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RESEARCH PAPER

Oncogenic RAS-induced downregulation of ATG12 is required for survival of malignant intestinal epithelial cells

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ABSTRACT

Activating mutations of RAS GTPase contribute to the progression of many cancers, including colorectal carcinoma. So far, attempts to develop treatments of mutant RAS-carrying cancers have been unsuccessful due to insufficient understanding of the salient mechanisms of RAS signaling. We found that RAS downregulates the protein ATG12 in colon cancer cells. ATG12 is a mediator of autophagy, a process of degradation and reutilization of cellular components. In addition, ATG12 can kill cells via autophagy-independent mechanisms. We established that RAS reduces ATG12 levels in cancer cells by accelerating its proteasomal degradation. We further observed that RAS-dependent ATG12 loss in these cells is mediated by protein kinases MAP2K/MEK and MAPK1/ERK2-MAPK3/ERK1, known effectors of RAS. We also demonstrated that the reversal of the effect of RAS on ATG12 achieved by the expression of exogenous ATG12 in cancer cells triggers both apoptotic and nonapoptotic signals and efficiently kills the cells. ATG12 is known to promote autophagy by forming covalent complexes with other autophagy mediators, such as ATG5. We found that the ability of ATG12 to kill oncogenic RAS-carrying malignant cells does not require covalent binding of ATG12 to other proteins. In summary, we have identified a novel mechanism by which oncogenic RAS promotes survival of malignant intestinal epithelial cells. This mechanism is driven by RAS-dependent loss of ATG12 in these cells.

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apoptosis; ATG12; autophagy; colorectal cancer; RAS

Introduction

Activating mutations of RAS are among the most frequently occurring oncogenic events in cancer. Such mutations were found in about 50% of colorectal,[1] 90% of pancreatic[2] and 30% of lung cancers.[3] RAS is a GTPase that binds GTP in response to various mitogenic signals.[4] GTP-bound and thus activated RAS triggers oncogenic signals in cancer cells by associating with proteins, such as RAF, the phosphoinositide 3-kinase (PI3K) or RAL-specific guanine nucleotide exchange factors.[5]

Activating mutations of RAS initiate lung,[6] pancreatic[7] and colon[8] cancer in mice, and mutant RAS is required for tumor maintenance in various mouse models of cancer.[6,9] Also importantly, treatments targeting other oncoproteins are often ineffective in cancers carrying mutant RAS.[10] Thus, RAS and RAS-induced signals are thought to be critical targets for cancer treatment.[10] However, so far attempts to develop therapies of mutant RAS-carrying cancers aimed at RAS or its effectors have been unsuccessful owing to insufficient understanding of the salient mechanisms of RAS signaling.[10] To address this knowledge gap the US National Cancer Institute has started a program aimed at studying the mechanisms of RAS-dependent cancers.[10]

It is now well established that RAS-dependent cell survival signals play a critical role in the progression of mutant RAS-carrying cancers.[11] For example, it is known that epithelial cells of many organs, including those of the intestine, grow in vivo as monolayers attached to the basement membrane, a form of the extracellular matrix (ECM). Detachment from the ECM causes growth arrest and apoptosis of such cells.[12-15] In contrast, detached tumor cells carrying oncogenic RAS can survive and grow,[16,17] and these properties of cancer cells are critical for tumor growth, invasion and metastasis.[18-20] We have observed that mutant RAS rescues detached cells from death by downregulating pro-apoptotic proteins BAK1/Bak[16] and CASP2/caspase-2[21] and upregulating apoptosis inhibitors BCL2L1/Bcl-X_L,[17] BIRC3/cIAP2 and XIAP.[22] We found that the reversal of the effect of RAS on BAK1, BCL2L1 and CASP2 blocks survival of detached cells in tissue culture and their tumorigenicity in mice.[16,17,21]

It is also known that autophagy, a process of cellular self-consumption,[23] plays a major role in the progression of oncogenic RAS-positive tumors. It has been found that RAS-driven progression of lung and pancreatic cancers is strongly reduced in mice lacking autophagy mediators ATG5 and ATG7.[24,25] To explain these results it was proposed that ATG7 or ATG5 loss blocks basal autophagy in tumor cells. Such autophagy is likely needed by these cells to adapt to nutrient deficiency which they often encounter in vivo, e.g. due to the disorganized nature of

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tumor vasculature.[26] It was proposed that basal autophagy causes degradation of nonessential cellular molecules and thus generates nutrients that are critical for cancer cell metabolism.[27,28]

Even though basal autophagy appears to be essential for RAS-driven tumor growth, uncontrolled, excessive autophagy represents a likely barrier for RAS-dependent cellular malignant transformation. We found that nonmalignant intestinal epithelial cells undergo growth arrest after detachment from the ECM due to the induction of autophagy which is triggered by detachment-dependent upregulation of autophagy mediators ATG3 and ATG7.[29] Furthermore, we observed that RAS blocks autophagy and growth arrest of detached colon cancer cells by downregulating the autophagy mediator BECN1/Beclin-1.[30] Thus, RAS-induced cellular malignant transformation requires basal autophagy, but RAS-dependent signals prevent such autophagy from exceeding certain levels, and these signals are critical for RAS-driven malignant cellular transformation.

Given that regulation of cellular apoptotic and autophagy machineries by RAS clearly plays a major role in the ability of RAS to promote solid tumor growth, we continued investigating the effect of RAS on the components of these machineries. We found that oncogenic RAS strongly downregulates the protein ATG12 in colon cancer cells. ATG12 is a critical mediator of autophagy which drives this process by forming a covalent complex with proteins, such as ATG5.[31] In addition, ATG12 has the ability to kill cells by autophagy-unrelated mechanisms.[32] We found that the reversal of the effect of RAS on ATG12 efficiently kills mutant RAS-carrying malignant intestinal epithelial cells and that the ability of ATG12 to exert this effect on cells does not require its ability to covalently bind other proteins. Thus, the mechanisms by which RAS downregulates ATG12 in such cells represent potential novel targets for treatment of oncogenic RAS-driven colon cancer.

Results

Oncogenic RAS downregulates ATG12 in intestinal epithelial cells

To explore the mechanisms by which RAS controls cellular autophagy machinery we used nonmalignant spontaneously immortalized rat intestinal epithelial cells IEC-18[33] and highly tumorigenic[21,34] clones of these cells ras-3 and ras-4[35] (referred to below as IEC-ras cells) obtained independently of each other by transfection of IEC-18 cells with an oncogenic HRAS-encoding vector.[36] Using this cell model we have discovered that RAS downregulates a multifunctional pro-apoptotic protein ATG12 in IEC-18 cells (Fig. 1A).

Covalent complexes between ATG12 and ATG5[31] and possibly between ATG12 and ATG3[37,38] promote autophagy. ATG12 can also cause autophagy-independent apoptosis.[32] Apoptosis is mediated by the release of CYCS/cytochrome c from the mitochondria to the cytoplasm where it triggers activation of caspases,[39] proteases that cleave vital cellular targets.[40] CYCS release is caused by the pro-apoptotic BCL2-family proteins using a "Bcl-2 homology 3" domain to bind and neutralize the anti-apoptotic BCL2 family members (which block CYCS release).[41] ATG12 contains such domain and kills cells by the same mechanisms.[32] This effect of ATG12 does not require the ability of ATG12 to covalently bind other autophagy mediators.[32]

The effect of RAS on ATG12 was not unique to rat cells as human colon cancer cells DLD1[42] carrying a mutant *KRAS* allele showed lower free ATG12 levels than their variants DKO3 and DKS8, in which this allele was ablated by homologous recombination (Fig. 1B).[42] Furthermore, mutant *KRAS*-positive human colon cancer cells LS180,[43] LoVo[43] and SKCO1[44] displayed lower free ATG12 levels than human colon cancer cells HT29[45] and CaCo2[43] expressing the wild-type *KRAS* (Fig. 1C). Finally, we observed that introduction of the mutant *KRAS* gene in mutant KRAS-negative cells CaCo2 resulted in a noticeable downregulation of free ATG12 (Fig. 1D). Thus, oncogenic RAS reduces free ATG12 levels in malignant intestinal epithelial cells.

RAS-induced ATG12 downregulation is critical for clonogenic survival of malignant intestinal epithelial cells

To test the role of ATG12 in cancer cell growth we infected ras-4 cells with a control murine stem cell virus (MSCV) or MCSV encoding ATG12. Infection efficiency was close to 100% as puromycin (resistance to which was encoded by MSCV) killed essentially all uninfected cells but essentially all cells were clonogenic in the presence of puromycin after being infected with a control MSCV (not shown). We found that ras-4 cells infected with ATG12-encoding viruses produced free ATG12 at levels that were significantly higher than those in the cells infected with a control virus and comparable to those in the parental IEC-18 cells (Fig. 2A). We also observed a band recognized by the anti-ATG12 and anti-ATG5 antibodies on the respective western blots that displayed a reduced mobility compared with the ATG12-ATG5 complex, most likely, due to the conjugation of ectopic ATG12 with endogenous ATG5 (Fig. 2A and B). We found that exogenous ATG12 strongly blocked clonogenicity of these cells (Fig. 2C). This observation was not unique to ras-4 cells as ectopic ATG12 also noticeably blocked clonogenicity of human mutant KRAS-positive cells LS180 (Fig. 2D and E). Unlike the case with mutant RAS-carrying cells, exogenous ATG12 did not have a significant effect on clonogenicity of nonmalignant IEC-18 cells (Fig. 2F and G).

Our data are consistent with the well-known fact that RAS and other major oncoproteins, e.g. EGFR, SRC, AKT, BCR-ABL and CTNNB1 (catenin β 1) simultaneously trigger both pro- and anti-apoptotic signals in malignant cells.[46-48] The cell death-promoting events represent an aspect of a phenomenon referred to as "oncogenic stress."[49] Oncogenic stress is oncogene-induced activation of anti-oncogenic signals that promote apoptosis or cell cycle arrest.[49] Oncogenic stress was proposed to be a safeguard mechanism that prevents tumor growth.[50] In those cells that succumb to malignant transformation, oncogene-triggered survival signals are believed to outbalance the death signals,[46] and the cells survive. Our data indicate that RAS-induced ATG12 loss triggers cell survival signals, which are critical for the viability of the respective cells. Once the wild-type ATG12 levels in the mutant RAS-carrying cells are restored, the pro-apoptotic signals outbalance the anti-apoptotic signals and the cells die.

