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BASIC RESEARCH PAPER

TPT1 (tumor protein, translationally-controlled 1) negatively regulates autophagy through the BECN1 interactome and an MTORC1-mediated pathway

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ABSTRACT

TPT1/TCTP (tumor protein, translationally-controlled 1) is highly expressed in tumor cells, known to participate in various cellular activities including protein synthesis, growth and cell survival. In addition, TPT1 was identified as a direct target of the tumor suppressor TP53/p53 although little is known about the mechanism underlying the anti-survival function of TPT1. Here, we describe a role of TPT1 in the regulation of the MTORC1 pathway through modulating the molecular machinery of macroautophagy/autophagy. TPT1 inhibition induced cellular autophagy via the MTORC1 and AMPK pathways, which are inhibited and activated, respectively, during treatment with the MTOR inhibitor rapamycin. We also found that the depletion of TPT1 potentiated rapamycin-induced autophagy by synergizing with MTORC1 inhibition. We further demonstrated that TPT1 knockdown altered the BECN1 interactome, a representative MTOR-independent pathway, to stimulate autophagosome formation, via downregulating BCL2 expression through activating MAPK8/JNK1, and thereby enhancing BECN1-phosphatidylinositol 3-kinase (PtdIns3K)-UVRAG complex formation. Furthermore, reduced TPT1 promoted autophagic flux by modulating not only early steps of autophagy but also autophagosome maturation. Consistent with in vitro findings, in vivo organ analysis using *Tpt1* heterozygote knockout mice showed that autophagy is enhanced because of haploinsufficient TPT1 expression. Overall, our study demonstrated the novel role of TPT1 as a negative regulator of autophagy that may have potential use in manipulating various diseases associated with autophagic dysfunction.

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Introduction

TPT1/TCTP (tumor protein, translationally-controlled 1) is a highly conserved multifunctional protein that controls various cellular processes, including cell growth, proliferation, and metabolism.[1-3] TPT1 is overexpressed in multiple human cancer types including breast cancer, liver cancer and prostate cancer.[4-6] Recently TPT1 was discovered as a direct target of tumor suppressor TP53/p53 and has been linked to controlling cell survival.[7]

Autophagy, a lysosome-dependent protein degradation pathway in mammalian cells is essential for cell survival, growth and homeostasis, and its abnormalities lead to pathologies including neurodegenerative diseases and cancer.[8,9] It has been reported that autophagy inhibits tumorigenesis in certain types of cancer, but some cancer cells are addicted to activated autophagy to ameliorate deficiencies in metabolism.[10-12]

It is well established that MTORC1 is the center of a signaling network that integrates a wide range of environmental signals and regulates an array of important cell growth processes. The best-characterized role of MTORC1 is in growth signaling and the regulation of protein synthesis. Growth factor signaling can directly regulate autophagy through MTORC1.[13] MTORC1 inhibits autophagy as a nutrient sensor by competing with AMPK in modulating the ULK1/Atg1 complex. A sustained metabolic starvation deactivates the MTORC pathway to stimulate autophagosome formation and facilitate the breakdown of more components which is necessary for cell survival.[14] It was also known that the cellular response to growth stimulation typically involves translational upregulation of TPT1 levels, suggesting that TPT1 is central for cell growth,[15] although until now the specific mechanisms involved are not yet characterized. Thus, in this report we investigated the role of TPT1 in autophagy through the regulation of the MTORC1 pathway. In addition, we examined the potential effect of TPT1 on the function of BECN1/Beclin-1, the mammalian ortholog of yeast Vps30/Atg6, which plays a central role in every major step in the autophagic pathway

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by altering its binding partners/interactome, which promotes autophagosome formation.[16] The complex of BECN1 with PIK3C3/VPS34, the catalytic subunit of the class III phosphatidylinositol 3-kinase (PtdIns3K), positively regulates the PtdIns3K to generate phosphatidylinositol-3-phosphate (PtdIns3P), which is essential for both vesicle nucleation and autophagosome formation.[16] UVRAG (UV radiation resistance associated gene) augments the BECN1-PIK3C3 interaction and PtdIns3K activity through its binding to BECN1, and thereby further promotes autophagosome formation.[17] In addition, UVRAG has been also reported to regulate autophagosome maturation by binding with the class C vacuolar protein sorting complex (C-Vps).[18] The autophagic activity of the BECN1-containing PtdIns3K complex, however, is inhibited by the anti-apoptotic BCL2 protein, which binds to BECN1 and disrupts the interaction between BECN1 and PIK3C3.[19]

Given the critical role of MTORC1 and the BECN1 interactome in autophagy, we hypothesized that TPT1 might play a key role in inhibiting autophagy. In this study, we proposed that TPT1 acts as a new negative regulator of the autophagy process by modulating MTORC1-AMPK and the BECN1 interactome, crucial players of the autophagic pathways.

Results

TPT1 acts as a negative regulator of basal autophagy

To investigate the involvement of TPT1 in autophagy, we transiently depleted TPT1 using shRNA transfection. Knocking down TPT1 in HeLa cells stably expressing GFP-LC3 (HeLa GFP-LC3) enhanced the number of GFP-LC3 puncta, a hallmark of autophagy induction (Fig. 1A).[20] Consistently, immunoblotting data showed that the lipid-bound GFP-LC3-II levels were increased after TPT1 silencing (Fig. 1B). SQSTM1/p62 is a well-known selective autophagic substrate and reduction in SQSTM1 indicates productive autophagic flux.[21,22] We found that SQSTM1 was reduced by TPT1 knockdown (Fig. 1B). To confirm the role of TPT1 in autophagy, we generated TPT1 stably silenced HeLa GFP-LC3 cells. As shown in Fig. 1C, GFP-LC3 puncta were significantly increased in TPT1-depleted cells. To rule out the possibility of nutrient consumption affecting autophagy levels during incubation, we harvested cells at various time points after renewing the culture media and assessed autophagy. As shown in Fig. 1D, reduction in TPT1 consistently induced autophagy at all time points. Of note, we observed that stable knockdown of TPT1 significantly enhanced the expression level of GFP-LC3, thereby maintaining high basal autophagy rates.[23] We further confirmed the role of TPT1 in autophagy regulation using mouse embryonic fibroblasts (MEFs). Since *tpt1*$^{-/-}$ MEFs are not available due to severe apoptosis and growth retardation,[24] we therefore used MEFs from *Tpt1* heterozygote knockout mice embryos (*Tpt1*$^{+/-}$). Endogenous LC3 puncta increased in *Tpt1*$^{+/-}$ MEFs compared with *Tpt1*$^{+/+}$ MEFs (Fig. 1E). We also confirmed an increase in LC3 conversion and SQSTM1 degradation in *Tpt1*$^{+/-}$ MEFs compared with *Tpt1*$^{+/+}$ (Fig. 1F). Taken together, these data suggest that TPT1 negatively regulates the basal autophagy process.

Silencing TPT1 enhances the on-rate of autophagy

Autophagy is a dynamic process and the complete process is also called the autophagic flux, which is a combination of 3 sequential steps: formation of autophagosomes, autolysosome generation, and the degradation phase.[25] Thus, the induction of GFP-LC3 puncta after knocking down TPT1 could be due to enhanced autophagosome formation (on-rate) or/and reduced autophagic degradation (off-rate). To further investigate the detailed mechanism of how TPT1 regulates autophagy, we used late-step autophagy inhibitors in combination with TPT1 silencing and examined their effect on autophagic flux. Knockdown of TPT1 further augmented the formation of GFP-LC3 puncta and GFP-LC3-II in the presence of bafilomycin A_1, a vacuolar-type H^+-translocating ATPase inhibitor that raises lysosomal pH (Fig. 2A and B). Also, TPT1 depletion-induced degradation of SQSTM1 was blocked by bafilomycin A_1 treatment (Fig. 2B). These results collectively indicate that a productive autophagic flux was induced by TPT1 knockdown. Another lysosomal inhibitor, chloroquine (CQ), also showed similar results further confirming that silencing TPT1 increases the on-rate of autophagy (Fig. 2C and B).

