YAP-Induced Resistance of Cancer Cells to Antitubulin Drugs Is Modulated by a Hippo-Independent Pathway

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Abstract

Although antitubulin drugs are used widely to treat human cancer, many patients display intrinsic or acquired drug resistance that imposes major obstacles to successful therapy. Mounting evidence argues that cancer cell apoptosis triggered by antitubulin drugs relies upon activation of the cell-cycle kinase Cdk1; however, mechanistic connections of this event to apoptosis remain obscure. In this study, we identified the antiapoptotic protein YAP, a core component of the Hippo signaling pathway implicated in tumorigenesis, as a critical linker coupling Cdk1 activation to apoptosis in the antitubulin drug response. Antitubulin drugs activated Cdk1, which directly phosphorylated YAP on five sites independent of the Hippo pathway. Mutations in these phosphorylation sites on YAP relieved its ability to block antitubulin drug-induced apoptosis, further suggesting that YAP was inactivated by Cdk1 phosphorylation. Notably, we found that YAP was not phosphorylated and inactivated after antitubulin drug treatment in taxol-resistant cancer cells. Our findings suggest YAP and its phosphorylation status as candidate prognostic markers in predicting antitubulin drug response in patients. Cancer Res; 74(16); 4493–503. ©2014 AACR.

Introduction

Antitubulin drugs, including paclitaxel (taxol), docetaxel, and vinblastine, have widely been used for the treatment of a variety of human cancers such as breast and ovarian cancers, non–small cell lung cancer, and head and neck cancer (1–3). These drugs can directly bind to β-tubulin and either promote or reduce β-tubulin polymerization, which changes the stability of microtubules (4). The change in microtubule stability can subsequently exert a mitotic block by altering kinetochore tension, which can activate the spindle assembly checkpoint, resulting in mitotic delay and subsequent apoptosis (5). Although antitubulin drugs kill cancer cells and can sometimes effectively suppress tumor growth in patients with cancer, a significant proportion of tumors is either intrinsically resistant to antitubulin drugs or later develop resistance after primary therapy, leading to disease relapse and patient mortality, which is a major obstacle to successful cancer treatments (2, 6). Therefore, identification and characterization of cellular genes or signaling pathways responsible for antitubulin drug resistance are critical for successful treatment of cancers. In addition, although tremendous progress has been made toward identifying the genes involved in antitubulin drug response, surprisingly, the signaling pathways by which antitubulin drugs cause mitotic arrest and cell death are poorly defined. YAP (Yes-associated protein) and TAZ (transcriptional coactivator with PDZ-binding motif) are paralogs of WW domain-containing transcriptional coactivators, which share 60% similarity and activate many transcriptional factors important for the development of various tissues in mammals (7, 8). Recently, TAZ and YAP have been identified as oncogenes and major components of an emerging Hippo signaling pathway that plays important roles in regulating animal size, cell proliferation, apoptosis, tumorigenesis, stem cell renewal and differentiation, neuronal growth, and mechanotransduction in both Drosophila and mammals (8–12). In this novel pathway, mammalian homologs of Drosophila Hippo, Mst1, and Mst2 phosphorylate and activate tumor suppressors and kinases LATS1 and LATS2, which in turn inactivate YAP and TAZ by phosphorylating multiple sites (five sites for YAP and four sites for TAZ) with consensus sequence of HxH/R/KxxS/T (H, histine; x, any amino acid; R, arginine; K, lysine; S, serine; T, threonine; Mst1/2→LATS1/2→YAP/TAZ). These core components also interact with many upstream (e.g., NF2, RASSF1A, and FAT4) and downstream (e.g., CTGF, Cyr61, and BMP4) cellular genes, forming a complex Hippo signaling pathway. Recently, we and others have identified YAP and TAZ as important players in response of breast cancer cells to apoptosis induced by antitubulin drug taxol (13, 14). It has been shown that overexpression of YAP or TAZ in MCF10A mammary cells causes transformation, epithelial–mesenchymal transition (EMT), and resistance to taxol, whereas knockdown of TAZ in MDA-MD231 breast cancer cells sensitizes these cells...
to taxol (14). However, whether YAP and TAZ overexpression causes resistance of cancer cells to other antitubulin drugs is unclear. In addition, the upstream signaling pathways or genes modulating YAP/TAZ in response to taxol remains to be identified. In this study, we have discovered that YAP is specifically phosphorylated by Cdk1/Cyclin B kinase after antitubulin drug treatment independent of the upstream Hippo signaling pathway. We have provided strong evidence that phosphorylation of YAP by Cdk1/Cyclin B inhibits YAP-induced resistance of cancer cells to antitubulin drug treatment.

