CDK1 Phosphorylation of YAP Promotes Mitotic Defects and Cell Motility and Is Essential for Neoplastic Transformation

Shuping Yang\textsuperscript{1,2}, Lin Zhang\textsuperscript{1,2}, Miao Liu\textsuperscript{2}, Rong Chong\textsuperscript{1}, Shi-Jian Ding\textsuperscript{2,3}, Yuanhong Chen\textsuperscript{1} and Jixin Dong\textsuperscript{1,*}

\textsuperscript{1}Eppley Institute for Research in Cancer and Allied Diseases,

\textsuperscript{2}Department of Pathology and Microbiology,

\textsuperscript{3}Mass Spectrometry and Proteomics Core Facility,

University of Nebraska Medical Center, Omaha, NE 68198

Running title: Mitotic phosphorylation regulates YAP activity

* To whom correspondence should be addressed

Eppley Institute for Research in Cancer and Allied Diseases
University of Nebraska Medical Center
985950 Nebraska Medical Center
Omaha, NE  68198-5950
phone: 402-559-5596; fax: 402-559-4651; email: dongj@unmc.edu

\begin{itemize}
\item No potential conflicts of interest were disclosed.
\item This work was supported in part by grant 8P20 GM103489 from the National Institutes of Health (to J.D.).
\item Word count: 5492. This manuscript contains 6 figures and 4 supplemental figures.
\end{itemize}
Abstract

The Yes-associated protein YAP is a downstream effector of the Hippo pathway of cell cycle control which plays important roles in tumorigenesis. Hippo-mediated phosphorylation YAP, mainly at S127, inactivates YAP function. In this study, we define a mechanism for positive regulation of YAP activity that is critical for its oncogenic function. Specifically, we found that YAP is phosphorylated in vitro and in vivo by the cell cycle kinase CDK1 at T119, S289, and S367 during G2/M phase of the cell cycle. We also found that ectopic expression of a phosphomimetic YAP mutant (YAP3D, harboring T119D/S289D/S367D) was sufficient to induce mitotic defects in immortalized epithelial cells, including centrosome amplification, multipolar spindles and chromosome missegregation. Finally, we documented that mitotic phosphorylation of YAP was sufficient to promote cell migration and invasion in a manner essential for neoplastic cell transformation. In support of our findings, CDK1 inhibitors largely suppressed cell motility mediated by activated YAP-S127A but not the phosphomimetic mutant YAP3D. Collectively, our results reveal a previously unrecognized mechanism for controlling the activity of YAP that is crucial for its oncogenic function mediated by mitotic dysregulation.
Keywords: YAP; Hippo pathway; mitotic phosphorylation; CDK1; mitotic defects

Introduction

Studies from Drosophila have defined the Hippo signaling pathway (1). Genetically engineered mouse models demonstrated that the Hippo pathway is highly conserved in mammals and controls organ size, tumorigenesis, cell contact inhibition, and stem cell self-renewal by regulating cell proliferation and apoptosis (2-4). The core of the Hippo pathway is a kinase cascade including the tumor suppressors Mst1/2 (Hippo in Drosophila), Lats1/2 (Warts in Drosophila) and the oncoproteins YAP/TAZ (Yorkie in Drosophila). Mst1/2, in complexes with WW45 (Salvador in Drosophila), phosphorylates and activates Lats1/2. Activated Lats1/2 (together with Mob1) phosphorylates and inactivates the transcriptional co-activators YAP/TAZ. The transcription factors TEAD1-4 (Scalloped in Drosophila) are the main mediators for YAP’s oncogenic function (5, 6).

Recent work has identified lysophosphatidic acid, sphingosine-1-phosphate as ligands, and G-protein coupled receptors as receptors for the Hippo pathway (7-9). YAP is phosphorylated at S127 and S381 by Lats1/2 kinases and inactivated by cytoplasmic sequestration and ubiquitination-dependent degradation (10-13).
YAP promotes tumorigenesis in several types of cancer, including hepatocellular carcinoma (HCC) (13) and skin cancer (14). It is not surprising that YAP is amplified or overexpressed/hyperactivated in many types of human cancer (13, 15). Accordingly, genetic ablation of upstream tumor suppressors (for example, Mst1/2, WW45, and Mob1a/1b) in mice also leads to formation of many types of tumors including HCC (16-20).

Although extensive studies have demonstrated the important roles for the Hippo pathway in tumorigenesis, the underlying mechanisms are still unclear. Interestingly, recent studies demonstrated that several key members of the Hippo pathway, such as Mst1/2, Lats1/2, WW45, Mob1 are involved in regulating mitosis (21). Aberration of mitosis often causes genome instability/aneuploidy and subsequent tumor formation. Thus, the Hippo pathway may contribute to cancer development by regulating mitosis-related events.

We recently reported that KIBRA (an upstream regulator of the Hippo pathway) is required for chromosome alignment and proper microtubule organization during mitosis (22). Furthermore, KIBRA is phosphorylated by mitotic kinases Aurora and CDK1 during mitosis (23, 24). These studies prompted us to examine whether YAP, the most critical
growth mediator of the Hippo pathway, is regulated during mitosis. We show that YAP is also hyperphosphorylated during G2/M phase. We further characterized the phosphorylation sites, the corresponding kinase, and the functional significance of the phosphorylation. Our data reveal a new layer of regulation for YAP activity, implicating that YAP exerts its oncogenic function through dysregulation of mitosis.

Materials and Methods

Expression constructs

YAP-S381A, YAP with mutations in the WW domains, and YAP-5SA plasmids were purchased from Addgene. pcDNA-YAP (no tag) expression constructs have been described (13). To make HA-tagged human YAP, full-length YAP cDNA (IMAGE clone 5747370, contains 2 WW domains and 504 amino acids) was subcloned into the pcDNA3.1+HA vector (25). To make the retroviral-mediated YAP expression construct, the above cDNA was cloned into MaRX™IV vector (25). Point mutations were generated by the QuickChange Site-Directed PCR mutagenesis kit (Stratagene) and verified by sequencing.

