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LIMD1 Phosphorylation in Mitosis Is Required for Mitotic Progression and its Tumor-suppressing Activity

Jiuli Zhou[1,2,*], Lin Zhang[3,*], Wei Zhou[3], Yuanhong Chen[1], Yufeng Cheng[3,4] and Jixin Dong[1,4]

[1]Eppley Institute for Research in Cancer and Allied Diseases, Fred & Pamela Buffett Cancer Center

[2]Department of Pathology and Microbiology,

University of Nebraska Medical Center, Omaha, NE 68198

[3]Department of Radiation Oncology, Qilu Hospital of Shandong University, Jinan, Shandong 250012, P. R. China.

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[4] To whom correspondence should be addressed:

Yufeng Cheng: qlcyf@163.com

and Jixin Dong, Ph.D.

Eppley Institute for Research in Cancer and Allied Diseases; Fred & Pamela Buffett Cancer Center

University of Nebraska Medical Center, 985950 Nebraska Medical Center, Omaha, NE68198-5950

Phone: 402-559-5596; Fax: 402-559-4651; Email: dongj@unmc.edu

*: Equal contribution to this work.

Abbreviations:

LIMD1: LIM domains containing 1; CDK1: cyclin-dependent kinase 1; JNK1/2: c-Jun NH2-terminal kinase 1/2; YAP: Yes-associated protein.

Abstract

LIM domains containing 1 (LIMD1) is a member of the Zyxin family proteins and functions as a tumor suppressor in lung cancer. LIMD1 has been shown to regulate Hippo-YAP signaling activity. Here, we report a novel regulatory mechanism for LIMD1. We found that cyclin-dependent kinase 1 (CDK1) and c-Jun NH2-terminal kinases 1/2 (JNK1/2) phosphorylate LIMD1 *in vitro* and in cells during anti-tubulin drug-induced mitotic arrest. Phosphorylation also occurs during normal mitosis. S272, S277, S421, and S424 were identified as the main phosphorylation sites in LIMD1. Deletion of LIMD1 resulted in a shortened mitotic cell cycle and phosphorylation of LIMD1 is required for proper mitotic progression. We further showed

that the phosphorylation-deficient mutant LIMD1-4A is less active in suppressing cell proliferation, anchorage-independent growth, cell migration, and invasion in lung cancer cells. Together, our findings suggest that LIMD1 is a key regulator of mitotic progression, and that dysregulation of LIMD1 contributes to tumorigenesis.

Key words: LIMD1; mitotic phosphorylation; CDK1; JNK1/2; Hippo; YAP

Introduction

Zyxin family members are adaptor/scaffold proteins with three LIM domains, which are involved in protein-protein interactions during cellular signaling transduction. In addition to Zyxin, this family also includes Ajuba, LIM domains containing 1 (LIMD1), Thyroid receptor-interacting protein 6 (TRIP6), Lipoma-preferred partner (LPP), and Wilms tumor 1 interacting protein (WTIP). The Zyxin family plays significant roles in many cellular processes including cell-cell adhesion, gene transcription, cell growth, and cell cycle regulation [1].

The *LIMD1* locus 3p21.3 is frequently down regulated in human lung tumors and loss of LIMD1 expression is mediated through a combination of gene deletion, loss of heterozygosity, and promoter methylation [2]. These studies suggest an abrogation of a tumor-suppressing role of LIMD1 in lung cancer, which was further supported by *in vitro* and genetic animal models [2,3]. LIMD1 interacts with pRb and inhibits E2F-mediated transcription of targets [3]. Recently, LIMD1 was shown to regulate miRNA-mediated silencing through coordinating the assembly of an Argonaute-trinucleotide repeat containing 6 (TNRC6)-containing miRNA-induced silencing complex (miRISC) [4]. Interestingly, some Zyxin family proteins, including LIMD1, Ajuba, and WTIP, represent a previously unrecognized group of hypoxic regulators [5]. For example, LIMD1 acts as a molecular

scaffold and assembles a prolyl hydroxylase domain (PHD)-LIMD1-Von Hippel-Lindau (VHL) protein complex that enables efficient degradation of hypoxia-induced factor-1α (HIF-1α) [5]. Furthermore, *LIMD1* itself is a HIF-1 target gene, which mediates a negative regulatory feedback mechanism for hypoxic HIF-α degradation in tumor growth by modulating PHD2-LIMD1-VHL complex formation [6]. Several reports suggest that LIMD1 controls tissue and cell growth through Hippo-YAP/Yki signaling [1,7-9], which plays critical roles in development and tumorigenesis [10-13].

Mitotic aberration can cause aneuploidy or genomic instability, which is highly associated with human malignancy [14,15]. Interestingly, several earlier studies have shown that some Zyxin family members (e.g. Zyxin, Ajuba, and LIMD1) are phosphorylated in mitosis and interact with the mitotic machinery [16-18]. LIMD1 is a novel BRCA2-interacting protein and is required for the centrosome localization of BRCA2 [19]. These studies suggest that Zyxin family proteins function as tumor suppressors or tumor promotors through, at least in part, dysregulation of mitosis. We recently characterized the phospho-regulation of Ajuba and Zyxin and determined their roles in mitosis and tumorigenesis [20,21]. Phosphorylation sites, corresponding kinases, and their functional significance for LIMD1 in mitosis remain elusive, however.

In this report, we showed that LIMD1 is phosphorylated in mitosis. We identified cyclin-dependent kinase 1 (CDK1) and c-Jun NH2-terminal kinases 1/2 (JNK1/2) as the upstream kinases for LIMD1 phosphorylation. We further demonstrated that LIMD1 phosphorylation controls proper completion of mitotic progression and is required for LIMD1 tumor-suppressing activity.

Results and Discussion

LIMD1 is phosphorylated during anti-tubulin drug-induced mitotic arrest

We recently found that many Hippo pathway proteins are phosphorylated in response to anti-tubulin drug (taxol or nocodazole)-induced mitotic arrest [20]. These studies revealed that LIMD1 was dramatically upshifted on a Phos-tag SDS-polyacrylamide gel during mitotic arrest in HeLa cervical cancer cells (Fig. 1A) [20]. The mobility shift was also evident in HPNE (an immortalized but not transformed human pancreatic nestin-expressing cell line) and other cancer cell lines (HCT116 colon cancer cells and Panc-1 pancreatic cancer cells) (Fig. 1B). Lambda phosphatase treatment completely abolished the mobility upshift of LIMD1, confirming that the mobility upshift of LIMD1 is due to phosphorylation during mitotic arrest (Fig. 1C).

