

# Mammalian Target of Rapamycin and S6 Kinase 1 Positively Regulate 6-thioguanine-Induced Autophagy

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## Abstract

**DNA mismatch repair (MMR) ensures the fidelity of DNA replication and is required for activation of cell cycle arrest and apoptosis in response to certain classes of DNA damage. We recently reported that MMR is also implicated in initiation of an autophagic response after MMR processing of 6-thioguanine (6-TG). It is now generally believed that autophagy is negatively controlled by mammalian target of rapamycin (mTOR) activity. To determine whether mTOR is involved in 6-TG-induced autophagy, we used rapamycin, a potential anticancer agent, to inhibit mTOR activity. Surprisingly, we find that rapamycin cotreatment inhibits 6-TG-induced autophagy in MMR-proficient human colorectal cancer HCT116 (MLH1<sup>+</sup>) and HT29 cells as measured by LC3 immunoblotting, GFP-LC3 relocalization, and acridine orange staining. Consistently, short interfering RNA silencing of the 70-kDa ribosomal S6 kinase 1 (S6K1), the downstream effector of mTOR, markedly reduces 6-TG-induced autophagy. Furthermore, we show that inhibition of mTOR by rapamycin induces the activation of Akt as shown by increased Akt phosphorylation at Ser<sup>473</sup> and the inhibition of 6-TG-induced apoptosis and cell death. Activated Akt is a well-known inhibitor of autophagy. In conclusion, our data indicate that mTOR-S6K1 positively regulates autophagy after MMR processing of 6-TG probably through its negative feedback inhibition of Akt.** [Cancer Res 2008;68(7):2384–90]

## Introduction

Macroautophagy (hereafter called autophagy) is an evolutionarily conserved catabolic process where cytoplasmic contents and organelles such as mitochondria are sequestered into a double-membrane vesicle, called an autophagosome. The autophagosome then fuses with a lysosome and releases the inner vesicle into the lysosome, where degradative enzymes break the vesicle down (1, 2). Degradation and recycling of the contents of the vesicle enable the cell to continue to carry out essential processes for cell survival under stress conditions (3, 4). However, excessive autophagy results in type II programmed cell death (3). Autophagy not only functions as a tumor suppressor mechanism (5, 6) but also has been observed as a novel response to some anticancer DNA-damaging agents such as ionizing radiation (7), temozolomide (8) and 6-thioguanine (6-TG; ref. 9).

DNA mismatch repair (MMR) system plays an essential role in repairing mismatches caused by DNA polymerase errors and

reduces mutagenesis (10). MMR has also been shown to be essential for mediating the antitumor effects of several classes of commonly used chemotherapy agents such as *N*-methyl-*N*-nitro-*N*-nitrosoguanidine, temozolomide, cisplatin, and 6-TG (10–12). These treatments induce cells to undergo cell cycle arrest and apoptosis in a MMR-dependent manner (10–12). In a recent report, we observed a novel role of MMR in initiation of an autophagic response to 6-TG (9).

In this study, we use the purine analogue 6-TG as a model compound to investigate the molecular machinery of autophagy after MMR processing of chemotherapy drugs (9, 13). Our previous report indicates that the cytotoxicity of 6-TG mainly results from the futile attempt of MMR to repair 6-TG-induced DNA mismatch damage (14). After intracellular processing and incorporation into DNA, 6-TG is chemically converted to form S<sup>6</sup>-methylthioguanine, which introduces S<sup>6</sup>-methylthioguanine–thymine mispairs during subsequent DNA replication. S<sup>6</sup>-methylthioguanine–thymine mispairs are then identified by MMR as replication errors (15). During the futile repair cycle, MMR attempts to process S<sup>6</sup>-methylthioguanine–thymine mismatches in the newly synthesized daughter strands. Because some S<sup>6</sup>-methylthioguanine is in the template strand, which is not excised by MMR, abortive repair cycles result in accumulating DNA strand breaks (14, 16). In other words, 6-TG-induced DNA damage is not fully MMR-repairable and the accumulating DNA strand breaks will continuously activate the autophagy pathway. Excessive autophagy may ultimately lead to type II programmed cell death (3). Therefore, it is of potential clinical importance to better understand the molecular mechanisms regulating the autophagic pathway after MMR processing of chemotherapeutic drugs such as 6-TG.

Mammalian target of rapamycin (mTOR) is a phosphatidylinositol 3 kinase (PI3K)-related serine/threonine protein kinase that regulates many aspects of cellular functions, including transcription, translation, cell size, cytoskeletal organization, and autophagy (17, 18). mTOR exists in two distinct protein complexes, termed TORC1 and TORC2. TORC1 is rapamycin-sensitive and consists of mTOR, raptor, and mLST8 (GβL; refs. 18, 19). TORC2 is rapamycin-insensitive and contains mTOR, rictor, and mLST8 (18, 19). TORC1 is primarily activated by the PI3K-Akt pathway (20, 21). After binding to growth factors including insulin or insulin-like growth factor (IGF)-I, insulin/IGF-I receptor tyrosine kinases (RTK) undergo autophosphorylation and become activated, leading to phosphorylation of insulin receptor substrate (IRS) proteins on several tyrosine residues with subsequent recruitment and activation of PI3K. PI3K together with TORC2 fully activates Akt (20, 21). PI3K-Akt activation is well-known to suppress autophagy in mammalian cells (22–25). Akt further activates the downstream rapamycin-sensitive TORC1 by inhibiting a protein complex formed by the tumor suppressor proteins, termed tuberous sclerosis complex. This complex acts as a key negative regulator of TORC1 activity (17, 18). Activated mTOR is generally believed to be involved in the negative control of mammalian autophagy,

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although the mTOR effectors that regulate autophagy have not been clearly elucidated (26–28).