Cell culture and transfection
HEK293T, HeLa, MCF-7, and MCF10A cell lines were purchased from American Type Culture Collection (ATCC). The cell lines were authenticated at ATCC and were used at low (<20) passages. MCF-10A cells were cultured as described (10). H2229, H1277, T47D, MDA-MB-231, and HCT-116 cell lines were maintained in DMEM media supplemented with 10% FBS. Attractene and HiPerFect (Qiagen) were used for transient overexpression and siRNA transfections, respectively following the manufacturer’s instructions. Nocodazole (100 ng/ml for 16-20 h) and Taxol (0.1 µM for 16 h) were used to arrest cells in G2/M phase unless otherwise indicated. Etoposide and doxorubicin were also from Sigma. YAP siRNA was synthesized by GenePharma based on the following target sequence (YAP-1: 5’-CAGGTGATACTATCAACCAAA-3’; YAP-2: 5’-GACCAATAGCTCAGATCCTTT (selected by Invitrogen online software). All other chemicals were either from Sigma or Thermo Fisher.

**Kinase inhibitors**

VX680 (Aurora-A, -B, -C inhibitor), ZM447439 (Aurora-B, -C inhibitor), and BI2536 (Plk1 inhibitor) were from Selleck Chemicals. U0126 (MEK-ERK inhibitor), SB203580 (p38 inhibitor), LY294002 (PI-3K inhibitor), rapamycin (mTOR inhibitor), and SP600125 (JNK inhibitor) were from LC Laboratories. RO-3306 (CDK1 inhibitor) and roscovitine (CDKs
inhibitor) were from ENZO Life Sciences. MK5108 (Aurora-A inhibitor) was from Merck. SB216763 (GSK3β inhibitor) and anisomycin (JNK activator) were from Sigma.

**Recombinant protein purification**

The GST-tagged proteins were bacterially expressed and purified on GSTrap FF affinity columns (GE Healthcare) following the manufacturer’s instructions. To make His-tagged human YAP, full-length YAP cDNA was subcloned into the pET-21c vector (Novagen/EMD Chemicals). The proteins were expressed and purified on HisPur™ Cobalt spin columns (Pierce) following the manufacturer’s instructions.

**Identification of phosphorylation sites by mass spectrometry**

Endogenous YAP from HeLa cells treated with Taxol was immunoprecipitated and stained with Coomassie blue. The up-shifted YAP bands were sliced and in-gel digested as described (26). NanoLC-MS/MS (nanoscale liquid chromatography followed by tandem mass spectrometry) was performed with an in-house built nanoLC system (27) coupled with an LTQ (linear ion trap)-Velos mass spectrometer (Thermo Scientific). Survey full scan MS spectra (from m/z 375 to 1,700) were acquired in the LTQ-Velos with resolution of 6,000. The 20 most intense ions (depending on signal intensity) were
sequentially isolated for fragmentation in the linear ion trap by collision-induced
dissociation. The capillary was maintained at 200°C, the spray voltage was kept at 2.3
kV. DeconMSn was used to determine and refine the monoisotopic mass and charge
state of parent ions from the LTQ-Velos raw data, and to create a peak list of these ions
in .mgf format. The “.mgf” files were searched against the human International Protein
Index (IPI) protein sequence database (Version 3.52) which contained the normal IPI
human proteins, commonly observed contaminants and the reverse sequences of all
proteins with the OMSSA search engine (version 2.1.9, NCBI) (28). In the database
search, carbamidomethylation of cysteine was set as the fixed modification. Oxidation of
methionine, phosphorylation of serine, threonine and tyrosine were set as the variable
modifications. The precursor tolerance was set as 1.5 Da and MS2 tolerance was 0.5
Da. E-value cut-off was set at 0.1. The false discovery rate (FDR) was made 1% by
filtering on the E-value of all forward and reversed peptide identifications. The spectra
were manually checked.

**In vitro kinase assay**

1-2 µg of His-YAP was incubated with 10 U recombinant CDK1/cyclin B complex (New
England Biolabs) or 100 ng CDK1/cyclin B (SignalChem) or HeLa cell total lysates
(treated with DMSO or Taxol) in kinase buffer (23) in the presence of 5 µCi γ-32P-ATP (3000 Ci/mmol, PerkinElmer). MEK1, ERK1, and p38α active kinases were purchased from SignalChem. Myelin basic protein (MBP) (Sigma) was used for positive control. The samples were resolved by SDS-PAGE, transferred onto PVDF (Millipore) and visualized by autoradiography followed by Western blotting or detected by phospho-specific antibodies.

**Antibodies**

The YAP antibodies from Abnova (H00010413-M01) and Abcam (52771) were used for immunoprecipitation of endogenous YAP and for Western blotting, respectively throughout the study. Rabbit polyclonal phospho-specific antibodies against YAP S367, S289, and T119 were generated and purified by AbMart. HA antibodies were from Sigma. Anti-β-actin, anti-ERK1/2, and anti-cyclin B antibodies were from Santa Cruz Biotechnology. Anti-Aurora-A, anti-glutathione S-transferase (GST), anti-His, anti-Mst1, anti-Mst2, anti-Lats1, and anti-Lats2 antibodies were from Bethyl Laboratories. Anti-phospho-Aurora-A,B,C, anti-phospho-S10 H3, anti-phospho-T202/Y204 ERK1/2, anti-phospho-S127 YAP, anti-phospho-T180/Y182 p38, anti-phospho-c-Jun, anti-phospho-Mst1/2, anti-phospho-Lats1/2, anti-phospho-S345 Chk1, anti-p38, anti-WW45, anti-TAZ,
anti-NF2, anti-Mob1, and anti-Cdc2 antibodies were from Cell Signaling Technology. Anti-Plk1 and anti-phospho-T210 Plk1 antibodies were obtained from Biolegend. Anti-α-tubulin (Abcam), anti-β-tubulin (Sigma), anti-γ-tubulin (Biolegend) antibodies were used for immunofluorescence staining.

