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Identifying Novel Regulators and Regulation of the Hippo-YAP Pathway

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by

Audrey Wei-Jin Hong

Committee in charge:

       Professor Kun-Liang Guan, Chair
       Professor Jorge Silvio Gutkind, Co-Chair
       Professor Tony Hunter
       Professor Bing Ren
       Professor Jin Zhang

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DEDICATION

This dissertation is dedicated to my grandma and family. They bring me love and joy.
They are my strongest support.

EPIGRAPH

The meaning of life is to create and sustain subsequent lives in the universe. The purpose of life is to improve the general life of humanity.

Chiang Kai-shek

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2008-2012	Bachelor of Arts, University of California Berkeley
2012-2013	Research Assistant, IBMS Academia Sinica, Taiwan
2013-2019	Graduate Student, University of California San Diego
2019	Doctor of Philosophy, University of California San Diego

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FIELDS OF STUDY

Major Field: Biomedical Sciences

      Studies in Pharmacology
      Professor Kun-Liang Guan

ABSTRACT OF THE DISSERTATION

Identifying Novel Regulators and Regulations of the Hippo Pathway

by

Audrey Wei-Jin Hong

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Professor Kun-Liang Guan, Chair

Professor Jorge Silvio Gutkind, Co-Chair

The recent TCGA study identified the Hippo pathway as one of the nine major signaling pathways that are frequently altered in human cancer. The Hippo pathway is a master regulator of cell proliferation, cell death, and cell differentiation. Its dysregulation has been implicated intensively in human diseases especially cancer. Exploring the regulatory network of the Hippo pathway answers basic biological questions such as

how organ size is controlled during development as well as contributes to cancer target therapy. My research focuses on elucidating how Hippo pathway is regulated by cellular stress, more specifically osmotic stress. Physiological effect of the Hippo pathway is carried through transcription co-activator YAP. We found that osmotic stress induces YAP nuclear translocation even though it is phosphorylated at Ser 127 site, where its phosphorylation has been implicated to cause YAP cytoplasmic retention. We identified a novel phosphorylation site on YAP and a novel upstream regulator NLK that lead to YAP activation and subsequent cell proliferation. This finding alters the well-established dogma that YAP is inhibited by phosphorylation.

The mechanisms by which osmotic stress activated the Hippo pathway remained unclear. We discovered that NF2 lipid binding is essential for osmotic stress-induced activation of the Hippo pathway. We showed that osmotic stress induces ARF6 and PIP5K family interaction, leading to PIP5K activation and enhanced PI(4,5)P2 membrane distribution. Membrane-associated PI(4,5)P2 interacts with NF2, which further induces downstream Hippo pathway activation. This work completes the missing piece in the field on how NF2 is involved in Hippo pathway activation. Completion of these projects not only address fundamental questions in Hippo regulation but also provide valuable leads for therapeutic intervention for cancer treatment.

Chapter 1: Introduction of the Hippo pathway

1.1 Hippo pathway overview

The Hippo pathway is a highly conserved signaling cascade that plays an important role in organ growth and tissue homeostasis through regulation of cell proliferation, death, and differentiation (Barry and Camargo 2013; Piccolo et al. 2014; Varelas 2014). Dysregulation of the Hippo pathway is an important mechanism in tumorigenesis and other diseases (Harvey et al. 2013; Moroishi et al. 2015; Plouffe et al. 2015). For instance, high YAP activities have been reported in many human cancers. However, genetic mutations of Hippo pathway components are relatively rare in cancer (Harvey et al. 2013). Therefore, identification of upstream regulators has become an extensively studied subject in the field.

The Hippo pathway in Drosophila melanogaster

The Hippo pathway is mostly conserved between Drosophila melanogaster and mammals (Bossuyt et al. 2014; Meng et al. 2016); it was initially identified in Drosophila during screens for genes that negatively regulate tissue growth. Loss of the serine/threonine-protein kinase warts (Wts) resulted in organ overgrowth in Drosophila wing and eye (Justice et al. 1995; Xu et al. 1995). Similar genetic screens later identified that mutation of the Hippo kinase (Hpo) resulted in a similar tissue overgrowth phenotype (Harvey et al. 2003; Jia et al. 2003; Pantalacci et al. 2003; Udan et al. 2003; Wu et al. 2003). In addition, the adaptor proteins salvador (Sav) and MOB kinase

activator-like 1 (Mats) were found to be partners of Hpo and Wts, respectively, with similar mutation phenotypes (Kango-Singh et al. 2002; Tapon et al. 2002; Lai et al. 2005). Five proteins comprise the core of the Hippo pathway: the Hpo kinase and its binding partner Sav; the Wts kinase and its binding partner Mats; and the transcriptional co-activator yorkie (Yki). Yki mediates the main functional output of the Hippo pathway and binds to the transcription factor Scalloped (Sd), which induces the expression of genes that promote cell proliferation, for example cyclin E (CycE) and the bantam microRNA (miRNA), or genes that prevent apoptosis, for example death-associated inhibitor of apoptosis (Diap1) (Huang et al. 2005; Thompson and Cohen 2006). The Hpo–Sav complex phosphorylates and activates the Wts–Mats complex (Harvey et al. 2003; Pantalacci et al. 2003; Udan et al. 2003; Wu et al. 2003; Lai et al. 2005; Wei et al. 2007), which in turn phosphorylates and inactivates Yki by sequestering Yki in the cytoplasm, thereby negatively regulating Yki–Sd interaction and Sd‑mediated gene expression (Wu et al. 2008). Three studies published between 2014 and 2015 show that, in addition to Hpo, two kinases known as Happyhour (Hppy) and Misshapen (Msn) also activate the Wts–Mats complex (Li et al. 2014; Meng et al. 2015; Zheng et al. 2015). Overexpression of Yki or deletion of Hpo, Sav, Wts or Mats leads to overgrowth phenotypes in Drosophila eye, wing, midgut and other tissues as a result of increased cell proliferation and decreased apoptosis (Harvey et al. 2003; Pantalacci et al. 2003; Udan et al. 2003; Wu et al. 2003; Staley and Irvine 2010).

The Hippo pathway in mammals

The Hippo pathway in mammals consists of the core components mammalian

STE20‑like protein kinase 1 and 2 (MST1 and MST2, homologues of Hpo, also known

as serine/threonine-protein kinase 4 and 3, respectively), salvador homologue 1 (SAV1,

a homologue of Sav), large tumour suppressor 1 and 2 (LATS1 and LATS2,

homologues of Wts), MOB kinase activator 1A and 1B (MOB1A and MOB1B,

homologues of Mats), and the two Yki homologues Yes-associated protein 1 (YAP1,

also known as transcriptional co-activator YAP1) and transcriptional co‑activator with

PDZ-binding motif (TAZ, also known as WW domain-containing transcription regulator

protein 1, WWTR1) (Yu and Guan 2013). YAP1 and TAZ have overlapping functions

when they are expressed in the same cells (Lei et al. 2008), but also have divergent

functional roles in different organs as YAP1 and TAZ knockout mice show different

developmental deficiencies (Varelas 2014). MST1 and MST2 form homodimers via

C‑terminal Sav/RassF/Hpo (SARAH) domains, resulting in activation loop

autophosphorylation (Creasy et al. 1996; Glantschnig et al. 2002; Lee and Yonehara

2002; Hwang et al. 2007). MST1 and MST2 can phosphorylate LATS1 and LATS2 at

the hydrophobic motif to induce their activation. The scaffold proteins SAV1 and MOB1

are also phosphorylated and activated by MST1 and MST2; activated SAV1 and MOB1

promote LATS1 and LATS2 activity (Chan et al. 2005; Callus et al. 2006; Ni et al. 2015).

The Hppy homologues MAP4K1, MAP4K2, MAP4K3 and MAP4K5, and the Msn

homologues MAP4K4, MAP4K6 and MAP4K7 act in parallel with MST1 and MST2 to

phosphorylate the hydrophobic motifs of LATS1 and LATS2, thereby leading to their

activation (Meng et al. 2015; Zheng et al. 2015). Activated LATS1 and LATS2 in turn

phosphorylate YAP1 and TAZ, leading to the retention of YAP1 and TAZ in the

cytoplasm by 14‑3‑3 family proteins. Cytoplasmic YAP1 and TAZ can undergo further

phosphorylation and ubiquitylation-dependent degradation (Zhao et al. 2007; Zhao et al.

2010), preventing their interaction with the mammalian homologues of Sd,

transcriptional enhancer factor TEF‑1 (also known TEA domain family member 1,

TEAD1), TEAD2, TEAD3 and TEAD4. Because the YAP1–TEAD complex activates the

transcription of genes involved in cell growth and survival, including CTGF, MYC and

BIRC5 (also known as survivin), the degradation of YAP1 as a result of phosphorylation

by LATS1 or LATS2 reduces expression of these genes (Dong et al. 2007; Zhao et al.

2008). Loss‑of‑function mutation of the Hippo kinase cascade components in

mammals, such as inactivation of Merlin (also known as NF2) and deletion of LATS2 in

mesothelioma (Murakami et al. 2011), also leads to overgrowth phenotypes and

consequently carcinogenesis in multiple tissues (Harvey et al. 2013; Plouffe et al. 2015).

1.2 The regulatory signals of the Hippo pathway

The mammalian Hippo pathway responds to a variety of extracellular signals,

including cell polarity, contact inhibition, stress, mechanotransduction, cell attachment,

hormones and growth factors (Meng et al. 2016). More than 20 upstream regulators of

the Hippo pathway (Johnson and Halder 2014) have been discovered, many of which

are involved in tissue homeostasis and carcinogenesis (Figure 1.1).

Polarity and cell-adherence signals

Intestinal epithelial cells, which display apical–basal polarity, are joined together by tight junctions and adherens junctions, forming the intestinal epithelium layer. Intestinal diseases are often associated with disruption of IEC polarity or epithelial junctions (Schulzke et al. 2009). The components of apical–basal polarity complexes attenuate the transcription activities of YAP1 and TAZ. For instance, in addition to stimulating LATS kinase activity, Angiomotin (AMOT) family proteins can directly bind to YAP1 and TAZ, sequestering them at tight junctions and inhibiting their transcriptional activity (Paramasivam et al. 2011; Zhao et al. 2011). Studies in Drosophila have shown that the FERM (Four-point-one protein, ezrin, radixin and moesin) domain proteins Merlin and Expanded colocalize with Kibra at the apical domain of polarized epithelial cells to promote the activation of Wts by Hpo (Yu et al. 2010). Many other key regulators of the Hippo pathway, such as α‑catenin, tyrosine-protein phosphatase non-receptor type 14 (also known as PTPN14), and cadherin‑1 (also known as E‑cadherin), are also components of junction complexes, and attenuate YAP1 and TAZ transcriptional activity by either increasing the kinase activity of LATS or directly sequestering YAP1 and TAZ at cell junctions (Yu and Guan 2013). A study published in 2014 showed that LKB1, which is a master regulator of the development and maintenance of cell polarity, activates the Hippo pathway in human cell lines through its substrates, the serine/threonine-protein kinase MARK protein family and Scribble (Mohseni et al. 2014). Another LKB1 substrate, 5′-AMP-activated protein kinase (AMPK), directly phosphorylates YAP1, disrupting YAP1–TEAD interaction and inhibiting YAP1 transcriptional activity (DeRan et al. 2014; Mo et al. 2015; Wang et al. 2015). In Drosophila, the planar cell polarity proteins cadherin-related tumour

suppressor (also known as Fat) and Dachsous inactivate Yki by promoting the abundance and localization of Expanded at the apical membrane, thereby stimulating Wts activity (Bennett and Harvey 2006; Cho et al. 2006; Silva et al. 2006; Willecke et al. 2006).

Regulation of contact inhibition

The Hippo pathway is believed to integrate signals from the spatial organization and the physical state of neighbouring cells, and can regulate contact inhibition of cell growth by phosphorylating and inactivating YAP1, leading to inhibition of YAP1‑mediated transcription (Zhao et al. 2007). Hippo pathway signaling is required for contact inhibition of cell growth mediated by the E‑cadherin–catenin complex (Kim et al. 2011). In addition, overexpression of constitutively active YAP1 leads to loss of contact inhibition in epithelial cells (Zhao et al. 2007). Contact inhibition has been shown to control miRNA biogenesis through the Hippo pathway. High YAP1 activity in cancer cells, in part due to loss of contact inhibition, led to a global downregulation of miRNA synthesis and potentially enhanced tumorigenesis (Mori et al. 2014). However, another study reported conflicting results (Chaulk et al. 2014), showing that YAP1 and TAZ promote processing and biogenesis of miRNA in human cells cultured at low density without contact inhibition. By contrast, cells under high density experiencing contact inhibition showed defective processing of miRNAs owing to loss of YAP1 and TAZ activities (Chaulk et al. 2014).

Regulation of Hippo signalling by stress

As a result of its physiological role in digestion, gastrointestinal tract tissue experiences many stresses, including oxidative stress, endoplasmic reticulum stress and hypoxia. These stresses regulate YAP1 and TAZ by various mechanisms (Shao et al. 2014; Ma et al. 2015; Wu et al. 2015). In addition, YAP1 and TAZ transcriptional activities are also controlled by mechanical signals through Rho GTPases or JNK1/2 (Dupont et al. 2011; Zhao et al. 2012; Rauskolb et al. 2014), although the involvement of the Hippo pathway in YAP1 and TAZ regulation in this context is still under debate (Codelia et al. 2014; Rauskolb et al. 2014). Mechanical forces affect many aspects of intestinal physiology, particularly by promoting the proliferation and migration of gut epithelial cells (Gayer and Basson 2009). However, whether YAP1 and TAZ play any part in the response of IECs to mechanical signals has not been studied.

G protein-coupled receptor regulation

Extracellular signalling molecules such as hormones can regulate the Hippo pathway via G protein-coupled receptors (GPCRs) (Mo et al. 2012; Yu et al. 2012; Yu et al. 2013). GPCR overexpression or mutations of guanine nucleotide-binding protein subunit alpha (Gα) proteins have been associated with carcinogenesis in a variety of tissues (O'Hayre et al. 2013). Many GPCRs known to promote cell growth and tumorigenesis, such as lysophosphatidic acid receptors (LPARs), sphingosine 1-phosphate receptors (S1PRs) and protease-activated receptors (PARs), are now recognized as cell surface receptors for Hippo signalling (Mo et al. 2012; Yu et al. 2012), suggesting that the Hippo pathway mediates GPCR-induced tumorigenesis. Indeed, GPCRs coupled to Gα12 or Gα13, or to Gαq/11, can activate YAP1 and TAZ, leading to tumorigenesis (Feng et al. 2014; Yu et al. 2014; Liu et al. 2015a). On the

other hand, Gαs activation was shown to inhibit YAP1 and TAZ (Bao et al. 2011; Yu et al. 2012; Iglesias-Bartolome et al. 2015). Intestinal GPCRs respond to many nutrients and other components in food, and therefore play a crucial part in regulating intestine physiology, particularly in metabolic homeostasis and appetite (Reimann et al. 2012). It would be interesting to evaluate whether the Hippo pathway is regulated by GPCRs that are highly expressed in the intestine, for instance the GPCRs that respond to fatty acids (which include free fatty acid receptors and GPR120) (Reimann et al. 2012).

1.3 The Hippo pathway in regeneration and homeostasis

1.3.1 The Hippo pathway in intestinal regeneration.

Although YAP1 is not required during normal intestinal homeostasis, its activity is essential for intestinal regeneration after injury. In mice, an increase in YAP1 protein level is observed 2–5 days after dextran sulfate sodium (DSS)- induced colitis and regeneration (Cai et al. 2010; Taniguchi et al. 2015). *In vivo* and *in vitro* studies show interleukin-6 receptor subunit beta (also known as gp130), a co-receptor for IL-6, activates YAP1 through Src family kinases (SFKs) upon DSS-induced intestinal colitis (Taniguchi et al. 2015). Mice with IEC-specific conditional knockout of *Yap1* had a higher mortality rate and more extensive loss of the crypt compartment than wild-type mice after both groups were subjected to DSS-induced intestinal injury (Cai et al. 2010). Whole-body irradiation is also widely used to study intestinal regeneration in mice. YAP1 is activated in IECs 2–4 days after irradiation (Gregorieff et al. 2015), and IEC-specific and ISC-specific *Yap1* knockout mice show reduced crypt proliferation after

irradiation-induced injury (Gregorieff et al. 2015). These studies suggest that YAP1 positively regulates intestinal tissue regeneration by regulating ISC proliferation. Furthermore, Gregorieff *et al.* (Gregorieff et al. 2015) found that YAP1 reprogrammes ISCs by transiently inactivating Wnt signalling and activating another regenerative programme, the EGFR pathway. They showed that the EGFR ligand epiregulin is upregulated in the intestines of *Yap1* conditional knockout mice after regeneration of the intestinal epithelia following whole-body exposure to ionizing radiation, which might serve as a compensatory mechanism for *Yap1* loss in some crypts. Indeed, they further showed that exogenous epiregulin can rescue YAP1-deficient organoid formation *ex vivo* (Gregorieff et al. 2015). By contrast, Barry *et al.* (Barry et al. 2013) found that whole-body irradiation of mice with intestine-specific *Yap1* conditional knockout resulted in crypt hyperplasia and overgrowth 7 days later, and suggested that Wnt signalling hyperactivation was responsible for this phenotype. In summary, functional YAP1 is important in the first few days of the regeneration phase. The hyperproliferative phase seen in *Yap1*-IEC-specific conditional knockout mice 7 days after injury could be the result of enhanced Wnt signalling (Figure 1.2). The divergent roles of YAP1 in the regenerative response at different time points after injury might be due to the distinct cellular repair mechanisms activated at these time points. For instance, pathways involved in cell cycle and DNA repair are active 2–5 days after injury whereas pathways for tissue development might be activated at subsequent time points, 1 week or later after injury (Stroncek and Reichert 2008). YAP1 and Wnt signalling work in coordination to maintain proper tissue regeneration after injuries. Mouse genetic studies clearly

demonstrate a role of the Hippo pathway in normal intestine homeostasis as well as in the response to tissue injury.

1.3.2 The Hippo pathway in liver regeneration

Liver regeneration and homeostasis are considered a classic model for mammalian organ size control (Fausto et al. 2006). The discovery that Hippo signalling is essential in the regulation of tissue homeostasis in *Drosophila* was followed immediately by speculation over whether the Hippo pathway similarly regulates mammalian organ size (Pan 2007). In mammalian models, overexpression of YAP1 in the liver leads to tissue overgrowth, and sustained overexpression of YAP1 eventually results in hepatocellular carcinogenesis (Camargo et al. 2007; Dong et al. 2007). In human hepatocellular carcinoma specimens, high YAP1 protein levels and increased YAP1 nuclear localization have been observed (Dong et al. 2007; Zhao et al. 2007; Xu et al. 2009). Because liver cancer results from uncontrolled proliferation of hepatocytes, increased YAP1 expression in human liver cancer and mouse models supports the notion that YAP1 can serve as a major driver of hepatocyte proliferation. Mouse models bearing deletion of Hippo pathway proteins, including MST1, MST2, SAV1, Merlin, LATS1 and LATS2, have been generated and characterized for the roles of these components in liver size control and carcinogenesis (Zhou et al. 2009; Lee et al. 2010; Lu et al. 2010; Song et al. 2010; Zhang et al. 2010; Chen et al. 2015) (Figure 1.3). Hepatomegaly and spontaneous hepatocellular carcinogenesis were consistently observed in liver-specific *Mst1* and *Mst2* knockout mice (Lee et al. 2010; Lu et al. 2010; Song et al. 2010; Zhou et al. 2011). Liver-specific *Sav1*-knockout mice exhibit more complex tumorigenesis, and display both hepatocellular carcinoma and

cholangiocarcinoma (Lee et al. 2010; Lu et al. 2010). SAV1-deficient mice have been speculated to be more prone to the malignant transformation of liver progenitor cells than wild-type mice, whereas hepatocytes are unaffected by loss of SAV1 (Lee et al. 2010). Similarly, liver-specific *Nf2*-knockout mice develop hepatocellular carcinoma and bile duct hamartoma at 1 year of age (Zhang et al. 2010). A study published in 2013 showed that combined liver-specific deletion of *Nf2* and *Sav1* causes an exaggerated liver overgrowth phenotype in mice at a very early age (8 days old), whereas deletion of either *Nf2* or *Sav1* alone did not result in any abnormality in liver at that age (Yin et al. 2013). This result indicates the synergistic actions of Merlin and SAV1 attenuate YAP1 and TAZ activity during liver development. Consistently, mice with knockout of other Hippo pathway components, such as *Mob1a* and *Mob1b*, also induce spontaneous hepatocellular carcinogenesis (Nishio et al. 2012) (Figure 1.3).

The role of Hippo signalling in liver development is further supported by evidence that the level of nuclear YAP1 correlates with cyclin D1 expression and hepatocyte proliferation during postnatal development in mice (Septer et al. 2012). Mice with liver-specific *Yap1* deficiency have smaller livers and a reduced hepatocyte proliferation rate compared with wild-type mice (Zhang et al. 2010). In addition, liver-specific *Yap1*-knockout mice show impaired bile duct formation, suggesting a critical role for YAP1 in bile duct development (Zhang et al. 2010). A study published in 2015 found that the Hippo pathway might be a major downstream effector of the transcription factors SOX4 and SOX9, which regulate liver and bile duct development (Poncy et al. 2015). Another study published in 2014 showed that the main function of the Hippo pathway in the liver is to maintain the differentiation status of hepatocytes (Yimlamai et al. 2014). The

activation of YAP1 dedifferentiates adult mouse hepatocytes into progenitor or ductal-like cells. In summary, YAP1 induces cell proliferation and suppresses differentiation during liver development, and reduced Hippo pathway activity causes uncontrolled organ growth and therefore tumorigenesis.

Hippo signalling is also involved in liver repair after injury in mouse models. A few studies have implicated that the Hippo signalling regulates liver regeneration after partial hepatectomy in rats and mice (Apte et al. 2009; Grijalva et al. 2014; Herr et al. 2014). In addition, studies in the past few years have demonstrated the essential role of YAP1 in liver repair after injury. Deletion of *Yap1* in mice potentiates cholestatic liver injury caused by bile-duct ligation (Brabletz et al. 1999). Similarly, mice with liver-specific knockout of *Yap1* exhibit deficient liver repair after carbon-tetrachloride-induced liver injury (Su et al. 2015). Notably, this study used a mosaic knockout mouse model to show that YAP1 activation alone in a fraction of hepatocytes is not sufficient to drive growth of the liver, whereas previous reports showed an organ hyperplasia phenotype as a result of YAP1 overexpression in the entire liver (Camargo et al. 2007; Dong et al. 2007). A possible explanation for this finding is that relative YAP1 activation in the two models is different. In conjunction with high YAP1 activity, inflammatory signals, which are always present in the context of liver injury, are essential to induce proliferation of hepatocytes (Su et al. 2015). Upon liver injury, YAP1 is also activated in cells besides hepatocytes. In the livers of carbon-tetrachloride-treated mice and tissue samples from human fibrotic livers, activated YAP1 causes activation of hepatic stellate cells. Prolonged activation of these cells leads to liver fibrogenesis (Mannaerts et al. 2015).

1.3.3 The Hippo pathway in heart regeneration

The four-chambered heart is a heterogeneous organ consists of cardiomyocytes, cardio fibroblasts, endothelial cells, epicardium cells, and the smooth muscle cells. During embryonic development, the mammalian heart undergoes dramatic change in size driven by cardiomyocyte and precursor cell proliferation. However, shortly after birth, cardiomyocyte proliferation stops and heart growth is primarily due to cardiomyocyte hypertrophy.

Several evidence show that the Hippo pathway is involved in mice embryonic heart development. YAP protein was highly expressed and mainly localized in the nucleus in neonatal mouse heart and decline with age (Varelas 2014). Active YAP overexpressing heart displayed a thickened myocardium and increased in size (Xin et al. 2013). it is also shown that YAP promoted proliferation of embryonic cardiomyocytes by activating the insulin-like growth factor and Wnt signaling pathways (Xin et al. 2011). Furthermore, conditional deletion of YAP in neonatal mice disrupts cardiomyocyte proliferation, leading to hypoplasia and death (Xin et al. 2011; von Gise et al. 2012). A combined deletion of YAP and TAZ exhibits gene dosage-dependent cardiac phenotype, suggesting that TAZ plays a redundant role in cardiomyocyte proliferation and survival (Xin et al. 2013). Other Hippo pathway components are also shown to be involved in heart development. Lats2, Mst1/2, and Salvador cardiac-conditional KO hearts had thickened ventricular wall and enlarged ventricular chamber without a change in myocardial cell size (Heallen et al. 2011). Together, the Hippo pathway plays a crucial role in heart development during embryogenesis.

The adult mammalian heart has only limited potential for regeneration. As a result, after injuries, the adult mammalian myocardium replaces lost cardiomyocytes

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with fibrotic scar tissues and the heart function is reduced. An attractive approach for heart injury would be enhancing cardiomyocyte proliferation to regenerate in adulthood. Manipulation of upstream components of the Hippo pathway can dramatically perturb cardiac growth. Mst1 overexpression in the heart resulted in cardiac dysfunction (Yamamoto et al. 2003), and overexpression of dominant-negative Mst1 or Lats2 improved cardiac function after injury (Odashima et al. 2007; Shao et al. 2014). Studies found that Hippo-deficient adult mouse cardiomyocytes re-enter the cell cycle and undergo cytokinesis. Hippo-deficient hearts had recovered function to a level comparable to that of control animals after ischemic damage, suggesting that Hippo-deficient cardiomyocytes can increase proliferation thus survival (Heallen et al. 2013). In addition, active YAP transgenic mice hearts regenerated with largely decreased level of fibrosis after injuries (Xin et al. 2013). Altogether, these data shows that the Hippo pathway is important in cardiomyocyte regeneration in adulthood.

1.4 The Hippo pathway in diseases and cancer

The Hippo pathway has been implicated in many human diseases especially cancer (Plouffe et al. 2015). Due to the length limitation, here we only pick up several diseases and cancer types for detailed discussions.

1.4.1 The Hippo pathway in colon cancer

Uncontrolled tissue regeneration can lead to malignant transformation (Terzic et al. 2010). Dysregulation of the Hippo pathway has been observed in many cancer types

(Plouffe et al. 2015), including CRC. Genetically engineered mouse models serve as useful tools to study how the Hippo pathway influences tumorigenesis. Because global knockout of many genes encoding Hippo pathway components, such as *Nf2*, *Sav1*, *Mst1*, *Mst2* and *Lats2*, results in embryonic lethality in mice, intestinal-epithelium-specific conditional knockout mice have been developed. Mice with conditional knockout of both *Mst1* and *Mst2* in the intestinal epithelium develop dysplastic small and large intestine and spontaneous adenomas in the colon (Zhou et al. 2011). The increased tumour risk might be the result of elevated YAP1 protein expression in these mice, resulting in strong activation of Wnt and Notch signalling. Intestinal-epithelium-specific *Sav1*-knockout mice develop colonic polyps by 13 months of age, whereas wild-type littermates do not develop polyps (Cai et al. 2010). This study further showed that DSS-induced intestinal injury and repair greatly exacerbated the tumorigenicity of the *Sav1*-deficient crypts. This effect was YAP1-dependent, as mice with intestinal epithelium-specific knockout of both *Sav1* and *Yap1* had no colonic polyp formation. In addition to canonical Hippo pathway components, the NDR protein kinases have been identified as tumour suppressors upstream of YAP1. Mice with intestinal epithelium-specific knockout of both *Ndr1* and *Ndr2* show increased YAP1 expression level and are more sensitive to colon carcinogenesis induced by DSS or azoxymethane (Zhang et al. 2015). These data support the notion that dysregulation of Hippo pathway leads to tumorigenesis and that active YAP1 is oncogenic in CRCs.

Studies of samples from patients with colon cancer and human colon-cancer-derived cell lines also support the oncogenic property of YAP1 and TAZ in CRCs. A positive correlation between YAP1 protein expression level and poor prognosis has

been observed in patients with CRC (Lam-Himlin et al. 2006; Steinhardt et al. 2008; Zhou et al. 2011), and TAZ has also been found to be a prognostic indicator for CRC outcome (Wang et al. 2013a; Yuen et al. 2013). In human colon cancer cell lines, YAP1 knockdown by small-hairpin RNA resulted in a dramatic decrease in cell proliferation, metastasis and invasion, whereas overexpression of YAP1 resulted in an increased proliferation rate (Zhou et al. 2011; Wang et al. 2013a). Knockdown of TAZ resulted in decreased cell proliferation, both *in vitro* and in xenograft mouse models (Pan et al. 2012). These studies support YAP1 and TAZ as oncogenes in CRCs.