Materials and Methods

Plasmid construction and site-directed mutagenesis

Plasmid construction and site-directed mutagenesis were performed as described in (15).

Cell culture and treatments of cells by chemotherapeutic drugs, kinase inhibitors, and anoikis

Cell culture was as described (15). HeLa cells were treated with antitubulin drugs (Supplementary Table S1) for various times, followed by protein extraction and Western blot analysis. For dose-dependent YAP phosphorylation, HeLa cells were treated with increasing concentration (0, 0.1, 0.5, 1, 5, 10, 50, 100, and 500 nmol/L) of taxol for 24 hours, followed by protein extraction and Western blot analysis. For kinase inhibitor treatments, HeLa cells were pretreated with inhibitors for Cdk, JNK, p38, GSK, MEK, DYRK, and mTOR (see Supplementary Table S1 for details) for 2 hours, followed by treatment with these inhibitors for 22 hours in the presence of 100 nmol/L taxol.

To test whether YAP phosphorylation is due to loss of anchorage of cells after antitubulin drug treatment, HeLa cells were plated onto a 60-mm plate coated with poly-HEMA (20 mg/mL; Sigma) and incubated at 37°C for 24 hours. The floating cells were then collected for protein extraction and Western blot analysis.

siRNA-mediated gene expression knockdown

siRNA duplexes targeting different region of human LATS1, LATS2, Mst1, Mst2, and CDK1 were purchased from IDT. Smartpool siRNAs targeting four different regions of Cyclin B mRNA (siCyclin B) were purchased from Dharmacon (Supplementary Table S2; Thermo Fisher). An siRNA with scrambled sequence (siControl) was used as a negative control. HeLa cells were transfected with 50nmol/L of siRNAs using RNAiMax (Invitrogen) according to manufacturer’s instructions. Two days posttransfection, cells were treated with 100 nmol/L Taxol for 24 hours, followed by protein extraction and Western blot analysis.

Lentivirus production, infection, and establishment of stable cell lines overexpressing or knocking down cellular genes

Lentiviral production, titration, and infection of overexpressing and shRNA constructs are as described (15). To establish JNK1 and JNK2 shRNA (shJNK1/shJNK2) knockdown cell lines in HeLa cells, a set of shJNK1 or shJNK2 in pTRIPZ lentiviral vector targeting different sequences of JNK1 or JNK2 mRNA (Open Biosoystmes) were screened (data not shown), and the cells infected with lentivirus expressing shJNK1/JNK2 with the best knockdown were used for functional studies. To establish CDK1 knockdown cell lines in HeLa cells, shCDK1 (Supplementary Table S2) was subcloned from pGIPZ into pTRIPZ lentiviral vector. To establish MCF10A cells overexpressing YAP and its phosphorylation site mutants, MCF10A cells were infected with lentivirus expressing WPI vector, YAP-WT-HA, YAP-5SA-HA, or YAP-5SD-HA at similar multiplicity of infection.

RNA extraction and qRT-PCR

RNA extraction and qRT-PCR were as described (15).

Antibodies, Western blot analysis, coimmunoprecipitation, immunostaining

The antibodies used in this study were purchased and used as listed in Supplementary Table S3. Rabbit anti-phospho-YAP-S367 polyclonal antibody was produced by injection of conjugated phospho-peptide (KQNPVSpSPGMSQ) into rabbit (Cocalico). Antibody was purified from antiserum using protein A beads. Protein extraction, Western blot analysis, coimmunoprecipitation (Co-IP), and immunostaining were as described previously (15).

Identification of YAP phosphorylation sites by mass spectrometry

HeLa cells were treated with 100 nmol/L taxol for 24 hours. Floating cells were collected and extracted for protein with 1% NP40 lysis buffer. About 30 mg of protein lysate were immunoprecipitated with anti-YAP (Santa Cruz Biotechnology) polyclonal antibody. The immunoprecipitated YAP was subjected to 10% SDS-PAGE and stained with commassie blue. The phosphorylated YAP band was cut and identification of YAP and phosphorylated YAP peptides was performed as described (16).

GST fusion protein production and in vitro kinase assay

GST fusion protein production and purification and in vitro kinase assays are as described (15). Cdk1/Cyclin B active kinase was purchased from New England Biolabs. The wild-type (WT) peptide containing S128 (PQHVRAHS128SP) and its mutants (S128V;PQHVRAHS128P) were purchased from GL Biochem.

