Biochemical and Biophysical Research Communications 443 (2014) 917–923

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

The PDZ-binding motif of Yes-associated protein is required for its co-activation of TEAD-mediated *CTGF* transcription and oncogenic cell transforming activity

Tadanori Shimomura, Norio Miyamura, Shoji Hata, Ryota Miura, Jun Hirayama *, Hiroshi Nishina *

Department of Developmental and Regenerative Biology, Medical Research Institute, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8510, Japan

ARTICLE INFO

Article history:
Received 11 December 2013
Available online 28 December 2013

Keywords:
YAP
Hippo pathway
CTGF
TEAD

ABSTRACT

YAP is a transcriptional co-activator that acts downstream of the Hippo signaling pathway and regulates multiple cellular processes, including proliferation. Hippo pathway-dependent phosphorylation of YAP negatively regulates its function. Conversely, attenuation of Hippo-mediated phosphorylation of YAP increases its ability to stimulate proliferation and eventually induces oncogenic transformation. The C-terminus of YAP contains a highly conserved PDZ-binding motif that regulates YAP's functions in multiple ways. However, to date, the importance of the PDZ-binding motif to the oncogenic cell transforming activity of YAP has not been determined. In this study, we disrupted the PDZ-binding motif in the YAP (5SA) protein, in which the sites normally targeted by Hippo pathway-dependent phosphorylation are mutated. We found that loss of the PDZ-binding motif significantly inhibited the oncogenic transformation of cultured cells induced by YAP (5SA). In addition, the increased nuclear localization of YAP (5SA) and its enhanced activation of TEAD-dependent transcription of the cell proliferation gene *CTGF* were strongly reduced when the PDZ-binding motif was deleted. Similarly, in mouse liver, deletion of the PDZ-binding motif suppressed nuclear localization of YAP (5SA) and YAP (5SA)-induced *CTGF* expression. Taken together, our results indicate that the PDZ-binding motif of YAP is critical for YAP-mediated oncogenesis, and that this effect is mediated by YAP's co-activation of TEAD-mediated *CTGF* transcription.

1. Introduction

The Yes-associated protein (YAP) is a transcriptional co-activator that regulates multiple cellular processes by activating several transcription factors [1]. Recently, YAP was shown to play an important role in organ size control and to be inhibited by the Hippo signaling pathway [2–4]. In mouse liver, either transgenic overexpression of YAP or knock-out of Hippo pathway genes causes enlargement of this organ and the eventual development of hepatic tumors [5,6]. In cultured cells, YAP overexpression promotes proliferation and induces oncogenic transformation by activating TEAD-mediated transcription of the cell proliferation gene connective tissue growth factor (*CTGF*) [7,8]. Thus, the proper control of YAP activity is critical for maintaining tissue homeostasis in animals.

YAP functions are regulated by multiple post-translational modifications, including phosphorylation, SUMOylation, acetylation, and methylation [9–13]. Among these, phosphorylation is the best characterized regulatory event. For example, cell–cell contact triggers the Hippo pathway to phosphorylate YAP and thereby inactivate it, inhibiting cell proliferation. This phosphorylation is mediated by Lats kinases, which are essential components of the Hippo pathway. Lats-mediated phosphorylation of serine 127 (Ser-127) of human YAP (hYAP) promotes its recognition and cytoplasmic retention by 14-3-3 protein [9], while phosphorylation of hYAP Ser-381 induces hYAP ubiquitination and degradation [10]. Thus, the Hippo pathway negatively regulates the transcriptional co-activation capacity of YAP by inducing its cytoplasmic localization and protein degradation.

YAP contains a highly conserved PDZ-binding motif in its C-terminal domain, and this motif reportedly contributes to the regulation of YAP's functions. For example, Oka et al. showed that the interaction of the tight junction protein zonula occludens 2 (ZO2) with YAP's PDZ-binding motif facilitates nuclear localization of YAP [14]. In another study, it was reported that YAP stabilizes p73 to promote apoptosis, and that YAP's PDZ-binding motif is required for this proapoptotic function [15]. Although these studies

Abbreviations: YAP, Yes-associated protein; CTGF, connective tissue growth factor; TEAD, TEA domain family member; TAZ, transcriptional co-activator with PDZ-binding motif.

* Corresponding authors. Fax: +81 3 5803 5829.
E-mail addresses: hirayama.dbio@mri.tmd.ac.jp (J. Hirayama), nishina.dbio@mri.tmd.ac.jp (H. Nishina).

0006-291X/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2013.12.100

demonstrate that the PDZ-binding motif is important for regulating YAP's normal functions, the role of this motif in controlling the oncogenic cell transforming activity of YAP has not been characterized.

In this study, we investigated whether the PDZ-binding motif of hYAP is involved in its ability to transform cultured cells using the hYAP (5SA) mutant, which is not under the negative control of the Hippo pathway due to mutation of key phosphorylation sites [9]. As a result, hYAP (5SA) is constitutively activated and abnormally increased in a cell's nucleus. YAP co-transcriptional activity is thus elevated, inducing the oncogenic transformation of cultured cells. We found that disruption of the PDZ-binding motif strongly inhibited these properties of hYAP (5SA) both in cultured cells and mouse liver. Our results thus provide several lines of evidence indicating that the PDZ-binding motif is involved in regulating the nuclear localization of YAP and its co-activator function. In particular, we show that loss of this motif reduces TEAD-mediated transcription of *CTGF*, damping down YAP's oncogenic transforming activity.

