Received: 10 June 2020 | Revised: 12 September 2020 | Accepted: 16 September 2020

DOI: 10.1111/cas.14662

ORIGINAL ARTICLE

Cancer Science WILEY

Autophagy regulates levels of tumor suppressor enzyme protein phosphatase 6

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Funding information
Extramural Collaborative Research Grant of Cancer Research Institute of Kanazawa University; Japan Society for the Promotion of Science, Grant/Award Number: 17H03915, 18K16364 and 20H03151; Research and Education Funds from Ehime Prefecture and Imabari City

Abstract

Protein phosphatase 6 (PP6) is an essential serine/threonine protein phosphatase that acts as an important tumor suppressor. However, increased protein levels of PP6 have been observed in some cancer types, and they correlate with poor prognosis in glioblastoma. This raises a question about how PP6 protein levels are regulated in normal and transformed cells. In this study, we show that PP6 protein levels increase in response to pharmacologic and genetic inhibition of autophagy. PP6 associates with autophagic adaptor protein p62/SQSTM1 and is degraded in a p62-dependent manner. Accordingly, protein levels of PP6 and p62 fluctuate in concert under different physiological and pathophysiological conditions. Our data reveal that PP6 is regulated by p62-dependent autophagy and suggest that accumulation of PP6 protein in tumor tissues is caused at least partially by deficiency in autophagy.

KEYWORDS

autophagy, cell biology, p62/SQSTM1, protein phosphatase 2A, protein phosphatase 6

Abbreviations: 4-HT, 4-hydroxytamoxifen; Atg, autophagy-related gene; EBSS, Earle's balanced salt solution; KO, knockout; LSD, least significant difference; MEF, mouse embryonic fibroblast; NT, nontarget; PP, protein phosphatase; SAPS, Sit4-associated protein subunits; sh, short hairpin; TMA, tissue microarray; VCP, valosin-containing protein; WT, wild-type; YPD, yeast extract-peptone-dextrose; YPGal, yeast extract-peptone-galactose.

1 | INTRODUCTION

The type 2A protein serine/threonine phosphatase family including PP2A, PP4, and PP6 are essential enzymes conserved from yeast to humans.[1] PP2A is a well-established tumor suppressor, and its activity is suppressed in cancer cells by various mechanisms, such as mutations, dysregulated holoenzyme assembly, and increased expression of endogenous inhibitory proteins.[2] The first evidence for the tumor-suppressive role of PP2A was obtained from the mouse two-stage skin carcinogenesis model. In this model, following one-time application of 7,12-dimethylbenz (a) anthracene (DMBA) as an initiator, 12-O-tetradecanoylphorbol 13-acetate (TPA) is repeatedly treated to induce papillomas. Suganuma et al found that TPA can be replaced by okadaic acid, a natural toxin isolated from *Halichondria*.[3] Okadaic acid has been widely utilized as a serine/threonine protein phosphatase inhibitor, more potent against PP2A compared with PP1. However, it is a type 2A protein phosphatase inhibitor, and it is not generally recognized that PP6 is even more sensitive to inhibition than PP2A.[4] PP6 (Sit4 in *Saccharomyces cerevisiae*) regulates a wide range of biological processes including cell cycle progression, DNA repair, inflammatory response, and cell/tissue volume.[1] Recently, okadaic acid in the mouse two-stage skin carcinogenesis model was replaced by targeted deletion of PP6 in the epidermis.[5] Consistent with this, mutational landscape analysis revealed that about 10% of melanoma patients harbored nonsynonymous PP6 gene mutations that are putative loss-of-function mutations.[6,7] These data indicate a tumor-suppressive role of PP6. However, in other studies PP6 expression is increased in some cancer types.[8,9] Particularly in glioblastoma, there is a positive correlation between PP6 protein level and poor prognosis.[8] These data raise a question how PP6 protein is regulated in cells and how its levels can be high in some tumors when it has tumor-suppressing activity.

The levels of PP2A protein in cells are relatively stable because of strict auto-regulatory control.[10] On the other hand, we observed that PP6 protein levels fluctuate compared with those of PP2A.[11] Relatively small changes in PP6 mRNA levels occur under circumstances where there are larger changes in protein levels, making it unlikely that transcription is the dominant regulatory mechanism for the control of PP6 protein levels. We reported previously that PP2A protein levels were regulated by the ubiquitin/proteasome system.[12] However, the regulatory mechanism for PP6 protein has not been revealed.

Intracellular proteins are degraded predominantly by the ubiquitin/proteasome system and autophagy. In autophagy, proteins are delivered by autophagosomes to the lysosome, where they are degraded and recycled.[13] Autophagy has been thought to be a bulk degradation system, but accumulating evidence revealed that it is a more selective process than originally anticipated.[14] Currently, autophagy is considered to be a cell survival pathway that plays roles in development, cell death, and aging. Deficient autophagy leads to various diseases including neurodegeneration, autoimmunity, and cancer.[15] Autophagy acts as both tumor suppressor and tumor promoter depending on the stage of cancer. In the early stage of tumorigenesis, autophagy acts as a suppressor by removing damaged proteins and organelles that were accumulated by various stresses.[16] For example, Beclin 1, an essential autophagy gene, is a haploinsufficient tumor suppressor.[17] Autophagy deficiency leads to an accumulation of p62/SQSTM1, an adaptor protein for selective autophagy, that contributes directly to tumorigenesis by promoting oxidative stress.[18,19] In the late stage of cancer, autophagy promotes survival and growth of the established tumors by protecting cancer cells from various stresses such as starvation and hypoxia.[16]

In the current study, we show that PP6 is degraded by p62-dependent selective autophagy, and the protein level of PP6 is positively correlated with p62 in physiological and pathophysiological conditions. Our data revealed the molecular mechanism for the instability of PP6 protein levels and suggested that the accumulation of PP6 protein in tumor tissues may be caused by autophagy deficiency.

2 | MATERIALS AND METHODS

2.1 | Animals

Male C57BL/6J mice purchased from Charles River Laboratories Japan were maintained in compliance with the guidelines of the Animal Care and Use Committee of Yamaguchi University. Mice were anesthetized and euthanized by exsanguination. All experiments and animal care procedures in this study were performed according to the Guide to Animal Use and Care of the Yamaguchi University and were approved by the ethics committee.

