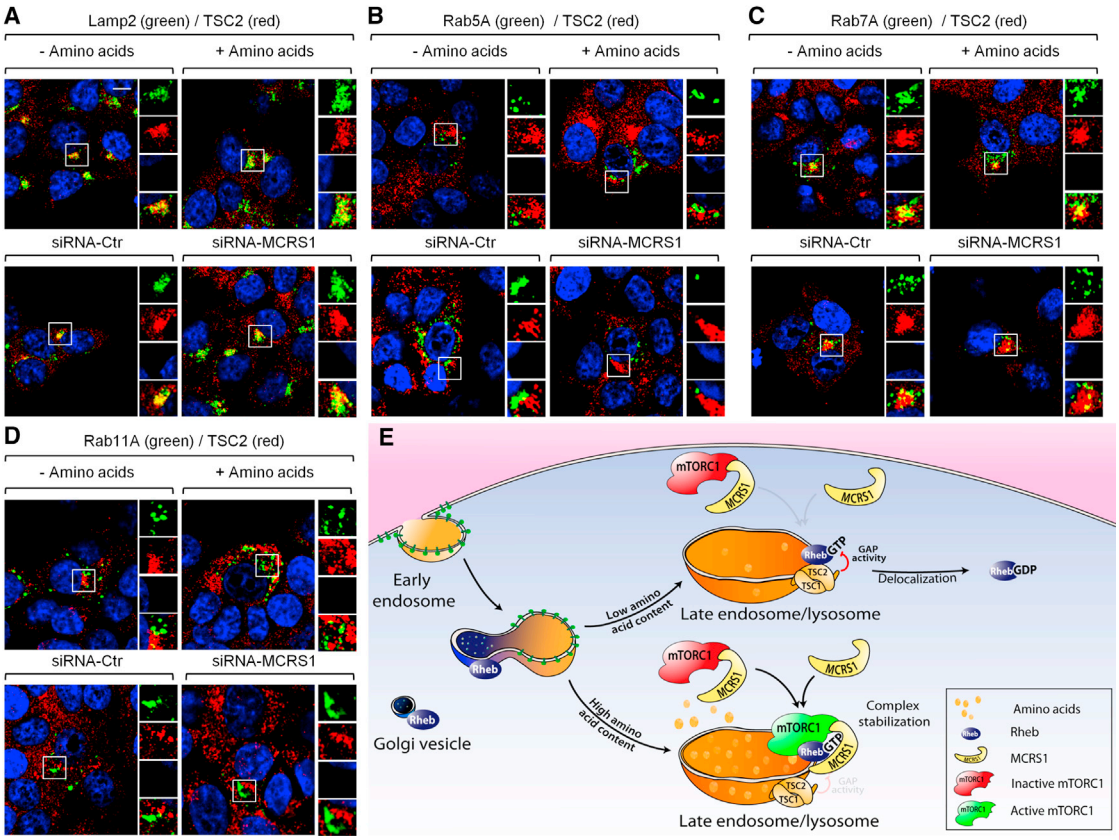

Figure 8. TSC2 Is Lysosomal and Does Not Shuttle in Response to AAs

(A) Immunofluorescence images of endogenous Lamp2 (green) and TSC2 (red) in HCT-116. Cells were either transfected with siRNA-Ctr (control) or siRNA-MCRS1 were serum-starved for 16 hr followed by 1 hr AA deprivation or by 1 hr AA deprivation and subsequently re-stimulated by addition of AAs for 10 min. Cells were then fixed with 4% PFA and then treated with 0.05% dodecyltrimethylammonium chloride for 15 min prior to blocking and labeling with primary and secondary antibodies.

(B–D) Immunofluorescence images of endogenous TSC2 in HCT-116 cells transiently expressing GFP-Rab5A (green) (B), GFP-Rab7A (green) (C), or GFP-Rab11A (green) (D) and treated as in (A).

(E) Model for the role of MCRS1 in AA-dependent TORC1 activation.

Scale bars represent 5 μm.

was recovered and submitted to the lysosomal extraction using the lysosomal isolation kit following the manufacturer's instructions. Protein concentrations of the fractions were determined (DC Protein Assay, Bio-Rad Laboratories) and equal amounts of each fraction were subjected for SDS-PAGE and western blotting with the indicated proteins.

Mass Spectrometry Analysis

GST pull-downs from cells transiently expressing GST or GST-Rheb were performed. Protein digestion was accomplished in solution or in gel, and desalted peptides were separated by reverse phase chromatography using a nanoLC Ultra 1D+ system (Eksigent) and directly coupled with a LTQ-Orbitrap Velos instrument (Thermo Fisher Scientific) via nanoelectrospray source (Proxeon-Biosystem). Data analysis of raw files was done using the Proteome Discoverer 1.3.0.339 software suite (Thermo Scientific). Specific details are provided in the Supplemental Experimental Procedures.

Size Exclusion Chromatography

Size exclusion chromatography was performed as detailed in the Supplemental Experimental Procedures.

Generation and Handling of MCRS1 Conditional Knockout Mouse

Generation of MCRS1 conditional knockout (cKO) was achieved as detailed in the Supplemental Experimental Procedures. All experiments were approved by the CNIO-ISCIII Ethics Committee and performed in accordance with the guidelines for ethical conduct in the care and use of animals as stated in the international guiding principles for biomedical research involving animals, developed by the Council for International Organizations of Medical Sciences.

Human Samples

Human samples were obtained from the CNIO-Biobank. The construction and analysis of a tissue microarray of colon cancer was approved by the appropriate ethics committee and informed consent was obtained from all subjects.

Statistical Analyses

Statistical analyses were performed using GraphPad Prism V5.0 software. Statistical significance was considered at $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, and $^{***}p \leq 0.001$ between the means of a minimum of three groups was determined using unpaired two-tailed Student's t test or one-way ANOVA or two-way ANOVA test as indicated in the figure legends. Results are expressed as the mean value ± SD. Immunoblots are representative of at least three independent experiments.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and eight figures and can be found with this article online at http://dx.doi.org/10.1016/j.devcel.2015.02.010.

AUTHOR CONTRIBUTIONS

M.A.F. and N.D. designed the experiments and analyzed the data. M.A.F. performed most of the experiments. M.B. and N.D. engineered and obtained the MCRS1 (lox/lox) allele. M.B. isolated and performed most of the experiments with MCRS1 MEFs. N.D conceived, developed and wrote the project and the manuscript. All authors edited the manuscript. Funding was secured by N.D.

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