Several underlying mechanisms of Hippo-pathway-driven tumour transformation have been proposed. The Wnt–β-catenin pathway has a crucial role in initiating CRC. Loss of function mutations in the gene encoding adenomatous polyposis coli (*APC*) are observed in most colorectal tumours (Powell et al. 1992). *APC* mutation increases the risk of CRC through the constitutive activation of the β-catenin–TCF4 complex in IECs (Bienz and Clevers 2000). Proteins encoded by β-catenin–TCF4 complex target genes, including matrilysin (also known as MMP-7), Myc, cyclin D1 and PPARδ, have been shown to contribute to CRC tumour progression (He et al. 1998; Brabletz et al. 1999; Crawford et al. 1999; He et al. 1999; Tetsu and McCormick 1999). YAP1 and TAZ are activated in APC-deficient cells both *in vitro* and *in vivo* (Azzolin et al. 2012; Azzolin et al. 2014; Cai et al. 2015), and mouse models with intestine-specific *Apc*-knockout do not show intestinal hyperplasia if TAZ or YAP1 are also knocked out in the intestines (Azzolin et al. 2014; Cai et al. 2015). Different mechanisms have been proposed for how APC regulates the activity of YAP1 and TAZ. Azzolin *et al.* (Azzolin et al. 2014) reported that YAP1 and TAZ are sequestered in the cytoplasm by the β-catenin

destruction complex when Wnt signalling is not active. When Wnt is activated by APC depletion, YAP1, TAZ and β-catenin separate from the destruction complex and become active. Cai *et al.* (Cai et al. 2015) reported that the activity of YAP1 and TAZ can also be regulated by APC via a mechanism independent of the β-catenin destruction complex. They showed that APC serves as a scaffolding protein to facilitate LATS activation. Loss of APC inactivates the Hippo pathway, leading to YAP1 and TAZ activation (Cai et al. 2015). Other studies have shown that the β-catenin–TCF4 complex directly binds to the *YAP1* enhancer region to promote *YAP1* gene expression, thereby identifying *YAP1* as a TCF4 target gene (Konsavage et al. 2012). In addition, β-catenin can complex with YAP1 and TBX5 to induce anti-apoptotic gene expression; this complex is essential for survival of β-catenin-driven colon cancers (Rosenbluh et al. 2012). Together, these results suggest that YAP1 and TAZ can be mediators downstream of APC, and that their activities contribute to colonic tumorigenesis. The dispensable nature of YAP1 and TAZ under normal conditions make them an attractive therapeutic target for treating CRC.

In addition to Wnt signalling, JNK signalling has also been implicated in inducing tumorigenesis in the intestine (Sancho et al. 2009; Chen 2012). Several studies indicate that JNK is an upstream regulator of the Hippo pathway. In the *Drosophila* midgut, bleomycin-induced injury activates JNK, leading to Yki nuclear translocation. Activated Yki upregulates Upd expression and Jak–Stat signalling, which induces cellular proliferation (Staley and Irvine 2010; Sun and Irvine 2011). In mammals, JNK induces YAP1 activity when cells are exposed to mechanical strain (Codelia et al. 2014) and DNA damage (Tomlinson et al. 2010). Mechanistically, JNK activates YAP1 through

inhibition of LATS by increasing binding between LATS proteins and Ajuba (the mammalian homologue of Jub) (Sun and Irvine 2013). Although no studies have established a connection between JNK signalling and the Hippo pathway in the context of the mammalian intestine, this mechanism could be worthy of further exploration in colorectal tumorigenesis.

1.4.2 The Hippo pathway in liver cancer

In the healthy adult liver, YAP1 is expressed and active in the bile duct cells, whereas it is expressed at low levels and is inactive in hepatocytes (Yimlamai et al. 2014). However, numerous immunohistochemistry and immunoblot analyses show raised YAP1 protein levels and increased nuclear localization in human liver cancer cells compared with adjacent normal tissues (Dong et al. 2007; Zhao et al. 2007; Xu et al. 2009; Bai et al. 2012; Li et al. 2012; Ahn et al. 2013; Li et al. 2015). YAP1 activity, defined either as protein levels or the degree of nuclear localization, can serve as a prognostic marker for overall survival and disease-free survival of patients with hepatocellular carcinoma (Xu et al. 2009; Sohn et al. 2016).

YAP1 activation in liver cancer could result from many factors. The *YAP1* gene was observed to be frequently amplified in human hepatocellular carcinoma tumours (Zender et al. 2006). YAP1 can also be activated by carcinogenic compounds such as 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene (Kowalik et al. 2011), which mimics the xenobiotic phenobarbital to activate the nuclear receptor constitutive androstane receptor (CAR). The tumour-promoting role of CAR has already been implicated in liver cancer (Huang et al. 2005; Dong et al. 2015). However, a direct link between YAP1 and

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CAR has not been clearly established. HBV is a major cause of liver cancer, and the viral HBx protein can increase hepatocyte protein levels of YAP1 and TAZ *in vitro*, which might contribute to tumorigenesis induced by HBV infection (Zhang et al. 2012; Liu et al. 2015b). In addition, bile acids, which play an important regulatory part in liver injury and injury-related carcinogenesis (Liu et al. 2012; Wang et al. 2013b), also activate YAP1 to promote spontaneous liver tumorigenesis in a mouse model of hepatocellular carcinoma (Anakk et al. 2013).

Mechanistically, the tumour-promoting property of YAP1 in liver cancer mainly relies on TEAD-mediated gene transcription. The YAP–TEAD complex can upregulate the expression of genes directly involved in promoting cell proliferation and tissue overgrowth, for instance the Notch ligand Jagged1 and the amino acid transporters SLC38A1 and SLC7A5 (Tschaharganeh et al. 2013; Park et al. 2016). In addition, YAP–TEAD also increases expression of the microRNA miR-130a, which in turn activates YAP1 by repressing the translation of the YAP1 antagonist VGLL4, resulting in a positive feedback loop that reinforces TEAD-mediated transcription (Shen et al. 2015). Using gene-set enrichment analysis of liver tissue from a mouse model of liver cancer, a study published in 2015 reported that the YAP–TEAD complex colocalizes with other transcription factors, such as E2F1, HNF4α and β-catenin, to coordinate the expression of genes that modulate hepatocyte quiescence and differentiation (Fitamant et al. 2015). Disruption of YAP–TEAD interaction using verteporfin abolishes tumour growth *in vitro* and in mouse models (Liu-Chittenden et al. 2012; Yu et al. 2014); targeting YAP–TEAD interaction could therefore be a potential therapeutic strategy for hepatocellular carcinoma.

1.4.3 The Hippo pathway in breast cancer

Several studies have suggested that dysregulation of the Hippo pathway plays an important role in breast cancer tumorigenesis. High nuclear YAP expression has been shown to correlate with E-cadherin deficient breast cancer subtype (Vlug et al. 2013). In addition, YAP/TAZ activity has also been reported to correlate with higher probability to develop metastasis and reduced survival in human breast cancer patients (Cordenonsi et al. 2011). TAZ is highly expressed in invasive breast cancer cell lines as well as primary breast cancers, and its overexpression induces cell proliferation, transformation, and epithelial-mesenchymal transition (EMT) in breast cancer cell lines (Chan et al. 2008; Lei et al. 2008). It is also shown that overexpression of YAP induces tumor formation and growth (Wang et al. 2012). In addition, leukemia inhibitory factor receptor (LIFR) has been identified as a tumor suppressor acting through the Hippo pathway by inactivating YAP *in vitro* and *in vivo* (Chen et al. 2012). These evidence supported the notion that YAP and TAZ act as oncogenes. However, there are still controversies of the role of Hippo pathway in breast cancer tumorigenesis. In contrast to the well-known oncogenic role of YAP, there is also evidence suggesting YAP as a tumor suppressor in breast cancers. YAP protein level is reported to be lost in luminal breast cancer tissues. In addition, YAP knockdown in breast cancer cell lines enhances tumor migration, invasion, and tumor growth in nude mice (Yuan et al. 2008). Further studies elucidating the paradox are needed. Notably, a recent study reported that hyperactivation of YAP alone is not sufficient drive mammary tumorigenesis *in vivo*, and that YAP-induced oncogenic growth may require other genetic lesions. However, loss of YAP is shown to suppress tumor growth in oncogene-induced breast cancer,

suggesting YAP as a potent therapeutic target in human breast cancers (Chen et al. 2014a).

1.4.4 The Hippo pathway in neurological disease

The nervous system consists of the central nervous system (CNS) and peripheral nervous system (PNS). The CNS includes spinal cord, retina, and brain, which are derived from the neural tube during embryonic development. The neural tube is formed of neuroepithelial cells, so-called neural stem cell in early stage of development. Many neurological diseases are due to disrupted regulation of neural stem cell proliferation and differentiation. Several studies have shown that the Hippo signaling pathway plays an important role in neuronal development, in the context of affecting cell proliferation, differentiation, and survival. In chick neural tube, YAP is shown to express in ventricular zone progenitor cells, and its interaction with TEAD induces neural progenitor cell proliferation and inhibits differentiation. In addition, the upstream components of the Hippo pathway Mst1/2 and Lats1/2 are also shown to regulate neural progenitor proliferation by regulating YAP activity (Cao et al. 2008). In mice, it is shown that YAP is mediated by bone morphogenetic protein-2 (BMP2) signaling and Sonic Hedgehog pathway acts downstream of YAP to regulate neuronal differentiation (Lin et al. 2012; Yao et al. 2014).

In addition to neuronal development, recent studies revealed that the Hippo pathway is also involved in many brain pathologies such as brain/nerve tumors. Loss of function mutation of the tumor suppressor *NF2* gene was identified as a cause of Neurofibromatosis Type 2 (NF2) disease, characterized by the development of

schwannomas (Schulz et al. 2014). In addition, it is documented that YAP was highly

expressed in many human brain tumors such as infiltrating gliomas, and its

overexpression promoted glioblastoma growth *in vitro* (Orr et al. 2011). Furthermore,

NF2 expression was significantly reduced in human malignant gliomas, and expression

of NF2 had shown to inhibit growth of human glioma both *in vitro* and *in vivo* (Lau et al.

2008).

In non-cancer neurological diseases, the Hippo pathway components are also

shown to be highly involved. YAP and TAZ have been identified as the mediator of the

amyloid-β protein precursor (AβPP), the precursor of the amyloid β that drives

Alzheimer's disease (Orcholski et al. 2011). Mst1 has shown to be a key mediator of

amyotrophic lateral sclerosis (ALS). Activation of Mst1 stimulated p38 MAPK, caspase-9

and -3, and led to autophagosome accumulation, resulting in the loss of motor neurons.

Mst1 deletion in ALS mouse model exhibited increased viability of motor neurons,

delayed symptom onset, and extended survival (Lee et al. 2013). These findings

revealed the importance of the Hippo pathway components in some neurodegenerative

diseases and could potentially be targeted therapeutically.

1.4.5 The Hippo pathway in Cardiovascular diseases

The Hippo pathway has also been shown to be involved in several heart

diseases. Arrhythmogenic cardiomyopathy (AC) is characterized by fibro-adipocytes

replacement of cardiomyocytes, cardiac enlargement and dysfunction, and sudden

death. Studies have shown that desmosome components concentrated at intercalated

discs leads to activation of the Hippo pathway, suppression of the canonical Wnt

signaling, and enhanced adipogenesis. Knockdown of Lats1/2 and expression of active

YAP in cardiomyocytes enhanced adipogenesis, indicating the Hippo pathway

components can potentially be therapeutic targets for AC (Chen et al. 2014b). In

addition to AC, studies have shown that the Hippo pathway is involved in the Holt-Oram

syndrome, which is characterized by upper limb abnormalities and heart defects. The T-

box transcription factor TBX5, essential in cardiac and limb development, is often

mutated (Basson et al. 1997) in Holt-Oram syndrome patients. Studies have shown that

TBX5 interacts with coactivators YAP and TAZ through interacting with p300 and PCAF,

and its mutations impede the binding of TAZ, resulting in heart dysfunction (Murakami et

al. 2005). Taken together, the Hippo pathway plays an important role in heart

development, regeneration and repair, and several heart diseases. Targeting the Hippo

pathway could potentially be a means of treatment for heart damage and disease.

1.4.6 The Hippo pathway in immunological disease

The immune system is a complex network made up of cells, tissues, and organs

that help defend the body from pathogens and toxins. Disorders in the immune system

can lead to immune diseases, such as inflammatory disease, autoimmune disease, and

cancer.

Studies revealed the importance of Mst1 in controlling lymphocyte function in the

aspect of adhesion, migration, proliferation, and apoptosis. It is found that Mst1 is highly

expressed in T and B lymphocytes in both human and mouse. Activation of Rap1-

RAPL-Mst1 signaling pathway in lymphocyte is shown to induce integrin LFA-1 cluster

at the leading edge, contributing to lymphocyte adhesion and migration. Mice deficient

in Mst1 exhibit impaired trafficking of immune cells, including lymphocytes (Katagiri et al. 2006; Nishikimi et al. 2014). Functionally, Mst1 deficient naïve T cells exhibit greater proliferation in response to stimulation of T cell receptor. However, it is shown that Mst1 deficient mice exhibit a lower number of mature naïve T cells in secondary lymphoid organs and a higher rate of apoptosis *in vitro*, which can be explained by lower threshold needed for naïve T cells stimulation, (Zhou et al. 2008). In Treg cells, Mst1 deletion leads to impaired interactions with dendritic cells (Tomiyama et al. 2013). Together, these data supporting the notion that Mst1 gene is highly involved in regulating lymphocyte functions.

Recent studies reported that Mst1 loss-of-function mutations in human patients is associated in immunodeficiency syndrome, characterized as recurrent bacterial and viral infections, and autoimmunity (Abdollahpour et al. 2012; Nehme et al. 2012). Several studies also indicated the importance of Mst1/2 in autoimmune diseases. Autoimmune phenotypes such as lymphocytes inflammatory infiltration, could be detected in Mst1 deficient mice, and further studies showed that Mst2 is partially redundant with Mst1 in autoimmunity (Ueda et al. 2012; Du et al. 2014). In addition to Mst1/2, the other Hippo pathway component TAZ had been identified to be involved in an autoimmune disease Sjogren's syndrome (SS). In NOD mice, a mouse model for SS, mislocalization of TAZ leads to impaired salivary gland development, causing the mice to develop features mimicking SS in human. In human labial biopsy, SS patients display mislocalization of TAZ (Enger et al. 2013). These data suggested that Mst1/2 and TAZ are involved in mammalian immune disease, while the role of YAP in immune diseases has not been reported.

1.5 Conclusion

The Hippo pathway has an important role in intestinal homeostasis and regeneration in both Drosophila and mammals, and its dysregulation often leads to tumorigenesis. In general, YAP1 and TAZ activity is associated with ISC maintenance and progenitor cell proliferation. Although YAP1 and TAZ might be dispensable under normal intestinal homeostasis, their activation is crucial during tissue regeneration, particularly in response to injury. Negative regulation of YAP1 and TAZ activity by upstream components of the Hippo pathway, such as MST1, MST2 and SAV1, is required to maintain normal intestine homeostasis and prevent tissue overgrowth. The Hippo pathway is integrated with other key pathways that are important for intestinal homeostasis, such as the Wnt signalling pathway. YAP1 acts either as a downstream effector and/or an inhibitor of Wnt signalling to contribute to ISC maintenance and differentiation of transit amplifying cells. The complex communications between the Hippo pathway and other pathways, especially the Wnt pathway, are still being explored and remain an important question in the gastrointestinal field. Notably, *in vitro* studies show the Hippo pathway can be activated by tight junctions and adherence junctions. Intestinal diseases are often associated with disruption of the intestine lining and junction structures. One can speculate that the intactness of IEC junction structure is a major signal acting via the Hippo pathway to modulate intestine homeostasis. Thus, it would be interesting to investigate the regulation of the Hippo pathway by junctions in the intestine, and how the junction structures contribute to intestinal tissue regeneration in the context of Hippo signalling pathway.
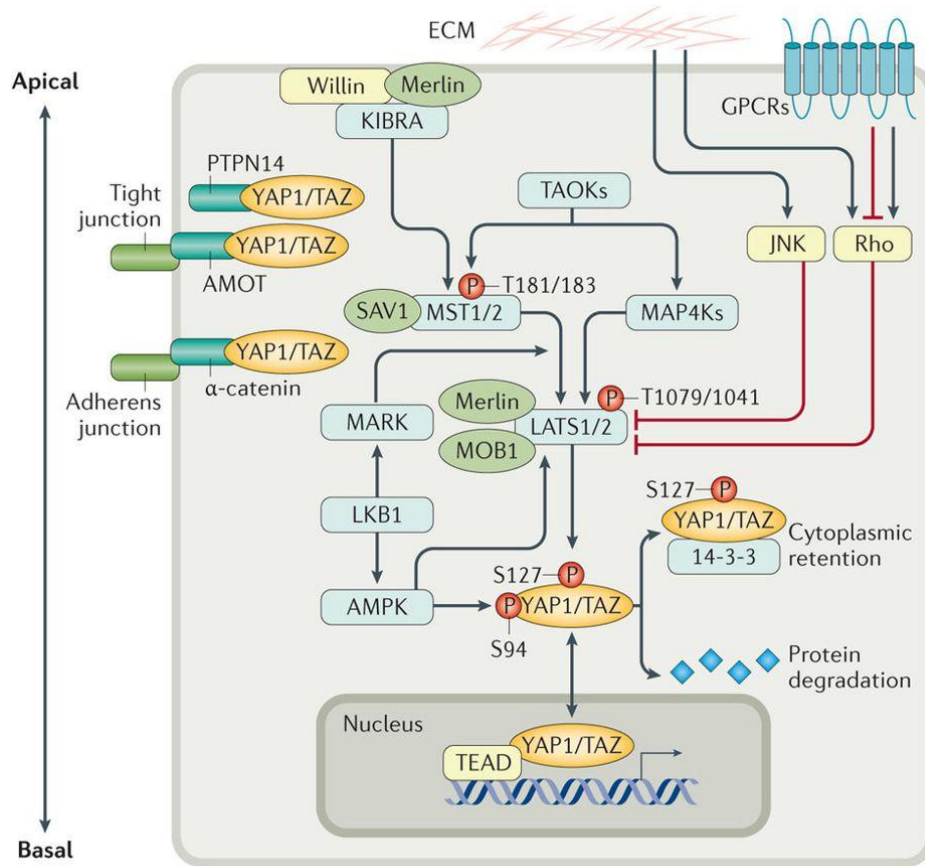
1.6 Acknowledgements

Figure 1.1: The Hippo pathway integrates signals to regulate the activity of YAP1 and TAZ. The core of the Hippo pathway is the kinase cascade of MST1 and MST2 and LATS1 and LATS2. MST proteins, activated by upstream signals, phosphorylate and activate LATS proteins directly, and also activate the scaffold proteins MOB1 and SAV1. MAP4Ks also phosphorylate and activate LATS proteins. Activated LATS proteins phosphorylate YAP1 and TAZ at multiple sites, triggering 14-3-3-mediated cytoplasmic retention and protein degradation. YAP1 and TAZ function as transcriptional co-activators of TEAD proteins to induce expression of genes involved in cell proliferation and apoptosis. Merlin, Willin and KIBRA form a complex to recruit MSTs and LATS to the apical plasma membrane, activating LATS proteins. AMOT and PTPN14 sequesters YAP1 and TAZ at tight junctions, and α-catenin sequesters YAP1 and TAZ at adherens junctions, to prevent their nuclear translocation. LKB1, which has important roles in both cell polarity and cellular stress response, regulates the activity of YAP1 and TAZ through activation of its substrates MARK and AMPK, both of which activate LATS. Activated AMPK also phosphorylates YAP1 and disrupts the YAP1–TEAD complex. The Hippo pathway is further regulated by ECM stiffness and mechanotransduction through Rho GTPases or JNK. GPCRs mediate many extracellular signals to either activate or inhibit LATS via Rho GTPase. AMOT, Angiomotin; AMPK, AMP-activated protein kinase; ECM, extracellular matrix; GPCRs, G protein-coupled receptors; JNK, c-Jun N-terminal kinase; LATS, large tumour suppressor; LKB1, liver kinase B1; MAP4K, mitogen-activated protein kinase kinase kinase kinase; MARK, microtubule affinity-regulating kinase; MOB1, Mob1 homologue; MST, mammalian ste20-like kinase; P, phosphorylation of stated amino acid; PTPN14, protein tyrosine phosphatase non-receptor type 14; SAV1, salvador family WW domain-containing protein 1; TAZ, transcriptional co-activator with PDZ-binding motif; TEAD, TEA domain family member; TAOK, thousand and one amino acid protein kinase; YAP1, Yes-associated protein 1.
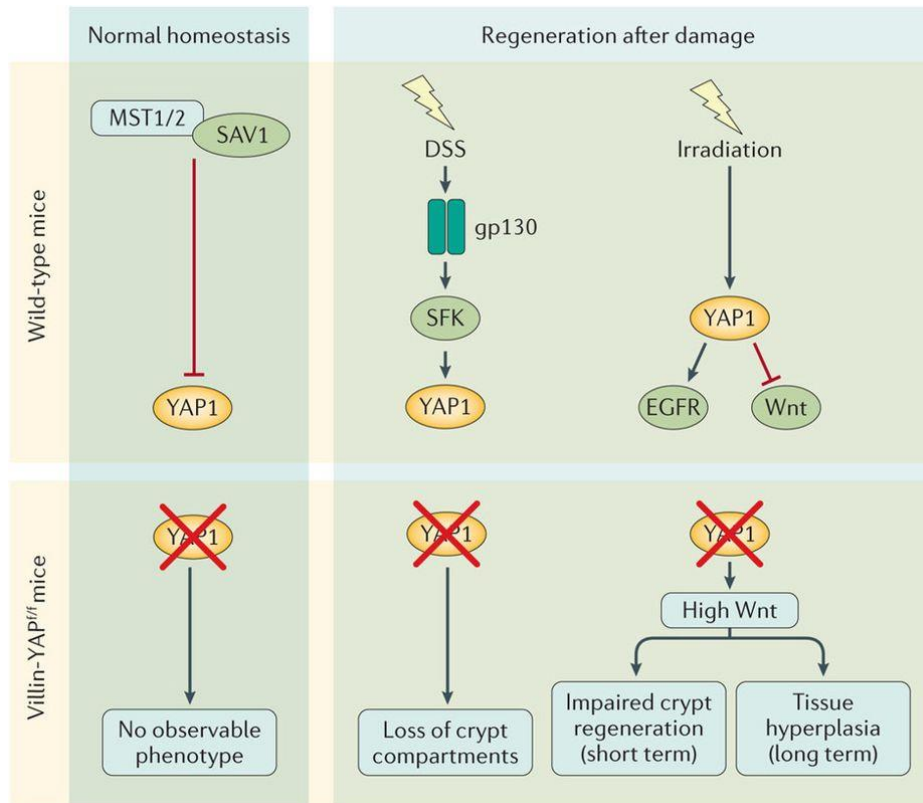
Figure 1.2: The Hippo pathway in intestinal homeostasis and injury-induced regeneration. In normal homeostasis, the Hippo pathway is constitutively active to keep YAP1 activity at low levels. Mice lacking YAP1 in intestinal epithelial cells (Villin-YAPf/f mice) have no observable phenotype. During tissue regeneration induced by damage caused by DSS, YAP1 is activated by gp130 signalling to stimulate cell proliferation. Mice with Yap1 deficiency in intestinal epithelial cells have higher mortality and greater loss of crypt compartments than wild-type mice. Whole-body irradiation of mice causes YAP1 activation, which transiently inhibits Wnt and stimulates EGFR signalling to promote regeneration. Loss of YAP1 in intestinal epithelial cells leads to Wnt hyperactivation, impairing crypt regeneration shortly after injury but inducing tissue hyperplasia in the long term. DSS, dextran sulfate sodium; EGFR, epidermal growth factor receptor; gp130, interleukin-6 receptor subunit beta; SAV1, salvador family WW domain-containing protein 1; SFK, Src family kinases; YAP1, Yes-associated protein 1.
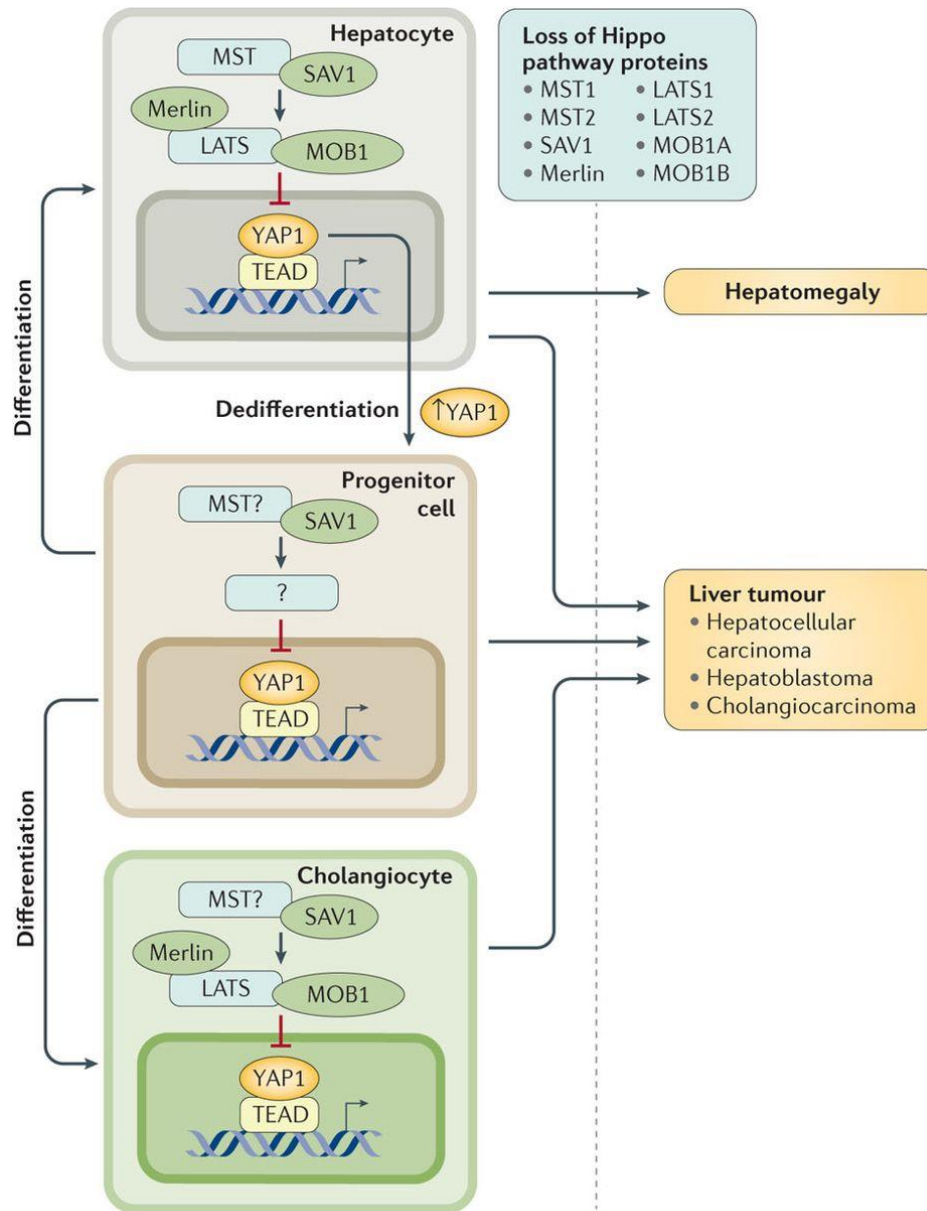
Figure 1.3: Hippo pathway has roles in organ size control and carcinogenesis in different types of liver cells. Disruption of Hippo signalling by deleting core kinase components (MST1, MST2, SAV1, MOB1A, MOB1B, LATS1, LATS2 and Merlin) leads to uncontrolled proliferation of hepatocytes and bile duct epithelial cells (cholangiocytes). Transgenic mice with deficiency in Hippo pathway show spontaneous liver tumours derived from hepatocytes and/or cholangiocytes, as well as hepatomegaly caused by expansion of hepatocytes. Notably, Sav1-knockout mice exhibit expansion of hepatic progenitor cells, which is not observed in mice with deficiency of other Hippo pathway components. On the other hand, induced ectopic expression of YAP1 converts hepatocytes into progenitor or ductal-like cells. These observations indicate a unique role of SAV1 in regulating YAP1 activities in hepatic progenitor roles. LATS, large tumour suppressor; MOB1, Mob1 homologue protein; MST, mammalian ste20-like kinase; SAV1, salvador family WW domain-containing protein 1; YAP1, Yes-associated protein 1.

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Chapter 2: Osmotic stress-induced phosphorylation by NLK at Ser128 activates YAP

2.1 Introduction

The mammalian Hippo pathway core kinase cascade consists of Mammalian Ste20-like kinases 1/2 (MST1/2), MAP kinase kinase kinase kinase (MAP4Ks), large tumor suppressor 1/2 (LATS1/2) and the downstream effectors Yes-associated protein (YAP) and transcriptional co-activator with PDZ-binding motif (TAZ) (Pan 2010; Meng et al. 2015; Zheng et al. 2015). MST1/2 or MAP4Ks activate LATS1/2 by phosphorylating the hydrophobic motif residues in LATS1/2 (Chan et al. 2005; Callus et al. 2006; Praskova et al. 2008; Meng et al. 2015; Ni et al. 2015; Zheng et al. 2015; Hoa et al. 2016). Activated LATS1/2 then phosphorylate YAP and TAZ, leading to 14-3-3 binding and cytoplasmic retention (Zhao et al. 2007). Because YAP and TAZ do not contain DNA-binding domains, they bind to transcription factors, primarily the TEA domain family members 1-4 (TEAD1-4), to induce transcription of downstream target genes, including CTGF, c-Myc, and BIRC5, which have been shown to promote cell proliferation and inhibit cell death (Vassilev et al. 2001; Dong et al. 2007; Zhao et al. 2008). Cytoplasmic YAP and YAZ are physically prevented from interaction with TEADs, and are therefore inactive. Extensive studies have established that the LATS-dependent YAP phosphorylation of Ser127 and resulting cytoplasmic localization is one of the most important mechanisms in physiological regulation of YAP activity. Phosphorylation of YAP Ser127 results in YAP-14-3-3 binding and therefore

cytoplasmic retention. This phosphorylationdependent cytoplasmic localization is highly conserved in Drosophila (Yki Ser 168) and mice (Yap S112) (Dong et al. 2007; Chen et al. 2015). Furthermore, phosphorylation on Ser397 (Ser381 in mice) by LATS leads to phosphodegron-mediated YAP degradation (Zhao et al. 2010).