2. Materials and methods

2.1. Plasmids

The mouse *CTGF* gene promoter was amplified by PCR and cloned into the pGL3-Basic vector (Promega). The *CTGF* promoter region spans codons −708 to −220 (+1 is the start codon) and contains the TEAD-binding element. Mutations were introduced into Myc-hYAP/pCS2 using PCR-based site-directed mutagenesis to generate mutant forms of hYAP described in each Figure. To inactivate the PDZ-binding motif, the *hYap* cDNA sequences corresponding to the last five amino acids of the hYAP protein were deleted. Other plasmids used in this study have been described elsewhere [12].

2.2. Cells, transfection, and luciferase assay

293T and NIH3T3 cells were grown in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum. For luciferase reporter assays, 293T cells were transfected with 40 ng firefly luciferase reporter plasmid, 20 ng sea pansy luciferase reporter plasmid [pRL-SV40 (Promega)], and the appropriate expression plasmids (indicated in each Figure) using Fugene HD (Promega). At 24 h post-transfection, cell lysates were prepared and dual luciferase assays performed using the dual-luciferase reporter assay system (Promega). Firefly and sea pansy luciferase activities were quantified by means of a luminometer, with the firefly luciferase activity normalized for transfection efficiency based on the sea pansy luciferase activity.

2.3. Antibodies

Anti-Myc, anti-lamin A/C, and anti-actin antibodies were purchased from Santa Cruz; rat anti-HA antibody from Roche Diagnostics Corp.; rabbit anti-HA antibody from Immunology Consultants Laboratory; and anti-β-tubulin antibody from Cell Signaling Technology.

2.4. Co-immunoprecipitation

Co-immunoprecipitation was performed as previously described [16], with some modifications. 293T cells were transfected with the expression plasmids described in each Figure. Transfected cells were washed with phosphate-buffered saline, homogenized in binding buffer (150 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 5% glycerol and 20 mM Tris–HCl, pH 7.4) containing protease inhibitor, and clarified by centrifugation. Total protein from the supernatant was incubated at 4 °C with rabbit anti-HA antibody and 20 μl protein G-Sepharose beads. The beads were washed four times with binding buffer, boiled in SDS sample buffer. The supernatant was fractionated by SDS–PAGE and analyzed by Western blotting as described below.

2.5. Western blotting

Immunoprecipitated materials and total cell extracts obtained as described above were fractionated by SDS–PAGE and transferred electrophoretically onto polyvinylidene difluoride membranes. Membranes were blocked with Blocking One (Nacalai Tesque) or 2% skim milk and incubated for 10 h at 4 °C with the antibodies indicated in each Figure. The blots were then incubated with the appropriate secondary antibodies plus peroxidase-conjugated anti-mouse, anti-rabbit, anti-rat, or anti-goat IgG antibodies (Santa Cruz) and developed with the ECL Western blotting detection system (Amersham Biosciences).

2.6. Immunofluorescence

Frozen sections of mouse liver were attached to APS-coated glass slides (Matsunami Glass). After blocking with 5% BSA in TBS, the slides were incubated with primary antibodies followed by fluorescent tag-conjugated secondary antibodies. Nuclei and plasma membranes were counterstained with Hoechst 33342 or phalloidin (both from Invitrogen), respectively.

2.7. Quantitation of nuclear protein in cultured cells

Levels of YAP localized in cellular nuclei were calculated as follows. The signal intensities of bands of YAP proteins appearing on Western blots were measured using the Quantity One (Bio-Rad) and normalized to the signal intensity of the band representing the nuclear protein laminA/C (value 1). The signal intensities of total hYAPs were normalized for protein loading based on the signal intensity of the band representing actin (value 2). Value 1 was divided by value 2 to obtain the value for nuclear hYAP protein.

2.8. Mice

All mice used in this study were of the C57BL/6J genetic background. All experimental procedures in this study were approved by the Institutional Animal Care and Use Committee of Tokyo Medical and Dental University.

2.9. Plasmid injections into living mice

Manipulated genes were expressed in hepatocytes of living mice by the hydrodynamic tail vein injection (HTVi) system. Briefly, each gene of interest was cloned into the pLIVE vector and suspended in *Trans*IT-EE Hydrodynamic Delivery Solution (Mirus Bio). Plasmids were injected into tail veins of mice.

