Identification of Cdk1–LATS–Pin1 as a Novel Signaling Axis in Anti-tubulin Drug Response of Cancer Cells

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Abstract

The Hippo pathway is a signaling cascade that plays important roles in organ size control, tumorigenesis, metastasis, stress response, stem cell differentiation, and renewal during development and tissue homeostasis and mechanotransduction. Recently, it has been observed that loss of the Hippo pathway core component LATS (large tumor suppressor) or overexpression of its downstream targets YAP and its paralog TAZ causes resistance of cancer cells to anti-tubulin drugs. However, YAP and TAZ mediate anti-tubulin drug-induced apoptosis independent of its upstream regulator LATS and the Hippo pathway. Thus, the underlying molecular mechanism of how LATS is involved in the anti-tubulin drug response remains unknown. Proteomic approaches, SILAC and BioID, were used to identify the isomerase Pin1 as a novel LATS-interacting protein after anti-tubulin drug treatment. Treatment with anti-tubulin drugs activated cyclin-dependent kinase 1 (CDK1), which phosphorylates LATS2 at five S/T-P motifs that functionally interact with the WW domain of Pin1 and inhibit its antiapoptotic function. Thus, these data identify Cdk1 and Pin1 as a novel upstream regulator and downstream mediator, respectively, of LATS in antitubulin drug response. Further studies on this novel Cdk1–LATS–Pin1 signaling axis will be important for understanding the molecular mechanisms of drug resistance and will provide useful information for targeting of this pathway in the future.

Implications: This study provides new insight on the molecular mechanism of anti-tubulin drug resistance and suggests novel therapeutic targets for drug-resistant cancers. Mol Cancer Res; 16(6); 1035–45. ©2018 AACR.

Introduction

Anti-tubulin drugs, which include taxanes and vinca alkaloids, have been a part of the cancer pharmacopoeia for decades and are one of the most successful classes of chemotherapeutics to date (1, 2). By inhibiting the polymerization dynamics of microtubules, anti-tubulin drugs are potent mitotic poisons that impede normal function of the mitotic spindle, leading to extended mitotic arrest and cell death (3). Cancer cells promote proliferation and invasion by means of an overactive cell cycle and increased mitotic division, thus making them particularly susceptible to microtubule disruption. Although anti-tubulin drugs are proven to be clinically efficacious, predicting patient response is often difficult and the development of drug-resistant tumor cells leading to relapse/patient mortality continues to be a major challenge to successful treatment (1). Therefore, identifying novel signaling networks controlling drug response is of critical importance in advancing current cancer therapies.

The Hippo pathway is an emerging signaling pathway that plays critical roles in organ size control, tumorigenesis, metastasis, stem cell differentiation and renewal, mechanotransduction (4–7). Recent studies have implicated the Hippo pathway in playing an important role in chemotherapeutic resistance (8–10). Classic mammalian Hippo signaling consists of a core kinase cascade in which Mst1/2 (mammalian Ste20-like 1/2) serine/threonine (S/T) kinases phosphorylate and activate LATS1/2 (large tumor suppressor 1/2) kinases, which subsequently phosphorylates and inhibits transcriptional coactivator/corepressors YAP (Yes-associated protein) and its paralog TAZ (transcriptional coactivator with PDZ-binding motif), preventing their translocation from cytoplasm to nucleus to bind TEAD family of transcription factors and induce expression of genes that promote cell proliferation, survival, differentiation, and migration (4, 11). Importantly this protein cassette integrates numerous upstream signals and translates them to downstream effectors while simultaneously cross-talking with other signaling pathways to maintain homeostasis and prevents tumorigenesis (4, 11). Unsurprisingly, dysregulation of the Hippo pathway, especially the well-established tumor suppressor genes LATS1/2, can lead to various human cancers (5, 12). Recently, we and others have found that many of the core components or upstream regulators of the Hippo pathway (e.g., RASSF1A, Expanded, MST, Ajuba, YAP/TAZ, and Vgll4) play pivotal roles in the resistance of cancer cells to chemotherapeutic drugs [e.g., taxol, doxorubicin, cisplatin, 5-FU, and EGFR, and MEK inhibitors; refs. 10, 11, 13–17].
Interestingly, studies by our laboratory and others showed that LATS plays a pivotal role in anti-tubulin drug resistance. Loss of LATS1/2 by siRNA knockdown renders HeLa cervical cancer cells resistant to taxol-induced cell death (18). In addition, LATS1 was identified in a functional genomic screen looking for genes involved in taxol sensitivity of A549 lung cancer cells (19). However, although we and others found that YAP and TAZ are also important in anti-tubulin drug response, this was independent of LATS1/2 or Hippo pathway regulation, suggesting that YAP and TAZ are not the proteins mediating MST1/2 or LATS1/2 function in anti-tubulin drug resistance (8, 9, 20). Moreover, there is no change on LATS1 phosphorylation status on MST phosphorylation site T1089 after taxol treatment (ref. 8 and data not shown), suggesting that MST1/2 is not the kinase regulating LATS in response to anti-tubulin drugs. Therefore, the upstream regulator(s) or downstream mediator(s) of LATS1/2 in anti-tubulin drug response remain unknown.

