

A Novel PTEN-Dependent Link to Ubiquitination Controls FLIP_S Stability and TRAIL Sensitivity in Glioblastoma Multiforme

Amith Panner, Courtney A. Crane, Changjiang Weng, Alberto Feletti, Andrew T. Parsa, and Russell O. Pieper

Brain Tumor Research Center, Department of Neurological Surgery and University of California San Francisco Comprehensive Cancer Center, University of California San Francisco, San Francisco, California

Abstract

Phosphatase and tensin homologue (PTEN) loss and activation of the Akt-mammalian target of rapamycin (mTOR) pathway increases mRNA translation, increases levels of the antiapoptotic protein FLIP_S, and confers resistance to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis in glioblastoma multiforme (GBM). In PTEN-deficient GBM cells, however, the FLIP_S protein also exhibited a longer half-life than in PTEN mutant GBM cells, and this longer half-life correlated with decreased FLIP_S polyubiquitination. FLIP_S half-life in PTEN mutant GBM cells was reduced by exposure to an Akt inhibitor, but not to rapamycin, suggesting the existence of a previously undescribed, mTOR-independent linkage between PTEN and the ubiquitin-dependent control of protein stability. Total levels of the candidate FLIP_S E3 ubiquitin ligase atrophin-interacting protein 4 (AIP4) were comparable in PTEN wild-type (WT) and PTEN mutant GBM cells, although in PTEN-deficient cells, AIP4 was maintained in a stable polyubiquitinated state that was less able to associate with FLIP_S or with the FLIP_S-containing death inducing signal complex. Small interfering RNA-mediated suppression of AIP4 levels in PTEN WT cells decreased FLIP_S ubiquitination, prolonged FLIP_S half-life, and increased TRAIL resistance. Similarly, the Akt activation that was previously shown to increase TRAIL resistance did not alter AIP4 levels, but increased AIP4 ubiquitination, increased FLIP_S steady-state levels, and suppressed FLIP_S ubiquitination. These results define the PTEN-Akt-AIP4 pathway as a key regulator of FLIP_S ubiquitination, FLIP_S stability, and TRAIL sensitivity and also define a novel link between PTEN and the ubiquitin-mediated control of protein stability. [Cancer Res 2009;69(20):7911-6]

Introduction

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a proapoptotic peptide that binds to the death receptors DR4/DR5 and induces formation of the death-inducing signaling complex and engagement of the type I extrinsic apoptotic pathway

(1, 2). Although many cancer cells are preferentially sensitive to TRAIL-induced apoptosis, the sensitivity of glioblastoma multiforme (GBM), the most aggressive form of brain cancer, is variable, and most short-term primary GBM cultures are TRAIL insensitive (3, 4). Although many factors contribute to TRAIL resistance in GBM, the levels of FLIP_S, a truncated splice variant of FLIP, have been shown to be of particular importance (5, 6). Levels of FLIP_S in TRAIL-resistant cells have in turn been shown to be regulated by the phosphatase and tensin homologue (PTEN)-Akt-mammalian target of rapamycin (mTOR) pathway, and PTEN loss and Akt activation correlate *in vitro*, in human GBM xenografts, and in primary human GBM samples with increased FLIP_S mRNA translation, high levels of FLIP_S expression, and TRAIL resistance (5).

In the process of completing studies related to the PTEN-dependent translational regulation of FLIP_S, we noted that the high amounts of FLIP_S in PTEN-defective TRAIL resistant GBM cells were also associated with a greatly prolonged half-life of the protein. Protein stability is frequently regulated by ubiquitination, a process by which the small protein ubiquitin is covalently attached to lysine residues in target proteins by E3 ubiquitin ligases (7, 8). Whereas the ligation of a single ubiquitin molecule at one or multiple lysines in the target protein (monoubiquitination) can change target protein activity and cellular location, chain-like addition of multiple ubiquitin molecules to the sites of monoubiquitination (at lysines 48 or 63 in ubiquitin itself; polyubiquitination) leads to alterations in protein sorting and activity (K63 polyubiquitination) or, more critically, to degradation of the targeted protein (K48 polyubiquitination; refs. 9, 10). Although PTEN has not, to date, been reported to regulate the ubiquitination process, the association between PTEN status and FLIP_S stability suggested that PTEN may use regulation of ubiquitination, in addition to regulation of protein production, to control the levels of FLIP_S and TRAIL sensitivity. In the present study, we explored this possibility.

