The role of POPX2 in the regulation of hippo pathway

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THE ROLE OF POPX2 IN THE REGULATION OF HIPPO PATHWAY

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ABBREVIATIONS

AGC	Protein kinase A, G, and C families
AMOT	Angiomotin
ANKRD	Ankyrin repeat domain-containing protein
CaMKII	Calcium/calmodulin-dependent protein kinase II
CaMKP	Ca ²⁺ /calmodulin-dependent protein kinase phosphatase
CRISPR	Clustered regularly interspaced short palindromic repeats
CTGF	Connective tissue growth factor
CYR61	Cysteine-rich 61
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
ECM	Extracellular matrix
EMT	Epithelial-Mesenchymal Transition
FBS	Fetal Bovine Serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GST	Glutathione S-transferases
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
KER	Lysine-Glutamic acid-rich domain
KIF3A	Kinesin Family Member 3A
LATS	Large tumor suppressor
MEGM	Mammary Epithelial Growth Medium
MET	Mesenchymal-Epithelial Transition
MOB	Mps One Binder Kinase Activator-Like
MST	Mammalian sterile 20-like
MTT	(3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide)
PAK	p21 protein (Cdc42/Rac)-activated kinase

PBS	Phosphate Buffered Saline
PFA	Paraformaldehyde
POPX	Partner of PIX
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SARAH	Sav, Rassf, Hippo
SAV	Salvador
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
siRNA	Small interfering Ribonucleic acid
STK	Serine/threonine protein kinase
STRIPAK	Striatin-interacting phosphatase and kinase
TAK	Transforming growth factor beta-activated kinase
TAZ	Transcriptional coactivator with PDZ-binding motif
TBS	Tris Buffered Saline
TBST	Tris Buffered Saline with 0.1% (v/v) Tween 20
TEAD	TEA domain family member
Vgll	Vestigial like family
YAP	Yes-associated protein

ABSTRACT

The Hippo pathway has gained immense attention due to its key role in regulating organ size as well as its relevance to tissue regeneration and cancer. The pathway is renowned for regulating the two transcriptional co-activators, YAP and TAZ, which are key players for cell proliferation, organogenesis, and maintenance of tissue-specific progenitor cells during tissue renewal and regeneration. During tumorigenesis YAP/TAZ reprogram cancer cells into cancer stem cells in addition to their involvement in tumor initiation, progression and metastasis. Not surprisingly, in the past few years, intense focus has been shown on YAP and TAZ as potential therapeutic targets in the study of cancer and regenerative medicine.

Herein, we investigate the role of POPX2 in the regulation of YAP/TAZ transcriptional co-activators through the Hippo pathway. We found that POPX2 interacts with several of the Hippo pathway core kinases, including LATS1 which is the direct kinase regulating YAP/TAZ. We also demonstrated that POPX2 could dephosphorylate LATS1, on Threonine-1079, the site required for LATS1 activity.

Furthermore, CRISPR knock-out of POPX2 in the highly invasive breast cancer cell line MDA-MB-231, X2KO cells, showed lower YAP/TAZ protein levels both in the cytoplasm as well as in the nucleus. When the X2KO cells are grown under low attachment condition, YAP/TAZ is lost from the nucleus, suggesting that POPX2 is required for YAP/TAZ nuclear retention. RT-PCR analysis on selected YAP/TAZ target genes, *CTGF* and *ANKRD1*, showed that the transcript levels of these genes are lower in the X2KO cells. These results support possible roles of POPX2 in YAP/TAZ stabilization. Consistent with these observations, loss of POPX2 further leads to the decrease in cell proliferation and anchorage independence growth, which phenocopies the loss of YAP/TAZ.

Our current study has identified POPX2 as a regulator of LATS1, revealing a novel point of modulation within the Hippo pathway i.e. through LATS dephosphorylation. Furthermore, since LATS inactivates the YAP/TAZ oncoprotein, the potential role of POPX2 in stabilizing YAP/TAZ is consistent with previous findings that implicate the role of POPX2 in breast cancer invasiveness and metastasis. Therefore, targeting POPX2 could prove beneficial for mitigating the growth and spread of cancer in the early stages of cancer progression.

1. INTRODUCTION

Cancer is one of the leading causes of morbidity and mortality worldwide, and metastasis which is the endpoint of its progression, constitutes more than 90% of all cancer-associated deaths. Metastasis is the spread of cancerous cells that causes death by disrupting the function of organs colonized by the cancer cells. Detecting a primary tumor site before it becomes metastatic is very challenging but increases the success of therapeutic intervention. Many events drive the transition from early- to late-stage cancer and understanding them would expedite the development of effective treatment. In line with this, it is vital to unravel the cellular mechanisms that initiate metastasis for future development of effective cancer therapeutics.

1.1 Carcinogenesis

Growth is a tightly regulated cellular process. Cells only divide when they receive the appropriate cues. Many checkpoints exist within the cell cycle to ensure that genomic integrity is preserved and that conditions are suitable before the cells divide.

Tumor is a mass of cells that display abnormal and uncontrolled growth. Tumor cells that invade other tissues or organs (i.e. malignant) are known as cancer cells. How or when these cancer cells decide to invade or spread depends on both intrinsic and extrinsic factors. As a tumor grows and more mutations are acquired, cells may remain benign or become malignant depending on the mutations they acquire (intrinsic factors). The acquisition of mutations generates a heterogeneous mix of cells that differ both in morphological and phenotypical profiles. The capabilities that these cells acquired that foster their cancerous state are known as the hallmarks of cancer (Hanahan and Weinberg, 2000).

Ten hallmarks of cancer are proposed by Hanahan and Weinberg (Hanahan and Weinberg, 2011) (Figure 1). They serve as the organizing principles to extenuate the complexities of carcinogenesis. The hallmarks include resisting apoptosis, dysregulation of cellular kinetics, sustaining proliferative signaling, evasion of growth suppressors, escaping immune destruction, enabling replicative immortality, tumor-promoting inflammation, triggering invasion and metastasis, promoting angiogenesis and acquiring genomic instability and mutation. Genome instability and mutation is an underlying hallmark which generates the genetic heterogeneity that promotes acquisition of the other hallmarks. In addition, inflammation also promotes the manifestation of multiple hallmarks.

Figure 1: The 10 Hallmarks of Cancer.

Two new features were added to the six hallmark of cancer proposed in 2000, they are; emerging hallmarks (deregulating cellular energetics & avoiding immune destruction) and enabling characteristics (tumor-promoting inflammation & genome instability and mutation)

Image adapted from "Hallmarks of cancer: the next generation" (Hanahan and Weinberg, 2011) with permission.

As mentioned earlier genomic instability and mutation underlie the acquisition of the various hallmarks, however not all mutations are equal. Driver mutations are defined as mutations that confer a selective growth advantage to the cell, whereas passenger mutations are mutations that do not alter the fitness of the cell, and are found in a cell that has acquired a driver mutation. Acquisition of driver mutations within a tumor has been known to lead to cancerous transformation enabling the cells to invade near and distant sites. Often, driver mutations occur from either the gain of function of an oncogene or the loss of function of a tumor suppressor gene. Acquisition of passenger mutations on the other hand, occurs from somatic mutations without functional consequences. Therefore, the acquisition of driver mutations enable invasion to a new site by allowing tumors to survive when existing resources are insufficient to meet their growing demand due to their abnormal growth rate. The new sites will then be exploited by the metastases, often affecting the growth of surrounding healthy cells and tissues leading to organ failure and consequently death. Tumor undergoes several monumental mutational changes that lead to its cancerous progression (as depicted below, Figure 2).

Figure 2: Histological classification of breast cancer progression.

Breast cancer progression is driven by epigenetic and genomic instability making it a heterogeneous disease. Normal phase: terminal ductal lobular unit (TDLU); Premalignant phase: Atypical ductal hyperplasia (ADH), Ductal carcinoma *in situ* (DCIS); and Malignant phase: Invasive breast cancer (IBC). As tumor progresses, the risk of transformation to invasive breast cancer (IBC) increases. Invasion to the lymph nodes is the primary means by which breast cancer metastasize to distant tissues and organs. Image adapted from "Modelling breast cancer: one size does not fit all" (Vargo-Gogola and Rosen, 2007) with permission.

Besides the intrinsic factors, tumors are also subjected to various micro-environmental stresses (extrinsic factors) that may accelerate the progression towards malignancy. For instance, the tumor microenvironment (TME) which constitutes the cellular environment in which the tumor exists, including the surrounding extracellular matrix (ECM), blood vessels, immune cells, fibroblasts, bone marrow-derived inflammatory cells, lymphocytes, and signaling molecules, may contribute to the acceleration of tumorigenesis. This may occur through cancer associated fibroblasts (CAFs), tumor-associated macrophage (TAMs), tumor infiltrating lymphocytes (TIL), hypoxic condition, and chronic inflammation.

CAFs are fibroblasts with phenotype resembling myofibroblast in cancerous wounds. CAFs can exert major influences on cancer progression through ECM remodeling (Glentis et al., 2017), promoting angiogenesis (Nagasaki et al., 2014), engaging inflammatory cells, and inducing cancer cell proliferation via the secretion of cytokines and growth factors (Erez et al., 2013). Similarly, Tumor-associated macrophages (TAMs) are Th2 cytokine-activated macrophages (IL-4, IL-10, and IL-13) which contribute to cancer-associated inflammation (Solinas et al., 2009), thus, promoting proliferation, invasion, angiogenesis (Badawi et al., 2015; Tjiu et al., 2009) and metastasis of tumor cells (Smith and Kang, 2013). Compared to the exacerbating role of CAFs in tumor progression, the presence of tumor infiltrating lymphocytes (TILs) are associated with favourable prognosis in several cancers (Haas et al., 2009; Sato et al., 2005), and in breast cancer in particular, through mediating response to chemotherapy and improving clinical outcomes (Denkert et al., 2010).

The diversity that arises from aberrant mutations in tumor cells and also from the nature of the tumor micro-environmental composition and stromal cell population makes cancer a heterogeneous disease. This in turn presents a significant challenge to the development of effective anticancer therapies.

1.2 Tumor heterogeneity

Intra-tumor heterogeneity (ITH) has been observed for many decades, originally from morphological approach (Fitzgerald, 1986; Hirsch et al., 1983). Today, all forms of tumors are recognized as extremely diverse populations of cells (Marusyk et al., 2012) with ITH manifesting at the genetic, epigenetic, and phenotypic levels.

As mentioned before, a tumor is made up of cells that proliferate uncontrollably resulting in a ballooned mass of cells. Tumor spheroids that exceed a critical size of 400–600 μm in diameter, tend to develop central necrosis where the innermost cells undergo apoptosis (Friedrich et al., 2009). However, most tumors are capable of growing beyond the size of 1-2mm³ due to a switch to an angiogenic phenotype that attracts blood vessels to commensurate to their metabolic demand. This in turn enables a tumor to grow beyond the size that would otherwise be impossible to sustain. Consequently, a tumor with the size of 1 cm³ (or 1 g) which, roughly contains 1×10^9 cells is not uncommon (DeVita et al., 1975). When such tumors are treated with anti-cancer drugs, it is presumed with an almost mathematical certainty that a small population of these cells will evolve a treatment-resistant phenotype (Gay et al., 2016).

ITH is a key factor for cancer lethality, therapeutic failure and drug resistance. A heterogeneous cell population can show distinct morphological, phenotypic and genotypic profiles, which in turn further diversify their metabolism, motility, proliferation, and metastatic potential. Many different approaches have been employed to suppress and overcome ITH. Radically, one even utilizes ITH itself to increase treatment efficacy. Using a mathematical model, Gatenby tried to model the best way to tackle cancer because he believes that oncologists are taking the wrong approach i.e. using the maximum tolerable dose of chemotherapy to kill as many cancerous cells as possible, to eradicate cancer before resistance evolves. However, several confounding evidences have revealed that therapy-

resistant cells were present even before they encounter therapy (Bhang et al., 2015; Su et al., 2012; Turke et al., 2010). Therefore, Gatenby proposed “Adaptive therapy” as an alternative approach where treatment is continuously modulated to achieve a fixed tumor population. This is achieved through imposing a stable tumor burden by permitting a significant population of chemosensitive cells to survive to suppress the proliferation of the less fit but chemoresistant subpopulations (less fit due to the phenotypic cost of resistance) (Gatenby et al., 2009). Many studies have embarked on understanding how tumor heterogeneity could arise, and currently two models are accepted to explain this phenomenon; they are: (1) the cancer stem cell model and (2) the clonal evolution model, where both models are not mutually exclusive but collectively contribute to the heterogeneity of the tumor (Shackleton et al., 2009).

1.2.1 Cancer stem cell model

Tissues and organs that undergo constant remodeling and regeneration are often sites where tumors develop. Since remodeling and cellular regeneration are activities maintained by stem cells, it is appealing to speculate that tumor initiation and progression are similarly maintained by cancer stem cells (CSCs). Here, cancer stem cells are defined as a subset of tumor cells endowed with the capacity to proliferate, differentiate and self-renew (Shackleton et al., 2009). The cancer stem cell (CSC) model assumes that a tumor is hierarchically organized, analogous to a normal tissue, and not all cancer cells are equally capable of producing a heterogeneous tumor cell population. The only cells capable of self-renewal and producing a heterogeneous tumor cell population are known as cancer stem cells (Figure 3).

Figure 3: The cancer stem cell model of tumor heterogeneity.

Cancer stem cells (grey) are the only cells capable of generating a heterogeneous tumor, whereas the more differentiated cell (blue) are not capable of generating a heterogeneous tumor. Blue arrows indicate differentiation, grey arrows indicate self-renewal and black arrows indicate potential for tumor heterogeneity recapitulation.

Data supporting the cancer stem cell model come from many different human cancers including germ cell cancers (Kleinsmith and Pierce, 1964), leukemias (Lapidot et al., 1994), breast cancers (Al-Hajj et al., 2003), brain cancers (Singh et al., 2004), and colon cancers (Dalerba et al., 2007). In each context, only the tumorigenic population of cells harboring CSC markers can recapitulate the disease upon transplantation into immunocompromised mice. In spite of robust data supporting the stem cell model in some cancers, it is imperative to acknowledge a number of limitations: (1) the lack of evidence that tumorigenic cells differ from non-tumorigenic cells as a result of epigenetic instead of genetic differences; and (2) the use of immunocompromised mice as model for transplantation assay underestimates the occurrence of human cancer cells with tumorigenic prospective in some cancers (Quintana et al., 2008).

1.2.2 Clonal evolution model

The clonal evolution model proposes that tumors arise from a single mutated cell which over time undergoes successive genetic and epigenetic changes generating outgrowth of sub-clones that best thrive in response to selection pressures, which ultimately determine the phenotype of the tumor (Shackleton et al., 2009) (Figure 4). This model enables understanding of not only heterogeneity, but also treatment failures and tumor aggression.

Figure 4: The clonal evolution (stochastic) model of tumor heterogeneity.

In the clonal evolution model, all cells within a tumor are capable of self-renewal and generating a heterogeneous tumor. Tumor arises from a single cell acquiring a mutation. Additional mutations are acquired along the way during subsequent cell divisions and eventually giving rise to different sub-populations. Each of these sub-populations continues to divide and further mutate. Some of the sub-populations may have growth advantage with respect to the environment and other selection pressures.

A common characteristic for the two models is the generation of a more tumorigenic cell population, and the difference lies in the mechanism through which these cells arise either through epigenetics (CSC model) or genetic selection (clonal evolution model).

Nonetheless, the defining feature of the tumorigenic cell population is the acquisition of stem-cell like characteristics, which are self-renewal, proliferation and differentiation.

Another stem-cell like characteristic that cancer cells could acquire to form tumor heterogeneity is phenotypic plasticity (Meacham and Morrison, 2013; Wahl and Spike, 2017). Phenotypic plasticity could fit with either of the above models, since it only confers dynamicity between the different cell lineages. Regardless of the number of mutations a cancer cell acquires, what makes cancer a lethal disease is in its ability to spread and colonize tissues. Spreading and colonizing tissues require a flexible program that allows cells to change during the course of invasion, migration and colonization. Such a program will require a certain degree of plasticity. Indeed, if plasticity is a wide-spread capability amongst cancer cells, it is then critical to distinguish situations in which intrinsic differences in tumorigenic capacity reflect reversible changes in cell state as compared to irreversible differentiation as these situations make very different experimental and clinical predictions (Marjanovic et al., 2013; Vlashi and Pajonk, 2015). More importantly, if the heterogeneity within tumors is due to cells that reversibly convert between tumorigenic and non-tumorigenic states it may be impossible to define what constitute a tumorigenic cell population, since a cell that is deemed non-tumorigenic could convert to a tumorigenic state under a different condition. The acquisition of cellular plasticity is an enabling factor for metastasis (Tsai and Yang, 2013). In addition to contributing to tumor heterogeneity, phenotypic plasticity enables phenotypic switching from one cell type to another, as exemplified by Epithelial-mesenchymal transition (EMT).

1.3 Tumor plasticity

The process of cellular differentiation has traditionally been view as the gradual restriction of developmental options until commitment to a specific fate is established. This is usually the case in normal physiological development. However, the differentiated state of mature cells is not permanent and stable as initially thought given the evidences from dedifferentiation and reprogramming studies (Friedmann-Morvinski and Verma, 2014).

Plasticity is defined as adaptability to changes to the environment. At the organismal level, adaptation occurs through mutations or genetic changes that promote survival, and these mutations or genetic changes have to first occur at the cellular level i.e. cellular adaptation precede organismal adaptation (Lopez-Maury et al., 2008). Cellular adaptation or plasticity is the program that underlies tissue patterning, morphogenesis and development which would later translate to organismal adaptation. In normal tissues, where hierarchical organization exists, stem cells are the only cells exhibiting plasticity. This enable regulated and coordinated execution of self-renewal and differentiation to maintain healthy tissue homeostasis and growth. However, when cells acquire mutations and genetic changes, certain plasticity genes may be activated in otherwise terminally differentiated mature cells. This in turn confers cells with plasticity which result in a population of cells with dedifferentiated states (Puri et al., 2015).

Cancer cells can become plastic to reversibly convert between states that differ in their competence for growth or spread (Tsai and Yang, 2013). For example, some cancer cells are capable of transiting between epithelial and mesenchymal states where the latter is more malignant. Hence it was proposed that, the discovery of distinct markers or molecular switches that foster the transition would provide a clear discrimination of these various states, and thus provide better treatment strategies. However, as more studies examine the different states, it is becoming clear that there is no absolute or discrete states but the

states themselves are continuous and move along a spectrum or sort, thus revealing certain intermediate/partial states (Ye and Weinberg, 2015).

In response to environmental triggers, plasticity molecular program(s) can enhance tumor cell invasion. An example of a well-defined plasticity program is the Epithelial-Mesenchymal Transition (EMT) (Figure 5).

Figure 5: Plasticity in Epithelial-Mesenchymal Transition.

EMT occurs in two stages: (1) epithelial cells lose cell-cell contact and dissociate from their matrix. Fostering these changes are the loss of epithelial markers such as E-cadherin and acquisition of Snail and Slug expression and activity; (2) cells acquire stress fibers and migration potential, fostering these changes are the the expression of vimentin and fibronectin. Between the two distinct phenotypes exists an intermediate/partial state in which cells are yet to become fully mesenchymal.

1.3.1 Epithelial-Mesenchymal Transition (EMT)

Defined as the acquisition of mesenchymal properties and loss of epithelial properties through a trans-differentiation process, EMT is a developmental program that functions physiologically to enable cells to translocate during embryogenesis and wound healing. However, this developmental program is hijacked by tumor during its transformation for metastatic spread. Cellular plasticity is reminiscent of early embryonic development characterized by reversible molecular programs. These events are fundamental for tissue formation during development and adult cell renewal. Characteristic of this process, both in physiological and cancer context, is that with various cues cells can bi-directionally slide along the spectrum going from mesenchymal to epithelial phenotype and reverse the course as situations warrant (Voon et al., 2017).

Epithelial and mesenchymal states are dictated by molecular determinants such as miRNAs and transcription factors, whereby changes to these determinants lead to an observable phenotype characterized by the expression of cytoskeletal proteins and cell surface proteins. Examples of EMT-inducing factors are the transcription factors: TWIST, SNAIL, SLUG and ZEB which promote the expression of mesenchymal markers such as vimentin, fibronectin and N-cadherin, and suppress the expression of epithelial markers such as E-cadherin, claudins, occludins, ZO-1, desmoplakin and cytokeratins (Kalluri and Weinberg, 2009).

It was proposed that epigenetic landscape directs the stability of epithelial-mesenchymal plasticity. EMT is regulated through contextual signals inducing epigenetic repression of epithelial markers by histone modifications, thus defining the plasticity of epithelial cells and the extent of the phenotypic state during the transition. The gain of a more stable mesenchymal phenotype relies on sustained and potent availability of EMT-inducing factors (Tam and Weinberg, 2013).

Rooted in both plasticity and EMT is the reactivation of developmental programs, which are otherwise highly active only during development. Examples of such programs include the TGF- β signaling pathway, Wnt signaling pathway, Notch signaling pathway and Hippo signaling pathway. Of particular interest to this study are the downstream targets of Hippo signaling pathway, YAP and its paralogue TAZ, where both proteins are involved in cellular activities governing stemness, plasticity, EMT and tumor growth.

Recent finding has also revealed the reprogramming capability of YAP/TAZ such as the induction of a tissue-specific stem/progenitor cell state upon transient expression of exogenous YAP/TAZ in primary differentiated mouse cells (Panciera et al., 2016). In addition TAZ has been shown to confer cancer stem cell traits in breast cancer (Cordenonsi et al., 2011), and promote EMT via the expression of N-cadherin, vimentin, and fibronectin (Lei et al., 2008).

1.4 Hippo pathway

During organogenesis, the final shape and size of an organ is determined by intrinsic information present within tissues and organs. In addition, cells also respond to spatial, physical and biochemical cues secreted by neighboring cells directing them to die, divide or differentiate. Therefore, a cell takes both intrinsic and extrinsic cues in directing its commitment fate. Depending on the nature of the stimuli, cellular response could be transient/permanent and reversible/irreversible. Many biochemical pathways exist within a cell, and they could work synergistically through cross-talk or antagonistically through negative modulation, establishing a fine tuning capability that allows the cell to adapt and survive under different circumstances.

The Hippo pathway is an evolutionarily conserved regulator of cell specification, tissue growth and organ development (Hansen et al., 2015). It forms a nexus from various inputs. The Hippo pathway consists of a large network of proteins (35 in both *D. melanogaster* and mammals are currently known) that control the growth of different tissues during development and regeneration, as well as in pathological states such as cancer. First discovered in *Drosophila*, four proteins constitute the core kinase cascade: Hpo, Sav, Wts and Mats which are orthologues of the human MST1/2, SAV1, LATS1/2, and MOB1, respectively (Meng et al., 2016).

The four proteins (Hpo, Sav, Wts and Mats) were discovered in genetic screens in *Drosophila* to identify potential tumor suppressor genes, where loss-of-function mutant clones for any of these four genes results in tissue overgrowth due to increased proliferation and decreased cell death (Pan, 2010). Following that, biochemical study reveals that the four tumor suppressors function in a kinase cascade in which the Hpo-Sav complex phosphorylates and activates Wts-Mats complex (Wu et al., 2003). Wts-Mats in

turn phosphorylate a downstream target, Yorkie (Yki), a transcriptional co-activator and oncogene. Overexpression of Yki phenocopies the loss of Hippo signaling.

Similarly, in mammalian cells, the cascade of serine/threonine kinases becomes activated through phosphorylation (Figure 6). MST together with SAV forms a complex to phosphorylate and activate the LATS/NDR-MOB1 complex (Chan et al., 2005; Praskova et al., 2008). Activated LATS/NDR then phosphorylates and inactivates the transcriptional co-activator Yes-associated protein (YAP) and/or Transcriptional coactivator with PDZ-binding motif (TAZ) resulting in YAP/TAZ cytoplasmic sequestration and degradation. Hence, YAP /TAZ are spatially regulated by their phosphorylation statuses. In the phosphorylated state, cytosolic YAP/TAZ are sequestered for degradation while in the dephosphorylated state nuclear YAP/TAZ interact with transcription factors such as TEAD1-4 and SMADs to promote gene expression (Varelas et al., 2008; Zhao et al., 2008). These interactions will lead to the expression of target genes promoting proliferation, survival and migration.

Figure 6: Overview of the Hippo pathway.

Core components of the kinase cascade are MST, SAV, MOB1, and LATS/NDR. (1) MST auto-phosphorylation and activation, (2) Activated MST phosphorylates SAV, MOB1, LATS and NDR, and (3) Activated LATS/NDR then phosphorylates YAP/TAZ. Phosphorylated YAP/TAZ are sequestered in the cytoplasm and targeted for degradation. On the other hand, non-phosphorylated YAP/TAZ translocate to the nucleus and partner a transcription factor (e.g. TEAD) to regulate the transcription of their target genes.

1.4.1 Mammalian Sterile-Twenty-like (MST)

MST kinases are involved in various developmental pathways such as the control of cell proliferation, differentiation, and apoptosis. The MST kinase family, consists of five proteins: MST1, MST2, MST3, MST4, and YSK1. MST1/2 were initially discovered in mammalian cells as members of the Ste20 family (Creasy and Chernoff, 1995) and subsequently isolated as kinases activated by extreme stress (Taylor et al., 1996). MST came into the limelight when its ortholog (Hpo) as well as other core kinases SAV (Pantalacci et al., 2003), LATS (Xu et al., 1995) and MOB1 (Lai et al., 2005) which were later known as the Hippo pathway were found through genetic screens to identify genes that regulate organ size in *Drosophila*. Following these studies, the conserved role of MST in regulating organ size was established and its potential as a tumor suppressor was later realized.

MST1 and MST2 are highly homologous with respect to sequence and domain organization. Structurally, MST1/2 consist of an N-terminal kinase domain, a coiled-coil domain, an autoinhibitory domain (AID), and a Salvador–Ras association (RA) domain family (RASSF)–Hippo (SARAH) domain (Figure 7). The presence of two caspase-cleavage sites between the kinase and the autoinhibitory domains enables MST1/2 to exist in two forms; a 54 kDa full length protein and a 36 kDa caspase cleaved form. The SARAH domain enables MST1-MST1 and MST2-MST2 homodimerization, MST1-MST2 heterodimerization, and dimerization with other SARAH domain containing proteins such as Ras-association domain family (RASSF) proteins (Rawat and Chernoff, 2015).

Figure 7: Schematic representation of MST1 domain organization.

MST1 comprises an N-terminal kinase domain (teal), a C-terminal Salvador–Ras association (RA) domain family (RASSF)–Hippo (SARAH) domain (blue), and an autoinhibitory domain between the kinase and the SARAH domain (red). MST1 is regulated by phosphorylation at T120, T183, T387, and Y433 and undergoes caspase cleavage at D326 and D349.