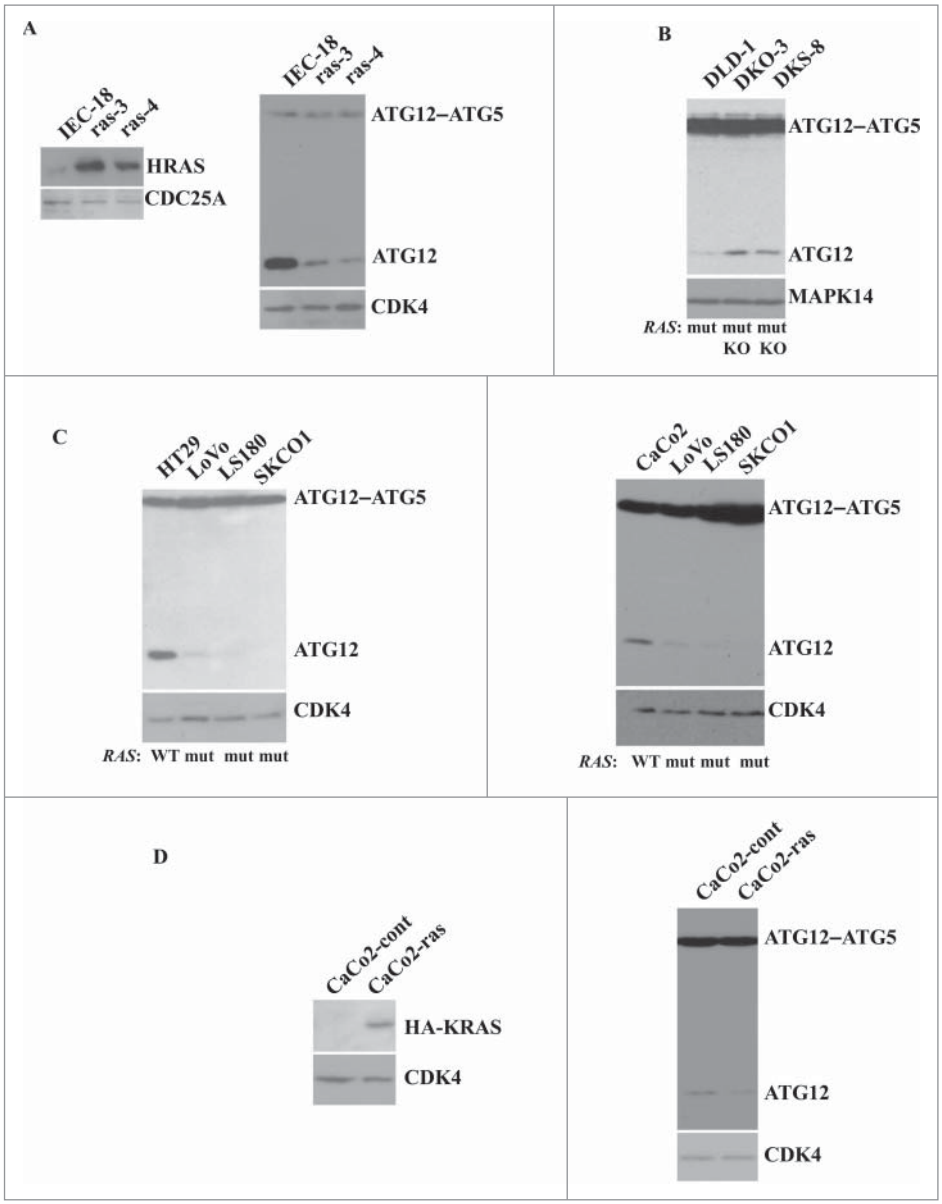

Figure 1. Oncogenic RAS downregulates unconjugated ATG12 in intestinal epithelial cells. (A) IEC-18 cells and 2 independently derived HRAS-transformed clones of these cells ras-3 and ras-4 were assayed for RAS (left) or ATG12 (right) expression by western blot. (B) Human colon carcinoma cells DLD-1 carrying a mutant *KRAS* allele and their mutant *KRAS* knockout derivatives DKO-3 and DKS-8 were assayed for ATG12 expression by western blot. (C) Human colon cancer cells HT29 (left) and CaCo2 (right) carrying the wild-type *KRAS* and human colon cancer cells LoVo, LS180 and SKCO1 carrying a mutant *KRAS* allele (left and right) were assayed for ATG12 expression by western blot. (D) Stable cell lines CaCo2-cont and CaCo2-ras generated by infection of human colon cancer cells CaCo2 with either a control retrovirus (CaCo2-cont) or HA-tagged an oncogenic KRAS mutant-encoding retrovirus (CaCo2-ras) were assayed for KRAS (left) or ATG12 (right) expression by western blot. CDC25 (A, left), CDK4 (A, right, C, and D) and MAPK14/p38 MAP kinase (B) served as loading controls. Positions of unconjugated ATG12 (ATG12), the ATG12-ATG5 (ATG12-ATG5) conjugate and that of HA-tagged KRAS on the blots are indicated.

ATG12-induced loss of clonogenicity by mutant RAS-carrying malignant intestinal epithelial cells does not require the ability of ATG12 to conjugate with other proteins

We found that IEC-ras cells infected with exogenous ATG12 contained increased levels of both free ATG12 and those of the complex between exogenous ATG12 and ATG5 (Fig. 2A and B). The ATG12-ATG5 complex promotes autophagy by mediating lipidation of critical mediators of autophagy, such as MAP1LC3/LC3, and autophagy causes cell death under certain circumstances.[51] Moreover, ATG12 can kill cells by autophagy-independent mechanisms.[32] Hence, ATG12-dependent death of cancer cells could, in principle, be caused by the increase in the cellular levels of the ATG12-ATG5 complex or those of the unconjugated ATG12. To distinguish between these possibilities, we first tested whether exogenous ATG12 promotes MAP1LC3 lipidation in ras-4 cells and did not observe any significant changes in the cellular levels of MAP1LC3B-II, the lipidated form of MAP1LC3B, in response to ectopic ATG12 at various times postinfection with the ATG12-encoding retrovirus (Fig. 3A and B). Moreover, exogenous ATG12 did not alter MAP1LC3 lipidation in the presence of the lysosomal inhibitor bafilomycin A_1 that disrupts the autophagosome-to-lysosome

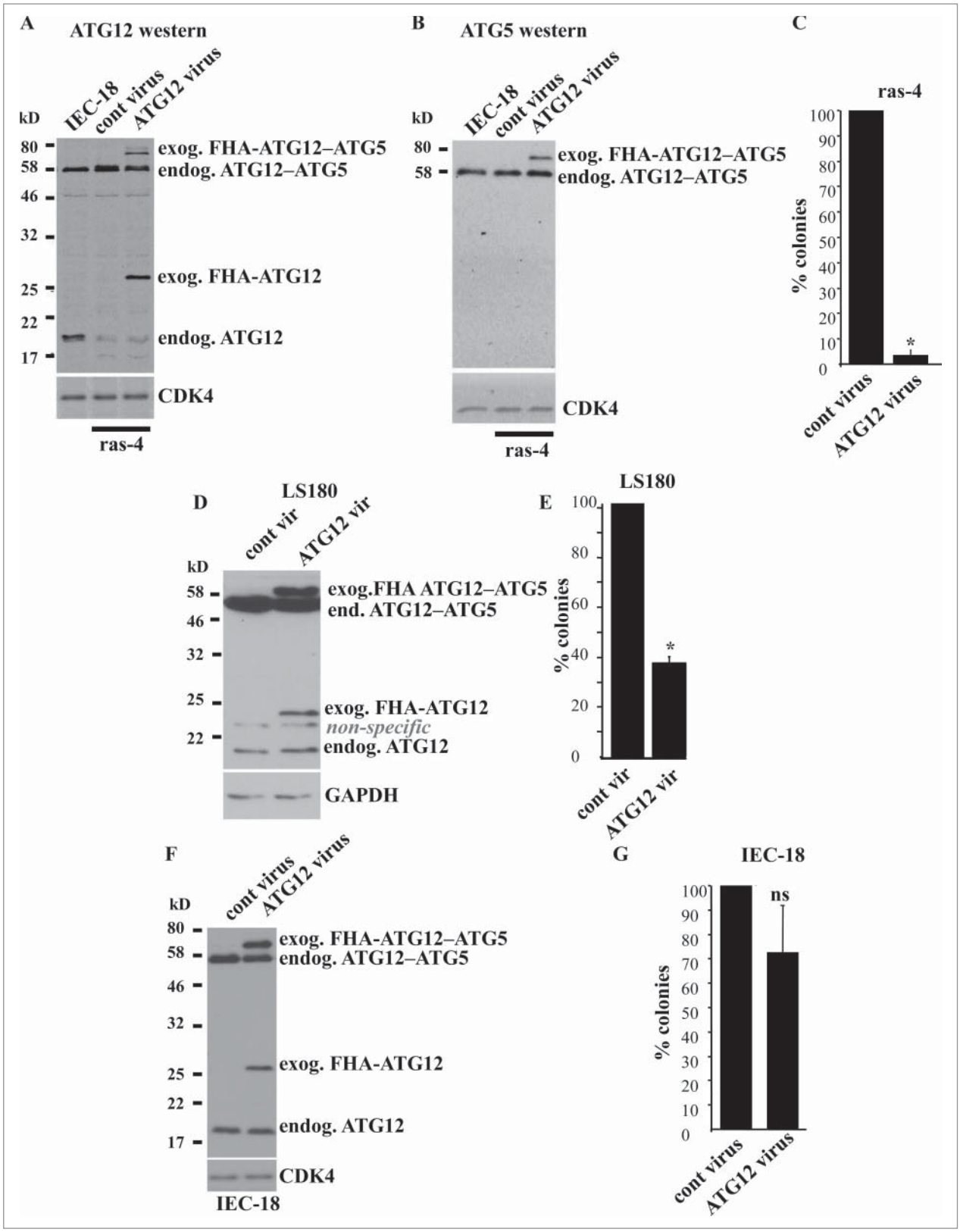

Figure 2. Exogenous ATG12 blocks clonogenicity of oncogenic RAS-carrying malignant intestinal epithelial cells. (A, B, D, and F) ras-4 cells (A and B), LS180 cells (D) and IEC-18 cells (F) were infected with the control MSCV (cont virus) or FLAG- and HA-tagged ATG12-encoding MSCV (ATG12 virus), treated for 24 h with 6 μg/ml puromycin and tested for ATG12 (A, D, and F) or ATG5 (B) expression by western blot along with the parental nonmalignant cells IEC-18 (A and B). Positions of the endogenous ATG12 (endog. ATG12), FLAG- and HA-tagged exogenous ATG12 (exog. FHA-ATG12), endogenous ATG12-ATG5 (endog. ATG12-ATG5) conjugate and that of the complex formed by the FLAG- and HA-tagged exogenous ATG12 and ATG5 (exog. FHA-ATG12-ATG5) complex on the gel are indicated. CDK4 (A, B, and F) and GAPDH (D) served as loading controls. Positions of molecular mass markers (kD) on the blots are indicated. (C, E, and G) ras-4 (C), LS180 (E) and IEC-18 (G) cells treated as in (A, D, and F), respectively were allowed to form colonies for 7 d in the presence of 6 μg/ml puromycin. The number of colonies formed by the cells infected with a control virus was designated as 100%. The numbers represent the average of 3 independent experiments plus SD. * indicates that P value was less than 0.05. ns - not significant.