2.2 | Cell culture

Lenti-X 293T (Takara Bio), HeLa (ATCC), NIH3T3 (ATCC), and mouse embryonic fibroblast (MEF) were grown in DMEM supplemented with 10% FBS and 1 × antibiotic/antimycotic solution (Life Technologies).

2.3 | Plasmids, transfection, and virus production

pLV 3 × FLAG human PP6c was generated using In-Fusion HD cloning kit (Takara Bio). Short hairpin RNA (shRNA)-expressing plasmids (pLV mCherry) were generated as described previously.[12] The 19-mer shRNA sequences were as follows: nontarget (NT), 5-CAACAAGATGAGAGCACCA-3; PP6 #1, 5-GCCTGGAG ATACTGTACCA-3; PP6 #2, 5-GACACAAACTACATATTTA-3; Beclin 1, 5-TGAGGATGACAGTGAACAG-3. The 21-mer shRNA sequences were as follows: NT, 5-TGAGGATGACAGTGAACAG-3; p62, 5-CCGAATCTACATTAAAGAGAA-3.

To produce lentiviruses 3 μg of pLV plasmids, 2.3 μg of a packaging plasmid (psPAX2), and 1.3 μg of a coat protein plasmid expressing

vesicular stomatitis virus G protein (pMD2.G) were transfected into Lenti-X 293T cells cultured in 60-mm dishes.

2.4 | Yeast media, plasmids, and transformation

Yeast cells were cultured in yeast extract-peptone-dextrose (YPD) medium (1% yeast extract, 2% peptone, and 2% glucose) at 30°C. For galactose-induced conditions, yeast cells were cultured in Yeast extract-peptone-galactose (YPGal) medium (1% yeast extract, 2% polypeptone, 2% galactose) at 30°C.

The plasmid for the expression of yEGFP-Sit4 fusion protein was constructed by gap-repair cloning method using the PCR-amplified DNAs. yEGFP-SIT4 sequence in YEp28025 was confirmed by DNA sequencing. Yeast cells were transformed using a lithium acetate method.[20]

2.5 | Immunoprecipitation and immunoblotting

For immunoprecipitation, the cells stably expressing 3 × FLAG-tagged PP6 were lysed in Triton X lysis buffer (50 mmol/L Tris-HCl [pH 8.0], 150 mmol/L NaCl, 5 mmol/L EDTA, 5 mmol/L EGTA, 1% Triton X-100, 1 mmol/L Na_3VO_4, 20 mmol/L sodium pyrophosphate, and Roche complete protease inhibitor mixture). The supernatants were incubated with FLAG-M2 affinity gel (Sigma).

For immunoblotting, cells and tissue samples were lysed in a buffer containing 50 mmol/L Tris-HCl (pH 8.0), 5 mmol/L EDTA, 5 mmol/L EGTA, 1% Triton X-100, 1 mmol/L Na_3VO_4, 20 mmol/L sodium pyrophosphate, and Roche complete protease inhibitor mixture. For tissue samples, Multi Beads Shocker (Yasui Kikai) was used to homogenate tissues according to the instructions of the manufacturer. A total of 20 μg of proteins was applied to SDS-PAGE. Yeast samples were lysed by boiling in SDS lysis buffer for 5 minutes (60 mmol/L Tris-HCl pH 6.8, 5% glycerol, 2% SDS, 4% β-mercaptoethanol, 0.0025% bromophenol blue) after 100 μmol/L NaOH treatment. Proteins were separated by SDS-PAGE and transferred onto a nitrocellulose membrane (Wako) or PVDF membrane (Bio-Rad). For immunoblotting, membranes were blocked with 3% skim milk and treated with primary antibodies, and immunoreactive bands were visualized using an ECL Pro (PerkinElmer) and LAS-3000 (Fujifilm) or Amersham Imager 680 (GE). Band densities were quantified using ImageJ densitometry analysis software (National Institutes of Health).

Antibodies used were as follows: anti-Actin (Santa Cruz, sc-1616-R), anti-GFP (Santa Cruz, sc-9996), anti-FLAG (Sigma, F7425), anti–valosin-containing protein (VCP, Gene Tex, GTX113030), anti-PP2A (Millipore, 07-324), anti-Beclin 1 (MBL, PD017), anti-PP4 (Bethyl, A300-835A), anti-p62 (MBL, PM-045), anti-Atg7 (Cell Signaling, 8558), anti-PP6R2 (Betyl, 970), and anti-PP6R3 (Bethyl, 972). Anti-PP6 and anti-PP6R1 antibody was kindly provided by Dr Brautigan (University of Virginia).

2.6 | Immunofluorescent staining

NIH3T3 cells and CreERT-PP6$^{flox/flox}$ MEFs were grown on glass coverslips and subsequently fixed with 4% paraformaldehyde for 20 minutes at room temperature and then methanol/acetone for 20 minutes at −20°C. Cells were permeabilized in 0.1% Triton X-100/PBS and blocked with 5% normal goat serum in PBS (137 mmol/L NaCl, 2.7 mmol/L KCl, 1.76 mmol/L KH_2PO_4, and 10 mmol/L Na_2HPO_4). After incubation with primary antibodies overnight at 4°C, Alexa Fluor 488, 568-conjugated secondary antibodies (Invitrogen) were added, and cells were incubated for 1 hour at room temperature. Nuclei were counterstained with Hoechst 33342 solution (Dojindo). Fluorescence images were captured by a confocal laser-scanning microscope (LSM880; Zeiss) and colocalization was analyzed by ZEN software (Zeiss).

2.7 | Immunohistochemical staining

Multiple-organ tumor and normal-tissue microarray (serial section) were purchased from US Biomax. After deparaffinization, the sections were treated with 3% peroxidase for 15 minutes and blocked with 1% bovine serum albumin in PBS or 5% skim milk at room temperature for 1 hour. The sections were then incubated with primary antibodies at 4°C overnight. After incubation with secondary antibodies (1:500) at room temperature for 1 hour, the sections were observed using an all-in-one fluorescence microscope (BZ-9000; Keyence). Signal intensity was scored as 0, +1, +2, +3, or +4.