The physiological effects of the Hippo pathway are mainly exerted through YAP and TAZ. Many upstream signals of YAP and TAZ have been identified (Meng et al. 2016). Most notably, cell contact inhibition, mechanotransduction, cellular energy status, and mitogens in serum can potently regulate YAP activity (Zhao et al. 2007; Dupont et al. 2011; Kim et al. 2011; Yu et al. 2012; DeRan et al. 2014; Mo et al. 2015; Wang et al. 2015). For example, serum deprivation induces YAP Ser127 phosphorylation through activation of LATS, resulting in increased YAP binding with 14-3-3 and cytoplasmic retention (Yu et al. 2012). Most upstream signals regulate YAP activity by influencing YAP Ser127 phosphorylation. Inhibition of Ser127 phosphorylation, by mutating YAP serine 127 to alanine (S127A), was shown to abolish 14-3-3 binding and increase nuclear localization (Zhao et al. 2007). Consistently, *in vivo* study also supports the notion that S127 (S112 in mouse YAP) phosphorylation is critical for YAP cytoplasmic localization as a more prominent nuclear YAP is found in the liver and the colon of YAP S112A knock-in mice (Chen et al. 2015). Therefore, YAP Ser127 phosphorylation has been widely used as an indicator of YAP inactivation. However, the researchers believe that there exist LATS dependent as well as LATS independent mechanisms that are both crucial for YAP/TAZ-driven tumorigenesis (Zanconato et al. 2016). Recently, studies have revealed several kinases that can directly regulate YAP/TAZ activity in addition to LATS, such as AMPK and β-catenin destruction complex. These kinases or

regulatory proteins are restricted to inhibiting YAP/TAZ activity, which are more difficult to serve as cancer therapeutic targets. Therefore, discovering novel regulators of the Hippo pathway, especially those that activate YAP/TAZ activity, may represent a new class of anti-cancer drugs.

In this study, we discovered that osmotic stress regulates YAP activity. Surprisingly, mild osmotic stress induces YAP nuclear localization and target gene expression despite the high level of Ser127 phosphorylation. We further show that the osmotic stress-induced YAP nuclear translocation is mediated through a mitogen-activated protein (MAP) kinase family member nemo-like kinase (NLK), which phosphorylates YAP at the Ser128 residue. Ser128 phosphorylation disrupts YAP binding with 14-3-3 even when Ser127 is phosphorylated, leading to YAP nuclear translocation. This report identifies osmotic stress and NLK as new upstream regulators of YAP, and reveals a mechanism that can override canonical YAP regulation by Hippo pathway-induced Ser127 phosphorylation. Our study also uncovers a functional interplay between osmotic stress response and the Hippo pathway.

2.2 Results

2.2.1 Osmotic stress activates LATS and induces YAP phosphorylation

A wide range of extracellular and intracellular signals, including stress signals, have been shown to regulate YAP and TAZ (Hansen et al. 2015). For example, energy stress activates AMPK to inhibit YAP via both LATS-dependent and LATS-independent

mechanisms (DeRan et al. 2014; Mo et al. 2015; Wang et al. 2015), and oxidative

stress inhibits YAP activity by activating the Hippo pathway (Shao et al. 2014). Here, we

investigated whether osmotic stress was able to affect YAP phosphorylation, and

whether the Hippo pathway was involved. Our previous studies have shown that YAP

phosphorylation is strongly regulated by serum (Yu et al. 2012). As expected, YAP was

dephosphorylated in the presence of serum whereas it was highly phosphorylated in the

absence of serum, as determined by mobility shift on phos-tag gel (Figure S2.1A).

Treatment of HEK293A cells with 0.2 M sorbitol induced rapid and robust YAP

phosphorylation in the presence of serum. In the absence of serum, YAP was already

highly phosphorylated and sorbitol treatment had no obvious effect on YAP

phosphorylation. The effect of YAP phosphorylation by sorbitol was dose-dependent.

We found that sorbitol concentrations lower than 100 mM sorbitol had little effect on

YAP phosphorylation (Figure S2.1A). Only when sorbitol concentrations reached 200

mM or higher was YAP phosphorylation induced, in addition to LATS phosphorylation.

YAP Ser127 is an important site phosphorylated by LATS, and its phosphorylation

inhibits YAP activity by inducing 14-3-3-mediated cytoplasmic retention. Western blot

analysis showed that osmotic stress induced YAP Ser127 phosphorylation (Figure

2.1A). TAZ is a YAP homolog similarly regulated by LATS. As expected, sorbitol

induced a mobility shift of TAZ, suggesting an increased phosphorylation (Figure 2.1A).

TAZ is known to be strongly destabilized upon phosphorylation by LATS (Liu et al.

2010). Consistently, TAZ protein levels were decreased upon prolonged sorbitol

treatment. In addition to HEK293A cells, osmotic stress induced YAP phosphorylation in

MCF10A cells (Figure S2.1B), indicating that osmotic stress induces YAP/TAZ phosphorylation in a cell type-independent manner.

To investigate whether the Hippo pathway is involved, we checked for LATS kinase activity in response to osmotic stress. We found that osmotic stress increased LATS1/2 phosphorylation in their hydrophobic motif (HM, Thr1079 for LATS1 and Thr1041 for LATS2) (Figure 2.1B), which is known to promote LATS kinase activity (Chan et al. 2005; Hergovich et al. 2006; Praskova et al. 2008; Yin et al. 2013; Hoa et al. 2016). LATS activation was further confirmed by LATS *in vitro* kinase assay using immunoprecipitated LATS1 from cells treated with or without sorbitol. Purified recombinant YAP was used as a substrate for LATS kinase assay. The result showed a significant increase of LATS1 activity by 0.2 M sorbitol treatment as measured by YAP Ser127 phosphorylation (Figure 2.1C). As expected, serum starvation strongly activated LATS1. In addition to sorbitol, NaCl was also used to test the effects of osmotic stress on YAP. Similar to sorbitol treatment, 0.1 M NaCl induced YAP phosphorylation at Ser127 and LATS phosphorylation at HM, indicating that the Hippo pathway activation by osmotic stress is not restricted to organic osmolyte-induced hyperosmolarity (Figure S2.1C). In summary, these observations demonstrate that osmotic stress activates the Hippo pathway component LATS and increases YAP phosphorylation.

YAP/TAZ are transcription co-activators and function by binding to transcription factors in the nucleus, such as TEAD, to induce gene expression. In order to determine YAP activity, we first checked its subcellular localization under sorbitol treatment. Immunofluorescence staining showed that YAP and TAZ remained in the nucleus 1 h after sorbitol treatment in the presence of serum although YAP Ser127 was highly

phosphorylated (Figure 2.1D). Similar results were observed when cells were treated with NaCl (Figure S2.1D). Notably, some YAP proteins eventually accumulated in the cytoplasm after prolonged osmotic stress (Figure S2.1E). In our immunofluorescence staining experiments, an antibody recognizing both YAP and TAZ was used. Our later result showed that TAZ behaved similarly to YAP in terms of localization, whereas in this study, we mainly focused on YAP. The above results are surprising and perplexing because it has been well established that YAP Ser127 phosphorylation promotes 14-3-3 binding and cytoplasmic localization. Here, the coupling between YAP Ser127 phosphorylation and cytoplasmic localization is obviously disrupted under osmotic stress condition; YAP Ser127 phosphorylation and nuclear localization are simultaneously induced. These observations are not consistent with the current dogma of YAP regulation.

To further test whether YAP is inhibited by osmotic stress, we measured expression of YAP target gene CTGF and CYR61. We found that expression of these two genes was not decreased 4 h after sorbitol treatment (Figure 2.1E). We then checked whether this sorbitol-induced YAP phosphorylation could disrupt its binding with TEAD. Co-immunoprecipitation (co-IP) results of endogenous proteins showed that YAP–TEAD interaction was not disrupted by 1 h of sorbitol treatment (Figure 2.1F). As a positive control, serum starvation reduced YAP–TEAD interaction. Furthermore, co-IP experiments showed that YAP-14-3-3 binding was not increased upon sorbitol treatment (Figure 2.1G). These data suggest that YAP is neither associated with 14-3-3 nor inhibited upon osmotic stress, despite activation of the Hippo pathway and high levels of YAP phosphorylation.

2.2.2 Osmotic stress induces YAP nuclear localization and activation

Phosphorylation of YAP Ser127 is widely used as a marker of YAP inactivation. However, YAP Ser127 phosphorylation induced by osmotic stress does not appear to inhibit YAP, as its nuclear localization is not affected by sorbitol. We then tested whether osmotic stress might activate YAP. To this end, we determined YAP localization upon sorbitol treatment in HEK293A cells under serum-free condition in which YAP is cytoplasmic. Immunofluorescence staining, using either an antibody recognizing both YAP and TAZ or a YAP-specific antibody, showed YAP was translocated into the nucleus upon sorbitol or NaCl treatment (Figures 2.2A and S2.2A). We also wanted to know whether TAZ was similarly regulated by osmotic stress. Since we did not have TAZ antibody suitable for immunofluorescence staining, we used the YAP/TAZ antibody in YAP KO cells; YAP/TAZ signals detected in YAP KO cells should reflect only endogenous TAZ localization. We found that sorbitol also induced TAZ nuclear translocation (Figures 2.2B and S2.2B). Western blot analysis showed that YAP was completely deleted in YAP KO cells (Figure S2.2C). The nuclear translocation of YAP by osmotic stress occurred at a short time point (peaking at 1 h upon osmotic stress), whereas at later time points YAP started to move to the cytoplasm (Figure 2.2C). Therefore, osmotic stress induces a transient YAP nuclear localization. In addition to subcellular localization of YAP, we queried whether osmotic stress activates YAP mediated gene transcription in the absence of serum. We measured the YAP downstream target gene CTGF and CYR61 expression and found a significant increase 4 h after sorbitol treatment (Figure 2.2D). This effect was abolished in YAP/TAZ knockout cells, suggesting that sorbitol-induced expression of CTGF and CYR61 was

YAP/TAZ-dependent. Consistent with YAP nuclear localization and activation, we found that sorbitol treatment significantly reduced the interaction between YAP and 14-3-3 (Figure 2.2E). Collectively, our data show that osmotic stress induces a transient activation of YAP in the absence of serum, while osmotic stress had a minor effect on YAP in the presence of serum. This osmotic stress-induced YAP regulation is uncoupled with YAP Ser127 phosphorylation.

2.2.3 NLK mediates osmotic regulation of YAP

To understand the mechanism of YAP regulation by osmotic stress, we searched for signaling molecules that may be responsible for YAP nuclear translocation induced by osmotic stress. p38 and JNK are members of the MAP kinase family, and they are activated by various cellular stresses, including osmotic stress (Han et al. 1994; Berl et al. 1997; Kyriakis and Avruch 2001). To test whether p38 and JNK are involved in osmotic stress-induced YAP activation, we pretreated cells with the p38 inhibitor SB203580 (2 µM) or JNK inhibitor SP600125 (20 µM) followed by sorbitol treatment, and YAP localization was determined. In both cases, inhibition of p38 or JNK did not abolish osmotic stress-induced YAP nuclear translocation (Figure 2.3A). Furthermore, a combined inhibition of both p38 and JNK did not block the osmotic stressinduced YAP nuclear localization (Figure S2.3A). The efficiency of these inhibitors was confirmed by checking the phosphorylation level of downstream substrates MK-2 at Thr334 for p38, and c-Jun at Ser63 for JNK (Figure 2.3A). Collectively, the above observations show that p38 and JNK are not required for YAP nuclear translocation in response to osmotic stress, indicating that other signaling molecules are involved in regulation of YAP upon osmotic stress.

Recently, we have discovered that nemo-like kinase (NLK), an atypical MAP kinase, can be activated by osmotic stress and plays a role in cellular response to osmotic stress (Yuan et al. 2015). In order to test the role of NLK, we used CRISPR/Cas9 system to knock out NLK by transfecting Cas9 and a guide RNA targeting NLK into HEK293A cells. Two NLK guide RNA sequences were used, and both resulted in an efficient deletion of NLK in transiently transfected cells (Figure S2.3B). YAP nuclear translocation by osmotic stress was blocked in the majority of these KO cell pools, which presumably had no NLK (Figure 2.3B). Similar results were observed in cells with two independent NLK guide sequences (Figures 2.3B and S2.3C). Quantification of the staining results showed a dramatic decrease of nuclear YAP in the NLK KO cell pool after sorbitol treatment (Figures 2.3B and S2.3C). We do not have a high-quality NLK antibody suitable for immunofluorescence staining to verify NLK KO in individual cells. However, Western blotting with a NLK antibody did confirm a strong reduction in NLK protein in the pooled cells transfected with the NLK CRISPR/Cas9 guide sequences (Figure S2.3B). Subcellular fractionation was performed to confirm the localization of YAP. Consistent with the immunofluorescence data, osmotic stress increased nuclear YAP in WT cells under serum starvation, and this effect was diminished in NLK KO cells (Figure S2.3D). These results suggest that NLK serves as a mediator between osmotic stress and YAP. In addition, overexpression of wild-type NLK (NLK-WT) but not the kinase-negative mutant (NLK-KN) induced nuclear localization of YAP in the absence of serum (Figure 2.3C), supporting the notion that NLK induces YAP nuclear localization. To further investigate the role of NLK in YAP regulation, NLK-WT and NLK-KN were overexpressed in HEK293A cells. We observed

that only wild-type NLK induced YAP mobility shift on a phos-tag gel, indicating that

NLK could promote YAP phosphorylation (Figure 2.3D). This notion was further

confirmed by *in vitro* kinase assays, which showed that immunoprecipitated NLK-WT,

but not NLK-KN, was able to phosphorylate purified GST-YAP (Figure 2.3E).

2.2.4 Osmotic stress disrupts YAP and 14-3-3 binding by inducing Ser128

phosphorylation

YAP phosphorylation at Ser127 site increases its binding with 14- 3-3 and results

in cytoplasmic retention (Zhao et al. 2007; Zhao et al. 2010). Here, we observed that

although osmotic stress increased YAP S127 phosphorylation (Figure 2.1A), YAP-14-3-

3 binding was actually decreased (Figure 2.2E). There is an apparent uncoupling

between YAP S127 phosphorylation and its interaction with 14-3-3 under osmotic

stress. Furthermore, this osmotic stress-induced uncoupling between YAP S127

phosphorylation and cytoplasmic localization requires NLK. In order to understand how

NLK regulates YAP localization under osmotic stress, it is critical to identify the NLK-

induced YAP phosphorylation site that may be responsible for the disruption of 14-3-3

binding. As a MAP kinase family member, NLK is a proline-directed kinase that

phosphorylates serines/threonines that are followed by a proline. We searched YAP

amino acid sequence and identified ten putative NLK phosphorylation sites. Notably, the

NLK consensus site Ser128 in YAP is adjacent to the Ser127 site and is within the 14-3-

3 binding pocket. Previous phosphoproteomic studies also showed that YAP Ser128 is

a phosphorylation site (Lee and Yonehara 2012; Zhao et al. 2014; Wang et al. 2015).

Thus, we hypothesized that NLK phosphorylates YAP Ser128 to disrupt YAP-14-3-3

binding, leading to the uncoupling event between S127 phosphorylation and cytoplasmic localization.

To test the above hypothesis, we obtained YAP Ser128 phosphospecific antibody, which is described in the accompanying paper by Moon et al (Moon S 2016). To confirm the specificity of the pYAP Ser128 antibody, we mutated this serine to alanine (S128A) to prevent phosphorylation. Flag-YAP wild-type (WT) and Flag-YAP S128A were expressed in HEK293A cells. YAP proteins were then immunoprecipitated, and samples were subjected to Western blot analysis. The result showed that this antibody recognizes Ser128 phosphorylated YAP, as there was no signal detected in the YAP S128A mutant (Figure S2.4A). To determine whether YAP Ser128 could be phosphorylated by NLK, NLK and YAP were ectopically co-expressed in HEK293A cells. Results showed that NLK expression indeed induced Ser128 phosphorylation of WT YAP, and this phosphorylation was abolished in YAP S128A mutant (Figure 2.4A). In addition, immunoprecipitated NLK, but not the kinase-inactive mutant NLK-KN, phosphorylated YAP Ser128 *in vitro* (Figure 2.4B). The low level of signal detected in the pcDNA control or NLK-KN by the YAP S128 phosphoantibody might be due to a weak recognition of the unphosphorylated YAP protein by the antibody. Next, we tested whether sorbitol could induce YAP Ser128 phosphorylation. We found that sorbitol increased Ser128 phosphorylation of the endogenous YAP in both HEK293A cells and MCF10A cells (Figures 2.4C and S2.4B). Consistently, sorbitol also induced Ser128 phosphorylation of the transfected YAP (right panel, Figure 2.4C). Furthermore, this sorbitol-induced YAP Ser128 phosphorylation was diminished in the NLK knockout cell

pool (Figure 2.4D). Collectively, these results suggest that osmotic stress induces YAP

Ser128 phosphorylation and this phosphorylation is mediated by NLK.

To examine the effect of Ser128 phosphorylation on YAP and 14-3-3 binding,

YAP Ser128 was mutated to nonphosphorylatable alanine (S128A) or phosphomimetic

aspartate (S128D). Myc-tagged 14-3-3 was co-transfected with WT or mutant YAP.

Coimmunoprecipitation showed that the phosphomimetic YAP S128D mutant abolished

its interaction with 14-3-3 (Figure 2.4E), supporting that Ser128 phosphorylation

interferes with YAP and 14-3-3 interaction. In contrast, YAP S128A showed stronger

interaction with 14-3-3, further supporting the notion that phosphorylation on YAP

Ser128 disrupts YAP-14-3-3 interaction (Figure 2.4E). As expected, YAP S127A

showed a weak 14-3-3 interaction because its phosphorylation is required for this

interaction (Zhao et al. 2007). We next tested the effect of sorbitol on the interaction

between 14-3-3 and wild-type YAP or YAP S128A mutant. Osmotic stress reduced the

interaction between 14-3-3 and wild-type YAP, but not the S128A mutant (Figure 2.4F).

These results indicate a model in which sorbitol induces YAP S128 phosphorylation to

disrupt YAP and 14-3-3 association.

2.2.5 YAP Ser128 phosphorylation is required for sorbitol-induced YAP nuclear localization

We then tested whether YAP Ser128 mutation would affect YAP subcellular

localization by NLK and osmotic stress. By co-transfection, NLK induced nuclear

accumulation of WT YAP, but failed to do so in the YAP S128A mutant (Figure 2.5A),

suggesting that phosphorylation on the Ser128 site is required for NLK to induce YAP

nuclear translocation. Furthermore, YAP WT and mutant stable cell lines were generated. WT, S128A mutant, and S128D mutant YAP were stably expressed in the YAP KO HEK293A cells (Figure S2.4C). The stably expressing Flag-tagged YAP WT behaved similarly as endogenous YAP. For instance, serum starvation induced YAP cytoplasmic localization, and sorbitol treatment induced its nuclear translocation in the absence of serum (Figure 2.5B). YAP S128D mutant was constitutively nuclear even in the absence of serum, consistent with the result that the YAP S128D mutant is defective in 14-3-3 binding. Of note, sorbitol treatment had a minor effect on the subcellular localization of YAP S128D mutant compared with WT YAP. To test whether YAP Ser128 phosphorylation is required for its osmotic stress-induced nuclear translocation, localization of YAP S128A was examined. We observed that YAP S128A mutant retained a normal serum response; that is, serum starvation induced its cytoplasmic localization (Figure 2.5B). Actually, upon serum starvation, YAP S128A mutant showed an even more cytoplasmic localization than the wild-type YAP. Importantly, YAP nuclear localization by sorbitol treatment was strongly diminished in YAP S128A mutant cells. Subcellular fractionation experiments showed a consistent result with the immunofluorescence observations (Figure S2.4D). Notably, sorbitol induces YAP Ser127 phosphorylation in both YAP S128A and YAP S128D mutants in the presence of serum, while in the absence of serum, Ser127 phosphorylation remained high (Figure S2.4E). These results show that YAP Ser127 phosphorylation does not correlate with its localization in the presence of osmotic stress, which is consistent with our model that S128 phosphorylation (mimicked by S128D mutation) overrides the S127 regulation on YAP. Collectively, we show that Ser128 plays a critical role in YAP subcellular

localization and its phosphorylation is required for sorbitol to induce YAP nuclear localization, consistent with the notion that NLK-induced YAP nuclear translocation requires phosphorylation of YAP on Ser128 (Figure 2.5A). In summary, when S128 is dephosphorylated, as mimicked by the S128A mutant, sorbitol could not induce YAP nuclear localization. When YAP S128 is phosphorylated, as mimicked by the S128D mutant, it is constitutively nuclear and serum starvation could not efficiently induce YAP cytoplasmic localization. Our data suggest a critical role of S128 in YAP regulation and that osmotic stress induces YAP nuclear localization by phosphorylating Ser128 and disrupting 14-3-3 binding.

2.2.6 YAP Ser128 phosphorylation is important for cell survival in hyperosmotic environment

Osmotic stress causes a wide spectrum of signaling events leading to alteration of gene expression in order for cells to adapt to the hyperosmotic environment. Cells may undergo apoptosis if adaptive mechanisms fail to balance the biochemical homeostasis, such as osmolyte concentrations (Burg et al. 2007). YAP activation has been implicated in inducing gene expression for cell survival. To investigate the function of YAP activation in cellular osmotic adaptation, we generated cells with re-expression of WT YAP, YAP S128A, and YAP S128D in the YAP/TAZ dKO HEK293A cells (Figure S2.4C). Cell growth was determined in the presence or absence of osmotic stress. Under normal osmotic conditions, these three cell pools showed similar growth rates (Figure 2.6A). However, in the presence of osmotic stress, YAP S128D cells were able to resume growth whereas the YAP WT or the S128A mutant expressing cells were more sensitive to osmotic stress (Figure 2.6A). Next, we performed cell cycle

distribution by FACS analysis and observed that WT YAP and YAP S128A mutant cells showed more severe cell cycle arrest and displayed much high levels of cell death as indicated by the sub-G1 phase (Figures 2.6B and S2.5). Annexin V staining indicated that the YAP S128D expressing cells were more resistant to apoptosis induced by hyperosmotic stress (Figure 2.6C). The above data indicate that the transient YAP activation may be advantageous for cell adaptation and survival under osmotic stress.

2.3 Discussion

The Hippo pathway is an exciting young field with great importance in both normal physiological regulations and pathological conditions such as tumorigenesis. YAP and TAZ are the major downstream effectors of the Hippo pathway, and their increased expression and activity are frequently associated with human cancers (Plouffe et al. 2015). Elucidating upstream regulators of the Hippo pathway remains an important research direction. Many studies have revealed that the Hippo–YAP pathway can integrate various upstream extracellular and intracellular signals. This report adds a new dimension to YAP/TAZ regulation. We show that NLK mediates osmotic signals to activate YAP, revealing an intricate interplay between osmotic stress via phosphorylation of YAP Ser128 and the Hippo pathway via phosphorylation of YAP Ser127.

As a transcription co-activator, YAP nuclear localization determines its activity. The current dogma of Hippo pathway regulation is that LATS inhibits YAP by phosphorylating Ser127, leading to increased YAP binding with 14-3-3 and cytoplasmic

retention (Zhao et al. 2007). Our results show that osmotic stress induces transient YAP nuclear localization and activation despite activation of LATS and YAP Ser127 phosphorylation. These observations demonstrate a LATS-independent mechanism utilized by osmotic stress to override the inhibitory effect of LATS on YAP. We show that YAP nuclear translocation induced by sorbitol treatment is mediated, at least in part, by NLK. Mechanistically, NLK phosphorylates YAP on Ser128, which is located in the 14-3-3 binding region of YAP. We propose that YAP Ser128 phosphorylation is not compatible with 14-3-3 binding, therefore, inhibiting YAP association with 14-3-3 and inducing its accumulation in nucleus (Figure 2.6D). Consistent with this model, osmotic stress cannot influence YAP subcellular localization when YAP Ser128 is mutated to alanine. Furthermore, the YAP S128D mutant appears to mimic the effect of phosphorylation as this mutant shows constitutively nuclear localization even under serum starvation, which normally induces Ser127 phosphorylation and cytoplasmic localization of YAP. Yorkie (Yki) is the Drosophila homolog of YAP. Consistent with the mammalian YAP protein, the phosphorylation of Yki Ser169, which is the analogous residue of YAP Ser128, has also been shown to promote Yki activity *in vivo* (Oh and Irvine 2009). In the accompanying paper by Moon et al, these authors suggest a model that YAP S128 phosphorylation inhibits S127 phosphorylation (Moon S 2016). However, we would like to propose that YAP S128 phosphorylation mainly interferes 14-3-3 binding to promote its nuclear accumulation. Nevertheless, our data could not exclude the possibility that S128 phosphorylation may interfere with S127 phosphorylation in YAP.

In addition to the regulation of YAP by NLK, it is possible that YAP is regulated by osmotic stress-induced cytoskeleton remodeling. One immediate osmotic stress response in mammalian cells is the rapid reorganization of the actin cytoskeleton. The pattern of actin remodeling varies between cell types, but typically, an increase in F-actin level is observed (Di Ciano et al. 2002; Bustamante et al. 2003; Yamamoto et al. 2006). Various studies have shown that YAP activity is associated with actin remodeling in response to different mechanical cues. For example, F-actin is known to be important for YAP regulation by cell shape and cell attachment/detachment (Dupont et al. 2011; Wada et al. 2011; Zhao et al. 2012). It will be interesting to explore the relationship between actin regulation and YAP activity under osmotic stress, and whether it is mediated by NLK.

It has also been reported that Ser127 may not be the only site regulating YAP localization although the mechanistic insights for this observation have not been provided (Wada et al. 2011; Barry et al. 2013). For example, YAP S127A protein is not restricted to the nucleus in the intestine of transgenic mice (Barry et al. 2013). It is possible that YAP Ser128 phosphorylation may regulate YAP localization under such conditions. It is worth noting that YAP Ser128 phosphorylation has been observed in many phosphoproteomic studies (Lee and Yonehara 2012; Zhao et al. 2014; Wang et al. 2015). We speculate that YAP Ser128 may be a common regulatory phosphorylation site for proline-directed kinases, such as CDK family and MAP kinase family. Notably, YAP Ser128 has been implicated as a phosphorylation target site of cyclin-dependent kinase 1 (CDK1) (Zhao et al. 2014). Future study is needed to determine the role of CDK1 in regulation of YAP activity and its involvement in Ser128 phosphorylation.

Phosphorylation of Ser128 in YAP would suppress the effect of the LATS-dependent

Ser127 phosphorylation and therefore, provides a mechanism for the integration of

numerous signaling pathways with Hippo.

A major function of YAP is to promote cell survival and proliferation. YAP

activation by osmotic stress might serve as an immediate stress response for cells to

adapt to the stressful environment. Consistently, cells expressing the more active YAP

S128D mutant show increased cell viability and proliferation under the osmotic stress.

The phosphorylation of Ser127 has been widely recognized as the indicator of YAP

cellular localization. Here, we report that YAP Ser128 phosphorylation can override the

inhibitory regulation of the canonical Hippo pathway and Ser127 phosphorylation,

leading to YAP nuclear localization and activation. Our study not only identifies osmotic

stress and NLK as novel regulators of YAP, but suggests that the current dogma of YAP

regulation needs to be modified. YAP Ser127 phosphorylation does not necessarily

equal to YAP cytoplasmic localization and inhibition. Additional layers of regulation,

such as Ser128 phosphorylation, can influence the outcome of Ser127 phosphorylation

on YAP activity.

2.4 Experimental procedures

Cell culture and transfection

HEK293A cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10%

fetal bovine serum (FBS) (Gibco) and 100 units/ml penicillin and streptomycin

(Invitrogen). Cells were incubated in a humidified incubator with 5% CO2. MCF10A cells were cultured in DMEM/F12 supplemented with 5% horse serum, 20 ng/ml EGF, 0.5 lg/ml hydrocortisone, 10 lg/ml insulin, 100 ng/ml cholera toxin, and 100 units/ml penicillin and streptomycin. Cells were harvested 24 h post-transfection for protein analysis. PolyJet *In Vitro* DNA Transfection Reagent (SignaGen Lab) was used for transfection.

Antibodies and chemicals

Anti-YAP/TAZ, GAPDH, and Myc-HRP antibodies were obtained from Santa Cruz Biotechnology. Anti-phospho-YAP (S127), LATS1, phospho-Lats1/2 (Thr 1079/1041), phospho-MK2 (T334), phospho-c- Jun (S63), and HA-HRP antibodies forWestern blot, and Myc and Flag (DYKDDDDK) tag antibodies for IF were obtained from Cell Signaling. Anti-YAP antibody for IP was obtained from Bethyl Laboratory. Anti- TEAD1 (TEF-1) and HSP90 antibodies were obtained from BD Biosciences. Anti-GST, Flag-HRP, and Flag (M2) for IP were obtained from Sigma-Aldrich. Anti-thioesterphosphate antibody was obtained from Abcam. Anti-Myc (9E10) antibody for IP was a homemade reagent. Anti-phospho-YAP (Ser128) was a kind gift from Professor Eek-hoon Jho. All pYAP S128 blotting were done with immunoprecipitated YAP. Alexa Fluor 488 and 546-conjugated secondary antibody for IF were obtained from Invitrogen. Chemicals SB203580 (p38 inhibitor) and SP600125 (JNK inhibitor) were from Tocris.

Immunofluorescence staining

HEK293A cells were plated on fibronectin-coated coverslips. After sorbitol or NaCl treatment, cells were fixed with 4% paraformaldehyde for 15 min and permeabilized with

0.1% Triton X-100 for 5 min. After blocking in 3% BSA in PBS for 30 min, cells were incubated with primary antibodies diluted in 3% BSA overnight at 4°C. After three washes with PBS, cells were incubated with Alexa Fluor secondary antibodies (Invitrogen, 1:1,000 dilution) for 1 h in the dark at room temperature. Coverslips were mounted with ProLong Gold antifade mountant with DAPI (Life Technologies). Slides were detected using Olympus FV1000 confocal microscopy. The final images were obtained and analyzed by using confocal microscopy with FLUOVIEW viewer software.