3. Results

3.1. Deletion of the PDZ-binding motif of hYAP (5SA) abolishes its oncogenic transforming activity

We first compared the effects of transient expression of Myc-tagged plasmids expressing wild type hYAP [hYAP (WT)] or several mutant forms of hYAP in NIH3T3 cells. NIH3T3 cells transfected with hYAP (WT) and cultured for an extended period stopped proliferating when they reached saturation density (Fig. 1A). Similar

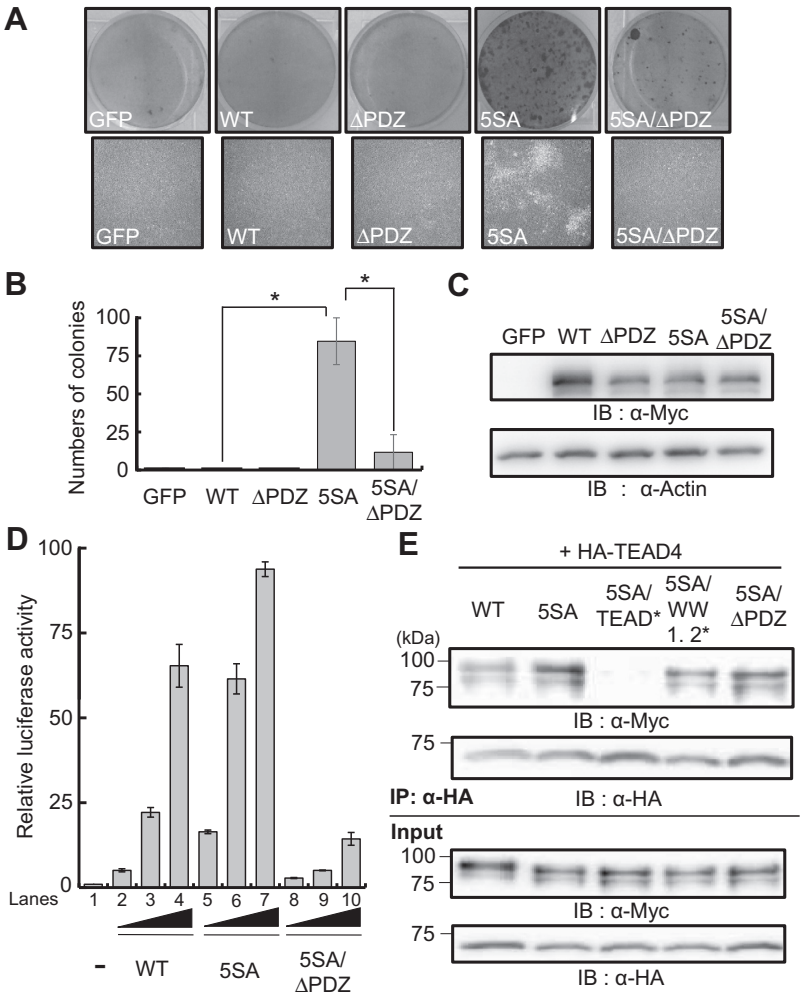

Fig. 1. The PDZ-binding motif of hYAP (5SA) is required for its oncogenic activity. (A) The cell transforming activity of wild type hYAP and various mutant forms of hYAP were determined by colony formation assays. Vectors expressing GFP (control), wild type hYAP (WT), WT hYAP lacking its PDZ-binding motif (ΔPDZ), the hYAP (5SA) mutant (5SA), or the hYAP (5SA) mutant lacking its PDZ-binding motif (5SA/ΔPDZ), were transiently expressed in NIH3T3 cells. At 18 days post-transfection, colonies were visualized by crystal violet staining (upper panels). The corresponding phase-contrast images of these NIH3T3 cells are also shown (lower panels). (B) The numbers of colonies in the plates in (A) were counted. Values are the mean ± S.E. ($n = 3$ plates/group). $^{*}P < 0.05$. (C) Protein levels of WT hYAP and its mutant forms in the cells in (A) were confirmed by Western blot (WB). (D) Transcriptional co-activation capacities of WT hYAP and its mutant forms were determined by luciferase reporter assays. Wild type hYAP, hYAP (5SA) or hYAP (5SA/ΔPDZ) was co-expressed in 293T cells with TEAD, and transactivation of a luciferase reporter plasmid containing the mouse *CTGF* promoter was examined. Values are the mean ± S.E. ($n = 3$) of luciferase activity relative to that in the sample containing only the reporter plasmid (set to 1). The difference between the results shown in lanes 2 and 5, 3 and 6, 5 and 8, 6 and 9, or 7 and 10 is statistically significant ($P < 0.01$). The difference between the results shown in lanes 4 and 7 is statistically significant ($P < 0.05$). (E) HA-tagged TEAD was co-expressed in 293T cells with Myc-tagged WT hYAP or its mutant forms. Lysates were immunoprecipitated (IP) with anti-HA antibody and analyzed by WB with anti-Myc antibody to detect hYAP proteins, or with anti-HA antibody to detect TEAD. Input: Total cell extracts were analyzed by WB with anti-Myc to detect WT hYAP and its mutant forms, or with anti-HA antibody to detect TEAD. 5SA/TEAD*, hYAP (5SA) with a mutated TEAD-binding domain; 5SA/WW1.2*, hYAP (5SA) with mutated WW domains.

results were observed for cells expressing GFP (control), or hYAP (WT) missing its PDZ-binding motif (hYAP-ΔPDZ). However, when NIH3T3 cells were transiently transfected with the hYAP (5SA) mutant, some of these cells re-initiated proliferation after confluence was reached. Nodules were formed in which the cells began to pile up, a feature of the oncogenic transformation phenotype (Fig. 1A–C). These findings are consistent with a previous report [10] and confirm that Hippo pathway-dependent suppression of hYAP activity inhibits its ability to transform cells. Strikingly, the oncogenic transforming activity of hYAP (5SA) was significantly reduced by deletion of its PDZ-binding motif [hYAP (5SA/ΔPDZ)] (Fig. 1A and B), suggesting that this motif plays an important role in the cell transforming activity of hYAP (5SA).

