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Lauren Rusnak Date

Regulation of apoptosis signal-regulating kinase 1 by LATS2

By

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Doctor of Philosophy

Graduate Division of Biological and Biomedical Science
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Date

Regulation of apoptosis signal-regulating kinase 1 by LATS2

By

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M.S., College of William & Mary, 2013
B.S., University of North Carolina at Chapel Hill, 2011

Advisor: Haian Fu, Ph.D.

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Abstract

Regulation of apoptosis signal-regulating kinase 1 by LATS2
By Lauren Rusnak

Apoptosis signal-regulating kinase 1 (ASK1) is a mitogen-activated protein kinase kinase kinase (MAP3K) protein which leads to phosphorylation and activation of mitogen activated protein kinases (MAPKs). ASK1 is as a pro-apoptotic signaling protein that is activated by a variety of cellular stressors. Because of its central role in promoting 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, viral infections, 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 within the Hippo signaling pathway and is commonly lost or downregulated in lung cancer. Lower LATS2 expression correlates with a worse prognosis in patients, highlighting the clinical importance of this protein to human cancer. We found that LATS2 interacts with ASK1 and increases downstream activation of c-Jun NH2-terminal kinase (JNK) MAPKs. These observed signaling changes are dependent on ASK1 kinase activity, indicating it is the upstream MAP3K protein responsible for transducing LATS2-mediated activation of JNK. Additionally, co-expression of LATS2 and ASK1 increase cell death. This work identifies LATS2 as a novel regulator of the ASK1-JNK signaling pathway.

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Abbreviations

AD: Alzheimer’s disease

Akt: protein kinase B

ASK1: apoptosis signal-regulating kinase 1

CC: coiled coil motif

Daxx: death domain associated protein

DJ-1: protein/nucleic acid deglycase DJ-1

ER: endoplasmic reticulum

ERK: extracellular signal–regulated kinase

FL: full-length

FOC: fold-over-control

GST: glutathione S-transferase

H_2O_2: hydrogen peroxide

HA: hemagglutinin

IKKβ: inhibitor of κB kinase β

IRE1: inositol-requiring enzyme 1

IP: immunoprecipitation

JNK: c-Jun NH_2-terminal kinase

KR: kinase-dead

LATS2: large tumor suppressor 2

MAPK: mitogen-activated protein kinase

MAP2K, MKK: mitogen-activated protein kinase kinase

MAP3K, MAPKKK: mitogen-activated protein kinase kinase kinase

MST1/2: mammalian sterile20 kinases 1 and 2

NanoPCA: nanoLuc-based protein-fragment complementation

NASH: nonalcoholic steatohepatitisis

PD: Parkinson's disease

PDK1: 3-phosphoinositide-dependent protein kinase 1

PPI: protein-protein interaction

PTM: post-translational modification

ROS: reactive oxygen species

S: serine

SA: serine to alanine mutation

T: threonine

TRAF: TNF receptor-associated factor

TNF-α: tumor necrosis factor α

TR-FRET: time-resolved fluorescence resonance energy transfer

Trx: thioredoxin

WT: wild-type

YAP: yes-associated protein

Chapter 1

Introduction

Parts of this chapter has been published: Rusnak L and Fu H. Regulation of ASK1 signaling by scaffold and adaptor proteins. Advances in Biological Regulation, 2017, 66:23-30 and Rusnak L, Qi Q, Tang C, Mo X-L, Fu H. Large tumor suppressor 2 activates JNK in a kinase-independent mechanism through apoptosis signal-regulating kinase 1. Journal of Molecular Cell Biology, submitted.

1. INTRODUCTION

Cell growth and death are tightly regulated through a network of signal transduction pathways in response to environment cues to determine cell fate. Such signaling pathways are formed by a cascade of protein-protein interactions and regulated by catalytic activation events and a complex array of post-translational modifications, such as phosphorylation. Well established signaling pathways that mediate growth and stress signals include evolutionarily conserved mitogen-activated protein kinases (MAPK) cascades, while developmental signal and mechanical signals engage the Hippo pathway. Understanding how these regulatory pathways interact with each other is expected to reveal new mechanisms to control cell growth and death and to identify novel approaches for potential therapeutic interventions. My research aims to establish such pathway cross-talks for enhanced understanding of physiological and pathological cell signaling.

1.1. The mitogen-activated protein kinase signaling pathway

The mitogen-activated protein kinase (MAPK) cascade is composed of many mitogen-activated protein kinase kinase kinases (MAP3K, MAPKKK), mitogen-activated protein kinase kinases (MAP2K, MKK) and MAPK proteins which can assemble into various pathways to drive a wide array of cellular outcomes. While the MAPK cascade is conserved in eukaryotic cells, the protein diversity and complexity of this pathway has expanded throughout evolution (Widmann et al., 1999). This can especially be seen in the MAP3K tier of the

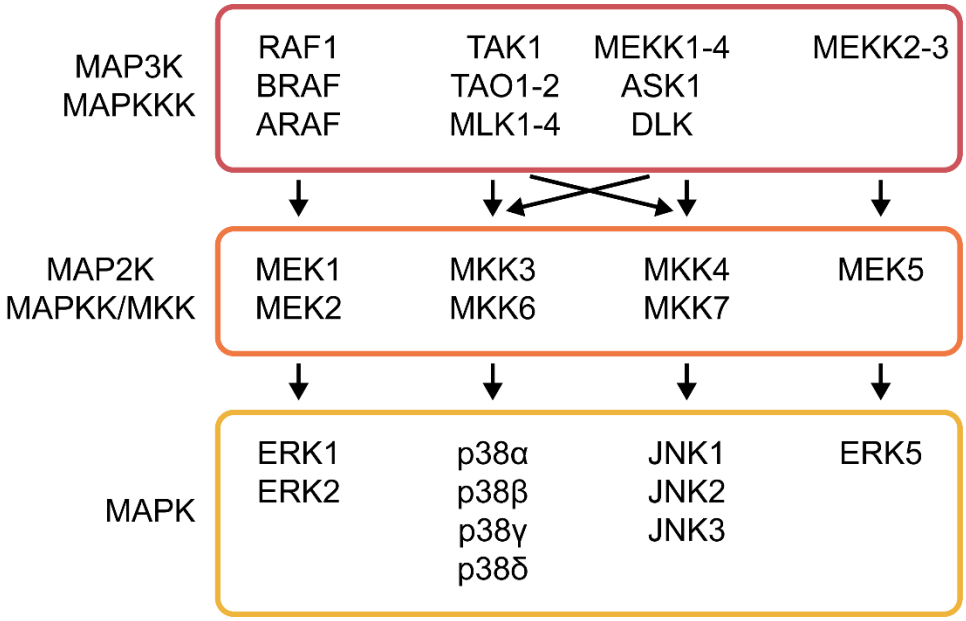
cascade. Yeast cells have four well-described MAP3Ks, while mammalian cells have at least 20, making it the most abundant and diverse level of the MAPK cascade (Uhlik et al., 2004; Johnson et al., 2005; Cuevas et al., 2007). As the initiators of MAPK signaling, the large expansion of MAP3K proteins likely mediates downstream MAPK activation in response to the many complex stimuli in mammalian cells (Widmann et al., 1999; Cuevas et al., 2007). These upstream kinases go on to activate 7 MAP2K proteins, which in turn activate the last tier of the kinase cascade, the MAPKs. The MAPK family is composed of four families, the extracellular signal–regulated kinases (ERKs), p38, c-Jun NH_2-terminal kinases (JNKs), and extracellular-signal-regulated kinase 5 (ERK5, also known as big MAP kinase 1) (Figure 1-1).

MAPK proteins can impact multiple cellular outcomes, such as proliferation, survival, and cell death. The duration of signaling is a key factor in determining what biological processes are affected. For example, tumor necrosis factor α (TNF-α) treatment can lead to the biphasic activation of JNK, where early transient activation of JNK promotes cell survival, and late sustained activation promotes apoptosis (Guo et al., 1998; Roulston et al., 1998; Ventura et al., 2006). Additionally, multiple MAP3K proteins can initiate the same downstream cascade, however the upstream MAP3K that initiates the cascade can have different biological outcomes (Song and Lee, 2007). For example, the MAP3K proteins mitogen-activated protein kinase kinase kinase 1 (MEKK1) and apoptosis signal-regulating kinase 1 (ASK1) can both activate p38 and JNK in response to TNF-α

and hydrogen peroxide (H_2O_2) treatment, however only ASK1 is required for sustained MAPK activation and subsequent cell death (Tobiume et al., 2001).

Figure 1-1: Proteins within the mammalian MAPK pathway.

MAP3K, MAP2K, MAPK proteins that compose the three-tiered MAPK pathway.


MAP3K
MAPKKK
RAF1
BRAF
ARAF
TAK1
TAO1-2
MLK1-4
MEKK1-4
ASK1
DLK
MEKK2-3
MAP2K
MAPKK/MKK
MEK1
MEK2
MKK3
MKK6
MKK4
MKK7
MEK5
MAPK
ERK1
ERK2
p38α
p38β
p38γ
p38δ
JNK1
JNK2
JNK3
ERK5

2. APOPTOSIS SIGNAL-REGULATING KINASE 1

2.1. The ASK family

The ASK family of proteins is composed of ASK1 (MAP3K5), ASK2 (MAP3K6) and ASK3 (MAP3K15). These proteins are STE kinases (homologs of the yeast sterile, or STE, family of kinases) that are involved in the MAPK pathway (Manning et al., 2002). They all have a highly conserved central kinase domain and coiled-coil motifs (section 2.2) (Ortner and Moelling, 2007; Naguro et al., 2012).

ASK1 is expressed throughout the body, while ASK2 and ASK3 are expressed only in select tissues (Tobiume et al., 1997). ASK2 is easily detectable in the skin, gastrointestinal tract, and lung, while ASK3 is highly expressed in the kidney (Iriyama et al., 2009; Naguro et al., 2012). Additionally, knockout (KO) mice for each gene present with different phenotypes. KO mice lacking either of the three ASK genes are viable and show no developmental defects but are deficient in H_2O_2 and TNF-α-induced apoptosis, as well as sustained activation of p38 and JNK (Tobiume et al., 2001; Matsuzawa et al., 2002). ASK2 knockout mice were prone to papillomas development in two-stage skin tumorigenesis experiments (Iriyama et al., 2009). ASK3 KO mice were hypertensive and had impaired blood pressure control and osmotic stress (Kaji et al., 2010; Naguro et al., 2012; Maruyama et al., 2016).

All three ASK family members can act as MAP3Ks to activate p38 and JNK and can also interact with ASK1 (Federspiel et al., 2016). ASK2 must dimerize with ASK1 to be stabilized and enzymatically active, but the role of ASK3 on ASK1 signaling has yet to be determined (Wang et al., 1998; Takeda et al., 2007).

2.2. ASK1 structure

ASK1 is composed of a central serine/threonine kinase domain that is flanked by N- and C- terminal coiled-coil motifs (NCC and CCC, respectively) (Figure 1-2A) (Bunkoczi et al., 2007). The structure of ASK1 is important to regulating its kinase activity. Like other MAP3K proteins, including Raf-1 (Luo et al., 1996; Inouye et al., 2000), MLK-3 (Leung and Lassam, 1998), and MEKK2 (Cheng et al., 2005), ASK1 kinase domains must dimerize to become active through trans-autophosphorylation of the kinase activation loop at threonine 838 (T838) (Gotoh and Cooper, 1998; Liu et al., 2000; Tobiume et al., 2002; Noguchi et al., 2005; Weijman et al., 2017). While there are some reports that show dimer formation between isolated ASK1 kinase domains (Bunkoczi et al., 2007; Petrvalska et al., 2016), it is generally believed that the coiled-coil motifs are important in mediating ASK1 kinase domain dimerization (Tobiume et al., 2002; Noguchi et al., 2005; Takeda et al., 2007). Under resting, unstressed conditions, ASK1 is dimerized via only CCC. Inhibitory protein binding and structural elements within the N-terminal region of ASK1 keep the proteins from fully dimerizing and in an inactive state (Saitoh et al., 1998; Liu et al., 2000; Fujino et al., 2007; Kosek et al., 2014; Weijman et al., 2017). Furthermore, deletion of the first 648 amino acids of ASK1 creates a constitutively active kinase which can freely dimerize via the CCC and kinase domain (Saitoh et al., 1998; Hatai et al., 2000).

2.3. Roles of ASK1

The most well-established role of ASK1 is as a pro-apoptotic signaling protein activated by a variety of cellular stressors, such as oxidative and endoplasmic reticulum (ER) stress (Hattori et al., 2009; Shiizaki et al., 2013). Over-expression of ASK1 or of a constitutively active form of the protein is sufficient to induce mitochondrial-dependent cell death under a variety of experimental conditions (Ichijo et al., 1997; Saitoh et al., 1998; Hatai et al., 2000).

2.3a. ASK1-induced signaling by p38 and JNK MAPKs

Activated ASK1 binds to MKK3/6 and MKK4/7 proteins and activates them through phosphorylation of two residues within the kinase domain (Zheng and Guan, 1994; Ichijo et al., 1997; Roskoski, 2012). MKK proteins are dual specificity kinases, meaning they can phosphorylate both serine/threonine and tyrosine residues. MKK3/6 and MKK4/7 activate p38 and JNK MAPKs, respectively, through dual phosphorylation of the tyrosine and threonine residues within the activation loop Thr-X-Tyr motif (Kyriakis et al., 1994; Zhang and Dong, 2007; Dhanasekaran and Reddy, 2008). p38 and JNK have a variety of cellular targets that mediate the effects of ASK1 activation.

p38 and JNK MAPKs are serine/threonine kinases that can phosphorylate cytoplasmic and nuclear substrates to impact a variety of biological outcomes. Multiple factors, such as the initial initiator of the MAPK pathway, duration of signaling, and composition of the MAP3K-MAP2K-MAPK pathway can all impact what kind of effect is elicited by MAPK activation (Chang and Karin, 2001; Ventura et al., 2006).

