PR55α regulatory subunit of PP2A inhibits the MOB1/LATS cascade and activates YAP in pancreatic cancer cells

Ashley L. Hein1, Nichole D. Brandquist1, Caroline Y. Ouellette1, Parthasarathy Seshacharyulu2, Charles A. Enke1, Michel M. Ouellette2,3, Surinder K. Batra2 and Ying Yan1,2

Abstract
PP2A holoenzyme complexes are responsible for the majority of Ser/Thr phosphatase activities in human cells. Each PP2A consists of a catalytic subunit (C), a scaffold subunit (A), and a regulatory subunit (B). While the A and C subunits each exists only in two highly conserved isoforms, a large number of B subunits share no homology, which determines PP2A substrate specificity and cellular localization. It is anticipated that different PP2A holoenzymes play distinct roles in cellular signaling networks, whereas PP2A has only generally been defined as a putative tumor suppressor, which is mostly based on the loss-of-function studies using pharmacological or biological inhibitors for the highly conserved A or C subunit of PP2A. Recent studies of specific pathways indicate that some PP2A complexes also possess tumor-promoting functions. We have previously reported an essential role of PR55α, a PP2A regulatory subunit, in the support of oncogenic phenotypes, including in vivo tumorigenicity/metastasis of pancreatic cancer cells. In this report, we have elucidated a novel role of PR55α-regulated PP2A in the activation of YAP oncoprotein, whose function is required for anchorage-independent growth during oncogenesis of solid tumors. Our data show two lines of YAP regulation by PR55α: (1) PR55α inhibits the MOB1-triggered autoactivation of LATS1/2 kinases, the core member of the Hippo pathway that inhibits YAP by inducing its proteasomal degradation and cytoplasmic retention and (2) PR55α directly interacts with and regulates YAP itself. Accordingly, PR55α is essential for YAP-promoted gene transcriptions, as well as for anchorage-independent growth, in which YAP plays a key role. In summary, current findings demonstrate a novel YAP activation mechanism based on the PR55α-regulated PP2A phosphatase.

Introduction
The PP2A (protein phosphatase 2A) family of heterotrimers accounts for the majority of serine/threonine phosphatase activities in human cells1,2. Each PP2A consists of one catalytic subunit (C), one scaffolding subunit (A), and one regulatory subunit (B)1,2 (Fig. 1).

While the A and C subunits each contain two highly conserved isoforms, a large number of the B subunits are classified into four distinct subfamilies (B, B′, B″, and B‴) and share no homology. It is the B subunit that determines the substrate specificities and cellular localizations of PP2A1,3.

PP2A regulates diversified cellular functions, including proliferation, migration, and survival, whereas its role in oncogenesis remains poorly defined1,2,4–7. Currently, PP2A is defined as a putative tumor suppressor, mostly based on the loss-of-function analyses using inhibitors for the highly conserved A/C subunits, such as okadaic acid (C-subunit inhibitor), short interfering RNA (siRNA)
Fig. 1 Protein phosphatase 2A (PP2A) heterotrimeric holoenzymes. One scaffold (PR65 or A) subunit binds to one catalytic (C) subunit to form an A/C heterodimer, which can further complex with one of the regulatory (B) subunits. The A and C subunits each contain two highly conserved isoforms (97% similarity between Ca and CB and 87% similarity between Aα and Aβ). A large number of the B subunits are classified into four distinct subfamilies (B, B′, B″, and B‴), which share no homology.13,15

against the A/C subunits, or viral oncoproteins displacing the B subunits (e.g., SV40 small-t)6,8,9. In addition, PP2A-B subunits PR61 (α/γ) and PR72 act as tumor suppressors preventing oncogenic transformation10–13. In contrast, PP2A-B subunits PR130 and PR55α function as tumor-promoters1,2,14. While PR130 sustains EGF-mediated survival signaling and supports metastasis preventing oncogenic transformation10–13. In contrast, PR55α activities for KRAS-loss in the KRAS-addicted pancreatic cancer cells to produce malignant phenotypes25.

Results
PR55α supports the activation of YAP in pancreatic cancer cells

We have shown that PR55α supports anchorage-independent growth and tumorigenicity of pancreatic cancer cells20, which is also the best-known function of YAP in cancer21,24. Immunoblotting detected a marked increase in YAP level in human pancreatic cancer cells relative to the HPNE human normal pancreatic cells immortalized with telomerase35 (Fig. 2). In contrast, YAP-S127 phosphorylation, which induces YAP cytoplasmic retention by 14-3-3, is only moderately increased in pancreatic cancer cells compared with HPNE.