TORC1 activation by the PI3K-Akt pathway also results in phosphorylation and activation of S6 kinase 1 (S6K1) and the eukaryotic initiation factor 4E-binding protein 1, which regulates protein synthesis (17, 18). Interestingly, S6K1 can, in turn, produce feedback inhibition of PI3K-Akt through phosphorylating IRS-1 and, thus, disrupting its interaction with insulin/IGF-I RTKs and destabilizing IRS-1 (29, 30).

In this study, we determine whether mTOR plays any role in regulating autophagy after MMR processing of 6-TG. In contrast with earlier reports (26–28), our results show that inhibition of mTOR activity by rapamycin impairs 6-TG-induced autophagy. In agreement, we find that short interfering RNA (siRNA)-mediated silencing of S6K1, one of the downstream effectors of mTOR, attenuates 6-TG-induced autophagy. Furthermore, inhibition of mTOR activity by rapamycin induces phosphorylation and activation of Akt, a well-established inhibitor of autophagy (22–25). Together, these data suggest that mTOR-S6K1 functions in positively modulating 6-TG-induced mammalian autophagy through negative feedback inhibition of PI3K-Akt.

## Materials and Methods

**Cell culture.** Human colorectal cancer (HCT116) cells with stable transfection of human MLH1 cDNA were kindly provided by Dr. Françoise Praz (Centre National de la Recherche Scientifique, Villejuif, France; ref. 31). HCT116 cells are MMR-deficient (MMR<sup>-</sup>) because the *hMLH1* gene in these cells contains a base substitution that results in a termination signal at codon 252 (TCATAA; ref. 32). Stable expression of a cloned wild-type *hMLH1* cDNA has been shown to restore MMR activity (MMR<sup>+</sup>) in the HCT116 cells (31). Human colorectal HT29 cell line, which is MMR proficient (14), was obtained from the American Type Culture Collection. The cells were maintained in DMEM (Mediatech, Inc.) and supplemented with 10% fetal bovine serum (Hyclone), penicillin (100 µg/mL), streptomycin (100 units/mL), l-glutamine (2 mmol/L), and nonessential amino acids (0.1 mmol/L; Invitrogen).

**Drug treatment.** Cells were seeded at 30,000 cells per 60-mm dish and allowed to attach and grow for 17 h. Three micromoles per liter of 6-TG (Sigma) were then added to the medium. After 24 h, 6-TG was removed and the cells were incubated in drug-free medium with or without 0.2 to 5 nmol/L rapamycin (EMD Chemicals, Inc.) for an additional 3 d to assess the role of mTOR in response to 6-TG-induced autophagy. Rapamycin is known to inhibit mTOR activity (27).

**Stable transfection of green fluorescent protein–light chain 3 in HCT116 cells.** HCT116 (MLH1<sup>+</sup>, MMR<sup>+</sup>) cells with stable expression of green fluorescent protein–light chain 3 (GFP-LC3) were generated as described recently (9). Briefly, pGFP-LC3 plasmids (a generous gift of Dr. Tamotsu Yoshimori, National Institute of Genetics, Shizuoka-ken, Japan; ref. 33) were transfected into HCT116 (MLH1 cDNA transfected) cells using FuGENE 6 (Roche), according to the manufacturer's protocol. Forty eight hours after the transfection, the cells were selected with 1 mg/mL G418 for 2 wk. GFP-LC3-expressing colonies were isolated and expanded.

**siRNA-mediated silencing of S6K1.** siRNA oligos were chemically synthesized by Dharmacon, Inc. The siRNA sequence specific for luciferase (5'-CGUACGCGAAUACUUCGAdTdT-3') was used as a control. The sense siRNA sequence for knocking down S6K1 was 5'-GGACAUGGCAGGAGU-GUUU dTdT-3'. siRNAs were transfected using Lipofectamine 2000 (Invitrogen), according to the manufacturer's protocol.

**Detection and quantification of acidic vesicular organelles with acridine orange.** Vital staining of cells with acridine orange (Invitrogen) was performed essentially as described (7–9). Briefly, cells were grown in 100-mm dishes and treated with 6-TG or vehicle (NaOH), as described above. Acridine orange was added for 15 min at a final concentration of 1 µg/mL, and unfixed cells were harvested by trypsinization and immediately analyzed by flow cytometry (Epics XL-MCL; Beckman Coulter, Inc.).

**Propidium iodide staining for apoptosis.** Cells were fixed with 90% ethanol at –20°C for 15 min, incubated with RNase, stained with propidium iodide, and then subjected to flow cytometry (Epics XL-MCL; Beckman Coulter, Inc.).

**Immunoblotting.** SDS-PAGE and immunoblot analyses were performed as described previously (9). The primary antibodies used for immunoblot analyses included a rabbit polyclonal antibody against LC3 (a generous gift of Dr. Tamotsu Yoshimori, National Institute of Genetics, Shizuoka-ken, Japan; ref. 33); a rabbit polyclonal antibody against cleaved poly(ADP)ribose polymerase (PARP; Promega); rabbit monoclonal antibodies against S6K1, phospho-Akt (Ser<sup>473</sup>), and Akt; a mouse monoclonal antibody against phospho-S6K1 (Thr<sup>389</sup>; Cell Signaling Technology, Inc.); and mouse monoclonal antibodies against β-Actin and α-Tubulin (Sigma).

