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PHLPP1 regulates contact inhibition by dephosphorylating Mst1 at the inhibitory site

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ABSTRACT

Contact inhibition has been largely elusive despite that a loss of contact inhibition is a critical event for cancer development and progression. Here, we report that PHLPP1 is a binding protein for Mst1 and it modulates the Hippo pathway by dephosphorylating Mst1 at the inhibitory Thr^{387} of Mst1. Yap1 was localized predominantly in the nucleus but marginally in the cytoplasm in HeLa cells under sparse conditions, whereas the functional protein was more directed to sequestration in the cytoplasm under dense environments. Furthermore, loss of PHLPP1 resulted in a failure of the apoptotic control. It is interesting that down-regulated expression of PHLPP1 appears to mimic the loss of contact inhibition, a hallmark of cancer.

1. Introduction

Normal cells regulate cell division in a cell density-dependent manner and, when in contact with adjacent cells, they halt the operation of cell proliferative machinery. This 'contact inhibition' of cell growth is universally observed for most cell types cultured *in vitro*. However, transformed cells are armed with molecular systems to break the regulatory machinery, thereby showing unchecked cell proliferation even under confluent states. This uncontrolled cell growth is the basis for a variety of subsequent malignant behaviors in cancer. Although the underlying concept was first coined several decades ago [1], relatively little is known about the molecular mechanism underlying the loss of contact inhibition by transformed cells. Because cellular contact is predictably perceived by cell-surface molecules, significant attention has been paid to several adhesion molecules on the plasma membrane including E-cadherin [2]. Direct evidence was provided by Croix et al. that neutralization by anti-E-cadherin antibody stimulated cancer cell proliferation and forced expression of E-cadherin caused contact-dependent growth inhibition [3]. Opposed to studies that have focused on cellular contact, little progress has been made regarding how the density signal is transduced through the cytoplasm to the nucleus.

Recently, the Hippo pathway has been shown to be the core pathway by which cell proliferation and size are coordinated following perception of external cellular cues [4,5]. Since it was first defined in *Drosophila* by genetic mosaic screens for tumor suppressor genes [6], the pathway has been also established in human by identifications of Mst1/2, Sav1, Lats1/2, and Mob1 [7]. The Hippo signal is subsequently transduced to Yap/Taz, the Yki orthologs, which normally translocate to the nucleus and act as a transcriptional coactivator to achieve an anti-apoptotic and proliferative outcome in conjunction with TEAD1-4 [8,9]. However, the signaled Yap is phosphorylated and destined for cytosolic sequestration and degradation, resulting in arrest of cell proliferation and apoptosis [10]. Mst1, also known as STK4 and Krs-2, is located at the core of the Hippo pathway, and shows an altered kinase activity depending on the phosphorylation states at Thr^{183} and Thr^{387} [11]. The two sites reciprocally regulate the kinase activity: the former site potentiates the Mst1-mediated signals and the latter plays an inhibitory role [12]. This suggests that the fate of Yap is closely linked to the activation/deactivation status of Mst1. Given the critical role of Mst1 in cellular regulation, it is anticipated that there is a certain repertoire of phosphatases and kinases for Mst1, and thus identification of the repertoire and its roles may provide deeper understanding of mechanisms about loss of contact inhibition by cancer cells as well as options for cancer treatments.

Herein, we identified pleckstrin homology domain leucine-rich repeat protein phosphatase 1 (PHLPP1) as an interacting protein for Mst1 in HeLa cells using photo-reactive amino acids. Known to have tumor suppressive activity, PHLPP1 was responsible for dephosphorylation of Thr^{387} of Mst1 and, subsequently, deactivation of Yap1 in HeLa cells under high-density conditions. It is tempting to speculate that PHLPP1 plays a key role in regulation of cellular contact inhibition and is central to the loss of contact inhibition by cancer cells.

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2. Materials and methods

2.1. Incorporation of photo-reactive amino acids into HeLa cells

HeLa cells were grown in methionine and leucine-depleted RPMI1640 media and then supplemented with either photo-reactive amino acids plus natural amino acids at 0.5 mM or natural amino acids alone as a control. Cells in PBS were subjected to UV illumination at 365 nm for 10 min prior to harvesting. Protein extracts were obtained by several rounds of brief sonication and centrifugation at 10,000*g* for 20 min.

