Involvement of the Tubulin Tyrosine Ligase-Like Family Member 4 Polyglutamylase in PELP1 Polyglutamylation and Chromatin Remodeling in Pancreatic Cancer Cells

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Abstract

Polyglutamylation is a new class of posttranslational modification in which glutamate side chains are formed in proteins, although its biological significance is not well known. Through our genome-wide gene expression profile analyses of pancreatic ductal adenocarcinoma (PDAC) cells, we identified the overexpression of tubulin tyrosine ligase-like family member 4 (TTLL4) in PDAC cells. Subsequent reverse transcription-PCR and Northern blot analyses confirmed its upregulation in several PDACs. TTLL4 belongs to the TTLL family which was reported to have polyglutamylase activity. Knockdown of TTLL4 by short hairpin RNA in PDAC cells attenuated the growth of PDAC cells and exogenous introduction of TTLL4 enhanced cell growth. We also found that TTLL4 expression was correlated with polyglutamylation levels of a glutamate stretch region of the proline, glutamate, and leucine–rich protein 1 (PELP1) that was shown to interact with various proteins such as histone H3, and was involved in several signaling pathways through its function as a scaffold protein. PELP1 polyglutamylation could influence its interaction with histone H3 and affect histone H3 acetylation. We also identified the interaction of PELP1 with LAS1L and SENP3, components of the MLL1-WDR5 supercomplex involving chromatin remodeling. Our findings imply that TTLL4 could play important roles in pancreatic carcinogenesis through its polyglutamylase activity and subsequent coordination of chromatin remodeling, and might be a good molecular candidate for the development of new therapeutic strategies for pancreatic cancer.

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Introduction

Pancreatic ductal adenocarcinoma (PDAC) is the fourth leading cause of cancer death in the western world and shows the worst mortality rate among common malignancies with a 5-year survival rate of only 5% (1, 2). The majority of patients with PDAC are diagnosed at an advanced stage, for which no effective therapy is available at present. Although only surgical resection offers a limited possibility for cure, 80% to 90% of patients with PDAC who undergo curative surgery die from their disseminated or metastatic diseases (1, 2). Recent advances in surgery and chemotherapy including 5-fluorouracil or gemcitabine, with or without radiation, could improve patients’ quality of life (1, 3). However, those treatments have a very limited effect on the long-term survival of patients with PDAC due to its extremely aggressive and chemoresistant nature. Hence, the management of most patients is focused on palliative measures (1). To overcome this dismal situation, the development of novel molecular therapies targeting a novel oncogenic molecule in PDAC cells is an urgent issue (3).

Toward this direction, we previously generated precise gene expression profiles of PDAC cells using genome-wide cDNA microarrays in combination with microdissection to enrich populations of PDAC cells (4). Among dozens of overexpressed genes identified by this strategy, we focused on tubulin tyrosine ligase-like family member 4 (TTLL4) as a novel molecular target for pancreatic cancer. TTLL4 belongs to a protein family that has a TTL homology domain and could catalyze ligations of diverse amino acids like tyrosination, polyglycylation, and polyglutamylation to various substrates (5). Recently, some TTLL family members were proven to have the activity to polyglutamylate tubulins and microtubule-associated proteins (6). Polyglutamylation is a new class posttranslational modification in which glutamate side chains are formed in proteins, although its biological significance is not well known.
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of posttranslational modification, which forms glutamate side chains of variable lengths on target proteins, and was first discovered on the tubulin proteins (7). Polyglutamylation is likely to influence the protein stability, cell cycle control (8, 9), and the interaction between microtubules and their associated proteins (10–12). Furthermore, TTLL4 and TTLL5 were shown to have the ability to polyglutamate several nontubulin proteins (13). However, the biological significance of polyglutamylation of these proteins is still not well understood.

In this study, we demonstrated TTLL4 transactivation in PDAC cells and showed that it could play some important roles in pancreatic cancer proliferation through polyglutamylation of a nontubulin protein, proline, glutamate, and leucine–rich protein 1 (PELP1; ref. 14). We also found that PELP1 polyglutamylation could affect its affinity to histone H3 and other proteins, and subsequent chromatin remodeling. Our data provide new insights into the biological and molecular roles of this new class of protein modification in pancreatic carcinogenesis.