**Clonogenic survival assay.** Cell survival after treatment with 6-TG was determined by a standard colony-forming assay. Briefly, 200 to 4,000 cells were plated into 60-mm dishes and allowed to adhere for 17 h. Cells were then treated with 6-TG (1–6 µmol/L) for 24 h and subsequently incubated in fresh medium with or without 5 nmol/L rapamycin for 14 d for colony formation. The colonies were stained with 0.5% crystal violet in methanol/acetic acid (3:1), and those with >50 cells were counted.

**Statistics.** Statistical analysis was performed using Student's *t* test, and a *P* value of <0.05 was considered significant. Data are expressed as the mean ± SE. The mean value was obtained from at least three independent experiments.

## Results

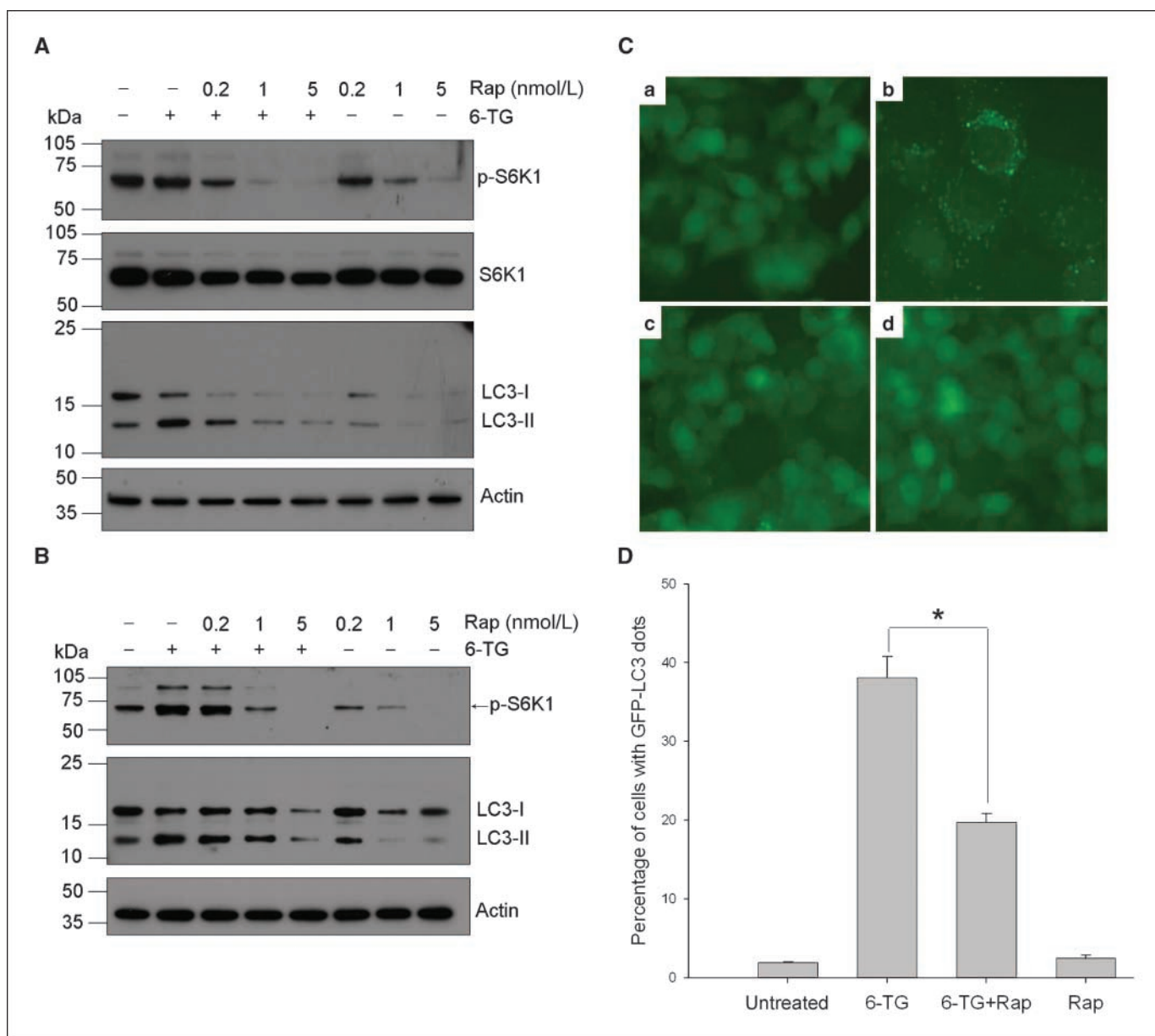
**Inhibition of mTOR by rapamycin impairs 6-TG-induced autophagy.** Our earlier studies have described that DNA MMR initiates 6-TG-induced autophagy in human colorectal cancer HCT116 (MLH1<sup>+</sup>, MMR<sup>+</sup>) cells (9, 13). Accumulating evidence suggests that mTOR is a negative regulator of mammalian autophagy (26–28). To better characterize how mTOR regulates an autophagy pathway after MMR processing of chemotherapy drugs such as 6-TG, we used rapamycin cotreatment to inhibit mTOR activity. In this study, microtubule-associated protein LC3 immunoblotting was first applied to monitor autophagosome biogenesis. LC3 exists in a cytosolic form (LC3-I) and a LC3-II form that is conjugated to phosphatidylethanolamine (33). LC3-II is present on isolation membranes, autophagosome membranes, and much less on autolysosomes during the autophagic process. The amount of LC3-II is closely correlated with the number of autophagosomes and serves as a good indicator of autophagosome formation (34). As shown by the immunoblots in Fig. 1A, rapamycin reduces mTOR activity in a dose-dependent manner as measured by the phosphorylation of S6K1 at Thr<sup>389</sup>, the active form of the downstream effector protein of mTOR. In contrast to earlier reports (26–28), the inhibition of mTOR activity by rapamycin impairs a 6-TG-induced increase in the amount of LC3-II in a dose-dependent manner in HCT116 (MLH1<sup>+</sup>, MMR<sup>+</sup>) cells (Fig. 1A). Similar results were obtained from another MMR-proficient human colorectal cancer HT29 cells (Fig. 1B) as well as in MMR<sup>+</sup> human endometrial (HEC59) and prostate (PC3) cancer cell lines (data not shown).

