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**Article**

# Large tumor suppressor 2, LATS2, activates JNK in a kinase-independent mechanism through ASK1

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**Apoptosis signal-regulating kinase 1 (ASK1) is an important mediator of the cell stress response pathways. Because of its central role in regulating cell death, the activity of ASK1 is tightly regulated by protein–protein interactions and post-translational modifications. Deregulation of ASK1 activity has been linked to human diseases, such as neurological disorders and cancer. Here we describe the identification and characterization of large tumor suppressor 2 (LATS2) as a novel binding partner for ASK1. LATS2 is a core kinase in the Hippo signaling pathway and is commonly downregulated in cancer. We found that LATS2 interacts with ASK1 and increases ASK1-mediated signaling to promote apoptosis and activate the JNK mitogen-activated protein kinase (MAPK). This change in MAPK signaling is dependent on the catalytic activity of ASK1 but does not require LATS2 kinase activity. This work identifies a novel role for LATS2 as a positive regulator of the ASK1–MKK–JNK signaling pathway and establishes a kinase-independent function of LATS2 that may be part of the intricate regulatory system for cellular response to diverse stress signals.**

**Keywords:** ASK1, LATS2, kinase regulation, signal transduction, mitogen-activated protein kinase

## Introduction

Apoptosis signal-regulating kinase 1 (ASK1) is a mitogen-activated protein kinase kinase kinase (MAP3K) that becomes activated by a wide variety of cellular stressors, such as reactive oxygen species, endoplasmic reticulum (ER) stress, and apoptosis inducers like Fas activation (Ichijo et al., 1997; Chang et al., 1998; Saitoh et al., 1998; Liu et al., 2000; Nishitoh et al., 2002; Tobiume et al., 2002). Once activated, ASK1 stimulates the mitogen-activated protein kinase (MAPK) cascade by directly phosphorylating the mitogen-activated kinase kinases (MAP2Ks), MKK3/6 and MKK4/7, which in turn phosphorylate the p38 and c-Jun N-terminal kinase (JNK) MAPKs, respectively (Ichijo et al., 1997). The p38 and JNK MAPKs can then promote a wide array of cellular processes such as apoptosis, inflammation, and proliferation (Dhillon et al., 2007; Wagner and Nebreda, 2009). While other MAP3K proteins can also lead to MAPK activation, ASK1 is essential for stress-induced cell death by p38 and JNK, as loss of ASK1 confers apoptotic resistance upon TNFα, oxidative, and ER stress conditions (Tobiume et al., 2001; Nishitoh et al., 2002).

As a mediator of the stress response and pro-apoptotic signaling, ASK1 activity is tightly regulated through multiple mechanisms, such as engaging in protein–protein interactions (PPIs) and through post-translational modifications (PTMs) (Tobiume et al., 2002; Sturchler et al., 2011). Many proteins have been identified that play critical roles in the regulation of ASK1, which can have positive or negative effects on its activity. For example, the redox-sensing protein thioredoxin binds to and inhibits ASK1 homodimerization that is needed for kinase activation (Bunkoczi et al., 2007; Fujino et al., 2007). Conversely, TNF receptor-associated factors 2 and 6 (TRAF2/6) are proteins that bind to ASK1 to promote ASK1 homodimerization and subsequent activation (Noguchi et al., 2005; Takeda et al., 2006). PTMs can also have positive or negative effects on ASK1 signaling. Autophosphorylation at threonine 838 in the activation loop of the kinase domain of ASK1 is required for full enzymatic activity, while phosphorylation at serine 967 by pro-survival

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kinases leads to ASK1 inhibition through 14-3-3 binding (Zhang et al., 1999; Tobiume et al., 2002; Goldman et al., 2004; Seong et al., 2010; Petrvalska et al., 2016).

The importance of maintaining proper regulation of ASK1 activity can be seen in human diseases, where perturbation of ASK1 signaling can impact disease progression and treatment. ASK1 is pathogenic in many neurodegenerative disorders, such as Alzheimer's disease and Parkinson's disease, where it promotes cell death (Nishitoh et al., 2002; Sturchler et al., 2011). In cancer, ASK1 can mediate apoptosis caused by common chemotherapeutic agents such as platinum-based drugs and microtubule disruptors (Chen et al., 1999; Yuan et al., 2003; Brozovic and Osmak, 2007). In many cases, loss or inactivation of ASK1 decreases the effectiveness of these agents to kill cancer cells. These findings highlight the importance of ASK1 to human health. Thus, it is critical to further our understanding into how the activity of this protein is regulated.

Through our examination of cancer-associated PPI networks, we found that ASK1 is associated with large tumor suppressor 2 (LATS2) (Li et al., 2017). LATS2 is a tumor suppressor protein and a core member of the Hippo signaling pathway where it phosphorylates and inhibits the oncogenic proteins, YAP and TAZ (Huang et al., 2005; Dong et al., 2007; Zhao et al., 2007; Yu et al., 2013; Mo et al., 2016). The kinase activity of LATS2 is required for Hippo pathway signaling, while other tumor suppressive functions of LATS2 are independent of its enzyme activity, such as the binding and inhibition of β-catenin (Li et al., 2013; Furth and Aylon, 2017). LATS2 also plays a role in cancer where it is commonly downregulated or lost in multiple tumor types (Yabuta et al., 2000; Takahashi et al., 2005; Lee et al., 2009; Strazisar et al., 2009; Liu et al., 2010; Fang et al., 2012; Lin et al., 2013; Yao et al., 2015).