Co-immunoprecipitation

Cells were lysed with mild lysis buffer (20 mM Tris–HCl pH 7.5, 100 mM NaCl, 50 mM NaF, 2 mM EDTA, 1% NP-40 substitute) supplemented with protease inhibitor (Roche), phosphatase inhibitor (Thermo Scientific), and 1 mM PMSF for 10 min on ice and centrifuged at 12,000 g for 15 min at 4°C. The supernatants were incubated with the appropriate antibodies overnight at 4°C, and protein A/G magnetic beads (Thermo Scientific) were added for 1 h. Proteins were washed with lysis buffer three times and were eluted with SDS–PAGE sample buffer. Samples were followed by Western blot analysis.

***In vitro* kinase assay**

For the LATS1 kinase assay, HEK293A cells were treated with sorbitol and were lysed with mild lysis buffer. For NLK kinase assay, cells were transfected with Flag-NLK-WT or Flag-NLK-KN constructs. Twenty-four hours after transfection, cells were lysed with mild lysis buffer. Proteins were immunoprecipitated using LATS1 antibody or FLAG antibody. The immunoprecipitates were washed three times with lysis buffer, followed

by a single wash with TBS. Immunoprecipitated kinases were subjected to kinase assay in the kinase buffer (NEBuffer for Protein Kinases) in the presence of 500 lM cold ATP or ATP-c-S and 1 lg GST-YAP. The reaction mixtures were incubated for 30 min at 30°C. For ATP-c-S reaction, p-Nitrobenzyl mesylate (PNBM) was added after the kinase reaction for 1 h to alkylate the thiophosphorylation site on the substrates. The reactions were terminated with SDS sample buffer, and subjected to SDS–PAGE. Phosphorylation of YAP was determined by phospho-YAP Ser127 or Ser128, and thiophosphate ester antibodies were used to detect total substrate phosphorylation.

RNA isolation and real-time PCR

Total RNAs were extracted using a RNeasy kit (Qiagen). cDNAs were synthesized by reverse transcription using iScript reverse transcriptase (Bio-Rad). cDNAs were then used for quantitative real-time PCR with gene-specific primers and KAPA SYBR FAST qPCR master mix (Kapa Biosystems) using the 7300 Real-time PCR system (Applied biosystems). The relative abundance of mRNAs was calculated by normalizing to GAPDH mRNA.

CRISPR

CRISPR genomic editing technology was used for the deletion of NLK. The guide RNA sequences were cloned into the px459 plasmid (Addgene 48319, a gift from Dr. Feng Zhang.). The constructed plasmids were transfected into HEK293A. 24 h after transfection, the transfected cells were enriched by 1 lg/ml puromycin selection for 2–3 days and then were used for experiments. Two guide RNA sequences were used, #1: 50-AAAATGA TGGCGGCTTACAA-30 and 50-TTGTAAGCCGCCATCATTTT-30, #2:

50-ACACCATCTTCATCCGGGGT-30 and 50-ACCCCGGATGAAGAT GGTGT-30.

Knockout efficiency of the cell pool was assayed with Western blot.

Stable cell lines

To generate YAP mutant expressing stable cells, retrovirus infection was performed by transfecting 293T cells with Retroviral gene (pQCXIH-Flag-YAP-WT/S128A/S128D), pCGP (pCMV-Gag-Pol), and pCMV-VSVG (envelop) constructs. Retroviral supernatant was collected at 12, 24 and 48 h, and filtered through 0.45-lm syringe filter. Filtered viral supernatant was used to infect HEK293A YAP KO or YAP/TAZ dKO cells with 10 lg/ml polybrene (Sigma- Aldrich). Infected cells were selected with 200 lg/ml hygromycin (Invitrogen).

Cell cycle analysis

After HEK293A cells were treated with sorbitol for indicated time points, cells were collected and fixed with cold 70% ethanol in PBS, incubated with 50 lg/ml propidium iodide (PI) and 0.5 lg/ml RNase A at 37°C for 30 min, and processed with the BD FACSCanto Flow Cytometer (BD Biosciences). The results were analyzed with FlowJo 7.6 software.

Annexin V staining

HEK293A cells were collected by trypsinization after sorbitol treatment for indicated time points. The PE Annexin V Apoptosis Detection Kit (BD Biosciences) was used as per manufacturer protocol. Samples were then processed by the FASCanto Flow Cytometer (BD Biosciences), and the results were analyzed with FlowJo 7.6 software.

Subcellular fractionation

Cells were lysed in hypotonic buffer A (10 mM HEPES pH 7.6, 1.5 mM MgCl2, 10 mM KCl, 1 mM DTT, 1× protease inhibitor cocktail (Roche), 1× phosphatase inhibitor cocktail (Thermo Fisher Scientific), 2 mM PMSF). Nuclei were pelleted at 800 g for 5 min and washed with hypotonic buffer A twice, then incubated in buffer B (20 mM HEPES pH 7.6, 5% glycerol, 420 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 1× protease inhibitor cocktail, 1× phosphatase inhibitor cocktail, 2 mM PMSF) with vigorous rotation for 30 min. Samples were then spun down at 18,000 g for 5 min, and supernatant was collected for nuclear proteins. Samples were handled at 4°C.

Statistical analysis

Each experiment was repeated at least three times. Data are presented as mean ± SEM. All statistical tests were performed using a Student's t-test (unpaired, two-tailed), *P < 0.05. No statistical method was used to predetermine sample size.

2.5 Acknowledgments

Chapter 2, in full, is a reprint of the material as it appears in EMBO Reports, 2017, Hong, A.W., Meng, Z., Yuan, H-X., Plouffe, S.W., Moon, S., Kim, W., Jho, E-H., Guan, K-L., EMBO Press, 2017. The dissertation author was the primary investigator and author of this paper.

Figure 2.1: Osmotic stress induces YAP phosphorylation and LATS activation but does not inhibit YAP. (A) Sorbitol stimulates YAP phosphorylation. HEK293A cells were cultured in the presence or absence of serum, and treated with 0.2 M sorbitol for the indicated time points. Phos-tag gel was used to assess total YAP phosphorylation based on mobility shift. Note that YAP and TAZ are recognized by the same antibody. l.e. denotes long exposure of YAP and TAZ. (B) Osmotic stress induces LATS phosphorylation. HEK293A cells were treated with 0.2 M sorbitol for 30 or 60 min in the presence or absence of serum. LATS phosphorylation at the hydrophobic motif (HM) was detected with the phosphospecific pLATS antibody. (C) Osmotic stress increases LATS kinase activity. HEK293A cells were treated with 0.2 M sorbitol for 30 min. Lats1 was immunoprecipitated, and an *in vitro* kinase assay was performed using recombinant GST-YAP as a substrate. Phosphorylation of YAP was determined by immunoblotting with phospho-YAP (S127) antibody. Data are presented as mean ± SEM. *$P < 0.05$ (two-tailed Student's t-test, n = 3). (D) Osmotic stress does not lead to YAP cytoplasmic localization. HEK293A cells were treated with 0.2 M sorbitol for 1 h in the presence of serum. YAP subcellular localization was determined by immunofluorescence staining (red). DAPI (blue) was used to stain for DNA. Scale bars: 20 µm. Quantification of more nuclear (N > C) or more cytosolic (N < C) YAP signal was determined with randomly chosen fields, each with approximately 100 cells. (E) Osmotic stress does not reduce YAP target gene expression. HEK293A cells were treated with 0.2 M sorbitol for 4 h. mRNA levels of CTGF and CYR61 were measured by quantitative RT–PCR and normalized to GAPDH control. Data are presented as mean ± SEM. n.s. means $P > 0.05$ (two-tailed Student's t-test, n = 3). (F) Osmotic stress does not disrupt the interaction between YAP and TEAD1. HEK293A cells were treated with 0.2 M sorbitol for 1 h. Endogenous YAP was immunoprecipitated with YAP antibody, and TEAD1 and YAP were detected by Western blot. Cells were serum starved for 1 h as indicated (- serum). (G) Osmotic stress does not increase the interaction between YAP and 14-3-3. HEK293A cells were transiently transfected with Flag-YAP and Myc-14-3-3, then treated with 0.2 M sorbitol for the indicated time points. Myc-14-3-3 was immunoprecipitated, and the co-precipitated Flag-YAP was detected.
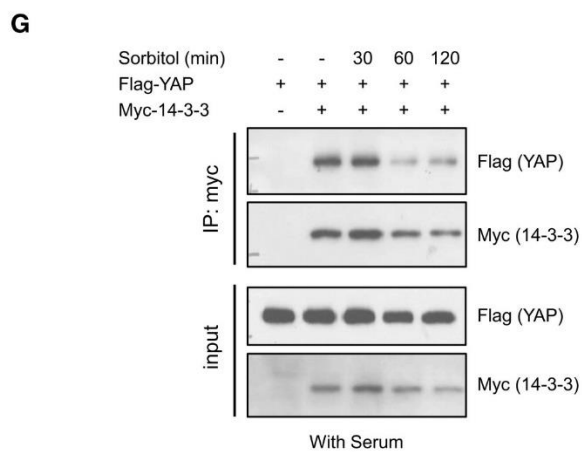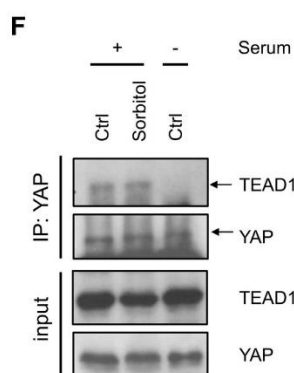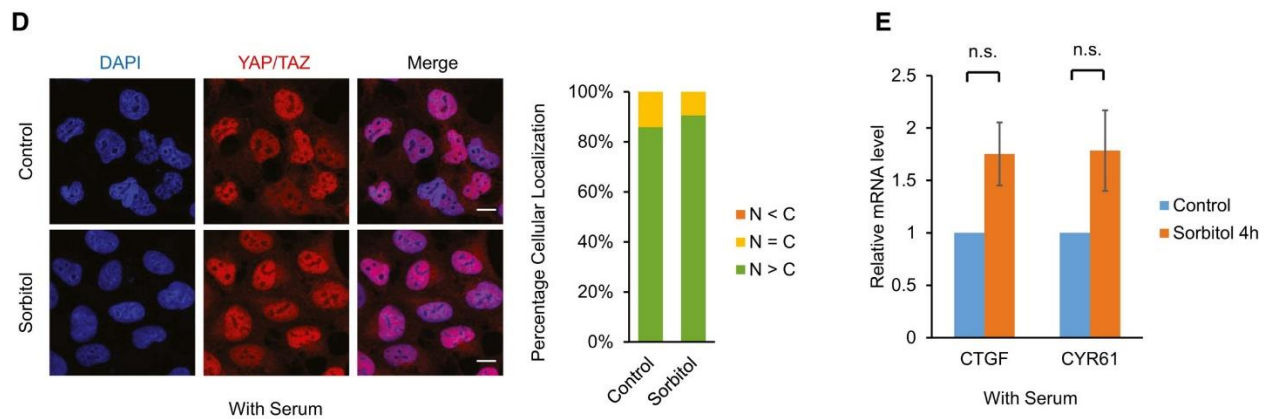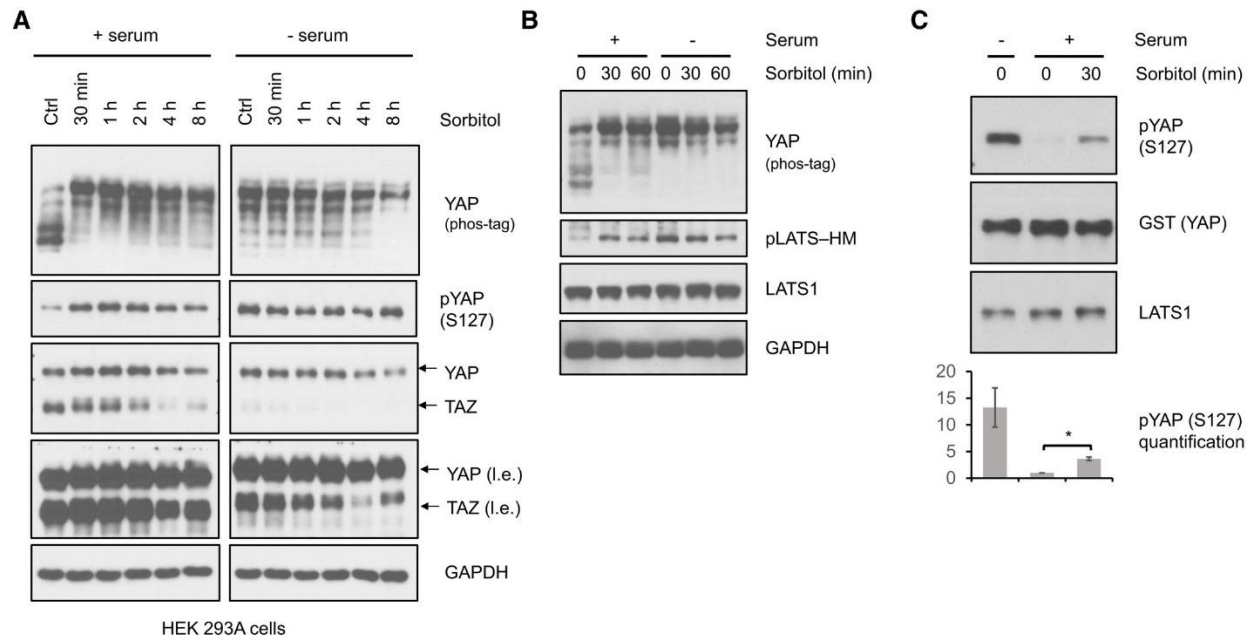
Figure 2.2: Osmotic stress induces YAP nuclear translocation and activation. (A) Osmotic stress induces YAP nuclear translocation. HEK293A cells were serum starved for 1 h followed by 0.2 M sorbitol treatment for 1 h. YAP/TAZ (red, upper panels) or YAP (red, lower panels) were stained with two specific antibodies. DAPI (blue) was used to visualize cell nuclei. Scale bars: 20 μm. Quantification of more nuclear (N > C) or more cytosolic (N < C) YAP signal is determined with randomly chosen fields. (B) Osmotic stress induces TAZ nuclear translocation. YAP knockout (KO) HEK293A cells were serum starved for 1 h followed by 0.2 M sorbitol treatment for 1 h. TAZ localization was determined by immunofluorescence staining with the YAP/TAZ antibody (red). Scale bars: 20 μm. (C) Osmotic stress induces a transient YAP nuclear translocation. HEK293A cells were serum starved for 1 h followed by 0.2 M sorbitol treatment for the indicated time points. Scale bars: 20 μm. (D) Osmotic stress induces YAP target gene expression. Wild-type (WT) or YAP/TAZ double knockout (Y/T KO) HEK293A cells were serum starved for 1 h and treated with 0.2 M sorbitol for 4 h. mRNA levels of CTGF and CYR61 were measured by quantitative RT–PCR and normalized to GAPDH control. Data are presented as mean ± SEM. *P < 0.05 (two-tailed Student's t-test, n = 3). (E) Osmotic stress decreases YAP and 14-3-3 interaction. HEK293A cells were transiently transfected with Flag-YAP and Myc-14-3-3, and were serum starved for 16 h. Cells were then treated with 0.2 M sorbitol for the indicated time points. Myc-14-3-3 was immunoprecipitated, and the associated Flag-YAP was detected by Western blot. Data are presented as mean ± SEM. *P < 0.05 (two-tailed Student's t-test, n = 3).

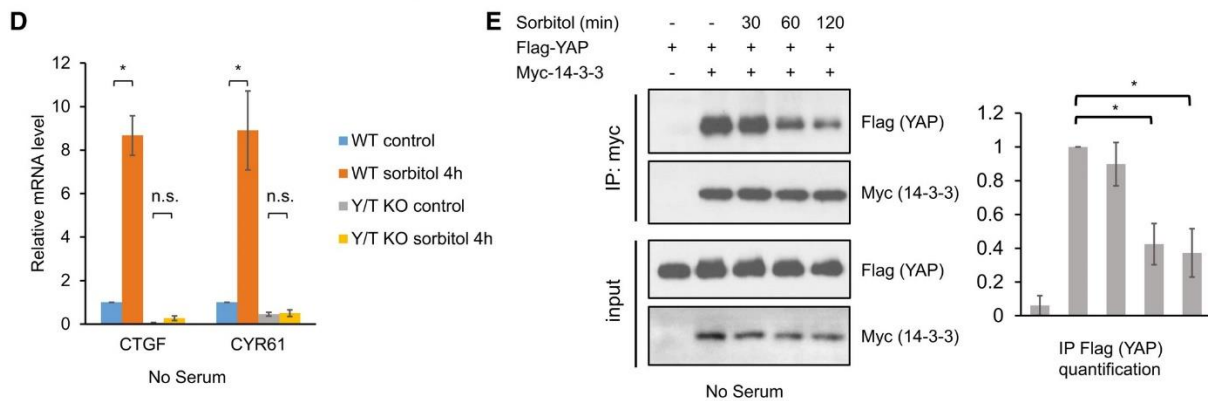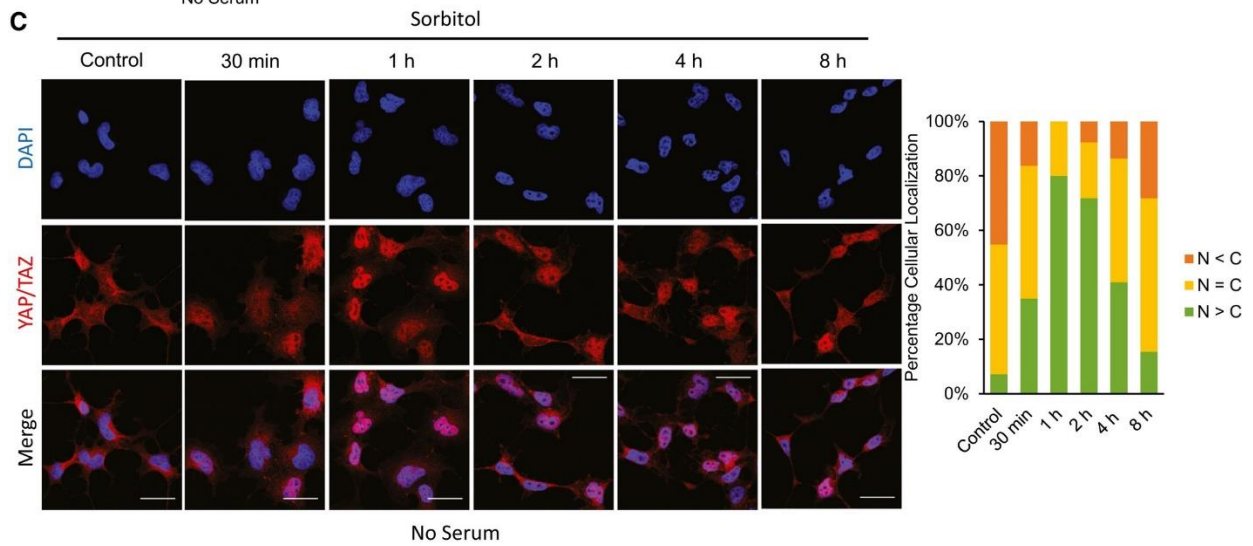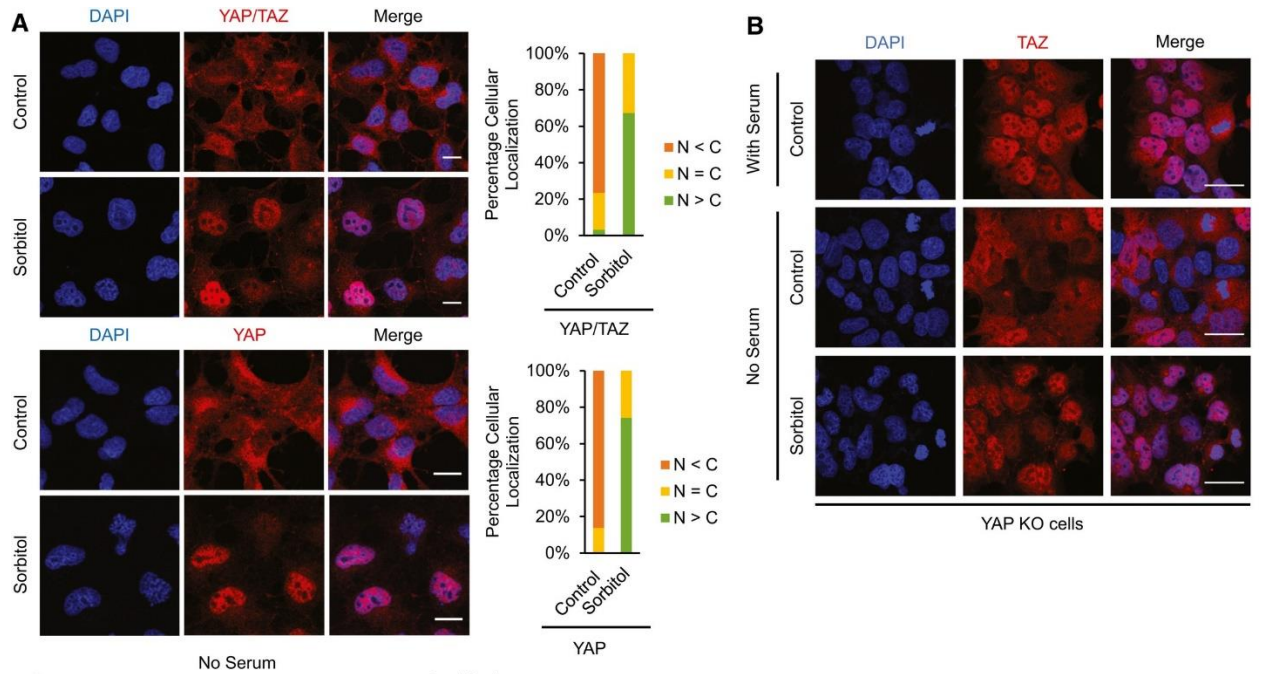Figure 2.3: NLK mediates osmotic stress signal to induce YAP nuclear localization. (A) Inhibition of p38 or JNK does not block osmotic stress-induced YAP nuclear localization. HEK293A cells were pretreated with 2 μM p38 inhibitor (SB203580) or 20 μM JNK inhibitor (SP600125) before treatment followed by 0.2 M sorbitol for 1 h in the absence of serum. Quantification of YAP/TAZ subcellular staining is shown. Scale bars: 20 μm. Cell lysates from identically treated samples were examined for phosphorylation of p38 substrate MK-2 and JNK substrate c-Jun. (B) NLK knockout blocks osmotic stress-induced YAP nuclear localization. HEK293A cells were transiently transfected with CRISPR/Cas9 to knock out NLK. Wild-type (WT) cells and the NLK KO cell pool were treated with 0.2 M sorbitol for 1 h in the absence of serum. YAP/TAZ subcellular localization was determined by immunofluorescence staining. Scale bars: 20 μm. (C) NLK induces YAP nuclear translocation. HEK293A cells were co-transfected with Flag-YAP together with vector control, Myc-NLK-WT (wild-type NLK), or NLK-KN (kinase-negative mutant). Cells were serum starved for 6 h, and YAP localization and NLK expression were determined by Flag (green) and Myc (red) antibodies, respectively. Scale bars: 20 μm. (D) NLK induces YAP phosphorylation. HA-YAP was co-transfected with NLK-WT or NLK-KN in HEK293A cells. The phos-tag gel showed NLK-WT but not NLK-KN caused a significant mobility shift of YAP. (E) NLK phosphorylates YAP in vitro. NLK-WT and NLK-KN were immunoprecipitated from HEK293A cells, and an *in vitro* kinase assay was performed using recombinant GST-YAP as a substrate in the presence of ATP-γ-S. Total phosphorylation of YAP was detected by immunoblotting with thiophosphate ester antibody, which identifies the alkylated thiophosphorylation on YAP.
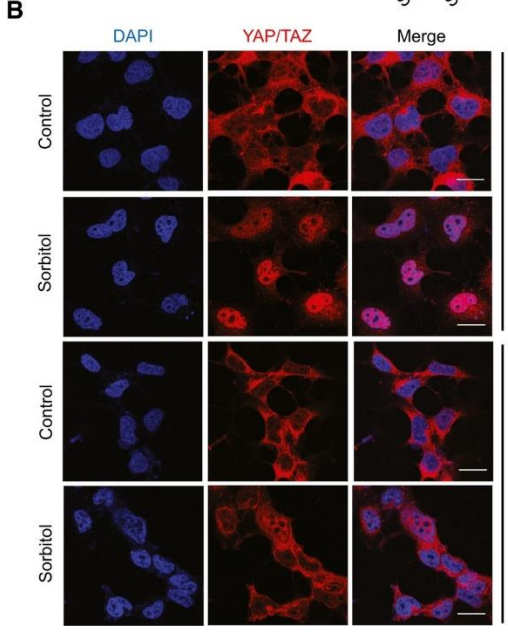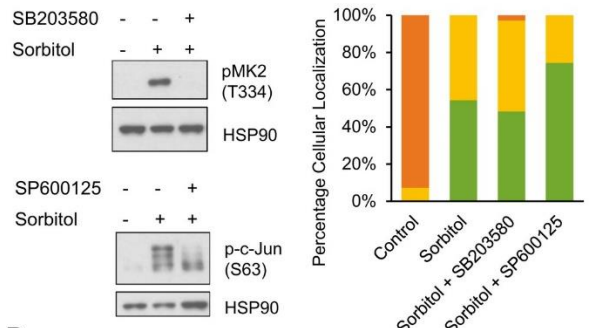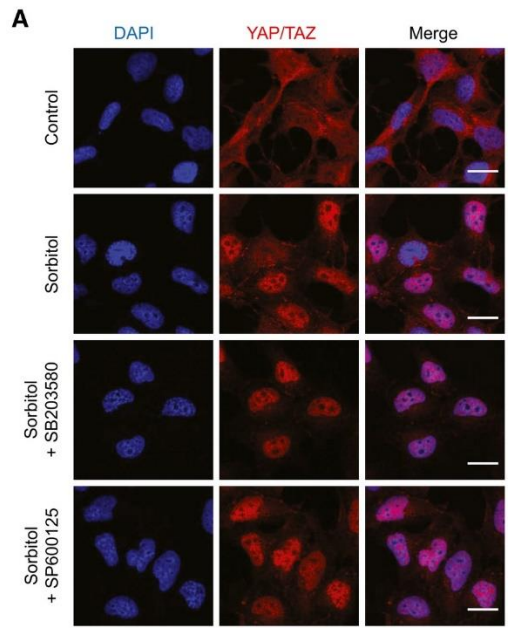
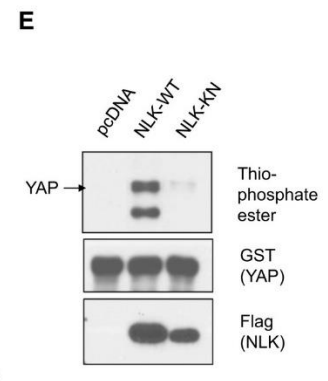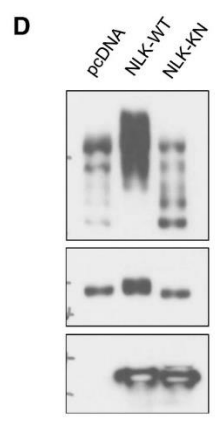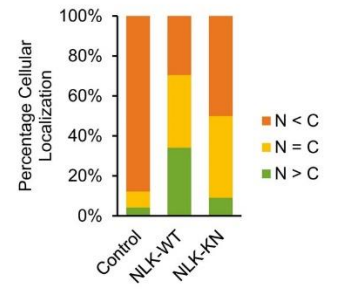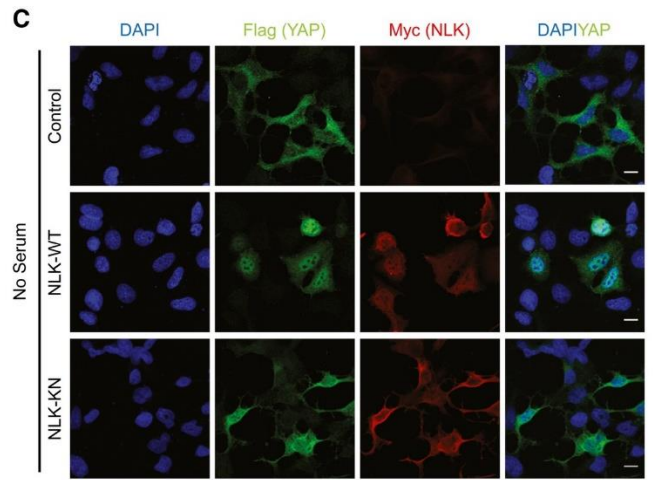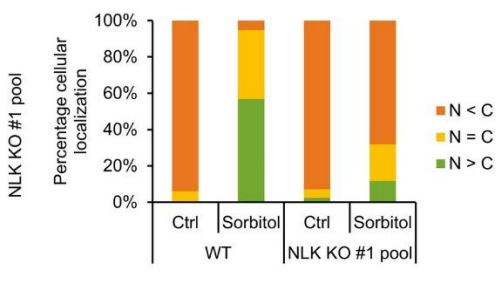Figure 2.4: Osmotic stress induces YAP Ser128 phosphorylation and inhibits its 14-3-3 binding. (A) NLK induces YAP Ser128 phosphorylation. Flag-YAP WT or S128A mutant was co-transfected with or without Myc-NLK. Phosphorylation was determined by Western blot using YAP S128 phosphospecific antibody. (B) NLK phosphorylates YAP Ser128 in vitro. NLK-WT or NLK-KN was transfected into HEK293A cells and immunoprecipitated. An *in vitro* NLK kinase assay was performed using recombinant GST-YAP as a substrate. Phosphorylation of YAP Ser128 was determined by immunoblotting with phospho-YAP (S128) antibody. (C) Osmotic stress induces YAP phosphorylation at Ser128. In the left panel, HEK293A cells were treated with sorbitol and endogenous YAP was immunoprecipitated. In the right panel, Flag-YAP was transfected into HEK293A cells, and Flag-YAP was immunoprecipitated. YAP Ser128 phosphorylation was detected by a phosphospecific antibody. Data are presented as mean ± SEM. *P < 0.05 (two-tailed Student's t-test, n = 3). (D) NLK deficiency reduces YAP S128 phosphorylation. Two CRISPR/Cas9 gRNA plasmids targeting different sites of NLK were transfected into HEK293A. WT cells and two pools of NLK CRISPR/Cas9-transfected cells were treated with 0.2 M sorbitol for the indicated time points. Data are presented as mean ± SEM. *P < 0.05 (two-tailed Student's t-test, n = 4). (E) The S128D phosphomimetic mutant abolishes YAP interaction with 14-3-3. Flag-YAP WT and mutant constructs were co-transfected with Myc-14-3-3 into HEK293A cells. Cells were serum starved for 16 h. 14-3-3 was immunoprecipitated with Myc antibody, and the associated YAP was detected with Flag antibody. (F) YAP Ser128 phosphorylation is required for disruption of YAP-14-3-3 interaction by osmotic stress. Flag-YAP WT or Flag-YAP S128A constructs were co-transfected with Myc-14-3-3 into HEK293A cells. Cells were serum starved for 16 h, and treated with 0.2 M sorbitol for the indicated time points or refreshed with serum containing medium for 1 h. Refreshing medium served as a control to disrupt YAP and 14-3-3 binding. 14-3-3 was immunoprecipitated with Myc antibody, and the associated YAP was detected with Flag antibody. Data are presented as mean ± SEM. *P < 0.05 (two-tailed Student's t-test, n = 3).