Previous work has demonstrated that YAP-dependent co-activation of TEAD-mediated transcription of the cell proliferation gene *CTGF* is an important step in the oncogenic cell transformation induced by hYAP (5SA) [7]. We then used a luciferase reporter assay to test whether the PDZ-binding motif of hYAP (5SA) could drive TEAD-mediated transcription from the *CTGF* promoter. As reported elsewhere [9], co-expression of hYAP (WT) enhanced TEAD-mediated *CTGF* transcription in a dose-dependent manner (Fig. 1D). As expected, *CTGF* transcription was significantly increased above this enhanced level when hYAP (5SA) was co-expressed. In contrast, the co-transcriptional activity of co-expressed hYAP (5SA/ΔPDZ) was markedly diminished, indicating that deletion of the PDZ-binding motif impairs the ability of hYAP (5SA) to co-activate TEAD-mediated *CTGF* transcription.

One explanation for our observations could be that deletion of hYAP (5SA)'s PDZ-binding motif affected its interaction with TEAD. To test this possibility, we used co-immunoprecipitation to examine the ability of WT and mutant forms of hYAP to interact with TEAD. HA-TEAD was co-expressed in cultured cells with Myc-hYAP (WT), Myc-hYAP (5SA), Myc-hYAP (5SA/WW1. 2*), or Myc-hYAP (5SA/ΔPDZ), all of which possess an intact TEAD-binding domain.

When cell lysates were subjected to immunoprecipitation with anti-HA antibody, we found that all four forms of hYAP successfully co-immunoprecipitated with HA-TEAD (Fig. 1E). In contrast, hYAP (5SA) bearing a mutated TEAD-binding domain (5SA/TEAD*; negative control) [8] did not co-immunoprecipitate with HA-TEAD. Because hYAP (5SA/ΔPDZ) maintained its TEAD-binding ability, we concluded that the reduced co-transcriptional capacity of hYAP (5SA/ΔPDZ) is not due to an inability to interact with TEAD.

3.2. Deletion of the PDZ-binding motif induces the cytoplasmic localization of hYAP (5SA)

We next tested the effect of PDZ-binding motif deletion on the subcellular localization of hYAP (5SA). Myc-hYAP (WT), Myc-hYAP (5SA) or Myc-hYAP (5SA/ΔPDZ) was expressed in cultured cells and hYAP sub-cellular localization was determined by cell fractionation and Western blotting. hYAP (WT) was detected in both the nucleus and cytoplasm (Fig. 2). Nuclear localization of hYAP (5SA) was significantly increased compared to that of hYAP (WT). These results are consistent with previous reports showing that Hippo-mediated YAP phosphorylation is necessary for its cytoplasmic retention [5,9]. Importantly, compared to Myc-hYAP (5SA), a higher concentration of Myc-hYAP (5SA/ΔPDZ) was present in the cytoplasm than in the nucleus (Fig. 2). Thus, deletion of the PDZ-binding motif blocks the nuclear localization of hYAP (5SA), an event that would account for its impaired promotion of TEAD-mediated *CTGF* transcription.

3.3. Addition of NLS does not rescue nuclear localization of hYAP (ΔPDZ)

To examine whether the addition of a nuclear localization sequence (NLS) to hYAP (ΔPDZ) could restore its nuclear localization, we generated a series of constructs in which the SV40 NLS sequence was inserted into the N-terminus of hYAP (WT) or hYAP (ΔPDZ). The subcellular localization of each of these NLS-fused hYAP proteins was then determined after transfection into 293T cells. We found that the addition of SV40 NLS significantly increased the nuclear localization of hYAP (WT) (Fig. 3A and B), indicating that the NLS was indeed functional. However, NLS addition did not increase the nuclear localization of hYAP (ΔPDZ) (Fig. 3A and B). We also investigated whether NLS fusion altered the co-transcriptional activity of hYAPs during TEAD-mediated *CTGF* transcription. As expected, the activity of NLS-fused hYAP (WT) was significantly greater than that of hYAP (WT) (Fig. 3C). On the other hand, the addition of SV40 NLS had no effect on the already limited co-transcriptional activity of hYAP (ΔPDZ). These results indicate that NLS-dependent nuclear import is insufficient to compensate for the defect in hYAP nuclear localization imposed by loss of its PDZ-binding motif.