Here we describe a novel role of LATS2 in promoting signaling and activation of the ASK1–MKK–JNK pathway. Mechanistically, LATS2 acts in a kinase-independent manner to promote ASK1-mediated signaling, potentially through the decrease in inhibitory phosphorylation of ASK1 at serine 967. Our work identifies LATS2 as a regulator of the ASK1-mediated stress response pathway that may lead to new strategies to control cellular response to stress in normal cells and in diseases.

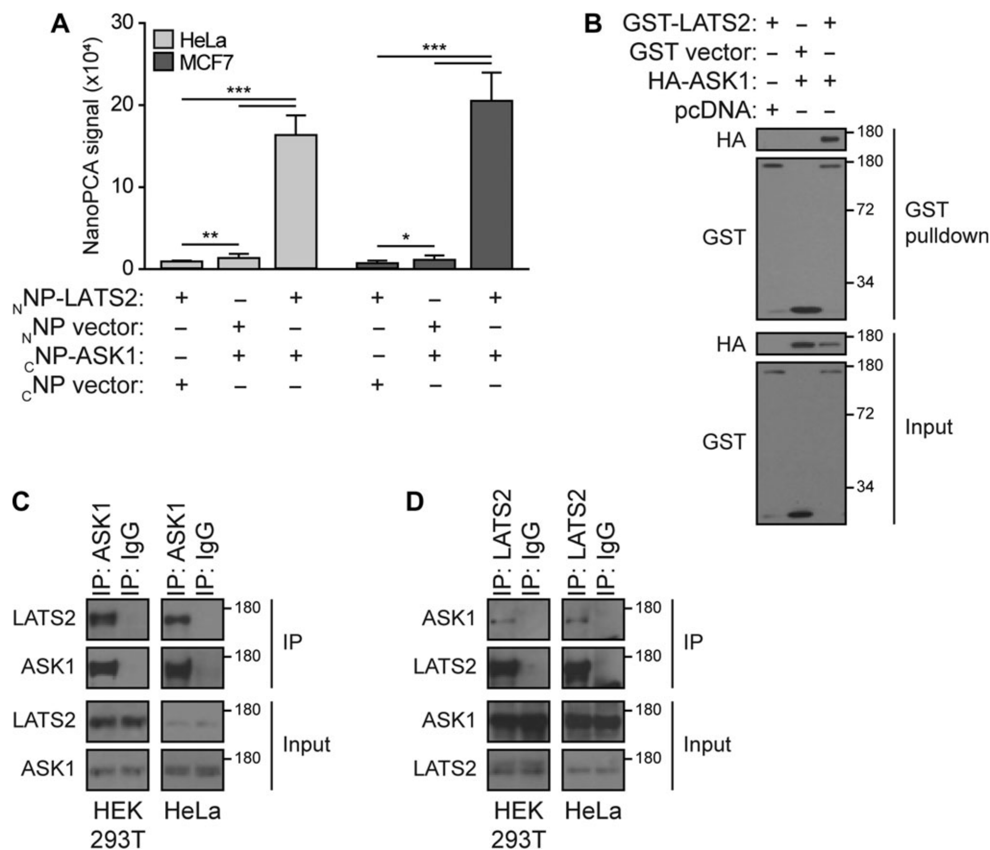


**Figure 1** Interaction between LATS2 and ASK1. (**A**) NanoPCA assays were performed in live HeLa and MCF7 cells. Results shown are average NanoPCA signals and standard deviation (SD) values from three independent replicates (**P* < 0.05, ***P* < 0.01, ****P* < 0.001). (**B**) GST-tagged protein complexes were isolated from lysates of co-transfected HEK293T cells by GST pulldown. The presence of HA-ASK1 in GST-protein complexes, as well as total protein levels, was determined by western blot analysis with HA and GST antibodies. (**C** and **D**) Endogenous immunoprecipitation of ASK1 (**C**) or LATS2 (**D**) was performed in HEK293T and HeLa and cells. Western blotting was used to detect proteins in the isolated immunocomplexes.

## Results

*LATS2 interacts with ASK1*

A high-throughput time-resolved fluorescence resonance energy transfer (TR-FRET) PPI screen detected an interaction between ASK1 and LATS2 when co-expressed in H1299 lung cancer cells (Li et al., 2017). Because ASK1 activity is known to be regulated through distinct interactions with both positive and negative binding partners, characterizing new ASK1 PPIs may reveal novel regulatory mechanisms which promote or inhibit ASK1 signaling.

To confirm the ASK1/LATS2 interaction, a panel of proximity- and affinity-based assays were employed to detect this PPI in various experimental conditions and cellular environments. First, a proximity-based assay, NanoLuc-based protein-fragment complementation (NanoPCA), was used to detect PPIs between overexpressed proteins in live human cancer cells (Mo et al., 2017). Here, the NanoLuc protein was split to generate N-terminal ($_N$NP) and C-terminal ($_C$NP) fragments that can reconstitute the luminescence of full-length protein when they are in close proximity. As expected, the co-expression of $_N$NP-LATS2 and $_C$NP-ASK1 in HeLa and MCF7 cells gave significantly higher luminescence signals than the negative controls, indicating these two proteins co-localize and interact in these experimental systems (fold-over-control (FOC) = 10.2 and 15.0 in HeLa and MCF7 cells, respectively) (Figure 1A).