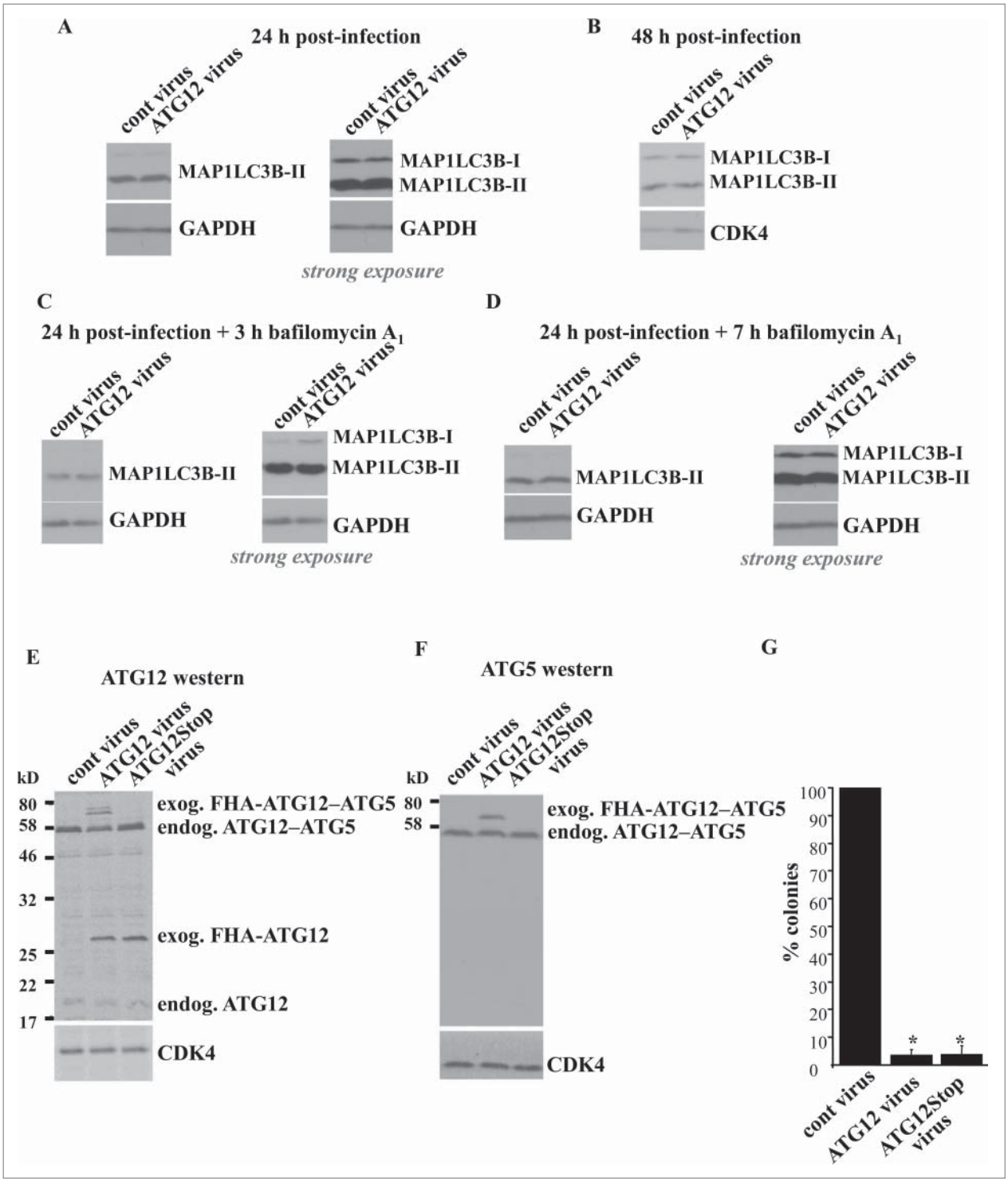

Figure 3. Exogenous ATG12 blocks clonogenicity of oncogenic RAS-carrying malignant intestinal epithelial cells regardless of the ability of ATG12 to form covalent complexes with other proteins. (A-D) ras-4 cells were infected with the control MSCV (cont virus) or FLAG- and HA-tagged ATG12-encoding MSCV (ATG12 virus), cultured for the indicated times in the presence of 6 μg/ml puromycin in the absence (A and B) or in the presence (C and D) of 50 nM bafilomycin A_1 and assayed for MAP1LC3B-I and MAP1LC3B-II expression by western blot. "Strong" exposure of the blots is shown to demonstrate our ability to distinguish between MAP1LC3B-I and MAP1LC3B-II. (E and F) ras-4 cells were infected with the control MSCV (cont virus) or FLAG- and HA-tagged ATG12-encoding MSCV (ATG12 virus), or FLAG- and HA-tagged ATG12Stop mutant-encoding MSCV (ATG12Stop virus), treated for 24 h with 6 μg/ml puromycin and tested for ATG12 (E) or ATG5 (F) expression by western blot. Positions of the endogenous ATG12 (endog. ATG12), FLAG- and HA-tagged exogenous ATG12 (exog. FHA-ATG12), endogenous Atg12-Atg5 (endog. ATG12-ATG5) conjugate and that of the complex formed by the FLAG- and HA-tagged exogenous ATG12 and ATG5 (exog. FHA-ATG12-ATG5) complex on the blots are indicated. Positions of molecular mass markers (kD) on the blots are indicated. GAPDH (A, C, and D) and CDK4 (B, E, and F) served as loading controls. (G) ras-4 cells treated as in (E) were allowed to form colonies for 7 d in the presence of 6 μg/ml puromycin. The number of colonies formed by the cells infected with a control virus was designated as 100%. * indicates that P value was less than 0.05.

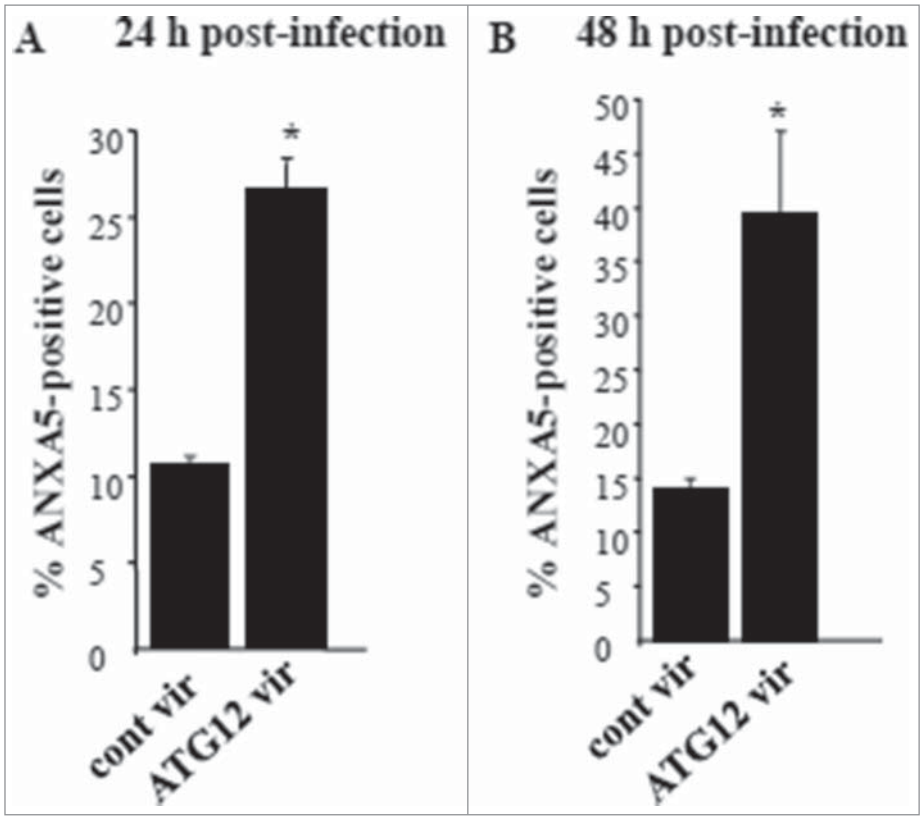

Figure 4. Exogenous ATG12 renders oncogenic RAS-carrying malignant intestinal epithelial cells ANXA5-positive (A and B) ras-4 cells were infected with the control MSCV (cont virus) or ATG12-encoding MSCV (ATG12 virus), cultured for 24 (A) or 48 (B) h in the presence of 6 μg/ml puromycin and assayed for ANXA5 and PI binding by flow cytometry. The data represent the average of the duplicates plus the SD. * indicates that P value was less than 0.05.

fusion and thus causes enrichment of the cells with the lipidated form of MAP1LC3[38] (Fig. 3C and D). As reported by others,[38] we observed some variability in the amounts of MAP1LC3B-I and provided "strong" exposures of blots in respective figures to demonstrate our ability to distinguish between MAP1LC3B-I and II (Fig. 3A, C, and D). Thus, exogenous ATG12 does not seem to alter basal levels of MAP1LC3B-II in these cells. One possible explanation of this result is that ATG12 is produced by IEC-ras cells in amounts sufficient for triggering maximally possible basal lipidation of MAP1LC3B, and further increase in ATG12 levels does not enhance this process.

We further infected ras-4 cells with a control MSCV or an MSCV encoding the ATG12Stop mutant in which the codon for the C-terminal Gly required for ATG12 conjugation to other proteins is deleted[52] (Fig. 3E and F). Loss of this Gly blocks the ability of ATG12 to promote autophagy.[53] As expected, unlike the wild-type ATG12, ATG12-Stop protein did not form detectable complexes with any cellular proteins (Fig. 3E and F). We further found that both the wild-type and the mutant ATG12 proteins, when produced by IEC-ras cells at similar levels, had the same ability to block clonogenicity of these cells (Fig. 3G). Thus, ATG12-dependent death of oncogenic RAS-carrying intestinal epithelial cells does not require the ability of ATG12 to form conjugates with other proteins and is not associated with the ability of ATG12 to promote MAP1LC3 lipidation.