Materials and Methods

Cell lines and clinical samples. PANC-1, MIA-PaCa2, COS-7, and HeLa cells were purchased from American Type Culture Collection. KLM-1, PK-59, PK-45P, and PK-1 were established by Tohoku University (Sendai, Japan). SUIT-2 and KP-1N cells were provided by the National Kyushu Cancer Center (Fukuoka, Japan). They were cultured under their respective depositors’ recommendations, RPMI 1640 (Invitrogen), or DMEM (Invitrogen) with 10% fetal bovine serum and 1% antibiotic/antimycotic solution. Frozen PDAC tissues were obtained from surgical specimens that were resected in Osaka Medical Center for Cancer and Cardiovascular Diseases under the appropriate informed consent. Using these clinical samples, this study was approved by the institutional review boards of the Institute of Medical Science, The University of Tokyo, and Osaka Medical Center for Cancer and Cardiovascular Diseases.

Semi-quantitative reverse transcription-PCR. Microdissection to enrich PDAC cells and normal pancreatic ductal epithelial cells from frozen PDAC tissues, T7-based RNA amplification, and cDNA synthesis were described previously (4). We prepared appropriate dilutions of each single-stranded cDNA for subsequent PCR amplification by monitoring α-tubulin (TUBA) for microdissected PDAC cells, or β2-MG for PDAC cell lines as a quantitative control. The sets of primer sequences we used were 5′-AAGGATTATGAG-GAGGTTGTGTTG-3′ and 5′-CTTGGTCTCTGTAACAGAAG-CATT-3′ for TUBA; 5′-TAGGCTGCTCCTGCCCAT-3′ and 5′-GCCCTACTCTCTCTGTAAC-3′ for β2-MG, 5′-GTAGGGTGCA-GCCTACTCTCTCTGTAAC-3′ and 5′-CAGGGAGGAT-CAGGAGGAT-3′ for TTLL4. All reactions involved initial denaturation at 94°C for 5 minutes followed by 24 cycles for TUBA, 28 cycles for β2-MG, or 29 cycles (for TTLL4) of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and elongation at 72°C for 1 minute, on a GeneAmp PCR system 9700 (Applied Biosystems).

Northern blotting analysis. We extracted total RNAs from eight PDAC cell lines using RNeasy Mini kit (Qiagen). Their RNAs were purified with mRNA Purification Kit (GE Healthcare). One microgram each of mRNA from PDAC cell lines, as well as those isolated from normal human adult pancreas, heart, lung, kidney, liver, brain, and testis (BD Biosciences), were separated on 1% denaturing agarose gels and transferred onto a nylon membrane. terminals and human Multiple Tissue Northern blot membrane (BD Biosciences) were hybridized for 16 hours with 32P-labeled TLL4 cDNA, which was labeled using Megaprime DNA Labeling System (GE Healthcare). Probe cDNA of TTLL4 was prepared as a 474-bp PCR product by using primers 5′-AT-CAAGGCGAGATGATTCC-3′ and 5′-CAAGACTCCCATGGAACC-3′. Prehybridization, hybridization, and washing were performed according to the instructions of the manufacturer. The blots were autoradiographed at −80°C for 10 days.

Knockdown of TTLL4 by short hairpin RNAs. To knock down endogenous TTLL4 expression in PDAC cells, we used psl568X30 vector for the expression of short hairpin RNAs (shRNA) against a target gene as described previously (15). The target sequences for TTLL4 were 5′-GAAGCACAGTGGAGA-CACTG-3′ (si466), 5′-GAAGCTTGGCAATATAGTTC-3′ (si2692), and 5′-CATTGGCAAGACCAT-3′ (si2146), and we used 5′-GAAGCAGACGACTCTTCC-3′ (siCON) as a negative control. PANC-1 and MIA-PaCa2 cells were seeded on 10 cm dishes, and transfected with these shRNA expression vectors using FuGENE6 (Roche Diagnostics) according to the instructions of the manufacturer, followed by 1,000 μg/mL (for PANC-1) or 800 μg/mL (for MIA-PaCa2) geneticin selection. The cells were harvested 7 days after the transfection and analyzed the knockdown effect on TTLL4 by reverse transcription-PCR (RT-PCR) using the primers as described above. After geneticin selection for 12 days, the cells were fixed with 100% methanol, stained with 0.1% of crystal violet-H2O for colony formation assay. In a 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, cell viability was measured using a Cell counting kit-8 (Dojindo) at 11 days after the transfection. Absorbance was measured at 490 nm and that at 630 nm was used as a reference with Microplate Reader 550 (Bio-Rad Laboratories).