3.4. Deletion of the PDZ-binding motif suppresses hYAP (5SA)-mediated co-activation of CTGF transcription in mouse liver

To determine if our results in cultured cells could be extended to the tissues of living mice, we used HTVi to test the effect of

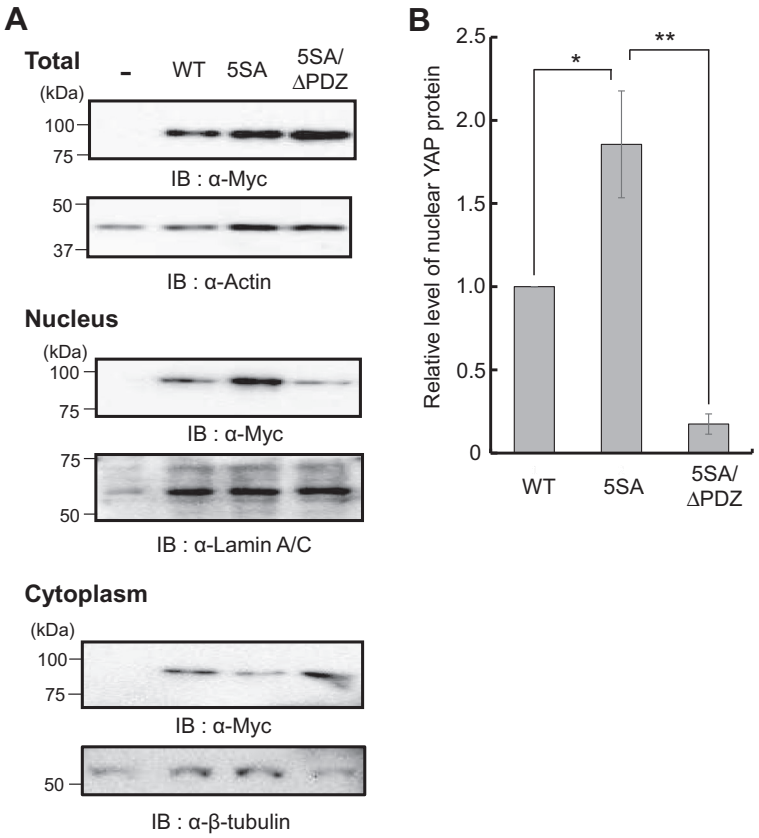

Fig. 2. The PDZ-binding motif of hYAP (5SA) is required for its nuclear localization in cultured cells. (A) Vectors expressing Myc-tagged hYAP (WT), hYAP (5SA), or hYAP (5SA/ΔPDZ) were individually transfected into 293T cells. Lysates were prepared, with some set aside (Total) and the rest subjected to subcellular fractionation to generate cytoplasmic and nuclear fractions. In each case, protein levels of Myc-tagged hYAP (WT) and the indicated mutant hYAP forms were determined by WB with anti-Myc antibody. The presence of the cytoplasmic marker β-tubulin and the nuclear marker lamin A/C in the appropriate fractions was confirmed by WB with the corresponding antibodies. Results shown are representative of three experiments. (B) Quantitative analysis of the nuclear localization of WT hYAP and its mutant forms in the cells in (A). The signal intensities in each lane of the "Nucleus" panel of (A) were measured as described in Section 2. Data are expressed relative to the value of the hYAP (WT) sample (set to 1). Values are the mean ± S.E. ($n = 3$). $^*P < 0.05$, $^*P < 0.01$.