The ASK1/LATS2 interaction was then tested by two affinity-based assays that require multiple wash steps, thereby complementing the proximity-based TR-FRET and NanoPCA biosensor methods. GST pulldown results showed that HA-tagged ASK1 was present only in the GST-LATS2 protein complex (Figure 1B). Lastly, we carried out a co-immunoprecipitation experiment in HEK293T and HeLa cervical carcinoma cells to test whether ASK1 associated with LATS2 under endogenous cellular environment. Here, LATS2 was detected in the ASK1 immunocomplex, while not present in the control IgG immunocomplex (Figure 1C). Similar results were also obtained from LATS2 co-immunoprecipitation experiments (Figure 1D). These results show that LATS2 and ASK1 can interact under physiological conditions and endogenous levels in both cancer and non-cancer cells, and providing robust evidence supporting the interaction between these two proteins.

*LATS2 increases MAPK activation in an ASK1 kinase-dependent manner*

To determine whether LATS2 regulated ASK1 activity, we examined whether downstream signaling was altered by overexpression of LATS2. HEK293T lysates co-expressing ASK1 with either LATS2 or a control vector were probed for phosphorylation of p38 (Thr180/Tyr182) and JNK (Thr183/Tyr185) as readouts of ASK1 activity (Ichijo et al., 1997). Interestingly, samples expressing both ASK1 and LATS2 showed a noticeable increase in phosphorylated JNK, compared to the expression of ASK1 alone (Figure 2A). The same trend was not observed for p38 phosphorylation, suggesting a potential selectivity for JNK activation. Selective activation of either p38 or JNK has been reported in other experimental conditions, where ASK1 preferentially activates one MAPK over the other (Matsuzawa et al., 2005; Hayakawa et al., 2013). Additionally, the co-expression of ASK1 did not affect LATS2 signaling, as determined by unchanging levels of YAP1 phosphorylation (Supplementary Figure S1).

To address the potential issue of LATS2 overexpression induced complications, we further investigated the effect of LATS2 on JNK activation using a LATS2 knockout cell line (Plouffe et al., 2016). The levels of phospho-JNK were compared between HEK293A cells expressing endogenous levels of LATS2, and HEK293A$^{LATS2\ KO}$ (LATS2 KO) cells. As shown in Figure 2B, the levels of phospho-JNK were correlated with that of LATS2. LATS2 KO cells with endogenous ASK1 showed reduced phospho-JNK when compared to HEK293A cells with endogenous levels of LATS2. As shown in Figure 2D, overexpression of wild-type ASK1 (ASK1$^{WT}$) significantly increased phospho-MKK4 and phospho-JNK in in HEK293A cells, but showed significantly reduced MKK4 and JNK activation in LATS2 KO cells. Thus, LATS2 appears to be required for the activation of ASK1-mediated signaling to MKK4 and JNK.

Because there are multiple upstream MAP3K proteins that can lead to the activation of MAPKs, we wanted to determine whether ASK1 was the upstream kinase responsible for transducing LATS2-evoked phosphorylation of JNK. To this end, we co-expressed LATS2 with either ASK1$^{WT}$ or a catalytically inactive kinase-dead mutant of ASK1 (ASK1$^{KR}$) in HEK293T cells. Western blot analysis was then used to assay downstream phosphorylation in the ASK1–MKK–JNK pathway. Co-expression of LATS2 with ASK1$^{WT}$ led to similar increases in phospho-JNK as seen in Figure 2A, as well as an increase in phospho-MKK4 (Ser257/Thr261), which is a direct substrate for ASK1 and the upstream kinase for JNK phosphorylation (Ichijo et al., 1997). Importantly, the expression of LATS2 with the catalytically inactive ASK1$^{KR}$ failed to induce phosphorylation of ASK1 effectors (Figure 2C). Similar results were also achieved in LATS2 KO cells where expressing ASK1$^{KR}$ did not lead to the same increase in phosphorylation of MKK4 or JNK as the expression of ASK1$^{WT}$ did (Figure 2D). Together these results suggest that LATS2 acts upstream of ASK1 to promote signaling and JNK activation.

*Co-expression of LATS2 with ASK1 increases apoptosis*

Having established the ability of LATS2 to enhance ASK1 signaling, we next sought to link this effect to a biological outcome. ASK1 can promote an array of biological processes, such as cell death, survival, and inflammation, with its best-known role is as a pro-apoptotic signaling protein (Ichijo et al., 1997; Hatai et al., 2000; Takeda et al., 2000, 2008; Hattori et al., 2009; Iriyama et al., 2009; Hayakawa et al., 2010, 2011). This effect has been observed in multiple cell lines where overexpression of a constitutively active form of ASK1 can be sufficient to trigger apoptosis (Ichijo et al., 1997; Hatai et al., 2000; Kanamoto et al., 2000; Pramanik and Srivastava, 2012). Additionally, LATS2 can also promote cell death when overexpressed (Ke et al., 2004; Suzuki et al., 2013). To determine whether the co-expression of LATS2 with ASK1 could increase