Besides LC3 immunoblotting, another specific and reliable method to monitor autophagy in mammalian cells is to visualize the intracellular localization of GFP-tagged LC3 by fluorescence microscopy (34). Before induction of autophagy, GFP-LC3 shows diffuse localization in the cytosol; whereas when autophagy is induced, GFP-LC3 will specifically relocalize to autophagic membranes and exhibit punctate dot cytoplasmic signals (34). Because transient transfection of GFP-LC3 causes a high background of punctate dot signals (35), we used HCT116 (MLH1<sup>+</sup>, MMR<sup>+</sup>) cells with stable expression of GFP-LC3 to further confirm if inhibition of mTOR by rapamycin attenuates 6-TG-induced autophagy as

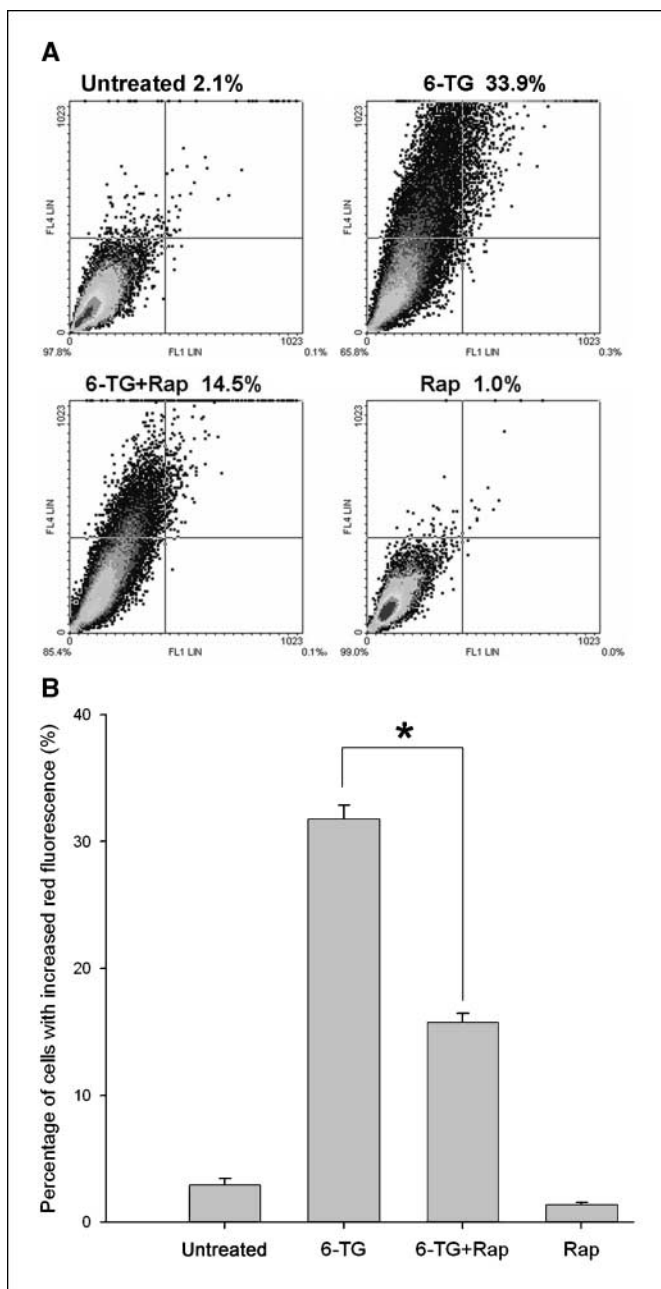
described in Fig. 1A. Consistent with our previous report (9), ~38% of the cells (MLH1<sup>+</sup>, MMR<sup>+</sup>) show punctate dot signals after MMR processing of 6-TG with little background in untreated cells. In agreement with the above results from LC3 immunoblotting (Fig. 1A and B), rapamycin cotreatment significantly reduces 6-TG-induced GFP-LC3 relocalization from the cytosol to the autophagic membranes ( $P = 0.00318$ , two-tailed  $t$  test; Fig. 1C and D).

