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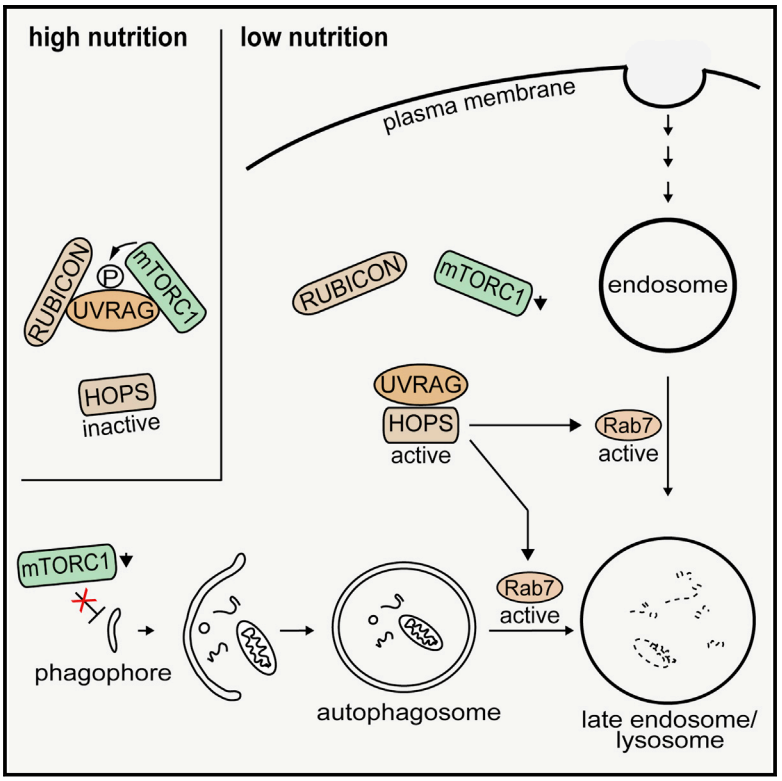
mTORC1 Phosphorylates UVRAG to Negatively Regulate Autophagosome and Endosome Maturation

Graphical Abstract



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In Brief

mTORC1 is known to regulate early stages of autophagy. In this study, Kim et al. report that mTORC1 also regulates late stages of autophagy as well as endosomal maturation by phosphorylating UVRAG. This finding defines a broad range of mTORC1 functions in the membrane-associated processes.

Highlights

- mTORC1 binds and phosphorylates UVRAG
- UVRAG Ser498 phosphorylation by mTORC1 enhances the UVRAG-RUBICON interaction
- UVRAG Ser498 phosphorylation suppresses autophagosome and endosome maturation
- Prevention of Ser498 phosphorylation enhances the lysosomal degradation of EGFR

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mTORC1 Phosphorylates UVRAG to Negatively Regulate Autophagosome and Endosome Maturation

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SUMMARY

mTORC1 plays a key role in autophagy as a negative regulator. The currently known targets of mTORC1 in the autophagy pathway mainly function at early stages of autophagosome formation. Here, we identify that mTORC1 inhibits later stages of autophagy by phosphorylating UVRAG. Under nutrient-enriched conditions, mTORC1 binds and phosphorylates UVRAG. The phosphorylation positively regulates the association of UVRAG with RUBICON, thereby enhancing the antagonizing effect of RUBICON on UVRAG-mediated autophagosome maturation. Upon dephosphorylation, UVRAG is released from RUBICON to interact with the HOPS complex, a component for the late endosome and lysosome fusion machinery, and enhances autophagosome and endosome maturation. Consequently, the dephosphorylation of UVRAG facilitates the lysosomal degradation of epidermal growth factor receptor (EGFR), reduces EGFR signaling, and suppresses cancer cell proliferation and tumor growth. These results demonstrate that mTORC1 engages in late stages of autophagy and endosome maturation, defining a broader range of mTORC1 functions in the membrane-associated processes.

INTRODUCTION

Autophagy is an evolutionarily conserved process through which eukaryotic cells degrade intracellular organelles and biomolecules in the lysosome. The intracellular degradation is important to maintain the balance between synthesis, degradation, and recycling of cellular constituents in response to changes in nutrient levels and other cellular environments. At the cellular level, autophagy is important to maintain the integrity of cellular structures during differentiation and under stress conditions. At the organism level, autophagy is important for the normal physiology and development of the body. Dys-regulation of autophagy has been implicated in aging, innate immunity, and many human diseases including cancers, neurodegenerative diseases, and immune disorders (Levine and Kroemer, 2008; Mizushima et al., 2008).

Autophagy is negatively regulated by mechanistic target of rapamycin (mTOR), the master controller of cell growth (Chang and Neufeld, 2009; Ganley et al., 2009; Hosokawa et al., 2009; Jung et al., 2009). mTOR forms a multiprotein complex called mTORC1 by interacting with raptor, GβL, PRAS40, and DEPTOR to regulate cell growth and autophagy in response to nutritional conditions and growth factor stimulation (Jung et al., 2010; Kim et al., 2002). A key event in the autophagy pathway regulated by mTORC1 is phosphorylation of Unc51-like kinase 1 (ULK1), a serine/threonine kinase that functions upstream in the autophagy pathway (Ganley et al., 2009; Hosokawa et al., 2009; Jung et al., 2009; Kim et al., 2011). Through phosphorylating ULK1, mTORC1 inhibits ULK1 activation by AMP-activated protein kinase (AMPK) (Egan et al., 2011; Kim et al., 2011; Shang and Wang, 2011). mTORC1 also targets proteins other than ULK1, such as Atg13 (Chang and Neufeld, 2009; Ganley et al., 2009; Hosokawa et al., 2009; Jung et al., 2009), Atg14L (Yuan et al., 2013), and Autophagy/beclin-1 regulator 1 (Ambra1) (Nazio et al., 2013). These mTORC1 targets are mainly known to function at early stages of autophagosome formation. Whether mTORC1 regulates autophagy at later stages, such as autophagosome maturation, remains unknown.

UV radiation resistance-associated gene product (UVRAG) is a protein localized in the endoplasmic reticulum (ER) and endosomes (He et al., 2013; Itakura et al., 2008; Liang et al., 2008). UVRAG is known to regulate autophagosome maturation (Liang et al., 2008) as well as early stages of autophagy (He et al., 2013; Liang et al., 2006; Takahashi et al., 2007). UVRAG regulates autophagosome maturation by binding to the homotypic fusion and vacuole protein sorting (HOPS) complex, which consists of the class C Vps complex (Vps11-Vps16-Vps18-Vps33) and two additional proteins (Vps39 and Vps41) (Liang et al., 2008). UVRAG binding to the HOPS complex stimulates lysosomal fusion with autophagosome and endosome (Liang et al., 2008; Sun et al., 2010). The function of UVRAG to regulate the HOPS complex is antagonized by RUN domain Beclin 1-interacting and cysteine-rich containing protein (RUBICON) (Sun et al., 2010). RUBICON also suppresses the UVRAG function in stimulating the kinase activity of Vps34 that contributes to autophagosome maturation (Sun et al., 2011).