**Immunoprecipitation, Western blot analysis, and lambda phosphatase treatment**

Immunoprecipitation, Western blotting, and lambda phosphatase treatment assays were done as previously described (23).

**Immunofluorescence staining and confocal microscopy**

Cell fixation, permeabilization, fluorescence staining, and microscopy were done as previously described (22). For peptide blocking, a protocol from Abcam website was used. Briefly, the phospho-YAP antibodies were first neutralized by an excess of immunizing (phosphorylated) peptides (1 µg/ml for 1 h at room temperature). The antibody (containing the phospho-peptide) was then used for staining in parallel with staining using antibodies with no peptide or non-phospho-peptide.

**Colony formation, cell migration, and invasion assays**
Colony formation assays in soft agar were performed as described (13). In vitro analysis of invasion and migration was assessed using the BioCoat invasion system (BD Biosciences) and Transwell system (Corning), respectively, according to the manufacturer's instructions. The invasive and migratory cells were stained with ProLong® Gold Antifade Reagent with DAPI. The relative invading and migrating rate were calculated by the number of cells invading and migrating through the membrane, divided by the number of cells that invaded and migrated in the control group.

**Statistical analysis**

Statistical significance was performed using a two-tailed, unpaired Student’s $t$-test.

**Results**

**YAP is phosphorylated during anti-mitotic drug-induced G2/M arrest**

To further explore whether other members of the Hippo pathway are regulated during mitosis, we treated HeLa cells with Taxol or nocodazole (both agents arrest cells in G2/M) and systematically examined the responses of the Hippo pathway during G2/M arrest. As shown in Figure 1A, the most prominent change we observed was the dramatic mobility up-shift of YAP and, to a lesser extent, of TAZ. The phosphorylation
level of YAP at S127 (the major phosphorylation site regulated by the Hippo pathway) was not altered, suggesting that the mobility shift of YAP was likely not due to the phosphorylation at S127. Taxol or nocodazole treatment did not cause any evident change in the expression levels for NF2, Mst1/2, WW45, Mob1, or in the activity of Mst1/2 and Lats1/2 revealed by phospho-antibodies (Fig. 1A). Consistent with previous reports, we detected a mobility up-shift of Lats1 (due to mitotic phosphorylation) (29, 30) and a significant increase of Lats2 expression (31) during Taxol or nocodazole treatment (Fig. 1A). The mobility up-shift of YAP was also evident in breast (MCF-7, T47D, and MDA-MB-231), colon (HCT-116) and lung (H1299 and H2227) cancer cell lines during Taxol-arrested mitosis (Fig. 1B). The shift was detectable as early as 4 h after 100 nM Taxol treatment, and only 10 nM of Taxol (for a 16 h treatment) was sufficient to induce the shift in HeLa cells (Fig. 1C). Taken together, YAP mobility is significantly retarded during anti-mitotic drug-induced G2/M arrest.

YAP is a phospho-protein whose mobility is retarded on SDS-polyacrylamide gels when phosphorylated (10, 13). Lambda phosphatase treatment completely converted all slow-migrating bands to fast-migrating bands, indicating that the mobility shift of YAP during G2/M is caused by phosphorylation (Fig. 1D). To further test whether YAP
phosphorylation is specific to mitosis, we collected mitotic cells by mechanical shake-off from Taxol-treated cells. As shown in Figure 2A, mitotic cells expressed exclusively phosphorylated YAP while YAP is not phosphorylated/shifted in attached non-mitotic cells, suggesting that YAP phosphorylation is specifically associated with G2/M cell cycle arrest.

Previous reports showed that YAP was phosphorylated by c-Abl, p38, and JNK kinases in response to DNA damage (32-34). We further explored whether DNA-damaging agents can cause such a mobility shift of YAP. As shown in Figure 2B, in contrast to YAP shift during mitosis, treatment with doxorubicin, etoposide, UV or ionizing radiation (IR) failed to cause any obvious change on YAP mobility. Increased activity of Chk1 revealed by phospho-S345 antibody indicated these treatments were effective. These data further suggest that the mobility shift/phosphorylation of YAP is a specific response during cell cycle G2/M.

Identification of the corresponding kinase(s) for YAP phosphorylation

Upon treatment of anti-mitotic agents, several mitotic kinases, including Aurora and Plk1, are activated during mitosis (35). Inhibitors for these kinases are widely used.
Inhibition of Aurora-A (with MK5108), or Aurora-B, C (with ZM447439), or Aurora-A, B, C (with VX-680) kinases did not alter the YAP phosphorylation. The treatments with these inhibitors were effective, as revealed by the phospho-Aurora antibody (Supplemental Fig. S1A). Addition of BI2536 (an inhibitor of Plk1 kinase) did not reverse the YAP mobility shift/phosphorylation either (Supplemental Fig. S1B). It has been reported that MEK-ERK signaling is also activated during Taxol treatment (36). However, in our hands, MEK-ERK activity was strongly inhibited upon treatment with Taxol (24, 37) (Supplemental Fig. S1C). Thus MEK-ERK kinases are not likely the kinases responsible for YAP phosphorylation under anti-mitotic drug-induced mitosis, and treatment with U0126 (an inhibitor for MEK-ERK kinases) did not affect the YAP phosphorylation (Supplemental Fig. S1C). Additionally, inhibition of PI-3K (with LY294002), mTOR (with rapamycin), MAPK-p38 (with SB203580) and GSK-3β (with SB216763) did not affect the phosphorylation of YAP induced by Taxol treatment (Supplemental Fig. S1D).