YAP inhibition by the LATS1/2 kinases is primarily via two mechanisms: phosphorylation of YAP-S397 leading to proteasomal degradation by β-TrCP and phosphorylation of YAP-S127 resulting in 14-3-3 cytoplasmic retention36. Therefore, we examined the impact of PR55α on YAP activation in pancreatic cancer cells using a series of Doxycycline (Dox)-inducible PR55α-shRNAs. As shown in Fig. 3a, PR55α-knockdown by short-hairpin RNAs (shRNAs) markedly decreased PR55α level in pancreatic cancer cells (CD18/HPAF and AsPC-1) compared with parental and Control-shRNA-transduced cells. Consequently, YAP level was largely reduced following PR55α-knockdown with a concomitant increase in YAP-S127 phosphorylation in the cells. On the other hand, the steady-state level of YAP-S397 phosphorylation was not particularly increased following PR55α-knockdown. Since YAP-S397 phosphorylation is specifically linked to YAP proteasomal degradation and cannot accumulate in the cells37, this outcome was anticipated. Collectively, these results suggest a role of PR55α in the maintenance of YAP protein and dephosphorylation of YAP-S127 in pancreatic cancer cells.
The effects of PR55α on the Hippo tumor suppressor pathway in pancreatic cancer cells

The Hippo pathway negatively regulates YAP level and activity. Therefore, we examined the effects of PR55α on the core members of this pathway (MST1/2, MOB1, and LATS1/2) in pancreatic cancer cells using Dox-inducible shRNAs.

We tested the effect of PR55α on the MST/LATS cascade in CD18/HPAF and AsPC-1 cells. Unexpectedly, PR55α-knockdown by shRNA resulted in decreases in MST1/2 levels and phosphorylation, along with the phosphorylation of its downstream targets LATS1-T1079/LATS2-T1041 (Fig. 3a).

Next, we analyzed the role of PR55α in the MOB1-triggered autophosphorylation of LATS1-S909/LATS2-S872, the key step leading to the LATS1/2 autoactivation loop37. In both CD18/HPAF and AsPC-1 cells, knockdown of PR55α induced the phosphorylation of MOB1-T35 and LATS1-S909/LATS2-S872 (MOB1 downstream targets), which indicates activation of the MOB1/LATS autoactivation loop28,38 (Fig. 3a). While LATS1 protein level remained unchanged, LATS2 protein was increased following PR55α-knockdown in CD18/HPAF cells. However, this effect was not detected in AsPC-1 cells. This difference probably attributes to cell-type specificity, as CD18/HPAF cells originated from metastatic liver lesions, while AsPC-1 cells were isolated from ascites39.

We validated the impact of PR55α on the MOB/LATS/YAP cascade with a time-course study. Following PR55α-knockdown by shRNA in CD18/HPAF cells, there was a time-dependent decrease in YAP level with a concurrent increase in YAP-S127 phosphorylation (Fig. 3b). Consistently, these changes in YAP were tightly associated with an increase in phosphorylation of both LATS1-S909/LATS2-S872 and MOB1-T35, an increase of protein level of LATS2, and the diminution of LATS1-T1079/LATS2-T1041 phosphorylation (Fig. 3b).

Since LATS2 was reported to be regulated by proteasomal degradation40, we evaluated the effect of PR55α on LATS2 protein stability using protein synthesis inhibitor cycloheximide (CHX), as described in our study41. The analysis indicated that LATS2 protein half-life is ~3.2 h in CD18/HPAF cells, while it elongated to ~6.7 h after the knockdown of PR55α (Fig. 3c).

Collectively, these results suggest an essential role for PR55α in the inhibition of the MOB1/LATS cascade that directly prevents YAP activation.

Ectopic PR55α expression induces YAP activation in normal human pancreatic ductal cells

To define the role of PR55α in normal pancreatic cells, we constructed the pREV-TRE-PR55α retroviral vector expressing Dox-inducible PR55α, which was further stably introduced into HPNE normal pancreatic ductal cells (Fig. 4a). Following 48 h induction with increasing doses of Dox, a marked increase in PR55α protein was detected in the pREV-TRE-PR55α-transduced cells but not in the control-vector-transduced cells (Fig. 4b). Associated with the PR55α induction were a concomitant increase of YAP protein levels and a simultaneous diminution of YAP-
S127/S397 phosphorylation (Fig. 4b). In contrast, control-vector-transduced cells receiving the same treatment showed little effect on either PR55α expression or on YAP protein level/phosphorylation.

Effects of ectopic PR55α on MOB1/LATS cascade in normal pancreatic ductal cells

We next examined the effect of PR55α on LATS1/2 phosphorylation and levels in HPNE cells. Ectopic PR55α expression in HPNE cells resulted in a marked decrease in LATS1-S909/LATS2-S872 phosphorylation along with a moderate increase in LATS1-T1079/LATS2-T1041 phosphorylation (Fig. 4b). Furthermore, LATS2 protein level was reduced in HPNE cells upon PR55α overexpression, while the effect is absent in the control cells (Fig. 4b). With a time-course study, we verified this effect of PR55α. Figure 4c shows that, following PR55α induction by Dox in HPNE cells, YAP protein level was increased, along with concomitant reductions in phosphorylation of YAP-S127, MOB1-T35, and LATS1-S909/LATS2-S872, and a decrease in LATS2 protein level. Immunoblotting also detected an increase in MST1/2 phosphorylation and level and LATS1-T1079/LATS2-T1041 phosphorylation (MST1/2 substrates) in HPNE cells.

Collectively, the results from both normal and malignant cells suggest a role of PR55α in YAP activation that involves the suppression of the MOB1/LATS autoactivation loop, leading to YAP phosphorylation/inhibition. Furthermore, this role of PR55α apparently does not require the MST/LATS cascade, since its activity increases in response to PR55α overexpression. This may implicate a feedback loop activation by PR55α.