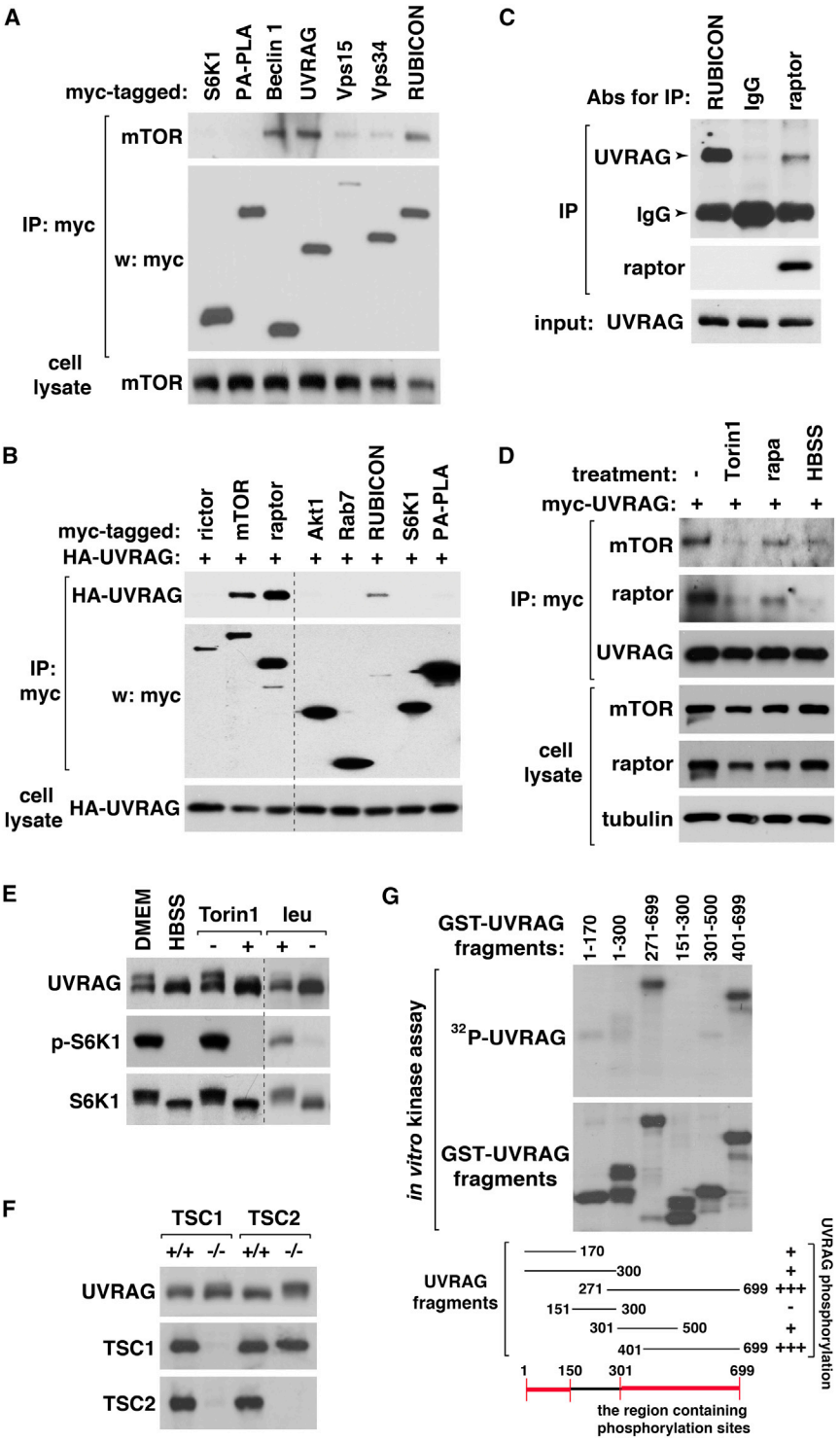

Figure 1. mTORC1 Binds to UVRAG and Induces UVRAG Phosphorylation

(A) mTOR binds to the UVRAG-containing Vps34 complex. Myc-tagged proteins were transiently expressed in HEK293T cells. Endogenous mTOR recovered with myc immunoprecipitate (IP) was analyzed by western blotting (WB).

(B) mTORC1 interacts with UVRAG. Myc-tagged proteins were transiently expressed with HA-UVRAG in HEK293T cells. UVRAG recovered with myc IP was analyzed by WB. An irrelevant lane in the middle was eliminated.

(C) Endogenous UVRAG recovered with RUBICON, raptor, and control IgG IP was analyzed by WB.

(D) The UVRAG-mTORC1 interaction is negatively regulated by Torin1 or HBSS, but not by rapamycin. HEK293T cells were transiently transduced to express myc-UVRAG and incubated in HBSS for 2 hr or treated with Torin1 or rapamycin for 1 hr. Endogenous mTOR or raptor isolated with myc-UVRAG was analyzed by WB.

(E) mTOR inhibition induces a mobility shift of UVRAG band on SDS-PAGE. HEK293T cells were incubated in DMEM or HBSS for 2 hr or treated with Torin1 for 1 hr. Cells were also incubated in leucine-deprived medium (–) and then supplemented with leucine (+).

(F) Deficiency of either TSC1 or TSC2 in MEFs induces a mobility shift of UVRAG.

(G) mTOR phosphorylates UVRAG in vitro. GST-tagged UVRAG fragments were prepared from bacteria and incubated with the active mTOR fragment in the presence of ^{32}P-ATP. Incorporation of ^{32}P into UVRAG fragments was analyzed by autoradiography. See also Figure S1.

In this study, we have identified that mTORC1 binds and phosphorylates UVRAG at Ser498 under nutrient-enriched conditions. We determined that the UVRAG phosphorylation has a positive effect on the interaction between UVRAG and RUBICON, whereas it has a negative effect on the kinase activity of Vps34 and the interaction between UVRAG and the HOPS complex. Preventing the UVRAG phosphorylation increased autophagosome maturation and lysosomal degradation of EGFR, reduced EGFR signaling, and suppressed cancer cell proliferation and tumor growth in vivo. These results demonstrate that mTORC1 has a broader range of functions in autophagy and endosomal pathways.

RESULTS

mTORC1 Interacts with UVRAG under Nutrient-Enriched Conditions

To understand the roles of mTOR in the autophagy pathway, we tested several autophagy proteins for their interaction with mTOR. We found that endogenous mTOR is coimmunoprecipitated with Beclin 1, UVRAG, and RUBICON (Figure 1A). mTOR also showed a relatively weak binding affinity toward Vps34 and Vps15. We were not able to detect any interaction of mTOR with its substrate S6K1 or a membrane-associated control protein PA-PLA, suggesting that the UVRAG-containing Vps34

complex might bind to mTOR relatively strongly compared to S6K1 binding to mTORC1 in the experimental condition. mTOR forms two distinct protein complexes, mTORC1 and mTORC2, for which raptor and rictor are the representative components, respectively (Kim et al., 2002; Sarbassov et al., 2004). UVRAG was coimmunoprecipitated with raptor (Figure 1B and Figure S1A available online), but barely with rictor. Consistently, rictor knockdown did not affect the interaction between mTOR and UVRAG (Figure S1B). We confirmed the interaction between raptor and UVRAG at endogenous levels (Figure 1C). We were not able to detect any interaction between UVRAG and RagB (Figure S1A), indicating that mTORC1 might bind to UVRAG independently of Rag GTPases that mediate amino acid-mTORC1 signaling on lysosomes (Kim et al., 2008a; Sancak et al., 2008).

To clarify whether the interaction is regulated by conditions that induce autophagy, we treated HEK293T cells with mTOR inhibitors, such as Torin1 or rapamycin, or incubated in nutrient-deprived Hank's balanced saline solution (HBSS). Torin1 or HBSS disrupted the interaction between mTORC1 and UVRAG (Figure 1D). Rapamycin moderately reduced the mTOR-UVRAG interaction while drastically reducing the raptor-UVRAG interaction. The drastic effect of rapamycin on the raptor-UVRAG interaction might be because rapamycin disturbs the mTOR-raptor interaction (Kim et al., 2002). mTOR required the full length of UVRAG for their stable association, whereas all the tested fragments except the C-terminal 501–699 fragment showed a weak binding affinity toward mTOR (Figure S1C). This indicates that the whole structural conformation of UVRAG might be important for the stable interaction. Alternatively, multiple sites of UVRAG scattered apart might be involved in the interaction with mTOR.

mTORC1 Induces UVRAG Phosphorylations

We observed that HBSS or Torin1, but not rapamycin, induced a downshift of UVRAG band on a low percent polyacrylamide gel (Figure 1E and Figure S1D). The downshift was also induced by deprivation of leucine, a potent stimulator of mTORC1 activity, in medium. Depletion of tuberous sclerosis complex 1 (TSC1) or TSC2, which negatively regulates mTORC1, in mouse embryonic fibroblasts (MEFs) or overexpression of Rheb, a positive regulator of mTORC1, in HEK293T cells caused an upshift of UVRAG band (Figure 1F and Figure S1E), confirming that mTORC1 is responsible for the mobility change. To determine whether the mobility shift is due to phosphorylation, we prepared several truncated fragments of UVRAG using a bacterial expression system (Figure S1F) and incubated the fragments with an active form of mTOR-containing residues 1,362–2,549 in the presence of ^{32}P-ATP. The active form of mTOR strongly induced phosphorylation of a fragment containing residues 271–699 and a fragment containing residues 401–699 (Figure 1G). mTOR also weakly phosphorylated fragments containing residues 1–170, 1–300, and 301–500, respectively. This result suggests that mTOR might phosphorylate multiple residues on UVRAG that are spread over the whole area of the protein, especially near the C terminus.