2.2. Identification of Mst1-interaction proteins

The protein spots of interest were excised, tryptic-digested using modified porcine trypsin (Promega), and dried *in vacuo*. A mass analysis was performed using a Nano-LC/MS system consisting of an ultimate HPLC system and a Q-TOF mass spectrometer (Waters) equipped with a nano-ESI source. The resulting peak lists were searched against NCBInr 20120319 (human) using a Mascot Daemon search engine (Matrix Science). Mascot was used with monoisotopic mass selected, a precursor mass tolerance of ± 1.5 Da and a fragment mass tolerance of ±0.8 Da. With regard to acceptance criteria for protein identification, proteins that were identified with 2 or more unique peptides with $p < 0.05$ (a peptide score $\geqslant 38$) were selected.

2.3. Western blot analysis

Western blot analysis was performed with primary antibodies raised against Mst1 (Abcam), Mst1 phospho-Thr183 (Cell Signaling Technology), Mst1 phospho-Thr387 (Upstate Biotechnology), Yap1 (Abcam), Yap1 phospho-S127 (Abcam), Lats1 (Cell Signaling Technology), Lats1 phospho-S909 (Cell Signaling Technology), PHLPP1 (Bethyl Laboratories) and β-actin (Abcam). After being treated with HRP-labeled secondary antibodies (Cell Signaling), membranes were allowed to react with ECL™ substrate solution (GE Healthcare) and exposed to an X-ray film for 1–2 min. The band intensity was calculated using ImageJ software.

2.4. Immunofluorescence

Cells were trypsinized and grown to various confluences on cover slips. Cell mono-layers were washed with PBS, fixed with 4% paraformaldehyde, and completely washed with excess PBS-Tween 20 (0.02% v/v). If necessary, cells were permeabilized with a commercial buffer containing 0.01% (v/v) TX-100 (Life Sciences). After treatment with primary antibodies, cells were washed extensively with PBS buffer and treated with secondary antibody conjugated with TRITC or FITC fluorescent dye (Cell Signaling Technology. Fluorescence was monitored by confocal fluorescence microscopy (LSM 510 Meta, Zeiss). Representative images were chosen and digitally recorded at a defined sensitivity and magnification.

2.5. Soft-agar assay

Cells were dispersed in 0.3% (w/v) soft-agar solutions and placed in 96-well plates. Agar solutions were allowed to solidify and cells were grown for up to 2 weeks in the presence of RPMI media supplemented with 10% (v/v) FBS. The size and number of colony were periodically measured on a microscope.

2.6. Stable and transient transfection

Human Mst1 and PHLPP1 vector constructs were transfected into HeLa cells using Lipofectamine reagents (Invitrogen). Stable transfectants of cells with silenced expression of the proteins were also established by transfecting the cells with shRNA vectors (Sigma). The insert DNA sequence for RNA interference of PHLPP1 gene was either 5′-CCGGCCGAGCTGTTTAACAAATAAACTCGAGTTTATTTGTTAAACAGCTCGGTTTTTG-3′ or 5′-CCGGCCTGATAGTATCATCTGTGAACTCGAGTTCACAGATGATACTATCAGGTTTTTG-3′. The insert DNAs were cloned into the p-silencer U6 vector (Life Technologies). Stable transfectant colonies were selected by an RT-PCR and immune-blot analysis.

2.7. Statistic analysis

Statistical differences between groups were determined by a Student's *t*-test. Values of $p < 0.05$ were considered as significant.