In this study, we have identified Cdk1 and Peptidyl-prolyl isomerase Pin1 as novel upstream regulator and downstream mediator of LATS1/2, respectively, in response to anti-tubulin drugs. This novel Cdk1–LATS–Pin1 signaling axis may play important roles in anti-tubulin drug resistance and represent a novel target for drug-resistant cancer therapy in the future.

Materials and Methods

Plasmid construction and site-directed mutagenesis

Plasmid construction and site-directed mutagenesis were performed as described in detail previously (21). Human PPIA, PPIB, and Pin1 cDNAs (obtained from Addgene) were subcloned into pcDNA3.1-HA and pEG4T1 vectors. Inducible TRIPZ-lentiviral shRNA vector (Dharmacon) was modified by cloning of human LATS2, Pin1, or BirA-R118G cDNAs (Addgene) into AgeI and MluI site of TRIPZ vector.

Cell culture and treatment of cells by drugs and transfection

Cos7 monkey embryonic kidney cells, HeLa cervical carcinoma, and H1299 lung carcinoma cell lines were purchased from the ATCC and cultured as instructed by ATCC. The cells were passaged 1:10 and cultured according to the manufacturer’s protocol. Cell culture and treatment of cells by drugs and transfection were normally passed 1:10 and cultured less than 2 months. HeLa and H1299 cells were treated with anti-tubulin drugs and kinase inhibitors as described previously. Transient transfection of expression constructs was performed using PolyJet in vitro transfection reagent (SigmaGen Laboratories) according to the manufacturer’s protocol.

Lentivirus production and establishment of stable cell lines

Lentivirus production and purification were performed as described previously (8). H1299 cells stably expressing TRIPZ-LATS2-WT, LATS2-5xA, or BirA-LATS2-WT were established by infection with lentivirus and subsequent selection with 1 μg/mL puromycin. For WPI-vector or -Pin1-WT overexpression, H1299 TRIPZ-LATS2-WT or -5xA cells were infected again and expression was examined by Western blot analysis using anti-LATS2 or -Pin1 antibodies.

SILAC labeling, BioID purification, and MS data analysis

SILAC Protein Quantitation kit was used and purchased from Pierce. HeLa N-BirA-LATS2-TRIPZ cells were cultured in light or heavy (containing 13C6-L-Lysine-2HCl) media with 2 μg/mL doxycycline for about 10 days, with media being changed every 2–3 days and passed onto larger plates. A total of 1 × 106 cells were passed onto each 150-mm plate (five for control and ten each for drug-treated) and grown to approximately 40–50% confluence. Media were changed containing 50 μmol/L biotin, 2 μg/mL doxycycline and either DMSO, 100 nmol/L taxol, or 1 μg/mL nocodazole and cultured for 24 hours. Adherent (control) or floating (drug-treated) cells were harvested and protein was extracted using modified RIPA buffer containing proteinase inhibitors/benzonase and sonicated. Supernatants were checked for expression of LATS2 by Western blot analysis and protein concentration measured by Bio-Rad DC protein assay. Twenty micrograms of lysate was used for overnight pulldown at 4°C using 30 μL of streptavidin-sephrose beads, which were then washed 2× with modified RIPA buffer, 2× with TAP lysis buffer (50 mmol/L Hepes-KOH, pH 8.0, 100 mmol/L KCl, 10% glycerol, 2 mmol/L EDTA, and 0.1% NP-40), and 3× with 25 mmol/L ammonium bicarbonate (pH 8.0) and prepared for mass spectrometry analysis.