Reduction of TPT1 stimulates autophagosome maturation

During autophagy, the autophagosome formation step is subsequently followed by autolysosome generation, a maturation step defined by the fusion of autophagosomes with lysosomes. In attempts to further elucidate the role of TPT1 on autophagic flux, we examined the effect of TPT1 knockdown on autolysosome formation, using the monomeric red fluorescent protein (mRFP)-GFP tandem fluorescent-tagged LC3 method.[26] As autolysosomes have low pH, the pH-sensitive GFP fluorescence is easily destabilized, whereas mRFP is relatively more stable. Consequently, only mRFP signals (red puncta) can be observed in autolysosomes. To perform this experiment, we generated HeLa cells in which TPT1 was stably knocked down, and transiently transfected them with mRFP-GFP-LC3. Silencing TPT1 exhibited an increase in both autophagosome and autolysosome levels, suggesting that TPT1 downregulation promotes not only autophagosome formation but also autophagosome maturation (Fig. 3A and B). Moreover, we confirmed the effects of TPT1 knockdown on autophagosome maturation by observing the colocalization efficiency of an autophagic marker, RFP-LC3, with a lysosomal marker, GFP-LAMP1, in the presence or absence of bafilomycin A_1, which inhibits fusion between autophagosomes and lysosomes.[27] Knockdown of TPT1, but not bafilomycin A_1 treatment, induced colocalization of RFP-LC3 and GFP-LAMP1 indicating that depletion of TPT1 promotes autophagosome interaction with lysosomes (Fig. 3C). Taken together, these findings indicate that reduction in TPT1 expression stimulates overall autophagic flux by promoting both autophagosome formation and maturation.

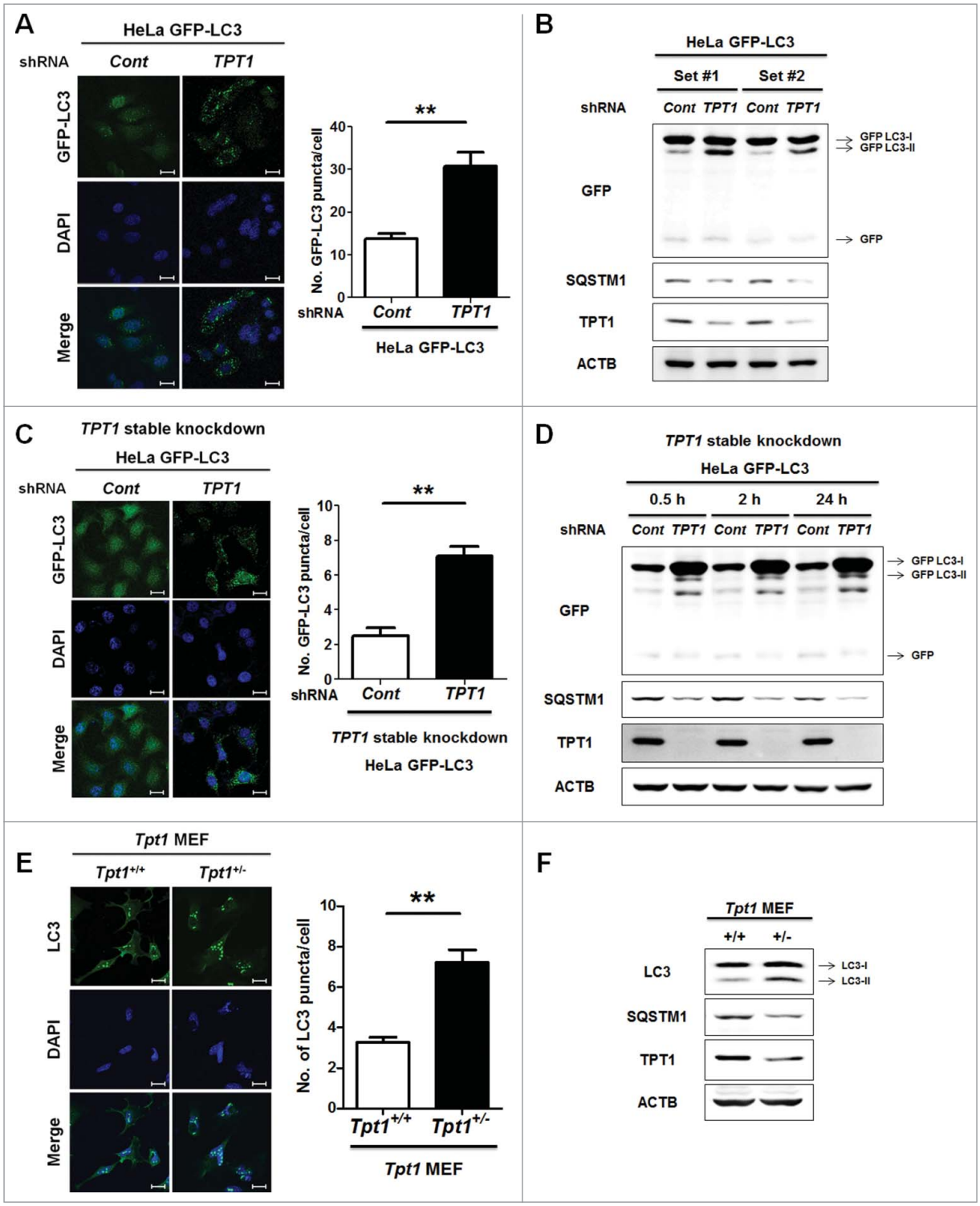

Figure 1. TPT1 negatively regulates basal autophagy. (A) GFP-LC3 puncta were analyzed in HeLa GFP-LC3 cells, transiently transfected with *TPT1* shRNA or *control* shRNA. Representative images were taken at x 600 magnification. Cells were stained with DAPI for the nucleus (blue). Scale bars: 20 μm. The number of GFP-LC3 dots per cell in each case was quantified. Data are presented as means ± S. E. M. (n = 3). P** < 0.0.1 (B) The lysates from either *control* shRNA or *TPT1* shRNA transiently transfected HeLa GFP-LC3 cells were immunblotted with the indicated antibodies. ACTB served as a loading control. (C) GFP-LC3 puncta were analyzed in *control* shRNA and *TPT1* shRNA stably transfected HeLa GFP-LC3 cells. The number of GFP-LC3 dots per cell in each case was quantified. Scale bars: 20 μm. Data are presented as means ± S. E. M. (n = 3). P** < 0.01. (D) Cell lysates from shRNA stably transduced HeLa GFP-LC3 cells were harvested at the indicated times after renewing the cell culture media and then subjected to immunoblotting analysis. ACTB served as a loading control. (E) $Tpt1^{+/+}$ and $Tpt1^{+/-}$ MEFs were stained with an antibody to endogenous LC3 (green). Scale bars: 20 μm. The number of LC3 puncta per cell in each condition was quantified. Data are presented as means ± S. E. M. (n = 3). P** < 0.01. (F) Cell lysates from $Tpt1^{+/+}$ and $Tpt1^{+/-}$ MEFs were immunoblotted. ACTB served as a loading control.