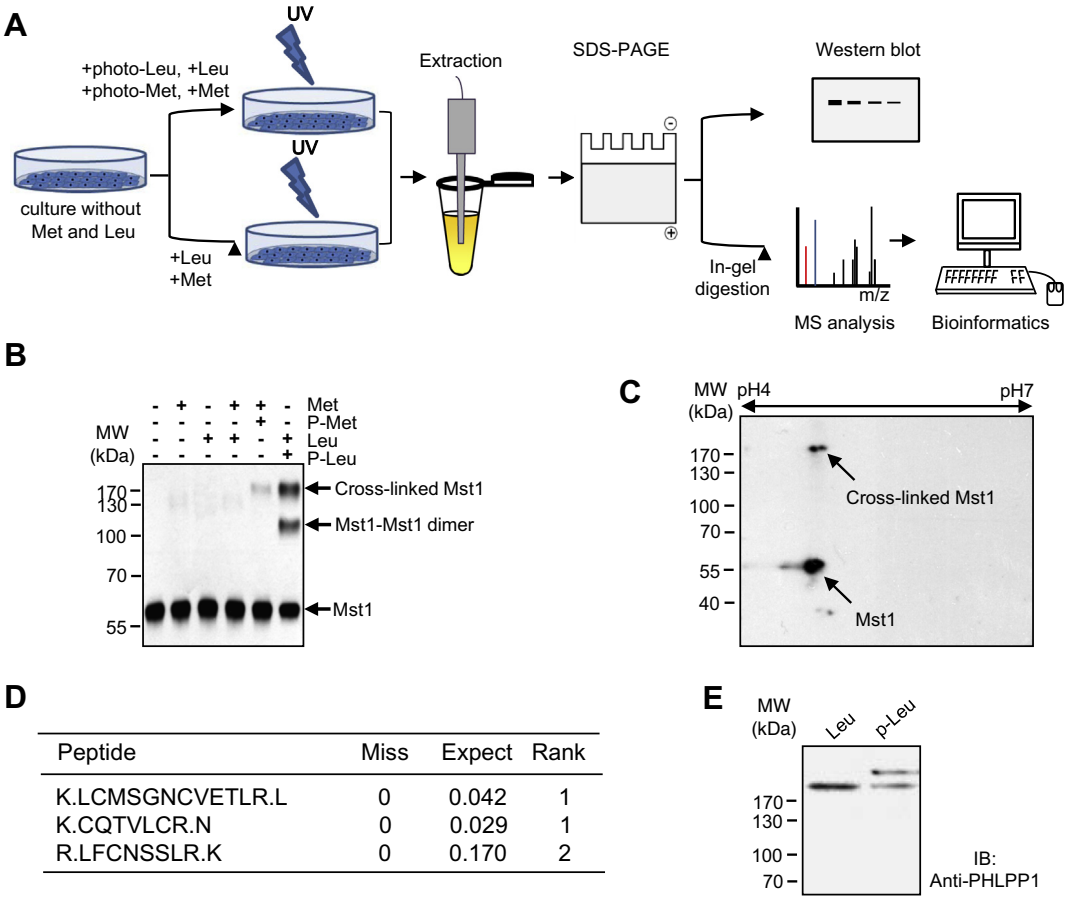
3. Results

3.1. PHLPP1 is a binding partner for Mst1 in HeLa cells

Rather than relying on conventional immunoprecipitation or pull-down assays, we attempted to apply a more robust tool to identify any binding protein for Mst1 as a regulator for the Hippo pathway. We used photo-reactive amino acids to covalently link associated proteins [13]. Illumination of UV light leads to an accumulation of covalently linked binding partners to a detectable level and this strategy is thought to be compatible with an MS-based identification.

Photo-leucine and photo-methionine were added in competition with their natural counterparts to culture media of HeLa cells that had been cultured for a day in the absence of methionine and leucine. After grown to confluence, cells were subjected to UV illumination at 365 nm at an interval where cell viability was not affected. The extracted protein preparations were resolved on SDS–PAGE gels and subjected to a western blot analysis using an anti-Mst1 antibody. Protein spots in a stained gel as located by Western blot were picked up and protein(s) were identified by a mass spectrometric analysis (Fig. 1A). We observed an accumulation of a cross-linked product that showed a rather low migration rate in SDS–PAGE gels (Fig. 2B). Depending on the illumination time and the distance between the light source and cells, additional bands were observed with various molecular masses and some of them were identified as the Mst1–Mst1 dimeric conjugate and another binding protein adducts (data not shown). Nonetheless, the large band with estimated mass over 180 kDa was invariably observed, as in Fig. 2B. Incorporation of photo-leucine followed by UV illumination was more efficient to cross-link an Mst1-interacting partner than using photo-methionine, suggesting that leucine may be involved in an interaction possibly near binding regions of either Mst1 or PHLPP1. To minimize any possible interference of identification by junk proteins, the cross-linked product was resolved on a second-dimensional SDS–PAGE gel (Fig. 1C). We used a pH4-7 IEF strip because the theoretical p*I* value of Mst1 is 4.97 and found a clear cross-linked product.

Results from the mass spectrometric analysis and bioinformatics revealed that PHLPP1 was the interacting protein for Mst1 in HeLa cells (Fig. 1D). Three independent peptides were identified without any miss cleavage and two of the peptides were identified with *p*-values of less than 0.05. Western blot analysis using an anti-PHLPP1 antibody confirmed an interaction between Mst1 and PHLPP1 (Fig. 1E).