MST regulation

Apoptotic or stress stimuli triggers MST auto-phosphorylation on multiple sites in the activation loop (Glantschnig et al., 2002; Graves et al., 2001). Activation of MST1/2 occurs through auto-phosphorylation at the activation loop mediated by its homodimerization via the C-terminal SARAH (Sav/Rassf/Hpo) domain (Jin et al., 2012; Ni et al., 2013). Two residues, Threonine 183 and 187 within the MST1 activation loop are essential for MST activation.

During apoptosis, caspase mediated cleavage of MST1/2 removes the autoinhibitory domain (AID) and SARAH domain, increasing its activity (Graves et al., 1998). Activated MST1 elicits cell detachment and JNK activation independent of caspase cleavage. In the absence of caspase-3, Serine 327 phosphorylation on MST modulates caspase cleavage. In addition, Threonine 387 is an inhibitory site where phosphorylation by AKT prevents MST apoptotic cleavage *in vitro* and in apoptotic cells (Jang et al., 2007), while dephosphorylation by PHLPP, activates Mst1 and its downstream effectors p38 and JNK to induce apoptosis.

MST1/2 substrates

MST1/2 phosphorylate LATS1 at Thr-1079, located in the hydrophobic motif (HM) C-terminal to the kinase domain. Upon phosphorylation by MST, LATS1 undergoes auto-phosphorylation on Ser-909 of the activation loop (T loop), leading to its activation (Chan et al., 2005). Phosphorylation on both residues Ser-909 and Thr-1079 are essential for LATS1 activation (Chan et al. 2005; Hergovich et al. 2006; Hergovich 2013). Other substrates of MST1/2 include the adaptor protein SAV1 (Callus et al., 2006), MOB1A/B (Praskova et al., 2008), histones H2B (Cheung et al., 2003), H2AX (Wen et al., 2010), FOXO3 (Lehtinen et al., 2006) and cardiac troponin (You et al., 2009).

MST1/2 cellular functions

MST1/2 are the serine/threonine kinases known to initiate the Hippo pathway cascade. MST1/2 belong to the germinal center kinase II (GCKII) family, while MST3 and MST4 are distantly related kinases belong to GCKIII family (Thompson and Sahai, 2015). MST3 regulates G1/S transition in mammalian cells via a MST3-NDR1/2-p21 axis (Cornils et al., 2011), while MST4 promotes growth and transformation via the MEK1/ERK pathway (Lin et al., 2001). Another related kinase, YSK1 regulates adhesion, polarity and migration via a 14-3-3 ζ dependent pathway (Preisinger et al., 2004).

1.4.2 Salvador Family WW Domain Containing Protein 1 (SAV1)

SAV1, also known as WW45 (ortholog to *Drosophila* Sav), functions as a scaffold protein that associates with MST1/2 via its SARAH domain to facilitate LATS1/2 phosphorylation (Figure 8). In addition to facilitating LATS phosphorylation, SAV1 also associates and stabilizes MST. SAV1 itself is a direct substrate of MST1 (Callus et al., 2006). The mechanism through which SAV1 promotes MST1/2 activation occurs via antagonizing the PP2A phosphatase activity of STRIPAK. SAV1 antagonistically binds to STRIPAK inhibiting its phosphatase activity, thus protecting the MST1/2 activation-loop phosphorylation (Bae et al., 2017). Besides its role in Hippo pathway, SAV1 functions as an effector of the tumor suppressor, RASSF1A. SAV1 mediates RASSF1A full apoptotic activity and the activation of p73 (Donninger et al., 2011).

Figure 8: Schematic representation of SAV1 domain organization.

SAV1 comprises of a C-terminal SARAH domain (blue), and two WW domains (yellow & peach).

1.4.3 Mps one binder kinase activator 1 (MOB1)

MOB1 proteins (ortholog to *Drosophila* Mats) are encoded by two closely-related genes MOB1A and MOB1B, where the proteins share 95% amino acid sequence similarity (Stavridi et al., 2003). Generally, MOB1 are cytoplasmic proteins (Hergovich et al., 2005), but when targeted to the plasma membrane via attaching a myristoylation/palmitoylation (mp) motif it can hyperactivate NDR/LATS kinases (Hergovich et al., 2006).

MOB1 is capable of binding to all four human NDR1/2 and LATS1/2 kinases (Hergovich, 2011) via a conserved domain from yeast to man known as the N-terminal regulatory (NTR) domain. In contrast to MOB1, MOB2 binds to NDR1/2 kinases, but not to LATS1/2 kinases (Devroe et al., 2004). Interestingly, the binding of MOB2 to NDR1/2 inhibits the activation of NDR1/2 as oppose to the binding of MOB1. MOB1 is autoinhibited by a switch helix which blocks its binding to LATS/NDR kinase. Its activation involves the dissociation of the switch helix via a “pull-the-string” mechanism. This is achieved through phosphorylation at both Threonines 12 and 35 by MST, leading to translocation of phosphorylated residues to their respective phosphorylation binding pockets, exposing the binding site for LATS/NDR kinase (Kim et al., 2016b).

When activated, MOB1A/B directly bind to LATS/NDR via an N-terminal SMA (S100B and MOB Association) domain, thus releasing the autoinhibition caused by the autoinhibitory sequence (AIS) on LATS/NDR (Bichsel et al., 2004). As an adapter protein, MOB1 regulates two phosphorylation reactions by binding concomitantly to MST and LATS kinases, which (1) enables the trans-phosphorylation reaction between MST and LATS, and (2) allosterically activates the catalytic function of LATS1/2 to stimulate YAP and TAZ phosphorylation.

1.4.4 Large tumor suppressor (LATS)

Large tumor suppressor (ortholog to *Drosophila* Warts, Wts) is a serine/threonine kinase belonging to the NDR family. It is also classified under the AGC class of protein kinases, which is named after Protein Kinase C, Protein Kinase A and Protein Kinase G. The paralogous proteins, LATS1 and LATS2 both display high sequence similarity and analogous means of post-translational modifications. Despite reports of the two kinases having functional differences, most converge to similar pathways and outcomes (Furth and Aylon, 2017).

LATS1 consists of a C-terminal hydrophobic motif (1075-1083), a kinase domain, a protein binding domain (PBD) which binds to MOB1, two PPxY motifs responsible for YAP interaction and a ubiquitin-associated domain (UBA), while LATS2 share similar domain organization except that it only has one PPxY motif (Figure 9).

Figure 9: Schematic representation of LATS1 domain organization.

LATS1 comprises a C-terminal hydrophobic motif domain (gold), kinase domain (teal), protein binding domain (green), two PPxY motifs (black), and an ubiquitin-associated domain (purple). LATS1 is phosphorylated at two regulatory sites, Ser-909 at the kinase domain and Thr-1079 at the hydrophobic motif.

LATS regulation

MST1/2 mediated phosphorylation at Thr-1079 on the hydrophobic motif (HM) stimulates LATS1 auto-phosphorylation at Ser-909 on the activation loop (T loop), resulting in LATS1 activation. In addition, MOB1 functions as an adaptor promoting MST-LATS interaction and phosphorylation. Binding of MOB1 to the LATS hydrophobic domain relieves LATS autoinhibition and promotes auto-phosphorylation (LATS1 residues S674, S909 and S1049; LATS2 residue S835).

Of note, MST1/2 is not the sole activator of LATS1/2, a recent study has implicated MAP4K family kinases acting in parallel by phosphorylating and activating LATS1/2 independently of MST1/2 to inhibit YAP/TAZ (Meng et al., 2015). Similarly, PKA mediated phosphorylation of LATS bypasses MST, enhancing LATS2 kinase activity for YAP. Other MST-independent phosphorylation of LATS may also result in cellular activities that are not linked to YAP/TAZ regulation. For instance, LATS2 phosphorylation by CHK1/2 on Ser408 leads to apoptosis. LATS proteins are stabilized by HSP90 and MOB1 (Huntoon et al., 2010), while LIM domain-containing proteins Ajuba, Dachshous and Zyxin function as LATS destabilizers to promote cell proliferation via reducing LATS protein levels and activity (Das Thakur et al., 2010; Rauskolb et al., 2011).

LATS substrates

YAP is phosphorylated by LATS on the following residues, Ser61/109/127/164/381 (Hao et al., 2008), and TAZ on Ser66/89/117/311 (Lei et al., 2008). The sequence similarity of these phosphorylation sites led to the identification of the HXRXXS/T consensus motif for LATS1/2 kinases. LATS1/2-mediated phosphorylation of YAP/TAZ promotes the inhibition of YAP/TAZ via (1) cytoplasmic sequestration of YAP/TAZ, and (2) increasing YAP/TAZ protein degradation. Besides YAP/TAZ, LATS is also known to phosphorylate Snail1 on Thr203 (Zhang et al., 2012), CDC26 on Thr7 (Masuda et al., 2015), and MYPT1 (myosin phosphatase-targeting subunit 1) on Ser445 (Chiyoda et al., 2012).

LATS1/2 cellular functions

Besides their role in the Hippo pathway, LATS1/2 is also involved in the regulation of breast cell fate, whereby ablation of LATS1/2 promotes the acquisition of luminal phenotype and increased luminal progenitor cells through the stabilization of ER α , independent of YAP/TAZ (Britschgi et al., 2017). LATS1/2 is also crucial for the preservation of genomic integrity, initiation of programmed cell death (Suzuki et al., 2013),

cell cycle and tetraploidy prevention (Aylon et al., 2006), suppression of migration (Furth et al., 2015) and regulation of stem cell differentiation (Aylon et al., 2014). All of which are beyond YAP/TAZ regulation.

LATS1 and LATS2 have several similar and unique binding partners. The similar binding partners of LATS1 and LATS 2 are proteins involved in Hippo signaling, tight junctions, gap junctions, and antigen processing. Exclusive binding partners of LATS1 are proteins involved in estrogen signaling, whereas exclusive binding partners of LATS2 are proteins involved in cell cycle metabolism (Furth and Aylon, 2017).

Both LATS kinases display dynamic localization depending on the cell type and context. They can be detected on centrosomes (Nishiyama et al., 1999), tethered to the plasma membrane (Yin et al., 2013) or localized to the cytoplasm (Li et al., 2003).

Dephosphorylation of LATS1 and subsequent activation of YAP/TAZ can occur both in the nucleus and cytoplasm (Zhang et al., 2016). LATS1/2 are localized in the cytoplasm or the nucleus, depending on cell lineage. The kinases can also be tethered to the plasma membrane for activation (Hergovich et al., 2006).

1.4.5 Nuclear Dbf2-related kinase (NDR)

Nuclear Dbf2-related kinases are the closest homologs of LATS1/2. NDR kinases exist in two isoforms, NDR1 and NDR2, (also known as STK38 and STK38L respectively), which are situated at discrete genomic loci (Stegert et al., 2004). Similar to LATS, NDR kinases consist of a C-terminal hydrophobic motif, a kinase domain and a protein binding domain which binds to S100B and MOB1. However, NDR kinases lack the PPxY motif present in LATS which cast doubt in its ability to bind YAP/TAZ via their WW domains (Figure 10).

Figure 10: Schematic representation of NDR domain organization.

NDR comprises of a C-terminal hydrophobic motif, a central Kinase domain and an N-terminal protein binding domain (PBD). NDR is phosphorylated on three residues: Thr-74, Thr-281 and Thr-444.

NDR regulation

NDR undergoes auto-phosphorylation on three residues *in vitro*. They are Thr-74, Ser-281 and Thr-444. Binding of MOB1A/B to the N-terminal regulatory (NTR) domain of NDR1/2 promotes the auto-phosphorylation and activation of NDR1/2. NDR1 is regulated by a Ca^{2+} -dependent, S100B-mediated auto-phosphorylation on Ser-281 and by phosphorylation by an as yet unidentified Ca^{2+} -dependent upstream kinase on Thr-444.

NDR substrates

NDR kinases have many substrates. NDR phosphorylates YAP1 on Ser-127 in mice intestinal epithelial cells (Zhang et al., 2015), p21/Cip1 on Ser-146 for regulating p21 protein stability (Cornils et al., 2011), AAK1 on Ser-635 and Rabin8 on Ser-240 for dendrite and spine development in neurons (Ultanir et al., 2012), and Ser-272 on Rabin8 for cilia development (Chiba et al., 2013)

NDR cellular functions

NDR kinases have been described to regulate apoptosis (Cornils et al., 2010; Vichalkovski et al., 2008), proliferation (Cornils et al., 2011), centrosome duplication (Hergovich et al., 2007), chromosome alignment (Chiba et al., 2009), and autophagy in response to starvation-induced stress (Hergovich, 2016). In mammalian cells, unlike LATS1/2 overexpression, NDR1/2 overexpression neither interacts with nor affects the phosphorylation status of YAP (Hao et al., 2008). However, ablation of *Ndr1* predisposes T cell lymphoma development in mice (Cornils et al., 2010), indicating a potential tumor-suppressive function.

A similar study with *Ndr1* discovered that genetic deletion of *Ndr1* results in the upregulation of Ndr2 protein, suggesting a compensatory effect between the two isoforms. Thus, to overcome this, they ablated both *Ndr1 and Ndr2* resulting in increased YAP protein level and decreased YAP phosphorylation on Ser-127 with no alterations in LATS1/2 protein levels and phosphorylation (Zhang et al., 2015). The increased in YAP levels in the NDR1/2-deficient tissue led to increase YAP activity *in vivo* as interpreted by the nuclear accumulation of YAP and increased YAP target gene expression. Thus, implicating NDR kinase in the regulation YAP function and phosphorylation in a kinase activity-dependent manner, and expanding the members of the Hippo kinase cassette.

1.4.6 YAP and TAZ

YAP and TAZ (orthologs to *Drosophila* Yorkie, Yki) are co-activators of transcription; the function of which are spatially regulated. Lacking a DNA binding domain, these co-activators require a transcription factor to regulate gene expression. The TEA domain (TEAD) transcription factors which regulates cell contact inhibition, epithelial-mesenchymal transition (EMT), oncogenic transformation and apoptosis inhibition represent a major interacting partner for YAP and TAZ to regulate gene expression. Shown below are the domain organization of YAP/TAZ, the interaction partners associated with the domains and the phosphorylation sites (Figure 11).

Figure 11: YAP and TAZ domains, serine phosphorylation sites and known interactions.

YAP and TAZ share several common domains; TEAD binding domain, 14-3-3 binding domain, WW domain, coiled-coil domain, Transcriptional Activation Domain (TAD) and PDZ binding domain. Phosphorylation at the 14-3-3 binding site induces cytoplasmic sequestration, and at the phosphodegion site induces β -TRCP mediated degradation.

YAP and TAZ share several domains:

TEAD binding domain binds to TEAD transcription factor which is crucial for development. TEAD together with its co-activator (e.g. YAP/TAZ, Vgll and p160) promotes cancer progression through the expression of proliferation genes such as c-myc, survivin, Axl, ANKRD1, CTGF and Cyr61.

The *14-3-3 binding domain* allows binding to 14-3-3 and thus the cytoplasmic retention of YAP/TAZ relieving their co-activator activity where subsequent phosphorylation tag for degradation.

The *WW domain* enables interaction with regulatory kinases such as LATS1/2 as well as PPxY motif-containing transcription factors such as RUNX, p73, and the cytoplasmic domain of ERBB4 or with the transcriptional cofactor WBP2.

The *coiled-coil domain* of TAZ interacts with Smad2/3 upon TGF- β stimulation. The complex is then recruited to the TGF- β response elements which sustains self-renewal in human embryonic stem cells (Varelas et al., 2008)

The *phosphodegrom domain* is a series of phosphorylated residues that enables interaction with an E3 ubiquitin-ligase (e.g. β -TrCP). YAP and TAZ are phospho-regulated; phosphorylation on S127 of YAP and S89 of TAZ mediates 14-3-3 binding, and subsequent phosphorylation on S397 of YAP or S314 of TAZ promotes E3 ubiquitin ligase-mediated degradation.

The *transcriptional activation domain* (TAD): no studies has been done to shed light on the functional role of this domain. The domain is largely unstructured based on secondary structure predictions.

The *PDZ binding domain* interacts with PDZ containing proteins forming multi-protein complexes that target proteins to specific submembranous sites essential for polarized protein distribution in epithelial cells.

TAZ shares homology with YAP, however, the differences between the two are: TAZ lacks a proline-rich region at its N-terminus, a SH3-binding motif and has only a single central WW domain which functions as a transcriptional co-activator by binding to the PPxY motif on transcription factors (Kanai et al., 2000).

Table 1 listed known transcriptional factors that interact with YAP/TAZ, their target genes and functions.

Transcription factors	Target genes	Function(s)	Reference(s)
TEADs	<i>ANKRD1</i>	Metastasis, Proliferation	(Zanconato et al., 2015)
	<i>AREG</i>	Metastasis, Proliferation	(Yang et al., 2012)
	<i>Axl</i>	Metastasis, Proliferation	(Xu et al., 2011)
	<i>BCL2/BIRC5</i>	Anti-apoptosis	(Heallen et al., 2011)
	<i>CDK6</i>	Senescence	(Xie et al., 2013)
	<i>Cdx2</i>	Trophoblast differentiation	(Nishioka et al., 2009)
	<i>CTGF</i>	Metastasis, Proliferation	(Vassilev et al., 2001)
	<i>Cyr61</i>	Proliferation, Angiogenesis	(Lai et al., 2011)
	<i>c-Myc</i>	Metastasis, Proliferation	(Neto-Silva et al., 2010)
	<i>EDN1/2</i>	Metastasis, Proliferation	(Zhang et al., 2014b)
	<i>Gata3</i>	Stemness	(Ralston et al., 2010)
	<i>Pax3</i>	Embryogenesis	(Murakami et al., 2006)
	<i>RHAMM</i>	Metastasis, Proliferation	(Wang et al., 2014)
	<i>SOX9</i>	Stemness	(Song et al., 2014)
	<i>Oct4/Nanog</i>	Stemness	(Lian et al., 2010)
	<i>ZEB2</i>	EMT	(Liu et al., 2010b)
β-catenin	<i>Sox2/Snai2</i>	Proliferation	(Azzolin et al., 2014)
FoxO1	<i>Catalase/Sod2</i>	Anti-apoptosis	(Shao et al., 2014)
p63/73	<i>PML</i>	Apoptosis	(Strano et al., 2001)
RUNX	-	Differentiation	(Vitolo et al., 2007)
SMAD1/7	-	Stemness	(Ferrigno et al., 2002)
SMAD2/3	<i>Snail/Twist1/Slug/NEGR1/UCA1/CTGF</i>	Proliferation & EMT	(Varelas et al., 2008)
TBX5	<i>Fgf10/Nppa</i>	Anti-apoptosis	(Murakami et al., 2005)

Table 1: Transcription factors that YAP/TAZ co-activates and their target genes.

YAP/TAZ partners TEADs, β -catenin, and SMADs to regulate proliferation; RUNX to promote differentiation and FoxO1 for survival.

1.5 Upstream regulators of the Hippo pathway

While the core kinase cassette of the Hippo pathway beginning from Hpo/MST to Yki/YAP is well-defined and highly conserved between insects and mammals, the upstream regulators of the pathway appear differently in different organisms. As such, we will only focus on the upstream regulators that are known in mammals.

1.5.1 Tight junctions/epithelial polarity complexes and cadherin.

Angiomotin (AMOT) is an angiostatin-binding protein that was discovered using yeast-two hybrid screening. AMOT is known to be involved in the maintenance of tight junction, endothelial migration and proliferation (Moleirinho et al., 2014). Acting upstream of the Hippo pathway, AMOT inhibits YAP/TAZ activity by regulating their localization via sequestration to the tight junction and actin cytoskeleton, and promoting Hippo pathway-mediated YAP/TAZ phosphorylation (Zhao et al., 2011). PARD3 is a PDZ-domain-containing scaffold protein that functions in the formation of epithelial tight junction and establishment of cell polarity and asymmetrical cell division, PARD3 regulates TAZ localization, and thus activity, via promoting LATS1 and protein phosphatase 1 (PP1) association (Lv et al. 2015). Crumbs polarity complex which includes PAL1, PATJ, MUPP1, LIN7C and AMOT was found to interact with YAP/TAZ when cells were grown at high cell densities and subjected to Flag-YAP/TAZ affinity purification-mass spectrometry (Varelas et al., 2010). At high cell density, crumbs-YAP/TAZ interaction functions to relay cell density information via promoting YAP/TAZ phosphorylation, cytosolic sequestration and inhibiting TGF- β signaling.

E-cadherin is a type of cell adhesion molecule (CAM) which depends on calcium ions (Ca^{2+}) to function. E-cadherin serves as a cell surface marker for epithelial cells. E-

cadherin/catenin complex regulates Hippo signaling pathway in mammalian cells, where the homophilic binding of E-cadherin between two epithelial cells induces Hippo signaling pathway via a Merlin-Kibra dependent activation of Hippo kinases, thus mediating cell-density inhibition of proliferation via regulating YAP activity (Kim et al., 2011).

1.5.2 Mechano-transduction

Mechano-transduction is the processes by which cells sense external forces and spatial constraints by translating them to biochemical signals that elicit adaptive cellular response. Pioneering work of Piccolo and colleagues showed that mechanical forces such as stretching, cell shape, and ECM stiffness can modulate YAP activity. Cell spreading, stiff ECM, and increasing contractile force lead to YAP/TAZ activation, conversely cellular confinement, soft ECM and decreasing contractile force lead to YAP/TAZ inactivation (Figure 12). Thus the study identifies YAP/TAZ as mechanotransducers that mediate mechanical inputs from the microenvironment to changes in gene expressions (Dupont et al., 2011).

YAP/TAZ Localization

Figure 12: YAP/TAZ localization in response to different form of mechano-stimulation.

Schematic illustration of high YAP and TAZ protein levels (brown) and low YAP and TAZ protein levels (beige) under various mechano-stimulations. YAP/TAZ localization is determined by cell shape, density, and physical environment.

Cell density (contact inhibition)

Figure 13: YAP/TAZ play a key role in controlling cell proliferation in response to cell contact.

In an expanding cell colony, YAP/TAZ localization is predominantly nuclear in cells at the edges, whereas cells nearer to the center display cytosolic YAP/TAZ localization. Brown represents high YAP and TAZ protein levels, whereas beige represents low YAP and TAZ protein levels.

YAP localization and phosphorylation are regulated by cell density. When cells were cultured to high cell density (to confluence), cells at the edge of a large colony displayed high nuclear YAP, whereas cells at the center displayed low nuclear YAP (Zhao et al., 2007). These observations suggest that cells at the edges with nuclear and active YAP/TAZ are more proliferative, whereas cells nearer to the center with cytosolic and inactive YAP/TAZ are less proliferative. When cells are cultured in low density, each cell is well-separated from another (absence of cell-cell contacts) and is capable of forming its own colony. Therefore, at low cell density, cells display nuclear YAP/TAZ localization. Meanwhile, in high cell density, cells are packed together side by side (presence of cell-cell contacts), thus, cells display cytosolic YAP/TAZ localization (Figure 13).

1.5.3 Receptor tyrosine kinases (RTKs) and Focal adhesion kinase (FAK)

It is known that contact inhibition can be relieved by mitogenic growth factor signaling. In confluent MCF10A cell culture, EGF treatment leads to YAP activation via the inhibition of Hippo pathway through a PI3K-PDK1 pathway, independent of AKT activity.

Mechanistically, in response to EGF signaling, PDK1 associates with the core Hippo kinase complex via Salvador causing the Hippo core complex to dissociate, thus leading to the activation of YAP (Fan et al., 2013).

Fibronectin-Integrin interaction control Hippo pathway via FAK signaling. Adhesion to fibronectin activates the FAK-Src-PI3K-PDK1 pathway which in turn inhibits LATS1/2 and activates YAP/TAZ (Kim and Gumbiner, 2015).

1.5.4 G-protein coupled receptors (GPCRs)

GPCRs play dual roles in the regulation of YAP/TAZ. Depending on the G-Protein and its regulation of LATS, the activity of YAP/TAZ could be positively or negatively regulated (Yu et al., 2012). Furthermore, Rho GTPases and the actin cytoskeleton appear to be mediating $G\alpha$ regulation of LATS1/2, although the mechanism remained unknown. We further described the different G-proteins and their effects on YAP/TAZ activities.

Activation of $G_{12/13}$ - or $G_{q/11}$ - or $G_{i/o}$ - coupled receptors either by lysophosphatidic acid (LPA) or sphingosine 1-phosphophate (S1P) inhibits LATS1/2, thereby activating YAP and TAZ. The mechanism by which the above GPCRs inhibit LATS1/2 however remains unknown. Activation of G_s -coupled receptor by epinephrine activates LATS1/2, thereby inhibiting YAP and TAZ. G_s -coupled receptor stimulates the cAMP-dependent pathway thereby activating Protein kinase A (PKA) and GTPases to stimulate LATS kinases and YAP phosphorylation (Yu et al., 2013). Consistent with this, GPCR activation has been linked to cell proliferation, and several mechanisms have been proposed (Bar-Shavit et al., 2016)

where Gq/11-, G12/13-, and Gi/o-coupled receptors usually promotes cell proliferation. Meanwhile the role of Gs-coupled receptors in cell proliferation is complicated with activation of Gs and PKA is usually associated with growth inhibition (Stork and Schmitt, 2002).

1.5.5 Energy states

One of the hallmarks of cancer is the deregulation of cellular energetics (metabolism). Besides reprogramming stem cell and plasticity programs, metabolism can also be reprogrammed via signaling pathways and oncogenes to meet cancer growth requirements during a metabolic switch. Recent findings have revealed that YAP/TAZ can be regulated by metabolic pathways and nutrient-sensing mechanisms. Under glucose deprivation, LATS1/2 is highly activated, thus inhibiting TAZ activity (Mo et al., 2015). Another report also demonstrated that glucose homeostasis can regulate YAP phospho-status and activity (Wang et al., 2015).

AMPK is a cellular energy sensor that functions to maintain energy homeostasis. AMPK could modulate YAP/TAZ activity in two ways: (1) phosphorylating AMOTL1, hence activating LATS1/2 which consequently inhibits YAP/TAZ (DeRan et al., 2014) and (2) phosphorylating YAP directly on Ser-94, inhibiting YAP activity by disrupting YAP-TEAD interaction (Mo et al., 2015).

These findings suggest that YAP/TAZ could respond and collaborate with the cellular nutrient sensors to regulate gene transcription and cell proliferation.