In mammalian cells, there are three genes encoding JNK proteins (*MAPK8, MAPK9, MAPK10),* which give rise to three main JNK proteins (JNK1, JNK2, JNK3), as well as other isoforms generated through alternative splicing. JNK signaling can have a pro-apoptotic effect by phosphorylating the pro-apoptotic BH3-only proteins, Bim and Bmf, which releases them from sequestration by the dynein and myosin V motor complexes, respectively, and allows them to translocate to the mitochondria where they can promote apoptosis (Puthalakath et al., 1999; Puthalakath et al., 2001; Puthalakath and Strasser, 2002; Lei and Davis, 2003). JNK proteins also regulate apoptosis through 14-3-3 protein binding. These kinases can promote cell survival by phosphorylate Bad at serine 112 to create a 14-3-3 binding site which keeps the pro-apoptotic Bad protein localized in the cytosol where is cannot antagonize Bcl-2 family proteins (Yu et al., 2004). Conversely, JNK can also directly phosphorylate 14-3-3 proteins which causes their release of Bad and the other pro-death protein, forkhead box protein O3a (FOXO3a). This allows for Bad to interact with B-cell lymphoma 2 (Bcl-2) family proteins to promote apoptosis, and for FOXO3a to translocate to the nucleus where it transcribes pro-apoptotic genes to drive cell death (Tsuruta et al., 2004; Sunayama et al., 2005; Das et al., 2016).

Within in the nucleus, JNK interacts and activates multiple transcription factors. JNK proteins were first identified based on their ability to phosphorylate the c-Jun transcription factors at serine 63 and 73 and threonine 91 and 93, which can stabilize c-Jun by inhibiting its ubiquitination (Dérijard et al., 1994; Hibi et al., 1993; Fuchs et al., 1996; Musti et al., 1997; Morton et al., 2003). Phosphorylation

also enhances c-Jun transcription and blocks the bindings of transcriptional repressors (Dérijard et al., 1994; Angel et al., 1988; Hibi et al., 1993; Weiss et al., 2003). In addition to c-Jun, JNK phosphorylates other transcription factors in the same family of activating protein 1 (AP-1) transcription factors, including Fos and activating transcription factor (ATF) proteins (Karin et al., 1997; Vesely et al., 2009). AP-1 proteins contain basic leucine zipper (bZIP) domains, where the basic region mediates DNA binding and the leucine zippers mediate hetero- and homo-dimerization with other AP-1 proteins that is required for activity (Angel and Karin, 1991). JNK-mediated activation of AP-1 transcription can promote expression of apoptotic genes, such as TNF-α, Fas ligand (Fas-L), transforming growth factor beta 1 (TGF-β1), and Bak (Faris et al., 1998; Fan and Chambers, 2001; Ventura et al., 2004; Jia et al., 2008; Yin et al., 2009; Zarzynska, 2014; Dong et al., 2016).

The p38 MAPK family is composed of 4 genes (*MAPK14, MAPK11, MAPK12, MAPK13*) which encode four main proteins (p38α, p38β, p38γ, p38δ). Like JNK, p38 can also promote apoptosis. Upon genotoxic stress, p38 can phosphorylate the p53 transcription factor within its transactivation domain to increase transcription of apoptotic genes and can also drive expression of the growth arrest and DNA damage (GADD)-inducible genes (Sanchez-Prieto et al., 2000; Sarkar et al., 2002).

p38 can promote cell survival and inflammation under different cellular contexts as well. For example, p38 can phosphorylate and inhibit the ability of glycogen synthase kinase 3 β (GSK3β) to block pro-survival signaling by β-catenin (Thornton et al., 2008). Additionally, p38 has a strong link to inflammation, where

it phosphorylates and activates MAPK-activated protein kinase 2 (MK2, MAPKAPK-2) which stabilizes the mRNA transcripts for multiple pro-inflammatory cytokines and signaling proteins, including TNF-α, interleukin (IL)-8, IL-6, IL-1β, and cyclooxygenase 2 (COX-2), thereby increasing protein expression (Clark et al., 2003). This inflammation can be pathogenic in diseases like nonalcoholic steatohepatitisis (NASH).

2.3b. ASK1 signaling in human diseases

ASK1 activity has been linked to many human diseases where ASK1-dependent cell death has both positive or negative effects on pathogenesis (Table 1-1). In the brain, ASK1 promotes neuronal cell death that is characteristic of many neurodegenerative diseases, such as Alzheimer's disease (AD) and Parkinson's disease (PD) (Nishitoh et al., 2002; Hashimoto et al., 2003; Harada et al., 2006; Sturchler et al., 2011; Lee et al., 2012; Bamji-Mirza et al., 2014; Guo et al., 2017). In both of these diseases, pathogenic cell death is caused by ER and oxidative stress (Imaizumi et al., 2001; Peel et al., 2004; Junn et al., 2005; Lee et al., 2012; Hwang, 2013; Song et al., 2014). Importantly, knockdown or inhibition of ASK1 is capable of reducing cell death, highlighting the importance of ASK1 in these disorders (Kadowaki et al., 2005; Karunakaran et al., 2007; Niu et al., 2011).

In cancer, ASK1 signaling promotes apoptosis induced by chemotherapeutic agents like cisplatin and microtubule disruptors (Chen et al., 1999; Wang et al., 1999; Yamamoto et al., 1999; Yuan et al., 2003; Brozovic and Osmak, 2007). Other anti-cancer drugs kill cancer cells through elevation of

reactive oxygen species (ROS) levels, which activate ASK1-mediated cell death (Cheng et al., 2014; Liang et al., 2014; Mao et al., 2014; Pan et al., 2014; Ma et al., 2017a). Conversely, ASK1 can promote cell proliferation and inflammation, two biological processes that can drive tumor development and inflammatory diseases (Iriyama et al., 2009; Guo et al., 2010; Hayakawa et al., 2010; Mnich et al., 2010; Hayakawa et al., 2011; Hayakawa et al., 2012b). In gastric cancer, ASK1-JNK signaling promotes the expression of cyclin D1 to drive cell cycle progression and tumor growth (Hayakawa et al., 2011). Importantly, different binding partners of ASK1 can influence its function. Using a two-stage skin tumorigenesis experiment where mice were treated once with a carcinogenic agent, followed by repeated treatments with tumor promoting chemicals, ASK1 was found to promote apoptosis when in the presence of ASK2, acting as a tumor suppressor during tumor initiation (Abel et al., 2009). However, without ASK2 expression, ASK1 enhanced tumorigenesis by increasing tumor-promoting inflammation and expression of TNF-α and IL-6 (Iriyama et al., 2009).

ASK1 shows a protective effect during viral infections (Geleziunas et al., 2001; Maruoka et al., 2003; Miyakawa et al., 2015; Okazaki et al., 2015). In influenza-infected cell, ASK1 promotes MAPK-dependent apoptosis, which is an important host defense mechanism (Hinshaw et al., 1994; Barber, 2001; Maruoka et al., 2003). ASK1 also interacts with two human immunodeficiency virus type 1 (HIV-1) proteins, Nef and Vif. Nef interacts with ASK1 to inhibit its kinase activity while also increasing S967 phosphorylation, protecting the HIV-infected cell from ASK1-mediated cell death (Geleziunas et al., 2001; Kumar et al., 2013). However,

ASK1 can promote death on HIV-infected cells by stabilizing APOBEC3G (A3G), an endogenous protein known to have anti-viral effects. It achieves this by blocking the interaction between Vif and A3G which prevents Vif-mediated ubiquitination and degradation of A3G (Miyakawa et al., 2015).

Nonalcoholic steatohepatitisis (NASH) is a form of nonalcoholic fatty liver disease (NAFLD) that is characterized by an accumulation of fat and inflammation of the liver. These symptoms can damage liver cells and cause fibrosis, potentially worsening to cirrhosis or hepatocellular carcinoma or requiring an organ transplant (Charlton et al., 2001). ASK1 is known to promote inflammation in the liver that contributes to fibrosis and cirrhosis (Yamamoto et al., 2008; Zhang et al., 2010; Noguchi et al., 2014; Yamada et al., 2017; Loomba et al., 2018). The incidence of NASH is increasing in the U.S. and it is most commonly seen in patients who are obese or have type 2 diabetes. Furthermore, NASH is expected to become the leading cause of liver transplantation in the U.S. between 2020 and 2025 (Charlton et al., 2001; Charlton et al., 2011). There are currently no FDA approved treatments for NASH and understanding the underlying biology of this disease is critical to developing therapeutics (Ratziu et al., 2015).

Table 1-1: The role of ASK1 in human diseases.

Disease promoting	Disease limiting
Neurodegenerative diseases	Viral infections
Alzheimer's disease	Influenza
Parkinson's disease	Human immunodeficiency virus
Amyotrophic lateral sclerosis	
Huntington's disease	Cancer
	Chemo-induced cell death
Cancer	
Gastric cancer	
Inflammatory diseases	
Nonalcoholic steatohepatitisis	
Multiple sclerosis	
Rheumatoid arthritis	

2.3c. Development of small molecule inhibitors of ASK1

Due to its strong connection to human diseases, ASK1 activity has been targeted for therapeutic exploration (Hattori et al., 2009; Sturchler et al., 2011; Hayakawa et al., 2012a; Soga et al., 2012; Kawarazaki et al., 2014; Song et al., 2014; Tesch et al., 2016; Fujisawa, 2017). GS-4997 is an ATP-competitive ASK1 inhibitor that is in phase 3 clinical trials for the treatment of NASH (Gibson et al., 2017; Loomba et al., 2018; Lovering et al., 2018). Here, patients treated with GS-4997 showed reductions in liver fibrosis and less disease progression, indicating ASK1 inhibition could provide effective treatment for NASH (Loomba et al., 2018). Additionally, GS-4997 is in phase 2 clinical trials for other diseases, including diabetic kidney disease, alcoholic hepatitis, and pulmonary arterial hypertension (Lin et al., 2015; Lanier et al., 2017)

2.4. Regulation of ASK1 activity

In cells, ASK1 forms a high molecular weight complex in the cytoplasm (>1500 kDa), termed the ASK1 signalosome (Noguchi et al., 2005). The components of the signalosome, as well as upstream phosphorylation, impact ASK1 activity. The binding of inhibitory proteins and phosphorylation by pro-survival kinases, such as p90 ribosomal S6 kinase 2 (RSK2), proviral integration site for Moloney murine leukemia virus-1 (PIM1), and protein kinase B (most commonly referred to as Akt), keep ASK1 in an inactive state (Kim et al., 2001; Zhang et al., 2005; Gu et al., 2009; Jin et al., 2013). Conversely, cellular stressors, such as ROS and ER stress, activate ASK1 through altering the composition of the ASK1 signalosome to promote ASK1 dimerization and subsequent kinase

activation (Figure 1-2B) (Takeda et al., 2007; Jung et al., 2008; Manoharan et al., 2013; Pleinis et al., 2017).

2.4a. Protein-protein interactions (PPIs)

Thioredoxin

Thioredoxin (Trx) is an oxidoreductase with a Cys-Gly-Pro-Cys active site motif that changes conformation based on cellular ROS levels (Holmgren, 1968; Lee et al., 2013). When cells have low or basal amounts of ROS, Trx is reduced (Trx(SH)2) and binds to the N-terminal of ASK1 (a.a. 46-277, thioredoxin binding domain, TBD) to prevent ASK1 dimerization required for kinase activation (Fujino et al., 2007; Kosek et al., 2014). Upon elevated ROS levels, Trx gets oxidized (Trx(S)2) and forms a disulfide bridge and releases ASK1. This allows for ASK1 to dimerize and interact with positive regulators (see TRAF2/6) to initiate the phosphorylation cascade that results in MAPK activation (Saitoh et al., 1998; Liu et al., 2000; Fujino et al., 2007; Kosek et al., 2014).

TRAF2/6

The TNF receptor-associated factor (TRAF) family is comprised of six structurally related, non-enzymatic adaptor proteins that are defined by a conserved C-terminal TRAF domain, with most proteins also containing RING and zinc finger domains (Chung et al., 2002). TRAF2/5/6 have been identified as important adaptors that help activate ASK1 in response to multiple stressors to enhance downstream MAPK activity (Song et al., 1997; Nishitoh et al., 1998;

Hoeflich et al., 1999). They do this by interacting with the N- and C-terminal regions of ASK1 to promote oligomerization required for full kinase activation and downstream signaling (Gotoh and Cooper, 1998; Hoeflich et al., 1999; Liu et al., 2000; Fujino et al., 2007; Nishida et al., 2017; Weijman et al., 2017).

Additionally, TRAF proteins can associate with other MAP3K proteins like MEKK1, but the TRAF-ASK1 interaction is especially important in mediating TNF-α and ER stress-induced cell death (Tobiume et al., 2001; Nishitoh et al., 2002; Kadowaki et al., 2005; Noguchi et al., 2005). Upon TNF-α treatment, ROS levels become elevated in the cell which dissociate ASK1 from its negative regulator, Trx (Liu et al., 2000; Chandel et al., 2001; Lin et al., 2004). Once Trx is no longer bound to ASK1, TRAF proteins can bind and promote ASK1 activation (Nishitoh et al., 1998; Liu et al., 2000). TRAFs also act as adaptors linking ASK1 activation to ER stress caused by the accumulation of unfolded proteins. Under these stress conditions, TRAF2 serves as an adaptor by linking the ER sensor protein, inositol-requiring enzyme 1 (IRE1), to ASK1 (Nishitoh et al., 1998; Liu et al., 2000; Urano et al., 2000; Nishitoh et al., 2002). Proper activation of ASK1 is essential to promoting apoptosis under these conditions, as loss of ASK1 impairs TNF-α and ER stress-induced cell death (Tobiume et al., 2001; Matsuzawa et al., 2002; Nishitoh et al., 2002; Kadowaki et al., 2005).