Expression constructs for TTLL4 and PELP1. The full-length cDNA encoding human TTLL4 was amplified by forward primer, 5′-AGAATTCTGTGGGGCCCCCTATGGGCTCAGCAG-3′ and reverse primer, 5′-TGGAGGCAGCAGGATGAGCT-CACAGCCAGAGGA3′ (underlined sequences indicate restriction enzyme sites), and was cloned into the EcoRI and NotI sites of pCAGGS vector. Enzyme-dead TTLL4 mutant (TTLL4 E906A), which was reported to lose its polyglutamylase enzyme activity (16), was generated by using QuikChange XL Site-Directed Mutagenesis kit (Stratagene) and the mismatch primer 5′-GCCCTGGGTCTCCTGGCAGTCAACATTCCC-3′. Full-length His-tagged PELP1 expression vector was kindly provided by Dr. Ratna K. Vadlamudi (Department of Obstetrics and Gynecology, The University of Texas Health Science Center at San Antonio, San Antonio, TX; ref. 17). For the mutant del 887- of PELP1, the PCR product was amplified by a set of primers

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(5′-TGAATTCATGGCGGCAGCGTTCTGAGT-3′ and 5′-TCAAGUGGAGACACUG-3′ and 5′-TCCAGGCCGCACTCCAGGTCTTCCACCTC-3′) and was cloned into the EcoRI and NotI sites of pCAGGS vector. For the mutant del 887–964 of PELP1, the PCR product was amplified by a set of primers (5′-TTGTGCCACAGCAGGAGGGGA-3′ and 5′-TCAAGUGGAGACACUG-3′) and was cloned into the EcoRI and NotI sites of pCAGGS vector. For the mutant del 1003–1065 of PELP1, the PCR product was amplified by a set of primers (5′-TGAATTCATGGCGGCAGCGTTCTGAGT-3′ and 5′-TCAAGUGGAGACACUG-3′) and was cloned into the EcoRI and NotI sites of pCAGGS vector. For cell growth assay, COS-7 cells were seeded on 10 cm dishes and transfected with wild-type TTLL4, enzyme-dead TTLL4 mutant (E906A), or an empty vector, or cotransfected with wild-type or mutant del 887–964 of PELP1 expression vectors 48 hours after the transfection, cell viability was evaluated by an MTT assay as described above.

**Western blot analysis and immunoprecipitation.** COS-7 or HeLa cells were transfected with TTLL4 expression vector and/or PELP1 expression vector. MiaPaCa2, or PANC-1 cells were transfected with siR6 RNA duplex (siTTLL4, 5′-GAAGCAAGGCGACACUG-3′) to knock down TTLL4 or siEGFP RNA duplex (5′-GAAGCAAGGCGACACUGUCUCC-3′) as a negative control. These cells were collected 48 hours after transfection and lysed with 0.4% NP40 lysis buffer [0.4% NP40, 150 mmol/L NaCl, and 50 mmol/L Tris-HCl, pH 8.0] and ultracentrifuged at 2,300 × g for 10 minutes at 4°C to separate the nuclear and cytosolic fractions. The pellet was washed twice with PBS, lysed with 40% NP40 lysis buffer, and incubated on ice for 30 minutes. The lysate was incubated with 5 μg of anti-PELP1 antibody (A300-876A, Bethyl Laboratories) or rabbit IgG (Santa Cruz Biotechnology) at 4°C for 1.5 hours. Immunocomplexes were incubated with 50 μL of protein G Sepharose (Invitrogen) for 1 hour and washed with lysis buffer. Coprecipitated proteins were separated in 7.5% SDS-PAGE gel and stained with silver staining kit (Invitrogen). We excised bands that specifically appeared in the precipitates with anti-PELP1 antibody, but not in those with the control rabbit IgG, and analyzed them by liquid chromatography-tandem mass spectrometry (LC/MS/MS) analysis. The excised bands were reduced in 10 mmol/L of tris(2-carboxyethyl)phosphine (Sigma-Aldrich) with 50 μmol/L of ammonium bicarbonate (Sigma-Aldrich) for 30 minutes at 37°C and alkylated in 50 mmol/L of iodoacetamide (Sigma-Aldrich) with 50 mmol/L of ammonium bicarbonate for 45 minutes in the dark at 25°C. These cells were digested with porcine trypsin (Promega) and their peptide mixture was separated on a 100 μm × 150 mm HiQ-Sil C18W-3 column (KYA Technologies) using a 30-minute linear gradient from 5.4% to 29.2% acetonitrile in 0.1% trifluoroacetic acid with total flow of 300 nL/min. The eluting peptides were automatically mixed with a matrix solution [4 mg/mL α-cyano-4-hydroxy-cinnamic acid (Sigma-Aldrich), 0.08 mg/mL ammonium citrate in 70% acetonitrile, and 0.1% trifluoroacetic acid] and spotted onto MALDI target plates (4 mg/mL α-cyano-4-hydroxy-cinnamic acid with total flow of 300 nL/min). The MS/MS peak list was generated by the Protein Pilot version 2.0.1 software (Applied Biosystems/MDS Sciex). The MS/MS peak list was exported to a local MASCOT database search engine version 2.2.03 (Matrix Science) for protein database search.