Identification of the upstream kinase(s) for LIMD1 phosphorylation

In order to identify the corresponding kinase(s) for LIMD1 phosphorylation, we treated the cells with various kinase inhibitors. Treatment with SB203580 (p38 inhibitor), U0126 (MEK-ERK inhibitor), MK2206 (Akt inhibitor), BI2536 (PLK1 inhibitor), VX680 (Aurora-A, -B, -C inhibitor), MK5108 (Aurora-A inhibitor), or Rapamycin (mTOR inhibitor), which were effective under the conditions [22,23], failed to alter the mobility/phosphorylation of LIMD1 during mitotic arrest (Fig. 1D). Interestingly, LIMD1 mobility shift was reverted by the addition of RO3306 (CDK1 inhibitor) or Purvalanol A (CDK1/2/5 inhibitor) (Fig. 1D, lanes 5, 6). Inhibition of JNK1/2 (with SP600125) partially blocked the mobility shift of LIMD1 (Fig. 1D). These data suggest that CDK1 and JNK1/2 are the potential upstream kinases for LIMD1 phosphorylation during taxol or nocodazole-induced mitotic arrest.

CDK1 and JNK1/2 phosphorylate LIMD1 in vitro

To determine whether CDK1 and JNK1/2 can directly phosphorylate LIMD1, we performed *in vitro* kinase assays with purified recombinant LIMD1 proteins as substrates. In the presence of purified CDK1/cyclin B kinase complex, ^{32}P-labled γ–ATP was robustly incorporated in GST-LIMD1 proteins (Fig. 2A). Consistent with a previous study [8], JNK1/2 kinases, to a lesser extent when compared with CDK1 kinase, also phosphorylated LIMD1 (Fig. 2A). We also included some other similar kinases including CDK2, CDK5, MEK1, ERK1, ERK2, or p38α in these assays and showed that none of them could phosphorylate LIMD1 (Fig. 2A). These results suggest that LIMD1 is a suitable substrate for CDK1 and JNK1/2 *in vitro*.

LIMD1 S272, S277, S421, and S424 are the main sites phosphorylated by CDK1 and JNK1/2 in vitro

CDK1 and JNK1/2 kinases phosphorylate a minimal S/TP consensus sequence [24]. Database analysis (WWW.phosphosite.org) revealed four sites (S272, S277, S421, and S424) that have been identified as potential mitotic phosphorylation sites through large-scale proteomic studies [25,26]. All these four sites fit the CDK1 and JNK1/2 phospho-consensus sequence and have been identified as JNK1/2 phosphorylation sites [8]. We tested the possibility that these sites are phosphorylated by CDK1. In the presence of CDK1 kinase, the ^{32}P incorporation in GST-LIMD1-4A (S272A/S277A/S421A/S424A) was largely eliminated, suggesting that S272, S277, S421, and S424 are the main CDK1 sites in LIMD1 *in vitro* (Fig. 2B). Similar observations were obtained when JNK1/2 kinases were used (Fig. 2C).

We have generated phospho-specific antibodies against S272, S277, and S421/S424. *In vitro* kinase assays confirmed that JNK1/2 kinases could phosphorylate LIMD1 at S272, S277, and S421/S424 (Fig. 2D). CDK1 also phosphorylated LIMD1 at S421/S424, but could not phosphorylate S272 and S277 of LIMD1 under these conditions (Fig. 2D). These data indicate that CDK1 and JNK1/2 phosphorylate LIMD1 at S272, S277, and S421/S424 *in vitro*.

CDK1 and JNK1/2 phosphorylate LIMD1 at S272 and S277 in cells

We further explored whether LIMD1 is phosphorylated within cells during taxol/nocodazole-induced mitosis. The phospho-signals of S272 and S277 of transfected LIMD1 were significantly increased after taxol or nocodazole treatment (Fig. 3A). Mutating serines to alanines abolished the phosphorylation signal, suggesting that these antibodies specifically recognize phosphorylated LIMD1 (Fig. 3A). Addition of RO3306 (CDK1 inhibitor) or SP600125 (JNK1/2 inhibitor) greatly inhibited LIMD1 S272 and S277 phosphorylation (Fig. 3B). Taxol treatment also increased phosphorylation at S272 and S277 of endogenous LIMD1 in a CDK1- and JNK1/2-dependent manner (Fig. 3C). Lambda phosphatase treatment eliminated the phospho-signal induced by taxol, further confirming the specificity of these antibodies (Fig. 3D). We could not detect a specific signal when the phospho-S421/S424 antibody was used in these experiments.

Since inhibition of either CDK1 or JNK1/2 eliminates most phosphorylation (Fig. 3B, C), it implies that they should not both be directly phosphorylating LIMD1 on these sites in cells and that SP600125 or RO3306 has an indirect effect on the other kinase. Overexpression of non-degradable (constitutive active) GFP-Cyclin B1-R42A (activator of CDK1 kinase) increased p-LIMD1 S272 and S277 (Fig. 4A). Moreover, inducible knockdown of CDK1 significantly reduced p-LIMD1 S272 and S277 levels induced by taxol treatment (Fig. 4B).

These observations suggest that CDK1 mediates LIMD1 phosphorylation. However, it is still unclear whether this phosphorylation occurs through a direct or indirect mechanism.

We next asked whether phosphorylation of LIMD1 occurs during normal mitosis. First, we examined the phospho-status of LIMD1 using a double thymidine block and release method [27]. We showed that the phospho-LIMD1 signal (at S272 and S277) was significantly increased in cells entering mitosis (10 hours post double thymidine block and release) (Fig. 4C). The phospho-level of LIMD1 decreased when cells exited from mitosis (24 h after release) (Fig. 4C). Second, we performed immunofluorescence staining with our phospho-specific antibodies in freely cycling cells. We found that LIMD1 phosphorylation at S277 and S421/S424 significantly increased when cells progressed into mitosis. The highest phosphorylation levels were detected in metaphase cells (Fig. 4D, E). The p-LIMD1 signal was almost undetectable in interphase cells. The staining signals were abolished in LIMD1-knockout (KO) cells, confirming the specificity of p-LIMD1 antibodies (Fig. 4D, E, bottom lows). Furthermore, treatment with RO3306 or Purvalanol A largely abolished the phospho-signal (Fig. 4D, E). Together, these observations indicate that LIMD1 phosphorylation occurs during normal mitosis likely in a CDK1-dependent manner.

Mitotic phosphorylation of LIMD1 affects mitotic progression

Next, we asked whether LIMD1 plays a role in mitotic progression. For this purpose, we deleted LIMD1 in U2OS osteosarcoma cells using a CRISPR-Cas9 approach. These cell lines were further transduced with RFP-H2B for live-cell imaging microscopy. We found that LIMD1 deletion shortened the duration of early mitotic phases [from nuclear envelope breakdown (NEBD) to anaphase onset] (Fig. 5A, B). Adding back of wild type LIMD1 largely restored the mitotic defects induced upon LIMD1 deletion (Fig. 5C). However, reexpression

of the LIMD1-4A mutant failed to do so, suggesting that mitotic phosphorylation is required for proper mitotic progression (Fig. 5C).

LIMD1 is a regulator of the Hippo-YAP signaling [1,7-9]. We thus tested whether LIMD1 phosphorylation affects YAP activity. S127 is a major phosphorylation site mediated by LATS1/2 kinases [28,29], and S397 phosphorylation promotes YAP degradation [30]. In HeLa and U2OS cells, the S127 and S397 phosphorylation levels of YAP were not altered in LIMD1-KO cells when compared with control cells (Fig. 5D). Transfection of LIMD1-WT or LIMD1-4A into LIMD1-KO cells did not affect YAP activity either, as shown by the lack of changes in phosphorylation (Fig. 5D). These observations suggest that unlike Zyxin [21], inactivation of LIMD1 does not significantly affect YAP phosphorylation, at least in the cells tested.