Peptide	Miss	Expect	Rank
K.LCMSGNCVETLR.L	0	0.042	1
K.CQTVLCR.N	0	0.029	1
R.LFCNSSLR.K	0	0.170	2

Fig. 1. Identification of PHLPP1 as an Mst1-interaction protein in HeLa cells. (A) Strategy for identification of Mst1-binding protein(s). Photo-leucine and photo-methionine and their natural counterparts were incorporated into culture media of HeLa cells, after which cells were UV-illuminated at 365 nm for cross-linking. Proteins were identified by Western blot analysis combined with mass analysis. (B) HeLa cells were grown in the presence of photo-amino acids and/or natural amino acids, and cell extracts were subject to western blot analysis using an anti-Mst1 antibody. (C) Proteins were resolved on a 2-DE gel and any Mst1-bound conjugate was traced by an anti-Mst1 antibody. (D) List of peptides for PHLPP1 that were mass-analyzed for protein identification and the identification criteria of each peptide. (E) PHLPP1 as an Mst1-interaction protein was confirmed by Western blot analysis using an anti-PHLPP1 antibody.

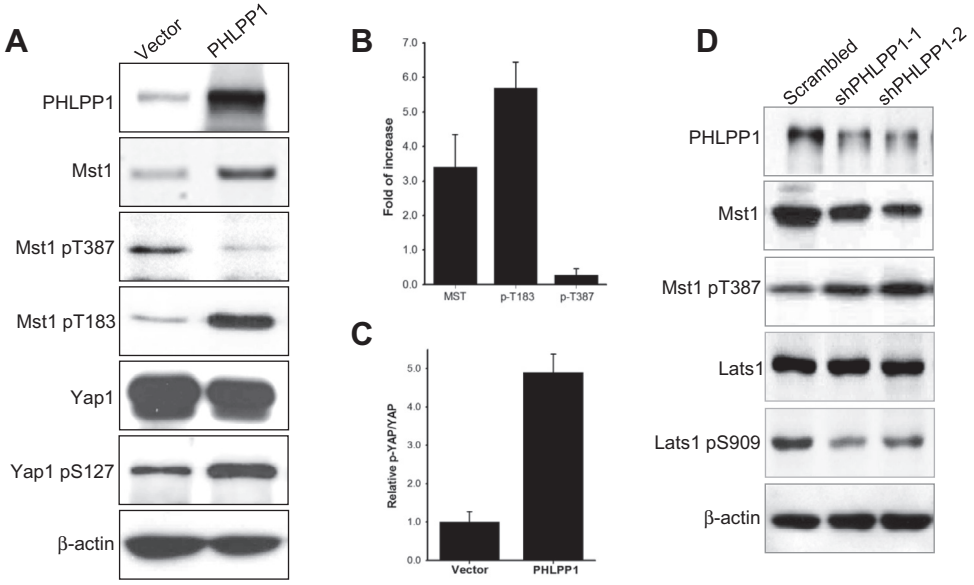


Fig. 2. Dephosphorylation of the inhibitory phospho-Thr^{183} of Mst1 by PHLPP1 and the subsequent increment of Yap1 phosphorylation. (A) Cell extracts were obtained from mock and PHLPP1-overexpressing HeLa cells, and Mst1 and Yap were investigated in terms of protein level and phosphorylation status. (B and C) Fold of increase in the levels of Mst1, Mst1 phospho-Thr^{183}, and Mst1 phospho-Thr^{387} (B) and the level of Yap1 phosphorylation at S381, as normalized by Yap1 expression level (C), was assessed from the normalized band intensities in A. Values are means with standard errors from 5 independent experiments. (D) Mst1 and Lats1 were investigated in terms of protein level and phosphorylation status in the control and shPHLPP1 cells.

3.2. PHLPP1 is responsible for dephosphorylation of the inhibitory phospho-Thr^{387} in Mst1

PHLPP1 is known to regulate Akt and protein kinase C (PKC) isoforms and to act as a tumor suppressor in several types of cancer by blocking growth factor-induced signaling in cancer cells [14]. Because Fig. 1 revealed an interaction of PHLPP1 with Mst1 (Fig. 1), effects of PHLPP1 expression on the phosphorylation of Mst1 and its down-stream signaling molecules were studied (Fig. 2A). Mst1 has two reciprocal phosphorylation sites, at Thr^{183} and Thr^{387}. The phosphorylation of Thr^{183} activates the Hippo pathway, whereas p-Thr^{387} is known to be inhibitory to the signaling [11]. Overexpression of PHLPP1 led to a mitigated level of Mst1 phospho-Thr^{387}, presumably through phosphatase activity (Fig. 2A). The quantitative analysis of Western blot revealed that the level of Mst1 phospho-Thr^{387} was inversely correlated to the expression level of PHLPP1 (Fig. 2B). In contrast, the activating phosphorylation at Thr^{183} was increased by an unknown mechanism, but may be explained by PHLPP1-catalyzed dephosphorylation of several kinases, including Akt and PKCα [12,15].