Daxx

Death domain associated protein (Daxx) was initially identified as a binding partner of the Fas/CD95 death receptor that has a well-defined role in promoting

apoptosis (Yang et al., 1997; Schulze-Osthoff et al., 1998; Peter and Krammer, 2003; Khelifi et al., 2005). Upon Fas ligand binding, the Fas/CD95 death receptor can interact with multiple adaptor proteins that lead to activation of different cell death pathways in the cell, such as Daxx, which triggers JNK-dependent cell death, especially under stress conditions (Kischkel et al., 1995; Yang et al., 1997; Chang et al., 1999; Song and Lee, 2003a; Khelifi et al., 2005; Sánchez-Capelo, 2005).

Further investigation found ASK1 to be critical in mediating Daxx-induced cell death (Chang et al., 1998). Daxx binds to ASK1 to induce conformational changes that promote oligomerization, leading to downstream signaling (Chang et al., 1998; Ko et al., 2001; Song and Lee, 2003b; Kitamura et al., 2009; Weijman et al., 2017). This initiates a positive feedback loop, as activated JNK enhances the nuclear export of Daxx to the cytoplasm where it can further drive ASK1-mediated signaling (Ko et al., 2001; Song and Lee, 2003b, a; Jung et al., 2007; Fukuyo et al., 2009; Kitamura et al., 2009; Niu et al., 2011).

2.4b. Post-translational modifications (PTMs)

Phosphorylation at serine 967

Akt pathway: The phosphoinositide-3-kinase/Akt (PI3K/Atk) pathway is a well-characterized pro-survival, growth, and proliferative pathway that is commonly altered in cancer (Vara et al., 2004). In this pathway, PI3K converts phosphatidylinositol (3,4)-bis-phosphate (PIP_2) to phosphatidylinositol (3,4,5)-tris-phosphate (PIP_3), which recruits Akt to the plasma membrane via its pleckstrin

homology (PH) domain. This membrane recruitment allows for the co-localization of Akt to its partial activator, 3-phosphoinositide-dependent protein kinase 1 (PDK1), which phosphorylates Akt in the activation loop (Alessi et al., 1997). An additional phosphorylation event in the hydrophobic motif of Akt is required for full kinase activation (Feng et al., 2004; Sarbassov et al., 2005).

Once activated, Akt phosphorylates many substrates to impact cell growth, proliferation, and survival. One of its targets is inhibitor of κB kinase β (IKKβ), a kinase within the nuclear factor-κB (NF-κB) pathway (Alessi et al., 1996; Bai et al., 2009; Puckett et al., 2013). The NF-κB pathway responds to a variety of stressors and inflammatory cytokines to activate the NF-κB transcription factors which can drive cell survival, proliferation, and the immune response (Oeckinghaus and Ghosh, 2009). Activated IKKβ then phosphorylates ASK1 at serine 967 (S967), which creates a 14-3-3 protein binding site (Zhang et al., 1999; Goldman et al., 2004; Puckett et al., 2013). Interestingly, another protein within the Akt pathway can also directly phosphorylate ASK1. PDK1, the upstream kinase responsible for phosphorylating and activating Akt, and can also inhibit ASK1 signaling through S967 phosphorylation (Seong et al., 2010).

14-3-3: The 14-3-3 family consists of seven non-enzymatic isoforms which bind specific phosphoserine and phosphothreonine motifs. Multiple 14-3-3 isoforms can bind ASK1 through their conserved amphipathic groove, which is also responsible for binding other substrates such as Raf-1 and Bad (Fu et al., 2000). 14-3-3 proteins bind phosphorylated ASK1 at S967, which is adjacent to the central

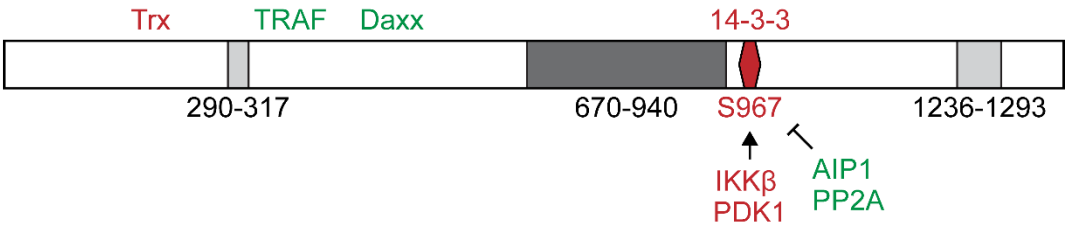
kinase domain (Zhang et al., 1999; Goldman et al., 2004; Subramanian et al., 2004). This allows 14-3-3 to make additional transient contacts with the ASK1 active site, as well as its substrate phospho-binding motif (Petrvalska et al., 2016). Such contacts may alter the conformation of the kinase domain, prevent activating auto-phosphorylation, or block substrate binding, all of which could serve as possible mechanisms for 14-3-3-mediated inhibition of ASK1 (Petrvalska et al., 2016).

AIP1 and PP2A: Other proteins can respond to pro-apoptotic stressors, such as ROS, and promote ASK1 dephosphorylation at S967, thereby enhancing ASK1-medating signaling. ASK1-interacting protein 1 (AIP1, also known as DAB2IP) is a Ras GTPase-activating protein (Ras-GAP) which associates with ASK1 in response to TNF-α treatment to trigger 14-3-3 dissociation and S967 dephosphorylation (Zhang et al., 2003). It does this by associating with protein phosphatase (PP)2A and acting as a scaffold to recruit the phosphatase to ASK1 (Min et al., 2008; Xie et al., 2009). Importantly, the control of ASK1 S967 phosphorylation is important in cancer therapy. Multiple chemotherapeutic agents can kill cancer cells through ASK1 activation and induce dephosphorylation of S967 to enhance apoptosis (Kuo et al., 2007; Hou et al., 2008; Palit et al., 2015).

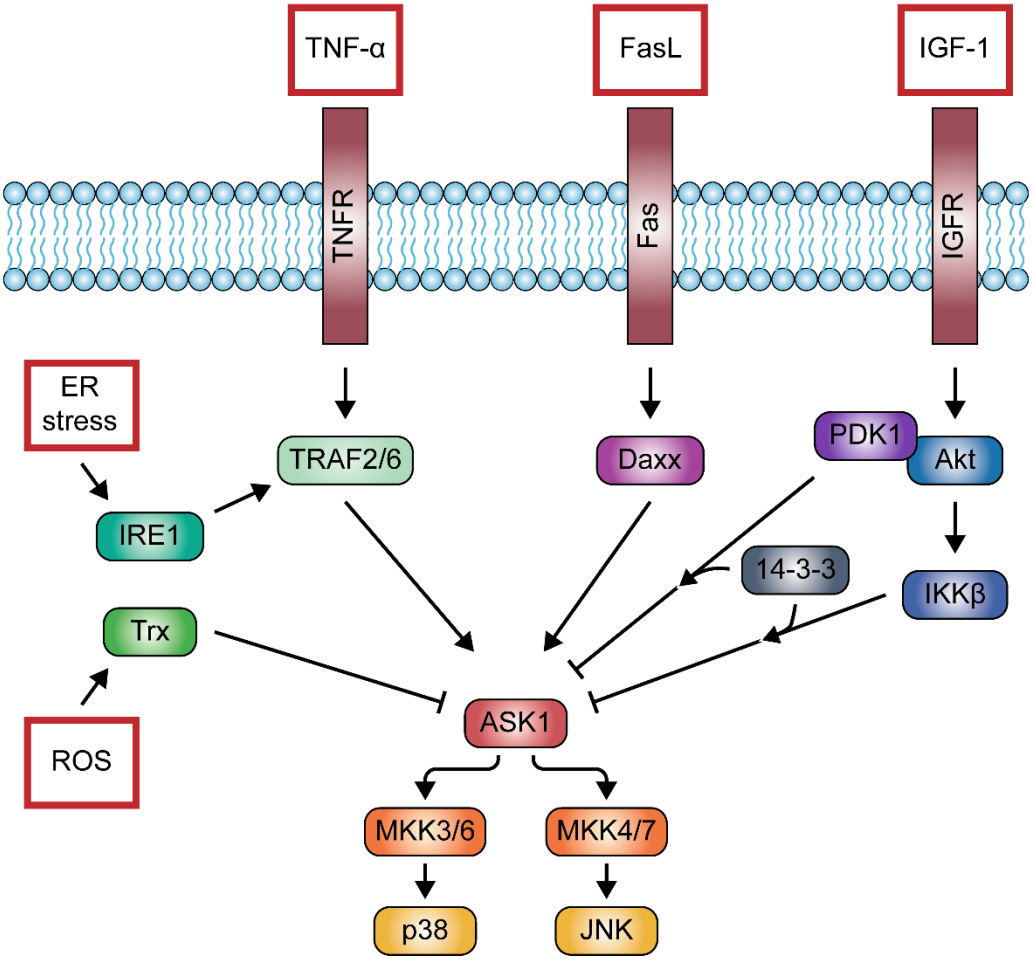
Figure 1-2: Regulation of ASK1 activity and signaling by protein-protein interactions and post-translational modification.

A) Schematic of ASK1 protein domains and binding sites for PPIs and PTMs (light grey: coiled-coil motifs, dark grey: kinase domain, green proteins: positive regulators of ASK1 activity, red proteins: negative regulators of ASK1 activity). B) Cellular pathways which impact ASK1 activity in the cell.

A


Trx
TRAF
Daxx
14-3-3
290-317
670-940
S967
1236-1293
IKKβ
PDK1
AIP1
PP2A

B


TNF-α
FasL
IGF-1
TNFR
Fas
IGFR
ER stress
TRAF2/6
Daxx
PDK1
Akt
IRE1
14-3-3
IKKβ
Trx
ROS
ASK1
MKK3/6
MKK4/7
p38
JNK

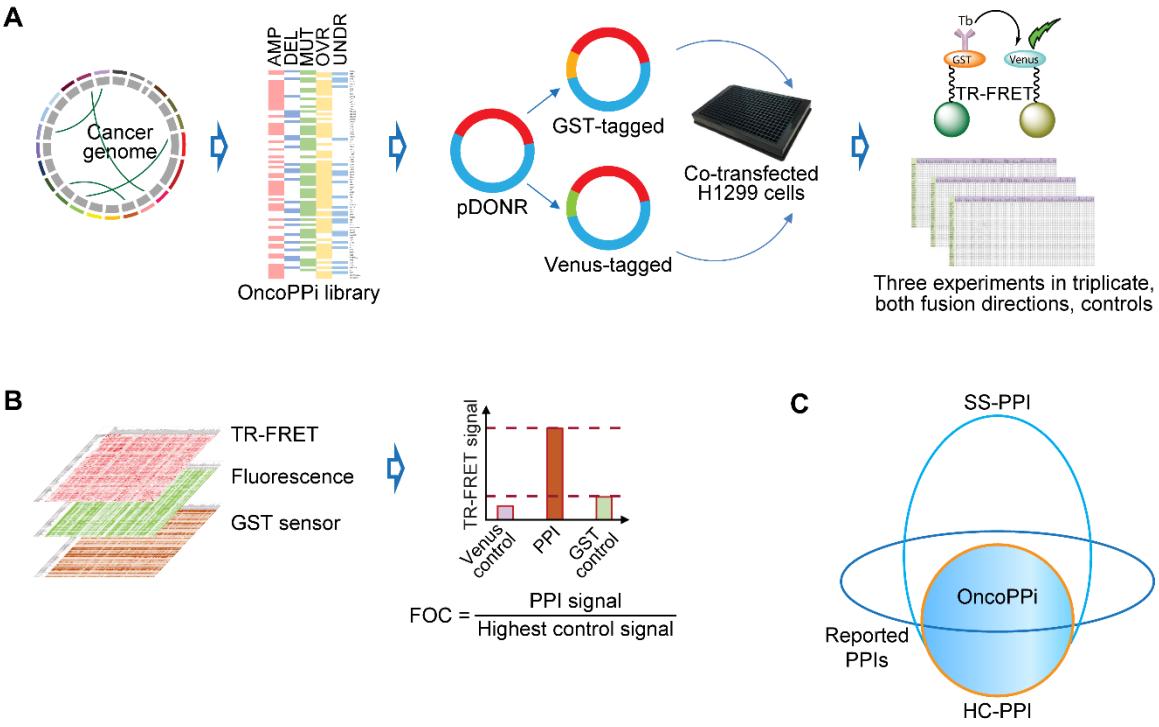
3. LATS2 DETECTED AS NOVEL ASK1 BINDING PARTNER

In order to identify new binding partners of ASK1, as well as to better characterize PPIs relevant to disease, our lab performed a high-throughput screen for PPIs between a lung cancer-associated gene set. Time-resolved fluorescence resonance energy transfer (TR-FRET) was used to detect interactions between over-expressed proteins transfected in H1299 non-small cell lung cancer cells. Additionally, negative control samples expressing empty vector plasmids were included for each protein pair and were used to calculate the fold-over-control (FOC) values (Li et al., 2017). We detected 348 PPIs which met our high-confidence criteria for a positive interaction (HC-PPI dataset, FOC ≥ 1.5, p-values ≤ 0.05, q-values ≤ 0.01) and make up part of the resulting high-confidence lung cancer-associated PPI network, termed OncoPPi (version 1) (Figure 1-3).

The screen detected a total of 16 statistically significant (p-values ≤ 0.05) PPIs. However only two interactions met the more stringent criteria to be included in OncoPPi, ASK1-ASK1 and ASK1-large tumor suppressor 2 (LATS2) (Li et al., 2017). The formation of ASK1 dimers has previously been reported and is well known to be an important step in the activation of ASK1, but the association between ASK1 and LATS2, a kinase within the Hippo signaling pathway, has never been reported.

Figure 1-3: Workflow for OncoPPi screening and data analysis.

A) Selection and generation of GST- and Venus-tagged plasmid library of lung cancer-associated genes. PPIs were screened in H1299 cells in triplicate, in three independent replicates. B) Expression of GST- and Venus-tagged plasmids was monitored by GST biosensor and fluorescence intensity of the fusion proteins, respectively. TR-FRET signals were measured, and FOC values were calculated. C) Generation of OncoPPi (version 1). Figure adapted from (Li et al., 2017).