Materials and Methods

Cell culture and manipulation. Flank xenografts of individual human GBM were established in mice as previously described (5). Freshly resected xenograft material was then obtained from the UCSF Brain Tumor Research Tissue Bank, dissected into small (<1 mm diameter) pieces, passed through a 100 μm pore size tissue culture sieve, and grown on reduced Matrigel-coated dishes (Fisher Scientific). Each resultant culture (e.g., 5, 10, 14) is therefore derived from a unique patient tumor. Transformed mouse astrocytes (TMA) and GBM xenograft cells (5) were cultured in DMEM (H-21) supplemented with 10% fetal bovine serum at 37°C in a 5% CO₂ atmosphere. Cells were incubated with vehicle (DMSO), MG132 (10 μmol/L, 24 h), Akt inhibitor III (50 μmol/L, 24 h; Calbiochem), rapamycin (100 nmol/L, 24 h), or TRAIL (800 ng/mL, 24 h; Genentech) and, where indicated, with cycloheximide (100 μg/mL) or vehicle for an additional 24 to 72 h. Pools of retrovirally infected or transfected (Lipofectamine) cells (11) were obtained by selection with neomycin (1 mg/mL, 7 d) or hygromycin B (400 μg/mL, 7 d).

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A. Panner and C.A. Crane contributed equally to this work.

Requests for reprints: Russell O. Pieper, Helen Diller Family Comprehensive Cancer Center, Room 287, Box 0520, University of California San Francisco, 1450 3rd Street, San Francisco, CA 94158-9001. Phone: 415-502-7132; Fax: 415-502-6779; E-mail: rpieper@cc.ucsf.edu or Andrew T. Parsa, Helen Diller Family Comprehensive Cancer Center, Room 286, Box 0520, University of California San Francisco, 1450 S. 3rd Street, San Francisco, CA 94158-9001. Phone: 415-353-2629; Fax: 415-353-2889; E-mail: parsaa@neurosurg.ucsf.edu.

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Atrophia-interacting protein 4 (AIP4)-targeted small interfering RNA (siRNA; 300 nmol/L; Ambion, identification no. 120674) or scramble control (300 nmol/L; silencer negative control #1, Ambion) was transfected, and target protein levels were analyzed 1 to 3 d after exposure to vehicle or cycloheximide.

Immunoprecipitation and Western blot analysis. Control or hemagglutinin (HA)-ubiquitin-expressing cells were lysed, and AIP4, FLIP_S, or death receptor 5 (DR5) was immunoprecipitated from lysates by incubation (1 h, 4°C) with the appropriate antibody pre-conjugated to protein-G beads (Santa Cruz Biotechnology). Levels of proteins in the cell lysate and the eluted immunoprecipitates were assessed by Western blot using the appropriate antibody. Levels of ubiquitinated protein were assessed by Western blot using a goat polyclonal antibody against FLIP_S (Santa Cruz

Biotechnology) or a rabbit polyclonal antibody against AIP4 or HA (Cell Signaling Technology), followed by detection with anti-goat IgG or anti-rabbit IgG (Santa Cruz Biotechnology) using enhanced chemiluminescence. Densitometric measurements were acquired using an AlphaImager 2200 (Alpha Innotech Corporation). Immunoprecipitations carried out using a nonspecific normal rabbit IgG antibody (for AIP4 immunoprecipitations) or a goat IgG antibody (for FLIP_S immunoprecipitations) were included as negative controls. Analysis of TRAIL-induced apoptosis was done as previously described (12).

Statistical analysis. Data presented are representative of at least three independent experiments. All statistical analyses were done using the Student *t* test, with significance defined as *P* < 0.05 (*).