CDK1 is a master regulator of cell cycle and is activated during normal and drug-arrested G2/M (35, 38). JNK1/2 kinases are also activated upon Taxol treatment (Fig. 2C, revealed by increased p-c-Jun levels). We tested whether CDK1 and/or JNK1/2
kinases are responsible for YAP phosphorylation. As shown in Figure 2C (left panel), both RO3306 (a CDK1 inhibitor) and SP600125 (a JNK1/2 inhibitor) completely reverted the mobility shift of YAP. These drugs are known to induce mitotic exit as shown by the complete loss of phospho-Aurora and degradation of cyclin B. We treated the cells with MG132 along with RO3306 or SP600125 to prevent cyclin B degradation and cells exiting from mitosis. Under these conditions, RO3306, but not SP600125, was still able to completely inhibit YAP phosphorylation (Fig. 2C). In the presence of RO3306, YAP was no longer phosphorylated even when JNK1/2 kinases were strongly activated (Fig. 2C, lanes 3 and 7), suggesting that CDK1, but not JNK1/2 kinases, is likely to be responsible for YAP phosphorylation. Furthermore, another CDK1 inhibitor (roscovitine) also completely inhibited YAP phosphorylation (Fig. 2D). Taken together, these data strongly suggest that YAP phosphorylation induced by Taxol treatment is CDK1 dependent and is independent of JNK1/2 kinases.

**CDK1 phosphorylates YAP in vitro**

To determine whether CDK1 kinase can directly phosphorylate YAP, we performed in vitro kinase assays with His-tagged YAP as substrates. Figure 2E shows that Taxol-treated mitotic lysates robustly phosphorylated YAP and that CDK1 depletion greatly
reduced phosphorylation of His-YAP (top row, compare lanes 4 to 3). As expected, purified CDK1/cyclin B complex phosphorylated His-YAP in vitro (Fig. 2F). These results indicate that CDK1 directly phosphorylated YAP in vitro. YAP is not a suitable substrate for MEK1, ERK1, and MAPK-p38α kinases in vitro (34) (Supplemental Fig. S1E).

**Identification of phosphorylation sites on YAP**

Next, we set out to map the phosphorylation site on YAP. YAP was immunoprecipitated from Taxol-treated HeLa cells, Coomassie stained (Supplemental Fig. S2A inset), and the shifted bands were excised and subjected to Mass/OB-LCA. The following four sites were identified: Serine-109, Threonine-119, Serine-289, and Serine-367 (Fig. 3A; Supplemental Fig. S2A-C). S109 is one of the Hippo-mediated phosphorylation sites (10, 12). The rest of the three sites all fit the proline-directed consensus sequence (Fig. 3A) of CDK1-phosphorylation sites (39). Interestingly, all these three sites have been identified as mitotic phosphorylation sites from large scale proteomic studies (40, 41).

We next examined whether these sites affect the mobility of YAP during Taxol treatment. YAP mutated S367 or T119 (to alanine) had a reduced mobility shift when compared to wild-type YAP (Fig. 3B, compare lanes 6, 4 to lane 2). S289A mutation had
no effect on YAP mobility induced by Taxol (Fig. 3B, compare lanes 8 to 2). No further decrease on YAP mobility was observed when T119 and S289, or S289 and S367 were mutated to non-phosphorylatable alanine (Fig. 3B, compare lanes 10 to 6; lanes 14 to 4). However, double mutation of T119A and S367A or triple mutation of all three sites largely abolished the mobility shift of YAP, suggesting that T119 and S367 are the main sites responsible for mobility shift of YAP upon Taxol treatment (Fig. 3B).

For comparison, we also tested whether some other known phosphorylation or binding sites are involved in the YAP mobility shift induced by Taxol treatment. Mutating the Hippo phosphorylation sites (S127, S381, 5SA: S381A/S164A/S127A/S109A/S61A) (10, 12, 13) or TEAD binding site (S94) (5) or the c-Abl phosphorylation site (Y407) (33) did not affect the YAP shift induced by Taxol in HEK293T cells (Supplemental Fig. S2D). The WW domain mutations (W199A/P202A and/or W258/P261A) did not affect the Taxol-induced YAP shift either (data not shown). Together, our data identified novel phosphorylation of YAP during Taxol-arrested G2/M.

**CDK1/cyclin B complex phosphorylates YAP at T119 and S289 in vitro and in cells**
We have generated phospho-specific antibodies against T119, S289, and S367. Using these antibodies we demonstrated that CDK1 robustly phosphorylated YAP at T119, S289 and at S367 as well *in vitro* (Fig. 3C). Addition of RO3306 abolished the phosphorylation (Fig. 3C). To explore whether T119 and S289 are also phosphorylated within cells during Taxol-induced G2/M arrest, we transfected YAP or corresponding non-phosphorylatable mutants into cells, treated the cells with Taxol, and determined levels of phosphorylation by phospho-antibodies. Taxol treatment significantly increased the phosphorylation of T119 and S289, and the signal was abolished by mutating T119 or S289 to alanine (Fig. 3D). Taxol treatment also significantly increased the phosphorylation of T119 and S289 in immunoprecipitated endogenous YAP (Fig. 3E). As expected, no signal was detected in control (IgG) immunoprecipitates, suggesting that these antibodies specifically recognize phosphorylated YAP. Lambda phosphatase treatment completely abolished the signal, further confirming the specificity of the phospho-specific antibodies (Fig. 3F). Using inhibitors for CDK1 kinase, we demonstrated that phosphorylation of YAP T119 and S289 is CDK1 kinase dependent (Fig. 3F). Taken together, these results indicate that YAP is phosphorylated at T119 and S289 in cells during anti-mitotic drug-induced G2/M arrest in a CDK1-dependent manner.
Phosphorylation of YAP occurs in cells during normal mitosis