A
Cancer genome
AMP
DEL
MUT
OVR
UNDR
OncoPPi library
pDONR
GST-tagged
Venus-tagged
Co-transfected H1299 cells
Tb
GST
Venus
TR-FRET
Three experiments in triplicate, both fusion directions, controls
B
TR-FRET
Fluorescence
GST sensor
TR-FRET signal
Venus control
PPI
GST control
FOC = PPI signal / Highest control signal
C
SS-PPI
OncoPPi
Reported PPIs
HC-PPI

4. THE HIPPO PATHWAY

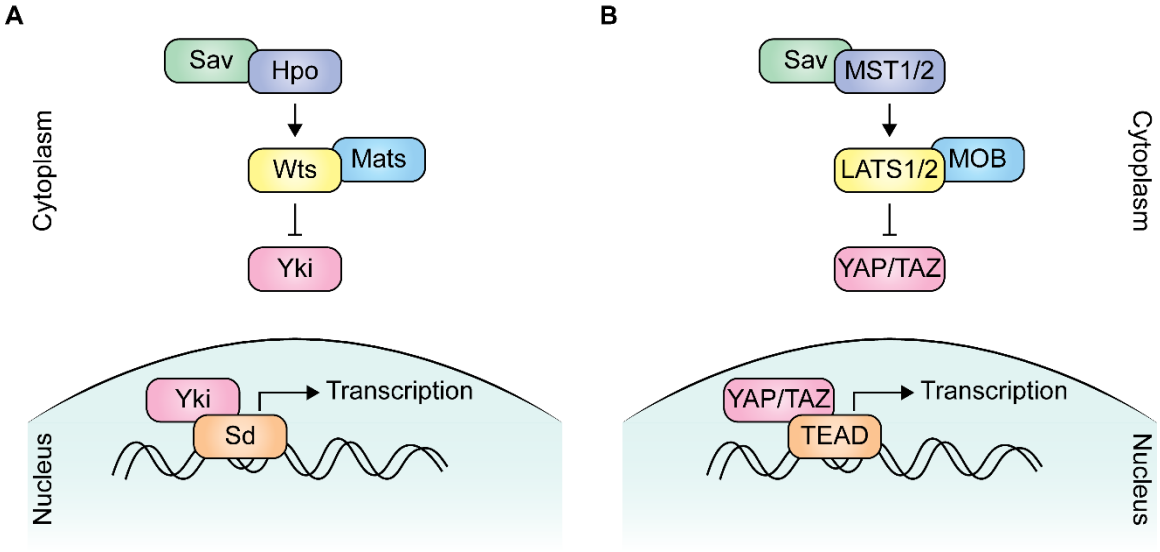
The Hippo pathway was first discovered in *Drosophila* where it controls cell growth and organ size. Here, loss of the *warts* gene in flies caused over-proliferation and developmental defects (Justice et al., 1995; Xu et al., 1995). Further experimentation led to the identification of the complete *Drosophila* Hippo pathway, composed of the serine/threonine kinases hippo and warts, as well as the scaffolding proteins sav and mats, which function to inhibit the binding of the transcriptional co-activator, yorkie, to the scalloped transcription factor (Figure 1-4A).

This pathway is conserved in mammals and is composed of a core kinase cascade where activated MST1/2 (mammalian sterile20 kinases 1 and 2, orthologs of hippo) phosphorylate the scaffolding proteins Sav (salvador, also called WW45, ortholog of sav) and MOB1A/1B (mps one binder proteins 1A and 1B, orthologs of mats). This promotes the binding between MST1/2 and its phospho-substrates, LATS1/2 (large tumor suppressor 1 and 2, orthologs of warts) (Callus et al., 2006; Praskova et al., 2008). LATS1/2 then phosphorylate the oncogenic YAP (yes-associated protein) and TAZ (transcriptional coactivator with PDZ-binding motif) transcriptional co-activators (orthologs of yorkie) which inhibits their action through 14-3-3-dependent cytoplasmic retention and ubiquitin-dependent degradation (Huang et al., 2005; Zhao et al., 2007; Hao et al., 2008; Lei et al., 2008; Oh and Irvine, 2008; Liu et al., 2010a; Ren et al., 2010; Schumacher et al., 2010; Zhao et al., 2010). This in turn blocks the nuclear translocation required for the binding of YAP/TAZ to TEAD (TEA/ATTS domain) transcription factors (orthologs of scalloped). TEAD transcription factors contain an N-terminal DNA-binding TEA

domain which recognize and bind specific DNA elements, but require co-activator binding to promote transcription of expression of genes involved in cell growth, survival, and proliferation, such as CTGF, AREG, survivin, Axl, CK6, Bcl-2, and Myc (Figure 1-4B) (Pan, 2007; Zhu et al., 2015).

Figure 1-4: Conservation of the Hippo signaling pathway.

Hippo pathways components in A) *Drosophila* and B) mammals.


A
Sav
Hpo
Wts
Mats
Yki
Cytoplasm
Yki
Sd
Transcription
Nucleus
B
Sav
MST1/2
LATS1/2
MOB
YAP/TAZ
Cytoplasm
YAP/TAZ
TEAD
Transcription
Nucleus

4.1. Activation of the Hippo pathway

The Hippo pathway is activated by a variety of upstream inputs, such as cell-cell contacts, apical-basal polarity, and mechanical forces. One key way in which Hippo signaling is linked to the upstream inputs is through the Merlin (Mer, also known as NF2 for neurofibromatosis-2), Expanded (Ex), and Kibra scaffolding proteins. These proteins interact with multiple members of the Hippo pathway, including MST1/2, Sav, and LATS1/2, and bring the different Hippo pathway components into close proximity to enhance LATS1/2 activation and downstream signaling to YAP (McClatchey and Giovannini, 2005; Hamaratoglu et al., 2006; Baumgartner et al., 2010; Genevet et al., 2010; Pan, 2010; Yu et al., 2010; Yin et al., 2013; Cooper and Giancotti, 2014; Li et al., 2015; Sun and Irvine, 2016; Su et al., 2017).

Mer, Ex, and Kibra can also be recruited to transmembrane proteins, such as Crumbs3 and E-cadherin that are components of apical polarity complexes and adherens junctions (AJs), respectively (Roh et al., 2003; Bulgakova and Knust, 2009; Baumgartner et al., 2010; Chen et al., 2010; Ling et al., 2010; Yu et al., 2010). This localizes Hippo pathway components to sites of cell junctions which sense cell density and positioning (Takahashi and Suzuki, 1996; Kim et al., 2011; Silvis et al., 2011; Irvine, 2012).

The composition and stiffness of the extracellular matrix (ECM) is one key mechanical force impacting a cell. For example, softening the ECM in breast cancer has been shown to have a significant impact on cell behavior by reducing tumor growth and progression (Levental et al., 2009). YAP and TAZ also impact the ECM and are over-expressed in cancer-associated fibroblasts (CAFs) which

enable matrix stiffening. The thickened microenvironment can then create a feedforward loop to enhance YAP/TAZ activation in CAFs (Calvo et al., 2013). Similarly, cytoskeletal mechanics also impact Hippo pathway activation and signaling, as cells treated with actin or microtubule destabilizing agents show reduced phosphorylation of YAP (Dupont et al., 2011; Sansores-Garcia et al., 2011; Zhao et al., 2012; Guo and Zhao, 2013).

4.2. Hippo pathway and disease

Members of the Hippo pathway have strong links to cancer. YAP and TAZ are oncogenic and enhance cancer cell stemness, proliferation, and metastasis in solid tumors (Zanconato et al., 2016). These proteins are often upregulated in cancers and correlate to a worse patient outcome (Xu et al., 2009; Lau et al., 2014; Malik et al., 2017). A small molecule drug, Verteporfin, is FDA approved for the treatment of macular degeneration and works by inhibiting the interaction between YAP and TEAD. Treatment of multiple cancers with this drug down-regulates cancer cell migration, invasion and stemness (Feng et al., 2016; Ma et al., 2016; Al-Moujahed et al., 2017).

LATS2 also plays a role in cancer where it is commonly down-regulated or lost in multiple tumor types through genetic deletion, promoter hypermethylation, and miRNA targeting (Yabuta et al., 2000; Takahashi et al., 2005; Jiang et al., 2006; Lee et al., 2009; Strazisar et al., 2009; Fang et al., 2012; Li et al., 2012; Lin et al., 2013; Yao et al., 2015). Lung cancer patients with lower expression of LATS2 show reduced overall survival rates (Takahashi et al., 2005; Lin et al., 2013; Gao

et al., 2017; Son et al., 2017). The MST1/2 kinases also are frequently hypermethylated in sarcomas, resulting in reduced expression of the initiator kinases within the Hippo pathway (Seidel et al., 2007).

While both YAP/TAZ and LATS1/2 have well documented oncogenic and tumor suppressive roles, respectively, they can each play an opposing role in tumorigenesis under certain contexts. The Wnt/β-catenin pathway promotes the development of colon cancer by driving the proliferation of intestinal crypt stem cells. YAP and TAZ are capable of binding Dishevelled (DVL), a positive regulator of Wnt/β-catenin signaling to reduce β-catenin expression and activity (Varelas et al., 2010; Barry et al., 2013). YAP achieves this by restricting DVL nuclear localization, while TAZ binds to and inhibits activating phosphorylation that is required for DVL activity (McKay et al., 2001; Wallingford and Habas, 2005; Bryja et al., 2007; Varelas et al., 2010; Barry et al., 2013). This function of TAZ is also dependent on Hippo signaling, as LATS1/2-mediated phosphorylation and cytoplasmic retention of TAZ is required for its co-localization and interaction with DVL (Varelas et al., 2010). LATS1/2 signaling can also have an opposing effect on cancer, where it aids in the evasion of immune destruction. This is achieved by reducing the secretion and altering the components of extracellular vesicles released from tumor cells, which contain less immunostimulators than those found in tumors harboring LATS1/2 deletions. Under this context, LATS1/2 signaling and YAP inactivation decreases the ability of the innate immune system to recognize and destroy cancer cells by reducing tumor immunogenicity (Moroishi et al., 2016).

Deletions of the *NF2* gene and loss of Mer expression can be inherited and result in the genetic disorder neurofibromatosis type 2. This disease is characterized by development of non-cancerous tumors of the nervous system, most often around the auditory nerve (Petrilli and Fernández-Valle, 2016). Sporadic mutations in *NF2* also lead to malignant tumor development and is exceptionally high in mesothelioma (Cheng et al., 1999; Baser et al., 2002; Petrilli and Fernández-Valle, 2016). In the Hippo pathway, loss of Mer results in reduced LATS1/2 activation due to impaired recruitment to the plasma membrane where it can be rapidly activated by MST1/2 (Yin et al., 2013).

4.3. Hippo and MAPK pathway cross-talk

While the interaction between ASK1 and LATS2 has not previously been reported, there are multiple reports of signaling cross-talk between the Hippo and MAPK pathways. Over-expression of the MEKK1-MKK3-p38 signaling pathway activates YAP through actin polymerization and activation of Ajuba LIM proteins (Huang et al., 2016). p38 phosphorylates Ajuba which increases Ajuba binding and inhibition of LATS proteins. This same effect has been observed with both ERK and JNK MAPK proteins, where they phosphorylate other members of the Ajuba family (Reddy and Irvine, 2013; Sun and Irvine, 2013). p38 can impact the localization of TEAD proteins and sequester these transcription factors in the cytosol to inhibit their gene expression upon osmotic stress (Lin et al., 2017).

Additionally, the Hippo pathway can also impact p38 and JNK signaling, as MST1 promotes activation of the MKK6-p38 and MKK7-JNK pathways (Graves et

al., 1998). JNK activation by MST proteins occurs in a kinase-dependent mechanism to regulate multiple processes, such apoptosis, cell cycle arrest, and invasion (Ura et al., 2001; Densham et al., 2009; Ma et al., 2017b). However, it has not been shown that MST1/2 directly phosphorylate MKK or MAPK proteins, and there may be other intermediate proteins which help mediate this effect.

5. LATS PROTEINS

The human LATS1 and LATS2 proteins are the orthologs of *Drosophila* warts and are part of the core Hippo pathway (Nishiyama et al., 1999; Tao et al., 1999; Hori et al., 2000; Yabuta et al., 2000). Two other proteins, NDR1/2 (nuclear Dbf2 related kinases 1/2, also known as STK38 and STK38L) are also in this group. Unlike LATS1/2, NDR1/2 lack the extended N-terminal region found in warts and LATS1/2. There are reports of NDR1/2 acting in the Hippo pathway, however their exact role is still unclear and they are not considered as part of the core pathway (Visser and Yang, 2010; Hergovich, 2016).

LATS1/2 are members of the AGC group of kinases, which is composed of structurally related serine/threonine kinases that require phosphorylation in both the activation loop and a conserved hydrophobic motif to become catalytically active (Pearce et al., 2010). MST1/2 phosphorylate LATS1/2 in the hydrophobic motif, which stabilizes the active kinase conformation (Pearce et al., 2010; Hoa et al., 2016). Activation loop phosphorylation is likely carried out by LATS1/2 autophosphorylation and is required for ATP positioning catalysis (Praskova et al., 2008; Pearce et al., 2010; Meng et al., 2016). Additionally, LATS1/2, as well as NDR1/2, share two structural regions not found in other AGC kinases; a 30-60

amino acid insertion in the kinase domain that serves an autoinhibitory function, and a N-terminal regulatory domain (NTR) which contains the MOB binding site (Pearce et al., 2010; Visser and Yang, 2010; Hergovich, 2013).

While the kinase domains of LATS1 and LATS2 are very similar, their N-terminal regions are more divergent. Both proteins share LATS conserved domains 1 and 2 (LCD1 and LCD2) which are not found in *Drosophila* warts, and deletion or mutation of these regions can impair the tumor suppressive role of LATS proteins and drive tumorigenesis (Visser and Yang, 2010; Okada et al., 2011; Yabuta et al., 2011; Yu et al., 2015). PPxY motifs found in LATS proteins are responsible for binding the hydrophobic WW motifs found in YAP/TAZ, which ultimately allows for phosphorylation of these target proteins at Ser127/Ser381 and Ser89/Ser311, respectively (Bork and Sudol, 1994; Chen and Sudol, 1995; Hao et al., 2008).

