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Heat Shock Protein 90 (HSP90) Inhibition Depletes LATS1 and LATS2, Two Regulators of the Mammalian Hippo Tumor Suppressor Pathway

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Abstract

Heat shock protein 90 (HSP90), which regulates the functions of multiple oncogenic signaling pathways, has emerged as a novel anticancer therapeutic target, and multiple small molecule HSP90 inhibitors are now in clinical trials. Although the effects of HSP90 inhibitors on oncogenic signaling pathways have been extensively studied, the impacts of these agents on tumor suppressor signaling pathways are currently unknown. Here, we have examined how HSP90 inhibitors affect LATS1 and the related protein LATS2, two kinases that relay antiproliferative signals in the Hippo tumor suppressor pathway. Both LATS1 and LATS2 were depleted from cells treated with the HSP90 inhibitors 17-allylamino-17-demethoxygeldanamycin (17-AAG), radicicol and PU-H71. Moreover, these kinases interacted with HSP90, and LATS1 isolated from 17-AAG-treated cells had reduced catalytic activity, thus demonstrating that the kinase is a *bona fide* HSP90 client. Importantly, LATS1 signaling was disrupted by 17-AAG in tumor cell lines *in vitro* and clinical ovarian cancers *in vivo* as shown by reduced levels of LATS1 and decreased phosphorylation of the LATS substrate YAP, an oncoprotein transcriptional coactivator that regulates genes involved in cell and tissue growth, including the *CTGF* gene. Consistent with the reduced YAP phosphorylation, there were increased levels of CTGF, a secreted protein that is implicated in tumor proliferation, metastasis, and angiogenesis. Taken together, these results identify LATS1 and LATS2 as novel HSP90 clients and demonstrate that HSP90 inhibitors can disrupt the LATS tumor suppressor pathway in human cancer cells.

Introduction

HSP90 is a central participant in a multistep chaperoning process that folds and stabilizes a wide range of cellular clients. Tumor cells express high levels of HSP90 and have increased reliance on the HSP90 chaperoning pathway compared to normal cells (1). This increased reliance on HSP90 has been attributed to several features of tumor cells. On the one hand, HSP90 plays critical roles in facilitating the survival and proliferation of tumors by chaperoning and supporting the activities of a multitude of key oncogenic proteins that promote tumorigenesis. These include wild-type and mutant receptor tyrosine kinases (e.g., EGFR family members, FLT-3, and BCR-ABL and NPM-ALK fusion proteins), signal-relaying

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serine-threonine kinases (AKT, Raf isoforms, Chk1, and CDK4), transcription factors (HIF-1 α , steroid receptors, and mutant p53), telomerase, and proteins involved in apoptosis (Apaf-1, Bcl-2). In addition, HSP90 helps tumors survive the stressful environmental conditions associated with tumor proliferation. As a result, HSP90 has attracted considerable attention as a potential cancer therapy target; and multiple HSP90 inhibitors are now in clinical trials worldwide (2).

Despite the intense interest in oncoprotein clients that are disabled by HSP90 inhibition and the potential of HSP90 inhibitors to treat malignancies, the effects of these inhibitors on tumor suppressor pathways have been largely unexplored. Given that HSP90 inhibition of tumor suppressor pathways may negatively impact the effectiveness of HSP90 inhibitors, here we have examined the effects of HSP90 inhibition on the Hippo tumor suppressor pathway, which was first discovered to control organ size in *Drosophila* by regulating proliferation, cell growth, and apoptosis (3,4). Central regulators of this pathway in mammals are the LATS1 and LATS2 kinases (known as Warts in *Drosophila*), which are members of the nuclear Dbf-2-related (NDR) serine-threonine kinase family. LATS1 and LATS2, which are activated by cell-cell contact, negatively regulate cell proliferation and organ size in mice (5,6). The activation states of LATS1 and LATS2 are regulated by the serine-threonine kinases MST1/2 (Hippo in *Drosophila*), which directly phosphorylate LATS, and by MOB (Mats in *Drosophila*) family members, small proteins that bind and activate LATS. Once activated, LATS kinases phosphorylate the transcriptional co-activators YAP and TAZ (7). Phosphorylation of these transcription factors creates binding sites for 14-3-3 proteins, which localize and anchor YAP/TAZ in the cytoplasm, and directs YAP for proteolytic degradation. In the absence of this inhibitory phosphorylation, these transcription factors enter the nucleus, where they interact with DNA-binding proteins, including those in the TEAD family (8,9), to trigger the transcription of genes that promote cell proliferation.

