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Therapeutics, Targets, and Chemical Biology

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Ubiquitin-Specific Protease 8 Links the PTEN-Akt-AIP4 Pathway to the Control of $FLIP_S$ Stability and TRAIL Sensitivity in Glioblastoma Multiforme

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

Abstract

The antiapoptotic protein $FLIP_S$ is a key suppressor of tumor necrosis factor–related apoptosis-inducing ligand (TRAIL)–induced apoptosis in human glioblastoma multiforme (GBM) cells. We previously reported that a novel phosphatase and tensin homologue (PTEN)–Akt–atrophin-interacting protein 4 (AIP4) pathway regulates $FLIP_S$ ubiquitination and stability, although the means by which PTEN and Akt were linked to AIP4 activity were unclear. Here, we report that a second regulator of ubiquitin metabolism, the ubiquitin-specific protease 8 (USP8), is a downstream target of Akt, and that USP8 links Akt to AIP4 and the regulation of $FLIP_S$ stability and TRAIL resistance. In human GBM xenografts, levels of USP8 correlated inversely with pAkt levels, and genetic or pharmacologic manipulation of Akt regulated USP8 levels in an inverse manner. Overexpression of wild-type USP8, but not catalytically inactive USP8, increased $FLIP_S$ ubiquitination, decreased $FLIP_S$ half-life, decreased $FLIP_S$ steady-state levels, and decreased TRAIL resistance, whereas short interfering RNA (siRNA)–mediated suppression of USP8 levels had the opposite effect. Because high levels of the USP8 deubiquitinase correlated with high levels of $FLIP_S$ ubiquitination, USP8 seemed to control $FLIP_S$ ubiquitination through an intermediate target. Consistent with this idea, overexpression of wild-type USP8 decreased the ubiquitination of the $FLIP_S$ E3 ubiquitin ligase AIP4, an event previously shown to increase AIP4-$FLIP_S$ interaction, whereas siRNA-mediated suppression of USP8 increased AIP4 ubiquitination. Furthermore, the suppression of $FLIP_S$ levels by USP8 overexpression was reversed by the introduction of siRNA targeting AIP4. These results show that USP8, a downstream target of Akt, regulates the ability of AIP4 to control $FLIP_S$ stability and TRAIL sensitivity. *Cancer Res; 70(12); 5046–53. ©2010 AACR.*

Introduction

Ubiquitination is a posttranslational modification used by cells to alter protein stability and function (1, 2). Protein ubiquitination is accomplished by the coordinated action of a series of proteins referred to as E1, E2, and E3 enzymes. The E3 ubiquitin ligases (of which over 1,000 are encoded in the human genome; ref. 3) catalyze the rate-limiting step of the process and facilitate the transfer of the activated ubiquitin protein to lysine (K) in the target protein. The ligation of a single ubiquitin molecule at one or multiple lysines in the target protein (monoubiquitination) changes target protein activity and/or cellular location, whereas chain-like addition of multiple ubiquitin molecules to the sites of monoubiquitination (at K48 or K63 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. 2, 4). Ubiquitination, however, is a reversible process, and in addition to ubiquitin-binding, ubiquitin-activating, and ubiquitin-ligating enzymes, deubiquitinating enzymes also exist. These ubiquitin-removing proteases, referred to as deubiquitinating proteins, exist in several categories including ubiquitin COOH-terminal hydrolases, sumo-specific proteases, and perhaps most importantly, ubiquitin-specific proteases (USP; ref. 5). The USPs comprise the bulk of deubiquitinating enzymes in the genome (6, 7), and by their ability to cleave the isopeptide bond between ubiquitin and substrate proteins or other ubiquitin molecules, are involved in both the generation of ubiquitin as well as the tailoring of patterns of ubiquitination in target proteins (8). Although the USPs play a clear and complementary role in the ubiquitination process, substrates have been identified for only a few of the more than 50 USPs that exist in the human genome, and processes known to be regulated by USPs are limited.

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In a previous study, we reported that ubiquitination plays a key role in the sensitivity of glioblastoma multiforme (GBM) cells to the proapoptotic tumor necrosis factor–related apoptosis-inducing ligand (TRAIL; ref. 9). TRAIL binds to the death receptors 4 and 5 and induces a tumor-selective, type I extrinsic apoptotic cell death (10, 11). The sensitivity of GBM, the most aggressive form of brain cancer, is variable, and in a previous report, we showed that levels of $FLIP_S$, a truncated splice variant of FLIP, were regulated by the phosphatase and tensin homologue (PTEN)–Akt–mammalian target of rapamycin pathway, and that PTEN loss and Akt activation correlated *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 (12). We also noted, however, that the PTEN pathway not only regulated $FLIP_S$ mRNA translation, but also the stability of the $FLIP_S$ protein. Specifically, we found that PTEN, via an Akt-dependent but mammalian target of rapamycin–independent mechanism, regulated the ubiquitination, localization, and activity of the E3 ubiquitin ligase atrophin-interacting protein 4 (AIP4), and that in response to PTEN loss and AIP4 inactivation, $FLIP_S$ was retained in a nonubiquitinated, stable form that accumulated and contributed to TRAIL resistance. AIP4, however, is not known to be a substrate of Akt, and the mechanism by which PTEN loss and Akt activation are linked to AIP4 activity was not defined. In this study, we provide evidence that a deubiquitinase, USP8, provides the link between Akt activation and loss of AIP4 function, and that PTEN uses this USP8-AIP4 ubiquitin switch to regulate the process of ubiquitination, protein stability, and TRAIL sensitivity.