Proteomics analysis was performed at the Ottawa Hospital Research Institute Proteomics Core Facility (Ottawa, Canada). Proteins were digested in-gel using trypsin (Promega) according to the method of Shevchenko (22). Peptide extracts were concentrated by Vacufuge (Eppendorf). LC/MS-MS was performed using a Dionex Ultimate 3000 RSLC nano HPLC (Thermo Scientific) and Orbitrap Fusion Lumos mass spectrometer (Thermo Scientific). Database searching (against the human Uniprot database) and quantitation were performed using MaxQuant software v1.2.7.4.14. The following criteria were used: peptide tolerance = 10 ppm, trypsin as the enzyme (two missed cleavages allowed), and carboxamidomethylation of cysteine as a fixed modification. Variable modifications were oxidation of methionine and N-terminal acetylation. The heavy SILAC label was Lys6 (K6). Minimum ratio count was 2, and quantitation was based on razor and unique peptides. Peptide and protein FDR was 0.01.

siRNA-mediated gene expression knockdown

On-Target SMARTpool siRNA for Cyclin B1 along with an siRNA with scrambled sequence (siControl) were purchased from Dharmacon. HeLa cells were transfected with 50 nmol/L of siRNAs using RNAiMax (Invitrogen) according to manufacturer’s instructions. Two days posttransfection, cells were treated with 1 μg/mL nocodazole or 100 nmol/L Taxol for 24 hours, followed by protein extraction and Western blot analysis.

GST fusion protein production, pull-down, and in vitro kinase assays

GST fusion proteins were produced and purified as previously described (21). For GST-pulldown assays, about 100–200 μg of each respective protein lysate was preclared overnight at 4°C with 20 μL glutathione sepharose 4B beads (CSB; GE Healthcare). Supernatants were transferred and mixed with 10 μg of appropriate GST fusion protein and incubated at 4°C for 2 hours. Twenty microliters of GST was then added and further incubated for another 1 hour. Beads were then washed four times with lysis buffer (50 mmol/L Tris-HCl pH 7.4, 150 mmol/L NaCl, 1 mmol/L EDTA and 1.0% Nonidet P-40), resuspended in 2× SDS sample buffer, boiled for 10 minutes, and centrifuged. Resultant supernatants were subjected to SDS-PAGE and Western blotting as described previously. For in vitro kinase assays, 20 U of Cdk1–cyclinB kinase (New England Biolabs, 13C6-Lysine-2HCl) media with 2 μg/mL doxycycline for about 10 days, with media being changed every 2–3 days and passed onto larger plates. A total of 1 × 106 cells were passed onto each 150-mm plate (five for control and ten each for drug-treated) and grown to approximately 40–50% confluence. Media were changed containing 50 μmol/L biotin, 2 μg/mL doxycycline and either DMSO, 100 nmol/L taxol, or 1 μg/mL nocodazole and cultured for 24 hours. Adherent (control) or floating (drug-treated) cells were harvested and protein was extracted using modified RIPA buffer containing proteinase inhibitors/benzonase and sonicated. Supernatants were checked for expression of LATS2 by Western blot analysis and protein concentration measured by Bio-Rad DC protein assay. Twenty micrograms of lysate was used for overnight pulldown at 4°C using 30 μL of streptavidin-sephrose beads, which were then washed 2× with modified RIPA buffer, 2× with TAP lysis buffer (50 mmol/L Hepes-KOH, pH 8.0, 100 mmol/L KCl, 10% glycerol, 2 mmol/L EDTA, and 0.1% NP-40), and 3× with 25 mmol/L ammonium bicarbonate (pH 8.0) and prepared for mass spectrometry analysis.

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P6020S) and 1 μg of fusion protein were mixed and incubated at 30°C for 20 minutes. Phosphorylated proteins were alkylated by adding p-nitrobenzyl mesylate (final concentration 2.5 mmol/L) and incubated at room temperature for 2 hours. Samples were prepared for Western blot analysis as described previously; phosphorylated proteins were detected by anti-thiophosphate ester 51-8 antibody (Abcam).

Antibodies, Phos-tag and Western blot analysis, and coimmunoprecipitation

Anti-LATS2 antibody was from Bethyl Laboratories. LATS1, phospho-Ser909 LATS1, phospho-Ser127-YAP, phospho-Tyr15-Cdk1 antibodies were from Cell Signaling Technology. Anti-Pin1, HA F7 (for co-IP) and Cyclin B1 antibodies were from Santa Cruz Biotechnology. FLAG (M2) and β-actin antibodies were from Sigma. Cdk1 and TAZ antibodies were from BD Transduction Laboratories. Antibodies against phospho-Ser/Thr (MPM2) were from Millipore and thiophosphate ester (51-8) from Abcam. Phos-tag was obtained from Wako Pure Chemical Industries and used at 40 μmol/L along with 100 μmol/L MnCl2 in 6%–8% SDS-acrylamide gels. Western blot analysis, coimmunoprecipitation, and phosphatase treatment were done as described previously (8).