75
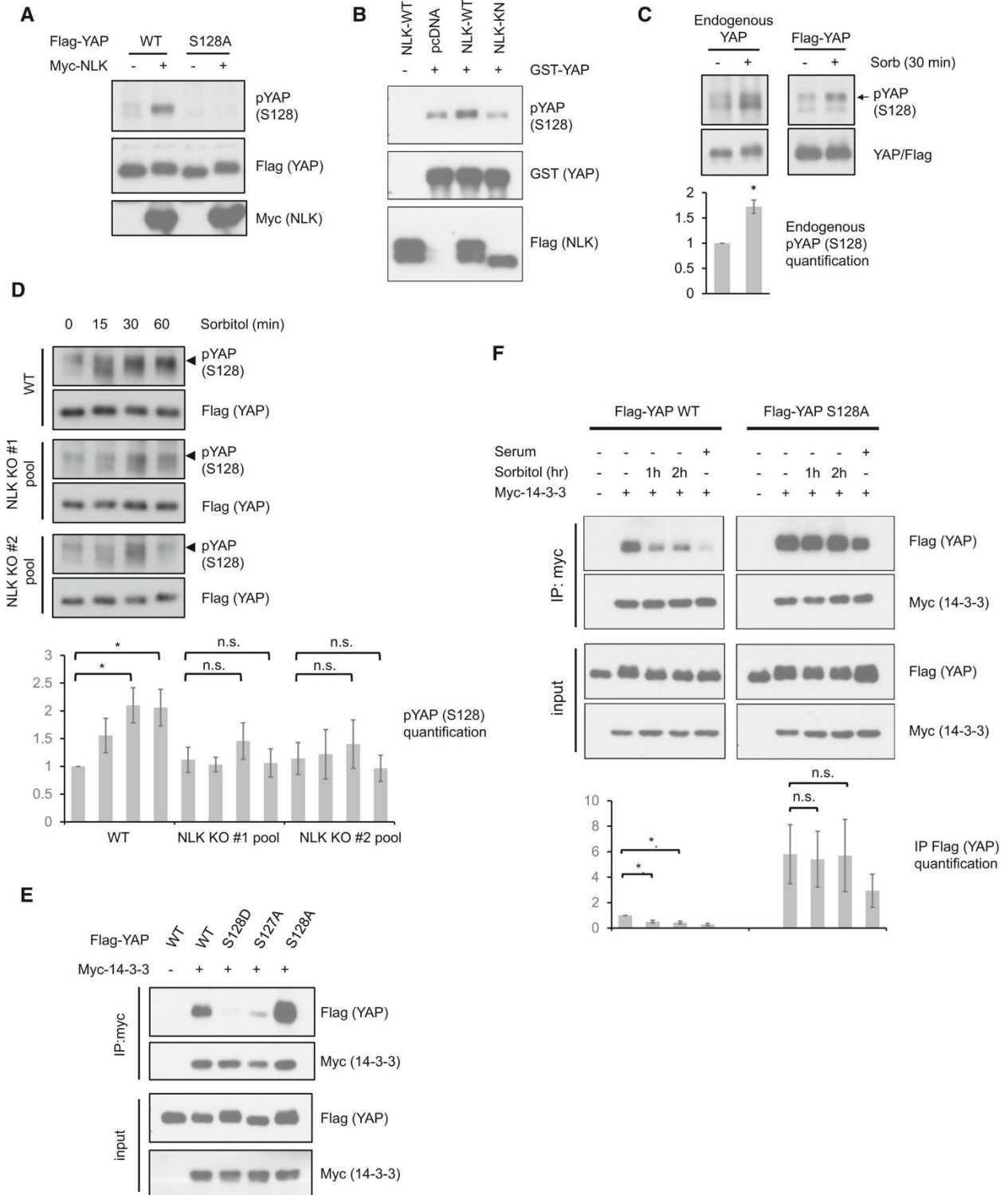
Figure 2.5: YAP Ser128 phosphorylation affects YAP subcellular localization. (A) NLK does not induce nuclear translocation of the S128A mutant YAP. HEK293A cells were co-transfected with Myc-NLK and Flag-YAP WT or S128A mutant. Cells were serum starved for 6 h, and YAP localization and NLK expression were determined by Flag (green) and Myc (red) antibodies, respectively. Scale bars: 20 μm. (B) YAP Ser128 phosphorylation is required for osmotic stress-induced YAP nuclear localization. HEK293A YAP KO cells reconstituted with Flag-YAP WT, YAP S128D, or YAP S128A mutant were treated with 0.2 M sorbitol for 1 h. YAP subcellular localization was determined by Flag immunofluorescence staining (green). Scale bars: 20 μm.

A

	DAPI	Flag (YAP)	Myc (NLK)	DAPIYAP

YAP WT

Control / NLK-WT (No Serum)

YAP S128A

Control / NLK-WT (No Serum)

B

	Flag (YAP)	Merge	Flag (YAP)	Merge	Flag (YAP)	Merge

With Serum: Control
No Serum: Control / Sorbitol

YAP WT   YAP S128D   YAP S128A

N < C
N = C
N > C

Figure 2.6: YAP Ser128 phosphorylation protects cells from hyperosmotic stress. (A) YAP S128D-reconstituted cells show growth advantage under hyperosmotic conditions. YAP/TAZ dKO HEK293A cells with stable expression of YAP WT, S128A, or S128D were cultured in the absence (left panel) or presence of 0.2 M sorbitol (right panel) for the indicated amount of time. Cell numbers were counted and normalized to day 0. Data are presented as mean ± SEM, n = 3. (B) YAP S128D-reconstituted cells have lower cell death in a hyperosmotic environment. Cell cycle analyses of YAP/TAZ dKO HEK293A cells with stable expression of YAP WT, S128A, or S128D after 0.2 M sorbitol treatment were determined using flow cytometry. Propidium iodine (PI) was used for DNA staining (Figure S2.5). Quantification of sub-G1 phase cells is shown. Data are presented as mean ± SEM. *P < 0.05 (two-tailed Student's t-test, n = 3). (C) YAP S128D-reconstituted cells show reduced apoptosis under hyperosmotic conditions. Annexin V analyses of YAP/TAZ dKO HEK293A cells with stable expression of YAP WT, S128A, or S128D after 0.2 M sorbitol treatment were determined using flow cytometry. PE-Annexin V and 7-AAD stained for phospholipid phosphatidylserine (PS) and DNA, respectively. Data are presented as mean ± SEM. *P < 0.05 (two-tailed Student's t-test, n = 3). (D) A proposed model for osmotic stress regulation of YAP via NLK kinase. See Discussion for details.
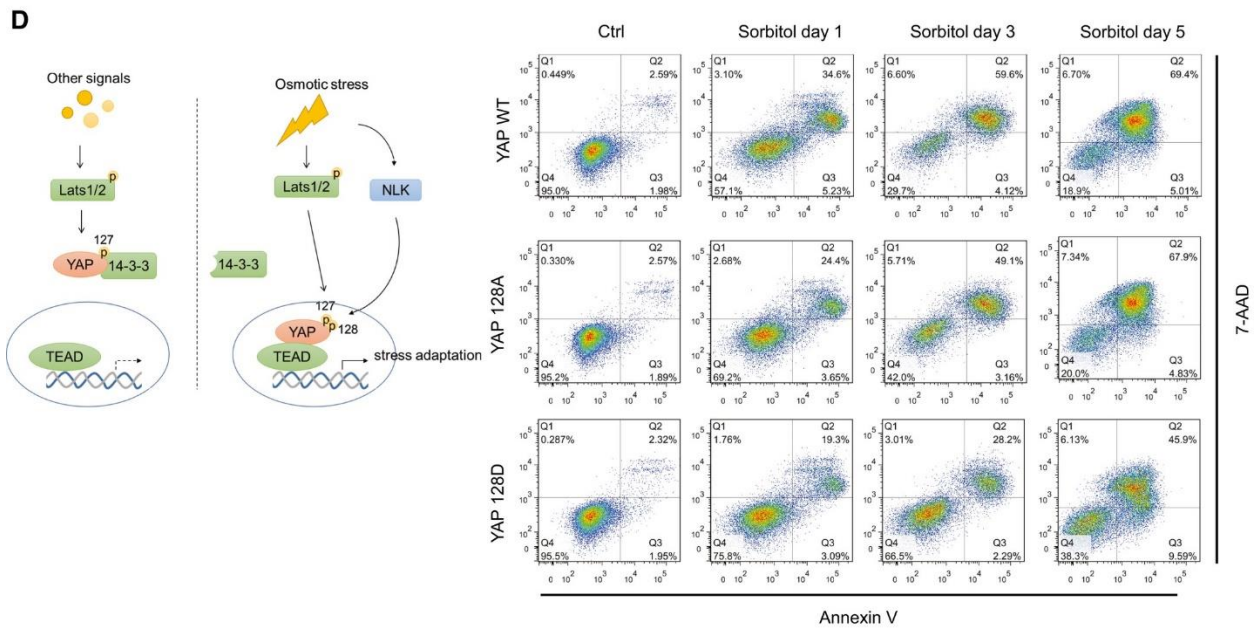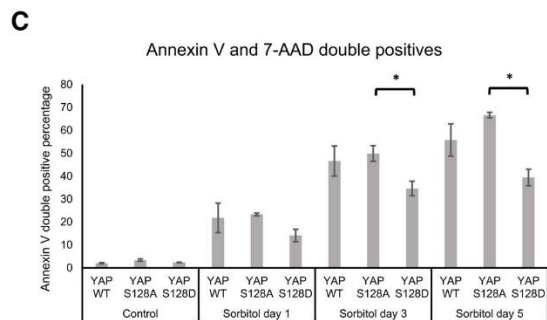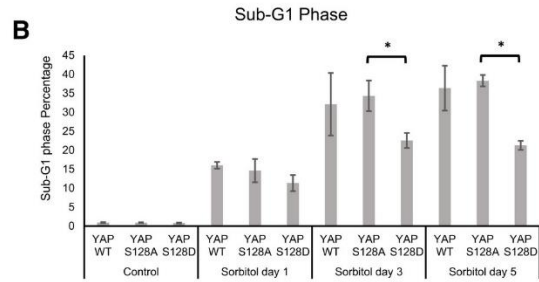
A

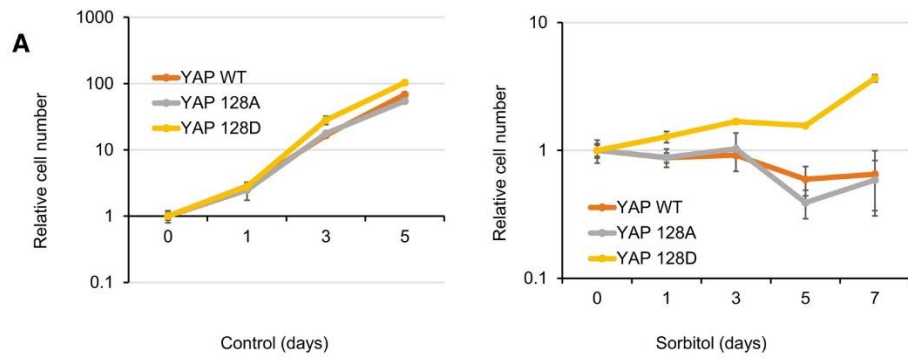**B** Sub-G1 Phase

C Annexin V and 7-AAD double positives

D

Figure S2.1: Osmotic stress induces YAP phosphorylation but not cytoplasmic retention. (A) Osmotic stress-induced YAP phosphorylation is dose-dependent. HEK293A cells were treated with different doses of sorbitol for 30 and 60 min. YAP phosphorylation is determined by mobility shift on phos-tag gel and a S127 phosphospecific antibody. (B) Sorbitol stimulates YAP phosphorylation in MCF10A cells. MCF10A cells were cultured in the presence or absence of serum, and were treated with 0.2 M sorbitol for the indicated time points. A phos-tag gel was used to assess total YAP phosphorylation. (C) NaCl stimulates YAP and LATS phosphorylation. HEK293A cells were cultured in the presence of serum and were treated with 0.1 M NaCl for the indicated time points. A phos-tag gel was used to assess YAP phosphorylation. (D) NaCl does not induce YAP cytoplasmic localization. HEK293A cells were treated with 0.1 M NaCl for 1 h in the presence of serum. YAP/TAZ subcellular localization was determined by immunofluorescence staining with an antibody that recognizes both YAP and TAZ (red). DAPI (blue) was used to stain for DNA (cell nuclei). Scale bars: 20 μm. Quantification of YAP/TAZ more nuclear (N > C) or more cytosolic (N < C) is determined with randomly chosen fields, each with approximately 100 cells. (E) Sustained osmotic stress induces YAP cytoplasmic localization in the presence of serum. HEK293A cells were treated with 0.2 M sorbitol from 30 min to 8 h in the presence of serum. YAP/TAZ subcellular localization was determined by immunofluorescence staining. Scale bars: 20 μm.

A

200 mM | 100 mM | 50 mM | 25 mM | Sorbitol (min)
0 30 60 | 0 30 60 | 0 30 60 | 0 30 60

YAP (phos-tag)

pYAP (S127)

YAP
TAZ

pLATS–HM

LATS1

GAPDH

B

+ | − | Serum
0 30 60 | 0 30 60 | Sorbitol (min)

YAP (phos-tag)

GAPDH

MCF10A

C

+ serum

Ctrl 30 60 | NaCl (min)

YAP (phos-tag)

pYAP (S127)

YAP
TAZ

pLATS–HM

LATS1

Actin

D

DAPI | YAP/TAZ | Merge

Control

NaCl

No Serum

N < C
N = C
N > C

Percentage Cellular Localization

Control NaCl

E

Sorbitol

Control | 30 min | 1 h | 2 h | 4 h | 8 h

DAPI

YAP/TAZ

Merge

With Serum

N < C
N = C
N > C

Percentage Cellular Localization

Control 30 min 1 h 2 h 4 h 8 h

81

Figure S2.2: Osmotic stress induces YAP and TAZ nuclear translocation in serum-free conditions. (A) NaCl induces YAP nuclear translocation under serum-starved conditions. HEK293A cells were serum starved for 1 h followed by 0.1 M NaCl treatment for 1 h. YAP/TAZ subcellular localization was determined by immunofluorescence staining (red). Scale bars: 20 μm. (B) Quantification of Figure 2.2B. YAP knockout (KO) HEK293A cells were serum starved for 1 h followed by 0.2 M sorbitol treatment for 1 h. Quantification of more nuclear (N > C) or more cytosolic (N < C) YAP signal was determined with randomly chosen fields. (C) YAP expression is completely abolished in YAP knockout (KO) cells. Western blot shows that YAP is absent and TAZ is still present in the YAP KO HEK293A cells. Vinculin serves as a loading control.
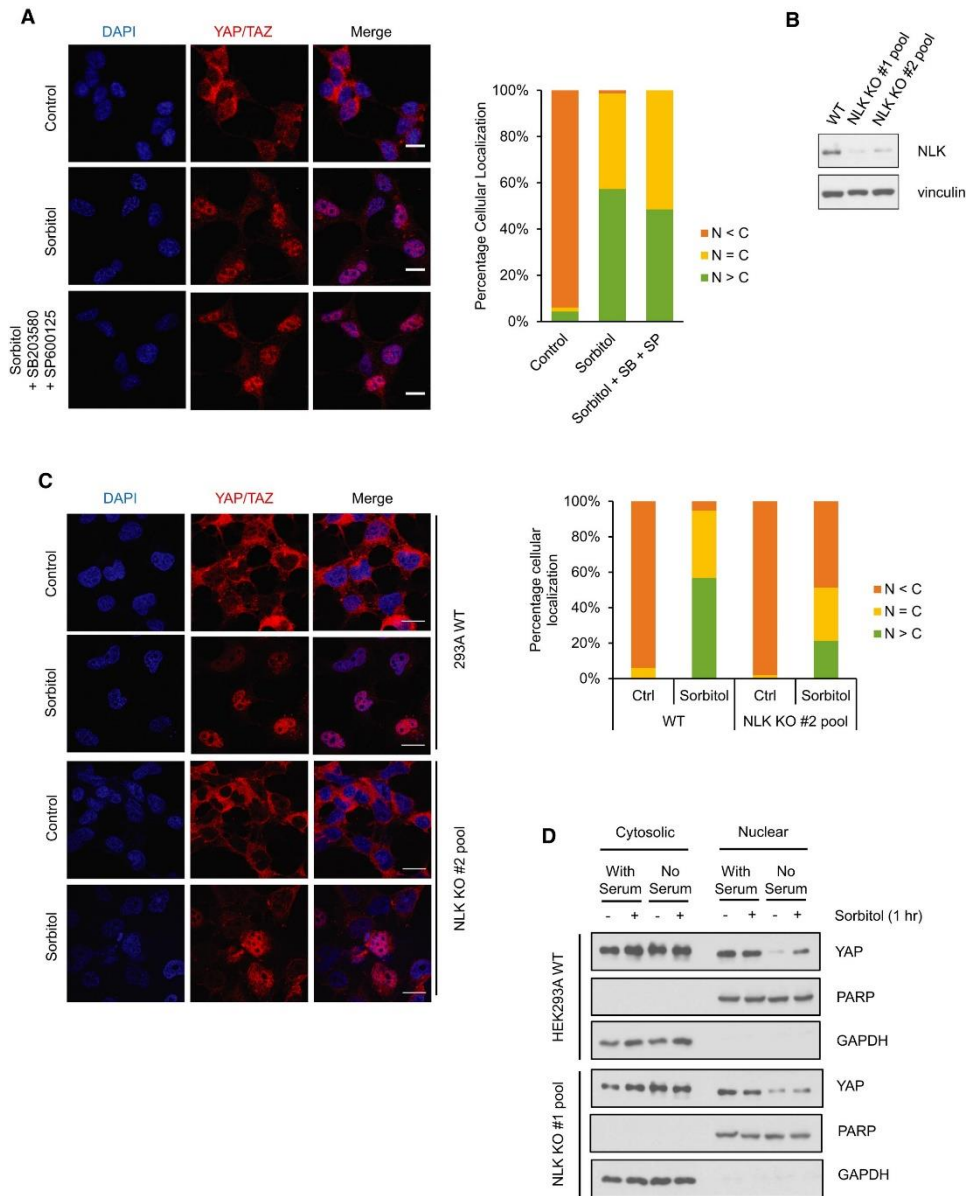
Figure S2.3: NLK mediates osmotic stress-induced YAP nuclear translocation. (A) Inhibition of both p38 and JNK does not block osmotic stress-induced YAP nuclear translocation. HEK293A cells were pretreated with 2 µM p38 inhibitor (SB203580) and 20 µM JNK inhibitor (SP600125) before treatment followed by 0.2 M sorbitol for 1 h in the absence of serum. Endogenous YAP/TAZ subcellular localization was determined by immunofluorescence staining (red). Scale bars: 20 µm. (B) Transient NLK CRISPR/Cas9 transfection reduces NLK expression levels. HEK293A cells were transiently transfected with CRISPR/Cas9 and guide RNAs to knock out NLK. Two NLK guide RNAs were used. NLK protein levels were measured by Western blot with vinculin as a loading control. (C) NLK knockout blocks osmotic stress-induced YAP/TAZ nuclear localization. HEK293A cells were transiently transfected with CRISPR/Cas9 to knock out NLK with gRNA #2. Wild-type (WT) cells and the NLK KO cell pool were treated with 0.2 M sorbitol for 1 h in the absence of serum. YAP/TAZ subcellular localization was determi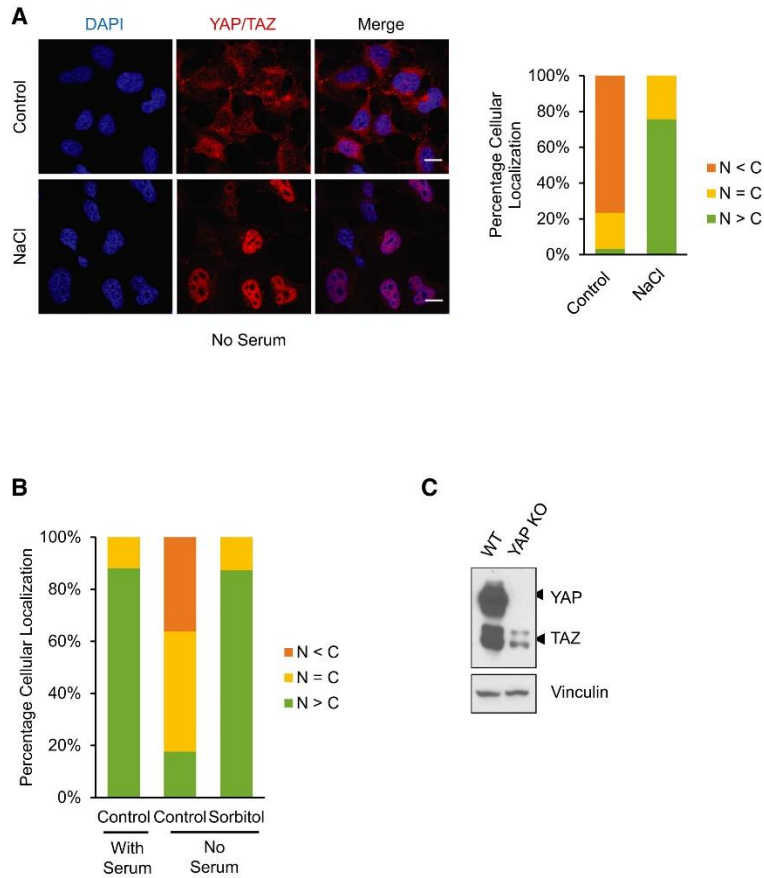ned by immunofluorescence staining (red). Scale bars: 20 µm. (D) Sorbitol-induced YAP nuclear accumulation requires NLK. Both WT and NLK KO HEK293A cells were cultured in the presence or absence and with or without sorbitol as indicated. Cytosolic and nuclear fractions were collected by differential fractionation. PARP and GAPDH were used as nuclear and cytosolic markers, respectively.
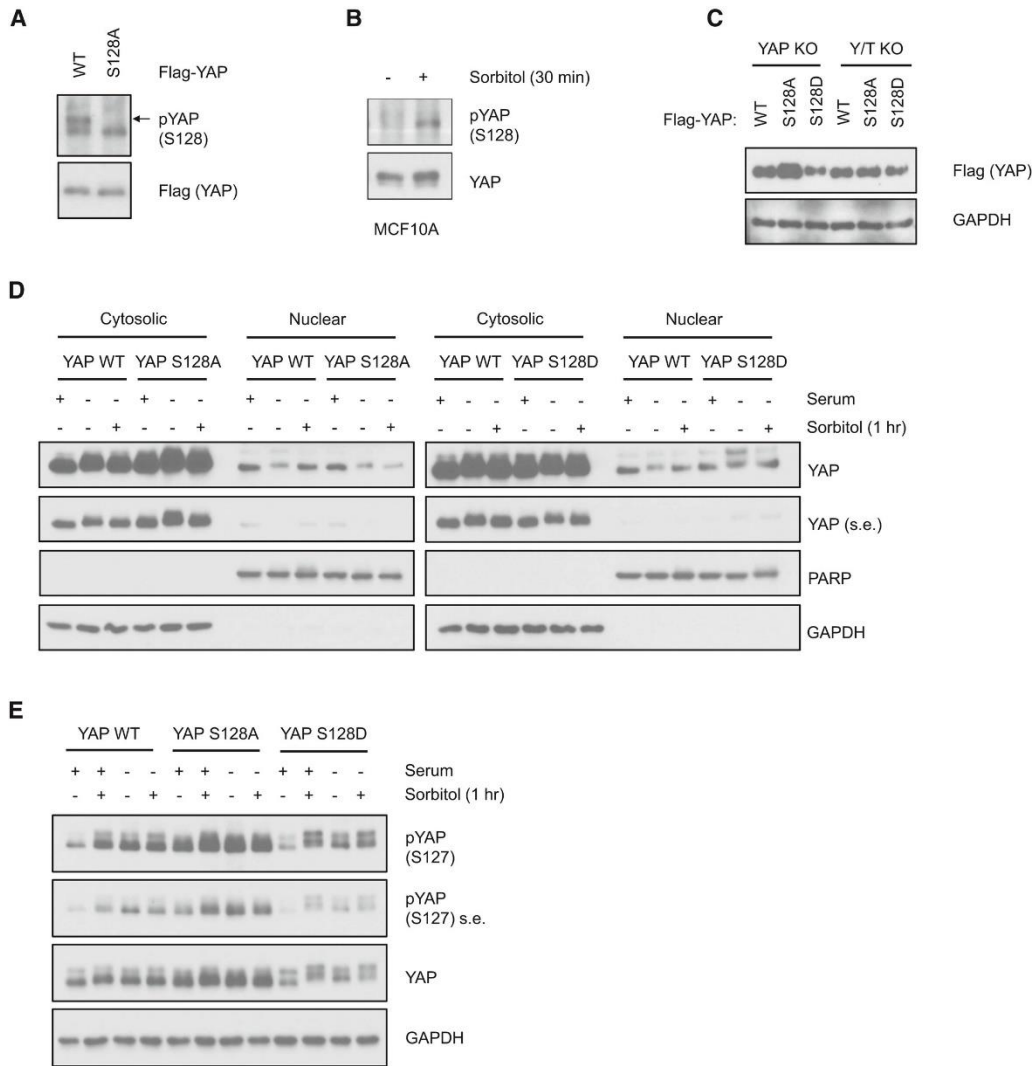
Figure S2.4: Osmotic stress induces YAP Ser128 phosphorylation to determine its subcellular localization. (A) pYAP S128 antibody is specific to YAP S128 site. Flag-YAP wild-type (WT) and S128A mutant constructs were transfected into HEK293A cells. Cell lysates were collected for Western blot analysis by indicated antibodies. (B) Osmotic stress induces endogenous YAP Ser128 phosphorylation in MCF10A cells. MCF10A cells were treated with sorbitol and endogenous YAP was immunoprecipitated. YAP Ser128 phosphorylation and protein levels were determined by Western blot. (C) Expressions of Flag-YAP WT, S128A, and S128D mutant stable cell lines are at similar levels. Stable cell lines were generated with retroviral infection of YAP WT and mutant constructs into YAP KO or YAP/TAZ dKO HEK293A cells. Cell lysates were collected for Western blot analysis. YAP expression level was detected by Flag antibody, with GAPDH as a loading control. (D) Subcellular fractionation of YAP WT-, S128D-, and S128A-reconstituted cells. YAP KO HEK293A cells were stably reconstituted with YAP WT, S128D, or S128A. Cytosolic and nuclear fractions were collected by differential fractionation. PARP and GAPDH were used as nuclear and cytosolic markers, respectively. s.e. denotes short exposure of the YAP Western blot. (E) Osmotic stress induces YAP Ser127 phosphorylation despite Ser128 phosphorylation status. YAP KO HEK293A cells were stably reconstituted with YAP WT, S128D, or S128A mutants and were treated with sorbitol in the presence or absence of serum. YAP Ser127 phosphorylation and protein levels were determined by Western blot. s.e. denotes short exposure.