Analysis of apoptosis by Trypan blue exclusion analysis

Analysis of cell death/apoptosis by Trypan blue exclusion assay was performed as described previously (14).

Results

YAP and TAZ are phosphorylated after antitubulin drug treatment

To understand how YAP and TAZ are involved in antitubulin drug response, we first examined their status after antitubulin drug taxol treatment. We collected both floating (F) and adherent (A) cells after taxol drug treatment for 6, 12, and...
24 hours. Interestingly, band-shifts of YAP and TAZ were detected in floating (F) cells, which are mitotic arrested [decreased phosphorylated Y15-Cdk1 (pCdk1)] and apoptotic [increased cleaved PARP (cPARP)] cells, rather than adherent (A) live cells (high pCdk1 and low cPARP; Fig. 1A and B). Besides band-shift, levels of TAZ rather than YAP were gradually decreased with time after taxol treatment (Fig. 1A). The band-shifts of both YAP and TAZ can be reversed after treatment of protein lysates from taxol-treated HeLa cells with calf intestine phosphatase (CIP; Fig. 1C), suggesting that the band-shifts of YAP and TAZ after taxol treatment are due to phosphorylation. To further examine whether YAP and TAZ changes are specific for taxol drug, we examined the status of YAP and TAZ after treatment of HeLa cells with a wide variety of chemotherapeutic drugs. YAP phosphorylation is only detected in antitubulin drug-treated cells, which specifically caused reduced pCdk1, whereas TAZ phosphorylation/degradation was found in cells treated by almost all of the drugs, as indicated by increased apoptotic marker cPARP (Fig. 1D). These results clearly demonstrated that YAP is specifically phosphorylated during mitotic arrest and apoptosis induced by antitubulin drugs in HeLa cells. To further explore whether YAP phosphorylation is dependent of drug dose, we treated HeLa cells with increasing concentration of taxol. As expected, increasing YAP phosphorylation was detected with the increase of taxol drug concentration, which is correlated with increasing levels of cPARP and decreasing levels of pCdk1 (Fig. 1E), especially when taxol concentration is higher than 10 nmol/L. Because antitubulin drugs can disrupt microtubule and cause loss of cell adhesion on the cell culture plate and previous studies have shown that the Hippo pathway plays important roles in anoikis (17), we tested whether YAP phosphorylation is due to loss-of-anchorage of cells after antitubulin drug treatment. Interestingly, although both taxol treatment and incubation of cells on HEMA-coated plate induced apoptosis as indicated by increased cPARP, the YAP band-shift/phosphorylation is only detected after taxol treatment (Fig. 1F), suggesting that YAP phosphorylation is not due to loss-of-anchorage of cells after antitubulin drug treatment. Next, we also tested to see whether YAP phosphorylation is specific for HeLa cells and found that YAP is phosphorylated in a wide variety of human cancer cells after taxol treatment (Fig. 1G), suggesting that YAP phosphorylation after antitubulin drug treatments occurs in various tissues. Finally, we showed that YAP is phosphorylated after taxol treatment only in taxol-sensitive (SK-BR3) cells rather than its taxol-resistant counterpart (SK-BR3-TR) cells.
counterpart (Sk-BR3-TR; Fig. 1H). Together, these findings strongly suggest that YAP can be specifically phosphorylated in apoptotic cells in a wide variety of cell types after treatment with antitubulin drugs such as taxol and vinblastine other than drugs such as doxorubicin and cisplatin.

**Phosphorylation of YAP is Hippo independent**

Next, we examined the expression of other Hippo core components and kinases Mst1, Mst2, LATS1, and LATS2. Except for LATS2, phosphorylation of Mst1/2 (pMst) and LATS1 (pLATS1), which normally activate their kinase activity, is significantly increased (Fig. 2A). Because our previous studies showed that LATS1 inactivates YAP by phosphorylating five sites (15), we examined whether increased phosphorylation/activity of LATS1 is responsible for YAP phosphorylation after taxol treatment by testing the levels of phosphorylated S127 (pS127) of YAP using anti-phospho-YAP-S127 antibody. Surprisingly, after taxol treatment, the levels of YAP-p127 gradually decreased after taxol treatment (Fig. 2B). Because LATS1/2 can phosphorylate YAP at five sites (15, 18), it is possible that LATS1 may phosphorylate YAP on other sites rather than S127. To test this possibility, we transfected WT YAP (YAP-HA) or YAP mutant with five LATS phosphorylation site mutations (YAP-5SA-HA) into HeLa cells. Unexpectedly, both YAP-HA and YAP-5SA-HA mutant are phosphorylated after taxol treatment (Fig. 2C). To exclude the possibility that LATS1 may phosphorylate other sites besides the Hippo-dependent five phosphorylation sites on YAP after taxol treatment, we treated LATS1−/− MEF with taxol. Western blot analysis clearly showed that YAP was still phosphorylated after taxol treatment (Fig. 2D). In addition, transient knockdown of LATS1 alone in HeLa cells by two different siLATS1 (siLATS1-A and siLATS1-B) or knockdown of both LATS1 and LATS2 by siRNAs also cannot block taxol-induced YAP phosphorylation (Fig. 2E and F). Moreover, YAP can be still phosphorylated after taxol treatment even when both Mst1 and Mst2, two major upstream kinases of LATS, were simultaneously knocked down (Fig. 2G). Together, our data convincingly demonstrate that antitubulin drugs induce YAP phosphorylation independent of the upstream Hippo signaling pathway and suggest that other kinase(s) may be responsible for YAP phosphorylation after antitubulin drug treatment.