2.8 | Realtime RT-PCR

Total RNA was extracted from cells by using TRIzol Reagents (Life Technologies). A total of 0.5 μg of RNA was reverse-transcribed in a final incubation volume of 10 μL using QuantiTect Reverse Transcription Kit (Qiagen). The resulting cDNA was subjected to qPCR using QuantiTect SYBRGreenI Kit (Qiagen) and StepOne Plus (Applied Biosystems). The sequences of primers were as follows: PP6 forward CGCCAGTAACAGTGTGTGGTGA, reverse GGCCACTTAGCCTTTAGTGCAAGA, β-actin forward GATTACTGCTCTGGCTCCTAGC, reverse GACTCATCGTACT CCTGCTTGC. PP6 expression was normalized to β-actin. The relative quantitative value for PP6 compared with β-actin was expressed as comparative Ct (2–(ΔCt-Cc)) method.

2.9 | Statistical analysis

The results are expressed as mean ± SE. Groups were compared using one-way analysis of variance, after which Fisher's Least Significant Difference test or Student–Newman–Keuls test was used. For all analyses, $P < 0.05$ was considered statistically significant.

3 | RESULTS

3.1 | Differential control of PP2A and PP6 degradation

We examined the effects of the proteasome inhibitor MG132 on the levels of type 2A protein phosphatases (Figure 1A,B), and, as expected, the PP2A protein accumulated in HeLa cells treated with MG132. In contrast, the PP6 protein level was decreased by MG132. We suspected this might have been due to autophagy because inhibition of the ubiquitin/proteasome system promotes compensatory autophagic activity.[21,22] Indeed, we observed that MG132 decreased p62/SQSTM1 protein levels, a marker for autophagic flux (Figure S1), and we speculated that autophagy was involved in degrading the PP6 protein. Treatment of HeLa cells with the endosomal acidification inhibitor bafilomycin A1 increased the p62 level, indicating suppression of autophagy (Figure S2A). PP6 protein accumulated in these bafilomycin A1–treated cells (Figure 2A,B). On the other hand, PP2A and PP4 protein levels were unaffected by bafilomycin A1 treatment. Accumulation of the PP6 protein by bafilomycin A1 was also observed in 293T cells, NIH3T3 cells, and MEFs (Figure S2B).

We utilized CreERT-PP6$^{flox/flox}$ MEFs to further analyze the role of autophagy vs the ubiquitin/proteasome system in PP6 degradation.[23] Treatment of CreERT-PP6$^{flox/flox}$ MEFs with 4-hydroxytamoxifen (4-HT) induced PP6 gene deletion, stopped mRNA synthesis, and decreased PP6 protein solely by protein degradation. We observed a reduction of PP6 protein and mRNA levels in response to the addition of 4-HT (Figure 2C-E). Although bafilomycin A1 did not block the suppression of PP6 mRNA by 4-HT, it blocked PP6 protein degradation. MG132 did not inhibit, but rather enhanced, 4-HT–induced PP6 degradation (Figure S2C). Conversely, PP6 reduction by MG132 treatment was blocked by bafilomycin A1 (Figure 2F,G). These data indicate that clearance of PP6 protein in the cell is dependent on autophagy.

PP6 forms a heterodimer with one of the three regulatory subunits (Sit4-associated protein subunits (SAPS): PP6R1, PP6R2, and PP6R3), but the protein levels of SAPS were not affected by bafilomycin A1 treatment (Figure 2H,I). We also observed that PP6 association with SAPS was not affected by bafilomycin A1 treatment (Figure S2D,E). These data suggest that a monomeric PP6, but not PP6 complex with SAPS, is a substrate of autophagy.

3.2 | Autophagy deficiency results in PP6 protein accumulation

We utilized knockdown (KD) and knockout (KO) systems targeting factors essential for autophagy: Beclin 1 and Atg7. We observed accumulation of the PP6 protein in autophagy-deficient 293T cells stably expressing shRNA-targeting Beclin 1 (shBeclin 1), compared with non-target shRNA (shNT)-expressing cells (Figure 3A,B). In contrast, PP2A and PP4 protein levels were not affected by autophagy deficiency. Accumulation of the PP6 protein in response to autophagy deficiency was also observed in Atg7 KO MEFs (Figure 3C,D). Unlike the acute inhibition of autophagy by bafilomycin A1, Atg7 KO MEFs expressed 1.5 times more SAPS proteins than WT MEFs (Figure S3A,B). Protein levels of SAPS are reported to be well correlated with PP6 protein[11,24], and we observed that PP6 KO led to a decrease in all SAPS proteins (Figure S3C,D). These data suggested that a long-term change of PP6 protein affects the SAPS protein levels.

We also investigated whether autophagic regulation of the PP6 protein was evolutionally conserved by examining whether Sit4, a PP6 homolog in *S. cerevisiae*, was degraded by autophagy. A galactose-dependent green fluorescent protein (GFP)-Sit4 expression plasmid was transformed into wild-type (WT) and ΔAtg8 yeast strains. The deletion of the *ATG8* gene was confirmed by PCR (Figure S3E). These yeast cells were cultured in galactose-based medium (YPGal) overnight to induce Sit4 expression. The culture medium was changed to glucose-based medium (YPD) to stop de novo synthesis of Sit4, and Sit4 degradation was measured. We found that the GFP-Sit4 protein was degraded slightly slower in ΔAtg8 yeast strains than WT (Figure S3F). This observation suggests that the Sit4 protein is partially degraded in an Atg8-dependent manner. However, Atg8 deficiency did not completely block Sit4 degradation, suggesting alternative mechanism(s) for Sit4 protein degradation in yeast.

3.3 | PP6 protein level is physiologically regulated by autophagy

Starvation is the most extensively studied physiological condition that induces autophagy. HeLa cells were treated with Earle's balanced salt solution (EBSS) to induce starvation. As expected, we observed that the PP6 protein level was decreased by EBSS treatment (Figure 4A,B). On the other hand, PP2A and PP4 protein levels were unaffected by

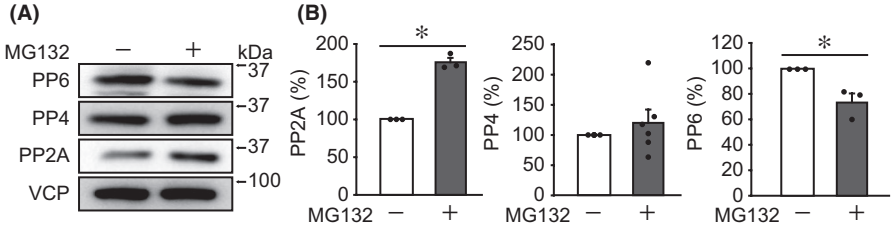

FIGURE 1 Treatment with a proteasome inhibitor decreases the protein phosphatase 6 (PP6) protein level. HeLa cells were treated with or without MG132 (10 μmol/L) for 24 h. Protein levels of type 2A protein phosphatases were analyzed by immunoblotting. Representative images (A) and quantitative data (B) from three to six independent experiments are shown. *$P < 0.05$ vs without MG132