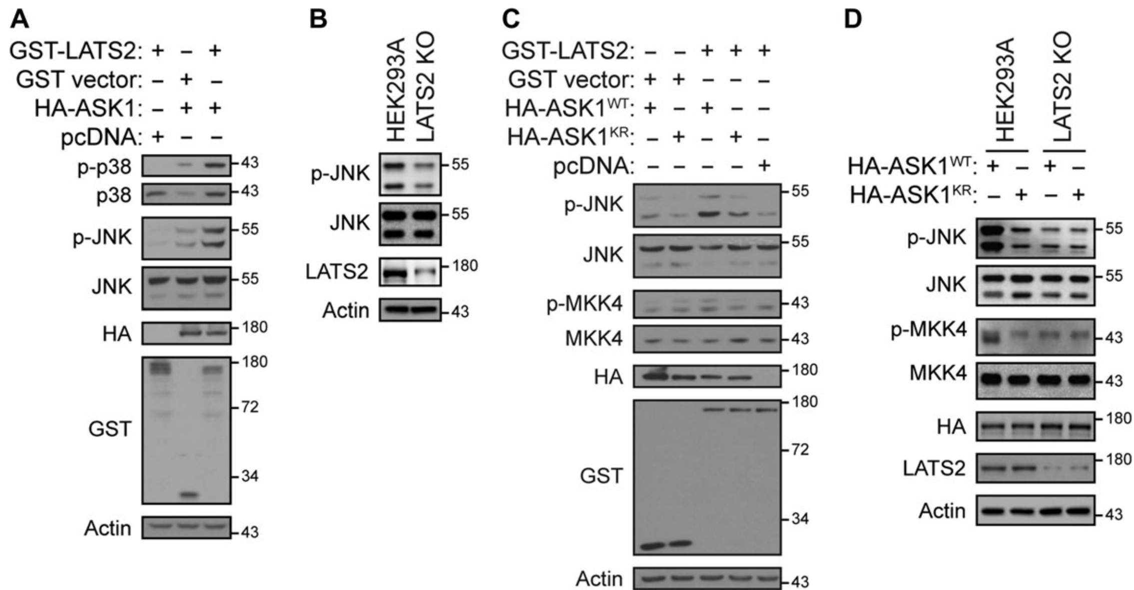


**Figure 2** LATS2 activation of JNK requires ASK1 kinase activity. (**A**) Cell lysate from HEK293T cells co-expressing GST-LATS2, HA-ASK1, or control vectors was probed for phosphorylated and total protein levels of p38 and JNK. (**B**) Phospho-JNK levels in HEK293A and HEK293A$^{LATS2\ KO}$ (LATS2 KO) cell lines. (**C**) Cell lysate was assayed for phosphorylation status of JNK and MKK4 in the presence of GST-LATS2 co-expressed with either HA-tagged wild-type (ASK1$^{WT}$) or kinase-dead (ASK1$^{KR}$) ASK1. (**D**) Phospho-MKK4 and phospho-JNK levels in HEK293A and HEK293A$^{LATS2\ KO}$ (LATS2 KO) cell lines expressing ASK1$^{WT}$ or ASK1$^{KR}$.

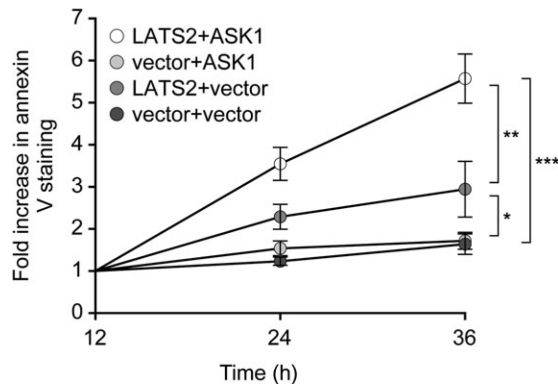


**Figure 3** Co-expression of LATS2 with ASK1 increases annexin V staining in HEK293T cells. Cells transfected with combinations of Venus-flag-LATS2 or Venus-flag vector, and HA-ASK1 or pcDNA were incubated with a cell permeable annexin V red fluorescent dye. Shown are the averaged values for fold increase in annexin V at 24 h and 36 h from three independent experiments and the SD values (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

cell death, HEK293T cells were transfected and incubated with a cell permeable annexin V red fluorescent dye, which binds to exposed phosphatidylserines on the outer leaflet of the plasma membrane of dying cells. Significantly more death, as measured by fold increase in annexin V, was observed in HEK293T cells transfected with both Venus-flag-LATS2 and HA-ASK1, compared to expression of either protein alone or the empty vectors at both 24 h and 36 h post-transfection (Figure 3). Furthermore, the fold increase in annexin V seen in the co-expression group was greater than the sum of the single expression groups, suggesting that LATS2 and ASK1 may have a synergistic effect on cell death.

*LATS2 interacts and activates ASK1 in a kinase-independent manner*

LATS2 is a Ser/Thr kinase that regulates multiple effector proteins through phosphorylation. To assess whether the kinase activity of LATS2 is required for binding to ASK1 and the subsequent signaling, we created a kinase-dead mutant of LATS2 (LATS2$^{KR}$) by introducing a point mutation to change lysine 697 in the conserved kinase domain to arginine (Xiao et al., 2011). Co-expression of Venus-flag-ASK1 with either GST-tagged wild-type LATS2 (LATS2$^{WT}$) or LATS2$^{KR}$ in HEK293T cells gave positive TR-FRET signals (FOC = 6.0 and 5.3, respectively) that were significantly different from samples expressing either protein alone (Figure 4A). Similar binding data were also achieved by GST pull-down, where both LATS2$^{WT}$ and LATS2$^{KR}$ were equally capable of pulling down ASK1$^{WT}$ and ASK1$^{KR}$ (Figure 4B). These data suggest that the kinase activity of neither LATS2 nor ASK1 is essential for their binding.