To summarize, multiple upstream cues can regulate the Hippo kinases and YAP/TAZ directly. YAP/TAZ activities are modulated by mechanosensors and polarity proteins at the membrane, mitogenic input from the environment and energy sensor in the cytoplasm. Together, these inputs reveal that YAP/TAZ are capable of responding to diverse cues to direct cellular gene expression. Moreover, these upstream regulators also have diverse functional roles, therefore their activities are subjected to crosstalk(s) with other pathways that may further fine tune the final outcome of YAP/TAZ-mediated gene expressions (Figure 14).

Figure 14: Overview of the various cell/tissue properties that regulate Hippo pathway.

(1) Sensing the integrity of the epithelial layer, the tight junction or epithelial polarity complexes or adherens junction (AJ) transduce signaling via the kinase cascade to regulate YAP/TAZ activity.

(2) Cell morphology and mechanotransduction regulate the activity of YAP/TAZ via both a canonical kinase cascade dependent and an independent pathway.

(3) Mitogenic growth factors promotes proliferation through well-known mechanisms, and in addition neutralize the growth inhibitory effects of Hippo kinase cascade.

(4) Energy states could regulate growth via modulating YAP/TAZ activity.

1.6 YAP/TAZ in organs and tissues

Since Hippo pathway is a regulator of organ size in insects and mammals, we next discuss the roles of YAP/TAZ in different organs and tissues under physiological condition.

Liver

Adult hepatocytes express low level of YAP/TAZ. Overexpression of YAP in the liver of transgenic mice causes increased liver mass by 4-fold through hepatocytes proliferation (Camargo et al., 2007), and critical to the observation of this phenotype is the activation of Notch signaling pathway (Yimlamai et al., 2014). The phenotype brought about by YAP overexpression is TEAD-dependent as treatment with the TEAD inhibitor, Verteporfin reverses the overgrowth phenotype (Liu-Chittenden et al., 2012).

Intestine

Overexpression of YAP in mice strongly increases intestinal cell proliferation without affecting organ size. Nuclear localization of YAP is exclusive to intestinal stem cells (ISCs) and Paneth cells, and overexpression of YAP in these cells forces them out of the intestinal crypts into the villi tips. Under physiological condition, protein kinase C zeta type (PKC ζ) acts as a tumor suppressor by phosphorylating and inactivating YAP in the ISCs. (Llado et al., 2015).

Epidermis

Nuclear YAP and TAZ is prominent in basal layer cells of the epidermis and hair follicle (Elbediwy et al., 2016). When active YAP is overexpressed in the basal layer, it causes keratinocyte proliferation and thickening. YAP/TAZ play an important role in skin homeostasis through maintaining basal epidermal progenitors and regulating hair follicle morphogenesis (Zhang et al., 2011), whereby the activation of YAP/TAZ is crucial for the

preservation of an undifferentiated population of epidermal progenitors via inhibition of Notch signaling (Totaro et al., 2017).

Nervous tissues

YAP is involved in the expansion of neural progenitor cells (NPCs) in the cortical hem, hippocampus, and neocortex. The tumor suppressor neurofibromatosis 2 (Nf2; merlin) limits the expansion of neural progenitor cells (NPCs) in the brain via inhibiting the Yap/Taz transcriptional coactivators, through a non-canonical Hippo pathway.

The evidence for the role of YAP in mammalian brain development comes from the observation that inactivation of NF2 causes severe deformation to the dorsal telencephalon which was attributed to expansion of neural progenitor cells (NPC) in the cortical hem, hippocampus, and neocortex. Overexpression of YAP led to a hippocampal phenotype resembling NF2 inactivation. The NF2 phenotype can be rescued by reducing YAP levels, suggesting NF2 could restrict YAP activity (Lavado et al., 2013).

Breast

The subcellular localization of YAP differs between virgin and pregnant glands. In the virgin glands, nuclear YAP is prominent in myoepithelial cells, while the luminal cells display a more diffused localization of YAP throughout the nucleus and cytosol. As for pregnant mammary glands, the expanding alveolar cells display increased YAP protein levels and prominent nuclear YAP. These observations suggest that Hippo signaling pathway may play a role during the pregnancy–lactation cycle (Chen et al., 2014).

Embryo

During mouse embryogenesis, the nuclear/cytoplasmic distribution of YAP/TAZ defines the initial cell fate determination to either trophectoderm (TE) or inner cell mass (ICM), where nuclear YAP/TAZ define the TE and cytosolic YAP/TAZ define the ICM (Nishioka et al., 2008). In addition, the specification of mesenchymal stem cell (MSC) is determined by YAP/TAZ, where stiff matrix or cell spreading promotes osteogenesis through TAZ co-activation of Runx2 while soft matrix or cell rounding promotes adipogenesis through TAZ inhibition of PPAR γ -dependent transcription. (Hong et al., 2005).

1.7 YAP/TAZ in clinical and experimental models

YAP/TAZ have been studied in different disease context. There are also clinical and experimental models which have been developed. A few examples will be discussed here.

Lung cancer

Lung cancer includes non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). Small cell lung cancer metastasize more rapidly to other organs. SCLC constitutes 15-20% of lung cancers, while NSCLC makes up about 80-85% of lung cancers. YAP/TAZ expression are elevated and are localized to the nucleus in malignant tumors of the lung (Wang et al., 2010). Consistent with this, elevated expression of YAP/TAZ signature gene targets correlates with poor prognosis in NSCLC patients (Lau et al., 2014).

Breast cancer

Breast cancer are tumorigenic mammary epithelial cells characterized by their site of origin; ductal or lobular, and the location of their growth; lumen (carcinoma *in situ*) or stroma (invasive carcinoma). In datasets of breast cancer patients, elevated expression of gene signatures for YAP/TAZ activity correlate with high histological grade, enrichment of stem cell signatures, metastasis tendency, and poor prognosis. TAZ overexpression increases the number of tumor-initiating cells in low-grade human breast cancer, and transforms them into high-grade cancers upon transplantation into the mammary fat pads of immunocompromised mice (Cordenonsi et al., 2011).

Gastric cancer

Most cases of gastric cancer are derived from glandular epithelial cells of the stomach, which constitutes about 90% of gastric cancers. YAP protein expression is upregulated in gastric cancer, and nuclear accumulation of YAP is associated with poor patient prognosis (Kang et al., 2011). Meanwhile, in a particular subtype of gastric cancer known as signet ring cell carcinoma (SRCC), TAZ expression is upregulated instead (Yue et al., 2014).

Pancreatic cancer

Pancreatic ductal adenocarcinoma (PDAC) is an aggressive cancer with poor survival rates and frequently harbors oncogenic *KRAS* mutation. YAP transcript expression is upregulated in PDAC tissue compared to normal pancreatic epithelia, where its function as an effector of *KRAS* is critical for the growth and progression from neoplastic pancreatic ductal cells to invasive PDAC in mice (Zhang et al., 2014a).

1.8 Role of YAP/TAZ in cancer

YAP/TAZ function may underlie some of the key hallmarks of cancer, such as uncontrolled proliferation, overcoming apoptosis, and acquisition of cancer stem cells traits. This may occur through the regulation of genes controlling proliferation, survival or stemness.

1.8.1 Proliferation

In *Drosophila*, clones of imaginal disk cells expressing hyperactive Yorkie multiply more rapidly than wild-type cells, while clones expressing inactive yorkie multiply less rapidly (Huang et al., 2005). The phenotype is conserved in mammalian cells. Overexpression of constitutively active forms of YAP or TAZ is capable of increasing the proliferation of several human and mouse cell lines *in vitro* (Mizuno et al., 2012; Ota and Sasaki, 2008; Overholtzer et al., 2006; Zhao et al., 2008). YAP/TAZ localization and phosphorylation are regulated by cell density. When cell colonies were grown on 2D culture, cells at the edge displayed nuclear YAP while cells toward the center displayed cytoplasmic YAP, as a consequence, cells at the edges were rapidly proliferating while cells towards the center were incapable of proliferating due to contact inhibition (Zhao et al., 2007). The study subsequently reveals that YAP/TAZ inactivation by the Hippo pathway promotes contact inhibition. In addition, overexpression of YAP resulted in cells overcoming cell contact inhibition and anoikis, leading to transformation (Zhao et al., 2012)

1.8.2 Survival

YAP and TAZ function to inhibit apoptosis induced by the loss of cell-substrate interactions (anoikis) (Zhao et al., 2012). Moreover, overexpression of YAP can inhibit TGF- β 1 induced apoptosis via promoting EMT instead through increased EGFR levels and activity (Liu et al., 2017). Conversely, TAZ knockdown in breast cancer cells strongly reduces their ability to survive treatments with chemotherapeutic drugs, such as taxol. However, the mechanism underlying these effects are unknown (Lai et al., 2011).

1.8.3 Endowment of Cancer stem cell traits

Cancer stem cells (CSCs) are characterized by several traits including tumor-initiation capability, plasticity, metastatic potential, and drug resistance. Multiple lines of evidence have shown that upregulation of YAP and TAZ is capable of endowing CSCs traits to cells (Cordenonsi et al., 2011; Zhao et al., 2014).

Tumor initiation

In mice, mutation of KRAS at the earliest stage of pancreas tumorigenesis activates YAP/TAZ. The activation of YAP/TAZ then leads to the up-regulation of JAK–STAT3 signaling, thus promoting the development of pancreatic ductal adenocarcinoma (PDAC) (Gruber et al., 2016).

Plasticity

TAZ promotes EMT through regulating the expression of Snail and FOXC2, which in turn down-regulate the expression of epithelial markers such as E-cadherin and occludin, and up-regulate the expression of mesenchymal markers such as N-cadherin, vimentin, and fibronectin. TAZ is known to promote EMT in oral (Li et al., 2015), pancreatic (Xie et al., 2015), and breast cancer cells (Cordenonsi et al., 2011).

Metastasis

In mice and human cancer cells, metastatic potential is associated with increased TEAD transcriptional activity. This often occurs through the disruption of the hippo pathway, thus removing its suppression of YAP/TEAD transcriptional activity. Consequently this leads to increased expression of TEAD-target genes conferring cells with metastatic potential (Lamar et al., 2012).

Drug resistance

There are several mechanisms that may underlie drug resistance in cancer. The drug may be inactivated, has its target altered, or removed by efflux pumps in the cells. In the case of DNA damage causing drugs, the damage could be repaired or cell death could be inhibited. In addition, cells could undergo a phenotypic change such as EMT or simply differ phenotypically/genotypically due to tumor heterogeneity which contribute to chemosensitive and chemoresistant population of cells (Housman et al., 2014).

The levels of YAP/TAZ proteins have been studied in several cases of cancer drug resistance. In most cases, YAP/TAZ expression or activity were increased to confer selective growth advantage after drug treatment. In melanoma cells that acquires resistant to the BRAF inhibitor PLX4032, changes in cell shape and cytoskeletal remodeling that followed resulted in YAP/TAZ activation (Kim et al., 2016a). In hepatocellular carcinoma (HCC) cells, YAP2/TEAD4 transcriptional activation is upregulated via SIRT1-mediated deacetylation of YAP2 (Mao et al., 2014). Meanwhile, TAZ is reported to be involved in conferring taxol resistance and chemoresistance of breast cancer cells (Bartucci et al., 2015; Lin et al., 2015)

1.8.4 YAP/TAZ and hallmarks of cancer

YAP/TAZ play multiple roles in conferring cells with the hallmarks of cancer amongst which are sustaining proliferative signaling (proliferation), resisting cell death (survival), and activating invasion/metastasis (endowment of CSCs traits). Depicted below are the cancer promoting attributes of YAP/TAZ (Figure 15).

Figure 15: Overview of YAP/TAZ roles in Cancer Cells.

YAP/TAZ promotes cell proliferation, survival, and in addition endow cancer stem cell traits to cancer cells. YAP/TAZ confer CSCs properties via promoting tumor initiation, phenotypic plasticity, drug resistance and metastasis.

1.9 Regulators of Hippo kinases

Hippo kinases are phosphoregulated. Activation occurs either by an upstream kinase or auto-phosphorylation, whereas dephosphorylation is carried out by phosphatases such as the PP1 and PP2A. Some common regulators of the Hippo pathways are discussed below.

1.9.1 MST regulators

Genetic experiments in *Drosophila* have uncovered several upstream regulators of the Hippo pathway, including the apical membrane proteins Crumbs, Expanded, Merlin, Kibra, and apical spectrins, which function as mechanosensors at the apical domain of epithelial cells. However, in mammalian cells, direct links between MST1/2 and these membrane proteins have not been established. Furthermore, recent evidence suggests that the mammalian Hippo pathway deviates considerably from the fly model in this aspect (Rawat and Chernoff, 2015).

MST auto-phosphorylation

MST phosphorylates itself at the C-terminal SARA (Sav, Rassf, Hippo) domain to self-regulate its activity. The SARA domain facilitates the formation of an anti-parallel homodimer that permits the trans-auto-phosphorylation of the activation loop of MST (Praskova et al., 2004).

Angiomotin family proteins

Tight junction protein Angiomotin (AMOT) has been identified to be an interacting partner of YAP and TAZ (Zhao et al., 2011). AMOT regulates YAP's localization in the cells by physical interaction as well as promoting its phosphorylation suggesting that AMOT could be a possible connection between cell contact inhibition and YAP function.

Protein Phosphatase 2A (PP2A)

PP2A dephosphorylates MST1 and MST2 at their auto-phosphorylation site Thr-183 and Thr-180 respectively (Guo et al., 2011). In contrast, cells treated with okadaic acid which inhibits both PP1 and PP2A will lead to MST1/2 phosphorylation on Thr-183/180. To date, PP2A is the only reported phosphatase which regulates the activities of Hippo kinases.

1.9.2 LATS regulators

Mammalian Sterile-Twenty-like (MST)

MST phosphorylates LATS1/2 at Thr-1079/Thr-1041 of the hydrophobic motif (Chan et al., 2005).

MAP4K family kinases

MAP4K family members MAP4K1/2/3 (orthologues of Happyhour) and MAP4K4/6/7 (orthologues of Misshapen) phosphorylate LATS1/2 at Thr-1079/Thr-1041 of the hydrophobic motif (Meng et al., 2015)

c-Jun N-terminal kinase (JNK)

JNK is a stress-activated kinase, which elicits varied responses including cytoskeletal modulation, apoptosis and cell proliferation. JNK phosphorylation of LIMD1 (inhibitor of LATS) promotes LIMD1-LATS interaction, thus inactivating LATS and activating YAP activity (Codelia et al., 2014). Despite the extensive study done to identify the regulators of Hippo kinases in both drosophila and mammalian cells, no LATS phosphatase has been identified.

1.10 Regulators of YAP and TAZ

1.10.1 YAP/TAZ kinases

Large tumor suppressor (LATS) kinase

The Hippo pathway itself is the best studied regulator of YAP/TAZ activity. Regulation of YAP/TAZ occurs through the kinase cascade phosphorylation with LATS1/2 as the direct kinases to phosphorylate YAP/TAZ. LATS1/2 phosphorylate YAP at Ser-127 (required for 14-3-3 mediated binding) and Ser-397 (implicated in E3 ubiquitin ligase-mediated degradation), and TAZ at Ser-89 (for 14-3-3 mediated binding) and Ser-314 (for E3 ubiquitin ligase-mediated degradation) (Huang et al., 2012).

Protein kinase B (PKB)/Akt

Also known as Akt, PKB is an important regulator of several cellular processes, including apoptosis, proliferation, and metabolism (Brazil and Hemmings, 2001). PKB preferential substrate sequence is the RxRxxS/T motif, and when phosphorylated, generates a sequence partially overlapping with one of two optimal binding motifs for 14-3-3 proteins, RxxS/T(phos)xP (Yaffe et al., 1997). In a study to identify novel substrates of PKB, proteins that interact with the 14-3-3 protein were analyzed. Amongst the binding partners of 14-3-3, YAP phosphorylation status changed when treated with Epidermal growth factor (EGF), a PKB activator. Further experiments revealed that PKB is able to phosphorylate YAP at Serine 127 leading to its cytoplasmic translocation and inactivation (Basu et al., 2003).

AMP-activated protein kinase (AMPK)

AMPK plays a role in cellular energy homeostasis. AMPK is capable of detecting shifts in AMP:ATP ratio, and is activated when cellular AMP levels is higher than ATP. Activation of AMPK due to cellular energy stress causes it to phosphorylate YAP on Ser-94, thereby disrupting YAP-TEAD interaction.

c-Abl kinase

Besides promoting cell proliferation, YAP could also induce apoptosis. The mechanism by which YAP promotes apoptosis is via interacting with p73 in response to DNA damage (Strano et al., 2005). Investigations elucidating the role of YAP in DNA damage response identifies that the activation of c-Abl through cisplatin treatment led to increased level of YAP via the stabilization of YAP. c-Abl was later found to phosphorylate YAP on Tyr-357, and that the Tyr-357 phosphorylation promotes YAP-p73 interaction. (Levy et al., 2008).

For years, the only known mechanism for the regulation of YAP/TAZ phospho-status and activity has been the Hippo pathway. This has lent support to the notion that dysregulation of the Hippo pathway may be the sole culprit of YAP/TAZ stabilization and activation in cancer (Meng et al., 2016; Pan, 2010). However, recent studies have expanded the regulatory proteins involved in the YAP/TAZ considerably, identifying new upstream regulatory components and revealing its crosstalk with other signaling pathways such as the EGF, GPCR, Notch, TGF β and Wnt. Mutations in Hippo pathway components can be selected in cancer but this usually occurs in specific tumor histotypes, such as in mesothelioma, schwannomas, and meningiomas. The fact that similar mutations in Hippo pathway components are not found in tumors presenting high YAP/TAZ activity appears to be inconsistent with the notion that Hippo signaling is the sole and main gatekeeper of YAP/TAZ activity in human tumors. Therefore it is crucial to note that Hippo pathway is one of the tumor suppressor pathways modulating YAP/TAZ in normal tissues, and it is neither the sole nor always the most pertinent one implicated in YAP/TAZ-driven tumorigenesis.

1.10.2 YAP/TAZ phosphatases

Protein phosphatase (PP1)

PP1 dephosphorylates TAZ at Ser-89 and Ser-311. Dephosphorylation at these sites promotes TAZ nuclear translocation, and stabilizes TAZ by disrupting its interaction with the SCF E3 ubiquitin ligase (Liu et al., 2011b). Similarly, YAP is dephosphorylated at Ser-127 by PP1 leading to its nuclear accumulation and transcriptional activation (Wang et al., 2011).

Protein phosphatase 2A (PP2A)

PP2A dephosphorylates YAP at Ser-127. It has been found that α -catenin serves to protect phosphorylated YAP from PP2A-mediated dephosphorylation. The loss of α -catenin either through knock-out or in the context of low cell density leads to PP2A-mediated YAP dephosphorylation (Schlegelmilch et al., 2011). The identification of PP1 and PP2A as YAP/TAZ phosphatases has enabled numerous studies to utilize okadaic acid and calyculin A to inhibit YAP and TAZ activities by maintaining their phosphorylation statuses through PP1 and PP2A inhibition.

1.10.3 Other post-translational modifications of YAP/TAZ

Set7

Set7 is a lysine methyltransferase (KMT) that was initially identified as a monomethylase of histone H3 lysine 4 (H3K4) (Wang et al., 2001). In a study using Set7 knockout mouse intestinal epithelial cells, it was observed that cells lacking Set7 expressed elevated amount of YAP/TAZ signature target genes, *Ctgf* and *Gli2*. Following that, biochemical analysis revealed that Set7 methylates YAP at lys-494 leading to its cytoplasmic translocation and inactivation. (Oudhoff et al., 2013).

1.10.4 Physical regulation of YAP/TAZ

Besides posttranslational modifications, YAP/TAZ could also be regulated through interaction with other proteins that promote their cytoplasmic or nuclear retention. Physical regulation of YAP/TAZ occurs through forming a complex with YAP/TAZ, thus sequestering and preventing their nuclear translocation. Regulators that modulate YAP/TAZ via this mechanism are Angiomotin (AMOT), protein tyrosine phosphatase 14 (PTPN14), and α -catenin. The PPxY motifs of AMOT and WW domains of YAP/TAZ mediates the AMOT-YAP/TAZ interaction which results in the latter sequestration to the tight junctions and actin cytoskeleton, independent of LATS1/2 mediated phosphorylation (Zhao et al., 2011). Similarly, PTPN14 also interacts with YAP via the PPxY-WW interaction to direct YAP cytoplasmic sequestration and thus modulate YAP activity (Michaloglou et al., 2013). Meanwhile, phospho-YAP enhances the binding of 14-3-3 to α -catenin, where 14-3-3 is known to bring together YAP to its phosphatase PP2A for activation. The presence of α -catenin serves to prevent this dephosphorylation from occurring thus maintaining YAP phosphorylation and deactivation (Schlegelmilch et al., 2011).

Altogether, these findings indicate that nuclear retention of YAP/TAZ is the summation of multiple, or parallel, regulatory layers. Furthermore, both YAP and TAZ lack the nuclear localization signal (NLS) required for nucleo-cytoplasmic translocation, hence the mechanism for their nuclear retention remains unknown. Seeking to resolve this, a recent study proposed a bold mechanism, whereby forces resulting in nuclear flattening that stretches nuclear pores leading to the reduction of mechanical resistance of nuclear transport could increase YAP nuclear translocation (Elosegui-Artola et al., 2017). However, if the proposed model is indeed one of many means whereby YAP/TAZ is transported to the nucleus it raises the question whether YAP/TAZ is still considered a mechano-transducer, since any protein could similarly be transported into the nucleus via such means.

1.10.5 Additional TAZ regulations

TAZ is regulated at several levels within the cell. At the post-translation level, its activity is phospho-regulated by LATS1/2, Cdk1, GSK3 and c-Abl. To maintain Hippo signaling homeostasis, a negative feedback loop exists between TAZ and YAP, where YAP negatively regulates TAZ abundance via GSK-3 mediated TAZ degradation (Finch-Edmondson et al., 2015). Meanwhile at the RNA level, TAZ transcripts are modulated by miRNAs as seen in gastric and liver cancer cells (Higashi et al., 2015; Zuo et al., 2015).

Compared to YAP, TAZ has two phosphodegrons (degradation switch) and one WW domain (regulatory switch), therefore it is more rapidly degraded and less stable than YAP (Finch-Edmondson et al., 2015; Liu et al., 2010a). The N-terminal and C-terminal phosphodegrons of TAZ are recognized by β -Transducin repeat containing protein (β -TrCP), a subunit of SCF E3 ubiquitin ligase. The C-terminal phosphodegron of TAZ is sequentially phosphorylated by LATS1/2 and CK1 (Liu et al., 2010a), whereas the N-terminal phosphodegron of TAZ is solely phosphorylated by GSK3 β on Ser-58/62 (Huang et al., 2012). Sequential phosphorylation of TAZ by LATS and CK1 creates a binding site for β -TrCP. Phosphorylation of TAZ at Ser-311 by LATS provides the priming site for subsequent CK1 phosphorylation at Ser-314 in TAZ, thereby creating a functional phosphodegron for β -TrCP binding. Thus, TAZ protein level can be modulated in response to a vast range of intracellular and extracellular signals by means of these pathways.

To summarise, Hippo kinases, which were the most-well studied regulator of YAP/TAZ can work in parallel with MAP4K to regulate YAP/TAZ. The two pathways converge at LATS. Several kinases act upstream of YAP/TAZ, they are LATS/NDR, Akt, AMPK, GSK3 β , CK1 and c-Abl. YAP/TAZ could be regulated by two forms of post-translational modification, which are phosphorylation and methylation. Both of which function to retain YAP/TAZ in the cytoplasm and thus kept the transcriptional co-activators inactive. The phosphatases, PP1 and PP2A can regulate YAP/TAZ to promote their activity. At the same time, PP2A can negatively regulate MST. However, to date no known phosphatase has been discovered to be regulating LATS activity (Figure 16).

Figure 16: Summary of the regulators of Hippo kinases and YAP/TAZ.

MST and MAP4K phosphorylates and activates LATS/NDR. Akt, AMPK, and c-Abl directly phosphorylates YAP at Ser-127, Ser-94, and Tyr-357 respectively. Meanwhile, Set7 methylates YAP at Lys-494. PP1 and PP2A regulate MST and YAP/TAZ phospho-statuses.

1.11 Partner of PIX 2 (POPX2)

Protein phosphorylation is a reversible post-translational modification that regulates protein activity both in prokaryotic and eukaryotic organisms. Phosphorylation plays a critical role in the transduction of signals in a myriad of signaling pathways. Kinase catalyzes the transfer of a phosphate (PO_4) to another protein (phosphorylation), whereas a phosphatase catalyzes the removal of a phosphate (PO_4) from a protein (dephosphorylation).

Protein phosphorylation usually occurs on serine, threonine, tyrosine and histidine residues in eukaryotic cells. Addition of a phosphate group imparts two negative charges onto the residue altering the characteristic of the residue and the vicinity surrounding it. Often this leads to a conformational change which in turn regulates the activity of the protein. Kinases and phosphatases maintain the homeostatic activity of many proteins involve in diverse signaling pathways.

Protein phosphatases are categorized into three main classes based on sequence, structure and catalytic function: (1) Phosphoprotein phosphatases (PPP) family and the protein phosphatase Mg^{2+} -/ Mn^{2+} -dependent (PPM) family, (2) Protein Tyr phosphatase (PTP) family, and (3) Aspartate-based protein phosphatases. Based on their substrate specificity, phosphatases can be further sub-divided into four classes: (1) Tyrosine-specific phosphatases, (2) Serine-/Threonine-specific phosphatases, (3) Dual-specific phosphatases, which dephosphorylate tyrosine, serine and threonine residues, and (4) Histidine phosphatases (den Hertog, 2003).

The protein phosphatase Mg^{2+} -/ Mn^{2+} -dependent (PPM) family consists of only the PP2C family. PP2C family are one of four major classes of Serine-/Threonine-specific phosphatases. The PP2C family is comprised of 12 protein phosphatases: PP2C α , PP2C β ,

PP2C γ , PP2C δ , PP2C ϵ , PP2C ζ , PP2C η , Wip1, POPX2, POPX1, NERPP-2C and PHLPP. A defining characteristic of members in this family is the presence of a conserved bi-nuclear metal ion active center that is critical for their catalytic activity.

Partner of PIX 2 (POPX2/CaMKP/PPM1F) phosphatase belongs to the PP2C family of serine/threonine protein phosphatase. POPX2 was first identified in rat brain as a unique protein phosphatase displaying calyculin A/okadaic acid-insensitivity, Mn²⁺-dependency, and poly-L-lysine-stimulated phosphatase activity that specifically dephosphorylates and regulates CaMKI, II, and IV (Ishida et al., 1998).