The reciprocal regulation of the Mst1 activity by phosphorylations at Thr^{183} and Thr^{387} was linked to phosphorylation of the effector protein for the Hippo pathway, Yap1 (Fig. 2A). The decreased phosphorylation at the inhibitory site of Mst1 was reflected by increased Yap1 phosphorylation, as normalized by the total Yap1 level (Fig. 2C). Interestingly, overexpression of PHLPP1 *per se* increased the Mst1 expression by an elusive mechanism. Taken together, these results suggest that PHLPP1 plays a tumor-suppressive role by directly dephosphorylating Thr^{387} of Mst1 as well as by regulating Mst1 expression.

The effect of PHLPP1 on the dephosphorylation of Mst1 was confirmed by suppressed expression of PHLPPL1 through small hairpin RNAs (shRNAs). Two independent regions of the PHLPP1 gene were targeted, as mentioned in Section 2, and Western blot analysis revealed a correlation of suppressed PHLPP1 expression with hyper-phosphorylation of Mst1 at Thr^{387} (Fig. 2D). Lats1 was found to mediate the Mst1-PHLPP1 cues to the effector Yap1 activity. These results suggest that PHLPP1 may act as a key regulator for the Hippo pathway by participating in the dephosphorylation of Mst1.

3.3. Down-regulation of PHLPP1 results in sustained cell proliferation even under confluent conditions

PHLPP1 is known to act as a tumor suppressor in several types of cancer [16,17], but little is known about how it regulates cellular contact inhibition. From Fig. 2, we obtained a clue that PHLPP1 may regulate the Hippo pathway through Mst1 dephosphorylation in a cellular contact-dependent manner. We investigated the localization of Yap1 because the effector protein shows oncogenic and proliferative activity by association with the TEAD family of transcription factors [8,9] and that the phosphorylated form is sequestered in the cytoplasm by 14-3-3 protein and enters a degradation pathway [18].

Immunofluorescence revealed that Yap1 is predominantly localized in the nucleus except nucleoli of HeLa cells under spare, low-density conditions (Fig. 3A). However, when cells were seeded at a high density to induce cellular contacts, cells showed, at least in part, shifts of Yap1 localization toward the cytoplasm, as indicated by arrows in the figure. Next, we monitored the cellular and molecular behaviors of PHLPP1 knock-down cells. Although no significant differences were observed under sparse growth conditions (data not shown), the Yap1 localization in shPHLPP1 cells was noticeably different from that of mock cells (Fig. 3B). The entire population of cells was occupied by an increased portion of cells that show the localization of Yap1 in the nuclei even under