**Identification of YAP phosphorylation sites**

To identify the kinase(s) phosphorylating YAP, we first attempted to map the YAP phosphorylation sites. Immunoprecipitated YAP from protein lysate extracted from taxol-treated HeLa cells was subjected to mass-spectrometric identification of phospho-peptides. Four YAP phosphorylation sites including S128, S138, S289, and S367 were identified and all of these sites contain a "SP" motif (Fig. 3A and Supplementary Fig. S1). To confirm these potential phosphorylation
sites, we constructed plasmids expressing various deletions and mutations of YAP (Fig. 3B and C) and transfected them into HeLa cells. Mutations of both S128 and S138 into alaines (A, S128A/S138A) rather than a single mutation alone (S128A or S138A) abolish taxol-induced YAP1-150-HA phosphorylation (Fig. 3B and C, top). Although mutation of S289 or S367 alone (S289A or S367A) only has minor effect, mutation of both sites (S289A/S367A) dramatically decreases YAP203-400-HA phosphorylation. Because S289A/S367A double mutants were unable to completely abolish YAP203-400-HA phosphorylation, it is possible that another phosphorylation site not identified by mass spectrometry may exist. By scanning YAP sequence, we identified another potential phosphorylation site, S217, which also contains "SP" motif within YAP203-400 sequence (Fig. 3B). Triple mutation S217A/S289A/S367A completely abolishes YAP203-400 phosphorylation (Fig. 3C, middle). Consistent with mass spectrometry analysis, mutation of S367 (S367A) rather than T412 (T412A), which also contains a "TP" motif, completely abolishes the phosphorylation of YAP300-504-HA. Together, through YAP deletion/mutation analysis, we have identified five novel YAP sites (YAP2L, a longer isoform of YAP, is used in the present study. Refer to Supplementary Table S4 for corresponding positions in other YAP isoforms) that are significantly phosphorylated after taxol treatment. We have further tested these five phosphorylation sites individually or in combination using full-length YAP. Mutation of only S367 rather than other five phosphorylation sites significantly but not completely reverses YAP-HA phosphorylation and band-shift, whereas mutation of all of the five phosphorylation sites (5SA-TX) completely abolishes YAP phosphorylation after taxol treatment (Fig. 3D). To further confirm this result in vivo, we raised antibody against the major YAP phosphorylation site, S367. The purified anti-pS367-YAP antibody specifically recognizes phosphorylated WT YAP rather than YAP-S367A with S367 mutation (Fig. 3E). YAP phosphorylation on S367 (pS367) is significantly increased in floating apoptotic rather than adherent live HeLa cells after taxol treatment (Figs. 1A and 3F). In addition, we found that YAP-pS367 is significantly increased in SK-BR3 cells rather than taxol-resistant SK-BR3-TR cells after taxol treatment (Fig. 3G). In summary, we have successfully identified 5 "SP" motif-containing phosphorylation sites that

Figure 3. Mapping the YAP phosphorylation sites. A, mass-spec identification of YAP phosphorylation sites (peptide sequences). The "SP" motifs are underlined. B, YAP structure, deletion constructs, and potential phosphorylation sites. TBD, tead-binding domain; TA, trans-activating domain. C, deletion/mutation mapping of YAP phosphorylation sites. D, phosphorylation of YAP and its mutants after taxol treatment. E, examination of anti-pS367 antibody specificity. YAP-WT-HA or YAP-S367A-HA was transfected into HeLa cells, followed by untreated (–) or treated (+) with 100 nmol/L taxol for 1 day. YAP-WT-HA or YAP-S367A-HA was immunoprecipitated by anti-HA antibody, followed by Western blotting using antibody specifically recognizing pYAP-S367. F, phosphorylation of YAP on S367 in vivo after taxol treatment. Western blot analysis of YAP phosphorylation by anti-phospho-YAP (S367) antibody (same lysates as used in Fig. 1B). G, loss of YAP phosphorylation on S367 after taxol treatment in drug-resistant SK-BR3-TR cells.
are responsible for YAP phosphorylation/band-shift after taxol treatment.