ATG12 triggers both apoptotic and nonapoptotic cell death-promoting signals in mutant RAS-carrying cells

To investigate the mechanisms by which ATG12 blocks clonogenicity of malignant cells we measured the ability of ectopic ATG12 to promote binding of ANXA5/Annexin V to phosphatidylserine on the surface of IEC-ras cells, which typically happens at the early stages of apoptosis.[54] ANXA5 can also bind such cells at the late stages of apoptosis associated with cytoplasmic swelling and damage of the plasma membrane.[55,56] The latter damage is normally assessed by cellular permeability to propidium iodide (PI)[21,57] Depending on which stage of apoptosis is captured in a particular experiment, ANXA5-positive cells can be PI-negative, PI-positive or both.[21,57] In addition, this assay allows to detect necrotic cells that that are PI-positive but ANXA5-negative.[56] We observed that exogenous ATG12 noticeably increased binding of ANXA5 to ras-4 cells at 24 h and 48 h postinfection with the ATG12-encoding retrovirus (Fig. 4A and B). Thus, our data are consistent with the documented ability of ATG12 to promote apoptosis.[32]

ATG12 triggers apoptotic cell death by neutralizing the anti-apoptotic BCL2 family members, such as MCL1, and thus blocking mitochondria-dependent ("intrinsic") apoptosis.[32] Thus, we reasoned that if ATG12 kills IEC-ras cells in this manner their ATG12-induced apoptosis should be suppressible by MCL1. Indeed, when ras-4 cells were co-infected with the retroviruses encoding both ATG12 and MCL1, their binding of ANXA5 was significantly reduced compared with that of the

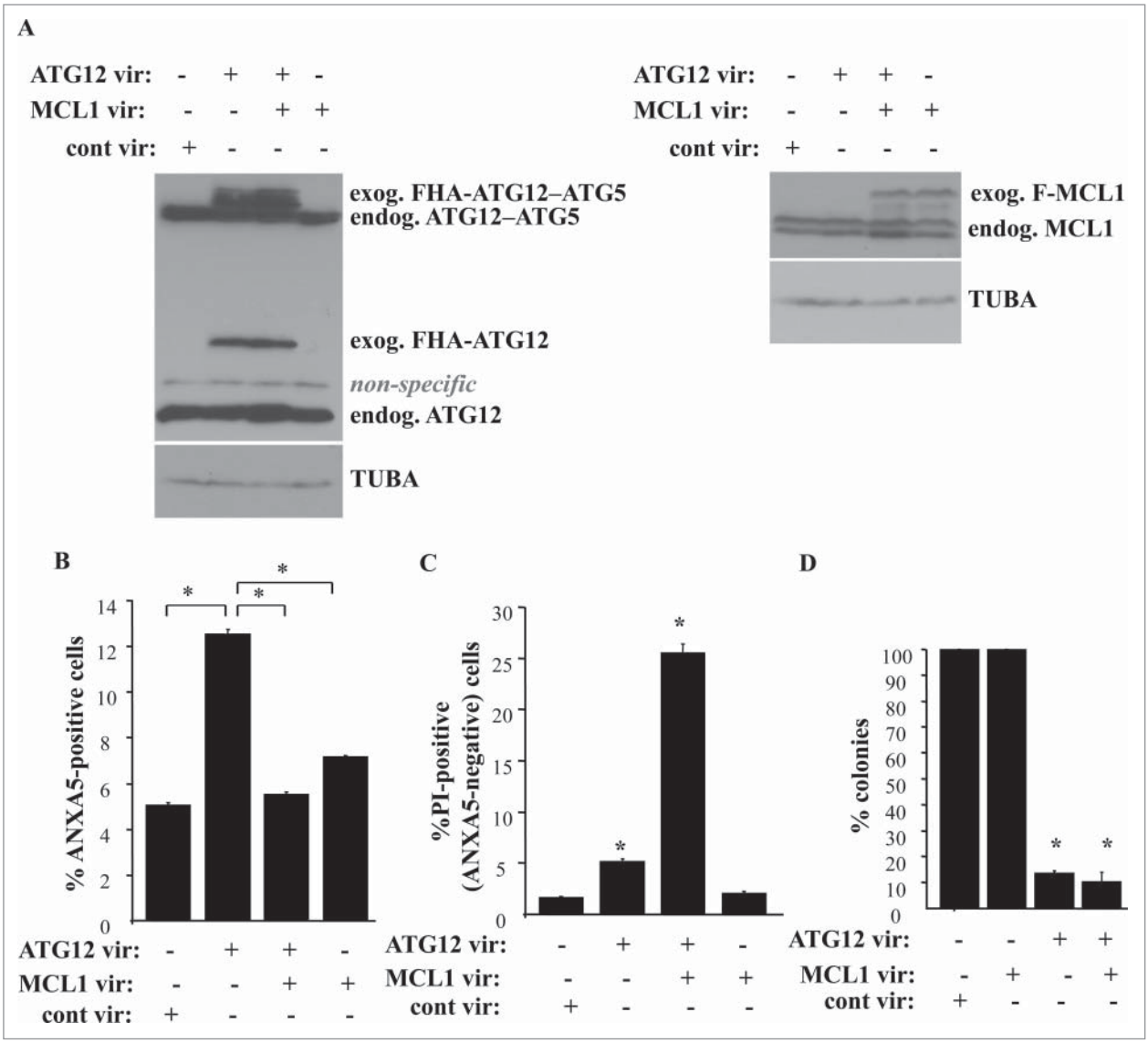

Figure 5. ATG12-induced ANXA5 binding but not clonogenic survival of oncogenic RAS-carrying malignant intestinal epithelial cells can be blocked by MCL1. (A) ras-4 cells were infected (+) or not infected (-) with the control (cont vir), FLAG- and HA-tagged ATG12- (ATG12 vir) or FLAG-tagged MCL1-(MCL1 vir)-encoding viruses, cultured for 24h in the presence of 6 μg/ml puromycin and assayed for ATG12 (left) or MCL1(right) expression by western blot. Positions of the endogenous ATG12 (endog. ATG12), FLAG- and HA-tagged exogenous ATG12 (exog. FHA-ATG12), endogenous ATG12-ATG5 (endog. ATG12-ATG5) conjugate, that of the complex formed by the FLAG- and HA-tagged exogenous ATG12 and ATG5 (exog. FHA-ATG12-ATG5) complex (A) as well as that of the endogenous MCL1 (endog. MCL1) and that of the exogenous FLAG-tagged MCL1 (exog. F-MCL1) on the blots are indicated. TUBA/α-tubulin served as a loading control. (B and C) Cells treated as in (A) were assayed for ANXA5 and PI binding by flow cytometry. Percentage of ANXA5-positive cells is shown in (B) and that of the PI-positive cells in (C). (D) Cells treated as in (A) were allowed to form colonies for 7 d in the presence of 6 μg/ml puromycin. The number of colonies formed by the cells infected with a control virus was designated as 100%. The numbers represent the average of the duplicates (B and C) and that of the triplicates (D) plus SD. * indicates that P value was less than 0.05.

cells infected only with an ATG12-encoding virus (Fig. 5A and B). These data are consistent with a scenario that ATG12 triggers the intrinsic mechanisms apoptosis of these cells. Remarkably, even though ATG12 was unable to promote ANXA5 binding of MCL1-expressing ras-4 cells, the presence of MCL1 noticeably increased the percentage of PI-positive, ANXA5-negative, i.e. necrotic,[55,56] cells in the population of ectopic ATG12-producing cells (Fig. 5C). (All relevant numbers derived from a representative experiment are also shown in Fig. S1). The presence of necrotic cells in this population was not the consequence of intrinsic apoptosis, as their emergence could not be blocked (but was rather increased) by MCL1 (Fig. 5C). Moreover, exogenous MCL1 did not prevent ATG12-induced loss of clonogenicity by the cells (Fig. 5D). Thus, our data indicate that ATG12 triggers both apoptotic and necrotic signals in cancer cells and that once the apoptotic signals are blocked, the cells die by necrosis. Scenarios when increased expression of the anti-apoptotic BCL2 family members in the cells triggers a switch from apoptotic to necrotic cell death in response to different stimuli have been described by others.[58,59] In addition, the fact that loss of a pro-apoptotic protease CASP8/caspase-8, another component of the cellular apoptotic machinery, by the cells promotes their necrosis is well established.[60,61] The mechanisms by which ATG12 triggers necrosis are not known and understanding them represents an important direction for our future studies.

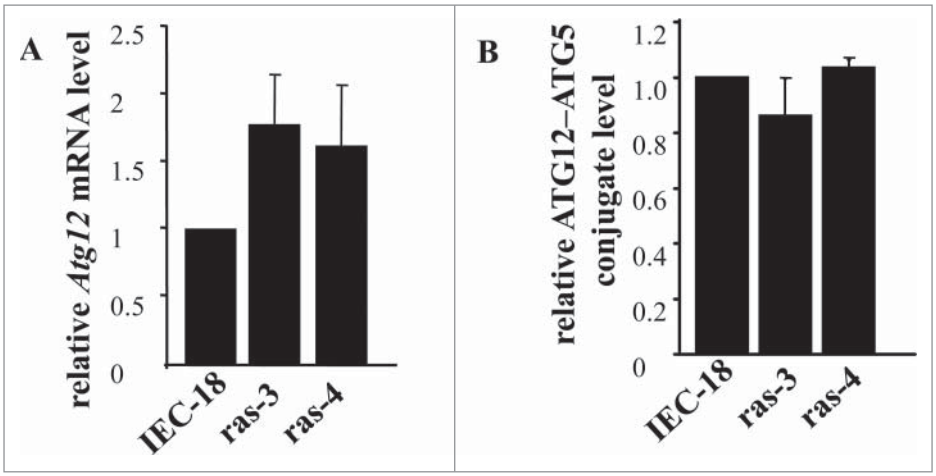

Figure 6. Oncogenic RAS does not downregulate the *Atg12* mRNA in intestinal epithelial cells. (A) IEC-18, ras-3 and ras-4 cells were assayed for *Atg12* mRNA expression by quantitative PCR (qPCR). The observed *Atg12* mRNA levels were normalized by the levels of *Rn18S/18S* rRNA which were also determined by qPCR. The resulting levels of the *Atg12* mRNA in IEC-18 cells were designated as 1.0. The data represent the average of 2 independent experiments plus the SD. (B) IEC-18, ras-3 and ras-4 cells were assayed for ATG12 expression by western blot as in Figure 1A and relative amounts of the ATG12-ATG5 complex were quantified by densitometry. The amount of the ATG12-ATG5 complex in IEC-18 cells was designated as 1.0. The data represent the average of 2 independent experiments plus SD.

