

# The Mfge8- $\alpha$ 8 $\beta$ 1-PTEN pathway regulates airway smooth muscle contraction in allergic inflammation

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**ABSTRACT:** Asthma affects ~300 million people worldwide. Despite multiple treatment options, asthma treatment remains unsatisfactory in a subset of patients. Airway obstruction is a hallmark of allergic asthma and is largely due to airway smooth muscle hypercontractility induced by airway inflammation. Identification of molecular pathways that regulate airway smooth muscle hypercontractility is of considerable therapeutic interest. We previously identified roles for milk fat globule epidermal growth factor-like 8 (Mfge8) in opposing the effects of allergic inflammation on increasing airway smooth muscle contractile force. In this study, we delineate the signaling pathway by which Mfge8 mediates these effects. By using genetic and pharmacologic approaches, we show that the  $\alpha$ 8 $\beta$ 1 integrin and the phosphatase and tensin homolog (PTEN) mediate the effects of Mfge8 on preventing IL-13-induced increases in airway contractility. Tracheal rings from mice with smooth muscle-specific deletion of  $\alpha$ 8 $\beta$ 1 or PTEN have enhanced contraction in response to treatment with IL-13. Enhanced IL-13-induced tracheal ring contraction in *Mfge8*<sup>-/-</sup> mice was abolished by treatment with the PI3K inhibitor. Mechanistically, IL-13 induces ubiquitination and degradation of PTEN protein. Our findings identify a role for the Mfge8- $\alpha$ 8 $\beta$ 1-PTEN pathway in regulating the force of airway smooth muscle contraction in the setting of allergic inflammation.—Khalifeh-Soltani, A., Gupta, D., Ha, A., Podolsky, M. J., Datta, R., Atabai, K. The Mfge8- $\alpha$ 8 $\beta$ 1-PTEN pathway regulates airway smooth muscle contraction in allergic inflammation. *FASEB J.* 32, 5927–5936 (2018). www.fasebj.org

**KEY WORDS:** integrins • airway hyperresponsiveness • cellular signaling

Approximately 300 million people worldwide are affected with asthma, and 1 in 12 people in the United States have this disease (1). Even with the current effective therapeutic options, symptoms remain poorly controlled in a significant number of patients with asthma. Airway obstruction leading to shortness of breath is a hallmark of allergic asthma and is in large part due to airway smooth muscle (ASM) hypercontractility. Although there has been an extensive effort to understand the immunologic mechanisms that cause asthma, less focus has been on investigating the abnormal contractile properties of ASM commonly found in patients with asthma. Exposure of

ASM to allergic inflammation leads to an increase in the contractile response to endogenous agonists due to enhanced ASM calcium sensitivity. Current treatment strategies for asthma primarily focus on dampening the immune response or dilating ASM. No current therapies specifically target ASM hypercontractility, although this strategy would address the main mechanism responsible for shortness of breath in asthma. Identification of endogenous molecules and pathways involved in ASM hypercontractility are therefore of considerable therapeutic interest.

We have previously identified one such pathway by which integrin-ligand milk fat globule epidermal growth factor-like 8 (Mfge8) regulates airway hyperresponsiveness (AHR) by preventing cytokine-induced increases in calcium sensitivity (2). Mice deficient in Mfge8 (*Mfge8*<sup>-/-</sup>) develop exaggerated AHR in experimental models of asthma. Ras homolog gene family member A (RhoA) has emerged as a key regulator of AHR and has a central role in enhancing ASM contraction (3–5). We have shown that under conditions of allergic inflammation, ligation of integrin receptors by Mfge8 suppresses RhoA activation and decreases the force of smooth muscle contraction in airways (2). The integrin receptors involved in this process and the

**ABBREVIATIONS:** AHR, airway hyperresponsiveness; ASM, airway smooth muscle; MCh, methacholine; Mfge8, milk fat globule epidermal growth factor-like 8 protein; PIP3, phosphatidylinositol 3,4,5-trisphosphate; PTEN, phosphatase and tensin homolog; RGE, Mfge8 construct where the RGD sequence was mutated to RGE; RhoA, Ras homolog gene family member A; WT, wild type

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mechanism by which integrin ligation prevents RhoA activation are, however, uncharacterized. Identification of signaling mechanisms and molecules involved in RhoA activation in the setting of allergic asthma could lead to specific and effective therapeutic targets.

The present study investigated the molecular mechanisms involved in Mfge8 regulation of RhoA in ASM contraction. Based on our recent report on the role of RGD-binding integrin  $\alpha 8 \beta 1$  in promoting nutrient absorption through regulation of antral smooth muscle contractile force (6), we hypothesized that an  $\alpha 8 \beta 1$ -PTEN pathway might be involved in mediating the effect of Mfge8 on ASM contraction in the setting of allergic inflammation. Toward this end, we observed that Mfge8 ligation of  $\alpha 8 \beta 1$  integrin modulates cytokine-mediated airway contraction in ASM by modulating the PI3K pathway. Specifically, we elucidated the role of phosphatase and tensin homolog deleted from chromosome 10 (PTEN), a major negative regulator of the PI3K pathway in this process. We found that mice with smooth muscle-specific deletion of PTEN have enhanced force of contraction in response to IL-13, and enhanced contraction was not rescued with the addition of rMfge8. Furthermore, Mfge8 ligation of  $\alpha 8 \beta 1$  on ASM prevents IL-13-mediated increase in RhoA activation by inhibiting PTEN ubiquitination and degradation.

## MATERIALS AND METHODS

### Mice

All animal experiments were approved by the University of California, San Francisco (UCSF) Institutional Animal Care and Use Committee in adherence to U.S. National Institutes of Health (Bethesda, MD, USA) guidelines and policies.  $\alpha \nu \beta 3$  and  $\alpha \nu \beta 5$  double knockout ( $\beta 3^{-/-}/\beta 5^{-/-}$ ) mice are in the 129SVEV background (7, 8). Wild-type (WT) mice in the 129SVEV background were used as controls for studies with  $\beta 3^{-/-}/\beta 5^{-/-}$  mice. All other mice were maintained on a C57BL/6 background. *Itga8*<sup>flox/flox</sup> mice have been previously described (9).  $\alpha 8 \text{sm}^{-/-}$  mice were created by crossing *Itga8*<sup>flox/flox</sup> mice with mice carrying *Tg(TetO-cre)1Jaw/J* and *Tg(Acta2-rtTA)#Des* transgenes. *PTEN*<sup>sm<sup>-/-</sup></sup> mice were created by crossing *pten*<sup>flox/flox</sup> mice (10) with mice carrying *Tg(TetO-cre)1Jaw/J* and *Tg(Acta2-rtTA)#Des* transgenes. For smooth muscle induction of Cre-mediated recombination of *Itga8*<sup>flox/flox</sup>—*Tg(Acta2-rtTA, TetO-Cre)* or *pten*<sup>flox/flox</sup>—*Tg(Acta2-rtTA, TetO-Cre)*, mice were fed doxycycline chow for 2 wk before the experiments.

### Tracheal ring contraction

Tracheal rings were obtained from 6- to 8-wk-old sex-matched mice as previously described (5), and were placed in DMEM supplemented with 20% fetal bovine serum. Rings were incubated with or without IL-13 (100 ng/ml; PeproTech, Rocky Hill, NJ, USA) for 1 h or overnight in a 37°C incubator with 5% CO<sub>2</sub>. They were then suspended in a 15-ml organ bath filled with