Mitotic phosphorylation of LIMD1 is required for its inhibitory activity in cell proliferation and anchorage-independent growth

LIMD1 is a tumor suppressor in lung cancer [2]. We next asked whether mitotic phosphorylation of LIMD1 plays a role in regulating its tumor-suppressing activity in lung cancer cells. We compared the effects from empty vector-, LIMD1-WT-, or LIMD1-4A-transfected lung cancer cells (Fig. 6A). As expected, enhanced expression of LIMD1-WT significantly inhibited proliferation and anchorage-independent growth in A549 lung cancer cells (Fig. 6B-D). Interestingly, overexpression of the LIMD1-4A mutant only partially reduced cell proliferation and colony formation when compared to LIMD1-WT-expressing cells (Fig. 6B-D). Similar results were obtained in H520 lung cancer cells (Fig. 6E-H). These data suggest that mitotic phosphorylation is indispensable for LIMD1 activity in suppressing cell proliferation and anchorage-independent growth.

Mitotic phosphorylation of LIMD1 is required for its inhibitory activity in cell migration and invasion

We further interrogated the biological significance of mitotic phosphorylation of LIMD1 on cell migration and invasion. Consistent with the observations in cell proliferation and anchorage-independent growth (Fig. 6), overexpression of LIMD1-WT greatly inhibited migration, as shown by the wound healing assay (Fig. 7A) and invasion (Fig. 7B, C) in H520 cells. Again, ectopic expression of LIMD1-4A suppressed migration and invasion to a significantly lesser extent when compared with LIMD1-WT transfected cells (Fig. 7A-C). Similar results were obtained in A549 cells (Fig. 7D, E). These results suggest that phosphorylation of LIMD1 enhances its tumor-suppressing activity in migration and invasion in lung cancer cells.

Taken together, our data show that the Zyxin family protein LIMD1, in addition to Zyxin itself and Ajuba [20,21], is phosphorylated in mitosis. Mitotic phosphorylation of LIMD1 regulates mitotic progression and is required for its tumor-suppressing activity in lung cancer cells. Future studies are needed to determine whether these three members have compensatory effects during the mitotic cell cycle. Our findings showed that LIMD1 and its mitotic phosphorylation did not affect the YAP activity in U2OS and HeLa cancer cells, and thus it will be interesting to unveil the downstream signaling and effector of LIMD1 in mitosis and tumor-suppressing activity.

Materials and Methods

Expression constructs, cell culture and transfection

His-LIMD1, His-LIMD1-4A (S272A/S277A/S421A/S424A), and V5-LIMD1 have been described [8]. To make the lentiviral LIMD1 expression constructs, the full-length LIMD1 cDNA (wild type or 4A) was cloned into the pSIN4-Flag-IRES-puro vector [31]. The lentiviral-

mRFP-H2B (18982) [32] and GFP-Cyclin B1-R42A (61849) [33] constructs were purchased from Addgene. HEK293T, HEK293GP, U2OS, HeLa, HCT116, and Panc-1 cell lines were purchased from American Type Culture Collection (ATCC) and cultured as ATCC instructed. The HPNE cell line was provided by Dr. Michel Ouellette (University of Nebraska Medical Center), who originally established the cell line [34]. The human lung cancer cell lines, A549 and H520, were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Both cell lines were maintained in RPMI-1640 medium with 10% fetal bovine serum and 1% penicillin/streptomycin (Invitrogen). All cells were cultured in incubators with 95% air and 5% CO_2 at 37 °C.

Attractene (Qiagen) was used for transient overexpression of proteins in HEK293T and HEK293GP cells following the manufacturer's instructions. Nocodazole (100 ng/ml for 16 h) and taxol (100 nM for 16 h) (Selleck Chemicals) were used to arrest cells in G2/M phase. VX680 (Aurora-A, -B, -C inhibitor), MK5108 (Aurora-A inhibitor), BI2536 (PLK1 inhibitor), Purvalanol A (CDK1/2/5 inhibitor), SP600125 (JNK1/2 inhibitor) and MK2206 (Akt inhibitor) were also from Selleck Chemicals. RO3306 (CDK1 inhibitor) was from ENZO Life Sciences. Kinase inhibitors for MEK-ERK (U0126), p38 (SB203580), and mTOR (Rapamycin) were from LC Laboratory. All other chemicals were either from Sigma or from Thermo Fisher.

Recombinant protein purification and in vitro kinase assay

GST-tagged LIMD1 or LIMD1-4A (cloned in pGEX-5X-1) was bacterially expressed and purified on GSTrap FF affinity columns (GE Healthcare) following the manufacturer's instructions. Recombinant proteins (GST-LIMD1/LIMD1-4A, 0.25 µg each) was incubated with 5-10 U purified CDK1/cyclin B complex (New England Biolabs) or 50-100 ng CDK1/cyclin B (SignalChem) in the presence of 10 µCi γ-^{32}P-ATP (3000 Ci/mmol,

PerkinElmer) as we previously described [22]. Active CDK2, CDK5, p38α, JNK1, JNK2, MEK1, ERK1, and ERK2 kinases were also purchased from SignalChem.

Antibodies

Rabbit polyclonal phospho-specific antibodies against human LIMD1 S272, S277, and S421/S424 were generated and purified by AbMart Inc. The peptides used for immunizing rabbits were: SSQRV-pS-PGLPSP (p-S272); SPGLP-pS-PNLEN (p-S277); SVLLD-pS-PS-pS-PRVRLP (p-S421/424). The corresponding non-phosphorylated peptide was also synthesized and used for antibody purification. Anti-β-actin (SC-47778) and anti-cyclin B (SC-752) antibodies were from Santa Cruz Biotechnology. Glutathione S-transferase (GST) (A190-122A) and His (A190-114A) antibodies were from Bethyl Laboratories. Phospho-S10 H3 (3377), phospho-S127 YAP (13008), phospho-S397 YAP (13619), YAP (12395), LIMD1 (13245), phospho-Aurora-A (T288)/Aurora-B (T232)/Aurora-C (T198) (2914), and Aurora-A (14475) antibodies were from Cell Signaling Technology.

Phos-tag and Western blot analysis

Phos-tag™ was obtained from Wako Pure Chemical Industries, Ltd. and used at 10-20 μM (with 100 μM $MnCl_2$) in 8% SDS-polyacrylamide gels as we previously described [20]. Western blotting, immunoprecipitation, and lambda phosphatase treatment assays were done as previously described [27,35].

Cell proliferation and colony formation assays

The LIMD1-wild type or LIMD1-4A plasmid was transfected to A549 and H520 cell lines by transfection reagent Lipo2000 (Invitrogen) according to the manufacturer's protocol. For cell

proliferation assays, 10,000 cells were seeded into each well of 24-well plates in triplicate and proliferation curves were made from three independent experiments [21]. Colony formation assays in soft agar were performed as described [28]. Cells were seeded into 6-well plates in triplicate (2,000 cells/well) for 14 days.