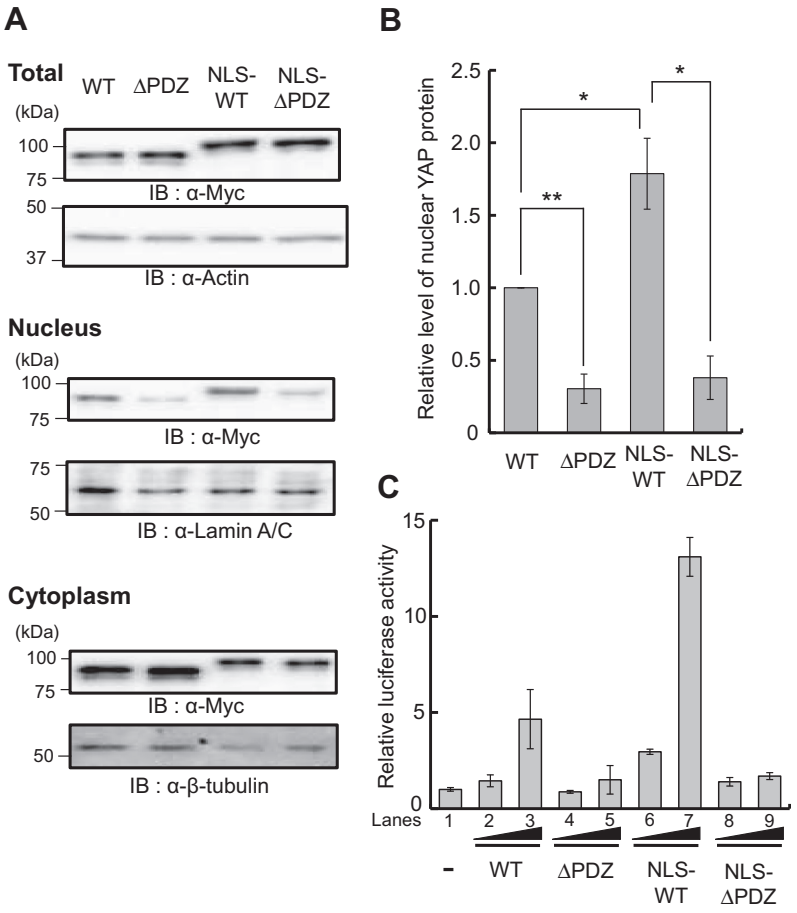

Fig. 3. The SV40 NLS has no effect on the cytoplasmic localization of hYAP (ΔPDZ). (A) Vectors expressing Myc-tagged hYAP (WT), hYAP (ΔPDZ), NLS-fused hYAP (WT), or NLS-fused YAP (ΔPDZ) were individually transfected into 293T cells and subcellular fractionation and analysis were performed as for Fig. 2A. (B) Quantitative analysis of the nuclear localization of hYAP (WT) and the indicated mutant hYAP forms was performed as for Fig. 2B. $^{*}P < 0.05$,$^{**}P < 0.01$. (C) The transcriptional co-activation capacities of hYAP (WT) and the indicated mutant forms were determined by luciferase reporter assay as for Fig. 1D. The difference between the results shown in lanes 7 and 9 is statistically significant ($P < 0.01$). The difference between the results shown in lanes 2 and 6, 3 and 7, or 6 and 8 is statistically significant ($P < 0.05$).

PDZ-binding motif deletion on YAP's subcellular localization in mouse liver. This system results in the safe and efficient delivery of naked nucleic acids to the liver of living mice. Myc-tagged plasmids expressing hYAP (WT), hYAP (5SA) or hYAP (5SA/ΔPDZ) were introduced into mouse liver by HTVi, and nuclear or cytoplasmic localization of each hYAP form was determined by immunofluorescence. When exogenously expressed in mouse liver, Myc-hYAP (WT) was almost exclusively localized in the cytoplasm (Fig. 4A and B). In contrast, Myc-hYAP (5SA) was concentrated in the nucleus and gave only a weak diffuse signal in the cytoplasm. These results confirm that, as in cultured cells, the Hippo pathway regulates YAP subcellular localization in mouse liver. Importantly, hYAP (5SA/ΔPDZ) was mainly located in the cytoplasm, replicating our earlier findings and showing that the PDZ-binding motif of hYAP is crucial for YAP nuclear localization *in vivo*.

We then examined expression levels of *CTGF* in livers of mice that had received HTVi introduction of hYAP (WT) or its mutant forms. As shown in Fig. 4C, levels of *CTGF* mRNA in mouse liver expressing exogenous hYAP (5SA) were much higher than in mouse liver expressing exogenous hYAP (WT). Once again, deletion of the PDZ-binding motif abolished the induction of *CTGF* expression by hYAP (5SA). Taken together, our results clearly demonstrate that the PDZ-binding motif of YAP is required for YAP's nuclear translocation *in vivo* and thus its ability to co-activate *CTGF* transcription.

4. Discussion

YAP can act as an oncoprotein that promotes excessive cell proliferation and induces tumorigenic transformation in both *in vitro* and *in vivo* systems [5,7,17]. It is known that the TEAD family of transcription factors plays an essential role in this YAP-induced proliferation and oncogenic transformation [8]. YAP activates TEAD-mediated transcription of *CTGF*, a cell proliferation gene, which triggers cell growth and eventually oncogenic transformation. Thus, in studies of the constitutively active YAP (5SA) mutant, disruption of either its TEAD-binding domain or transactivation domain suppresses tumorigenic cell transformation [8,9]. We have shown here that the PDZ-binding motif is also required for YAP's oncogenic cell transforming activity (Fig. 1A–C). Deletion of the PDZ-binding motif inhibited the nuclear translocation of hYAP (5SA), reducing its ability to co-activate TEAD-mediated transcription in the nucleus (Fig. 1D, Fig. 2). In addition, *CTGF* expression was significantly decreased in cells expressing hYAP (5SA/ΔPDZ) compared with cells expressing hYAP (5SA) *in vivo* (Fig. 4). Our results therefore imply that dysfunction of the PDZ-binding motif disrupts YAP/TEAD-dependent transcription of the *CTGF* gene in the nucleus, suppressing hYAP (5SA)-induced oncogenic transformation.

Previous studies have established that Hippo-mediated phosphorylation of hYAP promotes its recognition by 14-3-3 protein