It is well-documented that a substantial increase in the intensity of bright red fluorescence emitted by acridine orange occurs in conjunction with the induction of autophagy (7–9). Acridine orange, a lysosomotropic agent, enters an acidic compartment as

unchanged molecules but become protonated and trapped in the acidic compartments such as lysosomes in aggregates that fluoresce bright red or orange. When bound to DNA and RNA, acridine orange emits green fluorescence (36). It is thought that an increased autophagic influx tends to stimulate lysosomal proton pumping and increase lysosomal acidity, thereby establishing a correlation between autophagic activity and overall lysosomal acidity (37). The increased lysosomal acidity arising from induction of autophagy can be reflected by the intensity of bright red fluorescence emitted by acridine orange (37). Therefore, to further support our findings as described in Fig. 1, we next performed flow



**Figure 1.** Inhibition of mTOR by rapamycin reduces 6-TG-induced autophagy. HCT116 cells with stable expression of MLH1 (MLH1<sup>+</sup>, MMR<sup>+</sup>; A) or HT29 cells (B) were treated with 3  $\mu$ mol/L 6-TG for 24 h. Then, 0, 0.2, 1 or 5, nmol/L rapamycin (Rap) were added for another 3 d to inhibit mTOR activity. At day 3 after exposure to 6-TG, cells were harvested and analyzed by immunoblot analysis with antibodies against LC3, S6K1, and phospho-S6K1 (Thr<sup>389</sup>). C, HCT116 (MLH1<sup>+</sup>, MMR<sup>+</sup>) cells were stably transfected with pGFP-LC3 as described in the Materials and Methods. The GFP-LC3-expressing cells were treated with 3  $\mu$ mol/L 6-TG for 24 h. Then, 5 nmol/L rapamycin were added for another 3 d to inhibit mTOR activity. The cells were examined under fluorescence microscopy at day 3 after 6-TG treatment (a, untreated; b, 6-TG only; c, 6-TG + rapamycin; d, rapamycin only). The percentage of GFP-LC3-positive cells with GFP-LC3 punctate dots was then determined. A minimum of 250 cells per sample was counted from triplicate samples, and statistical analysis was performed using Student's  $t$  test (D). \*, rapamycin cotreatment significantly inhibits a 6-TG-induced increase in the percentage of cells with GFP-LC3 dots ( $P = 0.00318$ , two-tailed  $t$  test).



**Figure 2.** Inhibition of mTOR by rapamycin significantly reduces the 6-TG-induced increase in red fluorescence of acridine orange. **A**, HCT116 (MLH1<sup>+</sup>, MMR<sup>+</sup>) cells were treated with 3  $\mu\text{mol/L}$  6-TG for 24 h. Then, 5 nmol/L rapamycin were added for another 3 d to inhibit mTOR activity. Cells were stained with 1  $\mu\text{g/mL}$  acridine orange for 15 min at day 3 after exposure to 6-TG. The acridine orange-stained cells were then harvested by trypsinization and subjected to a flow cytometric analysis using the FL4 mode (Y axis; >650 nm) to measure bright red fluorescence and the FL1 mode (X axis; 500–550 nm) to measure green fluorescence. **B**, triplicate samples were done to quantitate the increase in acridine orange red fluorescence as described in (A) for statistical analysis. \*, rapamycin cotreatment significantly inhibits a 6-TG-induced increase in the percentage of cells with increased bright red fluorescence ( $P = 0.000226$ , two-tailed  $t$  test).

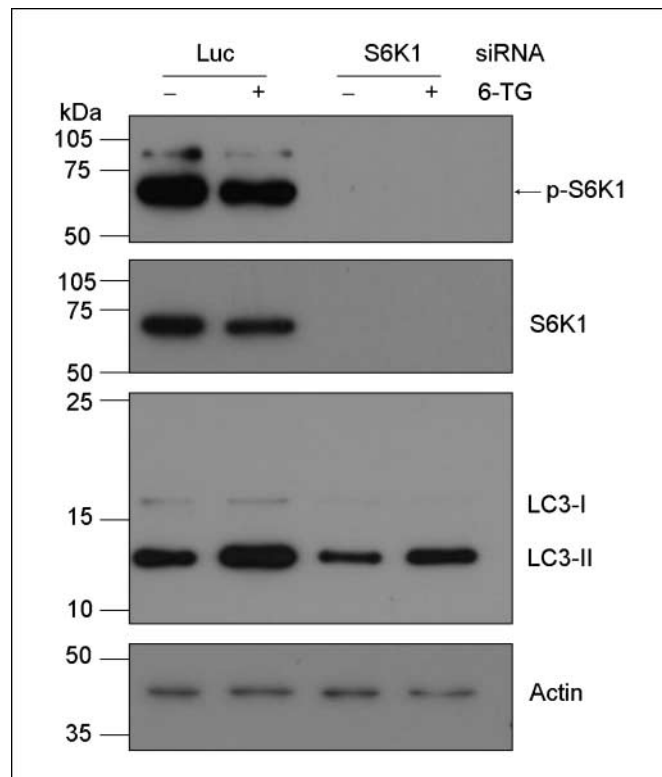
cytometric analyses of acridine orange-stained cells using the FL4 mode (>650 nm) to measure bright red fluorescence and the FL1 mode (500–550 nm) to measure green fluorescence. As shown in Fig. 2, 6-TG treatment induces a dramatic increase in the intensity of bright red fluorescence of acridine orange in HCT116 (MLH1<sup>+</sup>,

MMR<sup>+</sup>) cells, compared with that in cells treated with vehicle NaOH. In agreement with the findings as shown in Fig. 1, rapamycin cotreatment significantly impairs the 6-TG-induced increase in lysosomal acidity ( $P = 0.000226$ , two-tailed  $t$  test; Fig. 2).

Together, these findings (Figs. 1 and 2) show that inhibition of mTOR by rapamycin suppresses induction of autophagy after MMR processing of 6-TG in HCT116 (MLH1<sup>+</sup>, MMR<sup>+</sup>) and HT29 cells, indicating that mTOR is a positive regulator of 6-TG-induced autophagy.