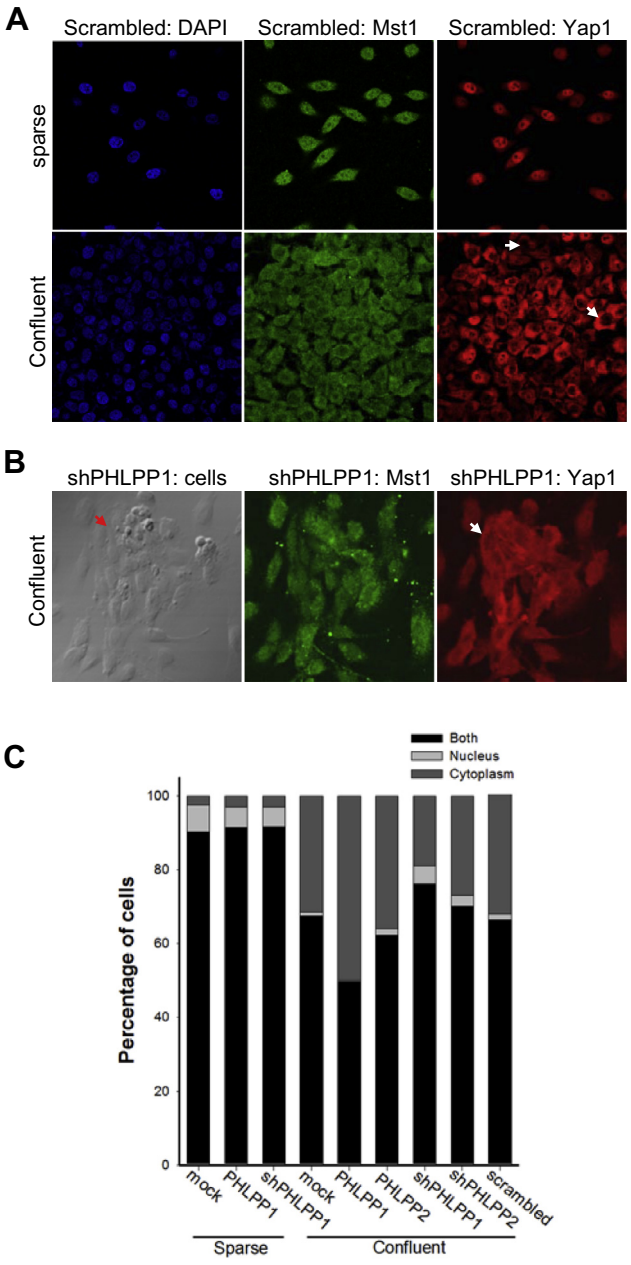


Fig. 3. Involvement of PHLPP1 in cell contact-dependent inhibition of cell proliferation. (A) Mst1 and Yap1 were traced by immunofluorescence under both sparse and confluent conditions. White arrows indicate the nucleus from which Yap1 is delocalized to cytoplasm. (B) Changes in the localization of Mst1 and Yap1 were assessed by immunofluorescence in HeLa cells with down-regulation of PHLPP1. Red and white arrows indicate cells that show deregulated growth. (C) Cells were classified into three groups in terms of Yap1 localization and were counted under both sparse and confluent conditions ($^{*}p < 0.01$, $n = 3$). Mock and scrambled indicate the pcDNA3.1 hygro(+) and p-silencer U6 vector-transfected control cells, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

contacted conditions. Interestingly, cells with suppressed PHLPP1 expression showed more aggressive phenotypes because some colonies were grown in layers as opposed to exclusive mono-layered growth for mock cells, as indicated by red arrow. A quantitative analysis indicates that suppressed expression of PHLPP1 resulted in a 14% increase in the number of cells whose nucleus was occupied by Yap1 (Fig. 3C). In contrast, PHLPP1 overexpression promoted translocation of Yap1 to the cytoplasm. Although PHLPP2 appeared to contribute to Yap1 localization, the effect was quite marginal.

3.4. PHLPP1 regulates Mst1-induced apoptosis in HeLa cells

Mst1 is reportedly pro-apoptotic in various cancer cells [19,20]. Roles of PHLPP1 in Mst1-induced apoptosis were investigated. GFP protein was allowed to be transiently co-expressed with Mst1, and GFP-positive cells were counted. Time-course counts indicated that fluorescent cells increased for up to 2 days after transfection and thereafter decreased over time as a natural decay. Several stably fluorescent cells that may arise from chromosomal random integration were observed (Fig. 4A). However, Mst1 overexpression resulted in a dramatic decrease in the number of fluorescent, viable cells from the first day of transfection and was ultimately responsible for rapid disappearance of fluorescent cells, which is explained by vigorous apoptotic cell death induced by overflowing Mst1 (Fig. 4B). Quantitatively, Mst1 overexpression accounted for a *ca.* 4 times increased death rate 72 h after transfection (Fig. 4C). Importantly, however, the apoptotic cell death stimulated by Mst1 was either rescued or exacerbated by down-regulation or forced expression of PHLPP1, respectively. Cells with expression of Mst1 T387D were refractory to the regulation of Mst1-induced apoptosis by PHLPP1 overexpression, which confirms that PHLPP1 regulates the Mst1-governed Hippo pathway by dephosphorylation of the inhibitory site (Thr^{387}) of Mst1. Overall cell counts supported this view (Fig. 4D). Conclusively, PHLPP1 regulates contact inhibition as well as apoptotic cell death of cells through dephosphorylation at Thr^{387} of Mst1. This implies that that any loss of PHLPP1 expression may cause impairment of oversight capability of cells through the Hippo pathway and ultimately lead to a loss of contact inhibition and an unchecked proliferation of tumor cells.