PR55α supports YAP protein stability

One of the primary mechanisms by which the Hippo pathway inhibits YAP is to induce its proteasomal degradation. We, therefore, assessed the effect of PR55α on YAP protein stability in both the cytoplasm and nuclei of CD18/HPAF cells using α-tubulin and Lamin A/C as...
cytoplasmic and nuclear markers, respectively. Immuno blotting showed that PR55α is ubiquitously expressed in both cytoplasm and nuclei of the cells and its level increases following proteasomal inhibition by MG132 (Fig. 5a). To test whether the decrease of YAP in PR55α-knockdown cells was due to proteasomal degradation, MG132 was used to block proteasome activity and assessed for its effect on YAP level. As shown in Fig. 5b, MG132 treatment of the PR55α-knockdown cells resulted in an induction of both cytoplasmic and nuclear YAP, while MG132 treatment of Control-shRNA knockdown cells caused a decrease in cytoplasmic YAP level and only a subtle increase in the nuclear YAP. This suggests that PR55α inhibits YAP proteasomal degradation in CD18/HPAF cells.

We tested the effect of PR55α on YAP protein half-life in the cytoplasm and nuclei of CD18/HPAF cells, which were treated with CHX to block protein synthesis, and analyzed for YAP protein decay over time by western blot. The results show that YAP protein half-life was ~15.5 and 20.6 h in the cytoplasm and nuclei of the control cells, respectively, whereas it was only ~8 and ~8.7 h in the cytoplasm and nuclei of the PR55α-knockdown cells, respectively (Fig. 5c). These findings suggest that PR55α supports YAP protein stability via a mechanism that involves the inhibition of the proteasomal degradation of YAP.
Interaction of PR55α and YAP in pancreatic normal and cancer cells

By reciprocal co-immunoprecipitation assay described previously⁴¹, we examined the interaction of PR55α and YAP in CD18/HPAF cells. As a control, immunoprecipitation with nonimmunized IgG was included in the study. Immunoblotting revealed the presence of both PR55α and YAP, along with PP2A-A and PP2A-C subunits in the immunoprecipitates obtained either with anti-PR55α or anti-YAP antibody (Fig. 6a). Furthermore, relative to control cells, PR55α-knockdown cells displayed a lesser amount of PR55α, PP2A-C, and PP2A-A subunits in the immunoprecipitates obtained with anti-PR55α or anti-YAP antibody (Fig. 6a). Unexpectedly, relative to control cells, a higher level of YAP was detected in the anti-PR55α immunoprecipitates obtained from PR55α-knockdown cells, which is inconsistent with a lesser amount of YAP revealed in the anti-YAP immunoprecipitates obtained from PR55α-knockdown cells relative to control cells (Fig. 6a).

To determine whether the high YAP level present in the anti-PR55α immunoprecipitates from PR55α-knockdown cells could be attributed to YAP hyperphosphorylation, the anti-PR55α immunoprecipitates from control and PR55α-knockdown cells were treated with/without shrimp alkaline phosphatase (SAP) and analyzed for PR55α and p-YAP/YAP levels. As shown in Fig. 6b, the high YAP level in the anti-PR55α immunoprecipitates from PR55α-knockdown cells was markedly diminished after SAP treatment (YAP, lane 4 vs. 2). Immunoblotting of YAP-Ser127 phosphorylation confirmed the effectiveness of SAP in YAP dephosphorylation (YAP-S127, lane 4 vs. 2).

We compared the interaction of PR55α with YAP [wild-type (WT)] versus YAP(5SA) (constitutive active mutant), in which the LATS phosphorylation sites (S61/S109/S127/S164/S397) were mutated to alanine³⁸,⁴². CD18/HPAF cells were stably transduced with Flag-YAP(WT) or Flag-YAP(5SA) and immunoprecipitated with anti-PR55α antibody. As shown in Fig. 6c, anti-Flag antibody detected the presence of Flag-YAP and Flag-YAP(5SA) in the anti-PR55α immunoprecipitates obtained from their respective lysate, whereas the Flag-YAP(WT) level was 28-fold higher than Flag-YAP(5SA). This result implicates a direct interaction of PR55α and YAP and further supports that PR55α expresses a higher affinity toward phosphorylated YAP.

Using immunofluorescence (IF) confocal microscopy, we analyzed the intracellular level, distribution, and colocalization of PR55α and YAP. The results show the detection of PR55α and YAP in both the cytoplasm and nucleus of CD18/HPAF cells, with PR55α slightly more concentrated in the cytoplasm and YAP slightly more in the nucleus (shControl, Fig. 6d and Supplementary Fig. S1a). In the PR55α-knockdown cells, both PR55α and YAP levels were markedly reduced and the residual YAP...
was now mainly present in the nucleus (shPR55α, Fig. 6d, and Supplementary Fig. S1a).

We next analyzed the intracellular distribution of PR55α and YAP in normal HPNE cells. Both PR55α and YAP were also detected in both the cytoplasm and nucleus of the cells (Fig. 6e and Supplementary Fig. S1b). Upon ectopic PR55α expression, YAP and PR55α protein levels were concurrently increased in the cells and the additional amounts of the proteins were predominantly detected in the nuclei.