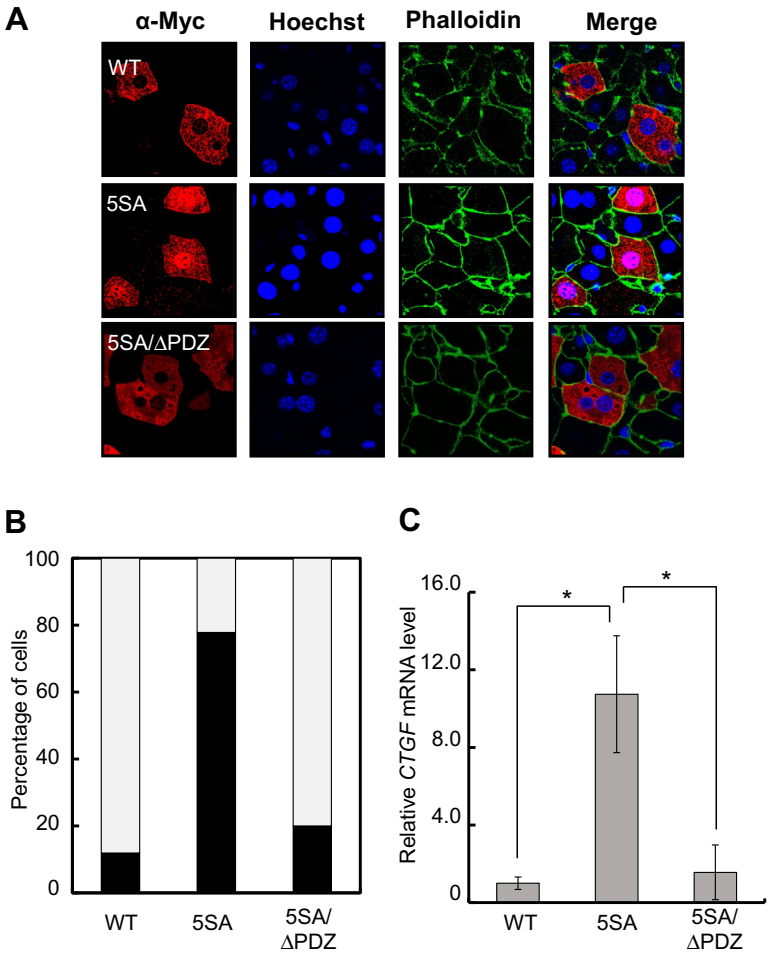

Fig. 4. The PDZ-binding motif is important for the nuclear localization and transcriptional co-activation capacity of hYAP (5SA) in mouse liver. (A) Vectors expressing Myc-tagged hYAP (WT), hYAP (5SA), or hYAP (5SA/ΔPDZ) were individually introduced into mouse liver by HTVi (see Section 2). Myc-tagged hYAPs were detected by staining with anti-Myc followed by fluorescein-conjugated secondary antibody (red). Nuclei and plasma membranes were counterstained with Hoechst 33342 (blue) or phalloidin (green), respectively. (B) Quantitative analysis of the subcellular localization of the exogenous hYAP proteins in (A). For each experimental group, 50–60 cells were evaluated to determine if the fluorescent hYAP protein was predominantly nuclear (black bars) or cytoplasmic (white bars) in localization. Data shown are the percentage of cells showing nuclear vs. cytoplasmic hYAP. (C) Extracts of livers from the mice in (A) were examined by RT-PCR analysis to detect *CTGF* mRNA levels. Data were normalized to expression of mouse *Gapdh* mRNA and are expressed relative to the value of the hYAP (WT) sample (set to 1). Results shown are the mean ± SEM ($n = 3$). $^*P < 0.05$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

and consequently its cytoplasmic retention [9]. We found that deletion of the PDZ-binding motif induced cytoplasmic localization of hYAP (5SA) (Fig. 2 and Fig. 4), suggesting that PDZ-binding motif-dependent nuclear localization of YAP is independent of Hippo-mediated regulation. TAZ is a paralog of YAP, and deletion of the PDZ-binding motif of TAZ inhibits its nuclear localization induced by disruption of Lats-mediated phosphorylation [18]. It is possible that the same subcellular localization control mechanisms operate for both TAZ and YAP.

It has been reported both that the tight junction protein ZO2 binds to YAP's PDZ-binding motif, facilitating YAP's nuclear localization, and that the NLS of ZO2 is required for the ZO2-mediated nuclear localization of YAP [14]. Our results indicate that this NLS-mediated mechanism is insufficient for PDZ-binding motif-dependent nuclear translocation of YAP (Fig. 3). Conceivably, ZO2 binding to YAP's PDZ-binding motif mediates the interaction of YAP with the other factor(s) promoting nuclear localization, or induces a conformational change in YAP that promotes its nuclear translocation. The identification of novel proteins interacting with the PDZ-binding motif of YAP and the delineation of their roles in the control of YAP activity will lead to a clearer understanding of YAP's biological functions.

Acknowledgments

This work was supported in part by research Grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan (J.H. and H.N.), and from the Ministry of Health, Labour and Welfare of Japan (H.N.).