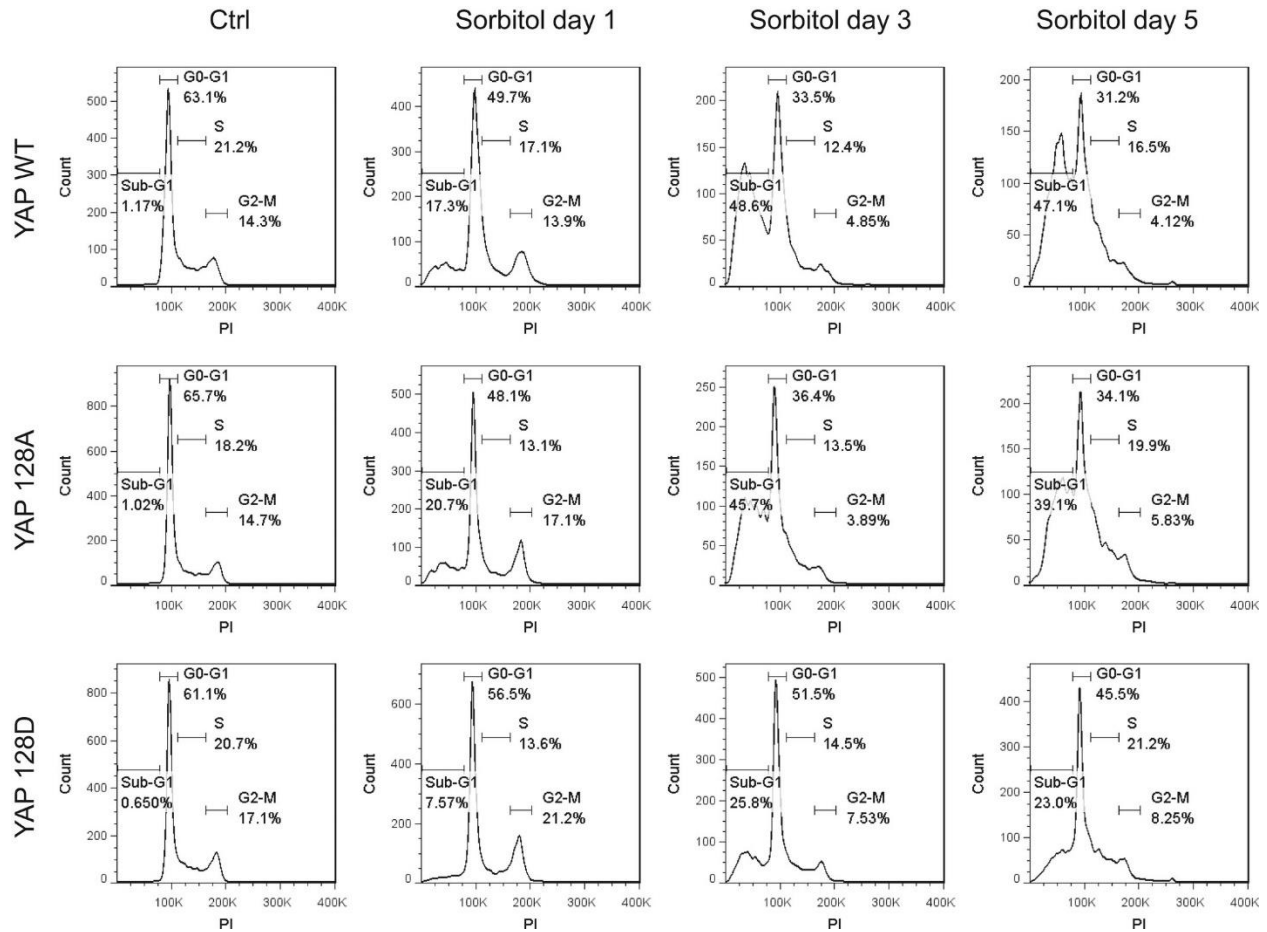Figure S2.5: YAP Ser128 phosphorylation prevents cell death under osmotic stress. Cell cycle analysis of YAP-reconstituted cells in hyperosmotic environment. Cell cycle analyses of YAP/TAZ dKO HEK293A cells with stable expression of YAP WT, S128A, or S128D after 0.2 M sorbitol treatment were determined using flow cytometry. Propidium iodine (PI) was used for DNA staining. Quantification of sub-G1 phase is shown in Figure 2.6B.

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Chapter 3: Critical roles of phosphoinositides and NF2 in Hippo pathway regulation

3.1 Introduction

The Hippo pathway plays an essential role in maintaining tissue homeostasis and organ size through regulating cell proliferation, migration, and differentiation (Pan, 2010, Piccolo et al., 2014, Yu et al., 2015). The mammalian Hippo pathway consists of a kinase cascade which negatively regulates the downstream effectors Yes-associated protein (YAP) and WW Domain-containing Transcription Factor (TAZ). The core components of this kinase cascade include mammalian STE20-like protein kinase (MST1/2), which act in parallel with the mitogen-activated protein kinase kinase kinase kinase (MAP4K) family to phosphorylate and activate the large tumor suppressor (LATS1/2). The activated LATS kinases then phosphorylate YAP and TAZ and result in YAP and TAZ binding to 14-3-3 and subsequent sequestration in the cytoplasm, where they are eventually targeted for further phosphorylation, ubiquitination, and degradation. Conversely, when the Hippo pathway kinase cascade is inactive, YAP and TAZ are dephosphorylated and translocate into the nucleus where they act as transcriptional co-activators, inducing expression of downstream target genes *CTGF*, *CYR61*, and other pro-survival genes by interacting with transcription factors such as the TEA domain (TEAD) family members.

Genetically disrupting components of this Hippo pathway kinase cascade results in uncontrolled tissue overgrowth in both *Drosophila* and mice (Halder and Johnson,

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2011). Similarly, dysregulation of the Hippo pathway has also been implicated in several human diseases and cancer (Harvey et al., 2013, Plouffe et al., 2015, Moroishi et al., 2015). For example, elevated YAP protein levels and increased nuclear localization are frequently observed in many types of tumors, suggesting a critical role for YAP in tumor progression (Pan, 2010, Plouffe et al., 2015). However, few somatic or germline mutations in any Hippo pathway core components have been identified in human patients (Harvey et al., 2013). As a result, many research efforts have been devoted to identifying novel upstream regulators of the Hippo pathway and to elucidating the Hippo pathway interactome in hopes of better understanding how YAP and TAZ become dysregulated in disease.

The *Neurofibromatosis type 2* (*NF2)* gene encodes the FERM-domain containing protein neurofibromin 2 (NF2, also known as Merlin). NF2 is a well-established tumor suppressor and regulator of the Hippo pathway (Hamaratoglu et al., 2006, Zhao et al., 2007, Harvey et al., 2013). In humans, genetic loss-of-function mutations in NF2 have been linked to increased incidence of schwannomas, meningiomas, ependymomas (Xiao et al., 2003), and mesothelioma (Sekido, 2011). Mice deficient in NF2 develop cancers such as osteosarcomas, fibrosarcomas and hepatocellular carcinomas (McClatchey et al., 1998). In the mouse liver, deleting YAP completely blocks tumorigenesis induced by NF2 knockout, suggesting that the Hippo pathway is the major signaling pathway mediating the tumorigenic potential of NF2 inactivation (Zhang et al., 2010). In cell culture, deleting NF2 is sufficient to severely compromise LATS and YAP activity in response to several stimuli known to activate the Hippo pathway (Plouffe et al., 2016). Taken together, these studies highlight the critical and physiologically-

relevant connection between NF2 and the Hippo pathway in tumorigenesis. However, the upstream regulators of NF2 and the underlying mechanisms by which NF2 regulates Hippo pathway activity remain unclear. In this study, we characterized the function of NF2 and explored the underlining mechanism of how NF2 may relay upstream signals to the Hippo pathway regulation.

Previously, we observed that osmotic stress induces YAP phosphorylation through both LATS-dependent and LATS-independent mechanisms (Hong et al., 2017), but the mechanisms by which osmotic stress activated LATS were unclear. Here, we discovered that NF2 lipid binding is essential for osmotic stress-induced activation of the Hippo pathway. Further, we show that osmotic stress induces ADP-Ribosylation Factor 6 (ARF6) and Type I Phosphatidylinositol 4-Phosphate 5-Kinase (PIP5K) interaction, leading to PIP5K activation and enhanced Phosphatidylinositol-4,5-bisphosphate $(PI(4,5)P_2)$ membrane distribution. $PI(4,5)P_2$ interacts with NF2 to induce downstream Hippo pathway activation. Our study identifies ARF6 as an osmotic sensor in mammalian cells, and PIP5K and phospholipid dynamics as upstream regulators of the Hippo pathway.

3.2 Results

3.2.1 Osmotic stress activates the Hippo pathway kinase cascade through NF2, but independent of NF2 Ser 518 phosphorylation

NF2 is a well-established regulator of the Hippo pathway, both through genetic experiments in *Drosophila* and mice, which have demonstrated that NF2 acts through the Hippo pathway to control tissue growth (Hamaratoglu et al., 2006, Zhang et al., 2010), as well as *in vitro*, where deletion of NF2 in human cell lines was sufficient to abolish the Hippo pathway response to serum deprivation, actin disruption, and glucose starvation (Plouffe et al., 2016). However, despite its obvious importance, the precise mechanism of how NF2 is activated under these circumstances is unknown.

We examined whether NF2 phosphorylation was altered in response to several stimuli known to activate the Hippo pathway. Among the various stimuli tested, including serum deprivation, osmotic stress (sorbitol treatment), energy starvation (2-DG treatment), and actin disruption (Latrunculin B treatment), only osmotic stress induced NF2 dephosphorylation based on the phos-tag gel analysis (Figure 3.1A). Hippo pathway activation was assessed by phosphorylation of the LATS hydrophobic motif (HM) and YAP.

It is well-documented that NF2 activity is modulated by phosphorylation at serine residue 518. The Ser 518 dephosphorylated NF2 is postulated to be more active in its growth suppressive function (Shaw et al., 2001, Surace et al., 2004, Rong et al., 2004, Sher et al., 2012). Therefore, we speculated that Ser 518 dephosphorylation may play a role in NF2 activation by osmotic stress. We used a phospho-specific antibody to check NF2 Ser 518 phosphorylation status and found that hyperosmotic stress led to a dramatic decrease in NF2 Ser 518 phosphorylation (Figure 3.1B). This NF2 dephosphorylation occurred very rapidly, as it was detected after only 2 minutes of sorbitol treatment, while YAP was not fully phosphorylated until 15 minutes after sorbitol

treatment (Figure S3.1A). These observations prompted us to examine the functional significance of NF2 Ser 518 phosphorylation in regulating the Hippo pathway in osmotic stress response.

Hyperosmotic stress, induced by different sorbitol concentrations and assessed at multiple time points, activated the Hippo pathway as indicated by increased LATS and YAP phosphorylation in HEK293A cells and other cell lines (Figure 3.1C, Figures S3.1B-C). To determine whether osmotic stress acts through either the Hippo pathway kinase cascade or NF2, we tested the sorbitol response in LATS, MST, and NF2 knockout (KO) cell lines, which were characterized in our previous studies (Meng et al., 2015, Plouffe et al., 2016). LATS and YAP phosphorylation in the MST and NF2 KO cells were completely abolished in response to low dose sorbitol treatment (Figure S3.1D) and were strongly compromised in response to high dose sorbitol treatment (Figure 3.1D). Thus, the Hippo pathway kinase cascade and NF2 are both involved in mediating the Hippo pathway response to osmotic stress.

Since both the Hippo pathway kinase cascade and NF2 are involved (Figure 3.1D), and because NF2 is dephosphorylated in response to osmotic stress (Figure 3.1A), it is possible that osmotic stress induces NF2 dephosphorylation at Ser 518 to activate the Hippo pathway. To test this hypothesis, we generated constructs containing either wild-type NF2, an NF2 phosphorylation-mimicking S518E mutant, or an NF2 phosphorylation-deficient S518A mutant. When expressed in the NF2 KO cells, the wild-type NF2 rescued the osmotic stress-induced LATS and YAP phosphorylation (Figure 3.1E). However, surprisingly, both the NF2 S518E and S518A mutants were also able to rescue the LATS and YAP phosphorylation in response to sorbitol treatment. This

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was unexpected due to the previous reports that Ser 518 phosphorylation inhibits NF2 (Shaw et al., 2001, Rong et al., 2004, Surace et al., 2004, Sher et al., 2012).

To avoid potential artifact due to ectopic expression of the NF2 phosphorylation mutants, we performed CRISPR-Cas9 gene editing to knock-in both the NF2 S518E and S518A mutations. The HEK293A cells have three alleles for NF2. The NF2 S518E KI cell line is homozygous, with all three alleles containing the S518E mutation (Figure S3.1E). The S518A KI cell line contains the S518A mutation in two alleles, while the third allele is an out-of-frame deletion (Figure S3.1F); therefore, the NF2 total protein expression in the S518A KI cells is slightly lower (Figure S3.1G). Similar to results from the rescue experiments, neither the S518E KI nor S518A KI cells showed observable difference in the phosphorylation of LATS or YAP in response to osmotic stress when compared to the wild-type control cells (Figure 3.1F). This clearly demonstrates that Ser 518 phosphorylation is not involved in mediating NF2 regulation of the Hippo pathway, although Ser 518 phosphorylation was reduced by osmotic stress. As regulating the Hippo pathway is the major function of NF2, our data also argue against the widely perceived notion that Ser 518 phosphorylation plays a critical role in NF2 regulation.

3.2.2 NF2 interacts with phospholipids via its FERM domain to regulate Hippo pathway activity

NF2 function may be determined by its post-translational modifications, localization, or interactions with binding partners (Cooper and Giancotti, 2014). Based on phosphorylation data from published literatures and high-throughput mass spectrometry cultivated on PhosphoSitePlus®, NF2 has several major phosphorylation

sites in addition to S518 (Hornbeck et al., 2019). We generated these NF2

phosphorylation mutations, however, NF2 mutant mimicking several of these

phosphorylation sites (NF2 6E: S10E, S13E, T230E, S315E, S518E, S581E) did not

preclude LATS and YAP phosphorylation either at the basal or in response to osmotic

stress when expressed in the NF2 KO cells (Figure S3.2). Therefore, NF2 regulation of

the Hippo pathway is most likely independent of NF2 phosphorylation status.

The NF2 protein structure consists of a FERM domain with three subdomains

(F1, F2, and F3), followed by a coiled-coil domain (CC) and a C-terminal domain (CTD)

(Figure 3.2A). The first 17 amino acids before the FERM domain are required for NF2

retention in the detergent insoluble membrane compartment and for consequent

junction enrichment (Cole et al., 2008). Furthermore, it has been shown that the F2

subdomain interacts with the LATS kinases (Li et al., 2015). To determine which protein

domains are essential for NF2 regulation of the Hippo pathway, we generated several

truncated NF2 mutants, expressed them in the NF2 KO cells, and monitored LATS and

YAP phosphorylation status following sorbitol treatment. Deleting the entire CTD (NF2

1-381) significantly blunted the LATS and YAP phosphorylation response, while deleting

the N-terminal FERM domains (NF2 382-595) completely blocked any LATS or YAP

phosphorylation (Figure 3.2B). To determine which FERM subdomains are most critical,

we individually deleted each of the F1, F2, and F3 subdomains. Interestingly, we found

that all three subdomains were equally important, as deleting any of the subdomains

was sufficient to compromise NF2 function as indicated by LATS and YAP

phosphorylation (Figure 3.2C). That the entire FERM domain is required for NF2

function raises the possibility that NF2 function in relation to the Hippo pathway is

primarily determined not by individual post-translational modifications but rather by the NF2 protein conformation and interactions with its binding partners mediated by the FERM domain.

In addition to binding to LATS, the NF2 FERM domain has also been shown to interact with the phospholipid PI(4,5)P$_2$ (Shimizu et al., 2002, Stickney et al., 2004). PI(4,5)P$_2$ binding has been shown to be important for Ezrin function (Nakamura et al., 1999, Barret et al., 2000, Fievet et al., 2004), which is a member of the ezrin–radixin–moesin (ERM) protein family and has high degree of sequence similarity with *NF2* (Trofatter et al., 1993, Rouleau et al., 1993). Two independent sets of mutations in the NF2 FERM domain have been shown to decrease the NF2 binding affinity to PI(4,5)P$_2$ (Mani et al., 2011, Chinthalapudi et al., 2018). In accordance with previous reports and incorporating these mutations, we generated two NF2 mutants: 6N (K79N, K80N, K269N, E270N, K278N, and K279N) and LBD (T59V, W60E, R309Q, and R310Q). We expressed each of these mutants in the NF2 KO cells and evaluated Hippo pathway activity in response to osmotic stress. Each of the mutants were partially defective, as there was a slight decrease in LATS phosphorylation and activity in each (Figure 3.2D); however, this reduced LATS activity was still sufficient to phosphorylate YAP.

Since the NF2 6N and LBD mutants were unable to completely abolish NF2 function on their own, we generated a third mutant, the NF2 10m, which combined the 6N and LBD mutations. We performed a protein-lipid binding assay to test the binding affinity of the NF2 10m mutant with an array of phospholipids. In brief, NF2 WT and 10m proteins were expressed, purified, and incubated with a membrane strip spotted with different types of phospholipids. The NF2 10m mutant exhibited decreased binding

affinity towards all of the phosphatidylinositol groups, especially towards $PI(3,4)P_2$, $PI(4,5)P_2$, and $PI(3,4,5)P_3$ (Figure 3.2E). Finally, to determine the functional activity of the NF2 10m mutant, we transfected both NF2 WT and the NF2 10m mutant into the NF2 KO cells. The NF2 10m mutant completely abolished LATS phosphorylation (Figure 3.2F), and similar to NF2 KO cells, YAP phosphorylation remained minimal even after 60 minutes of sorbitol treatment. These data strongly indicate that NF2 requires its lipid binding ability to regulate the Hippo pathway in response to sorbitol treatment.

3.2.3 Osmotic stress induces $PI(4,5)P_2$ membrane enrichment and NF2 colocalization

The phospholipids which showed the greatest difference in binding between the NF2 WT and NF2 10m mutant were the phosphoinositides, while other phospholipids PA and PS showed less difference (Figure 3.2E). Because the spatiotemporal regulation of phosphoinositides plays a critical role in their regulation and activity (Di Paolo and De Camilli, 2006), we utilized immunofluorescence staining (IF) to determine whether phosphoinositides are responsive to osmotic stress.

$PI(4,5)P_2$ and $PI(4)P$ are the two most abundant phosphoinositides in mammalian cells (Di Paolo and De Camilli, 2006). To visualize, GFP PLCδ-PH and GFP P4M-SidM were used as reporters for $PI(4,5)P_2$ and $PI(4)P$, respectively (Stauffer et al., 1998, Hammond et al., 2014). Distribution of $PI(4,5)P_2$ was strongly enriched in the plasma membrane compartment following sorbitol treatment (Figure 3.3A), while the distributions of $PI(4)P$ and the GFP-only control were largely unaffected (Figure 3.3B). Thus, it seems that hyperosmotic stress selectively induces $PI(4,5)P_2$ membrane

localization. A PI(4,5)P$_2$-specific antibody was also tested to further confirm the PI(4,5)P$_2$ enrichment in the plasma membrane compartment after sorbitol treatment (Figure 3.3C).

NF2 is mostly localized at the membrane, so PI(4,5)P$_2$ enrichment in the membrane compartment in response to sorbitol treatment may increase the likelihood of NF2-PI(4,5)P$_2$ interaction and NF2 activation. To test this, we transfected and stained for Flag-NF2 and found that osmotic stress caused not only increased PI(4,5)P$_2$ membrane localization, but also an increase in PI(4,5)P$_2$ colocalization with NF2 (Figure 3.3D). And consistent with the protein-lipid binding assay in which the NF2 10m mutant showed greatly reduced binding to PI(4,5)P$_2$, we found that the Flag-NF2 10m mutant failed to localize to the plasma membrane or colocalize with PI(4,5)P$_2$ following sorbitol treatment (Figure 3.3E). Both the NF2 6N and LBD mutants managed to partially localize to the plasma membrane, albeit to a lesser degree than the NF2 WT (Figure S3.3A); this may account for why the NF2 6N and LBD mutants retain some activity in activating the Hippo pathway and inducing LATS and YAP phosphorylation. As previously mentioned, the first 17 amino acids of NF2 are essential for NF2 retention in the membrane compartment (Cole et al., 2008). Removing the first 17 amino acids from the NF2 6N and LBD mutants abolished their ability to induce LATS and YAP phosphorylation (Figure S3.3B), supporting that NF2 membrane localization and PI(4,5)P$_2$ binding are critical for NF2 activity.

While it is clear that PI(4,5)P$_2$ membrane distribution plays an important role in regulating NF2 and Hippo pathway activity, we also investigated whether PI(4,5)P$_2$ levels play any role. PI(4,5)P$_2$ can be hydrolyzed by the phosphatase OCRL (also

known as Inositol polyphosphate 5-phosphatase, or INPP5F) to generate PI(4)P (Zhang et al., 1995, Zhang et al., 1998). First, when we transiently expressed OCRL, $PI(4,5)P_2$ levels were significantly lower in cells with high OCRL expression, as determined by immunofluorescence (Figure 3.3F). This also had clear implications on downstream Hippo pathway signaling, as over-expression of OCRL compromised sorbitol-induced YAP phosphorylation (Figure 3.3G). Thus, $PI(4,5)P_2$ levels positively contribute to Hippo pathway activity.

3.2.4 PIP5K is an upstream regulator of the Hippo pathway in response to osmotic stress

Hyperosmotic stress can be induced by a variety of methods, such as utilizing inorganic salts or organic osmolytes. However, these methods can trigger distinct downstream mechanisms. It has been reported that NaCl-induced osmotic stress, but not urea-induced osmotic stress, activates Phosphatidylinositol 4-Phosphate 5-Kinases (PIP5Ks), which are the primary kinases that synthesize $PI(4,5)P_2$ by phosphorylating PI(4)P (Yamamoto et al., 2006). If osmotic stress indeed relies specifically on $PI(4,5)P_2$ to activate the Hippo pathway, then we might expect that urea treatment would not induce Hippo pathway activation. And this prediction was supported by our observation that osmotic stress induced by urea failed to increase LATS or YAP phosphorylation (Figure 3.4A), implying a key role of PIP5Ks in osmotic stress-induced Hippo pathway activation.

There are three major isoforms of PIP5Ks: PIP5K1A, PIP5K1B, and PIP5K1C (Loijens and Anderson, 1996, Ishihara et al., 1996, Ishihara et al., 1998). To determine

which members of the PIP5K family could regulate the Hippo pathway, Flag-tagged PIP5Ks were transiently transfected into HEK293A cells. Ectopic expression of PIP5K1A, PIP5K1C γ87 and PIP5K1C γ90 all increased LATS and YAP phosphorylation (Figure 3.4B) and YAP cytoplasmic translocation (Figure 3.4C). The reduced YAP staining in the PIP5K transfected cells is consistent with our previous study that phosphorylation can induce YAP ubiquitination and degradation (Zhao et al., 2010). Notably, expression of PIP5K1B had no effect on Hippo pathway activation in our condition. Next, we examined the effect of osmotic stress on PIP5K subcellular localization. PIP5Ks are activated upon membrane association to produce $PI(4,5)P_2$ (van den Bout and Divecha, 2009, Liu et al., 2016). We found that the GFP-tagged PIP5K1C γ87 were enriched in the plasma membrane compartment, especially at the cell-cell junctions, following sorbitol treatment (Figure 3.4D). As expected, over-expression of PIP5K1C γ87 resulted in increased $PI(4,5)P_2$ levels and plasma membrane localization (Figure 3.4E). These data are consistent with previous report that PIP5K can be activated and thus increase $PI(4,5)P_2$ levels by NaCl-induced osmotic stress (Yamamoto et al., 2006).

In order to confirm that PIP5K activity is upstream of $PI(4,5)P_2$ and NF2, we further tested whether the lipid binding ability of NF2 is required for PIP5Ks to activate the Hippo pathway. Indeed, over-expressing PIP5K1A or PIP5K1C γ87 dramatically increased LATS and YAP phosphorylation in NF2 KO cells that were rescued with wild-type NF2, while NF2 10m mutant rescued cells only exhibited a blunted response (Figure 3.4F and Figure S3.4A). Together, these data further support that PIP5Ks act through $PI(4,5)P_2$ and NF2 to regulate the Hippo pathway.

Additionally, we generated PIP5K1A/B/C KO cell lines using CRISPR-Cas9 (Clone #B1-4, Figure S3.4B; and Clone #B1-8, Figure S3.4C). Both of these cell lines exhibited a marked decrease in LATS phosphorylation in response to sorbitol treatment compared to the wild-type cells (Figure 3.4G), supporting an important role for PIP5K in mediating the osmotic stress response to activate the Hippo pathway. Notably, the effect on YAP phosphorylation in these KO cell lines was relatively minor (Figure 3.4G). This could be due to the remaining levels of phosphorylated and active LATS, since even low levels of LATS kinase activity can result in strong YAP phosphorylation. In addition, although $PI(4,5)P_2$ is primarily synthesized by the PIP5K family, there are other pathways that can lead to increased $PI(4,5)P_2$ levels, such as Phosphatidylinositol 5-Phosphate 4-Kinase (PIP4K) phosphorylating PI(5)P or Phosphatase and Tensin Homologue (PTEN) dephosphorylating $PI(3,4,5)P_3$ (van den Bout and Divecha, 2009). These compensatory pathways may contribute to slight $PI(4,5)P_2$ production in the PIP5K KO cells.

3.2.5 ARF6 acts in coordination with PIP5K to stimulate the Hippo pathway

The small GTPase ARF6 has been reported to regulate $PI(4,5)P_2$ synthesis through direct activation of PIP5K (Honda et al., 1999, Jones et al., 2000, Funakoshi et al., 2011). Interestingly, ARF6 also responds to membrane curvature and is involved in cytoskeleton rearrangements (Lundmark et al., 2008, Boshans et al., 2000). Since cell volume change and cytoskeleton remodeling are key hallmarks of the osmotic stress response, we speculated that ARF6 may mediate the osmotic stress response to regulate PIP5K, hence the Hippo pathway. Similar to PIP5K, we found that ARF6 translocated to the plasma membrane following sorbitol treatment (Figure 3.5A).

Increased ARF6 and PIP5K interaction has also been reported to induce PIP5K activation (Aikawa and Martin, 2003). By co-immunoprecipitation, we found that osmotic stress rapidly induced ARF6 and PIP5K association (Figure 3.5B), suggesting a possible mechanism that osmotic stress promotes ARF6 interaction and thus activation of PIP5K.

To determine whether osmotic stress acts through ARF6 protein to activate the Hippo pathway, we used CRISPR-Cas9 gene editing to delete ARF6 and ARF1. ARF1 is the closest homolog of ARF6 and is also known to activate PIP5K in a cell type-dependent manner (Jones et al., 2000, Skippen et al., 2002). Complete ARF1/6 double KO cells were not viable (Figures S3.5A-B). However, we were able to obtain a complete ARF6 KO clone (Clone #1-1: ARF6-/-, ARF1+/+, Figure S3.5C) and a complete ARF6 KO clone with a heterozygous KO of ARF1 (Clone #2-3: ARF6-/-, ARF1-/+, Figure S3.5D). Both cell lines, especially the Clone #2-3, showed compromised Hippo pathway activation in response to low dose sorbitol treatment (Figure 3.5C), while only the ARF6-/-, ARF1-/+ cell line (Clone #2-3) showed diminished Hippo pathway activation in response to the high dose sorbitol treatment (Figure 3.5D). These results suggest that both ARF1 and ARF6 play a role in Hippo pathway regulation by osmotic stress, and that there exists some functional redundancy between ARF1 and ARF6. Finally, to further support the role of ARF6, we over-expressed the ARF6 constitutive-active Q67L mutant. ARF6 Q67L induced both LATS and YAP phosphorylation (Figure 3.5E). Collectively, our data indicate that ARF6 serves as an upstream regulator and activator of the Hippo pathway.

Next, we tested the function of NF2 for Hippo pathway activation by ARF6. ARF6 over-expression induced a weak phosphorylation of LATS and YAP in NF2 KO cells. This increased LATS and YAP phosphorylation by ARF6 can be potentiated by re-expression of wild type NF2, but not the NF2 10m mutant (Figure 3.5F). These data suggest that NF2 lipid binding is important for the Hippo pathway activation by ARF6, which is consistent with our model that ARF6 activates the Hippo pathway through regulating $PI(4,5)P_2$. Notably, over-expression of ARF6 was still able to induce some YAP phosphorylation in NF2 KO cells (Figure 3.5F), indicating that ARF6 may have additional mechanism to induce YAP phosphorylation. In summary, we found that ARF6 and PIP5K act together to generate plasma membrane domains enriched in $PI(4,5)P_2$, which then recruit NF2 to activate the Hippo pathway under hyperosmotic stress conditions (Figure 3.5G).

3.3 Discussion

The Hippo pathway plays an essential role in maintaining tissue homeostasis by balancing cell proliferation and differentiation. Exploring the upstream regulatory network of the Hippo pathway to gain a better and fuller understanding of how the Hippo pathway is regulated is critical for understanding its role in cellular signaling and how it becomes dysregulated in disease. NF2 is a well-established tumor suppressor and key component upstream of the Hippo pathway, and mutations in NF2 have been frequently observed in human cancer. Studies have demonstrated that NF2 is necessary for Hippo pathway activation, and that loss of NF2 significantly disrupts LATS and YAP

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phosphorylation. However, despite extensive research on NF2 regulation, the precise

mechanisms by which NF2 activity is controlled remain poorly understood. Our study

focused on characterizing the function of NF2 in response to osmotic stress and how

NF2 is involved in regulating Hippo pathway activity.

It is widely reported that NF2 phosphorylation at Serine 518 determines its

activity and tumor suppressor function; however, few of these studies have examined

NF2 activity in the context of Hippo pathway regulation. To investigate whether NF2

S518 phosphorylation is critical in regulating the Hippo pathway, we generated NF2

phospho-mimetic (S518E) and NF2 phospho-deficient (S518A) knock-in cell lines.

Ultimately, neither of these NF2 knock-in cell lines showed any difference in Hippo

pathway activity in response to various conditions, including serum starvation, cell

detachment (data not shown), or osmotic stress. This suggests that NF2 S518

dephosphorylation is not required, nor does it play an important role, for NF2 activity

towards the Hippo pathway. Activation of the Hippo pathway probably represents the

major mechanism for the tumor suppressor function of NF2. Our data question the

significance of S518 phosphorylation status in determining NF2 function in the context

of cell growth regulation.