Following that, a similar protein phosphatase with 52% identity in the catalytic domain to POPX2 was found in the human cDNA databases. When the cDNA was expressed in COS cells, the phosphatase was localized to the nucleus, in contrast to POPX2 which was localized exclusively in the cytosol. This nuclear homolog was named POPX1/PPM1E/CaMKP-N (Takeuchi et al., 2001). Henceforth, POPX comprises two isoforms POPX1 (or PPM1E) and POPX2 (or PPM1F). The larger isoform, POPX1, is mainly expressed in the brain and testes whereas the shorter isoform, POPX2, is ubiquitously expressed.

POPX1 is reported to be involved in the negative regulation of the p21-activated protein kinase (Koh et al., 2002) and the 5'-AMP-activated protein kinase (Voss et al., 2011). Genetic studies on zebrafish reveals that POPX1 is vital for early brain and spinal development (Nimura et al., 2007). A study conducted by Ishida et. al with a C-terminally truncated version of POPX1 (1-559) found that the proteolytic processed version of POPX1 exhibit higher phosphatase activity compared to the full length, suggesting that the proteolytic processing of POPX1 at the C-terminal serves as a post-translational regulatory mechanism to generate a highly active phosphatase (Ishida et al., 2013).

Figure 17: Schematic representations of the PP2Cα, POPX1 and POPX2.

The PP2C phosphatase domain in grey. Homology region between POPX1 and POPX2 represented by the hatched area. (Koh et al., 2002).

Image adapted from "The p21-Activated Kinase PAK Is Negatively Regulated by POPX1 and POPX2, a Pair of Serine/Threonine Phosphatases of the PP2C Family" with permission.

Sequence homology and the secondary structure predictions of various PP2C family enzymes and also by crystal structure analysis of PP2C, mapped the catalytic domain of POPX2 to the amino acids 156-413. The amino acid sequences of the N-terminal (amino acid residues 1-156) and C-terminal (amino acid residues 408- 454) regions are unique to POPX2 and show no homology to other protein phosphatases (Figure 17).

Ishida et al. reported that GAPDH binds to the N-terminal residues 1-98 of POPX2 (Ishida et al., 2005). Similarly, Koh et al. also reported that the N-terminal residues 65-191 and 150-193 of POPX2 are responsible for the binding of PIX and KAP3 respectively (Koh et al., 2002; Phang et al., 2014). Taken together, the N-terminal domain of POPX2 may function as a binding domain for substrates and regulatory proteins.

1.11.1 POPX2 regulation

Upstream factors or pathways that regulate POPX2 abundance during development or cancer have not been well-defined. However, several tumor suppressor microRNAs have been found to regulate and target POPX2 expression in various cancer. MicroRNA-200c (miR-200c) which is known to suppress EMT via targeting ZEB1/2 has been shown to target POPX2 mRNA. POPX2 expression is inversely correlated with the expression of miR-200c in breast cancer cell lines, breast cancer patient samples and 58 other cancer cell lines of various origin (Jurmeister et al., 2012). Another miRNA that is capable of targeting POPX2 is MicroRNA-149 (miR-149). miR-149 is a suppressor of invasion and metastasis in both breast and liver cancers (Chan et al., 2014; Luo et al., 2015). POPX2 expression is negatively correlated with the expression of miR-149 in Hepatocellular carcinoma tissues. Taken together, POPX2 appears to be regulated at the post-transcriptional level by tumor suppressing miRNAs which function to suppress EMT, thus, implicating the role of POPX2 as an effector of EMT. Despite the promising findings, more study will have to be carried out to elucidate the regulation of POPX2 at the transcriptional and post-translational levels.

1.11.2 POPX2 substrates

Biochemical studies of POPX2 have identified several substrates which are regulated through dephosphorylation; they include p21 activated kinase1/2 (PAK1/2) on Thr-423/Thr-402 (Koh et al., 2002), Calcium/calmodulin-dependent protein kinase II (CAMKII) on Thr-286 (Harvey et al., 2004), Kinesin Family Member 3A (KIF3A) on Ser-690 (Phang et al., 2014), and Transforming growth factor beta-activated kinase 1 (TAK1) on Thr-187 (Weng and Koh, 2017).

1.11.3 POPX2 cellular functions

POPX2 is involved in the regulation of several cellular functions such as transport by motor protein (Phang et al., 2014), centrosome localization and polarity (Hoon et al., 2014), and matrix attachment and metastasis (Susila et al., 2010). Below we have compiled several of POPX2 substrates, the pathway they are involved in and their cellular functions (Table 2).

POPX2	Substrates	Pathways	Functions	References
	PAK	PAK pathway	Stress fiber formation Invasiveness	(Koh et al., 2002) (Susila et al., 2010)
	?	GSK3-Erk pathway	Metastasis	(Singh et al., 2011)
	CaMKII KIF3	Protein transport pathway	Protein Transport	(Phang et al., 2014)
		CDC42-dependent polarity pathway	Cell polarity Centrosome positioning	(Hoon et al., 2014)

Table 2: Known cellular functions regulated by POPX2.

POPX2 regulates many cellular properties that have been linked to tumorigenesis, such as the regulation of cellular polarity, cytoskeleton, and migration.

1.12 POPX2-Hippo pathway functional links

Linking POPX2 with Hippo pathway are several factors inferred from various analytical approaches which we describe below.

Structural analysis

A known substrate of POPX2, PAK1 is dephosphorylated at Thr-423, a residue that aligns with Thr183 of MST1 (Glantschnig et al., 2002). Furthermore, the activation loop sequence within the kinase domains of MST and PAK are nearly identical (Lee and Yonehara, 2002), and the phosphorylation of the conserved threonine Thr423 in PAK1 and Thr183 in MST1 are required for autoactivation (Manser et al., 1997).

Biochemical analysis

Components of the core hippo kinase, MOB1 and NDR1 were identified to associate with POPX2 from a pulldown-mass spectrometry analysis (Weng and Koh, unpublished data).

Literature analysis

POPX2 has also been observed to increase the levels of cytosolic PAR3 (Hoon et al., 2014) and stress fiber formation (Susila et al., 2010), where both have been shown to regulate YAP/TAZ activity (Lv et al., 2015; Wada et al., 2011).

Functional analysis

In the context of breast cancer, POPX2 expression is low in *in situ* carcinoma such as MCF-7 but is high in metastatic carcinoma, MDA-MB-231. The high expression of POPX2 imparts invasiveness to breast cancer cells presumably via modulation of actin cytoskeleton and focal adhesion through regulation of MAPK1/3 and GSK3 α/β activations (Susila et al., 2010; Zhang et al., 2013), lending evidence that POPX2 may be an effector for the

mechanism governing breast cancer invasiveness. Similarly, TAZ expression has been shown to be correlated with breast cancer growth and invasiveness.

Below, we have collated data from several approaches such as structure analysis, mass-spectrometry, literature mining and functional studies to show possible functional links between POPX2 and the Hippo pathway (Figure 18). Altogether, these factors collectively prompts us to investigate if POPX2 indeed has a role in the regulation of Hippo pathway.

Figure 18: Functional links between POPX2 and the Hippo pathway.

POPX2 and the Hippo pathway appear to be connected on four levels. (1) PAK, the substrate of POPX2, shares a high structure similarity with MST. (2) Mass-spectrometry data of POPX2 interacting partners includes; NDR and MOB1. (3) POPX2 expression correlates with cytosolic PARD3 and stress fiber formation, both of which promotes YAP/TAZ activation. (4) Increased in POPX2 and YAP/TAZ expression share similar outcomes.

2. HYPOTHESIS AND AIMS

As mentioned in the previous section POPX2's roles, partners, and substrates appear to converge with the Hippo pathway on multiple instances. Therefore, we hypothesized that POPX2 may have a regulatory role within the Hippo pathway, and thus modulates YAP/TAZ activity.

The main objective of this study is to elucidate the role of POPX2 in the regulation of the Hippo pathway. The specific aims are listed below.

- (1) To identify POPX2 interacting partner(s) within the Hippo pathway.
- (2) To decipher POPX2 functional activity within the Hippo pathway.
- (3) To investigate the outcome of loss of function of POPX2 on the Hippo pathway and YAP/TAZ activity.

To verify POPX2 interaction with the kinases of the Hippo pathway, we performed both exogenous and endogenous immunoprecipitation assays. Thereafter upon identification of novel POPX2 partner(s), we performed phosphatase assay to determine the mechanism by which these proteins were regulated by POPX2. To complement this we also performed functional assays to determine the downstream consequence of the regulation.

3. MATERIALS AND METHODS

3.1 Cell culture and transfection

Both MDA-MB-231 and HEK293 cells were obtained from ATCC (HTB-26™ and CRL-3216™). MDA-MB-231 POPX2 CRISPR cells were generated by Zhang S (Koh lab). Both HEK293 and MDA-MB-231 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Transfections were carried out using Lipofectamine 2000 (Thermofisher) according to the manufacturer's instructions.

3.2 Antibodies and Plasmids

Antibodies specific for the following proteins were used at the indicated concentrations for western blot: POPX2 (1:1000, Koh); Flag (1:5000, Sigma); HA (1:2000, Sigma); Myc (1:5000, Cell Signaling); LATS1 #3477, MST1 #3682, Sav #13301, MOB1 #13730, YAP/TAZ #8418, TAZ #4883 (all 1:1000, Cell Signaling); Vimentin (1:1000, Abcam); GAPDH (1:5000, EMD Millipore) Actin (1:5000, EMD Millipore).

Antibodies specific for the following proteins were used at the indicated concentrations for immunostaining: Rhodamine-phalloidin was used for actin fiber staining (1:100, Life Technologies). TAZ #4883, YAP/TAZ #8418 (all 1:100, Cell Signaling).

The following plasmids were purchased from Addgene: pPS2977 was a gift from Pamela Silver (Addgene plasmid # 8927), pCIneoMyc human MOB1 was a gift from Yutaka Hata (Addgene plasmid # 37024), pcDNA3 Lats1 (Nigg HS189) was a gift from Erich Nigg (Addgene plasmid # 41156), pCIneoMyc-LATS2 was a gift from Yutaka Hata (Addgene plasmid # 66852), 3XFlag pCMV5-TOPO TAZ WT was a gift from Jeff Wrana (Addgene plasmid # 24809). Flag-YAP2 and Flag-LATS1 were gifts from Marius Sudol.

3.3 Western Blot

Cells were lysed with protein lysis buffer (PLB) containing 300mM Sodium chloride, 25mM HEPES, 1mM MgCl₂, 1mM EGTA, 20mM β -glycerophosphate, 10mM Sodium fluoride, 1mM Sodium orthovanadate, 0.5% (v/v) Glycerol, 0.5% (v/v) Triton X-100, and supplemented with protease inhibitor cocktail (Roche) and PhosSTOP™ (Sigma). Nitrocellulose membranes were used for protein transfer. Membranes were incubated with either 0.5% BSA or skimmed milk in TBST (Tris Buffered Saline + 0.1% (v/v) Tween 20) overnight, following which were washed 3 times with TBST and incubated with secondary antibody conjugated with HRP in 0.5% BSA or skimmed milk in TBST for 1 hour. Membranes were then washed 3 times with TBST.

3.5 Immunofluorescence staining

Cells were fixed with 4% paraformaldehyde for 30 min and washed with PBS three times for 5 minutes each. Cells were permeabilized with triton-X100 then incubated in blocking buffer (0.5% BSA/0.1% Triton X-100) for 1 hour, and following that with the appropriate primary antibody diluted in Antibody dilution buffer (0.5% BSA/0.3% Triton X-100) overnight in 4°C. After three washes with PBS for 5 minutes each, cells were incubated with Alexa Fluor 488- or 594-conjugated secondary antibodies (1:100 dilution) for 1 h. Cells were then washed with PBS three times for 5 minutes each and mounted with DAPI. Fluorescence were observed under Zeiss Axio Observer Z1 microscope.

3.6 Co-immunoprecipitation

Cells were lysed with immunoprecipitation lysis buffer (IPLB) containing 25mM Tris pH 7.5, 150mM NaCl, 0.5% Triton-X, protease inhibitor cocktail (Roche) and PhosSTOP™ (Sigma). For GFP/Flag-tagged protein immunoprecipitation, Glutathione sepharose beads or mouse Anti-Flag M2 beads were washed with (IPLB) three times. 1mg of protein lysate were then added to a bead volume of 40 μ l and incubated overnight in 4°C. Beads were then washed with IPLB three times and tagged proteins were eluted by adding SDS sample

buffer and boiling for 5 minutes. For endogenous immunoprecipitation, cell lysates were incubated with Protein G magnetic beads (Millipore) for 2 hours (pre-clearing). Primary antibody were then added to protein lysates at a concentration of 1:100 and incubated overnight in 4°C. 10µl of Protein G magnetic beads (Millipore) is then added to the Protein-Antibody complex and incubated in 4°C for 2 hours. The Protein G magnetic beads (Millipore) were then washed with IPLB three times and protein complex were eluted by adding SDS sample buffer and boiling for 5 minutes.

3.7 Cell proliferation assay

Cell proliferation was analyzed using tetrazolium salt 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT). Briefly, 24-well plates were seeded with 5×10^3 cells in 450µl growth medium per well. After 24 hours (Day 1) and every 48 hours (Day 3/5/7) 50µl of 10×MTT reagent (5mg/ml) were added to the growth medium of each wells. Plates were then incubated for 4 hours at 37°C, thereafter growth medium were removed and 200µl of DMSO were added into each well to solubilize the formazan crystals. Spectrophotometric absorbance were measured at 570 nm after 24 hours (Day 1) and every 48 hours (Day 3/5/7).

3.8 Soft agar assay

Cells were seeded at a density of 5×10^3 cells per well in 0.35% top agar over a layer of 0.5% base agar in a 60mm plate. Base agar were prepared by melting 0.1g (1%) Agarose into 10 ml sterile water (filtered through 0.2µm pore). 5ml of the 1% Agarose is then mixed with 5ml of 2× complete DMEM. 2 ml of the mixture is then added into 60 mm plates and allowed to solidify in a 37°C incubator for 30 minutes before adding the top agar. Top agar were prepared by melting 0.07g (0.7%) Agarose into 10 ml sterile water (filtered through 0.2µm pore). 1ml of the 0.7% Agarose is then mixed with 1ml of 2× complete DMEM containing 5×10^3 cells. The 2ml mixture is then added onto the base agar-coated 60 mm plates and allowed to solidify in a 37°C incubator for 30 minutes before adding 2ml

of DMEM to prevent desiccation of the top agar. 2ml of fresh media were replaced every week. After 4 weeks of incubation, colonies were stained with crystal violet. Colonies $\geq 50\mu\text{m}$ in diameter were counted.

3.9 Expression and purification of recombinant proteins

Full length POPX2 was cloned into the pGEX-4T-1 vector. pGEX-POPX2 plasmids were then transformed into Rosetta competent cells. Single cell colony were then selected and expanded into 5ml LB broth with ampicillin and grown overnight. 2ml of overnight culture was then incubated into 400ml of LB broth and incubated till its OD reaches 0.6. Upon reaching 0.6 OD, the culture was induced with 1mM IPTG for 16 hours at 25°C.

Bacterial pellets were resuspended in STE buffer (50mM Tris-HCL pH 7.5, 150mM NaCl, 0.5% Triton-X, 1mM EGTA) supplemented with 0.1mg/ml of lysozyme, 0.5mM PMSF, 1mM DTT, 1.5% of Sarkosyl powder and Protease inhibitor cocktail (Roche). Lysate was then sonicated at 30% amplitude for 20sec and 10sec break for a total of 3mins. Lysate was then centrifuged at 4,000 rpm for 30mins in 4°C.

Supernatant containing soluble proteins were incubated with glutathione sepharose beads for 2 hours at 4°C, rotating. The beads were washed thrice with immunoprecipitation lysis buffer (IPLB) containing 25mM Tris pH 7.5, 150mM NaCl, 0.5% Triton-X and protease inhibitor cocktail (Roche). GST fusion proteins were eluted with 10mM of reduced L-glutathione (Sigma) at room temperature for 30 minutes. Dialysis of eluted protein was performed using Slide-A-Lyzer Dialysis Cassette (Thermo scientific) with buffer containing 50mM Tris pH8, 1mM EGTA, 1mM PMSF and 0.1mM DTT. Dialysis was performed overnight in 4°C, following that dialyzed protein was collected and concentrated using Amicon- centrifugal unit (10ml) and centrifuged at 4,000 rpm for 10-15mins at 4°C. Protein concentration were measured and glycerol was then added to make a final concentration of 20%. Aliquots of purified proteins were then snapped freeze in liquid nitrogen and stored in -80°C for future use.

3.10 In vitro phosphatase assay

HEK293T cells were transfected with FLAG-MST1 or Flag-LATS1 with Myc-MOB1B. 48 hours later, cells were treated with 50nM Calyculin A for 1 hour. Cells were then collected and lysed in lysis buffer supplemented with protease inhibitor. The cell lysates were then incubated with 1:50 Anti-Flag M2 antibody overnight at 4°C with constant rotation. Tagged overexpressed proteins complexed with Anti-Flag M2 antibody were then pulled-down by incubating with Protein G magnetic beads (Millipore) for 2 hours at 4 °C . After washing with PBS, the magnetic beads were then incubated with different amount of bacterially expressed GST-POPX2 or CIP (New England Biolabs, Ipswich, MA) with phosphatase buffer (50 mM HEPES [4.pH 7.3], 10 mM MgCl₂, 5 mM MnCl₂, 1 mM DTT, 0.05% Triton X-100) to a final reaction volume of 25 µl. Phosphatase reactions were carried out at 30°C for 45 min on tube agitator. The reactions were stopped by adding 5 µl of 6X SDS sample buffer and boiling for 10 minutes.

3.11 Cell Fractionation

Cells were lysed with cytoplasmic extraction reagent (CER) containing 10 mM HEPES pH7.4, 10 mM KCl, 1% IGEPAL CA-630, 1 mM DTT, protease inhibitor cocktail (Roche) and PhosSTOP™ (Sigma). Cells were incubated in CER at 4°C for 15 minutes. Nuclear pellets were centrifuged out at 14,000 rpm (BOECO CENTRIFUGE M-24 A) for 10 min at 4°C, and cytoplasmic fraction were obtained from the supernatant. The nuclear pellets were washed with the CER before lysing with protein lysis buffer (PLB) supplemented with protease inhibitor cocktail (Roche) and PhosSTOP™ (Sigma). Nuclear lysates were incubated in PLB at 4°C for 15 minutes. Nuclear lysates were centrifuged at 14,000 rpm (BOECO CENTRIFUGE M-24 A) for 10 min at 4°C, nuclear fractions were obtained from the supernatants. Both fractions were analyzed by Western blot.

3.12 Cell cluster staining

Cells were grown in adherent condition for 48 hours, thereafter trypsinised and seeded onto 6-well Ultra Low Attachment plates (Corning) and allowed to grow for 48 hours. Cells were then transferred into fresh 6-well plate containing Poly-L-lysine coated coverslips and allowed to attach for 10 minutes. Cells were then fixed and stained with the appropriate antibodies.

3.13 Real-Time PCR (RT-PCR)

RNA extraction were performed using RNeasy Mini Kit (Qiagen). 2.5 µg of Purified RNA were reverse-transcribed to complementary DNA (cDNA) using SuperScript VILO Reaction Mix (Invitrogen). cDNA were diluted 25×. RT-PCR was performed on a StepOnePlus Real-Time PCR System (Applied Biosystems). The 20 µL PCR reaction mixture consisted of 2 µL of cDNA, 100 nM forward and reverse primers, 2×KAPA SYBR and 50× ROX (High). Cycling conditions were; initial denaturation at 95 °C for 5 minutes, followed by 40 cycles consisting of; 95 °C for 10 seconds, 60 °C for 30 seconds and 72 °C for 10 seconds.

The primers used for real-time RT-PCR were as follows:

Genes	Forward (5'-3')	Reverse (5'-3')
<i>β-ACTIN</i>	ATCAAGATCATTTGCTCCTCCTGAG	CTGCTTGCTGATCCACATCTG
<i>TATA box binding protein</i>	GCCAGCTTCGGAGAGTTCTGGGATT	CGGGCACGAAGTGCAATGGTCTTTA
<i>POPX2</i>	TGTTTGATGGTCACGGAGGC	TTCTCATCCTGCCGTTCTGGT
<i>CTGF</i>	AGGAGTGGGTGTGTGACGA	CCAGGCAGTTGGCTCTAATC
<i>CYR61</i>	CCTTGTGGACAGCCAGTGTA	ACTTGGGCCGGTATTTCTTC
<i>ANKRD</i>	AGTAGAGGAAGTGGTCACTGG	TGGGCTAGAAGTGTCTTCAGAT
<i>VIMENTIN</i>	GCAAAGATTCCACTTTGCGT	GAAATTGCAGGAGGAGATGC
<i>YAP</i>	GCACCTCTGTGTTTTAAGGGTCT	CAACTTTTGCCCTCCTCCAA
<i>TAZ</i>	GGCTGGGAGATGACCTTCAC	CTGAGTGGGGTGGTTCTGCT

Table 3: List of primers used for RT-PCR

3.14 General Statistical Analysis

Experimental data are presented as the mean $\pm$ SD. Two-tailed Student's t tests with unequal variance were performed using XLMiner Analysis ToolPak on Microsoft excel. Error bars represent standard deviation. Differences were considered statistically significant when p values were less than 0.05 (*p < 0.05, **p < 0.01, ***p < 0.001). Best represented images were shown.

4. RESULTS

4.1 POPX2 interacts with multiple core Hippo kinases

Previously we have identified several candidate proteins that may interact with POPX2 through immunoprecipitation of overexpressed tagged-POPX2 and the identification of its associated proteins using Mass spectrometry (Weng and Koh, unpublished data). Among the list of proteins, two were identified to be component of the Hippo core kinases, NDR1 and MOB1. This discovery prompts us to investigate whether POPX2 has a role in the regulation of Hippo kinases.

To confirm the interactions, we performed exogenous co-immunoprecipitation of POPX2 with NDR1 and MOB1. Since NDR1 and MOB1 are a part of the core Hippo kinase complex which regulates YAP and TAZ, we next investigated whether POPX2 also interacts with other members of the Hippo pathway by co-immunoprecipitation assays. We found that in addition to NDR1, POPX2 interacted with several members of the Hippo pathway including LATS1 and MST1, but not to MOB1, YAP, or TAZ (Figures 19 to 25), suggesting that the physical interactions between POPX2 and the Hippo pathway members are selective and restricted to the core kinases, as shown in the interaction hot-zone (Figure 26). The interactions were specific because associated proteins were not detected in immunoprecipitates from cells expressing vector (control). In the co-IP experiments, we used two different tagged versions of POPX2, GST-POPX2, GFP-POPX2 or Flag-POPX2, to make sure that the co-IP are specific and not influenced by the tags used (Figures 19 to 23). We also performed reciprocal-IP to confirmed interactions (Figures 24-25).

Here, we observed that POPX2 strongly interacts with three members of the NDR family including LATS1, LATS2 and NDR1 (Figures 20-22). Although both NDR1 and LATS1 are downstream kinases of MST1, we have decided to focus on the interaction of POPX2 with

the tumor suppressor LATS1 and its kinase, MST1 in this study. The reason is that MST1 and LATS1 are considered the core kinase cassette of the Hippo signaling pathway (Hergovich, 2013). Nevertheless, we cannot discount crosstalks between MST1-NDR1 and MST1-LATS1 signaling pathways. Together, our data show that POPX2 specifically interacted with multiple core Hippo kinases.

(A)

(B)

Figure 19: POPX2 interacts with MST1.

(A) Flag-MST is co-transfected with GST vector or GST-POPX2 in HEK293 cells. Vector expressing GST, co-transfected with Flag-MST is used as a negative control. Cell lysates were harvested and GST-vector and GST-POPX2 were pull down using glutathione beads.

(B) Flag-MST is co-transfected with GFP vector or GFP-POPX2 in HEK293 cells. Vector expressing GFP, co-transfected with Flag-MST is used as a negative control. Cell lysates were harvested and Flag-MST1 were pull down using Anti-FLAG M2 Affinity Agarose Gel.

Pull down proteins were separated by SDS-PAGE and analysed by Western analysis.

(A)

(B)

Figure 20: POPX2 interacts with LATS1.

(A) Flag-LATS1 is co-transfected with GST vector or GST-POPX2 in HEK293 cells. Vector expressing GST, co-transfected with Flag-MST is used as a negative control. Cell lysates were harvested and GST-vector and GST-POPX2 were pull down using glutathione beads.

(B) Myc-LATS1 is transfected alone or co-transfected with Flag-POPX2 in HEK293 cells. Myc-LATS1 or Flag-POPX2 transfected alone serve as negative controls. Cell lysates were harvested and Flag-POPX2 were pull down using Anti-FLAG M2 Affinity Agarose Gel.

Pull down proteins were separated by SDS-PAGE and analysed by Western analysis.

(A)

(B)

Figure 21: POPX2 interacts with LATS2.

(A) Myc-LATS2 is co-transfected with GST vector or GST-POPX2 in HEK293 cells. Vector expressing GST, co-transfected with Myc-LATS2 is used as a negative control. Cell lysates were harvested and GST-vector and GST-POPX2 were pull down using glutathione beads.

(B) Myc-LATS2 is co-transfected with Flag-GFP or Flag-POPX2 in HEK293 cells. Flag-GFP co-transfected with Myc-LATS2 is used as a negative control. Cell lysates were harvested and Flag-POPX2 were pull down using Anti-FLAG M2 Affinity Agarose Gel.

Pull down proteins were separated by SDS-PAGE and analysed by Western analysis.

(A)

(B)

Figure 22: POPX2 interacts with NDR1.

(A) Flag-NDR1 is co-transfected with GST vector or GST-POPX2 in HEK293 cells. Vector expressing GST, co-transfected with Flag-NDR1 is used as a negative control. Cell lysates were harvested and GST-vector and GST-POPX2 were pull down using glutathione beads.

(B) GST-NDR1 is co-transfected with Flag-GFP or Flag-POPX2 in HEK293 cells. Flag-GFP co-transfected with GST-NDR1 is used as a negative control. Cell lysates were harvested and Flag-POPX2 were pull down using Anti-FLAG M2 affinity Agarose Gel.

Pull down proteins were separated by SDS-PAGE and analysed by Western analysis.

(A)

(B)

Figure 23: POPX2 does not interact with MOB1.

(A) Myc-MOB1 is co-transfected with GST vector or GST-POPX2 in HEK293 cells. Vector expressing GST, co-transfected with Myc-MOB1 is used as a negative control. Cell lysates were harvested and GST-vector and GST-POPX2 were pull down using glutathione beads.

(B) Myc-MOB1 is co-transfected with Flag-GFP or Flag-POPX2 in HEK293 cells. Flag-GFP co-transfected with Myc-MOB1 is used as a negative control. Cell lysates were harvested and Flag-POPX2 were pull down using Anti-FLAG M2 Affinity Agarose Gel.