References

[1] R. Yagi, L.F. Chen, K. Shigesada, Y. Murakami, Y. Ito, A WW domain-containing yes-associated protein (YAP) is a novel transcriptional co-activator, EMBO J. 18 (1999) 2551–2562.
[2] G. Halder, R.L. Johnson, Hippo signaling: growth control and beyond, Development 138 (2011) 9–22.
[3] A. Genevet, N. Tapon, The Hippo pathway and apico-basal cell polarity, Biochem. J. 436 (2011) 213–224.
[4] B. Zhao, K. Tumaneng, K.L. Guan, The Hippo pathway in organ size control, tissue regeneration and stem cell self-renewal, Nat. Cell Biol. 13 (2011) 877–883.
[5] J. Dong, G. Feldmann, J. Huang, S. Wu, N. Zhang, S.A. Comerford, M.F. Gayyed, R.A. Anders, A. Maitra, D. Pan, Elucidation of a universal size-control mechanism in Drosophila and mammals, Cell 130 (2007) 1120–1133.
[6] D. Zhou, C. Conrad, F. Xia, J.S. Park, B. Payer, Y. Yin, G.Y. Lauwers, W. Thasler, J.T. Lee, J. Avruch, N. Bardeesy, Mst1 and Mst2 maintain hepatocyte quiescence and suppress hepatocellular carcinoma development through inactivation of the Yap1 oncogene, Cancer Cell 16 (2009) 425–438.

[7] M. Overholtzer, J. Zhang, G.A. Smolen, B. Muir, W. Li, D.C. Sgroi, C.X. Deng, J.S. Brugge, D.A. Haber, Transforming properties of YAP, a candidate oncogene on the chromosome 11q22 amplicon, Proc. Natl. Acad. Sci. USA 103 (2006) 12405–12410.

[8] B. Zhao, X. Ye, J. Yu, L. Li, W. Li, S. Li, J.D. Lin, C.Y. Wang, A.M. Chinnaiyan, Z.C. Lai, K.L. Guan, TEAD mediates YAP-dependent gene induction and growth control, Genes Dev. 22 (2008) 1962–1971.

[9] B. Zhao, X. Wei, W. Li, R.S. Udan, Q. Yang, J. Kim, J. Xie, T. Ikenoue, J. Yu, L. Li, P. Zheng, K. Ye, A. Chinnaiyan, G. Halder, Z.C. Lai, K.L. Guan, Inactivation of YAP oncoprotein by the Hippo pathway is involved in cell contact inhibition and tissue growth control, Genes Dev. 21 (2007) 2747–2761.

[10] B. Zhao, L. Li, K. Tumaneng, C.Y. Wang, K.L. Guan, A coordinated phosphorylation by Lats and CK1 regulates YAP stability through SCF(beta-TRCP), Genes Dev. 24 (2010) 72–85.

[11] E. Lapi, S. Di Agostino, S. Donzelli, H. Gal, E. Domany, G. Rechavi, P.P. Pandolfi, D. Givol, S. Strano, X. Lu, G. Blandino, PML, YAP, and p73 are components of a proapoptotic autoregulatory feedback loop, Mol. Cell 32 (2008) 803–814.

[12] S. Hata, J. Hirayama, H. Kajiho, K. Nakagawa, Y. Hata, T. Katada, M. Furutani-Seiki, H. Nishina, A novel acetylation cycle of transcription co-activator Yes-associated protein that is downstream of Hippo pathway is triggered in response to SN2 alkylating agents, J. Biol. Chem. 287 (2012) 22089–22098.

[13] M.J. Oudhoff, S.A. Freeman, A.L. Couzens, F. Antignano, E. Kuznetsova, P.H. Min, J.P. Northrop, B. Lehnertz, D. Barsyte-Lovejoy, M. Vedadi, C.H. Arrowsmith, H. Nishina, M.R. Gold, F.M. Rossi, A.C. Gingras, C. Zaph, Control of the hippo pathway by Set7-dependent methylation of Yap, Dev. Cell 26 (2013) 188–194.

[14] T. Oka, E. Remue, K. Meerschaert, B. Vanloo, C. Boucherie, D. Gfeller, G.D. Bader, S.S. Sidhu, J. Vandekerckhove, J. Gettemans, M. Sudol, Functional complexes between YAP2 and ZO-2 are PDZ domain-dependent, and regulate YAP2 nuclear localization and signalling1, Biochem. J. 432 (2010) 461–472.

[15] T. Oka, M. Sudol, Nuclear localization and pro-apoptotic signaling of YAP2 require intact PDZ-binding motif, Genes Cells 14 (2009) 607–615.

[16] Y. Uchida, T. Osaki, T. Yamasaki, T. Shimomura, S. Hata, K. Horikawa, S. Shibata, T. Todo, J. Hirayama, H. Nishina, Involvement of stress kinase mitogen-activated protein kinase kinase 7 in regulation of mammalian circadian clock, J. Biol. Chem. 287 (2012) 8318–8326.

[17] L. Zender, M.S. Spector, W. Xue, P. Flemming, C. Cordon-Cardo, J. Silke, S.T. Fan, J.M. Luk, M. Wigler, G.J. Hannon, D. Mu, R. Lucito, S. Powers, S.W. Lowe, Identification and validation of oncogenes in liver cancer using an integrative oncogenomic approach, Cell 125 (2006) 1253–1267.

[18] F. Kanai, P.A. Marignani, D. Sarbassova, R. Yagi, R.A. Hall, M. Donowitz, A. Hisaminato, T. Fujiwara, Y. Ito, L.C. Cantley, M.B. Yaffe, TAZ: a novel transcriptional co-activator regulated by interactions with 14-3-3 and PDZ domain proteins, EMBO J. 19 (2000) 6778–6791.