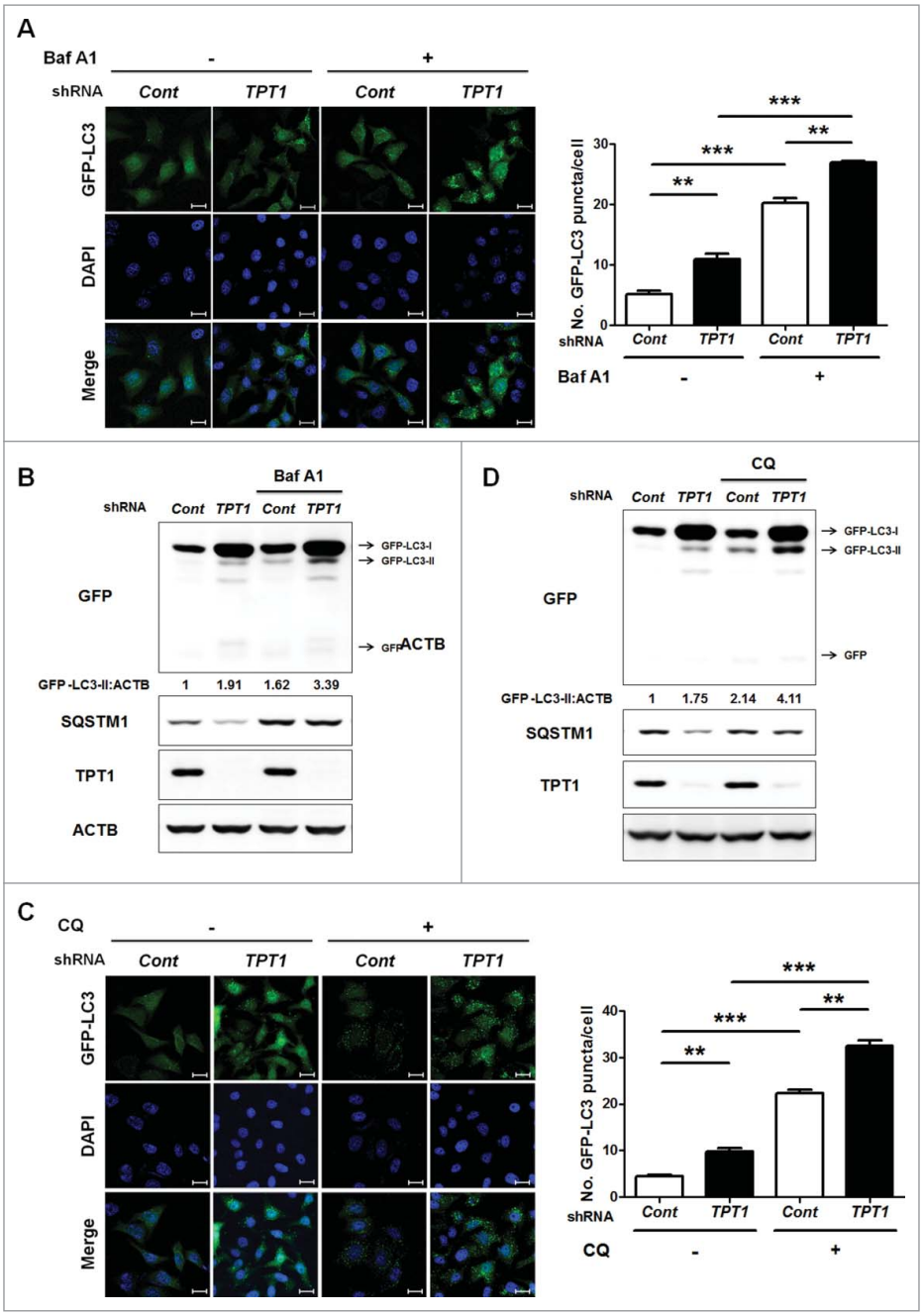

Figure 2. Reduction in TPT1 enhances the on-rate of autophagy. (A and B) HeLa GFP-LC3 cells, stably transduced with shRNA for *control* (sh*Cont*) or *TPT1* (sh*TPT1*) were cultured in the presence or absence of 100 nM of bafilomycin A_1 (Baf A1) for 8 h. (A) GFP-LC3 puncta were analyzed. Representative images were taken at x 600 magnification. The number of GFP-LC3 dots per cell in each condition was quantified. Scale bars: 20 μm. Data are presented as means ± S. E. M. (n = 3). $P^{**} < 0.01$, $P^{***} < 0.001$. (B) Cell lysates were immunoblotted with the indicated antibodies. ACTB served as a loading control. The experiments were repeated at least 6 times. The levels of GFP-LC3-II relative to ACTB were quantified by densitometry analysis. (C and D) Stable HeLa GFP-LC3-sh*Cont* and sh*TPT1* cells were cultured in the presence or absence of 50 μM of chloroquine (CQ) for 8 h. (C) GFP-LC3 puncta were analyzed. Representative images were taken at x 600 magnification. Scale bars: 20 μm. The number of GFP-LC3 dots per cell in each condition was quantified. Data are presented as means ± S. E. M. (n = 3). $P^{**} < 0.01$, $P^{***} < 0.001$. (D) Cell lysates were immunoblotted with the indicated antibodies. ACTB served as a loading control. The experiments were repeated at least 6 times. The levels of GFP-LC3-II relative to ACTB were quantified by densitometry analysis.

Downregulation of TPT1 alters the BECN1 interactome to promote autophagy

As suppressing TPT1 showed significant induction of autophagy, we investigated the molecular mechanism responsible. The anti-apoptotic protein BCL2 binds to BECN1 and inhibits autophagy by disrupting the interaction between BECN1 and the class III PtdIns3K.[19] Because TPT1 was previously reported to function as an anti-apoptotic protein, we hypothesized that modulating TPT1 expression might affect BCL2 expression and influence the autophagic flux. Hence, we evaluated BECN1 and BCL2 expression levels after transient knockdown of TPT1. As shown in Fig. 4A, we found that the knocking down TPT1 reduced BCL2 expression but not BECN1 expression. Similar results were also observed in TPT1 stably silenced cells (Fig. 4B). To directly evaluate the role of TPT1 in regulating the BECN1 interactome complex, we immunoprecipitated sh*TPT1* stable cells with a BCL2

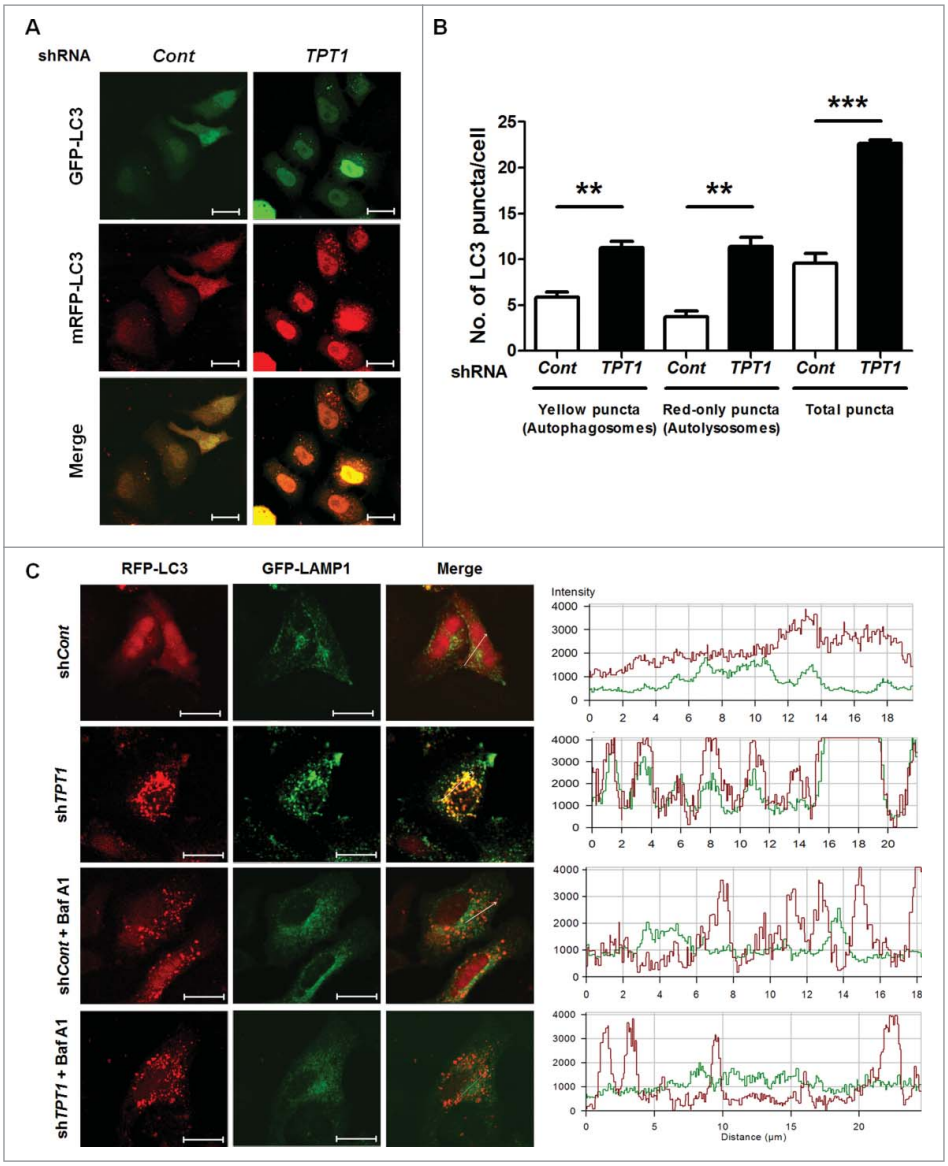

Figure 3. Reduction of TPT1 stimulates autophagosome maturation. (A and B) HeLa cells stably transduced with either *control* shRNA or *TPT1* shRNA were then transfected with mRFP-GFP-LC3 for 24 h. (A) Representative images were taken at x 800 magnification. Scale bars: 20 μm. (B) The number of yellow puncta and the number of mRFP-LC3-positive puncta (red) in the merged images were counted and the total number of puncta per cell was calculated. Data are presented as means ± S. E. M. (n = 3). $P^{**} < 0.01$, $P^{***} < 0.001$. (C) HeLa cells stably transduced with either *control* shRNA or *TPT1* shRNA were cotransfected with RFP-LC3 and GFP-LAMP1 for 24 h, in the presence or absence of 100 nM of Baf A1 for 8 h. The colocalization of LC3 and LAMP1 was analyzed. Representative fluorescence images are shown together with the profiles of colocalization. Scale bars: 20 μm.

antibody. Interaction between BECN1 and BCL2 was reduced in TPT1 knockdown cells (Fig. 4C).