The two LATS homologs share around 50% overall homology and perform the same role within the Hippo pathway, however these kinases also have functions outside the canonical Hippo pathways, some of which are unique to one homolog (Zhang et al., 2008; Yu et al., 2013). Evolutionary analysis indicates that vertebrate LATS1 is more related to warts, and that LATS2 may have originated from a gene duplication event (Chen et al., 2009; Hilman and Gat, 2011; Furth and Aylon, 2017). Additional analysis suggests that mammalian LATS2 may have a more expanded function outside the Hippo pathway because it interacts with a wider array of binding partners involved in multiple biological pathways, including

p53, β-catenin, and hormone signaling (Powzaniuk et al., 2004; Aylon et al., 2006; Aylon et al., 2010; Li et al., 2013).

6. SUMMARY

ASK1 is known to be one of the key upstream MAP3K proteins responsible for the sustained activation of p38 and JNK in response to certain stressors, as loss of ASK1 expression renders cells resistant to TNF-α and H_2O_2-induced apoptosis (Tobiume et al., 2001). Furthermore, ASK1 is heavily regulated by PPIs and PTMs within the cell that ultimately influence the activation state of ASK1.

From our high-throughput PPI screen, we detected the interaction between ASK1 and LATS2. While there have been previous links between the MAPK and Hippo pathways, there have been no reports of an interaction between these two proteins. By studying this interaction in more detail, we provide supporting data for the ASK1-LATS2 PPI, as well as functional effects of LATS2 on ASK1 activity, downstream MAPK activation, and apoptosis.

Chapter 2

Methods

Parts of this chapter has been published: Rusnak L, Qi Q, Tang C, Mo X-L, Fu H. Large tumor suppressor 2 activates JNK in a kinase-independent mechanism through apoptosis signal-regulating kinase 1. Journal of Molecular Cell Biology, submitted.

1. CELL CULTURE AND TRANSFECTION

Cell lines used in this study include HEK293T human embryonic kidney cells, HeLa human cervical cancer cells, and MCF7 human breast cancer cells. They were obtained from ATCC (Manassas, VA) and were maintained in 5% CO_2 and grown in Dulbecco's modified Eagle's medium (DMEM) (Corning, Manassas, VA) with 10% fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, MO). Media was supplemented with 1% Penicillin-Streptomycin (Corning) to reduce the risk of microbial contamination. Cell cultures were periodically tested for mycoplasma contamination using the MycoAlert Mycoplasma Detection Kit (Lonza, Basel, Switzerland, Cat# LT07). If not otherwise specified, cells were transfected with the X-tremeGENE cationic lipid reagent at a ratio of 3 uL reagent:1 ug DNA (Roche, Indianapolis, IN) according to the manufactures' instructions 24 hours after plating.

2. 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, Santa Clara, CA, Cat# 210515) and the pENTR223.1-LATS2 plasmid (DNASU, HsCD00353973) as template DNA (mutant primer 5'- ctcacgccctgtacgccatgagaaccctaaggaaaaag). Kinase-dead ASK1 ($ASK1^{KR}$) and S967A mutant ASK1 ($ASK1^{SA}$) were generated by site directed mutagenesis to mutate lysine 709 to arginine and serine 967 to alanine, respectively, using a hemagglutinin (HA)-tagged pcDNA3-ASK1 plasmid as template DNA (Zhang et al., 1999; Chen et al., 2001). 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, Carlsbad, CA) was used to generate glutathione S-transferase (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).

3. PROTEIN-PROTEIN INTERACTION DETECTION METHODS

3.1. Time resolved Forest resonance energy transfer FRET (TR-FRET)

Proteins under testing were expressed in mammalian cells. Cell lystates containing testing proteins were coupled to corresponding donor fluorophore and acceptor fluorophore with corresponding overlap excitation and emission spectrums. Briefly, HEK293T cells were lysed in 80 µL 1% NP-40 lysis buffer (1% NP-40, 10 mM HEPES, 150 mM NaCl, 1:1000 dilution of protease and phosphatase inhibitors (Sigma-Aldrich, Cat# P8340, P5726, and P0044)). Cell lysates were then serially diluted into a 384-well black plate (Corning, Cat# 3571) using TR-FRET buffer (0.01% NP-40, 20 mM Tris-HCl, pH 7.0, 50 mM NaCl). GST-Terbium (HTRF)-conjugated antibody (Cisbio Bioassays, Codolet, France, 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, Waltham, MA) (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 calculate fold-over-control (FOC) values for experimental PPIs ($FOC=Signal_{PPI}/Signal_{control}$) (Li et al., 2017).

3.2. NanoLuc-based protein-fragment complementation (nanoPCA)

NanoPCA detects binary PPIs in live, intact cells by splitting a reporter enzyme into two halves, each of which is then genetically fused to one of two proteins of interest. Upon protein-protein binding, the two halves of the split enzyme and brought into close proximity, allowing for the reconstitution of the full-length reporter enzyme (Michnick et al., 2000). The nanoLuc luciferase (NLuc) luminescent protein was selected due to its stability, bright signal, and small size (Mo et al., 2017). Here, the nanoPCA assay was performed as described previously (Mo et al., 2017). Briefly, HeLa and MCF7 cells were seeded in 384-well white plates (Corning, Cat# 3570) at 4,000 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 at a ratio of 3 uL reagent:1 ug DNA (Polysciences, Warrington, PA, Cat# 23966) 24 hours after plating. NanoPCA signals were measured 48 hours after transfection with an Envision Multilabel Plate Reader (PerkinElmer) immediately after Nano-Glo (Promega, Madison, WI) substrate had been added to cells.

3.3. Glutathione-S-transferase (GST) pulldown

GST pulldown assays utilize the interaction between GST and its ligand, glutathione (GSH). In this assay, a protein of interest is genetically fused with a

GST tag and is isolated from cell lysate by affinity-based capture to GSH-conjugated beads. Unbound proteins are washed away and discarded, while the GST-tagged protein and its binding partners remain bound to the beads and are purified. For our experiments, HEK293T cells were lysed in 1% NP-40 lysis buffer 48 hours after transfection. Lysate was incubated with Glutathione-Sepharose 4B Beads (bioWORLD, Dublin, OH, Cat# 20182003) for 3 hours at 4°C. The GST-protein complexes were washed 3 times in 1% NP-40 lysis buffer, and bound proteins were eluted in 2X SDS loading buffer. Samples were boiled and resolved on a 10% SDS-PAGE gel for western blot analysis.

3.4. Endogenous co-immunoprecipitation (coIP)

coIP experiments work on a similar principle as GST pulldowns, however instead of using tagged proteins and affinity beads, target-specific antibodies are used to isolate endogenous protein complexes. In this assay, 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 centrifuged to pellet cellular debris, and the supernatant was aliquoted in equal volumes to new tubes containing antibodies specific for ASK1 (Santa Cruz Biotechnology, sc-7931), LATS2 (Novus Biologicals, NB200-199) or IgG (Santa Cruz Biotechnology, sc-2027). After rotating overnight at 4°C, Protein A/G Agarose Beads (Sigma-Aldrich, sc-2003) were added to samples and rotated for an additional 4 hours. Immunocomplexes bound to the beads were pelleted and rinsed 4 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 2X SDS loading buffer, resolved by SDS-PAGE, and transferred to nitrocellulose membranes for western blotting with LATS2 (Abcam, ab54073) and ASK1 (Santa Cruz Biotechnology, sc-5294) specific antibodies.

3.5. *in vitro* GST pulldown assay

To examine the interaction of two proteins *in vitro*, plasmids were expressed individually in HEK293T cells plated in separate wells. Cells were lysed 48 hours after transfection in 1% NP-40 lysis buffer. Lysate from different wells was mixed in equal volumes and rotated at 4°C for 2 hours to allow protein complexes to form. Samples were then incubated with Glutathione-Sepharose 4B Beads for an additional 2 hours, and GST pulldown assays were performed as previously described to isolate the GST-tagged protein complexes.

4. WESTERN BLOTTING

All samples were resolved SDS-PAGE gels and transferred to polyvinylidene difluoride (PVDF) membranes by wet transfer at 100 volts for 2.5 hours at 4°C. Membranes were then blocked in 5% milk (w/v in 1X TBST) for 1 hour prior to the addition of primary antibodies. Next, membranes were washed 3 times in 1X TBST and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 hour. After 3 additional washes, enhanced

chemiluminescent HRP substrate was used to detect protein bands (Thermo Fisher Scientific, Carlsbad, CA, Cat# 34580 and 34075).

4.1. Antibodies

Primary antibodies for GST (sc-459, 1:2500), ASK1 (sc-5294, 1:300), HA (sc-7392, 1:2500), and YAP1 (sc-15407) were purchased from Santa Cruz Biotechnology (Dallas, TX). Actin antibody (A5441, 1:300) was purchased from Sigma-Aldrich. LATS2 antibody (NB200-199) was purchased from Novus Biologicals (Littleton, CO). Phospho-S967 ASK1 (3764), phospho-T838 ASK1 (3765), 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 (Danvers, MA). Secondary antibodies were purchased from Santa Cruz Biotechnology (anti-mouse: sc-2005, anti-rabbit: sc-2005, 1:2500 diluted in 1x TBST). Unless otherwise states, all antibodies were used at a final dilution of 1:1000 in 5% milk.

5. ANNEXIN V ASSAY

HEK293T cells were seeded in 384-well black plates (Corning) at 4,000 cells in 40 µL cell culture media per well. Cells were transfected with the indicated plasmids using FuGENE HD Transfection Reagent at a final ratio of 3 uL reagent:1 ug plasmid DNA (Promega, Cat# E2311) 24 hours after seeding. At the same time as transfection, IncuCyte Annexin V Red Reagent (Essen BioScience, Ann Arbor, MI, Cat# 4641) was added to wells according to the manufacturer's protocol at a

final concentration of 1:1000. Plates were imaged at 12-hour intervals post-transfection using the IncuCyte S3 Live-Cell Analysis System (Essen BioScience) and annexin V staining was measured as red object confluence (%) per well. All readings were normalized to the corresponding value from the initial 12-hour time point to obtain fold increase in percent red object confluence per sample. Lastly, the normalized results from three independent replicate experiments were averaged to get final values.

6. STATISTICAL ANALYSIS

For all TR-FRET, nanoPCA, and annexin V data, a two-tailed unpaired Student's t-test was used to determine whether there were any statistically significant differences between groups (ASK1+LATS2 vs. ASK1+vector; ASK1+LATS2 vs. vector+LATS2; and ASK1+vector vs. vector+LATS2). Statistical significance was defined as a p-value less than or equal to 0.05 (n.s.: $0.05<p$, *:$0.01<p<0.05$, **:$0.001<p<0.01$, ***$p<0.0001$).

Chapter 3

Large tumor suppressor 2 activates JNK in a kinase-independent mechanism through apoptosis signal-regulating kinase 1

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1. INTRODUCTION

Apoptosis signal-regulating kinase 1 (ASK1) is a mitogen-activated protein kinase kinase kinase (MAP3K) which 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 which 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 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 too much or too little ASK1 signaling can impact disease progression and treatment. ASK1 is pathogenic in many neurodegenerative disorders, such as Alzheimer's 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). 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 down-regulated 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., 2010b; 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 which may lead to new strategies to control cellular response to stress in normal cells and in diseases.

2. RESULTS

2.1. 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 could help provide a more detailed understanding of the 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, the TR-FRET assay was extended using a multipoint titration experiment in a cell lysate system that co-expressed ASK1 and LATS2 (Li et al., 2017). Results showed a significantly greater FRET signal for the ASK1/LATS2 PPI than for the negative controls, with a fold-over-control (FOC) of 5.8 (Figure 3-1A). Another proximity-based assay, NanoLuc-based protein-fragment complementation (NanoPCA), was used to detect PPIs between over-expressed 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, 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 (FOC=10.2 and 15.0 in HeLa and MCF7 cells, respectively) (Figure 3-1B).

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 methods. GST pulldown results showed HA-tagged ASK1 was present only in the GST-LATS2 protein complex (Figure 3-1C). 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 3-1D). Similar results were also obtained from LATS2 co-immunoprecipitation experiments (Figure 3-1E). 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.

2.2. 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 over-expression 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 expression of ASK1 alone (Figure 3-2A). 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).

Because there are multiple upstream MAP3K proteins which 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 wild-type ASK1 ($ASK1^{WT}$), 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 3-2A, as well as an increase in phospho-MKK4, which is a direct substrate for ASK1 and the upstream kinase for JNK phosphorylation (Ichijo et al., 1997). Importantly, expression of LATS2 with the catalytically inactive $ASK1^{KR}$ failed to induce phosphorylation of ASK1 effectors (Figure 3-2B).

Similar downstream analysis testing the effect of ASK1 on LATS2 kinase activity showed ASK1 having no effect on YAP1 phosphorylation (Ser127). A kinase-dead mutant of LATS2 ($LATS2^{KR}$) was used as a positive control for YAP1 phosphorylation, which was unchanged when co-expressed with $ASK1^{WT}$, $ASK1^{KR}$, or the pcDNA vector (Figure 3-3) (Xiao et al., 2011). Together these results suggest LATS2 acts upstream of ASK1 to promote signaling and JNK activation.

2.3. 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; Takeda et al., 2008; Hattori et al., 2009; Iriyama et al., 2009; Hayakawa et al., 2010; Hayakawa et al., 2011). This effect has been observed in multiple cell lines where over-expression of either wild-type or a constitutively active mutant 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 apoptosis when over-expressed (Ke et al., 2004; Suzuki et al., 2013). To determine whether co-expression of LATS2 with ASK1 could increase apoptosis, 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 apoptotic cells. Significantly more apoptosis, as measured by fold increase in percent annexin V area, was observed in HEK293T cells transfected with both Venus-flag-LATS2 and HA-ASK1, compared to expression of either protein alone at both 24 and 36-hours post-transfection (Figure 3-4). Furthermore, the fold increase in annexin V seen in the co-expression group was greater than the sum of the single expression groups at the 36-hour timepoint, suggesting LATS2 and ASK1 may have a synergistic effect on cell death.