**Interaction of PELP1 with SENP3 and LASIL.** To confirm the interaction of PELP1 with SENP3 or LASIL proteins, PELP1-HA expression vector and SENP3-Flag or LASIL-Flag expression vector were cotransfected into COS-7 cells. For full-length cDNA encoding human SENP3, the PCR product amplified by a set of primers (forward primer, 5′-TTGGCCACAGCAGGAGGGGA-3′ and reverse primer, 5′-TCAAGUGGAGACACUG-3′) was cloned into the NotI and XhoI sites of pcAGGS3FC vector. For full-length cDNA encoding human LASIL, the PCR product amplified by a set of primers (forward primer, 5′-TGGAATTCATTTCTGAGGAGAGACTTATACAA-3′ and reverse primer, 5′-TCAAGUGGAGACACUG-3′) was cloned into the EcoRI and NotI sites of pCAGGS vector. The transfected cells were lysed as described above and immunoprecipitated with rat anti-HA antibody (clone3F10, Roche) or Flag-M2 agarose affinity gel (Sigma-Aldrich). To examine the interaction of PELP1-HA with SENP3-Flag or LASIL-Flag proteins, we analyzed these immune complexes by Western blotting with rabbit anti-FLAG or anti-HA antibodies. To evaluate the effect of polyglutamylation of PELP1 on its affinity for protein interaction, COS-7 cells were seeded and cotransfected with wild-type TTLL4 expression vector or enzyme-dead TTLL4 mutant (E906A) vector, PELP1-HA expression vector, and LASIL-Flag or SENP3-Flag expression vector.
They were collected 48 hours after transfection, and their lysates were immunoprecipitated with anti-HA antibody. We analyzed these immune complexes by Western blotting with anti-FLAG M2 antibody (Sigma-Aldrich) or anti-HA antibody. Furthermore, PK-1 cells were seeded and transfected with siTTLL4 or siEGFP. After 24 hours, PELP1-HA expression vector and LASIL-Flag or SENP-Flag expression vector were co-transfected. They were collected 48 hours after the transfection, and their lysates were immunoprecipitated with anti-HA antibody. We analyzed these immune complexes by Western blotting as described above.

Results

Overexpression of TTLL4 in PDAC cells. Among a number of genes that were identified to be transactivated in PDAC cells through our genome-wide microarray analyses (4), we focused on the TTLL4 gene in this study. RT-PCR analysis confirmed TTLL4 overexpression in 5 of the 10 microdissected primary PDAC cells (Fig. 1A), and in all of the PDAC cell lines we examined (Supplementary Fig. S1A). Northern blot analysis of normal human tissues using a TTLL4 cDNA fragment as a probe identified a 4.4 kb transcript only in the testis and high levels of expression in all of the PDAC cell lines we examined (Supplementary Fig. S1B), but its expression was hardly detectable in any other adult normal organs (Fig. 1B). These findings implicated TTLL4 as a cancer-testis antigen.

Knockdown of TTLL4 by shRNA attenuated PDAC cell viability. To investigate the biological significance of TTLL4 overexpression in PDAC cells, we constructed three shRNA expression vectors specific to the TTLL4 transcript (si466, si2692, and si2146) and transfected each of them into two PDAC cell lines, PANC-1 or MIA-PaCa2, which endogenously expressed high levels of TTLL4. RT-PCR confirmed the knockdown effects of endogenous TTLL4 expression when we transfected si466 and si2692 shRNA expression constructs to PANC-1 cells. On the other hand, no effect was observed when we transfected si2146 or the negative control siCON (Fig. 2A, left). Colony formation assays (Fig. 2B, left) and MTT assays (Fig. 2C, left) using PANC-1 cells revealed a drastic reduction in the number of viable PDAC cells transfected with si466 and si2692, compared with those transfected with si2146 or siCON in which no knockdown effect was observed (Student’s t test, \( P < 0.01 \)). Similar results were obtained when we used another TTLL4-positive PDAC cell line, MIA-PaCa2 (Fig. 2A, B, and C, right), indicating that TTLL4 expression could be essential for PDAC cell proliferation.