Consistent with their roles in cell proliferation, the central players in this pathway have emerged as key regulators of tumorigenesis. *Lats1*^{-/-} mice develop ovarian cancers and sarcomas (10), and the *LATS1* and/or *LATS2* promoters are hypermethylated in multiple human tumor types, including breast cancer, astrocytomas, and sarcomas (11-14), with promoter hypermethylation corresponding to reduced mRNA expression and in some instances worse clinical outcome. In addition, there is loss of heterozygosity at the *LATS1* and *LATS2* loci in ovarian, cervical and breast cancer (15-18). Similarly, *MST1* and *MST2* are also tumor suppressors. Simultaneous genetic ablation of *Mst1* and *Mst2* in the liver of mice leads to stem cell accumulation, liver enlargement, and the rapid development of hepatocellular carcinomas (19-21). In humans, the *MST1* and *MST2* promoters are hypermethylated in some tumors (22,23). Collectively, these studies suggest that MST1/2 and LATS kinases are mammalian tumor suppressors.

In contrast, *YAP* is an oncogene. The human *YAP* locus (11q22) is amplified in multiple tumor types (24-28), and *YAP* is overexpressed in many tumors (6,29,30). Moreover, enforced *YAP* overexpression in the livers of transgenic mice causes rapid and dramatic increases in cell proliferation, organ size, cellular dysplasia, and the development of liver tumors (5,6). Consistent with the findings in mice, *YAP* and *TAZ* overexpression in nontransformed human cell lines induces multiple hallmarks of transformation, including epithelial-to-mesenchymal transformation, decreased apoptosis, and anchorage- and growth factor-independent proliferation (30-33). Given the pivotal role that the LATS-*YAP* pathway plays in cellular and organ homeostasis, the potential effects of HSP90 inhibitors on this pathway may be relevant to the development of these agents as anticancer agents.

Materials and Methods

Cell lines, cell culture, transfections, and cell cycle analyses

The human cell lines A549, MCF10A, H460, HCT-116, U2OS, and OVCAR5 were obtained from American Type Culture Collection (Manassas, VA). Every 3 months cell lines were re-initiated from cryopreserved stocks prepared immediately after receipt from ATCC. All cells were grown in RPMI supplemented with 10% fetal bovine serum, except MCF10A, which were cultured as recommended by American Type Culture Collection. Cell transfections and cell cycle analyses were as described previously (34).

Materials

17-AAG was from Kosan Biosciences (Hayward, CA). Reagents were purchased from the following suppliers: RPMI and Hams F12 media from MediaTech (Manassas, VA); DMEM/F12 medium from Life Technologies (Carlsbad, CA); fetal bovine and horse sera from Hyclone (Thermo Scientific, Waltham, MA) and American Type Culture Collection, respectively; PU-H71, ATP, bovine serum albumin, hydrocortisone, epidermal growth factor, insulin, propidium iodide, Tween 20, RNase A, Hoechst 33342, hydroxyurea, protein A-Sepharose, and bovine serum albumin from Sigma-Aldrich (St. Louis, MO); [γ ³²P]-ATP from MP Biomedicals (Solon, OH); streptavidin-agarose from Upstate Biotech (Millipore, Billerica, MA); LAB-TECH II chamber slides from Nalge Nunc (Rochester, NY); and SlowFade Gold from Invitrogen (Carlsbad, CA). Antibodies to the following antigens were purchased from the indicated suppliers: LATS1 (A300-477A) and LATS2 (A300-479A) from Bethyl Laboratories (Montgomery, TX); Chk1 (G4), YAP (H125), and CTGF (L20) from Santa Cruz Biotechnology (Santa Cruz, CA); AKT1 (9272) from Cell Signaling Technology (Danvers, MA); MST2 (1943-1) from Epitomics (Burlingame, CA); FITC-conjugated donkey anti-rabbit IgG from Jackson ImmunoResearch (West Grove, PA); and FLAG M2 from Sigma-Aldrich. Murine monoclonal antibody that recognizes HSP90 β (H9010) was a kind gift from David Toft (Mayo Clinic, Rochester, MN).