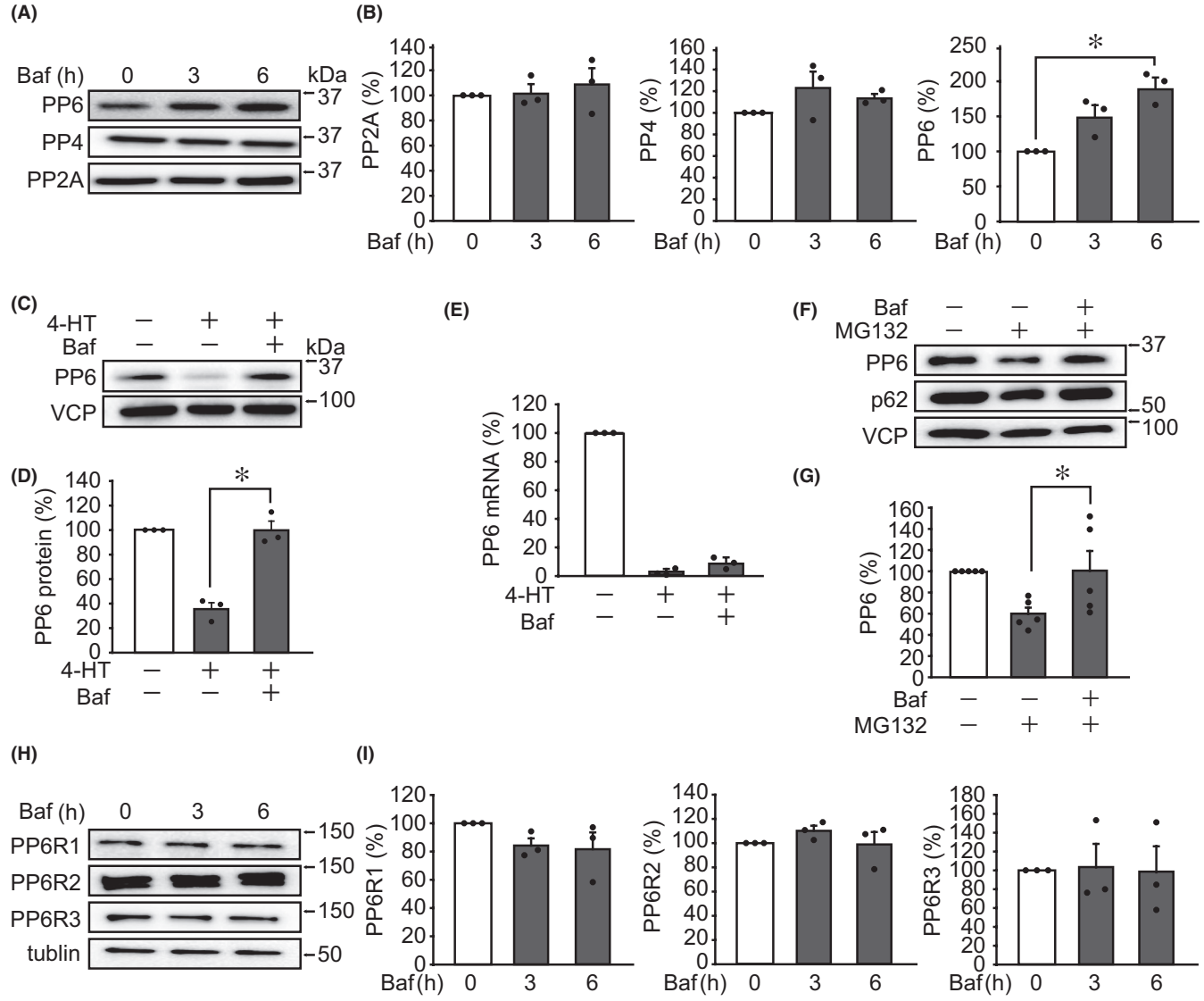

FIGURE 2 Treatment with a lysosomal inhibitor increases the protein phosphatase 6 (PP6) protein level. A, B, HeLa cells were treated with 100 nmol/L of bafilomycin A1 (Baf) for indicated time periods. Protein levels of type 2A protein phosphatases were analyzed by immunoblotting. Representative images (A) and quantitative data (B) from three independent experiments are shown. *$P < 0.05$ vs without Baf. C-E, CreERT-PP6 $^{flox/flox}$ mouse embryonic fibroblasts (MEFs) were treated with or without 500 nmol/L of 4-hydroxytamoxifen (4-HT) and 100 nmol/L of Baf for 24 h. Protein level (C, D) and mRNA expression level (E) of PP6 were analyzed by immunoblotting and qRT-PCR, respectively. Representative images (C) and quantitative data (D, E) from three independent experiments are shown. *$P < 0.05$. vs with 4-HT. F, G, HeLa cells were pretreated with 100 nmol/L of Baf for 1 h and then with MG132 (10 μmol/L) for 24 h. Protein levels of PP6 and p62 were analyzed by immunoblotting. Representative images (F) and quantitative data (G) from five independent experiments are shown. *$P < 0.05$ vs with MG132. H, I, HeLa cells were treated with 100 nmol/L of Baf for indicated time periods. Protein levels of SAPS were analyzed by immunoblotting. Representative images (H) and quantitative data (I) from three independent experiments are shown. *$P < 0.05$ vs without Baf

EBSS treatment. PP6 protein degradation by autophagy induced by starvation conditions was also observed in 293T cells, NIH3T3 cells, and MEFs (Figure S4A). We expressed FLAG-PP6 in 293T cells and treated these cells with EBSS. A decrease in the PP6 protein level was also observed with anti-FLAG antibody staining (Figure S4B). This showed that both endogenous PP6 and ectopically expressed FLAG-PP6 were degraded by starvation-induced autophagy. These results used the detection of PP6 levels by two different antibodies. To confirm that the autophagy is involved in starvation-induced PP6 downregulation, HeLa cells were pretreated with bafilomycin A1. We observed that bafilomycin A1 blocked the degradation of p62 and PP6 by starvation (Figure 4C,D). Moreover, EBSS-induced decrease in PP6 protein level was abolished in Atg7 KO cells (Figure 4E,F).