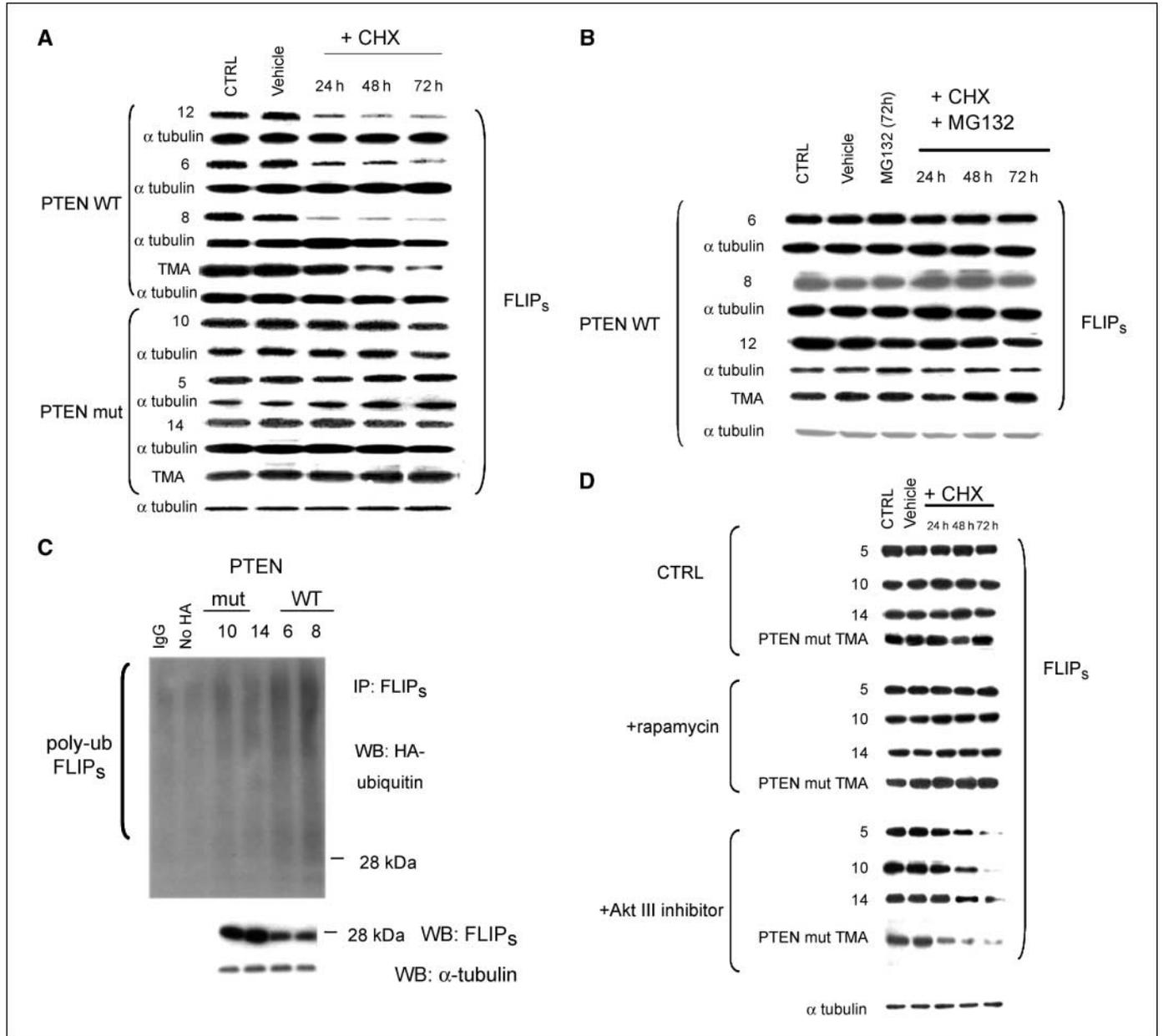


Figure 1. The PTEN-Akt pathway controls FLIP_S protein stability. Mouse PTEN knockout or WT TMA or PTEN WT or mutant human xenografted GBM cells, or the same cells infected with a blank vector or construct encoding HA-ubiquitin (C), were incubated with vehicle or MG132 (10 μmol/L, 24 h; B), rapamycin (100 nmol/L, 24 h; D), or Akt III inhibitor (50 μmol/L, 24 h; D), after which cells were incubated with either vehicle or cycloheximide (CHX; 100 μg/mL; A, B, and D), lysed at the indicated time points, and analyzed either for levels of FLIP_S and α-tubulin (A, B, D) or for the extent of HA ubiquitination in FLIP_S immunoprecipitates (C). The α-tubulin blot shown in D is representative of those for all experimental groups.