Analysis of apoptosis by caspase-3 activity measurement and Trypan blue exclusion assays

H1299 TRIPZ-LATS2-WT or -5xa cells were pretreated with or without 2 μg/mL doxycycline for 2 days prior to be seeded in triplicate for treatment with 100 nmol/L Taxol for 24 hours. Caspase-3 activity was assayed using the Caspase-Glo 3/7 kit (Promega, G8090) and measured by luminometer (Turner BioSystems). Absolute RUU values were then normalized to protein concentration using Bio-Rad DC Protein Assay. Measurement of cell death/apoptosis by Trypan blue exclusion analysis was as described previously (15). Briefly, H1299-stable lines were pretreated with 2 μg/mL doxycycline for 2 days before being seeded into 12-well plates in triplicate (2 × 10^5 cells/well). The next day, media was changed that contained DMSO or appropriate concentrations of Taxol and incubated for 24 hours. Alive and dead cells were collected and assayed as described before. The experiments were repeated 2–3 times and mean and SD of each experiment were presented.

Results

LATS2 is phosphorylated after anti-tubulin drug treatment

To further explore how LATS1/2 is involved in drug response, we treated HeLa cells with DMSO (vehicle control) or various anti-tubulin drugs for 24 hours and collected attached (DMSO) or floating (mitotic apoptotic) cells. Interestingly, treatment of HeLa cells with anti-tubulin drugs causes a mobility shift for LATS1 but not LATS2 on traditional SDS-PAGE gels (Fig. 1A; Supplementary Fig. S1). Thus, to test for the presence of differential migration of LATS2, we ran collected lysates on low percentage, Phos-tag gels, which can separate phosphorylated from unphosphorylated proteins more effectively. Surprisingly, treatment with anti-tubulin drugs caused dramatic retardation of endogenous LATS2 as compared with control (Fig. 1A). We also noted that there was no change in endogenous levels of YAP or its phosphorylation at S127 (a known LATS regulatory site; ref. 21), further supporting the notion that LATS1/2 is involved in drug response through YAP-independent means (Fig. 1A). Because of the functional similarities of LATS1 and LATS2, we mainly focused the following characterization study on LATS2, as its roles in anti-tubulin drug response are unclear. Similar to endogenous LATS2, we also found that FLAG-tagged LATS2 transfected into HeLa cells produced an upshift upon treatment with various anti-tubulin drugs (Fig. 1B), suggesting that the band-shift observed in HeLa cells is specific to LATS2. We further confirmed that the LATS2 mobility shift was due to phosphorylation as the band-shift can be reversed after treatment with lambda phosphatase (Fig. 1C). As we and others previously found that Cdk1 is a kinase that can complex with Cyclin B to phosphorylate other Hippo components under anti-tubulin drug treatment (8–10, 20), we asked whether LATS2 was also phosphorylated under such control. Indeed, pretreatment with Cdk1 inhibitor RO3306 or transient knockdown of Cyclin B by siRNA was sufficient to reverse LATS2 phosphorylation under various anti-tubulin drug conditions (Fig. 1D and E). Finally, we tested whether LATS2 phosphorylation is specific for HeLa cells and found that LATS2 is phosphorylated in a wide variety of human cancer cells after nocodazole or Taxol treatment (Supplementary Fig. S2). Taken together, these data strongly suggest that LATS2 can be phosphorylated by Cdk1 in apoptotic cells in a wide variety of cell types after treatment with anti-tubulin drugs.

Identification of LATS2 phosphorylation sites

To identify the phosphorylation site(s) on LATS2, we first performed in vitro kinase assays using purified Cdk1/Cyclin B as kinase and GST-fusion proteins expressing different regions of LATS2 as substrates. All of the LATS2 deletion fusion proteins, excluding the 601–800 region, were significantly phosphorylated by Cdk1/Cyclin B, suggesting that there are at least three distinct Cdk1 phosphorylation sites in LATS2 (Fig. 2A). As Cdk1 is a proline (P)-directed S/T kinase, we scanned the sequence of each phosphorylated LATS2 fragment (1–200, 201–400, 401–600, and 801–1089) for SP or TP motifs and found fifteen potential Cdk1 sites (Fig. 2B). We constructed plasmids expressing various deletions containing either consecutive or point mutations of LATS2 (Fig. 2C–F), transfected them into HeLa cells, and treated with Taxol for 24 hours. Using Phos-tag gels, we identified five potential Cdk1-phosphorylation sites that are necessary for Taxol-induced mobility shift: S157, S342, T349, S598 and S1027 (Fig. 2B–F). Mutation of these five sites (termed LATS2-5xa) completely abolished the mobility shift of LATS2 after nocodazole or Taxol treatment (Fig. 2G). Similar to LATS2, LATS1 is also phosphorylated by Cdk1 (Supplementary Fig. S3). In summary, we identified five S/T-P motif phosphorylation sites that are responsible for LATS2 band-shift after anti-tubulin drug treatment.