Several studies have demonstrated that MAPK8/JNK1 phosphorylates BCL2 under starvation conditions and leads to BCL2 degradation by the proteasome.[28,29] We examined whether reduction in TPT1 caused activation of the MAPK8 pathway and found that both phosphorylated MAPK8 as well as total MAPK8 levels were increased in TPT1 knockdown cells (Fig. 4C). We also confirmed the effects of TPT1 on BCL2-MAPK8 signaling both in MEFs (Fig. S1A) and in vivo (Fig. S1B). Since TPT1 knockdown resulted in the downregulation of BCL2 expression, we examined whether BECN1 binds more strongly with other binding partners including PIK3C3. We found that TPT1 knockdown increased BECN1 and PIK3C3 interaction, implying the possibility of enhanced PtdIns3K activity, which is involved in initiating vesicle nucleation. Of note, interaction between BECN1 and UVRAG, another core component of the BECN1 interactome, also significantly increased in TPT1 knockdown cells (Fig. 4D). A BECN1-PIK3C3-UVRAG interaction has been reported to play roles in both autophagosome biosynthesis[17] and maturation. Taken together, these results suggest that knockdown of TPT1 alters the BECN1 interactome to drive autophagy by suppressing the BECN1 and BCL2 interaction.

Knockdown of TPT1 potentiates rapamycin-induced autophagy

To further examine the underlying mechanism of TPT1-modulated autophagy, we performed experiments under 2 positive conditions for autophagy induction; starvation and rapamycin treatment. For starvation, cells were incubated in Hank's

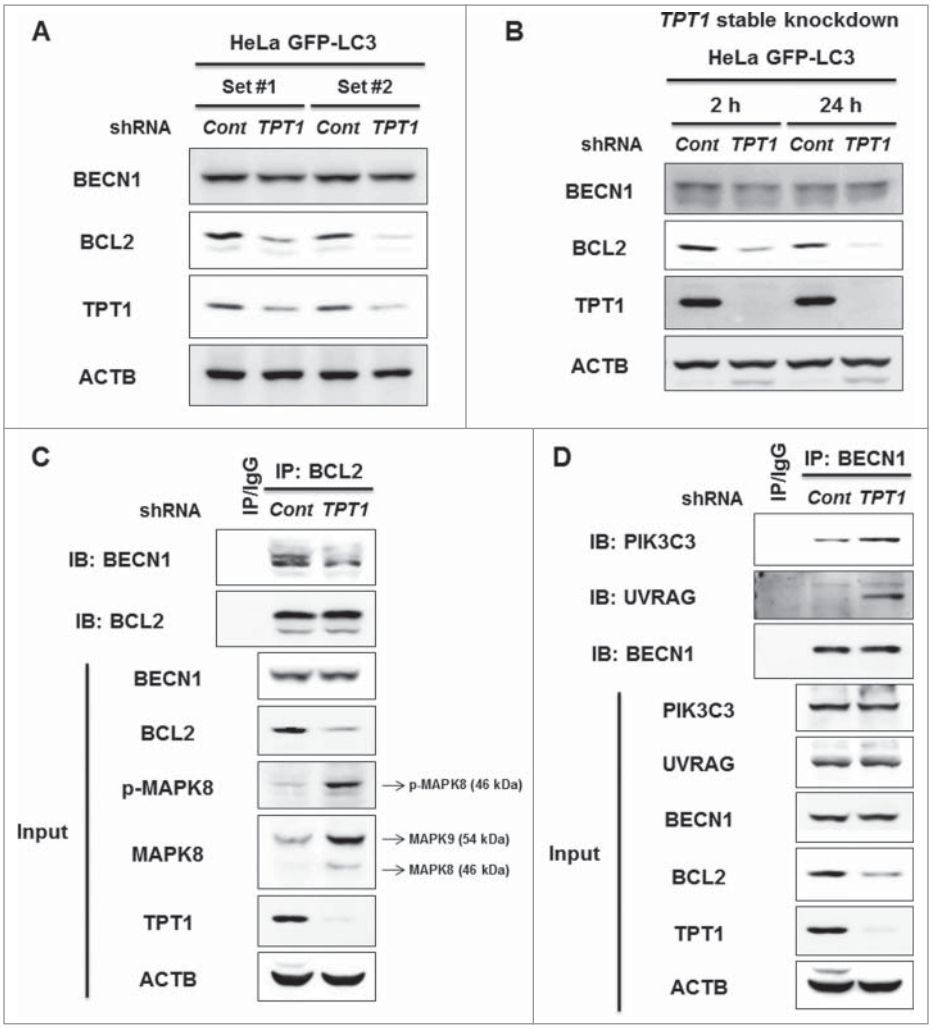

Figure 4. Downregulation of TPT1 alters the BECN1 interactome to promote autophagy. (A) HeLa GFP-LC3 cells were transiently transfected with shRNA for *control* and *TPT1*. After 24 h, cell lysates were immunoblotted with the indicated antibodies. ACTB served as a loading control. (B) HeLa GFP-LC3 cells, stably transduced with shRNA for *control* (sh*Cont*) or *TPT1* (sh*TPT1*), were harvested at the indicated times after changing the cell culture medium and then immunoblotted. ACTB served as a loading control. (C and D) Stable HeLa GFP-LC3-sh*Cont* and sh*TPT1* cells were harvested 2 h after changing the cell culture medium and then immunoprecipitated with BCL2 antibodies (C) and BECN1 antibodies (D) and then blotted with the indicated antibodies. ACTB served as a loading control.

balanced salt solution (HBSS) for 1 h. We observed that TPT1 knockdown enhanced GFP-LC3 puncta formation in non-starved conditions consistent with previous results. By contrast, upon induction of autophagy in response to the nutrient-starved condition, TPT1 knockdown caused only a slight increase in GFP-LC3 puncta (Fig. S2A) and GFP-LC3-II levels (Fig. S2B) compared with control cells. Also in MEFs, haploinsufficient TPT1 did not further enhance LC3 puncta (Fig. S2C) or increase the endogenous LC3-II level under starved conditions (Fig. S2D). We found that starved conditions diminished the expression of TPT1 as much as shRNA knockdown. Due to the effect of starvation on TPT1 level, it seems that knockdown of TPT1 was infeasible to potentiate starvation-induced autophagy. Previous studies, meanwhile, have suggested the involvement of TPT1 in activating the MTORC1 pathway,[30,31] and MTORC1 inhibits autophagy.[32] Rapamycin induces autophagy by acting as a potent MTORC1 inhibitor.[33] We therefore examined the effect of TPT1 knockdown on autophagy after treatment with rapamycin. As shown in Fig. 5A, TPT1 knockdown further increased GFP-LC3 puncta formation compared with the rapamycin-only treated group. Immunoblotting also showed that the knockdown of TPT1 potentiated rapamycin-induced conversion of GFP-LC3 and SQSTM1 degradation (Fig. 5B). We next confirmed these findings in *Tpt1* heterozygote MEFs. Silencing TPT1 further augmented the number of rapamycin-induced LC3 puncta (Fig. 5C) and the endogenous LC3-II level (Fig. 5D). Taken together, these results suggest that knockdown of TPT1 potentiates rapamycin-induced autophagy.

Downregulation of TPT1 modulates the MTORC1 and AMPK pathways

Hence, we hypothesized that in addition to TPT1s role in regulating the BECN1 interactome through MAPK8-BCL2, TPT1 knockdown might also induce autophagy by inhibiting the MTORC1 pathway. We analyzed the phosphorylation status of RPS6KB/p70S6K1, EIF4EBP1 and ULK1, which are downstream signaling molecules of MTORC1. Transiently silencing TPT1 reduced phosphorylation of RPS6KB and EIF4EBP1 (Fig. 6A). Phosphorylation of ULK1/Atg1 on the Ser757residue, which inhibits autophagy initiation, is also a direct target of MTORC1.[14]