We also induced autophagy in vivo, by performing a mouse starvation/refeeding experiment (Figure 4G,H). Liver tissues were collected at the following five time points: before starvation (0 hour), at 24 and 48 hours of starvation, and 2 and 4 hours after refeeding. Immunoblotting for p62 showed that starvation and refeeding enhanced

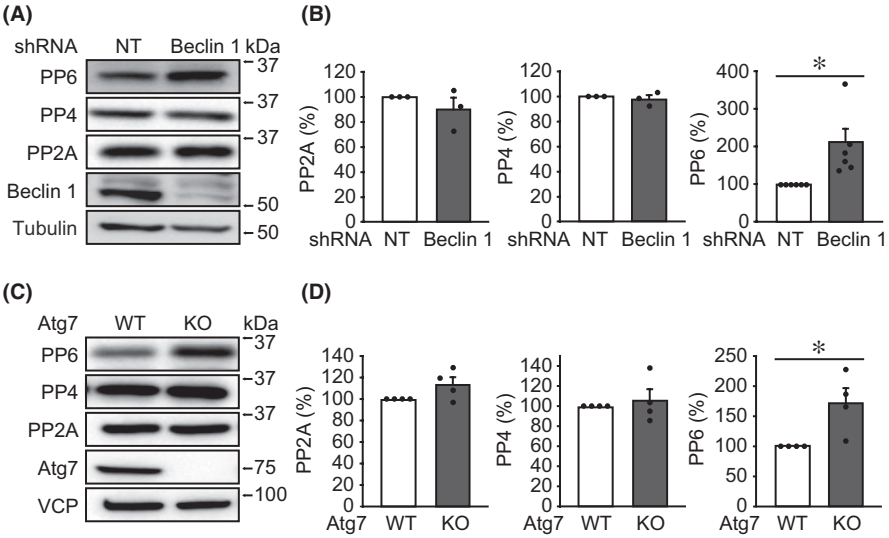

FIGURE 3 Autophagy deficiency results in protein phosphatase 6 (PP6) protein accumulation. A, B, 293T cells were expressed nontarget shRNA (shNT) or shRNA targeting Beclin 1 (shBeclin 1). Protein levels of type 2A protein phosphatases were analyzed by immunoblotting. Representative images (A) and quantitative data (B) from three to six independent experiments are shown. *$P < 0.05$ vs shNT. C, D, Protein levels of type 2A protein phosphatases were analyzed by immunoblotting in wild type (WT) and Atg7 knockout (KO) mouse embryonic fibroblasts (MEFs). Representative images (C) and quantitative data (D) from four independent experiments are shown. *$P < 0.05$ vs WT

and suppressed the autophagy pathway, respectively. The PP6 protein level was decreased by starvation and restored by refeeding.

3.4 | p62-mediated selective autophagy is involved in PP6 protein degradation

Selective autophagy is mediated by autophagic adapter proteins such as p62.[25] We found that PP6 physically associated with p62 and this association was enhanced by bafilomycin A1 treatment in HeLa cells (Figure 5A,B). PP6/p62 co-immunoprecipitation was also enhanced in 293T cells and NIH3T3 cells by bafilomycin A1 (Figure S5A). We also observed enhanced colocalization of PP6 with the lysosome marker LAMP1 and autophagosome marker LC-3 following bafilomycin A1 treatment (Figures 5C-F and S5B,C). CreERT-PP6$^{flox/flox}$ MEFs were used to confirm the specificity of anti-PP6 antibody in immunofluorescence (Figure S5D). These data indicate that PP6/p62 form a complex that is transported to lysosomes.

It is possible that p62 is a substrate of PP6. PP6 forms a heterodimer with one of the three regulatory subunits to be recruited to specific substrates. Therefore, we analyzed whether regulatory subunits were necessary for PP6 and p62 association, by using a phosphatase activity–deficient mutant of PP6 (H114A). As previously described,[26] PP6 the phosphatase activity–deficient mutant did not bind to SAPS regulatory subunits (Figure 5G). However, the PP6 H114A mutant bound to p62 as much as WT (Figure 5H). These data indicate that SAPS are not necessary for PP6 and p62 association, and therefore p62 is not a likely substrate of PP6.

Next, we analyzed whether p62 was necessary for starvation-induced PP6 degradation. p62 levels were knocked down by expressing shRNA-targeting p62 (shp62) (Figure S5E). In control shNT-expressing cells, starvation induced PP6 degradation (Figure 5I,J). However, starvation-induced PP6 degradation was almost completely blocked by the KD of p62. These data indicate p62-mediated selective autophagy for PP6 protein degradation.

3.5 | PP6 and p62 protein levels are positively correlated in tumor tissues

Accumulating evidence shows that autophagic activity is dysregulated in cancer.[27] We utilized gastric cancer model mice, *K19-Wnt1/C2mE* mice (*Gan* mice).[28] Gastric cancer and normal stomach tissues were isolated from ~50-week-old *Gan* mice and age-matched littermates. The PP6 and p62 protein levels were increased in parallel in *Gan* mouse tumor tissues compared with nontumor and normal stomach tissues (Figure 6A,B). To extend these experimental observations, we analyzed the PP6 and p62 protein levels by performing immunohistochemistry in multiple human organs, and tumor and normal tissues using tissue microarrays. The PP6 protein level between normal and tumor tissues was not significantly different (Figure 6C). However, PP6 showed a significant ($P = 0.001$) positive correlation with the p62 protein level (Figure 6D). These data suggest a negative correlation between the PP6 protein level and autophagic activity in vivo.