**Suppression of S6K1 expression disrupts 6-TG-induced autophagy.** It has been described that S6K1 is a critical downstream substrate and effector of the mTOR signaling pathway (17, 18). Next, we asked whether S6K1 is a downstream effector of mTOR in modulating 6-TG-induced autophagy. To test this, HCT116 (MLH1<sup>+</sup>, MMR<sup>+</sup>) cells were transiently transfected with siRNA targeting S6K1 to specifically knock down this protein expression. As shown in Fig. 3, S6K1 siRNA dramatically reduces the total protein level of S6K1 and accordingly reduces S6K1 phosphorylation at Thr<sup>389</sup> to an undetectable level compared with control luciferase siRNA-transfected cells. As a result, siRNA-mediated suppression of S6K1 expression reduces both basic and 6-TG-induced autophagy compared with that in control luciferase siRNA-transfected cells.

**Inhibition of mTOR-S6K1 increases Akt activity.** The above results show that inhibition of mTOR by rapamycin and S6K1 knockdown reduce 6-TG-induced autophagy. As discussed above, extensive studies have established that a negative feedback loop



**Figure 3.** siRNA-mediated silencing of S6K1 expression suppresses both basic and 6-TG-induced autophagy. HCT116 (MLH1<sup>+</sup>, MMR<sup>+</sup>) cells were treated with 3  $\mu\text{mol/L}$  6-TG for 24 h. Control luciferase (Luc) or S6K1 siRNA oligos were then transfected to the cells as described in the Materials and Methods. At day 3 after exposure to 6-TG, the cells were harvested and analyzed by immunoblot analysis with antibodies against LC3, S6K1, and phospho-S6K1 (Thr<sup>389</sup>).

from mTOR-S6K1 to PI3K-Akt is present not only in *Drosophila* (38) but also in mammalian cells (39). Furthermore, PI3K-Akt activation is well-known to suppress autophagy in mammalian cells (22–25). Next, we sought to determine if inhibition of mTOR-S6K1 by rapamycin relieves the negative feedback and increases Akt activity. As shown in Fig. 4, rapamycin cotreatment does not affect total Akt protein levels. However, it dramatically increases Akt phosphorylation at Ser<sup>473</sup> in a dose-dependent manner, indicating that mTOR-S6K1 exerts a strong negative feedback on Akt, an autophagy inhibitor, in HCT116 (MLH1<sup>+</sup>, MMR<sup>+</sup>) cells.

**Inhibition of mTOR-S6K1 suppresses 6-TG–induced apoptosis.** PI3K-Akt mediates a variety of biological functions, including glucose uptake, protein synthesis, and inhibition of cell death (40). Once activated, PI3K-Akt can promote cell survival by inhibiting type I (apoptosis) and type II (autophagy) programmed cell death (40). Thus, to show that phosphorylation and activation of Akt induced by rapamycin is functional, we measured 6-TG–induced apoptosis after rapamycin cotreatment. Consistent with the finding of rapamycin-induced Akt activation (Fig. 4), mTOR-S6K1 inhibition by rapamycin significantly attenuates a 6-TG–induced subG<sub>1</sub> compartment, a good indicator of apoptosis (Fig. 5A). In agreement, rapamycin also inhibits 6-TG–induced PARP cleavage, a marker of apoptosis, in a dose-dependent manner (Fig. 5B). These data indicate that activation of Akt by rapamycin blocks 6-TG–induced apoptosis.

**Inhibition of mTOR-S6K1 reduces 6-TG–induced cell death.** To further establish that Akt phosphorylation and activation induced by rapamycin has biological function, we measured clonogenic cell survival. Consistent with the findings of inhibition of autophagy (Figs. 1–3) and apoptosis (Fig. 5A and B), rapamycin cotreatment significantly reduces 6-TG–induced cell death ( $P < 0.05$ , two-tailed  $t$  test; Fig. 5C), indicating that inhibition of mTOR by rapamycin relieves the negative feedback, which activates Akt activity and promotes cell survival after exposure to 6-TG.

## Discussion

mTOR has been thought to be a negative regulator of autophagy in mammalian cells (26–28). In contrast, we show that mTOR positively modulates 6-TG–induced autophagy in human colorectal cancer HCT116 (MLH1<sup>+</sup>, MMR<sup>+</sup>) and HT29 cells. Using rapamycin, a chemical mTOR inhibitor, we found that rapamycin attenuates 6-TG–induced autophagy. siRNA-mediated silencing of S6K1 expression further reveals that S6K1 is the downstream effector protein of mTOR in modulating 6-TG–induced autophagy. Our results also indicate that mTOR-S6K1 positively regulates autophagy through negative feedback inhibition of Akt, a well-known inhibitor of mammalian autophagy (22–25). These studies expand our understanding of the roles of mTOR in regulating mammalian autophagy and provide important information on the molecular machinery of autophagy after MMR processes DNA damage from certain types of chemotherapy drugs.