Identification of mTORC1-Dependent Phosphorylation Sites of UVRAG

To identify mTORC1-dependent phosphorylation sites of UVRAG, we expressed myc-UVRAG with HA-Rheb in HEK293T cells and isolated UVRAG by immunoprecipitation. As a control, myc-UVRAG was isolated from HEK293T cells that were without Rheb overexpression and treated with Torin1 for 1 hr. The immunoprecipitated UVRAG was resolved by SDS-PAGE, and the UVRAG band was isolated from the gel. After trypsinization, phosphorylated peptides were enriched and analyzed by mass spectrometry. A total seven phosphorylation sites (S493, S498, S508, S522, S549, S550, and S582) were identified with UVRAG from Rheb-expressing cells but not from Torin1-treated cells. The surrounding sequences of S498, S522, and S550 showed a feature of the mTORC1 target consensus motifs (Kang et al., 2013) (Figure 2A and Figure S2A). We were able to successfully generate polyclonal antibodies that recognize the phosphorylated state of UVRAG S498 (Figures S2B and S2C).

mTORC1 Induces UVRAG Ser498 Phosphorylation

Using the phospho-specific antibody we developed, we found that UVRAG S498 phosphorylation is highly increased by Rheb overexpression in HEK293T cells (Figure 2B). Treatment of MEFs with Torin1 almost completely suppressed the phosphorylation of UVRAG at S497, the mouse equivalent of human UVRAG S498 (Figure 2C). The phosphorylation was also completely suppressed by an Akt inhibitor or HBSS, the conditions that inhibit mTORC1 (Figure 2C). By contrast, rapamycin barely suppressed the phosphorylation even at high concentrations (Figure 2C and Figure S2D). This indicates that Ser498 might be a rapamycin-insensitive site, like a few other mTORC1 target sites (Kang et al., 2013). Torin1 suppressed the induction of S498 phosphorylation by Rheb or a constitutively active mutant of Akt in HEK293T cells (Figure 2D), confirming that the Ser498 phosphorylation depends on mTORC1. Consistently, we found that deficiency of AMPK, phosphatase and tensin homolog (PTEN), or TSC1, which are negative regulators of mTORC1, in MEFs largely increased the UVRAG phosphorylation, and the increase was suppressed by Torin1 (Figure 2E and Figure S2E). Deficiency of ULK1, which functions both upstream and downstream of mTORC1 (Dunlop et al., 2011; Jung et al., 2011), in MEFs only marginally increased the UVRAG phosphorylation (Figure S2F). The increase was still suppressed by HBSS, indicating that mTORC1 induces the UVRAG phosphorylation independently of ULK1.

mTORC1 Phosphorylates UVRAG Ser498

To determine whether mTOR phosphorylates UVRAG S498, we conducted in vitro kinase assays. The active mTOR 1,362–2,549 fragment induced a large increase of S498 phosphorylation of UVRAG purified from bacteria (Figure 3A). We also found that mTOR wild-type (WT), but not its kinase-dead (KD) mutant, showed the kinase activity toward the UVRAG phosphorylation in vitro (Figure 3B). Endogenous mTOR IP also largely increased the phosphorylation of UVRAG 271–699 fragment in vitro (Figure 3C). mTOR or raptor IP induced multiple forms of UVRAG harboring S498 phosphorylation, which showed different mobilities on SDS-PAGE (Figure 3D), implying there might be other mTOR-mediated phosphorylations. mTOR IP, relative to raptor IP, induced more of slowly migrating UVRAG forms, implying that mTOR IP might contain a molecular factor important for UVRAG phosphorylations at sites other than S498. When rictor IP was isolated from HeLa cells that express rictor highly, we were able

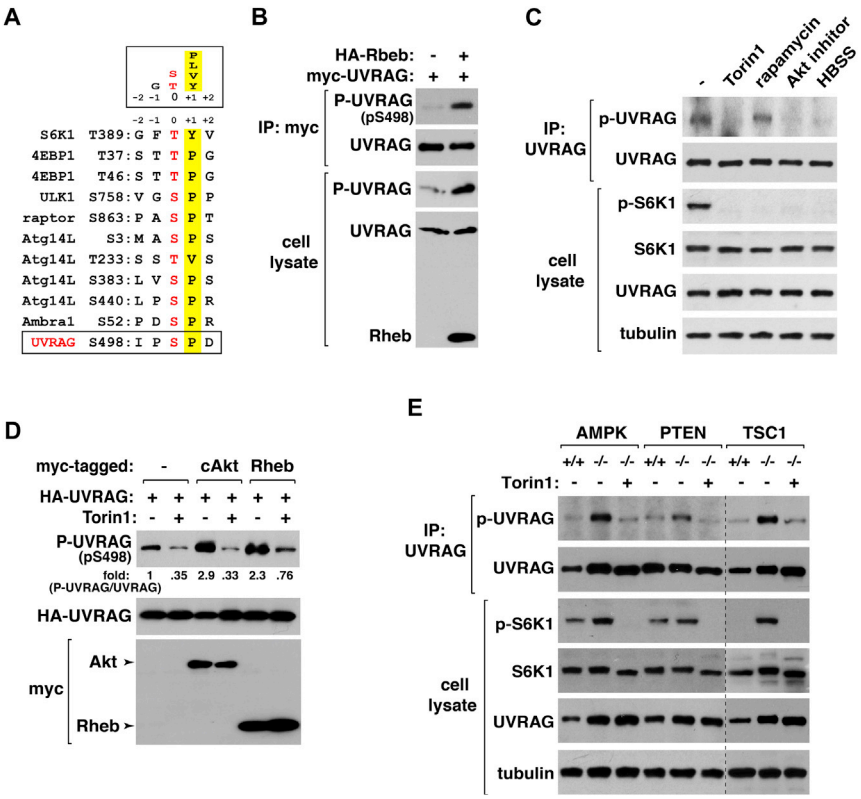

Figure 2. UVRAG Ser498 Is an mTORC1-Dependent Phosphorylation Site

(A) Alignment of the sequence surrounding UVRAG S498 with those of the known mTORC1 target sites.

(B) Rheb induces UVRAG S498 phosphorylation. HA-Rheb was expressed with myc-UVRAG in HEK293T cells. UVRAG S498 phosphorylation was analyzed by WB.

(C) UVRAG phosphorylation in MEFs is inhibited by Torin1, Akt inhibitor VIII, or HBSS, but not by rapamycin. Immunoprecipitated UVRAG was analyzed for the phosphorylation by WB.

(D) The S498 phosphorylation is enhanced by Akt and Rheb. HA-UVRAG was transiently expressed in HEK293T cells alone (–) or together with myc-tagged constitutively active Akt mutant (cAkt) or Rheb. Two days posttransfection, cells were treated with vehicle (–) or Torin1 (+) for 1 hr.

(E) Deficiency of AMPK, PTEN, or TSC1 in MEFs enhances UVRAG phosphorylation. MEFs were treated with vehicle (–) or Torin1 (+) for 1 hr. The UVRAG phosphorylation was analyzed by immunoprecipitation followed by WB. See also Figure S2.

to detect a marginal level of S498 phosphorylation but that was much less than that induced by raptor IP (Figure 3D). Knockdown of mTOR or raptor, but not rictor, in HEK293T cells drastically reduced S498 phosphorylation (Figure 3E). The in vitro phosphorylation of UVRAG by mTOR was not detected when S498 was replaced with alanine (S498A) or when Torin1 was added during the reaction (Figure 3F and Figure S3). Combined, these results demonstrate that mTORC1 phosphorylates UVRAG at S498.

UVRAG Ser498 Phosphorylation Positively Regulates the UVRAG-RUBICON Interaction

To understand the functional consequence of the UVRAG phosphorylation, we investigated whether the phosphorylation status could regulate the mTORC1-UVRAG interaction. The S498A mutation only marginally reduced the mTOR-UVRAG interaction, and the interaction was still disrupted by Torin1 (Figure S4A). This indicates that S498 phosphorylation might not be critical for the interaction but rather an event following their association under nutrient-rich conditions. Next, we examined whether S498A mutation has an effect on the UVRAG-RUBICON interaction. We expressed UVRAG WT, S498A, or S498D (a mutant with replacement of S498 with aspartate) in HEK293T cells where endogenous UVRAG was silenced by shRNA targeting UVRAG mRNA 3′ UTR (Figure S4B). S498A mutant interacted with RUBICON with a weaker affinity compared to WT (Figure 4A and Figure S4C). S498D mutant interacted with RUBICON more strongly than S498A mutant. However, S498D mutant could not fully recover the affinity of WT, indicating that aspartate is not a perfect mimic of phosphoserine at the residue. Combined, the results suggest that the dephosphorylation state of S498 might disturb the UVRAG-RUBICON interaction.