TTLL4 overexpression promotes cell growth. For further investigation into the oncogenic potential of TTLL4, COS-7 cells were transfected with wild-type TTLL4 or enzyme-dead TTLL4 (E906A) which contained a substitution in the ATP-binding site (the essential glutamate site at codon 906; ref. 16), and examined the growth-promoting effect by their overexpression. MTT assay showed that wild-type TTLL4 significantly promoted cell growth, compared with mock-transfected cells (Student’s t test, \( P < 0.01 \)), whereas enzyme-dead TTLL4 (E906A) revealed no growth-promoting effect (Fig. 2D, bottom). Western blot analysis using GT335 antibody, which could specifically detect polyglutamate side chains (18), showed that wild-type TTLL4 overexpression enhanced the polyglutamylation of multiple cellular proteins, whereas enzyme-dead TTLL4 (E906A) did not (Fig. 2D, top). These results suggest that TTLL4 could promote cell growth, possibly through its polyglutamylase activity.

TTLL4 polyglutamylated a nontubulin protein, PELP1. A previous proteomics approach identified several putative substrates including α-tubulin and β-tubulin for polyglutamylation by TTLL family members (13). To investigate
TTLL4 substrates, we induced TTLL4 into HeLa cells and screened proteins detected by polyglutamylation-specific GT335 antibodies. Western blot analysis using GT335 antibody detected increased levels of polyglutamylation in several proteins (Fig. 3A, left). When we knocked down TTLL4 expression in PDAC cells, a single 200 kDa band recognized by GT335 antibody was weakened in both PANC-1 and MIA-PaCa2 cells (Fig. 3A, middle and right, arrow). The intensity of this 200 kDa band was enhanced in HeLa cells when the exogenous TTLL4 was introduced (Fig. 3A, left). Hence, among the candidate polyglutamylated proteins identified by the previous proteomic approach (13), we focused on this 200 kDa protein and characterized it to be PELP1. To further validate the polyglutamylation of PELP1 by TTLL4, we immunoprecipitated PELP1 from the cell lysates of HeLa cells that were transfected with TTLL4 expression vector (Fig. 3B, top), or that of MIA-PaCa2 cells in which TTLL4 was knocked down (Fig. 3B, bottom). As shown in Fig. 3B, Western blot analysis using GT335 antibody indicated that overexpression of wild-type TTLL4 enhanced polyglutamylation of multiple cellular proteins, whereas that of enzyme-dead TTLL4 (E906A) did not (top). MTT assay at 48 hours after the transfection with wild-type TTLL4, TTL4 (E906A), or mock into COS-7 cells showed that wild-type TTLL4 significantly promoted cell growth (*, P < 0.001, Student’s t test, bottom), but TTLL4 (E906A) did not, compared with the growth of mock-transfected cells. ABS, absorbance at 490 nm measured with a microplate reader (that at 630 nm was used as reference).