Identification of Cdk1 as a novel upstream kinase phosphorylating YAP after taxol treatment

Because all of the five YAP phosphorylation sites contain a "SP" motif, we used PhosphoNet Kinase Predictor program to predict potential kinase(s) phosphorylating these motifs. Seven potential kinases with high prediction score were selected as candidate kinases. By using inhibitors for each specific kinase (Supplementary Table S1), we found that inhibition of Jnk or CDK kinase completely abolishes YAP phosphorylation after taxol treatment (Fig. 4A). Because inhibitors sometimes have nonspecific activity, we directly knocked down both JNK1 and JNK2, two major JNK family members, using doxycycline-inducible shJNK1 and shJNK2. As shown in Fig. 4B and C, YAP was still phosphorylated after knockdown of both JNK1 and JNK2 (both JNK1 and JNK2 have two isoforms/bands), suggesting that JNK is not the kinase responsible for YAP phosphorylation. Supporting our unexpected data, previous studies showed that the widely used "JNK" inhibitor used in our study, SP600125, can in fact inhibit Cdk kinase nonspecifically (19). Because Cdk inhibitor also abolishes YAP phosphorylation (Fig. 4A), it is possible that Cdk kinase, specifically Cdk1/Cyclin B, a kinase complex well known to be involved in taxol-induced mitotic arrest and apoptosis (20, 21), may be essential for YAP phosphorylation. We first used several general Cdk inhibitors (ROS, Pur A, OLO II) and a Cdk1-specific inhibitor (RO-3366) to test whether they can block taxol-induced phosphorylation. Significantly, treatment of HeLa cells with all of these inhibitors completely abolishes taxol-induced YAP phosphorylation (Fig. 4D). Second, to eliminate the possibility of nonspecific

Figure 4. Cdk1 as a novel upstream kinase phosphorylating YAP after taxol treatment. A, screen for kinase responsible for YAP phosphorylation. HeLa cells were untreated (−) or treated with taxol (+) alone or together with various kinase inhibitors (Supplementary Table S1). B and C, knockdown of JNK1/2 has no effect on YAP phosphorylation. shJNK1 and shJNK2 cloned in doxycycline-inducible vector pTRIPZ were stably expressed in HeLa cells. Cells were incubated in the absence (−) or presence (+) of 1 μg/mL doxycycline for 5 days, followed by protein extraction and Western blot analysis of JNK1 and JNK2 (B). HeLa-shJNK1/2 cells were also treated with doxycycline for 5 days, followed by untreated (−) or treated (+) with 100 nmol/L taxol for 24 hours (C). D, inhibition of Cdk1/Cyclin B activity by multiple Cdk inhibitors abolishes taxol-induced YAP phosphorylation. E, knockdown of Cdk1 by siCdk1 and shCdk1 abolishes YAP phosphorylation. F, knockdown of Cyclin B by siRNA abolishes YAP phosphorylation. G, phosphorylation of YAP by Cdk1/Cyclin B in vitro. About 1 μg of GST or YAP-GST fusion protein was incubated with 20 U of Cdk1/Cyclin B, and 10 μCi γ-ATP in a kinase buffer at 37°C for 60 minutes, followed by SDS-PAGE and transfer to nitrocellulose membrane. The membrane was stained with 0.1% Ponceau S (left) and exposed to film (middle). The same membrane was later subjected to Western blot analysis using anti-pS367-YAP antibody (right). H, peptide kinase assay. In vitro kinase assays were carried out using 20 U of Cdk1/Cyclin B.