If S498 phosphorylation positively regulates the UVRAG-RUBICON interaction, we predicted that Torin1 might suppress the interaction. As predicted, Torin1 suppressed the UVRAG-RUBICON interaction (Figure 4B). Similarly, leucine deprivation in medium suppressed the UVRAG-RUBICON interaction (Figure 4C). Neither Torin1 nor leucine altered the interaction of UVRAG or Atg14L with Beclin 1 (Figures 4B and 4C and Figure S4D). The S498A mutation also drastically suppressed the RUBICON-Vps34 interaction (Figure 4D). These results suggest that UVRAG S498 phosphorylation might facilitate binding of RUBICON to the UVRAG-containing Vps34 complex. The suppressive effects of Torin1 on the interactions of RUBICON with UVRAG and Vps34 were still observed with S498A mutant (Figure 4D). This suggests that there might be other mTOR-dependent phosphorylations that regulate the interaction between RUBICON and the Vps34 complex.

UVRAG Ser498 Phosphorylation Negatively Regulates Vps34

Knowing that S498 phosphorylation positively regulates the RUBICON-Vps34 interaction, we wondered whether the phosphorylation might regulate the kinase activity of Vps34. We expressed RFP-tagged 2xFYVE in WT or mutant UVRAG-reconstituted cells and monitored the formation of RFP-FYVE puncta, which reflects the production of phosphatidylinositide-3-phosphate (PI3P) (Sun et al., 2011; Zhong et al., 2009). We observed a higher induction of RFP-FYVE puncta in S498A cells compared to WT cells (Figures 5A and 5B and Figures S5A–S5C). The FYVE puncta formation was also increased in S498D cells but to a

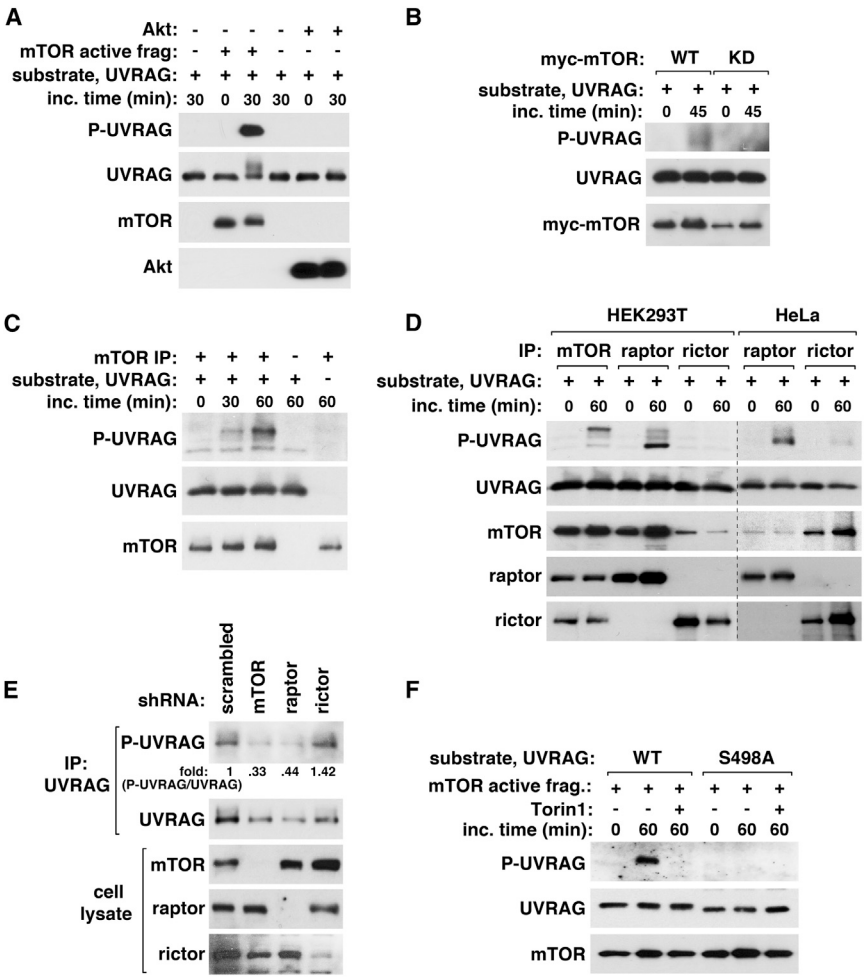

Figure 3. mTORC1 Phosphorylates UVRAG Ser498

(A) mTOR phosphorylates UVRAG S498 in vitro. The active form of mTOR was incubated with purified UVRAG in the presence of ATP. Akt1 was used as a negative control. UVRAG S498 phosphorylation was analyzed by WB.

(B) Myc-mTOR WT or its kinase-dead mutant (KD), transiently expressed in HEK293T cells, was isolated by immunoprecipitation using anti-myc antibody and incubated with UVRAG and ATP.

(C) Endogenous mTOR IP was isolated from HEK293T cells, and its kinase activity was analyzed using UVRAG 271–699 fragment as substrate.

(D) mTOR, raptor, or rictor IPs were obtained from HEK293T or HeLa cells, and the kinase reaction was analyzed as in (C).

(E) The phosphorylation state of S498 was analyzed for endogenous UVRAG isolated from shRNA-transduced HEK293T cells.

(F) Torin1 inhibits UVRAG S498 phosphorylation in vitro. The active fragment of mTOR was incubated with UVRAG WT or S498A purified from bacteria. The kinase reaction was performed in the presence or absence of Torin1. See also Figure S3.

significantly less extent compared to S498A cells. Although we could not observe any drastic change in the cellular distribution pattern of FYVE puncta between WT and mutant cells, FYVE-positive areas costained with the endosomal marker proteins, such as Rab5 and Rab7, were highly increased in S498A cells compared to WT cells (Figure S5D). This might be due to the higher accumulation of FYVE puncta in the mutant cells compared to WT cells.

To clarify whether the increase of PI3P in cells is due to an increase in the activity of Vps34, we analyzed the kinase activity of Vps34 in vitro. We isolated Vps34 from WT- and mutant-reconstituted HEK293T cells. The catalytic activity of Vps34 was analyzed by measuring the amount of a p40 phox domain-containing polypeptide that binds to PI3P following the procedure established previously (Farkas et al., 2011). Vps34 isolated from WT cells showed a higher production of PI3P compared to that from empty vector-transduced cells (Figures 5C and 5D and Figure S5E). This result is consistent with the positive effect of UVRAG on Vps34 as shown by other studies (Liang et al., 2006; Sun et al., 2011). PI3P production was dramatically increased with Vps34 isolated from S498A cells compared with that from WT cells (Figures 5C and 5D and Figure S5E). By contrast, Vps34 isolated from S498D cells showed an activity similar to that from WT cells. This suggests that S498 phosphorylation might suppress the UVRAG-mediated stimulation of the Vps34 kinase activity.

UVRAG Ser498 Phosphorylation Is Important for the Suppressive Effect of RUBICON on Vps34

Knowing that S498 phosphorylation has a negative effect on the Vps34 activity, we hypothesized that the phosphorylational effect might be through enhancing RUBICON binding to Vps34. Expression of RUBICON in WT-reconstituted HCT116 cells significantly reduced the number of FYVE puncta (Figures 5E and 5F and Figure S5F), a result consistent with the negative role of RUBICON on Vps34 (Sun et al., 2011). Such a suppressive effect of RUBICON was significantly attenuated in S498A cells without any drastic change in the puncta sizes (Figures 5E and 5F and Figures S5F and S5G). This result suggests that RUBICON might depend on S498 phosphorylation to suppress the Vps34 activity. We confirmed the result in vitro by observing that the kinase activity of Vps34 isolated from WT cells was drastically reduced by RUBICON, while such a drastic reduction was not observed with Vps34 isolated from S498A cells (Figures 5G–5I). Consistently, RUBICON knockdown largely increased the kinase activity in WT cells but the knockdown effect was reduced in S498A cells (Figures S5H–S5M). These results suggest that S498 phosphorylation might play an important role in RUBICON-mediated suppression of the Vps34 kinase activity.