Identification of Pin1 as a novel LATS2-interacting protein after anti-tubulin drug treatment using BioID-SILAC

Previous studies by our laboratory and others found that under Taxol treatment, the main Hippo pathway effector YAP could no longer be phosphorylated and regulated by LATS (8, 20), prompting us to seek novel proteins mediating LATS function under these conditions. We employed an approach that combined proximity-dependent labelling (BioID) and quantitative proteomics method called SILAC (stable isotope labeling by amino acids in cell culture), which has been applied successfully in studying a variety of proteins and processes (23–25). While BioID relies on the fusion of a protein of interest to a mutant form of biotin ligase BirA, which can promiscuously biotinylate proximal proteins.

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irrespective of whether they interact indirectly or transiently, SILAC uses “heavy” and “light” amino acids to label proteins of two populations and quantify differential abundance of each peptides of digested proteins by mass spectrometry. By coupling Bio-ID with SILAC, we could then identify novel anti-tubulin drug-induced LATS2-interacting proteins with high confidence.

The experimental workflow is briefly summarized in Fig. 3A. First, we generated stably expressing doxycycline-inducible N-terminal BirA-LATS2 in HeLa cells using lentivirus (Fig. 3B); C-terminal BirA-LATS2 cells were also generated but was found to impair LATS2 activity (data not shown). Next, we validated the system by a small-scale purification of known LATS2-interacting proteins. We treated HeLa-N-BirA-LATS2 cells with biotin alone or together with Taxol or nocodazole and subsequently performed pulldown with streptavidin agarose. Since BirA-LATS2 is able to biotinylate itself, we could detect LATS2 on the beads only when biotin was present (Fig. 3C). Furthermore, we probed for other known LATS2 interacting proteins (21, 26) and found that TAZ specifically binds to LATS2 when HeLa-N-BirA-LATS2 cells were treated with Dox to induce LATS2 in the absence or presence of TX or nocodazole (Fig. 3C and data not shown), suggesting that this BioID system worked for LATS2. We then proceeded to systematically profile LATS2 interacting proteins in large scale using the same procedure (Fig. 3A).

After mass spectrometry analysis, we identified candidate proteins that have enhanced binding to LATS2 after TX or nocodazole treatment (Supplementary Table S1). Most interestingly, we found various prolyl-isomerases (e.g., PPIB, PPIG and FKBP4) scattered in our identified list of proteins. Although Pin1 and PPIA were not detected in our screen most likely due to lack of efficient trypsin digestion, they are also members of the cis-trans isomerase family and were included in our analysis. To validate our findings, we performed coimmunoprecipitation (Co-IP) assays with HeLa cells transfected with FLAG-tagged LATS2 and HA-tagged isomerases in the presence of DMSO or Taxol. Interestingly, higher levels of LATS2-FLAG were immunoprecipitated with PPIA-HA, PPIB-HA, or Pin1-HA (with anti-HA antibody) in the presence of Taxol (Fig. 3D). We further tested whether enhanced levels of these isomerases induce Taxol resistance by overexpressing them in HeLa cells. Consistent with a previous publication (27), only overexpression of Pin1 causes resistance of cancer cells to Taxol-induced apoptosis (Supplementary Fig. S4). Therefore, we decided to further explore how interaction of LATS2 with Pin1 is involved in Taxol response.
Interaction of LATS and Pin1 in vitro and in vivo

To test whether the interaction between LATS and Pin1 was not cell type specific, we performed binding assays using other cell lines. In addition, as LATS1 and LATS2 have similar function, we also tested whether Pin1 also interact with LATS1. FLAG-tagged LATS1 or LATS2 expression vectors were transfected into Cos7 cells and GST pull-down assays were used to assess direct interaction in vitro. As expected, both LATS1 and LATS2 proteins were able to bind Pin1-GST (Fig. 4A and B). Next, we used co-IP assays to examine whether either LATS proteins interacted with Pin1 in vivo. FLAG-tagged LATS1 or -LATS2 and HA-tagged Pin1 were transfected alone or together into HEK293 cells. When cell lysates were immunoprecipitated with HA antibody, LATS1 and LATS2 was found in the immune complex confirming its interaction (Fig. 4C and D). We were unable to confirm the endogenous interaction of LATS and Pin1. Nevertheless, as Pin1 binds to known substrates via its WW domain, we asked whether this was also necessary for LATS1/2 interaction. To map the domains responsible for Pin1 binding, we made expression vectors containing either the Pin1-WW and PPIase domain. When GST pull-down assays were performed with cell lysates expressing FLAG-tagged LATS1 or LATS2 and Pin1 GST fusion proteins purified from bacteria, the WW rather than PPIase domain of Pin1 was found to bind with LATS1/2 (Fig. 4E and F). This was further verified with