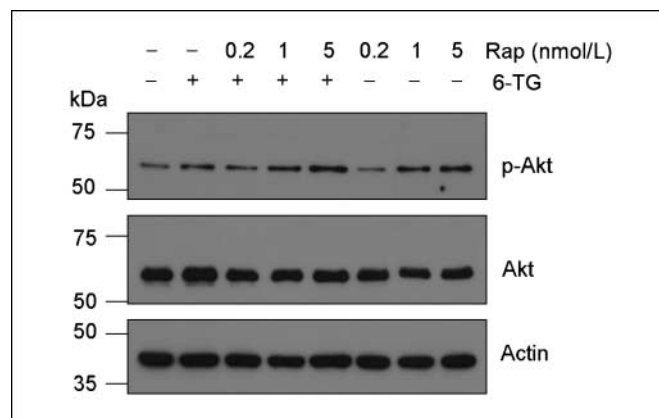
Extensive reports have described that mTOR is a negative regulator of mammalian autophagy (26–28). However, some recent studies have described that autophagy can also be induced in a mTOR-independent manner by lithium, inositol monophosphatase inhibitors (41), and trehalose (42). Our data show that mTOR inhibition by rapamycin and siRNA-mediated silencing of S6K1 expression reduces 6-TG–induced autophagy in human colorectal cancer HCT116 (MLH1<sup>+</sup>) cells, indicating that mTOR-S6K1 is needed for induction of autophagy (Figs. 1–3). Our results also show that rapamycin cotreatment reduces 6-TG–induced auto-

phagy (as measured by reduced LC3-II protein level) in another MMR<sup>+</sup> human colorectal cancer (HT29) cell line (Fig. 1B) as well as in MMR<sup>+</sup> human endometrial (HEC59) and prostate (PC3) cancer cell lines (data not shown). These discrepant reports indicate that the cellular effects of mTOR on autophagy may depend on cell type, cellular context, and nature of the treatment.

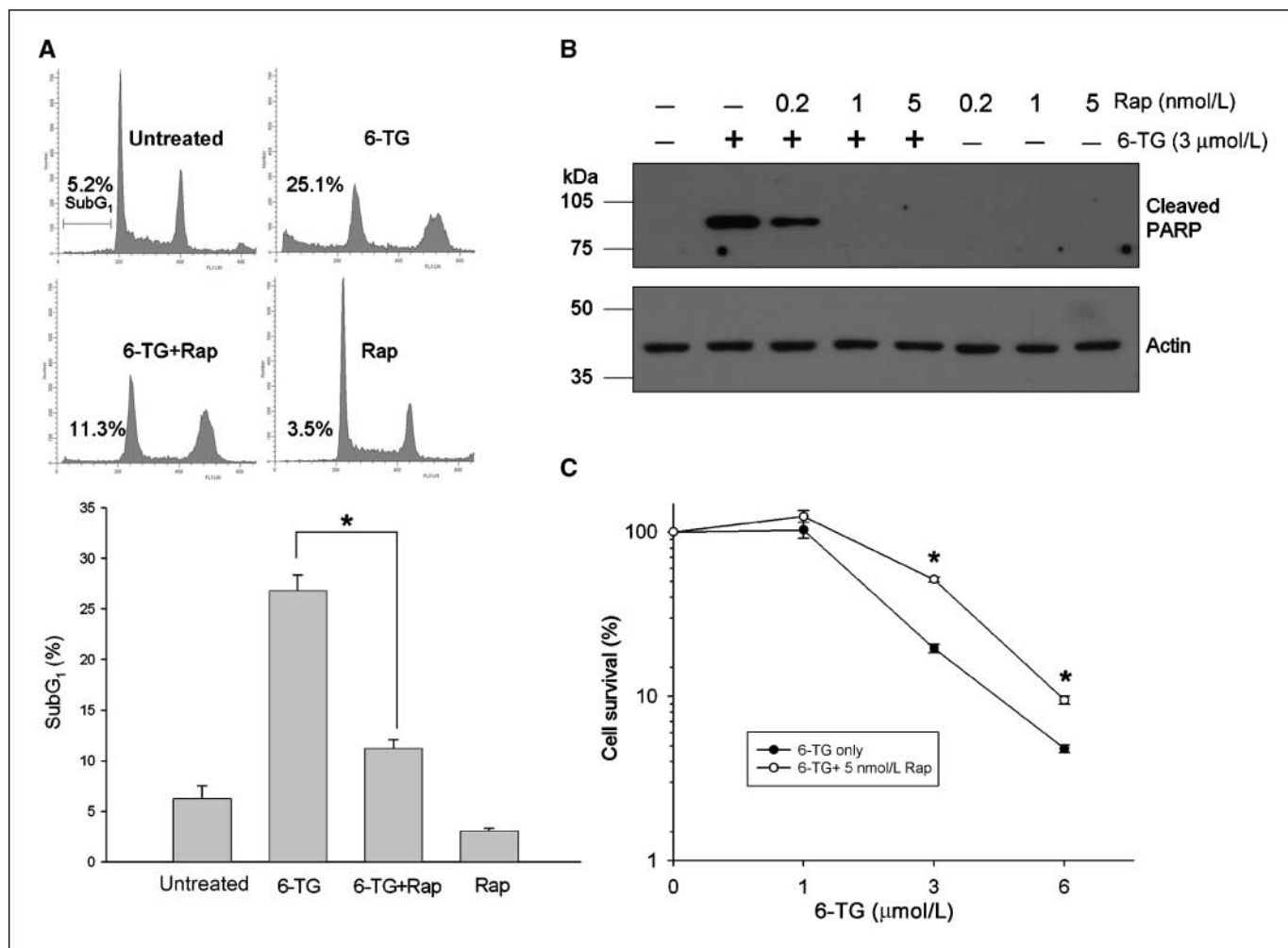
Our results further show that inhibition of mTOR-S6K1 by rapamycin induces Akt activation as shown by Akt phosphorylation at Ser<sup>473</sup>, inhibition of apoptosis, and promotion of cell survival after 6-TG treatment (Figs. 4 and 5). These data imply that one of the key factors determining how mTOR-S6K1 regulates autophagy could be whether the cell line possesses a sensitive negative feedback inhibition of PI3K-Akt by mTOR-S6K1. The negative feedback inhibition of PI3K-Akt by mTOR-S6K1 has been found in *Drosophila* (38), some normal mammalian cells (39), and some human cancer cell lines (43). Although the exact mechanism remains to be defined, activation of PI3K-Akt has been reported to suppress autophagy (22–25). Like the molecular mechanism for how Akt activity inhibits apoptosis (17, 18), Akt activation induced by rapamycin can block induction of autophagy through phosphorylating and inactivating its substrates such as Foxo, which has recently been found to be involved in autophagy in *Drosophila* (44). It will be very interesting to further investigate this possibility.