Oncogenic RAS does not downregulate Atg12 *mRNA in intestinal epithelial cells*

We further investigated the mechanisms by which oncogenic RAS downregulates free ATG12 in intestinal epithelial cells. We found that IEC-ras cells do not display reduced levels of *ATG12* mRNA compared with the parental IEC-18 cells. To the contrary, levels of this mRNA appeared to be somewhat higher in the oncogenic RAS-expressing cells compared with the nonmalignant cells (Fig. 6A). Hence, RAS-induced ATG12 loss by cancer cells is unlikely caused by the effect of RAS on the *ATG12* mRNA. Moreover, quantitative comparison of the levels of the ATG12-ATG5 conjugates between IEC-18 and IEC-*ras* cells by densitometry, indicated that oncogenic RAS does not increase the amount of this complex in the cells (Fig. 6B). These data indicate that the reduction of free ATG12 levels in the RAS-transformed cells compared with the parental cells is unlikely caused by increased ATG12-to-ATG5 conjugation.

A proteasome inhibitor upregulates ATG12 in intestinal epithelial cells

Since oncogenic RAS downregulates ATG12 in intestinal epithelial cells without causing any reduction of the cellular *ATG12* mRNA levels or changes in ATG12-to-ATG5 conjugation, we investigated whether RAS alters ATG12 protein stability in these cells. One major mediator of protein stability is the ubiquitin-proteasome system (UPS). Destruction by the UPS starts with covalent binding of a small protein ubiquitin to lysine residues of a target protein.[62] Several more ubiquitins are then linked to that attached to the target protein.[62] These events act as a signal for protein degradation by a multiprotein complex called the proteasome.[62] Others have found that the unconjugated ATG12 but not the ATG12-ATG5 complex can be degraded in cells by the UPS.[63] To test whether ATG12 levels in intestinal epithelial cells are controlled by the UPS we treated IEC-*ras* cells with MG132, a widely used proteasome inhibitor.[64,65] This strongly upregulated the unconjugated ATG12 in the cells (Fig. 7A and B) and killed most of them (Fig. 7C). We also observed that MG132 treatment of the cells resulted in the emergence of an extra band on the respective blot of a higher molecular weight than that corresponding to ATG12 (Fig. 7A and B). We further found that immunoprecipitation (IP) of ATG12 from lysates derived from MG132-treated cells followed by western blot using an anti-ubiquitin antibody caused the emergence of a smear on the blot which is characteristic of the polyubiquitinated proteins[66] (Fig. 7D). An extra band recognized by the anti-ATG12 antibody on the western blot in MG132-treated cells (Fig. 7A and B) was not detected in the ATG12 immunoprecipitation followed by ubiquitin western blot experiment (Fig. 7C) indicating that this band unlikely represents a ubiquitinated form of ATG12. While the nature of the band remains unclear, it cannot be excluded that the band is nonspecific (and is "unmasked" in MG132-treated cells). Indeed, similar to what was observed in IEC-ras cells, MG132-treatment of mutant KRAS-positive human colon cancer cells DLD1 and SKCO1 strongly upregulated free ATG12 but the extra band in question was not detected (Fig. 7E). Finally, we noticed that treatment of nonmalignant intestinal epithelial cells IEC-18 with MG132 also upregulates ATG12 (Fig. 7F). Thus, our data indicate that free ATG12 is turned over in the nonmalignant and mutant RAS-carrying intestinal epithelial cells by ubiquitination and further proteasomal degradation.

Oncogenic RAS reduces ATG12 protein stability in intestinal epithelial cells

Since oncogenic RAS downregulated ATG12 in intestinal epithelial cells by a mechanism that does not involve the reduction of the cellular *ATG12* mRNA levels (Fig. 6A) and since ATG12 protein seemed to be turned over in these cells via UPS-dependent degradation (Fig. 7), RAS likely accelerates this degradation. We reasoned that if this is the case then the reduction of the cellular *Atg12* mRNA levels by RNA interference (RNAi)

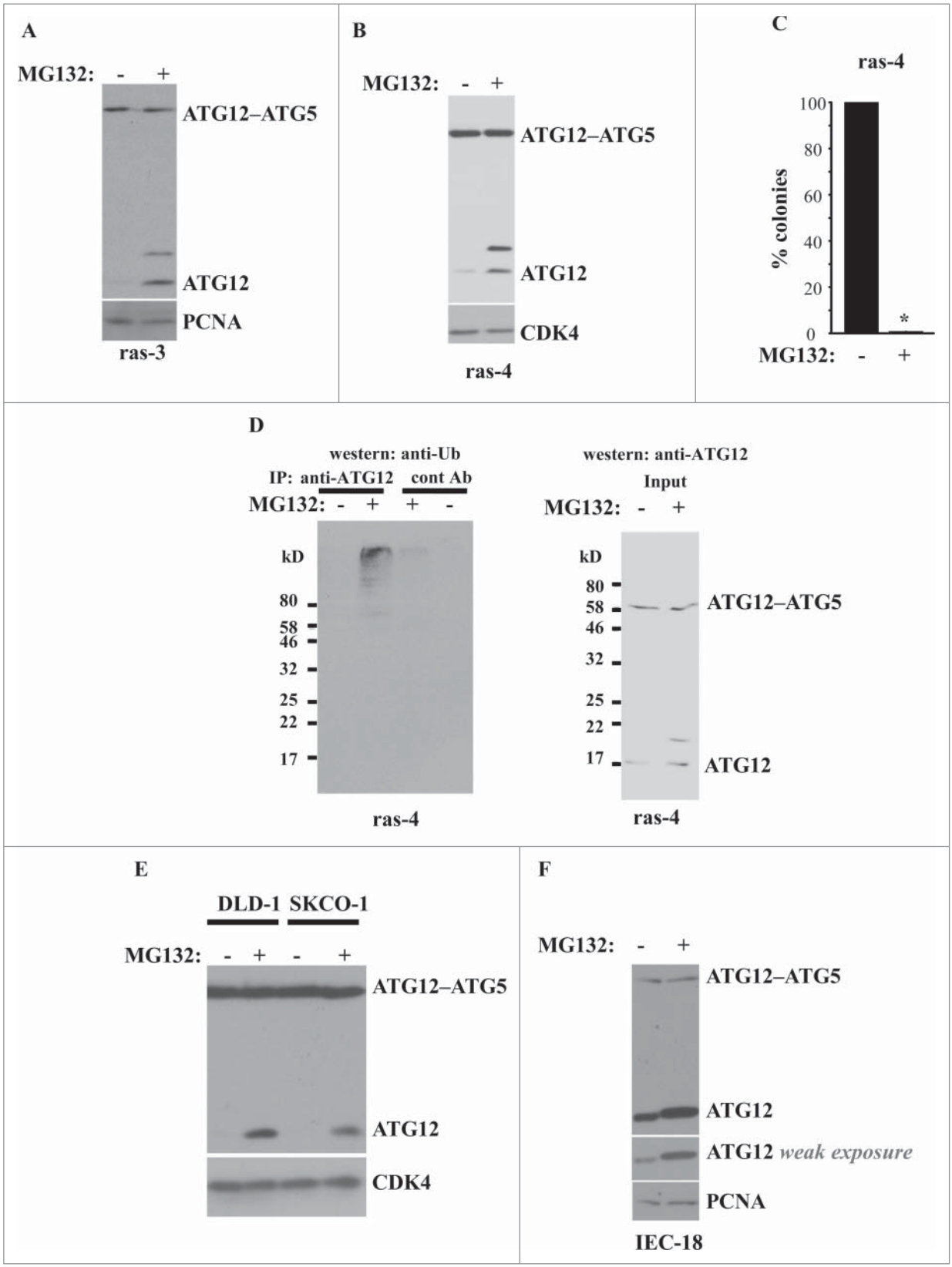

Figure 7. Treatment with a small molecule proteasome inhibitor MG132 upregulates ATG12 in intestinal epithelial cells. (A) ras-3 and (B) ras-4 cells were treated with either DMSO (-) or 10 μM MG132 (+) for 24 h and assayed for ATG12 expression by western blot. Positions of the unconjugated ATG12 (ATG12) and ATG12-ATG5 (ATG12-ATG5) conjugate on the gel are indicated. (C) ras-4 cells treated as in (B) were allowed to form colonies for 7 d. The number of colonies formed by the control cells was designated as 100%. The numbers represent the average of 3 independent experiments plus SD. * indicates that P value was less than 0.05. (D, left) Proteins were immunoprecipitated from lysates derived from ras-4 cells treated as in (B) with an anti-ATG12 antibody or the same isotype control antibody against a protein CDADD/RAIDD whose expression is not known to be controlled by ubiquitination and the precipitated proteins were analyzed by western blot with an anti-ubiquitin antibody. (D, right) Lysates derived from ras-4 cells treated as in (D, left) were tested for ATG12 expression by western blot (Input). (E and F) Human colon cancer cells DLD1 and SKCO1 carrying oncogenic KRAS (E) or nonmalignant rat intestinal epithelial cells IEC-18 (F) were treated and analyzed as in (A). "Weak" exposure of the blot is shown in (F) to better demonstrate the effect of MG132 on ATG12. PCNA (A and F) and CDK4 (B and E) served as loading controls.

