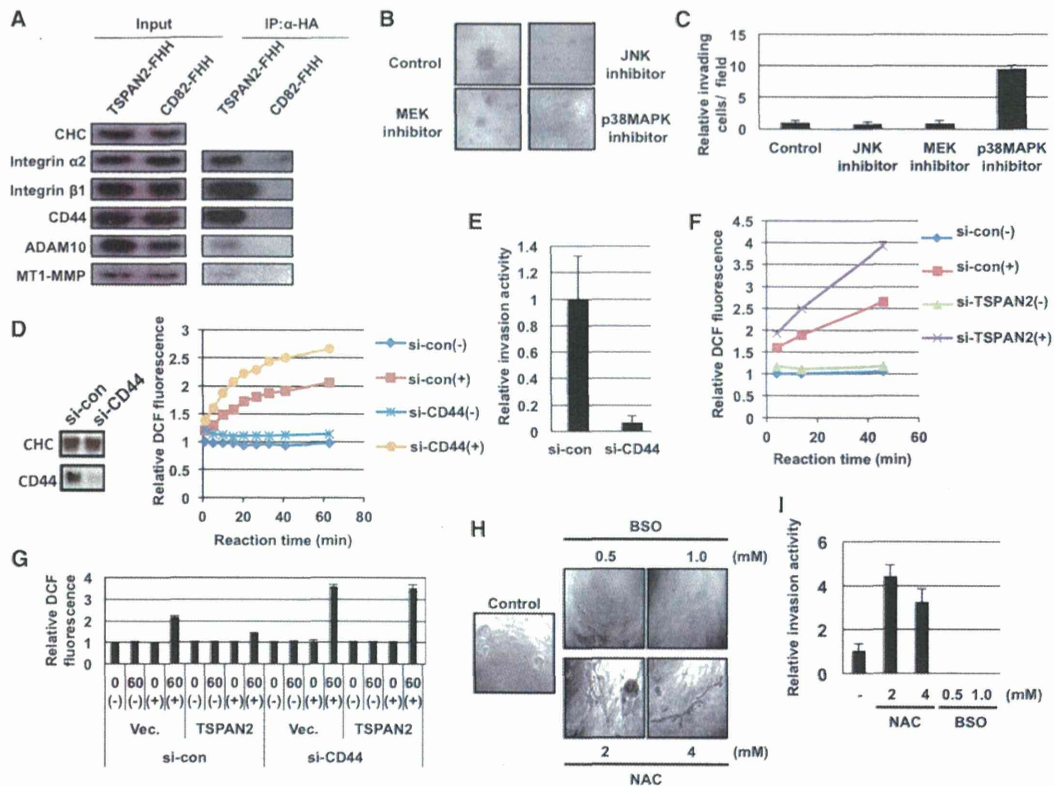

Figure 6. TSPAN2 Regulates the Production of Reactive Oxygen Species in Cooperation with CD44 for the Inhibition of Cell Motility and Invasion

(A) Identification of binding partners with TSPAN2. Lysates from cells stably expressing TSPAN2-FHH or CD82-FHH were sequentially immunoprecipitated with anti-FLAG and anti-HA antibodies, followed by immunoblotting with the indicated antibodies.

(B and C) Inhibition of the p38MAPK pathway, but not JNK and ERK pathways, enhances invasive activity in TSPAN2-depleted SAEC^{LSK}. Invasive activity of SAEC^{LSK} stably expressing shTSPAN2 was measured using 3D-Matrigel invasion assay. Photograph of invading cells under light microscopy (B). Quantification of invading cells (C).

(D) Increased ROS level by CD44 knockdown. Knockdown efficiency of si-CD44 was determined by immunoblotting (left panel). Clathrin heavy chain (CHC) is shown as a loading control. Cells were incubated with 10 μM DCFH-DA for 10 min at 37°C and treated with or without 500 μM H_2O_2 for the indicated periods. The fluorescence was measured using a fluorescence spectrophotometer and relative fluorescent values to the control are shown (right panel).

(E) Decreased invasive activity of SAEC^{LSK} by CD44 knockdown. Cells were transfected with si-CD44 and analyzed by cell invasion assays using cell-culture inserts.

(F) Increased ROS in SAEC^{LSK} by TSPAN2 knockdown. Cells were transfected with si-TSPAN2 and used for measurement of intracellular ROS.