An earlier report has described that S6K is required for starvation-induced autophagy in the *Drosophila* fat body (45). Consistent with this finding, we observed an essential role for S6K1 in 6-TG–induced autophagy in human colorectal cancer HCT116 cells (Fig. 3). Although the mechanism is still not well-defined, activated S6K1 by mTOR has been described to participate in protein synthesis (17, 18). Therefore, besides the effect on the negative feedback inhibition of PI3K-Akt as described above, S6K1 may also control 6-TG–induced autophagy through translation of some proteins essential for induction of autophagy.

Rapamycin and several analogues, such as CCI-779 and RAD1, are currently undergoing clinical evaluation as anticancer agents (46). The complex of rapamycin with its intracellular receptor FKBP12 binds directly to mTORC1 and then inhibits mTORC1-mediated phosphorylation of the substrates S6K1 and 4EBP1. The intrinsic sensitivity to rapamycin among different cancer cell



**Figure 4.** Inhibition of mTOR by rapamycin increases Akt phosphorylation at Ser<sup>473</sup> in a dose-dependent manner. HCT116 (MLH1<sup>+</sup>, MMR<sup>+</sup>) cells were treated with 3  $\mu$ M 6-TG for 24 h. Then, 0, 0.2, 1, or 5 nmol/L rapamycin were added for another 3 d to inhibit mTOR activity. At day 3 after exposure to 6-TG, cells were harvested and analyzed by immunoblot analysis with antibodies against Akt and phospho-Akt (Ser<sup>473</sup>).



**Figure 5.** Inhibition of mTOR by rapamycin inhibits induction of apoptosis and promotes cell survival after exposure to 6-TG. **A**, HCT116 (MLH1<sup>+</sup>, MMR<sup>+</sup>) cells were treated with 3 μmol/L 6-TG for 24 h. Five nanomoles per liter of rapamycin were then added for another 3 d to inhibit mTOR activity. At day 3 after exposure to 6-TG, cells were harvested, stained with propidium iodide as described in the Materials and Methods, and subjected to a flow cytometry analysis of DNA content (top). Data from three independent experiments were analyzed and statistical analysis was performed (bottom). \*, rapamycin cotreatment significantly inhibits a 6-TG-induced subG<sub>1</sub> population ( $P = 0.000884$ , two-tailed  $t$  test). **B**, cells were treated with 3 μmol/L 6-TG for 24 h. Then, 0, 0.2, 1, or 5 nmol/L rapamycin were added for another 3 d to inhibit mTOR activity. At day 3 after exposure to 6-TG, cells were harvested and analyzed by immunoblot analysis with antibodies against cleaved PARP. **C**, cells were treated with 1 to 6 μmol/L 6-TG for 24 h. Five nanomoles per liter of rapamycin were then added to inhibit mTOR activity. At day 14 after exposure to 6-TG, surviving colonies were stained with 0.5% crystal violet and colonies with >50 cells were counted (closed circles, 6-TG treated only; open circles, 6-TG + 5 nmol/L rapamycin). \*, rapamycin cotreatment significantly reduces 6-TG-induced cell death ( $P < 0.05$ , two-tailed  $t$  test).

lines may vary by several orders of magnitude ranging from 1 to 5,000 nmol/L (46), indicating that some cancer cell lines are actually resistant to rapamycin. In this study, we find that rapamycin antagonizes the anticancer effect of 6-TG in HCT116 colorectal cancer cells (Fig. 5C), probably through inhibiting 6-TG-induced apoptosis and excessive autophagy. These results suggest that caution should be used if future combination therapy with rapamycin and 6-TG or other chemotherapy drugs processed by MMR are used clinically, as modulation of the mTOR pathway by rapamycin could lead to reducing tumor cell death as opposed to enhancing tumor cell killing.

As described earlier, MMR is required for the cytotoxicity of many clinically important anticancer agents, including methylating agents such as temozolomide; platinum compounds such as cisplatin and carboplatin; and antimetabolites such as 6-TG, 5-fluorouracil, and 6-mercaptopurine (10–12). Our results indicate that PI3K-Akt activation induced by rapamycin attenuates the

antitumor effect of 6-TG in the HCT116 human colorectal cancer cell line probably through inhibiting induction of apoptosis and autophagy. Consequently, targeted inhibition of PI3K-Akt might potentially promote the antitumor efficacy of 6-TG or other MMR processed chemotherapy drugs in some human cancers.

In summary, we show that mTOR inhibition by rapamycin and siRNA-mediated silencing of S6K1 expression attenuates 6-TG-induced autophagy, and mTOR-S6K1 probably modulates 6-TG-induced autophagy through exerting a negative feedback inhibition of PI3K-Akt.

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