To determine whether mitotic phosphorylation of YAP occurs during unperturbed/normal mitosis, we collected samples from a double thymidine block and release (22) and determined the phospho-levels of YAP during different cell-cycle phases. Figure 4A shows that phosphorylated YAP was readily detected in cells 8-10 hours being released from double thymidine block, which is coincident with increased levels of cyclin B and phospho-H3 S10 (both of which are mitotic markers). The signal was diminished when cells exit mitosis (Fig. 4A, lane 4). These data strongly suggest that YAP is phosphorylated at T119 and S289 during normal mitosis.

To further investigate the dynamics of YAP phosphorylation in cells, we performed immunofluorescence microscopy with these phospho-specific antibodies. A strong signal was detected in nocodazole-arrested prometaphase cells for both antibodies against S289 and T119 (Supplemental Fig. S3A, B, top panels, red arrows). Very low or no signal was detected in interphase cells (Supplemental Fig. S3A, B, yellow arrows). Importantly, phosphopeptide-, but not non-phosphopeptide-, incubation completely blocked the signal, suggesting that these antibodies specifically detect phosphorylated YAP (Supplemental Fig. S3A, B, low panels). The specificity of the antibodies was
further confirmed by siRNA knockdown of YAP (Supplemental Fig. S3C, D). We found that the p-YAP-S289 signal was clearly increased in prophase and peaked in prometaphase/metaphase. The signal was then weakened during metaphase to anaphase transition and further diminished in telophase and cytokinesis (Fig. 4B). Similar staining patterns were generated with p-YAP-T119 antibody staining (Fig. 4C; Supplemental Fig. S4). These data further demonstrate that mitotic phosphorylation of YAP occurs dynamically in cells. Addition of RO3306 or roscovitine largely abolished the signals detected by p-YAP S289 and T119 antibodies in mitotic cells, further indicating that the phosphorylation is CDK1 dependent (Fig. 4D).

**Phospho-mimetic YAP induces mitotic abnormalities in immortalized cells**

Some of the Hippo components regulate mitotic events including chromosome alignment, centrosome duplication, and microtubule dynamics (21). We next examined whether YAP or its phosphorylation is able to trigger mitotic defects. The immortalized epithelial cell line MCF10A stably expressing vector, YAP, and YAP3D (a phospho-mimetic mutant) were used for this purpose (Fig. 5A). As expected, immunofluorescence staining with α-tubulin and γ-tubulin showed normal microtubule/spindle formation and centrosome number during mitosis in control cells (Fig. 5B). Overexpression of wild-type
YAP is not sufficient to cause significant mitotic defects in MCF10A cells (Fig. 5D-F). In contrast, mitotic abnormalities were detected in a significant higher percentage of cells expressing YAP3D (3-4 fold). There is a threefold increase of number of YAP3D-expressing cells with disorganization of microtubules and formation of multipolar spindles when compared with control cells (Fig. 5B, D). We also found the percentage of cells with more than 2 centrosomes (γ-tubulin staining) significantly increased in YAP3D-expressing cells (Fig. 5B, E). As expected, massive chromosome misalignment, chromosome lagging, and chromosome missegregation were observed in about 25% of YAP3D-expressing cells (Fig. 5B, C, F). These data suggest that ectopic expression of phospho-mimetic YAP, but not wild-type YAP, is sufficient to trigger mitotic abnormalities in immortalized epithelial cells.

Mitotic phosphorylation of YAP is required for cellular transformation

Overexpression of YAP transforms MCF10A cells (15). We further examined the biological significance of mitotic phosphorylation of YAP using these cell lines stably expressing YAP or YAP mutants (Fig. 6A). MCF10A cells expressing YAP-S127A formed colonies in soft agar, however, MCF10A-YAP4A (S127A/T119A/S289A/S367A) cells failed to produce any obvious colonies (Fig. 6B, C). YAP3D overexpression is not
sufficient to stimulate anchorage-independent growth in soft agar (data not shown). YAP or YAP-3A (T119A/S289A/S367A) overexpression failed to produce colonies in soft agar (Fig. 6B and data not shown). Together, these data strongly suggest that mitotic phosphorylation is required for YAP-mediated cellular transformation in MCF10A cells.

**Mitotic phosphorylation of YAP promotes cell migration and invasion**

Recent reports showed that YAP/YAP-S127A also promotes migration and invasion *in vitro* (42) and metastasis *in vivo* (43). Interestingly, centrosome amplification has been correlated with cancer invasiveness and enhances migration and invasion of malignant cells via modulation of microtubule cytoskeleton (44). We therefore tested whether mitotic phosphorylation of YAP is involved in cell motility. As expected, ectopic expression of YAP and YAP-S127A increased migration (Fig. 6D) and invasion (Fig. 6E) of MCF10A cells. Mutating CDK1-mediated phosphorylation sites to alanine (YAP-4A) dramatically suppressed YAP-S127A-mediated migration (Fig. 6D) and invasion (Fig. 6E). Interestingly, cells expressing YAP3D possess much higher migration and invasion activity than cells expressing wild-type YAP (Fig. 6D, E). These data suggest that mitotic phosphorylation of YAP promotes cell motility in immortalized epithelial cells.
The above observations indicate that elimination of phosphorylation of YAP by CDK1 inhibitors reduces YAP, but not YAP3D, -mediated cell motility. Indeed, addition of RO3306 almost completely suppressed YAP-S127A-driven migration (Fig. 6F) and invasion (Fig. 6G). Importantly, YAP3D-mediated migration and invasion was not affected by the presence of RO3306 (Fig. 6F, G). Collectively, these data strongly indicate that YAP promotes migration and invasion in a CDK1-phosphorylation dependent manner.