Cell lysis and immunoprecipitation

Cells were lysed in 50 mM HEPES, pH 7.6, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 10 mM NaF, 30 mM sodium pyrophosphate, 1 mM Na₃VO₄, 10 mM 2-glycerophosphate, 10 μ g/ml leupeptin, 5 μ g/ml aprotinin, 5 μ g/ml pepstatin, 20 nM microcystin-LR and 1 mM phenylmethylsulfonyl fluoride. For HSP90-LATS1 co-immunoprecipitation studies, cells were lysed on ice in 20 mM Tris, pH 7.4, 150 mM NaCl, 5 mM MgCl₂, 1% Triton X-100, and 10 mM Na₂MoO₄, and equal amounts of soluble protein from cleared lysates were immunoprecipitated overnight with anti-LATS1 antibody immobilized on protein A-Sepharose at 4°C. Washed immunoprecipitates were mixed with SDS-PAGE sample buffer, heated at 95°C for 10 min, separated by SDS-PAGE, and transferred to Immobilon P (Millipore, Billerica, MA) prior to immunoblotting.

In vitro kinase assays

A549 were transiently transfected with plasmids that express SFB-LATS1 and the indicated tagged proteins. Forty-eight h after transfection, the cells were lysed and SFB-LATS1 was purified from the lysates by binding to streptavidin-agarose for 1-3 h with rotation at 4°C. The beads were washed three times with lysis buffer followed by three washes with kinase buffer (50 mM HEPES, pH 7.4, 50 mM potassium acetate, 5 mM MgCl₂, and 1 mM dithiothreitol). Kinase reactions were initiated by the addition of 1 μ g GST-YAP (produced in *E. coli* by standard methods), 10 μ Ci [γ ³²P]-ATP, and 100 μ M unlabeled ATP to the washed beads. The reactions were incubated for 15 min at 30°C, stopped by the addition of SDS-PAGE sample buffer, heated to 95°C for 10 min, separated by SDS-PAGE, and transferred to Immobilon P.

Radioactivity on the membrane was detected and quantitated with a Storm Phosphorimaging system (GE Healthcare) prior to immunoblotting to detect tagged proteins.

Plasmids

cDNAs for *LATS1* (NM_004690.2), *MST2* (NM_006281.2), *MOB1B* (NM_018221.3), and *YAP* (NM_006106.1) were obtained from Open Biosystems (Thermo Scientific) and amplified by PCR using primers that appended appropriate restriction endonuclease sites. Following digestion with restriction endonucleases, the PCR fragments were ligated into the appropriate vectors to create pSFB-LATS1 (which appends an N terminal S-tag, a tandem FLAG epitope tag, and the streptavidin-binding peptide; (35)), pcDNA3-MST2-HA (which appends a C-terminal HA tag), pcDNA3-FLAG-MOB1B (which appends an N-terminal FLAG epitope tag), and pGEX-YAP (which fuses YAP to glutathione S-transferase, GST). All final constructs were sequenced to insure fidelity of the PCR amplifications and cloning manipulations.

Immunofluorescence

Cells were grown to confluence on chamber slides, treated with 1 μ M 17-AAG for 3 h, fixed in 4% paraformaldehyde in Dulbecco's calcium- and magnesium-free phosphate-buffered saline (PBS) for 20 min, and permeabilized in 0.1% Triton X-100 in PBS. Slides were blocked in PBS containing 3% bovine serum albumin, incubated with 1:250 dilution anti-YAP overnight at 4°C, stained with 1:400 dilution FITC-donkey anti-rabbit IgG, and counterstained with 1 μ g/ml Hoechst 33342. Images were acquired using a Zeiss LSM510 microscope and the manufacturer's software.

Ovarian cancer biopsy samples

Biopsies were obtained under the aegis of an Institutional Review Board-approved protocol as part of a phase II clinical trial assessing the effects of 17-AAG on ovarian cancer tumors that will be reported elsewhere. Paired core needle biopsies were obtained prior to treatment and 22-26 h after patients received 154 mg/m² 17-AAG intravenously as a 2-h infusion. Biopsy samples were immediately frozen on dry ice and processed as described previously (36).