Figure 2.
Mapping of LATS2 phosphorylation sites. A, In vitro kinase assay using 100 ng of Cdk1-CyclinB kinase complex and 1 μg of each purified GST-LATS2 fragment fusion proteins. B–F, Schematic of S/T/P sites in each LATS2 region (B). The potential Cdk1 phosphorylation sites are indicated by asterisks. Cdk1-phosphorylation sites are mapped by transfecting HeLa cells with different FLAG-LATS2 fragments/mutations with subsequent treatment of 100 nmol/L Taxol (TX) for 24 hours. Collected lysates were then run on Phos-tag gels for each region: LATS2-1-200, -201-400, -401-600, and -801-1089 (in C–F respectively). The positions of identified Cdk1 phosphorylation sites are indicated as framed numbers in B. G, Mutation of the identified five Cdk1 phosphorylation sites in HeLa cells transfected with LATS2 (5xA) abolishes mobility shift after 24-hour nocodazole (1 μg/mL) or Taxol (100 nmol/L) treatment. All protein lysates were run on phos-tag SDS-PAGE.
co-IP assays using lysates that were transfected with FLAG-tagged LATS1 or -LATS2 and either HA-tagged Pin1-WT, WW, or PPIase alone or together (Fig. 4G and H). In summary, these experiments confirm that Pin1 can bind to both LATS1 and LATS2 through its WW domain.

Phosphorylation-dependent interaction of LATS2 and Pin1
As the WW domain of Pin1 specifically recognizes phospho-S/T-P motifs (28) and LATS2 is phosphorylated by Cdk1 after anti-tubulin drug treatment (Figs. 1 and 2), we tested whether the binding between Pin1 and LATS is dependent on the phosphorylation status of LATS in the absence (endogenous CDK1 phosphorylation of LATS) of anti-tubulin drug. Treatment of calf intestinal phosphatase (CIP) on HEK293 lysates transfected with FLAG-tagged LATS1 or LATS2 greatly reduced binding between LATS and GST-Pin1 according to GST pull-down assays (Fig. 5A and B). Binding was not completely abolished due to residual LATS2 phosphorylation, as detected by anti-pS/T-P antibody (Fig. 5A and B). Next, we tested whether lysates from HeLa cells treated with various anti-tubulin drugs for 24 hours would increase interaction. In accordance, binding between FLAG-tagged LATS1 or LATS2 with GST-Pin1 was markedly higher in the anti-tubulin drug treated samples as compared with DMSO control (Fig. 5C and D). Most significantly, pretreatment with RO3306 (Cdk1 inhibitor) completely prevented the detection of FLAG-LATS2 after GST-pulldown with Pin1, despite treatment with anti-tubulin drugs (Fig. 5E), suggesting that phosphorylation of LATS by Cdk1 is essential for its interaction with Pin1. Finally, we tested the effect of the identified Cdk1-phosphorylation sites of LATS2 (Fig. 2B and G). Mutation of the five sites completely abolished LATS2 binding (FLAG-LATS2-WT vs. -5xA) to Pin1 by GST-pull-down assay (Fig. 5F). These results suggest that treatment with anti-tubulin drugs promotes Cdk1 phosphorylation of LATS2 and is necessary for priming it for recognition and binding to Pin1.

Characterization of Cdk1–LATS–Pin1 signaling axis in anti-tubulin drug-induced apoptosis
We next characterized the functional significance of Cdk1–LATS–Pin1 signaling axis. First, as LATS2 has roles in promoting apoptosis and drug sensitivity (5, 11, 18, 29), we examined the effects of Cdk1 phosphorylation site mutations of LATS2 (5xA) on its functions. We established H1299 lung cancer cell line (low endogenous LATS2) stably expressing doxycycline-inducible LATS2-WT or -5xA (Fig. 6A) and tested the effects of 5xA on LATS function. Using a caspase-3 activity assay and Taxol-treated conditions, LATS2-WT overexpression greatly increased caspase-3 activity, as opposed to LATS2-5xA which saw approximately half the level of activation (Fig. 6B). In addition, we
previously reported that siRNA knockdown of LATS1/2 can cause resistance to Taxol treatment in HeLa cells (18). Interestingly, overexpression of LATS2-WT in H1299 sensitized the cells through a range of Taxol treatment, an effect that was lost in LATS2-5xA overexpression cells (Fig. 6C). These results strongly suggest that mutation of the five Cdk1-phosphorylation sites of LATS2 greatly impairs its antitumorigenic functions and Taxol sensitivity.