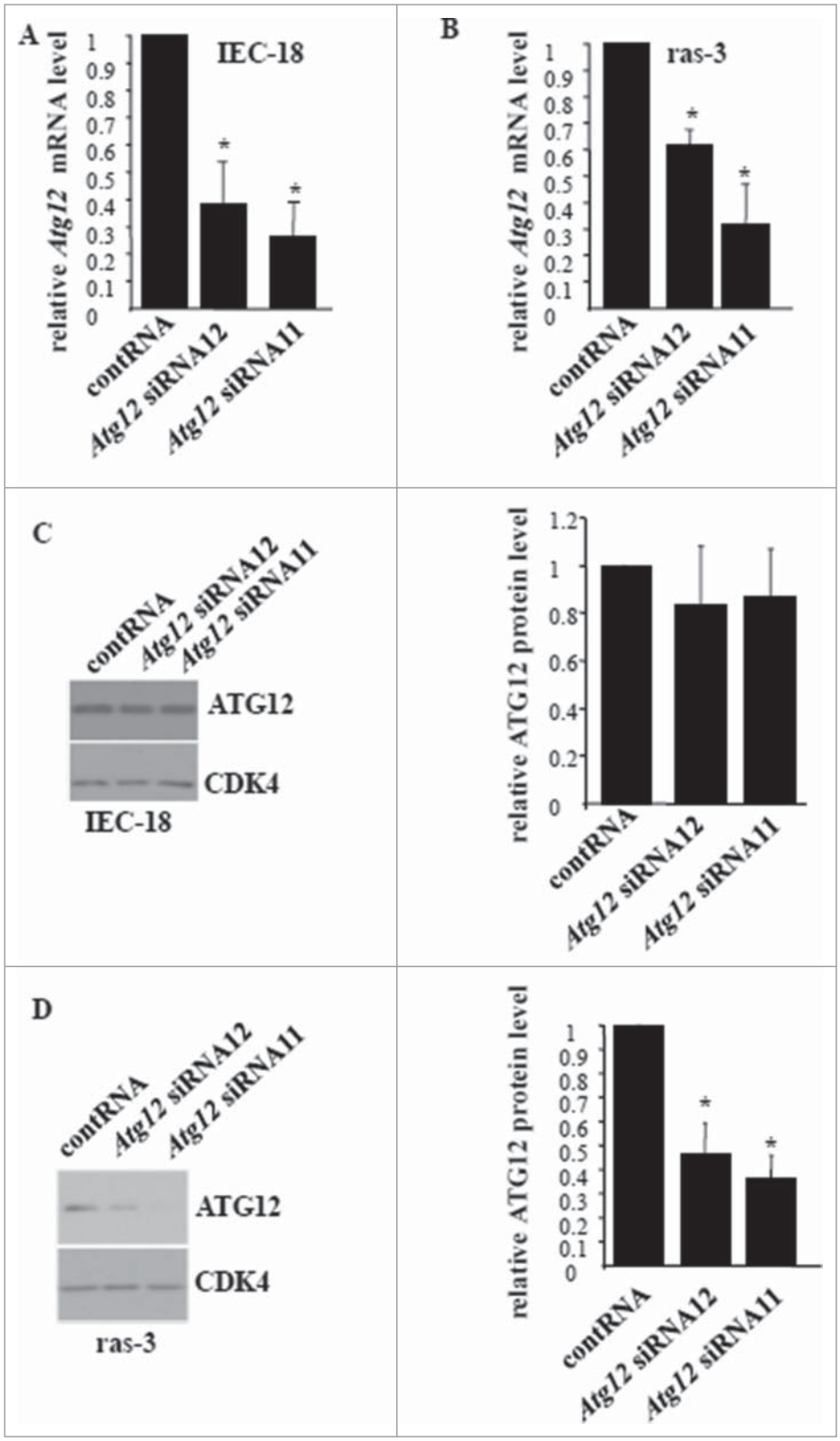

Figure 8. Oncogenic RAS reduces stability of the unconjugated ATG12 protein. (A) IEC-18 or (B) ras-3 cells were transfected with 100 nM control RNA (contRNA) or *Atg12*-specific small interfering RNA (*Atg12siRNA*) 11 or 12 and assayed for *Atg12* mRNA expression by qPCR. The observed *Atg*12 mRNA levels were normalized by the levels of *Rn18S/18S* rRNA which were also determined by qPCR. The levels of the *Atg12* mRNA in cells transfected with a control RNA were designated as 1.0. The data in (A) represent the average of 3 independent experiments plus the SE. The data in (B) represent the average of 4 independent experiments in case of ATGT12siRNA11 and that of 3 independent experiments in case of *Atg*12 siRNA12 plus the SE. (C) IEC-18 or (D) ras-3 cells were treated as in (A) or (B), respectively, and assayed for the expression of free ATG12 protein by western blot (C, left and D, left). CDK4 served as loading control. (C, right and D, right) Cells were assayed as in (C, left and D, left), and the amount of free ATG12 in the cells was quantified by densitometry. The levels of ATG12 protein in cells transfected with a control RNA were designated as 1.0. The data represent the average of 3 independent experiments plus SD. * indicates that p-value was less than 0.05.

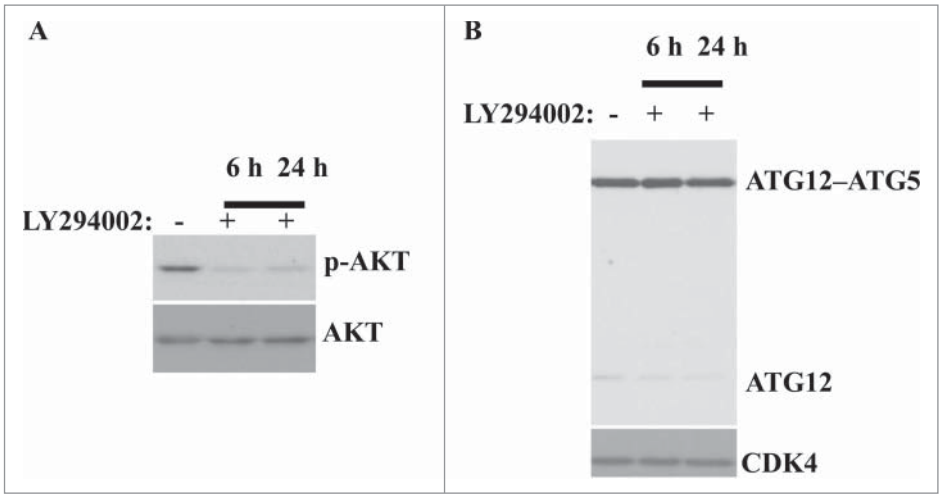

Figure 9. RAS-induced ATG12 downregulation in intestinal epithelial cells cannot be blocked by a PtdIns3K inhibitor. ras-4 cells were cultured in the presence of DMSO (-) for 24 h or 20 μM LY294002 (+) for the indicated times and assayed for phospho-AKT (p-AKT; A) or ATG12 (B) expression by western blot. Positions of the unconjugated ATG12 (ATG12) and ATG12-ATG5 conjugate (ATG12-ATG5) on the gel are indicated in (B). AKT (A) and CDK4 (B) served as loading controls.

and the resulting blockade of ATG12 protein synthesis can be expected to cause a more noticeable downregulation of the ATG12 protein in IEC-ras cells than in the parental IEC-18 cells. Indeed, we found that transfection with 2 separate *Atg12*-specific small interfering (si)RNAs caused a similar (2- to 3-fold) degree of reduction of the *Atg12* mRNA levels both in IEC-18 and IEC-ras cells (Fig. 8A and B). In contrast, the same siRNAs had little effect on ATG12 protein in IEC-18 cells (Fig. 8C) but noticeably reduced ATG12 protein levels in IEC-ras cells (Fig. 8D). Thus, oncogenic RAS reduces Atg12 protein stability in intestinal epithelial cells. Collectively (Fig. 7 and 8), our results indicate that RAS accelerates proteasomal degradation of ATG12 in intestinal epithelial cells.

The effect of RAS on ATG12 requires MAP2K/MEK activity

RAS promotes tumor growth by binding and activating proteins, such as the protein kinases RAF and the lipid kinase PI3K.[5] We found that treatment of IEC-*ras* cells with the PI3K inhibitor LY294002[67] blocked phosphorylation of the PI3K effector AKT which is normally observed when the inhibitor is used[68] but did not increase ATG12 levels in these cells which indicates that the effect of RAS on ATG12 in these cells is PI3K-independent (Fig. 9).

Once activated by RAS, RAF protein kinase phosphorylates and thus activates protein kinases MAP2K1/MEK1 and MAP2K2/MEK2, respectively, which mediate RAS-driven tumorigenesis.[69] We observed that treatment of IEC-ras cells with widely used specific MAP2K1/2 inhibitors PD98059[70-72] (Fig. 10A and B) or selumetinib[73,74] (Fig. 10C-F) blocked phosphorylation of MAP2K substrates MAPK1/ERK2 and MAPK3/ERK1, respectively (Fig. 10A, C, and E) and significantly upregulated the unconjugated ATG12 (Fig. 10B, D, and F) in these cells.

In an effort to confirm the role of MAP2K in the regulation of ATG12 expression by RAS in cancer cells by a genetic approach we found that IEC-ras cells, when infected with a retrovirus encoding a dominant-negative MAP2K1 mutant,[68] displayed significantly higher levels of total MAP2K (due to the presence of both the endogenous MAP2K and the exogenous dominant-negative MAP2K1 mutant which was not epitope-tagged), and noticeably higher free ATG12 levels than the control cells (Fig. 10G). We further observed that expression of an activated MAP2K mutant in the parental IEC-18 cells downregulates ATG12 (Fig. 10H and I). In addition, we found that treatment of IEC-18 cells (which already produce ATG12 at high levels) with the MAP2K inhibitor PD98059 did not upregulate ATG12 further (Fig. 10K). In summary, our data indicate that RAS downregulates ATG12 in intestinal epithelial cells in a MAP2K-dependent manner.

The effect of RAS on ATG12 requires MAPK activity

MAP2K can phosphorylate the protein kinases MAPK1/3 and other substrates, such as signaling proteins HSF1 and possibly SOS, all of which are thought to mediate RAS signaling.[69,75,76] Which of these proteins mediate the effect of RAS and MAPK2 on ATG12 is not known. In an effort to address this question we found that treatment with a recently developed highly specific small molecule MAPK1/3 inhibitor SCH772984[77,78] blocked phosphorylation of the MAPK1/3 substrate RPS6KA1/RSK1[77,77] (Fig. 11A, C, and E) and upregulated free ATG12 in IEC-ras cells (Fig. 11B) as well as in mutant KRAS-carrying human colon cancer cells DLD1 (Fig. 11D) but not in the variant of DLD1 cells DKS8 in which the mutant KRAS allele was knocked out[42] (Fig. 11F). These data indicate that MAPK1/3 activity is required for the ability of oncogenic RAS to downregulate ATG12.

In summary, we have identified here a novel critical mechanism of survival of malignant intestinal epithelial cells carrying oncogenic RAS. This mechanism is driven by RAS-induced activation of MAP2K and MAPK and subsequent proteasomal degradation of ATG12. This degradation prevents ATG12 from killing these cells.