Pull down proteins were separated by SDS-PAGE and analysed by Western analysis.

(A)

(B)

Figure 24: POPX2 does not interact with YAP.

(A) Flag-YAP is co-transfected with GST vector or GST-POPX2 in HEK293 cells. Vector expressing GST, co-transfected with Flag-YAP is used as a negative control. Cell lysates were harvested and GST-vector and GST-POPX2 were pull down using glutathione beads.

(B) Flag-YAP is co-transfected with GST vector or GST-POPX2 in HEK293 cells. Vector expressing GST, co-transfected with Flag-YAP is used as a negative control. Cell lysates were harvested and Flag-YAP were pull down using Anti-FLAG M2 Affinity Agarose Gel.

Pull down proteins were separated by SDS-PAGE and analysed by Western analysis.

(A)

(B)

Figure 25: POPX2 does not interact with TAZ.

(A) Flag-TAZ is co-transfected with GST vector or GST-POPX2 in HEK293 cells. Vector expressing GST, co-transfected with Flag-TAZ is used as a negative control. Cell lysates were harvested and GST-vector and GST-POPX2 were pull down using glutathione beads.

(B) Flag-TAZ is co-transfected with GST vector or GST-POPX2 in HEK293 cells. Vector expressing GST, co-transfected with Flag-TAZ is used as a negative control. Cell lysates were harvested and Flag-TAZ were pull down using Anti-FLAG M2 Affinity Agarose Gel.

Pull down proteins were separated by SDS-PAGE and analysed by Western analysis.

Based on the interaction profiles derived from the exogenous protein pull-down, we map the interaction of POPX2 within the Hippo pathway to the core kinases MST and LATS (Figures 19-21, 26). We also eliminated any possibility that POPX2 may interact to MST and LATS via the scaffold protein MOB1, due to the lack of interaction between the two proteins (Figure 23). However, we could not rule out the interaction of POPX2 with SAV1, which will be investigated in future.

Interaction Hot-Zone

Figure 26: POPX2 interacts with multiple core HIPPO kinases.

POPX2 selectively interacts with MST1, LATS1/2, and NDR1 but does not interact with MOB1B, YAP, and TAZ. The Hot-zone of POPX2-HIPPO kinase interaction maps to the core kinases, MST and LATS/NDR (red).

4.2 POPX2 interacts with MST/LATS endogenously

Next, to demonstrate that the interactions between POPX2-MST and POPX2-LATS also occur endogenously, we performed reciprocal endogenous POPX2 immunoprecipitation in HEK293 cells. Using antibodies specific for MST1 and LATS1, we immunoprecipitated MST1 (Figure 27A) and LATS1 (Figure 27B) respectively and probe for POPX2 within the pull-down fractions. Reciprocally, using antibody specific for POPX2 we immunoprecipitated POPX2 and probed for MST1 and LATS1 within the pull-down fraction (Figure 27C).

Figure 27: POPX2 interacts with MST1 and LATS1 endogenously.

(A) Endogenous MST1 from HEK293 cells was immunoprecipitated. The IP complexes were separated by SDS PAGE, followed by Western analysis using POPX2 antibody. The Western blot reveals endogenous MST-POPX2 interaction. (B) Similar experiment as in (A) was performed using LATS1 antibody for immunoprecipitation. The Western blot reveals endogenous LATS1-POPX2 interaction. (C) Reciprocal endogenous POPX2 immunoprecipitation followed by Western analysis using MST1 and LATS1 antibodies reveal endogenous POPX2-MST1 and POPX2-LATS1 interactions.

4.3 Mapping of POPX2 domain interacting with MST1

Pull-down experiment was performed with a series of truncated POPX2 mutants. MST1 was found to interact with the POPX2 truncation that contains the full catalytic domain of POPX2 (Figure 28A). Sequential deletion of the N-terminal residues of POPX2 revealed the importance of amino acids 150-194 for MST1 binding, thus, mapping the minimal domain required for MST1 binding to the catalytic domain of POPX2 (Figure 28B).

Figure 28: MST1 binds to POPX2 catalytic domain.

(A) Full length GST-POPX2 and its various truncated forms are co-transfected with Flag-MST. GST pulldown was separated by SDS-PAGE and subjected to western analysis using anti-Flag antibodies to detect co-precipitation of Flag-MST1. (B) Schematic bar map of various POPX2 truncation-MST interaction based on western blot densitometry normalized against full length GST-POPX2

4.4 POPX2 does not dephosphorylate MST1 on Threonine 183

Since POPX2 was found to interact with MST1 through its catalytic domain we proceeded to investigate if MST1 could be a substrate of POPX2. Interestingly, a known substrate of POPX2, PAK1 is dephosphorylated at Thr423 in the active loop. POPX2 dephosphorylates and inactivates PAK1 (Koh et al., 2002). PAK-Thr423 is a residue that aligns with Thr183 of MST1 (Glantschnig et al., 2002). Furthermore, the activation loop sequence within the kinase domains of MST and PAK are nearly identical (Figure 29), and the phosphorylation of the conserved threonine Thr423 in PAK1 and Thr183 in MST1 are required for autoactivation (Manser et al., 1997).

(A)

PAK1_HUMAN	ILLGMDGSVKLTDFGFCAQITPEQSKRS	TMVGTPYW
STK4_HUMAN	ILLNTEGHAKLADFGVAGQLTDTMAKRNT	TVIGTPFW
	. :* .** :...*:*	:**.*::***:*

(B)

Figure 29: Alignment of the activation loop within the kinase domain of PAK1 and MST1

(A) Amino acids within the kinase domains of PAK1 and MST1 aligned using Clustal Omega. Thr423 of PAK and Thr183 of MST1 are highlighted in red.

(B) PAK1 and STK4/MST1 activation sites alignment, Thr⁴²³ of PAK1 (Red stick) and Phospho-Thr¹⁸³ of MST (Blue stick).

Since MST1 activation requires phosphorylation on Thr-183 (Praskova et al., 2004), there is a possibility of POPX2 dephosphorylating and negatively regulates MST1. Therefore, we investigated whether POPX2 is capable of dephosphorylating MST1-Thr183. Bacterially purified GST-POPX2 is mixed with phosphorylated Flag-MST1 in an *in vitro* phosphatase assay (Figure 30A). To obtain phosphorylated Flag-MST1, HEK293 cells transfected with Flag-MST1 were treated with Calyculin A (50nM, 1hr) to stimulate Thr183 phosphorylation before immunoprecipitation using anti-Flag antibodies (Flag-IP). Calyculin A is an inhibitor to PP2A. Inhibition of PP2A can lead to MST1 auto-phosphorylation and activation (Guo et al., 2011). We also attempted to check for dephosphorylation of MST1-Thr183 *in vivo* by transfecting POPX2 cDNA construct into HEK293 cells and detect for phospho-MST1-Thr183 levels in the cell lysates (Figure 30B). In this experiment, we used Okadaic acid instead of Calyculin A for inhibition of PP2A (Hata et al., 2013) to induce MST1 auto-phosphorylation.

(A)

Figure 30: POPX2 does not dephosphorylate MST on Threonine-183.

(A) *In vitro* phosphatase assay. Bacterially expressed POPX2 (4 μ g and 8 μ g) was incubated with phospho-Flag-MST1 for 45 mins at 30°C. The reaction mixtures were subjected to SDS-PAGE and Western analysis using phospho-MST1-Thr-183 antibodies. Calf-intestinal phosphatase (CIP) was used as a negative control. (B) Overexpression of POPX2 in HEK293 does not affect endogenous MST1 phosphorylation status at Thr183 upon induction with okadaic acid. HEK293 cells were transfected with GFP-POPX2 or GFP-constructs. We then treated the cells with okadaic acid (1 μ M) for 1 hr to inhibit PP2A and induce MST1 autophosphorylation. Cell lysates were separated on SDS-PAGE and subjected to Western analysis.

We observed that POPX2 did not dephosphorylate MST1 on Thr183, the auto-phosphorylation site on the activation-loop of MST1 which is required for its activation. However, we could not rule out the possibility that POPX2 could dephosphorylate other MST phosphorylation sites due to the lack of availability of phospho-specific antibodies for these sites.

4.5 Mapping of POPX2 domain interacting with LATS1

Pull-down experiment was performed with a series of truncated POPX2 mutants. LATS1 was found to interact with the POPX2 truncation that contains the full catalytic domain of POPX2 (Figure 31A). Sequential deletion of the N-terminal residues of POPX2 revealed the importance of amino acids 65-194 for LATS1 binding, thus, mapping the minimal domain required for LATS1 binding to the N-terminal end of POPX2 containing the catalytic domain (Figure 31B).

Figure 31: LATS1 binds to the N-terminal end of POPX2 containing the catalytic domain. (A) GST pull-down of full length GST-POPX2 and its various truncated forms after co-expression with Flag-LATS1. (B) Schematic bar map of various POPX2 truncation-LATS1 interaction based on western blot densitometry normalized against full length POPX2.

4.6 Phosphorylation and activation of LATS requires MOB1

Since POPX2 does not dephosphorylate MST1, we asked whether POPX2 dephosphorylates LATS instead. To obtain phosphorylated LATS, we overexpressed Flag-LATS with MST1, MOB1 or both MST1 and MOB1. We observed that the co-expression of LATS1 with MOB1 was sufficient to induce LATS1 phosphorylation at Threonine 1079 (Figure 32). It has been reported that MOB1 acts as an adaptor to facilitate LATS activation (Ni et al., 2015).

Interestingly, addition of MST1 to LATS-MOB1 does not further increase phospho-LATS levels (Figure 32). This is because overexpressed MST1 is kept in a non-phosphorylated state in HEK293 cells unless stimulated with okadaic acid or calyculin A. Henceforth, to induce phosphorylation on Thr-1079 of LATS1, we co-expressed LATS1 with MOB1 for the subsequent experiments.

Figure 32: LATS1 phosphorylation on T1079 requires MOB1 expression.

Flag-LATS1 was co-transfected with either Flag-MST1 or Myc-MOB1 or Flag-MST1 + Myc-MOB1 constructs into HEK293 cells. Cell lysates were harvested and separated by SDS-PAGE and subjected to Western analysis. Total LATS1 and pLATS1 were probed on separate membranes with the loading control, GAPDH, shown on the right.

4.7 POPX2 dephosphorylates LATS on Threonine 1079

Since we determined that POPX2 and LATS1 interact, we investigated the function of this interaction. We hypothesized that LATS could be a substrate of POPX2 and its phosphorylation and hence activation is regulated by POPX2. LATS1 activation requires phosphorylation on threonine 1079. Therefore, we investigated whether POPX2 is capable of dephosphorylating this site. To validate this, bacterially purified GST-POPX2 was mixed with phosphorylated Flag-LATS1 in an *in vitro* phosphatase assay. To obtain phosphorylated Flag-LATS1, Flag-LATS1 was co-transfected with Myc-MOB1 into HEK293. The co-transfected cells were then treated with Calyculin A (50nM, 1hr) to further stimulate Thr1079 phosphorylation, thereafter phospho-LATS1 was pulldown using Anti-Flag M2 mouse antibody (Figure 33).

Figure 33: In vitro Phosphatase assay of Flag-LATS1 with GST-POPX2.

(A) *In vitro* phosphatase assay. Bacterially expressed POPX2 (4 and 8 μ g) were incubated with phospho-Flag-LATS1 for 45 mins at 30°C. The reaction mixtures were subjected to SDS-PAGE and Western analysis using phospho-LATS1-Thr1079 antibodies. Calf-intestinal phosphatase (CIP) was used as a negative control. (B) Bar chart representation of (A). Three independent experiments were conducted. The error bars represent standard deviation. Student t-test was performed to determine statistical significances.

POPX2 was observed to dephosphorylate LATS1 on Thr1079 (Figure 33A), the phosphorylation site on the hydrophobic motif (HM) of LATS1 which is required for its activation. The extent of dephosphorylation was increased in a POPX2 dose dependent manner (Figure 33B).

Next, we proceeded to validate the specificity of POPX2 as a LATS1 phosphatase. We included two phosphatases known to regulate the Hippo kinases, PP1 and PP2A in our experiment (Hata et al., 2013). We co-transfected the phosphatases together with Flag-LATS and Myc-MOB1. Here, we observed that neither PP1 nor PP2A overexpression was capable of reducing LATS phosphorylation (Figure 34A). The extent of dephosphorylation was quantitated (Figure 34B). Taken together, this indicates that POPX2 is a specific phosphatase that dephosphorylates LATS at Thr1079.

(A)

(B)

Figure 34: Overexpression of POPX2 decreases phosphorylation of LATS1 on Thr1079.

HEK293 cells were co-transfected with Flag-LATS1 + Myc-MOB1 + GST-vector or GST-PP1 or GST-PP2 or GST-POPX2. Cell lysates were harvested and separated by SDS-PAGE and subjected to Western analysis.

(A) Total LATS1 (left) and pLATS1 (right) were probed on separate membrane.

(B) Densitometry quantification of phospho-LATS1. Three independent experiments were performed. Error bars represent standard deviation. Student t-test was performed to determine statistical significances.

4.8 MDA-MB-231 cell density and YAP/TAZ expression

After establishing that POPX2 indeed regulates LATS1 phosphorylation (Figure 34), we wish to investigate further on the effects of POPX2 on the Hippo pathway. More specifically, we wish to determine if the levels of POPX2 can affect the activity of YAP/TAZ. To do so, we compare MDA-MB-231 wildtype cells with POPX2 CRISPR knock-out MDA-MB-231 cells [henceforth known as X2KO cells (Zhang and Koh, unpublished cell line)].

In non-transformed cells, YAP/TAZ expression is known to change with cell density, where YAP/TAZ expression is inversely correlated with cell density (Zhao et al., 2007). However, changes to the expression of YAP/TAZ with respect to cell density have not been studied in transformed cells such as MDA-MB-231. Thus, we studied both the morphological and biochemical changes of X2KO cells (Figure 35). Using bright-field microscopy and probing the levels of YAP/TAZ using (CST, #8418) and TAZ using (CST, #4883) at different cell densities, we studied the morphological changes and determined whether the loss of POPX2 affects the morphology and YAP/TAZ expression in MDA-MB-231 cells.

(A)

(B)

(C)

(D)

Figure 35: Loss of POPX2 affects MDA-MB-231 morphology as well as TAZ and Vimentin protein levels.

MDA-MB-231 wild type and X2KO cells (A) brightfield images at high cell density, scale bar: 50µm. (B) Brightfield images at increasing cell density, scale bar: 50µm (C) cell lysates at different cell densities were separated by SDS-PAGE, and subjected to western analysis. YAP/TAZ protein expression profiles at different cell density were probed using antibodies against YAP/TAZ (CST, #8418). (D) cell lysates at different cell densities were separated by SDS-PAGE, subjected to western analysis using antibodies against TAZ. TAZ protein expression profiles at different cell density were probed using (CST, #4883). (E) densitometry quantification of TAZ protein levels relative to actin at different cell density. Three independent experiments were performed. Error bars represent standard deviation. Student t-test was performed to determine statistical significances. (F) cell lysates were separated by SDS-PAGE, subjected to western analysis using vimentin antibody. (G) densitometry quantification of vimentin protein levels relative to GAPDH. Three independent experiments were performed. Error bars represent standard deviation. Student t-test was performed to determine statistical significances.

When grown in 2D we observed that compared to wild type MDA-MB231, X2KO cells displayed more cell-cell contacts and less spindle (mesenchymal) morphology (Figure 36A). X2KO cells also appeared to display contact inhibition of locomotion (CIL). In wild-type MDA-MB-231, the loss of CIL enable the cells to climb and grow above neighboring cells (Figure 35A). We also observed that in the highly metastatic breast cancer cell line MDA-MB-231, increasing cell density led to increased YAP/TAZ protein levels, while the levels of YAP/TAZ in X2KO cells did not increase with increasing cell density (Figure 35C-E). The increasing protein levels of YAP/TAZ in MDA-MB-231 reflect the highly malignant characteristic of this cell line, where contact inhibition via loss of YAP/TAZ is absent (Kim et al., 2011). Taken together, the loss of POPX2 in X2KO cells appears to prevent the accumulation of YAP/TAZ. We next check the level of an EMT marker, vimentin (Figure 28C). Here, we found that X2KO cells displayed lower level of vimentin compared to wild type MDA-MB-231 suggesting loss of EMT characteristic.

4.9 POPX2 knock out reduces TAZ protein level

Since TAZ protein level and activity are up-regulated in high-grade metastatic breast cancers, and overexpression of TAZ is sufficient to induce breast cancer cell proliferation, transformation and EMT(Chan et al., 2008; Cordenonsi et al., 2011), we focus on TAZ protein expression in our subsequent investigations. TAZ activity is spatially regulated depending on its phosphorylation status. When localized to the nucleus, TAZ is capable of regulating the expression of its target genes as a transcription co-activator (Liu et al., 2011a) while phosphorylated-TAZ is retained in the cytoplasm. Therefore, we investigated if loss of POPX2 affects TAZ localization. We assayed endogenous TAZ subcellular localization since its cytoplasmic localisation has been extensively used as a primary read-out for the Hippo pathway or for cell–cell contact inhibition by TAZ (LaQuaglia et al., 2016).

Cell fractionation experiment was performed on wild-type and X2KO MDA-MB-231 cells to determine the distribution of TAZ. Cytoplasmic and nuclear extracts were collected and together with total lysates were analyzed by western blotting (Figure 36A), and quantified by densitometry (Figure 36B). In parallel, immunofluorescence staining was performed to further complement the western analysis (Figure 36C).

Figure 36: Loss of POPX2 affects cytosolic and nuclear TAZ protein levels.

(A) MDA-MB-231 wild type and X2KO cells were lysed and fractionated into fractions of cytoplasmic extract, nuclear extract and total extract. Extracts were then separated by SDS-PAGE and subjected to western analysis.

(B) Western analysis from (A) quantified in a densitometry bar graph. Three independent experiments were performed. Error bars represent standard deviation. Student t-test was performed to determine statistical significances. (C) MDA-MB-231 wild type and X2KO cells grown to high cell density were immunostained with YAP/TAZ antibody (scale bar: 50μm)

From our western analysis, we observed that X2KO cells displayed lower total TAZ, a significant loss of cytoplasmic TAZ and lower nuclear TAZ compared to wild-type MDA-MB-231 (Figure 36A, B). Thus, supporting our earlier biochemical data (Figure 35C, D). Consistent with our biochemical analysis, we observed loss of cytoplasmic TAZ and lower nuclear TAZ in X2KO cells in cell staining experiment (Figure 36C).

4.10 X2KO cells displayed loss of nuclear TAZ when grown in cell clusters

After observing TAZ localization when grown in 2D culture we next investigate TAZ localization when cells were grown in low anchorage condition using ultra low attachment plate for 48 hours. We observed for differences in cell cluster morphology and immunostaining of TAZ between wildtype MDA-MB-231 and X2KO cells (Figure 37).

(A)

(B)

(C)

(D)

(E)

Figure 37: Loss of POPX2 affects TAZ localization when grown in low anchorage condition

(A) Schematic representation of experimental setup and (B) brightfield images of MDA-MB-231 cells (scale bar: 100μm) (C) Immunostaining of TAZ after growing on low attachment plate (scale bar: 50μm) (D) Fluorescence intensity between cytoplasmic and nucleus is measured using the Corrected Total Cell Fluorescence method. Cytoplasmic intensity = total cell fluorescence intensity - nucleus fluorescence intensity. CTCF = Integrated Density – (Area of selected cell X Mean fluorescence of background readings) (E) Graph depicting percentage of TAZ localization of wild type and X2KO cells clusters.

Here, we observed that wild type MDA-MB-231 displayed larger clustering of cells compared to X2KO cells, possibly due to the loss of cell-cell contact inhibition (Figure 37B). Meanwhile, the immunostaining of TAZ revealed little nuclear TAZ in X2KO cells (Figure 37C). TAZ was observed in wild-type MDA-MB-231. We quantify nuclear and cytoplasmic localization of TAZ by comparing the intensity of TAZ staining in the nucleus versus cytoplasm using the CTCF method reported by Burgess et al., Gavet et al., and McCloy et al., (Burgess et al., 2010; Gavet and Pines, 2010; McCloy et al., 2014) (Figure 37D). From differences between total cell fluorescence and nucleus fluorescence intensity (blue circles), we calculated the cytosolic fluorescence intensity. Cells were then classified as having higher cytoplasmic TAZ ($C > N$) or higher nuclear TAZ ($N > C$) based on the difference between the cytosolic and nuclear fluorescence intensity, and the percentage of $C > N$ and $N > C$ cells were then computed. Here we observed a higher number of MDA-MB-231 wild type cells displaying equal amount of cytoplasmic and nuclear TAZ ($C = N$) compared to cells displaying higher cytoplasmic than nuclear TAZ ($C > N$), whereas the opposite is observed in MDA-MB-231 X2KO cells (Figure 37E).

4.11 Loss of POPX2 affects MDA-MB-231 cell cluster morphology

Besides changes to the localization of TAZ, we also studied the differences in cluster morphology between wild type MDA-MB-231 and X2KO cells. Similar to many previous reports, wild-type MDA-MB-231 grown in low-anchorage condition displayed grape-like cell clusters morphology (Feng et al., 2017; Manuel Iglesias et al., 2013; Shaw et al., 2012). However, X2KO cells displayed a rather fragile planar cell clusters morphology.

Figure 38: Wild-type MDA-MB-231 cell clusters morphology.

(A) DAPI staining of wild-type MDA-MB-231 cell clusters after growing in low attachment condition (scale bar: 50µm)

(B) Interactive 3D surface plots of DAPI intensities using ImageJ.

Figure 39: X2KO MDA-MB-231 cell clusters morphology.

(A) DAPI staining of X2KO cell clusters after growing in low attachment condition (scale bar: 50µm).

(B) Interactive 3D surface plots of DAPI intensities using ImageJ.

Wild type MDA-MB-231 cells displayed robust grape-like cell clusters similar to the reported morphology of breast cancer circulating tumor cells (CTCs) (Vishnoi et al., 2015) as shown from the intercalation of DAPI intensity peaks (Figure 38B). Meanwhile X2KO cells displayed a fragile planar cell cluster morphology, as shown from the discrete DAPI intensity peaks (Figure 39B) suggesting cells detaching from the edges (red arrows).

4.12 POPX2 knockout reduces the expression of TAZ target genes

Following our observation that the loss of POPX2 resulted in lower TAZ protein levels in the nucleus (Figure 36A), we hypothesized that lower TAZ protein level would translate to lower expression of TAZ target genes. Therefore, we determined the expression of TAZ target gene expressions, *CTGF* and *ANKRD1*, using RT-PCR. Total RNA was extracted from wildtype MDA-MB-231 and X2KO cells. RT-PCR was performed and the fold change was computed using $\Delta\Delta CT$ method.

Figure 40: RT-PCR of YAP/TAZ target gene expression.

MDA-MB-231 wild type and X2KO cells were grown to high cell density. Cells were harvested and RNA were collected using RNeasy mini kit and converted to cDNA and subjected to RT-PCR. Data obtained were computed to fold change using the $\Delta\Delta CT$ method. X2KO cells exhibit lower amount of *CTGF* and *ANKRD1* transcripts levels. Three independent experiments were performed. Error bars represent standard deviation. Student t-test was performed to determine statistical significances.

Our RT-PCR analysis of two gene targets of TAZ reveals that X2KO cells exhibit lower expression of *CTGF* and *ANKRD1*, suggesting lower/loss of TAZ activity as a co-activator for transcription (Figure 40).

4.13 Loss of POPX2 affects proliferation and anchorage independence growth

Stable overexpression of TAZ in human MCF10A mammary epithelial cells induces cell proliferation and anchorage-independent growth (Lei et al., 2008). Since, POPX2 affects TAZ protein level and its gene targets, we next investigated how this biochemical change affects growth both in 2D and 3D culture. We hypothesized that loss of POPX2 in X2KO cells would result in reduced cell proliferation and affect anchorage-independent growth compared with wild type MDA-MB-231 cells. To determine loss of POPX2 growth fitness on 2D and 3D culture, we perform MTT assay and Soft Agar assay respectively.

(A)

(B)

(C)

Figure 41: Loss of POPX2 affects growth.

(A) Cells were grown on 2D culture plates for up to 7 days, where cell growth were monitored on days: 1,3,5 and 7 using the MTT coloumetric assay. Three independent experiments were performed. Error bars represent standard deviation. Student t-test was performed to determine statistical significances.

(B) Cells were grown in 3D soft agar assay, where cells were seeded onto the top agar and supplemented with fresh growth media every three days for up to 28 days. Three independent experiments were performed. Error bars represent standard deviation. Student t-test was performed to determine statistical significances.

(C) Bright field images of crystal violet stained cell colonies in soft agar assay (scale bar: 200µm)

Here, we observed that loss of POPX2 affects cell proliferation on 2D and 3D cell culture (Figure 41). Loss of POPX2 led to lower cell proliferation as shown in the MTT assay growth curves of control and X2KO cells (Figure 41A). Consistent with this, X2KO cells also showed less colonies in the soft agar assay (Figure 41B, C). Anchorage independent growth appeared to be affected in X2KO cells. Therefore, we concluded that X2KO cells are less fit when grown in low attachment condition suggesting that these cells might have lower tumorigenicity.

5. DISCUSSION

The Hippo pathway is an established regulator of cell fate and tissue growth (Pan, 2007). Thus, dysregulation of the pathway could lead to tumorigenesis via promoting cell proliferation and migration. The Hippo pathway receives input from various stimuli such as mechanosensors, mitogens, cell-cell and cell-substrate contacts. These stimuli eventually impact on YAP/TAZ to promote plasticity which is the key to cancer growth and metastasis. Plasticity is a prime characteristic of cancer, which enables them to respond to changes in the microenvironment, directing the cells to migrate, invade and colonize. As such, YAP/TAZ are molecular determinants imparting differentiated cells with plasticity and stemness through inducing EMT and self-renewal programs (Zanconato et al., 2016).

YAP/TAZ regulation occurs either via the MST/LATS kinase pathway or a MST-independent pathway, where both pathways converge onto LATS, indicating that the requirement of LATS is indispensable (Kim et al., 2013; Meng et al., 2015). Therefore, elucidating LATS regulation is critical to understanding the different modes of YAP/TAZ regulation.

To date, the only phosphatases regulating the Hippo pathway are the PP1 and PP2A phosphatases. These phosphatases dephosphorylate MST1 and YAP/TAZ (Hata et al., 2013). No phosphatase that targets LATS1/2 has been found despite its critical role in converging different pathways to regulate YAP/TAZ. In this current study, we demonstrated that POPX2 binds several of the core Hippo kinase members including MST1, NDR1 and LATS. As MST1 shares similar structure as PAK1 in its kinase active loop, this further fuel our hypothesis. Nevertheless, the phosphatase assay (Figure 30) demonstrated that MST1 is not a substrate of POPX2. Instead, we have identified a novel role of POPX2 as

a LATS1 phosphatase. POPX2 is capable of dephosphorylating LATS1 at Thr-1079 (Figures 33, 34), which is in its hydrophobic motif (HM).