To test whether fine-tuning of the Hippo pathway by PHLPP1 affects cell proliferation, cells were embedded in soft agar and colony formation and growth was monitored. Cells were allowed to grow for 14 days and the size of 50 colonies formed that were randomly selected was measured at ×200. As is seen in Fig. 4E, colony formation and growth were markedly accelerated by PHLPP1 down-regulation. In addition, PHLPP1 overexpression reduced the proliferation rate. This result was in line with inferences that could be made by the translocation of Yap1 by PHLPP1 in Fig. 3B. These

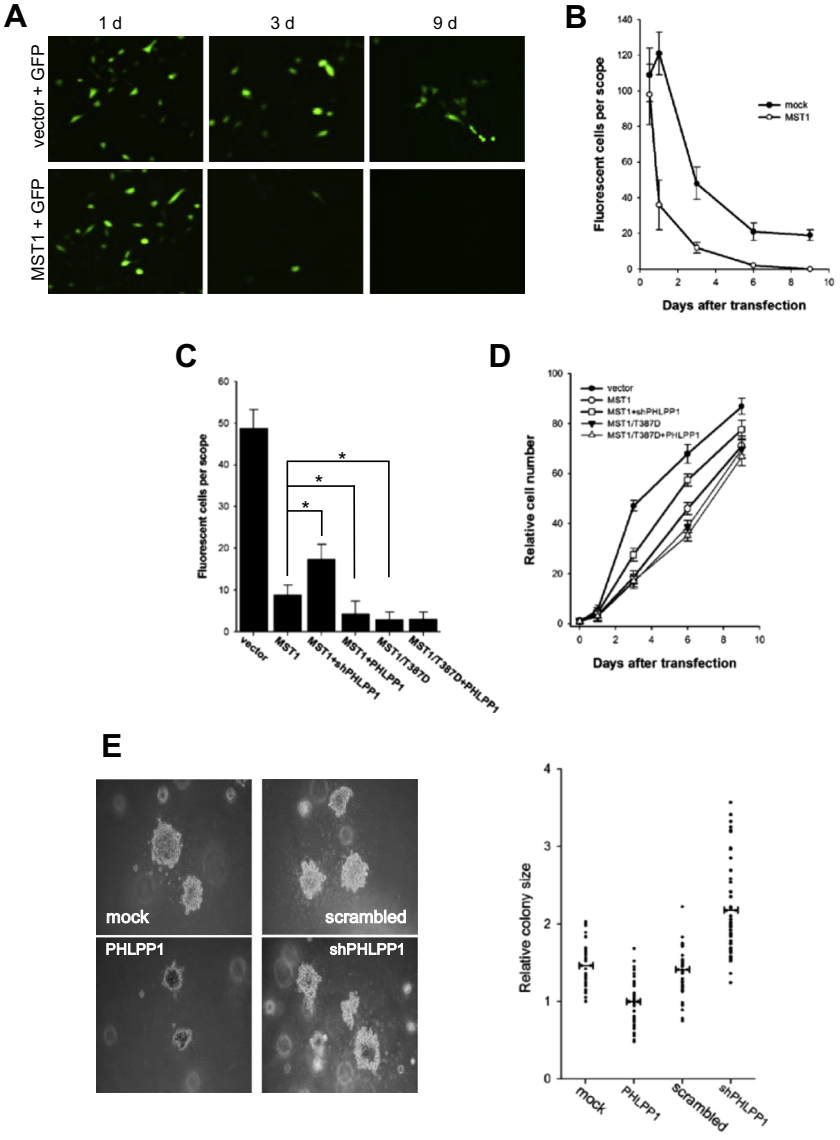


Fig. 4. Regulation of Mst1-induced apoptosis by PHLPP1 in HeLa cells. (A–D) The GFP gene was co-transfected with either the Mst1 gene construct or vector alone (A) and GFP-positive cells (B) and total cells (D) were counted for up to 9 days after transfection ($n = 3$). On the 3rd day after transfection, the number of fluorescent cells (C) was used as a barometer for Mst1-induced apoptotic death among various transfectant cells ($n = 3$). (E) Cells were embedded and grown for 14 days in 0.3% (w/v) soft agar. The mean values of the diameters for the *x*- and *y*-axes were plotted for 50 randomly chosen spheres.

results point to an identification of PHLPP1 as a key regulator for the Hippo pathway through Mst1 dephosphorylation and subsequent Yap1 translocation. Moreover, suppressed expression of PHLPP1 may drive a loss of contact inhibition. This is supported by our observation that increased methylation levels in the promoter region of Mst1 and PHLPP1 were observed in tumor tissues, and that hypermethylation was more pronounced in the promoter region of the PHLPP1 gene than in that of Mst1 (Supplementary 1).