**Figure 2.** TTLL4 expression was involved with PDAC cell viability. A, RT-PCR confirmed the knockdown effect on TTLL4 expression by si466 and si2692, but not by si2146 or a negative control siCON in PANC-1 (left) and MIA-PaCa2 (right) cells. β2-MG was used to quantify RNAs. B, colony formation assays of PANC-1 (left) and MIA-PaCa2 (right) cells transfected with each of the indicated shRNA-expressing vectors to TTLL4 and siCON. Cells were visualized with 0.1% crystal violet staining after 12 days of incubation with Geneticin. C, MTT assays of each of PANC-1 (left) and MIA-PaCa2 (right) cells transfected with each of the indicated shRNA-expressing vectors to TTLL4 and siCON. Each average is plotted with error bars indicating SD after 11 days of incubation with Geneticin. Absorbance (ABS) was measured at 490 nm with a microplate reader, and that at 630 nm was used as reference. These experiments were carried out in triplicate. Transfection of si466 and si2692 into PANC-1 (left) and MIA-PaCa2 (right) cells resulted in a drastic reduction in the number of viable cells, compared with that of si2146 or siCON (*, P < 0.001, Student’s t test). D, Western blot analysis using GT335 antibody indicated that overexpression of wild-type TTLL4 enhanced polyglutamylation of multiple cellular proteins, whereas that of enzyme-dead TTLL4 (E906A) did not (top). MTT assay at 48 hours after the transfection with wild-type TTLL4, TTL4 (E906A), or mock into COS-7 cells showed that wild-type TTLL4 significantly promoted cell growth (*, P < 0.001, Student’s t test, bottom), but TTLL4 (E906A) did not, compared with the growth of mock-transfected cells. ABS, absorbance at 490 nm measured with a microplate reader (that at 630 nm was used as reference).
region of the PELP1 protein (77% of amino acids in codons 887–964 are glutamate residues; Fig. 3C, top) as a candidate domain subject to polyglutamylation. To investigate this hypothesis, we constructed three deletion constructs of PELP1 (del 887-, del 887–964, and del 1003-), as shown in Fig. 3C (top); two of these PELP1 constructs, del 887- (lacking of codon 887 to the COOH-terminal end) and del 887–964 (lacking codons 887 and 964), lacked the glutamate-rich region. We cotransfected each of these PELP1 partial constructs with wild-type TTLL4 expression vector or enzyme-dead TTLL4 expression vector, and observed that GT335 antibody detected polyglutamylation when the full-length PELP1 or PELP1-del 1003-, but not PELP1-del 887- or PELP1-del 887–964, was cotransfected with wild-type TTLL4 (Fig. 3C, bottom). These findings indicated that TTLL4 could polyglutamylate PELP1 in its highly glutamate-rich region (codon 887–964). To examine whether TTLL4 could promote cell proliferation through PELP1 polyglutamylation, we performed MTT assay after PELP1 and TTLL4 expression vectors were cotransfected to COS7 cells (Fig. 3D). Overexpression of PELP1 itself could promote cell growth and over-expression of PELP1 deletion mutant (del 887–964) lacking the polyglutamylation site could also promote cell growth. Cotransfection of TTLL4 enhanced the growth-promoting

Figure 3. TTLL4 could polyglutamylate a nontubulin protein PELP1. A, GT335 antibody detected the enhancement of polyglutamylation in several proteins, including a protein of 200 kDa (arrow) which was likely to correspond with PELP1 when TTLL4 was overexpressed in HeLa cells (left, arrow). When TTLL4 was knocked down (siTTLL4) in PANC-1 (middle) and MiaPaCa2 (right) cells, polyglutamylation of a 200 kDa band (arrow) was significantly reduced in both cell lines. B, PELP1 was immunoprecipitated from the HeLa cells lysate transfected with TTLL4, and polyglutamylated PELP1 was detected by GT335 antibody. The polyglutamylation level of PELP1 was enhanced when TTLL4 was overexpressed (top). Western blot analysis using GT335 antibody followed by immunoprecipitation by anti-PELP1 antibody showed that the polyglutamylation level of PELP1 was decreased when TTLL4 was knocked down in MIA-PaCa2 cells (bottom). C, PELP1 contains a highly glutamate-rich region between codons 887 and 964. In addition to the full-length PELP1 protein, we constructed three plasmid clones of PELP1 (del 887–954, del 887-, and del 1003-) that lacked the glutamate-rich region (codon 887–964) or the COOH-terminal region (codon 887–, codon 1003–), respectively. Each of the PELP1 constructs was cotransfected with the wild-type TTLL4 or enzyme-dead TTLL4 (E906A). GT335 antibody detected the polyglutamylated PELP1 when the full-length (FL) PELP1 or PELP1 lacking COOH-terminal region (del 1003–) was cotransfected with wild-type TTLL4, but not when the two deletion PELP1 constructs lacking the glutamate-rich region was cotransfected. Immunoblot analysis using anti-HA antibody showed the expression of each PELP1 construct and that using anti-Flag antibody indicated TTLL4 or mutant TTLL4 expression. D, MTT assay when wild-type PELP1, mutant PELP1 (del 887–964), or mock was cotransfected with TTLL4 expression vectors into COS-7 cells showed that wild-type PELP1 significantly promoted cell growth (*, P < 0.001, Student’s t test, bottom), and this effect was enhanced when TTLL4 expression vector was cotransfected (P < 0.001).
effect of PELP1, but not that of PELP1 deletion mutant (del 887–964). This data indicated that the growth-promoting effect of PELP1 could be enhanced by polyglutamylation of PELP1 by TTLL4.