Results

LATS1 and LATS2 – but not MST2 – are depleted by 17-AAG

HSP90 inhibition causes the depletion of HSP90 clients by disrupting client chaperoning and targeting the clients for proteasomal degradation. Thus, treatment of cells with an HSP90 inhibitor provides a rapid and simple assay to assess whether a given protein depends either directly or indirectly on HSP90 chaperoning activity. To evaluate whether components of the LATS signaling pathway require HSP90, we treated subconfluent A549 cancer cells with 17-AAG and examined the levels of LATS1, LATS2, and MST2 at various time points after treatment with 1 μ M 17-AAG, a concentration that maximally inhibits HSP90 in many cell lines (34,37,38). While MST2 levels changed little over the 48-h time course (Fig. 1A), both LATS1 and LATS2 were depleted at rates similar to the rates at which Chk1 and AKT, two *bona fide* HSP90 clients (34,39-41), decreased. Similar effects on LATS1 and LATS2 were also observed when HeLa cells (data not shown) and MCF10A cells (Fig. 1A), a nontransformed breast cell line, were treated with 17-AAG for various times. Additional studies confirmed that a concentration of 1 μ M 17-AAG was maximally effective in depleting LATS1 and LATS2 in A549 and MCF10A cells (Fig. 1B) and that the LATS kinases were depleted by the same concentrations of 17-AAG that result in Chk1 loss. To confirm that the effects of 17-AAG were due to HSP90 inhibition, we also showed that radicicol (Fig. 1C), a structurally unrelated HSP90 inhibitor (42), and PU-H71 (Fig. 1D), a metabolically stable purine-scaffold HSP90 inhibitor (43), depleted LATS1 and LATS2. Taken together, these

results demonstrated that the levels of LATS1 and LATS2, but not MST2, are dramatically reduced in both transformed and untransformed human cell lines by HSP90 inhibitors.

LATS1 and LATS2 are HSP90 clients

Because HSP90 inhibitors cause cell cycle arrest in G1 and G2/M (38,44), it was possible that the effects seen in Fig. 1 were due to perturbations in the cell cycle, which might affect LATS1 and LATS2 levels. We assessed this possibility by arresting subconfluent A549 cells at the G1/S border with hydroxyurea (HU) for 24 h (Fig. 2A) and then treating them with 17-AAG for increasing times while in the continued presence of HU. As shown in Fig. 2A and B, 17-AAG did not relieve the cell arrest caused by HU; yet both LATS1 and LATS2 were depleted by 17-AAG in the HU-arrested cells, indicating that the effects of 17-AAG on LATS1 and LATS2 levels are not the result of cell cycle perturbations and suggesting that the LATS kinases might be HSP90 clients. In accord with that prediction, we found that LATS2 (Fig. 2C) and LATS1 (Fig. 2D) immunoprecipitates contained HSP90. Furthermore, the interaction between LATS1 and HSP90 was disrupted by 17-AAG (Fig. 2D) as has been observed with other HSP90 clients (34). Taken together, these results demonstrate that these kinases are novel HSP90 clients.