Co-localization studies revealed that, in both CD18/HPAF and HPNE cells, there was a significantly greater co-localization of PR55α and YAP in the PR55α-high cells (CD18/HPAF-shControl and HPNE-PR55α) compared with their respective isogeneic PR55α-low cells (CD18/HPAF-shPR55α and HPNE-PR55α) (Fig. 6d–e and Supplementary Fig. S1c).

Collectively, these results suggest a physical interaction and functional relationship of PR55α and YAP in pancreatic cancer and normal cells.

**Effect of MOB1 on the PR55α-promoted YAP activation**

MOB1-triggered LATS1-S909/LATS2-S872 autophosphorylation is the key event resulting in YAP phosphorylation.
inhibition\textsuperscript{38,44}. The results in Figs. 3–4 demonstrate that PR55α-promoted YAP activation in both malignant and normal cells is inversely associated with the activity of the MOB1/LATS axis. We, therefore, probed the role of MOB1 in YAP activation by PR55α using siRNA. MOB1 exists in two isoforms, MOB1A and MOB1B, which share 95% protein sequence identity and are functionally redundant\textsuperscript{44}. Since there is no antibody available to distinguish MOB1A and MOB1B, we analyzed their expressions by RT-PCR in a panel of pancreatic normal and cancer cells, which showed that MOB1A mRNA level is 5–15 fold higher than MOB1B in these cells (Supplementary Fig 2a, b). This result was confirmed by siRNA-knockdown studies, which showed that MOB1A-siRNA but not MOB1B-siRNA effectively reduced the total MOB1 protein level in the cells (Supplementary Fig 2c).

With MOB1A-siRNA, we assessed the role of MOB1 in the regulation of LATS1/2 and YAP by PR55α. MOB1-knockdown in HPNE-Control cells resulted in a decrease of LATS1-S909/LATS2-S872 phosphorylation and a concurrent increase in YAP protein level, as shown in Fig. 7a (lane 2 vs. 1, Bar graph). Similarly, MOB1-knockdown in HPNE-PR55α cells caused a marginal decrease in the already low LATS1-S909/LAT2-S872 phosphorylation and increase of YAP level compared with the HPNE-PR55α cells with control-knockdown (Fig. 7a, lane 4 vs. 3, Bar graph). However, MOB1-knockdown in HPNE cells produced no effect on YAP-S127 phosphorylation or LATS2 protein levels, which are negatively affected only by PR55α level.

We next evaluated the effect of MOB1 on the regulation of LATS and YAP by PR55α in malignant CD18/HPAF cells. While MOB1-knockdown by siRNA had very little effect on the low level of LATS1-S909/LATS2-S872 phosphorylation in CD18/HPAF cells, it markedly diminished the induction of LATS1-S909/LATS2-S872 phosphorylation caused by the PR55α-knockdown in CD18/HPAF cells (Fig. 7a, right panel, bar graph). In both control- and PR55α-shRNA-transduced CD18/HPAF cells, MOB1-knockdown by siRNA resulted in a subtle increase of YAP protein level but had little effect on YAP-S127 phosphorylation, or LATS2 protein level (Fig. 7a, lane 6 vs. 5 and lane 8 vs. 7), all of which were significantly affected by the level of PR55α, (Fig. 7a, right panel, lanes 7–8 vs. lanes 5–6).

These results indicate that PR55α suppresses the activation of MOB1/LATS cascade, while MOB1-inhibition cannot fully compensate for the loss of PR55α to restore YAP activation, suggesting that PR55α holds a dominant control on the magnitude of YAP activation.

**Effect of LATS1/2 in the PR55α-promoted YAP activation**

We investigated the role of LATS1/2 in the activation of YAP by PR55α in CD18/HPAF and AsPC-1 pancreatic cancer cells. In control-shRNA-transduced cells, knockdown of LATS1 and/or LATS2 by siRNA had only subtle effects on YAP phosphorylation and level in the cells (Fig. 7b, Control-shRNA). In PR55α-shRNA-transduced cells, knockdown of LATS1 or LATS2 alone by siRNA resulted in 1.6–3 fold increases in YAP protein levels relative to control cells (Fig. 7b, YAP, lanes 6–7 vs. lane 5). However, inhibition of both LATS1 and LATS2 by siRNA in the PR55α-knockdown cells resulted in a subtle, if any, decrease in YAP level compared with control cells (Fig. 7b, YAP, lane 8 vs. 5). Thus, in the PR55α-high (Control-shRNA) cells, manipulation of LATS1/2 levels apparently produced little effect on YAP level, whereas knockdown of either LATS1 or LATS2 in the PR55α-low (PR55α-shRNA) cells resulted in increases in YAP levels (Fig. 7b).

We also analyzed the effect of LATS1/2 on YAP-S127 phosphorylation in the presence/absence of PR55α-knockdown in pancreatic cancer cells. As shown in Fig. 7b, knockdown of LATS1 and/or LATS2 by siRNA had little effect on YAP-S127 phosphorylation in Control-shRNA-transduced cells, while it resulted in decreases in YAP-S127 phosphorylation in PR55α-shRNA-transduced cells. Furthermore, while knockdown of both LATS1 and LATS2 displayed an additive effect on inhibition of YAP-S127 phosphorylation in CD18/HPAF cells, this effect was not observed in AsPC-1 cells. These results suggest that PR55α plays a dominant role in the negative regulation of YAP-S127 phosphorylation.