(G) Cooperation of TSPAN2 with CD44. Suppressive effect of intracellular ROS production by TSPAN2 expression in SAEC^{LSK} was abolished by CD44 knockdown. Cells with or without ectopic expression of TSPAN2 were transfected with si-CD44 and fluorescence in cell lysates from cells treated with DCFH-DA in the absence (−) or presence (+) of 500 μM H_2O_2 for 60 min was measured.

(H and I) Low redox potential advantages of the invasive phenotype in SAEC^{LSK}. Cells were embedded in Matrigel in the absence or presence of NAC or BSO at the indicated concentrations, observed 10 days after incubation under a light microscope (H) and invading cells were quantified (I). Error bars indicate SD of three independent measurements.

See also Figures S5 and S6.

expression (Figure S5C), suggesting that TSPAN2 regulates alternative pathways to promote motility and invasion. To elucidate the signaling pathways by which TSPAN2 enhances tumor invasiveness, we next examined the effect of specific inhibitors of signaling pathways, including the JNK pathway, ERK/MEK pathway, and p38MAPK pathway, on invasiveness in TSPAN2-depleted SAECs because these pathways have been implicated in lung tumor malignancy (Kim and Choi, 2010; Malemud, 2007). The 3D Matrigel-invasion assay showed significant enhancement of invasiveness in TSPAN2-depleted SAEC^{LSK} by treatment with the p38MAPK inhibitor but not JNK and MEK inhibitors (Figures 6B and 6C). p38MAPK is activated by reactive oxygen species (ROS) (Zhang et al., 2003), and the CD44-mediated pathway is known to be involved in the defense against oxidative stress (Ishimoto et al., 2011; Zhao et al., 2008). Based on our data that TSPAN2 regulates the p38MAPK pathway and interacts with CD44, we examined whether TSPAN2 is involved in CD44-mediated ROS production in SAEC^{LSK}. CD44 knockdown exhibits increased ROS production following H_2O_2 treatment (Figure 6D) and a decreased invasive phenotype (Figure 6E),

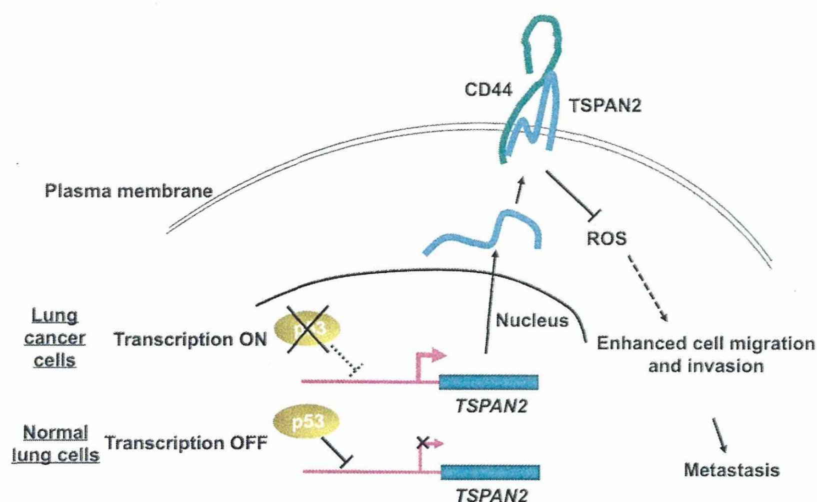

Figure 7. Proposed Model for the Mechanism of Invasiveness and Motility during Lung Tumor Progression

Model for the mechanism of invasiveness and motility elicited by p53 inactivation during lung tumor progression. In cells harboring wild-type p53, the expression level of TSPAN2 is maintained by p53; however, in p53 mutant cells, TSPAN2 is derepressed and binds CD44 to suppress ROS production. Reduced ROS by TSPAN2 induction enhances cell motility, invasion, and metastasis.