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effects caused by Cdk1 inhibitors, we transiently knockdown Cdk1 by siCdk1 or doxocycline-inducible shCdk1 alone or in combination. Surprisingly, although Cdk1 was knocked down significantly by siCdk1 or shCdk1, YAP was still partially phosphorylated, suggesting that residual Cdk1 may still be able to phosphorylate YAP. However, Cdk1 knockdown by combined effect of siCdk1 and shCdk1 plus doxocycline can completely abolish taxol-induced YAP phosphorylation (Fig. 4E). Similarly, inactivation of Cdk1/Cyclin B kinase complex by knocking down Cyclin B with siCyclin B also abolishes taxol-induced YAP phosphorylation (Fig. 4F). Our in vitro kinase assays also showed that purified Cdk1/Cyclin B kinase can directly phosphorylate purified YAP-GST rather than GST control in vitro (Fig. 4G, left and center). This result was further confirmed by Western blot analysis of the same blot using anti-phospho-YAP-S367 antibody (Fig. 4G, right) and in vitro peptide kinase assay in which WT containing S128 site rather than its mutant S128V peptide was significantly phosphorylated by Cdk1/Cyclin B in vitro (Fig. 4H). Altogether, our studies clearly demonstrated that Cdk1 is indeed the critical kinase phosphorylating YAP during antitubulin drug response. Next, we would like to know whether Cdk1 is also responsible for TAZ phosphorylation. Although only S128 in YAP is conserved in TAZ, there are other five potential "SP" or "TP" sites in TAZ that could be phosphorylated by Cdk1 after taxol treatment. To test this possibility, we have first tested TAZ phosphorylation/degradation after Cdk1 knockdown. Interestingly, TAZ phosphorylation/degradation is significantly blocked in Cdk1 knockdown HeLa cells after taxol treatment (Supplementary Fig. S2A). In addition, mutation of all potential Cdk1 phosphorylation sites containing "SP" or "TP" motif in TAZ (TAZ-6SA) also significantly block taxol-induced TAZ phosphorylation/degradation (Supplementary Fig. S2B), suggesting that TAZ may be also phosphorylated by Cdk1.

**Effect of YAP phosphorylation on its transactivating activity**

We have previously shown that interaction and activation of transcription factor TEAD4 is essential for TAZ-induced taxol resistance in mammary cells (14). To examine the effect of YAP phosphorylation on its function, we have performed Co-IP analysis of YAP and TEAD4 after taxol treatment. YAP-HA plasmid was transfected alone or together with TEAD4-FLAG into HeLa cells, followed by untreated (−) or treated (+) with taxol. B, Co-IP of YAP and its mutants with TEAD4. C, Western blot analysis of overexpressed YAP and its mutants in MCF10A cells. D, qRT-PCR analysis of Cyr61 and CTGF mRNA. Relative to the mRNA levels of Cyr61/CTGF in MCF10A–WPI (fold) was calculated for those in MCF10A–YAP-WT, SSA-TX, or SSD-TX cells. Statistical significance was performed using a two-tailed, unpaired Student t-test. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
FLAG-tagged TEAD4 in vivo in HeLa cells. Although treatment of HeLa cells has no effect on the levels of both YAP-HA and TEAD4-FLAG (for better quantification, high percentage of SDS-PAGE was run for Western blot analysis to eliminate YAP-HA band-shift in taxol-treated protein lysate), taxol treatment significantly reduced the interaction of YAP-HA with TEAD4-FLAG (Fig. 5A). However, this disruption of YAP-TEAD4 interaction was rescued after inactivation of Cdk1 by Cyclin B knockdown (Supplementary Fig. S3A). In addition, taxol treatment was unable to disrupt interaction between phosphorylation-deficient YAP-5SA-TX and TEAD4 (Supplementary Fig. S3B), suggesting that phosphorylation of YAP after taxol treatment is essential for its reduced interaction with TEAD after taxol treatment. To directly confirm that YAP phosphorylation reduces its interaction with TEAD4, we also constructed YAP mutants that change all five serine phosphorylation sites to either “A”’s (YAP-5SA-TX, inactivating mutation) or aspartic acid (YAP-5DD-TX, mimicking phosphorylation). YAP-5SA-TX is used to distinguish it from the LATS phosphorylation mutant YAP-5SA (Fig. 2C). Consistent with taxol treatment experiment, although similar levels of WT (YAP-HA) and mutant YAP were detected in cells, interaction of TEAD4-FLAG with YAP-5DD-TX-HA is significantly weaker than that with YAP-HA or YAP-5SA-TX-HA (Fig. 5B), suggesting that taxol-induced YAP phosphorylation may reduce its ability to interact with and activate transcription factor TEAD. To confirm that reduced interaction of YAP with TEAD after phosphorylation causes its decreased cotransactivating activity, we first established MCF10A mammary cells stably expressing WPI lentiviral vector, WT, or mutant YAP. Previous studies have shown that overexpression of YAP in MCF10A cells causes increased expression of CTGF and Cyr61 mRNA through interaction of YAP-5SA-TX-MCF10A (Fig. 5D). Although similar level of WT YAP and its mutants were expressed (Fig. 5C), significantly reduced levels of CTGF or Cyr61 mRNA were detected in YAP-5DD-TX-MCF10A (Fig. 5D). Together, these experiments clearly demonstrate that phosphorylation of YAP by Cdk1 after taxol treatment reduced its ability to interact with transcription factor TEAD and trans-activate its downstream genes important for taxol response.