4. Discussion

A biochemical strategy using photo-reactive amino acids and subsequent functional study allowed us to identify PHLPP1 as a binding partner for Mst1 and to elucidate the role of PHLPP1 as a functional modulator for Mst1 phosphorylation and the related contact inhibition of cells (Supplementary 2). Mst1 is a core regulator for the Hippo pathway, whereby the organ size and cell proliferation are controlled [21], and two phosphorylation sites of Mst1 compete with each other to regulate its anti-proliferative activity [11]. However, it was not fully elucidated how the modifications at both sites are modulated. Moreover, understanding of the involvement of Mst1 phosphorylation and the relation with PHLPP1 in contact inhibition has been elusive. PHLPP1 interacts with Mst1 (Fig. 1) and dephosphorylates Mst1 at the inhibitory Thr^{387} (Fig. 2). Yap1 was equilibrated into sequestration in the cytoplasm under dense environments. However, impairment of PHLPP1 expression is responsible for the dysfunction of the cellular contact-dependent regulation. This molecular fluctuation appears to mimic the loss of contact inhibition as a hallmark of cancer. In fact, the loss of PHLPP1 has been reported in several types of cancer, which may, at least in part, explain the unchecked proliferation during tumorigenesis [22]. Mst1-induced inhibition of cell proliferation was also rescued by down-regulation of PHLPP1, which was dependent on the phosphorylation at Thr^{387}. E-cadherin is involved in regulation of the Hippo pathway in terms of contact inhibition [23], but it remains unclear how E-cadherin communicates with and signals to PHLPP1 presumably near the cell contact site.

L-Photo-leucine and L-photo-methionine are the most frequently used analogs of L-leucine and L-methionine and are incorporated into proteins during peptide elongation in the endoplasmic reticulum. The photo-reactive amino acids carry an azirine group that is used to covalently link any associated proteins. We speculated that such characteristics could be applied to trap Mst1-binding phosphorylase(s) that might bind its substrates instantly and dissociate immediately after completion of dephosphorylation. Our result revealed that L-photo-leucine, not L-photo-methionine, could successfully capture PHLPP1, suggesting that leucine is involved in PHLPP1-Mst1 interaction. The photo-amino acids could be treated into cells that were cultured in the absence of the corresponding natural amino acids. In this case, it can be difficult to mass-analyze and identify proteins, because the analogs can incorporate into proteins in a multiple and random fashion. For this reason, competition between the natural and photo-reactive amino acids was allowed by treating both amino acids simultaneously into cells. Although the photolysis product from internal conversion of photoleucine has additional mass of 115.13 u [24], we neglected the contribution of the mass increase by analogs so that peptides comprising only natural amino acids are identified. Besides PHLPP1, PP2A was also identified as another binding molecule for Mst1 by repetitive identifications. However, it was revealed that the phosphatase was functionally independent of contact inhibition (data not shown).

PHLPP1 was found to have multiple targets including Akt [25], S6K1 [26], and protein kinase C [27]. PHLPP1 has also recently been shown to dephosphorylate the inhibitory residue (Thr^{387}) of Mst1 [11]. Interestingly, the inhibitory Thr^{387} is a common substrate residue of PHLPP1 and Akt [11,12]. Accordingly, phosphorylase and kinase appear to emulate each other to activate or inhibit Mst1 and thereby the Hippo pathway. Identified as a tumor suppressor *in vivo*, PHLPP1 expression is suppressed in a variety of human cancers [11,16,22] and degradation is enhanced by binding with beta-TrCP in cancer [28]. In line with this evidence, our preliminary results indicate that PHLPP1 expression can be suppressed in tumor tissues by hypermethylation in the promoter region (Supplementary 1). Hypermethylation in the promoter region was more dramatic for PHLPP1 than for Mst1. This may imply that a loss or suppression of PHLPP1 expression drives a loss of contact inhibition and unchecked proliferation of tumor cells. The relatively prevailing Akt1 activity may harness this process. In this regard, any attempt to restore the PHLPP1 activity may be relevant in precise treatment of cancer patients with defects in PHLPPL expression.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.12.129.

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