**PR55α enhances YAP-targeted gene transcriptions and anchorage-independent growth**

To evaluate the biological significance of PR55α in promoting YAP activation, we analyzed the effect of PR55α on YAP-targeted gene expressions in HPNE (normal) and CD18/HPAF (malignant) cells. Real-time (RT)-PCR analyses revealed that PR55α expression was positively associated with YAP-activated transcriptions of ANKRDI, CTGF, CYR61, and Survivin\textsuperscript{37} in both HPNE and CD18/HPAF cells (Fig. 8a). Thus, ectopic PR55α expression in HPNE cells resulted in 8–10 fold increases in mRNA expressions of the YAP targets compared with control cells (red bars), while PR55α-knockdown by shRNA in CD18/HPAF cells caused a 4–6-fold reduction in mRNA levels of the YAP targets relative to the control-shRNA-transduced cells (black bars). These functional data confirm the role of PR55α in the promotion of YAP activation.

Promoting anchorage independence is the predominant role of YAP in oncogenesis\textsuperscript{45–47}. YAP alone has been shown to induce anchorage-independent growth of HPNE normal cells by the soft-agar assay\textsuperscript{32}. Therefore, we tested the effect of PR55α on anchorage-independent growth using the soft-agar assay\textsuperscript{48}. The results in Fig. 8b show that, following Dox-induced ectopic PR55α expression,
there was a significant induction of the proliferation of HPNE normal cells in soft-agar, indicative of anchorage-independent growth. Conversely, PR55α-knockdown by shRNA abrogated the clonogenicity of pancreatic cancer cells in soft-agar, indicative of loss of anchorage independence (Fig. 8c). These results support a critical role of PR55α in the positive regulation of YAP oncogenic function.

In summary, the results of the current study (Figs. 2–8) reveal a novel mechanistic role of PR55α regulated PP2A in the activation of YAP oncoprotein. Figure 9 outlines the findings of this investigation, which indicates that PR55α specifically suppresses the MOB1-mediated LATS autophosphorylation/activation, which would otherwise promote YAP proteasomal degradation by β-TrCP and cytoplasmic retention by 14-3-3. Furthermore, PR55α also exhibited a Hippo pathway-independent role in YAP activation, as siRNA-knockdown of either MOB1 or LATS1/2 did not compensate completely for the effect of PR55α-loss or PR55α-overexpression on YAP activation in both normal and malignant pancreatic cells (see Fig. 7), which suggests a regulation of YAP activation directly by PR55α or by another unknown mechanism regulated by PR55α.

Discussion

PP2A has been suggested in the regulation of the Hippo pathway and YAP activation, while the specific PP2A holoenzyme(s) involved were not identified. We recently identified the PR55α regulatory subunit of PP2A in the support of anchorage independence and tumorigenicity of pancreatic cancer cells, which coincidentally is the main factor regulating YAP activation in these cells.
function of YAP\textsuperscript{20,49}. Thus, we investigated the role of PR55\textsubscript{α} in the regulation of the Hippo pathway and YAP activation in pancreatic cancer cells.

The results in this report elucidate a novel YAP activation mechanism based on the PR55\textsubscript{α}-regulated PP2A (Fig. 9), which engages PR55\textsubscript{α} at three levels of regulation\textsuperscript{1}: inhibition of MOB1-triggered LATS1/2 autoactivation loop (LATS1-S909/LATS2-S872)\textsuperscript{2}, destabilizing LATS2 protein and\textsuperscript{3} direct YAP activation (see Figs. 3–7). However, this PR55\textsubscript{α}-dependent mechanism of YAP activation apparently also activates MST1/2 (see Figs. 3–4), which may be through a feedback mechanism. Thus, the increase in MOB1-T35 phosphorylation in the PR55\textsubscript{α}-knockdown cells may attribute to the inhibition of the PR55\textsubscript{α}/PP2A phosphatase activity rather than the increase of MST1/2 kinase activity. Furthermore, MST1/2 level/activity is negatively associated with LATS2 protein stability in both HPNE normal and CD18/HPAF malignant cells in response to PR55\textsubscript{α} manipulation, implicating cross-talking or a feedback regulation mechanism among the Hippo pathway components.

PR55\textsubscript{α}-knockdown results in an increase of LATS2 stability in CD18/HPAF but not in AsPC-1 pancreatic cancer cells (see Fig. 3). Although the exact mechanism causing this difference is unclear, it is likely to be cell-type specific, as the two pancreatic cancer cell lines were originated from different metastatic sites, CD18/HPAF from the liver and AsPC-1 from ascites\textsuperscript{39}. In order to metastasize and recolonize at distant organ sites, primary cancer cells need to adapt and survive a cascade of the environmental challenges by undergoing the processes of invasion\rightarrow intravasation\rightarrow systemic transport\rightarrow extravasation\rightarrow distant colonization\textsuperscript{50}. Thus, pancreatic cancer cells metastasizing to the liver versus ascites would have gone through very different adaptive processes and LATS2 stability regulation could be one of those mechanisms needing to be altered to fit different processes. Future studies will be needed to elucidate the mechanism and biological significance of LATS2 regulation during metastasis.