Migration and invasion assays

Migration (wound healing) and invasion (Transwell plates with Matrigel, BD Biosciences) assays were done as described [36].

CRISPR-Cas9-mediated LIMD1 deletion

The CRISPR-LIMD1 and double nickase constructs were purchased from Santa Cruz Biotechnology (SC-404413-NIC). The plasmids were transiently transfected into cells and GFP-positive clones were selected by flow cytometry-based cell sorting [21].

Live-cell imaging microscopy

RFP-H2B-transduced U2OS cells were cultured in FluroBrite DMEM (Thermo Fisher) on black 96-well optical bottom plates (Thermo Fisher). Cells were monitored for 24 h and pictures taken every 5 min using an RFP filter on a Cellomics Arrayscan VTI HCS Reader.

Immunofluorescence staining and confocal microscopy

Immunofluorescence staining and microscopy were done as previously described [37].

Statistical analysis

Statistical significance was analyzed using a two-tailed, unpaired Student's *t*-test. The Mann Whitney test was used for comparing the mitotic length (Fig. 5). A p-value <0.05 was considered as statistically significant.

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Author contributions

JZ, LZ, and Y. Cheng designed experiments. JD and LZ wrote the paper. JZ, LZ, WZ, Y. Chen performed the experiments, analyzed the data and interpreted the results. Y. Chen also provided technical support. All authors reviewed and approved the manuscript prior to submission.

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Figure 1

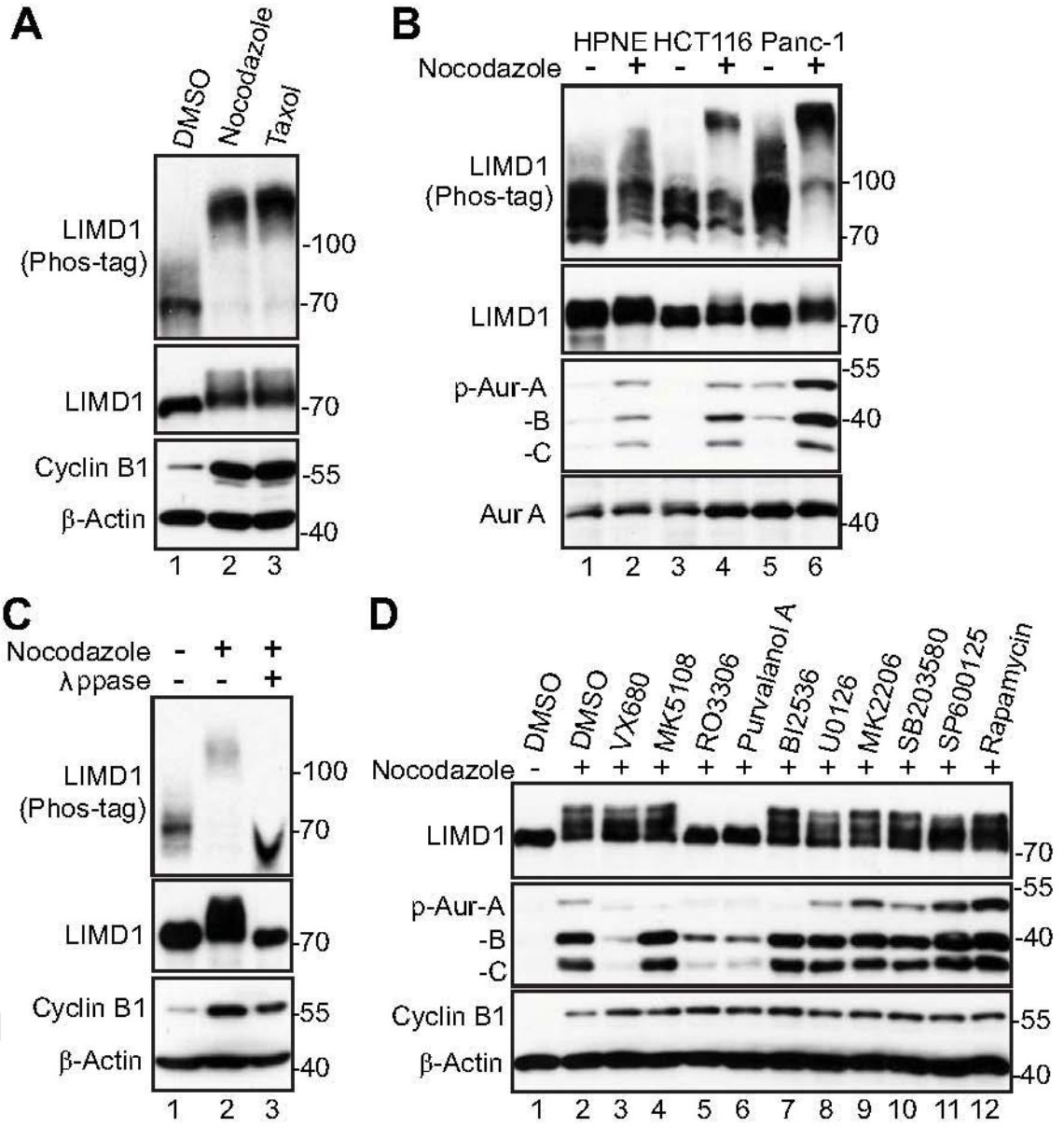

Fig. 1. CDK1- and JNK1/2-dependent phosphorylation of LIMD1 during anti-tubulin drug-induced mitotic arrest.

(A) HeLa cells were treated with DMSO, taxol or nocodazole. Total cell lysates were probed with the indicated antibodies on Phos-tag or regular SDS-polyacrylamide gels. Increased levels of cyclin B1 mark cells in mitosis.

(B) HPNE, HCT116, and Panc-1 cells were treated with DMSO or nocodazole. Total cell lysates were probed with the indicated antibodies on Phos-tag or regular SDS-polyacrylamide gels. Elevated levels of p-Aurora kinases indicate cells in mitosis.

(C) HeLa cells were treated with nocodazole as indicated and cell lysates were further treated with (+) or without (-) λ phosphatase (ppase). Total cell lysates were probed with anti-LIMD1 antibody on Phos-tag or regular SDS-polyacrylamide gels.

(D) HeLa cells were treated with nocodazole together with or without various kinase inhibitors as indicated. RO3306 (CDK1 inhibitor, 5 μM), Purvalanol A (CDK1/2/5 inhibitor, 10 μM), SB203580 (p38 inhibitor, 10 μM), SP600125 (JNK1/2 inhibitor, 20 μM), U0126 (MEK-ERK inhibitor, 20 μM), MK2206 (AKT inhibitor, 10 μM), BI2536 (PLK1 inhibitor, 100 nM), VX680 (Aurora-A, B, C inhibitor, 2 μM), and MK5108 (Aurora-A inhibitor, 5 μM) were used. Inhibitors were added 1.5 h before harvesting the cells (with MG132 to protect cyclin B1 from degradation and prevent cells from exiting from mitosis). Total cell lysates were subjected to Western blotting with the indicated antibodies. All Western blots are representative of three repeats.