Materials and Methods

Cell culture and drug treatment

Transformed mouse astrocytes (TMA) derived from wild-type or PTEN conditional knockout (KO) mice [P2 animals with genotypes $Pten^{+/+}$;GFAP-cre (wild-type) and PtenloxP/ loxP;GFAP-cre (Pten cKO) were provided by Suzanne Baker, St. Jude's Children's Research Hospital, Memphis, TN; ref. 13] and transformed with SV40 large T antigen and mutant V12 H-Ras (14, 15) were provided by Dr. Gabriele Bergers (University of California San Francisco, San Francisco, CA). Freshly resected human GBM xenografts were obtained from the University of California San Francisco 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). The PTEN status of GBM tissues was previously described (12). The TMA and GBM xenografts were incubated with either vehicle (DMSO, 24–72 hours), Akt inhibitor III (50 μmol/L, 24 hours; Calbiochem), or rapamycin (100 nmol/L, 24 hours; Cell Signaling Technology), after which cycloheximide (100 μg/mL) or vehicle was added for an additional 24 to 72 hours. For studies of apoptosis, cells were incubated with TRAIL (800 ng/mL, 24 hours) dissolved in a hypertonic solution containing Tris buffer (pH 7.2) and supplied as a gift from Avi Ashkenazi (Genentech). Both TMAs and GBM xenograft cell lines were cultured in DMEM (H-21) supplemented with 10% fetal bovine serum at 37°C in a 5% CO_2 atmosphere.

Retroviral infection, transfection of plasmids, and short interfering RNA

The retroviral construct pWzl-hygro-M(+)Akt-estrogen receptor was a generous gift from Martin McMahon (University of California San Francisco Comprehensive Cancer Center, San Francisco, CA). The construction of this vector has been previously described (12). Expression of the construct following infection of target cells was induced by the addition of 10 μmol/L of 4-hydroxytamoxifen (24 hours). The pcDNA mammalian expression construct encoding either a wild-type or catalytically inactive mutant (C478A) of USP8 was kindly provided by Kermit C. Carraway (University of California-Davis, Davis, CA). Cells were retrovirally infected as previously described (16–18), with pools of productively infected cells obtained by selection with neomycin (1 mg/mL, 7 days) or hygromycin B (400 μg/mL, 7 days). Mammalian expression constructs were transfected into target cells using LipofectAMINE (Invitrogen) according to the instructions of the manufacturer, after which stably transfected cells were selected using neomycin (1 mg/mL, 7 days), and verified for overexpression of the target protein by Western blotting. For ubiquitination studies, cells were similarly transiently transfected with a construct encoding hemagglutinin-tagged ubiquitin for 48 hours before any additional cell procedures. For short interfering RNA (siRNA) studies, AIP4-targeted siRNA (300 nmol/L, Ambion), USP8-targeted siRNA (300 nmol/L, Ambion), or scramble control (300 nmol/L, silencer negative control no. 1, Ambion) were transfected into cells using LipofectAMINE and target protein levels were analyzed 1 to 4 days later by Western blot. In studies involving cycloheximide, cells received the appropriate siRNA, scramble control, or vehicle (LipofectAMINE) control for 48 hours, after which cycloheximide was added (100 μg/mL) for an additional 24 to 72 hours. For Western blot studies, immunoprecipitation studies, and introduction of siRNA into stably infected/transfected cell lines, cells received the appropriate siRNA, scramble control, or vehicle for 48 hours prior to analysis. In studies using TRAIL, cells received either the target siRNA, scramble control, or vehicle for 48 hours, then TRAIL (800 ng/mL) for an additional 24 hours, after which cell death was assessed by flow cytometry.

Immunoblot analysis and analysis of apoptosis by flow cytometry

Cells were washed with ice-cold PBS, scraped from the culture dish, and incubated in tissue lysis buffer containing 10 mmol/L of KCl, 1 mmol/L of sucrose, 2 mmol/L of $MgCl_2$, 0.5% Igepal CA-630, 1 mmol/L of EDTA, 1 mmol/L of DTT, 10 mmol/L of β-glycerophosphate, 1 mmol/L of Na_3VO_4, 10 mmol/L of sodium fluoride, 100 μg/mL of phenylmethylsulfonyl fluoride, and 10 μg/mL of aprotinin for 30 minutes on ice. The cell lysate was centrifuged, and the supernatant was stored at −80°C until use. The protein concentration of