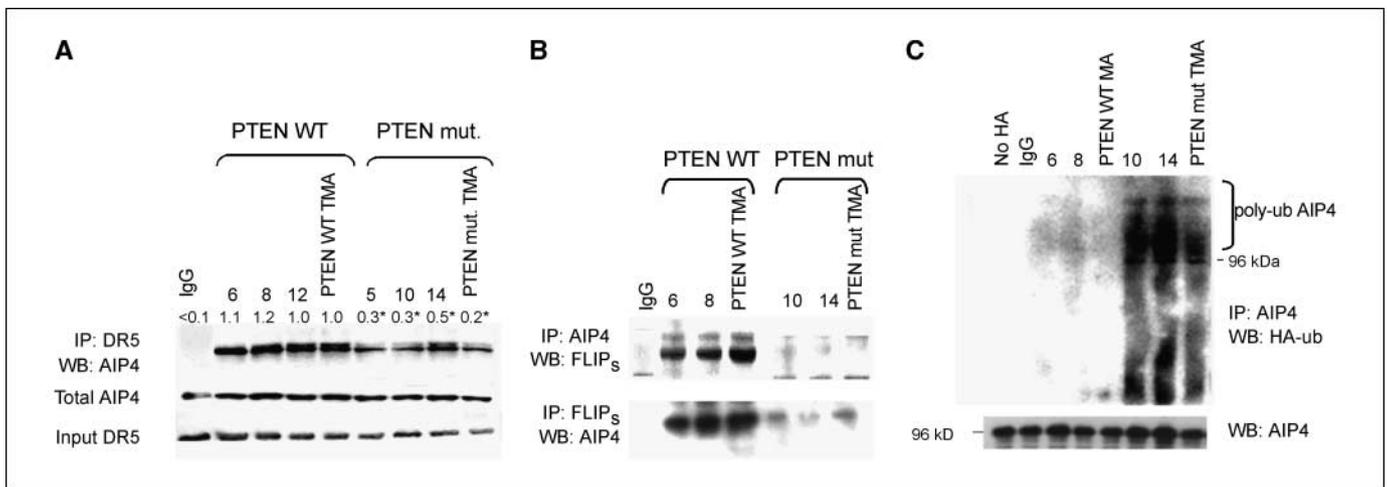


Figure 2. AIP4 interacts with FLIP_S in a PTEN- and ubiquitin-dependent manner. PTEN WT or mutant cells were transfected with a blank vector or a construct encoding HA-ubiquitin (C) or exposed to vehicle (B) or TRAIL (800 ng/mL, 24 h; A). Cells were then lysed and immunoprecipitated using antibodies specific for IgG or for DR5 (A), AIP4 or FLIP_S (B), or AIP4 (C). Lysates and immunoprecipitates were then subjected to Western blot analysis of AIP4 and DR5 (A), FLIP_S and AIP4 (B), and AIP4 and HA-AIP4 (C) levels.