Discussion

While the role of the Hippo pathway in tumorigenesis has been firmly demonstrated in several types of cancer, the underlying mechanisms are less clear. Recent reports support the notion that Hippo pathway plays critical roles in maintaining normal mitosis and that inactivation of key members of the Hippo pathway (including Lats2, Mst1/2, Mob1, WW45) leads to mitotic defects in multiple processes including centrosome maturation and disjunction, chromosome alignment, and cytokinesis (19, 45, 46). Mitotic aberrations cause aneuploidy or chromosome instability (47), which is often associated with tumorigenesis (48). The current study identified novel phosphorylation of YAP during mitosis and the mitotic phosphorylation controls YAP’s oncogenic activity.
Importantly, YAP-3D (a phosphorylation mimetic mutant), but not wild-type YAP, drives mitotic defects (Fig. 5). Thus, our data suggest there may be a positive layer of regulation for YAP activity during tumorigenesis, and highlight a previously unrevealed mechanism through which YAP exerts its oncogenic function (Fig. 6H). Interestingly, increased CDK1 activity also promotes defects in various mitosis-related processes and aneuploidy, and CDK1 overexpression is often observed in many types of cancer (49). Therefore, it is also possible YAP is one of the critical substrates of CDK1 that mediates CDK1-driven mitotic defects. There are several important questions need to be answered in the future. For example, does CDK1 phosphorylation of YAP impact its transcriptional activity? Does this phosphorylation affect YAP’s binding partners, including the transcription factors TEAD1-4? Furthermore, future studies are required to address the in vivo relevance of CDK1 phosphorylation of YAP and whether this phosphorylation occurs in human cancer patients. Addressing these unanswered questions should further strengthen the biological significance of CDK1 phosphorylation of YAP.

Compelling evidence clearly indicates that supernumerary centrosome is one of the hallmarks of cancer and has been correlated with metastatic progression (44, 50).
migration and invasion are critical processes for metastasis, which accounts for the majority of cancer-related deaths. YAP hyperphosphorylation induces supernumerary centrosomes (Fig. 5) and promotes migration and invasion (Fig. 6). These data suggest a novel mechanism in which YAP promotes migration and invasion through centrosome amplification in a CDK1-phosphorylation dependent manner. YAP plays important roles in cancer development and metastasis in several types of cancer and remains as an attractive target for cancer therapy (3). We found that inhibition of CDK1 activity substantially impaired YAP-driven migration and invasion (Fig. 6F, G). Since R-roscovitine (Selicilib) has been in phase I clinical trials with low toxicity (51), our findings support the feasibility of using CDK1 inhibitors in human cancers especially for those in which the Hippo-YAP signaling is dysregulated.

Upon treatment with anti-mitotic agents, one of the prominent responses of the Hippo pathway is the marked increase in the phosphorylation of YAP (Fig. 1) and KIBRA (23). Interestingly, the Hippo pathway core components Mst1/2 and Lats1/2 have also been shown to be regulated during mitosis. Autophosphorylation (kinase activity) of Mst and Lats is increased in U2OS cells upon nocodazole treatment (52). However, we could not detect significant change of Mst or Lats activity (by phospho-antibodies) in HeLa and
MCF-7 cells (Fig. 1A and data not shown), suggesting that the mitotic activation of Mst and Lats is cell-type specific. Lats2 was also phosphorylated by Aurora-A at the centrosomes during mitosis (53). YAP is a direct substrate of Lats1/2 (10, 12, 13) and KIBRA associates with Lats1/2 (25). Interestingly, CDK1 also phosphorylates KIBRA (24) and Lats1 (30) during mitosis, and one question is whether or how KIBRA/Lats1/2 is involved in the regulation of mitotic phosphorylation of YAP. Furthermore, YAP and TAZ are paralogs and have similar and distinct functions (2, 4). We also found that TAZ is up-shifted upon Taxol or nocodazole treatment (Fig. 1A). While all three phosphorylation sites on YAP are conserved in vertebrates, however, surprisingly, none of the phosphorylation sites of YAP exists on TAZ. We are currently investigating how TAZ is regulated and whether TAZ also plays a role in mitosis similar to YAP.

Acknowledgements

We wish to thank Dr. Nick George for designing siRNA oligos targeting YAP, Tom Dao for assistance with confocal microscopy at the imaging core facility at Nebraska Center for Cellular Signaling. We also thank Drs. Joyce Solheim, Robert Lewis and Keith Johnson for critical reading and comments on the manuscript.
References


Figure Legends

Figure 1. YAP is phosphorylated during G2/M arrest

A, HeLa cells were treated with DMSO (control), Taxol (0.1 µM for 16 h) or Nocodazole (Noco, 100 ng/ml for 16 h). Total cell lysates or immunoprecipitated products were probed with the indicated antibodies.

B, Various cancer cell lines were treated with (+) or without (-) Taxol as indicated.
C, HeLa cells were treated with Taxol at various concentrations and time points as indicated.

D, HeLa cells were treated with Taxol or Nocodazole (Noco) as indicated and cell lysates were further treated with (+) or without (-) λ phosphatase (ppase). Total cell lysates were probed with anti-YAP antibody.

**Figure 2.** Mitotic phosphorylation of YAP is CDK1-dependent

A, HeLa cells were treated with Taxol. Mitotic cells were collected by mechanical shake-off and total cell lysates from mitotic as well as attached cells were subjected to Western blot analysis with the indicated antibodies.