HSP90 inhibition reduces LATS1 catalytic activity

We also assessed how LATS1 kinase activity was affected by HSP90 inhibition. We first attempted to assess the activity of endogenous LATS1 in subconfluent and confluent A549 cells; however, we were unable to detect LATS1 kinase activity with *in vitro* kinase assays using immunopurified LATS1 (data not shown). As an alternative approach, cells were transfected with SFB-tagged LATS1 alone or with the LATS activators MST2 and MOB1B. MST2 is a serine-threonine kinase that phosphorylates LATS1 on a C-terminal hydrophobic motif required for LATS1 activation, and MOB1 is a small protein that binds LATS1 and increases LATS1 kinase activity by inducing autophosphorylation of the LATS1 activation loop in the catalytic domain (45). Following treatment of the cells with vehicle or 17-AAG, the SFB-tagged LATS1 was purified using streptavidin-agarose and the activity of the bead-bound LATS1 was assessed using GST-YAP as a substrate. In the absence of co-expressed MOB1B and MST2, LATS1 kinase activity was not above background levels (Fig. 3A), whereas the co-expression of MOB1B and MST2 dramatically activated LATS1, consistent with previous findings. Surprisingly, however, although 17-AAG caused reductions in LATS1 auto-kinase activity and YAP phosphorylation (Fig. 3A and 3B), LATS1 levels did not decrease with drug treatment. One possible explanation for this result is that high level expression of MOB1B and/or MST2 stabilized LATS1 in these experiments. To assess this possibility, we tested how MST2 alone, MOB1B alone, and the combination of MST2 and MOB1B affected the stability of LATS1 in cells treated with 17-AAG. Whereas MST2 alone had little effect on LATS1 stability in response to 17-AAG, MOB1B strongly stabilized LATS1 (Fig. 3C). Similarly, and consistent with our *in vitro* kinase results, co-expression of both MST2 and MOB1B further stabilized LATS1 in 17-AAG-treated cells. These results indicate that even when LATS1 is stabilized by MOB1B and MST2, LATS1 catalytic activity is reduced in 17-AAG-treated cells, suggesting that the LATS1 kinase retains a dependence on HSP90 even when stabilized by its physiological activators.

17-AAG reduces the phosphorylation of YAP, a physiological LATS substrate, and induces the accumulation of CTGF, a YAP target gene in cell lines and human tumors

The findings presented above demonstrate that HSP90 inhibition depletes LATS1 and reduces its kinase activity. We therefore asked how 17-AAG affects the phosphorylation of a physiological LATS1 substrate in cells treated with 17-AAG. For these studies, we examined YAP, a transcriptional co-activator oncoprotein that is phosphorylated and negatively regulated

by LATS (30), in a panel of cell lines derived from different tumors, including lung (A549, H460), colon (HCT-116), osteosarcoma (U2OS), and ovarian (OVCAR5) cancers. In all of these cell lines, LATS1 levels were reduced after 6 h of 17-AAG (Fig. 4A); in all but one cell line (H460), LATS1 levels continued to decline at 24 h. A different pattern emerged when we analyzed YAP phosphorylation on Ser127, a site phosphorylated by LATS1 (30). In most of the cell lines (HCT-116, U2OS, OVCAR5, and H460), YAP Ser127 phosphorylation was reduced after 6 and/or 24 h of 17-AAG exposure. However, this phosphorylation was essentially unchanged in A549 cells and slightly increased in MCF10A cells following 17-AAG, despite reduced levels of LATS1 and LATS2 (data not shown but see Fig. 1). Importantly, similar effects on LATS1 and YAP phosphorylation were also seen with the HSP90 inhibitor PU-H71 (Fig. S1). Consistent with a reduction in YAP phosphorylation, which prevents accumulation of YAP in the nucleus, 17-AAG caused increased YAP levels in the nuclei of density arrested U2OS cells (Fig. 4B), thus demonstrating that the 17-AAG-dependent YAP phosphorylation is promoting accumulation of the transcriptional coactivator.

We reasoned that if 17-AAG reduced the inhibitory phosphorylation of YAP and promoted its accumulation in the nucleus, then this agent should induce the expression of a YAP-regulated gene. One of the genes regulated by YAP-TEAD complexes is *CTGF* (9,46), which is important for YAP-induced transformation-associated phenotypes, including anchorage-independent cell proliferation and the ability to form colonies in soft agar (9). We therefore examined how HSP90 inhibition affects the levels of CTGF. In all the cell lines examined, including the cell lines in which 17-AAG had only modest effects on YAP Ser127 phosphorylation, we observed CTGF accumulation after 6 and/or 24 h of treatment with 17-AAG (Fig. 4C) or PU-H71 (Fig. S1).