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extracts was measured using Protein Assay reagent (Bio-Rad Laboratories). Proteins (30 μg) were subjected to SDS-PAGE and electroblotted onto Immobilon-P membrane (Millipore). The membrane was blocked in 5% nonfat skim milk/TBST [20 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 0.1% Tween 20] at 4°C overnight and incubated with goat polyclonal antibody against $FLIP_S$ or $FLIP_L$ (Santa Cruz Biotechnology), or rabbit polyclonal antibody against USP8 (Abcam), AIP4, or hemagglutinin (all from Cell Signaling Technology). Bound antibody was detected with anti-goat IgG or anti-rabbit IgG (Santa Cruz Biotechnology) using enhanced chemiluminescence Western blotting detection reagents (Amersham Pharmacia Biotech). Densitometric measurements of immunoreactive bands were acquired using an AlphaImager 2200 (Alpha Innotech Corporation). The extent of apoptosis in cultures (attached and floating cells) was determined by fluorescence-activated cell sorting analysis (sub-G_1 DNA content), with measurements verified by Annexin V staining (19).

Immunoprecipitation and analysis of ubiquitinated proteins

AIP4 or $FLIP_S$ was immunoprecipitated from cell lysates by a 1-hour incubation at 4°C with the appropriate antibody preconjugated to protein G beads (Santa Cruz Biotechnology). The beads were subsequently pelleted by centrifugation at 2,000 rpm for 5 minutes. and washed thrice using tissue lysis buffer. The samples were then boiled at 95°C for 10 minutes to elute the immunocomplexed proteins. Levels of protein in the eluate were assessed by Western blotting using the appropriate antibody. Levels of ubiquitinated protein were assessed by Western blotting using an antibody targeting hemagglutinin. Immunoprecipitations carried out using a nonspecific normal rabbit IgG antibody (for AIP4 immunoprecipitations) or a goat IgG antibody (for $FLIP_S$ immunoprecipitations; Santa Cruz Biotechnology) were included as negative controls.

Statistical analysis

All statistical analyses were performed using Student's *t* test, with significance defined as $P < 0.05$.

Results

We had previously shown that levels of the antiapoptotic protein $FLIP_S$ were higher in TRAIL-resistant, PTEN-deficient GBM cells and TMA than in TRAIL-sensitive PTEN wild-type cells, and that these higher levels of $FLIP_S$ were associated with a longer $FLIP_S$ half-life and lower levels of $FLIP_S$ ubiquitination (9). These data suggested an Akt-dependent, but mammalian target of rapamycin–independent link between PTEN and $FLIP_S$ ubiquitination, which we subsequently showed was the result of Akt-mediated regulation of the activity of the $FLIP_S$ E3 ubiquitin ligase AIP4. Because AIP4 is not known to be a substrate of Akt, we initiated a search for pathways that might link Akt to AIP4 regulation. The deubiquitinating enzyme USP8 has been suggested to be regulated by Akt (20), and has also been reported to play a broad role in growth factor receptor trafficking and degradation, in part, by its ability to stabilize the E3 ligase neuregulin receptor degradation pathway protein 1(Nrdp1; refs. 20, 21). We therefore considered the possibility that USP8 might be a link between the PTEN/Akt pathway and a ubiquitin E3 ligase involved in $FLIP_S$ protein stability and apoptotic sensitivity.

To begin to address this possibility, we first examined whether USP8 was regulated in a PTEN/Akt-dependent manner. Levels of USP8 were significantly higher in PTEN wild-type TMA than in PTEN KO TMA (lane 1 versus lane 2; Fig. 1A), and were also higher in PTEN wild-type human GBM cells than in PTEN mutant GBM cells (compare lane 1; Fig. 1B and C). Furthermore, 4-hydroxytamoxifen–mediated activation of a retrovirally encoded exogenous Akt-estrogen receptor protein in PTEN wild-type TMA (Fig. 1A, last lane) or PTEN wild-type human GBM cells (Fig. 1B, last lane) resulted in a decrease of USP8 levels to those noted in corresponding PTEN-deficient cells. Conversely, exposure of PTEN mutant human GBM cells to an Akt inhibitor enhanced USP8 levels (Fig. 1C, last lane). These results therefore show that PTEN loss and Akt activation were linked to the suppression of USP8 levels, and that USP8 is a target for PTEN-mediated regulation.