Next, we examined whether LATS2 kinase activity was required to enhance ASK1-mediated signaling. Lysates from HEK293T cells co-expressing ASK1 with LATS2$^{WT}$ or LATS2$^{KR}$ were probed for activation of ASK1 effectors. Here, both LATS2$^{WT}$ and LATS2$^{KR}$ were able to increase JNK phosphorylation when co-expressed with ASK1 (Figure 4C). Collectively, these findings show LATS2 acting as a positive regulator of ASK1-mediated signaling independently of its catalytic activity.

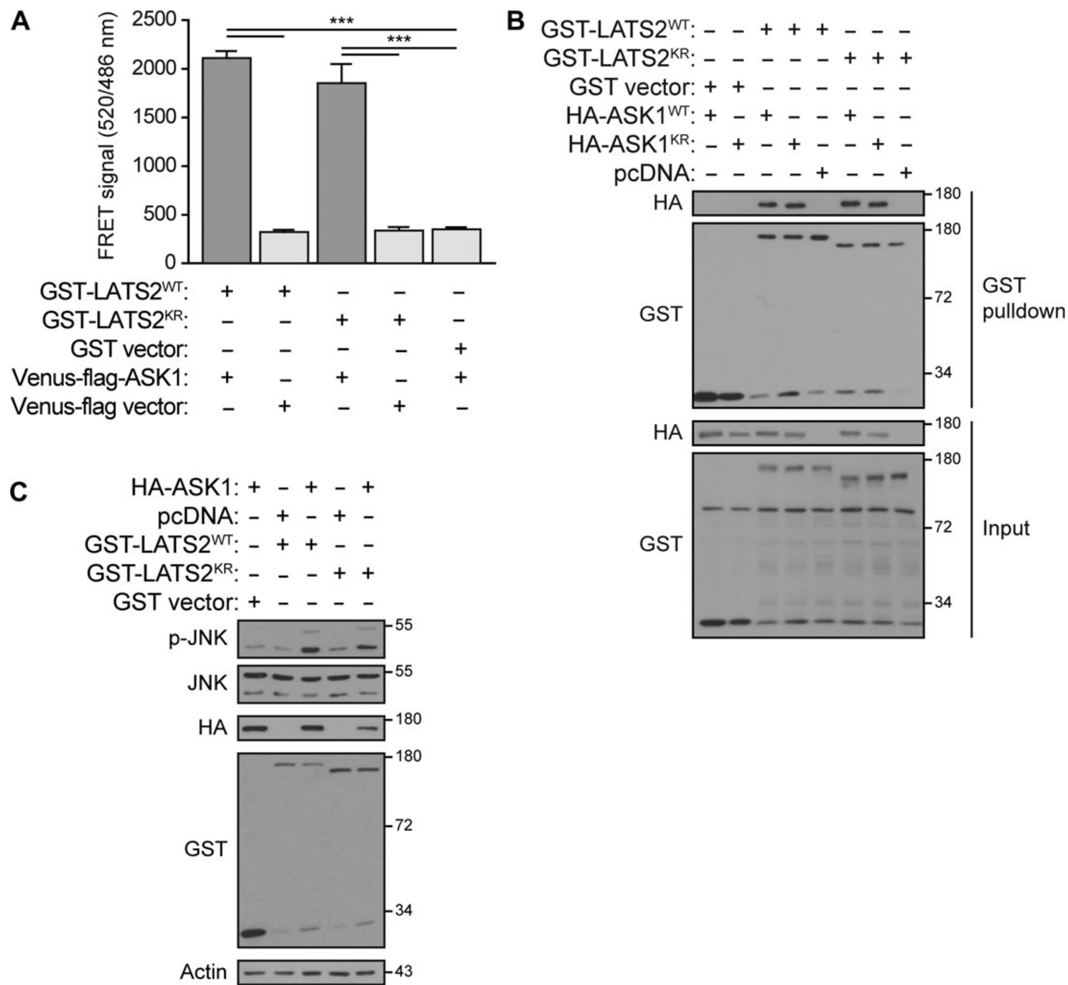


**Figure 4** LATS2 binds and activates ASK1 in a kinase-independent manner. (**A**) TR-FRET was used to detect the interaction between co-expressed Venus-flag-ASK1 and either wild-type LATS2 ($LATS2^{WT}$) or kinase-dead LATS2 ($LATS2^{KR}$). Results shown are averaged FRET and SD values from three independent experiments (***$P < 0.001$). (**B**) GST pulldown with GST-tagged $LATS2^{WT}$ or $LATS2^{KR}$, and HA-tagged $ASK1^{WT}$ or $ASK1^{KR}$ in HEK293T cells. (**C**) Lysate from HEK293T expressing HA-ASK1 with GST-$LATS2^{WT}$ or GST-$LATS2^{KR}$ was blotted for the status of JNK.

*LATS2 binds the C-terminal region of ASK1*

To better understand how LATS2 was activating ASK1, we mapped the LATS2-binding interface to determine which structural elements in ASK1 were important for mediating this effect. ASK1 is composed of three main regions, a N- and C-terminal coiled-coil (CC) domain flanking a central kinase domain. These domain boundaries were taken into consideration when designing truncations containing the N-terminal (N), the N-terminal and kinase domain (NK), and the kinase domain and C-terminal (KC) portion of the full-length (FL) ASK1 protein (Figure 5A) (Zhang et al., 1999; Chen et al., 2001). An *in vitro* GST pulldown assay was used to detect the binding between GST-tagged LATS2 and HA-ASK1 truncations. GST pulldown results showed LATS2 binding to both FL and the KC truncation of ASK1 (Figure 5B). The KC fragment of ASK1 contains a stretch of amino acids from 937 to 1374 that are not shared with the N or NK truncations, suggesting that this region of ASK1 may contain the LATS2 binding site.