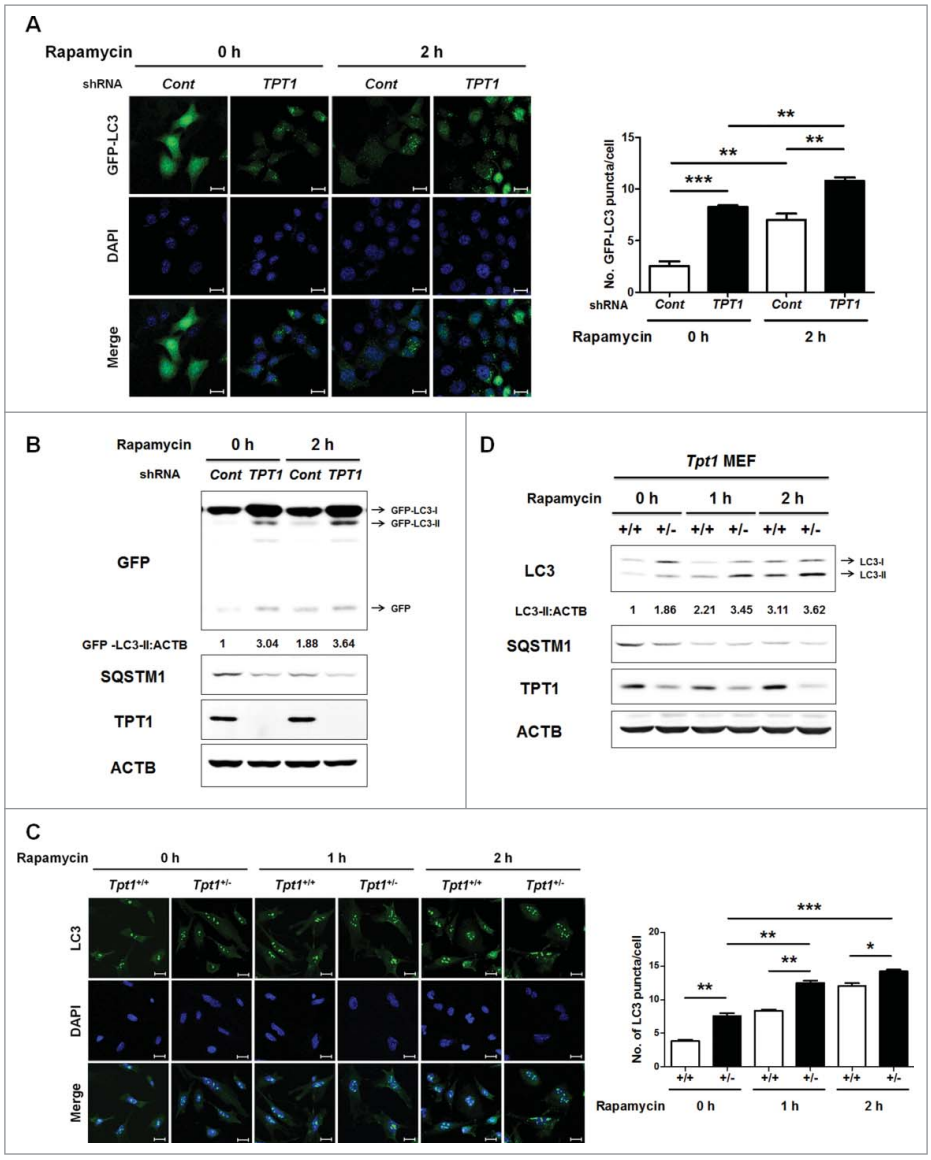

Figure 5. Knockdown of TPT1 potentiates rapamycin-induced autophagy. HeLa GFP-LC3 cells, stably transduced with shRNA for *control* (sh*Cont*) or *TPT1* (sh*TPT1*) were treated either with DMSO or 200 nM rapamycin for 2 h. (A) Analysis of GFP-LC3 puncta. Representative images were taken at x 600 magnification. Scale bars: 20 μm. The number of GFP-LC3 dots per cell in each condition was quantified. Data are presented as means ± S. E. M. (n = 3). $P^{**} < 0.01$, $P^{***} < 0.001$. (B) Immunoblotting of cell lysates with the indicated antibodies. The experiments were repeated at least 6 times. ACTB served as a loading control. The levels of GFP-LC3-II relative to ACTB were quantified by densitometry analysis. (C and D) *Tpt1*$^{+/+}$ and *Tpt1*$^{+/-}$ MEFs were treated with 200 nM rapamycin for the indicated times. (C) LC3 puncta were immunostained and observed by confocal microscopy. Representative images were taken at x 600 magnification. Scale bars: 20 μm. The number of LC3 dots per cell in each case was quantified. Data are presented as means ± S. E. M. (n = 3). $P^{*} < 0.05$, $P^{**} < 0.01$, $P^{***} < 0.001$. (D) Cell lysates from *Tpt1*$^{+/+}$ or *Tpt1*$^{+/-}$ MEFs were immunoblotted with the indicated antibodies. The experiments were repeated twice. ACTB served as a loading control. The levels of LC3-II relative to ACTB were quantified by densitometer analysis.

We consistently observed dephosphorylation of ULK1 Ser757 by TPT1 knockdown. Interestingly, we found that knockdown of TPT1 also activated the AMPK pathway (Fig. 6A). Similarly, stably knocking down TPT1 showed inhibition of MTORC1 signaling accompanied by an activation of AMPK and ACACA/ACC, a substrate of AMPK, at all time points (Fig. 6B).

Rapamycin induces autophagy through acting as a potent MTORC1 inhibitor.[33] Therefore, we next examined the effect of TPT1 downregulation on MTORC1 and AMPK signaling in the presence of rapamycin (Fig. 6C). Rapamycin is an allosteric inhibitor of MTOR, which partially suppresses part of MTORC1s function;[34] the EIF4EBP1-EIF4E effector pathway is reported to be partially resistant to rapamycin.[34-36] We found that rapamycin treatment resulted in the incomplete dephosphorylation of EIF4EBP1 and RPS6KB (Fig. 6C). However, knockdown of TPT1 resulted in further dephosphorylation of EIF4EBP1 and RPS6KB, thereby enhancing the rapamycin-induced inhibition of MTORC1 signaling. Intriguingly, silencing TPT1 resulted in reduction of total MTOR expression (Fig. 6D). Also, knockdown of TPT1 synergized with rapamycin in dephosphorylating MTOR (Fig. 6D). TPT1-modulated activation and total expression of MTOR were also confirmed in vivo in *Tpt1* heterozygote knockout mice livers (Fig. 6E). Though the mechanism should be further studied, TPT1 did not regulate