Results and Discussion

We previously showed that PTEN loss causes a mTOR-dependent increase in the translation of the FLIP_S mRNA levels, increased levels of the antiapoptotic protein FLIP_S, and increased TRAIL resistance (5). In cycloheximide-treated PTEN-deficient GBM xenograft cells and in cycloheximide-treated TMA derived from PTEN knockout mouse embryos, however, the half-life of preexisting FLIP_S was also significantly longer than in corresponding PTEN wild-type (WT) cells (Fig. 1A). Differences in FLIP_S protein stability were not due to effects of cycloheximide on FLIP_S mRNA levels (Supplementary Fig. S1), nor were they an artifact of the low initial levels of FLIP_S in PTEN WT cells because overexpression of FLIP_S did not alter FLIP_S half-life (Supplementary Fig. S2). MG132-mediated suppression of the proteasome, however, greatly prolonged the half-life of the FLIP_S protein in PTEN WT human GBM cells and PTEN WT TMA (Fig. 1B), suggesting that FLIP_S half-life was regulated by protein degradation in a PTEN-dependent manner. FLIP_S immunoprecipitates from PTEN WT cells transiently transfected with a construct encoding HA-ubiquitin and subjected to Western blot analysis using a HA-targeted antibody also contained more >28-kDa FLIP_S than FLIP_S immunoprecipitates from PTEN mutant cells (Fig. 1C; each ubiquitin added adds 7 kDa of mass), indicative of increased FLIP_S polyubiquitination and suggesting that the increased FLIP_S stability in PTEN mutant cells resulted from the inability of these cells to mark FLIP_S for proteasomal degradation. Because the PTEN-Akt-mTOR pathway regulates translation of FLIP_S mRNA (5), we questioned whether the PTEN-associated effects on FLIP_S protein stability were mediated by the same pathway. Incubation of PTEN-deficient GBM and TMA cells with an Akt inhibitor suppressed Akt phosphorylation (Supplementary Fig. S3) and significantly shortened FLIP_S half-life, although exposure of the cells to concentrations of rapamycin that suppressed S6 phosphorylation (Supplementary Fig. S3) did not (Fig. 1D). These results suggest that the PTEN-Akt pathway is linked to the control of FLIP_S stability in a novel, Akt-dependent but mTOR-independent manner.

To better define the linkage between PTEN loss, Akt activation, and ubiquitin-mediated regulation of FLIP_S stability, we took a candidate approach and ultimately focused on the E3 ubiquitin

ligase AIP4 (13). Levels of AIP4 were comparable in PTEN WT and PTEN-deficient cells, although less AIP4 was found in FLIP_S immunoprecipitates from PTEN-deficient cells than from PTEN-deficient cells (Fig. 2A). Similarly, DR5 immunoprecipitates of the death-inducing signaling complex (which contains both DR5 and FLIP_S; ref. 14) from TRAIL-treated PTEN mutant GBM cells also contained less AIP4 than those from PTEN WT cells (Fig. 2B). The immunoprecipitated AIP4 from PTEN-deficient cells was also maintained in a more highly polyubiquitinated form than that in PTEN WT cells (Fig. 2C), suggesting that PTEN loss and Akt activation lead to the generation of a (perhaps K63-) ubiquitinated AIP4, which, although not less stable, is decreased in its ability to interact with and ubiquitinate FLIP_S.

To more definitively link AIP4 to the control of FLIP_S stability and TRAIL sensitivity, PTEN WT cells were exposed to siRNA targeting AIP4, after which effects on FLIP_S levels, FLIP_S ubiquitination, FLIP_S stability, and TRAIL sensitivity were monitored. The siRNA-mediated suppression of AIP4 levels in PTEN WT cells (Fig. 3A, left) significantly increased the half-life of FLIP_S (Fig. 3A, middle) relative to that noted in cells receiving a nontargeted siRNA (Fig. 3A, right), increased steady-state levels of FLIP_S (Fig. 3B, bottom), decreased levels of polyubiquitinated FLIP_S (Fig. 3B, top), and increased resistance to TRAIL-induced apoptosis (Fig. 3C). These results show that AIP4 is directly linked to the control of FLIP_S ubiquitination and stability, and that AIP4 targets FLIP_S for ubiquitination and proteasomal destruction.