suggesting that CD44 has a ROS-scavenging function in lung cancer cells to promote invasiveness and cell motility. We next examined the effect of TSPAN2 on ROS production following H_2O_2 treatment of $SAEC^{LSK}$. Intriguingly, ROS production in TSPAN2-depleted cells treated with H_2O_2 was significantly higher than in control cells (Figure 6F), and decreased ROS production by forced expression of TSPAN2 was canceled by CD44 knockdown (Figure 6G). To further address the relationship between TSPAN2 and CD44 on the mechanism for ROS production, we first examined whether endogenous TSPAN2 interacts with CD44 by immunoprecipitation with anti-TSPAN2 antibody. Immunoblot analysis of immunoprecipitates showed that the interaction of TSPAN2 with CD44 was detected (Figure S6A). Next, to determine which region of TSPAN2 is required for the interaction with CD44, we constructed various expression vectors for deletion mutants of TSPAN2 (Figure S6B) and determined that the region designated as the cytoplasmic C-terminal tail in TSPAN2 protein (TSPAN2ΔC-tail) was a major binding site to CD44 (Figure S6C). Migration and invasion assays using cell-culture inserts showed that the expression of TSPAN2ΔC-tail (Figure S6D) did not enhance cell motility (Figure S6E) or invasion (Figure S6F) compared with wild-type TSPAN2. Furthermore, TSPAN2ΔC-tail had little or no ability to reduce intracellular ROS production (Figure S6G). Thus, these data suggest that the interaction of TSPAN2 with CD44 is crucial for maintenance of the intracellular ROS level. Indeed, this idea is supported by the fact that p53 knockdown decreases the oxidation level in SAECs (Figure S6H). Finally, we tested the effect of intracellular redox modulators on invasiveness into $SAEC^{LSK}$. We first determined appropriate concentrations of N-acetyl-cysteine (NAC), an antioxidant agent, and buthionine sulfoximine (BSO), a producer of oxidative stress in cells, by measurement of intracellular ROS activity. The level of intracellular ROS production was decreased by treating cells with NAC at a concentration of 2 mM, as efficiently as that of cells ectopically expressing TSPAN2 (Figure S6I). On the other hand, the level of intracellular ROS production was increased by treating cells with BSO at a concentration of 0.5 mM, as efficiently as that of TSPAN2-depleted cells (Figure S6J). Under these conditions, treatment of cells with NAC enhanced the invasive phenotype; in contrast, oxidative stresses elicited by BSO significantly decreased invasive activity (Figures 6H and 6I). These data demonstrate that TSPAN2 derepressed by p53 inactivation enhances cell motility and invasiveness through CD44 to scavenge intracellular ROS (Figure 7).

DISCUSSION

Based on clinical studies of lung cancer, p53 mutations are more often observed in invasive tumors than in noninvasive tumors (Iwakawa et al., 2008; Robles and Harris, 2010), implying that p53 regulates cancerous cell invasion and metastasis. Several critical factors and mechanisms for these phenomena have been identified, and several lines of recent evidence have shown that p53 regulates invasion and cell motility, which are associated with aggressive and metastatic phenotypes of cancer cells (Muller et al., 2011). In the present study, we established a mimetic system with genetic alternations in epithelial cells during lung carcinogenesis and found that KRAS activation was linked to EMT and that KRAS activation-induced EMT was not sufficient to give rise to invasiveness and motility, rather p53 inactivation, by which EMT-like phenotype was not induced, is the main cause of enhanced invasiveness and motility. These results are consistent with the fact that p53 inactivates during the transition from noninvasive to invasive tumors in lung ADCs. The relationship between EMT and tumor progression, including invasiveness and motility, is still controversial (Biddle and Mackenzie, 2012; Geiger and Peeper, 2009; Yilmaz and Christofori, 2009). Our present data support that p53 inactivation, but not EMT, is essential for invasiveness and motility, though these data cannot exclude the possibility that p53 inactivation cooperatively controls invasiveness and motility with EMT.

TSPAN2 derepressed by p53 inactivation is involved in lung cancer progression. Our data demonstrate that TSPAN2 expression is significantly higher in lung ADCs possessing mutant p53 than in those bearing wild-type p53 and that the basal level of p53 is sufficient to repress the expression of TSPAN2 mRNA. In addition, based on gene expression profiling databases in HLM and MSK (both are recognized as most reliable databases for gene expression profiling) (Shedden et al., 2008), high TSPAN2 expression is significantly associated with poor