4 | DISCUSSION

This study exposes profound differences in the regulation of the two most closely related protein phosphatases PP2A and PP6. The

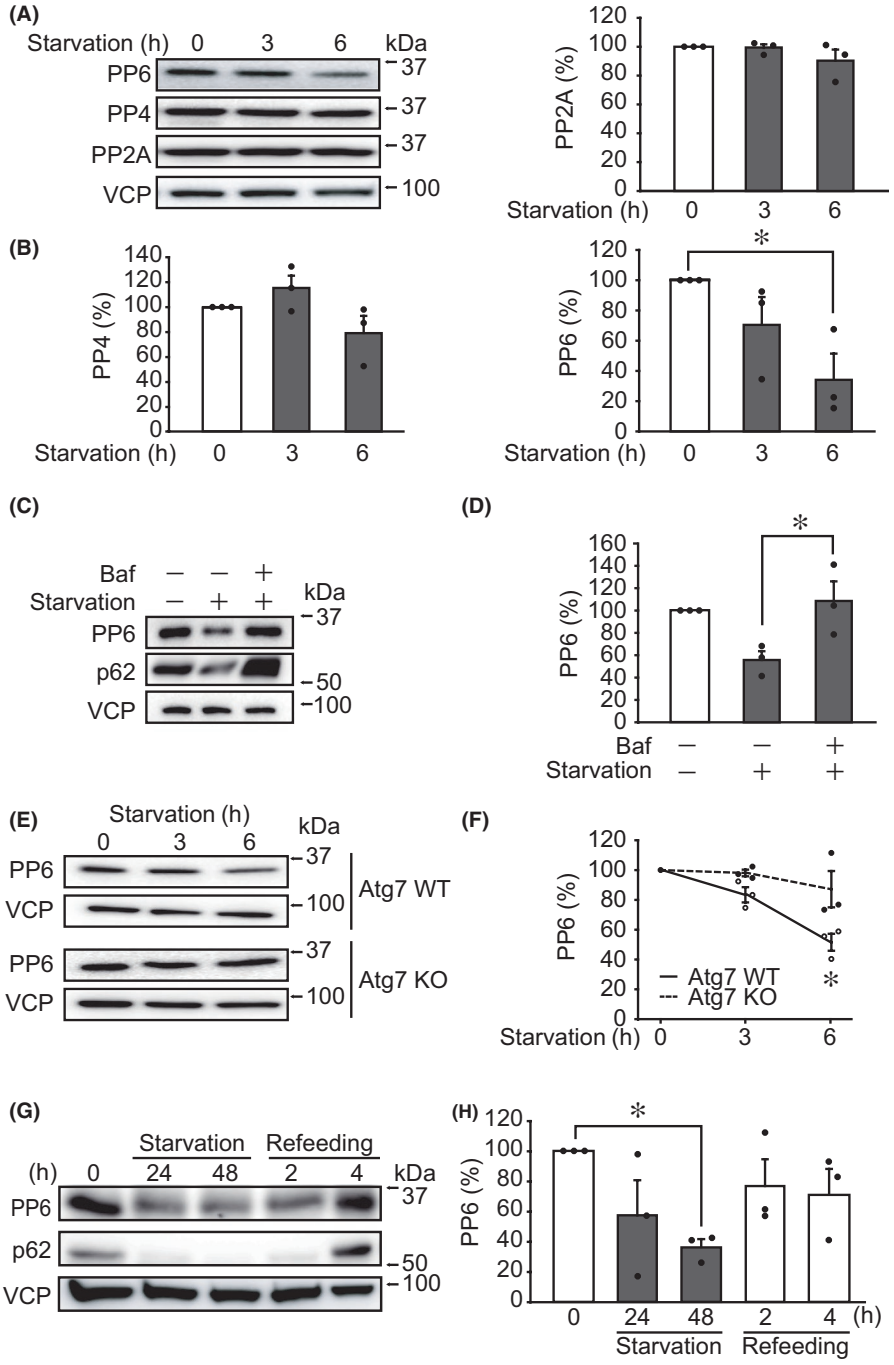

FIGURE 4 Protein phosphatase 6 (PP6) protein level is physiologically regulated by autophagy. A, B, HeLa cells were treated with Earle's balanced salt solution (EBSS, starvation) for indicated time periods. Protein levels of type 2A protein phosphatases were analyzed by immunoblotting. Representative images (A) and quantitative data (B) from three independent experiments are shown. *$P < 0.05$ vs without starvation. C, D, HeLa cells were pretreated with 100 nmol/L of bafilomycin A1 (Baf) for 1 h following the treatment with or without EBSS (starvation) for 6 h. Protein levels of PP6 and p62 were analyzed by immunoblotting. Representative images and quantitative data from three independent experiments are shown. *$P < 0.05$ vs without Baf. E, F, Wild type (WT) and Atg 7 knockout (KO) mouse embryonic fibroblasts (MEFs) were treated with EBSS (starvation) for indicated time periods. Protein levels of PP6 were analyzed by immunoblotting. Representative images and quantitative data from three independent experiments are shown. *$P < 0.05$ vs WT. G, H, Liver was isolated from mice before starvation, at 24 and 48 h of starvation, and 2 and 4 h after refeeding. PP6 and p62 protein levels were analyzed by immunoblotting. Representative images (G) and quantitative data (H) from three independent experiments are shown. *$P < 0.05$ vs 0 h

cellular PP2A protein level is relatively stable, and PP2A is degraded by the ubiquitin-proteasome system,[11,12] whereas PP6 levels are regulated by autophagy-mediated degradation. Although sharing high homology with PP2A, PP6 does not share the same regulatory subunits; the PP2A holoenzyme consists of a PP2A catalytic subunit and a scaffolding A subunit with one regulatory B subunit from one of four diverse families, while the PP6 holoenzyme consists of a PP6 catalytic subunit, one of the three SAPS proteins (PP6R1/2/3), and one of the three ankyrin repeat proteins (Ankrd28/44/52). We utilized both pharmacologic and genetic approaches to demonstrate the PP6 protein level is regulated in an autophagy-dependent manner in mammalian cells. Our data show that the protein degradation mechanism is totally different from that of other type 2A protein phosphatases, PP2A and PP4. Interestingly, degradation of the Sit4 protein, a PP6 homolog in *S. cerevisiae*, is only partially inhibited by genetic deficiency in autophagy, suggesting that autophagic regulation of the PP6 protein might have been acquired during the process of evolution.