*LATS2 decreases inhibitory phosphorylation of ASK1 at serine 967*

To begin to address how LATS2 activates ASK1 signaling, we focused on the ASK1 C-terminal region (a.a. 937–1374) that mediates the interaction (Figure 5B). This region contains multiple sites known to negatively affect ASK1 activity when phosphorylated (Fujii et al., 2004; Goldman et al., 2004; Seong et al., 2010). It is possible that LATS2 binding alters the phosphorylation of ASK1 at these sites, which also agrees with the kinase-independent mechanism for LATS2 activation of ASK1 signaling found in Figure 4B. We began to explore this model by examining the effect of LATS2 on one of the most well-characterized phosphosites in this region of ASK1, serine 967 (S967) (Goldman et al., 2004; Puckett et al., 2010, 2013; Seong et al., 2010). ASK1 is phosphorylated at S967 in resting cells to inhibit apoptosis, and as expected, ASK1 was phosphorylated at S967 when expressed with GST vector (Figure 6) (Goldman

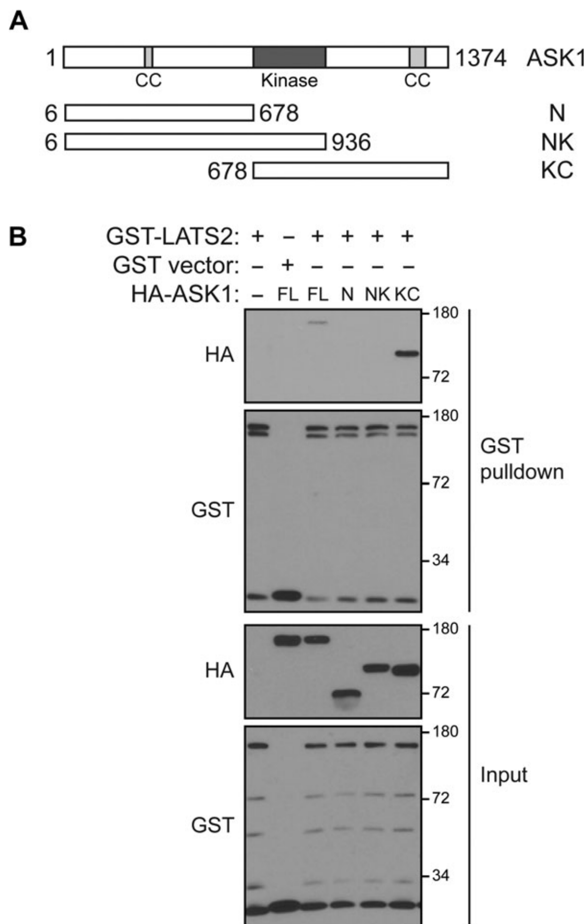


**Figure 5** LATS2 interacts with the C-terminal region of ASK1. (**A**) Schematic of ASK1 protein domains (light gray: coiled-coil domain; dark gray: kinase domain) and truncations used for deletion mapping. (**B**) An *in vitro* GST pulldown assay was used to isolate GST-LATS2 immunocomplexes with bound ASK1 truncations. The presence of HA-ASK1 in the purified GST-complex was determined by western blot analysis.

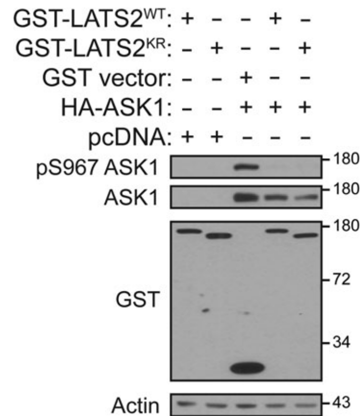


**Figure 6** LATS2 decreases phosphorylation of ASK1 at serine 967. HA-ASK1 was expressed with GST-LATS2$^{WT}$ or GST-LATS2$^{KR}$ in HEK293T cells, and levels of ASK1 phosphorylation at serine 967 (S967) were measured by western blot analysis.

et al., 2004). However, co-expression with wild-type or kinase-dead LATS2 resulted in a similar decrease in phosphorylation of ASK1 at this site (Figure 6). Our data support a model where LATS2 activates ASK1 at least in part by reducing the negative phosphorylation of ASK1 at S967 in a kinase-independent manner, leading to subsequent downstream activation of JNK.

## Discussion

Here we report LATS2 as a novel binding partner and positive regulator of ASK1, a stress-responsive signaling protein that is important in initiating a phosphorylation cascade to activate JNK. Furthermore, co-expression of LATS2 with ASK1 increases cell death compared to expression of either ASK1 or LATS2 alone. LATS2 interacts with the C-terminal region of ASK1 to promote downstream activation of JNK. Importantly, this signaling requires ASK1 kinase activity, indicating that it is the main MAP3K responsible for relaying signal transduction from LATS2 to the MKK–JNK pathway in this experimental system.