Although PR55\textsubscript{α} inhibits the MOB1-triggered LATS1/2 autoactivation that blocks YAP, knockdown of MOB1 in the PR55\textsubscript{α}-high cells (HPNE-PR55\textsubscript{α} or CD18/HPAF) had little effect on YAP level and phosphorylation (Fig. 7a, YAP and YAP-S127: lanes 3–4 and 5–6). In contrast, knockdown of MOB1 in the PR55\textsubscript{α}-low cells (HPNE-
control or CD18/HPAF-PR55α-shRNA) resulted in moderate but noticeable increases in YAP levels (Fig. 7a, YAP: lanes 1–2 and 7–8). Furthermore, knockdown of either LATS1 or LATS2 by siRNA only partially compensated for the loss of PR55α, marginally restoring YAP protein level in the PR55α-knockdown cells (see Fig. 7b, YAP: lane 6–7 vs. 5). However, such effects are lost when both LATS1 and LATS2 are inhibited by siRNA (see Fig. 7b, YAP: lane 8 vs. lanes 5–7), which suggests there might be an alternative YAP inhibitory pathway whose function is activated by the loss of both LATS1/2 and PR55α in the cells. Furthermore, the results of Fig. 7 suggest that the role of PR55α in YAP activation involves both Hippo pathway-dependent and -independent mechanisms, the latter of which could be direct or indirect.

PR55α directed PP2A activity has been shown to positively regulate several oncogenic pathways that play crucial roles in the oncogenesis of solid tumors, namely the Ras/Raf/MEK, Wnt/β-Catenin, and c-Myc signaling pathways17–19,51,52. While PR55α activates the Ras/Raf/MEK cascade through dephosphorylating KSR-S392 and RaS259/S295 inhibitory sites that block the pathway, its activation of β-Catenin and c-Myc is via the direct dephosphorylation of β-catenin-T41/S37/S33 and c-Myc-T58, respectively, preventing their proteasomal degradation by β-TrCP. Furthermore, recent studies indicate that cross-talk exists among the PR55α-promoted oncogenic pathways, such as the Ras/Raf/MEK/ERK signaling that promotes the activation of YAP and c-Myc by increasing their expression, β-catenin that synergizes with YAP/TAZ during cancer progression, and YAP that is required for KRAS-driving pancreatic tumorigenesis and can compensate for the loss of oncogenic KRAS in the KRAS-addicted pancreatic cells to sustain the malignant phenotypes25–27,53–55. These comprehensive data further highlight the significance of PR55α in tumor promotion and the potential of PR55α as a therapeutic target for cancer treatment.

Materials and methods

Cell culture and treatment

Human cancer cell lines AsPC-1, Capan-1, CD18/HPAF, L3.6, HeLa, and SH-SY5Y were obtained from ATCC. HPNE is a line of primary human pancreatic ductal cells immortalized by human telomerase hTERT35. Proteasome inhibitor MG132 (EMD Biosciences) was dissolved in DMSO and cells treated at 10 μM56,57. Protein synthesis inhibitor CHX (Sigma-Aldrich) was dissolved in water and cells treated at 15 μg/ml58.

Cytoplasmic and nuclear extracts were isolated using NE-PER™ Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific). Lamin A/C and α-tubulin were used as loading controls for nuclear and cytoplasmic extract, respectively59.

Additional details of cell culture/treatment are described in Supplementary Materials.

Antibodies

Antibodies are listed in Supplementary Materials.

Immunoblotting and immunoprecipitation

Immunoblotting and immunoprecipitation are described in Supplementary Materials20,41,60.

Short interfering RNA (siRNA) transfection

ON-TARGETplus SMARTpool of siRNA duplexes (Dharmacon) were used for silencing LATS1, LATS2, MOB1A, and MOB1B. Control siGENOME nontargeting siRNA (Dharmacon) was designed to target no known genes in human, mouse or rat. The siRNA sequences are described in Supplementary Materials.

Cells were transfected with 100 nmol/L of siRNA by DharmaFECT-1 (Thermo Fisher Scientific) as instructed by the manufacturer.

shRNA lentiviral vectors and viral infection

Dox-inducible lentiviral vector (TRIPZ) expressing shRNAs (Dharmacon) were used. shRNA sequences,
lentiviral production, and viral infection are described in Supplementary Materials.

Retroviral vectors and viral infection

pRevTet-On retroviral vector (Clontech) expresses the reverse tetracycline-controlled transactivator (rtTA) from the CMV promoter. pRevTRE retroviral vector (Clontech) expresses a gene of interest from the Tet-response element (TRE), which contains seven direct repeats of the tetO operator sequence upstream of a minimal CMV promoter that can be bound by the tTA or rtTA. The pRevTRE-PR55α retroviral vector contains the PR55α full-length cDNA sub-cloned from pBluescript-SK(-) vector by HindIII/ClaI digestion.

Flag-YAP and Flag-YAP(5SA) expression vectors were made respectively using plasmids p2xFlag-CMV2-YAP (Addgene #19045) and pCMV-flag-YAP-5SA (Addgene #27371), both of which encode N-terminally Flag-tagged versions of human YAP (NP_001181973). Coding sequences from both vectors were PCR-amplified using Platinum®-Pfx DNA-Polymerase (Thermo Fisher) using forward (5′-GTACGCCTGCAAGTGAACCCTGAGAAATGC-3′; Sall site underlined) and reverse (5′-CATGGAAGATCTCTATAACCATGTAAGAACCT-3′; BglII site underlined) primers. PCR fragments were then cut with BglII and Sall, gel-purified, and inserted into the BamHI/XhoI sites of pLXSH retroviral vector to produce the final constructs pLXSH-Flag-YAP (WT) and pLXSH-Flag-YAP(5SA). The 5SA mutant carries the following mutations eliminating all LATS1/2-phosphorylation sites in YAP: S61A, S109A, S127A/S164A, and S381A38,42.