Discussion

We have identified in this study a novel mechanism by which oncogenic RAS promotes growth of malignant intestinal

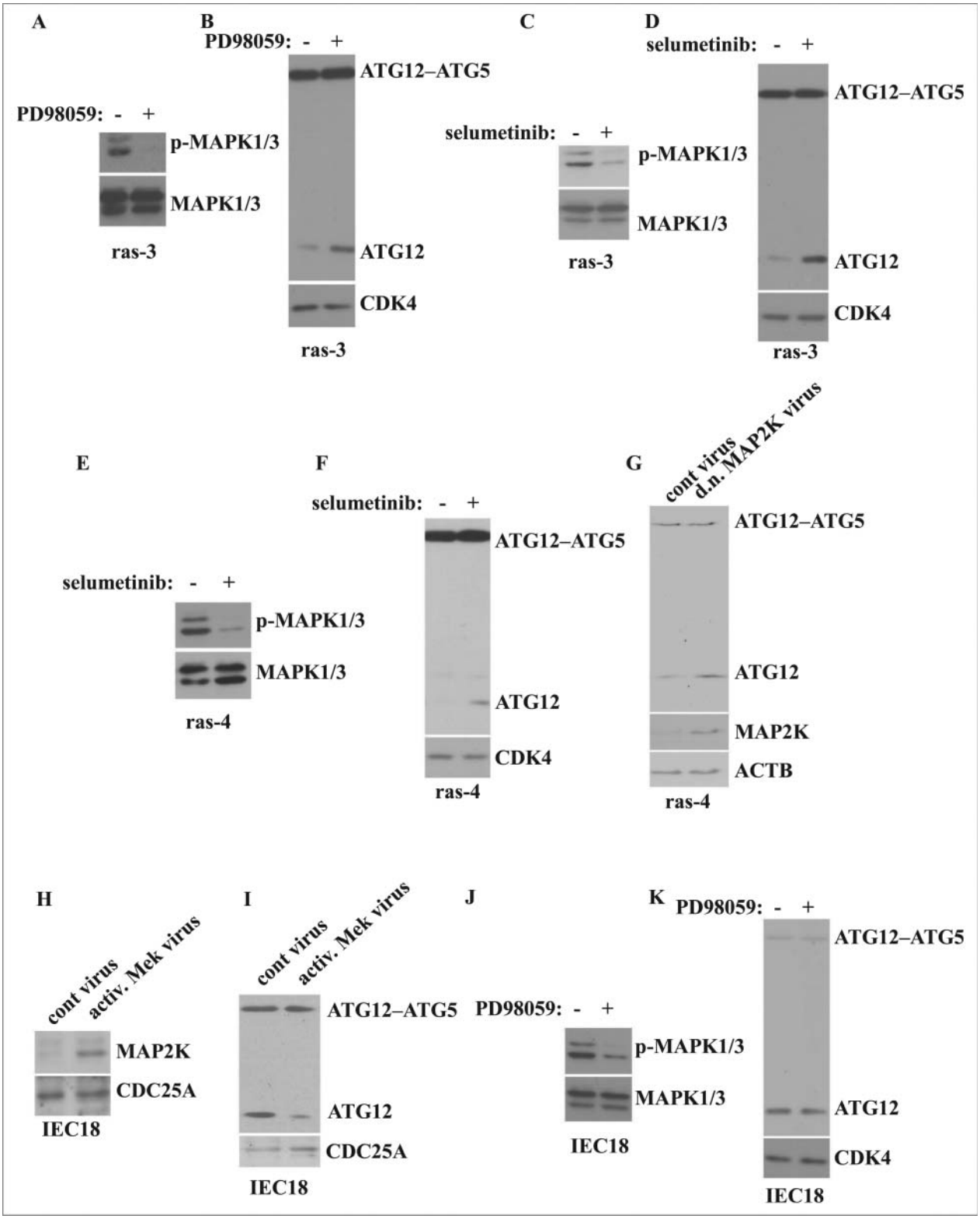

Figure 10. RAS downregulates ATG12 in intestinal epithelial cells in a MAP2K-dependent manner. (A-D) ras-3, (E and F) ras-4 and (J and K) IEC-18 cells were treated with DMSO (-) or 25 μM PD98059 (+; A, B, J, and K) or 1 μM selumetinib (+; C-F) for 24h and assayed for phospho-MAPK1/3 (pMAPK1/3) (A, C, E, and J) or ATG12 (B, D, F, and K) expression by western blot. (G) ras-4 cells were infected with a control retrovirus (cont virus) or a dominant-negative MAP2K-encoding retrovirus (d.n. Mek virus) and assayed for ATG12 expression by western blot. The membrane was reprobed with an anti-MAP2K antibody. (H and I) Stable cell lines (cont. and activ. Mek virus) generated by infection of IEC-18 cells with either a control retrovirus (cont) or an activated MAPK mutant-encoding retrovirus (activ. Mek virus) were assayed for MAP2K (H) or ATG12 (I) expression by western blot. MAPK1/3 (A, C, E, and J), CDK4 (B, D, F, and K), ACTB/β-actin (G) and CDC25 (H and I) served as loading controls. Positions of the unconjugated ATG12 (ATG12) and ATG12-ATG5 (ATG12-ATG5) conjugate on the gels are indicated.

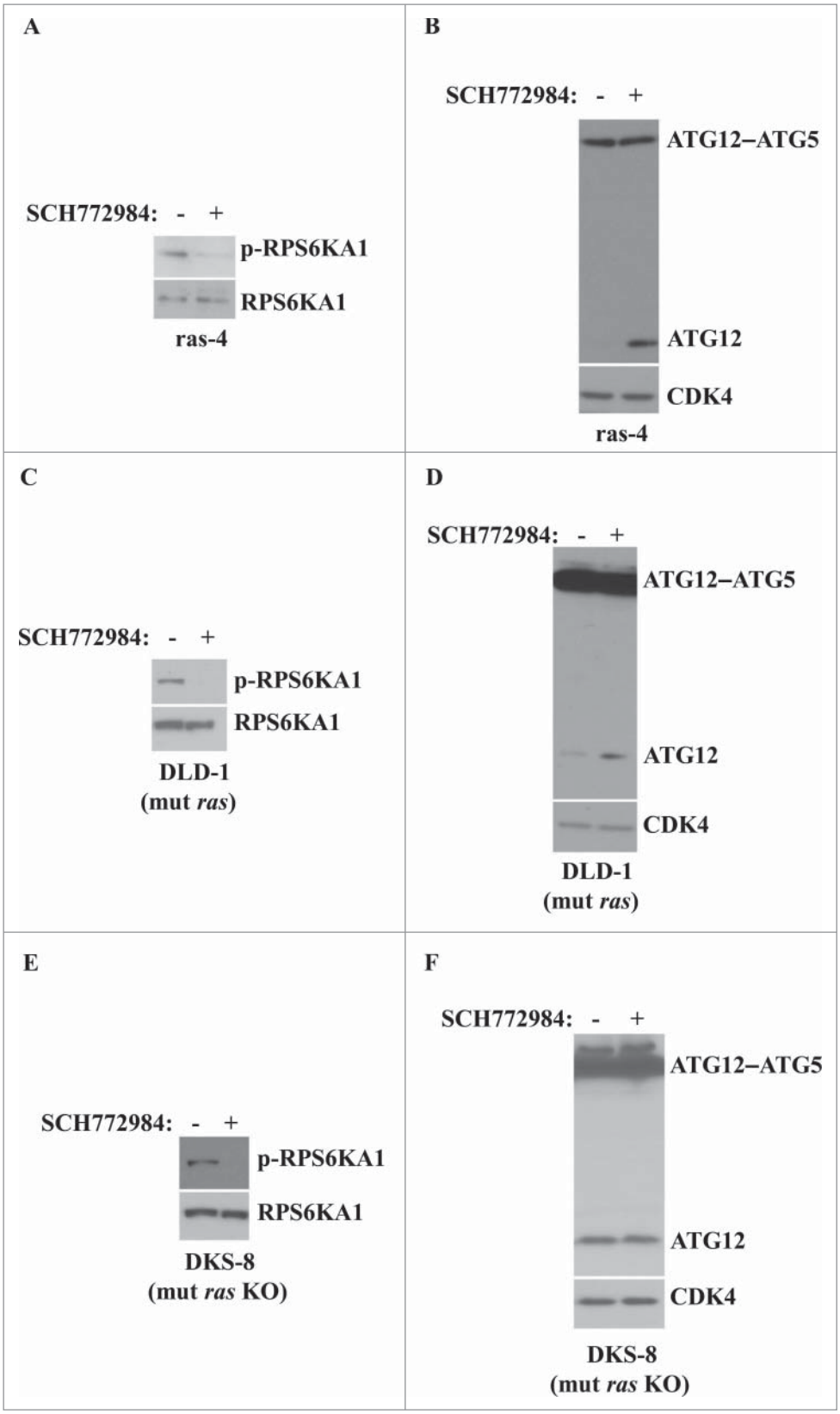

Figure 11. RAS downregulates ATG12 in intestinal epithelial cells in a MAPK1/3-dependent manner. (A and B) ras-3, (C and D) DLD1 and (E and F) DKS8 cells were treated with DMSO (-) or 1 μM SCH772984 (+) for 24 h (A and B) or 6 h (C-F) and assayed for phospho-RPS6KA1 (p-RPS6KA1) (A, C, E) or ATG12 (B, D, F) expression by Western blot. MAPK1/3 (A, C, and E) and CDK4 (B, D, and F) served as loading controls. Positions of the unconjugated ATG12 (ATG12) and ATG12-ATG5 (ATG12-ATG5) conjugate on the gels are indicated.

epithelial cells. RAS is well known to trigger death signals in cells.[11,50] Induction of these signals represents an aspect of a phenomenon called oncogenic stress[49] that is thought to preclude tumorigenesis in response to the emergence of oncogenic mutations of RAS and other proto-oncogenes.[50] It is believed that the likely reason why cancer cells survive, despite the presence of the antisurvival signals, is because in addition, oncoproteins, such as RAS, trigger the prosurvival signals which outbalance the death-promoting events in the indicated cells.[46] According to our data, RAS-dependent loss of ATG12 in cancer cells represents one prosurvival event that promotes viability of these cells. It seems likely that once this event is reversed (e.g. by introduction of ectopic ATG12 in the cells), the cell death-promoting signals outbalance the prosurvival signals, and malignant cells die. Thus, it is possible that despite the fact that RAS plays many important roles in physiology of not only cancer but normal cells as well,[5] the presence of death-promoting signals in mutant RAS-carrying tumor cells makes them vulnerable to treatments that upregulate ATG12 in the cells. By contrast, since the death signals are not present in the nonmalignant cells (e.g. IEC-18), these cells can survive in the presence of relatively high ATG12 levels. Whether ATG12 upregulation is toxic to other types of nonmalignant cells remains to be investigated.

Our data indicate that ATG12, once its levels in cancer cells are restored, kills them via autophagy-independent mechanisms. Others found that ATG12 can induce autophagy-independent apoptosis in cancer cells by physically binding and neutralizing the anti-apoptotic BCL2 family members, such as MCL1, and thus promoting the release of the pro-apoptotic mitochondrial factors to the cytoplasm.[32] Our results are consistent with these findings in that we observed that MCL1 can block ATG12-induced apoptosis of cancer cells. We also found that once ATG12-dependent apoptosis is blocked, ATG12 triggers necrotic signals in these cells. Situations when the presence of the anti-apoptotic BCL2 family members in the cells triggers a switch from apoptosis to necrosis have been described by others.[58,59] For example, it has been found that oxidized low density lipoproteins kill lymphocytes by apoptosis but when apoptosis is blocked by BCL2, the cells treated with the indicated lipoproteins die by necrosis.[58] In addition, it is well established that loss of a pro-apoptotic protease CASP8/caspase-8, another major component of the cellular apoptotic machinery, by the cells triggers programmed necrosis.[60,61] Various regulated forms of necrotic cell death, e.g. pyroptosis and necroptosis, are presently known,[79] and establishing the precise molecular mechanism by which ATG12 promotes necrosis represents an important direction for our future studies. So far, the physiological significance of the ability of ATG12 to kill cells remained unknown. Our work demonstrates that one physiologically relevant aspect of this ability is that ATG12 precludes RAS-driven growth of malignant intestinal epithelial cells by killing them.