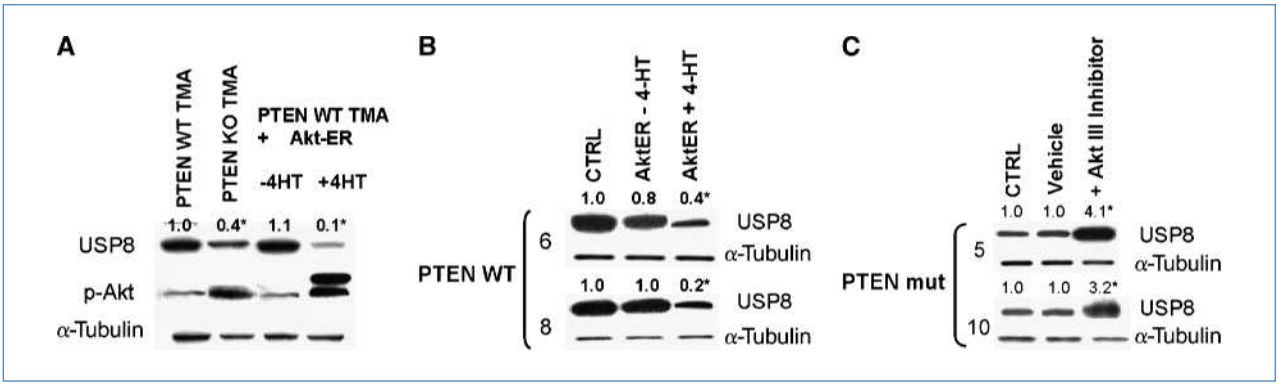

Figure 1. The PTEN-Akt pathway regulates levels of the deubiquitinase USP8. Mouse PTEN wild-type (WT) or KO TMA, human PTEN wild-type or mutant xenograft GBM cells, or the same cells infected with a construct encoding 4-hydroxytamoxifen–activated Akt-estrogen receptor were incubated with vehicle, 4-hydroxytamoxifen (4HT, 100 nmol/L, 24 h; A and B), or Akt III inhibitor (50 μmol/L, 24 h; C), then cells were lysed and analyzed for levels of USP8 and α-tubulin.

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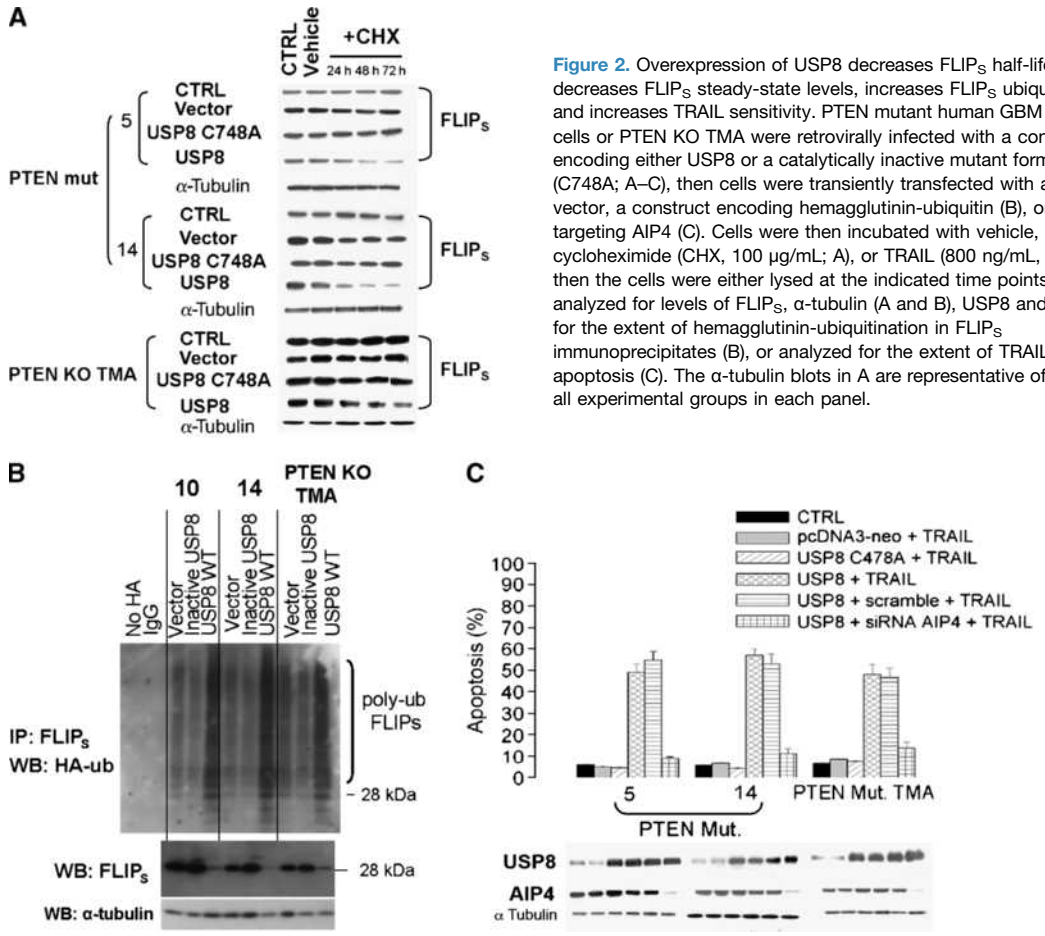

Figure 2. Overexpression of USP8 decreases FLIP$_S$ half-life, decreases FLIP$_S$ steady-state levels, increases FLIP$_S$ ubiquitination, and increases TRAIL sensitivity. PTEN mutant human GBM xenograft cells or PTEN KO TMA were retrovirally infected with a construct encoding either USP8 or a catalytically inactive mutant form of USP8 (C748A; A–C), then cells were transiently transfected with a blank vector, a construct encoding hemagglutinin-ubiquitin (B), or siRNA targeting AIP4 (C). Cells were then incubated with vehicle, cycloheximide (CHX, 100 μg/mL; A), or TRAIL (800 ng/mL, 24 h; C), then the cells were either lysed at the indicated time points and analyzed for levels of FLIP$_S$, α-tubulin (A and B), USP8 and AIP4 (C), for the extent of hemagglutinin-ubiquitination in FLIP$_S$ immunoprecipitates (B), or analyzed for the extent of TRAIL-induced apoptosis (C). The α-tubulin blots in A are representative of those for all experimental groups in each panel.