Retrovirus production and infection are described in Supplementary Materials.

Immunofluorescence and microscopy

IF and microscopy were performed as described with additional detail in Supplementary Materials. Images were taken using a Zeiss-810 confocal laser-scanning microscope. Nuclear/cytoplasmic YAP and PR55α and their co-localization were analyzed by ImageJ.63–65

RT-PCR analysis

Total RNA was isolated using the TRIzol RNA-Isolation Reagent (Invitrogen) and analyzed for human ANKRDR1, CTGF, CYR61, GAPDH, MOB1A, MOB1B, and Survivin mRNA levels by RT-PCR using the iScript Advanced cDNA Synthesis Kit and SsoAdvanced Universal SYBR Green Supermix (Bio-Rad). The mRNA expressions were normalized with GAPDH-mRNA levels. PCR-primer sequences are listed in Supplementary Materials.

Statistical analysis

SigmaPlot was used for statistical analyses. Multiple t-tests were used for comparison of experimental groups. P values ≤ 0.05 were considered significant.

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Author details

1Department of Radiation Oncology, University of Nebraska Medical Center, Omaha, NE, USA. 2Department of Biochemistry and Molecular Biology, University of Nebraska Medical Center, Omaha, NE, USA. 3Department of Internal Medicine, University of Nebraska Medical Center, Omaha, NE, USA.

Conflict of interest

The authors declare that they have no conflict of interest.