Finally, to formally establish the role of AIP4 in linking the PTEN-Akt pathway to the control of FLIP_S stability and TRAIL sensitivity, PTEN WT cells were retrovirally infected with a construct encoding Akt-ER (15) and incubated with vehicle or 4-hydroxytamoxifen (4HT), after which the known TRAIL-desensitizing effects of 4HT-mediated Akt activation were compared with the effects on AIP4 and FLIP_S levels and ubiquitination. The 4HT-mediated activation of Akt-ER in PTEN WT cells (Supplementary Fig. S4) that was previously shown to increase resistance to TRAIL-induced apoptosis (5) did not alter AIP4 levels (Fig. 4A, bottom), but significantly increased the extent of AIP4 polyubiquitination (Fig. 4A, top), suppressed levels of FLIP_S ubiquitination (Fig. 4B, top),

and increased levels of total FLIP_S (Fig. 4B, top). Taken as a whole, these results show the existence of a novel pathway that links PTEN to the control of the extrinsic apoptotic pathway.

The pathway that links PTEN to the control of FLIP_S ubiquitination described in this work is presented in Fig. 4C. In this model, PTEN suppresses levels of pAkt (left), which in turn retains AIP4 in a state in which it can interact with and (likely K48-) polyubiquitinate FLIP_S. K48-polyubiquitinated FLIP_S then undergoes ubiquitin-mediated degradation, leaving the cell susceptible to TRAIL-induced apoptosis. Loss of PTEN function (Fig. 4D, right), in contrast, increases pAkt levels and retains AIP4 in a (perhaps K63-) polyubiquitinated state in which it can no longer interact with and target FLIP_S for destruction, thereby allowing FLIP_S to accumulate and suppress TRAIL-induced apoptosis. Although the means by which Akt activation enhances AIP4 ubiquitination are not clear, many E3 ligases including AIP4 regulate their own ubiquitination (16–18), and Akt may directly modulate this process. Alternatively, because ubiquitination is a reversible process, Akt may interact with any of a number of deubiquitinases (19), which may in turn tailor

the pattern of AIP4 ubiquitination and serve to regulate AIP4 function. Cell type-specific factors that influence AIP4 ubiquitination and/or protein interactions may also help explain the ability of AIP4 to target FLIP_S in GBM cells, but not other cell types (20).

In light of the present work, PTEN seems to exert coordinate control on FLIP_S, suppressing FLIP_S mRNA translation (5) while at the same time contributing to the destabilization of the protein. This coordinate system could therefore allow both immediate resetting of the apoptotic threshold of cells (via rapid regulation of protein stability) and a more long-term restructuring of apoptotic potential (via changes in protein translation), and on inactivation (as in a significant percentage of PTEN-deficient gliomas; ref. 19), it could allow near-complete inhibition of the extrinsic apoptotic response. Whereas pharmacologic manipulation of either arm of this coordinate pathway could effectively resensitize tumor cells to TRAIL, manipulation of both arms may ultimately prove most effective. In a broader sense, the present work also has implications for PTEN function. It seems unlikely that PTEN has evolved the ability to regulate ubiquitination solely to control AIP4 activity, FLIP_S stability,