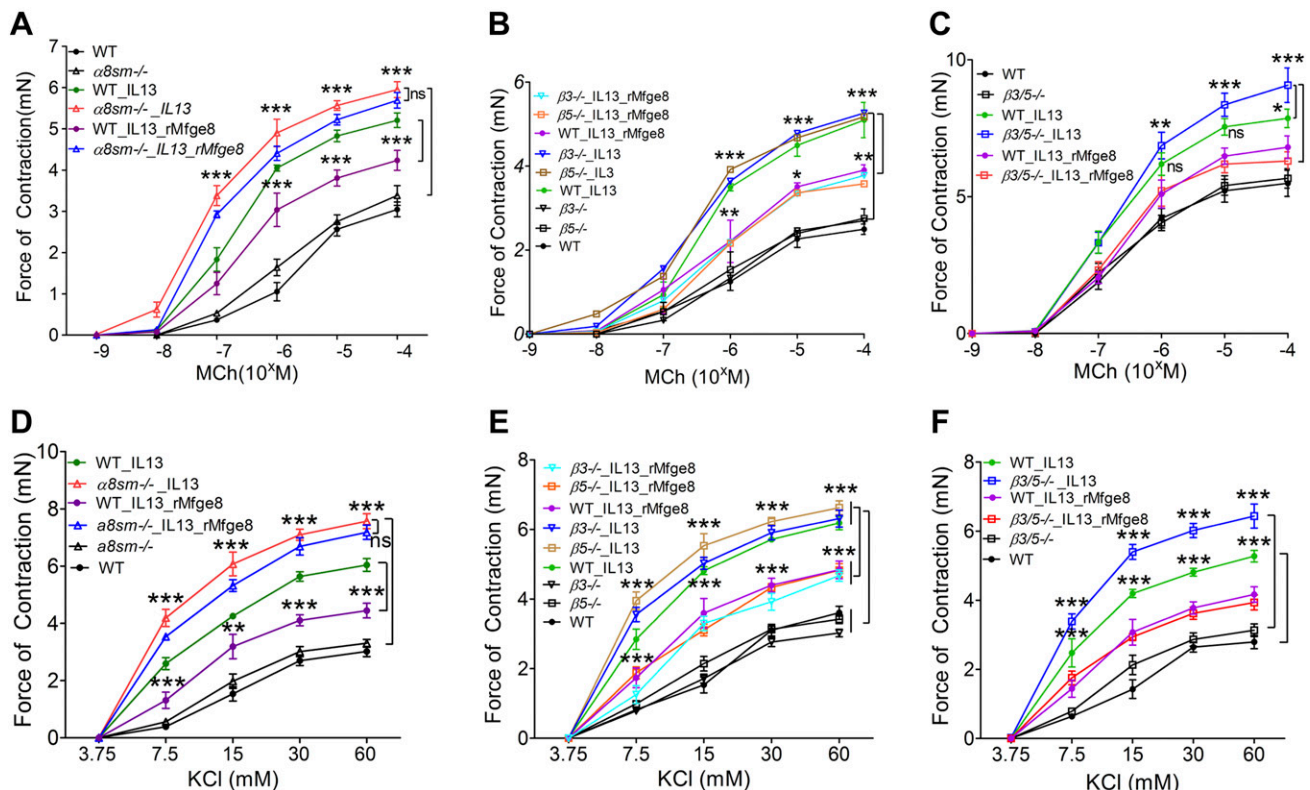


**Figure 1.** Mfge8 ligation of the  $\alpha 8 \beta 1$  integrin prevents the effect of IL-13 on ASM contraction. Tracheal rings isolated from  $\alpha 8 \text{sm}^{-/-}$  (A, D),  $\alpha \nu \beta 3$  ( $\beta 3^{-/-}$ ), or  $\alpha \nu \beta 5$  ( $\beta 5^{-/-}$ ) integrin knockout (B, E) and  $\alpha \nu \beta 3$  and  $\alpha \nu \beta 5$  double knockout ( $\beta 3^{-/-}/\beta 5^{-/-}$ ) (C, F) mice were treated with rMfge8 (10  $\mu\text{g}/\text{ml}$ ) and IL-13 (100 ng/ml) for 12 h. Force of contraction in response to MCh (A–C) ( $n = 3$ –4) and KCl (D–F) ( $n = 3$ ) was measured. Male mice were used for these experiments. Data are expressed as means  $\pm$  SEM. One-way ANOVA was used to make comparisons between multiple groups. When the ANOVA comparison was statistically significant ( $P < 0.05$ ), further pairwise analysis was conducted by using a Bonferroni  $t$  test. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

oxygenated modified Krebs-Henseleit solution and connected to a force displacement transducer (FT03; Grass Instrument, West Warwick, RI, USA), and a resting tension of 0.5 g was applied. After a 15 min equilibration period, concentration–response curves to MCh or KCl were obtained. For selected studies, wortmannin (100 ng/ml; Selleck Chemicals, Houston, TX, USA) and recombinant Mfge8 constructs (10 µg/ml; R&D Systems, Minneapolis, MN, USA) were added 20 min before the addition of IL-13.

### Primary ASM cells and treatments

Primary ASM cells were obtained from the tracheas of sex-matched 6- to 8-wk-old mice by dissecting out the trachea and placing 3-mm slices with the luminal side down on tissue culture plates in DMEM (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 20% fetal bovine serum and antibiotics. Cells from passages 5 to 8 were grown to near-confluence, serum-starved for 12 h before stimulation, and incubated with IL-13 (100 ng/ml) for 1 h at 37°C. Cells were then treated with 5-hydroxytryptamine (100 µM) (MilliporeSigma, Burlington, MA, USA) for 15 min and lysed for Western blotting. A total of 350 µg of total protein was used for evaluation of RhoA activation as previously described (5). For selected studies, wortmannin (100 ng/ml), recombinant Mfge8 constructs (10 µg/ml), and blocking antibodies (5 µg/ml) were added 20 min before the addition of IL-13. pAkt (S473) (4060; Cell Signaling Technology, Danvers, MA, USA), total Akt (9272; Cell Signaling Technology), RhoA (2117; Cell Signaling Technology), ROCK1 (4035; Cell Signaling Technology) and ROCK2 (9029; Cell Signaling Technology), and glyceraldehyde-3-phosphate dehydrogenase (5174; Cell Signaling Technology) antibodies were used in Western blotting.

### Immunocytochemistry and confocal imaging

ASM cells were cultured on the glass coverslips, fixed with ice-cold methanol for 5 min, and blocked in PBS with 0.5% Tween 20 and 10% goat serum for 1 h at room temperature. Cells were then treated with the antibody against  $\alpha$  smooth muscle actin (F3777; MilliporeSigma), calponin (ab46794; Abcam, Cambridge, MA, USA), and SM22 $\alpha$  (ab28811; Abcam) at 1:200 in blocking at 4°C for 16 h followed by secondary antibodies Alexa Fluor 568 and Alexa fluor 635 (A21069 and A31574, respectively; Molecular Probes, Eugene, OR, USA) at 1:500 in blocking for 1 h at room temperature. Coverslips were then mounted with fluorescent mounting medium (0100-01; SouthernBiotech, Birmingham, AL, USA). Confocal microscopy was performed by using a  $\times 40$  oil objective lens on a Leica TCS SPE confocal microscope (Leica Microsystems, Wetzlar, Germany). Images were processed with Photoshop CS6 software (Adobe Systems, San Jose, CA, USA).

### PTEN ubiquitination and degradation

Smooth muscle dissected from posterior mouse tracheas was treated with MG132 (50 µM; Selleck Chemicals) and chloroquine (50 µM; MilliporeSigma) for 4 h followed by the addition of IL-13 (100 ng/ml) for 1 h. Samples were then treated with MCh (100 µM; MilliporeSigma) for 15 min and lysed in NETN buffer (100 mM NaCl, 1 mM EDTA, 20 mM Tris-Cl [pH 8.0], and 0.5% NP-40) containing protease-phosphatase inhibitors (Roche, Basel, Switzerland). For immunoprecipitation, extracts were precleared by using protein G Sepharose beads (GE Healthcare, Waukesha, WI, USA) and incubated with PTEN antibody (9552; Cell Signaling Technology) or rabbit IgG (isotype control) (3900; Cell Signaling Technology) overnight at 4°C with constant shaking. The beads were then washed 3 times with the lysis buffer, and bound proteins were eluted by boiling in 1 $\times$  Laemmli buffer and

subjected to immunoblotting with the antibody against ubiquitin (sc-8017; Santa Cruz Biotechnology, Dallas, TX, USA).

### PTEN activity assay

Smooth muscle dissected from mouse posterior tracheas were used for quantification of PTEN activity by using a PTEN activity ELISA kit (Echelon, San Jose, CA, USA) as previously described (6). Briefly, PTEN activity ELISA kit quantifies the phosphatase activity of PTEN by detection of the product phosphatidylinositol 4,5-bisphosphate. This is a competitive ELISA in which the signal is inversely proportional to the amount of PI(4,5)P<sub>2</sub> produced by PTEN. After the PTEN reactions are complete, reaction products are added to the PI(4,5)P<sub>2</sub>-coated microplate, and a PI(4,5)P<sub>2</sub> detector protein is then added for competitive binding. A peroxidase-linked secondary detector and colorimetric detection are used to determine PI(4,5)P<sub>2</sub> detector binding to the plate.

### Statistical analyses

Data were assessed for normal distribution and similar variance between groups by using GraphPad Prism 6.0 (GraphPad Software, San Diego, CA, USA). A 1-way ANOVA was used to make comparisons between multiple groups. When the ANOVA comparison was statistically significant ( $P < 0.05$ ), further pairwise

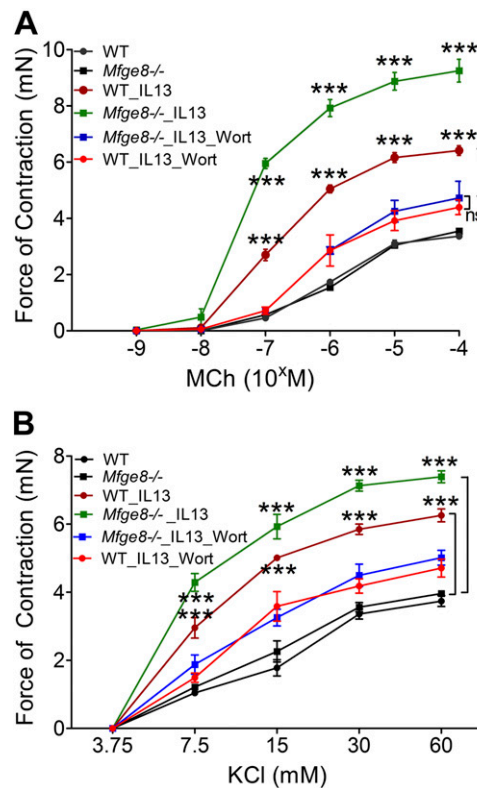


**Figure 2.** PI3K inhibition abrogates the effect of Mfge8 on IL-13-mediated ASM contraction. Force of smooth muscle contraction with and without the addition of PI3K inhibitor wortmannin (wort 100 ng/ml) in response to MCh (A) and KCl (B) in tracheal rings from WT and *Mfge8*<sup>-/-</sup> mice ( $n = 4-5$ ). Male mice were used for these experiments. Data are expressed as means  $\pm$  SEM. One-way ANOVA was used to make comparisons between multiple groups. When the ANOVA comparison was statistically significant ( $P < 0.05$ ), further pairwise analysis was conducted by using a Bonferroni  $t$  test. \*\*\* $P < 0.001$ .

analysis was performed by using a Bonferroni *t* test. A 2-sided Student's *t* test was used for comparisons between 2 groups.