As Pin1 is known to regulate other tumor suppressor genes by binding to pS/P-T motifs (27, 28, 30), we examined the functional
consequences of overexpression of both LATS2 and Pin1. From previously established H1299 TRIPZ-LATS2-WT cells, we stably expressed WPI vector control or Pin1-WT by infection with lentivirus (Fig. 6D). As expected, overexpression of LATS2-WT rather than LATS2-5xSA alone sensitized cells to Taxol treatment, whereas overexpression of Pin1 caused resistance of cells to Taxol (Fig. 6E). Most significantly, overexpression of LATS2-WT rather than LATS2-5xSA inhibited Pin1-induced Taxol resistance (Fig. 6E). Collectively, these findings indicate that Taxol induces apoptosis through regulation of Cdk1–LATS1/2–Pin1 signaling axis (Fig. 6F).

**Discussion**

LATS is a well-characterized major tumor suppressor, whose dysregulation can lead to various types of human cancers.

Figure 5.
Phosphorylation-dependent interaction of LATS1/2 and Pin1. A and B, GST-pulldown assays using GST or GST-Pin1 mixed with HEK293 lysates expressing FLAG-LATS1 (A) or –LATS2 (B) with (+) or without (–) treatment of calf intestinal phosphatase (CIP). Ponceau S was used to stain for equal loading of fusion proteins. Input lysates (10 μg) were probed with phospho-S/T antibody to check for CIP treatment. C and D, HeLa cells expressing FLAG-LATS1 (C) or –LATS2 (D) were treated with DMSO or anti-tubulin drugs as described in Fig. 1A and mixed with GST or GST-Pin1 for binding assay as in Fig. 4B. Expression levels of input were verified by Western blot analysis using FLAG-antibody and Ponceau S staining for fusion proteins. E, GST-pulldown assays of GST or Pin1-GST mixed with LATS2-FLAG-transfected HeLa cell lysates pretreated with Cdk1-inhibitor RO3306 for 2 hours and subsequently with anti-tubulin drugs (as in Fig. 1E). F, HeLa cells transfected with FLAG-LATS2-WT or -5xSA were treated with DMSO or 100 nmol/L Taxol for 24 hours. After cell collection and protein extraction, lysates were mixed with Pin1-GST in pulldown assay.
Although tremendous progress has been made in our understanding of how LATS controls organism homeostasis and prevents cancer formation, its role in drug response, particularly to anti-tubulin drugs, is not well defined. Our studies provide the first evidence that LATS can be specifically phosphorylated by Cdk1 independent of the upstream Hippo (MST1/2) signaling pathway. Significantly, we identified five sites (S157, S342, T349, S598, and S1027) that are responsible for enhanced LATS2 phosphorylation by Cdk1 after anti-tubulin drug treatment (Fig. 2G). Interestingly, out of the five Cdk1 phosphorylation sites, we identified on LATS2, only one has previously been reported (31). Under known conditions that trigger the Hippo pathway, cAMP-dependent protein kinase (PKA) was found to phosphorylate LATS2 at S598 (along with three other different sites), an event that induces full activation of LATS2 activity (31). This could partially explain why enhanced phosphorylation of S598 on LATS2 by Cdk1 activates LATS2 function, whereas mutation to alanine reduced LATS2 function, as revealed in our functional assays (Fig. 6B and C), a mechanism similar to the Mst1/2 phosphorylation and autophosphorylation sites on LATS2 (5). Aside from S598, the other 4 sites are also phosphorylated by Cdk1 and the functional significance of these sites is unclear. Therefore, it will be very
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interesting to further study how each individual phosphorylation site affects both the LATS2 activity and function. Moreover, as reduced expression of LATS1/2 causes anti-tubulin drug resistance (18) and mutation of Cdk1 phosphorylation sites (5xSA) significantly reduces its activation of Taxol-induced cell death, it will be also interesting to further examine the status of LATS2 phosphorylation in anti-tubulin drug-resistant cells and tissues to see whether reduced LATS2 phosphorylation may be responsible for anti-tubulin drug resistance.