To address whether the PTEN/Akt-mediated control of USP8 is in turn directly linked to the control of FLIP$_S$ stability and/or apoptotic sensitivity, we manipulated USP8 levels in PTEN wild-type and PTEN-deficient cells, after which effects on FLIP$_S$ half-life, FLIP$_S$ steady-state levels, FLIP$_S$ ubiquitination, and apoptotic sensitivity to TRAIL were measured. In control PTEN mutant GBM and PTEN-KO TMA (which have relatively low levels of endogenous USP8) in which new protein synthesis was inhibited by cycloheximide exposure, the preexisting FLIP$_S$ protein exhibited a relatively long half-life (Fig. 2A), consistent with previous data. Retroviral introduction of a wild-type USP8 into these cells increased the levels of USP8 (Fig. 2C, bottom), but also significantly reduced FLIP$_S$ half-life relative to that noted in cells receiving either a blank (pcDNA3-neo) vector or a vector encoding catalytically inactive USP (ref. 21; USP C748A; Fig. 2A). Consistent with this decrease in FLIP$_S$ stability, steady-state levels of FLIP$_S$ were also significantly decreased (Fig. 2B, bottom), and these cells were sensitized to TRAIL-induced apoptosis (Fig. 2C, fourth column versus first column in each group). Because PTEN-dependent regulation of FLIP$_S$ stability is mediated by changes in FLIP$_S$ ubiquitination, we took the above USP8-modulated cells, transiently transfected a construct encoding hemagglutinin-tagged ubiquitin, and following FLIP$_S$ immunoprecipitation, used Western blot analysis to monitor the effect of USP8 alteration on the extent of hemagglutinin-ubiquitin incorporated into FLIP$_S$. Immunoprecipitates generated using IgG, or from cells not transfected with the hemagglutinin-ubiquitin construct did not exhibit any hemagglutinin-ubiquitinated FLIP$_S$ (Fig. 2B, top). Levels of ubiquitinated FLIP$_S$ were also relatively low in control cells and cells expressing catalytically inactive USP8, consistent with the long half-life of FLIP$_S$ in these cells. Expression of wild-type USP8, however, significantly increased the amount of higher molecular weight, hemagglutinin-labeled FLIP$_S$ (each ubiquitin added increases the apparent mass of the target protein by 7 kDa). These results suggest that the PTEN-dependent regulation of USP8 is tied to the control of FLIP$_S$ ubiquitination and stability.

To confirm the apparent link between USP8, FLIP$_S$ stability, and TRAIL sensitivity, we also performed converse experiments in which PTEN wild-type GBM and TMA, which

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express relatively high levels of USP8, were transfected with scrambled siRNA or an siRNA targeting USP8, after which the effects on $FLIP_S$ steady-state levels, ubiquitination, and TRAIL sensitivity were similarly monitored. The PTEN wild-type cells transfected with scrambled siRNA had relatively low levels of endogenous $FLIP_S$ (Fig. 3A, bottom), consistent with the relatively high levels of ubiquitinated $FLIP_S$ noted in the $FLIP_S$ immunoprecipitates from the same cells engineered to express hemagglutinin-ubiquitin (Fig. 3A, top). Introduction of siRNA targeting USP8, however, not only decreased the levels of USP8 (Fig. 3B, bottom), but also increased $FLIP_S$ steady-state levels (Fig. 3A, bottom), decreased the extent of hemagglutinin-ubiquitinated $FLIP_S$ (Fig. 3A, top) and significantly reduced the extent of TRAIL-induced apoptosis in these cells (Fig. 3B) relative to controls. Taken as a whole, these results show that USP8 levels are regulated in a PTEN-dependent manner, and that loss of PTEN function leads to decreased USP8 levels, decreased $FLIP_S$ ubiquitination, increased $FLIP_S$ stability, and increased TRAIL resistance.