Figure 2

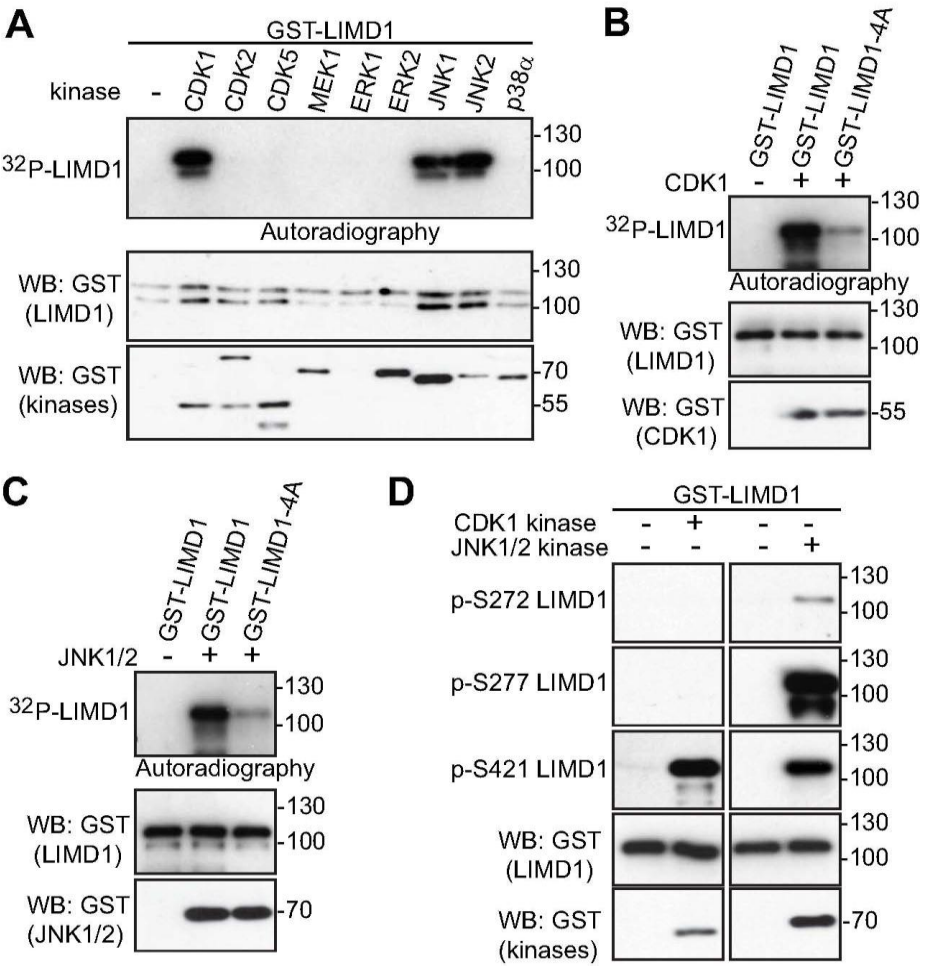

Fig. 2. CDK1 and JNK1/2 phosphorylate LIMD1 *in vitro*.

(A) *In vitro* kinase assays with kinases as indicated using GST-LIMD1 proteins as substrates. ERK1 kinase was not detectable in this context.

(B) *In vitro* kinase assays with CDK1/cyclin B complex using GST-LIMD1 or GST-LIMD1-4A proteins as substrates. 4A: S272A/S277A/S421A/S424A.

(C) *In vitro* kinase assays with JNK1/2 kinases using GST-LIMD1 or GST-LIMD1-4A proteins as substrates. 4A: S272A/S277A/S421A/S424A.

(D) *In vitro* kinase assays were done as in A except anti-p-S272, -S277, and -S421/S424 LIMD1 antibodies were used. All experiments are representative of two biological repeats.

Figure 3

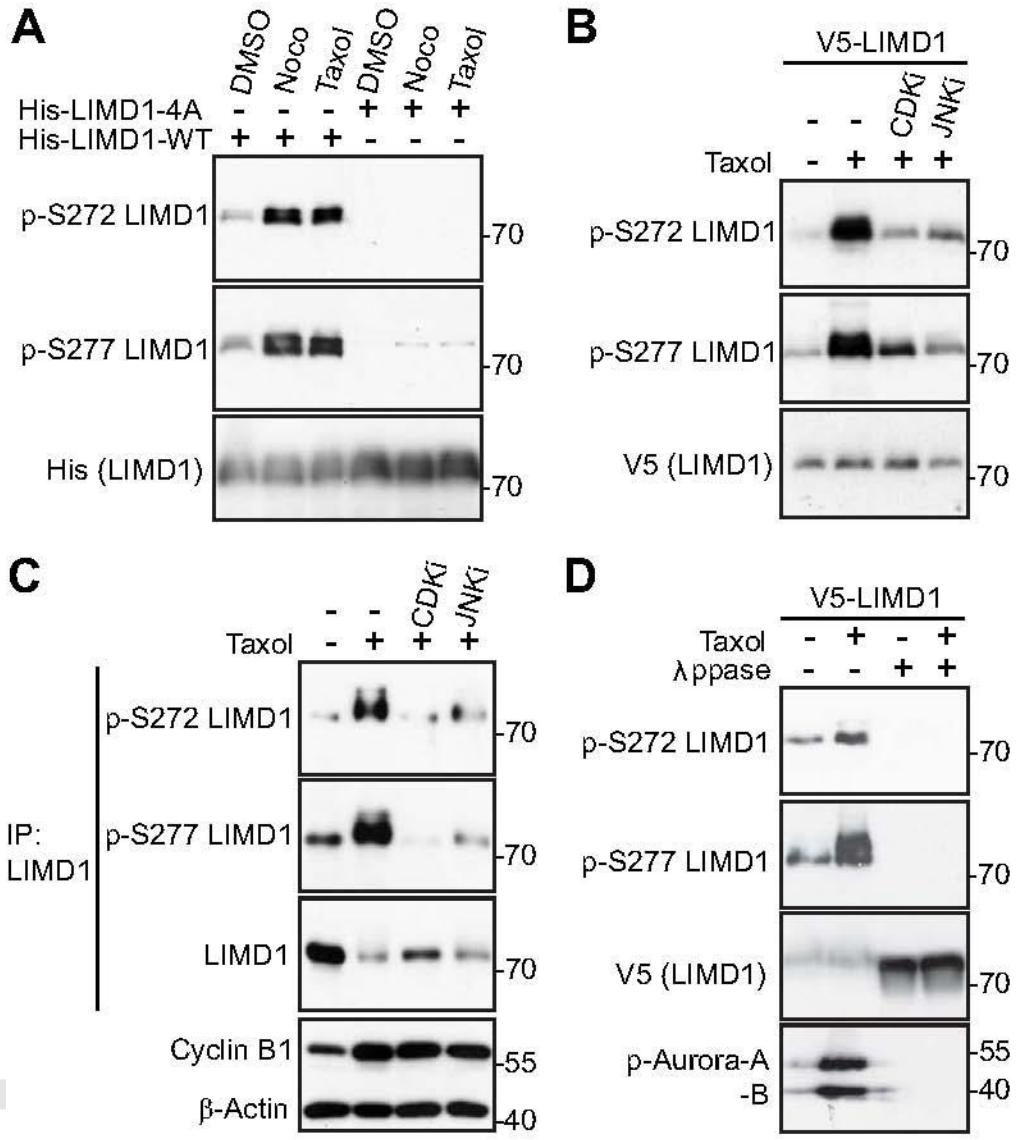

Fig. 3. CDK1 and JNK1/2 mediate the phosphorylation of LIMD1 in cells.