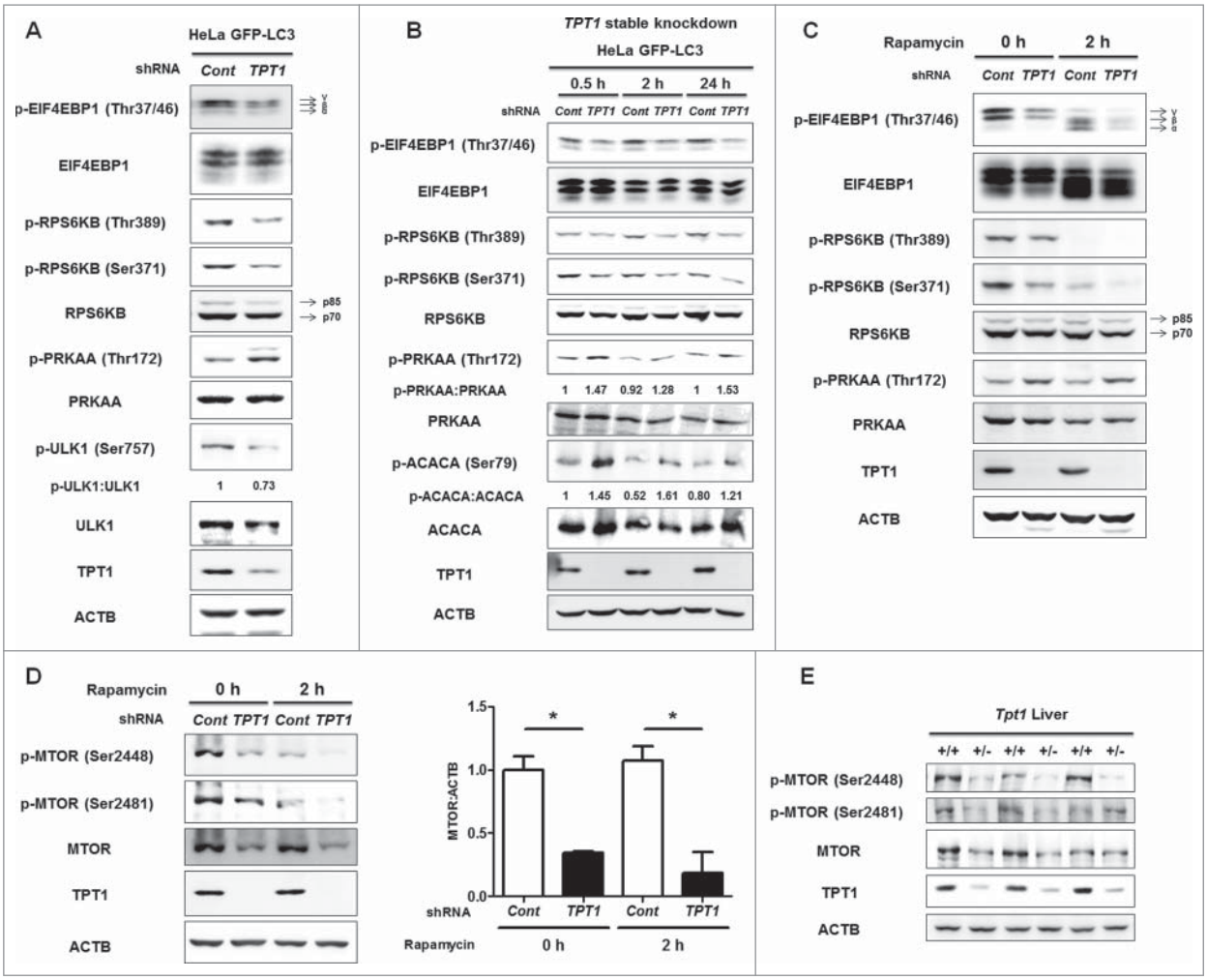

Figure 6. Downregulation of TPT1 modulates the MTORC1 and AMPK pathways. (A) HeLa GFP-LC3 cells were transiently transfected with shRNA for control or *TPT1*. After 24 h, cell lysates were immunoblotted with the indicated antibodies. (B) HeLa GFP-LC3 cells, stably transduced with shRNA for *control* (sh*Cont*) or *TPT1* (sh*TPT1*) were harvested at the indicated times after changing cell culture media and then immunoblotted. The levels of p-PRKAA/AMPKα and p-ACACA/ACC relative to each total forms were quantified by densitometry analysis. (C and D) Stable HeLa GFP-LC3-sh*Cont* and sh*TPT1* cells were treated with 200 nM rapamycin for 2 h and harvested for immunoblotting. Treatment with DMSO was used as a control. ACTB was used as a loading control. Quantification of total MTOR derived from western blotting is depicted in (D). Data are presented as means $\pm$ S. E. M. (n = 3). $P^* < 0.05$. (E) Livers were dissected from 3 representative sets of wild ($Tpt1^{+/+}$) and *Tpt1* heterozygote knockout mice ($Tpt1^{+/-}$). Autophagy-related molecules were immunoprobed with the indicated antibodies. ACTB served as a loading control.

Mtor expression at least at the transcriptional level (Fig. S3). More importantly, while rapamycin did not affect AMPK, downregulation of TPT1 led to an increase in AMPKphosphorylation (Fig. 6C). These results show that TPT1 regulates the MTORC1 and AMPK pathways. Our findings suggest that TPT1 exerts its inhibitory function on autophagy through controlling multiple pathways including MAPK8-BCL2-BECN1, MTORC1, and AMPK. However, rapamycin treatment did not affect MAPK8-BCL2 (Fig. S4), suggesting that TPT1-mediated inhibition of autophagy works at least in part through a rapamycin-independent mechanism. Therefore, these results suggested that TPT1 knockdown can enhance rapamycin-induced autophagy through both MTOR-dependent and -independent pathways.

Haploinsufficient TPT1 expression in vivo shows enhanced autophagy

We further attempted to investigate whether TPT1 plays a role in regulating autophagy in vivo. We examined autophagy markers in the livers and kidneys of *Tpt1* heterozygote mice, as the homeostasis of these organs are tightly regulated by autophagy.[37,38] The livers of $Tpt1^{+/-}$ mice displayed higher levels of LC3-I to -II conversions compared with that of $Tpt1^{+/+}$ mice (Fig. 7A and C). In addition, SQSTM1 levels were reduced in the livers of $Tpt1^{+/-}$ mice, demonstrating that haploinsufficient expression of TPT1 can promote autophagic flux (Fig. 7A and C). We also observed suppression of MTORC1 signaling and activation of AMPK in $Tpt1^{+/-}$ mice, in accordance with our in vitro data (Fig. 7A). Similar results were also observed in $Tpt1^{+/-}$ mouse kidneys (Fig. 7B). Although the conversion of LC3 showed a tendency to increase albeit not statistically significant (p-value = 0.1984) in kidney, SQSTM1 and TPT1 were significantly declined in $Tpt1^{+/-}$ mice (Fig. 7D). These results propose that the underlying mechanisms of TPT1-modulated LC3-I to -II conversion could be slightly varied by organs, and further study should be performed to figure out these phenomena. We also investigated the effect of TPT1 on in vivo autophagic flux by comparing the livers of $Tpt1^{+/+}$ and $Tpt1^{+/-}$

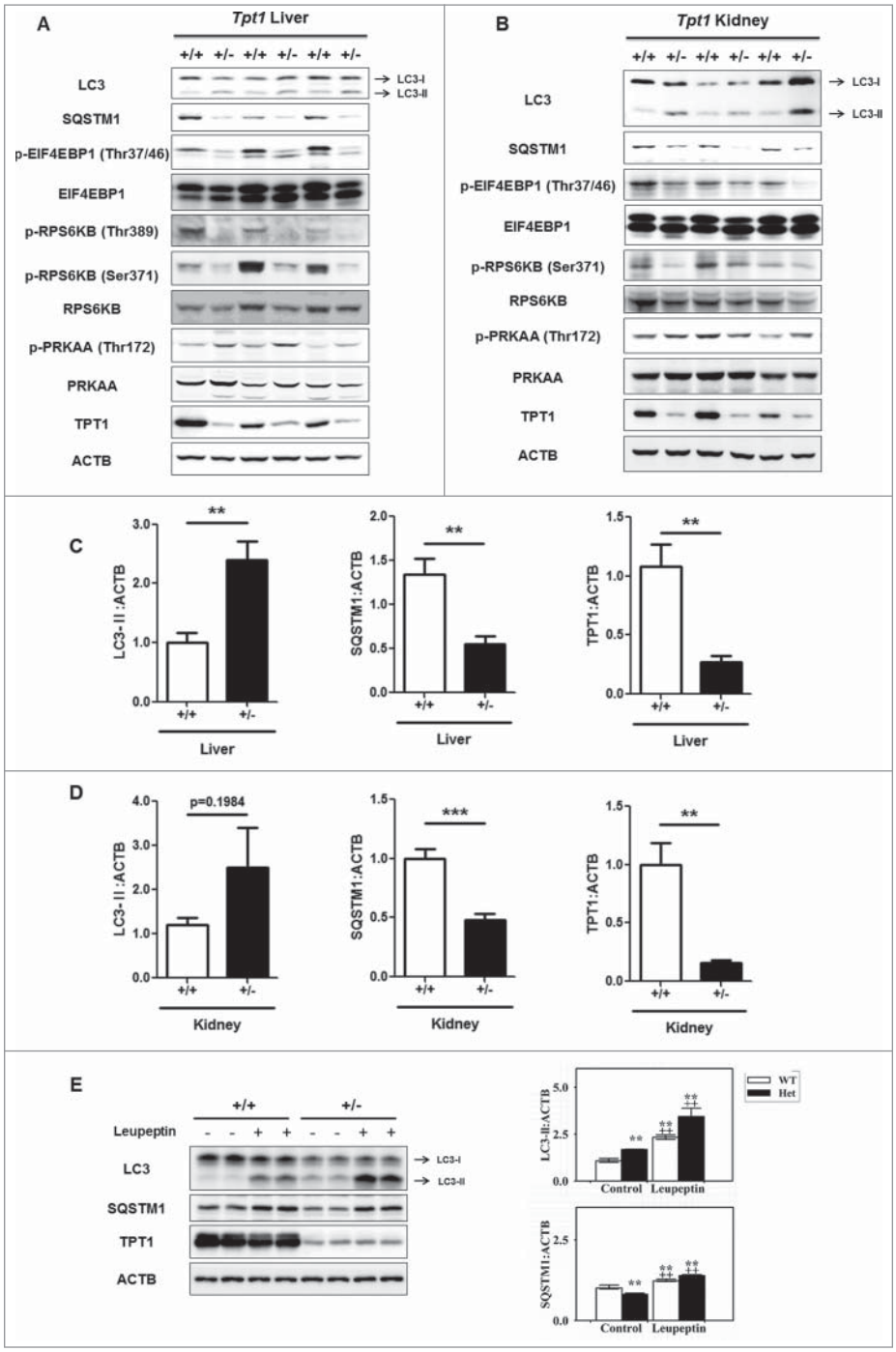

Figure 7. Haploinsufficient TPT1 expression in vivo leads to enhanced autophagy. Organs were dissected from 3 representative sets of wild-type (*Tpt1*$^{+/+}$) and *Tpt1* heterozygote knockout mice (*Tpt1*$^{+/-}$). Autophagy-related molecules were immunoprobed with the indicated antibodies in livers (A) and kidneys (B). ACTB served as a loading control. Quantification of LC3-II, SQSTM1 and TPT1 derived from western blotting depicted in (C) livers and (D) kidneys. Data are presented as means ± S. E. M. (n = 6). P** < 0.01, P*** < 0.001. (E) Basal autophagic flux in *Tpt1*$^{+/+}$ and *Tpt1*$^{+/-}$ mice was analyzed using the leupeptin assay. Mice were injected with PBS or 40 mg/kg leupeptin and killed 4 h later. Autophagy-related molecules were immunoprobed with the indicated antibodies in livers. ACTB served as a loading control. Data are presented as means ± S. E. M. (n = 3). P** < 0.01, P*** < 0.001.

mice using a leupeptin assay. We found that leupeptin treatment augmented LC3-II accumulation and blocked SQSTM1 degradation in *Tpt1*$^{+/-}$ mice livers (Fig. 7E). These results demonstrate that haploinsufficient expression of TPT1 induces early steps of autophagy in vivo. These in vivo data using *Tpt1* heterozygote knockout mice organs strengthened our in vitro data, which demonstrated the new role of TPT1 as a negative regulator of autophagy through the MTORC1 and AMPK pathways.