**Interaction of polyglutamylated PELP1 with histone H3 and its involvement in chromatin remodeling.** Because interaction of the glutamate-rich stretch region of PELP1 and histone H3 was reported previously (20, 21), we validated the interaction between PELP1 and histone H3 by immunoprecipitation using PK-1 cells (Fig. 4A). To investigate the effect of PELP1 polyglutamylation on the interaction with histone H3, we immunoprecipitated PELP1 from the lysates of PK-1 cells in which TTLL4 was knocked down with siTTLL4 or those treated with a control siRNA (siEGFP). As shown in Fig. 4B, the interaction of PELP1 with histone H3 was significantly reduced when TTLL4 was knocked down by siTTLL4. Concordantly, the interaction of PELP1 with histone H3 was enhanced in the presence of wild-type TTLL4, but not in the presence of enzyme-dead TTLL4 (Fig. 4C). In addition, because the interaction of PELP1 with histone H3 could inhibit the acetylation of histone H3 (21), we evaluated the acetylation status of histone H3 in PK-1 cells in which TTLL4 was knocked down and found that the acetylation level of histone H3 was drastically increased compared with those treated with the control siEGFP (Fig. 4D). On the other hand, we did not observe any significant change of methylation status of histone H3 in the TTLL4 knockdown cells (data not shown).

**Interaction of PELP1 with SENC3 and LASIL.** PELP1 protein has been shown to interact with various proteins such as estrogen receptor, Src, p85, etc., and to be involved in several signaling pathways by functioning as a scaffold protein (14, 17, 22, 23). To further clarify the function of PELP1 polyglutamylation in PDAC cells, we first attempted to identify proteins interacting with PELP1 in PDAC cells. Protein complexes were immunoprecipitated by anti-PELP1 antibody from the PK-1 cell lysates and characterized by LC-MS/MS analysis. We identified LASIL (LAS1-like, NP_112483) and SENC3 (SUMO1/sentrin/SMT3-specific protease 3, NP_056485) proteins as candidates interacting with PELP1 protein in PDAC cells. To validate these interactions, we transfected the vector expressing PELP1-HA, that expressing LAS1L-Flag, or both vectors together into COS-7 cells, and a protein complex containing PELP1-HA and/or LAS1L-Flag was immunoprecipitated from the cell extracts by anti-HA antibody (Fig. 5A, middle) or anti-Flag antibody (Fig. 5A, bottom). As shown in Fig. 5A (left), Western blot analysis using anti-HA antibody indicated that PELP1-HA

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**Figure 4. Interaction of PELP1 and histone H3.** A, interaction of PELP1 and histone H3 examined by immunoprecipitation. Western blotting using anti-histone H3 antibody indicated that histone H3 was coimmunoprecipitated with PELP1 in PK-1 cells. B, PELP1 was immunoprecipitated from PK-1 cell lysates in which TTLL4 was knocked down (siTTLL4). PELP1 and histone H3 were detected in the immunoprecipitated complex by Western blot. The interaction affinity of PELP1 with histone H3 was diminished when TTLL4 was knocked down (siTTLL4) in PK-1 cells whereas the control siEGFP revealed no effect. C, HA-tagged PELP1 was immunoprecipitated from COS-7 cell lysates in which PELP1 and TTLL4 was overexpressed. PELP1 and histone H3 were detected in the immunoprecipitated complex by Western blotting. The interaction of PELP1 with histone H3 was enhanced in the presence of wild-type TTLL4, but not in the presence of enzyme-dead TTLL4. D, acetylation level of histone H3 was significantly increased in TTLL4 knockdown (siTTLL4) in PK-1 cells, compared with cells transfected with the control (siEGFP).
was coimmunoprecipitated with LAS1L-Flag when both expression vectors were cotransfected. Concordantly, Western blot analysis using anti-Flag antibody indicated that LAS1L-Flag was coimmunoprecipitated with PELP1-HA when both expression vectors were coexpressed. Interaction between PELP1 and SENP3 was also validated by similar experiments (Fig. 5B). These two PELP1-interacting proteins (LAS1L and SENP3) as well as PELP1 itself were included as members of the MLL1-WDR5 complex (24, 25), which was reported to have both histone methyltransferase activity as well as acetyltransferase activity. Then, we examined the effect of PELP1 polyglutamylation on the interaction of PELP1 with LAS1L, SENP3, and histone H3. We transfected expression vectors for PELP1-HA, LAS1L, and SENP3 together with wild-type or enzyme-dead (E906A) TTLL4 expression vectors. Subsequently, a protein complex was immunoprecipitated from cell extracts by anti-HA antibody (middle) or anti-Flag antibody (bottom), respectively. Western blotting using anti-HA antibody indicated that PELP1-HA was coimmunoprecipitated with LAS1L-Flag when both expression vectors were cotransfected (left). Western blotting using anti-Flag antibody indicated that PELP1-HA was coimmunoprecipitated with LAS1L-Flag when both expression vectors were coexpressed (right). B, PELP1-HA vector and/or SENP3-Flag vector were cotransfected into COS-7 cells, and a protein complex containing PELP1-HA and/or SENP3-Flag was immunoprecipitated from the cell extracts by anti-HA antibody (middle) or anti-Flag antibody (bottom). Western blotting using anti-HA antibody indicated that PELP1-HA was coimmunoprecipitated with SENP3-Flag when both expression vectors were cotransfected (left). Western blotting using anti-Flag antibody indicated that SENP3-Flag was coimmunoprecipitated with PELP1-HA when both expression vectors were coexpressed (right). C, immunoprecipitation from the cell lysates when wild-type or enzyme-dead TTLL4 (E906A) was overexpressed (left) or when TTLL4 was knocked down (right). The binding affinity of PELP1 to LAS1L was increased when wild-type TTLL4 was overexpressed (right) and was reduced when TTLL4 was knocked down (left). D, the binding affinity of PELP1 to SENP3 was increased when enzyme-dead TTLL4 (E906A) was overexpressed (left) or when TTLL4 was knocked down (right). The binding affinity of PELP1 to SENP3 was decreased when wild-type TTLL4 was overexpressed (right) and was increased when TTLL4 was knocked down (left).
modulated by TTLL4 might be a regulator of the interaction of PELP1 with LAS1L and SENP3 as well as histone H3 in the MLL1-WDR5 complex.