(A) HEK293T cells were transfected with the indicated LIMD1 cDNA and at 48 h post-transfection, the cells were treated with taxol or nocodazole (noco) for an additional 18 h. Total cell lysates were subjected to Western blotting with the indicated antibodies.

(B) HEK293T cells were transfected with V5-LIMD1 cDNA, and at 36 h post-transfection the cells were treated with taxol as indicated for an additional 18 h. CDK1 inhibitor (CDKi, RO3306, 5 μM) or JNK1/2 inhibitor (JNKi, SP600125, 20 μM) together with MG132 (25 μM) were added 1.5 h before the cells were lysed. Total cell lysates were subjected to Western blotting with the indicated antibodies.

(C) HeLa cells were treated with taxol as indicated for 18 h. CDK1 inhibitor (CDKi, RO3306, 5 μM) or JNK1/2 inhibitor (JNKi, SP600125, 20 μM) together with MG132 (25 μM) were added 1.5 h before the cells were lysed. LIMD1 proteins were immunoprecipitated and the samples were probed with phospho-LIMD1 and subsequent LIMD1 antibodies.

(D) HEK293T cells were transfected with V5-LIMD1 and treated with taxol as indicated and cell lysates were further treated with (+) or without (-) λ phosphatase (ppase). Total cell lysates were probed with the indicated antibodies. P-Aurora A/B serves as a positive marker. All experiments are representative of three biological repeats.

Figure 4

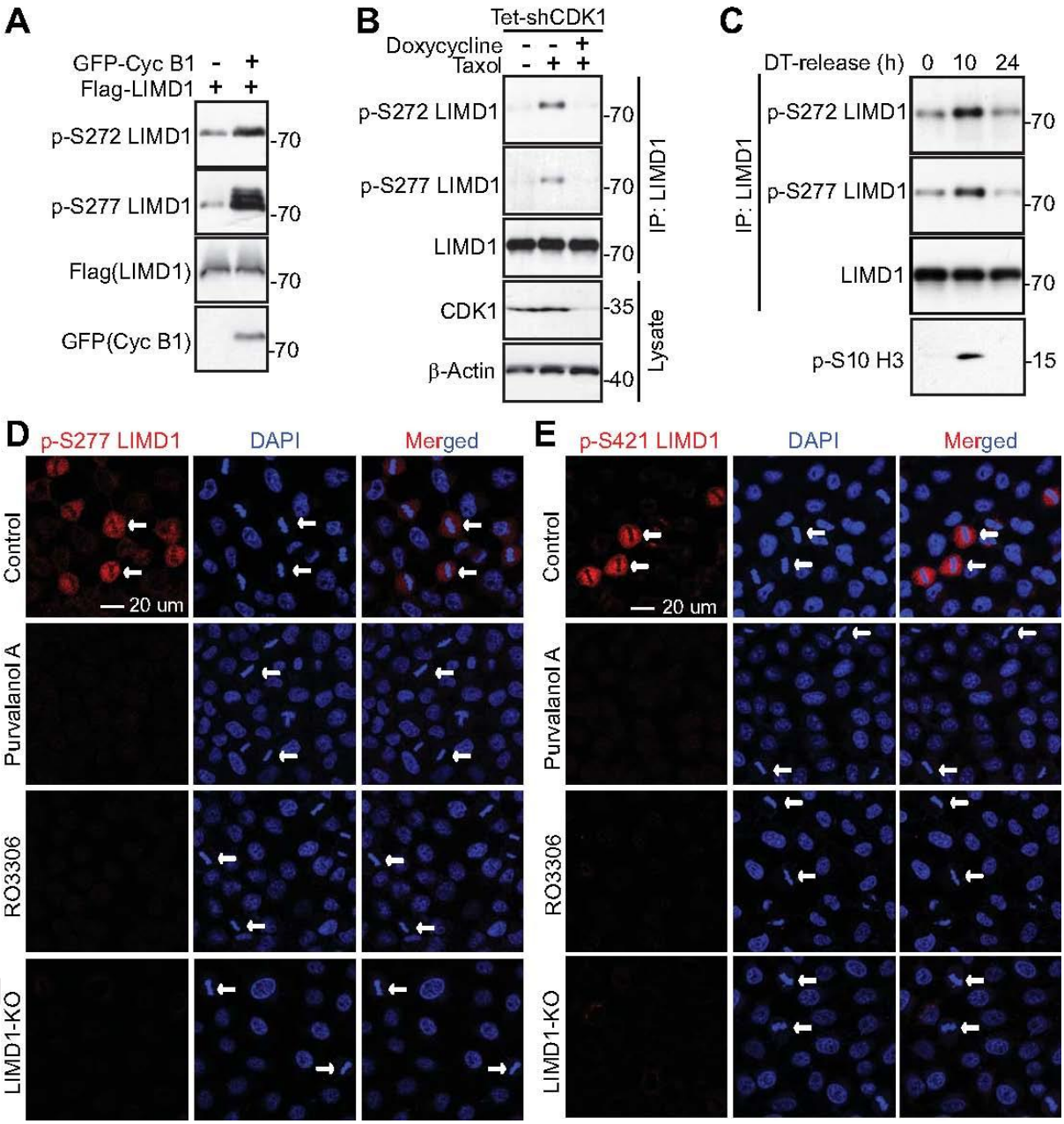

Fig. 4. Phosphorylation of LIMD1 occurs during normal mitosis in a CDK1-dependent manner.

(A) HEK293T cells were transfected with the indicated DNA constructs and total cell lysates were analyzed by Western blotting. GFP-Cyc B1: GFP-Cyclin B1-R42A (a non-degradable constitutive active mutant).

(B) CDK1 knockdown inhibited LIMD1 phosphorylation. TetOn-inducible shRNA targeting CDK1 was expressed in HeLa cells. The cells were treated with or without Doxycycline (Dox) for 2 days and were further treated with taxol for an additional 16 h. Endogenous LIMD1 was immunoprecipitated and subjected to Western blotting with the indicated antibodies. Increased p-H3 S10 levels mark the cells in mitosis.

(C) A double thymidine (DT) block and release was performed in HeLa cells and samples were collected at the indicated time points. LIMD1 proteins were immunoprecipitated and the samples were probed with the indicated antibodies. Phospho-S10 H3 serves as a mitotic marker.