Discussion

In the present study, we demonstrated that TPT1 inhibits autophagy by activating the MTORC1 pathway and deactivating

the AMPK pathway. In addition, silencing of TPT1 resulted in the downregulation of the anti-apoptotic protein, BCL2, and altered the BECN1 interactome interactions necessary for autophagy. We also showed that TPT1 depletion promoted the overall autophagic flux by stimulating both autophagosome formation and its maturation. We also confirmed these findings in vivo using *Tpt1* heterozygote knockout mice.

Of note, MTORC1 is a key interface, coordinately regulating the balance between cell growth and autophagy influenced by the nutritional status.[39] Because of the crucial role of the MTORC1 pathway in autophagy, it is not surprising that TPT1 is involved in negative regulation of autophagy via MTORC1 activation. In this regard, we recognize that a reported contrary finding that TPT1 positively regulates autophagy by activating the AMPK pathway[31] warrants an explanation from us of the reason for this discrepancy. Because of the hypoxic microenvironment surrounding oocytes, these authors investigated autophagy using TPT1 depleted COS-7 cells under both normoxic and hypoxic conditions. Under normoxic conditions, knockdown of TPT1 increases LC3-II and suppresses MTORC1, thereby activating the AMPK pathway similar to our data. Under hypoxic conditions, however, depletion of TPT1 decreases the LC3-II level. Since autophagy is a dynamic process and LC3-II levels can fluctuate during autophagy induction,[40] the LC3 data need to be carefully interpreted. As hypoxia has been widely shown to trigger autophagy,[41] it is possible that depletion of TPT1 under hypoxic condition might further facilitate autophagic flux and cause further degradation of LC3-II leading to the conclusion that TPT1 positively regulates autophagy by activating the AMPK pathway. Elucidation of the details of the mechanisms underlying modulation of autophagy by TPT1 under hypoxic conditions, using lysosomotropic agents could help confirm our explanation.

Rapamycin induces autophagy by modulating the MTORC1 pathway.[33] Like rapamycin, downregulation of TPT1 also negatively regulates the MTORC1 pathway and activates the AMPK pathway (Fig. 6). In addition, we found that TPT1 alters the BCL2-BECN1 interaction which is a representative MTOR-independent autophagic pathway.[42] We suggest that depletion of TPT1 potentiates rapamycin-induced autophagy (Fig. 5A and C) through an MTORC1-dependent pathway, by further dephosphorylating Ser371 residues of RPS6KB, EIF4EBP and MTOR itself (Fig. 6C and D), and an MTORC1-independent pathway, altering the BECN1 interactome by suppressing BCL2 (Fig. 4). Rapamycin has long been used as an immunosuppressant in cancer therapy.[43,44] A previous study revealed that cytotoxic effects of rapamycin on malignant glioma cells were based on autophagy induction but not apoptosis.[45] We suggest, based on the present study, that augmenting rapamycin-induced autophagy might help overcome rapamycin resistance of tumor cells and that combining rapamycin treatment with TPT1 depletion might offer an approach to overcoming rapamycin resistance in cancer therapy.

BCL2 is an anti-apoptotic protein that inhibits the release of CYCS/cytochrome c from mitochondria and prevents caspase activation.[46] Since it has been proposed that BCL2 inhibits autophagy by binding BECN1,[19] crosstalk between apoptosis and autophagy has become widely studied. Because TPT1 plays a key role in anti-apoptotic activity by interacting with other anti-apoptotic proteins such as MCL1 and BCL2L1/Bcl^{-}xL,[47-49] we investigated whether downregulation of TPT1 influences BCL2 expression. We found that knockdown of TPT1 downregulated both endogenous BCL2 (Fig. 4) and exogenous BCL2 (Fig. S5), without affecting mRNA level (Fig. S6). Unlike MCL1, which TPT1 directly interacts with thus interferring with proteasome-mediated degradation and stabilizing MCL1, BCL2 did not interact with TPT1 (Fig. S7). Recent studies revealed that the stress-activated signaling molecule MAPK8 causes the dissociation of the BECN1-BCL2 complex by phosphorylating BCL2 following starvation,[28] thereby leading to degradation of BCL2.[29,50,51] Our data showed that TPT1 knockdown results in increases in both MAPK8 and MAPK9/JNK2, and the phosphorylation of MAPK8. (Fig. 4C). These findings suggest that TPT1 possibly indirectly increases BCL2 by suppressing MAPK8 signaling. MAPK8 knockout or knockdown studies using siRNA approaches may provide direct evidence for this. The present study suggests that TPT1 might be potentially involved in crosstalk between apoptosis and autophagy similar to BCL2.

We found that depletion of TPT1 enhanced the association of the BECN1-PIK3C3-UVRAG complex (Fig. 4D). In addition, knockdown of TPT1 stimulated the early step of autophagy (on-rate), as shown by GFP-LC3 studies in the presence of lysosomotropic agents (Fig. 2). Though enzyme activity should be further measured to clarify the direct effect of TPT1 on PtdIns3K function, it seems that knockdown of TPT1 might promote an early step of autophagy by altering the BECN1 interactome. Our study showed that TPT1 knockdown promotes autophagosome formation as well as maturation (Fig. 3A and B). Also TPT1 knockdown caused autophagosomes to interact with lysosomes forming autolysosomes (Fig. 3C), indicating that overall autophagic flux was stimulated. To further elucidate the mechanism of direct effect of TPT1 on autophagosome maturation, the interaction between UVRAG and the class C-Vps/HOPS complex should be further studied.

Because autophagy plays key roles in maintaining cellular homeostasis, deregulation in autophagy may cause numerous human diseases, including cancer. The role of autophagy in cancer is complex and considered a double-edged sword in tumorigenesis.[10] On the one hand, autophagy enables cancer cells to survive in stress conditions, such as starvation, hypoxic microenvironment and following some therapies.[11] On the other hand, autophagy also acts as a tumor suppressor causing autophagic cell death.[12] This complexity tends to be dependent on the tumor type, stage, and genetic context. Although much needs to be known about the exact role of autophagy in cancer, it is clear that abnormal control of autophagy is one of the key hallmarks of cancer. Consequently, the modulation of autophagy can be a promising avenue for cancer therapy. While the role of TPT1 in cancer has been widely studied, these lines of research have been mainly focused on apoptotic inhibition.[47,48,52] This study, which identified the novel role of TPT1 as an autophagy suppressor, suggests that targeting TPT1 may be a possible new strategy in anticancer therapy and autophagy-related diseases.

Materials and methods

Cell culture

HeLa cells were purchased from the American Type Culture Collection (ATCC, CCL¯2) and maintained in Dulbecco's modified essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37°C and 5% CO_2. HeLa cells stably expressing GFP-LC3 (HeLa GFP-LC3) were also maintained in DMEM with 10% FBS.

Antibodies and reagents

The reagents, chemicals and antibodies used here (with their sources) are as follows: LC3 (Novus Biologicals, NB100–2331), GFP (Santa Cruz Biotechnology, sc-8334), goat polyclonal BECN1 (Santa Cruz Biotechnology, sc-10086), agarose-conjugated mouse monoclonal BCL2 (Santa Cruz Biotechnology, sc-7382 AC), PIK3C3 (Cell Signaling Technology, 4263), phospho-RPS6KB (Thr389 and Ser371; Cell Signaling Technology, 9234 and 9208), RPS6KB (Cell Signaling Technology, 2708), phospho-PRKAA/AMPKα (Thr172; Cell Signaling Technology: 2535), PRKAA/AMPKα (Cell Signaling Technology, 5832), p-MTOR (Ser2448 and Ser2481; Cell Signaling Technology, 9234 and 2974), MTOR (Cell Signaling Technology, 2983) phospho-EIF4EBP1 (Thr37/46; Cell Signaling Technology, 2855), rabbit monoclonal BECN1 (Cell Signaling Technology, 3495), BCL2 (Cell Signaling Technology, 2872), ACTB (Cell Signaling Technology, 4970), HA (Cell Signaling Technology, 3724), GAPDH (AbFrontier, LF-PA0018), SQSTM1/p62 (Sigma-Aldrich, P0067) and UVRAG (Sigma-Aldrich, U7508), FLAG (Sigma-Aldrich, F3165) and TPT1 (AbFrontier, YF-MA10968).