2.4. LATS2 interacts and activates ASK1 in a kinase-independent manner

LATS2 is a Ser/Thr kinase which 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 utilized the LATS2^{KR} mutant that was generated 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 both gave positive TR-FRET signals (FOC=6.0 and 5.3, respectively) that were significantly different from samples expressing either protein alone (Figure 3-5A). Similar binding data was also achieved by GST pulldown with both $ASK1^{WT}$ and $ASK1^{KR}$ (Figure 3-5B). These data suggest that the kinase activity of neither LATS2 or ASK1 is required 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 3-5C). Collectively these findings show LATS2 acting as a positive regulator of ASK1-mediated signaling independently of its catalytic activity.

2.5. 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) motifs 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 3-6A) (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 3-6B). The KC fragment of ASK1 contains a stretch of amino acids from 937-1374 that are not shared with the N or NK truncations, suggesting this region of ASK1 may contain the LATS2 binding site.

2.6. 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 3-6B). This region contains and is adjacent to two phosphorylation sites that are known to regulate ASK1 activity. Phosphorylation at threonine 838 (T838) in the kinase activation loop is an activating PTM which occurs upon ASK1 dimer formation and auto-transphosphorylation (Gotoh and Cooper, 1998; Liu et al., 2000; Tobiume et al., 2002; Noguchi et al., 2005; Weijman et al., 2017). Conversely, phosphorylation at serine 967 (S967) is an inhibitory PTM which creates a recognition motif for 14-3-3 proteins which block ASK1 kinase activity upon binding (Figure 3-7A) (Fujii et al., 2004; Goldman et al., 2004; Seong et al., 2010). Importantly, phosphorylation at these sites can be impacted by ASK1 mutants, which can help serve as experimental controls and validate the specificity of different phospho-antibodies. Cells transfected with $ASK1^{KR}$ have reduced phosphorylation at T838, because this kinase-dead construct of ASK1 lacks the

kinase activity needed for auto-transphosphorylation. A different mutant of ASK1 containing a point mutation converting serine 967 to alanine ($ASK1^{SA}$) shows no phosphorylation at S967 when expressed in cells.

When GST-LATS2 or GST vector were co-expressed with $ASK1^{WT}$, $ASK1^{KR}$, or $ASK1^{SA}$, we saw no changes in phosphorylation at T838. However, LATS2 caused a dramatic decrease in phosphorylation at S967 in both $ASK1^{WT}$ and $ASK1^{KR}$-expressing samples (Figure 3-7B). Furthermore, the kinase activity of LATS2 is not required for this effect, as kinase-dead LATS2 decreased S967 phosphorylation to the same degree as wild-type (Figure 3-7C). Our data support a model where LATS2 activates ASK1 at least in part by reducing the negative phosphorylation of ASK1 at S967, leading to subsequent activation of JNK.

3. DISCUSSION

Here we report LATS2 as a novel binding partner and positive regulator of ASK1, a stress-responsive signaling protein which 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 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 S967 could be an important residue for LATS2-mediated effects on ASK1 activity.

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). 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 not well understood, and there are reports of it acting as an oncogene or a tumor suppressor under different cellular contexts (Iriyama et al., 2009; Meurette et al., 2009; Hayakawa et al., 2011; Nakagawa et al., 2011; Hayakawa et al., 2012b; 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; Hayakawa et al., 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.

Figure 3-1: Interaction between LATS2 and ASK1.

A) TR-FRET titration assay performed in HEK293T lysate that had been co-transfected with experimental and control expression plasmids. GST-terbium served as the FRET donor and Venus protein as the acceptor. Results shown are average FRET signals and standard deviations (SD) from three independent replicates (***$p<0.0001$). B) NanoPCA assays were performed in live HeLa and MCF7 cells. Results shown are average NanoPCA signals and SD values from three independent replicates (*:$0.01<p<0.05$, **:$0.001<p<0.01$, ***$p<0.0001$). C) 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. D and E) Endogenous immunoprecipitation of ASK1 (D) or LATS2 (E) was performed in HEK293T and HeLa and cells. Western blotting was used to detect proteins in the isolated immunocomplexes.

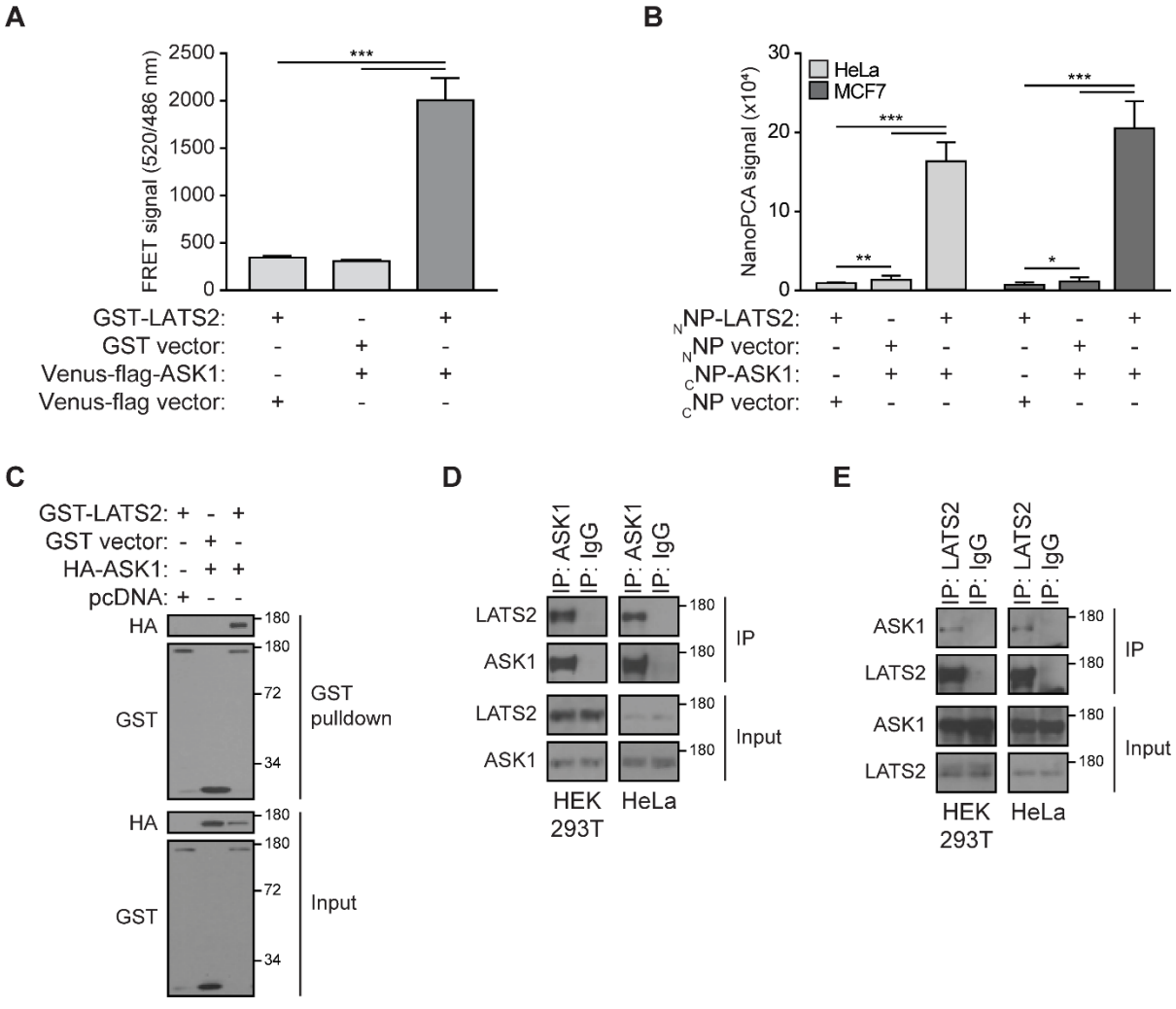

A
FRET signal (520/486 nm)
2500
2000
1500
1000
500
0
***
GST-LATS2: + - +
GST vector: - + -
Venus-flag-ASK1: - + +
Venus-flag vector: + - -
B
NanoPCA signal (x10⁴)
30
20
10
0
HeLa
MCF7
**
***
*
***
NNP-LATS2: + - + + - +
NNP vector: - + - - + -
CNP-ASK1: - + + - + +
CNP vector: + - - + - -
C
GST-LATS2: + - +
GST vector: - + -
HA-ASK1: - + +
pcDNA: + - -
HA
GST
180
72
34
GST pulldown
Input
D
IP: ASK1
IP: IgG
LATS2
ASK1
IP
Input
HEK 293T
HeLa
E
IP: LATS2
IP: IgG
ASK1
LATS2
IP
Input
HEK 293T
HeLa

Figure 3-2: 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) 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.

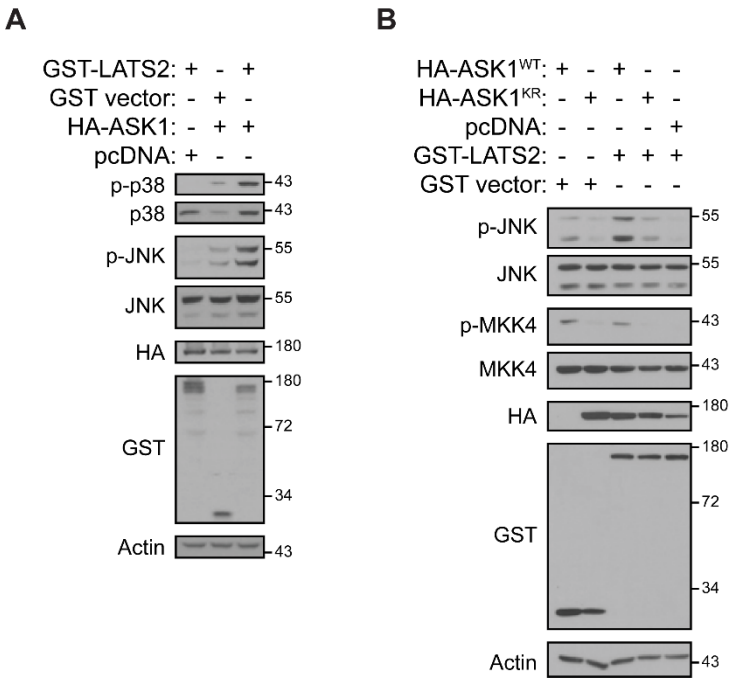

A
GST-LATS2: + - +
GST vector: - + -
HA-ASK1: - + +
pcDNA: + - -
p-p38
p38
p-JNK
JNK
HA
GST
Actin
43
43
55
55
180
180
72
34
43
B
HA-ASK1WT: + - + - -
HA-ASK1KR: - + - + -
pcDNA: - - - - +
GST-LATS2: - - + + +
GST vector: + + - - -
p-JNK
JNK
p-MKK4
MKK4
HA
GST
Actin
55
55
43
43
180
180
72
34
43

Figure 3-3: ASK1 does not impact YAP1 phosphorylation.

GST-tagged LATS2^{WT}, LATS2^{KR}, or vector was co-expressed with HA-tagged ASK1^{WT}, ASK1^{KR}, or pcDNA vector in HEK293T cells. Western blot analysis was performed on the resulting cell lysates to measure phospho- and total protein levels.

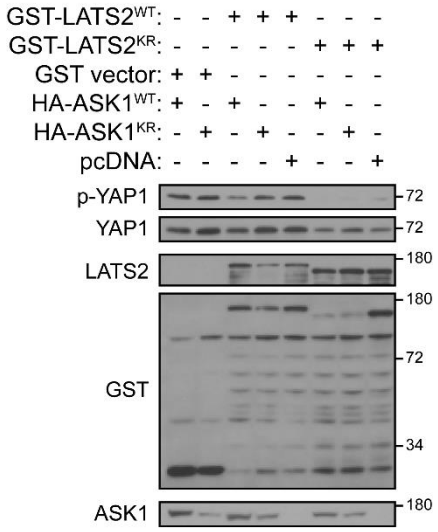

GST-LATS2WT: - - + + + - - -
GST-LATS2KR: - - - - - + + +
GST vector: + + - - - - - -
HA-ASK1WT: + - + - - + - -
HA-ASK1KR: - + - + - - + -
pcDNA: - - - - + - - +
p-YAP1
YAP1
LATS2
GST
ASK1
72
72
180
180
72
34
180

Figure 3-4: 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 percent (%) annexin V area at 24 and 36 hours from three independent experiments and the SD values (*:0.01<p<0.05, **:0.001<p<0.01, ***p<0.0001).