UVRAG Ser498 Phosphorylation Negatively Regulates the UVRAG-HOPS Interaction and Rab7

RUBICON is known to prevent UVRAG from binding to the HOPS complex (Sun et al., 2010). Since S498A mutation suppressed

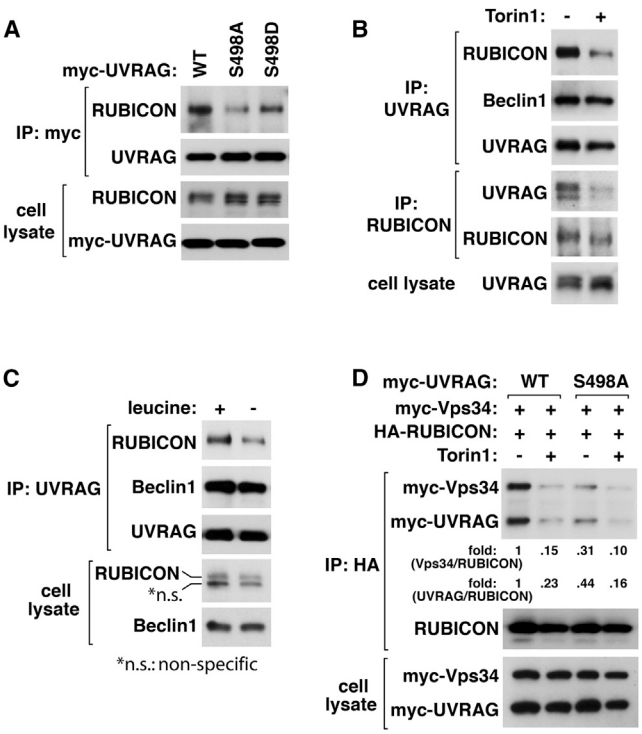

Figure 4. UVRAG Ser498 Phosphorylation Positively Regulates the UVRAG-RUBICON Interaction

(A) S498 phosphorylation positively regulates the UVRAG-RUBICON interaction. Myc-UVRAG WT or mutant was transiently expressed in UVRAG-silenced HEK293T cells. Endogenous RUBICON recovered with myc-UVRAG IP was analyzed by WB.

(B) Torin1 negatively regulates the UVRAG-RUBICON interaction. HEK293T cells were treated with Torin1 for 4 hr. The interaction between endogenous UVRAG and RUBICON was analyzed by immunoprecipitation and WB.

(C) Leucine deprivation reduces the UVRAG-RUBICON interaction. HEK293T cells were incubated in medium with (+) or without leucine (–) for 2 hr. Endogenous RUBICON recovered with UVRAG IP was analyzed by WB.

(D) S498A mutation has a negative effect on the interaction between RUBICON and the UVRAG-containing Vps34 complex. The indicated proteins were expressed in UVRAG-silenced HEK293T cells. Two days posttransfection, the cells were treated with Torin1 as in (B). See also Figure S4.

the UVRAG-RUBICON interaction, we predicted that the mutation might increase the UVRAG-HOPS interaction. As predicted, higher levels of Vps39 and Vps16 of the HOPS complex were coimmunoprecipitated with S498A mutant compared to WT (Figures 6A and 6B). Relative to S498A mutant, S498D mutant interacted with Vps39 and Vps16 with a weaker affinity. The positive effect of S498A mutation on the UVRAG-HOPS interaction was mimicked by Torin1 (Figure 6C). Consistently, the colocalization of UVRAG with HOPS was increased in S498A cells compared to WT cells (Figure S6A). The colocalization was further increased by Torin1 in both WT and S498A cells (Figure S6B), suggesting that other mTOR-dependent phosphorylations might also be involved in regulating the colocalization. RUBICON knockdown largely increased the binding of Vps16 with WT but only marginally with S498A mutant (Figure 6D and Figure S6C). Consistently, RUBICON overexpression drastically suppressed the interaction of Vps39 with WT but not with S498A mutant (Figure S6D). Combined, these results demonstrate that S498 phosphorylation is important for RUBICON-mediated suppression of the UVRAG-HOPS interaction.

UVRAG binding is known to enhance the HOPS activity as a guanine nucleotide exchange factor (GEF) toward Rab7, a small GTPase whose activation is important for autophagosome and endosome maturation (Liang et al., 2008; Sun et al., 2010). We assessed the active state of Rab7 by monitoring the recruitment of Rab7-interacting lysosomal protein (RILP) to Rab7. We found that the RILP-Rab7 interaction was largely increased in S498A cells compared to WT or S498D cells (Figure 6E). Consistently, a higher level of GTP-bound Rab7 was detected in S498A cells (Figures 6F and 6G and Figure S6E). We also asked whether S498 phosphorylation might regulate Rab7 via the Mon1-Ccz1 complex that was recently shown to act as a GEF toward Rab7 in yeast and plant cells (Cui et al., 2014; Nordmann et al., 2010). We found that UVRAG interacts with Ccz1 and Mon1B (Figures S6F and Figure S6G), but the interaction, unlike the UVRAG-HOPS interaction, was not altered by S498 mutations. Although the interaction implies a functional link between UVRAG and the Ccz1 complex, our result suggests that S498 phosphorylation probably has specific effects on HOPS rather than on the Ccz1 complex.

Prevention of UVRAG Ser498 Phosphorylation Facilitates Autophagosome Maturation

Knowing that S498 phosphorylation negatively regulates Vps34 and HOPS, we predicted that the phosphorylation might regulate autophagosome maturation. To address this possibility, we used the dual-tagged LC3 construct (mCherry-GFP-LC3) (Zhong et al., 2009). The formation of mCherry-LC3 puncta, which may reflect LC3 destined in autophagosomes or lysosomes, was significantly increased in S498A cells compared to WT cells (Figures 6H–6J and Figures S6H–S6J). Torin1 induced the formation of green and yellow LC3 puncta, which may reflect autophagosomes, to similar extents between WT and S498A cells, whereas Torin1 induced the formation of mCherry-LC3-only puncta to a greater extent in S498A cells compared to WT cells (Figures 6H–6K and Figures S6H–S6J). The higher induction of mCherry-LC3-only puncta in S498A cells compared to WT cells in response to Torin1 might be due to a residual amount of WT UVRAG that was not dephosphorylated at S498. Supporting this, Torin1 could not completely suppress S498 phosphorylation of recombinant WT UVRAG (Figure 2D), and Torin1 induced dephosphorylation of recombinant UVRAG S498 at a reduced rate relative to endogenous UVRAG (Figures S6K and S6L). Relevant to this, Torin1 more drastically impaired the interactions mediated by UVRAG for binding to RUBICON and mTOR when S498 was mutated to alanine (Figure 4D and Figure S4A). Thus, S498A mutation might somehow facilitate Torin1-induced changes in the protein interactions involved in UVRAG-mediated autophagosome maturation. It is possible that those additional phosphorylations of UVRAG that we identified (Figure S2A) might be involved in the Torin1 effects.

To further confirm that S498 phosphorylation negatively regulates UVRAG-mediated autophagosome maturation, we conducted an autophagy flux assay. Lysosomal inhibitors increased