Mechanistically, LATS2 could be enhancing ASK1 signaling by decreasing phosphorylation at S967. ASK1 is phosphorylated at S967 by survival kinases, such as PDK1 and IKKβ, to create a 14-3-3 docking site (Seong et al., 2010; Puckett et al., 2013). Subsequent 14-3-3 binding decreases ASK1 kinase activity and downstream signaling (Zhang et al., 1999; Subramanian et al., 2004). Our results suggest that S967 could be an important residue for LATS2-mediated effects on ASK1 activity.

It is possible that the physiological engagement of ASK1 by LATS2 is dictated by environmental factors as for other ASK1-mediated protein interactions. For example, elevated cellular ROS levels lead to the dissociation of negative binding partners, such as thioredoxin, from ASK1 (Saitoh et al., 1998). Other stress signals such as ER stress can lead to the interaction between ASK1 and positive binding partners, such as IRE1 (Nishitoh et al., 2002). Thus, understanding of whether and how the endogenous interaction between ASK1 and LATS2 is regulated by cellular and environmental signals is expected to have significant functional implications. Our proposed new pathway by which LATS2 interacts and impacts ASK1-mediate MKK–JNK pathway has set the stage for future examination of how this pathway is controlled in cells.

There are many reports of signaling cross-talk between the Hippo and MAPK pathways. For example, JNK serves as an activator of YAP1 by promoting the interaction between LATS2 and its negative regulator, LIMD1 (Sun and Irvine, 2013; Codelia et al., 2014). p38 signaling can also promote YAP1 nuclear localization and target gene expression (Huang et al., 2016). Conversely, MST1/2 proteins have been shown to enhance JNK and p38 activation (Graves et al., 1998; Ura et al., 2001). Our results present another link between these important cell signaling pathways, where LATS2, a central kinase in the Hippo pathway, can activate ASK1 in a kinase-independent manner.

The role of ASK1 in cancer is context dependent, and there are reports of it acting as an oncogene or a tumor suppressor under different cellular conditions (Iriyama et al., 2009; Meurette et al., 2009; Nakagawa et al., 2011; Hayakawa et al., 2011, 2012; Luo et al., 2016). ASK1-mediated signaling is known to regulate many cellular processes, including apoptosis and inflammation (Matsuzawa et al., 2005; Iriyama et al., 2009; Hayakawa et al., 2010, 2011; Kamiyama et al., 2013; Cheng et al., 2014). The pro-apoptotic arm of ASK1 signaling is likely to play a tumor suppressive role in cancer, while the pro-inflammatory arm of ASK1 signaling has been reported to facilitate tumorigenesis (Iriyama et al., 2009; Kamiyama et al., 2013). Importantly, protein-binding partners can directly influence whether ASK1 signaling has a more tumor suppressive or oncogenic effect. For example, when ASK1 is heterodimerized with its homolog, ASK2, it triggers ASK1-dependent apoptosis to suppress tumor initiation. However, ASK1 homodimers were found to enhance tumor-promoting inflammation in the same experimental model (Takeda et al., 2007; Iriyama et al., 2009; Kamiyama et al., 2013). As a well-known tumor suppressor, the binding of LATS2 to ASK1 could similarly drive pro-apoptotic signaling via ASK1 to induce cancer cell death. Future studies will aim to determine the physiological functions of the newly established LATS2–ASK1 pathway and what downstream processes are affected by the LATS2-mediated increase in ASK1 signaling.

## Materials and methods

### *Cell culture and transfection*

HEK293T human embryonic kidney cells, HeLa human cervical cancer cells, and MCF7 human breast cancer cells were obtained from ATCC. HEK293A and HEK293A$^{LATS2\ KO}$ cell lines were provided by Dr Kun-Liang Guan (Plouffe et al., 2016). All cells were maintained in 5% $CO_2$ and grown in Dulbecco's modified Eagle's medium (DMEM) (Corning) with 10% fetal bovine serum (FBS) (Sigma). Media was supplemented with 1% Penicillin-Streptomycin (Corning) to reduce the risk of microbial contamination. If not otherwise specified, cells were transfected with the X-tremeGENE cationic lipid reagent (Roche) according to the manufactures' instructions at 24 h after plating.

### *Molecular cloning and mutagenesis*

A point mutation was introduced into LATS2 to generate a kinase-dead mutant (LATS2$^{KR}$) by converting lysine 697 to arginine using QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies) and the pENTR223.1-LATS2 plasmid (HsCD00353973, DNASU) as template DNA (mutant primer 5′-ctcacgccctgtacgccatgagaaccctaaggaaaaag-3′). Kinase-dead ASK1 (ASK1$^{KR}$) was generated by site-directed mutagenesis to mutate lysine 709 to arginine using pcDNA3-HA-ASK1 as a template DNA, and the ASK1 truncations (N, NK, and KC) were generated in a pcDNA3 vector backbone as previously described (Zhang et al., 1999; Chen et al., 2001). Gateway cloning (Invitrogen) was used to generate GST-tagged and Venus-flag-tagged plasmids, as well as the N- and C-terminal NanoLuc luciferase plasmids ($_N$NP and $_C$NP) (Mo et al., 2017).