Autophagy is a nonselective bulk degradation process that is induced by starvation. However, accumulating evidence shows that autophagy can be highly selective in the elimination of pathogens and removal of damaged organelles or proteins. These selective processes require specific cargo recognition mechanisms. Some autophagy adaptors, such as p62/SQSTM1, NBR1, NDP52,

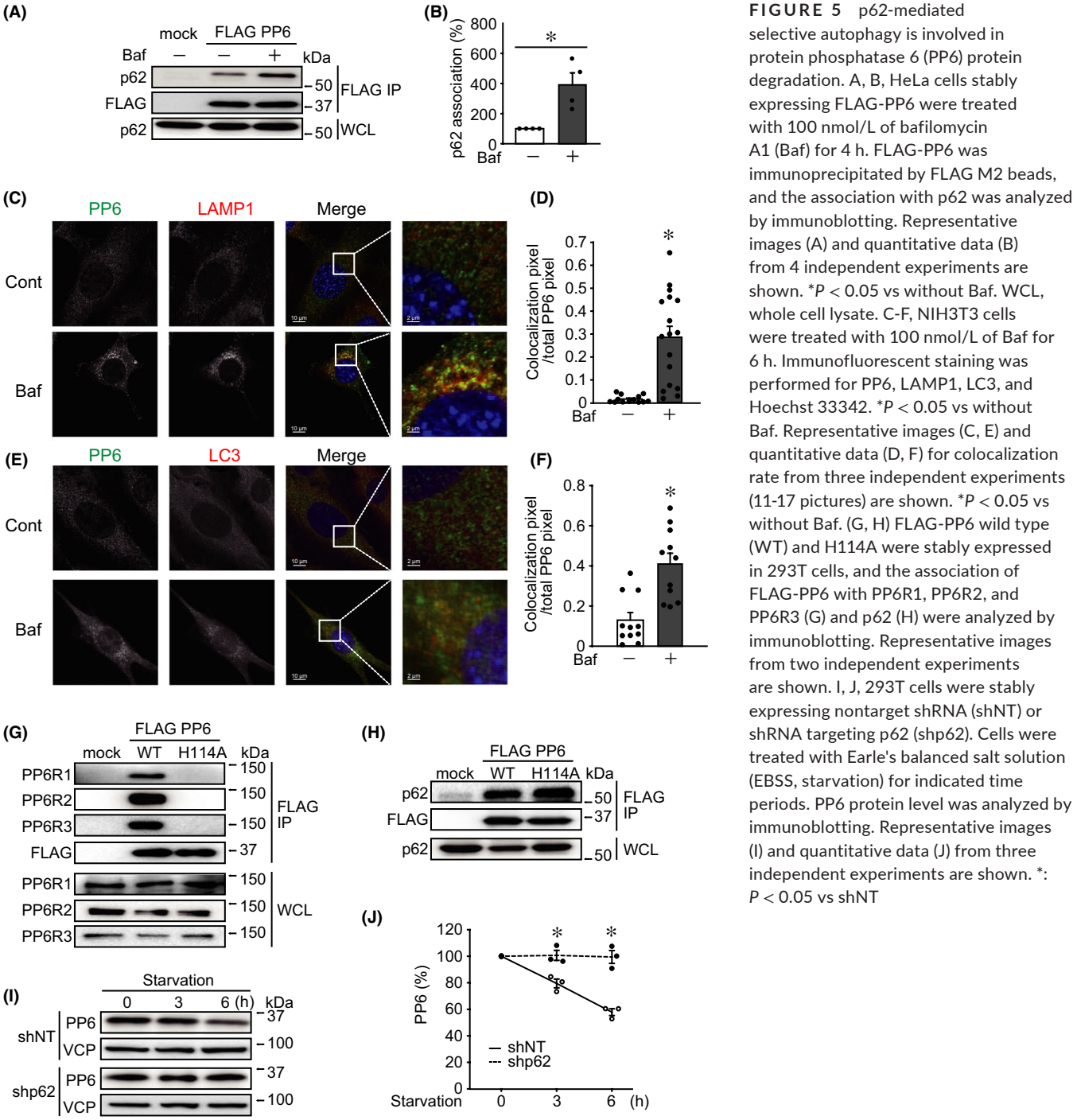

FIGURE 5 p62-mediated selective autophagy is involved in protein phosphatase 6 (PP6) protein degradation. A, B, HeLa cells stably expressing FLAG-PP6 were treated with 100 nmol/L of bafilomycin A1 (Baf) for 4 h. FLAG-PP6 was immunoprecipitated by FLAG M2 beads, and the association with p62 was analyzed by immunoblotting. Representative images (A) and quantitative data (B) from 4 independent experiments are shown. *$P < 0.05$ vs without Baf. WCL, whole cell lysate. C-F, NIH3T3 cells were treated with 100 nmol/L of Baf for 6 h. Immunofluorescent staining was performed for PP6, LAMP1, LC3, and Hoechst 33342. *$P < 0.05$ vs without Baf. Representative images (C, E) and quantitative data (D, F) for colocalization rate from three independent experiments (11-17 pictures) are shown. *$P < 0.05$ vs without Baf. (G, H) FLAG-PP6 wild type (WT) and H114A were stably expressed in 293T cells, and the association of FLAG-PP6 with PP6R1, PP6R2, and PP6R3 (G) and p62 (H) were analyzed by immunoblotting. Representative images from two independent experiments are shown. I, J, 293T cells were stably expressing nontarget shRNA (shNT) or shRNA targeting p62 (shp62). Cells were treated with Earle's balanced salt solution (EBSS, starvation) for indicated time periods. PP6 protein level was analyzed by immunoblotting. Representative images (I) and quantitative data (J) from three independent experiments are shown. *: $P < 0.05$ vs shNT

OPTN, and NIX, function in selective autophagy.[29-32] Autophagy adaptors recognize unnecessary proteins/organelles and move them to the autophagosomal membrane. We found that PP6 binds to p62, and the PP6 protein is degraded in a p62-dependent manner in mammalian cells. Moreover, we observed a positive correlation between PP6 and p62 protein levels in liver from mice during starvation/refeeding, normal and cancer tissues of a mouse gastric cancer model, and a panel of human normal/cancer tissues. In Tissue microarray analysis, PP6 protein levels between normal and tumor tissues were not different, suggesting that PP6 protein accumulation was caused by autophagy deficiency rather than tumor promotion. Because Sit4 was not degraded in an autophagy-dependent manner in yeast, and p62 is conserved only in metazoans, it appears that p62 is a key factor in the PP6 protein degradation mechanism.[33] p62 recruits ubiquitinated and aggregated proteins to autophagosomes.[25] There are several lysine residues in the PP6 sequence that do not exist in PP2A; however, we could not identify the ubiquitination sites of PP6 that are involved in PP6/p62 association (unpublished observation). More detailed analyses are necessary to clarify whether PP6 ubiquitination is necessary for association with p62, and why PP6, but not PP2A, is selectively degraded by autophagy.