prognosis in lung ADCs. Genes derepressed by p53 inactivation, such as MDR1 (Johnson et al., 2001), CD44 (Godar et al., 2008), and TCTP (Amson et al., 2012), have so far been identified, and we found TSPAN2 to be a derepressed gene by p53 loss under unstressed conditions. Unfortunately, the mechanism of transcriptional derepression of TSPAN2 by p53 inactivation is not simple, and we did not clarify that TSPAN2 is a direct p53 target gene, although a reporter assay using the TSPAN2 promoter region (−2,000–+36) and ChIP analysis by scanning the proximal TSPAN2 promoter region between −2,000 and +1,000 using various specific primers were carried out (data not shown). One reason is that p53 may inhibit the recruitment of some coactivators of TSPAN2 expression by binding p53 to these coactivators (Böhlig and Rother, 2011). Alternatively, TSPAN2 expression may be regulated by interference with the distal enhancer activity of p53 because the expressions of many genes suppressed by p53 in embryonic stem (ES) cells are affected by *cis* element far from promoter regions (Li et al., 2012). Although further investigation will be necessary to elucidate how p53 regulates TSPAN2 expression, we have demonstrated that TSPAN2 is a factor responsible for human lung cancer cell motility and invasion. In the future, antibodies and nucleic acid aptamers against TSPAN2 may be utilized as drugs for lung cancer therapy.

TSPAN2 is a four transmembrane-spanning protein belonging to the tetraspanin family and binds to various membrane proteins to modulate intracellular signaling (Hemler, 2008; Wang et al., 2011). Here, we found intriguing data that TSPAN2 is involved in lung cancer progression, including invasiveness, motility, and metastasis, and binds CD44 to suppress intracellular oxidative stresses. It has been reported that p53 controls the redox-oxidation state through transcription-dependent and -independent regulation of pro-oxidant and antioxidant enzymes (Liu et al., 2008). However, this regulation is presumably dependent on cellular contexts, and, if p53 inactivation occurs in lung tumor progression, lung tumor cells may evade oxidative stress, because stem cells protect against oxidative damage to maintain the cell population (Kobayashi and Suda, 2012). This idea is supported by the fact that p53 knockdown decreased the oxidation level in our system using human lung epithelial cells and that treatment of cells with NAC, an antioxidant agent, enhanced the invasive phenotype; in contrast, oxidative stresses elicited by BSO decreased invasion. According to previous reports (Ishimoto et al., 2011), the variant form of CD44 (CD44v) functions to scavenge oxidative stress through the interaction with cysteine transporter (xCT) on the transmembrane to stabilize xCT, and subsequently increases intracellular glutathione to maintain cancer-initiating cell-like properties in gastrointestinal cancers (Ishimoto et al., 2011); however, it has recently been reported that the standard form of CD44 (CD44s) is involved in the clinical outcome of patients with lung ADCs (Ko et al., 2011). Therefore, CD44s may also function in oxidative defense through the interaction with TSPAN2 because TSPAN2 knockdown increased the intracellular oxidative level, enhancement of the redox state elicited by TSPAN2 overexpression was abolished by CD44 knockdown, and TSPAN2 lacking CD44-binding capacity had no ability to suppress intracellular ROS production. What kind of CD44 isoforms TSPAN2 works with and how the interaction of CD44 with TSPAN2 transduces signals for oxidative defense will need further investigation, but TSPAN2 will be a potential target for cancer therapy by preventing the interaction with CD44. Because the decrease of intracellular ROS via the CD44 system contributed to the maintenance of cancer-initiating cell-like properties in gastrointestinal cancers, TSPAN2 may play a role in the acquisition of cancer-initiating cell-like properties in lung ADCs. Whether TSPAN2 is required for the maintenance of cancer-initiating cell-like properties will be the next significant question.

In conclusion, TSPAN2 derepressed by p53 inactivation is linked to cancer invasiveness and metastasis in lung ADCs and enhances intracellular redox potential in cooperation with CD44 to protect tumor cells from oxidative damage. Moreover, EMT-like features are insufficient for showing invasive and motile phenotypes, and the p53-TSPAN2 axis is a key pathway to the aggressiveness of lung ADCs. Our data presented here will provide new insights into a relevant mechanism by which p53 inactivation promotes tumor invasion and motility during lung carcinogenesis.

EXPERIMENTAL PROCEDURES

For more details, see Supplemental Information.