## RESULTS

### Mfge8 ligation of the $\alpha 8 \beta 1$ integrin prevents the effect of IL-13 on ASM contraction

We have previously shown that Mfge8 ligation of integrin receptors in airways prevents cytokine-mediated AHR (2). However, the integrin receptors that mediate this process are unknown.  $\alpha \nu \beta 3$  and  $\alpha \nu \beta 5$  integrins are the canonical receptors for Mfge8 (11, 12). In addition to these 2 receptors, we found that the RGD-binding integrin  $\alpha 8 \beta 1$ , which is highly expressed in smooth muscle cells (13–15), is also a receptor for Mfge8 and regulates the force of gastric antral smooth muscle contraction (6). We first examined if any of these integrin receptors ( $\alpha \nu \beta 3$ ,  $\alpha \nu \beta 5$ , and  $\alpha 8 \beta 1$ ) were involved in mediating the effects of Mfge8 on ASM contraction. We isolated the tracheal rings from  $\alpha \nu \beta 3$  ( $\beta 3^{-/-}$ ) and  $\alpha \nu \beta 5$  ( $\beta 5^{-/-}$ ) integrin knockout mice and mice with smooth muscle-specific deletion of  $\alpha 8 \beta 1$  ( $\alpha 8sm^{-/-}$ ). We then stimulated them with IL-13 *in vitro* and evaluated the force of contraction in an *ex vivo* muscle bath system. Tracheal rings from  $\alpha 8sm^{-/-}$  mice treated for 12 h with IL-13 had enhanced contraction in response to methacholine (MCh) and KCl (Fig. 1A, D). Recombinant Mfge8 (rMfge8) significantly reduced the force of contraction in rings from WT mice with no significant effect on tracheal rings from  $\alpha 8sm^{-/-}$  mice. Tracheal rings from  $\alpha 8sm^{-/-}$  mice did not

have increased contraction at baseline (without IL-13 treatment), similar to what we observed with tracheal rings from Mfge8<sup>-/-</sup> mice (2). Tracheal rings from  $\alpha \nu \beta 3$  ( $\beta 3^{-/-}$ ) and  $\alpha \nu \beta 5$  ( $\beta 5^{-/-}$ ) integrin knockout mice did not have enhanced contraction compared with WT mice at baseline or in response to IL-13. Furthermore, the force of IL-13-stimulated contraction in response to MCh and KCl was significantly reduced after incubation with rMfge8 (Fig. 1B, E). Interestingly, IL-13-treated tracheal smooth muscle rings from  $\alpha \nu \beta 3$  and  $\alpha \nu \beta 5$  double knockout mice ( $\beta 3^{-/-}/\beta 5^{-/-}$ ) had enhanced contraction (Fig. 1C, F) in response to MCh and KCl, similar to tracheal rings from Mfge8<sup>-/-</sup> mice (2). However, unlike IL-13-treated tracheal rings from  $\alpha 8sm^{-/-}$  mice, rMfge8 significantly reduced the force of contraction in tracheal rings from  $\beta 3^{-/-}/\beta 5^{-/-}$  mice. Taken together, these data indicate that the  $\alpha 8 \beta 1$  integrin mediates the effect of Mfge8 on smooth muscle contraction in ASM. Furthermore, the enhanced contraction seen in IL-13-treated tracheal rings from  $\beta 3^{-/-}/\beta 5^{-/-}$  mice occurs independent of the Mfge8 pathway.

### PI3K inhibition abrogates the effect of Mfge8 on IL-13-mediated ASM contraction and RhoA activation

We have shown that Mfge8 ligation of integrin receptors prevents cytokine-mediated increases in RhoA activation and RhoA protein expression in ASM cells (2). We next looked for the upstream molecular signaling pathways by which Mfge8 prevents RhoA activation in smooth muscle.

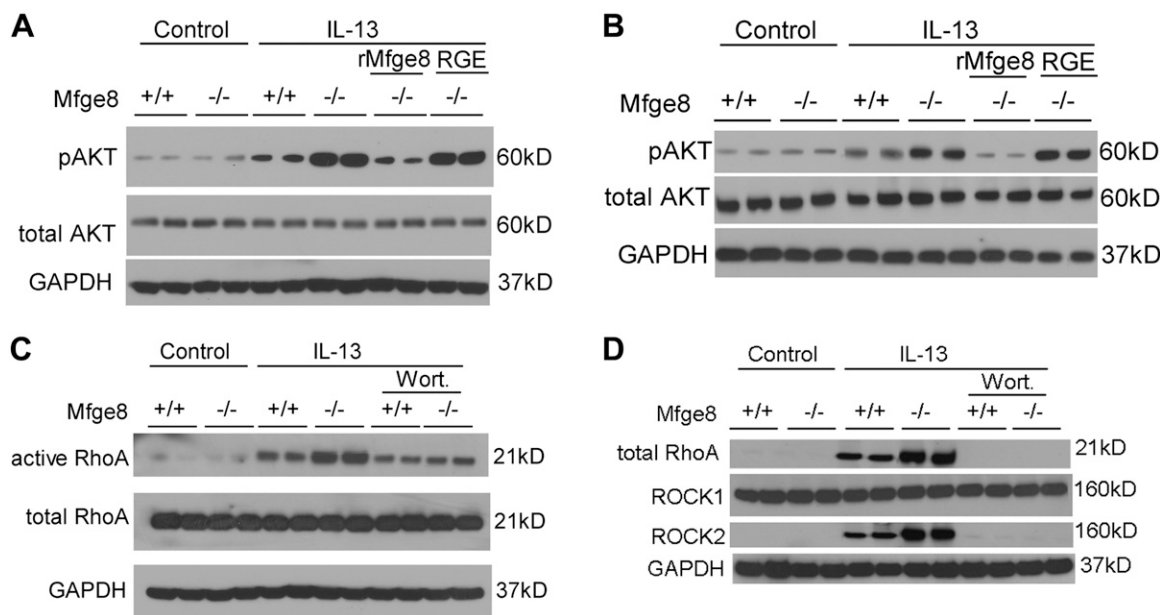


**Figure 3.** Mfge8 prevents IL-13-mediated Akt phosphorylation and RhoA activation in ASM. A, B) Western blot of tracheal smooth muscle strips (A) and primary ASM cells (B) from WT and Mfge8<sup>-/-</sup> mice, incubated with IL-13 in the presence of rMfge8 or RGE (10  $\mu$ g/ml) for 1 h followed by stimulation with MCh (A) or 5-hydroxytryptamine (5-HT) (B) (100  $\mu$ M) for 15 min. C) Western blot of tracheal smooth muscle strips obtained from WT and Mfge8<sup>-/-</sup> mice treated with IL-13 (100 ng/ml) for 1 h in the presence of the PI3K inhibitor wortmannin (100 ng/ml for 20 min before the addition of IL-13) demonstrating active and total RhoA using a glutathione S-transferase (GST) pull-down assay. D) Western blot of total proteins from tracheal smooth muscle strips obtained from WT and Mfge8<sup>-/-</sup> mice treated with IL-13 (100 ng/ml) for 12 h in the presence of the PI3K inhibitor wortmannin (100 ng/ml for 20 min before the addition of IL-13). Data are representative of 1 of the 3 independent experiments. Male mice were used for all the panels; *n* = 6 for all 4 panels. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Class I PI3Ks are enzymes modulated by Mfge8 (16) and are known to promote allergic airway diseases. To determine whether PI3K, also a known regulator of smooth muscle contraction (17, 18), mediates the effect of the Mfge8- $\alpha$ 8 $\beta$ 1 axis on ASM contraction, we first evaluated the effect of the PI3K inhibitor, wortmannin, on the contraction of tracheal rings from *Mfge8*<sup>-/-</sup> and WT mice. Wortmannin significantly reduced MCh- and KCl-induced contraction in IL-13-treated *Mfge8*<sup>-/-</sup> and WT tracheal rings with a proportionally greater effect in rings from *Mfge8*<sup>-/-</sup> mice compared with rings from WT mice (Fig. 2A, B). Treatment with wortmannin completely abrogated the difference in IL-13-mediated contraction in WT and *Mfge8*<sup>-/-</sup> mice.

We next evaluated whether AKT phosphorylation, a downstream marker of PI3K activation (19), was altered in tracheal rings from *Mfge8*<sup>-/-</sup> mice. AKT phosphorylation (at serine 473) was enhanced in IL-13-treated *Mfge8*<sup>-/-</sup> tracheal rings and primary ASM cells (Fig. 3A, B), and enhanced phosphorylation was rescued after incubation with rMfge8. ASM cells were stained with  $\alpha$  smooth muscle actin, calponin, and SM22 $\alpha$  to confirm the primary cell phenotype (Supplemental Fig. 1). Enhanced phosphorylation was not rescued with an Mfge8 construct in which the RGD sequence was mutated to RGE (20), thereby preventing integrin ligation. These data indicate that Mfge8 opposes the effect of IL-13 on PI3K activation in an integrin-dependent manner.