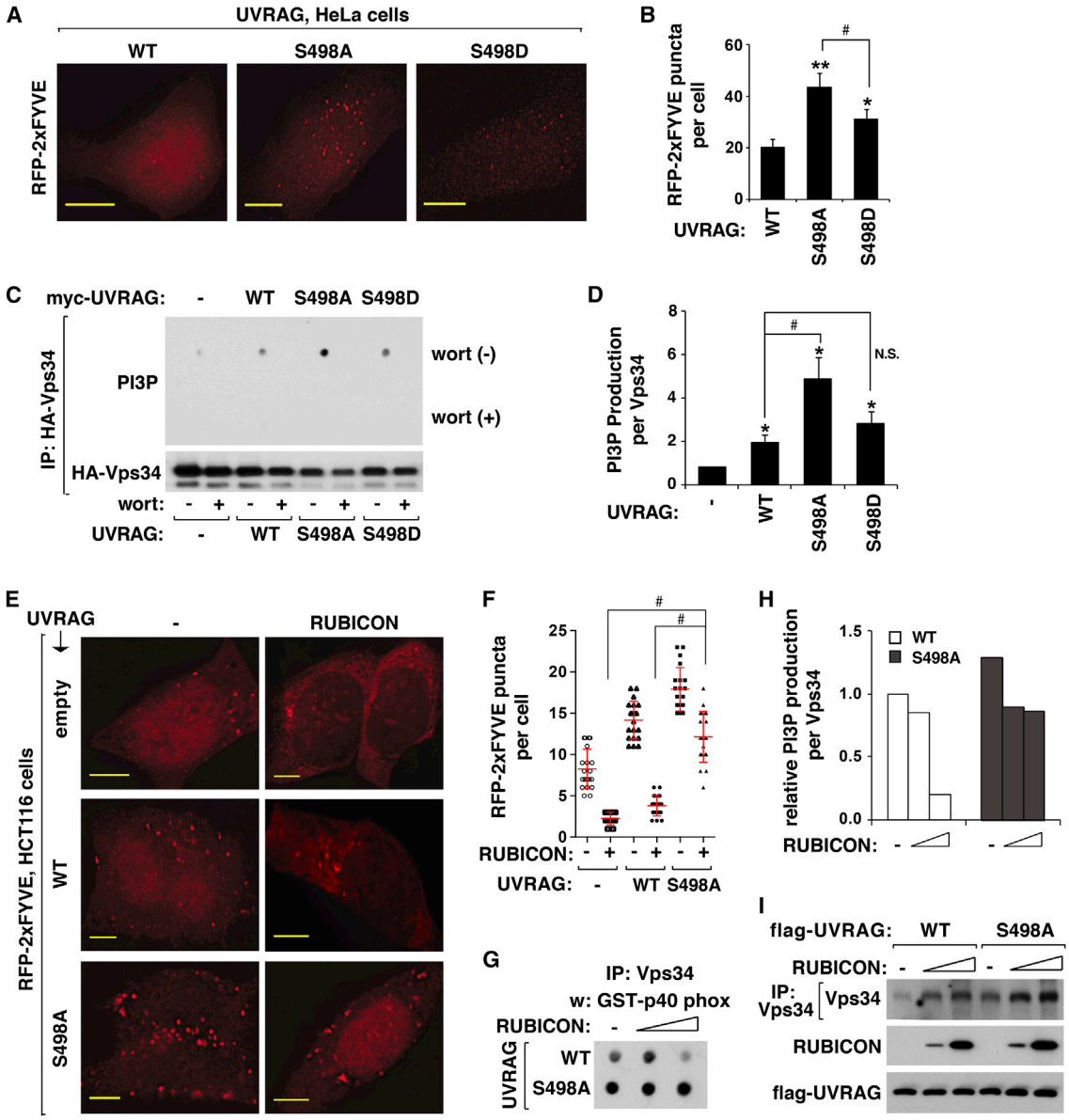

Figure 5. UVRAG Ser498 Phosphorylation Is Important for the Inhibitory Effect of RUBICON on Vps34

(A) Prevention of S498 phosphorylation increased the accumulation of PI3P in HeLa cells. RFP-tagged 2xFYVE, transiently expressed in WT or mutant UVRAG-reconstituted HeLa cells, was monitored by fluorescence microscope. Scale bar, 10 μm.

(B) Quantitative analysis of FYVE puncta formation. Results are represented as means ± SD (*$p < 0.05$ versus WT; **$p < 0.01$ versus WT; #$p < 0.01$; $n \geq 17$).

(C) Prevention of S498 phosphorylation increased the kinase activity of Vps34. HA-Vps34 was expressed alone or together with myc-UVRAG in UVRAG-depleted HEK293T cells. HA-Vps34 IP was obtained and incubated with phosphatidylinositol (PI) and ATP in the presence or absence of wortmannin (200 nM). The amount of PI3P was analyzed as described in Experimental Procedures.

(D) Quantitative analysis of Vps34 kinase activity. Results are represented as means ± SD (*$p < 0.01$ versus empty vector; #$p < 0.01$; NS, nonsignificant; $n = 3$).

(E) RUBICON depends on S498 phosphorylation to suppress UVRAG-induced production of PI3P. RFP-2xFYVE expressed alone (–) or together with RUBICON in empty vector- or UVRAG-transduced HCT116 cells was analyzed as described in (A). Scale bar, 5 μm.

(F) Quantitative analysis of PI3P puncta formation. The error bars represent means ± SD (#$p < 0.01$; $n = 20$).

(G and H) RUBICON depends on S498 phosphorylation to suppress UVRAG-mediated stimulation of Vps34 kinase activity. UVRAG-reconstituted HEK293T cells were transiently transfected with empty vector or RUBICON construct. The in vitro kinase assay was conducted as in (C). The graph is representative of two independent experiments.

(I) Vps34 recovered with Vps34 IP was analyzed by WB. See also Figure S5.

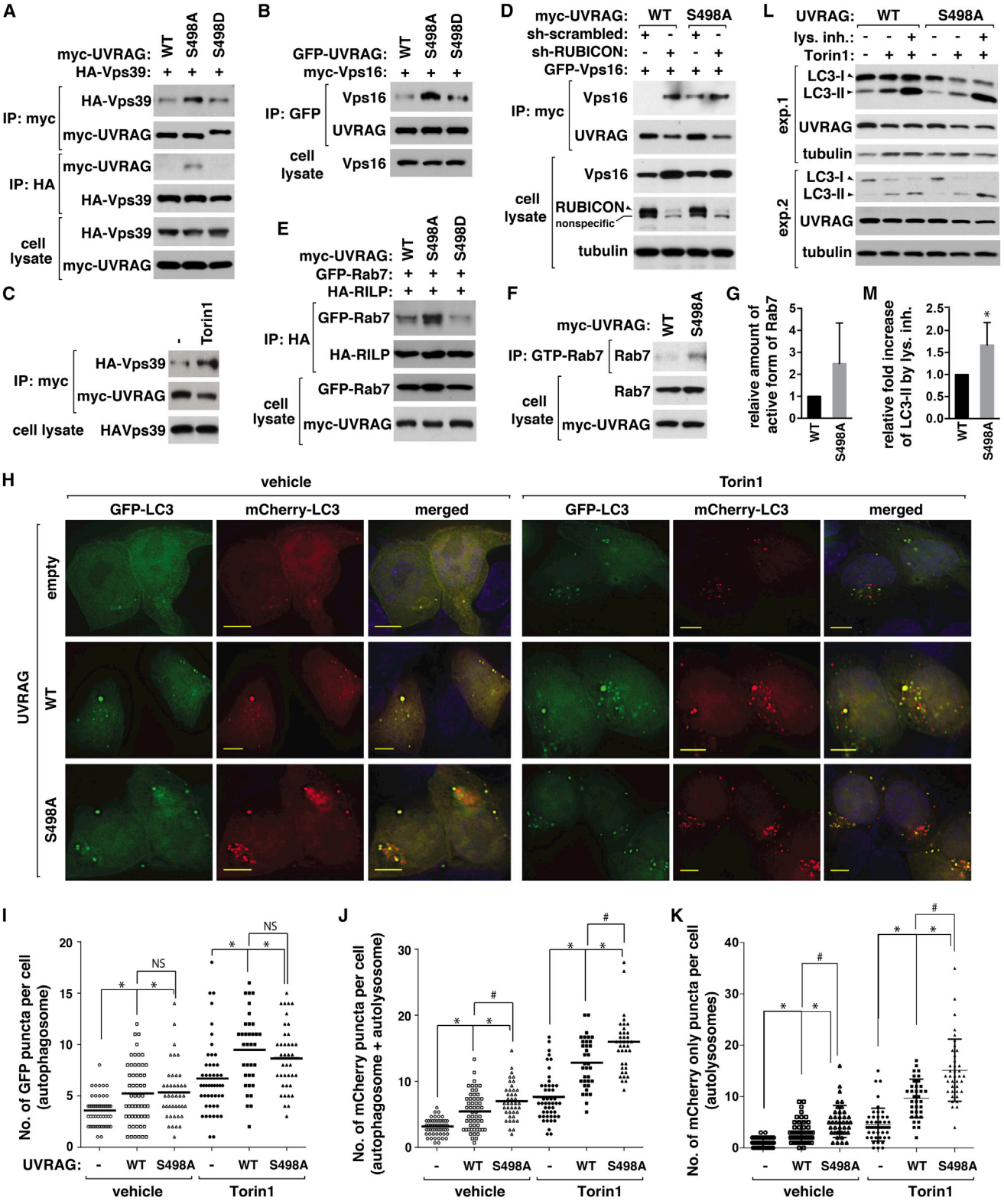

Figure 6. Prevention of UVRAG Ser498 Phosphorylation Facilitates Autophagosome Maturation

(A) S498 phosphorylation negatively regulates the UVRAG-Vps39 interaction. HA-Vps39 was transiently expressed in UVRAG-reconstituted HEK293T cells, and their interaction was analyzed by immunoprecipitation and WB.

(legend continued on next page)

the LC3-II level to a greater extent in S498A cells compared to WT cells either in nutrient-rich or Torin1-treated conditions (Figures 6L and 6M and Figures S6M and S6N). This result, in accordance with those from the analysis of LC3 puncta, suggests that autophagosomal maturation might be facilitated in mutant cells. The higher increase of LC3-II levels in mutant cells compared to WT cells in response to Torin1 could be interpreted as above where we interpreted the results of the higher accumulation of LC3 in autolysosomes in S498A mutant cells compared to WT cells. Consistent with no significant difference in autophagosome formation between WT and S498A cells (Figures 6H and 6I), S498A mutation did not have any significant effect on the formation of DFCP1 and WIPI2 puncta (Figures S6O–S6Q). Thus, the effects of S498A mutation are unlikely due to upregulation of early steps of autophagy.