We previously reported that the E3 ligase AIP4, like USP8, was regulated in a PTEN-dependent manner, and that AIP4 contributed to the ubiquitination of $FLIP_S$ (9). Given the apparent connection between PTEN, USP8, and AIP4, we questioned whether these components might be parts of a single pathway that controls $FLIP_S$ ubiquitination, and whether USP8 might act as a PTEN-dependent stimulator of AIP4 activity. To address this point, we first asked whether the modulation of USP8 that resulted in changes in $FLIP_S$ stability and ubiquitination also influenced AIP4. Levels of AIP4 were similar in PTEN wild-type and PTEN-deficient cells, and neither introduction of wild-type USP8 into PTEN mutant cells nor siRNA-mediated suppression of USP8 in PTEN wild-type cells altered total AIP4 levels (Fig. 4A and B). We previously noted, however, that although the PTEN loss did not alter total levels of AIP4, it did increase the ubiquitination of AIP4 and interfered with the ability of AIP4 to interact with its targets (9), raising the possibility that the PTEN loss interferes with AIP4 action not by lowering AIP4 levels, but by decreasing USP8 levels, which might in turn leave AIP4 in a ubiquitinated, inactive state. Consistent with this possibility, AIP4 in the PTEN mutant cells (which have low levels of USP8 and high levels of $FLIP_S$) was retained in a relatively highly ubiquitinated state, and introduction of wild-type USP8, but not catalytically inactive USP8, significantly decreased both AIP4 ubiquitination (Fig. 4A) as well as $FLIP_S$ stability (Fig. 2A). Conversely, AIP4 in the PTEN wild-type cells (which have high levels of USP8 and low levels of $FLIP_S$) was retained in a relatively under-ubiquitinated state, and the introduction of a siRNA targeting USP8 that significantly decreased $FLIP_S$ ubiquitination also significantly increased AIP4 ubiquitination (Fig. 4B). These data suggest that USP8, by modulating the ubiquitination status of AIP4, serves as a link between Akt and the AIP4-mediated regulation of $FLIP_S$ stability.

As a final test of the proposed linkage between PTEN, Akt, USP8, AIP4, $FLIP_S$ stability, and TRAIL sensitivity, USP8 and AIP4 levels were co-modulated in PTEN-deficient cells, after which effects on TRAIL sensitivity were monitored. PTEN-deficient cells, which have low levels of USP8 and high levels of $FLIP_S$, were relatively TRAIL resistant, and as previously noted, the introduction of wild-type USP8 (but not blank vector or catalytically inactive USP8) increased USP8 levels and significantly increased the extent of TRAIL-induced apoptosis (Fig. 2C). This USP8-induced increase in TRAIL sensitivity, however, could be reversed by the introduction of siRNA that targeted AIP4 but did not alter USP8 levels

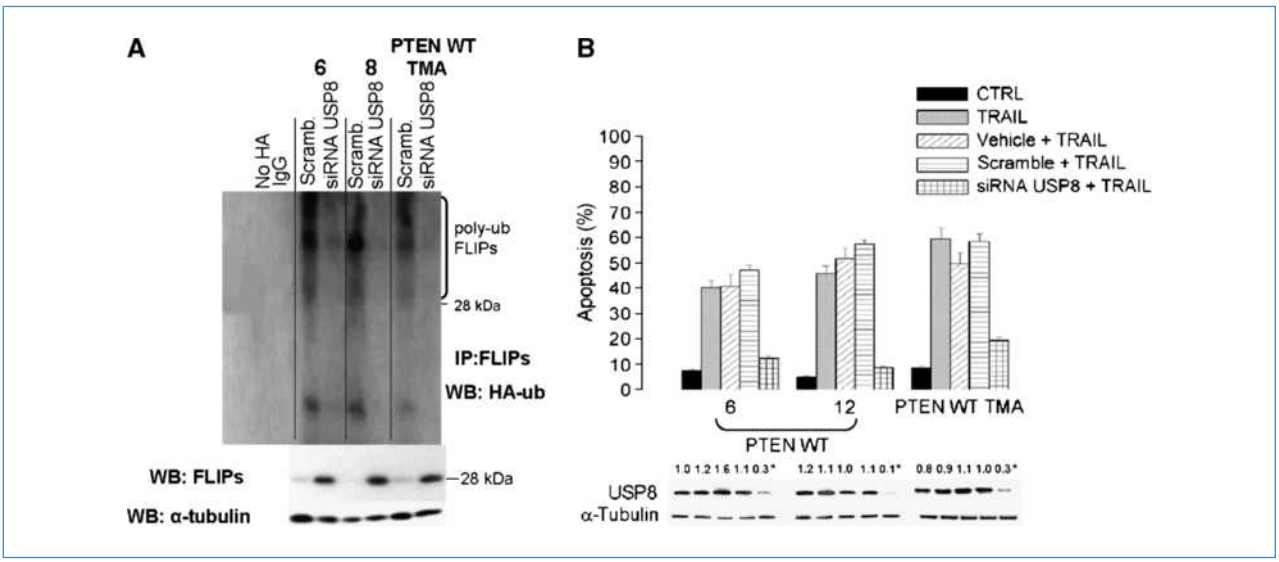

Figure 3. siRNA-mediated suppression of USP8 increases $FLIP_S$ steady-state levels, decreases $FLIP_S$ ubiquitination, and decreases TRAIL sensitivity. PTEN wild-type human GBM xenograft cells or PTEN wild-type TMA were transiently transfected with a scrambled siRNA or a siRNA targeting USP8, then cells were transiently transfected with a blank vector or a construct encoding hemagglutinin-ubiquitin (A), or incubated with TRAIL (800 ng/mL, 24 h; B). Cells were then either lysed and analyzed for levels of $FLIP_S$, α-tubulin, and for the extent of hemagglutinin-ubiquitination in $FLIP_S$ immunoprecipitates (A), or analyzed for levels of USP8 and the extent of TRAIL-induced apoptosis (B).