Mfge8 prevents RhoA activation in IL-13-treated tracheal rings, and *Mfge8*<sup>-/-</sup> mice have enhanced RhoA activity after cytokine treatment (2). We next tested whether wortmannin prevents the enhanced RhoA activity in IL-13-treated *Mfge8*<sup>-/-</sup> mice using a GST pull-down assay. Wortmannin reduced the enhanced RhoA activation in IL-13-treated *Mfge8*<sup>-/-</sup> and WT tracheal rings with a relatively greater effect in the *Mfge8*<sup>-/-</sup> samples (Fig. 3C), indicating that inhibition of PI3K activation prevents IL-13-induced RhoA activation. We also evaluated the effect of wortmannin on RhoA and ROCK2 protein expression levels. ROCK2 is a downstream kinase targeted by RhoA in smooth muscle and is responsible for phosphorylation of myosin light chain phosphatase causing ASM contraction (3, 5, 21). Wortmannin prevented the increase in IL-13-induced RhoA and ROCK2 expression in both WT and *Mfge8*<sup>-/-</sup> tracheal rings (Fig. 3D). These data indicate that Mfge8 prevents IL-13-mediated increase in PI3K activity and hence RhoA activation in ASM cells.

### Mfge8 regulates ASM contraction by preventing IL-13-induced PTEN degradation

We have found that the inhibition of PI3Ks by wortmannin prevents the exaggerated increase in contraction and RhoA activation induced by IL-13 in *Mfge8*<sup>-/-</sup> ASM. PI3Ks increase the production of the lipid mediator phosphatidylinositol 3,4,5-trisphosphate (PIP3), which then recruits 3-phosphoinositide-dependent kinase-1 and Akt to the plasma membrane, leading to kinase-mediated phosphorylation and activation of AKT. The tumor suppressor gene PTEN is known to be a major negative regulator of PI3Ks. It has important roles in smooth muscle function (22) and works by dephosphorylating PIP3 to phosphatidylinositol 4,5-bisphosphate. PTEN is a key

phosphatase that opposes the effect of PI3K on Akt phosphorylation (23). We next explored whether Mfge8 inhibits PI3K activation in IL-13-treated ASM by modulation of PTEN. To evaluate the role of PTEN in this pathway, we measured the tracheal strip PTEN activity after IL-13 stimulation using an ELISA-based assay that quantifies the ability of lysates to convert PI(3,4,5)P3 to PI(4,5)P2 (24). IL-13 reduced PTEN activity in WT and *Mfge8*<sup>-/-</sup> tracheal rings with greater effect in *Mfge8*<sup>-/-</sup> compared with WT (Fig. 4A, B). rMfge8 significantly increased PTEN activity in IL-13-treated tracheal rings from both WT and *Mfge8*<sup>-/-</sup> mice while RGE control construct had no effect. Previous treatment of tracheal rings with

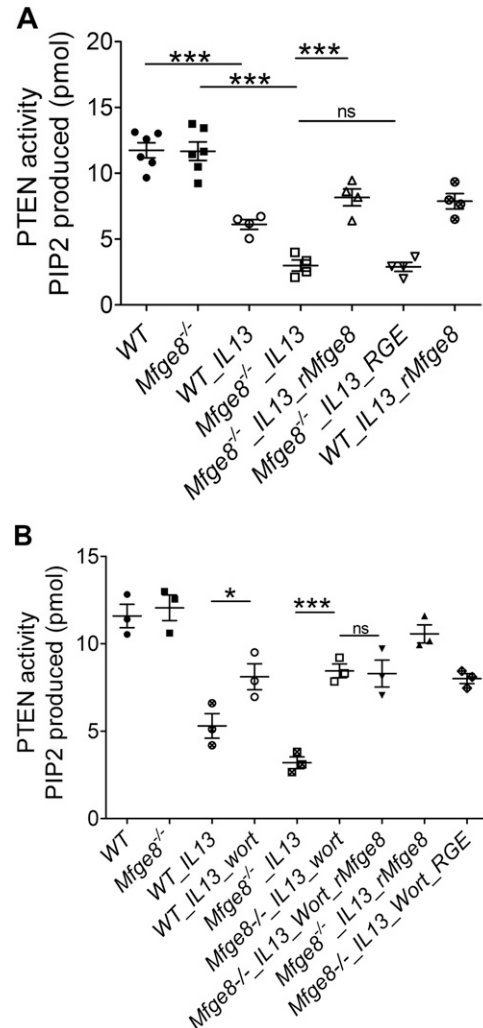


**Figure 4.** Mfge8 regulates IL-13-mediated activation of PI3K pathway by increasing PTEN activity. PTEN activity in tracheal smooth muscle strips of WT and *Mfge8*<sup>-/-</sup> ( $n = 3-6$ ) mice treated with IL-13 (100 ng/ml for 1 h), with and without the addition of rMfge8 and RGE construct (10  $\mu$ g/ml for 20 min before the addition of IL-13) followed by stimulation with MCh. Wortmannin (100 ng/ml for 20 min) was added to the respective groups 20 min before the addition of IL-13. Male mice were used for panel A and female mice were used for panel B. Data are expressed as means  $\pm$  SEM. One-way ANOVA was used to make comparisons between multiple groups. When the ANOVA comparison was statistically significant ( $P < 0.05$ ), further pairwise analysis was conducted by using a Bonferroni  $t$  test. Ns, not significant; PIP2, phosphatidylinositol 4,5-bisphosphate. \* $P < 0.05$ , \*\*\* $P < 0.001$ .

wortmannin prevented the reduction in PTEN activity upon IL-13 stimulation in tracheal rings from both WT and *Mfge8*<sup>-/-</sup> mice (Fig. 4B). Also, treatment of these rings with rMfge8 caused no further increase in PTEN activity. This finding indicates that Mfge8 regulates IL-13-mediated activation of PI3K pathway by increasing PTEN activity.

We next assessed the expression levels of PTEN protein upon IL-13 stimulation. One-hour treatment of tracheal rings with IL-13 led to a marked loss of PTEN protein in both WT and *Mfge8*<sup>-/-</sup> mice, an effect that was prevented by coinubation with rMfge8 but not the RGE control construct (Fig. 5A). Incubation with fibronectin or vitronectin, both  $\alpha 8 \beta 1$  integrin ligands, did not rescue IL-13-induced PTEN degradation, suggesting that this effect was specific for Mfge8 (Fig. 5B).

PTEN has a half-life of >8 h (25). We observed loss of PTEN protein after 1 h of treatment with IL-13, suggesting that IL-13 may modulate PTEN at a post-translational level rather than affecting PTEN transcription. PTEN is known to undergo extensive post-translational regulation, including ubiquitination (25, 26). We next investigated whether reduction of PTEN expression in ASM after IL-13 treatment was proteasome dependent. We therefore treated the tracheal rings from WT and *Mfge8*<sup>-/-</sup> mice with the proteasome inhibitor, MG132, for 4 h followed by treatment with IL-13 for 1 h and evaluated PTEN protein levels by Western blotting. Incubation with MG132 prevented the loss of PTEN protein after IL-13 treatment while

chloroquine (a lysosomal inhibitor) had no effect (Fig. 5C). To further confirm whether IL-13 induced ubiquitination and subsequent proteasomal degradation of PTEN, lysates of tracheal rings were immunoprecipitated with an anti-PTEN antibody after incubation with IL-13 in the presence of MG132 (Fig. 5D). We then probed lysates with an anti-ubiquitin antibody and anti-PTEN antibody. IL-13 treatment resulted in an increase in degradation bands, indicating that IL-13 induces ubiquitination of PTEN. We interpret these data to indicate that IL-13 promotes PTEN ubiquitination and degradation in ASM.

### Mfge8 ligation of the $\alpha 8 \beta 1$ integrin prevents PTEN degradation

To further confirm if Mfge8 prevents PTEN degradation by ligation to  $\alpha 8 \beta 1$  integrin, PTEN activity was measured in tracheal smooth muscle strips from  $\alpha 8 \text{sm}^{-/-}$  mice. IL-13 reduced PTEN activity in WT mice as well as in  $\alpha 8 \text{sm}^{-/-}$  mice (Fig. 6A). However, rMfge8 significantly increased PTEN activity in IL-13-treated tracheal rings from WT mice but not from  $\alpha 8 \text{sm}^{-/-}$  mice. Similar results were obtained in the presence of blocking antibodies (Fig. 6B). rMfge8 did not prevent the IL-13-mediated decrease in PTEN activity in tracheal rings treated with  $\alpha 8$  blocking antibody. These results confirmed our finding that Mfge8 ligation to  $\alpha 8 \beta 1$  integrin prevents IL-13-mediated decrease in PTEN activity in ASM.