Through domain mapping we identified that NF2 FERM domain is essential for

NF2 regulation of the Hippo pathway, as osmotic stress fails to induce LATS or YAP

phosphorylation if NF2 is missing any of its three FERM subdomains. Also, consistent

with previous reports that NF2 is localized to lipid rafts by binding to phospholipids

(Stickney et al., 2004, Mani et al., 2011), we found that NF2 lipid binding is required for

Hippo pathway activation and that an NF2 mutant deficient in phosphoinositide binding fails to activate the Hippo pathway in response to osmotic stress.

PI(4,5)P$_2$ levels are dynamically maintained through regulated synthesis and degradation. PI(4,5)P$_2$ is primarily synthesized by PIP5K phosphorylation of PI(4)P and can be hydrolyzed by the phosphatase OCRL. In this report, we present evidence that the PIP5K family, along with its activator ARF6, also serve as upstream regulators of the Hippo pathway. We propose a model that osmotic stress induces a series of signaling cascades, including ARF6 and PIP5K activation, which in turn enhances the membrane localization of PI(4,5)P$_2$ and increases PI(4,5)P$_2$ interaction with NF2. NF2 binding to PI(4,5)P$_2$ then induces downstream Hippo pathway activation. This report uncovers a novel pathway in which osmotic stress activates the Hippo pathway through phospholipids, which highlights the complexity of Hippo pathway regulation and opens new doors for future inquiries.

Although we identified PI(4,5)P$_2$ as an important contributor to NF2 activity and signaling, clearly there are other phospholipids interacting with NF2. It will be interesting to explore how other phospholipids may regulate the Hippo pathway in other contexts. We found that NF2 also interacts with phosphatidylserine (PS), which is a major structural phospholipid component for membrane bilayer. We speculate that the interaction with PS may provide a nonselective and low affinity binding for NF2 association with membrane bilayer while the interaction with PI(4,5)P$_2$ provides specificity for NF2 to be recruited to PI(4,5)P$_2$ enriched membrane subdomain, where NF2 recruits and activates Hippo pathway components such as LATS (Yin et al., 2013).

In addition to NF2, we also observed a critical role of MST1/2 in Hippo pathway activation by osmotic stress. Loss of MST1 and MST2 abolished osmotic stress-induced Hippo pathway activation even in the presence of MAP4Ks. This is a rather interesting observation because for many other stimuli, including serum deprivation, glucose starvation, or actin disruption, deleting both MST1/2 and MAP4K4/6/7 is required to completely abolish Hippo pathway activation (Meng et al., 2015). Therefore, osmotic stress may serve as a useful tool for future mechanistic and regulatory studies of the MST kinases.

3.4 Experimental procedures

Cell culture and transfection

Most cell lines (HEK293A, including KI and KO cells, MEF, and HaCaT) were cultured in Dulbecco's modified Eagle's medium DMEM (Gibco, #11965-092) with 10% FBS (Gibco, #A31606-02) and 100 units/ml penicillin-streptomycin (Gibco, # 15140-122). Cells were incubated in a humidified incubator with 5% CO_2 at 37˚C. MCF10A cells were cultured in DMEM-F12 (Gibco #11330-032) supplemented with 5% horse serum (Gibco, #26050088), 20 ng/ml EGF (Peprotech, #AF-100-15), 0.5 µg/ml hydrocortisone (Sigma, #H4001), 10 µg/ml insulin (Sigma, #I1882), 100 ng/ml cholera toxin (Sigma, #C8052), and 100 units/ml penicillin-streptomycin. MCF10A cells were also maintained at 37˚C with 5% CO_2.

Transfection

Cells were transfected with plasmid DNA using PolyJet Reagent (Signagen Laboratories, #50-478-8) according to the manufacturer's instructions. Cells were harvested 24 hr post-transfection for protein analysis.

Antibodies

The following antibodies were purchased from Cell Signaling and used at the indicated dilution for Western blot analysis: Merlin (NF2) (#6995, 1:1000), pMerlin-Ser518 (#9163, 1:1000), YAP (#14074, 1:1000), pYAP-Ser127 (#4911,1:1000), LATS1 (#3477,1:1000), pLATS-HM (#8654, 1:1000), and HA HRP conjugated (#2999, 1:10,000). GAPDH (#sc-25778, 1:2000) and YAP/TAZ (#sc-101199, 1:500) were obtained from Santa Cruz. Flag HRP conjugated (#A8592, 1:10,000) was from Sigma.

The following antibodies were used for immunofluorescent microscopy experiments at the indicated dilutions: GFP (#ab6673, 1:200) was from Abcam, $PI(4,5)P_2$ (#Z-G045, 1:200) was from Echelon Biosciences, Flag (#14793, 1:500) and HA (#3724, 1:500) were from Cell Signaling, and YAP was from Santa Cruz (#sc-101199, 1:200).

Secondary antibodies Alexa Fluor 488, 555, and phalloidin were from Invitrogen and used in 1:1000 dilution.

CRISPR knockout

CRISPR genomic editing technology was used for generating knockout (KO) cell lines. The gene-specific guide sequences were designed at https://benchling.com. Single guide RNA (sgRNA) sequences were cloned into expression vector pSpCas9(BB)-2A-Puro (PX459) (Addgene, # 48139). The constructed plasmids were transfected into

HEK293A. 24 hr after transfection, cells were enriched by 1 µg/ml puromycin (Gibco, #A11138-03) selection for 2 to 3 days and single-cell sorted by FACS into 96-well plate format. Single clones were expanded and genomic DNA were extracted using Purelink Genomic DNA Mini Kit (Invitrogen, # K182002). Genomic DNA were PCR amplified and sent out for sequencing. sgRNA sequences for each KO cell line are listed below.

Gene	sgRNA (5' → 3')
PIP5K1A	TCCCTTACCATGTAGTATCC
PIP5K1B	TGCATAAGAACATCTCGTTC
PIP5K1C	CGGATGCGTCCACACCTCGA
ARF1 for clone #1-3	CTTAAGCTTGTAGAGGATCG
ARF1 for clone #2-3	AGAACATCAGCTTCACTGTG
ARF6 for clone #1-1 and #1-3	GTGTAGTAATGCCGCCAGAG
ARF6 for clone #2-3	GAAACCCACAGTGGGAATGG

CRISPR knockin

CRISPR genomic editing technology was used for generating knockin (KI) cell lines. Gene-specific sgRNA and homologous recombination (HR) templates were designed using Benchling at https://benchling.com. Single guide RNA (sgRNA) sequence was cloned into expression vector pSpCas9(BB)-2A-Puro (PX459). sgRNA sequence for both NF2 S518E and S518A KI was 5'- TGACATGAAGCGGCTTTCCA- 3'. Below are the template sequence for S518E and S518A KI.

Template for S518E

5'-

AACCCAATTCCAGCACCGTTGCCTCCTGACATACCAAGCTTCAACCTCATTGGTGA

CAGCCTGTCTTTCGACTTCAAAGATACAGACATGAAGAGATTGGAAATGGAGATAG

AGAAAGAAAGTATGTAGCCCCCTGTGCCCTGCTGTGGGCAGCTGTGAACTAGAC

TGAGTGATTGGGGCCTTGGGAAGCTGGGGCAGA - 3'

Template for S518A

5'-

AACCCAATTCCAGCACCGTTGCCTCCTGACATACCAAGCTTCAACCTCATTGGTGA

CAGCCTGTCTTTCGACTTCAAAGATACAGATATGAAGAGGTTGGCTATGGAGATAG

AGAAAGAAAGTATGTAGCCCCCTGTGCCCTGCTGTGGGCAGCTGTGAACTAGAC

TGAGTGATTGGGGCCTTGGGAAGCTGGGGCAGA - 3'

HEK293A cells were transfected with sgRNA and HR template, selected with puromycin

for 2–3 days, and single-cell sorted by FACS into 96-well plate format. Single clones

were expanded and genomic DNA were extracted using Purelink Genomic DNA Mini

Kit. Genomic DNA were PCR amplified and sent out for sequencing.

Lipid binding assay

PIP strips membrane (Echelon Biosciences, #P-6001) was blocked in blocking solution

(3% BSA in TBST) for 1 hr at room temperature. 0.5 ug/ml of protein in blocking solution

was incubated with the membrane for 2 hr at room temperature with gentle mixing.

Membrane was then washed with TBST 3 times, and subjected to primary antibody

incubation in blocking solution with gentle mixing. Membrane was washed again as

above and lipid associated proteins were detected by ECL according to manufacturer's instructions.

Immunofluorescence staining

HEK293A cells were plated on fibronectin (Sigma, #F1141)-coated coverslips 1 day prior to experimentation. Cells were fixed with 4% paraformaldehyde (Electron Microscopy Sciences, # 15710) for 15 minutes and permeabilized with 0.1% saponin (Sigma, # 47036) for 5 min. Note that in Figure 4E, samples were permeabilized with 0.1% Triton X-100 for visualizing YAP localization. After blocking in 3% BSA in PBS for 30 minutes, cells were incubated with primary antibodies diluted in 3% BSA overnight in 4°C. After three washes of with PBS, cells were incubated with Alexa Fluor secondary antibodies at 1:1000 dilution (Invitrogen) for 1 hour in the dark at room temperature. Actin is stained with phalloidin conjugated with various fluorescent dye (Invitrogen). Phalloidin was added at 1:1000 dilution together with the secondary antibodies. Coverslips were mounted with ProLong Gold antifade mountant with DAPI (Invitrogen, #P36931). Images were captured with Nikon Eclipse Ti confocal microscopy. Figures were made and analyzed with ImageJ software.

Co-Immunoprecipitation

Cells were lysed with buffer (25 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 5 mM MgCl$_2$, 10 mM KCl, 1% Triton X-100) supplemented with 0.25% sodium deoxycholate, protease inhibitor (Roche, #11873580001), phosphatase inhibitor (Thermo Scientific, #88667), and 1 mM PMSF (Sigma, #P7626) for 10 minutes on ice and centrifuged at 12,000 g for 15 min at 4 °C. The supernatants were incubated with the Flag antibody

(Sigma, #F1804) overnight at 4 °C and protein A/G magnetic beads (Thermo Scientific, #88802) were added for 1 hour. Proteins were washed with lysis buffer without sodium deoxycholate three times and were eluted with SDS–PAGE sample buffer. Samples were followed by Western blot analysis.
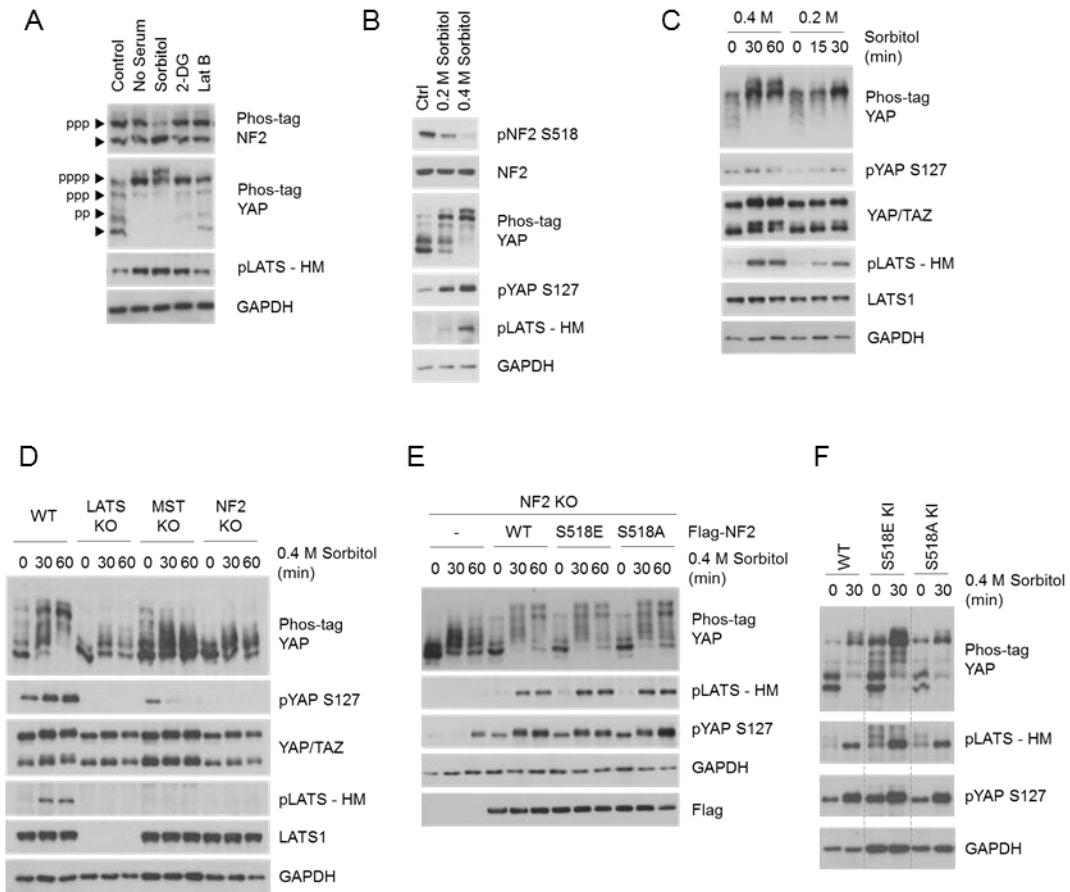
3.5 Acknowledgments

Figure 3.1: Osmotic stress activates the Hippo pathway through NF2, independent of Ser 518 phosphorylation. (A) Osmotic stress specifically induces NF2 dephosphorylation. HEK293A cells were treated with serum starvation, 0.4 M sorbitol, 25 mM 2-DG, or 250 ng/ml Latrunculin B (Lat B) for 30 min. Phos-tag gel was used to assess total NF2 and YAP phosphorylation based on mobility shift. (B) NF2 is dephosphorylated at Ser 518 site in response to increasing osmotic stress. HEK293A cells were treated with either 0.2 M or 0.4 M sorbitol for 30 min. Hippo pathway activation is assessed by YAP phosphorylation, based on mobility shift on the phos-tag gel and by a pSer 127-specific antibody, and LATS phosphorylation at its hydrophobic motif (HM). (C) Osmotic stress induces LATS and YAP phosphorylation in HEK293A cells. Cells were treated with 0.2 M or 0.4 M sorbitol for the indicated times (minutes). LATS and YAP phosphorylation levels were detected by Western blot. (D) NF2 and MST are important for Hippo pathway activation by osmotic stress. Western blot results for LATS and YAP phosphorylation in wild-type HEK293A, LATS KO, MST KO, and NF2 KO cells treated with 0.4 M sorbitol. (E) NF2 S518 mutants behave similarly as wild-type. NF2 WT, S518E, and S518A mutants were transiently expressed in NF2 KO cells, and were then treated with 0.4 M sorbitol. LATS and YAP phosphorylation levels were monitored by Western blot. (F) NF2 S518 dephosphorylation is not required for its function in Hippo regulation. HEK293A WT, S518E knockin, and S518A knockin cell lines were treated with 0.4 M sorbitol for 30 min. LATS and YAP phosphorylation levels were monitored by Western blot.

Figure 3.2: NF2 FERM domain interacts with phospholipids to regulate Hippo pathway activity. (A) Schematic diagram of the NF2 protein structure. NF2 consists of a FERM domain containing three subdomains (F1, F2, and F3), a coiled-coil (CC) domain, and a C-terminal domain (CTD). NF2 interacting partner and region are listed in the diagram. (B) The FERM domain is essential for NF2 activity. NF2 truncated constructs were transfected into NF2 KO cells. Flag-NF2 1-381 is missing the entire FERM domain, and Flag NF2 382-595 is missing the entire C-terminal domain (CTD). Cells were treated with 0.4 M sorbitol for the indicated times (minutes), and LATS and YAP phosphorylation was detected by Western blot. (C) Each of the F1, F2, and F3 subdomains is required for NF2 function. NF2 constructs with deletion of either the F1, F2, or F3 subdomains were transfected into the NF2 KO cells. Cells were treated with 0.4 M sorbitol for the indicated times (minutes), and LATS and YAP phosphorylation was detected by Western blot. (D) NF2 LBD and 6N mutants show slight decrease in their activities. The NF2 LBD mutant contains the T59V, W60E, R309Q, and R310Q mutations, and the NF2 6N mutant contains the K79N, K80N, K269N, E270N, K278N, K279N mutations. NF2 wild-type, LBD, and 6N were transiently expressed in NF2 KO cells and treated with 0.4 M sorbitol for the indicated times (minutes). LATS and YAP phosphorylation levels were measured by Western blot. (E) NF2 10m mutant has defect in binding with phosphoinositides. Flag NF2 WT and 10m proteins were purified and subjected to lipid binding assay. A strip of phospholipids was used to test for NF2 lipid binding ability. (F) Lipid binding is essential for NF2 function under osmotic stress. NF2 WT and the 10m mutant were transiently expressed in NF2 KO cells. Cells were treated with 0.4 M sorbitol for the indicated times (minutes), and LATS and YAP phosphorylation was detected by Western blot.
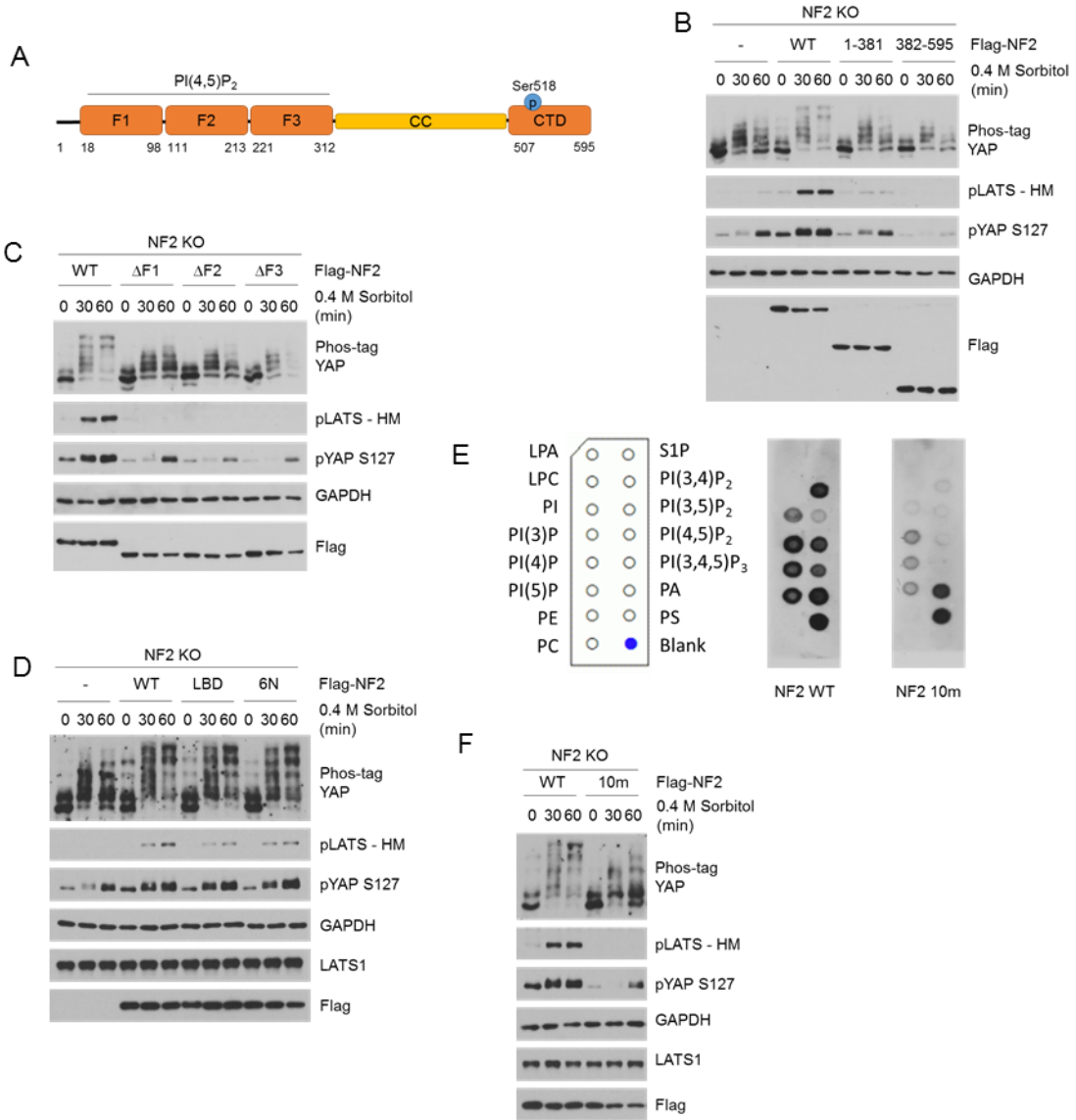
A

PI(4,5)P₂

F1 | F2 | F3 | CC | CTD
1 18 98 111 213 221 312   507 595

Ser518

B

NF2 KO

Flag-NF2: - | WT | 1-381 | 382-595
0.4 M Sorbitol (min): 0 30 60 | 0 30 60 | 0 30 60 | 0 30 60

Phos-tag YAP
pLATS - HM
pYAP S127
GAPDH
Flag

C

NF2 KO

Flag-NF2: WT | ΔF1 | ΔF2 | ΔF3
0.4 M Sorbitol (min): 0 30 60 | 0 30 60 | 0 30 60 | 0 30 60

Phos-tag YAP
pLATS - HM
pYAP S127
GAPDH
Flag

D

NF2 KO

Flag-NF2: - | WT | LBD | 6N
0.4 M Sorbitol (min): 0 30 60 | 0 30 60 | 0 30 60 | 0 30 60

Phos-tag YAP
pLATS - HM
pYAP S127
GAPDH
LATS1
Flag

E

LPA ○ ○ S1P
LPC ○ ○ PI(3,4)P₂
PI ○ ○ PI(3,5)P₂
PI(3)P ○ ○ PI(4,5)P₂
PI(4)P ○ ○ PI(3,4,5)P₃
PI(5)P ○ ○ PA
PE ○ ○ PS
PC ○ ● Blank