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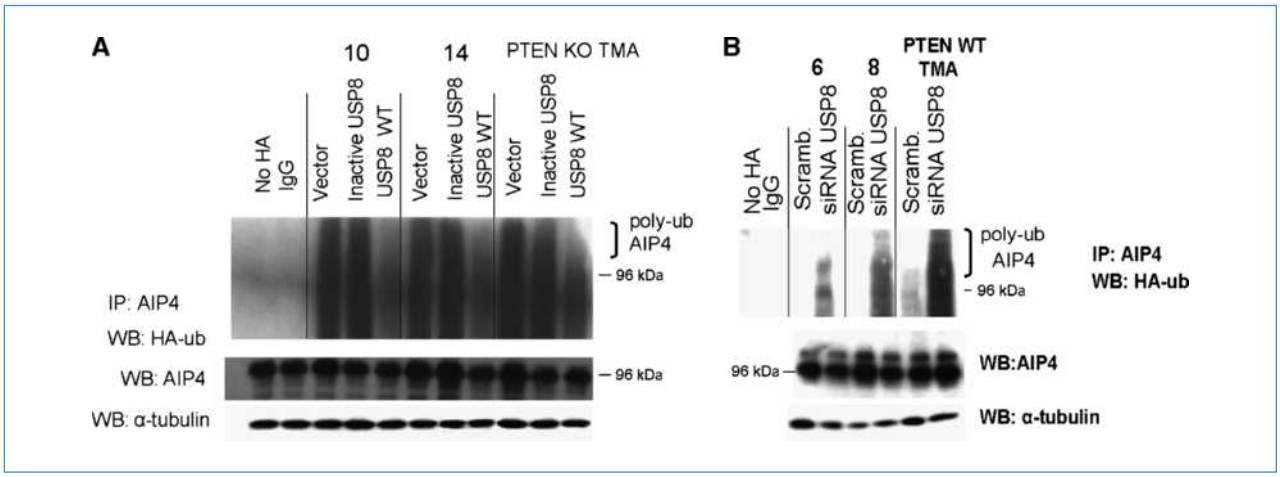

Figure 4. USP8 alterations are linked to changes in AIP4 ubiquitination. Cells (PTEN mutant human GBM xenograft cells or PTEN KO TMA, A; or PTEN wild-type human GBM xenograft cells or PTEN wild-type TMA, B) were either retrovirally infected with constructs encoding either USP8 or a catalytically inactive mutant form of USP8 (C748A; A), or transiently transfected with a scrambled siRNA or an siRNA targeting USP8 (B), then all cells were transiently transfected with a blank vector or a construct encoding hemagglutinin-ubiquitin. Cells were then lysed and analyzed for levels of AIP and α-tubulin, or for the extent of hemagglutinin-ubiquitination in AIP4 immunoprecipitates (A).

(Fig. 2C). These results, as a whole, show that USP8 is a PTEN-regulated deubiquitinase which, by altering the ubiquitination status of the E3 ligase AIP4, helps control FLIP$_S$ stability and TRAIL sensitivity in GBM cells.

Discussion

The pathway that links PTEN to the control of FLIP$_S$ ubiquitination and TRAIL sensitivity described in this work is presented in Fig. 5. In this model, PTEN suppresses the levels of pAkt (left), which in turn, leads to increased levels of USP8. USP8 interacts with AIP4 and retains this E3 ubiquitin ligase in a state in which it can interact with FLIP$_S$. Under these conditions, FLIP$_S$ undergoes ubiquitin-mediated degradation, leaving the cell susceptible to TRAIL-induced apoptosis. Loss of PTEN function (Fig. 5, right), in contrast, increases pAkt levels, decreases USP8 levels, and turns off the USP8/AIP4 ubiquitin switch, allowing FLIP$_S$ to accumulate and suppress TRAIL-induced apoptosis. In this manner, PTEN seems to use control of ubiquitination to help regulate TRAIL sensitivity in GBM cells.

Although the linkage between PTEN status and Akt activation state has been well described, the linkage between Akt and the deubiquitinase USP8 has been less well studied. In breast tumor cells, Akt activation led to USP8 (Thr^{907}) phosphorylation, which in turn, led to increased deubiquitinase activity toward the E3 ligase Nrdp1 (20). In the present study, the linkage between PTEN loss, Akt activation, and USP8 levels and activity seemed to be somewhat different, and in our work, Akt activation decreased, rather than enhanced, USP8