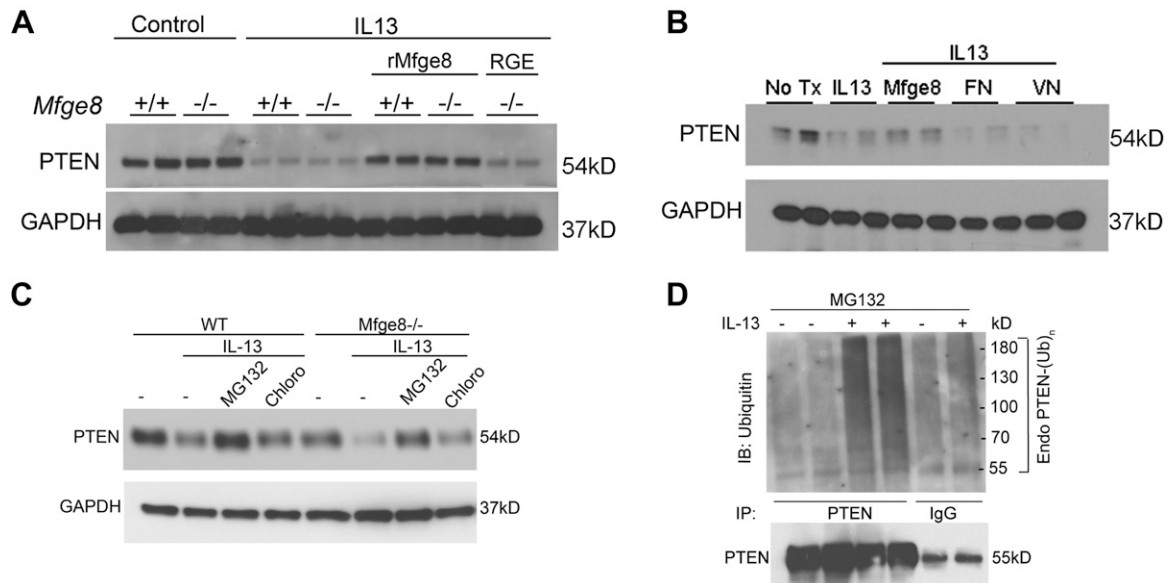


**Figure 5.** Mfge8 regulates ASM contraction by preventing IL-13-induced PTEN degradation. **A)** Western blot of tracheal smooth muscle strip from WT and *Mfge8*<sup>-/-</sup> mice treated with or without IL-13 (100 ng/ml for 1 h) followed by stimulation with MCh for 15 min. rMfge8 or RGE (10  $\mu$ g/ml) was added 20 min before the addition of IL-13 ( $n = 8$ ). Blot shown is representative of 1 of the 4 independent experiments. **B)** Western blot of tracheal smooth muscle strip from WT mice treated with or without IL-13 (100 ng/ml for 1 h) followed by stimulation with MCh for 15 min. rMfge8, fibronectin (FN), or vitronectin (VN) (10  $\mu$ g/ml) was added 20 min before the addition of IL-13 ( $n = 5$ ). Blot shown is representative of 1 of the 3 independent experiments. **C)** Western blot of tracheal smooth muscle strip from WT mice and *Mfge8*<sup>-/-</sup> mice treated with or without IL-13 (100 ng/ml for 1 h) followed by stimulation with MCh for 15 min. MG132 (50  $\mu$ M) and chloroquine (50  $\mu$ M) were added to respective groups 4 h before the addition of IL-13 ( $n = 4$ ). Blot shown is representative of 1 of the 4 independent experiments. **D)** Tracheal strips from WT mice were treated with MG132 for 4 h followed by treatment with IL-13 for 1 h. Lysates were immunoprecipitated with antibody against PTEN and immunoblotted with antibody against ubiquitin and PTEN. IgG was used as an isotype control for immunoprecipitation ( $n = 4$ ). Blot shown is representative of 1 of the 2 independent experiments. Female mice were used for all the panels. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

## Mfge8 prevents IL-13-mediated smooth muscle contraction via PTEN

Given the aforementioned data and as a proof-of-concept to establish that PTEN mediates the effect of Mfge8 and  $\alpha 8\beta 1$  on smooth muscle contraction, we created a mouse line carrying an  $\alpha$  smooth muscle actin-rtta transgene and a tetracycline-inducible Cre transgene to allow for smooth muscle-specific deletion of PTEN in mice homozygous for a PTEN floxed allele (10) ( $PTEN^{sm-/-}$ ).  $PTEN^{sm-/-}$  had a marked decrease in PTEN protein by Western blot of tracheal smooth muscle strips after 2 wk of doxycycline

administration (Fig. 7A) compared with single transgenic controls. Tracheal rings from  $PTEN^{sm-/-}$  had an increase in the force of tracheal ring contraction in response to MCh and KCl (Fig. 7B, C) after 12 h of treatment with IL-13. There was no difference in baseline  $PTEN^{sm-/-}$  tracheal ring contraction. Enhanced contraction after IL-13 treatment was not rescued with the addition of rMfge8, indicating that Mfge8 and PTEN function in the same pathway. We also analyzed the effect of Mfge8 on Akt phosphorylation in tracheal strips from  $PTEN^{sm-/-}$  mice (Fig. 7D). We found that rMfge8 prevents the increase in IL-13-mediated Akt phosphorylation in  $Mfge8^{-/-}$  mice but not in  $PTEN^{sm-/-}$  mice. These results confirm that Mfge8 prevents IL-13-mediated increase in ASM contraction by modulating PTEN.

## DISCUSSION

Airway obstruction caused by ASM hypercontractility is a major underlying feature of asthma (27, 28). Cytokines, growth factors, and other extracellular matrix proteins are known to modulate ASM contraction and function. Integrins and their ligands play an important role in regulating AHR. For example, the integrin  $\alpha 9\beta 1$ , highly expressed in ASM cells (29, 30), inhibits AHR. *In vivo* studies have shown that conditional knockout of the  $\alpha 9$  subunit leads to an increased contractile response to cholinergic agonists and enhanced AHR (30). Another study has suggested the role of  $\alpha v\beta 8$  in allergen-induced AHR. Tracheal rings from mice lacking  $\alpha v\beta 8$  on dendritic cells have decreased airway contractility after allergen challenge (5).

The role of the RGD-binding integrin  $\alpha 8\beta 1$  has previously not been characterized in ASM contractility. By using genetic and pharmacologic approaches, we identified the molecular mechanisms by which  $\alpha 8\beta 1$  ligation prevents cytokine-mediated ASM contraction (Fig. 8). Mice with smooth muscle-specific deletion of  $\alpha 8\beta 1$  integrin have enhanced force of tracheal smooth muscle contraction in response to IL-13, and Mfge8 binding to  $\alpha 8\beta 1$  integrin is essential to abrogate the enhanced contraction. Previous studies have identified the role of  $\alpha 8$  in vascular and gastric smooth muscle as well as in intestinal epithelial cells (6, 13, 31). However, the role of  $\alpha 8\beta 1$  in RhoA regulation differs among these cell types. In vascular smooth muscle cells (31, 32) and intestinal epithelial cells, knockdown of  $\alpha 8$  leads to less active RhoA. In contrast to this scenario,  $\alpha 8\beta 1$  ligation in gastric smooth muscle prevents RhoA activation (6). Similar to gastric smooth muscle, we found that  $\alpha 8\beta 1$  ligation in ASM prevents RhoA activation and contraction. However, unlike gastric smooth muscle, this effect of  $\alpha 8\beta 1$  ligation in ASM is seen in response to inflammatory cytokines and not under basal conditions.

Mechanistically, we found that Mfge8 ligation of  $\alpha 8\beta 1$  on ASM prevents IL-13-mediated increases in RhoA activation by regulating the PI3K pathway. Previous reports have established the role of the PI3K pathway in the pathogenesis of asthma such as in airway inflammation, mucus hypersecretion, and AHR. It has been shown that IL-13 binding to IL-13 receptors activates PI3K and downstream effector molecules (33, 34). Administration of wortmannin or LY294002, two broad-spectrum inhibitors

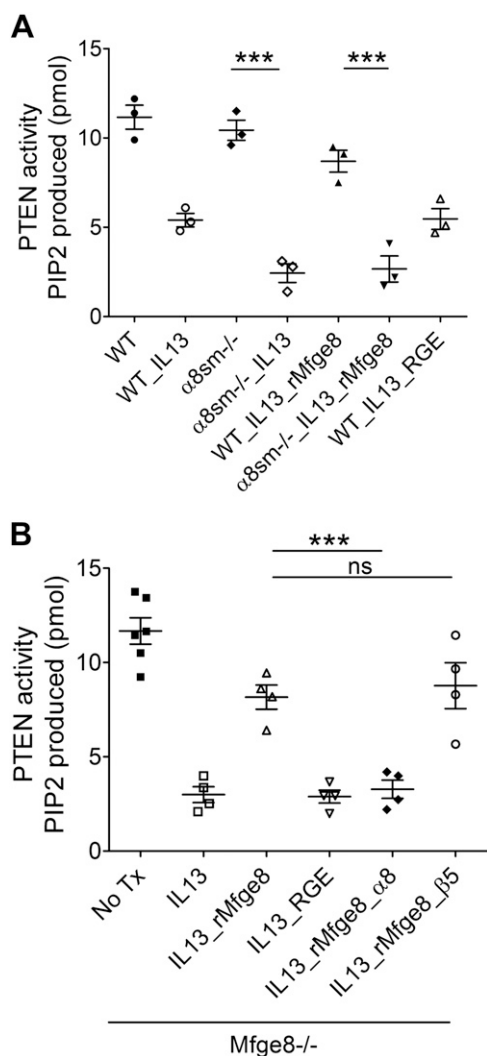


**Figure 6.** Mfge8 ligation to  $\alpha 8\beta 1$  integrin prevents IL-13-mediated decreases in PTEN activity in ASM. PTEN activity in tracheal smooth muscle strips of WT (single transgenic controls) and  $\alpha 8sm^{-/-}$  (A;  $n = 3$ ) and  $Mfge8^{-/-}$  (B;  $n = 4-6$ ) mice treated with IL-13 (100 ng/ml for 1 h), with and without the addition of rMfge8 and RGE construct (10  $\mu$ g/ml for 20 min before the addition of IL-13) followed by stimulation with MCh.  $\alpha 8$  and  $\beta 5$  blocking antibodies (5  $\mu$ g/ml) were added 20 min before the addition of rMfge8 in B. Male mice were used for both A and B. Data are expressed as means  $\pm$  SEM. One-way ANOVA was used to make comparisons between multiple groups. When the ANOVA comparison was statistically significant ( $P < 0.05$ ), further pairwise analysis was conducted by using a Bonferroni  $t$  test. Tx, treatment. \*\*\* $P < 0.001$ .