Rapamycin (Sigma-Aldrich, R8781), bafilomycin A_1 (Baf A1; Sigma-Aldrich, B1793), chloroquine (CQ; Sigma-Aldrich, C6628), and leupeptin (Sigma-Aldrich, L2884).

Plasmids and shRNA transfection

Monomeric RFP (mRFP)-GFP-LC3, RFP-LC3, GFP-LAMP1, Flag-BCL2 and HA-BECN1 constructs were described previously.[53] TPT1 was transiently knocked down using *Tpt1* shRNA as described[54] with minor modification. Transfection was performed by using Attractene transfection reagent (Qiagen, 301005).

Generation of stable cell lines by lentiviral transduction

Stable cells were generated as described[54] with minor modification. Transduced HeLa GFP-LC3 cells were selected with 1.4 μg/ml puromycin and infected HeLa cells were selected with 1 μg/ml of puromycin.

Autophagy analysis

Positive controls for autophagy were achieved with rapamycin treatment. Rapamycin was used to treat cells at 200 nM final concentration for the indicated times. Control cells were treated with DMSO.

Confocal microscopy and immunofluorescence staining

To monitor the formation of GFP-LC3 puncta, HeLa GFP-LC3 cells were fixed with prechilled 100% methanol then counterstained with DAPI performed by mounting in ProLong Gold antifade reagent (Invitrogen, P36931). For MEFs, endogenous LC3 was immunostained as described previously.[54] For the tandem fluorescence LC3 reporter assay, HeLa cells stably transduced with *control* or *Tpt1* shRNA, were transfected with the mRFP-GFP-LC3 construct.[26] After 24 h, cells were fixed with 3.7% paraformaldehyde, and then counterstained with DAPI. Cells were photographed with a fluorescence confocal microscope (LSM510, Zeiss, Germany). Quantification of autophagic vacuoles, was done by counting the numbers of LC3 puncta in at least 30 cells per sample. Mean values were obtained from at least 3 separate experiments. For analyzing colocalization of autophagosomes and lysosomes, RFP-LC3 and GFP-LAMP1 were cotransfected in stable TPT1 knockdown HeLa cells for 24 h.

Immunoblotting and immunoprecipitation

Immunoblotting was performed as described previously.[54] An endogenous BECN1 and BCL2 immunoprecipitation assay was performed as described previously[19] with minor modifications. Briefly, cells were lysed with 0.5% Triton X-100 lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM sodium chloride, 1 mM ethylenediaminetetraacetic acid/EDTA, 0.5% Triton X-100 [Sigma-Aldrich, T8787]) with protease inhibitor cocktail (Roche Molecular Biochemicals, 11 836 170 001) and phosphatase inhibitor cocktail (Sigma-Aldrich, P5726 and P0044), passed through a syringe 5 times and then incubated on ice for 30 min. The soluble fraction was isolated by centrifugation at 12,000 × g for 15 min at 4°C. Primary antibodies or agarose-conjugated antibodies were added to the lysates and rotated overnight at 4°C, and then 30 μl of a protein G agarose bead slurry (Roche, 1 719 416) was added for 2 h. Immunoprecipitates were washed 3 times with cold lysis buffer. Whole cell lysates and immunoprecipitated proteins were boiled in 30 μl sample buffer. Each sample (10–15 μl) was separated on 12% SDS-PAGE gels and transferred to nitrocellulose membrane (Whatman, BA85). Images were taken with the LAS-3000 (Fujifilm, Japan).

Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated using TRI Reagent® (Ambion, 15596026) according to the manufacturer's instructions. First-strand cDNA was synthesized from 1 μg of total RNA High-Capacity cDNA reverse transcription kit (Applied biosystems, 43–688–14). For reverse transcription PCR, the primers were *Bcl2*,[55] forward, 5′-CGACGACTTCTCCCGCCGCTACCGC-3′, reverse, 5′-CCGCATGCTGGGGCCGTACAGTTCC-3′; *Tpt1*,[56] forward, 5′-ATGATTATC TACCGGGACCTC-3′, reverse,5′-TACATTTTTCCATTTCTAAACCATCC-3′; *Actb*,[55] forward, 5′-CATCCTGCGTCTGGACCT-3′, reverse, 5′-CAGGAGGAGCAATGATCTTG-3′. The RT-PCR was performed with the following thermal cycling parameters: For *Bcl2*, 95°C for 10 min; 25 cycles of 94°C for 30 sec, 62°C for 30 sec, 72°C

for 20 sec; and final extension at 72°C for 10 min. For *Tpt1* and *Actb*, 94°C for 5 min; 25 cycles of 94°C for 15 sec, 55°C for 30 sec, 72°C for 1 min; and final extension at 72°C for 10 min. The PCR products were separated on a 1.6% agarose gel. The band intensities were measured using Image Lab Ver. 5.0 (Bio-Rad Laboratories; Hercules, CA, USA).

Mouse embryonic fibroblast isolation and animal tissue sample preparation

Tpt1 heterozygote knockout mice that have only a single copy of the *Tpt1* gene, *Tpt1*$^{+/-}$, were kindly provided by Hsin-Fang Yang-Yen (Institute of Molecular Biology, Academia Sinica, Taipei, Taiwan).[24] The male chimeric mice (C57BL/6 × 129/Svj) were backcrossed with C57BL/6 females for approximately 20 generations. Mice were housed in cages under a 12-h d/light cycle with free access to food and water. All animal experiments were performed according to protocols approved by the Institutional Animal Care and Use Committee of Ewha Womans University. MEFs were isolated at embryonic d 13.5 (E13.5) after the mating of heterozygous *Tpt1* knockout mice. The genotypes of MEFs were identified by PCR analysis using genomic DNA as described previously.[24] Isolated MEFs were cultured in DMEM containing 10% FBS. For organ analysis, livers and kidneys were dissected from 12-wk-old female mice (n = 6/group). Dissected tissues were lysed and analyzed by immunoblotting. The band intensities were calculated using Multi Gauge 3.0 photo software (Fuji Film, Tokyo, Japan) and normalized by ACTB. Statistical analysis was done using the Student *t* test by GraphPad Prism 5 (GraphPad Software, CA, USA). All animal studies were conducted in accordance with IACUC guidelines and were approved by the IACUC committee at Ewha Womans University (Approval ID: 14–093).

Leupeptin assay for measuring autophagic flux in vivo

A leupeptin assay was performed as described previously.[57] Briefly, either wild-type or heterozygous knockout mice (n = 3) were injected intraperitoneally with 0.5 ml sterile phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 2 mM KH_2PO_4) or 40 mg/kg leupeptin hemisulfate (Sigma-Aldrich, L2884) in 0.5 ml sterile PBS. After injection, the mice were returned to their cages and provided free access to food and water. Four h after intraperitoneal injection, the livers were extirpated and analyzed by immunoblotting.

Statistical analysis

Data are presented as means and their standard errors. Data were analyzed using GraphPad Prisms 5 software (GraphPad Software Inc., CA, USA). Statistical significance was determined using the Student *t* test.

Abbreviations

AMPK	adenosine monophosphate-activated protein kinase
Baf A1	bafilomycin A_1
CQ	chloroquine
EIF4EBP1/4E-BP1	eukaryotic initiation factor 4E binding protein 1
GFP	green fluorescent protein
LAMP1	lysosomal-associated membrane protein 1
MAP1LC3/LC3	microtubule-associated protein 1 light chain 3
MEF	mouse embryonic fibroblast
mRFP	monomeric red fluorescent protein
MTORC1	mechanistic target of rapamycin (serine/threonine kinase) complex 1
PIK3C3/VPS34	phosphatidylinositol 3-kinase, catalytic subunit type 3
PtdIns3K	class III phosphatidylinositol 3-kinase
RPS6KB/p70S6K	ribosomal protein S6 kinase
SQSTM1/p62	sequestosome 1
TPT1/TCTP	tumor protein, translationally-controlled1
UVRAG	UV radiation resistance associated gene.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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