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Supplementary Information

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References

1. Eichhorn, P. J., Creighton, M. P. & Bernards, R. Protein phosphatase 2A regulatory subunits and cancer. Biochim. Biophys. Acta 1795, 1–15 (2009).
2. Seshacharyulu, P., Pandey, P., Datta, K. & Bara, S. K. Phosphatase: PP2A structural importance, regulation and its aberrant expression in cancer. Cancer Lett. 335, 9–18 (2013).
3. Janssens, V., Longin, S. & Goris, J. PP2A holoenzyme assembly: in cauda venenum (the sting is in the tail). Trends Biochem. Sci. 33, 113 (2008).
4. Ruvolo, P. P. The broken “Off” switch in cancer signaling: PP2A as a regulator of tumorigenesis, drug resistance, and immune surveillance. BBA Clin. 6, 87–99 (2016).
5. Biket, I. et al. SV40 small t antigen enhances the transformation activity of limiting concentrations of SV40 large T antigen. Cell. 48, 321–330 (1987).
6. Palas, D. C. et al. Polyma small and middle T antigens and SV40 small t antigen form stable complexes with protein phosphatase 2A. Cell. 60, 167–176 (1990).
7. Skoczylas, C., Fahrbach, K. M. & Rundell, K. Cellular targets of the SV40 small-t antigen in human cell transformation. Cell Cycle. 3, 606–610 (2004).
8. Fuji, H. & Suganuma, M. Tumor promotion by inhibitors of protein phosphatases 1 and 2A: the okadaic acid class of compounds. Adv. Cancer Res. 61, 143–194 (1993).
9. Mateer, S. C., Fedorov, S. A. & Mumbly, M. C. Identification of structural elements involved in the interaction of simian virus 40 small tumor antigen with protein phosphatase 2A. J. Biol. Chem. 273, 35339–35346 (1998).
10. Nebumos, Y. et al. Characterization of B56γ tumor-associated mutations reveals mechanisms for inactivation of B56γ-PP2A. Mol. Cancer Res. 11, 995–1003 (2013).
11. Sablina, A. A., Hector, M., Colpaert, N. & Hahn, W. C. Identification of PP2A complexes and pathways involved in cell transformation. Cancer Res. 70, 10474–10484 (2010).
12. Yamamoto, H. et al. Inhibition of the Wnt signaling pathway by the PR61 subunit of protein phosphatase 2A J. Biol. Chem. 276, 26875–26882 (2001).
13. Yang, J. & Patel, C. Functions of B56-containing PP2As in major developmental and cancer signaling pathways. Life Sci. 87, 659–666 (2010).
14. Jansens, V. & Rebollo, A. The role and therapeutic potential of Ser/Thr phosphatase PP2A in apoptotic signalling networks in human cancer cells. Curr. Mol. Med. 12, 268–287 (2012).
15. Zvaneveld, K., Goris, J., Eunseu, C., Parker, P. J. & Janssens, V. Protein phosphatase 2A PR130/αB subunit binds to the SH2 domain-containing inositol polyphosphate 5-phosphatase 2 and prevents epidermal growth factor (EGF)-induced EGF receptor degradation sustaining EGF-mediated signaling. PASEB J. 24, 538–547 (2010).
16. Jansens, V. et al. PP2A binds to the LIM domains of Ipirma-preferred partner through its PR130/B subunit to regulate cell adhesion and migration. J. Cell. Sci. 129, 1665–1671 (2016).
17. Adams, D. G. et al. Positive regulation of Raf1-MEK1/2-ERK1/2 signaling by protein serine/threonine phosphatase 2A holoenzymes. J. Biol. Chem. 280, 42644–42654 (2005).
18. Ouy, S., Zhou, M., Conards, T. P., Veenstra, T. D. & Morrison, D. K. Protein phosphatase 2A positively regulates ras signaling by dephosphorylating KRAS1 and Raf1 on critical 14-3-3 binding sites. Curr. Biol. 13, 1356–1364 (2003).
19. MacDonald, B. T., Tamai, K. & He, X. Wnt/β-catenin signaling: components, mechanisms, and diseases. Dev. Cell. 17, 9–26 (2009).
20. Heir, A. et al. PRS5alpha subunit of protein phosphatase 2A supports the tumorigenic and metastatic potential of pancreatic cancer cells by sustaining hyperactive oncogenic signaling. Cancer Res. 76, 2243–2253 (2016).
21. Sebio, A. & Lenz, H.-J. Molecular pathways: hippo signaling, a critical tumor suppressor pathway. Oncogene 34, 12905–12912 (2015).
22. He, K. et al. Molecular pathways: hippo signaling, a critical tumor suppressor pathway. Oncogene 34, 12905–12912 (2015).
23. Pan, D. Hippo signaling in organ size control. Curr. Opin. Cell. Biol. 27, 1631–1636 (2015).
24. Zancanato, F., Cordenonsi, M. & Piccolo, S. YAP/TAZ as master ortho/er cancer drivers. Curr. Oncol. 22, 22–24 (2015).
25. Overholtzer, M. et al. Transforming properties of YAP, a candidate oncogene on the chromosome 11q22 amplicon. Proc. Natl. Acad. Sci. USA 103, 12405–12410 (2006).
26. Bata, S. K. et al. Epidermal growth factor ligand-independent, unregulated, cell-transforming potential of a naturally occurring human mutant EGFRvIII gene. Cell Growth Differ. 6, 1251–1259 (1995).
27. Yuan, Y., Li, D., Li, H., Wang, L., Tian, G. & Dong, Y. YAP overexpression promotes the epithelial-mesenchymal transition and chemoresistance in pancreatic cancer cells. Mol. Med. Rep. 13, 237–242 (2015).
28. Klein, C. A. The metastasis cascade. Science 321, 1785–1788 (2008).
29. Zhang, W. et al. PRS5a, a regulatory subunit of PP2A, specifically regulates PP2A-mediated β-catenin dephosphorylation. J. Biol. Chem. 284, 22649–22656 (2009).
30. Zhang, L. et al. Eya3 partners with P2PA to induce c-Myc stabilization and tumor progression. Nat. Commun. 9, 1047 (2018).
31. Lo Sardo, F., Strano, S. & Blandino, G. Y. A. P. and TAZ in lung cancer: oncogenic role and clinical targeting. Cancer Discov. 10, 137 (2018).
32. Huh, D. H., Kim, H. D., Jeong, H.-S. & Park, W. H. Regulation of TEAD transcription factors in cancer biology. Cells 8, 600 (2019).
33. Muranen, T. et al. PRK and p38 MAPK activities determine sensitivity to PI3K/mTOR inhibition via regulation of MYC and YAP. Cancer Res. 76, 7168 (2016).
34. Choudhury, A. D., Xu, H. & Bae, R. Ubiquitination and proteasomal degradation of the BRCA1 tumor suppressor is regulated during cell cycle progression. J. Biol. Chem. 279, 33909–33918 (2004).
35. Chen, P. et al. Arsenite-induced Cdc25C degradation is through the Xen-box and ubiquitin-proteasome pathway. Oncogene 28, 1995–2002 (2009).
36. Yuan, Y., Shay, J. W., Wright, W. E. & Mumbry, M. C. Inhibition of protein phosphatase activity induces p53-dependent apoptosis in the absence of p53 transcriptional activity. J. Biol. Chem. 272, 15220–15226 (1997).
37. Yasagawa, S. et al. The dynamic shuttling of SIRT1 between cytoplasm and nuclei in bronchial epithelial cells by single and repeated cigarette smoke exposure. PLoS ONE 13, e0193921 (2018).
38. Yan, Y. et al. Protein phosphatase 2A has an essential role in the activation of gamma-irradiation-induced G2/M checkpoint response. Oncogene 29, 4317–4329 (2010).
39. Oku, T., Masock, V. & Sudol, M. TAZ and Lats kinases regulate apoptotic function of yes kinase-associated protein (YAP). J. Biol. Chem. 283, 27534–27546 (2008).
40. Zhao, B. et al. Inactivation of YAP oncoprotein by the Hippo pathway is involved in cell contact inhibition and tissue growth control. Genes Dev. 21, 2747–2761 (2007).
41. Schindelin, J. et al. Fiji: an open-source platform for biological-image analysis. Nat. Methods 9, 676–682 (2012).
42. Thomsen, R. & Christensen, M. H. MoDock: a new technique for high-accuracy molecular docking. J. Med. Chem. 49, 3315–3321 (2006).
43. Xie, S. et al. The endocytic recycling compartment maintains cargo segregation acquired upon exit from the sorting endosome. Mol. Biol. Cell 27, 108–126 (2016).