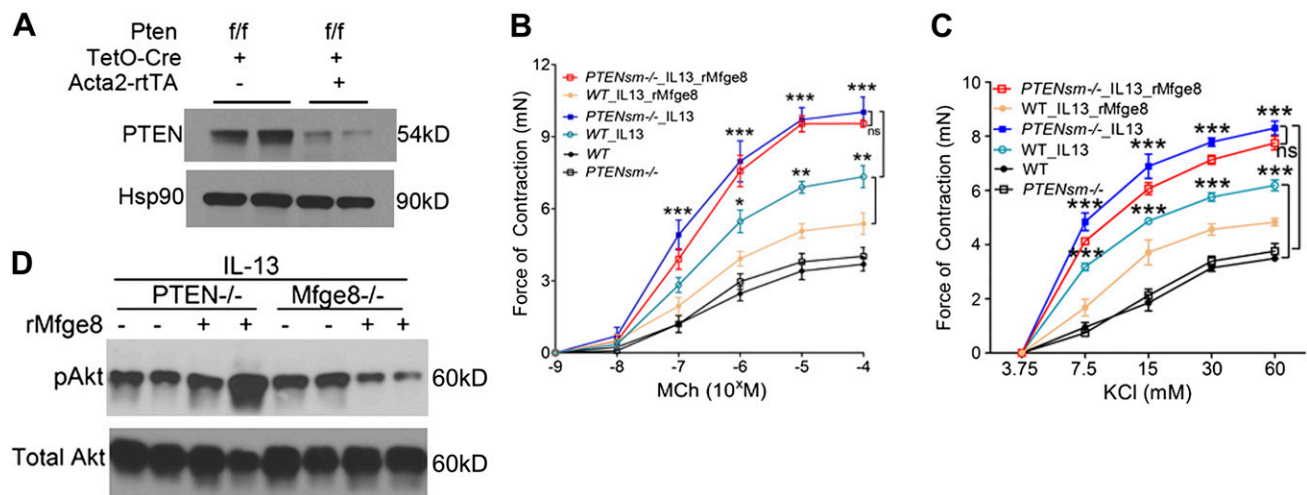
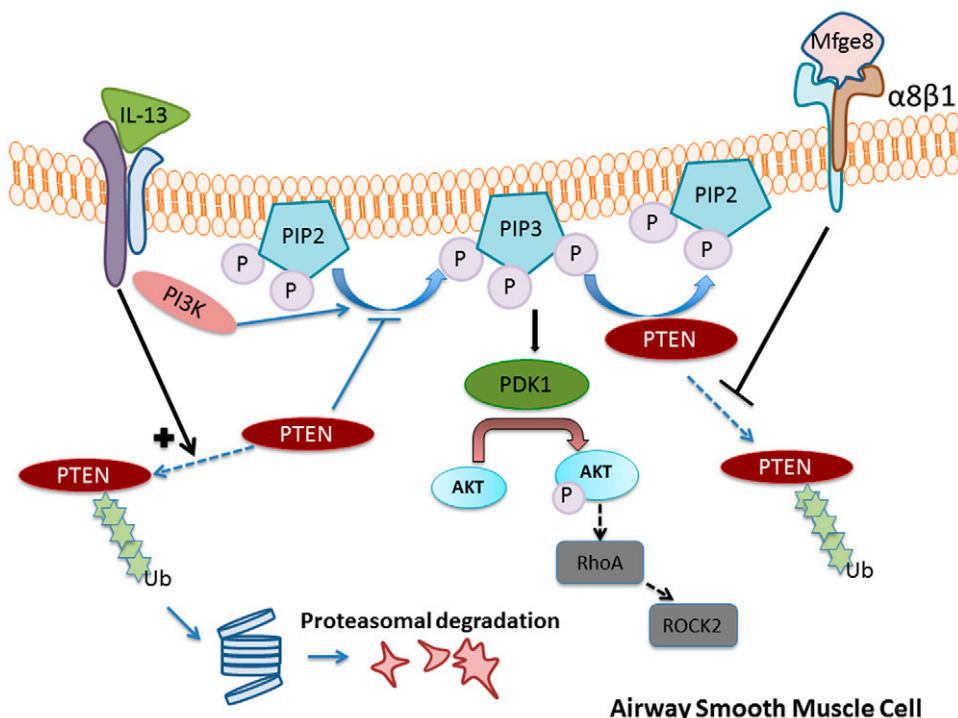


**Figure 7.** IL-13 mediates increases in ASM contraction by modulating PTEN. *A*) Smooth muscle-specific deletion of PTEN in tracheal smooth muscle strips. Western blot of tracheal strips from *PTEN<sup>sm-/-</sup>* mice after 2 wk of being fed a doxycycline diet. *B*, *C*) Force of tracheal smooth muscle contraction in WT (single transgenic controls) and *PTEN<sup>sm-/-</sup>* mice in the presence or absence of IL-13 and rMfge8 in response to MCh (*B*) and KCl (*C*) ( $n = 3-4$ ). *D*) Western blot of tracheal smooth muscle strip from *PTEN<sup>sm-/-</sup>* and *Mfge8<sup>-/-</sup>* mice treated with IL-13 (100 ng/ml for 1 h) followed by stimulation with MCh for 15 min. rMfge8 was added 20 min before the addition of IL-13 ( $n = 6$ ). Data are expressed as means  $\pm$  SEM. One-way ANOVA was used to make comparisons between multiple groups. When the ANOVA comparison was statistically significant ( $P < 0.05$ ), further pairwise analysis was conducted by using a Bonferroni  $t$  test. Male mice were used for all 4 panels. Data are representative of 1 of the 3 independent experiments (*A*, *D*). \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

of PI3K, attenuated inflammation in a murine model of allergic asthma (35). In another study, intratracheal administration of LY294002 significantly inhibited most of the pathologic characteristics in a mouse asthma model (36). Consistent with these findings, we found that treatment with wortmannin reduced IL-13-mediated increase in RhoA activation, increased PTEN activity, and abrogated the enhanced force of tracheal smooth muscle contraction. To extend this further, we found that Mfge8 prevents

cytokine-mediated airway contraction by inhibiting the PI3K/Akt pathway. Mfge8 regulates the PI3K pathway by inhibiting IL-13-induced PTEN ubiquitination and degradation. The mechanism by which IL-13 induces PTEN ubiquitination is a major focus of our future studies. One possibility is that IL-13 activates an E3 ligase(s), thereby promoting the addition of ubiquitin residues to PTEN. Alternatively, IL-13 may inhibit the functions of enzymes responsible for deubiquitination. Similarly,  $\alpha\beta 1$  could

**Figure 8.** Mfge8 ligation of  $\alpha\beta 1$  on ASM prevents IL-13-mediated increases in RhoA activation by regulating the PI3K pathway. Mfge8 binding to  $\alpha\beta 1$  prevents cytokine-mediated airway contraction by inhibiting the PI3K/Akt pathway. IL-13 binding to its cell surface receptor activates PI3K pathway and phosphorylates Akt. It also promotes PTEN ubiquitination and degradation. Mfge8 regulates the PI3K pathway by inhibiting IL-13-induced PTEN ubiquitination and degradation. PIP2, phosphatidylinositol 4,5-bisphosphate; Ub, ubiquitin.



prevent IL-13-induced PTEN degradation through either of these pathways.

IL-13 is an important cytokine implicated in the development of AHR and asthma. Other cytokines such as IL-17 and TNF- $\alpha$  are also implicated in the pathogenesis of asthma by inducing RhoA expression in ASM (37). We have previously shown that Mfge8 has a similar effect on airway contraction in response to IL-17 and TNF- $\alpha$  compared with IL-13. However, it is not known if Mfge8 regulates IL-17 and TNF- $\alpha$  mediated ASM contraction through a PTEN-PI3K-RhoA pathway or if there are other divergent pathways controlling RhoA activation in response to these cytokines.

PTEN has multiple and diverse roles in many biologic processes, including embryonic development, cell migration, cell survival, and apoptosis (38–40). It can act *via* the PI3K/Akt pathway and its protein tyrosine phosphatase activity (23, 24, 38, 41, 42), but the major function of PTEN appears to be down-regulation of PIP3 produced by PI3K. PTEN plays a pivotal role in the pathogenesis of the allergic airway disease in mice (43). A previous study found that somatic mutation or deletion of PTEN was observed in the epithelium of patients with chronic airway inflammatory disease (44). Airway PTEN expression is reduced after allergen challenge, and adenoviral overexpression of PTEN in the lung reduces bronchial inflammation and AHR (43). The research presented in this study suggests that smooth muscle PTEN expression modulates cytokine-induced airway constriction. Increased force of tracheal smooth muscle contraction in mice with the smooth muscle-specific deletion of PTEN, which Mfge8 could not rescue, provides compelling evidence that PTEN mediates the reduction in ASM contraction by Mfge8- $\alpha$ 8 $\beta$ 1 integrin ligation. Our results suggest that Mfge8 binding to the  $\alpha$ 8 $\beta$ 1 integrin on tracheal smooth muscle increases PTEN activity, thereby preventing RhoA activation and enhanced airway contraction in response to cytokine treatment.