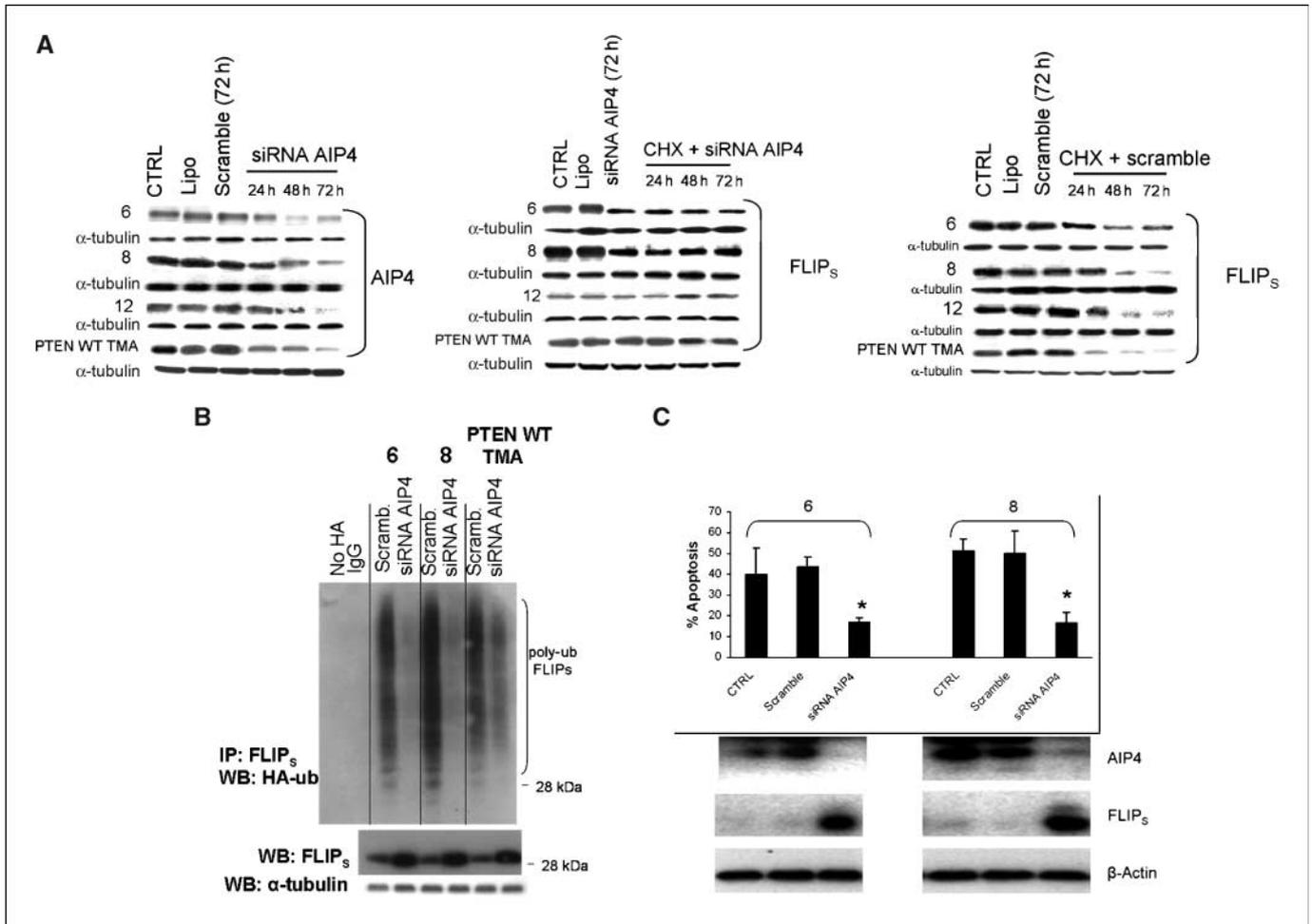


Figure 3. AIP4 is linked to the control of FLIP_S ubiquitination and stability and TRAIL sensitivity. PTEN WT or mutant cells were transfected with a scrambled siRNA or siRNA targeting AIP4. Cells were then incubated with vehicle or cycloheximide (A), transfected with a blank vector or a construct encoding HA-ubiquitin (B), or exposed to TRAIL (800 ng/mL, 24 h; C). Cells were then collected at the indicated time points and subjected to Western blot analysis of AIP4, FLIP_S, and α-tubulin levels (A and B); immunoprecipitated using antibodies specific for FLIP_S and subjected to analysis of extent of HA-ubiquitination of FLIP_S (B); or analyzed for extent of TRAIL-induced apoptosis.