Our results are also consistent with observations that ATG12 can control cellular autophagy-unrelated events. It has been found that ATG12 is involved in mitochondrial homeostasis,[52] endosome function and exosome biogenesis and these roles of ATG12 appear to be distinct from its ability to promote autophagosome formation.[37] Furthermore, the phenotype of mice lacking ATG12 in the brain (e.g. accelerated weight gain, adiposity, and glucose intolerance) cannot be explained exclusively by the ability of ATG12 to control autophagy.[80] However the mechanisms by which this phenotype is caused remain unknown.[80]

Our finding that RAS downregulates ATG12 in colon cancer cells is consistent with observations that ATG12 protein levels are reduced in a significant fraction of colorectal and pancreatic cancers compared with respective normal epithelial tissues.[81] Importantly, both types of cancer often carry oncogeneic mutations of RAS.[1,2]

Our results indicate that oncogenic RAS accelerates degradation of ATG12 by the UPS in intestinal epithelial cells. The UPS is composed of many elements. Two mammalian E1 enzymes,[82] approximately 30 E2 and about a 1000 E3 enzymes determine the specificity of target protein recognition by the UPS.[62] Ubiquitylated proteins are brought to the proteasome by "shuttle" proteins,[83] while protein degradation can be blocked by the deubiquitylating enzymes.[62] Our data are consistent with a scenario that RAS-induced activation of MAP2K and MAPK alters expression and/or activity of one or more elements of this system in such a way that ATG12 degradation by the cells is accelerated.

It is now known that pharmacological MAP2K inhibitors have a relatively weak effect on the growth of mutant RAS-carrying cancers[10,84] due to the fact that inhibition of MAP2K activates several feedback anti-apoptotic signals in tumor cells and that these signals antagonize the pro-apoptotic effects of the indicated drugs.[84] Hence, an alternative approach to treating these cancers would be to target MAP2K effectors. MAP2K has several substrates,[75,76] and we found that the effect of MAP2K on ATG12 is mediated by one of these substrates, such as MAPK. Importantly, others found that MAPK inhibitor SCH772984 that we used in this study does not trigger the indicated feedback mechanisms and kills mutant RAS-positive cancer cells more efficiently than the MAP2K inhibitors.[77] Whether MAPK inhibitors are active against such cancers in the clinic remains to be seen. MAPK has several effectors[85] and ATG12 represents one of them. Given that the reversal of the effect of RAS, MAP2K and MAPK on ATG12 potently kills cancer cells, ATG12 is likely an important mediator of the effect of MAPK on cancer cell survival. Thus, one way to develop therapies for mutant RAS-carrying malignancies would be to search for drugs that upregulate ATG12 in tumor cells. Using such drugs in combination with other antagonists of RAS signaling or with chemotherapeutic agents could represent a novel approach to treating these cancers.

Materials and methods

Cell culture

IEC-18 and IEC-*ras* cells were cultured as described.[17] Cell lines DLD1, DKS8, DKO3, CaCo2, HT29, LS180, SKCO1 and 293T were cultured in DMEM (GIBCO, 12800-082) and 10% fetal bovine serum (Sigma, F1051), 100 U/ml penicillin, 100 μg/ml streptomycin, 0.29 mg/ml L-glutamine (Pen-Strep-Glutanmine) (GIBCO, 10378-016). Cells LoVo were cultured in RPMI 1640 medium (GIBCO, 31800-022), 10% Fetal

Bovine Serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.29 mg/ml L-glutamine.

Western blot

This assay was performed as described.[17] The following antibodies were used in the study. Anti-ATG12 (Cell Signaling Technology, 4180), anti-MAP2K/MEK1 (Cell Signaling Technology, 9124), anti-CDK4 (Santa Cruz Biotechnology, sc-601), anti GAPDH (Cell Signaling Technology, 2118), anti-ACTB/β-actin (Santa Cruz Biotechnology, sc-130656), anti-ubiquitin (Santa Cruz Biotechnology, sc-8017), anti-MCL1 (Cell Signaling Technology, 5453), anti-RPS6KA/RSK (Cell Signaling Technology, 9355), anti phospho-RPS6KA/RSK (Cell Signaling Technology, 9344), anti-RAS (EMD Millipore, 05-516), anti-MAP2K1/MEK1 and MAP2K2/MEK (Cell Signaling Technology, 9102), anti-phospho-MAP2K1/MEK1 and MAP2K2/MEK (Cell Signaling Technology, 9101), anti-AKT2 (Cell Signaling Technology, 3063), anti-phospho-AKT (Cell Signaling Technology, 4060), anti-TUBA/α-tubulin (EMD Millipore 05-829), MAP1LC3B (Cell Signaling Technology3868), MAPK14/p38 MAP kinase (Cell Signaling Technology, 9212).

RNA interference

RNAi was performed as described previously.[22] The sequences ofthe sense strands of the RNAs used in this study were as follows:control RNA (siCONTROL non-targeting siRNA #1 (Dharmacon, D-001210-01-20)), UGUUGUUUGAGGGGAA CGGUU, *Atg12* siRNA11: CCAUAAUGAAAACGAAGAA; *Atg12* siRNA12: GGUCCUACAUUACUGCAAA.

Quantitative polymerase chain reaction (qPCR)

This procedure was performed as described previously.[21] Sequences of the primers used for the amplification of ATG12 mRNA were CTAGCCTGGAACTCAGAGATTG and CCTC AACGCCAGGTAAGAAT. The sequences of the primers used for the amplification of Rn18s/18S rRNA are published previously.[21]

Expression vectors

The following expression vectors were used in the study. pMSCV (Addgene, 24828, depositing laboratory: Lin He), pMSCVATG12 (Addgene, 27049; depositing laboratory: Jayanta Debnath), pMSCVAYG12Stop (Addgene, 27050; depositing laboratory: Jayanta Debnath), pBabe (Addgene, 1764; depositing laboratories: Hartmut Land, Jay Morgenstern, Bob Weinberg), pBabe-Flag-hMcl1 (Addgene, 25371; depositing laboratory: Roger Davis), pBABE-hygro (Addgene, 1765; depositing laboratories: Hartmut Land, Jay Morgenstern, Bob Weinberg). pBabehygro-dnMEK expression vector encoding the dominant negative MAP2K1 mutant carrying S217A and S221A mutations (Cell Biolabs, RTV111). pHIT and pVSVG vectors were provided by Dr. P. Lee, Dalhousie University, Canada. pBabe MEK2DD vector encoding an activated MAP2K mutant was provided by M. Reginato, Drexel University, PA, USA. A pBabe-Kras G12D vector encoding the $KRAS^{G12D}$ mutant was a gift from C. Der, the University of North Carolina at Chapel Hill, NC, USA.

Production of retroviruses

To generate ATG12-encoding MSCV retroviruses 3×10^6 293T cells were cultured on a 100-mm dish overnight and transfected with 5 μg of pMSCV or pMSCVATG12 or pMSCVATG12Stop vectors, 2.5 μg of pHIT and 2.5 μg of pVSVG vectors in the presence of 20 μl of Lipofectamine 2000 (Invitrogen, 11668-019) in 6 ml of OPTI MEM medium (GIBCO, 31989-070). The medium was changed 4 h later to the regular cell growth medium. The medium was collected 48 h later and filtered through a 0.45-micron filter unit. Viral concentration in the resulting solution was determined by use of the Retrovirus Quantitation Kit (Cell Biolabs, VPK-120) according to manufacturer's instructions. To generate MCL1-encoding Moloney murine leukemia viruses (MMLV) 293T cells were transfected as described above with 5 μg of pBabe or pBabe-Flag-hMcl1 vectors, 2.5 μg of pHIT and 2.5 μg of pVSVG vectors and processed further as described above.

Transduction of cells with retroviruses

3×10^5 ras-4 cells were grown overnight in a 60-mm dish. 6×10^4 viral particles per cell were then added to the cells in the presence of 8 μg/ml polybrene (Sigma-Aldrich, 107689), and the cells were cultured for 24 h. Medium was changed to the fresh medium and the cells were cultured for another 24 h in the presence of 6 μg/ml puromycin. In the experiments where the cells were cotransduced with ATG12- and MCL1-encoding retroviruses the cells were incubated with 6×10^4 control MSCV viral particles per cell together with 6×10^4 control MMLV viral particles per cell or 6×10^4 ATG12-encoding MSCV viral particles per cell together with 6×10^4 control MMLV viral particles per cell or 6×10^4 ATG12-encoding MSCV viral particles per cell together with 6×10^4 MCL1-encoding MMLV viral particles per cell or 6×10^4 control MSCV viral particles per cell together with 6×10^4 MCL1-encoding MMLV viral particles per cell. MAP2K-producing IEC-18 cells were generated as published by others.[86] To generate oncogenic RAS-producing CaCo2 cells, the cells were transduced with a control MMLV or $KRAS^{G12D}$ MMLV and selected for stable expression in the culture medium described above supplemented with 2 μg/ml puromycin. Cells producing the dominant negative MAP2K were generated as we described previously.[68]

Immunoprecipitation

Cells were lysed in a buffer containing 0.025 M TRIS, 0.15 M NaCl, 0.001 M EDTA, 1% NP40 (Roche, 11332473001), 5% glycerol, pH 7.4, and the lysates were precleared with Protein A beads (GE Healthcare, 17-0780-01) 3 times for 6 h. The lysates (1 mg of total protein) were incubated with the anti-ATG12 IgG indicated above or the anti RAIDD IgG (Santa Cruz Biotechnology, 7880) overnight, and proteins were precipitated overnight by use of protein A beads.

Detection of ANXA5 binding to the cells

ANXA5 binding to the cells was detected as described previously.[57]

Detection of clonogenic cell survival

Cell colonies were stained with Chrystal Violet.[13] Cells infected with retroviruses were maintained in the presence of 6 μg/ml puromycin for 7 to 10 d while the cell colonies were allowed to form.

Densitometry analysis of western blot images

This analysis was performed as we described previously.[22]

Statistical Analysis

Statistical analysis of the data in Figures 4 and 5B and C was performed by the chi-square test for goodness-of-fit. Statistical analysis of all other data was performed by the unpaired Student *t* test.

Western blot images

Western blot images were generated by Photoshop.

Abbreviations

ATG	autophagy related
BCL2L1	BCL-like 1
CDK4	cyclin-dependent kinase 4
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
IP	immunoprecipitation
MAP1LC3/LC3	microtubule-associated protein 1 light chain 3
MMLV	Moloney murine leukemia virus
MSCV	murine stem cell virus
PI	propidium iodide
qPCR	quantitative polymerase chain reaction
SD	standard deviation
siRNA	small interfering RNA
UPS	ubiquitin-proteasome system

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