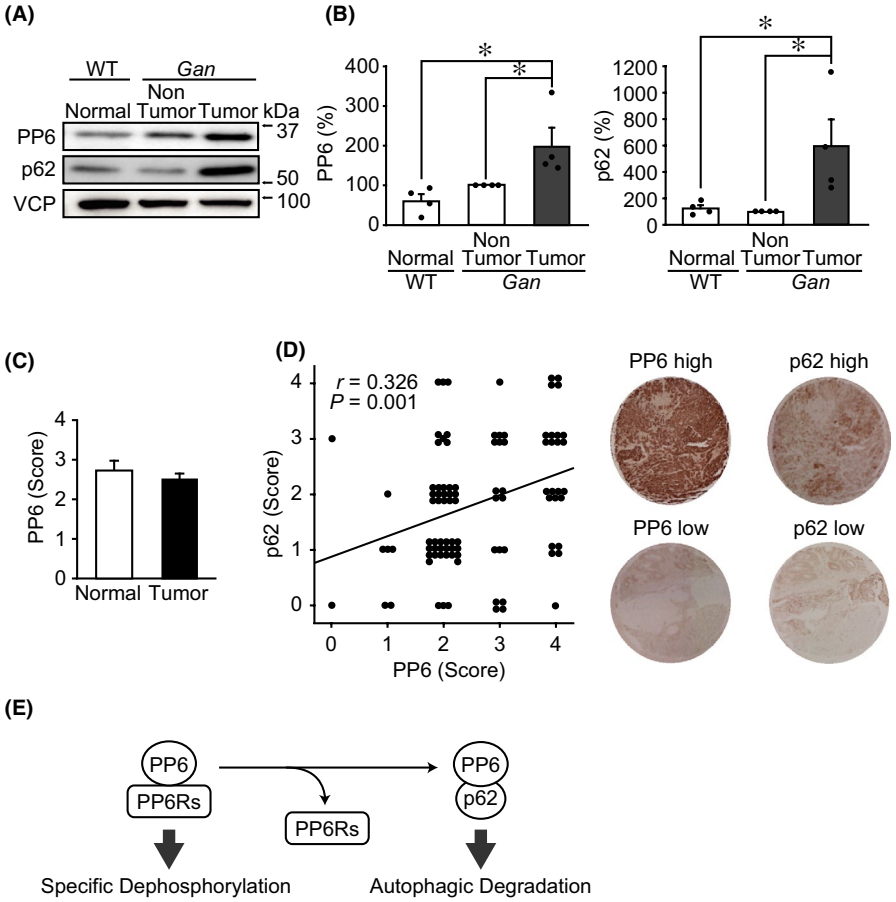

FIGURE 6 Protein phosphatase 6 (PP6) and p62 protein levels were positively correlated in tumor tissues. A, B, Stomach tissues were isolated from wild-type (WT) mice and the nontumor or tumor region of *K19-Wnt1/C2mE* mice (*Gan*), and protein levels were analyzed by immunoblotting. Representative images (A) and quantitative data (B) from four independent experiments are shown. $^*P < 0.05$ vs nontumor. C, D, PP6 and p62 protein levels in normal and cancer tissues were immunohistochemically analyzed using serial section tissue microarray (TMA). TMA contains 2-14 cases of esophagical, gastric, liver, gall bladder, large intestine, and small intestine cancer tissues and 2-4 cases each of normal tissues. C, PP6 protein levels in normal and cancer tissues. D, Correlation of PP6 and p62 protein levels. Correlation coefficient (*r*) and the *P* value were calculated using Pearson's trend test. E, Model for the regulation of PP6 protein level by autophagy

Accumulating evidence indicates that PP6 is an important suppressor of skin cancer.[5-7,23] However, previous reports showed that PP6 expression is increased in some cancer types.[8,9] Consistent with these reports, we observed the increase in PP6 protein level in tumor tissues of gastric cancer model mice, and the accumulation of p62 suggested that autophagy activity is reduced in the tumor tissues. Positive correlation between PP6 and p62 in human normal and tumor tissues suggests that increased PP6 protein observed in the previous reports was due to the reduced autophagic activity in cancer tissues.

Because p62 was reported to promote liver cancer by activating Nrf2-dependent pathway,[34] correlated increase in p62 and tumor-suppressor PP6 is contradictory. It is not clear why high levels of PP6 do not work as a tumor suppressor. Our results showed that a phosphatase activity–deficient mutant of PP6 (H114A) binds to p62 as much as WT PP6, but not to any regulatory SAPS subunits. A treatment with bafilomycin A1 for 6 hours accumulated PP6 protein but did not change the SAPS protein levels, and it also did not affect PP6/SAPS association. These observations suggest that p62-dependent selective autophagy degrades monomeric PP6, but not PP6 complex with regulatory subunits (Figure 6E). Because regulatory subunits recruit PP6 to specific substrates, PP6 accumulated by acute autophagy deficiency may not be functionally active. On the other hand, chronic deficiency of autophagy leads to coordinated upregulation of SAPS proteins, suggesting an increase in PP6 complex with SAPS. The substrate specificity of type 2A protein phosphatases is regulated by the assembled subunits. Although PP2A is a well-established tumor suppressor, a part of the PP2A complexes can work as a tumor promoter.[35] Therefore, it is necessary to know which regulatory subunit is important for the tumor-suppressive role of PP6.

ACKNOWLEDGMENTS

This work was partially supported by JSPS KAKENHI (grant numbers 17H03915, 18K16364, and 20H03151), the Extramural Collaborative Research Grant of the Cancer Research Institute of Kanazawa University, and the Research and Education Funds from Ehime Prefecture and Imabari City, Japan.

DISCLOSURE

One million yen or more total annual value of research funds provided. Takashi Ohama from the Mitsubishi Foundation and Shin-Nihon Senshin Iryo Kenkyu Zaidan. Issay Kitabayashi from Sumitomo Dainippon Pharma Co. Ltd.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Fujiwara N, Shibutani S, Sakai Y, et al. Autophagy regulates levels of tumor suppressor enzyme protein phosphatase 6. *Cancer Sci* 2020;111:4371–4380. https://doi.org/10.1111/cas.14662