Establishment of Immortalized SAECs

Plasmid Construction

cDNAs encoding human TSPAN2 and CD82 were amplified by PCR, and DNA fragments were subcloned into pLentiV5/DEST (Invitrogen). DNA fragments encoding $KRAS^{V12}$ were amplified by PCR using cDNA pools from H441 cells and subcloned into pBABE-puro (Addgene). For deletion constructs of TSPAN2, two-step PCR-based targeted mutagenesis was used (Armengaud and Jouanneau, 1993). Details about constructs and primer sequences can be found in Supplemental Experimental Procedures.

RNAi Experiments

Cells were transfected with the small interfering RNAs (siRNAs) using Lipofectamine 2000 or Lipofectamine RNAi MAX (Invitrogen), according to the manufacturer's instructions. Details about siRNA sequences and shRNA constructs can be found in Supplemental Experimental Procedures.

In Vitro Cell Migration and Invasion Assays

Migration and invasion assays were performed using 24-well transwell chambers with polyethylene terephthalate filters of 8 μm pore size with or without Matrigel (Becton Dickinson), according to the manufacturer's instructions. Details about these methodologies can be found in Supplemental Experimental Procedures.

RNA Isolation, Quantitative Real-Time PCR, and RT-PCR

Total RNA from cells was isolated using an RNeasy kit (QIAGEN) and reverse-transcribed with Super Script II First-Strand Synthesis System for RT-PCR kit (Invitrogen). Quantitative real-time RT-PCR was carried out in a ABI Prism 7900HT Sequence Detection system (Applied Biosystems). Semiquantitative RT-PCR was carried out in a PCR thermal cycler (MJ Research), and the PCR products were subjected to electrophoresis on 2% agarose gels. Details about these methodologies and primer sequences can be found in Supplemental Experimental Procedures.

Microarray Experiment

Total RNA was purified using an RNeasy kit (QIAGEN) and subjected to microarray experiment using a GeneChip Human Genome U133 plus 2.0 arrays (Affymetrix) according to the manufacturer's instructions. Details about these methodologies can be found in Supplemental Experimental Procedures.

Expression Data in Patients' Samples

The relationship of TSPAN2 expression and the p53 mutation status was analyzed using a data set of patients' lung cancer samples (n = 41: p53

mutation, 18; no p53 mutation, 23) (Ding et al., 2008), and information about this data set is available at Oncomine (https://www.oncomine.org/).

Immunoblot Analysis and Immunoprecipitation

Immunoblotting and immunoprecipitation were performed as described previously (Enari et al., 2006; Ohata et al., 2013), and FLAG-HA-His-tagged TSPAN2 and CD82 in cells were sequentially immunoprecipitated with anti-FLAG antibody (M2)-conjugated beads (Sigma-Aldrich) and anti-HA antibody-conjugated beads (Roche). Endogenous TSPAN2 was immnoprecipitated with anti-TSPAN2 (Avnova) antibody-conjugated Protein G Sepharose 4B (GE Healthcare) and normal IgG was used as a control. Details about these methodologies can be found in Supplemental Experimental Procedures.

Three-Dimensional Matrigel Invasion Assay

Ninety-six transwell plates were used for the 3D Matrigel invasion assay. Cells (1×10^3) were suspended in a mixture of culture medium and Matrigel (Becton Dickinson) (1:1, v/v) and layered onto solidified Matrigel. For 6–8 days after culturing, colonies were observed under a light microscope or confocal microscope, representative pictures of each experiment were taken, and cell numbers morphologically exhibiting the invasive outgrowth phenotype were counted.

Time-Lapse Motility Assay

Cells (2×10^5) were plated in 6-well plates 1 day before assay, and cell images under a light microscope were obtained every 15 min for 16 hr. Velocity of the cell movements was analyzed using AxioVision Version 4.8 Cell tracking software (Carl Zeiss).

Measurement of Cellular Reactive Oxygen Species

Cells (5×10^4) in suspension were incubated with 10 μM DCFH-DA (CELL BIOLABS) for 10 min at 37°C, washed with PBS, and treated with or without 500 μM H_2O_2. The fluorescence corresponding to the cellular ROS level was monitored in a black plate using a fluorescence spectrophotometer at excitation of 480 nm and emission of 530 nm.