PTEN is regulated by a number of post-translational modifications that modulate its phosphatase activity, stability, and intracellular localization. These modifications include phosphorylation (45), ubiquitination (46, 47), acetylation (48), oxidation (49), SUMOylation (50, 51), S-nitrosylation (52), and ADP-ribosylation (53). Protein-protein interactions also influence PTEN activity and stability. In the present study, we observed the loss of PTEN protein within 1 h of treatment with IL-13 in tracheal rings, indicating the modulation of PTEN at the post-translational level. Further investigation is needed to look at the mechanisms involved in IL-13-mediated ubiquitination and degradation of PTEN. Study of the role of the Mfge8- $\alpha$ 8 $\beta$ 1 interaction on these intermediaries will further elucidate the mechanism of cytokine-induced ASM hypercontractility. This information in turn will help design better therapeutic strategies that target the pathway specifically involved in the cytokine-mediated degradation of PTEN in the setting of allergic asthma. FJ

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## AUTHOR CONTRIBUTIONS

A. Khalifeh-Soltani designed and performed the experiments; D. Gupta performed the experiments and wrote the manuscript; A. Ha was responsible for help with the *in vivo* mouse experiments; M. J. Podolsky and R. Datta helped with writing the manuscript; and K. Atabai designed and oversaw all experiments and wrote the manuscript.

## REFERENCES

- Moorman, J. E., Rudd, R. A., Johnson, C. A., King, M., Minor, P., Bailey, C., Scalia, M. R., and Akinbami, L. J.; Centers for Disease Control and Prevention (CDC). (2007) National surveillance for asthma—United States, 1980–2004. *MMWR Surveill. Summ.* **56**, 1–54
- Kudo, M., Khalifeh Soltani, S. M., Sakuma, S. A., McKleroy, W., Lee, T. H., Woodruff, P. G., Lee, J. W., Huang, K., Chen, C., Arjomandi, M., Huang, X., and Atabai, K. (2013) Mfge8 suppresses airway hyperresponsiveness in asthma by regulating smooth muscle contraction. *Proc. Natl. Acad. Sci. USA* **110**, 660–665
- Chiba, Y., Takada, Y., Miyamoto, S., MitsuiSaito, M., Karaki, H., and Misawa, M. (1999) Augmented acetylcholine-induced, Rho-mediated Ca<sup>2+</sup> sensitization of bronchial smooth muscle contraction in antigen-induced airway hyperresponsive rats. *Br. J. Pharmacol.* **127**, 597–600
- Goto, K., Chiba, Y., Matsusue, K., Hattori, Y., Maitani, Y., Sakai, H., Kimura, S., and Misawa, M. (2010) The proximal STAT6 and NF-kappaB sites are responsible for IL-13- and TNF-alpha-induced RhoA transcriptions in human bronchial smooth muscle cells. *Pharmacol. Res.* **61**, 466–472
- Kudo, M., Melton, A. C., Chen, C., Engler, M. B., Huang, K. E., Ren, X., Wang, Y., Bernstein, X., Li, J. T., Atabai, K., Huang, X., and Sheppard, D. (2012) IL-17A produced by  $\alpha$  $\beta$  T cells drives airway hyper-responsiveness in mice and enhances mouse and human airway smooth muscle contraction. *Nat. Med.* **18**, 547–554
- Khalifeh-Soltani, A., Ha, A., Podolsky, M. J., McCarthy, D. A., McKleroy, W., Azary, S., Sakuma, S., Tharp, K. M., Wu, N., Yokosaki, Y., Hart, D., Stahl, A., and Atabai, K. (2016)  $\alpha$ 8 $\beta$ 1 Integrin regulates nutrient absorption through an Mfge8-PTEN dependent mechanism. *Elife* **5**
- Huang, X., Griffiths, M., Wu, J., Farese, R. V., Jr., and Sheppard, D. (2000) Normal development, wound healing, and adenovirus susceptibility in beta5-deficient mice. *Mol. Cell. Biol.* **20**, 755–759
- Sugimoto, K., Kudo, M., Sundaram, A., Ren, X., Huang, K., Bernstein, X., Wang, Y., Raymond, W. W., Erle, D. J., Abrink, M., Caughey, G. H., Huang, X., and Sheppard, D. (2012) The  $\alpha$ 6 $\beta$ 6 integrin modulates airway hyperresponsiveness in mice by regulating intraepithelial mast cells. *J. Clin. Invest.* **122**, 748–758
- Chan, C. S., Chen, H., Bradley, A., Dragatsis, I., Rosenmund, C., and Davis, R. L. (2010)  $\alpha$ 8-Integrins are required for hippocampal long-term potentiation but not for hippocampal-dependent learning. *Genes Brain Behav.* **9**, 402–410
- Backman, S. A., Stambolic, V., Suzuki, A., Haight, J., Elia, A., Pretorius, J., Tsao, M. S., Shannon, P., Bolon, B., Ivy, G. O., and Mak, T. W. (2001) Deletion of Pten in mouse brain causes seizures, ataxia and defects in soma size resembling Lhermitte-Duclos disease. *Nat. Genet.* **29**, 396–403
- Atabai, K., Fernandez, R., Huang, X., Ueki, I., Kline, A., Li, Y., Sadatmansoori, S., Smith-Steinhart, C., Zhu, W., Pytela, R., Werb, Z., and Sheppard, D. (2005) Mfge8 is critical for mammary gland remodeling during involution. *Mol. Biol. Cell* **16**, 5528–5537
- Hanayama, R., Tanaka, M., Miwa, K., Shinohara, A., Iwamatsu, A., and Nagata, S. (2002) Identification of a factor that links apoptotic cells to phagocytes. *Nature* **417**, 182–187
- Zargham, R., Wamhoff, B. R., and Thibault, G. (2007) RNA interference targeting alpha8 integrin attenuates smooth muscle cell growth. *FEBS Lett.* **581**, 939–943
- Zargham, R., and Thibault, G. (2006) Alpha 8 integrin expression is required for maintenance of the smooth muscle cell differentiated phenotype. *Cardiovasc. Res.* **71**, 170–178
- Schnapp, L. M., Breuss, J. M., Ramos, D. M., Sheppard, D., and Pytela, R. (1995) Sequence and tissue distribution of the human integrin alpha 8 subunit: a beta 1-associated alpha subunit expressed in smooth muscle cells. *J. Cell Sci.* **108**, 537–544
- Khalifeh-Soltani, A., McKleroy, W., Sakuma, S., Cheung, Y. Y., Tharp, K., Qiu, Y., Turner, S. M., Chawla, A., Stahl, A., and Atabai, K. (2014)