(D, E) Immunofluorescence staining of p-LIMD1 S277 (**D**) and p-LIMD1 S421 (p-S421/S424, **E**) in freely cycling HeLa cells. White arrows mark cells in metaphase (condensed and aligned chromosomes). CDK1 inhibitors (Purvalanol A or RO3306) were added 1.5 h before the cells were fixed. All experiments are representative of two biological repeats.

Figure 5

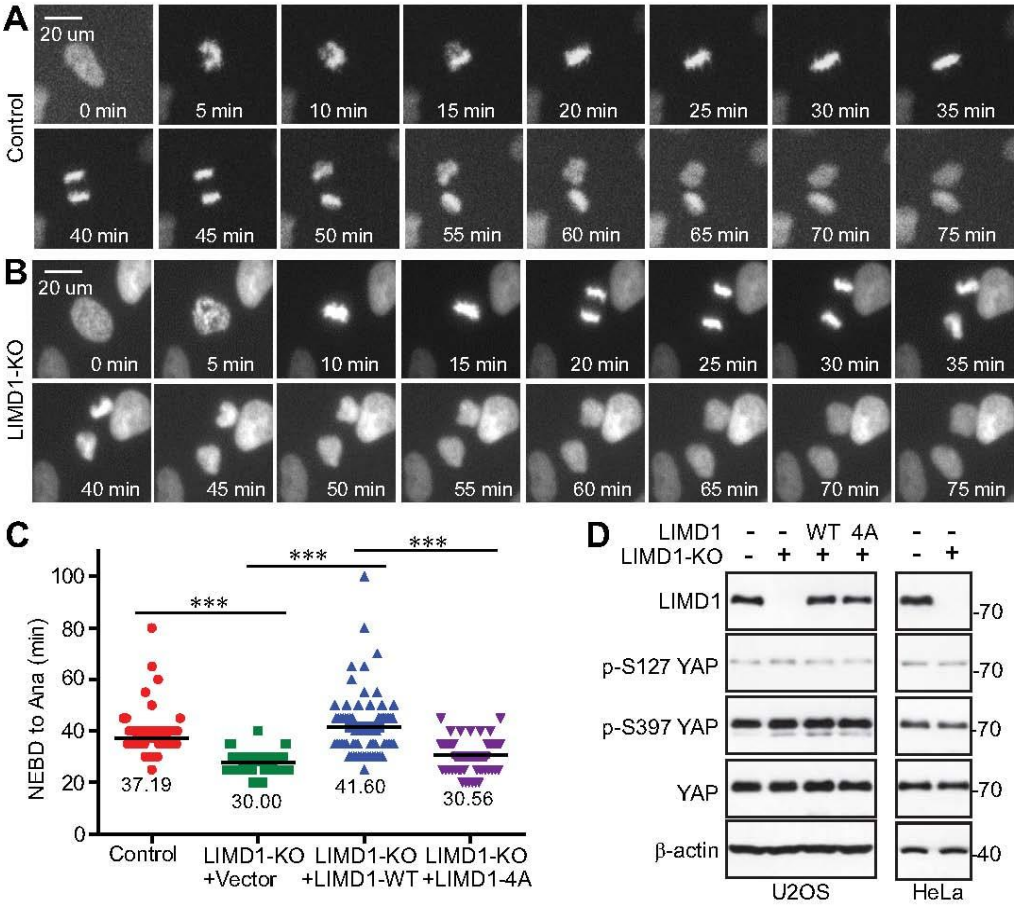

Fig. 5. Mitotic phosphorylation of LIMD1 is required for mitotic progression.

(A, B) Representative live images of control and LIMD1-KO cells.

(C) Quantification of mitotic length [from NEBD (nuclear envelope breakdown) to anaphase] from control (n=80), LIMD1-KO (n=80), LIMD1-KO+LIMD1-WT (n=72), and LIMD1-KO+LIMD1-4A (n=72) cells. ***: $p<0.001$ (Mann Whitney test).

(D) Deletion of LIMD1 does not affect YAP phosphorylation/activity in HeLa and U2OS cells. The U2OS LIMD1-KO cell line was used to establish cell lines expressing vector, LIMD1-WT, or LIMD1-4A. 4A: S272A/S277A/S421A/S424A. WT: wild type. Total cell lysates from these cell lines were probed with the indicated antibodies. Experiments were done at high cell density (>85% confluence). Data in panel D are representative of three biological repeats.