B, HeLa cells were treated with Taxol, nocodazole, etoposide (100 µM for 16 h), doxorubicin (50 nM for 16 h), IR (2 h after 10 Gray exposure), UV (2 h after 2 J/m² exposure). Total cell lysates were subjected to Western blotting with the indicated antibodies.

C, HeLa cells were treated with Taxol. RO3306 (CDK1 inhibitor) or SP600125 (JNK inhibitor) were added (with or without MG132) into the cells 2 h before harvesting the cells. Total cell lysates were subjected to Western blotting with the indicated antibodies.
D, Treatment and Western blot analysis were done as in (C). Roscovitine was used to inhibit CDK1 kinase activity.

E, *In vitro* kinase assays using HeLa cell lysates to phosphorylate recombinant His-YAP. Asy: asynchronized; Tax: Taxol-treated. Total cell lysates were probed with CDK1 and ERK antibodies.

F, *In vitro* kinase assays with purified CDK1/cyclin B complex. RO3306 (5 µM) was used to inhibit CDK1 kinase activity.

**Figure 3.** CDK1 phosphorylates YAP at T119 and S289 *in vitro* and in cells

A, HeLa cells were treated with Taxol. YAP was immunoprecipitated, electrophoresed, excised and subjected to mass spectrometry for phosphorylation site mapping (see ‘Materials and Methods’). Three phospho-peptides and the phospho-sites are shown.

B, HeLa cells were transfected with YAP and various non-phosphorylatable mutants. Cells were treated with Taxol at 24 h post-transfection. Total cell lysates were probed with the indicated antibodies.

C, *In vitro* kinase assays were done as in Figure 2F except anti-phospho-YAP T119, S289 and S367 antibodies were used.
D, HeLa (upper part) or HEK293T (lower part) cells were transfected with HA-YAP or HA-YAP mutants. At 30 h post-transfection, the cells were treated with Taxol for 16 h. The immunoprecipitates (with HA antibodies) were probed with anti-phospho-YAP and subsequent anti-HA antibodies. SE: short exposure; LE: long exposure.

E, HeLa cells were treated with Taxol and endogenous YAP was immunoprecipitated and probed with the indicated antibodies. Total cell lysates before immunoprecipitation were also included.

F, HeLa cells were treated with Taxol for 16 h and RO3306 (5 µM) or Roscovitine (Rosco, 10 µM) was added to cells 2 h before harvesting as indicated. Proteasome inhibitor MG132 was also added (together with inhibitors) to prevent cyclin B from degradation and cells from exiting from mitosis. The cells were treated with λ phosphatase (ppase) as needed. Total cell lysates before immunoprecipitation were also analyzed.

**Figure 4.** CDK1 mediates the phosphorylation of YAP at T119 and S289 during unperturbed mitosis

A, HeLa cells were synchronized by a double thymidine (DT) block and release method.

Endogenous YAP was immunoprecipitated at the indicated time points and probed with
the indicated antibodies. Total cell lysates before immunoprecipitation were also analyzed to confirm the cell phase status.

B and C, Exponentially growing HeLa cells were stained with DAPI, p-YAP S289, p-YAP T119, and β-tubulin.

D, HeLa cells were treated with nocodazole, roscovitine and MG132 was added 2 h before the cells were fixed. The cells were then stained with DAPI and p-YAP antibodies. Red and yellow arrows in (C, D) mark the metaphase/prometaphase and interphase cells, respectively.

**Figure 5.** Phosphorylation mimetic YAP (YAP3D) induces mitotic defects in MCF10A cells

A, MCF10A cells stably expressing vector, YAP, and YAP3D were established at the same time and maintained at similar passage (around 20 at the time of experiments conducted).

B and C, Representative photos of normal mitosis (vector control) and mitotic abnormalities (YAP3D) in MCF10A cells. Cells were stained with α-tubulin, γ-tubulin antibodies and DAPI to visualize microtubules (red), centrosomes (green), and chromosomes (blue), respectively.
D-F, Quantification of mitotic characteristics including microtubule organization/multipolar spindles (D), centrosome number (E), and chromosome alignment (F). Data were collected from n=296, 287, and 274 mitotic cells for control, YAP, and YAP3D-expressing cells, respectively. Data were expressed as the mean ± s.d. of at least three independent experiments.

**Figure 6.** Mitotic phosphorylation promotes YAP’s oncogenic activity


B and C, Colony assays in MCF10A cells.

D, Cell migration assays with Transwell with MCF10A cells expressing various YAP constructs. Migrated cells were stained with DAPI and representative fields were shown.

E, Cell invasion assays with MCF10A cells expressing various YAP constructs.

F and G, MCF10A cells expressing vector, YAP-S127A or YAP-3D were used for migration (F) and invasion (G) assays in the presence or absence of RO3306. Data were expressed as the mean ± s.d. of three independent experiments. ***: p< 0.001; **: p< 0.01(t-test).
H, A model for phosphorylation-mediated regulation of YAP.
Figure 1

A

<table>
<thead>
<tr>
<th></th>
<th>DMSO</th>
<th>Taxol</th>
<th>Noco</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-NF2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-Mst1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-Mst2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-Mst</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-p-Mst</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-WW45</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-Actin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-p-Lats1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-Lats1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-Lats2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-MOB1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-YAP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-p-S127 YAP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-TAZ</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-p-T288 Aurora-A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-Actin</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B

<table>
<thead>
<tr>
<th></th>
<th>MCF-7</th>
<th>HCT-116</th>
<th>T47D</th>
<th>MDA-231</th>
<th>H1299</th>
<th>H2227</th>
</tr>
</thead>
<tbody>
<tr>
<td>Taxol</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>α-YAP</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-Cyclin B</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-Aur-A</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-p-Aur-A-B-C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