Prevention of UVRAG Ser498 Phosphorylation Facilitates UVRAG-Mediated Endosome-Lysosomal Degradation of EGFR and Reduces EGFR Signaling

UVRAG positively regulates the endosome-lysosomal fusion through enhancing the HOPS activity (Liang et al., 2008; Sun et al., 2010). A consequence of the enhanced endosomal maturation is a facilitated degradation of EGFR in lysosomes (Liang et al., 2008). Since S498A mutation enhanced the UVRAG-HOPS interaction and the active state of Rab7, we predicted that S498A mutation might facilitate the endosome maturation and the lysosomal degradation of EGFR. Reconstitution of WT UVRAG in HEK293T cells enhanced EGFR degradation in response to EGF (Figures 7A and 7B). The degradation of EGFR was significantly increased in S498A cells compared to WT cells (Figures 7A and 7B and Figure S7A). The level of EGFR was lower in S498A cells compared to WT cells before EGF treatment, suggesting that the phosphorylation might also affect the basal endosome-lysosomal degradation of EGFR. The facilitated degradation of EGFR was accompanied by reduction in EGF-stimulated phosphorylations of ERK1/2 and AKT (Figure 7C and Figure S7B). Similarly as S498A mutation, Torin1 increased EGF-stimulated degradation of EGFR and reduced the level of EGFR even before cells were treated with EGF (Figures S7C–S7F), suggesting that mTORC1 inhibition might enhance the basal endosome maturation. The reduction of EGFR by Torin1 was marginal in S498A cells compared to WT cells (Figures S7G and S7H), supporting our interpretation that S498 dephosphorylation might contribute to Torin1-stimulated EGFR degradation. The further reduction of EGFR by Torin1 in mutant cells implies other mTORC1-dependent mechanisms involved in EGFR downregulation. Despite other potential mechanisms, the decreased basal level of EGFR in S498A cells might be due to facilitated endosome-lysosomal fusion.

Prevention of UVRAG Ser498 Phosphorylation Suppresses Cancer Cell Proliferation and Tumor Growth

Knowing that S498A mutation facilitates EGFR degradation and reduces EGFR signaling, we asked whether the mutation would suppress cancer cell proliferation. WT UVRAG expression in HCT116 colon cancer cells reduced cell proliferation (Figures 7D and 7E). This result is consistent with the known tumor suppressor function of UVRAG (Liang et al., 2006). The suppressive effect of UVRAG became greater when S498A mutant was expressed instead of WT. The proliferation rate of S498A cells was about 5 and 2.9 times lower than empty-vector-transduced cells and WT cells, respectively (Figure 7D). Expression of S498A mutant in HCT116 cells dramatically suppressed colony formation (about 70% and 54% reduction in the number of colonies compared to empty-vector-transduced cells and WT cells, respectively) in a clonogenic assay (Figure 7F and Figure S7I). Furthermore, the colonies of S498A cells were much smaller in size compared with those of empty-vector-transduced cells and WT cells (Figure S7I).

To address whether the suppressive effect of S498A mutation on cell proliferation could be recapitulated in vivo, we conducted a xenograft experiment. We subcutaneously injected WT or S498A HCT116 cells into the flank of immunity-deficient Balb/c mice and analyzed the sizes of tumors. The sizes of tumors formed by S498A cells were significantly smaller than those formed by WT cells (Figure 7G and Figure S7J). The average volume of S498A cell-derived tumor was about 47.3% of that of WT cell-derived tumor (Figure 7G). Tumor weight, relative to the whole body weight, was significantly reduced by S498A mutation (Figure 7H and Figures S7K and S7L). This result suggests

(B) S498 phosphorylation negatively regulates the UVRAG-Vps16 interaction. Myc-Vps16 recovered with GFP-UVRAG IP from HEK293T cells was analyzed by WB.

(C) Torin1 enhances the UVRAG-Vps39 interaction. HEK293T cells transiently expressing HA-Vps39 and myc-UVRAG were treated with Torin1 or vehicle (−). HA-Vps39 recovered with myc-UVRAG IP was analyzed by WB.

(D) RUBICON knockdown enhances the UVRAG-Vps16 interaction in WT cells but not in S498A cells. GFP-Vps16 recovered with myc-UVRAG IP from shRNA-transduced HEK293T cells was analyzed by WB.

(E) S498 phosphorylation negatively regulates the Rab7-RILP interaction. Rab7 recovered with HA-RILP from WT or mutant UVRAG-reconstituted HEK293T cells was analyzed by WB.

(F) S498 phosphorylation negatively regulates Rab7. The GTP-bound Rab7 was detected as described in Experimental Procedures.

(G) Quantitative analysis of (F). Data are represented as means ± SD (n = 2).

(H) S498 phosphorylation negatively regulates autophagosome maturation. mCherry-GFP-LC3 was expressed in empty vector- or UVRAG-transduced HCT116 cells. Cells were treated with Torin1 or vehicle. LC3 was monitored by fluorescence microscope. Scale bar, 5 μm.

(I and J) Quantitative analysis of GFP puncta (I) and mCherry puncta (J) (*$p < 0.01$ versus empty vector; #$p < 0.01$; NS; $n \geq 35$). Mean value is shown as a horizontal bar.

(K) Quantitative analysis of mCherry-only puncta (*$p < 0.01$ versus empty vector; #$p < 0.01$; $n \geq 35$). Mean and SD are shown as horizontal bars.

(L) S498A mutation enhances autophagy flux. HEK293T cells reconstituted with UVRAG constructs were treated with Torin1 in the presence or absence of Bafilomycin A1 (exp1) or E-64/Pepstatin A (exp2).

(M) Quantitative analysis of the fold increase of LC3-II level induced by the lysosomal inhibitors in Torin1-treated cells. Data are represented as means ± SD (*$p < 0.05$; $n = 5$). See also Figure S6.

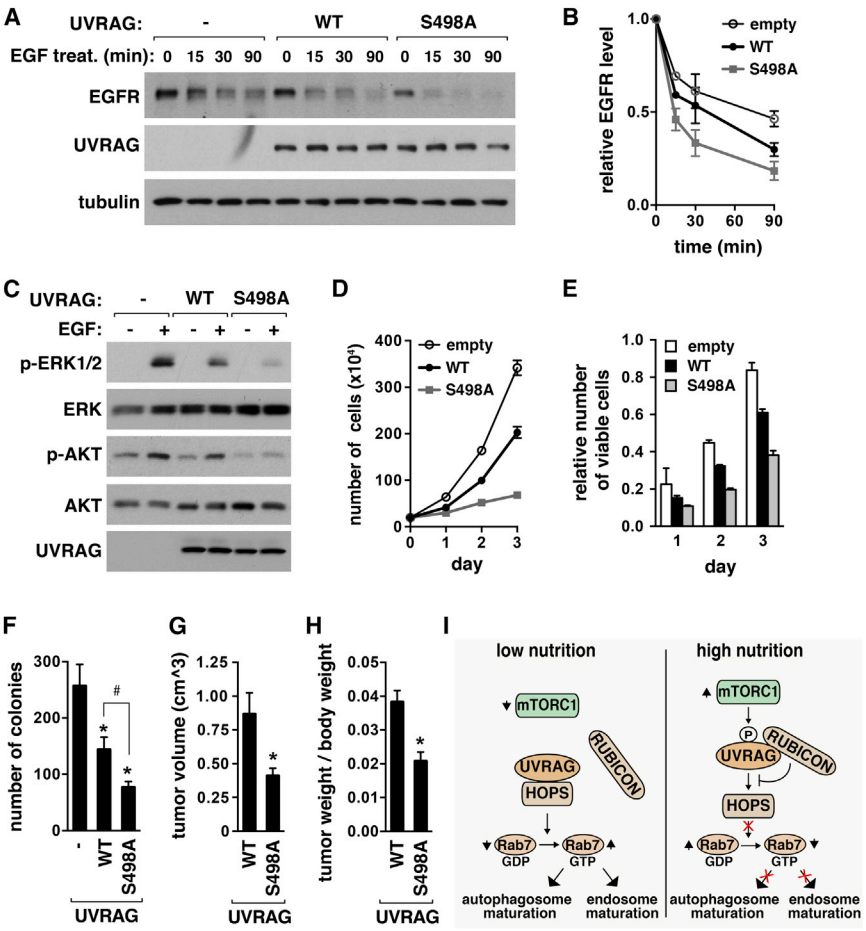

Figure 7. Prevention of UVRAG Ser498 Phosphorylation Enhances EGFR Degradation, Reduces EGFR Signaling, and Suppresses Cancer Cell Proliferation and Tumor Growth

(A) S498A mutation enhances EGFR degradation. HEK293T cells stably transduced by empty vector (–) or UVRAG construct were starved of serum overnight and treated with EGF. EGFR level in cell lysate was analyzed by WB.

(B) EGFR level was presented relative to that at time zero. Data are represented as means ± SD (n = 4).

(C) S498A mutation suppresses EGFR signaling. HEK293T cells, transduced as in (A), were serum starved overnight and treated with EGF. pERK1/2 (Thr202/Tyr204) or pAKT (Thr308) were analyzed by WB.