**Effect of YAP phosphorylation on its ability to inhibit antitubulin drug-induced apoptosis**

Previous studies have shown that overexpression of YAP inhibits taxol-induced apoptosis in MCF10A cells (13). To test whether YAP phosphorylation has any effect on its ability to reduce taxol-induced apoptosis, we have established MCF10A cells stably expressing WPI vector, YAP-WT, YAP-5SA-TX, or YAP-5DD-TX (Fig. 5C). Most interestingly, although both YAP-WT and YAP-5SA-TX cause morphologic changes from epithelial (normal cell–cell contact, high epithelial marker E-cadherin, and low mesenchymal marker vimentin) to mesenchymal phenotype (loss of cell–cell contact, reduced E-cadherin, and increased vimentin) as previously observed (13), YAP-5DD-TX mutant fails to cause significant morphologic changes (Fig. 6A–C). Most significantly, consistent to morphologic changes, analysis of apoptosis by Western blot analysis of cPARP and Trypan blue exclusion assay all showed that compared with WT and YAP-5SA-TX, YAP-5DD-TX has significant decreased ability to inhibit apoptosis induced by both taxol and vinblastine (Fig. 6D and E), two commonly used antitubulin drugs for cancer treatments. On the other hand, YAP-WT and YAP-5DD-TX have similar antiapoptotic effect in response to non-antitubulin drug, cisplatin (Supplementary Fig. S4), suggesting that YAP is only phosphorylated and inactivated during antitubulin drug response.

**Discussion**

Although tremendous progress has been made toward our understanding of the roles of the Hippo pathway in tumorigenesis, size control, and stem cell renewal and differentiation (8–11), its function in chemotherapeutic drug response is largely unknown (23). We and others have recently provided initial evidence that the core components of the Hippo pathway, LATS1/2, YAP, and TAZ, may be involved in the response of cancer cells to chemotherapeutic drugs such as taxol (14, 24–26). However, the upstream signaling pathway(s) or gene(s) transducing signals from drug treatment to the Hippo pathway remain elusive. In this study, we have shown for the first time that Cdk1 is a novel kinase responsible for inactivation of YAP during antitubulin drug-induced apoptosis. We found that YAP is specifically phosphorylated in floating apoptotic cells rather than adherent survival cells after antitubulin drug treatment (Fig. 1A and B). Significantly, we have shown that activated Cdk1 can directly phosphorylate YAP both in vitro and in vivo, which subsequently caused reduced cotransactivating and antiapoptotic activity of YAP. Although many genes have been identified to affect the sensitivity of cancer cells to antitubulin chemotherapeutics and it is well known that aberrant activation of Cdk1/Cyclin B is critical for antitubulin drug-induced apoptosis (20, 21), only a few genes are identified to be directly phosphorylated and regulated by Cdk1/Cyclin B (21, 27). Therefore, our studies provide the first biologic evidence that the Hippo core component YAP is a sensor for Cdk1 kinase activity and a critical link coupling Cdk1 activation to antitubulin drug-induced apoptosis.

Although the Hippo pathway (Mst1/2/LATS1/2-YAP/TAZ) has been shown to play important roles in many biologic processes, mounting evidence suggested that Hippo-independent pathways upstream of YAP/TAZ might be also important. For example, it was recently shown that YAP and TAZ are activated by cell shape, cytoskeletal, and mechanical cues independent of its upstream kinases Mst1/2 and LATS1/2 (11, 28, 29). Therefore, it has been suggested that YAP and TAZ are central players of the Hippo pathway and different stimuli may induce various biologic changes by regulating YAP/TAZ through multiple signaling pathways (30). In this study, we have identified another novel Hippo-independent pathway modulating YAP. In this pathway, antitubulin drugs induce microtubule tension by causing increased microtubule polymerization or depolymerization (22, 21), which subsequently causes increased Cyclin B levels (Fig. 4F) and activation of
Cdk1 kinase (Fig. 1D). Activated Cdk1 in turn phosphorylates YAP on five sites (S128, S138, S217, S289, and S367), which inactivate YAP function as a transcriptional coactivator and an antiapoptotic protein, leading to apoptotic cell death.