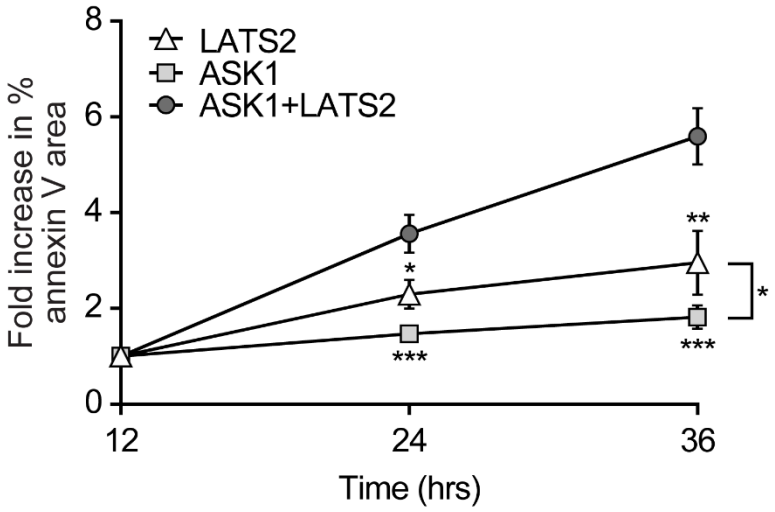
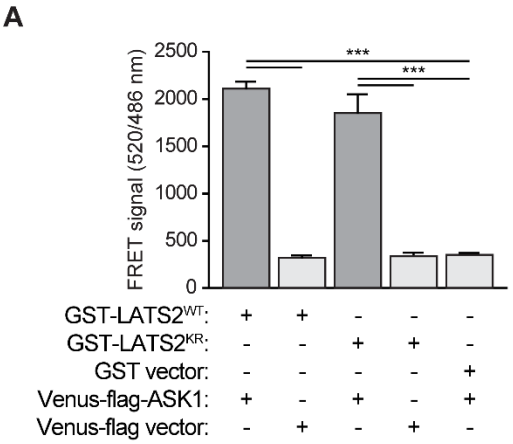
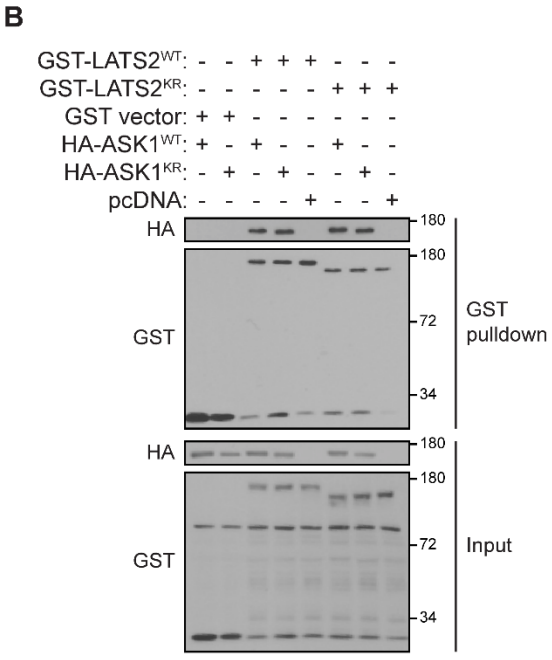

LATS2
ASK1
ASK1+LATS2
Fold increase in % annexin V area
8
6
4
2
0
12
24
36
Time (hrs)
*
**
***
***
*

Figure 3-5: 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 (***$p<0.0001$). B) GST pulldown with $LATS2^{WT}$ or $LATS2^{KR}$, and HA-ASK1 in HEK293T cells. C) Lysate from HEK293T expressing HA-ASK1 with GST-$LATS2^{WT}$ or GST-$LATS2^{KR}$ was blotted for status of JNK.


A
FRET signal (520/486 nm)
2500
2000
1500
1000
500
0
***
***
GST-LATS2WT: + + - - -
GST-LATS2KR: - - + + -
GST vector: - - - - +
Venus-flag-ASK1: + - + - +
Venus-flag vector: - + - + -


B
GST-LATS2WT: - - + + + - - -
GST-LATS2KR: - - - - - + + +
GST vector: + + - - - - - -
HA-ASK1WT: + - + - - + - -
HA-ASK1KR: - + - + - - + -
pcDNA: - - - - + - - +
HA
GST
180
180
72
34
GST pulldown
HA
GST
180
180
72
34
Input

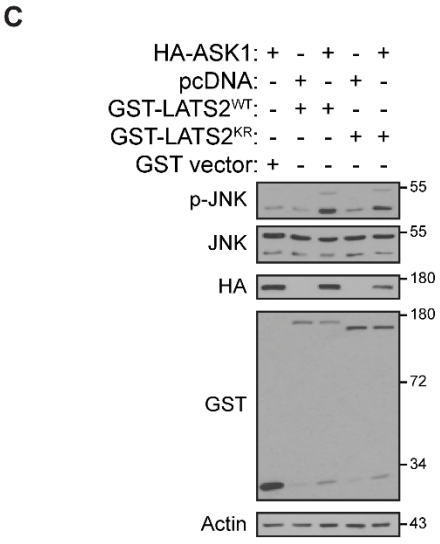

C
HA-ASK1: + - + - +
pcDNA: - + - + -
GST-LATS2WT: - + + - -
GST-LATS2KR: - - - + +
GST vector: + - - - -
p-JNK
JNK
HA
GST
Actin
55
55
180
180
72
34
43

Figure 3-6: LATS2 interacts with the C-terminal region of ASK1.

A) Schematic of ASK1 protein domains (light grey: coiled-coil motif, dark grey: 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.

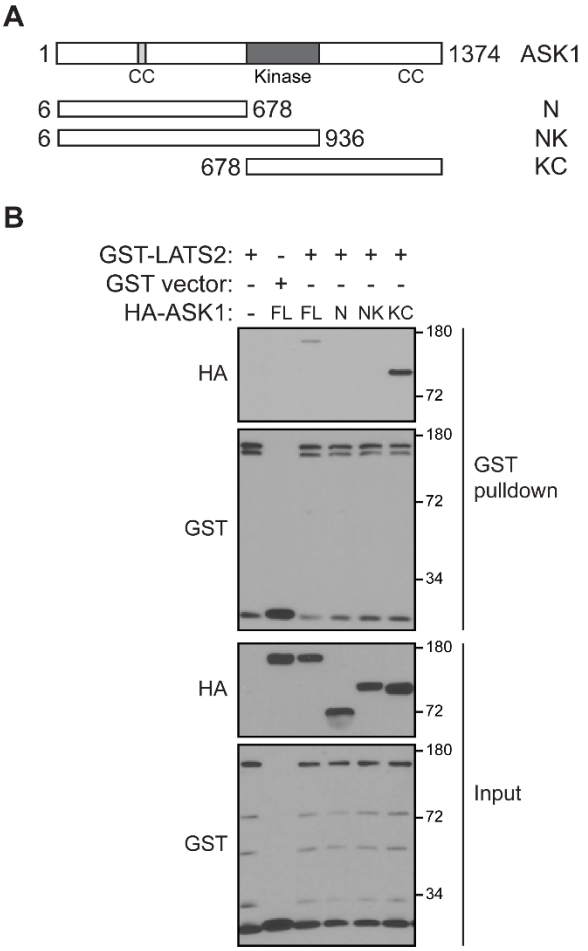
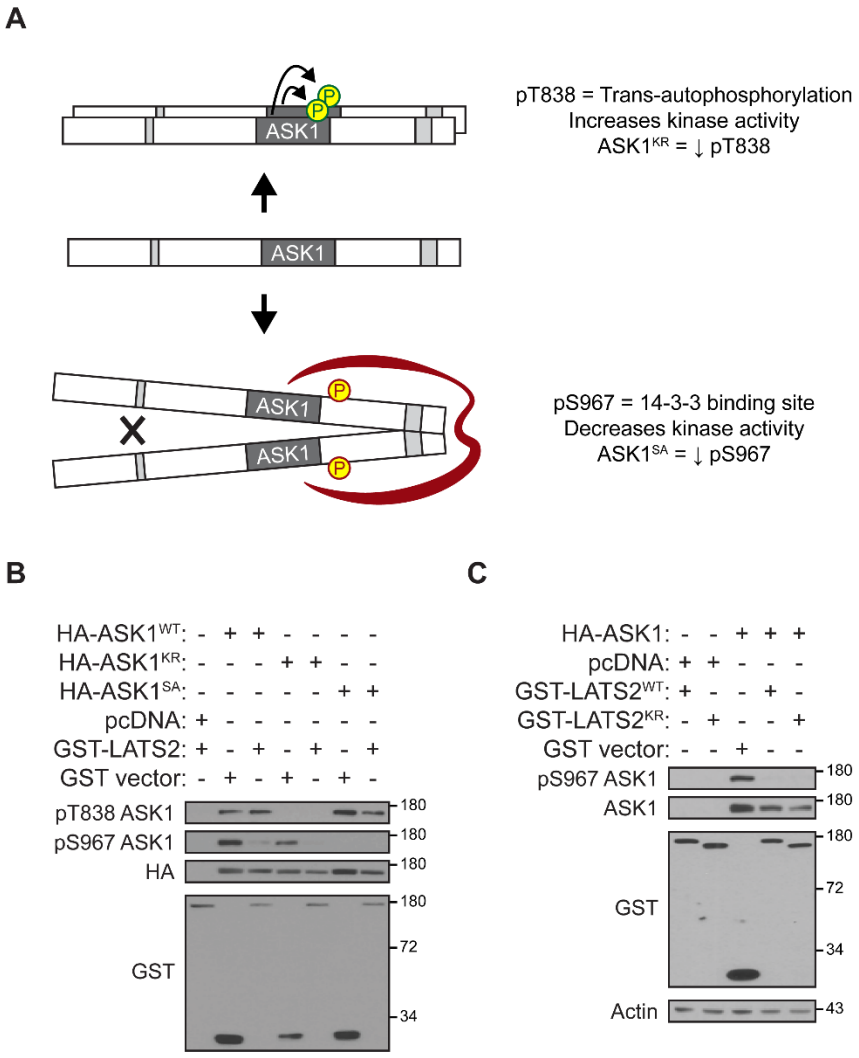

A
1
1374
ASK1
CC
Kinase
CC
6
678
N
6
936
NK
678
KC
B
GST-LATS2: + - + + + +
GST vector: - + - - - -
HA-ASK1: - FL FL N NK KC
HA
GST
180
72
34
GST pulldown
Input

Figure 3-7: LATS2 decreases phosphorylation of ASK1 at serine 967.

A) Representation of ASK1 phospho-sites at threonine 383 (T838) and serine 967 (S967). B) ASK1 phosphorylation at T838 and S967 was detected in cell lysates from HEK293T cells co-expressing GST-LATS2 or GST vector with either HA-tagged $ASK1^{WT}$, $ASK1^{KR}$ or $ASK1^{SA}$. C) HA-ASK1 was expressed with GST-$LATS2^{WT}$ or GST-$LATS2^{KR}$, and phospho-S967 ASK1 levels were measured by western blot analysis.


A
ASK1
pT838 = Trans-autophosphorylation
Increases kinase activity
ASK1KR = ↓ pT838
pS967 = 14-3-3 binding site
Decreases kinase activity
ASK1SA = ↓ pS967
B
HA-ASK1WT: - + + - - - -
HA-ASK1KR: - - - + + - -
HA-ASK1SA: - - - - - + +
pcDNA: + - - - - - -
GST-LATS2: + - + - + - +
GST vector: - + - + - + -
pT838 ASK1
pS967 ASK1
HA
GST
180
72
34
C
HA-ASK1: - - + + +
pcDNA: + + - - -
GST-LATS2WT: + - - + -
GST-LATS2KR: - + - - +
GST vector: - - + - -
pS967 ASK1
ASK1
GST
Actin
180
72
34
43

Chapter 4

Conclusions and future directions

Parts of this chapter has been published: Rusnak L and Fu H. Regulation of ASK1 signaling by scaffold and adaptor proteins. Advances in Biological Regulation, 2017, 66:23-30

1. CONCLUSIONS

ASK1 is an important mediator of the cell stress response. This kinase becomes activated under stress conditions, such as oxidative stress and ER stress. Once activated, ASK1 signals through the MAPK pathway to activate p38 and JNK to induce apoptosis. As such a potent pro-apoptotic signaling protein in the cell, the activity of ASK1 is tightly regulated. One of the main ways in which this control is achieved is through the formation of the ASK1 signalosome, which is a large molecular weight complex composed of ASK1 and various binding partners which either promote or inhibit downstream signaling. The composition of the signalosome and the cell stress level all control whether ASK1 is in an inactive or active state. Identifying binding partners which regulate ASK1 activity and how they impact ASK1 signaling will provide more insight into how the complex regulation of ASK1 is achieved.

Here we describe the discovery of LATS2 as a novel activator of ASK1 signaling. Importantly, ASK1-mediated apoptosis is known to have a therapeutic role in many cancer treatments, but a disease-promoting effect in many neurodegenerative disorders (see chapter 1). Future directions of this project will focus on further characterizing the mechanism by which LATS2 activates ASK1 and explore how this PPI impacts disease physiology.

1.2. Remaining questions

1.2a. Mechanism

We performed many protein-protein interaction assays, and while these provide strong evidence of the LATS2/ASK1 interaction, they do not definitively show direct binding between these two proteins. Additional binding assays such as surface plasmon resonance (SPR), bio-layer interferometry (BLI), or binding assays with purified proteins could be used to determine whether LATS2 and ASK1 interact directly.

Additionally, LATS2 was found to decrease the inhibitory phosphorylation of ASK1 at S967 in a kinase-independent mechanism. However, it is unclear how LATS2 mediates this effect. It is possible that LATS2 achieves reduction of S967 phosphorylation by altering the composition of the ASK1 signalosome. For example, another binding partner of ASK1, ASK1-interacting protein 1 (AIP1) has been shown to reduce phosphorylation of ASK1 at S967 in a similar enzyme-independent manner. AIP1 is a Ras-GTPase-activating protein (Ras-GAP). It activates ASK1 in response to TNF-α treatment by recruiting protein phosphatase (PP)2A to the signalosome to dephosphorylate ASK1 at S967, thereby blocking 14-3-3 binding (Min et al., 2008; Zhang et al., 2003). Conversely, LATS2 could be inhibiting the upstream phosphorylation of ASK1 at S967 by IKKβ or PDK1. LATS2 has been reported to directly and indirectly interact with both IKKβ and PDK1, respectively (Fan et al., 2013; Yao et al., 2015). By altering the components of the ASK1 signalosome, either by recruiting phosphatases or blocking kinases, LATS2 could directly impact phosphorylation at S967 independently of its kinase activity.

1.2b. Function

Our results showed the co-expression of LATS2 and ASK1 could increase cell death. However, our experiments were performed under normal cell culture conditions, while ASK1 is activated to enhance pro-apoptotic signaling in response to cell stress (Hattori et al., 2009; Shiizaki et al., 2013). It would be interesting to perform similar experiments under various stress conditions, such as elevated ROS levels, to see if the effect of LATS2 on ASK1 was altered. Additionally, because ASK1 activation has also been shown to promote proliferation and inflammation, we would like to test whether LATS2 impacts the effect of ASK1 on these other cellular processes.