To assess the impact of 17-AAG on LATS and LATS downstream signaling in a clinically relevant setting, we took advantage of paired ovarian cancer biopsy samples taken from patients before and 22-26 h after administration of single-agent 17-AAG as part of a recently completed phase II clinical trial in ovarian cancer. An analysis of these samples revealed several pertinent findings (Fig. 4D). First, all tumors expressed LATS1, but the levels were highly variable, with some expressing very low levels (Pt. 1) and others expressing far higher levels (Pt. 2). Second, LATS1 levels were reduced following 17-AAG treatment, but the magnitude of the reduction varied. Third, in at least one sample (Pt. 1) phosphorylation on YAP Ser127 was reduced, whereas there was no (or perhaps very modest) reduction in Pt. 2 or Pt. 3. Fourth, in all three patients, CTGF levels were increased, with the greatest increase in Pt. 1, who, notably, had the most pronounced decrease in phospho-YAP. Taken together, these results indicate that 17-AAG treatment *in vitro* and in the clinical setting can lead to LATS1 downregulation, reduced phosphorylation of YAP on sites that restrain this oncoprotein's transcriptional coactivator function, and coincident accumulation of CTGF, a protein implicated in tumorigenesis, metastasis, and angiogenesis.

Discussion

In this study we demonstrated for the first time that LATS1 and LATS2 are depleted from non-transformed and tumor cell lines as well as primary human tumors by HSP90 inhibitors and showed that LATS1 and LATS2 interact with HSP90, thus demonstrating that these kinases are HSP90 clients. We also observed that in many cell lines HSP90 inhibition reduced YAP phosphorylation on Ser127, a site phosphorylated by LATS1 and LATS2 in intact cells, showing that HSP90 inhibition reduces the ability of LATS to phosphorylate its cellular target. Consistent with the reduced YAP phosphorylation, we found that HSP90 inhibition caused increased levels of CTGF, which is encoded by a gene that is trans-activated by YAP-TEAD complexes.

These findings are important on several levels. First, we have discovered that even though 17-AAG caused the depletion of LATS in all the cell lines examined, 17-AAG-mediated reductions in LATS1 and LATS2 were not accompanied by decreases in YAP phosphorylation in all lines. Although this result was initially perplexing, a recent report found that another — as yet unidentified — kinase can also phosphorylate YAP on Ser127 (19). Thus, it is possible that in some cells YAP activity is restrained by a kinase(s) that is not depleted by, and may even be stimulated in response to, HSP90 inhibition. The assessment of this possibility will have to await the identification of the novel YAP kinase.

Second, we made the unexpected observation that MOB1, a small protein that binds and activates LATS1, dramatically reduces the HSP90 dependence of LATS1 and delays the loss of the kinase in cells treated with 17-AAG. Intriguingly, however, even though LATS1 was stabilized by MOB1, HSP90 inhibition still reduced the auto- and trans-phosphorylation activity of the stabilized kinase. Although we do not currently understand the biochemical basis for this observation, we suspect that the kinase domain requires HSP90 chaperoning to assume a catalytically competent state, but that even in the absence of appropriate kinase domain folding, MOB1B binding stabilizes LATS1. In contrast, in the absence of MOB1B, LATS1 can only acquire a stable state with a correctly folded kinase domain.

Third, our results raise important questions about HSP90 inhibitors as tumor treatments. The intense interest in HSP90 inhibitors as anti-cancer treatments is underscored by the fact that over 20 Phase I or II clinical trials are currently underway (as listed in ClinicalTrials.gov) to test the activities of a variety of HSP90 inhibitors in adult and pediatric neoplasms (2). Despite this intense interest, the compelling underlying basic biology of HSP90 inhibition in cancer cells, and exciting preclinical results (1), the effects of these inhibitors in the clinic have been disappointing to date (47). Although it remains unclear why these agents have shown such limited clinical activity, one possibility is that HSP90 inhibition has unanticipated effects that counter the anti-tumor effects of these agents. For example, HSP90 inhibition has been shown to promote prostate cancer cell growth (48), possibly by transiently stimulating activation of c-Src, a tyrosine kinase oncoprotein (49), and enhance bone metastasis of xenografted breast cancers (50). Similarly, the present results demonstrate that 17-AAG disrupts the LATS-MST2-YAP tumor suppressor pathway, raising additional questions regarding the effects of HSP90 inhibition on tumors and further suggesting that HSP90 inhibition may activate signaling pathways that fuel tumorigenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

HU	hydroxyurea
GST	glutathione S-transferase
17-AAG	17-allylamino-17-demethoxygeldanamycin
HSP90	heat shock protein 90

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Figure 1. LATS1 and LATS2 are depleted by HSP90 inhibition. **(A, B)** Subconfluent A549 and MCF10A cells were treated with 1 μ M 17-AAG (A) for the indicated times or with the indicated concentrations of 17-AAG for 24 h (B). **(C, D)** A549 cells were treated with the indicated concentrations of radicicol (C) or PU-H71 (D) for 24 h. Following treatment, cell lysates were analyzed by immunoblotting for the indicated antigens.