Although TAZ is a paralog of YAP and has overlapping function in various biologic functions (8), it is still unclear why YAP and TAZ have different response to antitubulin drug treatment. As shown in Fig. 1D, while YAP is phosphorylated only in mitotic arrested and apoptotic cells as indicated by reduced pCdk1 and increased cPARP, TAZ is phosphorylated and degraded in all apoptotic cells induced by almost all of the chemotherapeutics used independent of mitotic arrest, suggesting that YAP and TAZ may function differently in response to taxol. In addition, we also found that TAZ phosphorylation/degradation is significantly blocked in Cdk1 knockdown HeLa cells after taxol treatment (Supplementary Fig. S2A). These findings suggest that TAZ may be involved in apoptosis in Cdk1-dependent and/or Cdk1-independent pathways.

Most interestingly, three (S138, S289, and S367) out of five Cdk1 phosphorylation sites in YAP identified in our studies have already been identified previously in a mass spectrometry mapping of YAP phosphorylation sites under physiologic conditions (31). Because Cdk1/Cyclin B kinase complex is also activated during G2–M phase of the cell cycle, it is possible that YAP is phosphorylated by Cdk1 during mitosis. It will be very interesting to further study whether YAP is differentially phosphorylated during mitosis and under microtubule damage induced by antitubulin drugs. Moreover, we have found that phosphorylation of YAP abolishes its ability to induce morphologic changes such as EMT indicated by reduced E-cadherin and enhanced vimentin (Fig. 6A–C). Because there is a strong link between EMT and drug resistance (32), it is possible that phosphorylation and inactivation of YAP by Cdk1 may compromise its ability to trans-activate another transcription factor that suppresses E-cadherin and induces EMT. Moreover, because EMT plays important roles in stem cell renewal, cell migration, anoikis, invasion, and metastasis (33, 34), it will be also interesting to further explore how phosphorylation of YAP under physiologic condition regulates these important biologic processes.

In this study, we have established YAP as an antiapoptotic protein important for antitubulin drug resistance. We have shown that overexpression of YAP-WT and its phosphorylation mutant YAP-SSA-TX rather than its phosphomimic mutant YAP-SSD-TX causes resistance of MCF10A mammary cells to both taxol and vinblastine (Fig. 6D and E). Compared with YAP-WT, similar resistance to antitubulin drugs was found for YAP-SSA-TX in MCF10A cells. Because high levels of both YAP-WT and YAP-SSA-TX are expressed in MCF10A cells, most of the overexpressed YAP-WT and YAP-SSA-TX proteins may not be phosphorylated and inactivated by endogenous Cdk1, which protects MCF10A cells from

Figure 6. Phosphorylation of YAP by Cdk1 reduces its inhibition of antitubulin-induced apoptosis. A, morphologic changes after overexpression of YAP and its mutants in MCF10A cells. B, Western blot analysis of E-cadherin and vimentin in MCF10A cells expressing YAP and its mutants. C, immunofluorescent staining of E-cadherin (red) in MCF10A cells expressing YAP and its mutants. DAPI (3 μg/mL) was used to stain nuclear DNA (blue). D, Western blot analysis of cPARP after taxol and vinblastine treatment. E, Trypan blue exclusion analysis of cell death. Each drug treatment was repeated three times and the curve graph was made based on the average data of three times experiments. *, significant difference between YAP-WT and YAP-SSD-TX.
undergoing apoptosis. On the other hand, most of the phosphor- ylation mimicking YAP-5SD-TX proteins are already inactivated and have compromised antiapoptotic activity, which results in reduced protection from antitubulin drug-induced apoptosis. In addition, by using established taxol-resistant SK-BR3 breast cancer cells and their counterparts, we have found that YAP is not phosphorylated after taxol treatment in drug-resistant SK-BR3 cells (Figs. 1H and 3G), suggesting that resistance to Cdk1-induced YAP phosphorylation may be also important for taxol resistance in breast cancer. These findings have important clinical implications. Further examination of the relationship between the levels and phosphorylation status of YAP and the survival of clinical cancer patients before and after treatment with antitubulin drugs using tissues derived from patient with cancer will finally establish YAP and pYAP as prognostic biomarkers for predicting the sensitivity of patients with cancer to antitubulin drugs and potential therapeutic targets for the treatment of drug-resistant patients with cancer in the future.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

References


YAP-Induced Resistance of Cancer Cells to Antitubulin Drugs Is Modulated by a Hippo-Independent Pathway

Yulei Zhao, Prem Khanal, Paul Savage, et al.


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