- Mfge8 promotes obesity by mediating the uptake of dietary fats and serum fatty acids. *Nat. Med.* **20**, 175–183
17. Wang, Y., Yoshioka, K., Azam, M. A., Takuwa, N., Sakurada, S., Kayaba, Y., Sugimoto, N., Inoki, I., Kimura, T., Kuwaki, T., and Takuwa, Y. (2006) Class II phosphoinositide 3-kinase  $\alpha$ -isoform regulates Rho, myosin phosphatase and contraction in vascular smooth muscle. *Biochem. J.* **394**, 581–592
  18. Kawabata, A., Kuroda, R., Kuroki, N., Nishikawa, H., Kawai, K., and Araki, H. (2000) Characterization of the protease-activated receptor-1-mediated contraction and relaxation in the rat duodenal smooth muscle. *Life Sci.* **67**, 2521–2530
  19. Sarbassov, D. D., Guertin, D. A., Ali, S. M., and Sabatini, D. M. (2005) Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. *Science* **307**, 1098–1101
  20. Atabai, K., Jame, S., Azhar, N., Kuo, A., Lam, M., McKleroy, W., Dehart, G., Rahman, S., Xia, D. D., Melton, A. C., Wolters, P., Emson, C. L., Turner, S. M., Werb, Z., and Sheppard, D. (2009) Mfge8 diminishes the severity of tissue fibrosis in mice by binding and targeting collagen for uptake by macrophages. *J. Clin. Invest.* **119**, 3713–3722
  21. Schaafsma, D., Zuidhof, A. B., Nelemans, S. A., Zaagsma, J., and Meurs, H. (2006) Inhibition of Rho-kinase normalizes nonspecific hyperresponsiveness in passively sensitized airway smooth muscle preparations. *Eur. J. Pharmacol.* **531**, 145–150
  22. Leeners, S. J., Vanhaesebroeck, B., and Waterfield, M. D. (1999) Signalling through phosphoinositide 3-kinases: the lipids take centre stage. *Curr. Opin. Cell Biol.* **11**, 219–225
  23. Cantley, L. C., and Neel, B. G. (1999) New insights into tumor suppression: PTEN suppresses tumor formation by restraining the phosphoinositide 3-kinase/AKT pathway. *Proc. Natl. Acad. Sci. USA* **96**, 4240–4245
  24. Machama, T., and Dixon, J. E. (1998) The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate. *J. Biol. Chem.* **273**, 13375–13378
  25. Vazquez, F., Ramaswamy, S., Nakamura, N., and Sellers, W. R. (2000) Phosphorylation of the PTEN tail regulates protein stability and function. *Mol. Cell. Biol.* **20**, 5010–5018
  26. Leslie, N. R., Kriplani, N., Hermida, M. A., Alvarez-Garcia, V., and Wise, H. M. (2016) The PTEN protein: cellular localization and post-translational regulation. *Biochem. Soc. Trans.* **44**, 273–278
  27. Grainge, C. L., Lau, L. C., Ward, J. A., Dulay, V., Lahiff, G., Wilson, S., Holgate, S., Davies, D. E., and Howarth, P. H. (2011) Effect of bronchoconstriction on airway remodeling in asthma. *N. Engl. J. Med.* **364**, 2006–2015
  28. Janssen, L. J., and Killian, K. (2006) Airway smooth muscle as a target of asthma therapy: history and new directions. *Respir. Res.* **7**, 123
  29. Palmer, E. L., Rüegg, C., Ferrando, R., Pytela, R., and Sheppard, D. (1993) Sequence and tissue distribution of the integrin  $\alpha$ 9 subunit, a novel partner of beta 1 that is widely distributed in epithelia and muscle. *J. Cell Biol.* **123**, 1289–1297
  30. Chen, C., Kudo, M., Rutaganira, F., Takano, H., Lee, C., Atakilit, A., Robinett, K. S., Uede, T., Wolters, P. J., Shokat, K. M., Huang, X., and Sheppard, D. (2012) Integrin  $\alpha$ 9 $\beta$ 1 in airway smooth muscle suppresses exaggerated airway narrowing. *J. Clin. Invest.* **122**, 2916–2927
  31. Benoit, Y. D., Lussier, C., Ducharme, P. A., Sivret, S., Schnapp, L. M., Basora, N., and Beaulieu, J. F. (2009) Integrin  $\alpha$ 8 $\beta$ 1 regulates adhesion, migration and proliferation of human intestinal crypt cells via a predominant RhoA/ROCK-dependent mechanism. *Biol. Cell* **101**, 695–708
  32. Zargham, R., Touyz, R. M., and Thibault, G. (2007) Alpha 8 integrin overexpression in de-differentiated vascular smooth muscle cells attenuates migratory activity and restores the characteristics of the differentiated phenotype. *Atherosclerosis* **195**, 303–312
  33. Ceperonis, P. J., Botelho, F., Richards, C. D., and McKay, D. M. (2000) Interleukins 4 and 13 increase intestinal epithelial permeability by a phosphatidylinositol 3-kinase pathway. Lack of evidence for STAT 6 involvement. *J. Biol. Chem.* **275**, 29132–29137
  34. Wright, K., Ward, S. G., Kolios, G., and Westwick, J. (1997) Activation of phosphatidylinositol 3-kinase by interleukin-13. An inhibitory signal for inducible nitric-oxide synthase expression in epithelial cell line HT-29. *J. Biol. Chem.* **272**, 12626–12633
  35. Ezeamuzie, C. I., Sukumaran, J., and Philips, E. (2001) Effect of wortmannin on human eosinophil responses in vitro and on bronchial inflammation and airway hyperresponsiveness in guinea pigs in vivo. *Am. J. Respir. Crit. Care Med.* **164**, 1633–1639
  36. Duan, W., Aguinaldo Datiles, A. M., Leung, B. P., Vlahos, C. J., and Wong, W. S. (2005) An anti-inflammatory role for a phosphoinositide 3-kinase inhibitor LY294002 in a mouse asthma model. *Int. Immunopharmacol.* **5**, 495–502
  37. Chiba, Y., Nakazawa, S., Todoroki, M., Shinozaki, K., Sakai, H., and Misawa, M. (2009) Interleukin-13 augments bronchial smooth muscle contractility with an up-regulation of RhoA protein. *Am. J. Respir. Cell Mol. Biol.* **40**, 159–167
  38. Yamada, K. M., and Araki, M. (2001) Tumor suppressor PTEN: modulator of cell signaling, growth, migration and apoptosis. *J. Cell Sci.* **114**, 2375–2382
  39. Lu, Y., Lin, Y. Z., LaPushin, R., Cuevas, B., Fang, X., Yu, S. X., Davies, M. A., Khan, H., Furui, T., Mao, M., Zinner, R., Hung, M. C., Steck, P., Siminovich, K., and Mills, G. B. (1999) The PTEN/MMAC1/TEP tumor suppressor gene decreases cell growth and induces apoptosis and anoikis in breast cancer cells. *Oncogene* **18**, 7034–7045
  40. Di Cristofano, A., Pesce, B., Cordon-Cardo, C., and Pandolfi, P. P. (1998) Pten is essential for embryonic development and tumour suppression. *Nat. Genet.* **19**, 348–355
  41. Sun, H., Lesche, R., Li, D. M., Liliental, J., Zhang, H., Gao, J., Gavrilo, N., Mueller, B., Liu, X., and Wu, H. (1999) PTEN modulates cell cycle progression and cell survival by regulating phosphatidylinositol 3,4,5-trisphosphate and Akt/protein kinase B signaling pathway. *Proc. Natl. Acad. Sci. USA* **96**, 6199–6204
  42. Myers, M. P., Pass, I., Batty, I. H., Van der Kaay, J., Stolarov, J. P., Hemmings, B. A., Wigler, M. H., Downes, C. P., and Tonks, N. K. (1998) The lipid phosphatase activity of PTEN is critical for its tumor suppressor function. *Proc. Natl. Acad. Sci. USA* **95**, 13513–13518
  43. Kwak, Y. G., Song, C. H., Yi, H. K., Hwang, P. H., Kim, J. S., Lee, K. S., and Lee, Y. C. (2003) Involvement of PTEN in airway hyperresponsiveness and inflammation in bronchial asthma. *J. Clin. Invest.* **111**, 1083–1092
  44. Anderson, G. P., and Bozinovski, S. (2003) Acquired somatic mutations in the molecular pathogenesis of COPD. *Trends Pharmacol. Sci.* **24**, 71–76
  45. Torres, J., and Pulido, R. (2001) The tumor suppressor PTEN is phosphorylated by the protein kinase CK2 at its C terminus. Implications for PTEN stability to proteasome-mediated degradation. *J. Biol. Chem.* **276**, 993–998
  46. Wang, X., Trotman, L. C., Koppie, T., Alimonti, A., Chen, Z., Gao, Z., Wang, J., Erdjument-Bromage, H., Tempst, P., Cordon-Cardo, C., Pandolfi, P. P., and Jiang, X. (2007) NEDD4-1 is a proto-oncogenic ubiquitin ligase for PTEN. *Cell* **128**, 129–139
  47. Maddika, S., Kavela, S., Rani, N., Palicharla, V. R., Pokorny, J. L., Sarkaria, J. N., and Chen, J. (2011) WWP2 is an E3 ubiquitin ligase for PTEN. *Nat. Cell Biol.* **13**, 728–733
  48. Okumura, K., Mendoza, M., Bachoo, R. M., DePinto, R. A., Cavenee, W. K., and Furnari, F. B. (2006) PCAF modulates PTEN activity. *J. Biol. Chem.* **281**, 26562–26568
  49. Kwon, J., Lee, S. R., Yang, K. S., Ahn, Y., Kim, Y. J., Stadtman, E. R., and Rhee, S. G. (2004) Reversible oxidation and inactivation of the tumor suppressor PTEN in cells stimulated with peptide growth factors. *Proc. Natl. Acad. Sci. USA* **101**, 16419–16424
  50. Huang, S. M., Mishina, Y. M., Liu, S., Cheung, A., Stegmeier, F., Michaud, G. A., Charlat, O., Wiethe, E., Zhang, Y., Wiessner, S., Hild, M., Shi, X., Wilson, C. J., Mikanin, C., Myer, V., Fazal, A., Tomlinson, R., Serluca, F., Shao, W., Cheng, H., Shultz, M., Rau, C., Schirle, M., Schlegl, J., Ghidelli, S., Fawell, S., Lu, C., Curtis, D., Kirschner, M. W., Lengauer, C., Finan, P. M., Tallarico, J. A., Bouwmeester, T., Porter, J. A., Bauer, A., and Cong, F. (2009) Tankyrase inhibition stabilizes axin and antagonizes Wnt signalling. *Nature* **461**, 614–620
  51. Bassi, C., Ho, J., Srikumar, T., Dowling, R. J., Gorini, C., Miller, S. J., Mak, T. W., Neel, B. G., Raught, B., and Stambolic, V. (2013) Nuclear PTEN controls DNA repair and sensitivity to genotoxic stress. *Science* **341**, 395–399
  52. Li, W., Huang, R., Chen, Z., Yan, L. J., Simpkins, J. W., and Yang, S. H. (2014) PTEN degradation after ischemic stroke: a double-edged sword. *Neuroscience* **274**, 153–161
  53. Li, N., Zhang, Y., Han, X., Liang, K., Wang, J., Feng, L., Wang, W., Songyang, Z., Lin, C., Yang, L., Yu, Y., and Chen, J. (2015) Poly-ADP ribosylation of PTEN by tankyrase promotes PTEN degradation and tumor growth. *Genes Dev.* **29**, 157–170

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