Figure 2.

LATS1 is an HSP90 client. **(A, B)** Subconfluent A549 cells were treated with vehicle or 10 mM hydroxyurea (HU) for 24 h to induce arrest at the G1/S phase border. The HU-arrested cells were then incubated with vehicle (0.1% DMSO) or 1 μ M 17-AAG in the continued presence of HU for the indicated times. Following treatment, the cell samples were divided; one portion was used to assess cycle by staining the DNA with propidium iodide (A), and the remaining cells were lysed and immunoblotted for the indicated antigens (B). **(C, D)** A549 cell lysates were immunoprecipitated (IP) with non-reactive rabbit serum (NRS) or anti-LATS2 rabbit antiserum (C); or subconfluent A549 cells were treated with vehicle (-) or 1 μ M 17-AAG (+) for 24 h, and cell lysates were then immunoprecipitated with NRS or anti-LATS1 rabbit antiserum (D). The immunoprecipitates were then washed and sequentially immunoblotted for HSP90 β and LATS1 or LATS2. Cell lysates were immunoblotted to show equal amounts of HSP90 β in all lysates.

Figure 3.

MOB1B stabilizes LATS1 but does not prevent 17-AAG from reducing LATS1 kinase activity. (A, B) A549 cells were transfected with empty vector (EV) or plasmid encoding SFB-LATS1 alone or combination with vectors that express FLAG-MST2 and FLAG-MOB1B. Twenty-four h after transfection, cells were treated with 1 μ M 17-AAG for 24 h and SFB-LATS1 was recovered from cell lysates by reaction with streptavidin-agarose beads (which bind the streptavidin-binding peptide sequence of the SFB tag). Bead-bound SFB-LATS1 was subjected to *in vitro* kinase assays with GST-YAP as the substrate. Reactions were stopped with SDS-PAGE sample buffer and fractionated by SDS-PAGE, and transferred to a membrane. After membrane-bound radioactivity was detected and quantitated by phosphorimaging, the membrane was blotted with anti-FLAG antibody to detect precipitated LATS1. (A) Representative experiment of 5 independent experiments. (B) Relative ³²P-labeled GST-YAP, mean $\pm$ SD, n = 5 (0, 6, and 24-h time points) or n = 3 (1, and 3-h time points). (C) A549 cells were transiently transfected by electroporation with empty vector (EV) or plasmids that express SFB-LATS1, FLAG-MST2, and FLAG-MOB1B as indicated. Following electroporation, cells

were re-plated at low density, cultured for 24 h, and treated with 1 μ M 17-AAG for the indicated times. Cells lysates were immunoblotted with anti-FLAG monoclonal antibodies, which detect FLAG-tagged LATS1, MST2, and MOB1B.

Figure 4.

17-AAG depletes LATS1, reduces YAP Ser127 phosphorylation, and induces CTGF expression in tumor cell lines and human tumors. **(A)** Cell lines were treated with 0.1% DMSO vehicle (-) or 17-AAG for 6 or 24 h. Cell lysates were then immunoblotted for the indicated antigens. P-YAP, anti-phospho-Ser127-YAP. **(B)** Density-arrested U2OS cells were treated with vehicle (0.1% DMSO) or 1 μ M 17-AAG for 3 h and fixed. Green, anti-YAP immunostain; blue, DNA stained with Hoechst 33342. Bar = 20 μ m. **(C)** Samples from (A) were run on separate gels and immunoblotted for CTGF and HSP90. Due to low levels of CTGF in lysates, the A549 and MCF10A lysates were immunoprecipitated with anti-CTGF antibody and then immunoblotted with the same antibody; the HSP90 blot for these samples shows the starting lysates. **(D)** Paired patient ovarian cancer biopsies taken before (Pre) and 22-26 h after single-agent 17-AAG treatment (Post) were probed for the indicated antigens. To adjust for widely varying LATS1 levels, three exposures are shown for LATS1 immunoblots.