The implication of POPX2 dephosphorylating LATS1 could lie in the control of YAP/TAZ activity, the final members in the Hippo signaling pathway. We studied YAP/TAZ expression in the highly metastatic MDA-MB-231 cells with respect to cell density and observed that they displayed increased YAP/TAZ expression with increasing cell density, which is in contrast to the previous observations with NIH3T3 and MCF10A cells (Zhao et al., 2007). The difference observed could be attributed to the highly metastatic nature of the MDA-MB-231 cell line (Kim et al., 2011).

Loss of POPX2 results in decreased YAP/TAZ protein levels (Figure 36) possibly due to increased LATS1-mediated phosphorylation and consequently degradation.

Phosphorylation of YAP at Ser127 leads to its sequestration and retention in the cytoplasm by 14-3-3. Further phosphorylation at other residues and coordinated phosphorylation with Casein kinase 1 (CK1) lead to the ubiquitinylation of YAP by E3 ubiquitin ligase and its subsequent degradation (Liu et al., 2010a). Furthermore, loss of YAP/TAZ results in lower expression of their signature target genes, *CTGF* and *ANKRD1* suggesting lower YAP/TAZ activity as transcription co-activators (Figure 40).

Lastly we have also demonstrated the functional consequence POPX2-LATS1 interaction. POPX2 can function to oppose the tumour suppressor function of the Hippo pathway by dephosphorylating LATS1, leading to increased nuclear YAP/TAZ. Loss of POPX2, in turn, resulted in lower levels of YAP/TAZ and thus affected anchorage independent growth. Indeed, we observed fewer colonies of X2KO when grown in soft agar compared with wild type MDA-MB-231 cells (Figure 41B, C). This is consistent with removing a negative regulator of LATS1 leading to increased LATS1 activity and its tumour suppressor function.

Our data demonstrate that POPX2 functions at the level of LATS, suggesting that POPX2 may regulate the Hippo pathway downstream of multiple cues. Hence, we observed the requirement for POPX2 in regulating TAZ level in response to cell density and detachment events. In all the cases, loss of POPX2 expression resulted in loss of TAZ both in the nucleus and cytoplasm, leading to lower TAZ target genes expression. Thus, POPX2 could serve to stabilize YAP/TAZ through inhibiting their degradation, and therefore function as a strong tumorigenic factor in promoting the malignant progression of breast cancer cells, where, interestingly, the levels of POPX2 are found to be higher in more invasive cancers (Susila et al., 2010).

Altogether, our findings suggest a model in which POPX2 regulates the Hippo pathway activity by dephosphorylating LATS, leading to the accumulation of YAP/TAZ (Figure 42). The detailed mechanism by which POPX2-mediated dephosphorylation of LATS1 that could lead to the accumulation of YAP/TAZ requires further work.

(A)

(B)

Figure 42: Proposed mechanism for POPX2 regulation of YAP/TAZ.

(A) When POPX2 is present, it dephosphorylates LATS1 on Thr1079, thus inactivating LATS1 kinase activity leading to the accumulation of YAP/TAZ. (B) When POPX2 is absent, LATS1 is phosphorylated and activated, thus is capable of phosphorylating YAP/TAZ leading to their degradation.

5.1 Elusive nature of LATS phosphatase

For the first time, we have identified a direct LATS1 phosphatase which has eluded many studies on LATS1. The plausible explanation for this could be due to the presence of the abundant and well-studied phosphatases such as PP1 and PP2A acting both upstream (MST1 dephosphorylation) and downstream (YAP/TAZ dephosphorylation) of LATS, thus masking the discovery of other phosphatases that could regulate the Hippo pathway via LATS dephosphorylation. This is especially so when the PP1 and PP2A inhibitors are used to stimulate the Hippo pathway. Moreover, PP1 and PP2A account for >90% of phosphatase activities in eukaryotes (Virshup and Shenolikar, 2009). Therefore, it is often presumed that either of these phosphatases are involved whenever a phosphatase role is required to explain for the loss of phosphorylation, without taking an exhaustive approach to identify other phosphatases, such as in the case of a study conducted by Lv et al. (Lv et al., 2015). Since both PP1 and PP2A have promiscuous activities *in vitro* these assumption will hold true, overlooking other possible candidates that may regulate LATS kinase.

5.2 Loss of POPX2 may induce partial MET

Interestingly, loss of POPX2 also resulted in loss of mesenchymal marker vimentin in the highly metastatic breast cancer cell line, MDA-MB-231. The loss of mesenchymal marker together with morphological changes observed in the X2KO cells could indicate a partial/intermediate MET. Therefore, it will be interesting to determine the extent of MET following the loss of POPX2 in future studies.

5.3 Alternative mechanism of POPX2-Hippo regulation

Another plausible mechanism by which POPX2 could regulate the Hippo kinase would be through its interaction with β PIX (Koh et al. 2002). A study has proposed that β PIX may act as a scaffold, bringing together LATS and YAP upon upstream cytoskeletal signaling, resulting in LATS1-mediated phosphorylation and inactivation of YAP (Heidary Arash et al., 2014). Furthermore, they identified the Lysine-Glutamic acid-rich (KER) domain of β PIX to be critical for β PIX-LATS and YAP interaction, the same domain to which POPX1/2 binds β PIX (Figure 43B) (Koh et al., 2002).

Figure 43: LATS, YAP and POPX2 bind to β Pix via a shared domain.

(A) Model for β Pix role in Hippo signaling proposed by Attisano et al. The cytoplasmic β Pix which exists as a dimer or a trimer, acts as a scaffold promoting LATS-mediated phosphorylation of Yap/Taz, leading to Yap/Taz cytoplasmic sequestration and inactivation.

(B) All three proteins; LATS, YAP and POPX2 bind to β Pix via the KER domain.

In this hypothesis, POPX2 could (1) compete with LATS for β PIX interaction or (2) compete with YAP for β PIX interaction, both of which would lead to lower LATS-mediated phosphorylation and inactivation of YAP (Figure 44). Interestingly, this alternate mechanism would result in same the outcome as our proposed mechanism i.e. high POPX2 levels lead to the activation of YAP. Thus, further reinforcing the potential role of POPX2 as a negative regulator of the Hippo pathway.

Figure 44: Alternate mechanism of POPX2 regulation of Hippo pathway.

POPX2 competitive binding with (1) LATS and/or (2) YAP for binding with β PIX at its KIM domain.

5.4 Implication of increasing YAP/TAZ levels in highly metastatic breast cancer cells

Our observation that TAZ expression increases with increasing cell density in MDA-MB-231 suggests that when these cells form metastases they would likely display increased YAP/TAZ activity in response to physical constraints, as a consequence to the loss of contact inhibition. In response to this, the surrounding healthy cells which retain contact inhibition would suppress their YAP/TAZ activity. This in turn would lead to the apoptosis of surrounding healthy cells, and could in turn pave way for further cancer growth in a vicious cycle (Figure 45). A study with *drosophila* in 2016 has provide substantial experimental evidence for a similar competitive tumor-host interactions (Suijkerbuijk et al., 2016). Similarly, a study with NIH3T3 mouse fibroblast cell line showed that cells with higher TEAD activity became super-competitors, while cells with lower TEAD-activity became losers that were preferentially eliminated (Mamada et al., 2015).

Figure 45: Schematic illustration of the outcome of a highly metastatic cancer altering YAP/TAZ activity of surrounding cells.

The increasing level of YAP/TAZ in highly metastatic cells during tumor expansion may exert a mechanical force that renders neighbouring contact inhibited cells to inactivate their YAP/TAZ activity. With low YAP/TAZ activity these cells may eventually succumb to apoptosis and pave way for larger tumor expansion.

5.5 Implications of POPX2-mediated LATS regulation

The role of POPX2 as a regulator of LATS1 could lead to the regulation of other functions besides YAP/TAZ activity. LATS1 also functions as a negative regulator of CDC2/cyclin A to regulate G2/M transition and apoptosis (Xia et al., 2002). As a LATS1 phosphatase, POPX2 may function to relieve LATS1 suppression of cell proliferation and prevent LATS1-mediated apoptosis.

In addition, loss-of-function of LATS in the immortalized normal mammary epithelial cell line, MCF10A, reduces p53 phosphorylation and promotes its interaction with p52 NF- κ B subunit. Subsequently this led to changes to p53 conformation and shifted its transcriptional output towards an oncogenic state, with invasive potential (Furth et al., 2015). Similarly, the expression of POPX2 has been correlated to cancer invasiveness, therefore, high levels of POPX2 may inhibit LATS1 and consequently foster invasiveness via changes to p53 phospho-status and transcriptional output. Hence, the role of POPX2 in regulating LATS1 beyond the activity of YAP/TAZ could be elucidated in future studies.

6. CONCLUSION

In the current study, we have identified, for the first time, a phosphatase for LATS1. We found that POPX2 is a novel LATS1 phosphatase and is required for the stabilization of YAP/TAZ possibly through its regulation of LATS1. The role of POPX2 as a regulator of the Hippo pathway supports previous findings that implicate its role in promoting cancer metastasis.

The most crucial stage to contain and restrict tumor growth is the stage prior to tumor cells becoming invasive. At this stage tumor cells have yet to acquire invasive capability that could foster their metastatic spread to distant organ. Dysregulation of the Hippo pathway and thus constitutive activation of YAP/TAZ serve as monumental point in tumor growth enabling them to overcome cell contact inhibition and anoikis, the prerequisites for metastasis (Zanconato et al., 2016). POPX2 expression has been found to be correlated with breast cancer cells invasiveness (Susila et al., 2010). In addition we observed that POPX2 is required for YAP/TAZ stabilization and activity. Therefore, targeting POPX2 during early stages of cancer could potentially help mitigate both cancer spread and growth.

APPENDIX

Page	Figure 1: The Hallmarks of Cancer	License number: 4271691411988
Page	Figure 2: The biology of breast cancer.	License number: 4271741335659
Page	Figure 12: Schematic representations of the PP2C α , POPX1 and POPX2.	License number: 4292921352884

REFERENCES

- Al-Hajj, M., M.S. Wicha, A. Benito-Hernandez, S.J. Morrison, and M.F. Clarke. 2003. Prospective identification of tumorigenic breast cancer cells. *Proceedings of the National Academy of Sciences of the United States of America*. 100:3983-3988.
- Aylon, Y., D. Michael, A. Shmueli, N. Yabuta, H. Nojima, and M. Oren. 2006. A positive feedback loop between the p53 and Lats2 tumor suppressors prevents tetraploidization. *Genes & development*. 20:2687-2700.
- Aylon, Y., A. Sarver, A. Tovy, E. Ainbinder, and M. Oren. 2014. Lats2 is critical for the pluripotency and proper differentiation of stem cells. *Cell death and differentiation*. 21:624-633.
- Azzolin, L., T. Panciera, S. Soligo, E. Enzo, S. Bicciato, S. Dupont, S. Bresolin, C. Frasson, G. Basso, V. Guzzardo, A. Fassina, M. Cordenonsi, and S. Piccolo. 2014. YAP/TAZ incorporation in the beta-catenin destruction complex orchestrates the Wnt response. *Cell*. 158:157-170.
- Badawi, M.A., D.M. Abouelfadl, S.L. El-Sharkawy, W.E. El-Aal, and N.F. Abbas. 2015. Tumor-Associated Macrophage (TAM) and Angiogenesis in Human Colon Carcinoma. *Open access Macedonian journal of medical sciences*. 3:209-214.
- Bae, S.J., L. Ni, A. Osinski, D.R. Tomchick, C.A. Brautigam, and X. Luo. 2017. SAV1 promotes Hippo kinase activation through antagonizing the PP2A phosphatase STRIPAK. *Elife*. 6.
- Bar-Shavit, R., M. Maoz, A. Kancharla, J.K. Nag, D. Agranovich, S. Grisaru-Granovsky, and B. Uziely. 2016. G Protein-Coupled Receptors in Cancer. *International journal of molecular sciences*. 17.
- Bartucci, M., R. Dattilo, C. Moriconi, A. Pagliuca, M. Mottolese, G. Federici, A.D. Benedetto, M. Todaro, G. Stassi, F. Sperati, M.I. Amabile, E. Pillozzi, M. Patrizii, M. Biffoni, M. Maugeri-Sacca, S. Piccolo, and R. De Maria. 2015. TAZ is required for metastatic activity and chemoresistance of breast cancer stem cells. *Oncogene*. 34:681-690.
- Basu, S., N.F. Totty, M.S. Irwin, M. Sudol, and J. Downward. 2003. Akt phosphorylates the Yes-associated protein, YAP, to induce interaction with 14-3-3 and attenuation of p73-mediated apoptosis. *Molecular cell*. 11:11-23.
- Bhang, H.E., D.A. Ruddy, V. Krishnamurthy Radhakrishna, J.X. Caushi, R. Zhao, M.M. Hims, A.P. Singh, I. Kao, D. Rakiec, P. Shaw, M. Balak, A. Raza, E. Ackley, N. Keen, M.R. Schlabach, M. Palmer, R.J. Leary, D.Y. Chiang, W.R. Sellers, F. Michor, V.G. Cooke, J.M. Korn, and F. Stegmeier. 2015. Studying clonal dynamics in response to cancer therapy using high-complexity barcoding. *Nature medicine*. 21:440-448.
- Bichsel, S.J., R. Tamaskovic, M.R. Stegert, and B.A. Hemmings. 2004. Mechanism of activation of NDR (nuclear Dbf2-related) protein kinase by the hMOB1 protein. *The Journal of biological chemistry*. 279:35228-35235.
- Brazil, D.P., and B.A. Hemmings. 2001. Ten years of protein kinase B signalling: a hard Akt to follow. *Trends in biochemical sciences*. 26:657-664.
- Britschgi, A., S. Duss, S. Kim, J.P. Couto, H. Brinkhaus, S. Koren, D. De Silva, K.D. Mertz, D. Kaup, Z. Varga, H. Voshol, A. Vissieres, C. Leroy, T. Roloff, M.B. Stadler, C.H. Scheel, L.J. Miraglia, A.P. Orth, G.M. Bonamy, V.A. Reddy, and M. Bentires-Alj. 2017. The Hippo kinases LATS1 and 2 control human breast cell fate via crosstalk with ERalpha. *Nature*. 541:541-545.
- Burgess, A., S. Vigneron, E. Brioudes, J.C. Labbe, T. Lorca, and A. Castro. 2010. Loss of human Greatwall results in G2 arrest and multiple mitotic defects due to deregulation of the cyclin B-Cdc2/PP2A

- p>balance.
- Proceedings of the National Academy of Sciences of the United States of America*
- . 107:12564-12569.
- Callus, B.A., A.M. Verhagen, and D.L. Vaux. 2006. Association of mammalian sterile twenty kinases, Mst1 and Mst2, with hSalvador via C-terminal coiled-coil domains, leads to its stabilization and phosphorylation. *FEBS J*. 273:4264-4276.
- Camargo, F.D., S. Gokhale, J.B. Johnnidis, D. Fu, G.W. Bell, R. Jaenisch, and T.R. Brummelkamp. 2007. YAP1 increases organ size and expands undifferentiated progenitor cells. *Current biology : CB*. 17:2054-2060.
- Chan, E.H., M. Nousiainen, R.B. Chalamalasetty, A. Schafer, E.A. Nigg, and H.H. Sillje. 2005. The Ste20-like kinase Mst2 activates the human large tumor suppressor kinase Lats1. *Oncogene*. 24:2076-2086.
- Chan, S.H., W.C. Huang, J.W. Chang, K.J. Chang, W.H. Kuo, M.Y. Wang, K.Y. Lin, Y.H. Uen, M.F. Hou, C.M. Lin, T.H. Jang, C.W. Tu, Y.R. Lee, Y.H. Lee, M.T. Tien, and L.H. Wang. 2014. MicroRNA-149 targets GIT1 to suppress integrin signaling and breast cancer metastasis. *Oncogene*. 33:4496-4507.
- Chan, S.W., C.J. Lim, K. Guo, C.P. Ng, I. Lee, W. Hunziker, Q. Zeng, and W. Hong. 2008. A role for TAZ in migration, invasion, and tumorigenesis of breast cancer cells. *Cancer research*. 68:2592-2598.
- Chen, Q., N. Zhang, R.S. Gray, H. Li, A.J. Ewald, C.A. Zahnow, and D. Pan. 2014. A temporal requirement for Hippo signaling in mammary gland differentiation, growth, and tumorigenesis. *Genes & development*. 28:432-437.
- Cheung, W.L., K. Ajiro, K. Samejima, M. Kloc, P. Cheung, C.A. Mizzen, A. Beeser, L.D. Etkin, J. Chernoff, W.C. Earnshaw, and C.D. Allis. 2003. Apoptotic phosphorylation of histone H2B is mediated by mammalian sterile twenty kinase. *Cell*. 113:507-517.
- Chiba, S., Y. Amagai, Y. Homma, M. Fukuda, and K. Mizuno. 2013. NDR2-mediated Rabin8 phosphorylation is crucial for ciliogenesis by switching binding specificity from phosphatidylserine to Sec15. *The EMBO journal*. 32:874-885.
- Chiba, S., M. Ikeda, K. Katsunuma, K. Ohashi, and K. Mizuno. 2009. MST2- and Furry-mediated activation of NDR1 kinase is critical for precise alignment of mitotic chromosomes. *Current biology : CB*. 19:675-681.
- Chiyoda, T., N. Sugiyama, T. Shimizu, H. Naoe, Y. Kobayashi, J. Ishizawa, Y. Arima, H. Tsuda, M. Ito, K. Kaibuchi, D. Aoki, Y. Ishihama, H. Saya, and S. Kuninaka. 2012. LATS1/WARTS phosphorylates MYPT1 to counteract PLK1 and regulate mammalian mitotic progression. *The Journal of cell biology*. 197:625-641.
- Codelia, V.A., G. Sun, and K.D. Irvine. 2014. Regulation of YAP by mechanical strain through Jnk and Hippo signaling. *Current biology : CB*. 24:2012-2017.
- Cordenonsi, M., F. Zanconato, L. Azzolin, M. Forcato, A. Rosato, C. Frasson, M. Inui, M. Montagner, A.R. Parenti, A. Poletti, M.G. Daidone, S. Dupont, G. Basso, S. Bicciato, and S. Piccolo. 2011. The Hippo transducer TAZ confers cancer stem cell-related traits on breast cancer cells. *Cell*. 147:759-772.
- Cornils, H., R.S. Kohler, A. Hergovich, and B.A. Hemmings. 2011. Human NDR kinases control G(1)/S cell cycle transition by directly regulating p21 stability. *Molecular and cellular biology*. 31:1382-1395.
- Cornils, H., M.R. Stegert, A. Hergovich, D. Hynx, D. Schmitz, S. Dirnhofer, and B.A. Hemmings. 2010. Ablation of the kinase NDR1 predisposes mice to the development of T cell lymphoma. *Science signaling*. 3:ra47.
- Creasy, C.L., and J. Chernoff. 1995. Cloning and characterization of a human protein kinase with homology to Ste20. *The Journal of biological chemistry*. 270:21695-21700.

- Dalerba, P., S.J. Dylla, I.K. Park, R. Liu, X. Wang, R.W. Cho, T. Hoey, A. Gurney, E.H. Huang, D.M. Simeone, A.A. Shelton, G. Parmiani, C. Castelli, and M.F. Clarke. 2007. Phenotypic characterization of human colorectal cancer stem cells. *Proceedings of the National Academy of Sciences of the United States of America*. 104:10158-10163.
- Das Thakur, M., Y. Feng, R. Jagannathan, M.J. Seppa, J.B. Skeath, and G.D. Longmore. 2010. Ajuba LIM proteins are negative regulators of the Hippo signaling pathway. *Current biology : CB*. 20:657-662.
- den Hertog, J. 2003. Regulation of protein phosphatases in disease and behaviour. *EMBO reports*. 4:1027-1032.
- Denkert, C., S. Loibl, A. Noske, M. Roller, B.M. Muller, M. Komor, J. Budczies, S. Darb-Esfahani, R. Kronenwett, C. Hanusch, C. von Tonne, W. Weichert, K. Engels, C. Solbach, I. Schrader, M. Dietel, and G. von Minckwitz. 2010. Tumor-associated lymphocytes as an independent predictor of response to neoadjuvant chemotherapy in breast cancer. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology*. 28:105-113.
- DeRan, M., J. Yang, C.H. Shen, E.C. Peters, J. Fitamant, P. Chan, M. Hsieh, S. Zhu, J.M. Asara, B. Zheng, N. Bardeesy, J. Liu, and X. Wu. 2014. Energy stress regulates hippo-YAP signaling involving AMPK-mediated regulation of angiomin-like 1 protein. *Cell reports*. 9:495-503.
- DeVita, V.T., Jr., R.C. Young, and G.P. Canellos. 1975. Combination versus single agent chemotherapy: a review of the basis for selection of drug treatment of cancer. *Cancer*. 35:98-110.
- Devroe, E., H. Erdjument-Bromage, P. Tempst, and P.A. Silver. 2004. Human Mob proteins regulate the NDR1 and NDR2 serine-threonine kinases. *The Journal of biological chemistry*. 279:24444-24451.
- Donninger, H., N. Allen, A. Henson, J. Pogue, A. Williams, L. Gordon, S. Kassler, T. Dunwell, F. Latif, and G.J. Clark. 2011. Salvador protein is a tumor suppressor effector of RASSF1A with hippo pathway-independent functions. *The Journal of biological chemistry*. 286:18483-18491.
- Dupont, S., L. Morsut, M. Aragona, E. Enzo, S. Giullitti, M. Cordenonsi, F. Zanconato, J. Le Digabel, M. Forcato, S. Bicciato, N. Elvassore, and S. Piccolo. 2011. Role of YAP/TAZ in mechanotransduction. *Nature*. 474:179-183.
- Elbediwy, A., Z.I. Vincent-Mistiaen, B. Spencer-Dene, R.K. Stone, S. Boeing, S.K. Wculek, J. Cordero, E.H. Tan, R. Ridgway, V.G. Brunton, E. Sahai, H. Gerhardt, A. Behrens, I. Malanchi, O.J. Sansom, and B.J. Thompson. 2016. Integrin signalling regulates YAP and TAZ to control skin homeostasis. *Development*. 143:1674-1687.
- Elosegui-Artola, A., I. Andreu, A.E.M. Beedle, A. Lezamiz, M. Uroz, A.J. Kosmalka, R. Oria, J.Z. Kechagia, P. Rico-Lastres, A.L. Le Roux, C.M. Shanahan, X. Trepac, D. Navajas, S. Garcia-Manyes, and P. Roca-Cusachs. 2017. Force Triggers YAP Nuclear Entry by Regulating Transport across Nuclear Pores. *Cell*. 171:1397-1410 e1314.
- Erez, N., S. Glanz, Y. Raz, C. Avivi, and I. Barshack. 2013. Cancer associated fibroblasts express pro-inflammatory factors in human breast and ovarian tumors. *Biochemical and biophysical research communications*. 437:397-402.
- Fan, R., N.G. Kim, and B.M. Gumbiner. 2013. Regulation of Hippo pathway by mitogenic growth factors via phosphoinositide 3-kinase and phosphoinositide-dependent kinase-1. *Proceedings of the National Academy of Sciences of the United States of America*. 110:2569-2574.
- Feng, F., J. Zhang, X. Fan, F. Yuan, Y. Jiang, R. Lv, and Y. Ma. 2017. Downregulation of Rab27A contributes to metformin-induced suppression of breast cancer stem cells. *Oncology letters*. 14:2947-2953.

- Ferrigno, O., F. Lallemand, F. Verrecchia, S. L'Hoste, J. Camonis, A. Atfi, and A. Mauviel. 2002. Yes-associated protein (YAP65) interacts with Smad7 and potentiates its inhibitory activity against TGF-beta/Smad signaling. *Oncogene*. 21:4879-4884.
- Finch-Edmondson, M.L., R.P. Strauss, A.M. Passman, M. Sudol, G.C. Yeoh, and B.A. Callus. 2015. TAZ Protein Accumulation Is Negatively Regulated by YAP Abundance in Mammalian Cells. *The Journal of biological chemistry*. 290:27928-27938.
- Fitzgerald, P.J. 1986. Homogeneity and heterogeneity in pancreas cancer: presence of predominant and minor morphological types and implications. *International journal of pancreatology : official journal of the International Association of Pancreatology*. 1:91-94.
- Friedmann-Morvinski, D., and I.M. Verma. 2014. Dedifferentiation and reprogramming: origins of cancer stem cells. *EMBO reports*. 15:244-253.
- Friedrich, J., C. Seidel, R. Ebner, and L.A. Kunz-Schughart. 2009. Spheroid-based drug screen: considerations and practical approach. *Nature protocols*. 4:309-324.
- Furth, N., and Y. Aylon. 2017. The LATS1 and LATS2 tumor suppressors: beyond the Hippo pathway. *Cell death and differentiation*. 24:1488-1501.
- Furth, N., N. Bossel Ben-Moshe, Y. Pozniak, Z. Porat, T. Geiger, E. Domany, Y. Aylon, and M. Oren. 2015. Down-regulation of LATS kinases alters p53 to promote cell migration. *Genes & development*. 29:2325-2330.
- Gatenby, R.A., A.S. Silva, R.J. Gillies, and B.R. Frieden. 2009. Adaptive therapy. *Cancer research*. 69:4894-4903.
- Gavet, O., and J. Pines. 2010. Progressive activation of CyclinB1-Cdk1 coordinates entry to mitosis. *Developmental cell*. 18:533-543.
- Gay, L., A.M. Baker, and T.A. Graham. 2016. Tumour Cell Heterogeneity. *F1000Research*. 5.
- Glantschnig, H., G.A. Rodan, and A.A. Reszka. 2002. Mapping of MST1 kinase sites of phosphorylation. Activation and autophosphorylation. *The Journal of biological chemistry*. 277:42987-42996.
- Glentis, A., P. Oertle, P. Mariani, A. Chikina, F. El Marjou, Y. Attieh, F. Zaccarini, M. Lae, D. Loew, F. Dingli, P. Sirven, M. Schoumacher, B.G. Gurchenkov, M. Plodinec, and D.M. Vignjevic. 2017. Cancer-associated fibroblasts induce metalloprotease-independent cancer cell invasion of the basement membrane. *Nature communications*. 8:924.
- Graves, J.D., K.E. Draves, Y. Gotoh, E.G. Krebs, and E.A. Clark. 2001. Both phosphorylation and caspase-mediated cleavage contribute to regulation of the Ste20-like protein kinase Mst1 during CD95/Fas-induced apoptosis. *The Journal of biological chemistry*. 276:14909-14915.
- Graves, J.D., Y. Gotoh, K.E. Draves, D. Ambrose, D.K. Han, M. Wright, J. Chernoff, E.A. Clark, and E.G. Krebs. 1998. Caspase-mediated activation and induction of apoptosis by the mammalian Ste20-like kinase Mst1. *The EMBO journal*. 17:2224-2234.
- Gruber, R., R. Panayiotou, E. Nye, B. Spencer-Dene, G. Stamp, and A. Behrens. 2016. YAP1 and TAZ Control Pancreatic Cancer Initiation in Mice by Direct Up-regulation of JAK-STAT3 Signaling. *Gastroenterology*. 151:526-539.
- Guo, C., X. Zhang, and G.P. Pfeifer. 2011. The tumor suppressor RASSF1A prevents dephosphorylation of the mammalian STE20-like kinases MST1 and MST2. *The Journal of biological chemistry*. 286:6253-6261.
- Haas, M., A. Dimmler, W. Hohenberger, G.G. Grabenbauer, G. Niedobitek, and L.V. Distel. 2009. Stromal regulatory T-cells are associated with a favourable prognosis in gastric cancer of the cardia. *BMC gastroenterology*. 9:65.
- Hanahan, D., and R.A. Weinberg. 2000. The hallmarks of cancer. *Cell*. 100:57-70.