### *Time-resolved fluorescence resonance energy transfer*

HEK293T cells were lysed in 80 µl 1% NP-40 lysis buffer containing 1% NP-40, 10 mM HEPES, 150 mM NaCl, 1:1000 dilution of protease and phosphatase inhibitors (Sigma, Cat# P8340, P5726, and P0044). Cell lysates were serially diluted in a 384-well black plate (Corning, Cat# 3571) in TR-FRET buffer (0.01% NP-40, 20 mM Tris-HCl, pH 7.0, 50 mM NaCl). GST-Terbium (HTRF)-conjugated antibody (Cisbio Bioassays, Cat# 61GSTTLB) was used as the FRET donor at a final concentration of 1:1000, and the Venus tag on proteins served as the acceptor. FRET signals were measured by an Envision Multimode Plate Reader (PerkenElmer) (Ex/Em = 337/520 nm). Single protein expression with empty GST- or Venus-tagged vectors were used as negative controls to measure background fluorescence and to FOC values for experimental PPIs (FOC = $Signal_{PPI}/Signal_{control}$) (Li et al., 2017).

### *Nanoluc®-based protein-fragment complementation assay (NanoPCA)*

NanoPCA assay was performed similarly as described previously (Mo et al., 2017). Briefly, HeLa and MCF7 cells were seeded in 384-well white plates (Corning, Cat# 3570) at 4000 cells in 45 µl cell culture media per well. Co-transfection of $_N$NP-tagged LATS2 and $_C$NP-tagged ASK1 was performed with linear polyethylenimine (Polysciences, Cat# 23966) 24 h after plating. NanoPCA signals were measured 48 h after transfection with an Envision Multilabel Plate Reader (PerkinElmer) immediately after Nano-Glo (Promega) substrate had been added to cells.

### *GST pulldown*

Cells were lysed in 1% NP-40 lysis buffer 48 h after transfection. Lysate was incubated with Glutathione-Sepharose 4B Beads (bioWORLD, Cat# 20182003) for 3 h at 4°C. The GST-protein complexes were washed three times in 1% NP-40 lysis buffer, and bound proteins were eluted in 2× SDS loading buffer. Samples were boiled and resolved on a 10% SDS-PAGE gel and transferred to a PVDF membrane for western blot analysis.

### *Endogenous co-immunoprecipitation*

HEK293T and HeLa cells were grown in 10-cm dishes and lysed in immunoprecipitation lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5% glycerol, 5 mM NaF, 0.5% NP-40, 1:100 dilution of protease inhibitor). Lysate was collected and spun down to pellet cellular debris, and the supernatant was aliquoted in equal volumes to new tubes containing antibodies specific for ASK1 (Santa Cruz, sc-7931), LATS2 (Novus Biologicals, NB200-199), or IgG (Santa Cruz, sc-2027). After rotating overnight at 4°C, Protein A/G Agarose Beads (Sigma, sc-2003) were added to samples and rotated for an additional 4 h. Immunocomplexes bound to the beads were pelleted and rinsed four times in wash buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5% glycerol, 5 mM NaF, 1% NP-40, 1:100 dilution of protease inhibitor). Proteins were eluted from the beads using 2× SDS loading buffer, resolved by SDS-PAGE, and transferred to nitrocellulose membranes for western blotting with

LATS2 (Abcam, ab54073) and ASK1 (Santa Cruz, sc-5294) specific antibodies.

*In vitro GST pulldown assay*

To examine the interaction of two proteins *in vitro*, plasmids were expressed individually in separate wells. Cells were lysed in 1% NP-40 lysis buffer, and the lysate from different wells was mixed in equal volumes and allowed to rotate at 4°C for 2 h to allow protein complexes to form. Samples were then incubated with Glutathione-Sepharose 4B Beads for additional 2 h, and GST pulldown assays were performed as previously described to isolate the GST-tagged protein complexes.

*Antibodies*

Primary antibodies for GST (sc-459), ASK1 (sc-5294), HA (sc-7392), and YAP1 (sc-15407) were purchased from Santa Cruz Biotechnology Inc. Actin antibody (A5441) was purchased from Sigma. LATS2 antibody (NB200-199) was purchased from Novus Biologicals. For experiments using HEK293A and HEK293A$^{LATS2\ KO}$ cell lines, LATS2 antibody 20276-1-AP from Proteintech was used. Phospho-S967 ASK1 (3764), phospho-JNK (4668), JNK (9258), phospho-MKK4 (9156), MKK4 (9152), phospho-p38 (9211), p38 (9212), and phospho-YAP1 (13008) were purchased from Cell Signaling Technology. Secondary antibodies were purchased from Santa Cruz (anti-mouse: sc-2005, anti-rabbit: sc-2005).

*Annexin V assay*

HEK293T cells were seeded in 384-well black plates (Corning, Cat# 3571) at 4000 cells in 40 μl cell culture media per well. Cells were transfected with the indicated plasmids using FuGENE HD Transfection Reagent (Promega, Cat# E2311) 24 h after seeding. At the same time as transfection, IncuCyte Annexin V Red Reagent (Essen BioScience, Cat# 4641) was added to wells according to the manufacturer's protocol at a final concentration of 1:1000. Plates were initially imaged at 12 h post-transfection using the IncuCyte S3 Live-Cell Analysis System (Essen BioScience) to get baseline readings for cell death (red signal) and transfection efficiency (green signal). Plates were then imaged again at 24 h and 36 h post-transfection. The total red object area ($\mu m^2$/well) for each replicate was normalized to the corresponding value at 12 h to obtain a fold increase in annexin V for each condition. The presented results are averages from three independent replicates.

*Statistical analysis*

A two-tailed unpaired Student's *t*-test was used to determine whether the differences between groups were statistically significant ($^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

## Supplementary material

Supplementary material is available at *Journal of Molecular Cell Biology* online.

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