In Vivo Metastatic Assay

Cells stably expressing a luciferase gene were inoculated intravenously through the tail vein of 7-week-old female NOD-scid, IL-2Rγ-null (NOG) mice. NOG mice were obtained from the Central Institute for Experimental Animals (Kawasaki, Japan). Tumor growth in the lungs was monitored using the IVIS imaging system (Xenogen), and tissue samples were stained with hematoxylin and eosin. Details about these methodologies can be found in Supplemental Experimental Procedures. All animal care followed the Guideline for Animal Experiments at the National Cancer Center and was approved by the institutional committee for Ethics of Animal Experimentation.

Statistical Analysis

Data are represented as the means ± SD of three or more independent experiments. Statistical analysis was performed by Student's t test and unpaired t test at significance levels of $p < 0.05$.

ACCESSION NUMBERS

The GEO accession number for the row and processed microarray data presented in this paper is GSE47436.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.03.027.

ACKNOWLEDGMENTS

We are grateful to Dr. Teruhiko Yoshida (National Cancer Center Research Institute, Japan) and Ms. Sachiyo Mimaki (National Cancer Center Research Institute, Japan) for expression profiling. This work was supported in part by Grants-in-Aid from Ministry of Education, Culture, Sports, Science and Technology (MEXT) for Scientific Research on Priority Areas (17013088) and for Scientific Research (B) (21370083) from the Ministry of Health, Labour and Welfare (MHLW) for the third-term Comprehensive 10-year Strategy for Cancer Control (CSCC), the National Cancer Center (NCC) Research and Development Fund (M.E., 23-B-27[23-A-37]; Core Facility, 23-A-7), and a Grant-in-Aid for JSPS Fellows (10J02622) (C.O.).

Received: July 22, 2013
Revised: February 10, 2014
Accepted: March 10, 2014
Published: April 10, 2014

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I．がんゲノム解析の治療への応用

4．新規がん抑制遺伝子*PHLDA3*による Akt経路の制御機構と治療への展開
―膵神経内分泌腫瘍の個別化医療開発をめざして

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多くのがんで機能喪失が認められるp53経路の研究は，がん制御機構を解明するうえで重要である．われわれはp53の機能解析を通して，がん遺伝子*Akt*の抑制因子をコードする新規がん抑制遺伝子*PHLDA3*を同定した．*PHLDA3*遺伝子は，膵臓の神経内分泌腫瘍（neuroendocrine tumor：NET）において高頻度にヘテロ接合性の喪失LOHが認められ，その頻度は膵NETのがん抑制遺伝子として有名な*MEN1*遺伝子と同等であった[1]．本稿では，膵NETの新規がん抑制因子PHLDA3によるAkt抑制を介した膵NET抑制機能について解説した後，PHLDA3研究を応用した新規がん診断・治療法の開発について考察する．

はじめに

1）がん抑制遺伝子p53の機能

*p53*遺伝子はがんの約半数において変異や欠失が認められる．また，*p53*遺伝子が野生型である場合，p53の活性を抑制する因子や，p53の標的遺伝子が不活性化していることが報告されている[2]．このように*p53*は重要ながん抑制遺伝子であり，がんを理解するうえでp53研究は欠くことができない．

p53は転写因子であり，DNAダメージや低酸素状態，がん遺伝子の活性化などのさまざまなストレスに応答し特定の塩基配列に結合して，標的遺伝子の転写を誘導する．p53は標的遺伝子を介して細胞周期停止や細胞死を誘導し，細胞のがん化を防いでいる．p53タンパク質は，転写活性化ドメインを含むN末端ドメイン，DNA結合ドメイン，C末端ドメインから構成される（図1A）．がんにおいて*p53*遺伝子にみられる変異の多くがDNA結合ドメインに生じることが，p53の転

[キーワード＆略語]
p53，PHLDA3，Akt，膵神経内分泌腫瘍，エベロリムス

Gsk3β：glycogen synthase kinase 3-beta
Mdm2：mouse double minute 2
mTOR：mammalian target of rapamycin
PHLDA3：pleckstrin homology-like domain, family A, member 3
PTEN：phosphatase and tensin homolog
S6：ribosomal protein S6
S6k：ribosomal protein S6 kinase

Repression of the Akt pathway by a novel tumor suppressor gene *PHLDA3* towards development of tailor-made therapies for pancreatic neuroendocrine tumors

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