- Hanahan, D., and R.A. Weinberg. 2011. Hallmarks of cancer: the next generation. *Cell*. 144:646-674.
- Hansen, C.G., T. Moroishi, and K.L. Guan. 2015. YAP and TAZ: a nexus for Hippo signaling and beyond. *Trends in cell biology*. 25:499-513.
- Hao, Y., A. Chun, K. Cheung, B. Rashidi, and X. Yang. 2008. Tumor suppressor LATS1 is a negative regulator of oncogene YAP. *The Journal of biological chemistry*. 283:5496-5509.
- Harvey, B.P., S.S. Banga, and H.L. Ozer. 2004. Regulation of the multifunctional Ca²⁺/calmodulin-dependent protein kinase II by the PP2C phosphatase PPM1F in fibroblasts. *The Journal of biological chemistry*. 279:24889-24898.
- Hata, Y., S. Timalisina, and S. Maimaiti. 2013. Okadaic Acid: a tool to study the hippo pathway. *Marine drugs*. 11:896-902.
- Heallen, T., M. Zhang, J. Wang, M. Bonilla-Claudio, E. Klysik, R.L. Johnson, and J.F. Martin. 2011. Hippo pathway inhibits Wnt signaling to restrain cardiomyocyte proliferation and heart size. *Science*. 332:458-461.
- Heidary Arash, E., K.M. Song, S. Song, A. Shiban, and L. Attisano. 2014. Arhgef7 promotes activation of the Hippo pathway core kinase Lats. *The EMBO journal*. 33:2997-3011.
- Hergovich, A. 2011. MOB control: reviewing a conserved family of kinase regulators. *Cellular signalling*. 23:1433-1440.
- Hergovich, A. 2013. Regulation and functions of mammalian LATS/NDR kinases: looking beyond canonical Hippo signalling. *Cell & bioscience*. 3:32.
- Hergovich, A. 2016. The Roles of NDR Protein Kinases in Hippo Signalling. *Genes (Basel)*. 7.
- Hergovich, A., S.J. Bichsel, and B.A. Hemmings. 2005. Human NDR kinases are rapidly activated by MOB proteins through recruitment to the plasma membrane and phosphorylation. *Molecular and cellular biology*. 25:8259-8272.
- Hergovich, A., S. Lamla, E.A. Nigg, and B.A. Hemmings. 2007. Centrosome-associated NDR kinase regulates centrosome duplication. *Molecular cell*. 25:625-634.
- Hergovich, A., D. Schmitz, and B.A. Hemmings. 2006. The human tumour suppressor LATS1 is activated by human MOB1 at the membrane. *Biochemical and biophysical research communications*. 345:50-58.
- Higashi, T., H. Hayashi, T. Ishimoto, H. Takeyama, T. Kaida, K. Arima, K. Taki, K. Sakamoto, H. Kuroki, H. Okabe, H. Nitta, D. Hashimoto, A. Chikamoto, T. Beppu, and H. Baba. 2015. miR-9-3p plays a tumour-suppressor role by targeting TAZ (WWTR1) in hepatocellular carcinoma cells. *British journal of cancer*. 113:252-258.
- Hirsch, F.R., G. Ottesen, J. Podenphant, and J. Olsen. 1983. Tumor heterogeneity in lung cancer based on light microscopic features. A retrospective study of a consecutive series of 200 patients, treated surgically. *Virchows Archiv. A, Pathological anatomy and histopathology*. 402:147-153.
- Hong, J.H., E.S. Hwang, M.T. McManus, A. Amsterdam, Y. Tian, R. Kalmukova, E. Mueller, T. Benjamin, B.M. Spiegelman, P.A. Sharp, N. Hopkins, and M.B. Yaffe. 2005. TAZ, a transcriptional modulator of mesenchymal stem cell differentiation. *Science*. 309:1074-1078.
- Hoon, J.L., H.Y. Li, and C.G. Koh. 2014. POPX2 phosphatase regulates cell polarity and centrosome placement. *Cell cycle*. 13:2459-2468.
- Housman, G., S. Byler, S. Heerboth, K. Lapinska, M. Longacre, N. Snyder, and S. Sarkar. 2014. Drug resistance in cancer: an overview. *Cancers*. 6:1769-1792.
- Huang, J., S. Wu, J. Barrera, K. Matthews, and D. Pan. 2005. The Hippo signaling pathway coordinately regulates cell proliferation and apoptosis by inactivating Yorkie, the Drosophila Homolog of YAP. *Cell*. 122:421-434.

- Huang, W., X. Lv, C. Liu, Z. Zha, H. Zhang, Y. Jiang, Y. Xiong, Q.Y. Lei, and K.L. Guan. 2012. The N-terminal phosphodegron targets TAZ/WWTR1 protein for SCFbeta-TrCP-dependent degradation in response to phosphatidylinositol 3-kinase inhibition. *The Journal of biological chemistry*. 287:26245-26253.
- Huntoon, C.J., M.D. Nye, L. Geng, K.L. Peterson, K.S. Flatten, P. Haluska, S.H. Kaufmann, and L.M. Karnitz. 2010. Heat shock protein 90 inhibition depletes LATS1 and LATS2, two regulators of the mammalian hippo tumor suppressor pathway. *Cancer research*. 70:8642-8650.
- Ishida, A., I. Kameshita, and H. Fujisawa. 1998. A novel protein phosphatase that dephosphorylates and regulates Ca²⁺/calmodulin-dependent protein kinase II. *The Journal of biological chemistry*. 273:1904-1910.
- Ishida, A., Y. Tada, T. Nimura, N. Sueyoshi, T. Katoh, M. Takeuchi, H. Fujisawa, T. Taniguchi, and I. Kameshita. 2005. Identification of major Ca(2+)/calmodulin-dependent protein kinase phosphatase-binding proteins in brain: biochemical analysis of the interaction. *Archives of biochemistry and biophysics*. 435:134-146.
- Ishida, A., K. Tsumura, M. Oue, Y. Takenaka, Y. Shigeri, N. Goshima, Y. Ishihara, T. Hirano, H. Baba, N. Sueyoshi, I. Kameshita, and T. Yamazaki. 2013. An active C-terminally truncated form of Ca (2+) /calmodulin-dependent protein kinase phosphatase-N (CaMKP-N/PPM1E). *BioMed research international*. 2013:134813.
- Jang, S.W., S.J. Yang, S. Srinivasan, and K. Ye. 2007. Akt phosphorylates Mst1 and prevents its proteolytic activation, blocking FOXO3 phosphorylation and nuclear translocation. *The Journal of biological chemistry*. 282:30836-30844.
- Jin, Y., L. Dong, Y. Lu, W. Wu, Q. Hao, Z. Zhou, J. Jiang, Y. Zhao, and L. Zhang. 2012. Dimerization and cytoplasmic localization regulate Hippo kinase signaling activity in organ size control. *The Journal of biological chemistry*. 287:5784-5796.
- Jurmeister, S., M. Baumann, A. Balwierz, I. Keklikoglou, A. Ward, S. Uhlmann, J.D. Zhang, S. Wiemann, and O. Sahin. 2012. MicroRNA-200c represses migration and invasion of breast cancer cells by targeting actin-regulatory proteins FHOD1 and PPM1F. *Molecular and cellular biology*. 32:633-651.
- Kalluri, R., and R.A. Weinberg. 2009. The basics of epithelial-mesenchymal transition. *The Journal of clinical investigation*. 119:1420-1428.
- Kanai, F., P.A. Marignani, D. Sarbassova, R. Yagi, R.A. Hall, M. Donowitz, A. Hisaminato, T. Fujiwara, Y. Ito, L.C. Cantley, and M.B. Yaffe. 2000. TAZ: a novel transcriptional co-activator regulated by interactions with 14-3-3 and PDZ domain proteins. *The EMBO journal*. 19:6778-6791.
- Kang, W., J.H. Tong, A.W. Chan, T.L. Lee, R.W. Lung, P.P. Leung, K.K. So, K. Wu, D. Fan, J. Yu, J.J. Sung, and K.F. To. 2011. Yes-associated protein 1 exhibits oncogenic property in gastric cancer and its nuclear accumulation associates with poor prognosis. *Clinical cancer research : an official journal of the American Association for Cancer Research*. 17:2130-2139.
- Kim, M., M. Kim, S. Lee, S. Kuninaka, H. Saya, H. Lee, S. Lee, and D.S. Lim. 2013. cAMP/PKA signalling reinforces the LATS-YAP pathway to fully suppress YAP in response to actin cytoskeletal changes. *The EMBO journal*. 32:1543-1555.
- Kim, M.H., J. Kim, H. Hong, S.H. Lee, J.K. Lee, E. Jung, and J. Kim. 2016a. Actin remodeling confers BRAF inhibitor resistance to melanoma cells through YAP/TAZ activation. *The EMBO journal*. 35:462-478.
- Kim, N.G., and B.M. Gumbiner. 2015. Adhesion to fibronectin regulates Hippo signaling via the FAK-Src-PI3K pathway. *The Journal of cell biology*. 210:503-515.

- Kim, N.G., E. Koh, X. Chen, and B.M. Gumbiner. 2011. E-cadherin mediates contact inhibition of proliferation through Hippo signaling-pathway components. *Proceedings of the National Academy of Sciences of the United States of America*. 108:11930-11935.
- Kim, S.Y., Y. Tachioka, T. Mori, and T. Hakoshima. 2016b. Structural basis for autoinhibition and its relief of MOB1 in the Hippo pathway. *Scientific reports*. 6:28488.
- Kleinsmith, L.J., and G.B. Pierce, Jr. 1964. Multipotentiality of Single Embryonal Carcinoma Cells. *Cancer research*. 24:1544-1551.
- Koh, C.G., E.J. Tan, E. Manser, and L. Lim. 2002. The p21-activated kinase PAK is negatively regulated by POPX1 and POPX2, a pair of serine/threonine phosphatases of the PP2C family. *Current biology : CB*. 12:317-321.
- Lai, D., K.C. Ho, Y. Hao, and X. Yang. 2011. Taxol resistance in breast cancer cells is mediated by the hippo pathway component TAZ and its downstream transcriptional targets Cyr61 and CTGF. *Cancer research*. 71:2728-2738.
- Lai, Z.C., X. Wei, T. Shimizu, E. Ramos, M. Rohrbaugh, N. Nikolaidis, L.L. Ho, and Y. Li. 2005. Control of cell proliferation and apoptosis by mob as tumor suppressor, mats. *Cell*. 120:675-685.
- Lamar, J.M., P. Stern, H. Liu, J.W. Schindler, Z.G. Jiang, and R.O. Hynes. 2012. The Hippo pathway target, YAP, promotes metastasis through its TEAD-interaction domain. *Proceedings of the National Academy of Sciences of the United States of America*. 109:E2441-2450.
- Lapidot, T., C. Sirard, J. Vormoor, B. Murdoch, T. Hoang, J. Caceres-Cortes, M. Minden, B. Paterson, M.A. Caligiuri, and J.E. Dick. 1994. A cell initiating human acute myeloid leukaemia after transplantation into SCID mice. *Nature*. 367:645-648.
- LaQuaglia, M.J., J.L. Grijalva, K.A. Mueller, A.R. Perez-Atayde, H.B. Kim, G. Sadri-Vakili, and K. Vakili. 2016. YAP Subcellular Localization and Hippo Pathway Transcriptome Analysis in Pediatric Hepatocellular Carcinoma. *Scientific reports*. 6:30238.
- Lau, A.N., S.J. Curtis, C.M. Fillmore, S.P. Rowbotham, M. Mohseni, D.E. Wagner, A.M. Beede, D.T. Montoro, K.W. Sinkevicius, Z.E. Walton, J. Barrios, D.J. Weiss, F.D. Camargo, K.K. Wong, and C.F. Kim. 2014. Tumor-propagating cells and Yap/Taz activity contribute to lung tumor progression and metastasis. *The EMBO journal*. 33:468-481.
- Lavado, A., Y. He, J. Pare, G. Neale, E.N. Olson, M. Giovannini, and X. Cao. 2013. Tumor suppressor Nf2 limits expansion of the neural progenitor pool by inhibiting Yap/Taz transcriptional coactivators. *Development*. 140:3323-3334.
- Lee, K.K., and S. Yonehara. 2002. Phosphorylation and dimerization regulate nucleocytoplasmic shuttling of mammalian STE20-like kinase (MST). *The Journal of biological chemistry*. 277:12351-12358.
- Lehtinen, M.K., Z. Yuan, P.R. Boag, Y. Yang, J. Villen, E.B. Becker, S. DiBacco, N. de la Iglesia, S. Gygi, T.K. Blackwell, and A. Bonni. 2006. A conserved MST-FOXO signaling pathway mediates oxidative-stress responses and extends life span. *Cell*. 125:987-1001.
- Lei, Q.Y., H. Zhang, B. Zhao, Z.Y. Zha, F. Bai, X.H. Pei, S. Zhao, Y. Xiong, and K.L. Guan. 2008. TAZ promotes cell proliferation and epithelial-mesenchymal transition and is inhibited by the hippo pathway. *Molecular and cellular biology*. 28:2426-2436.
- Levy, D., Y. Adamovich, N. Reuven, and Y. Shaul. 2008. Yap1 phosphorylation by c-Abl is a critical step in selective activation of proapoptotic genes in response to DNA damage. *Molecular cell*. 29:350-361.
- Li, Y., J. Pei, H. Xia, H. Ke, H. Wang, and W. Tao. 2003. Lats2, a putative tumor suppressor, inhibits G1/S transition. *Oncogene*. 22:4398-4405.

- Li, Z., Y. Wang, Y. Zhu, C. Yuan, D. Wang, W. Zhang, B. Qi, J. Qiu, X. Song, J. Ye, H. Wu, H. Jiang, L. Liu, Y. Zhang, L.N. Song, J. Yang, and J. Cheng. 2015. The Hippo transducer TAZ promotes epithelial to mesenchymal transition and cancer stem cell maintenance in oral cancer. *Molecular oncology*. 9:1091-1105.
- Lian, I., J. Kim, H. Okazawa, J. Zhao, B. Zhao, J. Yu, A. Chinnaiyan, M.A. Israel, L.S. Goldstein, R. Abujarour, S. Ding, and K.L. Guan. 2010. The role of YAP transcription coactivator in regulating stem cell self-renewal and differentiation. *Genes & development*. 24:1106-1118.
- Lin, J.L., H.C. Chen, H.I. Fang, D. Robinson, H.J. Kung, and H.M. Shih. 2001. MST4, a new Ste20-related kinase that mediates cell growth and transformation via modulating ERK pathway. *Oncogene*. 20:6559-6569.
- Lin, L., A.J. Sabnis, E. Chan, V. Olivas, L. Cade, E. Pazarentzos, S. Asthana, D. Neel, J.J. Yan, X. Lu, L. Pham, M.M. Wang, N. Karachaliou, M.G. Cao, J.L. Manzano, J.L. Ramirez, J.M. Torres, F. Buttitta, C.M. Rudin, E.A. Collisson, A. Algazi, E. Robinson, I. Osman, E. Munoz-Couselo, J. Cortes, D.T. Frederick, Z.A. Cooper, M. McMahon, A. Marchetti, R. Rosell, K.T. Flaherty, J.A. Wargo, and T.G. Bivona. 2015. The Hippo effector YAP promotes resistance to RAF- and MEK-targeted cancer therapies. *Nature genetics*. 47:250-256.
- Liu-Chittenden, Y., B. Huang, J.S. Shim, Q. Chen, S.J. Lee, R.A. Anders, J.O. Liu, and D. Pan. 2012. Genetic and pharmacological disruption of the TEAD-YAP complex suppresses the oncogenic activity of YAP. *Genes & development*. 26:1300-1305.
- Liu, C., W. Huang, and Q. Lei. 2011a. Regulation and function of the TAZ transcription co-activator. *International journal of biochemistry and molecular biology*. 2:247-256.
- Liu, C.Y., X. Lv, T. Li, Y. Xu, X. Zhou, S. Zhao, Y. Xiong, Q.Y. Lei, and K.L. Guan. 2011b. PP1 cooperates with ASPP2 to dephosphorylate and activate TAZ. *The Journal of biological chemistry*. 286:5558-5566.
- Liu, C.Y., Z.Y. Zha, X. Zhou, H. Zhang, W. Huang, D. Zhao, T. Li, S.W. Chan, C.J. Lim, W. Hong, S. Zhao, Y. Xiong, Q.Y. Lei, and K.L. Guan. 2010a. The hippo tumor pathway promotes TAZ degradation by phosphorylating a phosphodegron and recruiting the SCF{beta}-TrCP E3 ligase. *The Journal of biological chemistry*. 285:37159-37169.
- Liu, Y., K. He, Y. Hu, X. Guo, D. Wang, W. Shi, J. Li, and J. Song. 2017. YAP modulates TGF-beta1-induced simultaneous apoptosis and EMT through upregulation of the EGF receptor. *Scientific reports*. 7:45523.
- Liu, Y., Y. Xin, F. Ye, W. Wang, Q. Lu, H.J. Kaplan, and D.C. Dean. 2010b. Taz-tead1 links cell-cell contact to zeb1 expression, proliferation, and dedifferentiation in retinal pigment epithelial cells. *Invest Ophthalmol Vis Sci*. 51:3372-3378.
- Llado, V., Y. Nakanishi, A. Duran, M. Reina-Campos, P.M. Shelton, J.F. Linares, T. Yajima, A. Campos, P. Aza-Blanc, M. Leitges, M.T. Diaz-Meco, and J. Moscat. 2015. Repression of Intestinal Stem Cell Function and Tumorigenesis through Direct Phosphorylation of beta-Catenin and Yap by PKCzeta. *Cell reports*.
- Lopez-Maurly, L., S. Marguerat, and J. Bahler. 2008. Tuning gene expression to changing environments: from rapid responses to evolutionary adaptation. *Nature reviews. Genetics*. 9:583-593.
- Luo, G., Y.L. Chao, B. Tang, B.S. Li, Y.F. Xiao, R. Xie, S.M. Wang, Y.Y. Wu, H. Dong, X.D. Liu, and S.M. Yang. 2015. miR-149 represses metastasis of hepatocellular carcinoma by targeting actin-regulatory proteins PPM1F. *Oncotarget*. 6:37808-37823.
- Lv, X.B., C.Y. Liu, Z. Wang, Y.P. Sun, Y. Xiong, Q.Y. Lei, and K.L. Guan. 2015. PARD3 induces TAZ activation and cell growth by promoting LATS1 and PP1 interaction. *EMBO reports*. 16:975-985.

- Mamada, H., T. Sato, M. Ota, and H. Sasaki. 2015. Cell competition in mouse NIH3T3 embryonic fibroblasts is controlled by the activity of Tead family proteins and Myc. *Journal of cell science*. 128:790-803.
- Manser, E., H.Y. Huang, T.H. Loo, X.Q. Chen, J.M. Dong, T. Leung, and L. Lim. 1997. Expression of constitutively active alpha-PAK reveals effects of the kinase on actin and focal complexes. *Molecular and cellular biology*. 17:1129-1143.
- Manuel Iglesias, J., I. Beloqui, F. Garcia-Garcia, O. Leis, A. Vazquez-Martin, A. Eguiara, S. Cufi, A. Pavon, J.A. Menendez, J. Dopazo, and A.G. Martin. 2013. Mammosphere formation in breast carcinoma cell lines depends upon expression of E-cadherin. *PloS one*. 8:e77281.
- Mao, B., F. Hu, J. Cheng, P. Wang, M. Xu, F. Yuan, S. Meng, Y. Wang, Z. Yuan, and W. Bi. 2014. SIRT1 regulates YAP2-mediated cell proliferation and chemoresistance in hepatocellular carcinoma. *Oncogene*. 33:1468-1474.
- Marjanovic, N.D., R.A. Weinberg, and C.L. Chaffer. 2013. Cell plasticity and heterogeneity in cancer. *Clinical chemistry*. 59:168-179.
- Marusyk, A., V. Almendro, and K. Polyak. 2012. Intra-tumour heterogeneity: a looking glass for cancer? *Nature reviews. Cancer*. 12:323-334.
- Masuda, K., T. Chiyoda, N. Sugiyama, A. Segura-Cabrera, Y. Kabe, A. Ueki, K. Banno, M. Suematsu, D. Aoki, Y. Ishihama, H. Saya, and S. Kuninaka. 2015. LATS1 and LATS2 phosphorylate CDC26 to modulate assembly of the tetratricopeptide repeat subcomplex of APC/C. *PloS one*. 10:e0118662.
- McCloy, R.A., S. Rogers, C.E. Caldon, T. Lorca, A. Castro, and A. Burgess. 2014. Partial inhibition of Cdk1 in G 2 phase overrides the SAC and decouples mitotic events. *Cell cycle*. 13:1400-1412.
- Meacham, C.E., and S.J. Morrison. 2013. Tumour heterogeneity and cancer cell plasticity. *Nature*. 501:328-337.
- Meng, Z., T. Moroishi, and K.L. Guan. 2016. Mechanisms of Hippo pathway regulation. *Genes & development*. 30:1-17.
- Meng, Z., T. Moroishi, V. Mottier-Pavie, S.W. Plouffe, C.G. Hansen, A.W. Hong, H.W. Park, J.S. Mo, W. Lu, S. Lu, F. Flores, F.X. Yu, G. Halder, and K.L. Guan. 2015. MAP4K family kinases act in parallel to MST1/2 to activate LATS1/2 in the Hippo pathway. *Nature communications*. 6:8357.
- Michaloglou, C., W. Lehmann, T. Martin, C. Delaunay, A. Hueber, L. Barys, H. Niu, E. Billy, M. Wartmann, M. Ito, C.J. Wilson, M.E. Digan, A. Bauer, H. Voshol, G. Christofori, W.R. Sellers, F. Hofmann, and T. Schmelzle. 2013. The tyrosine phosphatase PTPN14 is a negative regulator of YAP activity. *PloS one*. 8:e61916.
- Mizuno, T., H. Murakami, M. Fujii, F. Ishiguro, I. Tanaka, Y. Kondo, S. Akatsuka, S. Toyokuni, K. Yokoi, H. Osada, and Y. Sekido. 2012. YAP induces malignant mesothelioma cell proliferation by upregulating transcription of cell cycle-promoting genes. *Oncogene*. 31:5117-5122.
- Mo, J.S., Z. Meng, Y.C. Kim, H.W. Park, C.G. Hansen, S. Kim, D.S. Lim, and K.L. Guan. 2015. Cellular energy stress induces AMPK-mediated regulation of YAP and the Hippo pathway. *Nature cell biology*. 17:500-510.
- Moleirinho, S., W. Guarrant, and J.L. Kissil. 2014. The Angiomotins--from discovery to function. *FEBS letters*. 588:2693-2703.
- Murakami, M., M. Nakagawa, E.N. Olson, and O. Nakagawa. 2005. A WW domain protein TAZ is a critical coactivator for TBX5, a transcription factor implicated in Holt-Oram syndrome. *Proceedings of the National Academy of Sciences of the United States of America*. 102:18034-18039.

- Murakami, M., J. Tominaga, R. Makita, Y. Uchijima, Y. Kurihara, O. Nakagawa, T. Asano, and H. Kurihara. 2006. Transcriptional activity of Pax3 is co-activated by TAZ. *Biochemical and biophysical research communications*. 339:533-539.
- Nagasaki, T., M. Hara, H. Nakanishi, H. Takahashi, M. Sato, and H. Takeyama. 2014. Interleukin-6 released by colon cancer-associated fibroblasts is critical for tumour angiogenesis: anti-interleukin-6 receptor antibody suppressed angiogenesis and inhibited tumour-stroma interaction. *British journal of cancer*. 110:469-478.
- Neto-Silva, R.M., S. de Beco, and L.A. Johnston. 2010. Evidence for a growth-stabilizing regulatory feedback mechanism between Myc and Yorkie, the Drosophila homolog of Yap. *Developmental cell*. 19:507-520.
- Ni, L., S. Li, J. Yu, J. Min, C.A. Brautigam, D.R. Tomchick, D. Pan, and X. Luo. 2013. Structural basis for autoactivation of human Mst2 kinase and its regulation by RASSF5. *Structure*. 21:1757-1768.
- Ni, L., Y. Zheng, M. Hara, D. Pan, and X. Luo. 2015. Structural basis for Mob1-dependent activation of the core Mst-Lats kinase cascade in Hippo signaling. *Genes & development*. 29:1416-1431.
- Nimura, T., N. Sueyoshi, A. Ishida, Y. Yoshimura, M. Ito, H. Tokumitsu, Y. Shigeri, N. Nozaki, and I. Kameshita. 2007. Knockdown of nuclear Ca²⁺/calmodulin-dependent protein kinase phosphatase causes developmental abnormalities in zebrafish. *Archives of biochemistry and biophysics*. 457:205-216.
- Nishioka, N., K. Inoue, K. Adachi, H. Kiyonari, M. Ota, A. Ralston, N. Yabuta, S. Hirahara, R.O. Stephenson, N. Ogonuki, R. Makita, H. Kurihara, E.M. Morin-Kensicki, H. Nojima, J. Rossant, K. Nakao, H. Niwa, and H. Sasaki. 2009. The Hippo signaling pathway components Lats and Yap pattern Tead4 activity to distinguish mouse trophectoderm from inner cell mass. *Developmental cell*. 16:398-410.
- Nishioka, N., S. Yamamoto, H. Kiyonari, H. Sato, A. Sawada, M. Ota, K. Nakao, and H. Sasaki. 2008. Tead4 is required for specification of trophectoderm in pre-implantation mouse embryos. *Mechanisms of development*. 125:270-283.
- Nishiyama, Y., T. Hirota, T. Morisaki, T. Hara, T. Marumoto, S. Iida, K. Makino, H. Yamamoto, T. Hiraoka, N. Kitamura, and H. Saya. 1999. A human homolog of Drosophila warts tumor suppressor, h-warts, localized to mitotic apparatus and specifically phosphorylated during mitosis. *FEBS letters*. 459:159-165.
- Ota, M., and H. Sasaki. 2008. Mammalian Tead proteins regulate cell proliferation and contact inhibition as transcriptional mediators of Hippo signaling. *Development*. 135:4059-4069.
- Oudhoff, M.J., S.A. Freeman, A.L. Couzens, F. Antignano, E. Kuznetsova, P.H. Min, J.P. Northrop, B. Lehnertz, D. Barsyte-Lovejoy, M. Vedadi, C.H. Arrowsmith, H. Nishina, M.R. Gold, F.M. Rossi, A.C. Gingras, and C. Zaph. 2013. Control of the hippo pathway by Set7-dependent methylation of Yap. *Developmental cell*. 26:188-194.
- Overholtzer, M., J. Zhang, G.A. Smolen, B. Muir, W. Li, D.C. Sgroi, C.X. Deng, J.S. Brugge, and D.A. Haber. 2006. Transforming properties of YAP, a candidate oncogene on the chromosome 11q22 amplicon. *Proceedings of the National Academy of Sciences of the United States of America*. 103:12405-12410.
- Pan, D. 2007. Hippo signaling in organ size control. *Genes & development*. 21:886-897.
- Pan, D. 2010. The hippo signaling pathway in development and cancer. *Developmental cell*. 19:491-505.
- Panciera, T., L. Azzolin, A. Fujimura, D. Di Biagio, C. Frasson, S. Bresolin, S. Soligo, G. Basso, S. Bicciato, A. Rosato, M. Cordenonsi, and S. Piccolo. 2016. Induction of Expandable Tissue-Specific Stem/Progenitor Cells through Transient Expression of YAP/TAZ. *Cell stem cell*. 19:725-737.