C

<table>
<thead>
<tr>
<th>Taxol (nM)</th>
<th>0</th>
<th>10</th>
<th>20</th>
<th>50</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td>Taxol (h)</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>α-YAP</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-Cyclin B</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-Actin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

D

<table>
<thead>
<tr>
<th></th>
<th>Noco</th>
<th>Taxol</th>
<th>λppase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>α-YAP</td>
<td></td>
<td></td>
<td>-72</td>
</tr>
</tbody>
</table>
**Figure 2**

**A**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Taxol</th>
<th>α-YAP</th>
<th>α-Cyclin B</th>
<th>α-Actin</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-</td>
<td>-72</td>
<td>-55</td>
<td>-43</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**B**

<table>
<thead>
<tr>
<th>Condition</th>
<th>DMSO</th>
<th>Nocodazole</th>
<th>Taxol</th>
<th>Doxorubicin</th>
<th>Etoposide</th>
<th>UV</th>
<th>IR</th>
<th>α-YAP</th>
<th>α-Cyclin B</th>
<th>α-p-S345</th>
<th>Chk1</th>
<th>α-Actin</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-72</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-55</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+</td>
<td></td>
<td></td>
<td>-55</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td></td>
<td></td>
<td></td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td>-55</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td></td>
<td></td>
<td></td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td>-55</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td></td>
<td></td>
<td></td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td>-55</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td></td>
<td></td>
<td></td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td>-55</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td></td>
<td></td>
<td></td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td>43</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**C**

<table>
<thead>
<tr>
<th>Condition</th>
<th>DMSO</th>
<th>MG132</th>
<th>Taxol</th>
<th>MG132</th>
<th>SP600125</th>
<th>α-YAP</th>
<th>α-p-Aur-A-</th>
<th>α-Aur-A</th>
<th>α-p-c-Jun</th>
<th>α-Cyclin B</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-72</td>
<td>-55</td>
<td>-43</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td></td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td></td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**D**

<table>
<thead>
<tr>
<th>Condition</th>
<th>DMSO</th>
<th>Roscovitine</th>
<th>Taxol</th>
<th>MG132</th>
<th>Roscovitine</th>
<th>α-YAP</th>
<th>α-Cyclin B</th>
<th>α-Actin</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td>-</td>
<td></td>
<td>-72</td>
<td>-55</td>
<td>-43</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td></td>
<td></td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**E**

<table>
<thead>
<tr>
<th>Condition</th>
<th>His-YAP</th>
<th>CDK1-depletion</th>
<th>Asy</th>
<th>Tax</th>
<th>Tax</th>
<th>Lysates</th>
<th>Autoradiography</th>
<th>WB: α-His</th>
<th>α-CDK1</th>
<th>α-ERK</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**F**

<table>
<thead>
<tr>
<th>Condition</th>
<th>His-YAP</th>
<th>RO3306</th>
<th>CDK1/Cyclin B</th>
<th>Autoradiography</th>
<th>WB: α-His</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>+</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td>+</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
## Figure 4

### A

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>0</th>
<th>8</th>
<th>10</th>
<th>24</th>
</tr>
</thead>
<tbody>
<tr>
<td>DT and release</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-p-T119 YAP</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-YAP</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-p-S289 YAP</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-p-S10 H3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-Cyclin B</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-Actin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### B

- **DAPI**
- **p-YAP S289**
- **β-tubulin**
- **Merged**

### C

- **DAPI**
- **β-tubulin**

### D

- **p-YAP T119**
- **Roscovitine**
- **p-YAP S289**
- **Roscovitine**
- **Merged**

**Figure 4**

Research on October 20, 2017. © 2013 American Association for Cancer Research.
Figure 5

A. Vector YAP YAP3D

α-YAP
α-Actin
MCF10A

B. DAPI α-tubulin γ-tubulin Merged

Vector control Metaphase Anaphase

Metaphase Anaphase

Metaphase Anaphase

Vector YAP YAP3D

C. DAPI DAPI

YAP3D

Scale 10 um

D. Cells with spindle defects (%)

P = 9.5x10^{-4}

Vector YAP YAP3D

E. Cells with abnormal centrosomes (%)

P = 1.7x10^{-4}

Vector YAP YAP3D

F. Cells with chromosomal abnormalities (%)

P = 4.9x10^{-6}

Vector YAP YAP3D
Figure 6

A. Western blot showing the expression of α-YAP and α-Actin in MCF10A cells transfected with different vectors:
- Vector
- YAP
- YAP-S127A
- YAP-3A
- YAP-4A
- YAP-3D

B. Colony formation assay showing the number of colonies formed by transfected MCF10A cells:
- Vector
- YAP
- YAP-S127A
- YAP-4A

C. Bar graph showing the number of colonies formed by transfected MCF10A cells.

D. Graph showing the relative migrating rate of transfected MCF10A cells.

E. Graph showing the relative invading rate of transfected MCF10A cells.

F. Graph showing the relative migrating rate of transfected MCF10A cells in the presence of RO3306.

G. Graph showing the relative invading rate of transfected MCF10A cells in the presence of RO3306.

H. Diagram illustrating the Hippo-G2/Mitosis pathway and its targets:
- Lats1/2
- CDK1
- YAP
- S127
- S289

Legend:
- **: p < 0.01
- ***: p < 0.001

100 um scale bar indicating the size of the cells.
CDK1 phosphorylation of YAP promotes mitotic defects and cell motility and is essential for neoplastic transformation

Shuping Yang, Lin Zhang, Miao Liu, et al.

Cancer Res  Published OnlineFirst October 7, 2013.

Updated version  Access the most recent version of this article at: doi:10.1158/0008-5472.CAN-13-2049

Supplementary Material  Access the most recent supplemental material at: http://cancerres.aacrjournals.org/content/suppl/2013/10/07/0008-5472.CAN-13-2049.DC1

Author Manuscript  Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.