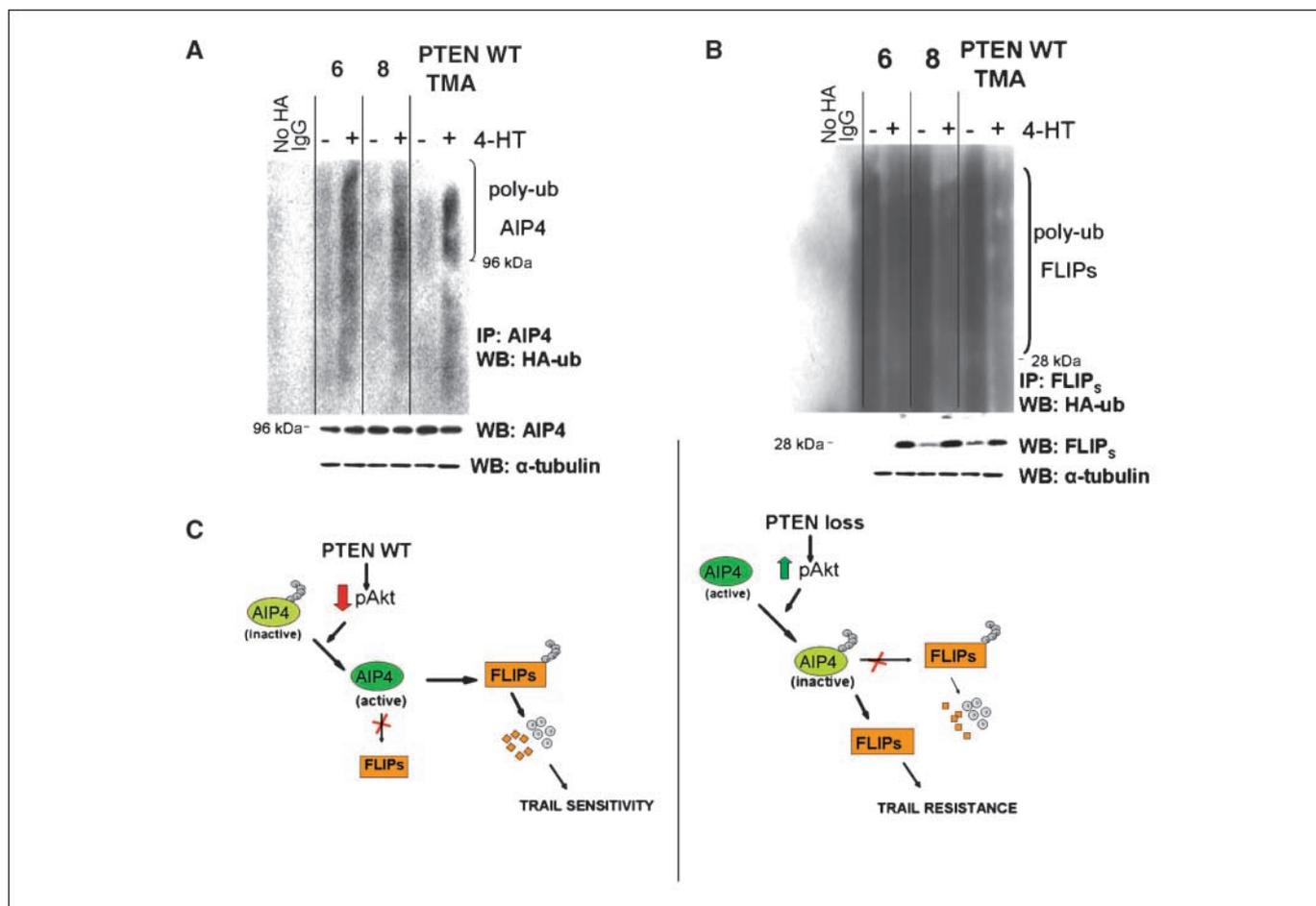


Figure 4. AIP4 links the PTEN-Akt pathway to FLIP_S ubiquitination and stability. PTEN WT or mutant cells were transfected with a blank construct or a construct encoding a 4HT-inducible Akt-ER (A and B). Cells were then incubated with vehicle or 4HT (100 μ mol/L, 24 h), transfected with a blank vector or a construct encoding HA-ubiquitin and lysed, then subjected either to Western blot analysis of FLIP_S, AIP4, and α -tubulin or to immunoprecipitation using antibodies specific for AIP4 or FLIP_S, and then analyzed for the extent of HA-ubiquitination of AIP4 (A) and FLIP_S (B). C, schematic representation of the PTEN-mediated control of FLIP_S ubiquitination and TRAIL sensitivity.

and TRAIL sensitivity. Rather, it seems more likely that PTEN may regulate the activity of many of the more than 1,000 E3 ubiquitin ligases in the human cell (8) and, in doing so, may control the stability of a wide range of proteins critical in regulating the transformed phenotype. These ideas are currently being investigated.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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