- Pantalacci, S., N. Tapon, and P. Leopold. 2003. The Salvador partner Hippo promotes apoptosis and cell-cycle exit in *Drosophila*. *Nature cell biology*. 5:921-927.
- Phang, H.Q., J.L. Hoon, S.K. Lai, Y. Zeng, K.H. Chiam, H.Y. Li, and C.G. Koh. 2014. POPX2 phosphatase regulates the KIF3 kinesin motor complex. *Journal of cell science*. 127:727-739.
- Praskova, M., A. Khoklatchev, S. Ortiz-Vega, and J. Avruch. 2004. Regulation of the MST1 kinase by autophosphorylation, by the growth inhibitory proteins, RASSF1 and NORE1, and by Ras. *The Biochemical journal*. 381:453-462.
- Praskova, M., F. Xia, and J. Avruch. 2008. MOBKL1A/MOBKL1B phosphorylation by MST1 and MST2 inhibits cell proliferation. *Current biology : CB*. 18:311-321.
- Preisinger, C., B. Short, V. De Corte, E. Bruyneel, A. Haas, R. Kopajtich, J. Gettemans, and F.A. Barr. 2004. YSK1 is activated by the Golgi matrix protein GM130 and plays a role in cell migration through its substrate 14-3-3zeta. *The Journal of cell biology*. 164:1009-1020.
- Puri, S., A.E. Folias, and M. Hebrok. 2015. Plasticity and dedifferentiation within the pancreas: development, homeostasis, and disease. *Cell stem cell*. 16:18-31.
- Ralston, A., B.J. Cox, N. Nishioka, H. Sasaki, E. Chea, P. Rugg-Gunn, G. Guo, P. Robson, J.S. Draper, and J. Rossant. 2010. Gata3 regulates trophoblast development downstream of Tead4 and in parallel to Cdx2. *Development*. 137:395-403.
- Rauskolb, C., G. Pan, B.V. Reddy, H. Oh, and K.D. Irvine. 2011. Zyxin links fat signaling to the hippo pathway. *PLoS Biol*. 9:e1000624.
- Rawat, S.J., and J. Chernoff. 2015. Regulation of mammalian Ste20 (Mst) kinases. *Trends in biochemical sciences*. 40:149-156.
- Sato, E., S.H. Olson, J. Ahn, B. Bundy, H. Nishikawa, F. Qian, A.A. Jungbluth, D. Frosina, S. Gnajatic, C. Ambrosone, J. Kepner, T. Odunsi, G. Ritter, S. Lele, Y.T. Chen, H. Ohtani, L.J. Old, and K. Odunsi. 2005. Intraepithelial CD8+ tumor-infiltrating lymphocytes and a high CD8+/regulatory T cell ratio are associated with favorable prognosis in ovarian cancer. *Proceedings of the National Academy of Sciences of the United States of America*. 102:18538-18543.
- Schlegelmilch, K., M. Mohseni, O. Kirak, J. Pruszk, J.R. Rodriguez, D. Zhou, B.T. Kreger, V. Vasioukhin, J. Avruch, T.R. Brummelkamp, and F.D. Camargo. 2011. Yap1 acts downstream of alpha-catenin to control epidermal proliferation. *Cell*. 144:782-795.
- Shackleton, M., E. Quintana, E.R. Fearon, and S.J. Morrison. 2009. Heterogeneity in cancer: cancer stem cells versus clonal evolution. *Cell*. 138:822-829.
- Shao, D., P. Zhai, D.P. Del Re, S. Sciarretta, N. Yabuta, H. Nojima, D.S. Lim, D. Pan, and J. Sadoshima. 2014. A functional interaction between Hippo-YAP signalling and FoxO1 mediates the oxidative stress response. *Nature communications*. 5:3315.
- Shaw, F.L., H. Harrison, K. Spence, M.P. Ablett, B.M. Simoes, G. Farnie, and R.B. Clarke. 2012. A detailed mammosphere assay protocol for the quantification of breast stem cell activity. *Journal of mammary gland biology and neoplasia*. 17:111-117.
- Singh, S.K., C. Hawkins, I.D. Clarke, J.A. Squire, J. Bayani, T. Hide, R.M. Henkelman, M.D. Cusimano, and P.B. Dirks. 2004. Identification of human brain tumour initiating cells. *Nature*. 432:396-401.
- Smith, H.A., and Y. Kang. 2013. The metastasis-promoting roles of tumor-associated immune cells. *Journal of molecular medicine*. 91:411-429.
- Solinas, G., G. Germano, A. Mantovani, and P. Allavena. 2009. Tumor-associated macrophages (TAM) as major players of the cancer-related inflammation. *Journal of leukocyte biology*. 86:1065-1073.
- Song, S., J.A. Ajani, S. Honjo, D.M. Maru, Q. Chen, A.W. Scott, T.R. Heallen, L. Xiao, W.L. Hofstetter, B. Weston, J.H. Lee, R. Wadhwa, K. Sudo, J.R. Stroehlein, J.F. Martin, M.C. Hung, and R.L. Johnson.

2014. Hippo coactivator YAP1 upregulates SOX9 and endows esophageal cancer cells with stem-like properties. *Cancer research*. 74:4170-4182.
- Stavridi, E.S., K.G. Harris, Y. Huyen, J. Bothos, P.M. Verwoerd, S.E. Stayrook, N.P. Pavletich, P.D. Jeffrey, and F.C. Luca. 2003. Crystal structure of a human Mob1 protein: toward understanding Mob-regulated cell cycle pathways. *Structure*. 11:1163-1170.
- Stegert, M.R., R. Tamaskovic, S.J. Bichsel, A. Hergovich, and B.A. Hemmings. 2004. Regulation of NDR2 protein kinase by multi-site phosphorylation and the S100B calcium-binding protein. *The Journal of biological chemistry*. 279:23806-23812.
- Stork, P.J., and J.M. Schmitt. 2002. Crosstalk between cAMP and MAP kinase signaling in the regulation of cell proliferation. *Trends in cell biology*. 12:258-266.
- Strano, S., O. Monti, N. Pediconi, A. Baccarini, G. Fontemaggi, E. Lapi, F. Mantovani, A. Damalas, G. Citro, A. Sacchi, G. Del Sal, M. Levrero, and G. Blandino. 2005. The transcriptional coactivator Yes-associated protein drives p73 gene-target specificity in response to DNA Damage. *Molecular cell*. 18:447-459.
- Strano, S., E. Munarriz, M. Rossi, L. Castagnoli, Y. Shaul, A. Sacchi, M. Oren, M. Sudol, G. Cesareni, and G. Blandino. 2001. Physical interaction with Yes-associated protein enhances p73 transcriptional activity. *The Journal of biological chemistry*. 276:15164-15173.
- Su, K.Y., H.Y. Chen, K.C. Li, M.L. Kuo, J.C. Yang, W.K. Chan, B.C. Ho, G.C. Chang, J.Y. Shih, S.L. Yu, and P.C. Yang. 2012. Pretreatment epidermal growth factor receptor (EGFR) T790M mutation predicts shorter EGFR tyrosine kinase inhibitor response duration in patients with non-small-cell lung cancer. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology*. 30:433-440.
- Suijkerbuijk, S.J., G. Kolahgar, I. Kucinski, and E. Piddini. 2016. Cell Competition Drives the Growth of Intestinal Adenomas in Drosophila. *Current biology : CB*. 26:428-438.
- Susila, A., H. Chan, A.X. Loh, H.Q. Phang, E.T. Wong, V. Tergaonkar, and C.G. Koh. 2010. The POPX2 phosphatase regulates cancer cell motility and invasiveness. *Cell cycle*. 9:179-187.
- Suzuki, H., N. Yabuta, N. Okada, K. Torigata, Y. Aylon, M. Oren, and H. Nojima. 2013. Lats2 phosphorylates p21/CDKN1A after UV irradiation and regulates apoptosis. *Journal of cell science*. 126:4358-4368.
- Takeuchi, M., A. Ishida, I. Kameshita, T. Kitani, S. Okuno, and H. Fujisawa. 2001. Identification and characterization of CaMKP-N, nuclear calmodulin-dependent protein kinase phosphatase. *Journal of biochemistry*. 130:833-840.
- Tam, W.L., and R.A. Weinberg. 2013. The epigenetics of epithelial-mesenchymal plasticity in cancer. *Nature medicine*. 19:1438-1449.
- Taylor, L.K., H.C. Wang, and R.L. Erikson. 1996. Newly identified stress-responsive protein kinases, Krs-1 and Krs-2. *Proceedings of the National Academy of Sciences of the United States of America*. 93:10099-10104.
- Thompson, B.J., and E. Sahai. 2015. MST kinases in development and disease. *The Journal of cell biology*. 210:871-882.
- Tjiu, J.W., J.S. Chen, C.T. Shun, S.J. Lin, Y.H. Liao, C.Y. Chu, T.F. Tsai, H.C. Chiu, Y.S. Dai, H. Inoue, P.C. Yang, M.L. Kuo, and S.H. Jee. 2009. Tumor-associated macrophage-induced invasion and angiogenesis of human basal cell carcinoma cells by cyclooxygenase-2 induction. *The Journal of investigative dermatology*. 129:1016-1025.

- Totaro, A., M. Castellan, G. Battilana, F. Zanconato, L. Azzolin, S. Giulitti, M. Cordenonsi, and S. Piccolo. 2017. YAP/TAZ link cell mechanics to Notch signalling to control epidermal stem cell fate. *Nature communications*. 8:15206.
- Tsai, J.H., and J. Yang. 2013. Epithelial-mesenchymal plasticity in carcinoma metastasis. *Genes & development*. 27:2192-2206.
- Turke, A.B., K. Zejnullahu, Y.L. Wu, Y. Song, D. Dias-Santagata, E. Lifshits, L. Toschi, A. Rogers, T. Mok, L. Sequist, N.I. Lindeman, C. Murphy, S. Akhavanfard, B.Y. Yeap, Y. Xiao, M. Capelletti, A.J. Iafrate, C. Lee, J.G. Christensen, J.A. Engelman, and P.A. Janne. 2010. Preexistence and clonal selection of MET amplification in EGFR mutant NSCLC. *Cancer cell*. 17:77-88.
- Ultanir, S.K., N.T. Hertz, G. Li, W.P. Ge, A.L. Burlingame, S.J. Pleasure, K.M. Shokat, L.Y. Jan, and Y.N. Jan. 2012. Chemical genetic identification of NDR1/2 kinase substrates AAK1 and Rabin8 Uncovers their roles in dendrite arborization and spine development. *Neuron*. 73:1127-1142.
- Varelas, X., R. Sakuma, P. Samavarchi-Tehrani, R. Peerani, B.M. Rao, J. Dembowy, M.B. Yaffe, P.W. Zandstra, and J.L. Wrana. 2008. TAZ controls Smad nucleocytoplasmic shuttling and regulates human embryonic stem-cell self-renewal. *Nature cell biology*. 10:837-848.
- Varelas, X., P. Samavarchi-Tehrani, M. Narimatsu, A. Weiss, K. Cockburn, B.G. Larsen, J. Rossant, and J.L. Wrana. 2010. The Crumbs complex couples cell density sensing to Hippo-dependent control of the TGF-beta-SMAD pathway. *Developmental cell*. 19:831-844.
- Vargo-Gogola, T., and J.M. Rosen. 2007. Modelling breast cancer: one size does not fit all. *Nat Rev Cancer*. 7:659-672.
- Vassilev, A., K.J. Kaneko, H. Shu, Y. Zhao, and M.L. DePamphilis. 2001. TEAD/TEF transcription factors utilize the activation domain of YAP65, a Src/Yes-associated protein localized in the cytoplasm. *Genes & development*. 15:1229-1241.
- Vichalkovski, A., E. Gresko, H. Cornils, A. Hergovich, D. Schmitz, and B.A. Hemmings. 2008. NDR kinase is activated by RASSF1A/MST1 in response to Fas receptor stimulation and promotes apoptosis. *Current biology : CB*. 18:1889-1895.
- Virshup, D.M., and S. Shenolikar. 2009. From promiscuity to precision: protein phosphatases get a makeover. *Molecular cell*. 33:537-545.
- Vishnoi, M., S. Peddibhotla, W. Yin, T.S. A, G.C. George, D.S. Hong, and D. Marchetti. 2015. The isolation and characterization of CTC subsets related to breast cancer dormancy. *Scientific reports*. 5:17533.
- Vitolo, M.I., I.E. Anglin, W.M. Mahoney, Jr., K.J. Renoud, R.B. Gartenhaus, K.E. Bachman, and A. Passaniti. 2007. The RUNX2 transcription factor cooperates with the YES-associated protein, YAP65, to promote cell transformation. *Cancer Biol Ther*. 6:856-863.
- Vlashi, E., and F. Pajonk. 2015. Cancer stem cells, cancer cell plasticity and radiation therapy. *Seminars in cancer biology*. 31:28-35.
- Voon, D.C., R.Y. Huang, R.A. Jackson, and J.P. Thiery. 2017. The EMT spectrum and therapeutic opportunities. *Molecular oncology*. 11:878-891.
- Voss, M., J. Paterson, I.R. Kelsall, C. Martin-Granados, C.J. Hastie, M.W. Pegg, and P.T. Cohen. 2011. Ppm1E is an in cellulo AMP-activated protein kinase phosphatase. *Cellular signalling*. 23:114-124.
- Wada, K., K. Itoga, T. Okano, S. Yonemura, and H. Sasaki. 2011. Hippo pathway regulation by cell morphology and stress fibers. *Development*. 138:3907-3914.
- Wahl, G.M., and B.T. Spike. 2017. Cell state plasticity, stem cells, EMT, and the generation of intra-tumoral heterogeneity. *NPI breast cancer*. 3:14.

- Wang, H., R. Cao, L. Xia, H. Erdjument-Bromage, C. Borchers, P. Tempst, and Y. Zhang. 2001. Purification and functional characterization of a histone H3-lysine 4-specific methyltransferase. *Molecular cell*. 8:1207-1217.
- Wang, P., Y. Bai, B. Song, Y. Wang, D. Liu, Y. Lai, X. Bi, and Z. Yuan. 2011. PP1A-mediated dephosphorylation positively regulates YAP2 activity. *PloS one*. 6:e24288.
- Wang, W., Z.D. Xiao, X. Li, K.E. Aziz, B. Gan, R.L. Johnson, and J. Chen. 2015. AMPK modulates Hippo pathway activity to regulate energy homeostasis. *Nature cell biology*. 17:490-499.
- Wang, Y., Q. Dong, Q. Zhang, Z. Li, E. Wang, and X. Qiu. 2010. Overexpression of yes-associated protein contributes to progression and poor prognosis of non-small-cell lung cancer. *Cancer science*. 101:1279-1285.
- Wang, Z., Y. Wu, H. Wang, Y. Zhang, L. Mei, X. Fang, X. Zhang, F. Zhang, H. Chen, Y. Liu, Y. Jiang, S. Sun, Y. Zheng, N. Li, and L. Huang. 2014. Interplay of mevalonate and Hippo pathways regulates RHAMM transcription via YAP to modulate breast cancer cell motility. *Proceedings of the National Academy of Sciences of the United States of America*. 111:E89-98.
- Wen, W., F. Zhu, J. Zhang, Y.S. Keum, T. Zykova, K. Yao, C. Peng, D. Zheng, Y.Y. Cho, W.Y. Ma, A.M. Bode, and Z. Dong. 2010. MST1 promotes apoptosis through phosphorylation of histone H2AX. *The Journal of biological chemistry*. 285:39108-39116.
- Weng, T., and C.G. Koh. 2017. POPX2 phosphatase regulates apoptosis through the TAK1-IKK-NF-kappaB pathway. *Cell death & disease*. 8:e3051.
- Wu, S., J. Huang, J. Dong, and D. Pan. 2003. hippo encodes a Ste-20 family protein kinase that restricts cell proliferation and promotes apoptosis in conjunction with salvador and warts. *Cell*. 114:445-456.
- Xia, H., H. Qi, Y. Li, J. Pei, J. Barton, M. Blackstad, T. Xu, and W. Tao. 2002. LATS1 tumor suppressor regulates G2/M transition and apoptosis. *Oncogene*. 21:1233-1241.
- Xie, D., J. Cui, T. Xia, Z. Jia, L. Wang, W. Wei, A. Zhu, Y. Gao, K. Xie, and M. Quan. 2015. Hippo transducer TAZ promotes epithelial mesenchymal transition and supports pancreatic cancer progression. *Oncotarget*. 6:35949-35963.
- Xie, Q., J. Chen, H. Feng, S. Peng, U. Adams, Y. Bai, L. Huang, J. Li, J. Huang, S. Meng, and Z. Yuan. 2013. YAP/TEAD-mediated transcription controls cellular senescence. *Cancer research*. 73:3615-3624.
- Xu, M.Z., S.W. Chan, A.M. Liu, K.F. Wong, S.T. Fan, J. Chen, R.T. Poon, L. Zender, S.W. Lowe, W. Hong, and J.M. Luk. 2011. AXL receptor kinase is a mediator of YAP-dependent oncogenic functions in hepatocellular carcinoma. *Oncogene*. 30:1229-1240.
- Xu, T., W. Wang, S. Zhang, R.A. Stewart, and W. Yu. 1995. Identifying tumor suppressors in genetic mosaics: the Drosophila lats gene encodes a putative protein kinase. *Development*. 121:1053-1063.
- Yaffe, M.B., K. Rittinger, S. Volinia, P.R. Caron, A. Aitken, H. Leffers, S.J. Gamblin, S.J. Smerdon, and L.C. Cantley. 1997. The structural basis for 14-3-3:phosphopeptide binding specificity. *Cell*. 91:961-971.
- Yang, N., C.D. Morrison, P. Liu, J. Miecznikowski, W. Bshara, S. Han, Q. Zhu, A.R. Omilian, X. Li, and J. Zhang. 2012. TAZ induces growth factor-independent proliferation through activation of EGFR ligand amphiregulin. *Cell cycle*. 11:2922-2930.
- Ye, X., and R.A. Weinberg. 2015. Epithelial-Mesenchymal Plasticity: A Central Regulator of Cancer Progression. *Trends in cell biology*. 25:675-686.

- Yimlamai, D., C. Christodoulou, G.G. Galli, K. Yanger, B. Pepe-Mooney, B. Gurung, K. Shrestha, P. Cahan, B.Z. Stanger, and F.D. Camargo. 2014. Hippo pathway activity influences liver cell fate. *Cell*. 157:1324-1338.
- Yin, F., J. Yu, Y. Zheng, Q. Chen, N. Zhang, and D. Pan. 2013. Spatial organization of Hippo signaling at the plasma membrane mediated by the tumor suppressor Merlin/NF2. *Cell*. 154:1342-1355.
- You, B., G. Yan, Z. Zhang, L. Yan, J. Li, Q. Ge, J.P. Jin, and J. Sun. 2009. Phosphorylation of cardiac troponin I by mammalian sterile 20-like kinase 1. *The Biochemical journal*. 418:93-101.
- Yu, F.X., Y. Zhang, H.W. Park, J.L. Jewell, Q. Chen, Y. Deng, D. Pan, S.S. Taylor, Z.C. Lai, and K.L. Guan. 2013. Protein kinase A activates the Hippo pathway to modulate cell proliferation and differentiation. *Genes & development*. 27:1223-1232.
- Yu, F.X., B. Zhao, N. Panupinthu, J.L. Jewell, I. Lian, L.H. Wang, J. Zhao, H. Yuan, K. Tumaneng, H. Li, X.D. Fu, G.B. Mills, and K.L. Guan. 2012. Regulation of the Hippo-YAP pathway by G-protein-coupled receptor signaling. *Cell*. 150:780-791.
- Yue, G., X. Sun, A. Gimenez-Capitan, J. Shen, L. Yu, C. Teixeira, W. Guan, R. Rosell, B. Liu, and J. Wei. 2014. TAZ is highly expressed in gastric signet ring cell carcinoma. *BioMed research international*. 2014:393064.
- Zanconato, F., M. Cordenonsi, and S. Piccolo. 2016. YAP/TAZ at the Roots of Cancer. *Cancer cell*. 29:783-803.
- Zanconato, F., M. Forcato, G. Battilana, L. Azzolin, E. Quaranta, B. Bodega, A. Rosato, S. Bicciato, M. Cordenonsi, and S. Piccolo. 2015. Genome-wide association between YAP/TAZ/TEAD and AP-1 at enhancers drives oncogenic growth. *Nature cell biology*. 17:1218-1227.
- Zhang, H., H.A. Pasolli, and E. Fuchs. 2011. Yes-associated protein (YAP) transcriptional coactivator functions in balancing growth and differentiation in skin. *Proceedings of the National Academy of Sciences of the United States of America*. 108:2270-2275.
- Zhang, K., E. Rodriguez-Aznar, N. Yabuta, R.J. Owen, J.M. Mingot, H. Nojima, M.A. Nieto, and G.D. Longmore. 2012. Lats2 kinase potentiates Snail1 activity by promoting nuclear retention upon phosphorylation. *The EMBO journal*. 31:29-43.
- Zhang, L., F. Tang, L. Terracciano, D. Hynx, R. Kohler, S. Bichet, D. Hess, P. Cron, B.A. Hemmings, A. Hergovich, and D. Schmitz-Rohmer. 2015. NDR functions as a physiological YAP1 kinase in the intestinal epithelium. *Current biology : CB*. 25:296-305.
- Zhang, P., S. Wang, S. Wang, J. Qiao, L. Zhang, Z. Zhang, and Z. Chen. 2016. Dual function of partitioning-defective 3 in the regulation of YAP phosphorylation and activation. *Cell Discov*. 2:16021.
- Zhang, S., T. Guo, H. Chan, S.K. Sze, and C.G. Koh. 2013. Integrative transcriptome and proteome study to identify the signaling network regulated by POPX2 phosphatase. *J Proteome Res*. 12:2525-2536.
- Zhang, W., N. Nandakumar, Y. Shi, M. Manzano, A. Smith, G. Graham, S. Gupta, E.E. Vietsch, S.Z. Laughlin, M. Wadhwa, M. Chetram, M. Joshi, F. Wang, B. Kallakury, J. Toretsky, A. Wellstein, and C. Yi. 2014a. Downstream of mutant KRAS, the transcription regulator YAP is essential for neoplastic progression to pancreatic ductal adenocarcinoma. *Science signaling*. 7:ra42.
- Zhang, Y., H. Xia, X. Ge, Q. Chen, D. Yuan, Q. Chen, W. Leng, L. Chen, Q. Tang, and F. Bi. 2014b. CD44 acts through RhoA to regulate YAP signaling. *Cellular signalling*. 26:2504-2513.
- Zhao, B., L. Li, Q. Lu, L.H. Wang, C.Y. Liu, Q. Lei, and K.L. Guan. 2011. Angiomotin is a novel Hippo pathway component that inhibits YAP oncoprotein. *Genes & development*. 25:51-63.
- Zhao, B., L. Li, L. Wang, C.Y. Wang, J. Yu, and K.L. Guan. 2012. Cell detachment activates the Hippo pathway via cytoskeleton reorganization to induce anoikis. *Genes & development*. 26:54-68.

- Zhao, B., X. Wei, W. Li, R.S. Udan, Q. Yang, J. Kim, J. Xie, T. Ikenoue, J. Yu, L. Li, P. Zheng, K. Ye, A. Chinnaiyan, G. Halder, Z.C. Lai, and K.L. Guan. 2007. Inactivation of YAP oncoprotein by the Hippo pathway is involved in cell contact inhibition and tissue growth control. *Genes & development*. 21:2747-2761.
- Zhao, B., X. Ye, J. Yu, L. Li, W. Li, S. Li, J. Yu, J.D. Lin, C.Y. Wang, A.M. Chinnaiyan, Z.C. Lai, and K.L. Guan. 2008. TEAD mediates YAP-dependent gene induction and growth control. *Genes & development*. 22:1962-1971.
- Zhao, Y., P. Khanal, P. Savage, Y.M. She, T.D. Cyr, and X. Yang. 2014. YAP-induced resistance of cancer cells to antitubulin drugs is modulated by a Hippo-independent pathway. *Cancer research*. 74:4493-4503.
- Zuo, Q.F., R. Zhang, B.S. Li, Y.L. Zhao, Y. Zhuang, T. Yu, L. Gong, S. Li, B. Xiao, and Q.M. Zou. 2015. MicroRNA-141 inhibits tumor growth and metastasis in gastric cancer by directly targeting transcriptional co-activator with PDZ-binding motif, TAZ. *Cell death & disease*. 6:e1623.