Discussion

In this study, we showed that TTLL4 was abundantly expressed in PDAC cells, but was not expressed in any of the adult normal organs except the testis. Because our data clearly indicated that the polyglutamylase activity of TTLL4 could be important in its oncogenic property, the development of drugs specifically inhibiting its activity should be a good therapeutic approach to achieve very specific cytotoxicity to pancreatic cancer cells with minimum risk of adverse effects to normal organs.

We also showed that TTLL4 could play an important role in pancreatic carcinogenesis through polyglutamylation of cellular proteins, including an oncogenic scaffold protein PELP1 that could interact with several key molecules of cancer cell proliferation or survival, such as Src, estrogen receptor, p85 subunit of phosphoinositide-3-kinase, and histones (14, 17, 22, 23). Polyglutamylation of PELP1 could change its scaffold property and affect the strength of these signaling pathways. In fact, our study showed that polyglutamylation of PELP1 could have a significant influence on its affinity to interact with histone H3 and subsequently affect the acetylation level of histone H3. The glutamate-rich stretch region of PELP1 was shown to bind to the hypoacetylated histone H3 protein and protect it to be processed as a substrate of histone acetyltransferase (21). Although how PELP1 polyglutamylation affects its binding affinity to histone H3 is unclear, PELP1 polyglutamylation in its glutamate-rich stretch region is speculated to affect its structure significantly because of alteration of the electric ionic charge of glutamate, the structure of PELP1, and then the binding affinity of PELP1 to histone H3 and other complex members, resulting in reduction of histone H3 acetylation.

In addition to histone H3, we identified additional PELP1-interacting proteins, LAS1L and SENP3. These PELP1-interacting proteins as well as PELP1 were considered as components of the MLL1-WDR5 supercomplex, which could coordinate chromatin remodeling through both methyltransferase and acetyltransferase activities to histone proteins (24, 25). The MLL1-WDR5 supercomplex consists of many components and its compositional changes might regulate the enzyme activity of histone modifications as well the selection of its modification sites (24, 25). Our study showed that polyglutamylation of PELP1 could affect the affinity of PELP1 to its interacting proteins such as LAS1L and SENP3, and it might change the composition or the conformation of the MLL1-WDR5 supercomplex, resulting in changes in histone modification and the chromatin structure. Epigenetic changes underlying chromatin remodeling could play important roles in tumorigenesis and cancer phenotypes through posttranslational modifications of histones such as acetylation and methylation and the subsequent transcriptional regulation of tumor suppressor genes or oncogenes (24, 26). TTLL4 overexpression and subsequent PELP1 polyglutamylation might exert an influence on the composition or conformation of highly organized chromatin remodeling complexes, such as the MLL1-WDR5 complex, and might be involved in cancer cell proliferation in part through histone modifications and chromatin remodeling.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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