NF2 WT   NF2 10m

F

NF2 KO

Flag-NF2: WT | 10m
0.4 M Sorbitol (min): 0 30 60 | 0 30 60

Phos-tag YAP
pLATS - HM
pYAP S127
GAPDH
LATS1
Flag

Figure 3.3: Osmotic stress induces PI(4,5)P$_2$ membrane enrichment and NF2 colocalization. (A) Osmotic stress induces PI(4,5)P$_2$ membrane localization assayed by PI(4,5)P$_2$ reporter. HEK293A cells were transiently transfected with the PI(4,5)P$_2$ reporter GFP-PLCd-PH and are shown in green. Actin (phalloidin) is shown in red, and the nuclei (DAPI) are shown in blue. Cells were treated with sorbitol for 30 min. The scale bar represents 20 um. (B) Localization of PI(4)P and GFP control protein do not change after sorbitol treatment. HEK293A cells were transiently transfected with GFP-P4M-SidM as a reporter for PI(4)P or GFP only construct as the control, as shown in green. The nuclei (DAPI) are shown in blue. Cells were treated with sorbitol for 30 min. The scale bar represents 20 um. (C) Osmotic stress induces PI(4,5)P$_2$ membrane localization assayed by PI(4,5)P$_2$ antibody. HEK293A cells were treated with sorbitol for 30 min. PI(4,5)P$_2$ is shown in green, Actin (phalloidin) is shown in red, and the nuclei (DAPI) are shown in blue. The scale bar represents 20 um. (D) Osmotic stress induces PI(4,5)P$_2$ and NF2 colocalization. Flag-NF2 was transiently transfected into HEK293A cells for 24 hrs, and then cells were treated with sorbitol for 30 min. NF2 was stained with an anti-Flag antibody and is shown in red. PI(4,5)P$_2$ is shown in green, and the nuclei (DAPI) are shown in blue. The scale bar represents 20 um. (E) NF2 10m mutant does not colocalize with PI(4,5)P$_2$ upon sorbitol treatment. Flag-NF2 WT and the 10m mutant were transiently transfected into cells for 24 hrs, and then cells were treated with sorbitol for 30 min. NF2 was stained with an anti-Flag antibody and is shown in red. PI(4,5)P$_2$ is shown in green, and the nuclei (DAPI) are shown in blue. Higher magnification images of the boxed area are shown on the right. The scale bar represents 20 um. (F) OCRL over-expression reduces PI(4,5)P$_2$ level. The PI(4,5)$_2$ phosphatase OCRL was expressed in HEK293A cells and PI(4,5)P$_2$ levels and distribution were detected by immunofluorescence. OCRL was stained with an anti-HA antibody and is shown in red. PI(4,5)P$_2$ is shown in green, and the nuclei (DAPI) are shown in blue. The scale bar represents 20 um. (G) OCRL impairs sorbitol induced Hippo pathway activation. HEK293A cells were transfected with HA-OCRL and treated with either 0.2 M or 0.4 M sorbitol for the indicated times (minutes). YAP phosphorylation and OCRL expression levels were detected by Western Blot.
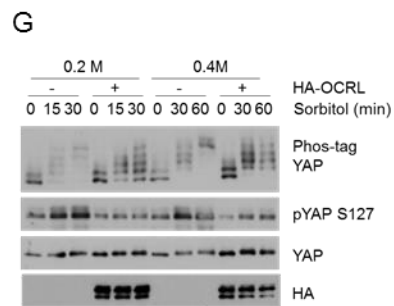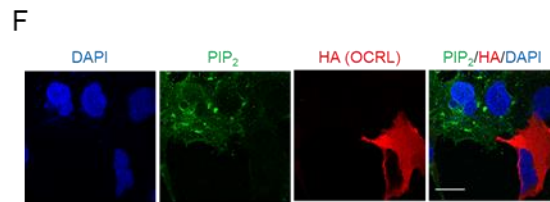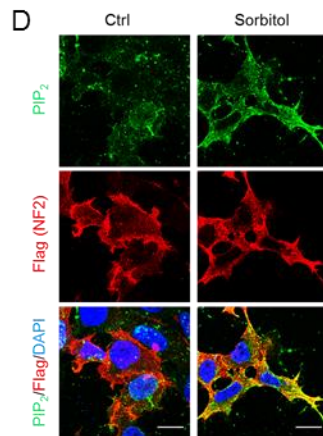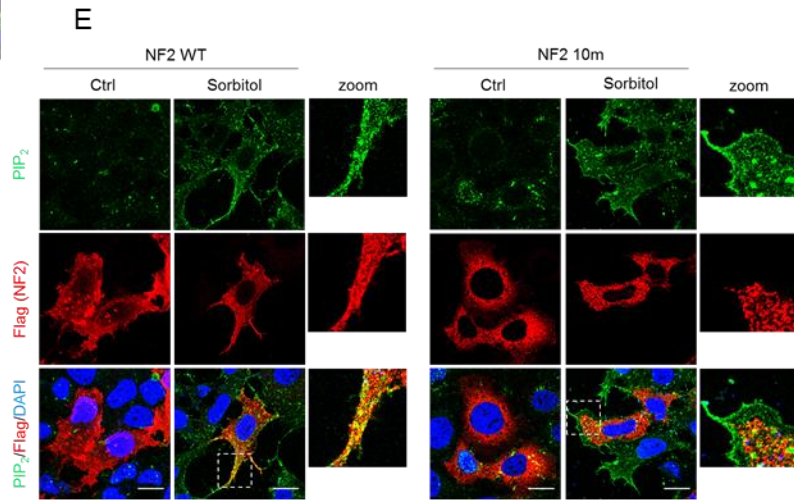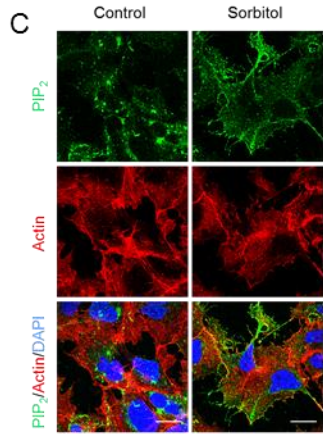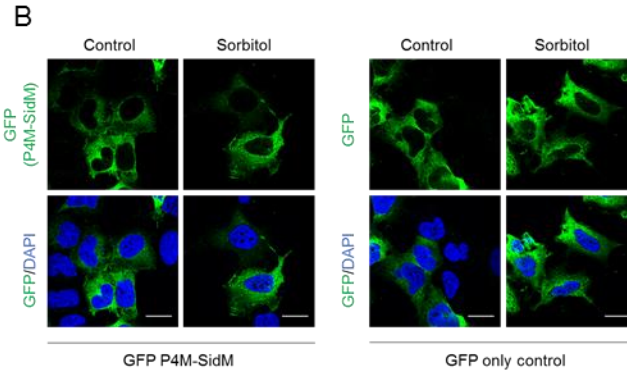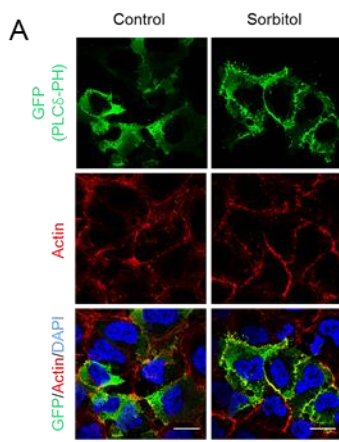
Figure 3.4: PIP5Ks act as upstream regulators of the Hippo pathway in response to osmotic stress. (A) Hyperosmotic stress induced by urea does not activate the Hippo pathway. HEK293A cells were treated with different dosages of sorbitol, NaCl, or urea for 30 min, and LATS and YAP phosphorylation was detected by Western blot. (B) PIP5K1A and PIP5K1C activate the Hippo pathway. PIP5K1A, PIP5K1B, PIP5K1C γ87, and PIP5K1C γ90 were over-expressed in HEK293A cells. Expression levels of PIP5Ks were detected using an anti-Flag antibody. LATS and YAP phosphorylation levels were detected by Western blot. (C) PIP5K1A and PIP5K1C induce YAP cytoplasmic translocation. Flag-PIP5K1A, Flag-PIP5K1C γ87 and Flag-PIP5K1C γ90 were transiently transfected into HEK293A cells. PIP5Ks were stained using an anti-Flag antibody and are shown in green, YAP is shown in red, and nuclei (DAPI) are shown in blue. The scale bar represents 20 um. (D) Osmotic stress induces PIP5K1C γ87 plasma membrane localization. GFP-PIP5K1C γ87 was transiently transfected into the cells. Cells were treated with 0.4 M sorbitol for 30 min. Nuclei (DAPI) are shown in blue. The scale bar represents 20 um. (E) PIP5K expression induces $PI(4,5)P_2$ levels and plasma membrane localization. Flag-PIP5K1C γ87 was transiently transfected into the cells. PIP5K1C γ87 was stained using an anti-Flag antibody and is shown in red, $PI(4,5)P_2$ is shown in green, and the nuclei (DAPI) are shown in blue. The scale bar represents 20 um. (F) NF2 lipid binding is required for PIP5K1C to activate the Hippo pathway. NF2 WT, NF2 10m mutant, and PIP5K1C γ87 were transiently transfected into NF2 KO cells in different combinations. LATS and YAP phosphorylation was determined by Western blot. (G) PIP5K1A/B/C tKO impairs Hippo pathway activation in response to osmotic stress. PIP5Ks KO cells with deletion of PIP5K1A, PIP5K1B, and PIP5K1C were generated with by CRISPR-Cas9 gene editing. LATS and YAP phosphorylation in wild-type, PIP5Ks KO clone #B1-4 and clone #B1-8 were detected by Western blot following treatment with 0.4 M or 0.2 M of sorbitol at indicated times (min).
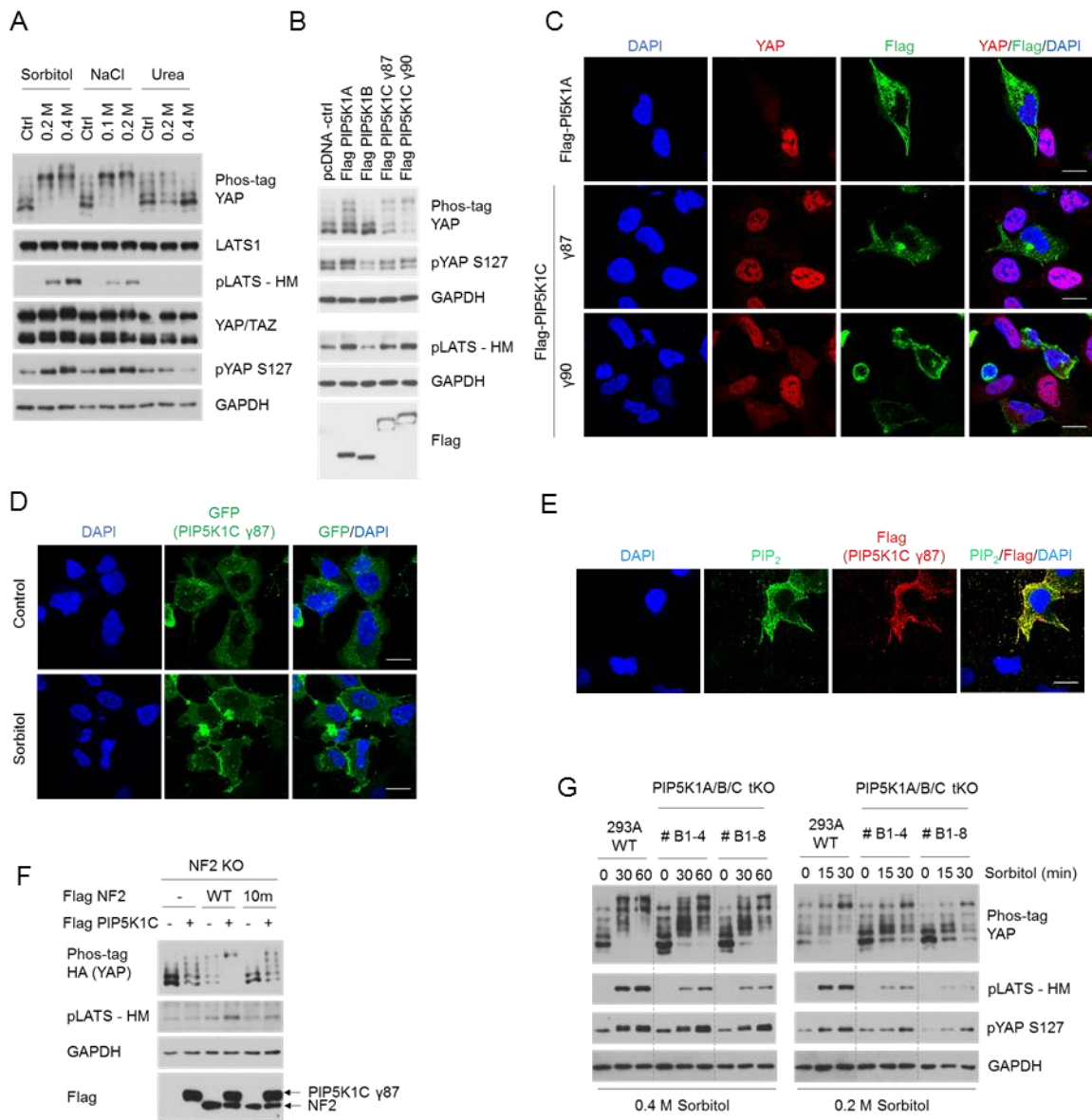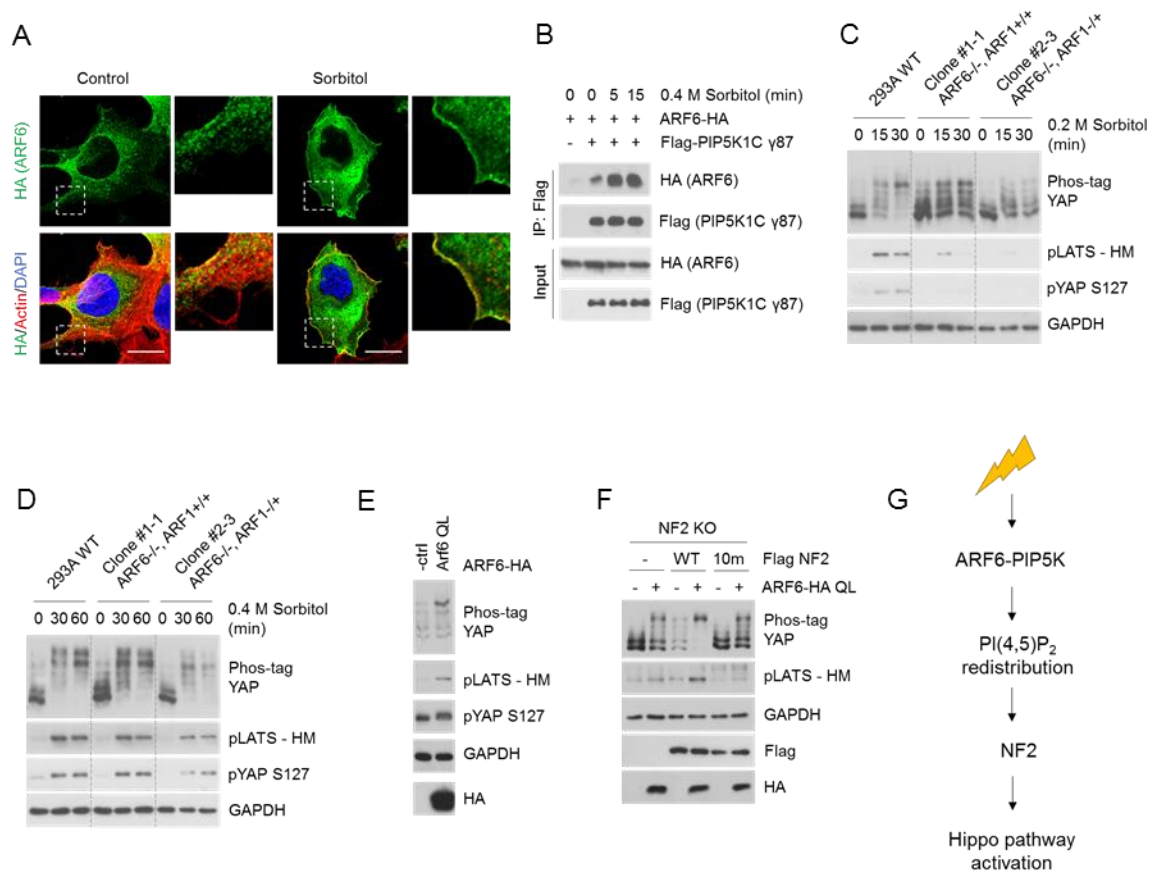
Figure 3.5: ARF6 acts in coordination with PIP5K to activate the Hippo pathway. (A) Osmotic stress induces ARF6 plasma membrane localization. ARF6-HA was transiently transfected into HEK293A cells. Cells were treated with 0.4 M sorbitol for 30 min. ARF6 was stained with an anti-HA antibody and is shown in green, actin (phalloidin) is shown in red, and the nuclei (DAPI) is shown in blue. Higher magnification images of the boxed area are shown on the right. The scale bar represents 20 um. (B) Osmotic stress induces ARF6 and PIP5K1C interaction. HEK293A cells were transiently transfected with HA-tagged ARF6 and Flag-tagged PIP5K1C γ87, and were treated with 0.4 M sorbitol at different time points (min). ARF6 was immunoprecipitated with the HA antibody, and ARF6 and PIP5K1C were detected using the anti-HA and anti-Flag antibodies, respectively, by Western blot. (C) ARF6 deletion compromises Hippo pathway activation under low dose sorbitol treatment. ARF1/6 KO Clone #1-1 cells have complete loss of ARF6, and Clone #2-3 has complete loss of ARF6 and partial deletion of ARF1. LATS and YAP phosphorylation in wild-type HEK293A, ARF1/6 KO Clone #1-1, and Clone #2-3 were detected by Western blot following treatment with 0.2 M sorbitol for the indicated times (in minutes). (D) ARF1/6 KO Clone #2-3 diminishes Hippo pathway activation under high dose sorbitol treatment. Experiments were similar to panel C except cells were treated with 0.4 M sorbitol. (E) ARF6 induces LATS and YAP phosphorylation. Constitutively-active ARF6 (ARF6 QL) was overexpressed in HEK293A cells. LATS and YAP phosphorylation was detected by Western blot. (F) NF2 lipid binding is important for ARF6 activation of the Hippo pathway. NF2 WT, NF2 10m mutant, and ARF6-QL were transiently transfected into NF2 KO cells in different combinations. LATS and YAP phosphorylation was detected by Western blot. (G) A proposed mechanistic model linking osmotic stress to the Hippo pathway.
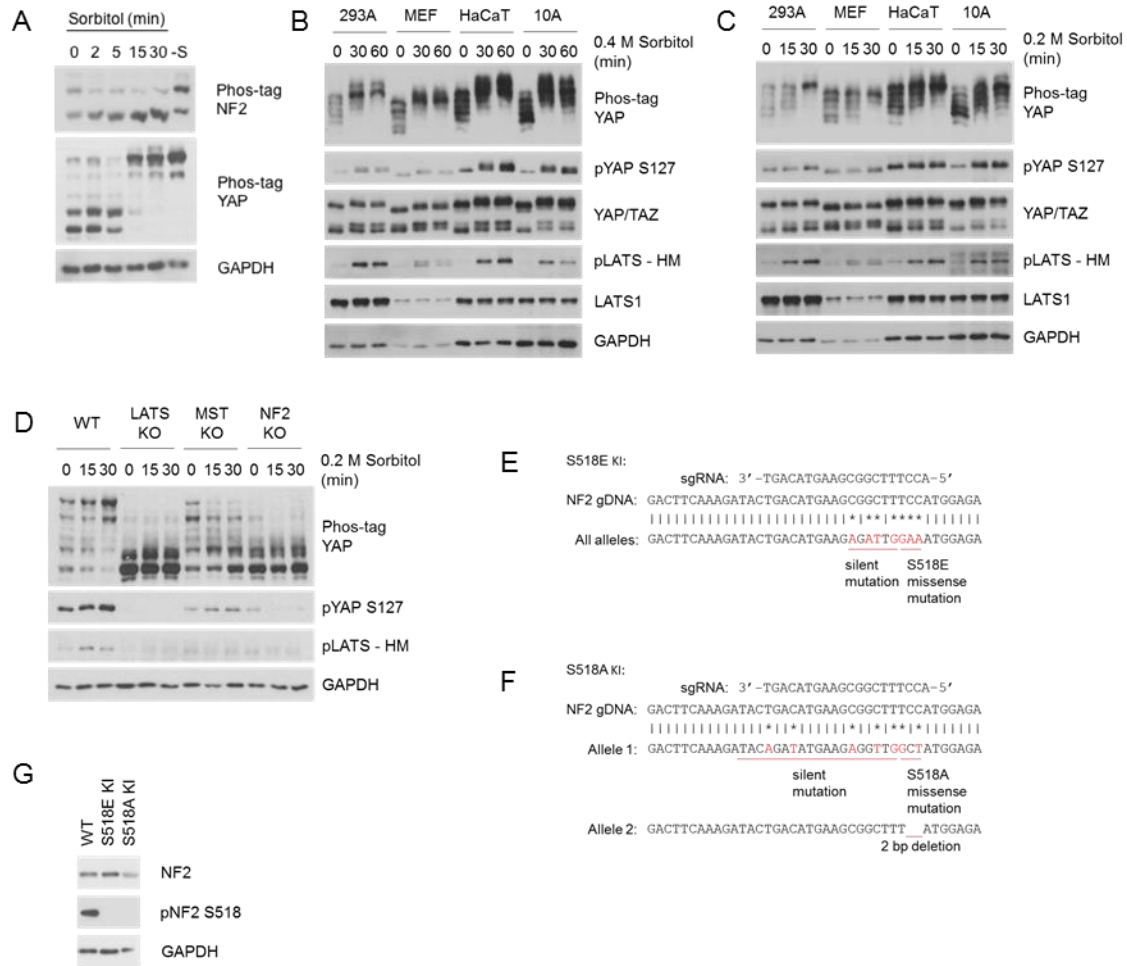
120

Figure S3.1: Effects of osmotic stress on different cell types and KO cell lines; Verification of NF2 S518E and S518A knockin cell lines. (A) Osmotic stress induces rapid NF2 dephosphorylation. HEK293A cells were treated with sorbitol for indicated times (minutes). –S indicates serum starvation for 30 min. Phos-tag gel was used to assess NF2 phosphorylation. (B) Osmotic stress induces LATS and YAP phosphorylation in a cell type independent manner under high dose sorbitol treatment. Different cell lines were treated with 0.4 M sorbitol for indicated times (minutes). LATS and YAP phosphorylation levels were detected by Western blot. (C) Osmotic stress induces LATS and YAP phosphorylation in a cell type independent manner under low dose sorbitol treatment. Experiments were similar to panel B except cells were treated with 0.2 M sorbitol. (D) NF2 and MST are required for Hippo pathway activation under low dose sorbitol treatment. Western blot results for LATS and YAP phosphorylation in wild-type HEK293A, LATS KO, MST KO, and NF2 KO cells treated with 0.2 M sorbitol. (E) Sequencing result of NF2 S518E knockin cell line. (F) Sequencing result of NF2 S518A knockin cell line. (G) NF2 protein level and phosphorylation status in NF2 S518E and S518A knockin cell lines. NF2 S518E and S518A knockin cell lines were generated with CRISPR-Cas9 in HEK293A cells. NF2 protein level and phosphorylation status were detected by Western blot.
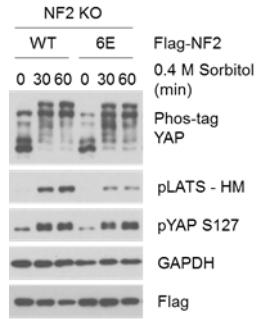
Figure S3.2: NF2 phosphorylation does not preclude Hippo pathway activation in response to osmotic stress. NF2 WT and 6E (S10E, S13E, T230E, S315E, S518E, T581E) mutant were transiently expressed in NF2 KO cells. Cells were treated with 0.4 M sorbitol for the indicated times (minutes). LATS and YAP phosphorylation levels were detected by Western blot.
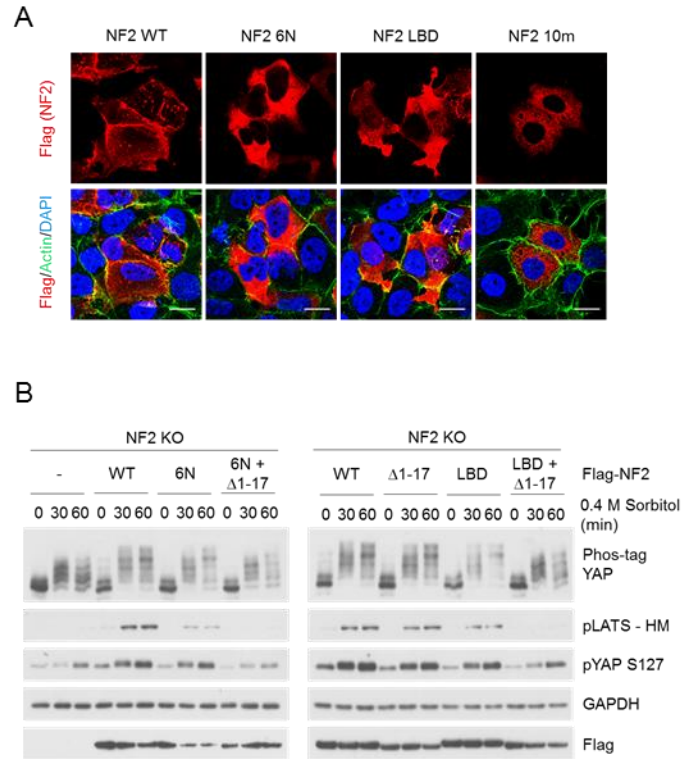
Figure S3.3: Further characterization of NF2 6N and LBD mutants. (A) Localization of different NF2 lipid binding deficient mutants. Flag-NF2 WT, 6N, LBD, and 10m mutants were transiently transfected into HEK293A cells for 24 hrs, and then cells were treated with sorbitol for 30 min. NF2 was stained with an anti-Flag antibody and is shown in red. Actin is shown in green, and the nuclei (DAPI) are show in blue. The scale bar represents 20 um. (B) Membrane association is important for the remaining activity of NF2 6N and LBD mutants. NF2 6N and LBD mutants were further truncated at the first 17 amino acids to generate NF2 6N+Δ1-17 and LBD+Δ1-17. Different mutation forms of NF2 constructs were transfected into NF2 KO cells. Cells were treated with 0.4 M sorbitol for the indicated times (minutes). LATS and YAP phosphorylation levels were detected by Western blot.
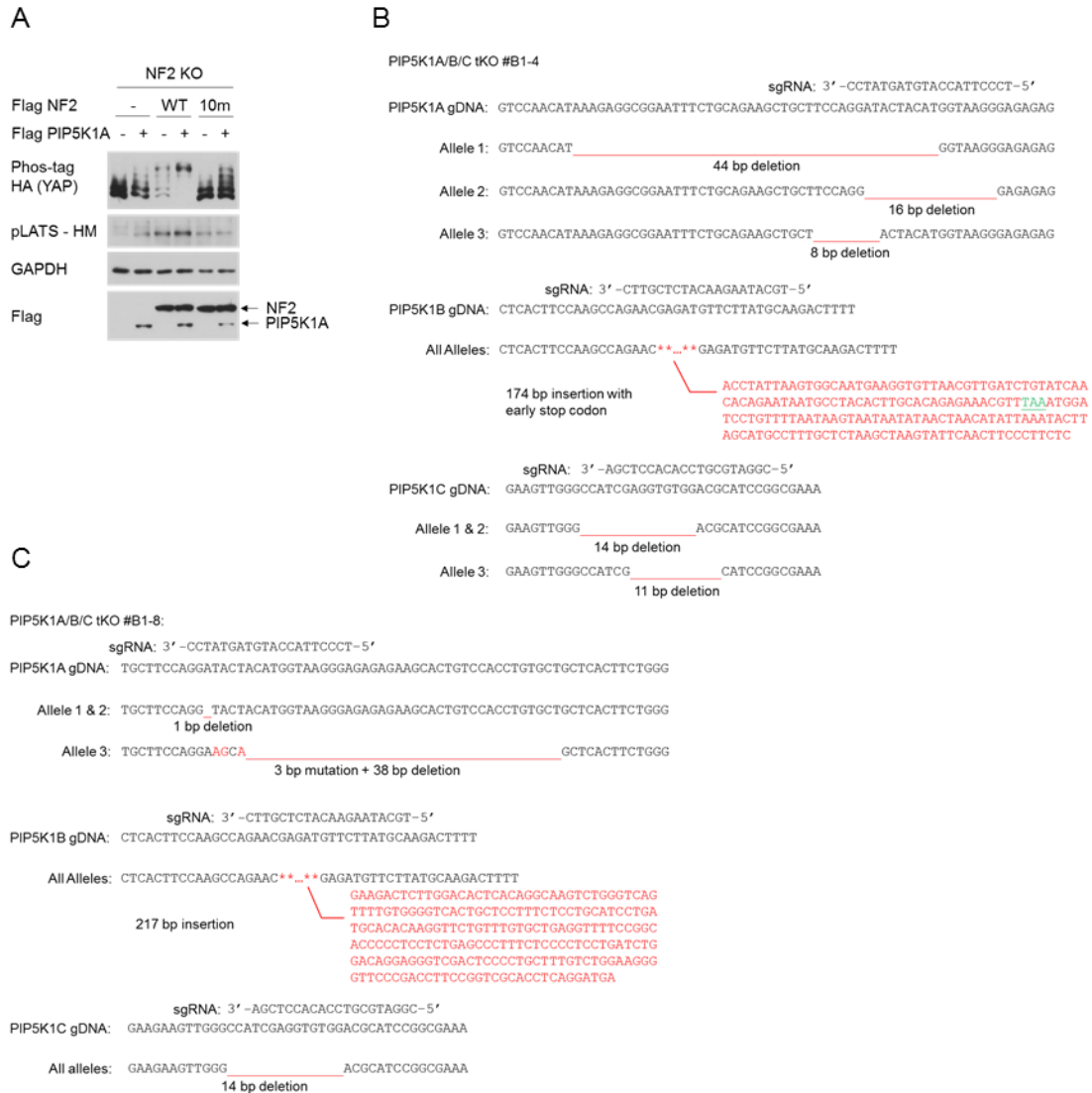
Figure S3.4: PIP5K1A activates the Hippo pathway through NF2 lipid-binding ability; Verification of PIP5K1A/B/C tKO cell lines. (A) PIP5K1A requires NF2 lipid binding ability to activate the Hippo pathway. NF2 WT, NF2 10m mutant, and PIP5K1A were transiently transfected into NF2 KO cells in different combinations. LATS and YAP phosphorylation was determined by Western blot. (B) Sequencing result of PIP5K1A/B/C tKO Clone #B1-4. (C) Sequencing result of PIP5K1A/B/C tKO Clone #B1-8.

A

Clone #1-3: ARF1/6 dKO

sgRNA: 3'-GAGACCGCCGTAATGATGTG-5'

ARF6 gDNA: ACAAGATCCGGCCGCTCTGGCGGCATTACTACACTGGGACCC

Allele 1: ACAAGATCCGGCCGCTCTTGGCGGCATTACTACACTGGGACCC
1 bp insertion

Allele 2: ACAAGATCCGGCCGCT_TGGCGGCATTACTACACTGGGACCC
1 bp deletion

sgRNA: 3'-GCTAGGAGATGTTCGAATTC-5'

ARF1 gDNA: GGATGCTGCAGGGAAGACCACGATCCTCTACAAGCTTAAGCTGGG

Allele 1: GGATG***********............***********TAAGCTGGG

396 bp insertion with early stop codon

CCACCTCTGACTTGAGCGTCGATTTTTGTGATGCTCGTCAGGGGGGCGG
AGCCTATGGAAAAACGCCAGCAACGCGGCCTTTTTACGGTTCCTGGCCT
TTTGCTGGCCTTTTGCTCACATGTGAGGGCCTATTTCCCATGATTCCTT
CATATTTGCATATACGATACAAGGCTGTTAGAGAGTAATTGGAATTAA
TTTGACTGTAAACACAAAGATATTAGTACAAAATACGTGACGTAGAAAG
TAATAATTTCTTGGGTAGTTTGCAGTTTTAAAATTATGTTTTAAAATGG
ACTATTATATGCTTACCGTAACTTGAAAGTATTTCGATTTCTTGGCTTT
ATATATCTTGTGGAAAGGACGAAACACCGGTGTAGTAATGCCGCCAGAG
GTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGC

B

C

Clone #1-1: ARF6-/-

sgRNA: 3'-GAGACCGCCGTAATGATGTG-5'

ARF6 gDNA: ACAAGATCCGGCCGCTCTGGCGGCATTACTACACTGGGACCC

All alleles: ACAAGATCCGGCCGCTCTTGGCGGCATTACTACACTGGGACCC
1 bp insertion

D
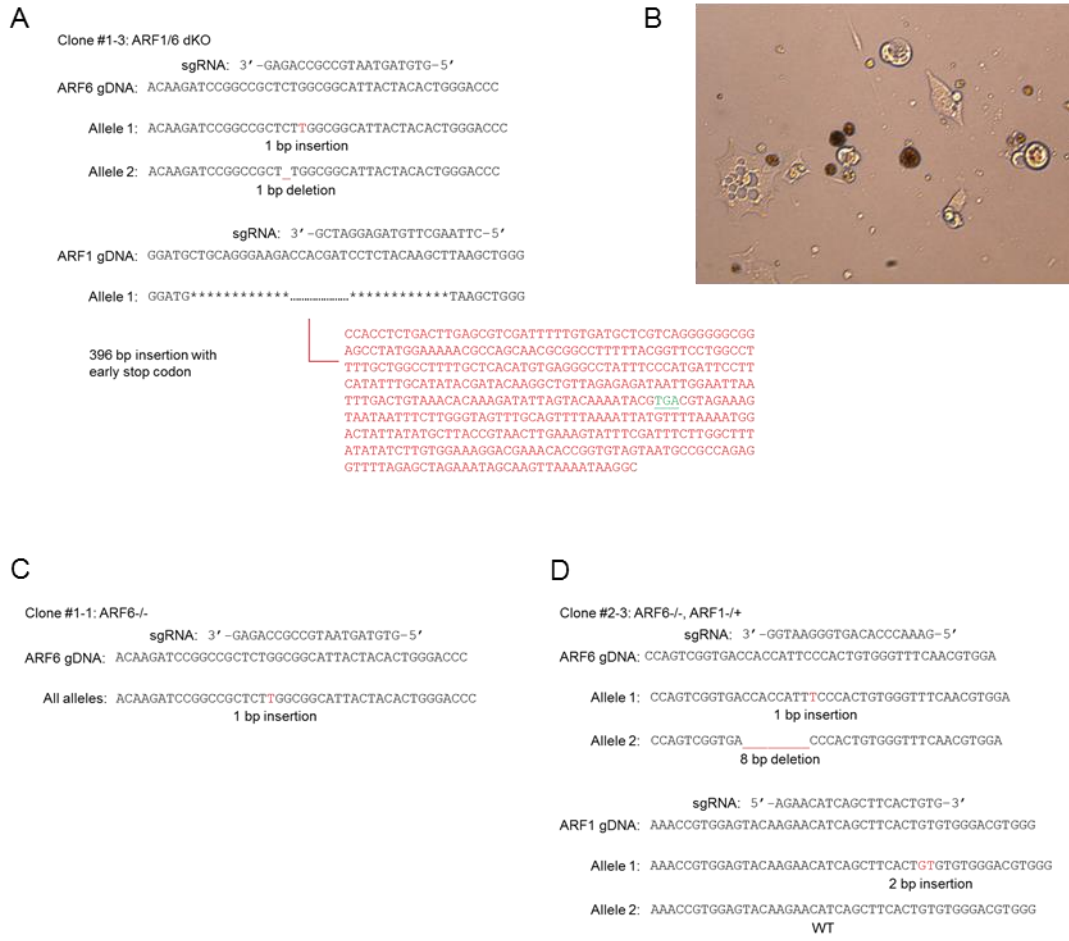
Clone #2-3: ARF6-/-, ARF1-/+

sgRNA: 3'-GGTAAGGGTGACACCCAAAG-5'

ARF6 gDNA: CCAGTCGGTGACCACCATTCCCACTGTGGGTTTCAACGTGGA

Allele 1: CCAGTCGGTGACCACCATTTCCCACTGTGGGTTTCAACGTGGA
1 bp insertion

Allele 2: CCAGTCGGTGA_____CCCACTGTGGGTTTCAACGTGGA
8 bp deletion

sgRNA: 5'-AGAACATCAGCTTCACTGTG-3'

ARF1 gDNA: AAACCGTGGAGTACAAGAACATCAGCTTCACTGTGTGGGACGTGGG

Allele 1: AAACCGTGGAGTACAAGAACATCAGCTTCACTGTGTGTGGGACGTGGG
2 bp insertion

Allele 2: AAACCGTGGAGTACAAGAACATCAGCTTCACTGTGTGGGACGTGGG
WT

Figure S3.5: Verification of ARF1/6 KO cell lines. (A) Sequencing result of ARF1/6 KO Clone #1-3 (ARF6-/-, ARF1-/-). (B) Cells with complete KO of ARF1 and ARF6 are not viable. Image shows cellular morphologies of ARF1/6 KO Clone #1-3, which has complete deletion in both ARF1 and ARF6. (C) Sequencing result of ARF1/6 KO Clone #1-1 (ARF6-/-, ARF1+/+). (D) Sequencing result of ARF1/6 KO Clone #2-3 (ARF6-/-, ARF1-/+).

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