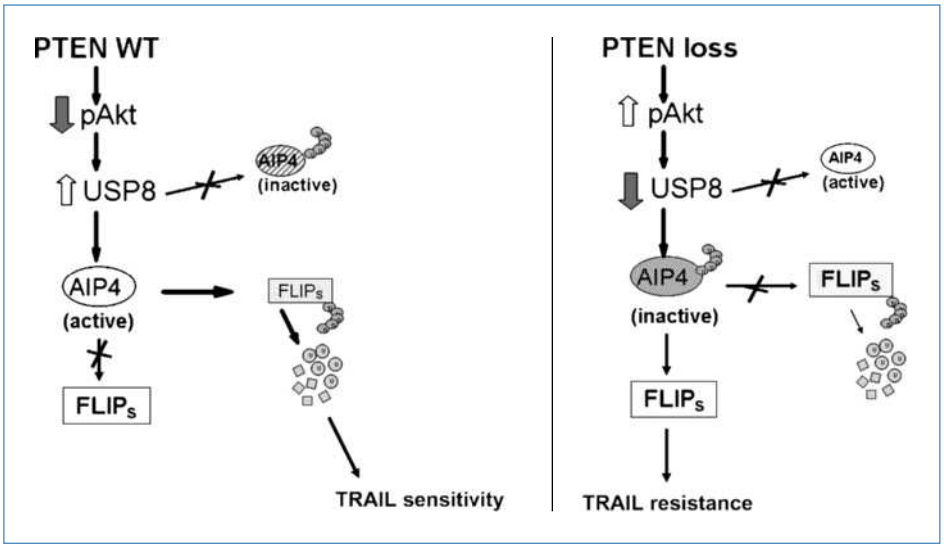

Figure 5. Schematic representation of the PTEN-Akt-USP8-AIP4 pathway which controls FLIP$_S$ ubiquitination and TRAIL sensitivity.

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function by increasing USP8 ubiquitination and decreasing steady-state USP8 levels (data not shown). Furthermore, a preliminary immunohistochemical analysis of paired tissue from 12 newly diagnosed GBMs revealed a statistically significant ($P < 0.05$) inverse relationship between levels of expression of USP8 and PRAS40 (a downstream marker of Akt activity). In addition to suppressing the levels of USP8, Akt may also stimulate the activity of USP8 toward select targets such as Nrdp1. Alternatively, Akt activation might directly suppress Nrdp1 function in addition to having effects mediated via USP8 control of ubiquitination. In the present work, and in that of Cao and colleagues (20), Akt kinase inhibition blocked the effect of Akt on USP8 function, suggesting that events related to Akt-mediated phosphorylation of USP8 control the ability of USP8 to regulate AIP4 and the ubiquitination process. USP8 contains at least three consensus sites for Akt phosphorylation, although the requirements for these sites for phosphorylation, stability, and/or function have not been defined.

Although the present studies show that USP8 regulates the E3 ligase function of AIP4, the exact means by which this occurs has only been partially defined. Because the deubiquitination-deficient USP8 mutant used in this study was unable to alter AIP4 ubiquitination and $FLIP_S$ stability, the USP8-mediated regulation of AIP function clearly involves the ubiquitination process. The decreased AIP4 ubiquitination noted following the introduction of wild-type USP8, combined with our previous work (9) showing mislocalization of ubiquitinated AIP4, strongly suggest that in the control PTEN wild-type setting, the deubiquitinating activity of USP8 prevents or reverses AIP4 K63 polyubiquitination, which in turn, allows the AIP4 E3 ligase to interact with K48 polyubiquitinate $FLIP_S$ in the DISC, leading to $FLIP_S$ degradation and enhanced sensitivity to apoptotic stimuli. A direct interaction between USP8 and AIP4 has not been reported, and it is possible that instead of directly deubiquitinating AIP4, USP8 might alter the ability of other E3 ligases (or perhaps of AIP4 itself) to stimulate AIP4 K63 polyubiquitination (22). Regardless of the exact mechanism, the present work is the first to clearly define a mechanism by which a pathway related to tumorigenesis also regulates apoptotic sensitivity.

The ubiquitin control pathway described in this work has broad implications, not only for our understanding of TRAIL resistance, but also for our understanding of the control of ubiquitination and PTEN function. TRAIL resistance in GBM is multifactorial and a number of alterations have been shown to contribute to TRAIL insensitivity (23–25). Although Akt activation has been linked to several of these alterations, and to TRAIL resistance (26–28), this effect has been typically ascribed to Akt-mediated, phosphorylation-dependent effects on the activity and/or translational regulation of specific (often antiapoptotic) proteins. The present study suggests that in addition to using phosphorylation, the PTEN-Akt pathway may use broader ubiquitin-based mechanisms to regulate protein stability, and in doing so, might be able to rapidly change a variety of cell characteristics including apoptotic sensitivity. The linkage of PTEN to USP8 and AIP4 also suggests that the range of proteins, the stability of which is controlled by PTEN-regulated ubiquitination, might be broader than expected. AIP4 is the E3 ligase for several proteins including $FLIP_L$ and Notch receptor 1 (29, 30), and our preliminary work suggests that several USPs, in addition to USP8, are regulated in a PTEN-dependent manner. PTEN may therefore use a modular approach to the regulation of protein ubiquitination and stability, recruiting different deubiquitinases linked to different E3 ligases to coordinately stabilize or destabilize families of proteins that share a common function. The USP8-AIP4 ubiquitin switch described in this work might therefore represent the first of many different ubiquitin switches used by PTEN to control cellular behavior.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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