(D) S498A mutation suppresses cancer cell proliferation. HCT116 cells stably transduced by empty vector (–) or UVRAG construct were cultured in DMEM. The number of viable cells was counted by hemacytometer. Data are represented as means ± SD (n = 3).

(E) Viable cells were assayed using MTT. Data are represented as means ± SD (n = 3).

(F) S498A mutation significantly inhibits anchorage-independent growth of cancer cells. HCT116 cells prepared as in (D) were used. Data are shown as mean ± SD (*$p < 0.01$ versus empty vector; #$p < 0.01$; n = 3).

(G) S498A mutation significantly inhibits tumor growth in mice. Values represent means ± SD (*$p = 0.012$; n = 5).

(H) Tumor weight relative to the whole body weight was analyzed. Values represent mean ± SD (*$p < 0.01$; n = 5).

(I) Regulatory function of UVRAG S498 phosphorylation in autophagosome and endosome maturation. See also Figure S7.

that Ser498 phosphorylation status might be important for the tumor suppressive function of UVRAG.

DISCUSSION

In this study, we have determined that mTORC1 phosphorylates UVRAG at Ser498 and thereby regulates late stages of autophagy and endosomal maturation. The mTORC1-mediated UVRAG phosphorylation may depend on the ability of mTORC1 to interact with UVRAG. With this regard, it is noteworthy that mTOR is localized to late endosomes and lysosomes in nutrient-enriched conditions (Sancak et al., 2008). The nutrient-regulated localization of mTOR fits our model that mTOR inhibits autophagosomal and endosomal maturation by binding and phosphorylating UVRAG in association with the HOPS complex, which also localizes to late endosomes and lysosomes (Itakura et al., 2008; Pols et al., 2013). According to our results, the phosphorylation positively regulates the UVRAG-RUBICON interaction and suppresses the UVRAG functions in autophagosome and endosome maturation (Figure 7I). Although our results support the role of S498 phosphorylation in regulating the lysosomal fusion machinery, we do not rule out a possibility that the effect of S498A mutation on autophagy could be secondary to a change in the lysosome function due to disturbance of the endocytic pathways.

Hyperactivation of mTORC1 is frequently found in many cancers (Laplante and Sabatini, 2012). The contribution of mTORC1 to cancer is mainly attributed to increases in protein synthesis. Our study suggests that mTORC1 might contribute to cancer by phosphorylating UVRAG and thereby protecting EGFR from the lysosomal degradation. Many genetic mutations that suppress the lysosomal degradation of EGFR have been implicated in oncogenesis and tumor progression (Grandal and Madshus, 2008; Kuan et al., 2001). Thus, mTORC1 inhibition might contribute to suppression of tumor growth at least in part through facilitating the lysosomal degradation of EGFR, although the extent of the contribution might vary depending on cancer types.

Several recent studies have raised a question about the function of UVRAG in the autophagosome-lysosome fusion (Farré et al., 2010; Jiang et al., 2014; Takáts et al., 2014). UVRAG participates in early autophagy events, such as recruitment of Bif-1 (N-BAR domain-containing protein) to the Beclin 1-Vps34 complex, which promotes the membrane curvature necessary for autophagosome formation (Takahashi et al., 2007). UVRAG

also plays a role in coordinating the Golgi-ER retrograde and autophagic membrane trafficking in early stages of the autophagy processes (He et al., 2013). However, since UVRAG S498A mutation did not alter the formation of omegasome and phagophore, S498 phosphorylation is unlikely involved in the early processes of autophagy. UVRAG also has a nonautophagic function, such as the maintenance of the chromosomal stability (Zhao et al., 2012). It remains to be explored whether mTORC1 regulates the nonautophagic function of UVRAG via the phosphorylation.

Our finding defines the mTORC1-UVRAG pathway as an important regulatory axis through which cells coordinate autophagy and the endosome-lysosomal degradation pathway. The coordinate regulation might be important to maintain normal cellular physiology under changing environments of growth factors and nutrients. Some cancer cells might disturb the coordination by reducing the level of UVRAG and suppressing the endosomal degradation pathway as implicated by frequent alterations of the *UVRAG* gene locus in breast, colorectal, and gastric cancers (Kim et al., 2008b; Knævelsrud et al., 2010). Alternatively, cancer cells might maintain a favorable cell growth condition through the mTORC1-mediated UVRAG phosphorylation. Elucidating further mTORC1-regulated interactions and phosphorylations in the membrane processes may shed light on the molecular network that coordinates autophagy and the endosomal pathway and provide insight into how the network is disturbed in cancers and other diseases.

EXPERIMENTAL PROCEDURES

Identification of UVRAG Phosphorylation Sites

Myc-UVRAG was expressed with HA-Rheb in HEK293T cells and immunoprecipitated using anti-myc antibody. As a control, myc-UVRAG was expressed alone and treated with Torin1 for 1 hr. Immunoprecipitated UVRAG was resolved by SDS-PAGE. UVRAG band was subjected to trypsinization followed by enrichment of phosphorylated peptides. Eluted fractions of tryptic digests of peptides were analyzed by mass spectrometry. A detailed procedure for mass spectrometry is described in the Supplemental Experimental Procedures.

Plasmids

Human UVRAG cDNA (IMAGE: 6186851) and its DNA fragments were cloned into pRK5 vector by PCR amplification. pLKO.1 vector was used to knock down UVRAG or RUBICON. CSII-EF-MCS was used to stably express UVRAG. The cDNAs for UVRAG, Vps16, Rab5a, Rab7, DFCP1, and WIPI2 were cloned into pEGFP-N3 and/or pmCherry-C1 (Clontech Lab) for cell-imaging experiments. The mCherry-GFP-LC3 and the RFP-2xFYVE constructs were kindly provided by T. Johansen and N. Mizushima, respectively. All other constructs have been previously described (Jung et al., 2009; Vander Haar et al., 2007).

Cell Image Analysis

Autophagosome maturation, omegasome and phagophore formation, and the protein colocalization were analyzed by monitoring the fluorescence protein-tagged constructs using a Deltavision fluorescence microscope. A detailed procedure is described in the Supplemental Experimental Procedures.

In Vitro Assay of UVRAG Phosphorylation by mTOR

The mTOR kinase activity toward UVRAG phosphorylation was analyzed in vitro using immunoprecipitated mTOR or a purified form of mTOR containing residues 1,362–2,549 (Millipore). As substrates, UVRAG fragments and its full-length WT or S498A were purified using the ArcticExpress system (Stratagene). The kinase reaction was performed as described previously (Jung et al., 2009).

In Vitro Assay of Vps34 Kinase Activity

The Vps34 kinase activity was assayed as described previously (Farkas et al., 2011). Briefly, Vps34 complex obtained by immunoprecipitation was incubated with PI and ATP. The amount of PI3P produced from the reaction was analyzed by use of GST-tagged p40 phox domain-containing polypeptide that binds to PI3P. A detailed procedure is described in the Supplemental Experimental Procedures.

Autophagy Flux Assay

HEK293T cells stably expressing UVRAG WT or S498A were treated with Torin1 (250 nM) for 4 hr in the presence or absence of Bafilomycin A1 (100 nM) or E-64/Pepstatin (10 μg/ml). The amounts of LC3-I and LC3-II in cell lysate were analyzed by WB.

Analysis of EGFR Degradation and EGFR Signaling

Endocytic degradation of EGFR was assayed as described previously (Liang et al., 2008; Sun et al., 2010). Briefly, cells starved of serum overnight were treated with EGF (100 ng/ml) for the indicated periods of time and lysed in 0.3% Chaps-containing lysis buffer. To analyze the activity of EGFR signaling, we stimulated serum-starved HCT116 or HEK293T cells with EGF for 10 min at 20 ng/ml or 10 ng/ml, respectively. The phosphorylation states of ERK1/2 and AKT were analyzed by WB.

Xenograft Analysis of Tumor Formation

WT and S498A HCT116 stable cells were injected subcutaneously into the flank of athymic nude male mice. Three weeks after the injection, tumors were removed and weighed. Tumor volume was evaluated by measurement of the length and width of the tumor mass in millimeters using electronic vernier calipers. A detailed procedure is described in the Supplemental Experimental Procedures.

Other Experimental Procedures

Other experimental procedures, including materials, cell culture, transfection, mutagenesis, immunoprecipitation, western blotting, lentiviral preparation, stable cell generation, preparation of recombinant proteins, cell proliferation assay, analysis of Rab7 active state, colony formation assay, and statistical analysis, are described in the Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and seven figures and can be found with this article online at http://dx.doi.org/10.1016/j.molcel.2014.11.013.

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