The exact binding interface of the ASK1-LATS2 PPI has yet to be defined, as well as the physiological effect caused by disrupting this interaction on the MAPK and Hippo pathways. Further characterizing the PPI interface and generating a specific disruption tool, such as a peptide mimetic or small molecule disrupter, would allow us to further asses the significance of this interaction on normal and disease physiology. We narrowed the LATS2 binding region to amino acids 937-1374 on ASK1. However, we have not determined where ASK1 interacts with LATS2. Preliminary data suggests that unlike LATS2, LATS1 does not interact with ASK1 (data not shown). While LATS1/2 have highly conserved kinase domains, their N-terminal regions are much different and contain domains that are not found in their homolog (Visser and Yang, 2010). It is likely that a unique region in the N-terminal of LATS2 is responsible for mediating binding to ASK1. Developing truncations of LATS2 would allow us to test this hypothesis and find the minimal binding region on LATS2 required for association with ASK1. This

fragment of LATS2 could then be used in functional studies to compete away the full-length binding between ASK1 and LATS2, enabling us to assay changes in signaling and cell behavior when the PPI is formed and disrupted. Additionally, because we have already optimized both the TR-FRET and nanoPCA assays for detection of the ASK1-LATS2 PPI, we could perform a high-throughput screen to identify a small molecule inhibitor of this PPI that would work similarly to the peptide fragment.

These disruption tools would allow us to determine the importance of LATS2 as a regulator of ASK1. PPI modulators have shown promise as therapeutics for the treatment of many diseases, some of which are impacted by ASK1 signaling. Future studies aimed at the ASK1-LATS2 connectivity would be expected to lead to the elucidation of the intertwined stress signaling network for enhanced understanding of growth regulatory signal transduction and for potential therapeutic strategies to target ASK1- and LATS2-mediated human diseases.

2. FUTURE APPLICATIONS

Our results show LATS2 and ASK1 can synergize to promote apoptosis. We have detected LATS2 as a novel activator of ASK1-mediated signaling that is capable of inducing apoptosis. Targeting this PPI could provide therapeutic benefits to patients suffering from diseases where ASK1 serves a pathogenic role (Table 1.1).

2.1. PPIs as drug targets

PPI interfaces are generally more difficult to target than catalytic pockets, due to their large, flat, and uncharged nature (Arkin and Wells, 2004). However, many different PPI modulating agents are in clinical trials for the treatment of cancer, some of which are discussed further below (Arkin et al., 2014; Ivanov et al., 2013).

The murine double minute (MDM) proteins, MDM2 and MDMX, are the negative regulators of the p53 tumor suppressor protein. Amplification of MDM genes and mutations in p53 are often mutually exclusive alterations in cancer, both leading to loss of p53 tumor suppressor signaling. In 2004, the first MDM2 antagonists (Nultin-1, Nultin-2, and Nultin-3) were discovered, which function by occupying the same hydrophobic pocket that is responsible for p53 binding (Joseph et al., 2010; Vassilev et al., 2004). Treatment with these compounds led to p53 accumulation and stabilization, with anti-cancer effects on human tumor xenografts in mice (Vassilev et al., 2004). Since 2004, further drug development has led to multiple MDM antagonists that are currently in phase I and II clinical trials (Burgess et al., 2016; Tisato et al., 2017).

BET inhibitors are a class of PPI disrupters that are in phase I and II clinical trials for the treatment of various cancers. These drugs target bromodomain and extra-terminal motif (BET) proteins which play role in many cancers by promoting the expression of oncogenes, such as Myc (Delmore et al., 2011). BET inhibitors work by interacting with the bromodomains responsible for binding acetylated lysine residues on histones. Use of these drugs can displace BET proteins from

chromatin, resulting in decreased proliferation and tumor growth (Filippakopoulos et al., 2010; Junwei and Vakoc, 2014).

In 2016, an intracellular PPI disruptor achieved FDA approval for the treatment of chronic lymphocytic leukemia. Venetoclax (also known as ABT-199) is a BH3 mimetic which occupies the same hydrophobic pocket in Bcl-2 family members that is responsible for binding BH3 only proteins. Importantly, Venetoclax is selective for Bcl-2 binding and does not inhibit Bcl-xL or Mcl-1. This allows the drug to avoid the thrombocytopenia caused by earlier versions of BH3 mimetics that were not selective (navitoclax and ABT-737) (Souers et al., 2013).

2.2. Targeting ASK1 binding partners as a therapeutic strategy

While GS-4997 has shown promise in treating NASH, this drug interacts with the ASK1 kinase domain which is a conserved structure found in many other protein kinases as well (Bunkoczi et al., 2007). Because of this, it is easy to get non-specific binding to other proteins in the cell which contain a homologous region, and potentially off-target side effects (Breen and Soellner, 2015). Another way to target ASK1 activity is through modulation of PPIs known to regulate its activity and downstream signaling, as described below (Figure 4-1).

2.2a. Daxx

As previous discussed in chapter 1, Daxx is a positive regulator of ASK1 signaling through promoting ASK1 dimerization, which ultimately can lead to apoptosis. ASK1 signaling also creates a positive feedback loop where JNK drives

the nuclear export of Daxx, allowing it to further activate ASK1 activity (Niu et al., 2011). Importantly, the cytoplasmic translocation of Daxx is significant to human disease. PD is the second most common neurodegenerative disease and is characterized by the loss of dopaminergic neurons in the substantia nigra pars compacta (Schulte and Gasser, 2011). While most cases of PD are sporadic, roughly 15% of cases occur in individuals with a previous family history of the disease. Inherited mutations in multiple genes have been linked to hereditary PD, one of which is the L166P point mutation in protein/nucleic acid deglycase DJ-1 (DJ-1) (Ariga et al., 2013; Bonifati et al., 2003; Olzmann et al., 2004). DJ-1 binds to Daxx and retains it in the nucleus, thereby preventing Daxx nuclear export to the cytosol where it can interact and activate ASK1. Additionally, the DJ-1 L166P mutant has impaired ability to bind and retain Daxx in the nucleus and results in increased activation of ASK1 and cell death (Junn et al., 2005). In cell culture experiments with SH-SY5Y dopaminergic neuroblastoma cells, over-expression of wild-type DJ-1 was sufficient to block Daxx cytosolic trafficking and inhibit ASK1 activation, ultimately blocking cell death (Junn et al., 2005) (Figure 4-1A).

Additionally, oxidative stress is a major contributor to the degeneration and cell death of dopaminergic neurons in PD, and ROS-dependent activation of ASK1 has been found to play a role in the pathogenesis of this disorder (Dias et al., 2013; Lee et al., 2012; Sturchler et al., 2011; Yoritaka et al., 1996; Zhang and Zhang, 2002). In animal models of PD, anti-oxidant treatment could increase levels of DJ-1 and block the cytosolic translocation of Daxx, thereby decreasing ASK1 signaling

and attenuating disease-causing cell death (Junn et al., 2005; Karunakaran et al., 2007).

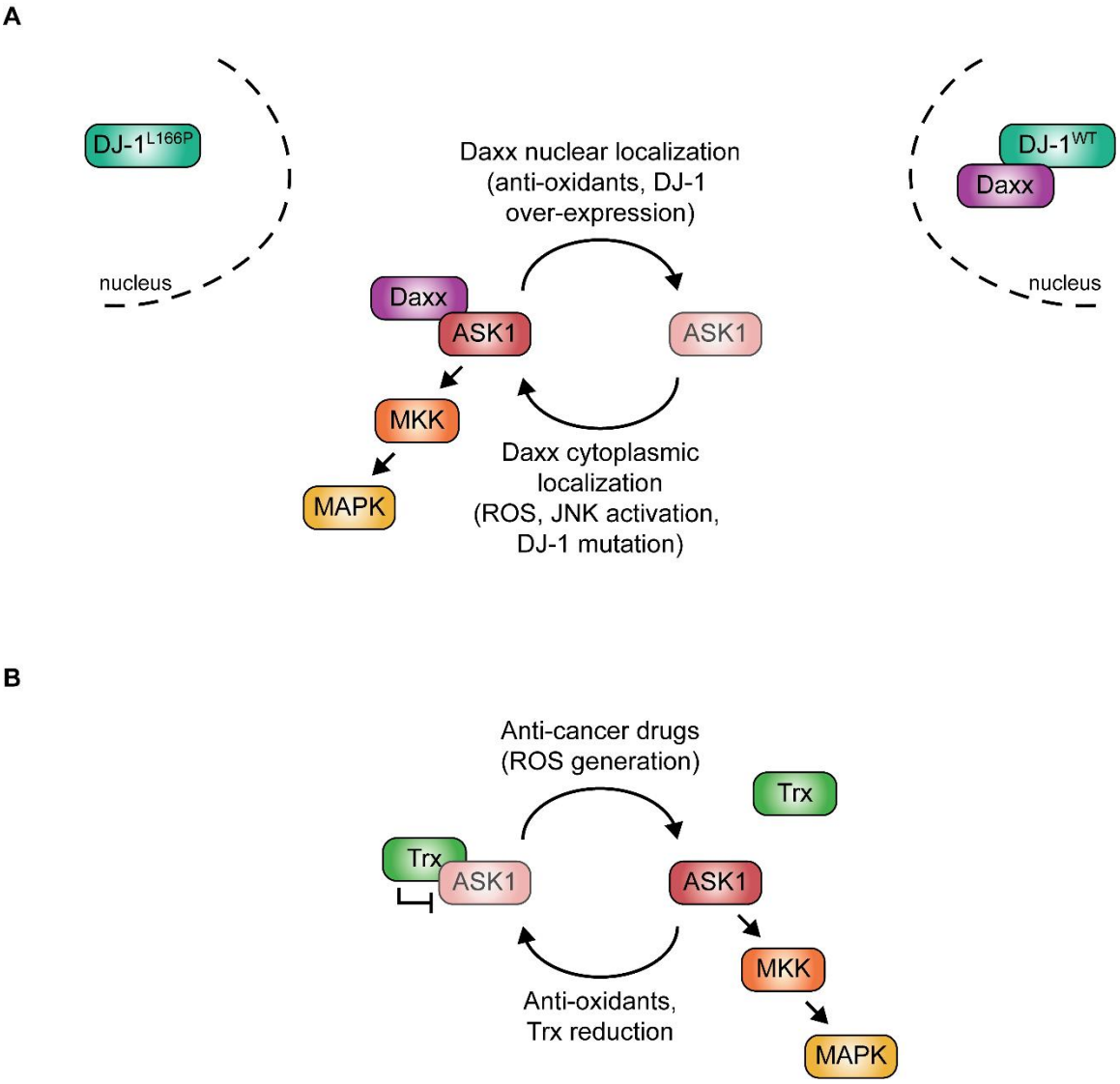
2.2b. Thioredoxin

Trx is one of the most well-characterized binding partners of ASK1 (see chapter 1). As a redox-sensitive protein, its interaction with ASK1 is regulated by intracellular ROS levels. Many cancers have elevated ROS levels which are critical in promoting tumor development by generating DNA damage that facilitates mutations and oncogenic transformation (Waris and Ahsan, 2006; Liou and Storz, 2010). This heightened ROS would trigger apoptosis in normal cells, but cancer cells accumulate alterations which allow them to continue to survive and proliferate under these conditions, such as upregulating Trx (Powis et al., 1995; Karlenius and Tonissen, 2010). Because of this, Trx can bind ASK1 and inhibit downstream apoptosis signaling. Many anti-cancer drugs can further increase cancer ROS levels to a toxic threshold and can disrupt the Trx-ASK1 interaction to trigger cell death. For example, treatment with 1,2-dipalmitoleoyl-sn-glycero-3-phosphoethanolamine (DPPE) in malignant pleural mesothelioma cells can increase intracellular ROS levels to inhibit Trx and induce cell death (Kaku et al., 2014; Kaku et al., 2015; Tsuchiya et al., 2015). Additionally, DPPE can activate the phosphatase activity of PP2A, the enzyme responsible for removing the inhibitory S967 phosphorylation from ASK1 (Kaku et al., 2014). This cytotoxic effect of DPPE is, at least in part, attributable to ASK1-p38 signaling, as knockdown of ASK1 prevented p38 activation and cell death. Many other

compounds such as isoobtusilactone A (IOA) and capsaicin act in a similar mechanism to increase ROS levels and free ASK1 from Trx inhibition to kill breast and pancreatic cancer cells lines, respectively (Kuo et al., 2007; Pramanik and Srivastava, 2012) (Figure 4-1B).

Figure 4-1: ASK1 PPIs are therapeutic targets.

A) Blocking the interaction between Daxx and ASK1 can decrease cell death in cell culture and animal models of Parkinson's disease. B) Many anti-cancer drugs increase cellular ROS levels, causing the dissociation of Trx from ASK1 and subsequent pro-apoptotic signaling.


A
DJ-1L166P
nucleus
Daxx nuclear localization
(anti-oxidants, DJ-1
over-expression)
Daxx
ASK1
ASK1
MKK
MAPK
Daxx cytoplasmic
localization
(ROS, JNK activation,
DJ-1 mutation)
DJ-1WT
Daxx
nucleus
B
Anti-cancer drugs
(ROS generation)
Trx
Trx
ASK1
ASK1
MKK
MAPK
Anti-oxidants,
Trx reduction

3. CLOSING REMARKS

As a strong inducer of apoptosis, ASK1 activity is intimately linked to human health, and alterations in ASK1-mediated signaling can directly promote or inhibit disease progression. Our work characterizing LATS2 as a novel positive regulator of ASK1-MKK-JNK signaling not only provides a more complete picture of the factors controlling ASK1 activity, but also could have clinical relevance in cancer patients who have decreased expression of LATS2. These patients could have impaired ASK1 signaling, and subsequently have tumors that are more resistant to cell death. additionally, targeting the interaction between LATS2 and ASK1 could have therapeutic benefits in gastric cancer and neurodegenerative diseases where ASK1 signaling promotes disease pathogenesis.

Chapter 5

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