Figure 6

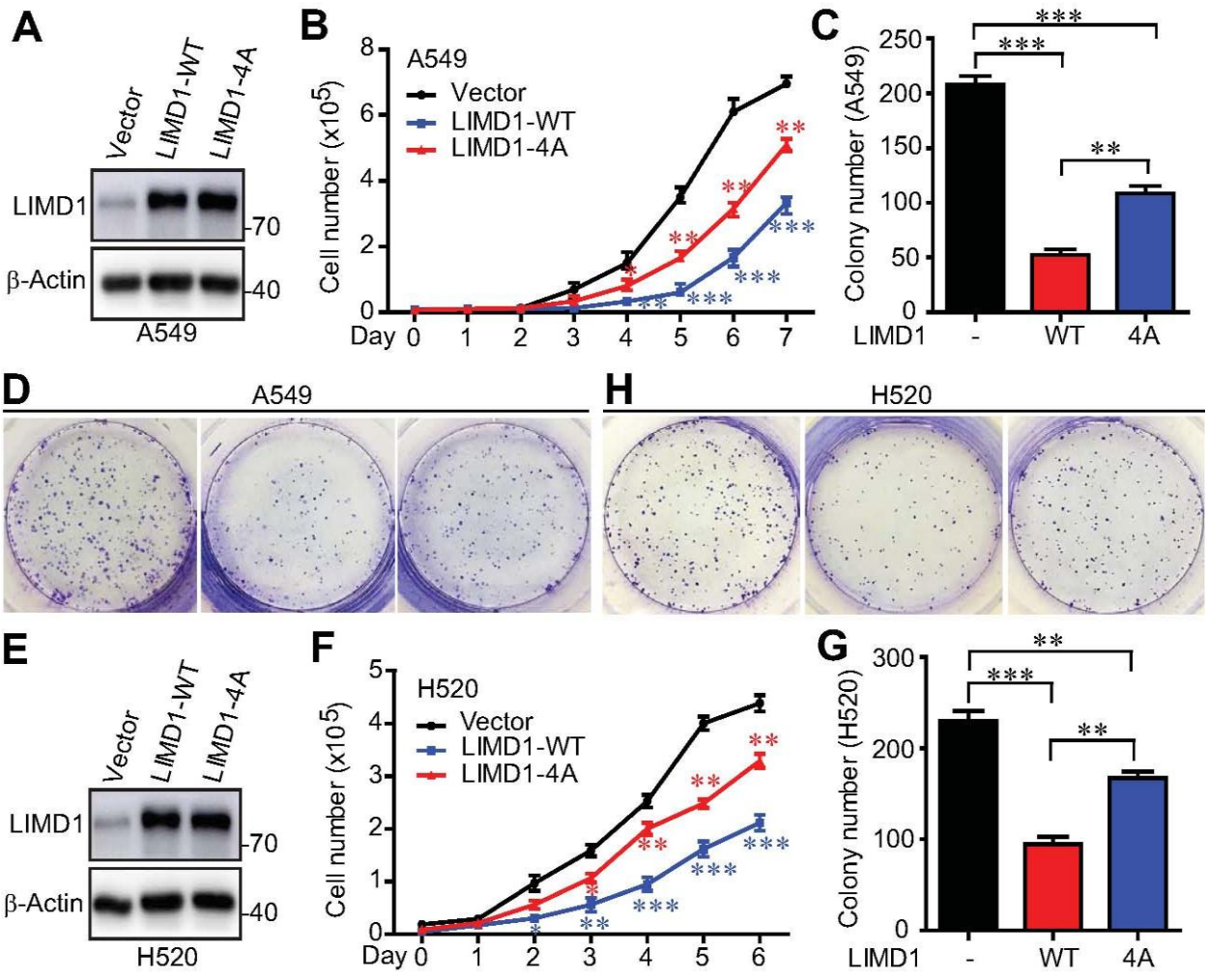

Fig. 6. Mitotic phosphorylation of LIMD1 is required for suppressing proliferation and anchorage-independent growth in lung cancer cells.

(A) Western blotting analysis of LIMD1 expression in A549 cells transfected with vector (control), LIMD1-WT, or LIMD1-4A DNA. 4A: S272A/S277A/S421A/S424A.

(**B-D**) Cell proliferation and anchorage-independent growth (colony formation assays in soft agar) assays with A549 cells from A. Data were expressed as the mean ± s.d. of three independent repeats. Representative images for colony assay were shown (D). Red asterisks mark the comparisons between LIMD1-4A and LIMD1-WT. Blue asterisks mark the

comparisons between Vector and LIMD1-WT. ***: p<0.001, **: p< 0.01; *: p< 0.05 (Student's t-test).

(E-H) Similar experiments were done as A-D in H520 cells. Data were expressed as the mean ± s.d. of three repeats. Representative images for colony assay were shown (H). Red asterisks mark the comparisons between LIMD1-4A and LIMD1-WT. Blue asterisks mark the comparisons between Vector and LIMD1-WT. ***: p<0.001, **: p< 0.01; *: p< 0.05 (Student's t-test).

Figure 7

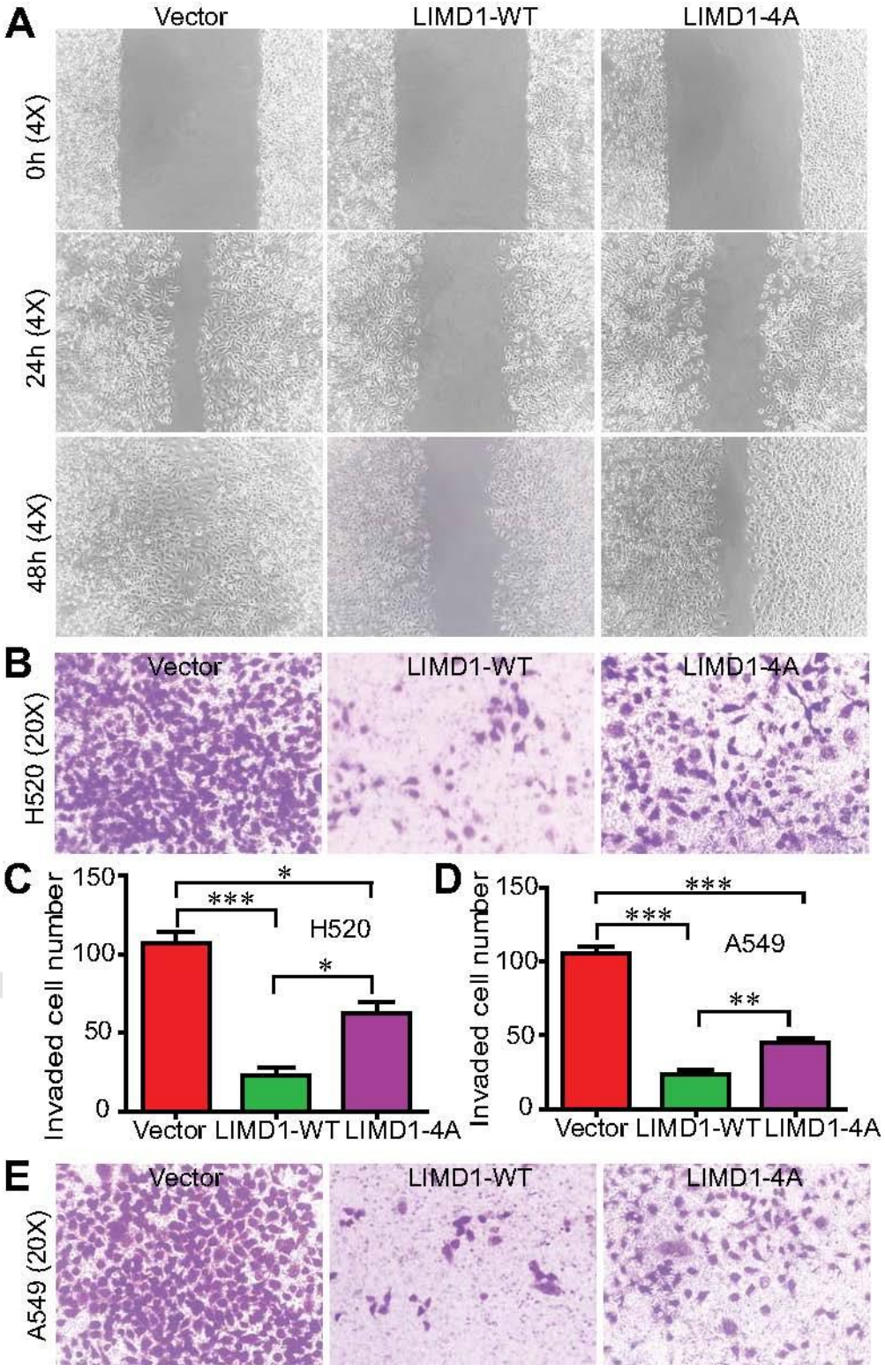

Fig. 7. Mitotic phosphorylation of LIMD1 is required for suppressing migration and invasion in lung cancer cells.

(A) Cell migration (wound healing) assays in H520 cells transfected with vector (control), LIMD1-WT, or LIMD1-4A. Representative images of three repeats were shown. 4A: S272A/S277A/S421A/S424A.

(B, C) Cell invasion assays of H520 cells transfected with vector (control), LIMD1-WT, or LIMD1-4A. Data were expressed as the mean ± s.d. of three repeats (C) and representative images were shown (B). ***: $p<0.001$, *: $p< 0.05$ (Student's t-test).

(D, E) Cell invasion assays of A549 cells transfected with vector (control), LIMD1-WT, or LIMD1-4A. Data were expressed as the mean ± s.d. of three repeats (D) and representative images were shown (E). ***: $p<0.001$, **: $p< 0.01$ (Student's t-test).