Previous studies suggest that phosphorylation of Hippo effectors YAP and its paralog TAZ by Cdk1 is important for both normal mitotic progression during cell division and apoptosis induced by anti-tubulin drugs (8, 9, 20, 32). While moderate activation of Cdk1 during mitosis induces YAP/TAZ phosphorylation/activation and subsequent mitosis progression, hyperactivation of Cdk1 by anti-tubulin drugs significantly inactivates their functions and causes apoptosis. Therefore, YAP/TAZ phosphorylation levels control the balance between cell proliferation and cell death (8, 9, 20, 32). Therefore, it is also possible that LATS can be phosphorylated on these 5 S/T sites during mitosis and play important role in cell proliferation. Consistent with this statement, LATS1 has already been shown to be phosphorylated during mitosis (33–35) and low levels of endogenous LATS2 phosphorylation/shift is also detected without drug treatment (Fig. 1A). In addition, we also found that mutation of Cdk1 phosphorylation sites on LATS2 (LATS2-5xA) significantly reduced LATS2-induced suppression of transformation in H1299 lung cancer cells (Supplementary Fig. S5). Moreover, Pin1 binding partner identification of mitotic protein (27, 36) The peptide-dy-prolyl cis/trans isomerase that interacts with numerous oncogenic or tumor suppressive phosphorylated proteins, causes conformational changes in target proteins, and eventually regulates the activities of such proteins (27, 28, 30, 37). Pin1 binds to S/T-P motif of its interacting protein through its WW domain. Overexpression/and/or activated forms of Pin1 has been observed in a variety of cancers including breast, ovary, prostate, lung, gastric, cervical cancers, and melanoma (28, 37). Moreover, Pin1 activates numerous oncogenes and also inactivates tumor suppressors (27, 28, 37). Recently, overexpression of Pin1 has been shown to induce resistance of cancer cells to anti-tubulin drug Taxol (27).

However, the upstream signaling pathway regulating Pin1 function in anti-tubulin drug response has not been elucidated. In this study, we have provided the first in vitro and in vivo evidence that Cdk1-LATS is a novel signaling axis negatively regulating Pin1. We have shown that both LATS1 and LATS2 can interact with WW domain of Pin1 in a phosphorylation-dependent manner (Fig. 5). In addition, we have shown that phosphorylation of 5 sites on LATS2 by Cdk1 is essential for its interactions with Pin1 and mutation of these sites not only abolishes its interaction with Pin1 but also significantly reduces its activation of anti-tubulin drug-induced apoptosis (Fig. 6E). Most significantly, we found that LATS2-WT rather than LATS2-5xA mutant lacking interaction with Pin1 is able to inhibit Pin1-induced anti-tubulin drug resistance (Fig. 6E). The molecular mechanism by which LATS inhibits Pin1 function is unknown. It has been previously shown that Pin1 is phosphorylated on several serine residues by a number of distinct kinases (e.g., S65 by Plk1, S138 by MLK3, and S71 by DAPK, respectively) that regulate Pin1 stability, substrate binding capability or isomerase activity (38–40). Therefore, it is possible that LATS may inhibit Pin1 by phosphorylating Pin1. Intriguingly we found that purified LATS2 is able to phosphorylate Pin1 in vitro in the PPlase region (Supplementary Fig. S6) even though it does not contain a consensus LATS phosphorylation motif. Future study to determine whether this phosphorylation occurs in vivo and its potential functional consequences need to be examined. Our data also showed that LATS can interact with YAP/TAZ in the absence or presence of Taxol (data not shown). Therefore, it is possible that phosphorylation of LATS1/2 by Cdk1 causes conformational changes to LATS1/2 and switches their preferred substrates from YAP/TAZ to Pin1. Moreover, it is also possible that Pin1 can inhibit LATS function as a negative feedback mechanism (Fig. 6F). As Pin1 is a cis-trans isomerase and modulate proteins by changing its conformation, it will be interesting to further explore whether Pin1 regulates LATS in various biological functions.

This study highlights a previously unrevealed mechanism through which LATS2 is phosphorylated and activated by Cdk1 in response to anti-tubulin drugs. Interestingly, we and others have previously shown that other core and regulatory Hippo pathway components (e.g., YAP, TAZ, Ajuba, Kibra, and Vglh4) are also phosphorylated by Cdk1 under various contexts. As Pin1 recognizes S/T-P phosphoproteins and is ubiquitously expressed, it is likely that Pin1 could bind and modulate the activities of these Hippo components, potentially the pathway as a whole. Indeed, Pin1 has already been shown to be intricately involved in other phosphorylation-dependent networks such as mitosis, acting as an added layer of regulatory complexity (28, 30). Further investigation into how Pin1 may affect other Hippo pathway components will provide insights into the underlying mechanisms in cancer development and treatment.

Taken together, we have discovered a novel Cdk1–LATS–Pin1 signaling axis mediating anti-tubulin drug response in cancer cells. Further exploration of this signaling in the development of cancer and drug resistance and targeting of this axis will have significant implication for successful cancer therapy in the future.

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

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): B. Yeung, P. Khanal, V. Mehta, I. Trinkle-Mulcahy
Writing, review, and/or revision of the manuscript: B. Yeung, P. Khanal, X. Yang
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): B. Yeung, P. Khanal
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